Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 4802

www.rsc.org/obc

PAPER

Synthesis, crystal structure and living cell imaging of a Cu^{2+}-specific molecular probe†

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Received 8th March 2011, Accepted 6th April 2011
DOI: 10.1039/c1ob05358b

We report the development of a rhodamine chromene-based fluorescence probe to monitor the intracellular Cu^{2+} level in living cells. The new fluorescent probe exhibits a fluorescence response towards Cu^{2+} under physiological conditions with high sensitivity and selectivity, and facilitates the naked-eye detection of Cu^{2+}. The fluorescence intensity was significantly increased by about 40-fold with 10 equiv. of added Cu^{2+}.

Introduction

Copper is an essential trace element that plays a critical role in the physiology of living organisms. Critical proteins such as cytochrome oxidase, zinc-copper superoxide dismutase, lysyl oxidase and several transcription factors require copper for their activities. Consequently, complex systems for acquiring and regulating a tight homeostasis of copper have evolved. However, disruption of copper homeostasis in cells results in severe disorders such as Menkes syndrome, Wilson's disease, amyotrophic lateral sclerosis and Alzheimer's disease.[1] Furthermore, higher levels of Cu^{2+} are detected in tumors with a possible role in promoting angiogenesis (new blood vessel growth).[2] Therefore, the quantitative detection of intracellular Cu^{2+} is of great importance for elucidating its complex physiological and pathological roles.

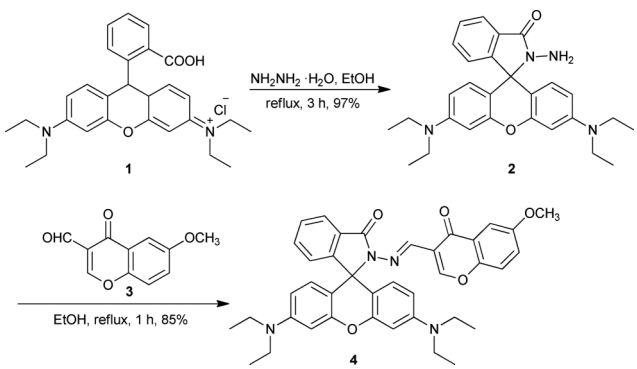
In recent years, the fluorescence method of detecting metal ions,[3] especially in living cells,[4] has attracted great attention. For this purpose, highly sensitive and selective probes that exhibit an enhanced fluorescence emission in aqueous media need to be developed.

Spirolactam derivatives of rhodamine dyes are useful sensing platforms because the spirolactam ring-opening process leads to a turn-on fluorescence change. An additional advantage of such a rhodamine-based sensing system is that the ring-opening process is also accompanied by a vivid color change from colorless to pink, thus enabling metal detection with the naked eye.[5]

Although a number of satisfactory rhodamine-based fluorescent probes for transition metals such as zinc,[6] iron,[7] chromium[8] and mercury[9] have been developed over past decades, relatively few copper-selective probes have been reported.[4d,10] Among these probes, membrane-permeable chemosensors that allow monitoring of intracellular copper are rare.[4d,10b,d,e] Thus, the development of turn-on fluorescent probes with high sensitivity and selectivity for monitoring Cu^{2+} in living cells remains a significant challenge.

Results and discussion

Probe **4** was synthesized in a facile manner from rhodamine B (**1**) and 6-methoxy-4-oxo-4*H*-chromene-3-carbaldehyde (**3**) by a two-step reaction, as shown in Scheme 1. Firstly, rhodamine B (**1**) reacted with hydrazine hydrate for 3 h under reflux in ethanol to afford (**2**) in 97% yield. Following a reported procedure,[11] **2** reacted with **3** to form **4** in 85% yield.



Scheme 1 Synthesis of compound **4**.

