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β-carboline-based pH fluorescent probe and its application for monitoring enzymatic ester hydrolysis

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A novel pH-activatable fluorescent probe L-1 based on β-carboline derivatives has been developed, which displays significant fluorescent response toward pH variation with high selectivity, good photo-stability and favorable pKa value. Moreover, L-1 can dynamically monitor the release of protons during ester hydrolysis reaction in consistent with enzymatic kinetics manner.

Keywords: pH probe • β-carboline • enzymatic • ester hydrolysis

Introduction

Enzymatic ester hydrolysis involves in a wide range of physiological processes, such as lipid metabolism and xenobiotics detoxification.[1-2] In a large number of therapeutic prodrugs, effective hydrolytic cleavage of ester linkage under varied esterases is a critical biotransformation and activation step.[3-4] Therefore, precise and sensitive monitoring of ester hydrolytic process would be beneficial for drug development and also facilitate further investigations on the lipid metabolism-disease pathogenesis.[5]

Traditional chromatographic techniques are discontinuous and time-consuming, which are not suitable for rapid kinetic reactions.[6] For those spectrophotometric methods based on p-nitrophenyl ester, a marked change in the absorption spectrum can be detected after the hydrolytic reaction.[7] Another widely-used approach is to design fluorescent activatable substrates to real-time probe endogenous esterase activity.[8-10] However, in above-mentioned methods, the requirement of certain chromogenic and fluorogenic substrates limits their applications to structurally diverse substrates and other enzyme mutants. Meanwhile, the strategy employing pH-sensitive indicators (including phenol red, carboxyfluorescein and quantum dot) to monitor the release of protons during the hydrolysis reactions have been established.[11-13] Those methods are rapid and straightforward, but still have some problems with regard to potential enzyme-dye interaction, optical instability and irreversible detection.

Desirable pH fluorescent probes should be highly sensitive and selective to pH variation without irreversible quenching or photo-bleaching,[14-18] so that the varying fluorescent response can be converted to the enzymatic rate of ester hydrolysis accordingly. β-Carboline alkaloids are important heterocyclic scaffolds for biological and medicinal applications owing to their extensive anti-HIV, anti-tumor and anti-bacterial activities.[19-20] Besides, the rigid conjugate structure of β-carboline ring is expected to be fluorescent-activatable with proper derivatization.[21] In our previous work, we identified a β-carboline alkaloid isolated from the fruiting bodies of *Suillus granulatus* with anti-HIV activity, [22-23], and then synthesized a series of β-carboline analogues.[24] Among those compounds, we focused on **L-1**, **L-2** and **L-3** (*Figure 1*) substituent with the same carboxylic group at C3-position but different groups at C1-position. It is anticipated that protonation of pyridine nitrogen atom under acidic condition would change the electronic distribution and fluorescent properties of those three compounds, which could serve as pH fluorescent probe for monitoring enzymatic ester hydrolysis.

Results and Discussion

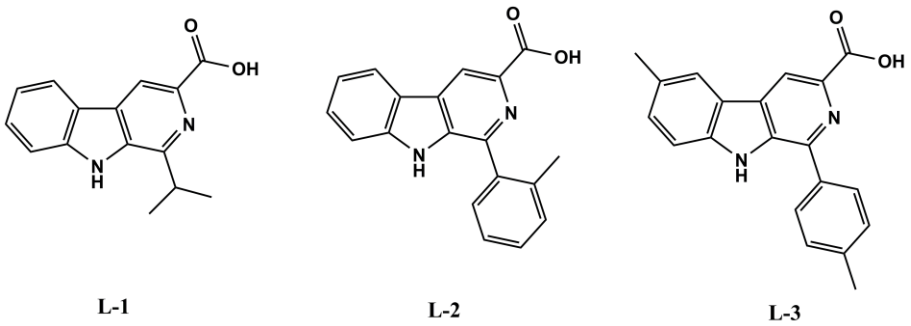

Figure 1. Structures of β-carboline alkaloid derivates **L-1** (1-Isopropyl-β-carboline-3-carboxylic acid), **L-2** (1-(3′-Methylphenyl)-β-carboline-3-carboxylic acid) and **L-3** (1-(4′-Methylphenyl)-6-methyl-β-carboline-3-carboxylic acid) as pH fluorescent probe.

