Fungal Metabolites. XVIII.[1,2] New Membrane-Modifying Peptides, Trichorozins I—IV, from the Fungus *Trichoderma harzianum*

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New membrane-modifying peptides, trichorozins I—IV, have been isolated from conidia of the fungus *Trichoderma harzianum*. Their amino acid sequences were clearly determined by spectrometric methods and, furthermore, they were synthesized by the solution-phase method. Trichorozins are a family of the class of peptaibols and are composed of 11 residues including an amino alcohol. Trichorozins exhibited voltage-dependent ion channel-like activity in lipid bilayers.

Key words *Trichoderma harzianum*; peptaibol; amino alcohol; α-aminoisobutyric acid; ion channel; trichorozin

Membrane-modifying fungal metabolites, peptaibols, provide insights into the structural principles of ion channel proteins, since they form voltage-gated ion channels in lipid bilayers.[3] Peptaibols are peptides composed of up to 20 residues and characterized by an acylated *N*-terminus, the presence of an amino alcohol and a high proportion of α-aminoisobutyric acid (Aib). Such structural properties give peptaibols an amphiphilic helix-favoring character,[4] which might make it easy for the peptides to aggregate in membranes and to form ion channels. Peptaibols such as alamethicin and trichosporins, for example, induce catecholamine secretion[5] from adrenal chromaffin cells and uncouple oxidative phosphorylation in mitochondria.[6] These activities are actually due to ion influx into the cells.

In the previous paper,[7] we described the isolation of two new peptaibol groups from the fungus *Trichoderma harzianum*, trichokindins (TK) and trichorozins (TZ), together with the structure determination and biological activity of the former peptide group. In this paper, we report the structures, synthesis and ion channel-forming properties of the 11-residue peptides, TZ-I—IV.

Results and Discussion

Isolation and Characterization of TZs A peptide mixture from the MeOH extract of conidia of *Trichoderma harzianum* was purified by semi-preparetive HPLC to afford four peptides, TZ-I—IV, which were all negative to ninhydrin reagent (the isolation procedure has been described[7]). Amino acid composition analysis was performed in the routine manner. The ^{1}H- and ^{13}C-NMR spectra showed the presence of an acetyl group and an amino alcohol [leucinol (Lol) for TZ-III and IV, and valinol (Vol) for TZ-I and II] together with three Aib residues in the respective molecules, suggesting that TZs are peptaibols which have an acetylated *N*-terminus and a *C*-terminal residue linked to Lol or Vol. For TZs, no methylation occurred with diazomethane. Thus, in view of the presence of Lol or Vol, Asp and Glu detected by the amino acid analysis exist as Asn and Gln in the TZ molecules, respectively. The L-configuration of the normal amino acids and amino alcohols was determined by HPLC analysis of the acid hydrolysate derivatives as previously reported.[7]

Sequence Determination of TZs The amino acid sequences of TZs were determined by using fast atom bombardment mass spectrometry (FAB-MS) and collision-induced dissociation (CID). The calculated molecular weight of the main component, TZ-III, is 1174 (nominal), assuming it to be a linear peptide consisting of Ac (1), Aib (3), Asn (1), Ile (2), Leu (2), Pro (2) and Lol (1). As shown in Fig. 1a, the positive-ion FAB-MS of TZ-III showed the predicted pseudomolecular ion (MH^+) only in very low abundance, but it gave a significantly abundant ion considered to be the sodium adduct at *m/z* 1197. On the other hand, pneumatically assisted electrospray mass spectrometry (ES-MS) exhibited a protonated molecular ion at *m/z* 1175, in accord with the postulated molecular weight (Fig. 1b). In the FAB-MS (Fig. 1), a b-type acylium ion series from the pseudomolecular ion is observed at *m/z* 961, 553, 468, 355 and 242, but there are two low-abundance ion regions A and B between *m/z* 553 and 961 (408 a.m.u.), and *m/z* 961 and 1175 (214 a.m.u.), respectively. This cleavage pattern suggests the presence of two labile Aib-Pro bonds in the molecule. The masses of regions A and B corresponding to the tetra- and dipeptides are observed at *m/z* 409 and 215 *via* cleavages by protonation steps as observed in the hypelcins, which also have two Aib–Pro bonds.[8] The complete amino acid sequence of TZ-III was obtained by CID. The product ions from the *m/z* 961 gave the *N*-terminal nonapeptide sequence (positions 1—9) as shown in Fig. 2. The spectrum shows the acylium ion series, which is generated through successive losses of Aib, 2 Lxx, Pro, Aib, 2 Lxx and Asn from the *m/z* 961 and gives the *N*-terminal Ac-Aib (Lxx = Ile or Leu). In addition, the CID spectrum of the *m/z* 215 ion arising from region B afforded the *m/z* 70 ion, which was produced by the losses of Lol and CO (28 a.m.u.) of the Pro acylium ion (*m/z* 98), and this characterized the *C*-terminal dipeptide as Pro–Lol (not shown). Thus, the amino acid sequence of TZ-III was determined as Ac–Aib–Asn–Lxx–Lxx–Aib–Pro–Lxx–Lxx–Aib–Pro–Lol.

