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Synthesis of [5-Bipyridylalanyl$^{2,2'}$]gramicidin S and Its Complexation with Divalent Metal Ions

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A gramicidin S (GS) analog ([Bpa$^{2,2'}$]GS), in which the Orn residues are replaced by L-5-bipyridylalanines, was synthesized by the solid-phase-synthesis and cyclization-cleavage method. The analog formed stable 1:1 complexes with transition metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} in CH_3OH without significant conformational change as shown by the ^{1}H-NMR and circular dichroism analyses. These peptide-metal complexes are water-soluble and will be used in various models of artificial metalloproteins based on the β-sheet structure.

The stable complexation of metal ions becomes more and more important in the *de novo* design of the artificial proteins. In this purpose, Imperiali *et al.* have synthesized the linear peptides with 6-bipyridylalanines and the Pro-D-Ala turn structure.[1,2] However, the metal complexes reported were not much stable probably because of the flexibility of their acyclic peptides and the steric hindrance of 6-bipyridylalanine toward the metal ions. On the other hand, Gramicidin S (GS) is an antimicrobial decapeptide with rigid cyclic structure containing two β-turns (D-Phe-Pro) and a set of antiparallel β-strands (Fig. 1).[3] GS has amphiphilic feature and the hydrophobic Val and Leu residues are considered to penetrate the lipid membrane. The Orn residues afford the hydrophilic property on the other side of the molecule. Because these Orn residues are faced with each other, the replacement of Orn to 5-bipyridylalanine (Bpa) would build a "metal ion's nest". Thus, we chose the cyclic framework of GS and Bpa for the design of a stable metalloprotein model based on the β-structure.

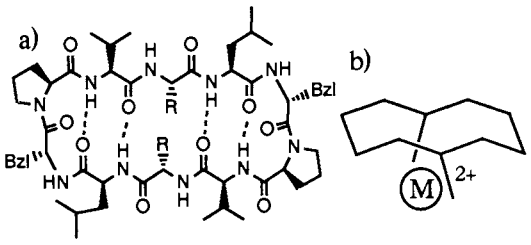

Fig. 1. a) GS (R =-$(CH_2)_3NH_2$) and [Bpa$^{2,2'}$]GS (1) (R = -CH_2-(2,2'-bipyrid-5-yl)). b) Metal-1 complex.

L-5-Bipyridylalanine was synthesized according to the literature.[1] The solid-phase-synthesis and cyclization-cleavage method with Kaiser's oxime resin was successfully applied for [Bpa$^{2,2'}$]GS (1), *via* *t*-butyloxycarbonyl-(D-Phe-Pro-Val-Bpa-Leu)$_2$-oxime resin.[4] The production of 1 during the cyclization-cleavage was monitored by HPLC and completed within 3 hours. The pure 1

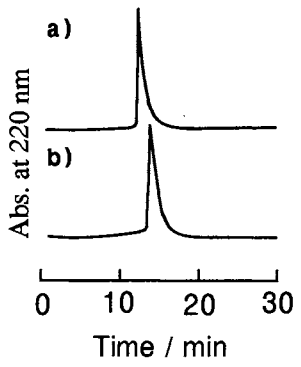

Fig. 2. HPLC profiles of a) 1 and b) Zn^{2+} (1). See ref. 5 for elution condition.

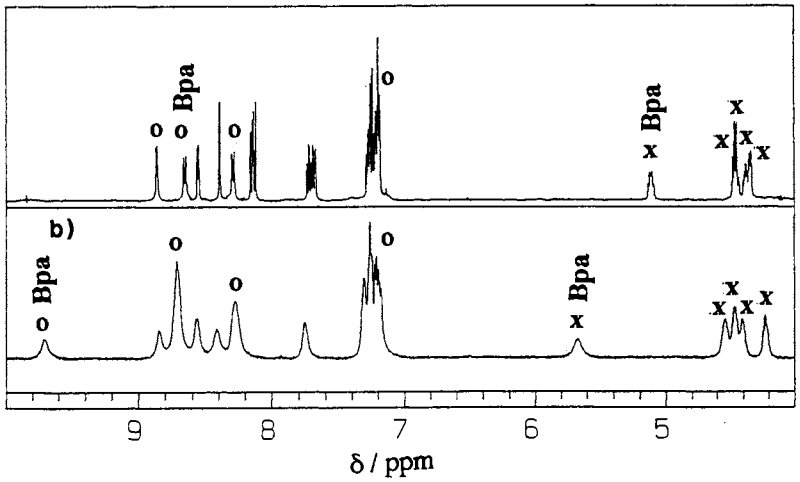

Fig. 3. ^{1}H-NMR spectra of (a) **1** and (b) Zn^{2+}/**1** in DMSO-d_6 at 298 K. [peptide] = 1.5 x 10^{-3} mol·dm^{-3}. o; amide protons, x; α-protons.

