

## Isolation, Chemical Structure, Acute Toxicity, and Some Physicochemical Properties of Territrems C from *Aspergillus terreus*

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Territrems C, a new tremorgenic mycotoxin ( $C_{28}H_{32}O_9$ ; molecular weight, 512.20) was isolated from the chloroform extract of rice cultures of *Aspergillus terreus* 23-1, which also produces territrems A and B. Isolation, acute toxicity, and some physicochemical properties of territrems C are discussed in this paper. The spectral and chemical evidence indicated that the structural difference between territrems C and territrems B ( $C_{29}H_{34}O_9$ ) was in their phenyl moieties: a 4-hydroxy-3,5-dimethoxy phenyl group in territrems C and a 3,4,5-trimethoxy phenyl group in territrems B. It was also demonstrated that territrems B was obtained by methylation of territrems C with dimethyl sulfate.

In our laboratory, two structure-related tremorgenic mycotoxins, territrems A (TRA) ( $C_{28}H_{30}O_9$ ) and territrems B (TRB) ( $C_{29}H_{34}O_9$ ), were isolated from the chloroform extract of rice cultures of *Aspergillus terreus* 23-1 (6). The structure of TRB was (4*aR*,6*aR*,12*aS*,12*bS*)-4*a*,6*a*,12*a*,12*b*-hexahydro-4*a*,12*a*,dihydroxy-4,4,6*a*,12*b*-tetramethyl-9-(3,4,5-trimethoxyphenyl)-4*H*,11*H*-naphtho(2,1-*b*)pyrano(3,4-*e*)pyran-1,11(5*H*)-dione by X-ray crystallography (1) (Fig. 1). It has also been observed that there are several blue fluorescent compounds which have lower  $R_f$  values than TRA and TRB by thin-layer chromatography (TLC) in the same chloroform extract of the rice culture (3). Using the procedure of isolation of TRA and TRB (5) and the parameter for screening territrems, e.g., by examining the blue fluorescent compound on its UV absorption spectra at 219 and 330 nm and on its tremor-inducing ability in mice, we succeeded in isolating a new territrems, territrems C (TRC). Some physicochemical properties, acute toxicity, and chemical structure of TRC are presented in this paper.

### MATERIAL AND METHODS

**Isolation.** Cultivation of *A. terreus* 23-1 and isolation of TRA and TRB followed the procedure of Ling et al. (5). Chloroform extracts (10 liters) were cleaned up on a column (3-cm inside diameter) packed with 100 g of Silica Gel 60 (Merck no. 7734) with 150 g of anhydrous sodium sulfate at the top portion. After the elution of TRA and TRB with 600 ml of chloroform-acetone (9:1 [vol/vol]), the column was further eluted with 600 ml of chloroform-acetone (4:1 [vol/vol]). The fractions containing TRC, monitored by TLC, were then pooled together and concentrated under reduced pressure. The subsequent silica gel column chromatography of TRC was performed under the same column conditions as those used in the clean-up step, except that benzene-ethyl acetate (1:1 [vol/vol]) was used as the eluent. The fraction of TRC was pooled and concentrated as described above and then applied to a column (inside diameter, 2 cm; length, 29 cm) which was packed with Sephadex LH-20 (particle size, 25 to 100  $\mu$ m; Pharmacia Fine Chemicals, Piscataway, N.J.) previously equilibrated with absolute ethanol. The column was eluted with absolute ethanol at a flow rate of 0.5 ml/min. The final step of purification was carried out by recrystallization in absolute ethanol. Because of the presence of the

phenolic group in TRC, it easily turned light yellow. However, when TRC was dissolved or suspended in absolute ethanol and kept in a refrigerator (at  $5 \pm 0.5^\circ\text{C}$ ) the solution or crystal remained colorless. The purity of the compound was confirmed by TLC as well as by high-pressure liquid chromatography. The reverse-phase high-pressure liquid chromatography of territrems will be reported elsewhere.

**TLC criteria.** A precoated aluminum sheet of Silica Gel 60F<sub>254</sub> (Merck no. 5554) was used. We followed the TLC procedure described previously (5). The following developing solvents were used: benzene-ethyl acetate (1:1 [vol/vol]), toluene-ethyl acetate-85% formic acid (5:4:1 [vol/vol/vol]), and benzene-ethyl acetate-acetic acid (55:40:5 [vol/vol/vol]).

