Oligopyridine ligands derived from amino acid precursors: Their Zn^{2+} complexation and effects on hepatic stellate cell functions†

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A series of oligopyridine ligands were derived from amino acid amides in which amide oxygen and ternary nitrogen atoms were combined with pyridine moieties. ^{1}H NMR and circular dichroism spectroscopic characterizations revealed that they formed stable Zn^{2+} complexes in neutral aqueous solutions and caused Zn^{2+} deficiency in the hepatic stellate cell systems. Since collagen synthesis was effectively promoted in the cells, the present oligopyridine derivatives worked as biocompatible ligands for Zn^{2+} complexation and cell activation.

Inroduction

Zn^{2+} ion is the second most abundant d-block metal cation in humans, and often plays important roles in DNA synthesis, apoptosis, gene expression, neurotransmission, signal transduction and other cell events.[1] Although several types of ligand have been designed to monitor the Zn^{2+} cation and to affect cell functions,[2] oligopyridine derivatives are promising candidates as biocompatible Zn^{2+} ligands.[3] Lippard *et al.* prepared fluorescent derivatives from tetrakis(2-pyridylmethyl)ethylenediamine **1** (see Fig. 1) as cell-permeable ligands for *in vivo* Zn^{2+} determination. Nagano *et al*., Smith *et al*., Hamachi *et al*. and others linked dipicolyamine **2**–Zn^{2+} complexes with dyes, cholesterols and proteins.[3] We have reported that ligand **1** and diethylenetriamine pentaacetic acid caused Zn^{2+} deficiency in hepatic stellate cell systems.[4] Since the Zn^{2+} cation is significantly involved in collagen synthesis and other hepatotoxic metabolic events, its abnormal metabolism and depletion caused liver fibrosis.

We describe here that a new series of mixed-donor type oligopyridines **3a–3c** form stable Zn^{2+} complexes in neutral aqueous solutions and promote collagen synthesis in hepatic stellate cells. Ligands **4a** and **4b** are compared, because they have two pyridine rings in addition to one amide oxygen and one tertiary nitrogen atom. Both types of ligand are derived from amino acid precursors, and have multidentate coordination modes and tunable lipophilic properties. NMR and circular dichroism (CD) characterizations revealed that ligands **3a–3c** and **4a** formed stable Zn^{2+} complexes in neutral aqueous solutions, though their complexation profiles significantly depended on the nature of the ligand substituents. Since they exhibited no cytotoxicity and promoted collagen synthesis in the hepatic stellate cells, the present oligopyridines worked as biocompatible ligands for Zn^{2+} complexation and cell activation.

Results and discussion

1. Ligand synthesis and characterization

Amino acids are useful building blocks for the synthesis of multidentate ligands. Alsfasser *et al.* prepared a series of 2,2′-bispicolylamino acid esters.[5] Ligand **5** was typically demonstrated to form a trigonal-bipyramidal Zn^{2+} complex, in which two pyridine, one tertiary nitrogen and one amide oxygen atom were significantly involved.[6] Although its solution structure and bioactivity were not detailed, hard amide and soft pyridine donor moieties were nicely arranged to offer stable Zn^{2+} complexes. We attached four pyridine rings to the amino acid amide skeletons of glycine, alanine and methionine (see **3a–3c** in Fig. 1). Their Zn^{2+} complexation properties were characterized in neutral aqueous solutions, and biological activities toward hepatic stellate cells were further investigated. Ligands **4a** and **4b** were synthesized for comparison. Since they have different arrangements of pyridine rings, one amide oxygen and one tertiary nitrogen atom, they provide markedly different coordination modes for the Zn^{2+} cation.

