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Fluorescence Enhancement *via* Enzymatic Cleavage of Internally Quenched Dendritic Peptides: A New Sensitive Assay for Proteases

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Supplementary Information:

General Methods. Analytical HPLC was carried out on a Hewlett Packard 1100 Chemstation with a Phenomenex Prodigy C_{18} 150x4.6mm column (analytical flow 0.5ml min^{-1}). The solvent gradient ran from water with 0.1% TFA to MeCN with 0.042% TFA over 20 minutes. Semi-preparative HPLC was performed on a HP1100 system equipped with a Phenomenex Prodigy C_{18} reverse phase column (250x10.0mm, flow rate 2.5 ml min^{-1}) eluting with water with 0.1% TFA to MeCN with 0.042% TFA over 20 minutes followed by 5 minutes in MeCN with 0.042% TFA and then a further 5 minutes to return to water containing 0.1% TFA. Electrospray mass spectra were recorded on a VG Platform Quadrupole Electrospray Ionisation mass spectrometer. MALDI spectra were recorded on a Micromass Tofspec 2E reflection matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometer. The fluorescence measurements were recorded using a Perkin Elmer Luminescence Spectrometer LS50B. Resin samples were agitated by spinning on a blood-tube rotor.

Peptide coupling. A solution of Fmoc-amino acid (4 equivalents) and HOBt (4 equivalents) in DCM (0.2 - 0.3M) with enough DMF to completely dissolve was stirred at room temperature for 10 minutes. DIC (4 equivalents) was then added and the resulting solution stirred for a further 10

minutes. The solution was then added to the resin (1 equivalent), preswollen in DCM, and the reaction mixture agitated. The solution was then drained and the resin washed with DMF (x3), DCM (x3), MeOH (x3) and Et_2O (x3). The coupling reactions were monitored by a qualitative ninhydrin test.

Fmoc deprotection. To the resin (preswollen in DCM) was added 20% piperidine in DMF and the reaction mixture agitated for 10 minutes. The solution was then drained and the piperidine treatment repeated. Finally the resin was washed with DMF (x3), DCM (x3), MeOH (x3) and Et_2O (x3).

Sulfonated Cy5 dye NHS ester coupling. To the resin (1 equivalent), preswollen in DCM, was added a solution of sulfonated Cy5 dye NHS ester (1.5 equivalents) and triethylamine (1.4 equivalents) in DMF (~0.03M). The reaction mixture was agitated for 3 days. The solution was drained and the resin washed with DMF x3, DCM x3, MeOH x3 and Et_2O x3.

Fluorescene Isothiocyanate coupling. To the resin (1 equivalent), preswollen in DCM, was added a solution of fluorescein isothiocyanate isomer I (2 equivalents) and triethylamine (2 equivalents) in DMF (~0.2M). The resulting

mixture was agitated overnight. The solution was then drained and the resin washed with DMF (x3), DCM (x3), MeOH (x3) and Et_2O (x3).

TFA cleavage. The resin was swollen in DCM and treated with 50% TFA and 3% TIS in DCM for 45 minutes. The solution was drained and the resin was washed with the cleavage cocktail (x2).

HFIP cleavage. To the resin (preswollen in DCM) was added 30% HFIP in DCM and the reaction mixture allowed to stand for 3 hours. The solution was drained and the resin washed with DCM and MeOH (x2). The solvent was removed *in vacuo*.

Purification of peptides. The crude peptides were dissolved in the minimum amount of cleavage cocktail and added to ice-cooled Et_2O. The mixture was centrifuged, the solvent was removed by decantation and the precipitate was washed with Et_2O (x2), before drying *in vacuo*. The precipitate was then purified by RP-HPLC and the sample lyophilized to afford the pure peptides.

Single peptide 4. The peptide was prepared from 1,4-diaminobutane trityl PS resin (0.22g, 0.33mmol, loading: 1.5mmol g^{-1}). Each Fmoc amino acid (Fmoc-Lys(Boc)-OH, Fmoc-

Val-OH), Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Ala-OH, Fmoc-Val-OH and Fmoc-Tyr(^{t}Bu)-OH) was coupled over 3 hours according to the general protocol above and was monitored by ninhydrin test with the exception of the coupling onto the proline residue which was monitored by the chloroanil test. The initial coupling was repeated 3 times and was followed by a capping step with acetic anhydride (10 equivalents) with catalytic pyridine in DCM overnight. After each coupling a small amount of resin was cleaved with 50% TFA, 3% TIS in DCM and analyzed by HPLC and ES-MS. Following the final peptide coupling the resin (7.1mg, 3.6μmol, theoretical loading NH_2: 0.51mmol g^{-1}) was subjected to Fmoc deprotection. The fluorescent peptide was then prepared by reaction of the peptide resin with the sulfonated Cy5 dye NHS ester, TFA cleavage and purification as described above to afford peptide **4** (74% from 1,4-diaminobutane trityl resin) as a blue solid. HPLC (600nm): 6.8 mins; MS (MALDI): 1571 (MH^+).

