생 약 학 회 지
Kor. J. Pharmacogn.
17(2):153~160(1986)

Isolation, Physicochemical Properties and Toxicities of Territrem A′ and B′

Kuo Huang Ling[1], Fu Chuo Peng[1], Bai Jiun Chen[1],
Yu Wang[2] and Gene Hsiang Lee[2]

Institute of Biochemistry, College of Medicine[1] and Department of Chemistry[2], National Taiwan University, Taipei 100, Taiwan, Republic of China

Abstracts—We have isolated two new metabolites of territrem, designated as territrem A′* (TRA′; $C_{28}H_{30}O_{10}$) and B′(TRB′; $C_{29}H_{34}O_{10}$) from chloroform extract of rice culture of *Aspergillus terreus* 23-1, using the same isolation procedure as that for territrem A, B and C(TRA, TRB, TRC). The present isolation procedure gave about 5 mg of TRA′ and 10 mg of TRB′ from 4 kg of rice culture per batch. Analysis of the high resolution mass spectrum showed that the molecular composition of TRA′ and TRB′ are $C_{28}H_{30}O_{10}$ and $C_{29}H_{34}O_{10}$ respectively. Some results of physicochemical properties were presented in this paper. Single crystal X-ray diffractometry of TRB′ showed that the three dimensional structure of TRB′ has not changed significantly from that of TRB ($C_{29}H_{34}O_9$), except for the insertion of one oxygen atom into TRB to make additional pyran in the E-ring. It is also suggested that the aromatic moiety of TRA′ is similar to that of TRA ($C_{28}H_{30}O_9$) and the rest non-aromatic portions resemble to those of TRB′. The tremorgenic activity, lethality and inhibitory effect on acetylcholine esterase of TRA′ and TRB′ are greatly reduced comparing to that of TRA and TRB.

Keywords—Territrem A′ and B′ · tremorgenic mycotoxin · metabolite of *Aspergillus terreus* · inhibitor of acetylcholine esterase · mycotoxin

From the significant discovery of aflatoxin B_1 like blue fluorescent compounds[1], a series of the structure related tremorgenic metabolites were isolated from the chloroform extract of a rice culture of *Aspergillus terreus* 23-1[2-5], which was isolated from the stored unhulled rice[6].

They were designated as territrem A, B and C respectively*. The complete structure of TRB was solved by a single crystal X-ray diffractometry[7]. The interpretation of spectral data of TRB was found to match well with the structure. The structures of TRA and TRC were determined by the comparison of their spectral data and chemical reaction with those of TRB.

More recently we have succeeded to isolate two additional blue fluorescent compounds designated as TRA′ and TRB′[5] from the fraction 1 of chloroform extract of rice culture of *Aspergillus terreus* 23-1.

The isolation, some physicochemical properties, acute toxicity, effect on acetylcholine esterase and chemical structure of TRA′ and TRB′ are presented in the paper.

* Abbreviation: Territrems A, A′, B, B′, C=TRA, TRA′, TRB, TRB′, TRC

Experimental

Material

For column chromatography, Silica Gel 60 (0.063~0.200 mm and 270~230 mesh of Merck no. 7734) and Sephadex LH 20 (25~100 μm) of Pharmacia Fine Chemicals (Piscataway, N.J.) were used. TLC plates used were precoated aluminum sheets of Silica Gel 60 F_{254}(Merck no. 5554). Eel acetylcholine esterase(EC 318) and acetylthiocholine were purchased from Sigma Co. Organic solvents and other reagents used were analytical grade.

Organism and Inoculum

Organism of *Aspergillus terreus* 23-1 was maintained on the modified Czapek Dox agar slant[8] with occasional transfer. The suspension of spores in 0.01% triton x-100 from the slant served as the inoculum.

Cultivation

4 kg of polished rice of Japonica type in 40 1-liter erlenmeyer flasks was used per batch. Each flask containing 100 g of rice was submerged in 200 ml water for overnight, and the excess water was discarded. The rice was autoclaved at 120°C for 15 min. After cooling the media was inoculated with 5×10^6 spores of *Aspergillus terreus* 23-1 and incubated at 28~30°C for 14 days as static culture.

