

Synthesis, Structure, and In Vitro and In Vivo Insulinomimetic Activities of the Zinc(II)–6-Ethylpicolinate Complex

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A new zinc(II) complex, bis(6-ethylpicolinato)zinc(II) ([Zn(6epa)$_2$]), was prepared by introducing an electron-donating ethyl group on the pyridine ring. [Zn(6epa)$_2$] was crystallized from an aqueous solution, and its structure was determined by X-ray analysis, in which both carboxylate O and pyridine N atoms of 6epa coordinated to a Zn^{2+} ion, forming a distorted trigonal bipyramidal geometry with two 6epa and a water. In an in vitro evaluation with regard to the inhibition of free fatty acid (FFA) release from isolated rat adipocytes in the presence of epinephrine, the insulinomimetic activity (IC_{50} = 0.37 mM) of [Zn(6epa)$_2$] was found to be higher than that (0.64 mM) of the bis(picolinato)-Zn(II) complex in terms of the IC_{50} value. When the [Zn(6epa)$_2$] complex was injected daily at a dose of 3 mg Zn/kg of body weight of KK-A^y mice for 14 d, the blood glucose levels of the mice were lowered to approximately 200 mg/dL (11.1 mM) from 400–500 mg/dL (22.2–27.8 mM). Based on the results, the [Zn(6epa)$_2$] complex was proposed to be a new candidate for treating type-2 diabetes in animals. However, it was proven that [Zn(6mpa)$_2$] has an advantage in having no side effect over [Zn(6epa)$_2$], when the effects on their serum parameters were considered.

Since the finding of an insulinomimetic activity of Zn^{2+} ion in 1980,[1] several research groups have attempted to confirm the insulinomimetic activity of the Zn^{2+} ion.[2–4] However, the insulinomimetic activity of Zn(II) complexes has not been studied for many years. During investigations to find insulinomimetic Zn(II) complexes,[5–8] we found that the bis(picolinato)zinc(II), [Zn(pa)$_2$], complex had a higher insulinomimetic activity than that of free Zn^{2+} ion.[9] Based on this finding, we used the complex as a leading compound for developing more active agents. By introducing an electron-donating group, such as a methyl group, into the pyridine ring of the picolinate ligand, bis(3- and 6-methylpicolinato)zinc(II) complexes ([Zn(3mpa)$_2$] and [Zn(6mpa)$_2$], respectively) were prepared, and their insulinomimetic activities were found to be higher than that of [Zn(pa)$_2$]; also the activity of [Zn(6mpa)$_2$] was higher than that of [Zn(3mpa)$_2$].[9] We then prepared the bis(6-ethylpicolinato)zinc(II) complex, [Zn(6epa)$_2$], in which an ethyl group with a higher lipophilicity than a methyl group was contained; the structure was analyzed by X-rays, and the in vitro insulinomimetic activity and in vivo blood glucose lowering effect in KK-A^y mice with type-2 diabetes mellitus of the complex were studied.

Experimental

Materials. All of the agents and solvents were commercially available and of the highest grade; they were used without more purification. $ZnSO_4$ (ZS), $ZnCl_2$, and picolinic acid (pa) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Epinephrine hydrochloride and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, Mo., USA).

Instrumentations. Elemental analyses of the compounds were carried out on a Perkin-Elmer 240C elemental analyzer (Tokyo, Japan). FT IR spectra were recorded with KBr pellets on a SHIMADZU FT IR-8600 Pc spectrometer (Kyoto, Japan). Melting points were determined on a Yanaco MP-J3 micro melting-point apparatus (Kyoto, Japan).

Preparation of Ligand. The ligand, 6epaH, was prepared according to a method from a reference.[10,11]

Preparation of Zn(6epa)$_2$. A ligand (6epa) and an equivalent mole of $LiOH\cdot H_2O$ were solved in water. Then, a half equivalent mole of a ZS aqueous solution was added to a solution of lithium salt of the ligand, and stirred over night at room temperature. A white precipitate was filtered off and washed with a methanol/ethanol (1:1) solution, and then dried in vacuo. Yield: 63%. Anal. Found: C, 47.86; H, 5.04; N, 7.00%. Calcd for $C_{16}H_{16}N_2O_4Zn\cdot 2.0H_2O$: C, 47.84; H, 5.02; N, 6.97%. IR (KBr): 1621 cm^{-1} for $\nu_{C=O}$. Mp: >300 °C (dec).

