Cyclic Peptides. IV.[1] Synthesis of Diastereomeric Dihydro-AM-toxin I and Its Analogs[2]

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Diastereomeric cyclotetradepsipeptides [L-Ala^2]- and [D-Ala^2]-AM-toxin I, corresponding to the hydrogenated products of AM-toxin I, were synthesized by the conventional method and their biological activities were tested. [D-Ala^2]-AM-toxin I exhibits weak but recognizable necrotic activity (5—10 μg/ml) on apple leaves. [L-Ala^2]-AM-toxin I is inactive even in a high concentration of 100 μg/ml. The results of NMR and ORD measurements of [D-Ala^2]-AM-toxin I and [L-Tyr(Me)^1, D-Ala^2]-AM-toxin indicates the presence of a common conformer. The results on [L-Ala^2]-AM-toxin I and [L-Tyr(Me)^1, L-Ala^2]-AM-toxin revealed the presence of more than two conformers, the predominant conformation differing from that of D-isomers. Difference in activity between [D-Ala^2]- and [L-Ala^2]-AM-toxin I can be attributed to the difference in conformation of the diastereomers.

AM-toxins are host-specific phytotoxins produced by *Alternaria mali* causing necrosis on apple leaves.[3] The structures of AM-toxin I (**1a**) and its analog [L-Tyr-(Me)^1]-AM-toxin (**1b**) are shown in Fig. 1.[4] Peptide **1a** and its analogs such as **1b** and di-AM-toxin I (cyclic octadepsipeptide) were synthesized in this laboratory, the minimum toxic activity of **1a**, **1b**, and di-AM-toxin I being determined to be 0.002, 20, and 10—20 μg/ml, respectively.[5]

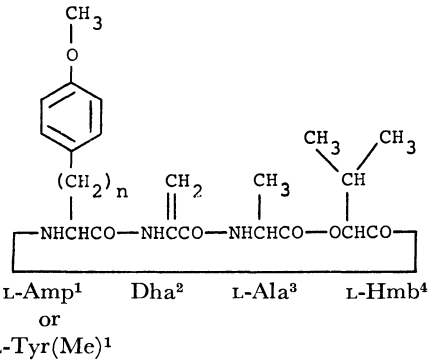

Fig. 1. Structure of AM-toxin I (**1a**) and [L-Tyr(Me)^1]-AM-toxin (**1b**). **1a**, $n=3$; **1b**, $n=1$.

This paper describes studies on the role of a double bond in the Dha^2 residue in **1a**. Since hydrogenation of natural **1a** produces a mixture of [L-Ala^2]- and [D-Ala^2]-AM-toxin I (**8a**-L and **8a**-D), pure **8a**-L and **8a**-D were prepared by the conventional method of peptide synthesis. The biological activities of the synthesized peptides were tested. Analogs (**8b**-L and **8b**-D) containing *O*-methyl-L-tyrosine (**2b**) in place of L-2-amino-5-(*p*-methoxyphenyl)pentanoic acid (**2a**) were also synthesized in order to investigate the influence of variation of the side chain in **2a** on necrotic activity and on conformation. When the synthesis of **1a** and **1b** was carried out in a previous study, a mixture of cyclic oligomers was obtained by the cyclization reaction of the corresponding linear tetradepsipeptides. The cyclic oligomers behaved chromatographically in a very similar way to each other, and thus purification with silica gel column was tedious and complete separation difficult.[5] In the present study, effective separation of cyclic oligomers was attained by fractional crystallization

