

A Fluorogenic Peptide-Based Smartprobe for Detection of Neutrophil Extracellular Traps and Inflammation

Maria R. Rios^a, Gloria Garoffolo^b, Giulia Rinaldi^c, Alicia Megia-Fernandez^a, Silvia Ferrari^b, Calum T. Robb^c, Adriano G. Rossi^c, Maurizio Pesce^b, Mark Bradley^{*,a}

Table of Contents

1. Supplementary Figures

- a. NHS ester of 5-Carboxyfluorescein diacetate vs 5-Carboxyfluorescein /DIC/Oxyma
- b. Cleavage specificity
- c. HL-60 differentiation
- d. Flow cytometry

2. Experimental Procedures. Chemistry

- a. General
- b. Synthesis of compound **4**
- c. General solid-phase synthesis methods
- d. Characterisation of the probe

3. Experimental Procedures. Biology

- a. Plate reader experiments
- b. Absorbance spectrum
- c. Fluorometric experiments
- d. Effect of pH and different biological microenvironments
- e. Catalytic constant determination
- f. HL-60 differentiation and microscopy
- g. Confocal images of NETosis in primary human neutrophils

1. Supplementary Figures

- a. **NHS ester of 5-Carboxyfluorescein diacetate vs 5-Carboxyfluorescein/DIC/Oxyma:** N-hydroxysuccinimide ester of 5-carboxyfluorescein diacetate in the final coupling step lead to a higher purity of the crude product dramatically increasing the yield and ease of purification.

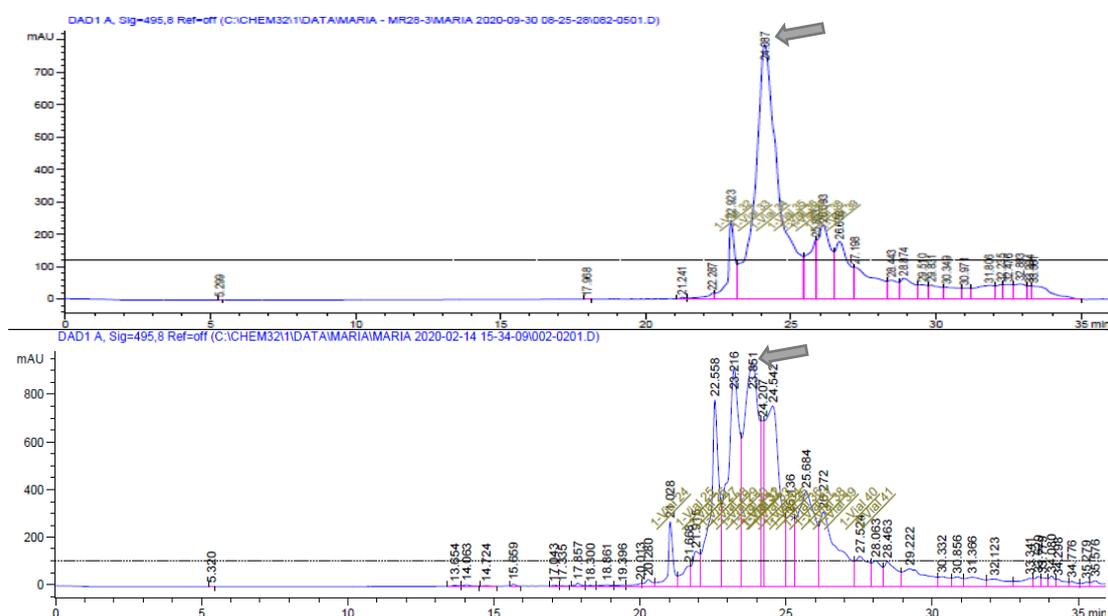
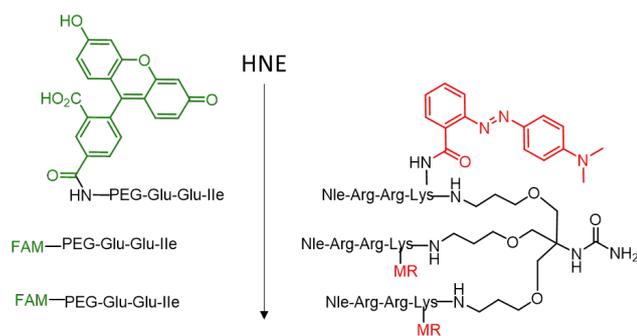


Fig. S1. Semi-prep HPLC chromatogram (detection 495nm) showing the differences in the purity of the crude peptide following the final coupling using NHS ester of 5-Carboxyfluorescein diacetate (top), and 5-Carboxyfluorescein/DIC/Oxyma (bottom). Arrows indicate the peak corresponding to the product.

b. Cleavage specificity



Specific hydrolytic cleavage between Ile↑Nle was confirmed following probe (5 μ M) incubation with hNE (100 nM) for 90 min at 37°C following analysis of the reaction mixture by MALDI-TOF MS and LC-MS confirmed the appearance of peaks with the expected masses:

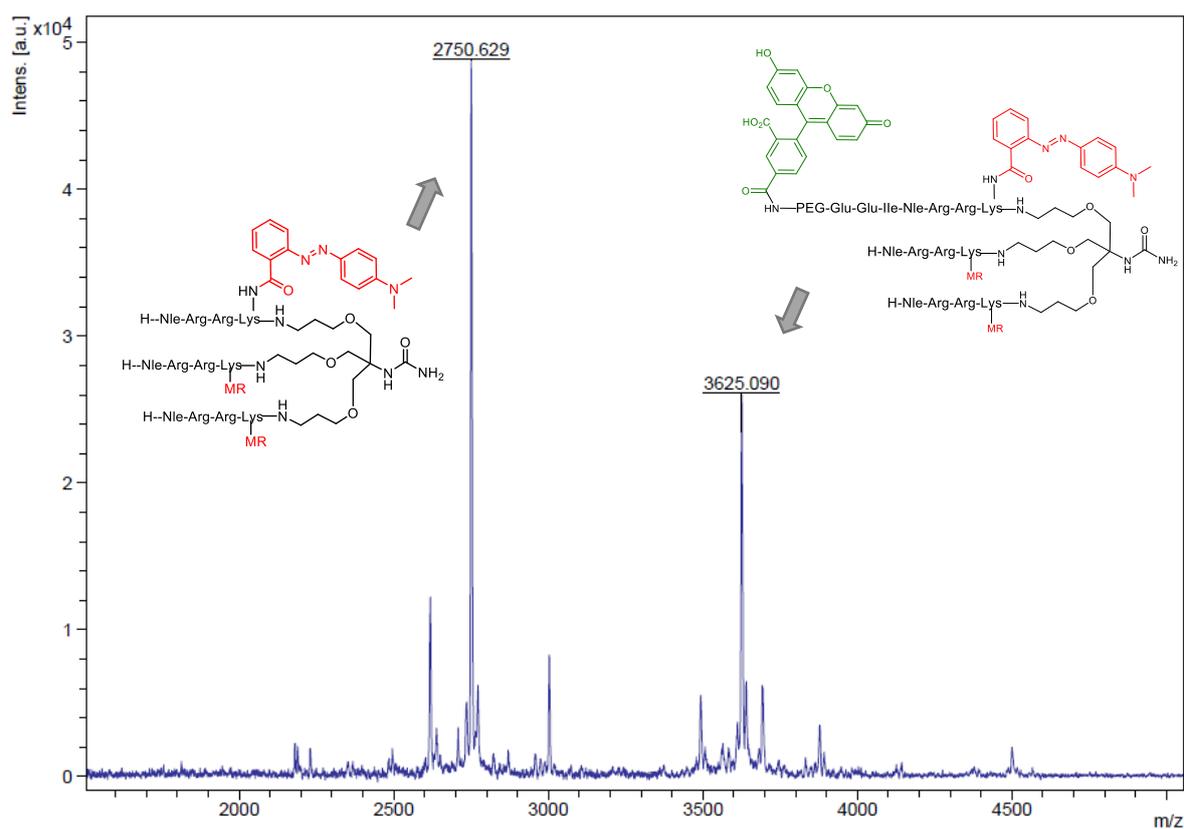


Fig. S2. MALDI-TOF analysis of the reaction mixture confirmed the presence of the fully cleaved probe (Expected: 2749.720 Found: 2750.629) and the probe with one remaining branch intact (Expected: 3625.010 Found: 3625.090).

