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Electronic Supplementary Information for

Chemical synthesis of membrane proteins: a model study on the influenza virus B proton channel

Andreas C. Baumruck, Daniel Tietze, Lena Steinacker and Alesia A. Tietze*

*To whom correspondence should be addressed,
Email: a.tietze@tietze-lab.de

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Table S1. Cleavable solubility tags used to increase the solubility and NCL described in the literature

Peptide length	Peptide name	For purification	For ligation	Strategy	Linker	Solubility tag	Ref.
12, 14	dodecaalanine (Ala12) and chemotactic protein 10	x		Boc-SPPS	glycoamid ester[a]	$(GlyArg_4)_4$	[1]
12-48	model peptides	x		Fmoc-SPPS	4-Hmb[a]	$Gly(ArgGlyGly)_3$ $Gly(LysGly)_6$	[2]
99	HIV-1 protease		x	Boc-SPPS	3-mercaptopropionic acid[b]	$(Arg)_6$	[3]
124	ribonuclease A		x	Boc-SPPS	3-mercptopropionic acid[b]	$(Arg)_6$	[4]
53	insulin glargine	x		Fmoc-SPPS	HMBA[a]	$(Lys)_5$	[5]
40	cancer protein NY-ESO-1		x	Fmoc-SPPS, Boc-SPPS	HMBA[a]	$(Arg)_6$	[6]
121	diacylglycerol kinase (DAGK1-121)		x	Boc-SPPS	thioglycolic acid[b]	PPO/$(Arg)_6$	[7]
79	dengue 2 capsid protein C (DEN2C)		x	Boc-SPPS	3-mercptopropionic acid[b]	$(Arg)_6$	[8]
32	conotoxins	x		Fmoc-SPPS	PAM[c]	$(Lys)_4$	[9]
42, 46	Aß42 and Aß46	x		Fmoc-SPPS	no linker	$(Lys)_2$, $(Lys)_3$,$(Lys)_6$	[10]
11	Q11		x[e]	Fmoc-SPPS	Mmsb-OH[d]	$(Lys)_6$	[11]

HMBA – 4-hydroxymethylbenzoic acid
PPO – poly(ethyleneglycol)–polyamide
PAM – phenylacetamido
a – pH-sensitive
b – self-cleavable during NCL
c – cleavable by HF
d – reductive acidolysis using NH_4I
e – fragment condensation of two fragments

Table S2. Analytical data for peptides used in this study

	Peptide	Calculated M_w [g/mol]	Experimental M_w[b] [g/mol]	t_R[c] [min]
1	ALHFL-Hmp[a]	703.36	703.40	16.97/17.66[d]
2	ALHFL-Hmp-ADO[a]	848.43	848.51	17.15/17.65[d]
3	ALHFL-Hmp-ADO_2[a]	993.50	993.61	17.55/17.90[d]
4	ALHFL-Hmp-ADO-Lys_5[a]	1489.91	1489.02	6.62/6.97[d]
5	[Cys^{22}]BM2(22-35)	1637.90	1637.91	7.98[e]
7	BM2(1-21)-NH_2	2421.30	2422.35	12.55[f]
8	[Cys^{11}(Acm)]BM2(1-21)-Hmp	2596.33	2597.32/2597.38	12.34/12.46[g]
9	[Cys^{11}(Acm)]BM2(1-21)-Hmp-ADO_2	2886.48	2887.56/2887.56	9.95/10.36[h]
10	[Cys^{11}(Acm)]BM2(1-21)-Hmp-ADO-Lys_5	3382.89	3384.27	10.36[g]
11	[Cys^{22}]BM2(22-51)	3530.97	3531.34	8.19[i]

[a] Corresponds to abbreviation BM2(17-21);

[b] mass peaks detected as $[M+H]^+$;

[c] retention times of peptides **1-5** and **8**, **9** are given for both diastereomers, if applicable;

[d] HPLC conditions: 15–45 % eluent B in 30 min;

[e] HPLC conditions: 20–80 % eluent B in 20 min;

[f] HPLC conditions: 50 – 99 % eluent B in 15 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column;

[g] 10 – 20% eluent B in 5 min followed by 20 – 70% eluent B in 20 min, at a flow rate of 2 mL/min, C4 column;

[h] 40 – 80 % eluent B in 20 min, at a flow rate of 1 mL/min, C18 column;

[i] 20-40 % eluent B in 30 min at a flow of 1 mL/min, C18 column.

Table S3. Ligation results for model peptides

	Ligated fragments	[^{22}Cys(Acm)]BM2(17-35) (**6**), [%][a]	Hydrolyzed ester (ALHFL-C^{α}OOH) [%][a]
1+5	ALHFL-Hmp + [Cys^{22}]BM2(22-35)	87	13
2+5	ALHFL-Hmp-ADO + [Cys^{22}]BM2(22-35)	86	14
3+5	ALHFL-Hmp-ADO_2 + [Cys^{22}]BM2(22-35)	85.4	14.6
4+5	ALHFL-Hmp-ADO-Lys_5 + [Cys^{22}]BM2(22-35)	84	16

[a] determined through integration of RP-HPLC chromatogram

Table S4. Ligation results for BM2 peptides in ligation buffers A, B and C

	Ligated fragments	[Cys^{11} (Acm)]BM2(1-51))(**12a**)/ hydrolyzed ester ([Cys^{11}(Acm)]BM2(1-21)-C$^{\alpha}$OOH) in **ligation buffer A**[a]	[Cys^{11}(Acm)]BM2(1-51) (**12a**)/ hydrolyzed ester ([Cys^{11}(Acm)]BM2(1-21)-C$^{\alpha}$OOH) in **ligation buffer B**[a]	[Cys^{11}(Acm)]BM2(1-51) **12a**)/ hydrolyzed ester ([Cys^{11}(Acm)]BM2(1-21)-C$^{\alpha}$OOH) in **ligation buffer C** [%][a]
8a+11	[Cys^{11}(Acm)]BM2(1-21)-Hmp diastereomer A+ [Cys^{22}]BM2(22-51)	n.d.	n.d.	96/4
8b+11	[Cys^{11}(Acm)]BM2(1-21)-Hmp diastereomer B+ [Cys^{22}]BM2(22-51)	n.d.	n.d.	97/3
9a+11	[Cys^{11}(Acm)]BM2(1-21)-Hmp-ADO$_2$ diastereomer A + [Cys^{22}]BM2(22-51)	n.d.	65/45	96/4
9b+11	[Cys^{11}(Acm)]BM2(1-21)-Hmp-ADO$_2$ diastereomer B + [Cys^{22}]BM2(22-51)	n.d.	n.d.	96/4
10+11	[Cys^{11}(Acm)]BM2(1-21)-Hmp-ADO-Lys$_5$ + [Cys^{22}]BM2(22-51)	80/20	n.d.	88/12

