Efficient Synthesis of Longer Aβ Peptides via Removable Backbone Modification

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Supplementary Information

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1. Peptide Synthesis

C S Bio Co. automated synthesizer. Use a CS136XT synthesizer running with a scale of 0.1 mmol. A general protocol as follow: 2×DMF, 30 s; 1×20% (vol/vol) piperidine in DMF, 5 min; 1×20% (vol/vol) piperidine in DMF, 20min; 2×DMF, 30s; 2×DCM, 30s; 3×DMF, 30s; amino acids (AA) activation by HBTU or HCTU in the presence of DIEA in DMF for ~0.5–1 min; Add AA solution to resin, couple 0.5–1 h; 2×DMF, 30 s; 1×DCM, 30 s.

Manual peptide-synthesis apparatus. Peptide synthesis vessels were purchased from Synthware Glass Co., Ltd.. DIEA, Oxyma, HCTU, HATU, HOAt, DIC, Fmoc-amino acids, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(^{t}Bu)-OH, Boc-Ala-OH, Boc-Met-OH, Boc-Cys(Trt)-OH, 2-Cl-(Trt)-Cl resin was from GL Biochem (Shanghai) Ltd. ChemMatrix Wang Resin was employed for the synthesis of peptide acid. Choose the proper coupling conditions for each amino acid, including the number of coupling reactions (single or double coupling), reaction time (0.5-1.5 h) and the type of coupling reagents. A single coupling reaction using HBTU or HCTU for 30-45 min is enough for most amino acids. However, a double coupling strategy is needed for amino acids located at hydrophobic C-terminal sequence of Aβ peptides or sterically hindered amino acids (for example, Fmoc-Thr(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Val-OH). It is necessary to perform a double coupling step for amino acids after Gly^{RBM} or Ala^{RBM} residue using DIC/Oxyma protocols. Piperidine (20% in DMF) was added to the resin for 20-30 min (twice) to remove the Fmoc protecting group. Note that the last Fmoc deprotection step should be treated by piperidine for 1h (15min+45 min) to completely aminolysis of the phenolic ester which was formed by esterification of Fmoc-Arg-OH and 2-OH. The deprotection of Side chain protecting groups and final cleavage from the resin were achieved by TFA cocktails for 3-4 h at room temperature. The cleavage cocktails (TFA/thioanisole/EDT/phenol/H_2O,

85/5/5/2.5/2.5) or (TFA/TIPS/H_2O, 95/2.5/2.5) can be used for the final cleavage. Finally, TFA was concentrated by N_2 blowing. The crude peptides were obtained by precipitation with cold ether and centrifugation. The crude peptides were dissolved in CH_3CN/H_2O and purified by semi-RP HPLC. Peptides were identified by ESI-MS.

2. Reversed-Phase HPLC (RP-HPLC)

The Vydac C4 or C18 column (5 μm, 4.6 mm×150 mm, 4.6 mm×250 mm) with a 1 mL/min flow rate was used for analytical RP HPLC, and the Vydac C4 or C18 column (10 μm, 10×250 mm or 22 mm×150 mm) with a 3-6mL/min flow rate was used for semi-preparative RP HPLC. Analytical and semi-preparative RP HPLC with a Prominence LC-20AT with SPD-20A UV/Vis detector were used. Two kinds of buffers for RP HPLC are buffer A (0.1% TFA in water) and buffer B (0.1% TFA in CH_3CN). Gradients for all peptides were detailed below. The temperature for all Aβ peptides was at 60 °C.

3. Mass Spectrometry (MS)

Products were identified by electrospray ionization mass spectrometry (ESI-MS). ESI-MS was measured on an Agilent 1200/6340 mass spectrometer in Center of Biomedical Analysis. The buffers for MS analysis were 50% CH_3CN/H_2O (v/v) containing 0.1% formic acid. MALDI-TOF mass spectra were measured on an Applied Biosystems 4700 Proteomics Analyzer 283.

4. Tricine-SDS-PAGE

The protocol for Tricine/Tris SDS–PAGE described previously (Klafki et al., 1996) has been modified here.

16% gel separation gel

AB-6 (1.67 ml) + Glycerol (0.50 g) + Gel buffer (1.67 ml) + 10% Ammonium persulfate (0.0167 μL) + TEMED (0.002 mL) + ddH2O

10% spacer gel

AB-3 (0.2 ml) + Glycerol (0.10 g) + Gel buffer (0.34 ml) + 10% Ammonium persulfate (0.005 μL) + TEMED (0.001 mL) + ddH2O

4% stacking gel

AB-3 (0.167 ml) + Gel buffer (0.5 ml) + 10% Ammonium persulfate (0.015 μL) + TEMED (0.002 mL) + ddH2O

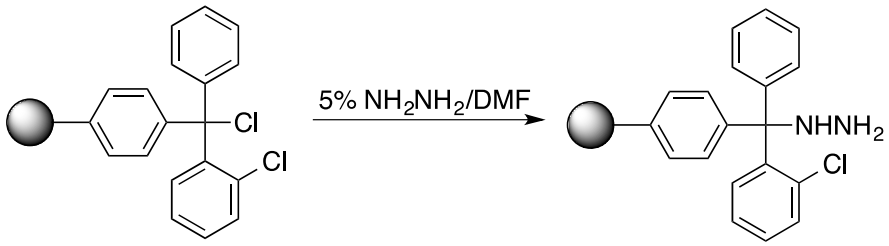
The dimensions of this slab gel were as follows: length of separation gel, 54 mm; length of spacer gel, 5 mm; length of stacking gel, 14 mm; width, 83 mm; thickness, 0.1 mm. (T indicates the total percentage concentration of acrylamide and bisacrylamide; C denotes the concentration of the crosslinker [bis] relative water to the total concentration).

For tricine SDS–PAGE, samples were made up in 50 mM Tris/HCl, pH 6.8; 4% (w/v) SDS; 12% (w/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.01% Coomassie blue G250, and heated to 95 ^{o}C for 5 min. Samples were loaded onto 4% SDS-PAGE gels and electrophoresed for 30 min at 100V and 150 min at 200 V. All the protein samples were prepared in 10% SDS buffer containing bromophenol blue.

5. Circular dichroism (CD) spectroscopy

CD spectra were recorded on a Pistar π-180 spectrometer from 250 nm to 200 nm at 37 °C in a quartz cell with 1 mm path length. The final concentration of samples was about 10 μM. The spectra for each peptide was performed in triplicate, averaged, subtracted from blank and smoothed.

