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CYCLIC PEPTIDES

III. Synthesis of AM-Toxin I*

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To confirm the structure of AM-toxin I (a phytotoxic cyclotetradepsipeptide) the proposed peptide was prepared by a conventional method. The synthetic peptide and natural AM-toxin I were identical as regards t.l.c., u.v., mass spectra and biological activity in causing necrosis on apple leaves. A prepared dimer of AM-toxin I showed extremely weak activity; the relationship between the ring size and biological activity of AM-toxin I is discussed.

Three congeners of AM-toxin are host-specific phytotoxic metabolites isolated from a culture filtrate of *Alternaria mali* which causes veinal necrosis on apple leaves. Ueno *et al.* (1975*a*) elucidated the primary structure of AM-toxin I, one of the congeners, by chemical and spectral methods as shown in Fig. 1. Okuno *et al.* (1974) reported the same structure for alternariolide, which was found to be identical with AM-toxin I, although they did not determine the configuration of α-carbons of three components in a molecule of alternariolide. AM-toxin II (Ueno *et al.*, 1975*a*) and III (Ueno *et al.*, 1975*b*) contain L-2-amino-5-phenylpentanoic acid and L-2-amino-5-(*p*-hydroxyphenyl)pentanoic acid residues, respectively, instead of the L-2-amino-5-(*p*-methoxyphenyl) pentanoic acid residue. We were interested in confirming the structure of AM-toxin I by synthesis. To search for an effective synthetic route [Tyr(Me)^1]-AM-toxin, an analog of AM-toxin I, was synthesized because *O*-methyl-L-tyrosine is more easily available than L-2-amino - 5 - (*p*-methoxyphenyl)pentanoic acid (Shimohigashi *et al.*, 1977). In the preliminary [Tyr(Me)^1]-AM-toxin synthesis, cyclization of several linear tetradepsipeptide precursors was studied, and only one precursor, H-Ser-Ala-Hmb-Tyr(Me)-ONSu, produced a cyclic mono-

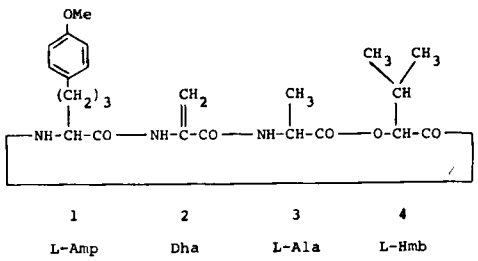

FIGURE 1
Structure of AM-toxin I.

* For a preliminary communication on this subject see reference (Lee *et al.*, 1976). Abbreviations according to IUPAC-IUB commission (1972), *Biochemistry* **11**, 1726–1732, are used throughout. Additional 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; DMF, *N, N*-dimethylformamide; HONSu, N-hydroxysuccinimide; TFA, trifluoroacetic acid; t.l.c., thin-layer chromatography.

mer ([Tyr(Me)[1], Ser[2]]-AM-toxin) in satisfactory yield (37%) as well as some cyclic dimer. The purified cyclic monomer was then converted into the desired [Tyr(Me)[1]]-AM-toxin containing a dehydroalanine residue by dehydration of a Ser residue in 16% yield. In this paper we describe the synthesis of a cyclopeptide (XI), corresponding to AM-toxin I, via a cyclic monomer (IX) from H-Ser-Ala-Hmb-Amp-ONSu (VII), and the comparison between synthetic and natural cyclopeptides. Since we anticipated the formation of cyclic dimer (X) as well as the monomer (IX) in the course of the cyclization reaction of VII, we additionally intended to prepare a dimerized form (XII) of AM-toxin I from the dimer (X) in order to elucidate the relationship between the ring size and biological activity of AM-toxin I.

