

Ascorbate as a pro-oxidant: Mild N-terminal modification with vinylboronic acid

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General information

All chemicals were purchased from commercial suppliers and used without purification. Peptide synthesis was conducted using standard solid-phase Fmoc protocols,¹ and crude peptide was purified by reverse-phase HPLC. HEPES buffer (0.1 M, pH 7.4) was prepared by dissolving 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (Oakwood Chemical, #047861) in MilliQ water, and pH was adjusted by addition of aq NaOH (1 M). *N*-methylmorpholine (NMM) buffer (5 mM, pH 7.4) was prepared by diluting NMM in MilliQ water, and pH was adjusted by addition of aq HCl (1 M). Coumarin **S1** was synthesized according to a literature.²

Mass spectroscopy

ESI-MS was performed on a Bruker Daltonics MicroTOF spectrometer. Sample was desalted using C18 tips (Thermo Scientific #87782) before analysis. Conversion of modification was assessed by using peak intensity.

MALDI-MS was performed on a Bruker Daltonics Autoflex-TOF/TOF spectrometer. Sinapic acid (Sigma-Aldrich, #85429) or α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich, #70990) soln in 1:1 water/acetonitrile with 0.1% v/v trifluoroacetic acid (20 mg/mL) was used as matrix.

General Procedure for MALDI: An aliquot of the crude reaction mixture (1 μ L) was diluted 5 \times with aq acetonitrile (4 μ L, 50% aq). The diluted soln (1 μ L) was spotted onto a MALDI plate with matrix soln (1 μ L), dried in air, and analyzed on the MALDI spectrometer. Conversion of modification reaction was assessed by peak area in mMass. For modification reaction of human insulin, peak intensity was used instead of peak area due to close proximity of the unmodified and modified protein peaks. α -cyano-4-hydroxycinnamic acid was used as matrix for all of MALDI-MS/MS experiments.

HPLC

Reverse-phase HPLC was performed on a Shimadzu CBM-20A instrument with Phenomenex Jupiter 4 μ Proteo 90A (250 \times 15 mm preparative) column at a flow rate of 8 ml/min. A gradient of acetonitrile in water with 0.1% v/v trifluoroacetic acid was used as an eluent. Compounds were detected by 220 and 254 nm UV lamp.

NMR

¹H NMR were performed on a Bruker AVANCE 600 spectrometer.

General procedure for peptide/protein modification reaction

Peptide/protein (100 μ M) was incubated with boronic acid (1 mM) and sodium ascorbate (9 or 50 mM) in HEPES buffer at rt overnight. Specifically, peptide (1 μ L, 5 nmol of 2.5-mM aq soln) was mixed with HEPES buffer (23.5 μ L), boronic acid (0.5 μ L, 50 nmol of 50-mM DMSO soln), and freshly prepared sodium ascorbate (1.25 μ L, 1.25 μ mol of 1-M aq soln for 50-mM final concn). The crude reaction mixture was analyzed by MALDI-MS, following the "General Procedure for MALDI".

Procedure for reduction of modified human insulin

After following the “General procedure for peptide/protein modification reactions”, the crude reaction mixture (10 μ L) was mixed with DTT (10 μ L, 0.2 μ mol of 20-mM aq soln). The mixture was incubated for 2 h at rt, desalted using a C18 zip-tip, and analyzed following the “General Procedure for MALDI”.

Overview of peptides and ascorbate derivatives.

Table S1. Sequence of peptide/protein studied.

Name	sequence	MW	Supplier (catalog number)
angiotensin I (1)	H-DRVYIHPFHL-OH	1296.5	Sigma-Aldrich (A9650)
DRVY	H-DRVY-NH ₂	550.6	home-made
angiotensin IV	H-VYIHPF-OH	774.9	Sigma-Aldrich (A0230)
amyloid β 35-25	H-MLGIIAGKNSG-OH	1060.3	Bachem (H-2964)
MF α 1-6	H-WHWLQL-OH	883.7	Sigma-Aldrich (T2903)
laminin 925-933	H-CDPGYIGSR-OH	967.1	APEXBIO (A1023)
hexahistidine	H-HHHHHH-OH	840.9	ANASPEC (AS-24420)
PAR1-AP	H-SFLLRN-NH ₂	747.9	Sigma-Aldrich (S1820)
HIV tat 47-57	H-YGRKKRRQRRR-OH	1559.8	Sigma-Aldrich (H0292)
neuromedin B	H-GNLWATGHFM-NH ₂	1019.1	Alfa Aesar (J66286)
neurotensin 8-13	H-RRPYIL-OH	817.0	Sigma-Aldrich (N5266)
bradykinin	H-RPPGFSPFR-OH	1059.6	Sigma-Aldrich (B3259)
human insulin	A: H-GIVEQCCTSICSLYQLENYCN-OH	5795.7	ROCHE (11376497001)
	B: H-FVNQHLCGSHLVEALYLVCGERGFFYTPKT-OH		

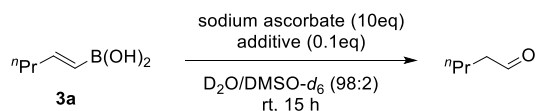
Table S2. A list of ascorbate analogues.

Name	Supplier (catalog number)
L-sodium ascorbate (3a)	Sigma-Aldrich (A7631)
L-dehydroascorbic acid (3b)	Sigma-Aldrich (261556)
L-dihydroascorbate (3c , (3S,4R,5R)-5-((S)-1,2-Dihydroxyethyl)-3,4-dihydroxydihydrofuran-2(3H)-one)	Ark Pharm (AK101076)
L-ascorbic acid acetone (3d)	Chem-Impex Intl. Inc (27243)
3- <i>o</i> -ethyl-L-ascorbic acid (3e)	Sigma-Aldrich (CDS009203)
2- <i>o</i> - α -D-glucopyranosyl-L-ascorbic acid (3f)	Alfa Aesar (J66601)

NMR study of the aldehyde generation

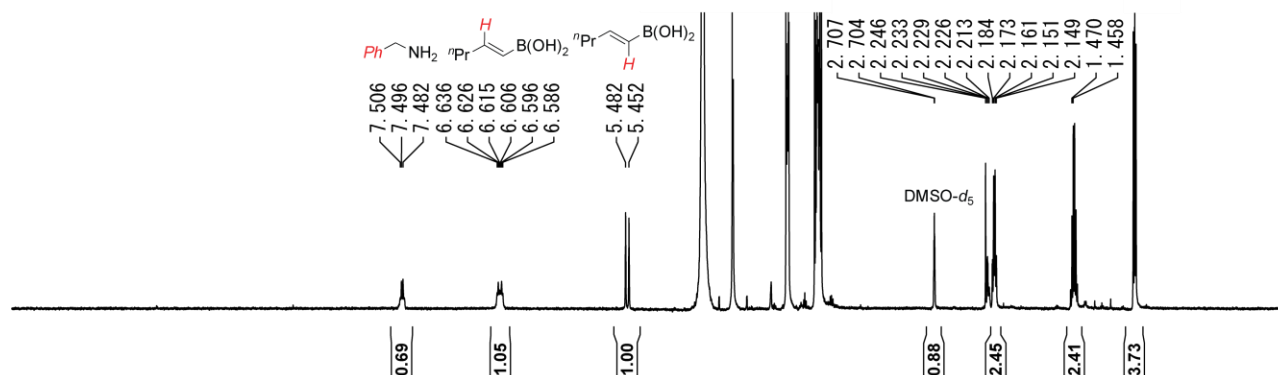
General procedure for the experiment (entry 4)

Sodium ascorbate (4.28 mg, 21.7 μmol) was dissolved in deuterium oxide (980 μL). Boronic acid **3a** (0.23 mg, 2.0 μmol) and *N*-benzylamine (0.02 mg, 0.2 μmol) in DMSO- d_6 were added to the soln. The mixture was stirred at rt for 15 h. The reaction was tracked by ^1H NMR with residual solvent peaks as an internal standard (2.70 ppm).



entry	C _{boronate} (mM)	additive	convn (%)
1	20	-	5
3	2	-	11
4	2	benzylamine	15
5	2	triethylamine	13
6	2	triethylamine	18 ^c

a) 5 min



b) 16 hrs

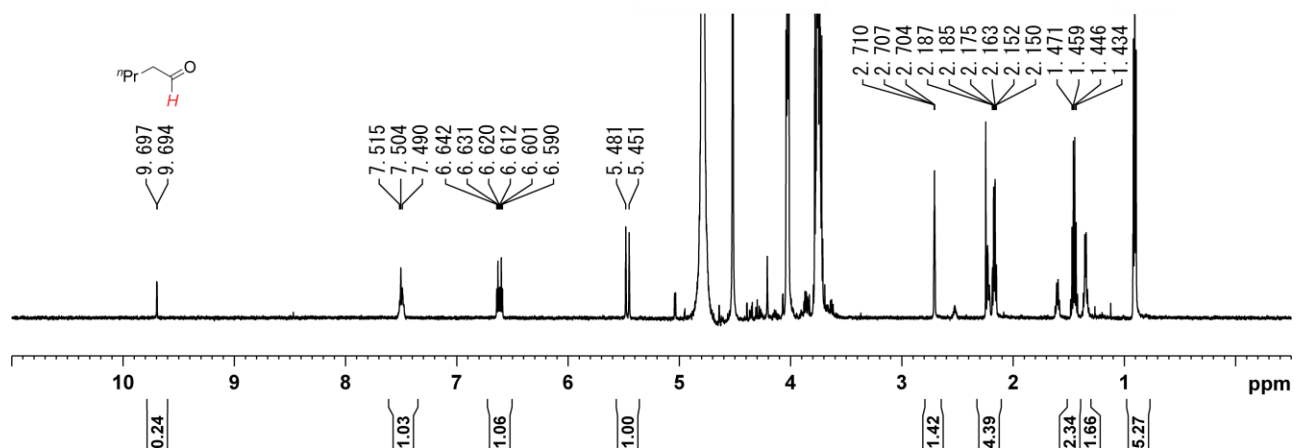


Figure S1. Representative ^1H NMR spectra of oxidation of *n*-propylvinylboronic acid (**3a**) by sodium ascorbate in the presence of benzylamine (entry 4). a) Spectrum after 5-min incubation. b) Spectrum after 16-h incubation.