First of all, the pH dependences of the spectral characteristics of the three β-carboline alkaloid derivates were evaluated (*Figure 2*). Standard fluorescence pH titrations were performed in potassium phosphate buffer ranging from pH 2.0 to pH 10.0 with compound concentrations of 0.25 μM at 25℃. For compound **L-1**, the excitation wavelength is 370 nm and the change of its fluorescent emission at 440 nm based on the pH variation is over 70 folds (*Figure 2a*). This significant enhancement can also be visually observed through the graph in abstract. Similarly, compound **L-2** (*Figure 2b*) had strong fluorescent response (>120 folds) at 465 nm under 380 nm irradiation, while **L-3** (*Figure 2c*) had moderate fluorescent response (≈ 45 folds) at 495 nm under 395 nm irradiation. It reveals that Photoinduced Electron Transfer (PET) quenching effect within β-carboline derivates was rendered after the protonation of pyridine nitrogen atom under acidic condition, resulting in highly fluorescent emission.[25]

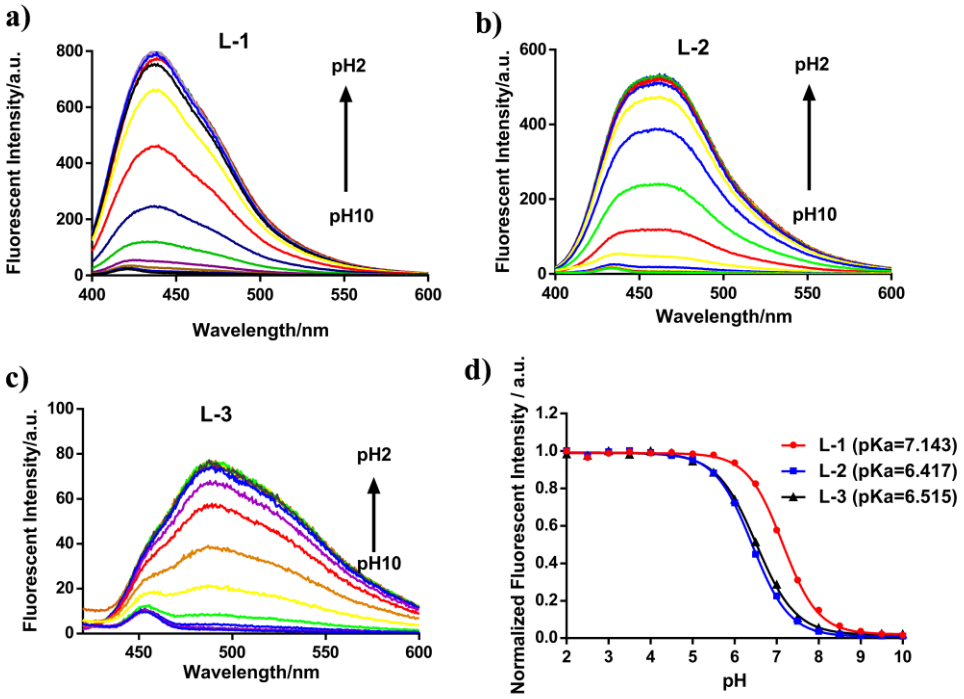

Figure 2. (a-c) Fluorescent emission changes with the pH titration curve of β-carboline alkaloid derivates **L-1** (a), **L-2** (b) and **L-3** (c). All samples were measured with concentration of 0.25 μM in 10 mM potassium phosphate buffer at pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. Excitation wavelength is 370 nm (a), 380 nm (b) and 395 nm (c) respectively. (d) pH-dependent changes in normalized fluorescent intensity at maximum emission wavelengths of the three β-carboline alkaloid derivates. Results are represented as mean from triplicates.

Table 1. Photochemical Properties of pH Probes Based on the β-carboline Scaffold

Compound[a]	Abs / Em (nm)	pKa	Quantum Yield	Extinction coefficient ($M^{-1}cm^{-1}$)
L-1 (protonated)	370/440	7.143	0.796	6.4×10^3
L-1 (deprotonated)	345/440		0.031	3.1×10^3
L-2 (protonated)	380/465	6.417	0.556	4.1×10^3
L-2 (deprotonated)	350/465		0.015	1.9×10^3
L-3 (protonated)	395/495	6.515	0.090	3.2×10^3
L-3 (deprotonated)	370/495		0.008	2.0×10^3

