Biosynthetic Studies of Fumonisin B_1 and AAL Toxins

Eloisa D. Caldas†

Department of Food Science and Technology, University of California, Davis, California 95616

Katerina Sadilkova and Barney L. Ward

Department of Plant Pathology, University of California, Davis, California 95616

A. Daniel Jones

Facility for Advanced Instrumentation, University of California, Davis, California 95616

Carl K. Winter

Department of Food Science and Technology, University of California, Davis, California 95616

David G. Gilchrist*

Department of Plant Pathology, University of California, Davis, California 95616

The biosynthesis of the sphinganine analogue mycotoxins (SAMs) fumonisin B_1 and the AAL toxins was studied by growing *Fusarium moniliforme* and *Alternaria alternata* f. sp. *lycopersici* in liquid culture. Radioactive and stable isotopically labeled amino acid, water, and molecular oxygen precursors were added to the culture media and toxins were analyzed using thin-layer chromatography, liquid scintillation counting, high-performance liquid chromatography (HPLC), ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and electrospray mass spectrometry (ESMS). Results indicated that glycine was incorporated directly into the AAL toxins and that methionine was incorporated into the AAL toxin methyl groups. Oxygens in tricarballylic acid moieties for fumonisin B_1 and the AAL toxins were derived from H_2O while the lipid backbone hydroxyls for fumonisin B_1 and the AAL toxins originated from molecular oxygen. Isotopic enrichment patterns for the various AAL toxins showed marked differences among toxins, suggesting a complex, rather than sequential, biosynthetic pattern.

Keywords: *Mycotoxins; sphinganine analogue mycotoxins; isotope enrichment*

INTRODUCTION

Fumonisins and AAL toxins are sphinganine analogue mycotoxins (SAMs) produced by *Fusarium moniliforme* and *Alternaria alternata* f. sp. *lycopersici*, respectively. Collectively, these fungi are ubiquitous saprophytes and successful plant pathogens associated with both pre- and postharvest tissues of a number of plant species. The toxicology of SAMs is fundamentally interesting because they appear to induce changes in cellular homeostasis, leading to either death or proliferation of target cells (Gelderblom et al., 1992; Gelderblom et al., 1988a,b; Marasas et al., 1988a; Merrill et al., 1996; Nelson et al., 1993; Tolleson et al., 1996). Because of the frequency and level of contamination of maize and the toxicity of fumonisins to animals, serious concern exists over the dual roles, inducing both proliferation and apoptosis, that these mycotoxins may play in animal and human disease. Part of this concern arises from emerging information linking the biological activity of SAMs to apoptosis in animal cells.

We demonstrated previously that SAM-induced cell death displays stereotypic hallmarks of apoptosis in both tomato cells (Wang et al., 1996a) and in African green monkey kidney CV-1 cells at 10−50 nM (Wang et al., 1996b). Morphological markers indicative of apoptosis in the tomato and the CV-1 cells included TUNEL positive cells, DNA ladders, Ca^{2+}-activated nucleosomal DNA cleavage, and formation of apoptotic-like bodies. The fact that the SAMs bear structural relationships to sphinganine and sphingosine suggested a connection to sphingolipids. Several reports now link the effects of the fumonisins on animals and cultured animal cells to altered sphingolipid metabolism (reviewed by Merrill et al., 1996, 1997).

Signal transduction involving ceramide and sphingoid bases has emerged as an important lipid-based second messenger system in development, cell proliferation, and degenerative diseases in animals through regulating a diversity of responses from gene expression to kinase cascades (Chao, 1995; Merrill et al., 1996, 1997; Obeid and Hannun, 1995; Pena et al., 1997). The linkage of ceramide signaling to apoptosis, cancer, and

* Author to whom correspondence should be addressed. [Fax, (530) 752-5674; e-mail, dggilchrist@ucdavis.edu]

† Present address: Pharmaceutical Science Department, University of Brasilia, Brasilia, DF, Brazil.

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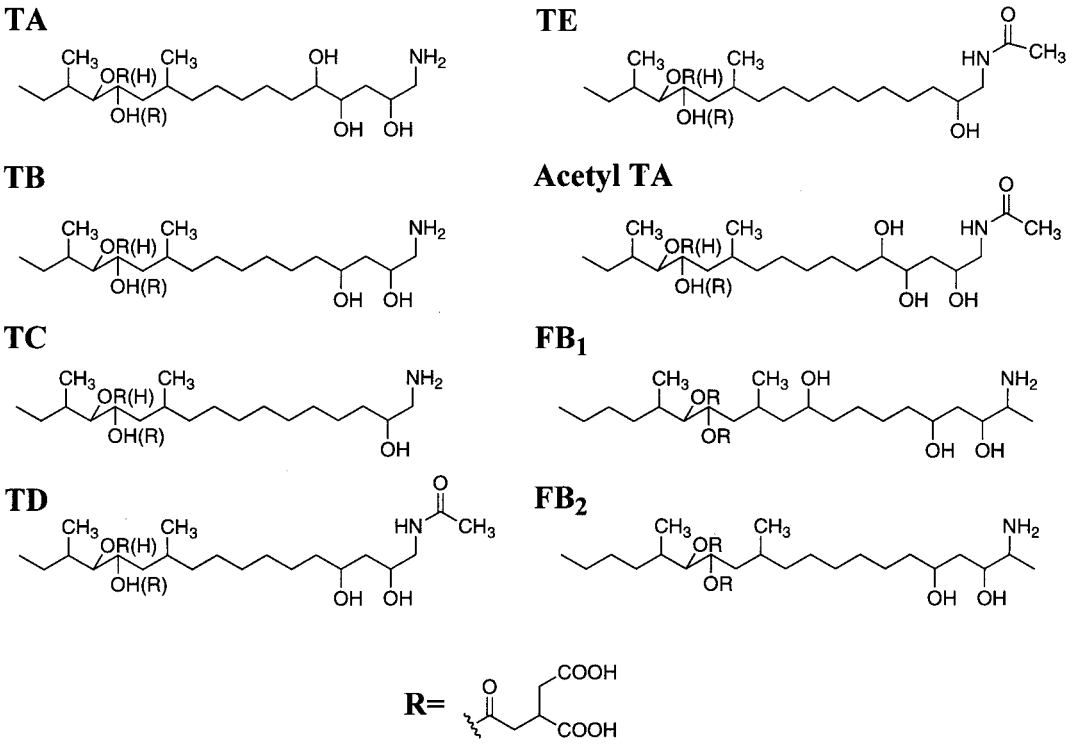


Figure 1. Structures of AAL toxins and fumonisins.

degenerative disease in animals is now driving a rapidly emerging novel area in signal transduction in animal biology (Hannun 1996; Gilchrist, 1997). The biosynthesis of the biologically active and inactive congeners of both the fumonisins and AAL toxins is of great interest in terms of the pathways involved, the mechanisms that regulate secretion of the toxins, and the mode of action of the various congeners.

Six analogues of AAL toxins have been reported (Caldas et al., 1995) that are mono-tricarballylic esters of long-chain polyhydroxyl alkylamines or alkylamides (Bottini and Gilchrist, 1981; Bottini et al., 1981; Caldas et al., 1994) (Figure 1). Fumonisins are di-tricarballylic esters of structurally similar alkylamines and alkylamides (Bezuidenhout et al., 1988) (Figure 1). In particular, fumonisin B_1 (FB_1) exhibits animal toxicity (Marasas et al., 1988a; Harrison et al., 1990) and has been linked to high incidences of esophageal cancer (Marasas et al., 1988b; Chu and Li, 1994). FB_1 has been evaluated by the International Agency for Research on Cancer as "possibly carcinogenic to humans" (IARC, 1993).

