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Tagetitoxin purification and partial characterization

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Abstract

Tagetitoxin is a non-host specific toxin produced *by Pseudomonas syringae* pv. *tagetis* in host species of the Asteraceae family and in liquid culture by certain strains of the bacterium. A purification protocol involving anion exchange and partition chromatography was developed that yielded tagetitoxin purified to homogeneity. Based on dilution end-point (nanograms injected in 50 μL that caused just detectable apical chlorosis in sunflower seedlings), the protocol resulted in a 2200-fold purification of tagetitoxin. The dilution end-point of purified tagititoxin was 10 ng/50 μL (295 nM) and the I_{50} for inhibition of *Escherichia coli* RNA polymerase was 0.1 μg/mL (147 nM). Electrospray ionization mass spectrometry in 50% methanol:H_2O indicated that the molecular weight of tagetitoxin is 678. Preliminary characterization of tagetitoxin structure was performed using 1D and 2D NMR spectroscopy. The results indicate that the previously proposed structure of tagetitoxin [Mitchell RE, Coddington JM, Young H. A revised structure for tagetitoxin. Tetrahedron Lett 1989; 30:501–504. [10]] is incorrect.

Keywords: Tagetitoxin; Pseudomonas syringae pv. tagetis; Bacterial toxin

1. Introduction

Tagetitoxin, a non-host-specific toxin produced by *Pseudomonas syringae* pv. *tagetis* (*Pst*), causes apical chlorosis and leaf spot in host species of the Asteraceae family [e.g. zinnia (*Zinnia elegans* Jacq.), sunflower *(Helianthus annuus*)] [8,11,13]. Toxin produced in planta is translocated to apical regions where it inhibits chloroplast RNA polymerase III which in turn blocks chloroplast biogenesis [3,6,7,12].

Mitchell and Durbin [8] described a protocol involving methanol precipitation, organic solvent partitioning, gel filtration, anion exchange and partition chromatography that yielded a tagetitoxin fraction reported to be purified to homogeneity. The purity of the final toxin fraction was based on two factors. First, the tagetitoxin fraction exhibited a high level of biological activity; 10 ng of purified tagetitoxin caused apical chlorosis of stem-inoculated zinnia [8]. Second, TLC of the fraction indicated the presence of a single ninhydrin- and molybdate-reactive spot ($R_c = 0.69$) that exhibited biological activity in stem-inoculated zinnia [8]. However, it appears that the purification protocol of Mitchell and Durbin [8] did not consistently yield pure tagetitoxin. In some isolations, the final tagetitoxin fraction contained ninhydrin-reactive contaminants and free phosphate in trace amounts. In a later study involving double-labeling $^{35}SO_4^{2-}$, $^{32}PO_4^{3-}$ of tagetitoxin in culture, Mitchell and Hart [9] reported that the purification protocol of Mitchell and Durbin [8] did not yield pure tagetitoxin. Additional purification steps involving partition chromatography and TLC were required to remove contaminants.

A modification of the tagetitoxin purification protocol of Mitchell and Durbin [8] was later developed by Lukens and coworkers [5,6]. In this protocol, the initial methanol precipitation and organic solvent partitioning steps used by Mitchell and Durbin [8] were replaced by a single step involving anion exchange chromatography. All subsequent purification steps were the same as previously reported [8]. This purification protocol yielded a fraction of variable purity, estimated to be in the range of 70–100% [5,6,12]. As in previous research [8,9], tagetitoxin purity was evaluated based on the presence of a single ninhydrin-reactive spot ($R_c = 0.69$) detected by TLC analysis and high biological activity of the fraction as measured by dilution end-point.

Two structures have been proposed for tagetitoxin purified by the protocol of Mitchell and Durbin [8]. In 1983, Mitchell and Hart [9] proposed that tagetitoxin was an 8-membered ring hemithioketal with a molecular weight of 435. The deduced

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structure was based on: (1) field desorption mass spectrometry, (2) 1D ^{1}H and ^{13}C NMR spectroscopy, and (3) chemical assays that indicated the presence of sulfur and phosphate in a ratio of 1:1 and a primary amine (ninhydrin-reactive group). Later, a revised structure of tagetitoxin was proposed based on fast atom bombardment mass spectrometry and 1D NMR spectroscopy. The revised structure (Fig. 1) consisted of two fused 6-membered heterocyclic rings (substituted 9-oxa-3-thiabicyclo[3.3.1] nonane) with a molecular weight of 416 [10]. The placement of an amide at C-11 in the proposed structure was considered equivocal.

In our initial attempts to purify tagetitoxin, we used a modification of the protocol of Lukens and Durbin [6] which involved anion (QAE-Sepharose) and partition (Sephadex LH-20) chromatography. TLC analysis using the protocol of Mitchell and Durbin [8] indicated that the tagetitoxin fraction obtained was not pure. Ninhydrin-reactive contaminants were present in the fraction. In close agreement with Mitchell and Durbin [8], TLC analysis of the semi-purified tagetitoxin fraction indicated that biological activity, as revealed by sunflower bioassay, was located at $R_c = 0.6$. Also, as previously reported [8], the material at this location on the TLC plate reacted with molybdate, indicating the presence of a phosphate ester. However, in contrast to Mitchell and Durbin [8], the compound located at $R_c = 0.6$ did not exhibit ninhydrin-reactivity. Furthermore, gel filtration (Sephadex G-15) chromatography of the tagetitoxin fraction indicated that the molecular weight of the compound exhibiting biological activity (tagetitoxin) was approximately 685. This value is in close agreement with the estimated molecular weight of approximately 700 that Mitchell and Durbin [8] obtained by gel filtration chromatography of tagetitoxin-containing fractions. Based on mass spectrometry, Mitchell et al. [10] later concluded that the molecular weight of tagetitoxin was 416.

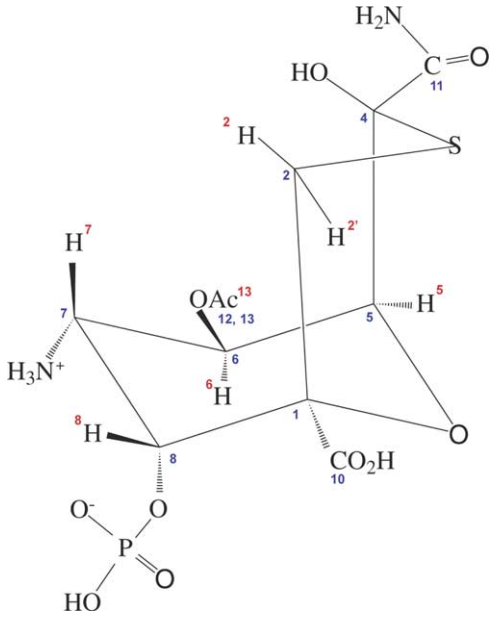

Fig. 1. Structure of tagetitoxin proposed by Mitchell et al. [10]. Numbers in blue represent assigned carbon numbers used by Mitchell et al. [10]. Numbers in red represent proton numbering system used in this paper. This figure is presented for orientation regarding the previously proposed structure [10]. Our results do not support this structure.

