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A Fluorogenic Probe with Aggregation-Induced Emission Characteristics for Carboxylesterase Assay through Formation of Supramolecular Microfibers

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Abstract: Herein, we report a novel fluorescent "light-up" probe useful for carboxylesterase assay that is based on a tetraphenylethylene derivative containing carboxylic ester groups. The specific cleavage of the carboxylic ester bonds by carboxylesterase results in the generation of a relatively hydrophobic moiety that self-assembles into supramolecular microfibers, thus giving rise to "turn-on" fluorescent signals. A high sensitivity towards carboxylesterase was achieved with a detection limit as low as 29 pM, which is much lower than the corresponding assays based on other fluorescent approaches.

Keywords: aggregation-induced emission · carboxylesterase · fluorescence · self-assembly · tetraphenylethylene

Introduction

Carboxylesterases are a group of isoenzymes commonly found in mammalian organs,[1] and play an important role in catalyzing the hydrolysis of carboxyl esters[2] for detoxification of narcotics or chemical toxin clearance.[3] Due to their broad substrate specificity and high enantioselectivity, carboxylesterases constitute attractive biocatalysts for the production of optically pure compounds in fine chemical synthesis[4] and represent important drug candidates for protein-based therapeutics or drug targets for chemotherapeutic prodrug activation.[5] Therefore, carboxylesterase assays would benefit various biochemical studies for understanding its mechanism of action and role in drug metabolism. A number of strategies have been established for carboxylesterase detection with the application of chromatography,[6] mass spectrometry,[7] chemiluminescence,[8] and fluorescence.[9] Among them, biomolecular probes based on different fluorogenic substrates have received much attention due to the simplicity, speed, and sensitivity of their assays.[10] Although these probes have been proven successful in carboxylesterase activity assays, the development of water-soluble[11] and synthetically readily accessible fluorogenic probes[12] with improved assay sensitivity and selectivity is still desirable.

Unlike conventional fluorophores that are easily subject to a fluorescence quenching effect at high concentrations or in the solid state due to intra- or intermolecular interactions,[13] tetraphenylethene (TPE) contains four propeller-shaped conjugated phenyl rings and shows an unusual fluorescent behavior; it is almost non-emissive when dissolved in solution but turns to be highly fluorescent in the aggregated form.[14] Because of this unique property, TPE-based molecules with aggregation-induced emission (AIE) properties have emerged as powerful and versatile building blocks[15] for the design of synthetically readily accessible and environmentally stable probes for the detection of various analytes.[16] Despite their remarkable advantages, a limited number of examples have so far been reported to apply AIE-type molecules in the design of fluorescence "light-up" probes for different enzymatic assays.[17] To the best of our knowledge, the development of new biomolecular probes for carboxylesterase detection based on the AIE feature has not been reported so far. Thus, we explored the potential of TPE conjugated with a carboxylic ester as a novel fluorogenic probe for carboxylesterase detection.

In this work, based on the tetraphenylethylene motif, we designed and synthesized a novel tetraphenylethylene derivative (**1**) that contains four carboxylic ester groups subject to carboxylesterase recognition and catalytic hydrolysis (Scheme 1). The design rationale and fluorescent sensing mechanism of **1** toward carboxylesterase are illustrated in Scheme 1 and are explained as follows: 1) **1** exhibits an excellent solubility in water due to the presence of four carboxylic acid groups in its molecular structure before enzymatic catalysis and displays very low fluorescence (emission quantum yield $\Phi_F = 0.02$) in aqueous solution at pH 7.4; 2) after catalytic hydrolysis of the carboxylic ester bonds, **1** is enzymatically converted into **2**, a relatively more hydrophobic entity, thus resulting in the self-assembly of the AIE

