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Non-Peptide-based Small-Molecule Probe for Fluorogenic and Chromogenic Detection of Chymotrypsin

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ABSTRACT

We report herein a non-peptide-based small molecule probe for fluorogenic and chromogenic detection of chymotrypsin, and the primary application. This probe was rationally designed by mimicking the peptide substrate and optimized by adjusting the recognization group. The refined probe **2** exhibits good specificity toward chymotrypsin, producing about 25-fold higher enhancement in both the fluorescence intensity and absorbance upon the catalysis by chymotrypsin. Compared with the most widely used peptide substrate (AMC-FPAA-Suc) of chymotrypsin, probe **2** shows about 5-fold higher binding affinity, and comparable catalytical efficiency against chymotrypsin. Furthermore, it was successfully applied for the inhibitor characterization. To the best of our knowledge, probe **2** is the first non-peptide-based small-molecule probe for chymotrypsin, with the advantages of simple structure and high sensitivity compared to the widely used peptide-based substrates. This small-molecule probe is expected to be a useful molecular tool for drug discovery and chymotrypsin-related diseases diagnosis.

Keywords: Chymotrypsin, non-peptide substrates, drug discovery, sensors, kinetics

INTRODUCTION

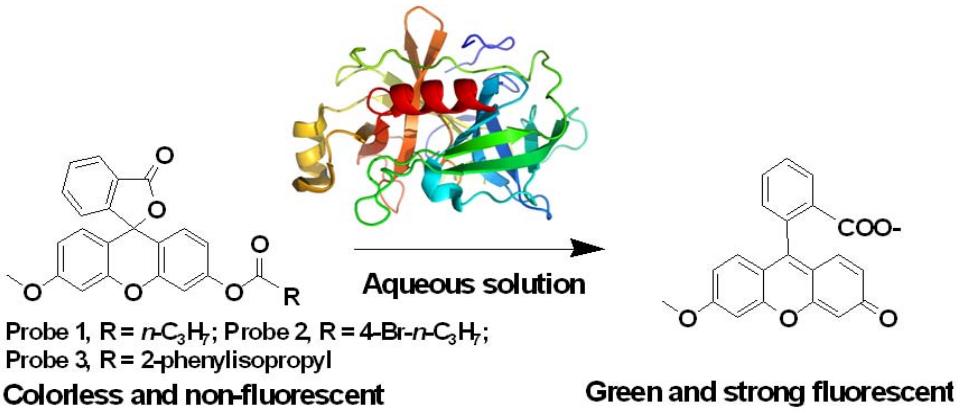
Chymotrypsin (EC 3.4.21.1), one of the most common serine proteases, is involved in many physiological processes, including digestion of dietary proteins, necrosis and apoptosis.[1-6] In addition, it has been implied in the pathogenesis of various diseases, such as pancreatic fibrosis, maldigestion, diabetes mellitus, hypertension, inflammation, and many types of cancer, particularly pancreatic cancer.[7-14] Moreover, the chymotrypsin itself is a medicine for reducing redness, swelling, or damages that associated with various situations likes infection and surgery.[15, 16] Therefore, the detection of chymotrypsin activity is highly important in drug discovery and clinical diagnostics.

To date, three major methods have been developed for assessing the activity of chymotrypsin and screening chymotrypsin inhibitors: 1) UV/Vis assays with direct chromogenic reaction catalyzed by chymotrypsin; 2) fluorometric assays with fluorogenic peptide substrates that yield fluorescence enhancement after the catalysis; 3) enzyme-linked immunosorbent assay (ELISA) and western blot methods using antibodies specific to chymotrypsin.[10, 17-26] Among them, ELISA and western blot methods are expensive and time-consuming. Moreover, other than the concentration of the active form, the total concentration of chymotrypsin was determined by this method. The colorimetric and fluorometric assays both used peptide substrates (identified decades ago) that are prone to degradation and thus require special storage. As far as we know, no small molecular probes have been reported for the specific detection of chymotrypsin. Herein, we report the discovery and the primary application of low-molecular weight non-peptide-based probes as substrates for the fluorogenic and chromogenic detection of chymotrypsin.

Similarly as other serine proteases, chymotrypsin uses its catalytic triad (consisted of the amino acid residues His-57, Asp-102, and Ser-195, human source) to cleave the peptide bond in peptide or protein substrate, in which the Ser-195 serves as the nucleophilic attacker.[4, 27] The chymotrypsin is specific for hydrolyzing the peptide or esters that are adjacent to aromatic residues (Phe, Tyr and Trp). Based on this mechanism, we made our effort in designing small organic molecules by mimicking the peptide substrate, in which a central ester bond flanked by a pair of fluorophore and quencher (**Scheme 1**, PDB entry is 2P8O).[28-30]

The desired probes were expected to show very weak absorbance background and exhibit very low fluorescence based on the mechanism of photoinduced electron transfer (PET), but upon cleavage of the ester bond by chymotrypsin, should produce a high level of optical signal. Accordingly, an "off-on" optical sensing of chymotrypsin activity was established. Recently, a peptide-based substrate with hydroxymethyl rhodamine green was designed as the fluorescent probe for chymotrypsin detection and enabled the good and rapid identification of chymotrypsin in pancreatic juice.[10] Since methylfluorescein shows very similar structure (compared with hydroxymethyl rhodamine green) and good optical properties, we chose methylfluorescein as the fluorophore. After structure and sensing property study, we finally identified a non-peptide-based small molecule as a specific chymotrypsin probe. To our delight, the new probe demonstrates good properties of specificity, sensitivity, binding affinity and catalytic efficiency against chymotrypsin. In addition, the small-molecule probe may be applied to kinetically characterize the chymotrypsin inhibitors for drug discovery.

