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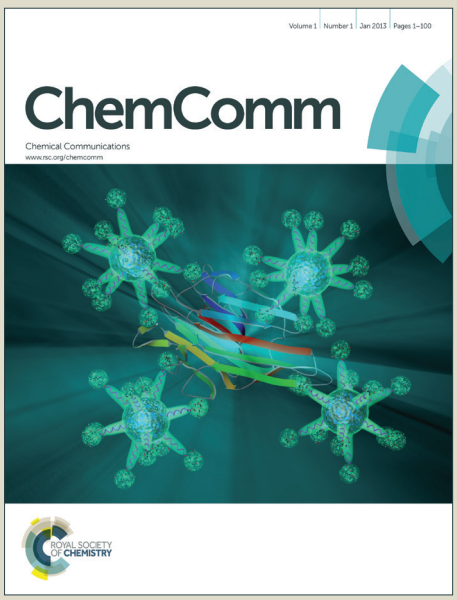

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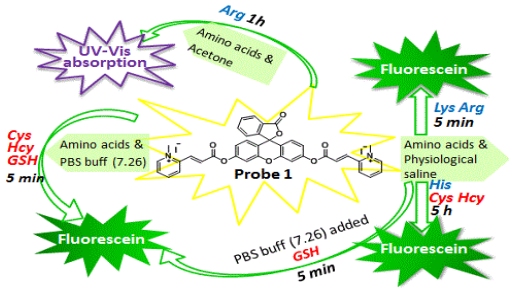
A smart probe to classify and differentiate basic amino acids, thiol-containing amino acids and GSH in selected environments.



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Multi-functional probe to discriminate Lys, Arg, His, Cys, Hcy and GSH from common amino acids

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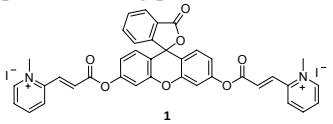
The co-existence and similar properties of amino acids result in the great challenge for the detection of individual amino acid. Herein we discovered that probe 1 may act as smart example to classify and differentiate basic amino acids (Arg/Lys/His), thiol-containing amino acids (Cys/HCy), and GSH through different sensing mechanism in selected environments.

Among common amino acids, basic amino acids e.g. lysine (Lys), arginine (Arg), histidine (His), and thiol-containing amino acids such as cysteine (Cys), homocysteine (Hcy), as well as reduced glutathione (GSH) play important roles in physiological processes.[1] Arg is the most basic natural amino acid and play crucial roles in cell division, immune function, the healing of wounds, and the release of hormones.[2] Lys is essential for the metabolic functions and weight gain of animals.[3] His is essential for the growth and repair of tissue, as well as for the control of metal transmission in biological bases.[4] Cys is a key amino acid and a precursor of GSH, CoA, and taurine. Abnormal levels of Cys have been associated with slow growth, hair depigmentation, liver damage, loss of muscle and fat, skin lesion, and cancer.[5] Hcy has been implicated in various types of vascular and renal diseases.[6,7] GSH level is biomarker of oxidative stress and the key factor for pre-venting serious damage to DNA, proteins, and lipid membranes.[8] Thus facile and selective detection of these amino acids is of vital significance in drug control,[9] food analysis,[10] medical diagnostics, and fundamental research.[11]

In the past few years, due to the high sensitivity of fluorimetric and colorimetric methods, some probes have been developed to recognize various amino acids.[12,13] Among them, probes for Cys and Hcy have been largely described, which take advantage of the unique nucleophilicity of the thiol groups.[12] However, great challenge was encountered to develop reaction-based probes for non-thiol-containing amino acids. A few number of fluorescent sensors were documented for other amino acids including phenylalanine (Phe),[14] tryptophan (Trp),[15] His,[16] Lys,[17] and Arg.[18] Unfortunately, most of the reported probes are not sensitive enough, difficult to synthesis and often poorly water soluble.[19] Furthermore, there is no single probe reported to discriminate simultaneously basic amino acids and thiol-containing amino acids from common amino acids. Herein we demonstrate that classifying and discrimination Lys, Arg, His, Cys, Hcy and GSH from common amino acids are possible using probes **1**.



