Supporting Information

Bypassing the need for cell permeabilization: Nanobody CDR3 peptide improves binding on living bacteria

A. Breine,[2,3,§] K. Van holsbeeck,[1,§] C. Martin,[1] S. Gonzalez,[4] M. Mannes,[1] E. Pardon,[3] J. Steyaert,[3] H. Remaut,[3,5] S. Ballet,[1,*] C. Van der Henst[2,3,*]

[1] Research Group of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium
[2] Microbial Resistance and Drug Discovery, VIB-VUB Center for Structural Biology, Pleinlaan 2, 1050 Brussels, Belgium
[3] Structural Biology Brussels, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium
[4] CNRS, BioCIS, CY Cergy-Paris Université, 95000 Neuville sur Oise, France
[5] Structural and Molecular Microbiology, Structural Biology Research Center, VIB, 1050 Brussels, Belgium.

***Corresponding authors:** Charles.Van.Der.Henst@vub.be**,** Steven.Ballet@vub.be

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1 Supplementary figures

1.1 Nb CDR3 determination

A

No°	1	2	3	4	5	6	7	8	9	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Nb	Q	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	**G**	**I**	**S**	**K**	**S**	**I**	**T**	**I**	M	G	W	Y	R	Q	A	P	G	K	Q	R
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6RTW	E	L	V	A	**E**	**I**	**T**	**S**	**S**	**-**	**G**	**T**	T	N	Y	A	D	S	V	K	G	R	F	S	I	S	R	D	N	A	K	N	T	V	P	L	Q	M	N	S	L	K	P	E	D
7O06	E	L	V	A	**N**	**I**	**N**	**S**	**G**	**-**	**G**	**S**	T	N	Y	I	D	S	V	K	G	R	F	T	I	S	R	D	N	A	K	N	M	V	Y	L	Q	M	N	S	L	K	P	E	D
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5MWN	E	L	V	A	**S**	**I**	**T**	**S**	**G**	**-**	**G**	**T**	T	N	Y	A	D	S	V	K	G	R	F	T	I	S	K	D	N	A	E	N	T	L	Y	L	R	M	N	S	L	K	P	E	D
3OGO	E	W	V	A	**G**	**M**	**S**	**S**	**A**	**G**	**D**	**R**	S	S	Y	E	D	S	V	K	G	R	F	T	I	S	R	D	D	A	R	N	T	V	Y	L	Q	M	N	S	L	K	P	E	D
6B20	E	L	V	A	**A**	**I**	**T**	**R**	**G**	**-**	**G**	**R**	T	N	Y	A	D	S	V	K	G	R	F	T	L	S	R	D	N	A	K	N	T	V	Y	L	Q	M	N	S	L	K	P	E	D

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Nb	T	A	V	Y	Y	C	**N**	**A**	**R**	**R**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**L**	**R**	**E**	Y	W	G	Q	G	T	Q	V	T	V	S	S	H	H	H	H	H	H	E	P	E	A
6RTW	T	A	I	Y	Y	C	**S**	**A**	**V**	**L**	**V**	**R**	**F**	**-**	**-**	**G**	**G**	**L**	**R**	**R**	**S**	Y	W	G	Q	G	T	Q	V	T	V	S	S	H	H	H	H	H	H	E	P	E	A
7O06	T	A	V	Y	F	C	**N**	**A**	**A**	**R**	**P**	**L**	**R**	**P**	**E**	**G**	**G**	**R**	**W**	**L**	**N**	Y	W	G	Q	G	T	Q	V	T	V	S	S	-	-	-	-	-	-	-	-	-	-	-
3EZJ	T	A	V	Y	Y	C	**N**	**A**	**N**	**V**	**K**	**T**	**W**	**-**	**-**	**A**	**G**	**M**	**T**	**R**	**D**	Y	W	G	Q	G	T	Q	V	T	V	S	S	H	H	H	H	H	H	-	-	-	-	-
5MWN	T	A	V	Y	Y	C	**K**	**A**	**F**	**F**	**R**	**R**	**D**	**-**	**-**	**-**	**Y**	**V**	**G**	**Y**	**D**	Y	W	G	Q	G	T	Q	V	T	V	S	S	-	-	-	-	-	-	-	-	-	-	-
3OGO	T	A	V	Y	Y	C	**N**	**V**	**N**	**V**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**G**	**F**	**E**	Y	W	G	Q	G	T	Q	V	T	V	S	S	K	H	H	H	H	H	H	-	-	-	-
6B20	T	A	V	Y	Y	C	**N**	**V**	**G**	**R**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**S**	**R**	**G**	Y	W	G	Q	G	T	Q	V	T	V	S	S	-	-	-	-	-	-	-	-	-	-	-

B

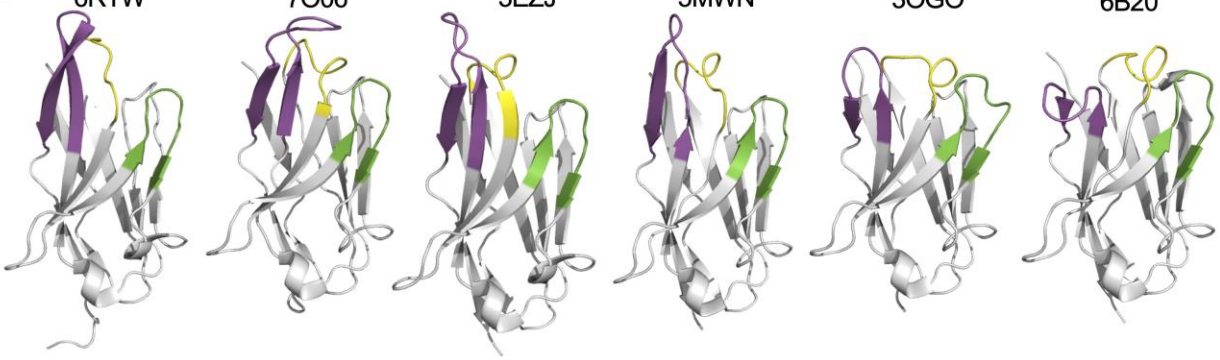


Figure S1. (A) Numbering of the studied Nb sequence compared to existing Nbs with some overlapping CDR residues. The Nb sequences were numbered following the standard IMGT scheme[1] using the online tool ANARCHI.[2] CDR residues identified by the IMGT CDR definitions are shown in bold. (B) 3D X-ray structures of previously reported Nbs (PDB ID 6RTW, 7O06, 3EZJ, 5MWN, 3OGO, 6B20) with some CDR sequence similarities to the investigated Nb. CDR sequence similarities were identified using the Antibody Modeller tool of MOE (Molecular Operating Environment). 3D figures were generated using the PyMOL Molecular Graphics System (Version 2.3 Schrödinger, LLC). Based on the structures of these Nbs, the CDR2 and CDR3 sequences were extended to increase the probability of taking the full loop structures. The CDR1-3 sequences were colored, respectively, in yellow, green and purple.