[a] determined through integration of RP HPLC chromatogram

Table S5. Characterization of BM2(1-51) products 12a, 12b and 12c

	Peptide	Molecular formula	Calculated M_w [g/mol]	Experimental M_w[a] [g/mol]	t_R[b] [min]
12a	[Cys^{11}(Acm), Cys^{22}]BM2(1-51)	$C_{273}H_{448}N_{77}O_{67}S_4$	1202,85	1202,45	16.33
12b	[Cys^{11}(Acm)]BM2(1-51)	$C_{273}H_{447}N_{77}O_{67}S_3$	1196,24	1196,08	14.96
12c	BM2(1-51)	$C_{270}H_{442}N_{76}O_{66}S_3$	1182,02	1181,86	19.47

[a] mass peaks detected as $[M+5H]^{5+}$;

[b] HPLC conditions:45 – 70 % eluent B in 30 min, detection was at 220 nm at a flow rate of 1 mL/min, C4 column

Table S6. Ligation buffers used for study

Ligation buffer A	8 M Urea, 0.2 M disodium phosphate, 150 mM MPAA, 100 mM TCEP, pH 7.0
Ligation buffer B	Ligation buffer A : TFE (2:1), pH 7.5
Ligation Buffer C	3 M Urea, 75 mM disodium phosphate , 120 mM MPAA, 75 mM TCEP : HFIP (2:1), pH 7.5

MPAA = 4-mercaptophenylacetic acid, TCEP = tris(2-carboxyethyl)phosphine, TFE = 2,2,2-trifluoroethanol, HFIP = hexafluoro-2-propanol

Table S7. Deconvolution of CD spectra: (a) in TFE at wavelength 190-260 nm and **(b)** in liposome membrane POPC at wavelength 190-260 nm. Spectra deconvolution was performed using CDNN (Circular Dichroism analysis using Neural Networks) software.

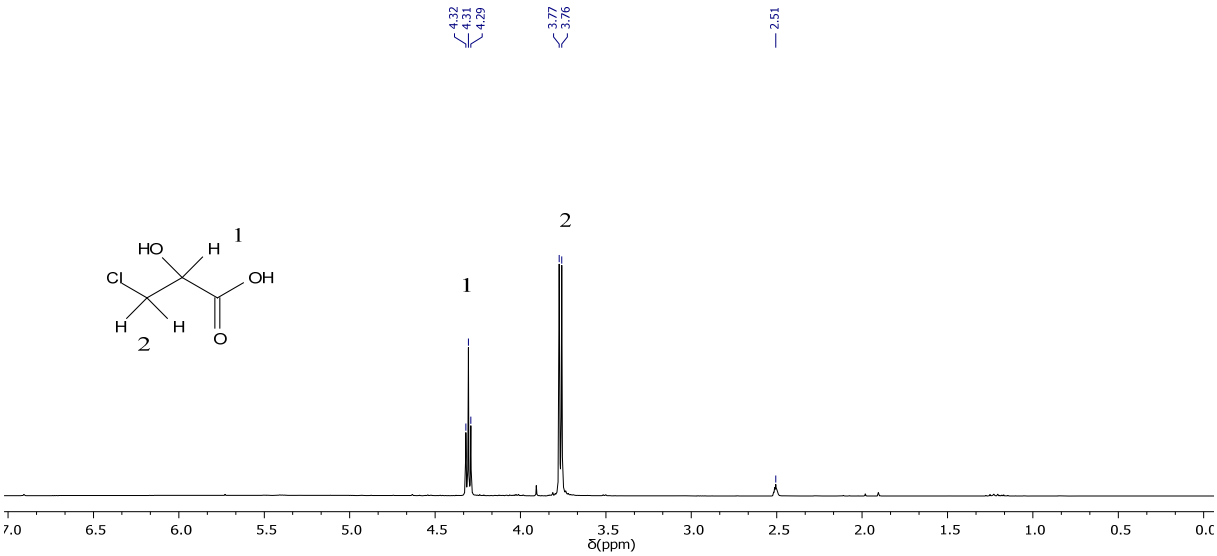
a)

	TFE				
Sec. element	BM2(1-21) (7), %	BM2(1-51) (12a), %	BM2(1-51) (12c), %	AM2(22-46), %	BM2(1-33), %
Helix	**76.6**	**66.3**	**63.30**	**92.3**	**55**
Antiparallel	0.2	0.6	0.50	0	1.4
Parallel	2.7	3.5	4.80	0.9	5.1
Beta-Turn	10.2	12.0	11.60	7.7	13.4
Rndm. Coil	10.1	13.0	21.10	2.0	20
Total Sum	99.8	95.5	101.30	102.9	94.9

b)

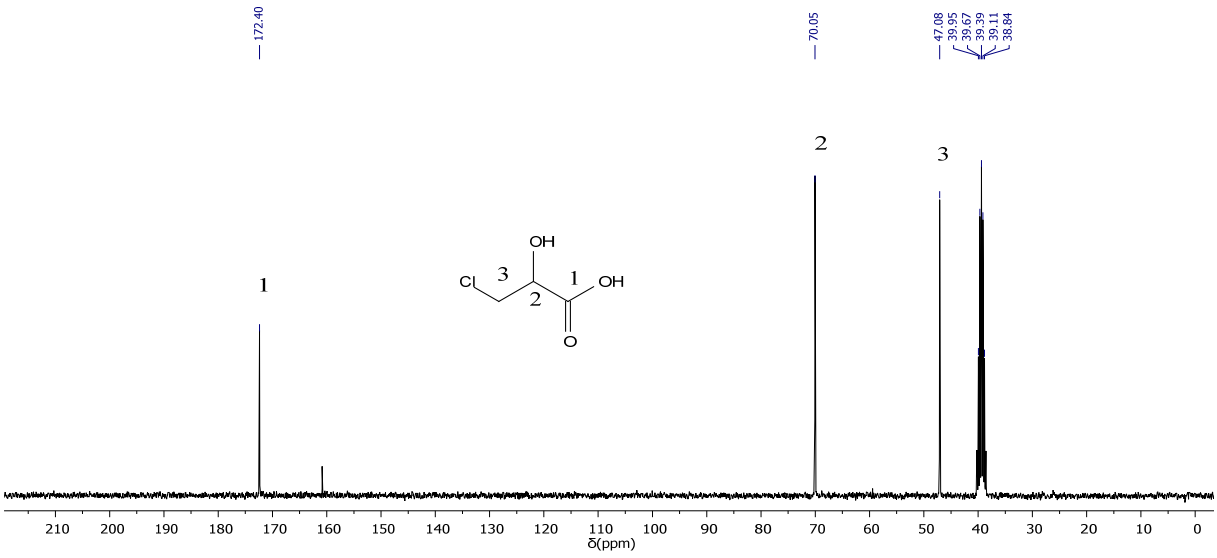
	Liposome membrane (POPC)		
Sec. element	BM2(1-21) (7), %	BM2(1-51) (12c), %	BM2(1-33), %
Helix	**53.20**	**59.20**	**61.20**
Antiparallel	0.30	0.30	0.40
Parallel	9.40	6.80	5.70
Beta-Turn	11.10	11.00	11.40
Rndm. Coil	50.10	35.40	27.80
Total Sum	124.20	112.70	106.50

a)



^{1}H NMR (300 MHz, DMSO-D6): δ = 4.32 – 4.29 (t, 2H), 3.77-3.76 (d, 1H), 2.51 (CDCl3).

