

New Bioactive Zinc(II) Complexes with Peptides and Their Derivatives: Synthesis, Structure, and In Vitro Insulinomimetic Activity

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Four Zn(II) complexes with dipeptides and three new Zn(II) complexes with pseudo-tripeptides were prepared and a complex, $[Zn(glythr)_2]_n$, was revealed to have a polymeric structure by X-ray structure analysis. The ligand, glythr$^-$, acts as a bridging ligand completely, a monodentate oxygen atom of the terminal carboxyl group of threonine residue and a didentate ligand containing the nitrogen and oxygen atoms of glycine one. Because the Zn^{2+} ion has a distorted six-coordinated octahedral geometry, it binds with two terminal carboxyl groups and two didentate ligands. Also, all Zn(II) complexes were found to have high in vitro insulinomimetic activity compared with that of zinc sulfate, as estimated by the inhibition of free fatty acid release in isolated rat adipocytes that had been treated with epinephrine (adrenalin).

In recent years, people all over the world have suffered from various diseases, such as cancer, ulcer, diabetes mellitus (DM), rheumatism, and osteoporosis etc.[1] Specifically, DM, which is well-known as a lifestyle-related disease, has been regarded as a serious problem, because it is difficult to fully recover from it. Once a patient is diagnosed with DM, he or she must undergo medical treatment throughout the rest of his or her life. Also, the number of patients suffering from DM is increasing year after year. New types of medicines to treat type 2 DM have been developed in many pharmaceutical companies, and some of them have been used in clinical fields. Medicines with metal ions have also been used to treat various diseases. For example, cisplatin (anti-cancer medicine: Pt complex), auranofin (anti-rheumatism medicine: Au complex), and polaprezinc (anti-gastric ulcer medicine: Zn complex) have been developed and clinically used.[2–4]

Zn^{2+} ion is known to be one of the essential trace elements in biological systems and to be less toxic than the other trace elements.[5,6] On the other hand, there were reports that the serum Zn^{2+} concentration of patients with the type 2 DM were relatively low compared to those of healthy people.[7,8] Therefore, it is very important to study the mutual relation between Zn^{2+} and DM. For this purpose, many researchers have found insulinomimetic activity of the Zn^{2+} ion.[9–11] However, the insulinomimetic activity of Zn(II) complexes were hardly reported. We thus prepared several Zn(II) complexes with natural products, such as amino acids, picolinic acid, and nicotinic acid, and found that many of them have high in vitro insulinomimetic activity and in vivo anti-diabetic activity.[12–15] Nevertheless, we have never studied Zn(II) complexes with peptides that have shown many important bioactive effects.

In this paper, we report on the synthesis, molecular structure analysis, and in vitro insulinomimetic activity of Zn(II) complexes with dipeptides and pseude-tripeptides. That is to say, we used to modified tripeptide compounds as the ligand by the alkylation of amide protons and molecular complexes with dipeptides to enhance the lipophilicity. In the human body, many peptides are essential constitutions for hormone and protein biosyntheses.[16,17] It has been said that peptides with physiological activities have corresponded to cure osteoporosis, hypertension, and others.[18] On the other hand, peptide transporters exist at the small intestine and various peptides are absorbed there.[19] From their considerations, Zn(II) complexes with peptides are expected to be highly absorbed, and thus exhibit high insulinomimetic activity.

Experimental

Materials. Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), zinc oxide ($Zn(OH)_2$), and NEFA-C test Wako were obtained from Wako Pure Chemicals (Osaka, Japan), and Hglygly and Hglypro, Hglyval and Hglythr were from Peptide Institute Inc. (Osaka, Japan) and Kokusan Laboratory Chemicals (Tokyo, Japan), respectively. (±)-Epinephrine hydrochloride, collagenase, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents were of analytical reagent quality and were used without further purification. The purity of $ZnSO_4 \cdot 7H_2O$ was determined by chelatometry using Cu–Pan (Cu–1-(2-pyridyl-azo-2-naphthol) (Dojindo, Kumamoto, Japan) as an indicator.

