Supplementary information

Separation of amyloid β fragment peptides with racemised and isomerised aspartic acid residues using an original chiral resolution labeling reagent

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CONTENTS

S1. General remarks

All the chemicals and solvents were of reagent or high-performance liquid chromatography (HPLC) grade and were used without further purification.

S2. Synthesis of the peptides

Aβ fragments were manually synthesized on Wang resin (Watanabe Chemical Industries, Hiroshima, Japan) by fluorenylmethoxycarbonyl (Fmoc) chemistry [1] with Fmoc-AA-OH (10 eq., Watanabe Chemical Industries) according to the *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, Nacalai Tesque, Kyoto, Japan) method. The *N*,*N*'-diisopropylcarbodiimide *N*,*N*-dimethyl-4-aminopyridine (DIPCI-DMAP) method was employed for the first residue. The side-chain-protecting groups used were as follows: *t*-butyloxy carbonyl (Boc) for lysine (Lys); 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine (Arg); *t*-butyl (tBu) for serine (Ser), tyrosine (Tyr), aspartic acid (Asp), and glutamic acid (Glu); and trityl (Trt) for histidine (His), asparagine (Asn), and glutamine (Gln). Fmoc-Asp-tBu, in which the carboxy group of the backbone was protected by a tBu group, was used for the synthesis of the Aβ fragments containing DL-iso-Asp. The Aβ fragments without Met residue were cleaved from the resins, and the side-chain protection was removed by incubating the peptides for 1 h in ultrapure water/triisopropylsilane (Nacalai Tesque)/thioanisole (Tokyo Chemical Industries, Tokyo, Japan)/trifluoroacetic acid (TFA, Nacalai Tesque) (1/1/1/50, vol./vol./vol./vol.). The Aβ fragments containing Met residue were cleaved from the resins, and the side-chain protection was removed by incubating the peptides for 1 h in ultrapure *m*-cresol (Nacalai Tesque)/ethandithiole (Nacalai Tesque)/thioanisole/TFA (1/2/2/50, vol./vol./vol./vol.). Thereafter, the peptides were precipitated by the addition of cold diethyl ether (Nacalai Tesque), collected by centrifugation, and dissolved in ultrapure water.

S3. Labeling protocol of Aβ fragments using D-FDLDA

The Aβ fragments were labeled using a DL-amino acid labeling kit (Nacalai Tesque) [2]. The labeling reagent solution (100 μL; D-FDLDA; enantiomer excess (ee), > 99.9%) and start solution (100 μL) were added to the sample solution (100 μL), and the mixture was incubated for 15 h (overnight) at 50 °C. Next, the side-chain delabeling reagent solution (100 μL) containing 6-mercapto-1-hexanol was added to the labeled sample solution, mixed using a vortex mixer for 5 s, and reacted at 50°C for 15 min. Stop solution (100 μL) was added, and the resulting solution was analyzed by HPLC and LC–mass spectroscopy (MS).

S4. HPLC measurements

HPLC was performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a LC20-AD intelligent pump. Separations were conducted on a COSMOSIL 3C_{18}-AR-II (3.0 mm internal diameter (I.D.) × 150 mm, Nacalai Tesque) column maintained at 40 °C. Solution A comprising 20% acetonitrile in ultrapure water (containing 0.1% formic acid) and solution B comprising 50% acetonitrile in ultrapure water (containing 0.1% formic acid) were used as the mobile phase for the labeled Aβ fragment analysis in the linear gradient elution mode. The flow rate was 0.4 mL/min with ultraviolet (UV) detection at 340 nm.

S5. LC–MS measurements and MS/MS analyses

An LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used. The sheath gas nitrogen flow was set at 4.5 L/min, and the block heater in the LC–MS equipment was heated to 400 °C. The mass range of m/z 100–2000 was covered with a scan time of 1.0 s, and data were collected in the positive ion mode using a detector voltage of 1.5 kV. The Aβ fragments containing racemised and isomerised Asp residues were detected at *m*/*z* 700.7 (labeled $Aβ_{1–3}$ fragments, molecular weight (MW) 699.7, z = +1, single charged peak); *m*/*z* 1074.1 (labeled $Aβ_{6–11}$ fragments, MW 1073.6, z = +1,

single charged peak); and *m*/*z* 675.8 (labeled $A\beta_{23–28}$ fragments, MW 1351.5, z = +2, double charged peak). MS/MS analysis was performed by fragmenting the peptides with a normalised collision energy of 35.0.

S6. Thioflavin T (ThT) fluorescence assay

A 1-mM Aβ fragment solution (20 μL) was mixed with 10× phosphate-buffered saline (PBS) (10 μL) and ultrapure water (70 μL). After incubation at 37 ℃ for 24 h, a 200-μM sample solution (10 μL) was mixed with 200-μM ThT (10 μL) and 1×PBS (80 μL). Fluorescence was measured (excitation at 450 nm and emission at 492 nm) using a fluorescence microplate reader (Infinite 200 Pro M Plex, Tecan Japan Co., Ltd., Kanagawa, Japan) [3].

S7. Supplemental references

[1] Chan, W.C., White, P.D.: Fmoc Solid phase peptide Synthesis; A Practical Approach, Oxford University Press: New York, 2000.

[2] Ozaki, M., Kuwayama, T., Hirose, T., Shimotsuma, M., Hashimoto, A., Kuranaga, T., Kakeya, H., *Anal*. *Bioanal*. *Chem*., **414**, 4039-4046 (2022).

[3] Usui, K., Hulleman, J.D., Paulsson, J.F., Siegel, S.J., Powers, E.T., Kelly, J.W., *Proc*. *Natl*. *Acad*. *Sci*. *USA*, **106**, 18563-18568 (2009).

