Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary information

Highly Selective and Rapidly Activatable Fluorogenic Thrombin Sensors and Application in Human Lung Tissue

Alicia Megia-Fernandez, Bethany Mills, Chesney Michels, Sunay V. Chankeshwara, Kevin Dhaliwal and Mark Bradley*

Contents:

Table S1

Figures S1-S4

MALDI-TOF MS spectra of probes before and after thrombin treatment

Materials and methods

Characterization table and HPLC chromatograms

Biology materials and methods

References

- **Table S1. Sequence and relative increase in fluorescence signal for probes 1-10 in the presence of proteolytic enzymes (compared to uncleaved probe).**

			Fold Change	Fold Change	Fold Change	Fold Change
			10 min	10 min	10 min	10 min
	Sequence		**rThrombin**	**Plasmin**	**rMMP-9 [83kDa]**	**Factor Xa**
Probe						
1	FAM-PEG2-**L-T-P-R-G-V-R-L-K**(MR)-NH2		2.29	1.45	1.12	1.08
2	FAM-PEG2-**L-W-P-R-G-V-R-L-K**(MR)-NH2		3.83	1.29	1.13	1.11
3	FAM-PEG2-**L-T-P-R-G-W-R-L-K**(MR)-NH2		4.77	1.82	1.18	1.20
4	FAM-PEG2-**L-W-P-R-G-W-R-L-K**(MR)-NH2		7.07	2.01	1.16	1.29
5	FAM-PEG2-**Ile-T-P-R-G-V-R-L-K**(MR)-NH2		15.05	6.24	1.18	1.29
6	FAM-PEG2-**Ile-T-P-R-G-W-R-L-K**(MR)-NH2		10.06	4.17	1.14	1.16
7	FAM-PEG2-**I-W-P-R-G-W-R-L-K**(MR)-NH2		2.53	1.39	1.16	1.16
8	FAM-PEG2-**Nle-T-P-R-G-V-R-L-K**(MR)-NH2		14.99	6.28	1.16	1.19
9	FAM-PEG2-**Nle-T-P-R-G-W-R-L-K**(MR)-NH2		9.62	5.33	1.12	1.06
10	FAM-PEG2-**Nle-W-P-R-G-W-R-L-K**(MR)-NH2		5.28	1.97	1.19	1.31

- **Figure S1: Background fluorescence of probes 1-10**

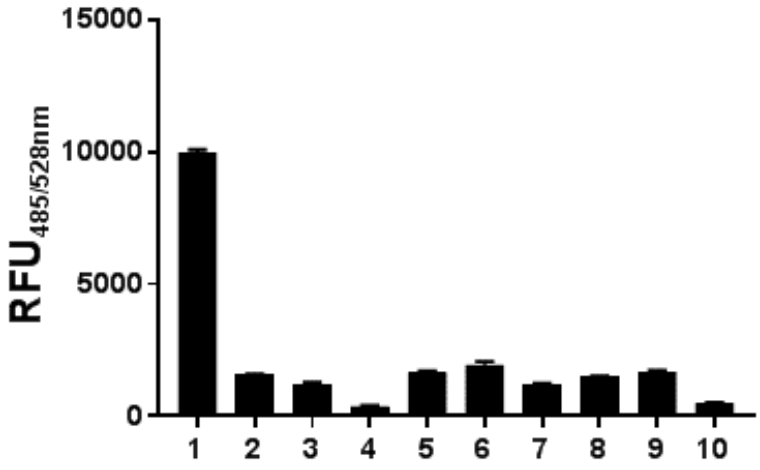


Figure S1. Relative fluorescent units (RFU) (ex/em 485/528nm) of probes **1-10** (10 µM) in buffer.

- **Figure S2: Relative increase (fold change) in fluorescence of probes 4, 7 and 10**

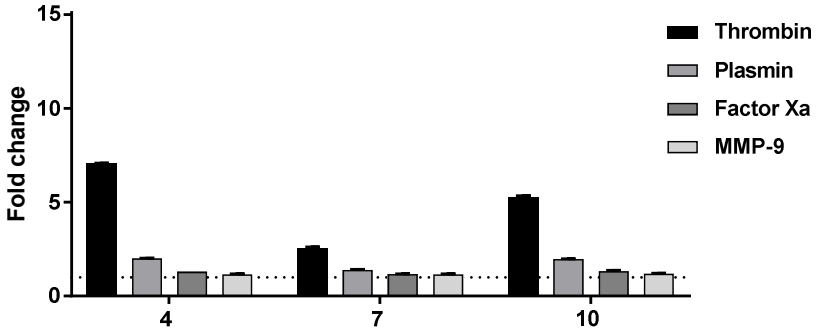


Figure S2. Relative increase (fold change) in fluorescence of probes **4**, **7** and **10** (10µM) after 10 min incubation with either Thrombin (5 U/ml), Plasmin (30 nM), Factor Xa (0.5 µM) or MMP-9 (83 kDa, 30 nM), compared to probe in buffer (ex/em 485/528nm).

- **Figure S3: Structure and MALDI-TOF MS of compound 8 before and after Thrombin and Neutrophil treatment.**

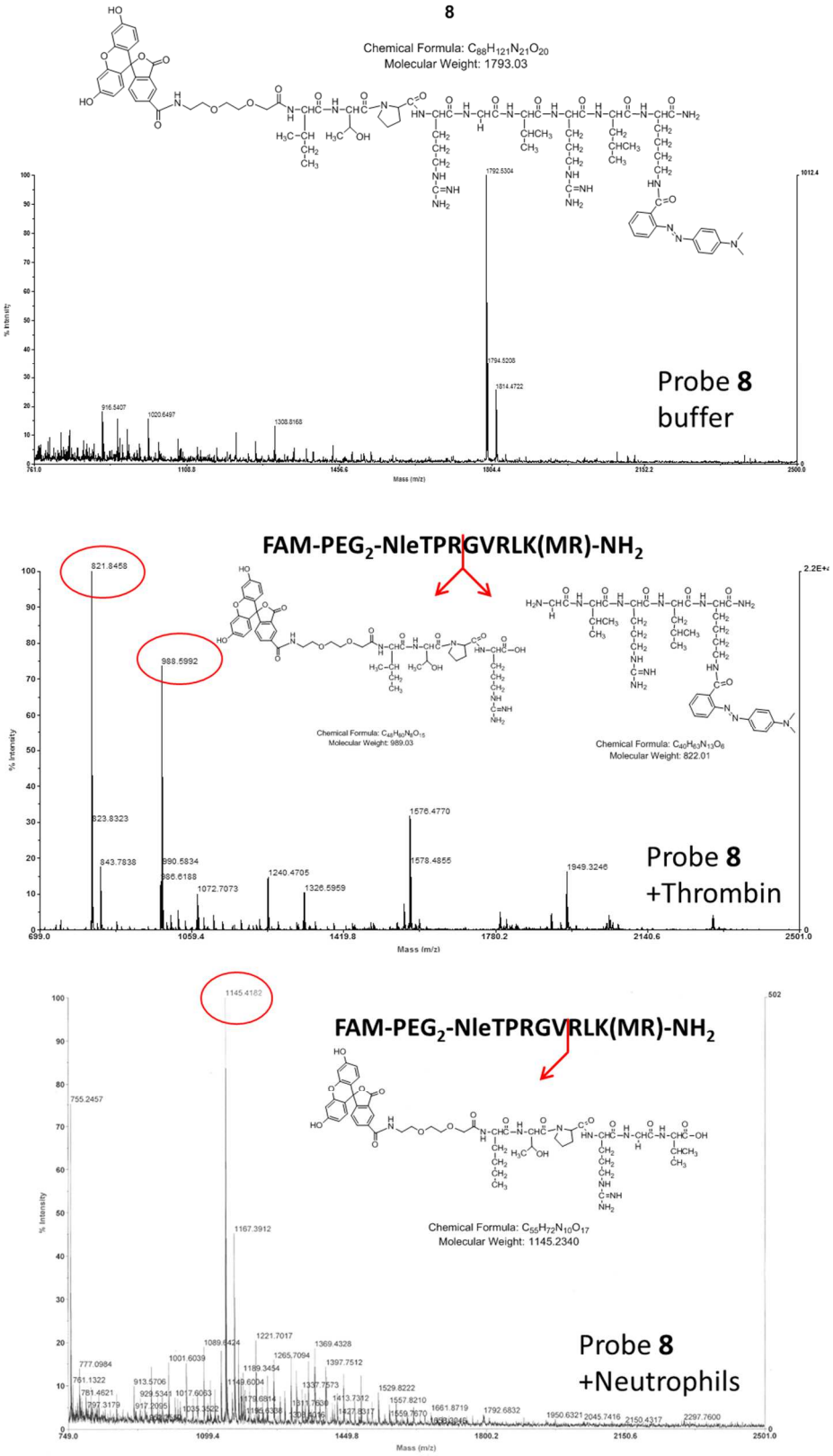


Figure S3. Peaks for fragments after selective cleavage (R-G) by Thrombin and non-selective cleavage (V-R) by neutrophils are highlighted.

Figure S4: Selectivity of probe 11 against panel of enzymes

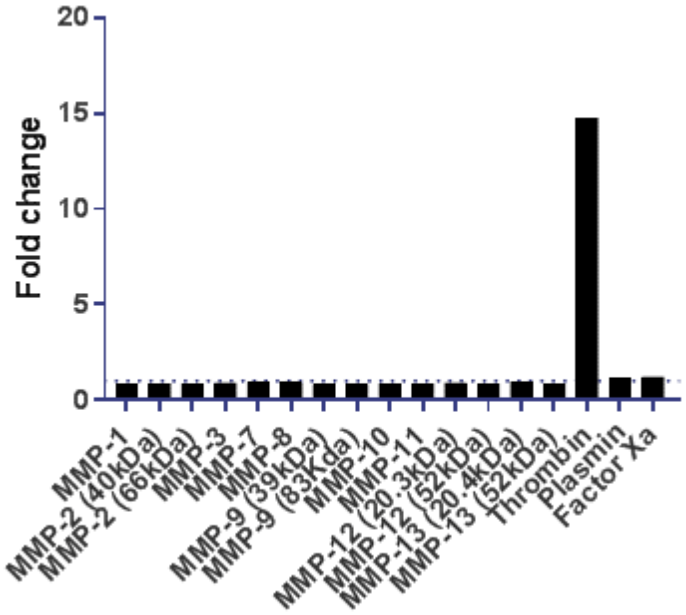
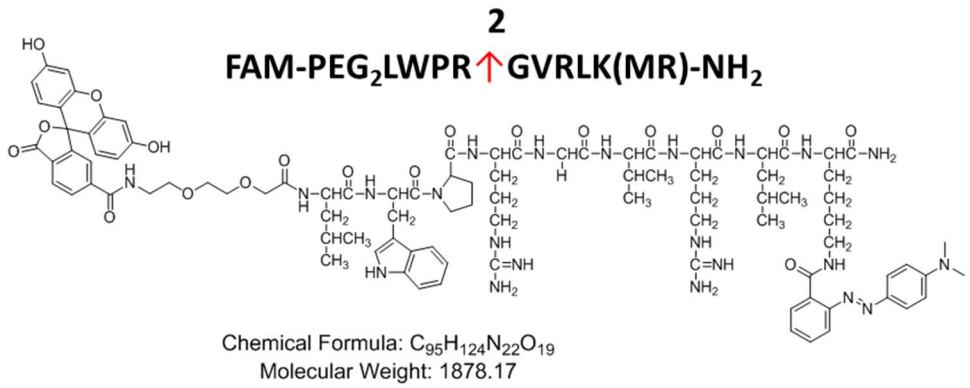
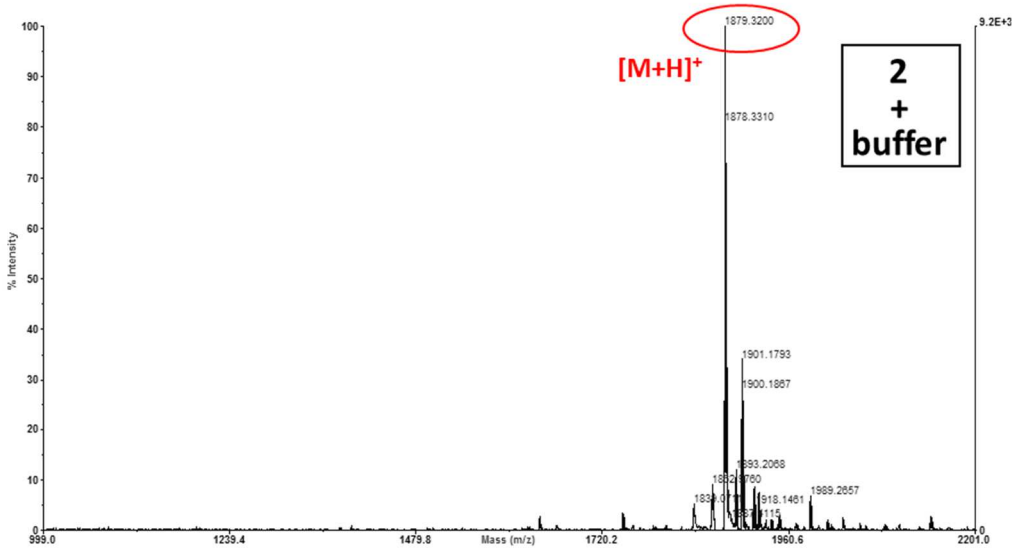
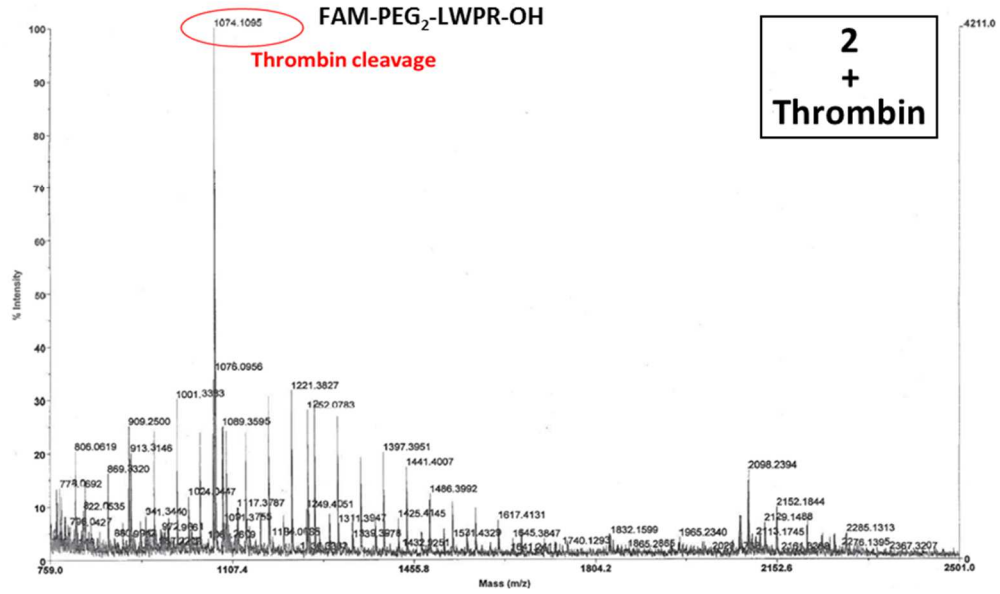


Figure S4. Data represents the change in fluorescence signal provided by probe **11** (10µM) with exogenous enzymes after 32 min using a multiwell plate fluorimeter at excitation/emission 485/528nm. Recombinant human MMPs -1, -2, -3, -7, -8, -9, -10, -11, -12, -13 were used at 30nM. Recombinant human Thrombin, Plasmin and Factor Xa were used at 5U/ml, 0.5µM and 30nM respectively.

