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Fluorescein-based amino acids for solid phase synthesis of fluorogenic protease substrates

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Abstract—An efficient synthesis of new type fluorescent amino acids is described. The Fmoc-protected dyes can be prepared in a four-step procedure with ~30% overall yield from aminofluoresceins and other inexpensive commercially available precursors. The dyes are much more photostable compared to fluorescein and exhibit constant pH-independent fluorescence that is advantageous in biological applications. The Fmoc-protected fluorescent amino acids are ready for use in solid phase peptide synthesis. As a proof of concept, a fluorogenic papain substrate was synthesized and employed for on-bead detection of the protease activity. By using a novel technique for quantitative analysis of bead fluorescence, a ~2.7-fold increase in mean bead brightness was measured and was attributed to substrate cleavage by papain. The new type fluorescent amino acids seem to be a promising tool for the synthesis of fluorescent peptide ligands and fluorogenic protease substrates.

1. Introduction

Proteases perform essential functions in all living organisms. The human genome data suggest that more than 550 genes encode proteases or protease homologues.[1–3] Besides mediating nonspecific protein hydrolysis, proteases also act as key post-translational modificators that perform highly selective, and efficient, cleavage of specific cellular substrates.[3] Therefore, an improved understanding of protease functions not only will provide insight into biological systems but will likely provide new important therapeutic targets.

Fluorogenic substrates allow for the continuous kinetic analysis of proteases and are useful reagents for screening potential protease inhibitors and for determining protease substrate specificity.[4–13] Two types of fluorogenic substrates are commonly used: one having an amino-fluorophore in the P1′ position,[5–8] and the other containing a fluorescence donor-acceptor couple.[9–13] Typically, P1′-fluorogenic substrates are based on amino-methylcoumarin, AMC[5] or ACC,[6] as well as on red-shifted amino-fluorophores such as rhodamine 110[7] or cresyl violet.[8] However, these substrates have limited value in studying proteases, which are sensitive toward substitution in P′ positions. For such specific endopeptidases, the complex fluorogenic substrates containing fluorescence donor–acceptor couple (two fluorophores or fluorophore-quencher) are generally employed.[9–13] These substrates can accommodate a peptide sequence mapping both P and P′ sites. The synthesis of such substrates is rather difficult and involves fluorescent dyes, which either are very expensive[10,11] or fluoresce in the UV region[12] not appropriate for inhibitor screening. Indeed, absorbance (inner-filter effect) and autofluorescence of library compounds are well-known problems when using UV-excited fluorophores for high-throughput screening of chemical libraries.[13] The stability toward chemical environment and photobleaching is another concern of the fluorophores. Peptide labeling with a dye is usually performed at the end of peptide assembly prior to cleavage from solid support that often requires the use of a complicated orthogonal protection strategy.[11] There is thus a pressing need to develop fluorescent amino acid monomers that could be incorporated in selected position during solid phase synthesis. To our knowledge, only one paper, which describes a synthesis of fluorescein-conjugated lysine, has addressed this issue.[14]

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We report herein a general method for the synthesis of fluorescent amino acids from commercially available fluorescein derivatives. These fluorescent compounds demonstrate excellent chemical- and photostability and can be used directly in solid phase peptide synthesis. As a proof of concept, we synthesized a fluorogenic papain substrate based on one of these fluorophores using Methyl red as a fluorescence quencher and demonstrated its application for directly measuring the papain activity on TentaGel beads.

2. Results and discussion

2.1. Design and synthesis of fluorescein-based amino acids

Because of their commercial availability and low cost, fluorescein and its derivatives provide an attractive starting material for the synthesis of fluorescent amino acids. One obvious way to the modification of fluorescein derivatives is a conjugation of the 2′ carboxylic group with a nucleophile. However, such functionalization chemistry is complicated by tautomeric equilibrium between a locked non-fluorescent spiro-lactone form and an open fluorescent quinoid form as well as by ionic equilibrium of multiple (de)protonated forms of fluorescein.[17,18,21] This results in a highly heterogeneous chemical composition, which depends on pH and solvent. Of note, the multiplicity of fluorescein forms having different spectral properties[17,18,21] also limits its application as a fluorescent probe.[22] Two methods to avoid the tautomerization problem described in the literature are a coupling of the 2′ carboxylic group to secondary amines[23,24] and its esterification.[14,16] While the formation of 2′ secondary amide of *N*,*N*,*N*′,*N*′-tetrasubstituted rhodamines proceeds with a relatively high yield,[24] the reaction is less efficient with fluorescein and requires an additional activation step.[23] On the other hand, the esterification of the 2′ carboxylic group of fluorescein has been reported to give an excellent yield.[16] Consequently, we chose to use esterification in our synthesis. Furthermore, we thought it appropriate to start from commercially available 5′- and 6′-aminofluorescein derivatives in the synthesis of fluorescent amino acids.

As shown in Figure 1, the esterification of aminofluoresceins in refluxing acidic ethanol afforded the corresponding esters **3** and **4** with >85% yield. A new carboxylic moiety was then introduced by alkylation of aminofluorescein ethyl ester with *tert*-butyl bromoacetate

