

Biosynthetic Pathway of Citrinin in the Filamentous Fungus *Monascus ruber* as Revealed by ^{13}C Nuclear Magnetic Resonance

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Carbon isotope distribution of [^{13}C]citrinin from *Monascus ruber* incubated with [^{13}C]acetate revealed that the biosynthesis of the toxin originated from a tetraketide, instead of a pentaketide as has been shown for *Penicillium* and *Aspergillus* species. The production of polyketide red pigments and citrinin by *M. ruber* may therefore be regulated at the level of the tetraketide branch point.

In filamentous fungi, the polyketide pathway is the major route for the formation of secondary metabolites (4, 16), including various mycotoxins (15). Citrinin is a typical toxin, isolated first in *Penicillium citrinum* and later in *Aspergillus* species (6) and *Monascus ruber* (3). It exhibits antibiotic activity against gram-positive bacteria, but its nephrotoxic properties prevent its use as a therapeutic drug (14). The production of citrinin together with red pigments (named “Anka”) rules out the use of *M. ruber* (3) as a producer of natural colorants for food technology (8). However, the fact that other fungi, like *Aspergillus* and *Penicillium*, do not produce these pigments but do synthesize mycotoxins suggests possible variations in the biosynthetic pathway of citrinin in *M. ruber*. It is known that in *Aspergillus*, citrinin is formed by the condensation of one acetyl coenzyme A (acetyl-CoA) molecule with four malonyl-CoA molecules, followed by the addition of three methyl units (2, 5, 9). We have therefore investigated the biosynthetic pathway of citrinin in *M. ruber* by ^{13}C nuclear magnetic resonance (NMR) analysis of carbon isotope distribution of ^{13}C -enriched citrinin after feeding the cultures with ^{13}C -labeled acetate, and we found that this pathway is different from the one previously identified in *Aspergillus terreus*. This finding may lead to new strategies to selectively control the production of pigments and uncouple the formation of them from that of citrinin.

A strain of *M. ruber* (ATCC 96218) was grown at 28°C in a chemically defined medium as described previously (8). A suspension of 10^8 spores was used to inoculate a 1-liter baffled Erlenmeyer flask containing 200 ml of glucose medium. The presence of red pigments and citrinin in the culture broth was determined spectrophotometrically as described previously (3, 8). For the ^{13}C labeling of citrinin, 1 ml of an aqueous solution (99.2% enriched with [$1\text{-}^{13}\text{C}$] [2- ^{13}C]sodium acetate [20 mg · ml⁻¹] or 98.6% enriched with [1,2- ^{13}C]sodium acetate [10 mg · ml⁻¹]) was added after 3, 4, 5, 6, and 7 days to a 200-ml culture. Citrinin was isolated from the medium by filtration of the mycelium cultures on M14 membranes (pore size of 0.8 μm ; Tech-sep, Bollene, France). The filtrate was lyophilized, resus-

ended in 60 ml of water, and extracted three times with water saturated with *n*-butanol. The organic phase was dried and vacuum concentrated, and the residue was dissolved in 50 ml of acidified water (pH 2.0). This solution was treated twice with 120 ml of ethyl acetate, and the retained organic phase was extracted twice with 150 ml of 0.4% NaHCO₃. The aqueous phase was adjusted to a pH of 3.0 with HCl and again extracted twice with 120 ml of ethyl acetate. The organic phase containing citrinin was evaporated to dryness and resuspended in a minimal volume of water. The toxin was isolated by thin-layer chromatography in chloroform-methanol-water (65/25/4, vol/vol/vol), and the band containing the toxin was solubilized in chloroform which was evaporated. ^{13}C and ^1H NMR spectra were recorded on a Bruker ARX 400-MHz spectrophotometer with CDCl₃ (99.6%) as a solvent. Spectra were referenced internally to the solvent for ^{13}C NMR and to trimethylsilyl for ^1H NMR. Minimization of the relative molecular energy of intermediates 2 and 3 (see Fig. 2) was carried out with the molecular mechanics programs Biosym and Discover (version 2.9.7-95.0-3.0.0 cvff force field) on a Silicon Graphics machine.

