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New Zinc(II) Complexes with Tetradentate Amino Acid Derivatives: Structure Characterization, Solution Chemistry, and in vitro Insulinomimetic Activity#

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Twelve new amino acid derivatives/Zn(II) complexes were prepared by the reactions of $ZnSO_4$ and nine tetradentate ligands, Gly, Ala, Val, Leu, Ser and His derivatives and three pentadentate ligands, Asp and His derivatives. The X-ray structure analysis of $[Zn_3(N\text{-pyridylmethyl-His})_3(H_2O)_2](ClO_4)_3\cdot 2H_2O$ revealed the formation of a trinuclear complex, in which three tetradentate ligands and two water molecules coordinate to three Zn(II), but the complex in an aqueous solution is present as a monomeric complex ion. Several Zn(II) complexes were found to have in vitro insulinomimetic activity which was estimated by the inhibition of free fatty acid release in isolated rat adipocytes treated with epinephrine. It was revealed that the insulinomimetic activities of Zn(II) complexes with the stability constants (log β) less than 11 were higher than that of $VOSO_4$ and were comparable to that of $ZnSO_4$ as positive controls except in the cases of two complexes with Gly derivatives, Zn(*N*,*N*-dipyridylmethyl-Gly)$^+$ and Zn(*N*,*N*′-trimethylene-bis-Gly).

Zinc(II) ion (Zn(II)) is known to be one of the most important essential trace elements found in living organisms as well as in many metalloproteins and metalloenzymes.[1] Among many pharmacological and nutritional roles of Zn(II),[2] this metal ion was found in 1980 to stimulate lipogenesis in rat adipocytes similarly to the action of insulin.[3] Based on these important results, $ZnCl_2$ was administered to streptozotocin-induced diabetes rats (STZ rats) or hereditary diabetic ob/ob mice and was found to normalize their high blood glucose levels, when a high dose[4] was given or a long-term (8 weeks) administration[5] of the compound was continued. However, the insulinomimetic activities of Zn(II) complexes have not yet been studied. During the investigations to find insulinomimetic metal complexes, we found that Zn(II) molecular complexes with maltol, amino acids, picolinic acid, and their derivatives have higher insulinomimetic activity than free Zn(II), as estimated by in vitro and in vivo experiments.[6–9] Furthermore, during our investigations on the development of insulinomimetic oxovanadium(IV) ($V^{IV}O$) complexes, we found that the $V^{IV}O$ complexes with tetradentate ligands exihibit excellent insulinomimetic activities in terms of the in vitro inhibition of free fatty acids (FFA) release in isolated rat adipocytes.[10,11] Therefore, it is expected that Zn(II) substitution of $V^{IV}O$/tetradentate amino acid complexes might open the window for developing a new type of insulinomimetic Zn(II) complexes. In this paper, we report several insulinomimetic Zn(II) complexes with tetradentate (pseudo-)tripodal type's mono-amino acid derivatives.

The esters of tetradentate ligands: ^{pm2}G, ^{pm2}A, ^{pm2}V, $^{pm2}V_R$, ^{pm2}L, and ^{pm2}S, ^{pm}D, and $^{6Me\text{-}pm}$D were prepared with Schiff bases that were made by a similar method to ^{pm}H[11] from each amino acid ester and pyridine-2-aldehyde or 6-methylpyridine-2-carbaldehyde by reducing with $Na[BH_3(CN)]$. Also, the ester of a pentadentate ligand, ^{pm2}D, was prepared from the tetradentate ligand, ^{pm}D, and pyridine-2-carbaldehyde, by reducing with $Na[BH_3(CN)]$. In addition, the methyl esters of two pentadentate, HeV and HeT, were prepared from L-histidine methyl ester and the methyl ester of L-valine or -threonine by a one pot method similar to that used for the preparation of the ester of HeY (Fig. 1).[11]

Thus, Zn(II) complexes with the ligands were synthesized and characterized by physical and analytical data. Their in vitro

General notice. In this article, abbreviations are used for the following compounds. ^{pm2}G: *N*,*N*-bis(2-pyridylmethyl)glycine, ^{pm2}A: *N*,*N*-bis(2-pyridylmethyl)-L-alanine, ^{pm2}V: *N*,*N*-bis(2-pyridylmethyl)-L-valine, $^{pm2}V_R$: *N*,*N*-bis(2-pyridylmethyl)-D-valine, ^{pm2}L: *N*,*N*-bis(2-pyridylmethyl)-L-leucine, ^{pm2}S: *N*,*N*-bis(2-pyridylmethyl)-L-serine, ^{pm}H: *N*-(2-pyridylmethyl)-L-histidine, ^{pm}D: *N*-2-pyridylmethyl-L-aspartic acid, $^{6Me\text{-}pm}$D: *N*-6-methyl-2-pyridylmethyl-L-aspartic acid, ^{pm2}D: *N*,*N*-bis(2-pyridylmethyl)-L-aspartic acid, HeV: *N*,*N*′-1,2-ethanediyl-*N*-L-histidine-*N*′-L-valine, HeT: *N*,*N*′-1,2-ethanediyl-*N*-L-histidine-*N*′-L-threonine, HeY: *N*,*N*′-1,2-ethanediyl-*N*-L-histidine-*N*′-L-tyrosine.

