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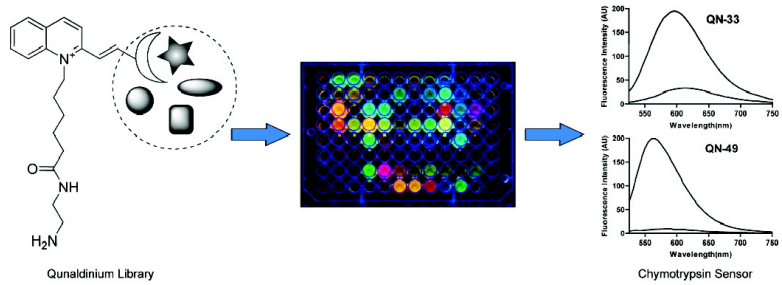

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Diversity-Oriented Fluorescence Library Approach (DOFLA) to the Discovery of Chymotrypsin Sensor

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The diversity-oriented fluorescence library approach (DOFLA) has emerged and found applications in various fields to meet the acute demands for novel fluorescence sensors. The power of this approach has been demonstrated with the impressive discoveries of novel sensors for polymers such as DNA and heparin or for small molecules such as GTP and glutathione (*J. Am. Chem. Soc.* **2003**, *125*, 1130−1131; *J. Am. Chem. Soc.* **2006**, *128*, 10380−10381; *J. Am. Chem. Soc.* **2007**, *129*, 4510−4511; *Chem. Commun.* [Online early access]. DOI: 10.1039/b717058k. Published online Dec 11, 2008. http://www.rsc.org/publishing/journals/CC/article.asp?doi=b717058k). Herein we report the application of this approach on quinaldinium fluorescent dye library synthesis on solid support and novel chymotrypsin sensor discovery. The new sensors are not only selective to chymotrypsin over other proteins but also only to the active conformation of chymotrypsin.

Introduction

Fluorescence sensors have become widely used as tools for chemical, biological, and medical applications,[2] thus the discovery and development of chemosensors have elicited scientists continuous interests and efforts. The conventional target-oriented strategy for the design of fluorescence sensors is to combine fluorescent dye molecules with designed receptors for specific analytes, in the hope that the recognition event between a receptor and an analyte will induce photophysical property changes of the dye moiety (Figure 1). While this approach has played the dominant role throughout the decades, it has limited the scope and speed of novel sensor discovery.

The application of combinatorial chemistry techniques and the diversity-oriented strategy have spurred the emergence of diversity-oriented fluorescence library approach (DOFLA) to the discovery of novel fluorescence sensors. This approach focuses on generation of a fluorescent dye library and bypasses specific analytes. The synthesis of a fluorophore scaffold can be designed where diversity is introduced in the process of assembling the fluorophore, and an efficient synthetic route can be developed to generate a diverse set of dyes. The design and preparation of the dye library is unbiased to any specific target analyte so the library can be evaluated with quite distinct analytes to maximize the chance of applications in different fields. Once a fluorescence response is observed, the selected dye and analyte pair can be identified as a potential lead for further development. A vast chemical parameter space can be explored with collections of building blocks, thus leads to higher likelihood to the discovery of new small molecule−target interactions.

In our previous development of the styryl fluorescent dye library,[3] we noticed that the quinaldine scaffold in general resulted in products with low purity, urging an improved synthetic strategy. To overcome the limitation, we designed a new solid-phase synthesis route for quinaldine scaffold compounds, from which a selective chymotrypsin sensing was demonstrated.

Results and Discussion

Synthesis of QN Library. Quinaldine was transformed to the quaternary salt by nucleophilic attack of alkyl alcohol triflic ester. The high activity of triflic ester at room temperature ensured the high yield and safely reduced the potential side reactions. The quinaldinium salt was loaded onto solid support by amide coupling of the deprotected carboxylic acid and ethylenediamine derivatized 2-chlorotrityl polystyrene resin. Different lengths of the linker were tested and optimized. The following secondary amine-catalyzed Knovenaegle condensation was performed at room temperature under nitrogen. The 96 aldehyde R_2 building blocks contain various functionalities, conjugation lengths, and electron-donating or -withdrawing properties. Products were cleaved from the resin under mild acidic conditions and 96 compounds were collected for further screening study (Scheme 1). These set of compounds were named the QN (quinaldinium) library. Because of the structural diversity, a broad range of exitation /emission wavelengths is covered by the library (see Supporting Information).