Verification of N-terminal modification by HPLC and NMR

HPLC comparison: Experimental procedures

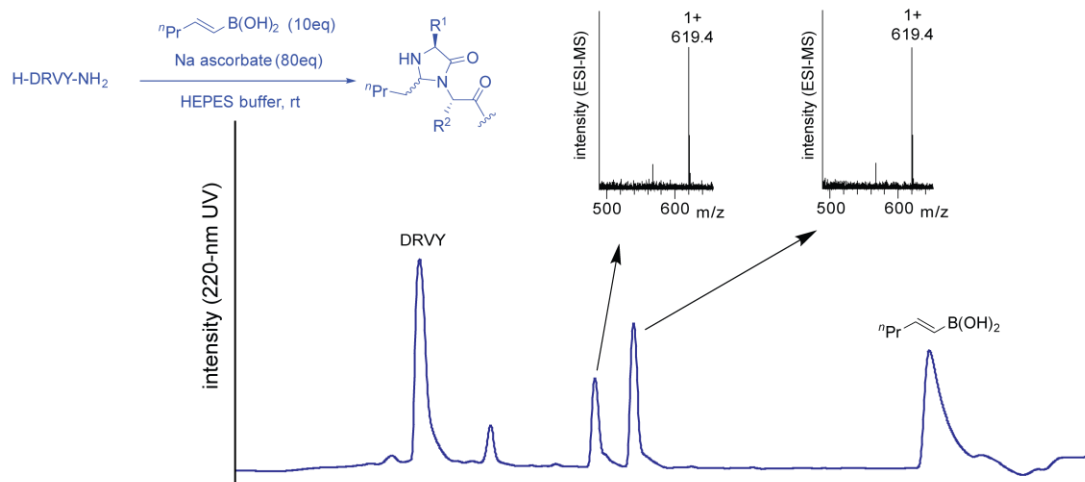
a) Boronic acid/vitamin C reaction

DRVY (6.0 mg, 0.011 mmol), *n*-propylvinylboronic acid (13.0 mg, 0.114 mmol), and sodium ascorbate (175.6 mg, 0.886 mmol) were dissolved in HEPES (0.1 M, pH 7.4)/MeCN (1:1, 16 mL). The soln was stirred at rt for 2 days. The soln was concentrated to ~8 mL by gentle flow of nitrogen gas, and the resulting soln was directly injected to HPLC (solvent gradient: 10-60 acetonitrile).

b) Aldehyde reaction

DRVY (3.5 mg, 0.006 mmol) was dissolved in HEPES (0.1 M, pH 7.4)/MeCN (1:1, 4 mL). Pentanal (58 μ L, 0.545 mmol) was added and the soln was stirred at rt overnight. The soln was concentrated to ~2 mL by gentle flow of nitrogen gas. The aq soln was washed with ether (3×5 mL) and remaining ether was removed by gentle flow of nitrogen gas to the aq soln.

a) boronic acid/vitamin C reaction



b) aldehyde reaction

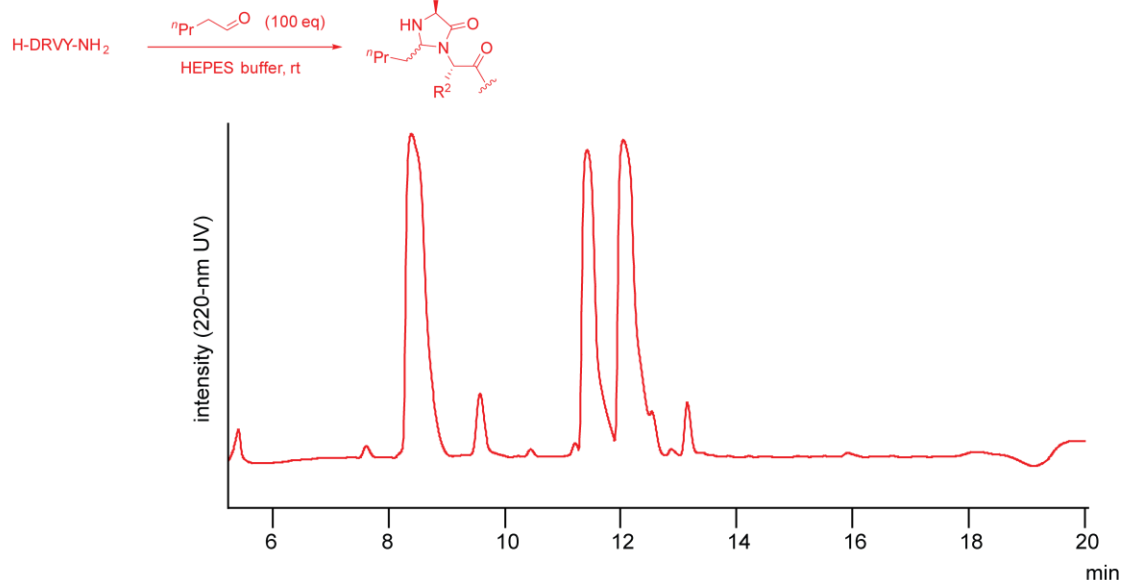


Figure S2. HPLC traces for the reaction of 4-amino acid peptide DRVY with a) *n*-propylvinylboronic acid /vitamin C b) pentanal. Detection: 220-nm UV lamp. Peaks at 11.5 min and 12.1 min corresponds to the products ([M]⁺ = 619.4), confirmed by ESI-MS.

NMR

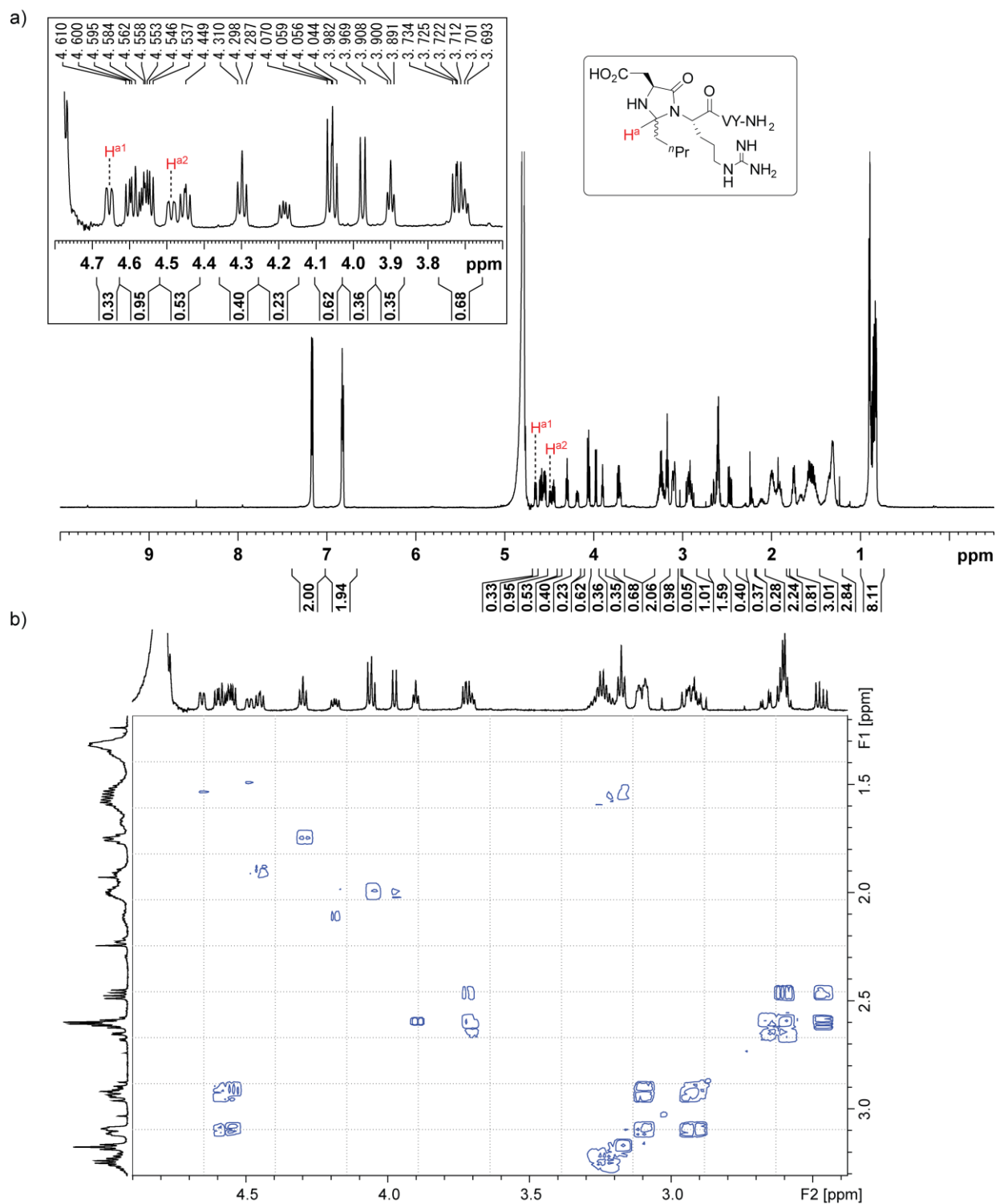


Figure S3. a) ^1H NMR (D_2O) of the isolated products as two diastomers by reaction of DRVY and *n*-propylvinylboronic acid with sodium ascorbate. Protons of the amination group were depicted as H^{a1} and H^{a2} in red. Inset: The magnified spectrum from 3.6 to 4.8 ppm. b) ^1H - ^1H COSY spectrum (F1: 1.2–3.3 ppm, F2: 2.4–4.9 ppm) of the isolated products.

MS spectra for screening and optimization

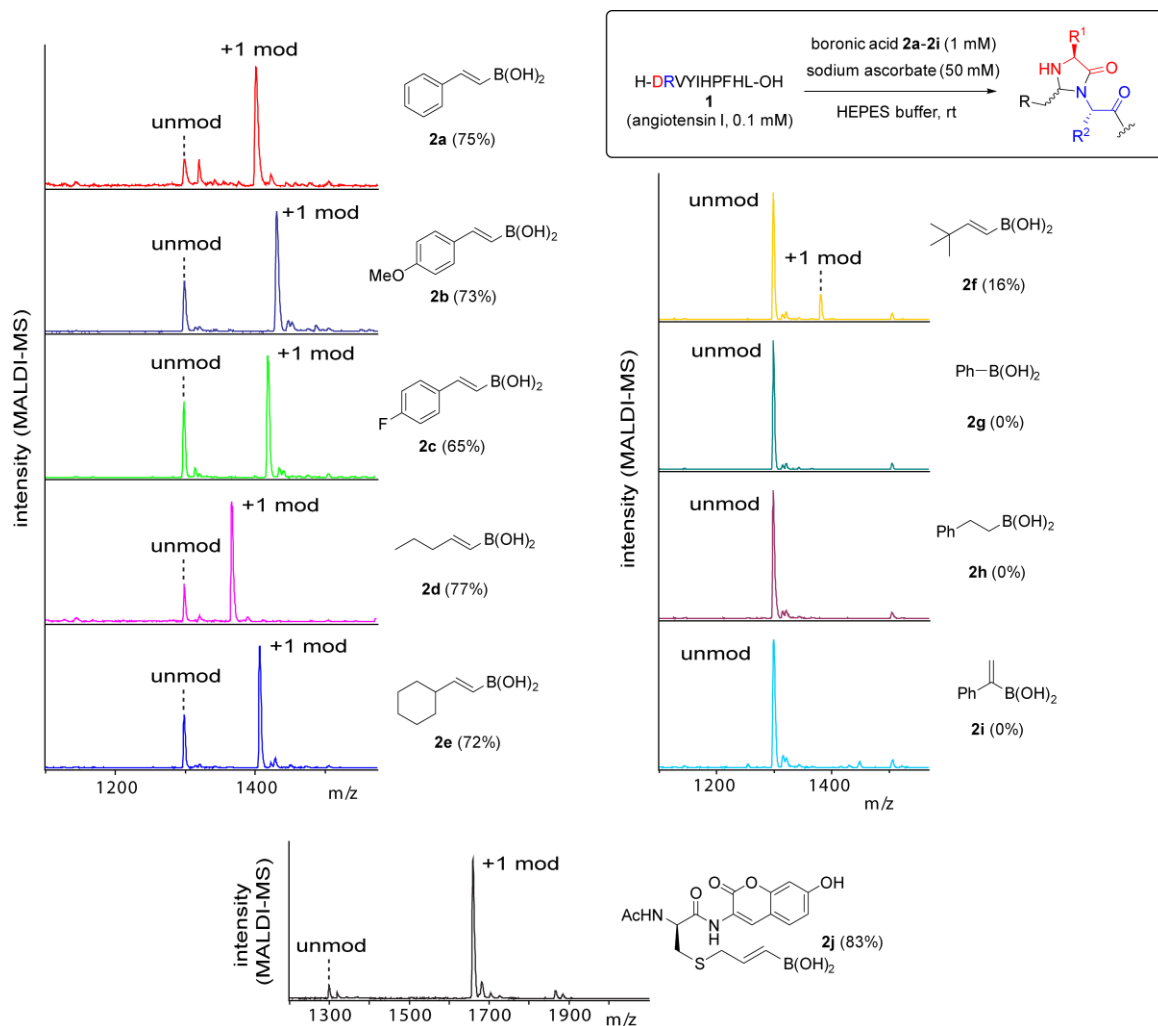


Figure S4. Spectra for Table 1. MALDI-MS spectra of the crude reaction mixture of angiotensin I (1) with various boronic acid and sodium ascorbate. Unmodified peptide was observed at 1297.5 ([M+H]⁺). Sinapic acid was used as matrix of MALDI-MS.

