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FRET-based fluorescence probes for hydrolysis study and pig liver esterase activity

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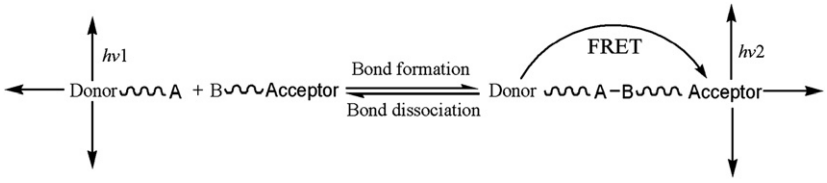
ABSTRACT

New fluorescent probes based on simple organic synthesis were designed and synthesized, and their hydrolysis catalyzed via base and pig liver esterase (PLE) was studied using FRET (fluorescence resonant energy transfer), with 1-naphthylacetic group as a donor and dansyl group as an acceptor. By simultaneous recording of changes of the donor fluorescence intensities, kinetic parameters for base-catalyzed and PLE-catalyzed hydrolysis can be determined. The presented FRET assay is a convenient and simple method and both fluorescent probes are good real-time indicators for the analysis of ester hydrolysis such as PLE activities.

1. Introduction

Fluorescence resonance energy transfer (FRET) as a physical phenomenon was first described by Förster,[1] to describe a non-radiative process whereby an excited state fluorophore donor transfers energy to a proximal ground state acceptor through long-range dipole–dipole interactions. The rate of energy transfer is highly dependent on many factors, such as the extent of spectral overlap, the relative orientation of the transition dipoles, and, most importantly, the distance between the donor and acceptor moieties (usually <6 nm).[2] The general rules for selecting appropriate FRET pairs include sufficient separation in excitation spectra for selective stimulation of the donor, a overlap (>30%) between the emission spectrum of the donor and the excitation spectrum of the acceptor to obtain efficient energy transfer, and reasonable separation in emission spectra between donor and acceptor to allow independent measurement of the fluorescence of each fluorophore.[3] FRET has been used over the past four decades as a versatile method for measuring noncovalent binding events in biochemical research such as structural elucidation of biological molecules and their interactions,[4] in vitro assays,[5] in vivo monitoring in cellular research,[6] nucleic acid analysis,[7] signal transduction,[8] light harvesting, and nanomaterials.[9] The FRET assay has some advantages including high-throughput screening, real-time detection, and in vivo ratiometric probes due to donor/acceptor fluorescence ratio change. However, the general investigation of the chemical bond formation and disassociation based on the FRET technique in organic synthesis (Scheme 1) has only been reported recently, such as in catalysts discovery[10,11] and hydrolysis reactions of phosphodiesters.[12] In this work, we have designed and synthesized a FRET pair with total separation of their emission



Scheme 1. General application of FRET in organic synthesis.

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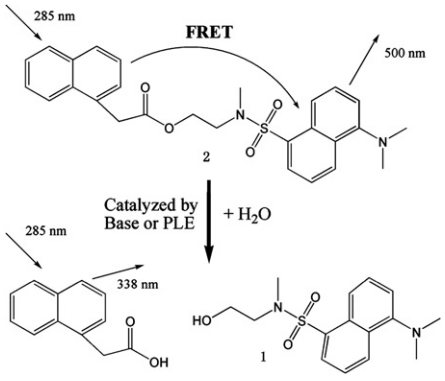
spectra to study base-catalyzed hydrolysis and pig liver esterase (PLE) activity, the widely used esterase in industry.[13]

PLE is a serine-type esterase with wide substrate tolerance.[14] The reported kinetics of PLE were measured by applying the pH-stat technique to unbuffered solution,[14,15] which is not very convenient and sensitive. Furthermore, there is few effective method to monitor PLE activity in real-time with high sensitivity. In order to conveniently probe this widely used enzyme, herein a sensitive ratiometric fluorescent probe, namely, observation of changes in the ratio of the fluorescent intensities of the emission at two wavelengths, were developed for quantitative detection of PLE activity. A commercial fluorescence reader is enough for the measurements and the hydrolysis of a carboxyl ester catalyzed by base and PLE was studied based on the FRET assay.

