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Fungal Metabolites. I. Isolation and Biological Activities of Hypelcins A and B (Growth Inhibitors against *Lentinus edodes*) from *Hypocrea peltata*[1)]

TETSURO FUJITA,*,[a] YOSHIHISA TAKAISHI,[a] HIDEKI MORITOKI,[a] TADASHI OGAWA,[b] and KYOUSUKE TOKIMOTO[c]

Faculty of Pharmaceutical Sciences, The University of Tokushima,[a] Shomachi, Tokushima 770, Japan, Department of Nutrition, School of Medicine, The University of Tokushima,[b] Kuramoto-cho, Tokushima 770, Japan, and The Tottori Mycological Institute,[c] Tottori 689–11, Japan

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Two new peptide antibiotics, hypelcins A and B, were isolated from *Hypocrea peltata*, which is antagonistic to *Lentinus edodes*. Hypelcins A and B are linear peptides which include nine or ten residues of the abnormal amino acid α-aminoisobutyric acid (Aib) and an amino alcohol, L-leucinol. Hypelcin A was confirmed to be an amide of hypelcin B by chemical conversion. Hypelcin A showed antifungal and antibacterial activities, and contractile action on guinea pig ileum.

Keywords——peptide antibiotic; α-aminoisobutyric acid; leucinol; hypelcin; *Hypocrea peltata*; *Lentinus edodes*; ileal contraction

Lentinus edodes (BERK.) SING. (Japanese name: Shiitake), the most important edible mushroom in Japan, is injured by many different wood-decaying fungi during its culture on the usual bed-logs (chiefly *Quercus* spp.). Some species of fungi, such as *Hypocrea*, *Trichoderma* and *Gliocladium*, are especially antagonistic to *L. edodes*.[2)] In 1974, *Hypocrea peltata* (JUNGH.) SACC. caused much damage to *L. edodes* cultivated in Tokushima Prefecture. We here report on the isolation and biological activities of the active substances, peptide antibiotics, hypelcins A and B, which caused the damage to *L. edodes*.

Fractionation of the methanolic extract of the dried stroma (Chart 1) based on the results of assay against *L. edodes*, revealed that crystalline fraction (fr.) 6 showed the greatest inhibitory activity. When the growth of *L. edodes* mycelia was inhibited by fr. 6, the color of the mycelia changed from white to brown on potato glucose agar plates. The same phenomenon on *vis-a-vis* culture[3)] of *L. edodes* with *H. peltata* was observed at the contact point of both mycelia on the plate. Further fractionation of crystalline fr. 6 by column chromatography (silica gel and Sephadex LH-20) resulted in the isolation of two new peptide antibiotics, hypelcin A as crystals (mp 283—286 °C) from dichloromethane and hypelcin B (mp 189—192 °C) as an amorphous solid, both of which were homogenous on silica gel thin layer chromatography (TLC) (Rf_1: hypelcin A 0.35, hypelcin B 0.18).

The antimicrobial and antifungal activities of hypelcins A and B are summarized in Table I. Hypelcins were found to be ineffective in inhibiting the growth of the following organisms at concentrations up to 100 μg/ml: *Escherichia coli, Salmonella typhosa, Shigella flexneri, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans, Penicillium chrysogenum, Aspergillus niger*, and *Mycobacterium* sp. Hypelcin A has growth-inhibitory activity against some fungi and gram-positive bacteria. Hypelcin B has weaker activity against these