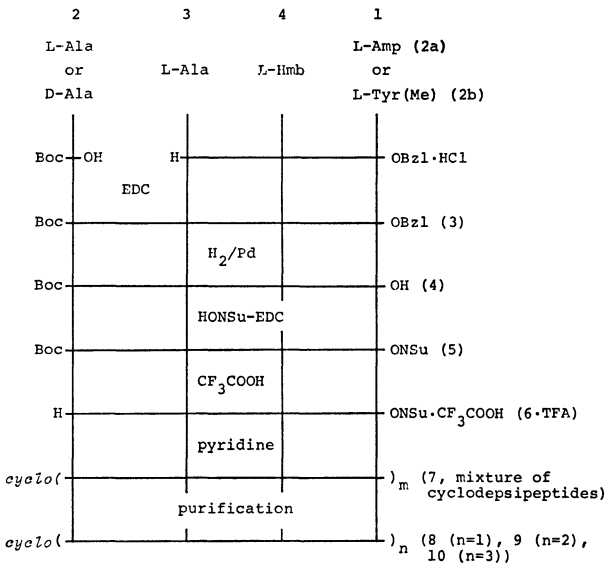

Fig. 2. Synthetic route for dihydro-AM-toxin I (**8a**-L and **8a**-D) an [DL-Tyr(Me)^1]-dihydro-AM-toxin (**8b**-L and **8b**-D).

with use of minute differences in the solubility of the oligomers.

Figure 2 shows the reaction route for the synthesis. L-Amp was prepared by the resolution of Ac-DL-Amp-OH with acylase.[6] Ester H-L-Ala-L-Hmb-L-Amp-OBzl·HCl[1] was coupled with Boc-L- or Boc-D-Ala-OH by the EDC method to give Boc-L- or Boc-D-Ala-L-Ala-L-Hmb-L-Amp-OBzl (**3a**-L or **3a**-D). Ester **3a**-L or **3a**-D was catalytically hydrogenated to give tetradepsipeptide acid (**4a**-L or **4a**-D), the acid being converted into active ester **5a**-L or **5a**-D with the use of HONSu and EDC. The active ester **5a**-L was treated with TFA at 0 °C for 20 min, the salt **6a**-L·TFA obtained was dissolved in DMF, and the solution was subjected to cyclization reaction in pyridine. A mixture (**7a**-L) of cyclodepsipeptides was obtained showing three spots on a thin layer chromatogram corresponding to cyclic monomer (**8a**-L), dimer (**9a**-L), and trimer (**10a**-L). In order to isolate each of the pure products, reprecipitation with dioxane was carried out. Less soluble fraction of **7a**-L was treated with DMF-dioxane to give pure **8a**-L and **10a**-L. Similar precipitation of

more soluble fraction of **7a**-L gave pure dimer **9a**-L. On the other hand, cyclization of **6a**-D gave monomer **8a**-D exclusively. Analogous cyclic depsipeptides containing L-Tyr(Me) (**2b**) were obtained similarly.

When natural **1a** was catalytically hydrogenated in acetic acid, a mixture consisting of **8a**-L and **8a**-D was found on a thin layer chromatogram. In order to determine the ratio of **8a**-L to **8a**-D in the mixture, the procedure of Shimohigashi *et al.*[6] was applied; the hydrogenated residue of the mixture was hydrolyzed in hydrochloric acid and the hydrolyzate was acylated with Z-L-Leu-ONSu in aqueous dioxane. Upon debenzyloxycarbonylation, leucyl dipeptides obtained were applied to an amino acid analyzer. The calculated ratio of L-Leu-L-Ala to L-Leu-D-Ala was 2.21: 1.00. From the result, the ratio of **8a**-L to **8a**-D in the hydrogenated mixture was determined to be 38: 62.

Experimental

All the melting points are uncorrected. TLC was carried out on silica gel G (Merck) with the following solvent systems, the ratio in parentheses being indicated by vol: R_f^1, $CHCl_3$–MeOH (5: 1); R_f^2, $CHCl_3$–MeOH–AcOH (95: 5: 1); R_f^3, $CHCl_3$–MeOH (9: 1). Optical rotations were measured with a Union high sensitivity polarimeter PM-71. Mass spectra were taken on a Hitachi RMS-4 mass spectrometer with a direct inlet system operating at 70 eV, UV spectra on a Hitachi 124 spectrophotometer, and NMR spectra on a JEOL JNM PS-100 spectrometer, tetramethylsilane being used as an internal reference. ORD measurements were carried out with a JASCO ORD/UV-5 spectropolarimeter using a 0.1 cm cell; sample concentration, 0.01%. Amino acid analyses were determined with a Hitachi amino acid analyzer KLA-5. Details of the synthetic procedures are described mainly for [L-Ala^2]-AM-toxin I and its derivatives as a representative case. Other compounds were synthesized in a similar way. Physical constants, yields, and the results of elemental analyses are summarized in Table 1.

