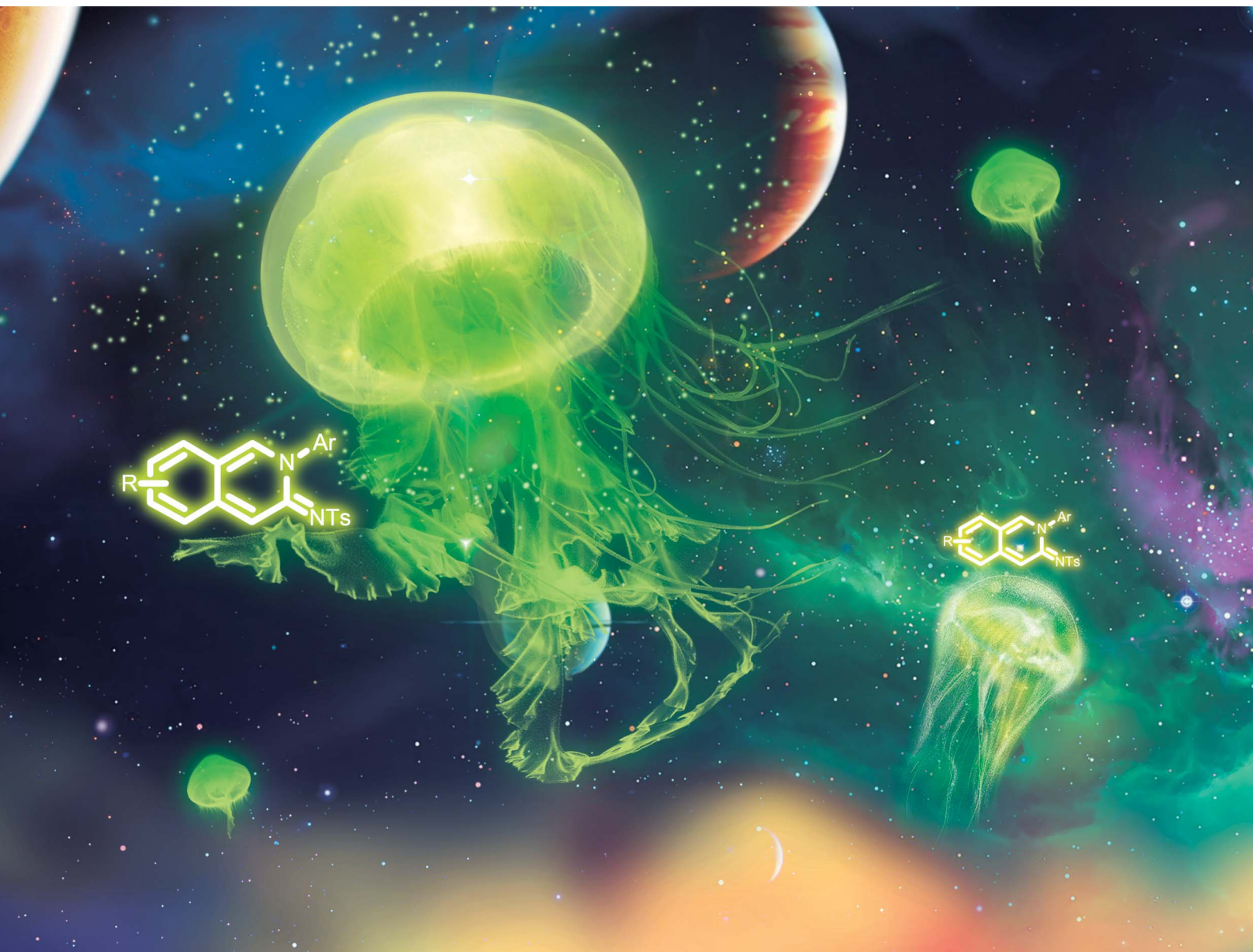


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Efficient synthesis of cyclic amidine-based fluorophores via 6π -electrocyclic ring closure†