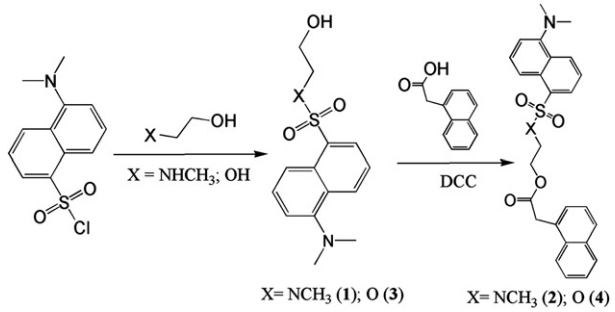
2. Results and discussion

1-Naphthylacetic moiety was selected as the fluorescent donor and a dansyl group as the acceptor.[16] Both donor and acceptor are located on opposite sides in a single molecule with ester bond as a linker, as shown in Scheme 2. For the FRET pair, the fluorescence from 1-naphthylacetic group is quenched by dansyl group with increased fluorescence from dansyl. If the ester bond is cleaved, no FRET occurs and the emission of donor increases. The changes of the ratio of the emission intensities at both acceptor and donor's peak wavelengths can then be converted to concentrations. If we follow the fluorescent data with time, kinetic parameters can be determined.



Scheme 2. The FRET-based detection mechanism.

The simple two-step syntheses successfully gave FRET-based fluorescence probes **2** and **4** (Scheme 3), which have a 1-naphthylacetic moiety as the donor and a dansyl moiety as the acceptor. Both donor and acceptor possess functional groups that are inert toward most types of coupling reactions. So a large number of nucleophiles and electrophiles could be attached to this FRET pair for investigation of a variety of reactions. The hydrolysis reactions of carboxyl esters were chosen in this work due to the importance of such reactions.[13–15,17]



Scheme 3. Synthesis of the fluorescent probes.

Figure 1 shows the absorption and photoluminescence of two pure hydrolysis products *N*-methyl-*N*-dansyl-2-aminoethylanol (**1**) and 1-naphthylacetic acid. 1-Naphthylacetic acid has strong emission at 326 and 338 nm excited at 285 nm, while compound **1** displays broad absorption band in the range of 290–410 nm. FRET efficiency is dependent on the spectral overlap integral between the donor emission and acceptor absorption. There is a significant spectral overlap between the two fluorophores, implying that they are good FRET pairs in an aqueous solution. The fluorescence quantum yields (Φ_f) of 1-naphthylacetic acid and 1-naphthylacetic group as a donor in compound **2** were 0.070 and 0.006, respectively, implying that the FRET efficiency in compound **2** is larger than 90%.

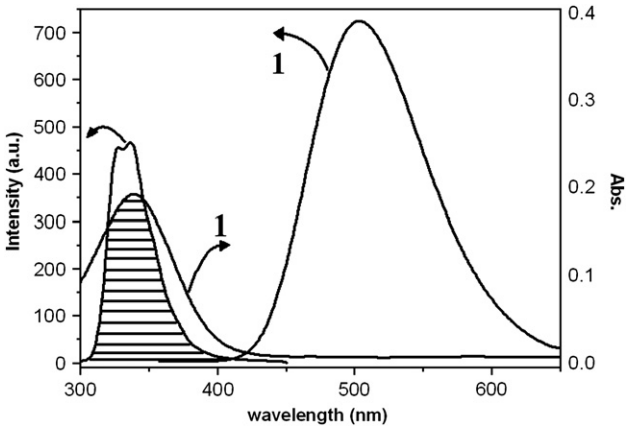


Figure 1. Spectral overlap (shaded area) of the emission of 1-naphthylacetic acid and the absorption of compound **1**; emission spectra of 1-naphthylacetic acid and compound **1** were excited at 285 and 338 nm, respectively; all spectra were in 3×10^{-5} M aqueous solution containing 2% CH_3CN. Left arrows represent fluorescence spectra; right arrow represents UV–vis absorption spectra of compound **1**.

The emission spectrum of compound **2** in aqueous solution excited at 285 nm exhibits that the donor at around 338 nm was strongly quenched and the acceptor at around 500 nm was observed. This emission spectrum demonstrates that energy transfer from the donor to the acceptor can proceed efficiently. Addition of compound **2** into a 0.1 M aqueous NaOH solution resulted in a rapid decrease in the acceptor fluorescence at around 500 nm and a rapid increase in the donor fluorescence at around 338 nm (Fig. 2). With enough reaction time, the emission intensity of the reaction solution nearly approached that of equimolar mixture of compound **1** and 1-naphthylacetic acid. Compound **2** displays a large shift (from 500 to 338 nm) in its emission spectrum after the base-catalyzed cleavage of ester bond within two fluorophores.

