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Biosynthesis of Territrems by *Aspergillus terreus*

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Different radioactive precursors were added to 8-day potato-dextrose liquid cultures of *Aspergillus terreus* 23-1. Territrems were isolated from chloroform extracts of the cultures at day 14 and purified by thin-layer chromatography and high-pressure liquid chromatography. The territrem B obtained was treated with alkaline hydrogen peroxide, and 3, 4, 5-trimethoxy benzoic acid was isolated from an ethyl acetate extract of the reaction mixture and purified by thin-layer chromatography and high-pressure liquid chromatography. By comparison of the specific radioactivities of territrem B and its cleaved aromatic product (disintegrations per minute per micromole of compound), it was demonstrated that the radioactivity of territrem B was located mainly on its aromatic moiety when [U-^{14}C]shikimate, L-[*methyl*-^{14}C]methionine, and L-[*methyl*-^{3}H]methionine were precursors; however, the radioactivity of territrem B was located mainly on its nonaromatic moiety when [2-^{14}C]mevalonate was the precursor. Mevinolin, a specific inhibitor of β-hydroxyl β-methyl glutaryl coenzyme A reductase, was shown to inhibit production of territrems by *A. terreus* 23-1. When [U-^{14}C]acetate was used as a precursor, mevinolin inhibited the incorporation of radioactive carbon into territrem but mevinolin did not inhibit incorporation of radioactive carbon from [2-^{14}C]mevalonate into territrem.

The chemical structure of territrem B was elucidated as (4a*R*,6a*R*,12a*S*,12b*S*)-4a,6,6a,12,12a,12b-hexahydro-4a,12a-dihydroxy-4,4,6a,12b-tetramethyl-9-(3,4,5-trimethoxyphenyl)-4*H*,11*H*-naphtho[2,1-*b*]pyrano[3,4-*e*]-pyran-1,11(5*H*)-dione (6). The differences among the chemical structures of territrems A, B, and C are due to different substitutions on their aromatic moieties (8, 10).

It has been shown that territrems A and B can be cleaved to give different derivatives of benzoic acid by treatment with alkaline hydrogen peroxide (K. H. Ling, B. J. Chen, Y. W. Peng, S. C. Tsai, F. C. Peng, and C. K. Yang, Mycotoxin Res., in press). 3,4,5-Trimethoxybenzoic acid and 3,4-methylenedioxy-5-methoxybenzoic acid (or 4-methoxy-6-carboxy-1,3-benzodioxole) were shown to be the cleaved aromatic products of territrems B and A, respectively (Ling et al., in press). Therefore, if territrem is biosynthesized with an appropriate radioactive precursor, the location of the isotope in the product, whether in the aromatic or nonaromatic moiety or in both, can be determined by comparison of the specific radioactivities of territrem and its cleaved aromatic compound.

Mevinolin, a fungal metabolite from *Aspergillus terreus* and *Penicillium citrinum* (3, 5), was demonstrated to be a specific inhibitor of mammalian β-hydroxy-β-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) (1, 2). A study using mevinolin as a tool showed HMG-CoA reductase to be a key enzyme in the biosynthesis of isoprenoid hormones in plants (11).

The present work attempts to define the pathway of carbon in the biosynthesis of territrem by utilizing both radioactive precursors, such as ^{3}H-labeled methionine and carbon-14-labeled methionine, acetate, mevalonate, and shikimate, and the HMG-CoA reductase inhibitor mevinolin.

In our previous study on the biosynthesis of territrem with [U-^{14}C]acetate by *A. terreus* 23-1 growing in rice and potato-dextrose liquid (PD) media, it was shown that the optimal time to add the labeled precursor to obtain the highest specific radioactivity in territrem at harvest (the day 14 culture) was on day 8 after the inoculation of spores (4). It was also demonstrated that, although the production of territrems in rice medium was superior to that in PD medium, the specific radioactivity of territrems obtained from PD culture was higher than that from rice culture (4). Therefore, the radioactive precursor was added to PD cultures on day 8 after inoculation in the present biosynthetic studies.

The radioactive precursors [U-^{14}C]acetate (sodium salt, 58 mCi/mmol), DL-[2-^{14}C]mevalonate lactone (22 mCi/mmol), L[*methyl*-^{14}C]methionine(60mCi/mmol),L-[*methyl*-^{3}H]methionine (85 mCi/mmol), and [U-^{14}C]shikimate (19.7 mCi/mmol) were purchased from New England Nuclear Corp. and Amersham. PPO (2,5-diphenyloxazole) and POPOP [1, 4-bis-[2]-(5-phenyloxazolyl)benzene] were purchased from Sigma. Mevinolin, the product of Merck, Sharp & Dohme Laboratory, was a gift from M. S. Shiao. 3,4,5-Trimethoxybenzoic acid was purchased from E. Merck. Chemicals used were all extra pure grade.

Territrems were isolated from chloroform extracts of rice cultures of *A. terreus* 23-1 according to our previous procedure (9). Their purities were assured by high-pressure liquid chromatography and two-dimensional thin-layer chromatography (7). The amounts of standard territrems in methanol were calculated from their molar absorption coefficients at 331 to 338 nm (10). The *A. terreus* 23-1 strain was maintained

TABLE 1. Incorporation of precursor label into territrem B and its cleaved product, 3,4,5-trimethoxybenzoic acid

Precursor	Sp act[a] (dpm/μmol)		Ratio, 3,4,5-TMB/TRB
	TRB	3,4,5-TMB	
[U-^{14}C]shikimate	5,065	4,082	80.60
DL-[2-^{14}C]mevalonate	13,782	ND	
L-[*methyl*-^{3}H]methionine	149,367	145,791	97.60
L-[*methyl*-^{14}C]methionine	74,282	62,597	84.27

[a] Mean values from triplicate cultures. TRB, Territrem B; 3,4,5-TMB; 3,4,5-trimethoxybenzoic acid. ND, Not detected.