Guofeng Li,^{‡a} Man Zhao,^{‡a} Junqiu Xie,^{‡b} Ying Yao,^a Lingyun Mou,^b Xiaowei Zhang,^b Xiaomin Guo,^b Wangsheng Sun,^b Zheng Wang,^c Jiecheng Xu,^a Jianzhong Xue,^a Tao Hu,^a Ming Zhang,^b Min Li^{*a} and Liang Hong^{‡*a}

Novel 10π -electron cyclic amidines with excellent fluorescence properties were synthesized by a general and efficient 6π -electrocyclic ring closure of ketenimine and imine starting from *N*-sulfonyl triazoles and arylamines. The photophysical properties of cyclic amidine fluorophores have been studied in detail and have shown good properties of a large Stokes shift, pH insensitivity, low cytotoxicity and higher photostability, which have great potential for biological imaging. Furthermore, this novel fluorophore was successfully applied to the localization of the NK-1 receptor in living systems.

Introduction

Small organic fluorophores have been used widely in biological science and drug discovery due to their easy handling, high sensitivity and minimal disruption to living systems.¹ Although a large variety of fluorescent small molecules have been developed, their core scaffolds are still limited to several types such as prodan, fluorescein, coumarin *etc.*,² most of which usually possess a π -conjugated system with an electron-donating and an electron-withdrawing group located at the opposite position of the fluorescent moiety (Fig. 1).³ On the other hand, the ideal fluorophores for living systems with good photophysical properties including a large Stokes shift, an applicable pH environment, good stability and low biological toxicity are in high demand. Consequently, the development of small organic fluorophores with a new framework and good photophysical properties is an appealing and challenging task.⁴

To develop a new fluorescent framework, we wondered whether it is possible to incorporate an electron-donating and an electron-withdrawing group at the adjacent position of a π -system. Considering the particularity of this structure, we tried

to choose π -system cyclic amidines as the core structure of novel fluorophores, not only due to their potent fluorescence properties,⁵ but also due to the electronic specificity of amidines (Fig. 1). However, how to incorporate an amidine into a π -system is a challenge, since the synthesis of such a scaffold is limited and difficult. Moreover, it usually needs a variety of cyclic amidines with structural diversity to study the fluorescence–structure relationship. Thus, it is necessary yet challenging to develop a simple and short synthetic route to access various cyclic amidines.

N-Sulfonyl-1,2,3-triazoles have become an important class of intermediates for accessing a wide variety of complex molecular scaffolds.⁶ Metal-bound imino carbenes, readily generated from *N*-sulfonyl 1,2,3-triazoles, have found wide application in many useful transformations, including cycloaddition, X–H insertion, alkyl migration, sigmatropic rearrangement and some other carbene induced reactions.^{6,7} In addition to imino carbenes, *N*-sulfonyl-1,2,3-triazoles could also form active ketenimine intermediates, which would readily undergo nucleophilic additions at C2.⁸ In particular, the ketenimines containing

^aGuangdong Key Laboratory of Chiral Molecular and Drug Discovery, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, China. E-mail: limin65@mail.sysu.edu.cn; hongliang@sysu.edu.cn

^bKey Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, Lanzhou 730000, China

^cGuangdong Key Lab of Nano-Micro Material Research, School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen 518055, China

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‡ These authors contributed equally to this work.