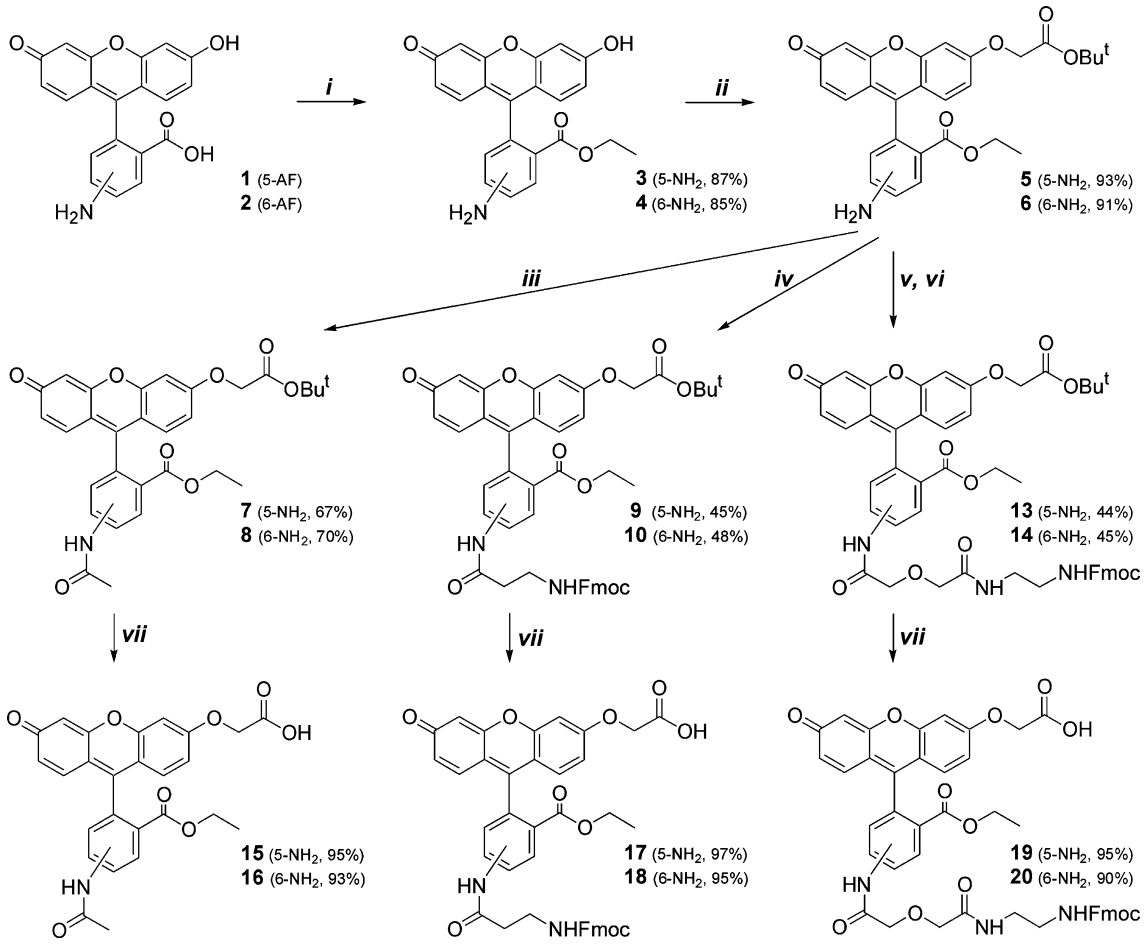

Figure 1. Synthetic scheme for Fmoc-protected fluorescent amino acids. Reagents: (i) H_2SO_4, EtOH; (ii) $BrCH_2COOBu^t$, K_2CO_3, DMF; (iii) Ac_2O, DIEA, DCM; (iv) Fmoc-β-Ala, DCC, DCM; (v) diglycolic anhydride, DMAP, DIEA, DCM; (vi) $NH_2(CH_2)_2NHFmoc$, DCC, DCM; (vii) TFA, DCM.

(Fig. 1(ii)). The alkylation proceeded selectively on the 3-phenol and did not involve the amino group. First we tried to obtain Fmoc-protected amino acid derivatives directly from the esters **5** and **6**. However, the acylation of these compounds with either Fmoc chloride or Fmoc-hydroxysuccinimide ester failed to produce appreciable yields of the desired Fmoc-amino acid esters. The reactions proceeded with low levels of conversion and the silica gel purification was ineffective due to the instability of the products. On the other hand, we have found that both 5′- and 6′-amino groups of aminofluoresceins could be acylated in reasonable yield with carboxylic acid anhydrides such as acetic acid anhydride (compounds **7** and **8**; Fig. 1(iii)). Therefore, Fmoc-protected amino acid esters **9** and **10** were synthesized by acylation of the compounds **5** and **6** with *N*-Fmoc-β-alanine and DCC as a coupling agent (Fig. 1(iv)). The reaction proceeded very slowly (~2 days), probably due to steric hindrance of the reacting compounds and low reactivity of the amino group, and, after silica gel chromatography, afforded the pure products with >45% yield. In parallel, the compounds **13** and **14** were synthesized with ~45% overall yield by a two-step procedure, which involves the acylation of **5** and **6** with diglycolic anhydride followed by the coupling of the resulting acids **11** and **12** to Fmoc-ethylene diamine (see Section 4 and Fig. 1(v and vi)). Compared with **9** and **10** the latter compounds have a longer polar linker between Fmoc-protected amino group and fluorophore that is often required in biological applications. Finally, Fmoc-protected fluorescent amino acids **15**–**20** were produced in a nearly quantitative yield by TFA hydrolysis of the corresponding *tert*-butyl esters **7**–**10**, **13**, and **14** (Fig. 1(vii)).

2.2. Physico-chemical characterization of the dyes

Low photostability and dependence of the fluorescence on pH are the two most important drawbacks limiting biological applications of fluorescein analogues.[22] We therefore examined the photochemical properties of the synthesized fluorescein derivatives.

The UV–vis absorbance spectra of compounds **15**–**20** exhibit two absorbance maxima at 456 and 481 nm (Fig. 2A). The maximum at 481 nm corresponds to the absorbance maximum of fluorescein at 490 nm, suggesting that the same light sources can be used for excitation of these fluorophores. The shorter wavelength maximum at 456 nm is characteristic of 3-*O*-fluorescein ethers[25] and has been observed in absorbance spectra of various fluorescein ethers having 2′-ester[14,16] or 2′-secondary amide residues.[23] The molar extinction coefficients determined at 456 nm for **15**–**20** ($2.0–2.4 \times 10^4$ L mol^{-1} cm^{-1}) are also close to reported values for 3-*O*-alkyl fluorescein 2′-esters[16] ($2.0–3.5 \times 10^4$ L mol^{-1} cm^{-1}). This means that the presence of the 5′- or 6′-amido groups does not significantly affect the electronic properties of 3-*O*-alkyl fluoresceins.

The fluorescence emission spectra of compounds **15**–**20** are broadened and redshifted (λ_{max} = 520 nm) relative to that of fluorescein (λ_{max} = 512 nm) (Fig. 2A). The

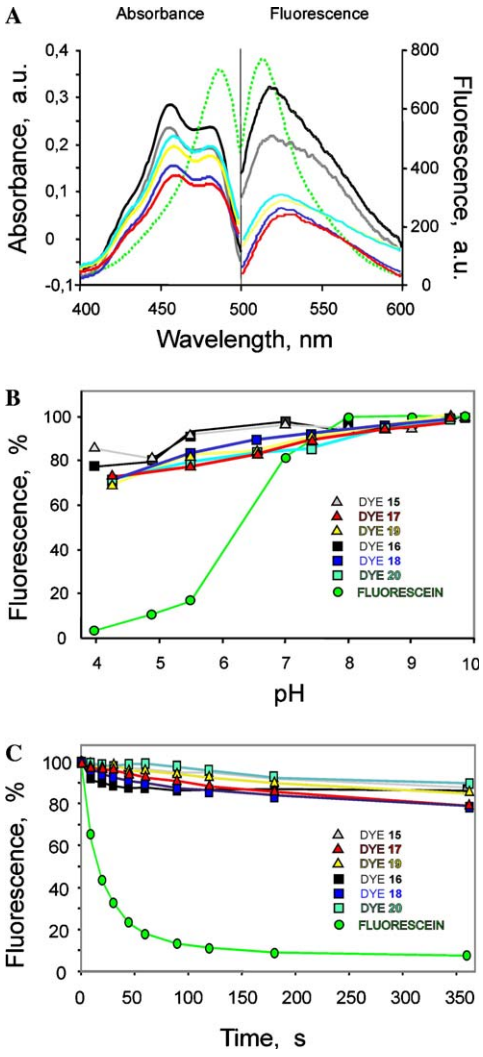

Figure 2. Photochemical properties of new dyes. (A) Absorbance and fluorescence spectra of dye **15** (grey), dye **16** (black), **17** (red), **18** (blue), **19** (yellow), **20** (cyan), and fluorescein (dotted green). The fluorescence quantum yields were measured to be 0.19 (**15**), 0.18 (**16**), 0.07 (**17**), 0.04 (**18**), 0.12 (**19**), and 0.07 (**20**). (B) pH-dependence of the fluorescence intensity of dye **15**–**20** and fluorescein (the color code is the same as in (A)). (C) Dye photobleaching.

