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Dendrimers and combinatorial chemistry—tools for fluorescent enhancement in protease assays

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Abstract—FRET based systems are some of the best methods available to detect and monitor proteolytic activity. To enhance fluorescent signals and hence assay sensitivity, two different systems were developed using two different dendrimeric constructs. In the first case, a triple branched dendrimer bearing three dansyl groups was used to enhance assay sensitivity and showed a significant enhancement of fluorescence following enzymatic cleavage. In another example, a *tris*-fluorescein probe, that undergoes self-quenching, was utilized in a combinatorial library synthesis to map the substrate specificity of proteases.

1. Introduction

Proteases are involved in numerous essential biological processes, and one of the first steps in understanding any new protease is the determination of its substrate specificity. Many assays for proteases are based on fluorescent probes due to the high sensitivity of this technique and fluorescence resonance energy transfer (FRET) methods in particular have been extensively exploited. In the FRET method, donor and quencher moieties are separated on a peptide chain to give rise to an internally quenched fluorescent peptide. This fluorescence is regained following cleavage of the peptide by a protease and it is this increase in fluorescence signal that forms the basis of the FRET assay. Many donor/quencher couples have been described in the literature with varying solubilities in aqueous or organic solvents and efficiency of energy transfer.[1]

Multiplying the number of dyes on an internally quenched peptide could increase the sensitivity of the assay and reduce detection levels. Although dendrimers[2] bearing fluorophores have been described in the literature,[3] it is only very recently that research groups have shown their possible use as a highly sensitive tool for biological assay amplification.[4] Multi-dyes derivatives however can show two types of behaviour: (a) significant amplification of fluorescence directly related to the number of chromophores, (b) a self-quenching phenomenon[5] for dendrimers displaying small Stoke's shift fluorophores.

Keywords: FRET; Enzymatic cleavage; Substrate specificity; Combinatorial libraries; Self-quenching.
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Combinatorial chemistry, including 'split and mix' synthesis, represents a key method for the identification and characterisation of new proteases. This methodology has allowed rapid progress to be made in the determination of the optimal proteolytic substrate for a protease[6] and provides essential information for the understanding of complex biological pathways and for the design of inhibitors.[7] We herein describe the use of multilabelled systems to amplify the fluorescent signal following enzymatic cleavage and their application to define the substrate specificity of proteases using combinatorial multi-chromophoric libraries.

2. Results and discussion

2.1. Dendrimers and amplification monomers

A multivalency system bearing a variety of chromophores should be able to reduce the detection limits of bioassays such as protease detection, or increase the length of sequencing runs in fluorescence based sequencing. The AB_3 type monomers, firstly developed by Newkome et al.,[8] were recently extended and enhanced in utility by our group[9,10] and these multivalent compounds were used as a basis to amplify signals by coupling chromophores to the terminal functional groups.[5] Two different strategies were chosen to monitor enzymatic cleavage. The first was based on a FRET tri-donor/mono-quencher system **1** and used Dansyl as the donor and Dabsyl as a quencher. The second involved a self-quenched derivative **2**, using a single fluorophore (fluorescein) in which the peptidic part consisted of a combinatorial library of 1000 peptides (10×10×10) and was used to define protease substrate specificity (Fig. 1).

The amplification isocyanates **8** and **9** were synthesised in six steps as shown in Scheme 1.[10] Thus, the Michael addition of 1,1,1-tris(hydroxymethyl)amino-methane onto acrylonitrile, followed by amino protection (Boc), and reduction of the nitrile groups with BH_3·THF gave **3**. This was reacted with either Dde-OH to give the Dde (2-acetyl-dimedone) protected amine **4** or the Dansyl donor group to give **5**. Following removal of the Boc protecting group, the isocyanates **8** and **9** were prepared following the procedure of Knölker (Scheme 1).[11] Monomer **8** was coupled directly to the resin via the isocyanate and the free amino groups of the amplification monomer were liberated with hydrazine. The fully formed tri-fluorophore construct **9** could be coupled directly to the free amine groups of the resin linked peptide or used in solution phase synthesis.

2.2. FRET-amplified substrate synthesis: 3-donors/1-quencher

The Dansyl/Dabsyl couple was described by Hartwig as a 'couple' to monitor Heck chemistry during catalyst screening.[12] These two dyes are easy to handle in solution or solid phase and display efficient energy transfer properties. Jakubke described the peptidic sequence Gly-Pro-Ala-Lys-Leu-Ala-Ile-Gly as a good substrate for trypsin with a cleavage site between lysine and leucine.[13] Peptides **10a** and **10b** were constructed on solid phase following an Fmoc-strategy using a hydroxymethylphenoxyacetic linker attached to aminomethyl PS resin. The tri-labelled isocyanate **9** was grafted onto the peptides *N*-terminus after a final Fmoc deprotection. The first amino acid (Lys(Ddiv)-OH),[14] at the beginning of the peptide sequence, allowed dabsyl derivatisation after Ddiv-deprotection with hydrazine. A

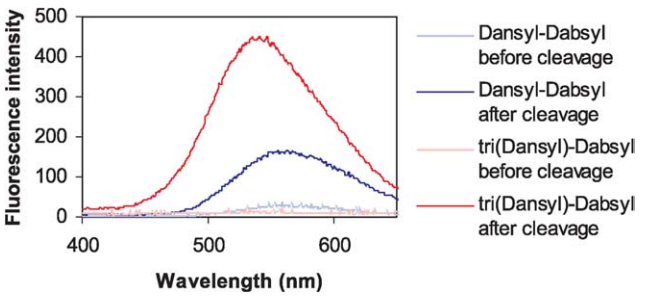


Figure 2. Fluorescence spectra of the peptides **10a** and **10b** (both at 10 μM). Ex=335 nm, Em=545 nm (**10a**) and 560 nm (**10b**). The tri-labelled peptide **10a** (red) showed a much more significant increase in fluorescence than the control peptide **10b** (blue).

control peptide of 1-donor/1-quencher **10b** was also prepared to compare the differences of fluorescence intensity between this and the tri-labelled derivative **10a**. Peptides **10a** and **10b** were precipitated in cold ether, centrifuged and purified by HPLC before analysis (>95%) (Scheme 2).

