Supporting Information

Lead optimization yields high affinity Frizzled 7-targeting peptides that modulate *Clostridium difficile* toxin B pathogenicity in epithelial cells

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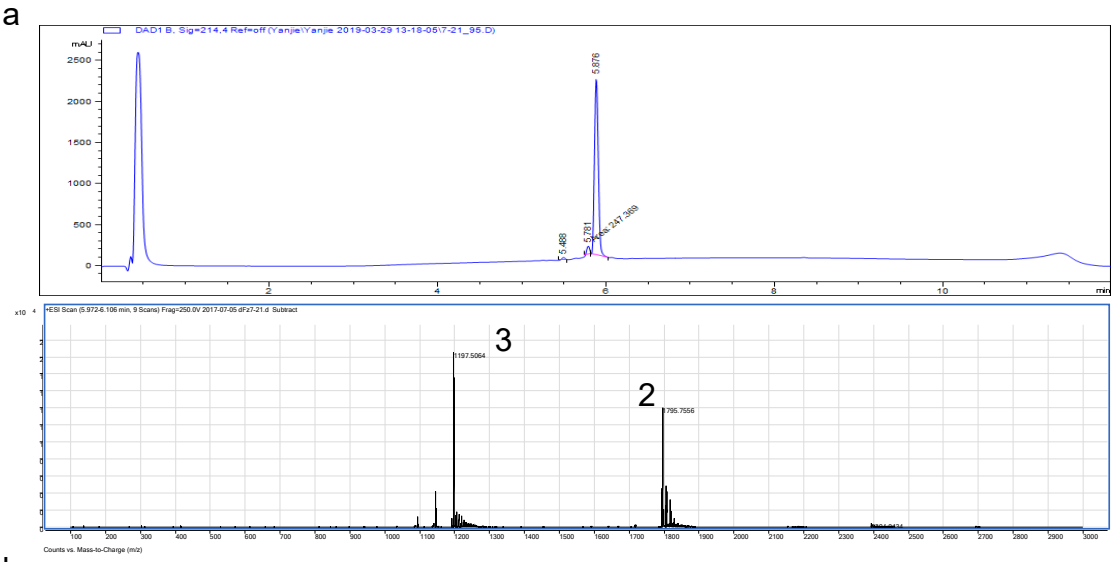
a

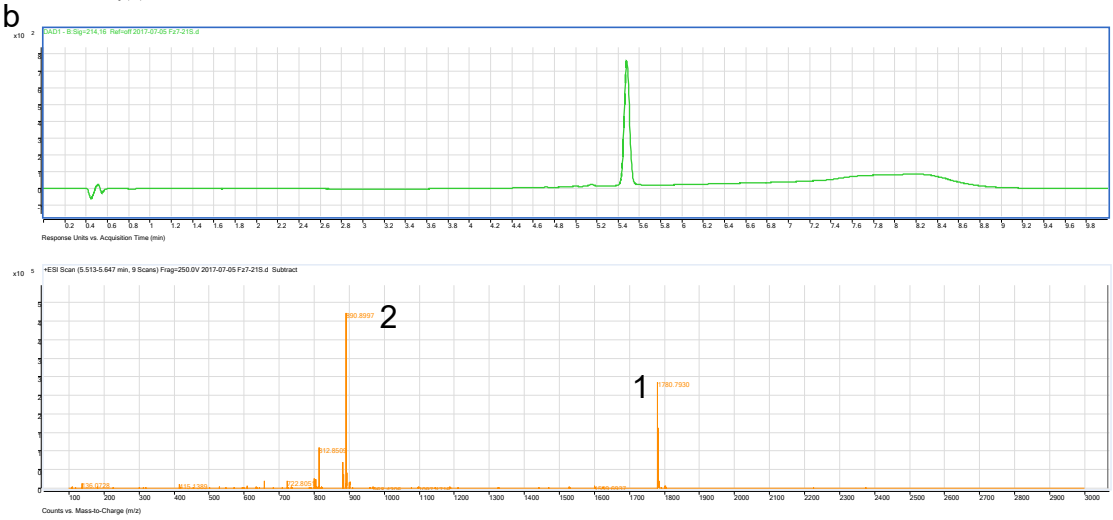


dFz7-21
purity: 95.37%
Expected MW: 3,590.0 Da

$(Ac\text{-}LPSDDLEFWCHVMY\text{-}NH_2)_2$

	Theoretical	Observed
1. $[M+1]^{+1}$	3,591.0	n.o.
2. $[M+2]^{+2}$	1,796.0	1,795.7
3. $[M+3]^{+3}$	1,197.7	1,197.5

b

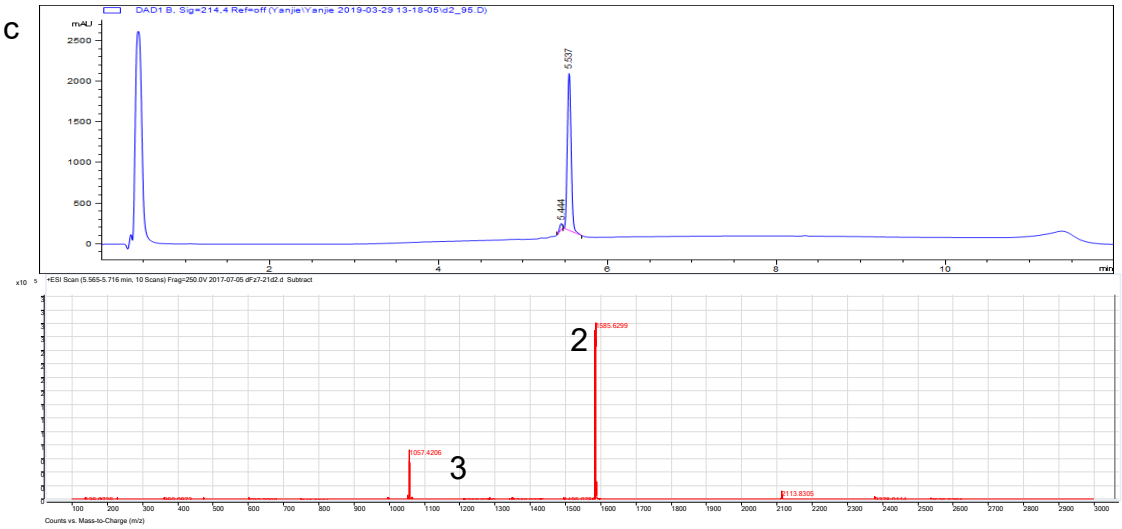


Fz7-21S
purity: 96.0%
Expected MW: 1,780.0 Da

$Ac\text{-}LPSDDLEFWSHVMY\text{-}NH_2$

	Theoretical	Observed
1. $[M+1]^{+1}$	1,781.0	1,780.8
2. $[M+2]^{+2}$	891.0	890.9
3. $[M+3]^{+3}$	594.3	n.o.

c



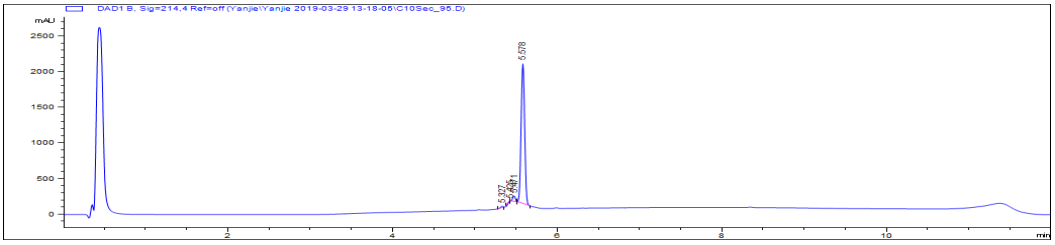
dFz7-21Δ2
purity: 95.23%
Expected MW: 3,169.6 Da

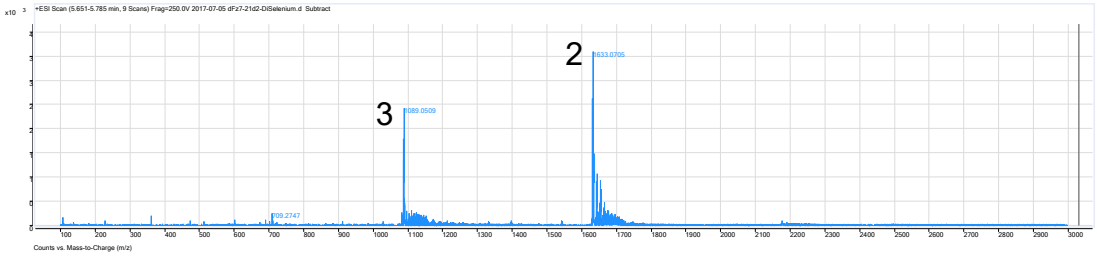
$(Ac\text{-}SDDLEFWCHVMY\text{-}NH_2)_2$

	Theoretical	Observed
1. $[M+1]^{+1}$	3,170.6	n.o.
2. $[M+2]^{+2}$	1,585.8	1,585.6
3. $[M+3]^{+3}$	1,057.5	1,057.4

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**a**) dFz7-21, (**b**) Fz7-21S or (**c**) dFz7-21Δ2. n.o., not observed.

d



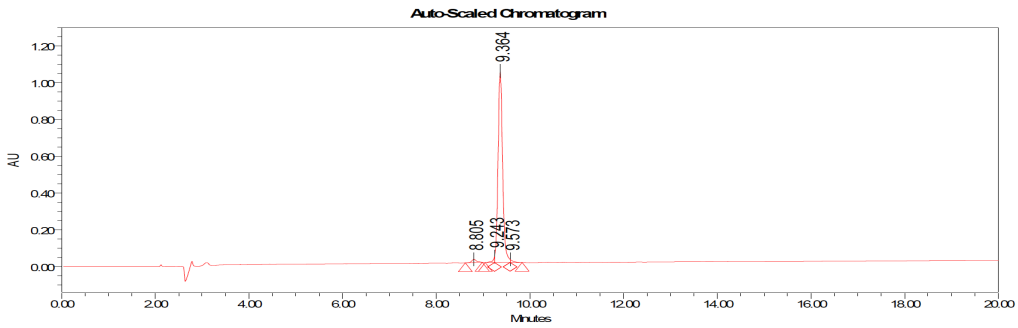


dFz7-21Δ2.C10Sec
purity: 95.82%
Expected MW: 3,263.3 Da

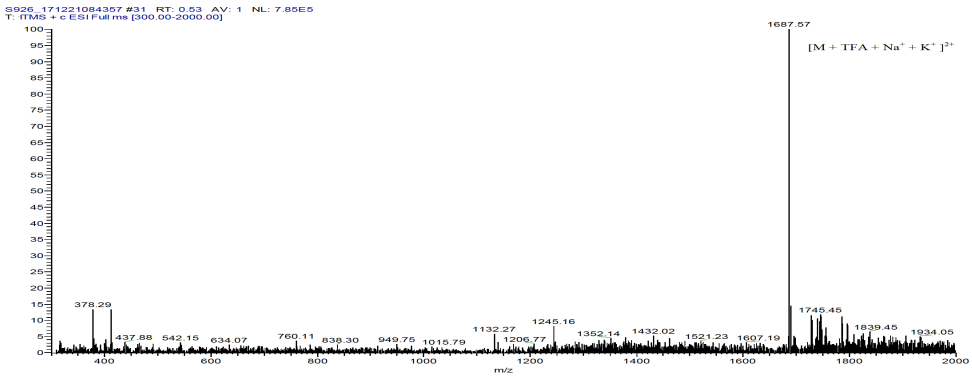
(Ac-SDDLEFW**X**HVMY-NH_2)$_2$
X= Selenocysteine

