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Two-Photon Fluorescent Probes for Biological Mg^{2+} Detection Based on 7-Substituted Coumarin

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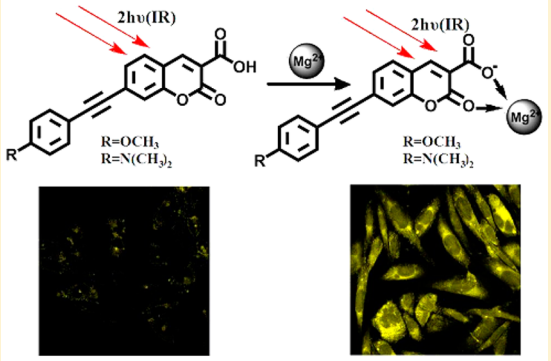
Supporting Information

ABSTRACT: Two novel water-soluble coumarin-based compounds (**OC7**, **NC7**) were designed and synthesized as two-photon fluorescent probes for biological Mg^{2+} detection. The compounds feature a β-keto acid as a high selective binding site for Mg^{2+} and the coumarin framework as the two-photon fluorophore. **OC7** and **NC7** show significant "off-on" detecting signals (9.05-fold and 23.8-fold fluorescence enhancement) and lower detection limits compared with previous reported two-photon fluorescent probes for Mg^{2+}. Moreover, **OC7**−Mg^{2+} and **NC7**−Mg^{2+} exhibit large two-photon absorption cross sections (340 and 615 GM) at the near-infrared wavelengths (740 and 860 nm), which indicates that the probes are very suitable for detection of Mg^{2+} *in vivo*. Both **OC7** and **NC7** are pH-insensitive and of low cytotoxicity and can be applied to image intracellular Mg^{2+} under two-photon microscopy (TPM). Our results provide a strategy to modify the coumarin fluorophore to get better two-photon fluorescent properties. And the results also suggest that electronic density of β-keto acid plays a very important role in the recognition of Mg^{2+}.



INTRODUCTION

Intracellular Mg^{2+} plays critical roles in biology system such as an enzyme cofactor, effector of signal transduction, various transporters, and so forth.[1] To understand these processes in biology, it is crucial to monitor Mg^{2+} in live cells and tissues.[2] At present, fluorescent probes provide the optimal choice to detect biological agents due to their high sensitivity and selectivity.[3] However, earlier Mg^{2+} fluorescent probes are mostly designed on the basis of the one-photon fluorescence technology.[4] Recently, two-photon microscopy (TPM) has become the leading imaging technology in biology research for its obvious advantages over one-photon microscopy (OPM) such as localized excitation, increased penetration depth (>500 μM), as well as the reduced photobleaching and photodamage.[5] Unfortunately, only four two-photon fluorescent probes for Mg^{2+} (**AMg1**, **CMg1**, **FMg1**, **FMg2**) were reported until now.[6] However, their selectivity and water solubility, as well as optical properties, still need to be improved. Thus, highly selective water-soluble two-photon probes for Mg^{2+} with large two-photon absorption cross sections are highly needed.

Herein, we report two novel 7-substituted coumarin-based two-photon fluorescent probes (**OC7**, **NC7**) for Mg^{2+}. Coumarin skeleton was chosen as the fluorophore because of its outstanding optical properties such as high photostability, good biological compatibility, a large Stokes shift and visible emission wavelength.[7] Previously reported results indicated that enlarging the conjugation system is an efficient way to improve the two-photon properties of fluophore quinoline.[8] Thus, two kinds of π-conjugatd electron donor (methoxy phenyl alkynyl and dimethylamino phenyl alkynyl) were introduced at 6-position (**OC6**, **NC6**) and 7-position (**OC7**, **NC7**) of the coumarin framework (Scheme 1) to enhance the two-photon properties. The β-keto acid was used as the binding group for its high selectivity for Mg^{2+} over other metal ions especially over Ca^{2+} to guarantee the selectivity.[9]

RESULTS AND DISCUSSION

All the target compounds were synthesized from simple starting materials with considerable yields (Scheme 2). High efficient Sonogashira coupling reaction was used to enlarge the conjugate system. The structures of the compounds and intermediates were characterized by ^{1}H NMR, ^{13}C NMR, and ESI-TOF MS. The single crystals of **NC6-AM**, **OC7-AM**, and **NC7-AM** were also collected from dichloromethane/methanol

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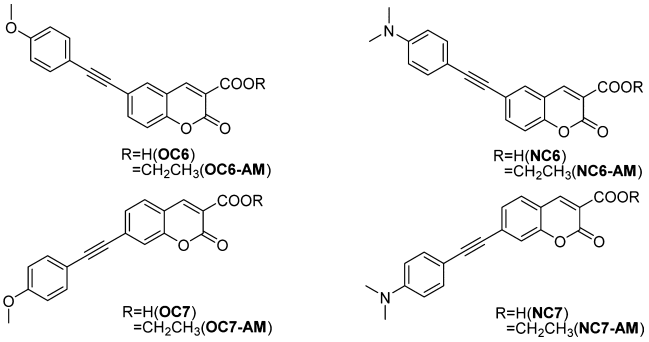
Scheme 1. Molecular Structures of OC6, NC6, OC7, and NC7