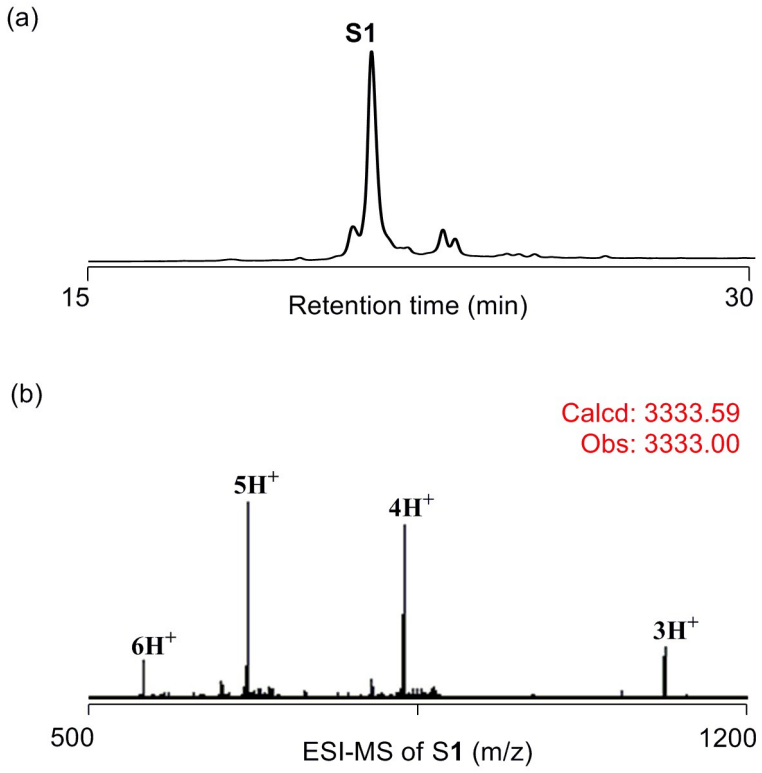
6. Chemical Synthesis of Aβ (Asp^{1}-Gly^{29})-MESNa



Scheme S1. The preparation of hydrazine resin.

The 2-Cl-(Trt)-Cl resin was used to prepare Aβ (Asp^{1}-Gly^{29})-NHNH2 **S1** by automated Fmoc SPPS. 2-Cl-(Trt)-Cl resin (0.2 mmol) was treated with 5% NH_2NH_2 in N,N-Dimethyformamide (DMF, 30min for twice) to prepare 2-Cl-(Trt)- $NHNH_2$

resin (**Scheme S1**). This resin was next used to prepare the corresponding peptide hydrazides **S1** for generating peptide thioesters (172 mg, 43% of isolated yield). Peptide hydrazides were dissolved using 0.2 M phosphate solution containing 6 M Gn·HCl (pH 3.0-3.1) and cooled to -15 °C. 0.5 M $NaNO_2$ (10-15 equiv) were added for 15 min at -15 °C to oxidize the peptide hydrazide to the corresponding azide. Subsequently, the phosphate solution containing 2-mercaptoethane sulfonate (MESNa, ~100 equiv) was added for the preparation of peptide-thioester equivalent Aβ (Asp^{1}-Lys^{28})-Gly-SR (R=$CH_2CH_2SO_3H$) **1** at 30 °C (pH 5.0-6.0) (130 mg, 73% of isolated yield). The reaction was traced by analytical RP HPLC and ESI-MS (**Figure S1**).



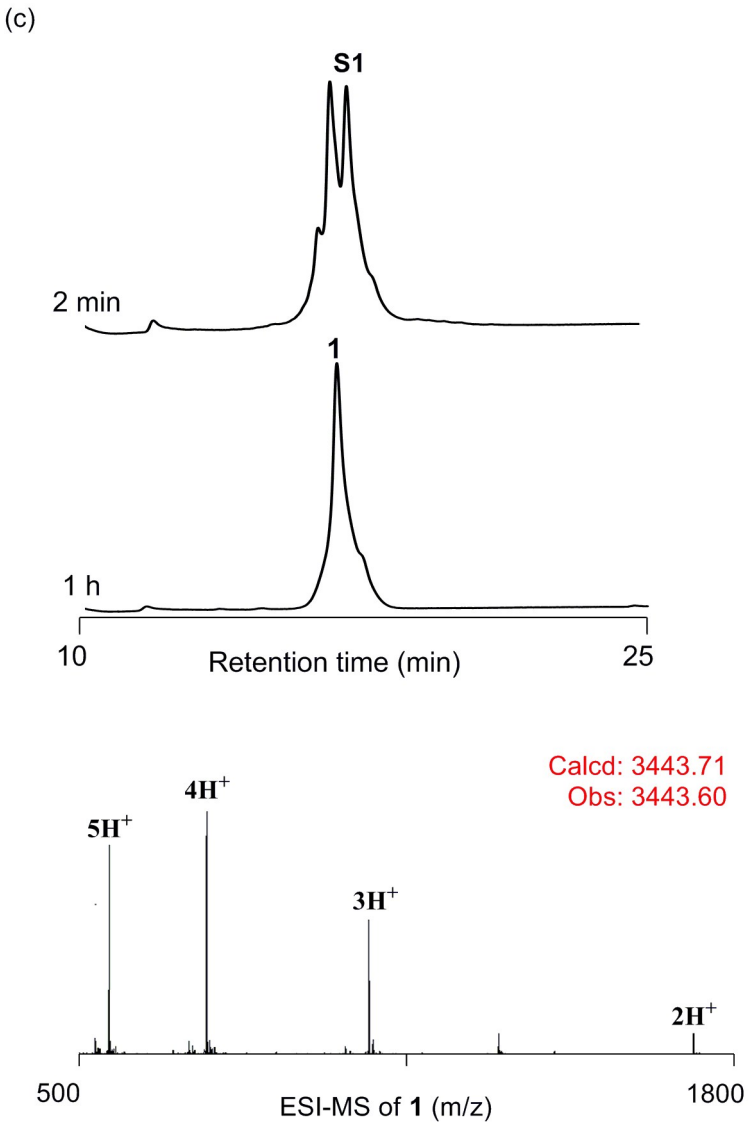


Figure S1. Analytical RP-HPLC and Mass spectra characterization of peptide **S1** and **1**. (a) Traces of the crude peptide **S1** by analytical RP HPLC using a gradient of 20% buffer B in A to 50% B in A over 30 min on a C4-column (4.6 mm×150 mm) and (b) its ESI-MS characterization. (c) Traces of the conversion of hydrazides groups of crude peptide **S1** (t= 2 min and 1 h) by analytical RP-HPLC using a gradient of 15% buffer B in A to 45% B in A over 30 min on a C4-column (4.6 mm×150 mm) and its ESI-MS characterization.

7. Chemical Synthesis of Gly ^{RBM}-Containing peptide 4

Chemical Synthesis of peptide 3. We next synthesized the peptide **3** using ChemMatrix Wang resin. The ChemMatrix Wang resin (400 mg, 0.2 mmol) was

