Bis(allixinato)oxovanadium(IV) Complex Is a Potent Antidiabetic Agent: Studies on Structure−Activity Relationship for a Series of Hydroxypyrone−Vanadium Complexes

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There is an urgent medical need for orally effective drugs to replace insulin injections for the treatment of diabetes mellitus. Vanadium complexes with insulin-mimetic activities have recently been proposed as candidates as new antidiabetic drugs. Following in vitro and in vivo studies on a group of bis(3-hydroxy-4-pyronato)oxovanadium(IV) (**1**) complexes with $VO(O_4)$ coordination mode, bis(allixinato)oxovanadium-(IV) (**3**) which contains allixin, a garlic component, was found to be the most potent antidiabetic agent among them. Complex **3** with a high in vitro insulin-mimetic activity in terms of both free fatty acid (FFA)-release inhibitory and glucose-uptake enhancing activities in isolated rat adipocytes exhibited a high hypoglycemic effect in type 1 diabetic model mice by both intraperitoneal injections and oral administrations. Complex **3** is thus proposed to be one of the most effective candidates for antidiabetic therapy.

Introduction

In the 21st century, the number of patients suffering from diabetes mellitus (DM) is increasing worldwide.[1] Though several injectable insulin preparations have been developed for patients with type 1 DM, which is characterized by hyperglycemia associated with progressive β-cell death and hypoinsulinemia, daily injections of these preparations are often associated with physical pain and mental stress. Thus, the development of new orally effective antidiabetic therapeutics is urgently required to replace insulin injections for the treatment of DM as well as other drugs for the treatment of type 2 DM in order to improve the quality of life of patients.

Recently, vanadium (V) salts were clinically examined to ascertain whether they improve the DM state in humans.[2] However, absorption and utilization of these inorganic salts are generally very low. In addition, vanadyl (VO^{2+}) compounds are less toxic than vanadate compounds as judged by LD_{50} values in rats,[3] and most of the vanadium in vanadate-treated normal rats actually exists in the vanadyl state.[4] On the basis of these findings, a large class of vanadyl complexes with different coordination modes have recently been proposed for anticipating their clinical use.[5]

Although hypoglycemic effects of vanadium are well established, there still exists a difference in opinion among researchers with regard to the mechanisms leading to these effects. Glucose homeostasis in the body largely depends on a balance between its production in the liver and its utilization in peripheral tissues. Under normal conditions, insulin inhibits gluconeogenesis in the liver and enhances glucose uptake in cells of the peripheral tissues; therefore, absolute (type 1) or relative (type 2) insulin deficiency leads to hyperglycemia. Insulin binding to the extracellular α-subunit of the insulin receptor causes conformational changes in the autophosphorylation at its intracellular β-subunits. This triggers phosphorylation of the insulin receptor substrate (IRS), and the signal is conveyed to downstream locations including phosphatidyl inositol-3-kinase (PI3-K) and cyclic nucleotide phosphodiesterase (PDE). The glucose transporter (GLUT) is then translocated to the inner surface of the cell membrane. Consequently, extracellular glucose is incorporated into the cell through GLUT.[6]

Recently, vanadium compounds have been revealed to be involved in such a mechanism through multiple intracellular modes of action, termed an "ensemble mechanism", similar to the action of zinc(II) complexes,[7,8] which includes protein tyrosine phosphatase (PTPase), PI3-K, GLUT, protein kinase B (PKB), and PDE acting simultaneously in cells.[9] On the basis of these facts, the permeability of cell membrane and incorporation of V components regulating insulin-targeting tissues are very important factors to exhibit hypoglycemic effects.

A bis(maltolato)oxovanadium(IV) complex, $VO(ma)_2$ (**2**), which was prepared in 1992, has been demonstrated to have high hypoglycemic activity in experimental animals,[10] the activity being approximately 1.5 times that of $VOSO_4$ in chronic experimental animal treatment and 3 times that of $VOSO_4$ in acute treatment protocols.[11]

Furthermore, its related complexes have been prepared and examined for hypoglycemic activity in diabetic animals.[12] However, no complexes with activity greater than complex **2** were yet found. We then attempted to develop vanadyl complexes with higher potency than this complex. For this purpose, we examined the in vitro as well as in vivo structure−activity relationships of bis(3-hydroxy-4-pyronato)oxovanadium(IV) complex (**1**) as a leading compound with $VO(O_4)$ coordination mode, proposing a novel more potent vanadyl complex with allixin (**3**), which was isolated from dried garlic (*Allium sativum* L., Figure 1) as a nonsulfur phytoalexin.[13]