Enzymatic cleavage was performed on the substrates (excitation at 335 nm and emission at 545 or 560 nm) as shown in Figure 1 and Table 1 demonstrating significant increases in fluorescence in both cases.

Despite the long interchromophore distance, the Dansyl/Dabsyl couple showed good quenching efficiency, which was enhanced for the tribranched-labelled peptide (97%). At equal concentrations (10 μM), the enzymatic cleavage of tri-branched dansyl derivative **10a** (amplification by 28.4) shows a significant enhancement over the control peptide **10b** (amplification by 6.1) and the signal-to-noise ratio

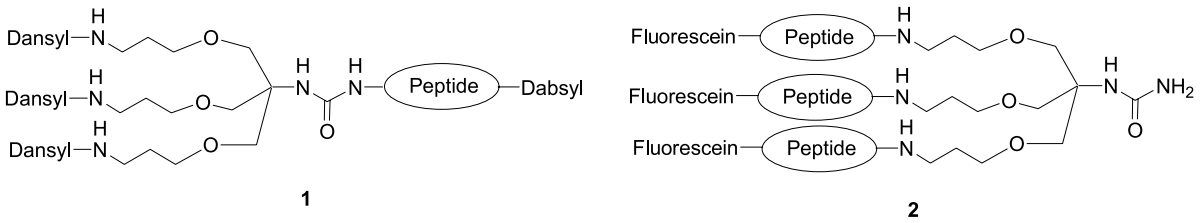
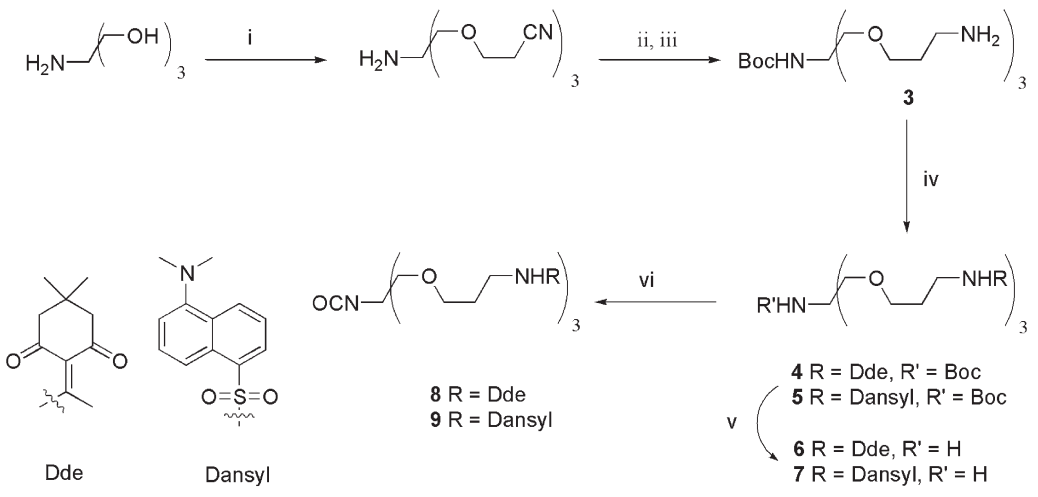
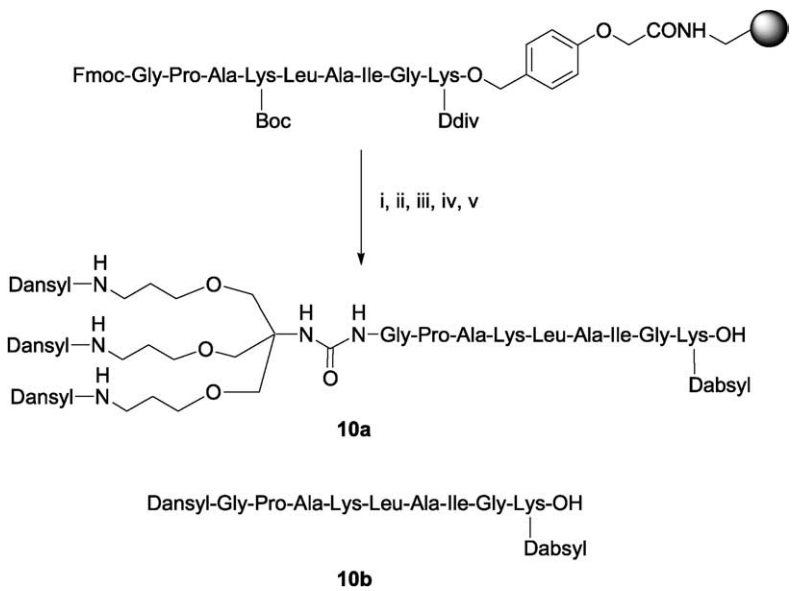


Figure 1. Internally quenched dendritic peptides **1** and **2** which undergo FRET (**1**) and self-quenching (**2**) processes.



Scheme 1. Synthesis of the 1→3 C-branched *tris*-based isocyanate amplification monomers **8** and **9**. (i) acrylonitrile, KOH 40%, dioxane; (ii) Boc_2O, Et_3N; (iii) BH_3·THF, dioxane, 55 °C; (iv) DdeOH, DIPEA for **4** or Dansyl chloride, Et_3N for **5**; (v) 20% TFA in CH_2Cl_2; (vi) Boc_2O, DMAP.



