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Design and synthesis of a new coumarin-based 'turn-on' fluorescent probe selective for Cu^{+2}

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ABSTRACT

The novel coumarin-based 'turn-on' fluorescent probe (*E*)-3-(2,5-dimethoxybenzylideneamino)-7-hydroxy-2*H*-chromen-2-one (**MGM**) was designed, synthesized, and characterized. This compound shows high selectivity for Cu^{+2}, combined with a large fluorescence enhancement upon binding to Cu^{2+}. Benesi–Hildebrand and Job plots demonstrate that the stoichiometry of the Cu^{2+} complex formed is 2:1. Preliminary studies employing epifluorescence microscopy demonstrated that Cu^{+2} could be imaged in human neuroblastoma SH-SY5Y cells treated with **MGM**.

Chemosensor development is an active field of research with excellent potential in clinical biochemistry, analytical chemistry, and environmental science.[1] Copper is a very important transition metal in the body, being the third most abundant metal after iron and zinc,[2] but it can also catalyze the formation of reactive oxygen species (ROS) capable of damaging biomolecules. Research has connected the cellular toxicity of copper ions with serious diseases, including prion disease.[3,4] In addition, alterations in cellular levels of copper have been associated with several neurodegenerative diseases, such as Menkes and Wilson diseases,[5–7] Alzheimer's and Parkinson's diseases.[8]

In this work, the novel fluorescence probe **MGM** was prepared via a conventional four-step synthesis from commercial precursors. Vilsmeier–Haack formylation of resorcinol gave 2,4-dihydroxybenzaldehyde, which was subsequently condensed with *N*-acetylglycine (Knoevenagel) and hydrolyzed in situ to afford 3-amino-7-hydroxycoumarin. The condensation of 3-amino-7-hydroxycoumarin with 2,5-dimethoxybenzaldehyde then provided **MGM** (Scheme 1), which was characterized by ^{1}H NMR and ^{13}C NMR spectroscopy (Supplementary Figs. S1A and S1B).

The molecular recognition of a variety of different metal cations by **MGM** was investigated by UV–vis and fluorescence spectroscopy. All absorption and emission spectral studies were performed in freshly purified CH_3CN at room temperature, while the corresponding metal chlorides were used as the source of the metal cations. The absorption spectrum of **MGM** in CH_3CN exhibits a band with a maximum at 375 nm, the intensity of which gradually decreases as the concentration of Cu^{+2} is increased (Supplementary Fig. S3). Concomitantly, a new absorption band builds up at 300 nm, which we originally ascribed to the **MGM**–Cu^{+2} complex.

The fluorescence spectrum of **MGM** (λ_{exc} = 375 nm) showed little or no enhancement in the presence of 200 μM cations such as Fe^{+2}, Fe^{+3}, Ca^{+2}, Co^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Cd^{+2}, Pb^{+2}, or Hg^{+2}. In sharp contrast, there was an enormous increase of its fluorescence intensity upon the addition of 200 μM Cu^{+2} (Fig. 1a). The specificity of the enhancement of the fluorescence of this 'turn-on' compound for Cu^{+2} and a visual indication of the Cu^{2+}-induced fluorescence enhancement are indicated in Figure 1b and Figure 1c, respectively.

Titration of **MGM** with Cu^{+2} resulted in an increase in the fluorescence intensity and a slight blue shift of the fluorescence emission at intermediate Cu^{2+} concentrations (Fig. 2). Upon 375 nm excitation, the emission quantum yield (Φ) of **MGM** was determined to be Φ = 0.0003, using quinine sulfate as standard. Upon addition of 200 μM Cu^{+2} (10 equiv), the quantum yield increased almost 50-fold to Φ = 0.014.

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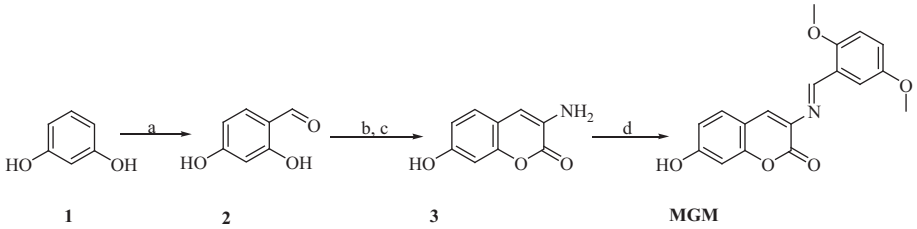

Scheme 1. Synthetic route to MGM. Reagents and conditions: a) $POCl_3$, DMF, acetonitrile, 0–5 °C, 2 h; b) acetylglycine, acetic anhydride, anhydrous sodium acetate, reflux 4 h; c) 2:1 HCl/H_2O reflux, 2 h; d) 2,5-dimethoxybenzaldehyde, EtOH, reflux, 4 h.

