N-Hydroxyamide-Containing Heterocycles. 4.[1] Synthesis and Fe^{III}-Chelating Properties of Novel Hexadentate Ligands Composed of N-Hydroxy-2(1H)-Pyrazinone, Amino Acid Residues, and Tris(2-aminoethyl)amine

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Synthesis of novel hexadentate ligands (**3-HOPR(X)**; X = Me and Bu^i) composed of *N*-hydroxy-2(1*H*)-pyrazinone, amino acids, and tris(2-aminoethyl)amine, and their Fe^{III}-chelating properties are described. *N*-(Benzyloxy)-2(1*H*)-pyrazinone (**6**) bearing the carboxyl group at C-3 position of the pyrazinone ring was synthesized from L-glutamic acid *via* five steps. The consecutive coupling of **6** with amino acids and tris(2-aminoethyl)amine, followed by the deprotection of the *O*-benzyl group, afforded target compounds (**3-HOPR(X)**). UV-vis spectral data of the 1:1 molar mixture of **3-HOPR(X)** and Fe^{III} in aqueous solution indicated the formation of intramolecular 1:3 Fe^{III} complex to the hydroxamate units. The relative stability constants (log *K* 21–22) of the complexes were influenced by the side chains of L-amino acid residues. Further, the absolute configuration around Fe^{III} was dramatically changed, *viz.*, Fe(**3-opr(Me)**) exists predominantly in the Λ-cis configuration, while Fe(**3-opr(Bu^i)**) exist in the Δ-cis in water. From the Fe^{III} removal experiment of **3-HOPR(X)** from transferrin at pH 7.4, it was concluded that **3-HOPR(X)** was a more effective Fe^{III} removal agent than the naturally occurring siderophore, desferrioxamine B.

Iron overload is a significant health problem. The most common cause of iron overload is regular blood transfusions, particularly in the treatment of a genetic disease such as β-thalassemia (Cooley's anemia).[2] The toxicity of the resulting excess iron in the body can be ameliorated by administration of an iron chelating agent. Many chemists have focused on siderophores and their analogs as promising candidates for therapeutic medicines for iron overload.[3]

Siderophores are low-molecular-weight Fe^{III}-chelating compounds secreted by microorganisms to solubilize Fe^{III} and transport it into the cell through the membrane. Two common functional groups found in siderophores are hydroxamate and catecholate, both of which act as strong bidentate chelators to Fe^{III}. Enterobactin, a typical tricatecholate natural siderophore produced by *Escherichia coli*, has been shown to have a high stability constant to Fe^{III} (log $K = 49$)[4] and to remove Fe^{III} from transferrin.[5–7] Unfortunately, the clinical use of chelation therapy is precluded due to the weak acidity of catechol and the extreme propensity toward hydrolysis.[8] On the other hand, the methanesulfonate salt of desferrioxamine B (Desferal: DFB), a typical trihydroxamate natural siderophore, has reigned for over 30 years as the only choice for clinical use in patients with β-thalassemia in spite of the fact that DFB has some disadvantages, i.e., a lack of oral activity and short plasma half-life[9–11] and severe side effects such as septicemia.[12] Thus, the full benefits of chelation therapy will not be realized until orally active iron chelators are available.

Recently, hydroxy group-containing heterocycles have received much attention owing to their strong acidities compared to catechol. These include hydroxypyridine,[13–15] 1-hydroxy-2(1*H*)-pyridinone,[16,17] 3-hydroxy-2(1*H*)-pyridinone,[16,18–20] and a variety of 3-hydroxy-4(1*H*)-pyridinones,[16,21–26] and 3-hydroxy-2-methyl-4*H*-pyran-4-one.[27] In addition, interesting examples for hexadentate ligands,

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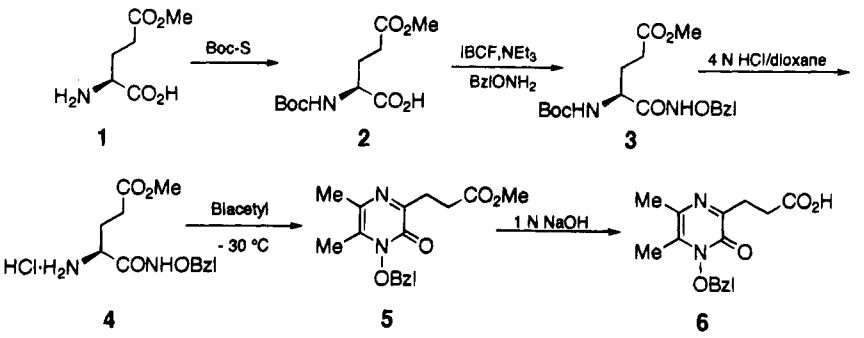
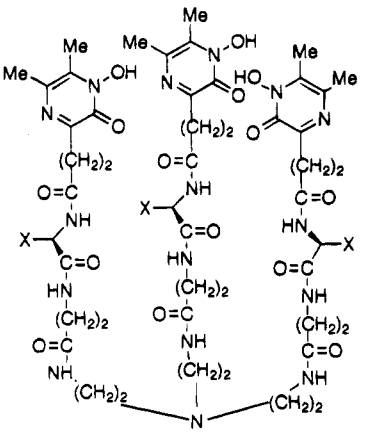
Scheme 1



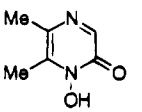
in which there are three sets of hydroxypyridinones in a molecule, have been reported by Streater[19] and Martell.[28] Among them, at least one, 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone, was found to be an orally active iron chelator although all toxicology and other preclinical safety studies were still ongoing.[29-31]

N-Hydroxyamide-containing diazinones would be expected to have higher solubility in water and lower pK_a values than the corresponding monoazinone by introducing the second electron-withdrawing nitrogen atom into the ring. We have investigated synthesis and Fe^{III}-chelating properties of *N*-hydroxy-2(1*H*)-pyrimidinone (**HOPY**) and -pyrazinone (**HOPR**).[32] They showed high water solubility, and lower pK_a values compared to hydroxypyridinones, and formed 3:1 Fe^{III} complexes under acidic to neutral conditions. However, the stability of these complexes was below that of 1-hydroxy-2(1*H*)-pyridinone.[16] To develop more effective Fe^{III}-chelating agents, the hexadentate ligands, **3-HOPR(X)**, were constructed. Their principal advantages are (1) the chelate effect of hexadentate ligand and C_3-symmetrical structure enhance the stability of the Fe^{III} complex, and (2) the selection of binding moiety with low pK_a values and its high water solubility allow an investigation of Fe^{III}-chelating properties under physiological pH.