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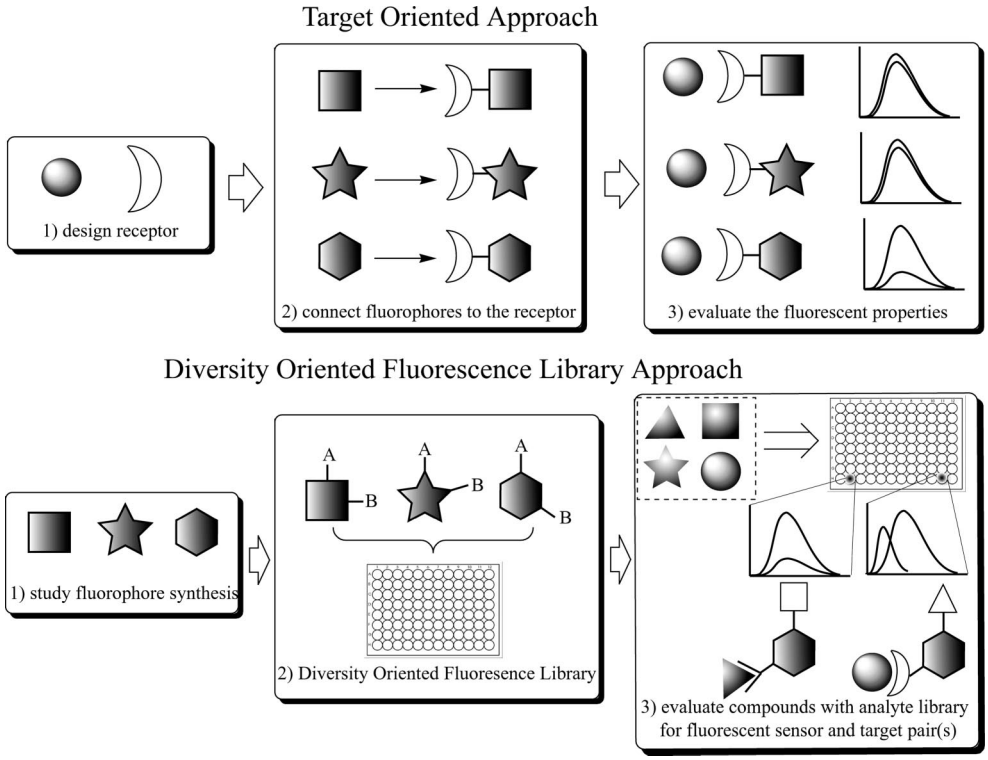


Figure 1. Comparison between target-oriented approach and diversity-oriented fluorescence library approach to the discovery of novel fluorescence sensors.

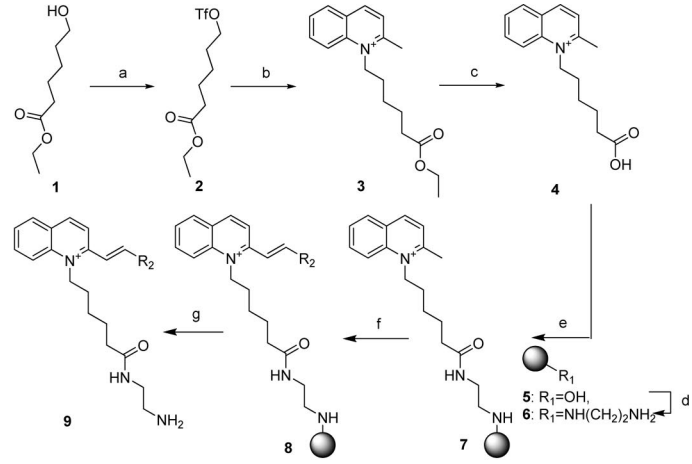
Primary Screening. The development of fluorescence protein sensors and detection methods has drawn wide influence on the detection and quantification of proteins and study of the modifications such as phosphorylation and glycosylation, as well as improved methods for separating and analyzing peptides and proteins.[4] Simple, sensitive, and specific protein-sensing methods are of great importance to clinical diagnostics and proteomic researches.[5,6] Thus the QN library was screened with a collection of 12 proteins (protease A, chymotrypsin, themolysin, lipase AY, cellulase A, hemicellulase, lysozyme, deamizyme, ribonuclease A, cytochrome c, hemoglobin, and insulin) in the hope of discovering novel fluorescence protein sensor. Out of the 96 library members, two compounds (QN-33 and QN-49) showed a distinctive fluorescence increase to chymotrypsin, and this stimulated our further study of the interactions between the hit compounds and chymotrypsin.

