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Solvent Systems for Improved Isolation and Separation of Territrems A and B

KUO HUANG LING,* CHUNG-KUANG YANG, CHIN-AN KUO, AND MING-DER KUO

Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei 100, Taiwan, Republic of China

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Territrems A and B, tremorgenic mycotoxins in the rice culture of *Aspergillus terreus*, were extracted with hot chloroform. The toxins were cleaned on a silica gel column by direct adsorption-concentration of the extracts followed by desorption with chloroform-acetone (9:1, vol/vol). Crude toxin mixtures were separated by silica gel column chromatography and eluted with benzene-ethyl acetate (65:35, vol/vol). The method gave 112 mg of territrem A, 379 mg of territrem B, and 80 mg of their mixture from 4 kg of rice per batch. The criteria of extraction, cleanup, and separation are provided.

Territrem A ($C_{28}H_{30}O_9$) and territrem B ($C_{29}H_{34}O_9$) are new tremorgenic mycotoxins isolated from the rice culture of *Aspergillus terreus* (5). The structure of territrem B (Fig. 1) has been elucidated by X-ray crystallography (2a). The structural differences (Fig. 1) have been determined from the spectral features and from chemical evidence, yet, in territrem A, the spatial configuration of the aryl methylene-dioxy group relative to the α-pyrone ring is unknown (K. H. Ling, C. K. Yang, M. D. Kuo, and C. M. Yang, Republic of China-Japan Seminar on Mycotoxins, 1981, Taipei).

Because the yield of territrems from the previous methods of isolation was too low, we present a modified procedure in this study for obtaining larger quantities in a more efficient manner. We also establish the criterion of extraction by solubility test and the criteria of cleanup and separation by thin-layer chromatography (TLC). Attention was focused on the choice of suitable solvents to fulfill the requirements of chromatography (8), but no attempts were made to examine the various characteristics of silica gel adsorbents.

MATERIALS AND METHODS

Production. A 1-liter Erlenmeyer flask containing 200 ml of modified Czapek-Dox liquid medium (3) was autoclaved at 121°C for 15 min. The cooled medium was sown with *A. terreus* 23-1 from the stock rice culture (5) and incubated at 28 to 30°C as static culture for 1 month. When sufficient sporulation appeared, the medium was kept at room temperature in a dark place. The aqueous spore suspension from this medium served as the inoculum throughout the study. On a large scale, 4 kg of polished rice of the Japonica type in 40 1-liter Erlenmeyer flasks was used per batch. To take up water more thoroughly, the 100 g of rice in each flask was submerged for 2 h in 100 ml of distilled water, which was then decanted, leaving about 20 ml. The rice was autoclaved at 121°C for 15 min, cooled, inoculated, and incubated at 28 to 30°C for 14 days as static culture.

Isolation. (i) Extraction. The resultant moldy rice in each flask was submerged in 200 ml of redistilled chloroform with 0.5% ethanol as stabilizer. The mixture was warmed at 50°C for 30 min. The solution was filtered through cheesecloth. The total residual rice was extracted again with 4 liters of chloroform. The combined extracts of about 10 liters were filtered through glass wool and stored overnight to separate the upper layer of trace water.

(ii) Cleanup. The chloroform extracts were passed at about 15 ml/min directly through a column (3 cm inside diameter) packed with 100 g of Silica Gel 60 (Merck no. 7734) and 150 g of anhydrous sodium sulfate at the top portion. After the application, territrems were adsorbed and thus concentrated within the upper half of silica gel, whereas most of the less polar substances were eluted out. After a wash with 1 liter of chloroform, the column was eluted with 600 ml of chloroform-acetone (9:1, vol/vol) at 1 ml/min. Each 20 ml of the effluent was collected and detected by TLC in toluene-ethyl acetate-formic acid (5:4:1, vol/vol/vol). The fractions of territrems were pooled, concentrated under reduced pressure to dryness, redissolved in chloroform, and precipitated with 10-fold volumes of *n*-hexane.

