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Nuclear Magnetic Resonance (NMR) Studies on the Biosynthesis of Fusaric Acid from *Fusarium oxysporum* f. sp. *vasinfectum*

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Supporting Information

ABSTRACT: *Fusarium oxysporum* is a fungal pathogen that attacks many important plants. Uniquely pathogenic strains of *F. oxysporum* f. sp. *vasinfectum* were inadvertently imported into the United States on live cottonseed for dairy cattle feed. These strains produce exceptionally high concentrations of the phytotoxin fusaric acid. Thus, fusaric acid may be a critical component in the pathogenicity of these biotypes. This study investigated the biosynthesis of fusaric acid using ^{13}C-labeled substrates including [1,2-$^{13}C_2$]acetate as well as ^{13}C- and ^{15}N-labeled aspartate and [^{15}N]glutamine. The incorporation of labeled substrates is consistent with the biosynthesis of fusaric acid from three acetate units at C5−C6, C7−C8, and C9−C10, with the remaining carbons being derived from aspartate via oxaloacetate and the TCA cycle; the oxaloacetate originates in part by transamination of aspartate, but most of the oxaloacetate is derived by deamination of aspartate to fumarate by aspartase. The nitrogen from glutamine is more readily incorporated into fusaric acid than that from aspartate.

KEYWORDS: *Fusarium oxysporum* f. sp. *vasinfectum*, fusaric acid, cotton, *Gossypium hirsutum*, phytotoxin, biosynthesis, ^{13}C-incorporation, ^{15}N incorporation, ^{13}C−^{15}N coupling in pyridine, aspartase shunt pathway

INTRODUCTION

Fusarium oxysporum is a fungal pathogen that attacks more than 100 species of plants. The formae speciales *vasinfectum* (Atk.) Sny. and Hans is a pathogen of cotton (*Gossypium hirsutum*) that significantly reduces yield and fiber quality in the United States.[1,2] *Fusarium* species produce a number of mycotoxins including fusaric acid.[3] Fusaric acid is a phytotoxin to which cotton is especially sensitive.[4]

A genetically unique strain of the *F. oxysporum* f. sp. *vasinfectum* (Fov) was first recognized in wilted and dead Upland cotton seedlings in Australia in 1993.[5,6] Since that time the pathogen has spread rapidly despite strict containment practices. On some Australian farms where it was first discovered, losses >90% have forced cotton production to be abandoned. The Australian biotype was inadvertently introduced into the United States in at least two shiploads of cottonseed imported into California for dairy feed in 2001 and 2002.[7]

In our studies with an Australian Fov isolate, we found that it produces exceptionally high concentrations of fusaric acid. Because fusaric acid may contribute to the pathogenicity of these Fov biotypes, we thought it appropriate to determine its biosynthetic origin. Several investigators conducted experiments in the mid-1960s to answer this question using for the most part ^{14}C-labeled precursors.[8−10] Because biosynthetic studies using ^{14}C-labeled substrates can sometimes be difficult to interpret, we conducted experiments using ^{13}C- and ^{15}N-labeled compounds to establish the biosynthetic origin of fusaric acid. This study investigated the biosynthesis of fusaric acid using doubly labeled [1,2-$^{13}C_2$]acetate, as well as ^{13}C- and ^{15}N-labeled D,L-aspartate and L-[$^{15}N_2$]glutamine.

