

**3-HYDROXY-4(1*H*)-PYRIDINONE-CONTAINING LINEAR AND CYCLIC  
HEXAPEPTIDES AND THEIR IRON(III) COMPLEXES – SYNTHESIS,  
PROPERTY, AND THE GROWTH-PROMOTION ACTIVITY –**

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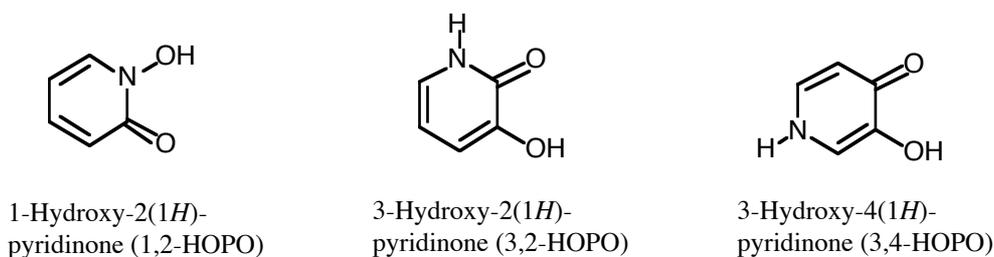
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**Abstract** — Linear and cyclic hexapeptides composed of  $\epsilon$ -(3-hydroxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucine and glycine or  $\beta$ -alanine residue have been successfully synthesized. These hexapeptides formed 1:1 iron(III) complexes at neutral pH region. Glycine linear (**8a**)- and  $\beta$ -alanine cyclic hexapeptide (**10**)-iron(III) complexes predominantly existed in  $\Delta$ -configuration, while  $\beta$ -alanine linear hexapeptide (**8b**)-iron(III) complex existed in  $\Lambda$ -one. The relative stability constants of **8a**, **8b**, and **10**-iron(III) complexes were estimated to be 31.6, 33.4, and 31.7, respectively. From iron(III) removal from human transferrin, linear hexapeptides (**8a,b**) were found to efficiently remove iron(III) compared with desferrioxamine B. The growth-promotion activity was also discussed.

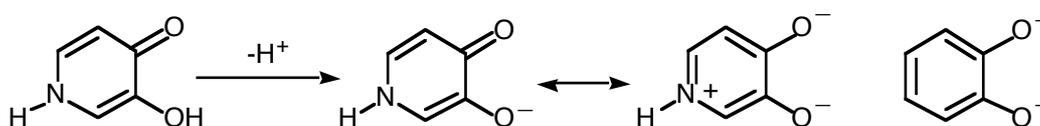
Iron is the fourth abundant element in the crust of the earth. Unfortunately, iron exists in extremely insoluble  $\text{Fe}(\text{OH})_3$  ( $K_{\text{sp}}=10^{-38}$ ) at physiological pH under the aerobic environment. In order to solubilize and uptake such low concentration of ferric iron, microorganisms excrete siderophores, low-molecular-weight iron-sequestering compounds. They solubilize ferric ion as the form of stable octahedral complexes and subsequently transport it into cell through the receptor on the surface of membrane. More than 200 siderophores have been isolated and characterized. They are primarily classified into two general structural classes, hydroxamates and catecholates. Three sets of these bidentate ligands scavenge iron(III) effectively and selectively by embedding it into an octahedral binding cavity.<sup>1-4</sup>

On the other hand, the iron overload disease arises from a long term blood transfusion for treatment of a certain anemia, particularly  $\beta$ -thalassemia. An excess iron deposit in liver and pancreas causes the damage of these organs or other serious diseases. Siderophores have paid much attention for treatment of the iron overload disease. Enterobactin, for example, has been shown to have a high stability constant toward iron(III) and to remove iron(III) from transferrin, but low acidity and difficulty in dissociation of two protons on the catechol ring at neutral pH limited the effectiveness of iron-sequestering.<sup>5</sup> Desferrioxamine

B (DFB, a commercial name: Desferal) has been shown to be an effective chelating agent for removing of an excess iron in body, and thus clinically used as a only choice of chemotherapeutic agent for a long time. Unfortunately it is orally inactive,<sup>6</sup> has a short half-life in blood plasma,<sup>7</sup> and possesses a number of side effects such as septicemia.<sup>8</sup> Many efforts, therefore, have been devoted to design and synthesize novel artificial siderophores in place of DFB. Recently, hydroxymonoazine-type ligands have received much attention because of an effective iron(III) removal, an oral activity, and no apparent toxicity.<sup>9</sup> Hexadentate ligands containing hydroxymonoazines such as 1,2-HOPO,<sup>10</sup> 3,2-HOPO,<sup>11</sup> and 3,4-HOPO,<sup>11b, 12</sup> which are expected to form stable iron(III) complexes by the chelate effect, have been intensively reported. However, no paper on the synthesis of chiral hexadentate ligands containing hydroxymonoazine-type heterocycles has been reported, to the best of our knowledge. In addition, *N*-substituted 2-alkyl-3-hydroxy-4(1*H*)-pyridinones were the first compounds among hydroxymonoazine-type heterocycles to enter clinical trials, and proved their potency as new chemotherapeutic agents for the iron overload disease.<sup>9, 13</sup>