A single crystal of **4** was obtained from a CH_2Cl_2–hexane (1 : 1, v/v) solution and was characterized using X-ray crystallography (Fig. 1). The crystal showed the formation of a unique spirolactam ring structure. Such a special conformation of the rhodamine group makes probe **4** colorless and non-fluorescent in solution. Therefore, probe **4** is expected to act as a signal switcher, which is envisioned to turn-on when the target cation is bound.

As expected, in buffered (20 mM HEPES, pH 7.2) CH_3CN/HEPES (3 : 7, v/v) solution, **4** scarcely shows absorption at 450–650 nm, indicating that **4** exists in a spirocycle-closed form.

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† Electronic supplementary information (ESI) available: Supplementary Figs. S1–S8. CCDC reference number 816990. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c1ob05358b

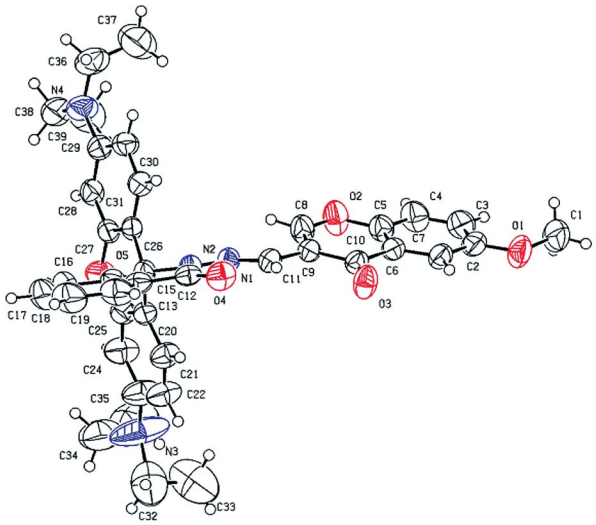

Fig. 1 The molecular structure of compound **4**.

The addition of Cu^{2+} to a solution of **4** (10 μM) caused a significant enhancement of the absorbance (Fig. 2) and fluorescence emission (Fig. 3) in the 500–650 nm range. This is a result of the Cu^{2+}-induced ring opening of the spirolactam form. Chetation with

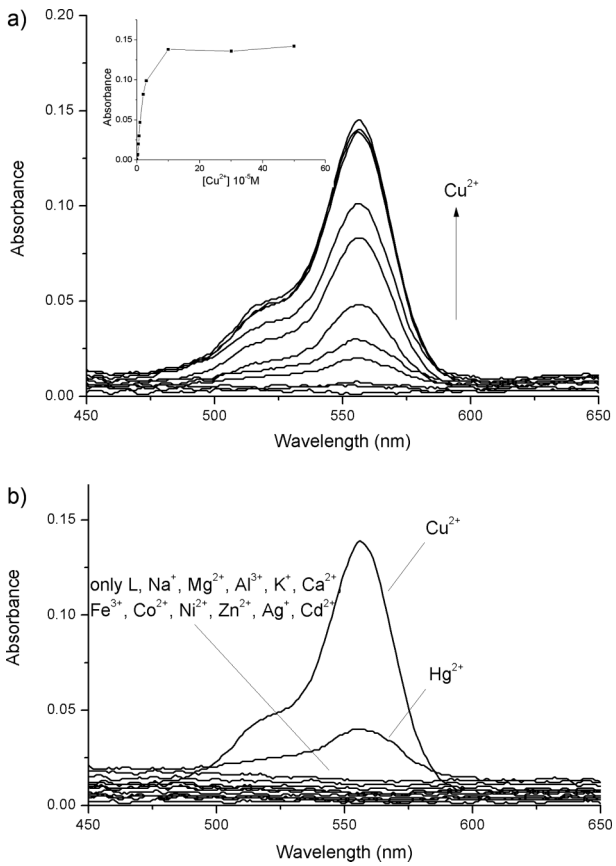

Fig. 2 (a) Absorption spectra of 10 μM **4** upon the addition of Cu^{2+} (0–50 equiv.) in CH_3CN/HEPES (20 mM, pH 7.2, 3 : 7, v/v) solution. The inset shows the absorbance of **4** at 554 nm as a function of Cu^{2+} concentration. (b) Absorption spectra of **4** (10 μM) in buffered CH_3CN/HEPES solution with 30 equiv. of metal ions: blank, Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^+, Cd^{2+} and Hg^{2+} ions.