	Theoretical	Observed
1. $[M+1]^{+1}$	3,264.3	3,264.3
2. $[M+2]^{+2}$	1,633.1	1,632.7
3. $[M+3]^{+3}$	1,089.1	1,089.1

e



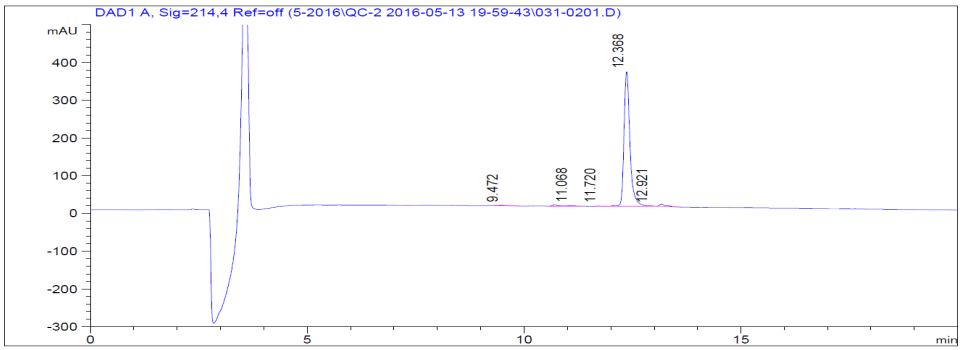
Compound: CS16244 dFz7-21A2.S3T (Ac-Thr-12-Tyr-NH_2)$_2$ Lot Number: S926 Expected M.W.: 3197.54 Found M.W.: 3199.14

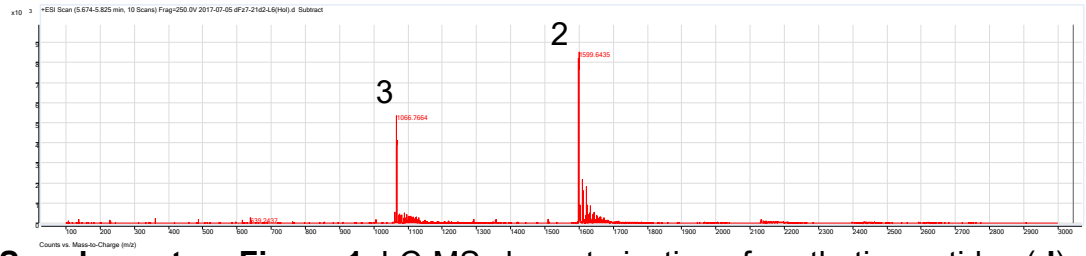


dFz7-21Δ2.S3T
purity: 96.36%
Expected MW: 3,197.54 Da
Observed MW: 3,199.14 Da

(Ac-**T**DDLEFWCHVMY-NH_2)$_2$

f





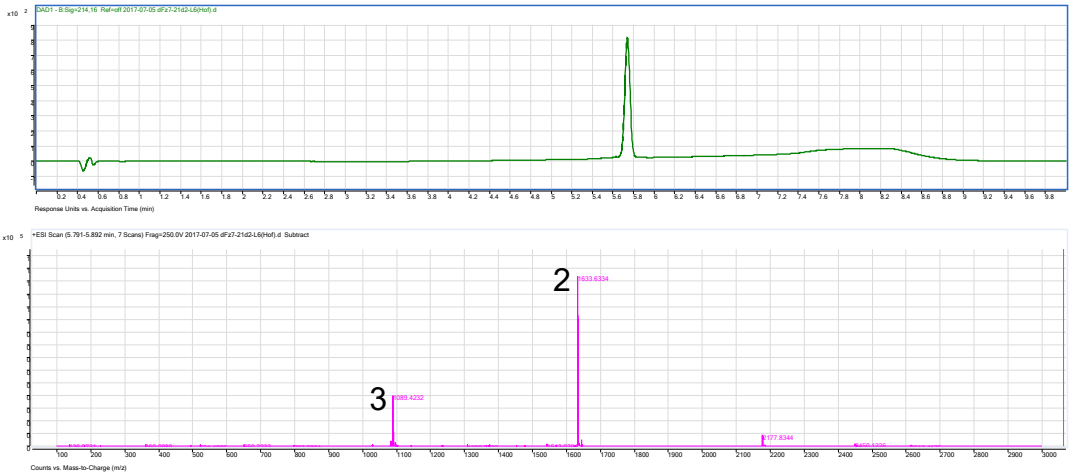
dFz7-21Δ2.L6Hol
purity: 95.16%
Expected MW: 3,197.52 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$
X= Homoleucine

	Theoretical	Observed
1. $[M+1]^{+1}$	3,198.5	n.o.
2. $[M+2]^{+2}$	1,599.8	1,599.6
3. $[M+3]^{+3}$	1,066.8	1,066.8

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**d**) dFz7-21Δ2.C10Sec, (**e**) dFz7-21Δ2.S3T or (**f**) Fz7-21Δ2.L6Hol. n.o., not observed.

g

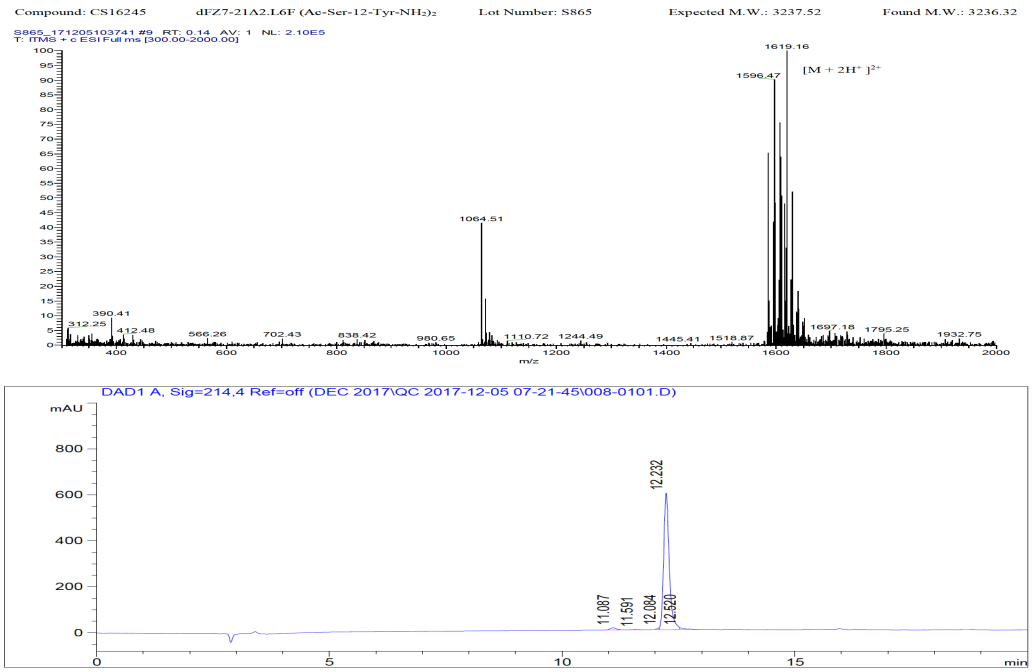


dFz7-21Δ2.L6Hof
purity: 95.07%
Expected MW: 3,265.66 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$
X= Homophenylalanine

	Theoretical	Observed
1. $[M+1]^{+1}$	3,266.7	n.o.
2. $[M+2]^{+2}$	1,633.8	1,633.6
3. $[M+3]^{+3}$	1,089.6	1,089.4

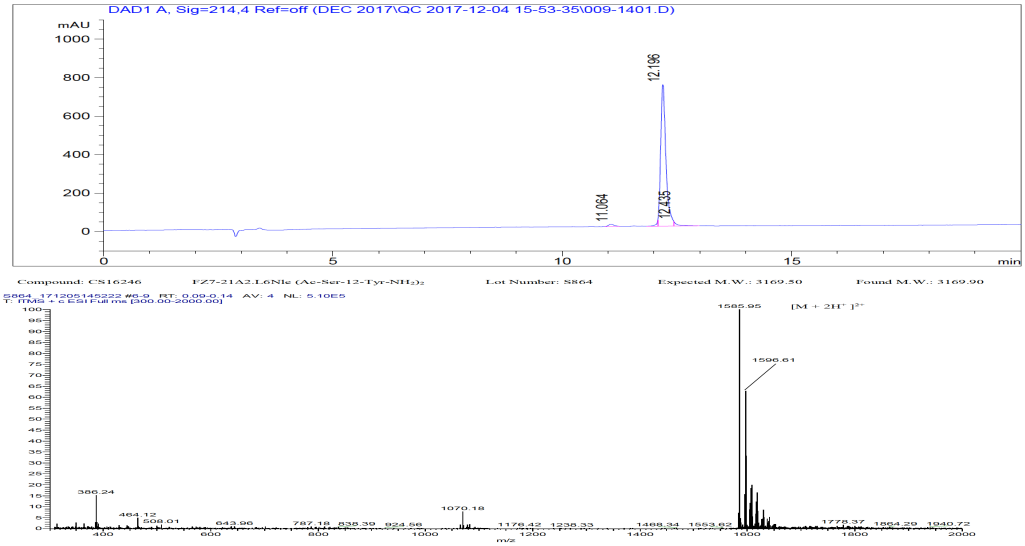
h



dFz7-21Δ2.L6F
purity: 96.02%
Expected MW: 3,237.52 Da
Observed MW: 3,236.32 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$

i

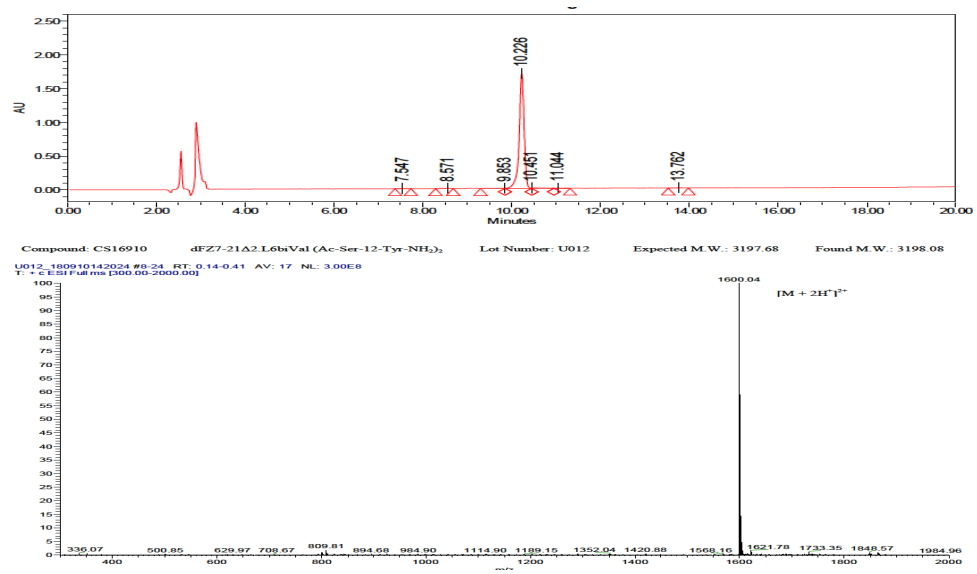


dFz7-21Δ2.L6Nle
purity: 95.36%
Expected MW: 3,169.5 Da
Observed MW: 3,169.9 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**g**) dFz7-21Δ2.L6Hof, (**h**) dFz7-21Δ2.L6F or (**i**) dFz7-21Δ2.L6Nle. n.o., not observed.