Scheme 2. Synthesis of the FRET-amplified substrate **10a** and the control peptide **10b**. (i) 20% piperidine; (ii) for **10a**: monomer **9**, DIPEA, DMAP. For **10b**: Dansyl-Cl, NEt_3; (iii) 2% hydrazine in DMF; (iv) Dabsyl-Cl, NEt_3; (v) TFA/CH_2Cl_2/TIS 95/3/2.

increased by a factor 4.6 (Fig. 2). Clearly, in this case, the FRET-amplified substrate was not subjected to a self-quenching process, due to its long Stoke's shift.

2.3. FRET-amplified substrate synthesis: self-quenching system

Another approach for assaying protease activity relies on multiple copies of a single type of fluorophore, giving rise internally quenched fluorescent peptides. This method offers a very simple method of 'internal FRET' and was initially described in our group as a method of assaying proteases but can be applied in variety of biological assays. The self-quenching process occurs for small Stoke's shift dyes, when the emission wavelength and the excitation wavelength of the fluorophore are close to each other (4(5)-carboxyfluorescein λ_{ex}=495 nm, λ_{em}=520 nm). In effect, the dendrimer generates a high-local concentration of fluorophore permitting efficient quenching while peptide hydrolysis releases multiple copies of the dyes causing a large increase in emission.

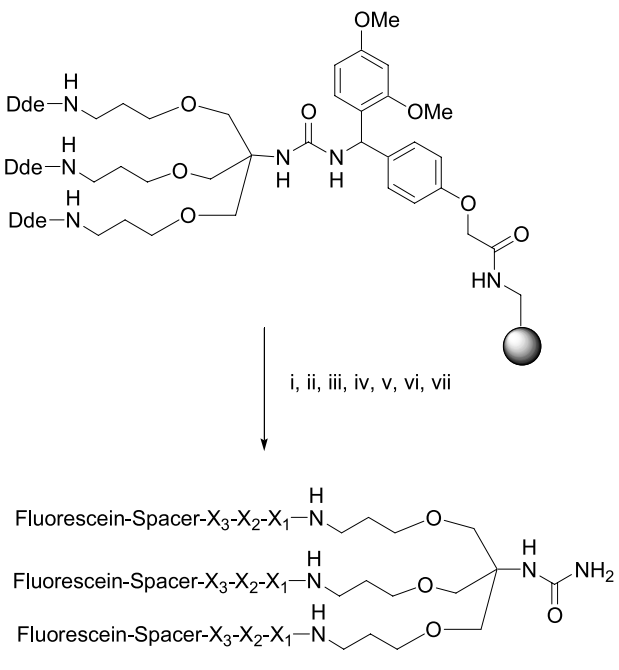
Herein, we show for the first time, that this process of 'internal quenching' can be used in a combinatorial chemistry screening sense, to profile protease specificity. FRET-based libraries of course have been widely exploited to investigate the design of the preferred enzyme substrate,[15,6] but a single fluorophore approach would simplify the whole process, the substrates would be chemically much more accessible, and cleavage could occur anywhere in the peptide chain.

Table 1. Fluorescence intensity of the peptides **10a** and **10b** before and after adding trypsin (arbitrary units)

Peptide	Starting fluorescence	Final fluorescence	Quenching efficiency	Amplification
10b	27	165	84	6.1
10a	16	455	97	28.4

The ability of an enzyme to recognise a substrate selectively in a pool of thousands of potential substrates provides vital information about its mode of action. Split and mix synthesis makes the synthesis rapid and subsequent screening of thousands of peptide targets possible.[16,17]

Here, the determination of the substrate specificity of two model proteases, papain and trypsin is reported by the



Scheme 3. Synthesis of the self-quenched substrate **2** (i) 2% hydrazine in DMF; (ii) (a) Fmoc-amino acid X_1 (10 equiv.), HOBt (10 equiv.), DIC (10 equiv.), (b) 20% piperidine in DMF; (iii) (a) Fmoc-amino acid X_2 (5 equiv.), HOBt (5 equiv.), DIC (5 equiv.), (b) 20% piperidine in DMF; (iv) (a) Fmoc-amino acid X_3 (5 equiv.), HOBt (5 equiv.), DIC (5 equiv.), (b) 20% piperidine in DMF; (v) (a) γ-aminobutyric acid (2 equiv.), HOBt (2 equiv.), DIC (2 equiv.) (b) 20% piperidine in DMF; (vi) 4(5)-carboxyfluorescein (10 equiv.), HOBt (10 equiv.), DIC (10 equiv.); (vii) TFA/TIS/CH_2Cl_2 (95/2/3).

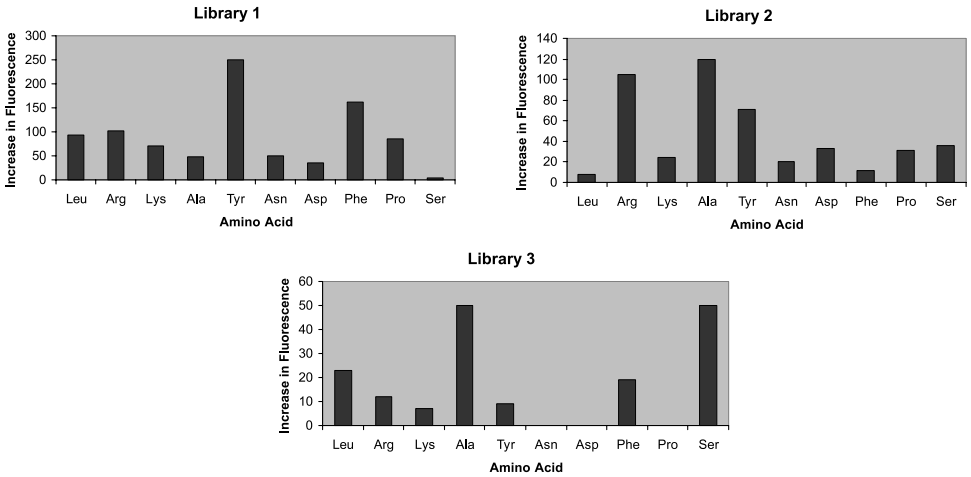


Figure 3. Screening of the 30 self-quenching sub-libraries against papain.