Scheme 1. Illustration of the one step reaction to assess chymotrypsin activity with designed probes.



EXPERIMENTAL SECTION

Organic Synthesis

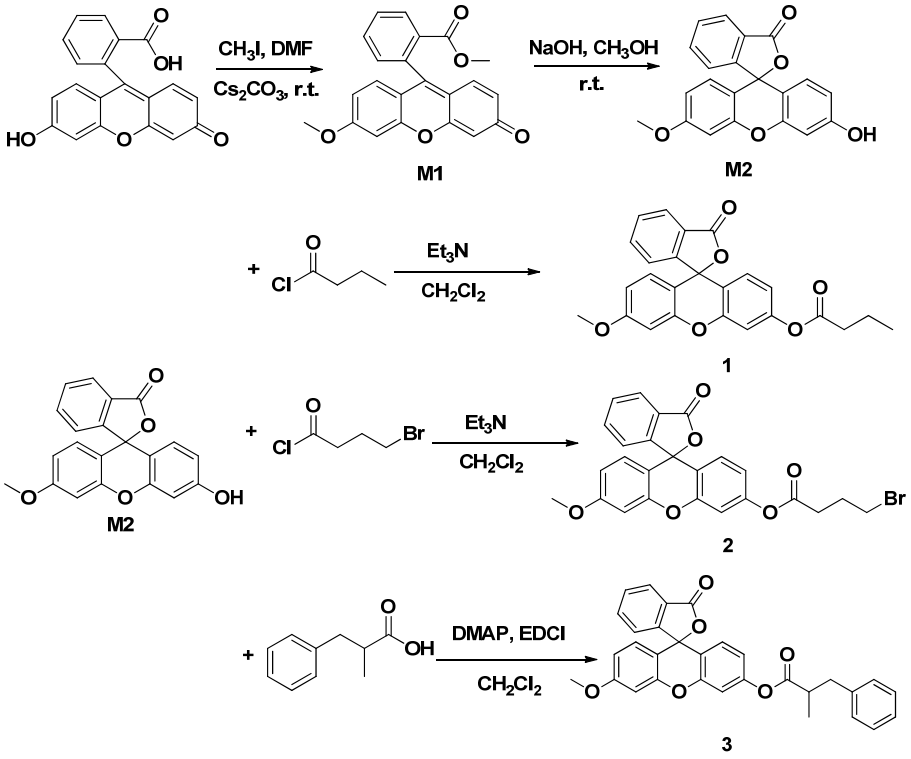
Unless otherwise noted, all chemical reagents were commercially available and treated with standard methods before use. Silica gel column chromatography (CC): silica gel (200-300 mesh); Qingdao Makall Group Co., Ltd (Qingdao; China). Solvents were dried in a routine way and redistilled. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a

Varian Mercury 400 MHz, 500 MHz and 600 MHz spectrometer and resonances (δ) are given in ppm relative to tetramethylsilane (TMS). The following abbreviations were used to designate chemical shift mutiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, q = quartet. High resolution Mass Spectra (HRMS) were acquired in positive mode on a WATERS MALDI SYNAPT G2 HDMS (MA, USA). Melting points were taken on a Buchi B-545 melting point apparatus and uncorrected.

Synthesis of the intermediates and probe 1 (Scheme 2)

The synthesis of **M2** was according to the literature as shown in **Scheme 2**. The synthesis of **M1**: CH_3I (2.84 g, 20 mmol) was added to the mixture of fluorescein (3.32 g, 10 mmol) and Cs_2CO_3 (2.77 g, 20 mmol) in DMF (20 mL) at room temperature. After stirring for 12 hours, the reaction mixture was filtered and washed with ethyl acetate. After extraction with water, the organic phase was dried over anhydrous Na_2SO_4 and then concentrated under reduced pressure. The residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (V:V = 50:1) to give **M1** as a yellow solid (yield: 90 %), which was used for next synthesis without further purification.

Sheme 2. The synthetic route of probes **1-3**.



The synthesis of M2: 10% aqueous solution of NaOH (10 mL) was added to the solution of **M1** (3.60 g, 10 mmol) in CH_3OH (36 mL) at room temperature. After stirring for 3 hours, CH_3OH was evaporated and the reaction mixture was diluted with H_2O (100 mL). The solution was acidified to pH = 5 with 1 M HCl, the resulting precipitate was filtered, washed with water and dried to give **M2** as a yellow solid (yield: 82%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.19 (s, 1H), 8.01 (d, J = 4.0 Hz, 1H), 7.80 (t, J = 3.4 Hz, 1H), 7.73 (t, J = 4.0 Hz), 7.28 (d, J = 4.0 Hz, 1H), 6.94(s, 1H), 6.72-6.58 (m, 5H), 3.83 (s, 3H). HRMS calcd. for $[M + H]^+$: 347.0914. Found: 347.0911.