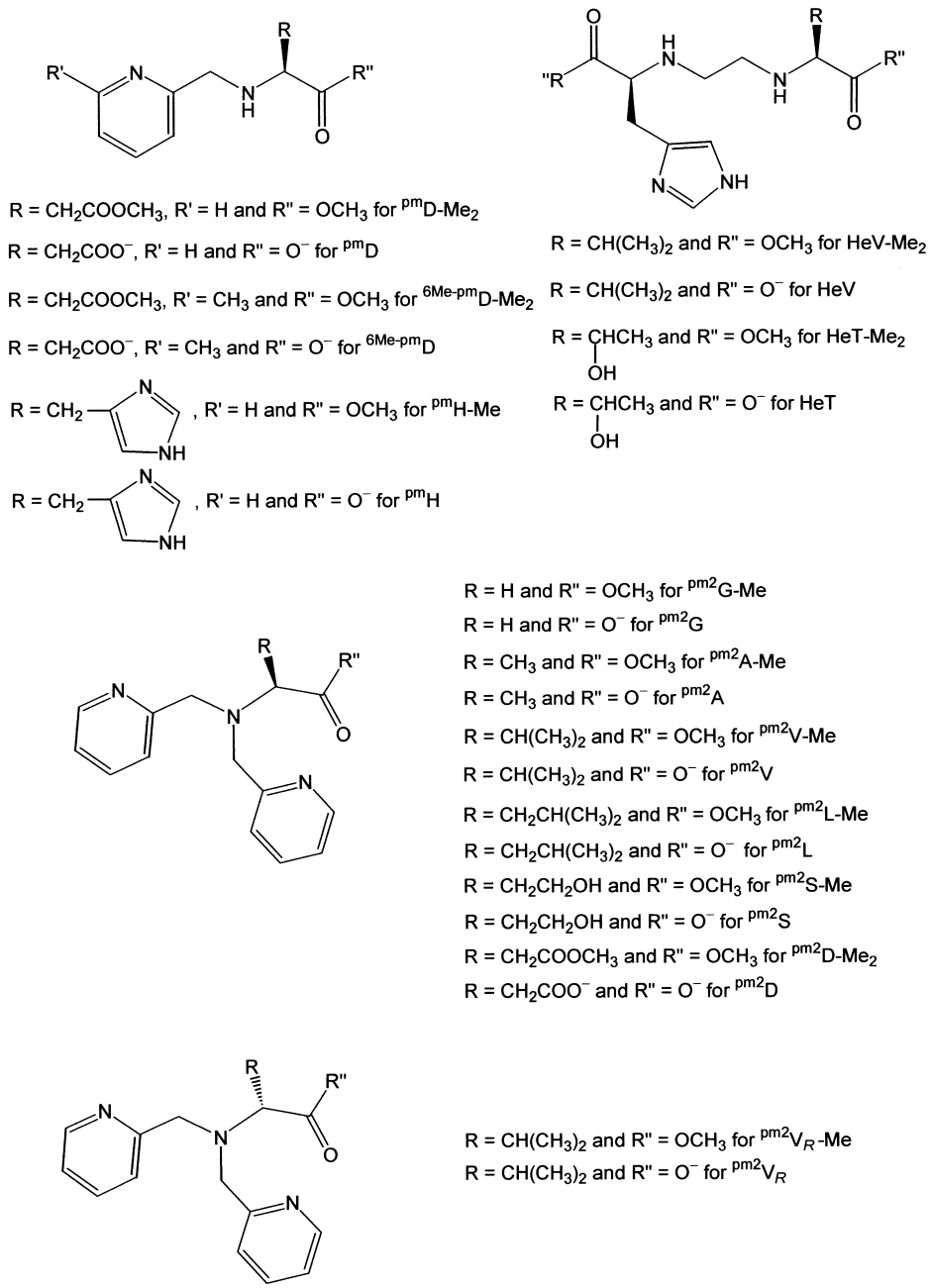

Fig. 1. The molecular formulas and abbreviations of ligands prepared.

insulinomimetic activities in regard to the inhibition of FFA release in isolated rat adipocytes treated with epinephrine were estimated. The evaluation method that has been proposed is simple and convenient for estimating the insulinomimetic activity of a compound.[12] The interrelationship between the insulinomimetic activities and the stability constants (log β) for the complex formations or the partition coefficients of Zn(II) complexes was also examined. In addition, the crystal structure of a complex was revealed by the X-ray structure analysis to be $[Zn_3(^{pm}H)_3(H_2O)_2](ClO_4)_3 \cdot 2H_2O$ (**1**). Interestingly, this complex was found to be present in a monomer in an aqueous solution by measuring the molecular weight of the complex.

Experimental

Materials. Reagents and solvents used in the experiments were of reagent grade, they were used without further purification unless otherwise noted. Amino acids and their hydrochloride esters were purchased from the Peptide Institute, Inc. (Osaka, Japan), Kokusan Laboratory Chemicals (Tokyo, Japan), Novabiochem (Tokyo, Japan), or Nacalai Tesque (Kyoto, Japan). Sodium cyanotrihydroborate ($Na[BH_3(CN)]$) was obtained from Aldrich (Tokyo, Japan). Acidic silica gel (BW-820MH) and a basic one (NH-DM1020) were obtained from Fuji Silysia Chemical (Aichi, Japan). For gel filtration, Sephadex LH-20 obtained from Amersham Pharmacia Biotech (Tokyo, Japan) was used. BSA (Bovine serum albumin; fraction V) and Epi ((±)-epinephrine monohydro-

chloride) were purchased from Sigma Chemical (Tokyo, Japan). $ZnSO_4 \cdot 7H_2O$ ($ZnSO_4$), $VOSO_4 \cdot 3.5H_2O$ ($VOSO_4$), and Collagenase was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Instrumentations. Elemental analyses of the complexes were carried out on a Perkin-Elmer 240C elemental analyzer (Tokyo, Japan). FT IR spectra were recorded on a JASCO FT/IR-420 spectrophotometer (Tokyo, Japan). ^{1}H NMR spectra were recorded on JEOL LA-300 or GX-400 FT NMR spectrometers (Tokyo, Japan). Me_4Si was used as an internal standard in the $CDCl_3$ solution. Melting points were determined on a Yanaco MP-J3 apparatus (Kyoto, Japan). Specific rotations $[\alpha]_D$ were measured on a Jasco DIP 300 polarimeter (Tokyo, Japan). FAB mass spectra were recorded on JEOL JMS-700T and AX500 mass spectrometers (Tokyo, Japan). Zn(II) concentrations for the measurement of partition coefficients of the complexes were determined by using a Shimadzu ICP MS 8600 (inductively coupled plasma-mass spectrometer) (Kyoto, Japan).

Preparation of Ligands. The molecular formulas and abbreviations of ligands prepared are shown in Fig. 1.

***N*-2-Pyridylmethyl-L-aspartic Acid Dimethyl Esters Dihydrochloride (^{pm}D-Me$_2$·2HCl).** 2-Pyridylaldehyde (5.40 g, 50.0 mmol) and L-Asp-$(OMe)_2$·HCl (9.89 g, 50.0 mmol) were dissolved in methanol and stirred at 0 °C. After 1 h, $Na[BH_3(CN)]$ (3.12 g, 50.0 mmol) was added at 0 °C and the mixture was stirred for 12 h at room temperature. White precipitate was filtered off and the solvent was evaporated. The residue was dissolved in chloroform and washed with saturated Na_2CO_3 aq. After evaporation of the solvent, the residue was diluted with methanol. Then, 4 mol dm^{-3} (M) HCl/AcOEt was added at 0 °C, and white precipitate was collected by filtration and washed with ethanol to yield 12.8 g of ^{pm}D-Me$_2$·2HCl. Yield: 80% with respect to L-aspartic acid dimethyl esters·HCl. The analytical and physical data of the ligands synthesized are summarized in Table 1.

***N*-6-Methyl-2-pyridylmethyl-L-aspartic Acid Dimethyl Esters Dihydrochlorides ($^{6Me\text{-}pm}$D-Me$_2$·2HCl).** $^{6Me\text{-}pm}$D-Me$_2$·2HCl was prepared by a method similar to the synthesis of ^{pm}D-Me$_2$·2HCl as described above, in which 6-methyl-2-pyridylaldehyde was used in place of 2-pyridylaldehyde. Yield: 50% with respect to L-aspartic acid dimethyl ester dihydrochloride.

***N*,*N*′-1,2-Ethanediyl-*N*-(L-histidine methyl ester)-*N*′-L-valine Methyl Ester (HeV-Me$_2$).** HeV-Me$_2$ was prepared by a method similar to the one pot synthesis of *N*,*N*′-ethanediyl-*N*-(L-histidine methyl ester)-*N*′-L-aspartic acid dimethyl esters.[13] A

