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A Fluorescent Probe for the Fe³⁺ Ion Pool in Endoplasmic Reticulum in Liver Cells

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Abstract

We have presented rhodamine-derived Schiff base probes (1-6) selective of the Fe³⁺ ion in biological systems. In the presence of Fe³⁺ ions, the probes display new absorption and emission bands at 526 nm and 551 nm, respectively. Confocal microscopic analysis using the HepG2 cell line showed that probes 1– 6 provide fluorescence Off–On changes caused by cellular Fe³⁺ ions, and demonstrated a largely enhanced fluorescence upon iron overloading of the cells. We also found that the Fe³⁺ ion-mediated fluorescence changes of probes 1–6 are mainly localized in the ER (Endoplasmic Reticulum) in HepG2 cells. Among them, probe **5** exhibits the highest selectivity for ER localization over the other organelles.

Key Words

Iron detection, fluorescent probe, rhodamine, cell imaging

1. Introduction

Iron is an essential trace element in human cells. It exists as a crucial cofactor for proteins that are involved in processes such as oxygen delivery, the electron transfer chain of mitochondria, and oxidation/reduction of biological substrates [1–3]. However, unbound Fe ions are potentially toxic species due to their high propensity to induce, through the Fenton reaction, reactive oxygen species that are responsible for oxidative cell damage [4]. In fact, the release of Fe ions from iron-containing proteins

induces iron (Fe³⁺)-mediated cytotoxicity that has been strongly implicated in many neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease [5,6]. However, how the Fe ions cause the cytotoxicity is still unclear. Therefore, detecting Fe ions in live cells would be essential for understanding its toxicity and the relationship with human diseases.

Currently, several fluorescent probes are being exploited for the selective detection of Fe³⁺ ions in human cells [7–11]. These probes have provided fluorescent images for cellular Fe³⁺ ions; however, investigations at the organelle level in cells are rare. Previously, we reported a rhodamine-based probe that gives a fluorescent enhancement for cellular Fe³⁺ ions through a Fe³⁺-mediated hydrolysis of the Schiff base and the spirolactam ring opening of the rhodamine moiety, as shown in Scheme 1 [11]. Based on this, we have synthesized rhodamine-based Schiff base derivatives (**1–6**), and assessed their abilities for Fe³⁺-sensing at the organelle level. Herein, we present rhodamine-based probes that allow for Fe³⁺ imaging, mainly localized in the ER (Endoplasmic Reticulum) of HepG2 cells.



Scheme 1.

2. Materials and Methods

2.1. Materials and instrumentation

All fluorescence and UV/Vis absorption data were collected using RF-5301PC (Shimadzu Corporation, Kyoto, Kyoto Prefecture, Japan) and S-3100 (Betatek Inc., Toronto, Ontario, Canada) spectrophotometers, respectively. NMR was recorded using a Varian INOVA spectrometer (400 MHz). All reagents and cationic compounds such as chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺, Fe²⁺, and Fe³⁺ were purchased from Aldrich (St. Louis, MO, USA). CH₃CN for

spectra detection was an HPLC grade reagent without fluorescent impurity, and deionized water was used.

2.2. UV/Vis and fluorescence spectroscopic methods

Stock solutions of metal chloride salts were prepared in deionized water. Stock solutions of synthetic probes were prepared in CH_3CN . All spectra were recorded in aqueous solution ($CH_3CN:H_2O = 5:95$). Excitation was carried out at 528 nm with all excitation and emission slit widths at 3 nm.

2.3. Cell culture and confocal microscopic methods

A human hepatoma cell line, HepG2, was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, and 100 μg/mL of streptomycin at 37 °C under 95% humidified air

containing 5% CO₂. A total of 1×10^5 cells were cultured and stabilized in a confocal dish and the medium was replaced with fresh medium containing 500 µM of ferric citrate. After 24 h of incubation, the medium was removed and the cells were briefly washed with 1 mL of PBS (phosphate buffered saline). Then, 1 mL of media containing each of the probes, **1–6** (final concentration was 1 µM), was added and incubated. The images were obtained using a confocal microscope from Carl Zeiss (LSM 510 META model; Oberkochen, Germany). Probes **1–6** were excited at 488 nm using an argon laser and the emission image was acquired using a band-path filter (505–550 nm). All images were collected under the same experimental parameters to minimize possible variations in fluorescence intensity.

2.4. Synthetic methods

Compounds 2 and 7 were prepared according to a previously described procedure [11] and compounds 1

and **3–6** were newly synthesized in this study by modifying the procedure (Scheme 2a).

2.4.1. Compound 1

Rhodamine-ethylene diamine (7) (0.3 g, 0.66 mmol) was stirred with salicylaldehyde (0.08 g, 0.66 mmol) in methanol for 26 h at room temperature. The precipitates were collected from the methanol. Finally, 0.25 g of probe 1 (69% yield) was obtained as a yellow solid. ESI-MS m/z (M⁺) calcd 560.3, found 561.2. ¹H NMR (CDCl₃, 400 MHz): δ 13.00 (s, 1H); 8.01 (s, 1H); 7.95 (d, 1H, J = 6.40 Hz); 7.45 (t, 2H, J = 3.60

Hz); 7.26–7.22 (m, 1H); 7.11 (d, 1H, *J* = 7.60 Hz); 7.05 (d, 1H, *J* = 7.20 Hz); 6.89 (d, 1H, *J* = 8.40 Hz); 6.80 (t, 1H, *J* = 7.60 Hz); 6.36 (s, 2H); 6.21 (s, 2H); 3.46–3.42 (m, 4H); 3.35 (t, 2H, *J* = 7.20 Hz); 3.15– 2.8 (m, 4H); 1.84 (s, 6H), 1.32 (t, 6H, *J* = 7.20 Hz). ¹³C NMR (CDCl₃, 100 MHz): 168.5, 165.9, 161.2, 153.8, 151.9, 147.6, 132.7, 132.1, 131.3, 131.2, 128.6, 128.2, 124.0, 123.0, 118.9, 118.4, 118.1, 117.1, 106.1, 96.7, 65.1, 57.3, 41.1, 38.5, 16.8, 14.9 ppm.

2.4.2. Compound 3

Rhodamine-ethylene diamine (**7**) (0.2 g, 0.44 mmol) was stirred with 2-hydroxy-3,5-dinitrobenzaldehyde (0.09 g, 0.44 mmol) in methanol for 15 h at room temperature. The precipitates were collected from methanol. Finally, 0.24 g of **3** (83% yield) was obtained as a yellow solid. ESI-MS *m/z* (M⁺) calcd 650.2, found 651.2. ¹H NMR (CDCl₃, 400 MHz): δ 8.93 (s, 1H); 8.23 (s, 1H); 7.94–7.92 (m, 1H); 7.83 (s, 1H); 7.53–7.48 (m, 2H); 7.08–7.06 (m, 1H); 6.35 (s, 2H); 6.13 (s, 2H); 3.56–3.52 (m, 6H); 3.23–3.18 (m, 4H); 1.84 (s, 6H); 1.33 (t, 6H, *J* = 6.80 Hz). ¹³C NMR (CDCl₃, 100 MHz): 170.6, 169.1, 166.8, 153.1, 152.0, 147.9, 135.9, 133.4, 130.6, 128.7, 128.5, 128.2, 124.2, 123.3, 118.5, 116.7, 105.5, 96.7, 65.4, 50.2, 39.5, 38.5, 16.8, 14.7 ppm.

2.4.3. Compound 4

Rhodamine-ethylene diamine (7) (0.2 g, 0.44 mmol) was stirred with 3-nitrobenzaldehyde (0.07 g, 0.44

mmol) in methanol for 14 h at room temperature. The precipitates were collected from methanol. Finally, 0.20 g of probe **4** (78% yield) was obtained as a yellow solid. FAB-MS *m/z* (M⁺) calcd 589.3, found 590.2. ¹H NMR (CDCl₃, 400 MHz): δ 8.33 (s, 1H); 8.21 (m, 1H); 7.95–7.92 (m, 3H); 7.52 (t, 1H, *J* = 8.01 Hz); 7.46–7.42 (m, 2H); 7.05–7.02 (m, 1H); 6.35 (s, 2H); 6.21 (s, 2H); 3.49–3.47 (m, 6H); 3.21–3.18 (m, 4H); 1.83 (s, 6H); 1.32 (t, 6H, *J* = 7.20 Hz). ¹³C NMR (CDCl₃, 100 MHz): 168.7, 159.5, 153.9, 151.9, 148.6, 147.5, 138.0, 133.5, 132.7, 131.1, 129.5, 128.7, 128.2, 124.9, 124.0, 123.1, 123.0, 118.0, 106.3,

96.8, 65.2, 59.2, 41.2, 38.5, 16.8, 14.9 ppm.

2.4.4. Compound 5

Rhodamine-ethylene diamine (**7**) (0.16 g, 0.35 mmol) was stirred benzaldehyde (0.04 g, 0.35 mmol) in methanol for 20 h at room temperature. The precipitates were collected from methanol. Finally, 0.07 g of probe **5** (38% yield) was obtained as a pink solid. FAB-MS *m*/*z* (M⁺) calcd 544.3, found 545.3. ¹H NMR (CDCl₃, 400 MHz): δ 8.00 (s, 1H); 7.94–7.92 (m, 1H); 7.58–7.56 (m, 2H); 7.45–7.42 (m, 2H); 7.36–7.31 (m, 3H); 7.05–7.03 (m, 1H); 6.35 (s, 2H); 6.23 (s, 2H); 3.47–3.39 (m, 6H); 3.21–3.20 (m, 4H); 1.85 (s, 6H); 1.32 (t, 6H, *J* = 7.20 Hz). ¹³C NMR (CDCl₃, 400 MHz): 196.3, 168.5, 162.5, 156.4, 154.0, 151.9, 147.5, 136.3, 132.6, 131.2, 130.6, 128.7, 128.5, 128.2, 128.1, 123.9, 122.9, 118.0, 106.2, 105.2, 96.8, 77.7, 65.2, 59.1, 41.4, 38.5, 16.8, 14.9 ppm.

