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Transformation of *Fusarium verticillioides* with a polyketide gene cluster isolated from a fungal endophyte activates the biosynthesis of fusaric acid

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Abstract

A large number of bioactive natural products have been isolated from plant endophytic fungi. However, molecular mechanisms for the biosynthesis of these metabolites have lagged behind because genetic and biochemical studies are difficult to perform within many of the endophytes. In this work, we describe our attempt to express a putative mycoepoxydiene (MED) biosynthetic gene cluster in *Fusarium verticillioides*, which has a well-developed genetic system for the study fungal polyketide biosynthesis. MED was isolated from *Phomopsis* sp. A123, a fungal endophyte of the mangrove plant, *Kandelia candel*. It has several unusual structural features and interesting biological activities. Integration of this *Phomopsis* gene cluster into the *F. verticillioides* genome led to the biosynthesis of multiple metabolites. The most highly activated metabolite was isolated and its structure was shown by 1D- and 2D-NMR to be fusaric acid, which is a mycotoxin in *Fusarium* species and is implicated in fungal pathogenesis. Although fusaric acid was isolated more than 70 years ago, its biosynthetic mechanism remains unclear. These transformants produced 30–35 mg fusaric acid per 100 ml culture. The high level production of fusaric acid will greatly facilitate the genetic and biochemical study of its biosynthetic mechanism. Although we have not detected MED or its analogs from the heterologous host, this work represents the first attempt to express a fungal endophytic gene cluster in a *Fusarium* species.

Keywords: endophyte, fusaric acid, biosynthesis, polyketide, *Fusarium verticillioides*, *Phomopsis*

Introduction

Plant endophytes represent a huge unexploited resource for new bioactive natural products (Strobel and Daisy 2003; Zhang et al. 2006). It is estimated that each of the ~300,000 plant species on the earth has at least one endophyte. Especially, the endophytes from organisms that occupy a special biological niche in the ecosystem, such as those from mangrove plants, are more likely to produce unusual natural products with new chemistry and new modes of action. We previously isolated a fungal endophyte, *Phomopsis* sp. A123, from the coastal mangrove plant, *Kandelia candel* (Lin et al. 2005; Wang et al. 2010). This endophyte is an outstanding source of new natural products. So far, we have isolated dozens of compounds from this strain. One of the compounds is mycoepoxydiene (MED), which was also found in other endophytes and possesses several rare structural features, including a cyclooctadiene with an oxygen bridge and an α,β -unsaturated δ -lactone (Cai et al. 1999; Takao et al. 2002, 2004; Prachya et al. 2007). MED exhibits a strong AMPK-activating activity and antitumor activity (Wang

et al. 2010). Owing to the interesting chemical structure and the biological activities of MED, we sought to study its biosynthetic mechanism and, as a first step, we identified a putative biosynthetic gene cluster responsible for MED.

However, genetic manipulation in the MED producer, *Phomopsis* sp. A123, has proven challenging, because the mycelia cells of this fungus contain multiple nuclei. This renders the gene knockout approach impractical. As a way around this problem, we transferred a cosmid clone (Cos711) that hosts the entire gene cluster into *Fusarium verticillioides*, which is a relatively user-friendly fungus. We have been using this fungus as a model system to study the biosynthetic mechanisms for fungal natural products, especially polyketides (Zhu et al. 2007, 2008; Du et al. 2008; Li et al. 2009; Huffman et al. 2010). Our initial intention was to use the heterologous expression approach to establish if the endophytic gene cluster is relevant to MED biosynthesis.

This paper describes the findings from the heterologous expression of the *Phomopsis* polyketide gene cluster in *F. verticillioides*. The results showed that integration of the endophytic genes into the chromosomes of *F. verticillioi-*

des activated the production of a number of metabolites which were silent in the wild type strain of *F. verticillioides*. We have further determined the structure of one of the major metabolites, which turned out to be fusaric acid (5-butylpicolinic acid). Fusaric acid is a mild mycotoxin found in many *Fusarium* species that contaminate foods and feeds (Bacon et al. 1996). It is a safety concern due to its potential synergistic effect with other more toxic *Fusarium* mycotoxins, such as fumonisins (Bacon et al. 1995; Smith et al. 1997). In addition, fusaric acid is implicated as a pathogenesis factor for *F. oxysporum* f. sp. *lycopersici* to cause tomato wilt symptoms. Despite the importance of the mycotoxin, its biosynthetic mechanism remains unclear. High level constitutive production of fusaric acid in the transformants will facilitate the study of its biosynthetic mechanism.