Table 1. Analytical and Physical Data of the Ligands

Ligands (Chemical formula)	Elemental Analysis Found (Calcd) /% C	H	N	FAB MS *m*/*z* $[M + H]^+$	IR spectra for $\nu_{C=O}$ / cm^{-1}(*)	$[\alpha]_D^{20}$ / deg dm^{-1} g^{-1} cm^3 (MeOH)
^{pm2}G-Me	61.15	6.21	14.27	272	1636(s)	—
($C_{15}H_{17}N_3O_2 \cdot 1.3H_2O$)	(61.13	6.70	14.26)			
^{pm2}A-Me	66.94	6.78	14.65	286	1736(s)	−48.6
($C_{16}H_{19}N_3O_2 \cdot 0.1H_2O$)	(66.93	6.74	14.63)			
^{pm2}V-Me	68.58	7.40	13.33	314	1730 (s)	−81.9
($C_{18}H_{23}N_3O_2 \cdot 0.1H_2O$)	(68.59	7.42	13.33)			
$^{pm2}V_R$-Me	68.26	7.44	13.30	314	1730(s)	+79.5
($C_{18}H_{23}N_3O_2 \cdot 0.2H_2O$)	(68.20	7.44	13.26)			
^{pm2}L-Me	66.58	7.60	12.26	328	1728(s)	−50.3
($C_{19}H_{25}N_3O_2 \cdot 0.8H_2O$)	(66.76	7.84	12.29)			
^{pm2}S-Me	60.94	6.40	13.65	302	1730(s)	−76.0
($C_{16}H_{19}N_3O_3 \cdot 0.75H_2O$)	(61.04	6.56	13.35)			
^{pm}D-Me$_2$·2HCl	44.34	5.69	8.46	253	1742(s)	+0.8
($C_{12}H_{16}N_2O_4 \cdot 2HCl$)	(44.32	5.58	8.61)			
$^{6Me\text{-}pm}$D-Me$_2$·2HCl	35.57	7.12	6.32	267	1749(s)	−3.4
($C_{13}H_{18}N_2O_4 \cdot 2HCl \cdot 2.75H_2O$)	(35.62	7.13	6.39)			
^{pm2}D-Me$_2$	62.12	6.17	12.08	344	1733(s)	−61.5
($C_{18}H_{21}N_3O_4 \cdot 0.25H_2O$)	(62.15	6.23	12.08)			
HeV-Me$_2$	51.46	8.30	15.76	327	1733(s)	−17.0
($C_{15}H_{26}N_4O_4 \cdot 1.4H_2O$)	(51.24	8.26	15.93)			
HeT-Me$_2$	47.14	7.87	15.57	329	1734(s)	−24.3
($C_{14}H_{24}N_4O_5 \cdot 1.6H_2O$)	(47.08	7.68	15.69)			

* s: strong.

methanol solution of L-valine methyl ester monohydrochloride (2.51 g, 15.0 mmol) and 40% glyoxal aqueous solution (2.18 g, 15.0 mmol) were stirred for 2 h at 0 °C, then L-histidine methyl ester dihydrochloride (3.63 g, 15.0 mmol) and sodium methoxide (0.87 g, 15.0 mmol) was added. After 1 h, Na[BH_3(CN)] (1.88 g, 30.0 mmol) was added and the reaction mixture was stirred overnight at room temperature. White precipitate was removed by filtration, and the filtrate was evaporated. The product was roughly purified by basic and acidic silica gel column chromatography (eluents: $CHCl_3$/MeOH). Further purification was achieved by gel filtration (eluent: MeOH) (yield: 0.99 g, 20% with respect to L-histidine methyl ester dihydrochloride).

***N*,*N*′-1,2-Ethanediyl-*N*-(L-histidine methyl ester)-*N*′-L-threonine methyl ester (HeT-Me$_2$).** HeT-Me_2 was prepared by a method similar to the one pot synthesis as described above, in which L-Thr-OMe·2HCl was used in place of L-Val-OMe·HCl. Yield: 10% with respect to L-histidine methyl ester dihydrochloride.

***N*,*N*-Bis(2-pyridylmethyl)-glycine, -L-alanine, -L-valine, -D-valine, -L-leucine, and -L-serine methyl ester, and -L-aspartic Acid Dimethyl Ester (^{pm2}G-, ^{pm2}A-, ^{pm2}V-, $^{pm2}V_R$-, ^{pm2}L-, and ^{pm2}S-Me, and ^{pm2}D-Me$_2$).** 2-Pyridylaldehyde (5.40 g, 50.0 mmol) and each amino acid methyl ester (50.0 mmol) were dissolved in methanol and stirred at 0 °C. After 1 h, Na[BH_3(CN)] (3.12 g, 50.0 mmol) was added at 0 °C and the mixture was stirred for 12 h at room temperature. White precipitate was filtered off and the solvent was evaporated. Each residue was dissolved in chloroform and washed with saturated Na_2CO_3 aq. After evaporation of the solvent, the residue was diluted with methanol. Then, 4 M HCl/AcOEt was added at 0 °C, and white precipitate was collected by filtration and washed with ethanol to yield *N*-2-pyridylmethyl-amino acid (di)methyl ester·2HCl (^{pm}X-Me$_{1\ or\ 2}$·2HCl).

2-Pyridylaldehyde (3.24 g, 30.0 mmol) and ^{pm}X-Me$_{1\ or\ 2}$·2HCl (30.0 mmol) were dissolved in methanol and stirred at 0 °C. After 1 h, Na[BH_3(CN)] (1.87 g, 30.0 mmol) was added and the mixture was stirred for 12 h at 0 °C–room temperature. White precipitate was filtered off and the solvent was evaporated. Each residue was dissolved in chloroform and washed with saturated Na_2CO_3 aq. After evaporation of the solvent, the residue was diluted with methanol. Then, each product was purified by acidic silica gel column chromatography (eluents: $CHCl_3$/MeOH) to yield *N*,*N*-bis(2-pyridylmethyl)-amino acid (di)methyl ester (^{pm2}X-Me$_{1or2}$). The yields of ^{pm2}G-, ^{pm2}A-, ^{pm2}V-, $^{pm2}V_R$-, ^{pm2}L-, and ^{pm2}S-Me, and ^{pm2}D-Me_2 were 67, 53, 60, 25, 53, 54, and 67%, respectively, with respect to each ^{pm}X-Me$_{1\ or\ 2}$.

Preparations of Zn(II) Complexes with Tetra- and Pentadentate Amino Acid Derivatives. These complexes: Zn(^{pm2}G)(ClO_4), Zn(^{pm2}A)(ClO_4), Zn(^{pm2}V)(ClO_4), Zn($^{pm2}V_R$)(ClO_4), Zn(^{pm2}L)(ClO_4), Zn(^{pm2}S)(SO_4)$_{1/2}$, Zn(^{pm}H)(ClO_4), Zn(^{pm}D), Zn($^{6Me\text{-}pm}$D), Zn(^{pm2}D), Zn(HeV), and Zn(HeT), were newly prepared. After the saponification of the ester of each ligand with an equivalent mole of $Ba(OH)_2$·$8H_2O$ or LiOH·H_2O, an equvalent mole of $ZnSO_4$ was added to the aqueous solution of barium or lithium salt of the ligand (generated in situ from each ligand and hydroxide anion) at room temperature. The choice of lithium or barium as the cation was made based upon the aqueous solubility of the desired complex. Lithium was preferable to barium, because the complex is prone to coprecipitate with barium sulfate in the reaction. All complexes were purified by recrystallization from hot water, and characterized by the elemental analyses and IR and ^{1}H NMR spectra. The yields of Zn(^{pm2}G)(ClO_4), Zn(^{pm2}A)(ClO_4), Zn(^{pm2}V)(ClO_4), Zn($^{pm2}V_R$)(ClO_4), Zn(^{pm2}L)(ClO_4), Zn(^{pm2}S)(SO_4)$_{1/2}$, Zn(^{pm}H)(ClO_4), Zn(^{pm}D), Zn($^{6Me\text{-}pm}$D), Zn(^{pm2}D), Zn(HeV), and Zn(HeT), were 42, 77, 87, 42, 34, 47, 22 95, 82, 86, 54, and 58%, respectively, with respect to each ligand. The analytical and physical data of these complexes are summarized in Table 2.