As a result of the disparities in our findings and previous reports, we initiated research with the objectives of: (1) developing a protocol that yielded pure tagetitoxin, (2) re-examining the composition and structure of purified tagetitoxin using mass spectrometry, 1D and 2D NMR. Our results indicate that the molecular weight of tagetitoxin is 678 and that the structure of the molecule previously proposed by Mitchell et al. [10] is incorrect.

2. Materials and methods

2.1. Chemicals

Sephadex LH-20, QAE Sephadex A-50, Q-Sepharose FF resins, and the Mono-Q(5/5) column were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Nitrocellulose and nylon microspin (0.2 μm) filters were purchased from Chromtech (Apple Valley, MN). *Escherichia coli* RNA Polymerase and calf thymus DNA were purchased from Sigma (St Louis, MO). Tagetin™ was purchased from Epicentre Technologies (Madison, WI). RNasin and polymerase assay components (Riboprobe® kit) were purchased from Promega (Madison, WI). [^{35}S]-UTP (specific activity, 600 Ci/mmol) was purchased from ICN (Costa Mesa, CA). Cellulose 300 TLC plates were purchased from Selecto Scientific (Suwanee, GA). Ecolume was purchased from MP Biomedicals (Irvine, CA). All other chemicals were purchased from Fisher Scientific (Hanover Park, IL).

2.2. P. syringae pv. tagetis culture

P. syringae pv. *tagetis* (*Pst*) strain EBO37, isolated from common ragweed (*Ambrosia artemisiifolia* L.), was provided by Encore Technologies (Minnetonka, MN). Fatty acid methyl ester (FAME) analysis of EBO37 was performed by Microcheck, Inc., (Northfield, VT). Multivariate cluster analysis of the FAME data indicated that EBO37 was identical to the *Pst* strain (ATCC#43127) isolated from common dandelion (*Taraxacum officinale* weber). Glycerol stocks of EBO37 were made from an overnight culture grown at 23 °C in modified 523 medium (glycerol substituted for sucrose) [4]. For tagetitoxin purification, *Pst* was grown in Fernbach flasks containing 1 L modified Woolley's medium (20 g/L dextrose, 0.2 g/L $MgSO_4 \cdot 7H_20$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.02 g/L $FeSO_4 \cdot 7H_2O$, 5 g/L KNO_3, 1.15 g/L NaH_2PO_4, 1.15 g/L K_2HPO_4, pH 6.5) in which 20 g/L dextrose was substituted for sucrose. Each flask was inoculated with a 1.0 mL glycerol stock of EBO37 and grown at 23 °C on an orbital shaker at 300 rpm for approximately 65 h. For experiments to measure *Pst* population dynamics, tagetitoxin production, and culture medium pH, 3-mL aliquots were removed from flasks at indicated intervals. To determine *Pst* population (cfu/mL), serial dilutions of the culture medium were made in sterile water and 100 μL of each dilution were plated in petri dishes

containing modified 523 medium. Plates were incubated at 23 °C for 48 h and individual *Pst* colonies were counted. The pH of the culture medium was measured using pH indicator paper (Sigma P-4536). For determination of tagetitoxin titer of the culture medium, a 1-mL aliquot of the medium was centrifuged (16,000*g*, 2 min) and 0.5 mL was filter-sterilized using nylon microspin filters (0.2 μm). Bioassays of the sterile fractions to determine dilution factor were conducted as described below.

2.3. Tagetitoxin purification

Ten liters of EBO37 culture grown for approximately 65 h were centrifuged (4100*g*, 5 min) at 4 °C to pellet cells. The resulting supernatant was applied to a QAE Sephadex A-50 column (10.5×50 cm) equilibrated in 0.05 M ammonium acetate, pH 8.0. All chromatography steps were conducted at room temperature (23 °C) and fractions containing tagetitoxin were identified by bioassay with sunflower as described below. The column was washed with 0.05 M ammonium acetate, pH 8.0, and the toxin was eluted with 0.4 M ammonium acetate pH 8.0. Active fractions (approximately 1.0 L) were pooled and lyophilized to dryness. The QAE fraction was dissolved in 25 mL of 0.05 M ammonium hydroxide: methanol (1:1) and applied to a LH-20 Sephadex column [LH-20(1), 5×96 cm] equilibrated in the same solvent. Tagetitoxin was eluted with 0.05 M ammonium hydroxide: methanol (1:1). Active fractions from the LH-20(1) column (~200 mL) were pooled and methanol was removed by rotary evaporation at 35 °C. The LH-20(1) pooled fractions were lyophilized to dryness and then brought up in 10 mL of 0.05 M ammonium hydroxide: methanol (1:1) and applied to a smaller LH-20 column [LH-20(2), 2.5×78 cm]. Active fractions were eluted with 0.05 M ammonium hydroxide:methanol (1:1), pooled, and the solvent was removed by rotary evaporation and lyophilization as described above. The LH-20(2) fraction was brought up in 10 mL 0.05 M ammonium acetate, pH 8.0, and applied to a Q-Sepharose FF (1.25×48 cm) column equilibrated in the same buffer. Tagetitoxin was eluted using a linear gradient (0.05–0.5 M ammonium acetate, pH 8.0, 425 mL). Active fractions were pooled, lyophilized, then brought up in 1.0 mL 0.05 M ammonium acetate, pH 8.0, and aliquots were applied to a Mono-Q (5/5) column equilibrated in the same buffer. Tagetitoxin was eluted using a two-step ammonium acetate linear gradient (0.05–0.2 M, 10 mL; 0.2–0.5 M, 45 mL). Multiple runs of the Mono-Q column were required since only a portion (approximately 15 mg) of the lyophilized Q-Sepharose toxin fraction could be applied to the column to prevent overloading. As indicated by plant bioassay, two peaks exhibiting activity eluted from the Mono-Q column. Peak 1, which eluted at 0.36 M ammonium acetate, exhibited high specific activity and was pure as determined by mass spectrometry. Peak 2, which eluted at 0.46 M ammonium acetate, exhibited lower specific activity and was not pure as determined by mass spectrometry. Mono-Q (peak 1) fractions were pooled, lyophilized and used in the characterization of tagetitoxin. A typical purification yielded approximately 2 mg of tagetitoxin.

