THE BIOSYNTHESIS OF FUSARIC ACID FROM ^{14}C-LABELLED ACETATE IN GIBBERELLA FUJIKUROI

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ABSTRACT

Acetate-1-^{14}C and acetate-2-^{14}C were supplied to cultures of *Gibberella fujikuroi* (Saw.) Wr., and the radioactive fusaric acid consequently formed was isolated. Acetate-1-^{14}C contributed activity mainly to C-4, C-6, C-7, C-8, and C-10 of fusaric acid, whereas acetate-2-^{14}C contributed activity to C-2, C-3, C-5, C-9, and C-11 of fusaric acid. These results are consistent with the hypothesis that fusaric acid is formed from a polyacetate unit and aspartic acid or closely related metabolites.

It is well established that more than one route exists for the biological formation of the pyridine ring. Nicotinic acid is formed from tryptophan in mammals and in the fungus *Neurospora crassa* (1). In higher plants (2) and certain bacteria (3), however, experiments with isotopically labelled tryptophan have failed to show any transformation to nicotinic acid. Glycerol and succinate have been shown to be precursors of nicotinic acid in *Escherichia coli* (4, 5). Aspartate and glycerol are incorporated into nicotinic acid in *Mycobacterium tuberculosis* (6).

Nicotinic acid is a precursor of the pyridine ring of nicotine (7) and anabasine (8) and of the pyridone-ring compound ricinine (9, 10). Nicotine derived from acetate-2-^{14}C (11) or aspartate-3-^{14}C (12) was shown to have one-half of the activity of the pyridine ring at C-3. Acetate-2-^{14}C (13) labelled C-2 and C-3 of the pyridine ring of anabasine equally, with little activity on C-4 and C-6. Administration of acetate-2-^{14}C or succinate-2,3-^{14}C to *Ricinus communis* plants yielded ricinine labelled equally at C-2 and C-3 (15); succinate-1,4-^{14}C gave radioactive ricinine in which most of the activity was located on the nitrile carbon (16). Glycerol-1,3-^{14}C and glycerol-2-^{14}C were also incorporated into the pyridone ring (17, 18), the pattern of labelling strongly suggesting that the three-carbon chain of glycerol formed C-4, C-5, and C-6 of ricinine. The accumulated evidence thus suggests that, in higher plants, nicotinic acid is formed from a condensation of glycerol and succinate or closely related metabolites.

The structure of the wilt toxin, fusaric acid (I), suggests that the pyridine ring of this compound may not be derived from glycerol and succinate. Leete (19) has suggested that the molecule may be formed biologically from a branched chain derived from five acetate units (structure I*a*). Vining[4] has proposed a slightly different condensation involving a polyacetate unit and aspartic acid (structure I*b*). The two hypotheses differ only in the origin of C-2, C-3, C-4, and C-7 of fusaric acid. In Leete's scheme, C-7 and C-3 would be derived from the methyl group of acetate, with C-2 and C-4 originating from the carboxyl group. Vining's suggestion requires C-2 and C-3 to be formed from the methyl group of acetate, with C-7 and C-4 being formed primarily from the carboxyl group.

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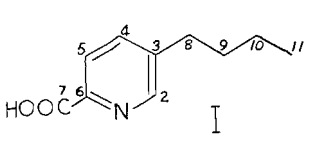
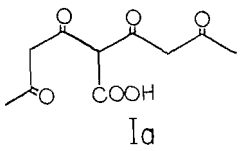
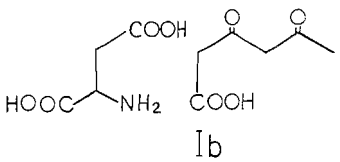
[4]*L. C. Vining, private communication.*



I



Ia



Ib

The incorporation of acetate-1-^{14}C, acetate-2-^{14}C, aspartate-1-^{14}C, and aspartate-4-^{14}C into fusaric acid by *Fusarium orthoceras* cultures has been demonstrated.[5] Aspartate contributed activity only to C-7 and the pyridine ring. Partial degradation of the labelled fusaric acid derived from acetate feedings suggested that the activity was probably distributed in a manner consistent with Vining's hypothesis.