**Solubility.** A saturated solution of TRC in *n*-hexane, benzene, acetone, or absolute ethanol made at  $30^\circ\text{C}$  was equilibrated further at room temperature ( $25^\circ\text{C}$ ) and then centrifuged to make a clear solution. The proper samples of each clear solution were evaporated to dryness under a stream of nitrogen. The dried specimens were redissolved in chloroform and quantified by UV absorbance based on 334 nm (in chloroform), where  $\Sigma = 1.845 \times 10^4$  mol/liter.

**Analyses.** Melting points were measured with a hot-stage melting apparatus (Shimadzu Seisakusho Ltd.) without correction of data. Optical rotation was measured with a Jasco Dip-180 polarimeter at 589 nm. Fluorescence was measured with a Hitachi model 204 fluorescence spectrophotometer. The UV absorption spectrum of the toxin in methanol, in 0.1 N methanolic NaOH, or in chloroform was recorded on a Jasco Uvidec-1 spectrophotometer. Infrared spectrometry was recorded with a Perkin-Elmer model 577 grating infrared spectrophotometer. Low-resolution mass spectrometry (MS) was analyzed with a Finnigan 4510 spectrophotometer. High-resolution MS was analyzed with an AEI MS-30 spectrometer at direct probe by Shrader Analytical and Consulting Laboratories, Inc., Detroit, Mich. PMR was done with a Varian EM 360 60-MHz nuclear magnetic resonance spectrophotometer.

**Ferric chloride test.** For the ferric chloride test (8) 2 mg of TRC in 1 ml of chloroform was treated with 2 drops of a saturated solution of ferric chloride in chloroform and 1 drop of pyridine.

**Methylation.** TRC (18 mg) in 6.5 ml of 0.2 N methanolic NaOH was warmed to  $68^\circ\text{C}$  and added to 500  $\mu$ l of dimethyl sulfate (Merck no. 3071) dropwise over 1 h. The temperature of the reaction mixture was maintained within  $68 \pm 2^\circ\text{C}$ . At the end of 1 h, the reaction mixture was added to 20 ml of

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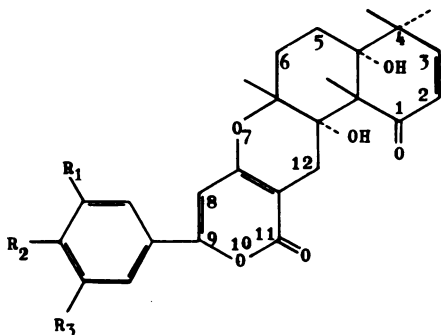


FIG. 1. The structure of territrems. The International Union of Pure and Applied Chemistry name of TRC is (4a*R*,6a*R*,12a*S*,12b*S*)-4a,6,6a,12,12a,12b-hexahydro-4a,12a-dihydroxy-4,4,6a,12b-tetramethyl-9-(3,5-dimethoxy,4-hydroxy phenyl)-4*H*,11*H*-naphtho (2,1-*b*) pyrano(3,4-*e*) pyran-1,11(5*H*)-dione. In TRB,  $R_1 = R_2 = R_3 = \text{OCH}_3$ .

distilled water and extracted five times with 20 ml of ethyl acetate. The extract was dried by a nitrogen stream. Isolation of the product was carried out by TLC with the precoated aluminum plate of Silica Gel 60F<sub>254</sub> (Merck no. 5715). After development in benzene-ethyl acetate, the blue fluorescent band at an  $R_f$  of 0.49 under long-wave UV light was scraped off and eluted with acetone. The product was identified by MS, nuclear magnetic resonance spectrum, TLC, and melting point as TRB. The total yield of TRB was ca. 6 mg (33.3%).

**Acute toxicity test.** A weighted sample of TRC was dissolved in chloroform and quantified by UV absorbance at 334 nm. The proper samples were dried under a stream of nitrogen and then redissolved in propylene glycol to give the desirable concentration. For each dose level, four male and four female ICR mice with body weights of  $17 \pm 2$  g were tested. Each mouse was treated intraperitoneally (i.p.) with various concentrations of TRC in 0.1 ml of propylene glycol. The onset of tremors of the hind limbs was recorded within 10 min. Mortality was observed within 24 h. The median tremulous dose and the median lethal dose were estimated by the methods of Inch et al. (2) and Litchfield and Wilcoxon (7), respectively.