X-ray Data Collection and Structure Determination. A colorless single crystal of [Zn(6epa)$_2$] was obtained by recrystallization from hot ethanol, and used for an X-ray structure analysis. The data collection was performed with monochromated Mo Kα radiation for [Zn(6epa)$_2$] on a Rigaku AFC7R diffractometer. All of the calculations, including data reduction, were performed using a teXsan crystallographic software package (Molecular Structure Corporation). The crystal data and experimental conditions are summarized in Table 1. Lorentz polarization and absorption corrections were applied to a crystal of [Zn(6epa)$_2$(H_2O)]. The structure of the crystal was solved by a direct method with the program SIR92, and refined by the full-matrix least-squares

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Table 1. Crystal Data and Experimental Conditions of [Zn(6epa)$_2$]

Empirical formula	$C_{32}H_{40}O_{12}N_4Zn_2$	Scan type	ω–2θ
Formula weight	803.45	Scan rate/° min^{-1}	16.0
Crystal dimensions/mm^3	$0.10 \times 0.10 \times 0.05$	Scan width/°	$1.73 + 0.3 \tan\theta$
Crystal system	Triclinic	$2\theta_{max}$/°	55.0
Lattice parameters		p-factor	0.05
a/Å	10.354(2)	No. of observations	3154
b/Å	10.679(3)	(All, $2\theta < 55.0°$)	
c/Å	8.335(2)	No. variables	451
α/°	95.28(2)	R/P ratio	6.99
β/°	108.04(2)	R; $R_w{}^2$	0.037; 0.111
γ/°	99.60(2)	Shift/Error	0.44
V/Å^3	853.9(3)	GOF	1.04
Space group	$P1$ (No. 1)	Max. peak in diff.	
Z value	1	map/e$^-$ Å^{-3}	0.59
D_{calcd}/g cm^3	1.562	Min. peak in diff.	
F_{000}	416.00	map/e$^-$ Å^{-3}	−0.60
μ(Mo Kα)/cm^{-1}	14.74		
Diffractometer	RigakuAFC7R		
Temperature/°C	23.0		
λ/Å	0.71069		

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.

Crystallographic data have been deposited with Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-256768. Copies of the data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge, CB2 1EZ, UK; Fax: +44 1223 336033; or deposit@ccdc.cam.ac.uk).

In Vitro Evaluation of the Inhibitory Effect of Zn(II) Complexes on Free Fatty Acid (FFA) Release in Isolated Rat Adipocytes. Isolated male rat adipocytes (1.0×10^6 cells/mL), prepared as described in literature,[5–9,12–14] were preincubated at 37 °C for 30 min with various concentrations (10^{-4}–10^{-3} M) of zinc(II) complexes dissolved in DMSO in a KRB buffer (118 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.6 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $NaHCO_3$, and 5 mM glucose; pH 7.4) containing 2% BSA. A 10^{-4} M epinephrine solution was then added to the reaction mixtures, and the resulting solutions were incubated at 37 °C for 120 min. The reactions were stopped by soaking in ice water, and the mixtures were centrifuged at 3000 rpm for 10 min. For the outer solution of the cells, the FFA levels were determined with an FFA kit (Wako Pure Chemical, Osaka, Japan).

In Vivo Evaluation of [Zn(6epa)$_2$] on the Blood Glucose Levels in KK-A^y Mice. KK-A^y mice as a congeneric strain, in which the A^y allele at the agouti locus (initially from C57B/6J-A^y) had been transferred to the inbred KK strain by repetitive back crossing, were used. KK-A^y mice (8 weeks old: CREA Japan Inc, Tokyo, Japan) with type-2 diabetes mellitus, which were housed in an air conditioned room at temperature of 23 ± 1 °C and 60 ± 10% humidity with lights on from 8:00 a.m. to 8:00 p.m., received daily intraperitoneal (*i.p.*) injections (5 mice in a group) of [Zn(6epa)$_2$] at a dose of 3.0 mg (45.9 μmol) Zn/kg body weight dissolved in 5% acacia vehicle at about 10 a.m. after the determination of their blood glucose levels for 2 weeks. The blood sample for an analysis of the glucose level was obtained from the tail vein of each mouse, and measured with a Glucocard (Arkray, Kyoto, Japan). The body weights of KK-A^y mice that were allowed free access to solid food and tap water, were measured daily during the administration of [Zn(6epa)$_2$]. The intakes of solid food and drinking water in each mouse were checked daily throughout the experiments. The blood samples for the analyses of blood urea nitrogen (BUN), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), total cholesterol (TCHO), and glycated hemoglobin (HbA_{1c}) were withdrawn from the cavernous sinus with a capillary under anesthesia with ether. The serum concentrations of BUN, GOT, GPT, and TCHO were determined by Fuji Dry Chem (Fuji Medical Co., Tokyo, Japan), and HbA_{1c} was measured by an immunoassay method (DCA2000 System, Bayer-Sankyo Co., Ltd. Tokyo, Japan). The animal study was approved by the Experimental Animal Research Committee at Osaka City University.