The purpose of this investigation was to determine the complete distribution of label in fusaric acid derived from acetate-^{14}C in *G. fujikuroi* (Saw.) Wr. cultures. The incorporation data for acetate-1-^{14}C and acetate-2-^{14}C presented in Table I demonstrate that the methyl carbon and carboxyl carbon of acetate are equally efficient precursors of the carbon skeleton.

TABLE I

Incorporation of acetate-^{14}C into fusaric acid by *G. fujikuroi*

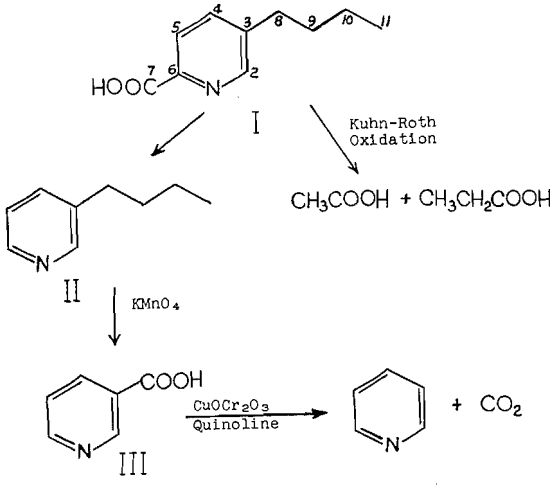
Compound supplied	Fusaric acid		Dilution of specific activity	Incorporation (%)
	Yield (g)	Specific activity (d.p.m.$\times 10^{-6}$/mmole)		
Acetate-1-^{14}C*	1.084	1.07	810	12.9
Acetate-2-^{14}C†	1.427	2.0	275	15.1

*5×10^7 d.p.m.; 8.5×10^8 d.p.m./mmole.
†1.06×10^8 d.p.m.; 5.5×10^8 d.p.m./mmole.

The radioactive fusaric acid was degraded as illustrated in Reaction Scheme 1. Chromic acid oxidation of fusaric acid (I) yielded a mixture of acetic and propionic acids which was separated by gas–liquid chromatography. A Schmidt reaction on the sodium salt of acetic acid gave carbon dioxide (C-10) and methylamine. The activity at C-11 was determined either by difference or by oxidation of the methylamine to yield carbon dioxide. The propionic acid was degraded by the Schmidt reaction to yield carbon dioxide (C-9). Decarboxylation of fusaric acid gave carbon dioxide (C-7) and 3-butylpyridine (II), which was oxidized with potassium permanganate to nicotinic acid (III). Decarboxylation of nicotinic acid with copper chromite in quinoline yielded carbon dioxide (C-7). Sequential degradation of the pyridine ring of nicotinic acid was accomplished by the method described by Friedman and Leete (13 14), with the exception that, in one case, the activity at C-4 was determined directly by a Schmidt reaction of the 2-methyl-3-phenylpropanoic acid obtained by permanganate oxidation of 2-methyl-3-phenylpropylamine.

The distribution of activity in fusaric acid from acetate-1-^{14}C is presented in Table II. The specific activity values reported are for undiluted material. It is apparent that the activity from acetate-1-^{14}C is concentrated on C-4, C-6, C-7, C-8, and C-10 of the molecule. The distribution of activity at three of the positions approaches the value expected if one-half of the carbon atoms of fusaric acid were derived from the carboxyl group of acetate. The low activity at C-10 may have resulted from contamination of the acetic

[5]*T. A. Dobson and L. C. Vining, unpublished results.*



REACTION SCHEME 1.

acid by propionic acid in the gas chromatographic separation. This would also account for the presence of activity at C-11 in this degradation.

The distribution of activity in fusaric acid from acetate-2-^{14}C (Table III) is compatible with the data obtained for the acetate-1-^{14}C feeding experiment. The activity is found mainly in positions 2, 3, 5, 9, and 11 of the fusaric acid. The low activity at C-2 and C-3 of the pyridine ring and the presence of activity at C-4 and C-7 are consistent with the participation of a four-carbon Krebs cycle acid or its equivalent.[6]

TABLE II

Distribution of activity in the degradation products of fusaric acid synthesized from acetate-1-^{14}C by *G. fujikuroi*