We describe here the synthesis of novel heterocyclic hexadentate ligands, **3-HOPR(X)**, in which three **HOPR-Me** units are linked to tris(2-aminoethyl)amine through the dipeptide chains, and their Fe^{III}-chelating properties in aqueous solution. In addition, we discuss whether the absolute configuration around Fe^{III} is controlled by the chiral L-amino acid residues. Kinetic results on Fe^{III} removal of these ligands from transferrin at physiological pH are also discussed.



3-HOPR (X)
X=Me, Bu^t



HOPR-Me

Results and Discussion

Synthesis. The synthetic procedure for 1-(benzyloxy)-3-(carboxyethyl)-5,6-dimethyl-2(1*H*)-pyrazinone (**6**) was depicted in Scheme 1. The use of L-glutamic acid γ-methyl ester instead of glycine[32] allows the introduction of the carboxyl functional group at C-3 position of the pyrazinone ring. *N-tert*-Butoxycarbonyl(Boc)-L-glutamic acid γ-methyl ester (**2**) was coupled with *O*-benzylhydroxylamine by the mixed anhydride method using isobutyl chloroformate (IBCF).[33] Deprotection of the Boc group with 4 N HCl in dioxane, followed by condensation of the hydrochloride salt (**4**) with biacetyl at −30 °C under basic condition gave 1-(benzyloxy)-3-[(methoxycarbonyl)-ethyl]-5,6-dimethyl-2(1*H*)-pyrazinone (**5**). The ester **5** was easily converted to the corresponding carboxylic acid **6** by treatment with 1 N NaOH in MeOH. As shown in Scheme 2, an L-amino acid (Ala or Leu) and β-alanine were successively introduced to compound **6** by the general peptide coupling method[33] using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (water soluble carbodiimide: **WSC·HCl**) and 1-hydroxybenzotriazole (**HOBt**). Dipeptide-linked pyrazinones **10** were converted to the corresponding *O*-succinimide esters **11** and then coupled with tris(2-aminoethyl)amine (**TREN**) at 38 °C to give the *O*-protected compounds **12**. Debenzylation by catalytic hydrogenation on 10% Pd−C and subsequent gel chromatographic purification afforded the desired hexadentate ligands **3-HOPR(X)**.

^{1}H NMR spectra of compound **12** and **3-HOPR(X)** in DMSO-d_6 at room temperature exhibited three sets of amide protons (δ 8.0−7.7), two of which are triplets and one a doublet, indicating that these molecules possess the pseudo-C_3-symmetry. The temperature dependence of the amide proton chemical shifts[34] was measured in the range from 22 to 90 °C. The plot gave a straight line (−4.6 to −5.4 × 10^{-3} ppm K^{-1}), but no particular hydrogen bonds were observed in DMSO-d_6 solution.

Fe^{III} Complex Formation. In UV-vis spectra of the 1:1 molar mixture of Fe^{III} and **3-HOPR(Me)** in water

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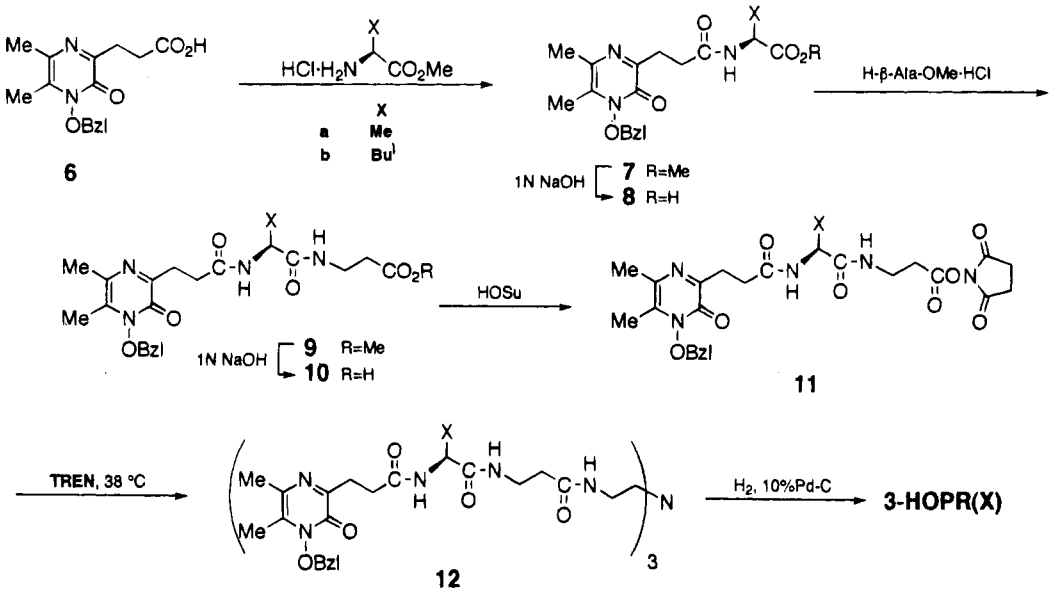
Scheme 2



under various pH conditions, the characteristic LMCT (ligand to metal charge transfer) band was observed around 450 nm in a wide pH range from 2 to 8 (pH 4.0, ϵ 3000 at 450 nm). These λ_{max} and ϵ values were comparable to those of Fe^{III}–**HOPR-Me** = 1:3 complex (ϵ 4237 at 425 nm),[32] suggesting the formation of 1:3 complex of Fe^{III} to the hydroxamate group of **3-HOPR-(Me)**. Formation of the 1:3 complex was also confirmed by the mole ratio plot.

