Structure-Activity Relationship Study of Host-Specific Phytotoxins (AM-Toxin Analogs) Using a New Assay Method with Leaves from Apple Meristem Culture

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Z. Naturforsch. **56 c,** 1029–1037 (2001); received May 2/July 23, 2001

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Host-Specific Toxin, Cyclic Depsipeptide, Meristem Culture

AM-toxins are host-specific phytotoxins of the *Alternaria alternata* apple pathotype, which induce necrosis on apple leaves. In this study, we developed a new assay to measure the necrotic activity of AM-toxin analogs using cultured leaves from meristem cells. This method was not only more sensitive to AM-toxin I, but also more reliable than the previous one that used tree leaves due to the homogeneous nature of cultured leaves and to the method of application of toxins. Using this assay method we investigated a structure-activity relationship of AM-toxin analogs synthesized in this study. Most residues and the macrocyclic ring structure were strictly recognized by AM-toxin putative receptor, whereas the L-Ala binding subsite of the receptor allowed for side chain structures with various stereoelectronic properties. These findings are important for designing ligands for further experimental probing of the nature of the receptor.

Introduction

AM-toxins I–III are host-specific phytotoxins of the *Alternaria alternata* apple pathotype that cause spot diseases on apple leaves and fruits of susceptible cultivars, such as Indo and Starking (Okuno *et al.,* 1974; Ueno *et al.,* 1975a). The spot disease against apples results in a loss of nutritional and market value, and is therefore of great importance in apple farming. AM-toxins are considered to be the primary determinant of pathogenicity because they selectively affect apple cultivars susceptible to the *A. alternata* apple pathotype and induce the same biochemical response of the host as by the inoculation of the pathogen (Kohmoto *et al.,* 1977).

The structures of AM-toxins have previously been determined to be cyclic tetradepsipeptides (Fig. 1) (Okuno *et al.,* 1974; Ueno *et al.,* 1975b, c). AM-toxin I contains an L-2-hydroxy-3-methylbutanoic acid (L-Hmb) residue, along with two unnatural amino acids, α,β-dehydroalanine (ΔAla) and L-2-amino-5-(*p*-methoxyphenyl)pentanoic acid (L-Amp). AM-toxins II and III contain L-2-amino-5-phenylpentanoic acid (L-App) and L-2-amino-5-(*p*-hydroxyphenyl)pentanoic acid (L-Ahp), respectively, instead of L-Amp in AM-toxin I.

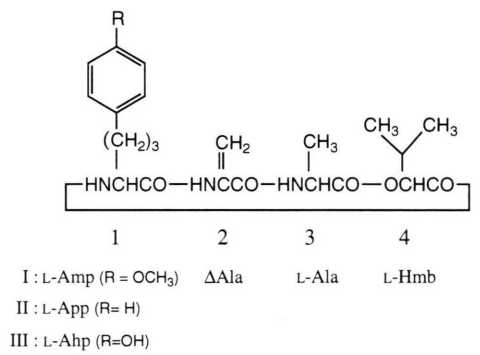


Fig. 1. Structures of AM-toxins I–III.
Abbreviations: L-Amp, L-2-amino-5-(*p*-methoxyphenyl)-pentanoic acid; L-App, L-2-amino-5-phenylpentanoic acid; L-Ahp, L-2-amino-5-(*p*-hydroxyphenyl)pentanoic acid; L-Hmb, L-2-hydroxy-3-methylbutanoic acid.

AM-toxins act on the chloroplasts and the plasma membranes (Park *et al.,* 1981), causing veinal necrosis and electrolyte loss (Shimomura *et al.,* 1991). The existence of the putative receptor of AM-toxins has been suggested by the fact that the host recognizes the chirality of AM-toxins (Aoyagi *et al.,* 1987b). However, the receptor of these toxins have not yet been identified, and the mechanism of their action and host-selectivity is not completely understood.

0939–5075/2001/1100–1029 $ 06.00

A number of AM-toxin analogs has been synthesized, and their necrotic activity has been assayed to elucidate structural factors which are critical to the biological activity (Aoyagi *et al.,* 1987a; Kozono *et al.,* 1983; Noda *et al.,* 1983; Shimohigashi and Izumiya, 1978). In these studies, young leaves removed from apple trees have been used for the assay (Ueno *et al.,* 1975a). The toxin samples were applied to the surface of the leaves as a water suspension. The efficiency of sample absorption into leaf tissue under these assay conditions is markedly affected by the age and growth conditions of the leaves. However, it is difficult to keep the growth conditions of the field trees constant. As a result, the sensitivity of leaves to the toxins fluctuates, giving rise to low reproducibility, and thus, low reliability of the assay data. For this reason, it is very likely that previous structure-activity studies have overlooked important factors for the expression of phytotoxicity.

