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Isolation and Structures of AK-Toxin I and II, Host-specific Phytotoxic Metabolites Produced by *Alternaria alternata* Japanese Pear Pathotype

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Two host-specific phytotoxic metabolites, AK-toxin I and II, were isolated from a culture broth of *Alternaria alternata* Japanese pear pathotype, the fungus causing black spot disease of susceptible Japanese pear cultivars. From chemical, spectral and X-ray crystallographic data, AK-toxin I was characterized as 8-[(2′*S*, 3′*S*)-2′-acetylamino-3′-methyl-3′-phenyl-propionyloxy]-(8*R*, 9*S*)-9,10-epoxy-9-methyl-deca-(2*E*, 4*Z*, 6*E*)-trienoic acid. The structure of AK-toxin II was also assigned to be 3′-demethyl derivative of AK-toxin I by comparing the spectral data with those of AK-toxin I.

At the beginning of this century, black spot disease of Japanese pear has become prevalent in the orchards of Japan immediately after widespread introduction of a new cultivar "Nijisseiki." The causal fungus for the disease, *Alternaria kikuchiana* Tanaka (at present regarded as *Alternaria alternata* Japanese pear pathotype[1]), showed the characteristic feature of being infective to Nijisseiki but not to other resistant cultivars of Japanese pear. In 1933 Tanaka[2] reported that *A. alternata* Japanese pear pathotype produced unique phytotoxic metabolite(s) responsible for the host specific pathogenicity, presenting the first evidence for the host-specific toxin which was proposed later as a descriptive word to define such phytotoxic metabolites.[3] Since then, many investigators have been prompted to isolate the active principle(s) from culture broth of the fungus and as the result several phytotoxic metabolites have been identified,[4~11] but none of them has shown host-specific activity. In the previous paper[12] we reported briefly the identification of AK-toxin I and II as the host-specific toxin produced by *A. alternata* Japanese pear pathotype. The present paper deals with the elucidation of their absolute configurations and some additional data for their isolation and identification and their biological activity.

The production of the host-specific toxins in the culture medium and every step of their isolation procedures were monitored by the bioassay method using fresh young leaves of both a susceptible Japanese pear cultivar *Pyrus serotina* Rehder *var. culta*, cv. Nijisseiki) and a resistant one (*P. serotine* Rehder *var. culta*, cv. Chojuro, see EXPERIMENTAL). The bioassay results showed that the maximal toxin production was attained when *A. alternata* Japanese pear pathotype (strain No. AS-1223) was cultured in a jar fermentor at 28°C for 3 days in a modified Richards' medium.

The isolation procedures are summarized in Fig. 1. The active principles in the culture filtrate inducing necrotic spots on the leaves of Nijisseiki but not on Chojuro were adsorbed

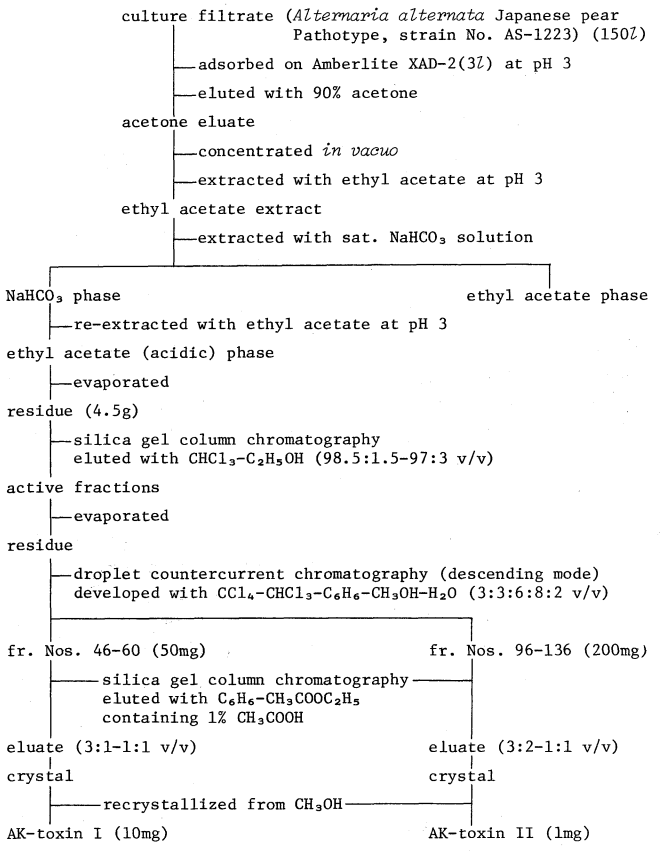

FIG. 1. Isolation of AK-Toxin I and II.