1.2 Membrane interaction image analysis

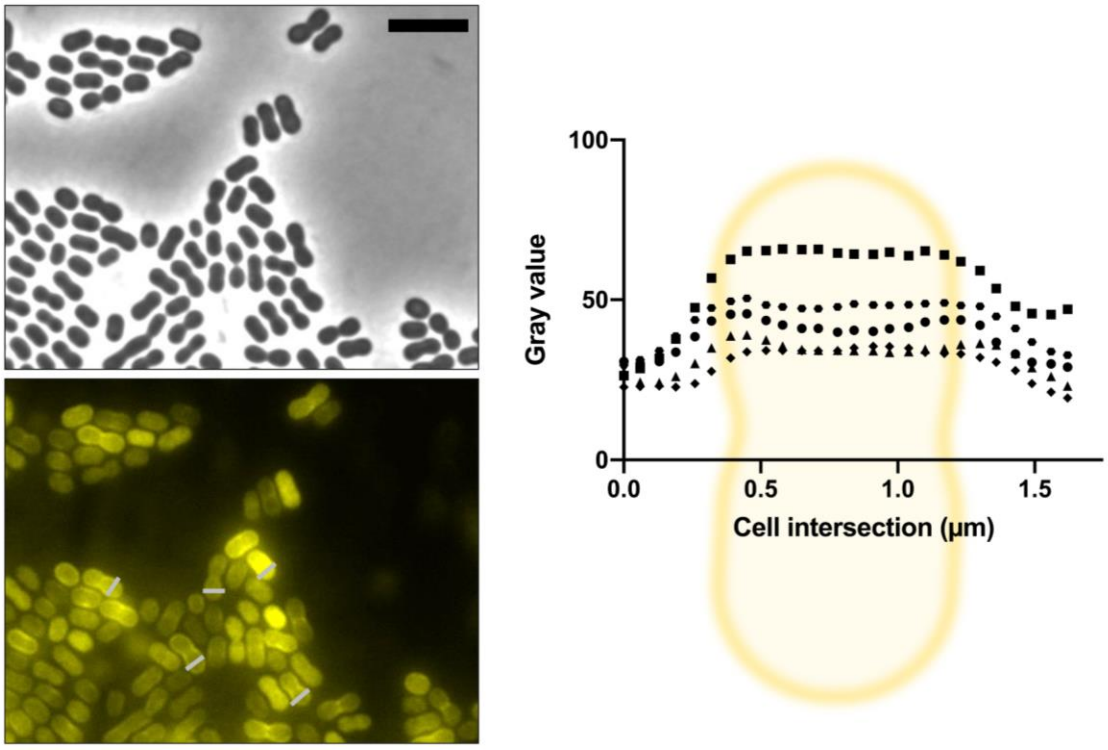
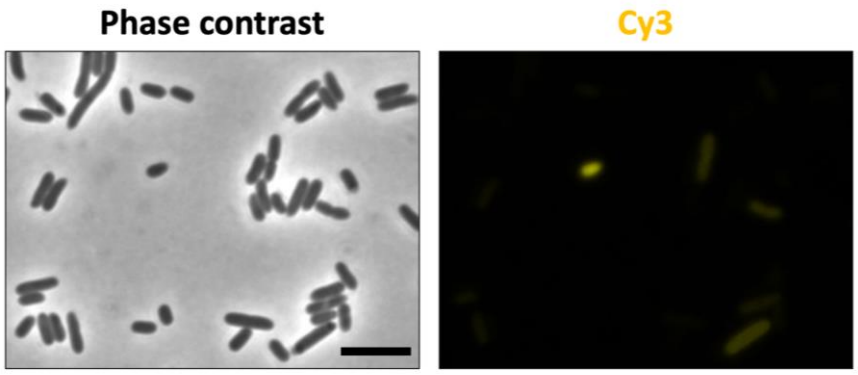


Figure S2. Membrane interaction of NbD4 with fixed *A. baumannii* cells. 5 bacterial cells of Figure 1A, indicated on the micrograph, were picked to illustrate the membrane labelling profile of NbD4. Scale bar is 5 µm.

1.3 Interaction of peptide 3 with *E. coli*

To test the interaction of peptide 3 with another Gram-negative bacterium, a clonal culture of *Escherichia coli* S17 was grown in the same conditions as the *A. baumannii* strains, normalized to OD_{600}=3 in PBS and tested by fluorescence microscopy as was done for *A. baumannii*.



	Cell total	Labelled cells	Ratio	Average (%)	SD (%)
BR1	101	18	17,82%		
BR2	114	38	33,33%	20,72%	0,11
BR3	100	11	11,00%		

Figure S3. Interaction of peptide 3 with another Gram-negative pathogen. The specificity of peptide 3 was tested by assessing its binding abilities on *E. coli* S17 through fluorescence microscopy. Micrographs of the bacteria with phase contrast and the cy3-labelled peptide are shown. The percentage of labelled cells was calculated by counting the labelled cells found in min. 100 cells total. BR = Biological replicate. Scale bar is 5 µm.

2 Peptide syntheses and characterization

2.1 General methods

Unless stated otherwise, all commercial chemicals were used without further purification. Fmoc-L-propargylglycine and *N,N,N′,N′*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Carbosynth. Rink amide AM resin, Sieber amide resin and all remaining protected standard and unnatural amino acids were obtained from Chem-Impex. Sulfonylated Cyanine 3 (SulfoCy3) under its *N*-hydroxysuccinimide (NHS) ester form was obtained from Lumiprobe GmbH. All other reagents and solvents were purchased from Sigma Aldrich (Merck).
Preparative RP-HPLC was performed on a Gilson semi-preparative HPLC equipped with a Supelco Discovery BioWide Pore C18 column (250 mm x 21.2 mm, 10 µm) and a UV detector set at 214 nm. The solvent system consisted of ultrapure water containing 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B). The flow rate was set at 20 mL/min.
Analytical HPLC was performed using a VWR-Hitachi Chromaster HPLC equipped with a Chromolith High Resolution RP-18C column from Merck (150 mm x 4.6 mm, 1.1 µm). Products were detected by a Chromaster HPLC 5430 diode array detector at a wavelength of 214 nm. The solvent system consisted of 0.1% TFA in ultrapure water (A) and 0.1% TFA in acetonitrile (B) with a linear gradient ranging from 1% to 99% B over 4.5 minutes at a flow rate of 2.8 mL/min.
HPLC-MS analyses were performed with a Waters 600 HPLC unit equipped with an EC 150/2 NUCLEODUR® 300-5 C18 column using a gradient ranging from 3% to 100% of acetonitrile containing 0.1% formic acid (C), in ultrapure water containing 0.1% formic acid (D), using a flow rate of 0.3 mL/min over 20 minutes. Monitoring was done via UV detection at 214 nm. Coupled MS analysis was performed on a Micromass QTOF-micro system. High resolution mass spectrometry (HRMS) spectra were recorded on the same MS system using a solution of reserpine (2 µg/mL) in water:acetonitrile 1:1 as the reference.

2.2 General SPPS protocols

General synthesis protocols

Peptides were synthesized on a Rink Amide AM resin or Sieber amide resin on 0.10 mmol scales by iterative cycles of N^{α}-Fmoc deprotection, amino acid couplings and washing steps, either in an automated fashion using a Liberty Lite™ Automated Microwave Peptide Synthesizer or manually to introduce the non-standard amino acids Fmoc-propargylglycine (Fmoc-Pra-OH) and Fmoc-azidolysine. Manual steps were carried out in polypropylene syringes equipped with a polyethylene frit. Amino acids were used in their N^{α}-Fmoc-protected form, combined with the following side chain protecting groups: trityl (Trt) for Asn; *tert*-butyl (*t*Bu) for Ser, Thr, Tyr and Glu; *tert*-butyloxycarbonyl (Boc) for Lys and Trp; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg.
On the Liberty Lite™ peptide synthesizer, couplings were performed using 4 equiv. of amino acids (0.5 M solution in DMF), *N,N′*-diisopropylcarbodiimide (DIC, 0.5 M solution in DMF), and Oxyma Pure (1 M solution in DMF). Whereas standard couplings were performed for 2.1 minutes at 90°C, Fmoc-Arg(Pbf)-OH was coupled twice at 75°C. Fmoc deprotections were carried out for 1.05 minutes at 90°C using 20 % (v/v) 4-methylpiperidine in DMF. Between every step, the resin was washed with DMF (3 times).
Manual syntheses were carried out in polypropylene syringes equipped with a polyethylene frit. Before use, the resin was swollen for 30 minutes in DMF. Between every step, the resin was washed thoroughly with DMF (3 times) and DCM (3 times). Fmoc deprotections were performed using 20 % (v/v) 4-methylpiperidine in DMF for 5 and 15 minutes. Amino acid residues were coupled using 3 equiv. of protected amino acid, 3 equiv. of *N,N,N′,N′*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and 6 equiv. of *N,N*-diisopropylethylamine (DIPEA) in DMF for 1 hour at room temperature. Completion of couplings was verified at all stages by Kaiser colorimetric tests.

Standard peptide cleavages (Rink amide AM resin)

Peptide cleavages were performed with a freshly prepared cocktail of TFA/TIS/H_2O 90/5/5 (v/v/v) for 2 h at room temperature, with an extension towards 4 h for the sequences containing a Pbf protecting group. Subsequently, the resin was filtered and washed with DCM and neat TFA. The combined filtrates were reduced in volume till ca. 1 mL under a stream of pressurized air, precipitated with 10 mL cold diethyl ether and centrifugated. After decantation of the supernatant, the crude white powder was again suspended in 10 mL cold diethyl ether, centrifugated and decanted (2 times). Subsequently, the crude peptides were dissolved in water, lyophilized, analyzed by analytical HPLC and HPLC-MS, and purified by preparative RP-HPLC.

General protocol for fluorophore linkage on unprotected purified peptides

Purified unprotected peptide (1.0 equiv) was dissolved in 1 mL dimethylsulfoxide (DMSO) in a glass vial and sealed from light. Subsequently, sulfonated cyanine 3 NHS (1.1 equiv) was dissolved in 1 mL DMSO and added to the vial, followed by DIPEA (10 equiv). After full conversion of the starting peptide was observed in analytical RP-HPLC, the pink solution was directly purified by preparative RP-HPLC.