2.4. RNA polymerase III inhibition assay

In vitro RNA Polymerase III transcription assays were performed in 20 μL of reaction mixture containing 40 mM Tris–HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 0.2 mM ATP, GTP, CTP, UTP with 1 μCi [^{35}S]-UTP, 20 U RNasin, 1 μg calf thymus DNA and 4 μL toxin fraction or RNase-free water. The reactions were started by the addition of 1.25 U *E. coli* RNA polymerase. After 30 min. at 37 °C, the reactions were stopped by the addition of 1 μL 0.5 M EDTA. The reaction mixtures were transferred to nitrocellulose microspin filters and spun in a microfuge (5000*g*, 2 min). The filters were washed three times with 0.2 mL of a solution containing 1.5 M NaCl and 0.15 M sodium citrate (10X SSC), pH 7.0 treated with diethyl pyrocarbonate (DEPC), and once with 0.2 mL 80% ethanol/DEPC. The filters were submerged in 15 mL Ecolume and radioactivity was determined by scintillation spectrometry.

2.5. Tagetitoxin bioassay

A sunflower *(Helianthus annuus*, var. Interstate) bioassay was used to: (1) measure tagetitoxin titer during growth of *Pst* in liquid culture, (2) locate tagetitoxin in eluted fractions collected during purification, and (3) determine the specific activity (dilution end-point) of the tagetitoxin fractions obtained during purification. Sunflowers were grown in Sunshine Mix#1 (SunGro Horticulture, Bellevue, WA) in a growth chamber [25/20 °C day/night temperatures, 16 h photoperiod, photosynthetic photon flux (PPF)= 300 μmol m^{-2} s^{-1}]. Fractions were injected into the cotyledons of sunflowers 7 days after planting using a tuberculin syringe with a 27 ga needle. A total volume of 0.05 mL was injected with approximately 0.025 mL injected into each cotyledon. Injected sunflowers were placed under continuous daylight fluorescent lights (PPF=150 μmol m^{-2} s^{-1}) at 23 °C for 4 days. Apical chlorosis, if present, was fully developed by this time. For bioassays to determine tagetitoxin titer in liquid culture, fractions were sterile-filtered as described above and diluted in sterile water as required prior to assay. Tagetitoxin titer in culture was measured by the dilution factor, which is defined as the highest dilution of the culture fraction that resulted in just detectable apical chlorosis in the sunflower bioassay. To determine the dilution factor, the following dilution series was used: 1:2, 1:5, 1:10; 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, 1:45, and 1:50. Fractions collected from the various chromatography steps during purification were injected directly into sunflowers without dilution. The purity of the fractions obtained during purification was determined by dilution end-point. Dilution end-point is defined as the amount (μg) of the purification fraction dissolved in 50 μL sterile water that resulted in just detectable apical chlorosis when injected into sunflower. Dilution end-point for each purification fraction was determined by diluting the fraction in sterile water prior to

bioassay. Samples were subjected to an initial tenfold dilution series followed by a twofold dilution series.

2.6. TLC analysis of purification fractions

A slight modification of the TLC protocol of Mitchell and Durbin [8] was used to evaluate the purity of fractions obtained during purification. Fractions obtained and aspartic acid were applied to cellulose 300 TLC plates. In contrast to Mitchell and Durbin [8], the plates were run twice instead of once in 1-butanol:acetic acid:water:pyridine (BAWP, 5:1:4:4). Running the plates twice improved the separation of tagetitoxin from impurities. R_c is defined as the ratio of distance traveled from the origin by the center of the zone of tagetitoxin to the distance simultaneously traveled by the center of the zone of the aspartic acid standard. Chromatographed components were visualized by spraying plates with either 1% (w/v) ninhydrin in absolute ethanol to detect primary amines or an acidic ammonium molydate spray (1% ammonium molybdate, 3% perchloric acid and 0.1 N HCl) to detect phosphate esters [1,2]. For ninhydrin color development, the plate was dried using hot air from a hair dryer. For phosphate ester detection, the plate was dried using cool air from a hair dryer and then placed in light (direct sunlight or growth chamber lights) for 10 min. To identify the location of tagetitoxin (biological activity) on TLC plates, 0.5-cm sections (measured from the origin) of an untreated lane containing the Mono-Q fraction, were scraped from the plate and placed in microfuge tubes containing 0.1 mL 0.05 M ammonium hydroxide. The microfuge tubes were vortexed and cellulose was pelleted by centrifugation (16,000*g*, 2 min). The supernatant was injected into sunflower cotyledons for bioassay. Injection of 0.1 mL 0.05 M ammonium hydroxide alone resulted in no symptoms.

2.7. Mass spectometry

Electrospray ionization (ESI) mass spectra were acquired using a QSTAR (Applied Biosystems, Inc., Foster, CA) quadrupole-TOF (time of flight) mass spectrometer equipped with an ESI source. The ESI voltage was 5200 V, the TOF region acceleration voltage was 9 kV, and the injection pulse repetition rate was 10.0 kHz. The nanospray ionization spectra were collected with an ion spray voltage of 1000 V. External calibration was performed using Renin (doubly charged ion at m/z 879.9705 and triply charged ion at m/z 586.9830). Mass spectra were the average of approximately 300 scans collected in positive mode over a 5 min acquisition period. Analysis was performed at the Mass Spectrometry Consortium for the Life Sciences Facility, University of Minnesota.