fluorescence quantum yields determined for **15** (ϕ = 0.19) and for **16** (ϕ = 0.18) were notably lower than the quantum yield of fluorescein dianion (ϕ = 0.92–0.93),[17,18,26] and were consistent with the values reported for other 3-*O*-alkyl fluorescein ethers (ϕ = 0.18–0.31).[14,16] The broadening of emission spectra and decrease in quantum efficiency is probably due to alteration of D_{2h} molecular symmetry of xanthene moiety in these compounds,[27] which results in changes in molecule vibrational modes[27] and in increased rate of non-radiative internal conversion from excited to ground states. The loss of D_{2h} symmetry in fluorescein monoanion results in a similar broadening of emission spectrum and in a comparable drop in quantum yield (ϕ = 0.25–0.37).[17,18] The fluorescence quantum yields of Fmoc-protected amino acids **17**–**20** (ϕ = 0.04–0.12, see Fig. 2A caption) are noticeably lower than those of the dyes **15** and **16**. This may result from fluorescence

quenching induced by Fmoc chromophore. Indeed, Fmoc-deprotection produced a significant increase in fluorescence of the compounds **17**–**20** (not shown).

Consistent with the previous studies on 3-*O*-alkyl fluorescein 2′-esters,[14,16,23] the fluorescence and the quantum yields determined for compounds **15**–**20** are virtually pH-independent throughout the range of pH 4.0–10.0 (Fig. 2B). This is explained by the absence of ionic equilibrium in 3-*O*-alkyl fluorescein derivatives trapped in a tautomeric quinoid form by 2′-esterification. The stability of 2′-ester toward an alkaline hydrolysis was also checked by measuring the absorbance and fluorescence spectra. No significant changes were observed after incubation of the fluorophores **15** and **16** for several hours in aqueous pH 9.8 buffer. The pH independence exhibited by these fluorophores is advantageous in applications requiring quantification and comparison of fluorescence intensity in different environments, as, for example, for the fluorescence measurement in acidic intracellular compartments,[28] or in the studies of the pH-dependence of enzymatic activity with fluorogenic substrates.[29]

In fluorescence measurements such as immunofluorescence, dealing with small amounts of fluorescent molecules and/or small sample volume, the irreversible destruction or photobleaching of the excited fluorophore becomes the factor limiting fluorescence detectability. We have developed an assay allowing comparison of the photostability of fluorophores under the condition of signal acquisition with a fluorescent microscope. The sample was placed in a well on the surface of a glass slide containing 40 circular wells of 2 mm diameter, separated by 30 μm-thick Teflon coating. The well was then enclosed with a coverslip producing a cylindrical sample chamber of ~380 nL volume. The slide was mounted on the microscope stage and the well was irradiated with light using standard 'fluorescein' filters set. In order to prevent a heterogeneous photobleaching and associated macrodiffusion phenomena, the microscope objective was adjusted to irradiate an entire sample chamber. The resulting light intensity at the chamber level was measured to be ~6 mW/mm^2. The fluorescence images of the well were taken at different intervals and the mean fluorescence intensity was calculated using IMSTAR software.[15] As seen in Figure 2C, under these conditions fluorescein was rapidly bleached with a characteristic time of 50% decay $t_{1/2} \sim 14$ s. Compounds **15** and **16** demonstrated remarkable photostability: only a ~10% decrease in fluorescence was observed for both fluorophores after 6 min of irradiation. This result is corroborated by a previous observation that under an exposure to white light some 3-*O*-alkyl 2′-amide fluorescein derivatives demonstrate up to 10-fold increased photostability compared to fluorescein.[23] The various photochemical reactions leading to fluorescein photobleaching have been investigated and described in considerable detail.[30] In all cases, photobleaching originates from the triplet excited state, which is created from the singlet state S_1 via an excited-state process called intersystem crossing. It seems probable that the same cause, which results in a drop in quantum yield, i.e., the alteration of D_{2h} molecular symmetry of xanthene moiety and reorganization of molecule vibrational modes, may be responsible for the increased photostability of fluorescein 3-O-ethers. Consistent with this hypothesis, it has been shown that the photostability of 3-*O*-alkyl 2′-amide fluorescein derivatives results mainly from the 3-etherification of the xanthene ring.[23]

2.3. Solid phase synthesis of fluorogenic substrate and measurement of proteolytic activity

To demonstrate the utility of the synthesized fluorophores for the solid phase synthesis of fluorogenic substrates, we have designed a fluorogenic substrate for cysteine protease papain. The substrate contains fluorescein amino acid **18** and Methyl red (MR) chromophore separated by peptide sequence GGFGLG (Fig. 3). This sequence has been described as a good papain substrate with a cleavage taking place at the G–L bond.[31] The specificity for the papain is determined by the phenylalanine at the P2 position and the leucine at the P1′ position.[32] The MR dye has been shown to quench efficiently the fluorescence of fluorescein derivatives and is widely used in the design of self-quenched oligonucleotide probes for real-time PCR.[33] The substrate was synthesized by Fmoc-chemistry on TentaGel resin (20 μm beads). For reasons of simplicity and potential high-throughput applications, we have evaluated the validity of on-bead protease screening. The major concern of such heterophase screening assay is the permeability of resins to biomolecules, which determines the accessibility of the resin-bound substrate for the enzymes. Although TentaGel resin has been shown not to be optimal for on-bead enzymatic screening,[31] it has some important advantages such as excellent swelling properties in organic and aqueous media, mechanical stability, and narrow size distribution.[34,35] Furthermore, we have found that the introduction of a PEG ($n = 7$) linker between the peptide and TentaGel functional group (Fig. 3) rendered the fluorogenic substrate more accessi-