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TABLE 2. Effect of mevinolin on incorporation of [U-^{14}C]acetate and DL-[2-^{14}C]mevalonate lactone into territrem by *A. terreus* 23-1 growing in PD liquid medium[a]

Precursor	Mevinolin (μM)	Mycelium (mg dry wt)	Territrem A			Territrem B			Territrem C		
			Sp act (dpm/μmol)	‰ Incorporated	% Inhibition	Sp act (dpm/μmol)	‰ Incorporated	% Inhibition	Sp act (dpm/μmol)	‰ Incorporated	% Inhibition
[U-^{14}C]acetate		582	21,370	0.057		10,040	0.156		29,880	0.043	
[U-^{14}C]acetate	50	523	17,090	0.033	42	7,810	0.102	35	18,000	0.030	30
DL-[2-^{14}C]mevalonate		550	20,960	0.051		15,060	0.214		24,950	0.049	
DL-[2-^{14}C]mevalonate	50	541	26,140	0.063	None[b]	15,910	0.235	None[b]	27,640	0.057	None[b]

[a] All values represent the means from triplicate cultures. Each culture contained 50 ml of PD liquid medium.
[b] The radioactive precursor and inhibitor were added on day 6 after inoculation.

on PD agar slants at 28 to 30°C for sporation (4). The spores from agar slants were made into a suspension containing 1.5 $\times 10^7$ to 2.0 $\times 10^7$ spores per ml of sterilized 0.01% Triton X-100 aqueous solution for inoculation. Approximately 10^6 spores each were inoculated into culture flasks which contained 50 ml of PD medium (Ling et al., in press). The cultures were incubated at 28 to 30°C in a static condition. The next day after inoculation was counted as day 1 of the culture. Radioactive precursors, such as 0.67 μCi of [U-^{14}C]shikimate, 15 μCi of DL-[2-^{14}C]mevalonate, 45 μCi of L-[*methyl*-^{3}H]methionine, and 15 μCi of L-[*methyl*-^{14}C]methionine, were added to the day 8 cultures with a microsyringe (Hamilton no. 1702) and mixed. The cultures were further incubated in a static condition and harvested on day 14 after inoculation. At harvest, the liquid portion of the PD culture was decanted off, and the residual mycelial mat in the culture flask was rinsed twice with 10 ml of deionized water, lyophilized, and kept in a desiccator until the next step of analysis. The dry weight of mycelium was weighed with a balance.

The procedures of extraction, preparative thin-layer chromatographic cleanups, and high-pressure liquid chromatographic quantitation of territrems followed the previous methods (4,7; Ling et al., in press). When an additional 0.91 to 1.88 μCi of territrem B was added to the dried mycelial mat, recovery of 91 to 96% was obtained. The radioactivity of each territrem was measured in 10 ml of scintillation fluid with a Packard liquid scintillation counter (Tricarb 460C and 460CD). The composition of the scintillation fluid was 3.3 g of PPO–0.08 g of POPOP–300 ml of Triton X-100–670 ml of benzene. For chemical cleavage of territrem B, approximately 1 mg of territrem B obtained from the triplicate cultures (see Table 1) was used. The procedures of chemical cleavage of territrem B and quantitation of its cleaved aromatic product, 3,4,5-trimethoxybenzoic acid, followed our previous methods (Ling et al., in press). The measurement of radioactivity of the cleaved aromatic product also followed the procedure for territrem as described above. In the experiment determining effect of mevinolin on fungal growth and territrem production, 25 or 50 μM mevinolin was added to day 6 or day 10 cultures. In the experiment examining the effect of mevinolin on incorporation of [U-^{14}C]acetate or DL-[2-^{14}C]mevalonate into territrem, 15 μCi of radioactive precursor was added to the day 6 cultures with or without the addition of 50 μM mevinolin. The cultures were harvested on day 14 after inoculation.

[U-^{14}C]shikimate, L-[*methyl*-^{3}H]methionine, and L-[*methyl*-^{14}C]methionine, but not DL[2-^{14}C]mevalonate, labeled territrem B mainly in its aromatic moiety; DL-[2-^{14}C]mevalonate labeled the compound exclusively in its nonaromatic moiety (Table 1). From these results we conclude that the benzene ring of territrem is biosynthesized from shikimate and that the methyl groups on the benzene ring of territrem are derived from the methyl group of methionine. Mevinolin added at 25 or 50 μM to the day 6 or day 10 cultures did not affect fungal growth but inhibited territrem production by 24 to 55% at harvest. Furthermore, mevinolin (50 μM) inhibited the incorporation of [U-^{14}C]acetate into territrems A, B, and C by 30 to 42% but did not affect the incorporation of [2-^{14}C]mevalonate into territrems (Table 2); this suggested that HMG-CoA reductase is one of the enzymes participating in the biosynthesis of territrem by *A. terreus*. To elucidate the origin of the carbon in territrem, ^{13}C-nuclear magnetic resonance studies of this compound are under way in our laboratory.

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