2.8. NMR spectroscopy

Purified tagetitoxin was dissolved in D_2O or 90% H_2O/10% D_2O. Proton (^1H) NMR spectra were acquired on Varian INOVA 600 and 800 MHz spectrometers at 25 °C using a relaxation delay of 2 s. Carbon (^{13}C) spectra were acquired on a Varian INOVA 600 MHz spectrometer equipped with a broadband probe. Characterization of tagetitoxin: ^1H NMR (600 MHz, D_2O) ppm d: 4.91(dd, 1H, H-6), 4.57 (s, 1H, H-8), 4.32 (d, 1H, H-5), 3.27 (dd, 1H, H-7), 2.83, 3.06 (d, 2H, $J=$ 14 Hz, H-2, H-2′), 2.53 (m, 1H), 1.82 (s, 3H, Me-13), 1.75 (s, 3H). ^{13}C NMR (600 MHz, D_2O) ppm d: 181.45, 173.71 (C-10), 172.74 (C-12), 170.46 (C-11), 85.10 (C-4), 79.26 (C-6), 76.65 (C-8), 72.38 (C-5), 70.46 (C-1), 42.51 (C-7), 32.57 (C-2), 23.23, 22.42 (C-13). Correlation spectroscopy (COSY) spectra were acquired using 1000 increments; Total Correlation Spectroscopy (TOCSY) spectra were acquired using 256 increments and a mixing time of 70 ms; heteronuclear multiple quantum coherence (HMQC) spectra were acquired using a $j1\times h$ of 140 Hz and 256 increments; and HMBC (heteronuclear multiple bond coherence) spectra were acquired using a $j1\times h$ of 140 Hz, a $jn\times h$ of 8 Hz and 400 increments. ^{31}P spectra analysis were conducted with a Varian INOVA 600 MHz spectrometer equipped with a broadband probe for 128 scans with a spectral width of 25,000 Hz at 25 °C.

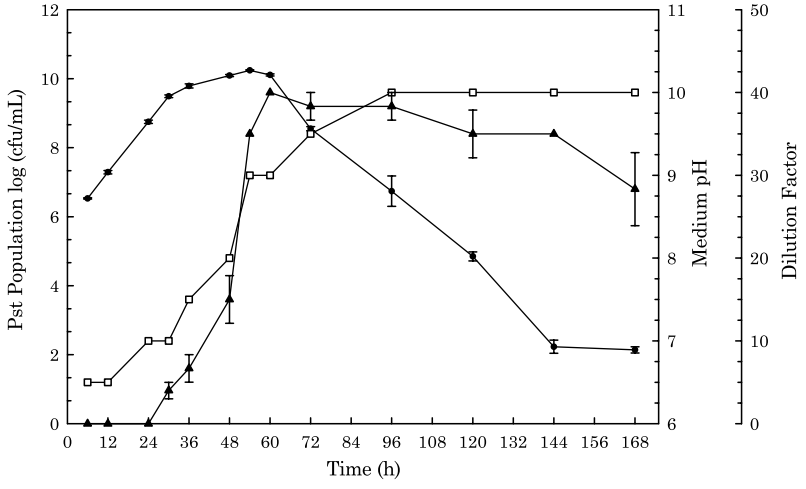

Fig. 2. *Pseudomonas syringae* pv. *tagetis* in liquid culture: population dynamics (●), tagetitoxin titer (▲), and medium pH (□). Data represent the mean ± S.E. ($n=3$). S.E. bars not shown are smaller than the data symbol. Tagetitoxin titer of the medium is defined in terms of dilution factor. Dilution factor is the maximum dilution of the sterile-filtered culture supernatant that resulted in detectable apical chlorosis in the sunflower bioassay.

3. Results and discussion

3.1. Tagetitoxin production in culture

Most *P. syringae* pv. *tagetis* (*Pst*) strains do not produce tagetitoxin in liquid culture [3,13]. However, selected strains [8,13], including the one used in this study (EBO37), produce tagetitoxin when grown in modified Woolley's medium. As determined by bioassay, rapid rates of tagetitoxin production occurred during late exponential and stationary phase growth (30–60 h) (Fig. 2). Maximum levels of tagetitoxin (dilution factor = 40) occurred after 60 h in culture. Tagetitoxin titer exhibited a gradual decline to a dilution factor of 28 after 168 h in culture. In contrast, *Pst* populations declined rapidly between 60 and 144 h in culture. In other time-course experiments conducted during the course of this investigation, population dynamics of *Pst* and the alkalization of the medium were very reproducible. However, the maximum titer of tagetitoxin produced in culture, as determined by dilution factor, ranged from 10 to 40. Tagetitoxin production in culture has also been examined for *Pst* stain DAR 26807. When grown in modified Woolley's medium, the dilution factor for tagetitoxin production ranged from less than five to 10 as determined by zinnia bioassay [8].

3.2. Tagetitoxin purification

A purification protocol involving partition (LH-20 Sephadex) and multiple-column, anion exchange chromatography (QAE-Sephadex A-50, Q-Sepharose, Mono-Q) yielded a tagetitoxin fraction purified to homogeneity (Table 1). The final step in the purification involving Mono-Q anion exchange chromatography was critical to obtaining a pure fraction. In agreement with Mitchell and Durbin [8], tagetitoxin exhibited no absorbance in the UV or visible spectrum, was unstable in dilute acid but stable at mildly basic pH (data not shown). The purification protocol resulted in a 2200-fold purification of tagetitoxin based on dilution end-point determination. Purified tagetitoxin exhibited a dilution end-point of 10 ng/plant (10 ng/50 μL injected or 295 nM) as determined by sunflower bioassay. The dilution end-point of the tagetitoxin isolated by Mitchell and Durbin [8] was 10 ng/plant as measured by a zinnia bioassay. Tagetitoxin yield, based on total units of activity in the starting material (culture supernatant) compared to the final (Mono-Q) fraction, was low (5%). The primary reason for the low yield was a significant loss of activity during the initial (QAE-Sephadex) purification step.

Purified tagetitoxin exhibited an I_{50} value for inhibition of *E. coli* RNA polymerase of 0.1 μg/mL or 147 nM based on a molecular weight for tagetitoxin of 678. *In vitro* activity of the purified tagetitoxin fraction, as determined by I_{50} values for *E. coli* RNA polymerase activity, was not evaluated with the tagetitoxin fraction isolated by Mitchell and Durbin [8]. However, I_{50} values for inhibition of *E. coli* RNA polymerase were determined for the tagetitoxin fraction isolated using the protocol described by Lukens and Durbin [6]. The purity of this fraction was reported to range from 70 to 100% [5,6,7,12]. Adjusting for the correct molecular weight of tagetitoxin, (678 instead of 416), Steinberg et al. [12] reported that the I_{50} value for inhibition of *E. coli* RNA polymerase using calf thymus DNA as template was approximately 1.6 μM. Using calf thymus as the DNA template, the I_{50} value for the tagetitoxin fraction that we purified was 147 nM which is consistent with the higher purity of the tagetitoxin fraction that we obtained.