[a] Acidic condition was pH 2, and basic condition was pH 10

Then, the constants pKa of the three probes were determined through analysis of fluorescence intensity changes as a function of pH (*Figure 2d*). By using Henderson-Hasselbalch equation (see SI), the calculated pKa values differed depending on the substituent group ranging from 6.4 to 7.1, in accordance with the results of the nonlinear regression of fluorescence data in *Figure 2d*. The photochemical properties of these three compounds based on β-carboline scaffold were provided in *Table 1*. In pH 10.0 buffer, the fluorescence quantum yield (QY) of **L-1** was very low (QY= 0.031), which was determined in reference to commercially available Rhodamine-6G (QY= 0.950). Whereas upon protonation, the PET process within β-carboline ring was rendered, strong fluorescence was obtained immediately and the fluorescence quantum yield increased to 0.769 in pH 2.0 buffer. Furthermore, **L-1** showed red shift of 25 nm for the maximum absorption under acidic conditions as the result of the decreasing electron-donating ability of pyridine (*Figure 3a*). The linear relationship between fluorescence and pH over the pH range 6.8-8.0 was verified (*Figure S1*, r^2= 0.991, n = 3). Through analysis and filtration, compound **L-1** was selected with its significant fluorescent response and near-neutral pKa value of 7.143 as optimal pH probe for biological applications.

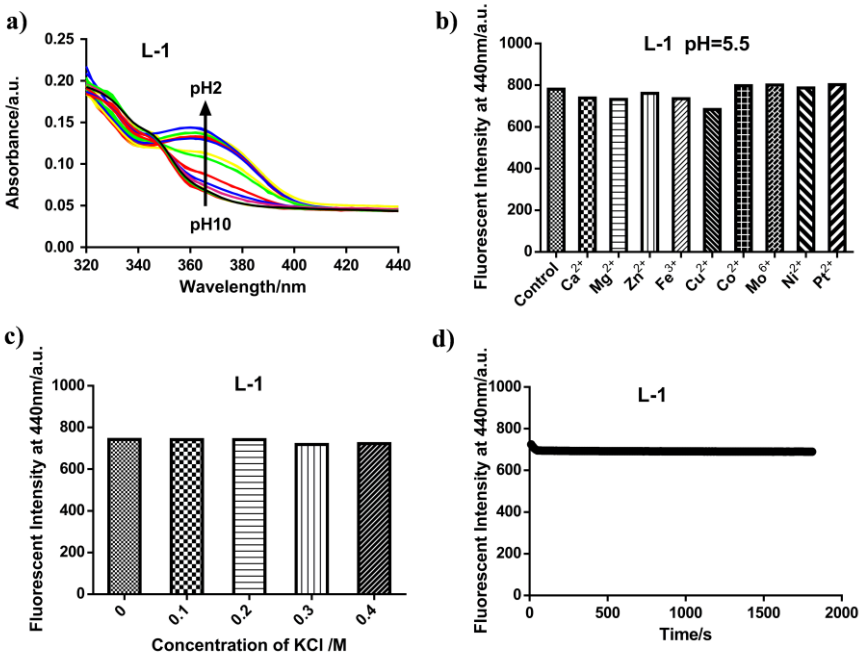

Figure 3. (a) Dependence of the UV absorption of **L-1** (20 μM) on pH in buffered aqueous solution (pH 2-10). (b) Fluorescence responses at 440 nm of **L-1** (0.25 μM) in the presence of excess 50 equiv of nine different metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Mo^{6+}, Ni^{2+} and Pt^{2+}) in pH 5.5 phosphate buffer (10 mM, pH 5.5). (c) Effects of ion strength on the fluorescence of **L-1** were measured. The concentration of **L-1** was 0.25 μM in phosphate buffer (10 mM, pH 5.5) and the concentration of KCl ranges from 0-0.4 M. (d) Time course of **L-1** were measured by spectrofluorometer (λex= 370 nm and λem= 440 nm) within 30 mins. The concentration of **L-1** was 0.25 μM in phosphate buffer (10 mM, pH 5.5). Data time interval is 10.00s.

Considering that amines can bind many metal cations, the selectivity assays of 0.25 μM **L-1** were conducted at pH 5.5 (*Figure 3b*) and pH 8.0 (*Figure S2*) in the presence of excess 50 equiv (12.5 μM) of different metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Mo^{6+}, Ni^{2+} and Pt^{2+}). As expected, the interference of those metals cations on pH fluorescent measurement is negligible. Additionally, almost no effects on fluorescent intensity were observed for different ion strength from KCl (*Figure 3c*) and the fluorescence response of **L-1** kept constant within 30 mins (Figure 3d). All above, it demonstrates that compound **L-1** can instantly respond to pH variation without potential interferents from metal cations, ion strength and photo-bleaching.

