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A novel hexahydroquinazolin-2-amine-based fluorescence sensor for Cu²⁺ from isolongifolanone and its biological applications[†]

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Pyrimidine-based derivatives 2a-2c were synthesized from renewable isolongifolanone, and compound 2c

exhibited high selectivity and sensitivity toward Cu^{2+} ions with a low detection limit of 4×10^{-8} M, a wide pH

range (5-11), and a short response time (30 s). The sensor still retained good fluorescence selectivity to Cu²⁺

ions when applied to fluorescence imaging in living black mice. Therefore, compound 2c can be used as an

effective fluorescence probe and imaging agent for detecting Cu²⁺ ions.

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Introduction

In recent years, selective chemo-sensors for recognizing specific metal ions have attracted more and more research interests. In particular, there have been advances in the development of sensors for detecting and tracking transition metal ions, such as Cu²⁺, Fe³⁺, Cr³⁺, and Cd²⁺, which are important in chemical and biological science, and the excellent properties of these sensors make them capable of being used as an efficient way to protect the environment.¹⁻⁹ Copper is an essential trace element and exists in the form of copper protein in the human body. Furthermore, copper mainly acts as biocatalyst in the human body and plays a pivotal role in hematopoiesis, angiomalacia, metabolism and hormone secretion.10 In spite of these good biological functions, excessive levels of copper can result in many kinds of diseases such as Menkes or Wilson disease,11 Alzheimer's disease,12-14 and prion disease.15 Copper can be ingested from polluted water and foods. Thus, the detection of copper ions in the environment and in vivo is important for human health.

A fluorescence probe is a simple, fast, and sensitive way of detecting copper ions compared with traditional methods such as electrochemistry,¹⁶ spectrophotometry,¹⁷ voltammetry,¹⁸ and atomic absorption spectroscopy.¹⁹ Many sensitive fluorescence probes for Cu²⁺ have been reported, such as ferrocenyl,²⁰ benzimidazole,²¹ benzothiazole,^{22,23} pyrene,^{24,25} BODIPY,^{26,27} rhodamine,^{28–31} *etc.* Pyrimidine is a typical nitrogen heterocyclic compound and has many structure sites that can be modified. Pyrimidine has been widely used in the fields of pesticides³² and medicine in the past few years,^{33,34} but there are few reports on its use in fluorescence probes. Due to the nitrogen heterocycle, pyrimidine derivatives can bind metal ions to serve as a potential probe.

Isolongifolanone is prepared from natural longifolene by oxidation,^{35,36} and it can serve as a renewable terpenoid material to synthesize various derivatives such as α,β -unsaturated ketones, pyrazole, and pyrimidine; these derivatives have been used in the fields of anophelifuges and antineoplastics.³⁷ However, there is no reported derivative that has been used for detecting metal ions and other substances as a florescence probe. In addition, longifolene is a main component of natural turpentine with great biological compatibility. Therefore, longifolene derivatives have good cell membrane permeability. This excellent cell membrane permeability enables the derivatives to be used for bio-imaging in organisms. In view of the above-mentioned merits, we believe that we can design some useful compounds as probes based on isolongifolanone.

In this study, we synthesized three pyrimidine-based derivatives **2a-2c** from isolongifolanone and investigated whether the fluorescence properties were affected by the locations of pyridine. After that, compound **2c** was selected as a moderate florescence probe from the three compounds. Compound **2c** showed obvious fluorescence quenching with the addition of Cu^{2+} ions. The optical properties of compound **2c** towards Cu^{2+} ions have been investigated using various instruments and

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means, and in addition, compound 2c has been successfully applied to the identification of Cu^{2+} ions *in vivo* in black mice.

Results and discussion

Synthesis

The synthetic route of compounds 2a-2c is shown in Scheme 1. Compounds 1a-1c were first synthesized by the aldol condensation reaction of isolongifolanone and pyridinecarboxaldehyde, and then compounds 2a-2c were synthesized by a reaction of compound 1a (1b or 1c) and guanidine hydrochloride in the presence of potassium tert-butylate in tert-butyl alcohol. The synthesized compounds 2a-2c were characterized by IR, NMR and HRMS techniques. Moreover, compound 2c was characterized by single-crystal X-ray diffraction (Table S1, ESI⁺ and Fig. 1). These analyses confirmed 2c to be 6,6,10,10-tetramethyl-4-(pyridine-4'-yl)-5,7,8,9,10,10a-hexahydro-6H-6a,9methanobenzo[*h*]quinazolin-2-amine.

UV-vis and fluorescence properties in solution

In order to investigate the fluorescence properties of the three compounds (2a-2c) in the solution state, the compounds were separately dissolved in 8/2 (v/v) ethanol-water solution (1 \times 10^{-4} M). The compounds (2a-2c) appeared as colourless and transparent solutions under sunlight (Fig. S1a[†]), while the compounds (2a-2c) could emit fluorescence under 365 nm UV light. Compounds 2a and 2c emitted strong blue light, but compound 2b emitted weaker light under UV light (Fig. S1b†), therefore the locations of the pyridine substituents have an influence on the fluorescence properties in solution. Compared to the insignificant colour change of compounds 2b and 2c with the addition of 1.5 mM copper ions, the colour of compound 2a changed from colourless to pale yellow under sunlight (Fig. S1c[†]); this may indicate that the coordinating ability of compound 2a with Cu²⁺ ions was superior to that of compounds 2b and 2c. Compounds 2a-2c quench fluorescence in the presence of Cu²⁺ ions under 365 nm UV light, but only compound 2b showed a faint quenching change due to its own weak fluorescence (Fig. S1d[†]). We concluded that compounds



Fig. 1 X-ray crystal structure of compound 2c.

2a and **2c** could serve as fluorescence probes for detecting Cu^{2+} ions, so compound **2c** was selected as a moderate sample to study its optical properties.

