

Territrems, Tremorgenic Mycotoxins of *Aspergillus terreus*

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The tremorgenic mycotoxins isolated from *Aspergillus terreus* were given the trivial names territrem A and territrem B instead of their previous designations of C₁ and C₂, respectively. High-resolution mass spectral data suggested the molecular formula of territrem A to be C₂₈H₃₀O₉ and that of territrem B, C₂₉H₃₄O₉. They were partially characterized by ultraviolet, infrared, proton magnetic resonance, and mass spectroscopy. The spectroscopic evidence indicated that their chemical structures were very similar. The procedures of purification were also revised for the complete separation of these two chemically related compounds.

During 1968, Tung et al. (8) and Chung et al. (1) carried out a survey of fungi contaminating stored unhulled rice in Taiwan. They showed that among 206 specimens of the aspergilli, 11 isolates of *Aspergillus terreus* were capable of producing toxic metabolites, which were thus accidentally discovered when they revealed blue fluorescence on thin-layer chromatograms irradiated with long-wave ultraviolet (UV) light. The compounds were further purified from rice media of *A. terreus* in two crystalline forms and designated compounds C₁ and C₂ (5). Ling and Huang (4, 5) demonstrated that both toxins induce tremor and convulsion in mice by intraperitoneal administration, but that they contain no nitrogen. This is the most distinctive feature of these compounds different from the known N-containing tremorgenic mycotoxins (2, 3). However, no other available information about their contamination of food or feeds or their intoxication to animals has been obtained since their discovery. In addition, it has never been reported that any tremorgen is produced by *A. terreus*. Therefore, in this report, the trivial names territrem A and B are given to emphasize both their tremor-inducing effect and their isolation from *A. terreus*.

Because territrems A and B are chemically related compounds, they were not completely separated by the previous method used (5). Although the method of isolation was improved to result in their clear resolution by thin-layer chromatography, there also appeared two additional blue fluorescent substances with lower R_f values on five thin-layer chromatography systems (4). We suspected that these additional substances were the decomposed products of original territrems when they were exposed to irradiation with long-wave UV light during the process of

separation by thin-layer chromatography, using toluene-ethyl acetate-90% formic acid (6:3:1, vol/vol/vol) as developer. It was the purpose of this investigation to provide an alternative procedure of purification in order to achieve the complete separation of territrems and avoid any possible decomposition. In addition, several physicochemical properties are provided for comparison.

MATERIALS AND METHODS

Organism. *A. terreus* 23-1, isolated from unhulled rice (1), was maintained on rice medium with occasional transfer and used throughout this study.

Cultivation. The rice medium, which consisted of 200 g of polished rice of the Japonica type and 80 ml of distilled water per 1-liter Erlenmeyer flask, was a modification of the method of Shotwell et al. (7). The flasks were autoclaved for 15 min at 121°C and then cooled. The media were inoculated with spores of *A. terreus* and incubated at 28 to 30°C for 21 days as stationary cultures.

Preparation of crude extracts. The procedures used, including the extraction of resultant moldy rice with chloroform and the primary fractionation of chloroform extracts with neutral alumina column chromatography, were the same as those previously described (4). Fractions of the eluates of column chromatography were monitored by thin-layer chromatography detected with long-wave UV light. The blue fluorescent components were pooled and concentrated under reduced pressure to dryness and subjected to the following steps of purification.

Purification of territrems. Crude territrems were redissolved in a small amount of chloroform and applied as a band on a thin-layer chromatographic plate (20- by 20-cm glass plate coated with a ca. 1-mm layer of Silica Gel G [Merck]), which was then developed in benzene-ethyl acetate (7:3, vol/vol). The ascending development distance was 10 cm. The blue fluorescent bands of R_f ca. 0.43 and 0.33 corresponding to territrems A and B, respectively, were scraped off and

eluted with acetone. The eluates were dried under a stream of nitrogen gas. Each kind of toxin was recrystallized by dissolving in chloroform and precipitating with *n*-hexane. Final crystallization of territrems B from chloroform gave a colorless needle form of crystals.

Physicochemical analyses. Melting points were determined with a melting-point apparatus (Shimadzu Seisakusho Ltd.) and were uncorrected. Optical rotation of each toxin in chloroform was measured with a Jasco Dip-180 polarimeter at 589 nm (sodium D line). The fluorescence wavelength of maximum emission (λ_{em}) was determined at 375 nm of maximum excitation (λ_{exc}) with a Hitachi model 204 fluorescence spectrophotometer. UV spectra were recorded with a Jasco Uvidec-1 spectrophotometer; the concentration was 20 μ g/ml in methanol. Molar absorptivities were calculated from the slopes of the regression lines calibrated at maximum wavelengths of UV absorbance. Infrared spectra were obtained with a Perkin-Elmer model 527 spectrophotometer. The toxin was mixed as a KBr disk. The proton magnetic resonance spectrum of each toxin in deuteriochloroform was obtained with a Jeolco 60-MHz spectrometer. High-resolution mass spectra were analyzed with an AEI MS-30 spectrometer (Shrader Analytical and Consulting Laboratories, Inc., Detroit, Mich.).

