Preparation and metal ion-binding properties of gramicidin S derivatives carrying picolinoyl groups on the ornithine side chains

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Derivatives of gramicidin S (GS) in which one or both of the two ornithine side chains were picolinoylated were prepared. The dipicolinoyl derivative [Orn(PyCO)$^{2,2'}$]GS **1** formed a 1 : 1 complex with Cu^{2+} and Zn^{2+} in MeOH, while in the case of the monopicolinoyl derivative [Orn(Boc)^{2},Orn(PyCO)$^{2'}$]GS (**2**) stepwise formation of 1 : 1 and 2 : 1 (**2** : Cu^{2+}) complexes was observed. The formation constant of the Cu^{2+}-mediated dimeric species of compound **2** was larger than those of the corresponding linear compounds possessing the partial structure of macrocycle **2**. The corresponding tetra-*N*-methyl derivative [MeOrn(Boc)^{2},MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS **3** also showed lower stability of the 2 : 1 complex compared with compound **2**, which suggested the presence of a β-sheet-type intermolecular H-bonding interaction between the two molecules of macrocycle **2** in the 2 : 1 complex.

Introduction

A great number of studies on metal ion-induced self-assembly systems have been carried out to construct functional supramolecules with well defined structures; *e.g.*, metal ion-induced assembly of α-helical peptides which possess a metal ion-binding site at N-termini was reported by Lieberman and Sasaki,[1a] and Ghadiri *et al.*[1b,c] However, studies using metal-binding peptides with β-structures are quite few. We attempted to incorporate metal ion-binding site(s) into the antibiotic cyclic decapeptide gramicidin S (GS), cyclo(Val-Orn-Leu-D-Phe-Pro)$_2$, whose conformation is established as an antiparallel β-sheet possessing two Orn side chains on the same side of the molecule. Nishino *et al.* reported the synthesis of the GS analogue possessing 5,5′-bipyridylalanine residues in place of the Orn residues, which was shown to bind a divalent metal ion.[2] Here, we have prepared analogues of GS possessing one or two picolinoylamido (pyridine-2-carbonylamino, PyCONH) group(s) by chemical modification of the Orn residue(s) of natural GS. The PyCONH group is known to coordinate a divalent metal ion and the bis(PyCO) derivatives of GS can be expected to form a tetradentate 1 : 1 complex with a metal ion such as Cu^{2+} or Zn^{2+}. In the case of the Cu^{2+} complex, pyridine N atoms and deprotonated amide N atoms constitute an N_4 tetradentate ligand, while in the Zn^{2+} complex the pyridine N atoms and amide O atoms are considered to coordinate Zn^{2+} ion.[3] The GS analogue containing only one PyCONH group is expected to undergo metal ion-mediated self assembly forming a 2 : 1 complex with a metal ion such as Cu^{2+} or Zn^{2+}. The metal ion-binding properties of these analogues are described in comparison with those of the corresponding linear peptides.