Isolation

The procedure was followed the method described before[9] (Fig. 1).

(a) Extraction

The moldy rice in each flask was submerged in 200 ml chloroform, which was warmed at 50°C for 30 min, filtered and pooled together. 10 liters of the combined chloroform extracts were stored overnight in order to separate the upper layer of water.

(b) Clean up on Silica Gel Column

The chloroform extracts were passed directly through a column (3 cm inside diameter) which was packed with 100 g of Silica Gel 60 and 150 g of anhydrous sodium sulfate at the top portion of the column, at a flow rate of about 15 ml/min. Territrems were absorbed and concentrated within the upper half of silica gel and most of the less polar substances were elutriated out. After washing with 1 liter of chloroform, the column was elutriated with 600 ml of a mixture of chloroform:acetone (9:1, v/v) at a flow rate of 1 ml/min. Each 20 ml of the effluent was collected. Territrems were detected by TLC developed in toluene: ethylacetate: formic acid (5:4:1, v/v). The tubes containing TRA, TRA′, TRB and TRB′ were pooled together (fraction 1), concentrated by a rotatory evaporator to dryness. The crude preparation was dissolved in a minimal volume of chloroform and precipitated with n-hexane.

(c) Silica Gel Column Chromatography

The precipitate, about 800 mg from one batch, was dissolved in a minimal volume of chloroform and applied to the other column of Silica Gel of the same condition used in the clean up step, except that benzene: ethylacetate (1:1, v/v) was used for elutriation. The flow rate was at 1 ml/min. Territrems in each tube were detected by TLC as described before. The tubes containing TRA, a mixture of TRA+TRA′+TRB, TRB and TRB′ were separately pooled together and dried by a rotary evaporator.

(d) Isolation of TRA′

The precipitate of a mixture of TRA+TRA′+TRB was dissolved in a minimal volume of absolute ethylalcohol and applied to a column of Sephadex LH 20 (2 cm inside diameter, 29 cm length, previously equilibrated with absolute ethylalcohol). The column was elutriated with absolute ethylalcohol at a flow rate of 0.8 ml/min. Each tube containing 16 ml of the effluent was

checked for territrems by TLC developed with benzene: ethylacetate (63:37, v/v). The tubes containing TRA′ were pooled together and dried as described before. The recrystallization was made in acetone.

(e) Isolation of TRB′

The precipitate of TRB′ obtained in the step (c) was dissolved in a mixture of chloroform: methanol (1:1, v/v) for crystallization. The solvent for recrystallization was chloroform: benzene (5:1, v/v)

Thin Layer Chromatography (TLC)

The precoated aluminum plates of Silica Gel 60 F_{254} (12 cm by 12 cm, thickness 0.2 mm, Merck no 5554) were used. The plates were activated for 1 hr at 120°C before use. To detect territrems in the moldy rice, 40 μl of chloroform extract was spotted with a microsyringe (Hamilton 701-N 80300) at a 2 cm distance from the bottom of the plate and developed for 10 cm ascending distance at 25±3°C in an unlined and unequilibrated tank. The fluorescent compounds on the plate were viewed under long wave (366 nm) UV light. Substances capable of quenching the fluorescent background of the plate were detected under short wave of UV light (254 nm) (UV lamp UV 56~58 Ultraviolet Product Inc., San Gabriel California, USA).

HPLC

A Tracer Instrument Model 900 series liquid chromatograph equipped with Model 950 pump, 907 variable volume valve loop injecter, Nucleosile 10 C_{18} colmn and Model 970 A variable wave length detector was used. 10 μl of the sample in methanol was injected and eluted by CH_3CN:H_2O (55:45, v/v) at a flow rate of 1.0 ml/min.