Compound	Carbon No.	Specific activity (d.p.m. × 10^{-5}/mmole)	Distribution of activity (%)
Fusaric acid	All	10.7	100
Barium carbonate*	7	1.52	14.2
Sodium propionate†	9, 10, 11	2.22	20.7
Sodium acetate†	10, 11	2.15	20.1
Barium carbonate‡	9	0.0	0.0
Barium carbonate§	10	1.4	13.1
Barium carbonate‖	11	0.78	7.3
Barium carbonate¶	8	2.08	19.4
Nicotinic acid	2, 3, 4, 5, 6, 8	6.49	60.4
Benzoic acid**	6	2.06	19.3
Barium carbonate††	5	0.0	0.0
Formaldehyde dimedone	2	0.37	0.35
Barium carbonate‡‡	3	2.90	2.71
By difference	4	—	18.6

*Obtained from the decarboxylation of fusaric acid.
†Obtained from the Kuhn–Roth oxidation of fusaric acid.
‡Obtained from a Schmidt reaction on sodium propionate.
§Obtained from a Schmidt reaction on sodium acetate.
‖Obtained by alkaline premanganate oxidation of the residue from the Schmidt reaction on sodium acetate.
¶Obtained from the decarboxylation of nicotinic acid.
**Obtained from the Kuhn–Roth oxidation of 1,3-dimethyl-2-phenylpiperidine.
††Obtained from a Schmidt reaction on sodium acetate derived from a Kuhn–Roth oxidation of 1,3-dimethyl-2-phenylpiperidine.
‡‡Obtained from a Schmidt reaction on the sodium salt of 3-methyl-4-phenylbutanoic acid.

[6]*For example, the equilibrium distribution of label in oxalacetate derived from acetate-2-^{14}C via the Krebs cycle is: C-1, 16.7%; C-2, 33.3%; C-3, 33.3%; and C-4, 16,7%. If C-7, C-2, C-3, and C-4 of fusaric acid are formed from a four-carbon dicarboxylic acid and contain 40% of the activity derived from acetate-2-^{14}C, then the percentage contribution of each carbon would be 6.65, 13.3, 13.3, and 6.65, respectively.*

TABLE III

Distribution of activity in the degradation products of fusaric acid synthesized from acetate-2-^{14}C by *G. fujikuroi*

Compound	Carbon No.	Specific activity (d.p.m. × 10^{-5}/mmole)	Distribution of activity (%)
Fusaric acid	All	20.0	100
Barium carbonate*	7	0.89	4.46
Sodium acetate†	10, 11	3.6	18.0
Barium carbonate‡	9	3.94	19.7
Barium carbonate§	10	0.12	0.6
By difference	11	—	17.4
Barium carbonate‖	8	0.0	0.0
3-Methylpyridine	2, 3, 4, 5, 6, 8	10.0	50.2
Benzoic acid¶	6	0.08	0.44
Barium carbonate**	5	3.34	16.7
Formaldehyde dimedone	2	2.1	10.5
Barium carbonate††	3	2.0	10.0
Barium carbonate‡‡	4	0.44	2.21

*Obtained from the decarboxylation of fusaric acid.
†Obtained from the Kuhn–Roth oxidation of fusaric acid.
‡Obtained from a Schmidt reaction on sodium propionate.
§Obtained from a Schmidt reaction on sodium acetate.
‖Obtained from the decarboxylation of nicotinic acid.
¶Obtained from the Kuhn–Roth oxidation of 1,3-dimethyl-2-phenylpiperidine.
**Obtained from a Schmidt reaction on sodium acetate derived from the Kuhn–Roth oxidation of 1,3-dimethyl-2-phenylpiperidine.
††Obtained from a Schmidt reaction on the sodium salt of 3-methyl-4-phenylbutanoic acid.
‡‡Obtained from a Schmidt reaction on the sodium salt of 2-methyl-3-phenylpropanoic acid.

The evidence presented in this communication thus indicates that all the carbons of fusaric acid are derived from acetate or closely related metabolites. The pattern of labelling is consistent with the aspartate–polyacetate hypothesis[4] for fusaric acid formation, and is not consistent with the polyacetate hypothesis (19). The incorporation of ^{14}C-labelled aspartate into fusaric acid[5] and the incorporation of aspartate-1,4-^{14}C,^{15}N into nicotinic acid in *M. tuberculosis* (6) suggest that aspartate may be incorporated intact into fusaric acid.