The mechanism of action of SAMs is believed to involve disruption of sphingolipid metabolism by inhibition of ceramide synthase (sphinganine *N*-acyltransferase) (Merrill et al., 1993), possibly leading to stimulation of DNA synthesis by accumulation of sphingoid bases (Schroeder et al., 1994). Changes in sphingolipid metabolism leading to increases of ceramide, a potent second messenger in animal systems, also could have dramatic effects on signal transduction pathways regulating cellular homeostasis (Merrill, 1991), including those regulating apoptosis. Structural relatedness of SAMs to intermediates in sphingolipid biosynthesis is the presumed molecular basis of ceramide synthase inhibition and subsequent disruption in this critical biosynthetic pathway. Similarly, structural relatedness is the basis for an unresolved hypothesis that these fungi produce SAMs via a pathway resembling the sphingolipid pathway.

The biosynthetic pathway for sphinganine leading to the formation of complex sphingolipids in all eukaryotic organisms begins with the condensation of L-serine with palmitoyl CoA to form 3-ketosphinganine followed by reduction to form sphinganine (Braun and Snell, 1968). There is currently limited direct evidence linking ceramide synthesis to AAL toxins or fumonisins. Both polyketide synthetic pathways and ceramide-related pathways have been suggested as possible routes for toxin biosynthesis. Branham and Plattner (1993) presented evidence indicating that alanine is incorporated intact into FB_1. In contrast, Blackwell et al. (1996), concluded from studies using labeled acetate in liquid culture of *F. moniliforme* that the backbone of the molecule is of polyketide origin. In addition, these authors found very poor incorporation of [^{13}C]alanine and serine into the FB_1 molecule.

Labeling studies remain the best approach to resolving the SAM biosynthetic pathway. Development of procedures to produce SAMs with high specific radioactivity also has a side benefit of providing labeled molecules for toxicological and metabolic studies. This report focuses on efficiently labeling FB_1 and AAL toxins using radioactive or isotopically labeled precursors added to liquid cultures of *Fusarium moniliforme* and *Alternaria alternata* f. sp. *lycopersici*, respectively. Separation of the stable isotope-labeled AAL toxin congeners was followed by use of NMR spectroscopy and electrospray ionization mass spectrometry to assign and quantify the level of label incorporation. The major goals of these experiments were to determine if amino acids are incorporated into the AAL toxins and to determine the biosynthetic nature of the tricarballylic acid and lipid backbones of FB_1 and the AAL toxins.

MATERIALS AND METHODS

Chemicals. [2-^{14}C]Glycine (specific activity, 57.5 mCi/mmol) and L-[*methyl*-^{14}C]methionine (specific activity, 40 mCi/mmol) were purchased from ICN Biomedicals, Inc (Irvine, CA). [2-^{13}C,^{15}N]Glycine (99% pure) was purchased from Isotec, Inc. (Miamisburg, OH); an electrospray mass spectrum confirmed that 99% of the glycine contained two heavy isotopes. L-[*methyl*-^{13}C]methionine was obtained from Cambridge Isotope (Andover, MA). Naphthalene-2,3-dicarboxaldehyde (NDA) was obtained from Molecular Probes, Inc. (Eugene, OR). Molecular oxygen $^{18}O_2$ (minimum 99 atom %) was purchased from Isotec and heavy oxygenated water $H_2{}^{18}O$ (70% atom ^{18}O) was purchased from Cambridge. Trifluoroacetic acid (TFA), methyl-d_3 alcohol-*d* (99.8% D), KCN, $Na_2B_4O_7$ and KH_2PO_4 were purchased from Aldrich Chemical Co. (Milwaukee, WI). All solvents used were HPLC grade (Fisher Scientific). Strong anion-exchange (SAX) cartridges were purchased from Varian Associates (Harbor City, CA). Liquid scintillation cocktail (Ready Safe) was obtained from Beckman Instruments (Fullerton, CA). Amberlite XAD-2 and *p*-anisaldehyde were purchased from Sigma Chemical Co. (St Louis, MO) and silica gel from Selecto Scientific (Kennesaw, GA). Silica TLC plates were purchased from Analtech (Newark, DE). The biologically synthesized toxins were treated as potential carcinogens in all experiments and should be handled with safety precautions reflecting their toxicity.

Amino Acid Medium Composition. To determine the effect of amino acid composition on toxin production, *A. alternata* f. sp. *lycopersici* was grown under standard culture conditions, on the laboratory bench, using media which includes yeast extract as a limiting source of amino nitrogen (Clouse et al., 1985). The standard media was modified by substitution of pectin for glucose (6 g/L) and 2.5−10 mM of glycine, or methionine for asparagine as will be indicated in the specific experiments. The liquid cultures were sampled, filtered in a 0.45 μm nylon filter and TA and TB concentration determined by HPLC as described below.

HPLC Analysis. KCN (300 nmol) was added to a 3-mL vial containing the sample. $Na_2B_4O_7{\cdot}10H_2O$ (260 μL, 0.05 M, pH 8.5) was added followed by 150 nmol of NDA (Winter et al., 1995). The sample was mixed thoroughly, and the reaction mixture was then incubated at 60 °C for 15 min in a ReactiTherm heating module (Pierce Chemical) followed by chromatography on a Ultracarb 5 ODS 30 (4.5 × 150 mm, Phenomenex) column using isocratic elution. The analysis was performed using a HPLC Beckman System Gold system coupled to a Perkin-Elmer 650 Fluorescence Detector (Ex 420 nm, 10 nm slit, Em 490 nm). The mobile phase consisted of 56% acetonitrile (44% 50 mM H_3PO_4 adjusted to pH 2.8 with NH_4OH) with a flow rate of 1 mL min^{-1}. Toxin concentration was determined against a standard curve of a mixture of TA and TB purified in our laboratory according to Caldas et al. (1995). Purity of standards was >95% on the basis of ^{1}H NMR spectra.

^{14}C Experiments. Preliminary experiments indicated that the surface of an *A. alternata* liquid culture flask was completely covered by mycelia within 2−4 days after inoculation with 1 mL of conidia at 10^6 mL^{-1}, and toxin production was logarithmic from 4 to 9 days (data not shown). Hence, 50 μCi of ^{14}C-labeled glycine or methionine was added to the culture flasks (300 mL per flask, experiments performed in duplicate) at day 3 to achieve maximum labeling of the toxins. The flasks were shaken gently to achieve mixing and maintained as still cultures. Each flask was sampled periodically from day 3 until day 24, when the cultures were harvested. Aliquots of 200 μL were taken for the measurement of total radioactivity in the media by liquid scintillation counting in a Beckman LS 6000SC instrument (Beckman Instruments, Inc, Palo Alto, CA). To a 1.7-mL sample of glycine culture filtrate was added 0.2 g of XAD-2, and the samples were shaken for 1.5 h (Caldas et al., 1994). The XAD-2 beads were washed 3 times with 1 mL of water, extracted 3 times with 1 mL of methanol, and the extract was evaporated to dryness and dissolved in 200 μL methanol. A 50-μL aliquot of this solution was used for analysis of TA and TB by HPLC as described previously, and a 30-μL aliquot was applied to a silica TLC plate and chomatographed in a solvent system consisting of ethyl acetate/acetic acid/hexane/water (6:2:2:1). The plate was scanned for 15 h in a AMBIS Radioanalytic Imaging Systems scanner (AMBIS Systems, Inc, San Diego, CA) to quantify the label associated with each AAL−toxin congener. Afterward the plates were sprayed with *p*-anisaldehyde to visualize the toxins. Methionine coelutes with TA using the solvent conditions described.