X-ray Crystallographic Data Collection and Refinement of a Complex, [Zn_3(^{pm}H)$_3$(H_2O)$_2$](ClO_4)$_3$·$2H_2O$ (1). The single crystal of **1** was obtained by recrystallization from hot water. The crystallographic data of **1** are summarized in Table 3. The data were collected 5120 reflections at 23 °C by the ω–2θ scan technique ($2\theta < 136°$) on a Rigaku AFC7R diffractometer with graphite monochromated Cu-$K\alpha$ radiation and a rotating anode generator (Tokyo, Japan). The refinement on 713 parameters was performed for all the 5019 data points ($I > 3\sigma(I)$) which resulted in $R = 0.058$ and $R_w = 0.080$ and GOF = 1.78, the values of minimum and maximum residual electron density became 0.88 and -1.13 eÅ^3, respectively. For computing a structure solution, PATTY was used, and for computing structure refinement, DIRDIF92 was used. Reflections were refined based on F_o by full matrix least squares. The non-hydrogen atoms were refined anisotropically but one in three ClO_4^- anions and one in two water molecules as crystalline solvents have heavy disorders at this temperature. Hydrogen atoms were placed in idealized positions and included in structural factor calculations.

Crystallographic data (without structure factors) for the Zn(II) complex **1** have been deposited at the Cambridge Crystallographic Data Centre as supplementary publication No. CCDC 167040. Copies of the data can be obtained free of charge from the CCDC (12 Union Road, Cambridge CB2 1EZ, UK; tel: (+44)1223-336-408; fax: (+44)1223-336-003; e-mail: deposit@ccdc.cam.ac.uk; http://www.ccdc.cam.ac.uk). The data are also deposited as Document No.75056 at the Office of the Editor of Bull. Chem. Soc. Jpn.

Molecular Weight Measurements. Molecular weights of Zn(^{pm}D) and Zn(^{pm}H)(ClO_4) were determined by using a vapor pressure osmometer, according to the method described previously.[14,15] Urea was used as a standard compound to calibrate the apparatus. The two Zn(II) complexes and urea were recrystallized from water before use. The measurements were performed in an aqueous solution at 30.0 ± 0.05 °C in vacuo.

Potentiometric Titrations. Potentiometric titrations were carried out by the use of a microburet in an aqueous KNO_3 solution at an ionic strength (μ) of 0.1 M and a controlled temperature of 25.0 ± 0.05 °C under a nitrogen atomosphere. The pH values were measured by TOA Electronics IM 40S pH combination electrodes (Tokyo, Japan). Calibration of the pH meter-electrode system was carried out by using commercially available buffer solution standards (pH = 4.01 and 6.86).

A 0.1 M NaOH solution was prepared by dissolving reagent grade NaOH in distilled water, which was prepared by boiling and cooling under a nitrogen flow, and standardized with potassium hydrogen phthalate. A 0.1 M HNO_3 solution standardized with Na_2CO_3 was titrated with the standardized 0.1 M NaOH solution at $\mu = 0.1$. Because almost all of the ligands were prepared as ester forms, they were quantitatively saponificated over-night in an equivalent alkaline solution under air at room temperature. Then, in order to neutralize and also to fully protonate the ligand, a slight excess amount of standardized HNO_3 solution was added to the solution. Finally, each thus-prepared solution was used for the potentiometric measurements.[13] The acid dissociation constants

Table 2. Analytical and Physical Data of Complexes

Complexes (Chemical formula)	Elemental Analysis Found (Calcd) /% C	H	N	Decomposition Temperature / °C	IR spectra for $\nu_{C=O}$ / cm^{-1}(*)	$[\alpha]_D^{20}$ / deg dm^{-1} g^{-1} cm^3 (H_2O)
Zn(^{pm2}G)(ClO_4) ($Zn(C_{14}H_{14}N_3O_6Cl)\cdot 0.5H_2O$)	39.28 (39.09	3.62 3.51	9.54 9.77)	> 300	1609(s)	—
Zn(^{pm2}A)(ClO_4) ($Zn(C_{15}H_{16}N_3O_6Cl)\cdot 0.5H_2O$)	40.81 (40.56	4.02 3.86	9.17 9.46)	265–275	1609(s)	−116.7
Zn(^{pm2}V)(ClO_4) ($Zn(C_{17}H_{20}N_3O_6Cl)\cdot 3.5H_2O$)	38.93 (38.80	4.35 5.17	7.95 7.98)	208–220	1610(s)	−101.0
Zn($^{pm2}V_R$)(ClO_4) ($Zn(C_{17}H_{20}N_3O_6Cl)\cdot 2H_2O$)	40.94 (40.90	4.21 4.85	8.38 8.42)	210–218	1610(s)	+107.4
Zn(^{pm2}L)(ClO_4) ($Zn(C_{18}H_{22}N_3O_6Cl)\cdot 0.3H_2O$)	44.82 (44.80	4.62 4.72	8.72 8.71)	185–188	1611(s)	−61.5
Zn(^{pm2}S)(SO_4)$_{1/2}$ ($Zn(C_{15}H_{16}N_3O_5S_{1/2})\cdot 3H_2O$)	39.75 (39.70	4.56 4.89	9.24 9.26)	240–262	1609(s)	−90.9
Zn(^{pm}H)(ClO_4) ($Zn(C_{12}H_{13}N_4O_6Cl)\cdot 2H_2O$)	32.39 (32.31	3.77 3.84	12.55 12.56)	> 300	1605(s)	+45.9
Zn(^{pm}D) ($Zn(C_{10}H_{10}N_2O_4)\cdot 3H_2O$)	35.20 (35.16	4.56 4.72	8.22 8.20)	272–292	1607(s)	+157.9
Zn(^{6Mepm}D) ($Zn(C_{11}H_{12}N_2O_4)\cdot 2.5H_2O$)	38.02 (38.11	4.70 4.94	8.07 8.08)	279–283	1590(s)	−44.8
Zn(^{pm2}D) ($Zn(C_{16}H_{15}N_3O_4)\cdot 3H_2O$)	44.15 (44.41	4.82 4.89	9.65 9.71)	191–207	1609(s)	−44.8
Zn(HeV) ($Zn(C_{13}H_{20}N_4O_4)\cdot 2.5H_2O$)	38.25 (38.39	6.02 6.20	13.64 13.77)	265–270	1595(s)	+30.4
Zn(HeT) ($Zn(C_{12}H_{18}N_4O_5)\cdot 3.5H_2O$)	33.64 (33.77	5.61 5.90	12.98 13.13)	270–282	1606(s)	+12.3

* s: strong.

were calculated by the use of the program PKAS.[16] A 0.05 M stock solution of Zn(II) was prepared from reagent grade $Zn(NO_3)_2\cdot 6H_2O$ and standardized by edta titration. Titrations for determining the formation constants of Zn(II) complexes were carried out at slightly excessive amounts of a ligand. More than 80 data points were collected for each titration. A slow equilibrium was observed for a few points at the unbuffered region; the longest time required for reaching equilibrium was 10 min. Except for these points, equilibrium was reached within a few minutes, and each titration was performed during the time in which the drift of the measuring system was tolerable. Stability constants for the Zn(II) complexes were calculated by using the program BEST.[16]

Partition Coefficient Measurements. The partition coefficients of the complexes were evaluated by a conventional method in an *n*-octanol/saline system.[10] The partition coefficients of the complexes were calculated by the equation $P = C_{oct}/C_w$, where C_{oct} and C_w are the equilibrium Zn(II) concentrations in *n*-octanol and saline, respectively, as determined by ICP-MS after shaking for 2 h at 37 °C.