The synthetic scheme is illustrated in Fig. 2. This scheme is similar to that of the synthesis of [Tyr(Me)[1]]-AM-toxin (Shimohigashi *et al.*, 1977), with the Tyr(Me) residue replaced by Amp. A precursor peptide (VII·TFA) was cyclized in pyridine and an isolated solid (VIII) showed two major and some fainter spots on t.l.c. After product VIII was treated with ethyl acetate, the less soluble component (IX) was purified by recrystallization and identified as a cyclic monomer by molecular weight determination. The more soluble component (X) was also purified by recrystallization and determined to be a cyclic dimer. The visual ratio of IX and X in the mixture (VIII) was found to be about 1:1 according to the intensity of two stains on t.l.c. when spraying with 10% sulfuric acid and heating, but the isolation yield of the dimer (X) was lower than that of the monomer (IX) because of difficulties in the purification of X. Compound IX was subjected to successive treatments with *p*-toluenesulfonyl chloride and diethylamine (see Fig. 2) to afford the desired cyclopeptide (XI), but the ensuing solid contained many products besides XI. Pure XI was isolated in low yield by silica gel column chromatography and subsequent preparative t.l.c. The dimerized form (XII) of AM-toxin I was prepared from X in the same manner. Thus, we isolated pure XI in only 1.8% yield, which was much lower than the 16% yield of pure [Tyr(Me)[1]]-AM-toxin obtained previously from cyclo [-Tyr(Me)-Ser-Ala-Hmb-] (Shimohigashi *et al.*, 1977). The low yield (1.8%) might be a consequence of streric hindrance of the bulky side chain of the Amp residue preventing access of diethylamine to the Ser(Tos) residue in cyclo[-Amp-Ser(Tos)-Ala-Hmb-].

Synthetic XI was compared with natural

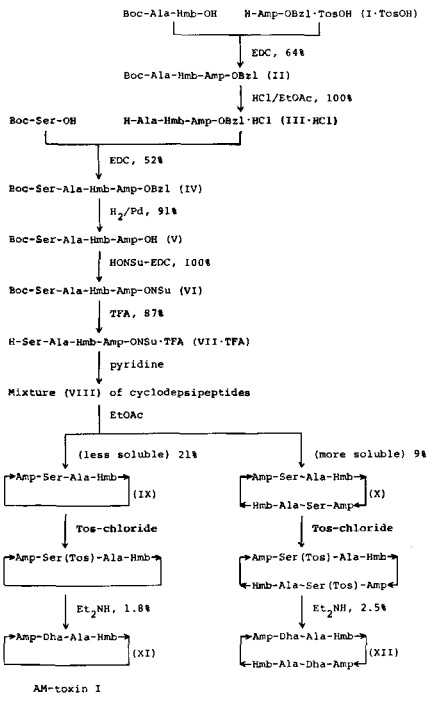

FIGURE 2

Synthesis of AM-toxin I. Preparation of cyclic monomer and cyclic dimer via the tetradepsipeptide active ester.

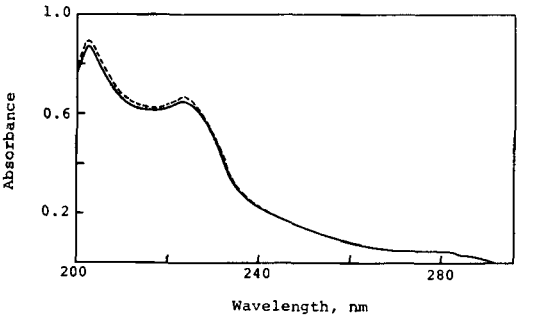

FIGURE 3

U.v. spectra of synthetic cyclopeptide (XI) (—) and AM-toxin I (- - -). Concentration of each cyclopeptide in MeOH, 4.3×10^{-3} M.