Analysis of Physicochemical Properties

Melting points were measured with a hot stage melting apparatus (Shimatzu Seisakusho Ltd.) without coprection of data. Optical rotation was measured with a Jasco Dip-181 digital polarimeter at 589 nm (sodium D line). Fluorescence was measured with a Hitachi Model 204 fluorescence spectrophotometer. The UV absorption spectrum of territrem in methanol or in chloroform was recorded on a Jasco Uvidec-1 spectrophotometer. Infrared spectrometry was recorded with a Perkin Elmer Model 577 grading infrared spectrophotometer. Low resolution mass spectrometry was analyzed with a Finnigan 4510 spectrophotometer. High resolution mass spectrometery was carried with an AEI MS-30 spectrometer at direct probe by Shrader Analytical and Consulting Laboratories Inc. (Detroit, Mich.USA). H^1-nmr was recorded with a Varian EM 360, 60 MHz nuclear magnetic resonance spectrophotometer.

Single Crystal X-Ray Diffractometry of TRB′

Territrem B′ crystallized into an orthorhombic configuration (space group $p2_12_12_1$) with the following cell dimensions: a=0.8840(3), b=1.2484 (2), c=2.4432(4) nm; Z=4, D=1.34, D_m= 1.33 g cm^{-1}. The intensity was measured with M_oK radiation on a Nonius CAD4 diffractometer by $\theta/2\theta$ scan techniques. The details were described somewhere[5].

Biological Tests

The procedure of acute toxicity test and the test animals were follwed the similar procedure and test animals of the previous reports[2,3] The method for determination of acetylcholine esterase was followed the method of Ellman et al[10]

Results and Discussion

Two dimensional TLC under detection of long wave and short wave UV light explored at least 15 spots. Only the Rf values of territrems are shown in the Table I. The descending order of Rf values from territrems A, A′, B, B′ and C were demonstrated in the table. The procedure of isolation of territrems is shown in Fig. 1. All

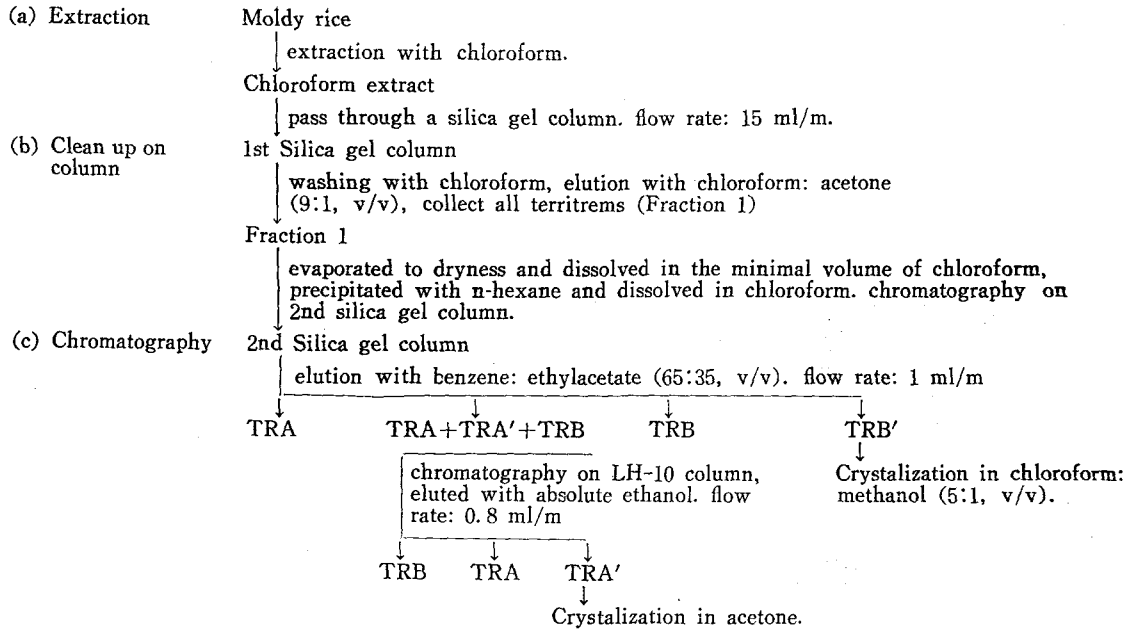


Fig. 1. A flow sheet of the isolation of territrem A, A', B and B'