screening of an internally quenched combinatorial peptide library. Three tripeptide sub-libraries were synthesised on the tri-branched resin using the split and mix method (Scheme 3). Monomer **8** was coupled to the Rink linker attached to the aminomethyl PS resin. This resin was deprotected with hydrazine (2% in DMF) and divided into 10 equal sized pools. The X_3 position was fixed in the first library, X_2 in the second library and X_1 position in the third library. To minimize the size of the libraries, ten amino acids with differing properties were chosen (Ala, Arg, Asn, Asp, Leu, Lys, Phe, Pro, Ser and Tyr). Although quite simple, logistically it becomes complex as 100 individual reactions have to be carried out at this split stage. The first amino acid was coupled following standard Fmoc strategy but because of the bulk of the dendrimer backbone, the reactivity of the terminal amine group was affected and some coupling reactions were performed a second time to ensure complete reaction. The second and the third amino acids were attached following the same procedure. The introduction of a spacer was vital; too long results in a decrease of the self-quenching efficiency and too small affects the access of the enzyme to the cleavage site. A 4-carbon spacer, γ-aminobutyric acid, was therefore attached to the backbone (a previous study showed poor hydrolysis kinetics for peptides on dendrimers without spacers). Carboxyfluorescein was finally coupled onto the *N*-termini of all the 30 sub-libraries. The multi labelled peptides were released from the resin with TFA removing, at the same time, side chain protecting groups. The sub-libraries were dissolved in buffer and the concentrations of each of the 30 sub-libraries (each of which contained 100 different tripeptides) were checked by UV spectroscopy (495 nm) to ensure identical concentrations of peptides in each assay.

2.4. Peptide libraries screenings

The 30 sub-libraries were screened against papain, with excitation at 495 nm (fluorescence at 520 nm). Fluorescent enhancement was monitored for 20 min after adding the enzyme (Fig. 3). Library 1 (X_3 fixed) showed selective cleavage for Tyr and Phe. The second library (X_2 fixed) demonstrated a preference for Ala, Arg and Tyr and the third library (X_1 fixed) for small amino acids such as Ser and Ala. These results were consistent with the literature,[18] (with library 1 representing the P_2 position, library 2 the P_1 position and library 3 the P_1' position).

To demonstrate the further potential of the library, the library was screened against another enzyme, trypsin. The results of the screening showed a high preference for basic

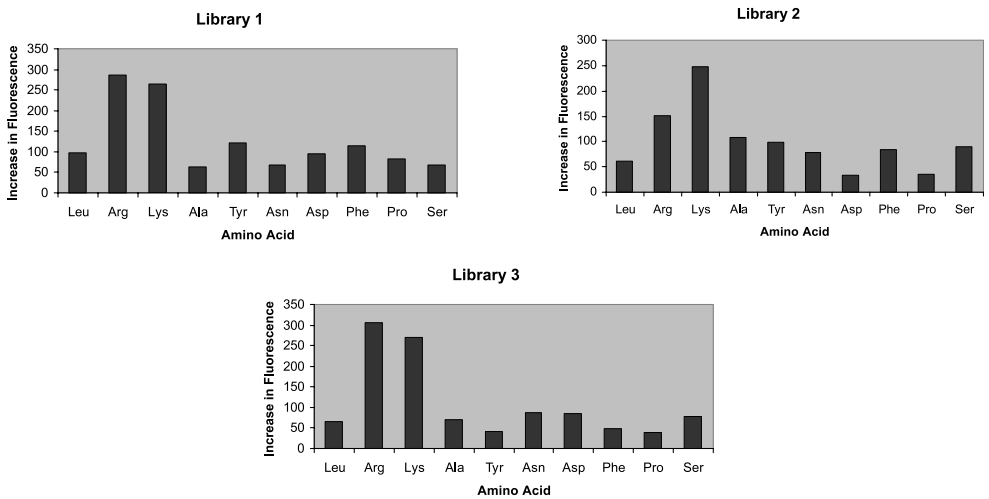


Figure 4. Screening of the 30 self-quenching sub-libraries against trypsin.

amino acid residues (Arg and Lys) as cleavage points in all three sub-libraries (Fig. 4). Trypsin reaches its full hydrolytic efficiency with positively charged side chain residues and presumably, this factor is causing bias with cleavage occurring at all three positions regardless of their location making it difficult to get reasonable data for the specific cleavage site a limitation of the method that has already been observed in other systems.[18]

3. Conclusion

A tri-branched amplification monomer has been developed as a multi-dye carrier to enhance fluorescence signals during protease assays. Peptides bearing a tri-dansyl derivative, internally quenched by a dabsyl group in a peptide, showed a significant increase in fluorescence following hydrolysis. A new 'self-quenched' split and mix peptide library was designed using fluorescein as the 'internally quenched dye', avoiding the need to use two fluorophores as in traditional FRET based protease substrates. Using this 'quenched' split and mix library, the substrate specificity (P_2 to P_1') of papain could be rapidly determined by screening the split and mix sub-libraries.

