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Synthesis of a fluorogenic probe for thiols based on a coumarin schiff base copper complex and its use for the detection of glutathione

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ABSTRACT

Glutathione is the most abundant non-protein thiols compound in cells and plays important metabolic roles. Changes in the amount of glutathione or its metabolic dysregulation can lead to a series of diseases. The determination of glutathione levels is very helpful to the diagnosis and treatment of the related diseases. A coumarin schiff base (compound **1**) was synthesized from coumarin hydrazide and 2,6-pyridine dicarboxaldehyde and the fluorogenic probe for thiols (compound **1-Cu^{2+}**) was prepared by coordinating compound **1** with copper ions. Compound **1** showed strong fluorescence, while compound **1-Cu^{2+}** hardly had fluorescence due to the paramagnetism and/or photoinduced electron transfer of Cu^{2+}. However, after the addition of thiols-containing compounds, the fluorescence of compound **1** was restored. The UV–vis absorption and fluorescence spectra indicated that the fluorogenic probe had good thiols selectivity and sensitivity, particularly for glutathione in CH_3CN:HEPES (3:2, v/v) buffer. It was successfully applied to the fluorescence imaging detection of glutathione in human cervical squamous cancer cells (SiHa cells).

1. Introduction

The thiols in cells, such as cysteine, homocysteine, and glutathione, play important roles in the regulation of various physiological and pathological processes in humans as vital bioactive compounds. The endogenous concentrations of these thiols suggest the functional state of the corresponding enzymes and proteins and their abnormal levels correlate with diseases.[1] Glutathione is the most abundant non-protein thiols compound in cells, with concentration ranges from 1 to 10 mmol/L.[2] Glutathione can remove toxic and hazardous compounds and metabolites, maintain the completeness of the erythrocyte membrane, reduce DNA damage and mutation by reducing radical attacks to DNA, participate in the reduction of methemoglobin, and promote iron absorption.[3] Metabolic dysregulation or the change in glutathione levels in organisms can lead to various diseases including cancers, Alzheimer's disease, cardiovascular diseases, leukocyte loss, psoriasis, liver damage and AIDS.[4] Measuring the level of glutathione in living organisms is helpful to diagnose relevant diseases.[5] Therefore, the development of fast, convenient, accurate and sensitive methods to measure physiological and clinical glutathione levels has attracted wide attention.

Current methods to detect thiols include high performance liquid chromatography, capillary electrophoresis, UV–vis spectrometry, mass spectrometry, electrochemical analysis, and surface enhanced Raman scattering. Fluorescence detection was considered to be a convenient method due to its simplicity, sensitivity, and high efficiency.[6] To date, researches on thiols fluorogenic probe s have shown significant development. Many organic reactions have been applied to the synthesis of such probes, namely: Michael addition,[7] nucleophilic substitution,[8] disulfide exchange reactions,[9] cyclization reactions between aldehydes and aminothiols,[10] and demetallation.[11] However, due to the fact that most thiols in vivo have very weak or no fluorescence, a high sensitivity fluorophore is required for this application. Because of the similarities in the structures and properties of various thiols compounds, design and preparation of a probe with high selectivity and sensitivity is still a great challenge.

Coumarins are often used as chromophores in the preparation of highly effective fluorogenic probes due to their highly intense fluorescence, good solubility, ease of preparation, relatively high

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molar absorption coefficient and fluorescence quantum yield.[12] The excitation wavelength of coumarins falls within the visible range, and its derivatization and modification are simple.[13] Herein, in this paper we report a new fluorescence probe **1-Cu^{2+}**, which was produced by coordination between a coumarin derivate **1** and Cu^{2+} ions. The precursor compound **1** was prepared by a simple schiff base condensation reaction of 7-(diethylamino)-2-oxo-2H-chromen-e-3-carbo- hydrazide (**2**) and pyridine-2,6-dicarbaldehyde. This probe could be used for rapid, highly selective and sensitive detection of thiols. The process and mechanism of detection by this probe were investigated by UV–vis, fluorescence spectra titration and mass spectrometry.