As carboxylesterase plays an important role in detoxification of exogenous toxins and metabolism of many ester drugs, an in-depth study of substrate recognition of carboxylesterase must lay a theoretical foundation for the successful design of ester prodrugs. We next sought to utilize our fluorimetric pH probe **L-1** to monitor the enzymatic hydrolysis of medicinal ester under carboxylesterase. Purified hog liver carboxylesterase and ethyl nicotinate were chosen as the model enzyme and substrate, time-course of the fluorescent response of **L-1** during enzymatic hydrolysis was evaluated (*Figure 4*). Different concentrations of ethyl nicotinate (0.11-3.50 mM) were preincubated with pH probe **L-1** (0.25 μM) in potassium phosphate buffer (1 mM, pH 8.0). Then, purified hog liver carboxylesterase (1.56 μg/mL) was added and the real-time fluorescent change of the mixtures was recorded. It shows that the fluorescence intensity gradually increased during the hydrolysis reaction, which means that stoichiometric production of protons can be measured by pH indicator present in the reaction mixture. Moreover, the higher rate of fluorescence enhancement indicating faster enzymatic reaction rate was observed with increasing concentrations of both ethyl nicotinate (*Figure 4a*) and carboxylesterase (*Figure 4b*). Kinetic analysis was investigated upon the present fluorimetric assay (*Figure S3*). It displayed a good linear correlation upon Lineweaver-Burk Plot, and Michaelis-Menten constant (Km) was calculated as 0.473 ± 0.023 mM, in consistent with the value previously obtained by a traditional time-consuming HPLC method (0.353 ± 0.024 mM).[26] The sensitive fluorescent response allowed low concentration of pH probe **L-1**, which minimized putative enzyme-indicator interactions or inhibition of enzyme activity. Considering the complexity of the reaction mixture, auto-fluorescence interference or quenching from the substrate and enzyme were excluded (*Figure S4*), indicating the measurements sensitive and specific.

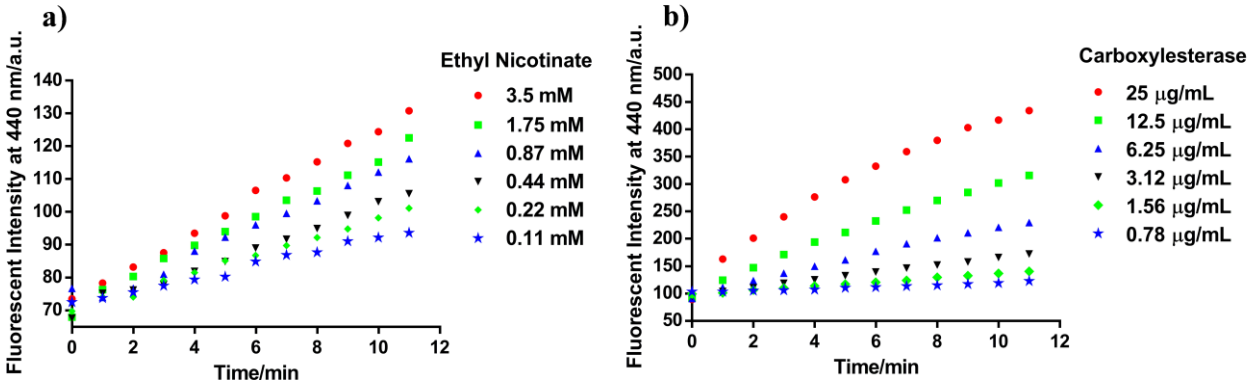

Figure 4. Time-course of the fluorescent response of **L-1** during enzymatic hydrolysis of ethyl nicotinate. (a) Varied concentrations of ethyl nicotinate (0.11, 0.22, 0.44, 0.87, 1.75 and 3.5 mM) were co-incubated with **L-1** (0.25 μM) and catalyzed by purified hog liver carboxylesterase (1.56 μg/mL). (b) Ethyl nicotinate (3.5 mM) were pre-incubated with **L-1** (0.25 μM) and catalyzed by varied concentrations of purified hog liver carboxylesterase (0.78, 1.56, 3.12, 6.25, 12.5 and 25 μg/mL).