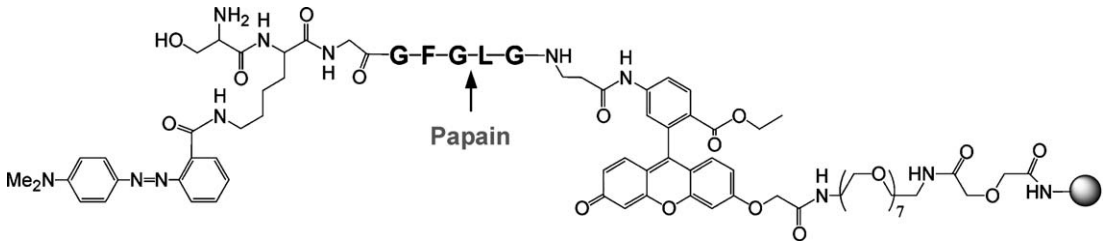

Figure 3. Structure of fluorogenic papain substrate.

ble for protease cleavage. The fluorophore **18** was coupled first after the linker followed by peptide sequence and the quencher MR, which was coupled to the side chain of the lysine at the end of the synthesis (Fig. 3).

Regardless of the biochemical model used, the on-bead screening assay requires methods for determination and analysis of the 'positive' beads. In our case, a peptide sequence susceptible to proteolysis should be cleaved from the solid phase support, thereby releasing the MR quenching moiety and resulting in an enhancement of the fluorescence of the resin-bound fluorophore. Therefore, to detect a proteolytic activity, the fluorescent proteolyzed beads should be distinguished from the control beads, which have not been exposed to a protease. Curiously, the majority of on-bead screening methods employ the visual assessment of the beads under a standard fluorescence microscope.[35] The subjective nature of this analysis does not allow us to discriminate a small difference in bead brightness and limits seriously the sensitivity of the method. This is particularly important when using quenched fluorogenic substrates, which possess significant intrinsic fluorescence. Furthermore, TentaGel, like many polystyrene-based resins, exhibits high-level, broad-wavelength autofluorescence complicating the analysis.[34] Finally, even under the same conditions, the population of beads shows significant brightness heterogeneity because of the bead-to-bead variation in polymer density, in functional group substitution, and in substrate loading.[35] All these considerations suggest a need for quantitative analysis of the distribution of bead brightness. The bead population can be analyzed and the positive beads isolated with a fluorescence-activated cell sorter;[35,36] however, the method becomes cumbersome when multiple bead samples have to be analyzed. Some modern techniques employed in genotyping and diagnostics, such as Luminex microfluidic technology[37] and Illumina fiber optic Bead Array,[38] provide quantitative measurements of bead fluorescence, but require special equipment and have not been tested for enzymatic screening.

We have developed a rather simple method to measure the distribution of bead fluorescence. The bead suspension was spotted on the surface of a microscope slide by using a piezo-electric dispenser. The 40-well patterned glass slides with 30 μm-thick Teflon frame were used to delimit the bead solution, although a higher density format (several thousands of hydrophilic wells patterned on a perfluorated glass surface[39]) can also be used. The well was then enclosed with a coverslip as described above for photobleaching assays. The measurements can also be performed in arrayed droplets without covering, but glycerol should be added to the bead suspension to prevent evaporation. To avoid the clogging of the piezoelectric pipette, the beads were maintained in suspension by sonication pulses. Under these conditions, rather homogeneous dispensing of the beads 5–500 per droplet, depending on the bead concentration, can be achieved. The beads were preincubated with protease prior to spotting or protease was added afterwards to the arrayed beads.

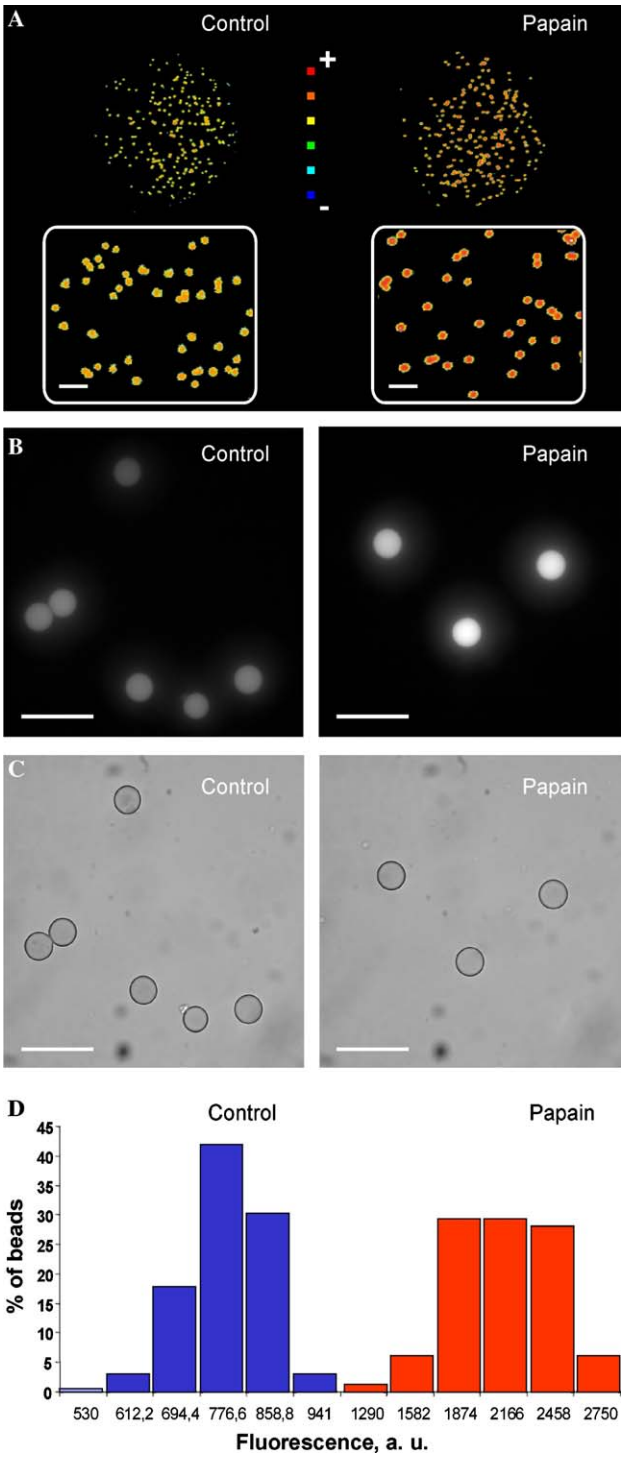

Figure 4. On-bead assay for proteolytic activity. The 20 μm TentaGel beads carrying the fluorogenic papain substrate (Fig. 3) were incubated with 0.5 μM papain (marked 'papain'), with mock solution (marked 'control') or with NEM-inhibited papain (results indistinguishable from 'control'). After 2 h, the beads were spotted on Super-Teflon glass slide and analyzed. (A) 5 μm-resolution scanner images of two bead populations. The relative fluorescence intensity scale is shown by color squares. The insets show 3× zoom images of bead populations. The white bars on the images A–C represent 50 μm. (B) Fluorescence micrographs of the beads obtained with fluorescein filter set. (C) Bright field images of the same bead populations as in B. (D) Histogram showing the distribution of the bead brightness in control and in papain-treated bead populations (200 beads in each case).