In the present study, we reexamined the phytotoxicity of available AM-toxin analogs, using cultured apple leaves. The culture was derived from meristem cells, and leaf tissue induced from this system have already been demonstrated to respond to AM-toxins with sufficient reproducibility (Komiya, 1992). We also synthesized several new AM-toxin analogs by the improved method reported previously (Miyashita *et al.,* 1998), in order to extend the structure-activity study for clarifying chemical factors that significantly affect the activity.

Materials and Methods

Apparatus

RP-HPLC was carried out under the following conditions: Column: Shim-Pack PREP-ODS (15 μm, 20 × 250 mm); mobile phase: 35–60% aqueous acetonitrile; flow rate: 10 ml/min; UV detector: 220 nm. ^{1}H-Nuclear magnetic resonance (NMR) spectra were measured with a Bruker AC 300 or ARX 500 spectrometer. High-resolution fast atom bombardment mass spectra (HR-FABMS) were measured with a JEOL JMS-HX110A spectrometer. CD spectra were measured by a Jasco J-720 spectropolarimeter.

Synthesis of AM-toxins and analogs

The synthesis of AM-toxin I (**1**) was carried out using the procedures as previously described (Miyashita *et al.,* 1998). AM-toxin analogs **2–3**, **5–16** were synthesized by the same procedure as described for the synthesis of **1**.

D-β-Phenylselenoalanine (D-Psa) was used as a precursor of dehydroalanine (Hashimoto *et al.,* 1996) for the synthesis of analog **4**. The precursor depsipeptide, cyclo[D-Psa-Gly-L-Hmb-L-Amp], which was synthesized by the same procedures as described for the synthesis of **1**, was dissolved in dichloromethane/trifluoroethanol (5:1 v/v) and *tert*-butylhydroperoxide in dichloromethane (6.0 M) was added dropwise to the solution while stirring on an ice bath. The solution was stirred overnight at room temperature. After evaporation of the solution, the residue was purified by RP-HPLC.

Table I contains MS and NMR data of the final products except for compound S **10**, **15** and **16**. Because of multiple conformers (**10**) and insufficient quantity for NMR measurement (**15**, **16**), only MS data are described for these compounds.

Meristem culture of apples

Apple cultivars which are susceptible (Indo), moderately resistant (Jonathan) and resistant (Gala) to *A. alternata* apple pathotype were used in this study. Explants used for initiating apple meristem cultures were obtained from dormant scion on an Indo tree. After removal of bark and bud scales, excised buds were sterilized with 70% ethanol in water for 1 min and 1% sodium hypochlorite in water for 3 min, and washed twice with sterilized water. The outer leaflets were dissected away and a shoot tip of approximately 1 mm in length was excised, and transferred to Murashige and Skoog (MS) medium (pH 5.8) containing 3% sucrose and 1% agar. After incubation at 25 °C for a week, explants not exhibiting contamination were transferred to MS medium (pH 5.8) containing 10^{-5} M 5-benzyladenine, 3% sucrose and 0.8% agar. Multiple shoots were obtained after incubation at 25 °C with a 16-hr photoperiod for 3 months. The Jonathan and Gala meristem cultures were kindly provided by Dr. R. Tao (Kyoto University, Kyoto, Japan).

Table I. MS and ^{1}H NMR data of AM-toxin analogs.