Statistical Treatment Data. Data are expressed as the means ± standard deviations for three repeated runs. A statistical analysis was performed using the Student's t-test at the 5% ($p < 0.05$), 1% ($p < 0.01$), or 0.5% ($p < 0.005$) significance level of the difference.

Results and Discussion

X-ray Structure of [Zn(6epa)$_2$(H$_2$O)]. Figure 1 shows an ORTEP view for the crystal structure of [Zn(6epa)$_2$(H$_2$O)] with the atom numbering. The selected bond distances and angles are listed in Table 2. The crystal was comprised of two independent complexes of [Zn(6epa)$_2$(H$_2$O)] and two water molecules in an asymmetric unit. The 6epa acted as a bidentate ligand to coordinate to metal ions through both a nitrogen atom of the pyridyl group and an oxygen atom of the terminal carboxyl group. Each coordinations sphere around the Zn^{2+} ion had a distorted trigonal bipyramidal geometry similar to that of [Zn(6mpa)$_2$(H$_2$O)],[9] constructed from coordination with two 6epa and a water molecule belonging to a *basal* plane. The coordinated two pyridyl nitrogen atoms in an *apical* position had coordination angles of 162.8(4)° for N1–Zn1–N2 and 166.9(4)° for N3–Zn2–N4, respectively. In the crystal, two [Zn(6epa)$_2$(H$_2$O)] molecules in the independent unit were connected by dual intermolecular hydrogen bonding between oxy-

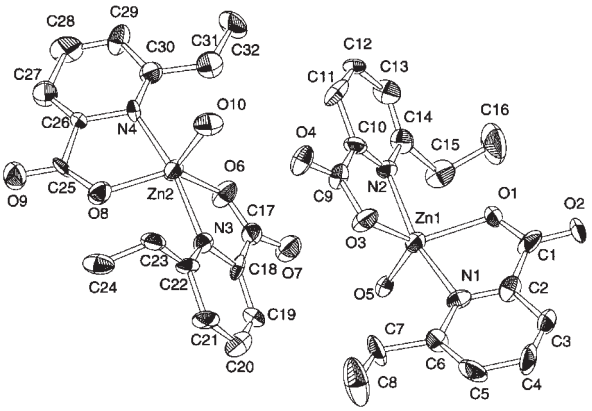

Fig. 1. ORTEP drawing of two independent $[Zn(6epa)_2(H_2O)]$ in an asymmetric unit.

Table 2. Selected Bond Lengths (Å) and Angles (°) for $[Zn(6epa)_2]$

Bond lengths/Å		Bond angles/°	
Zn(1)–O(1)	2.06(1)	O(1)–Zn(1)–N(1)	78.6(5)
Zn(1)–N(1)	2.17(1)	O(1)–Zn(1)–O(3)	126.3(5)
Zn(1)–O(3)	1.98(1)	O(1)–Zn(1)–O(5)	118.0(5)
Zn(1)–O(5)	1.971(10)	O(1)–Zn(1)–N(2)	90.1(5)
Zn(1)–N(2)	2.15(1)	N(1)–Zn(1)–O(3)	96.1(5)
Zn(2)–O(6)	2.02(1)	N(1)–Zn(1)–O(5)	102.4(4)
Zn(2)–N(3)	2.17(1)	N(1)–Zn(1)–N(2)′	162.8(4)
Zn(2)–O(8)	1.95(1)	O(3)–Zn(1)–O(5)	115.3(5)
Zn(2)–O(10)	2.04(1)	O(3)–Zn(1)–N(2)	80.1(5)
Zn(2)–N(4)	2.13(1)	O(5)–Zn(1)–N(2)	94.3(4)
		O(6)–Zn(2)–N(3)	79.2(5)
C(1)–O(1)	1.26(2)	O(6)–Zn(2)–O(8)	127.2(5)
C(1)–O(2)	1.24(2)	O(6)–Zn(2)–O(10)	113.3(5)
C(9)–O(3)	1.30(1)	O(6)–Zn(2)–N(4)	97.4(4)
C(9)–O(4)	1.24(2)	N(3)–Zn(2)–O(8)	91.5(5)
C(17)–O(6)	1.25(2)	N(3)–Zn(2)–O(10)′	93.0(4)
C(17)–O(7)	1.24(2)	N(3)–Zn(2)–N(4)	166.9(4)
C(25)–O(8)	1.28(2)	O(8)–Zn(2)–O(10)	119.0(5)
C(25)–O(9)	1.22(2)	O(8)–Zn(2)–N(4)	80.5(5)
		O(10)–Zn(2)–N(4)	99.9(4)