Table I. Rf values of two dimensional TLC

Solvents	TRA	TRA'	TRB	TRB'	TRC
1-D benzene: EtOAc (1:1, v/v)	0.60	0.54	0.49	0.41	0.29
2-D toluene: EtOAc: HCOOH (5:4:1, v/v)	0.59	0.56	0.52	0.49	0.40

blue fluorescent compounds in the chloroform extract of the moldy rice were absorbed in the upper half of the silica gel column. Territrems A, A', B and B' were elutriated in the solvent of a mixture of chloroform: acetone (9:1, v/v). The territrems in this fraction (fraction 1) were precipitated with n-hexane. The subsequent steps of chromatography and crystalization gave about 5 mg TRA', 10 mg TRB', 100 mg TRA and 370 mg TRB per batch of 4 kg rice culture. The purity of territrem was tested by TLC and HPLC. Some physicochemical properties of TRA' and TRB' are shown in Table II. Both TRA' and TRB' have similar UV absorption spectra and fluorescence spectra, which are also similar to those of TRA, TRB and TRC. The similarity of spectra in UV absorption and fluorescence in

Table II. Some physicochemical properties of territrem A' and B'

Properties	Territrem A'	Territrem B'
Molecular formula	$C_{28}H_{30}O_{10}$	$C_{29}H_{34}O_{10}$
Molecular weight	526	542
Melting point (°C)	264-266	222-224
Fluorescence(nm)		
λ_{exc}(MeOH)	375	375
λ_{em}(MeOH)	420	420
UV spectrum(nm)		
λ_{max}	219 (ε:31823)	219 (ε:33598)
	338 (ε:15573)	331 (ε:19953)

territrems indicated that their chromophores are very similar. No bathochromic shift of TRA' and TRB' (also TRA and TRB) in 0.1 N methanolic NaOH indicates absence of phenolic OH in their molecules. The major infrared absorption (cm^{-1}) of TRA and TRB were also observed in infrared spectra of TRA' and TRB' (Table III), which indicates that the same functional groups are present in all territrems. The high resolution mass spectra showed the observed molecular ion of TRA' and TRB' at

Table III. Infrared spectra of territrem A′ and B′ ν_{max}(KBr)Cm^{-1}

Territrem A′	Territrem B′	Assignment for TRA and TRB
3493, 3370	3694, 3385	C(4a, 12a) OH stretch
3113, 2964, 2919	3099, 2999, 2958	Ar. and Aliphatic: CH stretch
1725	1706	C(11)=O keton: C=O stretch
1629, 1577, 1517	1641, 1585, 1501	Ar and Olefinic: C=C stretch
1485, 1445, 1415 1404	1442, 1408, 1366	
1390, 1346, 1297	1334, 1297, 1245	
1261, 1201	1202, 1187, 1162	
1149	1127	C-O-C ether stretch
1101, 1058, 1046	1085, 1060, 1006	
1029, 983	972, 924, 895	
930, 897, 881 857	859, 804, 776	
750, 719, 668 543, 455	833, 801, 785, 748	

Table IV. Molecular formula of territrems A, A′, B and B′

Territrem	Molecular formula	Molecular found	Weight required	Difference
TRA′	$C_{28}H_{30}O_{10}$	526.1890	526.1888	TRA′-TRA= TRB′-TRB= 16(O)
TRA	$C_{28}H_{30}O_9$	510.1900	510.1888	
TRB′	$C_{29}H_{34}O_{10}$	542.2167	542.2200	TRB′-TRA′= TRB−TRA= 16(CH_4)
TRB	$C_{29}H_{34}O_9$	526.2285	526.2200	

m/z as 526.1890 and 542.2167 with the computerized elemental composition of $C_{28}H_{30}O_{10}$ and $C_{29}H_{34}O_{10}$, respectively (Table IV). The difference of molecular composition between TRA′ and TRA (and also between TRB′ and TRB) is 16(O). On the other hand the difference of molecular composition between TRA′ and TRB′ (and also between TRA and TRB) is 16 (CH_4). Three pairs of ion fragments with a difference of 16(O) between TRA′ and TRA (and also between TRB′ and TRB) are shown in Table V. These ion fragments were probably produced by the initial dehydration and subsequent demethylation of the molecule. In Table VI, a series