Materials and methods

Microbial strains, media and chemicals

Phomopsis sp. A123 was maintained on potato dextrose agar (PDA) slants containing 50% (v/v) sea water. Stock culture was transferred onto new PDA slants and allowed to develop for 7 days at 28°C. *Fusarium verticillioides* A0149 (FGSC number 7600) was the wild-type strain. Transformants and the wild type were maintained on V8 medium for conidiospore production or on YPD medium for mycelia growth (Proctor et al. 1999). All oligonucleotide primers for PCR were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Taq DNA polymerase and other molecular biology reagents were purchased from Promega (Madison, WI, USA). Hygromycin B was purchased from Calbiochem (La Jolla, CA, USA). Other chemicals were purchased either from Fisher Scientific (Springfield, NJ, USA) or Sigma-Aldrich (St. Louis, MO, USA).

Protoplasts preparation and transformation of *F. verticillioides*

F. verticillioides was grown on V8 solid medium at 28°C for 5 days to produce spores, as described (Yu et al. 2005). The spores were washed off from the plates using sterilized dd-H₂O and were subsequently inoculated into 50 ml YPD liquid medium. The liquid culture was grown at 28°C, 200 rpm for 7–8 h until the germinated hyphae were around 3–6 conidial diameters in length. The germlings were collected by centrifugation at 3000 rpm (1076 × *g*) at 4°C for 15 min and were resuspended in 25 ml of 0.7 M NaCl solution. The germlings were then washed with 12 ml of 0.7 M NaCl solution for the second time. The washed germlings were resuspended in an enzyme solution containing 0.5% Novozyme, 2.5% Driselase and 0.05% Chitinase in 0.7 M sodium chloride and incubated at 30°C, 100 rpm for 30 min to 1 h. The protoplasts released from the

germlings were collected and resuspended in 200–500 µl STC buffer (Yu et al. 2005). The protoplasts were diluted to 1 × 10⁸ /ml with STC/SPTC/DMSO (8:2:0.1, v/v).

To transform the protoplasts, 10 µg DNA (Cos711) dissolved in 100 µl STC buffer was mixed with 100 µl of protoplasts and 50 µl of 30% PEG 8000. After incubation at room temperature for 20 min, the mixture was added with 2 ml 30% PEG and continued to incubate at room temperature for another 5 min. Then, 4 ml STC and 45 ml regeneration medium were added to the mixture, which was poured onto plates. The plates were incubated at 28°C for 20 h, and then 15 ml of 1% water agar supplemented with 150 µg/ml hygromycin B was overlaid to each of the plates. After two more days of growth, another 15 ml of 1% water agar supplemented with 300 µg/ml hygromycin B was overlaid again. Single colonies that appeared on the second layer were individually transferred to liquid YPD medium containing 300 µg/ml hygromycin B and allowed to grow for 3 days.

Screening of *F. verticillioides* transformants

F. verticillioides colonies that showed a resistance to 300 µg/ml hygromycin B were screened by diagnostic PCR to identify true transformants containing an integrated cosmid (Cos711). Two sets of PCR reactions were carried out, one to amplify the hygromycin B resistance gene and the other to amplify the *PKS2* gene within Cos711. Genome DNA of the single colonies growing in the YPD liquid medium was extracted. One pair of primers, HYGFOR (5'-ACA GTT AAA TTG CTA ACG CAG-3') and HYG-REV (5'-ATC GGT CAA TAC ACT ACA TGG-3'), were used to amplify the hygromycin B resistance gene, and the other pair of primers, Pa₁ (5'-TGA AGA CGA ACC CGA ACC CGA TGC-3') and Ps₁ (5'-TTG CCC AGC CAA ACT CC-3') were used to amplify the *PKS2* gene. For both reactions, purified Cos711 served as the template of the positive control.