After incubation, the proteolysis of the bead-bound substrate was measured by using a microarray scanner or a fluorescent microscope. As shown in Figure 4A the beads incubated with papain appeared significantly brighter on scanner images than the control beads. Even though the scanning did not produce a high resolution image of each bead, it allowed us to obtain a quantitative comparison of two bead samples (not shown). Automated fluorescence microscopy provides even more precise data on brightness variation within the bead population (Fig. 4B and C). Due to narrow size distribution of TentaGel beads, the proteolytic activity can be characterized by a histogram representing the percentage of beads having fluorescence in the given intensity range (IMSTAR software, Fig. 4D). The obtained data are quantitative and allow detection of small changes in bead brightness, which is impossible by a visual examination. Thus, the analysis of a 200 beads population showed that the incubation of the beads with 0.5 μM solution of papain for 2 h results in ~2.7-fold increase in mean fluorescence with some increase in standard deviation (SD) of brightness distribution (Fig. 4D). This rise in bead brightness was not observed with *N*-ethylmaleimide-inhibited papain, suggesting that it results from the enzymatic cleavage of resin-bound substrate. Therefore, the increase in brightness heterogeneity measured through an increase of SD may be explained by the bead-to-bead variation in substrate cleavage.

3. Conclusion

We describe here cost-efficient synthesis, photochemical characterization, and application of new fluorescent amino acids. Being synthesized from inexpensive fluorescein derivatives, these fluorophores have several important advantages compared to fluorescein such as significantly increased photostability and pH-independent quantum yield. The synthesized fluorescent amino acids are ready for use in solid phase organic synthesis and can be employed directly in the synthesis of fluorescent peptide ligands and fluorogenic protease substrates. Collectively, these dyes seem to be very promising for various biological applications.

4. Experimental

4.1. Materials

All chemicals were from Fluka (Sigma–Aldrich Chimie, St. Quentin Fallavier, France). TentaGel beads (20 μm) were from Rapp Polymere GmbH (Tübingen, Germany). Column chromatography was carried out on Merck Silica Gel 60. Thin-layer chromatography was performed with Silica Gel 60 F_{254} plates. ^{1}H NMR spectra were recorded on a Bruker (300 MHz); high resolution mass spectra (HRMS) were recorded on a Finnigan MAT 8200 spectrometer (50–100 °C, 70 eV). UV–vis spectra were acquired with a Varian Cary 300 Scan spectrophotometer and fluorescence emission spectra with a Perkin-Elmer LS-50B luminescence spectrometer. The sciFLEXARRAYER piezoelectric dispensing system (Scienion, Berlin, Germany) was used for spotting of beads. Olympus BX51 microscope equipped with a Hamamatsu ORCA R camera and an Olympus U-RFL-T lamp was used for photobleaching study and beads imaging. Bead scanner images were obtained with a GeneTAC LS IV scanner (Genomic Solutions, Ann Arbor, MI, USA)). Microscope images were analyzed quantitatively using IMSTAR software (Imstar, Paris, France,[15]), and scanner images were analyzed with Genepix Pro 4.0 software (Axon Instruments, Union City, CA, USA).

4.2. 5-Amino-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 3)

H_2SO_4 (266 μL, 5.0 mmol) was added dropwise to the suspension of 5-aminofluorescein **1** (347 mg, 1.0 mmol) in 10 mL of EtOH at room temperature. After stirring at reflux for 24 h, EtOH was evaporated and the resulting mixture was diluted with $CHCl_3$. Solid $NaHCO_3$ was added to the solution until gas evolution ceased. Heterogeneous mixture was filtered and the organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was subjected to silica gel chromatography with EtOAc/hexane (1:1), MeOH/DCM (1:9), giving 326 mg of dye **3** (87%). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.80 (t, 3H, J = 7.5 Hz), 3.87 (q, 2H, J = 7.5 Hz), 5.86 (br s, 2H), 6.55 (m, 4H), 6.90 (m, 3H), 7.04 (m, 1H), 7.33 (m, 1H). HRMS calcd. for $C_{22}H_{17}NO_5$ $(M)^+$ 375.1107, found 375.1103.

4.3. 4-Amino-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 4)

Dye **4** was obtained by the same method from 6-aminofluorescein **2** (347 mg, 1.0 mmol) with 85% yield (319 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.83 (t, 3H, J = 7.5 Hz), 3.83 (q, 2H, J = 7.5 Hz), 5.85 (br s, 2H), 6.47 (m, 1H), 6.78 (m, 1H), 7.02 (m, 2H), 7.07 (m, 2H), 7.34 (m, 2H), 7.92 (m, 1H). HRMS calcd. for $C_{22}H_{17}NO_5$ $(M)^+$ 375.1107, found 375.1104.

4.4. 5-Amino-2-(6-*tert*-butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 5)

$BrCH_2COOBu^t$ (215 mg, 1.1 mmol) was added to the mixture of compound **3** (375 mg, 1.0 mmol) and K_2CO_3 (150 mg, 1.1 mmol) in 5 mL of DMF at room temperature. After stirring for 3 h, the reaction mixture was diluted with H_2O and extracted with ethyl acetate. The organic phase was washed with 1 M $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was subjected to silica gel chromatography with EtOAc/hexane (1:1), MeOH/DCM (1:9), giving 455 mg of dye **5** (93%). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.82 (t, 3H, J = 7.5 Hz), 1.42 (s, 9H), 3.88 (m, 2H), 4.85 (s, 2H), 5.87 (br s, 2H), 6.20 (m, 1H), 6.37 (m, 1H), 6.82–7.05 (m, 5H), 7.14 (m, 1H), 7.35 (s, 1H). HRMS calcd. for $C_{28}H_{27}NO_7$ $(M)^+$ 489.1788, found 489.1787.

4.5. 4-Amino-2-(6-*tert*-butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 6)

Dye **6** was synthesized by the same method from compound **4** (375 mg, 1.0 mmol) with 91% yield (445 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 1.42 (s, 9H), 3.84 (m, 2H), 4.85 (s, 2H), 6.22 (m, 1H), 6.30 (br s, 2H), 6.37 (m, 1H), 6.44 (m, 1H), 6.77 (m, 1H), 6.88–6.96 (m, 3H), 7.15 (m, 1H), 7.90 (m, 1H). HRMS calcd. for $C_{28}H_{27}NO_7$ $(M)^+$ 489.1788, found 489.1789.