EXPERIMENTAL

Conditions of Culture

Gibberella fujikuroi (Saw.) Wr., strain ETH M 82, was obtained from the Department of Special Botany, Swiss Federal Institute of Technology, Zurich, Switzerland. The fungus was maintained on malt agar or Difco potato-dextrose agar slants at 4 °C, with regular subculturing every 2 months.

Inoculum was prepared by transferring a suspension of the mycelium to 125 ml Erlenmeyer flasks containing sterile, water-saturated rice. After 5 days in the dark, the conidia were harvested by shaking the mixture with sterile, distilled water (60 ml). This spore suspension was transferred to a 250 ml Erlenmeyer flask and incubated on a shaker in the dark for 24 h. Ten milliliters of this inoculum was added to 250 ml of Czapek's medium in 1 l Erlenmeyer flasks, and the fungus was grown in the dark in shake culture.

Czapek's medium has the following composition: sucrose, 4%; sodium nitrate, 0.3%; monopotassium phosphate, 0.1%; magnesium sulfate, 0.05%; potassium chloride, 0.05%; ferrous sulfate, 0.001%; all in distilled water. The pH of the medium was adjusted to 5.5 before autoclaving.

Administration of Labelled Compounds

The fungus from shake cultures that were 3 days old was collected by low-speed centrifugation. The harvest from two culture flasks was resuspended in Czapek's medium (50 ml) and used to inoculate fresh medium (200 ml). Six flasks were normally used for each tracer experiment.

Aqueous solutions of sodium acetate-1-^{14}C or sodium acetate-2-^{14}C (Atomic Energy of Canada, Ltd.) were prepared and divided equally between the six flasks. After the flasks were shaken for 24 h in the dark, they were removed and the fusaric acid was extracted from the culture filtrate.

Assay of Radioactivity

Samples were counted in a Nuclear-Chicago model 725 liquid scintillation counter using, as solvents, either (*a*) toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.005% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP); or (*b*) dioxane – ethyl cellosolve (5:1) containing 5% naphthalene, 0.4% PPO, and 0.01% POPOP. Sample activities were determined in duplicate, with a counting error of 5% or less in each determination.

Extraction and Isolation of the Fusaric Acid

Celite was added to the culture flasks and the slurry filtered. The filtrate was reduced to one-quarter of its volume on a rotary evaporator and filtered. The filtrate (ca. 400 ml) was adjusted to pH 4.0 and extracted with ethyl acetate (3 × 300 ml). The ethyl acetate fraction was evaporated to dryness, the residue dissolved in water (50 ml), and the pH adjusted to 8.0 with 2 *N* sodium hydroxide. After extraction with ether (25 ml), the aqueous phase was adjusted to pH 4.0 with dilute hydrochloric acid and extracted with ether for 36 h in a continuous liquid–liquid extractor. The ether extract was dried with anhydrous magnesium sulfate, filtered, and evaporated to dryness on a rotary evaporator. The residue was extracted with petroleum ether (5 × 100 ml) and the extract evaporated to dryness. The residue was crystallized three times from petroleum ether to give colorless needles of fusaric acid, m.p. 101–102 °C.

Degradation of the Fusaric Acid

Dilutions with non-radioactive intermediates were made whenever the amounts available were too small for subsequent degradation steps.

Kuhn–Roth Oxidation of Fusaric Acid

Fusaric acid (123 mg) in water (2 ml) was added to a refluxing solution of chromium trioxide (0.8 g) in 10% sulfuric acid (14 ml). Distillation was started immediately. The volume of the reaction mixture was maintained by addition of distilled water through a dropping funnel. The distillate (130 ml) was titrated to pH 8.0 with 0.03227 *N* sodium hydroxide solution (20.1 ml) and evaporated to dryness. The residue was dissolved in water (5 ml), acidified (pH < 1) with sulfuric acid, and extracted with ether (4 × 5 ml). The ether extract, containing acetic and propionic acids, was dried, filtered, and evaporated to ca. 0.5 ml in an air stream at room temperature.