Table V. Comparison of mass spectra: Ion fragments with a difference of 16(O)

A′−A =16(O)	Territrem A′ ($C_{28}H_{30}O_{10}$)	Territrem A ($C_{28}H_{30}O_9$)	
	493(39.16)	477(61.8)	−15(CH_3)
	508(19.10)	492(34.5)	−18(H_2O)
	526(26.42)(M^+)	510(7.3)(M_+)	
B′−B =16(O)	Territrem B′ ($C_{29}H_{34}O_{10}$)	Territrem B ($C_{29}H_{34}O_9$)	
	509(7.11)	493(36.5)	−15(CH_3)
	524(30.7)	508(40.0)	−18(H_2O)
	542(6.33)(M^+)	526(8.8)(M^+)	

Table VI. Comparison of mass spectra: Ion fragments with a difference of 16(CH_4)

	Territrem A ($C_{28}H_{30}O_9$)	Territrem B ($C_{29}H_{34}O_9$)
B−A=16(CH_4)	179 (100)	195 (100)
	221 (8.7)	237 (6.9)
	275(14.3)	291(15.5)
	329 (5.3)	345(10.1)
	343(11.5)	359 (5.6)
	459 (4.6)	475((3.4)
	477(61.8)	493(36.5)
	492(34.5)	508(10.0)
	510 (7.3)M^+	526 (8.8)M^+
	Territrem A′ ($C_{28}H_{30}O_{10}$)	Territrem B′ ($C_{29}H_{34}O_{10}$)
B′−A′=16(CH_4)	179(91.45)	195(85.85)
	180 (6.9)	196 (8.11)
	202 (2.7)	205 (2.45)
	221(51.87)	218 (4.77)
	262 (2.17)	278 (5.91)
	275(11.23)	291 (9.59)
	493(39.16)	509(7.11)
	508(19.10)	524 (3.70)
	526(26.42)M^+	542(6.33)M^+

of ion fragments with a difference of 16(CH_4) between TRA and TRB′ were shown, which involves both molecular ions and the smallest ion fragments such as ***m/z*** 175 in TRA′ and ***m/z*** 195 in TRB′. The similar finding was also made between TRA and TRB (Table VI). It was suggested[2] that each phenyl group and CO

Table VII. Comparison of mass spectra: similar ion fragments
(TRB′=TRB)−(TRA′=TRA)=16

TRA′	TRA	TRB′	TRB
275(11.23)	275(14.30)	291 (9.59)	291(15.4)
262 (2.17)	262 (3.50)	278 (2.88)	278 (0.8)
221(51.87)	221 (8.70)	237 (5.92)	237 (6.9)
179(91.45)	179 (100)	195(85.85)	195 (100)

accounted for the respective base peak of a benzoyl cation, namely m/z 179 ($C_9H_7O_4^+$) for TRA′ (also for TRA), m/z 195 ($C_{10}H_{11}O_4^+$) for TRB′ (also for TRB). The largest common moiety, m/z 275 in TRA′ and TRA shown in Table VII would contain the same benzoyl cation, m/z 179. Likewise the largest common moiety of m/z 291 in TRB′ and TRB would contain

Table VIII. Comparison of proton magnetic resonance spectra δ_{ppm}.

