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Studies on Fungal Products. XI.[1] Isolation and Structures of Novel Cyclic Pentapeptides from *Aspergillus* sp. NE-45

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Three new compounds designated as cycloaspeptides A (**1**), B (**2**), and C (**3**) were isolated along with ergosterol from the mycelial chloroform extract of *Aspergillus* sp. NE-45. The structures of cycloaspeptides A, B, and C were established on the basis of chemical and spectroscopic evidence as **1**, **2**, and **3**, respectively. Cycloaspeptides are new cyclic pentapeptides containing an anthranilic acid residue and two *N*-methyl amino acid residues.

Keywords——*Aspergillus*; cyclic peptide; cycloaspeptide A; cycloaspeptide B; cycloaspeptide C; anthranylic acid; *N*-methyl amino acid; amino acid analysis

Aspergillus sp. NE-45 is a fungus isolated from soil of the forest between Chumro and Kuldi Ghar, Gandaki, Western Development Region in Nepal, collected by S. Udagawa in 1980.[2] Three new compounds, which were designated cycloaspeptides A (**1**), B (**2**), and C (**3**), along with ergosterol, were isolated from the mycelial chloroform extract of this fungus. The structural elucidation of the above compounds (**1**—**3**) is reported in this paper.

Cycloaspeptide A (**1**), mp 270—272 °C, $[\alpha]_D$ −228°, gave a molecular ion at *m/z* 641 on field desorption (FD) and electron impact ionization (EI) mass spectrometry, and elemental analysis confirmed its empirical formula as $C_{36}H_{43}N_5O_6$. The absorptions at 1680 and 1650 cm^{-1} in the infrared (IR) spectrum of **1**, and the positive chlorine–tolidine test (blue),[3] suggested the presence of amides, but not esters, in its structure. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) signals at δ 168.03, 168.13, 169.51, 169.59, and 174.13 were assigned to five amide carbonyls. On acetylation, **1** afforded a monoacetate (**4**), mp 178—180 °C, $[\alpha]_D$ −138°, $C_{38}H_{45}N_5O_7$, which showed a proton nuclear magnetic resonance (^{1}H-NMR) signal at δ 2.266 (3H, s), assigned to the methyl protons of an aromatic acetoxy group. No other significant changes were observed at **4** compared with **1**. Thus it is clear that the remaining one oxygen in **1** is a phenolic hydroxyl. On methylation with methyl iodide and sodium hydride, **1** gave a penta-*N*-methyl-*O*-methyl derivative (**5**), mp 166—168 °C, $C_{40}H_{51}N_5O_6$, which showed five *N*-methyl signals (δ 2.590, 2.616, 2.818, 3.075, and 3.621) and one aromatic *O*-methyl signal (δ 3.786). No IR absorption due to a hydroxyl, amino, or imino group was observed in the molecule of **5**. The negative reactivity in the ninhydrin test indicated the absence of an amino group in **1**. The above results suggested that **1** was a cyclic pentapeptide.

The homonuclear ^{1}H-{^{1}H} decoupling experiments and the analysis of the ^{13}C-NMR spectrum of **1** (Fig. 1) suggested the presence of alanine (Ala), leucine (Leu), *N*-methylphenylalanine (MePhe), *N*-methyltyrosine (MeTyr), and anthranilic acid (*o*-aminobenzoic acid, ABA) as amino acid residues. Me–L-Phe and Me–L-Tyr were synthesized from L-phenylalanine (Phe) and L-tyrosine (Tyr), respectively, in accordance with the method of