2.4.5. Compound 6

Rhodamine-ethylene diamine (7) (0.2 g, 0.44 mmol) was stirred 2,4-dinitrobenzaldehyde (0.09 g, 0.44 mmol) in methanol for 22 h at room temperature. The precipitates were collected from methanol. Finally, 0.08 g of probe **6** (28% yield) was obtained as an orange solid. FAB-MS m/z (M⁺) calcd 634.3, found 635.2. ¹H NMR (CDCl₃, 400 MHz): δ 8.81 (d, 1H, J = 1.60 Hz); 8.45–8.42 (m, 1H); 8.24–8.22 (d, 2H, J = 9.21 Hz); 7.93–7.91 (m, 1H); 7.45 (t, 2H, J = 3.20 Hz); 7.05–7.03 (m, 1H); 6.33 (s, 2H); 6.18 (s, 2H); 3.55 (t, 2H, J = 6.80 Hz); 3.48–3.45 (m, 4H); 3.22–3.16 (m, 4H); 1.82 (s, 6H); 1.33 (t, 6H, J = 6.80 Hz). ¹³C NMR (CDCl₃, 400 MHz): 196.3, 168.5, 162.5, 156.4, 154.0, 151.9, 147.5, 136.3, 132.6, 131.2, 130.6, 128.7, 128.5, 128.2, 128.1, 123.9, 122.9, 118.0, 106.2, 105.2, 96.8, 77.7, 65.2, 59.1, 41.4, 38.5, 16.8, 14.9 ppm.

3. Results and Discussion

3.1. Absorption and fluorescence spectra of probes 1-6 responding to Fe^{3+} ions

The synthetic routes for the iron probes 1-6 and their chemical structures are displayed in Scheme 2.

Among the probes presented here, probe **2** was previously reported by our group and probes **1** and **3–6** were newly synthesized by modifying the synthetic procedure for probe **2** [11]. All chemical structures of the probes were confirmed by ¹H NMR, ¹³C NMR, and ESI–MS (Figs. S1–S15). The probes **1** and **4** were also confirmed by X-ray crystallographic analysis as displayed in Fig. 1 (see also Tables S1–S10).





Fig. 1.

For the Fe³⁺-sensing mechanism of compound **2**, we previously demonstrated that the irreversible binding of Fe³⁺ with probe **2** resulted in the ring opening of spirolactam in the rhodamine moiety followed by the hydrolysis of the Schiff base linkage (Scheme 1) [11]. This was confirmed by ¹H-NMR and ESI-MS spectroscopy, and X-ray crystallography. Similarly, in ESI-MS analysis experiments, the mass peaks for the probes **1** and **3–6** disappeared upon the addition of Fe³⁺ ions, whereas rhodamine-ethylene diamine (**7**) was mainly detected (Figs. S16–S21). These results tell us that probes **1–6** provide a fluorescence

change due to the addition of the Fe³⁺ ions through a Fe³⁺-mediated hydrolysis of the Schiff base, which leads to spirolactam ring opening of rhodamine moiety as depicted in Scheme 1.

We tested the selectivity of the probes (**1**–**6**) for Fe³⁺ ions over other metal ions in aqueous solution. As seen in Figs. 2a and 2b, probe **5** alone displayed no distinct absorption and emission bands, whereas upon the addition of Fe³⁺ ions to a solution of probe **5**, new absorption and emission bands appeared at 526 nm and 551 nm, respectively. Upon the addition of Hg²⁺, similar fluorescence change was observed. However, in the case of other metal ions, including Na⁺, K⁺, Li⁺, Ag⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺, and Fe²⁺, the changes were relatively negligible. In addition, the absorption and emission bands at 526 nm and 551 nm were gradually enhanced with an increasing concentration of Fe³⁺ ions (Fig. 3). Similar photophysical changes were also seen in the case of the other probes (Figs. S22–S33). These results imply that the probes **1–6** are predominantly selective for Fe³⁺ over the other metal ions, including

 Fe^{2+} ions.





Fig. 3.

Fluorescence changes caused by a Fe^{3+} -induced ring opening of the rhodamine part in probes 1–6

were also monitored as a function of time. As seen in Fig. 4, upon the addition of 100 equiv. of Fe³⁺ ions

to a solution of probe 5, the emission band at 552 nm was gradually increased over the course of 60 min

(Fig. 4 inset). Similar changes were also monitored upon the addition of Fe³⁺ ions to other probes (Figs.

S35–S39).





In order to investigate the interference of other biologically active metals, the fluorescence changes of probes **1–6** were tested in the presence of various essential metal ions, including K⁺, Na⁺, Ca²⁺, Fe²⁺, Mg²⁺, and Mn²⁺. In Fig. 5, the Fe³⁺-mediated fluorescence enhancements at 552 nm were readily observed with probes **1–6** in the presence of high concentrations of other biologically relevant metal ions such as K^+ , Na⁺, Ca²⁺, Fe²⁺, Mg²⁺, and Mn²⁺ (5 mM each). The levels of enhanced fluorescence intensities were also similar to those of probes **1–6** recorded upon the addition of 50 μ M of Fe³⁺ ions. This indicates that probes **1–6** can be employed to detect Fe³⁺ in biological systems without interference from other

competitive metal ions.



3.2. Sensing abilities of probes 1-6 to the Fe^{3+} ions in live cells

The Fe³⁺-sensing abilities of probes **1–6** were tested in iron-overloaded HepG2 cells where the iron overloading was achieved by treatment with ferric citrate. It is reported that the hepatocytes are important in iron metabolism, and ferric citrate is the major iron carrier in the blood system to the liver cells in iron overload and related diseases [12]. Therefore, in the presence of ferric citrate, the fluorescent intensity of the probe in the cells was anticipated to be increased. Indeed, upon incubation with probe **5**, ferric citrate-treated cells display a stronger fluorescence than the cells in the absence of ferric citrate (Fig. 6). The fluorescence images were also monitored as a function of incubation time upon the addition of 500 μ M of ferric citrate (Fig. S40). Thus, we demonstrated that probe **5** provides a fluorescence Off–On image for the cellular Fe³⁺ ions.

Fig. 5.

Cytotoxicity tests using probes 1–6 were also carried out to determine their cell compatibilities. As

inferred from an MTT assay (Fig. S41), the probes 1-6 displayed negligible cytotoxicity for the cellular

Fe³⁺ imaging in HepG2 cells.

Concentration of ferric citrate (μM)

0
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200
500
1000

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Fig. 6.

To identify the cellular location for the fluorogenic reactions of **1–6**, co-localization experiments were performed using selective fluorescent dyes for the ER (Endoplasmic Reticulum), lysosome (Lyso), and mitochondria (Mito), in HepG2 cells. As revealed in Figs. 7 and S42–S46, the green images of the probes **1–6** are found to be more co-localized with the red image of the ER tracker than those of Mito and Lyso trackers. The colocalization analysis for each probe was performed using the Pearson coefficient (Rr value) [13, 14]. We found that the values of **1-6** for ER are 0.6718, 0.5294, 0.5425, 0.5527, 0.7854, and 0.6587, respectively (Figs. S42–S46), suggesting that the variable substituents (-H, -OH, or -NO₂) on the phenyl group of Schiff base linkage of probes **1-6** affect the ER-accumulation ability of the probe without major changes of the Fe³⁺ ion selectivity or reactivity. Interestingly, the fluorescence image of probe **5** is

highly overlapped with that of the ER. The Pearson coefficients of probe **5** for ER, Lyso, and Mito trackers were also determined to be 0.7854, 0.4709, and 0.2034, respectively (Fig. 7). From this, we could infer that probes **1–6** are mainly localized in the ER and give a fluorescence Off–On change with Fe^{3+} ions. In addition, probe **5** was highly selective for the ER-localized Fe^{3+} ions. This would be useful for the imaging of Fe^{3+} involved in the ER in iron-overloaded HepG2 cells.



Fig. 7.

4. Conclusions

In summary, we have presented rhodamine-derived Schiff base probes (1-6) that allow for Fe³⁺ imaging in HepG2 cells. Probes 1–6 alone displayed no distinct absorption and emission bands, whereas upon the addition of Fe³⁺ ions to the solutions of probes 1–6, new absorption and emission bands appeared at 526

nm and 551 nm, respectively. The Fe³⁺ ions irreversibly bind to the probes and induce the ring opening of spirolactam in the rhodamine part. However, in the case of other biologically relevant metal ions, including Na⁺, K⁺, Fe²⁺, Cu²⁺, Ca²⁺, Mg²⁺, Zn²⁺, and Mn²⁺, a negligible change was observed under similar conditions. These results indicate that probes **1–6** can be used for the detection of Fe³⁺ in biological systems without interference from other competitive metal ions. In confocal microscopic analysis using the HepG2 cell line, we demonstrated that the probes **1–6** provide a fluorescence Off–On image for cellular Fe³⁺ ions and it could be used to discriminate between normal and iron-overloaded cells. We also found that probes **1–6** give rise to the fluorescence changes, due to the Fe³⁺ ions, mainly localized in the ER in HepG2 cells. Particularly, probe **5** exhibits a highly selective fluorescent image for the ER-localized Fe³⁺ ions over other organelles. We thus believe that probe **5** would be further useful for

the imaging of Fe³⁺ involved in the ER of iron-overloaded HepG2 cells.

Acknowledgments

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Supplementary data

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Supporting Information

A Fluorescent Probe for the Fe³⁺ Ion Pool in Endoplasmic Reticulum in Liver Cells

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1. Materials and instrumentation

All fluorescence and UV/Vis absorption spectra were recorded in RF-5301PC and S-3100 spectrophotometer, respectively. NMR was recorded at Varian instrument (400 MHz). All reagents and cationic compounds such as chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺ Fe²⁺, and Fe³⁺ were purchased from Aldrich and used as received. All solvents were analytical reagents. CH₃CN for spectra detection was HPLC reagent without fluorescent impurity and water was deionized water.

