T-2 Toxin Biosynthesis: Origin of the Isovalerate Side Chain

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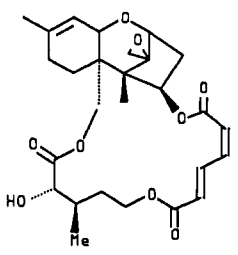
In biosynthetic incorporation experiments ^{13}C-labeled leucine was shown to be incorporated into the C-8 isovalerate side chain of T-2 toxin (2). Other incorporation experiments and implications to the mevalonate shunt pathway as well as to directed biosynthetic studies are also discussed.

The trichothecenes are a large class of mycotoxins which as protein-synthesis inhibitors exhibit a variety of biological activities.[1] Members of the trichothecene class of natural products differ mainly in their levels of oxidation and in the composition of their ester side chains. Various natural and synthetic side chains have profound effects on the biological activities of the >140 members of this family.[2] These effects are presumably due to differences in solubility, membrane permeability, ion complexation, and 3D site recognition. An understanding of the biosynthetic attachment of these side chains is essential to the rational control of the biological production of the trichothecenes.

While the sesquiterpenoid nature of the trichothecene carbon skeleton has been well documented,[3] only limited studies have been carried out concerning the origin of the side chains. In one study involving radiolabeled incorporations and extensive chemical degradations, the macrocyclic triester side chain of Verrucarin A (1) was shown to be derived from a mevalonic acid unit and a polyacetate unit.[4] Even though T-2 toxin (2) is one of the most potent of the trichothecene protein synthesis inhibitors, only preliminary biosynthetic studies in connection with a radiolabeling study of 2 have been reported.[5] Studies concerning the origin of the oxygen atoms and speculation concerning the biosynthesis of 2 have appeared.[6] We describe herein studies elucidating the biogenesis from leucine of the C-8 isovaleroxy side chain of T-2 toxin as well as attempts to incorporate alternative subunits.[7]



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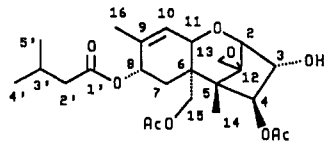

2

Since the ^{13}C NMR spectrum of 2 has been completely assigned,[8] ^{13}C-labeled precursor incorporation studies were particularly appealing. One remaining ambiguity in the ^{13}C NMR assignment for 2 is in the carbonyl region. Since the carbonyl carbons were of interest in this study and are not well-resolved (170, 172.5 and 172.6 ppm), a direct spectral analysis was undertaken in order to attempt to distinguish the isovalerate from the acetate carbonyls. A gated decoupled ^{13}C NMR spectrum showed the 170 ppm signal to be a quartet, which implied that it arose from one of the acetate carbonyls (probably the C-15 based on homologue comparisons). The signals at 172.5 and 172.6 ppm overlapped in this experiment and in most of the spectra obtained, so the isovalerate carbonyl and one of the acetate carbonyls were indistinguishable in this study.

Table I. ^{13}C Enrichments in T-2 Toxin Derived from ^{13}C-Labeled Acetate and Mevalonic Acid[a]

carbon	ppm[b]	[1-^{13}C]-NaOAc[c]	[2-^{13}C]-NaOAc[c]	[2-^{13}C]-MVA[c]
2	78.7	0.8	**2.5**	0.8
3	78.3	**4.3**	1.0	1.1
4	84.5	1.2	**3.1**	**3.1**
5	48.4	1.7	0.9	0.6
6	43.0	0.6	**2.5**	0.8
7	27.8	**5.6**	1.0	1.1
8	68.0	0.8	**2.5**	**2.7**
9	136.2	**6.3**	0.5	1.1
10	123.7	0.7	**2.7**	0.7
11	67.3	**5.2**	1.0	1.1
12	64.3	**2.6**	1.0	1.0
13	47.1	0.9	**2.8**	1.0
14	6.8	1.2	**4.0**	**3.8**
15	64.6	1.2	**2.3**	1.1
16	20.3	1.3	**3.2**	1.3
2′	43.6	0.9	0.8	0.9
3′	25.7	1.1	0.6	1.0
4′,5′	22.3	1.1	1.3	1.0
4′,5′	22.4	1.2	1.4	1.2
acetyl(Me)	21.0	1.0	**2.8**	1.0
acetate(CO)	170.0	**10.7**	1.5	1.4
acetate(CO) & 1′	172.5	**2.7**	1.2	1.0