3.3. TLC analysis

The purity of the fractions obtained during purification was initially evaluated by determining the presence of ninhydrin- and molybdate-reactive components. Fig. 3(A)–(D) show TLC of the purification fractions measured 2 or 96 h after treatment with molybdate or ninhydrin. At 2 h after treatment with molybdate (Fig. 3(A)), free phosphate (P_i), indicated by the areas of gray-green color, was detectable in all fractions except the Mono-Q fraction. In the Q-Sepharose purified fraction, in addition to the large gray-green spot indicative of free P_i, there is a smaller blue spot closer to the origin. In the Mono-Q fraction, free P_i is not detected, but a single blue spot indicating the presence of a phosphate ester is present. Sections (0.5-cm measured from the origin) were removed from an untreated

Table 1
Purification of tagetitoxin

Fraction	Dry weight (g)[a]	Yield		RNA polymerase I_{50} μg/mL[b]	Bioassay end-point μg[c]
		Millions of activity units[d]	(%)		
Culture supernatant	81.1	3.69	100	896	22
QAE-Sephadex A-50	6.63	0.55	14.9	127	12
LH-20 Sephadex (1)	0.591	0.35	9.5	20.2	1.7
LH-20 Sephadex (2)	0.248	0.30	8.1	12.7	0.83
Q-Sepharose	0.014	0.28	7.6	0.9	0.05
Mono-Q	0.002	0.20	5.4	0.1	0.01

[a] Determined after fractions were lyophilized.
[b] μg/mL of fraction required to inhibited *E. coli* RNA polymerase assay by 50%.
[c] End-point defined as μg of fraction that caused just detectable apical chlorosis in the sunflower bioassay when injected in a volume of 50 μL sterile water.
[d] One activity unit is equivalent to the bioassay end-point: the μg of the fraction that caused just detectable apical chlorosis in the sunflower bioassay.

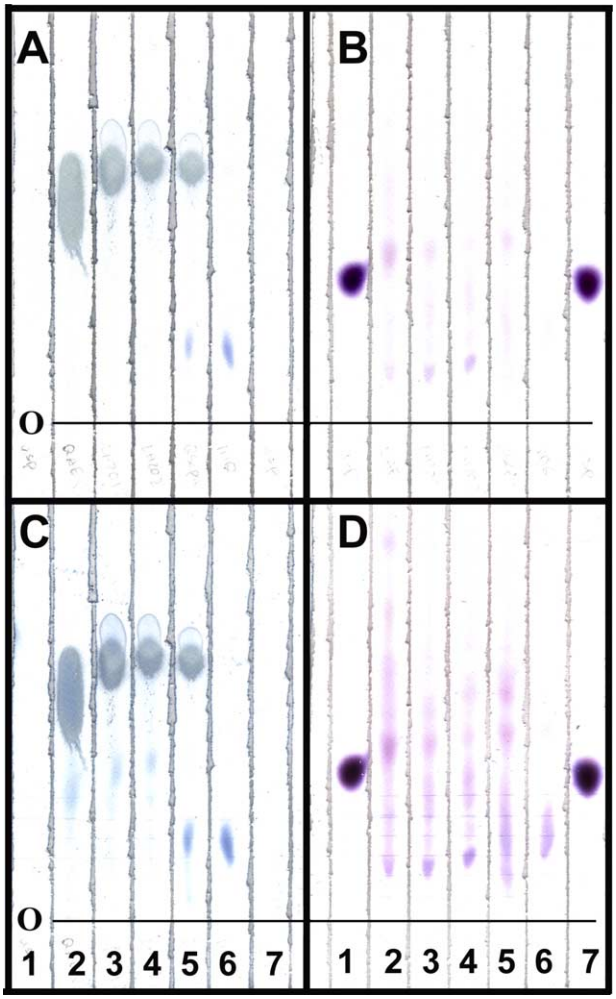

Fig. 3. TLC analysis of fractions obtained during tagetitoxin purification. Photographs of TLC plates were taken 2 h after spraying with molybdate (A) or ninhydrin (B), or 96 h after spraying with molybdate (C) or ninhydrin (D). Lanes: (1) and (7), 5 μg aspartic acid; (2) 72 μg QAE-Sephadex; (3) 30 μg Sephadex LH-20(1); (4) 13 μg Sephadex LH-20(2); (5) 14 μg Q-Sepharose; (6) 4 μg Mono-Q.

lane containing the Mono-Q fraction. The matrix was treated with 0.05 M ammonium hydroxide and the eluent was injected into sunflower. The bioassay indicated that tagetitoxin was located at $R_c = 0.6$. In the ninhydrin-treated plate viewed 2 h after treatment (Fig. 3(B)), lanes 1 and 7 show strong ninhydrin reactivity (purple color) for aspartic acid (5 μg). In all purification fractions, except the Mono-Q fraction, ninhydrin-reactive spots (light purple/pink) were detected. Although a ninhydrin-reactive spot was not located in the Mono-Q lane 2 h after treatment, tagetitoxin, as measured by bioassay, was located in the lane at $R_c = 0.6$. In agreement with Mitchell and Durbin [8], our results indicate that tagetitoxin contains a phosphate ester. However, in contrast to the report by Mitchell and Durbin [8], our results indicate that tagetitoxin does not contain a primary amine.

When viewed at 96 h after treatment (Fig. 3(C)), the pattern and intensity of molybdate reactivity for the different purification fractions remains largely unchanged. However, for the ninhydrin-treated plate (Fig. 3(D)), additional ninhydrin-reactive regions are detectable in lanes 2–6. For the Mono-Q fraction (lane 6), a single purple spot at $R_c = 0.65$ is now detectable. However, the color intensity of tagetitoxin is considerably less than the aspartic acid standard. Color development for tagetitoxin was detectable 24 h after ninhydrin treatment with maximum expressed at approximately 96 h after treatment. The time-dependence of color development and the faint purple color that appeared are not consistent with tagetitoxin containing a primary amine. The acid labile nature of tagetitoxin and the acidic conditions on the TLC plate may be responsible for the time-dependence of color development. Exposure to dilute acid results in hydrolysis of tagetitoxin and loss of activity [8]. It is possible that slow degradation of tagetitoxin on the acidic plates yielded a ninhydrin reactive product.