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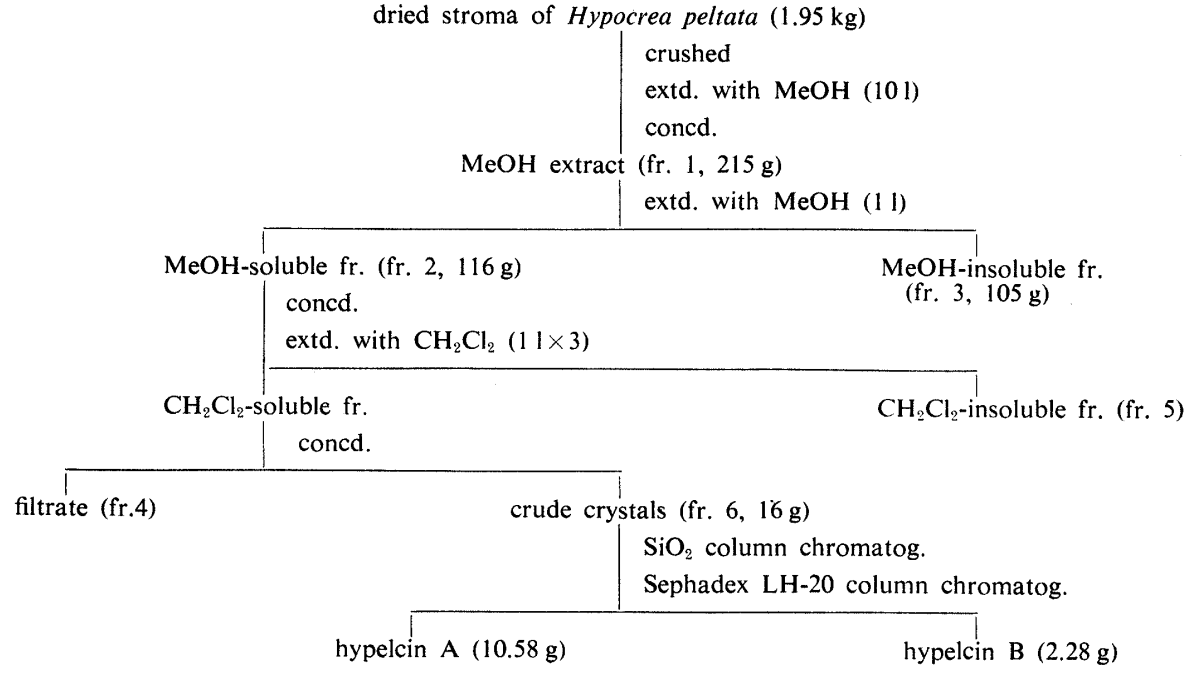

Chart 1

organisms than hypelcin A. The minimum inhibitory concentration (MIC) of hypelcins for *L. edodes* was 300 μg/ml as determined by the paper disk method. We recently reported[4] that hypelcins are unique uncouplers of oxidative phosphorylation in mitochondria of rat liver. Peptide antibiotics, trichopolyns,[5] which were isolated by us from *Trichoderma polysporum* and are antagonistic to *L. edodes*, also have uncoupling activity in mitochondria. Hence, growth inhibition by these peptides, hypelcins and trichopolyns, towards *L. edodes* was tested in comparison with those of uncouplers, 2,4-dinitrophenol,[6] and SF-6847,[7] and oligomycin,[8] an inhibitor of ATP formation in mitochondria. SF-6847 and oligomycin were found to be ineffective at concentrations up to 125 μg/ml. The minimum concentrations of hypelcins and trichopolyns needed to attain maximum effect on state 4 mitochondria are similar (hypelcins 0.2—0.5 μM, trichopolyns 0.5 μM[9]). The ED_{50}s of hypelcins were ten times those of the trichopolyns. These results suggest that uncoupling activity in rat liver mitochondria does not reflect the inhibitory activity of these peptides towards *L. edodes*.

It is well known that a peptide antibiotic with high content of α-aminoisobutyric acid (Aib) induces a potential-dependent ionic conductance from the effects observed on the synaptic transmission of nerve signals.[10] It was thus of interest to study the pharmacological action of the present compounds on several smooth muscle preparations. Hypelcin A produced dose-dependent contractions of guinea-pig ileum[11] when added cumulatively at concentrations of above 3×10^{-6} g/ml. The contractions developed rapidly and were sometimes followed by spontaneous wave-like movement. The contractions produced by 4×10^{-5} g/ml of hypelcin A corresponded to those produced by 10^{-5} g/ml of X537A[12] or 3×10^{-7} g/ml of acetylcholine. The contractile action of hypelcin A was suppressed by atropine (10^{-8} M) at a concentration sufficient to inhibit the action of acetylcholine. The contractile action of X537A (used as a reference compound) was similarly inhibited by atropine. Trichopolyn B also produced contractions at concentrations above 3×10^{-6} g/ml, the effect being comparable to that produced by 3×10^{-8} M acetylcholine. Sixty min after the last application of trichopolyn B or hypelcin A, the maximal contraction induced by acetylcholine was suppressed to less than 50%. Thereafter the ileum gradually recovered its sensitivity to acetylcholine.