Results and discussion

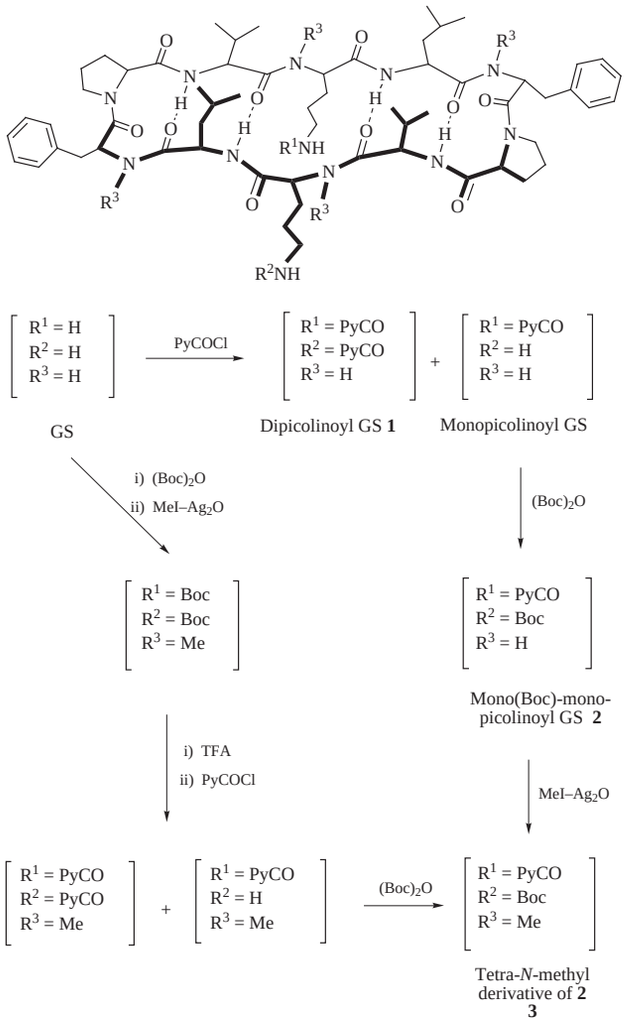
Synthesis

Introduction of a picolinoyl group as an amino-protecting group to afford α-amino acid esters was reported by Koul *et al.*[4] using picolinic acid (pyridine-2-carboxylic acid, $PyCO_2H$) and 1,1′-carbonyldiimidazole (CDI). Treatment of GS·2HCl with picolinoylimidazole reagent prepared from $PyCO_2H$ and CDI was found to afford only dipicolinoyl derivatives of GS, namely [Orn(PyCO)$^{2,2'}$]GS **1**, while no formation of monopicolinoyl derivative [Orn(PyCO)^{2}]GS was observed in the reaction mixture. Reaction of GS·2HCl with the acid chloride PyCOCl yielded a mixture of di- and mono-acylated products and unreacted GS, from which [Orn(PyCO)^{2}]GS was isolated in 32% yield by column chromatographic separation. The monopicolinoyl derivative was then treated with $(Boc)_2O$ to afford [Orn(Boc)^{2},Orn(PyCO)$^{2'}$]GS **2** as summarized in Scheme 1.

The low yield of the monoacyl derivative **2** was not unexpected, since modification of one of the two δ-amino groups of the Orn residues in GS is usually quite difficult. For example, 2,4-dinitrophenylation of GS using one equivalent of 1-fluoro-2,4-dinitrobenzene resulted in an equimolar mixture of bis-2,4-dinitrophenyl derivative and unchanged GS, which is quite analogous to the acylation using picolinoylimidazole described above. Very recently, we have found that trifluoroacetylation of GS with trifluoroacetic anhydride (TFAA) afforded a ~70% yield of mono-TFA derivative, a versatile intermediate for the preparation of mono-substituted GS.[5]

CD spectra of the GS derivatives **1** and **2**, possessing one and two PyCONH group(s), respectively, were very similar to that of natural GS·2HCl, indicating that both peptides also adopt the same β-sheet conformation as that of GS.

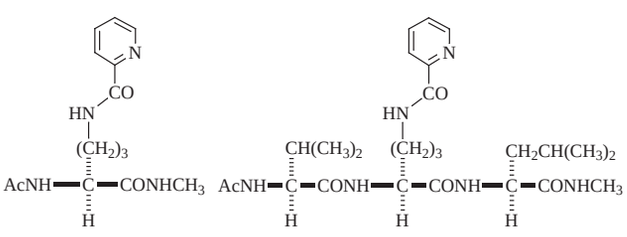
As a derivative of compound **2** lacking α-NH groups required for intermolecular inter-β-sheet H-bonding interaction, tetra-*N*-methyl derivative [MeOrn(Boc)^{2},MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS **3** was also prepared, in which solvent-exposed α-NH groups of the Orn and D-Phe residues of structure **2** were methylated. As shown in Scheme 1, preparation of the tetra-N-methylated derivative **3** made use of selective N-methylation with MeI–Ag_2O in DMF.[6] N-Methylation of the di-Boc derivative [Orn(Boc)$^{2,2'}$]GS yielded a tetramethyl derivative [MeOrn(Boc)$^{2,2'}$,D-MePhe$^{4,4'}$]GS, which was deprotected and picolinoylated in essentially the same manner as the preparation of compound **2** to yield di- and mono-picolinoyl derivatives, [MeOrn(PyCO)$^{2,2'}$,D-MePhe$^{4,4'}$]GS and [MeOrn(PyCO)^{2},MeOrn$^{2'}$,D-MePhe$^{4,4'}$]GS, respectively. The latter compound was then treated with $(Boc)_2O$ to furnish [MeOrn(Boc)^{2},MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS **3** possessing four *N*-methyl groups instead of α-NH groups which could participate in intermolecular H-bonding interaction. Since not only the



Scheme 1 Preparation of picolinoyl derivatives [Orn(PyCO)$^{2,2'}$]GS **1**, [Orn(Boc)^{2},Orn(PyCO)$^{2'}$] **2** and [MeOrn(Boc)^{2},MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS **3** from gramicidin S (GS).

BocNH group but also the PyCONH group in the Orn side chain in GS was found to be methylation-resistant,[7] [Orn-(Boc)^{2},Orn(PyCO)$^{2'}$]GS **2** was directly subjected to selective N-methylation to yield compound **3**, which was indistinguishable from the compound prepared from [Orn(Boc)$^{2,2'}$]GS as described above.

Linear peptide derivatives corresponding to partial structures of the picolinoylamide-containing GS, namely Ac-Orn(PyCO)-NHMe **4** and Ac-Val-Orn(PyCO)-Leu-NHMe **5**, were also prepared to compare the dimeric Cu^{2+}-complex-forming ability. Introduction of a PyCO group to the δ-amino group of the Orn residue in these compounds was accomplished by treatment with picolinoylimidazole reagent.[4] Their structures were confirmed by ^{1}H NMR, liquid secondary-ion mass (LSIMS) and UV-visible spectroscopy.