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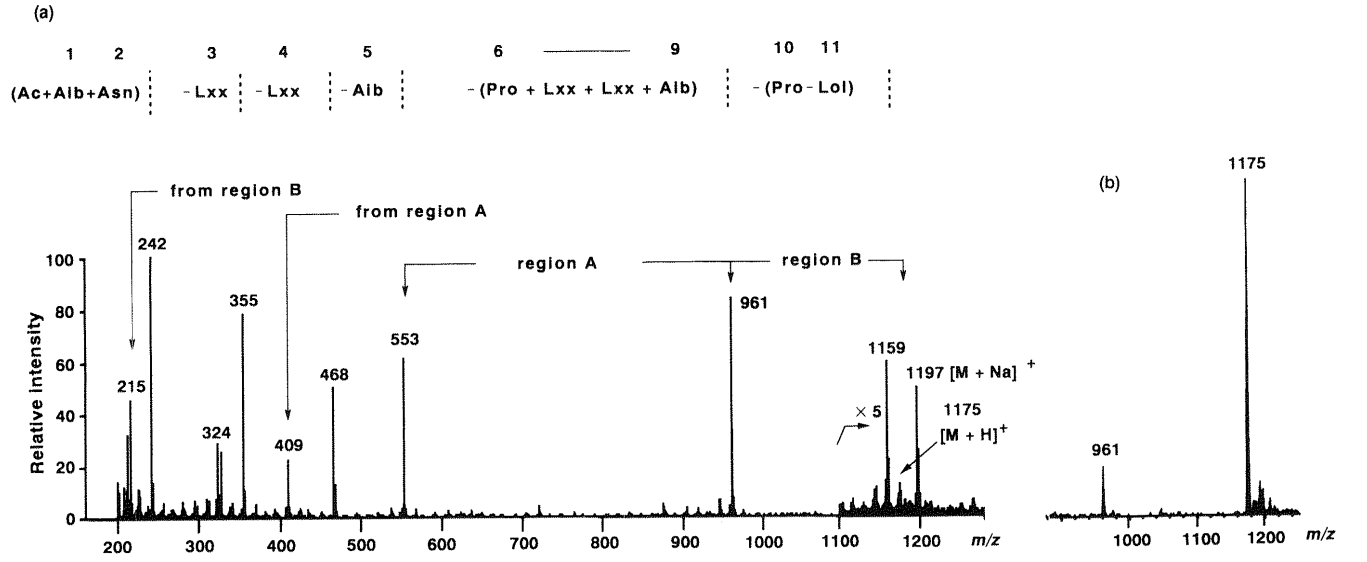


Fig. 1. Positive-Ion FAB (a) and ES (b) Mass Spectra of TZ-III

Lxx stands for Leu or Ile. In b, only the molecular ion region is shown. The *m/z* 1159 ion, which was also observed in the FAB-MS of synthetic TZ-III, was unassignable even by CID, but was found to arise from the loss (15 a.m.u.) of one of the side chains in the C-terminal hexapeptide.

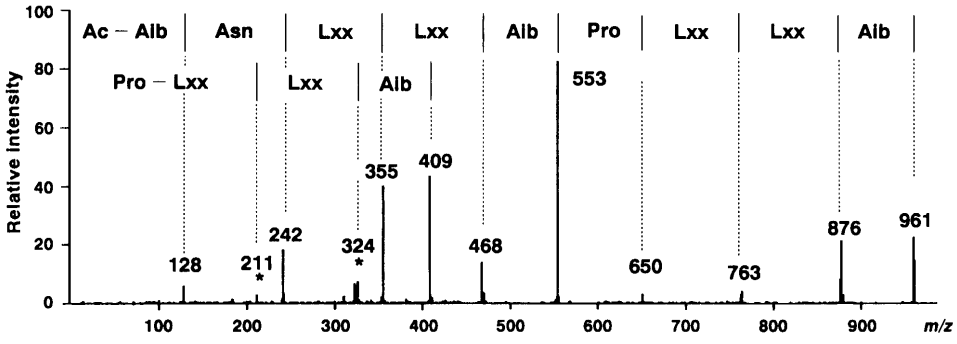


Fig. 2. Product Ions from the *m/z* 961 Ion by CID

The * ions are acylium ions arising from the *m/z* 409 ion.

The remaining problem is differentiation of the isomeric amino acids, Ile and Leu. This problem was solved by the analysis of through-space couplings between backbone NH protons in the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum.[9] After identification of the spin systems of normal amino acids, the sequence-specific assignment of the NH protons was carried out. As shown in Fig. 3, sequential NH–NH connectivities were observed between Aib[1] and Aib[5], and Ile[7] and Aib[9]. This allows us to place Ile–Leu–Aib in positions 3—5 and 7—9. Therefore, the complete structure of TZ-III is Ac–Aib–Asn–Ile–Leu–Aib–Pro–Ile–Leu–Aib–Pro–Lol. The structures of TZ-I, II and IV were characterized in the same manner, as summarized in Table I. These new peptides have a molecular weight up to *ca.* 1200 and are the shortest members of the peptaibol family, excluding the lipopeptaibols such as trikoningins KB[10] and trichodecenins.[11]

Synthesis of TZs With the aim of making available a sufficient amount for further investigation of the biological activities, we synthesized all the TZs by the solution-phase method with *N*,*N*′-dicyclohexylcarbodiimide (DCC)–1-hydroxybenzotriazole (HOBt), which is still useful for the synthesis for peptaibols. The synthetic route to TZ-I—IV is shown in Fig. 4. Each fragment was prepared in a stepwise manner and coupled after deprotection of the protecting groups. In order to avoid racemization, the Aib residue, which does not have chirality, was assigned to the *C*-terminus of [**2**] and [**3**]. The synthetic TZs were finally purified by semi-preparative HPLC and showed a single peak on the analytical HPLC profiles. The retention times (t_R) and ^{1}H- and ^{13}C-NMR spectra of these peptides were in agreement with those of the natural products. The CD absorptions of the synthetic peptides at 207 and 221 nm are larger than those of the corresponding natural products. However, the ratio of the values, $[\theta]_{221}/[\theta]_{207}$, is almost identical in the four pairs of synthetic and natural peptides. It is implied that the isolated peptides contain some impurities.