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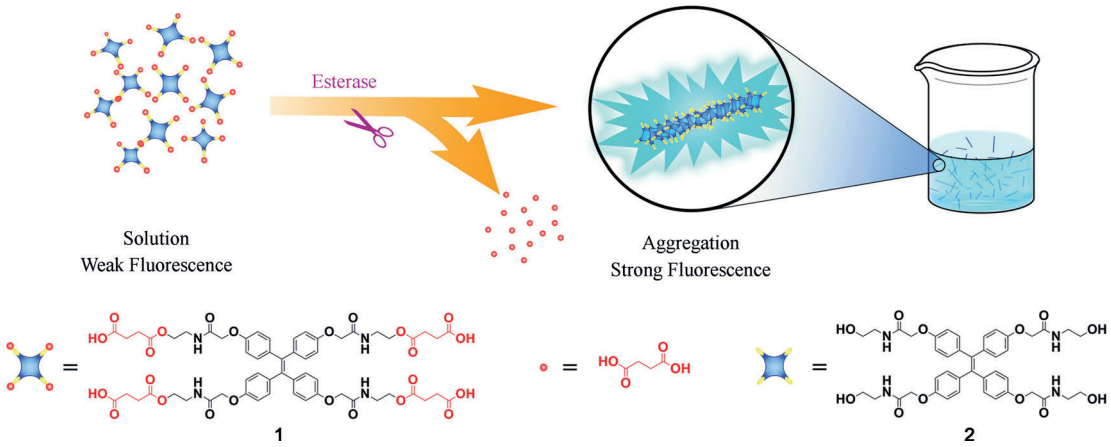


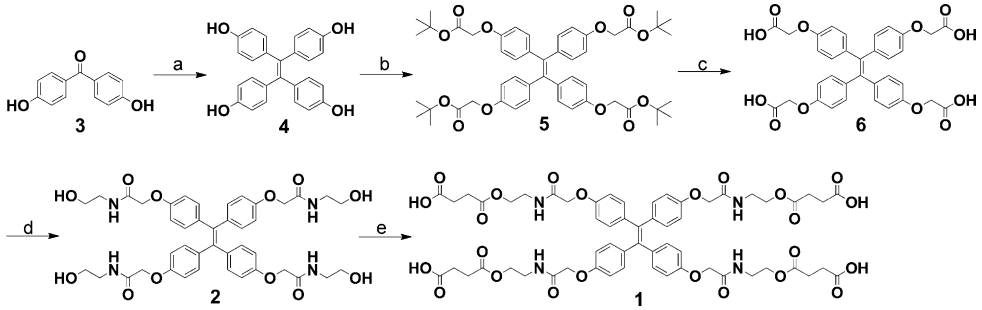
Scheme 1. Illustration of the fluorometric detection of carboxylesterase through the formation of supramolecular microfibers by carboxylic ester hydrolysis of the AIE probe.

residues in aqueous solution with enhanced fluorescent signals. In this way, **1** can be used for the fluorescence turn-on detection of carboxylesterase with a detection limit as low as 29 pM. This result clearly demonstrates that **1** could not only be applied to the real-time monitoring of carboxylesterase activity but also to the screening of potential carboxylesterase inhibitors.

Results and Discussion

Scheme 2 shows the synthetic route for the preparation of fluorogenic probe **1**. By following the reaction conditions and procedures described by Tang et al. in a previous report,[18] we synthesized **4** from 4, 4′-dihydroxybenzophenone as the starting material through a McMurry coupling reaction. The next step consisted of a condensation reaction with *tert*-butyl bromoacetate, and the subsequent treatment with TFA afforded **6** in a yield of 42% over two steps. Upon activation by *N*-hydroxysuccinimide (NHS), **6** reacted with 2-aminoethanol to afford **2** in 63% yield. Further reaction with succinic anhydride in the presence of *N,N*′-diisopropylethylamine led to **1** in a yield of 83%. The chemical structures of these new products were verified by NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS; see Figure S1 in the Supporting Information). As expected, **1** was soluble in water, and an aqueous solution of **1** (300 μM) in 10 mM Tris-HCl buffer (pH 7.4) was prepared for the following studies.