In vitro Insulinomimetic Activity of Zn(II) Complexes. Isolated male Wistar rat adipocytes (1.0×10^6 cells/mL) prepared as described in Ref. 12 were preincubated at 37 °C for 30 min with various concentrations (10^{-4}–10^{-3} M) of each Zn(II) complex in KRB buffer (120 mM NaCl, 1.27 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.75 mM KCl, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$ and 5 mM glucose: pH 7.4) containing 2% BSA. A 10^{-4} M epinephrine was then added to the reaction mixtures and the resulting solutions were incubated at 37 °C for 180 min. The reactions were stopped by soaking in ice water and the mixtures were centrifuged at 3000 rpm for 10 min. FFA levels for the outer solution of the cells were determined with an FFA kit (Wako Pure Chemical Industries). The IC_{50} values were obtained on the basis of the concentration of Zn(II) complex to inhibit 50% of the FFA released from the adipocytes.

The animal study was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University (KPU) and was performed according to the Guideline for Animal Experi-

Table 3. Crystallographic Data of $[Zn_3(^{pm}H)_3(H_2O)_2](ClO_4)_3$ (**1**)

Formula	$C_{36}H_{51}O_{24}N_1Zn_3Cl_3$
Fw	1338.36
Crystal system	Orthorhombic
Space group	$P2_12_12_1$ (NO. 19)
a/Å	16.056(2)
b/Å	23.318(3)
c/Å	13.712(2)
V/Å^3	5133(1)
Z	8
Scan method	ω–2θ
Scan speed/min^{-1}	8
T/°C	23
D_{calcd}/g cm^{-1}	1.731
μ(Cu $K\alpha$)	78.31
Trans. Factor	0.3184–0.9973
$2\theta_{max}$/deg	136.0
No. of unique data (R_{int})	5120 (0.593)
No. of observed data ($I > 3\sigma(I)$)	5019
No. of variables	713
R	0.058
R_w	0.086
Goodness of Fit Indicator	1.78

$R = \Sigma\ ||F_o| - |F_c||/\Sigma|F_o|$
$R_w = [\Sigma\ w\ (|F_o| - |F_c|)^2 / \Sigma\ w\ F_o^2]^{1/2}$

mental of KPU.

Results and Discussion

Syntheses and Structure Determinations of Zn(II) Complexes. Twelve new Zn(II) complexes were prepared to promote the development of insulinomimetic metal complexes; their analytical and physical properties are summarized in Table 2. Among them, the Zn(^{pm}H) complex was found to be a trinuclear complex, $[Zn_3(^{pm}H)_3(H_2O)_2](ClO_4)_3\cdot 2H_2O$ **1**, after the X-ray structure analysis of its crystal. Figure 2 shows the ORTEP view of a trinuclear complex cation of **1**. Tables 3 and 4 show the crystallographic data and the selected bond lengths and angles, respectively. The chiral crystal was constructed from **1**, being two discrete water molecules and three ClO_4^- anions. Coordination geometries for three Zn(II) of **1** exhibit distorted octahedral configurations with N_3O_3 donor sets. The coordination spheres of three Zn(II), except for the coordination of pyridyl and imidazolyl groups of ^{pm}H, are formed in a plane. Each Zn1, Zn2 and Zn3 of the mean deviations from a least-square plane is within ca. 0.06 Å. Three Zn(II) of **1** have no symmetrical structure due to Zn1 and Zn3 being bridged by two carboxyl oxygens O1 and O5, while Zn2 and Zn3 are bridged by one O3 (Zn1···Zn2: 5.82 Å, Zn2···Zn3: 3.95 Å and Zn3···Zn1: 3.50 Å). Water oxygens O7W and O8W coordinate to Zn1 and Zn2, respectively. Interestingly, the coordinate conformations of the three ligands of **1** have all the same orientations, indicating that all three pyridyl groups of **1** are oppositely directed to the three imidazolyl groups.

On the other hand, single crystals of tetra- and penta-dentate amino acids/VO complexes were all revealed to have monomeric structures by X-ray analyses.[10,11]

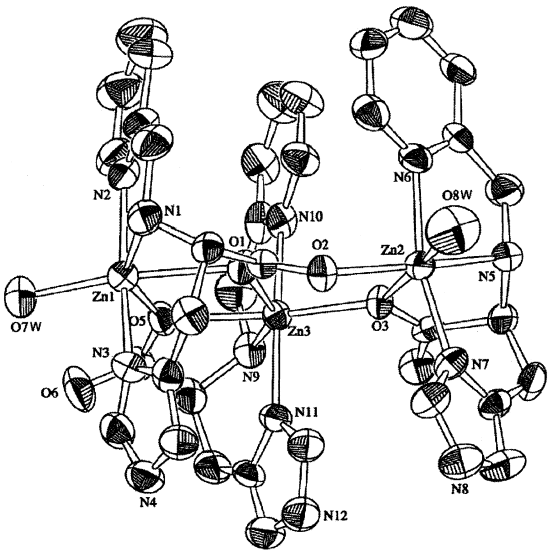

Fig. 2. The side ORTEP view of the complex cation in $[Zn_3(^{pm}H)_3(H_2O)_2](ClO_4)_3$ (**1**). Three ClO_4^- anions as crystalline solvents are omitted for clarity.

Determination of Average Dissociation Number of Zn(II) Complexes in Aqueous Solution. In a series of Zn(II) complexes prepared in the present study, two different types of complexes, i.e., Zn(^{pm}D)^0 and Zn(^{pm}H)$^+$, were chosen as representative Zn(II) complexes from the standpoint of the structural composition. Then the average dissociation number for those complexes in aqueous solution was determined by means of vapor pressure osmometry in vacuo. The reasons why we chose the two complexes are as follows: (1) The structure of Zn(^{pm}D)^0 is unknown in a solid state and aqueous solution, but it is a molecular complex; (2) Zn(^{pm}H)$^+$ has an ionic structure and is present in a trinuclear complex in the solid state. Therefore, it was necessary to verify whether or not the complex exists in the same chemical species in aqueous solution as in solid state.

Firstly, the correlation between the bridge potential difference (ΔE (mV)) and the solute concentration ([urea] (mol kg^{-1})) was examined, in which ΔE is proportional to the temperature difference between a pure solvent and a solution. It was found that a good linear relationship between ΔE and [urea] persists up to 0.080 mol kg^{-1}. A least-squares treatment showed the equation, $\Delta E = 20.80$[urea] $\pm$ 0.15. On the other hand, the ΔE values for each complex at a known concentration were found as follows. ΔE (mV) = 1.16 and 1.18 for Zn(^{pm}D)^0 = 0.050 mol kg^{-1}, and ΔE = 1.74 and 1.76 for Zn(^{pm}H)$^+$ = 0.044 mol kg^{-1}.