Configuration of Fe^{III} Complex. The Λ-cis configuration of hydroxamate ligands around the central Fe^{III} has been proved for ferrichrome by means of X-ray crystallographic and CD analyses both in the solid state and in solution.[35-37] From molecular model examination of the metal complex of the hexadentate ligand, it is evident that the trans isomer can be precluded due to the steric hindrance, and that Δ and Λ configurations are equally possible for the cis isomer. Therefore, the absolute configuration of Fe(**3-opr(X)**) in solution was examined by CD spectroscopy. The spectra of Fe(**3-opr-(X)**) in water were shown in Figure 1. Fe(**3-opr(Me)**) showed a negative band at 360 nm and a positive band at 478 nm. The spectroscopic results allow the configuration of Fe(**3-opr(Me)**) to be assigned to the Λ-cis configuration in aqueous solution. On the other hand, Fe(**3-opr(Bu^i)**) showed the opposite CD pattern to Fe-(**3-opr(Me)**) (λ(nm) 369 and 485), indicating the Δ-cis configuration. Recently, Shanzer and co-workers have synthesized L-amino acid-containing enterobactin and ferrichrome analogs[38,39] and investigated the configuration of their Fe^{III} complexes. It was suggested that strong intramolecular hydrogen-bonds in the ligand could constrain the molecule's conformation and the orientation of the side chains to stabilize particular configuration of Fe^{III} complex (enterobactin models: Δ type, ferrichrome models: Λ type). As mentioned above, however, no particular hydrogen bond was detected in the ligands. It is quite interesting that the difference of amino acid residues in a molecule causes dramatic change in the absolute configuration although the mechanism remains unclear.

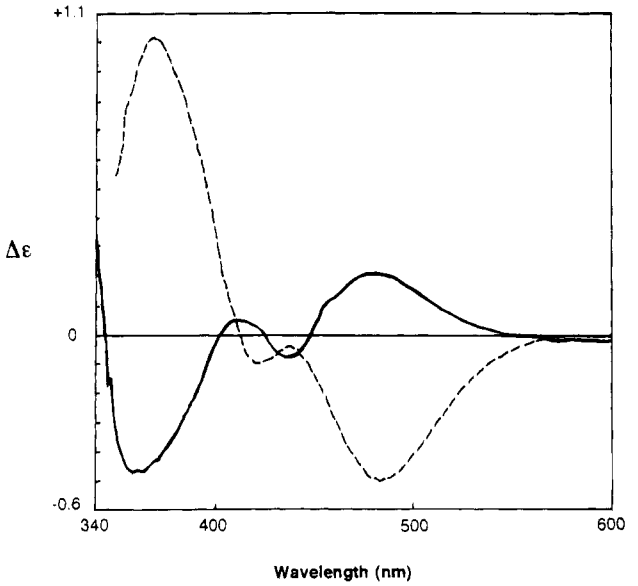


Figure 1. CD spectra of Fe^{III} complex of **3-HOPR(X)** in water at pH 6.0: [Fe(**3-opr(Me)**)] = 0.3 mM (—); [Fe(**3-opr(Bu^i)**)] = 0.6 mM (- - -).

Gallium Complex of 3-HOPR(X). To obtain the information on the conformation in solution, ^{1}H NMR spectra of diamagnetic Ga^{III} complexes were measured in 10% CD_3OD in D_2O (pD 6) at room temperature. Two singlets due to methyl protons apparently shifted to down field compared to those of free ligands; Δδ 0.13 for 5-Me and 0.06 for 6-Me in Ga(**3-opr(Me)**), and Δδ 0.13 for 5-Me and 0.07 for 6-Me in Ga(**3-opr(Bu^i)**). The overlapped signal due to two methylene protons of the $CONHCH_2$ moiety in β-Ala and **TREN** was separated into two signals: a signal at 3.43 ppm into 3.42 and 3.57 ppm in Ga(**3-opr(Me)**) and a signal at 3.42 ppm into two signals at 3.45 and 3.60 ppm in Ga(**3-opr(Bu^i)**). These

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Table 1. Stability Constants with Fe^{III} of 3-HOPR(X)[a]

ligand (L)	K_{eq}[b]	log K
3-HOPR(Me)	0.019	22.5
3-HOPR(Bu^i)	0.167	21.7
DFB[c]	–	30.5

[a] T = 20 °C, μ = 0.04 with KNO_3 solution, pH 6.0 (McIlvain's buffer). $C_{Fe^{III}}$ 0.07–0.36 mM, C_L 0.07–0.36 mM, C_{EDTA} 0.15–0.20 mM. [b] K_{eq} is the equilibrium constant for the reaction Fe(**3-opr(X)**) + H_2**EDTA**$^{2-}$ + H^+ ⇌ Fe**EDTA**$^-$ + **3-HOPR(X)**. [c] Reference 41.

spectral changes indicated the formation of the Ga^{III} complex. The only simple set of signals for these ligands indicated that the complexes fold a C_3-symmetric structure. More detailed ^1H NMR study on the complex in aprotic solvent such as $CDCl_3$ was impossible due to its low solubility.

Fe^{III} Binding Stability of 3-HOPR(X). The stability constant of Fe(**3-opr(X)**) is defined by the following equilibrium:

$$Fe^{3+} + 3\text{-opr(X)}^{3-} \overset{K}{\rightleftharpoons} Fe(3\text{-opr(X)}) \qquad (1)$$

The stability constants of Fe(**3-opr(X)**) were determined by competition against **EDTA**.[40] Three proton dissociation constants of hydroxamic acid groups were important parameters for this calculation. These values, however, were approximated by pK_a value of the bidentate ligand **HOPR-Me** (pK_a 4.7)[32] due to experimental limitation. The relative stability constants were obtained from the acid dissociation constants[41] and the stability constant[42] of Fe^{III}–**EDTA** complex, after determination of an equilibrium point for the Fe^{III} exchange reaction at pH 6.15 for **3-HOPR(Me)** and at pH 6.30 for **3-HOPR(Bu^i)**, and the results are shown in Table 1. The stability constant of Fe(**3-opr(Me)**) was 1 order greater than that of Fe(**3-opr(Bu^i)**), suggesting that the substituent at α-carbon of the amino acid residue affected the stability of the complex. However, these stability constants were below that of ferrioxamine B.[41]