gen atoms of the coordinated water molecules and those of non-coordinated carboxyl groups of each other (O5···O7 = 2.70(1) Å, O4···O10 = 2.65(1) Å). Figure 2 shows the arrangements in a crystal consisting of four $[Zn(6epa)_2(H_2O)]$ molecules in a unit cell. However, O11 or O12 of a water molecule with no relation to the metal coordination in the crystal was stabilized by intermolecular hydrogen bonding between a coordinated water molecule and two oxygen atoms of carboxyl groups to give three-connected coordination. However, no hydrogen bonds between O11 and O12 occurred (O5–O12 = 2.66(1) Å, O9–O12 = 2.77(1) Å, and O2–O12 = 2.81(2) Å for a water oxygen atom of O12; O10–O11 = 2.66(1) Å, O9–O11 = 2.78(2) Å, O2–O11 = 2.78(1) Å, and O11–O12 = 3.74(2) Å for O11).

Insulinomimetic Activity of Zinc(II) Compounds. The in vitro insulinomimetic activity of $[Zn(6epa)_2]$ was examined with regard to the inhibition of free fatty acid (FFA) release from isolated rat adipocytes treated with epinephrine.[5–9,12–16]

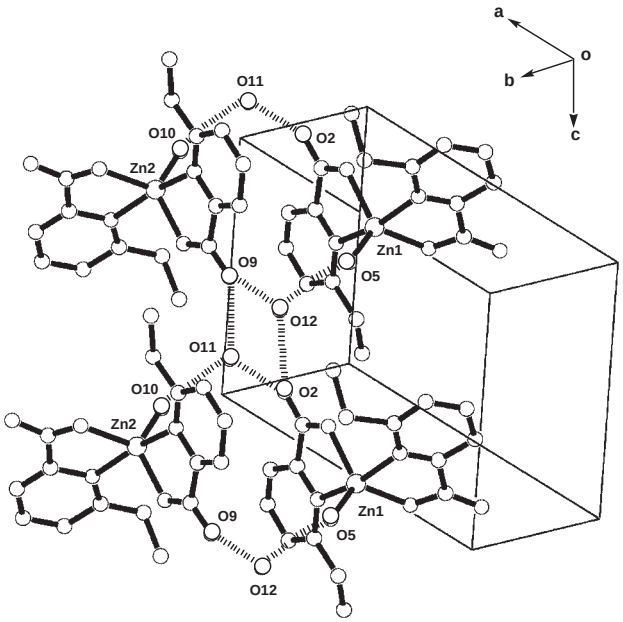

Fig. 2. Tetrameric structure of $[Zn(6epa)_2(H_2O)]$ complex connected by hydrogen bondings of two water molecules.

Table 3. Estimated IC_{50} (mM) Values of Zinc(II) Complexes in the Presence of Glucose

Complexes	IC_{50}/mM (±S.D.[a)])
$ZnSO_4$	0.81 (0.10)
$ZnCl_2$	0.68 (0.07)
$[Zn(pa)_2]$	0.64 (0.13)[b,c)]
$[Zn(6mpa)_2]$	0.39 (0.15)[b,c,d)]
$[Zn(6epa)_2]$	0.37 (0.07)[c,e)]

a) Each values are expressed as the means ± S.D.s for three experiments. b) Significance at $P < 0.05$. c) Significance at $P < 0.02$. d) Significance at $P < 0.05$ vs $ZnCl_2$. e) Significance at $P < 0.01$ vs $ZnCl_2$.

