chemosensors

MDPI

Supplementary Materials

Fluorescence-Based On-Resin Detection of Three Model Proteases

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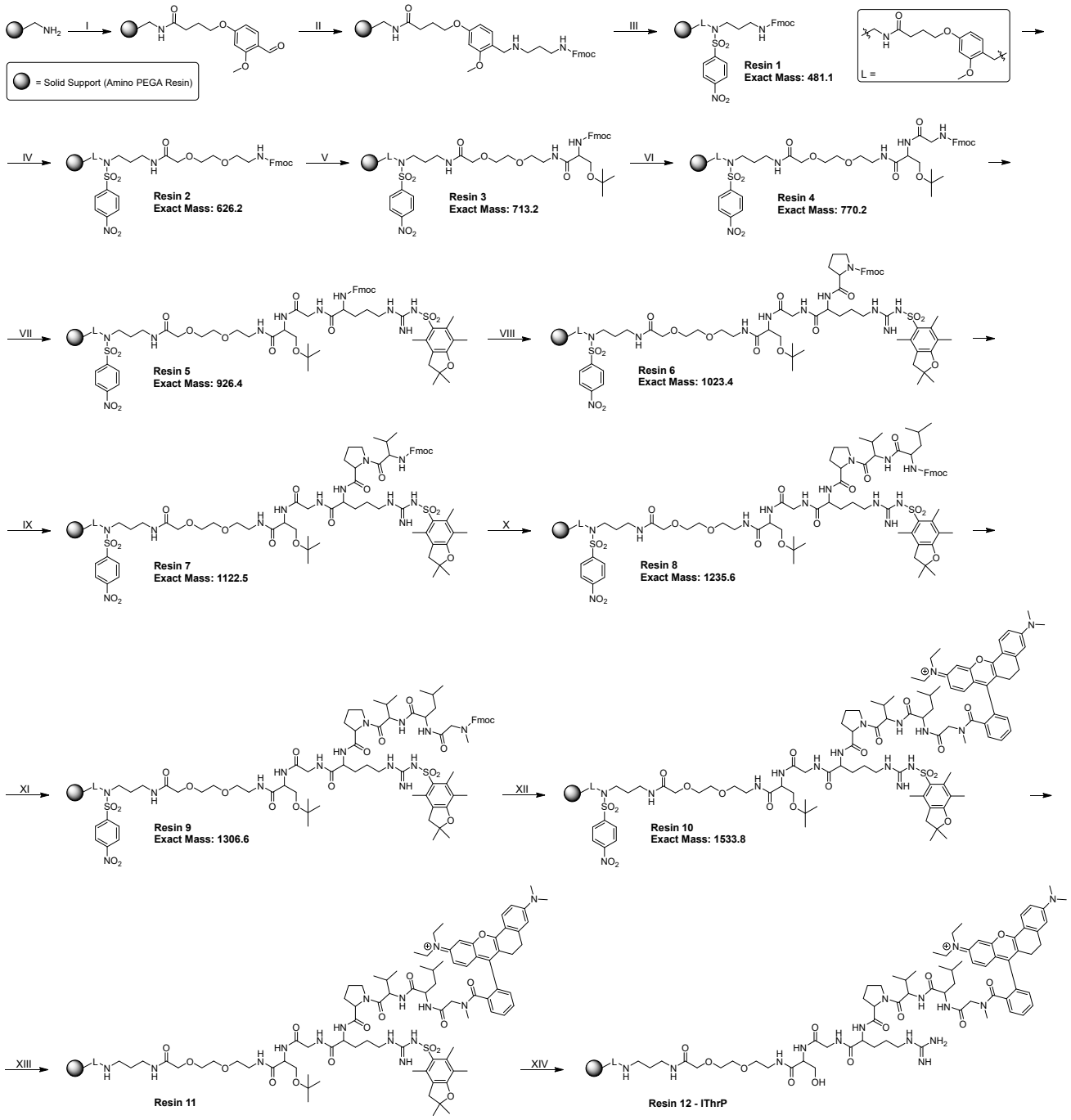
1. Synthesis – Immobilized Thrombin Probe (**IThrP**)

In all reaction steps, the purity of synthesized peptides on Amino PEGA resin exceeded 90% according to LC-MS.

Scheme S1. Synthesis of immobilized thrombin probe (**IThrP**) on Amino PEGA resin (yield: 71%).



I. 4-(4-Formyl-3-methoxyphenoxy)-butyric acid, HOBt, DIC, DMF:DCM 1:1, rt, 16h; **II.** a.) Fmoc-1,3-diaminopropane·HCl, 10% CH_3COOH in dry DMF, rt, 16h; b.) $NaBH(AcO)_3$, rt, 6h; **III.** 4-Nos-Cl, 2,6-dimethylpyridine in dry DCM, rt, 2h; **IV.** a.) 50% piperidine in DMF, rt, 30 min; b.) PEG, HOBt, DIC, DMF:DCM 1:1, rt, 2h; **V.** a.) 50% piperidine in DMF, rt, 30 min; b.) Fmoc-Ser(tBu)-OH, HOBt, DIC, DMF:DCM 1:1, rt, 2h; **VI.** a.) 50% piperidine in DMF, rt, 30 min; b.) Fmoc-Gly-OH, HOBt, DIC, DMF:DCM:DMSO 1:2:2, rt, 2h; **VII.** a.) 50% piperidine in DMF, rt, 30 min; b.) Fmoc-Arg(Pbf)-OH, HOBt, DIC, DMF:DCM 1:1, rt, 2h; **VIII.** a.) 50% piperidine in DMF, rt, 30 min; b.) Fmoc-Pro-OH, HOBt, DIC, DMF:DCM 1:1, rt, 2h; **IX.** a.) 50% piperidine in DMF, rt, 30 min; b.) Fmoc-Val-OH, HOBt, DIC, DMF:DCM 1:1, rt, 3h; **X.** a.) 50% piperidine in DMF, rt, 30 min; b.) Fmoc-Leu-OH, HOBt, DIC, DMF:DCM 1:1, rt, 3h; **XI.** a.) 50% piperidine in DMF, rt, 30 min; b.) Fmoc-Sar-OH, HOBt, DIC, DMF:DCM 1:1, rt, 3h; **XII.** a.) 50% piperidine in DMF, rt, 30 min; b.) HN6, HOBt, DIC, DMAP, DMF:DCM:DMSO 1:2:2, rt, 16h; **XIII.** DBU, 2-mercaptoethanol, dry DMF, rt, 3h; **XIV.** 50% TFA in DCM, rt, 3h.

Molecular weights of unanchored peptides are reported. Tert-butyl (tBu) and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting groups are removed during the chemical cleavage using 50% TFA in DCM.

Embedding of BAL linker to a solid support

Amino PEGA resin was prewashed with DCM (5x) and then a solution of 4-(4-Formyl-3-methoxyphenoxy)-butyric acid (1.1 mmol), HOBt (1.1 mmol) and DIC (1.1 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added. Heterogeneous reaction mixture was shaken overnight at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

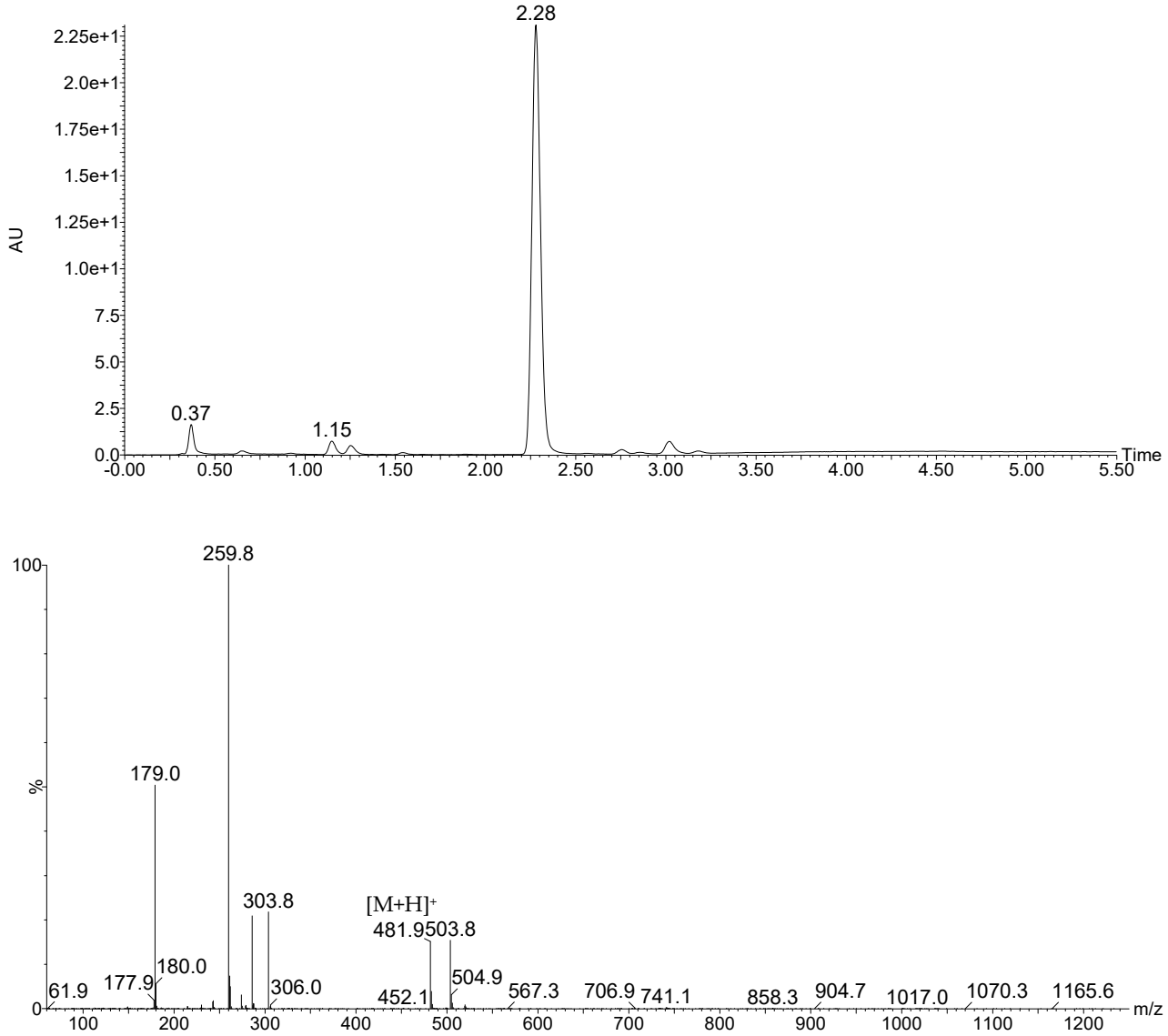
Introduction of Fmoc-1,3-diaminopropane

Obtained BAL resin was washed with dry THF (5x) and dry DCM (5x) and then a solution of Fmoc-1,3-diaminopropane·HCl (2.5 mmol) in 10% acetic acid in dry DMF (5 mL) was added. The mixture was shaken overnight at lab temperature. Afterwards, $NaBH(AcO)_3$ (2.5 mmol) in solid state was added directly into a syringe in three equal portions, and the heterogeneous mixture was shaken for 2 hours after each addition. The resin was then washed with 5% acetic acid in DMF (30x), DMF (10x) and DCM (10x).