Fe^{III} Removal from Transferrin. The possibility of Fe^{III} removal of **3-HOPR(X)** from human diferric transferrin ($Fe_{2.0}$**Tf**) was evaluated at pH 7.4 and 6.0. $Fe_{2.0}$**Tf** was prepared from commercially available apotransferrin (98%, Sigma) according to the literature.[17,43,44] The reaction was initiated by mixing solutions of **3-HOPR(X)** (0.2 mM, 2 mL) and $Fe_{2.0}$**Tf** (0.04 mM, 2 mL), and absorbance at 430 nm was monitored. The plots of log $[(A_\infty - \text{Abs})/(A_\infty - A_0)]$ as a function of time[17] at pH 7.4 gave a straight line as shown in Figure 2, indicating that the reaction of Fe^{III} removal from transferrin by **3-HOPR(Me)** proceeded according to the pseudo-first-order kinetics. From the slope k_{obsd} was obtained, and the results are summarized in Table 2. In the case of **3-HOPR(Me)**, the rate of Fe^{III} removal at pH 6.0 was 15 times faster than that at pH 7.4. This observation strongly suggests that the low pK_a value of *N*-hydroxy-2(1*H*)-pyrazinone is advantageous for the completeness of the reaction with transferrin. The presence of bulky substituent in **3-HOPR(Bu^i)** lowers the pseudo-first-order rate constant to one-fourth that of **3-HOPR(Me)**.

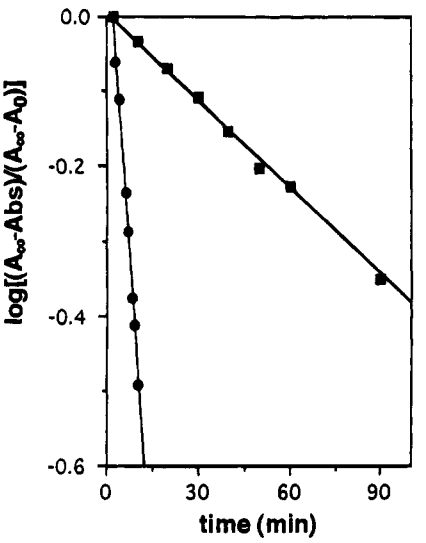

Figure 2. The plots of log $[(A_\infty - \text{Abs})]/(A_\infty - A_0)]$ vs time on Fe^{III} removal of **3-HOPR(Me)** from $Fe_{2.0}$**Tf**; pH 6.0 (●), pH 7.4 (♦). The absorbance at 430 nm was taken at 30 s after mixing **3-HOPR(Me)** in Tris buffer solution with $Fe_{2.0}$**Tf** stock solution. The absorbance at 300 min was used for A_∞.

Table 2. Fe^{III} Removal from Transferrin at pH 7.4

ligand (L)	[L]/[$Fe_{2.0}$**Tf**]	k_{obsd} (× 10^{-3} min^{-1})	% Fe^{III} removed[a]
3-HOPR(Me)	5	6.12[b]	
	5	0.38	23
3-HOPR(Bu^i)	6	0.09	9
DFB[c]	100		5

[a] At a point 30 min after the reaction was initiated. [b] pH 6.0. [c] Reference 45.

It is noteworthy that **3-HOPR(Me)** efficiently removed Fe^{III} from transferrin compared to **DFB** even at a small excess of the ligand to transferrin ([L]/[$Fe_{2.0}$**Tf**] 5–6) at 30 min after the reaction was initiated. In conclusion, the present novel ligands act as more efficient Fe^{III} removal agents than conventional **DFB**.

Summary and Conclusion

N-(Benzyloxy)-2(1*H*)-pyrazinone bearing the carboxyl group at C-3 position was synthesized from L-glutamic acid. The successive coupling of the pyrazinone with amino acids and tris(2-aminoethyl)amine and subsequent removal of the benzyl group afforded novel hexadentate ligands. The spectrophotometric analysis in aqueous solution revealed the 3:1 stoichiometrical complexation of the hydroxamate in **3-HOPR(X)** with Fe^{III}. In addition, it is suggested that the low pK_a of *N*-hydroxyamide of the 2(1*H*)-pyrazinone enables them to bind Fe^{III} under acidic to neutral conditions. ^1H NMR analysis of Ga^{III} complex indicated that the complex possessed a C_3-symmetry. CD spectra demonstrated that Fe(**3-opr(Me)**) exists predominantly in the Λ-cis configuration, while Fe(**3-opr(Bu^i)**) exists in the Δ-cis configuration in water. Further, it is worthy of note that these novel hexadentate ligands efficiently removed Fe^{III} from transferrin compared to **DFB**.

Experimental Section

General. Melting points were determined on a Mel-Temp apparatus in open capillaries and are uncorrected. IR and UV-vis spectra were recorded on a JASCO A-100 infrared and on

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a JASCO Ubest V-550 spectrophotometers, respectively. ^{1}H NMR spectra were obtained on a JEOL GX-270 spectrometer in $CDCl_3$, CD_3OD, and DMSO-d_6 solutions. Chemical shifts are reported in ppm (δ) downfield from internal TMS. Thin layer chromatography (TLC) was performed on silica gel 60 F-254 with a 0.2 mm layer thickness. Column chromatography was carried out with Merck Kieselgel 60 (230–400 mesh). Optical rotations were determined on a JASCO PIP-370 digital polarimeter. CD spectra were measured with a JASCO J-720 spectropolarimeter. HPLC was carried out on a JASCO 880-PU and a 875-UV equipped with a JASCO IT integrator by using a column packed with a Finepak SIL $C_{12}S$. Combustion analyses were performed on a YANACO MT-3 CHN corder. *N*-(*tert*-Butoxycarbonyl)-L-glutamic acid γ-methyl ester (**2**) was prepared according to the literature.[46,47]