Instrumentations. Elemental analysis was carried out on a Perkin-Elmer 240C Elemental Analyzer (MA, USA) or a FISONS instruments EA 1108 Elemental Analyzer (Manchester UK). FT-IR spectra were recorded on a Jasco FT/IR-420 spectrophotometer

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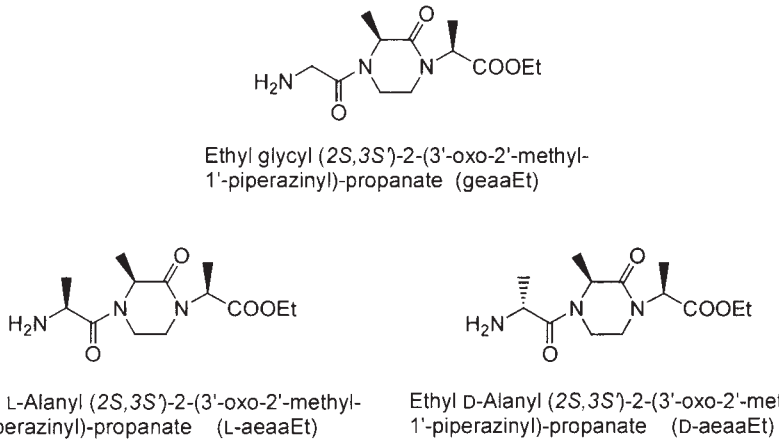

Fig. 1. Pseudo-tripeptide ligands of Zn(II) complexes.

(Tokyo, Japan). The melting point was taken with a Yanako MP-J3 Micro Point Aparatus (Kyoto, Japan). Specific rotatory power, $[\alpha]_D$, was determined by a JASCO P-1030 Polarimeter (Tokyo, Japan).

Preparations of geaaEt, L-aeaaEt, and D-aeaaEt. These three compounds were synthesized by previously described methods.[20] The molecular formulas and abbreviations of the prepared ligands are shown in Fig. 1.

Preparation of [Zn(glygly)$_2$] 1. To an aqueous solution of Hglygly (0.53 g, 4.0 mmol), an aqueous solution of $ZnSO_4 \cdot 7H_2O$ (0.58 g, 2.0 mmol) was added. This was followed by adding an aqueous solution of $Ba(OH)_2 \cdot 8H_2O$ (0.63 g, 2.0 mmol), and filtering the precipitated $BaSO_4$ at room temperature. After removing the solvent, **1** was recrystallized from hot water.

Preparation of [Zn(glythr)$_2$] 2, [Zn(glyval)$_2$] 3, and [Zn(glypro)$_2$] 4. To an aqueous solution of Hglythr (0.42 g, 2.0 mmol), $Zn(OH)_2$ (0.10 g, 1.0 mmol) was added and stirred at 60 °C for 6 h and continuously stirred overnight at room temperature. After filtration of the precipitation and removal of the solvent, **2** was obtained as a residue and then washed with hot water. **3** and **4** were prepared by referring to the method of **2**. However, as for complex **4**, after filtration of the precipitate and removal of the solvent, we dissolved the residue in a methanol/ether (1:1) mixture solvent to remove the water. Complex **4** was obtained as a powder by removing the mixture solvent.

Preparation of [Zn(geaaEt)$_2$]Cl$_2$ 5, [Zn(L-aeaaEt)$_2$]Cl$_2$ 6, and [Zn(D-aeaaEt)$_2$]Cl$_2$ 7. To water:methanol = 1:1 mixed solution of geaaEt·HCl (0.41 g, 1.3 mmol), an aqueous solution of $ZnSO_4 \cdot 7H_2O$ (0.19 g, 0.65 mmol) was added, which was followed by adding an aqueous solution of $Ba(OH)_2 \cdot 8H_2O$ (0.63 g, 0.65 mmol). After stirring at room temperature, **5** was obtained as a residue by removing the solvent. **6** and **7** were prepared by referring to the method of **5**. The ether including complex **6** was brought from the ligand. Thus, complex **6** also contained a little ether.