The synthetic routes of the employed ligands were straightforward as reported for related ligands (Fig. 2).[7] Ligands **3a–3c** were derived from the corresponding glycine, L-alanine and L-methionine amides. Boc-amino acids were first reacted with dipicolylamine to give amide precursors. After deprotection, the treatment with chloromethylpyridine yielded ligands **3a–3c**. Reference ligands **4a** and **4b** were prepared from *N*,*N*-diethyl glycine amide and *N*,*N*-dimethylglycine. The ligands obtained were fully characterized by ^{1}H and ^{13}C NMR, IR and HRMS methods. The log *D* (distribution coefficient) value of each ligand was calculated in an *n*-octanol–water system at pH = 6.3 as an indication of ligand hydrophobicity. The PALLAS program (Windows version 3.0, CompuDrug International Inc., Sedona,

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† Electronic supplementary information (ESI) available: ^{1}H NMR spectral changes of **3a** upon $Zn(ClO_4)_2$ complexation in aqueous solution, ESI-MS spectrum of an equimolar mixture of **3b** and $Zn(ClO_4)_2$ in H_2O, CD spectral changes and titration curves for log *K*′ determination of **2**, **3a**, **3c** and **4a**, ^{1}H and ^{13}C NMR spectra of **3a–3c** and **4b**. See DOI: 10.1039/b806548a

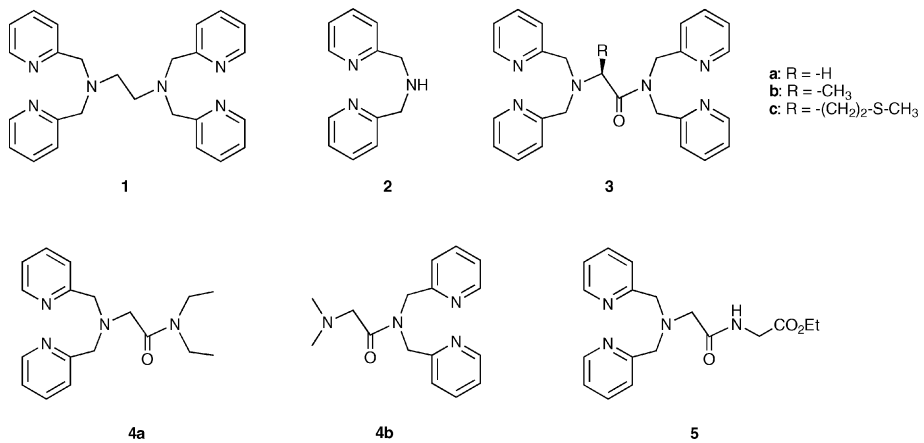

Fig. 1 Structures of ligands **1**, **2**, **3a–3c**, **4a**, **4b** and **5**.