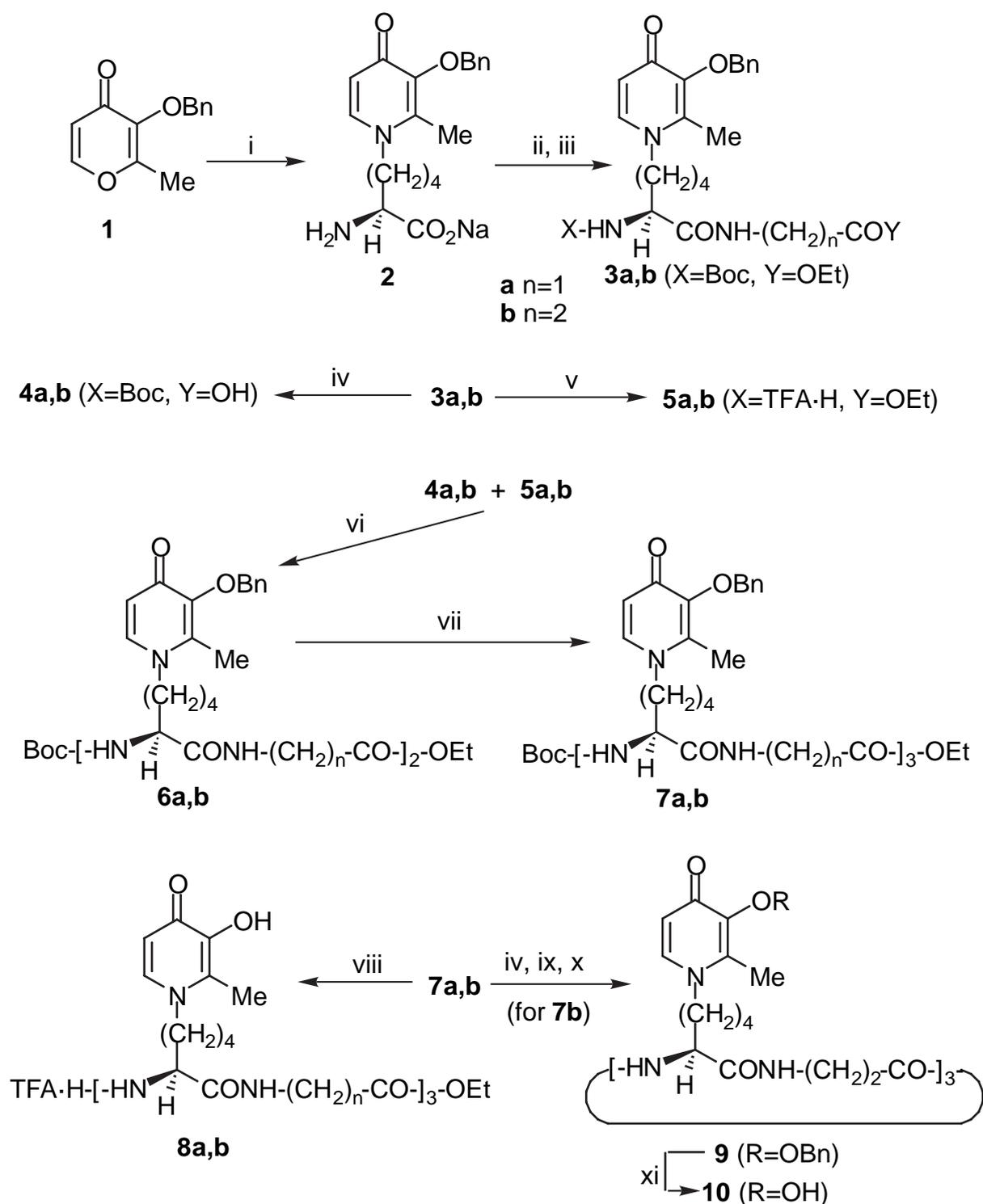
A resonance form of monoanion of 3,4-HOPO is isoelectronic to catechol dianion as shown below. It is, therefore, quite interesting to examine whether the present hexadentate ligands act as siderophores. A number of synthetic analogues have been prepared and the growth-promotion activity of them has been tested using siderophore auxotroph strains.<sup>12</sup> Two types of strains, *Aureobacterium flavescence* (ATCC 25091) and *Escherichia coli* RW193 (ATCC 33475), are generally used for testing the growth-promotion activity.<sup>14</sup>



In the past decade, we have intensively investigated design of functional molecules based on hydroxyazine-type heterocycles and application of the heterocycles to chemotherapeutic agents.<sup>15,16</sup> We describe here synthesis and iron(III) complex-forming property of chiral linear and cyclic hexadentate ligands composed of three sets of  $\epsilon$ -(3-hydroxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-*L*-norleucine and glycine or  $\beta$ -alanine in a molecule, including the growth-promotion activity.

## RESULTS AND DISCUSSION

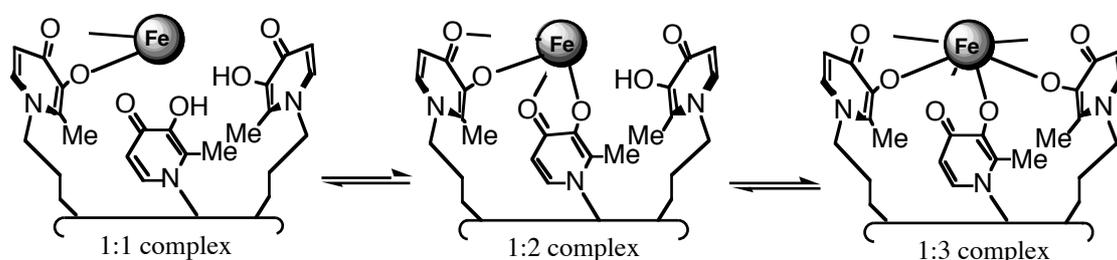
**Synthesis of Hexapeptide Ligands:** The synthetic procedure for linear and cyclic hexapeptide ligands is depicted in Scheme 1. 3-Benzoyloxy-2-methyl-4-pyrone (**1**) was allowed to react with *L*-lysine to give  $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-*L*-norleucine (**2**). The *N*-*tert*-butoxy-carbonyl



**Scheme 1** *Reagents and conditions:* i) L-lysine, NaOH, H<sub>2</sub>O-EtOH, rt for 4 days; ii) Boc<sub>2</sub>O, dioxane, 0 °C for 8 h, and then rt for 2 days; iii) HCl·H<sub>2</sub>N-(CH<sub>2</sub>)<sub>n</sub>-CO<sub>2</sub>Et, *N*-methylmorpholine, WSC·HCl·HOBt, CH<sub>2</sub>Cl<sub>2</sub>, rt for 3 days; iv) 1M NaOH, MeOH, rt for 2 h; v) TFA, CH<sub>2</sub>Cl<sub>2</sub>, ice-bath for 1 h; vi) *N*-methylmorpholine, WSC·HCl·HOBt, CH<sub>2</sub>Cl<sub>2</sub>, rt for 3 days; vii) **4a,b**, TFA, CH<sub>2</sub>Cl<sub>2</sub>, ice-bath for 2 h, and then *N*-methylmorpholine, CDI, DMF, rt for 3 days; viii) H<sub>2</sub>/10% Pd-C, MeOH, rt for 4 h, and then TFA, CH<sub>2</sub>Cl<sub>2</sub>, ice-bath for 2 h; ix) WSC·HCl·HOSu, DMF, rt for 3 days; x) TFA, CH<sub>2</sub>Cl<sub>2</sub>, ice-bath for 2 h, and then pyridine, 75 °C for 5 days (high dilution); xi) H<sub>2</sub>/10% Pd-C, MeOH, rt for 3 days.

(Boc) protection of compound (2), and subsequent coupling with glycine or  $\beta$ -alanine by using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (WSC·HCl) and 1-hydroxybenzotriazole (HOBt) gave the dipeptides (3a,b) as the repeating unit of hexapeptides. Dipeptides (3a,b) were converted to C-terminal free dipeptides (4a,b) by treatment with 1M NaOH. On the other hand, deprotection of the Boc group of compounds (3a,b) with TFA gave N-terminal free dipeptides (5a,b). Two components (4a,b) and (5a,b) were coupled by means of WSC·HCl-HOBt method to afford the tetrapeptides (6a,b). The Boc group of tetrapeptides (6a,b) were removed by treatment with TFA to give the corresponding TFA salts, and then the salts were coupled with dipeptides (4a,b) by using 1,1'-carbonyldiimidazole (CDI) to give the hexapeptides (7a,b). Debenzoylation by the catalytic hydrogenation, followed by deprotection of the Boc group with TFA gave the desired linear hexapeptide ligands (8a,b). Compounds (7a,b) were treated with 1M NaOH to give the C-terminal free hexapeptides, and then they were converted into the corresponding O-succinimide esters. After removal of Boc groups of the O-succinimide esters and subsequent cyclization of the resulting TFA salts were carried out under high dilution conditions. Any attempts to synthesize cyclic hexapeptide containing the glycine residue were unsuccessful. It may be attributable to 1) the steric hindrance of the bulky side chain including 4(1H)-pyridinone ring, 2) the rigidity of the peptide backbone originated from the glycine residue, and 3) the lability of the methylene protons of the glycine residue by virtue of the activated ester. On the other hand, cyclization of the hexapeptide containing the  $\beta$ -alanine residue smoothly proceeded to afford the cyclic hexapeptide (9). Compound (9) was subjected to the catalytic hydrogenation on 10% Pd-C to give the desired cyclic hexapeptide ligand (10).

