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An intermediary role for the tremorgenic mycotoxin TR-2 in the biosynthesis of verruculogen

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^{14}C-Labelled compound TR-2, a tremorgenic mycotoxin, was administered to *Penicillium raistrickii* in submerged fermentation. Half of the added radiolabel was taken up by the fungus during the 60h incubation period and the secondary metabolites subsequently isolated, principally verruculogen but also fumitremorgin B, were found to be radiolabelled. The efficiency of biosynthetic incorporation of TR-2 into verruculogen within the mycelium was at least 35%, demonstrating for the first time an intermediary role for TR-2. Fumitremorgin B was also TR-2-derived but may not be an important intermediate in verruculogen biosynthesis.

The structurally related indoles V and FB are secondary metabolites of several *Penicillium* and *Aspergillus* spp. and in some of these fungi they have been shown to be co-metabolites (Mantle & Penny, 1981; Patterson *et al.*, 1981). Another related compound, designated TR-2 (Cole *et al.*, 1975) but not since given a trivial name, has also been isolated as a minor metabolite from *A. fumigatus* (Cole *et al.*, 1977). Consideration of the structures of these metabolites (Perera *et al.*, 1982) suggests biosynthetic inter-relationships with V, the most potent of all tremorgenic mycotoxins, as the end-product.

Materials and methods

Preparation of [^{14}C]*TR-2*

[^{14}C]V (sp. radioactivity 0.589Ci/mol; 780μg), prepared biosynthetically radiolabelled from [U-^{14}C]proline and [2-^{14}C]mevalonic acid (Day & Mantle, 1982) was subjected to catalytic hydrogenation in ethanol over a palladium/carbon catalyst for 25min at atmospheric pressure, a modification of the method previously described (Perera *et al.*, 1982*a*). [^{14}C]TR-2 was isolated by preparative h.p.l.c. on reversed-phase silica (Ultrasphere ODS; 5μm particles) in a methanol/water (5:1, v/v) solvent system, and its purity confirmed by electron-impact mass spectrometry.

Incubation of [^{14}C]*TR-2 with Penicillium raistrickii*

The strain of *P. raistrickii* used was isolated from soil (Patterson *et al.*, 1979) and was subsequently shown (Mantle & Wertheim, 1982) to produce V closely coincident with growth in submerged liquid culture. Czapek Dox broth (100ml), supplemented with yeast extract (0.5%), in a 500ml Erlenmeyer flask was inoculated with a 10% transfer from a 25h seed stage (spore-inoculated) shaken flask. After 7h incubation on a rotary shaker at 27°C [^{14}C]TR-2 (1.32×10^5d.p.m.), dissolved in ethanol (200μl) and diluted with water (1.8ml), was added. After incubation for a further 60h the culture was separated into washed mycelium and culture filtrate, both of which were freeze-dried. The total radioactivity of freeze-dried culture filtrate, and the mycelium before and after extraction, was determined by tissue oxidation (Perera *et al.*, 1982*a*) of samples (50mg).

Extraction and analysis of cell-associated indolic secondary metabolites

Dry mycelium was extracted exhaustively, first with chloroform/acetate (1:1, v/v), then with methanol. Combined extracts were taken to dryness *in vacuo* and the residue was subjected to preparative t.l.c. on silica gel GF_{254} using chloroform/acetone (93:7, v/v) as developing solvent. Two regions, corresponding to V + FB (ratio 4:1) and TR-2, were eluted with acetone and methanol respectively, and the products were separated and/or purified by h.p.l.c. [methanol/water, 5:1 (v/v) and 3:1 (v/v) respectively]. The efficiency of the h.p.l.c. step was found to be 76% for V and 47% for FB, derived from spectrofluorimetric assay (Day *et al.*, 1980) of the separated products and of the combined V and FB eluted from preparative t.l.c.

The application of the spectrofluorimetric assay, described by Day *et al.* (1980) for V but not for FB,

Abbreviations used: V, verruculogen; FB, fumitremorgin B; h.p.l.c., high-performance liquid chromatography.

was shown to be valid for FB by demonstrating that FB fluorescence generated by acid treatment (3 ml of ethanolic solution of the tremorgen heated with 0.2 ml of conc. H_2SO_4 for 40 min at 70°C) was equivalent to that of V on an equimolar basis. In the process of obtaining, with difficulty, pure FB by h.p.l.c. it became apparent that FB is spontaneously oxidized to V in the presence of water. For example, 12.4% of pure FB, in solution in the methanol/water (4 : 1, v/v) eluate from h.p.l.c., was converted into V within 6 h at room temperature.