Boc-L-Ala-L-Ala-L-Hmb-L-Amp-OBzl (3a-L). Boc-L-Ala-OH (0.45 g, 2.37 mmol), H-L-Ala-L-Hmb-L-Amp-OBzl·HCl[1] (1.23 g, 2.37 mmol), and Et_3N (0.33 ml, 2.37 mmol) were dissolved in $CHCl_3$ (15 ml). To the solution was added EDC·HCl (0.50 g, 2.61 mmol) at 0 °C. The mixture was stirred for 2 h at 0 °C and overnight at room temperature, evaporated *in vacuo*, and the residue was treated with a mixture of water and EtOAc. The organic layer was washed with 4% $NaHCO_3$, 10% citric acid and water, dried (Na_2SO_4), and evaporated to leave an oil, which was crystallized by the addition of ether and petroleum ether; yield, 1.39 g. The solid was contaminated with a small amount of by-products and was purified with a silica gel column (3.3×40 cm) using a solvent of $CHCl_3$–EtOAc (1: 1). The fractions (260—380 ml) were collected and evaporated, and the residual solid was recrystallized from EtOAc–ether–petroleum ether; yield, 1.24 g (79%).

Boc-L-Ala-L-Ala-L-Hmb-L-Amp-OH (4a-L). Ester **3a**-L (1.12 g, 1.70 mmol) was dissolved in THF (20 ml) and hydrogenated in the presence of Pd black. The filtrate from the catalyst was evaporated and the residue was crystallized from $CHCl_3$–petroleum ether; yield, 0.89 g (93%).

Boc-L-Ala-L-Ala-L-Hmb-L-Amp-ONSu (5a-L). To a chilled solution of **4a**-L (453 mg, 0.80 mmol) in DMF (5 ml), HONSu (138 mg, 1.20 mmol) and EDC·HCl (184 mg, 0.96 mmol) were added at 0 °C. After 24 h at 0 °C the solution was evaporated, and the residual oil was solidified by the addition of water. Powder obtained was filtered off quickly and dried over P_2O_5; yield, 531 mg (100%); R_f^1 0.87.

A Mixture of Cyclopeptides (7a-L). Ester **5a**-L (531 mg, 0.80 mmol) was dissolved in TFA (5 ml) at 0 °C. After 20 min the solution was evaporated to leave an oil, which was solidified by the addition of ether. The solid (**6a**-L·TFA) obtained was dissolved in DMF (5 ml) and stirred in pyridine (260 ml) at room temperature. After 24 h the solution was evaporated, and the residue was triturated with cold water and 10% citric acid; yield of solid (**7a**-L), 300 mg (84%). TLC of **7a**-L showed three spots by monomer (**8a**-L), dimer (**9a**-L), and trimer (**10a**-L).

Cyclo(-L-Amp-L-Ala-L-Ala-L-Hmb-) ([L-Ala^2]-AM-toxin I) (8a-L). Mixture **7a**-L (300 mg) was added to dioxane (20 ml) and the suspension was stirred at room temperature for 1 day. The insoluble solid was collected and washed with hot dioxane (yield of the solid, 180 mg), the filtrate (**7a**-L-**f**)