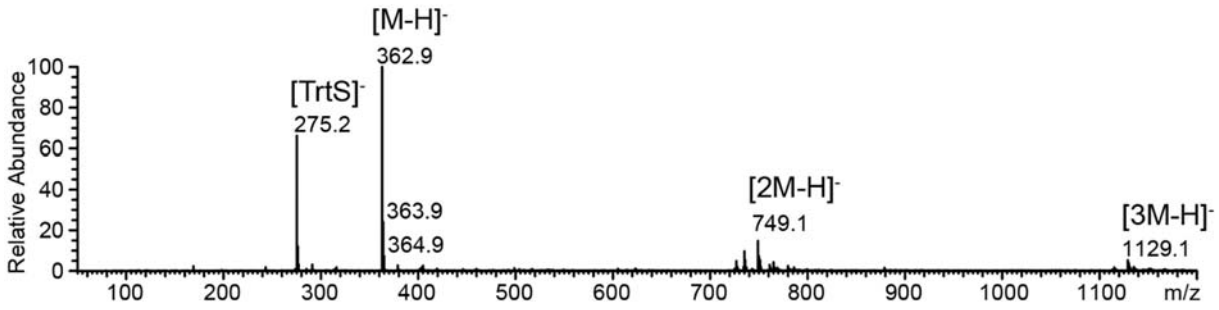
b)



^{13}C NMR (75 MHz, DMSO-D6): δ = 172.40, 70.05, 47.08, 39.95-38.84 (DMSO).

Figure S1. Characterization of 3-chloro-2-hydroxypropanoic acid (Hmp). (**a**) ^{1}H-NMR spectrum of Hmp in DMSO-d6, (**b**) ^{13}C-NMR spectrum of Hmp in DMSO-d6

a)



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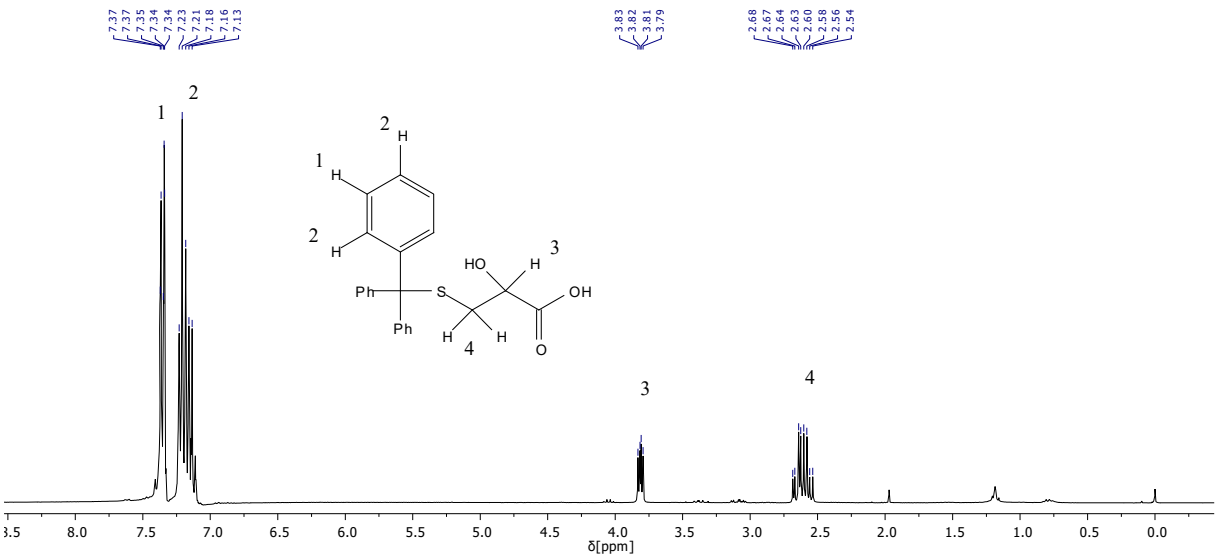
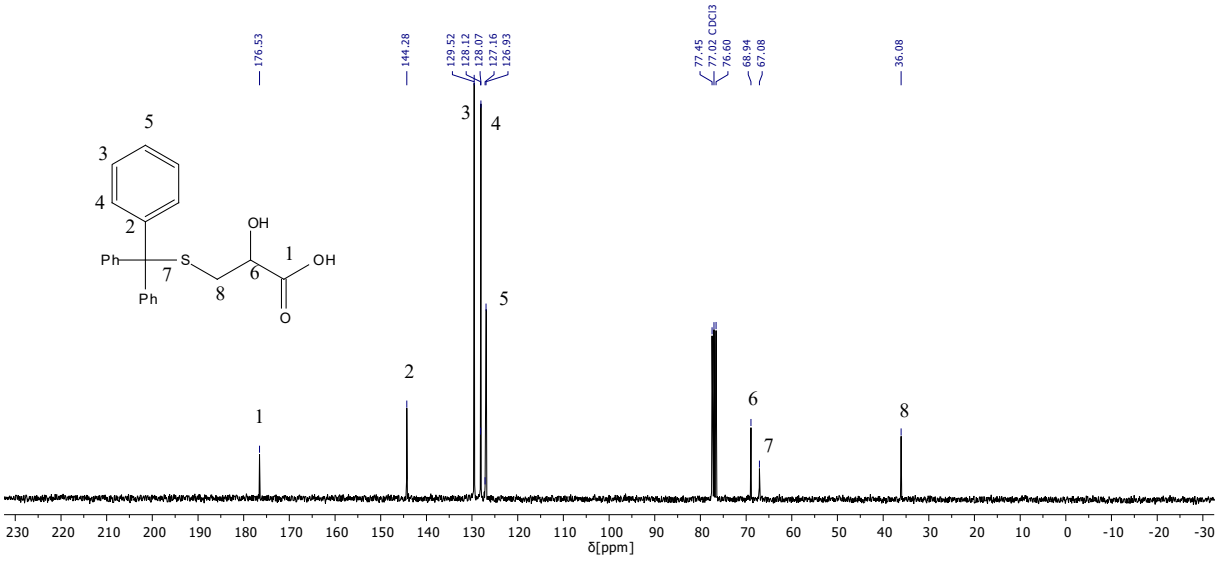


^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.37 – 7.34 (m, 2H, H_{Ar}), 7.23 – 7.13 (m, 4H, H_{Ar}), 3.83 – 3.79 (m, 1H), 2.68 – 2.54 (m, 2H).

c)



^{13}C NMR (75 MHz, $CDCl_3$): δ = 176.53, 144.28, 129.52, 128.12, 127.16, 68.94, 67.08, 36.08, 77.45-76.60 ($CDCl_3$).