Interaction of TZ-IV with Planar Lipid Bilayers Peptaibols such as alamethicin are well-known as membrane modifiers,[12] and their channel-forming properties have been investigated using planar lipid bilayers. It is, in general, considered that peptaibol ion channels are formed by aggregation of amphiphilic helical peptide molecules in lipid membranes. CD properties of TZs indicate that these 11-residue peptides take a helical structure. Thus, in view of their amphiphilic property, TZs are expected to have channel-forming ability. In macroscopic experiments,

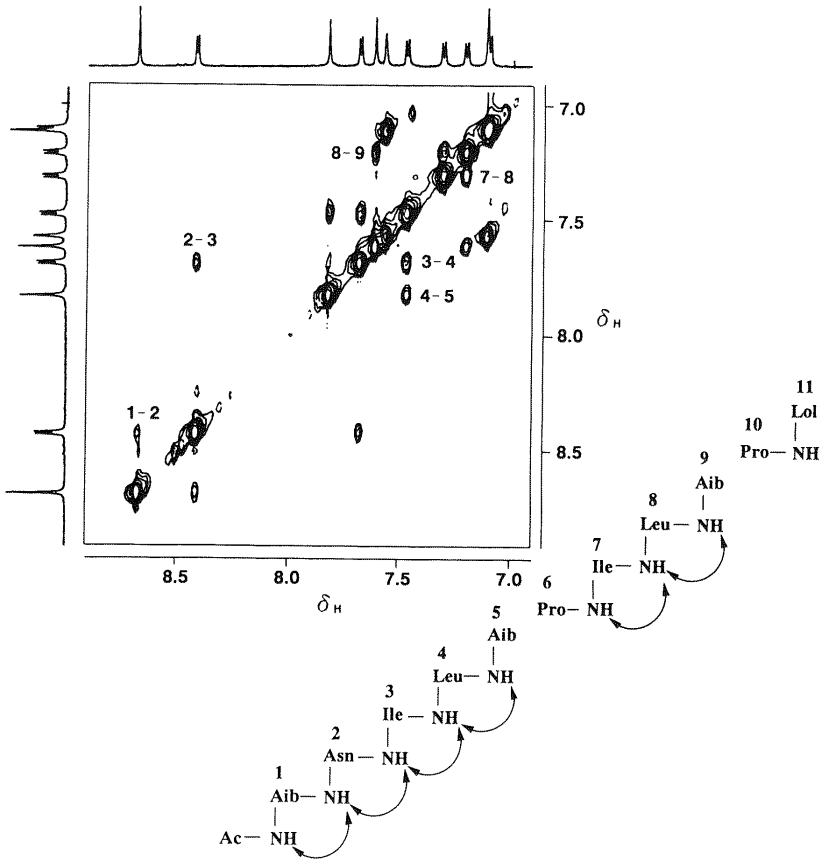


Fig. 3. The NH–NH Region of the 600 MHz NOESY Spectrum of TZ-III

The spectrum was obtained at 20 °C in $(CD_3)_2SO$ and was unsymmetrized (mixing time: 350 ms). Sequential NH–NH cross-peaks are observed between Aib^1 and Aib^5, and Ile^7 and Aib^9. The sequence-specific assignment is shown by the sequence numbers (· – ·) around each cross-peak.

TABLE I. Primary Structures of Trichorozins

Position	1	2	3	4	5	6	7	8	9	10	11
Trichorozin											
I	Ac–	Aib–	Asn–	Ile–	Leu–	Aib–	Pro–	Ile–	Leu–	Aib–	Pro–Vol
II	Ac–	Aib–	Gln–	Ile–	Leu–	Aib–	Pro–	Ile–	Leu–	Aib–	Pro–Vol
III	Ac–	Aib–	Asn–	Ile–	Leu–	Aib–	Pro–	Ile–	Leu–	Aib–	Pro–Lol
IV	Ac–	Aib–	Gln–	Ile–	Leu–	Aib–	Pro–	Ile–	Leu–	Aib–	Pro–Lol

TZ-IV (synthetic) at concentrations of 1.0 and 1.3 μM showed the current–voltage relationship illustrated in Fig. 5. Increase of such a voltage-dependent membrane current suggests that TZ-IV forms ion channels in the lipid bilayers like alamethicin. From extensive electrophysiological studies on peptaibols and their synthetic analogues, their ion channel-forming abilities are primarily dependent upon peptide length and molecular charge. The length of the helical rod of TZ-IV is apparently insufficient to span the membranes because of the 3-nm thick hydrophobic region of the lipid. Thus, the channel-like activity of TZ-IV sugggests that long helical rods might be formed by head-to-tail dimerization of TZ-IV helices, or TZ-IV monomers might provoke local bilayer distortion as was discussed in connection with other short channel-forming peptides,[3a] mastoparan and bombolitins.