Similarities in spectral data and color reactions of hypelcins A and B indicated a close

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TABLE I. Antibacterial and Antifungal Activity Spectra of Hypelcins A and B

Organisms	MIC (μg/ml)		
	Hypelcin A	Hepelcin B	Condition
Staphylococcus aureus FDA 209P	50	>100	A
Staphylococcus aureus 308A-1	50	>100	A
Bacillus subtilis 1840	25	>100	A
Bacillus cereus 219	50	100	A
Sarcina lutea PCI 1001	25	100	A
Micrococcus flavus IFO 3242	25	100	A
Trichophyton mentagrophytes IFO 7522	100	>100	B
Trichophyton rubrum IFO 5467	100	>100	B
Mycobacterium phlei IFO 3158	25	100	B

A: trypticase soy agar (BBL) medium with incubation at 37 °C for 18 h.
B: 1% glucose bouillon agar medium with incubation at 28 °C for 40 h.

TABLE II. Molar Ratios of Amino Acids in Hypelcins A and B

	Hypelcin A		Hypelcin B	
	Amino acid[a] analyzer	IP[b]	Amino acid analyzer	IP[c]
Glu	3.05±0.06	3.00	3.09	3.00
Pro	2.04±0.05		2.02	
Gly	0.95±0.03		0.92	
Ala	1.20±0.04		1.15	
Aib	9.77±0.33	9.66±0.21	9.70	9.69±0.15
Val	1.00		1.00	
Leu	0.78±0.03		0.76	
Ile	0.14±0.02		0.15	

The values are means±S.D. *a*) $n=4$, *b*) $n=7$, and *c*) $n=3$.

structural relationship. Both infrared (IR) spectra showed peptide bands (1660 and 1540—1530 cm^{-1}). The proton nuclear magnetic resonance (^{1}H-NMR) spectra suggested the presence of an acetyl group (δ 2.28, s). The ultraviolet (UV) spectra above 210 nm showed no absorption maximum. There was a negative ninhydrin reaction, indicating that hypelcins have no free amino group. Amino acids of the complete acid hydrolysates (6 N HCl, 110 °C, 24 h) of both hypelcins were identified as Aib, Ala, Val, Gly, Leu, Pro, and Glu[13] by cellulose TLC, gas liquid chromatography (GC), and GC-mass spectrometry (MS). The results of amino acid analysis and isotachophoresis (IP)[14] of the hydrolysate of hypelcins are shown in Table II. These data imply that our samples of hypelcins are a mixture of two or more forms, like alamethicins[15] and trichotoxins,[16] which are peptides containing large amounts of Aib. The predominant form of hypelcins contains $(Glu)_3$, $(Pro)_2$, $(Gly)_1$, $(Ala)_1$, $(Aib)_{9-10}$, $(Val)_1$, and $(Leu)_1$, whereas in the minor forms Aib and Leu residues are probably replaced by Ala and Ile. The samples we employed were not separated into individual components and the present work was carried out on the mixtures. Hypelcin A contained three $CONH_2$ groups while hypelcin B contained two $CONH_2$ groups (on hydrolysis to ammonia) as determined by the Conway microdiffusion method.[17]

A peak at a retention time of 8.9 min in the GC of the *N*-trifluoroacetyl (TFA) butyl ester

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and the *N*-TFA isopropyl ester of hypelcin A hydrolysate was also observed in that of the hydrolysate of hypelcin B. Paper electrophoresis (PE) (pH 4.7) of the hydrolysates of hypelcins A and B showed a ninhydrin-positive spot which moved faster to the cathode than did known amino acids. This component was prepared by PE from hypelcin A hydrolysate and trifluoroacetylated. The TFA derivative showed the same retention time of 8.9 min in GC. The MS of the peak suggested it to be *N,O*-diTFA leucinol. An ethereal extract of the hydrolysate of hypelcin A yielded L-leucinol, mp 66—70 °C, which was identical with an authentic sample.