2. UV/Vis and Fluorescence Spectroscopic Methods

Stock solutions of metal chloride salts were prepared in deionized water. Stock solutions of synthetic probes were prepared in CH₃CN. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95). Excitation was carried out at 528 nm with all excitation and emission slit widths 3 nm.

3. Cell Culture and Confocal Microscopic Methods

A human hepatoma cell line, HepG2, was grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 100 unit/ml penicillin and 100 µg/ml of streptomycin at 37 °C under 95 % humidified air containing 5% CO₂. The 1 x 10^5 cells were located and stabilized in a confocal dish and the medium was replaced with a new one containing 500 µM of ferric citrate. After 24 h of incubation at the incubator, the medium was removed and the cells were briefly washed with 1 mL of phosphate buffered saline (1×PBS). And 1 mL of probes **1-6** containing medium (the final concentration was 1 µM) was placed and incubated at the incubator. Then, the images were obtained using a confocal microscope from Carl Zeiss (LSM 510 META model). Probes **1-6** were excited at 488 nm using an argon laser and emission image was acquired using a band-path filter (505-550 nm). All images were taken under the same experimental parameters to minimize possible variations in fluorescence intensity.

4. Experimental Section

Compound 2 was prepared according to the literature procedure¹ and compounds 1 and 3-6 were newly synthesized in this study by modifying the procedure.

Compound 1

Rhodamine-ethylene diamine (7) (0.3 g, 0.66 mmol) was stirred with salicylaldehyde (0.08 g, 0.66 mmol) in methanol for 26 h at room temperature. The precipitates were collected from methanol, finally 0.25 g of **1** (69% yield) was obtained as a yellow solid. ESI-MS m/z (M^+) calcd 560.3, found 561.2. ¹H NMR (CDCl₃, 400 MHz): δ 13.00 (s, 1H); 8.01 (s, 1H); 7.95 (d, 1H, *J* = 6.40 Hz); 7.45 (t, 2H, *J* = 3.60 Hz); 7.26~7.22 (m, 1H); 7.11 (d, 1H, *J* = 7.60 Hz); 7.05 (d, 1H, *J* = 7.20 Hz); 6.89 (d, 1H, *J* = 8.40 Hz); 6.80 (t, 1H, *J* = 7.60 Hz); 6.36 (s, 2H); 6.21 (s, 2H); 3.46~3.42 (m, 4H); 3.35 (t, 2H, *J* = 7.20 Hz); 3.15~2.8 (m, 4H); 1.84 (s, 6H), 1.32 (t, 6H, *J* = 7.20 Hz). ¹³C NMR (CDCl₃, 100 MHz): 168.5, 165.9, 161.2, 153.8, 151.9, 147.6, 132.7, 132.1, 131.3, 131.2, 128.6, 128.2, 124.0, 123.0, 118.9, 118.4, 118.1, 117.1, 106.1, 96.7, 65.1, 57.3, 41.1, 38.5, 16.8, 14.9 ppm.



Compound 3

Rhodamine-ethylene diamine (7) (0.2 g, 0.44 mmol) was stirred with 2-hydroxy-3,5-dinitrobenzaldehyde (0.09 g, 0.44 mmol) in methanol for 15 h at room temperature. The precipitates were collected from methanol, finally 0.24 g of **3** (83% yield) was obtained as a yellow solid. ESI-MS m/z (M⁺) calcd 650.2, found 651.2. ¹H NMR (CDCl₃, 400 MHz): δ 8.93 (s, 1H); 8.23 (s, 1H); 7.94~7.92 (m, 1H); 7.83 (s, 1H); 7.53~7.48 (m, 2H); 7.08~7.06 (m, 1H); 6.35 (s, 2H); 6.13 (s, 2H); 3.56~3.52 (m, 6H); 3.23~3.18 (m, 4H); 1.84 (s, 6H); 1.33 (t, 6H, *J* = 6.80 Hz). ¹³C NMR (CDCl₃, 100 MHz): 170.6, 169.1, 166.8, 153.1, 152.0, 147.9, 135.9, 133.4, 130.6, 128.7, 128.5, 128.2, 124.2, 123.3, 118.5, 116.7, 105.5, 96.7, 65.4, 50.2, 39.5, 38.5, 16.8, 14.7 ppm.

^{1.} M. H. Lee, T. V. Giap, S. H. Kim, Y. H. Lee, C. Kang and J. S. Kim, *Chem. Commun.* 2010, 46, 1407.



Compound 4

Rhodamine-ethylene diamine (7) (0.2 g, 0.44 mmol) was stirred with 3-nitrobenzaldehyde (0.07 g, 0.44 mmol) in methanol for 14 h at room temperature. The precipitates were collected from methanol, finally 0.20 g of **4** (78% yield) was obtained as a yellow solid. FAB-MS m/z (M^+) calcd 589.3, found 590.2. ¹H NMR (CDCl₃, 400 MHz): δ 8.33 (s, 1H); 8.21 (m, 1H); 7.95~7.92 (m, 3H); 7.52 (t, 1H, *J* = 8.01 Hz); 7.46~7.42 (m, 2H); 7.05~7.02 (m, 1H); 6.35 (s, 2H); 6.21 (s, 2H); 3.49~3.47 (m, 6H); 3.21~3.18 (m, 4H); 1.83 (s, 6H); 1.32 (t, 6H, *J* = 7.20 Hz). ¹³C NMR (CDCl₃, 100 MHz): 168.7, 159.5, 153.9, 151.9, 148.6, 147.5, 138.0, 133.5, 132.7, 131.1, 129.5, 128.7, 128.2, 124.9, 124.0, 123.1, 123.0, 118.0, 106.3, 96.8, 65.2, 59.2, 41.2, 38.5, 16.8, 14.9 ppm.



Compound 5

Rhodamine-ethylene diamine (**7**) (0.16 g, 0.35 mmol) was stirred benzaldehyde (0.04 g, 0.35 mmol) in methanol for 20 h at room temperature. The precipitates were collected from methanol, finally 0.07 g of **5** (38% yield) was obtained as a pink solid. FAB-MS m/z (M⁺) calcd 544.3, found 545.3. ¹H NMR (CDCl₃, 400 MHz): δ 8.00 (s, 1H); 7.94~7.92 (m, 1H); 7.58~7.56 (m, 2H); 7.45~7.42 (m, 2H); 7.36~7.31 (m, 3H); 7.05~7.03 (m, 1H); 6.35 (s, 2H); 6.23 (s, 2H); 3.47~3.39 (m, 6H); 3.21~3.20 (m, 4H); 1.85 (s, 6H); 1.32 (t, 6H, *J* = 7.20 Hz). ¹³C NMR (CDCl₃, 400 MHz): 196.3, 168.5, 162.5, 156.4, 154.0, 151.9, 147.5, 136.3, 132.6, 131.2, 130.6, 128.7, 128.5, 128.2, 128.1, 123.9, 122.9, 118.0, 106.2, 105.2, 96.8, 77.7, 65.2, 59.1, 41.4, 38.5, 16.8, 14.9 ppm.



Compound 6

Rhodamine-ethylene diamine (7) (0.2 g, 0.44 mmol) was stirred 2,4-dinitrobenzaldehyde (0.09 g, 0.44 mmol) in methanol for 22 h at room temperature. The precipitates were collected from methanol, finally 0.08 g of **6** (28% yield) was obtained as an orange solid. FAB-MS m/z (M^+) calcd 634.3, found 635.2. ¹H NMR (CDCl₃, 400 MHz): δ 8.81 (d, 1H, *J* = 1.60 Hz); 8.45~8.42 (m, 1H); 8.24~8.22 (d, 2H, *J* = 9.21 Hz); 7.93~7.91 (m, 1H); 7.45 (t, 2H, *J* = 3.20 Hz); 7.05~7.03 (m, 1H); 6.33 (s, 2H); 6.18 (s, 2H); 3.55 (t, 2H, *J* = 6.80 Hz); 3.48~3.45 (m, 4H); 3.22~3.16 (m, 4H); 1.82 (s, 6H); 1.33 (t, 6H, *J* = 6.80 Hz). ¹³C NMR (CDCl₃, 400 MHz): 196.3, 168.5, 162.5, 156.4, 154.0, 151.9, 147.5, 136.3, 132.6, 131.2, 130.6, 128.7, 128.5, 128.2, 128.1, 123.9, 122.9, 118.0, 106.2, 105.2, 96.8, 77.7, 65.2, 59.1, 41.4, 38.5, 16.8, 14.9 ppm.









Figure S2. ¹³C-NMR spectrum of **1** in CDCl₃.

==== Shimadzu LabSolutions Data Report ====

<Spectrum>

Line#1 R.Time:0.517(Scan#:63) MassPeaks:801 RawMode:Averaged 0.367-0.767(45-93) BasePeak:561.20(1342587) BG Mode:None Segment 1 - Event 1



C:\Documents and Settings\Administrator\Desktop\data file\Hyeong Seok, kim\20150725_JMJ_6-1.lcd





Figure S4. ¹H-NMR spectrum of 3 in CDCl₃.



7/25/2015 3:23:27 PM 1/2

==== Shimadzu LabSolutions Data Report ====



C:\Documents and Settings\Administrator\Desktop\data file\Hyeong Seok, kim\20150725_LMH orange-5.lcd

Figure S6. ESI-MS spectrum of 3.





Figure S8. ¹³C-NMR spectrum of 4 in CDCl₃.

==== Shimadzu LabSolutions Data Report ====

<Spectrum>

Line#:1 R.Time:0.500(Scan#:61) MassPeaks:879 RawMode:Single 0.500(61) BasePeak:590.20(185006) BG Mode:None Segment 1 - Event 1



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Figure S10. ¹H-NMR spectrum of 5 in CDCl₃.



Figure S11. ¹³C-NMR spectrum of 5 in CDCl₃.