swollen with CH2Cl2/DMF (1/1, v/v) for 15 min. The resin was washed (3×DMF, 3×CH_2Cl_2, 3×DMF), and followed by substitution reaction with Fmoc-Ala-OH (622 mg, 2.0 mmol), DIC (320 mg, 2.0 mmol) and Oxyma (285 mg, 2.0 mmol) (2×4 h). A standard Fmoc SPPS procedure was performed for the next five residues. Then, the 4-methoxy-5-nitrosalicylaldehyde **2** was quantitatively installed onto the α-NH_2 of the Gly via on-resin reductive amination protocol. In the first imine formation step, the salicylaldehyde derivative **2** was dissolved in DMF and added into peptide-resin (1.0 eq., double, 25 min for each imidization) to give an intense yellow coloration. The reductive amination step was achieved by using $NaBH_4$ in DMF (5.0 eq., double, 5-10 min for each reduction). The resin was then drained and washed successively with DMF, H_2O, MeOH, DCM, and DMF for the following amino acid coupling. The coupling of the following amino acid (Fmoc-Val-OH) to the relatively inert secondary amino group was accomplished through an O-to-N intramolecular acyl migration. The coupling of Val following the $Gly^{37,\ RBM}$ was achieved by a double coupling step using Fmoc-Val-OH (679 mg, 2.0 mmol), DIC (320 mg, 2.0 mmol) and Oxyma (285 mg, 2.0 mmol) with 2-3 h each and a following Ac_2O capping step with Ac_2O/DIEA/DMF (1/1/8, v/v/v). Subsequent six amino acids still followed the standard Fmoc-SPPS. After completing peptides assembly, a solution of 6 M $SnCl_2$ and 10 mM HCl/methanol in DMF (4 mL) was added into the resin to convert the nitro group to an amine group. The conversion step was repeated twice for 120 min each time. The Gly-Arg_4-tag was coupled by standard Fmoc-SPPS protocols. It should be noted that the phenol OH of the compound **2** must be acetylated with cetic anhydride to avoid the removal of Arg_4-tagged RBM in the trifluoroacetic acid (TFA) condition. A capping step with Ac_2O/DIEA/DMF (1/1/8, v/v/v) was performed after the Gly-Arg_4-tag coupling step. Finally, the Aβ (Cys^{30}-Ala^{42})-OH **3** was cleaved from the resin with TFA/PhOH/TIPS/H_2O cocktails and isolated by analytical RP-HPLC to obtain the target peptide **3** (108 mg, 25% of isolated yield) which was identified by ESI-MS.

Deacetylation of Peptide 3. To release the phenol group, the purified peptide **3** (1 mM) was dissolved in the neutral aqueous buffer (0.4 M Cys, 6M Gn·HCl, 0.2 M

phosphate salt, pH 7.2). The analytical RP-HPLC traces and MSI-MS spectrum revealed that the peptide **3** was quantitatively converted into **4** within 30 min at 30 °C (64 mg, 60% isolated yield).

8. Total Chemical Synthesis of Aβ42

Native chemical ligation. With peptide **1** and peptide **4** in hand, we carried out a ligation reaction between two peptide segments under standard condition. The native chemical ligation of peptide **1** (12.40 mg, 3.6 μmol) and peptide **4** (6.36 mg, 3.0 μmol) preformed smoothly in the neutral aqueous buffer (6M Gn·HCl, 0.2 M phosphate salt, 50 mM TCEP, 50 mM MPAA, pH 7.0) to yield the full-length peptide Aβ42 (Cys^{30}, $Gly^{37, RBM}$) **5** (10.9 mg) in 67% yield by RP HPLC purification.

Desulfurization: Peptide **5** (10.9 mg, 2 μmol) was dissolved in 1.0 mL solution of 6.0 M Gn·HCl, 0.2 M Na_2HPO_4 (pH 7.3) followed by mixing with 1.0 mL of neutral aqueous buffer (6M Gn·HCl, 0.2 M phosphate salt, 300 mM TCEP, pH 3.8). Then *t*BuSH (20 μmol) and VA-044 (v/v, 17%~20%) were added. The reaction mixture was adjusted to pH 6.6 and then stirred on amagnetic stirrer at 37 °C. Under this reaction condition, peptide **5** was mainly converted to Aβ42 ($Gly^{37, RBM}$) **6** within 15 h. After isolation, peptide **6** was obtained, giving 6.0 mg of product with a yield of 55%.

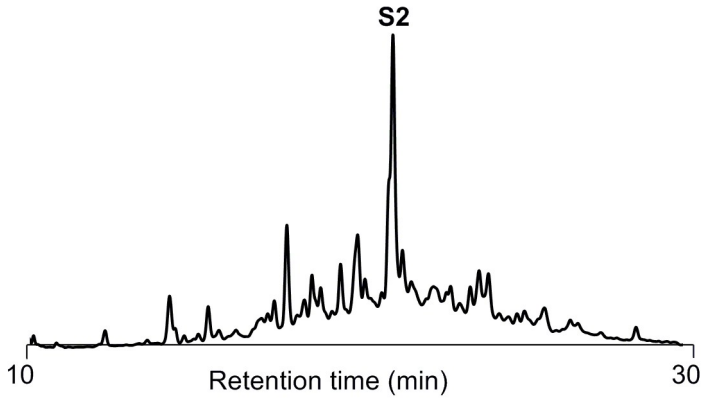
Removal of Gly-Arg_4-tag: Peptide **6** was treated with TFA/Thioanisole/DTT/PhOH/H2O (87.5/2.5/2.5/2.5/5, v/v/w/w/v) to remove the backbone modification group in 5 h at 30 °C. After the cleavage, the reaction mixture was concentrated by N_2 blowing and precipitated with Et_2O to yield the final crude product. The crude product can be washed and then precipitated with water to remove the water-soluble Gly-Arg_4-agged form the Aβ peptide. The final wild-type Aβ42 **7** was obtained through a further RP HPLC separation and characterized by ESI-MS.

9. Total Chemical Synthesis of Aβ48

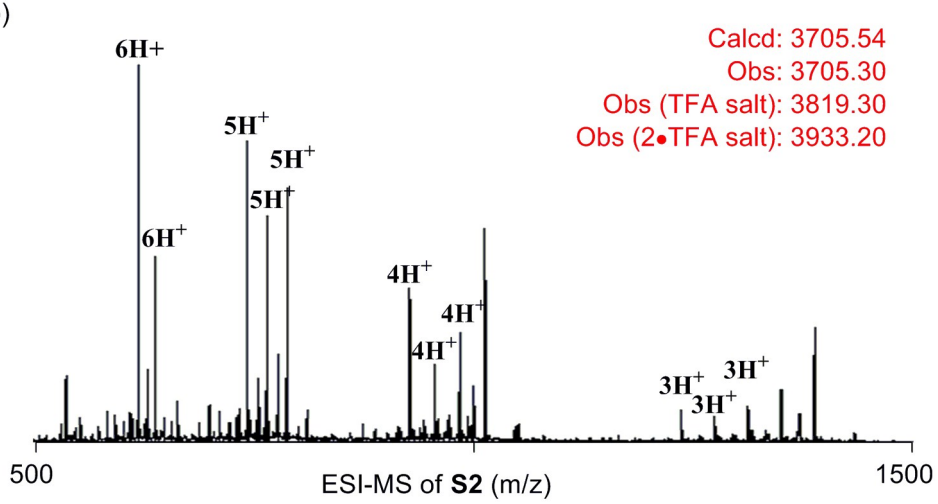
These successful results encouraged us to test the Arg_4-tagged RBM method for the synthesis of the longer Aβ species, Aβ48. Similarly, we divided the Aβ48 into two segments and Ala^{30} was selected as the ligation site. Initially, we examined the