Conclusions

In summary, β-carboline derivative **L-1** exhibits remarkable changes of fluorescent emission accompanying with its protonation under acidic condition. With a favourable pKa value, it can instantly respond to pH variation regardless of potential disturbances from metal ions, proteins and optical instability. Furthermore, this pH-activatable fluorescent probe **L-1** is applicable of dynamically monitoring and visualizing the enzymatic ester hydrolysis process, which will facilitate the rapid and accurate high-throughput screening of therapeutic esters.

Experimental Section

General

β-carboline alkaloid derivates **L-1**, **L-2** and **L-3** were synthesize by Dr J. G. Tang in our group**.** All solvents and reagents were commercially available and used without further purification unless for special needs. Ethy nicotinate was purchased from Macklin (shanghai, China), purified hog liver carboxylesterase were purchased from Sigma–Aldrich (St. Louis, MO, USA), and inorganic and organic salts (such as KCl, K_2HPO_4, KH_2PO_4, $NaMoO_4$, $FeCl_3$, $ZnCl_2$, $MgSO_4$, $CaCl_2$ and so on) were purchased from Adamas (China). The ultra-pure water used throughout the experiments was purified with a Milli-Q A10 filtration system (Millipore, Billerica, MA, USA).

Optical Properties Study

Fluorescent emission spectra were collected from 400-600 nm on PerkinElmer LS 55 with different excitation wavelength (440 nm for **L-1**, 465 for **L-2** and 495nm for **L-3**), the excitation and emission slit widths were 10 and 8 nm, respectively. Quartz cuvettes with 2 mL mixture volume used for emission measurements. UV-Vis absorption spectra were collected in 96-well plate on microplate reader (Tecan spark 10M) from 300-500 nm with 200 μL mixture volume. Unless otherwise specified, all spectra were taken at 25 ℃ in 10 mM potassium phosphate buffers. All pH measurements were performed with a pB-10 pH-meter (Sartorius, Shanghai, China) with a combined glass-calomel electrode.

Enzymatic ester hydrolysis

(1) Fluorescent response vs different concentrations of substrate. Different concentrations of ethyl nicotinate (0.11, 0.22, 0.44, 0.87, 1.75 and 3.5 mM) were preincubated with pH probe **L-1** (0.25 μM) in potassium phosphate buffer (1 mM, pH 8.0) and catalyzed by purified hog liver carboxylesterase (1.56 μg/mL). (2) Fluorescent response vs different concentrations of enzyme. Ethyl nicotinate (3.5 mM) were pre-incubated with **L-1** (0.25 μM) and catalyzed by varied concentrations of purified hog liver carboxylesterase (0.78, 1.56, 3.12, 6.25, 12.5 and 25 μg/mL). All the measurements were conducted in potassium phosphate buffer (1 mM, pH 8.0) with the final volume of 2 mL at 25 ºC. The real-time fluorescent change at 440 nm was recorded as soon as the carboxylesterase was added and stopped within 15 mins.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

R. Huang was responsible for experiment design, result, discussion, and preparing the manuscript. X.-D. Zhang and X. Wu carried out the bioassays. J.-K. Liu provided the compounds and supervised this research.

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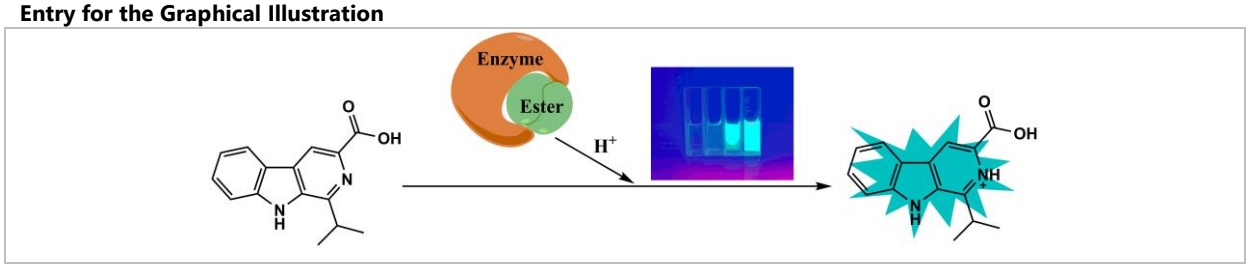
Entry for the Graphical Illustration



Twitter Text

β-carboline derivative JKL-1 can serve as pH-activatable fluorescent probe and dynamically monitor enzymatic ester hydrolysis reaction.