**Iron(III) Complex Formation:** UV-VIS spectra of a 1:1 molar mixture of a hexapeptide and iron(III) in aqueous solution under various pH conditions were measured, and spectra of 8b-iron(III) complex are shown in Figure 1. The absorption maxima due to the ligand-to-metal charge transfer were observed at 460-520 nm. With an increase of pH in acidic region, the absorption maximum was shifted to shorter wavelength with an increase of absorbance, suggesting the transformation of 3-hydroxy-4(1H)-pyridinone : iron(III) (1:1) complex into a 1:2 and then into a 1:3 complex.



The absorption maximum and extinction coefficient of the complex at pH 7.47 were 460 nm and  $4300 \text{ M}^{-1} \text{ cm}^{-1}$  as shown in Table 1. These values are comparable to the reported value<sup>17</sup> of a 3,4-HOPO-iron(III) (3:1) complex, indicating that synthetic linear and cyclic hexapeptides formed stable intramolecular 1:1 iron(III) complexes [in other word, iron(III) : 3-hydroxy-4(1H)-pyridinone (1:3) complexes] in the wide

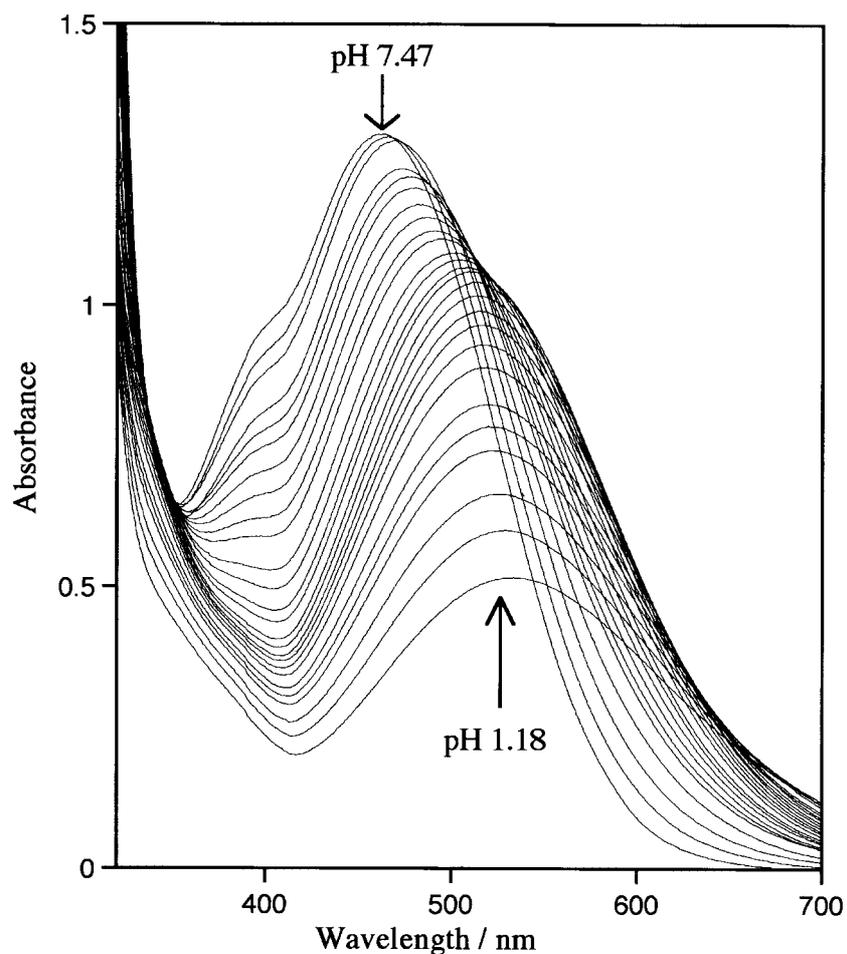


Figure 1 Spectral change of **8b**-iron(III) complex in aqueous solution under various pH conditions

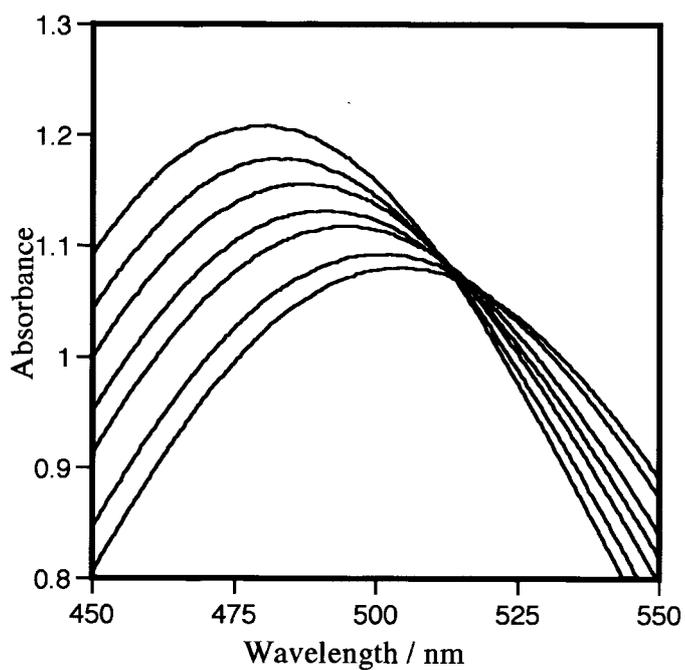


Figure 2 UV-VIS spectra of **8b**-iron(III) in water covering the pH range 2.36-2.93

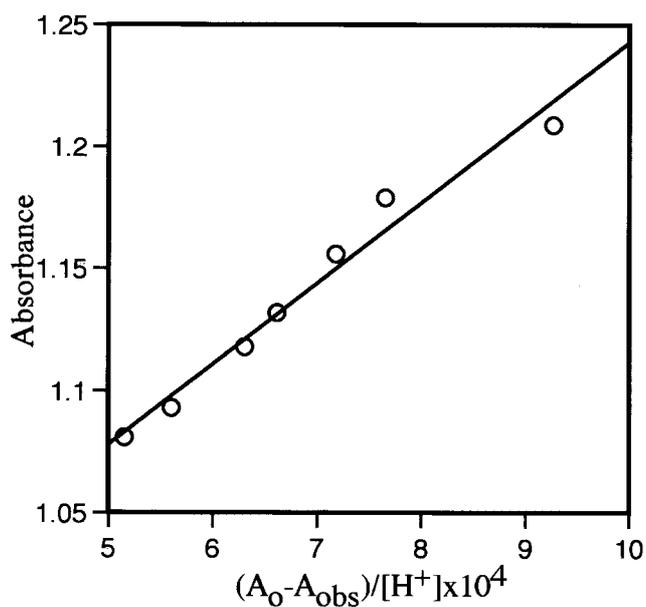


Figure 3 The Schwarzenbach plot for the same pH range as shown in Figure 2

pH range. The mole-ratio plots were also supported 1:1 stoichiometry. (not shown here)

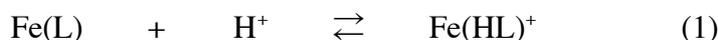
In the case of **8b**-iron(III) complex, the absorption spectra showed an isosbestic point at 515 nm in the pH

Table 1 UV-VIS spectral data of iron(III) complexes

Ligand	$\lambda_{\text{max}}/\text{nm}$	$\epsilon / \text{M}^{-1} \text{cm}^{-1}$
<b>8a</b>	460	4500
<b>8b</b>	461	4300
<b>10</b>	460	4300
3,4-HOPO*	446	4320

\* Ref. 17

range of 2.36-2.93 (Figure 2), reflecting the following equilibrium between Fe(L) and Fe(HL)<sup>+</sup> (eq 1);



where Fe(L) and Fe(HL)<sup>+</sup> are tris(1,4-dihydro-4-oxo-3-pyridinolato)iron(III) complex and its mono-protonated one, respectively. Equation 1 is also expressed by eq 2,  $K_{\text{Fe(HL)}}$  being the protonation equilibrium constant of the reaction.