- **MALDI TOF MS spectra of probes 2-10 before and after Thrombin treatment**

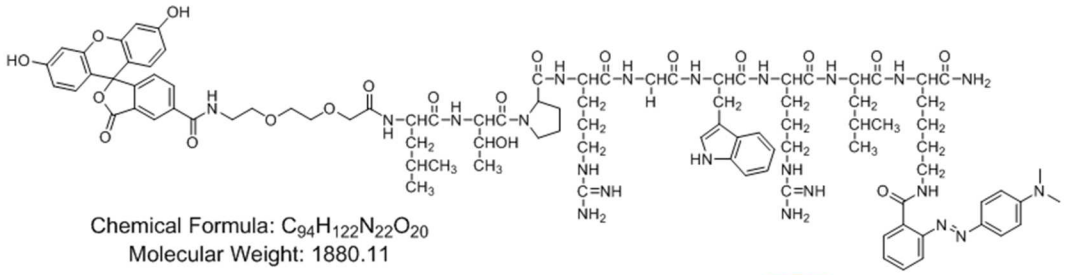




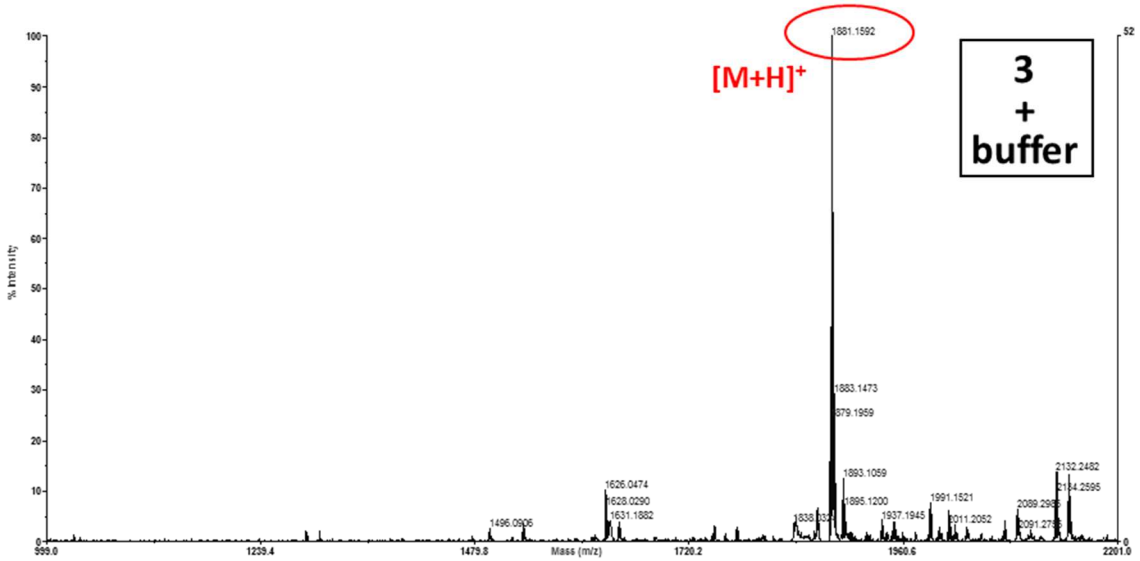


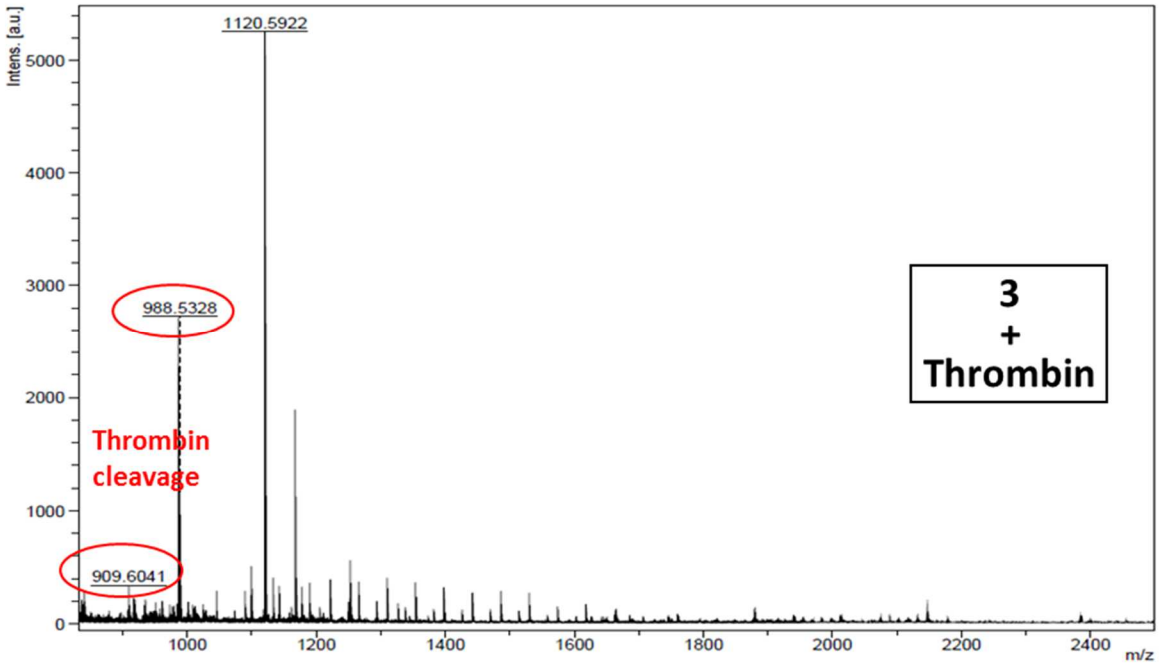
3

FAM-PEG$_2$LTPR↑GWRLK(MR)-NH$_2$



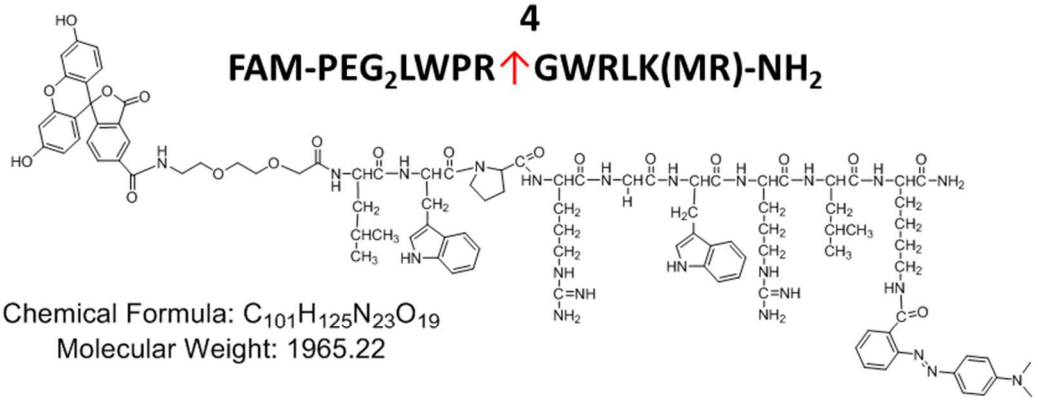
Chemical Formula: $C_{94}H_{122}N_{22}O_{20}$
Molecular Weight: 1880.11



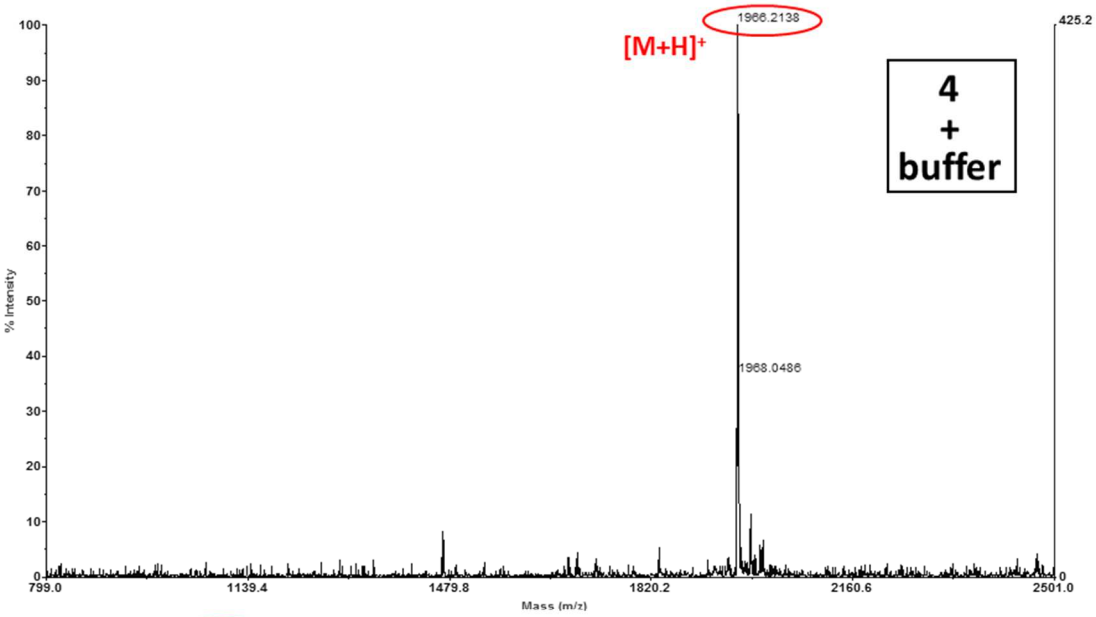


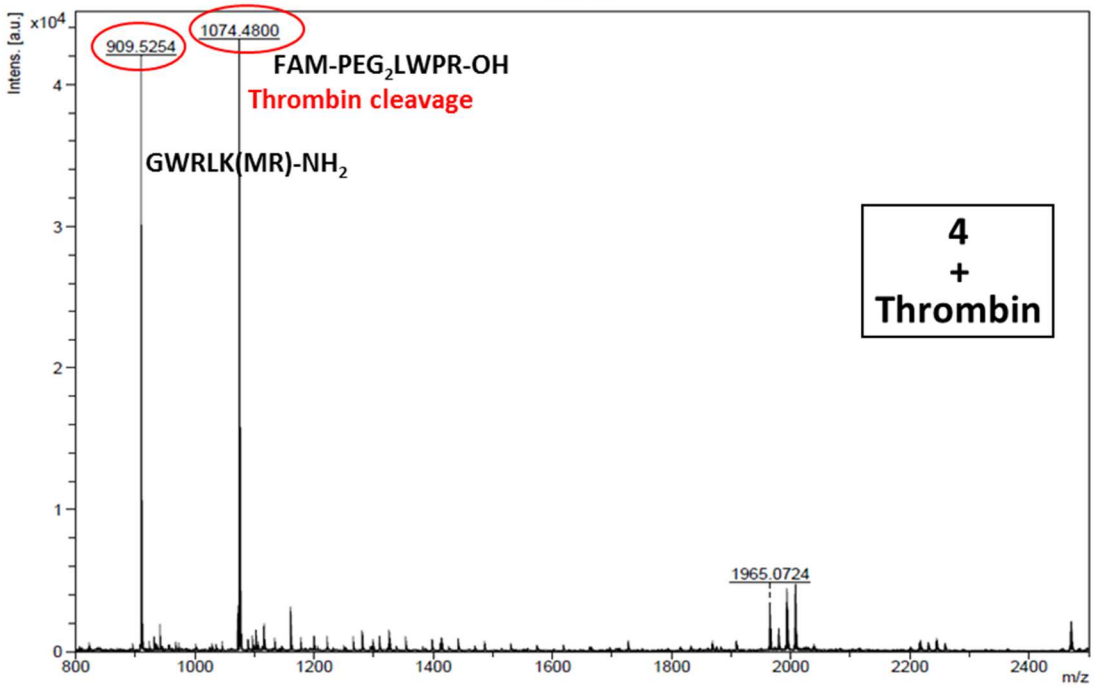
4

FAM-PEG$_2$LWPR↑GWRLK(MR)-NH$_2$



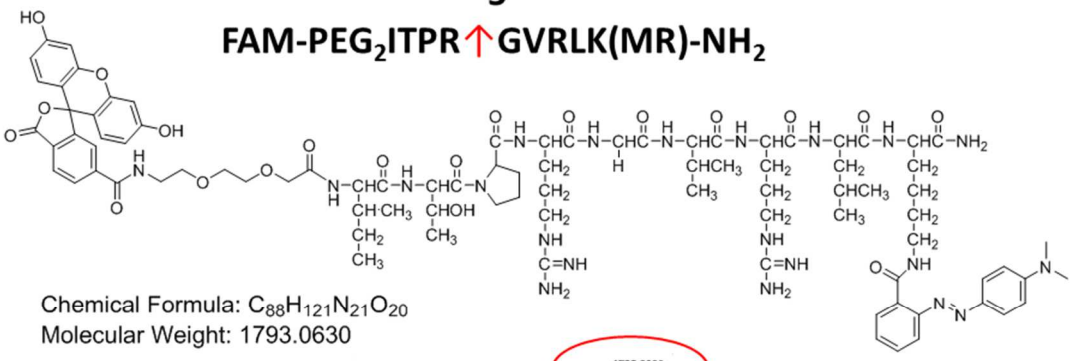
Chemical Formula: $C_{101}H_{125}N_{23}O_{19}$
Molecular Weight: 1965.22