Compound 2c in the liquid state $(1 \times 10^{-5} \text{ M})$ emitted bright blue light when it was dissolved in different organic solvents such as ethanol, methanol, acetonitrile, ethyl acetate, and dichloromethane. The sunlight and fluorescence responses of compound 2c were further investigated with the addition of 150 μ M of various metal ions such as Zn^{2+} , Hg^{2+} , Fe^{2+} , Fe^{3+} , Cu^{2+} , Cr^{3+} , Co^{2+} , La^{3+} , Ag^+ , Al^{3+} , and Pb²⁺ in ethanol-water media (v/ v = 8/2, pH = 7.2). The solutions of compound 2c maintained their original colours in the presence of metal ions under sunlight (Fig. 2a). However, the solution changed from bright blue to colourless after the addition of Cu²⁺ under 365 nm UV light (Fig. 2b). Meanwhile, the addition of the other metal ions did not lead to fluorescence quenching. Thus, the results showed that the new compound could be used as a novel fluorescence sensor for identifying copper ions.

The UV-visible spectrum of compound **2c** $(1 \times 10^{-4} \text{ M})$ was measured in 8/2 (v/v) ethanol–water solution (20 mM HEPES, pH = 7.2). It had two main absorption peaks; the narrow band was strong at 225 nm and the broad band at 325 nm was very



Scheme 1 Synthesis of compounds 2a-2c.



Fig. 2 Photographs of compound $2c (1 \times 10^{-5} \text{ M})$ with the addition of various metal ions (1.5 \times 10⁻⁴ M) in CH₃CH₂OH-H₂O (v/v = 8/2, 20 mM HEPES buffer, pH = 7.2) solution under sunlight (a) and 365 nm UV light (b).

weak (Fig. S2[†]). The complexing interaction of compound **2c** with metal ions (1 mM) such as Zn^{2+} , Hg^{2+} , Fe^{2+} , Fe^{3+} , Cu^{2+} , Cr^{3+} , Co^{2+} , La^{3+} , Ag^+ , Al^{3+} , and Pb^{2+} was observed using UV-vis spectroscopy. The results showed that the absorbance of compound **2c** at 225 nm obviously increased with the addition of La^{3+} , Ag^+ , Co^{2+} , and Pb^{2+} and the weak band in the range 280–325 nm became more intense in the case of Cu^{2+} ions.

To investigate the binding properties of compound **2c** with Cu^{2+} ions, we measured the UV-vis absorption spectra of compound **2c** $(1 \times 10^{-4} \text{ M})$ with the addition of various amounts of Cu^{2+} ions $(0-1.6 \times 10^{-3} \text{ M})$ in 8/2 (v/v) ethanol-water solution (20 mM HEPES, pH = 7.2). As shown in Fig. S3,† with increasing concentrations of Cu^{2+} ions $(0-1.6 \times 10^{-3} \text{ M})$, the UV-vis absorption spectra of compound **2c** show a gradually enhanced intensity at 325 nm, whereas another absorption peak at 225 nm had no obvious change with the increasing concentrations of Cu^{2+} ions. It is possible that the complexation of compound **2c** with Cu^{2+} ions led to this tendency of the absorption peak.

Next, the selectivity of compound $2c (1 \times 10^{-5} \text{ M})$ was also evaluated after adding 10 equivalents of different metal ions using a fluorescence spectrophotometer (Fig. 3a and b). All the salts of the Pb²⁺, Ag⁺, Al³⁺, Co²⁺, Cr³⁺, Fe³⁺, Hg²⁺, La³⁺, Zn²⁺, Fe²⁺, and Cu²⁺ ions and compound **2c** were dissolved in 8/2 (v/v) ethanol-water solution (20 mM HEPES, pH = 7.2). A slight



Fig. 3 (a) Fluorescence intensity of compound $2c (1 \times 10^{-5} \text{ M})$ in response to different metal ions $(1 \times 10^{-4} \text{ M})$ in CH₃CH₂OH-H₂O (v/ v = 8/2, 20 mM HEPES buffer, pH = 7.2) solution. (b) Fluorescence intensity ($(F_0 - F)/F_0$) of compound $2c (1 \times 10^{-5} \text{ M})$ at 450 nm in the HEPES buffer solution upon addition of various metal ions. Excitation wavelength of 325 nm; em. slit of 12 nm; ex. slit of 5 nm.

fluorescence enhancement occurred with the addition of La^{3+} , Al^{3+} , and Ag^+ , while the addition of Co^{2+} , Cr^{3+} , Fe^{3+} , Hg^{2+} , Zn^{2+} , and Fe^{2+} into the solution of compound **2c** led to a tiny fluorescence decrease. Compound **2c** only displayed sharp fluorescence quenching after the addition of Cu^{2+} ions. Compared to Cu^{2+} ions, the other metal ions could not induce such an obvious change of the fluorescence spectrum. Therefore, compound **2c** could be used as a highly selective fluorescence quenching probe for Cu^{2+} ions.

Compound **2c** (1 × 10⁻⁵ M) was dissolved in HEPES (20 mM, v/v = 8/2, CH₃CH₂OH-H₂O, pH = 7.2) buffer solution with the addition of Cu²⁺ ions (Fig. 4). With the addition of increasing concentrations of Cu²⁺ ions (0-1 × 10⁻⁴ M), the fluorescence intensity of compound **2c** gradually weakened until it almost disappeared along with a colour change from blue to colourless.