No.	Analogs	MS (*m/z*) Calcd.	$[M+H]^+$ Found	^{1}H NMR δ (DMSO-d_6)
1	AM-toxin I	446.2283	446.2300	0.89 (3H, d, *J* = 6.7 Hz), 0.90 (3H, d, *J* = 6.8 Hz), 1.35 (3H, d, *J* = 7.2 Hz), 1.47 (1H, m), 1.56 (2H, m), 1.83 (1H, m), 1.97 (1H, m), 2.50 (1H, m), 2.59 (1H, m), 3.71 (3H, s), 4.31 (2H, m) 4.69 (1H, d, *J* = 6.3 Hz), 5.29 (1H, bs), 5.41 (1H,), 6.83 and 7.09 (4H, A_2B_2), 7.98 (1H, bs), 8.11 (1H, d, *J* = 9.4 Hz), 9.06 (1H, s).
2	AM-toxin II	416.2185	416.2201	0.89 (3H, d, *J* = 6.6 Hz), 0.90 (3H, d, *J* = 6.8 Hz), 1.35 (3H, d, *J* = 7.2 Hz), 1.51 (1H, m), 1.61 (2H, m), 1.86 (1H, m), 1.97 (1H, m), 2.56 (1H, m), 2.66 (1H, m), 4.33 (2H, m), 4.69 (1H, d, *J* = 6.4 Hz), 5.29 (1H, bs), 5.41 (1H, s), 7.14–7.30 (5H, m), 7.99 (1H, bs), 8.11 (1H, d, *J* = 9.1 Hz), 9.07 (1H, s).
3	[L-Phe^1]AM-toxin	388.1872	388.1880	0.63 (3H, d, *J* = 6.8 Hz), 0.79 (3H, d, *J* = 6.7 Hz), 1.36 (3H, d, *J* = 7.2 Hz), 1.83 (1H, m), 2.79 and 3.23 (2H, m), 4.35 (1H, m), 4.53 (1H, d, *J* = 7.6), 4.58 (1H, m), 5.35 (1H, bs), 5.42 (1H, s), 7.19 and 7.25 (5H, m), 8.10 (1H, bs), 8.11 (1H, d, *J* = 8.9 Hz), 9.19 (1H, s).
4	[Gly^3]AM-toxin	432.2134	432.2129	0.90 (6H, m), 1.55 (3H, m), 1.80 (1H, m), 1.97 (1H, m), 2.49 (1H, m), 2.57 (1H, m), 3.71 (3H, s), 3.83 and 3.98 (2H, m), 4.32 and 4.46 (1H, m), 4.64 and 4.84 (1H, m), 5.45 (1H, bs), 6.83 and 7.07 (4H, A_2B_2), 7.91 and 8.20 (2H, b), 8.98 (1H, bs).
5	[L-Abu^3]AM-toxin	460.2447	460.2444	0.91 (9H, m), 1.48 (1H, m), 1.57 (2H, m), 1.72 (2H, m), 1.84 (1H, m), 1.96 (1H, m), 2.49 (1H, m), 2.61 (1H, m), 3.71 (3H, s), 4.18 (1H, m), 4.31 (1H, m), 4.72 (1H, d, *J* = 6.2 Hz), 5.23 (1H, bs), 5.40 (1H, s), 6.83 and 7.09 (4H, A_2B_2), 8.00 (1H, d, *J* = 9.5 Hz), 8.12 (1H, bs), 9.12 (1H, s).
6	[L-Nva^3]AM-toxin	474.2604	474.2616	0.90 (9H, m), 1.35 (2H, m), 1.47 (1H, m), 1.55 (2H, m), 1.66 (2H, m), 1.83 (1H, m), 1.96 (1H, m), 2.50 (1H, m), 2.61 (1H, m), 3.71 (3H, s), 4.28 (2H, m), 4.72 (1H, d, *J* = 6.3 Hz), 5.23 (1H, bs), 5.39 (1H, s), 6.83 and 7.09 (4H, A_2B_2), 8.00 (1H, d, *J* = 9.3 Hz), 8.03 (1H, bs), 9.12 (1H, s).
7	[L-Nle^3]AM-toxin	488.2760	488.2755	0.88 (9H, m), 1.31 (4H, m), 1.49 (1H, m), 1.57 (2H, m), 1.66 (2H, m), 1.84 (1H, m), 1.96 (1H, m), 2.50 (1H, m), 2.60 (1H, m), 3.70 (3H, s), 4.24 (1H, m), 4.31 (1H, m), 4.72 (1H, d, *J* = 6.2 Hz), 5.25 (1H, bs), 5.39 (1H, s), 6.83 and 7.09 (4H, A_2B_2), 7.99 (1H, d, *J* = 9.3 Hz), 8.03 (1H, bs), 9.10 (1H, s).
8	[L-Leu^3]AM-toxin	488.2760	488.2758	0.91 (12H, m), 1.53 (6H, m), 1.84 (1H, m), 1.96 (1H, m), 2.50 (1H, m), 2.61 (1H, m), 3.71 (3H, s), 4.31 (2H, m), 4.72 (1H, d, *J* = 6.3 Hz), 5.22 (1H, bs), 5.38 (1H, s), 6.83 and 7.09 (4H, A_2B_2), 8.00 (1H, d, *J* = 9.8 Hz), 8.02 (1H, bs), 9.13 (1H, s).
9	[L-Tyr^3]AM-toxin	538.2553	538.2557	0.91 (6H, m), 1.50 (1H, m), 1.57 (2H, m), 1.84 (1H, m), 1.98 (1H, m), 2.50 (1H, m), 2.60 (1H, m), 2.91 (2H, m), 3.71 (3H, s), 4.32 (1H, m), 4.48 (1H, m), 4.72 (1H, d, *J* = 6.2 Hz), 5.22 (1H, bs), 5.36 (1H, s), 6.67 and 7.02 (4H, A_2B_2), 6.84 and 7.09 (4H, A_2B_2), 8.07 (1H, bs), 8.11 (1H, d, *J* = 9.6 Hz), 9.18 (1H, s), 9.28 (1H, s).
10	[L-Val^4]AM-toxin	445.2451	445.2446	ND[a]
11	[L-Lac^4]AM-toxin	418.1978	418.1970	1.34 (3H, d, *J* = 7.3), 1.36 (3H, d, *J* = 7.0), 1.50 (1H, m), 1.57 (2H, m), 1.84 (1H, m), 2.50 (1H, m), 2.58 (1H, m), 3.71 (3H, s), 4.28 (1H, m) 4.33 (1H, m), 4.93 (1H, m), 5.34 (1H, bs), 5.42 (1H, s), 6.84 and 7.10 (4H, A_2B_2), 8.04 (1H, bs), 8.08 (1H, d, *J* = 9.2 Hz), 9.00 (1H, s).
12	[L-Hic^4]AM-toxin	460.2447	460.2452	0.92 (6H, m), 1.33 (3H, d, *J* = 7.00), 1.51–1.63 (6H, m), 1.82 (1H, m), 2.53 (1H, m), 2.58 (1H, m), 3.71 (3H, s), 4.29 (2H, m), 4.94 (1H, s), 5.41 (2H, s), 6.84 and 7.09 (4H, A_2B_2), 8.06 (2H, d, *J* = 8.75), 9.05 (1H, s)