MATERIALS AND METHODS

Culture Preparation and Compound Isolation. A 200 μL aliquot of a conidial suspension (1.0×10^6 conidia/mL) of Fov (isolate 1089) was added to a 250 mL Erlenmeyer flask containing 50 mL of Czapek Dox broth with 0.8 ppm Zn^{2+} (35 g of CDB in 1.0 L of water with 8 mL of 10 ppm aqueous $ZnSO_4 \cdot 7H_2O$ solution). The culture was allowed to grow in an incubator/shaker set at 28 °C and 200 rpm. After 48 h, 0.5 mL of absolute ethanol was added to the culture to stimulate fusaric acid production, and 250 μL of culture was removed for analysis to determine the concentration of fusaric acid; this provided a baseline concentration of fusaric acid in the culture. The culture was placed back on the shaker and allowed to grow. The culture was sampled and the fusaric acid content determined at intervals of 6−12 h depending on the change in fusaric acid concentration as determined by HPLC. When the rate of change in fusaric acid content began to accelerate, a sterile substrate solution or sterile water (the latter included as a control) was added to the culture. The sterile substrate solution was prepared by dissolving a weighed substrate in water in a 30 mL test tube. The solution meniscus was marked on the tube, the solution was autoclaved for 15 min; then, after cooling, enough sterile water was added to bring the solution back up to the mark. The substrate solution was prepared such that 60 or 80 mg of substrate was added to the culture in 2 or 3 mL, respectively, of water. Thus, [1,2-$^{13}C_2$]acetate (60 mg/flask), D,L-[4-^{13}C]aspartic acid (99 atom % ^{13}C, 80 mg/flask), D,L-[2-^{13}C,^{15}N]aspartic acid (99 atom % ^{13}C; 98 atom % ^{15}N, 80 mg/flask), or L-[^{15}N]glutamine (98 atom % ^{15}N,

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Table 1. ^{1}H and ^{13}C NMR Chemical Shifts and the C−C Coupling Constants of Methyl Fusarate

C no.	proton (δ)	carbon (δ)	J_{C-C} (Hz)
2		144.9	J_{C11-C2} 77.4
3	8.17	124.5	J_{C3-C4} 54.7
4	7.75	138.5	J_{C3-C4} 54.7
5		143.1	J_{C5-C6} 55.9
6	8.70	147.6	J_{C5-C6} 55.9
7	2.71	32.7	ND[a]
8	1.62	32.9	ND[a]
9	1.34	22.2	J_{C9-C10} 34.7
10	0.90	13.7	J_{C9-C10} 34.7
11		165.2	J_{C11-C2} 77.4

[a] ND, not determined because the chemical shifts and coupling constants for these two carbon atoms are nearly identical, preventing coupling constant measurement.

80 mg/flask) was added in one portion. Three flasks were treated with substrate and three with sterile water (control). The six flasks were allowed to grow, and each flask was sampled at intervals of 6−12 h depending on the rate of fusaric acid production as determined by HPLC analysis. When the fusaric acid content was past the rapid production portion of the curve, the culture was removed from the incubator/shaker and held at 2 °C to reduce fungal growth until the cultures could be autoclaved to kill the fungus; this was done within 48 h. Autoclaving did not affect the fusaric acid concentration. The culture media were passed through filter paper (Whatman no. 4), and the filtrate was diluted with water and brine to 100 mL and acidified to pH 2−3 with concentrated H_3PO_4. The acidified filtrate was extracted four times with one-fifth its volume of chloroform, and the fusaric acid content in the aqueous layer was determined by HPLC. This first set of extractions usually recovered 80−85% of the fusaric acid in the original filtrate. The extraction was repeated twice more so that 95−99% of the fusaric acid was recovered. The $CHCl_3$ solution was reduced to dryness on a rotary evaporator under reduced pressure. This dried residue was washed five to eight times with boiling heptane. The combined heptane washes were reduced on the rotary evaporator, and the fusaric acid was crystallized from the heptane. This crude fusaric acid was recrystallized twice from heptane.

When glutamine was used as a substrate, 9,10-dehydrofusaric acid was also produced. The 9,10-dehydrofusaric acid was less soluble than the fusaric acid in the heptane. Thus, to separate these compounds the crude product was recrystallized three times from excess heptane; therefore, a significant amount of fusaric acid remained in solution with each recrystallization. This provided a product (16 mg) that was ∼90% fusaric acid and 10% 9,10-dehydrofusaric acid. This mixture of acids was methylated using a solution of (trimethylsilyl)diazomethane (Aldrich Chemical Co., St. Louis, MO) in methanol[11] and chromatographed on a short column of silica gel (15 g). The two compounds eluted from the column at almost the same rate, but the 9,10-dehydrofusaric acid methyl ester eluted in the first fractions, and later fractions contained 98% fusaric acid methyl ester (3 mg) that was used to determine the degree of incorporation of ^{15}N.