A linear relationship between the fluorescence intensity of compound 2c and Cu2+ concentration was also obtained. According to the fluorescence intensity at 450 nm, we established a linear quenching relation with increasing Cu²⁺ concentrations from 0 M to 1 \times 10⁻⁴ M (y = -61.667x + 774.935) (Fig. 5), and the fitting constant was up to 0.9949. The limit of detection (LOD) was calculated from the formula LOD = $3\sigma_{\rm bi}/m$, where $\sigma_{\rm bi}$ is the standard deviation of the blank data, and m is the slope of the intensity reduction before and after quenching versus Cu²⁺ concentration (Fig. S4[†]). The LOD of Cu^{2+} with compound 2c was 4.0 \times 10⁻⁸ M. The quenching mechanism could be divided into a static quenching mechanism and a dynamic quenching mechanism; the UV-vis spectra changes at 325 nm indicated that the quenching effect should be attributed to a static quenching mechanism. The Stern-Volmer equation was introduced in the following section as shown in eqn (1) to further explain the static quenching mechanism:

$$\frac{F_0}{F} = 1 + K_s \left[C u^{2+} \right] \tag{1}$$

where F_0 and F respectively represent the fluorescence intensity of compound **2c** in the absence and presence of Cu²⁺ ions, and K_s is the static quenching constant of the Stern–Volmer



Fig. 4 Fluorescence quenching spectra of compound **2c** (1×10^{-5} M) with increasing concentrations of Cu²⁺ ions in CH₃CH₂OH-H₂O (v/ v = 8/2, 20 mM HEPES buffer, pH = 7.2) solution. Excitation wavelength of 325 nm; em. slit of 12 nm; ex. slit of 5 nm.



Fig. 5 Fluorescence intensity of compound 2c (1×10^{-5} M) at 450 nm with increasing concentrations of Cu²⁺ ions in CH₃CH₂OH-H₂O (v/ v = 8/2, 20 mM HEPES buffer, pH = 7.2) solution. Excitation wavelength of 325 nm; em. slit of 12 nm; ex. slit of 5 nm.

equation. Based on the linear relation of F_0/F versus Cu²⁺ concentration (Fig. S5†), K_s was computed to be 1.21×10^4 L M⁻¹.

The binding stoichiometry between compound **2c** and Cu²⁺ was observed using the rate of intensity change on adjusting the concentration proportions of compound **2c** and Cu²⁺ ions. A Job plot was recorded in 8/2 (v/v) ethanol-water solution (20 mM HEPES, pH = 7.2) (Fig. S6†). The binding stoichiometry of compound **2c** and Cu²⁺ ions was 2 : 1. The ESI-mass spectrum of compound **2c** binding with Cu²⁺ ions also shows a peak at 760.8 *m*/*z*, which was interpreted as $[2 \times 2\mathbf{c} + \mathrm{Cu}^{2+}-\mathrm{H}]^+$ (Fig. S7†). In addition, the binding constant (*K*_a) of compound **2c** and Cu²⁺ was evaluated from the intensity titration data using the Benesi–Hildebrand method (eqn (2)):³⁸

$$\frac{\alpha^2}{(1-\alpha)} = \frac{1}{2K_{\rm a}C_{\rm F}[{\rm M}]} \tag{2}$$

In eqn (2), $C_{\rm F}$ is the total concentration of compound **2c** in the system, and α is defined as the ratio between the concentration of free **2c** and the total concentration of compound **2c**. α was obtained using eqn (3):

$$\alpha = \frac{[F - F_0]}{[F_1 - F_0]} \tag{3}$$

where *F* is the fluorescence intensity of compound **2c** at 450 nm with increasing concentrations of Cu^{2+} ions, and F_1 and F_0 are the fluorescence intensity of free **2c** at 450 nm and the minimal fluorescence intensity of compound **2c** at 450 nm in the presence of Cu^{2+} ions.

The binding constant (K_a) of compound 2c and Cu²⁺ was obtained according to $\alpha^2/(1 - \alpha)$ against $1/[Cu^{2+}]$. The binding constant (K_a) of compound 2c and Cu²⁺ was calculated to be $1 \times 10^8 \text{ M}^{-2}$ (Fig. S8†). In addition, the binding mode of compound 2c with Cu²⁺ was investigated using IR spectroscopy. As shown in Fig. S9 and S10,† the characteristic stretching band at 3155 cm⁻¹, which corresponds to the amino (ν_{N-H} , NH₂) absorption of compound 2c, disappeared upon chelation with Cu²⁺.

Moreover, a typical scissor bending peak at 1567 cm⁻¹, which corresponds to the amino (δ_{N-H}, NH_2) absorption of compound **2c**, shifted to a higher frequency (1604 cm⁻¹) in the **2c**-Cu²⁺ complex. The stretching peak corresponding to $\nu_{C=N}$ (pyrimidine) was measured at 1647 cm⁻¹ in compound **2c**, while the band strengthened and shifted to a higher frequency (1666 cm⁻¹) in the complex. With significant evidence of characteristic peaks in free **2c** and the complex, the optimized binding mode of **2c** and Cu²⁺ could be concluded on the basis of the above binding study (Fig. S11†).

A performance comparison with some existing Cu²⁺ fluorescence probes is listed in Table S2.† Almost all of the enhanced fluorescence probes or quenched fluorescence probes show excellent selectivity to Cu²⁺ and an extremely low detection limit (μ M).^{39–46} Fluorescence imaging towards Cu²⁺ has been widely applied in live cells in completed studies, while compound **2c** maintained a good fluorescence intensity until copper ions were injected in the mice. Compound **2c** could be used as a sensitive and specific probe for Cu²⁺. In addition, the performance of compound **2c** was not inferior to that of other Cu²⁺ fluorescence probes.

Based on the coexistence of various metal ions in soil, rivers, and animals, the interference from other metal ions has to be taken into account. The competition of compound $2c (1 \times 10^{-5} \text{ M})$ with Cu²⁺ ions $(1 \times 10^{-4} \text{ M})$ was examined in the presence of other metal ions $(1 \times 10^{-4} \text{ M})$ by recording successive fluorescence intensity changes in CH₃CH₂OH-H₂O (v/v = 8/2, 20 mM HEPES buffer, pH = 7.2) solution. As shown in Fig. 6, the quenching system of compound 2c towards Cu²⁺ ions showed a very slight growth with the addition of different metal ions (Pb²⁺, Ag⁺, Al³⁺, Co²⁺, Cr³⁺, Fe³⁺, Hg²⁺, La³⁺, Zn²⁺, and Fe²⁺) in the presence of Cu²⁺ ions. The detection of Cu²⁺ ions using compound 2c was not affected by other metal ions. Thus, compound 2c could serve as a specific probe for Cu²⁺ ions in buffer solution.