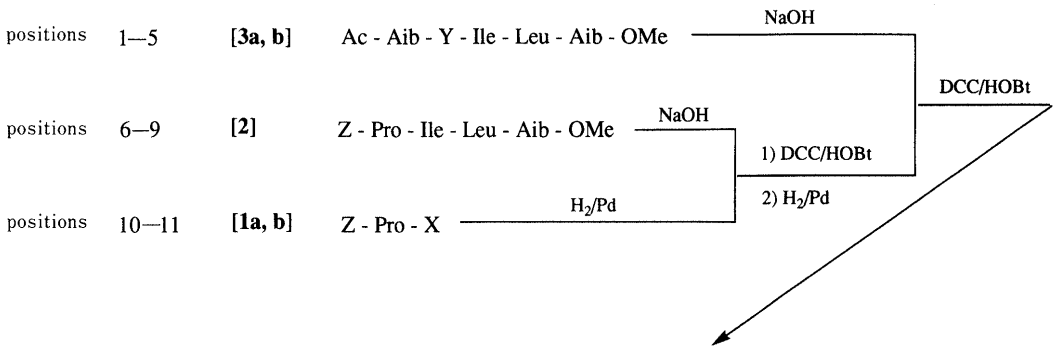
Experimental

General Methods All melting points are uncorrected. Optical rotations were measured with a JASCO DIP-181 digital polarimeter at 27 °C. All NMR experiments were performed on Bruker AC-300 and AM-600 spectrometers. Samples were dissolved in CD_3OH or $(CD_3)_2SO$ containing tetramethylsilane as an internal standard. CD spectra were recorded on a JASCO J-720 spectropolarimeter. HPLC was performed on Shimadzu LC-6A and LC-8A systems using YMC packed ODS columns (YMC Co., Ltd.), AM-313 (6 mm i.d. × 250 mm) for analytical HPLC and SH-345 (20 mm i.d. × 250 mm) for semi-preparative HPLC. FAB-MS and CID were carried out on a Finnigan MAT 70 triple-stage quadrupole mass spectrometer. Glycerol–thioglycerol (1 : 1) was used as a matrix. Samples were bombarded with 8 kV xenon atoms and then product ions were collided with argon atoms whose energy was 20—40 eV at a pressure of 0.8—1.2 mTorr. Pneumatically assisted ES-MS was performed on an API III (Perkin Elmer Sciex). Samples were dissolved in CH_3CN–H_2O (1 : 1) containing 0.1% TFA. EI-MS was performed on a JEOL OI-SG. Amino acid analyses were done with a Hitachi Model 835 amino acid analyzer. TLC was performed on silica gel (Kieselgel 60F254, Merck). The *Rf* values refer to the following solvent systems (v/v): Rf_1 = $CHCl_3$–MeOH (95 : 5), Rf_2 = $CHCl_3$–MeOH (9 : 1), Rf_3 = $CHCl_3$–MeOH (8 : 2), Rf_4 = $CHCl_3$–MeOH (7 : 3), Rf_5 = $CHCl_3$–MeOH–H_2O (60 : 40 : 5). For column chromatography, Silica gel 60 (70—230 mesh, Merck) and Sephadex LH-20 (Pharmacia) were used.

Current–voltage curves were taken by imposing a triangular wave voltage (100 s per cycle) on the membrane at room temperature. A planar lipid bilayer was painted from a lipid solution on a *ca.* 1 mm diameter hole in a Teflon septum, pretreated with the lipid solution. The lipid solution was composed of eggphosphatidylcholine (167.7 mg, Merck) and cholesterol (46.2 mg) in *n*-decane (20 ml). A pair of Ag/AgCl electrodes was inserted into a solution of 0.1 M KCl on both sides of the membrane. The peptide sample was added to the aqueous phase before the membrane formation.

Charcteristics of TZs TZ-I ($C_{57}H_{100}N_{12}O_{13}$): ES-MS *m/z* 1162.1 ($MH^+$, monoisotopic). mp 163—165 °C. λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−132000) 221 (−47900). Amino acid ratios (6 N HCl, 24 h): Asp 1.00 (1), Ile 1.93 (2), Leu 2.21 (2), Pro 2.12 (2).

TZ-II ($C_{58}H_{102}N_{12}O_{13}$): ES-MS *m/z* 1176.2 ($MH^+$). mp 167—170 °C.



Ac - Aib -Y - Ile - Leu - Aib - Pro - Ile - Leu - Aib - Pro - X

	X	Y
TZ - I	Vol	Asn
TZ - II	Vol	Gln
TZ - III	Lol	Asn
TZ - IV	Lol	Gln

Fig. 4. Synthetic Route to TZs

X stands for Lol for **1a** and Vol for **1b**, while Y represents Gln for **3a** and Asn for **3b**.

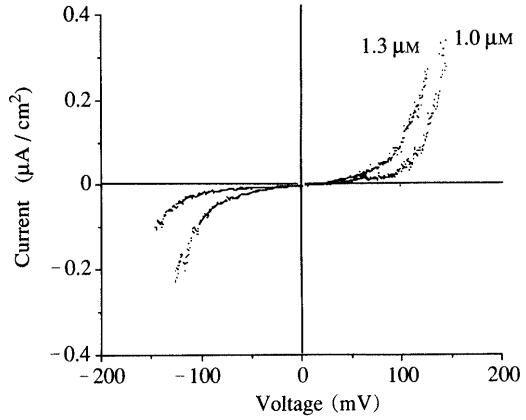


Fig. 5. Macroscopic Current–Voltage Relationships for TZ-IV

λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−135000) 221 (−44800). Amino acid ratios; Glu 1.00 (1), Ile 1.85 (2), Leu 1.98 (2), Pro 2.03 (2).

TZ-III ($C_{58}H_{102}N_{12}O_{13}$): ES-MS *m/z* 1176.1 ($MH^+$). mp 159—162 °C. λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−133000) 221 (−38300). Amino acid ratios; Asp 1.00 (1), Ile 1.87 (2), Leu 2.11 (2), Pro 2.10 (2).

TZ-IV ($C_{59}H_{104}N_{12}O_{13}$): ES-MS *m/z* 1190.2 ($MH^+$). mp 153—156 °C. λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−124000) 221 (−36900). Amino acid ratios; Glu 1.00 (1), Ile 1.95 (2), Leu 2.25 (2), Pro 1.98 (2).

Coupling Reactions Unless otherwise stated, coupling reactions were performed by the DCC–HOBt method at room temperature for 12—72 h and the mixtures were worked up according to procedure A or B after removal of DCU and the solvent.