Resin 1

The solid support was washed with dry DCM (5x), and subsequently reacted with a solution of 4-nitrobenzenesulfonyl chloride (4-Nos-Cl) (2.5 mmol) and 2,6-dimethylpyridine (2.5 mmol) in dry DCM (5 mL). The reaction mixture was shaken for 2 hours at lab temperature. Subsequently, the resin was washed with dichloromethane (10x).

Figure S1. LC-MS analysis of chemically cleaved compound from Resin 1.



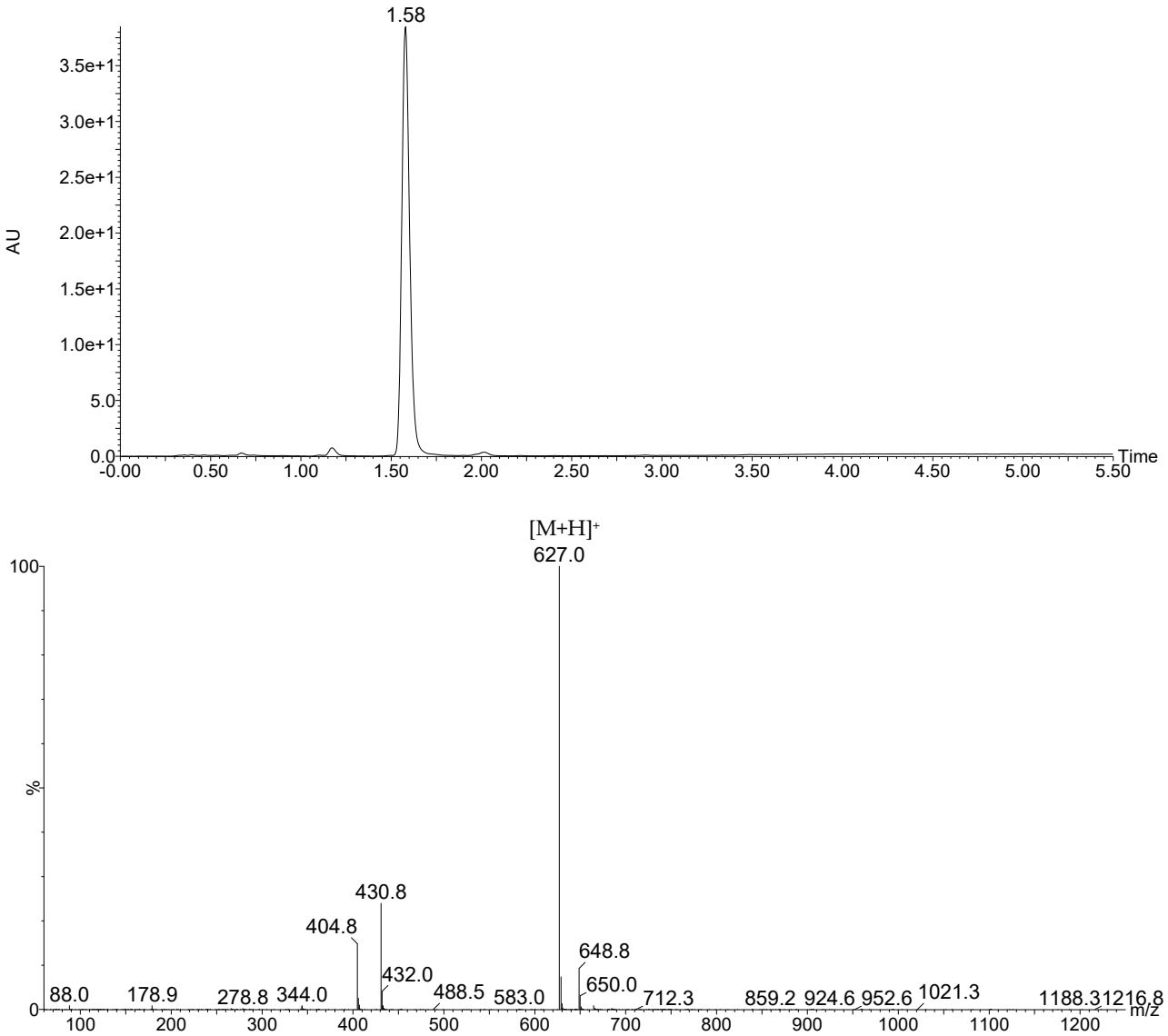
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 50–80% during the first 3 min).

Resin 2

Resin 1 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of PEG (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added to the resin and heterogeneous reaction mixture was shaken for 2 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S2. LC-MS analysis of chemically cleaved compound from Resin 2.



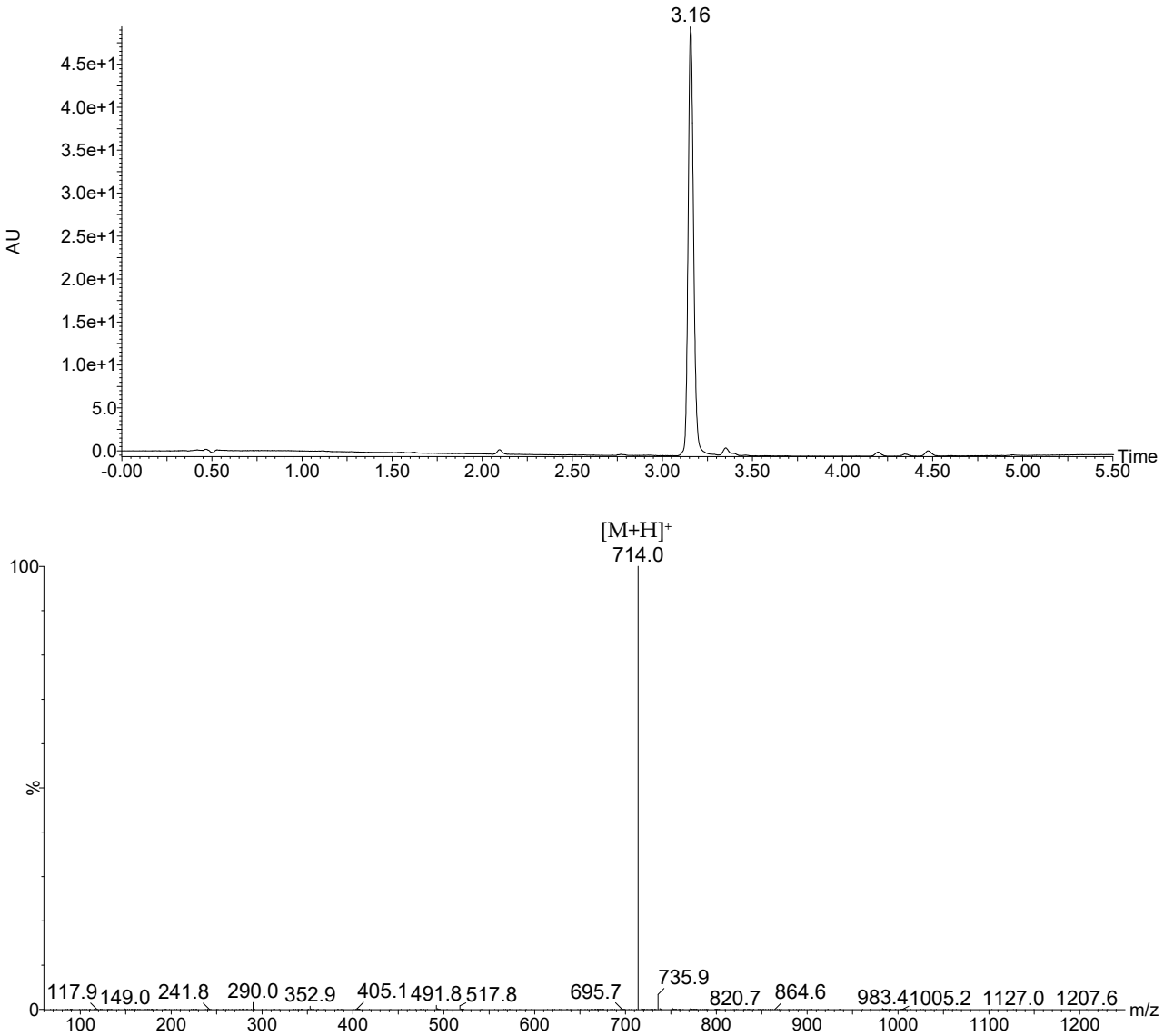
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 50–80% during the first 3 min).

Resin 3

Resin 2 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of Fmoc-Ser(tBu)-OH (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added to the resin and heterogeneous reaction mixture was shaken for 2 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S3. LC-MS analysis of chemically cleaved compound from Resin 3.