[a] Entries in bold are >2σ above 1.0; standard deviations were 0.28 for [1-^{13}C]NaOAc, 0.48 for [2-^{13}C]NaOAc, and 0.42 for [2-^{13}C]MVA (see Experimental Section). [b] All chemical shift values are for $CDCl_3$ solutions referenced to $CHCl_3$ at 77.1 ppm. [c] Enrichment factors are the ratio of signals between labeled and natural abundance spectra normalized to the mean of the unlabeled positions.[13]

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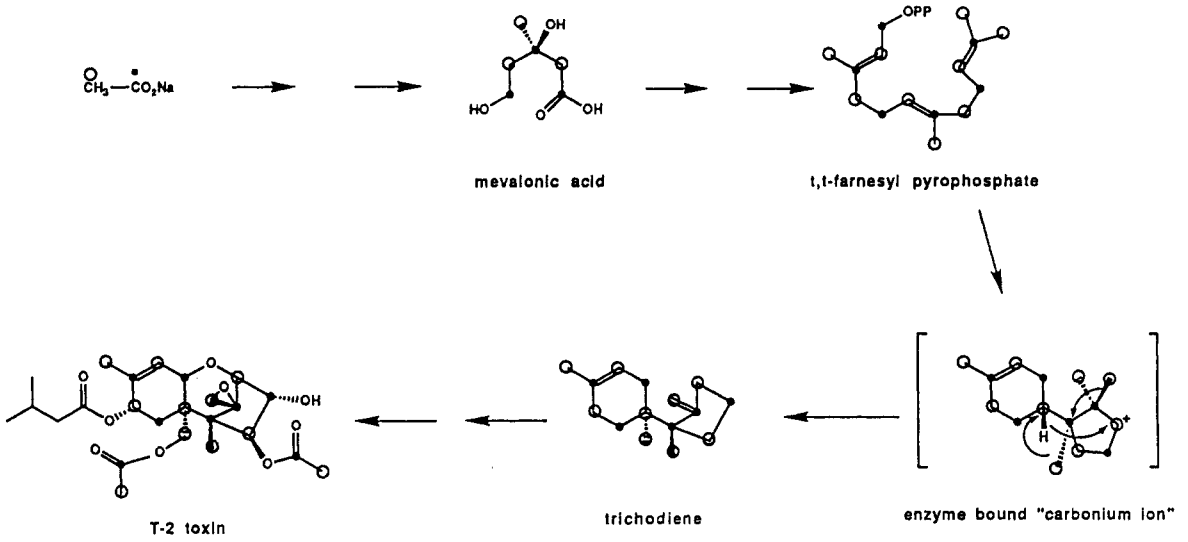
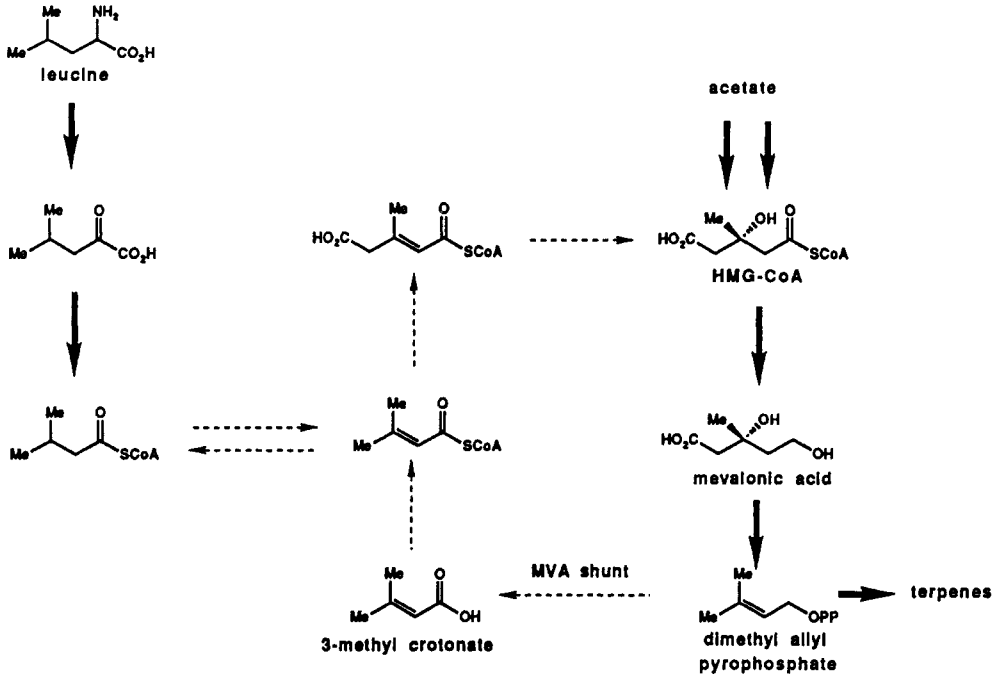
Scheme I. Labeled Acetate and Mevalonate Incorporation into T-2 Toxin