Figure S2. Characterization of 2-hydroxy-3-(triphenylmethyl)thio-propanoic acid (Hmp(Trt)-OH). (**a**) ESI-MS spectrum in negative mode, (**b**) 1H-NMR spectrum of Hmp(Trt)-OH in $CDCl_3$, (**c**) 13C-NMR spectrum of Hmp(Trt)-OH in $CDCl_3$

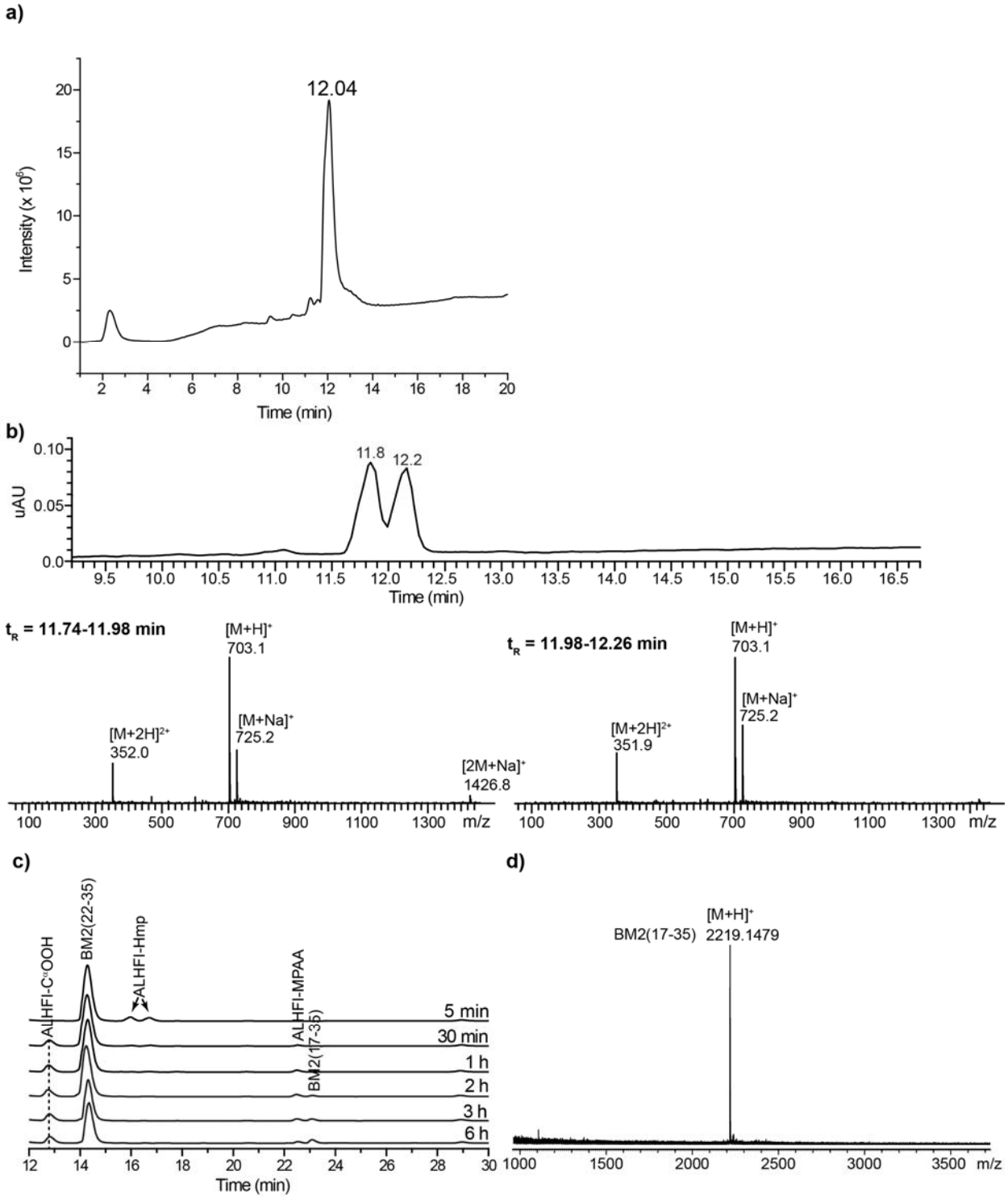
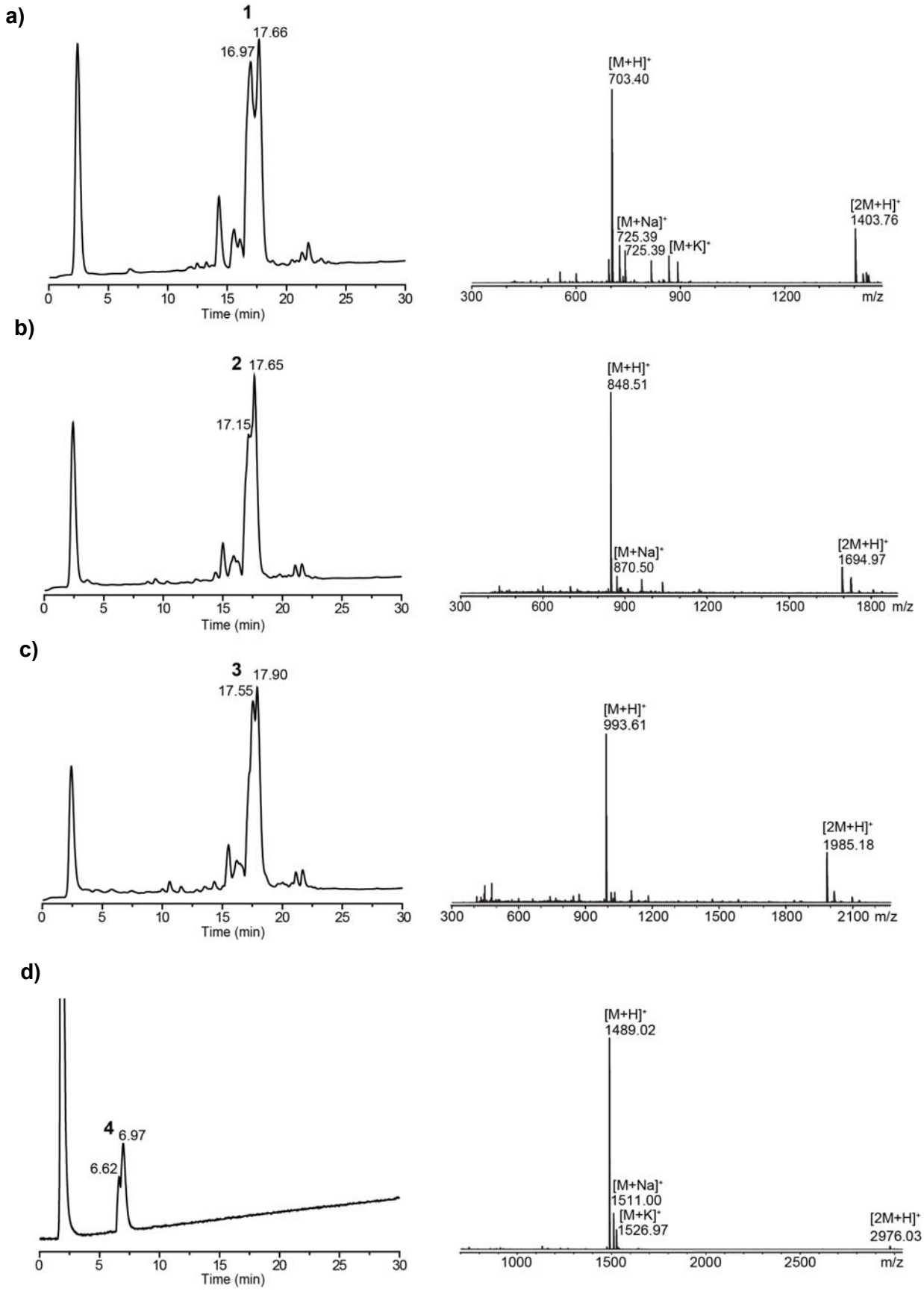