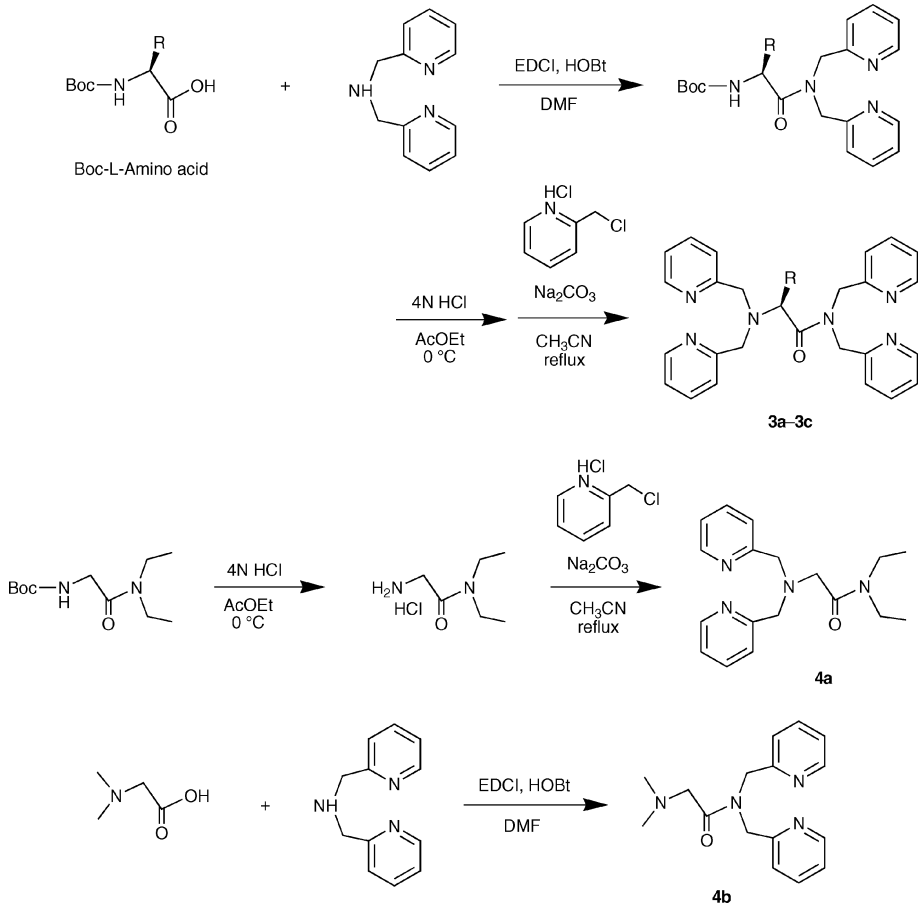

Fig. 2 Synthesis of ligands **3a–3c**, **4a** and **4b**. Boc = *N*-*tert*-butoxycarbonyl, HOBt = 1-hydroxybenzotriazole, EDCI = 1-[3-(dimethylamino)-propyl]-3-ethyl carbodiimide.

USA) was employed. Ligands **3a–3c** exhibited comparable log *D* values to that of ligand **1**, while ligand **4b** had a lower value: log *D* = 1.91 for **1**; 1.26 for **3a**; 1.80 for **3b**; 2.14 for **3c**; 1.18 for **4a**; and 0.03 for **4b**. Table 1 summarizes some spectroscopic characteristics of the ligands. Ligand **4a** exhibited an NMR signal for C=O carbon at higher field (δ = 169.3 ppm) than ligands **3a** (δ = 171.5 ppm) and **4b** (δ = 171.0 ppm). A similar trend was observed in their IR characteristic C=O bands.[8] Thus, the amide oxygen atom of ligand **4a** was confirmed to provide stronger coordination with the Zn^{2+} cation than those of ligands **3a** and **4b**.

2. Zn^{2+} Complexation profiles in aqueous solution

When ligand **3a** formed a $Zn(ClO_4)_2$ complex in D_2O, several ^1H NMR signals appeared separately at $[Zn^{2+}]/[\mathbf{3a}] = 0.5$, which were assigned to the free and 1 : 1 complex forms (Fig. S1 in ESI†). In

Table 1 Characteristics of ligands and conditional formation constants of their Zn^{2+} complexes in neutral aqueous solutions (pH = 6.3)

Ligand	δ_C(C=O) in $CDCl_3$ (ppm)	ν_{max}(C=O) in KBr/cm^{-1}	Conditional formation constant (log K')
1			14.6 ± 0.1[a]
2			6.6 ± 0.2
3a	171.5	1651	7.2 ± 0.2
3b	173.6	1648	6.1 ± 0.2
3c	172.3	1648	5.4 ± 0.1
4a	169.3	1643	8.3 ± 0.2
4b	171.0	1651	[b]

[a] Calculated from protonation and formation constants reported in ref. 9 and 10. [b] Formation constants of 1 : 1 and 1 : 2 complexes were too small to be determined.

contrast, only one set of NMR peaks remained at $[Zn^{2+}]/[\mathbf{3a}] = 1.0$ and 2.0, indicating that ligand **3a** formed a 1 : 1 Zn^{2+} complex. ^{1}H NMR and ESI-MS characterizations revealed that ligands **3b**, **3c** and **4a** also formed 1 : 1 complexes with $Zn(ClO_4)_2$. Ligand **3b** typically exhibited ESI-MS signals due to 1 : 1 complexation: $[\mathbf{3b} + Zn^{2+} + ClO_4^-]^+$ and $[\mathbf{3b} + Zn^{2+}]^{2+}$ were detected at m/z 615.3 and 258.2 (Fig. S2 in ESI†). Ligand **4b** rarely formed Zn^{2+} complexes. A mixture of ligand **4b** and $Zn(ClO_4)_2$ gave somewhat different ^{1}H NMR spectral changes. Further addition of equimolar amounts of ligand **4a** to the mixture gave only signals due to the **4a**–Zn^{2+} complex. Since no ESI-MS signal for **4b**–Zn^{2+} complex species was observed, it is assumed that the two pyridine units attached to the amide nitrogen atom rarely coordinate the Zn^{2+} cation.