Scheme II. Crossover of Leucine Metabolism, HMG-CoA Utilization, and MVA Shunt



The organism used for these investigations was the well-studied *Fusarium sporotrichioides* NRRL 3299 from which titers of **2** as high as 300 mg/L have been observed in liquid culture.[9] The onset of trichothecene biosynthesis in this organism has been studied,[10] and precursor additions were made at estimated optimal incorporation times.

The complex transformations involved in the biosynthetic formation of the trichothecene carbon framework have been extensively investigated by precursor incorporations.[3] The generally accepted pathway is outlined in Scheme I. The condensations and reductions common to all terpene pathways are followed up to *trans,trans*-farnesyl pyrophosphate (FPP). The formation of trichodiene from FPP has recently been shown to be catalyzed by a single enzyme.[11] This transformation is postulated[3] to involve an isomerization and cyclization to the intermediate depicted, followed by a double 1,2-methyl shift and a 1,4-hydride shift to form trichodiene. Little is known concerning the later stages of trichothecene biosynthesis.

As shown in Table I, the ^{13}C NMR spectra of **2** biosynthesized in the presence of sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, and [2-^{13}C]mevalonic acid showed significant and specific enrichments over natural abundance. The expected incorporations[3] following the well-documented transformations were observed (Scheme I). Numerous other trichothecenes have been shown to incorporate labeled acetate and MVA in exactly the same core positions as were observed for T-2.[3] In addition, the acetates at C-4 and C-15 also showed high enrichments

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Table II. ^{13}C Enrichments in T-2 Toxin Derived from ^{13}C-Labeled Leucine and Alanine[a]

carbon	[3-^{13}C]leucine[b]	[3-^{13}C]alanine[b]
2	0.9	**1.5**
3	1.0	1.1
4	1.1	**2.3**
5	c	1.0
6	0.8	**1.6**
7	c	1.1
8	0.9	**1.4**
9	c	0.8
10	1.0	**1.5**
11	0.7	1.1
12	c	0.9
13	1.0	**1.8**
14	c	**1.9**
15	1.2	1.3
16	1.5	1.2
2′	**4.6**	1.0
3′	0.5	0.9
4′,5′	1.2	**2.0**
4′,5′	1.4	**2.5**
acetyl(Me)	1.1	**1.5**
acetate(CO)	1.0	0.7
acetate(CO) & 1′	c	1.0

[a] Entries in bold are >2σ above 1.0; standard deviations were 0.85 for [3-^{13}C]leucine and 0.21 for [3-^{13}C]alanine (see Experimental Section). [b] Enrichment factors are the ratio of signals between labeled and natural abundance spectra normalized to the mean of the unlabeled positions.[13] [c] Due to the small sample size, these signals were not 4σ above the noise level.

from labeled sodium acetate. The lack of incorporation of labeled acetate into the C-8 3-methylbutanoyl moiety implies that a different biosynthetic pathway is followed for the formation of the side chain of **2** than for the similar 2-methylbutanoyl side chain of the fungal metabolite mevinolin. The side chain of mevinolin has recently been shown to be derived from two acetate units and a methyl from methionine.[12]

We are aware of no report of a specific labeling study testing an amino acid as a biosynthetic precursor to any of the trichothecenes. However, if fungi metabolize leucine similarly to bacteria, isovaleryl-CoA could be produced by an analogous transamination and decarboxylation of leucine[14] (Scheme II). Based on this precedent leucine seemed to be a likely candidate as a precursor to the C-8 isovalerate unit of T-2 toxin.[15]

A complicating factor in leucine incorporation studies could be the existence of a common intermediate, HMG-CoA, in both the leucine degradation pathway and the terpene biosynthetic pathway. The dashed arrows in Scheme II represent enzymatic reactions from various sources[14,16-18] which have been reported in the literature. Overton has demonstrated[16] that in many cases there is no cross-over between these two pathways at the level of HMG-CoA, but very few cases have been studied. If leucine catabolites were to enter the terpene pathway in fungi, singly labeled [3-^{13}C]leucine would be expected to label the 2-, 6-, and 10-positions of the trichothecene carbon skeleton.