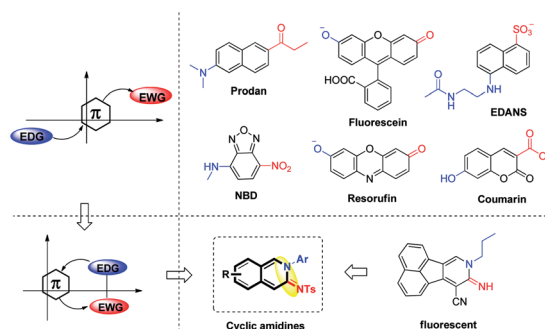
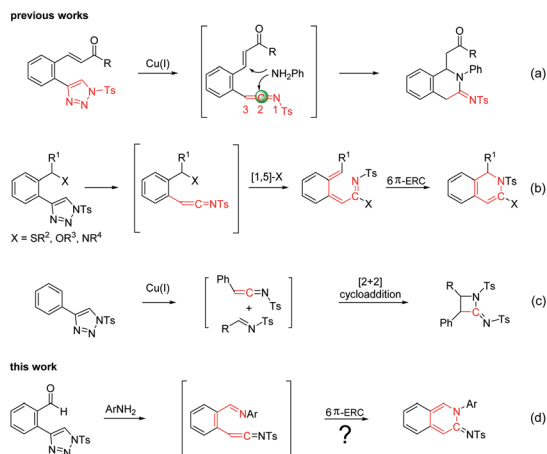


Fig. 1 Structural characteristics of traditional fluorophores and amidines.



Scheme 1 Related research and this work.

another electrophilic site would undergo double nucleophilic addition with amines to give cyclic amidines (Scheme 1a).⁹ Moreover, ketenimines could also in whole or in part participate in some cyclization reactions. For example, in the [1,5]-X sigmatropic shift/6 π -electrocyclic ring closure (6 π -ERC), the whole ketenimines (C=C=NTs) take part in the reaction to give dihydroisoquinolines (Scheme 1b),^{8f,10} while part of the ketenimines (C=C) in the [2 + 2] cycloadditions reacts to give amidines (Scheme 1c).¹¹ From these studies, we found that cyclic amidines could be constructed by the intramolecular conjunction of a nitrogen atom to the C2 of the ketenimine. Accordingly, we designed the intermediate **A** functionalized with an adjacent imine and ketenimine. The imine bond (C=NAr) was introduced for the formation of 6 π -substrates suitable for ERC and might react with part of the ketenimines to give cyclic amidine products (Scheme 1d).

Results and discussion

With this reaction design in mind, we first investigated the reaction of *N*-sulfonyl triazoles **1a** with phenylamine **2a** as the model reaction in the presence of a catalyst at 120 °C in a sealed tube for 2 h (Table 1). A low yield of 17% was observed in the presence of a CuI catalyst (entry 1). We also examined some other catalysts including Cu(OAc)₂, CuTc, Rh₂(OAc)₄, Rh₂(Oct)₄ and Pd(OAc)₂, and they gave yields ranging from 12% to 54% (entries 2–6). Interestingly, when the reaction was conducted in the absence of any catalyst, the reaction could give a significant improvement of the yield to 83% (entry 7). Increasing the reaction time to 4 h could further improve the yield to 91% (entry 8), but further increasing the reaction time to 10 h reduced the yield to 86% (entry 9). After screening several solvents, CHCl₃ was found to be the optimum solvent for this reaction (entries 8–13).

Under optimal conditions, the scopes of the reaction were examined as shown in Scheme 2. We first examined the scope of benzenamines with various substituents at different positions. The reaction proceeded efficiently to afford the corresponding cyclic amidines **3aa–3ar** in good to excellent yields, though

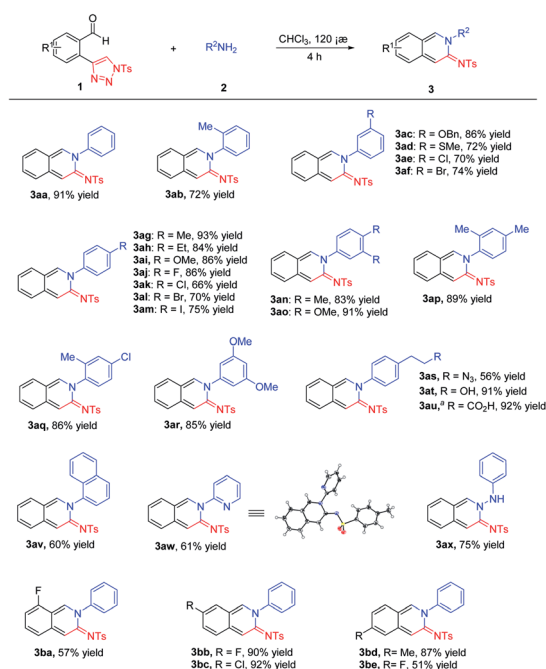
Table 1 Optimization of the reaction conditions^a