3.4. Mass spectrometry

Positive ion electrospray ionization (ESI) mass spectrometry of purified tagetitoxin (Fig. 4(A)–(C)) was performed in 50% methanol:H_2O. Preliminary experiments indicated that tagetitoxin was difficult to detect when mass spectrometry was performed in aqueous solution. An organic solvent was necessary for volatilization (detection) with good results obtained with 50% methanol:H_20. Mass spectrometric analysis of purified tagetitoxin in 50% methanol indicated that the molecular weight of the compound was 678 (MH^+ m/z = 679.5216) (Fig. 4(A)). The molecular species at m/z = 701.5055 and 717.4756 represent MNa^+ and MK^+ ions, respectively of tagetitoxin. Product ion analysis (MS/MS) of the m/z = 679.5 ion produced fragment ions m/z = 661.5 and 643.5 (Fig. 4(B)). This fragmentation pattern is consistent with the consecutive loss of two water molecules. MS/MS of m/z = 679.5 also produced fragment ions m/z = 552.4 (loss of 127 mass units) and m/z = 453.3 (loss of 226 mass units) (Fig. 4(B)). MS/MS of m/z = 453.3 produced fragment ions m/z = 435.3 and 417.3 (Fig. 4(C)) similar to the consecutive loss of water seen in the MS/MS of m/z = 679.5. In addition, MS/MS of m/z = 453.3 produced fragment ion m/z = 326.3 (loss of 127 mass units). MS/MS analysis using ion trap ESI confirmed that ions m/z = 453, 435 and 417 all come from the molecular ion m/z = 679 (data not shown). These results indicate that ions m/z = 679.5 and 453.3 are part of the same molecule and contain a similar structural element.

Mass spectrometry was also performed on the biologically active, molybdate-reactive, but ninhydrin-unreactive compound located at $R_c = 0.65$ on TLC plates (Fig. 3(A) and (B)). The region containing tagetitoxin was scraped from the TLC plate, eluted from the cellulose matrix, and subjected to nanospray ESI TOF (time of flight) mass spectrometry. Although chemical contaminants associated with the cellulose matrix were present, the spectra contained the molecular m/z = 679 ion and MS/MS of m/z = 679 produced the m/z = 453, 435 and 417 ions that were detected in the tagetitoxin fraction obtained by the purification protocol described in Table 1 (data not shown). In addition, a sample from the commercially

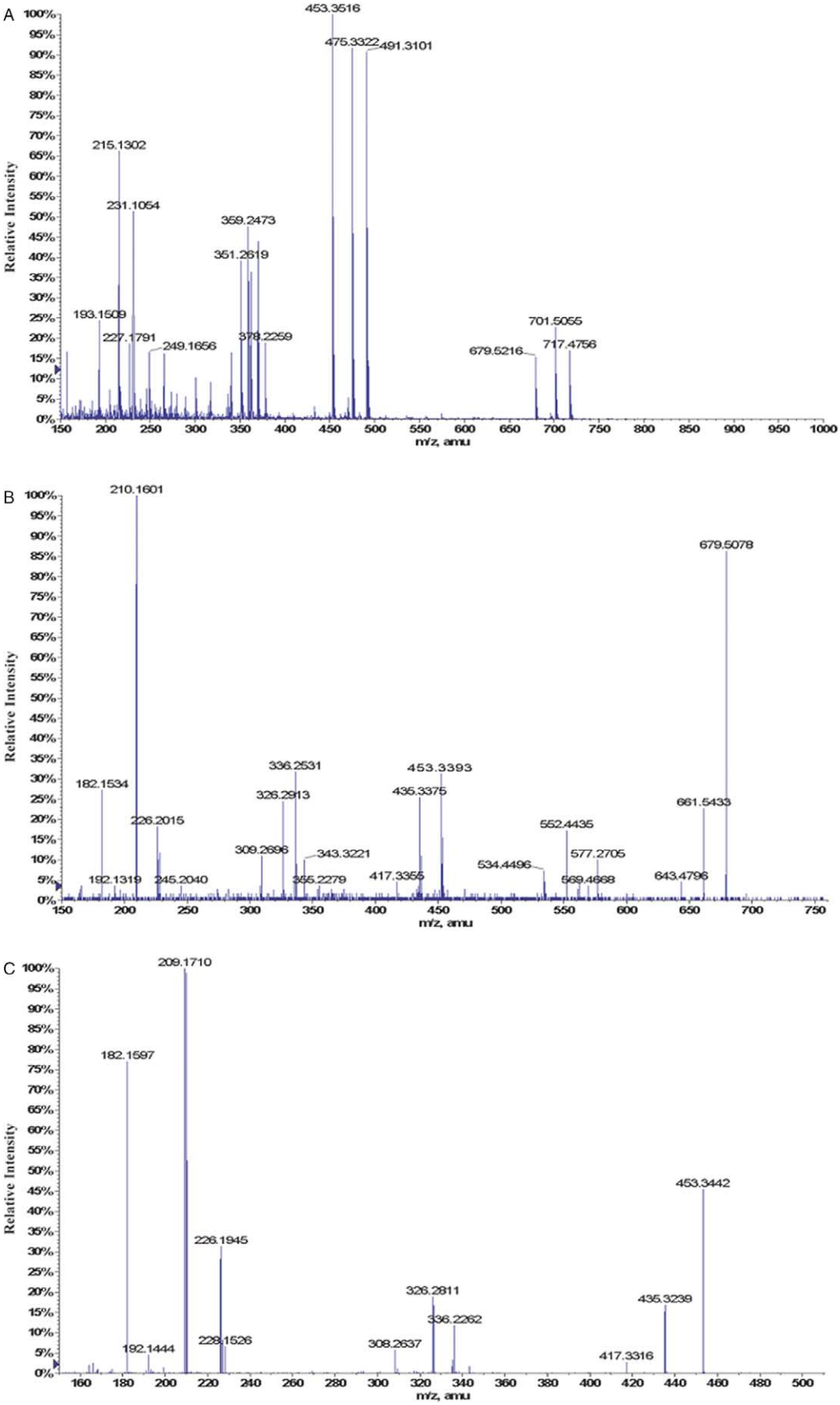

Fig. 4. Electrospray ionization (ESI) TOF mass spectra: (A) purified tagetitoxin; (B) MS/MS of tagetitoxin (MH^+, $m/z=679$); and (C) MS/MS of $m/z=453$.

available preparation of tagetitoxin (Tagetin™) was run in a separate lane on the same TLC plate with purified tagetitoxin. The only biologically-active spot was located at $R_c = 0.65$. It was eluted from the cellulose matrix and nanospray ESI TOF (time of flight) mass spectrometry was performed. The results confirm the presence of the molecular ion (MH^+, $m/z = 679.5$) and the MS/MS fragmentation ions observed for purified tagetitoxin obtained using the protocol described in Table 1 (data not shown).