(iii) Separation. About 700 mg of crude territrems in chloroform was applied to a column of silica gel under the same conditions as in cleanup, except that benzene-ethyl acetate (65:35, vol/vol) was used for both column packing and elution. Collection of each territrem A or B followed the same operation as in cleanup. Final recrystallization was in chloroform.

TLC criteria. Proper aliquots of testing sample were spotted 2 cm from the bottom of a precoated aluminum sheet of Silica Gel $60F_{254}$ (Merck no. 5554). Each plate

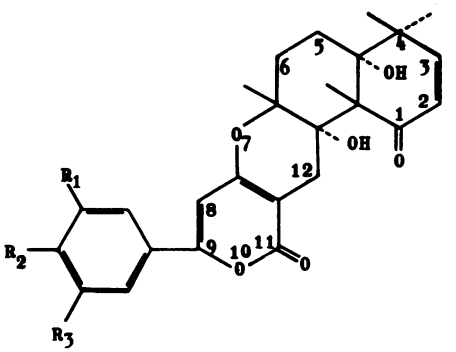

FIG. 1. The structure of territrems. Territrem A: $R_1 + R_2 = OCH_2O$, $R_3 = OCH_3$. Territrem B: $R_1 = R_2 = R_3 = OCH_3$. The IUPAC name of territrem B is (4a*R*,6a*R*,12a*S*,12b*S*)-4a,6,6a,12,12a,12b-hexahydro-4a,12a-dihydroxy-4,4,6a,12b-tetramethyl-9-(3,4,5-trimethoxyphenyl)-4*H*,11*H*-naphtho[2,1-*b*]pyrano[3,4-*e*]pyran-1,11(5*H*)-dione. The aryl moiety in territrem A is [3-methoxy-4,5-(methylenedioxy)phenyl].

was developed for 10 cm of ascending distance at room temperature in an unlined and unequilibrated tank. Solvents of analytical grade were used. The compositions of solvent systems were expressed in volume ratio. Territrems A and B were located by their blue fluorescence on chromatograms under longwave UV light. Substances capable of quenching the fluorescent background of the plate were monitored under shortwave UV light. The R_f values were averaged from triplicate experiments.

(i) Scope of single solvent. A 10-μl sample of the chloroform extracts was spotted and developed in the first dimension in benzene-ethyl acetate (65:35) and in the second dimension in acetone, benzene, chloroform, diethyl ether, or ethyl acetate. The resultant R_f values of each component provided the information about the proper choice of solvent mixture.

(ii) Load of solvent system. Territrems from cleanup were dissolved in chloroform with various concentrations ranging from 0.2 to 40 mg/ml. A 5-μl sample was spotted as a circle about 0.3 cm in diameter. The difference in concentration of various samples was 1, 2, or 10 μg per spot. The solvent systems were benzene-ethyl acetate (7:3), chloroform-acetone (93:7), diethyl ether (7:3), or toluene-ethyl acetate-formic acid (6:3:1). The load of each solvent system for sufficient separation of territrem A from B was determined from Giddings' definition of resolution (2, 10): $Rs = \Delta z/\bar{w}$, where Rs is resolution, Δz is the distance between the centers of the two spots under investigation, and $\bar{w}$ is the mean longitudinal length averaged from the individual spot length. The maximal load of a solvent system is restricted by unity resolution: $Rs = 1$, which implies that the outer edge of the two spots is just overlapped.

(iii) Resolving power of benzene-ethyl acetate. Benzene-ethyl acetate was the best choice in the loadability test. Its optimal composition was estimated from the optimal volume of benzene by the formula (10)

$$(x - \Delta x) + \Delta x \cdot \frac{(Rs)_2 - 4(Rs)_1 + 3(Rs)_0}{2(Rs)_2 - 4(Rs)_1 + 2(Rs)_0}$$

Each experiment used 5 μl of territrems (100 μg per spot) from cleanup and was developed in benzene-ethyl acetate of (ml/ml) 50:50, 70:50, and 90:50, respectively; that is, the volume of benzene $x = 70$ ml, the variation of volume $\Delta x = 20$ ml. The R_f value and longitudinal spot length of each territrem in each solvent mixture were used to calculate the $(Rs)_0$, $(Rs)_1$, and $(Rs)_2$ corresponding to the composition of 50:50, 70:50, and 90:50, respectively.