Synthesis of probe 1 as shown in Scheme 2: **M2** (346 mg, 1.0 mmol) was dissolved in dichloromethane, trimethylamine (151.5 mg, 1.5 mmol) was added, the resulting solution was cooled to 0 ^{o}C in ice bath. Then the solution of n-butyryl chloride (222 mg, 1.2 mmol) in CH_2Cl_2 was added drop wise. The reaction mixture was stirred for 2 h at room temperature, then diluted with H_2O and extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The combined residue was subjected to silica gel chromatography to give probe **1** as white solid (yield: 74%). m. p. 87-88 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.05 (d, J = 7.2 Hz, 1H), 7.82 (t, J = 7.2 Hz, 1H), 7.75 (t, J = 6.6 Hz, 1H), 7.34 (d, J = 6.6 Hz, 1H), 7.24 (s, 1H), 6.96 (s, 1H), 6.94-6.86 (m, 2H), 6.75 (q, J = 9.2 Hz, 2H), 3.82 (s, 3H), 2.58 (t, J = 6.6 Hz, 2H), 1.66 (q, J = 7.2 Hz, 2H), 0.97 (t, J = 6.9 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 170.59, 167.76, 160.43, 151.58, 151.41, 150.81, 150.31, 135.19, 129.73, 128.44, 128.39, 124.99, 124.23, 123.39, 117.71, 115.73, 111.71, 109.86, 109.71, 100.16, 81.12, 55.20, 34.76, 17.36, 12.97. HRMS calcd. for $[M + H]^+$: 417.1333. Found: 417.1331.

The synthesis of probe 2 (Scheme 2)

Probe **2** was synthesized in the similar strategy as used in probe **1**, and the yield is about 71 %. m. p. 198-199 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.04 (d, J = 7.8 Hz, 1H), 7.68 (t, J = 7.5 Hz, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.18 (d, J = 7.2 Hz, 1H), 7.08 (s, 1H), 6.82-6.78 (m, 3H), 6.70 (q, J = 16.4 Hz, 2H), 3.84 (s, 3H), 3.54 (t, J = 6.0 Hz, 2H), 2.81 (t, J = 7.2 Hz, 2H), 2.30 (quintuplet, J = 6.9 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 171.09, 168.96, 161.62,

152.75, 152.22, 151.98, 151.46, 136.29, 130.82, 129.53, 129.47, 126.09, 125.33, 124.45, 118.74, 116.91, 112.76, 110.93, 110.80, 101.22, 82.11, 56.12, 34.31, 32.57, 27.86. HRMS calcd. for $[M + H]^+$: 495.0438. Found: 495.0437.

The synthesis of probe 3 (Scheme 2)

The synthesis of probe 3 as shown in Scheme 2: **M2** (346 mg, 1.0 mmol) was dissolved in 20 mL dichloromethane, (388 mg, 2.0 mmol) 2-methyl-3-phenylpropanoic acid was added. The result solution was stirred in room temperature with (60 mg, 0.5 mmol) DMAP and (384 mg, 2 mmol) EDCI for 5 hours, then diluted with H_2O and extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The combined residue was subjected to silica gel chromatography to give **2** as brown oil. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.06 (d, J = 7.5 Hz, 1H), 7.79 (t, J = 5.0 Hz, 1H), 7.74 (d, J = 6.0 Hz, 1H), 7.34-7.19 (m, 6H), 7.05 (s, 1H), 6.95 (d, J = 2.5 Hz, 1H), 6.86 (d, J = 9.0 Hz, 1H), 6.76-6.70 (m, 3H), 3.81 (s, 3H), 3.08-2.98 (m, 1H), 2.87-2.83 (m, 1H), 2.66-2.59 (m, 1H), 1.24 (d, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 177.34, 174.18, 169.01, 161.68, 152.79, 152.22, 152.02, 151.51, 140.07, 139.23, 136.31, 130.85, 129.47 (2C), 129.34, 128.82 (2C), 128.65, 126.15, 125.37, 124.46, 118.57, 116.96, 112.81, 110.99, 101.27, 82.12, 56.14, 41.21, 41.11, 16.93. HRMS calcd. for $[M + Na]^+$: 515.1521. Found: 515.1528.

Determination of Quantum Yield

The quantum yields for both probes and their fluorophores were determined with rhodamine B as reference by the previously reported method[31-33], using rhodamine B as the reference. The quantum yields were estimated with the same equation showed below.

$$\phi_s = \frac{F_s \cdot A_c}{F_c \cdot A_s}\phi_c \qquad (1)$$

Colormetric assay

The chromogenic assay for chymotrypsin activity was measured with the following protocol. Purified human chymotrypsin obtained from Sigma-Aldrich Company was dissolved in 0.1 M HEPES (pH 8.0, 0.5% DMSO) and aliquots of them were frozen at -20 °C. The reaction mixture in a total assay volume of 200 μL contained appropriate amounts of our probe, 0.1 M HEPES buffer (pH 8.0, 0.5% DMSO), and chymotrypsin. Enzymatic hydrolysis of the probe was monitored by microplate reader (BioTek) in the presence or absence of various concentrations of inhibitor at 30 °C, in 0.1 M HEPES at different pH. Each experiment was repeated at least three times and the values were averaged.