Preparation of [Zn(pro-a)$_3$](ClO$_4$)$_2$ 8. To an aqueous solution of pro-a (0.46 g, 4.0 mmol), an aqueous solution of $ZnSO_4 \cdot 7H_2O$ (0.58 g, 2.0 mmol) and $Ba(ClO_4)_2 \cdot 3H_2O$ (0.78 g, 2.0 mmol) were added and stirred overnight at room temperature. After filtration of $BaSO_4$ and removal of the solvent, **8** was recrystallized from a small amount of hot water.

Preparation of [Zn(gly-a)$_2$](SO$_4$) 9. To an aqueous solution of gly-a·HCl (0.44 g, 4.0 mmol), an aqueous solution of $ZnSO_4 \cdot 7H_2O$ (0.58 g, 2.0 mmol) was added, followed by adding an aqueous solution of $LiOH \cdot H_2O$ (0.17 g, 4.0 mmol), and stirred for 6 h at room temperature. After the solution became uniform, the solvent was evaporated under reduced pressure. The obtained oil residue was dissolved with ethanol. The obtained powder was filtered off, and **9** was obtained as a residue by washing with ethanol.

Preparation of [Zn(ala-a)$_3$]Cl$_2$ 10. To an aqueous solution of ala-a·HCl (0.25 g, 2.0 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (0.32 g, 1.0 mmol), an aqueous solution of $ZnSO_4 \cdot 7H_2O$ (0.29 g, 1.0 mmol) was added and stirred for 6 h at room temperature. After filtration of the precipitate, **10** was obtained as the residue by removing the solvent.

Preparation of [Zn(met-a)$_2$]Cl$_2$ 11. To a methanol solution of met-a·HCl (0.59 g, 4.0 mmol), an aqueous solution of $LiOH \cdot H_2O$ (0.17 g, 4.0 mmol) and $ZnCl_2$ (0.27 g, 2.0 mmol) was added and stirred overnight at room temperature. A complex **11** was obtained as the residue by removing the solvent.

The analytical and physical data of these complexes synthesized are summarized in Table 1.

X-ray Chrystallographic Data Collection and Refinement of a Complex, {[Zn(glythr)$_2$]·(H$_2$O)$_2$}$_n$. A single crystal of $\{[Zn(glythr)_2] \cdot (H_2O)\}_n$ was obtained by recrystallization from hot water. The crystal data and experimental condition are summarized in Table 2. Lorentz polarization and absorption corrections were applied to a crystal of $\{[Zn(glythr)_2] \cdot (H_2O)\}_n$. The data were collected 1212 reflections at 23 °C by the ω–2θ scan technique ($2\theta < 55°$) on a Rigaku AFC7R diffractometer with graphite monochromated Mo Kα radiation and a rotating anode generator (Tokyo, Japan). A refinement on 168 parameters was performed for all of the 1120 data points ($I > 3.00\sigma(I)$) which resulted in $R = 0.031$ and $R_w = 0.089$. The values of the maximum peak in the final differential map was 0.34 e Å^{-3}. The structure of the crystal was solved by a direct method with the program SIR92 and refined by a full-matrix least-squares method with the program DIRDIF94. The positions of all the hydrogen atoms were calculated. The anisotropic temperature factors were applied to non-hydrogen atoms in the final refinement.

Inhibitory Effects of Zn(II) Complexes on Free Fatty Acid (FFA) Release from Isolated Rat Adipocytes Treated with Epinephrine. Isolated male rat adipocytes (1.0×10^6 cells/mL), prepared as described,[21] were preincubated at 37 °C for 30 min with various concentrations (10^{-4}–10^{-3} mol dm^{-3}) of Zn(II) complexes in Krebs–Ringer bicarbonate buffer (120 mmol dm^{-3} NaCl, 1.27 mmol dm^{-3} $CaCl_2$, 1.2 mmol dm^{-3} $MgSO_4$, 4.75 mmol dm^{-3} KCl, 1.2 mmol dm^{-3} KH_2PO_4, 24 mmol dm^{-3} $NaHCO_3$: pH 7.4) containing 2% BSA. A 10^{-4} mol dm^{-3} epi-