Toxin Stability in Culture. A 2-day old fungus mat, grown on 25 mL of glycine-amended medium in a 250-mL flask, was washed 2 times with standard liquid medium lacking both a carbon source and amino acids and then placed back into 10 mL of this same medium. A mixture of ^{14}C-AAL toxins TA and TB (200 μL, 80% pure according to TLC/AMBIS) was added to the medium, and a 100 μL sample was taken every 2 days until day 12 for analysis of toxin stability in the presence of the fungus and the absence of any other carbon source. A 10-mL beaker containing 0.5 mL of 0.5 N NaOH was placed inside the flask to trap $^{14}CO_2$. The radioactivity of the medium and of the NaOH was determined by liquid scintillation counting.

^{13}C and ^{15}N Experiments. [2-^{13}C,^{15}N]Glycine (10 mmol) was added to a 3-day *A. alternata* f. sp. *lycopersici* liquid culture (four flasks) of the standard media without asparagine. Two of the flasks were harvested at day 9 and two were harvested at day 15. The harvested media were filtered and processed using XAD-2 and SAX columns according to Caldas et al. (1994). The toxins were then purified by HPLC (Beckman System Gold, Beckman Instruments, Inc., San Ramon, CA), monitoring absorbance at 210 nm using a reverse-phase C18 column (Ultracarb 7 ODS (30), 10 × 250 mm, Phenomenex, Rancho Palos Verdes, CA), and a gradient of acetonitrile/water (containing 0.1% TFA), 20−50% for 40 min at a flow rate of 5 mL min^{-1}. Each toxin was then repurified in a gradient optimized separately for each toxin.

In a different experiment, 5 mM L-[*methyl*-^{13}C]methionine was added to a 4-day liquid culture (2 flasks) of the standard media without asparagine. The flasks were harvested at day 12, and the toxins were processed as described above.

$^{18}O_2$ and $H_2{}^{18}O$ Experiments. The single conidial isolate of *A. alternata* f. sp. *lycopersici* (AS27-3), grown on corn meal agar (Difco, Detroit, MI), was used for AAL toxin accumulation studies after several modifications of our previously described method (Clouse et al., 1985). A suspension of AS27-3 conidia in sterile water containing 0.05% Tween-20 was used to inoculate sterile medium at a final concentration of 3.3×10^4 conidia mL^{-1}. A liter of 1× medium contained 0.75 g of glycine, 0.1 g of NaCl, 1.31 g of $K_2HPO_4{\cdot}3H_2O$, 0.5 g of $MgSO_4{\cdot}7H_2O$, 0.13 g of $CaCl_2{\cdot}2H_2O$, 0.67 g of L-malic acid, 0.5 g of yeast extract, and 20.7 g of D-glucose. The medium was adjusted to pH 3.1 with 5 N HCl prior to autoclaving for 20 min at 19 psi (121 °C) and remained at pH 3.1 after autoclaving. Preliminary experiments indicated toxin secretion by both *A. alternata* and *F. moniliforme* was very sensitive to pH with a peak secretion occurring at pH 2.4−3.1. Liquid cultures were grown on a laboratory shelf at room temperature (22−26 °C) under cool-white fluorescent lighting (~12 h per day). $^{18}O_2$, $^{16}O_2$, (see apparatus below), and control air experiments were conducted in 250-mL flat-bottom flasks containing 25 mL of medium stirred at 110 rpm. Sterile tissue culture plates (25830, Corning Inc, Corning, NY) were used to conduct stationary $H_2{}^{18}O$ and control air experiments by thoroughly mixing 1 g of $H_2{}^{18}O$, 70% ^{18}O, or unlabeled water; 111 μL of 10× medium, and 4 μL of inoculum were added per well.

F. moniliforme Sheldon strain A00149 (gift of John Leslie) was grown on V-8 agar, flooded with sterile H_2O containing 0.05% Tween-20, and the Petri dish was gently rocked to produce a suspension of microconidia. Sterile medium (see above) pH 2.4 was inoculated to a final concentration of 3.3×10^4 microconidia mL^{-1}. Growth conditions and handling were identical to the *Alternaria* studies.

After 6−11 days of culture, cell-free culture filtrates were prepared by sequential filtration through filter paper (no. 1,

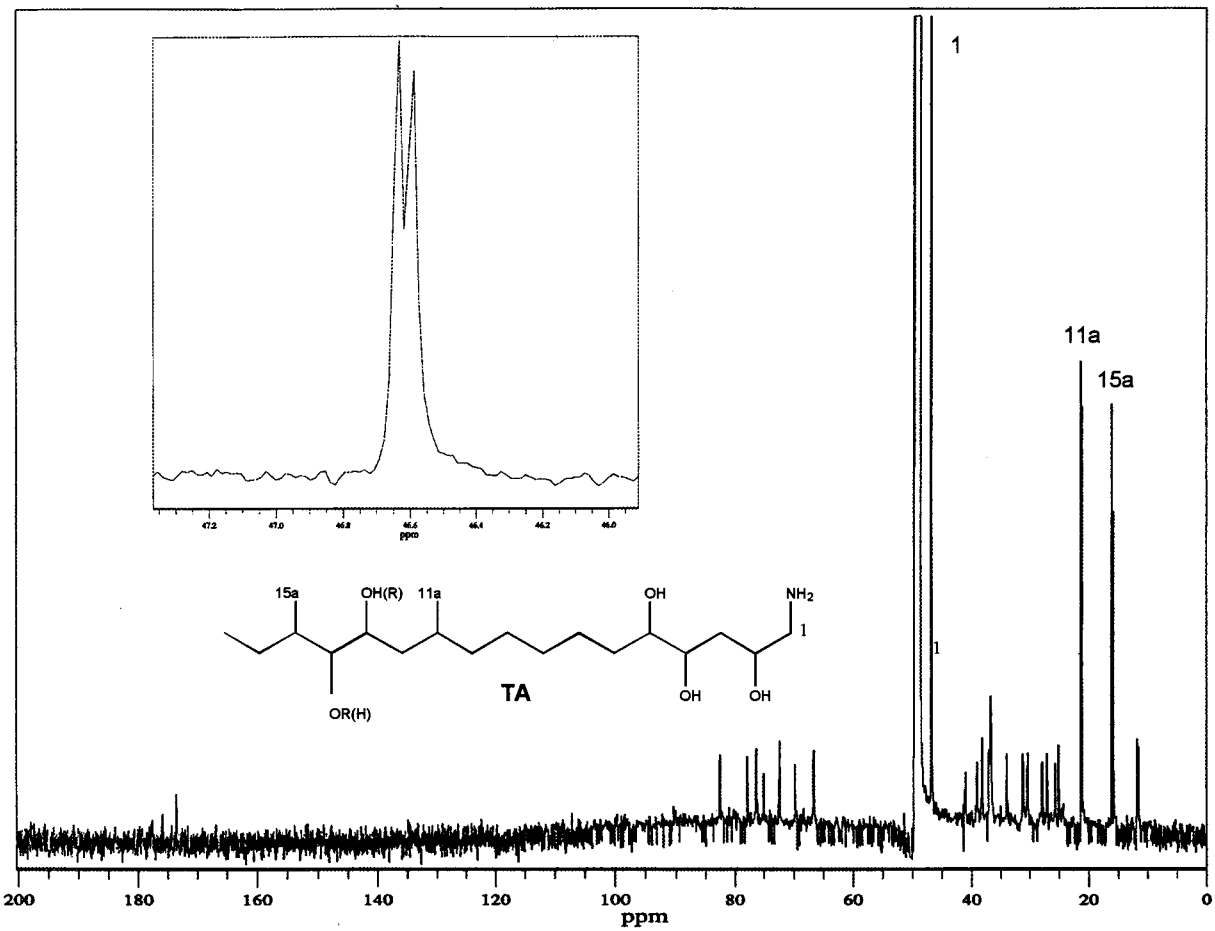


Figure 2. ^{13}C NMR spectrum of AAL toxin TA isolated from cultures of *A. alternata* f. sp. *lycopersici* supplemented with [2-^{13}C,^{15}N]-glycine. Insert illustrates coupling between ^{15}N and C_1.