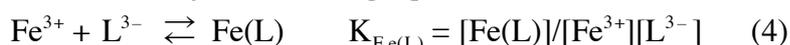
$$K_{\text{Fe(HL)}} = [\text{Fe(HL)}^+]/[\text{Fe(L)}][\text{H}^+] \quad (2)$$

The spectral data was further analyzed using the Schwarzenbach equation (eq 3), given as follows;

$$A_{\text{obs}} = (A_0 - A_{\text{obs}})/[\text{H}^+]K_{\text{Fe(HL)}} + \epsilon_{\text{Fe(HL)}}c_{\text{total}} \quad (3)$$

where  $A_{\text{obs}}$  is the observed absorbance at each pH value,  $A_0$  is the absorbance at pH 7.47, both at 460 nm,  $\epsilon_{\text{Fe(HL)}}$  is the molar absorption coefficient at 460 nm for the monoprotonated species, and  $c_{\text{total}}$  is the total concentration of the ligand. A straight line was obtained when  $A_{\text{obs}}$  was plotted against  $(A_0 - A_{\text{obs}})/[\text{H}^+]$ , as shown in Figure 3.  $K_{\text{Fe(HL)}}$  and  $\epsilon_{\text{Fe(HL)}}$  values were calculated using the slope and intercept, respectively. The value of 2800 for  $\epsilon_{\text{Fe(HL)}}$  indicates that average increments of 1400 are assignable to the first and second 3-hydroxy- $\gamma$ -pirinonato ligands; hence, the almost same contribution of 1500 is estimated for the third ligand upon 1:3 complex. The  $K_{\text{Fe(HL)}}$  value, whose logarithmic value corresponds half protonation pH value ( $\text{pH}_{1/2}$ ), was calculated to be  $1.2 \times 10^2$ , being much smaller than the reported peptide-based tris(hydroxamate) iron(III) complexes.<sup>18</sup> These data implied that -norleucyl- $\beta$ -Ala- sequence supplies the sufficient spacing to form a stable 1:3 complex.

**The Relative Stability of Iron(III) Complexes:** The relative stability constant ( $K_{\text{Fe(L)}}$ ) of hexapeptide ligands with iron(III) is defined by the following equation 4.



The competitive reaction between EDTA and these ligands was carried out in order to obtain the stability constants of iron(III) complexes. Three pKa values of ligands are necessary for calculation. However, due to the experimental limitation, pKa value (9.8) of *N*-substituted 3,4-HOPO<sup>19</sup> was used for calculation. The relative stability constants were obtained from the acid dissociation constants<sup>20</sup> of EDTA, the stability constant<sup>21</sup> of EDTA-iron(III) complex ( $\log K = 25.1$ ), and the equilibrium constant, and the results are summarized in Table 2, together with the data<sup>19</sup> for DFB. The stability constants were found to be over 30 in logK. These relatively high values are attributable to the pKa value. The relative stability constant of

**8b**-iron(III) complex increased two orders of magnitude over that of **8a**-iron(III) one, indicating that the change in the peptide chain length remarkably affected the stability of the complex. Considering from the molecular model, only four isomers are possible for **8a**-iron(III) complex, while all *cis* and *trans* isomers are possible for **8b**-iron(III) complex. Further, the relative stability constant of **10**-iron(III) complex was not so high as expected. It may be attributed that cyclic structure is so rigid that iron(III) ion can not be tightly held in the form of 3:1 complex.

Table 2 Stability constants of iron(III) complexes

Ligand	log K
<b>8a</b>	31.6
<b>8b</b>	33.4
<b>10</b>	31.7
DFB*	30.5

\* Ref. 19

**Configuration of Iron(III) Complexes:** The absolute configuration of iron(III) complexes in aqueous solutions was examined by means of CD spectroscopy. The CD spectra of iron(III) complexes were shown in Figure 4. The iron(III) complex of linear hexapeptide (**8a**) showed a negative band at 488 nm and a positive band at 349 nm and, indicating that **8a**-iron(III) complex predominantly exists in a  $\Delta$ -configuration in an aqueous solution. A cyclic hexapeptide **10**-iron(III) complex also gave the similar CD pattern. On the contrary, **8b**-iron(III) complex showed the opposite CD pattern (a positive band at 485 nm and a negative band at 366 nm), indicating the preference of a  $\Lambda$ -configuration.

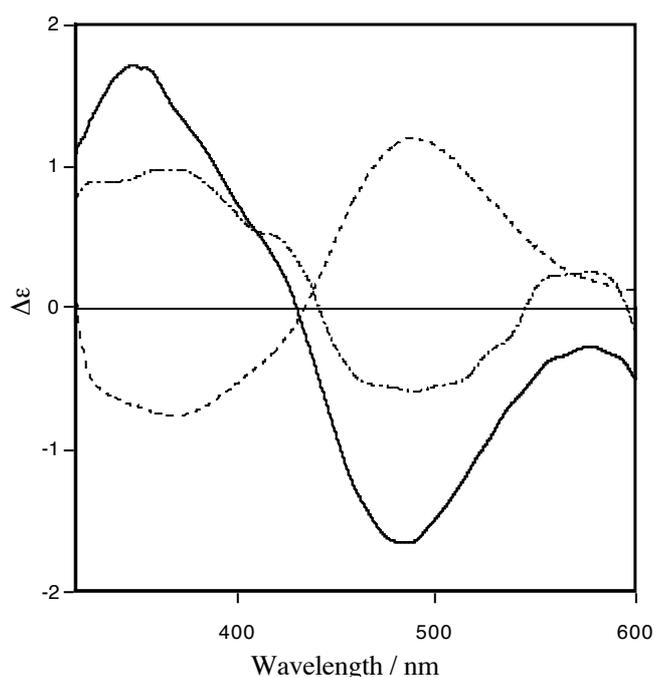


Figure 4 CD spectra of iron(III) complexes in aqueous solution at pH 7.4; (—); **8a**-iron(III), ( - - ); **8b**-iron(III); (-•-); **10**-iron(III)