## RESULTS AND DISCUSSION

The present procedure of isolation gave about 55 to 60 mg of TRC from 4 kg of rice culture per batch. TRC exhibited light-blue fluorescence on TLC under long-wave UV light (336 nm) at  $R_f$  values of 0.25 in benzene-ethyl acetate, 0.43 in toluene-ethyl acetate, and 0.42 in benzene-ethyl acetate-acetic acid. The fluorescence intensity of TRC was quenched when the concentration was higher than 20  $\mu\text{g}$  per spot. The fluorescence intensity also gradually faded after development in benzene-ethyl acetate, but was enhanced and turned somewhat greenish in an acidic solvent system. TRC was not as soluble in *n*-hexane as TRA and TRB. The solubilities, expressed as milligrams of TRC in milliliters of acetone, absolute ethanol, or benzene were 5.55, 1.37, and 1.0, respectively. TLC results and solubility of TRC showed that it has a higher polarity than TRA and TRB. The melting point of TRC was 172.5 to 173.5°C (uncorrected), and the optical rotation was +120 at a concentration of 0.1 g/100 ml of chloroform.

The major infrared absorptions ( $\text{centimeter}^{-1}$ ) of TRC were 3,320 (m, broad), 1,705 (vs), 1,685 (vs), 1,515 (vs), and

1,120 (vs). These functionalities also appeared in infrared spectra of TRA and TRB (6). The excitation and emission wavelengths of fluorescence spectra of TRC in methanol were 375 and 430 nm, respectively. The maximum UV absorption spectra of TRC in methanol were 219 ( $\Sigma = 36,000$ ) and 344 nm ( $\Sigma = 18,800$ ). The similarity of spectra in UV absorption and fluorescence of TRA, TRB, and TRC indicated that their chromophores are very similar. However, one notable feature of TRC discriminable from TRA and TRB was that the UV absorbance at 344 nm of TRC was shifted to 398 nm ( $\Sigma = 21,300$ ) in 0.1 N methanolic NaOH, which suggested the presence of phenolic OH in TRC. The high-resolution MS spectrum showed the observed molecular ion of TRC at  $m/e$  512.20 with the computerized composition of  $\text{C}_{28}\text{H}_{32}\text{O}_9$ . In the comparison of high-resolution MS spectra of TRA and TRB in our previous study (6), there are a series of fragment ions with a difference of 16 ( $\text{CH}_4$ ) between TRA and TRB, including both basal peaks  $m/e$  179 ( $\text{C}_9\text{H}_7\text{O}_4$ ) in TRA and  $m/e$  195 ( $\text{C}_{10}\text{H}_{11}\text{O}_4$ ) in TRB and molecular ions 510 (TRA) and 526 (TRB). In addition, there was a series of common fragment ions in TRA and TRB in which the largest common ion was  $m/e$  254 ( $\text{C}_{17}\text{H}_{18}\text{O}_4$ ). Similarly, there was a series of  $m/e$  values which showed that the difference in  $m/e$  values between TRB and TRC is 14 ( $\text{CH}_2$ ), including both basal peaks 195  $m/e$  in TRB and 181  $m/e$  in TRC and molecular ions. Again, there was a series of common ions between TRB and TRC, including  $m/e$  254. It was suggested that each phenyl group and CO accounted for the respective base peak of a benzoyl cation, namely,  $m/e$  179 ( $\text{C}_9\text{H}_7\text{O}_4+$ ) for TRA,  $m/e$  195 ( $\text{C}_{10}\text{H}_{11}\text{O}_4+$ ) for TRB, and  $m/e$  181 ( $\text{C}_9\text{H}_9\text{O}_4+$ ) for TRC. Therefore, the structural difference between these three toxins was due to different substitutions in their phenyl moieties. The ion of the largest common moiety should be derived from the nonaromatic moiety R ( $\text{C}_{20}\text{H}_{23}\text{O}_6$ ), which accounts for 4*H*,11*H*-naphtho (2,1-*b*) pyrano(3,4-*e*) pyran (1). In comparison of PMR signals in  $\text{CDCl}_3$  from  $\text{Me}_4\text{Si}$  of three toxins, the data also correlated well with the results of the mass spectra. The common moiety R exhibited PMR signals for the 23 total protons with very similar values, splitting patterns, and proton numbers for three toxins. For TRC these signals ( $\delta$ ) were 1.22, 1.32, 1.48, 1.55 (each s, 3H), 1.82 to 2.12 (m, 3H), 2.20 to 2.42 (m, 1H), 2.80 (d,  $J = 18$  Hz, 1H), 3.43 (d,  $J = 18$  Hz, 1H), 3.97 (s, 1H,  $\text{D}_2\text{O}$  exchangeable), 5.80 (d,  $J = 10$  Hz, 1H), 5.98 (s, 1H,  $\text{D}_2\text{O}$  exchangeable), 6.28 (d,  $J = 10$  Hz, 1H), and 6.32 (s, 1H). The rest of nine protons of TRC were located in the phenyl moiety, and their signals were at  $\delta$  3.92 (s, 6H, two  $\text{OCH}_3$ ),  $\delta$  5.82 (s, 1H,  $\text{D}_2\text{O}$  exchangeable, OH), and  $\delta$  6.95 (s, 2H, Ar- $\text{H}_2$ ). The corresponding signals of PMR located in the phenyl moiety of TRB were at  $\delta$  3.90 (s, 9H, three  $\text{OCH}_3$ ) and  $\delta$  6.95 (s, 2H, Ar- $\text{H}_2$ ). The presence of a phenol group in TRC was supported by the positive result of the ferric chloride test. The most direct evidence was the chemical conversion of TRC to TRB by methylation with dimethylsulfate. TRC had a median tremulous dose of  $0.24 \pm 0.03$  mg/kg in mice i.p. The median tremulous doses for TRA and TRB under identical experimental conditions were  $0.31 \pm 0.05$  and  $0.21 \pm 0.03$  mg/kg, respectively, in mice i.p. The median lethal doses for TRA, TRB, and TRC were  $17.60 \pm 1.91$ ,  $9.06 \pm 1.07$  and  $6.28 \pm 1.56$  mg/kg, respectively, in mice i.p. Our previous study on tremulous hyperkinesia of TRB indicated that the onset of trembling in mice (i.p.) was within 10 min, the frequency ranged from 4 to 7 cycles per second, and congestion of the liver and kidney was conspicuous at postmortem. These manifestations also occurred with TRC.