c. HL-60 differentiation

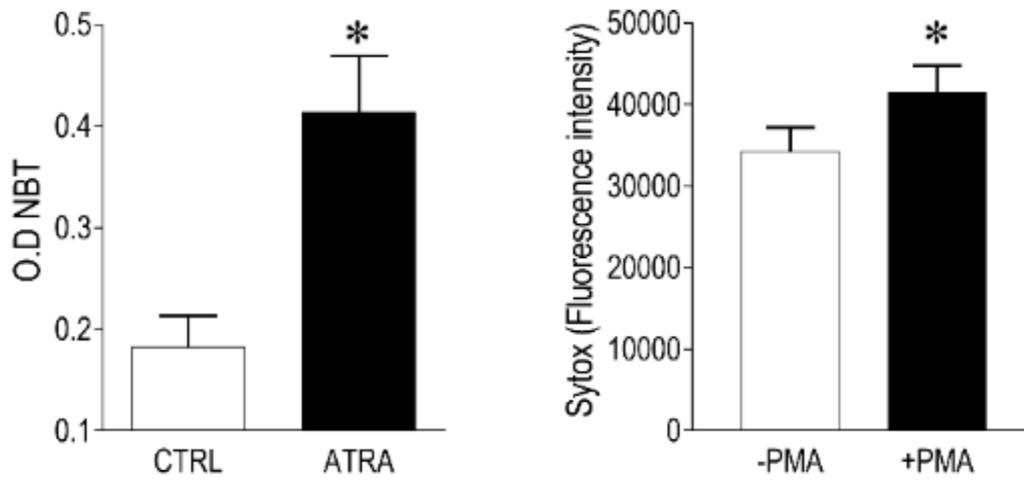


Fig. S4. Quantification of HL-60 cell line differentiation into neutrophils and neutrophil activation as detected by nitro-blue tetrazolium (NBT) and SYTOX Orange, respectively. * indicates $P < 0.05$ by paired Student's t-test ($n=6$ for NBT and $n=3$ for SYTOX Orange).

d. Flow cytometry

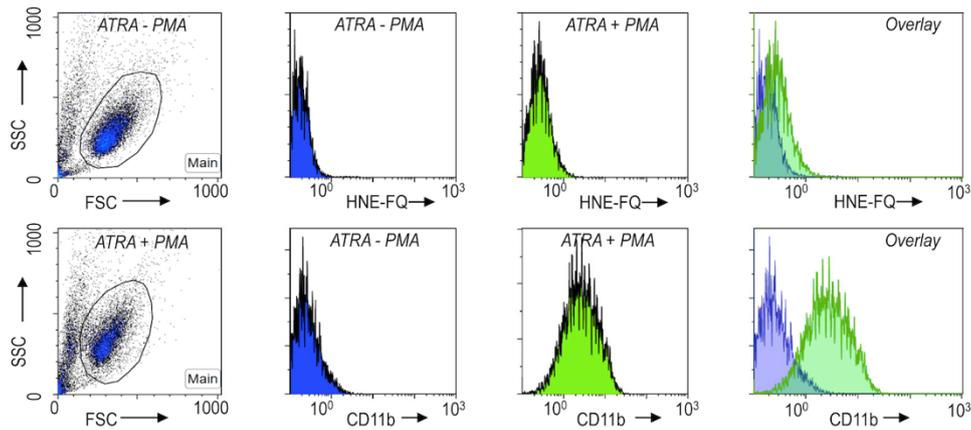


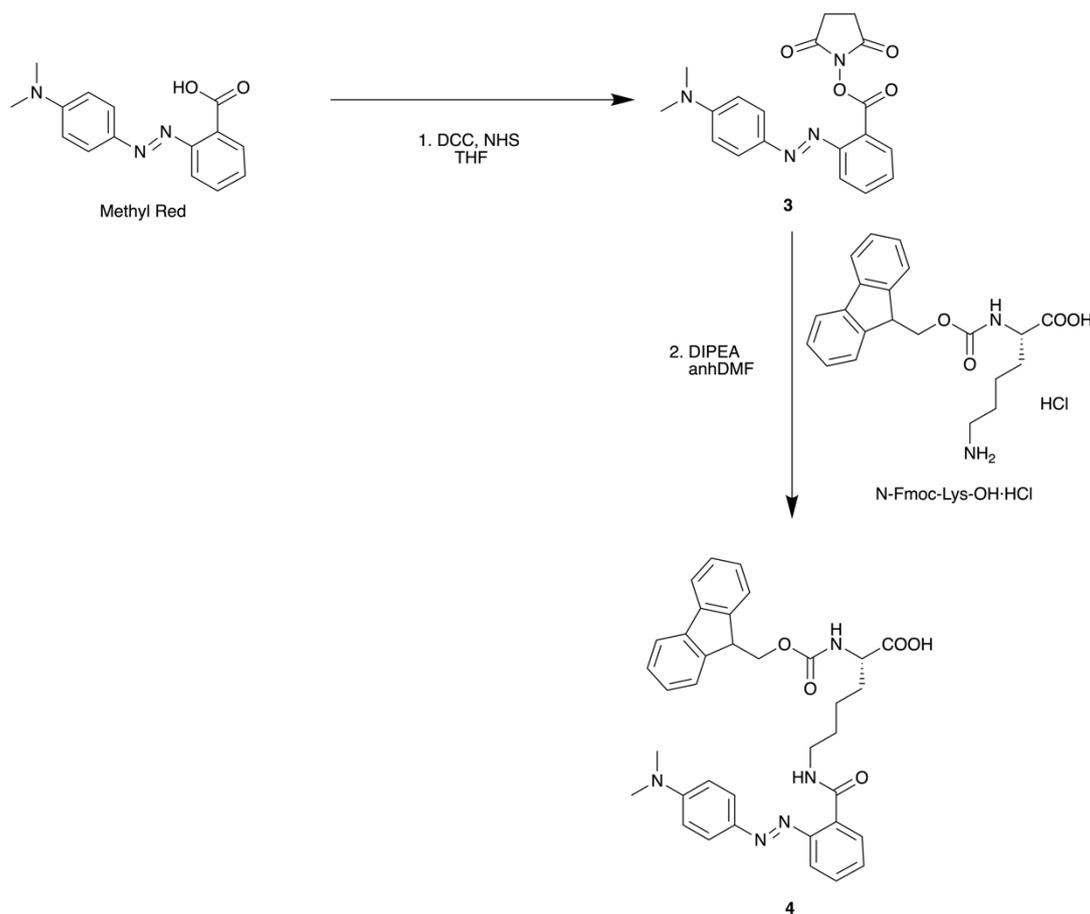
Fig. S5 Detection of activated neutrophils by flow cytometry. Upper panels: fluorescence profile of control and PMA-activated HL60 cells after staining with the probe. Lower panels: fluorescent profile of CD11b a marker of activated neutrophils.