The kinetics of pigment and citrinin production during the growth of *M. ruber* in the presence of 6.5 g of glucose · liter⁻¹ and 4.5 g of monosodium glutamate · liter⁻¹ are presented in

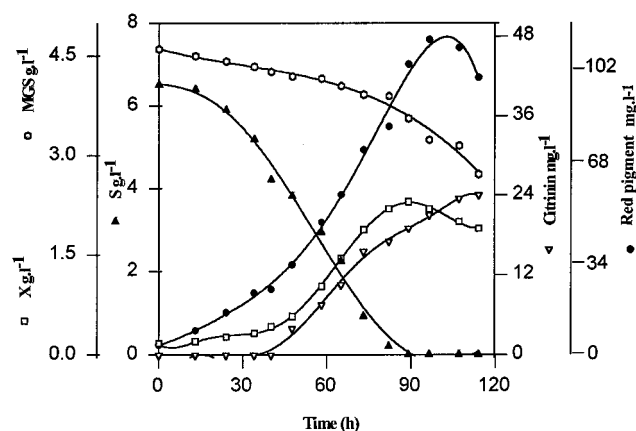


FIG. 1. Culture development of *M. ruber* in a 2-liter fermentor at 28°C. X, biomass; S, glucose; MGS, monosodium glutamate.

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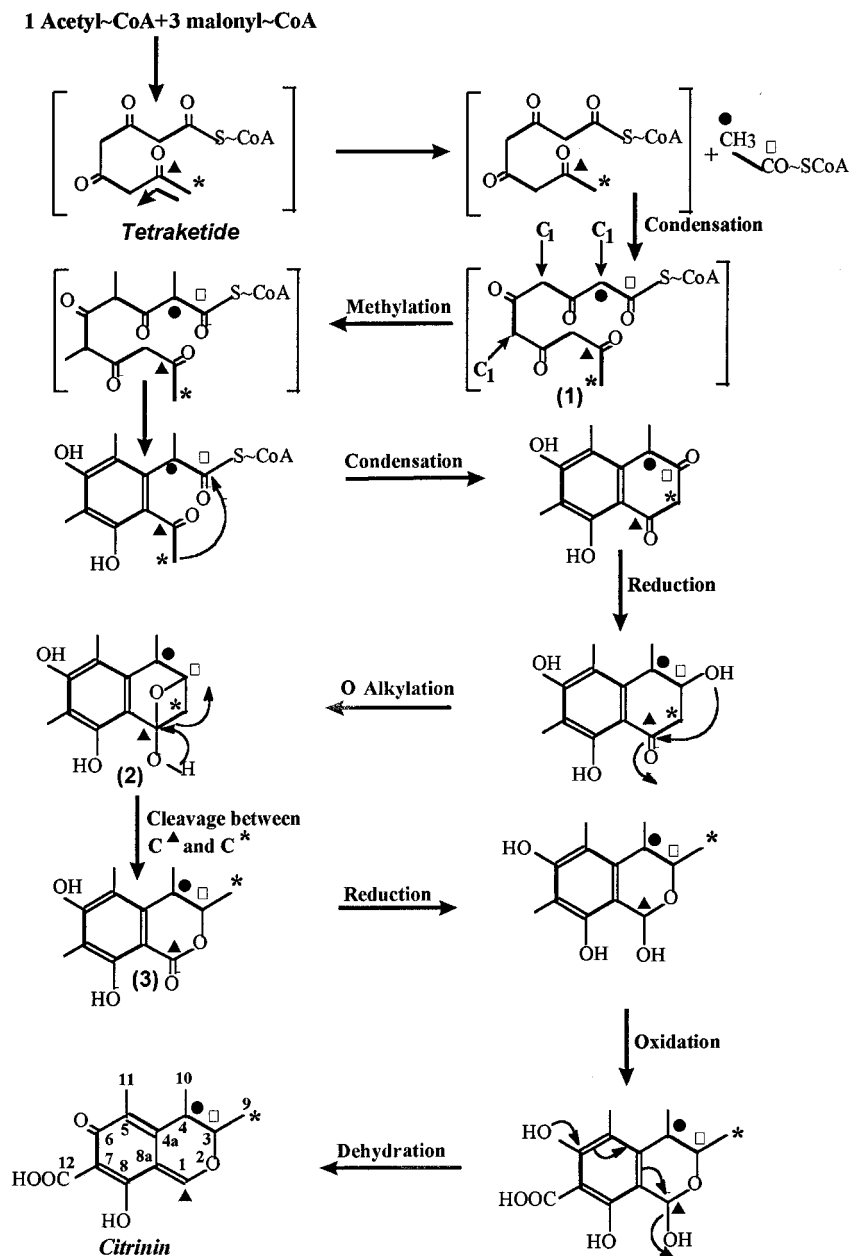