The fluorescence intensity of compound $2c (1 \times 10^{-5} \text{ M})$ in different solvents could be regarded as an important indicator of its applicability; compound 2c was capable of keeping



Fig. 6 Fluorescence intensity changes $((F_0 - F)/F_0)$ of compound **2c** (1 \times 10⁻⁵ M) with Cu²⁺ ions (1 \times 10⁻⁴ M) in the absence and presence of other metal ions (1 \times 10⁻⁴ M) in CH₃CH₂OH-H₂O (v/v = 8/2, 20 mM HEPES buffer, pH = 7.2) solution. Excitation wavelength of 325 nm; em. slit of 12 nm; ex. slit of 5 nm.

Paper

a relatively higher fluorescence intensity in test solvents (Fig. S12†). The intensity of compound 2c in DMF was much stronger than that in other solvents, but the intensity was obviously weaker in low-polarity solutions such as methylbenzene and *n*-hexane. When compound 2c was dissolved in acetonitrile, HEPES buffer solution, 1,4-dioxane, trichloromethane, and ethyl acetate separately, compound 2c exhibited a moderate fluorescence intensity. In addition, the maximum emission wavelength of compound 2c showed a tiny red-shift from 435 nm to 450 nm in the test solutions and the fluorescence intensity of compound 2c had no significant quenching in most of the organic solvents.

Because the fluorescence intensity of compound 2c could be affected by solvents according to the above research, the fluorescence quenching efficiency of compound 2c $(1 \times 10^{-5} \text{ M})$ to Cu^{2+} ions $(1 \times 10^{-4} \text{ M})$ at 450 nm was evaluated in different solvents (Fig. S13†). The fluorescence of compound 2c was quenched in DMF, acetonitrile, HEPES buffer solution, 1,4-dioxane, trichloromethane, ethyl acetate, methylbenzene, and *n*-hexane in the presence of Cu^{2+} ions. The fluorescence quenching efficiency $(F_0 - F)/F_0$ of compound 2c reached over 80% in the test solvents, and in particular, the quenching efficiency could reach up to 90% with the addition of Cu^{2+} ions in DMF, acetonitrile, trichloromethane, and ethyl acetate. Compound 2c could act as a sensitive probe for the determination of Cu^{2+} in different solvents.

We examined the fluorescence properties of compound 2c (1 \times 10⁻⁵ M) in the absence and presence of Cu²⁺ ions (1 \times 10⁻⁴ M) with time. The fluorescence intensity of compound 2c and the response of compound 2c towards Cu²⁺ were recorded in HEPES (20 mM, v/v = 8/2, CH₃CH₂OH-H₂O, pH = 7.2) buffer solution (Fig. S14[†]). With the increase of time from 0 to 3.0 min, the fluorescence intensity of compound 2c was unchanged in principle. The response time of the probe to the substrate determines whether the substrate could be detected quickly or not; compound 2c had a very fast response time towards Cu²⁺ in our study. The fluorescence intensity of compound 2c did not decline after 30 s in the presence of Cu²⁺ ions indicating that the quenching reaction between compound 2c and Cu²⁺ had been balanced at the moment. The excellent fluorescence stability and the rapid response time towards Cu²⁺ would extend the utilization of compound 2c in detection.

The fluorescence intensity at 450 nm of compound **2c** (1 × 10^{-5} M) in the absence and presence of Cu²⁺ ions (1 × 10^{-4} M) was measured with different pH values (1–13) in 8/2 (v/v) ethanol-water solution (Fig. 7). The fluorescence intensity of compound **2c** was relatively weak in acidic solution and gradually enhanced with increasing pH values until the pH value reached 11. An overly acidic or alkali environment was unfavourable for detecting Cu²⁺ ions; the optimum pH range for compound **2c** to respond to Cu²⁺ ions was between 5 and 11. In view of the wide pH range for detecting Cu²⁺ ions, compound **2c** could be used as a working Cu²⁺ probe for practical application.

To evaluate whether the fluorescence intensity of compound **2c** towards copper ions was affected by the anions of the copper salts, fluorescence curves of compound **2c** with the addition of cupric chloride, cupric bromide, and cupric nitrate were



Fig. 7 Fluorescence intensity changes of compound **2c** (1×10^{-5} M) in the absence and presence of Cu²⁺ (1×10^{-4} M) at 450 nm with different pH values. Excitation wavelength of 325 nm; em. slit of 12 nm; ex. slit of 5 nm.

obtained in HEPES (20 mM, v/v = 8/2, CH₃CH₂OH-H₂O, pH = 7.2) buffer solution (Fig. S15†). The quenching intensity showed an obvious difference under the action of the corresponding copper salts, and the optimal fluorescence quenching efficiency of compound **2c** towards copper ions was found in the presence of cupric nitrate. With the addition of Cu²⁺ ions, the fluorescence intensity of compound **2c** was quenched effectively.

The reversible and reusable response of compound 2c was investigated by performing four alternate cycles of titration of compound 2c with Cu^{2+} followed by the addition of EDTA (Fig. S16†). An obvious decrease of the fluorescence intensity resulted from the formation of the 2c– Cu^{2+} complex. However, the fluorescence intensity of the system returned to a level close to that of the free compound 2c with the addition of EDTA. The repeated OFF/ON behavior verified the remarkable reversibility and reusability of compound 2c in detecting Cu^{2+} ions.

The photo-stability of compound **2c** was evaluated upon continuous illumination in HEPES (20 mM, v/v = 8/2, CH₃CH₂-OH-H₂O, pH = 7.2) buffer solution. As shown in Fig. S17,† the fluorescence intensity of compound **2c** at 450 nm showed no significant decrease upon continuous illumination for 60 h with a fluorescent lamp. The excellent photo-stability of compound **2c** indicated that it could be used as a practical fluorescence probe.