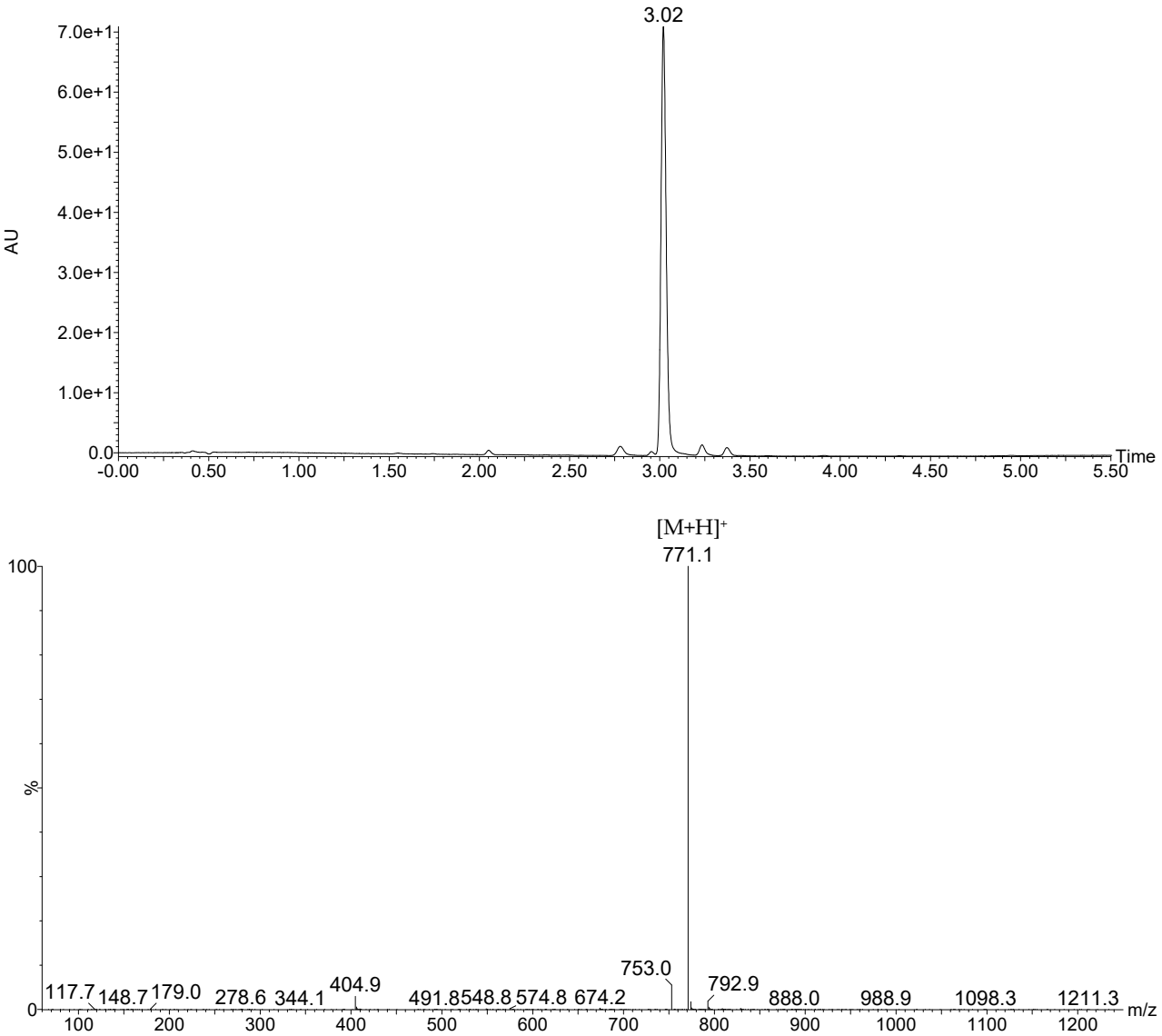
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 4

Resin 3 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of Fmoc-Gly-OH (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (1.0 mL), DCM (2.0 mL) and DMSO (2.0 mL) was added to the resin and heterogeneous reaction mixture was shaken for 2 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S4. LC-MS analysis of chemically cleaved compound from Resin 4.



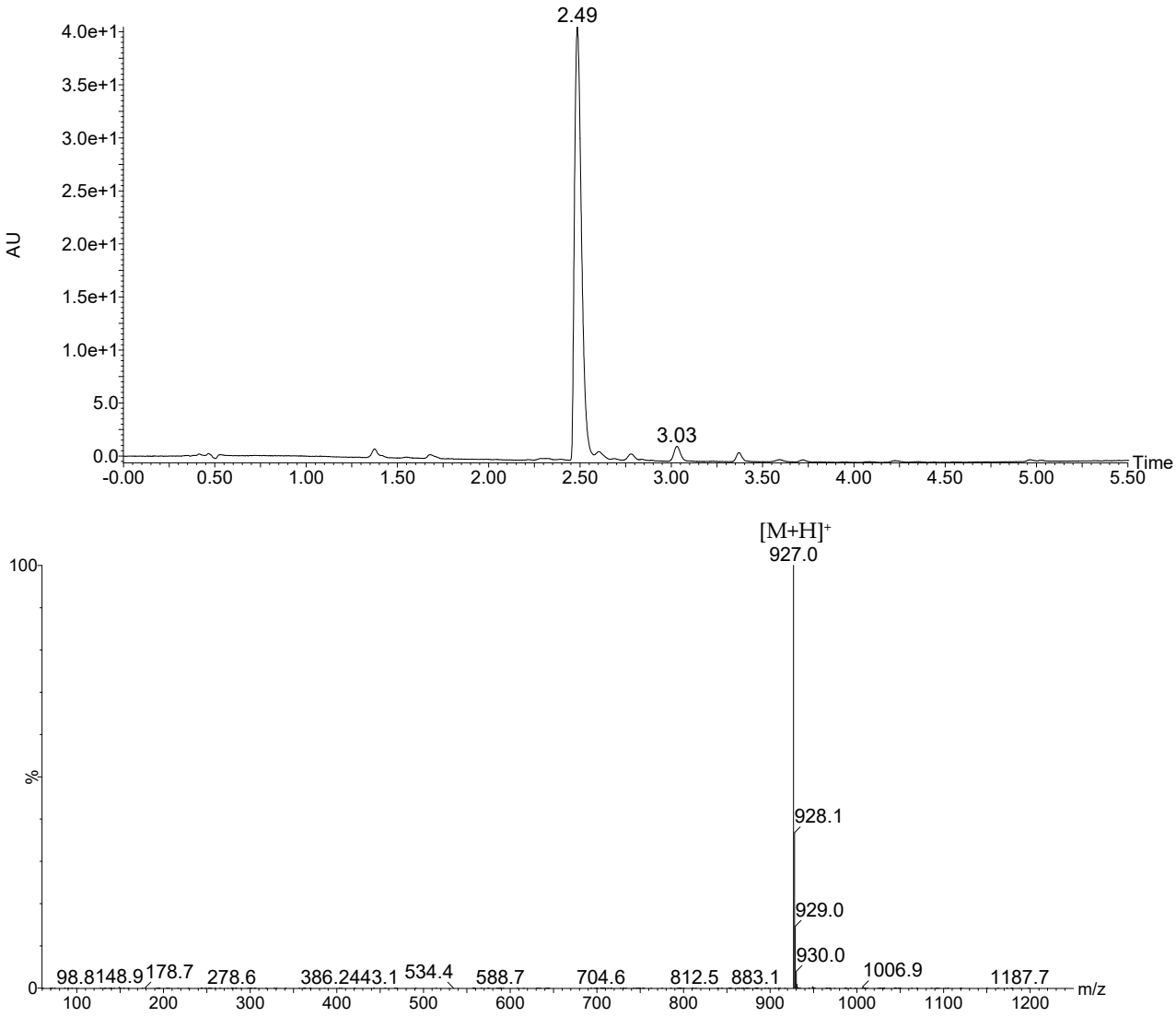
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 5

Resin 4 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of Fmoc-Arg(Pbf)-OH (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added to the resin and heterogeneous reaction mixture was shaken for 2 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S5. LC-MS analysis of chemically cleaved compound from Resin 5.



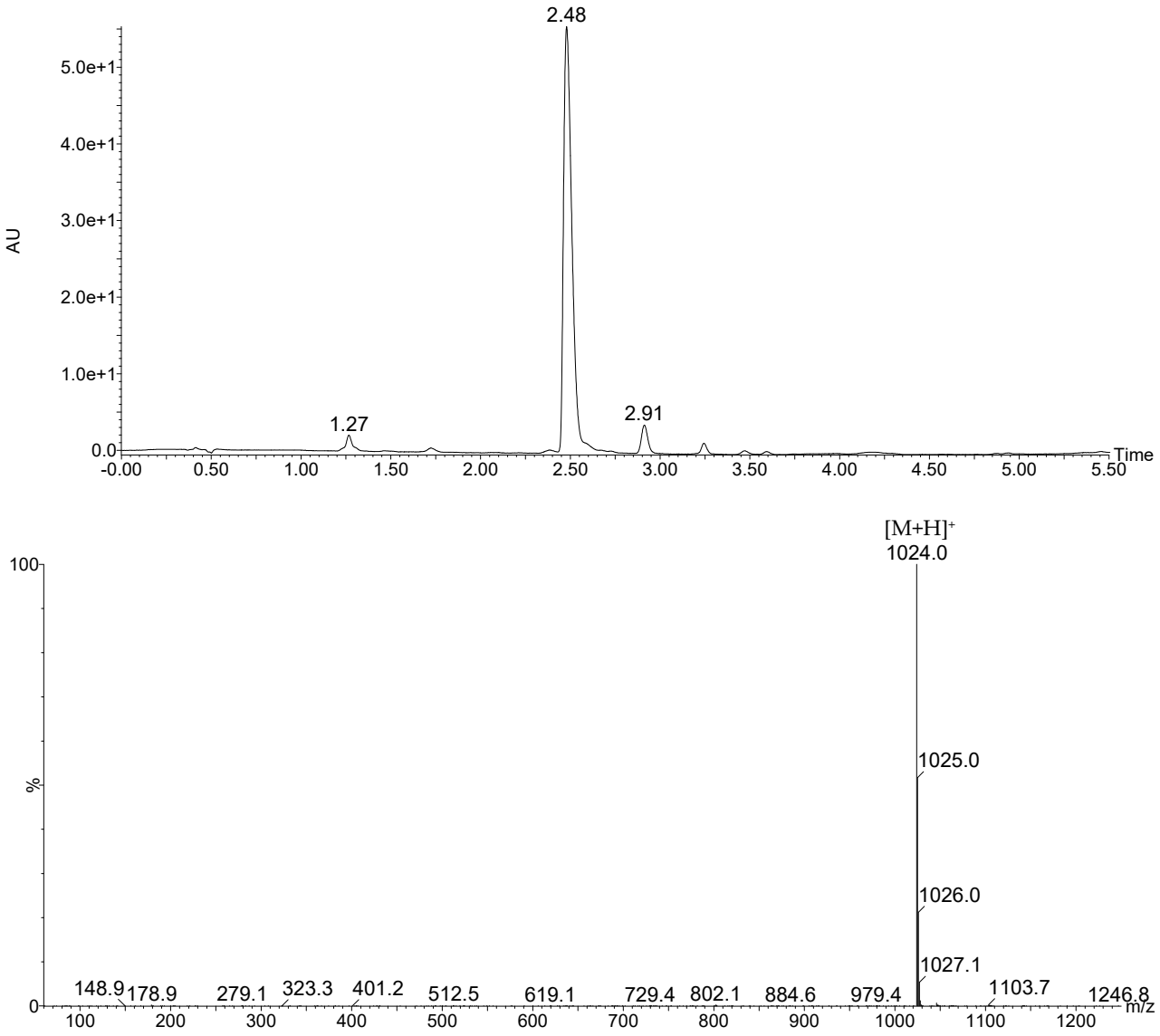
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 6

Resin 5 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of Fmoc-Pro-OH (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added to the resin and heterogeneous reaction mixture was shaken for 2 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S6. LC-MS analysis of chemically cleaved compound from Resin 6.