The reaction rate can be determined by relying on either the donor fluorescence or acceptor fluorescence only. Since the donor fluorescence is a 'turn-on' signal and is linearly proportional to the product formed, its change may be a better indicator of the initial reaction rate. As shown in Figure 3, the donor fluorescence has better linear correction (correlation coefficient R=0.9979) in the standard curve than that of the acceptor fluorescence (R=0.9779). Therefore, we used the donor fluorescence to determine kinetic parameters.

The concentration of compound **2** in the reaction mixture was determined by changes of the fluorescence at 338 nm. A plot of concentration of compound **2** versus time in the base-catalyzed hydrolysis (0.1 M NaOH) is shown in Figure 4. Due to excessive OH^- in the reaction, the hydrolysis reaction can be seen as pseudo-first-order. The observed kinetic constant k_{obs}, kinetic constant k_2, and

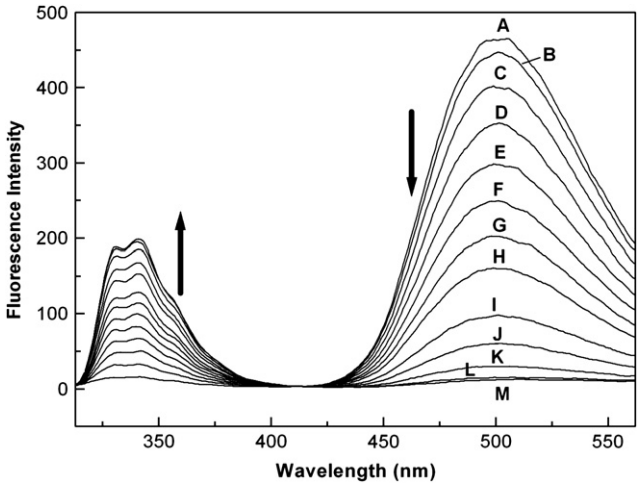


Figure 2. The emission spectra of compound **2** (9 μM) in 0.1 M aqueous NaOH solution at 20 °C: (A) 0 min, (B) 2 min, (C) 4 min, (D) 6 min, (E) 8 min, (F) 10 min, (G) 12 min, (H) 14 min, (I) 18 min, (G) 22 min, (K) 30 min, (L) 44 min, after addition of compound **2**; (M) equimolar mixture of **1** and 1-naphthylacetic acid in 0.1 M aqueous NaOH.

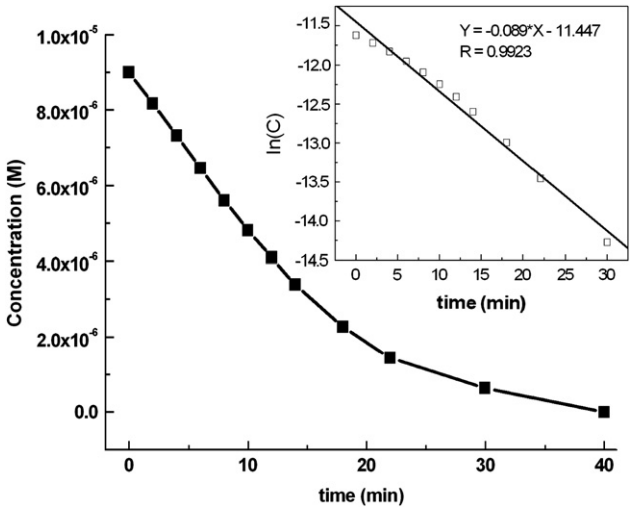


Figure 4. The concentration *C* versus time for the hydrolysis reaction in Figure 2 and ln(*C*) versus time (inset) of compound **2**.

half life time $T_{1/2}$ can be calculated by equations $\ln(C)=-k_{obs}t+B$ (B is a constant), $k_2=k_{obs}/C_1$, and $T_{1/2}=\ln 2/k_{obs}$, where C and C_1 are the concentration of compound **2** and OH^-, respectively. Curve fitting of the plots gives $k_{obs}=0.089\ min^{-1}$, $k_2=0.89\ M^{-1}\ min^{-1}$, and $T_{1/2}=7.79$ min (Fig. 4).