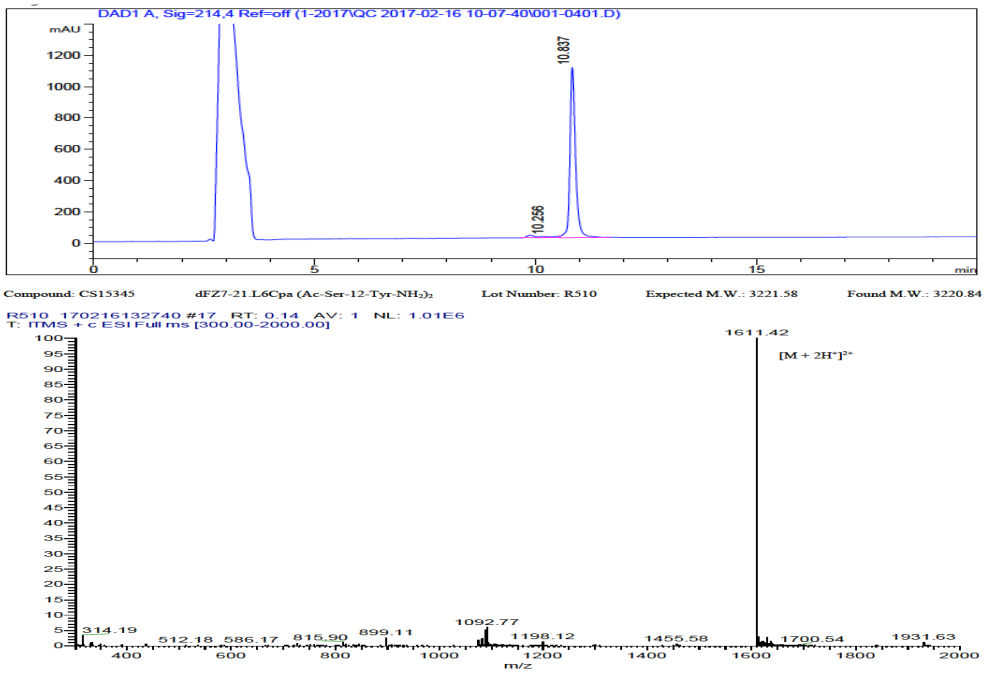
j



dFz7-21Δ2.L6biVal
purity: 97.87%
Expected MW: 3,197.68 Da
Observed MW: 3,198.08 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$

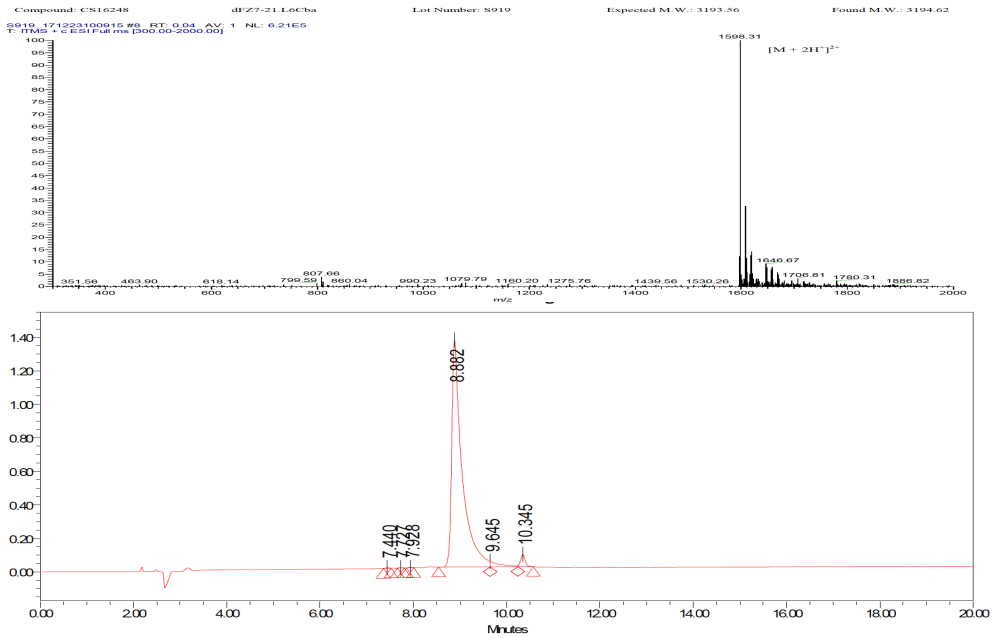
k



dFz7-21Δ2.L6Cpa
purity: 95.83%
Expected MW: 3,221.58 Da
Observed MW: 3,220.84 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$

l

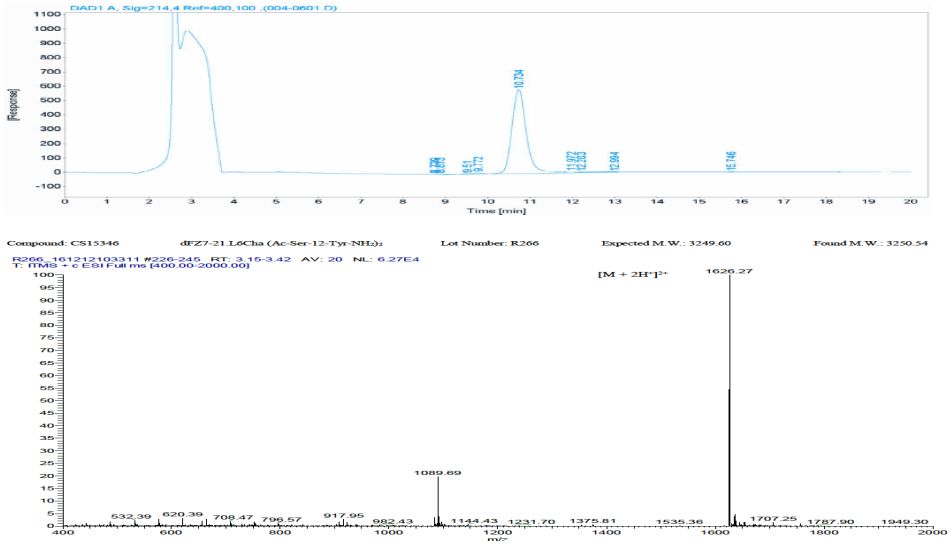


dFz7-21Δ2.L6Cba
purity: 95.48%
Expected MW: 3,193.56 Da
Observed MW: 3,194.62 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**j**) dFz7-21Δ2.L6biVal, (**k**) dFz7-21Δ2.L6Cpa or (**l**) dFz7-21Δ2.L6Cba. n.o., not observed.

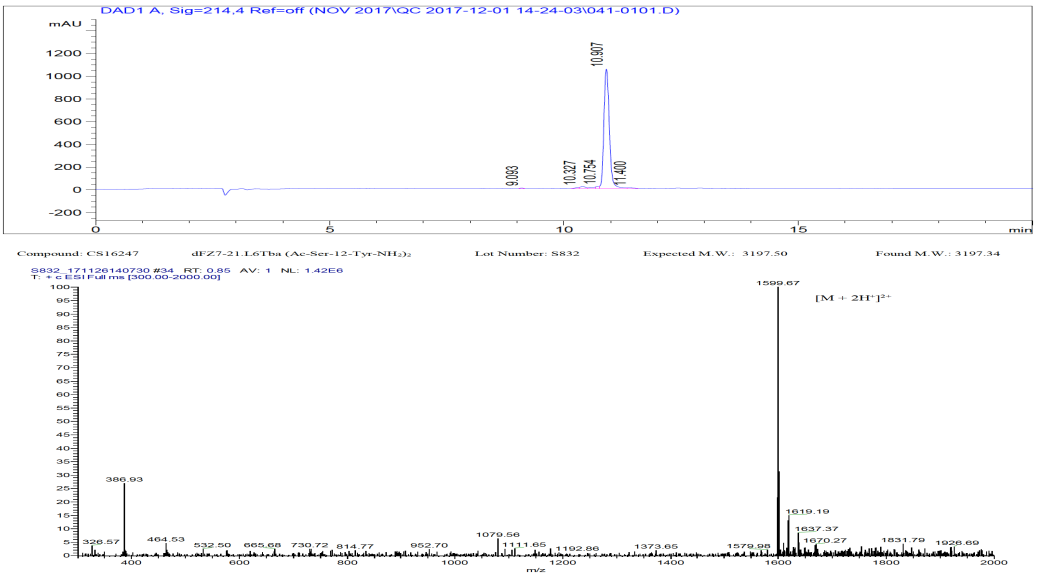
m



dFz7-21Δ2.L6Cha
purity: 96.68%
Expected MW: 3,249.60 Da
Observed MW: 3,250.54 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$

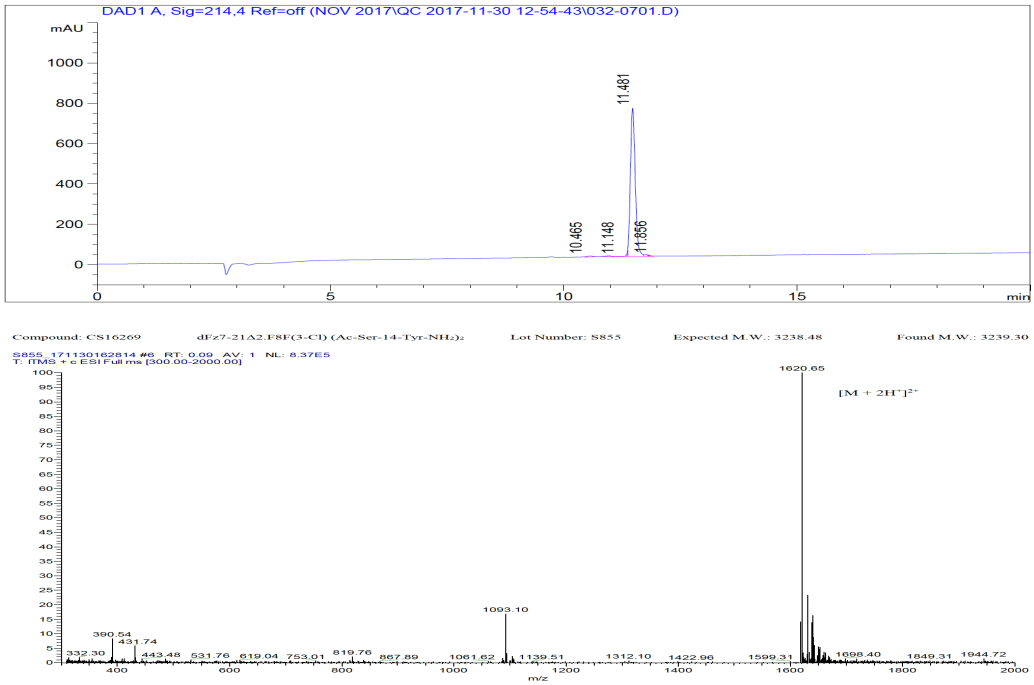
n



dFz7-21Δ2.L6Tba
purity: 95.11%
Expected MW: 3,197.5 Da
Observed MW: 3,197.34 Da

(Ac-SDD**X**EFWCHVMY-NH_2)$_2$
X = tBu-Ala

o

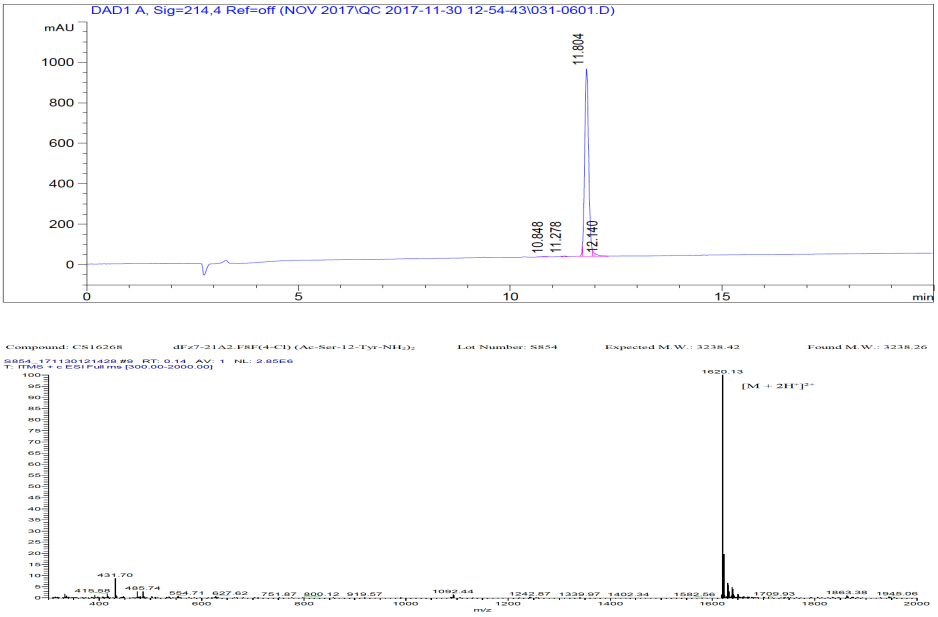


dFz7-21Δ2.F8F(3-Cl)
purity: 96.18%
Expected MW: 3,238.48 Da
Observed MW: 3,239.30 Da

(Ac-SDDLE**X**WCHVMY-NH_2)$_2$

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**m**) dFz7-21Δ2.L6Cha, (**n**) dFz7-21Δ2.L6Tba or (**o**) dFz7-21Δ2.F8F(3-Cl). n.o., not observed.