synthesis of Aβ48 by introducing a single Arg_4-tagged RBM at the same site Gly^{37}. Unfortunately, we could not acquire the target product due to the addition of C-teminal strongly hydrophobic sequence lead to incomplete acylation and deprotection in the process of SPPS. To overcome the problem, we decided to incorporate two Arg_4-tagged RBMs into the Aβ (Cys^{30}-Thr^{48})-OH at Gly^{37} and Ala^{42}, respectively. As expected, the synthesis of Aβ (Cys^{30}-Thr^{48})-OH ($Gly^{37,\ RBM\text{-}Ac}$, $Ala^{42,\ RBM\text{-}Ac}$) **S2** with two backbone modification groups could be successfully achieved using a similar method for the peptide **3**. The deacetylation of peptide **S2** was performed at 0.4 M Cys aqueous solution (pH 7.2). The two acetyl groups could also be completely removed to generate desired Aβ (Cys^{30}-Thr^{48})-OH ($Gly^{37,\ RBM}$, $Ala^{42,\ RBM}$) **8** within 30 min (21% isolated yield). The processes were monitored by analytical RP HPLC and ESI-MS (**Figure S2**). NCL between peptide **1** and peptide **8** to generate Aβ48 (Cys^{30}, $Gly^{37,\ RBM}$, $Ala^{42,\ RBM}$) **9**, followed by desulfurization yielded Aβ48 ($Gly^{37,\ RBM}$, $Ala^{42,\ RBM}$) **10**. The sharp and symmetric peaks of the analytic RP-HPLC traces of target peptide **9** and peptide **10** indicated the Arg_4-tagged RBM indeed provided benefits for the total synthetic yield and purity of longer Aβ species. Finally, the cleavage reaction of two backbone modification groups of peptide **10** was performed under TFA/Thioanisole/DTT/PhOH/H2O (87.5/2.5/2.5/2.5/5, v/v/w/w/v) conditions. Unexpectedly, the peptide **11** was refractory to elute with standard RP-HPLC butter and be characterized by ESI-MS (no ionization). An explanation for the phenomenon is that the hydrophobicity and aggregation of the peptide **11** significantly reduce the handing properties. To characterize the synthetic peptide **11**, we performed MALDI-TOF after dissolving **11** in 100% hexafluoroisopropanol (HFIP) for 24 h to disaggregate it completely. In the MALDI-TOF mass spectrum, the major peak was assigned to peptide **11** but a peak corresponding to an intermediate, peptide **11** with a single Arg_4-tagged RBM, still appeared. A plausible explanation for this result was that the ionization of the intermediate was better than that of peptide **11**, owing to the presence of Arg_4-tag. This data indicated that the two backbone modification groups of peptide peptide **9** were almost cleaved within 5 h under TFA/Thioanisole/DTT/PhOH/H2O (87.5/2.5/2.5/2.5/5, v/v/w/w/v) conditions.

(a)


S2
10
Retention time (min)
30

(b)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5H+
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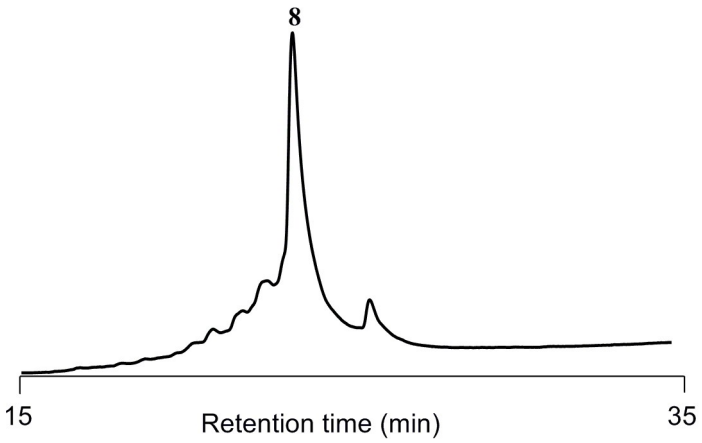
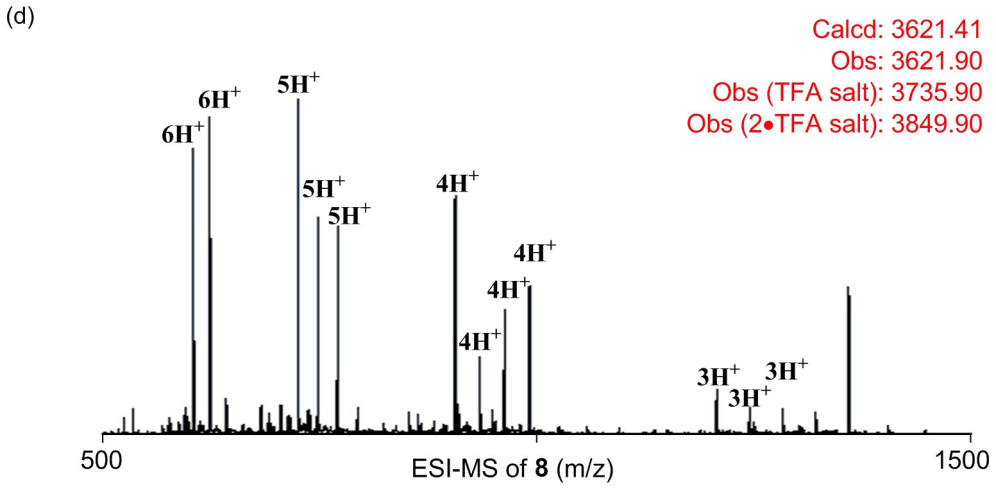
4H+
4H+
3H+
3H+
3H+
500
ESI-MS of S2 (m/z)
1500

(c)


8
15
Retention time (min)
35

(d)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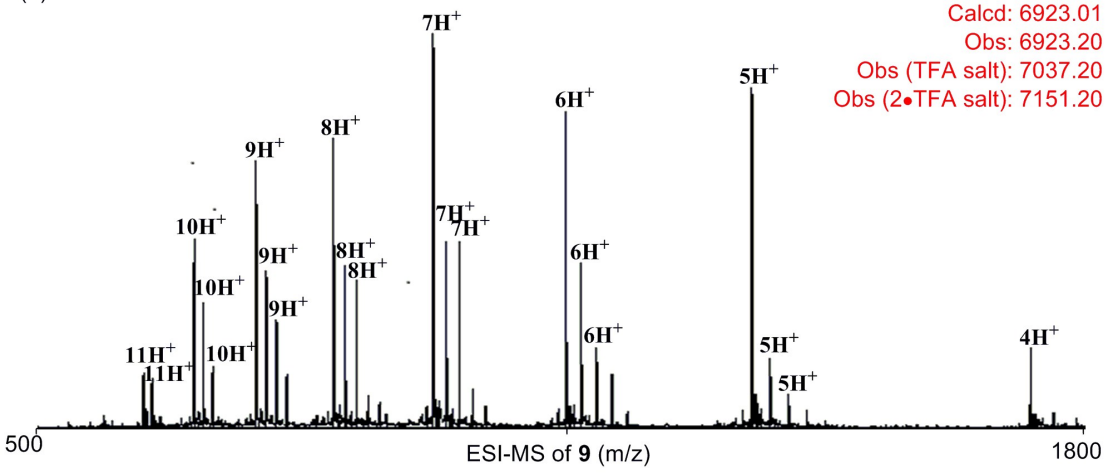
5H+
5H+
4H+
4H+
4H+
4H+
3H+
3H+
3H+
500
ESI-MS of 8 (m/z)
1500