4. Experimental

4.1. General information

NMR spectra were recorded using Bruker AC 300 or DPX 400 spectrometers operating at 300 or 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts are reported on the δ scale in ppm and are referenced to residual non-deuterated solvent resonances. Electrospray mass spectra were obtained on a VG Platform single quadripole mass spectrometer. MALDI spectra were recorded on a Micromass Tofspec 2E reflection matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometer. IR spectra were obtained on a Biorad FTS 135 spectrometer with a Golden Gate accessory with neat compounds as oil or solids. Fluorescent measurements were recorded using a Perkin Elmer Luminescence Spectrometer LS50B. Commercially available reagents were used without further purification. THF was freshly distilled under nitrogen from a solution of sodium and benzophenone. Purifications by column chromatography were carried out on silica gel 60 (230–400 mesh) purchased from Merck. Analytical HPLC was performed on a Hewlett Packard HP1100 Chemstation equipped with a C_{18} ODS analytical column, (4.6×3 mm i.d. 5 μm, flow rate 0.5 ml min^{-1}) eluting with H_2O/MeCN/TFA (90/10/0.1) to H_2O/MeCN/TFA (10/90/0.05) over 3 min, detection by UV at 254 nm. Semi-preparative HPLC was performed on a HP1100 system equipped with a Phenomenex Prodigy C_{18} reverse phase column (250×10.0 mm, flow rate 2.5 ml min^{-1}) eluting with water (0.1% TFA) to MeCN (0.042% TFA) over 20 min. Resin samples were agitated by spinning on a blood-tube rotor. Compounds **3**, **4** and **8** were synthesized according to literature procedures.[10]

4.1.1. [2-{3-[5-Dimethylaminonaphthalene-1-sulfonic acid]propoxy amide}-1,1-bis-{3-[5-dimethylaminonaphthalene-1-sulfonic acid]propoxymethyl amide}-ethyl] carbamic acid *tert*-butyl ester (5). A solution of **3** (400 mg, 1 mmol), dansyl chloride (891 mg, 3.3 mmol, 1.1 equiv.) and triethylamine (335 mg, 3.3 mmol, 1.1 equiv.) in CH_2Cl_2 (20 ml) was stirred for 4 days. The residue was poured into brine (20 ml) and extracted with CH_2Cl_2 (2×10 ml). The combined organic layers were washed with water (20 ml), dried over $MgSO_4$ and the solvent removed *in vacuo*. The crude product was purified by column chromatography (CH_2Cl_2/MeOH 98:2) to give **5** (524 mg, 48%) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30 (s, 9H), 1.58 (quint, J=6.0 Hz, 6H), 2.80 (s, 18H), 2.94 (t, J=6.0 Hz, 6H), 3.22 (s, 6H), 3.38 (t, J=5.5 Hz, 6H), 5.50 (br s, 3H), 7.08 (d, J=7.0 Hz, 3H), 7.38–7.49 (m, 6H), 8.15 (d, J=7.5 Hz, 3H), 8.24 (d, J=8.5 Hz, 3H), 8.44 (d, J=8.5 Hz, 3H); ^{13}C NMR (100 MHz $CDCl_3$) δ 27.5, 28.2, 40.7, 44.6, 52.6, 57.5, 69.0, 69.6, 78.7, 114.4, 118.2, 122.4, 127.5, 128.7, 128.9, 129.1, 129.4, 134.2, 151.1, 154.1; IR υ 3290, 1716, 1666, 1580; MS (ES$^+$): 1092 (M+H)$^+$.

4.1.2. [2-{3-[5-Dimethylaminonaphthalene-1-sulfonic acid]propoxy amide}-1,1-bis-{3-[5-dimethylaminonaphthalene-1-sulfonic acid]propoxymethyl amide}-ethyl] amine (7). The protected amine **5** (524 mg, 0.48 mmol) was treated with 20% TFA in CH_2Cl_2 (10 ml) and stirred for 45 min. The solvent was removed in vacuo and CH_2Cl_2 (50 ml) was added to the crude product which was washed with saturated aqueous $NaHCO_3$ (2×50 ml) and water (50 ml). The organic layer was dried over Na_2SO_4 and the solvent was removed in vacuo to give the amine **7** as a white solid (470 mg, quantitative yield) which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.59 (quint, J=6.0 Hz, 6H), 2.50 (br s, 2H), 2.80 (s, 18H), 2.96 (t, J=6.0 Hz, 6H), 3.22 (s, 6H), 3.40 (t, J=5.5 Hz, 6H), 6.0 (br s, 3H), 7.07 (d, J=7.0 Hz, 3H), 7.42 (dt, J=7.5, 4.0 Hz, 6H), 8.15 (d, J=7.5 Hz, 3H), 8.24 (d, J=8.5 Hz, 3H), 8.44 (d, J=8.5 Hz, 3H); ^{13}C NMR (100 MHz $CDCl_3$) δ 28.0, 40.3, 44.4, 56.4, 68.7, 70.8, 114.2, 118.1, 122.2, 127.2, 128.2, 128.6, 128.9, 129.1, 134.2, 150.8; IR υ 3290, 1662, 1565; MS (ES$^+$): 992 (M+H)$^+$.

4.1.3. [2-{3-[5-Dimethylaminonaphthalene-1-sulfonic acid]propoxy amide}-1,1-bis-{3-[5-dimethylaminonaphthalene-1-sulfonic acid]propoxymethyl amide}-ethyl] isocyanate (9). 7 (470 mg, 0.48 mmol) and DMAP (61 mg, 0.5 mmol) were dissolved in THF (20 ml) and stirred at −10 °C for 5 min under nitrogen To this solution was added dropwise a solution of Boc_2O (152 mg, 0.7 mmol) in THF (2 ml). The reaction mixture was stirred for 90 min and the solvent removed *in vacuo*. Because of its instability, the crude product was rapidly used without further purification for coupling to the peptide. IR υ 2253, 1732, 1609. LC-MS (ES$^-$): 1016 (M−H)$^-$.

4.2. Peptide synthesis

Peptide synthesis was carried out using a hydroxylmethyl-phenoxy-acetic acid linker attached to aminomethyl PS resin (1.11 mmol g^{-1}, 1% DVB, 75–150 μm).