Table I. (cont.)

No.	Analogs	MS (*m/z*) Calcd.	$[M+H]^+$ Found	^1H NMR δ (DMSO-d_6)
13	[L-Pla[4]]AM-toxin	494.2291	494.2300	1.27 (3H, d, *J* = 7.2), 1.42 (1H, m), 1.53 (2H, m), 1.79 (1H, m), 2.50 (1H, m), 2.58 (1H, m), 2.96 and 3.06 (2H, m), 3.72 (3H, s), 4.25 (2H, m), 5.06 (1H, m), 5.28 (1H, bs), 5.39 (1H, s), 6.86 and 7.12 (4H, A_2B_2), 7.16 and 7.25 (5H, m), 7.96 (1H, bs), 8.12 (1H, d, *J* = 9.5 Hz), 9.03 (1H, s).
14	[βAla[3]]AM-toxin	446.2291	446.2279	0.90 (3H, d, *J* = 6.6 Hz), 0.91 (3H, d, *J* = 6.3 Hz), 1.45 (1H, m), 1.54 (2H, m), 1.87 (1H, m), 2.06 (1H, m), 2.48 (1H, m), 2.58 (1H, m), 2.64 (2H, b), 3.41 (2H, b), 3.71 (3H, s), 4.43 (1H, bs), 4.85 (1H, d, *J* = 5.3 Hz), 5.32 (1H, s), 5.44 (1H, s), 6.83 and 7.08 (4H, A_2B_2), 7.49 (1H, bs), 8.09 (1H, d, *J* = 8.3 Hz), 8.56 (1H, s).
15	[L-Ala-Gly[3]]AM-toxin	503.2506	503.2501	ND[b]
16	[Gly-L-Ala[3]]AM-toxin	503.2506	503.2514	ND[b]

[a] ND; not determined because of multiple conformers; [b] ND; not determined because of low quantities.

Measurement of necrotic activity of AM-toxin analogs

AM-toxin analogs were dissolved in acetone, and diluted in successive 10-fold concentrations with distilled water. The final acetone concentration in the toxin solution was below 10%, and no solvent effects were observed. Fifty microliters of 1% agar in hot distilled water were pipetted into each well of a 96-well tissue culture plate (Becton Dickinson Labware; Franklin Lakes, NJ, USA), which was cooled at room temperature to coagulate. Small leaves, which were cut off at the stem from a shoot of meristem cultures, were put into each well. The surface of the cut section was placed in contact with the agar, and then 20 µl of toxin solution at various concentrations was layered over the agar. After incubation at 25 °C for 48 hours in the dark, the occurrence of leaf necrosis was examined by the visual observation. In a typical necrotic reaction, the leaf color turned into dark brown: the color change spread from the leaf stem to the whole leaf, depending on the severity of the necrosis. No necrosis occurred in the leaves which were treated with H_2O. Three leaves were used for the assay at one specified concentration, and the result was judged as positive when more than two leaves exhibited necrosis. The final concentration of the toxin analogs in each well was varied from 10^{-4} to 10^{-12} M. The minimum concentration (MC) for induction of necrosis was used as an index for the activity of each toxin analog.