In 1983, Mitchell and Hart [9] reported that field desorption (FD) mass spectrometry of tagetitoxin indicated a molecular weight of 435, $[M]^+ = 435$. Later, based on fast atom bombardment (FAB) spectrometry, Mitchell et al. [10] proposed a revised molecular weight for tagetitoxin of 416, $[M+H]^+ = 417.0361$. The reason why the $m/z = 679.5$ $[MH]^+$ molecular ion of tagetitoxin was not detected in previous reports is difficult to ascertain. In neither publication were the mass spectra of tagetitoxin published nor were the conditions used in the mass spectrometric analysis reported. It is also not clear if mass spectrometry was performed on a recently purified tagetitoxin fraction or on a fraction purified years earlier [8].

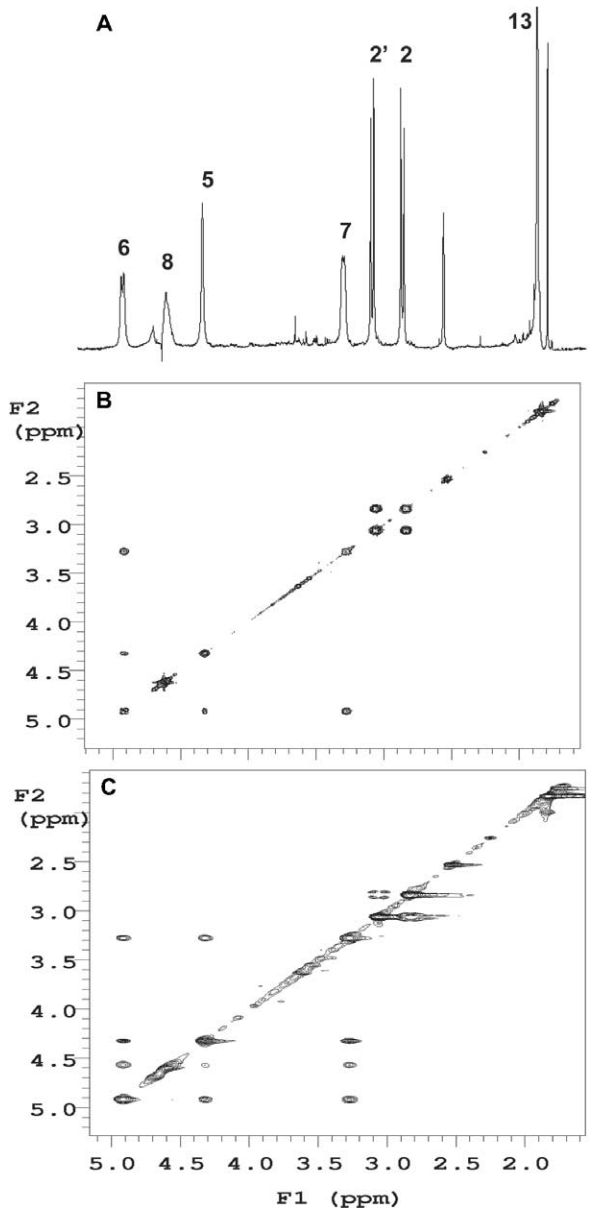

Fig. 5. ^{1}H NMR of non-exchangeable protons on tagetitoxin. (A) 1D ^{1}H NMR of tagetitoxin. ^{1}H identified were assigned generic numbers (See Fig. 1) on the tagetitoxin structure proposed by Mitchell et al. [10]; (B) 2D ^{1}H NMR COSY analysis of tagetitoxin; (C) 2D ^{1}H NMR TOCSY analysis of tagetitoxin.

Our results show that fragmentation ion $m/z = 417.3316$ is generated from the molecular ion of tagetitoxin ($m/z = 679.5216$). Our results also indicate that the fragmentation ion $m/z = 417.0361$, reported to be tagetitoxin by Mitchell et al. [10], is not formed from the molecular ion $m/z = 679.5$. However, we observed an intense peak at $m/z = 417.0377$ in the mass spectrum of some partially purified tagetitoxin fractions (data not shown). This peak was never observed in the spectrum of purified tagetitoxin (Fig. 4(A)) nor in MS/MS of $m/z = 679.5$ (Fig. 4(B)). A precursor ion study of the $m/z = 417.03$ ion observed in semi-purified tagetitoxin fractions indicated that it was largely derived from ions $m/z = 531.9$ and 647.5, and not from $m/z = 679.5$ (data not shown). These results indicate that the $m/z = 417.0361$ ion detected by Mitchell et al. [10] does not represent a fragmentation ion of the $m/z = 679.5$ parent molecule, but instead represents a fragmentation ion that is generated from contaminants in tagetitoxin-enriched fractions. These results raise the question as to why FAB MS analysis of a fraction that was highly enriched in tagetitoxin [10], as indicated by bioassay end-point, yielded an incorrect molecular ion ($m/z = 417.0361$). We can only speculate that Mitchell et al. [10] did not detect the $m/z = 679.5$ molecular ion using FAB MS. Instead an intense ion ($m/z = 417.0361$), readily generated from minor contaminants, was detected and misinterpreted as the molecular ion for tagetitoxin.

3.5. NMR analysis

One-dimensional (1D) NMR analysis of purified tagetitoxin was performed to determine the number of non-exchangeable hydrogen atoms and the number of carbon atoms, and their functionality in the molecule. Our results confirm the presence of hydrogen and carbon atoms previously identified by Mitchell et al. [10] (see Section 2.8). However, in both the 1D ^{1}H and ^{13}C spectra, signals not identified by Mitchell et al. [10] were also present (δ 1.75, 2.53 and δ 23.23, 181.45, respectively). The non-exchangeable protons identified by 1D ^{1}H NMR that are consistent with the structure proposed by Mitchell et al. [10] were assigned generic numbers (Fig. 5(A), see Fig. 1 for assigned numbers). A 1D ^{31}P spectrum of tagetitoxin confirmed that the molecule contains phosphate as reported by Mitchell et al. [10] (data not shown). Although the results of our 1D NMR analysis are similar to Mitchell et al. [10], our MS data (Fig. 4) indicate that tagetitoxin has a molecular mass of 678, which is considerably higher than 416 reported by Mitchell et al. [10]. This additional mass may be accounted for by the presence of atoms (oxygen, nitrogen, sulfur) and exchangeable protons that are not detected by 1D NMR.