The thermostability of compounds 2a-2c

The thermostability is an important parameter for a fluorescence probe to evaluate its applicability. We tested the thermostability of compounds **2a–2c** using TGA (Fig. S18†). All of compounds **2a–2c** had excellent thermostabilities, and the thermostabilities of compounds **2b** and **2c** were much better than that of compound **2a**. The weight loss of compounds **2a–2c** attained 10% at the temperature points approaching 255.3 °C, 277.0 °C, and 275.9 °C. Compared to compound **2a**, compounds **2b** and **2c** had better thermostabilities.

Fluorescence response to various metal ions on filter paper

The detection of a fluorescence probe towards a substrate should ensure accuracy as simply as possible, so we attempted



Fig. 8 Fluorescence photographs of compound $2c~(1\times10^{-5}~\text{M})$ with drops of various metal ions (1.5 \times 10^{-4} M) on filter paper.}

to use compound 2c to distinguish Cu^{2+} ions from other metal ions on filter paper. The solution of compound 2c $(1 \times 10^{-5} \text{ M})$ was added dropwise and evenly on the filter paper, then water solutions of metal salts $(1.5 \times 10^{-4} \text{ M})$ were respectively dropped in the previous circles and dried on the filter paper. As shown in Fig. 8, compound 2c in the absence or presence of Zn^{2+} , Hg^{2+} , Fe^{3+} , Ag^+ , Al^{3+} , and Pb^{2+} exhibits a bright fluorescent circle on the filter paper under 365 nm UV light, while the fluorescent circles of compound 2c were quenched slightly with Co^{2+} and Cr^{3+} ions and enhanced mildly with La^{3+} ions. With the drop of Cu^{2+} ions, the fluorescent circles of compound 2c changed from bright blue to a dark colour. Compound 2c could quickly and simply recognize Cu^{2+} ions from other metal ions on filter paper, so it was likely to be a feasible way to detect Cu^{2+} ions.



Fig. 9 The optimized structures and molecular orbitals (LUMO and HOMO) of compounds 2a-2c.

DFT calculations

For further understanding optical properties of synthetic compounds with density functional theory (DFT), the



Fig. 10 (a) The fluorescence images of untreated black mice and mice given a skin-pop injection of compound **2c** (50 μ l × 20 μ M), then incubated for 30 min. (b) The fluorescence images of black mice injected with compound **2c** and 1.5 equivalents Cu²⁺ ions and incubated for 0, 10, 30, and 70 min. (c and d) Quantified fluorescence signals of (a) and (d), respectively.

Paper

geometries of compounds 2a-2c were optimized at the B3LYP level with the 6-31G(d) basis set using the Gaussian 09 program. The optimized structures of compounds 2a-2c were obtained. In addition, the lowest unoccupied molecular orbital (LUMO) and the highest occupied molecular orbital (HOMO) are also shown in Fig. 9. The HOMOs of compounds 2a-2c were distributed over the molecules except for the partial isolongifolanone units, while the LUMOs were mainly situated on the pyridine and pyrimidine groups. The HOMO-LUMO band gaps (ΔE) acted as the theoretical basis of the molecular fluorescence properties. The band gaps (ΔE) of compounds 2a-2c were 4.710 eV, 4.672 eV, and 4.590 eV, and they decreased with the increase of the distance of the nitrogen atoms on the pyridine groups from the pyrimidine units. The lowest ΔE indicated that compound 2c could show fluorescence after electron transition more easily than the other compounds could.

Bio-imaging in vivo of compound 2c

The part around a mouse's hip with loose muscle tissues and abundant blood vessels enables an imaging agent to be absorbed as quickly as possible. Therefore, the fluorescence imaging efficacy of compound 2c towards copper ions was evaluated in vivo in black mice using a near-infrared (NIR) fluorescence imaging system. A solution of compound 2c (50 μ l \times 20 μ M in saline, containing 1% DMSO) was injected into the subcutaneous tissues on the hip of the black mice. The fluorescence variation at the same region of the black mice was obvious after the injection of compound 2c (Fig. 10a and S43a[†]); the florescence intensity in the tissue sharply increased from 7.52 to 11.11% (Fig. 10c). Compared with the initial fluorescence imaging (immediately recorded after compound 2c was injected), the fluorescence durability of compound 2c was also investigated within 30 min. As shown in the diagrams, the fluorescence signal after 30 min at the same injecting region had no significant change; the % florescence intensity almost did not weaken during the testing time. The fluorescence imaging of compound 2c was visualized after the black mice were treated with an injection of 1.5 equivalents Cu^{2+} after 0, 10, 30, and 70 min, and the graphs at the same region show a quenching reaction over time (Fig. 10b and S43b[†]). The % fluorescence intensity of compound 2c towards Cu²⁺ ions exhibited a constant decrease in the black mice within the testing time; the quenching velocity of the % fluorescence intensity $(\Delta \% F/\Delta t)$ was fastest ten minutes before and then mildly slowed down with time (Fig. 10d). Compound 2c had a good florescence durability and short response time to Cu^{2+} in *vivo*, so it could be used as an excellent probe for imaging Cu²⁺ ions in living black mice.

Conclusions

In summary, a novel fluorescence probe has been synthesized from isolongifolanone. The sensor exhibited highly selective and sensitive fluorescence quenching towards Cu^{2+} ions, and the detection limit for Cu^{2+} ions was 4.0×10^{-8} M. In addition, the quenching constant $(1.21 \times 10^4 \text{ L M}^{-1})$ and the binding constant $(1 \times 10^8 \text{ M}^{-2})$ were also obtained according to the linear relationship between the fluorescence intensity and Cu²⁺ ion concentration. The sensor could detect Cu²⁺ ions in a wide pH range of 5–11 and the detection could be achieved in different solvents. The sensor could respond to Cu²⁺ ions in a short time (0.5 min) and sustain a good quenching efficiency during the testing time. A simple method was provided for detecting Cu²⁺ ions using the sensor on filter paper. The sensor was applied to the detection Cu²⁺ ions in living mice, and the fluorescence signals were quantified to visualize the quenching effect *in vivo*. Moreover, the fluorescence sensor was synthesized from isolongifolanone, which is an important derivative of turpentine. Therefore, the synthesized sensor has exploited the utilization of turpentine and provided a possible route for deep processing of forest resources.