4.6. 5-Acetylamino-2-(6-*tert*-butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 7)

Ac_2O (47 μL, 0.5 mmol) was added to the solution of amine **5** (98 mg, 0.2 mmol) and DIEA (88 μL, 0.5 mmol) in 5 mL of DCM at room temperature. After stirring overnight, DCM was evaporated, the residue was diluted with H_2O and EtOAc, and the mixture was stirred. Organic layer was separated and washed with 1 M HCl, 1 M $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was subjected to silica gel chromatography with EtOAc/hexane (1:1), MeOH/DCM (1:9), giving 71 mg of dye **7** (67%). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 1.42 (s, 9H), 2.12 (s, 3H), 3.94 (m, 2H), 4.86 (s, 2H), 6.22 (m, 1H), 6.38 (m, 1H), 6.85–6.95 (m, 3H), 7.13 (m, 1H), 7.46 (m, 1H), 8.08 (m, 1H), 8.41 (s, 1H), 10.46 (s, 1H). HRMS calcd. for $C_{30}H_{29}NO_8$ $(M)^+$ 531.1894, found 531.1892.

4.7. 4-Acetylamino-2-(6-*tert*-butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 8)

Dye **8** was obtained by the same method from amine **6** (98 mg, 0.2 mmol) with 70% yield (74 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 1.41 (s, 9H), 2.08 (s, 3H), 3.85 (m, 2H), 4.93 (s, 2H), 6.41 (m, 1H), 6.54 (m, 1H), 7.95–7.02 (m, 3H), 7.29 (m, 1H), 7.72 (m, 1H), 7.88 (m, 1H), 8.19 (m, 1H), 10.52 (s, 1H). HRMS calcd. for $C_{30}H_{29}NO_8$ $(M)^+$ 531.1894, found 531.1890.

4.8. 2-(6-*tert*-Butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-5-[3-(9*H*-fluoren-9-ylmethoxy-carbonyl-amino)-propionylamino]-benzoic acid ethyl ester (dye 9)

DCC (412 mg, 2.0 mmol) was added to the solution of Fmoc-β-alanine (622 mg, 2.0 mmol) in 10 mL DCM at room temperature, after stirring for 30 min a solution of amine **5** (489 mg, 1.0 mmol) in 5 mL DCM was added to the mixture. After stirring for 2 days, DCM was evaporated, the residue was diluted with H_2O, and the mixture was extracted with ethyl acetate. The organic phase was washed with 1 M HCl, 1 M $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was subjected to silica gel chromatography with EtOAc/hexane (1:1), EtOAc, giving 352 mg of dye **9** (45%). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 1.41 (s, 9H), 2.55 (m, 2H), 3.35 (m, 2H), 3.96 (m, 2H), 4.21 (m, 1H), 4.60 (m, 2H), 4.92 (s, 2H), 6.18 (m, 1H), 6.22 (s, 1H), 6.38 (m, 1H), 6.49–6.61 (m, 3H), 6.69 (m, 1H), 6.96 (m, 1H), 7.15 (m, 1H), 7.26–7.39 (m, 4H), 7.64 (m, 2H), 7.86 (m, 1H), 7.88 (m, 2H), 10.48 (s, 1H). HRMS calcd. for $C_{46}H_{42}N_2O_{10}$ $(M)^+$ 782.2840, found 782.2842.

4.9. 2-(6-*tert*-Butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-4-[3-(9*H*-fluoren-9-ylmethoxycarbonyl-amino)-propionylamino]-benzoic acid ethyl ester (dye 10)

Dye **10** was obtained by the same method from amine **6** (489 mg, 1.0 mmol) with 48% yield (375 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.86 (t, 3H, $J = 7.5$ Hz), 1.42 (s, 9H), 2.38 (m, 2H), 3.39 (m, 2H), 3.92 (m, 2H), 4.19 (m, 1H), 4.58 (m, 2H), 4.85 (s, 2H), 6.30 (s, 1H), 6.42 (m, 1H), 6.78 (m, 1H), 6.84–6.97 (m, 3H), 7.17 (m, 1H), 7.23 (m, 2H), 7.25–7.42 (m, 4H), 7.64 (m, 2H), 7.87 (m, 2H), 10.53 (s,1H). HRMS calcd. for $C_{46}H_{42}N_2O_{10}$ $(M)^+$ 782.2840, found 782.2841.

4.10. 2-(6-*tert*-Butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-5-(2-carboxymethoxy-acetyl-amino)-benzoic acid ethyl ester (dye 11)

Diglycolic anhydride (23 mg, 0.2 mmol) was added to the solution of amine **5** (98 mg, 0.2 mmol), DIEA (35 μL, 0.2 mmol) and DMAP (24 mg, 0.2 mmol) in 5 mL of DCM at room temperature. After stirring overnight, DCM was evaporated, the residue was diluted with H_2O and extracted with ethyl acetate. The organic phase was washed with 1 M HCl, brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was diluted with Et_2O, and precipitate was filtered off, washed with Et_2O, and dried to afford 109 mg of dye **11** (90%). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.83 (t, 3H, $J = 7.5$ Hz), 1.42 (s, 9H), 3.95 (m, 2H), 4.22 (s, 2H), 4.24 (s, 2H), 4.87 (s, 2H), 6.23 (m, 1H), 6.39 (m, 1H), 6.84–6.92 (m, 3H), 7.17 (m, 1H), 7.44 (m, 1H), 8.12 (m, 1H), 8.52 (m, 1H), 10.42 (s, 1H). HRMS calcd. for $C_{32}H_{31}NO_{11}$ $(M)^+$ 605.1898, found 605.1894.

4.11. 2-(6-*tert*-Butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-4-(2-carboxymethoxy-acetylamino)-benzoic acid ethyl ester (dye 12)

Dye **12** was obtained by the same method from amine **6** (98 mg, 0.2 mmol) with 92% yield (112 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.87 (t, 3H, $J = 7.5$ Hz), 1.42 (s, 9H), 3.93 (m, 2H), 4.11 (s, 2H), 4.18 (s, 2H), 4.86 (s, 2H), 6.24 (m, 1H), 6.39 (m, 1H), 6.83–6.96 (m, 3H), 7.19 (m, 1H), 7.76 (m, 1H), 8.03 (m, 1H), 8.18 (m, 1H), 10.48 (s, 1H). HRMS calcd. for $C_{32}H_{31}NO_{11}$ $(M)^+$ 605.1898, found 605.1897.