Ac-Orn(PyCO)-NHMe — **4**

Ac-Val-Orn(PyCO)-Leu-NHMe — **5**

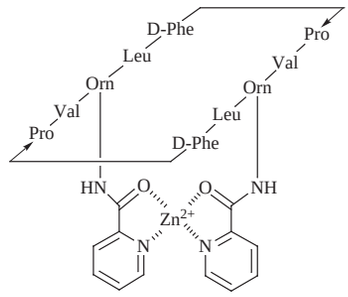


Fig. 1 Proposed structure of 1 : 1 [Orn(PyCO)$^{2,2'}$]GS **1**–Zn^{2+} complex.

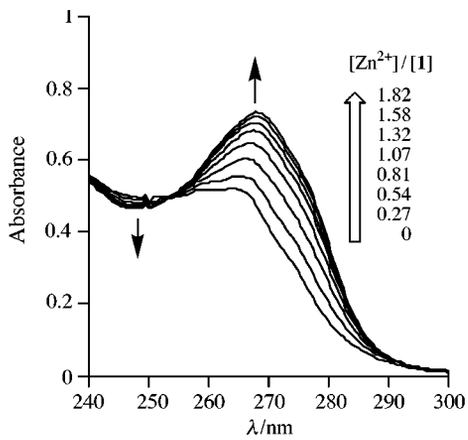


Fig. 2 Absorption spectra of [Orn(PyCO)$^{2,2'}$]GS **1** with the addition of $ZnCl_2$ (MeOH; 25 °C; [**1**] = 5.0 × 10^{-5} M, [$ZnCl_2$] = 0–9.1 × 10^{-5} M).

Metal ion-binding analysis

Complexation ability of the picolinoyl peptides **1**–**5** with Zn^{2+} and Cu^{2+} ions was examined in MeOH at 25 °C using UV-visible absorption spectral changes induced by complexation. The following equilibria were assumed and the binding constants, $K_{1:1}$, $K_{1:2}$ and $K_{2:1}$, were defined according to equations (1)–(3).

$$L + M = ML\ (1:1\ \text{complex}) \quad K_{1:1} = [LM]/[L][M] \quad (1)$$

$$LM + M = LM_2\ (1:2\ \text{complex}) \quad K_{1:2} = [LM_2]/[LM][M] \quad (2)$$

$$LM + L = L_2M\ (2:1\ \text{complex}) \quad K_{2:1} = [L_2M]/[LM][L] \quad (3)$$

L: PyCO-carrying peptide derivatives.
M: metal ion (Cu^{2+} or Zn^{2+}).

Dipicolinoyl GS derivative 1. The pyridine N atoms and amide O atoms of the two PyCONH groups of [Orn(PyCO)$^{2,2'}$]GS **1** are assumed to participate in the coordination to a Zn^{2+} ion forming a 1 : 1 complex[3] as schematically shown in Fig. 1. Upon addition of $ZnCl_2$, the absorption maximum of the dipicolinoyl derivative **1** at 264 nm (ε 11 200 dm^3 mol^{-1} cm^{-1}) showed hyperchromic and bathochromic shifts, exhibiting an isosbestic point at 253 nm (Fig. 2). Nonlinear least-squares curve-fitting analysis[8] based on the absorbance change at 272 nm indicated the formation of a 1 : 1 complex with the log $K_{1:1}$ value of 5.28. The CD spectrum of compound **1** with a weak positive maximum around 290 nm ([θ] + 800) and a negative shoulder around 265 nm also changed upon the addition of $ZnCl_2$ to a distinct negative maximum at 273 nm ([θ] − 6200) with an isoelliptic point at 270 nm (Fig. 3). The log $K_{1:1}$-value obtained from the change of ellipticity at 280 nm was in good agreement with that obtained by absorption spectral analysis. No evidence of formation of a 1 : 2 or 2 : 1 complex was observed over the range of [**1**] : [Zn^{2+}] from 1 : 0.24 to 1 : 1.9.

Addition of $CuCl_2$ to a solution of compound **1** was also

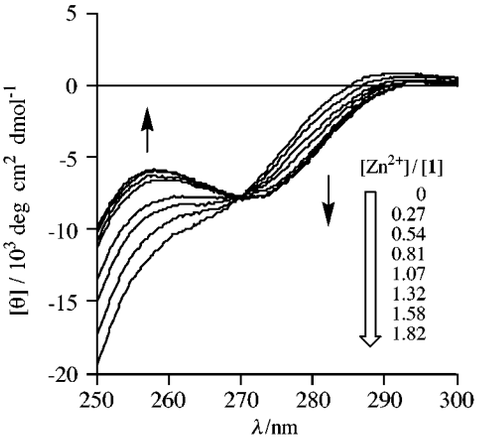


Fig. 3 CD spectra of [Orn(PyCO)2,2′]GS **1** with the addition of $ZnCl_2$ (MeOH; 25 °C; [**1**] = 5.0×10^{-5} M, [$ZnCl_2$] = 0–9.1×10^{-5} M).

shown to produce a 1 : 1 complex, but in the presence of an excess of Cu^{2+} additional spectral change was observed indicating the formation of a 1 : 2 complex. The formation constants of the 1 : 1 and 1 : 2 complexes with Cu^{2+} were determined similarly by the curve-fitting analysis using equations (1) and (2), and the log $K_{1:1}$- and log $K_{1:2}$-values were obtained as 7.64 and 5.72, respectively.