HPLC Analysis. An aliquot was placed in a 1.5 mL microfuge tube and centrifuged at 11000 rpm for 1 min, and a portion of the resulting clear supernatant was transferred to a HPLC vial. The supernatant was analyzed on an Agilent Technologies 1200 LC (Wilmington, DE) equipped with a diode array detector module [signal at 275 ± 10 nm, reference at 550 ± 50 nm, and spectra stored (190−600 nm)]. The column was a 250 mm × 4.6 mm i.d., 5 μm, Hypersil-C18 (Phenomenex, Torrance, CA) equipped with a column prefilter (Upchurch Scientific, Oak Harbor, WA) self-packed with a CO:PELL ODS (Whatman Inc., Clifton, NJ). The column was maintained at 40 °C, and the mobile phase was a gradient of water and methanol, both with 0.07% H_3PO_4. The gradient was linear, beginning at 25% MeOH and progressing to 100% MeOH over 4 min and then held at 100% MeOH for 7 min. Flow was 1.25 mL/min.

Chemical Analysis. NMR spectra were recorded on Bruker Avance 300 or Bruker Avance 500 spectrometers (Bruker Analytical, The Woodlands, TX) in $CDCl_3$. Mass spectra were recorded by a Direct Exposure Probe using a Thermo Electron Trace DSQ mass spectrometer (Thermo Electron Corp., Austin, TX). Commercially available fusaric acid (Aldrich Chemical Co., St. Louis, MO) was converted to its methyl ester[11] and subjected to ^{1}H NMR and ^{13}C NMR analysis (1D and 2D experiments) to unequivocally assign chemical shifts for all proton and carbon atoms. Analysis of ^{13}C NMR spectra obtained from the ^{13}C-labeled fusaric acid confirmed all chemical shift assignments. The hydrogen observed $^{1}H-^{15}N$ gradient HMBC experiment was performed with a single bond coupling for peak removal set to 90° and a long-range coupling constant for observation optimized for 12.5 Hz. Cross peaks were detected from the nitrogen to hydrogens on the adjacent carbons. Sweep width was 350 ppm in the nitrogen dimension. The identity of the 9,10-dehydrofusaric acid was established by mass spectrometric analysis [m/z (relative %) 191 (3.6), 161 (25), 135 (61), 133 (100), 121 (33), 90 (25)] and agreed with published ^{1}H NMR and ^{13}C NMR data.[12]

To determine the amount of labeled compound incorporated, the fusaric acid was converted into its methyl ester,[11] and the ^{13}C NMR spectra of all methyl esters were run under identical conditions. In the case of spectra of methyl fusarate derived by feeding [1,2-$^{13}C_2$]acetate, [^{15}N]aspartate, or [^{15}N]glutamine, the incorporation rate was calculated according to the method of McInnes et al.[13] When singly labeled ^{13}C precursors including D,L-[4-^{13}C]aspartate and D,L-[2-^{13}C,^{15}N]aspartate were fed, the peak areas of all methyl fusarate carbons were compared to that of the area of the methyl group in the methyl ester. This ratio was then used to determine the amount of label incorporated into each methyl fusarate site by reference to the natural abundance ^{13}C at the individual positions in unlabeled methyl fusarate compared to that of the natural abundance of the ^{13}C of the methyl group of the ester.

RESULTS AND DISCUSSION

NMR Analysis of Fusaric Acid. Detailed 1D- and 2D-NMR analysis was used to assign all proton and carbon chemical shifts (Table 1). In addition, we could measure some $^{13}C-^{13}C$ coupling constants when [1,2-$^{13}C_2$]acetate was fed to the cultures. Thus, the coupling constants for C_2-C_{11}, C_3-C_4, C_5-C_6, and C_9-C_{10} were measured and are reported in Table 1. Because the chemical shift difference between C_7 and C_8 is small compared to the magnitude of their coupling constant, the C_7-C_8 coupling constant was not determined. The percent ^{13}C incorporation and sites of incorporation for all experiments are shown in Table 2, and the percent ^{15}N incorporation is shown in Table 3.