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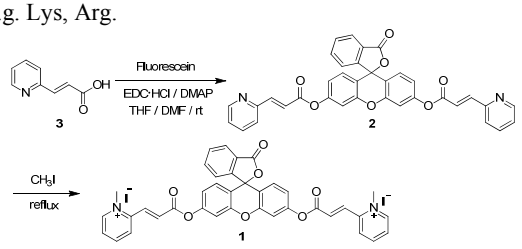
Our design concept was inspired by the recent advances in 1,4-addition of thiols to α, β-unsaturated ketones,[20] nitroolefin,[21] and esters.[22] Through conjugate addition/cyclization cascade sequence in fluorescein-bis-acrylate, apparent fluorescence enhancement to discriminate Cys from other amino acids was noticed.[22c,22d] One recent publication from Wu et al. revealed fast response to Cys owing to the electrostatic attraction.[20e] We speculated that fluorescein-bis-acrylate carrying pyridinium moiety at the terminal olefin site of each acrylate portion would possibly offer a number of advantages including complete water solubility and enhanced sensitivity to biothiols. In addition, the strong electron-withdrawing pyridinium would result in increased sensitivity to hydrolysis of the ester group, which might enable required reactivity with basic amino acids e.g. Lys, Arg.



Scheme 1 Synthesis of probe **1**.

Probe **1** was synthesized by a straight forward way as shown in Scheme 1. As expected, good water solubility of **1** was noticed. Probe **1** was subjected to various testing conditions against 18 of

amino acids carrying various side chains, and potential interfering species including metal ions (Fe^{3+}, Zn^{2+}), hydrogen sulfide (H_2S), as well as thiosemicarbazide (TSC). To our delight, when probe **1** in saline was tested against various analytes, simultaneous enhancement of fluorescence with the characteristics of fluorescein was observed only in the case of Lys, Arg.

Fluorescence responses at 513 nm were shown in Fig. 1. In short period of time (5 min), only Lys and Arg exhibited strong fluorescence (Fig. 1a). However, under long exposure time (e.g. 5 h), Cys, Hcy also presented significant responses, and His showed moderate fluorescence enhancement (Fig 1b). The absorption and emission spectra from various analytes were shown in Fig.S1.

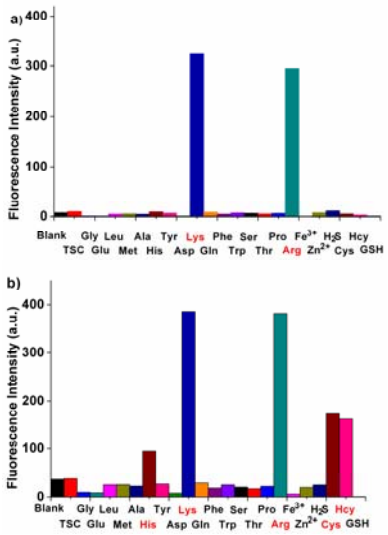


Figure 1. Emission response (λ_{ex} = 478nm) at 513 nm of **1** (10 μM) upon addition of various analytes (100 μM) in physiological saline at room temperature. a) Data recorded 5 min after addition; b) Data recorded 5 h after addition.

Time course fluorescence responses as shown in Fig. S2 indicated that highly reactive Lys and Arg were clearly differentiated from other analytes, and Lys was observed to be even more reactive than Arg in saline. These changes could be visualized clearly through photographs shown in Fig. S3. The pale solution of probe **1** turned yellowish upon addition of Lys or Arg and green emission could be recognized under UV irradiation immediately after addition. Under long exposure time up to 5 h, enhanced fluorescence was observed in the case of Cys, Hcy, as well as His.

