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Leucine Auxotrophy Specifically Alters the Pattern of Trichothecene Production in a T-2 Toxin–Producing Strain of *Fusarium sporotrichioides*

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The biosynthetic pathway for trichothecenes in the filamentous fungus *Fusarium sporotrichioides* NRRL 3299 has been further characterized. Experiments using the techniques of mutational analysis and the incorporation of radiolabeled precursors indicated that leucine is a direct precursor to the isovalerate moiety present in the trichothecene, T-2 toxin. Analysis of trichothecene production in a UV-induced leucine auxotroph also revealed the existence of a branched biosynthetic pathway which results in the coproduction of T-2 toxin and the T-2 toxin analogs neosolaniol, 8-isobutyryl-neosolaniol, and 8-propionyl-neosolaniol. Leucine limitation imposed by the leucine auxotroph simultaneously led to underproduction of T-2 toxin and overproduction of these T-2 toxin analogs, which are produced in small amounts by the wild-type parent. Furthermore, it was shown that the ratio of T-2 toxin to T-2 toxin analogs produced by the leucine auxotroph can be modulated by the concentration of leucine in the medium. These results suggest that the four trichothecenes mentioned above are derived from a common intermediate and that there is competition for this intermediate among the branched pathways leading to these four cometabolites.

The trichothecenes are a family of toxic fungal secondary metabolites which possess a characteristic 12,13-epoxytrichothecene nucleus. The subsequent addition of several different oxygen-containing substituents to this nucleus leads to the production of over 60 different trichothecenes (13), including T-2 toxin, which contains an isovaleroxy side group located at position C-8 (Fig. 1). While it is known that the trichothecene nucleus is derived from three molecules of mevalonate via farnesyl pyrophosphate (13), additional information regarding the biochemical origin of the trichothecenes is incomplete. The primary goal of the present study was to investigate the biosynthetic origin of the isovalerate moiety in T-2 toxin.

T-2 toxin is the major trichothecene produced by *Fusarium sporotrichioides* NRRL 3299 (1, 4, 11). Results from $^{18}O_2$ and $H_2{}^{18}O$ incorporation experiments performed with this organism revealed that the isovalerate carbonyl oxygen in T-2 is derived from water (4). This suggested that the isovalerate moiety may be derived from leucine via decarboxylative transamination (4, 9). We have tested this hypothesis in the present study by using both mutant analysis and radiolabeled L-[^{3}H]leucine incorporation studies.

The data presented here provide evidence that, in *F. sporotrichioides* NRRL 3299, L-leucine is a direct precursor to the isovalerate moiety in T-2. Consequently, T-2 is the product of a mixed biogenesis that involves two primary metabolic pathways, one for isoprenoids and the other for an amino acid. In this paper, we also report the purification and characterization of two additional trichothecenes, 8-propionyl-neosolaniol (P-Neo) and 8-isobutyryl-neosolaniol (B-Neo). In addition, the results from this study indicate that the trichothecene biosynthetic pathway in *F. sporotrichioides* NRRL 3299 is branched such that P-Neo, B-Neo, and T-2 are alternative end products synthesized from a common intermediate(s).

MATERIALS AND METHODS

Strains. The strains of *F. sporotrichioides* used in this study are listed in Table 1. Mutants were derived from the wild-type, T-2 toxin–producing parent NRRL 3299 (ATCC 24043) (11) by UV treatment (1). All of the auxotrophs failed to grow on minimal medium. MB1716 is a trichothecene toxin synthesis mutant that is completely blocked in the biosynthesis of T-2 toxin and accumulates another trichothecene, diacetoxyscirpenol (DAS) (1).

Media and culture conditions. Solid-medium cultures were grown under conditions previously described (3). All strains were maintained as conidial suspensions at −70°C in 10 to 15% glycerol and as cultures at 4°C on either V-8 juice agar (12) slants or, in the case of auxotrophs, on V-8 complete agar (1) slants supplemented with an additional 40 to 80 μg of the required amino acid per ml (V-8C+aa). For auxotrophy tests and reversion frequency determinations, the complete medium was YEPD-2G-5T (1) and the minimal medium was M-100 (12), modified by the addition of 0.05% Triton X-100. The reversion frequencies for the auxotrophic strains are presented in Table 1.

A complete medium, designated YEPD-5G and containing 0.1% yeast extract, 0.1% peptone, and 5.0% glucose (14), was used for trichothecene production in liquid shake cultures; in certain experiments, extra L-leucine (25, 50, or 100 μg/ml) was added to the YEPD-5G medium. All of the wild-type and mutant strains used in this study grew well on the unsupplemented YEPD-5G medium. Liquid cultures were inoculated to a final density of 1.0×10^4 to 7.0×10^4 conidia per ml of medium with conidia washed from strains grown for 6 to 7 days on V-8 or V-8C+aa agar plates. The liquid cultures were incubated at 28°C and 180 to 200 rpm on

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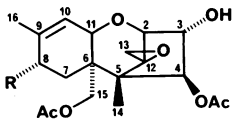

Trichothecene	R
Diacetoxyscirpenol	H
Neosolaniol	OH
8-propionyl-neosolaniol (I)	$OCOCH_2CH_3$
8-isobutyryl-neosolaniol (II)	$OCOCH(CH_3)_2$
T-2 toxin	$OCOCH_2CH(CH_3)_2$

FIG. 1. Trichothecene chemical structures of DAS, Neo, P-Neo, B-Neo, and T-2 toxin.

a rotary shaker. In the experiments where toxin production and growth rate were monitored, samples (5 to 10 ml) were removed at 24-h intervals and filtered; the culture filtrates were used to determine the types and concentrations of the trichothecenes present, and the collected mycelia were used to monitor cell growth by measuring the dry weight of the fungal biomass as previously described (1).