2. Experiment section

2.1. Instruments

^{1}HNMR and ^{13}CNMR spectra were measured on a Bruker Ascend™ 400 spectrometer with chemical shifts reported as ppm with TMS as internal standard. Mass spectrometric data were obtained with a Bruker Microtof-QIII spectrometry. UV–vis absorption spectra were recorded with Shimadzu UV2550 spectrophotometer. Fluorescence spectra were recorded with Edinburgh Instruments FS-5 fluorescence spectrophotometer. Cell imaging was recorded with Nikon Eclipse TE2000-S.

2.2. Reagents

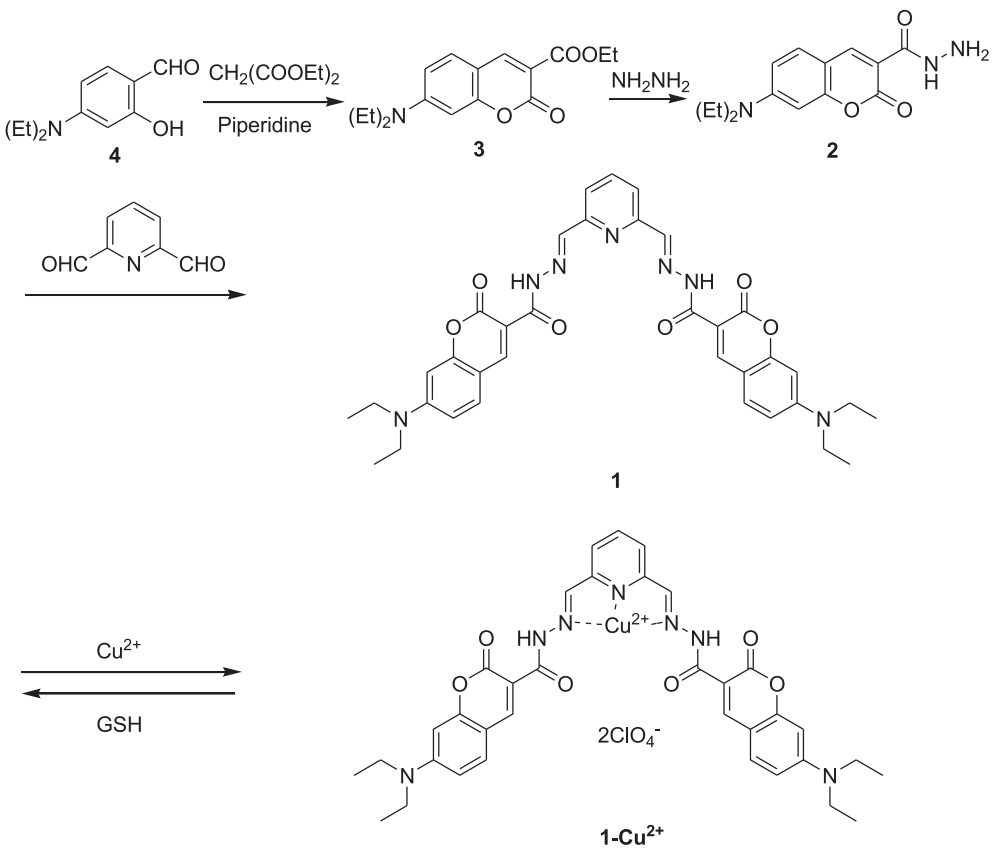
All the chemicals were of analytical grade and used as received. 2,6-pyridine dicarboxaldehyde was purchased from Tianjin Heowns Biochemical Technology Co., Ltd. 4-(Diethylamino)-2-hydroxybenzaldehyde, diethyl malonate, piperidine, hydrazine hydrate (80%) and the other reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. Stock solutions (2.0×10^{-2} M) of the perchlorate Na^+, Mg^{2+}, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Pb^{2+} and the amino acids plus GSH were prepared in aqueous solutions. Stock solutions of compound **1** and compound **1-Cu^{2+}** (10 μM) for spectral measurement were prepared in CH_3CN:HEPES (3:2, v/v) solution. Stock solutions of compound **1** and compound **1-Cu^{2+}** for fluorescence imaging in cells were prepared in DMSO solution. DMSO has a better solubility for compound **1** and compound **1-Cu^{2+}** and the lower cytotoxicity. Each time a 3 mL compound **1** or compound **1-Cu^{2+}** was filled in a quartz cell of 1 cm optical path length, and different stock solutions of metal ions or amino acids were added into the quartz cell gradually by using a micro-syringe.