Results and Discussion

Chemisty. As shown in Chart 1, we prepared five complexes related to **1** by a previously reported method for the synthesis of complex **2**.[10] The structures of the complexes were characterized by elemental analysis, IR, visible spectrum, MS spectrum, and ESR spectrum. IR absorption bands of the ligands due to O−H and C═O stretching frequencies at approximately 3000−

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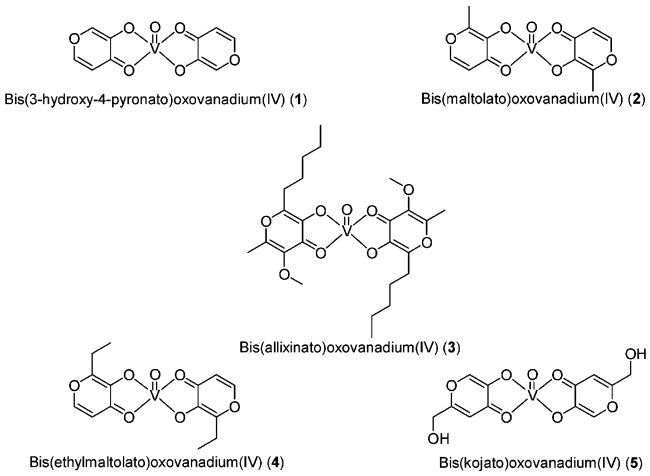
Figure 1. Dried garlic with allixin adhering to it after approximately 2 years of storage.

Chart 1. Chemical Structures of Vanadyl Complexes



3250 and 1650 cm^{-1}, respectively, disappeared and shifted in each vanadyl complex, indicating the coordination of the deprotonate hydroxyl as well as the carbonyl group of ligands to VO^{2+}. In addition, the IR absorption band due to V=O stretching frequency was detected at around 950−997 cm^{-1}. The high-resolution mass spectra (HR MS) of all vanadyl complexes indicated molecular weights corresponding to the structure consisting of VO^{2+}:ligand = 1:2, as supported by elemental analyses. ESR spectra of the vanadyl complexes in solution gave eight-line hyperfine splitting patterns due to an unpaired electron of the ^{51}V nucleus ($I = 7/2$), supporting the presence of mononuclear vanadyl species in each complex. The spectra obtained at liquid nitrogen temperature exhibited anisotropic; two sets of eight lines were distinguished, indicating the formation of single species of vanadyl complexes. From these spectra, the ESR parameters such as *g*-values and hyperfine coupling constants (*A*-values) were calculated. To estimate the coordination mode of vanadyl complexes, we compared the ESR parameters of several vanadyl complexes with different coordination modes around VO^{2+} and confirmed that the ESR parameters of these complexes were similar to those of the vanadyl complexes of the $VO(O_4)$ type,[14] indicating the $VO(O_4)$ coordination type (see Chart 1). Previously the complex **2** was analyzed in square pyramidal structure in the trans form, as evaluated by X-ray structure analysis.[15] These data suggested that the structures of the present vanadyl complexes including complex **2** were estimated to have a common $VO(O_4)$ coordination mode at the binding ratio of VO^{2+}:ligand = 1:2 with the trans forms.

In Vitro Insulin-Mimetic Activity. To evaluate in vitro insulin-mimetic activity of these complex, we first performed an in vitro assay based on inhibition of free fatty acid (FFA) release from isolated rat adipocytes treated with epinephrine (adrenaline), which is a simple and convenient method compared with the use of radioisotope reagents.[16] With this assay, it was found that among the prepared vanadyl complexes, complex **3** ($IC_{50} = 553\ \mu M$) was found to be the most effective in inhibiting FFA release (Table 1). Activities of the hydrophilic complexes such as complexes **1** ($IC_{50} = 1179\ \mu M$) and **5** ($IC_{50} = 2370\ \mu M$) were lower than those of the complexes **2** ($IC_{50} = 676\ \mu M$), **3**, and **4** ($IC_{50} = 632\ \mu M$). Interestingly, a good linear correlation ($r = 0.99$) was observed between FFA-release inhibitory activity (reciprocal IC_{50}) value and partition coefficients (log *P*) of the complexes, indicating that within this series of complexes, insulin-mimetic activity correlates positively with lipophilicity of the complex (Figure 2). Increased lipophilicity may cause increased cell membrane penetration. The in vitro activity of **3** was further evaluated in regard to the glucose uptake, the activity of **3** ($EC_{50} = 24 \pm 3\ \mu M$) being significantly higher than that of **2** ($EC_{50} = 95 \pm 4\ \mu M$, $P < 0.001$) (Figure 3). These results suggest a possibility that vanadium-dependent active sites are primarily present in the cells, thus supporting the hypothesis that lipophilicity of the