Table 1. Analytical data of synthetic peptides

Compound	Formula	Yield (%)	Mp(°C)	R_f^1	R_f^2	R_f^3	$[\alpha]^{20}$	Found C(%)	Found H(%)	Found N(%)	Found M+	Calcd C(%)	Calcd H(%)	Calcd N(%)	Calcd mol wt
3a-L	$C_{35}H_{49}O_9N_3$	79	119—120	0.70	0.56		−40.8[a]	63.78	7.53	6.42		64.10	7.53	6.41	
3a-D	$C_{35}H_{49}O_9N_3$	77	131—133	0.69	0.57		+2.8[a]	63.86	7.55	6.42		64.10	7.53	6.41	
3b-L	$C_{33}H_{45}O_9N_3$	66	140—141	0.86	0.56		−40.4[a]	63.00	7.22	6.69		63.14	7.23	6.69	
3b-D	$C_{33}H_{45}O_9N_3$	71	119—120	0.81	0.58		−0.2[a]	63.02	7.25	6.63		63.14	7.23	6.69	
4a-L	$C_{28}H_{43}O_9N_3$	93	136—138	0.57	0.42		−29.0[a]	59.32	7.67	7.42		59.45	7.66	7.43	
4a-D	$C_{28}H_{43}O_9N_3$	95	137—139	0.56	0.40		+0.2[a]	59.28	7.64	7.34		59.45	7.66	7.43	
4b-L	$C_{26}H_{39}O_9N_3$	100	138—140	0.29	0.38		−20.4[a]	57.75	7.35	8.12		58.08	7.31	7.82	
4b-D	$C_{26}H_{39}O_9N_3$	99	107—108	0.33	0.43		+8.6[a]	57.48	7.34	7.40		57.13[b]	7.38[b]	7.69[b]	
8a-L	$C_{23}H_{33}O_6N_3$	27	277—278[c]		0.17	0.31	−6500[d]	61.52	7.45	9.37	447	61.72	7.43	9.39	447
8a-D	$C_{23}H_{33}O_6N_3$	72	>300[c]		0.60	0.80	−6800[d]	61.48	7.41	9.32	447	61.72	7.43	9.39	447
8b-L	$C_{21}H_{29}O_6N_3$	36	>300[c]		0.15	0.30	−5700[d]	59.93	6.94	10.14	419	60.13	6.97	10.02	419
8b-D	$C_{21}H_{29}O_6N_3$	50	>300[c]		0.61	0.82	−6800[d]	60.02	6.94	9.96	419	60.13	6.97	10.02	419
9a-L	$C_{46}H_{66}O_{12}N_6$	17	217—219[c]		0.36	0.51	−1600[d]	60.77	7.37	9.30	894	60.50[e]	7.51[e]	9.21[e]	894
9b-L[f]	$C_{42}H_{58}O_{12}N_6$	4	255—256[c]		0.52	0.63	−1900[d]	59.20	6.97	9.71	838	59.49[g]	7.01[g]	9.91[g]	838
10a-L	$C_{69}H_{99}O_{18}N_9$	8	>300[c]		0.41	0.51	−1100[d]	60.71	7.36	9.32	1342	60.90[e]	7.48[e]	9.27[e]	1342

a) $[\alpha]_D$ (*c* 1, $CHCl_3$). b) $1/2H_2O$. c) With decomp. d) $[\alpha]_{250}$ (*c* 0.01, CF_3COOH). e) Monohydrate. f) $[\alpha]_D^{27}$ −83° (*c* 0.3, DMF). g) $1/4H_2O$.