α-Chymotrypin is a well-studied member of the serine protease family. The catalytic triad mechanism is of classical importance and was widely discussed and updated through the decades.[7] It was recently discovered that α-chymotrypin could form the amyloid-like aggregation structure under specific conditions,[8] attracting researchers to revisit this enzyme from a structure point of view. Moreover, the comparably small size, known crystal structure, and easy availability of chymotrypsin make it one of the ideal models for protein and proteomics studies.[6c,9] In addition to the various activity-based fluorescent substrates and covalent labeling reagents,[10] there are only two fluorescent compounds that have been thoroughly studied on the noncovalent interactions with chymotrypsin: 1-anilinonaphthalene-8-sulfonate (ANS)[11] and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS),[12] both of which are nonspecific to chymotrypsin and were usually used as general protein fluorescent probes. Therefore, we further studied our two hit compounds' specificities and binding properties to α-chymotrypin as below.

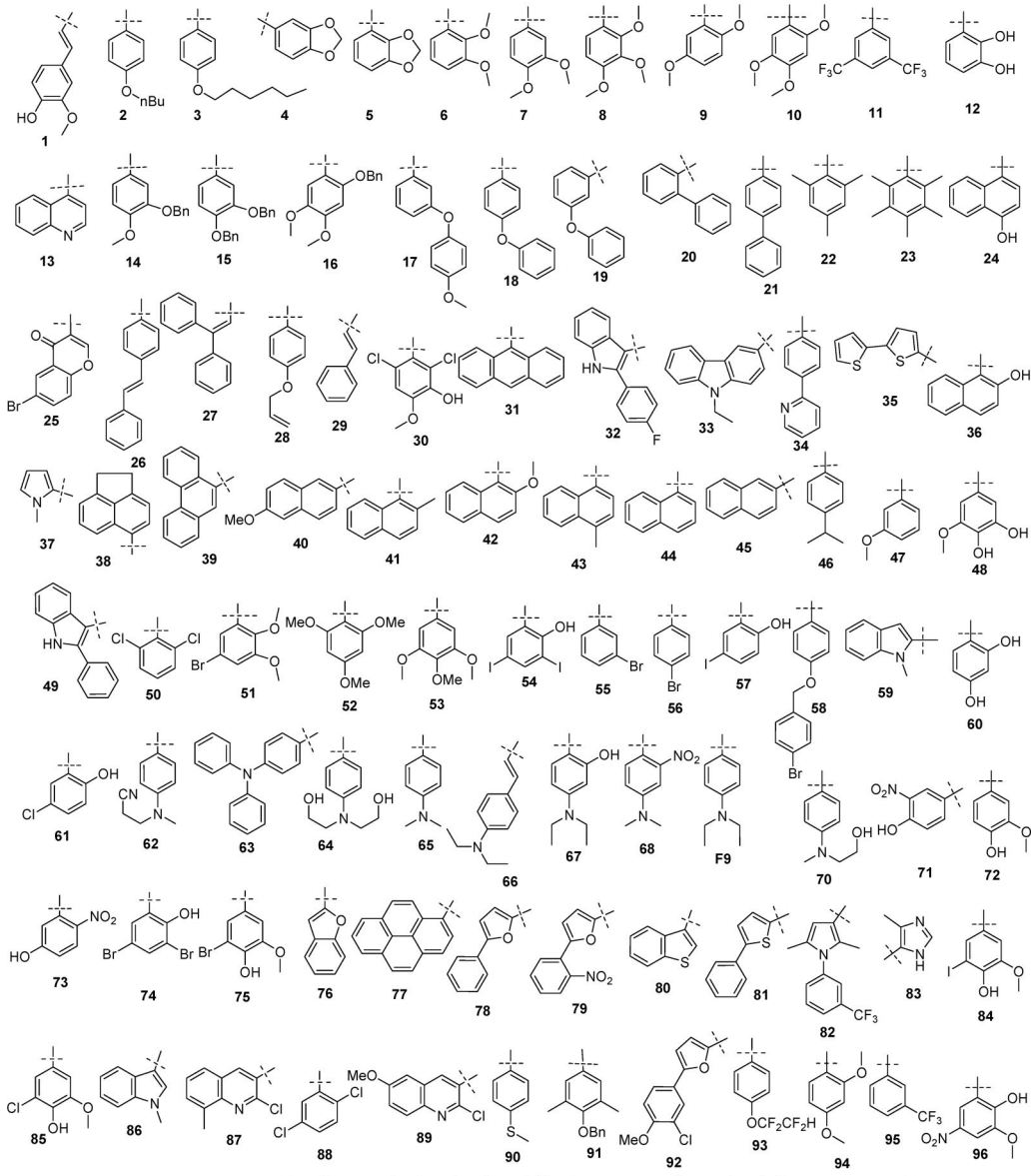
Binding of QN-33 and QN-49 to Chymotrypsin. Sensitivity and selectivity to the target are the major criteria for a fluorescence sensor. We first tested QN-33 and QN-49 on the selectivity to α-chymotrypsin (Figure 2). Major enzymes from the serine protease family, such as trypsin, elastase, and subtilisin, as well as the precursor chymotrypsinogen, were selected as the analytes to check the selectivity of the two hit compounds (Figure 2). These two compounds showed very unique selectivity only toward chymotrypsin. To our best knowledge, thus far there is no report on the fluorescence sensors to chymotrypsin with such a high selectivity. While the exact binding site and mode is not clear at the moment, the Klotz plot showed 1:1 interaction between chymotrypsin and QN-33/QN-49 (see Supporting Information). QN-33 or QN-49 also did not compete with bromoenol lactone, an irreversible inhibitor of chymotrypsin at active site, so it seems that the sensors bind to allosteric site of chymotrypsin (see Supporting Information).