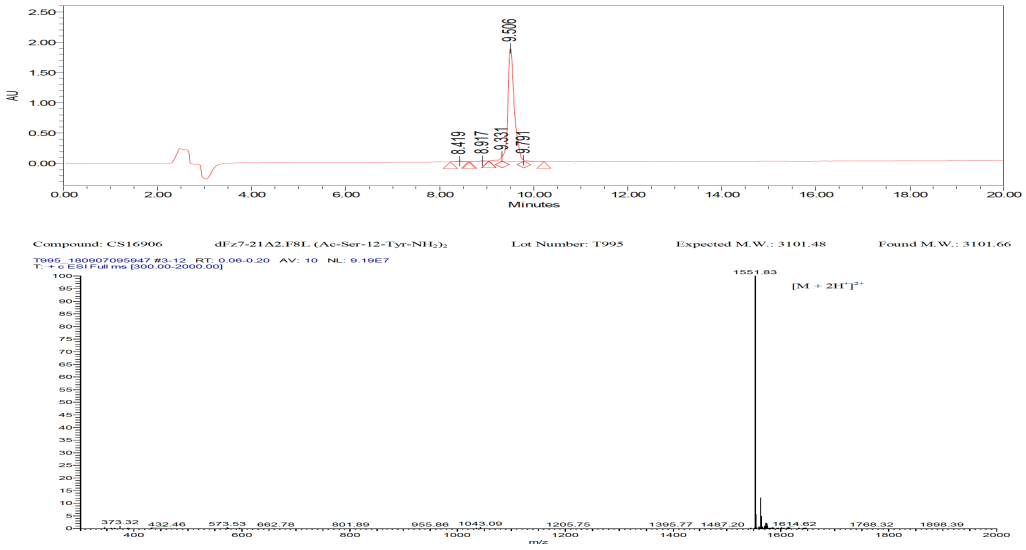
p



dFz7-21Δ2.F8F(4-Cl)
purity: 95.68%
Expected MW: 3,238.42 Da
Obsrved MW: 3,238.26 Da

(Ac-SDDLE**X**WCHVMY-NH_2)$_2$

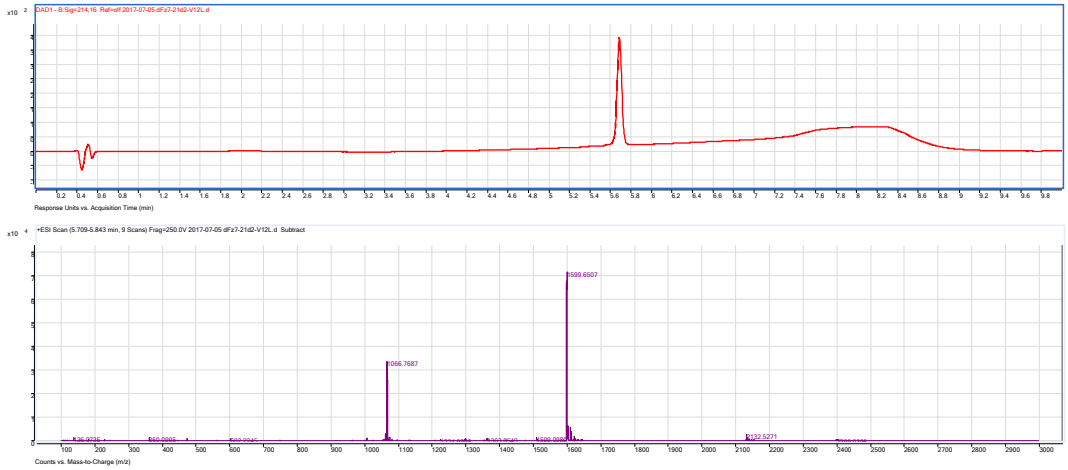
q



dFz7-21Δ2.F8L
purity: 96.32%
Expected MW: 3,101.48 Da
Obsrved MW: 3,101.66 Da

(Ac-SDDLE**L**WCHVMY-NH_2)$_2$

r



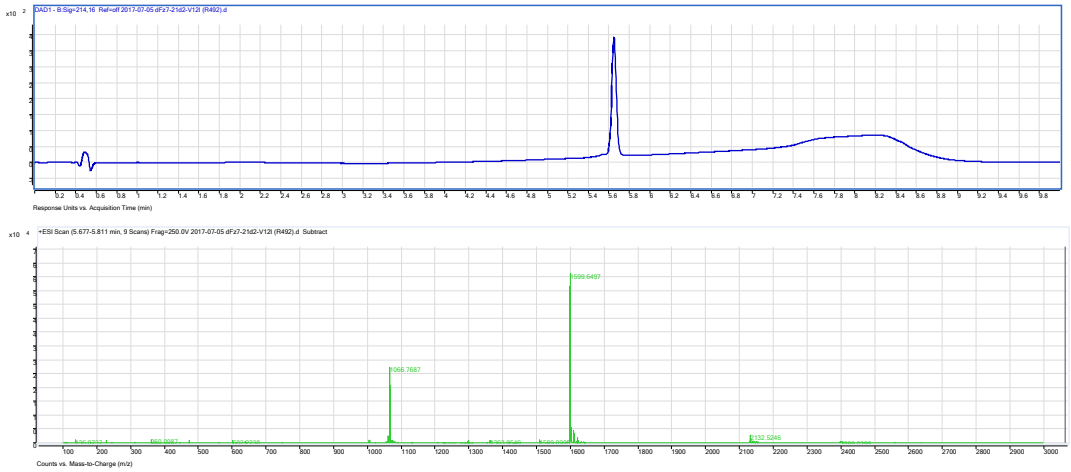
dFz7-21Δ2.V12L
purity: 95.83%
Expected MW: 3,197.54 Da

(Ac-SDDLEFWCH**L**MY-NH_2)$_2$

	Theoretical	Observed
1. $[M+1]^{+1}$	3,198.5	n.o.
2. $[M+2]^{+2}$	1,599.8	1,599.7
3. $[M+3]^{+3}$	1,066.8	1,066.8

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**p**) dFz7-21Δ2.F8F(4-Cl), (**q**) dFz7-21Δ2.F8L or (**r**) dFz7-21Δ2.V12L.

s

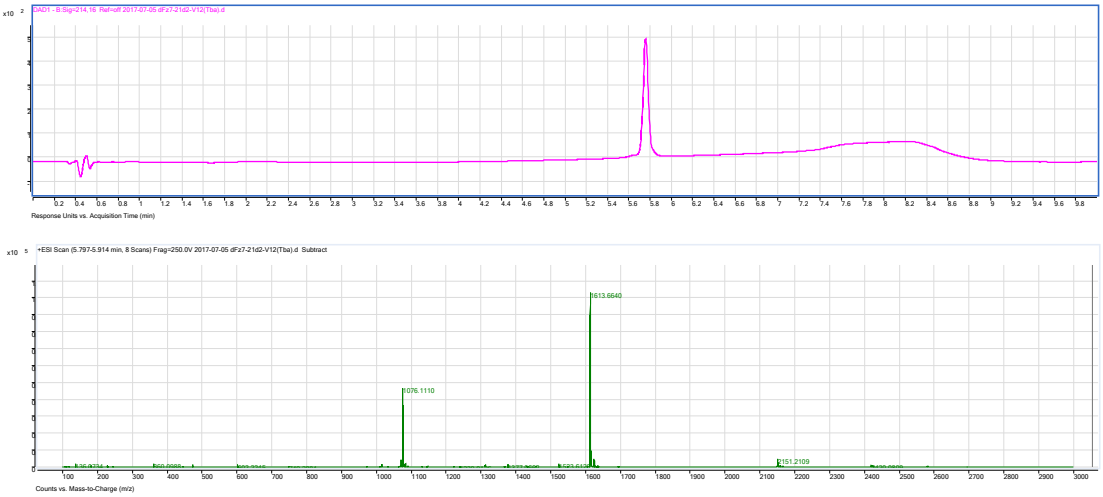


dFz7-21Δ2.V12I
purity: 95.36%
Expected MW: 3197.54 Da

(Ac-SDDLEFWCH**I**MY-NH_2)$_2$

	Theoretical	Observed
1. $[M+1]^{+1}$	3,198.54	n.o.
2. $[M+2]^{+2}$	1,599.77	1,599.65
3. $[M+3]^{+3}$	1,066.85	1,066.77

t

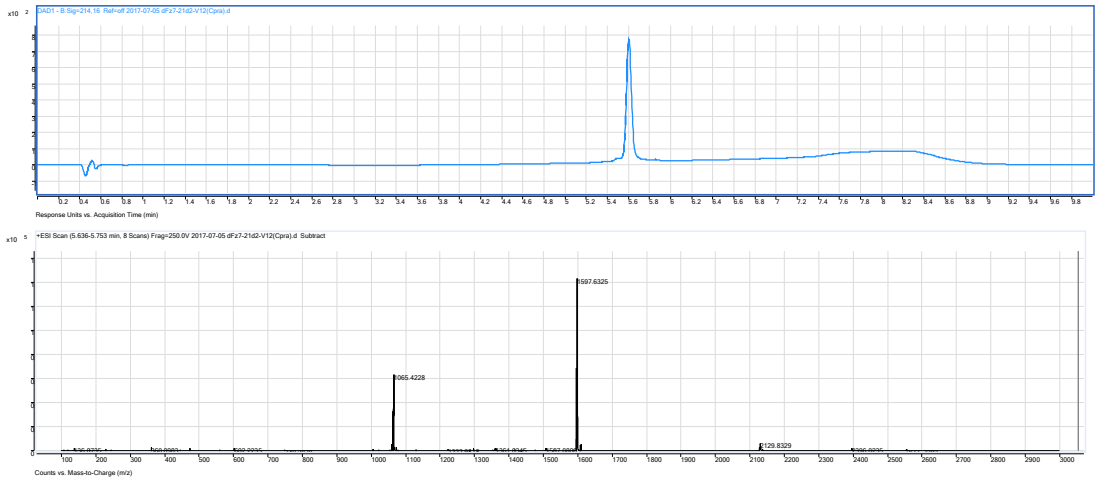


dFz7-21Δ2.V12Tba
purity: 96.81%
Expected MW: 3225.64 Da

(Ac-SDDLEFWCH**X**MY-NH_2)$_2$
X= t-butyl-L-alanine

	Theoretical	Observed
1. $[M+1]^{+1}$	3,226.64	n.o.
2. $[M+2]^{+2}$	1,613.82	1,613.7
3. $[M+3]^{+3}$	1,076.21	1,076.11

u



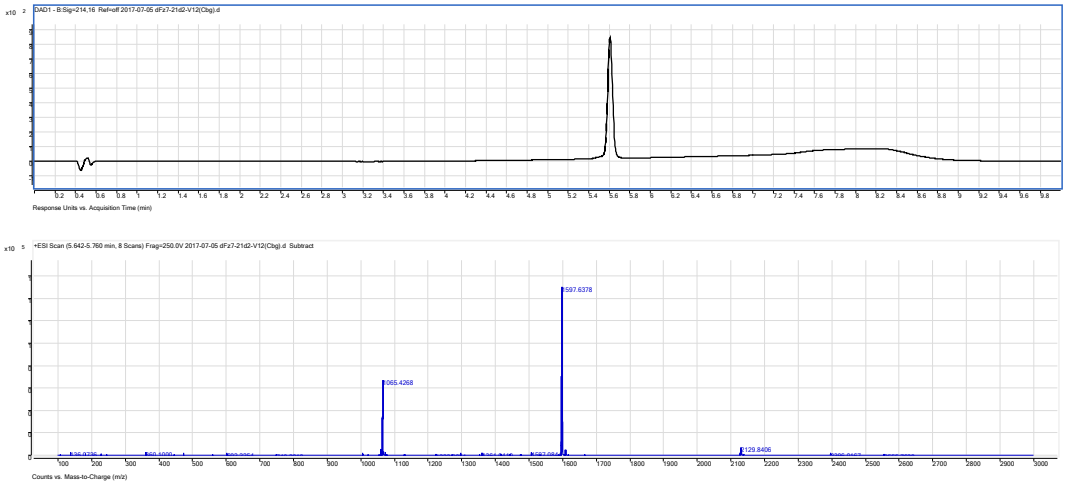
dFz7-21Δ2.V12Cpra
purity: 95.50%
Expected MW: 3193.34 Da