RESULTS AND DISCUSSION

High-resolution mass analyses showed the molecular ion peak of each toxin to be $C_{28}H_{30}O_9$ for territrems A (found, 510.1900; required, 510.1888) and $C_{29}H_{34}O_9$ for territrems B (found, 526.2285; required, 526.2200). The proton numbers could be matched with proton magnetic resonance proton counting (Table 3). For a more comprehensive comparison, the molecular formula, molecular weight, melting point, specific optical rotation, fluorescence, and UV spectrum are presented in Table 1. It was found that the chromophores for UV absorption and fluorescence of both compounds were very similar.

Major infrared absorptions of territrems B (Table 2) were at: 3,470 and 3,340 cm^{-1} (OH); 1,705 and 1,685 cm^{-1} (C=O); 1,640, 1,585, and 1,505 cm^{-1} (C=C); and 1,130 cm^{-1} (C—O—C). These functionalities also appeared in the infrared spectrum of territrems A, but with reduced intensity at 1,580 and 1,130 cm^{-1} . The fingerprint region revealed slight differences in absorption pattern and relative intensity.

Proton magnetic resonance spectra are summarized in Table 3. There were 23 protons present in both compounds, which closely corresponded to each other in spite of a little variation in values of chemical shift. The distinctive peaks of territrems A consisted of 3H at δ 3.93, 2H at δ 5.97, 1H at δ 6.88, and 1H at δ 7.02, whereas 11 protons of territrems B included 9H at δ 3.90 and 2H at δ 6.95. These data differentiated territrems A from territrems B.

TABLE 1. Some physicochemical properties of territrems^a

Property	Territrems A	Territrems B
Molecular formula	$C_{28}H_{30}O_9$	$C_{29}H_{34}O_9$
Molecular weight	510	526
Melting point ($^{\circ}C$)	288–290 (dec.)	200–203
$[\alpha]_D^{25}$ ($CHCl_3$)	+102 (c 0.10)	+131 (c 0.60)
Fluorescence (nm)		
λ_{exc} (MeOH)	375	375
λ_{em} (MeOH)	420	420
UV spectrum (nm)		
λ_{max} (MeOH)	219	219
	(ϵ 43,000)	(ϵ 39,000)
	338	331
	(ϵ 19,600)	(ϵ 18,400)

^a dec., Decomposed. c = Concentration of solute in grams per 100 ml of solution (6).

TABLE 2. Infrared spectra of territrems: ν_{max} (KBr) cm^{-1}

Territrems A	Territrems B
3,520, 3,350 (m, broad),	3,470, 3,340 (m, broad),
3,000, 2,930, 2,860,	3,000, 2,950, 2,840,
1,710 (vs), 1,680 (vs),	1,705 (vs), 1,685 (vs),
1,630 (s), 1,580 (s), 1,510 (s)	1,640 (m), 1,585 (vs), 1,505 (s),
1,450, 1,435, 1,400,	1,460, 1,430, 1,410
1,375, 1,345, 1,295, 1,260,	1,365, 1,335, 1,300, 1,250,
1,230, 1,200, 1,150,	1,225, 1,200, 1,190, 1,170,
1,130 (m), 1,100, 1,075, 1,050,	1,130 (vs), 1,075,
1,025, 1,000, 985,	1,025, 1,000, 985,
970, 940, 895, 850,	975, 930, 895, 830
820, 800, 785, 750	820, 810, 780, 750

TABLE 3. Proton magnetic resonance spectra of territrems:^a δ ppm (in $CDCl_3$ from tetramethylsilane)

Territrems A	Territrems B
1.21 (s, 3H)	1.23 (s, 3H)
1.30 (s, 3H)	1.31 (s, 3H)
1.47 (s, 3H)	1.49 (s, 3H)
1.53 (s, 3H)	1.55 (s, 3H)
1.70–2.07 (m, 3H)	1.80–2.08 (m, 3H)
2.27 (m, 1H)	2.28 (m, 1H)
2.78 (d, J = 17 Hz, 1H)	2.83 (d, J = 17 Hz, 1H)
3.41 (d, J = 17 Hz, 1H)	3.45 (d, J = 17 Hz, 1H)
— ^b	3.90 (s, 9H) ^c
3.83 (s, 1H) ^d	4.03 (s, 1H)
3.93 (s, 3H)	—
5.74 (d, J = 10 Hz, 1H)	5.77 (d, J = 10 Hz, 1H)
5.83 (s, 1H) ^d	5.95 (s, 1H)
5.97 (s, 2H)	—
6.23 (s, 1H)	6.32 (s, 1H) ^c
6.26 (d, J = 10 Hz, 1H)	6.28 (d, J = 10 Hz, 1H) ^c
6.88 (d, J = 2 Hz, 1H)	6.95 (s, 2H)
7.02 (d, J = 2 Hz, 1H)	—

^a The data were also confirmed by proton magnetic resonance spectra analyzed by H. K. Schnoes (University of Wisconsin-Madison, Madison, Wis.).

