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STRUCTURE ELUCIDATION OF AK-TOXINS, HOST-SPECIFIC PHYTOTOXIC METABOLITES PRODUCED BY *ALTERNARIA KIKUCHIANA* TANAKA

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Abstract: The structures of two host-specific phytotoxic metabolites, AK-toxin I and II, isolated from culture broth of *Alternaria kikuchiana* Tanaka (the fungus causing black spot disease of Japanese pear) were determined to be the ester consisting of N-acetyl-β-methyl-phenylalanine and 9,10-epoxy-8-hydroxy-9-methyl-2*E*,4*Z*,6*E*-decatrienoic acid (1) and its β-demethyl derivative (2).

At present, "host-specificity" of plant pathogens is the most intriguing problem in plant pathology[1]. Host-specific toxins were demonstrated to be the primary determinants for host recognition in several diseases caused by *Alternaria* species[2-5]. *Alternaria kikuchiana* Tanaka is the causal fungus of black spot disease of Japanese pear. The fact that a droplet of the culture filtrate induces necrosis on the leaves and fruits of susceptible cultivars (e.g. *Pyrus serotina* Rehder *var. culta*, cv. Nijisseiki) but not on those of resistant cultivars (e.g. *P. serotina* Rehd. *var. culta*, cv. Chojuro) suggests production of the host-specific toxin(s) by the fungus[6]. Sugiyama et al. reported the isolation, structure elucidation and synthesis of altenin as a necrosis inducing factor on the pear leaves, but its host-specificity was not reported[7-9]. Based on the strict plant pathological experiments, we report here the isolation and structure elucidation of the two host-specific toxins (1) and (2) from culture broth of the causal fungus.

Every step of the isolation procedures was monitored by the bioassay on young leaves of the susceptible cultivar. The active principle was extracted from the culture broth of *A. kikuchiana* (the strain No. AS-1223) which was cultured in a jar fermenter at 28°C in a modified Richards' medium. Repeated chromatographic purification using Amberlite XAD-2, silica gel, droplet counter-current and Sephadex LH-20, and recrystallization from methanol gave two acidic compounds, named AK-toxin I [mp. 168°(decomp.), $[\alpha]_D^{25}$ 164°(c=0.128 in methanol)] and II [mp. 168°(decomp.), $[\alpha]_D^{23}$ 125°(c=0.132 in methanol)].

The high resolution mass spectrum (In-Beam EI) of AK-toxin I ($M^{+\cdot}$ at m/z 413.1827) suggested the molecular formula $C_{23}H_{27}NO_6$ (calcd. 413.1836) which was consistent with the result of elemental analysis (found: C; 67.26, H; 6.70 and N; 3.27%, calcd. for $C_{23}H_{27}NO_6$: C; 66.81, H; 6.58 and N; 3.39%) and the ^{13}C-NMR data shown in Table 1. Three carbonyl groups (see Table 1) were infered from the IR spectrum (in KBr) to be an ester ($\nu_{C=O}$ 1750cm^{-1}), an amide ($\nu_{C=O}$ 1680 and 1550cm^{-1}) and a conjugated acid ($\nu_{C=O}$ 1690cm^{-1}).

The presence of N-acetyl-β-methyl-phenylalanyl moiety in AK-toxin I was deduced from the ^{1}H-NMR spectrum (see Table 2) where acetyl methyl protons (3H, s, δ 1.84), phenyl protons (5H, broad s, δ 7.23), methyl protons (3H, d, δ 1.30, *J*=7.1Hz) coupling with a methine proton (double q, δ 3.22, *J*=7.1 and 8.8Hz), the latter coupling with another methine proton (d, δ