Figure S3. Characterization of ALHFI-Hmp peptide ligation behavior. (**a**) RP-HPLC elution profile of the crude product ALHFI-Hmp, Eluent A was 0.1% TFA in water, eluent B was 0.1 % TFA in acetonitrile and a gradient of 10–80 % eluent B in 20 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column (**b**) LC-MS of ALHFI-Hmp diastereomers A and B. , Eluent A was 0.1% TFA in water, eluent B was 0.1 % TFA in acetonitrile and a gradient of 20–80 % eluent B in 25 min using a C8 column and (**c**) RP-HPLC ligation monitoring of ALHFI-Hmp with BM2(22-35) at different time points and (**d**) MALDI-TOF MS of ligation product [Cys^{22}]BM2(17-35). HPLC conditions: 15–45 % eluent B in 30 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column


a)
1
17.66
16.97
Time (min)
[M+H]+
703.40
[M+Na]+
725.39
725.39
[M+K]+
[2M+H]+
1403.76
m/z
b)
2
17.65
17.15
Time (min)
[M+H]+
848.51
[M+Na]+
870.50
[2M+H]+
1694.97
m/z
c)
3
17.90
17.55
Time (min)
[M+H]+
993.61
[2M+H]+
1985.18
m/z
d)
4
6.97
6.62
Time (min)
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1511.00
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1526.97
[2M+H]+
2976.03
m/z

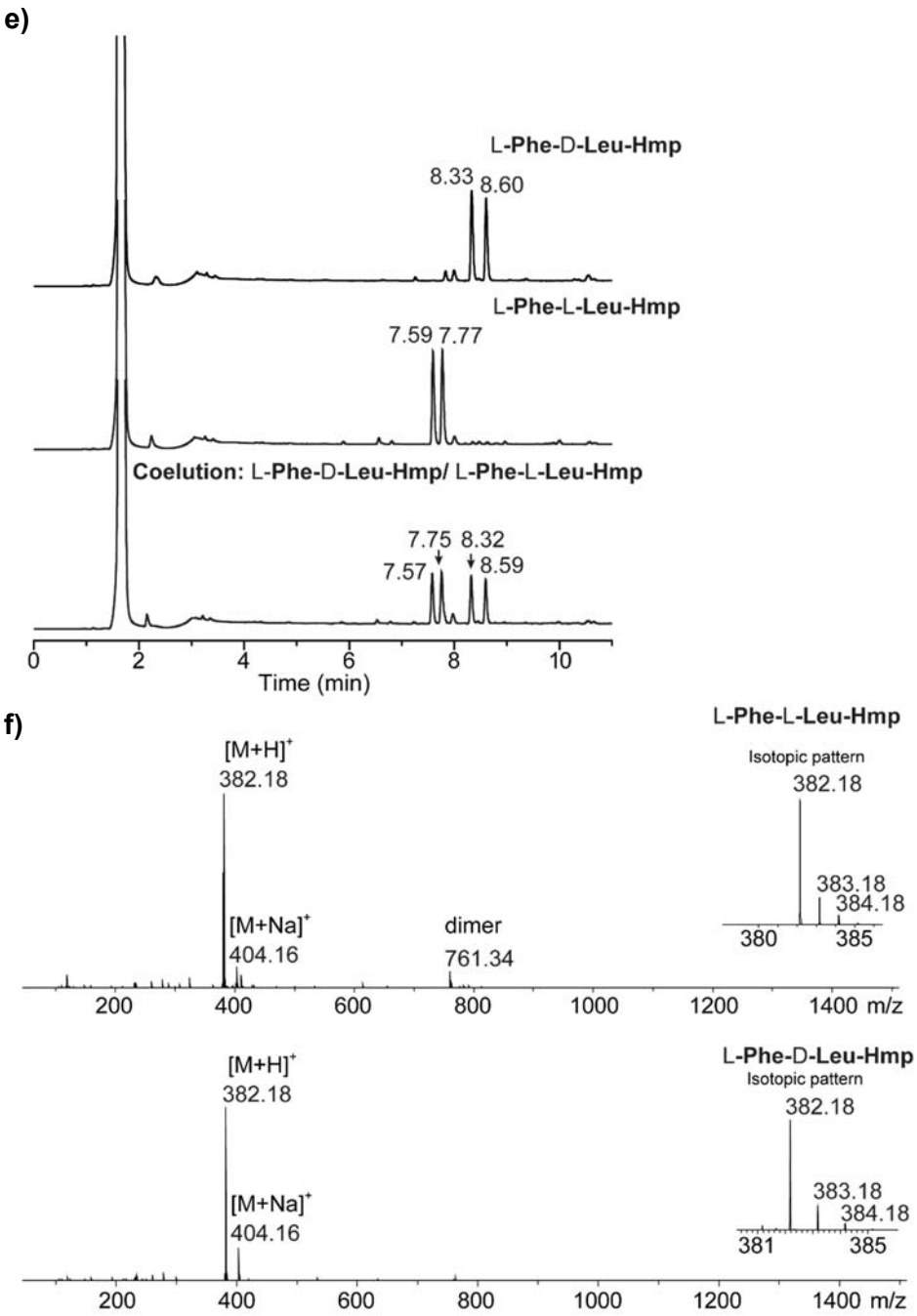


Figure S4. RP-HPLC and MALDI-TOF MS characterization of crude peptides and racemization behavior of Leu (directly attached to Hmp). (**a**) ALHFL-Hmp (**1**), (**b**) ALHFL-Hmp-ADO (**2**), (**c**) ALHFL-Hmp-ADO_2 (**3**) and purified (**d**) ALHFL-Hmp-ADO-Lys_5 (**4**). Eluent A was 0.1% TFA in water, eluent B was 0.1 % TFA in acetonitrile and a gradient for (**a-d**) 15–45 % eluent B in 30 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column; MALDI-TOF MS spectra for (**a-d**) were recorded at a reflector mode (500 shots/min) and as matrix α-cyano-4-hydroxycinnamic acid (HCCA) was used. (**e**) Stacked HPLC chromatograms of crude L-Phe-D-Leu-Hmp (upper chromatogram) and L-Phe-L-Leu-Hmp (middle) and and the coelution of two dipeptides. HPLC conditions: 0–80 % eluent B in 15 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column. (**f**) ESI MS spectra of L-Phe-L-Leu-Hmp and L-Phe-D-Leu-Hmp with isotopic patterns as insert.