CD measurements

CD spectra were measured from 200 to 250 nm in methanol at room temperature with a 1 mm path-length cell. Data were collected at a 0.2 nm interval with a scan rate of 100 nm·min^{-1} and a time constant of 0.5 s. The concentration of each AM-toxin analog was 200 µM.

Results and Discussion

Comparison of the activity obtained from meristem cultured leaves versus that obtained from tree leaves

Table II shows the MC values of AM-toxin I (**1**) on necrosis induction measured using small leaves of meristem cultures of three apple cultivars; Indo (susceptible), Jonathan (moderately resistant) and Gala (resistant). The necrosis of meristem cul-

Table II. Effect of AM-toxin I on necrosis induction in apple cultivars having different susceptibility.

Cultivars	MC[a] [M] Cultured leaf tissues[b]	Tree leaves[c]
Indo	10^{-10}	2×10^{-9}
Jonathan	10^{-5}	10^{-5}
Gala	$>10^{-4}$	$>10^{-4}$

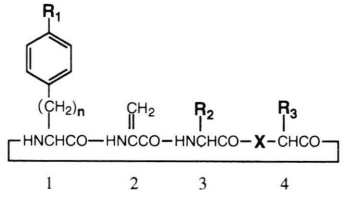
[a] Minimum concentration required for necrosis induction [M]; [b] measured using small meristem cultured leaves; [c] measured using young tree leaves. Values are taken from literature (Shimohigashi *et al.*, 1977; Kohmoto *et al.*, 1977; Tabira *et al.*, 1998)

tured leaves was induced by AM-toxin I at levels as low as 10^{-10} M for the susceptible cultivar (Indo). Necrosis appeared along the veins of meristem cultured leaves after treatment with low concentrations of AM-toxin I, and the entire part of leaves were necrotized following treatment with high concentrations of AM-toxin I. The necrosis of leaves was induced by AM-toxin I at 10^{-5} M for meristem cultures of the moderately resistant cultivar (Jonathan) and no necrosis was observed even at 10^{-4} M for the resistant cultivar (Gala). These results are in good agreement with previous studies using tree leaves. The MC values obtained from the use of tree leaves in previous studies (Kohmoto *et al.*, 1977; Shimohigashi *et al.*, 1977; Tabira *et al.*, 1998) are listed for comparison in Table II. In the previous studies no necrosis was induced by AM-toxin I at concentrations less than 2×10^{-9} M, indicating that the assay method using meristem cultured leaves is 20 times more sensitive to AM-toxin I.

In this study three leaves were treated by AM-toxins at each concentration and two or three of them reproducibly exhibited necrosis at concentrations from 10^{-4} to 10^{-10} M AM-toxin I for the susceptible cultivar (Indo). Since the novel method developed in this study utilized cultured leaf tissues that were always grown under identical conditions, our results were more reproducible than those from traditional methods using tree leaves. These results indicate that the assay method developed in this study can be useful for evaluating the host selectivity of AM-toxin analogs as well as for measuring their potency.

The structures and necrotic activity of AM-toxin I, II and their analogs against the susceptible cultivar (Indo) are shown in Tables III and IV. All analogs except **13** demonstrated activity against Indo at 10^{-4}–10^{-11} M, whereas they had no effect against the resistant cultivar (Gala) at 10^{-4} M [data not shown except for AM-toxin I (Table II)], indicating that they retain host selectivity. The necrotic activity of AM-toxin analogs **1–3**, **5**, **8**, **10–12**, which was examined using young Indo leaves in previous studies (Aoyagi *et al.*, 1985; Kozono *et al.*, 1983; Mihara *et al.*, 1986; Shimohigashi and Izumiya, 1978; Shimohigashi *et al.*, 1977), is also listed in Table III. The necrotic activity of analog **8**, **10**

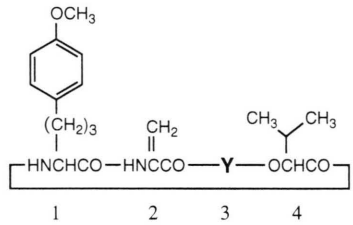
Table III. Structures and necrotic activity of AM-toxin analogs.