To study the relationship between the structure and fluorescence response, analogues of QN-33 and QN-49 were prepared. Methyl, sulfonate, and neutral versions of the compounds were synthesized and tested with different concentrations of chymotrypsin, and the binding affinities were compared (Figure 3). At a neutral condition (pH 7.4), both QN-33 and QN-49 are positive charged at the linker termini because of the amino group and showed highest binding affinity. The methyl analogues showed lower-binding affinity compared to that of the original compounds, but it was higher than that of the sulfonate compounds. This is

Scheme 1. Solid-Phase Synthesis of Quinaldinium Dyes[a]



R_2-CHO, R_2=



[a] Reagents and conditions: (a) Tf_2O, poly(4-vinylpyridine), DCM; (b) quinaldine, DCM; (c) 48% HBr, 65°C; (d) 2-chlorotrityl alcohol resin sequentially treated with thionyl chloride in DCM and ethylenediamine in DCM; (e) **6**, HATU, DIPEA, 25% DMF/DCM; (f) R_2-CHO, pyrrolidine, NMP; (g) 5% TFA/DCM.

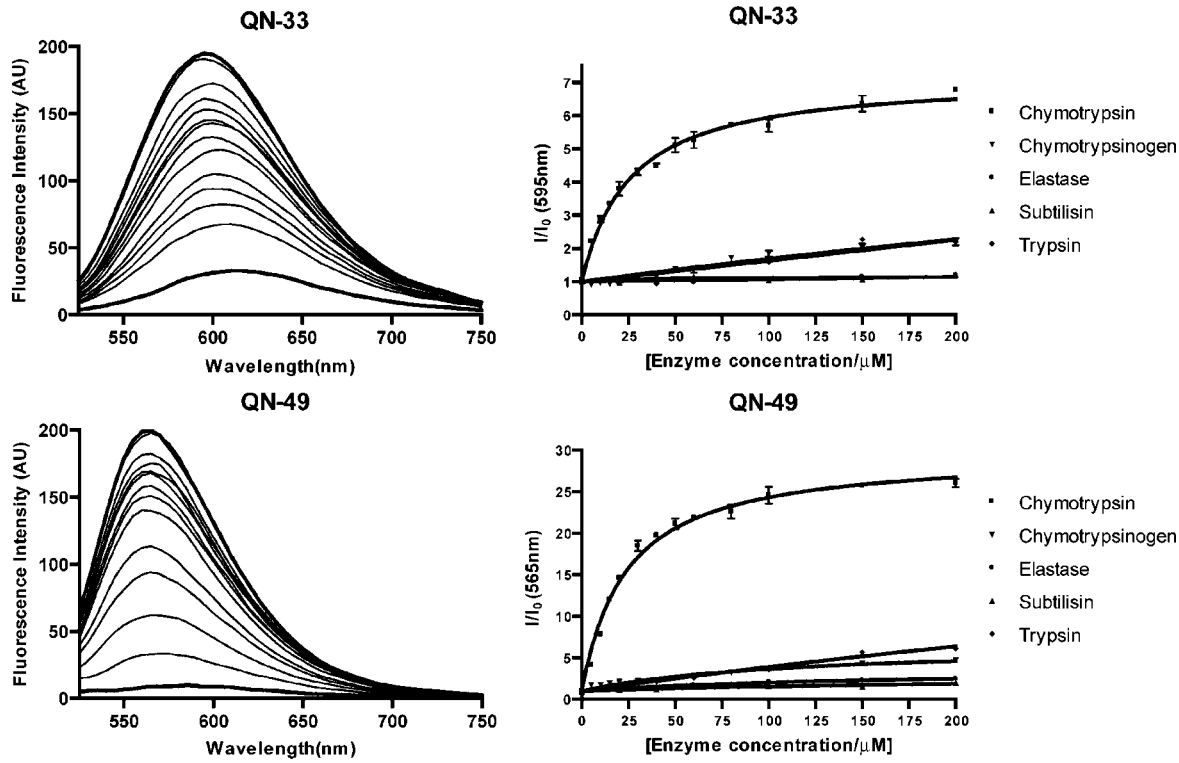


Figure 2. Fluorescence emission spectra of QN-33 (1.67 μM, excitation 460 nm) and QN-49 (2 μM excitation 490 nm) with 0–200 μM of α-chymotrypsin in 10 mM HEPES buffer (pH 7.4) in left panel and binding isotherm from the fluorescence titration experiment with α-chymotrypsin, chymotrypsinogen, elastase, subtilisin, and trypsin in right panel.