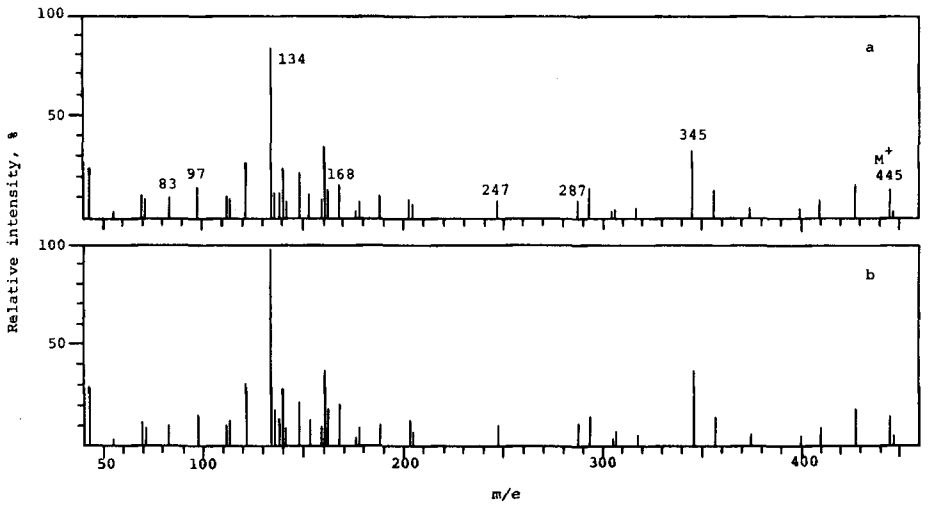

FIGURE 4

Mass spectra of synthetic cyclopeptide (XI) (a) and AM-toxin I (b).

AM-toxin I. In addition to having the same R_f values on t.l.c., both materials exhibited superimposable u.v. and mass spectra (Figs. 3 and 4) and identical levels of minimum toxic activity of 2 ng/ml for the induction of necrosis on apple leaves. Thus, the structure of AM-toxin I proposed by Ueno *et al.* (1975*a*) has been further confirmed by the present synthesis.

The dimer (XII) of AM-toxin I showed extremely low activity (at levels of 20–40 μg/ml on an apple leaf), and [Tyr(Me)^1]-AM-toxin also had low activity at the same level (Shimohigashi *et al.*, 1977). These results indicate that both the ring size and the presence of a bulky and/or hydrophobic side chain of an Amp residue in AM-toxin I are important for its biological activity.

EXPERIMENTAL PROCEDURES

T.l.c was carried out on silica gel G (Merck) with the following solvent systems, the ratios in parentheses being indicated by vol.: R_f^1, $CHCl_3$-MeOH (5:1); R_f^2, *n*-BuOH-AcOH-pyridine-H_2O (4:1:1:2); R_f^3, $CHCl_3$-EtOAc (7:3); R_f^4, $CHCl_3$-MeOH (9:1); and R_f^5, benzene-acetone (2:1). 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. U.v. spectra were determined on a Hitachi 124 spectrophotometer.

H-Amp-OBzl·TosOH (I·TosOH). This compound was prepared from L-2-amino-5-(*p*-methoxyphenyl)pentanoic acid (1.34 g, 6 mmol) (Shimohigashi *et al.*, 1976), benzyl alcohol (5 ml) and TosOH·H_2O (1.26 g, 6.6 mmol) by the procedure of Izumiya & Makisumi (1957). The solid product was recrystallized from ethanol-ether; yield, 2.73 g (94%); m.p. 129°; $[\alpha]_D^{20}$ −27.0° (*c* 2, $CHCl_3$); R_f^2 0.77.

Anal. Calc. for $C_{26}H_{31}O_6NS$ (485.6): C, 64.30; H, 6.44; N, 2.89. Found: C, 64.32; H, 6.38; N, 2.94.