Before the addition of carboxylesterase, the solution of **1** was completely transparent, colorless, and non-fluorescent under illumination with visible light or UV light (365 nm; Figure 1). However, the addition of carboxylesterase at a final concentration of 3.2 nM transformed the solution of **1** into a suspension owing to the generation of insoluble **2** (Figures S2 and S3 in the Supporting Information), which was emissive under irradiation with UV light. The obvious transition from a clear solution of **1** to a suspension of **2** triggered by carboxylesterase could be observed with the naked eye. From the fluorescence spectroscopic analysis shown in Figure 1C, we observed that the solution of **1** was weakly fluorescent at 475 nm ($\Phi_F = 0.02$), which is consistent with the non-emissive character of AIE fluorogens in the soluble state. However, an emission band at 475 nm appeared and increased with time after the addition of carboxylesterase, and the enhancements were attributed to the generation



Scheme 2. The synthetic routes for the preparation of fluorogenic probe **1** and enzymatic generation of **2** through carboxylesterase hydrolysis. Reagents and conditions: a) $TiCl_4$, Zn powder, reflux, 12 h; b) *tert*-butyl bromoacetate, K_2CO_3, reflux, 72 h; c) TFA, RT, 12 h; d) DIC, NHS, 2-aminoethanol, RT, 12 h; e) succinic anhydride, DIEA, RT, 12 h.

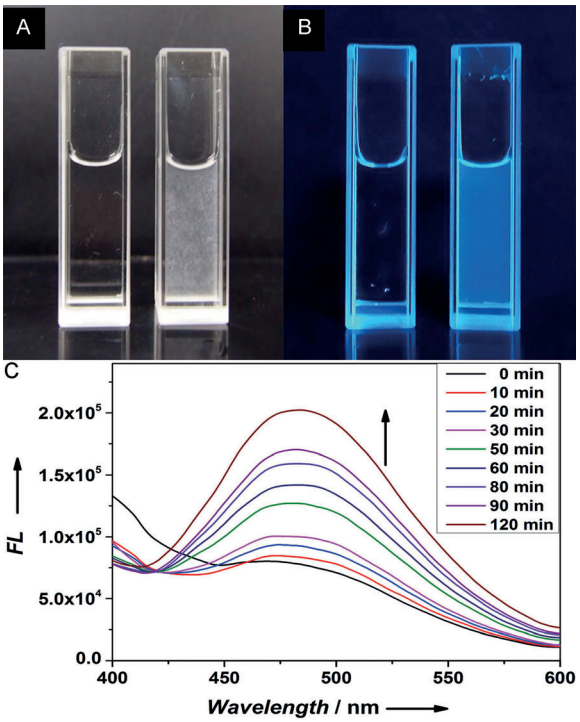


Figure 1. Optical images of solutions of **1** (300 μM, pH 7.4) in the absence (left) or presence (right) of carboxylesterase (3.2 nM) under A) visible and B) UV light (365 nm). C) Time evolution of fluorescence emission spectra (λ_{ex} = 375 nm) of the solution of **1** (300 μM, pH 7.4) upon addition of carboxylesterase (3.2 nM).

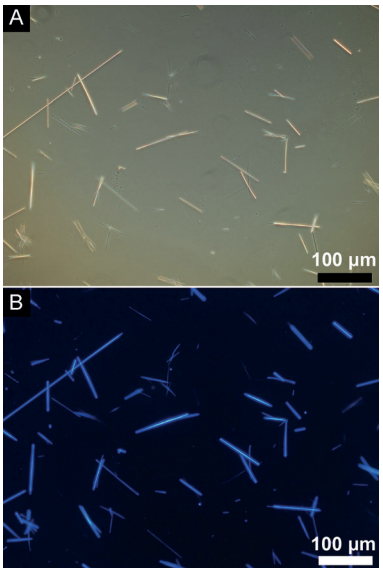


Figure 2. Bright field (A) and fluorescence (B) microscopy images of molecular aggregates observed after incubation of a solution of **1** (300 μM, pH 7.4) with carboxylesterase (3.2 nM) for 120 min.

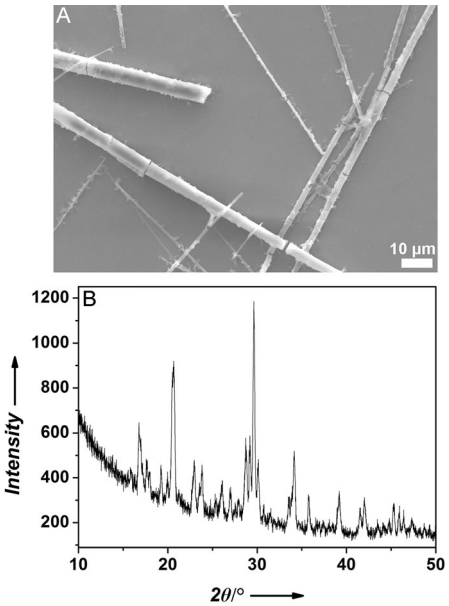


Figure 3. A) SEM image and B) XRD pattern of microfibers observed after incubation of a solution of **1** (300 μM, pH 7.4) with carboxylesterase (3.2 nM) for 120 min.

and accumulation of insoluble **2** (Figures S2 and S3 in the Supporting Information), which aggregated and self-assembled in water.