***N*-(Benzyloxy)-*N*$^{\alpha}$-(*tert*-butoxycarbonyl)-L-glutamamide γ-Methyl Ester (3).** To a solution of **2** (6.78 g, 26 mmol), and Et_3N (2.91 g, 29 mmol) in THF (50 mL) was added isobutyl chloroformate (3.56 g, 26 mmol) in THF (20 mL) at −17 °C. After 15 min, *O*-benzylhydroxylamine (2.96 g, 27 mmol) in THF (20 mL) was added to the mixture at −15 °C, and then the reaction mixture was stirred overnight at room temperature. The precipitated Et_3N·HCl was filtered off and the filtrate was then evaporated. The residue was dissolved in EtOAc (300 mL), and the organic layer was washed with 10% citric acid, 4% $NaHCO_3$, brine, and dried (Na_2SO_4). Evaporation of the solvent gave the product **3** as colorless crystals, 8.29 g (87%): mp 88–90 °C; IR (KBr) 1720, 1650, 740, 700 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 2.05–2.39 (m, 4H), 3.65 (s, 3H), 4.07 (br s, 1H), 4.89 (s, 2H), 5.18 (br s, 1H), 7.37 (m, 5H), 9.51 (br s, 1H). Anal. Calcd for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.65. Found: C, 59.24; H, 6.82; N, 7.29.

***N*-(Benzyloxy)-L-glutamamide γ-Methyl Ester Hydrochloride (4).** Compound **3** (7.8 g, 21.3 mmol) was dissolved in 4 N HCl in dioxane (100 mL) at 0 °C. Disappearance of **3** was monitored by TLC. The reaction mixture was evaporated to remove HCl and dioxane. Dry benzene was added to the residue and evaporated. Addition and evaporation of benzene were repeated three times to give the product (ca. 100%).

1-(Benzyloxy)-3-[(methoxycarbonyl)ethyl]-5,6-dimethyl-2(1*H*)-pyrazinone (5). To a solution of compound **4** (6.83 g, 22.6 mmol) in MeOH–H_2O (2:1) mixture (60 mL) was added biacetyl (1.94 g, 22.5 mmol) in a dry ice–acetone bath (−30 °C). The reaction mixture was adjusted to pH 8 with 4 N NaOH solution and then stirred for 2 h at room temperature. After evaporation of the solvent, the residue was dissolved in $CHCl_3$ (500 mL). The organic layer was washed with 10% citric acid, 4% $NaHCO_3$, brine, and dried (Na_2SO_4). The residue was purified by column chromatography on silica gel with $CHCl_3$–acetone–EtOH (100:5:1) mixture to give the product **5** as pale yellow crystals, 3.1 g (43%): mp 118–121 °C, IR ($CHCl_3$) 1730, 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.21 (s, 3H), 2.25 (s, 3H), 2.80 (t, J = 7 Hz, 2H), 3.20 (t, J = 7 Hz, 2H), 3.71 (s, 3H), 5.26 (s, 2H), 7.43–7.49 (m, 5H). Anal. Calcd for $C_{17}H_{20}N_2O_4$: C, 64.54; 6.37; N, 8.85. Found: C, 64.41; H, 6.26; N, 8.58.

1-(Benzyloxy)-3-(carboxyethyl)-5,6-dimethyl-2(1*H*)-pyrazinone (6). To a solution of compound **5** (350 mg, 1.1 mmol) in MeOH (10 mL) was added 1 N NaOH (1.5 mL, 1.5 mmol) at 0 °C. After stirring for 6.5 h at room temperature, the solution was evaporated to remove the bulk of MeOH. The residual aqueous solution was adjusted to pH 2 with 5 N HCl at 0 °C and then extracted with $CHCl_3$ (100 mL × 3). The organic layer was washed with 10% citric acid, brine, and dried ($MgSO_4$). Evaporation of the solvent gave the product as pale yellow crystals, 297 mg (89%): ^{1}H NMR ($CDCl_3$) δ 2.15 (s, 3H), 2.18 (s, 3H), 2.68 (t, J = 7 Hz, 2H), 3.24 (t, J = 7 Hz, 2H), 5.20 (s, 2H), 7.40 (m, 5H), 9.79 (br s, 1H). Anal. Calcd for $C_{16}H_{18}N_2O_4$: C, 63.56; H, 6.00; N, 9.27. Found: C, 63.84; H, 5.89; N, 9.02.

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***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-alanine Methyl Ester (7a).** WSC·HCl (1.86 g, 9.7 mmol) in CH_2Cl_2 (40 mL) was added to a mixture of compound **6** (2.35 g, 7.8 mmol), H-L-Ala-OMe·HCl (1.46 g, 10.4 mmol), *N*-methylmorpholine (1.08 g, 10.7 mmol), and HOBt (1.90 g, 12.4 mmol) in DMF (20 mL) at −10 °C. The mixture was stirred overnight at room temperature. After removal of DMF under reduced pressure, the residue was dissolved in EtOAc (300 mL). The organic layer was washed with water, 4% $NaHCO_3$, 10% citric acid, brine, and dried ($MgSO_4$). Evaporation of the solvent, followed by purification by column chromatography on silica gel with $CHCl_3$–acetone–EtOH (100:10:1) mixture gave the product, 2.2 g (71%): mp 116–120 °C; ^{1}H NMR ($CDCl_3$) δ 1.38 (d, J = 7 Hz, 3H), 2.16 (s, 3H), 2.23 (s, 3H), 2.69 (m, 2H), 3.16 (m, 2H), 3.69 (s, 3H), 4.57 (m, 1H), 5.20 (s, 3H), 7.30–7.44 (m, 5H). Anal. Calcd for $C_{20}H_{25}N_3O_5$: C, 62.00; H, 6.50; N, 10.85. Found: C, 61.69; H, 6.50; N, 10.85.

***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-leucine Methyl Ester (7b).** The coupling of compound **6** (320 mg, 1.1 mmol) and H-L-Leu-OMe·HCl (278 mg, 1.5 mmol) was carried out by a similar procedure to **7a** to give the product **7b**, 350 mg (60%): mp 85–97 °C; ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.1 Hz, 6H), 1.54–1.62 (m, 3H), 2.20 (s, 3H), 2.25 (s, 3H), 2.71 (t, J = 6.6 Hz, 2H), 3.19 (t, J = 6.6 Hz, 2H), 3.70 (s, 3H), 4.64 (m, 1H), 5.24 (s, 2H), 6.71 (d, J = 8.0 Hz, 1H), 7.40–7.48 (m, 5H). Anal. Calcd for $C_{23}H_{31}N_3O_5 \cdot 0.5H_2O$: C, 62.99; H, 7.36; N, 9.58. Found: C, 62.86; H, 7.10; N, 9.68.