2.3. Synthetic procedure

The synthetic pathway for compound **1** was shown in Scheme 1. 7-N,N-dimethylamino-2-oxo-2H-3-coumarate (compound **3**) and coumarin hydrazine (compound **2**) were prepared according to previous reports.[14] Compound **1** was synthesized from compound **2** and 2,6-pyridine dicarboxaldehyde, and characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS and IR spectra (Figs. S1–S4). Compound **1-Cu^{2+}** was prepared from compound **1** and $Cu(ClO_4)_2$ in ethanol and characterized by ESI-MS and IR spectra (Figs S5–S7, S11). ESI-MS showed a 1:1 M ratio between compound **1** and $Cu(ClO_4)_2$.

2.3.1. Synthesis of ethyl 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylate[14a]

Compound **4** (3.8648 g, 0.02 mol), diethyl malonate (6.4067 g,



Scheme 1. Syntheses of compound **1** and compound **1-Cu^{2+}**.

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0.04 mol) and piperidine (2 mL) were mixed in 60 mL anhydrous ethanol. The mixture was refluxed for 6 h, cooled to room temperature, and evaporated to give compound **3** as orange oil, which was used directly for the next step.

2.3.2. Synthesis of 7-(diethylamino)-2-oxo-2H-chromene-3-carbohydrazide[14b]

Compound **3** (4.3399 g, 15 mmol) and hydrazine monohydrate (3.64 mL, 60 mmol, 80%) were added into an ethanol (40 mL) solution. The mixture was stirred for 12 min at room temperature. After cooling in an ice bath for 15 min, the precipitate was filtered and purified by chromatography (mobile phase, ethyl acetate) to give compound **2** as orange needles, mp 161–162 °C. Yield: 83%.

2.3.3. Synthesis of compound *1*

Compound **2** (0.3028 g, 1.1 mmol) and 2,6-pyridine dicarboxaldehyde (0.0676 g, 0.5 mmol) were dissolved in ethanol (30 mL). After refluxed for 12 h under nitrogen and cooled to room temperature, the obtained yellow precipitate was filtered, washed and dried to afford compound **1**. ^{1}H NMR (400 MHz, CF_3COOD): δ (ppm) 9.53 (s, 2H), 8.95 (s, 2H), 8.92–8.79 (m, 1H), 8.73–8.56 (m, 2H), 8.44–8.31 (m, 2H), 8.04–7.94 (m, 2H), 7.86–7.81 (m, 2H), 3.99 (q, J = 6.9 Hz, 8H), 1.40 (t, J = 7.0 Hz, 12H); ^{13}C NMR (100 MHz, CF_3COOD): δ (ppm) 155.1, 152.2, 148.7, 145.9, 142.2, 140.4, 139.9, 133.8, 127.9, 120.3, 119.7, 117.5, 115.2, 111.7, 55.5, 9.0. ESI-MS: m/z: 650.2672, [compound **1** + H]$^+$; 672.2513, [compound **1** + Na]$^+$.