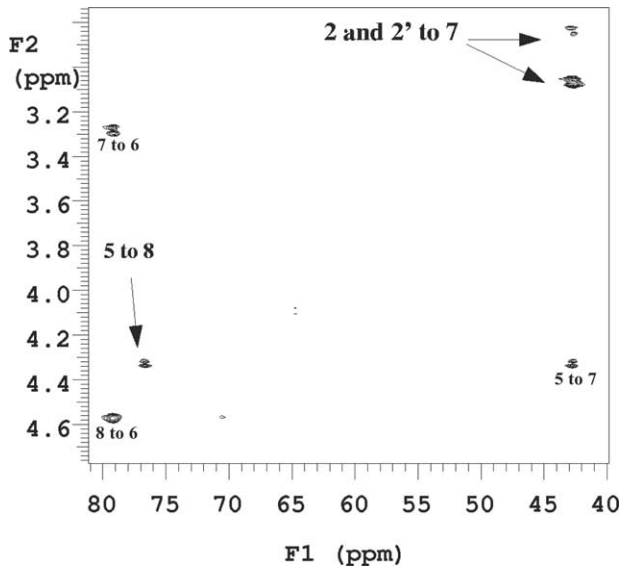

Fig. 6. Expanded region of the HMBC spectrum of tagetitoxin. The labels with arrows represent correlations that are discussed in the text.

Two-dimensional (2D) NMR analysis was performed to identify linkages. COSY experiments show that protons 2 and 2′ are coupled to each other and are likely attached to the same carbon based on the coupling constant, $J = 14$ Hz (Fig. 5(B)). In addition, the COSY spectrum shows that proton 6 is coupled to both protons 5 and 7. TOCSY experiments show that proton 8 is coupled to proton 7, and that protons 5, 6, 7 and 8 are in a spin system consistent with the structure proposed by Mitchell et al. [10] (Fig. 5(C)). The TOCSY spectrum also shows that protons 2 and 2′ are isolated from the other protons identified. Although the results confirm the linkages of carbons 5, 6, 7 and 8 proposed by Mitchell et al. [10], they do not confirm the attachment of specific heteroatoms.

HMQC and HMBC NMR spectroscopy experiments were performed with tagetitoxin to examine heteronuclear correlations. HMQC analysis confirmed that protons 2 and 2′ are attached to the same carbon and correlated the carbon shifts with their attached protons (data not shown). In addition, HMBC analysis confirmed the presence of an acetate group (methyl 13 is correlated to carbon 12). However, the HMBC experiment demonstrated strong correlations for protons and carbons within two to three bonds that are not consistent with the structure proposed by Mitchell et al. [10]. Specifically, HMBC indicated strong correlations between carbon 8 and proton 5, and carbon 7 and protons 2 and 2′(Fig. 6).

The results of this investigation confirm some of the previously reported properties of tagetitoxin. In agreement with Mitchell and Durbin [8], we found that tagetitoxin: (1) does not absorb in the UV or visible spectrum, (2) is unstable in dilute acid, but stable at mildly alkaline pH, and (3) exhibits a similar specific activity as measured by plant bioassay. However, in contrast to previous reports, our results indicate that tagetitoxin: (1) does not contain a primary amine, and (2) has a molecular weight of 678, not 435 [9] or 416 [10]. Furthermore, 2D NMR correlations do not support the deduced structure proposed by Mitchell et al. [10].

The results of this investigation indicate that the previously proposed structures of tagetitoxin are erroneous primarily because the results obtained from NMR and chemical analysis were ‘fitted’ to structures of molecular weight 435 [9] or 416 [10]. For unknown reasons, the parent ion of tagetitoxin (molecular weight = 678) was apparently not detected by field desorption or fast atom bombardment spectrometry. Other inconsistencies between our results and those of Mitchell and colleagues [8,9,10] may reflect contamination in the tagetitoxin fraction isolated using the protocol of Mitchell and Durbin [8]. Although our work clearly indicates that the previously proposed structures of tagetitoxin are incorrect, the results do not allow for a definitive structure to be proposed. Additional experiments involving NMR analysis of ^{15}N labeled tagetitoxin and complete elemental analysis will be required to describe the structure of tagetitoxin unequivocally.

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References

[1] Bandurski RS, Axelrod B. The chromatographic identification of some biologically important phosphate esters. J Biol Chem 1951;193:405–10.
[2] Bieleski RL, Young RE. Extraction and separation of phosphate esters from plant tissues. Anal Biochem 1963;6:54–68.
[3] Durbin R.D. Biochemistry of non-host-selective phytotoxins. In: Hoagland RE, (ed), Microbes and microbial products as herbicides, ACS Symposium 439. Washington, DC: American Chemical Society; 1990, p. 63–71.
[4] Kado CI, Heskett MG. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas*, and *Xanthomonas*. Phytopathology 1970;60:969–76.
[5] Lukens JH. Investigations into the mode of action of tagetitoxin in plants. PhD Dissertation. Madison, WI: University Wisconsin; 1983. 135 p.
[6] Lukens JH, Durbin RD. Tagetitoxin affects plastid development in seedling leaves of wheat. Planta 1985;165:311–21.
[7] Mathews DE, Durbin RD. Tagetitoxin inhibits RNA synthesis directed by RNA polymerases from chloroplasts and *Escherichia coli*. J Biol Chem 1990;265:493–8.
[8] Mitchell RE, Durbin RD. Tagetitoxin, a toxin produced by *Pseudomonas syringae* pv. *tagetis*: purification and partial characterization. Physiol Plant Pathol 1981;18:157–68.

[9] Mitchell RE, Hart PA. The structure of tagetitoxin, a phytotoxin of *Pseudomonas syringae* pv. *tagetis*. Phytochemistry 1983;22:1425–8.
[10] Mitchell RE, Coddington JM, Young H. A revised structure for tagetitoxin. Tetrahedron Lett 1989;30:501–4.
[11] Rhodehamel NH, Durbin RD. Host range of strains of *Pseudomonas syringae* pv. *tagetis*. Plant Dis 1985;69:589–91.
[12] Steinberg TH, Mathews DE, Durbin RD, Burgess RR. Tagetitoxin: a new inhibitor of eukaryotic transcription by RNA polymerase III. J Biol Chem 1990;265:499–505.
[13] Styer DJ. *Pseudomonas syringae* pv. *tagetis*: hosts, symptomatology and toxin production. PhD Thesis. Madison: University Wisconsin; 1982, 156 p.