The complexation between chiral ligand **3b** and $Zn(ClO_4)_2$ was quantitatively characterized in neutral aqueous solution by monitoring CD spectral changes (Fig. 3). When the CD intensity at 262 nm was plotted against a function of the added amount of $Zn(ClO_4)_2$, the obtained titration curve gave a good fit for 1 : 1 complexation between the ligand and $Zn(ClO_4)_2$. To characterize Zn^{2+} complexation properties in neutral aqueous solution, the conditional formation constant K' was calculated using the curve-fitting method: log $K' = 6.1$ at pH = 6.3. log K' of ligand **3c** was similarly estimated as 5.4 (Fig. S3 in ESI†). Since achiral ligand **2** was also confirmed to form 1 : 1 complexes with $Zn(ClO_4)_2$ by NMR experiments, its K' value was determined by competitive binding experiments with chiral ligand **3b** (Fig. S4 in ESI†). When achiral ligand **2** was added to an equimolar solution of chiral **3b** and $Zn(ClO_4)_2$, the CD signal intensity of the **3b**–Zn^{2+} complex around 262 nm decreased. Since both ligands practically

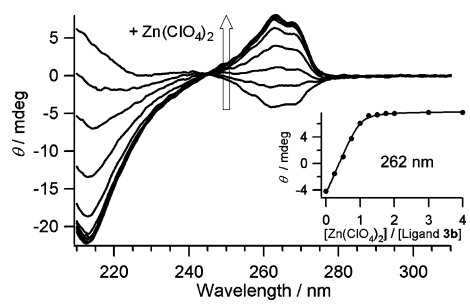

Fig. 3 CD spectral changes and titration curve for the log K' determination of the **3b**–$Zn(ClO_4)_2$ complex in aqueous solutions. [**3b**] = 3.0 × 10^{-5} mol L^{-1}.

exist in their monoprotonated forms at pH = 6.3,[9] the following competitive displacement took place:

[**3b**–Zn^{2+}] (*CD active*) + **2**–H^+ ⇆ **3b**–H^+ + [**2**–Zn^{2+}] (*CD inactive*)

The concentration of the **2**–Zn^{2+} complex was evaluated by monitoring the CD signal intensity at 262 nm, and the log K' value for the **2**–Zn^{2+} complex was estimated as 6.6.[10] Other combinations of chiral and achiral ligands were examined and the determined log K' values are listed in Table 1. When ligands **3a–3c** were compared, their conditional formation constants with $Zn(ClO_4)_2$ clearly depended on the bulkiness of the ligand substituents: log $K' = 7.2$ for **3a** (R = –H), > 6.1 for **3b** (R = $-CH_3$), > 5.4 for **3c** (R = $-CH_2CH_2SCH_3$). Ligand **4a** exhibited a much larger log K' value, indicating that its two pyridine moieties effectively coordinated the Zn^{2+} ion. In contrast, the log K' value of **4b** was too small to be spectroscopically determined. Since ligand **1** formed a stable Zn^{2+} complex, the two pyridine moieties on the amide sides of ligands **3a–3c** and **4b** were thought to inhibit coordination. Interestingly, the CD spectrum of an equimolar mixture of ligand **3b** and $Zn(ClO_4)_2$ was only slightly changed (< 10%) by the addition of a large excess (2000 equivalents) of NaCl. This is a common salt in biological cell systems, but has hardly perturbed the structure of the **3b**–Zn^{2+} complex.