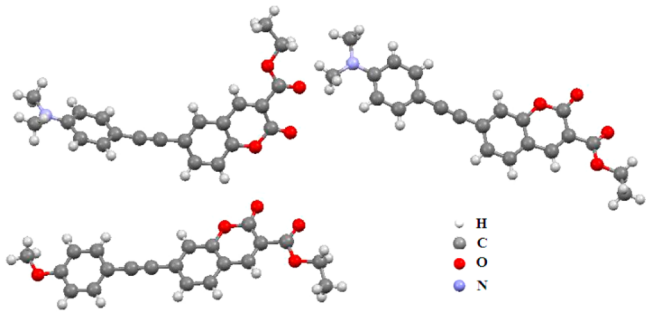


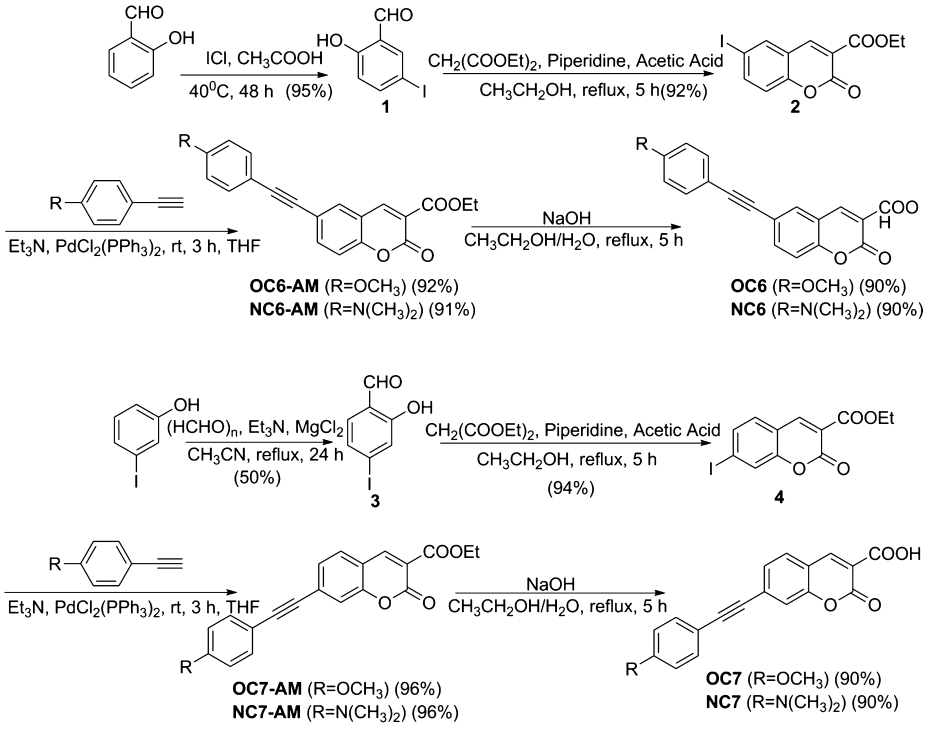
Figure 1. X-ray crystal structures for **NC6-AM**, **OC7-AM**, and **NC7-AM**.

solution. Crystal structures of **NC6-AM**, **OC7-AM**, and **NC7-AM** were depicted in Figure 1.

The absorbance and fluorescence emission spectra of the compounds were recorded at a concentration of 20 μM in aqueous buffer solution (pH 7.4, 10 mM HEPES, 10 mM SDS). The absorption of the **NC6** ($\varepsilon = 4.59 \times 10^4$ $M^{-1}\cdot cm^{-1}$) and **OC6** ($\varepsilon = 4.27 \times 10^4$ $M^{-1}\cdot cm^{-1}$) are both centered at 294 nm (Supporting Information Figure S1); meanwhile, the absorptions of **NC7** and **OC7** are centered at 394 nm ($\varepsilon = 1.09 \times 10^4$ $M^{-1}\cdot cm^{-1}$) and 357 nm ($\varepsilon = 2.08 \times 10^4$ $M^{-1}\cdot cm^{-1}$) (Supporting Information Figure S2). The maxium fluorescence emissions of **NC6** and **OC6** are located at 430 and 430 nm; however, the maxium emissions of **NC7** and **OC7** are located at 520 and 500 nm. The difference of absorption and emission maybe arose from the different structures of these two types of compounds. Density functional theory (DFT) calculations of the energy gapes between HOMO (the highest occupied molecular orbitals) and LUMO (the lowest unoccupied molecular orbitals) of **NC6** and **NC7** were performed. As shown in Figure 2, the energy gaps between HOMO and LUMO of **NC6** and **NC7** were calculated to be 2.80 and 2.58 eV, respectively. Therefore, the 7-substituted coumarins showed more red-shifted spectrum (about 100 nm) compared with 6-substituted ones for their more efficient conjugated structure. The crystal data also confirmed the result. From the X-ray crystal structures (Figure 1), in **NC6-AM** the dihedral angle between the coumarin and terminal benzene is 68.11°, but **NC7-AM** and **OC7-AM** which substituted at 7-position of coumarin fluorophore ,adopt an almost coplanar structure with dihedral angles of 3.55° and 7.42°, respectively. These suggested a more efficient π conjugation system firmed within the 7-substituted coumarins than 6-substituted ones, which will result in better fluorescence properties and the larger TP cross sections.[10]

Upon addition of Mg^{2+} (0−20 mM), the absorption spectra of **OC7** and **NC7** showed a bathochromic shift from 357 to 372 nm and 394 to 424 nm, respectively (Supporting Information Figure S2). This result can be attributed to the binding of Mg^{2+} with β-keto acid moiety. As shown in Figure 3, **OC7** and **NC7** displayed the weak and broad fluorescence emission bands centered at 500 and 520 nm, respectively. **NC7** showed lower