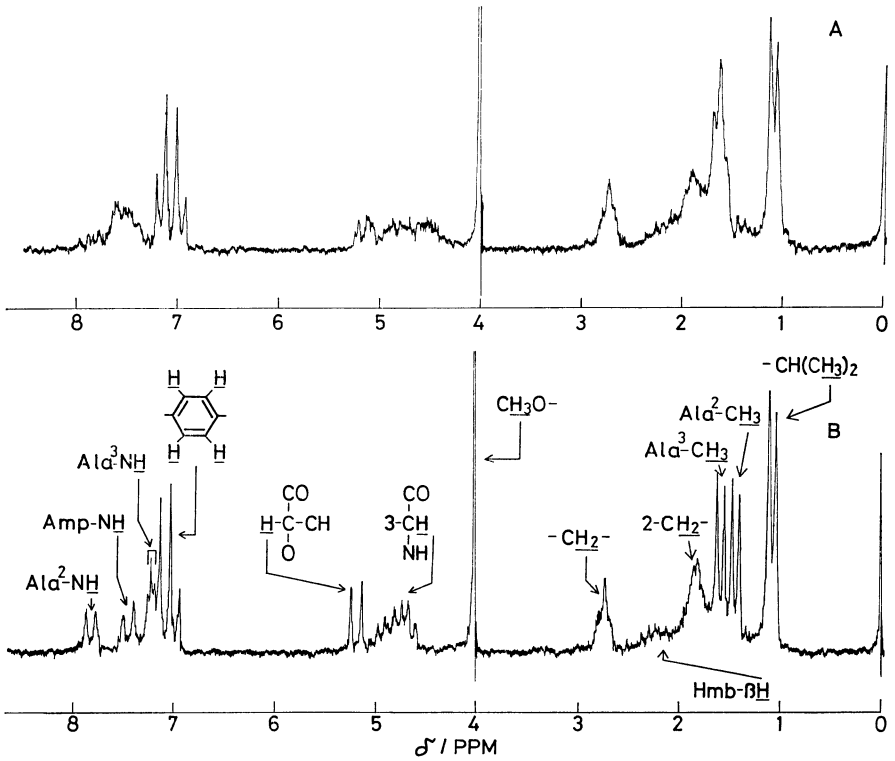

Fig. 3. NMR spectra of dihydro-AM-toxin I in TFA. A, [L-Ala^2]-AM-toxin I (**8a**-L); B, [D-Ala^2]-AM-toxin I (**8a**-D).

being put aside for the isolation of **9a**-L. The solid was dissolved in hot DMF (2 ml) and cooled to room temperature. The resulting precipitate was collected with the aid of dioxane (yield of the precipitate, 34 mg), the precipitate (**7a**-L-**p**) being used for the isolation of **10a**-L. The filtrate was evaporated, and the residue was repeatedly recrystallized from the following solvent systems: DMF–EtOAc, DMF–dioxane, and DMF–EtOAc–ether; yield of **8a**-L, 95 mg (27%).

Cyclo[–(L-*Amp*–L-*Ala*–L-*Ala*–L-*Hmb*)$_2$–] (*9a*-L). The filtrate **7a**-L-**f** mentioned above was evaporated, and the residue (107 mg) was crystallized from dioxane–EtOAc–ether–petroleum ether; yield, 66 mg. Further recrystallization from dioxane–ether–petroleum ether gave 60 mg (17%).

Cyclo[–(L-*Amp*–L-*Ala*–L-*Ala*–L-*Hmb*)$_3$–] (*10a*-L). The precipitate **7a**-L-**p** mentioned above was crystallized from DMF–EtOAc–ether; yield, 29 mg (8%).

Cyclo(–L-*Amp*–D-*Ala*–L-*Ala*–L-*Hmb*–) ([D-*Ala*^{2}]-*AM-toxin I*) (*8a*-D). Ester **5a**-D (332 mg) obtained from **4a**-D (283 mg, 0.50 mmol) was treated with TFA as described for **7a**-L, and **6a**-D·TFA obtained was subjected to cyclization reaction for three days. After evaporation of pyridine the residue was triturated with cold water and filtered off. Recrystallization of the solid from DMF–EtOAc gave 161 mg (72%).

Hydrogenation of Natural AM-toxin I. Natural **1a** (4 mg, 9 μmol) was dissolved in AcOH (5 ml) and hydrogenated in the presence of Pd black. The filtrate from the catalyst was evaporated, and the residue (3.2 mg) showed two spots on TLC corresponding to **8a**-L and **8a**-D. A portion (1.6 mg) of this residue was dissolved in 6 M HCl (1 ml) and hydrolyzed at 110 °C for 24 h. After evaporation the residue was dissolved in a mixture of water (0.5 ml) and dioxane (0.5 ml) and the mixture was neutralized by the addition of Et_3N. To this mixture were added a solution of Z-L-Leu–ONSu (7.8 mg, 21 μmol) in dioxane (0.5 ml) and Et_3N (14 μmol) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature. After evaporation, the residue was dissolved in a mixture (3 ml) of MeOH–AcOH–water (5:1:1) and hydrogenated in the presence of Pd black. The product was evaporated, and an aliquot of the diastereomeric dipeptides obtained was applied to an amino acid analyzer.[6] The molar ratio of L-Leu–L-Ala to L-Leu–D-Ala was determined to be 2.21: 1.00. After the correction of one equivalent of L-alanine present in **1a** as L-Ala^3, the ratio of **8a**-L to **8a**-D in the hydrogenated product was calculated to be 0.38: 0.62. When natural **1a** was hydrogenated in dioxane instead of AcOH, the ratio was 0.41: 0.59.