2.3.4. Synthesis of probe compound *1-Cu^{2+}* and other complexes

Compound **1** (0.0065 g, 0.01 mmol) and excess $Cu(ClO_4)_2\cdot 6H_2O$ (0.0074 g, 0.02 mmol) were dissolved in ethanol (20 mL). After refluxed for 30 min, the obtained precipitate was filtered, washed and dried to afford compound **1-Cu^{2+}**. Yield: 79%. ESI-MS: m/z: 711.1933. In order to investigate the behaviors of compound **1** with other metal ions, complexes such as compound **1-Co^{2+}**, compound **1-Zn^{2+}**, and compound **1-Mg^{2+}** were also prepared in a similar method, and further characterized by ESI-MS (Figs. S8–S10).

3. Results and discussions

3.1. The experiment of water solubility

Compound **1** has a relatively high fluorescence intensity and dissolved completely in CH_3CN:HEPES (3:2, v/v) buffer, as shown in Fig. S12. Therefore, this ratio is suitable for the spectral experiments.

3.2. The dependence of pH

The effect of pH on the fluorescence intensity for compound **1** (10 μM) treated with Cu^{2+} (4 equiv.) and GSH (2 equiv.) in CH_3CN:HEPES (3:2, v/v) buffer was showed in Fig. S13. The fluorescence intensity changed less than 10% in the range of 6.0–8.0, which indicated that compound **1** could be applied in physiological condition.

3.3. The UV–vis absorption and fluorescence spectra responses of compound *1* to Cu^{2+} ions

The UV–vis absorption spectrum of compound **1** (10 μM) in CH_3CN:HEPES (3:2, v/v) solution was shown in Fig. 1. The maximum UV–vis absorption peak of compound **1** was at 440 nm. Upon addition of $Cu(ClO_4)_2$, the UV–vis absorption peak of compound **1** at 440 nm gradually decreased. After the addition of 6 equivalents of Cu^{2+}, its peak value reached equilibrium. The UV–vis absorption spectra of compound **1** (10 μM) with other metal ions (such as Na^+, Mg^{2+}, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cu^+, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Pb^{2+}, as ClO_4^- salt) were shown in Fig. S14. The result indicated that Cu^{2+} showed the most significant response to compound **1** in the UV–vis absorption spectrum, other metal ions had weak impacts on the absorption.

The fluorometric titration experiment of compound **1** (10 μM) with Cu^{2+} was proceeded in CH_3CN:HEPES (3:2, v/v) solution (Fig. 2). compound **1** exhibited a strong fluorescence emission peak at 485 nm (excited at 440 nm). Upon addition of $Cu(ClO_4)_2$, the fluorescence intensity of compound **1** gradually decreased. After the addition of 4 equivalents of Cu^{2+}, the peak intensity reached equilibrium. The low fluorescence intensity of the compound **1-Cu^{2+}** might be due to the quenching effect resulted from the paramagnetism and photoinduced electron transfer of the copper ion.[15] As a comparison, the fluorescence responses of compound **1** to the other metal ions (such as Na^+, Mg^{2+}, K^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cu^+, Zn^{2+}, Ag^+, Cd^{2+}, Hg^{2+}, Pb^{2+}, as ClO_4^- salt.) were also investigated (Fig. S15), the results were

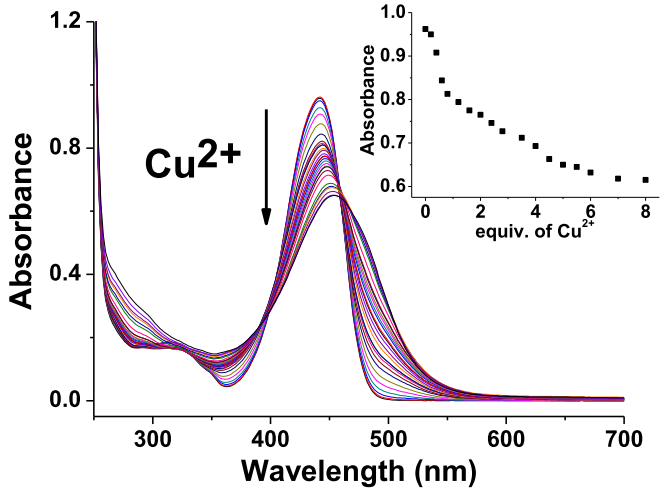

Fig. 1. The UV–vis spectra titration of compound **1** (10 μM) upon addition of Cu^{2+} in CH_3CN:HEPES (3:2, v/v) solution. Inset: UV–vis titration profile of compounds **1** upon addition of Cu^{2+}, the absorption spectra were recorded at 440 nm.