Trichothecenes. The production of five trichothecenes, T-2 toxin, DAS, neosolaniol (Neo), P-Neo, and B-Neo, was examined in detail. Their chemical structures are presented in Fig. 1.

Analysis of trichothecenes by GC and GC-mass spectrometry. Samples (5 ml) of the cultures or culture filtrates were extracted with ethyl acetate (EtOAc; three 10-ml samples); the combined EtOAc extracts were filtered through a charcoal column (Romer Labs) and eluted under slight vacuum (600 mm Hg [7,980 Pa] pressure). The columns were eluted with additional EtOAc (two 10-ml samples), and the EtOAc was removed from the pooled eluents at 35°C in vacuo; the resulting residue was dissolved in 2 ml of toluene-acetone-methanol (2:1:1), and 400-μl aliquots were evaporated to dryness. Trimethylsilyl (TMS) derivatives were prepared for gas chromatography (GC) or GC-mass spectrometry analysis by treating the residues described above with 100 μl of Tri-Sil/TBT (Pierce Chemical Co., Rockford, Ill.) at 80°C for 1 h. The derivatized samples were then diluted to 1 ml with hexane. For GC analysis, 2 μl of the derivatized samples was injected (in the splitless mode) onto a Spectra Physics 7100 GC equipped with a flame ionization detector and a DB-1 coated (0.25-μm) capillary column (30 m by 0.25 mm; J. & W. Scientific, Folsom, Ga.). The temperature program consisted of an initial oven temperature of 120°C, followed by a 15°C/min gradient to 210°C with 1 min at 210°C, and a 5°C/min gradient to 260°C with an additional 10 min at 260°C. The retention times (in minutes) observed were as follows: DAS, 12.37; Neo, 14.68; P-Neo, 16.1; B-Neo, 16.5; and T-2, 17.9. For GC-mass spectrometry analysis, the derivatized sample was chromatographed by using a DB-1 capillary column (30 m by 0.225 mm; J. & W. Scientific) which was coupled directly to the source of a Finnigan TSQ 46 mass spectrometer. Spectra were recorded in the electron ionization mode or the chemical ionization (CI) mode. For CI spectra, isobutane (0.3 terr [~40 Pa]) was the reagent gas and the temperature was 100°C. Precisely measured, high-resolution mass spectra were obtained from analysis of pure samples inlet via the solids probe into a VG 7070 mass spectrometer and analyzed in the CI mode with ammonia as the reagent gas. T-2, DAS, Neo, and T2-tetraol standards were obtained from Sigma (St. Louis, Mo.).

TABLE 1. Strains of *F. sporotrichioides* used in this study

UV-induced mutant[a]	Phenotype	Genotype	Reversion frequency
MB1026	Gln^-	*Gln1-1*	5.7×10^{-7}
MB1083	Met^-	*Met1-1*	3.4×10^{-7}
MB1572	Arg^-	*Arg1-1*	1.1×10^{-6}
MB2402	Lys^-	*Lys1-1*	$<1.0 \times 10^{-6}$
MB2915	Leu^-	*Leu1-1*	2.7×10^{-7}
MB1716	Tox^-	*Tox1-2*	ND[b]

[a] The parent strain was NRRL 3299.
[b] ND, Not determined.

Isolation of P-Neo and B-Neo. P-Neo and B-Neo were isolated from a 4-liter fermentation of MB2915. By using the procedures described above, four replica 1-liter liquid cultures in 2-liter flasks were inoculated to a final density of 10^4 conidia per ml. After 8 days of incubation at 28°C and 200 rpm, each 1-liter culture was extracted with EtOAc (three 400-ml samples), and the combined organic solvents derived from all four cultures were removed in vacuo, leaving 1.15 g of crude residue. The residue was applied to a silica gel (250 g) column. The column was then washed with 200 ml of CH_2Cl_2, and the trichothecenes were eluted with three 500-ml washes of ethyl acetate. The final fraction, eluted with methanol (400 ml), contained no trichothecenes and was discarded. The residue (665 mg) obtained from concentration of the combined EtOAc fractions was divided into two portions of 265 and 400 mg. Exploratory methods of purification were performed with the 265-mg portion, and the 400-mg portion was purified by preparatory reverse-phase high-pressure liquid chromatography (HPLC) in batches of 60 mg each. The 400-mg fraction gave rise to purified compound I or P-Neo (36 mg) and compound II or B-Neo (43 mg). The HPLC was conducted on a Spectra Physics 8100 HPLC equipped with a C-18 reverse-phase Rainin Dynamax column (30 cm by 21.4 mm [inner diameter]). An isocratic solvent system of CH_3OH-H_2O (60:40) was used at a flow rate of 6 ml/min, and the column was held at 50°C. The trichothecenes were detected by their UV absorption at 210 nm by using a Spectra Physics 8440 UV detector. Under these conditions, the following retention times (in minutes) were observed: DAS, 19.7; P-Neo, 21.5; B-Neo, 33.4; and T-2, 54.8.

NMR analysis. Nuclear magnetic resonance (NMR) spectra were obtained with a Bruker WM-300 WB spectrometer. A 5-mm dual ($^1H/^{13}C$) probe was used to obtain ^1H, ^{13}C, and distortionless enhancement by polarization transfer (5) spectra.