Fluorometric assay

Fluorometric assay for chymotrypsin was performed as described in the following procedures by employing probes **1-3** as substrates and the peptide substrate (AMC-FPAA-Suc) according to PET mechanism. The weakly fluorescent substrate becomes highly fluorescent once the chymotrypsin cleaves the ester bond or amide bond, and the fluorescence enhancement could be observed and the fluorescence intensity is linearly correlated with the rate of hydrolysis at the early stage of the enzymatic reaction. Although the probes for elastase were designed based the mechanism of PET, the internal filter effect as well as other possible factors that may affect fluorescent signal should be calibrated for accurate kinetic measurement. The dose dependent fluorescence intensity of fluorophores was measured in the absence and presence of various concentrations of substrate. The concentrations of probe **2** were set to be 0.2 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM and 60 μM, while the concentrations of peptide substrate were set to be 5 μM, 10 μM, 50 μM, 100 μM, 200 μM, 300 μM and 400 μM. The slope obtained from the fluorescence enhancement as the function of its concentration was converted as the correction factor of fluorometric assay. For the inhibitory kinetic study, various concentrations of inhibitor, and appropriate amounts of probe **2** or peptide substrate were preincubated in 100 mM HEPES (pH 8.0, 0.5% DMSO) at 30 °C. Enzyme (50 μL) was then added to start the reaction and the fluorescence signal was recorded at λ_{em} = 515 nm (λ_{ex} = 450 nm) for our probe and λ_{em} = 445 nm (λ_{ex} = 350 nm) for peptide substrate, with microplate reader (SpectraMax M5, Molecular

Devices) using black microplate (96 wells). Each experiment was repeated at least three times and the values were averaged.

Molecular Docking

The three dimensional structures of the probes **1-3** was constructed and primarily optimized by Sybyl 2.0 software. Then the structural optimizations for all the probes were carried out in tripos force field. The optimized probes were docked into the crystal structures of chymotrypsin (PDB entry: 2P8O) [29] with Autodock software, respectively. The gird size was set to be 60×64×68 for both of the Autodock and Vina methods. The Lamarkian genetic algorithm (LGA) was applied for the conformational search of the Autodock. The best poses were selected for the binding model analysis for all the probes.

RESULTS AND DISCUSSION

Probe design and optimization

Considering the structural diversity of recognition group having different steric and electronic effect, three acyl groups (butyryl, 4-bromobutyryl and 2-phenylbutyryl) were selected as recognition site to establish the target probes (probes **1-3**, **Scheme 1**). In general, target compounds were prepared by straightforward synthetic routes shown in **Scheme 2**. The chemical structures of all the three probes and their intermediates were fully characterized (See **Figures S1-S7**). After structural validation, the properties of the synthesized probes and the corresponding chromosphore methylfluorescein were characterized by UV-Vis and fluorescence spectra, respectively. To our delight, it was found that all three probes (Φ<0.01) exhibited extremely low background signal on both the absorbance and the fluorescence. On the contrary, the hydrolytic product is a good chromophore with maximum absorbance at 450 nm, and an excellent fluorophore (Φ= 0.43) with the maximum emission at 515 nm (see **Figure S8**). It is noteworthy that all probes showed good water solubility and were very stable in aqueous solution and no significant change of color and the fluorescence intensity (FI) was observed before the addition of chymotrypsin. We then

performed the primary screening by measuring the UV-Vis and fluorescence spectra of each probe in HEPES buffer (100 mM, pH 7.0, 0.5% DMSO) after the addition of chymotrypsin, respectively. The most remarkable increase on the absorbance and the FI were identified for probe **2** and relatively lower response for probe **3**, whereas no significant response for probe **1**. In our understanding, the bromo-substitution of probe **2** may make the nucleophilic attack of Ser much easier than that of probes **1** and **3**. In addition, we performed the molecular docking and obtained the simulated binding modes of probes **1-3** in the active site of chymotrypsin. As shown in **Figure 1**, both probes **1** and **2** formed H bonds with Gly216 in the active site, respectively, whereas no H bond was observed in the binding mode of probe **3**. More importantly, the Ser195 showed the closest distance to the ester bond of probe **2** than those of probes **1** and **3**, which is expected to facilitate the nucleophilic attack in the active site. Therefore, probe **2** was picked for the follow-up studies.

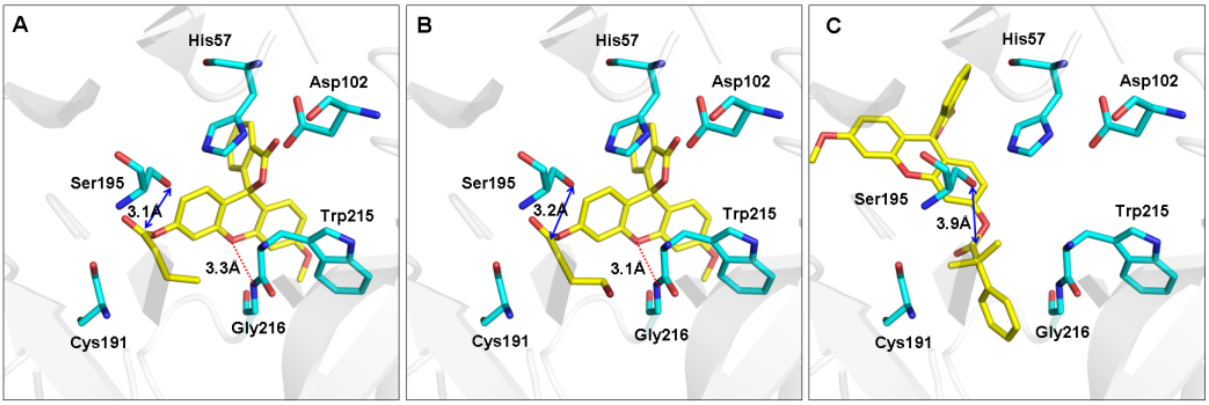


Figure 1. Simulated binding models of probe **1** (A), probe **2** (B) and probe **3** (C) in the active sites of chymotrypsin, respectively. The distances between the key residue Ser195 and the carbonyl group of probes were showed with arrow line in blue, and the H bonds were depicted in red dash line.