FIG. 2. Scheme of the biosynthesis of citrinin by *M. ruber*. The start of the condensing reaction is indicated by the bent arrow in the upper left panel. Intermediates are numbered. Enrichment of C-1 (▲), C-3 (□), C-9 (*), and C-4 (●) is indicated.

Fig. 1. After a lag of about 20 h, cell biomass and red pigments increased in parallel to reach $2.3 \text{ g (dry weight) } \cdot \text{ liter}^{-1}$ and $112 \text{ mg} \cdot \text{ liter}^{-1}$, respectively, at the time of glucose exhaustion. The maximal rate of pigment production was observed 20 to 60 h after the initiation of the fermentation. In contrast, the production of citrinin started after about 45 h of cultivation and did not appear to stop when the glucose from the medium was consumed. The uncoupling in the production of these two polyketide derivatives suggested that they might not follow the same metabolic pathway. Feeding the culture medium with $[1-^{13}\text{C}]$ acetate resulted in the enrichment of C-1, C-8, C-6, C-4a, and C-3 (Table 1), while the enrichment of C-8a, C-7, C-5, C-4, and C-9 was obtained with $[2-^{13}\text{C}]$ acetate (Table 1).

These experiments confirmed that citrinin arose from the polyketide pathway, by a route apparently similar to that found in *P. citrinum* and *A. terreus* (1, 14). Another experiment carried out with $[1,2-^{13}\text{C}]$ acetate resulted in the enrichment of all the carbons coming from the acetate pool, except for C-10, C-11, and C-12, which came from the endogenous C_1 pool (most likely CO_2). However, we found that the spectra from labeled citrinin were strikingly different from those obtained for *P. citrinum* and *A. terreus*, incubated under the same conditions (1, 14) (results not shown). The relative enrichment of ^{13}C peaks was estimated with respect to its natural abundance. Since C-10 on the citrinin molecule came from the endogenous C_1 (methyl) pool and could be considered an internal refer-

TABLE 1. Chemical shifts and coupling constants for [¹³C]citrinin and RE with labeled [1,2-¹³C₂]acetate^a

Carbon atom	Chemical shift (ppm)	¹ J(¹³ C- ¹³ C) (Hz)	Enrichment	RE ^b
1	162.9	69.6	a	3.1
3	81.8	37.6	a	0.6
4	34.7	40.7	b	0.7
4a	139.2	40.7	a	1.3
5	123.2	57.0	b	2.1
6	183.9	57.0	a	2.4
7	100.4	63.9	b	2.0
8	177.3	63.9	a	2.5
8a	107.5	69.6	b	1.6
9	18.3	37.6	b	1.1
10	9.6	s	s	1.0
11	18.4	s	s	
12	174.7	s	s	

^a a, incorporation of [1-¹³C]acetate; b, incorporation of [2-¹³C]acetate; s, singlet.