Table 1. FFA-Releasing IC_{50} Data and Partition Coefficient[a]

		lipophilicity (log *P*)	
compd	IC_{50} (μM)	vanadyl complex	ligand
1	$1179 \pm 217^{*\#}$	$-0.43 \pm 0.02^{*\#}$	$-0.19 \pm 0.01^{*\#}$
2	676 ± 5	0.46 ± 0.11	0.60 ± 0.01
3	$553 \pm 33^{*\#}$	$1.02 \pm 0.06^{*\#}$	$1.65 \pm 0.02^{*\#}$
4	632 ± 57	$0.83 \pm 0.04^{*}$	$1.24 \pm 0.03^{*}$
5	$2370 \pm 971^{*\#}$	$-1.48 \pm 0.07^{*\#}$	$-1.32 \pm 0.10^{*\#}$

[a] Data are expressed as mean ± SD for three experiments. Significance: $^{*}p < 0.01$ vs complex **2**. Significance: $^{\#}p < 0.01$ vs complex **4**.

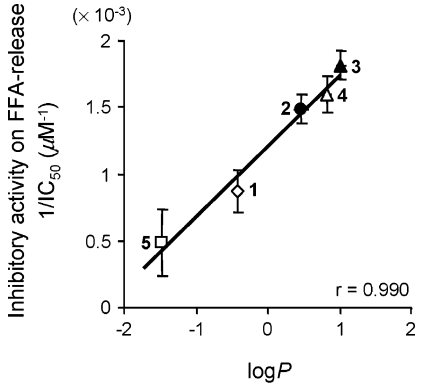


Figure 2. Relationship between reciprocal IC_{50} value and partition coefficient of vanadyl complexes ($n = 3$/complex).

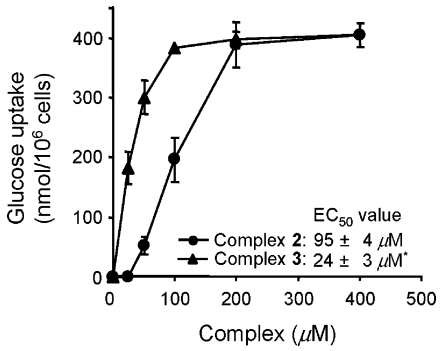


Figure 3. Concentration-dependent glucose-uptake enhancement by complexes **2** or **3** in isolated rat adipocytes. Significance: $^{*}p < 0.01$ vs complex **2**.

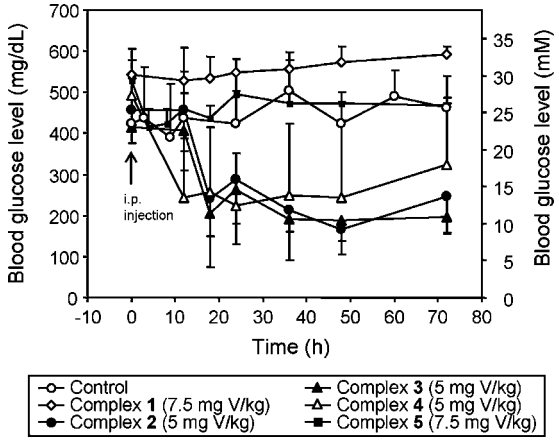


Figure 4. Effect of a single intraperitoneal injection of vanadyl complexes on blood glucose levels in STZ diabetic mice ($n = 5$ to 8 mice/group).