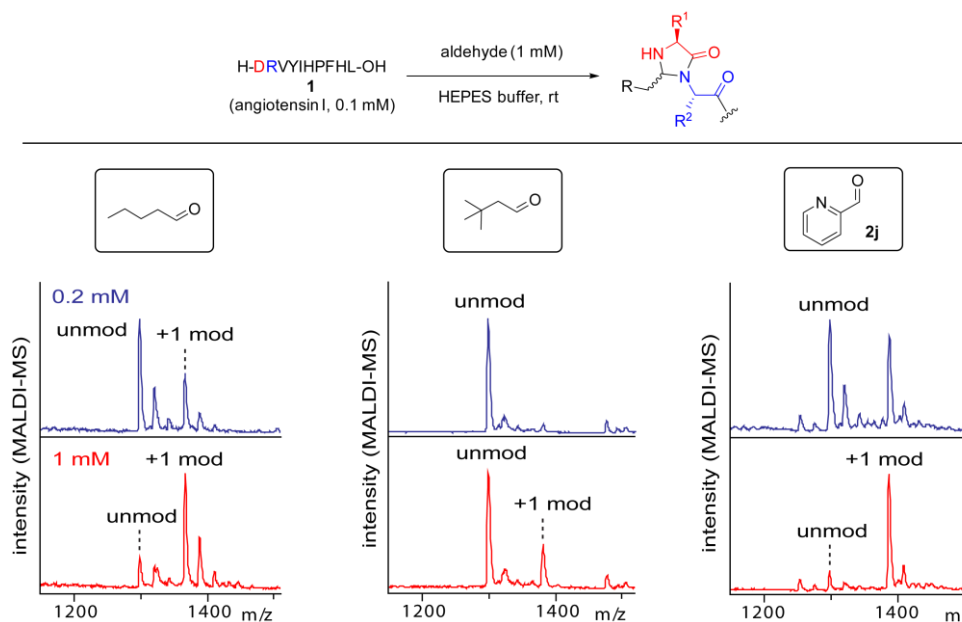


Figure S5. MALDI-MS spectra of the crude reaction mixture of angiotensin I **1** (0.1 mM) with different concn of aldehyde. Top: 0.2 mM aldehyde. Bottom: 1 mM aldehyde. Unmodified peptide was observed at 1297.5 ($[M+H]^+$). Sinapic acid was used as matrix of MALDI-MS.

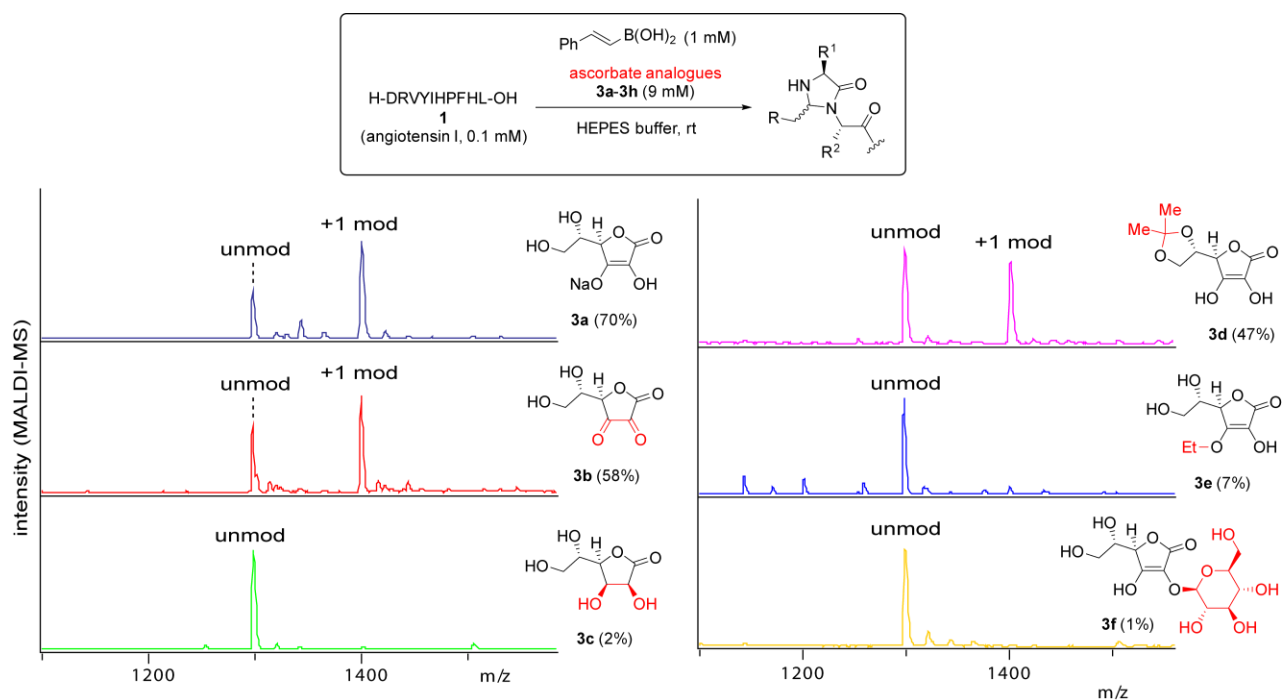


Figure S6. Spectra for Table 2. MALDI-MS spectra for the crude reaction mixture of angiotensin I (**1**) with styrylboronic acid (**2a**) and ascorbate derivatives. Unmodified peptide was observed at 1297.5 ($[M+H]^+$). Sinapic acid was used as matrix of MALDI-MS.

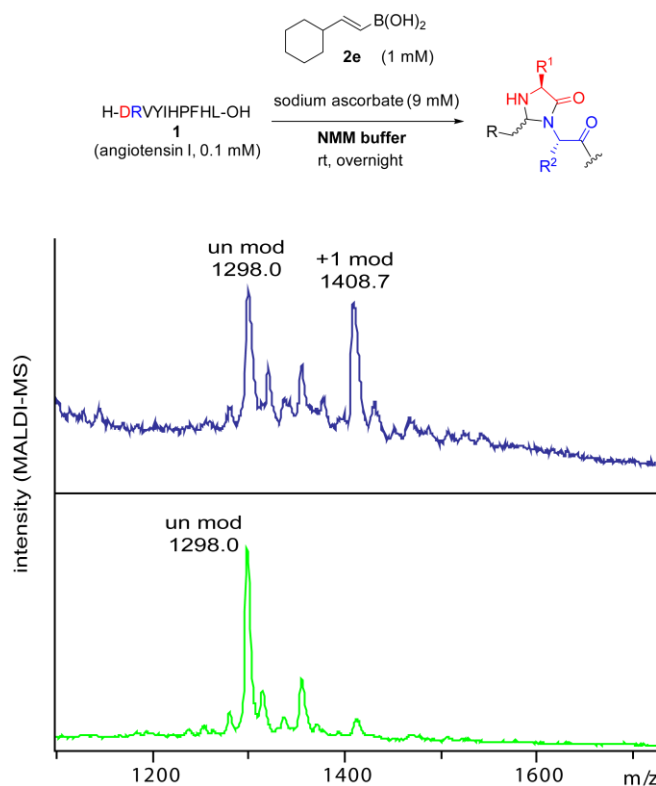


Figure S7. Different buffer. MALDI-MS spectra for the crude reaction mixture of angiotensin I (**1**) with boronate **2e** (1 mM) and sodium ascorbate (9 mM) in *N*-methylmorpholine (NMM) buffer. Reaction with (top) and without (bottom) sodium ascorbate. Unmodified peptide was observed at 1297.5 ($[\text{M}+\text{H}]^+$). Sinapic acid was used as matrix of MALDI-MS.

MS-based characterization of modification and product structure for various peptide substrates

Angiotensin I

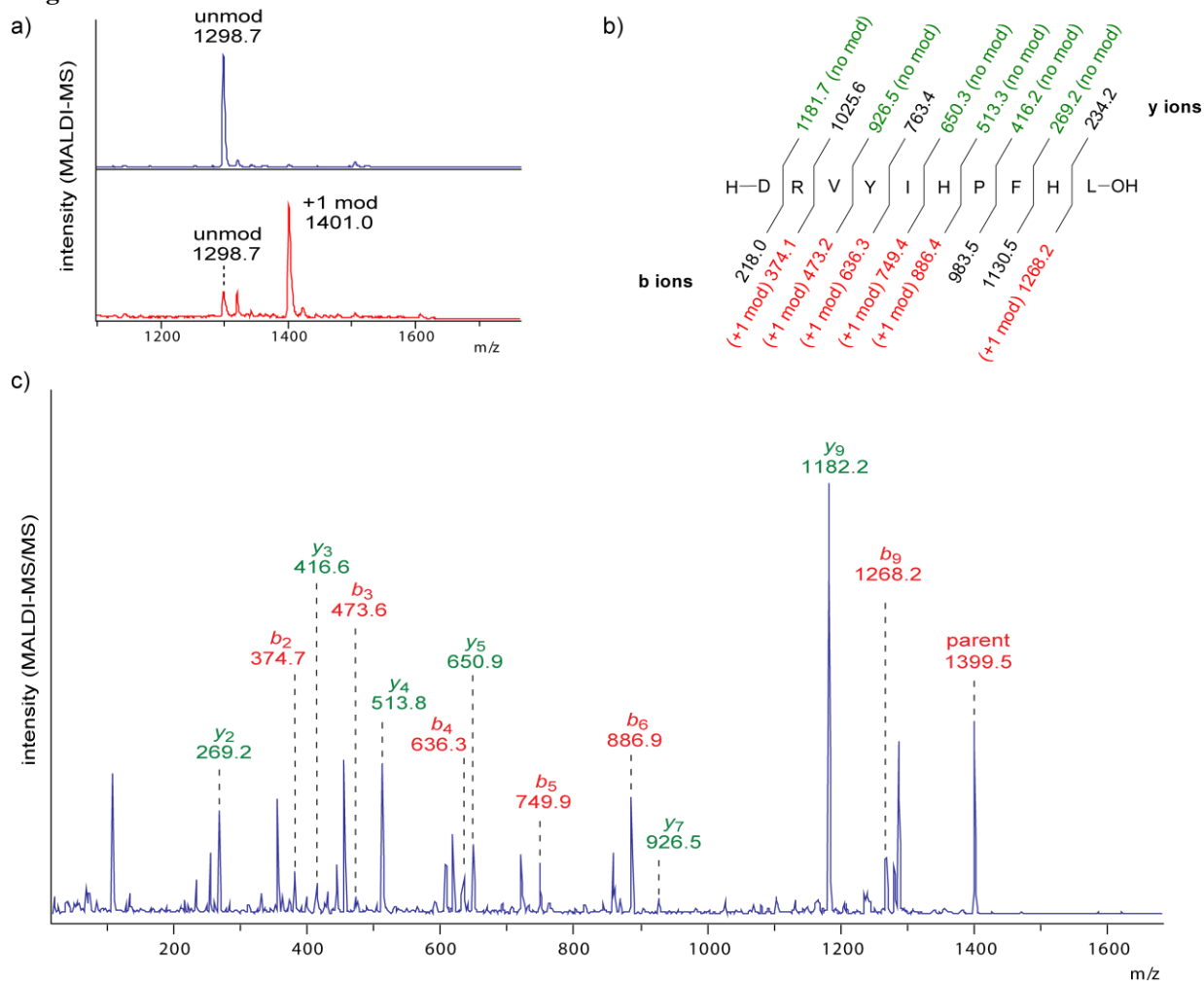


Figure S8. a) MALDI-MS spectrum of the crude modification reaction of angiotensin I (**1**) with styrylboronic acid (**2a**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for angiotensin I (**1**) modified with styrylboronic acid (**2a**) at Asp1. c) MALDI-MS/MS spectrum of angiotensin I (**1**) modified with styrylboronic acid (**2a**). Sinapic acid was used as matrix of MALDI-MS.