The absolute configurations of the amino acids and amino alcohol were determined as L-form by a combination of methods using GC,[18] L-Leu-*N*-carboxyanhydride,[19] and D-amino acid oxidase.[20]

Hypelcins A and B were acetylated to give their acetates. Hypelcin B gave a methyl ester on treatment with diazomethane, but hypelcin A was recovered unchanged. The methyl ester of hypelcin B gave the methyl ester acetate on pyridine–acetic anhydride treatment. The same methyl ester acetate of hypelcin B was also obtained by methylation of the acetate. Amidation of the methyl ester of hypelcin B gave the amide, mp 283—286 °C (amide N: 3), which was identical with hypelcin A (*Rf* value, mixed mp, IR, and ^{1}H-NMR). These results indicate that hypelcin A is an amide of hypelcin B. In addition to methylation, dansylation and dinitrofluorophenylation of hypelcin A resulted in recovery of the starting material, and hydrazinolysis gave no amino acids, suggesting that hypelcin A contains neither amino nor carboxy groups.

The presence of an acetyl group, an esterifyable hydroxy group, and three primary amide groups in hypelcin A and the conversion of hypelcin B into hypelcin A suggest that (i) hypelcins are not cyclic but linear; (ii) the terminal N is blocked by an acetyl group; and (iii) the C-terminal group is linked with L-leucinol as an amide.

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were obtained on a JEOL PS-100 spectrometer. Chemical shifts (δ) are reported in ppm from internal tetramethylsilane. IR spectra were measured on a Hitachi EPI-G_2 spectrometer, GC-MS were obtained with a Shimadzu LKB-9000 instrument, UV spectra were taken on a Hitachi 124 spectrometer, GC were obtained with a Shimadzu GC-6A and GC-4BM, IP was done on a Shimadzu IP-1B, and amino acid autoanalysis was done with JEOL JIC-6AH and Yanagimoto LC-5S instruments. Column chromatography was carried out on Silica gel 60 (0.063—0.20 mm, Merck) and Sephadex LH-20 (Pharmacia). Thin layer chromatography was performed on silica gel [PF_{254}, Merck; Rf_1 refers to MeOH–acetone–CH_2Cl_2 (1 : 1 : 3 v/v)] and on cellulose [Avicel, Funakoshi; Rf_2 refers to BuOH–AcOH–H_2O (3 : 1 : 1 v/v)].

Isolation of Hypelcins A and B——The dried and crushed stroma (1.95 kg) of *Hypocrea peltata* collected in Tokushima city were extracted with MeOH (10 l) at room temperature for 30 d. The methanolic solution was concentrated to yield a brown oily residue (219 g), which was triturated in MeOH (1 l) and separated into soluble and insoluble fractions in MeOH. The fraction soluble in MeOH was concentrated to give a brown oily residue (116 g), which was separated into soluble and insoluble fractions in CH_2Cl_2. The fraction soluble in CH_2Cl_2 was concentrated to obtain a mixture, fr. 6 (16 g), as colorless crystals. Fraction 6 was subjected to silica gel (300 g) column chromatography and eluted with CH_2Cl_2–acetone–MeOH (70 : 15 : 15—50 : 15 : 15, v/v) followed by acetone. The eluates with CH_2Cl_2–acetone–MeOH were combined, concentrated and chromatographed on a Sephadex LH-20 column (MeOH). The eluates which showed the same Rf_1 values on TLC were collected, combined and concentrated to afford hypelcin A (10.58 g) as needles (from CH_2Cl_2) Rf_1: 0.35, mp 283—286 °C, $[\alpha]_D^{28}$ −9.75° (c = 1.0, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3320, 1660, 1530, 1460, 1380. UV λ_{max}^{MeOH} nm: no maximum absorption above 210. ^{1}H-NMR (C_5D_5N) δ: 2.28 (s, CH_3CO–). Ninhydrin reaction: negative. Dragendorff test: positive. Hypelcin A with the composition $(Gln)_3(Pro)_2(Gly)_1(Ala)_1(Aib)_{10}(Val)_1(Leu)_1(leucinol)_1(acetyl)_1$ has the following theoretical elemental composition for $C_{89}H_{153}N_{23}O_{24}\cdot 5H_2O$: C, 52.95; H, 8.09; N, 15.96. Found: C, 53.05; H, 8.05; N, 16.05. Amide nitrogen: Calcd. 3, Found 2.83. On silica gel column chromatography, eluates with acetone were collected, combined, concentrated, and chromatographed on a Sephadex LH-20 column (MeOH) to afford hypelcin B (2.28 g) as an amorphous powder Rf_1 0.18, mp 189—192 °C, $[\alpha]_D^{28}$ −7.25° (c = 1.0, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3320, 1660, 1540, 1460, 1380. UV λ_{max}^{MeOH} nm: no