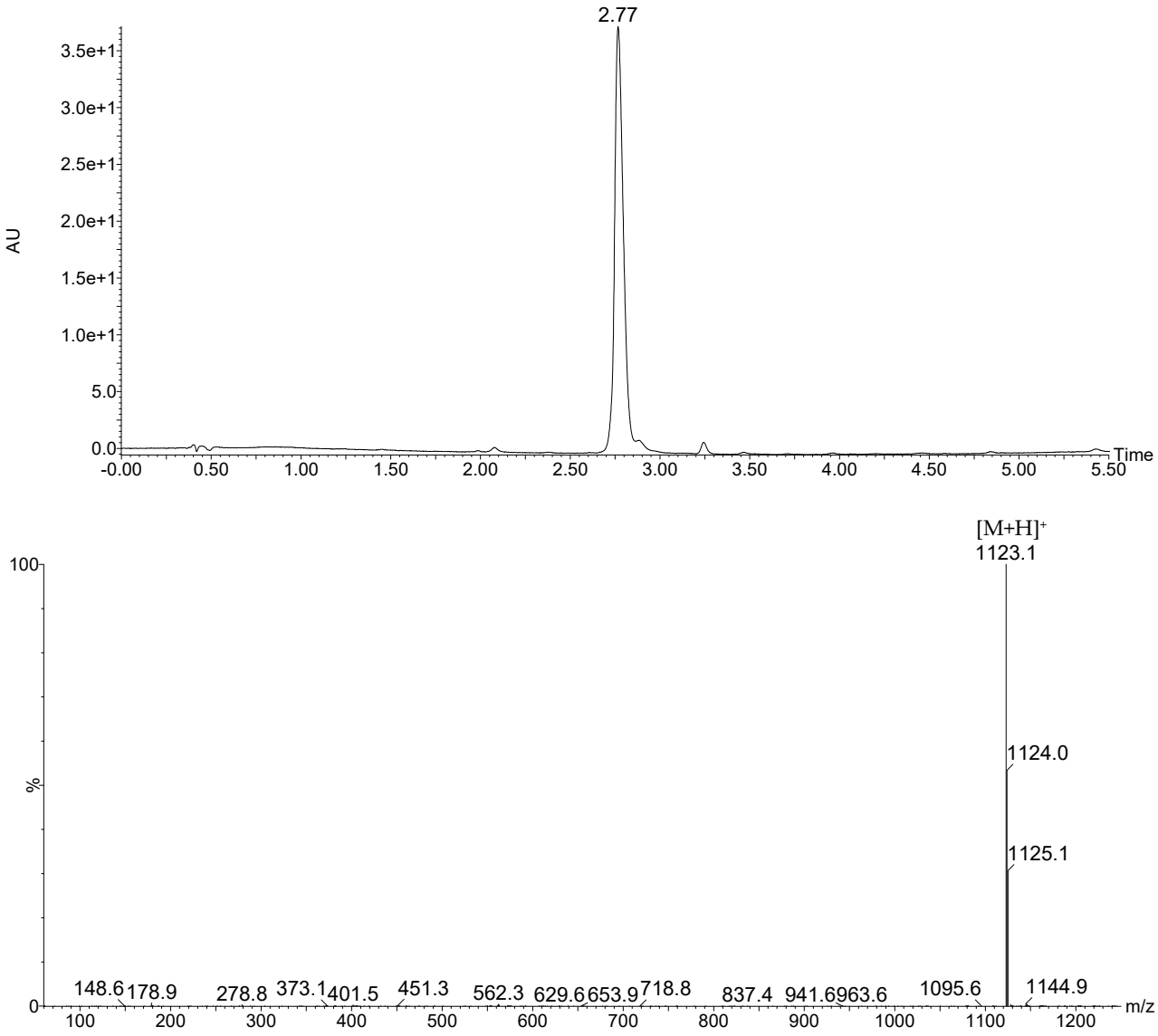
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 7

Resin 6 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of Fmoc-Val-OH (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added to the resin and heterogeneous reaction mixture was shaken for 3 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S7. LC-MS analysis of chemically cleaved compound from Resin 7.



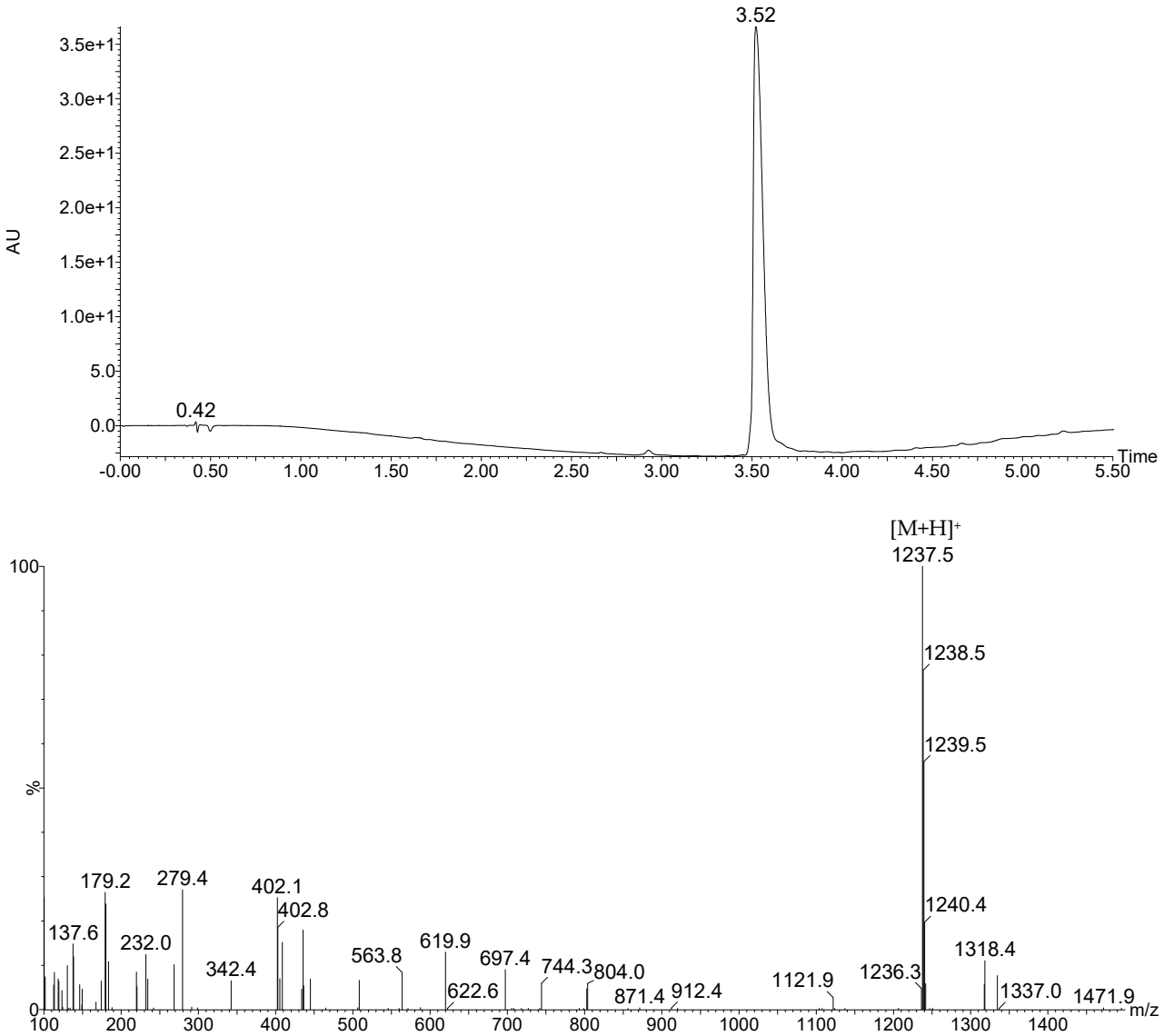
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 8

Resin 7 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of Fmoc-Leu-OH (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added to the resin and heterogeneous reaction mixture was shaken for 3 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S8. LC-MS analysis of chemically cleaved compound from Resin 8.



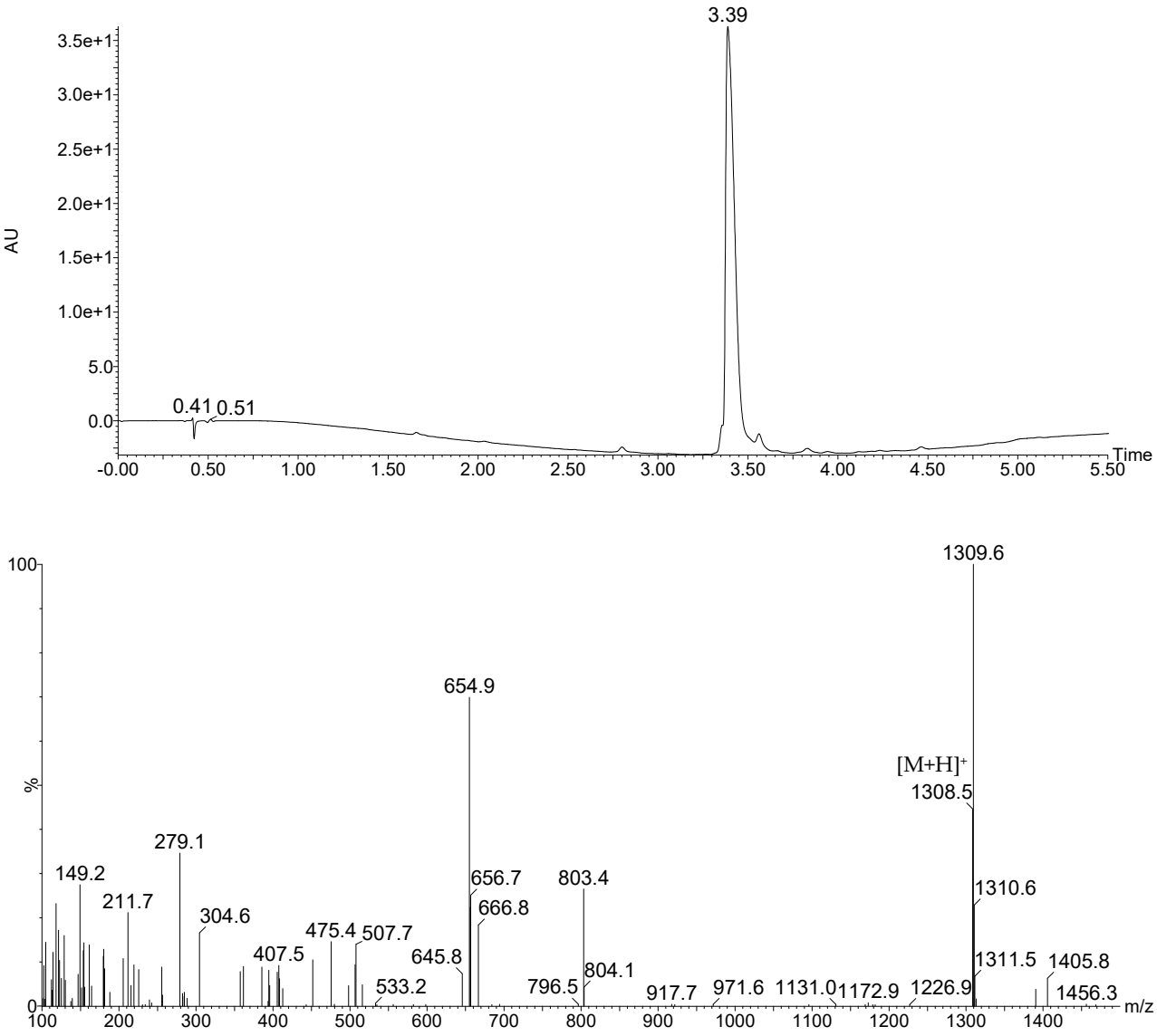
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 9

Resin 8 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of Fmoc-Sar-OH (1.0 mmol), HOBt (1.0 mmol) and DIC (1.0 mmol) in DMF (2.5 mL) and DCM (2.5 mL) was added to the resin and heterogeneous reaction mixture was shaken for 3 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Figure S9. LC-MS analysis of chemically cleaved compound from Resin 9.