Procedure A: EtOAc-soluble protected peptides were each dissolved in EtOAc and the solution was washed successively with 1 N HCl, 5% $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4 and concentrated. The residue was reprecipitated from EtOAc–*n*-hexane.

Procedure B: EtOAc-insoluble protected peptides were purified by gel-filtration on Sephadex LH-20 in MeOH.

Catalytic Hydrogenation The benzyloxycarbonyl group, Z, was removed by the use of H_2 gas over 10% palladium-on-charcoal with stirring. After removal of the catalyst by filtration, the filtrate was concentrated and used in the next step without further purification (procedure C).

Z–Pro–Lol [1a] Z–Pro–OH (8.74 g, 35 mmol), HOBt (4.74 g, 1 eq), and DCC (7.24 g, 1 eq) were added successively to a stirred solution of Lol (4.11 g, 1 eq) in EtOAc (75 ml). After 24 h, the solution was worked up according to procedure A. The residue was recrystallized from EtOAc to give the title compound; yield 7.19 g (59%). mp 97—98 °C. $[\alpha]_D$ −68.7° (c=1.0, MeOH). Rf_1 0.39. EI-MS *m/z*: 348 (M^+), 317 ($M^+ - CH_2OH$), 204 (M^+ − Lol − CO). *Anal.* Calcd for: $C_{19}H_{28}N_2O_4$: C, 65.19; H, 8.10; N, 8.04. Found: C, 65.43; H, 8.14; N, 8.15.

Z–Pro–Vol [1b] Z–Pro–Vol was prepared in the same mannner as the above dipeptide using Vol (1.00 g); yield 1.80 g (64%), mp 113—114 °C (EtOAc). $[\alpha]_D$ −60.8° (c=1.0, MeOH). Rf_1 0.33. EI-MS *m/z*: 334 (M^+), 303 ($M^+ - CH_2OH$), 204 (M^+ − Vol − CO). *Anal.* Calcd for: $C_{18}H_{26}N_2O_4$: C, 64.65; H, 7.84; N, 8.38. Found: C, 64.35; H, 7.78; N, 8.40.

HCl·H–Pro–Lol Z–Pro–Lol (2.00 g) was hydrogenated in MeOH (20 ml) containing 1 N HCl (2.87 ml) according to procedure C; yield 1.23 g (85%), Rf_5 0.36.

HCl·H–Pro–Vol Z–Pro–Vol (639 mg) was hydrogenated in MeOH (5 ml) containing 1 N HCl (1.91 ml) according to procedure C; yield 411 mg (91%), Rf_5 0.34.

Z–Leu–Aib–OMe The title compound was prepared according to the procedure reported previously.[13]

HCl·H–Leu–Aib–OMe The above dipeptide (7.71 g) was hydrogenated in MeOH (50 ml) containing 1 N HCl (21 ml) according to procedure C; yield 5.60 g (100%), Rf_5 0.62.

Z–Ile–Leu–Aib–OMe To a stirred solution of HCl·H–Leu–Aib–OMe (4.03 g, 1 eq) in EtOAc (150 ml) containing TEA (2.12 ml) were added Z–Ile–OH (4.01 g, 15.1 mmol), HOBt (2.04 g, 1 eq) and DCC (3.12 g, 1 eq). After 24 h, work-up was done according to procedure A; yield 7.44 g (74%), mp 124—125 °C. $[\alpha]_D$ −52.4° (c=1.0, MeOH). Rf_1 0.58. EI-MS *m/z*: 477 (M^+), 446 (M^+ − OMe), 361 (446 − Aib), 248 (361 − Leu). *Anal.* Calcd for: $C_{25}H_{39}N_3O_6$: C, 62.87; H, 8.23; N, 8.80. Found: C, 62.60; H, 8.17; N, 8.75.

HCl·H–Ile–Leu–Aib–OMe Z–Ile–Leu–Aib–OMe (7.44 g) was hydrogenated in MeOH (50 ml) containing 1 N HCl (15.6 ml) according to procedure C; yield 5.92 g (100%), Rf_5 0.72.

Z–Pro–Ile–Leu–Aib–OMe [2] Z–Pro–OH (1.64 g, 6.58 mmol), HOBt (0.89 g, 1 eq) and DCC (1.36 g, 1 eq) were added successively to a stirred solution of HCl·H–Ile–Leu–Aib–OMe (2.50 g, 1 eq) in EtOAc (60 ml) containing TEA (0.92 ml). After 28 h, the solution was worked up according to procedure A; yield 2.70 g (71%), mp 146—147 °C. $[\alpha]_D$ −94.4° (c=1.0, MeOH). Rf_1 0.58. EI-MS *m/z*: 574 (M^+), 430 (M^+ − OMe − Aib − CO), 345 (M^+ − OMe − Aib − Leu), 232 (345 − Ile). *Anal.* Calcd for: $C_{30}H_{46}N_4O_7$: C, 62.70; H, 8.07; N, 9.75. Found: C, 62.61; H, 8.03; N, 9.71.

Z–Gln–Ile–Leu–Aib–OMe Z–Gln–OH (0.89 g, 3.18 mmol), HOBt (0.43 g, 1 eq) and DCC (0.66 g, 1 eq) were added successively to a stirred solution of HCl·H–Ile–Leu–Aib–OMe (1.21 g, 1 eq) in DMF (20 ml) containing TEA (0.44 ml). After 30 h, DCU was removed by filtration and the filtrate was concentrated. The residue was suspended in EtOAc,

filtered and washed with the same solvent. The residue was washed again with H_2O and dried over KOH; yield 1.23 g (64%), mp 246—248 °C. $[\alpha]_D$ −38.4° (c=0.1, MeOH). Rf_2 0.42. EI-MS m/z: 605 (M^+), 489 (M^+−OMe−Aib), 376 (489−Leu), 263 (376−Ile). *Anal.* Calcd for: $C_{30}H_{47}N_5O_8$: C, 59.49; H, 7.82; N, 11.56. Found: C, 59.21; H, 8.00; N, 11.62.