Thus, the dipicolinoyl derivative **1**, as expected, formed a 1 : 1 complex both with Zn^{2+} and Cu^{2+}, indicating the coordination of the two PyCONH groups to the divalent metal ions. In the case of Cu^{2+}, however, the 1 : 2 species was also formed in which each of the two picolinoylamido groups of **1** was considered to bind a Cu^{2+} ion. The pyridine N atom and ionized amide N atom of the picolinoylamide group are assumed to coordinate Cu^{2+} ion[3] although no structural study of these complexes was undertaken.

Monopicolinoyl GS derivatives 2 and 3. In order to investigate Cu^{2+}-mediated assembly of the monopicolinoyl derivative [Orn(Boc)^2,Orn(PyCO)$^{2'}$]GS **2**, the absorption spectral change of the *d*–*d** band of Cu^{2+} ion due to complexation was monitored. Keeping the total concentration of Cu^{2+} species constant ([$CuCl_2$] = 1 mM), aliquots of MeOH solution of compound **2** containing $CuCl_2$ were added to the solution of $CuCl_2$. As shown in the difference spectra, complex-formation-induced increase of the absorption around 650 nm was observed upon the addition of compound **2** (Fig. 4a). The plots of the absorbance change (ΔA) at 800, 750, 700 and 650 nm *vs.* [**2**]/[$CuCl_2$] gave an inflection point around [**2**]/[$CuCl_2$] = 1, suggesting the stepwise formation of 1 : 1 and 2 : 1 complexes (Fig. 4b). Nonlinear least-squares curve-fitting analysis based on equations (1) and (3) yielded the complex-formation constants as log $K_{1:1}$ = 5.22 and log $K_{2:1}$ = 3.08.

The reference compounds Ac-Orn(PyCO)-NHMe **4** and Ac-Val-Orn(PyCO)-Leu-NHMe **5**, possessing the partial structures of compound **2**, were also subjected to the Cu^{2+} ion-binding analysis, and the results are summarized in Table 1. The $K_{2:1}$ value of the GS derivative **2** is larger than those of the linear compounds **4** and **5** (log $K_{2:1}$ = 2.53 and 2.62, respectively). The relative stability of the Cu^{2+}-mediated dimeric GS species could be attributed to inter-β-sheet H-bonding interaction between the two GS units in the complex. The 'cross-β' aggregation tendency of the GS molecule in oriented poly(oxyethylene) was indicated by an IR dichroism study.[9] Seto and Whitesides also reported intermolecular H-bonding-mediated aggregation of the di-Boc derivative [Orn(Boc)$^{2,2'}$]GS in $CHCl_3$ solution studied by vapour pressure osmometry.[10] The possible structure of the 2 : 1 complex composed of the antiparallel β-sheet type assembly of four Val-Orn(PyCO)-Leu strands is illustrated in Fig. 5, the structure might also be stabilized by hydrophobic

Table 1 The formation constants of Cu^{2+} complex with PyCONH-containing peptides in MeOH at 25 °C

Peptide	log $K_{1:1}$	log $K_{2:1}$	log $K_{1:2}$
1	7.64 ± 0.2		5.72 ± 0.15
2	5.22 ± 0.1	3.08 ± 0.05	
3	5.18 ± 0.1	2.67 ± 0.1	
4	5.15 ± 0.15	2.53 ± 0.1	
5	5.06 ± 0.1	2.62 ± 0.05	