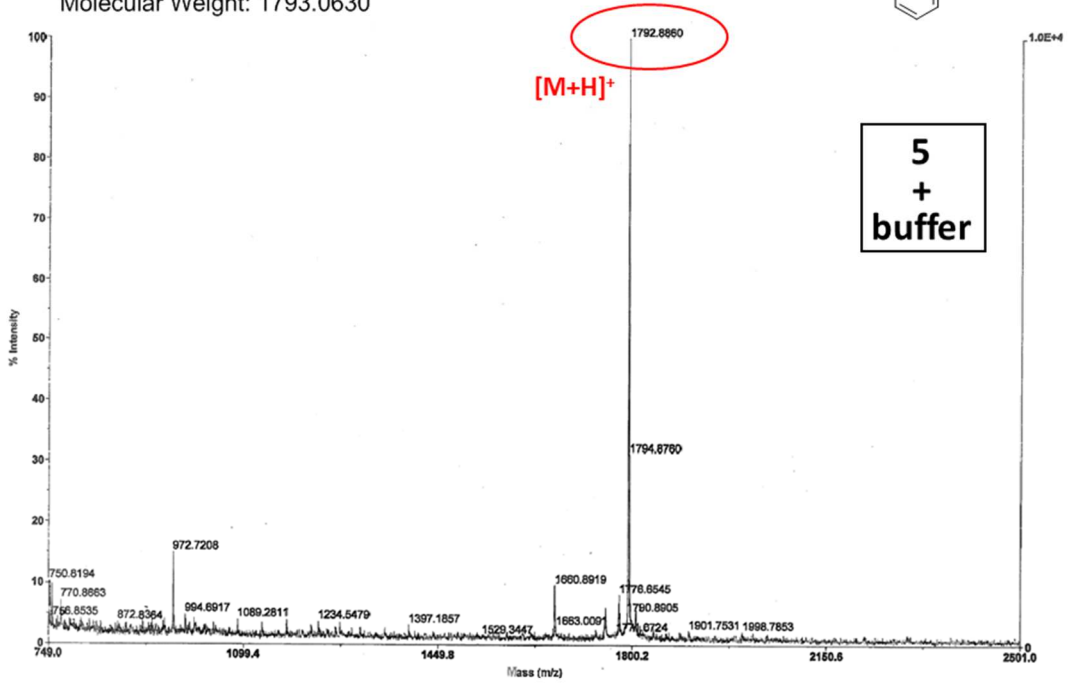


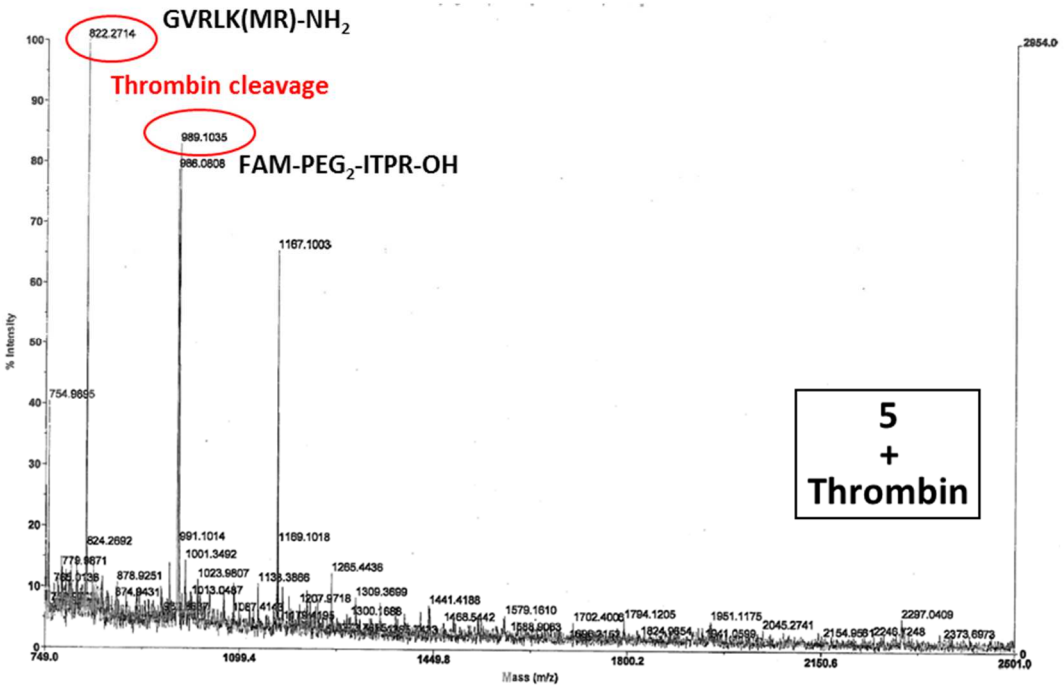
5

FAM-PEG$_2$ITPR↑GVRLK(MR)-NH$_2$



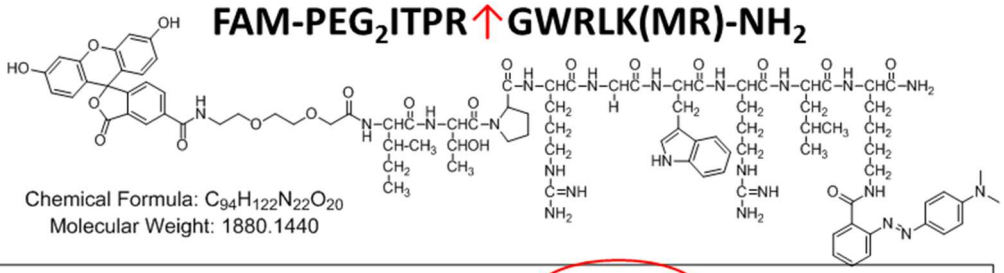
Chemical Formula: $C_{88}H_{121}N_{21}O_{20}$
Molecular Weight: 1793.0630



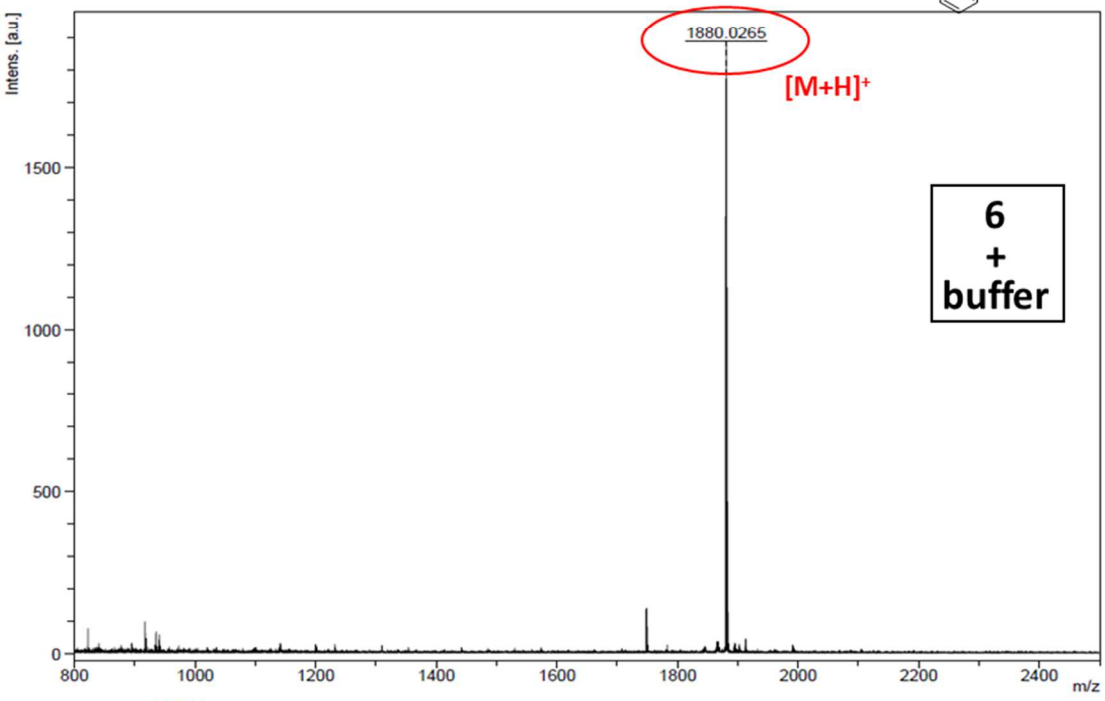


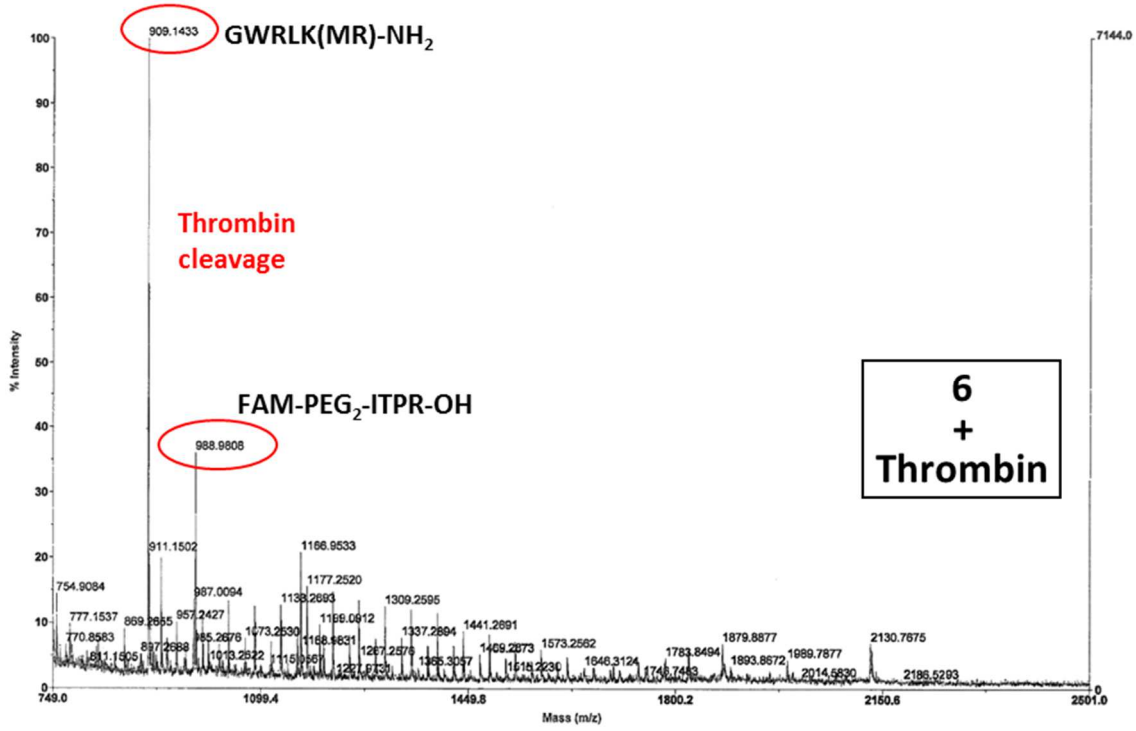
6

FAM-PEG$_2$ITPR↑GWRLK(MR)-NH$_2$



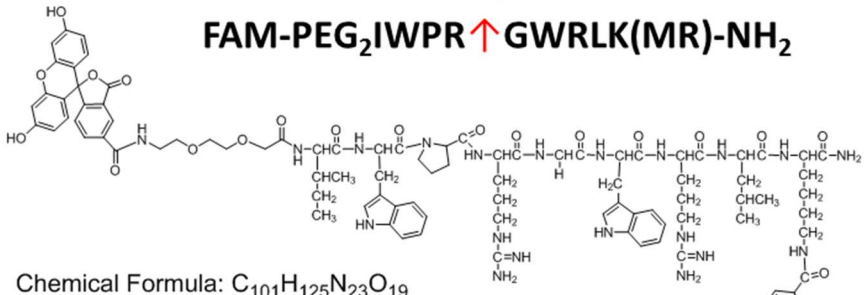
Chemical Formula: $C_{94}H_{122}N_{22}O_{20}$
Molecular Weight: 1880.1440