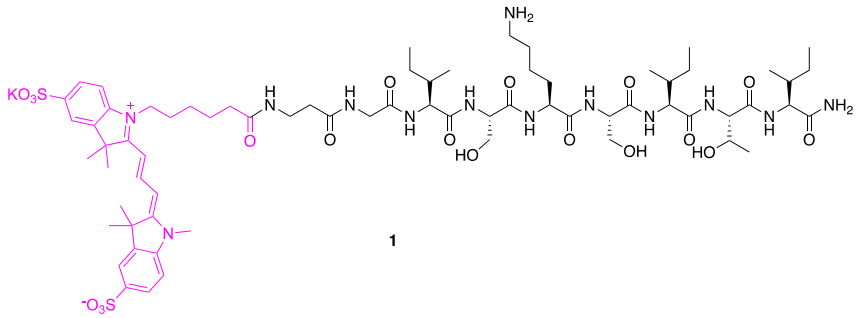
Peptide purifications

Purifications were performed by preparative RP-HPLC using a linear gradient of acetonitrile containing 0.1% TFA (B) in water containing 0.1% TFA (A). The crude peptides were solubilized in DMSO. Fractions with an HPLC purity exceeding 95% were pooled and lyophilized, yielding the corresponding peptides under their TFA salt form.

2.3 Synthesis & characterization of linear peptides 1-3

Synthesis & characterization of peptide 1

The peptide was assembled on a Sieber amide resin (0.6 mmol/g, 0.1 mmol) using the Liberty Lite™ peptide synthesizer, followed by cleavage of a side chain protected precursor (H-β-Ala-Gly-Ile-Ser(*t*Bu)-Lys(Boc)-Ser(*t*Bu)-Ile-Thr(*t*Bu)-Ile-NH_2) using 1% TFA in DCM (5 x 1 min). The combined filtrates were pooled and directly evaporated under reduced pressure. Given the high hydrophobicity of the protected peptide fragment, it was directly labelled. Therefore, sulfonated cyanine 3 NHS ester (1.0 equiv) was coupled to a small fraction of the obtained protected peptide (5.70 mg, 0.0078 mmol) by dissolving the in 0.8 mL DMSO:DCM 1:3 containing DIPEA (10 equiv). After overnight reaction, the side chain protecting groups were removed by stirring the peptide in a solution of TFA/TIS/H_2O 90/5/5 (v/v/v) for 2 h at room temperature. After concentration *in vacuo*, the peptide was directly purified by preparative RP-HPLC using a linear gradient of 10 to 60% B in A, and peptide **1** was obtained under its TFA salt form after lyophilization as a pink powder (4.3 mg).

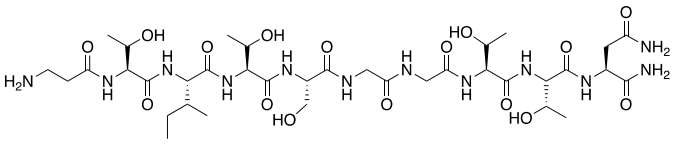


1

Sequence: SulfoCy3-β-Ala-Gly-Ile-Ser-Lys-Ser-Ile-Thr-Ile-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.73 min (> 99% purity); **HRMS (ESI+)** m/z calc. for $C_{69}H_{106}N_{13}O_{19}S_2Na$ $[M+2H]^{2+}$ = 754.8612, found 754.8577.

Synthesis & characterization of peptide 2

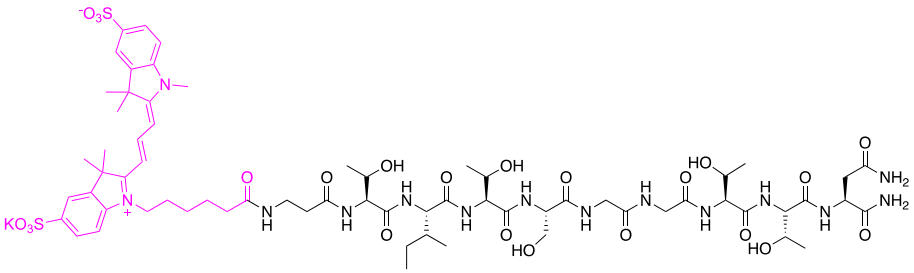
The peptide was assembled on a Rink amide AM resin (0.64 mmol/g, 0.1 mmol) using the Liberty Lite™ peptide synthesizer, followed by isolation of the non-labelled precursor **7** (H-β-Ala-Thr-Ile-Thr-Ser-Gly-Gly-Thr-Thr-Asn-NH_2) by the conditions outlined above. The non-labelled precursor **7** was purified by preparative RP-HPLC using a linear gradient of 1 to 30% B in A. After lyophilization, the peptide precursor **7** was obtained under its TFA salt form as a white powder in 6% yield (6.2 mg).



7

Sequence: H-β-Ala-Thr-Ile-Thr-Ser-Gly-Gly-Thr-Thr-Asn-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 1.92 min (> 99% purity); **HRMS (ESI+)** m/z calc. for $C_{36}H_{64}N_{12}O_{16}$ $[M+H]^+$ = 921.4642, found 921.4636.

Subsequently, peptide **7** (4 mg, 0.0043 mmol) was labelled using the procedure outlined above, and purified by preparative RP-HPLC using a linear gradient of 10 to 50% B in A. After lyophilization, peptide **2** was obtained as a pink powder in 83% yield (5.4 mg).

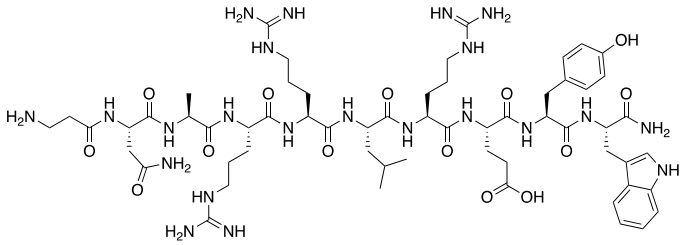


2

Sequence: SulfoCy3-β-Ala-Thr-Ile-Thr-Ser-Gly-Gly-Thr-Thr-Asn-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.51 min (> 99% purity); **HRMS (ESI+)** m/z calc. for $C_{66}H_{96}N_{14}O_{23}S_2Na_2$ $[M+H]^+$ = 1563.6088, found 1563.6031.

Synthesis & characterization of peptide 3

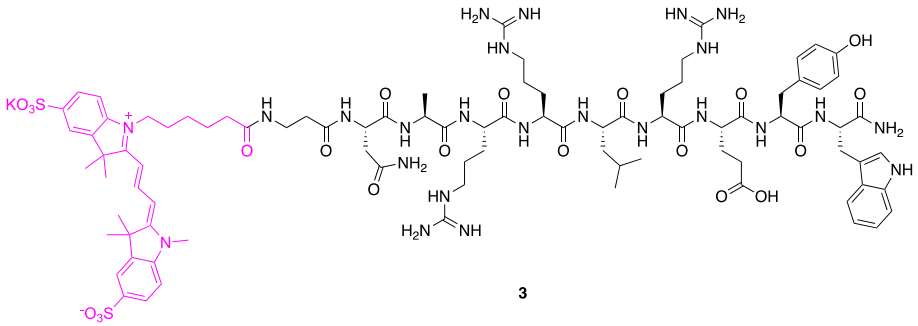
The peptide was assembled on a Rink amide AM resin (0.41 mmol/g, 0.1 mmol) using the Liberty Lite™ peptide synthesizer, followed by isolation of the non-labelled precursor **8** (H-β-Ala-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-NH_2) by the conditions outlined above. The non-labelled precursor **8** was purified by preparative RP-HPLC using a linear gradient of 10 to 40% B in A. After lyophilization, the peptide precursor **8** was obtained under its TFA salt form as a white powder in 33% yield (59.3 mg).



8

Sequence: H-β-Ala-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.51 min (99% purity); **HRMS (ESI+)** m/z calc. for $C_{59}H_{92}N_{22}O_{14}$ $[M+H]^+$ = 1333.7241, found 1333.7321.

Subsequently, peptide **8** (18.3 mg, 0.01 mmol) was labelled using the procedure outlined above, and purified by preparative RP-HPLC using a linear gradient of 15 to 60% B in A. After lyophilization, peptide **3** was obtained under its TFA salt form as a pink powder in 83% yield (19.6 mg).

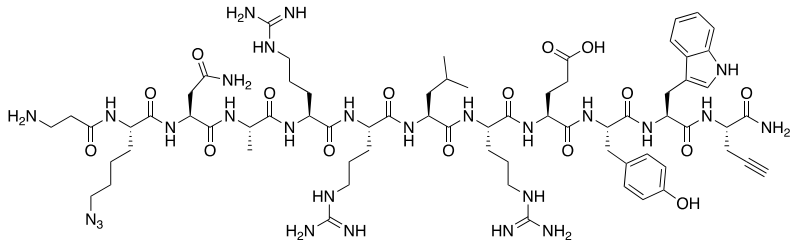


3

Sequence: SulfoCy3-β-Ala-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.74 min (> 99% purity); **HRMS (ESI+)** m/z calc. for $C_{89}H_{126}N_{24}O_{21}S_2$ $[M+2H]^{2+}$ = 966.4564, found 966.4481.