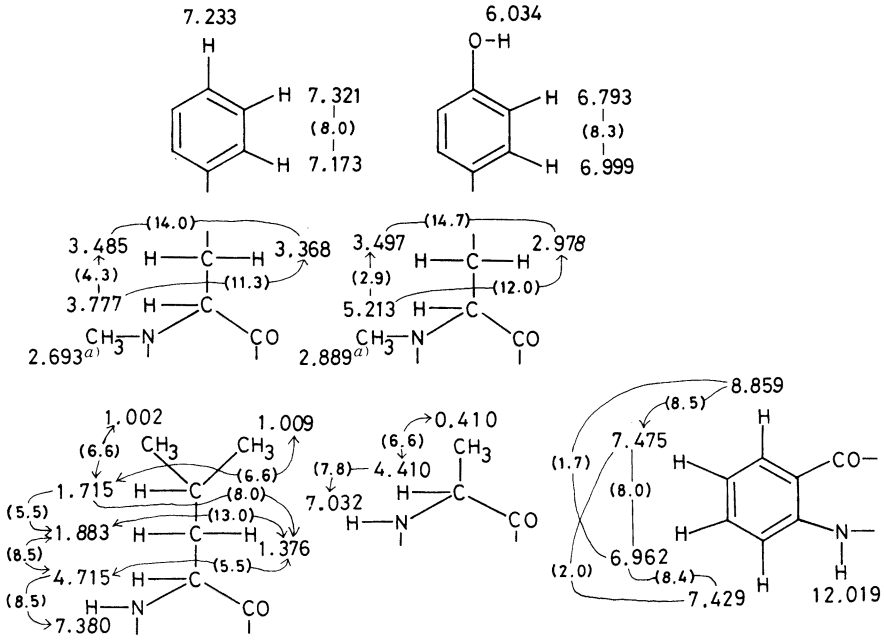


Fig. 1. ^{1}H-NMR Chemical Shift Assignments of Cycloaspeptide A (**1**)

An arrow ($H_a \rightarrow H_b$) indicates that proton b was decoupled when proton a was irradiated. The coupling constants are given in parentheses.
a) The assignments may be reversed.

TABLE I. Amino Acid Components of Complete Hydrolysates of Cycloaspeptides A (**1**), B (**2**), and C (**3**)

Hydrolysates	Amino acid components[a)]				
1	Ala[b)]	MePhe[c)]	Leu[b)]	MeTyr[c)]	ABA[c)]
2	Ala[b)]	MePhe[c)]	Leu[b)]	Tyr[b)]	ABA[c)]
3	Ala[b)]	Phe[b)]	Leu[b)]	MeTyr[c)]	ABA[c)]

a) Detected by cellulose TLC with ninhydrin reagent. *b*) Also detected by amino acid analysis. *c*) Also detected by HPLC with detection at 254 nm.

Coggins and Benoiton[4)] for use as standard samples in amino acid analysis. L-Phe and *O*-benzyl-L-Tyr (L-Tyr was benzylated preliminarily[5)]) were reacted with benzyloxycarbonyl chloride, methylated with methyl iodide and sodium hydride, and then hydrolyzed and hydrogenated to give Me–L-Phe and Me–L-Tyr. The acid hydrolysates of **1** were analyzed by cellulose thin layer chromatography (TLC), and Ala, MeTyr, Leu, and MePhe were detected with ninhydrin reagent (Table I). In addition, at the top of the TLC plate, a strong blue fluorescent spot, which showed no coloration with ninhydrin, was detected under ultraviolet (UV) light. This compound was shown to be identical with authentic ABA on silica gel TLC after extraction with chloroform. The result of the amino acid analysis and high performance liquid chromatography (HPLC) also confirmed that **1** was composed of Ala, Leu, MePhe, MeTyr, and ABA.

It was necessary to obtain a linear peptide by the partial hydrolysis of **1** in order to determine the arrangement of the amino acid residues, because **1** has neither a C-terminal nor an N-terminal. Partial acid hydrolyses under various conditions were examined in order to get

TABLE II. Amino Acid Components of Partial Acid Hydrolysates Derived from Cycloaspeptide A (**1**)

Peptide	Amino acid components[a]			
IA	Ala[b]	MePhe[c]	Leu[b]	
IB	MePhe[c]	Leu[b]	MeTyr[c]	
IC	Ala[b]	MePhe[c]	ABA[c]	
ID	Ala[b]	MePhe[c]	Leu[b]	ABA[c]

a) Detected by cellulose TLC with ninhydrin reagent. *b*) Also detected by amino acid analysis. *c*) Also detected by HPLC with detection at 254 nm.