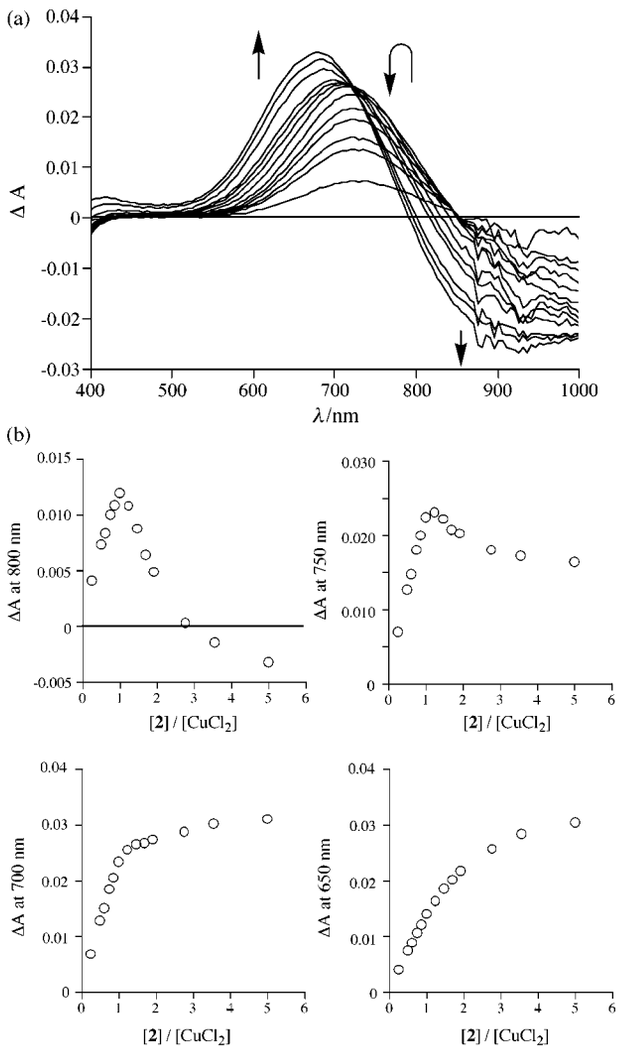


Fig. 4 Absorption spectral change of $CuCl_2$ in MeOH with the addition of the monopicolinoyl derivative **2** (25 °C; [$CuCl_2$] = 1.0×10^{-3} M, [**2**] = 0.26–5.0×10^{-3} M). (a) Difference spectra are given. ΔA refers to the absorbance of the solution less the absorbances of the component solutions of $CuCl_2$ and compound **2** at the corresponding concentrations. (b) Plots of ΔA *vs.* [**2**]/[$CuCl_2$] at 800, 750, 700 and 650 nm are shown.

interaction between ^{i}Pr and ^{i}Bu groups of Val and Leu residues.

In order to study the importance of the intermolecular H-bonding interaction for the formation of the 2 : 1 complex, the tetra-*N*-methyl derivative of compound **2**, namely [MeOrn-(Boc)^2,MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS **3**, was prepared. Prevention of aggregation of β-strands by the introduction of *N*-methyl group(s) was reported for a three-stranded β-sheet peptide[11] and an interleukin monomer.[12] It is known that tetra-*N*-methylation does not affect the β-sheet conformation of GS.[6] The tetra-*N*-methyl derivative **3** lacking α-NH groups of the Orn and D-Phe residues required for intermolecular H-bonding interaction was subjected to the metal ion-binding analysis. As

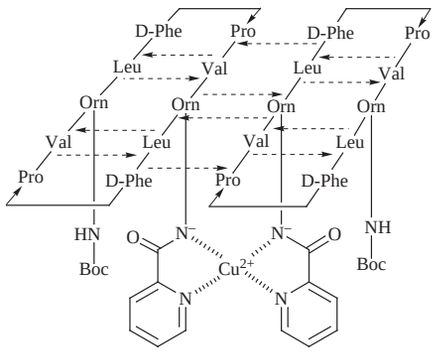


Fig. 5 Illustration of the proposed structure of 2:1 [Orn(Boc)^2,Orn-(PyCO)$^{2'}$]GS **2**–Cu^{2+} complex. Broken arrows indicate H-bonds constituting a four-strand antiparallel β-sheet. The intermolecular H-bonds are impossible for the tetra-*N*-methylated derivative **3**.

expected, compound **3** exhibited lower stability of its 2:1 complex (log $K_{2:1} = 2.67$) than did analogue **2**, which is in agreement with the proposed antiparallel β-sheet-type assembly of the two GS units in the case of compound **2**. Very recently we have synthesized dimeric analogues of GS which actually possess a methylene-chain bridge between the δ-N atoms of the two GS units.[13] Preliminary ^{1}H NMR spectral studies of the synthetic trimethylene-bridged precursor molecules have indicated the presence of β-sheet-type H-bonding interaction between the two cyclic decapeptide modules. The dimeric derivatives can be considered as synthetic models of the inter-Orn side chain interaction-mediated assembly of GS molecules validating the proposed structure of the 2:1 complex illustrated in Fig. 5. Detailed studies on the conformational characteristics of these δ-*N*(Orn)–δ-*N*(Orn′) covalently linked dimeric compounds are in progress.

In the present study the GS derivative **2** carries a metal ion-binding group on the δ-N atom of an Orn residue while another Orn side chain is protected with a Boc group which can be replaced with different functional groups to construct a functional assembly system. However, although formation of the expected metal ion-mediated dimeric complex was observed for the GS derivative–Cu^{2+} system, the stability of the 2:1 complex was not high enough compared with that of the 1:1 complex. Thus, stepwise formation of the 1:1 and 2:1 complexes was observed when the GS derivative was added to the metal ion solution and the 2:1 complex became prominent only after the addition of more than 1 equivalent of the peptide to Cu^{2+}. In order to realize the system in which formation of the dimeric species can be controlled by the addition of a metal ion, a higher tendency to form a metal ion-mediated dimeric complex is necessary. Studies on the construction of self-assembly systems using metal ion-binding peptide derivatives which carry pyridine ring-containing groups other than PyCO are in progress.