The mixture of acetic and propionic acids was separated by gas chromatography, with a copper column (6 ft × 0.25 in. outside diameter) packed with 20% neopentyl glycol succinate on 60–80 mesh firebrick treated with 2% phosphoric acid. The column temperature was 128 °C and the helium flow rate was 50 ml/min. The pure acids were collected by bubbling the exit gas through distilled water, and the solutions were then titrated to pH 8.0 with 0.03227 *N* sodium hydroxide solution (4.56 ml for propionic acid; 3.32 ml for acetic acid). The solutions were each evaporated to dryness *in vacuo*, the residues dissolved in absolute ethanol (10 ml), and the solutions evaporated to dryness again. Treatment with absolute ethanol was repeated twice, followed by final treatment with absolute ether (10 ml) and evaporation to dryness to yield the dry sodium salts of acetic and propionic acids.

Schmidt Reaction on Sodium Acetate or Sodium Propionate

The flask containing the dry sodium salt of the acid (0.1–0.15 mmole) was cooled in an ice bath, 100% sulfuric acid (0.1 ml) was added, and the contents were dissolved by rotating the flask. Sodium azide (15 mg) was added and the flask was connected to a gas train containing a 5% potassium permanganate in 5% sulfuric acid scrubber (to remove sulfur dioxide). The flask was flushed for 2 min with carbon dioxide free nitrogen, and a carbon dioxide trap of ethanolamine or barium hydroxide was attached after the permanganate scrubber. The nitrogen flow was stopped and the reaction flask was heated on a water bath at 75–80 °C for 1 h. The system was flushed with nitrogen for 15 min, after which the carbon dioxide trap was removed and analyzed for carbon dioxide and radioactivity.

Degradation of Methylamine from Schmidt Reaction on Sodium Acetate

A solution of 3% potassium permanganate (5ml) was added to the residue from the Schmidt reaction, followed by the addition of a 40% sodium hydroxide solution (0.2 ml). The flask was connected to the gas train, containing a fresh carbon dioxide trap, and was heated for 15 min on a boiling water bath. The system was flushed with nitrogen, the water bath removed, and the reaction mixture acidified with sulfuric acid to release carbon dioxide. After the system was flushed with nitrogen for 15 min, the carbon dioxide trap was removed and the contents were analyzed for radioactivity and carbon dioxide.

3-n-Butylpyridine

A flask containing fusaric acid (2.95 g) was connected to a reflux condenser and flushed with carbon dioxide free nitrogen. A gas train containing a dilute sulfuric acid scrubber and a carbon dioxide trap was connected after the condenser. The nitrogen flow was stopped, and the reaction flask heated at 200 °C on an oil bath. After 5 h the oil bath was removed and the system flushed with nitrogen for 10 min. The carbon dioxide trap was removed and the contents were analyzed for carbon dioxide and radioactivity. The condenser was set for distillation and the flask contents were distilled under reduced pressure to give 3-*n*-butylpyridine (2.04 g, 94%).

Nicotinic Acid

3-*n*-Butylpyridine (2.04 g), mixed with water (50 ml), was reacted with potassium permanganate (4 g). The potassium permanganate was added in 0.5 g portions over a 4 h period to the stirred solution. The temperature of the reaction mixture was raised to 40 °C, and additional portions of permanganate (4 g) were added after 12 and 24 h. After 48 h the excess permanganate was decomposed by the addition of methanol. The manganese dioxide was filtered off and thoroughly washed with hot water. The combined filtrates were evaporated to dryness, the residue was redissolved in water (25 ml), and the pH of the solution was adjusted to 8.0 with dilute hydrochloric acid. A saturated aqueous solution of cupric acetate (50 ml)

was added, and the resultant precipitate of cupric nicotinate was filtered off and washed with water. The copper salt was suspended in water and decomposed with hydrogen sulfide. The mixture was filtered and the filtrate evaporated to dryness. The residue was crystallized from ethanol to yield nicotinic acid (862 mg, 47%).

Decarboxylation of Nicotinic Acid

Nicotinic acid (35 mg) was refluxed with quinoline (1 ml) and copper chromite (40 mg) for 20 min in a stream of nitrogen. The liberated carbon dioxide was passed through a solution of 2 *N* sulfuric acid (to remove pyridine vapors), and then trapped in ethanolamine – methyl cellosolve or barium hydroxide solution. The yield of carbon dioxide in the reaction was 91% of the theoretical yield.

Degradation of the Pyridine Ring

Sequential degradation of the pyridine ring was accomplished by previously described methods (36, 37).

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