***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-alanine (8a).** To a solution of compound **7a** (2.2 g, 5.6 mmol) in MeOH (100 mL) was added 1 N NaOH (11 mL, 11 mmol) at 0 °C. After 3 h, the mixture was neutralized and then evaporated. The residual aqueous solution was acidified with 5 N HCl at 0 °C and then extracted with $CHCl_3$ (200 mL). The organic layer was washed with 10% citric acid, brine, and dried ($MgSO_4$). Evaporation of the solvent, followed by recrystallization from benzene–hexane mixture gave the product **8a** as pale yellow crystals, 1.90 g (92%): mp 158–162 °C; ^{1}H NMR ($CDCl_3$) δ 1.43 (d, J = 7 Hz, 3H), 2.20 (s, 3H), 2.24 (s, 3H), 2.70 (t, J = 7 Hz, 2H), 3.18 (t, J = 7 Hz, 2H), 4.57 (m, 1H), 5.23 (s, 2H), 7.38–7.48 (m, 5H), 7.95 (d, J = 6 Hz, 1H, NH). Anal. Calcd for $C_{19}H_{23}N_3O_5 \cdot 0.3H_2O$: C, 60.25; H, 6.24; N, 11.10. Found: C, 60.18; H, 6.52; N, 10.99.

***N*-[3-(1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-leucine (8b).** The hydrolysis of compound **7b** (350 mg, 0.81 mmol) with 1 N NaOH was carried out by a similar procedure to **8a** to give the product **8b** as yellow crystals, 330 mg (90%): mp 147–149 °C; ^{1}H NMR ($CDCl_3$) δ 0.92 (d, J = 5.0 Hz, 6H), 1.55–1.68 (m, 3H), 2.20 (s, 3H), 2.24 (s, 3H), 2.72 (t, J = 6.8 Hz, 2H), 3.19 (t, J = 6.8 Hz, 2H), 4.60 (m, 1H), 5.23 (s, 2H), 6.97 (d, J = 8.0 Hz, 1H), 7.38–7.49 (m, 5H). Anal. Calcd for $C_{22}H_{29}N_3O_5 \cdot 1.0H_2O$: C, 60.96; H, 7.21; N, 9.69. Found: C, 61.09; H, 7.23; N, 9.38.

***N*-3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-alanyl-β-alanine Methyl Ester (9a).** Compound **8a** (1.45 g, 3.89 mmol) was coupled with H-β-Ala-OMe·HCl (706 mg, 5.06 mmol) in the presence of HOBt (1.17 g, 7.65 mmol), *N*-methylmorpholine (529 mg, 5.23 mmol), and WSC·HCl (904 mg, 4.71 mmol) by the same procedure as for **7a**. The crude product was purified by column chromatography on silica gel with $CHCl_3$-acetone-EtOH (100:40:8) mixture to give the pure product **9a**, 1.44 g (81%): mp 134–137 °C; $[\alpha]^{22}_D$ −17.3° (*c* 0.38, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.35 (d, J = 7 Hz, 3H), 2.19 (s, 3H), 2.24 (s, 3H), 2.52 (t, J = 6 Hz, 2H), 2.67 (t, J = 6 Hz, 2H), 3.17 (m, 2H), 3.49 (t, J = 6 Hz, 2H), 3.67 (s, 3H), 4.47 (m, 1H), 5.23 (s, 2H), 7.01 (d, J = 7 Hz, 1H, NH), 7.09 (br s, 1H, NH), 7.30–7.49 (m, 5H). Anal. Calcd for $C_{23}H_{30}N_4O_6 \cdot 0.5H_2O$: C, 59.08; H, 6.68; N, 11.98. Found: C, 58.97; H, 6.54; N, 12.14.

***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-leucyl-β-alanine Methyl Ester (9b).** The coupling of compound **8b** (330 mg, 0.79 mmol) and H-β-Ala-OMe·HCl (127 mg, 0.91 mmol) was carried out by a similar procedure to **9a** to give the product **9b**, 360 mg (91%): mp

142–148 °C; ^{1}H NMR ($CDCl_3$) δ 0.90 (d, J = 4.3 Hz, 3H), 0.92 (d, J = 4.3 Hz, 3H), 1.51–1.70 (m, 3H), 2.20 (s, 3H), 2.25 (s, 3H), 2.51 (t, J = 6.3 Hz, 2H), 2.68 (t, J = 7.3 Hz, 2H), 3.17 (t, J = 7.3 Hz, 2H), 3.48 (m, 2H), 3.68 (s, 3H), 4.40 (m, 1H), 5.24 (s, 2H), 6.70 (d, J = 9.0 Hz, 1H), 6.82 (br s, 1H), 7.39–7.50 (m, 5H). Anal. Calcd for $C_{26}H_{36}N_4O_6$·1.0H_2O: C, 60.21; H, 7.39; N, 10.80. Found: C, 60.22; H, 7.19; N, 10.79.

***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-alanyl-β-alanine (10a).** Compound **9a** (350 mg, 0.76 mmol) was hydrolyzed by a similar procedure to **8a** to give the product **10a**, 311 mg (92%); mp 172–173 °C; $[\alpha]^{22}_D$ −17.2° (c 0.52, MeOH); IR (KBr) 3550–2930, 1732, 1658 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.34 (m, 3H), 2.27 (s, 6H), 2.52 (m, 2H), 2.71 (m, 2H); 3.12 (m, 2H), 3.44 (m, 2H), 4.35 (m, 1H), 5.26 (s, 2H), 7.43–7.52 (m, 5H). Anal. Calcd for $C_{22}H_{28}N_4O_6$·0.5H_2O: C, 58.28; H, 6.40; N, 12.36 Found: C, 58.30; H, 6.32; N, 12.25.