(e)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10H+
7H+
7H+
8H+
8H+
9H+
6H+
10H+
9H+
6H+
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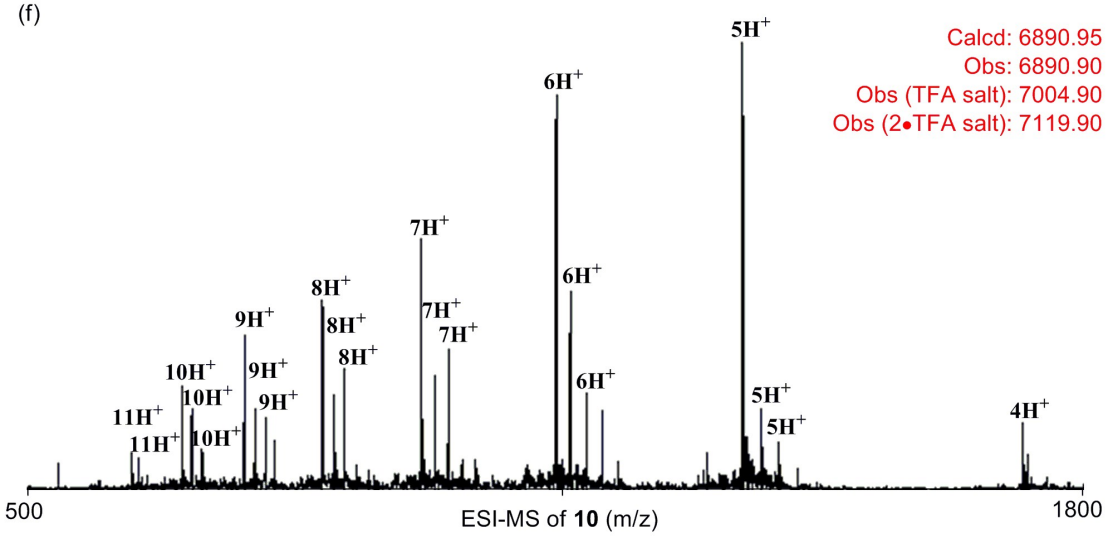
5H+
500
ESI-MS of 9 (m/z)
1800

(f)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7H+
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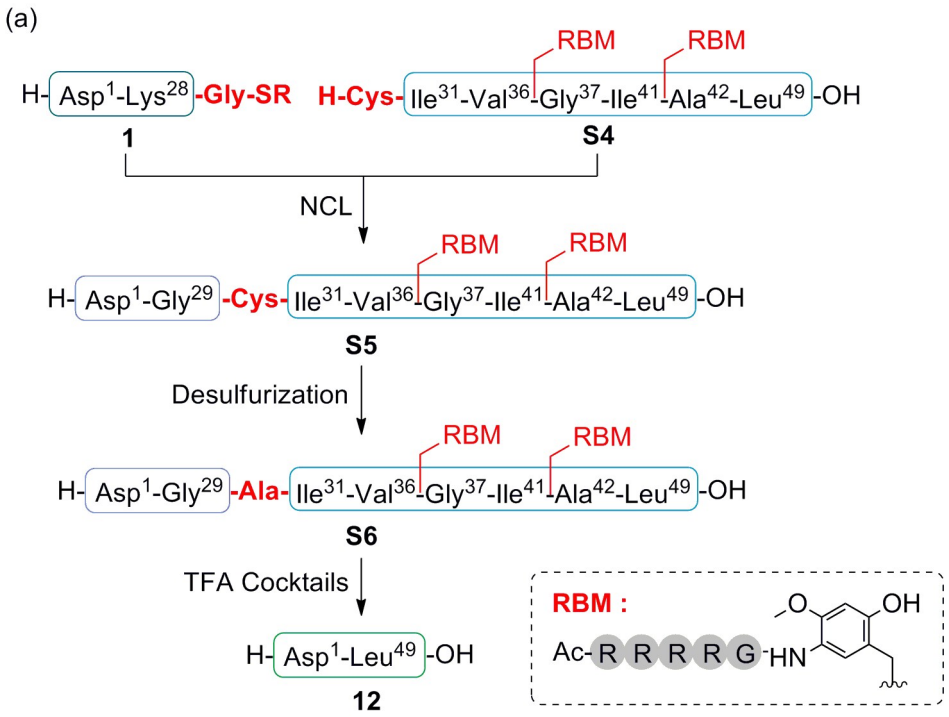
8H+
9H+
8H+
7H+
7H+
8H+
10H+
9H+
6H+
10H+
9H+
5H+
11H+
10H+
9H+
5H+
4H+
11H+
10H+
500
ESI-MS of 10 (m/z)
1800

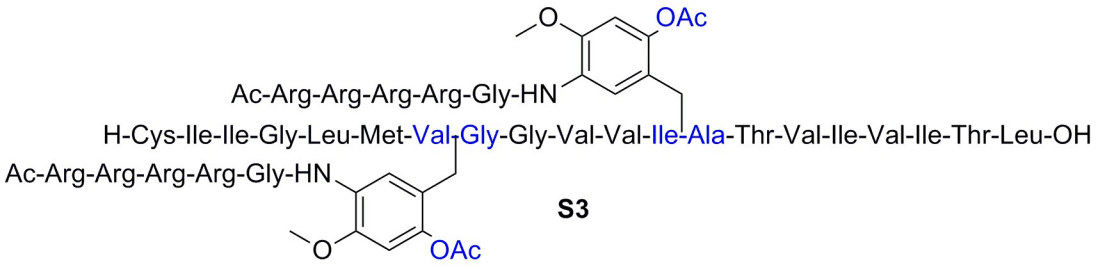
Figure S2. Characterization of peptide **9** and **10** by analytical RP-HPLC and Mass spectra. (a) Traces of crude peptide **S2** by analytical RP HPLC using a gradient of 15% buffer B in A to 55% B in A over 30 min on a C18-column (4.6 mm×250 mm) and (b) its ESI-MS characterization. (c) Traces of the deacetylation of purified peptide **S2** by analytical RP-HPLC using a gradient of 15% buffer B in A to 45% B in A over 30 min on a C4-column (4.6 mm×150 mm) and (d) its ESI-MS characterization. The ESI-MS characterizations of (e) peptide **9** and (f) peptide **10**.

10. Total Chemical Synthesis of Aβ49

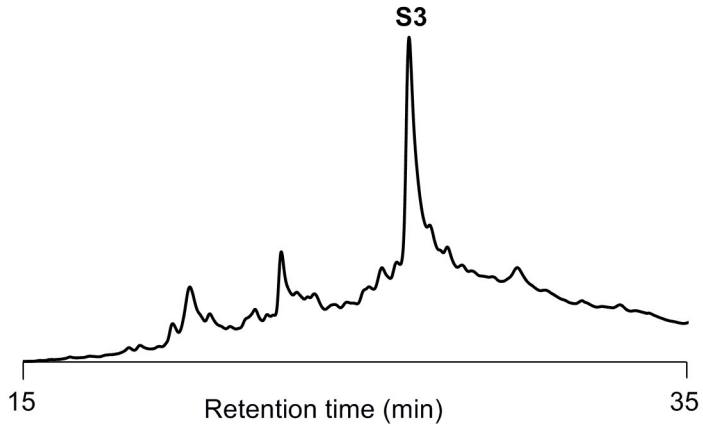
Furthermore, the synthesis of **Aβ49 12** can be successfully achieved using a similar method for the peptide **11** except for adding one Leu at the C-terminal. We incorporated two Arg_4-tagged RBMs into the Aβ (Cys^{30}-Thr^{48})-OH at Gly^{37} and Ala^{42}, respectively. Using the same procedure as the synthesis of peptide **S2**, the desired Aβ (Cys^{30}-Leu^{49})-OH ($Gly^{37,\ RBM}$, $Ala^{42,\ RBM}$) **S3** carrying two Arg_4-tagged RBMs was successfully obtained. The deacetylation of peptide **S2** was performed at 0.4 M Cys aqueous solution (pH 7.2) and two acetyl groups could also be completely removed to generate desired Aβ (Cys^{30}-Thr^{48})-OH ($Gly^{37,\ RBM}$, $Ala^{42,\ RBM}$) **S4** within 30 min. NCL between peptide **1** and peptide **S4** to generate Aβ49 (Cys^{30}, $Gly^{37,\ RBM}$, $Ala^{42,\ RBM}$) **S5**, followed by desulfurization yielded Aβ49 ($Gly^{37,\ RBM}$, $Ala^{42,\ RBM}$) **S6**. Using the new method we successfully prepared Aβ49 **12** after the cleavage of the Arg_4-tagged RBM by TFA cocktails (**Figure S3)**.