Fmoc-amino acids (3.76 mmol, 2 equiv.) (Fmoc-Lys(Ddiv)-OH (2.15 g), Fmoc-Gly-OH (1.12 g), Fmoc-Ile-OH

(1.33 g), Fmoc-Ala-OH (1.17 g), Fmoc-Leu-OH (1.3 g), Fmoc-Lys(Boc)-OH (1.76 g), Fmoc-Ala-OH (1.17 g), Fmoc-Pro-OH (1.27 g), Fmoc-Gly-OH) (1.12 g) and HOBt (507 mg, 3.76 mmol) were dissolved in CH_2Cl_2 (10 ml) (with enough DMF to get complete dissolution) and stirred at room temperature for 10 min. DIC (0.63 ml, 3.76 mmol) was then added and the resulting solution stirred for a further 10 min. The solution was then added to the resin (approx. 2 g, 1.88 mmol), pre-swollen in CH_2Cl_2, and the reaction mixture agitated for 2 h. The solution was then drained and the resin washed with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3). The coupling reactions were monitored by the qualitative ninhydrin test with the exception of the coupling onto proline which was monitored by a chloranil test. A small amount of resin was cleaved with TFA/CH_2Cl_2/TIS (95/3/2) ratio and analyzed by analytical HLPC (254 nm) and mass spectroscopy to check peptide synthesis.

4.3. Fmoc-deprotection

To the resin (preswollen in CH_2Cl_2) was added 20% piperidine in DMF (15 ml) and the reaction mixture agitated for 20 min. The solution was then drained and the resin was washed with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3).

4.4. Dansyl chromophores coupling

Peptide **10a**. Isocyanate **9** (335 mg, 0.33 mmol), DMAP (2 mg, 0.02 mmol) and DIPEA (64 mg, 0.33 mmol) were dissolved in CH_2Cl_2/DMF (1:1) (4 ml). The resulting solution was added to the resin (500 mg, 0.22 mmol) and the mixture was shaken overnight and monitored by a qualitative ninhydrin test. The solution was then drained and the resin was washed with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3).

Peptide **10b**. Dansyl chloride (119 mg, 0.44 mmol) and triethylamine (44 mg, 0.44 mmol) were added to the pre-swollen resin (500 mg, 0.22 mmol) in CH_2Cl_2 and the reaction mixture was agitated overnight. The solution was then drained and the resin was washed with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3). The coupling reaction was checked by a qualitative ninhydrin test.

4.5. Ddiv deprotection

To the resin (pre-swollen in CH_2Cl_2) (approx. 500 mg) was added 2% hydrazine in DMF (5 ml) and the reaction mixture agitated for 2 h. The solution was then drained and the resin was washed with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3).

4.6. Dabsyl group coupling

Dabsyl chloride (142 mg, 0.44 mmol) and triethylamine (44 mg, 0.44 mmol) were added to the pre-swollen resin (approx. 500 mg, 0.22 mmol) in CH_2Cl_2 and the reaction mixture was agitated overnight. The solution was then drained and the resin was washed with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3). The coupling reaction was checked by a qualitative ninhydrin test.

4.7. TFA cleavage

The resin (approx. 500 mg) was swollen in CH_2Cl_2 and treated with TFA/CH_2Cl_2/TIS (95:3:2) (12 ml) for 45 min. The solution was drained and the resin was washed with the cleavage cocktail (2×5 ml) and the mixture solution was removed in vacuo.

4.8. Purification of peptides 10a and 10b

The crude cleaved peptides **10a** and **10b** were dissolved in the minimum amount of cleavage cocktail and added dropwise to ice-cooled Et_2O. The mixture was centrifuged, the solvent was removed by decantation and the precipitate was washed with Et_2O (×3), before drying in vacuo. The precipitate was purified by RP-HPLC and lyophilized to afford the peptides as red solids.

Peptide **10a**: Yield: 32%. HPLC (254 nm): 3.98 min. MALDI-TOF-MS: 2159 $(M+H)^+$.

Peptide **10b**: Yield: 42%. HPLC (254 nm): 3.52 min. LC-MS: 1374 $(M+H)^+$.

4.9. Library synthesis

The libraries were carried out using a Rink linker attached to aminomethyl PS resin (1.11 mmol g^{-1}, 1% DVB, 75–150 μm). Isocyanate **8** was synthesized according to literature procedures[10] and coupled to the resin (5.0 g, 4.15 mmol). The Dde protecting group was cleaved using 5% hydrazine in DMF (20 ml) for 2 h, the resin was drained and washed using DMF (×3), MeOH (×3), CH_2Cl_2 (×3) and Et_2O (×3).

4.10. Library 1

The resin was divided into 10 portions (each 30 mg, 60 μmol, 2.0 mmol g^{-1} loading). HOBt (81 mg, 600 μmol) was dissolved in CH_2Cl_2/DMF (200 μL: 200 μL) along with one of each of the following amino acids (600 μmol): Fmoc-Pro-OH (202 mg), Fmoc-Ser(^tBu)-OH (230 mg), Fmoc-Asn(Trt)-OH (358 mg), Fmoc-Asp-(O^tBu)-OH (246 mg), Fmoc-Tyr(^tBu)-OH (275 mg), Fmoc-Arg(Pbf)-OH (388 mg), Fmoc-Lys (Boc)-OH (281 mg), Fmoc-Phe-OH (232 mg), Fmoc-Ala-OH (186 mg) and Fmoc-Leu-OH (212 mg). DIC (100 μL, 600 μmol) was added to each mixture. The resulting solution stirred for a further 10 min and added to the pre-swollen resins. The 10 resin samples were shaken overnight. The reaction mixture was drained off and washed using DMF (×3), MeOH (×3), CH_2Cl_2 (×3) and Et_2O (×3). The ninhydrin test was still positive for some of pools and the coupling was repeated under the same conditions as above until completion of reaction. The resin was then combined, mixed and Fmoc cleavage was performed as previously described. The resin was split into 10 portions and the same procedure was employed in order to couple each one of the 10 amino acids onto the second and third positions. Following the coupling of the third amino acid, the pools of resin were labelled according to their third position and the Fmoc group was cleaved using 20% piperidine in DMF.