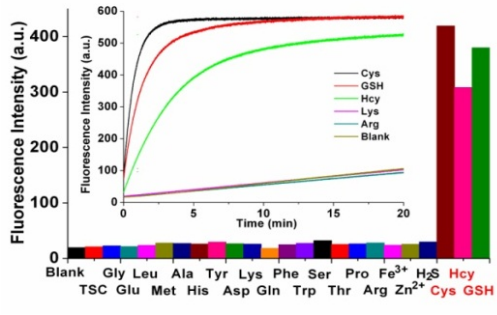


Figure 2. Emission responses at 513 nm (λ_{ex} = 478 nm, slit: 2.5 nm/2.5 nm) of **1** (10 μM) to various analytes (100 μM each) in PBS buffer (pH 7.26) collected 5 min after addition of various analytes at ambient temperature. Inset: Time course fluorescence response of probe **1** upon addition of various analytes.

Surprisingly, when the experiments were conducted in PBS buffer (pH 7.26), thiol-containing amino acids (Cys, Hcy, GSH) turned out to be very reactive. Cys, Hcy and GSH were demonstrated to induce intense fluorescence enhancement within 5 min. The data of fluorescence response at 513 nm were shown in Fig. 2. The kinetic studies shown in Fig. 2 (Inset) indicated that Cys reacted faster than Hcy. Accordingly, the absorption and fluorescence spectra were recorded (see Fig. S4).

In addition, GSH as tripeptide carrying thiol functional group exhibited fast response and large fluorescence enhancement. Thus GSH can be discriminated from other amino acids through no fluorescence in saline over 5 h (GSH was completely inactive in saline), however to be strongly fluorescent once PBS was added in the saline solution.

We strive to find method to distinguish Lys and Arg. We eventually discovered that probe **1** can act as colorimetric probe to specifically respond to Arg in acetone (see Fig. S5 and Fig. S6). Probe **1** and Arg in acetone displayed absorption band around 510 nm. Other water miscible solvent e.g. MeOH, EtOH, CH_3CN, or DMF did not produce this colored species. Moreover, the co-existence of Lys did not interfere with the discrimination of Arg (see Fig. S7.). The detection mechanism was unclear so far, which may need further investigation.

The interesting responses of probe **1** to basic amino acids and thiol-containing amino acids promoted us to study the behaviour of probe **1** in details. Thus pH effect of probe **1** in comparison with the precursor **2** was studied (see Fig. S8). As expected that probe **1** carrying pyridinium moiety was more susceptible to ester hydrolysis under alkaline conditions.

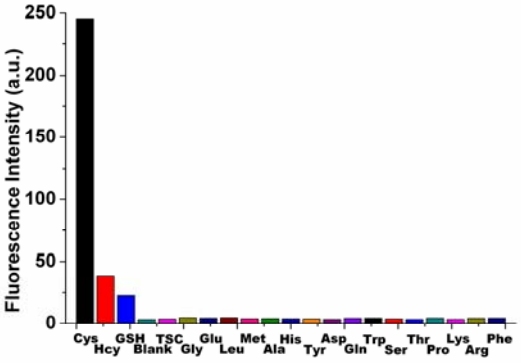


Figure 3. Emission response (λ_{ex} = 478nm, slit: 2.5 nm/2.5 nm) at 513 nm of **2** (10 μM) upon addition of various analytes (100 μM each) in CH_3CN-PBS buffer (pH 7.26, 2:8 v/v). Data were recorded 1 h after addition.

In an effort to discriminate Cys from Hcy, probe **1** was observed to respond too quickly to any thiol-containing amino acid. We were happy to discover that less reactive probe **2** which was the precursor of probe **1** was able to provide suitable differentiation. In CH_3CN-PBS buffer (pH 7.26), probe **2** was observed to respond to Cys much faster than Hcy, GSH, and other amino acids (see Fig. 3).