Whatman, Hillsboro, OR) and nylon 0.45 μm filters (Acrodisc 4426, Gelman, Ann Arbor, MI) followed by storage at −20 °C until analyzed.

Molecular Oxygen Culture Apparatus. Schlenk-type glassware with airtight, threaded ground-glass joints that connected flasks and adaptors was purchased from Aldrich (St. Louis, MO) and modified for fungal culture growth with stirred liquid medium. The design permitted the evacuation, flushing with inert nitrogen gas, and the introduction of gaseous oxygen while maintaining a slight positive pressure on the system during growth without removing or disconnecting any part of the system. The apparatus was configured to permit attachment of a solid CO_2 trap and attachment of a manometer. The culture flask with stir bar was sterilized by autoclaving. Previously sterilized culture medium (25 mL) was added to the flask, and inoculum was added subsequently. The flask was then attached to the apparatus, evacuated, and filled with nitrogen gas, and the evacuation−nitrogen purge was repeated several times. During this procedure, the culture medium was stirred to release air bubbles during the evacuation. Following the final evacuation, $^{18}O_2$ or $^{16}O_2$ gas was introduced into the closed system.

Toxin Purification and Hydrolysis. Cell-free culture filtrates (1−2 mL) obtained from the $^{18}O_2$ and $H_2{}^{18}O$ experiments were loaded onto C18 cartridges containing 0.5 g of packing material (Sep-Pak Vac 3 cm^3, 20805, Waters Corp, Milford, MA), preconditioned with 8 mL of MeOH, and followed by 8 mL of H_2O. The cartridge was washed with 10 mL of H_2O after which the toxins were eluted with 5 mL MeOH, evaporated to dryness at 50 °C under a N_2 stream, and redissolved in MeOH.

Toxin samples, after cleanup on solid-phase extraction C18 cartridges, were diluted to 50% MeOH with H_2O and hydrolyzed with an equal volume of 2 N KOH for 1 h at 70 °C (Plattner et al., 1990), cooled to room temperature, and adjusted to pH 4 with 5 N HCl. The toxin hydrolysis products were then recovered on C18 cartridges as described above.

Spectroscopic Analysis. All negative electrospray spectra were acquired using a VG Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, UK), connected to a micro flow pump (μL-500, Isco, Inc., Lincoln, NE). The flow rate was held constant at 10 μL/min. Loop injections of 10 μL were made using a solvent system of 50% CH_3OH in water. The transport region temperature was maintained at 75 °C, and the capillary voltage was adjusted to give optimum ion yield at about −2.5 kV. The resolution of the mass analyzer was adjusted to give a peak width at half-height of about *m*/*z* 0.25. Full scan spectra were acquired using MCA mode spectrum summation. NMR spectra were obtained using a GE Ω-500 spectrometer (General Electric, Fremont, CA) at 125.758 MHz (^{13}C) using either a 5- or 3-mm probe at room temperature. Operating conditions included a 30 kHz spectral window, 2.0-s recycle time, and a 15-μs pulse. Chemical shifts are reported based upon the chemical shift of the CD_3OD solvent peak at 49.00 ppm.

RESULTS AND DISCUSSION

Effect of Amino Acid Addition on AAL Toxin Production. The level of TA produced from the glycine-amended medium was 463 nmol/mL while the level of TA produced from the methionine-amended medium was 220 nmol/mL at 21 days after inoculation, representing a significant difference ($p < 0.01$) in the amounts produced. In contrast, TB levels were similar in the two media with 127 nmol/mL produced in the glycine medium and 101 nmol/mL in the methionine

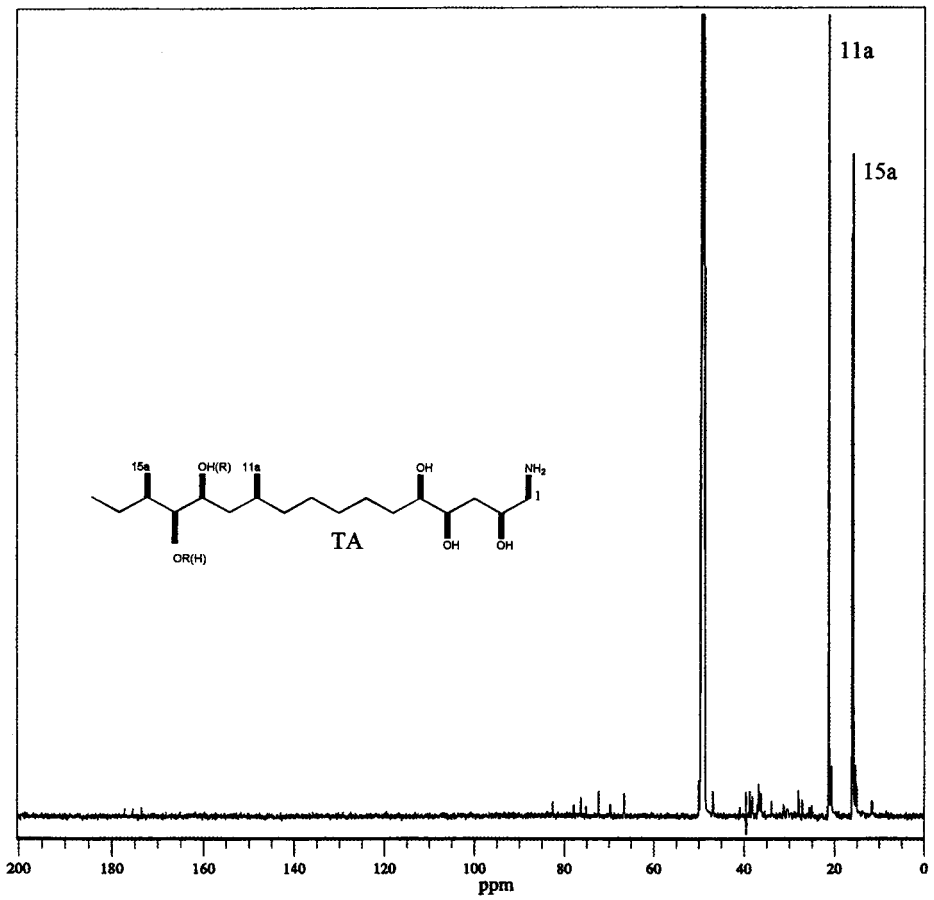


Figure 3. ^{13}C NMR spectrum of AAL toxin TA isolated from cultures of *A. alternata* f. sp. *lycopersici* supplemented with L-[*methyl*-^{13}C]methionine.

medium. The results indicate that the ratio of TA to TB is differentially affected by the choice of amino acid added and that TA is secreted in greater amounts than is TB.