2. Experimental Procedures. Chemistry

a. General:

All Fmoc-amino acids, Methyl Red, 5-Carboxyfluorescein, DIC, Oxyma and Aminomethyl ChemMatrix Resin (1 mmol/g) and Fmoc-Rink Amide Linker were purchased from GL Biochem, Sigma, Fluorochem or Apollo Scientific and used without further purification. Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on an HP1100 system equipped with a Kinetex 5 μm XB-C18 reverse-phase column (5 cm \times 4.6 mm, 5 μm) with a flow rate of 1 mL/min and eluting with H₂O/CH₃CN/HCOOH (95/5/0.1) to H₂O/CH₃CN/HCOOH (5/95/0.1), over 6 min, holding at 95% CH₃CN for 3 min, with detection at 495 nm and by evaporative light scattering. Electrospray ionization mass spectrometry (ESI-MS) analyses were carried out on an Agilent Technologies LC/MSD quadrupole 1100 series mass spectrometer (QMS) in an ESI mode. High-resolution mass spectra were recorded on a Bruker Solarix Fourier transform ion cyclotron resonance mass spectrometer (FT-MS). MALDI-TOF spectra were acquired on a Bruker Ultraflextreme MALDI TOF/TOF with a matrix solution of sinapic acid (10 mg/mL) in H₂O/CH₃CN/TFA (69.9/30/0.1) or α -cyano-4-hydroxycinnamic acid (10 mg/mL) in H₂O/CH₃CN/TFA (49.9/50/0.1). The synthesis of isocyanate **1** has been reported previously¹.

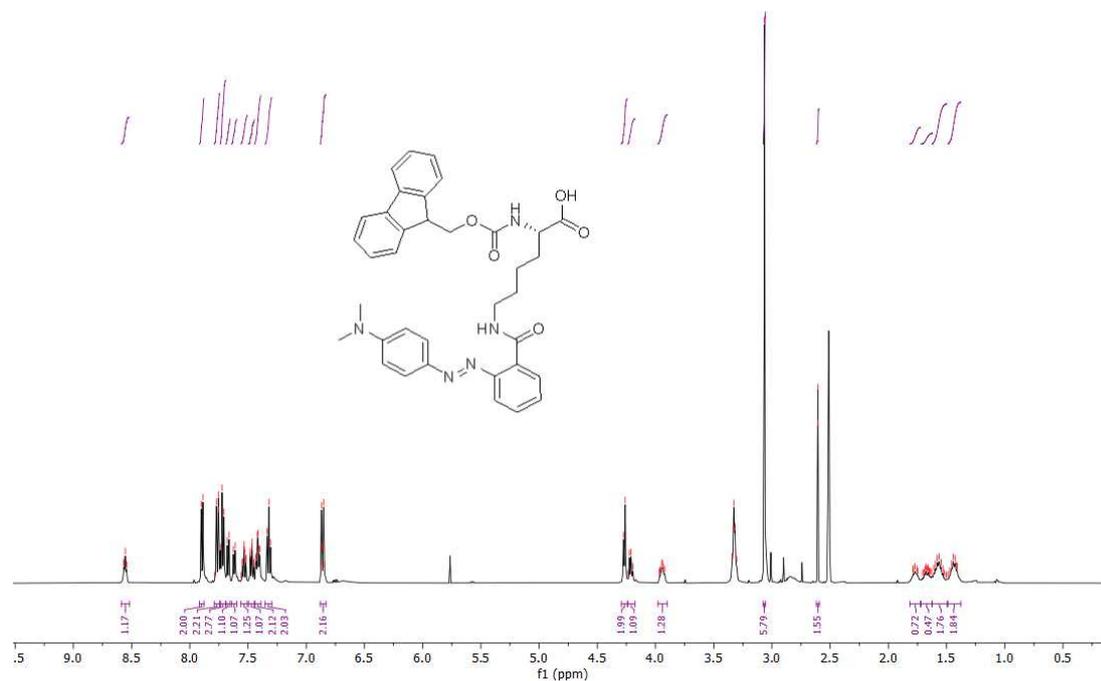
b. Synthesis of compound **4**



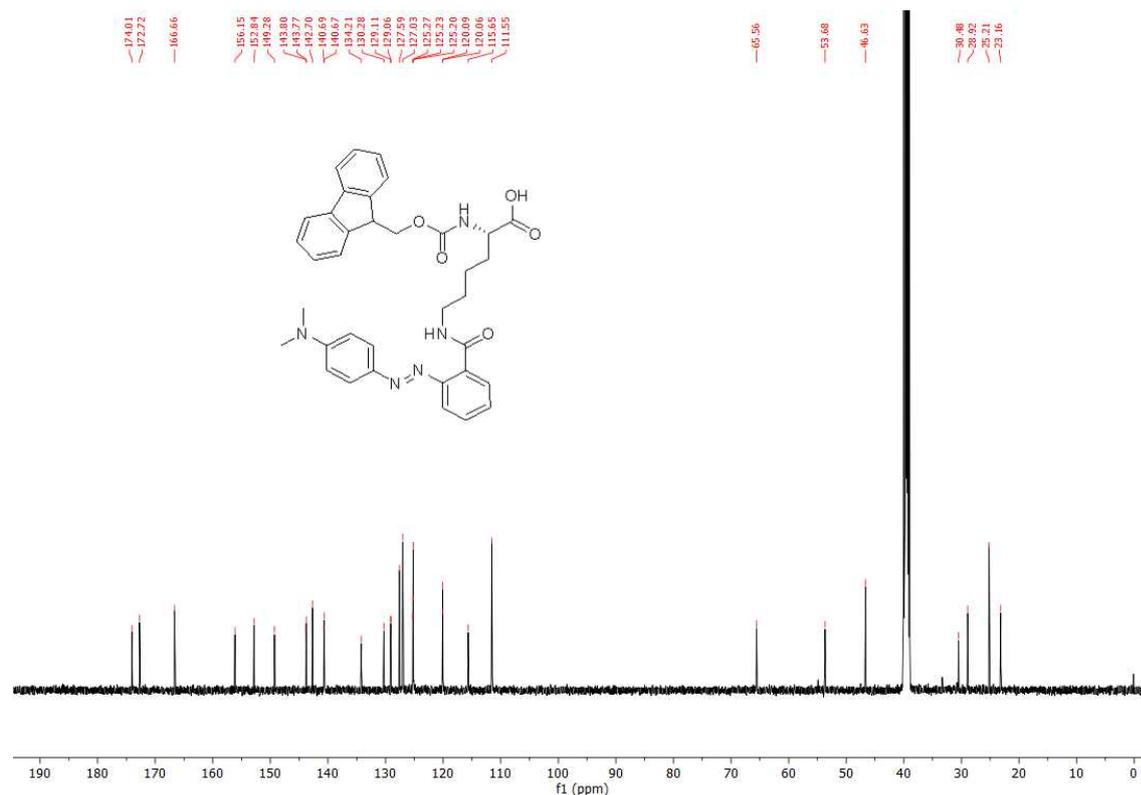
Methyl Red (2.5 g) was cooled to 0°C in THF (60 mL) and DCC (2.206 g) was added followed by N-Hydroxysuccinimide (1.136 g). After stirring 2h at 0°C the reaction was maintained at room temperature overnight. After filtration the filtrate was evaporated to give compound **3** as a red solid that can be recrystallized from acetone-Et₂O. The solid obtained (MR-NHS) was dissolved in anhydrous DMF (10 mL) and added dropwise to a solution of Fmoc-Lys-OH.HCl (4.886 g) in anhydrous DMF (10 mL) together with DIPEA (4.8 mL). After stirring overnight the solvent was evaporate under vacuum and the crude purified by column chromatography: DCM->DCM-MeOH (15:1) to obtain compound **4** as a red solid (4.51 g, 78%).