Urea is one of the most suitable compounds as a standard solute when vapor pressure osmometry is performed in an aqueous solution, because urea is neither a dissociable nor an associable compound at relatively low concentration ranges in aqueous solution. In other words, urea exists as a monomeric species in aqueous solution. Taking this point into consideration, one can define the average dissociation number, n, as: n = (ΔE value of the complex)/(ΔE value of urea) at a given concentration of the solute. The resulting n values were $n = 1.13$ and 1.15 for Zn(^{pm}D)^0 at 0.050 mol kg^{-1}, and $n = 1.89$ and

Table 4. Selected Bond Lengths (Å), Angles (°), and Distance with Plane (Å) of **1**

	Length (Å)		
Zn1–O1	2.332(4)	Zn1–N1	2.167(5)
Zn1–O7W	2.126(4)	Zn1–N3	2.093(5)
Zn1–N2	2.097(5)	Zn1– O5	2.095(4)
Zn2–O3	2.258(3)	Zn2–O2	2.031(4)
Zn2–N5	2.134(5)	Zn2–O8W	2.214(5)
Zn2–N7	2.055(4)	Zn2–N6	2.109(5)
Zn3–O3	2.117(3)	Zn3–O1	2.140(4)
Zn3–N9	2.206(4)	Zn3–O2	2.222(3)
Zn3–N11	2.085(4)	Zn3–O5	2.332(4)
	Angles (°)		
Zn1–O1– Zn3	102.9(1)	O1–Zn1–O7W	162.0(2)
Zn1–O5–Zn3	108.2(2)	O1–Zn1–N2	87.1(2)
Zn2–O3–Zn3	128.8(2)	O5–Zn1–O7W	88.3(2)
O1–Zn1–O5	73.7(1)	O5–Zn1–N2	93.6(2)
O1–Zn1–N1	73.9(2)	O7W–Zn1–N1	123.9(2)
O1–Zn1–N3	88.1(2)	O7W–Zn1–N3	94.7(2)
O5–Zn1–N1	147.1(2)	N1–Zn1–N3	88.7(2)
O5–Zn1–N3	95.7(2)	O2–Zn2–O3	90.7(1)
O7W–Zn1–N2	93.3(2)	O2–Zn2–N5	164.5(2)
N1–Zn1–N2	79.3(2)	O2–Zn2–N7	97.0(2)
N2–Zn1–N3	167.9(2)	O3–Zn2–N5	74.8(2)
O2–Zn2–O8W	86.3(3)	O3–Zn2–N7	97.0(2)
O2–Zn2–N6	96.8(2)	O8W–Zn2–N6	85.8(2)
O3–Zn2–O8W	177.0(2)	N5–Zn2–N6	79.3(2)
O3–Zn2–N6	94.8(2)	N6–Zn2–N7	165.1(2)
O8W–Zn2–N5	108.2(3)	O1–Zn3–O5	75.2(1)
O8W–Zn2–N7	89.4(2)	O1–Zn3–N10	93.6(2)
N5–Zn2–N2	88.8(2)	O3–Zn3–O5	163.7(1)
O1–Zn3–O3	88.7(1)	O3–Zn3–N10	91.0(2)
O1–Zn3–N9	147.3(2)	O5–Zn3–N9	73.3(1)
O1–Zn3–N11	100.2(2)	O5–Zn3–N11	88.7(2)
O3–Zn3–N9	123.0(1)	N9–Zn3–N11	87.6(2)
O3–Zn3–N11	91.9(2)	N9–Zn3–N10	79.3(2)
O5–Zn3–N10	92.3(2)	N10–Zn3–N11	166.0(2)
	Distance with plane (Å)		
Zn1	0.1847		
Zn2	1.2793		
Zn3	0.1847		

1.91 for $Zn(^{pm}H)^{+}$ at 0.044 mol kg^{-1}.

Since the average dissociation number for $Zn(^{pm}D)^{0}$ is nearly $n = 1$, one can conclude that this complex forms a monomeric molecular species in aqueous solution. On the other hand, it should be noted that the n value for the trinuclear complex, $[Zn_3(^{pm}H)_3(H_2O)_2](ClO_4)_3$, in the solid state was found to be almost $n = 2$ in aqueous solution. It is reasonably deduced that this complex is no longer a trinuclear species, but exists as a monomer and dissociates into an ion pair of $Zn(^{pm}H)^{+}$ and ClO_4^{-}.

Thus, the chemical species of two structurally different typical tetradentate Zn(II) complexes in aqueous solution were successfully revealed. In the present study, it can be generally predicted that the molecular Zn(II) complex as well as the ionic Zn(II) complex in the solid state exists in monomeric form in an aqueous solution.

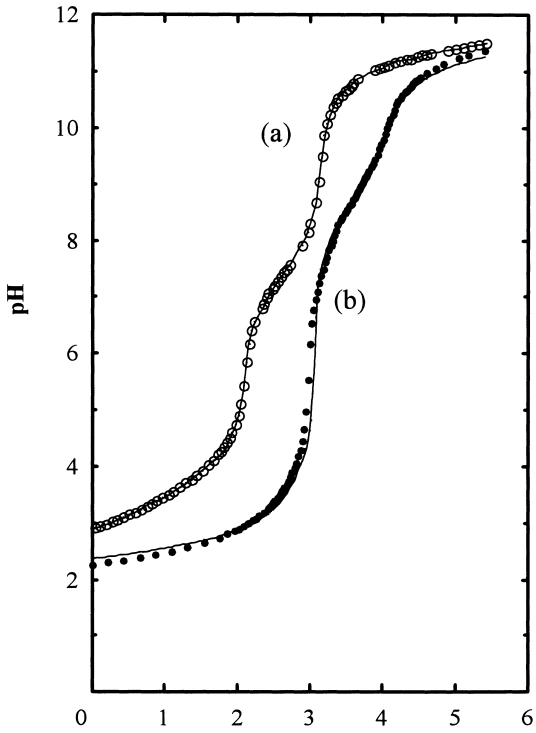

Fig. 3. Potentiometric titration curves for (a) ^{pm2}A ligand only ($[^{pm2}A] = 2.39 \times 10^{-3}$ mol dm^{-3}) and (b) ^{pm2}A in the presence of $ZnSO_4$ ($[ZnSO_4] = 1.99 \times 10^{-3}$ mol dm^{-3}) ; $[OH^-]$, [L], and [M] represent the concentrations of NaOH, ^{pm2}A, and Zn^{2+}, respectively. Open and closed circles indicate the experimental data and the solid curves represent the simulated ones.

Determination of Stability Constants of Zn(II) Complexes with Amino Acid Derivatives. In order to determine the stability constants of nine Zn(II) complexes with tetradentate amino acid derivatives prepared in this study, the dissociation constants of the free ligands were firstly determined by a potentiometric titration method. However, because the structures of these ligands are closely similar to each other, ^{pm2}A was chosen as a typical ligand and its dissociation constants as well as the stability constant of its Zn(II) complex are briefly discussed below.

As a typical example, the observed potentiometric titration curve of ^{pm2}A is shown as open circles in Fig. 3(a). On the basis of the experimental data, the dissociation constants for ^{pm2}A were calculated with a computer program PKAS.[16] Thus obtained pK_a values of ^{pm2}A are listed in Table 5 together with those of other ligands. Using the pK_a values of ^{pm2}A, the calculated pH values were obtained, and these are illustrated as a solid line in Fig. 3(a). As shown in the figure, the experimental values and the calculated curve were in good agreement with each other.