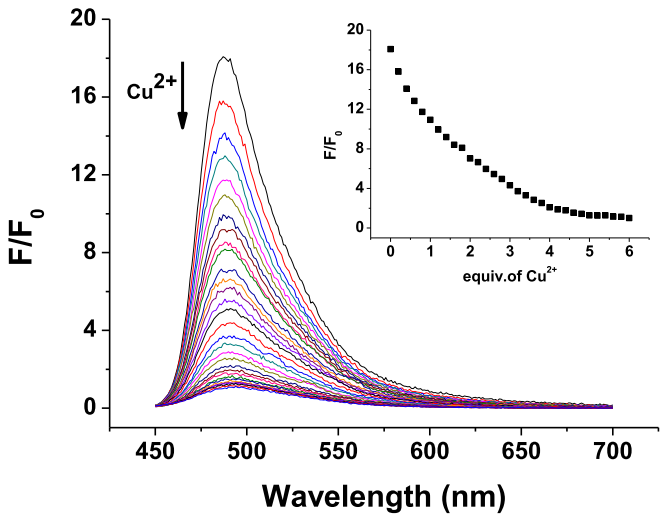

Fig. 2. The fluorescence spectra titration of compound **1** (10 μM) upon addition of Cu^{2+} in CH_3CN:HEPES (3:2, v/v) solution. Insert: fluorescence titration profile at 485 nm upon the addition of Cu^{2+} (excited at 440 nm).

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consistent with that observed in UV–vis titration experiments, in which Cu^{2+} showed the most significant quenching effect to compound **1.** Other metal ions especially Cu^{+} had no influence to compound **1**, and Co^{2+} ion had relative weak influence than Cu^{2+} ion.

Furthermore, the solid form of compound **1-Cu^{2+}** was prepared in ethanol and characterized by ESI-MS and IR spectra. The evidence of complexation behavior of compound **1** ($C_{35}H_{35}N_7O_6$) with Cu^{2+} came directly from ESI-MS spectrum. As shown in Fig. S5, the predominated peak at m/z = 711.1933 corresponding to [(compound **1**)+Cu^{2+}-H^{+}] = $C_{35}H_{34}CuN_7O_6^{+}$ was observed in ESI-MS spectrum. The experimental isotopic patterns (Fig. S6) fit well with the theoretical simulation result (the predominated peak at m/z = 711.1966, Fig. S7) calculated by using the IsoPro 3.0 program. Such results indicated that the composition of compound **1-Cu^{2+}** is 1: 1 M ratio between compound **1** and Cu^{2+}.

IR spectrum of compound **1-Cu^{2+}** revealed the possible coordination model of compound **1** with copper ions (Scheme 1). IR spectrum of compound **1** exhibited characteristic peaks of certain groups such as -C=N- group (1582.63 cm^{-1}), the carbonyl group of -CO-NH- unit (1618.05 cm^{-1}) and the carbonyl group of the coumarin unit (1700.47 cm^{-1}).[11a,16] After coordinated with copper ions, the characteristic stretching band of the -C=N- group was shifted to lower value (1573.50 cm^{-1}) while the carbonyl stretching bands of the -CO-NH- unit (1617.82 cm^{-1}) and coumarin unit (1701.39 cm^{-1}) only shifted slightly. It was assumed in all probability that the -C=N- group participated in the coordination while the carbonyl groups of the -CO-NH- and coumarin units were not involved in the coordination.[17]