Analysis of [1,2-$^{13}C_2$]Acetate Feeding Experiment. Specific incorporation was readily discernible in the methyl fusarate derived from feeding [1,2-$^{13}C_2$]acetate by the $^{13}C-^{13}C$ coupling observed in the ^{13}C NMR spectrum (see the Supporting Information). Coupling between C_5-C_6, C_7-C_8, and C_9-C_{10} was readily observed in the spectrum. Lower incorporation was also observed between C_2-C_{11} and C_3-C_4. Other than these carbon−carbon coupling constants, no other carbon−carbon coupling was obvious in the $^{13}C-^{13}C$ COSY spectrum. This confirmed that acetate was incorporated intact between C_5-C_6, C_7-C_8, and C_9-C_{10} (Figure 1), as well as between C_2-C_{11} and C_3-C_4. The higher percent enrichment at C_5-C_6, C_7-C_8, and C_9-C_{10} compared to that at C_2-C_{11} and C_3-C_4 is consistent with the six-carbon chain C-5 to C-10 being derived via a polyketide synthase. The lower percent enrichment at C_2-C_{11} and C_3-C_4

Table 2. Percent ^{13}C Incorporation of [1,2-$^{13}C_2$]Acetate, D,L-[4-^{13}C]Aspartate, and D,L-[2-^{13}C,^{15}N]Aspartate into Methyl Fusarate

	% ^{13}C enrichment[a]	% ^{13}C enrichment[b]	
C no. FA	[1,2-$^{13}C_2$]acetate	[4-^{13}C]aspartate	[2-^{13}C,^{15}N]aspartate
2	–[c]	–	8.8
3	–	–	5.7
4	–	9.3[d]	2.6
5	C_5–C_6 8.9	–	–
6	C_5–C_6 9.0	–	–
7	ND[e]	–	–
8	ND	–	–
9	C_9–C_{10} 9.5	–	–
10	C_9–C_{10} 7.2	–	–
11	–	4.6[f]	2.2

[a] Calculated by integrating the total peak area and subtracting the area for the naturally occurring ^{13}C as determined in unlabeled methyl fusarate. [b] Percent enrichment calculated according to the method of McInnes et al.;[13] for ^{15}N incorporation see Table 3. [c] Enrichment of 1.4% or less. [d] Derived by transamination and aspartase. [e] Not determined; chemical shifts are nearly equal for C-7 and C-8, and individual percent enrichment could not be calculated. [f] Derived only from aspartase.

Table 3. Percent ^{15}N Enrichment[a] in Methyl Fusarate from Feeding D,L-[2-^{13}C,^{15}N]Aspartate and L-[^{15}N]Glutamine

	% ^{15}N enrichment	
C no. FA	[2-^{13}C,^{15}N]aspartate	[^{15}N]glutamine
2	0.6	2.0
3	0.5	2.1
4	0.7	2.2[b]
5	0.4	2.1
6	0.3	2.2
7	–	–
8	–	–
9	–	–
10	–	–
11	0.6	2.5

[a] Calculated according to the method of McInnes et al.[13] [b] Natural abundance peak overlapped with ^{13}C satellite peak; value was determined by integration rather than intensity.

is consistent with these positions being derived via the tricarboxylic acid cycle (TCA) (see the Supporting Information), where acetate is incorporated into oxaloacetate at C_1–C_2 and C_3–C_4. This is consistent with enrichment at C_2–C_{11} and C_3–C_4 in fusaric acid from oxaloacetate during incorporation of acetate into citrate.