| Entry | Cat. | Solvent | <i>t</i> (h) | Yield ^b (%) |
|-------|------------------------------------|-------------------|--------------|------------------------|
| 1 | CuI | CHCl ₃ | 2 | 17 |
| 2 | Cu(OAc) ₂ | CHCl ₃ | 2 | 26 |
| 3 | CuTc | CHCl ₃ | 2 | 46 |
| 4 | Rh ₂ (OAc) ₄ | CHCl ₃ | 2 | 23 |
| 5 | Rh ₂ (Oct) ₄ | CHCl ₃ | 2 | 54 |
| 6 | Pd(OAc) ₂ | CHCl ₃ | 2 | 54 |
| 7 | — | CHCl ₃ | 2 | 83 |
| 8 | — | CHCl ₃ | 4 | 91 |
| 9 | — | CHCl ₃ | 10 | 86 |
| 10 | — | Toluene | 4 | 40 |
| 11 | — | DCE | 4 | 77 |
| 12 | — | THF | 4 | 30 |
| 13 | — | 1,4-Dioxane | 4 | 44 |

^a Conditions: **1a** (0.10 mmol, 1.0 equiv.), **2a** (0.12 mmol, 1.2 equiv.), the catalyst (0.005 mmol, 5 mol%), and solvent (2.0 mL) in a sealed tube.

^b Isolated yield.

higher reactivity was observed with electron-donating groups. Significantly, the benzenamines with functional groups such as azide, hydroxy and carboxyl groups were well tolerated to give **3as–3au**, which could be modified easily for the purpose of bioconjugate chemistry. Some other amines, including naphthyl amine, 2-aminopyridine and phenylhydrazine were also



Scheme 2 Scope of the reaction. Conditions: **1** (0.10 mmol, 1.0 equiv.), **2** (0.12 mmol, 1.2 equiv.) and the solvent (2.0 mL) in a sealed tube for 4 h at 120 °C. ^a 1 mmol scale.



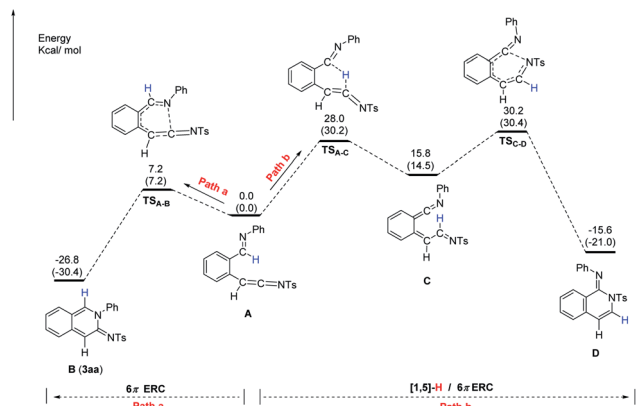


Fig. 2 DFT calculations for the transformation of ketenimine A.

suitable substrates for the reaction, leading to the desired products **3av–3ax** in moderate yields. The structure of **3aw** was determined by X-ray crystallography.¹² The scope of *N*-sulfonyl-1,2,3-triazoles was subsequently investigated. The reaction took place with moderate to excellent yields with substrates bearing different substituents **3ba–3be**.