on Amberlite XAD-2 at pH 3, eluted with 90% acetone and concentrated under reduced pressure. The residue was extracted with ethyl acetate at pH 3. The acidic fraction in the extract was extracted with saturated $NaHCO_3$ aqueous solution and collected by re-extraction with ethyl acetate after acidification to pH 3. The acidic fraction was subjected to silica gel column chromatography and subsequent droplet countercurrent chromatography to give two active fractions, each of which was purified by silica gel chromatography and recrystallization from methanol to isolate two active compounds: AK-toxin I, mp 168°C (decomp.), $[\alpha]_D^{25}+164$ ($c=0.128$ in methanol) and AK-toxin II, mp. 163°C (decomp.), $[\alpha]_D^{23}+125$ ($c=0.132$ in methanol). The isolated crystalline AK-toxin I and II showed the same level of host-specific phytotoxic activity to that of the causal fungus itself. The

TABLE I. CORRELATION BETWEEN THE SUSCEPTIBILITY TO *Alternaria alternata* JAPANESE PAPER PATHOTYPE AND THE SENSITIVITY TO AK-TOXIN I AND II AMONG CULTIVARS OF JAPANESE PEAR

Clutivar	Spore inoculation*	Toxin treatment (10^{-5} M)*	
		AK-toxin I	AK-toxin II
Chojuro	−	−	−
Doitsu	+	+	+
Kikusui	−	−	−
Kosui	−	−	−
Nijisseiki	+	+	+
Shinsui	+	+	+

* Leaf symptoms are rated: +, positive; −, negative.

threshold concentration inducing veinal necrosis on the susceptible cultivar, Nijisseiki, was 5×10^{-9} M and 10^{-7} M of AK-toxin I and II respectively, but the leaves of the resistant cultivar, Chojuro, were not affected even by

TABLE II. SIGNIFICANT IONS OBSERVED IN HR-MS SPECTRA OF AK-TOXIN I AND DERIVATIVES (in-beam EI 70 eV, ion source temp., 180°C)

Compound	*m/z* (obs.)	Elements	er. (MU)	int. (%)	Comments*
AK-toxin I	413.1853	$C_{23}H_{27}O_6N$	1.6	2	M^+
	204.1021	$C_{12}H_{14}O_2N$	−0.2	34	(A)
	176.1077	$C_{11}H_{14}ON$	0.3	55	(B)
	134.0941	$C_9H_{12}N$	−2.7	54	(C)
	105.0678	C_8H_9	−2.5	100	(D)
Hexahydro AK-toxin I	419.2289	$C_{23}H_{33}O_6N$	−1.6	4	M^+
	204.1014	$C_{12}H_{14}O_2N$	−0.9	6	(A)
	176.1107	$C_{11}H_{14}ON$	3.2	18	(B)
	134.0980	$C_9H_{12}N$	1.1	24	(C)
	105.0685	C_8H_9	−1.8	100	(D)
Hexahydro AK-toxin I Me-ester	433.2430	$C_{24}H_{35}O_6N$	−3.1	4	M^+
	204.1018	$C_{12}H_{14}O_2N$	−0.5	6	(A)
	176.1075	$C_{11}H_{14}ON$	0.0	16	(B)
	134.0952	$C_9H_{12}N$	−1.6	37	(C)
	105.0705	C_8H_9	0.2	100	(D)

* See Fig. 2.

application of 10^{-4} M of AK-toxin I and II. Among the tested Japanese pear cultivars, susceptible ones to the causal fungus were sensitive to AK-toxins, while resistant ones were completely tolerant as shown Table I. More detail biological data and the phytopathological role of these compounds will be published elsewhere in near furure.

The molecular formula of AK-toxin I was determined to be $C_{23}H_{27}O_6N$ from data of high resolution mass spectrometry (HR-MS, see Table II) and its elemental analysis. The ^{13}C-NMR spectrum revealed chemical features of all 23 carbon atoms in the molecule as shown in Table III, indicating the presence of three carbonyl groups which were confirmed by the IR spectrum to be an ester, a conjugated carboxylic acid and an amide (see EXPERIMENTAL).

AK-toxin I was converted by catalytic hydrogenation to its hexahydro-derivative, which was, in turn, treated with diazomethane to give the methyl ester. In their HR-MS spectra (Table II) four significant fragment ions were observed at *m/z* 204, 176, 134 and 105 as in the HR-MS spectrum of AK-toxin I itself, which suggested the presence of an *N*-acetyl-*β*-methyl-phenylalanyl moiety as shown in Fig. 2. This suggestion was consistent with the ^{1}H-NMR spectral data of AK-toxin I (Table IV) where following protons were observed: acety methyl (3H, s, δ 1.84), phenyl (5H, broad s, δ 7.23) and methine (1H, dq, δ 3.22, J=7.1 and 8.8 Hz) coupling with both another methine (1H, d, δ 4.73, J=8.8 Hz) and methyl (3H, d, δ 1.30, J=7.1 Hz). Firm evidence was given by