(Ac-SDDLEFWCH**X**MY-NH_2)$_2$
X= Cyclopropyl-L-alanine

	Theoretical	Observed
1. $[M+1]^{+1}$	3,194.34	n.o.
2. $[M+2]^{+2}$	1,597.67	1,597.6
3. $[M+3]^{+3}$	1,065.45	1,065.4

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**s**) dFz7-21Δ2.V12I, (**t**) dFz7-21Δ 2.V12Tba or (**u**) dFz7-21Δ2.V12Cpra.

v

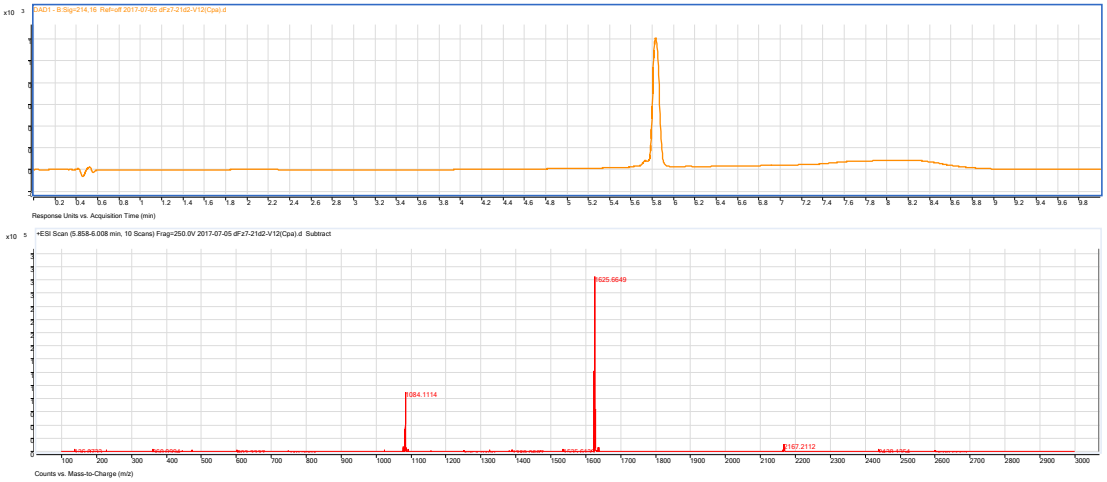


dFz7-21Δ2.V12Cbg
purity: 95.22%
Expected MW: 3,193.54 Da

(Ac-SDDLEFWCH**X**MY-NH_2)$_2$
X= cyclobutyl-glycine

	Theoretical	Observed
1. $[M+1]^{+1}$	3,194.5	n.o.
2. $[M+2]^{+2}$	1,597.8	1,597.6
3. $[M+3]^{+3}$	1,065.5	1,065.4

w

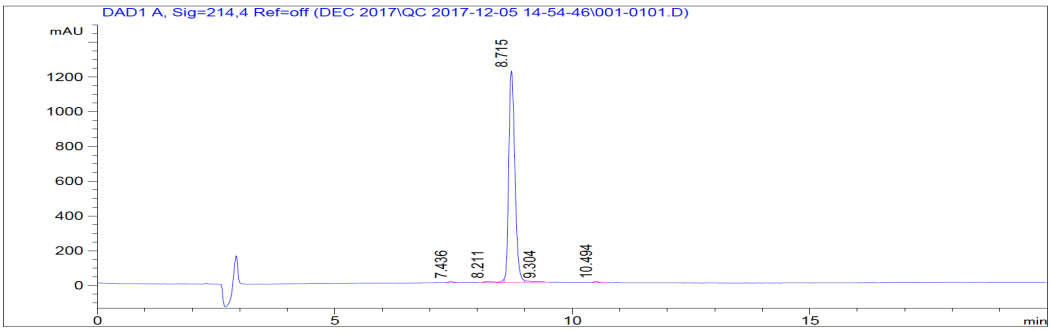


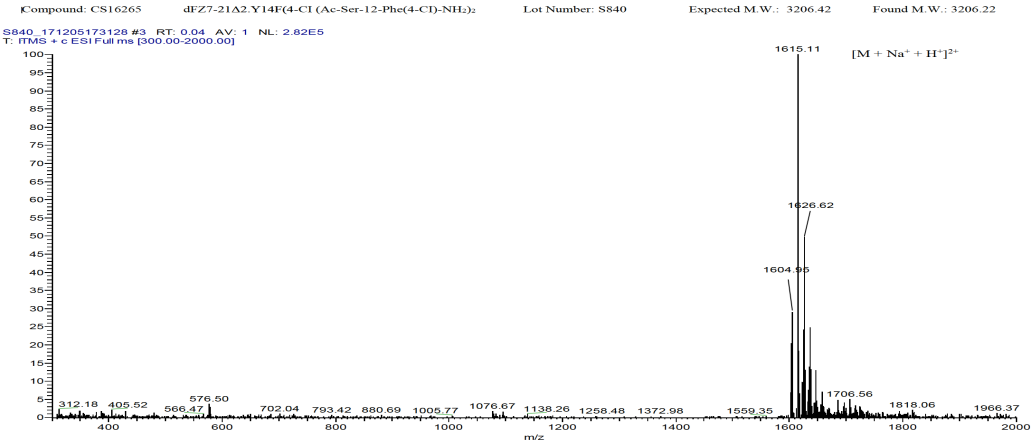
dFz7-21Δ2.V12Cpa
purity: 97.24%
Expected MW: 3249.64 Da

(Ac-SDDLEFWCH**X**MY-NH_2)$_2$
X= Cyclopentyl-L-alanine

	Theoretical	Observed
1. $[M+1]^{+1}$	3,250.64	n.o.
2. $[M+2]^{+2}$	1,625.82	1,625.7
3. $[M+3]^{+3}$	1,084.21	1,084.1

x



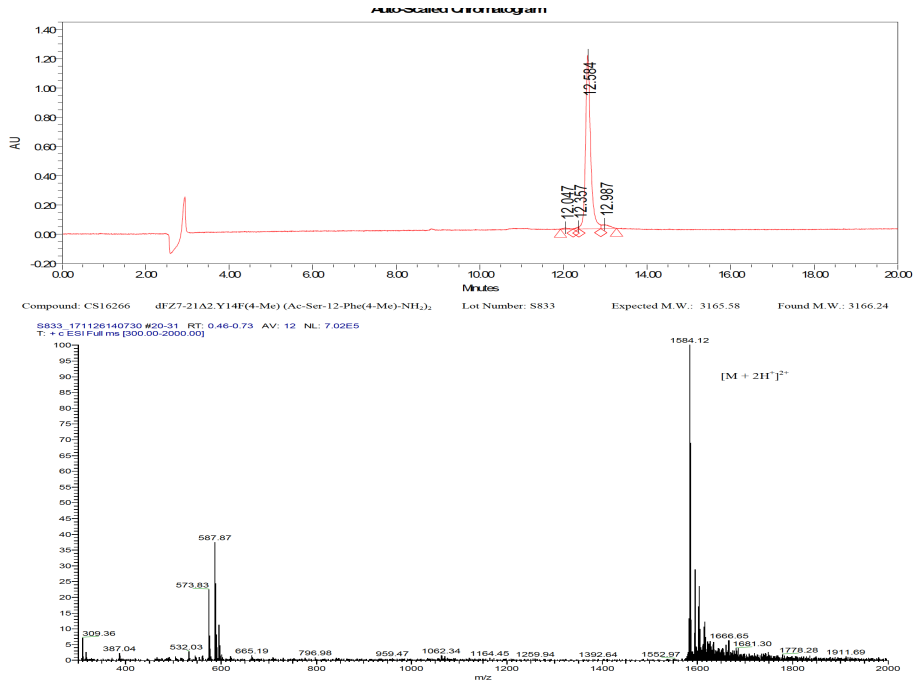


dFz7-21Δ2.Y14F(4-Cl)
purity: 96.40%
Expected MW: 3,206.42 Da
Observed MW: 3,206.22 Da

(Ac-SDDLEFWCHVM**X**-NH_2)$_2$

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**v**) dFz7-21Δ2.V12Cbg, (**w**) dFz7-21 Δ2.V12Cpa or (**x**) dFz7-21Δ2.Y14F(4-Cl).

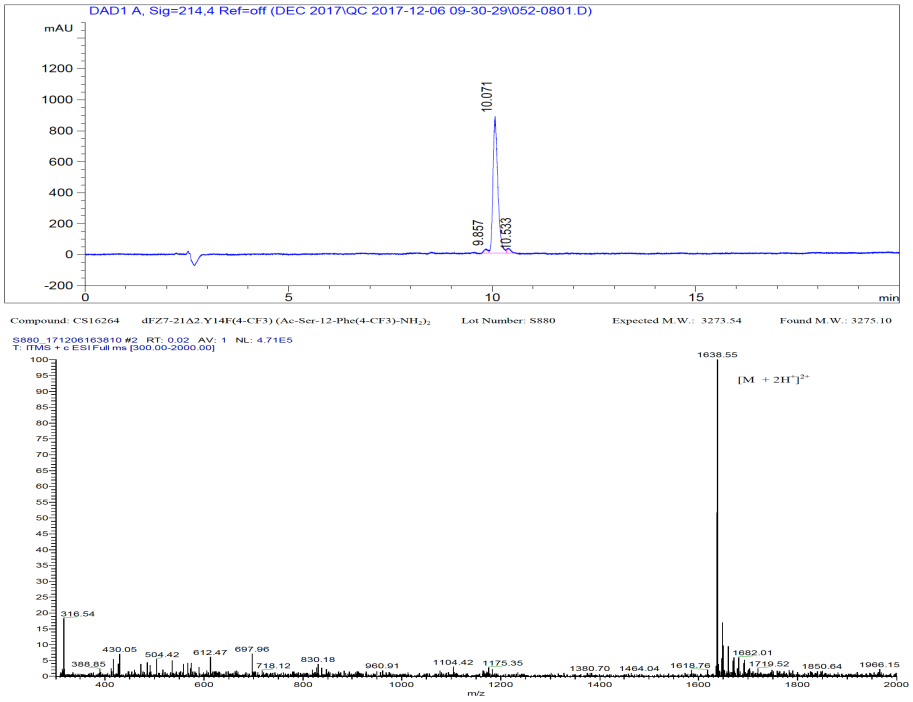
y



dFz7-21Δ2.Y14F(4-Me)
purity: 95.14%
Expected MW: 3,165.58 Da
Observed MW: 3,166.24 Da