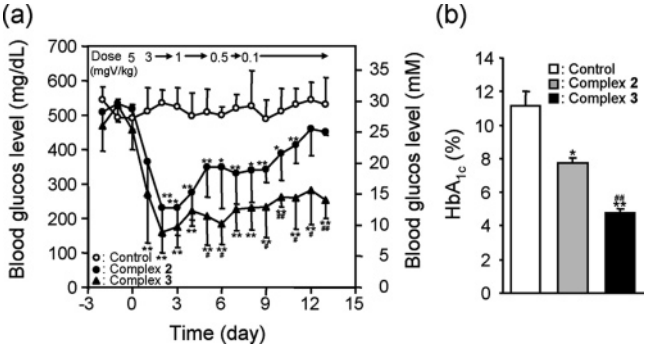


Figure 5. (a) Changes of blood glucose level and HbA_{1c} level in STZ mice treated with complex **2** or **3** by daily i.p. injections for 14 days ($n = 4$ to 6 mice/group); (b) HbA_{1c} levels of STZ mice after treatment of vanadyl complexes. Significance: $*p < 0.05$, $**p < 0.01$ vs control STZ mice. Significance: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs STZ mice treated with complex **2**.

complex is an important factor for developing insulin-mimetic activity of vanadyl complexes. It was thus revealed that complex **3** exhibited the highest in vitro insulin-mimetic activity among all the vanadyl complexes examined in this study. This relatively high level of activity was associated with the high partition coefficient of the complex **3** ($\log P = 1.65 \pm 0.02$) contributed by the allixin ligand. Previously, we suggested that the overall stability constant ($\log \beta$) of a metallocomplex was one of the important factors in developing insulin-mimetic activity.[17] However, no difference was observed between the stability constants of complexes **2** and **3** (data to be reported), indicating that insulin-mimetic activity of the present complexes predominantly depends on the permeability of the complex through the cell membranes. These results of in vitro experiments demonstrated that complex **3** has the most potent insulin-mimetic activity among the vanadyl complexes examined.

In Vivo Study. Following the in vitro experiments, we examined the in vivo insulin-mimetic activities in terms of hypoglycemic activity of these complexes in streptozotocin-induced diabetic mice (STZ mice), a typical rodent model of type 1 DM. The mice were treated with the individual complex by an intraperitoneal (i.p.) injection at the doses indicated in Figure 4. A single bolus administration of complexes **2**, **3**, and **4** resulted in a dramatic lowering of blood glucose levels within 18 h after the injection (Figure 4). In contrast, hydrophilic complexes such as **1** and **5** with low in vitro activities did not show hypoglycemic effects at a dose of 7.5 mg V/kg body mass. These results were consistent with those of the in vitro evaluations. However, a significant difference was not observed between the activities of complex **3** and **2** in the acute study.

Then the in vivo abilities of complexes **2** and **3** were compared in a chronic study at low doses of vanadyl complexes for 14 days (Figure 5). STZ mice were treated with complex **2** or **3** by daily i.p. injections at doses indicated in Figure 5a. Complex **3** produced a significant reduction in high blood glucose levels together with a remarkably lowered glycated hemoglobin (HbA_{1c}) (Figure 5b). After treatment, compared with those of complex **2**, the oral glucose tolerance test (OGTT) which indicates glucose tolerance ability was performed on both complexes (see Supporting information). In the OGTT, blood glucose levels of the control STZ mice as well as those of complex **2** treated STZ mice were elevated to a maximal concentration of approximately 500 mg/dL (28 mM) at 30 to 45 min after the glucose loading. In contrast, blood glucose elevation in complex **3** treated STZ mice was significantly lower than those of control STZ mice and complex **2** treated STZ mice. As expected, the insulin-mimetic activity of complex **3** was found to be considerably higher than that of complex **2**. On the other hand, we determined the vanadium level in the tissues of mice treated with vanadyl complexes by the neutron activation analysis (NAA), which is the most reliable method for vanadium determination.[18] Vanadium was detected in almost all tissues, particularly bone, spleen, liver, pancreas, and skeletal muscle, in this order, in STZ mice treated with complex **2** or **3**, but it was not detectable in the control STZ mice (Table 2). Among the insulin-sensitive tissues, skeletal muscle accounts for the high uptake of vanadium. Interestingly, complex **3** showed a higher distribution of vanadium in the skeletal muscle than complex **2**. These results suggest a possibility that complex **3** has a potent hypoglycemic effect due to the higher distribution of V to skeletal muscle, which is an important target tissue for insulin, than that of complex **2**. In contrast, the vanadium accumulation in the pancreas due to complex **3** was significantly lower than that due to complex **2**. Several data indicated that treatment with vanadium compounds had no effect on insulin release from the pancreas in type 1 diabetic animals.[19] These observations are consistent with the results of our chronic study. In type 1 DM, the pancreas might be less important with respect to hypoglycemic effects of vanadium compounds.