Since the pH value may affect the reactivity of probe, we therefore studied the influence of pH on the chymotrypsin-catalyzed hydrolysis with probe **2**. It revealed that the fluorescence of probe **2** in the presence of chymotrypsin significantly increased as the pH increased from 6.0 to 9.0, and almost reached the maximum at 8.0 (see **Figure S9**). Beside, previous report claimed that the optimum pH range for

chymotrypsin is 7.8-8.0. As a whole, the most suitable pH for the reaction of probes with chymotrypsin is 8.0, which was adopted in the following studies. We selected the moderate temperature 30 ^{o}C as the temperature without further optimization. Under these conditions, the time-dependent increase on the absorbance (**Figure 2A**) and the FI (**Figure 2B**) were obtained for probe **2** in the presence of 10 μg/mL chymotrypsin in HEPES buffer (100 mM, pH 8.0, 0.5% DMSO). Apparently, after incubation with chymotrypsin for 35 min, the color of reaction mixture changed from colorless to green and no fluorescence to strong green fluorescence was observed. The maximum absorbance at 450 nm and the maximum FI at 515 nm jointly indicated around 25-fold increase over the basal level (**Figure 2**). The data showed that probe **2** offered a sensitive colormetric and fluorometric "off-on" system for sensing chymotrypsin activity, which could be visualized by naked-eye and spectrophotometers.

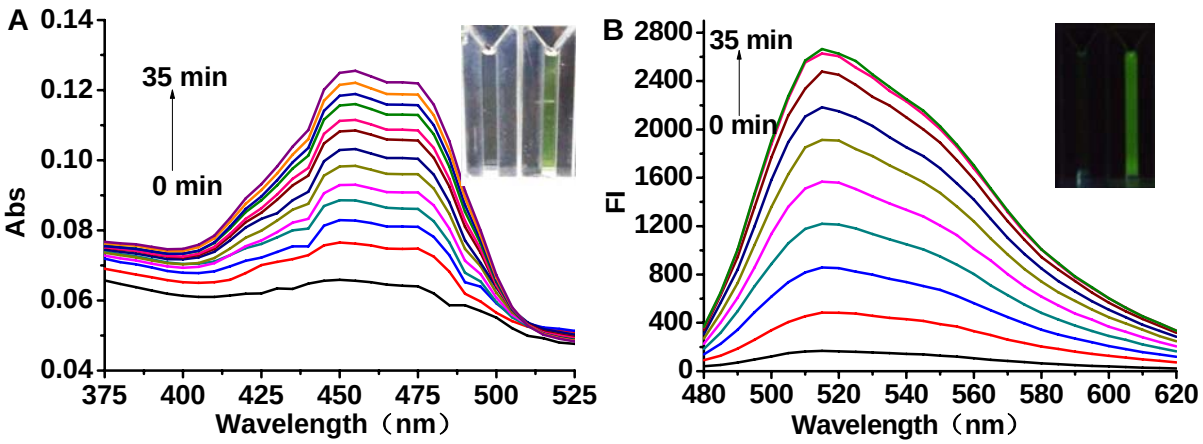


Figure 2. The time-dependent absorbance (A) and fluorescence (λ_{ex} = 455 nm, B) of probe **2** (10 μM) in HEPES buffer (100 mM, pH 8.0, 0.5% DMSO) in the presence of chymotrypsin (10 μg/mL) at 30 °C; Left Insets show the photos of the corresponding reaction mixtures in the absence and presence of chymotrypsin (0.02 U/mL) after 35 min incubation at 30 °C. Photos in the right inset were taken under UV light (365 nm) illumination.

Since the sensitivity of fluorometric method is usually three orders of magnitude higher than colormetric method, we mainly focus on the fluorometric "off-on" sensing in the rest experiments. To further elucidate the applicability of the refined probe to

quantitatively detect chymotrypsin, the fluorescence of the assay system was carried out in the presence of various concentrations of chymotrypsin (0-40 μg/mL). As depicted in **Figure S10**, the FI of probe **2** increased notably and sharply with the increase of the concentration of chymotrypsin. Furthermore, a linear correlation was obtained between the slope of the probe **2** and the concentration of chymotrypsin from 0 μg/mL to 10 μg/mL. According to the limit of detection (LOD)=3σ/k method, in which σ means the standard deviation of blank experiment and k means the slope of the calibration curve, the LOD for this probe to detect chymotrypsin was determined to be ~50 ng/mL based on three independent measurements.

To shed light on the reaction mechanism of chymotrypsin-catalyzed hydrolysis of the probes, the reaction mixture (100 mM HEPES buffer at pH 8.0, 10 μg/mL chymotrypsin) was analyzed by HRMS, with the similar reaction system containing no chymotrypsin as the negative control. As shown in **Figure 3**, the peaks for the probe **2** alone and its pre-incubation with chymotrypsin in HEPES buffer (100 mM, pH 8.0, 0.5% DMSO) after 60 min were obtained. The molecular ion peak of m/z 495.0437 corresponds to the probe (**2**+H^+), while the molecular ion peak (m/z 347.0916) correlates to the fluorophore that was released from cleavage of the ester bond of the designed probe. Thus, the above observation indicated that the hydrolysis was caused by the chymotrypsin-catalyzed cleavage at the ester bond.