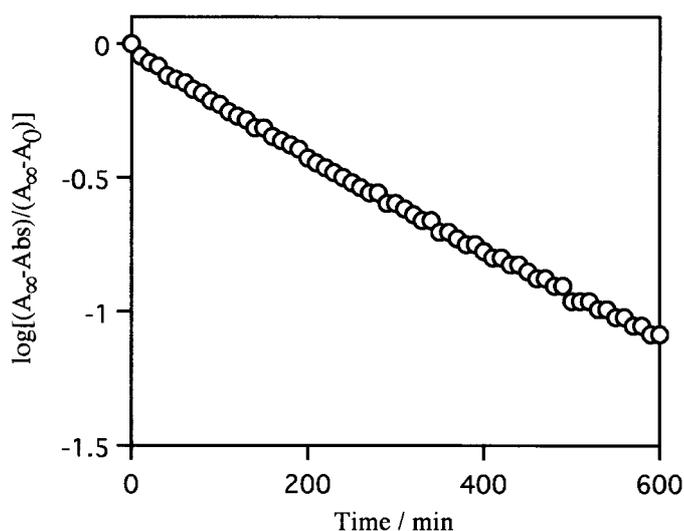


Figure 5 The plots of  $\log[(A_{\infty}-Abs)/(A_{\infty}-A_0)]$  vs time on iron(III) removal of **8b** from TfFe2.00

Previously, Shanzer and co-workers demonstrated factors for determining and stabilizing the configuration of iron(III) complex by using synthetic ferrichrome analogues in terms of 1) the chirality of amino acid residues in a molecule and 2) a strong intramolecular hydrogen bond.<sup>22</sup> Further, we showed that the difference in the side-chain bulkiness of the amino acid residue of ligands causes change in absolute configuration in the case of tripodal ligands.<sup>23</sup> It is noteworthy that the difference in the amino acid residue (Gly vs.  $\beta$ -Ala) in the peptide chain causes change in absolute configuration, although the reason has remained obscure.

**Iron(III) Removal from Transferrin:** Diferric transferrin was prepared from commercially available human apotransferrin according to the literatures.<sup>24</sup> An addition of hexapeptides to a solution of diferric transferrin (0.002 mM) result in an increase of absorbance at 460 nm characteristic of hexapeptide-iron(III) complexes. The concentration of ligands was 7-fold excess over diferric transferrin. Plots of  $\log[(A_{\infty}-A_{\text{obs}})/(A_{\infty}-A_0)]$  as a function of time gave a good linear relationship as shown in Figure 5, indicating that the reaction of iron removal from transferrin by **8a** proceeded in the pseudo-first-order kinetics. The kinetic results were summarized in Table 3, together with the data for DFB. It is noteworthy that these ligands efficiently removed iron from transferrin even at less concentration of the ligand after the reaction was performed for 30 min. Further, there was remarkable difference between **8a** and **8b**. As mentioned above, **8a**- and **8b**-iron(III) complexes had  $\Delta$ - and  $\Lambda$ -configuration, respectively. We have demonstrated that the rates of iron(III) removal from transferrin by L- or D-alanine residue-containing tripodal ligands were affected by the absolute configuration ( $\Delta$  or  $\Lambda$ ) of iron(III) complexes.<sup>23</sup> The present data was consistent with the reported phenomenon. Unfortunately, kinetic data for cyclic hexapeptide (**10**) could not be obtained because of low water solubility under the employed conditions.

Table 3 Iron(III) removal from transferrin at pH 7.4

Ligand	[ligand]/[Fe <sub>2.00</sub> Tf]	$k_{\text{obs}}$ ( $\times 10^{-3} \text{ min}^{-1}$ )	% Fe removed (at a point 30 min)
<b>8 a</b>	7	1.72	7
<b>8b</b>	7	3.08	14
DFB*	100	0.66	5

\* Ref. 24a

**The Growth-Promotion Activity:** As mentioned above, *Aureobacterium flavescence* (ATCC 25091) and *Escherichia coli* RW193 (ATCC 33475) were commonly used for assay of the growth-promotion activity. *A. flavescence* was shown to require hydroxamate-type siderophores and has a ferrichrome receptor, although it does not synthesize any detectable siderophore and does not response to catecholate-type siderophores. *E. coli* RW193 showed activity against both hydroxamate- and catecholate-type siderophores. These strains, therefore, are useful for diagnosis whether a synthetic compound acts as an artificial siderophore. The assay was performed according to the standard procedure.<sup>14,22,25,26</sup> The activity was evaluated by measuring the diameter of the halo of exhibition of growth, and the results are presented in Table 4. Natural siderophore DFB exhibited 55 mm halo of growth, but **8b**-iron(III)

complex showed no response, indicating that synthetic hexapeptide (**8b**) is not an auxotrophe for *A. flavescence*. On the other hand, although less than DFB, *E. coli* RW193 showed significant growth-promotion activity against **8b**-iron(III) complex. This result may be attributable that microorganism recognizes 3-hydroxy-4(1*H*)-pyridinone as catecholate-type ligand. The assay was performed only using **8b**-iron(III) complex, because other complexes were insoluble in water under the employed conditions.

Table 4 The growth-promotion activity

Ligand	Diameter (mm)	
	<i>A. flavescence</i>	<i>E. coli</i>
<b>8b</b>	<6	12
DW	<6	<6
DFB	55	18

\*20  $\mu$ L iron(III) complex solutions (1 mM) were absorbed onto a 6 mm paper discs. DFB-iron(III) solution was used as a reference, and distilled water (DW) as a blank. The diameter of the halo of growth was measured.

## EXPERIMENTAL

Melting points were recorded on a Mel-Temp apparatus in open capillaries and are uncorrected. IR spectra were recorded on JASCO FT/IR-230 infrared spectrophotometer. <sup>1</sup>H-NMR spectra were recorded on a JEOL GX-270 and JNM-LA400D spectrometers using Me<sub>4</sub>Si or sodium 3-trimethylsilyl-1-propane-sulfonate sodium salt as an internal standard. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. UV-VIS spectra were recorded on a JASCO Ubest V-550 spectrophotometer. CD spectra were measured with a JASCO J-500 spectropolarimeter. Column chromatography was carried out with Merck Kieselgel 60 (230-400 mesh). Combustion analysis was performed on a Perkin Elmer 2400 II CHNS Analyzer. 3-Benzyloxy-2-methyl-4-pyrone (**1**) was prepared according to literature methods.<sup>13, 27</sup>  $\epsilon$ -(**3-Benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl**)-**L-norleucine sodium salt (2)**: To a solution of NaOH (0.64 g, 16 mmol) in H<sub>2</sub>O : EtOH (2:1) mixture (100 mL) was added compound (**1**) (1.4 g, 6.5 mmol) and H-L-Lys-OH·HCl (1.1 g, 6.1 mmol), and then the reaction mixture was stirred at rt for 4 days. The reaction mixture was washed with AcOEt (45 mL x 2), and then the aqueous layer was evaporated under reduced pressure to give the sodium salt, which was used for the next reaction without further purification: <sup>1</sup>H-NMR ( $\delta$ , D<sub>2</sub>O, 270 MHz): 1.33 (2H, m), 1.57-1.86 (4H, m), 1.93 (3H, s), 3.54 (1H, t, *J*=7.0 Hz), 3.59 (2H, t, *J*=7.8 Hz), 5.02 (2H, s), 6.56 (1H, d, *J*=8.7 Hz), 7.40 (5H, m), and 7.72 (1H, d, *J*=8.7 Hz).