Alsfasser *et al.* isolated the trigonal-bipyramidal [**5**–ZnCl]$^+$ complex.[6] Each ligand exhibited a similar log K' value with $ZnCl_2$ to that with $Zn(ClO_4)_2$. For ligand **3b**, log $K' = 6.1 \pm 0.2$ for $Zn(ClO_4)_2$ and 6.0 ± 0.4 for $ZnCl_2$; for ligand **4a**, log $K' = 8.3 \pm 0.2$ for $Zn(ClO_4)_2$ and 8.4 ± 0.4 for $ZnCl_2$. Since these ligands exhibited similar ^{1}H NMR spectra upon complexation with $Zn(ClO_4)_2$ and $ZnCl_2$, they were proposed to form complexes of the type [ligand–Zn–H_2O]$^{2+}$. Based on this assumption, their Zn^{2+} complex structures were optimized using the DFT B3LYP/6-31G* method.[11] The crystal structure of the [**5**–ZnCl]$^+$ complex[6] was used in the construction of the initial geometry with CAChe PM3 calculations (Version 3.2, Oxford Molecular Ltd.), in which the Zn^{2+} cation was coordinated by a tetradentate ligand and one H_2O molecule.[12,13] Fig. 4a typically illustrates the optimized structure of the [**3b**–Zn–H_2O]$^{2+}$ complex, and ligands **3a** and **3c** gave similar complex structures. In the penta-coordinated complex, three nitrogen atoms of the dipicolylamine unit and the amide oxygen atom are coordinated, while the two pyridine units connected to the amide nitrogen atom were not involved in coordinating the Zn^{2+} center. As shown in Fig. 4b, five-membered ring chelation was suggested to stabilize the Zn^{2+} complex and lock the rotation around the C–N bond indicated. Since ligand **3c** derived from a methionine precursor exhibited a smaller log K' value than ligands **3a** and **3b** from glycine and alanine ones, its bulky subustituent $-(CH_2)_2-S-CH_3$ was proposed to cause the large steric repulsion with one of the $-CH_2Py$ groups of the dipicolylamine part.

3. Zn^{2+} Deficiency in hepatic stellate cells

When Zn^{2+} cation homeostasis is not maintained, a broad range of defects occur in living cells. We have previously reported that ligand **1** caused Zn^{2+} deficiency in the hepatic stellate cells and induced several cell events followed by collagen synthesis.[4] Although ligands **3a–3c** have different complex structures from ligand **1**, their cytotoxic and cell activation abilities toward the hepatic stellate cells were characterized.

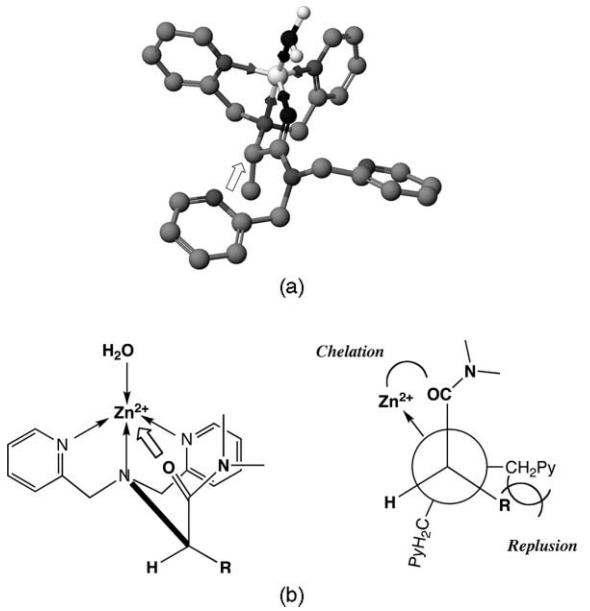

Fig. 4 (a) Optimized structure of the [**3b**–Zn–H_2O]$^{2+}$ complex and (b) schematic illustration of its geometry.

The hepatic stellate cells were isolated from the liver of male Wistar rats, incubated and their purity confirmed to be higher than 95% (see Experimetnal section). They were incubated in a culture solution containing 1.6×10^{-5} mol L^{-1} of Zn^{2+} and other essential ingredients.[14] After incubation with the ligand, the expression of type I collagen was detected using immunohistochemical staining (Fig. 5). When 1.0×10^{-5} mol L^{-1} of ligand **3a**, **3b** or **3c** was added, no ligand exhibited cytotoxicity in the hepatic stellate cell system. Rather strong immunoreactivity was observed in filaments in each ligand system, though the control cells were almost negative. When 1.0×10^{-5} mol L^{-1} of $ZnSO_4$ was supplemented after treatment with the ligand, the staining was comparable to that at control level. Thus, ligands **3a–3c** induced progression of collagen synthesis in a similar fashion to ligand **1**.[4] Based on the log K' values determined above, the free Zn^{2+} concentration in the culture was roughly estimated to be lower than 1.6×10^{-5} mol L^{-1}: 6.0×10^{-6} mol L^{-1} for ligand **1**; 6.1×10^{-6} mol L^{-1} for ligand **3a**; 7.0×10^{-6} mol L^{-1} for ligand **3b**; and 9.1×10^{-6} mol L^{-1} for ligand **3c**. Although the employed ligands had various log D values, they had high enough activities to cause Zn^{2+} deficiency in the hepatic stellate cells. Further biological analysis is required, but previous biochemical studies supported the fact that the depletion of intracellular glutathione levels triggered the progression of collagen synthesis in Zn^{2+} deficient cells.[4]