Table 1. Analytical and Physical Data of Complexes

Complexes (Chemical formula)	Elemental analysis Calcd (Found)/% C	H	N	Decomposition temperature /°C	IR spectra for $\nu_{C=O}$ /cm^{-1}[a)]	$[\alpha]_D^{20}$/deg dm^{-1} g^{-1} cm^3 (H_2O)	Yield /%
1·3H_2O	28.11	5.31	16.39	220–230	1644 (s)	—	23
	(28.36	5.33	16.13)				
2·H_2O	33.23	5.58	12.92	185–190	1663 (s)	−29.4	19
	(33.44	5.65	12.79)				
3·0.2H_2O	40.48	6.41	13.49	>300	1660 (s)	−111.8	29
	(40.54	6.39	13.34)				
4·H_2O·0.1Et_2O	40.48	5.82	12.93	188–197	1604 (s)	−102.7	96
	(40.38	6.06	12.95)				
5·4H_2O	38.38	6.71	11.19	102–110	1609 (s)	+59.8	82
	(37.92	6.64	11.56)				
6·0.6Et_2O	45.39	6.97	11.18	92–95	1638 (s)	+48.4	99
	(45.61	7.27	11.54)				
7	44.11	6.56	11.89	94–97	1638 (s)	+66.4	94
	(44.11	7.21	12.35)				
8·2H_2O	28.03	5.33	13.07	168–170	1692 (s)	−55.2	8
	(27.86	5.22	13.05)				
9·0.3H_2O	15.25	4.03	17.79	210–220	1644 (s)	—	81
	(15.30	3.97	17.74)				
10·3.5H_2O	23.31	6.74	18.12	163–169	1671 (s)	+5.0	80
	(23.42	6.27	18.10)				
11·1.2H_2O	26.43	5.86	12.33	75–80	1661 (s)	−1.62	95
	(26.57	5.93	12.30)				

a) s; strong.

Table 2. Crystal Data and Experimental Conditions of $\{[Zn(glythr)_2]\cdot(H_2O)_2\}_n$

Empirical formula	$C_{12}H_{26}O_{10}N_4Zn$	Scan type	ω–2θ
Formula weight	451.74	Scan rate/° min^{-1}	16.0
Crystal dimensions/mm^3	0.40 × 0.40 × 0.1	Scan width/°	1.73 + 0.3 tanθ
Crystal system	Monoclinic	$2\theta_{max}$/°	55.0
Lattice parameters		p-factor	0.05
a/Å	11.590(2)	No. of observations	1120
b/Å	12.628(2)	(All, $2\theta > 54.98°$)	
c/Å	8.047(5)	No. variables	167
β/°	126.69(2)	R/P ratio	6.71
V/Å^3	944.5(8)	R; R_w	0.030; 0.084
Space group	$C2$ (# 5)	Shift/Error	0.02
Z value	2	GOF	1.10
D_{calc}/g cm^{-3}	1.588	Max. peak in Diff. Map/e$^-$ Å^{-3}	0.34
F_{000}	472.00	Min. peak in Diff. Map/e$^-$ Å^{-3}	−0.86
μ(Mo Kα)/cm^{-1}	13.59		
Diffractometer	Rigaku AFC7R		
Temperature/°C	23.0		
λ/Å	0.71069		

nephrine was then added to the reaction mixtures and the resulting solutions were incubated at 37 °C for 3 h. The reactions were stopped by soaking it in ice water and the mixtures were centrifuged at 3000 rpm for 10 min. For the outer solutions of the cells, free fatty acid (FFA) levels were determined with a NEFA-C test Wako.

Statistical Treatment Data. Data are shown as the means ± standard errors for three repeated runs. Comparisons were made using the Student's t-test.