**General procedure for compounds (3a,b); A typical example:  $\epsilon$ -(3-Benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-*N*-*tert*-butoxycarbonyl-L-norleucylglycine ethyl ester (3a)**: To a solution of compound (**2**) (9.32 mmol) in H<sub>2</sub>O (30 mL) was added Boc<sub>2</sub>O (2.43 g, 11.0 mmol) in dioxane (20 mL) on an ice-bath. The reaction mixture was stirred at 0 °C for 8 h and then at rt for another 2 days. H<sub>2</sub>O (50 mL) was added to the mixture and the solution was washed with CHCl<sub>3</sub> (100 mL x 2). The aqueous solution was adjusted to pH 4 with 5% citric acid, and then extracted with CHCl<sub>3</sub>

(200 mL x 3). The organic layer was washed with saturated NaCl solution (200 mL), and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Removal of the solvent by evaporation under reduced pressure and subsequent recrystallization from EtOH gave the product,  $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-*N*-*tert*-butoxycarbonyl-L-norleucine (1.67 g, 37%): mp 172-174 °C; [ $\alpha$ ]<sub>D</sub><sup>20</sup> -2.9° (c=1 in MeOH); IR (KBr): 1705, 1624, 753, and 704 cm<sup>-1</sup>; <sup>1</sup>H-NMR ( $\delta$ , CDCl<sub>3</sub>, 270 MHz): 1.30-1.39 (2H, m), 1.44 (9H, s), 1.61-1.72 (2H, m), 1.80-1.92 (2H, m), 2.13 (3H, s), 3.48 (1H, m), 3.80-3.91 (2H, m), 4.20 (1H, br s), 5.14 (2H, s), 5.4 (1H, d, *J*=8.1 Hz), 6.6 (1H, d, *J*=8.1 Hz), and 7.30-7.42 ppm (5H, m). *Anal.* Calcd for C<sub>23</sub>H<sub>32</sub>N<sub>2</sub>O<sub>6</sub>: C, 63.87; H, 7.45; N, 6.47. Found: C, 64.07; H, 7.24; N, 6.23.

To a solution of HCl·H-Gly-OEt (252 mg, 1.80 mmol) and *N*-methylmorpholine (192 mg, 1.90 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1.5 mL) was added the above Boc-norleucine (801 mg, 1.8 mmol) and HOBt (257 mg, 1.90 mmol) at -10 °C. A solution of WSC·HCl (346 mg, 1.80 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (3 mL) was added to the reaction mixture at -10 °C, and then the reaction mixture was stirred for 3 days at rt. After removal of the solvent under reduced pressure, the residue was dissolved in CHCl<sub>3</sub> (150 mL). The organic layer was successively washed with 5% citric acid (80 mL x 3), 5% NaHCO<sub>3</sub> (80 mL x 3), water (80 mL x 3), saturated NaCl solution (100 mL), and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography on silica gel with CHCl<sub>3</sub> : MeOH (10:1) mixture as an eluent to afford the pure product (**3a**) (701 mg, 75%): [ $\alpha$ ]<sub>D</sub><sup>20</sup> -3.3° (c=1 in MeOH); IR(KBr): 1738, 1718, 1679, 1628, 754, and 703 cm<sup>-1</sup>; <sup>1</sup>H-NMR ( $\delta$ , CDCl<sub>3</sub>, 400 MHz): 1.26 (3H, t, *J*=7.6 Hz), 1.35-1.40 (2H, m), 1.43 (9H, s), 1.58-1.72 (2H, m), 1.86-1.98 (2H, m), 2.07 (3H, s), 3.72 (2H, t, *J*=7.9 Hz), 3.96 and 4.02 (2H, ABq, *J*=19 Hz), 4.17 (2H, q, *J*=7.6 Hz), 4.19 (1H, m), 5.18 (2H, s), 5.26-5.33 (1H, m), 6.39 (1H, d, *J*=7.3 Hz), 7.15-7.17 (1H, m), 7.19 (1H, d, *J*=7.3 Hz), and 7.29-7.42 ppm (5H, m). *Anal.* Calcd for C<sub>28</sub>H<sub>39</sub>N<sub>3</sub>O<sub>7</sub>·2H<sub>2</sub>O: C, 58.52; H, 7.27; N, 7.31. Found: C, 58.68; H, 7.18; N, 7.27.

***N*-*tert*-Butoxycarbonyl- $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanine ethyl ester (**3b**):** 85%; mp 101-103 °C; [ $\alpha$ ]<sub>D</sub><sup>22</sup> -6.5° (c=0.5 in MeOH); IR(KBr): 1735, 1707, 1661, 1626, 754, and 704 cm<sup>-1</sup>; <sup>1</sup>H-NMR ( $\delta$ , CDCl<sub>3</sub>, 400 MHz): 1.26 (3H, t, *J*=7.2 Hz), 1.35 (2H, m), 1.44 (9H, s), 1.62 (2H, m), 1.84 (2H, m), 2.08 (3H, s), 2.52 (2H, t, *J*=6 Hz), 3.53 (2H, t, *J*=6 Hz), 3.70 (2H, t, *J*=7 Hz), 4.07 (1H, m), 4.14 (2H, q, *J*=7.2 Hz), 5.20 (2H, s), 5.22 (1H, br s), 6.38 (1H, d, *J*=7.6 Hz), 6.90 (1H, br s), 7.17 (1H, d, *J*=7.6 Hz), and 7.33 ppm (5H, m). *Anal.* Calcd for C<sub>29</sub>H<sub>41</sub>N<sub>3</sub>O<sub>7</sub>: C, 64.09; H, 7.55; N, 7.73. Found: C, 63.84; H, 7.87; N, 8.07.

**General procedure for compounds (4a,b); A typical example: *N*-*tert*-Butoxycarbonyl- $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucylglycine (**4a**):** To a solution of compound (**3a**) (260 mg, 0.49 mmol) in MeOH (5 mL) was added 1M NaOH (0.75 mL, 0.75 mmol), and then the reaction mixture was stirred at rt for 2 h. The solution was adjusted to pH 8 with 5% citric acid, and then the solvent was removed under reduced pressure. The solution of the residue in H<sub>2</sub>O (5 mL) was adjusted pH 4 with 6M HCl on an ice-bath, and then extracted with CHCl<sub>3</sub> (15 mL x 4). The organic layer was washed with saturated NaCl solution (20 mL), and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Removal of

the solvent under reduced pressure afforded the dipeptide (**4a**) (223 mg, 94%): mp 124-127 °C;  $[\alpha]_D^{23}$  -3.2° (c=0.5 in MeOH); IR(KBr): 1706, 1659, 1614, 754, and 704  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ , 400 MHz): 1.31 (2H, m), 1.39 (9H, s), 1.63 (2H, m), 1.76 (2H, m), 2.10 (3H, s), 3.81 (2H, m), 3.99 (2H, m), 4.24 (1H, m), 5.11 (2H, s), 5.79 (1H, m), 6.68 (1H, d,  $J=7.8$  Hz), 7.34 (5H, m), 7.29-7.38 (1H, m), and 7.55 ppm (1H, d,  $J=7.8$  Hz). Anal. Calcd for  $\text{C}_{26}\text{H}_{35}\text{N}_3\text{O}_7 \cdot 1.5\text{H}_2\text{O}$ : C, 59.08; H, 6.96; N, 7.95. Found: C, 59.21, H, 6.98; N, 7.88.