***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-)-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-leucyl-β-alanine (10b).** Compound **9b** (230 mg, 0.46 mmol) was hydrolyzed with 1 N NaOH (1 mL, 1 mmol) by a similar procedure for **10a** to give the product **10b**, 180 mg (80%): mp 144–149 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (d, J = 6.4 Hz, 3H), 0.88 (d, J = 6.4 Hz, 3H), 1.55 (m, 3H), 2.25 (s, 3H), 2.52 (m, 2H), 2.70 (m, 2H), 3.16 (m, 2H), 3.49 (m, 2H), 4.70 (m, 1H), 5.23 (s, 2H), 7.11 (d, J = 8.8 Hz, 1H), 7.35–7.48 (m, 5H), 7.66 (m, 1H). Anal. Calcd for $C_{25}H_{34}N_4O_6$·0.5H_2O: C, 60.59; H, 7.19; N, 11.31. Found: C, 60.25; H, 7.02; N, 11.20.

***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-alanyl-β-alanine *O*-Succinimide Ester (11a).** WSC·HCl (374 mg 1.95 mmol) in CH_2Cl_2 (9 mL) was added to a mixture of compound **10a** (352 mg, 0.94 mmol) and HOSu (219 mg, 1.9 mmol) in DMF (4.5 mL) at −10 °C. After stirring for 24 h at room temperature, the solvent was removed under reduced pressure, and the residue was dissolved in $CHCl_3$ (300 mL). The organic layer was washed with H_2O, 4% $NaHCO_3$, and brine and dried ($MgSO_4$). Evaporation of the solvent gave the *O*-succinimide ester **11a** as colorless crystals, which was used for the next reaction without further purification, 805 mg (100%); IR ($CHCl_3$) 1816, 1792, 1740 cm^{-1}.

***N*-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanoyl]-L-leucyl-β-alanine *O*-Succinimide Ester (11b).** Compound **10b** (180 mg, 0.37 mmol) was coupled with HOSu (54 mg, 0.47 mmol) by a similar procedure to **11a** to give the corresponding *O*-succinimide ester **11b** as yellow crystals, which was used directly for the next reaction, 230 mg (ca. 100%): IR ($CHCl_3$) 1810, 1740, 1710 cm^{-1}.

Tris-[2-[3-[2-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanamido]-2(*R*)-methylethanamido]propanamido]ethyl]amine (12a). A solution of compound **11a** (511 mg, 0.94 mmol) and tris(2-aminoethyl)amine (40 mg, 0.27 mmol) in DMF (12 mL) was stirred for 48 h at 38 °C. After removal of the solvent, $CHCl_3$ (400 mL) and 0.1 N NaOH (100 mL) were added to the residue. The organic layer was washed with H_2O and brine and dried (Na_2SO_4). Purification by column chromatography on silica gel with $CHCl_3$–MeOH (6:1) mixture and subsequent gel chromatography on TOYOPEARL HW-40 with MeOH as an eluent gave the product **12a**, 283 mg (74%): mp 158–160 °C dec; $[\alpha]^{22}_D$ −15.6° (c 0.36, MeOH); IR (KBr) 1648 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.32 (d, J = 7 Hz, 6H), 2.24 (s, 18H), 2.42 (t, J = 6 Hz, 6H), 2.60 (m, 6H), 2.67 (t, J = 7 Hz, 6H), 3.08 (m, 6H), 3.25 (m, 6H), 3.43 (t, 6H), 4.28 (q, J = 7 Hz, 6H), 5.23 (s, 6H), 7.40–7.51 (m, 15H). Anal. Calcd for $C_{72}H_{96}N_{16}O_{15}$·2.5H_2O: C, 58.80; H, 6.92; N, 15.24. Found: C, 58.59; H, 7.18; N, 15.76.

Tris-[2-[3-[2-[3-[1-(Benzyloxy)-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl]propanamido]-2(*R*)-*sec*-butylethanamido]propanamido]ethyl]amine (12b). The coupling of compound **11b** (1.26 g, 2.16 mmol) and tris(2-aminoethyl)-amine (104 mg, 0.71 mmol) in DMF (25 mL) was carried out at 38 °C for 96 h. The crude product was purified by column and gel chromatography as described for compound **12a** to give the product **12b**, 940 mg (85%): mp 165–172 °C dec; $[\alpha]^{20}_D$ −4.15° (c 0.58, MeOH); ^{1}H NMR (DMSO-d_6) δ 0.80 (d, J = 6 Hz, 9H), 0.86 (d, J = 6 Hz, 9H), 1.41 (m, 6H), 1.55 (m, 3H), 2.18 (s, 9H), 2.24 (s, 9H), 2.24 (m, 6H), 2.50 (m, 6H), 2.90 (m, 6H), 3.07 (m, 6H), 3.30 (m, 12 H), 4.22 (m, 3H), 5.20 (s, 6H), 7.42–7.52 (m, 15H), 7.77 (br s, 3H), 7.90 (br s, 3H), 7.95 (d, J = 8 Hz, 3H). Anal. Calcd for $C_{81}H_{114}N_{16}O_{15}$·3$H_2O$: C, 60.58; H, 7.53; N, 13.95. Found: C, 60.44; H, 7.40; N, 13.99.

Tris-[2-[3-[2-[3-(1-Hydroxy-5,6-dimethyl-2-oxo-1,2-dihydropyraz-3-yl)propanamido]-2(*R*)-methylethanamido]propanamido]ethyl]amine (3-HOPR(Me)). A suspension of 10% Pd–C (86 mg) suspended in MeOH (10 mL) was prehydrogenated with H_2 for 0.5 h. To the suspension was added a solution of compound **12a** (157 mg, 0.11 mmol) in MeOH (40 mL). After hydrogenation with H_2 under atmospheric pressure for 1 h under reflux, the catalyst was removed by filtration. The filtrate was evaporated to give the residue, which was purified by gel chromatography on Shephadex LH-20 with MeOH to afford the product (**3-HOPR(Me)**), 104 mg (84%): $[\alpha]^{25}_D$ −16.5° (c 0.11, MeOH); ^{1}H NMR (CD_3OD) δ 1.33 (d, J = 7.1 Hz, 9H), 2.31 (s, 9H), 2.38 (s, 9H), 2.40 (m, 6H), 2.64 (t, J = 8.9 Hz, 6H), 3.01–3.05 (m, 12H), 3.43 (m, 12H), 4.27 (q, J = 7.1 Hz, 3H). Anal. Calcd for $C_{51}H_{78}N_{16}O_{15}$·8.5H_2O: C, 46.82; H, 7.32; N, 17.13. Found: C, 46.98; H, 6.99; N, 16.79.