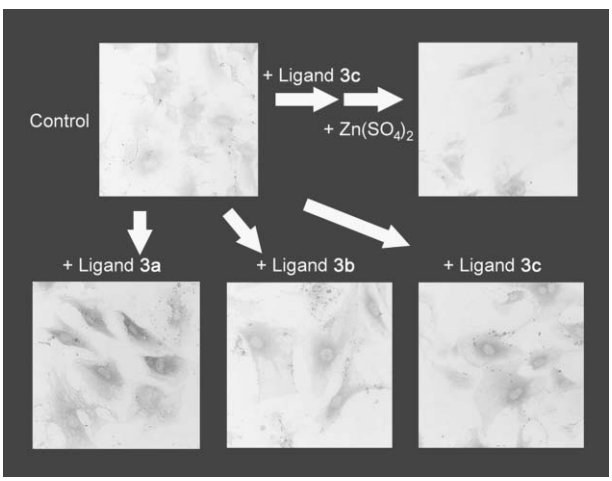

Fig. 5 Collagen synthesis of hepatic stellate cells upon addition of ligand **3a, 3b**, or **3c**. The produced collagen was stained.

Experimental section

General

^{1}H and ^{13}C NMR spectra were recorded on JEOL LA-300 and 400 spectrometers. CD spectra were obtained on a JASCO J-820 spectrometer and UV-visible spectra were recorded with a Hitachi U-3500 apparatus. ESI-MS spectra were recorded with a JEOL JMS-700 instrument. TLC plates were purchased from Merck KGaA (neutral alumina, No. 5550).

Ligand synthesis

Ligands **1** and **2** were commercially available, while ligands **3a–3c** and **4b** were synthesized in a similar fashion to ligand **4a** as reported earlier.[7] Their synthetic routes are summarized in Fig. 2 and selected specroscopic data are described below.

***N*,*N*,*N*²,*N*²-Tetrakis(pyridin-2-ylmethyl)glycinamide (3a).** Yellow oil (15%); R_f = 0.22 (EtOAc), ν_{max}(neat)/cm^{-1} 1651 (C=O) and 1593, 1571, 1476, 1436 and 764 (py); δ_H(300 MHz; $CDCl_3$; Me_4Si) 3.62 (2 H, s, CH_2), 3.95 (4 H, s, CH_2N_{amine}), 4.73 (2 H, s, CH_2N_{amide}), 4.78 (2 H, s, CH_2N_{amide}), 7.00 (1 H, d, J 7.8, py), 7.08–7.12 (4 H, m, py), 7.28 (1 H, d, J 7.8, py), 7.50–7.61 (6 H, m, py) and 8.42–8.46 (4 H, m, py); δ_C(75 MHz; $CDCl_3$; $CDCl_3$) 51.4, 52.6, 56.3, 60.5, 120.8, 122.1, 122.3, 122.3, 122.6, 123.8, 136.5, 136.7, 136.8, 149.1, 149.3, 149.8, 156.9, 157.5, 159.1 and 171.5; MS(EI) m/z 438.2170 (M^+. $C_{26}H_{26}ON_6$ requires 438.2168).

***N*,*N*,*N*²,*N*²-Tetrakis(pyridin-2-ylmethyl)-L-alaninamide (3b).** Colorless oil (8%); R_f = 0.30 (EtOAc), ν_{max}(neat)/cm^{-1} 1648 (C=O) and 1591, 1570, 1475, 1433 and 764 (py); δ_H(300 MHz; $CDCl_3$; Me_4Si) 1.40 (3 H, d, J 6.8, CH_3), 3.85 (2 H, d, J 14, CH_2N_{amine}), 4.05 (3 H, d, J 14, CH_2N_{amine} + CH), 4.13 (1 H, d, J 14, CH_2N_{amide}), 4.41 (1 H, d, J 17, CH_2N_{amide}), 5.23 (1 H, d, J 14, CH_2N_{amide}), 5.34 (1 H, d, J 17, CH_2N_{amide}), 6.95 (1 H, d, J 9.0, py), 7.05–7.13 (4 H, m, py), 7.24–7.40 (4 H, m, py), 7.50–7.55 (3 H, m, py), 8.39–8.46 (4 H, m, py); δ_C(100 MHz; $CDCl_3$; $CDCl_3$) 9.4, 51.4, 52.3, 55.4, 56.9, 120.5, 121.9, 122.0, 122.2, 122.5, 123.5, 136.4, 136.6, 136.7, 149.0, 149.1, 149.6, 157.3, 157.6, 159.4 and 173.6; MS(FAB) m/z 453.2417 (M + H^+. $C_{27}H_{29}ON_6$ requires 453.2403).