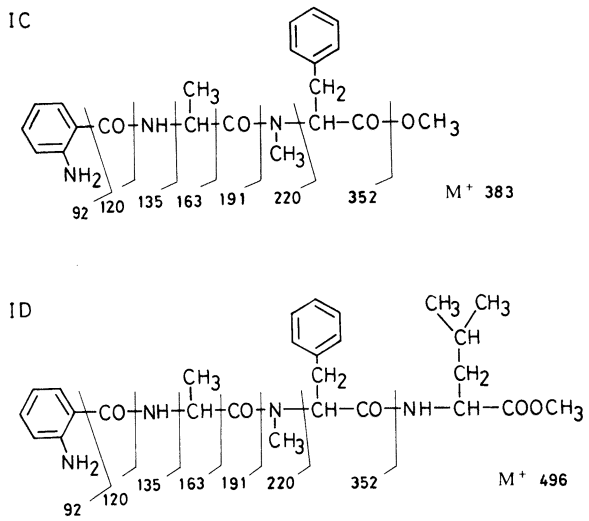
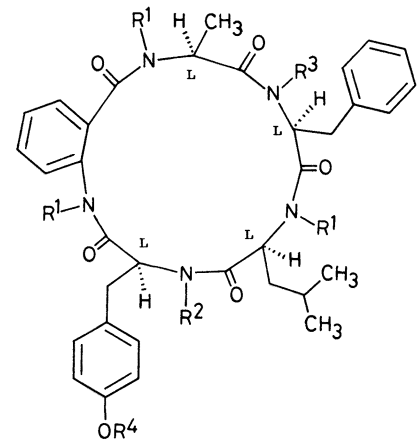


Fig. 2. Mass Fragmentation of Methyl Esters of Peptides IC and ID (*m/z*)



1: $R^1=R^4=H$, $R^2=R^3=CH_3$
2: $R^1=R^2=R^4=H$, $R^3=CH_3$
3: $R^1=R^3=R^4=H$, $R^2=CH_3$
4: $R^1=H$, $R^2=R^3=CH_3$, $R^4=COCH_3$
5: $R^1=R^2=R^3=R^4=CH_3$

Chart 1

a single peptide, but two or more peptides were obtained in each experiment. Thus the best partial hydrolysate (with 12 N HCl–AcOH at 27 °C for 48 h) was analyzed by amino acid analysis, HPLC, and cellulose TLC. The results are shown in Table II. The C-terminals of the peptide fragments, IC and ID, were methylated with diazomethane, and the mass spectra (MS) of the methyl esters prepared from peptides IC and ID showed favorable fragmentation (Fig. 2). The structures of the methyl esters of IC and ID were determined as H–ABA–Ala–MePhe–OH and H–ABA–Ala–MePhe–Leu–OH, respectively. The above results confirmed the structure of cycloaspeptide A to be **1**.

Cycloaspeptides B (**2**), mp 250—252 °C, $[\alpha]_D$ −138° [tetrahydrofuran (THF)–dimethylformamide (DMF)], and C (**3**), mp 281—283 °C, $[\alpha]_D$ −168° (THF–DMF), were confirmed to have the molecular formula $C_{35}H_{41}N_5O_6$. Both compounds were positive in the chlorine–tolidine test (blue) and negative in the ninhydrin test. The ^{1}H-NMR spectra of **2** and **3** were closely similar to that of **1** except for the absence of one *N*-methyl group in **2** and **3**. The ^{1}H-NMR signals assigned to *N*-methyl groups were observed at δ 2.693 and 2.889 in **1**, whereas signals were observed at δ 2.797 in **2** and at δ 2.552 in **3**. The permethylation of **2** and **3** gave the same compound (**5**) as that of **1**. Thus, it is clear that **2** and **3** are cyclic pentapeptides identical with **1**, except for the number of *N*-methyl groups. On complete acid hydrolysis (Table I), **2** gave Tyr and MePhe, and **3** gave MeTyr and Phe, whereas **1** afforded

MeTyr and MePhe. Compounds **2** and **3** are thus the demethyl derivatives at MeTyr and MePhe in the structure of **1**, respectively. The above results confirmed the structures of cycloaspeptides B and C as **2** and **3**, respectively.