Solubility test. Saturated solution of each pure toxin in the proper volume of *n*-hexane, benzene, chloroform, tetrahydrofuran, ethyl acetate, acetone, or methanol was kept at 30°C for 15 min. The solution was equilibrated at 25°C and centrifuged to settle the residual sample. The proper samples of each clear solution were evaporated to dryness under a stream of nitrogen. The sample redissolved in chloroform was quantified by UV molar absorptivity of 18,300 at 340 nm for territrem A and 17,700 at 334 nm for territrem B (4). The original solubility was calculated with the dilution factor involved.

RESULTS

The cleanup step gave 696 mg of crude mixture of at least 80% territrems. Further column chromatography gave 112 mg of territrem A, 379 mg of territrem B, and 80 mg of their mixture. The coefficient of variance in yield from five batches was 12%. Each pure toxin showed the same properties as previously reported (5).

Solubility tests indicated that territrem A was more soluble in tetrahydrofuran at 9.5 mg/ml and in chloroform at 7.8 mg/ml; territrem B was more soluble in chloroform at 40.6 mg/ml; both toxins were insoluble in *n*-hexane. The results suggested that chloroform was the most suitable extractant tested and that *n*-hexane precipitation of chloroform solution of territrems could be used for crystallization. Nevertheless, the complete extracts also contained many interfering substances. Two-dimensional TLC under the detection of longwave or shortwave UV light exhibited at least 14 spots. Only the R_f values of territrems A and B are shown in Table 1. Both territrems had R_f values of about 0.1 after development in chloroform. Therefore, territrems would be adsorbed and thus concentrated on a column of silica gel through which the 10 liters of chloroform extracts were passed. Territrems were developed in acetone at about R_f 0.9 (Table 1). This meant that territrems in a column of cleanup would be easily desorbed with acetone. Combining both sets of data established the criterion of cleanup. A restricted fraction of territrems was obtained for further purification.

Comparisons among the various solvent systems were made to test the loadability for crude mixture of territrems at or nearly at the resolution of unity (Table 2). When the applied quantity was as low as 2 μg per spot, diethyl ether was proper for resolution of both toxins. However, a yellow pigment and a quenching spot under 254 nm adhered to territrem B. When the applied

TABLE 1. Scope of single solvent on TLC for development of territrems[a]

Solvent	TLC R_f	
	TRA	TRB
Benzene	0	0
Chloroform	0.10	0.07
Diethyl ether	0.66	0.43
Ethyl acetate	0.88	0.81
Acetone	0.90	0.89

[a] An ascending technique was used. Each 10 μl of chloroform extracts was applied. The dried chromatogram developed in the first dimension in benzene-ethyl acetate (65:35) was rechromatographed in the second dimension in single solvent. Territrems A and B are indicated as TRA and TRB, respectively. The other spots (see the text) were omitted from this table.

quantity was increased, resolution of each solvent system was decreased because of the extension of the longitudinal length of each spot. At approximating unity resolution, benzene-ethyl acetate was able to separate territrem A from territrem B at 200 μg per spot. This solvent system mixture could be loaded with a much greater quantity of crude territrems than the other solvent system tested. The optimal composition, 63:37, of benzene-ethyl acetate was determined from solvent compositions and their resolution (Table 3). When the composition of 65:35 was applied to silica gel column chromatography, each kind of territrem was obtained in an efficient manner in spite of minor overlapping fractions.