(Ac-SDDLEFWCHVM**X**-NH_2)$_2$

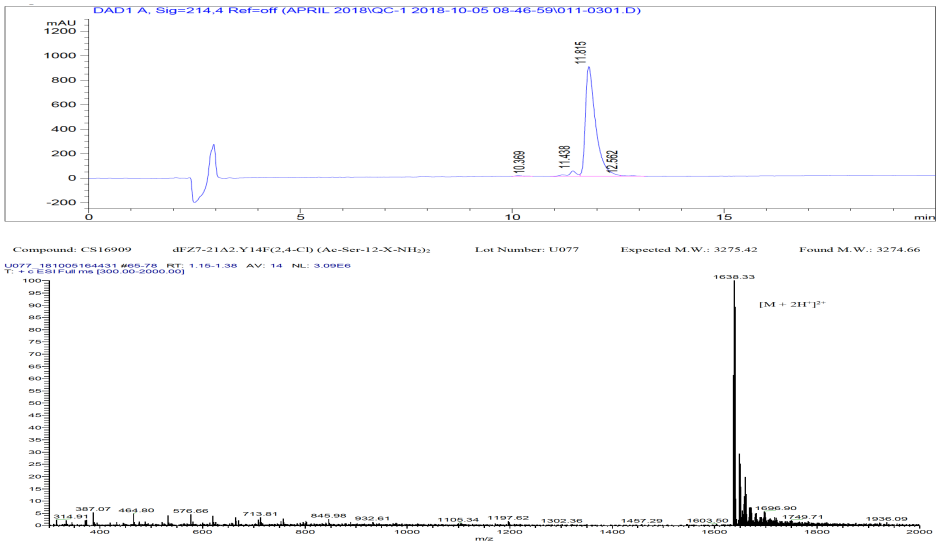
z



dFz7-21Δ2.Y14F(4-CF3)
purity: 95.07%
Expected MW: 3,273.54 Da
Observed MW: 3,275.10 Da

(Ac-SDDLEFWCHVM**X**-NH_2)$_2$

aa

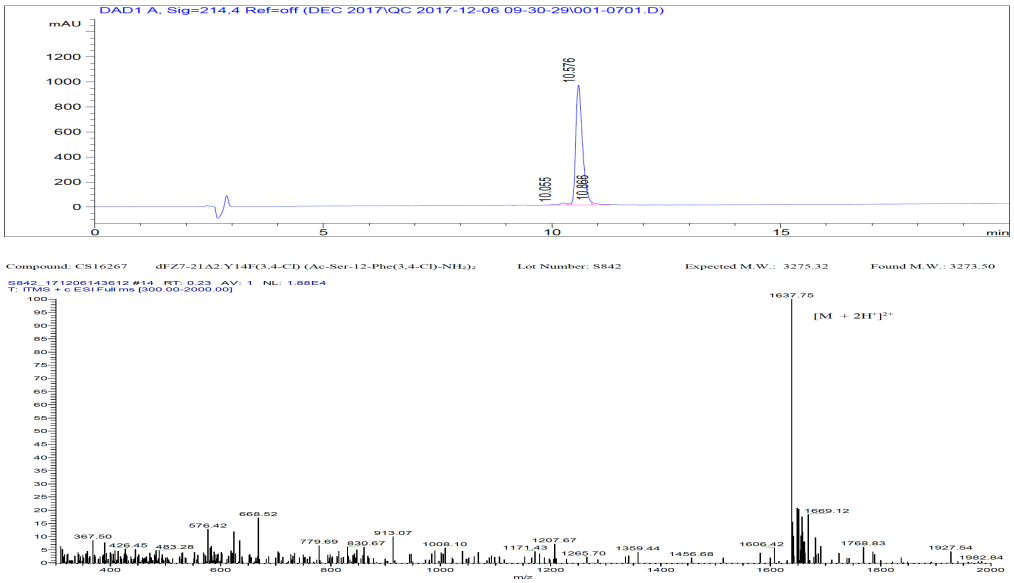


dFz7-21Δ2.Y14F(2,4-Cl)
purity: 95.29%
Expected MW: 3,275.42 Da
Observed MW: 3,274.66 Da

(Ac-SDDLEFWCHVM**X**-NH_2)$_2$

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**y**) dFz7-21Δ2.Y14F(4-Me), (**z**) dFz7-21Δ2.Y14F(4-CF3) or (**aa**) dFz7-21Δ2.Y14F(2,4-Cl).

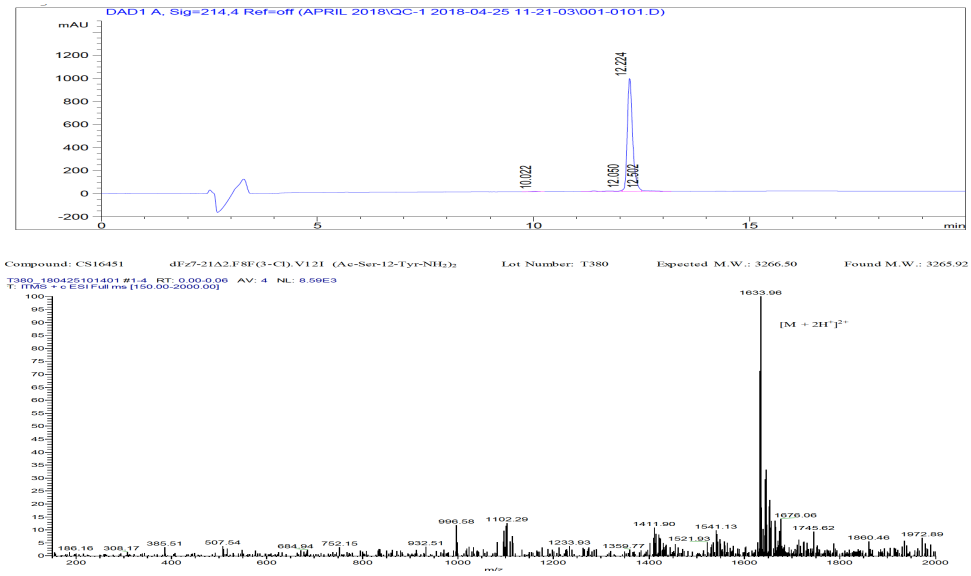
ab



dFz7-21Δ2.Y14F(3,4-Cl)
purity: 95.61%
Expected MW: 3,275.32 Da
Observed MW: 3,273.50 Da

(Ac-SDDLEFWCHVM**X**-NH_2)$_2$

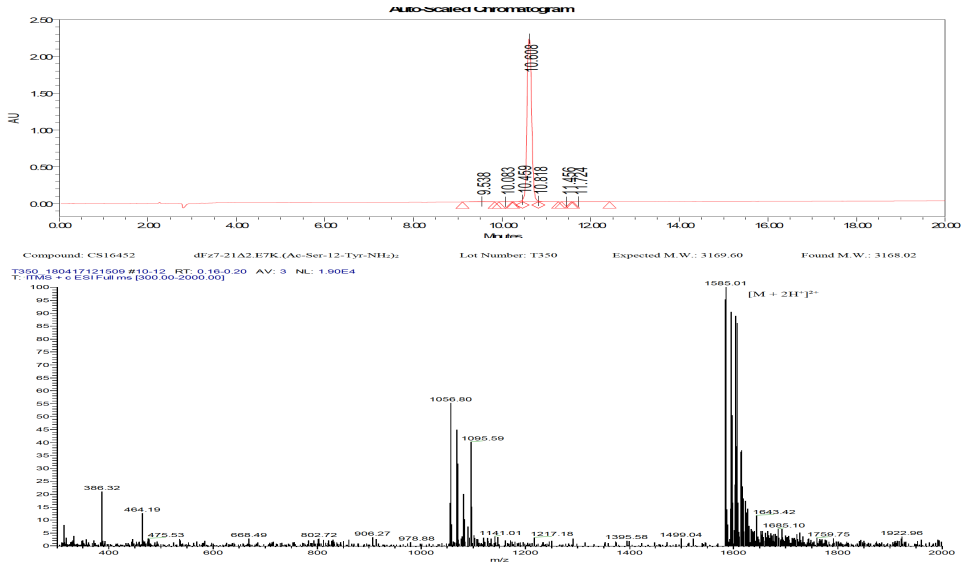
ac



dFz7-21Δ2.F8F(3-Cl).V12I
purity: 95.52%
Expected MW: 3,266.50 Da
Observed MW: 3,265.92 Da

(Ac-SDDLE**X**WCH**I**MY-NH_2)$_2$

ad

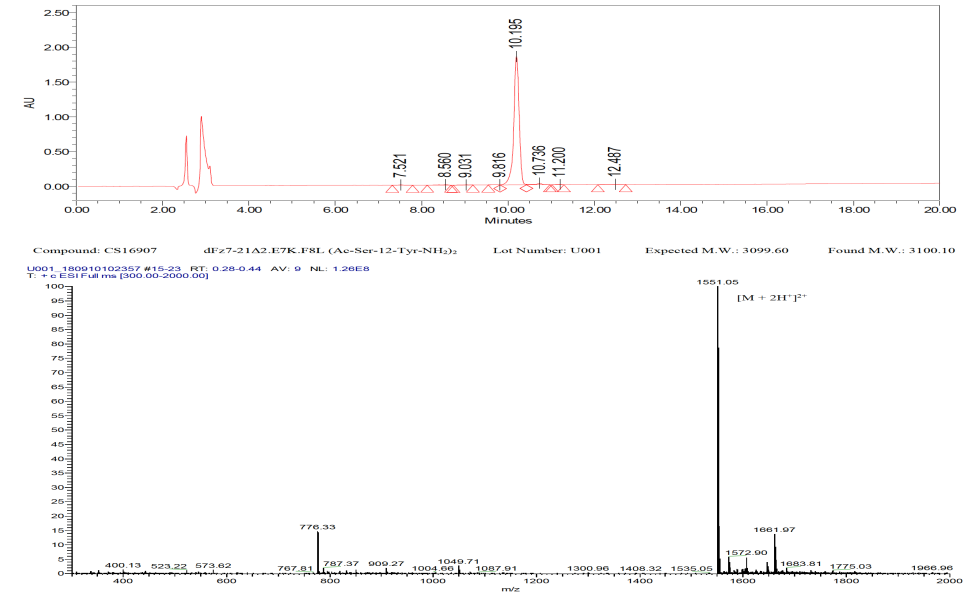


dFz7-21Δ2.E7K
purity: 98.47%
Expected MW: 3,169.60
Observed MW: 3,168.02

(Ac-SDDL**K**FWCHVMY-NH_2)$_2$

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**ab**) dFz7-21Δ2.Y14F(3,4-Cl), (**ac**) dFz7-21Δ2.F8F(3-Cl).V12I or (**ad**) dFz7-21Δ2.E7K.