4.12. 2-(6-*tert*-Butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-5-(2-{[2-(9*H*-fluoren-9-ylmethoxy-carbonyl-amino)-ethylcarbamoyl]-methoxy}-acetylamino]-benzoic acid ethyl ester (dye 13)

DCC (41 mg, 0.2 mmol) was added to the solution of acid **11** (121 mg, 0.2 mmol) in 5 mL of DCM at room temperature, after stirring for 30 min, the solution of Fmoc-ethylenediamine (56 mg, 0.2 mmol) in 2 mL of

DCM was added to the mixture. After stirring overnight, DCM was evaporated, the residue was diluted with H_2O and extracted with ethyl acetate. The organic phase was washed with 1 M HCl, 1 M $NaHCO_3$, brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to silica gel chromatography with EtOAc/hexane (1:1), EtOAc, MeOH/DCM (1:20), giving 101 mg of dye **13** (58%). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 1.42 (s, 9H), 3.11 (m, 2H), 3.19 (m, 2H), 3.92 (m, 2H), 4.08 (s, 2H), 4.16 (m, 1H), 4.21 (s, 2H), 4.28 (m, 2H), 4.86 (s, 2H), 6.24 (m, 1H), 6.37 (m, 1H), 6.80–6.97 (m, 3H), 7.19 (m, 1H), 7.27–7.42 (m, 4H), 7.65 (m, 2H), 7.64 (m, 1H), 7.88 (m, 2H), 7.82 (m, 1H), 8.05 (m, 1H), 10.49 (s, 1H). HRMS calcd. for $C_{49}H_{47}N_3O_{12}$ $(M)^+$ 869.3160, found 869.3164.

4.13. 2-(6-*tert*-Butoxycarbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-4-(2-{[2-(9*H*-fluoren-9-ylmethoxycarbonyl-amino)-ethylcarbamoyl]-methoxy}-acetylamino]-benzoic acid ethyl ester (dye 14)

Dye **14** was obtained by the same method from acid **12** (121 mg, 0.2 mmol) with 64% yield (111 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.86 (t, 3H, $J = 7.5$ Hz), 1.42 (s, 9H), 3.07 (m, 2H), 3.15 (m, 2H), 3.91 (m, 2H), 4.04 (s, 2H), 4.16 (m, 1H), 4.19 (s, 2H), 4.26 (m, 2H), 4.85 (s, 2H), 6.22 (m, 1H), 6.48 (m, 1H), 6.82–6.95 (m, 3H), 7.17 (m, 1H), 7.27–7.41 (m, 4H), 7.64 (m, 2H), 7.77 (m, 1H), 7.86 (m, 2H), 8.00 (m, 1H), 8.16 (m, 1H), 10.49 (s, 1H). HRMS calcd. for $C_{49}H_{47}N_3O_{12}$ $(M)^+$ 869.3160, found 869.3162.

4.14. Removal of Boc-protection (dyes 15–20)

TFA (2.0 g, 1.3 mL) was added to the solution of ester in DCM (2.0 g, 1.5 mL) at room temperature. After 3 h, DCM and TFA were evaporated, the residue was solidified by addition of diethyl ester, washed thoroughly with the same solvent, and dried.

4.15. 5-Acetylamino-2-(6-carboxymethoxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 15)

Dye **15** was obtained by ester **7** (53 mg, 0.1 mmol) deprotection with 95% yield (45 mg). NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 2.12 (s, 3H), 3.96 (m, 2H), 4.87 (s, 2H), 6.22 (m, 1H), 6.39 (m, 1H), 6.84–6.92 (m, 3H), 7.17 (m, 1H), 7.40 (m, 1H), 8.04 (m, 1H), 8.40 (s, 1H), 10.45 (s, 1H). HRMS calcd. for $C_{26}H_{21}NO_8$ $(M)^+$ 475.1268, found 475.1269.

4.16. 4-Acetylamino-2-(6-carboxymethoxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid ethyl ester (dye 16)

Dye **16** was obtained from ester **8** (53 mg, 0.1 mmol) with 93% yield (44 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.83 (t, 3H, $J = 7.5$ Hz), 2.10 (s, 3H), 3.83 (m, 2H), 4.93 (s, 2H), 6.41 (m, 1H), 6.55 (m, 1H), 6.95–7.02 (m, 3H), 7.29 (m, 1H), 7.72 (m, 1H), 7.89 (m, 1H), 8.19 (m, 1H), 10.52 (s, 1H). HRMS calcd. for $C_{26}H_{21}NO_8$ $(M)^+$ 475.1268, found 475.1267.

4.17. 2-(6-Carbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-5-[3-(9*H*-fluoren-9-ylmethoxycarbonyl-amino)-propionyl-amino]-benzoic acid ethyl ester (dye 17)

Dye **17** was obtained from ester **9** (782 mg, 1.0 mmol) with 97% yield (704 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 2.58 (m, 2H), 3.39 (m, 2H), 3.93 (m, 2H), 4.32 (m, 1H), 4.66 (m, 2H), 4.87 (s, 2H), 6.21 (m, 1H), 6.40 (m, 1H), 6.50–6.59 (m, 3H), 6.69 (m, 1H), 6.70 (m, 1H), 7.15 (m, 1H), 7.26–7.39 (m, 4H), 7.64 (m, 2H), 7.88 (m, 1H), 7.89 (m, 2H), 10.49 (s, 1H). HRMS calcd. for $C_{42}H_{34}N_2O_{10}$ $(M)^+$ 726.2214, found 726.2212.

4.18. 2-(6-Carbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-4-[3-(9*H*-fluoren-9-ylmethoxycarbonyl-amino)-propionyl-amino]-benzoic acid ethyl ester (dye 18)

Dye **18** was obtained from ester **10** (782 mg, 1.0 mmol) with 95% yield (690 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 2.36 (m, 2H), 3.23 (m, 2H), 3.92 (m, 2H), 4.25 (m, 1H), 4.51 (m, 2H), 4.85 (s, 2H), 6.21 (m, 1H), 6.33 (s, 1H), 6.44 (m, 1H), 6.80 (m, 1H), 6.83–6.95 (m, 3H), 7.15 (m, 1H), 7.22 (m, 2H), 7.26–7.42 (m, 4H), 7.64 (m, 2H), 7.85 (m, 2H), 10.50 (s, 1H). HRMS calcd. for $C_{42}H_{34}N_2O_{10}$ $(M)^+$ 726.2214, found 726.2211.