Incorporation of L-[^{3}H]leucine. Erlenmeyer flasks (50 ml) containing 15 ml of medium were inoculated in parallel with 5.25×10^5 conidia from either the parent strain (NRRL 3299), the Leu^- mutant (MB2915), or the toxin mutant (MB1716). After 44 h of incubation at 28°C and 200 rpm, a mixture of L-[4,5-^{3}H]leucine (58 Ci/mmol in 2% ethanol; ICN Pharmaceuticals Inc., Irvine, Calif.) and L-leucine (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 1 μCi/ml and 250 μM additional leucine. The cultures were incubated another 48 h and then frozen at −20°C until they were analyzed for trichothecenes. EtOAc extracts were prepared, concentrated to dryness, and dissolved in the toluene-acetone-methanol solution as described above. A sample equal to 1 ml of the original culture was taken to dryness, and *p*-nitrobenzoate derivatives were prepared for HPLC analysis by dissolving the resulting

residue in 1 ml of CH_2Cl_2 and then adding *p*-nitrobenzoyl chloride (3.7 mg, 0.02 mmol) and *N*,*N*-dimethylaminopyridine (4.9 mg, 0.04 mmol). After heating for 1 h at 80°C in sealed vials, the reaction products were collected and washed sequentially with 1 N HCl and 5% $NaHCO_3$. The organic layer was dried ($MgSO_4$), evaporated to dryness, and taken up in the initial HPLC mobile phase (CH_3CN-H_2O, 60:40). HPLC separations were performed by using a Spectra Physics 8100 system equipped with a C-18 reverse-phase column (25 cm by 4.6 mm [inner diameter]) and UV detection at 260 nm (0.02 absorbance units, full scale). The mobile phase used a 15-min linear gradient of CH_3CN-H_2O from 60:40 to 100:0. Retention times (in minutes) were: DAS, 8.14; P-Neo and B-Neo, 9.53; Neo, 10.38; and T-2, 10.86. The peaks from 50-μl injections were manually collected in scintillation vials and diluted with 10 ml of scintillation cocktail (OCS; Amersham). The radioactivity was then measured by scintillation counting with a Beckman LS-3801 counter.

RESULTS

Unique alteration of trichothecene biosynthesis by leucine auxotrophy. We chose to use a leucine mutant derived from a T-2-producing strain to help determine whether leucine is a precursor to T-2. The rationale for this was twofold. If leucine is a precursor to T-2, then a leucine auxotroph might display reduced levels of T-2 and correspondingly increased levels of T-2 analogs, and could facilitate labeling T-2 in precursor feeding studies with radiolabeled leucine. Comparison of trichothecene synthesis by the leucine auxotroph MB2915 and the wild-type parent NRRL 3299 revealed that the Leu^- mutant displayed reduced production of T-2 and increased production of Neo and two additional trichothecenes, I and II, which were present as minor constituents in the NRRL 3299 cultures (Fig. 2). In marked contrast, four other UV-induced auxotrophs, Arg^-, Gln^-, Met^-, and Lys^-, displayed patterns of trichothecene production identical to that observed for the wild-type parent (Fig. 2). These results indicate that the relative decrease in the production of T-2 is directly related to leucine auxotrophy and are consistent with leucine being a biosynthetic precursor of T-2.

If leucine is the major source of the isovalerate moiety of T-2, then a decrease in T-2 caused by a leucine limitation could lead to the observed increase in the level of Neo, which is identical to T-2 except for the absence of the isovalerate side chain (Fig. 1). The increase in the other two compounds was unexpected. The structures for these two trichothecenes were determined in the following experiments.

Isolation and structural identification of P-Neo and B-Neo. The increased production of I and II by the leucine auxotroph allowed for their efficient isolation and characterization. Compounds I and II were identified as trichothec-9-ene-8α-propionyl-4β,15-acetyl-3αol (common name: 8-propionyl-neosolaniol or P-Neo) and trichothec-9-ene-8α-isobutyryl-4β,15-acetyl-3αol (common name: 8-iso-butyryl-neosolaniol or B-Neo), respectively. P-Neo and B-Neo are related to T-2, with P-Neo containing a propionyl moiety and B-Neo containing an iso-butyryl moiety, instead of the isovalerate at C-8 (Fig. 1).

The structural similarity of these two compounds to T-2 was obvious from the GC-mass (CI) spectra of the TMS-derivatized crude (EtOAc) extract of the MB2915 culture filtrate (Fig. 3). All three compounds contain a common base peak at *m/z* 377, which in each case arose from the loss of the C-8 side chain moiety and one of the two acetates at either C-4 or C-15. Weak molecular ions were also observed for T-2-TMS (MH^+, *m/z* 539), B-Neo-TMS (MH^+, *m/z* 525), and P-Neo-TMS (MH^+, *m/z* 511), and the fragmentations were virtually identical below *m/z* 377.

Purification of the trichothecenes from the crude EtOAc extract was accomplished by using a combination of two chromatographic columns. Three trichothecene-containing fractions were obtained following chromatography on a silica gel column. One fraction consisted of purified Neo, while the other two fractions contained a mixture of T-2, B-Neo, P-Neo, and DAS. These four compounds, which exhibit extremely similar chromatographic mobilities on normal phase particles, were separated via HPLC on a C-18 reverse-phase column.

Definitive structures for B-Neo and P-Neo were derived from the spectral and chemical analyses of the purified compounds. Precise ammonia CI mass spectral analysis of the underivatized samples confirmed the hypothesized empirical formulas for B-Neo (MH_4^+, *m/z* 470.2394, calculated for $C_{23}H_{36}NO_9$, 470.2388) and P-Neo (MH_4^+, *m/z* 456.2241, calculated for $C_{22}H_{34}NO_9$, 456.2230). The basic carbon skeletons of the newly isolated compounds were chemically shown to be identical with each other and with that of T-2 by the hydrolysis (NH_4OH, CH_3OH) of each of the three to T-2 tetraol (identical by thin-layer chromatography or GC [TMS] with reference samples). As shown in Tables 2 and 3, the ^1H- and ^{13}C-NMR spectra of P-Neo, B-Neo, and T-2 are extremely similar and differ only in the alkyl regions (C-2′, C-3′, C-4′, C-5′, and associated protons), which corroborates the evidence that they are analogs. The only ambiguity left to be resolved was whether the C-8 butyryl of B-Neo was *n*-butyryl or isobutyryl. The fact that it is isobutyryl can be ascertained from the observed ^1H-NMR methyl doublet for C-3′ and C-4′ at 1.16 ppm (J = 7 Hz). The ^{13}C-NMR distortionless enhancement by polarization transfer (5) experiment also shows that the C-8 side chain of B-Neo consists of two CH_3 groups and one CH but lacks any CH_2 groups.