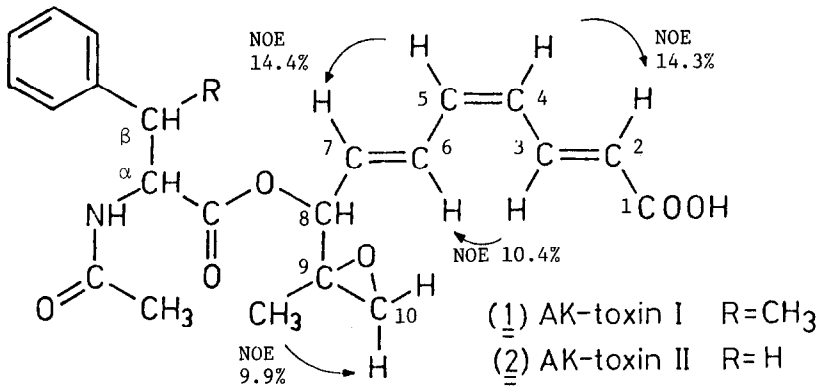

Fig. 1 Structure of AK-toxin I and II

4.73, J=8.8Hz) were observed and also from the spectrum obtained in DMSO-d_6 where a doublet signal of the amide proton (δ 8.29, J=6.5Hz) appeared and coupled with the methine proton (donble d, δ 4.86, J=6.1 and 6.5Hz) that was observed as a doublet signal at δ 4.73 in the spectrum in CD_3OD. This conclusion was also confirmed from the following characteristic ions observed in the high resolution mass spectrum of AK-toxin I (In-Beam EI 70eV); methyl tropylium cation at m/z 105.0672 (100%, calcd. for C_8H_9 105.0703), Ph-CH(CH_3)-CH=$\overset{+}{N}H_2$ at m/z 134.0974 (52%, calcd. for $C_9H_{12}N$ 134.0968), Ph-CH(CH_3)-CH=$\overset{+}{N}H$-CO-CH_3 at m/z 176.1100 (43%, calcd. for $C_{11}H_{14}NO$ 176.1075) and Ph-CH(CH_3)-CH(C=$\overset{+}{O}$)-NH-CO-CH_3 at m/z 204.1011 (22%, calcd. for $C_{12}H_{14}NO_2$ 204.1023).

Another structural moiety of AK-toxin I was concluded to be 9,10-epoxy-8-hydroxy-9-methyl-2*E*,4*Z*,6*E*-decatrienoic acid from the following experimental results. AK-toxin I gave a monomethyl ester ($M^{+\cdot}$ m/z 427.2024, In-Beam EI, calcd. for $C_{24}H_{29}NO_6$ 427.1993) by treatment with diazomethane in methanol and a hexahydro derivative [$(M+1)^{+}$ m/z 420, FD-MS; $M^{+\cdot}$ m/z 419.2304, In-Beam EI, calcd. for $C_{23}H_{33}NO_6$ 419.2306] by catalytic hydrogenation on PtO_2 in methanol. Taking account of rather lower value of chemical shift of an olefinic proton (δ 7.77), the presence of C=O streching band of conjugated acid at 1690cm^{-1} in the IR spectrum and UV λ_{max} 284nm (ε=27600 in methanol), all of the six olefinic protons and a methine proton in ^{1}H-NMR spectrum of AK-toxin I were reasonably assigned to H_{C-2} (δ 5.95), H_{C-3} (δ 7.77), H_{C-4} (δ 6.24), H_{C-5} (δ 6.39), H_{C-6} (δ 6.95), H_{C-7} (δ 5.87) and H_{C-8} (δ 5.35) (see Table 2) of 8-oxy-2*E*,4*Z*,6*E*-trienoic acid. The fact that large paramagnetic shifts were observed on H_{C-2} and H_{C-3} by addition of $Pr(NO_3)_3$ shift reagent in D_2O indicated that the two protons should locate on α and β-position from the terminal carboxyl group. Informations about their geometrical arrangements of the conjugated olefinic protons were obtained from their coupling constants and enhancement of proton signals on NOE experiments. The values of the coupling constants, J_{H2-3}=15.1(*trans*)[10], J_{H3-4}=11.2(*vicinal*), J_{H4-5}=11.1(*cis*), J_{H5-6}=11.0(*vicinal*), J_{H6-7}=15.2(*trans*) and J_{H7-8}=7.1Hz(*vicinal*), suggested the arrangement of *trans*, *cis*, *trans* trienoic structure, which was confirmed by observation of NOE enhancement between H_{C-2} and H_{C-4}, H_{C-3} and H_{C-6}, and H_{C-5} and H_{C-7} as shown in Fig. 1. Remaining one methyl (s, δ 1.31) and one methylene signals with AB type (1H, d, δ 2.61 and 1H, d, δ 2.76, J=4.8Hz) should be assigned to methyl-ethyleneoxide group attached to C_8 of the oxy-trienoic acid by the fact that NOE enhancement was also observed between the methyl protons and one of the methylene

Table 1. ^{13}C-NMR spectrum of AK-toxin I (50MHz in CD_3OD)

No.	Chemical shift(δ ppm)	Remarks**	No.	Chemical shift(δ ppm)	Remarks**
1	17.75	$-CH_3$	12	129.43	-CH=
2	18.99	$-CH_3$	13	129.55	-CH= x 2
3	22.18	$-CH_3$	14	130.64	-CH=
4	42.98	>CH-	15	132.23	-CH=
5	52.83	$-CH_2-$	16	136.48	-CH=
6	57.77	>C<	17	140.06	-CH=
7	59.40	>CH-	18	143.37	>C=
8	77.57	>CH-	19	170.22	-CO-
9	124.20	-CH=	20	171.70	-CO-
10	128.04	-CH=	21	173.04	-CO-
11	128.80	-CH= x 2			

** Assigned by off-resonance decoupling and peak hight.

Table 2. ^{1}H-NMR spectra of AK-toxin I and II (200MHz in CD_3OD)