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maximum absorption above 210. ^{1}H-NMR (C_5D_5N) δ: 2.28 (s, C$\underline{H}_3$CO–). Ninhydrin reaction: negative. Dragendorff test: positive. Hypelcin B with the composition $(Gln)_2(Glu)_1(Pro)_2(Gly)_1(Ala)_1(Aib)_{10}(Val)_1(Leu)_1$-$(leucinol)_1(acetyl)_1$ has the following theoretical elemental composition for $C_{89}H_{152}N_{22}O_{25}\cdot 5H_2O$: C, 52.92; H, 8.08; N, 15.25. Found: C, 53.22; H, 7.85; N, 15.08. Amide nitrogen: Calcd. 2, Found 1.84.

Bioassay for Isolated Fractions——Potato agar medium (10 ml) was poured into a Petri dish (90 mm in a diameter). After the agar surface had dried, *L. edodes* was inoculated into the center of the Petri dish. During precultivation at 23 °C for 7 d, *L. edodes* grew to about 20 mm in diameter. Paper disks (8 mm in a diameter) containing a given amount of each sample were placed on the agar plate. After cultivation at 23 °C for 7 d, the minimum concentration at which no growth occurred was regarded as the minimum inhibitory concentration (MIC). MIC (ppm): fr. 1 (2000), fr. 2 (3000), fr. 3 (1000), fr. 4 (1000), fr. 5 (2000), fr. 6 (300), hypelcin A (300), hypelcin B (300).

Inhibitory Activity against *L. edodes* of Mitochondrial Uncouplers and an Inhibitor——*L. edodes* was incubated in a glucose medium containing a given amount of each sample at 25 °C in the dark for 15 d. After the incubation the mycelia were obtained by filtration, dried, and weighed. The inhibition ratios were calculated as [mycelia weight of challenge]/[mycelia weight of control] (percent). ED_{50} (μg/ml): hypelcin A (40), hypelcin B (46), trichopolyn A (3), trichopolyn B (4), 2,4-dinitrophenol (2), SF-6847 (>125), and oligomycin (>125).

General Method of Hydrolysis of Peptides with Acid——Hypelcins A and B were each heated in a sealed tube with 6 N HCl at 110 °C for 24—72 h. The hydrolysate was evaporated *in vacuo* to give a residue.

Amino Acid Constituents of Hypelcins A and B——1) A hydrolysate of hypelcin A was subjected to cellulose TLC to give spots of Ile, Val, Aib, Pro, Ala, Glu, and Gly (Rf_2: 0.78, 0.62, 0.52, 0.44, 0.39, 0.32, and 0.16). A hydrolysate of hypelcin B gave the same results. 2) Hydrolysates of hypelcins A and B were analyzed on an amino acid autoanalyzer (Table II). 3) Preparation of *N*-TFA butyl esters of amino acids and hydrolysates of hypelcins A and B for GC was carried out by the method described by Gehrke.[21] The chromatography was carried out under the following conditions: 3% silicon OV-225 chromosorb W(AW-DMCS), 1.5 m × 3 mm glass column; temperature program, (a) 0—18 min 130 °C isotherm, 18—35 min 5 °C/min for *N*-TFA butyl ester derivatives, (b) 0—16 min 130 °C isotherm, 16—35 min 5 °C/min for *N*-TFA isopropyl ester derivatives carrier flow, N_2 50 ml/min. The retention times (min) of *N*-TFA butyl esters derived from the hydrolysate of hypelcin A were: Aib (5.6), Ala (6.4), leucinol (Lol) (8.9), Gly (11.1), Leu (14.5), Pro (24.3), and Glu (34.6). The retention times (min) of *N*-TFA isopropyl esters derived from the hydrolysate of hypelcin A were: Aib (1.9), Ala (2.4), Val (3.2), Gly (4.4), Leu (5.2), Lol (8.9), Pro (14.4), and Glu (28.7). The same results were obtained after similar treatment of hypelcin B.