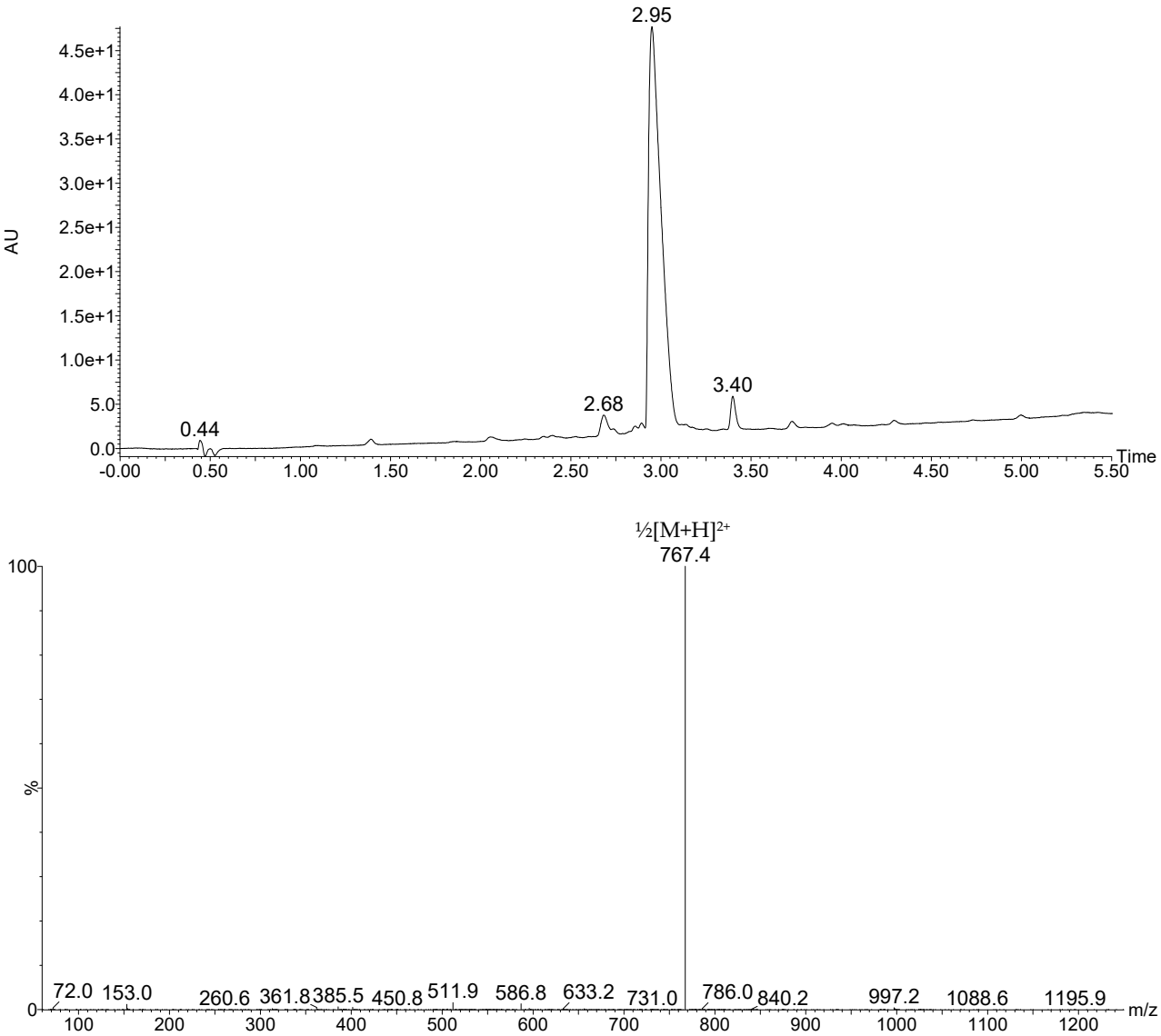
Method: Ammonium acetate (10 mM) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 10

Resin 9 was treated with 50% piperidine in DMF for 30 minutes, and subsequently washed with DMF (10x) and DCM (10x).

A solution of HN6 dye (1.0 mmol), HOBt (1.0 mmol), DIC (1.0 mmol) and DMAP (1.0 mmol) in DMF (1.0 mL), DCM (2.0 mL) and DMSO (2.0 mL) was added to the resin and heterogeneous reaction mixture was shaken overnight at lab temperature. A solid support was then washed with DMF (15x) and DCM (15x).

Figure S10. LC-MS analysis of chemically cleaved compound from Resin 10.



Method: Formic acid (0.1% *V*/*V*) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Resin 11

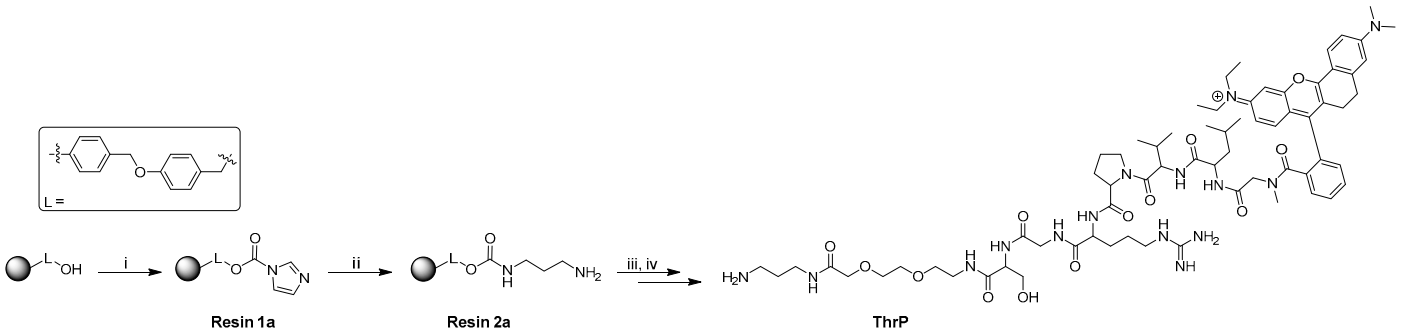
Resin 10 was prewashed with dry DMF (5x) and reacted with a solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (1.0 mmol) and 2-mercaptoethanol (3.0 mmol) in dry DMF (5 mL). A reaction mixture was shaken for 3 hours at lab temperature. A solid support was then washed with DMF (10x) and DCM (10x).

Resin 12 – **IThrP**

To remove tBu and Pbf protecting groups, Resin 11 was treated with 50% TFA in DCM for 3 hours, subsequently washed with DCM (15x), dried under a stream of nitrogen, and finally stored at −80 °C.

2. Synthesis – Soluble Thrombin Probe (**ThrP**)

Scheme S2. Synthesis of soluble thrombin probe (**ThrP**) on Polystyrene Wang resin.



i. Carbonyldiimidazole, pyridine, DCM, rt, 3h; **ii.** Diaminopropane, DCM, rt, 16h; **iii.** Reaction conditions from Scheme S1 **IV(b)**–**XII**; **iv.** 50% TFA in DCM, rt, 3h.

Resin 1a

The solid support was washed with DCM (5x), and subsequently reacted with a solution of carbonyldiimidazole (5 mmol) and pyridine (5 mmol) in DCM (10 mL). The heterogeneous reaction mixture was shaken for 3 hours at lab temperature. Subsequently, the resin was washed with dichloromethane (10x).

Resin 2a

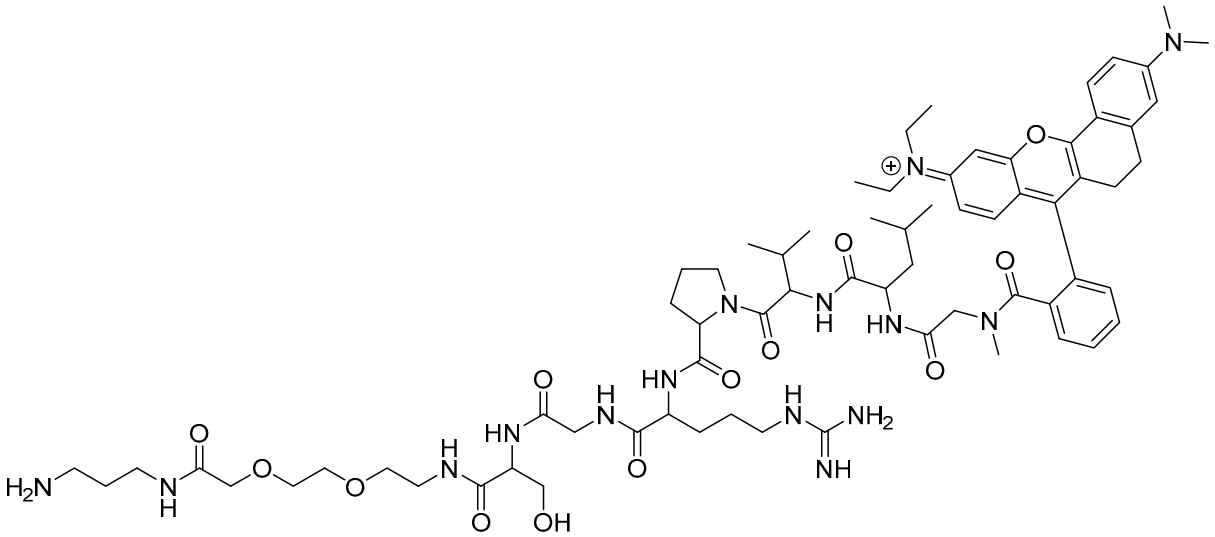
Resin 1a was reacted with a solution of diaminopropane (5 mmol) in DCM (10 mL). The heterogenoeus reaction mixture was shaken for 16 hours at lab temperature. Subsequently, the resin was washed with dichloromethane (10x).