No.	Analogs	X	R_1	n	R_2	R_3	MC[a] [M] Cultured leaf tissues[b]	MC[a] [M] Tree leaves[c]
1	AM-toxin I	O	OCH_3	3	CH_3	$CH(CH_3)_2$	10^{-10}	2×10^{-9}
2	AM-toxin II	O	H	3	CH_3	$CH(CH_3)_2$	10^{-8}	5×10^{-8}
3	[L-Phe[1]]AM-toxin	O	H	1	CH_3	$CH(CH_3)_2$	10^{-5}	5×10^{-5}
4	[Gly[3]]AM-toxin	O	OCH_3	3	H	$CH(CH_3)_2$	10^{-7}	
5	[L-Abu[3]]AM-toxin	O	OCH_3	3	CH_2CH_3	$CH(CH_3)_2$	10^{-9}	2×10^{-9}
6	[L-Nva[3]]AM-toxin	O	OCH_3	3	$CH_2CH_2CH_3$	$CH(CH_3)_2$	10^{-9}	
7	[L-Nle[3]]AM-toxin	O	OCH_3	3	$CH_2CH_2CH_2CH_3$	$CH(CH_3)_2$	10^{-9}	
8	[L-Leu[3]]AM-toxin	O	OCH_3	3	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	10^{-11}	2×10^{-8}
9	[L-Tyr[3]]AM-toxin	O	OCH_3	3	$CH_2Ph(4\text{-}OH)$	$CH(CH_3)_2$	10^{-8}	
10	[L-Val[4]]AM-toxin	NH	OCH_3	3	CH_3	$CH(CH_3)_2$	10^{-9}	2×10^{-7}
11	[L-Lac[4]]AM-toxin	O	OCH_3	3	CH_3	CH_3	10^{-7}	2×10^{-4}
12	[L-Hic[4]]AM-toxin	O	OCH_3	3	CH_3	$CH_2CH_2(CH_3)_2$	10^{-7}	2×10^{-7}
13	[L-Pla[4]]AM-toxin	O	OCH_3	3	CH_3	CH_2Ph	$>10^{-4}$	

[a] Minimum concentration required for necrosis induction [M]; [b] measured using small meristem cultured leaves; [c] measured using young tree leaves. Values are taken from literature (Aoyagi *et al.*, 1985; Kozono *et al.*, 1983; Mihara *et al.*, 1986; Shimohigashi *et al.*, 1977 and 1978).

Table IV. Structures and necrotic activity of AM-toxin analogs having larger macrocyclic ring.



No.	Analogs		MC[a] [M]
14	[βAla^3]AM-toxin I	$HNCH_2CH_2CO$	10^{-6}
		CH_3	
		|	
15	[L-Ala-Gly^3]AM-toxin I	$HNCHCO-HNCH_2CO$	10^{-4}
		CH_3	
		|	
16	[Gly-L-Ala^3]AM-toxin I	$HNCH_2CO-HNCHCO$	10^{-4}

[a] Minimum concentration required for necrosis induction using meristem cultured leaves [M].

and **11** obtained in the present study by using meristem cultured leaves is 200–2000 times higher than the data obtained by using tree leaves, whereas other analogs showed almost the same activity in both assay methods. The difference in the activity between these two assay methods can be attributed to the low reproducibility of the previous method, because the sensitivity of tree leaves to AM-toxins depends on the growth conditions. It also depends on the age of leaves, with old one being less sensitive to toxins (Saito *et al.,* 1989). Another significant factor that may contribute to the difference between the present assay method and the previous one is the method of toxin application. In the previous assay method, AM-toxin analogs were first adsorbed to silica gel for application to the leaf surface. The silica gel was suspended in water such that the toxins were slowly released from the silica gel and penetrated into leaves. Under these conditions, the applied compounds were likely to permeate into the leaf tissues through the stomata, the abundance of which in the leaves depends on the growth conditions. This can result in the difference in the susceptibility of each leaf against toxins applied to its surface. On the other hand, using the methods developed in this study the toxin analogs were applied as an aqueous solution, which can be taken up from the petioles and transported through the vascular system. The applied toxin can therefore permeate into the leaves without being affected by differences in each leaf.

Thus, the assay method using cultured leaf tissues is more sensitive to AM-toxins and provides more reproducible results. Subsequently, we measured the activity of newly synthesized AM-toxin analogs by this assay method.