As demonstrated in Figure 1, such fluorescence generation and enhancement may be ascribed to the formation of molecular aggregates of **2** that were produced from the reaction of **1** with carboxylesterase. We used fluorescence microscopy to identify the aggregates self-assembled from molecules of **2** moieties in its suspension. As displayed in Figure 2A, fluorescence microscopy analysis revealed that the aggregates were one-dimensional microfibers with length of more than 50 μm. The microfibers were highly fluorescent, emitting a bright blue light upon photo-excitation (λ_{ex} = 365 nm) (Figure 2B). By comparison, almost no fluorescent aggregates could be observed in the solution of **1** before the addition of carboxylesterase (Figure S4A and S4B in the Supporting Information), thereby indicating that carboxylesterase catalyzed the transformation of **1** to **2** for the formation of supramolecular microfibers and turn-on of fluorescence (Figure S7 in the Supporting Information).

Due to the capability of self-assembly, **2** was packed in a one-dimensional fashion to afford crystalline microfibers through enzymatic catalysis (Figure S7 in the Supporting Information). Figure 3A shows a representative scanning electron microscopy (SEM) image of the microfibers, which are several micrometers in width and up to hundreds of micrometers in length. In comparison, for the solution of **1** before incubation with carboxylesterase, only highly dispersed micellar microstructures with an average diameter of around 10 μm were observed by SEM (Figure S4C in the Supporting Information). From the X-ray diffraction (XRD) pattern displayed in Figure 3B, we observed multiple strong and sharp diffraction peaks at 16.76°, 20.65°, 29.62°, and 34.16°, implying both a polymorphous and microcrystalline nature of the microfibers[19] self-assembled from **2**.

To address the selectivity of the probe to carboxylesterase, we conducted control experiments by treating **1** with other nonspecific proteins, such as pepsin, trypsin, lysozyme, human serum albumin (HSA), alkaline phosphatase, and exonuclease I under the same conditions. As shown in Figure 4, only the solution containing carboxylesterase led to an intense fluorescence signal at 475 nm that was about 9 times higher than that for the other proteins. These data

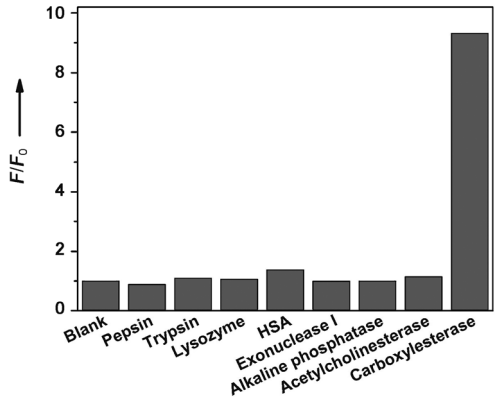


Figure 4. Fluorescence intensity changes (λ_{em} = 475 nm) of solutions of **1** in 10 mM Tris-HCl buffer (pH 7.4) after incubation with different proteins (3.2 nM each) for 100 min at 37 °C.

confirmed that these proteins cannot recognize **1** as a substrate for carboxylic ester hydrolysis. Moreover, we also tested the response of **1** to acetylcholinesterase, one kind of commercially available esterase that hydrolyses the carboxylic ester bond of acetylcholine.[20] The fact that we did not observe an obvious increase in fluorescence emission under the same conditions provides further support of the selectivity of **1** towards carboxylesterase (Figure 4).