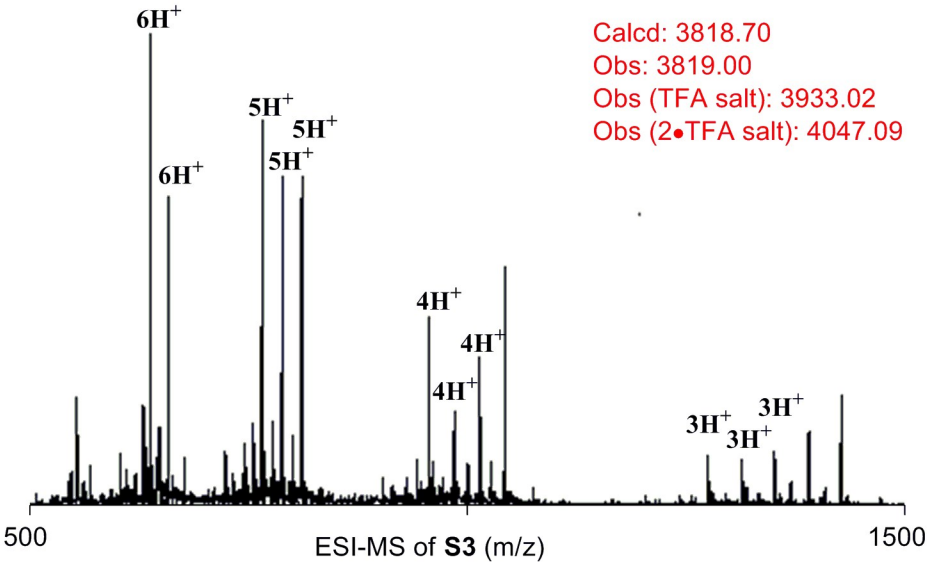

(a)
RBM
RBM
H- Asp1-Lys28 -Gly-SR
H-Cys- Ile31-Val36-Gly37-Ile41-Ala42-Leu49 -OH
1
S4
NCL
RBM
RBM
H- Asp1-Gly29 -Cys- Ile31-Val36-Gly37-Ile41-Ala42-Leu49 -OH
S5
Desulfurization
RBM
RBM
H- Asp1-Gly29 -Ala- Ile31-Val36-Gly37-Ile41-Ala42-Leu49 -OH
S6
TFA Cocktails
H- Asp1-Leu49 -OH
12
RBM :
Ac-RRRRG-HN
O
OH

(b)



S3





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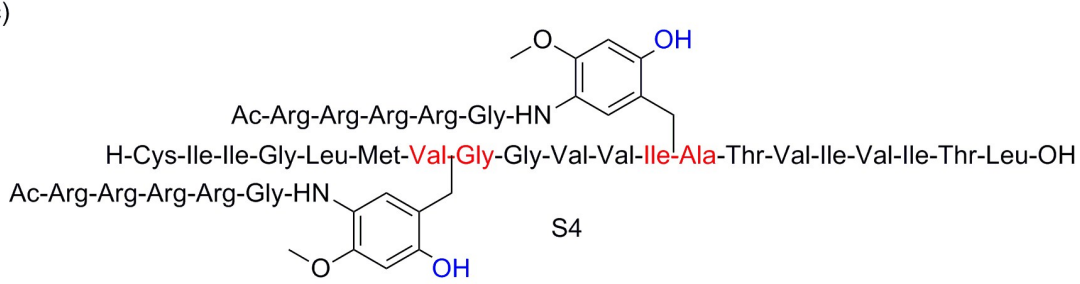
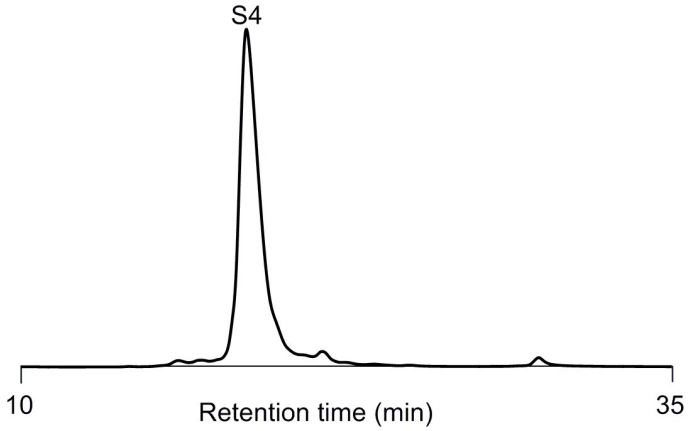
(c)