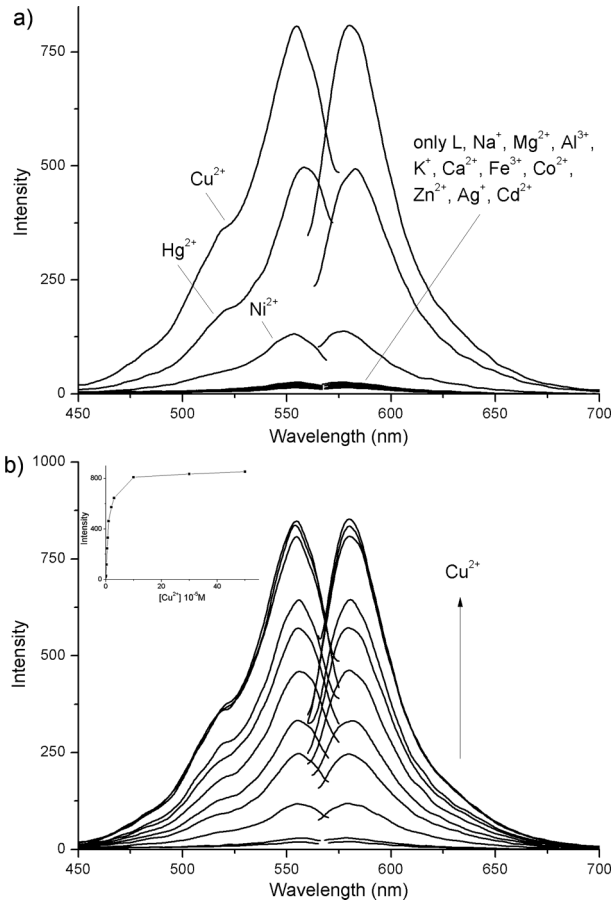

Fig. 3 (a) Fluorescence excitation and fluorescence emission spectra of 10 μM **4** in buffered CH_3CN/HEPES solution at pH 7.2 with 10 equiv. (for Cu^{2+} and Hg^{2+}) and 30 equiv. of metal ions: blank, Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+ and Cd^{2+} ions. (b) Fluorescence excitation and fluorescence emission spectra of 10 μM **4** upon the addition of Cu^{2+} (0–50 equiv.) in buffered CH_3CN/HEPES solution. The inset shows the fluorescence intensity of **4** at 580 nm as a function of Cu^{2+} concentration (excitation wavelength (λ_{ex}), 554 nm; slit width, 10 nm; emission wavelength (λ_{em}), 580 nm; slit width, 4.5 nm).

Cu^{2+} also induced the development of a purple color when 10 μM of **4** was used. About ten other metal ions showed little interference (Fig. 4). These results showed that **4** was a Cu^{2+}-specific fluorescence probe that also facilitated the visual detection of Cu^{2+}.

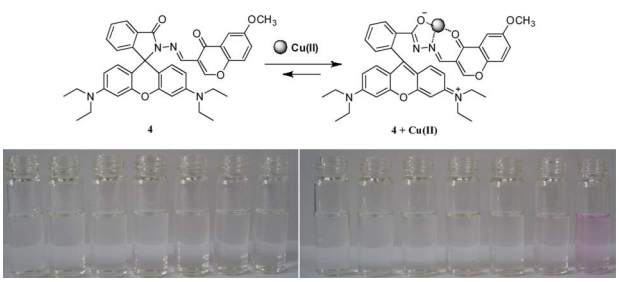

Fig. 4 Top: the proposed binding mechanism for **4** with Cu^{2+}. Bottom: change in color of **4** (10 μM) in buffered CH_3CN/HEPES solution with 30 equiv. of metal ions from left to right: blank, Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+} and Cu^{2+} ions.