To further understand the formation of cyclic amidine **3aa**, analysis by DFT calculations was performed. As shown in Fig. 2, starting from ketenimine intermediate **A**, there are two pathways to proceed. By path a, the intermediate **A** could undergo 6 π -electrocyclic ring closure (6 π -ERC) *via* TS_{A-B} to give **B** (**3aa**); while by path b, the intermediate **A** could undergo the first [1,5]-H shift to form the intermediate **C**, followed by the 6 π -ERC *via* TS_{C-D} to afford the isoquinolone **D**. The energy for the formation of TS_{A-B} ($\Delta G = 7.2$ kcal mol^{−1}) is lower than that for the TS_{C-D} ($\Delta G = 30.2$ kcal mol^{−1}). Therefore, the intermediate **A** favors path a to furnish the observed product **3aa**.

With a series of 10 π -electron cyclic amidines in hand, we measured their photophysical properties including absorptions, emissions, extinction coefficients, Stokes shifts and quantum yields. As shown in Table 2, the maximum absorption wavelengths (λ_{max}) varied from 424 to 443 nm with moderate extinction coefficients (2110–4364 M^{−1} cm^{−1}), while the maximum emission wavelengths (λ_{em}) varied from 525 to 552 nm with quantum yields from 1% to 23%. In addition, cyclic amidines **3as–3au** with functional groups exhibited similar photophysical properties, which could be conveniently modified for further fluorophore tagging.

Table 2 Spectral properties of fluorophores

| Compound | λ_{max}^a (nm) | λ_{em}^a (nm) | ϵ^b (M ^{−1} cm ^{−1}) | Stokes shift (nm) | Φ^c (%) |
|----------------------------|-------------------------------|------------------------------|--|-------------------|--------------|
| Coumarin ^{13d} | 386 | 448 | 36 700 | 62 | 70 |
| Fluorescein ^{3bd} | 490 | 512 | 93 000 | 22 | 95 |
| 3aa | 433 | 535 | 2110 | 102 | 17 |
| 3ab | 432 | 527 | 3066 | 95 | 23 |
| 3ac | 434 | 542 | 3613 | 108 | 14 |
| 3ad | 434 | 538 | 3790 | 104 | 13 |
| 3ae | 436 | 543 | 3588 | 107 | 11 |
| 3af | 437 | 545 | 3440 | 108 | 12 |
| 3ag | 434 | 538 | 3558 | 104 | 16 |
| 3ah | 434 | 541 | 3430 | 107 | 17 |
| 3ai | 434 | 535 | 3312 | 101 | 19 |
| 3aj | 435 | 536 | 2770 | 101 | 15 |
| 3ak | 435 | 540 | 3209 | 105 | 14 |
| 3al | 436 | 542 | 3786 | 106 | 14 |
| 3am | 435 | 543 | 3418 | 108 | 12 |
| 3an | 433 | 536 | 4364 | 103 | 19 |
| 3ao | 434 | 537 | 3874 | 103 | 18 |
| 3ap | 432 | 525 | 2804 | 93 | 22 |
| 3aq | 434 | 530 | 3917 | 96 | 18 |
| 3ar | 433 | 539 | 3578 | 106 | 14 |
| 3as | 435 | 535 | 2915 | 100 | 18 |
| 3at | 434 | 538 | 3250 | 104 | 18 |
| 3au | 434 | 536 | 2935 | 102 | 17 |
| 3av | 434 | 532 | 3170 | 98 | 18 |
| 3aw | 437 | 552 | 3099 | 115 | 1 |
| 3ax | 425 | 528 | 3865 | 103 | 16 |
| 3ba | 440 | 542 | 2285 | 102 | 09 |
| 3bb | 443 | 550 | 3771 | 107 | 09 |
| 3bc | 443 | 547 | 2796 | 104 | 10 |
| 3bd | 427 | 536 | 3853 | 109 | 17 |
| 3be | 424 | 535 | 2458 | 111 | 12 |

^a Measured in CH₃CN at 200 μ M. ^b Molar extinction coefficient. ^c Absolute fluorescence quantum yield determined with an integrating sphere system. ^d The structures of coumarin and fluorescein are shown in Fig. 1.