==== Shimadzu LabSolutions Data Report ====



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Figure S12. ESI-MS spectrum of 5.







Figure S14. ¹³C-NMR spectrum of 6 in CDCl₃.



==== Shimadzu LabSolutions Data Report ====

<Spectrum>

Line#:1 R.Time:0.550(Scan#:67) MassPeaks:852 RawMode:Single 0.550(67) BasePeak:635.20(116198) BG Mode:None Segment 1 - Event 1



C:\Documents and Settings\Administrator\Desktop\data file\Hyeong Seok, kim\20150725_JMJ_5-2.lcd



<Spectrum>

Line#:1 R.Time:0.583(Scan#:71) MassPeaks:881 RawMode:Single 0.583(71) BasePeak:520.30(54100) BG Mode:None Segment 1 - Event 1



Figure S16. ESI-MS spectrum of reaction mixture of **1** and FeCl₃ in aqueous solution. The peak at 457.2 m/z is corresponding to the rhodamine-ethylene diamine (**7**).



Figure S17. ESI-MS spectrum of reaction mixture of 2 and FeCl₃ in aqueous solution. The peak at 457.2 m/z is corresponding to the rhodamine-ethylene diamine (7).



Figure S18. ESI-MS spectrum of reaction mixture of **3** and FeCl₃ in aqueous solution. The peak at 457.2 m/z is corresponding to the rhodamine-ethylene diamine (**7**).





Figure S19. ESI-MS spectrum of reaction mixture of **4** and FeCl₃ in aqueous solution. The peak at 457.2 m/z is corresponding to the rhodamine-ethylene diamine (**7**).



Figure S20. ESI-MS spectrum of reaction mixture of **5** and FeCl₃ in aqueous solution. The peak at 457.2 m/z is corresponding to the rhodamine-ethylene diamine (**7**).


Line#.1 R.Time:0.533(Scan#:65) MassPeaks:856 RawMode:Single 0.533(65) BasePeak:316.95(2283998) BG Mode:None Segment 1 - Event 1



Figure S21. ESI-MS spectrum of reaction mixture of **6** and FeCl₃ in aqueous solution. The peak at 457.2 m/z is corresponding to the rhodamine-ethylene diamine (**7**).



Figure S22. (a) UV/Vis absorption and (b) fluorescence spectra of **1** (10.0 μ M or 1.0 μ M, respectively) upon addition of chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺ Fe²⁺, and Fe³⁺ (50 equiv., respectively). All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S23. (a) UV/Vis absorption and (b) fluorescence spectra of **2** (10.0 μ M or 1.0 μ M, respectively) upon addition of chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺ Fe²⁺, and Fe³⁺ (50 equiv., respectively). All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S24. (a) UV/Vis absorption and (b) fluorescence spectra of **3** (10.0 μ M or 1.0 μ M, respectively) upon addition of chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺ Fe²⁺, and Fe³⁺ (50 equiv., respectively). All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S25. (a) UV/Vis absorption and (b) fluorescence spectra of **4** (10.0 μ M or 1.0 μ M, respectively) upon addition of chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺ Fe²⁺, and Fe³⁺ (50 equiv., respectively). All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S26. (a) UV/Vis absorption and (b) fluorescence spectra of **5** (10.0 μ M or 1.0 μ M, respectively) upon addition of chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺ Fe²⁺, and Fe³⁺ (50 equiv., respectively). All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S27. (a) UV/Vis absorption and (b) fluorescence spectra of **6** (10.0 μ M or 1.0 μ M, respectively) upon addition of chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺ Fe²⁺, and Fe³⁺ (50 equiv., respectively). All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S28. (a) UV/Vis absorption and (b) fluorescence titration spectra of **1** (10.0 μ M or 1.0 μ M, respectively) with an increase of [FeCl₃]. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S29. (a) UV/Vis absorption and (b) fluorescence titration spectra of **1** (10.0 μ M or 1.0 μ M, respectively) with an increase of [FeCl₃]. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S30. (a) UV/Vis absorption and (b) fluorescence titration spectra of **3** (10.0 μ M or 1.0 μ M, respectively) with an increase of [FeCl₃]. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S31. (a) UV/Vis absorption and (b) fluorescence titration spectra of 4 (10.0 μ M or 1.0 μ M, respectively) with an increase of [FeCl₃]. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S32. (a) UV/Vis absorption and (b) fluorescence titration spectra of **5** (10.0 μ M or 1.0 μ M, respectively) with an increase of [FeCl₃]. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S33. (a) UV/Vis absorption and (b) fluorescence titration spectra of **6** (10.0 μ M or 1.0 μ M, respectively) with an increase of [FeCl₃]. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S34. Fluorescence enhancements of probes **1-6** (1.0 μ M, respectively) in the presence of Fe³⁺ (5.0 × 10⁻⁵ M). The fluorescence intensities at 552 nm were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.



Figure S35. Time-dependent fluorescence spectral changes observed when 1 was treated with Fe^{3+} (100 equiv.) in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm. Inset: time course of the normalized fluorescence intensity at 552 nm.



Figure S36. Time-dependent fluorescence spectral changes observed when **2** was treated with Fe^{3+} (100 equiv.) in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm. Inset: time course of the normalized fluorescence intensity at 552 nm.



Figure S37. Time-dependent fluorescence spectral changes observed when **3** was treated with Fe^{3+} (100 equiv.) in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm. Inset: time course of the normalized fluorescence intensity at 552 nm.



Figure S38. Time-dependent fluorescence spectral changes observed when **4** was treated with Fe^{3+} (100 equiv.) in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm. Inset: time course of the normalized fluorescence intensity at 552 nm.



Figure S39. Time-dependent fluorescence spectral changes observed when **6** was treated with Fe^{3+} (100 equiv.) in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm. Inset: time course of the normalized fluorescence intensity at 552 nm.

Confocal Microscopic Analysis

Incubation time (min)



Figure S40. Confocal microscopic images of HepG2 cells treated with **5**. Cells were pretreated with ferric citrate (500 μ M) for 24 h at 37 °C, and then images of **5** (1.0 μ M) were collected at time points consisting of 5, 10, 20, 30, and 60 min incubation. Fluorescent images and the corresponding contrast ones were collected upon 30 min incubation. The fluorescent images were obtained using an excitation wavelength of 488 nm and a band-path (505–550 nm) emission filter.



Figure S41. Cell viability of probes **1-6** in HepG2 cells. The cells were treated with media containing the corresponding probes (1.0 and 3.0 μ M, respectively) for 24 h, and MTT assay was then performed.



Figure S42. Colocalization experiments of **1** carried out using ER, Lyso, and Mito trackers in iron-overloaded HepG2 cells. Cells were pretreated with media containing 500 μ M of ferric citrate for 24 h at 37 °C. The iron citrate-treated cells were incubated with serum free media containing ER (0.2 μ M), Lyso (0.1 μ M), and Mito (0.1 μ M) trackers, respectively, for 10 min at 37 °C. The media were replaced with PBS containing **1** (1.0 μ M) and incubated for 5 min at 37 °C. In the merged image, the yellow regions highlight the colocalized areas of the corresponding trackers and **1**. The fluorescent confocal images were obtained using excitation wavelengths of 488 nm and 543 nm and a band-path (505–550 nm, green signal) and a long-path (> 650 nm, red signal) emission filter.



Figure S43. Colocalization experiments of **2** carried out using ER, Lyso, and Mito trackers in iron-overloaded HepG2 cells. Cells were pretreated with media containing 500 μ M of ferric citrate for 24 h at 37 °C. The iron citrate-treated cells were incubated with serum free media containing ER (0.2 μ M), Lyso (0.1 μ M), and Mito (0.1 μ M) trackers, respectively, for 10 min at 37 °C. The media were replaced with PBS containing **2** (1.0 μ M) and incubated for 5 min at 37 °C. In the merged image, the yellow regions highlight the colocalized areas of the corresponding trackers and **2**. The fluorescent confocal images were obtained using excitation wavelengths of 488 nm and 543 nm and a band-path (505–550 nm, green signal) and a long-path (> 650 nm, red signal) emission filter.



Figure S44. Colocalization experiments of **3** carried out using ER, Lyso, and Mito trackers in iron-overloaded HepG2 cells. Cells were pretreated with media containing 500 μ M of ferric citrate for 24 h at 37 °C. The iron citrate-treated cells were incubated with serum free media containing ER (0.2 μ M), Lyso (0.1 μ M), and Mito (0.1 μ M) trackers, respectively, for 10 min at 37 °C. The media were replaced with PBS containing **3** (1.0 μ M) and incubated for 5 min at 37 °C. In the merged image, the yellow regions highlight the colocalized areas of the corresponding trackers and **3**. The fluorescent confocal images were obtained using excitation wavelengths of 488 nm and 543 nm and a band-path (505–550 nm, green signal) and a long-path (> 650 nm, red signal) emission filter.



Figure S45. Colocalization experiments of **4** carried out using ER, Lyso, and Mito trackers in iron-overloaded HepG2 cells. Cells were pretreated with media containing 500 μ M of ferric citrate for 24 h at 37 °C. The iron citrate-treated cells were incubated with serum free media containing ER (0.2 μ M), Lyso (0.1 μ M), and Mito (0.1 μ M) trackers, respectively, for 10 min at 37 °C. The media were replaced with PBS containing **4** (1.0 μ M) and incubated for 5 min at 37 °C. In the merged image, the yellow regions highlight the colocalized areas of the corresponding trackers and **4**. The fluorescent confocal images were obtained using excitation wavelengths of 488 nm and 543 nm and a band-path (505–550 nm, green signal) and a long-path (> 650 nm, red signal) emission filter.