Soluble Thrombin Probe (**ThrP**)

After applying the above described reaction procedure (Scheme S1 **IV(b)**–**XII**), the synthesized compound was cleaved from the solid support using 50% TFA in DCM. During the treatment with the cleavage cocktail, the protecting gtoups (tBu and Pbf) were removed as well. After evaporation of volatile liquids under a stream of nitrogen, the resulting sticky residue was purified to provide the final compound – soluble thrombin probe (**ThrP**).

3. Characterization

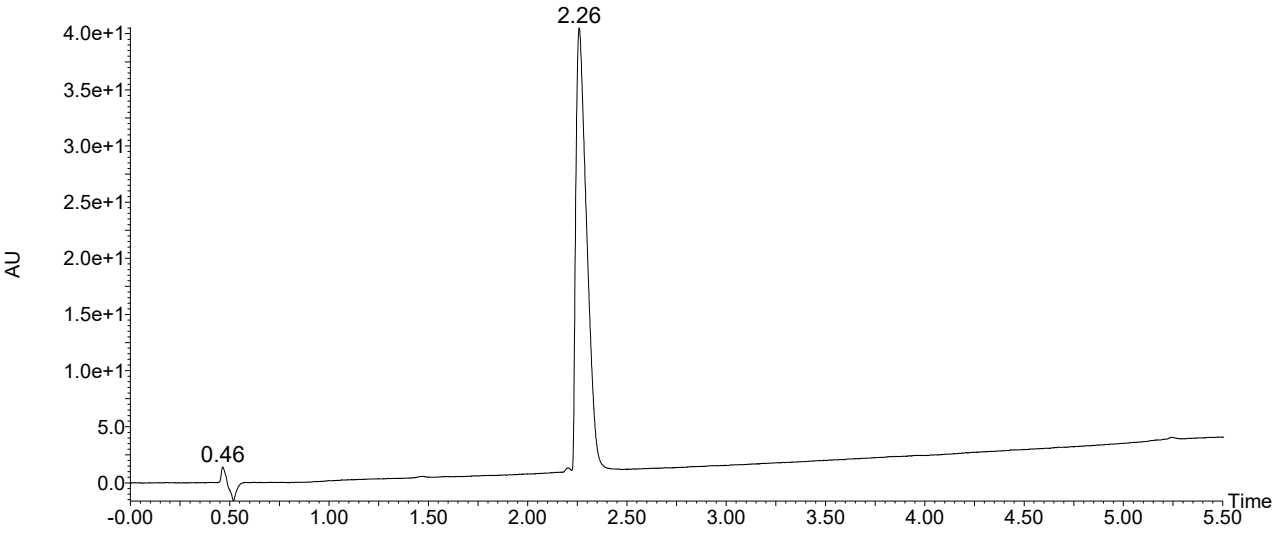
Figure S11. Thrombin probe (chemically cleaved from Polystyrene Wang resin).

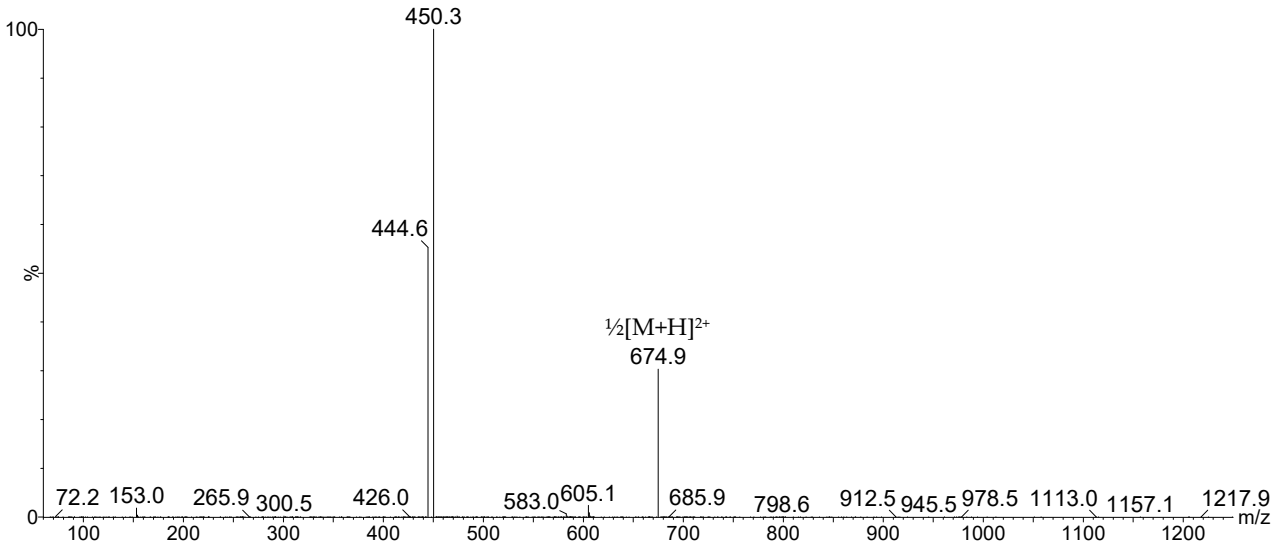


HRMS (ESI+): m/z calcd for $C_{69}H_{102}N_{15}O_{13}^{+}$ $[M]^{+}$ 1348.7776, found 1348.7722.

Quantum yield: φf = 91% (reference: Rhodamine B in distilled water).

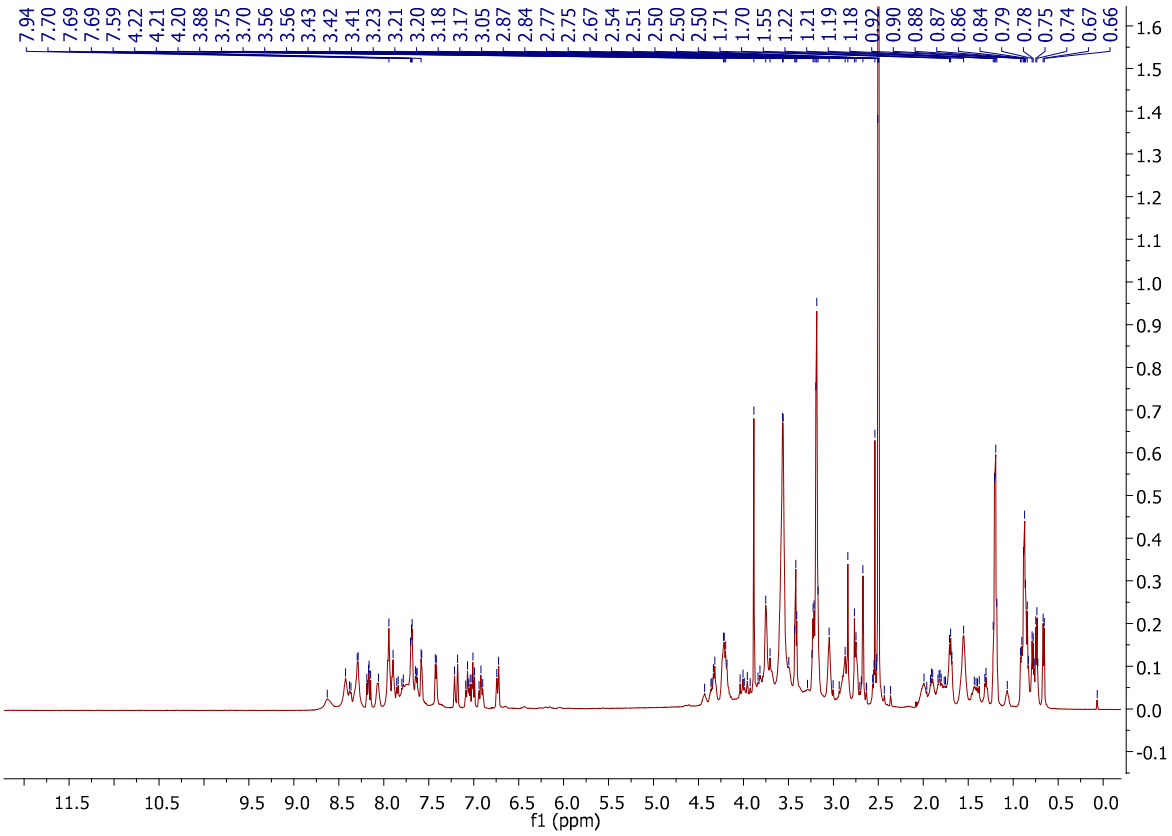
Figure S12. LC-MS analysis of purified thrombin probe.





Method: Formic acid (0.1% *V*/*V*) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Figure S13. ^{1}H NMR spectrum of purified thrombin probe.



4. Enzyme Assays

Figure S14. Graphical representation of on-resin enzyme assay.