A good fluorophore for living systems has good properties including a remarkable Stokes shift, an applicable pH environment, high photostability and low biological toxicity. Keeping this in mind, we further examined these photophysical properties (Fig. 3). Based on the absorption and emission wavelengths in Table 2, all cyclic amidines exhibited remarkable Stokes shifts of ~ 100 nm, which could minimize self-absorption and provide better fluorescence imaging. We next studied the effect of pH on cyclic amidine **3aa**. The results showed that the pH value of the environment ranging from 2.8–11.4 had no effect on the emissive properties (Fig. 3c). In addition, amidine **3aa** showed excellent resistance to photobleaching with only 3.4% fluorescence lost after one hour at the wavelength of maximum excitation (Fig. 3d).¹⁴ Subsequently, the impact of different solvent environments was investigated. As shown in Fig. 3e, **3aa** worked well in aqueous environments, indicating that it could be used as a polarity probe. In addition, the emission intensity could be maintained in aqueous solution without loss after 24 h (see the ESI†). Moreover, cytotoxicity was another important factor for its application in living systems. We evaluated the cytotoxicity of **3aa** by using CCK-8 assays for HeLa cells, and it exhibited no significant cytotoxicity at concentrations of up to 100 μM (Fig. 3f). These results demonstrated that the novel amidine fluorophore has good properties of a large Stokes shift, pH insensitivity, low cytotoxicity and good photostability, which has great potential for biological imaging.

Ligand-based probes¹⁵ have received extensive attention and have extensive applications due to their high selectivity and

affinity in the visualization of receptor–ligand interactions and drug evaluation. In order to evaluate the potential of cyclic amidines for cell imaging, we synthesized a fluorescent probe **4** by conjugating cyclic amidine **3au** with the hemokinin-1 (HK-1) peptide (TGKASQFFGLM-NH₂), which was highly selective to the NK-1 receptor (Fig. 4a).¹⁶ The neurokinin-1 (NK-1) receptor, as a member of the G-protein-coupled receptor (GPCR) family, is located at the cell membrane. If a fluorescent ligand binds to the NK-1 receptor, it would give fluorescence signals at the cell membrane. To test whether fluorescent probe **4** could bind to the NK-1 receptor, WT 22RV1 and NK-1-overexpressing 22RV1 cells were treated with **4** respectively. As Fig. 4 shows, green fluorescence could only be observed in the cell membrane of NK-1-overexpressing 22RV1, not in the cell membrane of WT 22RV1. Furthermore, the fluorescence could be blocked with

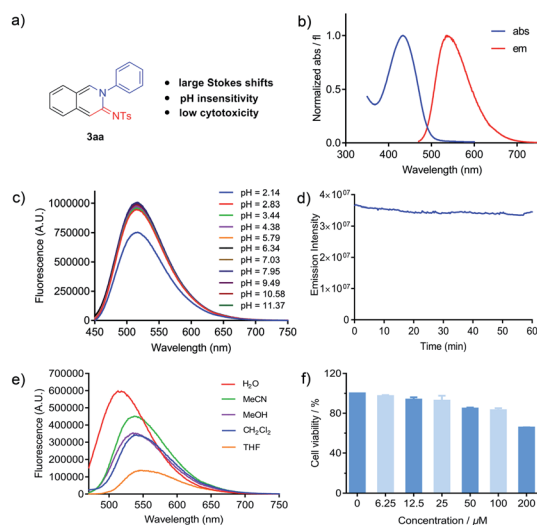


Fig. 3 (a) The photophysical properties were examined using **3aa** as a representative. (b) Normalized absorbance (abs) and emission (em) spectra of **3aa** in CH₃CN. (c) **3aa** was prepared as a 1 mM stock solution in DMSO and then diluted to a concentration of 10 μM with phosphate buffers of different pH values prepared in advance. Emission spectra were measured at 433 nm excitation. (d) Test of the photostability of **3aa** ($\lambda_{\text{ex}} = 433$ nm) in CH₃CN at 100.0 μM . (e) Emission spectra of **3aa** in various solvents at 20 μM . (f) Cell viability (%) was measured by using CCK-8 assays, treated in the presence of 6.25–200 μM of **3aa** using WT HeLa cells for 24 h at 37 °C.