TABLE III. ^{13}C-NMR SPECTRUM OF AK-TOXIN I (50 MHz in CD_3OD)

Chemical shift (δ ppm)	Assignment*	Chemical shift (δ ppm)	Assignment*
17.75	$-CH_3$ C (11)**	129.43	–CH=
18.99	$-CH_3$ C (12′)	129.55	–CH= ×2
22.18	$-CH_3$ C (11′)	130.64	–CH=
42.98	>CH– C (3′)	132.23	–CH=
52.83	$-CH_2-$ C (10)	136.48	–CH=
57.77	>C< C (9)	140.06	–CH=
59.40	>CH– C (2′)	143.37	>C= C (4′)
77.57	>CH– C (8)	170.22	–CO–
124.20	–CH=	171.70	–CO–
128.04	–CH=	173.04	–CO–
128.80	–CH= ×2		

* Assigned by off-resonance decoupling and peak height.

** The numbering of carbon atoms is according to that of Fig. 4.

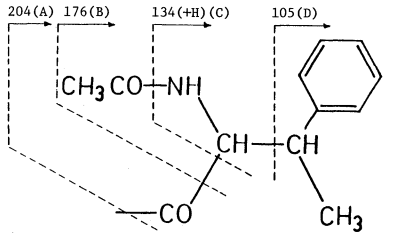

FIG. 2. Mass Fragment Patterns of AK-Toxin I and Derivatives.

* Each mass number in HR-MS spectra of the fragment ions [(A), (B), (C) and (D)] is given in Table II.

TABLE IV. ^{1}H-NMR SPECTRUM OF AK-TOXIN I (200 MHz in CD_3OD)

Chemical shift (δ ppm)	Number of protons			Remarks
1.30	3	d	J=7.1 Hz	H (12′ABC)*
1.31	3	s		H (11ABC)
1.84	3	s		H (11′ABC)
2.61	1	d	J=4.8 Hz	H (10A)
2.76	1	d	J=4.8 Hz	H (10B)
3.22	1	dq	J=8.8, 7.1 Hz	H (3′)
4.73	1	d	J=8.8 Hz	H (2′)
5.35	1	d	J=7.1 Hz	H (8)
5.87	1	dd	J=15.2, 7.1 Hz	H (7)
5.95	1	d	J=15.1 Hz	H (2)
6.24	1	dd	J=11.2, 11.1 Hz	H (4)
6.39	1	dd	J=11.1, 11.0 Hz	H (5)
6.95	1	dd	J=11.0, 15.2 Hz	H (6)
7.23	5	bs		H (5′~9′)
7.77	1	dd	J=15.1, 11.2 Hz	H (3)

* Numbering of protons is according to that of Fig. 4.

the fact that acid hydrolysis of AK-toxin I gave L-erythro-β-methyl-phenylalanine (*i.e.* 2′*S*,3′*S*-configuration), which was identified by comparing its physicochemical properties with those of the reported values[13] (see Table V).

The remaining structural moiety in the molecule of AK-toxin I should be $C_{11}H_{13}O_4$, containing a methyl, a methylene, a methine, a quaternary carbon, three carbon-carbon double bonds, a carboxylic acid and two oxygen atoms (see Tables III and IV). Although no experiments on the acid hydrolysis of AK-toxin I were successful in isolating component(s) other than β-methyl-phenyl-

TABLE V. IDENTIFICATION OF β-METHYL-PHENYLALANINE

	From AK-toxin I	*erythro*-L*	*threo*-L*
$[\alpha]_D$ (H_2O)	−27.4° (c=0.15, 18°C)	−30.6° (c=1, 20°C)	−7.5° (c=1, 20°C)
NMR (D_2O)**			
δ_{CH_3}	1.43		
δ_{H_β}	3.31	3.31	3.55
δ_{H_α}	3.85	3.81	3.94
$\delta_{H_{Ph}}$	7.38		
$J_{CH_3\text{-}H_\beta}$	7.0	6.9	7.2
$J_{H_\alpha\text{-}H_\beta}$	7.5	7.5	4.8

* Reported values.[13]