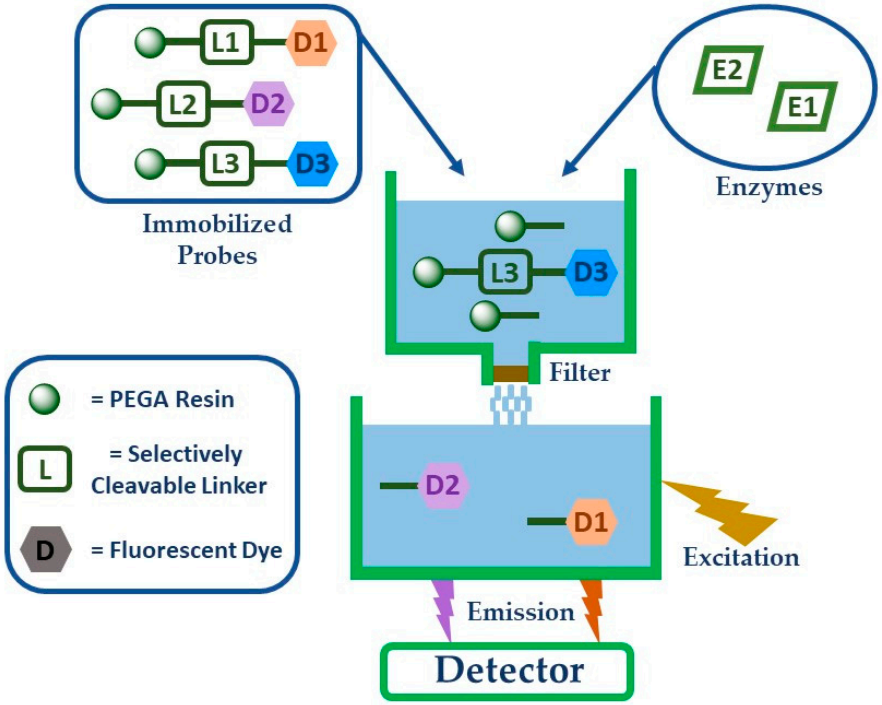
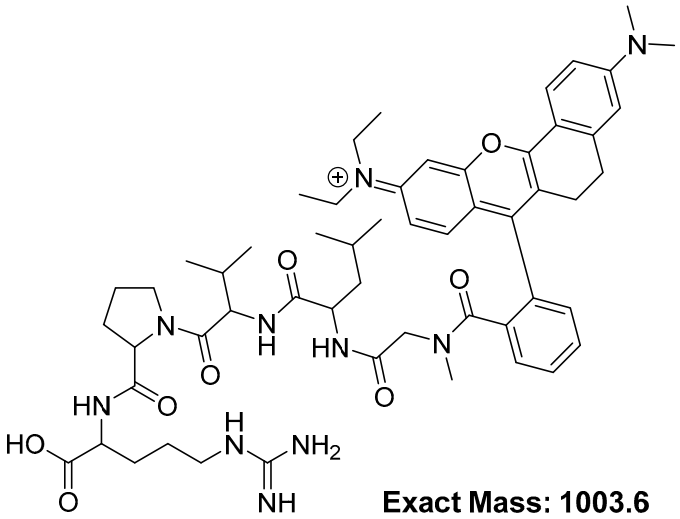
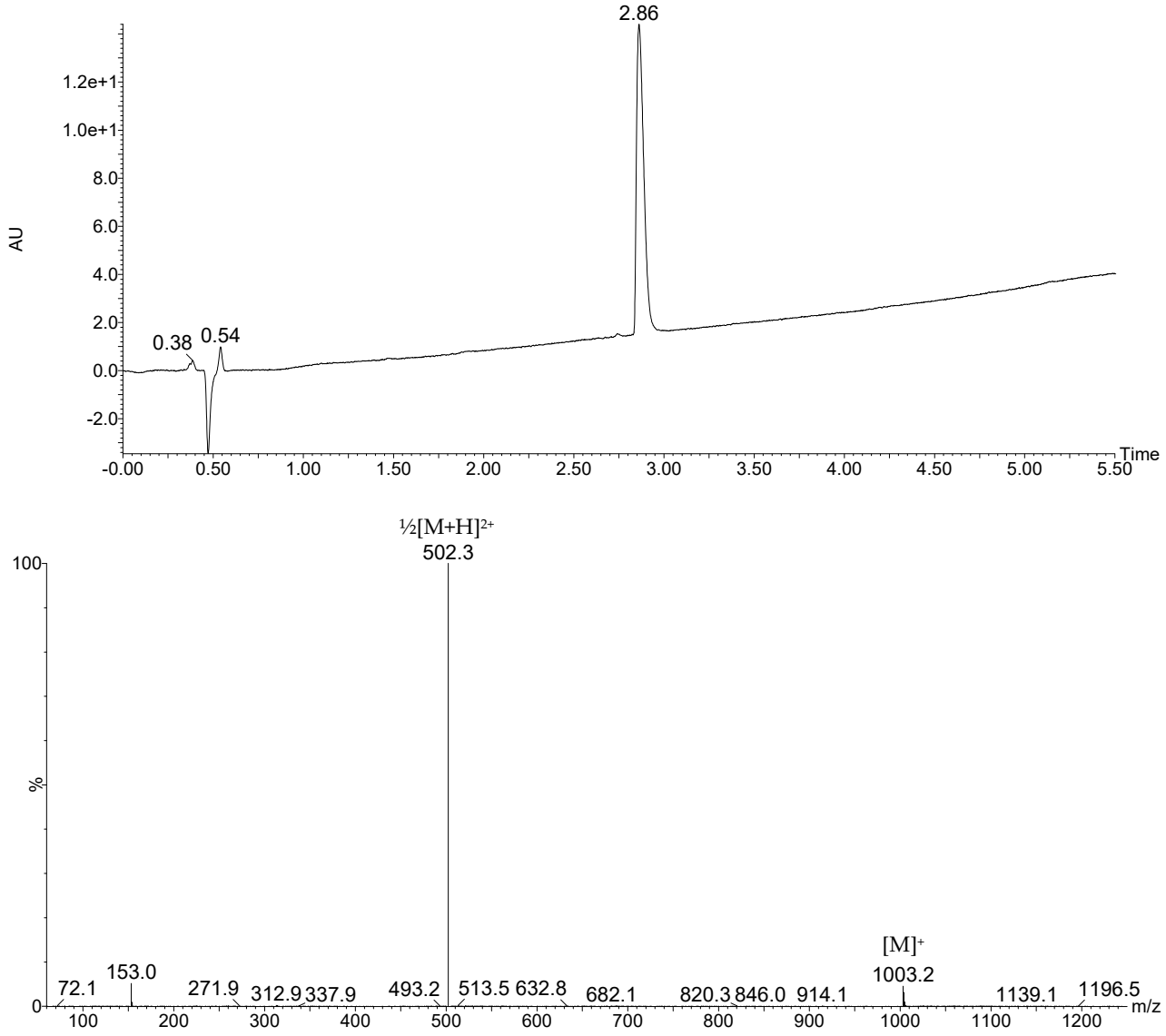


Figure S15. Enzymatically cleaved thrombin probe fragment.



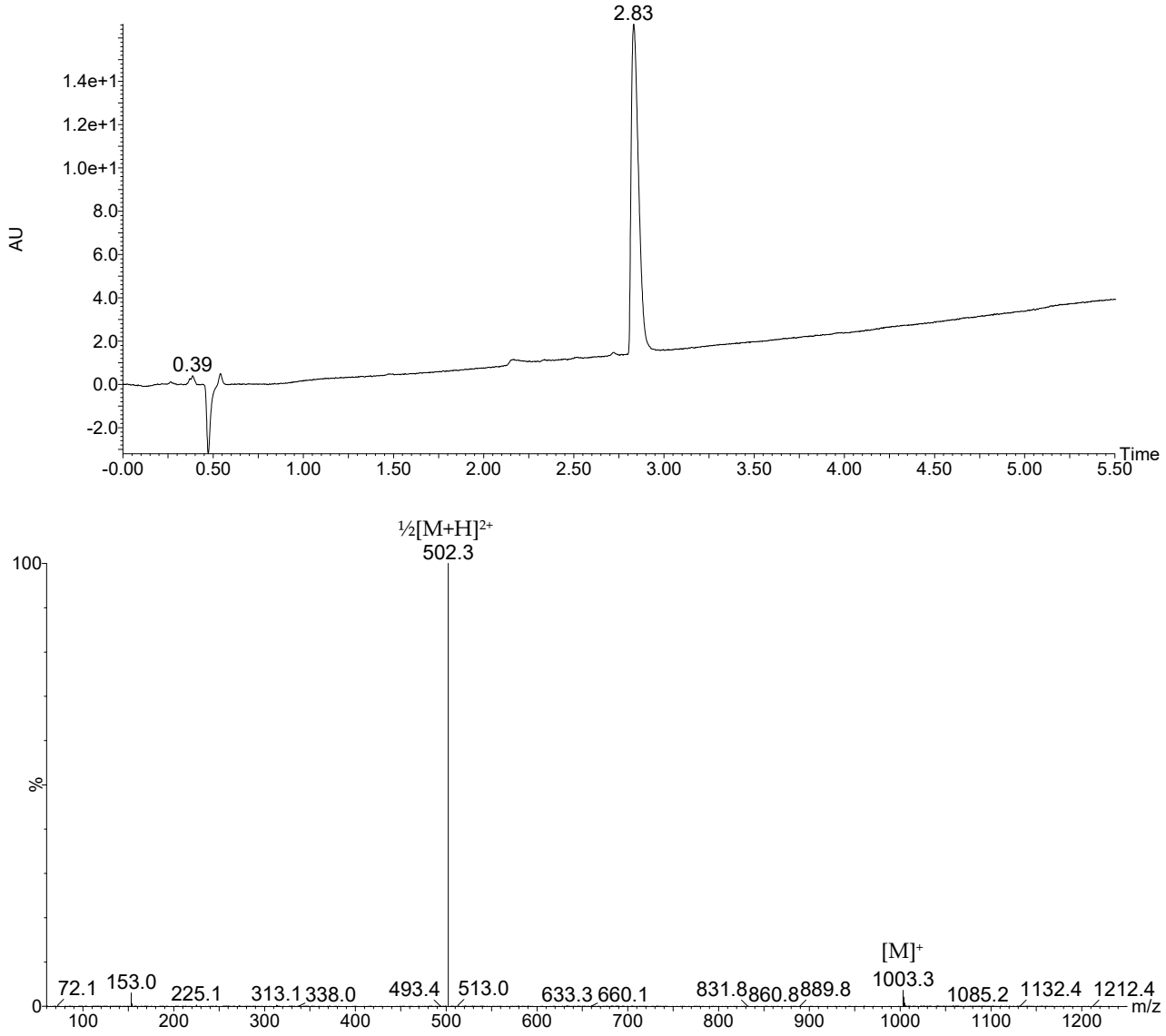
Thrombin probe was cleaved by thrombin, trypsin and chymotrypsin between *N*-terminus of glycine and *C*-terminus of arginine.

Figure S16. LC-MS analysis of thrombin probe fragment (trypsin cleavage).