Structure-activity relationship

We synthesized analogs in which the L-Amp^1, L-Ala^3 or L-Hmb^4 residues of AM-toxin I were replaced with other amino acids or α-hydroxy acids and measured their activity by the method using meristem cultured leaves. We also synthesized analogs in which the ΔAla^2 of AM-toxin I was replaced with L-Ala or D-Ala. However, we were unable to evaluate the activity of these analogs due to their low H_2O solubility. Shimohigashi *et al.* determined that the activity of these analogs was low using tree leaves (Shimohigashi *et al.,* 1978). The decrease of the activity was explained in terms of the conformational change of the main chain for the case of L-Ala^2 analog, and of the unfavorable orientation of the methyl group of D-Ala^2 for the case of D-Ala^2 analog (Higashijima *et al.,* 1983).

Substitution of L-Amp^1 residue

Analogs **2** and **3** showed 10^2 and 10^5 times lower activity than that of AM-toxin I, respectively. AM-toxin II (**2**) contains L-App^1 which lacks the methoxy group on the benzene ring of L-Amp^1 of AM-toxin I (**1**), whereas **3** contains L-Phe^1 which has a shorter side chain than that of L-App^1. This indicates that both the methoxy group and the length of the L-Amp^1 side chain are important for high activity and that the L-Amp^1 side chain is strictly recognized in the binding subsite of the putative AM-toxin receptor. The methoxy group of the L-Amp^1 residue is possibly interact with the putative receptor either electrostatically or by forming a hydrogen bond.

Substitution of L-Ala^3 residue

Analogs **4–9** were synthesized to evaluate the effect of the side chain of the L-Ala^3 residue on the activity. Substitution of the methyl group of L-Ala^3 with longer normal alkyl chains, ethyl (L-

Abu, **5**), *n*-propyl (L-Nva, **6**) and *n*-butyl (L-Nle, **7**) groups had a only small effect on the activity. This suggests that the space of the L-Ala^3 binding subsite of the receptor is relatively large and can accommodate long side chains such as the *n*-butyl group of L-Nle^3. The analog containing L-Leu^3 at this position (**8**) showed 10 times higher activity than AM-toxin I. This is notable because the activity of this analog was reported to be much lower on the basis of an assay using tree leaves (Aoyagi *et al.,* 1985). The substitution of L-Ala^3 with L-Tyr^3 (**9**) decreased the activity by a factor of 100. This is consistent with the previous assay data of an analog containing L-Phe^3 instead of L-Ala^3, which showed only a low activity against tree leaves (Aoyagi *et al.,* 1985). It is likely that the aromatic nature of the L-Tyr^3 (and L-Phe^3) side chain, rather than the bulkiness, is unfavorable for the interaction with the putative receptor since the L-Leu^3 analog **8** containing a bulky *i*-butyl side chain retained its high potency. The replacement of L-Ala^3 with Gly^3 (**4**) also decreased the activity by 1000-fold. The Gly^3 residue has no substituent on the α-carbon atom. This may provide a substantial flexibility to the backbone ring structure, resulting in a different stable conformation from that of AM-toxin I. The specific orientation of the C=O and N–H moieties of the amide groups and the C=O of ester groups on the backbone ring structure has been reported to be important for the expression of high activity. This is probably due to the fact that these groups significantly contribute to the receptor binding (Higashijima *et al.,* 1983). In the most active conformation, three of the four C=O moieties are thought to be oriented to the same side of the ring structure of AM-toxin I. In the low-energy stable conformers of the Gly^3 analog, some of these C=O moieties may have a different orientation from that of AM-toxin I due to the flexibility of the ring structure.

Substitution of L-Hmb^4 residue

Effect of the structural modification of the L-Hmb^4 residue in AM-toxin I was examined by using analogs **10–13**. In these analogs the corresponding residues were substituted with an amino acid: L-Val (**10**), and α-hydroxy acids: L-lactic acid (L-Lac) (**11**), L-hydroxyisocaproic acid (L-Hic) (**12**) and L-phenyllactic acid (L-Pla) (**13**). Replacement of the ester bond between L-Hmb^4 and L-Ala^3 in AM-toxin I with an amide bond in **10** resulted in a 10-fold decrease in the activity. The slight decrease in the activity of this analog (**10**) is probably due to differences in the orientation of the C=O moieties on the backbone structure between **10** and AM-toxin I, which was previously measured by NMR (Kozono *et al.,* 1983). Our results show that the effects of this conformational change upon the activity are smaller than that indicated in previous studies. Substitution of the *i*-propyl group of L-Hmb^4 residue with methyl (**11**) or *i*-butyl (**12**) groups lowered the activity by a factor of 10^3. Furthermore, the analog **13** containing a benzyl group exhibited no activity even at 10^{-4} M. This suggests that there is an optimum bulkiness required for this residue to favorably interact with the putative receptor. The notable deleterious effect of benzyl group may be related to differences in its electronic properties compared to that of alkyl groups as well as steric effects.