Effect of leucine concentration on trichothecene biosynthesis. The preliminary results with the leucine auxotroph suggested that the biosynthesis of T-2 is dependent upon leucine and that leucine deprivation results in the utilization of an alternative branch pathway(s). These possibilities were further examined by testing whether trichothecene production could be altered by varying the leucine concentration in the growth medium.

Growth and trichothecene production were measured for wild-type and Leu^- cells cultured in YEPD-5G complete medium, which contains leucine in the form of peptone, and in YEPD-5G complete medium supplemented with additional leucine. Neither the wild-type nor the Leu^- mutant showed any change in growth rate in response to the different levels of leucine (Fig. 4). Likewise, the pattern, rate, and level of trichothecene production remained constant in the wild-type culture over the time period and the range of leucine concentrations tested (Fig. 5A and 6A). This was not observed for the Leu^- mutant. The leucine concentration in the medium dramatically affected not only the pattern of trichothecene production by the Leu^- mutant (Fig. 5B) but also the rate and level of production (Fig. 6B).

In the leucine auxotroph, the proportion of the T-2 analogs (Neo, P-Neo, and B-Neo) was inversely related to the proportion of T-2 and the concentration of leucine in the medium (Fig. 5B). At the highest leucine concentration,

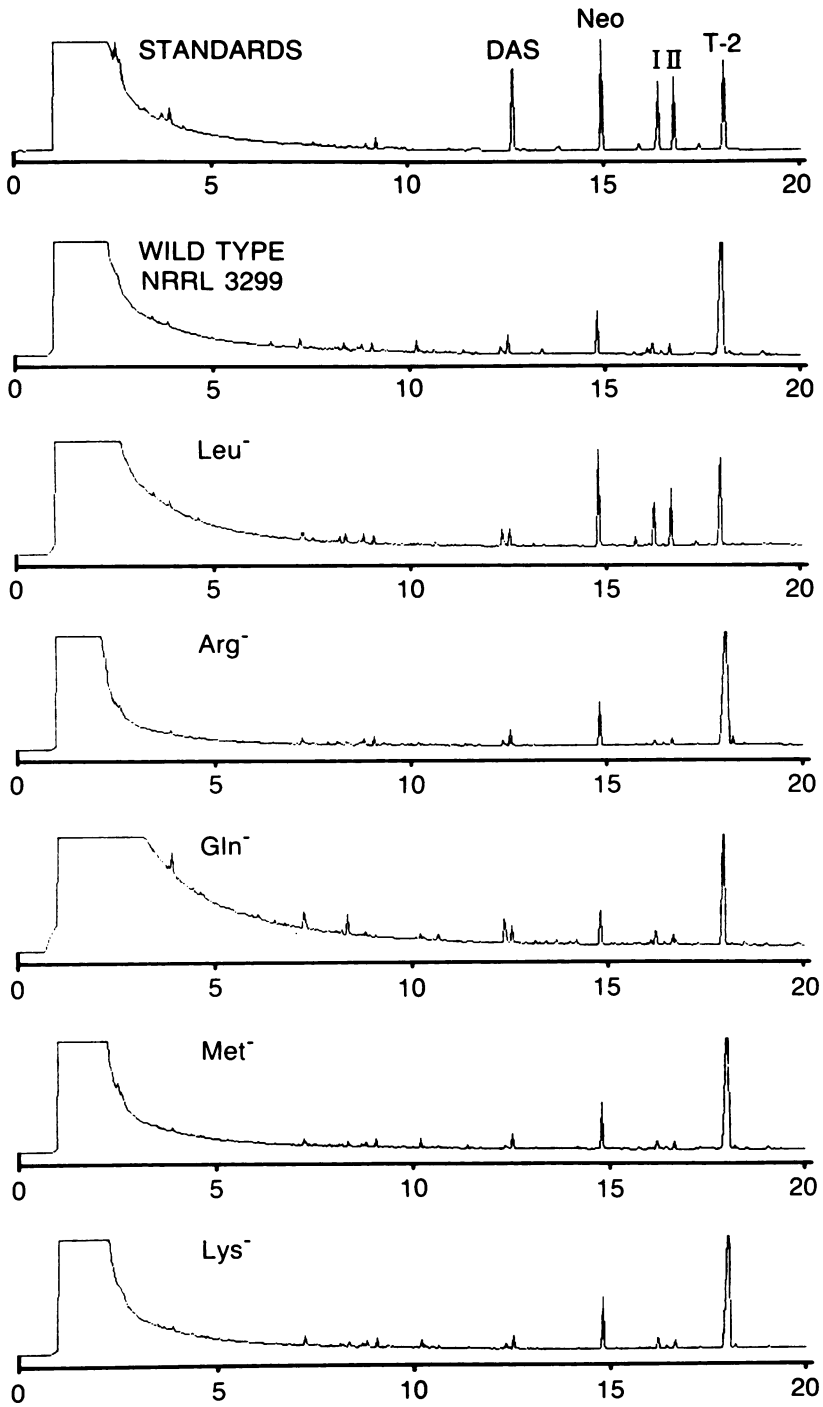

FIG. 2. GC analysis of trichothecenes produced by wild-type and auxotrophic mutant strains of *F. sporotrichioides* NRRL 3299. Shown are standards, NRRL 3299, MB2915 (*Leu1-1*), MB1572 (*Arg1-1*), MB1026 (*Gln1-1*), MB1083 (*Met1-1*), and MB2420 (*Lys1-1*). Strains were grown for 8 days at 28°C and 180 rpm in 50-ml flasks containing 25 ml of YEPD-5G that had been inoculated with 1.25×10^6 conidia collected from cultures grown on V-8 plates for 7 days. Culture filtrates were stored at −20°C and prepared for GC analysis as described in Materials and Methods. Following formation of the TMS derivatives, samples equivalent to 2 μl of the original culture filtrate were analyzed by capillary gas chromatography.