Supplemental figures

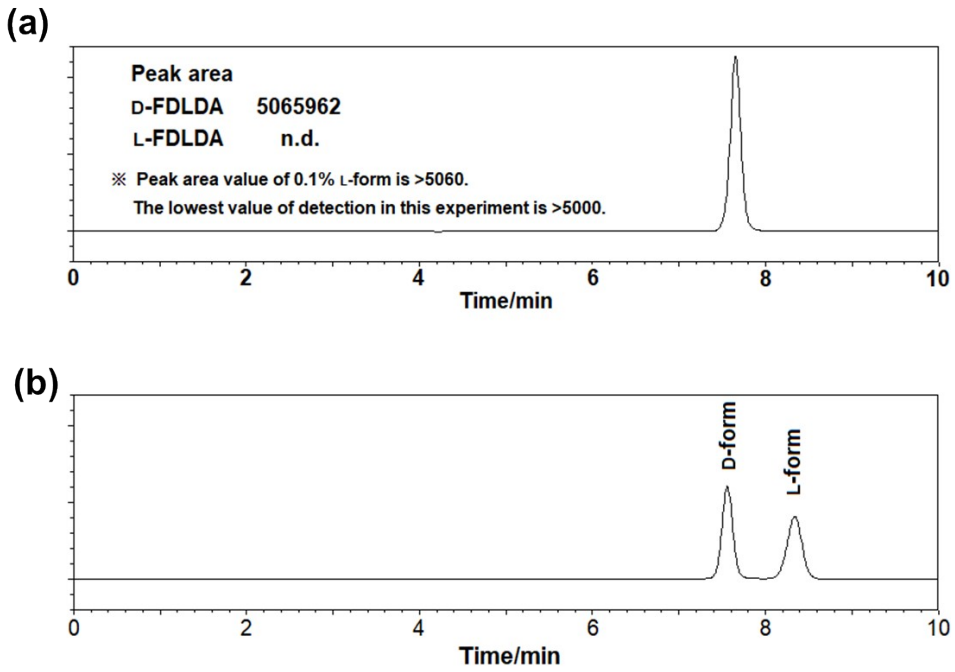


Fig. S1 HPLC chromatograms of (a) D-FDLDA alone and (b) D-FDLDA and L-FDLDA (1:1) mixtures. The HPLC measurement was performed using a COSMOSIL CHiRAL 3C column (4.6 mm I. D. × 250 mm) for analysis with triethylamine/ethanol/*n*-hexane (0.1/30/70, vol/vol/vol) using isocratic mode at a flow rate of 1.0 mL/min. UV detection was performed at 340 nm.

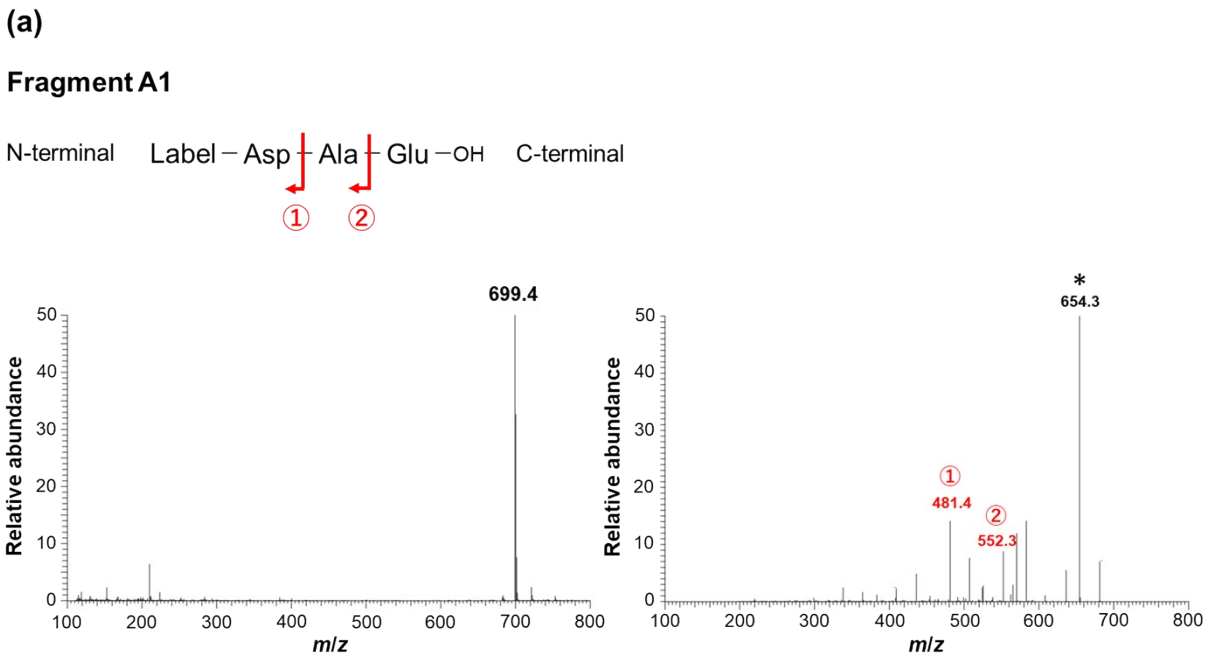
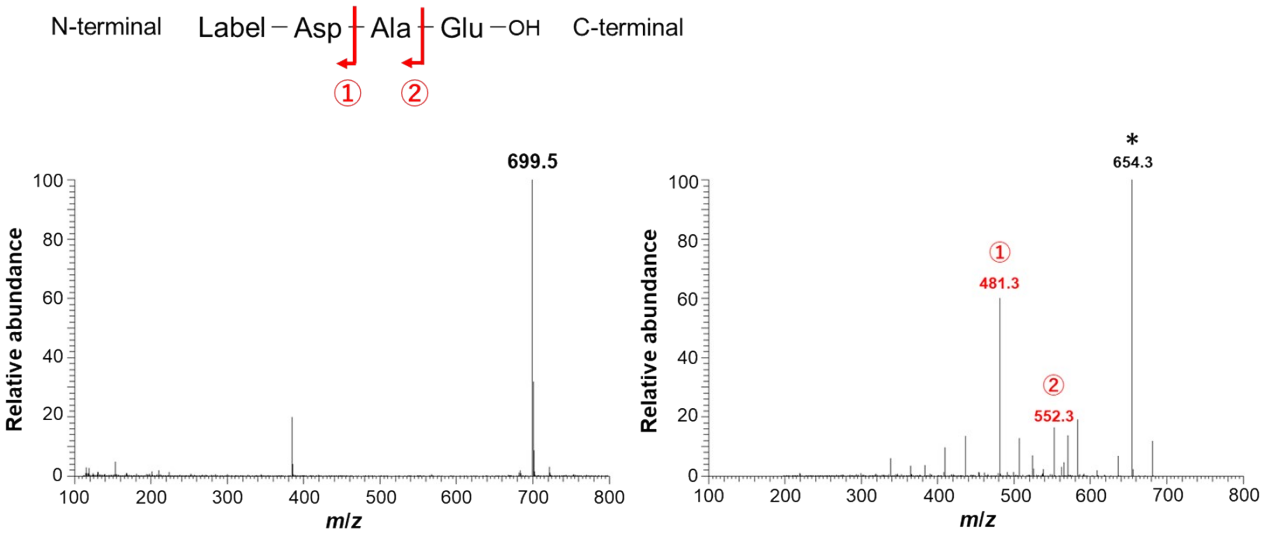


Fig. S2 MS spectra and MS/MS spectra of (a) fragment A1, (b) fragment A2, (c) fragment A3, and (d) fragment A4 in ESI-positive mode. *Peptide fragment without highly sensitive tag [-$N(CH_3)_2$] of D-FDLDA.