Experimental

Instruments

The mass spectra were recorded on an America Agilent 5975c mass spectrometer. The purity was measured on an America Agilent 7890A gas chromatograph. The ¹³C NMR and ¹H NMR spectra were recorded in DMSO-d₆ or CDCl₃ solutions on a Bruker AV 500 spectrometer. The UV-vis absorption spectra were determined on a Shimadzu UV-2450 spectrophotometer. The fluorescence spectra were obtained on a Perkin Elmer LS 55 fluorescence spectrophotometer with the excitation wavelength at 325 nm. The infrared spectra were measured on a Nicolet 380 FTIR infrared spectrometer. The melting points were recorded on an X-6 microscopic melting point apparatus. Single-crystal X-ray diffraction measurements were done on a Bruker D8 Venture area diffractometer. The fluorescence images of living mice were taken using a Maestro *In Vivo* Imaging System.

Materials

All the reagents and solvents were purchased from various commercial sources and used without further purification. Deionized water was used throughout the experiment. The most common solutions of the sensor were prepared in HEPES buffer solution (20 mM, pH = 7.2, CH₃CH₂OH-H₂O, v/v = 8/2). The variety of pH solutions from 1 to 13 were prepared using HCl and NaOH solutions at a concentration of 1 M for slight pH adjustments. The stock solutions of metal ions were prepared from various metal salts (ZnCl₂, HgSO₄, Fe(NO₃)₃·9H₂O, FeCl₂, CuCl₂·H₂O, CrCl₃·6H₂O, Co(NO₃)₂·6H₂O, La(NO₃)₃·6H₂O, AgNO₃, AlCl₃, Pb(NO₃)₂, CuBr₂, and Cu(NO₃)₂·3H₂O). The sensor used in the mice was dissolved in saline (containing 1% DMSO) and the CuCl₂·H₂O used in the mice was dissolved in saline.

Methods

All experiments involving living animals and their care were performed in strict accordance with the National Care and Use of Laboratory Animals by the National Animal Research Authority (China) and guidelines of Animal Care and Use issued by the Medical School of Southeast University Institutional Animal Care and Use Committee, and the experiments were approved by the Institutional Animal Care and Use Committee of the Medical School of Southeast University.

Synthesis of 7-(pyridine-2'-ylmethylene)-isolongifolanone (1a). Isolongifolanone (2.2 g, 8 mmol), tert-butyl alcohol (30 mL), potassium tert-butoxide (0.84 g, 7.5 mmol), and pyridine-2carboxaldehyde (1.07 g, 10 mmol) were successively added in a 50 mL dried three-necked flask equipped with a stirrer, condenser, and thermometer. The mixture was stirred and refluxed for 4 h until the conversion ratio of isolongifolanone exceeded 95% (monitored by GC). The reacted solution was evaporated under vacuum and extracted three times with 20 mL ethyl acetate, then the merged organic layers were washed to neutrality with saturated salt water, dried with sodium sulfate, filtered, and evaporated to afford a crude product, then recrystallized with ethanol in a refrigerator. Pale yellow crystals were afforded (67.5% yield). Mp: 102.5–102.6 °C; FT-IR (KBr, cm⁻¹) v: 2964, 2871, 1670, 1600, 1471, 1426, 1385, 1238, 1198, 1176, 921, 816, 736; ¹H NMR (400 MHz, CDCl₃, ppm) δ: 0.85 (s, 2H), 1.06 (s, 3H), 1.09-1.16 (m, 1H), 1.22 (s, 3H), 1.26-1.30 (m, 1H), 1.45-1.53 (m, 1H), 1.61-1.68 (m, 1H), 1.74 (d, J = 4 Hz), 1.80-1.83 (m, 2H),1.99 (s, 1H), 2.97-3.02 (m, 1H), 3.10-3.15 (m, 1H), 7.15 (t, J = 4 Hz, 1H), 7.39 (d, J = 4 Hz, 2H), 7.66 (t, J = 8 Hz, 1H), 8.67 (d, J = 4 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃, ppm) δ: 24.13, 24.48, 25.27, 25.43, 28.14, 30.05, 31.14, 37.45, 41.62, 44.62, 47.88, 55.49, 62.89, 122.07, 126.92, 133.40, 135.85, 139.36, 149.28, 155.39, 203.35; EIMS m/z (%): 309 (M⁺, 100), 294 (56), 281 (19), 266 (26), 252 (7), 200 (45), 131 (73), 93 (68); HRMS (m/z): $[M + H]^+$ calcd for C₂₁H₂₇NO + H⁺, 310.2171; found, 310.2178.