Extraction and isolation of metabolites from the transformants

After the true transformants were identified, they were transferred to potato dextrose agar (PDA) medium and allowed to grow at 28°C for 28 days. The untransformed wild type strain was also similarly transferred to serve as a control. The cultures were extracted with MeOH three times and the organic phase was combined and concentrated with a rotary evaporator. The crude extract was redissolved in 4 ml methanol. A 100-µl aliquot was injected into a HPLC column (Alltima C18LL, 250 × 4.6 mm, 5 µm) to analyze the metabolites in the extract. The HPLC system was ProStar model 210 (Varian, Walnut Creek, CA, USA), with a detection wavelength of 220 nm and a flow rate of 1 ml/min. The mobile phases were water/trifluoroacetic acid (TFA; 100:0.1, v/v) (mobile phase A) and

acetonitrile/TFA (100:0.1, v/v) (mobile phase B) with a gradient of 5–20% of phase A in phase B from 0 to 5 min, 20–80% from 5 to 25 min, 80–100% from 25 to 27 min, 100% from 27 to 29 min, 100–5% from 29 to 30 min, 5% from 30 to 35 min.

To isolate the major metabolite (fusaric acid) from the extract, a total of 100 mg crude extract from 100 ml of solid culture was loaded onto a C₁₈ reversed-phase cartridge (10 g, RP-18). The cartridge was eluted stepwise with 100 ml of H₂O containing 0, 30, 50, 70, and 100% MeOH. This yielded five fractions, Fr-S1 to Fr-S5. Fr-S1 (8 mg) was subjected to HPLC analysis, which showed that it contained one major component (R_t = 11.57 min).

Results and discussion

Integration of the endophytic PKS gene cluster into the genome of *F. verticillioides*

During our study of the biosynthesis of MED, we obtained a cosmid clone, Cos711, which contains two polyketide synthase (PKS) genes in addition to a number of genes encoding tailoring enzymes. Cos711 was obtained from a genomic library of *Phomopsis* sp. A123 using a specific PKS fragment as probe. This specific PKS fragment was the only PKS identified from a suppression subtractive hybridization (SSH) library (Wang 2008). This gene's high level expression paralleled the high yield of MED in the "driver strain", but not in the low yield "tester strain" of *Phomopsis*. Thus, this PKS gene is possibly relevant to MED biosynthesis.

To obtain more direct evidence, we transferred the cosmid clone into *F. verticillioides*. Colonies that showed resistance to hygromycin B on the initial YPD plants were transferred to YPD liquid medium containing 300 µg/ml hygromycin B to ensure the colonies have true resistance. Among them, four putative transformants were subjected to PCR assay using primers HYG-FOR/HYG-REV and primers Pa₁/Ps₁. The first pair of primers was designed to detect the presence of a hygromycin B-resistant gene within the cosmid vector, and the expected size of the PCR product is 1220 bp. As shown in Figure 1, a band at 1220 bp was amplified from the genomic DNA of the transformant, which was identical to that from the positive control. The second pair of primers was designed to detect the presence of the *PKS2* gene within the biosynthetic gene cluster, and the expected size of the PCR product is 230 bp. As shown in Figure 1, a band at 230 bp was amplified from the genomic DNA of the transformant, which was identical to that from the positive control. These results showed that true transformants carrying Cos711 in the genome have been obtained.

Transformants produced multiple metabolites that were absent in wild type *F. verticillioides*

Three independent transformants were subjected to metabolite analysis by HPLC. Under the conditions used,

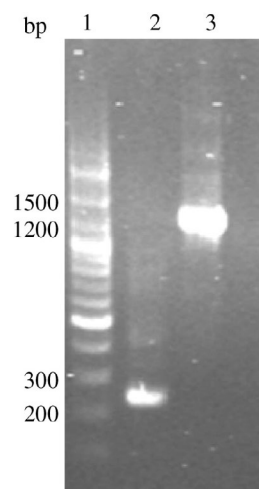


Figure 1. Screening of transformants by PCR. **Lane 1:** DNA size markers. **Lane 2:** fragment (expected size 230 bp) amplified from *PKS2* gene using primers Ps₁/Pa₁, with the genomic DNA of transformant as template. **Lane 3:** fragment (expected size 1220 bp) amplified from the hygromycin B-resistant gene using primers HYG-FOR/HYG-REV, with the genomic DNA of transformant as the template.