the wild-type DAS/Neo/P-Neo/B-Neo/T-2 ratio was approached; 100 μg of additional leucine per ml initially shifted the ratio of DAS/Neo/P-Neo/B-Neo/T-2 in MB2915 from approximately 4:18:19:19:40 to 3:8:10:7:72, which is closer to the wild-type ratio of approximately 2:7:5:4:82. Values obtained for 10 and 50 μg of additional leucine per ml followed a similar shift but at levels that corresponded to the leucine concentration. At all leucine concentrations, the steady decrease in the relative proportions of T-2 with increasing growth time most likely reflects a progressive

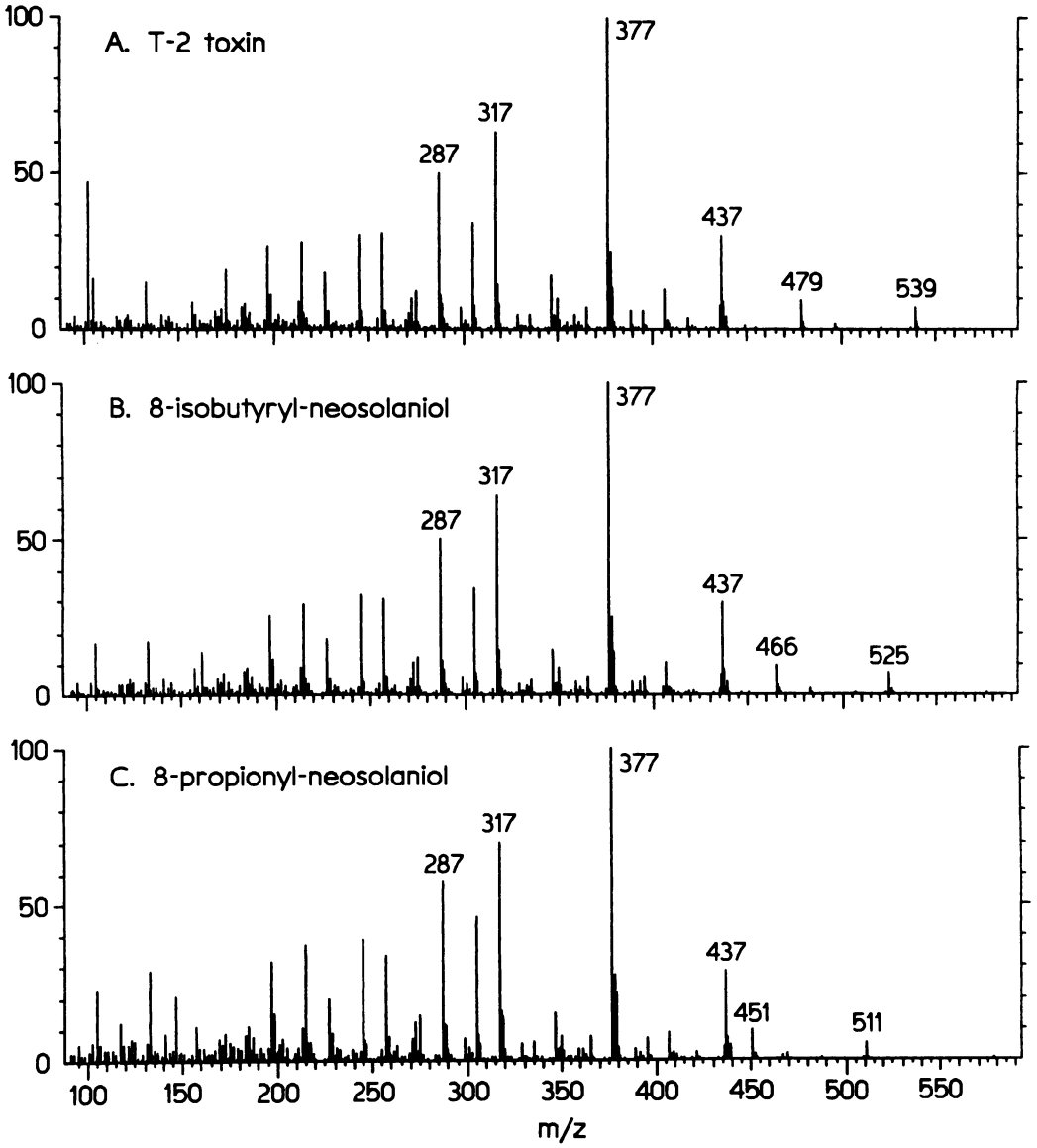

FIG. 3. Mass spectral CI for TMS derivatives of T-2 toxin, B-Neo, and P-Neo.

exhaustion of leucine from the medium. The results discussed above support the hypotheses that leucine is a precursor to T-2 and confirm that, in response to leucine deprivation, alternative pathways are utilized which lead to the production of T-2 analogs.