On the basis of the results, we examined whether complex **3** is effective on oral administration in type 1 diabetic STZ mice at a dose of 10 mg V/kg. A single oral gavage of complex **3** clearly exhibited the hypoglycemic effects in type 1 diabetic STZ mice, as shown in Figure 6, finding that complex **3** is an orally active vanadyl complex with high insulin-mimetic activity

Table 2. Organ Distribution of Vanadium in STZ Mice Treated with Vanadyl Complex[a]

	vanadium content (μg/g of wet tissue)					
group	liver	muscle	spleen	kidney	pancreas	bone
control	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.
complex **2**	1.1 ± 0.6	0.10 ± 0.03	1.2 ± 0.3	0.76 ± 0.31	0.91 ± 0.13	4.6 ± 0.3
complex **3**	1.0 ± 0.3	0.16 ± 0.01*	1.1 ± 0.4	0.87 ± 0.20	0.56 ± 0.09*	4.0 ± 0.6

[a] Data are expressed as mean $\pm$ SD for three or four experiments. Significance: $*p < 0.05$ vs complex **2**. [b] n.d. = not detected.

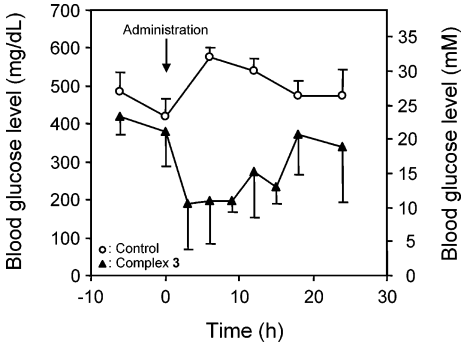


Figure 6. Effect of a single oral administration of complex **3** at a dose of 10 mg V/kg body mass on blood glucose levels in STZ mice ($n = 7$ mice/group).

in both cell and animal levels. Since vanadium compounds may induce some side effects such as diarrhea, green tongues, haemotological changes, and nervous system abnormalities,[20] further studies on the complex **3** are required.

Conclusion

A newly prepared complex **3** is found to be a potent insulin-mimetic and antidiabetic vanadyl complex in type 1 diabetic animals in which the lipophilicity of the complex is proposed to be an important factor for developing the insulin-mimetic vanadyl complexes. Based on in vitro as well as in vivo evaluations, complex **3** could be a candidate as an orally active hypoglycemic medicine which is useful for the treatment of type 1 DM. To enhance the bioavailability more effectively on oral administration of compound **3**, we are trying to develop a suitable preparation of this compound in the next stage.

Experimental Section

Chemicals. Vanadyl sulfate ($VOSO_4 \cdot nH_2O$), maltol (3-hydroxy-2-methyl-4-pyrone, Hma), and kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone, Hka) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The $VOSO_4 \cdot nH_2O$ was standardized complexometrically with ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) and determined as the 2.8 H_2O adduct and then used in all the experiments. Ethyl maltol (2-ethyl-3-hydroxy-4-pyrone, Hema) was obtained from Tokyo Kasei Industry (Tokyo, Japan). Allixin (3-hydroxy-5-methoxy-6-methyl-2-pentyl-4-pyrone, Halx), which was isolated from garlic and purified by silica gel, was a product of Wakunaga Pharmaceutical Co. (Hiroshima, Japan). Bovine serum albumin (BSA; fraction V), collagenase (Type II), (±)-epinephrine monohydrochloride (adrenaline), and streptozotocin (STZ) were purchased from Sigma Chemical (St. Louis, MO). 3-Hydroxy-4-pyone was synthesized by the existing methods.[21]

Animal. Male Wistar rats (7−8 weeks old) used for in vitro tests and male ddY mice (7 weeks old) used for the in vivo study were obtained from Shimizu Experimental Material Co. (Kyoto, Japan). All animals were allowed free access to solid food (MF, Oriental Yeast Co., Tokyo, Japan) and tap water. All of the animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and were performed according to the Guidelines for Animal Experimentation at KPU.