***N*,*N*,*N*²,*N*²-Tetrakis(pyridin-2-ylmethyl)-L-methioninamide (3c).** Yellow oil (25%); R_f = 0.30 (EtOAc–MeOH = 50 : 1), ν_{max}(neat)/cm^{-1} 1638 (C=O) and 1591, 1474, 1433 and 764 (py); δ_H(400 MHz; $CDCl_3$; Me_4Si) 2.04–2.14 (1 H, m, CH_2), 2.09 (3 H, s, SCH_3), 2.37–2.43 (2 H, m, CH_2), 2.59–2.66 (1 H, m, CH_2), 3.85 (2 H, d, J 14, CH_2N_{amine}), 3.93 (1 H, m, CH), 4.03 (2 H, d, J 14, CH_2N_{amine}), 4.10 (1 H, d, J 15, CH_2N_{amide}), 4.39 (1 H, d, J 17, CH_2N_{amide}), 5.26 (1 H, d, J 18, CH_2N_{amide}), 5.27 (1 H, d, J 15, CH_2N_{amide}), 6.96–7.30 (8 H, m, py), 7.37–7.58 (4 H, m, py) and 8.38–8.46 (4 H, m, py); δ_C(75 MHz; $CDCl_3$; $CDCl_3$) 15.5, 23.8,

31.4, 51.6, 52.6, 57.2, 59.2, 121.0, 122.0, 122.2, 122.3, 122.6, 123.5, 136.4, 136.5, 136.7, 149.0, 149.1, 149.5, 156.0, 157.6, 159.4 and 172.3; MS(FAB) *m/z* 513.2436 (M + H$^+$. $C_{29}H_{33}ON_6S$ requires 513.2437).

***N*^{2},*N*^{2}-Dimethyl-*N*,*N*-bis(pyridin-2-ylmethyl)glycinamide (4b).** Colorless oil (49%); R_f = 0.17 (EtOAc), ν_{max}(neat)/cm^{-1} 1651 (C=O) and 1592, 1571, 1475, 1436 and 757 (py); δ_H(400 MHz; $CDCl_3$; Me_4Si) 2.32 (6 H, s, CH_3), 3.30 (2 H, s, CH_2), 4.75 (2 H, s, CH_2N_{amine}), 4.87 (2 H, s, CH_2N_{amine}), 7.14–7.20 (3 H, m, py), 7.31 (1 H, d, *J* 7.8, py), 7.61–7.67 (2 H, m, py), 8.49 (1 H, d, *J* 4.5, py) and 8.56 (1 H, d, *J* 4.5, py). δ_C(100 MHz; $CDCl_3$; $CDCl_3$) 45.7, 51.3, 52.7, 62.2, 121.1, 122.4, 122.5, 122.5, 136.8, 136.8, 149.2, 149.9, 157.1, 157.5 and 171.0; MS(FAB) *m/z* 285.1705 (M + H$^+$. $C_{16}H_{21}ON_4$ requires 285.1715).

Determination of conditional formation constants

CD spectra were recorded with non-buffered aqueous solutions containing ligand **3b** and $Zn(ClO_4)_2$ after stirring for 2 h at room temperature. Since the observed UV spectral changes were small, the CD intensity at 262 nm was plotted against the mole ratio of $Zn(ClO_4)_2$ to ligand **3b**, and the log *K*′ value for 1 : 1 complexation was calculated. Similar procedures were done to determine the log *K*′ value for ligand **3c**. Two independent experiments were conducted for this combination and the calculations were carried out using IGOR Pro (version 4, WaveMetrics Inc.).