** δ, ppm from DSS; J, Hz.

alanine, the spectral data of AK-toxin I revealed it to be 9,10-epoxy-8-hydroxy-9-methyl-deca-(2*E*,4*Z*,6*E*)-trienoic acid. Absorbtion band at 1690 cm^{-1} in the IR- and 284 nm (ε=27,600) in the UV-spectrum of AK-toxin I suggested a conjugated trienoic acid structure. Six signals of olefinic protons in the ^{1}H-NMR spectrum of AK-toxin I (Table IV) were assigned to each proton as shown in Fig. 3 based on their chemical shifts, coupling constants and on the following experimental results. The addition of $Pr(NO_3)_3$ shift reagent resulted in a large paramagnetic shift of two signals of δ 5.95 and 7.77, indicating that these signals were assigned to H(2) and H(3) respectively. The coupling constants of olefinic protons suggested geometric arrangements between each pair of the triene moiety: H(2)–H(3), *trans* (J=15.1 Hz); H(4)–H(5), *cis* (J=11.1 Hz); H(6)–H(7), *trans* (J=15.2 Hz); and H(3)–H(4), H(5)–H(6) and H(7)–H(8), *vicinal* (J=11.2, 11.0 and 7.1 Hz, respectively). These were supported by observation of the NOE enhancement between each pair of H(2) and H(4), H(3) and H(6), and H(5) and H(7), as shown in Fig. 3.

The doublet signal of one proton at δ 5.35 was assigned to >CH–O– at the C(8) position on the basis of its chemical shift and coupling with H(7) (J=7.1 Hz). The remaining CH_3– (δ 1.31, s), –CH_2– (δ 2.61 and 2.76, each d), >C< (δ 57.77, s, in the ^{13}C-NMR) and –O– were arranged into a 9,10-epoxy-9-methyl

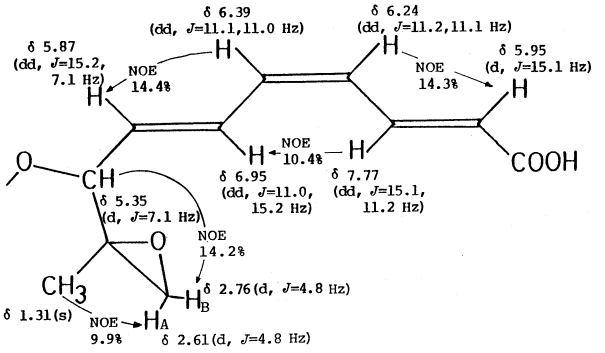

FIG. 3. Assignment of ^{1}H-NMR Signals of AK-Toxin I.

structure on the basis of NOE enhancement in both cases between H(10A) and C(9)-methyl protons and between H(10B) and H(8), as shown in Fig. 3.

Consequently the structure of AK-toxin I was concluded to be 8-[(2′*S*,3′*S*)-2′-acetylamino-3′-methyl-3′-phenyl-propionyloxy]-9,10-epoxy-9-methyl-deca-(2*E*,4*Z*,6*E*)-trienoic acid, except for the absolute configurations of C(8) and C(9). Their absolute configurations were confirmed unambiguously by a single crystal X-ray analysis. The structure of AK-toxin I was solved by the direct method and refined by block-diagonal least squares method to $R=0.042$. A perspective computer-drawn model of AK-toxin I is shown in Fig. 4. The absolute configurations of C(8) and C(9) were determined by the relationship to (2*S*,3*S*)-2-amino-3-methyl-3-phenyl-propionic acid obtained from the acid hydrolyzate of AK-toxin I. On the basis of these experimental results, the structure of AK-toxin I is proposed to be that in Fig. 5 including the absolute configurations of C(8*R*), C(9*S*), C(2′*S*) and C(3′*S*).

The molecular formula of AK-toxin II was determined as $C_{22}H_{25}O_6N$ from HR-MS data. The characteristic fragment ions observed in the lower mass region of the mass spectrum of AK-toxin II were by 14 mass units (CH_2) less than those of AK-toxin I: *m/z* 190.0863 [C_6H_5–CH_2–$CH(C\equiv\overset{+}{O})$–NH–CO–$CH_3$, calcd. 190.0867], *m/z* 162.0912 (C_6H_5–CH_2–$CH=\overset{+}{N}H$–CO–CH_3, calcd. 162.0917), *m/z*

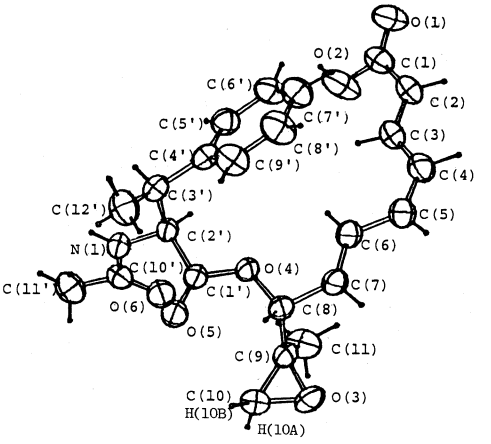

FIG. 4. Perspective Computer-drawn Model of AK-Toxin I.