6,6,10,10-tetramethyl-4-(pyridine-2'-yl)-Synthesis of 5,7,8,9,10,10a-hexahydro-6H-6a,9-methanobenzo[h]quinazolin-2-amine (2a). 7-(Pyridine-2'-ylmethylene)-isolongifolanone (1.54 g, 5 mmol), guanidine hydrochloride (1.91 g, 20 mmol), tert-butyl alcohol (60 mL), and tert-butoxide (2.8 g, 25 mmol) were successively added in a 100 mL dried threenecked flask equipped with a stirrer, condenser, and thermometer. The mixture was stirred and refluxed for 18 h until the conversion ratio of 7-(pyridine-2'-ylmethylene)isolongifolanone was over 95% (monitored by GC). The reacted mixture was evaporated under vacuum and extracted three times with ethyl acetate, then the combined organic layers were washed to neutrality with saturated brine, dried with sodium sulfate, filtered, and evaporated to afford a viscous liquid, then recrystallized with ethanol and ethyl acetate in a refrigerator. The final product was a white powder (35.0%, yield). Mp: 176.8–177.1 °C; FT-IR (KBr, cm⁻¹) v: 3489, 3265, 3139, 2959, 2934, 2869, 1618, 1552, 1458, 1408, 1372, 1209, 992, 797; ¹H NMR (300 MHz, DMSO-*d*₆, ppm) δ: 0.61 (s, 3H), 0.72 (s, 3H), 0.91 (s, 3H), 1.06–1.09 (m, 1H), 1.17 (d, J = 9 Hz, 1H), 1.32 (s, 3H), 1.46–1.50 (m, 2H, 1H), 1.59 (d, J = 12 Hz, 1H), 1.69 (s, 2H), 1.82 (s, 1H), 2.21–2.29 (m, 2H), 2.79 (d, J = 15 Hz, 1H), 3.34 (s, 1H), 6.19 (s, 2H), 7.41 (t, *J* = 6 Hz, 1H), 7.78 (d, J = 6 Hz, 1H), 7.89 (t, J = 6 Hz, 1H), 8.63 (d, J = 3 Hz, 1H);¹³C NMR (75 MHz, DMSO- d_6 , ppm) δ : 22.88, 24.32, 25.20, 25.52, 28.06, 29.68, 31.74, 36.69, 38.64, 43.67, 47.41, 54.57, 57.10, 114.66, 123.36, 123.31, 136.49, 148.05, 157.17, 160.68,

162.72, 168.81; EIMS m/z (%): 348 (M⁺, 37), 333 (100), 250 (4), 239 (2), 105 (1), 78 (2); HRMS (m/z): [M + H]⁺ calcd for C₂₂H₂₈N₄ + H⁺, 349.2392; found, 349.2402.

Synthesis of 7-(pyridine-3'-ylmethylene)-isolongifolanone (1b). Isolongifolanone (2.2 g, 8 mmol), tert-butyl alcohol (30 mL), potassium tert-butoxide (0.84 g, 7.5 mmol), and pyridine-3carboxaldehyde (1.07 g, 10 mmol) were successively added in a 50 mL dried three-necked flask equipped with a stirrer, condenser, and thermometer. The mixture was stirred and refluxed for 5 h until the conversion ratio of isolongifolanone exceeded 95% (monitored by GC). The reacted solution was evaporated under vacuum and extracted three times with 20 mL ethyl acetate, then the merged organic layers were washed to neutrality with saturated salt water, dried with sodium sulfate, filtered, and evaporated to afford a crude product, then recrystallized with ethanol in a refrigerator. Pale yellow crystals were afforded (66.8%, yield). Mp: 73.5–73.7 °C; FT-IR (KBr, cm⁻¹) *v*: 2964, 2873, 1674, 1597, 1468, 1412, 1367, 1237, 1180, 960, 816, 707; ¹H NMR (400 MHz, CDCl₃, ppm) δ: 0.83 (s, 3H), 0.85 (s, 3H), 0.92-0.95 (m, 1H), 1.03 (s, 3H), 1.21 (s, 3H), 1.29 (d, J = 4 Hz, 1H), 1.48-1.54 (m, 1H), 1.60-1.67 (m, 1H), 1.76-1.83 (m, 3H), 1.97 (d, J = 4 Hz), 2.52–2.57 (m, 1H) 2.81–2.86 (m, 1H), 7.31–7.34 (m, 1H), 7.44 (s, 1H), 7.75 (d, J = 4 Hz, 1H), 8.51–8.53 (m, 1H), 8.69 (d, J = 4 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃, ppm) δ : 24.28, 24.78, 25.67, 25.79, 28.45, 30.43, 31.90, 37.78, 41.80, 45.03, 48.21, 55.70, 63.09, 123.52, 132.05, 132.74, 137.47, 137.67, 148.96, 151.23, 202.35; EIMS m/z (%): 309 (M⁺, 71), 294 (27), 280 (24), 266 (100), 252 (32), 240 (37), 117 (54), 93 (24); HRMS (*m/z*): $[M + H]^+$ calcd for C₂₁H₂₇NO + H⁺, 310.2171; found, 310.2177.

Synthesis of 6,6,10,10-tetramethyl-4-(pyridine-3'-yl)-5,7,8,9,10,10*a*-hexahydro-6*H*-6*a*,9-methanobenzo[*h*]quinazolin-2-amine (2b). 7-(Pyridine-3'-ylmethylene)-isolongifolanone (1.54 g, 5 mmol), guanidine hydrochloride (1.91 g, 20 mmol), tert-butyl alcohol (60 mL), and tert-butoxide (2.8 g, 25 mmol) were successively added in a 100 mL dried three-necked flask equipped with a stirrer, condenser, and thermometer. The mixture was stirred and refluxed for 20 h until the conversion ratio of 7-(pyridine-3'-ylmethylene)-isolongifolanone was over 95% (monitored by GC). The reacted mixture was evaporated under vacuum and extracted three times with ethyl acetate, then the combined organic layers were washed to neutrality with saturated brine, dried with sodium sulfate, filtered, and evaporated to afford a viscous liquid, then recrystallized with ethanol and ethyl acetate in a refrigerator. The final product was a white powder (33.2%, yield). Mp: 245.3-245.7 °C; FT-IR (KBr, cm⁻¹) v: 3482, 3281, 3157, 2961, 2870, 1618, 1552, 1455, 1400, 1375, 1210, 1024, 797; ¹H NMR (400 MHz, DMSO d_6 , ppm) δ : 0.59 (s, 3H), 0.75 (s, 3H), 0.93 (s, 3H), 1.05 (d, J =4 Hz, 1H), 1.15–1.19 (m, 1H), 1.31 (s, 3H), 1.47–1.52 (m, 1H), 1.55–1.64 (m, 2H), 1.70 (d, J = 4 Hz, 1H), 1.78–1.86 (m, 1H), 1.98 (d, J = 16 Hz, 1H), 2.19 (s, 1H), 2.84 (d, J = 16 Hz, 1H), 6.26 (s, 2H), 7.46–7.49 (m, 1H), 7.95–7.98 (m, 1H), 8.61 (d, *J* = 4 Hz, 1H), 8.74 (d, J = 4 Hz, 1H); ¹³C NMR (75 MHz, DMSO- d_6 , ppm) δ: 23.45, 24.06, 25.04, 25.79, 26.23, 28.74, 30.36, 32.86, 37.42, 39.39, 44.41, 48.04, 55.42, 57.82, 114.91, 123.69, 134.88, 137.00, 150.13, 161.63, 162.95, 169.38; EIMS m/z (%): 348 (M⁺, 100), 333 (26), 319 (32), 266 (88), 251 (20), 237 (12), 105 (8), 78