At the same time, the rate and total level of trichothecene production were directly related to the leucine concentration in the medium (Fig. 6B). At 0 and 10 μg of leucine per ml, total toxin production was less for MB2915 than for the wild-type parent, but, at 50 and 100 μg of additional leucine per ml, total toxin production exceeded that of the wild-type parent.

The data reported in Table 4 tabulate the yields (in milligrams per liter) of the individual toxins produced by the wild-type and mutant strains at the minimum and maximum leucine concentrations tested. Under the appropriate conditions, the leucine auxotroph can be expected to produce 67 mg of Neo per liter, 37 mg of P-Neo per liter, and 36 mg of B-Neo per liter.

Incorporation of [^{3}H]leucine into T-2 toxin. L-[^{3}H]leucine incorporation studies provided additional evidence that leucine is a direct precursor to the isovalerate on T-2. As shown in Table 5, L-[^{3}H]leucine was utilized efficiently by both the wild-type and Leu$^-$ strains as a precursor to T-2, but not to DAS or the T-2 analogs Neo, P-Neo, or B-Neo. These results suggest that the radiolabeled leucine primarily enters T-2 via the isovalerate moiety which distinguishes T-2 from the other four trichothecenes. If the label were entering the trichothecenes via the common trichothecene nucleus, then all of the trichothecenes would be equally labeled. To further prove this point, we used another mutant of NRRL 3299, MB1716, which makes no T-2 or T-2 analogs but accumulates an equal amount of DAS (1; M. N. Beremand and R. D. Plattner, unpublished results). The data confirmed that L-[^{3}H]leucine is very inefficiently incorporated into DAS as compared to T-2. In addition, ^{13}C-NMR data obtained from the analysis of T-2 produced by cultures grown in the presence of L-[^{13}C]leucine have definitively demonstrated that leucine is a precursor to the isovalerate on T-2 (F. Van-

TABLE 2. ^{1}H chemical shift assignments and coupling constants for epoxy trichothecene skeleton

Proton position	^{1}H chemical shift (coupling constant) for:		
	T-2	B-Neo	P-Neo
2	3.67 d ($J_{2,3}$ = 4.9)	3.68 d ($J_{2,3}$ = 4.9)	3.68 d ($J_{2,3}$ = 4.9)
3	4.14 m ($J_{3,4}$ = 2.9)	4.14 m ($J_{3,4}$ = 2.9)	4.15 m ($J_{3,4}$ = 3.0)
4	5.30 d	5.38 d	5.26 d
7a	2.38 dd (J_{AB} = 15.1) ($J_{7,8}$ = 5.8)	2.41 dd (J_{AB} = 15.1) ($J_{7,8}$ = 5.8)	2.39 dd (J_{AB} = 16.3) ($J_{7,8}$ = 5.6)
7b	1.88 m	1.83 m	1.93 m
8	5.27 br d	5.26 br d	5.26 br d
10	5.78 m ($J_{10,11}$ = 5.9)	5.80 m ($J_{10,11}$ = 6.0)	5.79 m ($J_{10,11}$ = 5.9)
11	4.33 br d	4.38 br d	4.31 br d
13a	3.04 d (J_{AB} = 3.9)	3.05 d (J_{AB} = 3.9)	3.05 d (J_{AB} = 3.9)
13b	2.78 d	2.78 d	2.78 d
14	0.79 s	0.80 s	0.80 s
15a	4.27 d (J_{AB} = 12.6)	4.31 d (J_{AB} = 12.6)	4.28 d (J_{28} = 12.6)
15b	4.04 d	4.02 d	4.04 d
16	1.73 br s	1.73 br s	1.73 br s
2′	2.05–2.15	2.48 m	2.27 m (J = 7.6)
3′	2.05–2.15	1.16 d (J = 7.0)	1.13 t
4′	0.95 d (J = 6.3)	1.16 d (J = 7.0)	
5′	0.94 d (J = 6.3)		
$OCOCH_3$	2.02, 2.12	2.02, 2.13	2.01, 2.13

Middlesworth, M. N. Beremand, T. Isbell, and D. Weisleder, unpublished results).

DISCUSSION

In this paper, we report the production of the trichothecenes P-Neo and B-Neo by *F. sporotrichioides* NRRL 3299. These two trichothecenes are overproduced in response to a

TABLE 3. ^{13}C chemical shift assignments for epoxytrichothecene skeleton

Carbon no.	^{13}C chemical shift for:		
	T-2	B-Neo	P-Neo
2	78.7 d	78.8 d	78.7 d
3	78.3 d	78.4 d	78.4 d
4	84.5 d	84.5 d	84.6 d
5	48.4 s	48.3 s	48.5 s
6	43.0 s	43.0 s	43.0 s
7	27.8 t	28.1 t	27.8 t
8	68.0 d	68.0 d	68.2 d
9	136.2 s	136.4 s	136.3 s
10	123.7 d	123.6 d	123.7 d
11	67.3 d	67.3 d	67.4 d
12	64.3 s	64.4 s	64.3 s
13	47.2 t	47.2 t	47.2 t
14	6.8 q	6.8 q	6.9 q
15	64.6 t	64.8 t	64.6 t
16	20.3 q	20.2 q	20.3 q
1′	172.6 s	176.5 s	173.9 s
2′	43.6 t	34.2 d	27.7 t
3′	25.7 d	[18.6 q	9.1 q
4′	[22.3 q	[19.1 q	
5′	[22.4 q		
CH_3	[172.5 s	172.5 s	172.6 s
O-C=O	[170.0 s	170.1 s	170.1 s
O-C-CH_3	21.0 q	21.0 q	21.0 q