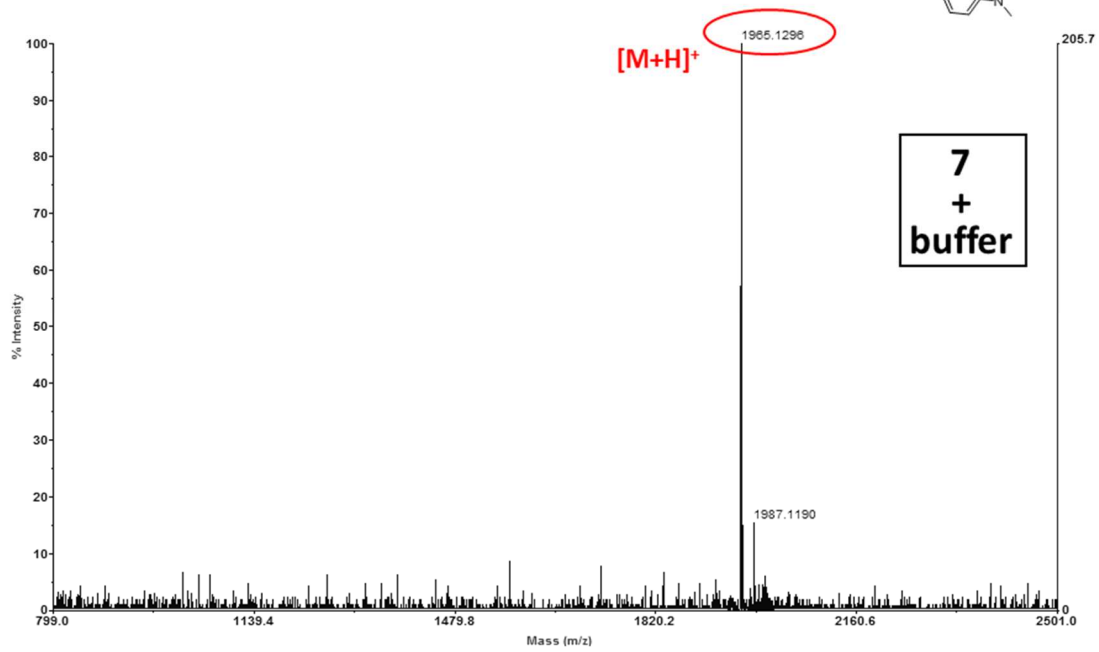


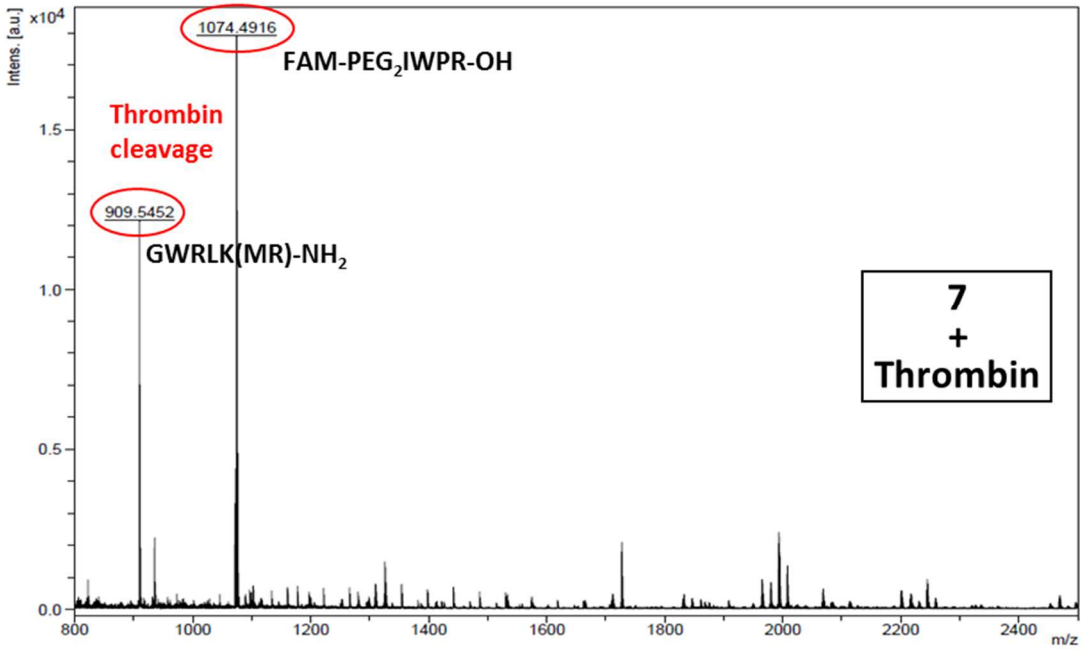
7

FAM-PEG$_2$IWPR↑GWRLK(MR)-NH$_2$



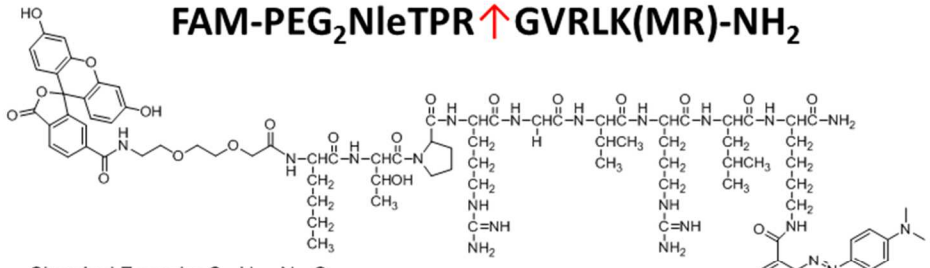
Chemical Formula: $C_{101}H_{125}N_{23}O_{19}$
Molecular Weight: 1965.2530



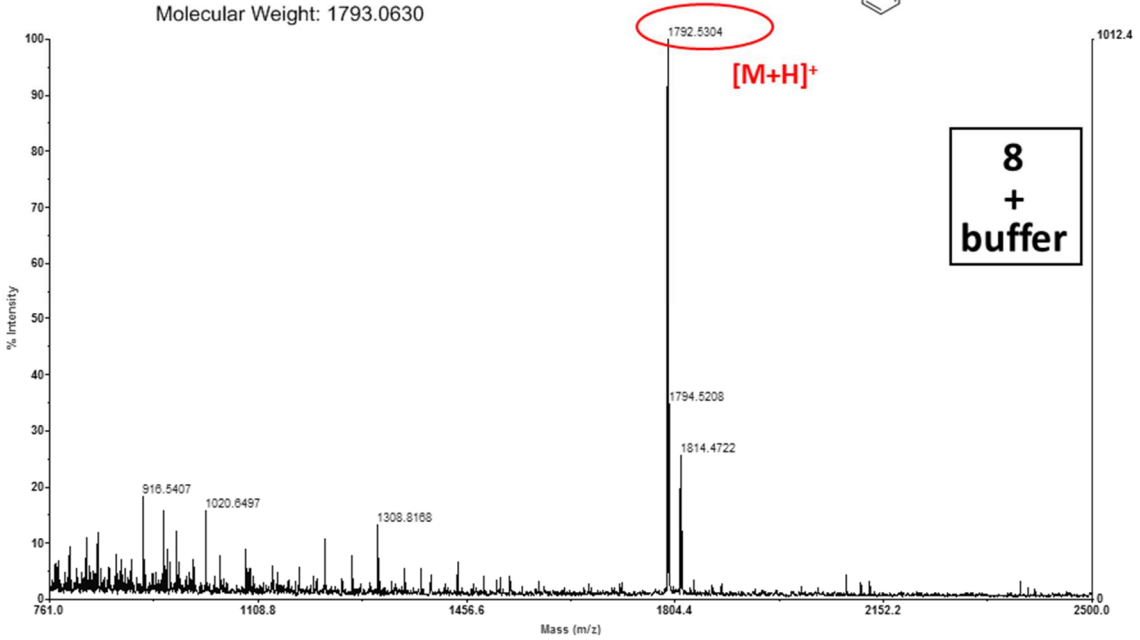


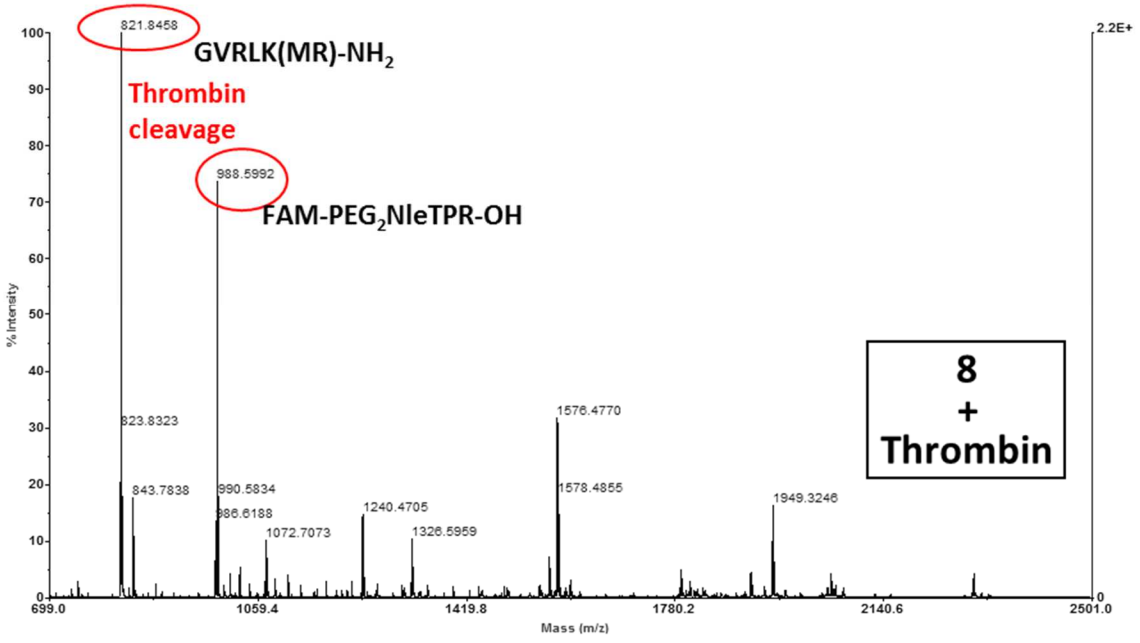
8

FAM-PEG$_2$NleTPR↑GVRLK(MR)-NH$_2$



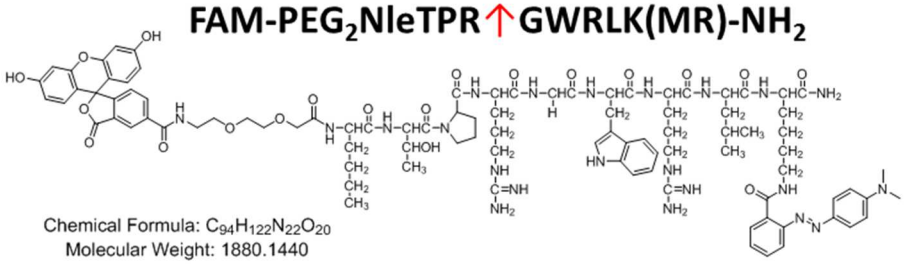
Chemical Formula: $C_{88}H_{121}N_{21}O_{20}$
Molecular Weight: 1793.0630



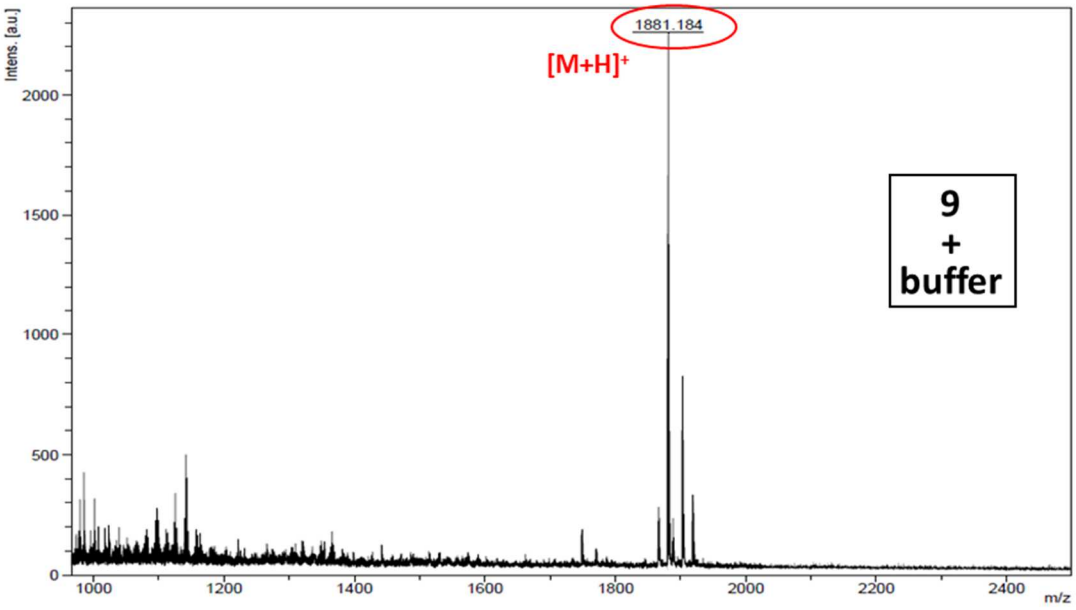


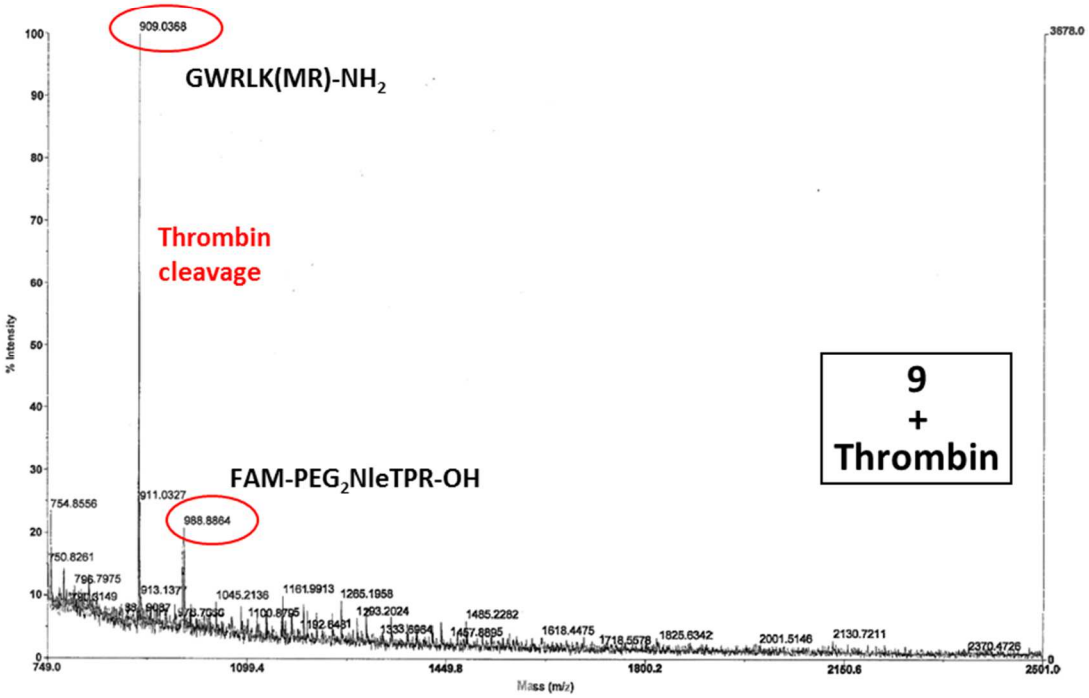
9

FAM-PEG$_2$NleTPR↑GWRLK(MR)-NH$_2$



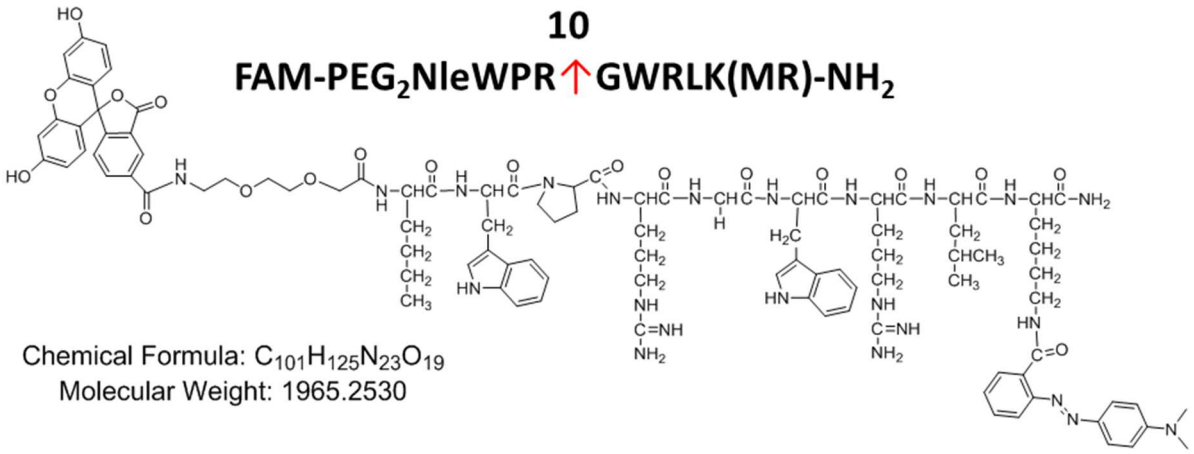
Chemical Formula: $C_{94}H_{122}N_{22}O_{20}$
Molecular Weight: 1880.1440