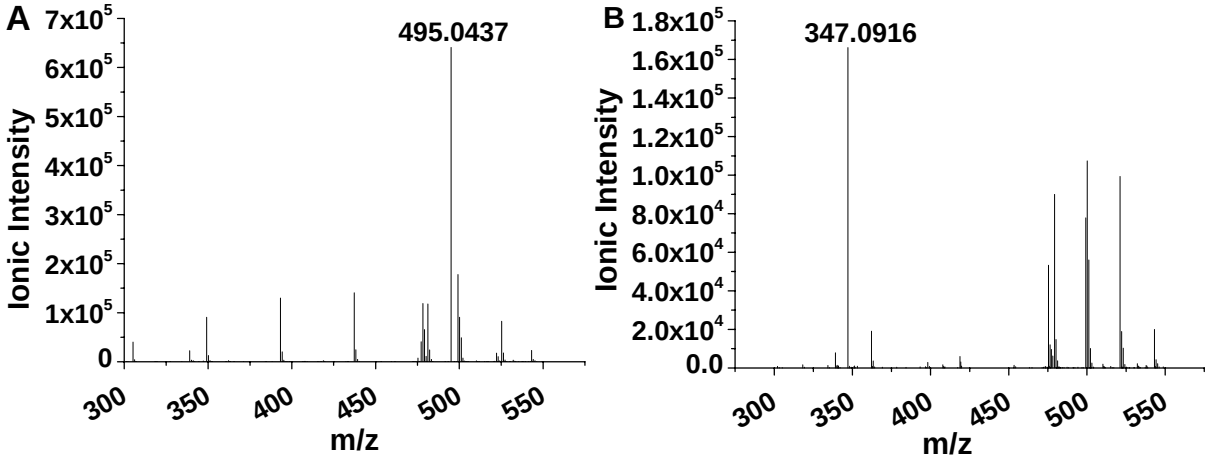


Figure 3. HRMS spectrum of probe **2** (20 μM) without (A) and with (B) the addition of chymotrypsin (10 μg/mL) and incubated for 1 h in HEPES buffer (100 mM, pH 8.0, 0.5%

DMSO) at 30 °C.

Probe Specificity

To disclose the specificity of probe **2**, its hydrolysis was examined under the interference of various analytes, including metal ions (K^+, Na^+, Mg^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}), anions (HS^-, HSO_3^-, SO_3^{2-}, HCO_3^- , HPO_4^{2-} and CO_3^{2-}), amino acids and biothiols (Met, GSH, Phe, Lys, Trp, Ala and His), as well as the common esterases or proteins (elastase, AChE, BChE, trypsin, carboxypeptidase and BSA). The data presented in **Figure 4A and Figure S11** shows that this probe is highly selective for chymotrypsin over competing amino acids, metal ions, and biothiols. More interestingly, the relative reaction rate of chymotrypsin-catalyzed hydrolysis for probe **2** is much higher than that of other hydrolases and proteins (**Figure 4B**), whereas none of those hydrolases and proteins showed significant interference against chymotrypsin. The above study indicated that probe **2** is highly specific toward chymotrypsin.

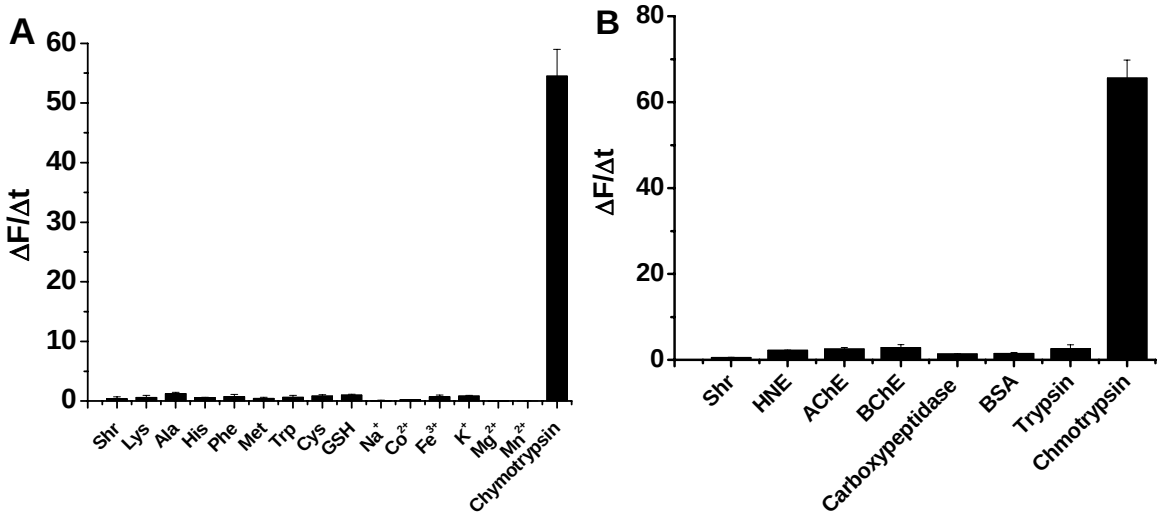


Figure 4. (A) The specificity profile of probe **2** (10 μM) toward different metal ions (K^+, Na^+, Mg^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}), amino acids and biothiols (Met, GSH, Phe, Lys, Trp, Ala and His). All the analytes except chymotrypsin (10 μg/mL) are in the concentration of 1 mM. (B) The specificity profile of probe **2** (10 μM) toward different esterases or proteins (elastase, AChE, BChE, trypsin, BSA, carboxypeptidase) in HEPES buffer (100 mM, pH 8.0, 0.5% DMSO). Shr refers the rate of spontaneous hydrolysis; AChE, BChE, and BSA are the abbreviations of acetylcholinesterase, butyrylcholinesterase, and bovine serum albumin, respectively.