HPLC: t_R 7.78 min (254 nm); **IR:** 2932, 1716, 1601, 1522, 1366, 1141 cm^{-1} ; **^1H NMR** (500 MHz, DMSO-d_6) δ 8.55 (t, $J = 5.6$ Hz, 1H), 7.89 (d, $J = 7.6$ Hz, 2H), 7.76 (d, $J = 8.9$ Hz, 2H), 7.71 (d, $J = 7.7$ Hz, 2H), 7.67 (d, $J = 8.0$ Hz, 1H), 7.62 (d, $J = 8.1$ Hz, 1H), 7.53 (t, $J = 7.6$ Hz, 1H), 7.46 (t, $J = 7.3$ Hz, 1H), 7.42 (td, $J = 7.5, 2.8$ Hz, 2H), 7.32 (t, $J = 7.4$ Hz, 2H), 6.86 (d, $J = 9.0$ Hz, 2H), 4.27 (d, $J = 7.1$ Hz, 2H), 4.21 (t, $J = 7.2$ Hz, 1H), 3.94 (m, 1H), 3.06 (s, 6H), 2.61 (m, 2H), 1.76 (m, 1H), 1.67 (m, 1H), 1.56 (m, 2H), 1.44 (m, 2H). **^{13}C NMR** (125 MHz, DMSO-d_6) δ 174.0, 172.7, 166.7, 156.1, 152.8, 149.3, 143.8, 143.8, 142.7, 140.7, 140.7, 134.2, 130.3, 129.1, 129.1, 127.6, 127.0, 125.3, 125.2, 120.1, 120.1, 115.7, 111.6, 65.6, 53.7, 46.6, 30.5, 28.9, 25.2, 23.2. **HR-MS** (ESI): calcd for $\text{C}_{36}\text{H}_{38}\text{O}_5\text{N}_5$ 620.2867; found, 620.2876 ($\text{M}+\text{H}$)⁺

^1H -NMR



^{13}C -NMR



c. General solid-phase synthesis methods

Rink amide linker attachment to ChemMatrix Resin: Fmoc-Rink-amide linker (1.61 g, 3.0 mmol, 3eq) was dissolved in DMF (10 mL) and Oxyma (0.43 g, 3.0 mmol, 3eq) was added and the mixture was stirred for 10 min. DIC (0.5 mL, 3.0 mmol, 3eq) was added and the mixture was stirred for further 5 min. The solution was added to the resin (1.0 g, 1 mmol/g, 1eq, pre-swollen in DCM) and shaken for 2 h. The resin was washed with DMF (3 × 10mL), DCM (3 × 10mL) and MeOH (3 × 10mL). The coupling reaction was monitored by a Kaiser test.

Isocyanate coupling: A solution of isocyanate **1** (0.9 g, 1.1 mmol), DIPEA (0.21 mL, 1.2 mmol) and DMAP (3 mg, 0.1 mmol, 0.06 eq) in a 1:1 mixture of DCM/DMF (4 mL) was added to the resin (400 mg, 0.4 mmol, pre-swollen in DCM) and the mixture was shaken overnight (the reaction monitored by a Kaiser test). The solution was drained and the resin was washed with DMF (3 × 20mL), DCM (3 × 20mL), MeOH (3 × 20mL) and ether (3 × 20mL).

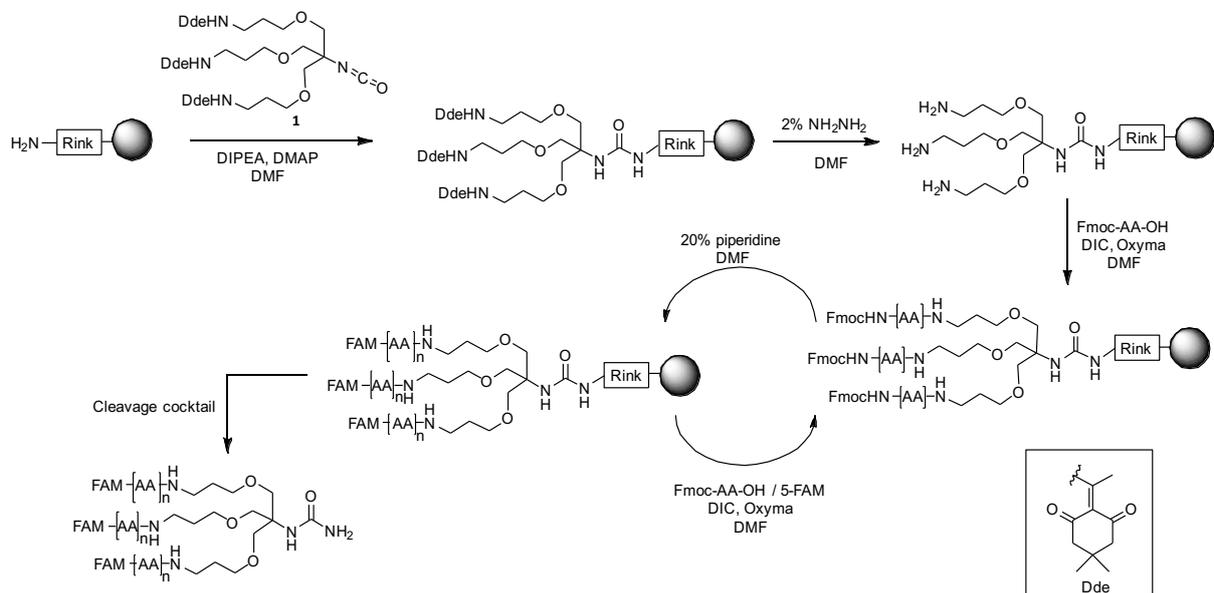
Dde deprotection: To the resin (100 mg, pre-swollen in DCM), 2% hydrazine in DMF (3 mL) was added and the reaction mixture was shaken for 2h. The resin was filtered and washed with DMF (3 × 20 mL), DCM (3 × 20 mL) and MeOH (3 × 20 mL).

Fmoc deprotection: To the resin (100 mg, pre-swollen in DCM), 20% piperidine in DMF (5 mL) was added and the reaction mixture was shaken for 10 min. The solution was drained and the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL).

Amino acid and dye couplings: A solution of the *N*-Fmoc-protected amino acid, 5-Carboxyfluorescein, Fmoc-(EG)₂-OH (4.3 eq) or Fmoc-Lys(MR)-OH (1.7 eq) with Oxyma (4.3 eq or 1.7 eq) in DMF (0.07 M) was stirred for 10 min. DIC (4.3 eq or 1.7 eq) was added and the solution was stirred for further 5 min. The solution was added to the resin (1 eq, pre-swollen in DCM) and the reaction mixture was shaken for 30 min at 50° C, except for Fmoc-Lys(MR)-OH that was shaken for 1 hour at 50° C. The solution was drained and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10mL). Following the last coupling with 5-Carboxyfluorescein, the resin was washed with 20 % piperidine to remove any esters formed. The coupling reactions were monitored by a Kaiser test.

N-hydroxysuccinimide ester of 5-carboxyfluorescein diacetate² coupling: A solution of N-hydroxysuccinimide ester of 5-carboxyfluorescein diacetate (1.7 eq) in DMF (0.07 M) were stirred for 10 min and added to the resin followed by addition of DIPEA (1 eq) and the mixture was shaken for 30 min at 50° C followed by shaking at room temperature overnight. The solution was drained and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10mL). The resin was washed with 20 % piperidine to remove the acetates or esters. The coupling reaction was monitored by a Kaiser test.

Cleavage and purification: The resin (200 mg, pre-swollen in DCM) was shaken 3 h in TFA/TIS/DCM (95:2.5:2.5, 5 mL). The solution was collected by filtration and the resin was washed with the cleavage cocktail (1 mL). The combined filtrates were added to ice-cold ether (25 mL), and the precipitated solid was collected by centrifugation, and washed repeatedly with cold ether (3 × 50mL). Purification of the probe was performed on a Semi-preparative HPLC HP1100 system equipped with a reverse-phase column (Zorbax Eclipse XDB-C18 250 × 4.6 mm, 5 μm). The flow rate was 2.5 mL/min eluting with 0.1% HCOOH in H₂O (A) and 0.1% HCOOH in CH₃CN (B), with a gradient of 5 to 60% B over 25 min and from 60% to 95% B over 5 min with an initial and final isocratic periods of 2 min (*t_R* = 24.4 min). Fractions containing the product were combined and the solvent removed *via* freeze-drying to give **HNE-FQ** as an orange solid.