Table 1. Temperature coefficients of amide proton chemical shifts of GS, **1**, and Zn^{2+}(**1**) in DMSO-d_6.[a]

Amide proton	Temperature coefficient / ppb·K^{-1}		
	GS[b]	**1**	Zn^{2+}(**1**)
Val	-2.2	-1.3	-1.0
Orn	-5.4	-	-
Bpa	-	-9.1	-11.7
Leu	-3.3	-2.4	-2.9
D-Phe	-8.1	-6.2	-7.6

a) [peptide] = 1.5x10^{-3} mol·dm^{-3}.
b) Data from Ref. 6.

was obtained in 46% yield after the silica gel chromatography with 3% (v/v) CH_3OH in $CHCl_3$ (Fig. 2a).[5]

In the ^{1}H-NMR spectrum of **1** in DMSO-d_6 (Fig. 3a), only one set of the amide proton signals is observed for four amino acid residues (the assignments of the signals were determined by the ^{1}H-^{1}H COSY spectrum). The replacement of the Orn residues to Bpa retained the C_2 symmetry of GS. Table 1 summarizes the temperature coefficients of the amide proton chemical shifts (298-338 K). The chemical shift of the amide proton occupied in the intramolecular hydrogen bonding is known to show a little temperature dependence (*ca.* > -3 ppb·K^{-1}).[6] As depicted in Figure 1a, the Val and Leu residues of **1** participated in the intramolecular hydrogen bonding, and the Bpa and D-Phe groups not. Probably D-Phe-Pro moieties in **1** constructed the type

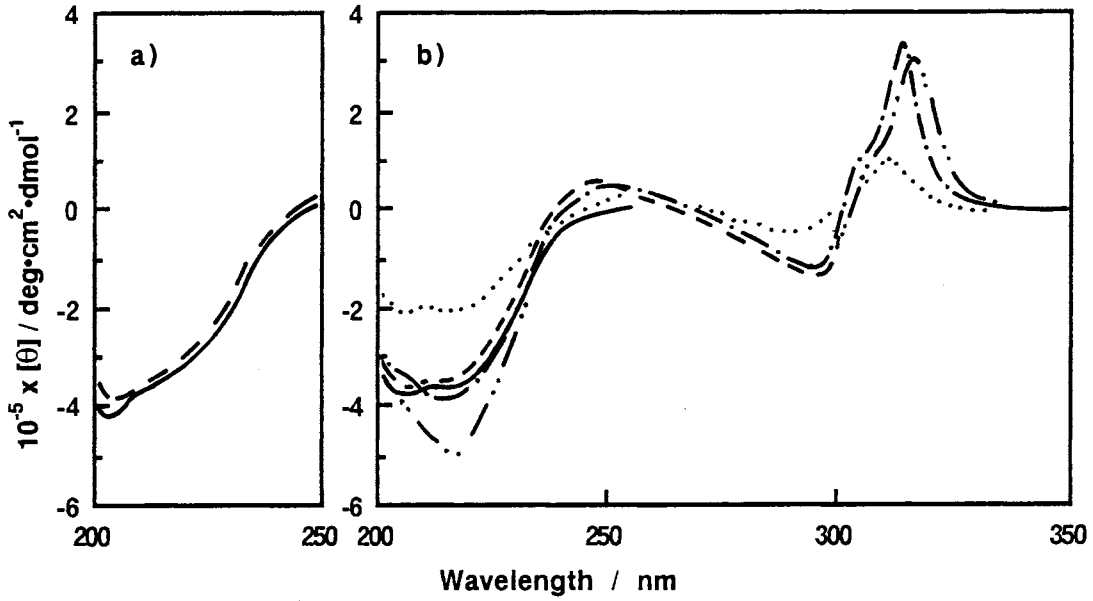

Fig. 4. CD spectra of a) (——) GS and (– –) **1** in CH_3OH, b) (——) GS, (·····) Co^{2+}/**1**, (– –) Ni^{2+}/**1**, (–··–) Cu^{2+}/**1**, and (–·–) Zn^{2+}/**1** in H_2O. [peptide] = 3.0 x 10^{-5} mol·dm^{-3}.