A dose-dependent activity was confirmed in concentrations of 10^{-4}, 5×10^{-4}, and 10^{-3} mol dm^{-3} of the Zn(II) complex. The apparent IC_{50} value, the 50% inhibitory concentration of $[Zn(6epa)_2]$ on FFA release was lower than those of $ZnSO_4$, $ZnCl_2$, and the lead complex, $[Zn(pa)_2]$ (Table 3). In the past research, we reported that bis(kinic acid) or tris(betain)/Zn(II) complexes retained the structure of the Zn:ligand = 1:2 or 1:3 in the solution.[17] Therefore, Zn(II) complexes with picolinic acid derivatives may also be supposed to retain the structure of the Zn:ligand = 1:2 in the solution; we consider that they affect the structure of the Zn:ligand = 1:2. Generally, it has been known that an increase in the chain length of the alkyl group enhances the lipophilicity, if the basic structure is fixed.[18,19] In this study, $[Zn(6epa)_2]$ didn't melt in the water solution. Therefore, we estimated that solubility to water of these complexes was on the order of the $[Zn(pa)_2] > [Zn(6mpa)_2] > [Zn(6epa)_2]$. Previously, we proposed a correlation between the hydrophobicity of the complex and the IC_{50} value,[9] which did not contradict the present result.

Blood Glucose Lowering Effect of the $[Zn(6epa)_2]$ Complex in KK-A^y Mice. $[Zn(6epa)_2]$ complex was subjected to

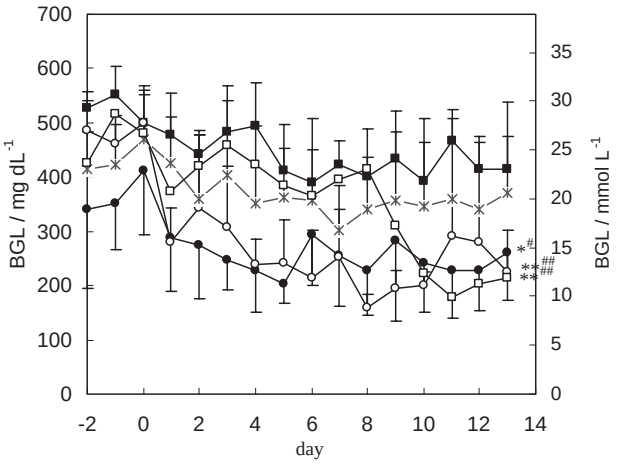

Fig. 3. Changes of blood glucose level of KK-A^y mice given zinc complexes. Hyperglycemic KK-A^y mice received daily *i.p.* injection of 5% acasia (control) ($n = 5$) (■), $ZnCl_2$ (*),[6] [Zn(pa)$_2$] (●), [Zn(6mpa)$_2$] (○),[9] and [Zn(6epa)$_2$] (□) at a dose of 3.0 mg Zn/kg body weight for 14 days (numbers of animals; control, $ZnCl_2$, [Zn(pa)$_2$], [Zn(6mpa)$_2$]; $n = 5$, and [Zn(6epa)$_2$]; $n = 4$). Each point is expressed as the means ± S.D.s for 5 or 4 mice. * $P < 0.01$ vs control, ** $P < 0.005$ vs control. # $P < 0.05$ vs before *i.p.* injections, ## $P < 0.001$ vs before *i.p.* injections.

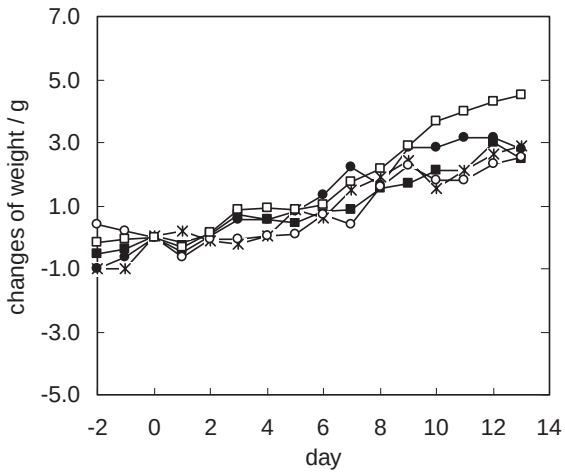

Fig. 4. Changes of body weight of KK-A^y mice treated with the complexes. Hyperglycemic KK-A^y mice received daily *i.p.* injections of 5% acasia (control) ($n = 5$) (■), $ZnCl_2$ (*),[6] [Zn(pa)$_2$] (●),[9] [Zn(6mpa)$_2$] (○),[9] and [Zn(6epa)$_2$] (□) at a dose of 3.0 mg Zn/kg body weight for 14 days (number of animals; control, $ZnCl_2$, [Zn(pa)$_2$], [Zn(6mpa)$_2$]; $n = 5$, and [Zn(6epa)$_2$]; $n = 4$).