In order to further investigate the binding mode of **4** and Cu^{2+}, an absorption spectrum titration and Job's method were carried out. As illustrated in Fig. 2a, upon the addition of Cu^{2+} up to 1.0 equiv. into 10 μM of **4** in buffered CH_3CN/HEPES solution, the absorbance of the absorption band peaked at 554 nm, increased linearly at first and then reached its maximum when the amount of added Cu^{2+} was above 1.0 equiv. The non-linear fit of the data revealed that the binding of **4** to Cu^{2+} was most probably of 1 : 1 stoichiometry with an association constant (K_a) of about 3.7×10^4 M^{-1}, which corresponds to a stronger binding capability toward Cu^{2+} in comparison with a Rhodamine 6G derivative binding to Cu^{2+} (with a K_a value of 2.08×10^4 M^{-1}).[10e] The data from Job's method exhibited a maximum absorbance when the molecular fraction of **4** was close to 50%, which also suggests a 1 : 1 stoichiometry for the **4**-Cu^{2+} complex; the extinction coefficient of the **4**-Cu^{2+} complex (1 : 1 stoichiometry) at 554 nm was 4570 L M^{-1} cm^{-1} (Fig. S1 and S2, ESI†). The proposed binding mode is shown in Fig. 4.

Cu^{2+} selectivity is the most important property for developing a Cu^{2+}-specific cellular probe, and this was tested next. In the presence of Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+ and Cd^{2+} ions (30 equiv.) in turn, no significant absorbance was observed at 554 nm, except that Hg^{2+} had a small absorption (Fig. 2b). This suggested that **4** was a highly selective Cu^{2+} probe.

Fig. 3a shows fluorescence spectra (λ_{ex} 554 nm) of **4** with respective metal cations. Without cations, **4** showed no fluorescence at 450–650 nm. However, Cu^{2+} addition created a remarkable enhancement at 580 nm. Although Hg^{2+} and Ni^{2+} also induced some fluorescence, the other metal ions, including Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ag^+ and Cd^{2+}, did not cause fluorescence, even when 30 equiv. was used. The binding between **4** and Hg^{2+}, however, is not a problem for Cu^{2+} detection. An obvious purple color developed in the presence of an equal equiv. of Cu^{2+}, but no color change could be found when up to 10 equiv. of Hg^{2+} was used (Fig. S3, ESI†).

The fluorescence titration was carried out by adding increasing amounts of Cu^{2+} to **4** (10 μM). The fluorescence intensity at 580 nm increased proportionally upon Cu^{2+} addition.[12] Cu^{2+} chelation induced as much as a 40-fold fluorescence enhancement (Fig. 3b). Moreover, this complex was stable in buffered CH_3CN/HEPES solution for at least 2 d. This supports our expectation that compound **4** could serve as a sensitive fluorescent switcher, as well as a naked-eye chemosensor, for Cu^{2+}.

The effect of pH on the fluorescence of the probe was also evaluated. The relative fluorescence intensity was not strongly dependent on pH between 6.9 to 7.5, close to physiological conditions (Fig. S4, ESI†).

We proceeded to investigate the applicability of **4** as a Cu^{2+} probe in living cells. Incubation of HeLa cells with 10 μM of **4** for 5 h at 37 °C gave almost no intracellular fluorescence, as monitored by fluorescence microscopy (Fig. 5a). This was consistent with previous findings that cancer cells in cell cultures contain little Cu^{2+}.[13] When HeLa cells were incubated with growth medium containing 50 μM of Cu^{2+}, the same treatment with **4** generated remarkable intracellular fluorescence (Fig. 5e). Bright-field measurements after treatment with Cu^{2+} and **4** confirmed that the cells were viable throughout the imaging experiments (Fig. 5b and 5f). The overlay of fluorescence and images revealed that the fluorescence signals were localized in the perinuclear region of the cytosol (Fig. 5g), indicating the subcellular distribution of Cu^{2+} internalized into the living cells from the growth medium. The results of the fluorescence microscopic analyses of treated cells show that the probe can be used for monitoring Cu^{2+} within biological samples.