Angiotensin IV

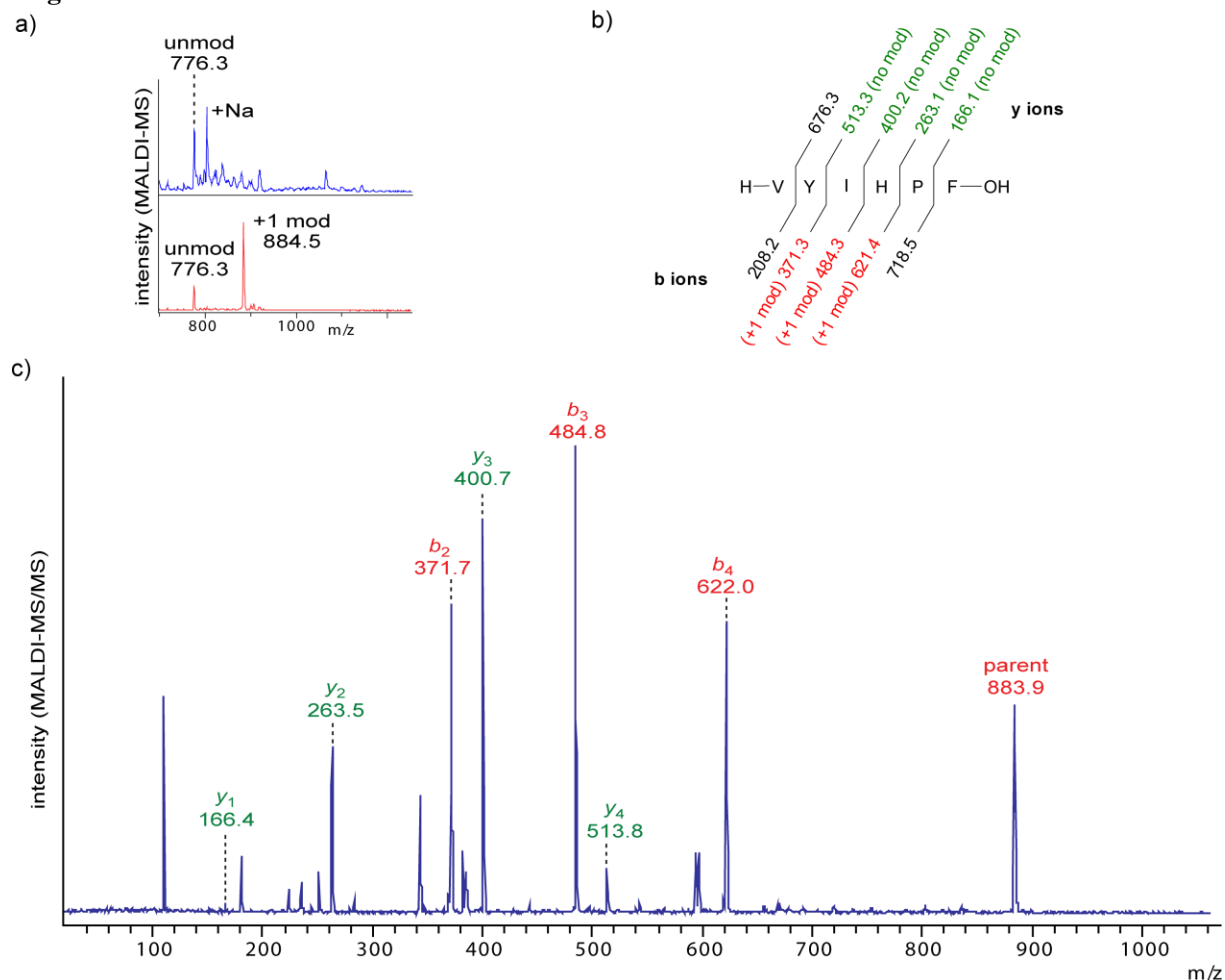


Figure S9. a) MALDI-MS spectrum of the crude modification reaction of angiotensin IV with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for angiotensin IV modified with cyclohexylboronic acid (**2e**) at Val1. c) MALDI-MS/MS spectrum of angiotensin IV modified with cyclohexylboronic acid (**2e**). Sinapic acid was used as matrix of MALDI-MS.

Amyloid β 35-25

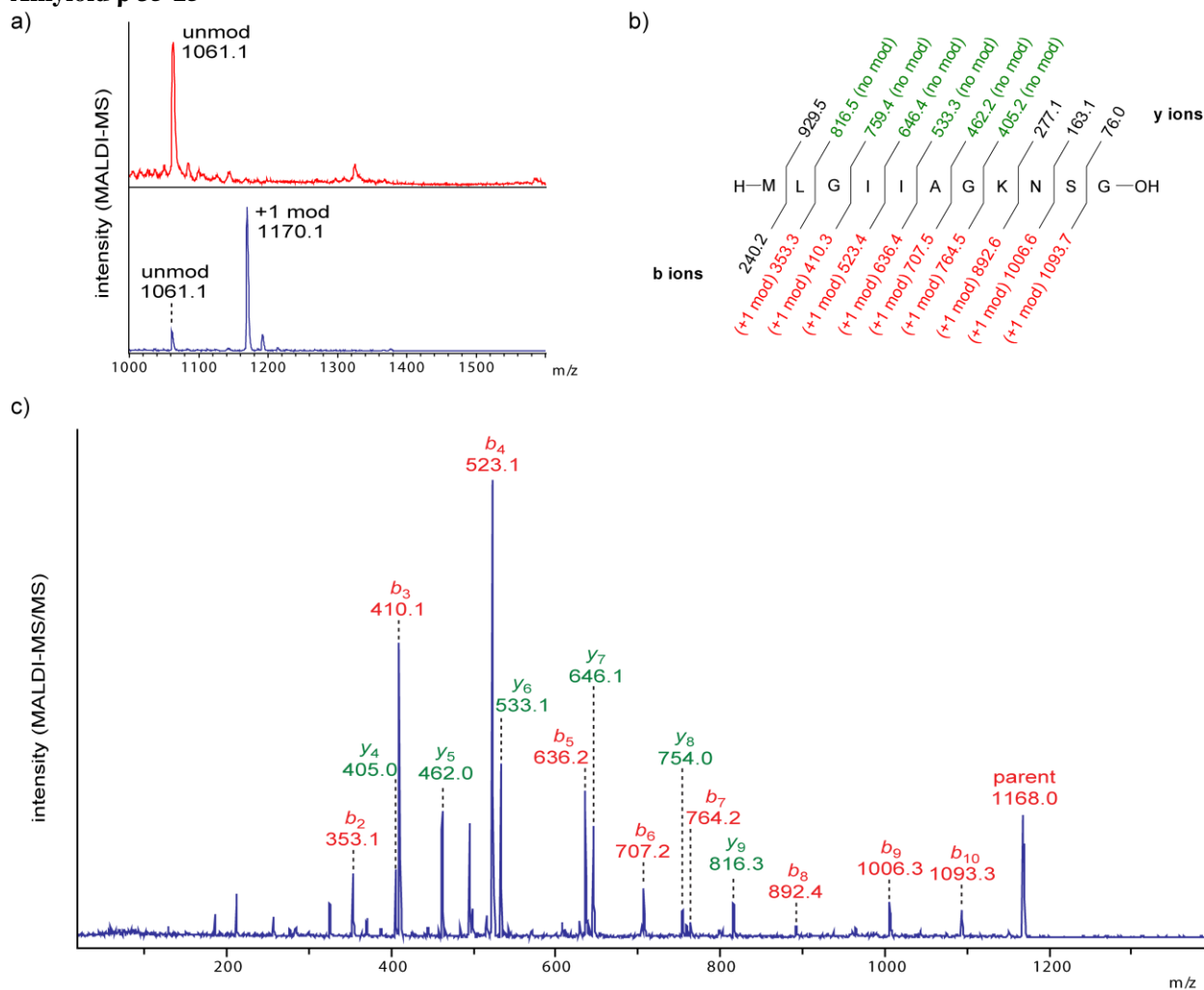


Figure S10. a) MALDI-MS spectrum of the crude modification reaction of amyloid β 35-25 with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for amyloid β 35-25 modified with cyclohexylboronic acid (**2e**) at Met1. c) MALDI-MS/MS spectrum of amyloid β 35-25 modified with cyclohexylboronic acid (**2e**). Sinapic acid was used as matrix of MALDI-MS.