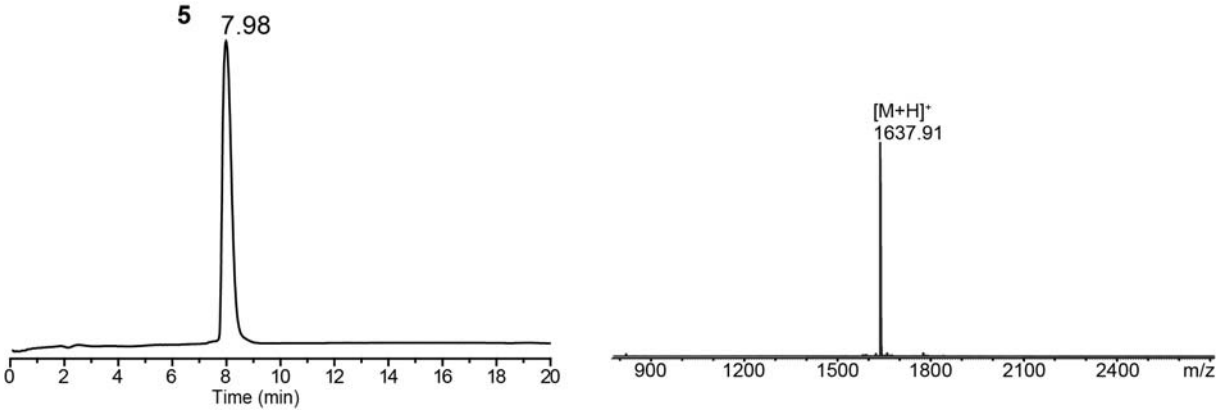
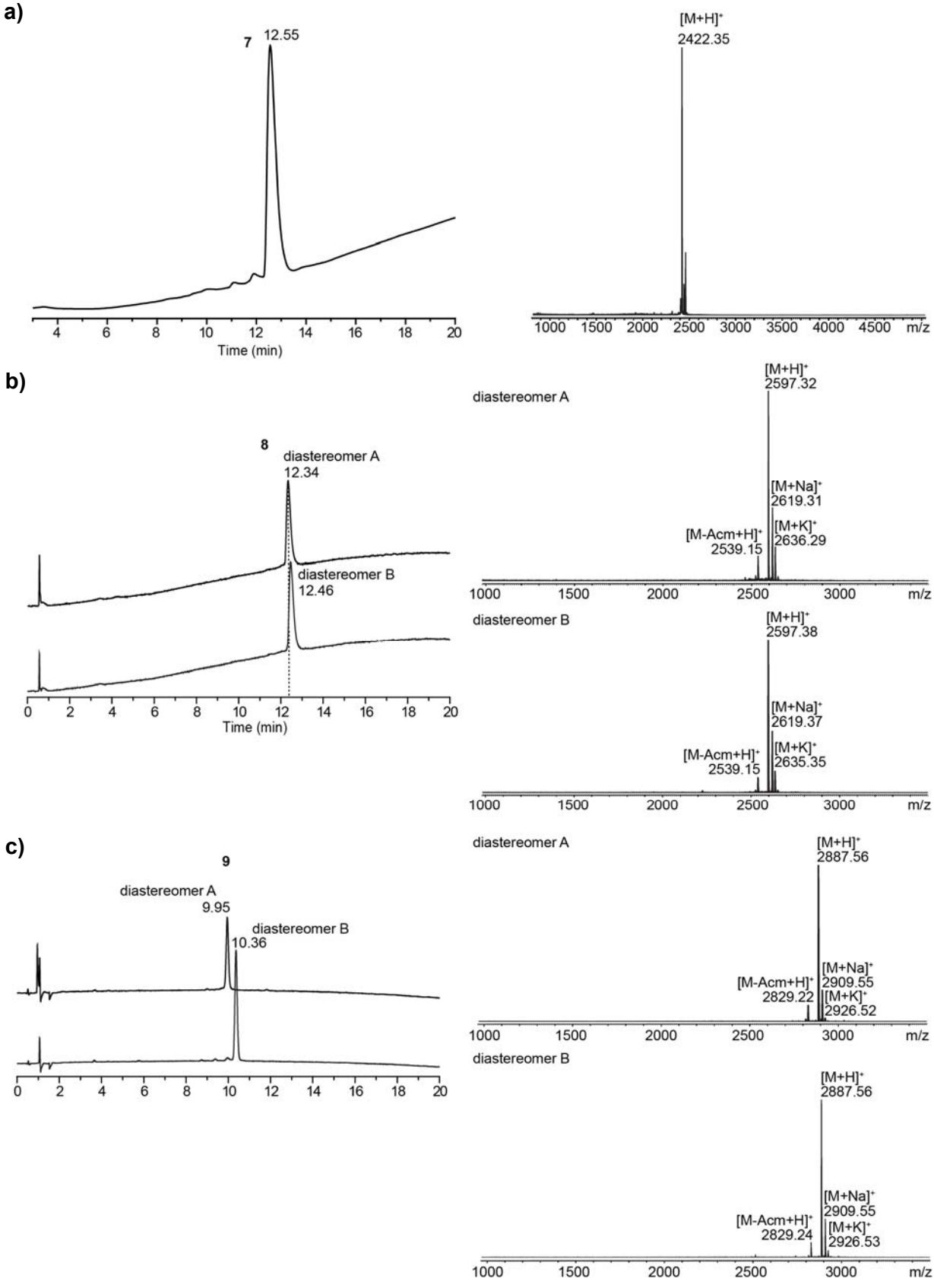


Figure S5. RP-HPLC and MSLDI-TOF MS characterization of purified [^{22}Cys]BM2(22-35) Cys-fragment (5). Eluent A was 0.1% TFA in water, eluent B was 0.1 % TFA in acetonitrile and a gradient: 20–80 % eluent B in 20 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column. MALDI-TOF MS spectra for (A-D) were recorded at a reflector mode (500 shots/min) and as matrix α-cyano-4-hydroxycinnamic acid (HCCA) was used.


a)
7
12.55
Time (min)
[M+H]+
2422.35
m/z
b)
8
diastereomer A
12.34
diastereomer B
12.46
Time (min)
diastereomer A
[M+H]+
2597.32
[M+Na]+
2619.31
[M-Acm+H]+
2539.15
[M+K]+
2636.29
diastereomer B
[M+H]+
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2539.15
[M+K]+
2635.35
c)
9
diastereomer A
9.95
diastereomer B
10.36
diastereomer A
[M+H]+
2887.56
[M+Na]+
2909.55
[M-Acm+H]+
2829.22
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2926.52
diastereomer B
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2829.24
[M+K]+
2926.53