Michaelis–Menten Kinetics

To further elucidate the feasibility of the developed probe as a substrate for evaluating the enzymatic mechanism of chymotrypsin, we performed the kinetic characterization. Here we still only took the fluorogenic measurement, considering the higher sensitivity of fluorometric assay compared to colorimetric method. Generally, the initial reaction rates for the enzymatic hydrolysis of target probe were derived from the fluorescence response depending on the concentration of probe **2** (0-50 μM), while the widely used method with the peptide substrate (AMC-FPAA-Suc) as the reference. As anticipated, the kinetic curve describing the velocity versus the concentration of substrate obeys the Michaelis–Menten equation and the specific curves as shown in **Figure 5A** and **5B**. On the basis of the above kinetics, the Michaelis constant (K_m) and the catalytic rate constant (k_{cat}) of chymotrypsin for probe **2** were determined to be 11.09 ± 2.08 μM and 3.45 ± 0.26 min^{-1}, respectively. Thus, the total catalytic efficiency (k_{cat}/K_m) of chymotrypsin in hydrolyzing probe **2** is about 0.22 $\mu M^{-1} \cdot min^{-1}$, indicating the probe **2** is a suitable substrate for assessing the chymotrypsin activity. For clear comparison, we also tested the K_m (58.84 ± 6.47 μM) and k_{cat} (210 ± 15 min^{-1}) of chymotrypsin against the existing method with peptide substrate (AMC-FPAA-Suc) under the same condition. In all, the binding affinity of probe **2** with chymotrypsin is ~5-fold higher than that of AMC-FPAA-Suc, although the catalytic efficiency for probe **2** is relatively lower. More importantly, the synthetic cost of probe **2** is ~40-50 ￥/g without further optimization, whereas the price of AMC-FPAA-Suc is about 2500 ￥ per 5 mg according to the Wuhan Fine Peptide Co., Ltd. Hence the cost of probe **2** is more than 10000-fold lower than that of AMC-FPAA-Suc. The above analyses implied that probe **2** could be a suitable substrate for chymotrypsin activity assessment.

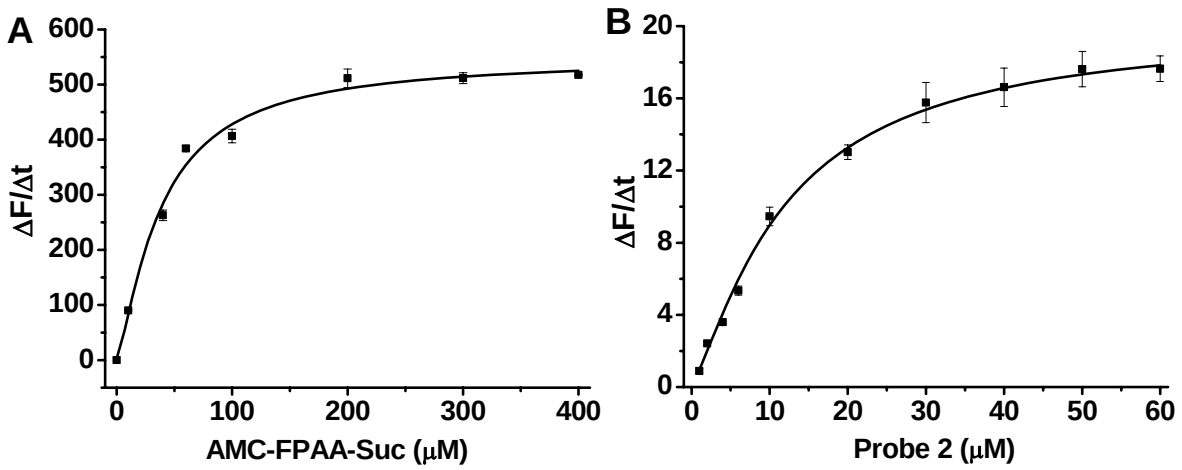


Figure 5. Michaelis-Menten curve of probe **2** (A) and AMC-FPAA-Suc (B) catalyzed by chymotrypsin (5 μg /mL) in HEPES buffer (100 mM, pH 8.0, 0.5% DMSO) with fluorometric method.