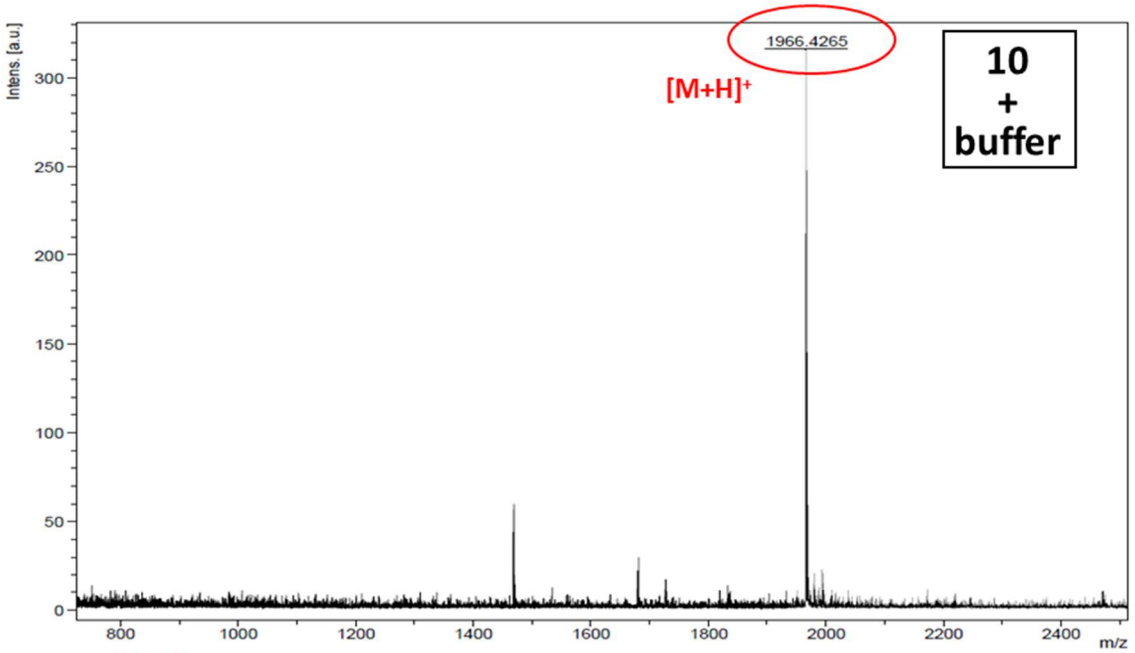


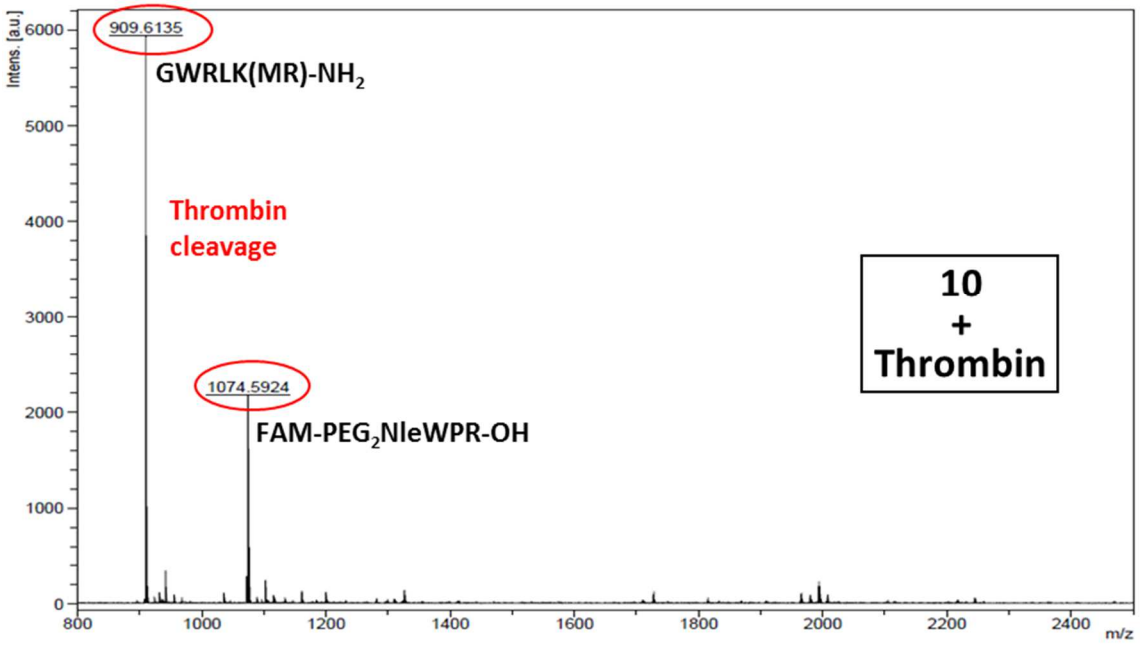
10

FAM-PEG$_2$NleWPR↑GWRLK(MR)-NH$_2$



Chemical Formula: $C_{101}H_{125}N_{23}O_{19}$
Molecular Weight: 1965.2530





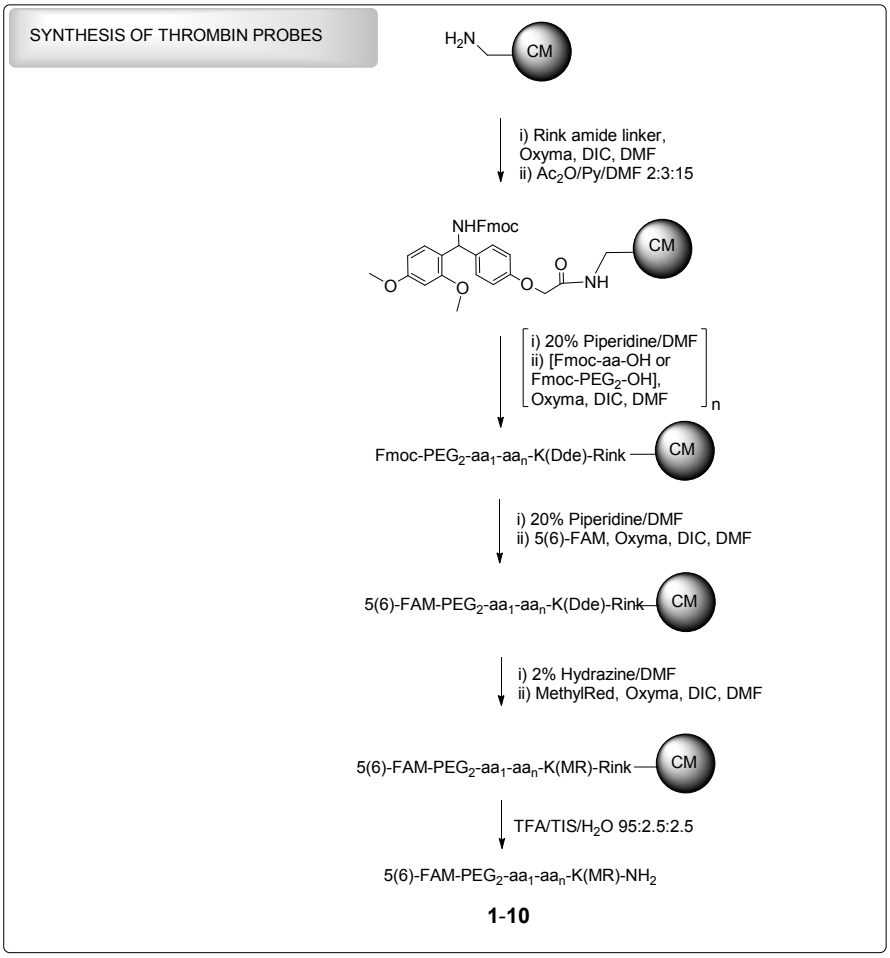
- ## Materials and methods

General

Commercially available reagents were used without further purification. NMR spectra were recorded using Brüker AC spectrometers operating at 500MHz for 1H. Chemical shifts are reported on the δ scale in ppm and are referenced to residual non-deuterated solvent resonances. Normal phase purifications by column chromatography were carried out on silica gel 60 (230-400 mesh). Analytical reverse-phase high-performance liquid chromatography (RP–HPLC) was performed on an HP1100 system equipped with a Discovery C18 reverse-phase column (5 cm x 4.6 mm, 5 μm) with a flow rate of 1 mL/min and eluting with 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B), with a gradient of 5 to 95% B over 10 min and additional isocratic period of 4 min (1) or over 6 min, holding at (B) for 2 min (2), with detection at 495nm and by evaporative light scattering (ELSD). Semi-preparative RP–HPLC was performed on an HP1100 system equipped with a Zorbax Eclipse XDB-C18 reverse-phase column (250 x 9.4 mm, 5 μm) with a flow rate 2.0 mL/min and eluting with 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B), with a gradient of 5 to 95% B over 30 min and additional isocratic period of 5 min. Electrospray ionization mass spectrometry (ESI–MS) analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) in an ESI mode. MALDI spectra were acquired on a Brüker Ultraflextreme MALDI TOF/TOF with a matrix solution of sinapic acid (10 mg/mL) in H_2O/CH_3CN/TFA (50/50/0.1).

Synthesis

The FRET peptides were synthesized by standard Fmoc solid-phase peptide chemistry. Dyes and quenchers were also coupled while the probe was on the solid support. General procedures are as follows. Peptide synthesis was performed on Aminomethyl-ChemMatrix resin using a Rink amide linker.



Scheme S1. Synthesis of Thrombin Probes **1-10**

Coupling of Rink amide linker: Fmoc-Rink linker (4-[(R,S)-a-[1-(9H-Fluoren-9-yl)-methoxy-formamido]-2,4-dimethoxybenzyl-phenoxyacetic acid) (0.54 g, 1.0 eq) was dissolved in DMF (10 mL) and Oxyma (0.14 g, 1.0 eq) was added and the mixture was stirred for 10 min. Diisopropylcarbodiimide (DIC, 155 µL, 1.0 eq.) was then added and the solution stirred for 1 min before adding it to Aminomethyl-Polystyrene resin (1.0 eq). The resulting mixture was stirred at 50^0C for 45 min and the after that the resin was washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL). Finally the resin was treated with Ac_2O:Py:DMF (2:3:15) for 30 min in order to cap any remaining free amino groups and it was washed again with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL).

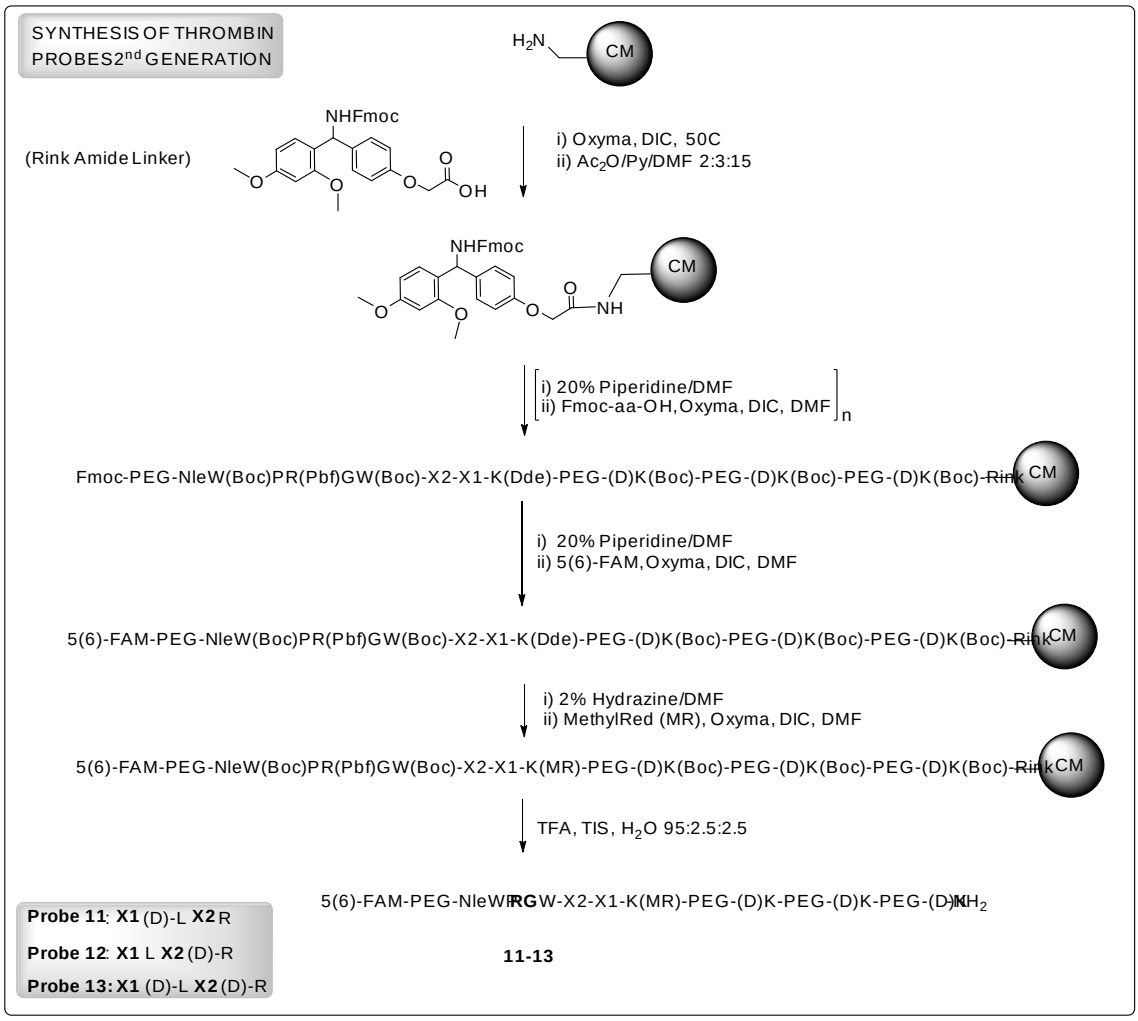
Fmoc deprotection: In general, to the resin pre-swollen in DCM was added 20% piperidine in DMF and stirred (2x10 min). The solution was drained and the resin washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL).