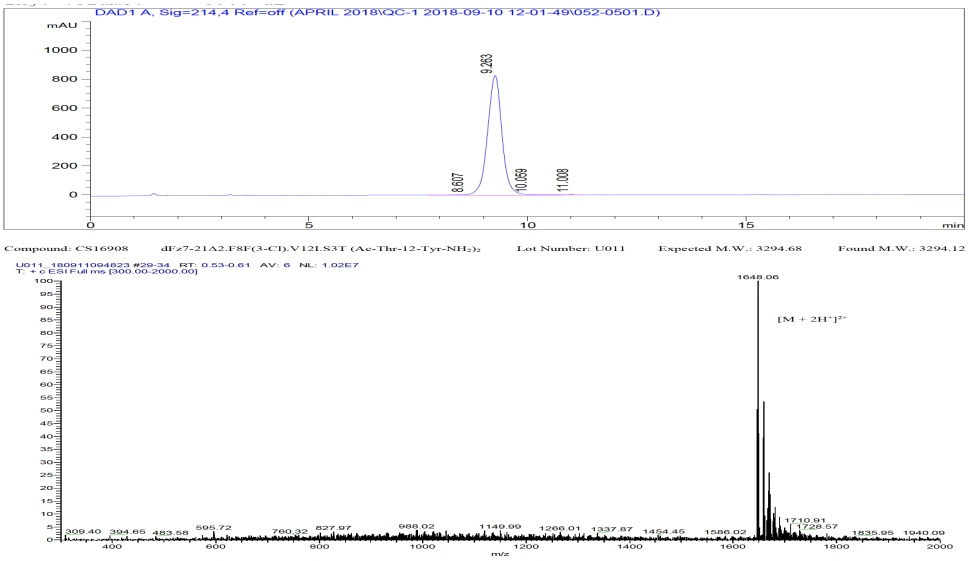
ae



dFz7-21Δ2.E7K.F8L
purity: 97.08%
Expected MW: 3,099.60 Da
Observed MW: 3,100.10 Da

(Ac-SDDL**KL**WCHVMY-NH_2)$_2$

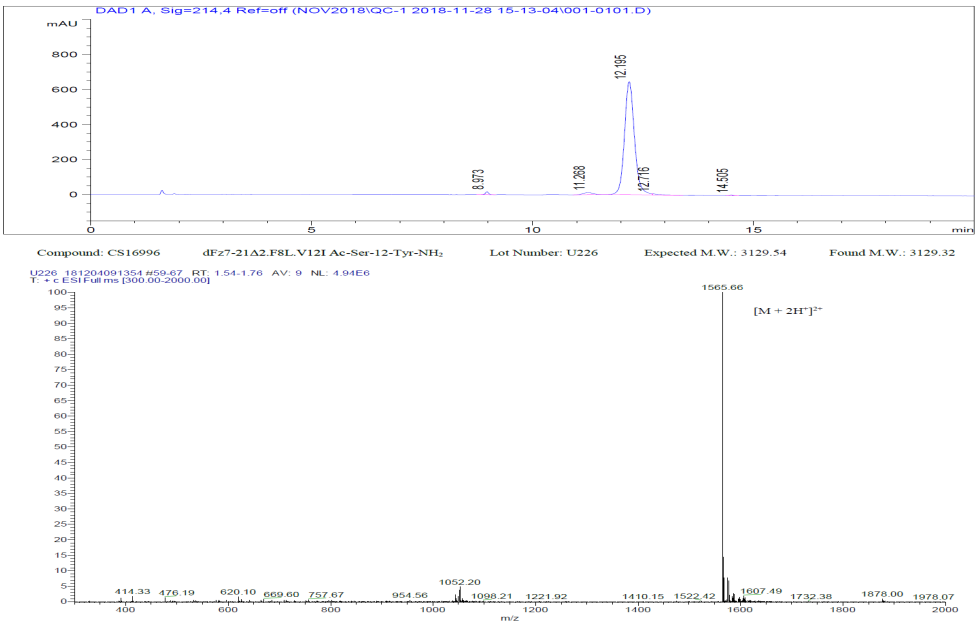
af



dFz7-21Δ2.F8F(3-Cl).V12I.S3T
purity: 99.08%
Expected MW: 3,294.68 Da
Observed MW: 3,294.12 Da

(Ac-**T**DDLE**X**WCH**I**MY-NH_2)$_2$

ag

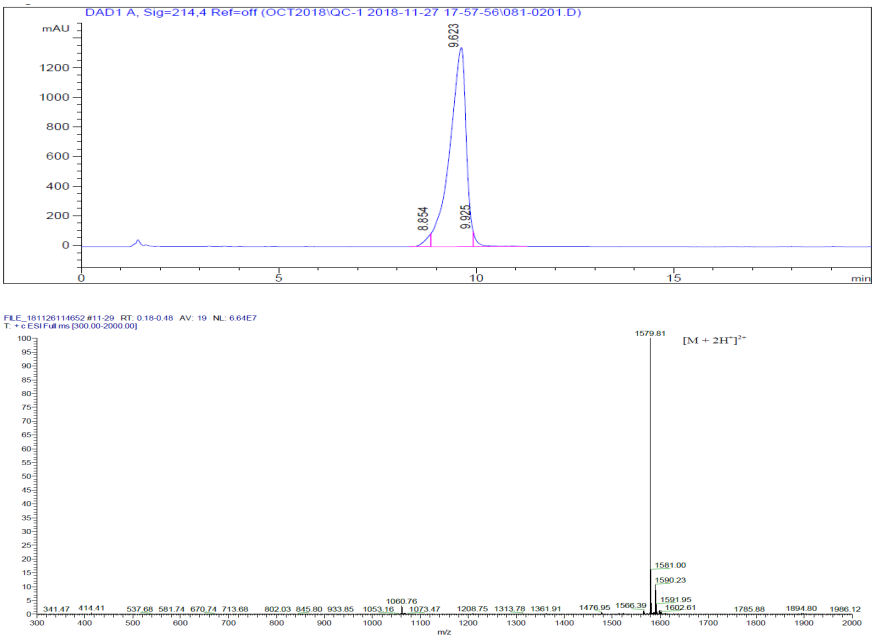


dFz7-21Δ2.F8L.V12I
purity: 96.43%
Expected MW: 3,129.54 Da
Observed MW: 3,129.32 Da

(Ac-SDDLE**L**WCH**I**MY-NH_2)$_2$

Supplementary Figure 1. LC-MS characterization of synthetic peptides (**ae**) dFz7-21Δ2.E7K.F8L (**af**) dFz7-21Δ2.F8F(3-Cl).V12I.S3T and (**ag**) dFz7-21Δ2.F8L.V12I.

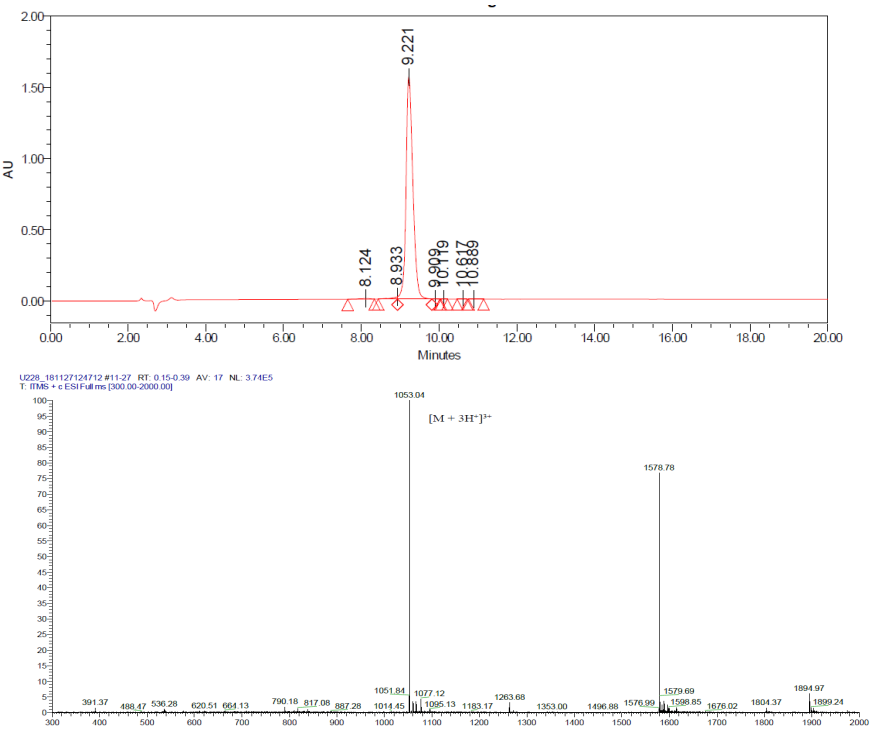
ah



dFz7-21Δ2.F8L.V12I.S3T
purity: 96.19%
Expected MW: 3,157.60 Da
Observed MW: 3,157.62 Da

(Ac-**T**DDLE**L**WCH**I**MY-NH_2)$_2$

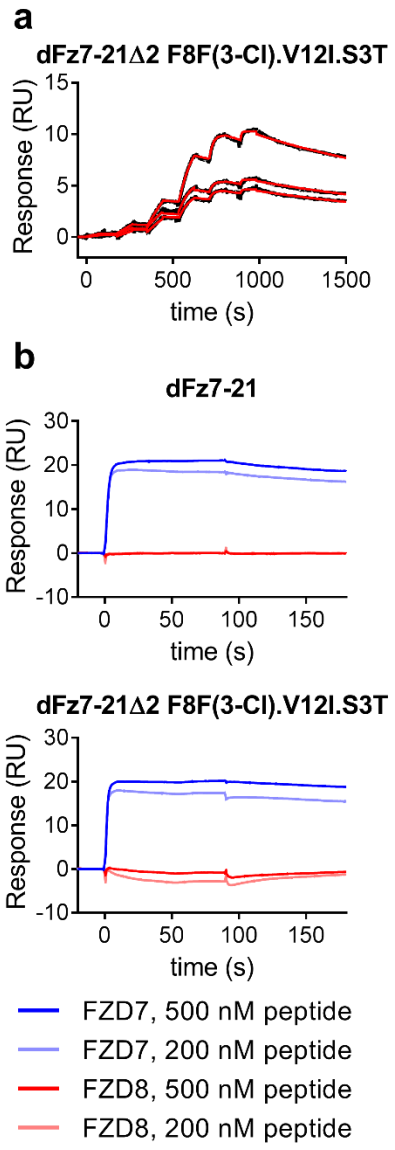
ai



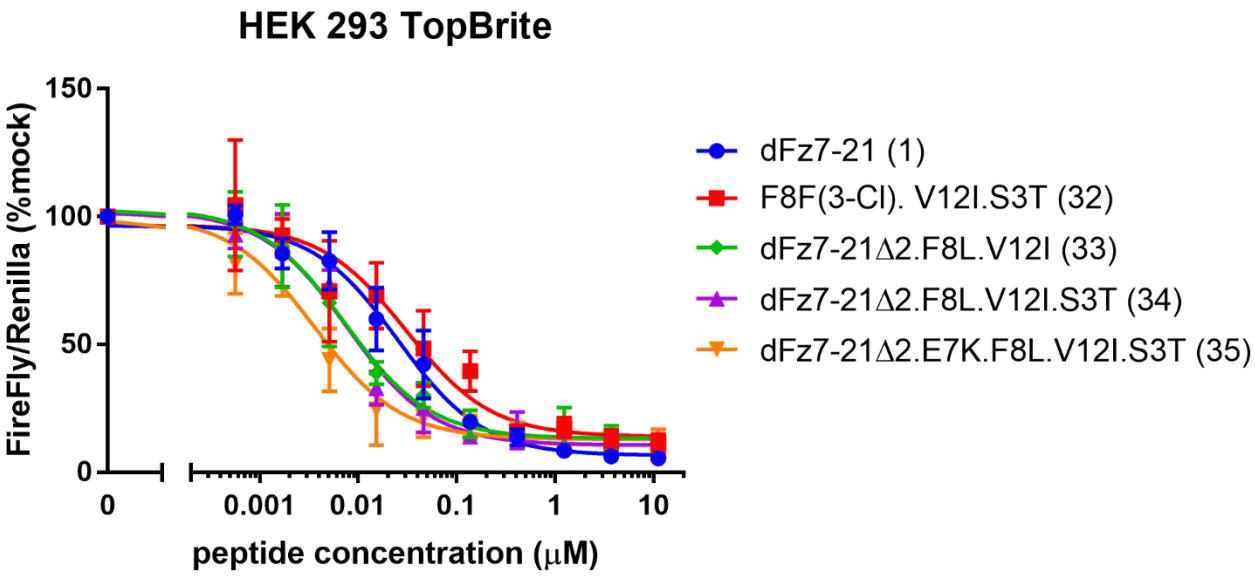
dFz7-21Δ2.E7K.F8L.V12I.S3T
purity: 98.67%
Expected MW: 3,155.72 Da
Observed MW: 3,256.12 Da

(Ac-**T**DDL**KL**WCH**I**MY-NH_2)$_2$

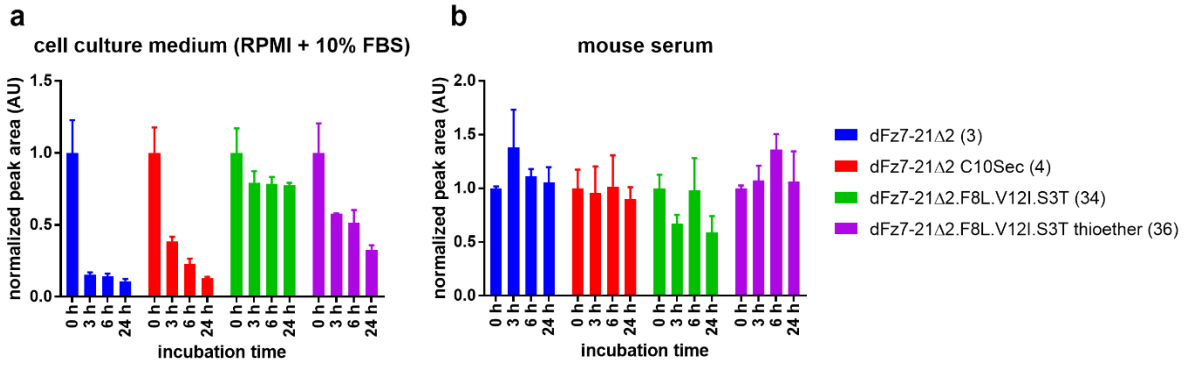
Supplementary Figure 1. LC-MS characterization of synthetic peptides (**ah**) dFz7-21Δ2.F8L.V12L.S3T and (**ai**) dFz7-21Δ2.E7K.F8L.V12I.S3T.