In order to determine the stereochemistry of **1**, each amino acid separated after the complete acid hydrolysis of **1** was converted to the 2,4-dinitrophenyl (DNP) derivative.[6] Comparison of the circular dichroism (CD) curves[7] of these compounds with those of the standard derivatives, prepared from L-amino acids, confirmed that the stereochemistry of the amino acid components of **1** was L-ala, Me–L-Phe, L-Leu, and Me–L-Tyr. Consequently the structure of **1** was determined as cyclo (L-alanyl-*N*-methyl-L-phenylalanyl-L-leucyl-*N*-methyl-L-tyrosyl-*o*-aminobenzoyl) as shown in **1**, including its absolute stereochemistry.

Anthranilic acid (ABA) derivatives have been isolated from various fungi, and cyclopenin[8] and its derivatives, dioxopiperazines including an ABA residue, were isolated from *Penicillium cyclopium*, but cyclic peptides containing ABA in the molecule are very rate. Cycloaspeptide A (**1**) had no antifungal or antibacterial activity[9] at concentrations lower than 100 μg/disc. Since many of the cyclic peptides isolated from fungi and known to be toxic, cycloaspeptides may have some biological activity. Further tests are planned.

Experimental

Melting points are uncorrected. IR and UV spectra were taken with a Hitachi 215 spectrophotometer and a Hitachi 124 spectrophotometer, respectively. EI, FD, and chemical ionization (CI) MS were obtained on a JEOL JMS-D 300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-GX 400 spectrometer at 399.78 and 100.43 MHz, respectively, using tetramethylsilane as an internal standard. The coupling patterns are indicated as follows: singlet=S or s, doublet=D or d, Triplet=T or t, quarter=Q or q, multiplet=m, and broad=br. Capital letters refer to the pattern resulting from directly bonded coupling ($^1J_{C,H}$). Optical rotations were measured with a JASCO DIP-181 spectrometer. CD curves were determined on a JASCO J-40 spectrophotometer. Amino acid analyses were performed on a JEOL JLC-5AH automatic amino acid analyzer equipped with a Shimadzu Chromatopac C-RIB, on a Nihon Seimitsu NSLC-100 HPLC, and/or Merck HPTLC precoated cellulose plates.

Isolation of Cycloaspeptides A (1), B (2), and C (3)——*Aspergillus* sp., strain NE-45, was cultivated at 27 °C for 14—17 d in Czapek–Dox medium using 300 Roux flasks containing 250 ml of the above medium in each flask. The dried mycelia (980 g) were pulverized, defatted with hexane, and extracted with chloroform at room temperature. The evaporated residue (19 g) was chromatographed on silica gel successively with benzene–acetone (10:1) to obtain ergosterol (180 mg), with benzene–acetone (5:1) to give cycloaspeptide A (**1**) (700 mg), with benzene–acetone (3:1) to obtain cycloaspeptide B (**2**) (36 mg), and finally with benzene–acetone (2:1) to afford cycloaspeptide C (**3**) (27 mg).