Temporal measurement of the amount of total radioactivity in the media indicated that glycine was taken up into the mat rapidly between days 3 and 6 after which 20% of radioactivity remained in the media. Uptake of methionine was much slower, with removal of label from the medium continuing throughout the 24-day incubation. In contrast to glycine, 95% of the radioactivity was still in the medium at day 6. The overwhelming majority of ^{14}C from the culture media and mycelial mat extracts represented either toxins or the precursor amino acids (glycine or methionine) as determined by TLC/AMBIS. Nitrogen is limiting in media containing only yeast extract, at least for optimal toxin production, as only 10.2 nmol/mL of TA and no detectable TB were produced after 15 days in the amino acid-free medium. These observations suggest that the low uptake of methionine by the fungus, and the consequent low availability of this amino acid as a nitrogen source, might explain why TA production was lower in methionine amended medium, compared with glycine. However, this low uptake was sufficient to sustain TB production, which was produced at the same level in the two amended media. Similar results were found by Alberts et al. (1993), who observed that increased methionine in corn patty cultures of *F. moniliforme* had an inhibitory effect on biosynthesis of FB_1 but not on FB_2.

Incorporation of Glycine into AAL Toxins. The maximum radioactivity found in TA and TB extracted from the [^{14}C]glycine medium was reached at 12 days with no significant change ($p > 0.01$) from that point through day 24. Toxin radioactivity and toxin concentrations in growth medium did not change over time, which suggested that the toxins are not further metabolized after being secreted into the medium. Studies using ^{14}C-labeled toxins TA and TB, added to a carbon-free medium with a 2-day-old fungal mat, did not reveal any toxin turnover or support release of the radiolabeled CO_2 (<1% released after 12 days). The change in the total radioactivity in the medium and in toxin concentration was < 10% during the period from day 12 to day 24. These results indicate that even when the toxins were the sole carbon source, the fungus did not appear to use the toxin as a carbon source or alter the ratio of toxins once secreted.

The specific activity of TA and TB reached a maximum (~110 μCi/mmol) at day 6, after which it dropped to a constant value, remaining stable until day 24 (~20 μCi/mmol). After 6 days, toxin production increased substantially but most likely the radioactive pool is very diluted, yielding toxins with low specific activity. Our interpretation of these results is that toxin biosynthesis continues until about day 12, but the pool of precursors is increasingly diluted with unlabeled precursors after day 6. As a result, the total activity peaked at day 12 at which time the rate of toxin production slowed to nearly zero.

Sites of ^{13}C and ^{15}N Incorporation. Since glycine appeared to be an effective precursor for toxin production in liquid culture, [^{13}C,^{15}N]glycine was added to the standard medium to determine whether both labels were incorporated equally into the toxins as would be

Table 1. Mole Percentage[a] of AAL Toxins Obtained from a [^{13}C,^{15}N]Glycine Medium

	9 days				15 days			
	TA	TB	TD	TE	TA	TB	TD	TE
M	32.0 ± 2.2	52.6 ± 0.6	46.5 ± 0.4	18.4 ± 0.9	16.9 ± 4.3	25.8 ± 0.7	50.9 ± 1.0	17.4 ± 1.0
M + 1	30.7 ± 0.7	28.6 ± 1.5	33.0 ± 0.4	34.4 ± 0.4	32.9 ± 5.1	34.2 ± 0.1	26.0 ± 1.2	32.2 ± 0.5
M + 2	23.4 ± 1.9	12.7 ± 0.5	14.4 ± 0.5	27.9 ± 0.6	25.9 ± 2.9	23.9 ± 0.9	16.9 ± 2.5	27.6 ± 1.7
M + 3	10.0 ± 0.3	3.6 ± 0.4	3.3 ± 0.5	12.9 ± 0.1	15.7 ± 4.6	11.1 ± 0.4	1.6 ± 1.4	14.4 ± 0.7
M + 4	2.7 ± 0.3	1.9 ± 0.6	2.2 ± 0.4	5.1 ± 0.1	6.0 ± 2.4	3.5 ± 0.3	4.8 ± 2.7	6.5 ± 0.5
M + 5	1.4 ± 0.4	0.4 ± 0.4	0.6 ± 0.1	1.4 ± 0.3	2.7 ± 0.8	1.4 ± 0.4	0.2 ± 0.2	1.9 ± 1.4

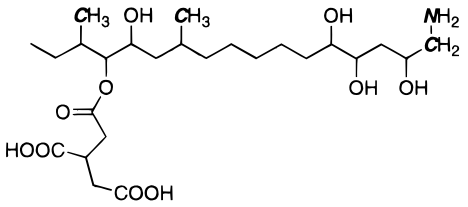
[a] Obtained by subtracting the compound natural abundance from the apparent level of enrichment and expressed as atom % excess ^{13}C.

expected if the C−N bond of glycine remained intact. The ^{13}C NMR spectrum of TA toxin isolated from a 9-day liquid culture (Figure 2) indicates that signal intensities at carbon C_1, C_{11a}, and C_{15a} are highly enhanced relative to the other signals, consistent with a selective incorporation of the C-2 carbon from ^{13}C,^{15}N-labeled glycine into these specific carbons. The peak at C_1 appeared in the spectrum as a doublet (Figure 2, insert), which is attributed to the ^{13}C−^{15}N coupling (J = 5.5 Hz), indicating that both atoms are incorporated together as the amino nitrogen and C_1 of TA. While the possibility exists that ^{15}N could be incorporated into singly labeled molecules without concomitant incorporation of ^{13}C, the clean splitting of the C_1 peak into a doublet provides strong evidence that nearly all, if not all, toxin molecules labeled at C_1 also contain ^{15}N. The ^{13}C spectra of TA from a 15-day culture and of TB and TE from a 9-day culture showed similar enhancements at C_1, C_{11a}, and C_{15a}. This result agrees well with the findings of Branham and Plattner (1993) in which alanine was incorporated specifically at C_1 and C_2 of FB_1. ^{13}C NMR assignments were made in reference to Winter et al. (1995).

Plattner and Shackleford (1992) and Alberts et al. (1993) have shown that the methyl group of methionine is incorporated at C_{12} and C_{16} of FB_1. Our results indicate that the C_2 of glycine is incorporated into analogous methyl groups (C_{11a} and C_{15a}) of TA (Figure 2). It is well-known from numerous biosynthetic studies that the C_2 of glycine can be the source of the methyl group in methionine and, as is the case for FB_1, methionine is the potential donor of the methyl groups in TA. To test whether methionine is the precursor of the methyl group in the AAL toxins, L- [*methyl*-^{13}C]-methionine was added to a liquid culture. The ^{13}C NMR spectrum of TA toxin produced in this medium (Figure 3) shows a selective incorporation of the methyl group of methionine at C_{11a} and C_{15a} of the toxin molecule. L-[*methyl*-^{13}C]Methionine also specifically incorporates the methyl groups into TB and TE at the same position, indicating a common pathway among the various congeners of AAL toxins and fumonisins.

Comparison of Label Incorporation for the Toxin Congeners. Negative mode electrospray mass spectra of the toxins obtained from the [^{13}C,^{15}N]glycine medium at day 9 (not shown) revealed TB and its acetylated analogue TD exhibited similar enrichment at day 9 for all isotopic populations (Table 1). Since TD is the *N*-acetylated form of TB, it is likely that the latter is the precursor of TD, and their similar enrichments suggest acetylation of TB is a fast process. On the other hand, the fraction of TA molecules not containing any heavy isotope (32%) differ substantially from the corresponding fraction of unlabeled TB (52.6%). This finding argues against the prospect that TA forms via rapid hydroxylation of TB.