^b RE was calculated by the following equation: $\alpha \cdot IC-XE/IC-XN$, where α is determined by the equation $IC-10N/IC-10E$, C-X is the carbon number, C-10 is the reference carbon, IC-XN is the intensity of the NMR signal of carbon X naturally enriched, and IC-XE is the intensity of the NMR signal of carbon X enriched in the presence of [1,2-¹³C₂]acetate. The calculation takes into account the experimental ¹³C NMR conditions.

ence, the relative enrichment (RE) of each carbon was measured in reference to that of C-10. It can be seen in Table 1 that the RE value of C-1 (Fig. 2) in the citrinin structure was the highest, while that of C-3 (Fig. 2) was the lowest, which was exactly the opposite to what has been observed in *A. terreus* (14). Similar to C-1 from acetate moieties, the condensation reaction progressed from C-1, as the most enriched, to C-8, C-6, C-4a, and then C-3. With carbon arising from the CH₃ unit of acetate, the enrichment increased from C-9 (Fig. 2) to C-5 (Table 1). Such an enrichment towards the polyketide condensing reactions has been reported previously for the biosynthesis of botryodiplodine (13). The last two carbons which closed the ring were C-4 (Fig. 2) and C-3, which had comparable but very low levels of isotopic enrichment. This situation contrasted with the high and uniform enrichment of the same carbon obtained in *Penicillium* and *Aspergillus* (1, 14).

To account for these striking differences, we suggest in Fig. 2 that the precursor for citrinin formation is a tetraketide arising from the condensation of one acetyl-CoA molecule with three malonyl-CoA molecules instead of a pentaketide (one acetyl-CoA molecule and four malonyl-CoA molecules). Then, an additional acetyl-CoA molecule is added to the tetraketide to form intermediate 1. Furthermore, one cannot exclude the possibility that a malonyl-CoA molecule condenses to the tetraketide and that this is accompanied by a decarboxylation. Subsequent reactions include O alkylation and the cleavage of the single bond between C-1 and C-9 (Fig. 2) in a way similar to that which occurs in the formation of bovilactone or gomphilactone (10, 11). This cleavage also agrees with the proximity of C-3 and C-9. In addition, intermediates 2 and 3 were energetically acceptable, with molecular energy levels of 100.6 and 93.8 kcal · mol⁻¹, respectively. In summary, the occurrence of a tetraketide as the precursor for both citrinin and red pigments may account for the differential production of these two polyketides during the growth of *M. ruber*. It will be interesting to further characterize the enzymatic reactions at the tetraketide branch point (Fig. 3) in order to develop strategies aimed at a selective production of red pigments.

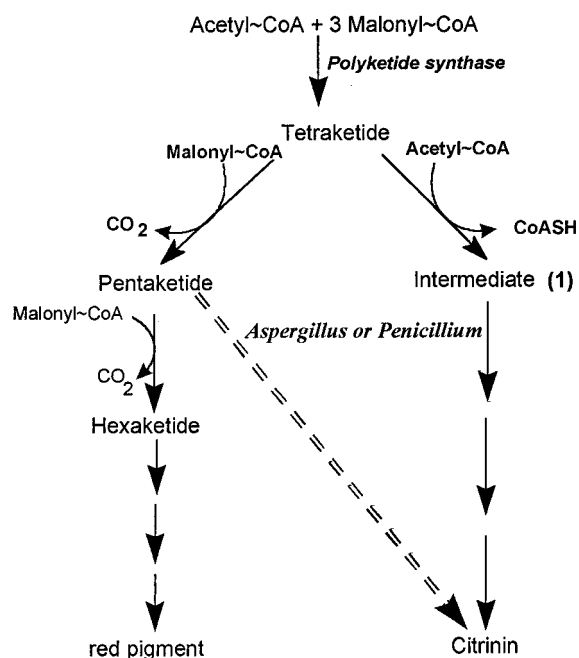


FIG. 3. Biosynthesis of citrinin and red pigment in *M. ruber*. The toxin pathway in *Aspergillus* and *Penicillium* is indicated by the dashed arrow.

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