Scheme S1. Fmoc-SPPS synthesis of the probe

d. Characterisation

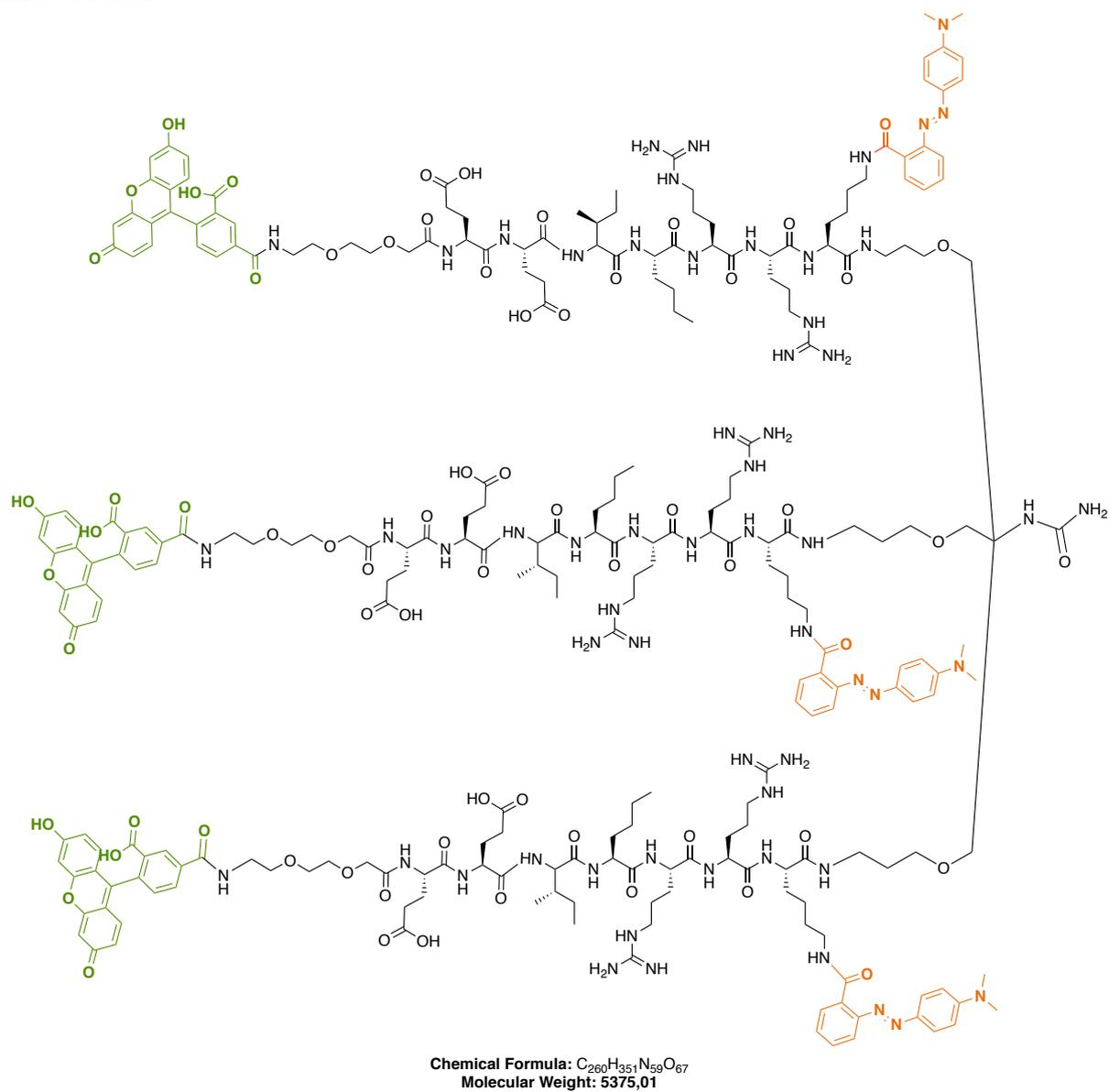


Fig. S6 . Full structure of the HNE-FQ probe.

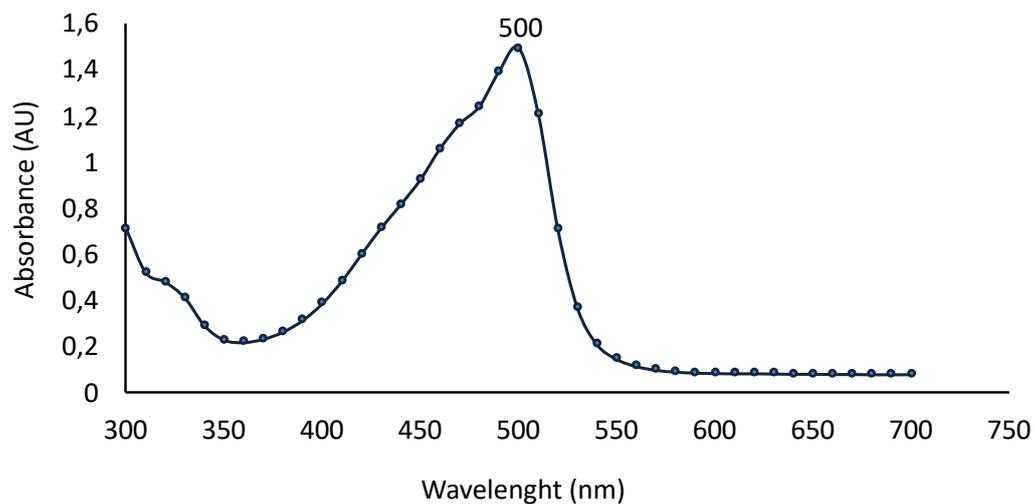


Fig. S7. Absorbance spectrum (300-700 nm) of the probe (5 μ M) in 50 mM Hepes buffer (pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) showing absorbance λ_{max} at 500 nm.

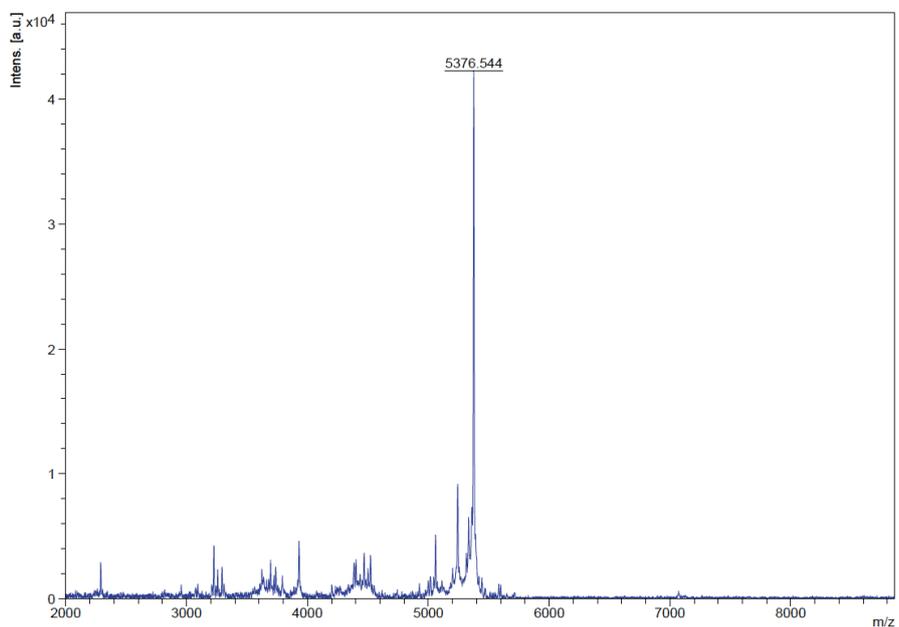


Fig. S8. MALDI-TOF MS analysis of the probe showing the molecular ion $[M+H]^+$ Expected: 5375.603 Found: 5376.544