[2-^{13}C,^{15}N]Glycine



[^{13}C-Methyl]methionine

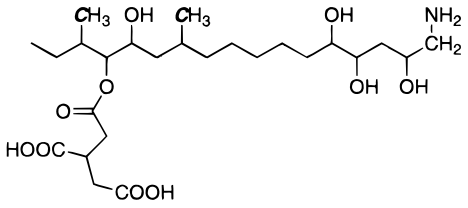


Figure 4. Sites of incorporation of ^{13}C and ^{15}N from labeled glycine and methionine into AAL toxins. Sites of label incorporation are in italics.

By using [^{13}C,^{15}N]glycine medium, the fraction of molecules containing no heavy isotopes ([M]) of TA and TB dropped significantly from day 9 to 15 (Table 1). Toxin concentrations increased substantially during that period (TA increased from 13.8 to 22.6 nmol/mL). Label incorporation during this period is distributed among multiple sites in the toxin molecules. Enrichments for acetylated toxins TD and TE did not undergo substantial change during the same period, suggesting that biosynthesis of acetylated toxins stopped by day 9 while synthesis of the nonacetylated analogues continued. Owing to the possibility that ^{13}C may be present in the acetyl group of acetylated congeners TD and TE, direct comparisons of labeling of nonacetylated toxin TB with its acetylated analogue TD are difficult to interpret.

From the results shown here (Figures 2 and 3 and Table 1), we conclude that the ^{13}C and ^{15}N labels from glycine are incorporated together and equally into the amino group and C-1 carbon of AAL toxins and that methionine is the direct donor of the methyl group at positions C_{11a} and C_{15a} (Figure 4). These findings, in addition to the similar absolute stereochemistry of AAL TA and fumonisins FB_1 and FB_2 (Boyle et al., 1994; Oikawa et al., 1994; Harmange et al., 1994; Hoye et al., 1994; Shier et al., 1995; Boyle and Kishi, 1995) suggest that both fungi, *A. alternata* f. sp. *lycopersici* and *F. moniliforme*, use similar biosynthetic routes for the production of SAMs.

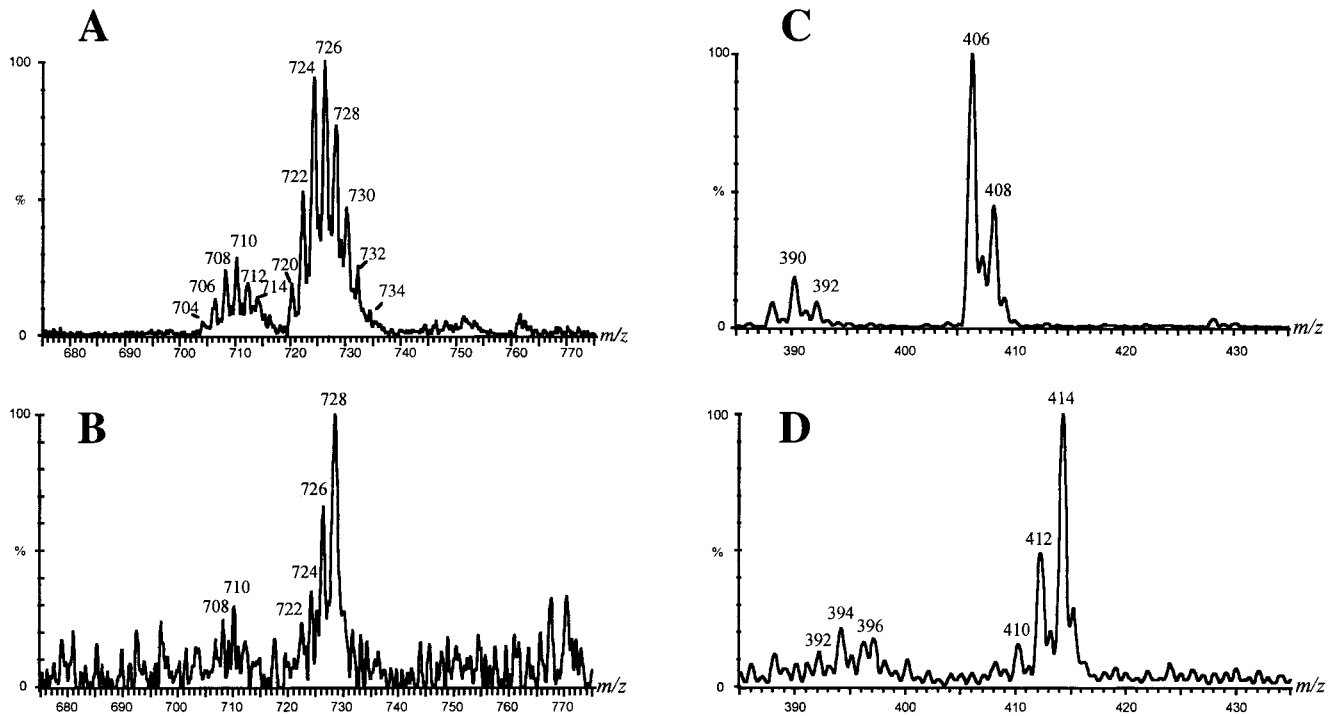


Figure 5. Negative ion electrospray mass spectra of fumonisins FB_1 and FB_2 generated by *F. moniliforme* cultured in $H_2{}^{18}O$ (panel A) and under $^{18}O_2$ atmosphere (panel B). Unlabeled FB_1 gives $[M - H]^-$ at *m/z* 720. Positive ion electrospray mass spectra of aminopolyol hydrolysis products from $H_2{}^{18}O$ cultures (panel C) and $^{18}O_2$ cultures (panel D) show the extent of ^{18}O incorporation.

Incorporation of Labeled Oxygen. The incorporation of isotopically enriched oxygen from water or molecular oxygen was determined by ESMS for FB_1, the AAL toxins, and their related hydrolysis products. Parts A and B of Figure 5 compare the ESMS spectra of the molecular anion of FB_1 from the experiment using $H_2{}^{18}O$-enriched water with that obtained using $^{18}O_2$. The molecular ion $[M - H]^-$ of unlabeled FB_1 would appear at *m/z* 720. The isotopically enriched product from the $H_2{}^{18}O$ experiment (Figure 5A) shows significant incorporation of ^{18}O as is evidenced by isotopomer peaks at *m/z* 722, 724, 726, 728, 730, 732, and 734, revealing incorporation of as many as seven heavy oxygen atoms. As the FB_1 backbone possesses only five oxygens, it is concluded that the isotopic enrichment must involve incorporation of the label in the tricarballylic acid side chains, consistent with the findings of Blackwell et al. (1994). While some exchange between $H_2{}^{18}O$ cannot be ruled out, there is considerable evidence that exchange rates are slow even at pH 4 when solutions are not heated (Murphy and Clay, 1979; Tsikas et al., 1992; Borhan et al., 1995). Significant isotopic enrichment for the molecular anion of FB_1 was also observed in the $^{18}O_2$ study (Figure 5B). Although the number of heavy isotopes incorporated using $^{18}O_2$ was less than observed from the $H_2{}^{18}O$ experiment (Figure 5A), the maximum number of ^{18}O atoms incorporated corresponded to the number of backbone hydroxyl groups, suggesting backbone molecular oxygen incorporation.