By varying the reaction media and time scale, with the assistance of probe **2**, the individual amino acid from the class of basic amino acid and thiol-containing amino acid could be discriminated (see Fig. S9, and Table 1). When probe **1** was exposed to various analytes in saline, Lys/Arg can be identified immediately. When probe **1** exposed to analytes was allowed to stand for 5 h, Cys, Hcy and His

were recognized according to the increased fluorescence. And then PBS was added to the individual saline solution containing the probe **1** and analyte. Lys/Arg remained strong fluorescence with slight decrease in intensity due to the dilution effect. The one which was initially non-fluorescent in short period of time, became gradually fluorescent and almost kept the brightness under UV irradiation after PBS addition may belong to His. Using the conditions shown in Fig. S5, Arg can be discriminated from Lys. Once the solution containing probe **1** and analyte became gradually fluorescent in saline and turned intense fluorescent once PBS was added, thiol-containing amino acid of Cys or Hcy should be contained. With the help of probe **2**, Cys can be discriminated from Hcy (Fig. 3).

Table 1. Response of Lys, Arg, His, Cys, Hcy and GSH with probe **1** and **2** in various test conditions.

Amino acid	**1** in saline		**1** in PBS 5 min	**1** in Acetone 1 h	**2** in CH_3CN-PBS
	5 min	5 h			
Lys	+	+	–	–	–
Arg	+	+	–	+	–
His	–	+	–	–	–
Cys	–	+	+	–	+
Hcy	–	+	+	–	–
GSH	–	–	+	–	–

To measure detection limit of probe **1** for Lys, Arg, Cys, Hcy, GSH respectively, **1** (10 μM) was treated with various concentration of Lys, Arg, Cys, Hcy, and GSH (0−30 μM for each). (Fig. S10). The fluorescence intensity at 513 nM was plotted against a function of the analyte concentration. The fluorescence intensity of **1** was linearly proportional to analytes concentration range of 1−30 μM. The detection limits of probe **1** for Lys, Arg, Cys, Hcy, and GSH were determined to be 20 μM, 15 μM, 2 μM, 4 μM and 2 μM respectively based on signal-to-noise ratio of 3.

The displayed unique properties of probe **1** promoted us to study the reaction mechanism. Thus responses of probe **1** to common nucleophiles, carbohydrates, nucleobases, fetal bovine serum (FBS), as well as albumin from bovine serum (BSA) were investigated in physiological saline and in PBS buffer (pH 7.26) (see Fig. S11). Various carbohydrates and nucleobases did not affect the probe. FBS and BSA containing various thiol-amino acids and basic amino acids induced fluorescence both in saline and in PBS. While toxic thiophenol and benzylmercaptan induced strong fluorescence in saline and PBS, benzylamine induced moderate fluorescence change only in saline, which suggested that PBS effectively retarded the reactivity of amine functionality. Spectroscopic studies were subsequently applied to follow up the various species involved during sensing process (see Fig S12 to S18). In saline, it was observed as expected that fluorescein was generated in the presence of Arg and Lys (see Figures S12 and S13). However, Arg mainly resulted in the basic hydrolysis of the ester bond and released N-methylpyridinium-2-yl acrylic acid (MS: 164; HRMS: 164.0706). While Arg mainly resulted in basic hydrolysis reflected by the presence of two sets of trans double bonds in NMR spectra, Lys not only induced the hydrolysis of the ester but also may participate the aminolysis of the ester to generate the amide (MS: 292, three sets of trans double bonds present in NMR spectra).. Cys and Hcy followed the anticipated electrostatic attraction-assisted conjugate addition/cyclization processes in PBS buffer as evidenced by the presence of the lactam species generated (see Fig. S14 and S15, MS: 267, HRMS: 267.0804 for Cys generated lactam; MS: 281, HRMS: 281.0961 for Hcy generated lactam, as well as the lack of trans double bond signals in NMR spectra).[22a] No direct hydrolysis of the ester was observed due to the lack of the signals from N-methylpyridinium-2-yl acrylic acid moiety both in HRMS and NMR spectra. In the case of GSH, the thiolate anion possibly attacked the ester center and resulted in the hydrolysis of the ester as evidenced by the presence of (N-methylpyridinium-2-yl) acrylic acid which was believed to be generated from the unstable thioester intermediate produced (see Fig. S16). The sensing mechanism for Cys/Hcy in saline was believed to follow conjugate addition/cyclization processes (see Figures S17 and S18). Additionally, the presence of N-methylpyridinium-2-yl acrylic acid signals suggested that the direct hydrolysis of the ester occurred presumably due to the inefficient conjugated addition/cyclization processes in saline. Based on the spectroscopic investigations, the sensing processes are summarized in Scheme S1. The mechanism of the specific response of probe **2** to Cys in CH_3CN-PBS buffer has been illustrated by Chen *et al*.[22c]