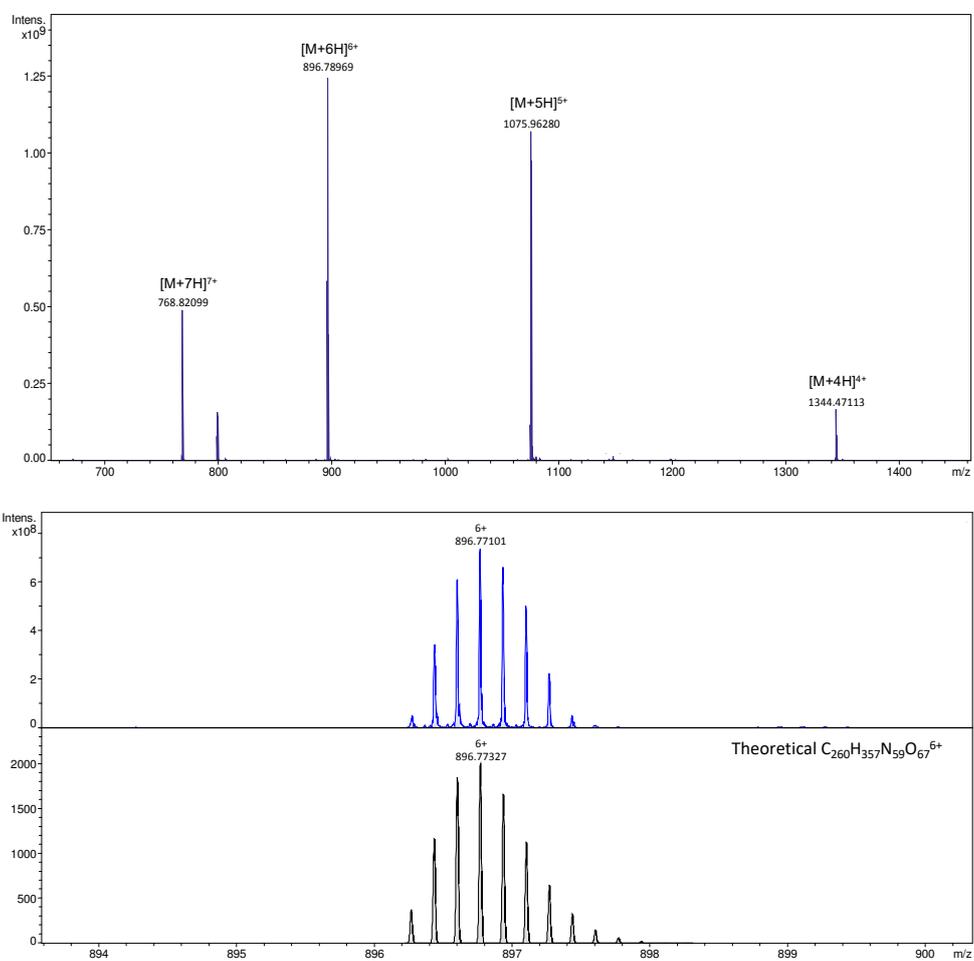


Fig. S9. FT-HRMS (ESI) analysis showing the most abundant molecular ions (top) and expanded to show the $[M+6H]^{6+}$ molecular ion (lower) Expected: 896.7733 Found: 896.7710.

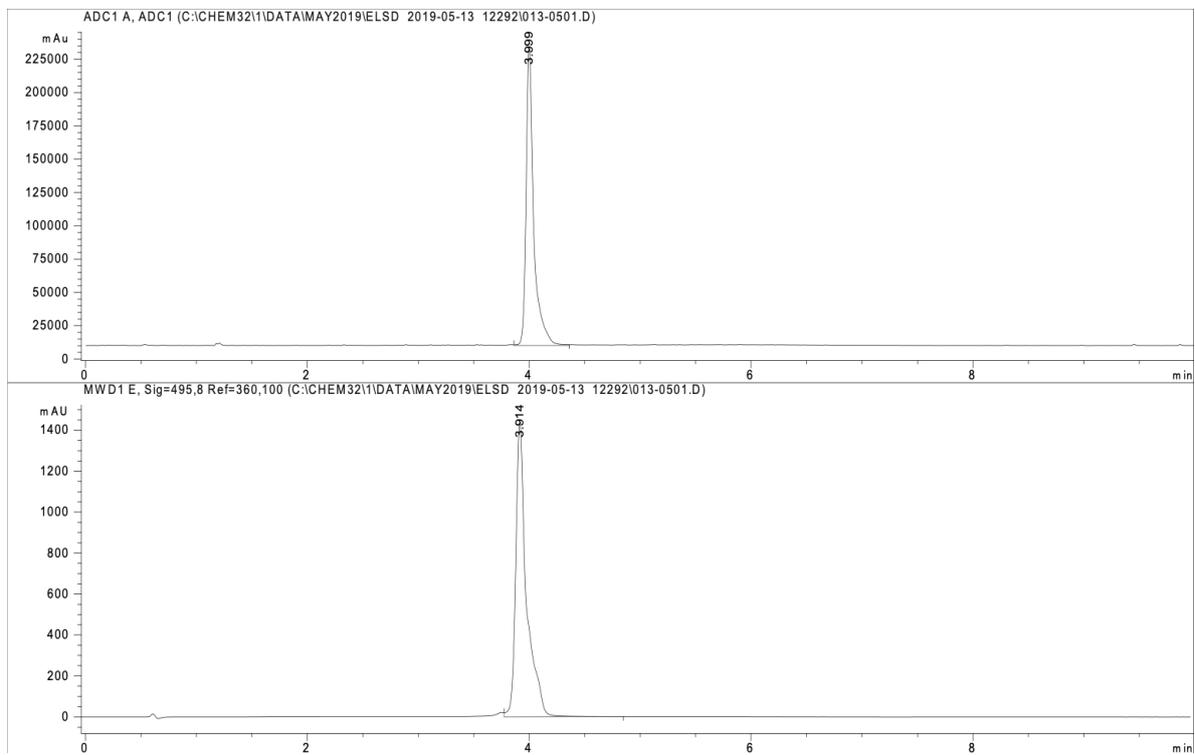


Fig. S10 . RP-HPLC chromatogram of the probe with detection by evaporative light scattering (upper) and by absorbance at 495 nm (lower).

Experimental Procedures. Biology

a. Plate reader experiments

Probe (5 or 15 μM) was incubated with hNE (100 nM), Cathepsin G (330 nM) or PR3 (100 nM) (Athens Biotechnology) in reaction buffer (50 mM HEPES buffer, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume of 50 μL in a 96-well plate (Life Technologies). The time-dependent increase in fluorescence was monitored over 1.5 h using a fluorescence microplate reader (Biotek Synergy HT multi-mode reader) ($\lambda_{\text{ex/em}}$ 480/20, emission 528/25) at 37°C. Buffer, enzymes and inhibitor where appropriate were incubated in the wells for 30 min at 37°C before adding the probe. Readings were taken immediately after addition of the probe every 30 seconds and the plate was shaken for 10 seconds before the start of the readings. Data was normalised to buffer alone and the fold-change in signal (Relative Fluorescent Units) compared to enzyme-free controls was calculated. Data was plotted using Prism5 (GraphPad Software Inc., La Jolla, CA, USA).

b. Absorbance spectrum

Probe (5 μM) was incubated in reaction buffer (50 mM HEPES buffer, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume of 50 μL in a 96-well plate (Life Technologies) and absorbance was measured across the wavelength spectrum range 300 to 700 nm.

c. Fluorometric experiments

Probe (5 μM) was incubated with or without hNE (100 nM) (Athens Biotechnology), in reaction buffer (50 mM HEPES buffer, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume of 150 μL in a quartz cuvette. Emission spectrum readings were recorded in a spectrofluorimetric range from 500 to 700 nm after 40 minutes incubation, with the excitation wavelength of 490 nm.

d. Effect of pH, temperature and different biological environments on fluorescence signal.

pH effect on the fluorescence signal: The fluorescence intensity of the cleaved vs uncleaved probe was assessed between pH 4.0 and 8.5 (Fig. S11). As expected, at more acidic pH's (less than 4.0) the intensity of fluorescence was reduced due to the nature of 5-carboxyfluorescein (n=2).