the wild-type strain produced very few metabolites (Figure 2, bottom trace). In contrast, the three transformants gave four to eight new peaks that did not appear in the wild type (Figure 2, top three traces). The results indicate that the biosynthesis of a number of metabolites was activated by the introduction of the endophytic genes. Since the transformants were obtained through a random insertion of the *Phomopsis* PKS gene cluster into *F. verticillioides* genome, it is likely that the transformants were independent from each other (the genes integrated into a different location of the genome). Despite this, the general metabolite profile on HPLC was similar among the transformants. This suggests that the integration of the endophytic PKS gene cluster into the genome of *F. verticillioides* may have a common impact on the biosynthesis of these new metabolites. Among the metabolites was a peak (**Y10-D1**) with a retention time of 11.57 min, which was the dominant peak in all transformants (Figure 2). The transformants gave 30–35 mg of **Y10-D1** per 100 ml solid culture, whereas the wild type had no detectable **Y10-D1** under the same conditions. This is a significant activation of the biosynthesis of this metabolite in this filamentous fungus.

Fusaric acid was the major metabolite produced by the transformants of *F. verticillioides*

To evaluate the effect of the endophytic genes on the metabolite biosynthesis in the host, we set out to identify the nature of the metabolites. When a standard sample of MED was used in the HPLC, it did not co-migrate with any of the peaks. We then purified the major peak (**Y10-D1**) and determined its structure using spectroscopic methods (Table 1).

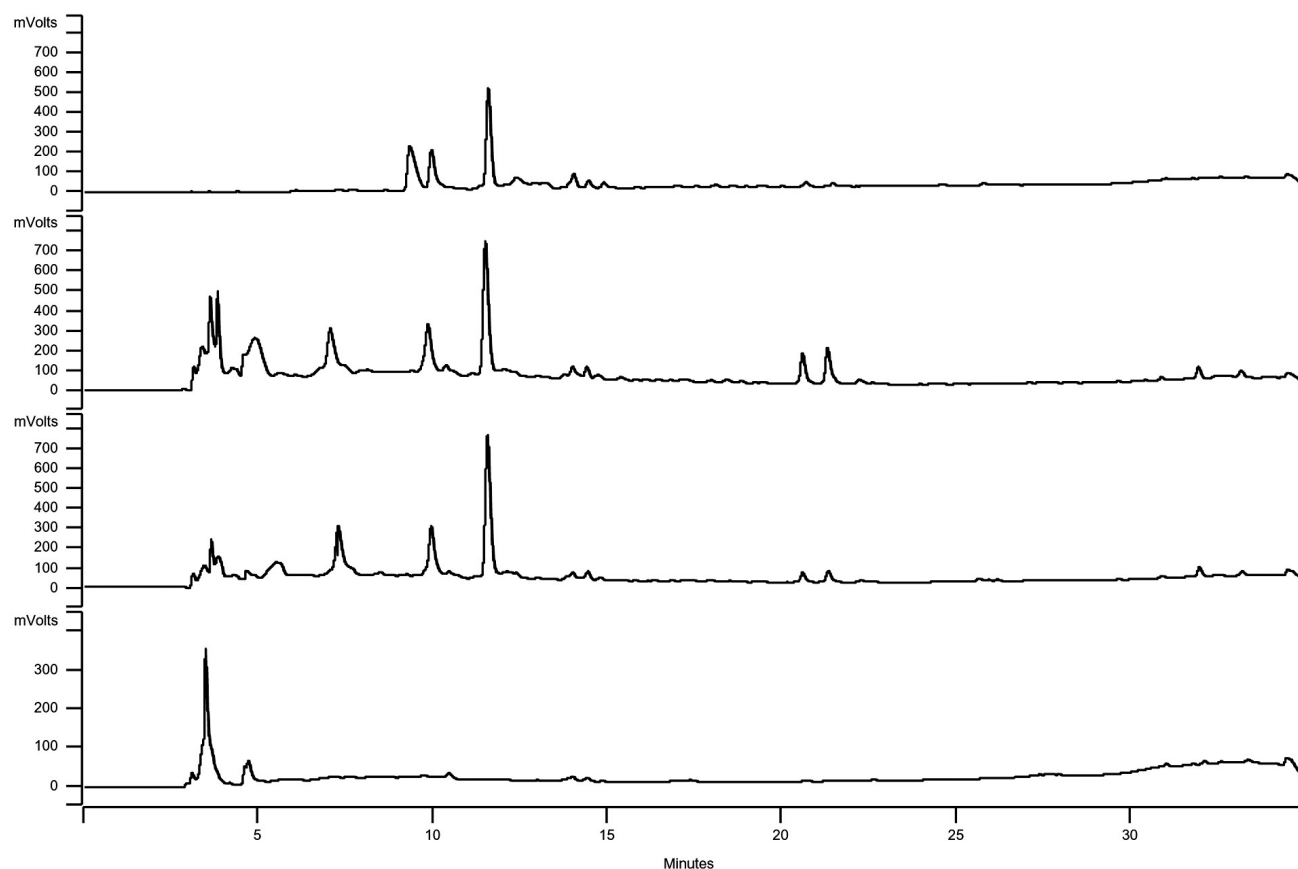
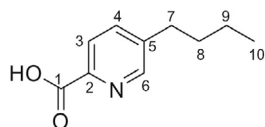