Analysis of D,L-[4-^{13}C]Aspartate Feeding Experiment. To further explore the possible involvement of the TCA cycle in the biosynthesis of fusaric acid, D,L-[4-^{13}C]aspartate was fed to the cultures. In the NMR spectrum (see the Supporting Information), enrichment was observed only at C-4 (9.3%) and C-11 (4.6%) (Table 2). Enrichment at C-11 indicates at least some aspartate was not converted directly to fusaric acid, and subsequent

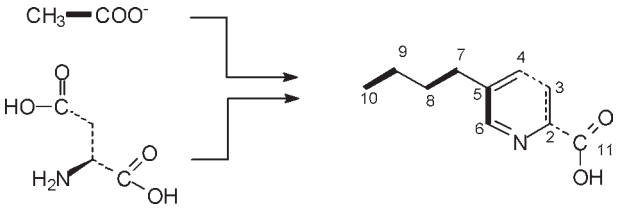

Figure 1. Biosynthesis of fusaric acid from [1,2-$^{13}C_2$]acetate and [2-^{13}C]aspartate.

experiments described below indicate there is little or no intact conversion of aspartate into fusaric acid. Thus, the high enrichment at C-4 in fusaric acid is consistent with the conversion of aspartate to [4-^{13}C]oxaloacetate via transamination with α-ketoglutarate. Enrichment of [4-^{13}C]oxaloacetate into fusaric acid by this pathway will provide a label only at C-4 in fusaric acid. That is, the first turn of the TCA cycle leads to loss of the ^{13}C from [4-^{13}C]oxaloacetate as $^{13}CO_2$, and no enrichment would be observed at C-4 or C-11 in fusaric acid. Thus, incorporation at C-11 indicates that an additional alternative pathway such as intervention of aspartase (see the Supporting Information) must be operating.[14] Aspartase converts aspartate to fumarate with elimination of ammonia; this alternative pathway leads to scrambling of C-1 and C-4 in the resulting oxaloacetate, giving an equal distribution of label at C-4 and C-11 in fusaric acid. If this same oxaloacetate enters the TCA cycle, labels are lost from both positions as CO_2. The observation of label exclusively at C-4 and C-11 with higher enrichment at C-4 strongly supports the involvement of these two pathways. The conversions to explain the enrichments are diagramed at the top of Figure 2. Thus, transamination of [4-^{13}C]aspartate leads to [4-^{13}C]fusaric acid (percent B in Figure 2), whereas aspartase leads to both [4-^{13}C]fusaric acid and [11-^{13}C]fusaric acid in equal concentrations (percent *A*), and the ratio between labeled fusaric acid production via transamination and aspartase is 4.7 to 9.2.

Analysis of D,L-[2-^{13}C,^{15}N]Aspartate Feeding Experiment. To delve more deeply into the role of TCA products in this biosynthesis, D,L-[2-^{13}C,^{15}N]aspartate was added as substrate. The ^{13}C observed ^{15}N coupled spectra (see the Supporting Information) provide the percent ^{15}N enrichment as calculated by I_S/I_C, where I_S is the intensity of the two satellite peaks due to coupling between the ^{15}N and ^{13}C and I_C is the intensity of the "central" ^{13}C peak (Table 3).[13] If [2-^{13}C,^{15}N]aspartate were incorporated intact into fusaric acid, the satellite peaks for C-2 would be significantly greater than those for the other pyridine ring carbons. However, the percent enrichment was nearly equal at all positions; thus, there was no indication of intact incorporation of ^{15}N and ^{13}C from aspartate. This indicates that little if any aspartate is directly converted into fusaric acid. Thus, the organism appears to use nitrogen from the aspartate, but the nitrogen is first lost from aspartate and then used in subsequent biosynthetic steps.