S4



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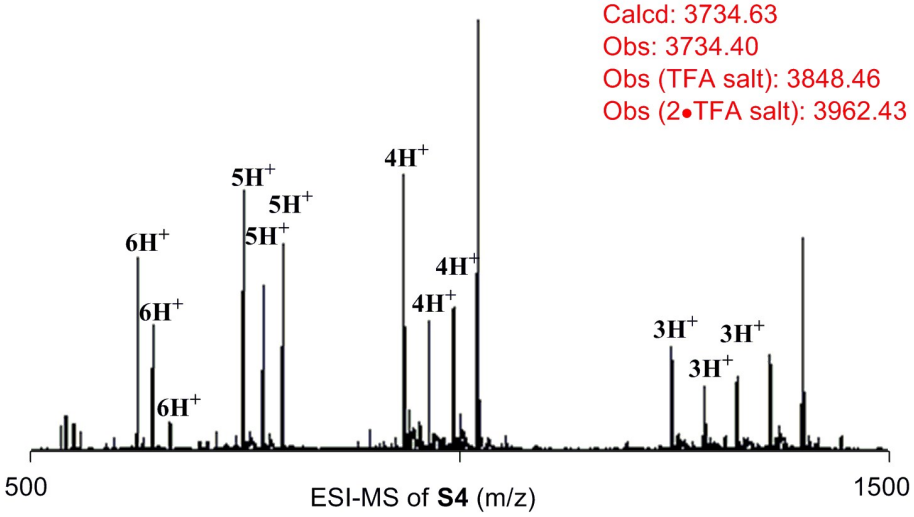
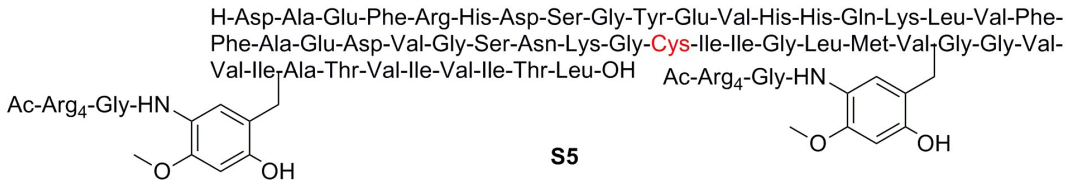
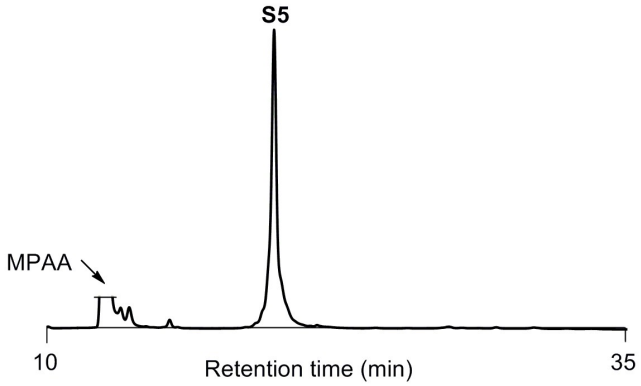
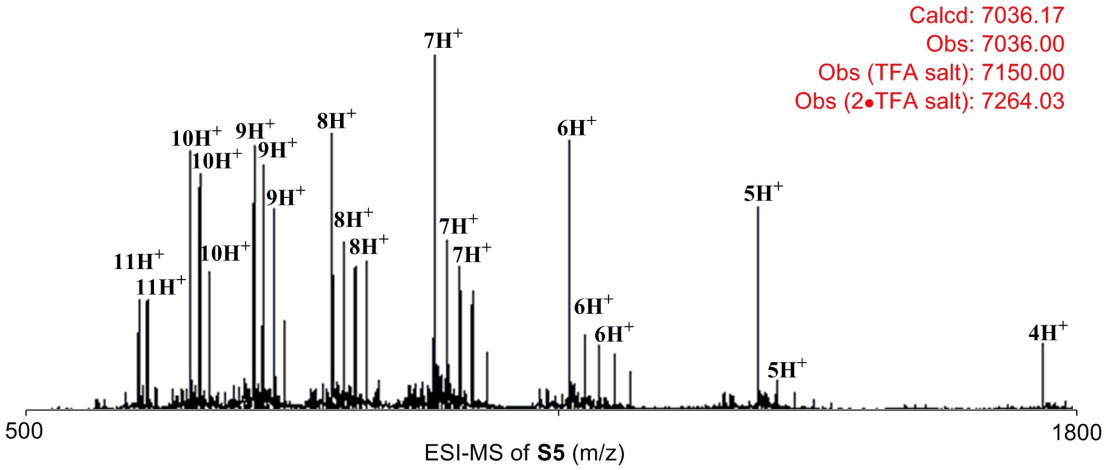


(d)


H-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-
Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Cys-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-
Val-Ile-Ala-Thr-Val-Ile-Val-Ile-Thr-Leu-OH
Ac-Arg4-Gly-HN
O
OH
Ac-Arg4-Gly-HN
O
OH

S5


S5
MPAA
10
Retention time (min)
35


Calcd: 7036.17
Obs: 7036.00
Obs (TFA salt): 7150.00
Obs (2•TFA salt): 7264.03
11H+
11H+
10H+
10H+
10H+
9H+
9H+
9H+
8H+
8H+
8H+
7H+
7H+
7H+
6H+
6H+
6H+
5H+
5H+
4H+
500
1800
ESI-MS of S5 (m/z)