Experimental

Mps were determined on a hot-plate apparatus and are uncorrected. Column chromatography was performed using SiO_2 (Fuji Sylisia, FL60D). Gel filtration chromatography (GFC) was performed using Sephadex LH-20 (Pharmacia Biotech) with MeOH as eluent. Monitoring of reactions and analysis of chromatographic fractions were undertaken by means of TLC with precoated SiO_2 plate (Merck, Silica gel $60F_{254}$). Purity of all of the synthetic compounds was ascertained by HPLC analysis (JASCO, Finepak-SIL, 4.6 × 50 mm, elution with $CHCl_3$–MeOH). ^{1}H NMR spectra were recorded in [$^2H_6$]-DMSO solutions at 25 °C on a Varian Gemini-200 (200 MHz) or a JEOL α-500 (500 MHz) spectrometer. *J*-Values are in Hz. UV-visible and CD spectra were recorded in MeOH solution on a HITACHI U-3500 spectrometer and a JASCO J-600 spectropolarimeter, respectively. SIMS were measured on a Hitachi M-2000 spectrometer. Elemental analyses were done at the Elemental Analysis Center of Kyoto University.

[Orn(PyCO)$^{2,2'}$]GS 1

A solution of $PyCO_2H$ (372 mg, 3.0 mmol) and 1,1′-carbonyldiimidazole (CDI, 487 mg, 3.0 mmol) in dry THF (10 cm^3) was stirred at rt under argon for 30 min, to which was added a solution of GS·2HCl (413 mg, 0.5 mmol) in dry THF and reaction mixture was stirred at rt for 2 h. After removal of precipitated imidazole hydrochloride by filtration the filtrate was evaporated *in vacuo* and the residue was subjected to GFC. The crude product was purified by SiO_2 column chromatography using $CHCl_3$–MeOH (95:5 v/v) as eluent to afford *title compound* **1** (512 mg, 76%) as a powder (lyophilized); λ_{max}(MeOH)/nm 264 (ε/dm^3 mol^{-1} cm^{-1} 11 200); $[\theta]_{max}$(MeOH)/nm 206 ([θ]/deg cm^2 dmol^{-1} -4.4×10^5), 217 (-4.3×10^5), 265_{sh} (-9.5×10^3), 285 (0) and 292 (800) [Found: C, 62.3; H, 7.1; N, 14.0. $C_{72}H_{98}N_{14}O_{12}\cdot 2H_2O$ requires C, 62.3; H, 7.4; N, 14.1%; *m*/*z* (SIMS) 1351.7642 (M + H)$^+$. ($C_{72}H_{98}N_{14}O_{12}$ + H) requires *m*/*z*, 1351.7544].

[Orn(Boc)^2,Orn(PyCO)$^{2'}$]GS 2

To a stirred solution of GS·2HCl (242 mg, 0.2 mmol) in DMF were added PyCOCl·HCl (76 mg, 0.4 mmol), Et_3N (0.172 cm^3, 1.2 mmol) and DMAP (28 mg, 0.2 mmol) at rt. After being stirred for 2 days at rt the reaction mixture was lyophilized and was subjected to gel filtration chromatography. The crude product was then chromatographed over SiO_2 using $CHCl_3$–MeOH (9:1 v/v) as eluent to yield **1** (31 mg, 12%) and [Orn(PyCO)^2]GS (79 mg, 32%); λ_{max}(MeOH)/nm 264 (ε/dm^3 mol^{-1} cm^{-1} 6400); *m*/*z* (SIMS) 1248.1 (M + H)$^+$]. The latter compound (34 mg, 0.027 mmol) was dissolved in $CHCl_3$–MeOH (3:1 v/v) (4 cm^3), to which were added $(Boc)_2O$ (41 mg, 0.19 mmol) and Et_3N (0.027 cm^3, 0.19 mmol). After stirring the mixture at rt for 5 h, the solvent was evaporated *in vacuo*. The crude product was purified by GFC and then SiO_2 column chromatography using $CHCl_3$–MeOH (95:5 v/v) as eluent to afford *title compound* **2** (36 mg, 98%) as a powder (lyophilized); λ_{max}(MeOH)/nm 264 (ε/dm^3 mol^{-1} cm^{-1} 7000); $[\theta]_{max}$(MeOH)/nm 206 ([θ]/deg cm^2 dmol^{-1} -5.2×10^5), 216 (-4.8×10^5) and 264_{sh} (-2.3×10^3) [Found: C, 61.3; H, 7.7; N, 13.25. $C_{71}H_{103}N_{13}O_{13}\cdot 2.5H_2O$ requires C, 61.3; H, 7.8; N, 13.1%; *m*/*z* (SIMS) 1346.7842 (M + H)$^+$. ($C_{71}H_{103}N_{13}O_{13}$ + H) requires *m*/*z*, 1346.7852].