Incorporation of the ^{13}C from [2-^{13}C, ^{15}N]aspartate proved to be quite revealing. The highest enrichments were at C-2 (8.8%) and C-3 (5.7%) with almost equal enrichments at C-4 (2.6%) and C-11 (2.2%) (Table 2). The conversions to explain the enrichments are diagramed in the middle of Figure 2 (these diagrams and equations assume there is no intact incorporation of aspartate into fusaric acid). Thus, transamination of [2-^{13}C]aspartate leads to [2-^{13}C]oxaloacetate, which on conversion to [2-^{13}C]fusaric acid gives enrichment percent *W* (Figure 2). The [2-^{13}C]oxaloacetate derived by this pathway that enters the TCA cycle leads to

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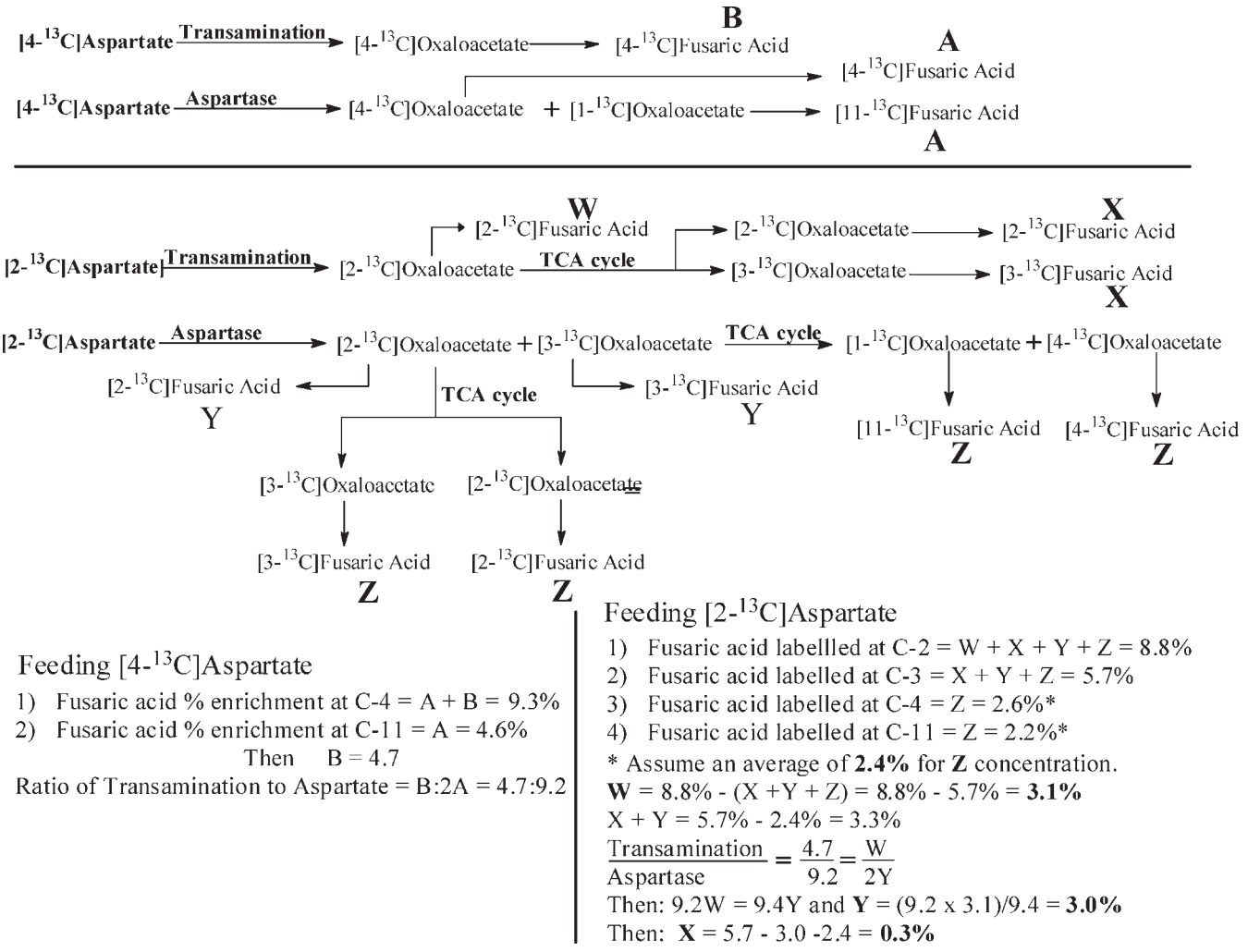