Following the six-step route of Overton,[19] [3-^{13}C]leucine was synthesized and tested in incorporation studies. The results of these labeling experiments are presented in Table II. From this data it is evident that [3-^{13}C]leucine was incorporated into the 2′-position of the isovalerate side chain of T-2 with specific and high enrichment. If leucine was also incorporated into T-2 toxin at positions 2, 6, and 10 via HMG-CoA, it was to an indetectable extent (<1%) in this experiment. This observation could be due to the lack of this pathway, or to the presence of a much larger or inaccessable internal pool of HMG-CoA compared to isovaleryl-CoA.

Commercially available [3-^{13}C]alanine was also tested as a precursor to determine whether amino acid transport mechanisms could be partly responsible for the high incorporation of leucine. As shown in Table II, the incorporation of [3-^{13}C]alanine into T-2 was very low; however, the pattern of incorporation remained consistent for the three concentrations of [3-^{13}C]alanine tested (data only shown for 0.25 mg/mL). T-2 positions which showed enrichments could be explained by metabolism of [3-^{13}C]-alanine to [2-^{13}C]acetate or to [3-^{13}C]pyruvate. [2-^{13}C]-Acetate could be incorporated into T-2 as in Table I, and [3-^{13}C]pyruvate could enter the leucine metabolic pathway[17] and show enrichments in the 4′- and 5′-methyl groups of **2**.

Another source of isovaleryl-CoA could hypothetically be from the mevalonate metabolism shunt,[18] shown in Scheme II on the right, in which 3,3-dimethylallyl pyrophosphate can be converted to 3-methylcrotonyl-CoA and presumably to isovaleryl-CoA. If this were a predominant pathway in fungi, [2-^{13}C]mevalonic acid and [3-^{13}C]methyl crotonate would each be expected to label **2** in the 4′- or 5′-position. No enrichments over natural abundance were observed for attempted incorporations of [3-^{13}C]methyl crotonate, and the enrichments from [2-^{13}C]MVA (Table I) were in the trichothecene core.

This observation that leucine is a direct biosynthetic precursor to T-2 toxin extends our previous analysis of a leucine auxotroph derived from *F. sporotrichioides* NRRL 3299.[20] Under standard growth conditions this UV-induced mutant forms very low titers of **2** and in turn produces increased titers of two novel trichothecene alternate end products identified as the propionyl and isobutyryl analogues of **2**. Directed biosynthetic studies with this mutant indicated that leucine was a T-2 precursor and that the altered trichothecene production was a direct result of reduced levels of leucine available to serve as a precursor to the isovalerate moiety in T-2. The results from the present study support this mixed biosynthesis hypothesis.

Experimental Section

General Fermentation Conditions. YEPD-5G medium, containing 0.1% yeast extract, 0.1% peptone, and 5.0% glucose, was used to support trichothecene production in liquid shake cultures. Liquid cultures were inoculated to a final density of 7×10^4 conidia/mL of medium using conidia washed from the surface of *F. sporotrichioides* NRRL 3299 cultures grown for 7 days on V-8 juice agar plates.[21] All liquid cultures were incubated

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at 28 °C and 180 rpm on a rotary shaker.

Preparation of Labeled Substrates. Samples of sodium [1-^{13}C]acetate (99% ^{13}C) and sodium [2-^{13}C]acetate (99% ^{13}C) were obtained from Aldrich Chemical Co. [3-^{13}C]Alanine (98% ^{13}C) was obtained from Merck and Co. [2-^{13}C]Mevalonic acid was synthesized in four steps from 3-buten-1-ol and [2-^{13}C]ethyl bromoacetate (99% ^{13}C, Aldrich) essentially by the procedure of Tanabe.[22] [3-^{13}C]Leucine was synthesized by the procedure of Overton,[15] and the label was introduced from $Ba^{13}CO_3$ (99% ^{13}C, Aldrich). 3-(2*E*)-[3-^{13}C]Methylcrotonic acid was prepared from [^{13}C]iodomethane (99% ^{13}C, Aldrich) by the method of Aberhart.[23]