Results and Discussions

X-ray Structure of Zn(II) Complex, {[Zn(glythr)$_2$]·(H$_2$O)$_2$}$_n$. Figure 2 shows an ORTEP view of $\{[Zn(glythr)_2]\cdot(H_2O)_2\}_n$ crystal structure along with the atom numbering. The selected bond distances and angles are listed in Table 3. The bridging ligand glythr$^-$ acts as a tridentate ligand completely, a monodentate oxygen atom of the terminal carboxyl group

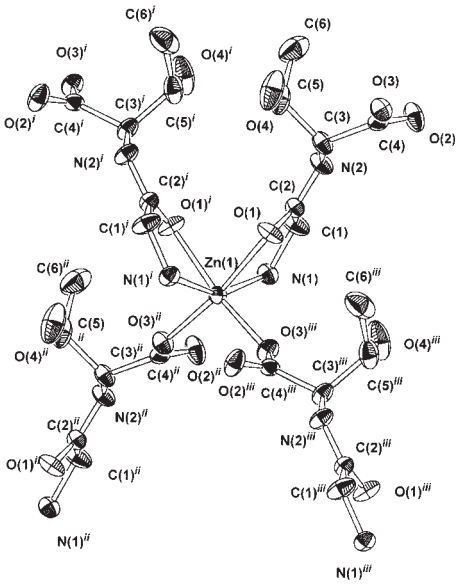

Fig. 2. ORTEP view on the molecular structure of $\{[Zn(glythr)_2]\cdot(H_2O)_2\}_n$. The italic letters indicate the symmetry related atoms. The symmetric operators are as follows: *i*: $-x-1,\ y,\ -z-2$, *ii*: $-x-3/2,\ y+1/2,\ -z-3$, *iii*: $x+1/2,\ y+1/2,\ z+1$.

Table 3. Selected Bond Length (Å) and Angles (°) for $\{[Zn(glythr)_2]\cdot(H_2O)_2\}_n$

Zn(1)–O(1)	2.213(4)	O(1)–Zn(1)–O(1)^{i}	85.5(2)
Zn(1)–N(1)	2.101(6)	O(1)–Zn(1)–O(3)^{iii}	88.4(2)
Zn(1)–O(3)^{ii}	2.071(4)	O(1)–Zn(1)–N(1)^{i}	83.6(2)
O(1)–C(2)	1.233(8)	O(1)^{i}–Zn(1)–O(3)^{iii}	173.6(2)
O(3)–C(4)	1.290(6)	O(1)^{i}–Zn(1)–N(1)^{i}	78.2(2)
O(2)–C(4)	1.223(8)	O(3)^{ii}–Zn(1)–N(1)	93.7(2)
N(1)–C(1)	1.460(7)	O(3)^{iii}–Zn(1)–N(1)	102.7(1)
C(1)–C(2)	1.523(7)	N(1)–Zn(1)–N(1)^{i}	155.1(2)
		O(3)^{ii}–Zn(1)–N(3)^{iii}	97.9(2)

i: $-x-1,\ y,\ -z-2$, *ii*: $-x-3/2,\ y+1/2,\ -z-3$, *iii*: $x+1/2,\ y+1/2,\ z+1$.

of threonine residue and a didentate ligand containing a nitrogen atom of the amino group and the oxygen atom of the peptide group of glycine one. Zn^{2+} ion in the structure has a distorted octahedral six-coordinate geometry. It binds with two terminal carboxyl groups and two didentate ligands with the *cis*-configuration. The Zn^{2+} ion is located at the crystallographic inversion center, and is distorted 0.103 Å from the least-squares plane with Zn(1), O(1), C(2), C(1), and N(1). The bond length of Zn(1)–O(1) (2.213(4) Å) in the chelate ring is longer than those of of Zn(1)–O(3) (2.071(4) Å) and Zn(1)–N(1) (2.101(6) Å). Figure 3 shows the crystal structure of the crystal along the *c* axis. The network formed with Zn^{2+} ions and the bridged ligand glythr^{-} constructs two-dimensional sheets along the *ab* plane with the cavities containing two water molecules. The whole structure indicates a porous crystal with one-dimensional channels, as shown in Fig. 4. The water molecules with the degree of high disorders are located into the channels due to weak hydrogen bonding (O(4)–H(8)···O(5) 3.10(1) Å) between