ESMS analysis of the hydrolyzed FB_1 (aminopolyol) provided further evidence supporting tricarballylic acid enrichment from $H_2{}^{18}O$ and backbone hydroxyl enrichment from $^{18}O_2$. Figure 5C shows the ESMS spectrum of hydrolyzed FB_1 obtained from the $H_2{}^{18}O$ experiment. Minimal isotopic enrichment is noted, indicating again that water is the source of oxygens for the tricarballylic acid side chains but not for the backbone hydroxyl groups. Figure 5D shows that the hydrolyzed FB_1

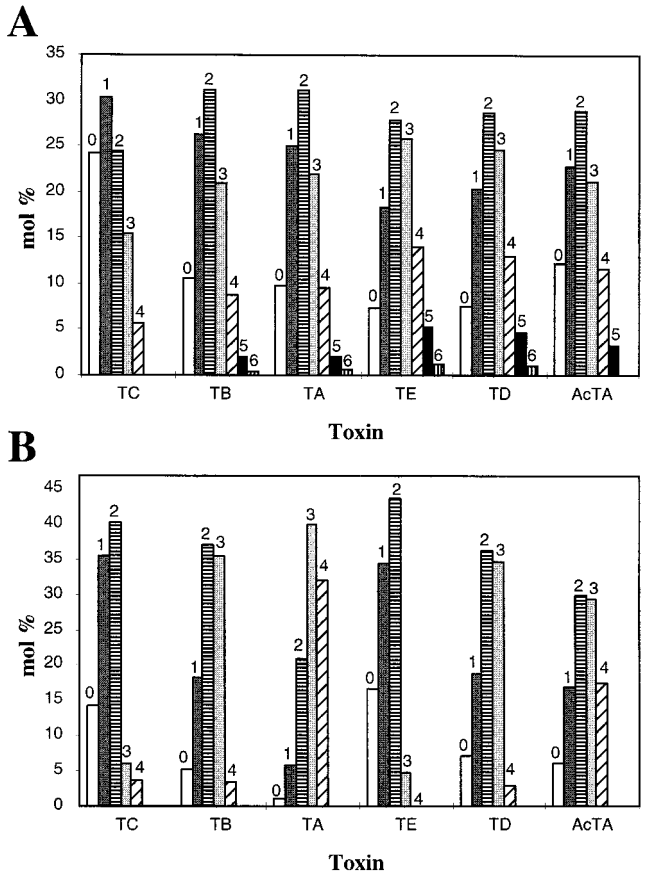


Figure 6. Fractions of isotopomers (mol %) showing incorporation of ^{18}O into all six AAL toxins from 10-day cultures using $H_2{}^{18}O$ (panel A) and $^{18}O_2$ (panel B) as determined using negative mode electrospray ionization mass spectrometry. Labels denote the number of heavy oxygens for each isotopomer.

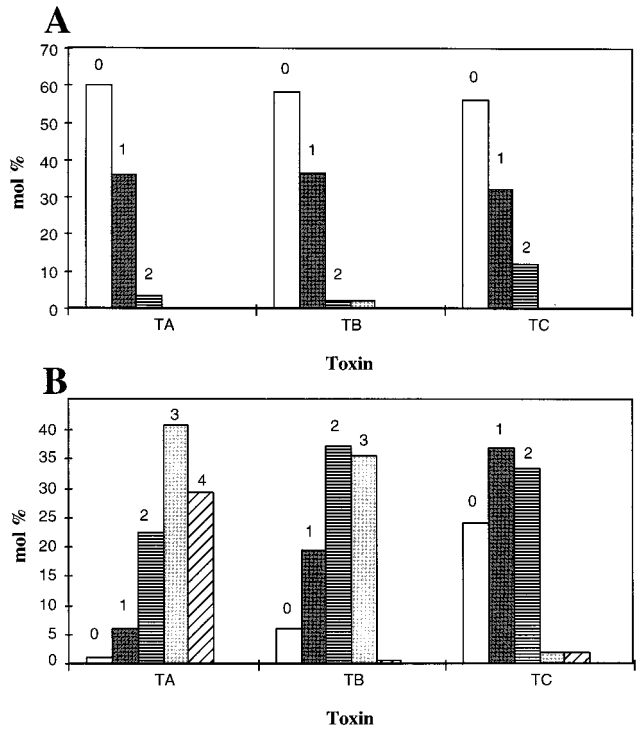


Figure 7. Fractions of isotopomers (mol %) showing incorporation of ^{18}O into aminopolyols generated by hydrolysis of AAL toxins TA, TB, and TC from 10-day cultures using $H_2{}^{18}O$ (panel A) and $^{18}O_2$ (panel B) as determined using positive mode electrospray ionization mass spectrometry. Labels denote the number of heavy oxygens for each isotopomer.

obtained from the $^{18}O_2$ experiment retains significant isotopic enrichment, indicating that the incorporation of molecular oxygen occurred on the FB_1 lipid backbone rather than on the tricarballylic acid side chain.

Similar findings are seen for the isotopic enrichment for the AAL toxins. Figure 6A documents significant enrichment of molecular anions of all six AAL toxins arising from incubations with $H_2{}^{18}O$. Spectra of amino- and amidopolyols derived from hydrolysis of AAL toxins obtained from the $H_2{}^{18}O$ experiment (Figure 7A) revealed minimal incorporation of ^{18}O into the backbone oxygens, with approximately 30% of aminopolyols containing a single heavy oxygen and the remaining aminopolyol molecules contained no heavy isotope.

Isotopically enriched molecular oxygen ($^{18}O_2$) produced enriched AAL toxins (Figure 6B), and the enrichment was also maintained following hydrolysis of the toxins (Figure 7B). Again, as was the case with FB_1, the maximum number of ^{18}O atoms corresponded to the number of hydroxyl groups in the aminopolyols. The fraction of oxygen labeled in each aminopolyol, $^{18}O/(^{18}O + {}^{16}O)$, was similar in aminopolyols produced after hydrolysis of the three toxins but showed a systematic increase with the number of hydroxyl groups (50%, 54%, and 59% in TC, TB, and TA, respectively). The sites of incorporation of oxygens from H_2O and O_2 are illustrated in Figure 8. Assignment of partial incorporation of one oxygen from H_2O into a single site at the ester linkage at either C-13 or C-14 is based on indirect evidence, namely that the maximum extent of label incorporation from $^{18}O_2$ was equal to the number of backbone hydroxyl groups. Attempts to determine sites