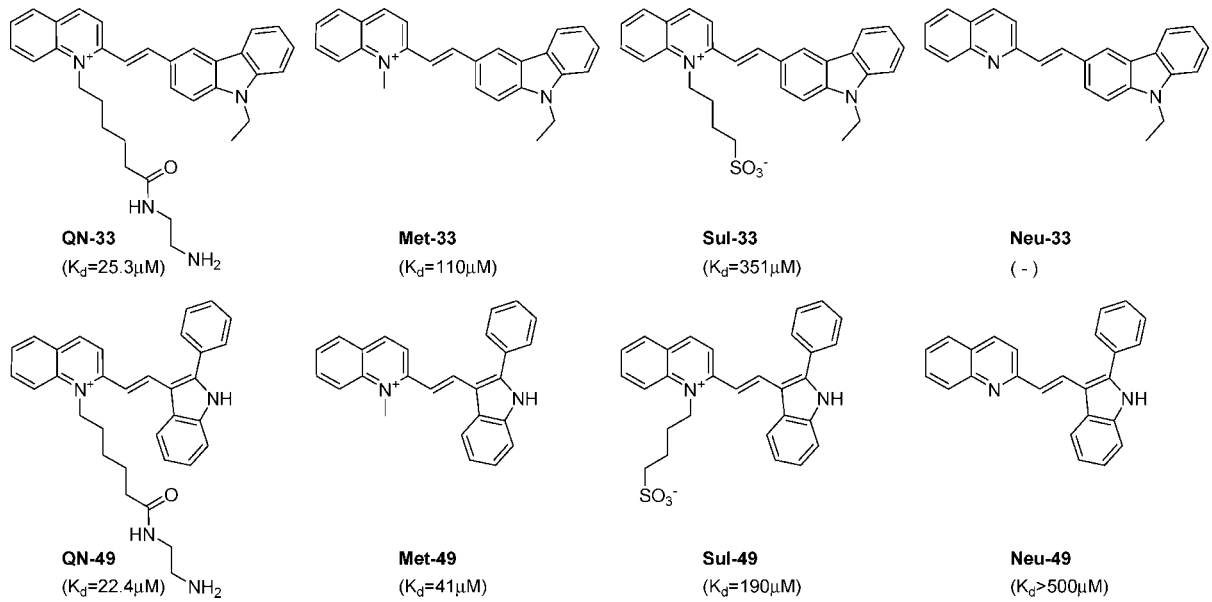


Figure 3. Structure of QN-33, QN-49, and analogues prepared and their dissociation affinities to α-chymotrypsin based on fluorescence titration in 10 mM HEPES buffer (pH 7.4). Neu-33 showed no obvious fluorescence response.

congruent with the charge status that when the charge of the linker part is reduced from +1 to 0 and −1, the binding affinity decreased. A titration experiment with the neutral compounds showed a similar trend. Because of this and the fact that all other members of the QN library showed lower binding affinity to chymotrypsin, we propose that the 2-phenylindole and the carbazole moiety play an important role in the recognition and binding to chymotrypsin. The positive charge of quinaldinium part and the linker also contribute to the binding affinity. The whole conjugated system binds to protein, and this event leads to a fluorescence response caused by structural recognition.

Finally we tested the fluorescence response of QN-33 and QN-49 with heat-denatured chymotrypsin to demonstrate that maintenance of the correct protein conformation is crucial to the binding events. α-chymotrypsin (100 μM) in 10 mM HEPES buffer was maintained at 65 °C, and samples were drawn every 5 min and tested with QN-33 and QN-49 for fluorescence response. These samples were also tested for their enzyme activity with N-succinyl-Ala-Ala-Pro-Phe *p*-