120.0823 (C_6H_5–CH_2–$CH=\overset{+}{N}H_2$, calcd. 120.0813) and *m/z* 91.0566 [$(C_7H_7)^+$, calcd. 91.0547]. These fragment ions suggested that there was an *N*-acetyl-phenylalanyl residue as a structural component of AK-toxin II in the place of the *N*-acetyl-*β*-methyl-phenylalanyl moiety in AK-toxin I. Signals in the ^{1}H-NMR spectrum of AK-toxin II showed a pattern quite similar to those of AK-toxin I with almost the same chemical shifts, except the fact that a multiplet methine signal at δ 2.97 ~ 3.16 (2H, AB parts of an ABX system) and a multiplet methine signal at δ 4.66 (1H, X part of an ABX system) were observed in AK-toxin II instead of the double quartet methine signal at δ 3.22 [1H, H(2′)] in AK-toxin I (see Table IV). These data clearly demonstrate that AK-

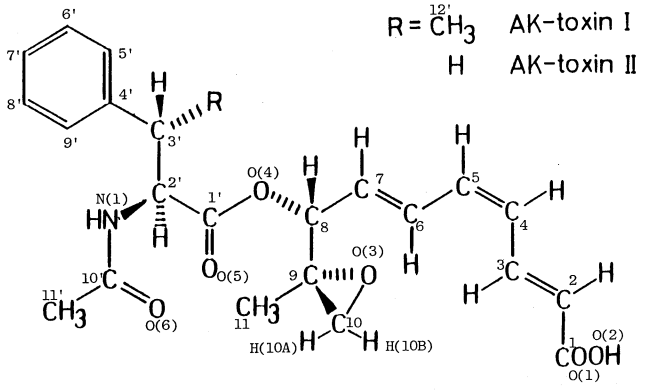

FIG. 5. Structures of AK-Toxin I and II.
The numbering of atoms is according to that of Fig. 4.

toxin II contained a 2-acetylamino-3-phenylpropionyl moiety as the structural component, and consequently AK-toxin II was the 3′-demethyl derivative of AK-toxin I as shown in Fig. 5. Absolute configurations of C(8), C(9) and C(2′) in AK-toxin II seem to be the same as AK-toxin I without any definitive evidence, since both toxins indicate the same host-specific phytotoxic activity (see Table I).

EXPERIMENTAL

Melting points are uncorrected. Spectral data were recorded as follows: UV spectra on a Shimadzu UV-360, IR spectra on a Shimadzu IR-279 spectrometer, optical rotation on a JASCO ORD UV-5, ^{1}H-NMR spectra on a JEOL FX-100, FX-200 or Brucker WH-270 spectrometer, and ^{13}C-NMR spectra on a JOEL FX-200 spectrometer (the internal standard was tetramethylsilane in organic solvents and DSS in deutrated water). Low resolution and high resolution mass spectral data were obtained by the In-Beam EI (70 eV) method on Hitachi M-70 and M-80 instruments coupled with a 003-B data system, respectively, and FD-MS were obtained on the latter. X-ray diffraction data were obtained on a Rigaku AFC-5 difractometer and computations were performed on a FACOM M-382 computer in the Data Processing Center of Kyoto University.

Rf values on TLC were measured with Kieselgel GF_{254} plate (E. Merck, 0.25 mm thick) using a solvent system of benzene–ethyl acetate–acetic acid (99 : 99 : 2, v/v). Silica gel column chromatography was performed with Kieselgel 60 (E. Merck, 70 ~ 270 mesh ASTM).

Bioassay.

(a) Impregnated silica gel method: A small square section (10 × 10 × 0.25 mm) on a thin layer silica gel plate (Kieselgel GF_{254}, E. Merck) was impregnated with 10 μl of the test solution, scraped onto the back of a fresh young leaf of Japanese pear, moistened with small quantity of water and then incubated at 28°C. After 20 hr an induced necrotic spot was observed. Every isolation procedure of AK-toxins was monitored by this method using both a susceptible and a resistant cultivar (Nijisseiki and Chojuro, respectively).

(b) Droplet method: Fresh young leaves of various cultivars of pear were slightly injured by needles and a droplet of the AK-toxins aqueous solution at a defined concentration was placed on the back of each. After the same incubation procedure as above, induced necrotic spots were observed and compared with each other. This method was used to determine the threshold concentration of AK-toxins to various Japanese pear cultivars.

Culture condition of Alternaria alternata Japanese pear pathotype. A. alternata Japanese pear pathotype (the strain number AS-1223, isolated from an orchard in Nagano prefecture, 1974) was maintained on potato-sucrose-agar slants. Mass production of the host-specific toxins was carried out by the following procedures. The culture medium were sucrose, 25 g; KNO_3, 5 g; KH_2PO_4, 2.5 g; $MgSO_4 \cdot 7H_2O$, 1.25 g in one liter of water. Precultures were grown for 2 days at 28°C by shaking (500 ml medium in a two liter Sakaguchi flask). Thirty liters of medium in a jar fermenter was inoculated with 3 liters of the precultured fungus, and cultured for 3 days (28°C, air at 20 liters/min, agitation at 130 rpm, pressure at 0.7 kg/cm^2).