Z–Asn–Ile–Leu–Aib–OMe Z–Asn–OH (0.70 g, 2.63 mmol), HOBt (0.36 g, 1 eq) and DCC (0.54 g, 1 eq) were added successively to a stirred solution of HCl·H–Ile–Leu–Aib–OMe (1.00 g, 1 eq) in DMF (20 ml) containing TEA (0.37 ml). After 34 h, the solution was worked up by the same procedure described above; yield 1.22 g (78%), mp 225—227°C. $[\alpha]_D$ −55.7° (c=1.0, MeOH). Rf_2 0.36. EI-MS m/z: 592 (M^+), 560 (M^+−OMe), 475 (560−Aib) 362 (475−Leu), 249 (362−Ile). *Anal.* Calcd for: $C_{29}H_{45}N_5O_8$: C, 58.87; H, 7.67; N, 11.83. Found: C, 59.07; H, 7.68; N, 11.84.

Z–Pro–Ile–Leu–Aib–OH Z–Pro–Ile–Leu–Aib–OMe (1.50 g) was hydrolyzed with 1 N NaOH (2 eq) in MeOH at 0 °C. After complete saponification, the solution was neutralized with 1 N HCl and evaporated to remove MeOH. The residual solution was acidified to pH 3 and extracted with EtOAc. The extract was washed with saturated NaCl, dried over Na_2SO_4 and concentrated. The residue was employed without further purification; yield 1.45 g (99%), Rf_5 0.66.

Z–Pro–Ile–Leu–Aib–Pro–Lol (Positions 6—11) The above tetrapeptide acid (1.11 g, 1.98 mmol), HOBt (0.27 g, 1 eq) and DCC (0.41 g, 1 eq) were added successively to a stirred solution of HCl·H–Pro–Lol (0.50 g, 1 eq) in DMF (10 ml) containing TEA (0.28 ml). After 48 h, the solution was worked up according to procedure A; yield 1.20 g (80%), mp 179—181 °C. $[\alpha]_D$ −75.8° (c=0.7, MeOH). Rf_2 0.41. EI-MS m/z: 757 (M^+), 726 (M^+−CH_2OH), 640 (M^+−Lol), 543 (640−Pro), 458 (543−Aib), 345 (458−Leu), 232 (345−Ile). *Anal.* Calcd for: $C_{40}H_{64}N_6O_8 \cdot 1/2H_2O$: C, 62.72; H, 8.55; N, 10.97. Found: C, 63.00; H, 8.45; N, 10.97.

Z–Pro–Ile–Leu–Aib–Pro–Vol (Positions 6—11) Z–Pro–Ile–Leu–Aib–OH (0.80 g), HOBt (0.19 g, 1 eq) and DCC (0.31 g, 1 eq) were added successively to a stirred solution of HCl·H–Pro–Vol (0.34 g, 1 eq) in DMF (10 ml) containing TEA (0.21 ml). After 48 h, the solution was worked up according to procedure A; yield 874 mg (79%), mp 92—95 °C. $[\alpha]_D$ −68.5° (c=0.9, MeOH). Rf_2 0.39. EI-MS m/z: 743 (M^+), 712 (M^+−CH_2OH), 543 (M^+−Vol−Pro), 458 (543−Aib), 345 (458−Leu), 232 (345−Ile). *Anal.* Calcd for: $C_{39}H_{62}N_6O_8 \cdot 1/2H_2O$: C, 62.29; H, 8.44; N, 11.17. Found: C, 62.23; H, 8.26; N, 11.16.

HCl·H–Pro–Ile–Leu–Aib–Pro–Lol Z–Pro–Ile–Leu–Aib–Pro–Lol (1.16 g) was hydrogenated in MeOH (10 ml) containing 1 N HCl (1.5 ml) according to procedure C; yield 0.87 g (92%), Rf_5 0.62.

HCl·H–Pro–Ile–Leu–Aib–Pro–Vol Z–Pro–Ile–Leu–Aib–Pro–Vol (818 mg) was hydrogenated in MeOH (10 ml) containing 1 N HCl (1.1 ml) according to procedure C; yield 620 mg (93%), Rf_5 0.60.

HCl·H–Gln–Ile–Leu–Aib–OMe Z–Gln–Ile–Leu–Aib–OMe (1.20 g) was hydrogenated in MeOH (50 ml) containing 1 HCl (1.53 ml) according to procedure C; yield 1.00 g (100%), Rf_5 0.52.

Ac–Aib–Gln–Ile–Leu–Aib–OMe [3a] Ac–Aib–OH (0.29 g, 2.00 mmol), HOBt (0.27 g, 1 eq) and DCC (0.41 g, 1 eq) were added successively to a stirred solution of HCl·H–Gln–Ile–Leu–Aib–OMe (1.01 g, 1 eq) in DMF (20 ml) containing TEA (0.28 ml). After 48 h, work-up was done as described in procedure B. The residue was chromatographed on silica gel ($CHCl_3$: MeOH = 8 : 2); yield 0.61 g (52%), mp 152—154 °C. $[\alpha]_D$ −27.1° (c= 0.1, MeOH). Rf_3 0.44. EI-MS m/z: 598 (M^+), 567 (M^+−OMe), 482 (566−Aib), 369 (482−Leu), 256 (368−Ile), 128 (256−Gln). *Anal.* Calcd for: $C_{28}H_{50}N_6O_8 \cdot 1/2H_2O$: C, 55.34; H, 8.46; N, 13.83. Found: C, 55.40; H, 8.50; N, 13.85.