II' β-turns like the natural GS. The circular dichroism (CD) spectra in CH_3OH also suggested that the conformation of **1** was not so much different from the natural GS (Fig. 4a). The characteristic CD bands were observed with double minima at 207 and 217 nm for both **1** and GS, which have been attributed to the GS backbone conformation (type II' β-turn and β-sheet structure).[3,6]

In the presence of the various divalent metal ions such as $Co(OAc)_2$ (OAc = acetate ion), $Ni(OAc)_2$, $Cu(OAc)_2$, and $Zn(OAc)_2$, **1** formed the peptide-metal (1:1) complexes. Figure 5a-d show the UV spectra of **1** with the addition of Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. By adding Zn^{2+} for instance, the absorption bands at 242 (ε = 21200) and 288 nm (ε = 30500) of **1** decreased and new absorption bands appeared at 257, 302, and 313 nm. The clear isosbetic points indicated that only one species was formed in the reaction of **1** with metal ion. The (1:1) molar ratios of M^{2+} to **1** in the products were determined by the UV-titration method (Fig. 5e). The formation of $M^{2+}(Bpa)_2$ moiety on the GS framework (Fig, 1b) was thus ascertained. The Zn^{2+}(**1**) (Zn^{2+}[$Bpa^{2,2'}$]GS) showed fluorescence spectra (excitation at 313 nm, emission maximum at 340 nm) characteristic to the d^{10} complexes.[7] Moreover, these metal complexes were water-soluble, while **1** or **1**/NaOAc mixture was not soluble. The FAB-MS also indicated the formation of the stable Zn^{2+}(**1**) complex: 1428 (M^+) and 1430 ($M+2^+$) were detected in (2:1) ratio, which is the isotope pattern of mono-zinc compounds. No signal was observed for the free **1** or Zn_2/**1** (*m/z* = 1492). Furthermore, the HPLC analysis of Zn^{2+}(**1**) only showed a peak at 14.66 min (Fig. 1b), in which the free **1** (if existed) should show its peak at 13.25 min. Thus, Zn^{2+}(**1**) was stable as to be detected by the FAB-MS and HPLC. The dissociation constant K_d for Zn^{2+}(**1**) was measured by the UV-Vis method, adding Zn^{2+} to the large excess of **1** in CH_3OH. K_d was estimated to be 8.8×10^{-5} mol·dm^{-3} in CH_3OH. This value of K_d implies the stability of Zn^{2+}(**1**) compared with the Zn^{2+} complex reported by