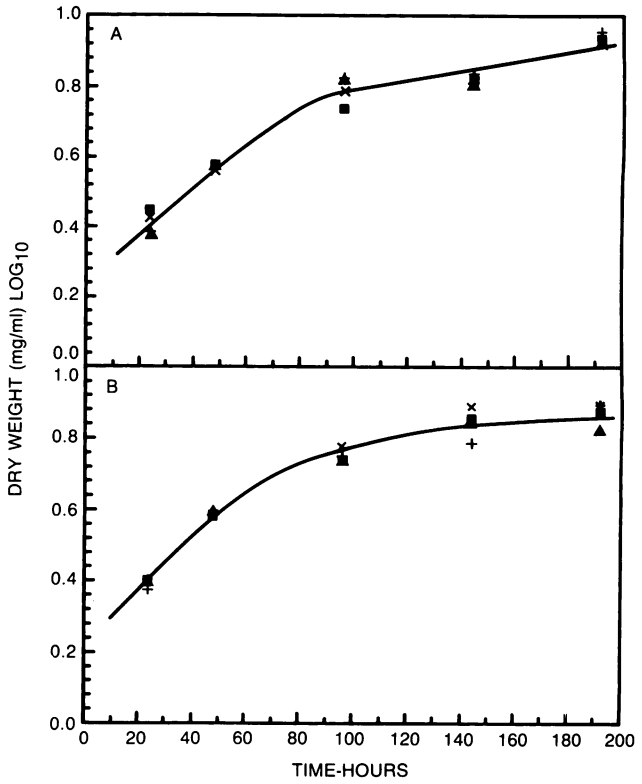

FIG. 4. Growth of *F. sporotrichioides* NRRL 3299 wild type (A) and MB2915 Leu$^-$ mutant. (B) Strains were cultivated in liquid shake cultures at 28°C and 180 rpm by using YEPD-5G medium supplemented with 0 (■), 10 (▲), 50 (×) and 100 (+) μg of L-leucine per ml. Following inoculation at a density of 4 × 10^4 conidia per ml with conidia from 7-day-old V-8 and V8C+Leu agar cultures, the liquid cultures (150 ml of media in 300-ml flasks) were sampled at intervals for growth as measured by mycelial dry weight. Each point is the average of 4 determinations consisting of duplicate samples from duplicate cultures.

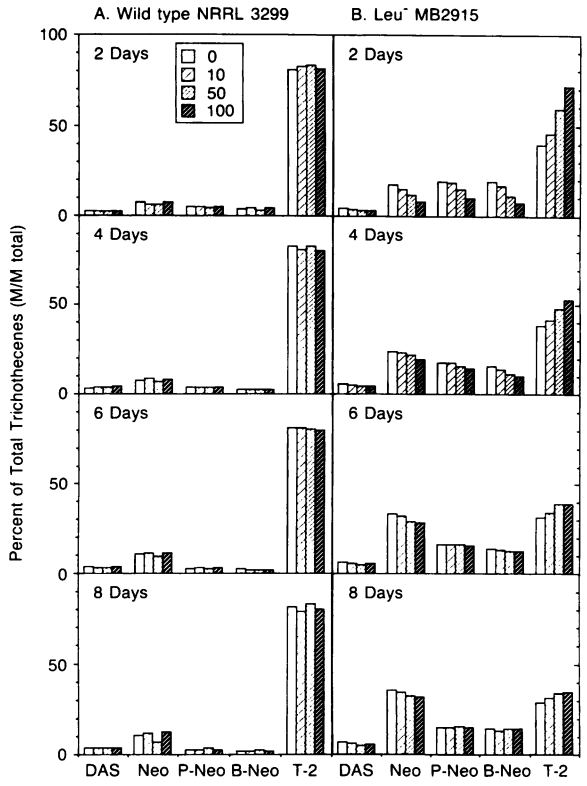

FIG. 5. Effect of leucine concentration on the pattern of trichothecene production by the NRRL 3299 wild-type and MB2915 Leu⁻ mutant strains of *F. sporotrichioides*. Data were obtained for cultures grown in YEPD-5G complete medium amended with 0, 10, 50, or 100 μg of additional L-leucine per ml. Samples, collected from the same cultures used to measure growth in Fig. 4, were assayed for trichothecene content by GC analysis. Samples were prepared and analyzed as described for Fig. 2. Duplicate MB2915 cultures were analyzed, and the averages were plotted; the average range between the two data points for each sample was ±0.65, with the maximum difference being ±2.8. Data for a single set of wild-type cultures are shown. All values are reported as molar ratios (M/M).

leucine limitation imposed by leucine auxotrophy. This overproduction led us to identify, purify, and chemically characterize these two compounds which proved to be T-2 analogs. During the course of this work, others independently reported the production of trace amounts of compounds assigned as B-Neo and P-Neo by various strains of *F. sporotrichioides* (2, 6, 15).

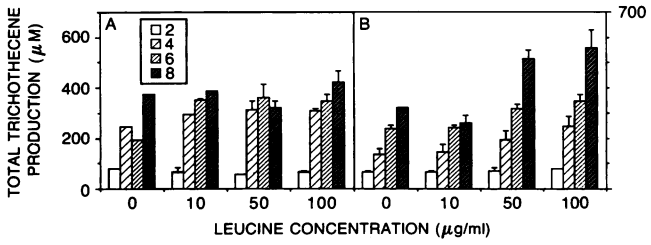

FIG. 6. Effect of leucine concentration on the kinetics and level of trichothecene production by the NRRL 3299 wild type (A) and MB2915 Leu⁻ mutant (B) strains of *F. sporotrichioides*. Data were obtained for cultures grown for 2, 4, 6, and 8 days in YEPD-5G complete medium amended with 0, 10, 50, or 100 μg of additional L-leucine per ml as described for Fig. 5. Data represent the averages of values from duplicate cultures (error bars show range of duplicates), except for the wild-type strain grown with 0 μg of additional leucine per ml; in this case, data were obtained for a single culture.