Amino acid coupling: A solution of the appropriate L-amino acid (3.0 eq per amine) and Oxyma (3.0 eq) in DMF (0.1M) was stirred for 10 min. DIC (3.0 eq) was added and stirred for 1 min. The pre-activated mixture was then added to the resin previously swollen in DCM and the reaction heated at 50^0C for 30 min. The solution was drained and washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL). The completion of the coupling reactions were monitored by the Kaiser test (or Chloranil test when secondary amines are involved). The side chain protecting groups used were Boc for Lysine and Tryptophan and Pbf for Arginine. Fmoc-Lys(Dde)-OH was used to allow dye introduction.

Coupling of other carboxylic acids: Coupling of [2-[2-(Fmoc-amino)ethoxy]ethoxy] acetic acid (PEG), MethylRed (MR) and 5(6)-Carboxyfluorescein (FAM) was carried-out following the same procedures as described for the amino acid couplings.

Dde deprotection: to the resin (pre-swollen in DCM) was added 2% hydrazine in DMF and stirred (5x10 min). The solution was drained and the resin washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL).

Cleavage and purification: Before cleavage, the resin was washed with 20% piperidine to remove any fluorescein phenol esters.[1] The resin pre-swollen in DCM was treated with a cleavage cocktail of TFA:triisopropylsilane(TIS):water (95:2.5:2.5) for 3h. The reaction solution was drained and the resin washed again with the cleavage cocktail. The combined TFA solutions were treated with cold ether to precipitate the peptide, centrifuged (x3) and purified by RP-HPLC on a C_{18} semi-preparative column. The desired fractions containing the product were collected and lyophilized to afford the products (characterized by MALDI and analytical HPLC).



Scheme S2. Synthesis of Thrombin Probes **11-13**

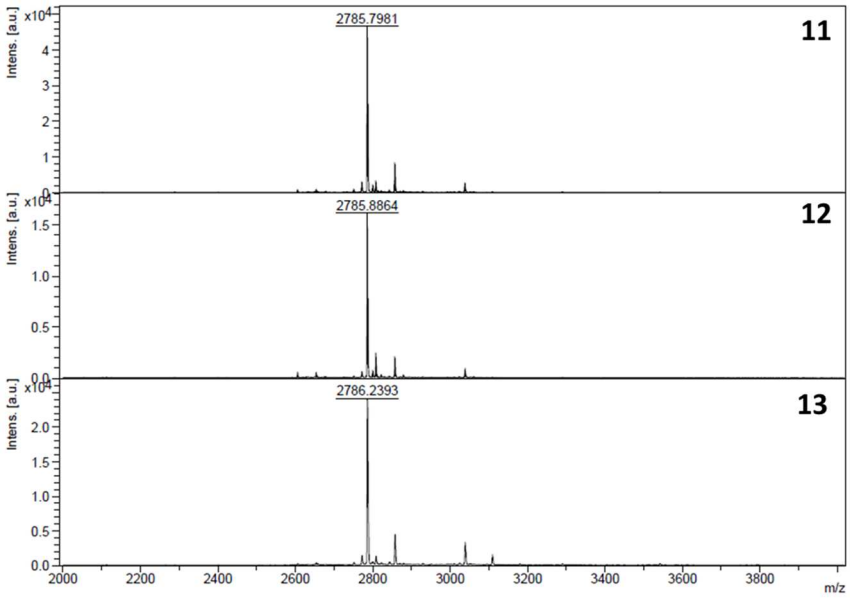


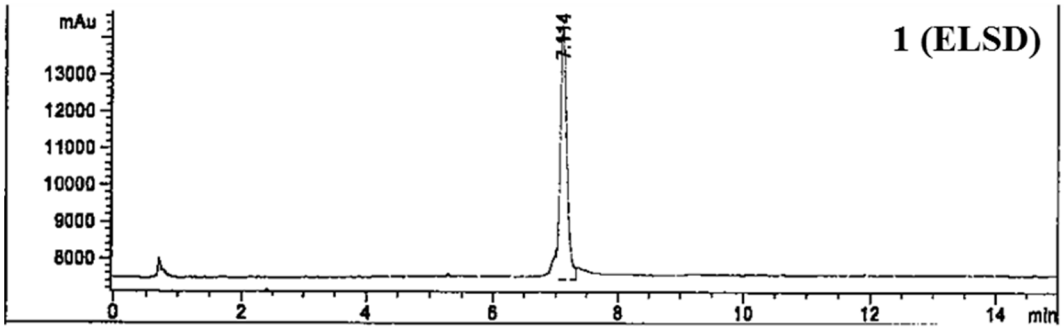
Figure S5. MALDI-MS spectra of synthesised probes **11-13**

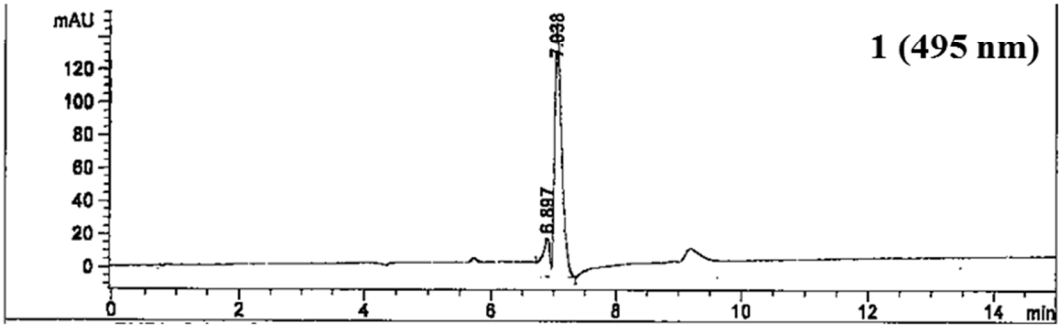
- Characterization table

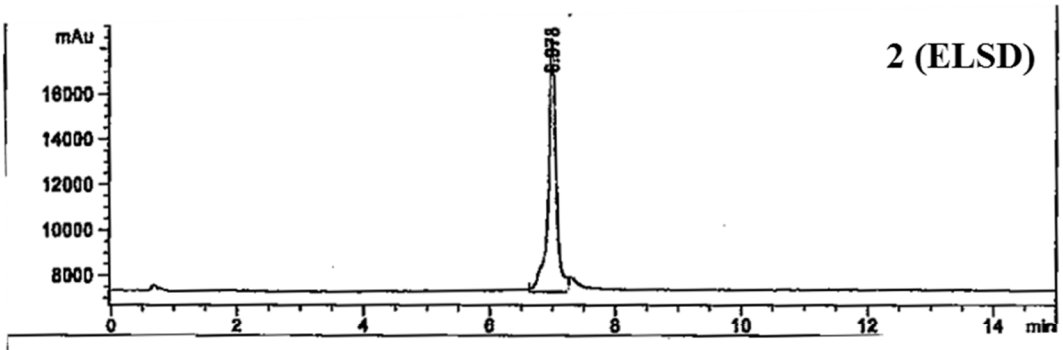
Probe	5(6)-FAM-PEG_2-**Sequence**	**MW** (Da)	**MALDI** m/z_{obs} (Da)[a]	**HPLC** t_R (min)
1	-L-T-P-R-G-V-R-L-K(MR)-NH_2	1793.06	1794.07	7.114[b]
2	-L-W-P-R-G-V-R-L-K(MR)-NH_2	1878.17	1879.32	6.915[b]
3	-L-T-P-R-G-W-R-L-K(MR)-NH_2	1880.14	1881.16	7.551[b]
4	-L-W-P-R-G-W-R-L-K(MR)-NH_2	1965.25	1966.21	5.611[b]
5	-I-T-P-R-G-V-R-L-K(MR)-NH_2	1793.06	1792.89	3.511[c]
6	-I-T-P-R-G-W-R-L-K(MR)-NH_2	1880.14	1880.03	5.621[c]
7	-I-W-P-R-G-W-R-L-K(MR)-NH_2	1965.25	1965.13	4.063[c]
8	-Nle-T-P-R-G-V-R-L-K(MR)-NH_2	1793.06	1792.53	3.618[c]
9	-Nle-T-P-R-G-W-R-L-K(MR)-NH_2	1880.14	1881.18	6.030[c]
10	-Nle-W-P-R-G-W-R-L-K(MR)-NH_2	1965.25	1966.43	4.177[c]
11	-Nle-W-P-R-G-W-R-(*D*)L-K(MR)-(PEG_2-(*D*)K)$_3$-NH_2	2785.25	2785.80	6.590[b]
12	-Nle-W-P-R-G-W-(*D*)R-L-K(MR)-(PEG_2-(*D*)K)$_3$-NH_2	2785.25	2785.89	4.035[c]
13	-Nle-W-P-R-G-W-(*D*)R-(*D*)L-K(MR)-(PEG_2-(*D*)K)$_3$-NH_2	2785.25	2786.24	4.086[c]

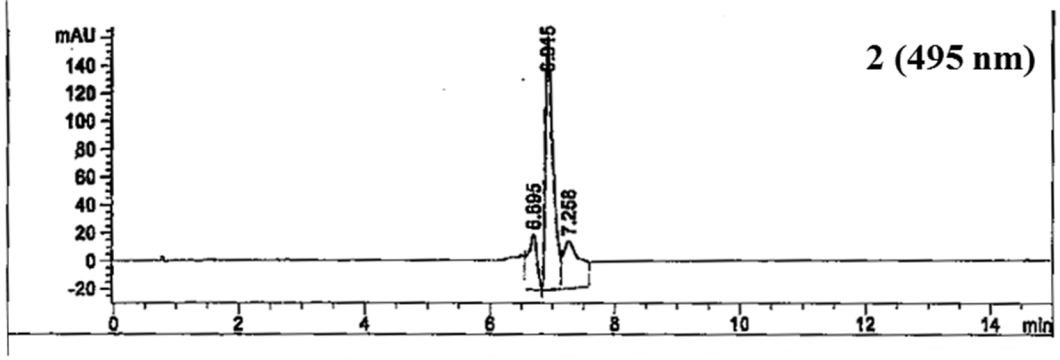
[a] m/z observed corresponding to $[M+H]^+$; [b]Elution with 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B), with a gradient of 5 to 95% B over 10 min and additional isocratic period of 4 min. [c]Elution with 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B), with a gradient of 5 to 95% B over 6 min, holding at (B) for 2 min.