Potentiometric data (Fig. 3(a)) exhibited two separate dissociation steps. The first step was the low pH buffer region terminated by a sharp inflection point at a = 2 (moles of base/moles of ligand). The other step was followed by a second buffer region with a weak inflection point at a = 3. The order

Table 5. Acid Dissociation Constants of Tetradentate Amino Acid Derivatives

Ligand	pK_{a1}(± SD[b)])	pK_{a2}(± SD[b)])	pK_{a3}(± SD[b)])
^{pm2}G	3.08(± 0.06)	4.30(± 0.08)	7.21(± 0.11)
^{pm2}A	3.16(± 0.04)	4.14(± 0.14)	7.97(± 0.25)
^{pm2}V, $^{pm2}V_R$	3.94(± 0.18)	4.56(± 0.26)	7.38(± 0.32)
^{pm2}L	3.24(± 0.04)	5.45(± 0.04)	11.30(± 0.17)
^{pm2}S	2.91(± 0.06)	4.23(± 0.29)	7.15(± 0.22)
^{pm}H	2.41(± 0.04)	5.80(± 0.09)	7.41(± 0.14)
^{pm}D	2.73(± 0.01)	3.01(± 0.01)	8.18(± 0.05)
(Ref.)[a)]	2.23	3.63	8.72
$^{6Me\text{-}pm}$D	2.72(± 0.05)	3.80(± 0.06)	8.81(± 0.14)
(Ref.)[a)]	2.72	3.72	8.68

a) Refer to Ref. 17. b) SD = standard deviation in logarithm unit.

of deprotonation for ^{pm2}A was determined by comparing the pK_a values with those of ^{pm}D and $^{6Me\text{-}pm}$D, because the deprotonation order from these ligands was unambiguously assigned in the literature.[17] Although there were small differences in the pK_a values between the literature and the present study, the tendencies between them were almost the same. Based on the study,[17] the similarity of the pK_{a2} values for ^{pm}D and $^{6Me\text{-}pm}$D as well as the difference of 0.49 log unit in their pK_{a1} values indicated that the pK_{a2} values can be assigned to the deprotonation from the aspartic acid side chain carboxylate and the pK_{a1} values to the deprotonation from the pyridine nitrogen. Consequently, deprotonation from the aminonium nitrogen was associated with the pK_{a3} Thus, it was reasonably concluded that pK_{a1} and pK_{a2} and the last pK_{a3} value for ^{pm2}A are assigned to be the two pyridine nitrogens and the amino nitrogen, respectively. Therefore, the following deprotonation equilibrium can be proposed for ^{pm2}A: $K_{a0} = [H_3L^{2+}][H^+]/[H_4L^{3+}]$, $K_{a1} = [H_2L^+][H^+]/[H_3L^{2+}]$, $K_{a2} = [HL][H^+]/[H_2L^+]$, and p$K_{a3} = [L^-][H^+]/[HL]$, where H_4L^{3+} and L^- represent the fully protonated and entirely deprotonated ^{pm2}A species, respectively. By means of computer calculation, pK_{a0} = 2.12, was obtained. This may correspond to the dissociation of carbonic moiety of ^{pm2}A. This value is, however, much lower than the starting pH point, i.e., the lowest pH value of titration measurements. Therefore, carboxylic acid must already be dissociated under the experimental conditions applied in this study, and pK_{a0} of the carboxylic acid should not be considered to be an observed value. With the same argument, the pK_{a0} values were not obtained for all the rest of ligands used in this study.

Evaluation of the chelating ability of ^{pm2}A with Zn(II) was then examined. The potentiometic titration curve is depicted as closed circles in Fig. 3(b). The curve consisted of a low pH buffer zone terminated by a sharp inflection at a = 3, indicating that the complex is formed with 1:1 molar ratio of Zn(II) to the ligand ^{pm2}A. At high pH, a weak buffer zone was observed, suggesting a hydrolysis of the Zn(II) complex. On the basis of the experimental data together with pK_a values of ^{pm2}A, the stability constant (log K_1 = log β) of the 1:1 complex of Zn(II) with ^{pm2}A was calculated with a computer program BEST[16] and found to be 11.12 ± 0.10. The calculated values were used to simulate the experimental titration curve, as shown in a solid line (Fig. 3(b)). A good agreement between the experimental and calculated curves was observed. By using the log β and pK_a values, the species distribution for the $[Zn^{2+}]/[L] = 1$ system was calculated; the results is shown in Fig. 4. In this system, the ML^+ (i.e., $Zn(^{pm2}A)^+$) was found to be the predominant species, forming 100% in the region of pH = 3–7.5. However, this species gradually underwent hydrolysis beyond pH = 7.5 to yield [MLOH] and came to be the main one in the alkaline region. Assuming $K_{OH} = [MLOH][H^+]/[ML^+]$, we estimated the value of log K_{OH} to be -8.89 ± 0.09. Although, in the process of the calculation, it was postulated that an additional hydrolyzed species like a $[ML(OH)_2^-]$ exists in the system, the amount of such species was too low to consider.

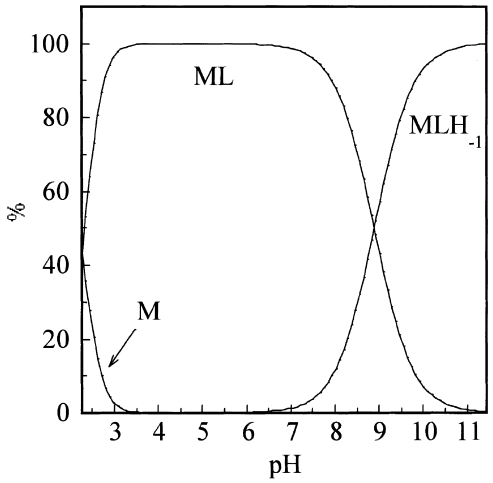

Fig. 4. Species distribution curve in the system [Zn(II)]/[L] = 1 as a function of pH; the initial concentration of $[^{pm2}A] = 2.39 \times 10^{-3}$ mol dm^{-3}, the initial concentration of $[Zn^{2+}] = 1.99 \times 10^{-3}$ mol dm^{-3}, μ = 0.10 mol dm^{-3}, temp = 25.0 °C. The abbreviations are as follows: M = Zn^{2+}, L = ^{pm2}A, ML = Zn(^{pm2}A), and MLH_{-1} = Zn(^{pm2}A)(OH)

The above potentiometric measurements showed that each of the amino acid derivatives used in the present study forms an essentially 1:1 complex with Zn(II) in aqueous solution. The stability constants (log β) of the Zn(II) complexes are summarized in Table 6.

Correlation between the IC_{50} and Stability Constant log β or Partition Coefficients of Zinc(II) Complexes. In vitro insulinomimetic activities of Zn(II) complexes were estimated by inhibition of the FFA release from isolated rat adipocytes treated with epinephrine[12] and were confirmed to be dose-dependent in the concentration range of 10^{-4}–10^{-3} M of the complexes. The apparent IC_{50} value, 50% inhibitory concentration of the complex on the FFA release, was estimated as shown in Table 6.