Figure S46. Colocalization experiments of **6** carried out using ER, Lyso, and Mito trackers in iron-overloaded HepG2 cells. Cells were pretreated with media containing 500 μ M of ferric citrate for 24 h at 37 °C. The iron citrate-treated cells were incubated with serum free media containing ER (0.2 μ M), Lyso (0.1 μ M), and Mito (0.1 μ M) trackers, respectively, for 10 min at 37 °C. The media were replaced with PBS containing **6** (1.0 μ M) and incubated for 5 min at 37 °C. In the merged image, the yellow regions highlight the colocalized areas of the corresponding trackers and **6**. The fluorescent confocal images were obtained using excitation wavelengths of 488 nm and 543 nm and a band-path (505–550 nm, green signal) and a long-path (> 650 nm, red signal) emission filter.

X-ray Crystallographic Analysis

Table 1. Crystal data and structure refinement for 1 (CCDC number: 1443864).

Identification code	1
Empirical formula	C ₃₅ H ₃₆ N ₄ O ₃
Formula weight	560.68
Temperature	273(2) K
Wavelength	0.71073 Å
Crystal system	monoclinic
Space group	P2(1)/c
Unit cell dimensions	$a = 12.4990(15) \text{ Å}$ $\Box = 90.00^{\circ}.$
	$b = 15.5495(18) \text{ Å}$ $\Box = 93.427(5)^{\circ}.$
	$c = 15.5176(18)$ Å $\Box = 90.00^{\circ}.$
Volume	3010.5(6) Å ³
Ζ	4
Density (calculated)	1.237 Mg/m ³
Absorption coefficient	0.080 mm ⁻¹
F(000)	1192
Crystal size	$0.13\times0.08\times0.04~mm^3$
□ range for data collection	1.63 to 28.41°.
Index ranges	$-16 \le h \le 16, -19 \le k \le 20, -20 \le l \le 20$
Reflections collected	47351
Independent reflections	7479 [$R(int) = 0.2774$]
Completeness to theta = 28.41°	98.7 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9968 and 0.9897
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	7479 / 0 / 380
Goodness-of-fit on F^2	0.943
Final <i>R</i> indices $[I > 2 \Box (I)]$	R1 = 0.0702, wR2 = 0.1791
<i>R</i> indices (all data)	R1 = 0.2282, wR2 = 0.2492
Largest diff. peak and hole	0.405 and –0.381 e·Å ⁻³

Table 2. Atomic coordinates ($x \ 10^4$) and equivalent isotropic displacement parameters
$(\text{\AA}^2 x \ 10^3)$ for 1. U(eq) is defined as one third of the trace of the orthogonalized U ^{ij}
tensor.

	х	У	Z	U(eq)
O(1)	9835(19)	10127(14)	6280(17)	59(7)
O(2)	5077(2)	8920(2)	6103(18)	81(9)
O(3)	7657(2)	10356(18)	9765(19)	78(9)
N(1)	11278(3)	7379(19)	6887(19)	57(9)
N(2)	8901(2)	13005(17)	5631(18)	50(8)
N(3)	6839(2)	9351(18)	6171(18)	48(8)
N(4)	7107(3)	9245(2)	8572(2)	65(9)
C(1)	13042(4)	7081(3)	7566(3)	82(14)
C(2)	12245(3)	7757(3)	7266(3)	63(11)
C(3)	10421(3)	7877(2)	6583(2)	47(9)
C(4)	9450(3)	7482(2)	6293(2)	49(9)
C(5)	8623(3)	8002(2)	5972(2)	50(9)
C(6)	8694(3)	8907(2)	5932(2)	45(9)
C(7)	9651(3)	9254(2)	6241(2)	46(9)
C(8)	10506(3)	8769(2)	6556(2)	51(9)
C(9)	7402(3)	11008(2)	5183(2)	47(9)
C(10)	7649(3)	11876(2)	5190(2)	43(9)
C(11)	8644(3)	12143(2)	5579(2)	42(9)
C(12)	9354(3)	11527(2)	5930(2)	47(9)
C(13)	9058(3)	10668(2)	5911(2)	44(8)
C(14)	8097(3)	10383(2)	5551(2)	43(8)
C(15)	9904(3)	13307(2)	6025(2)	55(10)
C(16)	9902(4)	14276(2)	6119(3)	71(12)
C(17)	7745(3)	9448(2)	5601(2)	45(9)
C(18)	6862(3)	12519(2)	4822(2)	545(10)
C(19)	9324(4)	6521(2)	6323(3)	73(13)
C(20)	5919(3)	9041(2)	5761(3)	56(10)
C(21)	6186(3)	8873(2)	4863(2)	51(9)
C(22)	7225(3)	9113(2)	4765(2)	45(9)
C(23)	7681(3)	9026(2)	3980(3)	56(10)

C(24)	7038(3)	8682(3)	3304(3)	649(11)
C(25)	5995(4)	8441(3)	3404(3)	71(12)
C(26)	5547(3)	8543(3)	4185(3)	66(11)
C(27)	6886(3)	9622(2)	7061(2)	55(10)
C(28)	7127(4)	8900(3)	7691(2)	73(13)
C(29)	6457(4)	8937(3)	9084(3)	73(13)
C(30)	6348(3)	9273(3)	9944(2)	63(11)
C(31)	5596(4)	8918(4)	10472(3)	97(17)
C(32)	5437(5)	9262(4)	11280(3)	102(18)
C(33)	6044(5)	9939(4)	11561(3)	91(16)
C(34)	6772(4)	10299(3)	11066(3)	73(12)
C(35)	6934(3)	9970(2)	10252(3)	58(10)

Table 3. Bond lengths [Å] and angles [°] for 1. C(1)-C(2) 1.503(5) C(2)-N(1) 1.42017

C(1)-C(2)	1.503(5)
C(2)-N(1)	1.438(5)
C(3)-N(1)	1.381(4)
C(3)-C(8)	1.392(4)
C(3)-C(4)	1.409(5)
C(4)-C(5)	1.382(5)
C(4)-C(19)	1.504(5)
C(5)-C(6)	1.411(4)
C(6)-C(7)	1.372(5)
C(6)-C(17)	1.518(5)
C(7)-C(8)	1.375(5)
C(7)-O(1)	1.378(4)
C(9)-C(10)	1.385(5)
C(9)-C(14)	1.402(5)
C(10)-C(11)	1.413(5)
C(10)-C(18)	1.492(5)
C(11)-N(2)	1.380(4)
C(11)-C(12)	1.395(5)

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2	C(12)-C(13)	1.385(4)		
з 4	C(13)-C(14)	1.368(5)		
5	C(13)-O(1)	1 383(4)		
6 7	C(14) C(17)	1.503(4)		
8	C(14)-C(17)	1.522(4)		
9	C(15)-N(2)	1.440(5)		
10	C(15)-C(16)	1.515(5)		
12	C(17)-N(3)	1.486(4)		
13 14	C(17)-C(22)	1.509(5)		
15	C(20)-O(2)	1.221(4)		
16 17	C(20)-N(3)	1.368(5)		
18	C(20)-C(21)	1.476(5)		
19 20	C(21) - C(22)	1 368(5)		
20 21	C(21) - C(22)	1.200(3)		
22	C(21)-C(26)	1.381(5)		
23 24	C(22)-C(23)	1.382(5)		
25	C(23)-C(24)	1.390(5)		
26 27	C(24)-C(25)	1.374(5)	7	
28	C(25)-C(26)	1.374(5)		X
29 30	C(27)-N(3)	1.442(4)		
31	C(27)-C(28)	1.508(5)	Y	
32	C(28)-N(4)	1.469(5)		
33 34	C(20) N(4)	1.707(5)		
35	C(29)-IN(4)	1.204(3)	7	
36 37	C(29)-C(30)	1.448(6)		
38	C(30)-C(35)	1.378(5)		
39 40	C(30)-C(31)	1.397(6)		
41	C(31)-C(32)	1.388(7)		
42	C(32)-C(33)	1.354(7)		
43 44	C(33)-C(34)	1.347(6)		
45	C(34)-C(35)	1.389(5)		
46 47	C(35)-O(3)	1.354(4)		
48	N(1)-C(2)-C(1)	111 5(4)		
49 50	N(1) - C(2) - C(1)	111.3(4)		
51	N(1)-C(3)-C(8)	120.7(4)		
52 52	N(1)-C(3)-C(4)	120.0(3)		
53 54	C(8)-C(3)C(4)	119.3(3)		
55	C(5)-C(4)-C(3)	118.2(3)		
56 57	C(5)-C(4)-C(19)	121.0(4)		
58	C(3)-C(4)-C(19)	120.8(3)		
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2	C(4)-C(5)-C(6)	123.5(4)
4	C(7)-C(6)-C(5)	115.6(3)
5	C(7)-C(6)-C(17)	123.2(3)
7	C(5)-C(6)-C(17)	121.2(3)
8 9	C(6)-C(7)-C(8)	123.5(3)
10	C(6)-C(7)-O(1)	122.9(3)
12	C(8)-C(7)-O(1)	113.6(3)
13 14	C(7)-C(8)-C(3)	119.9(4)
15	C(10)-C(9)-C(14)	122.7(3)
16 17	C(9)-C(10)-C(11)	118.7(3)
18	C(9)-C(10)-C(18)	120.6(3)
20	C(11)-C(10)-C(18)	120.6(3)
21	N(2)-C(11)-C(12)	120.3(3)
23	N(2)-C(11)-C(10)	120.4(3)
24 25	C(12)-C(11)-C(10)	119.3(3)
26	C(13)-C(12)-C(11)	119.3(3)
27 28	C(14)-C(13)-O(1)	123.3(3)
29	C(14)-C(13)-C(12)	123.3(3)
31	O(1)-C(13)-C(12)	113.4(3)
32 33	C(13)-C(14)-C(9)	116.7(3)
34	C(13)-C(14)-C(17)	122.6(3)
35 36	C(9)-C(14)-C(17)	120.5(3)
37	N(2)-C(15)-C(16)	111.0(3)
38	N(3)-C(17)-C(22)	99.9(3)
40 41	N(3)-C(17)-C(6)	110.5(3)
42	C(22)-C(17)-C(6)	112.8(3)
13 14	N(3)-C(17)-C(14)	111.1(3)
45 16	C(22)-C(17)-C(14)	113.4(3)
47	C(6)-C(17)-C(14)	108.9(3)
48 49	O(2)-C(20)-N(3)	125.0(4)
50	O(2)-C(20)-C(21)	129.3(4)
51 52	N(3)-C(20)-C(21)	105.7(3)
53	C(22)-C(21)-C(26)	121.8(3)
55	C(22)-C(21)-C(20)	109.0(3)
56 57	C(26)-C(21)-C(20)	129.2(3)
58	C(21)-C(22)-C(23)	121.0(3)
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2 3	C(21)-C(22)-C(17)	111.2(3)	
4	C(23)-C(22)-C(17)	127.8(3)	
5 6	C(22)-C(23)-C(24)	116.9(4)	
7	C(25)-C(24)-C(23)	121.9(4)	
8 9	C(26)-C(25)-C(24)	120.5(4)	~
0	C(25)-C(26)-C(21)	117.8(4)	
1 2	N(3)-C(27)-C(28)	113.5(3)	
3	N(4)-C(28)-C(27)	108.5(3)	1
.4 .5	N(4)-C(29)-C(30)	122.9(4)	
.6	C(35)-C(30)-C(31)	118.0(4)	
. 7 .8	C(35) - C(30) - C(29)	121 8(4)	
9	C(33)-C(30)-C(23)	121.0(4)	
1	C(31)-C(30)-C(29)		
2	C(32)-C(31)-C(30)	121.0(5)	
3 4	C(33)-C(32)-C(31)	118.9(5)	
5	C(34)-C(33)-C(32)	121.6(5)	
6 7	C(33)-C(34)-C(35)	120.4(5)	
8	O(3)-C(35)-C(30)	120.9(4)	
19 10	O(3)-C(35)-C(34)	118.9(4)	
1	C(30)-C(35)-C(34)	120.1(4)	
2	C(3)-N(1)-C(2)	121.7(3)	
4	C(11)-N(2)-C(15)	122.3(3)	
6	C(20)-N(3)-C(27)	122.2(3)	
7	C(20)-N(3)-C(17)	114.1(3)	
8 9	C(27)-N(3)-C(17)	123.5(3)	
0	C(29)-N(4)-C(28)	119.6(4)	
:⊥ :2	C(7)-O(1)-C(13)	118.0(3)	
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Symmetry transformations used to generate equivalent atoms:

Table 4. Anisotropic displacement parameters $(Å^2x \ 10^3)$ for **1**. The anisotropic displacement factor exponent takes the form: $-2p^2[h^2 \ a^{*2}U^{11} + ... + 2h \ k \ a^{*} \ b^{*} U^{12}]$

	U ¹¹	U ²²	U ³³	U ²³	U ¹³	U ¹²
O(1)	43(15)	33(13)	99(2)	2(13)	-14(14)	-2(11)
O(2)	52(18)	112(2)	81(2)	-7(17)	16(16)	-24(17)
O(3)	87(2)	74(2)	76(2)	-12(15)	25(18)	-30(17)
N(1)	63(2)	42(18)	67(2)	3(15)	0(17)	15(17)
N(2)	49(2)	33(16)	67(2)	2(14)	-3(16)	5(14)
N(3)	44(19)	50(18)	50(19)	-3(14)	4(14)	-6(14)
N(4)	77(2)	57(2)	58(2)	-5(16)	-2(18)	-11(18)
C(1)	84(3)	87(3)	73(3)	13(2)	-4(2)	35(3)
C(2)	60(3)	63(3)	65(3)	5(2)	0(2)	22(2)
C(3)	52(2)	41(2)	49(2)	3(16)	6(18)	11(18)
C(4)	57(2)	36(2)	55(2)	1(17)	6(19)	2(19)
C(5)	51(2)	38(2)	62(2)	-1(17)	6(19)	-6(18)
C(6)	40(2)	40(2)	55(2)	1(16)	6(17)	4(16)
C(7)	42(2)	36(2)	59(2)	1(16)	3(18)	1(17)
C(8)	44(2)	43(2)	64(2)	3(17)	2(19)	-1(18)
C(9)	41(2)	46(2)	53(2)	-2(17)	7(17)	0(17)
C(10)	41(2)	40(2)	48(2)	3(16)	6(17)	8(17)
C(11)	43(2)	39(2)	45(2)	1(15)	9(17)	3(16)
C(12)	41(2)	36(2)	65(2)	0(17)	0(18)	0(16)
C(13)	35(2)	37(19)	59(2)	3(16)	-1(17)	2(16)
C(14)	41(2)	37(19)	51(2)	0(16)	2(17)	3(17)
C(15)	59(3)	43(2)	63(2)	-1(18)	9(2)	-11(18)
C(16)	78(3)	51(2)	85(3)	5(2)	0(2)	-12(2)
C(17)	43(2)	38(19)	54(2)	-1(16)	3(17)	-4(16)
C(18)	55(2)	48(2)	61(2)	6(18)	1(19)	7(18)
C(19)	81(3)	39(2)	98(3)	4(2)	3(3)	3(2)
C(20)	43(2)	57(2)	68(3)	1(19)	4(2)	-10(19)
C(21)	49(2)	47(2)	56(2)	-2(18)	0(2)	-8(18)
C(22)	44(2)	35(19)	55(2)	3(16)	1(18)	1(16)
C(23)	43(2)	54(2)	72(3)	0(2)	5(2)	1(19)

C(24)	64(3)	73(3)	58(3)	-4(2)	3(2)	2(2)	
C(25)	66(3)	85(3)	62(3)	-5(2)	-9(2)	-11(2)	
C(26)	53(3)	72(3)	72(3)	3(2)	-1(2)	-15(2)	
C(27)	59(3)	44(2)	61(3)	-6(18)	9(2)	-8(19)	
C(28)	101(4)	58(3)	60(3)	-4(2)	-2(2)	-3(2)	
C(29)	87(3)	61(3)	71(3)	5(2)	-6(3)	-27(2)	
C(30)	68(3)	66(3)	51(3)	9(2)	-9(2)	-22(2)	
C(31)	96(4)	122(4)	71(3)	20(3)	-6(3)	-58(3)	
C(32)	93(4)	149(5)	66(4)	21(3)	7(3)	-35(4)	
C(33)	93(4)	121(5)	60(3)	10(3)	5(3)	-5(3)	
C(34)	81(3)	74(3)	64(3)	-1(2)	6(2)	-3(3)	
C(35)	58(3)	60(3)	56(3)	3(2)	2(2)	-4(2)	

Table 5. Hydrogen coordinates ($x \ 10^4$) and isotropic displacement parameters (Å²x 10³) for **1**.

		/		
	X	у	Z	U(eq)
H(1A)	13680	7353	7811	122
H(1B)	13219	6732	7084	122
H(1C)	12738	6726	7995	122
H(2A)	12072	8112	7753	75
H(2B)	12561	8124	6845	75
H(5)	7986	7742	5771	60
H(8)	11141	9036	6750	61
H(9)	6748	10832	4924	56
H(12)	10020	11691	6175	57
H(15A)	10484	13135	5674	66
H(15B)	10026	13044	6590	66
H(16A)	10576	14461	6385	107
H(16B)	9333	14446	6472	107
H(16C)	9796	14536	5559	107
H(18A)	7163	13086	4882	82

82	5126	12489	6217	H(18B)
82	4222	12397	6701	H(18C)
109	6564	6267	9975	H(19A)
109	5749	6306	9172	H(19B)
109	6676	6377	8744	H(19C)
6	3907	9191	8385	H(23)
7	2767	8613	7322	H(24)
8	2940	8206	5590	H(25)
7	4255	8394	4837	H(26)
6.	7184	9880	6205	H(27A)
6.	7146	10061	7434	H(27B)
8	7605	8448	6595	H(28A)
8	7600	8658	7826	H(28B)
8	8900	8474	6030	H(29)
11	10280	8443	5196	H(31)
12	11623	9031	4923	H(32)
11	12108	10161	5955	H(33)
8	11271	10770	7169	H(34)
6	6847	6828	11232	H(1)
6	5420	13376	8446	H(2)
11′	9297	10111	7666	H(3)

Table 6. Crystal data and structure refinement for **4** (CCDC number: 1443863).

Identification code	4
Empirical formula	$C_{35}H_{35}N_5O_4$
Formula weight	589.68
Temperature	300(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	Pbca
Unit cell dimensions	$a = 16.6232(8) \text{ Å}$ $\Box = 90.00^{\circ}.$
	$b = 17.1570(9) \text{ Å}$ $\Box = 90.00^{\circ}.$
	$c = 21.2584(11) \text{ Å}$ $\Box = 90.00^{\circ}.$
Volume	6063.0(5) Å ³
Ζ	8
Density (calculated)	1.292 Mg/m ³
Absorption coefficient	0.086 mm ⁻¹
<i>F</i> (000)	2496
Crystal size	$0.13 \times 0.12 \times 0.12 \text{ mm}^3$
□ range for data collection	1.92 to 21.98°.
Index ranges	$-17 \le h \le 17, -18 \le k \le 18, -22 \le l \le 22$
Reflections collected	60773
Independent reflections	3710 [<i>R</i> (int) = 0.2113]
Completeness to theta = 21.98°	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9897 and 0.9889
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3710 / 0 / 397
Goodness-of-fit on F^2	0.948
Final <i>R</i> indices $[I > 2 \Box(I)]$	R1 = 0.0579, wR2 = 0.1474
R indices (all data)	R1 = 0.1062, wR2 = 0.1651
Largest diff. peak and hole	$0.384 \text{ and } -0.411 \text{ e} \cdot \text{\AA}^{-3}$

Table 7. Atomic coordinates ($x \ 10^4$) and equivalent isotropic displacement parameters
$(\text{\AA}^2 x \ 10^3)$ for 4. U(eq) is defined as one third of the trace of the orthogonalized U ^{ij}
tensor.