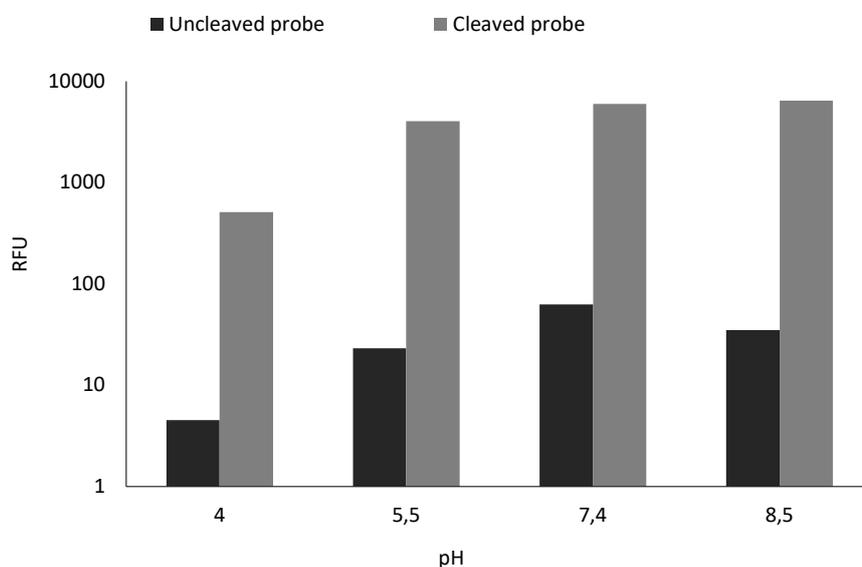


Fig. S11. Effect of pH on the fluorescence signal of the cleaved and uncleaved probe. Stocks of cleaved and uncleaved probe were generated by incubating the probe (85 μM) in reaction buffer (50 mM HEPES pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume of 200 μL , with or without hNE (Athens Biotechnology), for

1.5 hours. Cleaved or uncleaved probe solutions (15 μM) were added to the different pH buffers (4.0, 5.5, 7.4 and 8.5) and fluorescent intensities measured using a fluorescence microplate reader.

Effect of complex media on probe fluorescence: The fluorescence of cleaved and uncleaved probe was monitored in either 10% FBS, complete cell media or a HeLa cell lysate for 1.5 h. Results demonstrate no further activation of the uncleaved probe, with fluorescence intensities unaltered over time.

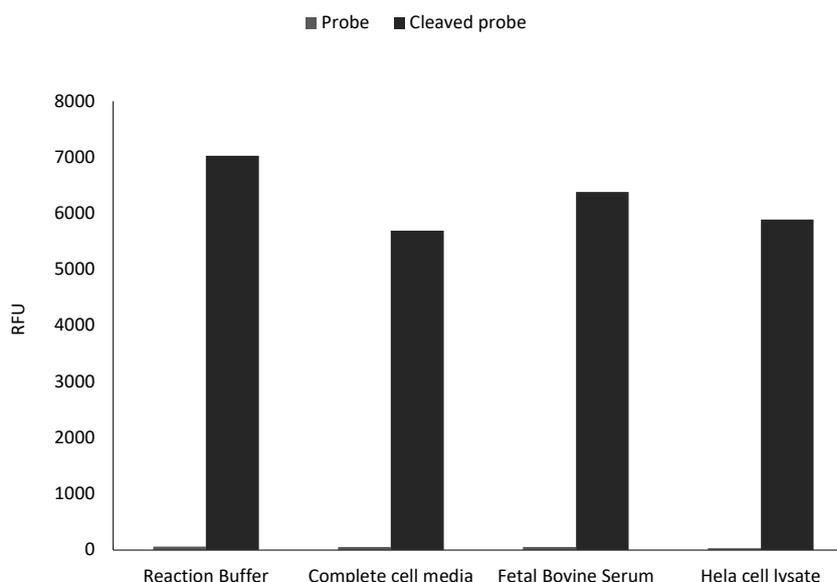


Fig. S12 . Effect of different biological environments on the fluorescence signal. The effect of different biological microenvironment on the fluorescence signal was evaluated by incubating intact or hNE cleaved probe (15 μM) in HEPES buffer (control), complete cell media , 10% Fetal Bovine Serum (FBS) or HeLa cell lysate) and incubated for 2 hours at 37 $^{\circ}\text{C}$ in a final volume of 50 μL . Fluorescence intensities were read using a fluorescence microplate reader (for the HeLa cell lysate cells, a T25 at confluency was used, cells were resuspended in sterile water (5 mL), approximately 1 million cells/mL, and incubated for 30 min at 37 $^{\circ}\text{C}$). The resulting lysate was centrifuged at 13000 g for 10 min and the supernatant collected and used immediately.

e. Catalytic constant determination

hNE was added to 50 μL of HEPES buffer (50 mM, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) with final probe concentrations ranging from 0.04 μM to 50 μM in a 96-well plate (n=3). Fluorescence ($\lambda_{\text{ex/em}}$ 485/528) was recorded on a Biotek Synergy HT multi-mode reader every 30 seconds. Control samples (n=3) had the same composition but no enzyme.

The RFU was plotted against time (min) to obtain initial velocity values (V_0) on the first 10 minutes of reaction. Velocity units were normalised to M/min by the factor $\text{conc}/\text{RFU}_{\text{max}}$ at 5 μM . The generated value of $1/V$ were plotted against time (1/min) to give a Lineweaver-Burk Plot to generate values of K_M , V_{max} and k_{cat} .

[Probe] (μM)	E_T (nM)	V_0 (RFU/min)	V_0^* (M/s)	$1/[\text{Probe}]$	$1/V_0$
12,5	100	169.8	6.8×10^{-9}	8.0×10^4	1.5×10^8
5	100	88.4	3.6×10^{-9}	2.0×10^5	2.8×10^8
1	100	25.0	1.0×10^{-9}	1.0×10^6	10.0×10^8
0.2	100	6.3	2.5×10^{-10}	5.0×10^6	4.0×10^9
0.04	100	1.4	5.8×10^{-9}	2.5×10^7	1.7×10^{10}

*Normalisation factor using 5 μM data: $[\text{M}] / \text{RFU}_{\text{max}}$; 0.000005/2071

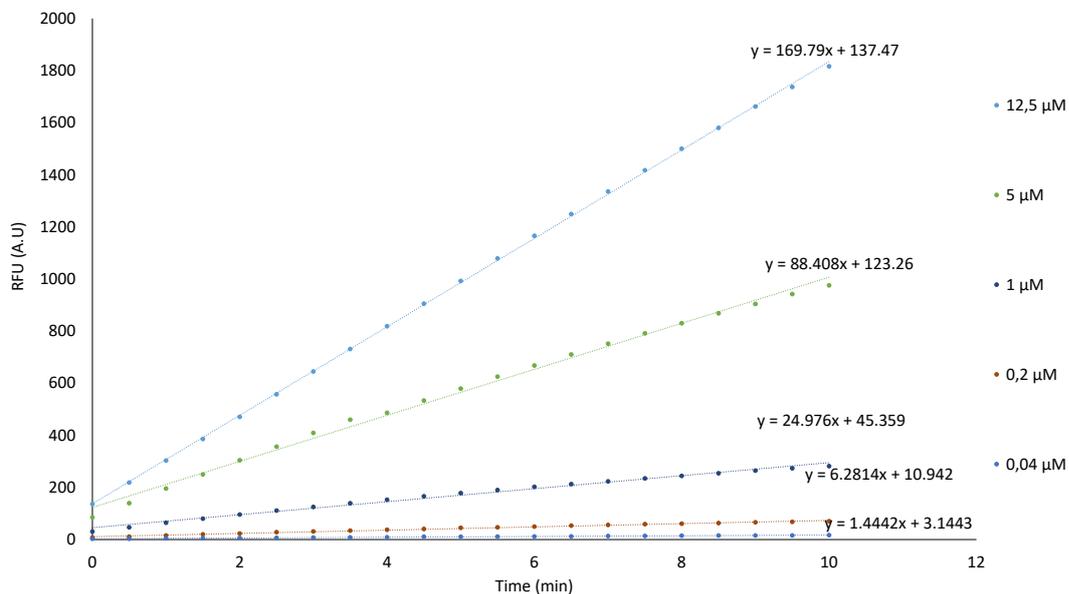


Fig. S13 . Initial velocity plots. RFU increase over the first 10 minutes of reaction