TRA	TRA′	TRB	TRB′	Pattern	Assingment for TRA, TRB	
1.21	1.075	1.185	1.075	3H	C(4)-CH_3	
1.30	1.273	1.276	1.273	3H	C(4)-CH_3	
1.47	1.327	1.457	1.327	3H	C(6a)-CH_3	
1.53	—	1.518	—	3H	C(12a)-CH_3	
—	—	3.90	3.90	9H, S	Ar-$(OCH_3)_3$	aryl moiety
3.93	3.87	—	—	3H, S	Ar-OCH_3	aryl moiety
5.97	5.96	—	—	2H, S	Ar-OCH_2O-	aryl moiety
—	—	6.95	6.98	2H, S	$Ar(H)_2$	aryl moiety
6.88	6.84	—	—	1H, d, J=2Hz	Ar-H	aryl moiety
7.02	6.96	—	—	1H, d, J=2Hz	Ar-H	aryl moiety
5.74	—	5.77	—	1H, d, J=10Hz	C(2)H	
6.26	—	6.28	—	1H, d, J=10Hz	C(3)H	

Table IX. The dose response relation of territrems versus mortality and tremor in mice by i.p. injection. The median effective dose (mg/kg body weight):

Territrems	TRA	TRB	TRC	TRA′	TRB′
Lethality	17.60±1.91	9.06±1.90	6.28±1.45	>100	>100
Tremor	0.3±0.05	0.21±0.03	0.24±0.03	>100	>100

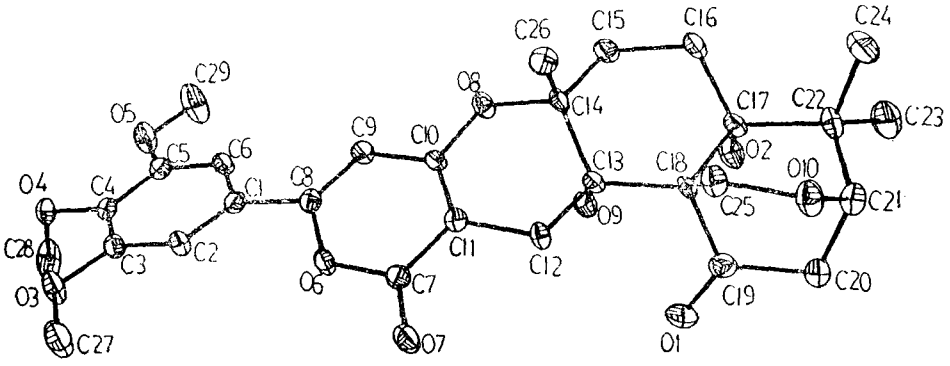
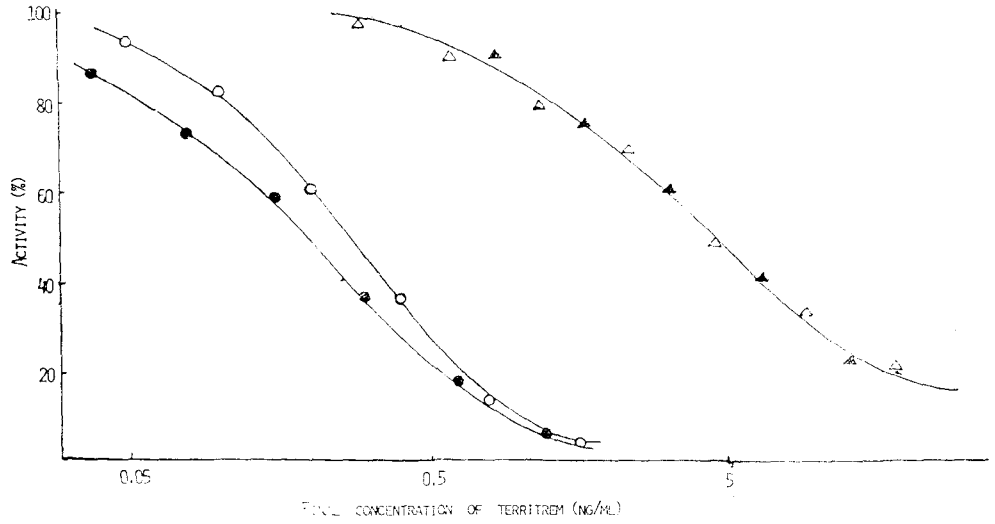