Figure 2. HPLC analysis of metabolites from the wild type (bottom trace) and three independent Cos711 transformants (top three traces) of *F. verticillioides*. The major peak in the three transformants was isolated for structural determination.

Table 1. ^1H -NMR (500 MHz) and ^{13}C -NMR (125 MHz) data for **Y10-D1**.

No.	δH (mult., J in Hz)	δC	HMBC	^1H - ^1H COSY
1		166.5s		
2		146.4s		
3	8.30(d,8.0)	125.5d	143.1s, 166.5s	H-3
4	8.22(d,8.0)	138.5d	32.7t, 146.4s	H-4
5		143.1s		
6	8.64(s)	147.3d	138.5d, 143.1s, 146.4s	H-7
7	2.88(t,7.5,8.0)	32.7t	21.8t, 32.7t, 138.5d, 143.1s, 147.3d	H-6, H-8
8	1.74(quintet,7.5)	33.3t	12.5q, 21.8t, 32.7t, 143.1s	H-7, H-9
9	1.46(sextet,7.5)	21.8t	12.5q, 33.3t	H-8, H-10
10	1.01(t,7.0,7.5)	12.5q	21.8t, 32.7t, 33.3t	H-10



Numbering of carbons in the proposed structure of **Y10-D1**.

Compound **Y10-D1** was obtained as a colorless oil, whose molecular formula was determined as $\text{C}_{10}\text{H}_{13}\text{NO}_2$ by HR-ESI-MS data with m/z 180.0115 ($[\text{M} + \text{H}]^+$) and 197.0384 ($[\text{M} + \text{NH}_4]^+$). From the ^1H -NMR and HSQC spectra, three methine, three methylene and one methyl groups were observed. DEPT experiments showed the existence of three quaternary carbons at δ 143.1s, 146.4s and 166.5s, respectively. The presence of a 5-substituted 2-pyridinecarboxylic acid moiety was indicated on the basis of the HR-ESI-MS data, ^1H - ^1H COSY ($J = 8.0$ Hz) between H-C(3) and H-C(4), and HMBC correlations from H-C(3) to C(1) and C(5), HC(4) to C(2) and C(7), and H-C(6) to C(2), C(4) and C(5). The presence of a *n*-butyl moiety was revealed by ^1H - ^1H COSY experiments and the HMBC correlations from protons of the only methyl group HC(9) and three methylenes to corresponding carbons. Moreover, the HMBC correlations from H-C(7) to C(4), C(5) and C(6) indicated the linkage of *n*-butyl group at C(5) of 2-pyridinecarboxylic acid. Therefore, the structure of **Y10-D1** was determined to be 5-*n*-butyl 2-pyridinecarboxylic acid, namely fusaric acid (Figure 3), a known metabolite from *Fusarium* fungi (Bacon et al. 1996).

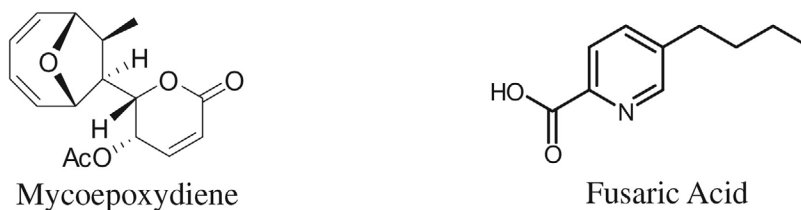


Figure 3. Structures of MED and Y10-D1, which was determined to be fusaric acid in this work.