Cycloaspeptide A (**1**): Colorless needles with violet fluorescence under UV, mp 270—272 °C from MeOH. $[\alpha]_D^{20}$ −228° (c=1.01, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH, NH), 1680, 1650 (amide), 1630, 760. UV λ_{max}^{EtOH} nm (log ε): 223 (4.62), 242 (4.29), 248 (4.29), 254 (4.37), 260 (4.30), 286 (3.65), 308 (3.76). EI-MS m/z(%): 641 (M^+, 6), 497 (14), 352 (11), 351 (10), 285 (51), 184 (80), 134 (98), 120 (100), 113 (40), 107 (73), 91 (55), 44 (58). FD-MS m/z (%): 680 [$(M+K)^+$, 11], 664 [$(M+Na)^+$, 56], 641 (M^+, 100). *Anal.* Calcd for $C_{36}H_{43}N_5O_6 \cdot H_2O$: C, 65.53; H, 6.88; N, 10.62. Found: C, 65.67; H, 6.73; N, 10.34. ^{1}H-NMR ($CDCl_3$) δ: 0.410 (3H, d, J=6.6 Hz), 1.002 (3H, d, J=6.6 Hz), 1.009 (3H, d, J=6.6 Hz), 1.376 (1H, ddd, J=13.0, 8.0, 5.5 Hz), 1.715 (1H, m), 1.883 (1H, ddd, J=13.0, 8.5, 5.5 Hz), 2.693 (3H, s, NMe), 2.889 (3H, s, NMe), 2.978 (1H, dd, J=14.7, 12.0 Hz), 3.368 (1H, dd, J=14.0, 11.3 Hz), 3.485 (1H, dd, J=14.0, 4.3 Hz), 3.497 (1H, dd, J=14.7, 2.9 Hz), 3.777 (1H, dd, J=11.3, 4.3 Hz), 4.410 (1H, qd, J=6.6, 7.8 Hz), 4.715 (1H, ddd, J=8.5, 8.5, 5.5 Hz), 5.213 (1H, dd, J=12.0, 2.9 Hz), 6.034 (1H, s, OH), 6.793 (2H, d, J=8.3 Hz), 6.962 (1H, br dd, J=8.4, 8.0 Hz), 6.999 (2H, d, J=8.3 Hz), 7.032 (1H, d, J=7.8 Hz, –CONH–), 7.173 (2H, br d, J=8.0 Hz), 7.233 (1H, t-like), 7.321 (2H, t-like), 7.380 (1H, d, J=8.5 Hz, –CONH–), 7.429 (1H, dd, J=8.4, 2.0 Hz), 7.475 (1H, ddd, J=8.5, 8.0, 2.0 Hz), 8.859 (1H, dd, J=8.5, 1.7 Hz), 12.019 (1H, s, –CONH–). ^{13}C-NMR ($CDCl_3$) δ: 16.02 (Q), 21.84 (Q), 23.26 (Q), 24.76 (D), 30.02 (Q, NMe), 31.98 (T), 33.97 (T), 39.04 (Q, NMe), 41.12 (T), 44.15 (D), 48.84 (D), 63.29 (D), 69.94 (D), 114.90 (S), 115.63 (D×2), 120.72 (D), 122.18 (D), 126.83 (D), 127.14 (D), 129.07 (D×2), 129.28 (D×2), 130.19 (D×2), 130.23 (S), 134.22 (D), 137.46 (S), 141.25 (S), 154.90 (S), 168.03 (S, CON), 168.13 (S, CON), 169.51 (S, CON), 169.59 (S, CON), 174.13 (S, CON). CD (c=8.97×10^{-4}, MeOH) [θ]^{20} (nm): −9.14×10^3 (231), −4.46×10^3 (254), −3.79×10^3 (262), +0.45×10^3 (297).

Cycloaspeptide B (**2**): Colorless needles with violet fluorescence under UV, mp 250—252 °C from MeOH. $[\alpha]_D^{15}$ −138° (c=0.10, THF–DMF). IR ν_{max}^{KBr} cm^{-1}: 3480, 3360, 3280 (OH, NH), 1660, 1630 (amide). UV λ_{max}^{EtOH} nm (log ε): 222 (3.84), 257 (3.82), 278 (3.75). EI-MS m/z (%): 627 (M^+, 0.5), 497 (10), 368 (11), 271 (15), 134 (57), 120 (40), 113 (38), 107 (30), 91 (37), 44 (100). FD-MS m/z (%): 666 [$(M+K)^+$, 12], 650 [$(M+Na)^+$, 25], 627 (M^+, 100). *Anal.* Calcd