Determination of Amino Acid Ratio by IP——Hydrolysates of hypelcins A and B were analyzed by IP. The IP was carried out under the following conditions: constant current of 200 μA; leading electrolyte, 0.05 M 2-amino-2-methylpropanol, 0.5% methylcellulose, HCl (pH 9.0); terminal electrolyte, 0.1 M γ-aminobutyric acid, Ba $(OH)_2$ (pH 10.9). The molar ratio of Aib to Glu was determined by using the linear relationship between the ratio of zone length and the molar ratio of standard Aib to Glu (Table II).

Paper Electrophoresis of Hydrolysates of Hypelcins A and B——The hydrolysate of hypelcin A was analyzed by PE. The PE was carried out under the following conditions: buffer, AcOH–pyridine–H_2O = 1 : 1 : 100 (pH 4.7); paper, Toyo No. 51; 3000 V for 25 min; detection, 1% ninhydrin–acetone. Electrophoretic mobilities are indicated in cm from the origin towards the cathode (−) or anode (+). Leucinol, −10.5; Ala, Aib, Pro, Val and Leu, −1.5; Glu, +4.2. The same results were obtained in PE of hypelcin B hydrolysate.

GC and GC-MS of Leucinol from Hydrolysates of Hypelcins A and B——The hydrolysate of hypelcin A (20 mg) was subjected to preparative PE as described above to give crude leucinol (1 mg). The crude leucinol (1 mg), trifluoroacetic anhydride (0.5 ml), and CH_2Cl_2 (3 ml) were placed in a Pyrex culture tube which was capped with a Teflon-lined cap and allowed to stand at 110 °C for 1 h. The reaction mixture was evaporated to give the TFA derivative. The TFA derivative was analyzed by GC and GC-MS. The GC was carried out under the conditions described above. Retention time: 9 min. The GC-MS was carried out under the following conditions: 3% OV-225, 1.5 m × 3 mm, glass column; column temperature, 130 °C; carrier flow, He 50 ml/min. Retention time: 5.2 min. MS *m/z*: 240 ($M^+ - CF_3$), 196 ($M^+ - CF_3COO$), 182 ($M^+ - CF_3COOCH_2$), 140 ($M^+ - C_6H_8F_3O$), 139 ($M^+ - C_6H_9F_3O$). The same results were obtained after similar treatment of hypelcin B.

Isolation of L-Leucinol from a Hydrolysate of Hypelcin A——Hypelcin A (1 g) was hydrolyzed with 6 N HCl (20 ml) at 110 °C for 24 h. The reaction mixture was diluted with H_2O (30 ml), extracted with Et_2O, and basified with 1 N NaOH. The basified solution was extracted with Et_2O (40 ml × 5). The Et_2O layer was washed with brine, dried over $MgSO_4$ and evaporated to give an oil (45 mg). The oil was crystallized from Et_2O to give L-leucinol (34 mg) as needles, mp 66—70 °C. IR ν_{max}^{KBr} cm^{-1}: 3340, 3250, 1605, 1565, 1530, 1480, 1085. ^{1}H-NMR ($CDCl_3$) δ: 0.90, 0.92 [each 3H, d, J = 7 Hz, (C$\underline{H}_3$)$_2$CH–], 1.18 (2H, t, J = 7 Hz, >CH–C$\underline{H}_2$CH<), 1.70 [1H, m, $(CH_3)_2$C$\underline{H}$–]. *Anal.* Calcd for $C_6H_{11}NO$: C, 61.49; H, 12.90; N, 11.95. Found: C, 61.26; H, 12.98; N, 11.98. This compound was identical with synthetic L-leucinol[22] on direct comparison (TLC, IR, and mixed mp).