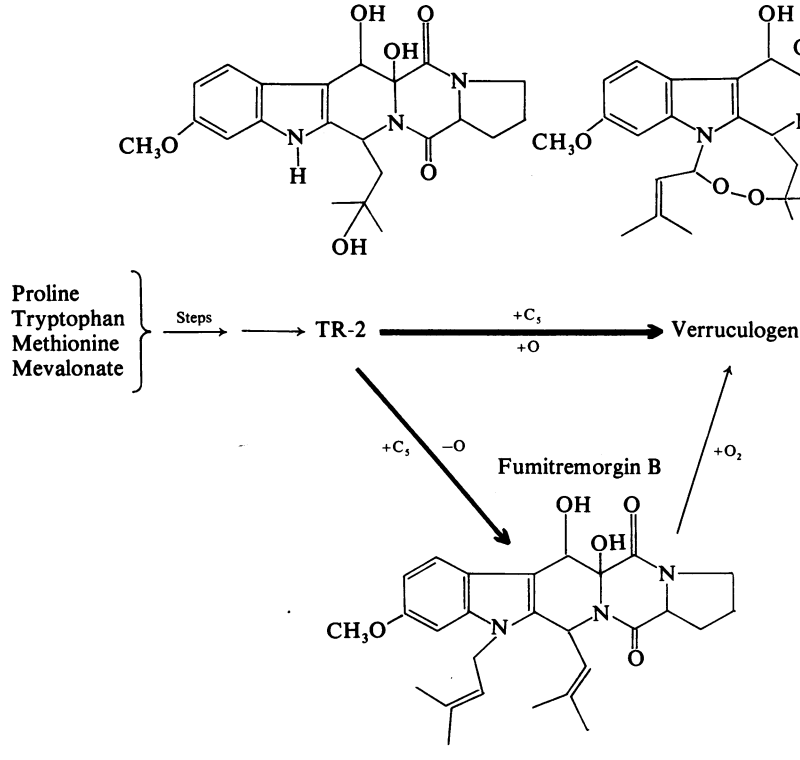
Half of the purified V and FB, and all of the TR-2, was deposited on planchets and counted for radioactivity in a Nuclear Chicago low-background counter (model C155), with reference to standard n-[^{14}C]hexadecane. The other half of V and FB was used for spectrofluorimetric assay.

Results

The results are summarized in Table 1. Half of the radioactivity fed remained in the culture filtrate and, of the remainder taken up by the fungus, most was extracted efficiently. Preparative t.l.c. of the extract

Table 1. *Incorporation of [^{14}C]TR-2 into verruculogen and fumitremorgin B by Penicillium raistrickii*

Amount of [^{14}C]TR-2 fed	1.32×10^5 d.p.m. ($50\,\mu g$)
Mycelial dry weight after incubation with radiolabelled precursor for 60 h	1.1 g
Radioactivity remaining in culture filtrate	6.4×10^4 d.p.m. (48.7% of radiolabel fed)
Radioactivity remaining in mycelium after solvent extraction	2.1×10^3 d.p.m. (1.5% of radiolabel fed)
Radioactivity in combined mycelial extracts	6.7×10^4 d.p.m. (50.8% of radiolabel fed)
Total V and FB recovered after preparative t.l.c.	8.5 mg
Amount of h.p.l.c.-purified V	5.2 mg (1.8×10^4 d.p.m.)
Amount of h.p.l.c.-purified FB	0.8 mg (1.6×10^3 d.p.m.)
Efficiency of incorporation of the [^{14}C]TR-2 taken up by the mycelium into purified V and FB (combined)	29.9%



Scheme 1. *Biosynthesis of V*
The C_5 unit is derived from mevalonate.

gave a small amount of [^{14}C]TR-2, which, when subsequently purified by h.p.l.c., showed 3.2×10^3d.p.m. (equivalent to 2.4% of the [^{14}C]-TR-2 fed, presumably unmetabolized. However, the principal indolic products were V and FB (co-chromatographing on preparative t.l.c.) and their total weight was estimated by spectrofluorimetric assay to be 8.5 mg. Preparative resolution of V and FB by h.p.l.c. gave pure material of each, which was clearly radiolabelled. In spite of inevitable losses of products during purification, the radioactivity measured in V corresponded to 27% of the [^{14}C]-TR-2 taken up into the mycelium (35% if the efficiency of the h.p.l.c. step is taken into account), compelling evidence of an intermediary role for TR-2 in the biosynthesis of V.

Discussion

The yield of purified [^{14}C]FB, relative to [^{14}C]V, was rather less than the usual proportion of this metabolite would suggest, but reflected both the greater difficulty of separating a small component of the mixture and the tendency of FB to give V by spontaneous addition of O_2. This implies both that FB can be derived from TR-2 and that in aerobic culture some may become converted into V, mediated at least partly through a non-biological mechanism. Although it does not necessarily follow therefore that V is an artefact, it may not derive entirely from fungal biosynthesis. Thus, the biosynthesis of V may be summarized as shown in Scheme 1.

Further, the capacity for prenylation of the indolic nitrogenation of TR-2 by *P. raistrickii* to give V contrasts with the opposite mechanism shown to occur in the hepatic metabolism of V in the rat (Perera *et al.*, 1982*a*), and in the sheep (Perera *et al.*, 1982*b*), in which TR-2 is the principal metabolite eliminated in the bile.

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