## ACKNOWLEDGMENT

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## LITERATURE CITED

1. Heu, T. H., C. K. Yang, K. H. Ling, C. J. Wang, and C. P. Tang. 1982. (4a*R*, 6a*R*, 12a*S*, 12b*S*)-4a,6,6a,12,12a,12b-hexahydro-4a,12a,-dihydroxy-4,4,6a,12b-tetra methyl-9-(3,4,5-trimethoxyphenyl)-4*H*,11*H*-naphtho(2,1-*b*)pyrano(3,4-*e*) pyran-1,11(5*H*)-dione, territrem B, C<sub>29</sub>H<sub>34</sub>O<sub>9</sub>. Cryst. Struct. Comm. 11:199-206.
2. Inch, T. D., D. M. Green, and P. B. J. Thompson. 1972. The central and peripheral activities of anti-acetylcholine drug. Some concepts of practical relevance. J. Pharm. Pharmacol. 25:359-370.
3. Ling, K. H. 1976. Study on mycotoxins contaminated in food in Taiwan. (2) Tremor inducing compounds from *Aspergillus terreus*, p. 121-130. In Proceedings of the National Science Council, Republic of China, no. 9., part 2.
4. Ling, K. H., C.-K. Yang, and H.-C. Huang. 1979. Differentiation of aflatoxins from territrems. Appl. Environ. Microbiol. 37:358-361.
5. Ling, K. H., C.-K. Yang, C.-A. Kuo, and M.-D. Kuo. 1982. Solvent systems for improved isolation and separation of territrems A and B. Appl. Environ. Microbiol. 44:860-863.
6. Ling, K. H., C.-K. Yang, and F.-T. Peng. 1979. Territrems, tremorgenic mycotoxin of *Aspergillus terreus*. Appl. Environ. Microbiol. 37:355-357.
7. Litchfield, J. T., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Therap. 96:99-113.
8. Pasto, D. J., and C. R. Johnson. 1969. Laboratory test for organic chemistry, p. 391-393. Prentice-Hall Inc., Englewood Cliffs, N.J.