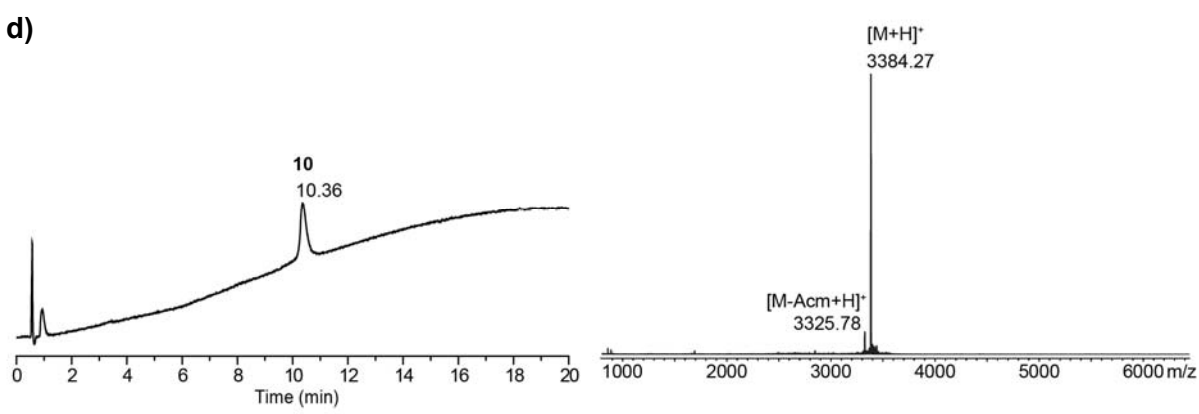
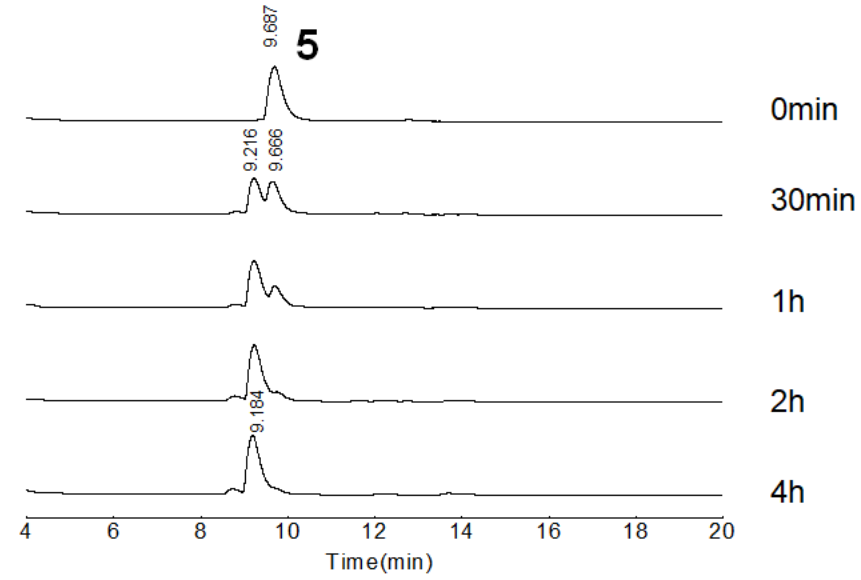


Figure S6. RP-HPLC and MALDI-TOF MS characterization of purified (**a**) [Cys^{11}(Acm)]BM2(1-21) (**7**), (**b**) [Cys^{11}(Acm)]BM2(1-21)-Hmp diastrereomer A/diastereomer B (**8a/8b**), (**c**) [Cys^{11}(Acm)]BM2(1-21)-Hmp-ADO$_2$ diastrereomer A/ diastereomer B (**9a/9b**), (**d**) [Cys^{11}(Acm)]BM2(1-21)-Hmp-ADO$_2$-Lys$_5$ (**10**). Eluent A was 0.1% TFA in water, eluent B was 0.1 % TFA in acetonitrile and a gradient: (**a**) 50 – 99 % eluent B in 15 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column; (**b**) 10 – 20% eluent B in 5 min followed by 20 – 70% eluent B in 20 min, at a flow rate of 2 mL/min, C4 column; (**c**) 40 – 80 % eluent B in 20 min, at a flow rate of 1 mL/min, C18 column; (**d**) 10 – 20 % eluent B in 5 min followed by 20 – 70% eluent B in 20 min, at a flow rate of 2 mL/min, C4 column. MALDI-TOF MS spectra for (**a-d**) were recorded at a reflector mode (500 shots/min) and as matrix α-cyano-4-hydroxycinnamic acid (HCCA) was used.

a)



b)

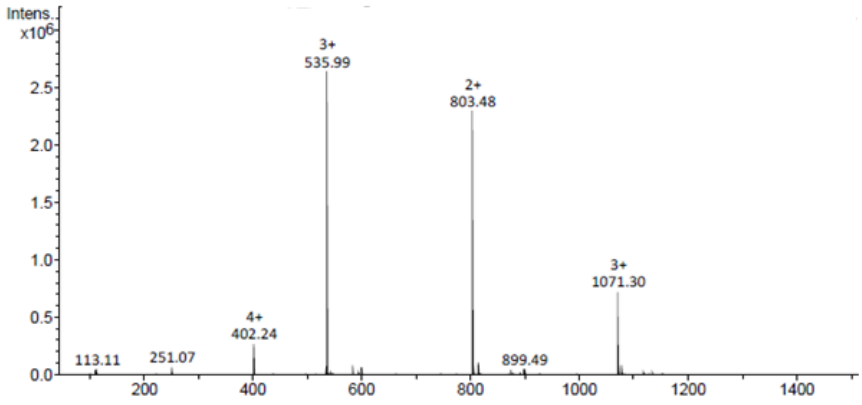
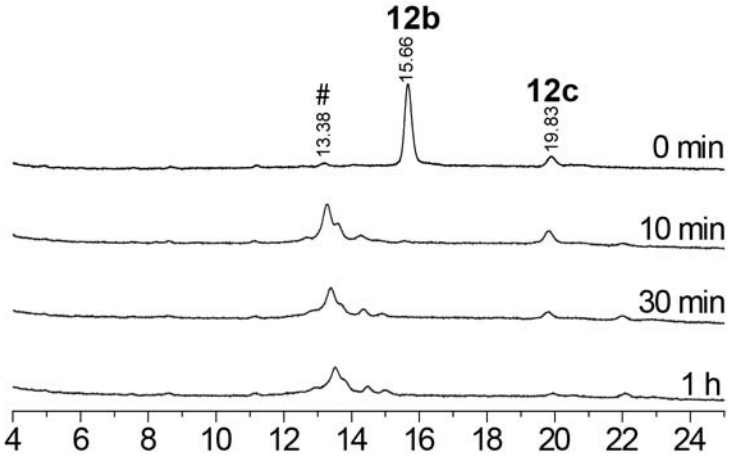
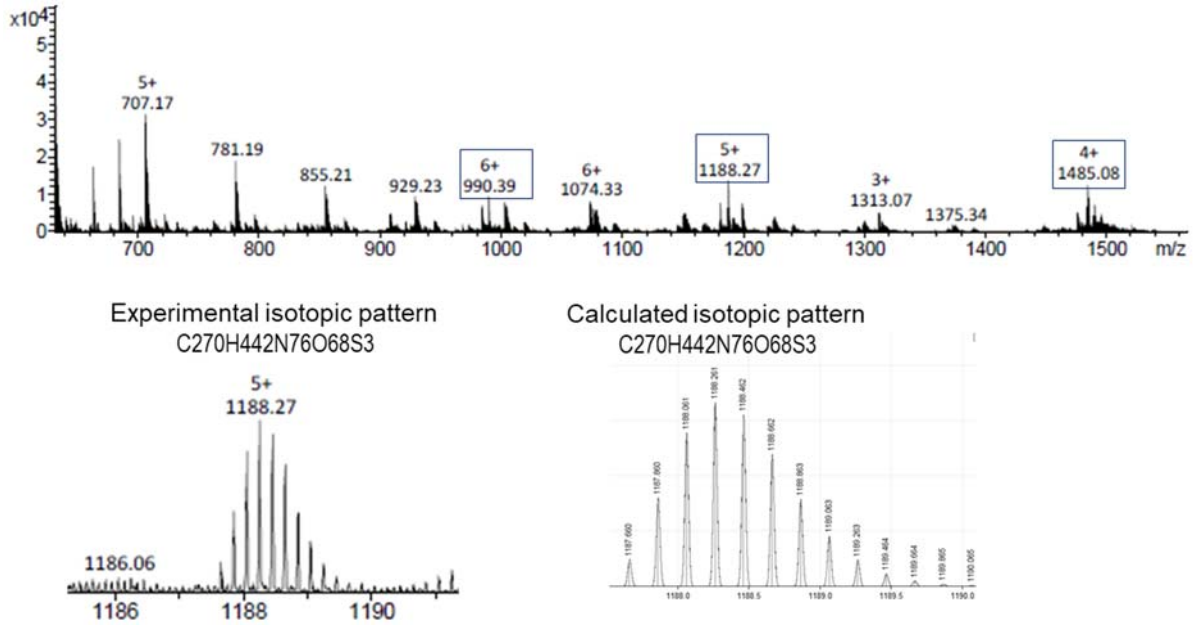