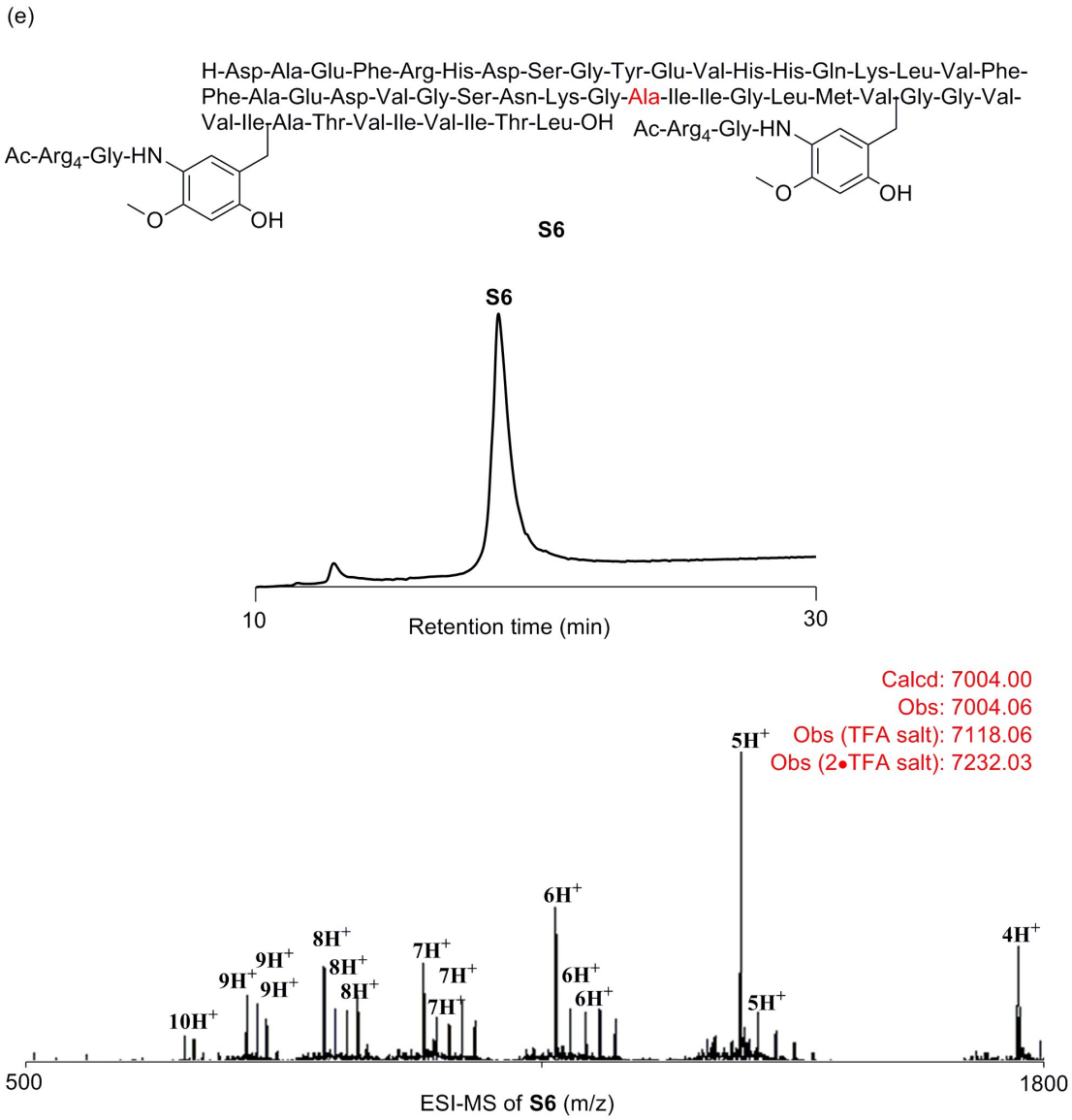


Figure S3. Chemical synthesis of peptide **12**. (a) Synthesis route of peptide **12**. (b) Analytical RP-HPLC traces and ESI-MS spectra of peptide **S3**. (c) Analytical RP-HPLC trace for the deacetylation of **S3** in neutral buffer (pH 7.2) at 30 °C with an observed ESI-MS of 3734.400 Da (calcd: 3734.63 Da). (d) Analytical RP-HPLC trace for the ligation between **S4** and **7** and ESI-MS spectra of peptide **S5**. (e) Analytical RP-HPLC trace for the desulfurization of **S5** to **S6** (t=15 h) and ESI-MS spectra of **S6**.

11. Characterization of the Longer Aβ Species using Tricine-SDS-PAGE and Standard SDS-PAGE

We further confirmed the high efficiency of the cleavages of the two Arg_4-tagged RBMs by Tricine-SDS-PAGE analysis. On the gel, we found that there were several bands for the peptide **12**. The bands at 10 KDa, 15 KDa and 20 KDa corresponded to the dimers, trimers and tetramers of peptide **12**, respectively (**Figure S4**). In contrast, we observed a single band for the peptide **S6** with a molecular weight above 10 KDa corresponding to the monomers of peptide **S6.** To demonstrate the band at the site above 10 KDa on the Tricine gel was the monomers of peptide **S6** and **10**, we further characterized using the standard 15% SDS-PAGE and found the band corresponding to the **S6** and **10** were detected at the site below 10 KDa.

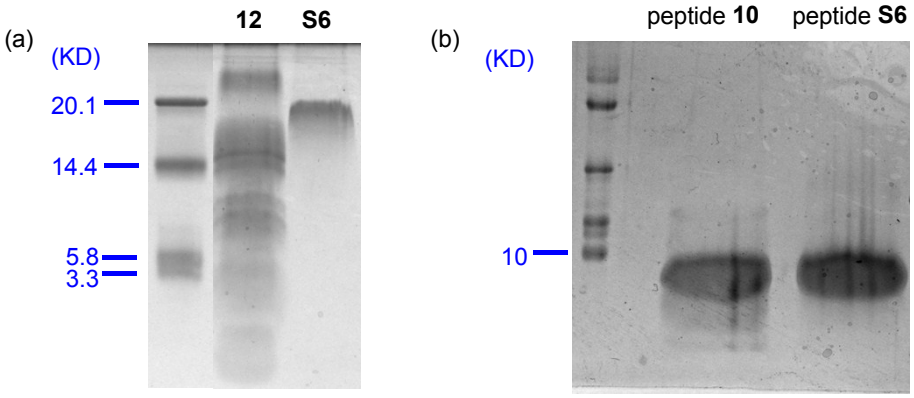


Figure S4. (a) The Tricine-SDS-PAGE analysis of peptide **12** and peptide **S6**: lane 1, the de-tagged products **12**; lane 2, the synthetic products **S6**. (b) The Standard 15% SDS-PAGE analysis of peptide **10** and peptide **S6**; lane 1, the de-tagged products **10**; lane 2, the de-tagged products **S6**.

12. Characterization of the Aggregation of Aβ with and without Gly-Arg_4-tagged RBM

Finally, we explored the influence of RBM groups upon the peptide conformation in 2,2,2-Trifluoroethanol (TFE) and PBS buffer by circular dichroism (CD). To this end, peptide **S6** and **12** were respectively diluted in phosphate buffer (PBS) from 40% TFE solution to the final concentration of 10 μM. It was noted that,

to ensure the peptide **12** remained monomeric, peptide **12** was pretreated with 100% HFIP followed by N_2 blowing and vacuum drying. The residues was suspended with 40% TFE. The CD spectrum of peptide **S6** showed a clear random-coil structure with a small negative minimum at 208 nm, whereas peptide **12** showed a strong positive minimum at 217 nm indicating a typical β-sheet structure profile (**Figure S5**). These studies revealed that the introduction of Arg_4-tagged RBM groups could disrupt of the formation of β-strand structure.

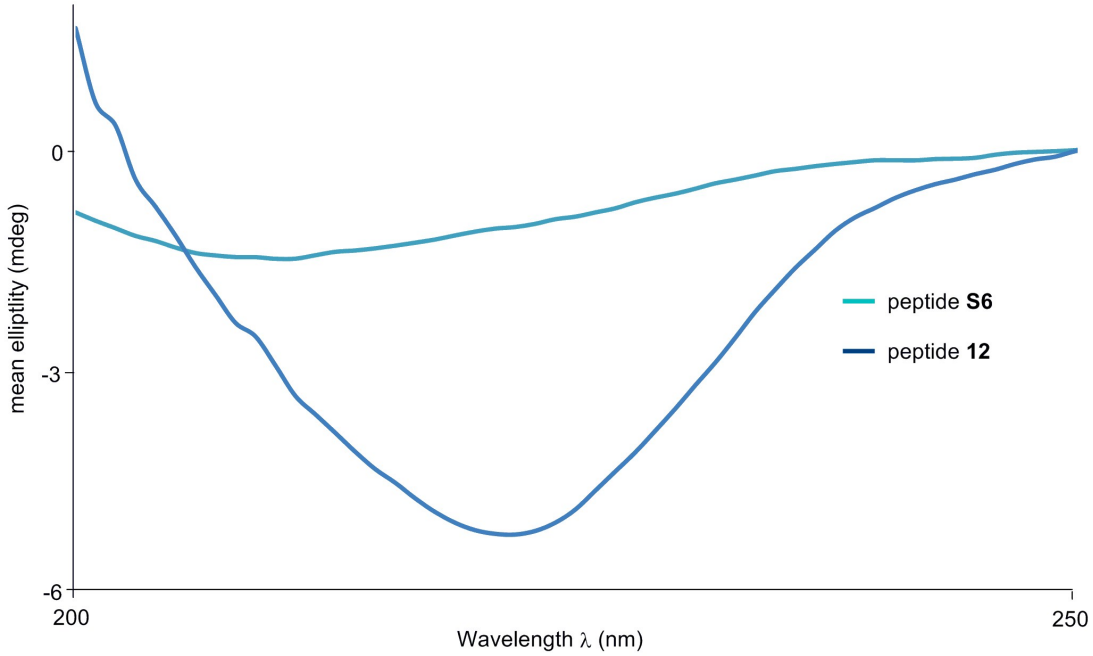


Figure S5. The CD spectra of peptides **S6** and **12** (10 μM, in a 1 mm quartz cell) in 1:1 2,2,2-Trifluoroethanol (TFE) and PBS buffer. Secondary structures of peptides were detected immediately after dilution into 20 mM phosphate-buffered saline (pH 7.3). CD spectra were recorded on a Chirascan-plus spectrometer (Applied Photophysics) from 200 nm to 250 nm using a 1 mm path length quartz cuvette.