HCl·H–Asn–Ile–Leu–Aib–OMe Z–Asn–Ile–Leu–Aib–OMe (1.16 g) was hydrogenated in MeOH (40 ml) containing 1 N HCl (1.96 ml) according to procedure C; yield 0.97 g (100%). Rf_5 0.59.

Ac–Aib–Asn–Ile–Leu–Aib–OMe [3b] Ac–Aib–OH (0.2 g, 1.86 mmol), HOBt (0.25 g, 1 eq) and DCC (0.38 g, 1 eq) were added successively to a stirred solution of HCl·H–Asn–Ile–Leu–Aib–OMe (0.92 g, 1 eq) in DMF (15 ml) containing TEA (0.26 ml). After 24 h, the solution was worked up according to procedure B. The residue was chromatographed on silica gel ($CHCl_3$: MeOH = 8 : 2); yield 0.83 g (78%), mp 177 −188 °C. $[\alpha]_D$ −29.6° (c=1.0, MeOH). Rf_3 0.40. EI-MS m/z: 584 (M^+), 553 (M^+−OMe), 468 (553−Aib), 355 (468−Leu), 242 (355−Ile), 128 (242−Asn). *Anal.* Calcd for: $C_{27}H_{48}N_6O_8 \cdot 1/2H_2O$: C, 54.62; H, 8.32; N, 14.15. Found: C, 54.82; H, 8.35; N, 13.98.

Ac–Aib–Gln–Ile–Leu–Aib–OH Ac–Aib–Gln–Ile–Leu–Aib–OMe (560 mg) was saponified and the solution was neutralized with Amberlite IR-120. The solvent was removed *in vacuo* to give the title pentapeptide acid; yield 540 mg (99%), Rf_5 0.44.

Ac–Aib–Asn–Ile–Leu–Aib–OH Ac–Aib–Asn–Ile–Leu–Aib–OMe (780 mg) was saponified and the solution was neutralized with Amberlite IR-120. The solvent was evaporated *in vacuo* to give the title compound; yield 715 mg (94%), Rf_5 0.44.

Ac–Aib–Asn–Ile–Leu–Aib–Pro–Ile–Leu–Aib–Pro–Vol [TZ-I] The *N*-terminal pentapeptide acid (375 mg, 0.66 mmol), HOBt (89 mg, 1 eq) and DCC (136 mg, 1 eq) were added successively to a stirred solution of HCl·H–Pro–Ile–Leu–Aib–Pro–Vol (424 mg, 1 eq) in DMF (5 ml) containing TEA (92 μl). After 44 h, the solution was worked up according to procedure B. The residue was purified by preparative HPLC [conditions: mobile phase, MeOH–H_2O (85 : 15, v/v); flow rate, 5 ml/min; UV detection, 220 nm; column, YMC packed column SH-345-5 (20 mm i.d. × 250 mm); column temperature, 40 °C] to give TZ-I; yield 198 mg (26%), mp 167—170 °C. $[\alpha]_D$ −3.3° (c=1.0, MeOH). λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−142000) 221 (−49800), t_Rs (min): 16.14 (synthesis), 16.18 (natural) [conditions: mobile phase, MeOH–H_2O (85 : 15, v/v); flow rate, 1 ml/min; UV detection, 220 nm; column, YMC packed ODS AM-313 (6 mm i.d. × 250 mm); column temperature, 40 °C], Rf_4 0.58. ES-MS m/z: 1162.1 (MH^+, monoisotopic). *Anal.* Calcd for: $C_{57}H_{100}N_{12}O_{13} \cdot H_2O$: C, 58.04; H, 8.72; N, 14.25. Found: C, 57.90; H, 8.72; N, 14.19.

Ac–Aib–Gln–Ile–Leu–Aib–Pro–Ile–Leu–Aib–Pro–Vol [TZ-II] The *N*-terminal pentapeptide acid (193 mg, 0.33 mmol), HOBt (45 mg, 1 eq) and DCC (68 mg, 1 eq) were added successively to a stirred solution of HCl·H–Pro–Ile–Leu–Aib–Pro–Vol (213 mg, 1 eq) in DMF (5 ml) containing TEA (46 μl). After 48 h, the solution was worked up according to procedure B. The residue was purified in the same manner as in the case of TZ-I; yield 150 mg (39%), mp 174—177 °C. $[\alpha]_D$ −6.6° (c=1.0, MeOH). λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−151000) 221 (−46100). t_Rs (min): 17.02 (synthesis), 17.17 (natural), Rf_4 0.58. ES-MS m/z: 1176.1 (MH^+). *Anal.* Calcd for: $C_{58}H_{102}N_{12}O_{13} \cdot 3/2H_2O$: C, 57.93; H, 8.80; N, 13.98. Found: C, 57.82; H, 8.77; N, 13.88.

Ac–Aib–Asn–Ile–Leu–Aib–Pro–Ile–Leu–Aib–Pro–Lol [TZ-III] The *N*-terminal pentapeptide acid (175 mg, 0.31 mmol), HOBt (42 mg, 1 eq) and DCC (64 mg, 1 eq) were added successively to a stirred solution of HCl·H–Pro–Ile–Leu–Aib–Pro–Lol (202 mg, 1 eq) in DMF (5 ml) containing TEA (43 μl). After 48 h, the solution was worked up according to procedure B. The residue was purified in the same manner as in the case of TZ-I; yield 109 mg (31%), mp 164—167 °C. $[\alpha]_D$ −7.7° (c=1.0, MeOH). λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−156000) 221 (−43200). t_Rs (min): 18.24 (synthesis), 18.33 (natural), Rf_4 0.58. ES-MS m/z: 1176.1 (MH^+). *Anal.* Calcd for: $C_{58}H_{102}N_{12}O_{13} \cdot H_2O$: C, 58.37; H, 8.78; N, 14.08. Found: C, 58.16; H, 8.85; N, 13.99.