^b —, No corresponding peak in the other toxin.

^c δ ppm ($CDCl_3$ + Me_2SO-d_6): 3.83 (s, 3H) and 3.90 (s, 6H).

^d Disappeared on shaking with D_2O .

^e δ values were arranged in reverse to match data of territrems A.

High-resolution mass spectra of individual toxins provided measured masses as well as their corresponding computerized elemental compositions, which could be presented in the form of an element map (6). A comparison made between the element maps of territrems A and B led to the classification of the fragment ions into the following three groups (Table 4): (i) this group included the ions with a difference of 16 (CH₄) between territrems B and A; (ii) this group included the ions which could be found in both spectra; and (iii) this group included the other prominent ions which could not be definitely ascribed to group i or ii because of low intensity or lack of corresponding mass in either compound. Group ii showed that a moiety of at least 17 carbons should be arranged in the same structural feature in both toxins, because of the concomitant occurrence of *m/e* 254 (C₁₇H₁₈O₂). Group i showed that the difference of CH₄ would

be located in a portion of, at most, 10 carbons which made up the individual base peak. The absence of the four protons in territrem A, when tentatively correlated with proton magnetic resonance data, could be attributed to the difference between 5H (δ3.93, 3H, and δ5.97, 2H) of territrem A and 9H (δ3.90) of territrem B, provided that the peaks of territrem A at δ6.88 and 7.02 were derived from the splitting of the singlet peak of territrem B at δ6.95. This structural difference will be clearly elucidated by chemical derivatives of territrems.

Ciegler and co-workers (2, 3) have reviewed several tremorgenic mycotoxins which appear to act at the level of the central nervous system. All the tremorgens of known chemical structures contain nitrogen and have an indole nucleus in common. On the other hand, territrems contain no nitrogen but do induce tremor in experimental animals. The questions concerned with their structures and toxic effect are under investigation.

TABLE 4. Mass spectra of territrems:^a *m/e* (relative intensity) *x-y-z*^b

Group	Territrem A		Territrem B	
(i) B - A = 16 (CH ₄)	179 (100)	9-7-4	195 (100)	10-11-4
	221 (8.7)	11-9-5	237 (6.9)	12-13-5
	275 (14.3)	14-11-6	291 (15.4)	15-15-6
	329 (5.3)	17-13-7	345 (10.1)	18-17-7
	343 (11.5)	18-15-7	359 (5.6)	19-19-7
	459 (4.6)	27-23-7	475 (3.4)	28-27-7
	477 (61.8)	27-25-8	493 (36.5)	28-29-8
(ii) A = B	492 (34.5)	28-28-8	508 (30.0)	29-32-8
	510 (7.3)	28-30-9 (M ⁺)	526 (8.8)	29-34-9 (M ⁺)
	131 (2.1)	10-11-0	131 (4.9)	10-11-0
	135 (8.9)	9-11-1	135 (6.4)	9-11-1
	149 (6.5)	10-13-1	149 (9.6)	10-13-1
	150 (5.9)	10-14-1	150 (11.8)	10-14-1
	159 (4.3)	11-11-1	159 (4.2)	11-11-1
(iii) Other peaks	175 (2.4)	12-15-1	175 (6.2)	12-15-1
	189 (5.7)	13-17-1	189 (14.4)	13-17-1
	239 (4.1)	16-15-2	239 (3.9)	16-15-2
	254 (7.0)	17-18-2	254 (2.9)	17-18-2
	135 (3.8)	8-7-2	137 (4.4)	7-5-3
	151 (12.3)	8-7-3	152 (4.5)	8-8-3
	153 (3.2)	8-9-3	154 (17.0)	8-10-3
165 (3.9)	9-9-3	165 (4.0)	9-9-3	
211 (2.9)	15-15-1	169 (3.3)	9-13-3	
262 (3.5)	13-10-6	173 (4.4)	13-17-0	
363 (5.3)	21-15-6	181 (3.5)	10-13-3	
381 (3.0)	21-17-7	181 (5.1)	9-9-4	
		191 (9.2)	13-19-1	
		210 (6.4)	11-14-4	
		212 (4.7)	15-16-1	
		217 (3.2)	14-17-2	
		272 (9.0)	17-20-3	
		412 (4.5)	23-24-7	

^a The original electron mass values with four decimals were rounded and presented as the whole numbers, which were also confirmed by low-resolution mass spectra. Several less intense -1 or +1 peaks were omitted from the table. The prominent masses below *m/e* 130 in both spectra included 18, 29, 41, 43, 55, 69, 77, 83, 91, 95, 105, and 125.

^b *x-y-z* in C_xH_yO_z denotes the computerized elemental composition of the fragment ion.

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