Boc-Ala-Hmb-Amp-OBzl (II). Boc-Ala-Hmb-OH (1.59 g, 5.5 mmol) (Shimohigashi *et al.*, 1977), I·TosOH (2.67 g, 5.5 mmol) and Et_3N (0.77 ml, 5.5 mmol) were dissolved in $CHCl_3$ (60 ml). To the solution was added EDC·HCl (1.15 g, 6.0 mmol) at −10°. The reaction mixture was stirred for 2 h at 0° and overnight at room temperature, and evaporated to leave an oil, which was dissolved in EtOAc. The solution was washed successively with 4% $NaHCO_3$, 10% citric acid and H_2O, dried over Na_2SO_4, and evaporated. The residual solid was recrystallized from EtOAc-ether-petroleum ether; yield, 2.10 g (64%); m.p. 94-95°C; $[\alpha]_D^{20}$ −25.2° (*c* 1, $CHCl_3$); R_f^1, 0.92.

Anal. Calc. for $C_{32}H_{44}O_8N_2 \cdot 1/2H_2O$ (593.7):

C, 64.73; H, 7.64; N, 4.72. Found: C, 64.69; H, 7.39; N, 4.54.

H-Ala-Hmb-Amp-OBzl·HCl (III·HCl). Compound II (2.02 g, 3.4 mmol) was dissolved in 2.5 M HCl-EtOAc (13.6 ml). After 2 h at room temperature the solution was evaporated to yield an oil, 1.77 g (100%); R_f^1 0.55.

Boc-Ser-Ala-Hmb-Amp-OBzl (IV). Boc-Ser-OH (0.70 g, 3.4 mmol) was coupled with III·HCl (1.77 g, 3.4 mmol) by EDC·HCl (0.71 g, 3.7 mmol) as described for II to yield a solid (2.20 g). The solid was contaminated with small amounts of by-products and the purification was carried out by silica gel column chromatography (4.5 × 22 cm) using $CHCl_3$-EtOAc (2:1) as an eluant. The fractions (380–560 ml) were evaporated and the residual solid was recrystallized from EtOAc-ether-petroleum ether; yield, 1.19 g (52%); m.p. 112–114°; $[\alpha]_D^{20}$ −36.0° (*c* 2, $CHCl_3$); R_f^1 0.68, R_f^3 0.50. *Anal.* Calc. for $C_{35}H_{49}O_{10}N_3$ (671.8): C, 62.57; H, 7.35; N, 6.26. Found: C, 62.53; H, 7.17; N, 5.99.

Boc-Ser-Ala-Hmb-Amp-OH (V). Compound IV (1.14 g, 1.7 mmol) was dissolved in *t*-butanol (30 ml) and treated with hydrogen in the presence of Pd black. The filtrate from the catalyst was evaporated and the residual solid was recrystallized from EtOAc-petroleum ether; yield, 0.91 g (91%); m.p. 85°; $[\alpha]_D^{20}$ −22.0° (*c* 2, $CHCl_3$); R_f^1 0.52.
Anal. Calc. for $C_{28}H_{43}O_{10}N_3 \cdot 1/2H_2O$ (590.7): C, 56.93; H, 7.51; N, 7.11. Found: C, 57.06; H, 7.36; N, 7.06.

Boc-Ser-Ala-Hmb-Amp-ONSu (VI). To a chilled solution of V (0.59 g. 1 mmol) in DMF (5 ml) were added HONSu (0.17 g, 1.5 mmol) and EDC·HCl (0.29 g, 1.5 mmol) at 0°. After 24 h at 4° the solution was evaporated and the residual oil was solidified by the addition of cold water; yield, 0.68 g (100%); R_f^1 0.75.

H-Ser-Ala-Hmb-Amp-ONSu · TFA (VII · TFA). Ester VI (0.68 g, 1 mmol) was dissolved in TFA (5 ml) at 0°. After 15 min the solution was evaporated to leave an oil which was solidified by the addition of ether; yield, 0.60 g (87%). The solid was used for the next step without further purification.