Scheme 2. Synthetic routes of designed compounds



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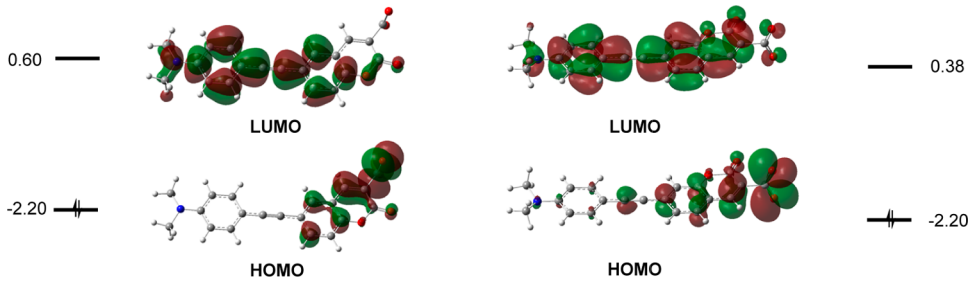

Figure 2. Calculated LUMOs and HOMOs of **NC6** (left column) and **NC7** (right column).

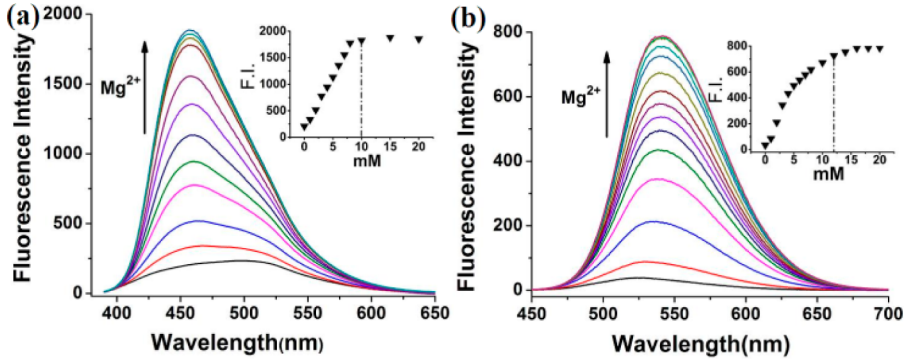

Figure 3. (a) Emission spectra of **OC7** (20 μM) after addition of Mg^{2+} (0–20 mM) in pH 7.4 (10 mM HEPES, 10 mM SDS, λ_{ex} = 370 nm). Inset: Fluorescence intensity calibration curve as a function of Mg^{2+} concentration. (b) Emission spectra of **NC7** (20 μM) after addition of Mg^{2+} (0–20 mM) in pH 7.4 (10 mM HEPES, 10 mM SDS, λ_{ex} = 420 nm). Inset: Fluorescence intensity calibration curve as a function of Mg^{2+} concentration.

baseline fluorescence intensity (Φ = 0.0017) in the absence of magnesium ions compared to **OC7** with a methoxy donor (Φ = 0.065). The low quantum yield may arise from the mixing of π/π^* and n/π^* excited states in the system, which allow rapid deactivation through intersystem crossing, resulting the quenching of the fluorescence. Upon the addition of Mg^{2+}, **NC7** showed a 23.8-fold enhancement of the fluorescence emission intensity at 540 nm (Φ = 0.125). However, a 9.1-fold enhancement was observed for **OC7** at 460 nm (Φ = 0.209). Job's plot verified that both **NC7** and **OC7** formed complexes with Mg^{2+} complex in 1:1 binding mode (Supporting Information Figure S3).The detection limits of **OC7** and **NC7** for Mg^{2+} were calculated to be 1.74×10^{-5} and 2.79×10^{-6} M, respectively.[11] The more efficient fluorescent quenching processes in **NC7** resulted in better "off-on" detection signals for Mg^{2+} and lower detection limits compared with previous reported two-photon fluorescent probes for Mg^{2+} such as **CMg1** (2.2-fold enhancement), **FMg1** (19.0-fold), **FMg2** (21.0-fold), **AMg1** (12.3-fold).[6]

The addition of Mg^{2+} did not cause any change of fluorescence emission of **OC6** and **NC6** (Supporting Information Figure S4). These may be caused by the lower electron density of the β-keto acid in 6-substituted coumarins compared with 7-substituted ones, which results in lower binding ability to Mg^{2+}. As shown in Figure 2, the orbital interaction between dimethylamino phenyl alkynyl and coumarin was obviously observed in **NC7**, but it is complete absent in **NC6**. These results suggested that the electron density is an important factor and should be considered in the design of β-keto acid type fluorescent probe for Mg^{2+}.

The TP cross sections of **OC7**–Mg^{2+} and **NC7**–Mg^{2+} were determined by using the two-photon induced fluorescence measurement technique. As shown in Figure 4, upon saturating with Mg^{2+}, **OC7** and **NC7** exhibited the TP cross sections

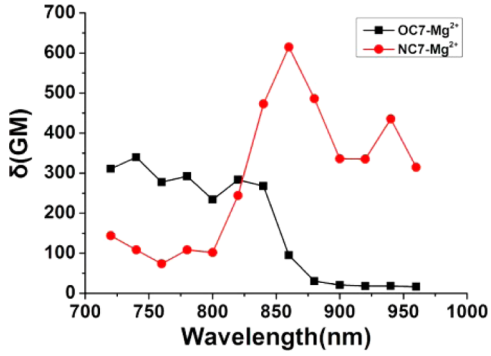

Figure 4. TP cross sections of **OC7** and **NC7** with Mg^{2+} under different exciting wavelengths of identical energy of 0.5 w.