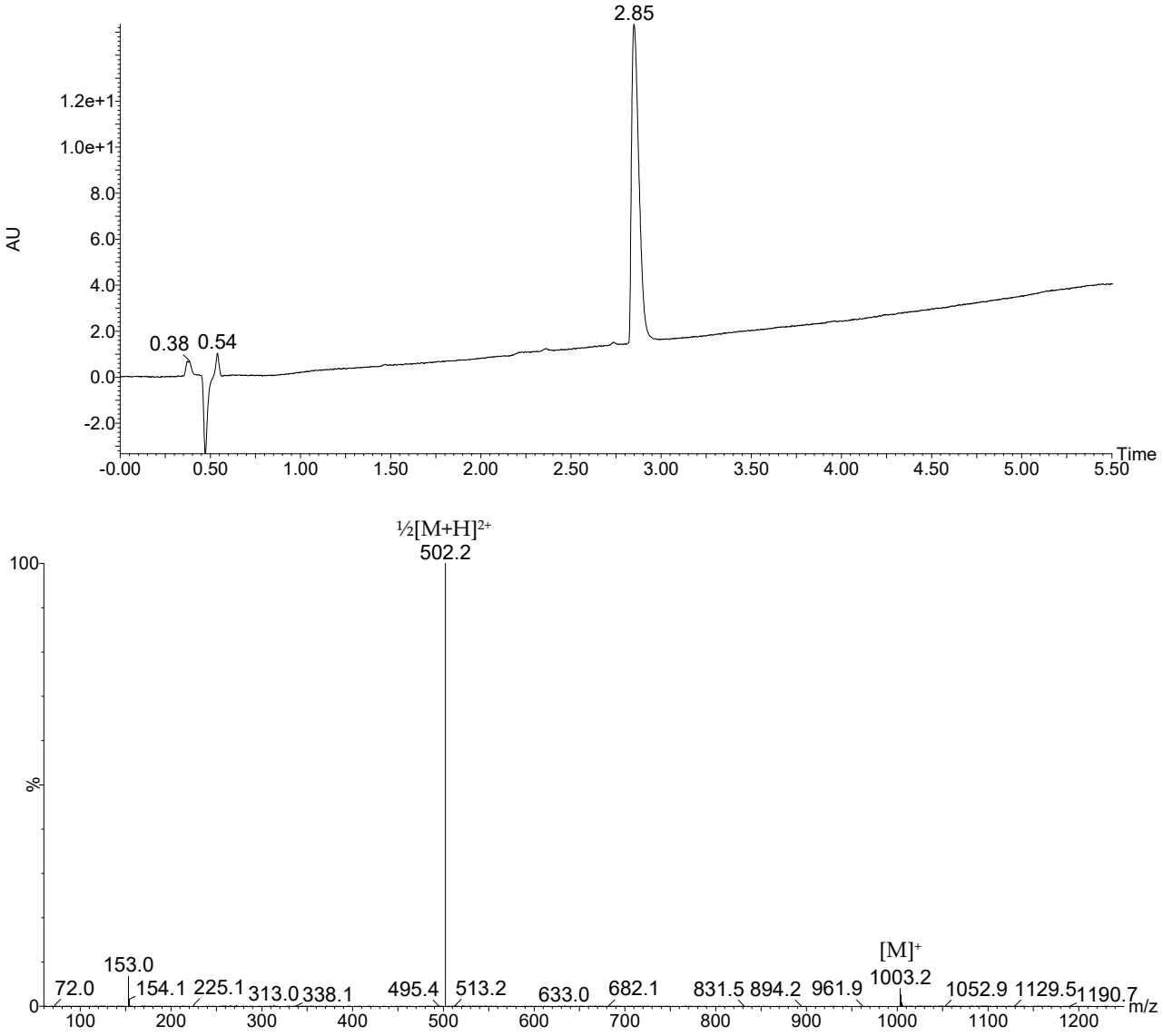
Method: Formic acid (0.1% *V*/*V*) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Figure S17. LC-MS analysis of thrombin probe fragment (chymotrypsin cleavage).



Method: Formic acid (0.1% *V*/*V*) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Figure S18. LC-MS analysis of thrombin probe fragment (thrombin cleavage).



Method: Formic acid (0.1% *V*/*V*) in ultrapure water and acetonitrile (gradient 20–80% during the first 4.5 min).

Table S1. Fluorescence response – determination of the lowest detectable concentration of thrombin.

Thrombin [μg/mL]	Avg. Resp. (Inhibitor)	Stand. Dev. (Inhibitor)	Avg. Resp. (No Inhibitor)	Stand. Dev. (No Inhibitor)
0	1.0	0.00	/	/
5	2.0	0.00	/	/
7	4.0	0.00	/	/
10	7.0	0.82	1.0	0.00
15	14.7	1.25	2.0	0.00
20	46.0	6.53	3.0	0.82
30	93.3	8.96	15.7	1.25

Preparation: 1.0–1.1 mg of dried **IThrP** resin; Bowman-Birk inhibitor (0 or 120 μg/mL); Tris-HCl buffer (pH=8.0) (700 μL) + 1 mM HCl (200 μL) + 0.9% *w*/*V* NaCl (100 μL); Incubation: t=15 min; T=37 °C; ν=210 min^{-1}; Wavelengths: λ_{EXC}=600 nm; λ_{EMS}=652 nm; $Slit_{EXC}$/$Slit_{EMS}$=5/5 nm. All measurements were performed in five parallels. The average value and standard deviation of three middle measurements (after discharging the minimum and maximum values) are reported for each individual set.
Avg. Resp. – average fluorescence response; Stand. Dev. – standard deviation.

Table S2. Fluorescence response – detection of thrombin in the presence of Bowman-Birk inhibitor.

Thrombin [μg/mL]	Inhibitor [μg/mL]	Avg. Resp.	Stand. Dev.
30	/	28.0	10.71
30	120	91.0	2.16
30	200	130.0	15.77

Preparation: 1.0–1.1 mg of dried **IThrP** resin; Bowman-Birk inhibitor (0, 120 or 200 μg/mL); Tris-HCl buffer (pH=8.0) (700 μL) + 1 mM HCl (200 μL) + 0.9% *w*/*V* NaCl (100 μL); Incubation: t=15 min; T=37 °C; ν=210 min^{-1}; Wavelengths: λ_{EXC}=600 nm; λ_{EMS}=652 nm; $Slit_{EXC}$/$Slit_{EMS}$=5/5 nm. All measurements were performed in five parallels. The average value and standard deviation of three middle measurements (after discharging the minimum and maximum values) are reported for each individual set.
Avg. Resp. – average fluorescence response; Stand. Dev. – standard deviation.

Table S3. Fluorescence response – simultaneous detection of trypsin and chymotrypsin in the presence of thrombin.

Trypsin [μg/mL]	Chymotrypsin [μg/mL]	Thrombin [μg/mL]	Avg. Resp. (Trypsin)	Stand. Dev. (Trypsin)	Avg. Resp. (Chymotrypsin)	Stand. Dev. (Chymotrypsin)
/	/	/	33.0	0.82	3.0	0.00
/	/	50	38.3	0.94	6.0	0.00
/	10	/	38.0	0.00	12.3	0.47
/	10	50	33.7	0.94	13.0	1.41
/	50	/	45.3	1.89	26.7	1.70
/	50	50	44.0	3.74	46.7	3.77
/	100	/	46.0	1.63	57.3	4.03
/	100	50	48.0	0.82	88.3	2.87
1	/	/	60.7	3.86	4.0	0.00
1	/	50	51.7	1.70	5.7	0.47
1	10	/	75.0	1.63	12.7	0.47
1	10	50	66.0	2.16	15.3	0.47
1	50	/	74.7	1.89	29.0	0.82
1	50	50	61.7	1.70	35.3	3.30
1	100	/	81.3	0.47	43.7	2.05
1	100	50	60.7	1.25	64.0	4.32
5	/	/	102.7	6.18	7.3	0.47
5	/	50	101.0	3.56	8.0	0.00
5	10	/	111.3	5.91	14.0	0.82
5	10	50	110.0	7.26	13.7	0.47
5	50	/	122.0	6.16	32.0	2.83
5	50	50	115.7	3.30	34.3	2.62
5	100	/	134.3	7.04	55.7	2.05
5	100	50	106.0	2.16	59.3	3.68
10	/	/	139.0	8.52	7.3	0.47
10	/	50	143.3	5.31	8.7	0.47
10	10	/	142.3	8.96	11.7	0.47
10	10	50	136.0	6.16	13.0	0.82
10	50	/	150.3	9.67	27.3	1.25
10	50	50	134.3	4.92	30.0	2.45
10	100	/	156.7	3.68	35.0	0.82
10	100	50	139.0	5.35	37.3	1.70

Preparation: 2.0–2.1 mg of dried **ITP** and **ICP** resins; Tris-HCl buffer (pH=8.0) (700 μL) + 1 mM HCl (200 μL) + 0.9% *w*/*V* NaCl (100 μL); Incubation: t=15 min; T=37 °C; ν=210 min^{-1}; Wavelengths: λ_{EXC}=410 nm; λ_{EMS1}=480 nm; λ_{EMS2}=590 nm; $Slit_{EXC}$/$Slit_{EMS}$=5/5 nm. All measurements were performed in five parallels. The average value and standard deviation of three middle measurements (after discharging the minimum and maximum values) are reported for each individual set.

Avg. Resp. – average fluorescence response; Stand. Dev. – standard deviation.

Table S4. Fluorescence response – detection of thrombin in the presence of trypsin and chymotrypsin.

Trypsin [μg/mL]	Chymotrypsin [μg/mL]	Thrombin [μg/mL]	Avg. Resp. (Thrombin)	Stand. Dev. (Thrombin)
/	/	/	1.0	0.00
10	/	/	1.0	0.00
/	100	/	1.0	0.00
10	100	/	1.0	0.00
/	/	10	21.3	0.94
10	/	10	15.0	4.32
/	100	10	40.0	12.36
10	100	10	58.0	10.71
/	/	25	154.7	9.74
10	/	25	108.0	6.38
/	100	25	206.7	11.15
10	100	25	169.0	45.22
/	/	50	452.3	2.49
10	/	50	494.3	67.38
/	100	50	574.7	156.24
10	100	50	581.0	62.25

Preparation: 1.0–1.1 mg of dried **IThrP** resin; Bowman-Birk inhibitor (120 μg/mL); Tris-HCl buffer (pH=8.0) (700 μL) + 1 mM HCl (200 μL) + 0.9% *w*/*V* NaCl (100 μL); Incubation: t=15 min; T=37 °C; ν=210 min^{-1}; Wavelengths: λ_{EXC}=600 nm; λ_{EMS}=652 nm; $Slit_{EXC}$/$Slit_{EMS}$=5/5 nm. All measurements were performed in five parallels. The average value and standard deviation of three middle measurements (after discharging the minimum and maximum values) are reported for each individual set.
Avg. Resp. – average fluorescence response; Stand. Dev. – standard deviation.

5. Stability Testing

Table S5. Fluorescence response – stability of Amino PEGA resin-anchored thrombin probe (**IThrP**).

Time	Temp. of storage [°C]	Avg. Resp.	Stand. Dev.
Day 0	/	1.0	0.00
Day 7	-80 °C	1.0	0.00
Day 15	-80 °C	1.0	0.00
Day 30	-80 °C	1.0	0.00
Day 5	25 °C	21.7	8.06
Day 10	25 °C	495.7	52.04
Day 14	25 °C	Out of range	Out of range

Preparation: 1.0–1.1 mg of dried **IThrP** resin; Tris-HCl buffer (pH=8.0) (700 μL) + 1 mM HCl (200 μL) + 0.9% *w*/*V* NaCl (100 μL); Incubation: t=15 min; T=37 °C; ν=210 min^{-1}; Wavelengths: λ_{EXC}=600 nm; λ_{EMS}=652 nm; $Slit_{EXC}$/$Slit_{EMS}$=5/5 nm. All measurements were performed in five parallels. The average value and standard deviation of three middle measurements (after discharging the minimum and maximum values) are reported for each individual set.
Avg. Resp. – average fluorescence response; Stand. Dev. – standard deviation.