SI Figure 2. Affinity and selectivity of lead peptides assessed by SPR**.** (**a**) Representative sensogram (black) of the tightest rationally designed peptide (**32**) overlaid with its fit to 1:1 binding model (red). (**b**) Representative sensograms of specificity screening for dFz7-21 (**1**) and dFz7-21Δ2.F8F(3-Cl).V12I.S3T (**32**) against two selected receptors, hFZD7 and hFZD8 CRDs as measured by SPR. Results of full specificity screen are summarized in **Table 6**.

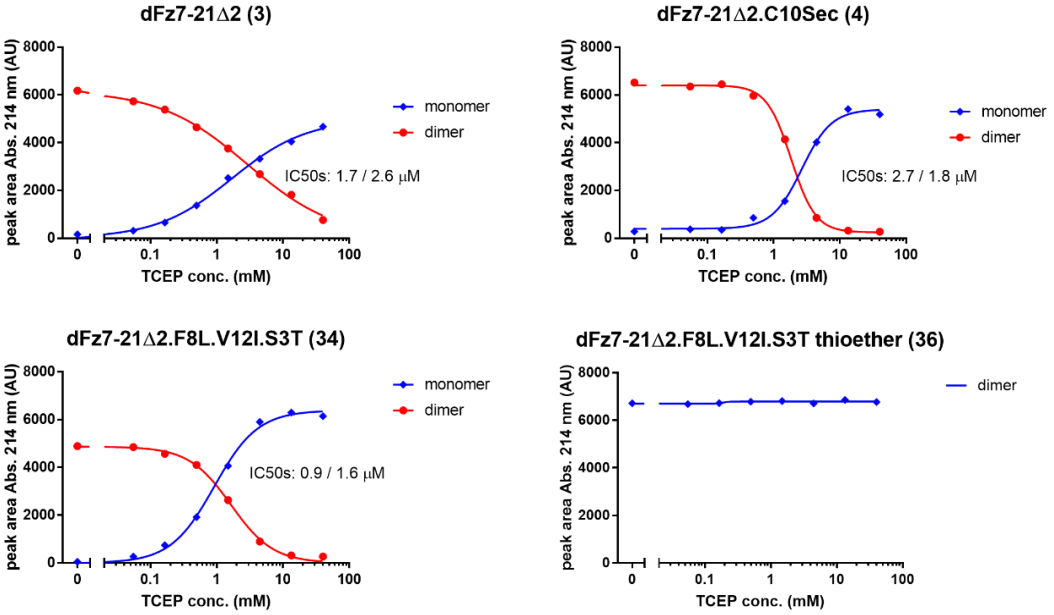


SI Figure 3. Inhibition of Wnt signaling by lead peptides. HEK293 cells stably integrated with a firefly luciferase Wnt reporter were used to determine the inhibition of Wnt signaling by the indicated lead peptides. One representative example is shown out of four independent experiments that were performed, each with technical triplicates. Error bars show standard deviations.

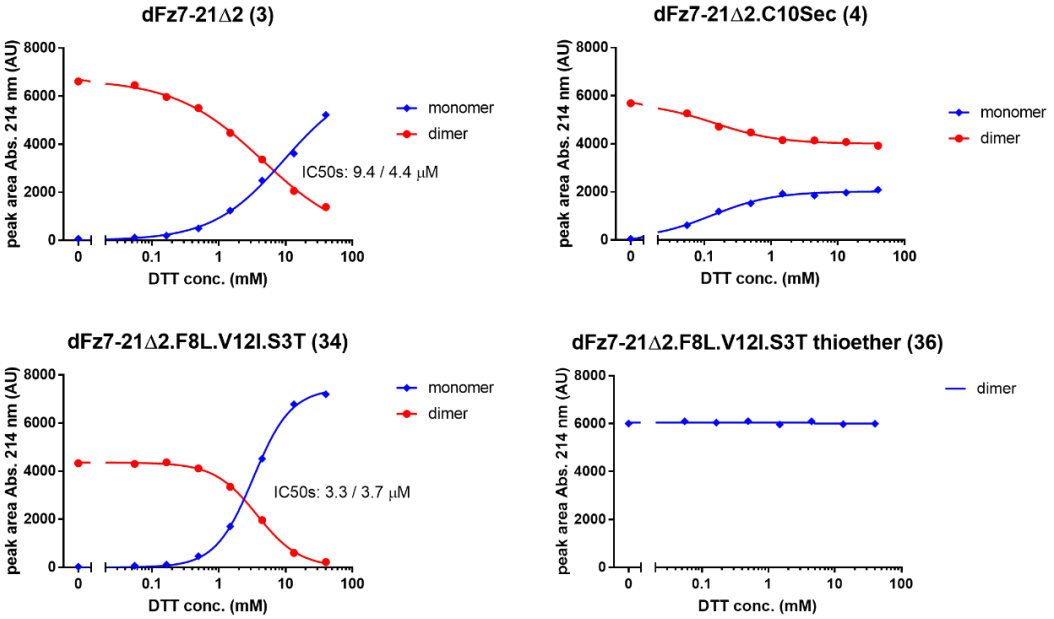


SI Figure 4. Stability of selected peptides in cell culture medium and mouse serum. (**a**) Stability of peptides 3, 4, 34 and 36 in cell culture medium (**b**) Stability of peptides 3, 4, 34 and 36 in mouse serum. All values represent the mean of three independent experiments with standard deviations. Experimental details are described in methods.

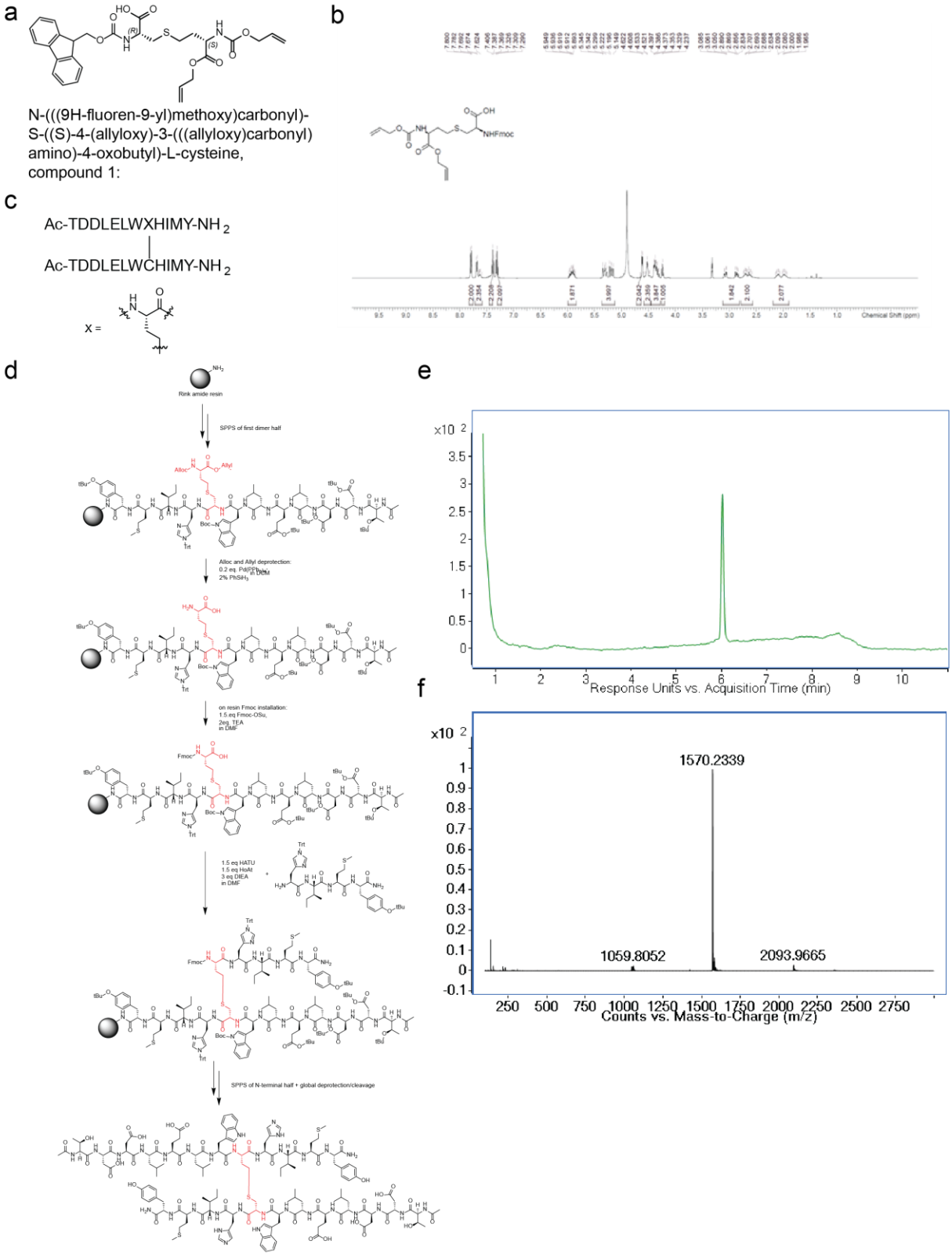
a



b



SI Figure 5. Stability of selected peptides under reducing conditions. (**a**) Stability of peptides 3, 4, 34 and 36 in TCEP. (b) Stability of peptides 3, 4, 34 and 36 in DTT. Peptides were incubated for 40 min at 37 °C in buffer containing reducing agents as described in methods. All measurements were done as two independent experiments. Plots are representative of one experiment.



SI Figure 6: Synthesis and characterization of peptide **36**. (**a**) Structure of the Fmoc, Alloc and Allyl protected cystathionine building block (compound 1). (**b**) NMR spectra of compound 1; ^{1}H

NMR (Methanol-d4, 400 MHz), δ(ppm): 7.79 (d, J = 7.5 Hz, 2H), 7.68 (d, J = 7.5 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.3 Hz, 2H), 5.95-5.88 (m, 2H), 5.35-5.15 (m, 4H), 4.62-4.60 (m, 2H), 4.52-4.50 (m, 2H), 4.40-4.32 (m, 4H), 4.24 (t, J = 6.8, 1H), 3.12-2.83 (m, 2H), 2.75-2.62 (m, 2H), 2.10-1.96 (m, 2H). (**c**) Schematic of peptide **36**. (**d**) Synthesis steps of peptide **36**. (**e**) LC trace of the final peptide product (97% purity) (**f**) HRMS (ESI-TOF-MS) spectrum of peptide **36**. Expected mass for $C_{143}H_{204}N_{32}O_{42}S_3$ $(M + 2H)^{2+}$: 1570.2076 Da, observed mass: 1570.2339 Da.