2.4 Synthesis of cyclic peptide 6

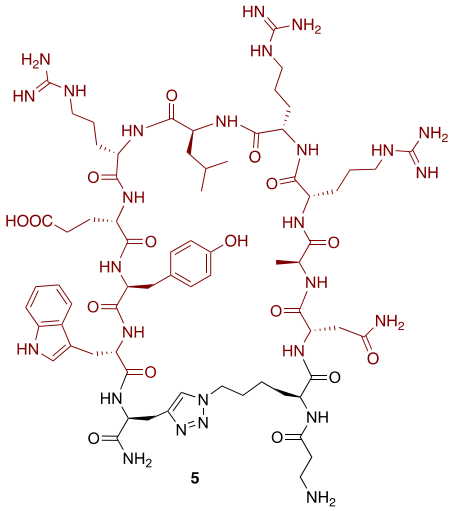
The labelled cyclic peptide **6** was synthesized following Scheme 1 provided in the manuscript. Linear peptide precursor **4** was first assembled by SPPS on a Rink amide AM resin (0.41 mmol/g, 0.10 mmol). Fmoc-Pra-OH, Fmoc-Lys(N_3)-OH and Fmoc-β-Ala-OH were manually coupled to the resin, whereas the standard amino acid residues were introduced using the Liberty Lite™ peptide synthesizer. After cleavage from the resin, precursor **4** was purified by preparative RP-HPLC using a linear gradient of 15 to 50% B in A. After lyophilization, the linear peptide precursor **4** was obtained under its TFA salt form as a white powder in 41% yield (83.9 mg).



4

Sequence: H-β-Ala-Lys(N_3)-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-Pra-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.74 min (99% purity); **HRMS (ESI+)** m/z calc. for $C_{70}H_{107}N_{27}O_{16}$ $[M+H]^+$ = 1582.8467, found 1582.8425.

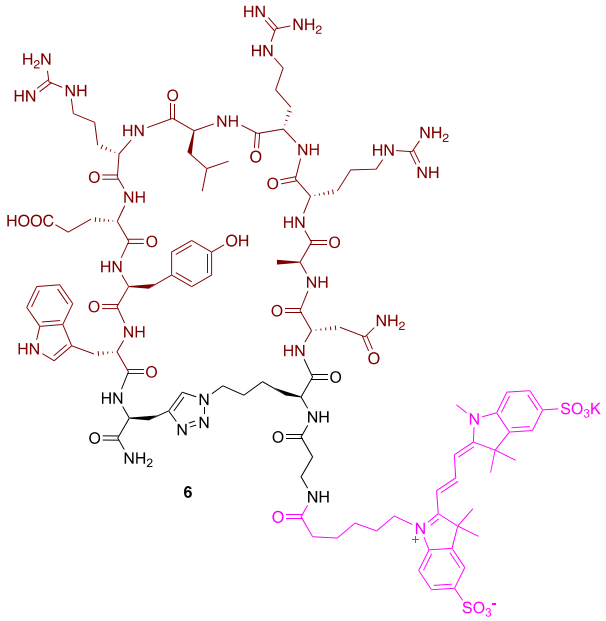
Subsequently, peptide **4** (66.0 mg, 0.032 mmol, 1.0 equiv) was stirred in a round-bottom flask in 5 mL H_2O:MeCN 1:1 and diluted with 50 mL H_2O. Subsequently, copper(II) sulfate pentahydrate (26.2 mg, 0.105 mmol, 3.2 equiv), sodium ascorbate (21.4 mg, 0.108 mmol, 3.4 equiv) and ammonium hydrogencarbonate (31.4 mg, 0.324 mmol, 10.0 equiv) were each solubilized in 1 mL water and separately added to the peptide solution, followed by washing with a total of 2 mL water (final peptide concentration of 0.75 mM). After stirring the mixture for 2 hours at room temperature, it was lyophilized. The resulting green powder was solubilized in DMSO and acidified with a few drops of TFA, followed by purification by preparative RP-HPLC using a linear gradient of 10 to 50% B in A. After lyophilization, cyclic peptide **5** was obtained under its TFA salt form as a white powder in 42% yield (28.0 mg).



5

Sequence: H-β-Ala-c[Lys(N_3)-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-Pra]-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.48 min (99% purity); **HRMS (ESI+)** m/z calc. for $C_{70}H_{107}N_{27}O_{16}$ $[M+H]^+$ = 1582.8467, found 1582.8425.

Subsequently, peptide **5** (18.2 mg, 0.009 mmol) was labelled using the procedure outlined above, and purified by preparative RP-HPLC using a linear gradient of 15 to 60% B in A. After lyophilization, peptide **6** was obtained as a pink powder in 86% yield (19.8 mg).



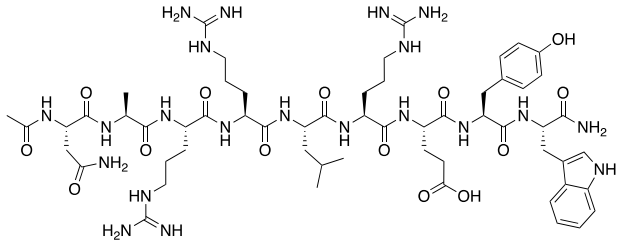
6

Sequence: SulfoCy3-β-Ala-c[Lys(N_3)-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-Pra]-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.76 min (> 99% purity); **HRMS (ESI+)** m/z calc. for $C_{100}H_{141}N_{29}O_{23}S_2$ $[M+2H]^{2+}$ = 1091.0177, found 1091.0271.

2.5 Synthesis of acetylated analogues

Synthesis & characterization of peptide 9

The peptide was assembled on a Rink amide AM resin (0.41 mmol/g, 0.1 mmol) using the Liberty Lite™ peptide synthesizer, followed by isolation by preparative RP-HPLC using the conditions outlined above. After lyophilization, peptide **9** was obtained under its TFA salt form as a white powder in 19% yield (34 mg).

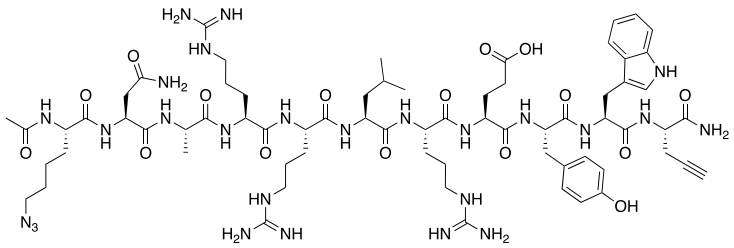


9

Sequence: Ac-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 3.08 min (98% purity); **HRMS (ESI+)** m/z calc. for $C_{58}H_{89}N_{21}O_{14}$ $[M+H]^+$ = 1304.6975, found 1304.6998.

Synthesis & characterization of cylic peptide 11

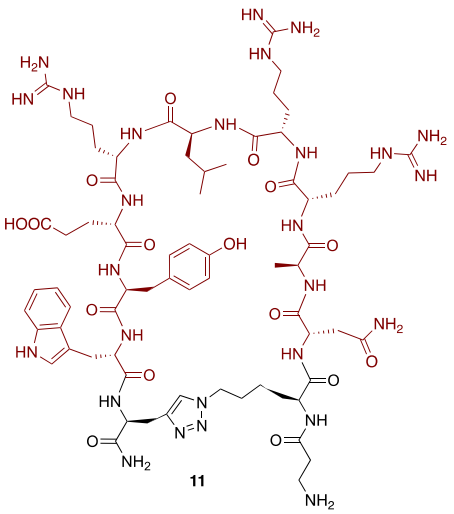
The cyclic acetylated variant **11** of peptide **6** was synthesized in an analogous manner. Linear peptide precursor **10** was first assembled by SPPS on a Rink amide AM resin (0.41 mmol/g, 0.10 mmol). Fmoc-Pra-OH and Fmoc-Lys(N_3)-OH were manually coupled to the resin, whereas the standard amino acid residues were introduced using the Liberty Lite™ peptide synthesizer. After cleavage from the resin, precursor **10** was purified by preparative RP-HPLC. After lyophilization, the linear peptide precursor **10** was obtained under its TFA salt form as a white powder in 44% yield (85 mg).



10

Sequence: Ac-Lys(N_3)-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-Pra-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.84 min (97% purity); **HRMS (ESI+)** m/z calc. for $C_{69}H_{104}N_{26}O_{16}$ $[M+H]^+$ = 1553.8202, found 1553.8265.