DISCUSSION

The discovery of territrem-producing strains of *A. terreus* was initiated by Ling and his coworkers, starting from a mycological survey of aflatoxin-producing fungi. Consequently, the initial procedures for cultivation of *A. terreus* 23-1 and the subsequent purification of territrems were similar to those for aflatoxins (5, 9). When the natural substrates served as cultural media of aflatoxins, difficulties arose from (i) interfering components in the crude extracts, (ii) close similarity of the chemical structures, and (iii) great relative differences in the amounts of each toxin (7). These problems were also encountered in the isolation of territrems.

First, too many components in chloroform extracts could not be easily fractionated when they were condensed to a small volume and precipitated with *n*-hexane. This step rendered a sticky mixture, some loss of territrems in the mother liquid, and several interfering substances in the precipitates. The similar manipulation contained about 40 to 60% of aflatoxins (1, 6, 7, 9). In this study, the problem was solved by serial unit operation of adsorption, concentration, and desorption of toxins on a silica gel column. Moreover, the high percentage of territrems in the crude sample led to the subsequent efficient purification.

Second, concerning the structural difference of $-CH_4$ between territrems A and B (Fig. 1), the main problem was to search for a solvent system with the maximal load and the optimal resolution on TLC or column chromatography. By trial and error, many solvent systems were tested. The present best choice was benzene-ethyl acetate (63:37) on TLC. However, column chromatography with the composition of 65:35 resulted in a yield nearly equivalent to that from 35 bands on TLC (5).

Third, in addition to territrems A and B, there were territrem-related compounds which exhibited similar UV spectra in methanol (5), elicited tremors in mice, showed higher polarity, and

TABLE 2. Load of solvent system on TLC for separation of territrems

Solvent system[a]	TR(A+B)[a] (μg per spot)	TLC R_f		Spot length (cm)		Rs[b]
		TRA	TRB	TRA	TRB	
BE	2	0.33	0.23	0.40	0.40	2.50
	200	0.30	0.21	0.80	0.80	1.12
TEF	2	0.50	0.45	0.40	0.45	1.18
	40	0.50	0.45	0.45	0.55	1.00
Et_2O	2	0.47	0.29	0.60	0.65	2.88
	14	0.35	0.20	1.50	1.50	1.00
CA	2	0.37	0.30	0.40	0.50	1.56
	12	0.34	0.28	0.40	0.80	1.00

[a] Abbreviations: BE, benzene-ethyl acetate (7:3); TEF, toluene-ethyl acetate-formic acid (6:3:1); Et_2O, diethyl ether; CA, chloroform-acetone (93:7). TR(A+B) was the mixture of territrems A and B from cleanup.

[b] *Rs* indicates resolution, defined as the distance between two spot centers divided by the longitudinal mean spot length; the dividend was the 10-fold difference of R_f in this study. The maximal load for territrems was limited at unity resolution.

TABLE 3. Resolving power of benzene-ethyl acetate on TLC for separation of territrems[a]

Benzene-EtOAc (ml:ml)	TLC R_f		Spot length (cm)		Rs
	TRA	TRB	TRA	TRB	
50:50	0.61	0.49	0.57	0.75	1.88
70:50	0.54	0.41	0.58	0.72	2.05
90:50	0.43	0.31	0.52	0.70	2.09

[a] See footnotes to Table 2 for abbreviations. Each 100 μg per spot of TR(A+B) was used. The optimal composition was 63:37 as calculated by the method of Turina et al. (10). For details, see the text.

were fewer in quantity. The problem of the great differences in the amounts could be solved by column cleanup. After the elution of territrems A and B by chloroform-acetone (9:1), the other territrem-related toxins were desorbed with the same solvent mixture with higher eluotropic power. Their isolation and characterization are in progress.

In summary, the present method could be integrated by chromatography tetrahedron: resolution, scope, load, and speed (8), at a preparative level. The rapid cleanup gave a more restricted fraction of territrems within the resolvable scope of silica gel column eluted with benzene-ethyl acetate, which was demonstrated to be maximal in loadability and was adjusted in composition to be optimal in resolution.

The efficient isolation of mycotoxins has two major aspects: bioproduction and purification. The cultivation conditions necessary to reach the maximal production of territrems require further examination.

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