AK-toxin I				AK-toxin II			
Chemical shift (δ ppm)	No. of protons	Remarks		Chemical shift (δ ppm)	No.of protons	Remarks	
1.30	3	d	J=7.1Hz				
1.31	3	s		1.29	3	s	
1.84	3	s		1.93	3	s	
2.61	1	d	J=4.8Hz	2.61	1	d	J=4.6Hz
2.76	1	d	J=4.8Hz	2.75	1	d	J=4.6Hz
3.22	1	dq	J=8.8, 7.1Hz	2.97 - 3.16	2	m	
4.73	1	d	J=8.8Hz	4.66	1	t	
5.35	1	d	J=7.1Hz	5.22	1	d	J=7.0Hz
5.87	1	dd	J=15.2, 7.1Hz	5.70	1	dd	J=15.2, 7.0Hz
5.95	1	d	J=15.1Hz	5.94	1	d	J=15.1Hz
6.24	1	dd	J=11.2, 11.1Hz	6.21	1	dd	J=10.7, 10.7Hz
6.39	1	dd	J=11.1, 11.0Hz	6.27	1	dd	J=10.7, 10.7Hz
6.95	1	dd	J=15.2, 11.0Hz	6.81	1	dd	J=15.2, 10.7Hz
7.23	5	bs		7.25	5	bs	
7.77	1	dd	J=15.1, 11.2Hz	7.73	1	dd	J=15.1, 10.7Hz

protons (δ 2.61) on C_{10}. This investigation was also supported from the chemical shifts of a singlet carbon (δ 57.77) and a triplet carbon signal (δ 52.83) in the ^{13}C-NMR spectrum of AK-toxin I (see Table 1). On the basis of these experimental results, the structure of AK-toxin I was determined to be 8-(α-acetylamino-β-methyl-β-phenyl-propionyloxy)-9.10-epoxy-9-methyl-2*E*,4*Z*,6*E*-decatrienoic acid.

The molecular formula of AK-toxin II was determined to be $C_{22}H_{25}NO_6$ ($M^{+\cdot}$ m/z 399.1676, calcd. 399.1679) by high resolution mass spectrometry (In-Beam EI 70eV). The ^{1}H-NMR spectra of AK-toxin I and II (see Table 2) indicated that their difference was in the substitution on the β-position of N-acetyl-phenylalanyl moiety with or without a methyl group. In the ^{1}H-NMR spectrum of AK-toxin II (see Table 2), a multiplet methylene (2H on C_β) and a triplet methine

signal (1H on C_α) with an ABX system were observed at δ 2.97 - 3.16 and 4.66 instead of the doublet methyl (CH_3 on C_β, δ 1.30), the double quartet methine (1H on C_β, δ 3.22) and the doublet methine signal (1H on C_α, δ 4.73) of AK-toxin I, and other all of the proton signals showed quite similar patterns with almost the same chemical shifts as those of AK-toxin I. The high resolution mass spectrum of AK-toxin II (In-Beam EI 70eV), 14 mass unit (CH_2) decreased peaks were observed in the lower mass region, compared with the characteristic ions for N-acetyl-β-methyl-phenylalanyl moiety of AK-toxin I; tropylium cation at m/z 91.0566 (50%, calcd. for C_7H_7 91.0547), Ph-CH_2-CH=$\overset{+}{N}H_2$ at m/z 120.0823 (100%, calcd. for $C_8H_{10}N$, 120.0813), Ph-CH_2-CH=$\overset{+}{N}H$-CO-CH_3 at m/z 162.0912 (62%, calcd. for $C_{10}H_{12}NO$ 162.0917) and Ph-CH_2-CH(C≡$\overset{+}{O}$)-NH-CO-CH_3 at m/z 190.0863 (42%, calcd. for $C_{11}H_{12}NO_2$ 190.0867). These data clearly demonstrated that AK-toxin II contained N-acetyl-phenylalanine as the structural moiety, and consequently AK-toxin II was the β-demethyl derivative of AK-toxin I (2).

The results of biological assay showed the crystalline AK-toxin I and II had a host-specific phytotoxic property resemble to that of the causal fungus when they were applied to young fresh leaves of Japanese pear cultivars. The threshold concentrations of these toxins inducing necrosis observed after 16hr at room temperature were 0.065μg/ml respectively when droplets of the toxin solution were placed on the leaves of a susceptible cultivar, Nijisseiki. But in the case of a resistant cultivar, Chojuro, even 6.5μg/ml solutions of the toxins did not induce any necrosis on the leaves. These results show apparently that AK-toxin I and II have a host-specific phytotoxic activity for Japanese pear cultivars which are susceptible to *A. kikuchiana* Tanaka.

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10) J_{Hm-n} means the coupling constant between H on C_m and H on C_n.

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