3.4. The fluorescence spectra response of the probe (compound 1-Cu^{2+}) to thiols

The fluorometric titration experiment of probe (compound **1-Cu^{2+}**) with glutathione was proceeded in CH_3CN:HEPES (3:2, v/v) solution. As shown in Fig. 3, along with glutathione added, the fluorescence intensity of this probe (compound **1-Cu^{2+}**) was gradually restored, which may caused by demetallation effect of the probe to released compound **1** into the solution. After 2 equivalents of glutathione added, the fluorescence intensity of the solution reached equilibrium. Therefore, compound **1-Cu^{2+}** could be used for the detection of glutathione with fluorescence response "turn-on" manner.

To further investigate the selectivity of the probe **1-Cu^{2+}** for thiols, the fluorescence responses of the probe to different amino acids were measured (Fig. 4). The fluorescence intensity of the thiols probe itself was very weak. When 2 equivalents of thiols-containing amino acids (excess), such as N-acetyl-L-cysteine, DL-homocystine, L-homocysteine, L-cysteine and GSH, were added respectively, the fluorescence intensity increased. Among these thiols-containing compounds, GSH exhibited the most obvious enhancement. The reason might be that GSH has the propensity of generating Cu–S bond, which may grab the Cu^{2+} ions from probe and lead to a demetallation reaction of the probe, however Cys and

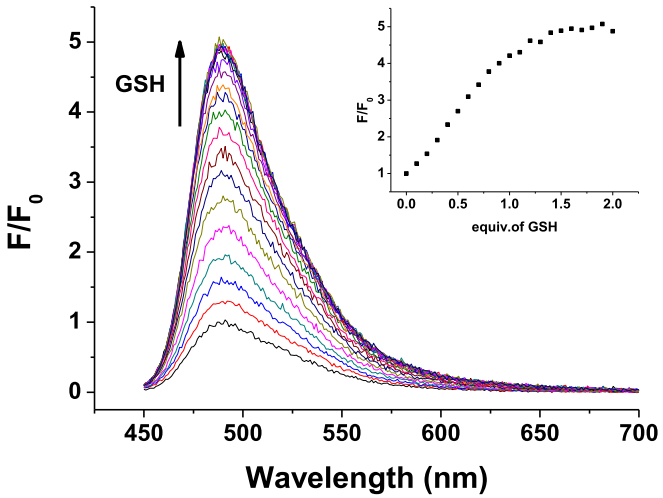

Fig. 3. The fluorescence responses of compound **1-Cu^{2+}** (10 μM) upon addition of GSH in CH_3CN:HEPES (3:2, v/v) solution upon the addition of increasing GSH. Insert: fluorescence titration profile of compound **1-Cu^{2+}** at 485 nm upon the addition of GSH (excited at 440 nm).

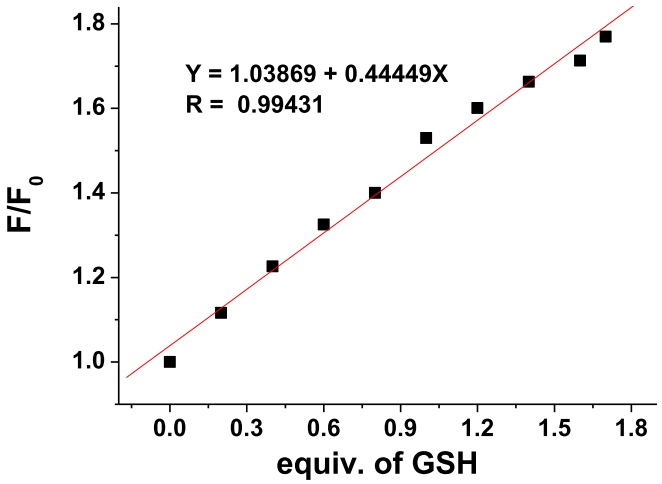

Fig. 5. Fluorescence intensity (485 nm) of compound **1-Cu^{2+}** (1 μM) in CH_3CN:HEPES (3:2, v/v) solution upon additions of GSH with an excitation wavelength at 440 nm.