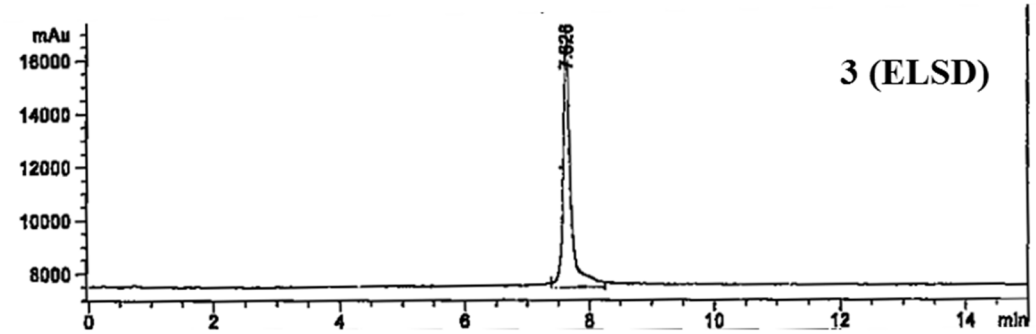
HPLC Chromatogram of compounds 1-13



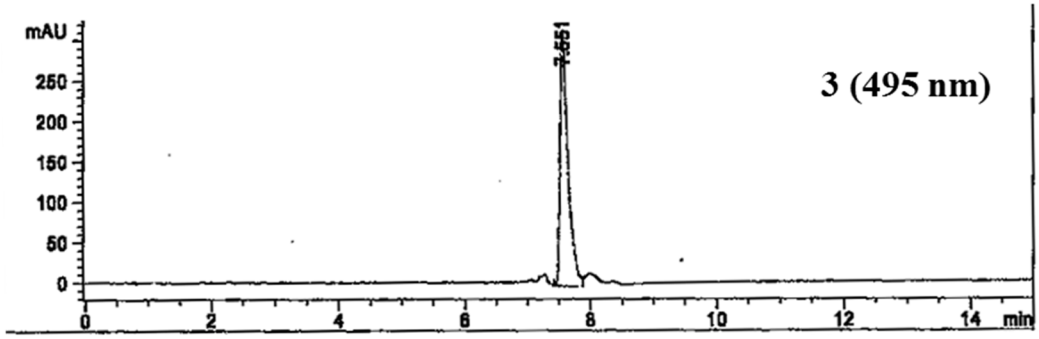




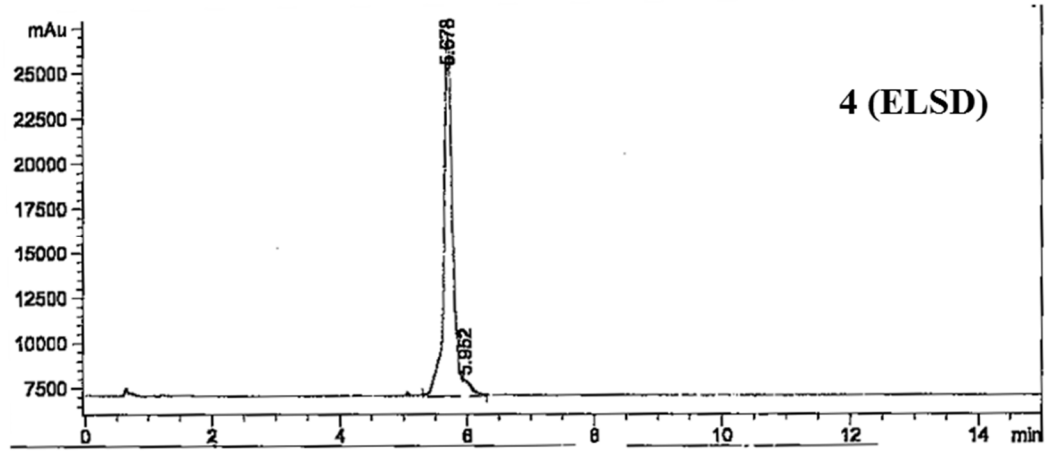




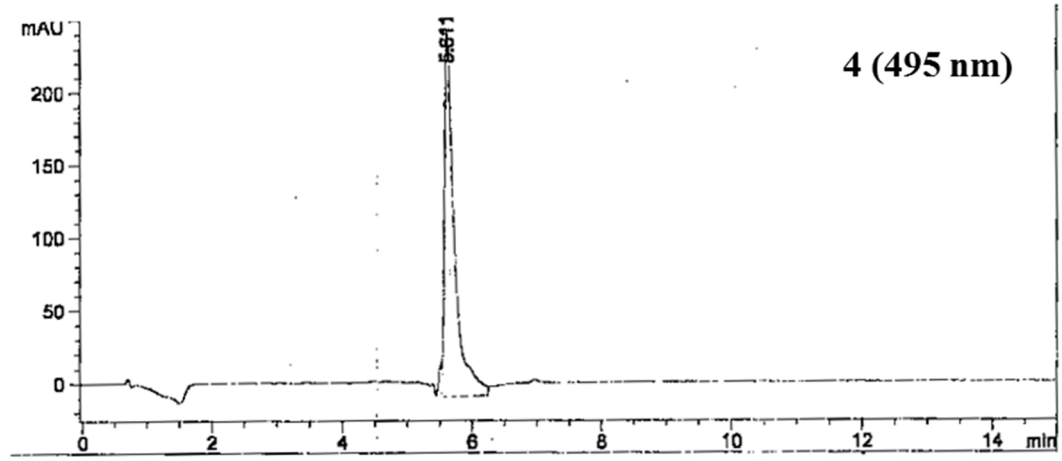
3 (ELSD)



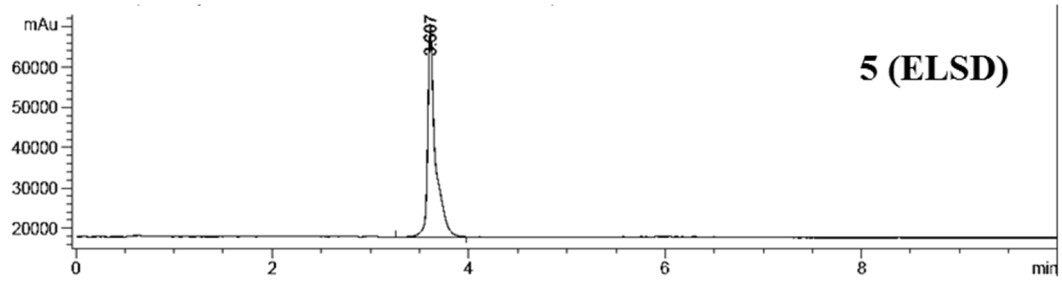
3 (495 nm)



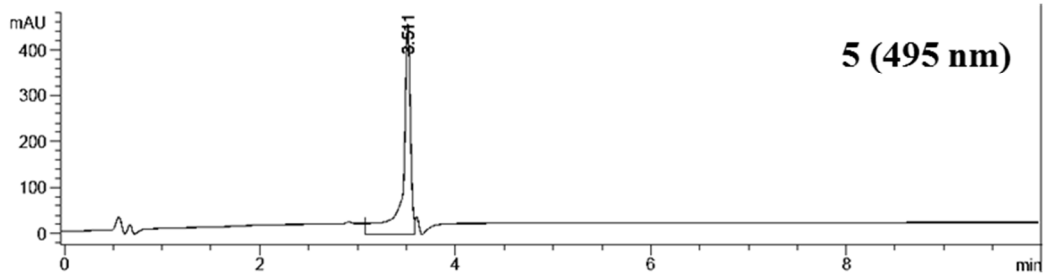
4 (ELSD)



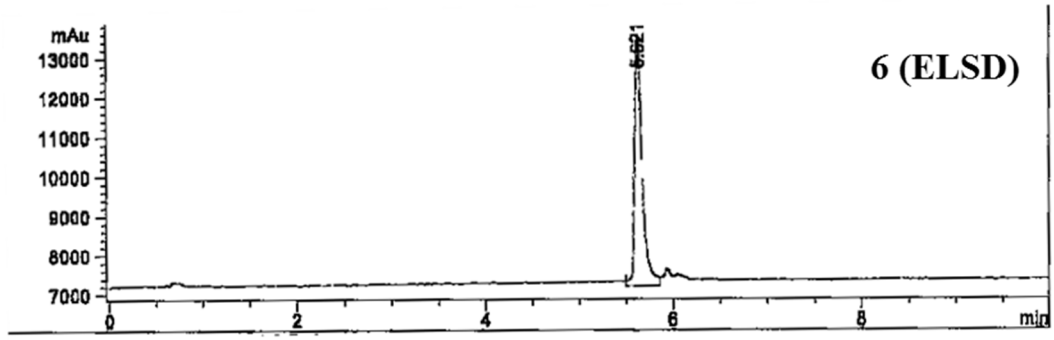
4 (495 nm)



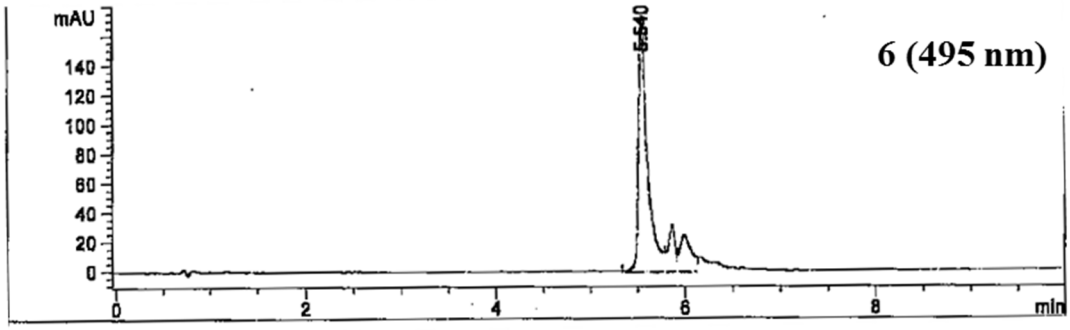
5 (ELSD)



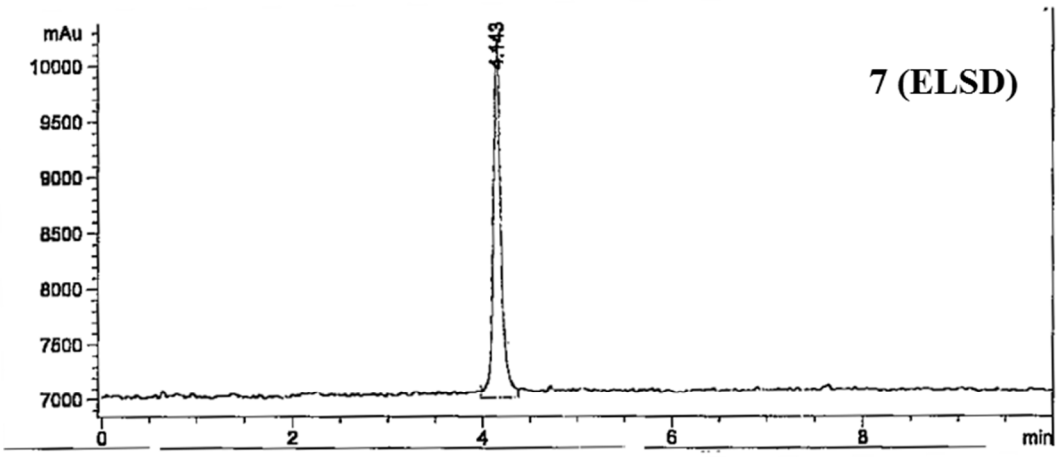
5 (495 nm)



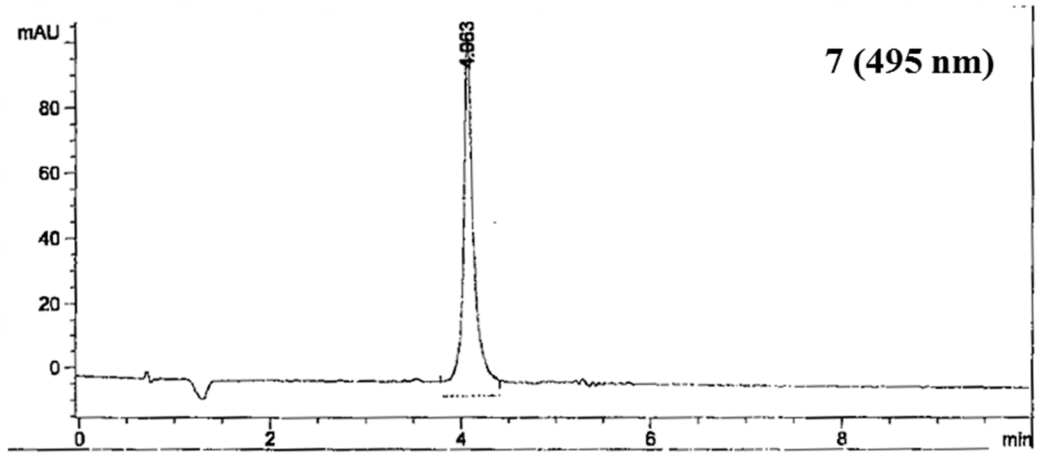
6 (ELSD)



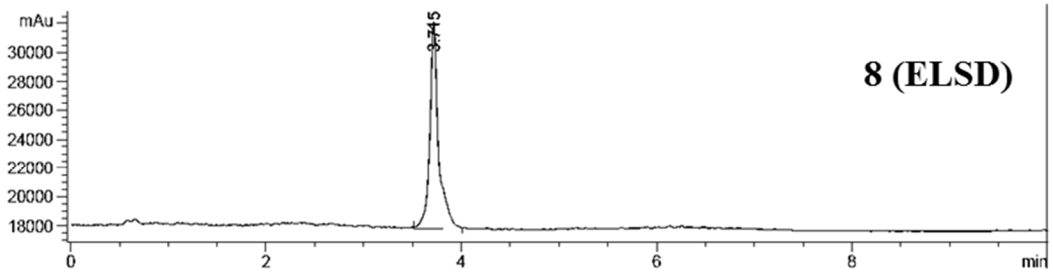
6 (495 nm)



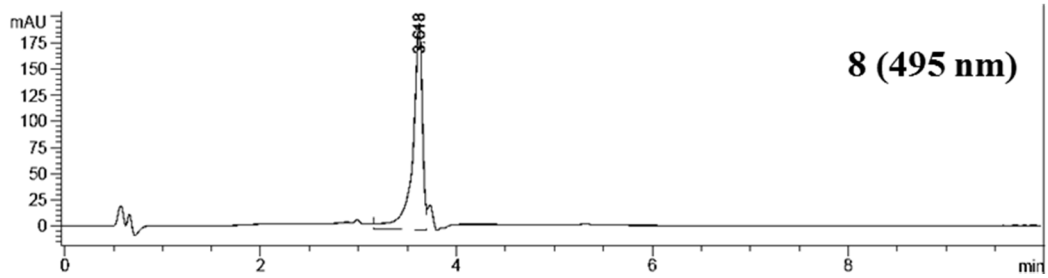
7 (ELSD)



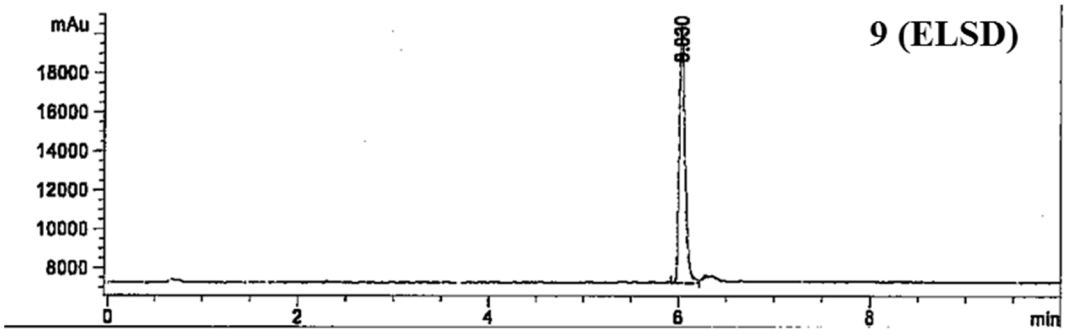
7 (495 nm)