[MeOrn(Boc)^2,MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS 3

***via* selective N-methylation of [Orn(Boc)$^{2,2'}$]GS.** [MeOrn-(Boc)$^{2,2'}$,D-MePhe$^{4,4'}$]GS was prepared from [Orn(Boc)$^{2,2'}$]GS as described in ref. 5. The tetra-*N*-methylated Boc derivative (104 mg, 0.074 mmol) was treated with TFA (1 cm^3) at 0 °C for 1 h. TFA was evaporated off *in vacuo* and the residue was dissolved in 1 M HCl (10 cm^3) and lyophilized to give [MeOrn$^{2,2'}$,D-MePhe$^{4,4'}$]GS·2HCl (112 mg, ≈100%). Picolinoylation of the tetra-*N*-methyl derivative (102 mg, 0.080 mmol) using PyCOCl·HCl (29 mg, 0.16 mmol) in the same manner as described above, followed by chromatographic separation (SiO_2; $CHCl_3$–MeOH, 9:1 v/v), yielded *[MeOrn(PyCO)$^{2,2'}$,D-MePhe$^{4,4'}$]GS* (20 mg, 23%) (Found: C, 63.6; H, 7.5; N, 13.3. $C_{76}H_{108}N_{14}O_{12}\cdot 1.5H_2O$ requires C, 63.5; H, 7.8; N, 13.6%) and [MeOrn^2,MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS (20 mg, 19%). The monopicolinoyl derivative (40 mg, 0.030 mmol) was treated with $(Boc)_2O$ and Et_3N to afford *[MeOrn(Boc)^2,MeOrn(PyCO)$^{2'}$,D-MePhe$^{4,4'}$]GS* **3** (26 mg, 62%) [Found: C, 63.2; H, 7.8; N, 12.65. $C_{75}H_{111}N_{13}O_{13}\cdot H_2O$ requires C, 63.4; H, 8.0; N, 12.8%. *m*/*z* (SIMS) 1403.4 (M + H)$^+$. $C_{75}H_{111}N_{13}O_{13}$ requires *m*/*z*, 1403.9].

***via* selective N-methylation of compound 2.** To a stirred solution of compound **2** (68 mg, 0.050 mmol) in DMF were added

Ag_2O (232 mg, 1.00 mmol) and methyl iodide (2.5 cm^3, 40 mmol) at rt. The reaction mixture was stirred for 1 day and lyophilized to remove the solvent. After GFC, the crude product was chromatographed with aminopropyl-modified SiO_2 (Fuji Sylisia, Chromatorex NH-DM1020) using $CHCl_3$–MeOH (98 : 2 v/v) as eluent to afford compound **3** (48 mg, 68%), which was indistinguishable from the compound prepared from [MeOrn$^{2,2'}$,D-MePhe$^{4,4'}$]GS·2HCl as described above.

Ac-Orn(PyCO)-NHMe 4

A mixture of Boc-Orn(Cbz)-OH (1.43 g, 3.69 mmol), $MeNH_2$·HCl (621 mg, 9.20 mmol) and HOBt (450 mg, 3.33 mmol) in CH_2Cl_2 (15 cm^3) was neutralized by the addition of Et_3N, to which DCC (1.49 g, 7.21 mmol) was added under ice-cooling. After stirring of the reaction mixture at rt for 23 h the solvent was evaporated off *in vacuo* and to the residue were added AcOEt and 10% aq. citric acid. After removal of precipitated dicyclohexylurea by filtration the AcOEt layer was washed successively with 10% aq. citric acid, 5% aq. $NaHCO_3$ and saturated aq. NaCl and was then dried over Na_2SO_4. The solvent was evaporated off and the residue was subjected to SiO_2 column chromatography using $CHCl_3$ as eluent to afford Boc-Orn(Cbz)-NHMe as a solid, mp 142–143.5 °C (1.00 g, 68%).

The N-methylamide (1.00 g, 2.65 mmol) as a solution in MeOH (5 cm^3) was hydrogenolysed under atmospheric H_2 over Pd-black (0.35 g) for 2 h. Filtration, and evaporation off of the solvent, yielded crude Boc-Orn-NHMe, which was added to a solution of picolinoylimidazole reagent prepared by stirring $PyCO_2H$ (0.509 g, 4.14 mmol) and CDI (0.559 g, 3.45 mmol) in dry THF (5 cm^3) for 1 h. After stirring of the mixture for 15 h at rt the solvent was evaporated off *in vacuo* and the residue was chromatographed over SiO_2 using $CHCl_3$–MeOH as eluent to give Boc-Orn(PyCO)-NHMe as an oil (0.958 g, 94%).

The picolinoyl compound (0.956 g, 2.50 mmol) was treated with TFA (3 cm^3) at 0 °C for 2 h. After evaporation off of TFA *in vacuo* the crude product was dissolved in dry pyridine (10 cm^3) and excess of TFA was neutralized by addition of Et_3N to which Ac_2O (4 cm^3) had been added, and the mixture was stirred for 1.5 h. The volatiles were removed *in vacuo*, and the resulting crude product was purified by SiO_2 column chromatography using $CHCl_3$ as eluent and crystallization from MeOH–Et_2O to yield *title compound* **4** as crystals (0.419 g, 57%), mp 190–192 °C; δ_H(200 MHz; [$^2H_6$]DMSO) 1.82–1.64 (4H, m), 1.84 (3H, s), 2.80 (3H, d, *J* 4.8), 3.45 (1H, m), 3.75 (1H, m), 4.65 (1H, m), 6.71 (1H, d, *J* 8.2), 6.82 (1H, q, *J* 4.8), 7.44 (1H, ddd, *J* 7.7, 4.8 and 1.2), 7.86 (1H, dt, *J* 7.7 and 1.7), 8.18 (1H, ddd, *J* 7.7, 1.2 and 0.9), 8.24 (1H, br t, *J* 6.6) and 8.55 (1H, ddd, *J* 4.8, 1.7 and 0.9) {Found: C, 57.65; H, 6.9; N, 19.1%; $[M + H]^+$ (SIMS), 292.9. $C_{14}H_{20}N_4O_3$ requires C, 57.5; H, 6.9; N, 19.2%; $[M + H]^+$, *m*/*z*, 292.2}.