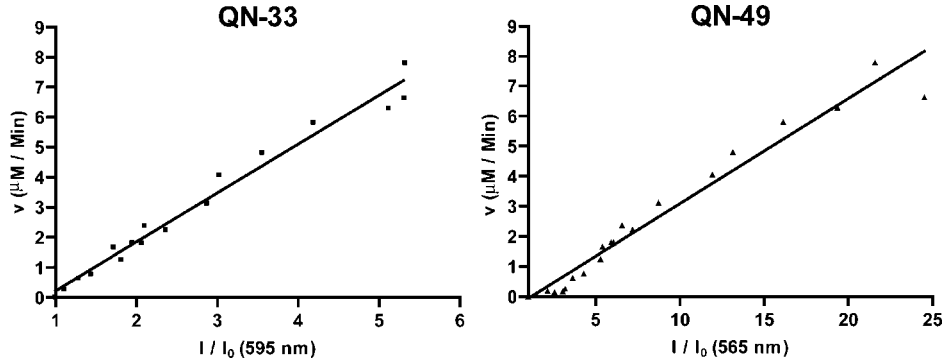


Figure 4. QN-33 and QN-49 fluorescence intensity fold change plotted with the initial velocity of the heat denatured α-chymotrypsin (100 μM) in 10 mM HEPES buffer (pH 7.4). 100 μL of the protein solution were drawn and tested with QN-33 (1.67 μM) and QN-49 (2 μM) every 5 min. Meanwhile, samples were diluted 1000 times and subjected to Suc-AAPF-pNA-based initial velocity test by monitoring absorbance at 405 nm.

nitroanilide (Suc-AAPF-pNA) substrate. The initial velocities were plotted with the corresponding fluorescence emission (Figure 4). The activity of the enzyme is directly related to the active conformation of the protein, and the heat-denatured protein loses its activity, as well as the active conformation. It is clearly demonstrated that the activity of α-chymotrypsin showed a linear relationship with fluorescence response. The fluorescence of QN-33 and QN-49 changed in response to the protein active conformation decrease, demonstrating that only chymotrypsin with an active conformation was recognized by QN-33 and QN-49.