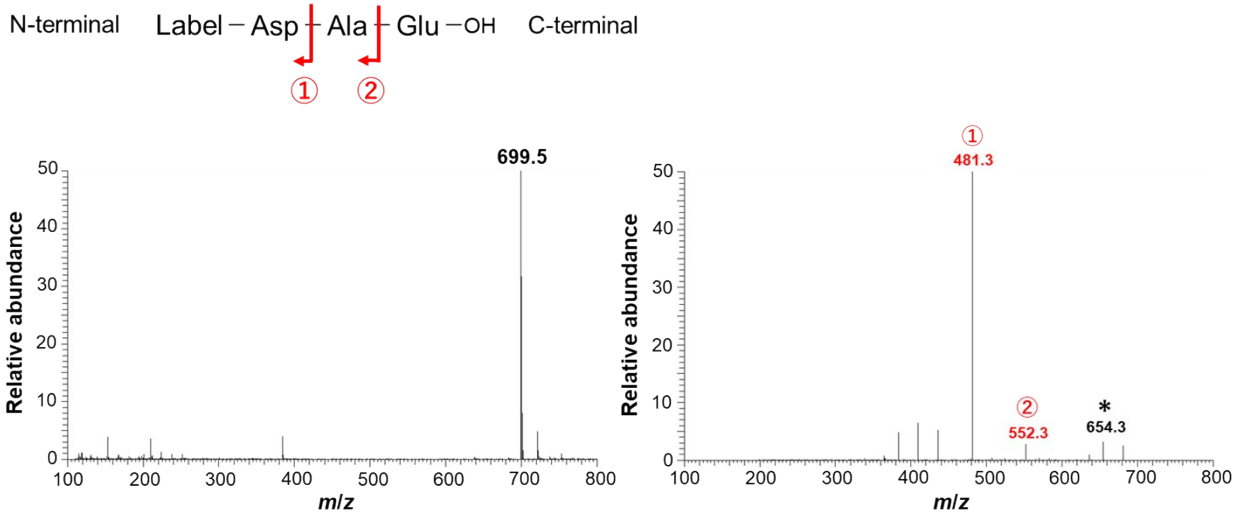
(b)

Fragment A2



(c)

Fragment A3



(d)

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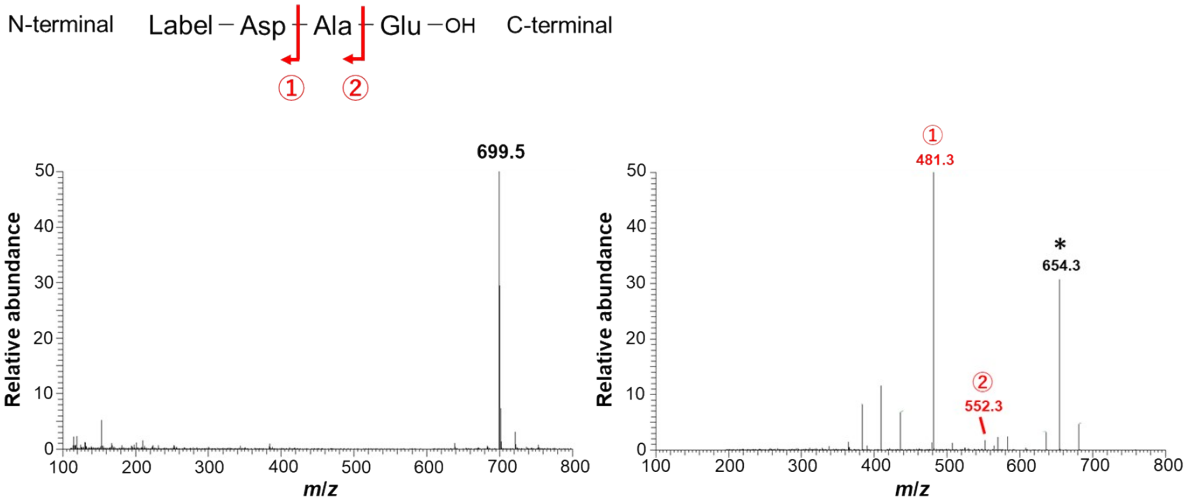


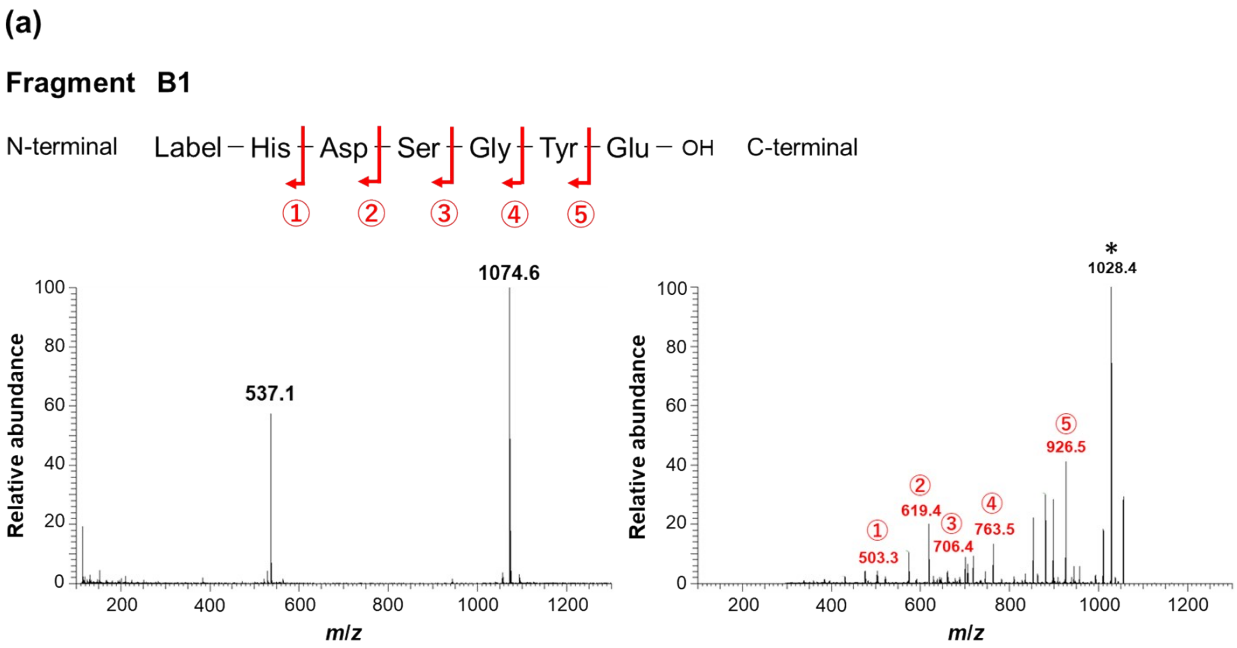
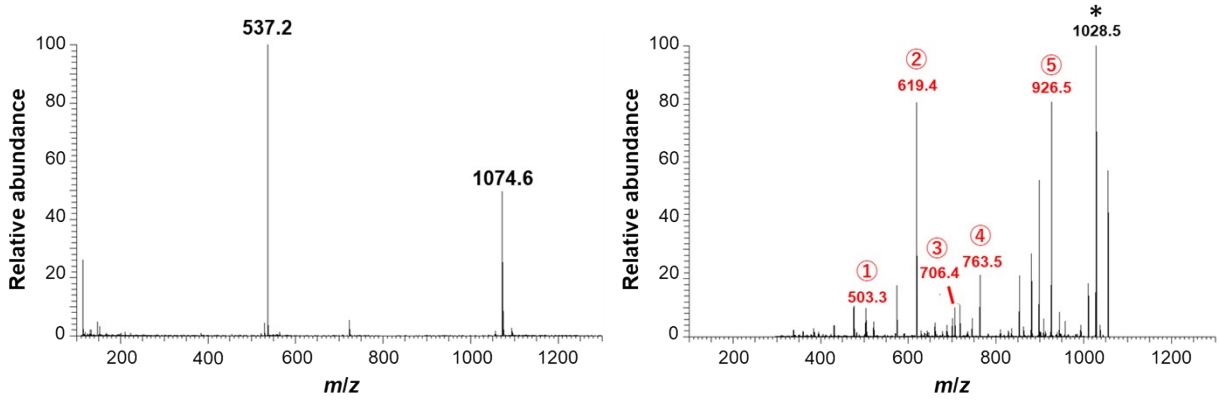