NMR Spectroscopy. Figure 3 shows the NMR spectra of **8a**-L and **8a**-D in TFA at ambient temperature with sample concentration at 15 mg/0.4 ml. Signals of **8b**-D were assigned as follows: δ=0.92 (3H, d, J=7.0 Hz, HmbCH$_3$), 1.04 (3H, d, J=7.0 Hz, HmbCH$_3$), 1.38 (3H, d, J=7.0 Hz, D-Ala^2-CH$_3$), 1.58 (3H, d, J=7.0 Hz, L-Ala^{3}CH$_3$), 2.2 (1H, m, HmbβH), 3.0—3.4 (2H, ABX, Tyr(Me)CH$_2$), 4.00 (3H, s, OCH$_3$), 4.5—5.0 (3H, m, αH), 5.12 (1H, d, HmbαH), 6.98—7.30 (5H, aromatic H and NH), 7.58 (1H, d, J=10.0 Hz, Tyr(Me)NH), 7.80 (1H, d, J=9.0 Hz, D-Ala^{2}NH). Because of poor solubility of **8a**-D and **8b**-D the use of other solvents was not attempted.

Biological Assay. Biological assay on apple leaves (susceptible cultivar, Indo) was carried out as described previously.[7] The minimum toxic concentrations of **8a**-D and **1a** were 5—10 and 0.002 μg/ml, respectively. **8a**-L, **8b**-L, and **8b**-D showed no activity in high concentration up to 100 μg/ml.

Results and Discussion

Since the results of the bioassay revealed a distinct difference between the activity of **8a**-L and that of **8a**-D, we investigated the conformations of these diastereomeric peptides in order to clarify the structure-activity relationship. The NMR spectra of **8a**-D and **8b**-D

showed well resolved but very similar patterns (one difference between them being the splitting of the Hmb side chain), suggesting the presence of a common and stable conformation for **8a**-D and **8b**-D. On the other hand, **8a**-L and **8b**-L displayed complex spectra indicating that each of these L-peptides is a mixture of at least two conformers. For example, **8a**-L and **8b**-L gave the complex signals of α-CH and NH protons in contrast with the well resolved signals of the corresponding protons of **8a**-D and **8b**-D. Furthermore, **8a**-L revealed weak signals with the same chemical shifts at those obtained in **8a**-D or **8b**-D spectra, such as doublets at 1.40 ppm and at 7.80 ppm.

These observations suggest that predominant conformations of **8a**-L and **8b**-L differ from those of **8a**-D and **8b**-D. Unfortunately, information obtained from the NMR spectra was not sufficient to deduce a definite conformation for **8a**-D and **8b**-D. At least two conformations were in line with the results of NMR study; one with the all-*trans* backbone system[8] and the other with the *cis-trans-cis-trans* backbone.[9] In general the presence of *cis* peptide bond is observed only for α-imino acid such as proline. In case of cyclotetrapeptides, however, the all-*trans* backbone system has to be strained because of steric constraint.[10] Thus, the presence of a conformation containing a *cis* bond can not be excluded. Natural **1a** showed similar NMR spectra to those of **8a**-D. However, rapid decomposition of **1a** in TFA made further study difficult.