	Х	У	Z	U(eq)
O(1)	245(14)	1252(13)	3516(13)	48(7)
O(2)	-884(16)	-2304(15)	3662(14)	57(8)
O(3)	2749(3)	1142(2)	4979(19)	105(13)
O(4)	4027(3)	899(3)	5032(2)	1301(17)
N(1)	-2450(2)	2114(2)	3416(2)	93(15)
N(2)	3046(17)	709(19)	3515(15)	54(10)
N(3)	-412(16)	-1043(17)	3713(14)	38(8)
N(4)	766(2)	-1026(2)	4858(16)	59(10)
N(5)	3321(3)	680(3)	5014(2)	91(14)
C(1)	-1962(4)	3212(4)	3033(3)	143(3)
C(2)	-2167(3)	2979(4)	3631(3)	115(2)
C(3)	-1907(2)	1519(2)	3384(19)	49(11)
C(4)	-2187(2)	750(2)	3283(17)	40(10)
C(5)	-1622(2)	165(2)	3258(18)	42(10)
C(6)	-1089(2)	1646(2)	3457(19)	49(11)
C(7)	-551(2)	1031(2)	3433(17)	40(10)
C(8)	-791(2)	278(2)	3329(16)	35(9)
C(9)	4138(3)	1646(3)	3407(2)	95(18)
C(10)	3237(2)	1532(3)	3462(2)	66(13)
C(11)	2253(2)	446(2)	3489(17)	40(10)
C(12)	2099(2)	-357(2)	3421(17)	39(9)
C(13)	1316(2)	-598(2)	3375(17)	39(9)
C(14)	1612(2)	955(2)	3536(17)	43(10)
C(15)	652(19)	-94(2)	3398(16)	35(9)
C(16)	827(2)	683(2)	3489(16)	37(9)
C(17)	2784(2)	-930(2)	3398(18)	52(11)
C(18)	-3073(2)	593(2)	3199(2)	55(12)
C(19)	-193(2)	-386(19)	3281(17)	36(9)
C(20)	-237(2)	-809(2)	2653(18)	38(9)
C(21)	-623(2)	-1711(2)	3404(19)	40(10)

C(22)	-482(2)	-1567(2)	2732(19)	41(10)
C(23)	-560(2)	-2069(2)	2226(2)	55(12)
C(24)	-384(2)	-1784(3)	1633(2)	61(13)
C(25)	-133(2)	-1025(3)	1554(2)	58(12)
C(26)	-55(2)	-521(2)	2065(2)	48(11)
C(27)	-576(2)	-934(2)	4382(18)	51(11)
C(28)	-24(3)	-1374(2)	4824(2)	64(13)
C(29)	1365(3)	-1472(3)	4902(2)	67(13)
C(30)	2201(3)	-1195(3)	4966(2)	62(13)
C(31)	2831(3)	-1718(3)	5039(2)	82(16)
C(32)	3621(4)	-1447(4)	5101(2)	92(18)
C(33)	3784(3)	-667(4)	5091(2)	88(17)
C(34)	3149(3)	-156(3)	5021(2)	67(13)
C(35)	2365(3)	-402(3)	4963(18)	62(13)

Table 8. Bond lengths [Å] and angles [°] for 4.

C(1)-C(2)	1.375(8)
C(2)-N(1)	1.624(7)
C(3)-N(1)	1.365(5)
C(3)-C(6)	1.386(5)
C(3)-C(4)	1.415(5)
C(4)-C(5)	1.376(5)
C(4)-C(18)	1.508(5)
C(5)-C(8)	1.404(5)
C(6)-C(7)	1.384(5)
C(7)-C(8)	1.369(5)
C(7)-O(1)	1.387(4)
C(8)-C(19)	1.515(5)
C(9)-C(10)	1.514(5)
C(10)-N(2)	1.452(5)
C(11)-C(14)	1.381(5)
C(11)-N(2)	1,394(4)

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2	C(11)-C(12)	1.409(5)	
4	C(12)-C(13)	1.368(5)	
5 6	C(12)-C(17)	1.505(5)	
7	C(13)-C(15)	1.403(5)	
8 9	C(14)-C(16)	1.390(5)	
10	C(15)-C(16)	1.377(5)	
11 12	C(15) - C(19)	1 513(5)	
13	C(16) - O(1)	1 375(4)	
14 15	C(10) - O(1)	1.499(4)	
16	C(19) - N(3)	1.499(4)	
17 18	C(19)-C(20)	1.521(5)	
19	C(20)- $C(22)$	1.374(5)	
20 21	C(20)-C(26)	1.377(5)	
22	C(21)-O(2)	1.234(4)	
23 24	C(21)-N(3)	1.367(4)	
25	C(21)-C(22)	1.468(5)	
26 27	C(22)-C(23)	1.384(5)	
28	C(23)-C(24)	1.383(6)	
29 30	C(24)-C(25)	1.377(6)	
31	C(25)-C(26)	1.393(5)	
32 33	C(27)-N(3)	1.461(4)	
34	C(27)-C(28)	1.515(5)	
35 36	C(28)-N(4)	1.443(5)	
37	C(29)-N(4)	1.259(5)	
38 39	C(29)-C(30)	1.475(6)	
40	C(30)-C(35)	1.387(6)	
42	C(30)-C(31)	1.389(6)	
43 44	C(31)-C(32)	1.400(7)	
45	C(32)-C(33)	1.365(7)	
46 47	C(33)-C(34)	1.380(7)	
48	C(34)-C(35)	1.376(6)	
49 50	C(34)-N(5)	1.462(7)	
51	N(5) O(4)	1.702(7)	
52 53	N(3) - O(4)	1.200(5)	
54	N(3)-O(3)	1.240(5)	
55 56		04.470	
57	C(1)-C(2)-N(1)	94.4(6)	
58 59	N(1)-C(3)-C(6)	121.8(4)	
60			

1				
2 3	N(1)-C(3)-C(4)	119.1(3)		
4	C(6)-C(3)-C(4)	119.1(3)		
5 6	C(5)-C(4)-C(3)	117.5(3)		
7	C(5)-C(4)-C(18)	122.1(3)		
8 9	C(3)-C(4)-C(18)	120.4(3)		
LO	C(4)-C(5)-C(8)	124.5(3)		
L1 L2	C(7)-C(6)-C(3)	120.6(3)		
L3	C(8)-C(7)-C(6)	122.5(3)		
15	C(8)-C(7)-O(1)	123.8(3)		
L6 L7	C(6)-C(7)-O(1)	113.8(3)		
L8	C(7)-C(8)-C(5)	115.8(3)		
19 20	C(7)-C(8)-C(19)	122.0(3)		
21	C(5)-C(8)-C(19)	122.2(3)		
23	N(2)-C(10)-C(9)	110.4(4))
24	C(14)-C(11)-N(2)	121.5(3)		
26	C(14)-C(11)-C(12)	119.1(3)		
27 28	N(2)-C(11)-C(12)	119.5(3)		
29	C(13)-C(12)-C(11)	118.4(3)		
30 31	C(13)-C(12)-C(17)	121.3(3)	Y	
32	C(11)-C(12)-C(17)	120.3(3)		
34	C(12)-C(13)-C(15)	124.0(3)		
35 36	C(11)-C(14)-C(16)	120.5(3)		
37	C(16)-C(15)-C(13)	115.8(3)		
38 39	C(16)-C(15)-C(19)	122.6(3)		
10	C(13)-C(15)-C(19)	121.4(3)		
±⊥ 12	O(1)-C(16)-C(15)	123.0(3)		
13 14	O(1)-C(16)-C(14)	114.8(3)		
15 15	C(15)-C(16)-C(14)	122.2(3)		
16 17	N(3)-C(19)-C(15)	111.9(3)		
18	N(3)-C(19)-C(8)	111.4(3)		
19 50	C(15)-C(19)-C(8)	110.4(3)		
51	N(3)-C(19)-C(20)	99.6(3)		
53	C(15) - C(19) - C(20)	110 3(3)		
54	C(13) = C(13) = C(20)	112.8(3)		
56	C(0) - C(1) - C(20)	12.0(3)		
57 58	C(22) - C(20) - C(20)	121.1(4) 111.0(2)		
59	U(22)-U(20)-U(19)	111.0(5)		
50				

1			
2	C(26)-C(20)-C(19)	127.9(3)	
4	O(2)-C(21)-N(3)	124.6(4)	
5 6	O(2)-C(21)-C(22)	128.8(4)	
7	N(3)-C(21)-C(22)	106.5(3)	
8 9	C(20)-C(22)-C(23)	121.4(4)	
LO	C(20)-C(22)-C(21)	109.1(3)	
2	C(23)-C(22)-C(21)	129.6(4)	
.3	C(24)-C(23)-C(22)	118.0(4)	
. 4 .5	C(25)-C(24)-C(23)	120.6(4)	
.6 7	C(24)-C(25)-C(26)	121.3(4)	
.8	C(20)-C(26)-C(25)	117.6(4)	
9 0	N(3)-C(27)-C(28)	115.3(3)	
1	N(4)-C(28)-C(27)	112.1(3)	
2 3	N(4)-C(29)-C(30)	123 8(5)	
4	C(35)-C(30)-C(31)	119 1(5)	
5 б	C(35)-C(30)-C(31)	120.1(4)	
7	C(31) C(30) C(27)	120.1(4)	
o 9	C(31)-C(30)-C(29)	120.0(3)	
)	C(30)-C(31)-C(32)	120.2(3)	
2	C(33) - C(32) - C(31)	120.7(5)	
¦ F	C(32)- $C(33)$ - $C(34)$	118.2(5)	
5	C(35)-C(34)-C(33)	122.6(5)	
5 7	C(35)-C(34)-N(5)	119.0(5)	
}	C(33)-C(34)-N(5)	118.4(5)	
۶)	C(34)-C(35)-C(30)	119.1(4)	
1	C(3)-N(1)-C(2)	120.4(4)	
∠ 3	C(11)-N(2)-C(10)	121.2(3)	
4 5	C(21)-N(3)-C(27)	121.8(3)	
46	C(21)-N(3)-C(19)	113.5(3)	
.7 .8	C(27)-N(3)-C(19)	123.1(3)	
9	C(29)-N(4)-C(28)	118.2(4)	
50 51	O(4)-N(5)-O(3)	122.5(6)	
2	O(4)-N(5)-C(34)	119.0(6)	
53 54	O(3)-N(5)-C(34)	118.5(5)	
55	C(16)-O(1)-C(7)	118.1(3)	
о 57			