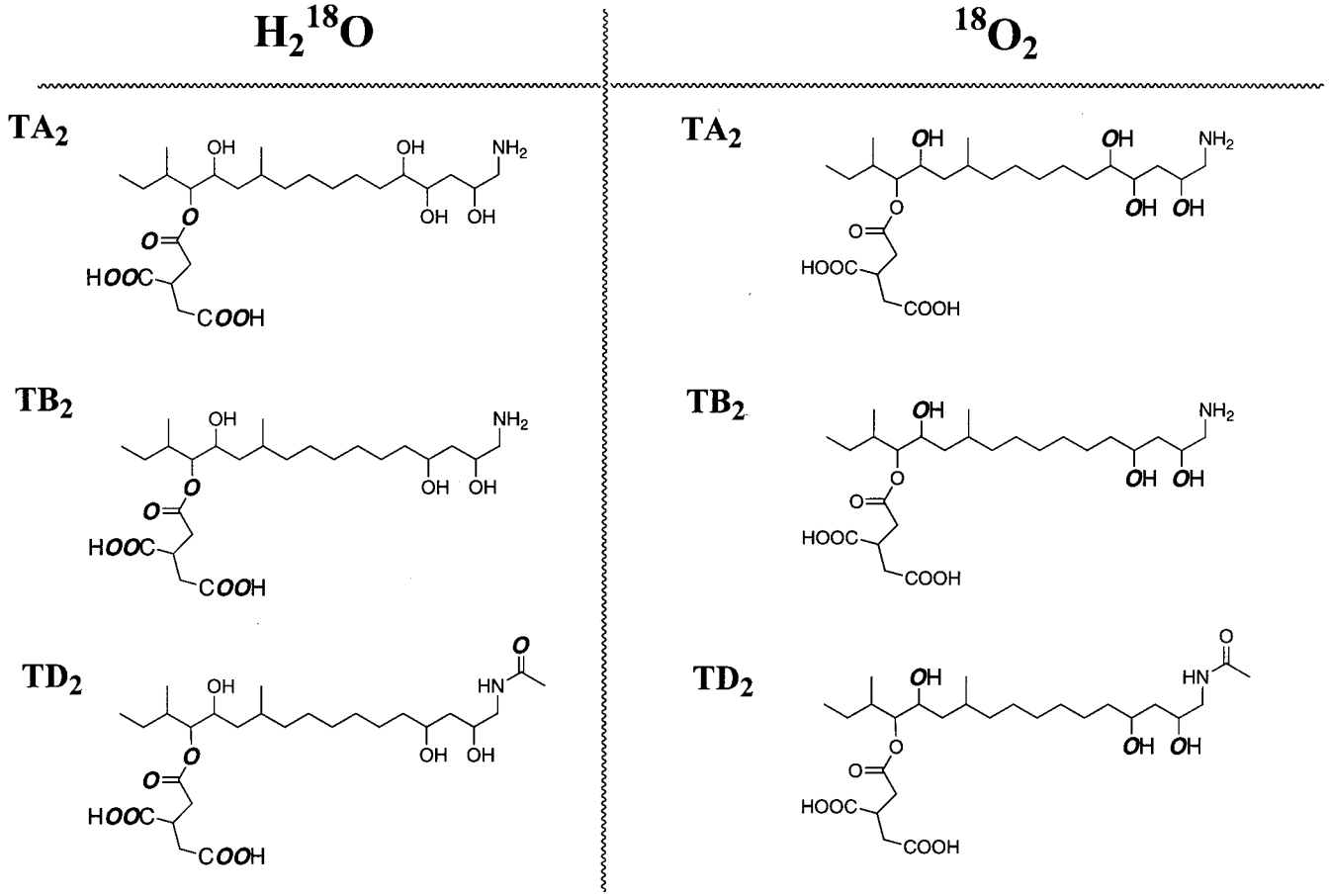


Figure 8. Site of incorporation of oxygens from H_2O and from O_2 into AAL toxins. Sites of label incorporation are in italics.

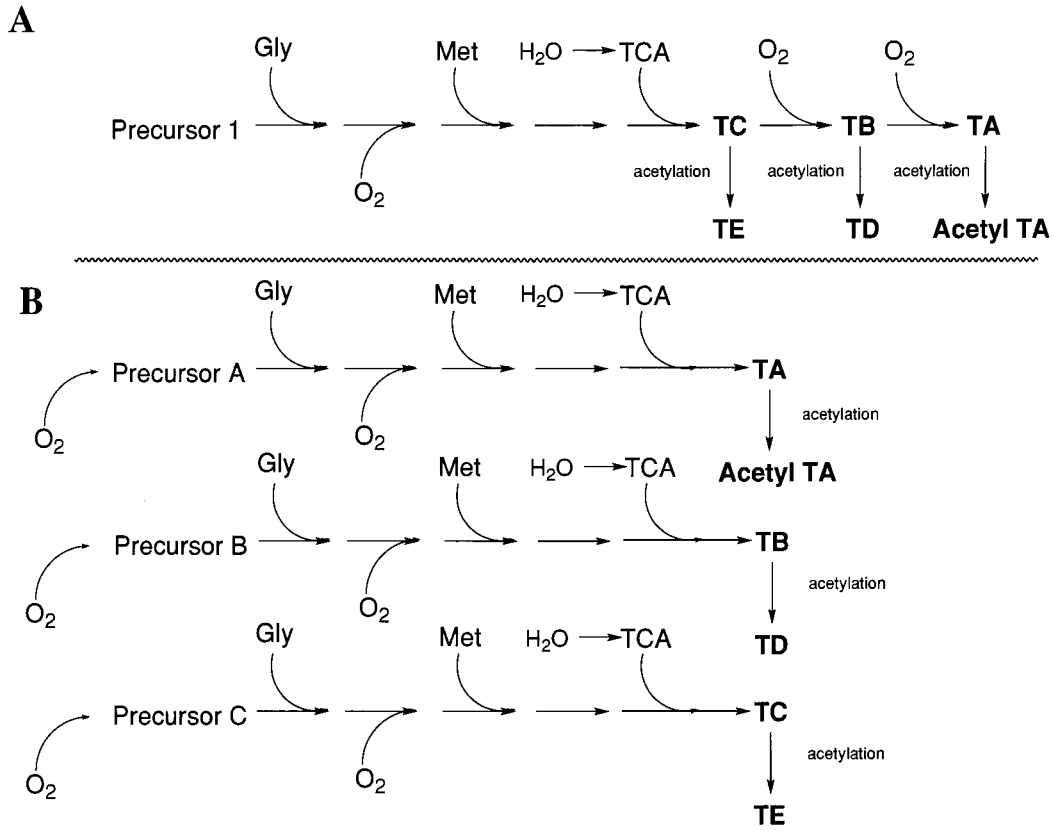


Figure 9. Schematic representing sequential (A) and parallel (B) biosynthetic schemes for AAL toxins.

of label incorporation from fragmentation in the mass spectrometer were unsuccessful.

An analysis of isotopomer patterns is also informative. The distributions of the isotopomers show distinct differences among toxins. Upon incubation under $^{18}O_2$, AAL toxins TC and TE, which have the fewest number of hydroxyl groups, showed substantially greater populations of molecules containing no heavy isotope (~16%) than toxin TA, for which 1% of TA molecules contained no heavy isotope in the same extract. Such differences in ^{18}O incorporation among toxins suggest parallel biosynthetic schemes for TA, TB, and TC which go through pools of intermediates which are labeled to different extents (Figure 9), rather than sequential conversion of TC to TB to TA. Such observations would be explained if toxins which show higher proportion of unlabeled toxin (e.g., TC) are derived from larger pools of TC precursors than occurs for precursors of TA. Addition of the isotopic label would create higher fractional labeling of a precursor to TA than would occur for precursors of TC. Rapid conversion of TA precursors to TA toxin, expected with greater production of TA relative to TC, may prevent accumulation of TA precursors and lead to more extensive labeling of TA.

Since O_2 provides the oxygens in the alcohol groups and H_2O provides oxygens in the tricarballylic acid groups of fumonisins and AAL toxins, it is unlikely that the backbone hydroxyls are remnants from acetate precursors in the proposed polyketide biosynthetic path. The current experiments suggest an important role for oxidases, perhaps including cytochrome P-450s, in the biosynthesis of both classes of SAMs and point to potential mechanisms by which toxin biosynthesis might be regulated.

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