Chemistry. The prepared complexes were characterized by elemental analysis, IR absorption, and HR MS spectra. UV and ESR spectra were measured using Agilent-8453 spectrometer (Yokogawa Analytical Systems Co., Tokyo, Japan) and JEOL JES-RE1X (Tokyo, Japan) spectrometers, respectively. ESR spectra were recorded at room and liquid nitrogen temperature. Instrumental conditions were as follows: modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; microwave power, 5 mW; standards, tetracyanoquinodimethane-lithium salt (TCNQ-Li) ($g = 2.00252$) and Mn(II) in MgO (magnetic field between the third and fourth signals due to Mn(II), 8.69 mT). The magnetic field was calibrated with a Takeda Riken frequency counter, TR 5212 (Tokyo, Japan). IR spectra were measured with a Shimadzu FT-IR 8100A (Kyoto, Japan) on KBr pellet. Elemental analyses were performed by the Analytical Center of KPU. HR MS spectra (JEOL JMS-SX 102AQQ; Tokyo, Japan) were measured in FAB mode using thioglycerol as a matrix material by the Analytical Center of KPU. The partition coefficients of the complexes were determined by a conventional method in a 10 mM HEPES buffer (pH 7.4)/chloroform system at a concentration of 1.0 mM of the complex using an ESR spectrometer.

Bis(3-hydroxy-4-pyronato)oxovanadium(IV) Complex (1). To a solution of 3-hydroxy-4-pyrone (7 mmol) in H_2O (10 mL) was added dropwise $VOSO_4 \cdot 2.8H_2O$ (3.5 mmol) in H_2O (5 mL). The pH of the reaction mixture was adjusted to 8.5 with 10 M KOH, and the reaction mixture was refluxed for 12 h. After the mixture was cooled to room temperature, the precipitate was filtered off, and the filtrate was concentrated to dryness. The crude product was purified twice by gel chromatography on Sephadex G-10 with water as an eluant to give the vanadyl complex as pale green solids. Yield: 49% based on V. ESR (H_2O): $g_0 = 1.968$, $g_\perp = 1.982$, $g_\parallel = 1.942$, $A_0 = 95 \times 10^{-4}$, $A_\perp = 56 \times 10^{-4}$, $A_\parallel = 172 \times 10^{-4}$ cm^{-1}. IR (cm^{-1}, KBr disk): 1600, 1560, 1465 ($\nu_{C=O}$, $\nu_{C=C}$), 959 cm^{-1} ($\nu_{V=O}$). UV/vis (H_2O): $\lambda_{max} = 270$ ($\epsilon = 9077$ M^{-1} cm^{-1}) and 820 (25) nm. HRMS (*m*/*z*): $[M]^+$ calcd for $C_{10}H_6O_7V$, 289.9631; found, 289.9642. Analysis (calcd, found for $C_{10}H_6O_7V$): C (39.09, 38.78), H (2.61, 2.54).

Bis(maltolato)oxovanadium(IV) Complex (2). This complex was made for the preparation according to a previously reported method.[15] Yield: 74% based on V. ESR (H_2O): $g_0 = 1.969$, $g_\perp = 1.982$, $g_\parallel = 1.942$, $A_0 = 94 \times 10^{-4}$, $A_\perp = 56 \times 10^{-4}$, $A_\parallel = 171 \times 10^{-4}$ cm^{-1}. IR (cm^{-1}, KBr disk): 1610, 1550, 1460 ($\nu_{C=O}$, $\nu_{C=C}$), 993 ($\nu_{V=O}$). UV/vis (H_2O): $\lambda_{max} = 275$ nm ($\epsilon = 12000$ M^{-1} cm^{-1}), 327 (7600), 633 (19), and 873 (27). HRMS (*m*/*z*): $[M]^+$ calcd for $C_{12}H_{10}O_7V$, 317.9944; found, 317.9950. Analysis (calcd, found for $C_{12}H_{10}O_7V$): C (45.45, 45.17), H (3.18, 3.31).