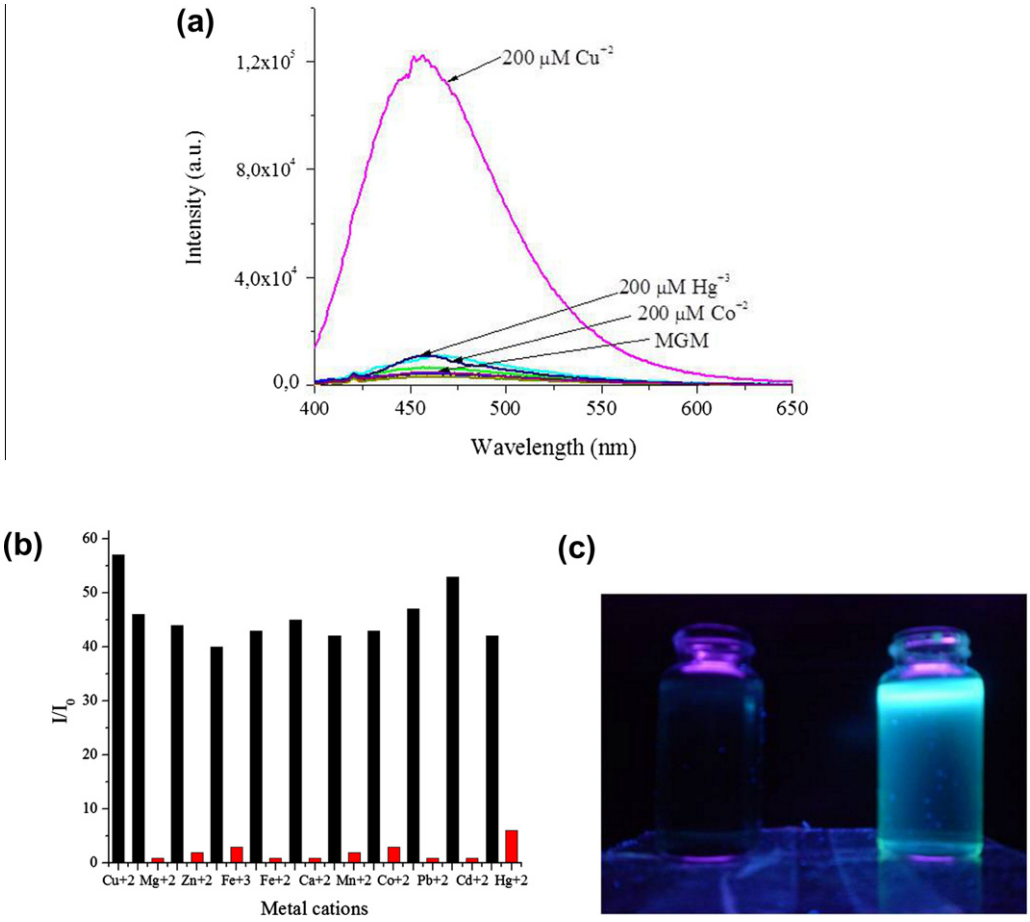

Figure 1. (a) Fluorescence spectra of **MGM** (20 μM) alone and in the presence of several different metal salts (200 μM Fe^{+2}, Fe^{+3}, Ca^{+2}, Cu^{+2}, Co^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Cd^{+2}, Pb^{+2} or Hg^{+2}) in 90% ACN-H_2O mixture. (b) Selectivity of the fluorescence enhancement for **MGM** with Cu^{+2} ion in 90% acetonitrile-H_2O mixture. Red bars indicate the fluorometric responses of **MGM** with 10 equiv of Fe^{+2}, Fe^{+3}, Ca^{+2}, Co^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Cd^{+2}, Pb^{+2}, and Hg^{+2} and black bars represent the fluorescence response after the addition of the same ions and 10 equiv of Cu^{+2}. (c) Photographs of the fluorescence of acetonitrile solutions of **MGM** (20 μM) in the absence (left) and presence (right) of Cu^{+2} (200 μM). All the metal ion solutions were prepared by dissolving their corresponding chloride salts in water.