The inhibitory effects of the complexes with tetradentate ligands were compared with those of $VOSO_4$ and $ZnSO_4$ as positive controls. It was revealed that Zn(II) complexes with lower stability constants (log β) than 11 exhibited higher insulinomimetic activities than that of $VOSO_4$ or were comparable to that of $ZnSO_4$ except for Zn(GtG) (IC_{50} = 3.18) and Zn(^{pm2}G) (IC_{50} = none) as shown in Fig. 5(a). On the other hand, Zn(II) complexes with higher partition coefficients of 0.17 gave higher insulinomimetic activities than that of $VOSO_4$

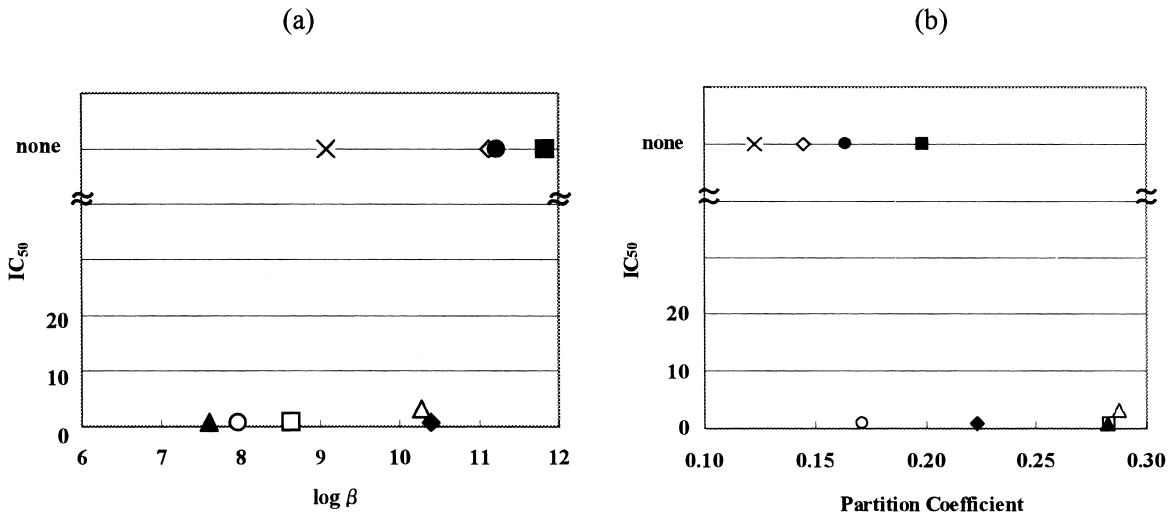

Fig. 5. Correlation between IC_{50} and stability constant (log β) (a) or partition coefficient (b) of Zn(II) complexes. Zn($^{6Me\text{-}pm}$D) ○, Zn(^{pm2}G)(ClO_4) ×, Zn(^{pm2}A)(ClO_4) ◇, Zn(^{pm2}V)(ClO_4) ◆, Zn(GeG) ●, Zn(mGeGm) ■, Zn(βAeAβ) ▲, Zn(GtG) △, Zn(VtV) □. "None" of the *y*-axis means essentially no insulinomimetic activity.

Table 6. Estimated IC_{50} (mM) Value for the Free Faty Acids (FFA) Release from Isolated Rat Adipocytes in the Presence of Glucose, the Stability Constant (log β)[a] and Partition Coefficient of Zn(II) Complexes

Complex	IC_{50} / mM(± SD[b])	log β [c] (± SD[d])	Partition Coefficient(± SD[e])
$ZnSO_4$	0.81 (0.10)		
$VOSO_4$	1.00 (0.08)		
Zn(II) complexes with tetra-dentate ligands			
Zn(^{pm2}G)$^+$	none	9.08(0.19)	0.12(0.01)
Zn(^{pm2}A)$^+$	none	11.12(0.11)	0.14(0.01)
Zn(^{pm2}V)$^+$	0.79(0.08)	10.39(0.23)	0.22(0.01)
Zn($^{pm2}V_R$)$^+$	0.92(0.04)	10.39(0.23)	0.22(0.01)
Zn(^{pm2}L)$^+$	none	14.44(0.42)	
Zn(^{pm2}S)$^+$	none	15.60(0.20)	
Zn(^{pm}H)$^+$	none	15.31(0.14)	
Zn(^{pm}D)	none	16.89 (0.16)	
Zn($^{6Me\text{-}pm}$D)	0.86(0.06)	7.96(0.05)	0.17(0.01)
Zn(GeG[f])	none[g]	11.22[h]	0.16(0.01)
Zn (mGeGm[i])	none[g]	11.83(0.05)	0.20(0.01)
Zn(βAeAβ[j])	0.82(0.04)[g]	7.6[h]	0.28(0.01)
Zn(GtG[k])	3.18(0.04)[g]	10.27(0.07)	0.29(0.01)
Zn(VtV[l])	0.92(0.04)[g]	8.63(0.11)	0.28(0.02)

a) Refer to ref. 16. b) Each value is expressed as the mean ± SD for 3 experiments. c) The stability constants of the complexes without the symbols of superscripts are unpublished data, refer to Ref. 16. d) The standard deviation is expressed in logarithm unit. e) Each value is expressed as the mean ± SD for 3 experiments. f) GeG; *N*,*N*'-Ethylene-bis-glycine = EDDA. g) Refer to Ref. 7. h) Refer to Ref. 18. i) mGeGm; *N*,*N*'-Ethylene-bis-sarcosine. j) βAeAβ; *N*,*N*'-Ethylene-bis-β-alanine. k) GtG; *N*,*N*'-Trimethylene-bis-glycine. l) VtV; *N*,*N*'-Trimethylene-bis-(*S*)-valine.

or were comparable to that of $ZnSO_4$ except for Zn(GtG) (IC_{50} = 3.18) and Zn(mGeGm) (IC_{50} = none) as shown in Fig. 5(b). Zn(^{pm2}G) complex showed essentially no insulinomimetic activity, one possible reason is because this complex might not be able to transmit the membrane of isolated rat adipocytes due to its low partition coefficient, 0.12. Also, Zn(mGeGm) showed essentially no insulinomimetic activity because of its high stability constant (log β = 11.83).

The IC_{50} values of Zn(II) complexes with pentadentate ligands, HeT, HeV, and ^{pm2}D, were not obtained. Each log β of two bis-amino acid type's complexes, Zn(HeT) and Zn(HeV), was predicted to be higher than that (log β =11.22) of a key complex, Zn(GeG) with a tetradentate bis-amino acid type's ligand. The log β of the complex, Zn(^{pm2}D), was also expected to be higher than that (log β = 16.89) of Zn(^{pm}D) with a similar tetradentate ligand. The IC_{50} values of the above three complexes with pentadentate ligands were expected to be none and therefore agreed with their experimental results. In vivo blood glucose normalizing effects of the complexes are now being examined, and the results will be reported later.

In conclusion, we synthesized 12 Zn(II) complexes with tetra- and penta-dentate amino acid derivatives and found that several Zn(II) complexes have in vitro insulinomimetic activity as estimated by the inhibition of free fatty acids release in isolated rat adipocytes treated with epinephrine. It was revealed that the six Zn(II) complexes with tetradentate amino acid derivatives with log β less than 11 and higher partition coefficients than 0.17 exihibit the insulinomimetic activities. Also, the X-ray structure analysis of a complex, $[Zn_3(^{pm}H)_3(H_2O)_2](ClO_4)_3 \cdot 2H_2O$, in a solid state revealed the formation of a trinuclear complex, but the complex in an aqueous solution exists as a monomer and dissociates into an ion pair of Zn(^{pm}H)$^+$ and ClO_4^-. All Zn(II) complexes prepared are proposed to be present in monomer form in aqueous solutions.

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