Fig. S3 MS spectra and MS/MS spectra of (a) fragment B1, (b) fragment B2, (c) fragment B3, and (d) fragment B4 in ESI-positive mode. *Peptide fragment without highly sensitive tag [-N(CH_3)$_2$] of D-FDLDA.

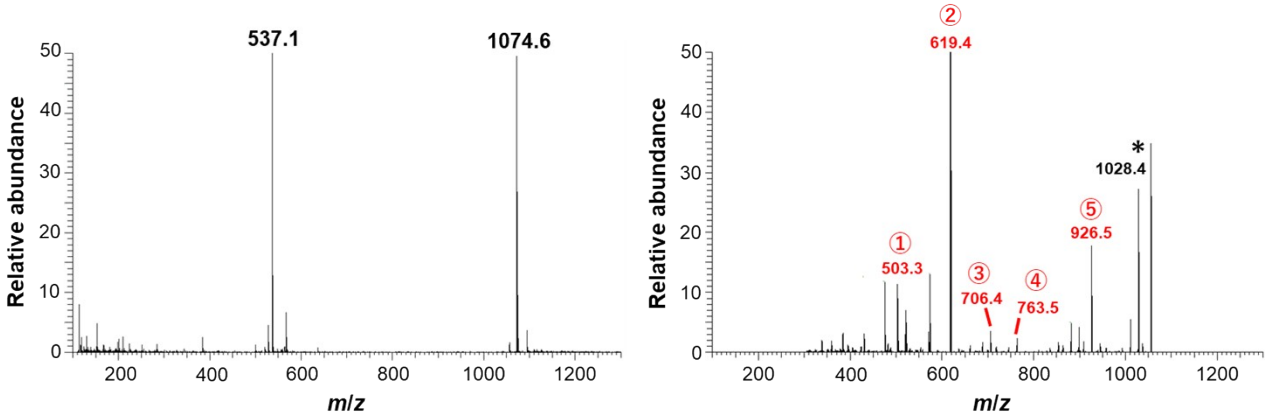
(b)

Fragment B2



(c)

Fragment B3



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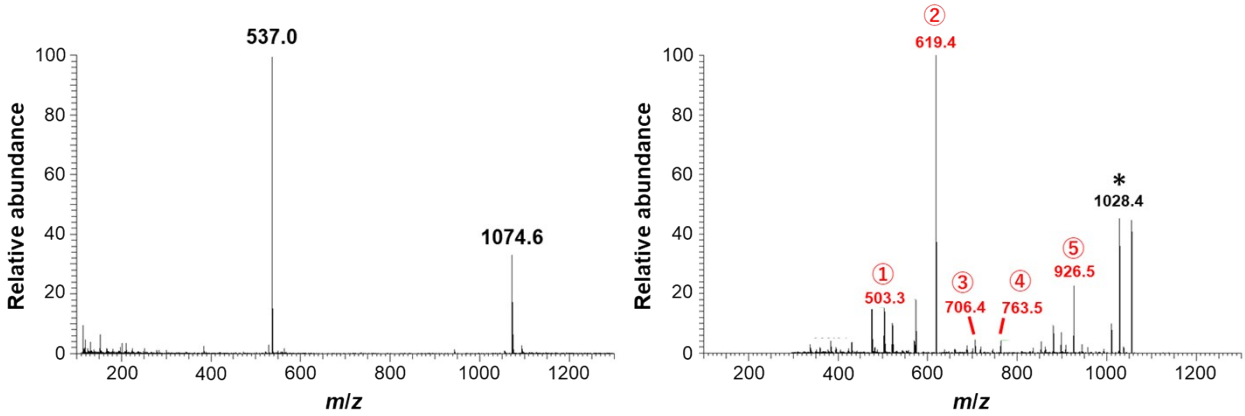