Subsequently, peptide **10** (83.7 mg, 0.041 mmol, 1.0 equiv) was stirred in a round-bottom flask in 5 mL H_2O:MeCN 1:1 and diluted with 70 mL H_2O. Subsequently, copper(II) sulfate pentahydrate (40.1 mg, 0.161 mmol, 3.9 equiv), sodium ascorbate (28.2 mg, 0.142 mmol, 3.5 equiv) and ammonium hydrogencarbonate (42.0 mg, 0.531 mmol, 13.0 equiv) were each solubilized in 1 mL water and separately added to the peptide solution, followed by washing with a total of 2 mL water (final peptide concentration of 0.75 mM). After stirring the mixture for 1 hour at room temperature, it was lyophilized. The resulting green powder was solubilized in DMSO and acidified with a few drops of TFA, followed by purification by preparative RP-HPLC. After lyophilization, cyclic peptide **11** was obtained under its TFA salt form as a white powder in 52% yield (43 mg).



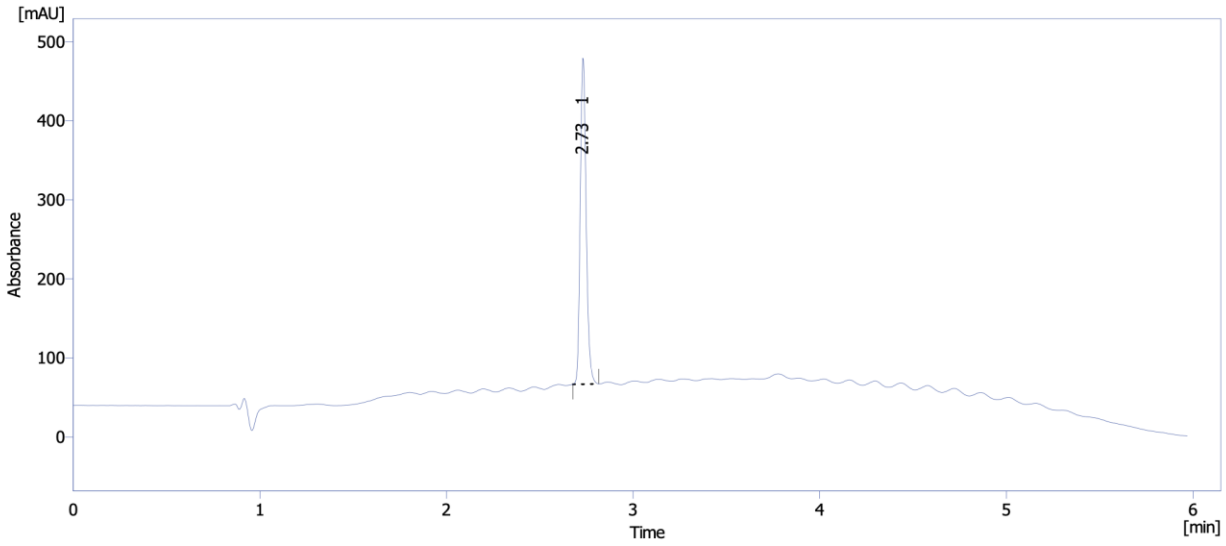
11

Sequence: Ac-c[Lys(N_3)-Asn-Ala-Arg-Arg-Leu-Arg-Glu-Tyr-Trp-Pra]-NH_2; **HPLC** (Chromolith RP-18C, 1-99% B in A over 4.5 min, flow rate = 2.8 mL/min, wavelength = 214 nm): $\mathbf{t_R}$ = 2.58 min (99% purity); **HRMS (ESI+)** m/z calc. for $C_{69}H_{104}N_{26}O_{16}$ $[M+H]^+$ = 1553.8202, found 1553.8298.

2.6 Copies of HPLC spectra

Spectra were recorded on an analytical HPLC (VWR) equipped with a Chromolith High Resolution RP-18C column from Merck (150 mm x 4.6 mm, 1.1 μm). Product were eluted using a linear gradient ranging from 1% to 99% B over 4.5 minutes at a flow rate of 2.8 mL/min.

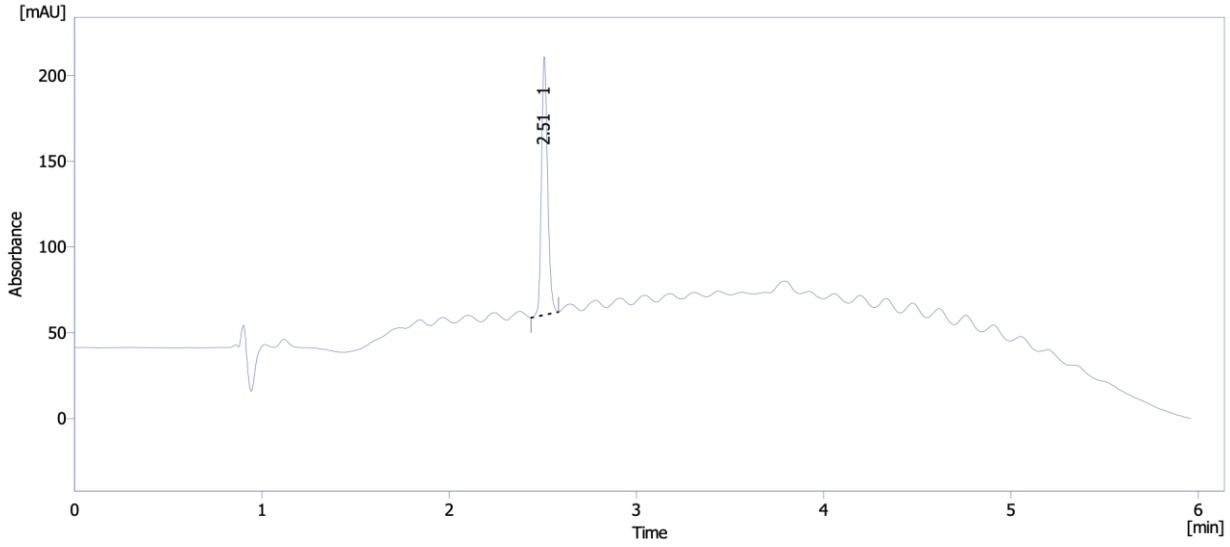
Peptide **1**



Result Table (Uncal - MM72 SulfoCy3 Pure_11_17_2022 4_24_28 PM_023 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.730	908.467	412.406	100.0	100.0
		Total	908.467	412.406	100.0	100.0

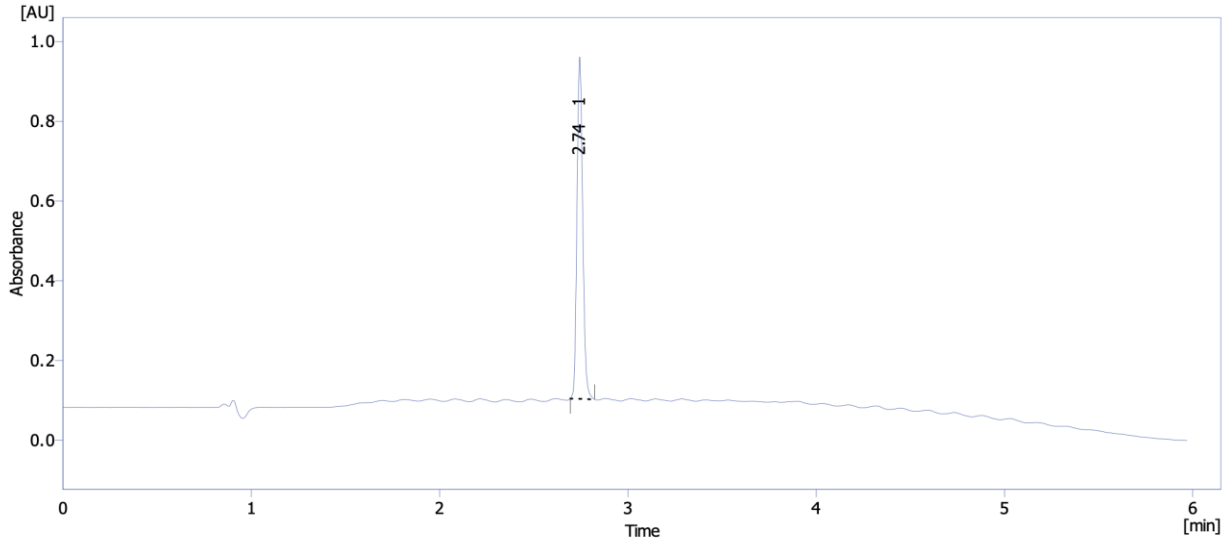
Peptide **2**



Result Table (Uncal - MM71 SulfoCy3 Pure_11_10_2022 1_28_13 PM_010 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.507	344.020	150.576	100.0	100.0
		Total	344.020	150.576	100.0	100.0