Compounds **2** and **4** were further chosen as ratiometric fluorescent probes for pig liver esterase activity. Without the enzyme, the fluorescence intensities of both compounds were hardly affected in the buffer (100 mM phosphate, pH 8.0 at 20 °C) for more than 48 h. Addition of PLE to an aqueous solution of these fluorescent probes resulted in an increase in the donor fluorescence and a decrease in the acceptor fluorescence, which is similar to the hydrolysis reaction in aqueous NaOH solution. Standard curves of the donor fluorescence were made at the same conditions with enzyme-catalyzed hydrolysis in the absence of enzyme.

Varying substrate concentrations and simultaneous recording of donor fluorescence intensity with time yield the kinetic data (Fig. 5). The plot of inverse initial velocity ($1/v$) of hydrolysis versus inverse substrate concentrations ($1/[S]$) was fitted by Lineweaver–Burk equation: $1/v=((K_m/V_{max})/[S])+1/V_{max}$, where V_{max} is the apparent maximum rate and K_m is the apparent Michaelis constant. The K_m and V_{max} for compounds **2** and **4** were determined to be 12.22×10^{-5} and 1.56×10^{-6} M/min/unit enzyme, 3.42×10^{-5} and 0.82×10^{-6} M/min/unit enzyme, respectively. The K_m value of **2** is larger than that of **4**, which may be due to the steric effect of methyl group in **2** influencing its affinity for PLE. The reported kinetic parameters of PLE varied widely according to the various structures of substrates (K_m of 4.14×10^{-5} to 5.3×10^{-3} M and V_{max} of 1.40×10^{-6} to 11.8×10^{-6} M/min/unit enzyme).[14,15] Comparing the experimentally obtained kinetic parameters of **2** and **4** with the literature data, K_m value of both compounds is at lower end of reported reactions, implying that both probes have relatively high affinity for PLE with a relative fast catalytic rate for hydrolysis. These results suggest that compounds **2** and **4** should be good ratiometric fluorescent probes for PLE activity.

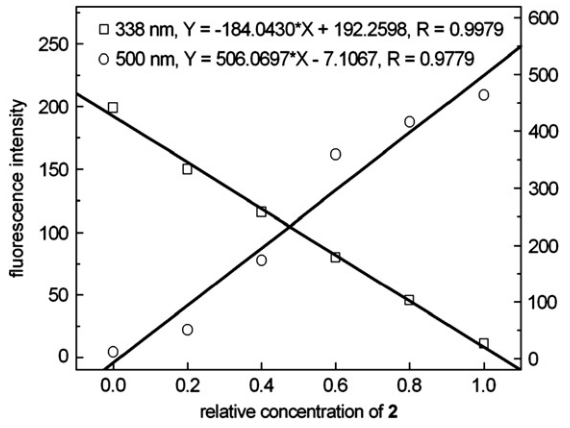


Figure 3. Standard curves for base-catalyzed hydrolysis. Conditions: compound **2** varied from 0 to 9 μM, the total concentration of **2** and 1-naphthylacetic acid was 9 μM, equimolar mixture of compound **1** and 1-naphthylacetic acid in 0.1 M aqueous NaOH. The donor fluorescence is a turn-on signal recorded at 338 nm, while the acceptor fluorescence is a turn-off signal recorded at 500 nm.

3. Conclusions

In conclusion, we have developed two new FRET-based fluorescent probes that can be easily used in the study of ester hydrolysis. 1-Naphthylacetic group as donor and dansyl group as acceptor were chosen as a FRET pair because (1) both possess functional groups to tether various types of coupling reactions for study; (2) they have good FRET efficiency; and especially (3) both emission spectra are totally separated for precision ratiometric measurements. Used this FRET pair, the hydrolysis of a carboxyl ester catalyzed by base and PLE was studied and the kinetic parameters were obtained. We believe that this FRET pair can be used for investigation of a variety of chemical reactions and both fluorescence probes **2** and **4** are practically useful for the real-time analysis of ester hydrolysis such as PLE activities. Since the study of protein dynamics in living cells is critical to an understanding of sophisticated process of life,[18] our further work will focus on developing small-molecular FRET probe to monitor specific proteins in vivo.