Ac–Aib–Gln–Ile–Leu–Aib–Pro–Ile–Leu–Aib–Pro–Lol [TZ-IV] The *N*-terminal pentapeptide acid (178 mg, 0.30 mmol), HOBt (41 mg, 1 eq) and DCC (63 mg, 1 eq) were added successively to a stirred solution of HCl·H–Pro–Ile–Leu–Aib–Pro–Lol (201 mg, 1 eq) in DMF (5 ml) containing TEA (42 μl). After 48 h, the solution was worked up according to procedure B. The residue was purified in the same manner as in the case of TZ-I; yield 145 mg (40%), mp 158—161 °C. $[\alpha]_D$ −10.2° (c=1.0, MeOH). λ (nm) $[\theta]_M$ (deg·cm^2·dmol^{-1}) 207 (−161000) 221 (−44300). t_Rs (min): 19.51 (synthesis), 19.50 (natural), Rf_4 0.58. ES-MS m/z: 1190.2 (MH^+). *Anal.* Calcd for: $C_{59}H_{104}N_{12}O_{13} \cdot 3/2H_2O$: C, 58.25; H, 8.86; N, 13.81. Found: C, 58.24; H, 8.77; N, 13.77.

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References and Notes

1) Part XVII: Y. Nagaoka, A. Iida, T. Fujita, *Chem. Pharm. Bull.*, **42**, 1258 (1994).
2) The following abbreviations are used: FAB = fast atom bombardment, CID = collision-induced dissociation, ES-MS = pneumatically assisted electrospray mass spectormetry, EI-MS = electron impact mass spectrometry, NOESY = nuclear Overhauser enhancement spectroscopy, Z = benzyloxycarbonyl, DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole, DCU = dicyclohexylurea, DMF = dimethylformamide, EtOAc = ethyl acetate, TEA = triethylamine, TFA = trifluoroacetic acid.
3) For recent reviews, see *a*) M. S. P. Sansom, *Prog. Biophys. Molec.*

Biol., **55**, 139 (1991); *b*) G. A. Wooley, B. A. Wallace, *J. Membrane Biol.*, **129**, 109 (1992); *c*) M. S. P. Sansom, *Eur. Biophys. J.*, **22**, 105 (1993).
4) R. O. Fox, F. M. Richards, *Nature* (London), **300**, 325 (1982); G. Esposito, J. A. Carver, J. Boyd, I. D. Campbell, *Biochemistry*, **26**, 1043 (1987); S. Rebuffat, L. Conraux, M. Massias, C. Auvin-Guette, B. Bodo, *Int. J. Peptide Protein Res.*, **41**, 74 (1993); A. Iida, S. Uesato, T. Shingu, Y. Nagaoka, Y. Kuroda, T. Fujita, *J. Chem. Soc., Perkin Trans. 1*, **1993**, 375.
5) R. I. Fonteriz, M. G. Lopez, J. Garcia-Sancho, A. G. Garcia, *FEBS Lett.*, **283**, 89 (1991); E. Tachikawa, S. Takahashi, K. Furumachi, T. Kashimoto, A. Iida, Y. Nagaoka, T. Fujita, Y. Takaishi, *Mol. Pharmacol.*, **40**, 790 (1991).
6) Y. Takaishi, H. Terada, T. Fujita, *Experientia*, **36**, 550 (1980); M. K. Mathew, R. Nagaraj, P. Balaram, *Biochem. Biophys. Res. Commun.*, **98**, 548 (1981); M. K. Das, S. Raghothama, P. Balaram, *Biochemistry*, **25**, 7110 (1986); M. Okuda, A. Iida, S. Uesato, Y. Nagaoka, T. Fujita, Y. Takaishi H. Terada, *Biol. Pharm. Bull.*, **17**, 482 (1994).
7) A. Iida, M. Sanekata, T. Fujita, H. Tanaka, A. Enoki, G. Fuse, M. Kanai, P. J. Rudewicz, E. Tachikawa, *Chem. Pharm. Bull.*, **42**, 1070 (1994).
8) K. Matsuura, A. Yesilada, A. Iida, Y. Takaishi, M. Kanai, T. Fujita, *J. Chem. Soc., Perkin Trans. 1*, **1993**, 381.
9) G. Wagner, K. Wuthrich, *J. Mol. Biol.*, **155**, 347 (1982).
10) C. Auvin-Guette, S. Rebuffat, I. Vuidepot, M. Massias, B. Bodo, *J. Chem. Soc., Perkin Trans. 1*, **1993**, 249.
11) T. Fujita, S. Wada, A. Iida, T. Nishimura, M. Kanai, N. Toyama, *Chem. Pharm. Bull.*, **42**, 489 (1994).
12) K. Matsuzaki, S. Nakai, T. Handa, Y. Takaishi, T. Fujita, K. Miyajima, *Biochemistry*, **28**, 9392 (1989); G. Irmscher G. Jung, *Eur. J. Biochem.*, **80**, 165 (1977); A. L. Y. Lau, S. I. Chan, *Biochemistry*, **13**, 4942 (1974).
13) A. Iida, S. Yoshumatsu, M. Sanekata, T. Fujita, *Chem. Pharm. Bull.*, **38**, 2997 (1990).