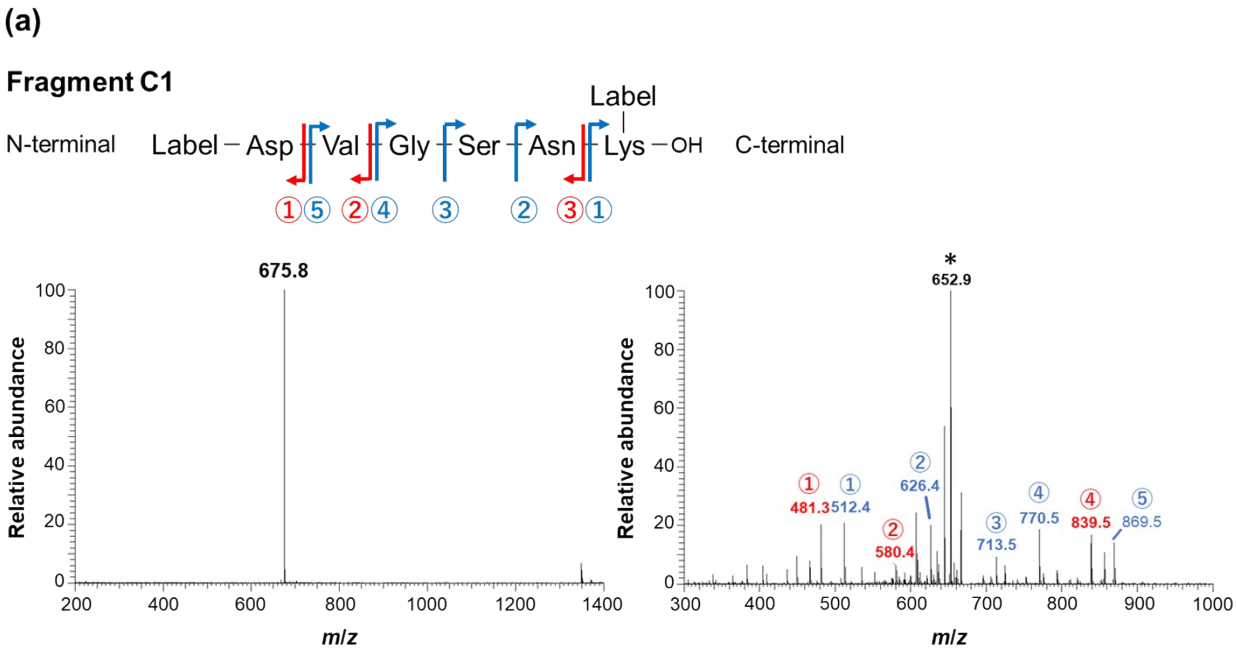
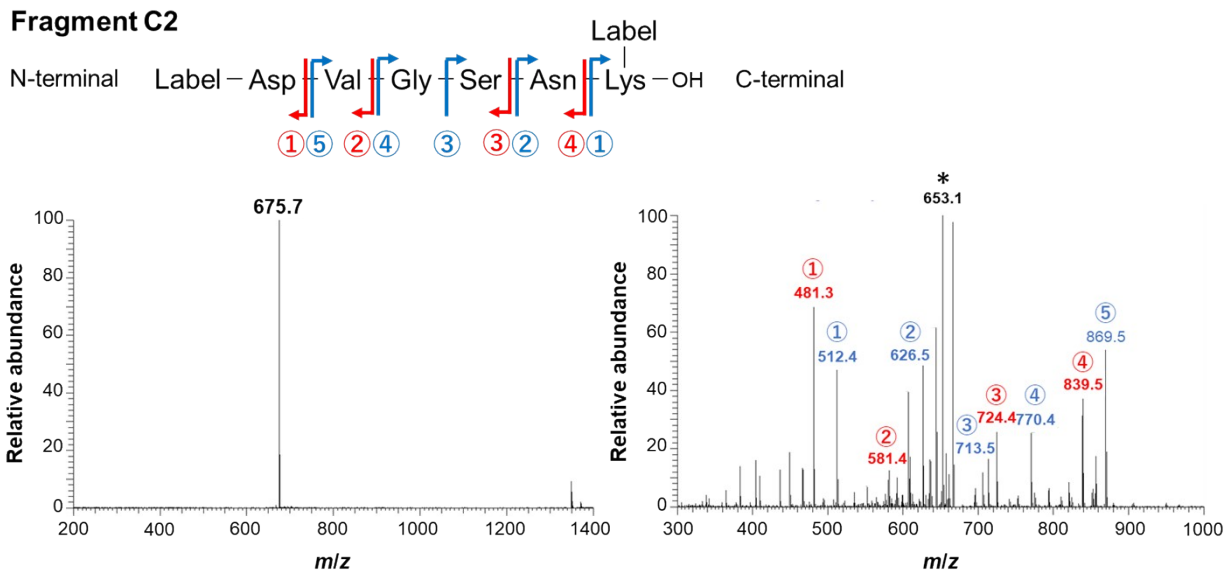


Fig. S4 MS spectra and MS/MS spectra of (a) fragment C1, (b) fragment C2, (c) fragment C3, and (d) fragment C4 in ESI-positive mode. *Peptide fragment without one highly sensitive tag [-$N(CH_3)_2$] of D-FDLDA (double charged peak).

(b)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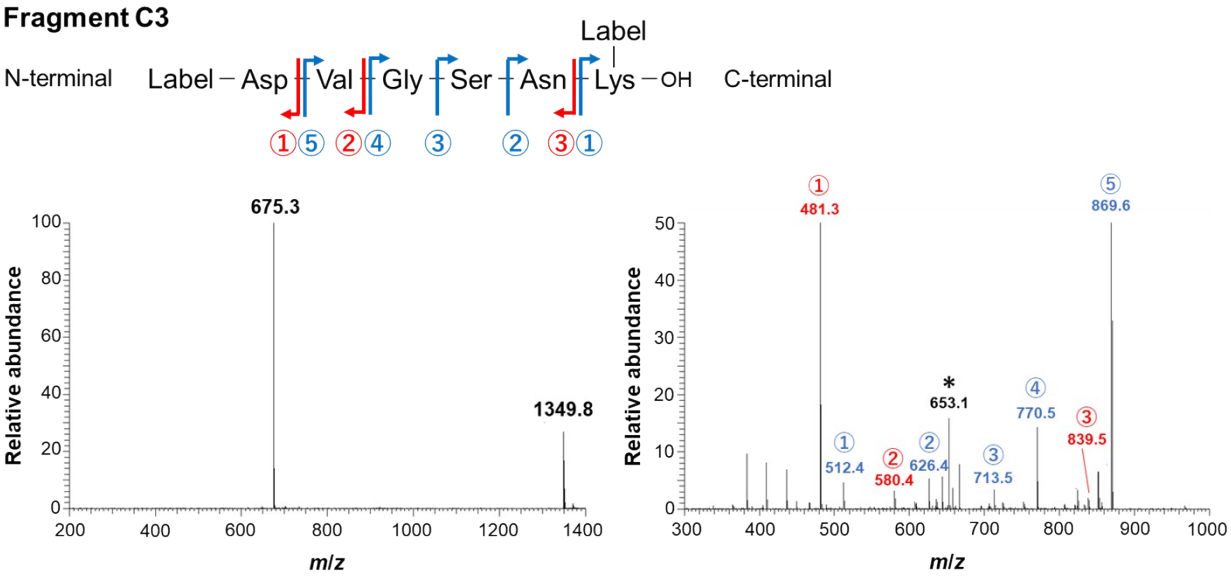
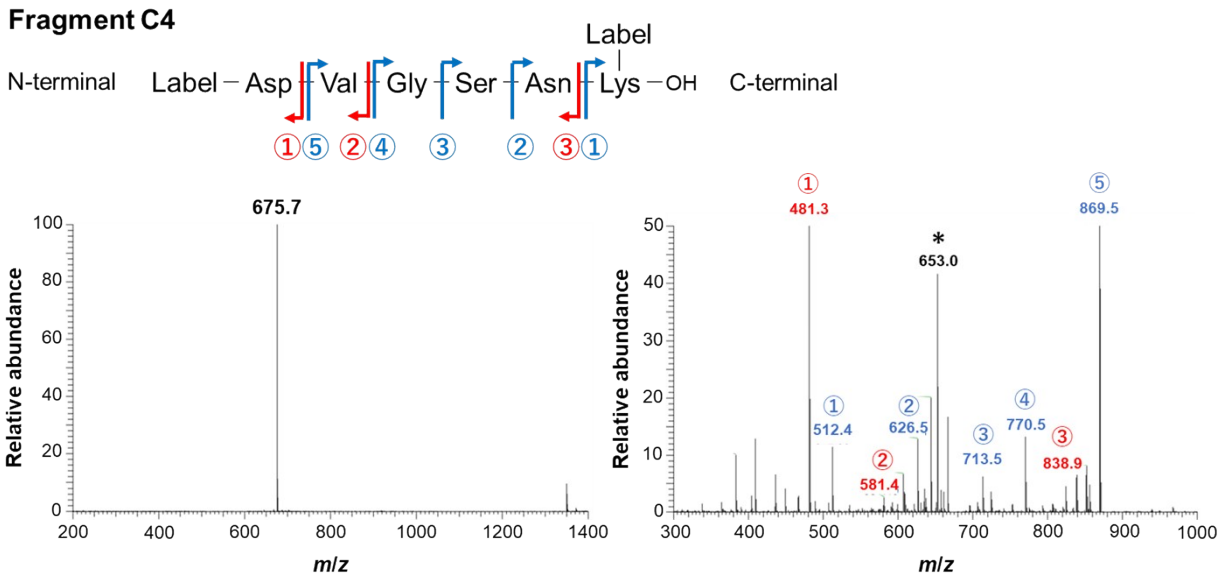
N-terminal
Label – Asp – Val – Gly – Ser – Asn – Lys – OH
Label
C-terminal
① ⑤ ② ④ ③ ③ ② ④ ①
675.7
Relative abundance
m/z
*
653.1
① 481.3
① 512.4
② 626.5
② 581.4
③ 724.4
③ 713.5
④ 770.4
④ 839.5
⑤ 869.5