Bis(allxinato)oxovanadium(IV) Complex (3). $VOSO_4 \cdot 2.8H_2O$ (1 mmol) dissolved in 10 mL of H_2O was added slowly to a suspension of allixin (2 mmol) in 20 mL of H_2O. The pH of the mixture was adjusted to 8.0 with 2 M KOH, and the mixture was heated at 80 °C for 10 h. After the mixture was cooled at room temperature, a black solid precipitated and was collected. The solid was washed with water several times and dried overnight in vacuo. Yield: 67% based on V. ESR (DMSO): $g_0 = 1.969$, $g_\perp = 1.981$, $g_\parallel = 1.944$, $A_0 = 97 \times 10^{-4}$, $A_\perp = 57 \times 10^{-4}$, $A_\parallel = 176 \times 10^{-4}$ cm^{-1}. IR (cm^{-1}, KBr disk): 1610, 1550, 1430 ($\nu_{C=O}$, $\nu_{C=C}$), 997 cm^{-1} ($\nu_{V=O}$). UV/vis (DMSO): λ_{max} 277 nm ($\epsilon = 13600$ M^{-1} cm^{-1}), 327 (5300), and 819 (27) nm. HRMS (*m*/*z*): $[M]^+$ calcd for $C_{24}H_{34}O_9V$, 517.1643; found, 517.1635. Analysis (calcd, found for $C_{24}H_{34}O_9V$): C (55.71, 55.49), H (6.62, 6.47).

Bis(ethylmaltolato)oxovanadium(IV) Complex (4). This complex was made for the preparation by an analogous method for the complex **2**.[15] Yield: 80% based on V. ESR (DMSO): $g_0 = 1.970$, $g_\perp = 1.983$, $g_\parallel = 1.943$, $A_0 = 95 \times 10^{-4}$, $A_\perp = 56 \times 10^{-4}$, $A_\parallel = 174 \times 10^{-4}$ cm^{-1}. IR (cm^{-1}, KBr disk): 1600, 1550, 1470 ($\nu_{C=O}$, $\nu_{C=C}$), 992 cm^{-1} ($\nu_{V=O}$). UV/vis (H_2O): $\lambda_{max} = 277$ ($\epsilon = 13000$ M^{-1} cm^{-1}), 329 (9000), 631 (23), and 873 (32) nm. HRMS (*m*/*z*): $[M]^+$ calcd for $C_{14}H_{14}O_7V$, 346.0257; found, 346.0251. Analysis (calcd, found for $C_{14}H_{14}O_7V$): C (48.71, 49.01), H (4.09, 4.27).

Bis(kojato)oxovanadium(IV) Complex (5). $VOSO_4 \cdot 2.8H_2O$ (3 mmol) dissolved in 10 mL of water was added to a suspension of kojic acid (6 mmol) in 20 mL of water. The pH of the mixture was adjusted to 6.0 with 2 M KOH, and the mixture was stirred at room temperature for 30 min under nitrogen gas. The deep green solution was concentrated and cooled. A formed green precipitate was collected, washed with small amount of water, and dried overnight in vacuo. Yield: 56% based on V. ESR (H_2O): $g_0 = 1.972$, $g_\perp = 1.987$, $g_\parallel = 1.940$, $A_0 = 94 \times 10^{-4}$, $A_\perp = 54 \times 10^{-4}$, $A_\parallel = 175 \times 10^{-4}$ cm^{-1}. IR (cm^{-1}, KBr disk): 1620, 1560, 1470 ($\nu_{C=O}$, $\nu_{C=C}$), 950 cm^{-1} ($\nu_{V=O}$). UV/vis (H_2O): $\lambda_{max} = 262$ ($\epsilon = 14453$ M^{-1} cm^{-1}), 627 (14), and 857 (32) nm. HRMS (*m*/*z*): $[M]^+$ calcd for

$C_{12}H_{10}O_9V$, 349.9843; found, 349.9837. Analysis (calcd, found for $C_{12}H_{10}O_9V \cdot 1.3H_2O$): C (38.69, 38.60), H (3.41, 3.36).