for $C_{35}H_{41}N_5O_6 \cdot 2H_2O$: C, 63.83; H, 6.83; N, 10.55. Found: C, 63.67; H, 6.89; N, 10.52. ^{1}H-NMR (Me_2SO-d_6) δ: 0.258 (3H, d, J=6.6 Hz), 0.749 (3H, d, J=6.6 Hz), 0.776 (3H, d, J=6.6 Hz), 1.023 (1H, m), 1.352 (2H, m), 2.797 (3H, s, NMe), 2.843 (1H, dd, J=14.0, 3.3 Hz), 2.857 (1H, dd, J=14.2, 12.1 Hz), 3.320 (1H, dd, J=14.2, 3.7 Hz), 3.330 (1H, dd, J=14.0, 2.9 Hz), 4.334 (2H, m), 4.694 (1H, dd, J=10.6, 4.0 Hz), 5.222 (1H, dd, J=12.1, 3.7 Hz), 6.663 (2H, d, J=8.4 Hz), 7.011 (2H, d, J=8.4 Hz), 7.155 (1H, ddd, J=7.9, 7.4, 1.1 Hz), 7.289 (5H, m), 7.558 (1H, ddd, J=8.4, 7.4, 1.4 Hz), 7.883 (1H, dd, J=7.9, 1.4 Hz), 8.070 (1H, br d, J=8.5 Hz, –CONH–), 8.450 (1H, br d, J=8.9 Hz, –CONH–), 8.660 (1H, br d, J=8.4 Hz), 8.713 (1H, br d, J=3.0 Hz, –CONH–), 9.373 (1H, s, OH), 11.877 (1H, s, –CONH–).

Cycloaspeptide C (**3**): Colorless needles with violet fluorescence under UV, mp 281—283 °C from MeOH. $[\alpha]_D^{15}$ −168° (c=0.09, THF–DMF). IR ν_{max}^{KBr} cm^{-1}: 3350, 3240 (OH, NH), 1665, 1630 (amide). UV λ_{max}^{EtOH} nm (log ε): 222 (4.27), 257 (4.17), 263 sh (4.16), 278 sh (4.01). FD-MS m/z (%): 628 [(M+H)$^+$, 100], 627 (M$^+$, 53). *Anal.* Calcd for $C_{35}H_{41}N_5O_6 \cdot 11/3H_2O$: C, 64.68; H, 6.75; N, 10.75. Found: C, 64.49; H, 6.45; N, 10.62. ^{1}H-NMR (Me_2SO-d_6) δ: 0.866 (3H, d, J=6.7 Hz), 0.902 (3H, d, J=6.4 Hz), 1.079 (3H, d, J=7.2 Hz), 1.179 (1H, ddd, J=13.3, 7.8, 5.7 Hz), 1.511 (1H, m), 1.694 (1H, ddd, J=13.3, 7.8, 5.7 Hz), 2.552 (3H, s, NMe), 2.806 (1H, dd, J=12.7, 11.5 Hz), 2.918 (1H, dd, J=12.7, 4.4 Hz), 3.086 (1H, m), 3.330 (1H, m), 3.810 (1H, qd, J=7.2, 4.8 Hz), 4.127 (1H, dd, J=9.3, 5.8 Hz), 4.345 (1H, ddd, J=8.5, 7.8, 5.7 Hz), 4.418 (1H, ddd, J=11.5, 10.5, 4.4 Hz), 6.666 (2H, d, J=8.2 Hz), 6.934 (2H, d, J=8.2 Hz), 7.113 (1H, br t, J=7.8 Hz), 7.139 (2H, br d, J=7.8 Hz), 7.196 (1H, br t, J=7.8 Hz), 7.258 (2H, br t, J=7.8 Hz), 7.527 (1H, br dd, J=8.5, 7.8 Hz), 7.773 (1H, d, J=10.5 Hz, –CONH–), 7.897 (1H, br d, J=7.8 Hz), 8.224 (1H, d, J=8.5 Hz, –CONH–), 8.802 (1H, br d, J=8.5 Hz), 8.842 (1H, d, J=4.8 Hz, –CONH–), 9.232 (1H, s, OH), 11.424 (1H, s, –CONH–).