MF α 1-6

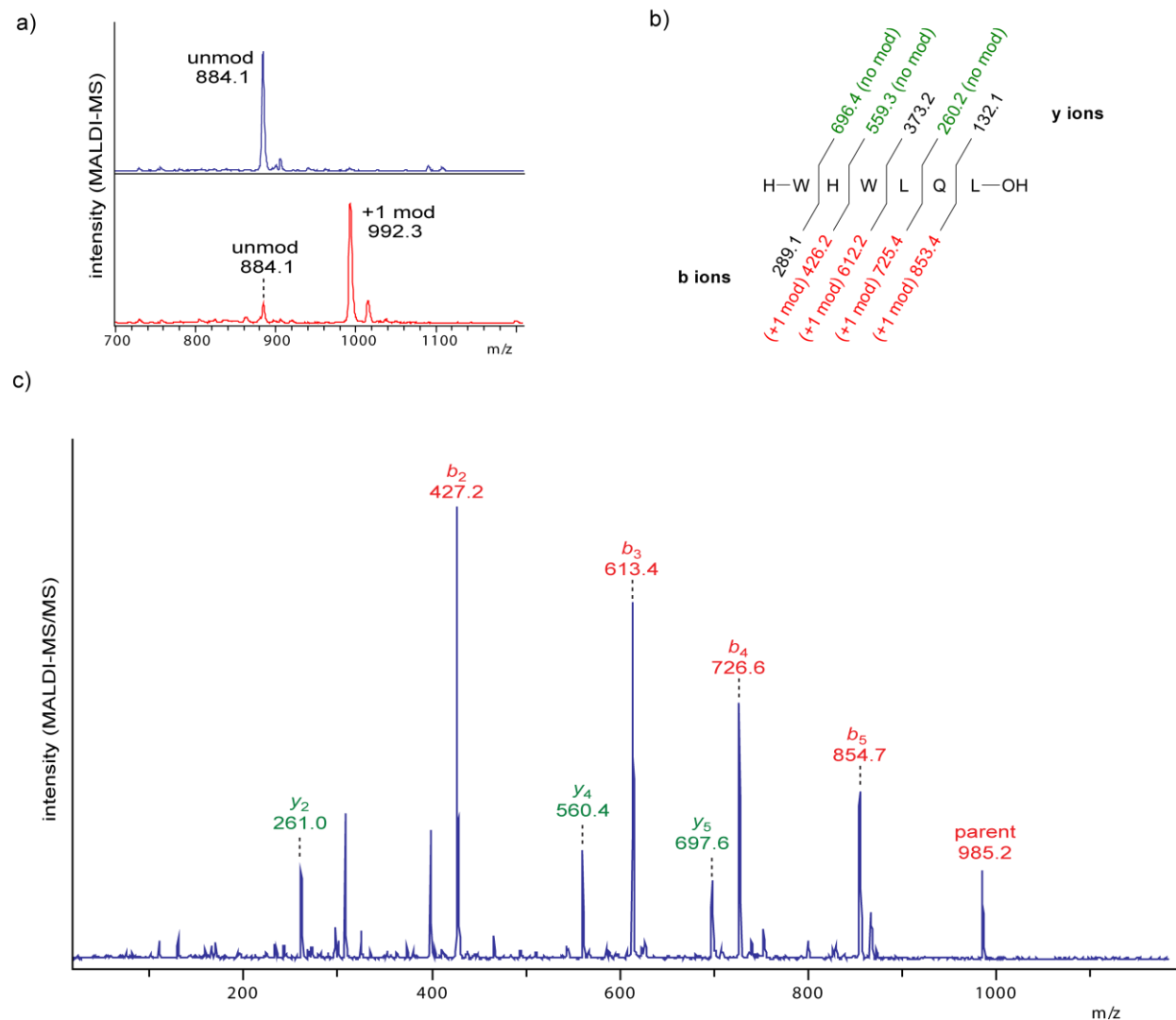


Figure S11. a) MALDI-MS spectrum of the crude modification reaction of MF α 1-6 with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for MF α 1-6 modified with styrylboronic acid (**2a**) at Trp1. c) MALDI-MS/MS spectrum of MF α 1-6 modified with styrylboronic acid (**2a**). Sinapic acid was used as matrix of MALDI-MS.

Laminin 925-933

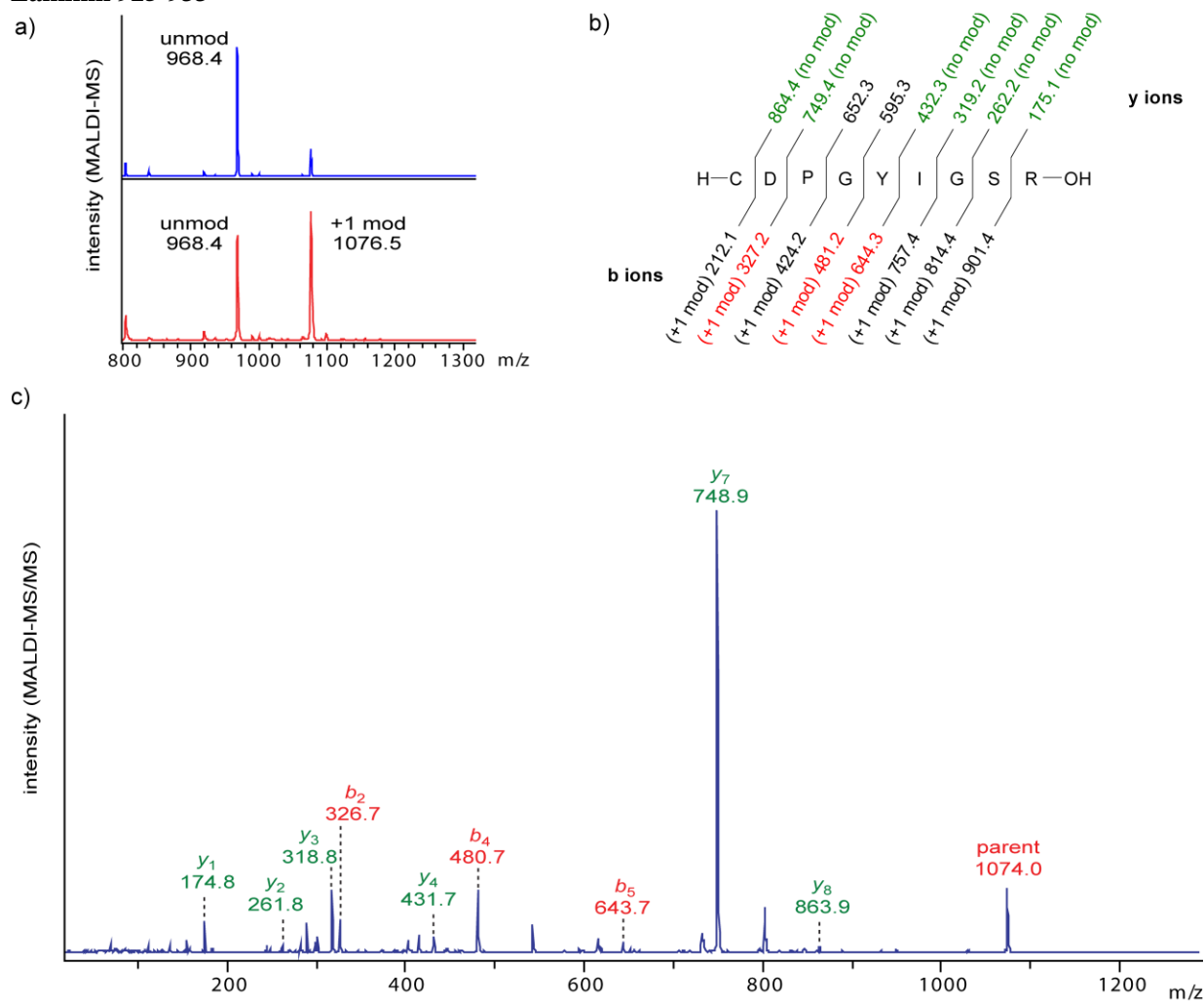


Figure S12. a) MALDI-MS spectrum of the crude modification reaction of laminin 925-933 with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for laminin 925-933 modified with cyclohexylboronic acid (**2e**) at Cys1. c) MALDI-MS/MS spectrum of laminin 925-933 modified with cyclohexylboronic acid (**2e**). α -cyano-4-hydroxycinnamic acid was used as matrix of MALDI-MS.

Hexahistidine

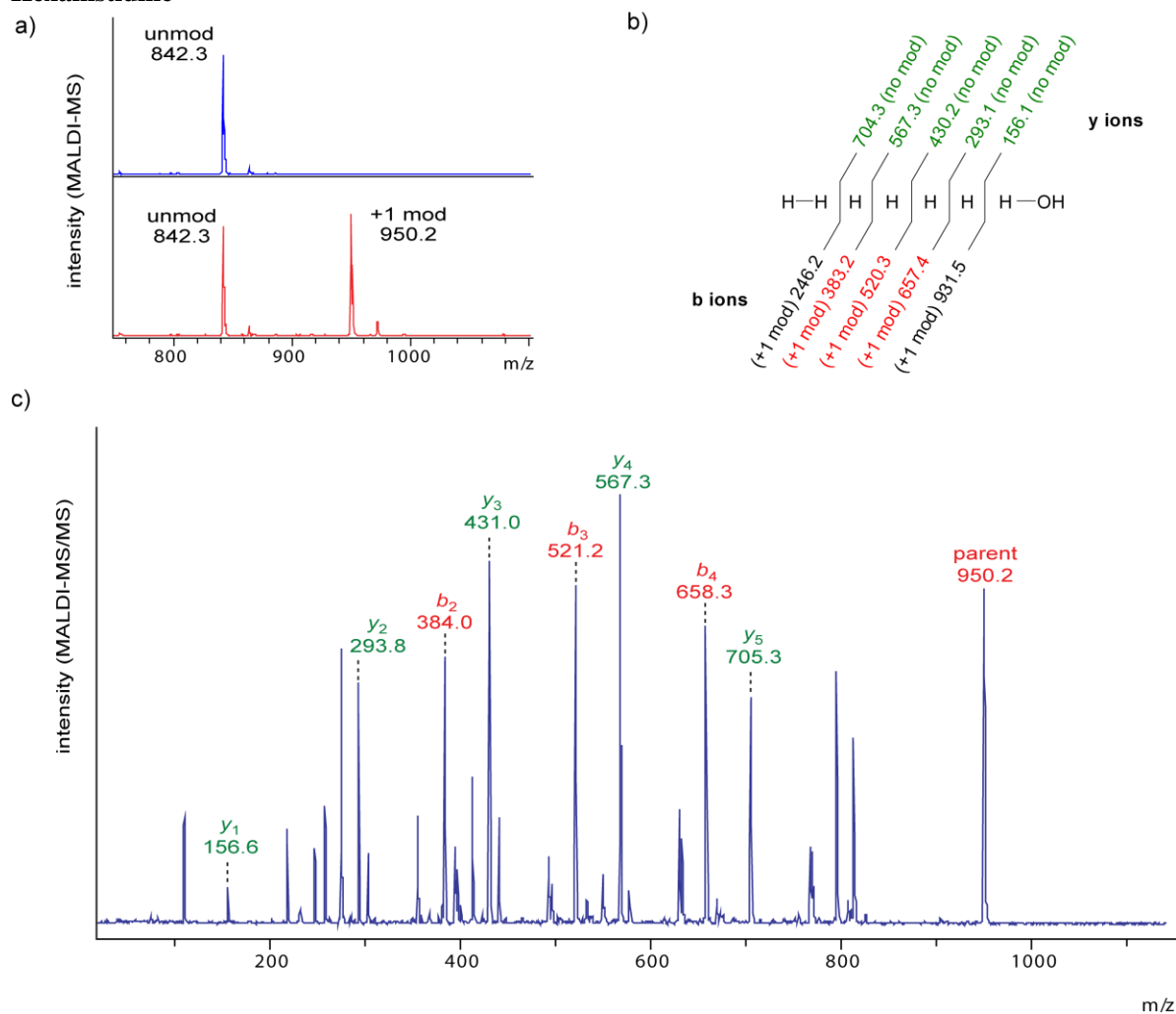
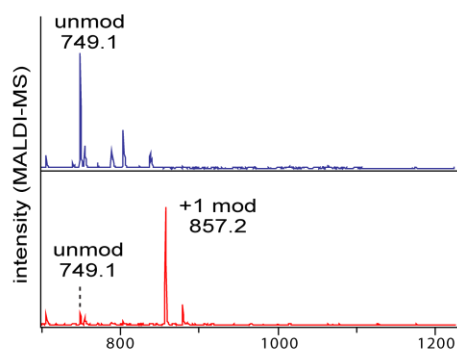


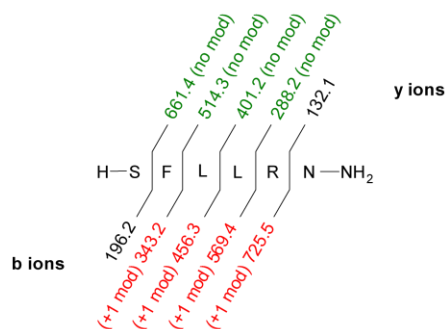
Figure S 13. a) MALDI-MS spectrum of the crude modification reaction of hexahistidine with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for hexahistidine modified with cyclohexylboronic acid (**2e**) at His1. c) MALDI-MS/MS spectrum of hexahistidine modified with cyclohexylboronic acid (**2e**). The reaction was conducted at higher concn than one described in the general procedure. Reaction conditions: hexahistidine (0.2 mM), cyclohexylvinylboronic acid (2 mM), sodium ascorbate (50 mM) in HEPES buffer at rt overnight. α -cyano-4-hydroxycinnamic acid was used as matrix of MALDI-MS.