Cyclization of VII: Mixture (VIII) of cyclopeptides. A solution of VII·TFA (0.60 g, 0.87 mmol) in DMF (10 ml) was added to pyridine (290 ml) at room temperature. After 24 h the solution was evaporated and the residue was treated with cold water. The resulting solid was collected and washed successively with 10% citric acid and H_2O to yield 0.31 g of a solid (VIII). T.l.c. of VIII showed the two major spots of IX (R_f^4 0.42) and X (R_f^4 0.55), besides additional faint spots of the by-products.

cyclo(-Amp-Ser-Ala-Hmb-) (IX). Compound VIII (0.31 g) was added to EtOAc (30 ml) and the insoluble solid was collected and washed with cold EtOAc (the filtrate was put aside for the isolation of the dimer). The solid was recrystallized from DMF-EtOAc-ether; yield, 84 mg (21%); m.p. 193–195°; $[\alpha]_D^{20}$ −113° (*c* 0.9, DMF); R_f^1 0.57, R_f^4 0.42; mass spectrum, m/e 463 (calc. 463.2).

cyclo[-(Amp-Ser-Ala-Hmb)$_2$-] (X). The filtrate mentioned above was evaporated and the residual solid was recrystallized from EtOAc-ether; yield, 36 mg (9%); m.p. 222–225°; R_f^1 0.63, R_f^4 0.55; mass spectrum, m/e 926 (calc. 926.4).

cyclo(-Amp-Dha-Ala-Hmb-) (XI). To a chilled solution of IX (69 mg, 0.15 mmol) in pyridine (1.5 ml) was added Tos-chloride (57 mg, 0.3 mmol) at 0°. After 24 h cold water was added to yield a solid precipitate. The collected solid was washed with a mixture (5 ml) of ether-petroleum ether (3:1) to remove excess Tos-chloride. The yield of cyclo[-Amp-Ser(Tos)-Ala-Hmb-] was 69 mg; R_f^1 0.62. To a solution of the solid (69 mg) in DMF (0.4 ml) was added 1 M Et_2NH in dioxane (0.2 ml) at room temperature. After 6 h the solution was evaporated to leave a solid which was collected with the aid of cold water; yield, 49 mg. T.l.c. of this solid showed many spots including that of XI. The purification was carried out with silica gel column chromatography using a 1.8 × 30 cm column and $CHCl_3$-EtOAc (1:1) as an eluant. Each fraction was assayed by t.l.c. and u.v.,

natural AM-toxin I being used as a reference compound. The fractions (180–224 ml) were evaporated and the residual solid was recrystallized from $CHCl_3$-petroleum ether. Since the solid obtained was still contaminated with several by-products, it was further purified by preparative t.l.c. using a solvent of $CHCl_3$-EtOAc (7:3). A portion of R_f^3 0.16 was extracted with EtOAc and the solution was evaporated to leave a powder; yield, 1.20 mg (1.8% from XI); mass spectrum, m/e 445 (calc. 445.2).

cyclo[-(Amp-Dha-Ala-Hmb)$_2$-] (XII). Compound X (30 mg, 0.032 mmol) was treated with Tos-chloride (12 mg, 0.064 mmol) and the ensuing cyclo [- (Amp - Ser(Tos) - Ala - Hmb)$_2$-] (26 mg) was treated with 0.1 M Et_2NH in dioxane (0.48 ml) as described above. The obtained solid was purified by silica gel column chromatography. The purified product was further subjected to preparative t.l.c. and a portion of R_f^3 0.20 afforded a powder (XII); yield, 0.71 mg (2.5% from X); mass spectrum, m/e 890 (calc. 890.4). Compound XII possessed very low activity of induction of necrosis on an apple leaf i.e. a level of 20–40 μg/ml.

Comparison of synthetic cyclopeptide (XI) and natural AM-toxin I. Compound XI and AM-toxin I showed single spots with the same R_f values on t.l.c. R_f^1 0.65, R_f^3 0.16, and R_f^5 0.50. The u.v. and mass spectrum of XI were superimposable with those of AM-toxin I (Figs. 3 and 4, respectively).

Biological assay on an apple leaf (susceptible cultivar, Indo) was carried out as described in the previous paper (Shimohigashi *et al.*, 1977). Compound XI and AM-toxin I possessed the same minimum toxic activity of induction of necrosis at a level of 2 ng/ml.

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