(δ_{max}) of 340 GM at 740 nm (Φ = 0.209) and 615 GM at 860 nm (Φ = 0.125), respectively. It is obvious that **NC7** shows better two-photon activity than **OC7** at NIR wavelengths, because dimethylamino group is a stronger electron donor than methoxyl group to achieve high TP cross sections (δ_{TPA}).[12] However, the results suggest that both **NC7** and **OC7** are suitable for imaging Mg^{2+} in living cells under two photon excitation.

Since **OC7** and **NC7** showed similar selectivity response to Mg^{2+}, for clarity, the data of **NC7** were chosen to illustrate the ion selectivity, cell imaging application. The data of selectivity, pH dependence and cell culture of **OC7** were shown in the Supporting Information.

The selectivity test of **NC7** was conducted in fully buffered aqueous solution. The absorption spectra (Supporting Information Figure S6b) and emission intensity (Figure 5a) of **NC7** were examined on addition of various cations. **NC7** showed a strong response toward alkaline earth metal ions

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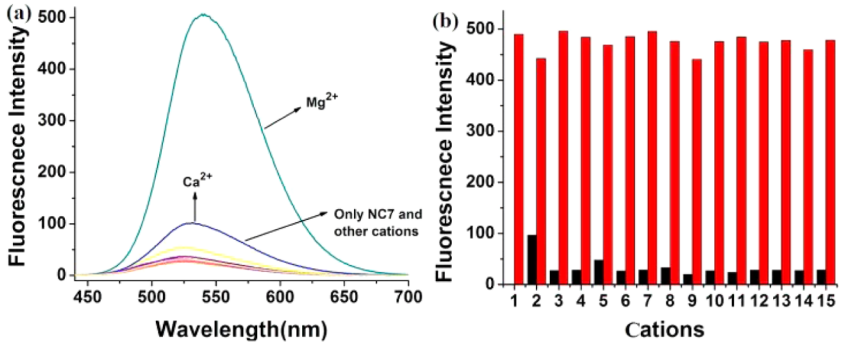

Figure 5. (a) Fluorescence spectra of **NC7** (20 μM) upon addition of Mg^{2+} and other metal ions (λ_{ex} = 420 nm). (b) Fluorescence intensity at 540 nm of **NC7** (20 μM) upon addition of various metal ions (black bars, only **NC7** and **NC7** with other metals; red, **NC7** with Mg^{2+} and other metals). Experimental conditions: buffer (pH 7.4, 10 mM HEPES 10 mM SDS), 5 mM for Mg^{2+} (1), 1 mM for Ca^{2+} (2), 10 mM for Na^{+} (3), K^{+} (4), 20 μM for Ba^{2+} (5), Co^{2+} (6), Cr^{3+} (7), Fe^{2+} (8), Mn^{2+} (9), Pb^{2+} (10), Zn^{2+} (11), Cu^{2+} (12), Ag^{+} (13), Fe^{3+} (14), only **NC7** (15) (λ_{ex} = 420 nm).

(Ca^{2+}, Mg^{2+}), whereas no noticeable changes were observed with other cations (Na^{+}, K^{+}, Ba^{2+}, Co^{2+}, Cr^{3+}, Fe^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Ag^{+}, Fe^{3+}). The dissociation constant K_d of **NC7** with Mg^{2+} was 2.7 mM and with Ca^{2+} was 2.9 mM. However, the intracellular Mg^{2+} concentration (0.1−6.0 mM) is much higher than that of Ca^{2+} (10 nM−1 mM), so these probes can be applied to measure the intracellular free Mg^{2+} ion concentration without the interference by Ca^{2+}.[13] Competitive experiments indicated that **NC7** could selectively detect Mg^{2+} in the presence of other cations (Figure 5b). Owing to the use of β-keto acid as the binding group, the selectivity over Ca^{2+} and other metal ions of our probe are among the best of reported two-photon fluorescent probes for Mg^{2+}.[6a,c] The color change of **NC7** in water with different metal ions is shown in Figure 6. Among the metal ions tested, only Mg^{2+} can arise the

Figure 6. Top: color of **NC7** and **NC7** with different metal ions. Bottom: fluorescence (λ_{ex} = 365 nm) change upon addition of different metal ions. Mg^{2+} (1), Ca^{2+} (2), Na^{+} (3), K^{+} (4), Ba^{2+} (5), Co^{2+} (6), Cr^{3+} (7), Fe^{2+} (8), Mn^{2+} (9), Pb^{2+} (10), Zn^{2+} (11), Cu^{2+} (12), Ag^{+} (13), Fe^{3+} (14), only NC7 (15).

yellow fluorescence of **NC7** under a UV-lamp and yellow in light. Therefore, **NC7** can also be served as a "naked-eye" probe for Mg^{2+} with remarkable selectivity in water.

The pH-dependence of **NC7** and **NC7**−Mg^{2+} were also examined. In the biological relevant pH range (e.g., 6−9.5), the fluorescence intensities of **NC7** and **NC7**−Mg^{2+} (Figure 7) show almost no response to the change of pH. This indicates that the probe is pH-insensitive in the biologically relevant pH range, and suitable for application to physiological studies.