Figure S7. Desulfurization of peptide [Cys^{22}]BM2(22-35) (5). (**a**) RP-HPLC elution profile of the reaction mixture at different time points: 0 min (directly after reaction start) – 4 h. Initial peptide eluting at t_R = 9.68 min and reaction product [^{22}Ala]BM2(22-35) at t_R = 9.18 min. Eluent A was 0.1% TFA in water, eluent B was 0.1 % TFA in acetonitrile at gradient: 15 – 45 % eluent B in 30 min, detection was at 220 nm at a flow rate of 1 mL/min, C18 column. (**b**) ESI-MS of the reaction mixture after 4 h of reaction time. Following mass peaks belong to the product [Ala^{22}]BM2(22-35): 402.24 $[M+4H]^{4+}$, 535.99 $[M+3H]^{3+}$, 803.48 $[M+2H]^{2+}$. The mass peak at 1073.30 $[M+3H]^{3+}$belongs the **dimerized peptide 5** (BM2(22-35)[Cys^{22}]–[Cys^{22}]BM2(22-35)).

a)


12b
15.66
#
13.38
12c
19.83
0 min
10 min
30 min
1 h
4 6 8 10 12 14 16 18 20 22 24

b)


x10^4
5+
707.17
781.19
855.21
929.23
6+
990.39
6+
1074.33
5+
1188.27
3+
1313.07
1375.34
4+
1485.08
700 800 900 1000 1100 1200 1300 1400 1500 m/z
Experimental isotopic pattern
C270H442N76O68S3
5+
1188.27
1186.06
1186 1188 1190
Calculated isotopic pattern
C270H442N76O68S3

c)

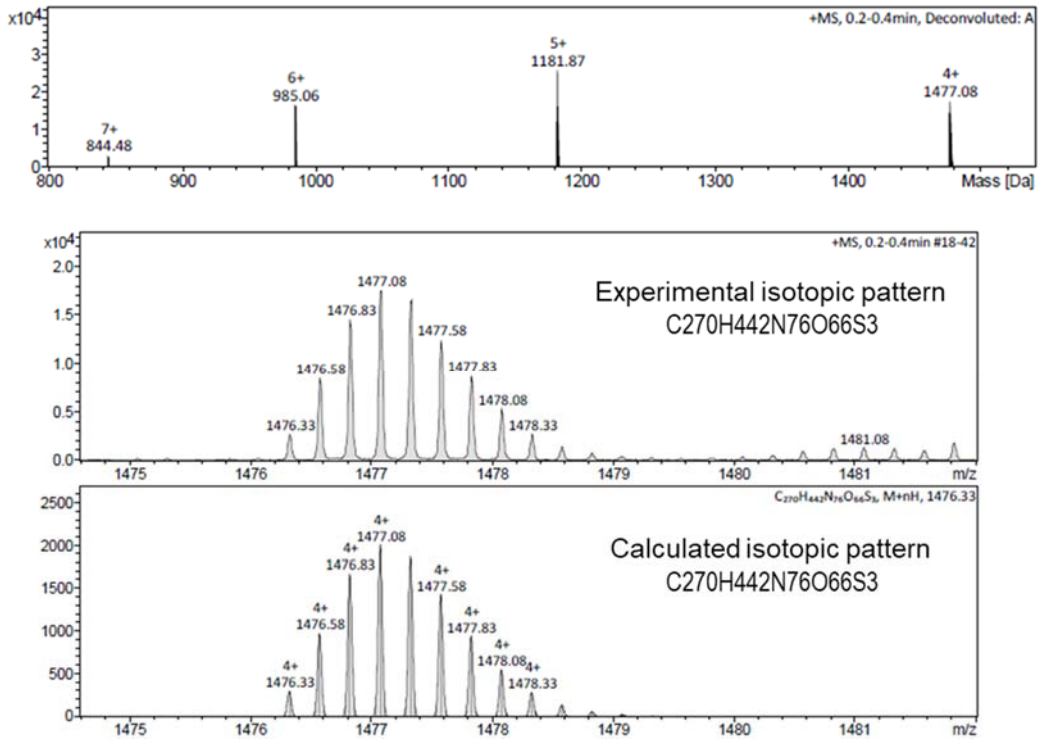


Figure S8. (a) RP-HPLC elution profiles of Acm-group cleavage from [Cys^{11}(Acm)]BM2(1-51) (**12b**) in iodine/acetic acid solution at different time points 0 min (directly after reaction start) – 1 h. Reaction products are highlighted as **12c** and #. According to peak integration the yield of product **12c** after 10 min is ~ 16 %. HPLC conditions: 45 – 70 % eluent B in 30 min, detection was at 220 nm at a flow rate of 1 mL/min, C4 column (**b**) ESI-MS of isolated reaction products t_R=13.38 min: # - BM2(1-51) where sulfur group of cysteine is present as sulfone and corresponding experimental and calculated isotopic patterns. (**c**) ESI-MS of deconvoluted product t_R=19.83 min: BM2(1-51) (**12c**) and corresponding experimental and calculated isotopic patterns.

References

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