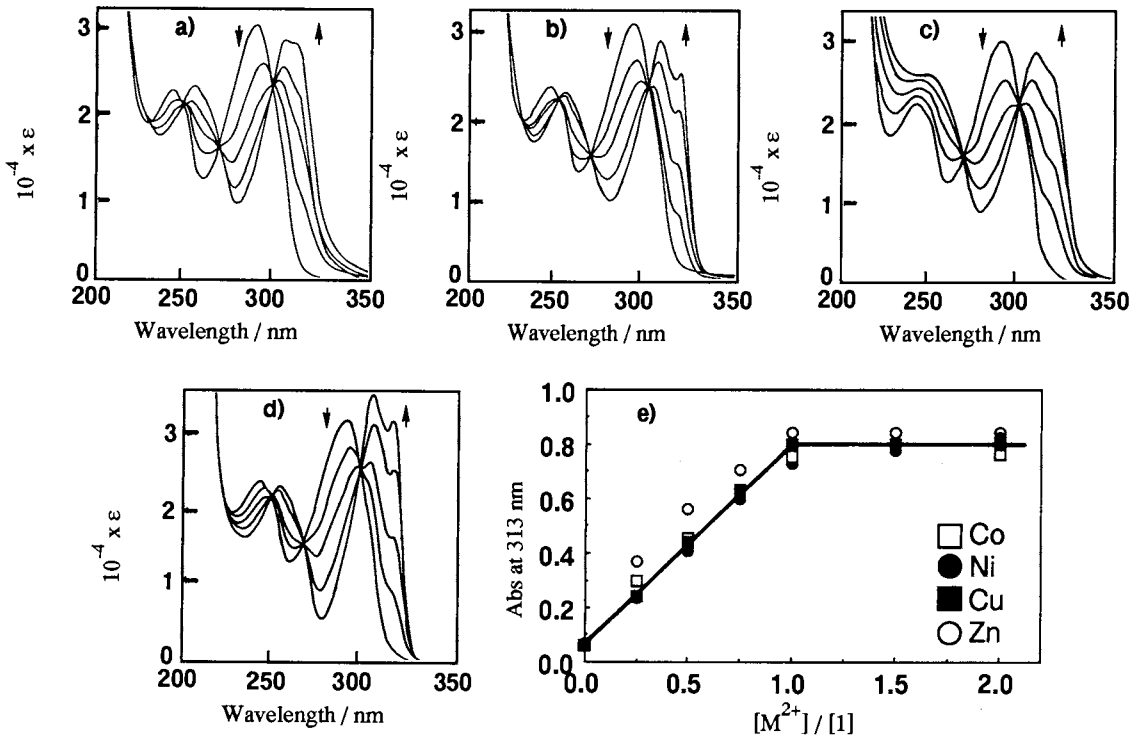

Fig. 5. UV-Vis spectra of **1** with the addition of a) $Co(OAc)_2$, b) $Ni(OAc)_2$, c) $Cu(OAc)_2$, and d) $Zn(OAc)_2$ in CH_3OH. **1** = 3.0×10^{-5} mol·dm^{-3} and the metal ions are 0, 0.25, 0.5, 0.75, and 1.0 equiv. to **1**. e) Absorption maxima of metal-**1** complexes vs. the molar ratio of metal ions to **1**.

Imperiali *et al.* (9.6×10^{-4} mol·dm^{-3} in H_2O).[1] The cyclic framework of GS enabled the stable formation of the metal complex. Zn^{2+}(**1**) was isolated by concentrating the CH_3OH solution of **1** and $Zn(OAc)_2$.[8]

^{1}H-NMR spectra of Zn^{2+}(**1**) were measured in the presence of excess $Zn(OAc)_2$ (5 equivalent to **1**). By using the ^{1}H-^{1}H COSY technique, all the signals were unambiguously assigned to the protons of Zn^{2+}(**1**), except that of the acetate ion (Fig. 3b). The signals of Bpa residue were shifted to the lower field by the addition of Zn^{2+}. For instance, amide proton of the Bpa residue showed its signal at 8.65 ppm in **1** and at 9.72 ppm in Zn^{2+}/**1**, and the α-proton at 5.11 ppm in **1** and 5.68 ppm in Zn^{2+}/**1**. The temperature coefficients of the amide protons (Table 1) indicated that the hydrogen bonding of Zn^{2+}(**1**) was all the same for the metal-free **1**. This fact suggested that the Zn^{2+} captured at the bipyridyl groups of **1** did not alter the conformation of the cyclic decapeptide unit. The CD spectra of **1** and the metal complexes in H_2O (Fig. 4b) also supported that the conformation of M^{2+}(**1**) was similar to the natural GS. The CD bands at 207 (shoulder) and 217 nm are typical to GS analogs. The CD bands at the longer wavelength region (257, 295, and 314 nm) suggested that the formation of the metal-bipyridine unit fixed the side chain of Bpa on the chiral GS framework. In addition, Zn^{2+}(**1**) exhibited antimicrobial activity as strong as the natural GS toward Gram-positive bacteria (*B. subtilis* ATCC 6633 and *S. aureus* ATCC 6538P; IC_{50}, 3.16×10^{-6} g·cm^{-3}). This fact also suggests the stability of the complex and the structural similarity between Zn^{2+}(**1**) and natural GS.

The *de novo* design of artificial proteins based on the β-sheet structure is little reported so far. The successful metalation of a pair of β-strands in GS may be expanded to stitch several β-strands in more sophisticated three-dimensional structure such as β-burrel.

References

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2) All amino acids are of L-configuration unless otherwise noted.
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5) **1**. TLC R_f 0.20 (5% (v/v) CH_3OH in $CHCl_3$); HPLC retention time 13.25 min on MS GEL C-18 (4.6x150 mm), CH_3CN 55-100% in H_2O (30 min), detected at 220 and 280 nm; FAB-MS (glycerol) 1365 ($M+1^+$); mp 285 °C (dec); Anal. Found:C, 64.00; H, 7.49; N. 13.33%. Calcd for $C_{76}H_{94}N_{14}O_{10}\cdot 3H_2O$:C, 64.39; H, 7.11; N. 13.83%.
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8) Zn^{2+}(**1**). mp > 360 °C; Anal. Found:C, 60.41; H, 7.02; N. 12.01%. Calcd for $C_{80}H_{102}N_{14}O_{14}Zn\cdot 3H_2O$: C, 60.01; H, 6.67; N. 12.25%.

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