TABLE 4. Level of trichothecene production by wild-type NRRL 3299 and Leu⁻ MB2915 strains of *F. sporotrichioides*

Strain	Leucine added (μg/ml)	Trichothecene level (μg/ml)[a]					Total concn (μM)
		DAS	Neo	P-Neo	B-Neo	T-2	
NRRL 3299	0	5	15	4	4	141	374
	100	6	18	4	5	150	378
MB2915	0	8	44	21	20	43	320
	100	11	67	37	36	89	558

[a] All values represent amounts present in 8-day-old liquid shake cultures and are averages from duplicate cultures, except for NRRL 3299 at 0 μg of leucine per ml, which is from a single culture.

Mutants are useful for studying biosynthetic pathways. It is well known that if several metabolites are derived from branches of a common biosynthetic pathway, then a mutation in one of the branches can result in the overproduction of the end products from the other interconnecting pathways (8, 10). Production of the wild-type end product phenotype may be restored by supplying the metabolite that the mutant is unable to synthesize. This type of directed channeling of metabolites to alternative end products can provide information about the biosynthetic pathway(s) and their associated regulatory mechanisms. The utilization of a leucine-requiring mutant in the present study has provided such new information about the trichothecene biosynthetic pathway.

On the basis of the results reported here, a partial sequence of the trichothecene biosynthetic pathway has been elucidated. The data demonstrate that L-leucine is a biosynthetic precursor to T-2 toxin and, more specifically, that it is a direct precursor to the isovalerate moiety present at the C-8 position. Additional data further suggest that the trichothecene biosynthetic pathway in this strain is branched such that Neo, P-Neo, B-Neo, and T-2 are alternative end products synthesized from a common intermediate(s) (Fig. 7). Analogous to the isovalerate side group in T-2, the isobutyryl side group in B-Neo may be derived from valine; this would suggest that other trichothecenes may also result from a mixed biogenesis involving both the isoprenoid and an amino acid biosynthetic pathway.

The findings from this research have also contributed to the understanding of the mechanisms which regulate the production of the trichothecene secondary metabolites. We have shown that the flow of intermediates through the metabolic pathway leading to the trichothecenes in *F. sporotrichioides* can be modified by the availability of leucine, and that the availability of leucine can be controlled by an

TABLE 5. Specific activity of trichothecenes from L-[^{3}H]leucine incorporation[a]

Strain	Trichothecene	[^{3}H] cpm/HPLC unit of trichothecene
NRRL 3299	T-2	302.0
MB2915	T-2	222.0
	P-Neo + B-Neo	8.6
	Neo	2.5
	DAS	3.7
MB1716	DAS	15.0

[a] ^{3}H incorporation was quantified for 92-h cultures which had been labeled by the addition of [^{3}H]leucine following 44 h of incubation.

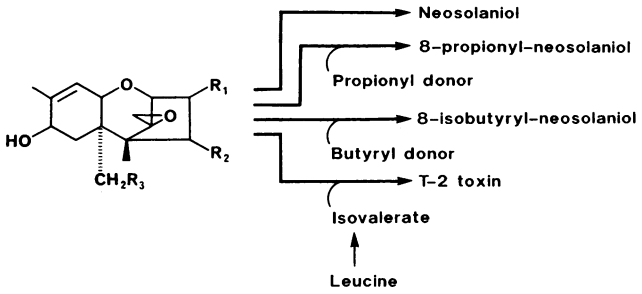

FIG. 7. Proposed biosynthetic scheme for the coproduction of T-2 and T-2 analogs by *F. sporotrichioides*.

environmental factor (leucine concentration in the medium) when superimposed on a genetic factor (leucine auxotrophy). The fact that the concentration of leucine can determine the amount of T-2 produced, relative to the amounts of Neo, P-Neo, and B-Neo produced, suggests that not only are these four compounds derived from a common intermediate, but that there is competition for this intermediate among the branched pathways leading to these cometabolites. Furthermore, that competition must be related, at least in part, to the availability of the different C-8 side group precursors. Presumably, the intracellular pool size of leucine can regulate the biosynthetic rates of Neo, P-Neo, B-Neo, and T-2, and in the absence of a threshold level of leucine, propionyl and butyryl donor groups are more efficiently incorporated into the trichothecene biosynthetic pathway. Finally, the pathway to Neo could either be an active, alternative competing route, or Neo could simply accumulate because substrates for the side groups at C-8 are depleted or limited.

The utilization of leucine in the biosynthesis of T-2 provides another link between the production of these secondary metabolites and primary metabolism. This also potentially places the production of T-2 in competition with primary metabolic processes (most notably protein synthesis) that depend upon leucine. When primary and secondary metabolites are derived from a common precursor, then both biosynthetic pathways may compete for the intracellular pool of that compound. Katz and Weissbach (7) presented data which indicated that such competition occurs during the biosynthesis of the *Streptomyces antibioticus* amino acid-derived actinomycin antibiotics. In the present case, it would appear that the amount of leucine available to the leucine-requiring cells from the peptone in the unsupplemented complete medium is sufficient to support a wild-type growth rate and, thus, primary metabolism; however, there is only enough leucine to support limited production of T-2. This suggests that the exogenously supplied leucine may be preferentially channeled into primary metabolic pathways. This channeling may be largely the result of the temporal difference between primary metabolism and secondary metabolism (7).

The knowledge gained from this study can be used to manipulate the amount of kinds of trichothecenes produced by the Leu$^-$ mutant. Under the proper conditions, large amounts of P-Neo, B-Neo, and Neo can be readily obtained. Future studies involving mutants and the manipulation of growth conditions to alter trichothecene production will continue to provide more information about the trichothecenes and their synthesis.

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