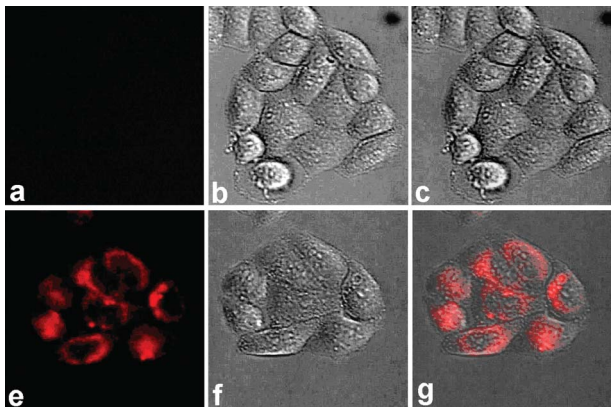

Fig. 5 Fluorescence microscope images of living HeLa cells. (a) Cells incubated with 10 μM probe **4** for 5 h at 37 °C. (b) Bright-field view of panel (a). (c) Overlay image of (a) and (b). (e) Cells with 50 μM Cu^{2+} added to the growth medium for 2 h at 37 °C and then incubated with probe **4** under the same conditions. (f) Bright-field image of the live HeLa cells shown in panel (e). (g) Overlay image of (e) and (f).

Conclusions

In summary, we have developed a novel fluorescence probe to monitor intracellular Cu^{2+} levels in living cells. The probe switches to a highly fluorescent complex upon Cu^{2+} chelation under physiological conditions. The high sensitivity and selectivity of the probe are demonstrated by the 40-fold fluorescence enhancement and the lack of interference from up to ten other metal ions. Furthermore, we have demonstrated the application of this probe in biology by imaging intracellular Cu^{2+} in HeLa cells.

Experimental section

Materials and characterization

De-ionized water was used throughout the experiments. All reagents were purchased from commercial suppliers and used without further purification. The solutions of metal ions were prepared from $CuCl_2 \cdot 2H_2O$, $CdCl_2 \cdot 2.5H_2O$, $AgNO_3$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, $HgCl_2$, $ZnCl_2$, NaCl, KCl, $CaCl_2$, $MgSO_4$ and $AlCl_3$, respectively, and were dissolved in distilled water. All samples were prepared at room temperature, shaken for 10 s and stood for 18 h before UV-vis and fluorescence determination. Thin-layer chromatography (TLC) was conducted on silica gel 60 F254 plates (Merck KGaA). HEPES buffer solutions (pH 7.2) were prepared using 20 mM HEPES and the appropriate amount of aqueous sodium hydroxide using a pH meter. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard. Melting points were determined on an XD-4 digital micro melting point apparatus. IR spectra were recorded with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). HRMS spectra were recorded on a Q-TOF6510

spectrograph (Agilent). UV-vis spectra were recorded on a U-4100 (Hitachi) instrument. Fluorescent measurements were recorded on a Perkin-Elmer LS-55 luminescence spectrophotometer.

Cell culture and imaging

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% calf bovine serum (HyClone) at 37 °C in humidified air and 5% CO_2. For fluorescence imaging, the cells (5×10^4 mL^{-1}) were seeded into 24-well plates, and experiments to assay Cu^{2+} uptake were performed in the same media supplemented with 50 μM of $CuCl_2$ for 5 h. The cells were washed twice with PBS buffer before staining experiments and incubated with 10 μM of probe **4** for 2 h in the incubator. After washing twice with PBS, the cells were imaged under a phase contrast microscope (Nikon, Japan).