4. Experimental

4.1. General

Pig liver esterase (PLE, 27 unit/mg) was purchased from Sigma (Beijin, China), lyophilized powder containing less than 5% buffer salts, 24 unit/mg. One unit will hydrolyze 1.0 mol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25 °C. All chemical reagents were commercially available and were used

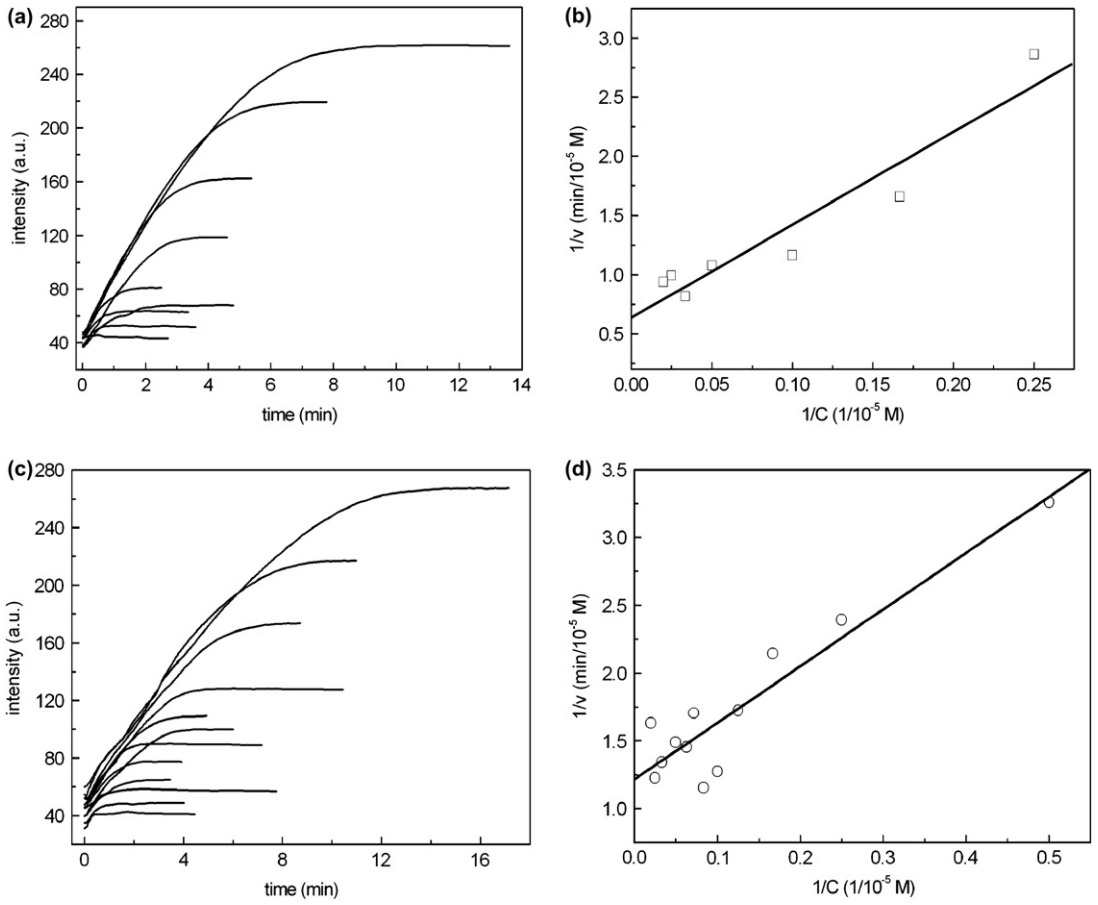


Figure 5. Determination of kinetic constants of fluorescence probes **2** and **4** for PLE. Conditions: pig liver esterase (0.2 unit enzyme/ml) in 0.1 M sodium phosphate buffer (pH 8.0) at 20 °C, compounds **2** or **4** with increased concentrations from 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 30.0, 40.0 to 50.0 μM. (a) and (c) changes in the donor emission at 338 nm. (b) and (d) Lineweaver–Burk plot of enzyme-catalytic hydrolysis reactions measured as a function of the concentration of substrates; the solid lines represent the best fitting results.

without further purification. Solvents were distilled from the appropriate drying agents before use. All organic reactions were monitored by TLC on silica gel GF_{254} (0.5 mm). Spots were detected under UV light. Flash column chromatography was carried out on silica gel H (400 mesh, Qingdao, China) or silica gel (200–300 mesh, Qingdao, China). Yields refer to chromatographically and spectroscopically homogeneous material. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 spectrometer at 300 and 75.5 MHz, respectively. Chemical shifts are given in parts per million downfield from tetramethylsilane (0.0 ppm) for spectra in $CDCl_3$. The following abbreviations are used to explain multiplicities: s=single, d=doublet, t=triplet, m=multiplet, br=broad. High-resolution mass spectra (HRMS) were obtained on a Varian QFT-ESI mass spectrometer. UV–vis spectra were recorded on a CARY 100 Bio spectrophotometer (Varian, USA). Fluorescence study was carried out using Varian Cary Eclipse spectrophotometer at 20 °C. The slit width was 5 nm for both excitation and emission and the data recorded each minute in a fast mode. The photomultiplier voltage was 600 V.