Isolation of AK-toxin I and II. The preliminary experiment using a small amount of the culture filtrate indicated that the active principle(s) was contained in the acidic fraction. Based on this finding the isolation procedure was designed as shown in Fig. 1. The filtrate of culture medium was acidified to pH 3 with 6 N-HCl and mixed with Amberlite XAD-2 by stirring. The Amberlite was filtered, washed with water and then eluated with 90% acetone.

The acetone eluate was concentrated *in vacuo* to give an aqueous solution, which was adjusted to pH 3 and extracted with ethyl acetate. The obtained ethyl acetate layer was extracted with $NaHCO_3$-saturated water, and then the aqueous layer was acidified to pH 3 before being re-extracted with ethyl acetate. The ethyl acetate extract was evaporated under reduced pressure to give an oily residue, which was chromatographed on silica gel column eluted with a chloroform–ethanol mixture increasing stepwise the mixing ratio of the latter. Repeating these procedures with 150 liters in total of the culture filtrate gave 2 g of active material, which was subjected to droplet countercurrent chromatography [descending mode using a solvent system of tetrachloromethane–chloroform–benzene–methanol–water (3:3:6:8:2, v/v)] to give two active fractions: A, fraction Nos. 46~60 (50 mg) and B, fraction Nos. 96~136 (200 mg). The fraction A was chromatographed on silica gel column eluted with a benzene-ethyl acetate mixture (containing 1% acetic acid) increasing stepwise the mixing ratio of the latter. The fractions eluted with the solvent system containing 25~50% ethyl acetate gave, on evaporation, a crystalline active compound which was recrystallized from methanol to isolate pure AK-toxin I (10 mg). The fraction B was chromatographed according to the same procedure as that used for fraction A, and the fractions eluted with the solvent system containing 40~50% ethyl acetate gave another crystalline active compound, which was recrystallized from methanol to afford AK-toxin II (1 mg).

Physicochemical properties of AK-toxin I and II. The *Rf*-values of AK-toxin I and II on TLC developed with the conditions described above were 0.32 and 0.24 respectively. These spots on TLC were detectable with a 254 nm UV-lamp or with iodine vapour.

AK-toxin I. mp 168°C (decomp.); $[\alpha]_D^{25}+164$ ($c=0.128$ in methanol). UV λ_{max}^{MeOH}: 284 nm (ε 27,600); IR ν_{max}^{KBr}: 3350 cm^{-1} (ν_{N-H} amide), 1750 cm^{-1} ($\nu_{C=O}$ ester), 1690 cm^{-1} ($\nu_{C=O}$ conj. acid), 1680, 1550 cm^{-1} ($\nu_{C=O}$ amide). Elemental analysis. Found: C, 67.26; H, 6.70; N, 3.27%. Calcd. for $C_{23}H_{27}O_6N$: C, 66.18; H, 6.58; N, 3.39%. FD-MS: $(M+H)^+$ m/z 414. HR-MS and ^{13}C-NMR and ^1H-NMR data are shown in Tables II, III and IV, respectively.

AK-toxin II. mp 163°C (decomp.); $[\alpha]_D^{23}+125$ ($c=0.132$ in methanol). UV λ_{max}^{MeOH}: 286 nm (ε 28,100); FD-MS: $(M+H)^+$ m/z 400; HR-MS (In-Beam EI, 70 eV): M^+, m/z 399.1676 (calcd. for $C_{22}H_{25}O_6N$, 399.1679); ^1H-NMR $\delta_{Me_4Si}^{CD_3OD}$ 1.29 [3H, s, H(11ABC)], 1.93 [3H, s, H(11'ABC)], 2.61 [1H, d, $J=4.6$ Hz, H(10A)], 2.75 [1H, d, $J=4.6$ Hz, H(10B)], 2.96~3.16 [2H, m, H(3'AB)], 4.66 [1H, m, H(2')], 5.22 [1H, d, $J=7.0$ Hz, H(8)], 5.70 [1H, dd, $J=15.2$, 7.0 Hz, H(7)], 5.94 [1H, d, $J=15.1$ Hz, H(2)], 6.21 [1H, dd, $J=10.7$, 10.7 Hz, H(4)], 6.27 [1H, dd, $J=10.7$, 10.7 Hz, H(5)], 6.81 [1H, dd, $J=15.2$, 10.7 Hz, H(6)], 7.25 [5H, bs, H(5'~9')] and 7.73 [1H, dd, $J=10.7$, 15.1 Hz)].