The ORD curves of selected synthetic peptides are shown in Figs. 4 and 5. All cyclic tetradepsipeptides show a significant trough, $[\alpha] = -6000 \sim -9000°$, around 245 nm region in their ORD curves. In contrast, ORD curves of dimers (**9a**-L and **9b**-L) and a trimer(**10a**-L) show a shallow trough around 245 nm, the depth being less than −2500°. The results suggest that peptide

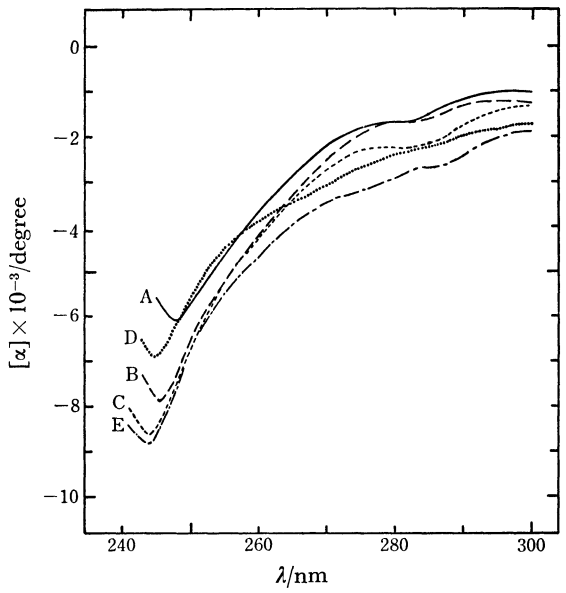

Fig. 4. ORD curves of AM-toxin I and its analogs in TFA. A, natural AM-toxin I (**1a**); B, [L-Ala²]-AM-toxin I (**8a**-L); C, [D-Ala²]-AM-toxin I (**8a**-D); D, [L-Tyr(Me)¹,L-Ala²]-AM-toxin(**8b**-L); E, [L-Tyr(Me)¹, D-Ala²]-AM-toxin (**8b**-D).

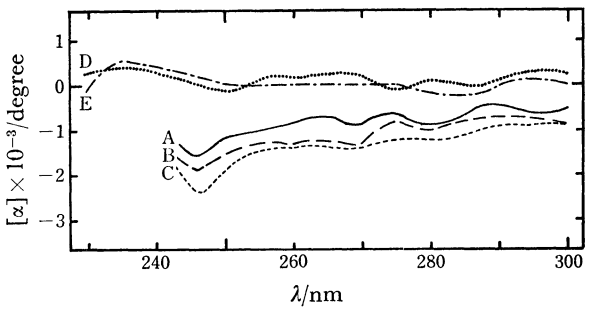

Fig. 5. ORD curves of cyclic polymers (A, B, and C in TFA) and linear peptides (D and E in MeOH). A, cyclo[-(L-Tyr(Me)-L-Ala-L-Ala-L-Hmb)$_2$-]; B, cyclo-[-(L-Amp-L-Amp-L-Ala-L-Ala-L-Hmb)$_2$-]; C, cyclo-[-(L-Amp-L-Ala-L-Ala-L-Hmb)$_3$-]; D, Boc-D-Ala-L-Ala-L-Hmb-L-Amp-OBzl; E, Boc-D-Ala-L-Ala-L-Hmb-L-Amp-OH.

chromophores in cyclic tetradepsipeptides have a certain distinct spatial orientation, but chromophores in peptides with larger ring structure together with linear tetradepsipeptide derivatives have no such definite orientation. In contrast to NMR spectra, there is no