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Synthesis of 7-(pyridine-4'-ylmethylene)-isolongifolanone (1c). Isolongifolanone (2.2 g, 8 mmol), tert-butyl alcohol (30 mL), potassium tert-butoxide (0.84 g, 7.5 mmol), and pyridine-4-carboxaldehyde (1.07 g, 10 mmol) were successively added in a 50 mL dried three-necked flask equipped with a stirrer, condenser, and thermometer. The mixture was stirred and refluxed for 5 h until the conversion ratio of isolongifolanone exceeded 95% (monitored by GC). The reacted solution was evaporated under vacuum and extracted three times with 20 mL ethyl acetate, then the merged organic layers were washed to neutrality with saturated salt water, dried with sodium sulfate, filtered, and evaporated to afford a crude product, then recrystallized with ethanol in a refrigerator. Pale vellow crystals were afforded (64.2%, yield). Mp: 88.5-88.6 °C; FT-IR (KBr, cm⁻¹) ν : 2965, 2933, 2874, 1672, 1588, 1467, 1411, 1364, 1233, 1186, 957, 814, 787; ¹H NMR (400 MHz, CDCl₃, ppm) δ: 0.85 (s, 3H), 0.87 (s, 3H), 0.95-1.01 (m, 1H), 1.06 (s, 3H), 1.24 (s, 3H), 1.32 (d, J = 4 Hz, 1H), 1.50–1.57 (m, 1H), 1.63– 1.70 (m, 1H), 1.79–1.88 (m, 3H), 2.00 (d, J = 4 Hz, 1H), 2.56 (d, J = 16 Hz, 1H), 2.82–2.87 (m, 1H), 7.30 (d, J = 8 Hz, 2H), 7.39 (s, 1H), 8.63 (d, J = 8 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, ppm) δ : 23.99, 24.49, 25.40, 25.49, 28.13, 30.19, 31.59, 37.52, 41.33, 44.80, 47.95, 55.46, 62.88, 124.10, 133.12, 134.25, 139.16, 143.30, 149.73, 149.95, 202.04; EIMS m/z (%): 309 (M⁺, 100), 294 (52), 280 (12), 266 (37), 252 (63), 238 (50), 135 (37), 93 (63); HRMS (m/z): $[M + H]^+$ calcd for C₂₁H₂₇NO + H⁺, 310.2171; found, 310.2171.

Synthesis of 6,6,10,10-tetramethyl-4-(pyridine-4'-yl)-5,7,8,9,10,10a-hexahydro-6H-6a,9-methanobenzo[h]quinazolin-(2c). 7-(Pyridine-4'-ylmethylene)-isolongifolanone 2-amine (1.54 g, 5 mmol), guanidine hydrochloride (1.91 g, 20 mmol), tert-butyl alcohol (60 mL), and tert-butoxide (2.8 g, 25 mmol) were successively added in a 100 mL dried three-necked flask equipped with a stirrer, condenser, and thermometer. The mixture was stirred and refluxed for 18 h until the conversion ratio of 7-(pyridine-4'-ylmethylene)-isolongifolanone was over 95% (monitored by GC). The reacted mixture was evaporated under vacuum and extracted three times with ethyl acetate, then the combined organic layers were washed to neutrality with saturated brine, dried with sodium sulfate, filtered, and evaporated to afford a viscous liquid, then recrystallized with ethanol and ethyl acetate in a refrigerator. The final product was a white powder (33.2%, yield). Mp: 272.2-272.4 °C; FT-IR (KBr, cm^{-1}) v: 3327, 3156, 2960, 2925, 2872, 1647, 1567, 1544, 1465, 1407, 1386, 1213, 998, 825, 793; ¹H NMR (400 MHz, CDCl₃, ppm) δ: 0.65 (s, 3H), 0.77 (s, 3H), 0.97 (s, 3H), 1.12 (s, 1H), 1.24 (s, 3H), 1.36 (s, 3H), 1.49–1.56 (m, 1H), 1.61–1.65 (m, 1H), 1.71 (d, J = 8 Hz, 1H), 1.76 (s, 1H), 1.90 (t, J = 8 Hz, 1H), 2.07–2.13 (m, 1H), 2.31 (s, 1H), 2.69 (d, J = 16 Hz, 1H), 4.98 (s, 2H), 7.42 (d, J = 4 Hz, 2H), 8.70 (d, J = 8 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, ppm) δ : 23.16, 24.34, 24.95, 25.59, 26.10, 28.56, 29.88, 30.33, 32.86, 37.46, 39.32, 44.63, 48.20, 55.45, 58.13, 116.38, 123.59, 146.56, 150.04, 160.65, 163.57, 170.86; EIMS m/z (%): 348 (M⁺, 100), 333 (17), 319 (30), 266 (76), 251 (14), 237 (10), 105 (7), 78 (6); HRMS

(m/z): $[M + H]^+$ calcd for $C_{22}H_{28}N_4 + H^+$, 349.2392; found, 349.2393.

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