Fusaric acid is a widespread mycotoxin in *Fusarium* species and is implicated in fungal pathogenesis (Bacon et al. 1996). Although it was isolated more than 70 years ago (Yabuta et al. 1937), the molecular mechanism for its biosynthesis remains unclear. One of the reasons for this situation is that fusaric acid is produced only in certain conditions; for example, in maize kernel media but not in a regular PDA medium. It is inconvenient to perform genetic and biochemical manipulations on maize kernels due to the uncertainty of the seed ingredients and the difficulty in isolating DNA/proteins from fungi growing on the seeds. Our results showed that the transformants constitutively express a high level of fusaric acid in PDA medium. This will enable future manipulation of putative fusaric acid biosynthetic genes in the strains growing in regular media and greatly facilitate the genetic and biochemical study of fusaric acid biosynthetic mechanism.

Previous feeding experiments using ^{14}C -labeled acetate and aspartate as substrates supported an “aspartate–polyacetate” hypothesis for fusaric acid biosynthesis (Hill et al. 1966). Based on the labeling pattern, we propose that the biosynthetic mechanism for fusaric acid involve a hybrid system of polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS), which assembles the “aspartate–polyacetate” precursor of fusaric acid, as shown in Figure 4.

In addition to fusaric acid, several other metabolites were produced in *F. verticillioides* resulted from the integration of the *Phomopsis* polyketide gene cluster. This suggests that multiple biosynthetic pathways in *F. ver-*

ticillioides have been activated. We are currently in the process of identifying the structure of these metabolites. In this study, we used a hygromycin-resistant gene as the selective marker for fungal transformation. It is more likely that the activation of the new metabolites was due to the endophytic genes rather than the resistance gene, because this resistance gene had been used previously to transform *F. verticillioides* and we had not observed the production of fusaric acid (Yu et al. 2005; Zhu et al. 2007, 2008; Li et al. 2009). Our initial purpose was to test the feasibility of *F. verticillioides* as a host for genetic manipulation of endophytic genes. Although we have not detected MED or its analogs from the heterologous host, this work represents the first attempt to express a fungal endophytic gene cluster in a *Fusarium* species. The reason for failure of producing MED in the transformants is not clear at this moment, but several possibilities exist. One could be that the *Phomopsis* PKS gene cluster did not contain the entire regulatory genes/elements needed for the expression of the biosynthetic genes. Another may be that this PKS gene cluster produced precursors or intermediates of MED which were not detected or among those new peaks on HPLC. The *Phomopsis* genes may have integrated into a region in the host *Fusarium* genome, where a negative regulator for the biosynthesis of a number of metabolites, including fusaric acid, was located. This may have led to inactivation of the negative regulator and, consequently, activation of the metabolite biosynthetic genes. Further experiments are needed to answer these questions.

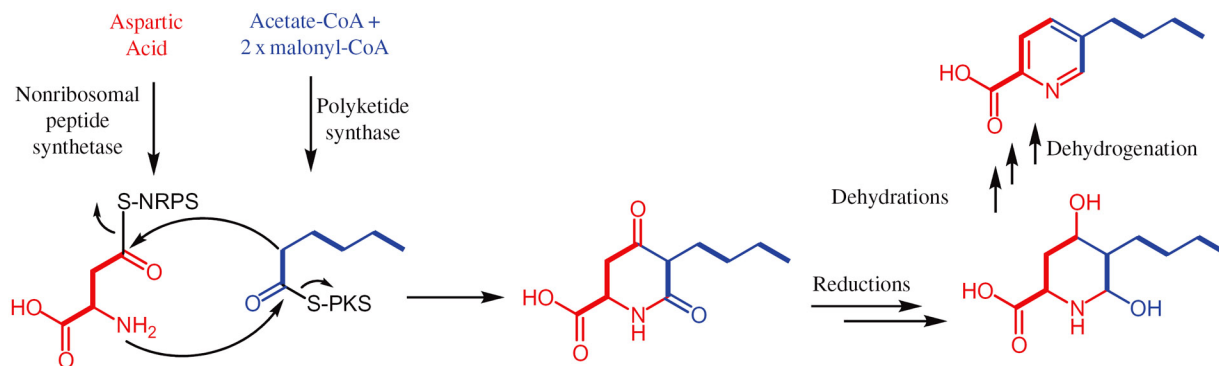


Figure 4. Proposed biosynthetic pathway for fusaric acid.

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