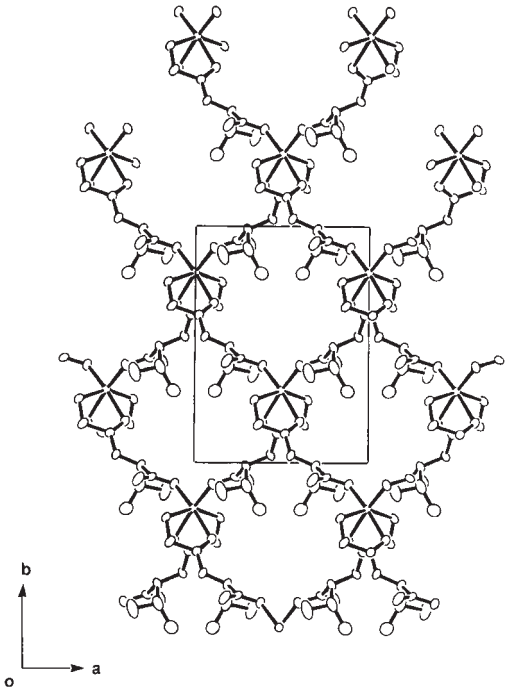

Fig. 3. Crystal structure of $\{[Zn(glythr)_2]\cdot(H_2O)_2\}_n$ viewed along the *c*-axis.

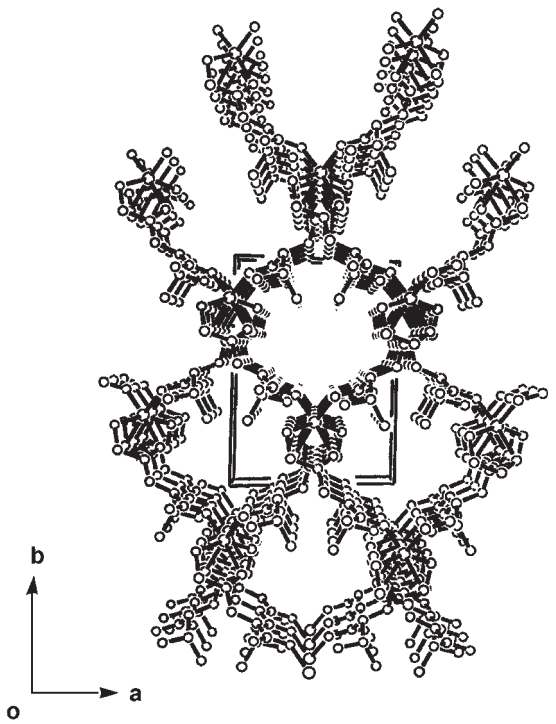

Fig. 4. Open-channel structures along the *c*-axis of $\{[Zn(glythr)_2]\cdot(H_2O)_2\}_n$.

the oxygen atom (O(4)) of the terminal hydroxy groups on the ligand and the confined water oxygen atom (O(5)) through the hydrogen atom H(8). Takayama and his co-workers reported on the crystal structure of the Zn(II) complex ($[Zn(glygly)_2]_n$) coordinating dipeptide ligands of a glygly^{-}, like the glythr^{-}.[22] The Zn(II) complex also forms a distorted octahedral six-coor-

dinate geometry constructed from two oxygen atoms of the carboxyl groups and two five-membered didentate chalets. However, its coordination sphere has a *trans*-configuration formed from the *equatorial* positions with two chelate rings and the *axial* positions with two terminal carboxylic oxygen atoms. Thus, the difference between the *cis*-configration and *trans*-one on coordination spheres is caused by different side-chain groups of the threonine and glycine residues. As a result, the deference on the coordination spheres between [Zn(glythr)$_2$] and [Zn(glygly)$_2$] also has an influence on the formed crystal structures three-dimensionally. One *trans*-[Zn(glygly)$_2$] forms a unique three-dimensional polymer seat aligned perpendicular to the *a* axis, and the other *cis*-[Zn(glythr)$_2$] forms a porous crystal with one-dimensional channels.