PAR-1AP

a)



b)



c)

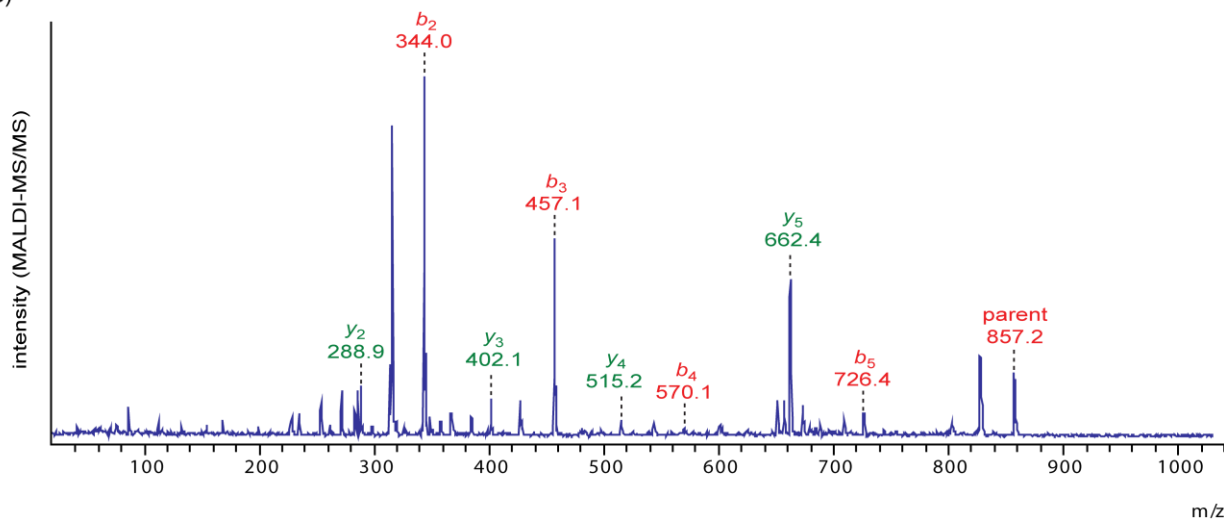


Figure S14. a) MALDI-MS spectrum of the crude modification reaction of PAR-1AP with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for PAR-1AP modified with cyclohexylboronic acid (**2e**) at Ser1. c) MALDI-MS/MS spectrum of PAR-1AP modified with cyclohexylboronic acid (**2e**). α -cyano-4-hydroxycinnamic acid was used as matrix of MALDI-MS.

HIV Tat 47-57

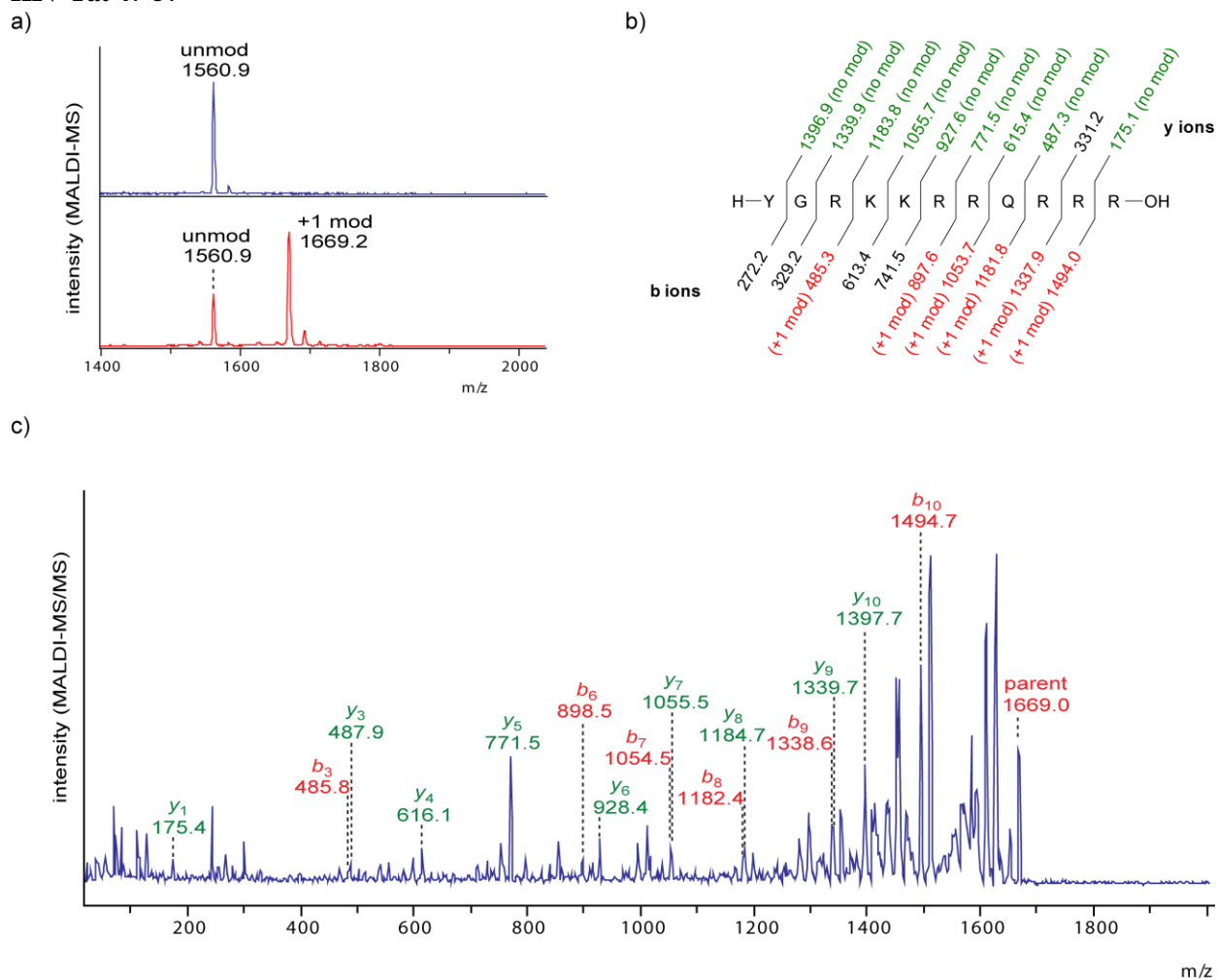


Figure S15. a) MALDI-MS spectrum of the crude modification reaction of HIV Tat 47-57 with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for HIV Tat 47-57 modified with cyclohexylboronic acid (**2e**) at Tyr1. c) MALDI-MS/MS spectrum of HIV Tat 47-57 modified with cyclohexylboronic acid (**2e**). α -cyano-4-hydroxycinnamic acid was used as matrix of MALDI-MS.

Neuromedin B

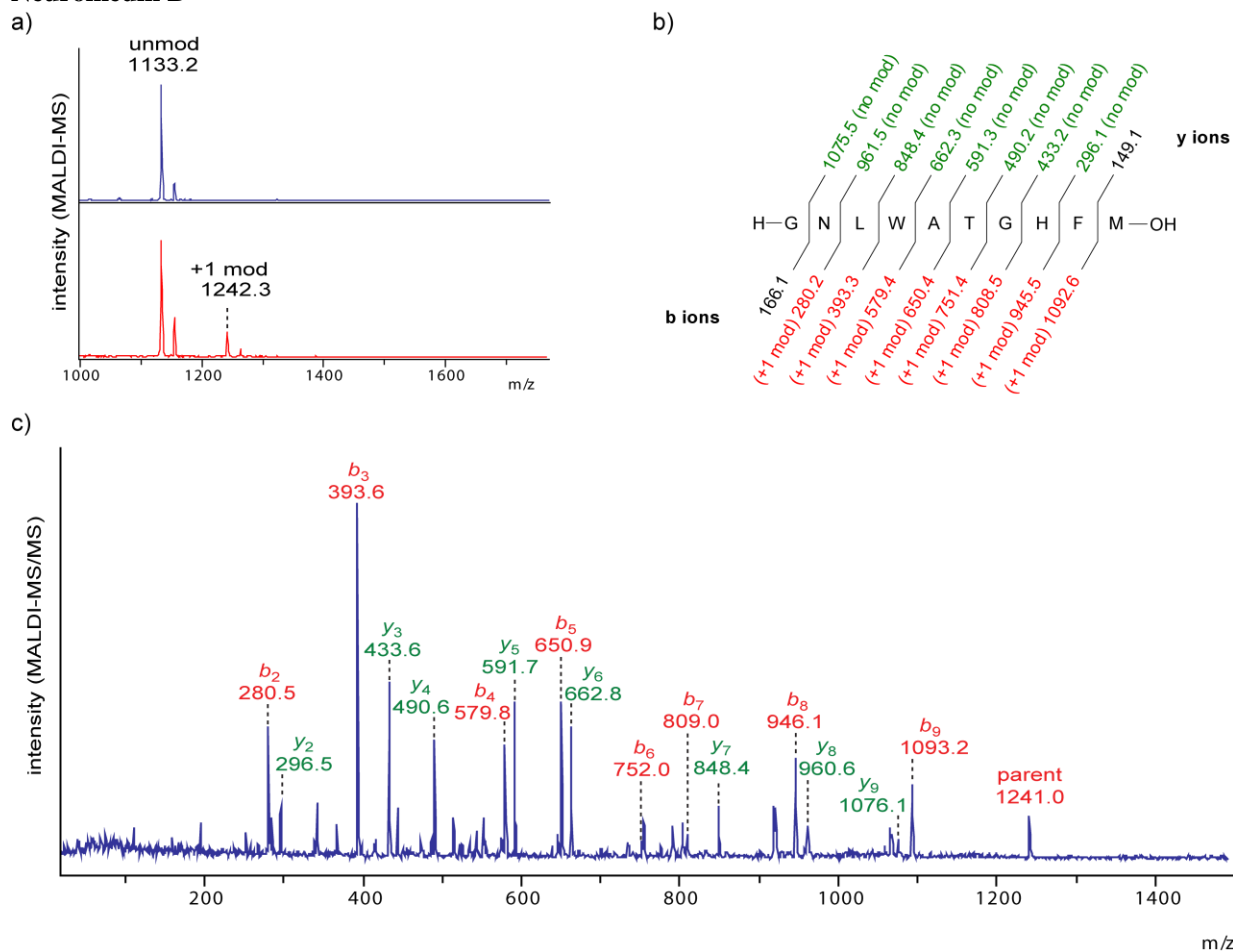


Figure S16. a) MALDI-MS spectrum of the crude modification reaction of neuromedin B with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for neuromedin B modified with cyclohexylboronic acid (**2e**) at Gly1. c) MALDI-MS/MS spectrum of neuromedin B modified with cyclohexylboronic acid (**2e**). Sinapic acid was used as matrix of MALDI-MS.