***N-tert*-Butoxycarbonyl- $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanine (**4b**):** 98%; mp 63-65 °C;  $[\alpha]_D^{22}$  -12.1° (c=0.5 in MeOH); IR(KBr): 1706, 1659, 1625, 754, and 704  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ , 400 MHz): 1.26 (2H, m), 1.45 (9H, s), 1.70 (4H, m), 2.08 (3H, s), 2.53 (2H, m), 3.33 (1H, m), 3.49 (1H, m), 3.7 (2H, m), 4.11 (1H, m), 5.10 (2H, s), 5.51 (1H, br s), 6.59 (1H, d,  $J=7$  Hz), 6.91 (1H, br s), 7.31 (5H, m), and 7.44 ppm (1H, d,  $J=7$  Hz). Anal. Calcd for  $\text{C}_{27}\text{H}_{37}\text{N}_3\text{O}_7 \cdot \text{H}_2\text{O}$ : C, 60.77; H, 7.37; N, 7.87. Found: C, 60.94, H, 7.23; N, 7.78.

**General procedure for compounds (**5a,b**); A typical example:  $\epsilon$ -(3-Benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucine ethyl ester TFA salt (**5a**):** To a solution of compound (**3a**) (350 mg, 0.66 mmol) in  $\text{CH}_2\text{Cl}_2$  (2.5 mL) was added TFA (3.39 g, 29.7 mmol), and then the reaction mixture was stirred on an ice-bath for 1 h. After removal of the solvent under reduced pressure, dry EtOH (20 mL) was added to the residue, and then the solvent was removed under reduced pressure to afford the TFA salt (**5a**). This salt was used for the next reaction without further purification. Compound (**3b**) was similarly converted into the corresponding TFA salt (**5b**).

**General preparation procedure for compounds (**6a,b**); A typical example: *N-tert*-Butoxycarbonyl- $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucylglycyl- $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucylglycine ethyl ester (**6a**):** To a solution of compound (**5a**) (789 mg, 1.04 mmol) and *N*-methylmorpholine (304 mg, 3.01 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (1.5 mL) was added compound (**4a**) (515 mg, 1.03 mmol) and HOBt (154 mg, 1.14 mmol) at -10 °C. A solution of WSC·HCl (219 mg, 1.14 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (1.5 mL) was added to the mixture at -10 °C, and then the reaction mixture was stirred for 3 days at rt. After removal of the solvent under reduced pressure, the residue was dissolved in  $\text{CHCl}_3$  (250 mL). The organic layer was successively washed with 5% citric acid (100 mL x 3), 5%  $\text{NaHCO}_3$  (100 mL x 3), saturated NaCl solution (150 mL), and then dried over anhydrous  $\text{Na}_2\text{SO}_4$ . After removal of the solvent under reduced pressure, the crude product was purified by column chromatography on silica gel with  $\text{CHCl}_3$ :MeOH (6:1) mixture as an eluent to afford the tetrapeptide (**6a**) (729 mg, 75%): mp 98-101 °C;  $[\alpha]_D^{27}$  -8.6° (c=0.5 in MeOH); IR(KBr): 1671, 1664, 1651, 1626, 754, and 703  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CDCl}_3$ , 400 MHz): 1.22 (3H, t,  $J=7$  Hz), 1.24- 1.39 (4H, m), 1.40 (9H, s), 1.53-1.98 (8H, m), 2.06 (6H, s), 3.71 (4H, m), 3.80-3.98 (4H, m), 4.12 (2H, q,  $J=7$  Hz), 4.19 (1H, m), 4.49 (1H, q,  $J=8$  Hz), 5.12 (4H, s), 5.93 (1H, d,  $J=8$  Hz), 6.37 (2H, d,  $J=7$  Hz), 7.35 (10H, m), 7.37 (2H, d,  $J=7$  Hz), 7.49 (1H, br s), 8.03 (1H, br s), and 8.28 ppm (1H, br s). Anal. Calcd for  $\text{C}_{49}\text{H}_{64}\text{N}_6\text{O}_{11} \cdot \text{H}_2\text{O}$ : C, 60.29; H, 6.97; N, 8.61. Found: C, 60.23; H, 6.79; N, 8.21.





br s), and 8.01 ppm (3H, br s). *Anal.* Calcd for  $C_{49}H_{71}N_9O_{15} \cdot 2H_2O$ : C, 55.41; H, 7.12; N, 11.87. Found: C, 55.44; H, 7.20; N, 11.79.

A removal of the Boc protecting group of the above debenzoylation product with TFA afforded the desired linear hexapeptide (**8b**) as an oil: 100%;  $[\alpha]_D^{22}$   $-4.9^\circ$  ( $c=0.1$  in MeOH);  $^1H$ -NMR ( $\delta$ , DMSO- $d_6$ , 400 MHz): 1.17 (3H, t,  $J=7$  Hz), 1.32 (6H, m), 1.73 (12H, m), 2.33 (4H, m), 2.43 (2H, t,  $J=8$  Hz), 2.51 (9H, s), 3.28 (6H, m), 3.73 (6H, m), 4.04 (2H, q,  $J=7$  Hz), 4.17 (2H, m), 4.28 (6H, m), 7.17 (3H, d,  $J=8$  Hz), 8.06 (4H, m), 8.20 ppm (3H, d,  $J=8$  Hz), and 8.53 ppm (1H br s). This TFA salt was used for spectral measurement without further purification.

**Cyclo[ $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanyl- $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanyl- $\epsilon$ -(3-benzyloxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanyl]** (**9**): To a solution of compound (**7b**) (180 mg, 0.135 mmol) in MeOH (3 mL) was added 1M NaOH (0.75 mL, 0.75 mmol), and then the reaction mixture was stirred at rt for 2 h. The solution was adjusted to pH 8 with 5% citric acid, and then the solvent was removed under reduced pressure. The solution of the residue in  $H_2O$  (5 mL) was adjusted to pH 2 with 6M HCl on an ice-bath, and then extracted with  $CHCl_3$  (250 mL) by using liquid-liquid Soxlet's extractor. The organic layer was dried over anhydrous  $Na_2SO_4$ . Removal of the solvent under reduced pressure afforded the corresponding C-terminal free hexapeptide (171 mg 97%): mp 135-136  $^\circ C$ ;  $[\alpha]_D^{23}$   $-1.7^\circ$  ( $c=0.5$  in MeOH); IR(KBr): 1708, 1657, 1640, 1625, 753, and 705  $cm^{-1}$ ;  $^1H$ -NMR ( $\delta$ ,  $CD_3OD$ , 400 MHz): 1.37 (6H, m), 1.42 (9H, s), 1.73 (12H, m), 2.37 (9H, s), 2.50 (6H, m), 3.43 (6H, m), 3.99 (1H, m), 4.19 (6H, t,  $J=7$  Hz), 4.49 (2H, m), 5.14 (6H, s), 6.94 (3H, d,  $J=8$  Hz), 7.39 (15H, m), and 8.12 ppm (3H, d,  $J=8$  Hz). *Anal.* Calcd for  $C_{71}H_{91}N_9O_{15} \cdot 3H_2O$ : C, 62.49; H, 7.16; N, 9.24. Found: C, 62.49; H, 7.04; N, 9.08.