Furthermore, we examined the kinetics of the carboxylesterase-catalyzed hydrolysis of **1** using different amounts of carboxylesterase. Figure 5 A and Figure S6 in the Supporting Information show an increase in fluorescence intensity at 475 nm with increasing carboxylesterase concentration (0–3.2 nM) over 4 hours. Obviously, at higher concentrations of carboxylesterase, a greater fluorescence enhancement was observed, and the fluorescence intensity increased more quickly when the probe was incubated with higher concentrations of enzyme. These results indicate that increasing the enzyme concentration gave rise to a higher cleavage reaction rate so that less time was required to complete the hydrolysis process. Furthermore, on the basis of the plot of the relative fluorescence intensity (F/F_0) of **1** in solution at 475 nm as a function of the concentration of carboxylesterase ranging from 0.05 to 3.2 nM, the detection limit was calculated to be 29 pM under these conditions (Figure 5 B), which was much lower than the previously reported data for carboxylesterase assays.[21] In addition, the kinetic parameters of carboxylesterase-catalysed hydrolysis of **1** were determined. The K_m and V_{max} values were estimated to be 55.3 μM and 0.15 μM min^{-1}, respectively (Figure S5 in the Supporting Information).

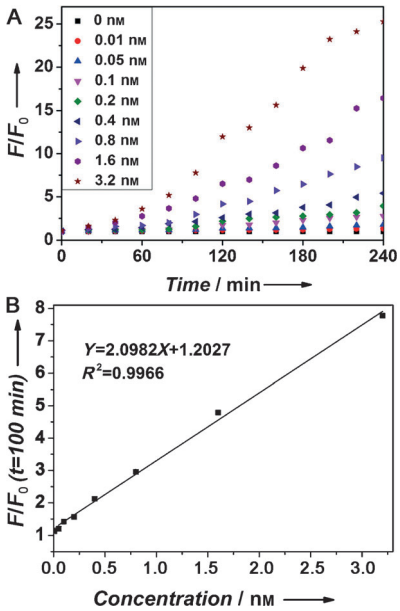


Figure 5. A) Time evolution of the fluorescence intensities of a solution of **1** (300 μM, pH 7.4) at 475 nm upon addition of different concentrations of carboxylesterase. B) Linear plot of the relative fluorescence intensity (F/F_0, λ_{em} = 475 nm) of a solution of **1** as a function of the concentration of carboxylesterase (0.05–3.2 nM). The fluorescence intensities were measured after incubation for 100 min at 37 °C.

It is well known that the hydrolytic activity of carboxylesterase is greatly reduced in the presence of inhibitors. In order to examine the possibility to use our probe for inhibitor screening assays, we selected two common carboxylesterase inhibitors, 4-chlorobenzenesulfonamide and 2-thenoyltrifluoroacetone, to investigate their inhibitory effects on the hydrolysis of **1**. We found that the enhancement of fluorescence intensity by our probe was inhibited in a dose-dependent manner. As shown in Figure 6, on the basis of the plot of the relative activity of enzyme as a function of the concentration of inhibitors, the IC_{50} values were calculated to be 8.21 μM for 4-chlorobenzenesulfonamide and 0.73 μM for

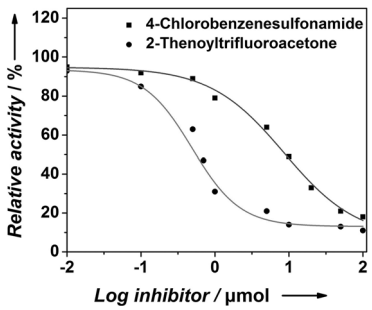


Figure 6. Relative activity of carboxylesterase (3.2 nM) as a function of different concentrations of carboxylesterase inhibitors in 10 mM Tris-HCl buffer (pH 7.4) at 37 °C.

2-thenoyltrifluoroacetone, which are in good agreement with previously reported values.[22] These results clearly demonstrated that our system can be used not only for the real-time monitoring of carboxylesterase activity but also for the screening of potential carboxylesterase inhibitors.

Conclusions

In summary, we have demonstrated the development of a novel, simple, and sensitive carboxylesterase assay system relying on the aggregation-induced emission characteristics of tetraphenylethylene luminogens for the first time. Upon the addition of carboxylesterase, which induced the specific cleavage of the carboxylic ester bond in a tetraphenylethylene derivative, **1** was enzymatically converted into **2**, a relatively more hydrophobic entity, to result in the supramolecualr self-assembly and aggregation of the AIE residues in aqueous solutions and enhancement of the fluorescent signals at 475 nm. Under these conditions, a high sensitivity for carboxylesterase detection was achieved with a detection limit as low as 29 pM, which was much lower than those of previously reported assays.[21] Moreover, probe **1** also exhibited the potential for carboxylesterase inhibitor screenings.