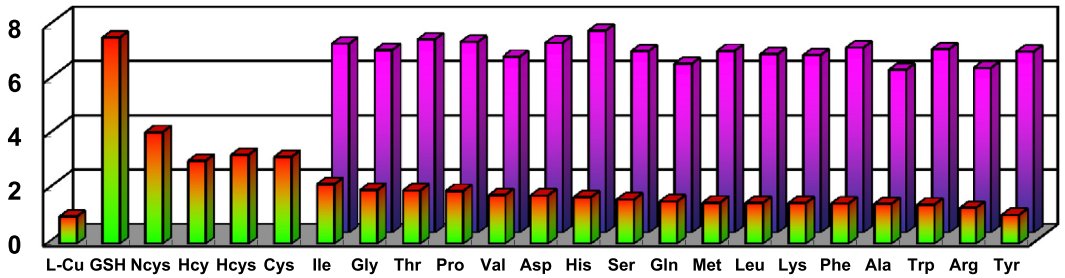

Fig. 4. The fluorescence intensities of compound **1-Cu^{2+}** (10 μM) to different amino acids (λ_{em} = 485 nm). The first row showed the fluorescence intensities upon addition of 2 equivalents of different amino acids; the second row showed the fluorescence intensities after subsequent addition of 2 equivalents of GSH to the non-sulfhydryl amino acids solution, respectively.

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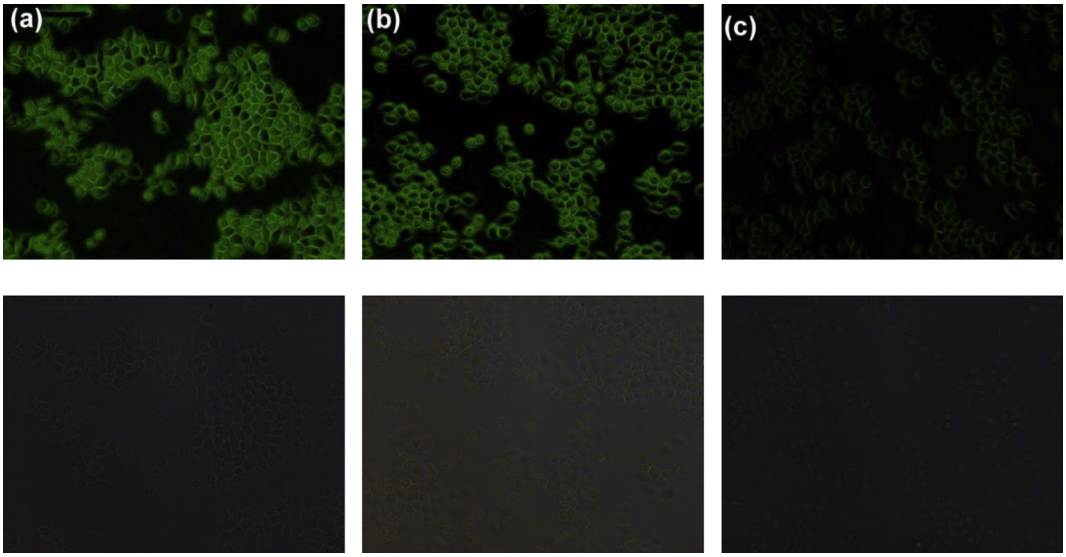

Fig. 6. The fluorescence images of SiHa cells incubated with **1** (a), SiHa cells incubated with probe (b), SiHa cells pretreated by erlotinib then incubated with probe (c) and their corresponding bright field images.