The competitive method was applied to determine the conditional formation constants (*K*′ values) of some ligand–Zn^{2+} complexes. When achiral ligand **3a** was added to the **3b**–Zn^{2+} complex solution, competitive displacement was successfully followed by monitoring the decreased CD signal around 262 nm. Since the resulting **3a**–Zn^{2+} complex was a CD inactive species, the decreased CD signal was employed in the calculation of the log *K*′ value. We usually added three different amounts of competitive ligand and averaged the three estimated log *K*′ values. Once the log *K*′ value was estimated for the achiral **3a**–Zn^{2+} complex, further combinations allowed log *K*′ estimations for Zn^{2+} complexes with ligands **2**, **4a** and **4b**. The reproducibility was confirmed to be 0.2 or better in log scale for ligands **2**, **3a**, **3b**, **3c** and **4a**.

DFT Calculations

A geometry optimization was performed for the [ligand–Zn–H_2O]$^{2+}$ complex using Gaussian 03 (D.01)[11] starting from coordinates based on the crystal structure of the [**5**–Zn–Cl]$^+$ complex previously reported.[6] After replacement of Cl^- with H_2O, the initial coordinate was obtained for each complex with the CAChe program (Version 3.2, Oxford Molecular Ltd.). The DFT calculation was performed on a singlet electronic state. The method used was B3LYP with 6-31G* as the basis set.

Cell activation experiments

Male Wistar rats, 300–350 g, were purchased from Japan SLC Inc. (Shizuoka, Japan), housed at constant temperature, and allowed free access to water and standard rat chow. Our animal experiments completely followed the guidelines of Osaka City University. Hepatic stellate cells were isolated from the liver by density gradient centrifugation in Nycodenz after digestion of the liver with pronase and collagenase, as previously described.[4] The cells were identified by the typical star-like configuration and vitamin A autofluorescence, and their purity was confirmed as higher than 95%. They were plated at 5 × 10^5 cells mL^{-1} on uncoated culture dishes in 1.5 mL of DMEM (Nissui Pharmaceutical, Tokyo, Japan)[14] containing 10% fetal bovine serum (Equitech-Bio, Inc., TX, USA) and supplemented with antibiotics (10^5 units L^{-1} of penicillin G and 500 mg L^{-1} of streptomycin) for 2 d, then cultured in fresh medium without serum and Zn^{2+} salt for 18 h. After pre-incubation, the cells were divided into three groups: (1) control; (2) ligand (Zn^{2+} deficient group); and (3) ligand + Zn^{2+} salt (Zn^{2+} supplement group). Each culture contained 1.6 × 10^{-5} mol L^{-1} $ZnSO_4$ and the essentials for cell existence. For the Zn^{2+} deficient group, 1.0 × 10^{-5} mol L^{-1} ligand was added. For the Zn^{2+} supplement group, 1.0 × 10^{-5} mol L^{-1} $ZnSO_4$ was further added to the culture 1 h after ligand addition. After incubation for 24 h, the cells were fixed with 4% paraformaldehyde fixative overnight at 4 °C. We used antitype I collagen polyclonal antibody (1 : 200 dilution) as the primary antibody. After incubation with 0.3% hydrogen peroxide to block endogenous peroxidase and subsequently with normal goat serum to inhibit non-specific reactions, samples were incubated with primary antibody for 1 h at room temperature and then with biotinylated anti-rabbit goat immunoglobulin for 30 min followed by incubation with horseradish peroxidase-labelled streptavidin-biotin complex for 30 min. For the peroxidase reaction, 3,3′-diaminobenzidine tetrahydrochloride (0.2 mg mL^{-1}) with $NiCl_2$ color modification was incubated for 5 min until the desired color intensity was obtained. The reproducibility was confirmed by two independent experiments.

Conclusions

We present a new series of mixed donor-type oligopyridines as biocompatible ligands for Zn^{2+} complexation and cell activation. Since the ligands were derived from amino acid precursors, they had versatile coordination modes and tunable lipophilic properties. NMR and CD characterization revealed that they formed stable Zn^{2+} complexes in neutral aqueous solution, although their conditional formation constants significantly depended on the ligand substituents. The present type of oligopyridines exhibited no cytotoxicity and promoted collagen synthesis in hepatic stellate cells.

Notes and references

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