Effect of the macrocyclic ring size on the activity

The importance of the macrocyclic ring structure for activity was further investigated with analogs **14–16**, which have a larger ring size than that of AM-toxin I (Table IV). The analog **14,** in which the L-Ala^3 residue was replaced with β-Ala^3, has a 13-membered ring structure, while AM-toxin I has a 12-membered one. Analogs **15** and **16,** in which Gly residues were inserted between L-Ala^3 and L-Hmb^4, and between ΔAla^2 and L-Ala^3, respectively, have 15-membered rings. The replacement of the L-Ala^3 residue with βAla^3 (**14**) diminished the activity by a factor of 10^4. Both analogs **15** and **16**, containing an additional Gly residue in the ring structure, exhibited much lower activity than AM-toxin I. The low activity of analogs **14–16** can be attributed to differences in the conformation of their ring structure as discussed for analog **4**. Supportingly, [βAla^3]AM-toxin (**14**) showed a different CD spectrum from that of AM-toxin I, whereas [L-Abu^3] AM-toxin (**5**), which retained high activity, showed a CD spectrum similar to that of AM-toxin I (Fig. 2). This suggests that the extension of the ring size caused an increase in the flexibility of the backbone ring structure. As a result, the population of the active conformation, which probably has a substantial similarity to that of AM-toxin I, could have decreased.

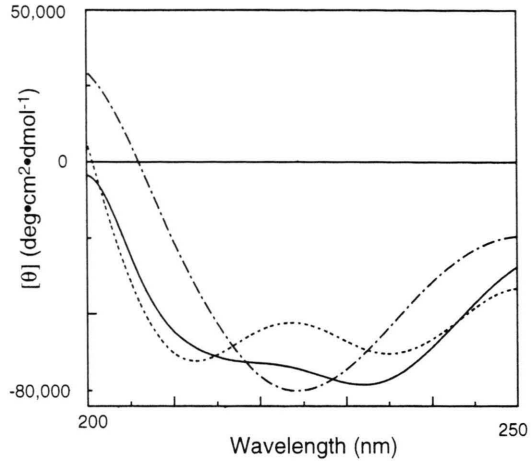


Fig. 2. CD spectra of AM-toxin I (—), [βAla³]AM-toxin (···) and [Abu³]AM-toxin (---).

Figure 3 represents the schematized structure of the binding site of the AM-toxin I in the putative receptor based upon the information obtained in this and previous SAR studies. The receptor recognizes L-Ala³ residue much less strictly than other three residues in AM-toxins. These findings are important for designing ligands for further experimental probing of the nature of the receptor (Hashimoto *et al.*, 1997; Hashimoto *et al.*, 1998). Utilizing these probes will lead to a better understanding of the mechanism of action of AM-toxins.

Acknoledgement

We thank Dr. Ryutaro Tao of Kyoto University for providing some meristem cultures of apple and Dr. Kimiko Hashimoto of Kwansei Gakuin University for invaluable suggestions for the synthesis. We also thank Dr. Naoshige Akimoto of Kyoto University for the mass spectral measurements and Dr. Kazunari Akiyoshi of Kyoto University for CD spectral measurements. This research was in part supported by Grant-in-Aid of Ministry of Education, Science and Culture of Japan (No. 10460048).

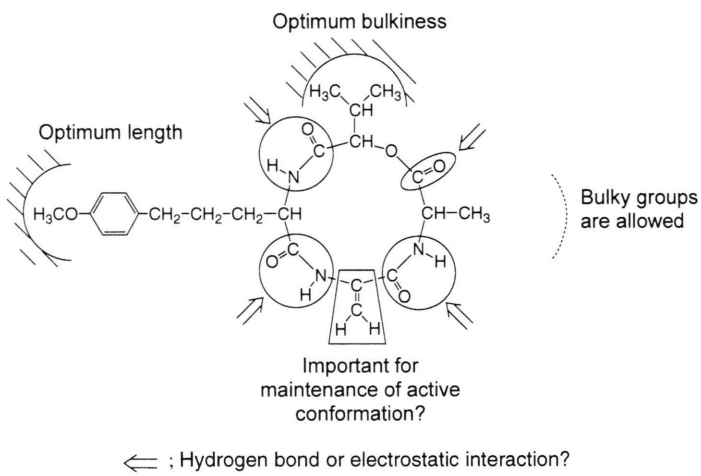


Fig. 3. Schematized structure of the binding site of AM-toxin I in its putative receptor.

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