Acetylation of 1——Compound **1** (50 mg) was dissolved in pyridine (0.5 ml) containing acetic anhydride (1.0 ml), and the solution was kept overnight at room temperature. Then the reaction mixture was poured into ice-water and extracted with chloroform. The evaporated extract was chromatographed on silica gel with benzene–acetone (10 : 1) to give a monoacetate (**4**) (34 mg). Colorless needles with violet fluorescence under UV, mp 178—180 °C from MeOH. $[\alpha]_D^{15}$ −138° (c=0.10, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500, 3300 (NH), 1760 (COO), 1660, 1630 (amide). UV λ_{max}^{EtOH} nm (log ε): 257 (4.39), 264 sh (4.37), 282 (4.12). EI-MS m/z (%): 683 (M$^+$, 12), 540 (12), 421 (7), 352 (15), 315 (14), 308 (7), 192 (26), 189 (23), 134 (100), 120 (32). CI-MS m/z (%): 684 [(M+1)$^+$, 20], 195 (100). *Anal.* Calcd for $C_{38}H_{45}N_5O_7 \cdot CH_3OH$: C, 65.43; H, 6.90; N, 9.78. Found: C, 65.66; H, 6.75; N, 9.85. ^{1}H-NMR (Me_2SO-d_6) δ: 0.233 (3H, d, J=6.6 Hz), 0.879 (3H, d, J=6.6 Hz), 0.908 (3H, d, J=6.6 Hz), 1.275 (1H, m), 1.531 (1H, m), 1.668 (1H, m), 2.266 (3H, s, OAc), 2.659 (3H, s, NMe), 2.688 (3H, s, NMe), 2.860 (1H, dd, J=14.4, 12.0 Hz), 3.20—3.42 (3H, m), 4.283 (1H, m), 4.445 (2H, m), 5.306 (1H, dd, J=11.5, 2.9 Hz), 7.061 (2H, d, J=8.5 Hz), 7.065 (1H, m), 7.202 (2H, d, J=8.5 Hz), 7.275 (5H, m), 7.514 (1H, br dd, J=8.4, 7.3 Hz), 7.979 (1H, br d, J=7.3 Hz), 8.763 (1H, d, J=8.4 Hz), 8.770 (1H, d, J=8.4 Hz, –CONH–), 8.839 (1H, d, J=5.1 Hz, –CONH–), 11.952 (1H, s, –CONH–).

Permethylation of 1——Compound **1** (50 mg) was dissolved in anhydrous THF (10 ml) and added to a suspension of sodium hydride (300 mg) in anhydrous THF (10 ml). The mixture was stirred at room temperature for 3 h. After addition of methyl iodide (5 ml), the mixture was further stirred at room temperature for 24 h, and the solvent was evaporated off. The residue was chromatographed on silica gel with chloroform–methanol (50 : 1) to give a penta-*N*-methyl-*O*-methyl derivative (**5**) (27 mg). Colorless crystalline powder, mp 166—168 °C from *n*-hexane. IR ν_{max}^{KBr} cm^{-1}: 1650 sh, 1630 (amide). EI-MS m/z (%): 697.3825 (M$^+$, 24) (697.3837 Calcd for $C_{40}H_{51}N_5O_6$), 596 (58), 563 (23), 507 (17), 449 (16), 379 (32), 296 (31), 219 (48), 134 (70), 111 (100). ^{1}H-NMR ($CDCl_3$) δ: 0.663 (3H, d, J=6.7 Hz), 0.809 (3H, d, J=6.6 Hz), 0.819 (3H, d, J=6.6 Hz), 1.314 (1H, m), 1.825 (2H, m), 2.567 (1H, m), 2.590 (3H, s, NMe), 2.616 (3H, s, NMe), 2.812 (1H, m), 2.818 (3H, s, NMe), 3.075 (3H, s, NMe), 3.095 (2H, m), 3.621 (3H, s, NMe), 3.725 (1H, m), 3.786 (3H, s, OMe), 4.583 (1H, q, J=6.7 Hz), 4.920 (1H, dd, J=11.7, 2.3 Hz), 5.236 (1H, dd, J=11.0, 3.4 Hz), 5.582 (1H, dd, J=6.3, 2.1 Hz), 6.903 (2H, d, J=8.7 Hz), 7.227 (2H, d, J=8.7 Hz), 7.20—7.37 (7H, m), 7.473 (1H, br t, J=8.1 Hz), 8.964 (1H, br d, J=8.1 Hz).