Determination of Absolute Configuration of Amino Acids and Amino Alcohol——1) GC Method: The derivatives (amino acid as *N*-TFA isopropyl ester, amino alcohol as *N,O*-diTFA) were obtained as described above. The GC was carried out under the following conditions: OA-201,[14] 20 m × 0.25 mm glass WCOT column; carrier flow, N_2 0.5 ml/

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min. The retention times (min) of *N*-TFA isopropyl esters derived from the hydrolysate of hypelcin A were: Aib (2.25), L-Ala (3.75), L-Val (4.30), Gly (5.50), L-Leu (6.15) at 110 °C, L-Lol (7.55) at 130 °C, and L-Glu (11.20) at 150 °C under isothermal conditions. The retention times (min) of reference compounds were: D-Ala (3.50), L-Ala (3.70), D-Val (4.05), L-Val (4.30), Gly (5.50), D-Leu (5.85), L-Leu (6.20) at 110 °C, L-Lol (7.55), D-Lol (7.75) at 130 °C, and D-Glu (10.60), L-Glu (11.30) at 150 °C under isothermal conditions.

2) L-Leucine-*N*-carboxyanhydride (L-Leu-NCA) Method: A hydrolysate of hypelcin A (20 mg) was subjected to preparative paper chromatography (PhOH–H_2O = 4 : 1, v/v) to give Pro. A mixture of Pro and L-Leu-NCA (10 mg) was vigorously stirred in 0.2 M Na-borate buffer (2 ml) at 0 °C for 2 min and then 6 N HCl (50 μl) was added. The reaction mixture was analyzed on an amino acid autoanalyzer (0.2 M Na-citrate buffer, pH 4.25, flow rate 80 ml/h, Aminex A-4 column). The retention time was 88 min. Retention times (min) of references were: L-Leu-D-Pro (78), L-Leu-L-Pro (90). The hydrolysate of hypelcin B (20 mg) was treated in a similar manner and gave a retention time of 88 min.

3) D-Amino Acid Oxidase (D-AOD) Method: The assay procedure used was as follows. A hydrolysate of hypelcin A (0.5 mg) was incubated in 0.2 M Tris-HCl buffer (0.5 ml, pH 8.3) containing D-AOD (2.0 mg), 10^{-5} M FAD (20 μl) and catalase (5 μg) at 37 °C for 2 h. After incubation, 1 N HCl (20 μl) was added to the reaction mixture. Amino acid analysis of an aliquot of the reaction mixture was carried out to confirm the disappearance of amino acids. L-Pro recovery yield: 98%. The hydrolysate of hypelcin B was treated in the same manner as described above. L-Pro recovery yield: 104%.

Hypelcin A Acetate——A solution of hypelcin A (20 mg) in pyridine (1 ml) and acetic anhydride (1 ml) was stirred at room temperature for 24 h, then MeOH (3 ml) was added to the reaction mixture. The solvent was evaporated off *in vacuo* to give hypelcin A acetate (17 mg) as needles (from hexane–CH_2Cl_2), mp 254—256 °C. IR ν_{max}^{KBr} cm^{-1}: 3320, 1740, 1660, 1540, 1380. ^{1}H-NMR (C_5D_5N) δ: 2.18 (s, C$\underline{H}_3$CO–), 2.32 (s, C$\underline{H}_3$CO–). *Anal.* Calcd for $C_{91}H_{155}N_{23}O_{25}\cdot 5H_2O$: C, 53.02; H, 8.07; N, 15.63. Found: C, 52.93; H, 7.79; N, 15.44.

Hypelcin B Acetate——A solution of hypelcin B (60 mg) in pyridine (1 ml) and acetic anhydride (1 ml) was stirred at room temperature for 24 h. The reaction mixture was poured into ice water, acidified with 5% HCl, and extracted with EtOAc (70 ml × 2). The EtOAc layer was washed with brine, dried over Na_2SO_4, and evaporated to give a residue, which was crystallized from EtOAc to give hypelcin B acetate (46 mg) as colorless granules, mp 269—271 °C. IR ν_{max}^{KBr} cm^{-1}: 3320, 1740, 1660, 1530, 1460, 1380. ^{1}H-NMR (C_5D_5N) δ: 2.11 (s, C$\underline{H}_3$CO–), 2.24 (s, C$\underline{H}_3$CO–). *Anal.* Calcd for $C_{91}H_{154}N_{22}O_{26}\cdot 3H_2O$: C, 53.93; H, 7.96; N, 15.21. Found: C, 54.02; H, 7.72; N, 14.92.