Table 4. Serum Parameters and HbA_{1c} Level of KK-A^y Mice after Daily *i.p.* Injections of 5% Acasia (Control) and Zn(6epa)$_2$ for 14 Days[a)]

Treatment	BUN/mg dL^{-1}	GOT (U/L)	GPT (U/L)	TCHO/mg dL^{-1}	TG/mg dL^{-1}	HbA_{1c}/%
Control	32.9 (2.4)	61 (15)	30 (9)	176 (48)	181 (27)	8.3 (0.3)
[Zn(6epa)$_2$]	20.9 (1.2)[d)]	109 (18)[b)]	50 (30)	149 (13)	147 (16)	6.2 (0.8)[c)]

a) Values are mean are expressed as the ±S.D.s for 3 or 4 mice (control; 4 mice, [Zn(6epa)$_2$]; 3 mice). b) Significance at $P < 0.05$ vs control KK-A^y mice. c) Significance at $P < 0.01$ vs control KK-A^y mice. d) Significance at $P < 0.005$ vs control KK-A^y mice.

an in vivo evaluation in terms of the blood glucose lowering effect. When [Zn(6epa)$_2$] was given to KK-A^y mice at a dose of 3.0 mg Zn/kg of body weight for 10 days, the high blood glucose levels (approximately 400 mg/dL) were lowered to about 200 mg/dL (11.1 mol/L), and was maintained by daily administrations of 3.0 mg (45.9 μmol) Zn/kg of body weight (Fig. 3). Moreover, the blood glucose level of the animals given [Zn(pa)$_2$] was also lowered. However, its blood glucose lowering effect was weaker than those of [Zn(6mpa)$_2$][9] and [Zn(6epa)$_2$] complexes between day 0 and day 13. (The difference of blood glucose level between day 0 and day 13 were [Zn(pa)$_2$]; 150 ± 103 mg/dL, [Zn(6mpa)$_2$]; 274 ± 72 mg/dL, and [Zn(6epa)$_2$]; 265 ± 38 mg/dL.) The body weight of the animals didn't decrease during complex administration, but increased in all groups (Fig. 4). The food and water intake didn't change in the [Zn(6epa)$_2$] administrated group (data not shown).

HbA_{1c} and Serum Parameters. The level of HbA_{1c}, which shows the number of glucose molecules attached to the hemoglobin in the erythrocytes over a long period,[20] was measured. In KK-A^y mice untreated and treated with [Zn(6epa)$_2$], the HbA_{1c} levels changed from 7.6 ± 0.5 to 8.3 ± 0.3 (%) and from 8.0 ± 1.0 to 6.2 ± 0.8 (%) before and after administration of the complex, respectively (Table 4), indicating that the blood glucose lowering effect of the [Zn(6epa)$_2$] complex continued for a long term. When KK-A^y mice were administered the complex for 14 d, the animals were fasted for 14 h after the final administration day; all of the several serum parameters were measured. The GOT, GPT, BUN, TG, and TCHO levels in the control KK-A^y mice (untreated) and KK-A^y mice treated with [Zn(6epa)$_2$] for 14 d are summarized in Table 4. TG and TCHO levels, which indicate the degree of cholesterol metabolism,[21] did not change compared with those of the control KK-A^y mice. The BUN level was lowered by a treatment with [Zn(6epa)$_2$], suggesting that the synthesis of urea in the liver was decreased by the complex administration. Moreover, the GOT and GPT levels, which indicate the degree of liver disturbance,[22] showed a tendency to increase by the complex administration, indicating that [Zn(6epa)$_2$] affected the hepatic functions. Previously, we reported that the administration of [Zn(6mpa)$_2$] didn't show any disorder in the serum parameters.[9] From these observations, we suggest that if the lipophilicity of [Zn(pa)$_2$] is increased by introducing some substituents, the complex will affect the internal organs, which

in turn develop some side effects.

In conclusion, the $[Zn(pa)_2]$ derivatives, such as $[Zn(6mpa)_2]$ and $[Zn(6epa)_2]$ with an electron-donating group, were found to exhibit higher in vitro insulinomimetic activity than that of the leading compound. Moreover, as for the in vivo blood glucose lowering effect, we found that the lipophilicity of the $[Zn(pa)_2]$ derivatives didn't always show good effects. That is to say, the moderate hydrophobicity of $[Zn(6mpa)_2]$ may afford the best complex for in vivo blood glucose lowering effect, where the serum parameters don't change compared with those of $[Zn(pa)_2]$. We will use these results effectively to choose the most suitable ligands.

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