4.2. Fluorescence quantum yield

Fluorescence quantum yield (Φ_f) was determined in absolute ethanol using optically matching solution of rhodamine B (Φ_f=0.50) as the standard at an excitation wavelength of 495 nm,[19] and the quantum yield was calculated using Eq. 1:

$$\Phi_f = \Phi_r(A_r F_S/A_S F_r)(\eta_S/\eta_r)^2 \quad (1)$$

where A_s and A_r are the absorbance of the sample and reference solutions, respectively, at the same excitation wavelength, F_s and F_r are the corresponding relative integrated fluorescence intensities, and η is the refractive index of the solvent.

4.3. Kinetic analysis of hydrolysis reactions

Compounds **2** and **4** were dissolved in CH_3CN to make a 2.1 mM stock solution, which was diluted to the required concentration for measurement. The base-catalyzed kinetic parameters were determined in 0.1 M aqueous NaOH solution containing 9 μM compound **2** at 20 °C. Pig liver esterase activity was determined in 3 ml sodium phosphate buffer (100 mM, pH 8.0) with different concentrations (2.0–50.0 μM) of substrates (compounds **2** or **4**) and 0.2 unit/ml esterase at 20 °C. The real-time concentration of **2** in the reaction mixture was determined by simultaneous recording of changes of the donor fluorescence intensities at 338 nm. From Lineweaver–Burk equation, K_m and V_{max} were calculated.

4.4. Preparation of compounds 1–4

*4.4.1. Syntheses of N-methyl-N-dansyl-2-aminoethylanol (**1**)*

N-Methylmonoethanolamine (450 μl, 5.6 mmol) was added into dansylchloride (50 mg, 0.18 mmol) in 4 ml dry CH_2Cl_2 with stirring

at room temperature for 15 min. The mixture was washed with saturated Na_2CO_3 solution (2×5 ml) and then dried over sodium sulfate. After evaporation of the CH_2Cl_2, the residue was purified by silica gel column chromatography using chloroform/methanol (5:1, v/v) as eluent to afford **1** (52 mg, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.24 (br, 1H), 2.88 (s, 6H), 2.94 (s, 3H), 3.34 (t, 2H, J=5.3), 3.75 (t, 2H, J=5.3), 7.18 (d, 1H, J=7.5), 7.45–7.57 (m, 2H), 8.14–8.20 (m, 1H), 8.33 (d, 1H, J=9.1), 8.55 (d, 1H, J=9.1); ^{13}C NMR (75 MHz, $CDCl_3$): δ 151.83, 133.82, 130.56, 130.20, 130.11, 129.88, 128.22, 123.16, 119.37, 115.28, 60.10, 51.84, 45.39, 35.34; HRMS (ESI): m/z=309.1264 $[M+H]^+$ (calcd for $C_{15}H_{21}N_2O_3S$, 309.1273).

*4.4.2. Syntheses of 1-naphthylacetic acid(N-methyl-N-dansyl-2-amino)ethylester (**2**)*

1-Naphthylacetic acid (20 mg, 0.11 mmol), DCC (23 mg, 0.11 mmol), DMAP (14 mg, 0.11 mmol), and **1** (30 mg, 0.1 mmol) in 5 ml dry CH_2Cl_2 were stirred at room temperature for 5 h and then cooled to 0 °C. The reaction mixture was filtered and the filtrate was purified by silica gel column chromatography using dichloromethane as eluent to afford **2** (42 mg, 89%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.67 (s, 3H), 2.86 (s, 6H), 3.43 (t, 2H, J=5.3), 3.96 (s, 2H), 4.23 (t, 2H, J=5.3), 7.17 (d, 1H, J=7.5), 7.30–7.38 (m, 2H), 7.40–7.62 (m, 4H), 7.77 (d, 1H, J=8.3), 7.78–7.83 (m, 2H), 7.90–7.95 (m, 2H), 8.14 (d, 1H, J=7.5), 8.28 (d, 1H, J=9.1), 8.54 (d, 1H, J=9.1); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.20, 151.83, 134.10, 133.79, 132.07, 130.54, 130.27, 130.16, 129.89, 128.70, 128.15, 128.10, 126.44, 125.82, 125.46, 123.77, 119.50, 115.28, 62.39, 48.27, 46.11, 45.40, 34.92; HRMS (ESI): m/z=477.1838 $[M+H]^+$ (calcd for $C_{27}H_{29}N_2O_4S$, 477.1848).