4.11. Library 2

The resin was divided into 10 portions (each 100 mg, 200 μmol, 2.0 mmol g^{-1} loading). HOBt (270 mg, 2 mmol) was dissolved in CH_2Cl_2/DMF (600 μL: 600 μL) along with one of each of the following amino acids (2 mmol): Fmoc-Pro-OH (674 mg), Fmoc-Ser(*t*Bu)-OH (766 mg), Fmoc-Asn(Trt)-OH (1.20 g), Fmoc-Asp-O*t*Bu (823 mg), Fmoc-Tyr(*t*Bu)-OH (918 mg), Fmoc-Arg(Pbf)-OH (1.30 g), Fmoc-Lys(Boc)-OH (936 mg), Fmoc-Phe-OH (774 mg), Fmoc-Ala-OH (622 mg) and Fmoc-Leu-OH (706 mg). DIC (315 μL, 2 mmol) was added to each mixture. The resulting solutions were stirred for a further 10 min and added to the pre-swollen resin. The pools of resin were shaken overnight. The solution was drained off and the resin washed using DMF (×3), MeOH (×3), CH_2Cl_2 (×3) and Et_2O (×3). The ninhydrin test was still positive for some of pools and the coupling was repeated as above until completion of the reaction. The resin was then combined and mixed and Fmoc cleavage was performed as previously described. The resin was split into 10 portions. The same procedure as above was employed in order to couple each of the amino acid (1 mmol) to each of the portions of resin. The solutions were drained off, the resin washed using DMF (×3), MeOH (×3), CH_2Cl_2 (×3) and Et_2O (×3) and dried and 10 Fmoc cleavages performed. Each pool of resin was labelled according to the second position amino acid and each pool split into 10 further pools (100 in total). The third amino acid was coupled employing the same procedure (100 μmol) before recombining back into 10 pools. The Fmoc group was cleaved using 20% piperidine in DMF.

4.12. Library 3

The resin was divided into 10 portions (each 100 mg, 200 μmol, 2.0 mmol g^{-1} loading). HOBt (270 mg, 2 mmol) was dissolved in CH_2Cl_2/DMF (600 μL: 600 μL) along with each of the following amino acids (2 mmol): Fmoc-Pro-OH (674 mg), Fmoc-Ser(*t*Bu)-OH (766 mg), Fmoc-Asn(trt)-OH (1.20 g), Fmoc-Asp-O*t*Bu (823 mg), Fmoc-Tyr(*t*Bu)-OH (918 mg), Fmoc-Arg(Pbf)-OH (1.30 g), Fmoc-Lys(Boc)-OH (936 mg), Fmoc-Phe-OH (774 mg), Fmoc-Ala-OH (622 mg) and Fmoc-Leu-OH (706 mg). DIC (315 μL, 2 mmol) was added to each mixture. The resulting solution stirred for a further 10 min and added to the pre-swollen resin. The pools of resin were shaken overnight. The solutions were drained off and the resin washed using DMF (×3), MeOH (×3), CH_2Cl_2 (×3) and Et_2O (×3). The ninhydrin test was still positive for some of pools and the coupling was repeated as above until completion of the reaction. The pools of resin were labelled according to the first position amino acids and each pool split into 10 sub-pools. The second amino acid was coupled following the same procedure as above. The 10 sub-pools of resin were mixed in accordance with their labels, Fmoc deprotected and each pool was again split into 10 sub-pools. The third amino acid was coupled employing the same procedures as above. The sub-pools were then recombined to give the 10 pools. The Fmoc group was cleaved using 20% piperidine in DMF.

4.13. Spacer coupling

A stock solution of HOBt (2 equiv.) and Fmoc-γ-amino-butyric acid spacer (2 equiv.) in CH_2Cl_2/DMF along DIC (2 equiv.) was prepared. To each pool, a tenth of this stock solution was added and the mixture was shaken overnight. The solutions were drained off, the resin washed using DMF (×3), MeOH (×3), CH_2Cl_2 (×3) and Et_2O (×3). Qualitative ninhydrin tests were negative for all 10 pools. An Fmoc cleavage was performed in each pool.

4.14. 4(5)-Carboxyfluorescein coupling

A stock solution of HOBt (10 equiv.) and 4(5)-carboxy-fluorescein (10 equiv.) in CH_2Cl_2/DMF along DIC (10 equiv.) was prepared. To each pool, a tenth of this stock solution was added and the mixture was shaken for 24 h. The ninhydrin test was still positive for some of pools and the coupling was repeated.

4.15. General procedure for library cleavage

The resins (15 mg) were swollen in CH_2Cl_2 (1 ml) and treated with TFA/CH_2Cl_2/TIS (95:3:2) (0.5 ml) mixture for 45 min. The solutions were drained and the resins were washed with the cleavage cocktail (2×0.5 ml). The residue was evaporated to dryness and DMSO (1 mL) was added. These stock solutions were sonicated for 30 min to facilitate peptide solubilization. All of the pools were diluted by a factor of 300 in HEPES (50 mM, pH 8) or potassium phosphate (50 mM, pH 6.8) buffer depending on the enzyme under investigation and the final concentrations of the peptide were checked by absorbance intensities at 495 nm (the absorbance wavelength of the fluorophore) and adjusted as necessary.