To a solution of the above C-terminal free hexapeptide (170 mg, 0.128 mmol) in dry DMF (5 mL) was added HOSu (29.4 mg, 0.256 mmol) at  $-10^\circ C$ . A solution of WSC $\cdot HCl$  (42.2 mg, 0.256 mmol) in dry  $CH_2Cl_2$  (2 mL) was added to the reaction mixture at  $-10^\circ C$ , and then the reaction mixture was stirred for 3 days at rt. After removal of the solvent under reduced pressure, the residue was dissolved in  $CHCl_3$  (200 mL). The organic layer was successively washed with water (80 mL x 3), saturated NaCl solution (100 mL), and then dried over anhydrous  $Na_2SO_4$ . Removal of the solvent under reduced pressure afforded the corresponding O-succinimide ester (100%): IR(KBr): 1813, 1782, and 1737  $cm^{-1}$ . This product was used for the next reaction without further purification.

To a solution of the O-succinimide ester (171 mg, 0.12 mmol) in dry  $CH_2Cl_2$  (1.5 mL) was added TFA (684 mg, 6.0 mmol), and then the reaction mixture was stirred for 2 h on an ice-bath. Removal of the solvent under reduced pressure afforded the corresponding TFA salt. This salt was used for the next reaction without further purification.

A solution of the TFA salt (220 mg, 0.153 mmol) in dry DMF (5 mL) was added dropwise to pyridine (175 mL), and then the reaction mixture was stirred for 5 days at  $75^\circ C$ . After removal of the solvent, the crude product was purified by column chromatography on silica gel with MeOH followed by gel chromatography on Sephadex LH-20 with MeOH as an eluent to give the cyclic peptide (**9**) (60 mg, 33%): mp 141-144  $^\circ C$ ;

$[\alpha]_D^{22} + 11.7^\circ$  ( $c=0.1$  in MeOH); IR(KBr): 1655, 1624, 753, and 703  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{CD}_3\text{OD}$ , 400 MHz): 1.39 (6H, m), 1.67 (12H, m), 2.17 (9H, s), 2.30 (3H, m), 2.55 (3H, m), 3.30 (3H, m), 3.53 (3H, m), 3.94 (6H, t,  $J=7$  Hz), 4.00 (3H, t,  $J=6$  Hz), 5.06 (6H, s), 6.44 (3H, d,  $J=8$  Hz), 7.34 (15H, m), and 7.67 ppm (3H, t,  $J=8$  Hz). *Anal.* Calcd for  $\text{C}_{66}\text{H}_{81}\text{N}_9\text{O}_{12}\cdot 2\text{H}_2\text{O}$ : C, 64.53; H, 6.97; N, 10.26. Found: C, 64.33; H, 6.78; N, 10.11.

**Cyclo[ $\epsilon$ -(3-hydroxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanyl- $\epsilon$ -(3-hydroxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanyl- $\epsilon$ -(3-hydroxy-1,4-dihydro-2-methyl-4-oxo-1-pyridyl)-L-norleucyl- $\beta$ -alanyl] (10):** A suspension of 10% Pd-C (15 mg) in MeOH (2 mL) was prehydrogenated with  $\text{H}_2$  for 0.5 h. To the suspension was added a solution of compound (9) (90 mg, 0.08 mmol) in MeOH (3 mL). After hydrogenation under  $\text{H}_2$  atmospheric pressure for 4 h, the catalyst was removed by filtration. The filtrate was evaporated, and the residue was purified by gel chromatography on Sephadex LH-20 with DMF as an eluent to give the desired cyclic hexapeptide **10** (40 mg, 57%): mp 183-185  $^\circ\text{C}$  (decomp);  $[\alpha]_D^{22} + 12.3^\circ$  ( $c=0.1$  in MeOH); IR(KBr): 1654, and 1637  $\text{cm}^{-1}$ ;  $^1\text{H-NMR}$  ( $\delta$ ,  $\text{DMSO-d}_6$ , 400 MHz): 1.30 (6H, m), 1.60 (12H, m), 2.14 (3H, m), 2.26 (9H, s), 2.40 (3H, m), 3.13 (3H, m), 3.43 (3H, m), 3.88 (6H, t,  $J=8$  Hz), 3.91 (3H, m), 6.10 (3H, d,  $J=8$  Hz), 7.53 ppm (3H, d,  $J=8$  Hz), 7.71 (3H, m), and 8.06 (3H, m). *Anal.* Calcd for  $\text{C}_{45}\text{H}_{63}\text{N}_9\text{O}_{12}\cdot 4\text{H}_2\text{O}$ : C, 54.37; H, 7.20; N, 12.68. Found: C, 54.33; H, 7.06; N, 12.45.

**General Procedure for Spectral Measurement of 1:1 Mixtures of Hexapeptide Ligands and Iron(III)**<sup>23</sup>: 3 mg of each hexapeptide ligand and an equimolar amount of  $\text{Fe}(\text{NO}_3)_3$  ( $2.99 \times 10^{-3}\text{M}$ ) were mixed and diluted to 10.0 mL with distilled water. The pH was adjusted to an appropriate pH value with 0.01 or 0.1 M NaOH and 0.01 or 0.1 M  $\text{HNO}_3$  solutions before spectral measurement.

**Iron(III) Exchange Reaction**<sup>23</sup>: A buffered solution (2 mL Tris buffer (pH 7.4), and  $[\text{KNO}_3] = 0.04$  M) containing  $1 \times 10^{-4}$  M of iron(III) complex was mixed with EDTA solution (1 mL Tris buffer (pH 7.4), and  $[\text{EDTA}] = 0.1$  M). Iron(III) exchange reaction was monitored by a decrease of absorbance at 460 nm. The relative stability constant of iron(III) complex was calculated by using the stability constant of  $\text{Fe}(\text{edta})$ , pKa values of EDTA, pKa value of 3,4-HOPO, and the equilibrium point.

**Iron(III) Removal from Transferrin**<sup>23</sup>: The solutions of each hexapeptide ligand (1.0 mL, 1 mM) and  $\text{Tf}_{\text{Fe}2.00}$  (2.0 mL, 0.02 mM) in Tris buffer were mixed, and then the absorbance of the solution was monitored at 460 nm. The pseudo-first-order rate constant ( $k_{\text{obs}}$ ) was calculated from the slope of the plots of  $\log[(A_\infty - \text{Abs})/(A_\infty - A_0)]$  as a function of time.

**The Growth-Promotion Activity**<sup>26</sup>: The biological activity of **8b**-iron(III) complex was evaluated by the standard paper-disc method by using *Aureobacterium flavescens* and *E. Coli*. On nutrient agar (ATCC Medium No. 424: *A. flavescens*, ATCC Medium No. 33475: *E. coli*) (0.7 % agar) containing the strain ( $\sim 10^8$  cells  $\text{mL}^{-1}$ ) laid over nutrient agar plate (1.5 % agar) was placed filter paper discs (6 mm diam.).

Each disc was impregnated with aliquots (20  $\mu$ L) of 1 mM complex solutions in water. The diameter of halo of growth was measured after incubation at 30 °C for 2 days.

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