Tris[2-[3-[2-[3-(1-Hydroxy-5,6-dimethyl-2-oxo-1,2-dihydro-pyraz-3-yl)propanamido]-2(*R*)-*sec*-butylethanamido]propanamido]ethyl]amine (3-HOPR(Bu^i)). Compound **12b** (100 mg, 0.064 mmol) was hydrogenated with 10% Pd–C (45 mg) in MeOH (50 mL) in the presence of conc. HCl (17 μL) for 25 min at room temperature. Purification by gel chromatography on Shephadex LH-20 with MeOH gave the product (**3-HOPR(Bu^i)**), 56 mg (68%): mp 170–190 °C dec; $[\alpha]^{22}_D$ −7.79° (c 1.0, MeOH); ^{1}H NMR (DMSO-d_6) δ 0.80 (d, J = 7 Hz, 9H), 0.86 (d, J = 7 Hz, 9H), 1.41 (t, J = 7 Hz, 6H), 1.54 (m, 3H), 2.22 (s, 9H), 2.27 (s, 9H), 2.27 (m, 6H), 2.52 (m, 6H), 2.75 (m, 6H), 2.89 (m, 6H), 3.15 (m, 6H), 3.27 (m, 6H), 4.21 (q, J = 7 Hz, 3H), 7.95 (br s, 3H), 8.30 (br s, 6H); (CD_3OD) δ 0.86 (d, J = 7 Hz, 9H), 0.93 (d, J = 7 Hz, 9H), 1.55 (m, 9H), 2.30 (s, 9H), 2.40 (s, 15H), 2.66 (m, 6H), 3.05 (m, 6H), 3.16 (m, 6H), 3.42 (m, 12H), 4.30 (t, J = 7 Hz, 3H). Anal. Calcd for $C_{60}H_{96}N_{16}O_{15}$·3.0H_2O: C, 53.95; H, 7.70. Found: C, 53.86; H, 7.47.

Gallium Complex Formation. A sample (8 mg) of each hexadentate ligand and $Ga(NO_3)_3$ (4–5 mg) was dissolved in 10% CD_3OD in D_2O (0.5 mL). pD was adjusted to 6 with flesh 0.4% NaOD in D_2O.[48] ^{1}H NMR spectra were measured at room temperature.

Ga(3-opr(Me)): δ 1.31 (d, J = 7 Hz, 9H), 2.24 (m, 6H), 2.44 (s, 24H), 2.67 (m, 6H), 3.06 (m, 6H), 3.42 (m, 6H), 3.57 (m, 6H), 4.20 (m, 3H).

Ga(3-opr(Bu^i)): δ 0.86 (d, J = 6 Hz, 9H), 0.92 (d, J = 6 Hz, 9H), 1.53 (m, 6H), 2.23–2.27 (m, 6H), 2.43 (s, 21H), 2.47 (s, 9H), 2.75 (m, 12H), 3.10 (m, 6H), 3.45 (br s, 6H), 3.60 (m, 6H), 4.25 (m, 3H).

Spectral Determination of the 1:1 Mixture. A sample (13–15 mg) of each hexadantate ligand was dissolved in deionized water (5.0 mL). A 1.0 mL volume of the sample solution was mixed with an equimolar amount of ferric nitrate solution (3.28 mM) and diluted to 10.0 mL (0.3 mM). The pH of the solution was adjusted to an appropriate value with 0.1 or 0.01 N NaOH or 0.1 or 0.01 N HNO_3 before spectral measurement.

Fe^{III} Binding Ratio. A sample of each hexadentate ligand was dissolved in deionized water (5.0 mL; 1.53 mM). A 0.5 mL volume of the sample solution was mixed with an appropriate amount of standard aqueous ferric nitrate solution (3.28 mM) and 0.5 mL of 0.4 M KNO_3. The pH of the mixture was adjusted to 4.0 with 0.01 or 0.1 N NaOH and diluted to 5.0 mL with acetate buffer (pH 4.0), and the visible spectra were measured.

Fe^{III} Exchange Reaction. Each Fe^{III} complex solution (0.195 mM) of hexadentate ligand was prepared by mixing the stock ligand solution (1.3 mM) with an equimolar amount of ferric nitrate solution (3.28 mM), and 0.5 mL of 0.4 M KNO_3, and then diluting to 5.0 mL with McIlvaine's buffer solution. **EDTA** in the buffer solution was prepared by dissolving **EDTA**·2Na^+·2H_2O in McIlvaine's buffer solution (ionic strength 0.04, pH 6.0) to give a concentration of 0.3 mM. Fe^{III} exchange

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reaction was followed by monitoring the decrease of absorbance at 450 nm. The relative stability constant of Fe^{III} complex was calculated by using the stability constant of Fe^{III}-**EDTA**,[42] the pK_a of the **HOPR-Me**,[32] and an equilibrium point at pH 6.0 at 20 °C.

Fe^{III} Removal from Transferrin. A commercially available human serum transferrin (98%, Sigma) was used. $Fe_{2.0}$**Tf** was prepared according to the literature reported in detail by Raymond.[17] The stock solutions of **3-HOPR(X)** (2 mL, 0.2 mM, pH 7.4) and $Fe_{2.0}$**Tf** (2 mL, 0.04 mM) in Tris buffer were combined, and then the absorbance of the solution was monitored at 430 nm. The pseudo-first-order-rate constant (k_{obsd}) was calculated from the slope of the plots of log $[(A_\infty - \text{Abs})/(A_\infty - A_0)]$ as a function of time.

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