Ac-Val-Orn(PyCO)-Leu-NHMe 5

Boc-Leu-NHMe (1.10 g, 4.51 mmol), which was prepared in 90% yield from Boc-Leu-OH and $MeNH_2$·HCl in a similar manner to the preparation of Boc-Orn(Cbz)-NHMe as described above, was treated with TFA (2.5 cm^3) at rt for 2 h. TFA was evaporated off *in vacuo* and the residue was dissolved in CH_2Cl_2 (6 cm^3) and neutralized by addition of Et_3N.

Boc-Orn(Cbz)-OH (2.03 g, 5.54 mmol) was added to the solution of H-Leu-NHMe and the mixture was cooled to 0 °C, to which HOBt (450 mg, 3.33 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.13 g, 5.90 mmol) were added. After stirring of the reaction mixture at rt for 21 h the solvent was evaporated off *in vacuo* and the residue was dissolved in AcOEt. The solution was washed successively with 10% aq. citric acid, 5% aq. $NaHCO_3$ and saturated aq. NaCl and was then dried over Na_2SO_4. The solvent was evaporated off and the residue was chromatographed over SiO_2 using $CHCl_3$–MeOH as eluent to give Boc-Orn(Cbz)-Leu-NHMe as a solid (1.52 g, 69%).

After treatment of the protected dipeptide methylamide (465 mg, 0.945 mmol) with TFA as described above, the resulting H-Orn(Cbz)-Leu-NHMe was subjected to a coupling reaction with Boc-Val-OH (294 mg, 1.35 mmol), using DCC (542 mg, 2.63 mmol) and HOBt (101 mg, 0.75 mmol) in CH_2Cl_2 (2 cm^3) in a similar manner to the preparation of Boc-Orn(Cbz)-NHMe. After stirring of the reaction mixture for 4 h it was treated similarly and the crude product was subjected to SiO_2 column chromatography using $CHCl_3$ as eluent to afford Boc-Val-Orn(Cbz)-Leu-NHMe as crystals (467 mg, 84%), mp 176–178 °C (from Et_2O–MeOH).

The Cbz group of the protected tripeptide was replaced by a PyCO group in a similar manner as described for the preparation of Boc-Orn(Cbz)-NHMe to give Boc-Val-Orn(PyCO)-Leu-NHMe in 66% yield. The Boc group was then replaced by an Ac group also in a similar manner as above to yield the *desired product* **5** as a powder (66%), mp 256–256.8 °C (lyophilized) [Found: C, 58.7; H, 7.8; N, 16.1. $C_{25}H_{40}N_6O_5$·0.5H_2O requires C, 58.45; H, 8.05; N, 16.35%; *m*/*z* (SIMS) 505.0 $(M + H)^+$. $C_{25}H_{40}N_6O_5$ requires *M*, 504.3].

Metal ion-binding analysis

Analyses were repeated several times with varying concentrations of the initial solution and the added solution of the peptide and metal ion. Typical examples are given below.

Dipicolinoyl derivative 1. To a MeOH solution of compound **1** (5.2×10^{-5} M; 2.5 cm^3) were added aliquots (1.2×10^{-4} cm^3) of a MeOH solution of $CuCl_2$ (2.8×10^{-3} M) containing compound **1** (5.2×10^{-5} M) at 25 °C. Thus, while the concentration of **1** was kept constant, that of $CuCl_2$ was changed from 0.11 to 1.4×10^{-4} M. After each addition absorption spectrum was recorded and the absorbance changes at 272 nm were subjected to curve-fitting analysis.[8] Essentially the same method was applied to the analysis of CD spectral changes of compound **1** with the addition of the metal ion.

Monopicolinoyl derivatives 2 and 3. To a MeOH solution of $CuCl_2$ (1.00×10^{-3} M; 2.5 cm^3) were added aliquots (7.50×10^{-4} cm^3) of a MeOH solution of a compound **2** or **3** (2.50×10^{-2} M) containing $CuCl_2$ (1.00×10^{-3} M) at 25 °C. The concentration of the peptide ranged from 0.26 to 4.99×10^{-3} M. The absorbance changes at 650, 700, 750 and 800 nm were used for the analysis. Spectral changes upon the addition of $CuCl_2$ to the peptide solution as described for compound **1** were also recorded (peptide 9.90×10^{-6} M, $CuCl_2$ 0.049–1.30×10^{-4} M). All these data were included for the estimation of the $K_{1:1}$- and $K_{2:1}$-values.

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