Experimental Section

General Experimental Details

Materials

Esterase from porcine liver was obtained from Sigma–Aldrich (unit size: 218 units mg^{-1} protein, 0.163 mL, 28.1 mg mL^{-1} (biuret)). Alkaline phosphatase was purchased from Fermentas (unit size: 300 units, 1.0 unit μL^{-1}). Exonuclease I was obtained from Shanghai Shifeng Biological Technology Company (unit size: 750 units, 5 units μL^{-1}). All other starting materials were obtained from Sigma and J&K Chemical. Compound **6** was synthesized by following the reported procedures.[18] Other commercially available reagents were used without further purification, unless noted otherwise.

Measurements

^{1}H NMR spectra were obtained on a Varian Unity Inova 400 spectrometer by using [D_6]DMSO as the solvent. LC-MS analyses were performed on Agilent 6220 Quadrupole LC/MS system with an ESI resource. HPLC purification and analysis were carried out on a Waters 600E Multi-solvent Delivery System using a YMC C_{18} RP column with CH_3CN (0.1 v% of TFA) and water (0.1 v% of TFA) as the eluents. SEM images were recorded on a Hitachi S-4800 scanning electron microscope. X-ray diffraction experiments were performed on a PANalytical X′Pert PRO MRD diffractometer using Cu Kα radiation operated at 40 kV and 20 mA and an X′celerator detector. Fluorescence spectroscopy measurements were taken on a FluoroMax-4 spectrofluorometer and a Thermo Scientific Varioskan Flash spectral scanning multimode reader. Fluorescence microscopy images were recorded on an Olympus IX71 fluorescence microscope.

Fluorogenic Probe Synthesis

Synthesis of 4: 4, 4′-Dihydroxybenzophenone (2.1 g, 10 mmol) and Zn power (1.44 g, 22 mmol) were dissolved in dry THF under a nitrogen atmosphere. The solution was cooled to 0 °C, followed by the dropwise addition of $TiCl_4$ (1.3 mL, 12 mmol). After refluxing overnight, the solution was cooled to room temperature, treated with 40 mL HCl solution (1 mol L^{-1}), and then extracted with CH_2Cl_2. The organic layer was collected and concentrated under reduced pressure. The crude product was further purified by silica gel column chromatography (MeOH/CH_2Cl_2 1:16) to give **4** as a white solid (1.5 g, 78%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 9.23 (s, 4H), 7.26–7.35 (d, 8H), 6.54–6.65 ppm (d, 8H); MS (*m*/*z*) calcd for $C_{26}H_{20}O_4$: 395.14 [*M*−H]$^-$; found: 395.20.

Synthesis of 5: Briefly, under a nitrogen atmosphere, *tert*-butyl bromoacetate (7.88 g, 40.3 mmol) was added dropwise to dry acetone (80 mL) containing tetrakis(4-hydroxyphenyl)ethane (TPE-OH_4; 2.0 g, 5.04 mmol) and K_2CO_3 (11.2 g, 80.9 mmol), and the reaction mixture was heated at reflux for 72 h. After filtration and removal of the solvent, the residue was purified by silica gel column chromatography (EtOAc/*n*-hexane 1:15) to afford **5** as a yellow powder (2.3 g, 54.7%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 7.46–7.57 (m, 8H), 7.12–7.02 (m, 8H), 4.66–4.74 (m, 8H), 4.52–4.63 ppm (m, 36H); MS (*m*/*z*) calcd for $C_{50}H_{60}O_{12}$: 851.41 [*M*−H]$^-$; found: 851.30.

Synthesis of 6: To 30 mL of 10% trifluoroacetic acid in dry CH_2Cl_2, TPE-COO*t*Bu_4 (1.3 g, 1.5 mmol) was added. The reaction mixture was stirred at room temperature overnight and then concentrated in vacuo. The resulting residue was purified by silica gel column chromatography (EtOAc/*n*-hexane 5:1) to afford **6** as a white powder (684 mg, 73.7%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 6.87–6.90 (m, 8H), 6.61–6.63 (m, 8H), 4.49–4.53 ppm (m, 8H). MS (*m*/*z*) calcd for $C_{34}H_{28}O_{12}$: 627.16 [*M*−H]$^-$; found: 627.20.