In conclusion, a diversity-oriented fluorescence quinaldinium library was successfully prepared with high purity, and two unique chymotrypsin selective sensors were discovered. Furthermore, these two sensors are only responding to active conformation of the enzyme and thus would be very useful for further study of chymotrypsin and its mechanistic studies. With this successful example of selective protein sensor discovery, the diversity-oriented fluorescence library approach (DOFLA) will be applied to a systematic search for proteomic scale of fluorescence ligand-protein pair discovery in due course.

Experimental Section

General Information. All chemicals were purchased from Sigma-Aldrich or Acros and used without further purification. 2-Chlorotrityl alcohol resin (1.37 mmol/g) was purchased from BeadTech Inc., Korea. All compounds were tested with LC-MSD (ChemStation 1100, Agilent Technologies.) equipped with a Phenomenex Luna 3μC18 column (20 × 4.0 mm), with 4 min elution using a gradient solution from 5% CH_3CN/H_2O(0.1% acetic acid) to 95% CH_3CN/H_2O (0.1% acetic acid), with UV detector and an electrospray ionization source. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker Avance 400 NMR spectrometer and were recorded at 400 and 100 MHz, respectively. Chemical shifts are reported relative to internal chloroform (^{1}H δ 7.26 ppm, ^{13}C δ 77.0 ppm) or dimethyl sulfoxide (^{1}H δ 2.50 ppm, ^{13}C δ 39.43 ppm). Lipase AY, protease A, cellulose A, hemicellulase, and deamizyme were purchased from Amano Enzyme Inc., Nagoya, Japan, and all the rest proteins were purchased from Sigma.

Ethyl 6-(trifluoromethylsulfonyloxy)hexanoate (2). To the suspension of poly(4-methylpyridine) (2.24 g, 2 equiv) in newly dried dichloromethane (DCM, 25 mL) at 0 °C was added trifluoromethanesulfonic anhydride (5 g, 1 equiv) dropwise over 30 min. Ethyl 6-hydroxyhexanoate (2.76 g, 0.95 equiv) was then added in dropwise via a syringe. The mixture was stirred at 0 °C for 4 h, and the polymer was filtered off by a frit. The solvent was evaporated under reduced pressure (without heating), and the crude product was subjected to a short silica gel pad with 1:4 (v/v) diethyl ether/hexane as the eluent solvent. The solvent was evaporated (without heating), and the product was used directly in the following step. (3.9 g, 75% yield based on ethyl 6-hydroxyhexanoate).

1-(6-Ethoxy-6-oxohexyl)-2-methylquinolinium trifluoromethanesulfonate (3). To quinaldine (1.4 g, 1 equiv) in 10 mL of dichloromethane was added **2** (2.9 g, 1 equiv) dropwise, and the reaction mixture was stirred in dark for 24 h at room temperature. The solvent was evaporated under reduced pressure, and the residue was crystallized from diethyl ether to get the white powder as product. (3.5 g, 82% yield.) ^{1}H NMR ($CDCl_3$): δ 8.776 (d, 1H), 8.228 (d, 1H), 8.144 (dd, 1H), 8.077 (t, 1H), 7.8672 (d, 1H), 7.784 (t, 1H), 4.868 (t, 2H), 4.036 (q, 2H), 3.068 (s, 3H), 2.268 (t, 2H), 1.899 (m, 2H), 1.644 (m, 2H), 1.578 (m, 2H), 1.167 (t, 3H). ^{13}C NMR ($CDCl_3$): δ 173.171, 159.677, 146.201, 138.644, 135.854, 130.888, 129.339, 128.657, 125.532, 118.215, 60.286, 51.964, 33.66, 28.472, 25.842, 24.199, 22.922, 14.11. ESI-MS (*m*/*z*) Calcd (found): 286.2 (286.2) for $[M]^+$.