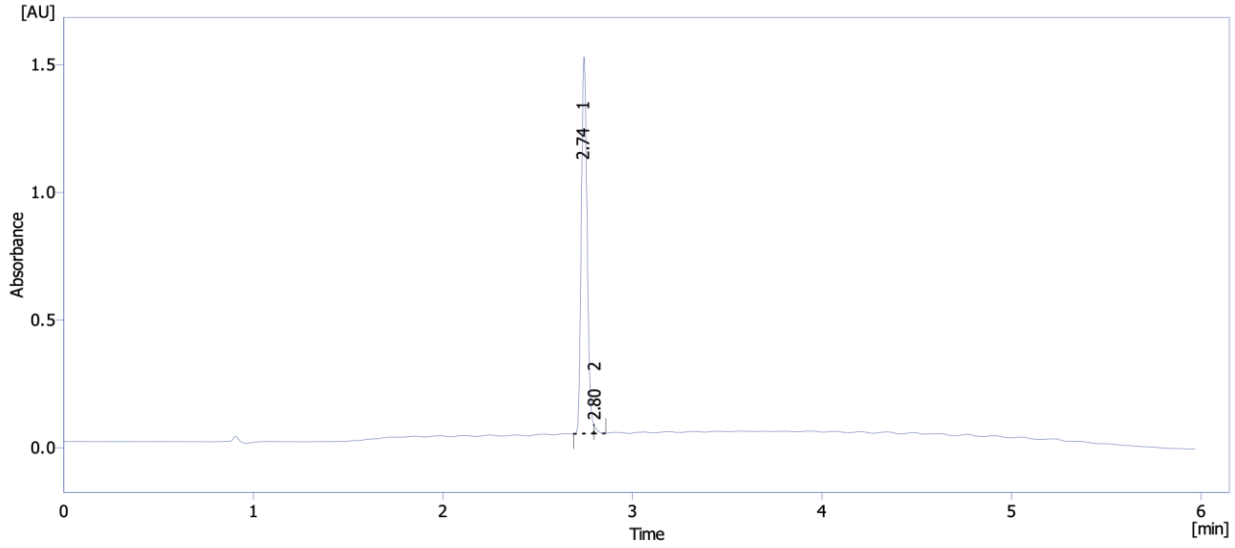
Peptide **3**



Result Table (Uncal - C:\Clarity VA\DataFiles\orgc\Data\KVH\KVH347-pure_9_13_2021 9_25_56 AM_007 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.743	1819.851	857.742	100.0	100.0
		Total	1819.851	857.742	100.0	100.0

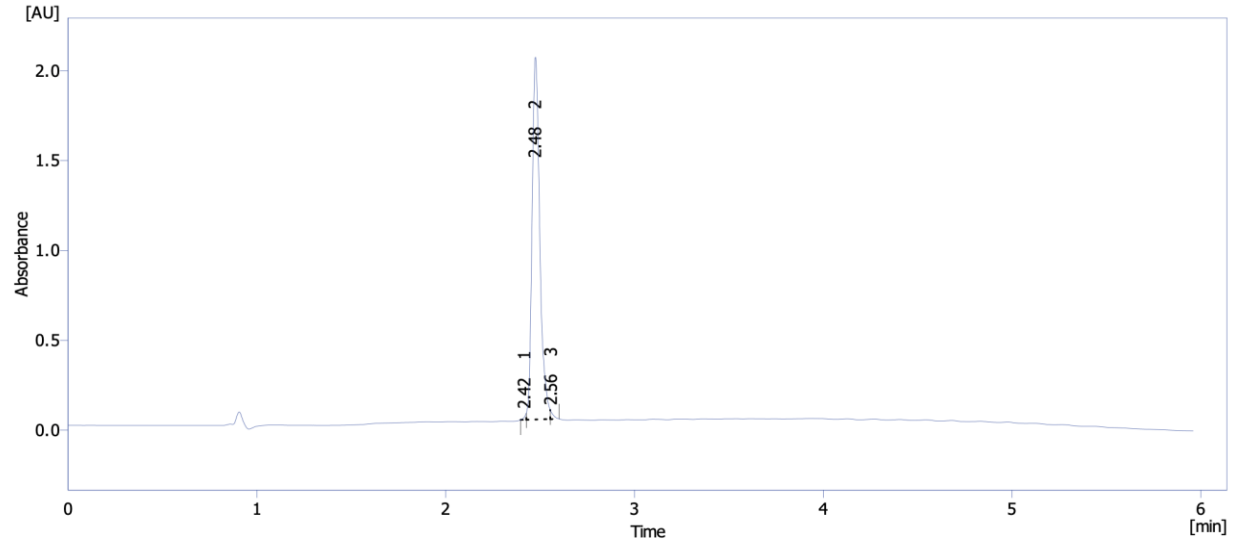
Peptide **4**



Result Table (Uncal - KVH354B-i2-f8_10_12_2021 10_34_46 AM_012 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.743	3154.512	1475.673	98.9	98.4
2		2.803	36.534	23.578	1.1	1.6
		Total	3191.046	1499.251	100.0	100.0

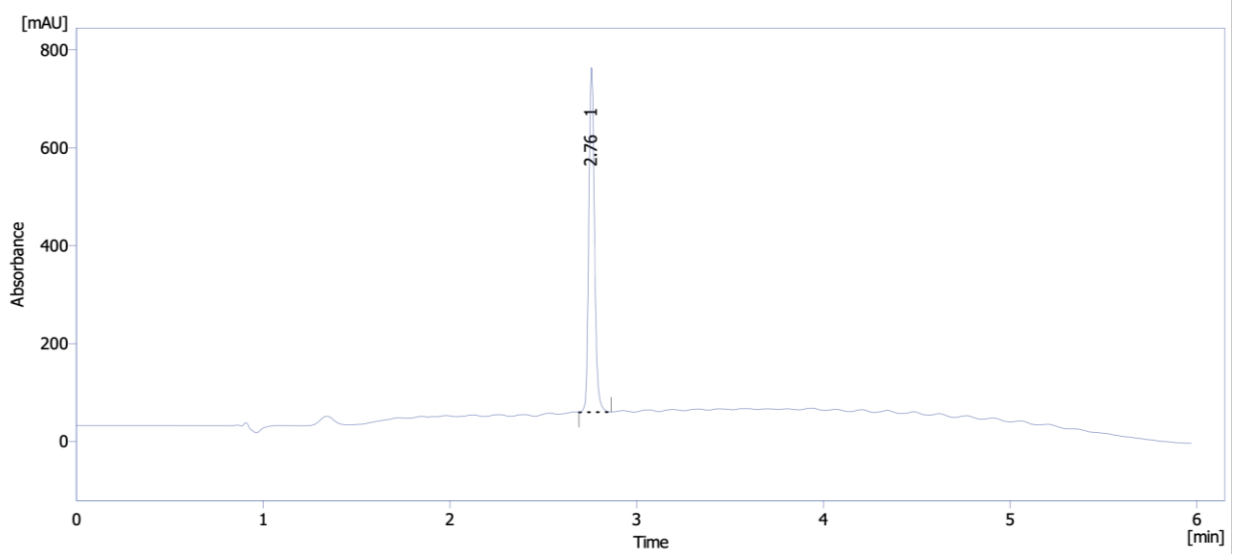
Peptide **5**



Result Table (Uncal - KVH356-i2-f2_10_19_2021 9_35_11 AM_007 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.420	19.019	21.059	0.3	1.0
2		2.477	5744.554	2015.426	98.7	97.2
3		2.560	54.819	36.856	0.9	1.8
		Total	5818.392	2073.341	100.0	100.0

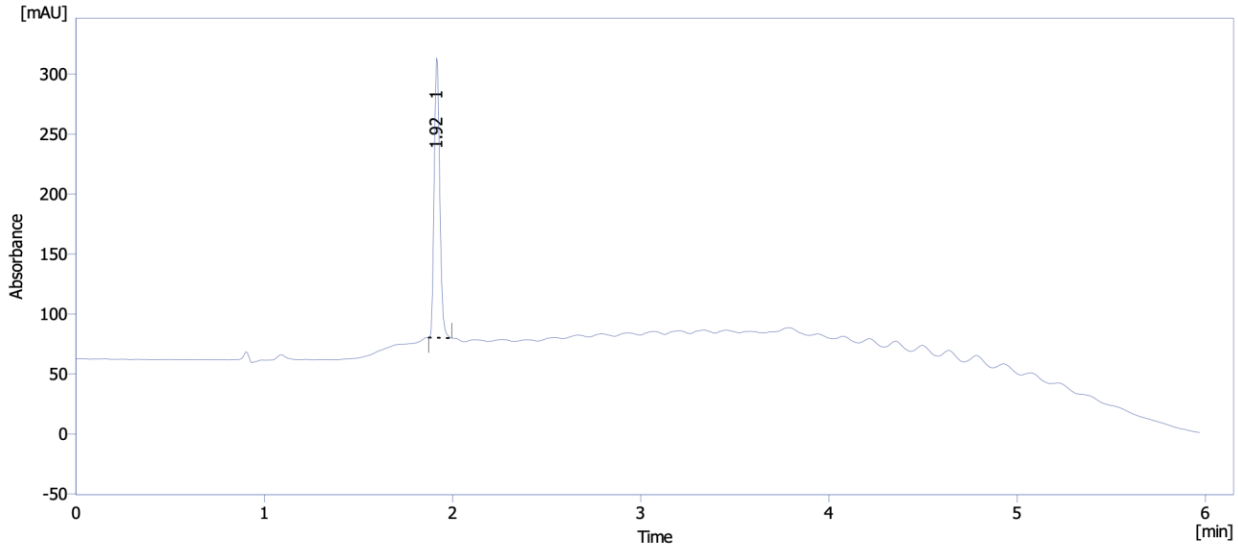
Peptide **6**



Result Table (Uncal - KVH374-pure_10_25_2021 3_29_29 PM_022 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.757	1498.560	704.055	100.0	100.0
		Total	1498.560	704.055	100.0	100.0