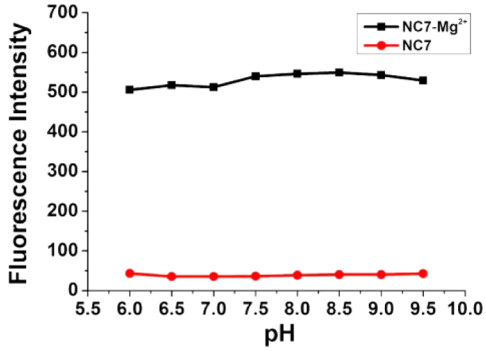

Figure 7. pH sensitivity of **NC7** and **NC7**−Mg^{2+}.

Finally, the utility of **NC7** for monitoring the intracellular Mg^{2+} flux under two-photon excitation was investigated. To improve the cell permeability, **NC7** was converted to acetoxymethyl ester (**NC7-AM**). Acetoxymethyl group was hydrolyzed by the intracellular esterase after membrane permeation, resulting in the regeneration of the free binding site of the probe.[14] MTT (5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide) assay was performed to test the cytotoxicity of **NC7-AM** against CHO cells over a 24 h period. As shown in Figure 8, the CHO cells incubated with concentration of 10 μM of **NC7-AM** retain 90% viability after 24 h of feeding time. The results indicated that **NC7-AM** exhibited almost no cytotoxicity at the working concentration (10 μM) and was suitable for the cell imaging of Mg^{2+}. TPM images are obtained by exciting the samples with a mode-

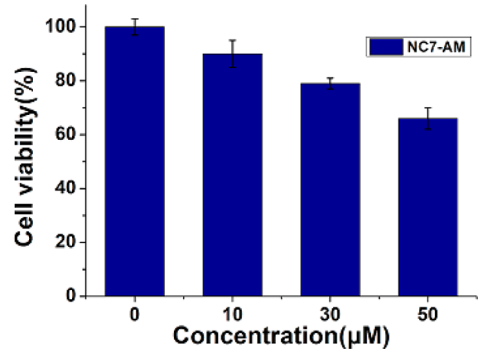

Figure 8. Cytotoxicity data of **NC7-AM** (CHO cells incubated for 24 h).

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locked titanium−sapphire laser source set at wavelength of 840 nm. As shown in Figure 9, before addition of Mg^{2+}, **NC7-AM**

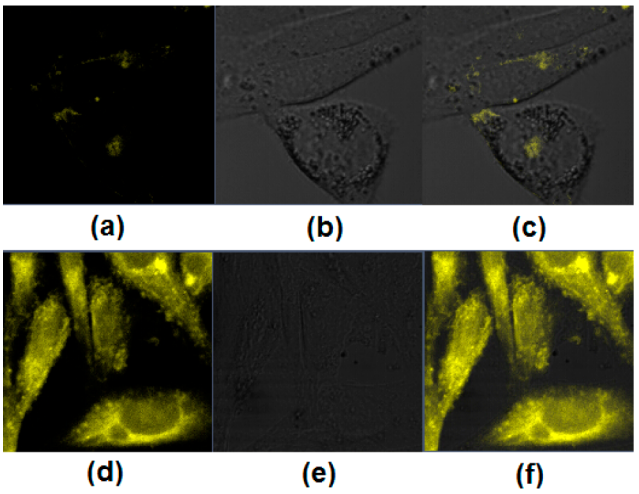

Figure 9. Two-photon images of CHO cells labeled with 20 μM **NC7-AM** after 30 min of incubation, washed with PBS buffer. λ_{ex} = 840 nm. (a) Emission wavelength from 520 to 560 nm; (b) bright-field of CHO cells; (c) the overlay of panels a and b. TPF image **NC7-AM** labeled CHO after 0.5 h treated with 20 mM $MgCl_2$ water solution, λ_{ex} = 840 nm. (d) Emission wavelength from 520 to 560 nm; (e) bright-field of CHO cells; (f) the overlay of panels d and e.

labeled CHO cells displayed weak intracellular fluorescence. After incubation with Mg^{2+} (20 mM) for 0.5 h, the cells exhibited a greatly increased fluorescence from optical observation window at 520−560 nm. These results demonstrate that **NC7** can reveal the variation of intracellular Mg^{2+} flux under two-photon excitation.

CONCLUSIONS

In summary, we have developed two biocompatible two-photon fluorescent probes (**OC7**, **NC7**) for biological Mg^{2+} with low detection limits 1.74×10^{-5} and 2.79×10^{-6} M. **NC7** and **OC7** showed excellent two-photon properties and "off-on" detecting signals upon the addition of Mg^{2+}, (23.8-fold and 9.05-fold fluorescence enhancement for **NC7** and **OC7**, respectively) which is much better than previously reported two-photon fluorescent probes for Mg^{2+}. The test results indicated that both **OC7** and **NC7** exhibited excellent biocompatibility and can be used to monitor the Mg^{2+} flux in living cells under TPM. Moreover, our results indicated that modification of coumarin at 7-position rather than at 6-position will be an efficient way to enhance the two-photon optical properties of the fluorophore. And the electron density of the β-keto acids plays an important role in their response to Mg^{2+} in this high selective type of fluorescent probe for Mg^{2+}.

EXPERIMENTAL SECTION

General. All reagents and solvents were commercially purchased. ^{1}H NMR spectra were recorded on 400 MHz spectrometers, and ^{13}C NMR spectra were recorded on 100 MHz spectrometers. MS spectra were recorded on a ESI-TOF mass spectrometer.