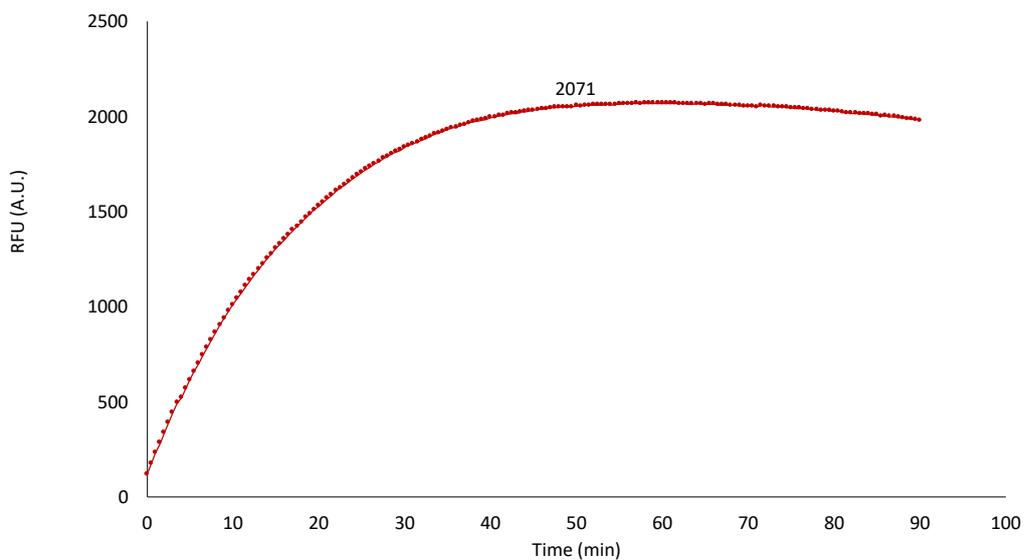


Fig. S14. Full de-quenching of the probe at 5 μM . RFU_{max} at 5 μM : 2071.

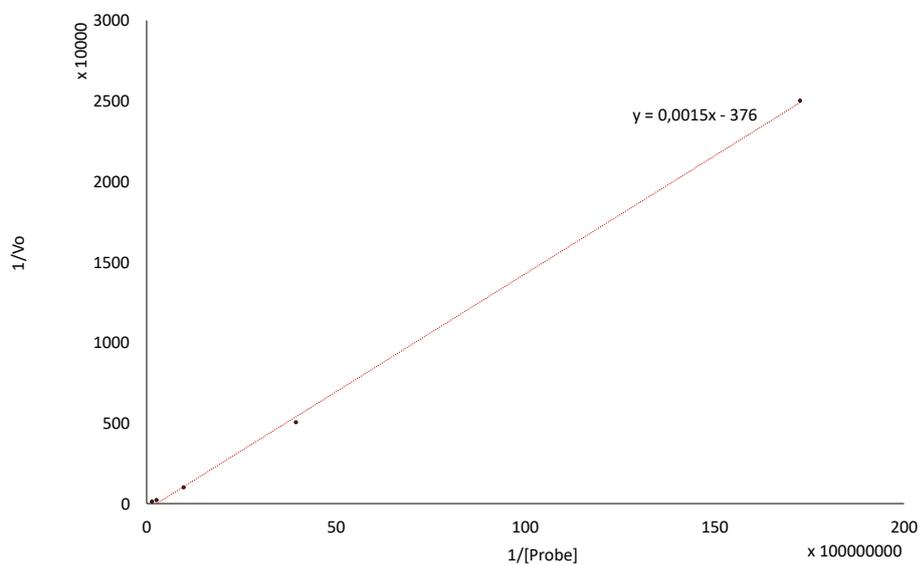


Fig. S15 . Initial velocity plots. RFU increase over the first 10 minutes of reaction

$$y \text{ intercept} = \frac{1}{V_{max}}$$

$$V_{max} = 4.0 \times 10^{-9} \text{ M/min}$$

$$x \text{ intercept} = \frac{-1}{K_M}$$

$$K_M = 2,7 \mu\text{M}$$

$$k_{cat} = \frac{V_{max}}{E_T}$$

$$k_{cat} = 0,04 \text{ s}^{-1}$$

$$\text{catalytic efficiency} = \frac{k_{cat}}{K_M}$$

$$\frac{k_{cat}}{K_M} = 1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$$

f. HL-60 differentiation and fluorescence microscopy

HL-60 were grown in RPMI-1640 medium + 10% FBS at 37°C in a humidified atmosphere (95% air, 5% CO₂). Cells, at the density of 2x10⁵/ml, were treated with all-trans retinoic acid (ATRA) (2 μM) for 5 days in order to induce neutrophil-like differentiation. Differentiation into neutrophils was assessed by an NBT assay³. After 3 hours of incubation, NBT (1 mM) was dissolved in 90% (v/v) DMSO, 0.1% (v/v) SDS and optical density (550 nm) was measured using an Infinite M200 PRO reader (Tecan). NET formation was monitored by SYTOX Orange quantification. In brief, after PMA treatment, cells were collected and incubated with micrococcal nuclease⁴ (0.5 U/ml) for 10 minutes at 37°C. EDTA (5 mM) was used to stop the reaction. Finally, supernatants containing NET-associated DNA were incubated with SYTOX Orange (5 μM) for 15 minutes at room temperature and fluorescence intensities were read using Infinite M200 PRO reader. Imaging of HL-60 NETS was performed with a Zeiss LSM710 confocal microscope, using cells cultured into chambered glass coverslips.

g. Confocal images of NETosis in primary human neutrophils

Neutrophils from healthy volunteers were isolated using a protocol approved by the Accredited Medical Regional Ethics Committee (AMREC, reference number 15-HV-013)⁵, (5 x 10⁴) and HNE-FQ (5 μM) were cultured in RPMI + 5% FBS media and stimulated with PMA (10 nM) in monolayer in an ibidi μ-Slide 8 well (3 h, 37°C, 5% CO₂). After culture, media was removed and cells were fixed with 2% PFA (30 min, RT). Cells were then washed with PBS and stained with SYTOX orange (1 μM) to stain extracellular DNA and Hoechst 33342 (100 nM) to stain the nuclei (30 min, RT, protected from light). The cell chamber was deconstructed, following manufacturer instructions and coverslip was mounted with ProLong Gold Antifade mounting media. NETs were imaged using Confocal Leica SP8 microscope.

References

1. S. Lebreton, S. E. How, M. Buchholz, B. E. Yingyongnarongkul and M. Bradley, *Tetrahedron*, 2003, **59**, 3945-3953.
2. A. Brunet, T. Aslam and M. Bradley, *Bioorg Med Chem Lett*, 2014, **24**, 3186-3188.
3. H. S. Choi, J. W. Kim, Y. N. Cha and C. Kim, *J Immunoassay Immunochem*, 2006, **27**, 31-44.
4. E. Pieterse, N. Rother, M. Garsen, J. M. Hofstra, S. C. Satchell, M. Hoffmann, M. A. Loeven, H. K. Knaapen, O. W. H. van der Heijden, J. H. M. Berden, L. B. Hilbrands and J. van der Vlag, *Arterioscler Thromb Vasc Biol*, 2017, **37**, 1371-1379.
5. R. D. Gray, C. D. Lucas, A. MacKellar, F. Li, K. Hiersemenzel, C. Haslett, D. J. Davidson and A. G. Rossi, *J Inflamm*, 2013, **10**, 12.