Hexahydro AK-toxin I. AK-toxin I (3 mg) was shaken over in methanol solution for 1 hr over a small amount of platinum oxide in an atmosphere of hydrogen. The MS spectrum of the product indicated that AK-toxin I was converted to its hexahydro-derivative; The *Rf*-value on TLC was 0.42 in the same conditions described above. FD-MS: $(M+H)^+$, m/z 420. The HR-MS data are shown in Table II.

Methyl ester of hexahydro AK-toxin I. Hexahydro AK-toxin I (3 mg) was dissolved in methanol (1 ml) and estrified with diazomethane to give a product [*Rf*-value on TLC, 0.43; solvent system, benzene–ethyl acetate (4:1, v/v)], the structure of which was confirmed by the HR-MS data shown in Table II.

Identification of L-erythro-β-methyl-phenylalanine. AK-toxin I (18 mg) in 2 N-hydrochloric acid (2 ml) was heated in a sealed tube at 110°C for 16 hr. The resulting hy-

TABLE VI. ATOMIC PARAMETERS FOR NON-HYDROGEN ATOMS IN AK-TOXIN I

Estimated standard deviations are given in parentheses. B_{eq} is the isotropic equivalent of the anisotropic thermal parameter (Hamilton, 1959).

Atom	x	y	z	B_{eq}
C(1)	0.9855 (1)	0.8969 (3)	0.9213 (4)	4.33 (16)
C(2)	0.9955 (1)	0.8577 (3)	0.7595 (5)	4.18 (15)
C(3)	0.9495 (1)	0.8422 (3)	0.6575 (4)	3.87 (15)
C(4)	0.9570 (1)	0.8071 (3)	0.4989 (4)	4.45 (17)
C(5)	0.9095 (1)	0.7880 (3)	0.3986 (4)	4.47 (16)
C(6)	0.8437 (1)	0.7888 (3)	0.4332 (3)	3.65 (14)
C(7)	0.8009 (1)	0.7786 (3)	0.3241 (3)	3.70 (14)
C(8)	0.7313 (1)	0.7790 (2)	0.3476 (3)	3.27 (14)
C(9)	0.7015 (1)	0.8777 (2)	0.2627 (3)	3.41 (14)
C(10)	0.6431 (1)	0.8600 (3)	0.1821 (4)	4.50 (17)
C(11)	0.7212 (2)	0.9929 (3)	0.3156 (5)	5.38 (18)
C(1')	0.6647 (1)	0.7484 (2)	0.5640 (3)	3.13 (14)
C(2')	0.6638 (1)	0.7489 (2)	0.7419 (3)	2.87 (14)
C(3')	0.6847 (1)	0.6326 (2)	0.8060 (3)	3.35 (14)
C(4')	0.7546 (1)	0.6177 (2)	0.7931 (3)	3.28 (14)
C(5')	0.7930 (1)	0.6811 (2)	0.8919 (4)	3.88 (14)
C(6')	0.8575 (1)	0.6675 (3)	0.8889 (4)	4.62 (17)
C(7')	0.8846 (2)	0.5905 (3)	0.7880 (5)	4.84 (18)
C(8')	0.8466 (2)	0.5288 (3)	0.6894 (5)	5.23 (18)
C(9')	0.7825 (1)	0.5420 (3)	0.6921 (4)	4.44 (15)
C(10')	0.5762 (1)	0.8754 (2)	0.7705 (4)	3.71 (14)
C(11')	0.5144 (2)	0.8995 (3)	0.8412 (5)	5.26 (19)
C(12')	0.6476 (1)	0.5336 (3)	0.7455 (5)	4.91 (17)
N(1)	0.6018 (1)	0.7757 (2)	0.7987 (3)	3.18 (7)
O(1)	1.0250 (1)	0.8916 (2)	1.0201 (3)	5.93 (14)
O(2)	0.9300 (1)	0.9368 (2)	0.9464 (3)	6.46 (17)
O(3)	0.7006 (1)	0.8592 (2)	0.0969 (2)	4.82 (13)
O(4)	0.7199 (1)	0.7850 (2)	0.5125 (2)	3.14 (4)
O(5)	0.6233 (1)	0.7171 (2)	0.4820 (3)	4.89 (7)
O(6)	0.6041 (1)	0.9459 (2)	0.6875 (3)	4.85 (8)