Stock solutions of **OC7** and **NC7** (1 mM) were prepared in DMSO. The test solutions of **OC7** and **NC7** (20 μM) in pH 7.4 buffer (10 mM HEPES, 10 mM SDS) were prepared.

Measurement of Two-Photon Cross Section (δ). All measurements were carried out in air at room temperature. TPA cross sections were measured using two-photon-induced fluorescence measurement technique. The TPA cross sections (δ) are determined by comparing their TPEF to that of fluorescein in different solvents, according to the following equation:

$$\delta = \delta_{ref}\frac{\phi_{ref}}{\phi}\frac{c}{c_{ref}}\frac{n_{ref}}{n}\frac{F}{F_{ref}}$$

Here, the subscripts ref stand for the reference molecule. δ is the TPA cross-section value, c is the concentration of solution, n is the refractive index of the solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The δ_{ref} value of reference was taken from the literature.[15]

Cytotoxicity Assays. To test the cytotoxic effect of the probe in cells for over a 24 h period, MTT (5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) assay was performed as previously reported.[16] HeLa cells were passed and plated to ca. 70% confluence in 96-well plates 24 h before treatment. Prior to **NC7-AM** treatment, DMEM (Dulbecco's Modified Eagle Medium) with 10% FCS (Fetal Calf Serum) was removed and replaced with fresh DMEM, and aliquots of **NC7-AM** stock solutions (1 mM DMSO) were added to obtain final concentrations of 10, 30, and 50 μM, respectively. The treated cells were incubated for 24 h at 37 °C under 5% CO_2. Subsequently, cells were treated with 5 mg/mL MTT (40 μL/well) and incubated for an additional 4 h (37 °C, 5% CO_2). Then the cells were dissolved in DMSO (150 μL/well), and the absorbance at 570 nm was recorded. The cell viability (%) was calculated according to the following equation: Cell viability% = OD_{570} (sample)/OD_{570}(control) × 100, where OD_{570}(sample) represents the optical density of the wells treated with various concentration of **NC7-AM** and OD_{570}(control) represents that of the wells treated with DMEM. The percent of cell survival values is relative to untreated control cells.

Cell Culture and Two-Photon Fluorescence Microscopy Imaging. For two-photon bioimaging, CHO cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Cytotoxicity assays show that **NC7-AM** is safe enough for two-photon bioimaging at low concentrations, so that the cells were incubated with 20 μM **NC7-AM** at 37 °C under 5% CO_2 for 30 min, washed once and bathed in DMEM containing no FCS prior to imaging and/or Mg^{2+} addition. Then 20 mM Mg^{2+} was added in the growth medium for 0.5 h at 37 °C, and washed 3 times with PBS buffer. Then, cells were imaged on a confocal microscope. Two-photon fluorescence microscopy images of labeled cells were obtained by exciting the probe with a mode-locked titanium−sapphire laser source set at wavelength 840 nm.

Synthesis of Compound 1. Compound **1** was prepared according to the literature method.[17] ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.95 (s, 1H), 9.83 (s, 1H), 7.84 (d, J = 2.0 Hz, 1H), 7.76 (dd, J = 8.8, 2.0 Hz, 1H), 6.80 (d, J = 8.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 195.4, 161.2, 145.3, 141.9, 122.6, 120.2, 80.4.

Synthesis of Compound 2. Compound **1** (5 g, 20 mmol), diethyl malonate (3.9 g, 24 mmol), piperidine (200 μL, 2 mmol), and acetic acid (5 droplets) were added to ethanol (50 mL). The mixture was heated under reflux for 5 h. Then it was cooled to room temperature, and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, EtOAc:PE = 1:5) to give compound **2** (6.4 g, 92%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 1H), 7.94 (d, J = 1.9 Hz, 1H), 7.89 (dd, J = 8.7, 1.9 Hz, 1H), 7.13 (d, J = 8.7 Hz, 1H), 4.42 (q, J = 7.1 Hz, 2H), 1.41 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.6, 156.0, 154.7, 146.9, 142.7, 137.7, 119.9, 119.3, 118.7, 87.4, 62.2, 14.2.

Synthesis of Compound OC6-AM. A mixture of **2** (2 g, 6 mmol), 1-ethynyl-4-methoxybenzene (0.92 g, 7.2 mmol), $PdCl_2(PPh_3)_2$ (42 mg, 0.06 mmol), CuI (23 mg, 0.12 mmol), $N(Et)_3$ (10 mL) and THF (30 mL) was stirred at room temperature for 3 h under N_2 atmosphere. Then it was concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, EtOAc: PE = 1:3) to give compound **OC6-AM** (1.86 g, 92%) as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.48 (s, 1H), 7.74 (d, J = 1.8 Hz, 1H), 7.72(s,1H), 7.48 (d, J = 8.4 Hz, 2H), 7.33 (d,

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J. Org. Chem. 2015, 80, 4306−4312

J = 9.1 Hz, 1H), 6.90 (d, J = 8.4 Hz, 2H), 4.43 (q, J = 7.1 Hz, 2H), 3.84 (s,3H), 1.42 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.9, 160.0, 156.3, 154.3, 147.9, 137.0, 133.2, 131.9, 120.8, 119.0, 117.9, 117.0, 114.5, 114.2, 90.7, 85.8, 62.1, 55.4, 14.2.