In Vitro Insulin-Mimetic Activity. The in vitro insulin-mimetic activity of vanadyl complex was determined by both FFA-release inhibitory (FFA-releasing assay) and glucose-uptake enhancing activities (glucose-uptake assay) in isolated rat adipoctyes treated with epinephrine.[16] Male Wistar rats (weighing 200−250 g) under ether anaesthesia were sacrificed, and the adipose tissues were excised, cut with scissors, and digested with collagenase in Krebs−Ringer bicarbonate (KRB) buffer (pH 7.4) containing 2% BSA, with gentle shaking at 100 cycles/min for 1 h at 37 °C. At the end of the incubation period, the prepared cells were filtered by passing through a sterilized cotton gauze and washed three times with the KRB buffer. The cells ($1.5-2.0 \times 10^6$ cells/mL) were incubated at 37 °C for 30 min with vanadyl complexes at various concentrations in KRB buffer containing 2% BSA, 2% DMSO, and 500 μM ascorbic acid. A 10 μM dose of epinephrine was then added to the reaction mixtures, and the resulting solutions were incubated at 37 °C for 3 h. The mixtures were centrifuged at 3000 rpm for 10 min at 4 °C. With respect to the outer solution of the cells, FFA and glucose levels were determined by using an FFA kit (NEFA C-test Wako; Wako Pure Chemicals, Osaka, Japan) and an automatic glucose analyzer (Fuji DryChem; Fuji Medical Co., Tokyo, Japan), respectively. The glucose-uptake levels were evaluated according to the decrease in glucose concentration in the medium. The inhibitory activity of FFA release was evaluated with respect to the apparent IC_{50} value, i.e., the 50% inhibitory concentration of the vanadyl complex on the release of FFA from isolated rat adipocytes treated with epinephrine during a 3 h incubation period. The enhancement of glucose uptake by the vanadyl complex was evaluated using the EC_{50} value, i.e., the 50% enhancing concentration of the vanadyl complex on the maximal glucose-uptake level in glucose uptake during a 3 h incubation.

In Vivo Studies. Preparation of Streptozotocin (STZ)-Induced Type 1 Diabetic Mice. STZ was dissolved in 0.1 M cold sodium citrate buffer, pH 5, and was used within 2 min after preparation. Male ddY mice (7 weeks) fasted for 12 h received i.p. injections of STZ (100 mg/kg body weight) twice at intervals of a week.

Hypoglycemic Activity of Vanadyl Complexes by Single Administration in Diabetic Mice (Acute Study). The STZ mice (12 weeks old) were given vanadyl complexes in 1% carboxy methyl cellulose (CMC) by single i.p. injection or oral administration (1:00 p.m.). Blood samples for analysis of blood glucose level were obtained from the tail vein of the mice, and blood glucose levels were measured by using the glucose oxidase method (Glucocard; Arkray, Kyoto, Japan). Mice were allowed free access to both food and water throughout the course of study.

Effects of Vanadyl Complexes by Chronic Administrations in Diabetic Mice (Chronic Study). STZ mice with type 1 DM received daily i.p. injections of vanadyl complexes for 14 days, and the blood glucose levels were monitored daily at 2:00 p.m. Blood samples used for the analysis of blood glucose levels were obtained from the tail vein of STZ mice, the blood glucose levels were measured by Glucocard. As regards i.p. injections of vanadyl complexes suspended in 1% CMC, the doses were 5 mg (98 μmol) V/kg body weight at the first day and gradually decreased to 0.1 mg (2 μmol) V/kg body weight following 13 days (Figure 5). HbA_{1c} level in the blood obtained from the tail vein of the mice after the treatment by vanadyl complexes was determined by using a DCA 2000 system (Bayer Medical Co., Tokyo, Japan). After treatment of the vanadyl complexes for 14 days, oral glucose tolerance test (OGTT) was performed. The STZ mice were fasted for 12 h, and glucose solution at a dose 1 g/kg body weight was given orally. Blood samples were obtained from the tail vein at 0, 30, 45, 60, 90, and 120 min after the load of glucose. Following the OGTT test, the STZ mice were sacrificed under anesthesia with ether, and organs such as the liver, muscle (right femur), bone, kidney, spleen, and pancreas were immediately removed. Vanadium was determined by NAA, which is the most reliable for vanadium determination among many methods, at the Research Reactor Institute of Kyoto University using the peak area of 1434.1 keV based on the $^{51}V(n,\gamma)^{52}V$ reaction (half-life of ^{52}V, 3.75 min).[18]

Statistical Analysis. All experimental results are presented as the mean value ± standard deviation. Statistical analysis was performed by analysis of variance (ANOVA) at a 1% or 5% significance level of the difference.

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Supporting Information Available: Results of OGTT tests and organ distributions of V in STZ mice after treatments of vanadyl complexes by i.p. injections for 14 days, analytical and spectroscopic data, and elemental analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

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