Symmetry transformations used to generate equivalent atoms:

Table 9. Anisotropic displacement parameters ($Å^2x$ 10³) for 4. The anisotropic displacement factor exponent takes the form: $-2p^2[h^2 a^{*2}U^{11} + ... + 2h k a^{*} b^{*}]$ U¹²]

	U ¹¹	U ²²	U ³³	U ²³	U ¹³	U ¹²
0(1)	30(16)	31(15)	84(2)	-2(13)	-4(14)	1(12)
O(2)	49(18)	35(17)	86(2)	6(15)	-2(15)	-8(14)
O(3)	104(3)	90(3)	120(3)	-28(2)	2(3)	-8(3)
O(4)	81(3)	170(4)	140(4)	-13(3)	-18(3)	-47(3)
N(1)	41(2)	28(2)	210(5)	-7(3)	-2(3)	2(18)
N(2)	29(2)	54(2)	79(3)	7(18)	-7(17)	-4(17)
N(3)	37(19)	31(19)	47(2)	1(16)	2(15)	-4(15)
N(4)	59(3)	59(2)	59(2)	2(19)	-4(19)	0(2)
N(5)	81(4)	108(5)	82(3)	-16(3)	-7(3)	-13(4)
C(1)	137(6)	138(6)	153(7)	-3(5)	-33(5)	77(5)
C(2)	61(4)	168(7)	117(5)	51(5)	24(3)	56(4)
C(3)	38(3)	30(2)	80(3)	0(2)	4(2)	8(2)
C(4)	28(2)	43(3)	50(2)	-3(19)	3(18)	3(19)
C(5)	35(2)	34(2)	56(3)	-7(19)	-1(18)	-2(19)
C(6)	34(2)	27(2)	86(3)	-1(2)	3(2)	-1(19)
C(7)	29(2)	33(2)	59(3)	0(2)	2(19)	3(19)
C(8)	29(2)	31(2)	45(2)	-4(18)	0(17)	1(18)
C(9)	53(3)	92(4)	140(5)	6(4)	4(3)	-28(3)
C(10)	48(3)	58(3)	93(4)	8(3)	-3(2)	-16(2)
C(11)	28(2)	45(3)	48(3)	2(2)	-5(18)	-3(19)
C(12)	30(2)	44(3)	44(2)	1(19)	-2(18)	4(19)
C(13)	36(2)	31(2)	50(3)	4(19)	1(19)	1(19)
C(14)	32(2)	37(2)	60(3)	1(2)	-1(19)	-9(2)
C(15)	27(2)	36(2)	41(2)	1(18)	3(17)	3(18)
C(16)	32(2)	32(2)	46(2)	2(18)	1(18)	4(19)
C(17)	34(2)	60(3)	63(3)	0(2)	6(2)	6(2)
C(18)	33(2)	57(3)	75(3)	-5(2)	1(2)	4(2)
C(19)	29(2)	32(2)	46(2)	3(19)	1(17)	-2(17)
C(20)	24(2)	42(3)	46(3)	-2(2)	0(18)	5(18)
C(21)	23(2)	33(3)	65(3)	-5(2)	-1(2)	3(18)

C(22)	29(2)	33(2)	60(3)	-9(2)	-4(2)	7(18)
C(23)	46(3)	43(3)	75(3)	-16(3)	-11(2)	6(2)
C(24)	50(3)	60(3)	71(4)	-23(3)	-11(3)	19(2)
C(25)	43(3)	80(4)	51(3)	-5(3)	-3(2)	20(2)
C(26)	34(2)	52(3)	58(3)	-1(2)	-1(2)	4(2)
C(27)	45(3)	51(3)	56(3)	-6(2)	10(2)	-6(2)
C(28)	70(3)	63(3)	58(3)	9(2)	-1(2)	-12(3)
C(29)	84(4)	60(3)	58(3)	4(2)	5(3)	-3(3)
C(30)	59(3)	73(4)	54(3)	11(2)	2(2)	12(3)
C(31)	87(4)	85(4)	73(4)	25(3)	9(3)	29(4)
C(32)	69(4)	126(6)	80(4)	30(4)	3(3)	39(4)
C(33)	66(4)	136(6)	63(4)	12(4)	-5(3)	18(4)
C(34)	62(4)	83(4)	57(3)	3(3)	-5(3)	5(3)
C(35)	59(3)	76(4)	49(3)	-2(3)	0(2)	13(3)

Table 10. Hydrogen coordinates ($x \ 10^4$) and isotropic displacement parameters (Å²x 10³) for **4**.

		x y	Z	U(eq)			
H(1A)	-176	1 3736	3046	214			
H(1B)	-242	9 3191	2767	214			
H(1C)	-155	5 2872	2869	214			
H(2A)	-260	2 3285	3810	139			
H(2B)	-171	1 2970	3916	139			
H(5)	-180	4 -341	3190	50			
H(6)	-89	9 2150	3522	59			
H(9A)	425	6 2192	3376	142			
H(9B)	433	1 1383	3038	142			
H(9C)	439	8 1434	3773	142			
H(10A)	303	9 1806	3830	80			
H(10B)	297	4 1748	3094	80			
H(13)	121	9 -1128	3325	47			
H(14)	170	7 1483	3599	52			
H(17A)	328	5 -656	3437	79			

H(17B)	0770				
	2112	-1204	3004	79	
H(17C)	2732	-1295	3737	79	
H(18A)	-3366	1074	3230	82	
H(18B)	-3253	241	3520	82	
H(18C)	-3164	365	2792	82	
H(23)	-726	-2582	2282	66	
H(24)	-434	-2107	1284	73	
H(25)	-14	-845	1152	70	
H(26)	115	-9	2011	58	
H(27A)	-1125	-1095	4465	61	
H(27B)	-538	-382	4478	61	
H(28A)	-260	-1382	5241	76	
H(28B)	27	-1908	4681	76	
H(29)	1275	-2007	4895	81	
H(31)	2728	-2251	5047	98	
H(32)	4040	-1801	5149	110	
H(33)	4308	-485	5131	106	
H(35)	1950	-41	4922	74	
H(1)	-2946	2030	3321	112	
H(2)	3429	377	3564	65	
	CER S				

Captions

Scheme 1. Illustration for the Fe³⁺-sensing mechanism of the rhodamine-derived Schiff base.

Scheme 2. (a) Synthetic routes and (b) chemical structures of probes 1-6.

Fig. 1. X-ray crystal structures of probe **1** (a) and probe **4** (b). Displacement ellipsoids are scaled to a 30% probability level.

Fig. 2. UV/Vis absorption (a) and fluorescence spectra (b) of probe **5** (10.0 μ M or 1.0 μ M, respectively) upon the addition of the chloride salts of Na⁺, K⁺, Li⁺, Ag⁺, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺, Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺, Fe²⁺, and Fe³⁺ ions (50 equiv., respectively). All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.

Fig. 3. UV/Vis absorption (a) and fluorescence titration spectra (b) of probe **5** (10.0 μ M or 1.0 μ M, respectively) with an increasing [Fe³⁺]. All spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.

Fig. 4. Time-dependent fluorescence changes of probe **5** (1.0 μ M) upon addition of Fe³⁺ ions (100 equiv.). Inset: the time course of the normalized fluorescence intensity at 552 nm. The fluorescence spectra were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.

Fig. 5. Fluorescence enhancements at 552 nm of probes **1–6** to Fe³⁺ ions in the presence of biologically relevant metal ions. Gray bars represent fluorescence intensities (FI) of the probes (1.0 μ M) upon the addition of Fe³⁺ (50 μ M); orange bars represent fluorescence intensities (FI) upon the subsequent addition of Fe³⁺ (50 μ M) in the presence of biologically relevant metal ions (M = K⁺, Na⁺, Ca²⁺, Fe²⁺, Mg²⁺, and Mn²⁺; 5.0 mM, respectively). The FI were recorded in aqueous solution (CH₃CN:H₂O = 5:95) with an excitation at 528 nm.

Fig. 6. Confocal microscopy images of HepG2 cells treated with probe **5**. Cells were pretreated with various concentrations of ferric citrate (0, 50, 100, 200, 500, and 1000 μ M, respectively) for 24 h at 37 °C, and the media were replaced with PBS containing **5** (1.0 μ M). Fluorescence images and the corresponding contrast images were collected after 30 min incubation. The fluorescent images were obtained using an excitation wavelength of 488 nm and a band-path (505–550 nm) emission filter.

Fig. 7. Co-localization experiments of probe **5** carried out using ER, Lyso, and Mito trackers in iron-overloaded HepG2 cells. Cells were pretreated with media containing 500 μ M of ferric citrate for 24 h at 37 °C. The ferric citrate-treated cells were incubated with serum-free media containing ER (0.2 μ M), Lyso (0.1 μ M), and Mito (0.1 μ M) trackers, respectively, for 10 min at 37 °C. The media were replaced with PBS containing **5** (1.0 μ M) and incubated for 5 min at 37 °C. In the merged image, the yellow regions indicate the co-existed areas of the trackers and **5**. The fluorescent confocal images were obtained using excitation wavelengths of 488 nm and 543 nm and a band-path (505–550 nm, green signal) and a long-path (>650 nm, red signal) emission filter.

<Highlights>

- \checkmark The probe 5 exhibited the selective Fe³⁺-sensing at the organelle level.
- ✓ The probe **5** is mainly localized in the Endoplasmic Reticulum in HepG2 cells.
- ✓ It would be very useful for the imaging of Fe^{3+} in the ER of iron-overloaded cells.

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