Synthesis of Compound NC6-AM. Compound **2** (2 g, 5.8 mmol), 1-ethynyl-4-dimethylaminobenzene (0.92 g, 7.2 mmol), $PdCl_2(PPh_3)_2$ (42 mg, 0.06 mmol), CuI (23 mg, 0.12 mmol), $N(Et)_3$ (10 mL) and THF (30 mL) were stirred at room temperature for 3 h in N_2 atmosphere. Then the mixture was concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, EtOAc:PE = 1:3) to give compound **NC6-AM** (1.91 g, 91%) as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.48 (s, 1H), 7.73 (d, J = 7.9 Hz, 1H), 7.72 (s, 1H), 7.41 (d, J = 8.3 Hz, 2H), 7.31 (d, J = 8.6 Hz, 1H), 6.67 (d, J = 8.3 Hz, 2H), 4.43 (q, J = 7.1 Hz, 2H), 3.01 (s, 6H), 1.42 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 156.4, 154.1, 150.4, 148.1, 137.0, 132.8, 131.6, 121.4, 118.9, 117.9, 116.9, 111.8, 108.9, 92.1, 85.2, 62.1, 40.2, 14.2.

Synthesis of Compound OC6. To the solution of **OC6-AM** (2 g, 6 mmol) in ethanol (20 mL), 20 mL of 10% NaOH solution was added. Then the mixture was refluxed for 5 h. After reaction, the solution was acidified by hydrochloric acid to pH = 1 under 0 °C. The product was collected by filtration yielding **OC6** (1.67 g, 91%). ^{1}H NMR (400 MHz, DMSO): δ 13.33 (s, 1H), 8.73 (s, 1H), 8.09 (s, 1H), 7.84 (d, J = 8.6 Hz, 1H), 7.52 (d, J = 7.8 Hz, 2H), 7.47 (d, J = 8.6 Hz, 1H), 7.01 (d, J = 8.0 Hz, 2H), 3.34 (s, 3H). ^{13}C NMR (100 MHz, DMSO): δ 163.8, 159.7, 156.2, 153.9, 147.5, 136.5, 133.0, 132.5, 119.2, 119.1, 118.3, 116.8, 114.5, 113.7, 89.9, 86.3, 55.3. HRMS (ESI-TOF) m/z: $[M - H]^-$ $C_{19}H_{11}O_5$ calcd, 319.0606; found, 319.0600.

Synthesis of Compound NC6. To a solution of **NC6-AM** (2 g, 5.5 mmol) in ethanol (20 mL) was added 20 mL of 10% NaOH solution. Then the mixture was refluxed for 5 h. After reaction, the solution was acidified by hydrochloric acid to pH = 1 under 0 °C. The product was collected by filtration yielding **NC6** (1.66 g, 90%). ^{1}H NMR (400 MHz, DMSO): δ 8.70 (s, 1H), 8.04 (s, 1H), 7.79 (d, J = 8.7 Hz, 1H), 7.45 (d, J = 8.6 Hz, 1H), 7.38 (d, J = 8.3 Hz, 2H), 6.73 (d, J = 8.4 Hz, 2H), 2.96 (s, 6H). ^{13}C NMR (100 MHz, DMSO): δ 163.9, 156.5, 154.5, 150.6, 147.8, 132.9, 130.3, 129.0, 127.1, 117.7, 117.5, 117.4, 111.8, 107.1, 96.3, 87.0, 55.2. HRMS (ESI-TOF) m/z: $[M - H]^-$ $C_{20}H_{14}NO_4$ calcd, 332.0923; found, 332.0926.

Synthesis of Compound 3. 3-Iodophenol (10 g, 45 mmol) was dissolved in anhydrous acetonitrile (160 mL). The resulted solution was cooled in an ice bath. Then, magnesium chloride (12.8 g, 134 mmol) was added portion wise to the solution over 10 min. Triethylamine (25.3 mL, 363 mmol) was added to this mixture over 5 min, followed by the addition of paraformaldehyde (5.47 g, 636 mmol). The result mixture was heated at reflux for 18.5 h, and then was saturated by aqueous 1 M HCl. The crude product was extracted with EtOAc, dried over Na_2SO_4, and then concentrated in vacuum. The residue was purified by column chromatography (silica gel, pure PE) to give compound **3** (5.6 g, 50%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 11.01 (s, 1H), 9.85 (s, 1H), 7.44 (s, 1H), 7.39 (d, J = 8.1 Hz, 1H), 7.23 (d, J = 8.1 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 196.1, 161.3, 134.2, 129.4, 127.3, 120.0, 105.2.

Synthesis of Compound 4. Compound **3** (5 g, 20 mmol), diethyl malonate (3.9 g, 24 mmol), piperidine (200 mL, 2 mmol), and acetic acid (5 droplets) were added to ethanol (50 mL). The mixture was heated under reflux for 5 h. Then it was cooled to room temperature, and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, EtOAc:PE = 1:5) to give compound **4** (6.5 g, 94%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.46 (s, 1H), 7.76 (s, 1H), 7.68 (d, J = 8.2 Hz, 1H), 7.30 (d, J = 8.1 Hz, 1H), 4.41 (q, J = 7.1 Hz, 2H), 1.41 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 162.8, 155.9, 154.8, 148.0, 134.2, 130.1, 126.1, 118.8, 117.2, 100.9, 62.2, 14.2.