4.19. 2-(6-Carbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-5-(2-{[2-(9*H*-fluoren-9-ylmethoxycarbonyl-amino)-ethyl-carbamoyl]-methoxy}- acetylamino]-benzoic acid ethyl ester (dye 19)

Dye **19** was obtained from ester **13** (87 mg, 0.1 mmol) with 95% yield (77 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.85 (t, 3H, $J = 7.5$ Hz), 3.07 (m, 2H), 3.15 (m, 2H), 3.95 (m, 2H), 4.04 (s, 2H), 4.16 (m, 1H), 4.19 (s, 2H), 4.26 (m, 2H), 4.85 (s, 2H), 6.32 (m, 1H), 6.43 (m, 1H), 6.94–7.00 (m, 3H), 7.26 (m, 1H), 7.24–7.36 (m, 4H), 7.64 (m, 2H), 7.78 (m, 1H), 7.86 (m, 2H), 8.01 (m, 1H), 8.28 (m, 1H), 10.49 (s, 1H). HRMS calcd. for $C_{45}H_{39}N_3O_{12}$ $(M)^+$ 813.2534, found 813.2537.

4.20. 2-(6-Carbonylmethoxy-3-oxo-3*H*-xanthen-9-yl)-4-(2-{[2-(9*H*-fluoren-9-ylmethoxycarbonyl-amino)-ethyl-carbamoyl]-methoxy}-acetylamino]-benzoic acid ethyl ester (dye 20)

Dye **20** was obtained from ester**14** (87 mg, 0.1 mmol) with 90% yield (73 mg). ^{1}H NMR ($(CD_3)_2SO$, ppm): 0.87 (t, 3H, $J = 7.5$ Hz), 3.05 (m, 2H), 3.15 (m, 2H), 3.93 (m, 2H), 4.02 (s, 2H), 4.18 (m, 1H), 4.19 (s, 2H), 4.25 (m, 2H), 4.86 (s, 2H), 6.42 (m, 1H), 6.53 (m, 1H), 6.93–6.99 (m, 3H), 7.25 (m, 1H), 7.29–7.40 (m, 4H), 7.64 (m, 2H), 7.80 (m, 1H), 7.85 (m, 2H), 7.99 (m, 1H), 8.18 (m, 1H), 10.51 (s, 1H). HRMS calcd. for $C_{45}H_{39}N_3O_{12}$ $(M)^+$ 813.2534, found 813.2534.

4.21. Physico-chemical characterization of the dyes

Standard UV–vis and fluorescence spectra in 50 mM sodium tetraborate buffer, pH 9.8, were recorded at 0.5–10 μM dye concentration. The fluorescence

quantum yield of the dyes was determined as described (16) using alkaline solution of fluorescein as a reference ($\phi = 0.92$) (14, 16–18). To measure an effect of pH on fluorescence, 50 nM dye solution in 50 mM sodium tetraborate buffer (pH 9.8) was slowly titrated with HCl, aliquots were taken between pH 9.8 and 4, and fluorescence was measured at λ_{ex} 488 nm/λ_{em} 515 nm.

For studying dye photostability Super-Teflon glass slides (VWR International) were used, which carry 40 circular wells of 2 mm diameter, separated by 30 μm-thick Teflon coating. A well was filled with 1 μL of 5 μM dye solution in 50 mM sodium tetraborate buffer, pH 9.8, and was enclosed with a coverslip. The entire well volume was irradiated under the microscope using standard 'fluorescein' filters set. The intensity delivered by the source on the surface of the slide was measured with a COHERENT LaserCheck probe and found to be ~6 mW/cm^2 at 488 nm. Well images were recorded regularly with IMSTAR software and the mean fluorescence intensity was calculated. The snapshots took 8–120 ms, that was considerably shorter than the characteristic time of photobleaching kinetics $t_{1/2} > 14$ s.

4.22. Solid phase synthesis of fluorogenic substrate

The substrate was synthesized semi-manually on TentaGel S NH_2 resin (20 μm beads, 0.25 mmol/g) using Bohdan MiniBlock synthesizer (Mettler Toledo, Viroflay France). The resin (100 mg, 0.025 mmol) preswollen for 3 h in dry DCM was resuspended in 2 mL of dry DCM, and the solution of Boc-PEG-acid ($n = 7$) (44 mg, 0.075 mmol), DIC (16 mg, 0.125 mmol), HOBt (17 mg, 0.125 mmol), and DMAP (3 mg, 0.025 mmol) in 0.5 mL of dry DMF was added. The reaction mixture was shaken overnight at room temperature. The resin was washed with dry DMF (3× 3 mL) and dry DCM (3× 3 mL), and treated with a mixture of acetic anhydride (18 μL, 0.2 mmol), pyridine (16 μL, 0.2 mmol) in 2 mL of dry DMF for 2 h. As revealed by ninhydrin test, this procedure capped all non-reacted amino groups remaining on the resin. After thorough washing with dry DMF and dry DCM, the resin was deprotected with 20% TFA in DCM.

The resulting TentaGel-PEG(7)-NH_2 resin was used for substrate synthesis by standard Fmoc protocol.[19] Ten-fold excess of Fmoc-protected amino acids for 2 h was used in each coupling cycle to ensure the completion of coupling reaction (3-fold excess overnight for Fmoc-protected fluorescent amino-acid **18**). Each successive coupling was verified by ninhydrin test and by measuring the resin weight. Prior to Methyl red coupling, the synthesized sequence was confirmed by Edman sequencing. In order to attach Methyl red, Nε-Boc lysine was introduced in the peptide chain. Prior to final Nα-Fmoc deprotection the N^{ε}-Boc-protecting group was removed with 20% TFA in DCM. The resin was resuspended in 2 mL of dry DCM and was treated with a solution of Methyl red (34 mg,0.125 mmol), DIC (16 mg, 0.125 mmol), HOBt (17 mg, 0.125 mmol), and DMAP (3 mg, 0.025 mmol) in 0.5 mL of dry DMF. The reaction mixture was stirred for 6 h at room temperature. The resin was washed thoroughly with dry DMF and dry DCM, and fully deprotected.

4.23. On-bead proteolysis assay

The beads were swollen overnight in 0.4 M sodium phosphate buffer (pH 6.8), centrifuged, and resuspended in the same buffer containing 8 mM DTT and 4 mM EDTA.[20] The samples of bead suspension were incubated with 0.5 μM papain for 2 h, then washed and examined for fluorescence. For inhibitor assays, 5 μM stock papain solution was preincubated with 1 mM *N*-ethylmaleimide for 15 min at 37 °C before adding to beads.

For bead imaging, the bead samples were spotted in the wells of Super-Teflon glass slides and were enclosed with coverslips. The scanning was performed on GeneTAC LS IV scanner using a 488 nm argon ion laser and a 512 nm emission filter (512BP). The usual instrument settings were gain = 26.0, black = 1.5, and scanning resolution of 5 μm. The obtained images were analyzed with Genepix Pro 4.0 software. Microscope measurements were performed on an Olympus BX51 fluorescent microscope with standard 'fluorescein' filters set. A random population of 200 beads was analyzed for each sample. Individual beads were localized automatically with IMSTAR software, which was also used to measure fluorescence intensity. Data were converted into histograms representing a percentage of bead population with a fluorescence in a given range.

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