A Benesi–Hildebrand graph of the fluorescence data (Supplementary Fig. S3) was non-linear, indicating that the stoichiometry of the Cu^{+2} complex formed is different from 1:1. Consistent with this, application of the Method of Continuous Variation resulted in a Job plot (Supplementary Fig. S5) with a maximum at a mole fraction of Cu^{2+} close to 0.33, indicating a preferred 2:1 stoichiometry for the complex.

To determine if the observed changes in absorption and fluorescence are due to chelation of Cu^{2+} by **MGM** the ^1H NMR spectra of the probe in the absence and presence of Cu^{+2} were recorded. As shown in Figure 3, upon addition of Cu^{+2}, the H_b proton signal (the proton at C4 of the coumarin) at 7.81 ppm, shifts upfield to 7.2 ppm, accompanied by more subtle changes in the rest of the aromatic region of the spectrum. More disturbingly, the resonance of the imine proton H_a, at δ 9.31 disappears and a typical aldehyde signal arises at δ 10.25. This was interpreted as indicating that **MGM** undergoes hydrolysis in the presence of Cu^{+2}, releasing 2,5-dimethoxybenzaldehyde and 3-amino-7-hydroxycoumarin.

In fact, we were able to show that 2,5-dimethoxybenzaldehyde, which itself is unusually fluorescent for a benzaldehyde derivative,[9] becomes much more strongly fluorescent upon the addition of Cu^{+2} (Supplementary Fig. S6; Supporting Information), giving the same emission spectrum observed when **MGM** was treated with Cu^{+2}. Moreover, the fluorescence of 3-amino-7-hydroxy-coumarin is quenched by Cu^{+2} (Supplementary Fig. S7), and therefore does not interfere with the emission of the 2,5-dimethoxybenzaldehyde-Cu^{+2} complex (Supplementary Fig. S8). Recent work presented similar imino systems, which are not fluorescent.[10] However, in the

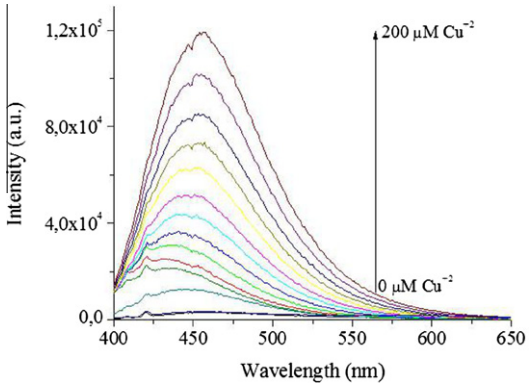

Figure 2. Fluorescence spectra of **MGM** (20 μM) with different concentrations of Cu^{+2}.

presence of Cu^{+2} they are hydrolyzed to regenerate the amine and 3-formylcoumarin reactants. In these cases complexation of the free 3-formylcoumarin with Cu^{+2} is responsible for the increase in fluorescence.

Copper-selective probes are relatively few in number,[11] and those that can be employed in biological systems are even scarcer. **MGM** was therefore evaluated for its potential as a probe for intracellular Cu^{+2} in SH-SY5Y human neuroblastoma cells. The cells were initially incubated with **MGM** under physiological conditions and then subsequently treated with the histidine-Cu^{+2} complex as the source of Cu^{2+}. Fluorescence was monitored using a microplate fluorescence reader and by epifluorescence microscopy (Fig. 4). The fluorescence was monitored using a microplate fluorescence reader and by epifluorescence microscopy (Fig. 4). The fluorescence was significantly increased (Fig. 4B) following the addition of histidine-Cu^{+2}, with most of the fluorescent species residing near the cell periphery.