Figure 2. Proposed steps to account for the incorporation of ^{13}C into fusaric acid when Fov is fed [4-^{13}C]aspartate (top) or [2-^{13}C]aspartate (middle).

equal incorporation to give [2-^{13}C] and [3-^{13}C]oxaloacetate, which gives equal amounts of [2-^{13}C] and [3-^{13}C]fusaric acid with enrichment percent *X*. The transformation of [2-^{13}C]aspartate via aspartase gives equal enrichment to [2-^{13}C] and [3-^{13}C]oxaloacetate. This pool of labeled oxaloacetate may differ from the labeled oxaloacetate derived from transamination. Direct conversion of this [2-^{13}C] and [3-^{13}C]oxaloacetate to [2-^{13}C] and [3-^{13}C]fusaric gives equal incorporation at [2-^{13}C] and [3-^{13}C]-fusaric acid with enrichment percent *Y*. Finally, conversion of [2-^{13}C]oxaloacetate via the TCA cycle from this same pool gives equal incorporation at [2-^{13}C] and [3-^{13}C]fusaric acid (enrichment percent *Z*), but the [3-^{13}C]oxaloacetate in this pool gives an equal incorporation at both [4-^{13}C] and [11-^{13}C]fusaric acid via the TCA cycle (enrichment percent *Z*). Again, *Z* and *X* may be different. This leads to development of eqs 1−4 shown at the bottom right of Figure 2. Thus, the [11-^{13}C] and [4-^{13}C]fusaric acid derived from the TCA cycle is derived exclusively from [3-^{13}C]oxaloacetate and is equal to ~2.4% (i.e., average percent enrichment at C-4 and C-11 from Table 1), and *Z* = 2.4%. Knowing the percent enrichment for *Z* gives a percent enrichment of 3.1% for *W* [$W = 8.8\% - (X + Y + Z) = 8.8\% - 5.7\% = 3.1\%$].

Calculating Fusaric Acid Produced via Transamination or Aspartase. Total productions of fusaric acid were very similar in experiments when either D,L-[4-^{13}C]aspartate or D,L-[2-^{13}C, ^{15}N]aspartate was fed to the cultures (Figure 3). If one assumes that the ratio of transamination versus aspartase is similar for the two experiments, then it is possible to solve for enrichment percentages *X* and *Y* in Figure 2. That is, in the experiment with [4-^{13}C]aspartate the percent enrichments *A* and *B* are due to direct conversion of oxaloacetate to fusaric acid (i.e., any labeled oxaloacetate entering the TCA cycle is lost), and *A*/2*B* is equal to 4.7/9.2. This ratio should be equal to the corresponding ratio of *W*/2*Y* in the [2-^{13}C,^{15}N]aspartate experiment, which represents the ratio for direct conversion of oxaloacetate to fusaric acid by transamination (i.e., not considering any conversion via the TCA cycle) versus the direct conversion by aspartase (again, no conversion via the TCA cycle). Thus, *A*/2*B* = *W*/2*Y* = 4.7/9.2. Substituting 3.1% for the value of *W* allows one to calculate the values for *X* and *Y* (i.e., *X* = 0.3% and *Y* = 3.0%).