Synthesis of 2: Compund **6** (400 mg, 0.64 mmol), *N*,*N*′-diisopropylcarbodiimide (710 mg, 5.63 mmol), and *N*-hydroxysuccinimide (589 mg, 5.12 mmol) were added to dry CH_2Cl_2 (20 mL), and the reaction mixture was stirred at room temperature for 6 h. The crude product was then used in the next reaction without purification. Ethanolamine (313 mg, 5.12 mmol) was dissolved in deionized water (10 mL), and the aforementioned solution containing activated compound **6** was added dropwise. The reaction mixture was stirred overnight at room temperature. Subsequently, the solvent was evaporated, and the residue was purified by silica gel chromatography ($CHCl_3$/MeOH 12:1) to afford **2** as a pale yellow powder (322 mg, 63%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.00 (s, 4H), 6.86–6.88 (d, 4H), 6.73–6.76 (d, 4H), 4.74 (s, 4H), 4.40 (s, 8H), 3.41 (s, 8H), 3.20 ppm (s, 8H); MS (*m*/*z*) calcd for $C_{42}H_{48}N_4O_{12}$: 845.33 [*M*+HCOOH−H]$^-$; found: 845.30.

Synthesis of 1: Compound **2** (400 mg, 0.50 mmol), succinic anhydride (600 mg, 6 mmol), and *N*,*N*-diisopropylethylamine (DIPEA, 517 mg, 4 mmol) were dissolved in DMF 25 mL, and the mixture was stirred overnight at room temperature. After the reaction was completed, the solvent was removed under reduced pressure, and the solid residue was washed with diluted HCl (1 mM), dried, and then purified by HPLC to yield **1** as a pale yellow powder (996 mg, 83%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.19 (s, 4H), 8.13 (s, 4H), 6.83–6.86 (d, 8H), 6.69–6.72 (d, 8H), 4.37 (s, 8H), 4.00 (s, 8H), 3.30 (s, 8H), 2.48 (s, 8H), 2.45 ppm (s, 8H); MS (*m*/*z*) calcd for $C_{58}H_{64}N_4O_{24}$: 1199.39 [*M*−H]$^-$; found: 1199.4.

*Kinetic Studies of the Transformation of **1** to **2** by Carboxylesterase*

For the studies of the hydrolysis reaction kinetics, different concentrations of probe **1** (20, 50, 100, 200, and 300 μM) in 10 mM Tris-HCl buffer were hydrolyzed by carboxylesterase (3 nM). The reaction was monitored by measuring the fluorescence change at 475 nm (excitation at 375 nm) at 37 °C. The kinetic parameters for the enzymatic hydrolysis reaction of probe **1** were determined by using the Lineweaver–Burk analysis, thus yielding values of K_m = 55.3 μM and V_{max} = 0.15 μM min^{-1}.

*Determination of the Detection Limit of Carboxylesterase with Probe **1***

To assess the sensitivity of the fluorescent assays, we prepared a series of solutions in 10 mM Tris-HCl buffer (pH 7.4) containing 300 μM of **1** and different concentrations of carboxylesterase (0, 0.05, 0.2, 0.8, 1.6, and 3.2 nM), and incubated them at 37 °C. The fluorescence intensities of the mixtures were collected at various time points (0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 min), and the relationships between known concentrations of carboxylesterase and the fluorescent response at different times were analyzed by using a calibration curve. A good linear relationship between the carboxylesterase concentration and the relative fluorescence intensity (F/F_0) was obtained in the range between

0.05 and 3.2 nM at 100 min. Its regression equation is $Y=2.0928X+1.2099$ (where Y is the relative fluorescence intensity and X is the concentration of carboxylesterase in nM) with a correlation coefficient of 0.9986. The limit of detection was calculated from the standard deviation of the response (SD = 0.0184) and the slope of the calibration curve ($S=2.0928$) according to the following formula: LOD = 3.3(SD/S).[23]

Acknowledgements

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