Neurotensin 8-13

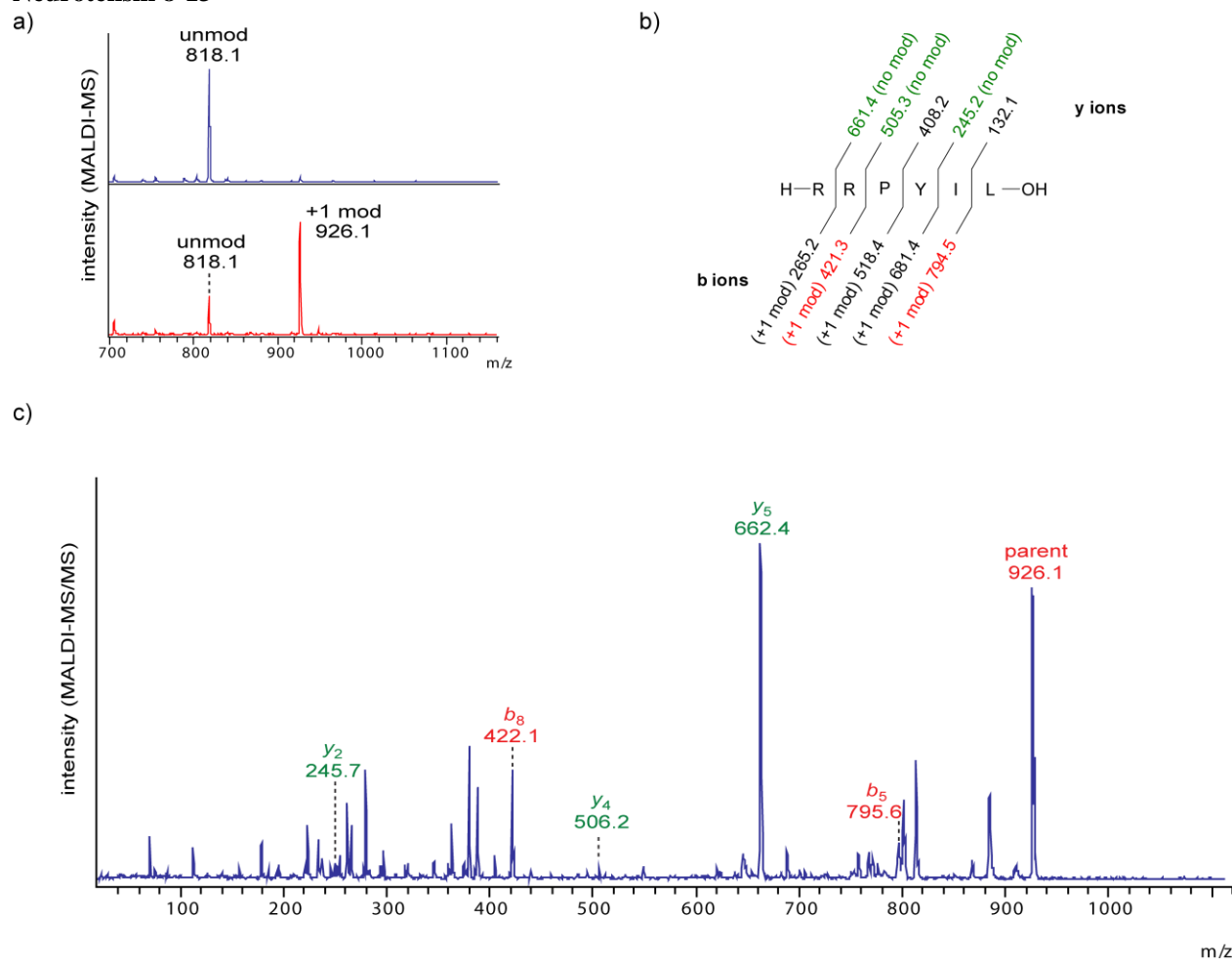


Figure S17. a) MALDI-MS spectrum of the crude modification reaction of neurotensin 8-13 with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) Fragmentation ladder for neurotensin 8-13 modified with cyclohexylboronic acid (**2e**) at Arg1. c) MALDI-MS/MS spectrum of neurotensin 8-13 modified with cyclohexylboronic acid (**2e**). α -cyano-4-hydroxycinnamic acid was used as matrix of MALDI-MS.

Bradykinin

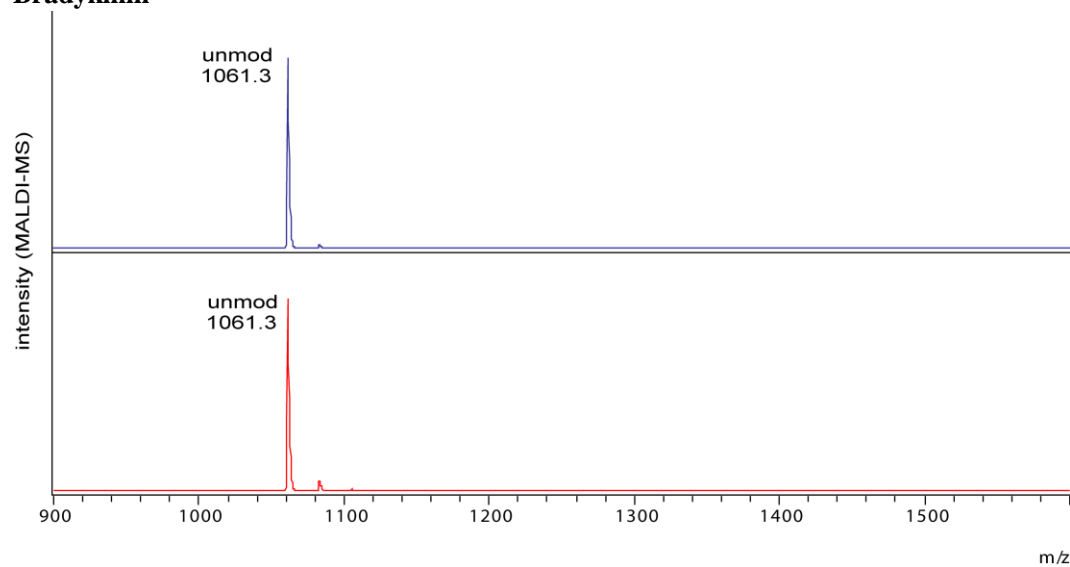


Figure S18. a) MALDI-MS spectrum of the crude modification reaction of bradykinin with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. α -cyano-4-hydroxycinnamic acid was used as matrix of MALDI-MS.

Human insulin

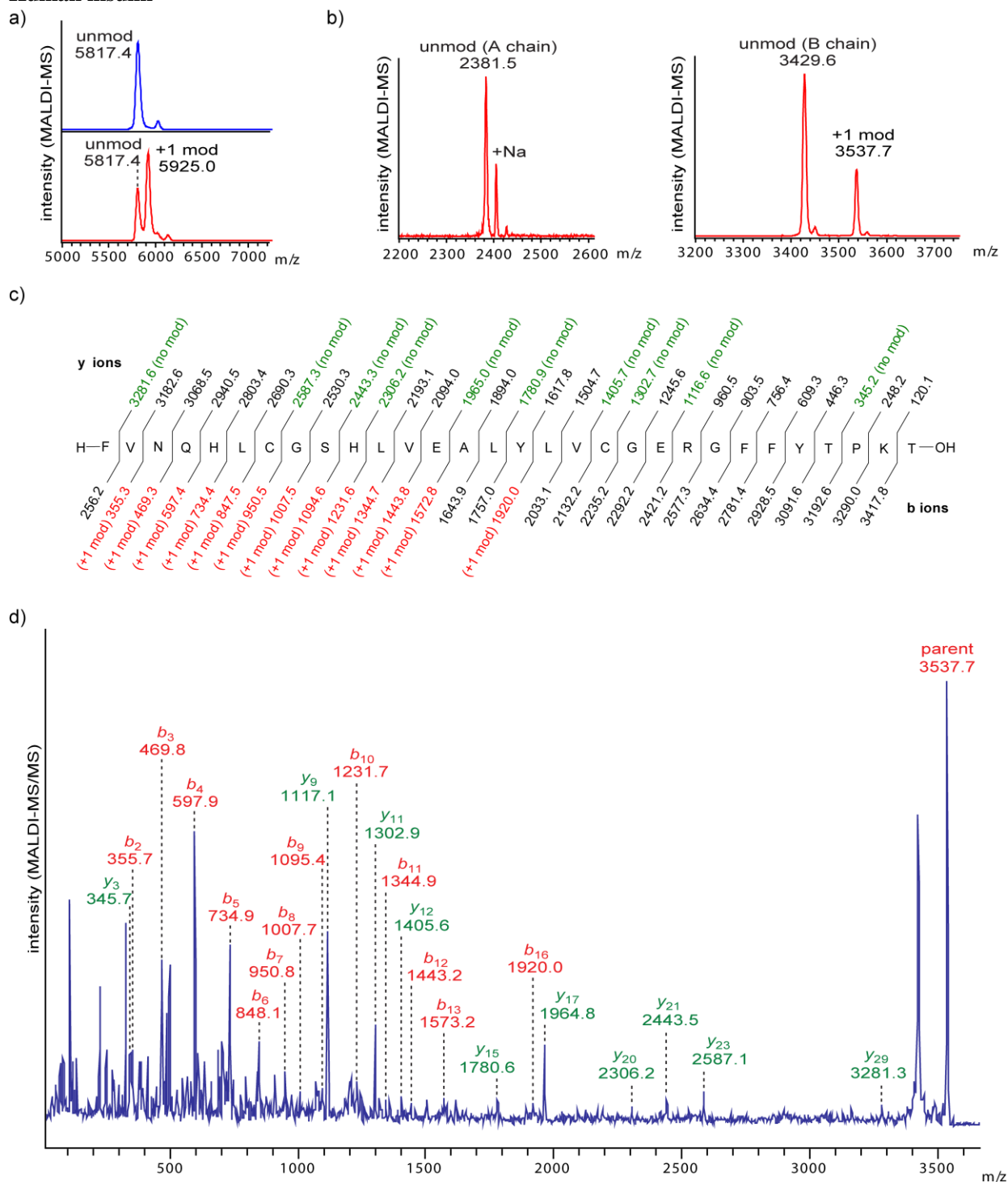
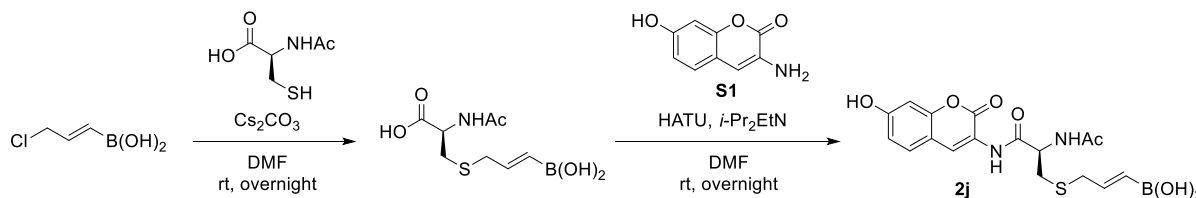


Figure S19. a) MALDI-MS spectrum of the crude modification reaction of human insulin with cyclohexylboronic acid (**2e**). Top: reaction without sodium ascorbate. Bottom: reaction with sodium ascorbate. b) MALDI-MS spectra of the modified insulin after reduction with DTT (20 mM) and desalting process. c) Fragmentation ladder for human insulin modified with cyclohexylboronic acid (**2e**) at PheB1 d) MALDI-MS/MS spectrum of human insulin modified with cyclohexylboronic acid (**2e**). Sinapic acid was used as matrix of MALDI-MS for the crude modification reaction mixture and α -cyano-4-hydroxycinnamic acid was used for the reduced samples.