Hcy tend to form Cu–N bond, which is less stable than Cu–S bond formed by GSH.[18] Thus GSH manifested a higher fluorescence response than Cys/Hcy. Meanwhile, as a control group, addition of 2 equivalents of non-sulfhydryl amino acid did not result in obvious change of the fluorescence intensity. Subsequently 2 equivalents of GSH were added, the fluorescence intensity significantly increased, which indicated that compound **1-Cu^{2+}** had a particular response to GSH, and the response was not disrupted by other competitive non-thiols amino acids. The above experiments demonstrated that compound **1-Cu^{2+}** had good selectivity for GSH.

3.5. The detection limit of the probe (compound *1-Cu^{2+}*) for GSH

To further investigate the sensitivity of the probe **1-Cu^{2+}** for GSH, the detection limit experiment for GSH was performed. When the probe was employed at 1 μM and the slits were adjusted to 1.5 nm/1.5 nm, the fluorescence intensity at 485 nm was linearly proportional to the concentration of GSH in the range of 0.0–1.8 μM (Fig. 5), indicating the potential capability of compound **1-Cu^{2+}** for quantitative detection for GSH. The detection limit for GSH was determined to be 0.2 μM, which was significantly below the physiological levels of GSH in live cells (1–10 mM).[19] In vivo experiments, we utilize the 10^{-7} order of magnitude probe to incubate the SiHa cells at 37 °C for 60 min. Under Nikon Eclipse TE2000-S fluorescent inverted microscope, green fluorescence could still be observed. The result indicated that in vivo the probe still keep very sensitive to GSH (Fig. S17).

3.6. The fluorescence imaging of the probe (compound *1-Cu^{2+}*) for GSH

Cell imaging with a fluorogenic probe had important significance and application.[20] The fluorescence imaging of the probe (compound **1-Cu^{2+}**) for GSH was performed in cervical cancer SiHa cells. Before the fluorescence imaging, SiHa cells were cultured in a 12-well cell culture plate for 24 h, incubated with compound **1** (3 μM) and the probe (3 μM) at 37 °C for 60 min respectively, and subsequently washed twice with PBS solution. Under Nikon Eclipse TE2000-S fluorescent inverted microscope, the images of the SiHa cells were shown in Fig. 6 (Fig. 6a as control). Compound **1** showed significant intracellular green fluorescence in SiHa cells when excited with blue light (Fig. 6a), which indicated that compound **1** had strong cell permeability. The probe (compound **1-Cu^{2+}**) was used directly to stain the SiHa cells (Fig. 6b), and the significant intracellular green fluorescence in SiHa cells was also observed, which indicated that the fluorescence of probe could be restored by GSH in SiHa cells. Another well was pretreated by erlotinib (Fig. 6c) (an anticancer drug) for 1 h. The intracellular green fluorescence intensity is relative weak than that without pretreated by erlotinib, which might be due to treatment by erlotinib could create a redox stress environment and decrease the intracellular GSH content.[21] The results above proved that the probe (compound **1-Cu^{2+}**) could determine the intracellular GSH.

Then MTT experiments (Fig. S16, Table 1) were proceeded, and the results demonstrated that the cells were visible and showed no significant toxicity or side effects when the probe concentration was less than 10 μM (viability was higher than 75%, approximately 16 h). The fluorescent imaging experiment indicated that the thiols fluorogenic probe (compound **1-Cu^{2+}**) could be used for the detection of GSH in living cells.

4. Conclusion

In summary, a new thiols fluorogenic probe (compound **1-Cu^{2+}**) was prepared. The fluorescence spectra indicated that the probe had relatively good selectivity and sensitivity to GSH in CH_3CN:HEPES (3:2, v/v) solution. And the detection limit for GSH was determined as 0.2 μM, suggesting that the probe is sensitive enough for the determination of GSH. On the other hand, the fluorogenic probe at pH 6.0–8.0 conditions was stable, indicating that the probe could be used under physiological condition. In addition, the probe showed good cell membrane permeability and low toxicity to cells and could be used for the detection of GSH in living cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2016.12.012.

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