(c)


Fragment C3
N-terminal
Label – Asp – Val – Gly – Ser – Asn – Lys – OH
Label
C-terminal
① ⑤ ② ④ ③ ② ③ ①
675.3
1349.8
Relative abundance
m/z
① 481.3
⑤ 869.6
* 653.1
④ 770.5
③ 839.5
① 512.4
② 580.4
② 626.4
③ 713.5
Relative abundance
m/z

(d)


Fragment C4
N-terminal
Label – Asp – Val – Gly – Ser – Asn – Lys – OH
Label
C-terminal
① ⑤ ② ④ ③ ② ③ ①
675.7
Relative abundance
m/z
① 481.3
⑤ 869.5
* 653.0
① 512.4
② 626.5
② 581.4
③ 713.5
④ 770.5
③ 838.9
Relative abundance
m/z

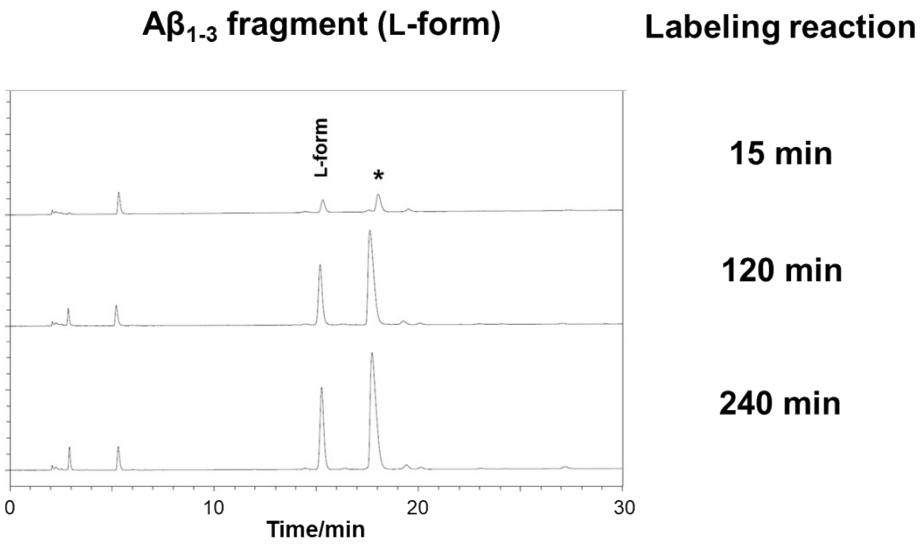
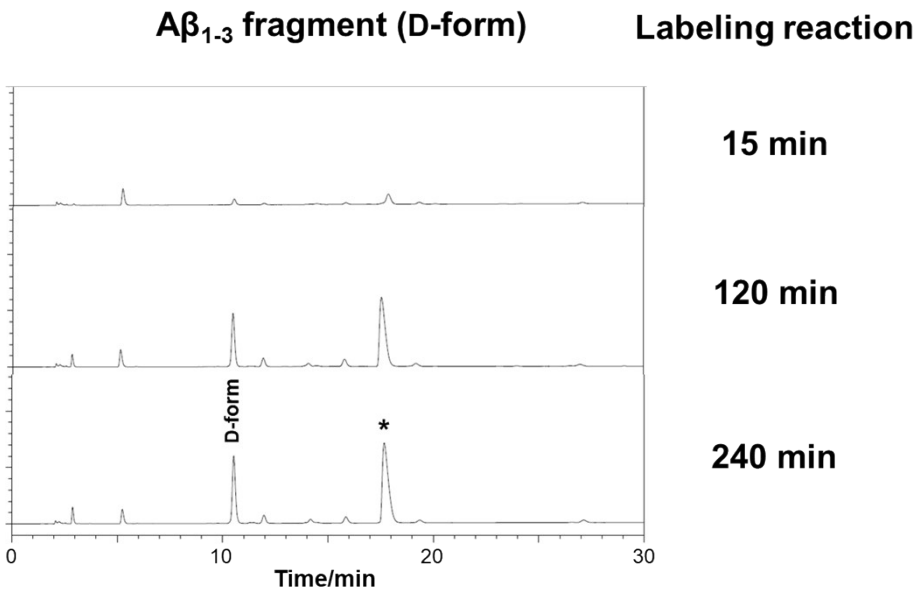
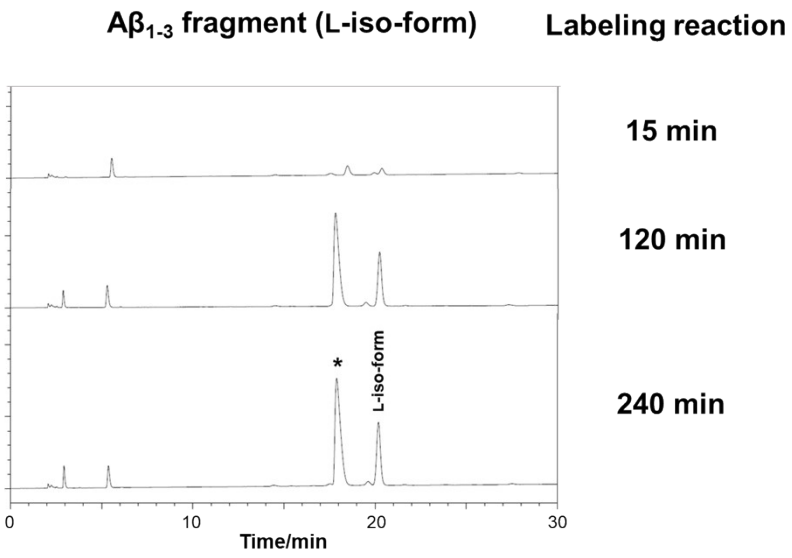
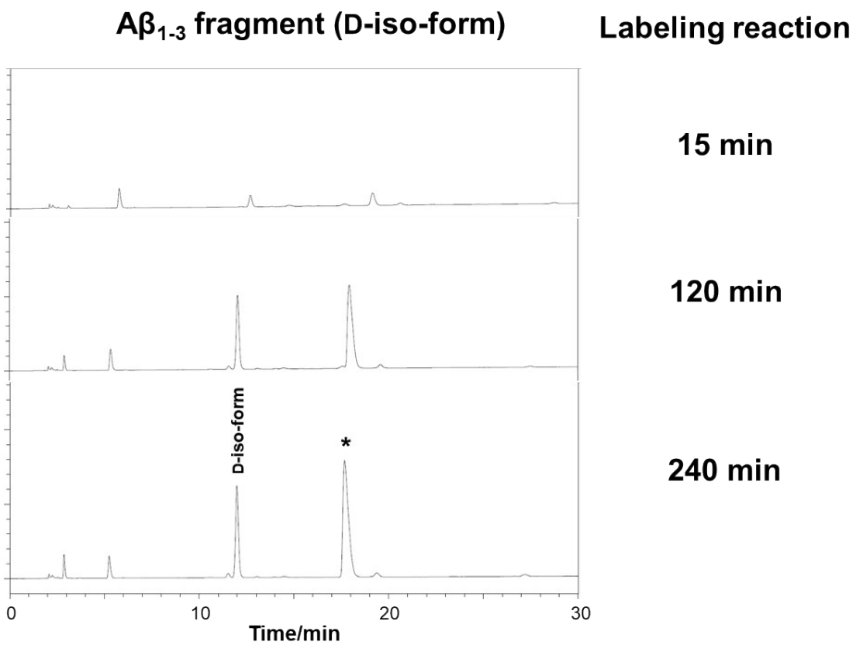