Synthesis and characterization of fluorophore-borate **2j**

Synthesis



N-Acetyl-cysteine (61.3 mg, 0.376 mmol), (*E*)-(chloromethyl)vinylboronic acid (79.1 mg, 0.657 mmol), and cesium carbonate (276.5 mg, 0.849 mmol) were mixed with DMF (1 mL). The mixture was stirred at rt overnight. Acetic acid (600 μ L) was added, and the suspension was filtered through cotton. The volatiles were removed by gentle flow of nitrogen gas. The resulting solid was dissolved in aq trifluoroacetic acid (0.1% v/v) and purified by reverse-phase HPLC (5-21% acetonitrile/H₂O over 14.5 min). Lyophilization of the obtained fractions afforded the carboxylic acid intermediate as a white solid (84.3 mg). The carboxylic acid intermediate (23.2 mg, 0.094 mmol) and HATU (41.1 mg, 0.108 mmol) were dissolved in DMF (1 mL), and *i*-Pr₂EtN (20 μ L, 0.113 mmol) was added to the soln at 0 $^{\circ}$ C. The mixture was stirred at rt for 10 min. Coumarin **S1** (61.1 mg, 0.345 mmol) was added to the soln in one portion at 0 $^{\circ}$ C, and the soln was stirred at rt overnight. The precipitate was removed by filtration, washing with acetonitrile (2 mL), and all of volatiles were removed by gentle flow of nitrogen gas. The resulting solid was dissolved in 9:1 H₂O/MeCN with 0.1% v/v trifluoroacetic acid, purified by reverse-phase HPLC (10-60% acetonitrile/H₂O over 22 min), and lyophilized to afford **2j** as an off-white solid (12.2 mg, two steps 29%). ¹H NMR (600 MHz, DMSO-*d*₆): δ 10.43 (br, 1H), 9.61 (br, 1H), 8.49 (s, 1H), 8.35 (d, *J* = 7.8 Hz, 1H), 7.62 (br, 2H), 7.52 (d, *J* = 8.4 Hz, 1H), 6.80 (dd, *J* = 8.4, 1.8 Hz, 1H), 6.74 (d, *J* = 1.2 Hz, 1H), 6.33 (dt, *J* = 17.4, 6.6 Hz, 1H), 5.42 (d, *J* = 17.4 Hz, 1H), 4.73 (m, 1H), 3.25 (d, *J* = 6.6 Hz, 2H), 2.81 (m, 1H), 2.64 (m, 1H), 1.89 (s, 3H). ¹³C NMR (151 MHz, DMSO-*d*₆): δ 170.5, 169.7, 159.8, 157.8, 151.6, 144.5, 129.1, 126.2, 120.4, 113.7, 111.2, 102.0, 53.0, 35.6, 31.9, 22.4. ESI-MS: *m/z* calcd for C₁₇H₁₉BN₂O₇S [M-H]⁻ 405.1, found 405.3.

ESI-MS

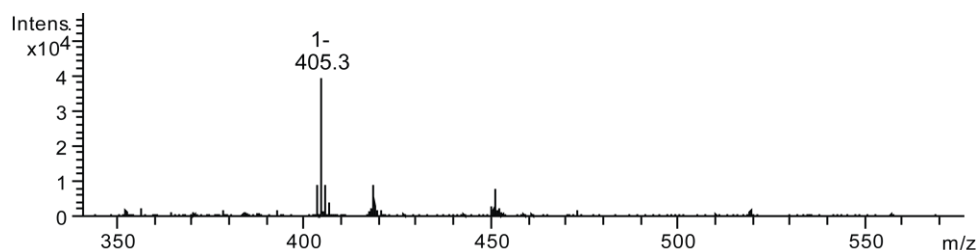


Figure S20. Boronate **2j** (negative mode). *m/z* 405.3 corresponds to [M-H]⁻.

NMR

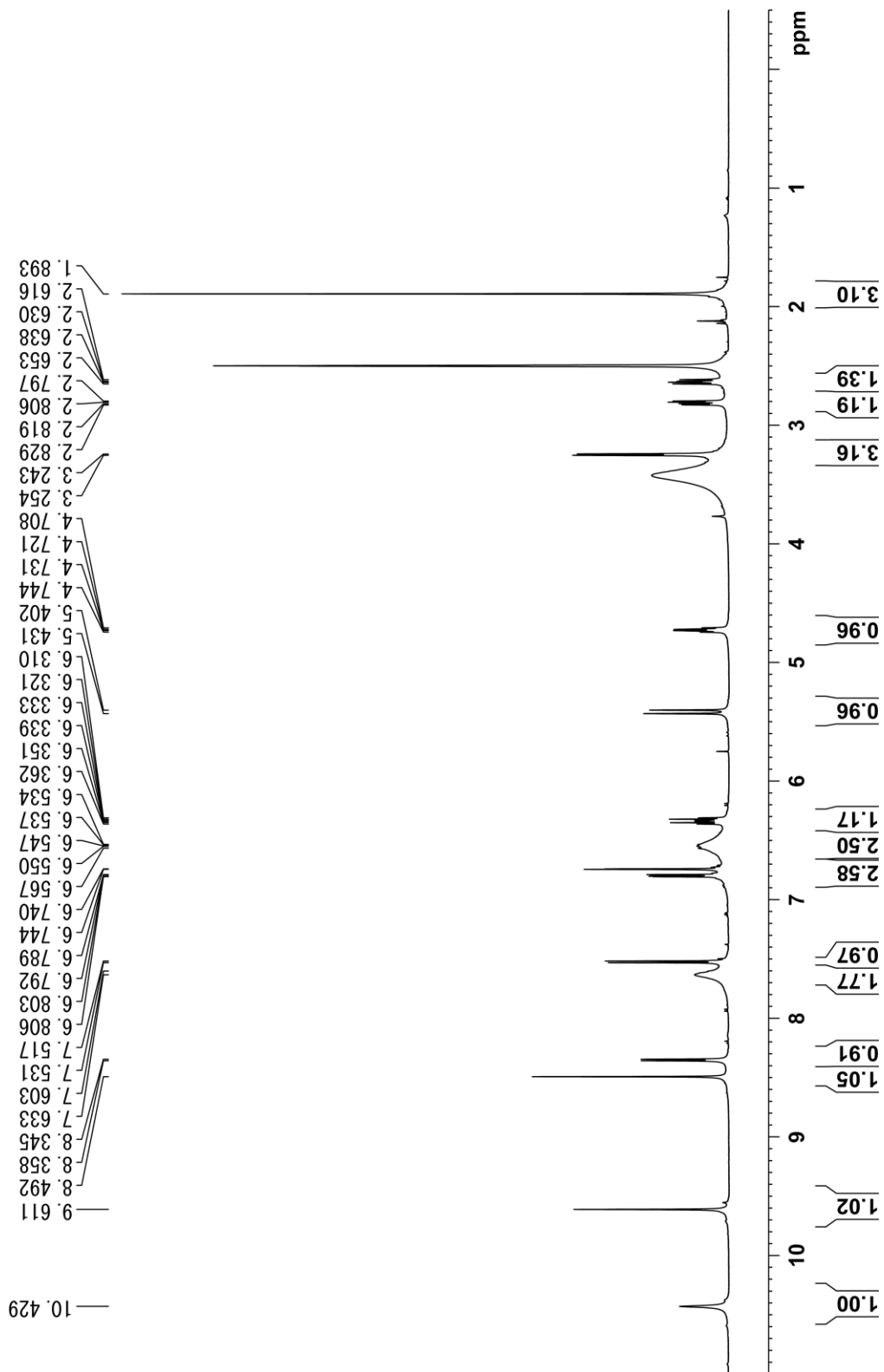


Figure S21. ^1H NMR spectrum of coumarin-boronate (**2j**) in $\text{DMSO-}d_6$.

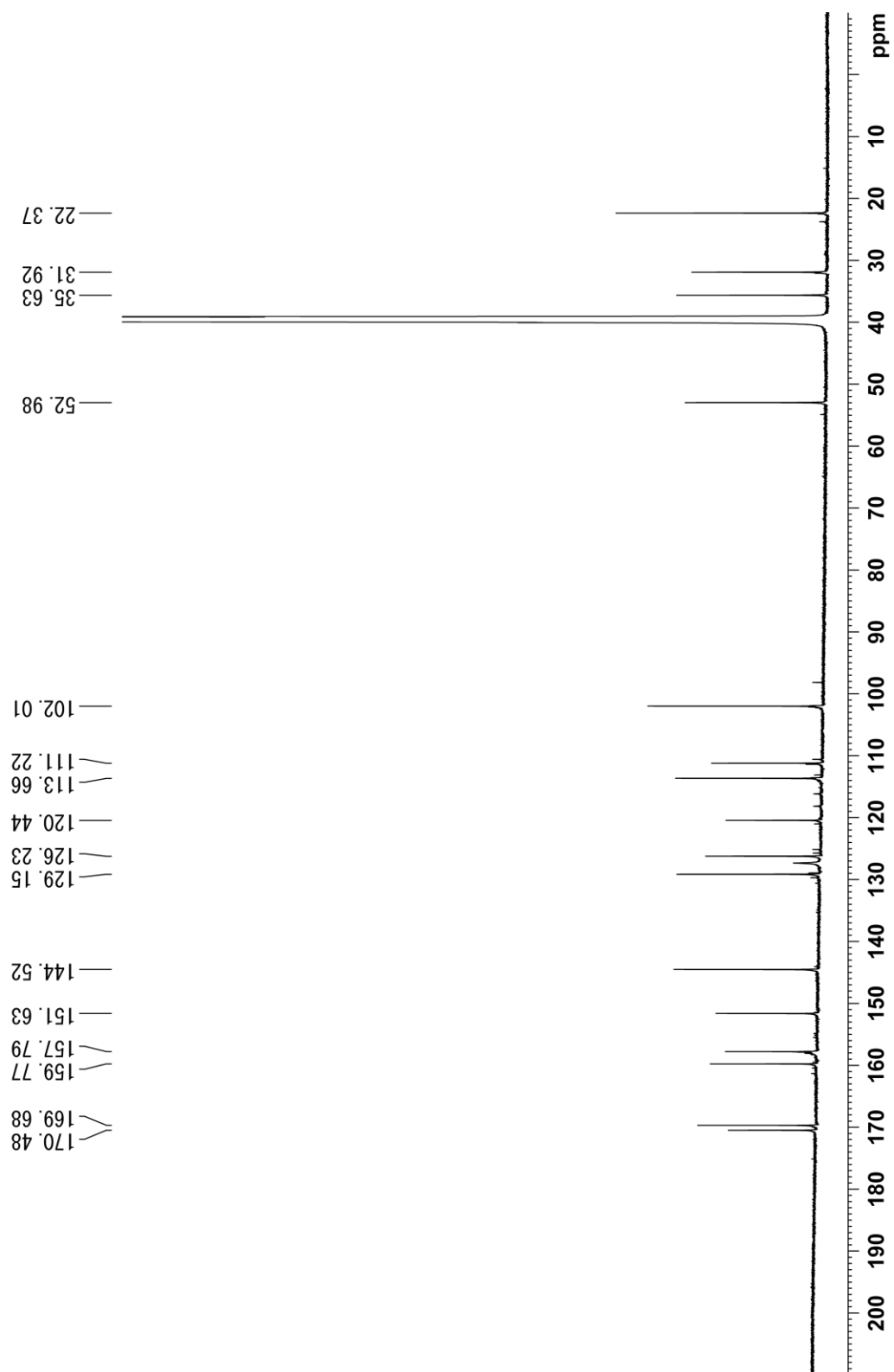


Figure S22. ¹³C NMR spectrum of coumarin-boronate (**2j**) in DMSO-*d*₆.

References

- (1) Wellings, D. A.; Atherton, E. *Method Enzymol* **1997**, 289, 44.
- (2) Yi, L.; Li, H. Y.; Sun, L.; Liu, L. L.; Zhang, C. H.; Xi, Z. *Angew. Chem., Int. Edit.* **2009**, 48, 4034.