Analysis of L-[^{15}N]Glutamine Feeding Experiment. To further investigate ^{15}N incorporation, L-[^{15}N]glutamine was fed to the fungus. The percent ^{15}N enrichment from this experiment as determined from the $^{15}N-^{13}C$-coupled ^{13}C observed spectra from the I_S/I_C ratio[13] is shown in Table 3. Significant enrichment was observed, with an approximately 4-fold higher enrichment than attained when [2-^{13}C,^{15}N]aspartate was used. Therefore, glutamine is a more immediate provider of ^{15}N than aspartate. This also agrees with our conclusion that aspartate is not converted intact

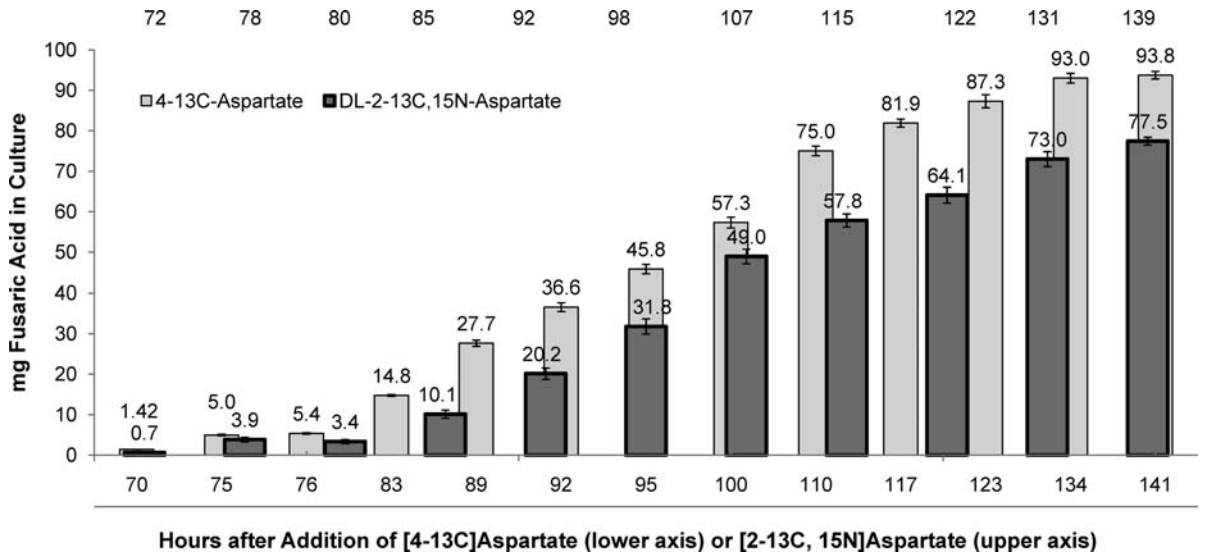

Figure 3. Mean production of fusaric acid (mg of fusaric acid/50 mL of culture) from three experiments after the addition of [2-^{13}C,^{15}N]aspartate or [4-^{13}C]aspartate (± standard errors).

into fusaric acid. In addition, these cultures also produced, 9,10-dehydrofusaric acid in small but significant amounts compared to cultures grown without glutamine.

In conclusion, the results of these feeding studies are consistent with the hypothesis that fusaric acid is biosynthesized via a polyketide synthase gene and an amino acid kinase gene cluster in Fov. In fact, we have recently identified a PKS and amino acid kinase gene cluster in the Australian Fov biotype. The percent ^{15}N enrichment when [2-^{13}C,^{15}N]aspartate was fed to the cultures indicates that little if any aspartate is converted intact into fusaric acid but rather is converted to oxaloacetate. Approximately half of the labeled oxaloacetate is converted directly into fusaric acid (47%), whereas the remaining originates from oxaloacetate that has completed one turn of the TCA cycle. Furthermore, only ~20% of the oxaloacetate converted to fusaric acid is derived from transamination. Finally, glutamine is a major participant in contributing nitrogen to the pool from which fusaric acid is derived.

These results confirm and extend results from previous investigators.[8−10] That is, they demonstrate that new pathways are operating in Fov in the conversion of aspartate to oxaloacetate, reveal relative product ratios derived from different pathways, and conclusively prove that the nitrogen in fusaric acid is derived from an endogenous pool to which both aspartate and glutamine contribute, with the latter making a greater contribution.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figure 1, a color version of the TCA cycle, and Figures 2−6, ^{13}C NMR spectra. This material is available free of charge via Internet at http://pubs.acs.org.

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