***Tris* dendrimer peptide 5.** Prepared from generation [1.0] *tris* dendrimer resin **2** (0.29g, 0.24mmol NH_2, theoretical loading NH_2: 0.84mmol g^{-1}) according to the method for the synthesis of **4**. Following final Fmoc deprotection of the peptide resin (14.2mg, 5.8μmol NH_2, theoretical loading NH-

$_2$: 0.41mmol g^{-1}) to afford **5** (20% from 1,4-diaminobutane trityl resin) as a blue solid. HPLC (600nm): 7.3 mins; MS (MALDI): 5066 (MH^+).

Endoproteinase Asp-N cleavage. The reaction mixture contained 40nM Endoproteinase Asp-N (Sigma), 10μM peptide and 100mM phosphate buffer pH 8.0. The solution was suspended in a water bath at 37°C. Samples for fluorescence measurements (excitation 640nm, emission 670nm) were prepared by taking a 3μl aliquot and quenching with 3000μl of sodium tetraborate buffer ph 9.0. Control experiments were run simultaneously with water added in place of the enzyme solution. Cleavage product HPLC (600nm): 7.5 mins.

Endoproteinase Asp-N kinetics. Cleavage kinetics were determined by measuring the initial rate of fluorescence increase over a range of substrate concentrations [0.5-5xKm]. The assays were conducted by incubating the enzyme (0.6nM) in 100mM pH 8.0 K_2HPO_4 buffer containing varying substrate concentrations at 37°C. The fluorescence was followed for 600 seconds. Each assay was repeated in triplicate.

Single peptide 6. The peptide was synthesised from 1,4-diaminobutane trityl polystyrene resin **3** (0.2g, 0.3mmol, loading 1.5mmol g^{-1}). Coupling of Fmoc-amino acid residues (Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH and Fmoc-Ala-OH) according to the peptide coupling procedure above. Once the peptide had been prepared fluorescein was coupled and the product cleaved from the resin using the general HFIP cleavage procedure and purified as described above to afford **6** (54% from 1,4-diaminobutane trityl resin) as a yellow solid. HPLC (440nm): 8.3 mins; MS (ES^+): 961 (MH^+).

'PAMAM' dendrimer peptide 7. Prepared from TentaGel generation [1.0] 'PAMAM' dendrimer resin **1** (0.3g, 0.13mmol NH_2, theoretical loading NH_2: 0.42mmol g^{-1}) according to the method for the synthesis of **6** to afford **7** (9% from TentaGel hydroxytrityl resin) as a yellow solid. HPLC (440nm): 8.8 mins; MS (MALDI): 2091 (MH^+).

***Tris* dendrimer peptide 8.** Prepared from generation [1.0] *tris* dendrimer resin **2** (0.3g, 0.25mmol NH_2, theoretical loading NH_2: 0.84mmol g^{-1}) according to the method for the synthesis of **6** to afford **8** (19% from 1,4-diaminobutane trityl resin) as a yellow solid. HPLC (440nm): 9.5 mins; MS (MALDI): 3240 (M^+).

Chymotrypsin cleavage. The reaction mixture contained: 50mM pH 8.1 HEPES buffer, 10mM $CaCl_2$, 0.1M NaCl, 10μM peptide and 0.3μM chymotrypsin (α-chymotryspin, bovine pancreas – Calbiochem). An eppendorf tube containing the reaction mixture (1 ml) was suspended in a water bath at 25 °C. Samples for fluorescence measurements (excitation 492nm, emission 520nm) were prepared by taking 3μl of the reaction mixture and quenching with 3000μl of a pH 9 buffer solution (sodium tetraborate buffer). The control experiments were run in an identical manner simultaneously to the corresponding enzyme experiment but with water added in place of the chymotrypsin solution. Enzyme cleavage afforded peptide FITC-Ala-Lys(Boc)-Leu-OH **9**. HPLC (440nm): 9.4 mins; MS (ES+): 820 (MH^+).