As 2,5-dimethoxybenzaldehyde might conceivably be released in the extracellular medium by hydrolysis of **MGM**, we incubated SH-SY5Y cells with the aldehyde (10 μM, 20 min) under the same

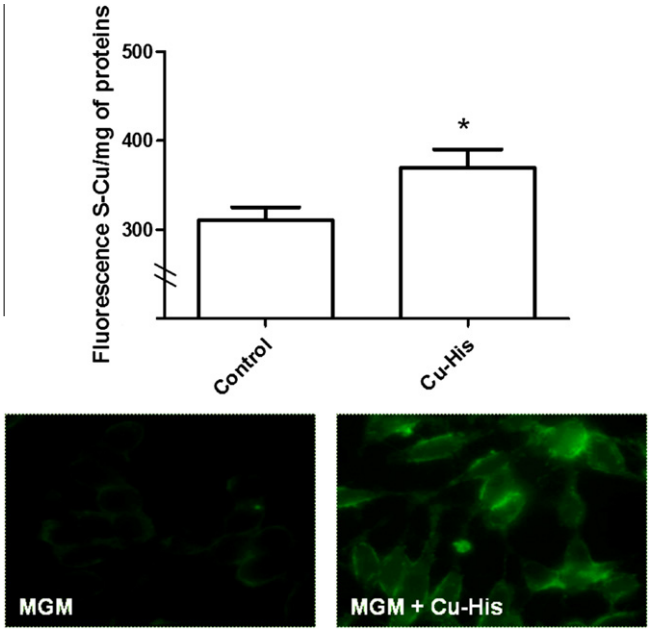

Figure 4. In vitro tests of the potential of **MGM** as an intracellular probe for Cu^{2+}. SH-SY5Y cells were incubated with the probe (10 μM, 20 min), washed, and the basal fluorescence measured (A). The cells were then incubated with Cu-His (200 μM, 15 min) and fluorescence measured (B) in a microplate fluorescence reader and by epifluorescence microscopy. The bar graph represents the mean ± -SEM of the data; $n = 6$, $P < 0.01$(*).

conditions as the experiments carried out with **MGM**. After washing the cells no fluorescence was observed and, more importantly, none was observed after treatment with histidine-Cu^{+2} complex. We therefore concluded that 2,5-dimethoxybenzaldehyde does not enter the cells. On the contrary, **MGM** must act as a cell-penetrating carrier that enters the cells and, once inside, releases 2,5-dimethoxybenzaldehyde which fluoresces strongly when copper is present in the intracellular medium.

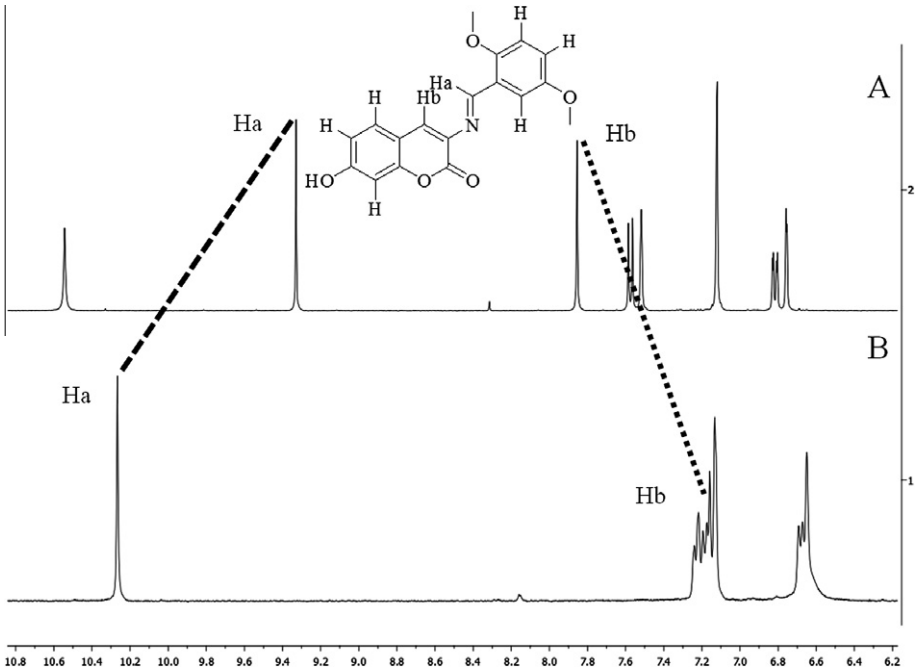

Figure 3. Changes in the ^1H NMR spectrum of **MGM** (20 mM) upon addition of Cu^{+2} (10 equiv) in DMSO–d_6 at 25 °C. (A) **MGM** only; (B) **MGM** + Cu^{+2} (after 10 min).

In conclusion, we have prepared and characterized the novel fluorescence 'turn-on' sensor imine **MGM** that exhibits a strong, highly selective response to Cu^{+2} in vitro and in cultured cells mediated by the Cu-mediated release of its aldehyde constituent.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.07.082.

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