*4.4.3. Syntheses of dansyl-2-ol-ethylester (**3**)*

Ethylene glycol (1 ml, 18 mmol) was added into dansylchloride (100 mg, 0.36 mmol) in 8 ml dry CH_2Cl_2 with stirring at room temperature for 15 min. The mixture was washed with saturated Na_2CO_3 solution (2×5 ml) and then dried over sodium sulfate. After evaporation of the CH_2Cl_2, the residue was purified by silica gel column chromatography using ethyl ether/dichloromethane (1:1, v/v) as eluent to afford **3** (37 mg, 35%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.88 (br, 1H), 2.89 (s, 6H), 3.76 (t, 2H, J=4.1), 4.11 (t, 2H, J=4.6), 7.21 (d, 1H, J=7.5), 7.53–7.62 (m, 2H), 8.24–8.29 (m, 2H), 8.61 (d, 1H, J=8.3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 45.39, 60.75, 71.88, 115.63, 118.98, 123.05, 128.94, 129.89, 130.67, 130.98, 131.808, 151.96; HRMS (ESI): m/z=318.0772 $[M+Na]^+$ (calcd for $C_{14}H_{17}NO_4SNa$, 318.0776).

*4.4.4. Syntheses of 1-naphthylacetic acid(2-dansyloxy)-ethylester (**4**)*

1-Naphthylacetic acid (20 mg, 0.11 mmol), DCC (23 mg, 0.11 mmol), DMAP (14 mg, 0.11 mmol), and **3** (30 mg, 0.1 mmol) in 5 ml dry CH_2Cl_2 were stirred at room temperature for 5 h and then cooled to 0 °C. The reaction mixture was filtered and the filtrate was purified by silica gel column chromatography using dichloromethane as eluent to afford **4** (37 mg, 78%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.87 (s, 6H), 3.76 (s, 2H), 4.21 (s, 4H), 7.22 (t, 2H, J=6.8), 7.38 (t, 1H, J=7.5), 7.46–7.64 (m, 4H), 7.76–7.86 (m, 3H), 8.29–8.33 (m, 2H), 8.65 (d, 1H, J=9.1); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.85, 151.71, 133.78, 131.76, 130.62, 129.90, 128.77, 128.69, 128.15, 127.95, 126.42, 125.76, 125.39, 123.61, 123.14, 119.61, 115.74, 67.82, 61.85, 45.95, 45.41; HRMS (ESI): m/z=486.1346 $[M+Na]^+$ (calcd for $C_{26}H_{25}NO_5SNa$, 486.1351).