Addition of Labeled Substrates. Labeled substrates were administered to 44–48 h liquid cultures (50–100 mL), which were then incubated for an additional 48 h to allow incorporation of labeled compounds. Sodium [1-^{13}C]acetate and sodium [2-^{13}C]acetate were each added at three different final concentrations of 0.25, 0.50, and 1.00 mg/mL. Results from the 1.00 mg/mL experiment are reported in Table I. [2-^{13}C]Mevalonic acid was also added at three final concentrations: 0.05, 0.10, and 0.25 mg/mL. Results from the 0.25 mg/mL experiment are reported in Table I. [3-^{13}C]Leucine was added at a final concentration of 0.32 mg/mL (0.25 mM). [3-^{13}C]Alanine was added at final concentrations of 0.05, 0.10, and 0.25 mg/mL, and the results from the 0.25 mg/mL experiment are shown in Table II. 3-(2*E*)-[3-^{13}C]Methylcrotonic acid was added at 0.05 and 0.25 mg/mL.

^{13}C NMR Spectra. ^{13}C nuclear magnetic resonance spectra were acquired in $CDCl_3$ at 75 MHz on a Bruker WM-300WB instrument. ^{13}C-NMR measurements were made under the following conditions: 30° pulse; acquisition time, 0.54 s; spectral width, 15.15 kHz; 105 000 scans; and continuous broad-band ^{1}H decoupling. Relative ^{13}C abundances were determined by calculating the ratio of peak heights (1-Hz line broadening) of labeled and natural abundance spectra and normalizing on the mean of the positions which appeared to be unlabeled.[13] For each experiment, a standard deviation was calculated for the set of unlabeled signals plus the smallest "labeled" signal. In Tables I and II, the entries in bold are two standard deviations above 1.0.

Trichothecene Analysis. Fermentations were monitored for trichothecene production by GC analysis of TMS-derivatized ethyl acetate extracts. Dried extracts were derivatized with TBT (Pierce) at 80 °C for 1 h. A Spectra-Physics 7100 GC equipped with a DB-1 coated (0.25-μm) capillary column (30 m × 0.25 mm) and FID detector were used for the analysis. The GC temperature program consisted of an initial oven temperature of 120 °C with an immediate 15 deg/min gradient to 210 °C for 1 min and a 5 deg/min gradient to 260 °C for 10 min. The retention time observed for T-2(TMS) in this system was 17.9 min.

General Isolation Procedure. The above fermentations (50–100 mL) were extracted with equal volumes of ethyl acetate (3×), and the solvents were removed in vacuo. The residue was then applied directly to a silica TLC plate (2mm, Merck) and developed with 98% $CHCl_3$ 2% MeOH. The band with an R_f = 0.35–0.45 was then scraped and eluted with ethyl acetate. Final purification was carried out by reverse-phase HPLC in a Spectra-Physics 8100 HPLC equipped with a Zorbax ODS column (25 cm × 0.46 cm i.d.) and was followed by UV detection at 205 nm. The mobile phase employed was an isocratic system of 70% MeOH/H_2O. Under these conditions the retention time for T-2 toxin was 20 min. Yields of T-2 toxin obtained from the 50–100-mL cultures varied from 1.7 to 11.7 mg.

Registry No. 2, 21259-20-1; leucine, 61-90-5; isovaleric acid, 503-74-2.

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Reduction of *o*-Hydroxybenzaldehydes by Aqueous Titanium Trichloride. A New Route to 2-(Benzofuran-2-yl)phenols

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Reduction of *o*-hydroxybenzaldehydes **1a–k** by aqueous titanium trichloride is a new simple way to the synthesis of the title compounds **2a–k**. The temperature at which the reduction occurs (50 or 80 °C) is related to the nature and position of the R group in the benzene ring. When the reduction is performed at 0 °C in the presence of acetaldehyde, stereoselective formation of 1,3-dioxolanes **7** occurs, due to in situ condensation with the intermediate diols. A mechanism is proposed to account for the formation of both **2** and **7**. Two-dimensional NMR methods have been used to completely assign ^{1}H and ^{13}C NMR resonances of the 2-(benzofuran-2-yl)phenols **2a–d,f–h**.

In recent years much attention has been given to the antifungal activities of certain hydroxy- and methoxy-substituted 2-phenylbenzofurans,[1] and different methods have been reported[2] for the synthesis of these compounds, structurally related to the phytoalexins Vignafuran[3] and Moracin.[4] On continuation of our studies on the reducing properties of aqueous titanium trichloride,[5] we report herein a new, one-step synthesis of 2-(benzofuran-2-yl)-

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