4.16. Enzymatic assays

4.16.1. Trypsin assays. Enzyme activity was monitored at 25 °C in 50 mM HEPES at pH 8.0, 10 mM $CaCl_2$, 100 mM NaCl, DMSO (15% for peptides **10a** and **10b** and less than 1% for the peptide libraries). Reactions were initiated by the addition of trypsin and monitored fluorimetrically for 20 min with excitation at 335 nm (**10a** and **10b**) or 495 nm (libraries) and emission at 545 nm (**10a**), 560 nm (**10b**) or 520 nm (libraries).

4.16.2. Papain assays. Enzyme activity was monitored at 25 °C in potassium phosphate buffer at pH 6.8, cysteine (2 mM), EDTA (1 mM) and the pool of peptides. Reactions were initiated by the addition of papain and monitored fluorimetrically for 20 min with excitation at 495 nm and emission at 520 nm.

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References and notes

1. (a) Meldal, M.; Breddam, K. *Anal. Biochem.* **1991**, *195*, 141–147. (b) Matayoshi, E. D.; Wang, G. T.; Krafft, G. A.;

Erikson, J. *Science* **1990**, *247*, 954–958. (c) Nagase, H.; Fields, C. G.; Fields, G. B. *J. Biol. Chem.* **1994**, *269*, 20952–20957. (d) Rossé, G.; Kueng, E.; Page, M. G. P.; Schauer-Vukasinovic, V.; Giller, T.; Lahm, H. W.; Hunziker, P.; Schlatter, D. *J. Comb. Chem.* **2000**, *2*, 461–466.

2. Chow, H.-F.; Mong, T. K.-K.; Nongrum, M. F.; Wan, C.-W. *Tetrahedron* **1998**, *54*, 8543–8660.
3. (a) Vögtle, F.; Gestermann, S.; Hesse, R.; Schwierz, H.; Windish, B. *Prog. Polym. Sci.* **2000**, *25*, 987–1041. (b) Balzani, V.; Ceroni, P.; Gestermann, S.; Gorka, M.; Kauffmann, C.; Vögtle, F. *Tetrahedron* **2002**, 629–637. (c) Serin, J. M.; Brousmiche, D. W.; Fréchet, J. M. *J. Am. Chem. Soc.* **2002**, *124*, 11848–11849. (d) Weil, T.; Reuther, E.; Müllen, K. *Angew. Chem., Int. Ed.* **2002**, *41*, 1900–1904. (e) Zhou, M.; Roovers, J. *Macromolecules* **2001**, *34*, 244–252. (f) Sato, T.; Jiang, D.-L.; Aida, T. *J. Am. Chem. Soc.* **1999**, *121*, 10658–10659. (g) Hahn, U.; Gorka, M.; Vögtle, F.; Vicinelli, V.; Ceroni, P.; Maestri, M.; Balzani, V. *Angew. Chem., Int. Ed.* **2002**, *41*, 3595–3598.
4. (a) Minard-Basquin, C.; Weil, T.; Hohner, A.; Rädler, J. O.; Müllen, K. *J. Am. Chem. Soc.* **2003**, *125*, 5832–5838. (b) Ong, K. K.; Jenkins, A. L.; Cheng, R.; Tomalia, D. A.; Durst, H. D.; Jensen, J. L.; Emanuel, P. A.; Swim, C. R.; Yin, R. *Anal. Chem. Acta* **2001**, *444*, 143–148. (c) Staffilani, M.; Höss, E.; Giesen, U.; Schneider, E.; Hartl, F.; Josel, H.-P.; De Cola, L. *Inorg. Chem.* **2003**, *42*, 7789–7798. (d) Blackburn, G. F.; Shah, H. P.; Kenten, J. H.; Leland, J.; Kamin, R. A.; Link, J.; Peterman, J.; Powell, M. J.; Shah, A.; Talley, D. B. *Clin. Chem.* **1991**, *37*, 1534–1539.
5. Ellard, J. M.; Zollitsh, T.; Jon Cummins, W.; Hamilton, A. L.; Bradley, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 3233–3236.
6. Maly, D. J.; Huang, L.; Ellman, J. A. *Chem. BioChem.* **2002**, *3*, 16–37, and references therein.
7. Whittaker, M. *Curr. Opin. Chem. Biol.* **1998**, *2*, 386–396.
8. Newkome, G. R.; Weis, C. D.; Childs, B. J. *Des. Monom. Polym.* **1998**, *1*, 3–14.
9. Fromont, C.; Bradley, M. *Chem. Commun.* **2000**, 283–284.
10. Lebreton, S.; How, S.-E.; Buchholz, M.; Yingyongnarongkul, B.; Bradley, M. *Tetrahedron* **2003**, *59*, 3945–3953.
11. Knölker, H. J.; Braxmeier, T.; Schlechtingen, G. *Angew. Chem., Int. Ed.* **1995**, *34*, 2497–2500.
12. (a) Stambuli, J. P.; Stauffer, S. R.; Shaughnessy, K. H.; Hartwig, J. F. *J. Am. Chem. Soc.* **2001**, *123*, 2677–2678. (b) Stauffer, S. R.; Beare, N. A.; Stambuli, J. P.; Hartwig, J. F. *J. Am. Chem. Soc.* **2001**, *123*, 4641–4642.
13. Grahn, S.; Ullmann, D.; Jakubke, H.-D. *Anal. Biochem.* **1998**, *265*, 225–231.
14. Chhabra, S. R.; Hothi, B.; Evans, D. J.; White, P. D.; Bycroft, B. W.; Chan, W. C. *Tetrahedron Lett.* **1998**, *39*, 1603–1606.
15. Meldal, M.; Svendsen, I.; Breddam, K.; Auzanneau, F.-I. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3314–3318.
16. Furka, A. *Drug Disc. Today* **2002**, *7*, 1–5.
17. Kassel, D. B. *Chem. Rev.* **2001**, *101*, 255–267.
18. Hilaire, St. P. M.; Willert, M.; Juliano, M. A.; Juliano, L.; Meldal, M. *J. Comb. Chem.* **1999**, *1*, 509–523.