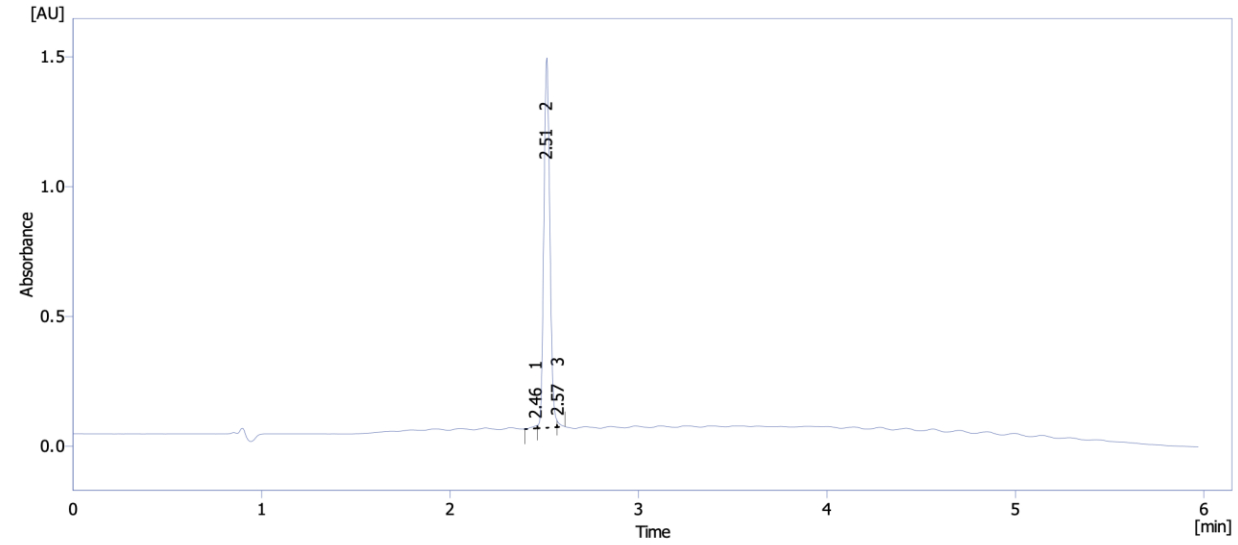
Peptide **7**



Result Table (Uncal - MM74 P1B NU_12_7_2022 10_59_59 AM_019 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		1.917	479.486	233.261	100.0	100.0
		Total	479.486	233.261	100.0	100.0

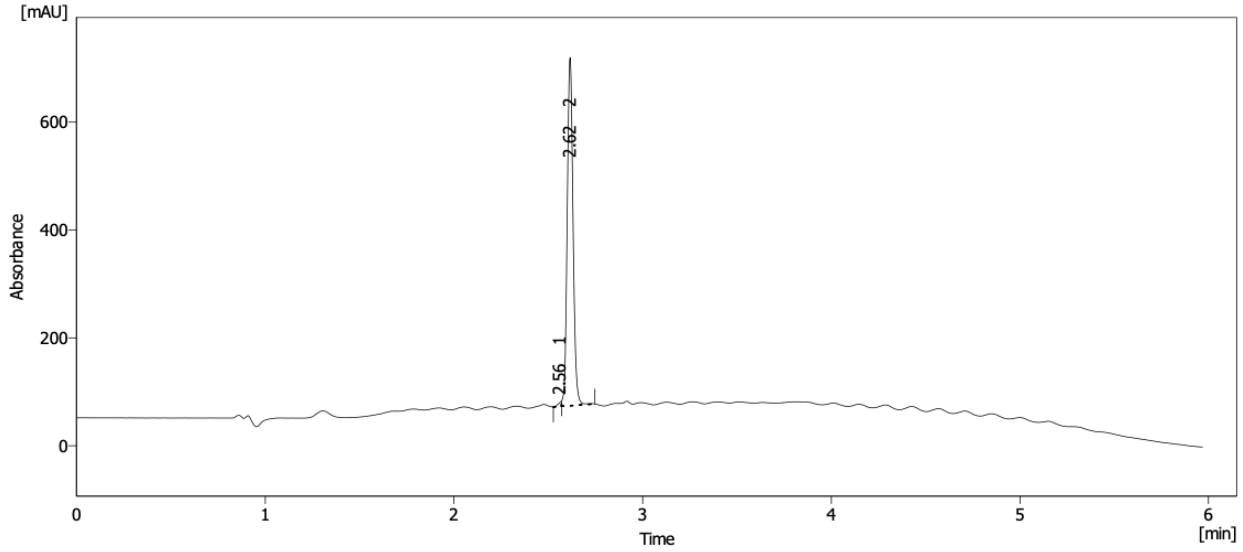
Peptide **8**



Result Table (Uncal - C:\ClarityVA\DataFiles\orgc\Data\KVH\KVH343-pure-lyo_9_8_2021 2_23_07 PM_027 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.457	19.060	10.118	0.6	0.7
2		2.513	3149.651	1424.166	98.6	98.1
3		2.573	26.089	17.349	0.8	1.2
		Total	3194.800	1451.633	100.0	100.0

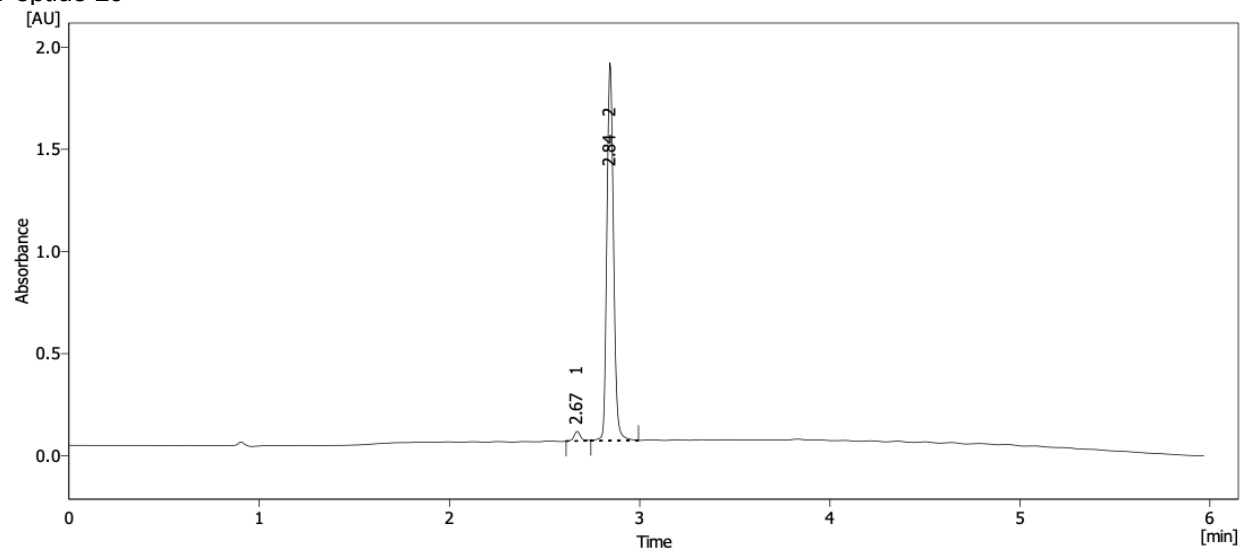
Peptide **9**



Result Table (Uncal - KVH544-pure_8_1_2022 11_52_24 AM_020 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.563	9.038	7.579	0.7	1.2
2		2.617	1378.720	645.589	99.3	98.8
		Total	1387.758	653.168	100.0	100.0