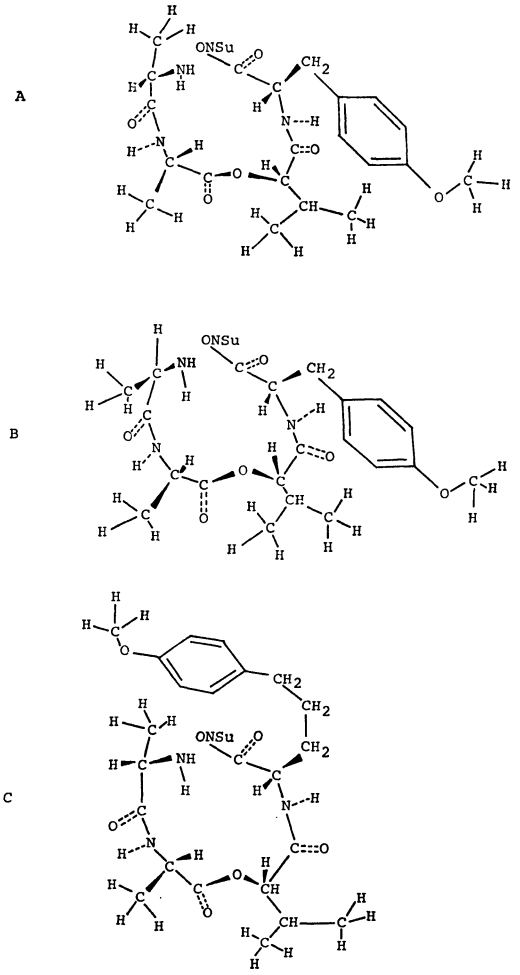

Fig. 6. Supposed conformation of reaction-intermediates in cyclization reaction starting from: A, H-L-Ala-L-Ala-L-Hmb-L-Tyr(Me)-ONSu (**6b**-L); B, H-D-Ala-L-Hmb-L-Tyr(Me)-ONSu (**6b**-D); C, H-L-Ala-L-Ala-L-Hmb-L-Amp-ONSu (**6a**-L).

distinct difference between **8a**-D or **8b**-D and **8a**-L or **8b**-L in their ORD curves. All these cyclotetradepsipeptides should have a similar orientation of chromophores.

Previous results showed that cyclic tetradepsipeptides containing one ester bond are obtained in high yields by cyclization reaction when a hydroxy acid residue occupies the third position from N-terminus in linear precursors.[5] An explanation for this finding has been presented.[7] Results obtained in this study indicate that not only the sequence of linear peptides but also the presence of D-amino acid in the N-terminus is important for giving a cyclic monomer in high yield. Exclusive formation of monomers (**8a**-D and **8b**-D) is of particular interest. This and the corresponding NMR data suggest that monomeric cyclization can readily proceed when a D-amino acid residue is present in a linear precursor. A possible substance of the intermediate having a *cis-trans-cis-trans* backbone is shown in Fig. 6. The methyl group in N-terminal L-Ala may hinder the access of the active ester group in Tyr(Me) (Fig. 6-A). In case of **6b**-D, the methyl group in D-Ala leads to a leftward direction away from the active ester (Fig. 6-B). Not only the active ester but also the bulky side chain in Amp was sterically hindered by the methyl group in L-Ala (Fig. 6-C). The fact that **6a**-L yielded much more dimer and trimer than **6b**-L supports this view. A similar discussion holds for another conformer with all-*trans* backbone.

Rich *et al.* reported that tentoxin, a similar phytotoxic cyclotetrapeptide, loses its activity upon hydrogenation even in high concentrations.[11] Since saturation of the double bond in the Dha^2 residue in **1a** results in about 2000—5000 times lowering in activity, the presence of the double bond in Dha^2 is undoubtedly important. However, while **8a**-D still retains recognizable activity (5—10 μg/ml), **8a**-L is inactive even in 100 μg/ml. This shows that the presence of a double bond is not an exclusive factor for revealing activity and that some other unknown factor seems to play a role in the activity. NMR studies indicate the presence of a common conformer of **8a**-D and **8b**-D. On the other hand, the content of the same conformer of **8a**-L and **8b**-L, if any, must be extremely low. This suggests the specific conformation is related to biological activity.

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2) A part of this paper was briefly communicated: Y. Shimohigashi, S. Lee, T. Kato, N. Izumiya, T. Ueno, and H. Fukami, *Agric. Biol. Chem.*, **41**, 1533 (1977).

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4) Abbreviations given by the IUPAC-IUB Commission (*J. Biol. Chem.*, **247**, 977 (1972)) have been used throughout. Other abbreviations: Amp, 2-amino-5-(*p*-methoxyphenyl)-pentanoic acid; Dha, dehydroalanine; Hmb, 2-hydroxy-3-methylbutanoic acid; Tyr(Me), *O*-methyltyrosine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HONSu, *N*-hydroxysuccinimide; DMF, *N,N*-dimethylformamide; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

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