Synthesis of 2-amino-3′,6′-bis(diethylamino)spiro[isoindoline-1,9′-xanthen]-3-one (2)

Rhodamine B (**1**) (1.0 g, 2.1 mmol), 80% hydrazine hydrate (2.7 g, 43 mmol) and EtOH (12 mL) were added to a flask, stirred and heated to reflux for 3 h, and then water (40 mL) added to the mixture. The mixture was extracted with ethyl acetate (40 mL) three times. The combined organic layer was dried over anhydrous magnesium sulfate and then filtered. The filtrate was concentrated to give product **2** 0.95 g (97%); mp 166–170 °C (lit.[8c] 176–177 °C, from water); ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 1.17 (t, 12H, NCH_2CH_3, J = 7.0 Hz), 3.34 (q, 8H, NCH_2CH_3, J = 7.0 Hz), 3.62 (bs, 2H, NH_2), 6.29 (d, 2H, xanthene-H, J = 8.7 Hz), 6.42 (s, 2H, xanthene-H), 6.46 (d, 2H, xanthene-H, J = 8.7 Hz), 7.10 (dd, 1H, Ar–H, J = 8.5, 3.9 Hz), 7.44–7.46 (m, 2H, Ar–H), 7.94 (dd, 1H, Ar–H, J = 8.7, 3.7 Hz).

Synthesis of (*E*)-3′,6′-bis(diethylamino)-2-((6-methoxy-4-oxo-4*H*-chromen-3-yl)methyleneamino)spiro[isoindoline-1,9′-xanthen]-3-one (4)

2-Amino-3′,6′-bis(diethylamino)spiro[isoindoline-1,9′-xanthen]-3-one (**2**) (608 mg, 1.33 mmol) was dissolved in EtOH (10 mL). Then, 6-methoxy-4-oxo-4*H*-chromene-3-carbaldehyde (**3**) (300 mg, 1.46 mmol) was added. The reaction mixture was stirred and heated to reflux for 1 h, at the end of which time it was cooled and filtered. The solid was washed with EtOH and dried to afford **4** as a grey-yellow solid: 735 mg (85%); mp 254–258 °C; IR (KBr), υ: 3075, 3037, 2967, 2929, 2894, 2868, 1693, 1613, 1515, 1484, 1306, 1213, 1115, 823, 784, 713 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 1.15 (t, 12H, NCH_2CH_3, J = 7.0 Hz), 3.32 (q, 8H, NCH_2CH_3, J = 7.0 Hz), 3.84 (s, 3H, OCH_3), 6.25 (dd, 2H, xanthene-H, J = 8.9, 2.5 Hz), 6.47 (d, 2H, xanthene-H, J = 2.4 Hz), 6.51 (d, 2H, xanthene-H, J = 8.8 Hz), 7.11 (d, 1H, Ar–H, J = 6.8 Hz), 7.19 (dd, 1H, chromone-H, J = 9.1, 3.0 Hz), 7.33 (d, 1H, chromone-H, J = 9.1 Hz), 7.44–7.51 (m, 2H, Ar–H), 7.52 (d, 1H, chromone-H, J = 3.0 Hz), 7.99 (d, 1H, Ar–H, J = 7.2 Hz), 8.42 (s, 1H, N═CH), 8.74 (s, 1H, chromone-H); ^{13}C NMR ($CDCl_3$, 100 MHz), δ (ppm): 175.4, 165.0, 157.1, 153.7, 153.2, 152.1, 150.9, 148.9, 140.1, 133.5, 128.9, 128.3, 127.9, 124.8, 123.9, 123.7, 123.4, 119.6, 119.0, 107.9, 105.6, 105.0, 98.1, 65.9, 55.9, 44.3, 12.6; HRESIMS calc. for $[M + H]^+$ $C_{39}H_{39}N_4O_5^+$: 643.2915, found: 643.2917.

Acknowledgements

The authors acknowledge Dr Bing Yan (Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, USA) who edited the manuscript. This study was supported by the 973 Program (2010CB933504) and the National Natural Science Foundation of China (20972088 and 90813022).

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