Application in Inhibitor Characterizing

One of the most important applications for the bioanalytical methods is the inhibitor screening and characterization in drug discovery. Thus, the new fluorometric method with probe **2** as the substrate was employed to study the inhibitory kinetics for the well-known chymotrypsin inhibitors. Two typical chymotrypsin inhibitors, a covalent inhibitor phenylmethanesulfonyl fluoride (PMSF, purchased from J&K Chemical Company) and a reversible inhibitor chymostatin (CAS number 9076-44-2, purchased from Sigma-Aldrich), were chosen as the two representatives for kinetic study with the newly developed method. We incubated all the reaction systems composing of probe **2** (10 μM), chymotrypsin (20 μg /mL) and different amounts of PMSF (0.1 to 40 μM) and chymostatin (0 to 40 μM) in the 96-well plates at 30 °C and the FI at 515 nm was continually recorded for 5 min by microplate reader. One thing to note is that the PMSF was preincubated with enzyme for 10 min due to its irreversible binding manner. As expected, the enhancement of FI was retarded gradually when the concentration of inhibitors increased, and the kinetic curves of the residual activity against the concentration of inhibitors were obtained accordingly (See **Figure 6A** and **6B**). Then the IC_{50} values for PMSF and chymostatin inhibitor were calculated to be 22.89 ± 3.27 nM and 3.12 ± 0.34 μM, respectively. The above values are very close to those (16.65 ± 2.34 nM for chymostatin and 5.53 ± 1.49 μM) obtained with the peptide-based substrate

(AMC-FPAA-Suc) under the same conditions. To further explore the selectivity of probe **2**, the fluorescence changes were studied in the presence of some other inhibitors, including the acetylcholinesterase inhibitors (tacrine and donepezil), the elastase inhibitor (sivelestat) and *p*-hydroxyphenylpyruvate dioxygenase inhibitor (sulcotrione). In general, no significant fluorescence change was observed by non-chymotrypsin inhibitors against the probe **2**-based assay (see **Figure S12**). The above results demonstrated that probe **2** has a great potential to be used as a reliable non-peptide substrate for discovering chymotrypsin inhibitors under mild conditions.

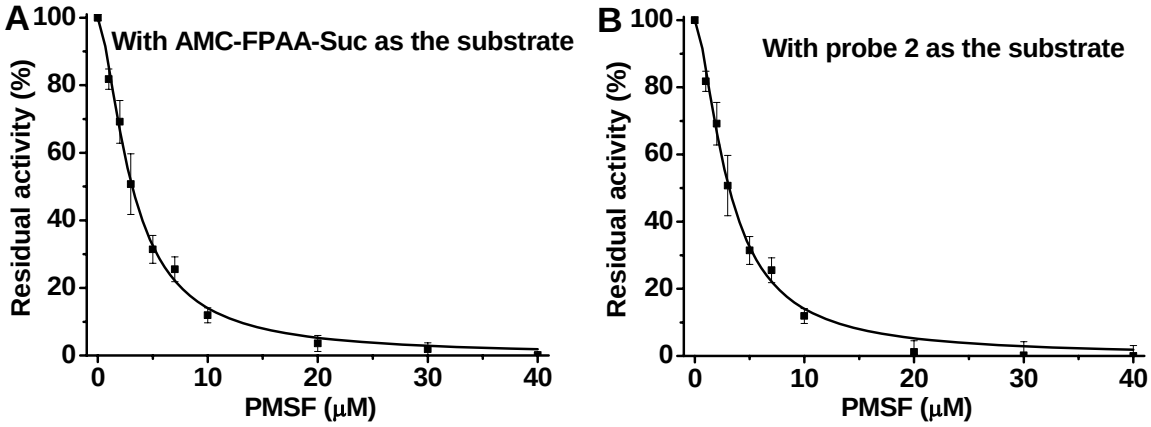


Figure 6. Plots of inhibitory efficiency of phenylmethanesulfonyl fluoride (PMSF) toward the chymotrypsin with peptide (AMC-FPAA-Suc)-based method (A) and probe **2**-based method (B).

CONCLUSION

In conclusion, a non-peptide-based small-molecule probe (**2**), was discovered as a specific chromogenic and fluorogenic substrate for chymotrypsin. Probe **2** showed very low background in both absorbance and fluorescence, but exhibited remarkable enhancement in bright green signal upon the catalysis of chymotrypsin. In addition, chymotrypsin showed about 5-fold higher binding affinity against probe **2** than the most widely used peptide substrate (AMC-FPAA-Suc), although the overall catalytical efficiency for probe **2** was relatively lower. More interestingly, this refined probe exhibited excellent specificity for chymotrypsin over other proteins or hydrolases. To our knowledge, probe **2** is the first-ever

non-peptide-based chromogenic and fluorogenic substrate for chymotrypsin. Accordingly, a turn-on colormetric and fluorescent assay was established for evaluating chymotrypsin activity in aqueous solution, and subsequently applied in chymotrypsin inhibitors screening and characterization. The current study demonstrated that the small-molecule probe **2** could be potentially applied in future high-throughput studies in drug discovery and clinical diagnosis of chymotrypsin-associated diseases.

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SUPPORTING INFORMATION

The general synthesis and NMR and HRMS spectra for the newly synthesized compounds and some figures for probe**s** characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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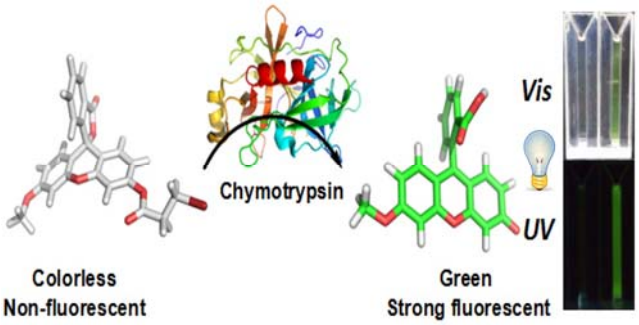
Chymotrypsin
Vis
UV
Colorless
Non-fluorescent
Green
Strong fluorescent