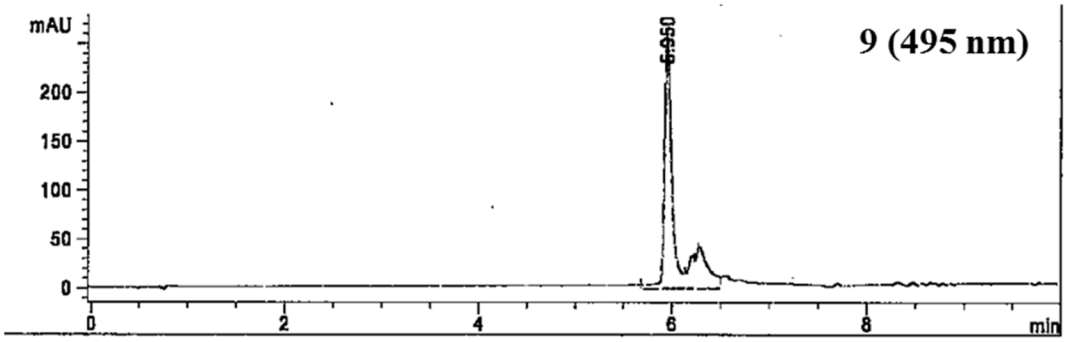
8 (ELSD)



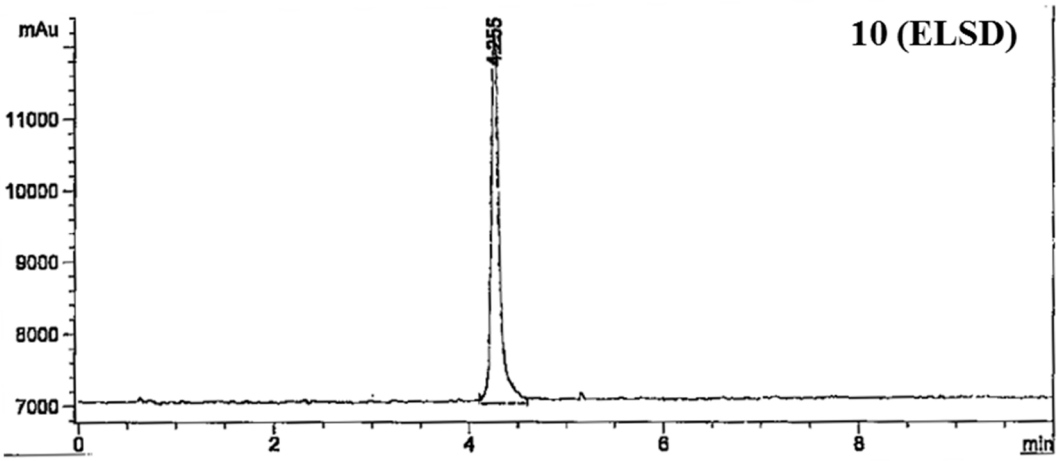
8 (495 nm)



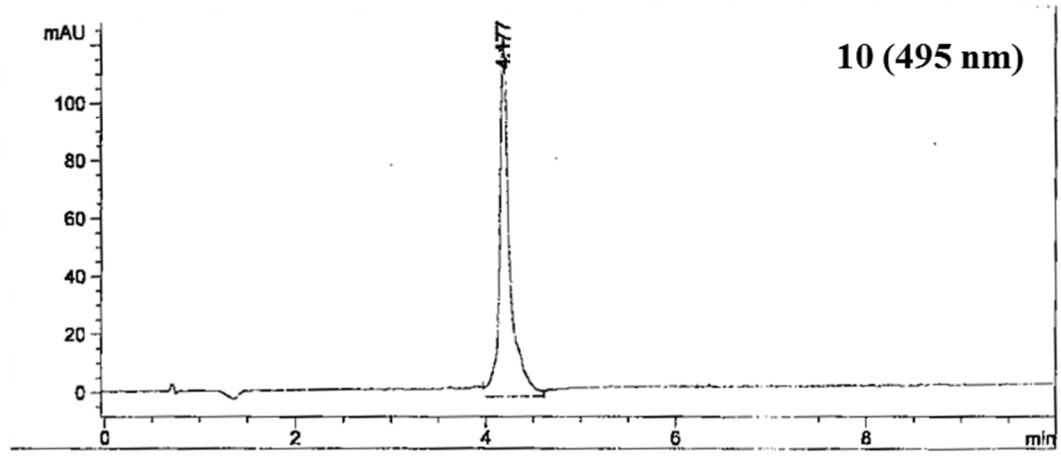
9 (ELSD)



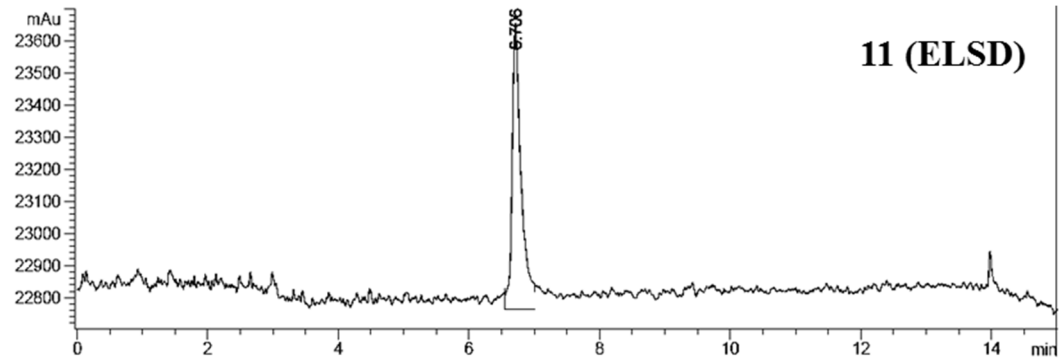
9 (495 nm)



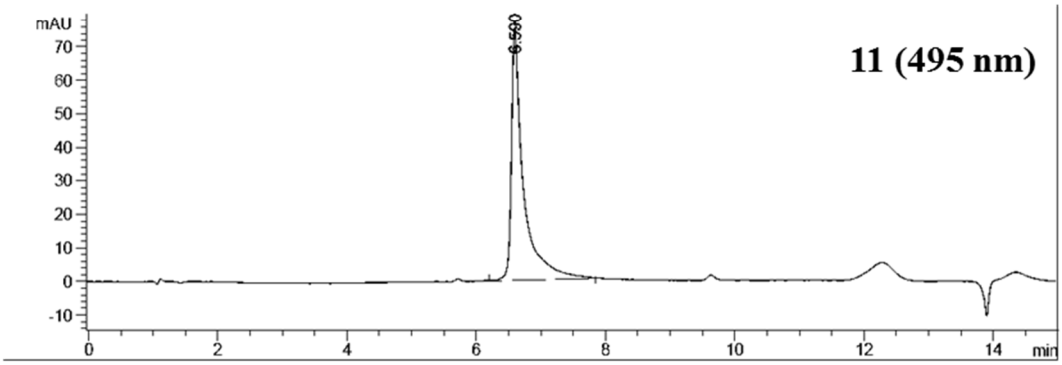
10 (ELSD)



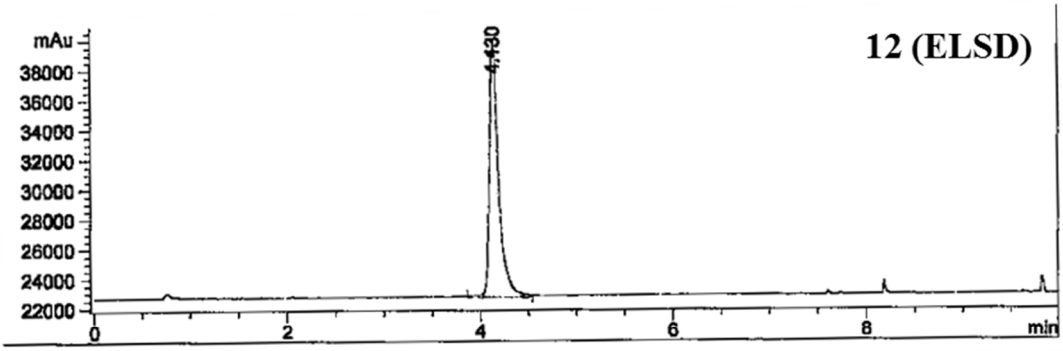
10 (495 nm)



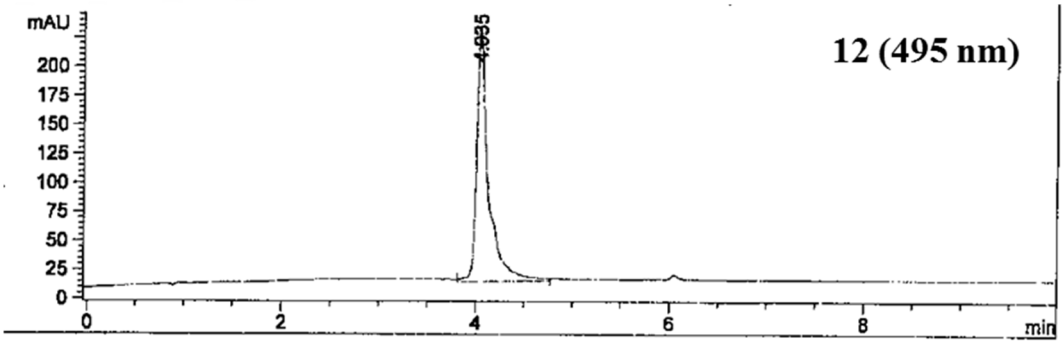
11 (ELSD)



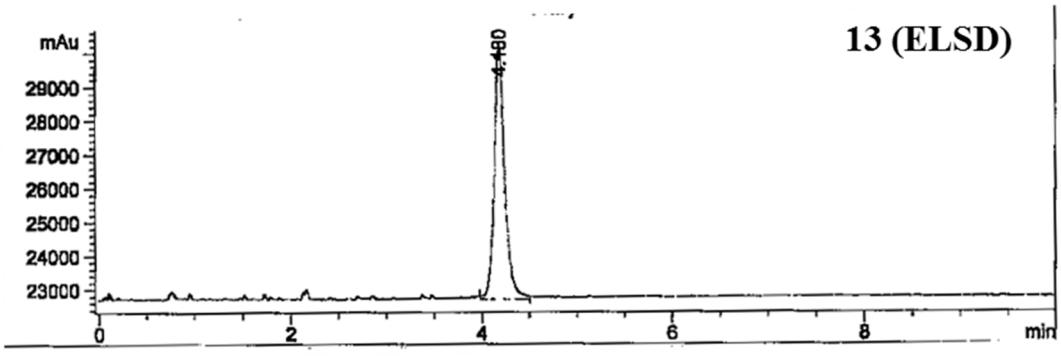
11 (495 nm)

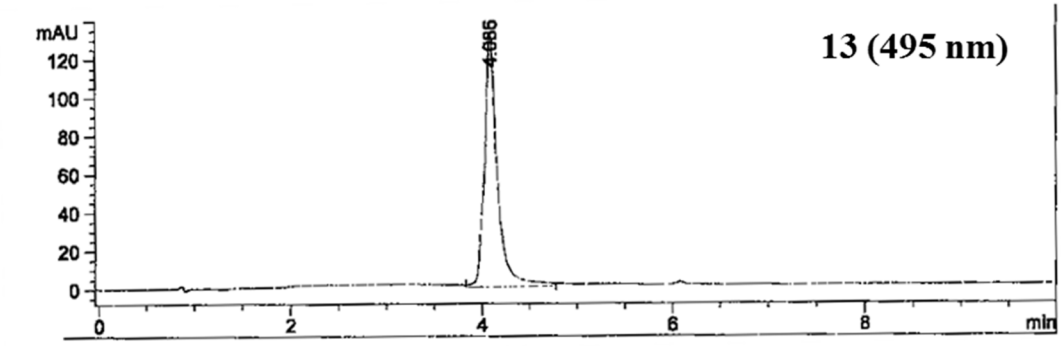


12 (ELSD)



12 (495 nm)


13 (ELSD)
mAu
29000
28000
27000
26000
25000
24000
23000
4.180
0
2
4
6
8
min


13 (495 nm)
mAU
120
100
80
60
40
20
0
4.085
0
2
4
6
8
min

- Biology materials and methods

Neutrophil preparation: The number of retrieved neutrophils from whole human blood was determined with NucleoCounter NC-1000 (Chemo Metec). Neutrophils were resuspended at a concentration of 20 x 10^6 per mL in 0.9 % NaCl with 0.9 mM $CaCl_2$. Cells were incubated for 30 minutes at 37°C. Cells to be stimulated were subsequently activated with 5 µM calcium ionophore A23187 (Tocris Bioscience) for 30 minutes at 37 °C. Neutrophils were harvested by centrifugation at 400 x *g* for 5 min. Supernatants were removed and stored. The cell pellets were suspended in an original volume of 0.9 % NaCl and lysates were harvested by repeated cycles of snap freeze-thaw in dry-ice and acetone followed by a further centrifugation step.

Monocyte Derived Macrophage (MDM): Mononuclear cells recovered from blood prep and washed x2 with PBS [without Calcium, Magnesium] and counted with NucleoCounter NC-1000. Monocytes were resuspended at 4x10^6 cells per mL in IMDM (containing 1% Pen/strep, no serum) and seeded into a T-25 tissue culture flask. Cells were incubated for 1 hour at 37°C, then washed gently 3x to remove non-adherent cells using IMDM (+ pen/strep). IMDM (containing 10% autolog. [human] Serum and 1% pen/strep) made up as appropriate and added to cells. The flask was incubated for 5-7 days. The growth media was replaced (by 50 % volume) every 48 hours. MDMs were harvested from the flask and lysed as described above for neutrophils.

Determining k_{cat}/K_m: Stock solutions of probe **11** were prepared in MMP buffer at concentrations of 2x10^{-5}, 1x10^{-5}, 5x10^{-6}, 2.5x10^{-6}, 1.25x10^{-6} and 6.25x10^{-7} M. Probe solutions were mixed 1:1 with 0.8 nM (8x10^{-10} M), final enzyme concentration was 4x10^{-10}M. All conditions were plated in triplicate and repeated twice. Fluorescence increase was measured by plate reader, with measurements taken every 20 seconds for 60 minutes. Initial reaction rates were calculated from the first 10 minutes of reaction. The gradient of each slope was calculated by plotting linear regression, with r^2 values measured at 0.97 or above. k_{cat} and K_m values were calculated using software features of Prism 7 (GraphPad).

- References

1. R. Fischer, O. Mader, G. Jung and R. Brock, *Bioconjugate Chem.*, 2003, **14**, 653-660.