Fig. S5. HPLC chromatograms of $A\beta_{1-3}$ fragments labeled with D-FDLDA. Analysis was performed using a COSMOSIL $3C_{18}$-AR-II column (3.0 mm I.D.×150 mm; particle size, 3 μm) for analysis with 20% acetonitrile in ultrapure water (containing 0.1% formic acid) and a linear gradient from 0% to 20% with 50% acetonitrile in ultrapure water (containing 0.1% formic acid) over 30 min at a flow rate of 0.4 mL/min at 40 °C. UV detection was performed at 340 nm. *Peak derived from hydrolysis.


Aβ1-3 fragment (D-form)
Labeling reaction
15 min
120 min
240 min
D-form
*
0
10
20
30
Time/min


Aβ1-3 fragment (L-iso-form)
Labeling reaction
15 min
120 min
240 min
*
L-iso-form
0
10
20
30
Time/min


Aβ1-3 fragment (D-iso-form)
Labeling reaction
15 min
120 min
240 min
D-iso-form
*
0
10
20
30
Time/min

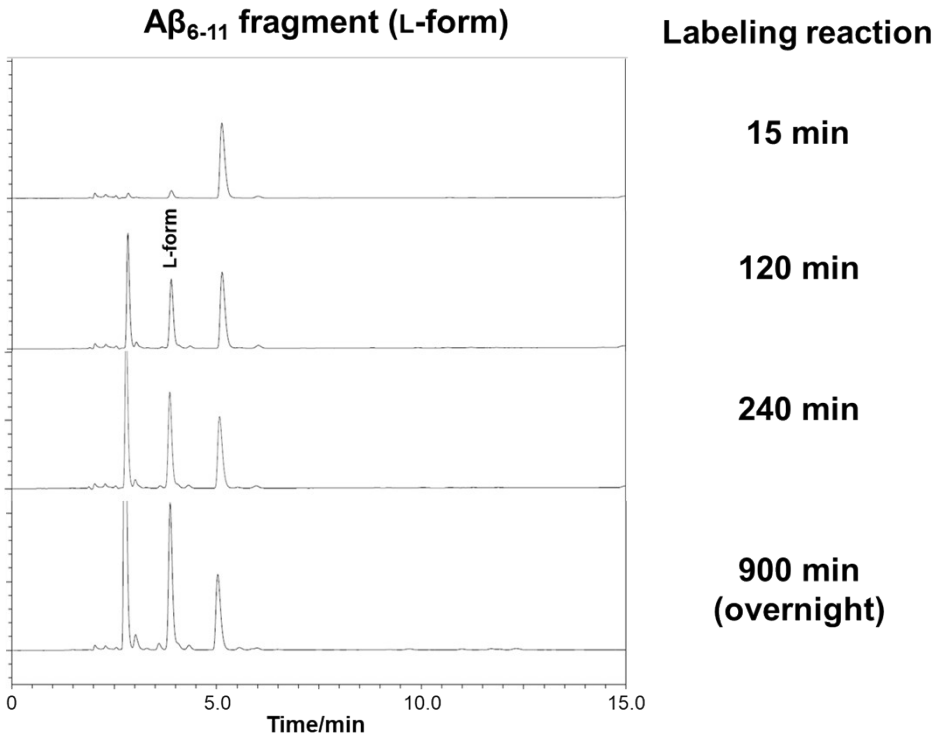
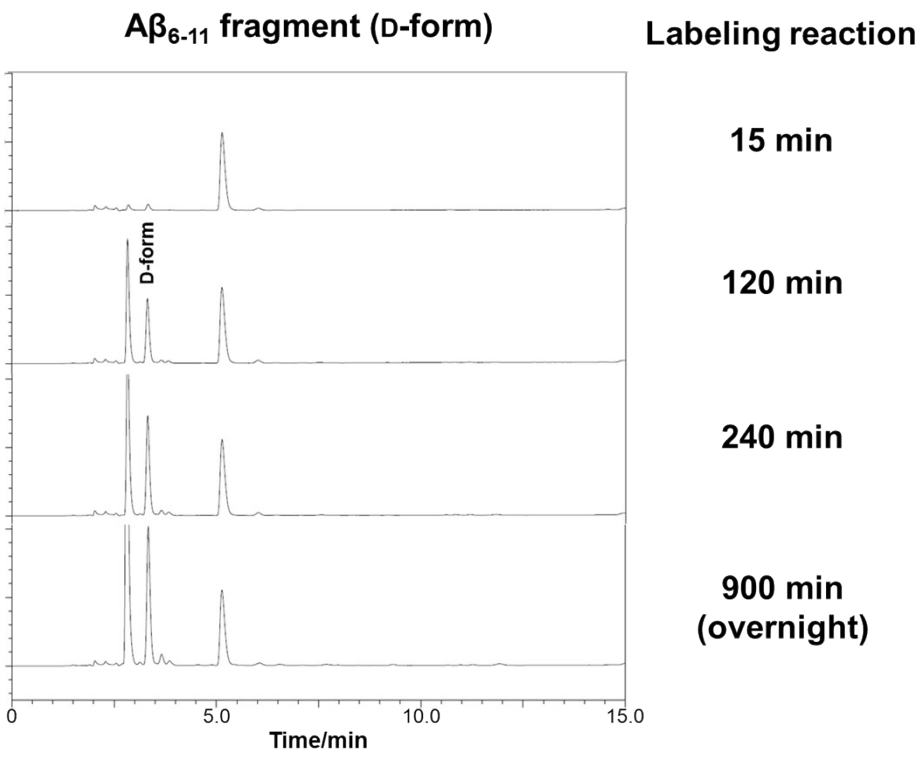