Fig. 2. Three dimensional structure of territrem B′.



final concentration of territrem(ng/ml)

Fig. 3. Inhibition of acetylcholine estrase by territrem. TRA (○), TRB (●), TRA′ (△)), TRB′ (▲)

the same benzoyl cation, *m/z* 195. The signals of H^1-nmr located in their aromatic moiety of TRA and TRB were compared with the corresponding H^1-nmr signals in TRA′ and TRB′ (Table VIII). The data indicated that the same aryl moiety presents in TRA and TRA′ (and also in TRB and TRB′). The H^1-nmr signals of four isolated methyl protons in both TRA and TRB were replaced by three isolated methyl protons in both TRA′ and TRB′. It is worthwhile to indicate that the H^1-nmr signals assigned for C(2)H and C(3)H in TRA and TRB disappeared in TRA′ and TRB′. The single crystal X-ray diffractometry of TRB′[5] indicated that the three dimensional structure of TRB′ (Fig. 2) has not changed significantly from that of TRB[7], except for the insertion of one oxygen atom into TRB to make additional pyran ring in the E-ring of TRB. It is also suggested that the aromatic moiety of TRA′ is similar to the aromatic moiety of TRA and the rest non-aromatic portion resembles to the non aromatic portion of TRB′. No tremor or other symptoms were observed in mice after ip injection of TRA′ or TRB′ up to 1 mg/mice. A higher dose of TRA′ or TRB′ at 2 mg/mice did not show tremor and other symptoms. However, the median effective dose (mg/kg body weight) by ip injection in mice of lethality or tremor is quite lower in TRA, TRB and TRC. (Table IX.). The inhibitory effect on acetylcholine esterase of TRA′ and TRB′ are greatly reduced comparing to that of TRA and TRB (Fig 3). The mechanism of induction of tremor and of inhibitory effect on acetylcholine esterase by territrem is under investigation in our laboratory.

Acknowledgement—This work was supported by grant to K.H.L. from the National Science Council, Taipei, Taiwan, Republic of China.

Literature Cited

1. Chung, C.H., Tung, S.S. and Tung, T.C.: *J. Formosan Med. Assoc.* **70**, 258 (1971)
2. Ling, K.H., Yang, C.K. and Peng, F.T.: *Appl. and Environ. Microbiol.* **37**, 355 (1979)
3. Ling, K.H., Liou, H.H., Yang, C.M. and Yang, C.K.: *Appl. and Environ. Microbiol.* **47**, 98 (1984)
4. Ling, K.H., Yang, C.K., Liou, H.H., Yang, C.M., Kuo, M.D. and Peng, F.C.: *Proceeding of ROC-ROK 1st Symposium on Natural Product Chemistry*, Taipei, Taiwan ROC, Nov. 16-20

(1984)
5. Peng, F.C., Ling, K.H., Wang, Y. and Lee, G.H.: *Appl. and Environ. Microbiol.* **49**, 721 (1985)
6. Tung, S.S., Ling, K.H., Tsai, S.E., Chung, C.H., Wang, J.J. and Tung, T.C.: *J. Formosan Med. Assoc.* **79**, 251 (1971)
7. Hseu, T.H., Yang, C.K., Ling, K.H., Wang, C.J. and Tang, C.P.: *Cryst. Struct. Comm.* **11**, 199 (1982)
8. Wang, C.T., Ling, K.H. and Tung, T.C.: *J. Formosan Med. Assoc.* 65, 348 (1966)
9. Ling, K.H., Yang, C.K., Kuo, C.A. and Kuo, M.D.: *Appl. and Environ. Microbiol.* **44**, 860 (1982)
10. Ellman, G.L., Courtney, K.D., Andres, V. and Feathersone, R.M.: *Biochem. Pharmacol.* **7**, (1961)