In Vitro Insulinomimetic Activity of Zn(II) Complexes. In vitro insulinomimetic activities of seven Zn(II) complexes with peptides were examined with regard to the inhibition of free fatty acid (FFA) release from isolated rat adipocytes treated with epinephrine. In evaluating the insulinomimetic activity of the compounds, we used the inhibition of FFA release from rat adipocytes.[5,21,23] The complexes were confirmed to act dose-dependently at the concentrations of 10^{-4}, 5×10^{-4} and 10^{-3} mol dm^{-3} of the Zn(II) complexes. The apparent IC_{50} values, the 50% inhibitory concentration of the Zn(II) complexes on the FFA release, are given in Table 4. These Zn(II) complexes showed higher insulinomimetic activities than that of the standard $ZnSO_4$. Consequently, we concluded that the Zn(II) complexes with peptides show higher insulinomimetic activities compared with that of Zn^{2+} ion, such as $ZnSO_4$.

For a comparison, we prepared four Zn(II) complexes (Table 1) with amino acid amides (gly-a, ala-a, pro-a, and met-a) and attempted to evaluate their insulinomimetic activities. However, their insulinomimetic activities didn't show any higher activity compared with that of $ZnSO_4$ (Table 4). The reason why these Zn(II) complexes didn't have high insulinomimetic activities is speculated to be as follows. They are highly hydroscopic and rapidly soluble in water. We previously reported that there is a correlation between the partition coefficients of Zn(II) complexes and the insulinomimetic activities,[13] which does not contradict the present results.

Table 4. Estimated IC_{50} Values of Zn(II) Complexes Free Fatty Acid Release from Isolated Rat Adipocytes Treated with Epinephrine

Zn(II) complexes	IC_{50}/mmol dm^{-3}
$ZnSO_4$	1.58 ± 0.05
1	1.08 ± 0.06[b)]
2	0.96 ± 0.05[b)]
3	1.23 ± 0.06[b)]
4	1.21 ± 0.07[b)]
5	0.85 ± 0.02[c)]
6	1.37 ± 0.05[a)]
7	1.32 ± 0.06[a)]
8	>10.0
9	8.17 ± 0.05
10	3.12 ± 0.04
11	2.24 ± 0.05

Values are means ± S.D. for three runs. a) Significance at $p < 0.05$ vs $ZnSO_4$. b) Significance at $p < 0.01$ vs $ZnSO_4$. c) Significance at $p < 0.005$ vs $ZnSO_4$.

Accordingly, we attempted to enhance the lipophilicity by alkylation of the amide protons or the formation of molecular complexes. In conclusion, we synthesized Zn(II) complexes with four dipeptides (molecular complexes) and three *N*,*N*′-ethylene bridged tripeptides (alkylation of adjacent amide protons), and revealed that they have high insulinomimetic activities in an in vitro study. Also, an X-ray structure analysis of a complex, $\{[Zn(glythr)_2]\cdot(H_2O)\}_n$, in the solid state revealed the formation of a polymer complex. Peptides show various functions in organisms. Moreover, because peptide transporters exist in the small intestine, some peptides are absorbed there.[19] In previous research, we reported that Zn(II) complexs with amino acid derivatives, picolinic acid derivatives, and nicotine amide derivatives have high insulinomimetic activities. In this paper, we propose other candidates for new types of insulinomimetic Zn(II) complexes with peptides. In addition, the result of Zn(II) complexes with peptides shows not only insulinomimetic activity in in vitro experiments but also the possibility to develop new clinically useful complexes that will be transported through the peptide transporter in cells.

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Abbreviations

geaaEt: Ethyl glycyl-(2*S*,3*S*′)-2-(3′-oxo-2′-methyl-1′-piperazinyl)-propanoate, L-aeaaEt: Ethyl L-alanyl-(2*S*,3*S*′)-2-(3′-oxo-2′-methyl-1′-piperazinyl)-propanoate, D-aeaaEt: Ethyl D-alanyl-(2*S*,3*S*′)-2-(3′-oxo-2′-methyl-1′-piperazinyl)-propanoate, Hglygly: Glycylglycine, Hglythr: Glycyl-L-threonine, Hglyval: Glycyl-L-valine, Hglypro: Glycyl-L-proline, gly-a: glycinamide, ala-a: L-alaninamide, pro-a: L-prolinamide, and met-a: L-metioninamide.

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