1-(5-Carboxypentyl)-2-methylquinolinium bromide (4). Compound **3** (3 g) was dissolved in 48% HBr aqueous solution (5 mL), and the mixture heated at 65 °C for 2 h and then evaporated to dryness. The residue was recrystallized from acetone to yield 1.6 g of pale crystals, yield 70%. ^{1}H NMR (DMSO): δ 9.106 (d, 1H), 8.582 (d, 1H), 8.412 (dd, 1H), 8.229 (t, 1H), 8.128 (d, 1H), 7.992 (t, 1H), 4.910 (t, 2H), 3.115 (s, 3H), 2.259 (t, 2H), 1.897 (m, 2H), 1.598 (m, 4H). ^{13}C NMR (DMSO): δ 174.217, 160.512, 145.592, 138.157, 135.176, 130.506, 128.936, 128.146, 125.455, 118.836, 51.178, 33.414, 27.653, 25.385, 23.942, 22.363. ESI-MS (*m*/*z*) Calcd (found): 258.1 (258.1) for $[M]^+$.

Resin-Bound 2-Amino-ethylamine (6). To 2-chlorotrityl alcohol resin (**5**) (1 g, 1.37 mmol/g) presuspended in dichloromethane (10 mL) in a 20 mL vial for 10 min was added thionyl chloride (1 mL, 10 equiv), and the vial was shaken for 2 h at room temperature. The resin was filtered

and washed with dichloromethane and acetonitrile alternatively 5 times and dried in vacuum. The resin was then suspended in dichloromethane (10 mL) for 10 min, and ethylenediamine (400 μL, 4 equiv) was added; the mixture was shaken at room temperature for 3 h. The resin was filtered and washed with dichloromethane and methanol alternatively 5 times and dried in vacuum.

Resin-Bound 2-Methylquinolinium (7). A mixture of DIPEA (800 μL, 3 equiv), HATU (1.6 g, 3 equiv), and **4** (1.4 g, 3 equiv) in 60 mL of dichlormethane and 20 mL of DMF was shaken at room temperature for 30 min. To this solution was added resin **6** (1 g, 1 equiv), and the mixture was shaken at room temperature overnight. The resin was filtered and washed with dichloromethane and methanol alternatively 5 times and dried in vacuum. To an aliquot of the resin was added 100 μL of 5% trifluoroacetic acid/dichloromethane cleavage cocktail solution, and the mixture was shaken for 15 min. The solution was subjected to LC-MS. ESI-MS (*m*/*z*) Calcd (found): 300.2 (300.2) for $[M]^+$.

Resin-Bound Quinaldinium Dyes (8). To resin **7** (10 mg, 1 equiv) was added each aldehyde (10 equiv) in 1-methyl-2-pyrrolidinone (NMP, 300 μL) solution and pyrrolidine (2 μL). The reaction mixture was shaken in dark and under a positive pressure of nitrogen for 24 h. The resin was filtered and washed with DMF (5 times), alternatively dichloromethane and methanol (5 times), and dichloromethane (5 times) and dried in vacuum.

Cleavage of Quinaldinium Dyes from Resin (9). Resin **8** (10 mg) was suspended in 5% trifluoroacetic acid/dichloromethane cleavage cocktail solution (0.5 mL) and shaken for 15 min. The resin was filtered off and washed with dichloromethane (1 mL) and methanol (1 mL). The solutions were collected and evaporated to dryness to obtain the quinaldinium dyes.

General Procedure for Fluorescence Measurement. All fluorescence measurements were performed with on a Gemini XS fluorescent plate reader with Grainer 96-well black polypropylene plate. Briefly, fluorescence compounds were prepared as DMSO stock solutions. One microliter of the DMSO solution was mixed with 100 μL of corresponding concentrations of analytes in 10 mM HEPES buffer (pH 7.4) or specified solvents. The binding affinity was obtained by fitting fluorescence titration experiment data with equation

$$F = F_0 + (F_{max} - F_0) \times \frac{([L_0] + [M_0] + K_d) - \sqrt{([L_0] + [M_0] + K_d)^2 - 4[L_0][M_0]}}{2[L_0]} \quad (1)$$

in which F_0 and F_{max} are the minimum and maximum fluorescence fold change, respectively, and $[L_0]$, $[M_0]$, K_d are the dye concentration, protein concentration, and dissociation constant, respectively.

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Supporting Information Available. LC-MS and fluorescence–excitation/emission data of QN library members, synthesis of QN-33 and QN-49 analogues, spectral data of QN-33 and QN-49, and titration experiment data with QN-33 and QN-49. This information is available free of charge via the Internet at http://pubs.acs.org.

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