TABLE VII. FRACTIONAL COORDINATES AND ISOTROPIC THERMAL PARAMETERS (Å^2) FOR HYDROGEN ATOMS IN AK-TOXIN I

Atom	x	y	z	B_{iso}
H(2)	1.035 (1)	0.848 (3)	0.715 (3)	4.1 (6)
H(3)	0.910 (1)	0.845 (2)	0.699 (3)	3.5 (6)
H(4)	1.002 (1)	0.801 (2)	0.466 (3)	3.1 (6)
H(5)	0.921 (1)	0.773 (2)	0.299 (3)	3.5 (6)
H(6)	0.835 (1)	0.804 (2)	0.536 (3)	3.3 (6)
H(7)	0.813 (1)	0.766 (2)	0.215 (4)	3.9 (6)
H(8)	0.712 (1)	0.708 (2)	0.306 (3)	2.9 (6)
H(10A)	0.615 (1)	0.928 (2)	0.163 (4)	3.8 (6)
H(10B)	0.622 (1)	0.785 (3)	0.177 (4)	3.8 (6)
H(11A)	0.703 (1)	1.052 (2)	0.249 (3)	4.0 (6)
H(11B)	0.767 (1)	1.000 (3)	0.312 (3)	3.6 (7)
H(11C)	0.707 (1)	1.006 (3)	0.425 (3)	3.9 (6)
H(2′)	0.693 (1)	0.803 (2)	0.778 (3)	2.7 (6)
H(3′)	0.675 (1)	0.645 (2)	0.908 (3)	3.0 (6)
H(5′)	0.772 (1)	0.735 (3)	0.964 (3)	3.5 (6)
H(6′)	0.885 (1)	0.709 (3)	0.968 (3)	3.8 (6)
H(7′)	0.930 (1)	0.579 (2)	0.776 (3)	3.6 (6)
H(8′)	0.870 (1)	0.475 (3)	0.626 (4)	4.1 (6)
H(9′)	0.761 (1)	0.503 (3)	0.633 (4)	3.3 (6)
H(11′A)	0.491 (1)	0.922 (2)	0.762 (4)	3.9 (6)
H(11′B)	0.516 (1)	0.962 (3)	0.918 (3)	3.8 (6)
H(11′C)	0.498 (1)	0.825 (2)	0.893 (4)	4.0 (6)
H(12′A)	0.649 (1)	0.529 (2)	0.654 (3)	3.4 (6)
H(12′B)	0.612 (1)	0.541 (2)	0.749 (3)	3.5 (6)
H(12′C)	0.654 (1)	0.468 (2)	0.807 (4)	3.7 (6)
H(N1)	0.586 (1)	0.723 (3)	0.852 (4)	3.4 (6)
H(O2)	0.920 (1)	0.962 (2)	1.053 (3)	3.6 (6)

drolyzate was washed with ethyl acetate. The residual aqueous layer was evaporated to dryness *in vacuo* and subjected to HPLC [Nucleosil $_5C_{18}$ (0.8 × 30 cm), eluting with a methanol–water mixture (1 : 1, v/v) at a flow rate of 1.5 ml/min] to give only one peak (t_R 10.7 min) detected under UV-lamp (254 nm). The eluate corresponding to the peak was collected and evaporated to dryness to give a solid (5.8 mg), which showed the following physicochemical properties: $[\alpha]_D^{18}-27.4$ ($c=0.15$ in water); FD-MS: $(M+H)^+$, m/z 180; MS (In-Beam EI, 20 eV): m/z 179 (0.7%, M^+.), 134 (8.8%), 105 (base peak), 75 (45%). ^{1}H-NMR data (100 MHz, in D_2O, DSS) are shown in Table V. Based on these data this compound was identified to be L-*erythro*-β-methyl-phenylalanine.

X-ray analysis of AK-toxin I. AK-toxin I was recrystallized from a mixture of benzene and methanol to give colorless plates. Determination of the cell parameters and collection of the intensity data were performed on Rigaku AFC-5 diffractometer with graphite-monochromated CuK_α radiation ($\lambda=1.54173$ Å) using a crystal of 0.2 × 0.2 × 0.25 mm in size.

Crystal data: $C_{23}H_{27}O_6N$, orthorhombic, space group $P2_12_12_1$, $a=21.463$ (3), $b=11.879$ (2), $c=8.576$ (1) Å, $V=2184.2$ Å^3, $Z=4$, $D_{cal}=1.257$ g·cm^{-1}, $\mu(CuK_\alpha)=7.10$ cm^{-1}.

Intensities were measured within the range $0<\theta<120°$ by the ω–2θ scan mode. A total number of 1843 independent reflections with $|F_o|>3\sigma(F_o)$ were retained as observed and employed in solving and refineing the structure. The structure was solved by the direct method using the RANTAN computer program.[14] Several cycles of refinements by the block-diagonal least-squares method, including hydrogen atoms obtained from the difference Fourier map, reduced the R-value to 0.042. Atomic scattering factors used for all atoms were obtained from the International Table for X-Ray Crystallography.[15] The final positional parameters for the non-hydrogen atoms and hydrogen atoms are listed in Tables VI and VII, respectively.

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