Hypelcin B Methyl Ester——A solution of hypelcin B (50 mg) in MeOH was treated with diazomethane–ether and the reaction mixture was left overnight at room temperature, then evaporated at room temperature to give a white powder (52 mg), which showed two spots on TLC. The powder was separated on preparative silica gel TLC (CH_2Cl_2–acetone–MeOH = 4 : 1 : 1, v/v) to give a main product. The main product was crystallized from CH_2Cl_2 to give hypelcin B methyl ester (32 mg) as needles, mp 255—257 °C. IR ν_{max}^{KBr} cm^{-1}: 3300, 1740, 1650, 1530, 1460, 1380, 1360. ^{1}H-NMR (C_5D_5N) δ: 2.23 (s, C$\underline{H}_3$CO–), 3.51 (s, C$\underline{H}_3$OCO–). *Anal.* Calcd for $C_{90}H_{154}N_{22}O_{25}\cdot 4H_2O$: C, 53.61; H, 8.10; N, 15.21. Found: C, 53.71; H, 7.87; N, 15.01.

Hypelcin B Acetyl Methyl Ester——1) A solution of hypelcin B methyl ester (50 mg) in pyridine (1 ml) and acetic anhydride (1 ml) was stirred at room temperature for 24 h. The reaction mixture was poured into ice water, acidified with 5% HCl, and extracted with EtOAc (70 ml × 2). The EtOAc layer was washed with brine, dried over Na_2SO_4, and evaporated to give a residue, which was crystallized from EtOAc to give hypelcin B acetyl methyl ester (43 mg) as colorless needles, mp 251—253 °C. IR ν_{max}^{KBr} cm^{-1}: 3330, 1740, 1660, 1530, 1460, 1380, 1360, 1220. ^{1}H-NMR (C_5D_5N) δ: 2.10 (s, C$\underline{H}_3$CO–), 2.21 (s, C$\underline{H}_3$CO–), 3.52 (s, C$\underline{H}_3$OCO–). *Anal.* Calcd for $C_{92}H_{156}N_{22}O_{26}\cdot 4H_2O$: C, 53.68; H, 8.03; N, 14.97. Found: C, 53.89; H, 8.12; N, 14.86.

2) A solution of hypelcin B acetate (5 mg) in MeOH (1 ml) was treated with diazomethane–ether. The reaction mixture was left overnight at room temperature, then evaporated, and the residue was crystallized from EtOAc to give the ester (4 mg) as colorless needles. This ester was identical with the above hypelcin B acetyl methyl ester.

Conversion of Hypelcin B Methyl Ester to Hypelcin A——A mixture of hypelcin B methyl ester (70 mg), MeOH (0.5 ml) and liquid ammonia (7 ml) was placed in a Pyrex culture tube at −50 °C. The tube was capped with a Teflon-lined cap and allowed to stand at −50 °C for 1 h and at room temperature for one week. The reaction mixture was then evaporated *in vacuo* to give a residue (72 mg), which was purified by silica gel (2 g) column chromatography to give an amide (32 mg) as needles (from CH_2Cl_2), mp 283—286 °C. IR ν_{max}^{KBr} cm^{-1}: 3320, 1660, 1530. ^{1}H-NMR (C_5D_5N) δ: 2.28 (s, C$\underline{H}_3$CO–). *Anal.* Calcd for $C_{89}H_{153}N_{23}O_{24}\cdot 5H_2O$: C, 52.95; H, 8.09; N, 15.96. Found: C, 53.02; H, 8.01; N, 16.00. Amide nitrogen: Calcd. 3, Found: 2.78. The amide was identical with hypelcin A (TLC, IR, ^{1}H-NMR and mixed mp).

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