Synthesis of Compound OC7-AM. A mixture of **4** (2 g, 6 mmol), 1-ethynyl-4-methoxybenzene (0.92 g, 7.2 mmol), $PdCl_2(PPh_3)_2$ (42 mg, 0.06 mmol), CuI (23 mg, 0.1 2 mmol), $N(Et)_3$ (10 mL) and THF (30 mL) was stirred at room temperature for 3 h under N_2 atmosphere. Then it was concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, EtOAc:PE = 1:3) to give **OC7-AM** (1.84 g, 91%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.50 (s, 1H), 7.56 (d, J = 7.9 Hz, 1H), 7.51 (d, J = 8.1 Hz, 2H), 7.44 (s, 1H), 7.41 (s, 1H), 6.91 (d, J = 8.1 Hz, 2H), 4.42 (q, J = 7.1 Hz, 2H), 3.85 (s, 3H), 1.42 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 163.1, 160.6, 156.7, 155.2, 148.0, 133.7, 130.2, 129.3, 128.0, 119.1, 117.9, 117.3, 114.2, 114.1, 95.2, 87.1, 62.1, 55.4, 14.3.

Synthesis of Compound OC7. To a solution of **OC7-AM** (2 g, 6 mmol) in ethanol (20 mL) was added 20 mL of 10% NaOH aqueous solution. The result mixture was refluxed for 5 h. The reaction solution was acidified to pH = 1 by hydrochloric acid under 0 °C. The product was collected by filtration (1.61 g, 88%). ^{1}H NMR (400 MHz, DMSO): δ 13.27 (s, 1H), 8.75 (s, 1H), 7.92 (d, J = 8.1 Hz, 1H), 7.57 (s, 1H), 7.56 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.0 Hz, 1H), 7.03 (d, J = 8.6 Hz, 2H), 3.35 (s, 3H). ^{13}C NMR (100 MHz, DMSO): δ 163.8,159.7,156.2, 153.9, 147.5, 136.5, 133.0, 132.5, 119.2, 119.1,118.3, 116.82, 114.5, 113.7, 89.9, 86.3, 55.3. HRMS (ESI-TOF) m/z: $[M - H]^-$ $C_{19}H_{11}O_5$ calcd, 319.0606; found, 319.0609.

Synthesis of Compound NC7-AM. Compound **4** (2 g, 6 mmol), 1-ethynyl-4-dimethylaminobenzene (0.92 g, 7.2 mmol), $PdCl_2(PPh_3)_2$ (42 mg, 0.06 mmol), CuI (23 mg, 0.12 mmol), $N(Et)_3$ (10 mL) and THF (30 mL) were stirred at room temperature for 3 h under N_2 atmosphere. Then it was concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, EtOAc:PE = 1:3) to give **NC7-AM** (1.87 g, 89%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.49 (s, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.44 (d, J = 8.6 Hz, 2H), 7.41 (s, 1H), 7.40 (d, J = 8.8 Hz, 1H), 6.67 (d, J = 8.7 Hz, 2H), 4.42 (q, J = 7.1 Hz, 2H), 3.03 (s, 6H), 1.41 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 163.2, 156.9, 155.2, 150.8, 148.2, 133.5, 130.9, 129.2, 118.7, 117.3, 116.9, 111.7, 108.5, 97.4, 86.9, 61.8, 40.2, 14.2.

Synthesis of Compound NC7. To a solution of **NC7-AM** (2 g, 5.5 mmol) in ethanol (20 mL) was added 20 mL of 10% NaOH aqueous solution, and the mixture was refluxed for 5 h. Then, reaction solution was acidified to pH = 1 hydrochloric acid under 0 °C. The product was collected by filtration (1.71 g, 90%). ^{1}H NMR (400 MHz, DMSO): δ 8.74 (s, 1H), 7.89 (d, J = 8.1 Hz, 1H), 7.50 (s, 1H), 7.47 (d, J = 8.1 Hz, 1H), 7.42 (d, J = 8.4 Hz, 2H), 6.74 (d, J = 8.6 Hz, 2H), 2.98 (s, 6H). ^{13}C NMR (100 MHz, CF_3CO_2D): δ 167.5, 165.5, 155.0, 154.3, 142.1, 134.7, 132.5, 131.3, 130.4, 126.4, 120.4, 120.1, 118.8, 113.2, 93.1, 89.6, 47.5. HRMS (ESI-TOF) m/z: $[M - H]^-$ $C_{20}H_{14}NO_4$ calcd, 332.0923; found, 332.0910.

X-ray Crystal Structure Analysis. Calculations were performed with SHELXTL-97 program package.[18] Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC: 1030851, 1034459, 1030852.

■ ASSOCIATED CONTENT

Supporting Information

UV−vis spectra response, Job's plot, UV−vis ion selectivity, fluorescent ion selectivity, pH stability of **OC7**. Cytotoxicity assay, cell culture of **OC7-AM**. UV−vis spectra response, Job's plot, molecular orbital plots, UV−vis ion selectivity of **NC7**. UV−vis spectra, fluorescent spectra response of **OC6**. UV−vis spectra, fluorescent spectra response, molecular orbital plots of **NC6**. NMR spectra for compounds **1**, **2**, **3**, **4**, **OC6-AM**, **OC7-AM**, **NC6-AM**, **NC7-AM**, **OC6**, **OC7**, **NC6**, **NC7**. Mass spectra for compounds **OC6**, **OC7**, **NC6**, **NC7**. Crystallographic data for **NC6-AM**, **OC7-AM**, **NC7-AM**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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