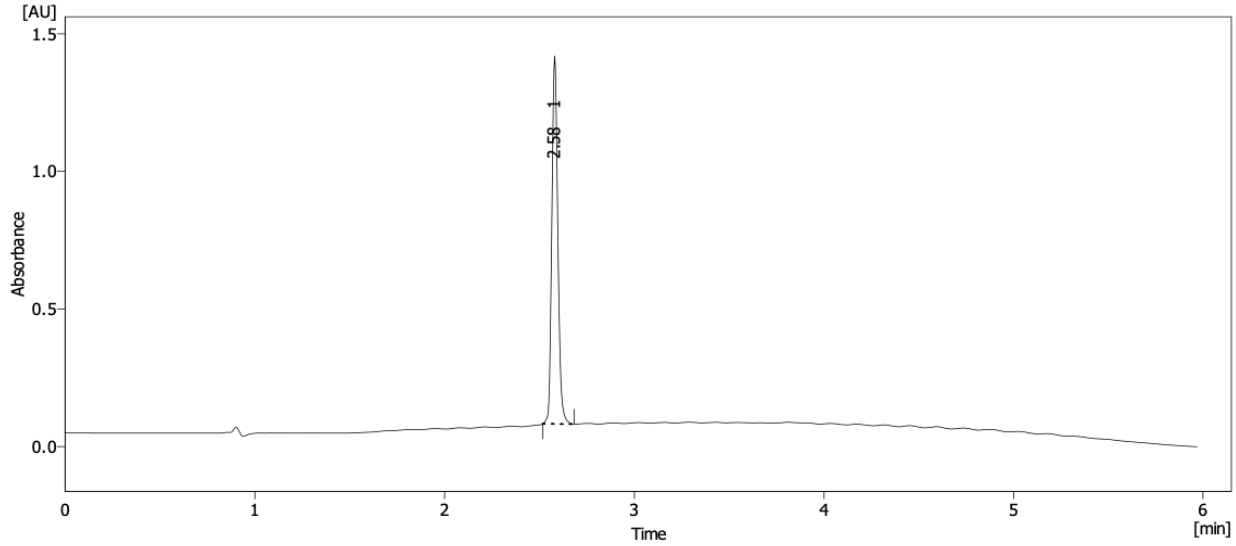
Peptide **10**



Result Table (Uncal - KVH541-f3 new vial_7_18_2022 9_31_01 AM_005 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.670	112.972	46.862	2.5	2.5
2		2.843	4489.813	1849.525	97.5	97.5
		Total	4602.785	1896.387	100.0	100.0

Peptide **11**



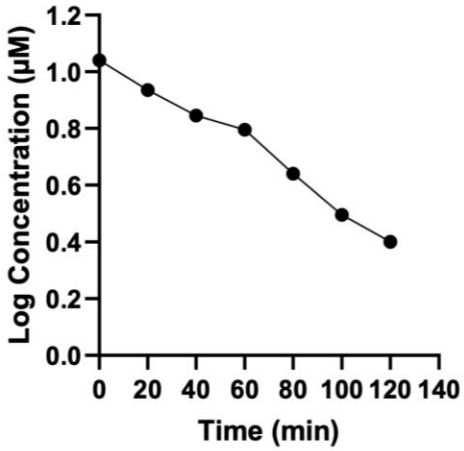
Result Table (Uncal - KVH553-f1_7_25_2022 9_39_28 AM_002 - PDA1-214nm-at-4nm)

	Compound Name	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]
1		2.580	2942.695	1336.151	100.0	100.0
		Total	2942.695	1336.151	100.0	100.0

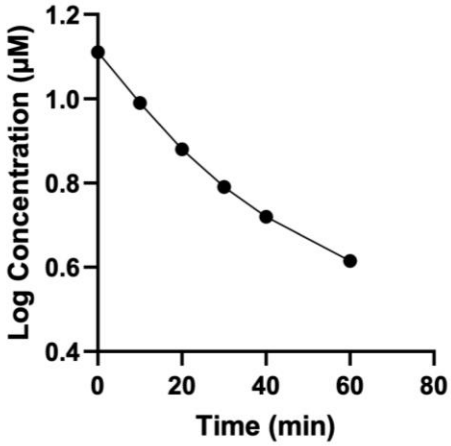
2.7 Proteolytic stability

Proteolytic stability was assessed using an *in vitro* human plasma stability assay. First, lyophilized peptides **9** and **11** were dissolved in milliQ water (2mM stock solution) and consecutive dilutions were prepared. Assessment of the linearity, accuracy and precision of the method have been validated using 3 independent calibration curves. Stability experiments have been performed in duplicate. Frozen human plasma samples (from the Belgian Red Cross (Vlaams-Brabant, Mechelen)) were thawed and heated to 37°C in an incubator for at least 30 min. Then peptide solutions (1.12 mM) were spiked in human plasma (10:90 v/v peptide solution/plasma). Samples of 100 µL were taken at different time points, depending on the peptide stability, and 300 µL of cold (4°C) precipitation solvent (methanol containing 0.1% TFA v/v) was added, allowing a protein crash. The resulting suspensions were vortexed (2000 x g) for 15 s and placed at 4°C for 30 min. After centrifugation for 15 min, 100 µL supernatant was diluted with 100 µL water in the injection vial. Sample analysis was performed using an Agilent 1200 series gradient HPLC system in combination with an EC HPLC column EC 150/2 NUCLEODUR (C18 HTec, 150 mm x 2 mm, 3 µm, Macherey-Nagel). The half-life of the peptides was calculated by interpolating the data based on the calibration curves. Concentrations were calculated by use of the calibration curve and transferred to a semi-log chart presenting the log concentrations as a function of time. The optimum curve was used to calculate the peptide half-life. Data analysis was performed using Microsoft® Office 365 Excel.

A



B



C

Peptide	Half-life (min)	Half-life SD (min)
9	36,25	0,48
11	55,74	0,34

Figure S4. Proteolytic stability of acetylated analogues of peptides 3 and 6. The semi-log charts of peptide **9** (A) and **11** (B) are shown. These were used to calculate their peptide half-life (C).

2.8 Cytotoxicity on living *A. baumannii* cells

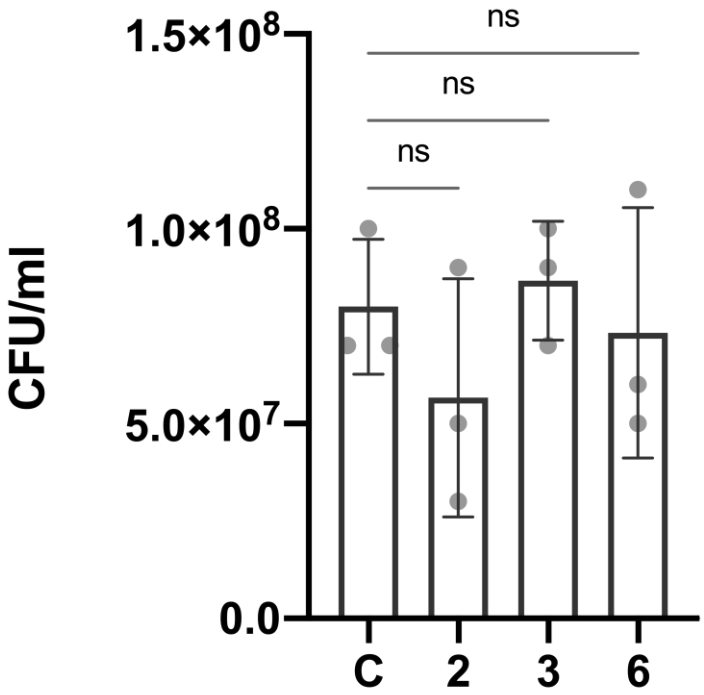


Figure S5. **Peptide cytotoxicity on living AB5075-VUB.** Colony forming units (CFU) of AB5075-VUB were compared after 30 min incubation with peptides **2**, **3** and **6** or buffer only (C). No statistically significant difference between the peptides and the control was observed. Statistics were tested by a one-way ANOVA test using Graphpad Prism software.

3 Quantification labelled cells

	BR1	BR2	BR3
Phase contrast (all cells)	571	344	297
SulfoCy3 (labelled cells)	22	9	7
Percentage labelled	3,85%	2,62%	2,36%
Average (%)		2,94%	
SD (%)		0,65%	

Table S1. Quantification of AB5075-VUB cells labelled by SulfoCy3-labelled NbD4. BR= Biological Replicate.

4 References

(1) Lefranc, M.-P.; Pommié, C.; Riuz, M.; Giudicelli, V.; Foulquier, E.; Truong, L.; Thouvenin-Contet, V.; Lefranc, G. IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. *Dev. Comp. Immunol.* **2003,** *27*, 55-77.

(2) Dunbar, J.; Deane, C. M. ANARCI: antigen receptor numbering and receptor classification. *Bioinformatics* **2016,** *32* (2), 298-300.