The observed unique experimental results of probe **1** to differentiate basic amino acids and bio-thiols can be explained well with the suggested mechanism. In saline, basic hydrolysis plays the major role for the sensing speed. With the highest isoelectric point (PI) (10.76 for Arg),[23] the basic guanidinyl group resulted in hydrolysis of ester bond in probe **1** and led to fast response. The surprisingly fastest response to Lys (PI 9.74)[23] in saline was also understandable because amine side chain acted as both a base and a nucleophile so that basic hydrolysis and nucleophilic attack by amine side chain functionality to the ester bond operated concurrently. For His, the weak basicity (PI 7.59)[23] was responsible for the long responding time. In saline, bio-thiol side chain existed in neutral form and possessed relatively weak nucleophilicity, so that it took long time for Cys/Hcy to react with the probe. GSH was completely inert, which suggested that the conjugation/cyclization did not happen in saline for GSH presumably due to the bulkiness and difficulty for large ring cyclization supported by the fact that *tert*-butyl mercaptan was completely inactive both in saline and in PBS as shown in Fig. S11.[22b] However, in PBS buffer (pH 7.26), the side chain of Arg, Lys, and even His was protonated and lost the nucleophilicity, thus was much less reactive. Cys, Hcy, and GSH instead, existed largely in the thiolate form as dominant species (The side chain pKa values for Cys, Hcy and GSH are 8.3, 8.87, and 9.2 respectively) which was strongly nucleophilic,[24] and led to the quick turn-on of the fluorescence. The lowest pKa of Cys resulted in the fastest response accordingly. Hcy possesses a slightly higher pKa, thus less content of thiolate was formed in PBS, which led to decreased sensing speed. Surprisingly, GSH was observed to display fast response with the rate between Cys and Hcy. Such interesting phenomenon may be attributed to the bulkiness of GSH, which leads to the direct hydrolysis of the ester bond without touching the unsaturated acrylate. Although such direct attach of thiolate to ester bond could not be excluded during the sensing process of Cys/Hcy, Michael addition/cyclization cascade reaction pathway was believed to play major role from the evidences of LC-MS, HRMS and NMR spectroscopic studies.

In summary, we have successfully developed easily synthesized, probes **1** to differentiate Arg, Lys, His, Cys, Hcy, GSH from common amino acids. Probe **1** can also classify the basic amino

acids, thiol-containing amino acid, GSH as witnessed with naked eyes. The good linear response of **1** to Lys, Arg, Cys, Hcy, or GSH in 1−30 μM may be applied in drug and food control.

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Notes and references

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Electronic Supplementary Information (ESI) available: The synthesis, UV-vis absorption, emission spectra and images upon addition of analytes; The fluorescence intensity linear relationship, LC-MS analysis; The sensing mechanism; Copies of NMR spectra.. See DOI: 10.1039/c000000x/

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