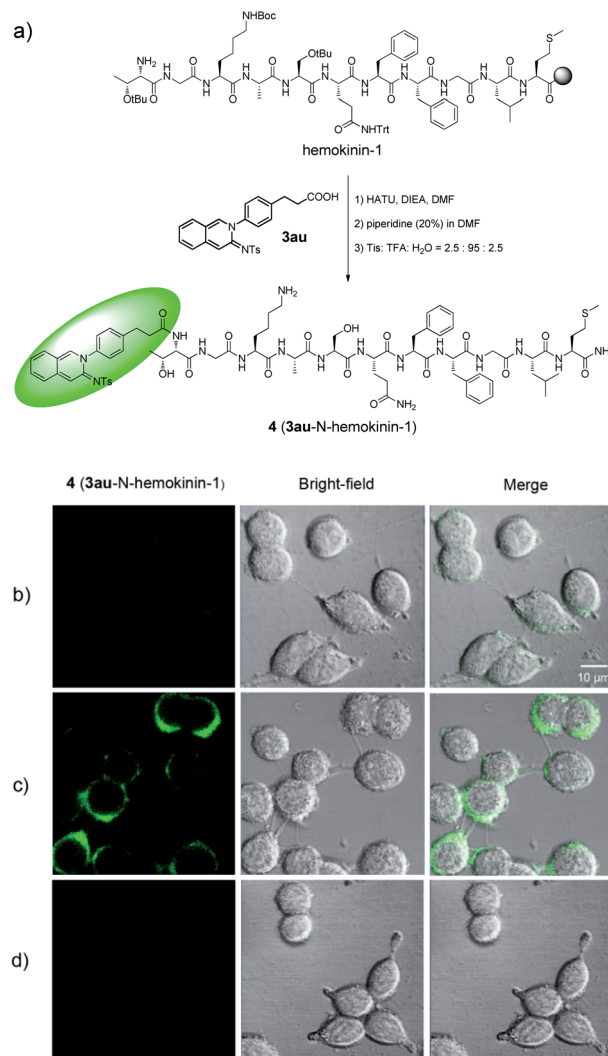


Fig. 4 (a) Synthesis and structure of fluorescent ligand **4** (**3au**-N-hemokinin-1). (b) WT 22RV1 cells and fluorescence images of 22RV1 cells treated with **4** (1 μM) at 37 °C for 30 min. (c) NK-1-overexpressing 22RV1 cells and fluorescence images of 22RV1 cells treated with **4** (1 μM) at 37 °C for 30 min. (d) NK-1-overexpressed 22RV1 cells treated with the NK1R inhibitor aprepitant (1 μM) at 37 °C for 30 min and then incubated with fluorescent ligand **4** (1 μM) for 30 min at 37 °C. Excitation wavelength 488 nm and emission wavelength 520 nm.



aprepitant, a selective NK-1 receptor antagonist, indicating that the fluorescent ligand **4** was specifically bound to the NK-1 receptor. These preliminary results demonstrated that amidine fluorophores could be used as potential bioprobes.

Conclusions

In summary, we have developed a general and efficient 6π -electrocyclic ring closure of ketenimine and imine starting from *N*-sulfonyl triazoles and arylamines. The method provides expeditious access to a variety of 10π -electron cyclic amidines in moderate to excellent yields. Through a fluorescence–structure relationship study, we found that cyclic amidine fluorophores have the advantages of large Stokes shifts, pH insensitivity, low cytotoxicity and higher photostability. Furthermore, they can be used efficiently in developing new fluorescent probes for imaging in living systems.

Conflicts of interest

There are no conflicts to declare.

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