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References and notes

1. (a) Förster, T. *Ann. Phys.* **1948**, *2*, 55–75; (b) Förster, T. *Discuss. Faraday Soc.* **1959**, *27*, 7.
2. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Kluwer Academic/Plenum: New York, NY, 1999.
3. (a) Pollok, B. A.; Heim, R. *Trends Cell Biol.* **1999**, *9*, 57–60; (b) *The Single-Molecule Behaviors in Life Science and Real-Time Detection in Living Cells*; Chen, Y. Z., Lin, Q. S., Eds.; China Science: Beijing, 2005; (Chinese version).
4. Endoh, T.; Funabashi, H.; Mie, M.; Kobatake, E. *Anal. Chem.* **2005**, *77*, 4308–4314.
5. (a) Wang, Q.; Liu, Y.; Ke, Y.; Yan, H. *Angew. Chem., Int. Ed.* **2007**, *47*, 316–319; (b) Sasajima, Y.; Aburatani, T.; Sakamoto, K.; Ueda, H. *Biotechnol. Prog.* **2006**, *22*, 968–973; (c) He, F.; Tang, Y.; Wang, S.; Li, Y.; Zhu, D. *J. Am. Chem. Soc.* **2005**, *127*, 12343–12346.
6. (a) Sekar, R. B.; Periasamy, A. *J. Cell Biol.* **2003**, *160*, 629–633; (b) Awais, M.; Sato, M.; Lee, X.; Umezawa, Y. *Angew. Chem., Int. Ed.* **2006**, *45*, 2707–2712.
7. (a) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303–308; (b) Tan, W.; Wang, K.; Drake, T. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 547–553; (c) Grossmann, T. N.; Röglin, L.; Seitz, O. *Angew. Chem., Int. Ed.* **2007**, *46*, 5223–5225.
8. Brownlee, C. *ACS Chem. Biol.* **2007**, *2*, 149–151.
9. Sapsford, K. E.; Berti, L.; Medintz, I. L. *Angew. Chem., Int. Ed.* **2006**, *45*, 4562–4588.
10. (a) Lewis, W. G.; Magallon, F. G.; Fokin, V. V.; Finn, M. G. *J. Am. Chem. Soc.* **2004**, *126*, 9152–9153; (b) Beletskaya, I. P.; Chaprakov, A. V. *Chem. Rev.* **2000**, *100*, 3009–3066.
11. (a) Stauffer, S. R.; Beare, N. A.; Stambuli, J. P.; Hartwig, J. F. *J. Am. Chem. Soc.* **2001**, *123*, 4641–4642; (b) Stambuli, J. P.; Stauffer, S. R.; Shaughnessy, K. H.; Hartwig, J. F. *J. Am. Chem. Soc.* **2001**, *123*, 2677–2678; (c) Stauffer, S. R.; Hartwig, J. F. *J. Am. Chem. Soc.* **2003**, *125*, 6977–6985.
12. (a) Kawanishi, Y.; Kikuchi, K.; Takakusa, H.; Mizukami, S.; Urano, Y.; Higuchi, T.; Nagano, T. *Angew. Chem., Int. Ed.* **2000**, *39*, 3438–3440; (b) Takakusa, H.; Kikuchi, K.; Urano, Y.; Sakamoto, S.; Yamaguchi, K.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 1653–1657; (c) Takakusa, H.; Kikuchi, K.; Urano, Y.; Kojima, H.; Nagano, T. *Chem.—Eur. J.* **2003**, *9*, 1479–1485.
13. (a) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regio and Stereoselective Biotransformations*; Wiley-VCH: Weinheim, 1999; (b) Faber, K. *Biotransformations in Organic Chemistry*; Springer: Berlin, 2000; (c) Toone, E. J.; Werth, M. J.; Jones, J. B. *J. Am. Chem. Soc.* **1990**, *112*, 4946–4952; (d) Musidlowska, A.; Lange, S.; Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2001**, *40*, 2851–2853; (e) Annalen, L. *Eur. J. Org. Chem.* **1995**, 1901–1902; (f) Hultin, P. G.; Mueseler, F. J.; Jones, J. B. *J. Org. Chem.* **1992**, *57*, 2978.
14. Lange, S.; Musidlowska, A.; Schmidt-Dannert, C.; Schmitt, J.; Bornscheuer, U. T. *ChemBioChem* **2001**, *2*, 576–582.
15. (a) Adler, A. J.; Kistiakowsky, G. B. *J. Am. Chem. Soc.* **1962**, *84*, 695–703; (b) Stoops, J. K.; Horgan, D. J.; Runnegar, M. T. C.; Jersey, J.; DeWebb, E. C.; Zerner, B. *Biochemistry* **1969**, *8*, 2026–2033; (c) Allen, K. N.; Abeles, R. H. *Biochemistry* **1989**, *28*, 135–140.
16. Stella, L.; Venanzi, M.; Carafa, M.; Maccaroni, E.; Straccamore, M. E.; Zanotti, G.; Palleschi, A.; Pispisa, B. *Biopolymers* **2002**, *64*, 44–56.
17. (a) Goldsmith, H. A. *Chem. Rev.* **1943**, *33*, 257–349; (b) Fersht, A. R.; Kirby, A. J. *J. Am. Chem. Soc.* **1967**, *89*, 5960–5961; (c) von Bennekum, A. M.; Fisher, E. A.; Blaner, W. S.; Harrison, E. H. *Biochemistry* **2000**, *39*, 4900–4906.
18. Komatsu, T.; Kikuchi, K.; Takakusa, H.; Hanaoka, K.; Ueno, T.; Kamiya, M.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 15946–15947.
19. Karstens, T.; Kobs, K. *J. Phys. Chem.* **1980**, *84*, 1871–1872.