Permethylation of 2 and 3——Compounds **2** (5 mg) and **3** (5 mg) were methylated with sodium hydride and methyl iodide in the same manner as described above to give a permethylate (**5**) (3 mg and 2.5 mg, respectively). The products were identical with that derived from **1** (TLC and IR comparison, and mixed melting point determination).

Complete Acid Hydrolysis of 1, 2, and 3——The compound (**1**, **2**, or **3**) (1 mg each) was hydrolyzed with 5.7 N HCl (0.3 ml) at 110 °C for 24 h in a sealed tube. The hydrolysate was analyzed on cellulose TLC using the solvent system of *n*-butanol–acetic acid–pyridine–water (3 : 3 : 3 : 1). Detection was done with ninhydrin reagent, by using an amino acid analyzer, and/or by HPLC system which consisted of a 70 × 2.1-mm guard column packed with Co:Pell ODS (Whatman Inc., Clifton, N.J.) in series with a 100 × 4.6-mm Spherisorb ODS-2 column (particle size: 3 μm, Senshu Scientific Co., Tokyo) using the solvent system of acetonitrile–0.1% trifluoroacetic acid (22 : 78) at a flow rate of 1 ml/min, with monitering at 254 nm. The results are summarized in Table I. ABA was also detected by silica gel TLC using the solvent system of chloroform–methanol (10 : 1).

Partial Acid Hydrolysis of 1——Compound **1** (10 mg) was hydrolyzed with a mixture of 12 N HCl (0.3 ml) and acetic acid (0.2 ml) at 27 °C for 48 h in a sealed tube. The hydrolyzed mixture was separated by preparative cellulose TLC with *n*-butanol–acetic acid–pyridine–water (3 : 3 : 3 : 1) to afford the fragment peptides IA, IB, IC, and ID. These peptide fragments were hydrolyzed with 5.7 N HCl at 110 °C by the same procedure as described above, and the hydrolysates were analyzed by cellulose TLC, amino acid analyzer, and/or HPLC. The results are summarized in

Table II.

Methylation of Fragment Peptides IC and ID——An ether solution of diazomethane was added to a methanol solution of peptide IC or ID. The solvent was removed by evaporation, and the reaction mixture was purified by HPLC. The MS of purified peptides IC and ID were measured.

Peptide IC: EI-MS *m/z* (%): 383 (M^+, 12), 352 (0.5), 221 (6), 220 (1.4), 194 (3), 191 (7), 190 (5), 163 (32), 135 (1.4), 134 (11), 120 (100), 92 (12).

Peptide ID: EI-MS *m/z* (%): 496 (1.3), 425 (4), 352 (1.5), 351 (3), 263 (9), 233 (11), 221 (13), 220 (3), 201 (9), 192 (6), 191 (5), 163 (18), 162 (100), 147 (13), 135 (4), 134 (34), 120 (46), 92 (15), 91 (11), 44 (40).

2,4-Dinitrophenylation of Amino Acids——The DNP-derivatives were synthesized by the procedure of Sanger,[6] and purified by column chromatography using chloroform–methanol–acetic acid (95 : 5 : 1). The CD curve of each DNP-amino acid between 280—430 nm was measured. CD (MeOH) $[\theta]^{20}$ (nm): DNP-L-Ala: −2500 (328), +2100 (390). DNP-Me-L-Phe: +13400 (316), +12800 (404). DNP-L-Leu: −2000 (328), +1800 (385). DNP-Me-L-Tyr: +18300 (320), +11500 (400). DNP-Ala from **1**: −2800 (328), +1900 (390). DNP-MePhe from **1**: +14600 (318), +12300 (402). DNP-Leu from **1**: −1700 (324), +1800 (384). DNP-MeTyr from **1**: +19700 (320), +11600 (397).

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