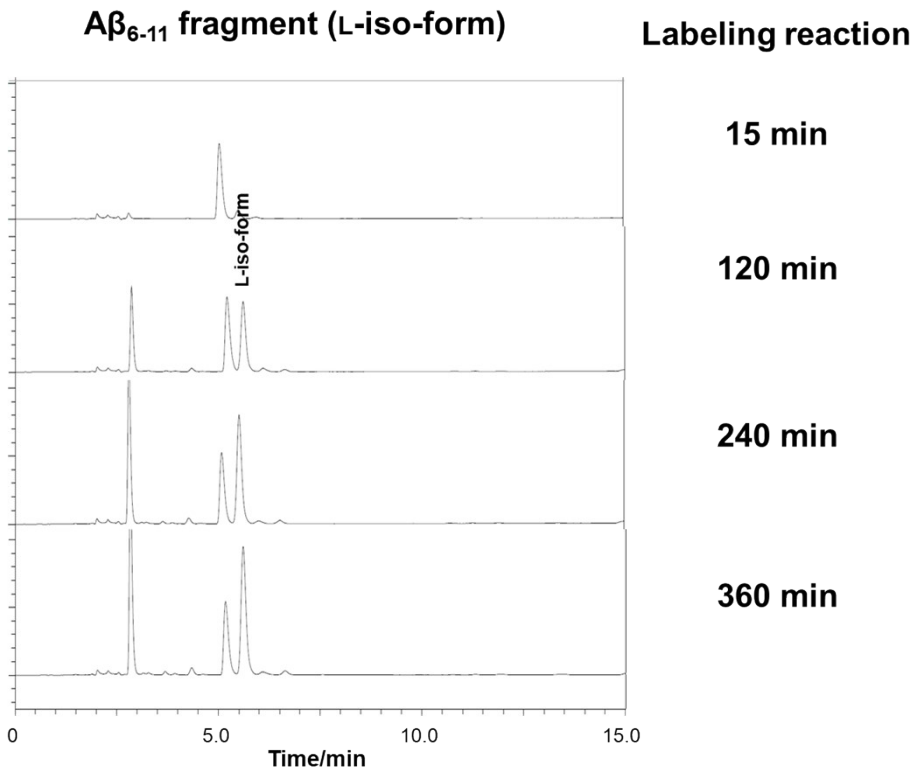
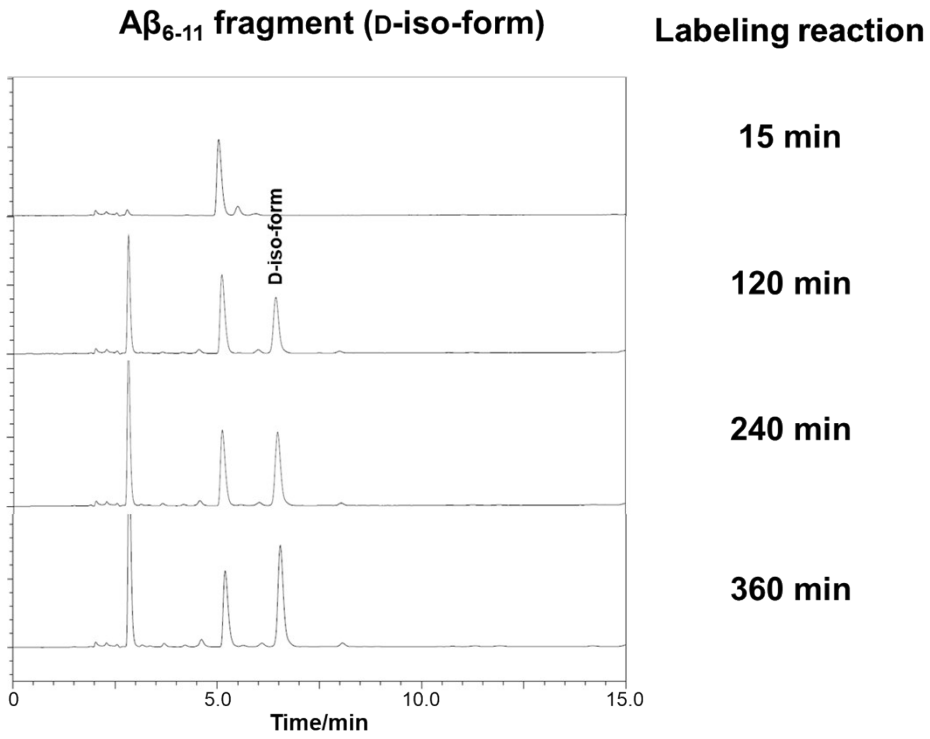


Fig. S6. HPLC chromatograms of $A\beta_{6–11}$ fragments labeled with D-FDLDA. Analysis was performed using a COSMOSIL $3C_{18}$-AR-II column (3.0 mm I.D.×150 mm; particle size, 3 μm) for analysis with 20% acetonitrile in ultrapure water (containing 0.1% formic acid) and a linear gradient from 0% to 10% with 50% acetonitrile in ultrapure water (containing 0.1% formic acid) over 15 min at a flow rate of 0.4 mL/min at 40 °C. UV detection was performed at 340 nm.


Aβ6-11 fragment (D-form)
Labeling reaction
D-form
15 min
120 min
240 min
900 min
(overnight)
0
5.0
10.0
15.0
Time/min


Aβ6-11 fragment (L-iso-form)
Labeling reaction
L-iso-form
15 min
120 min
240 min
360 min
0
5.0
10.0
15.0
Time/min


Aβ6-11 fragment (D-iso-form)
Labeling reaction
D-iso-form
15 min
120 min
240 min
360 min
0
5.0
10.0
15.0
Time/min

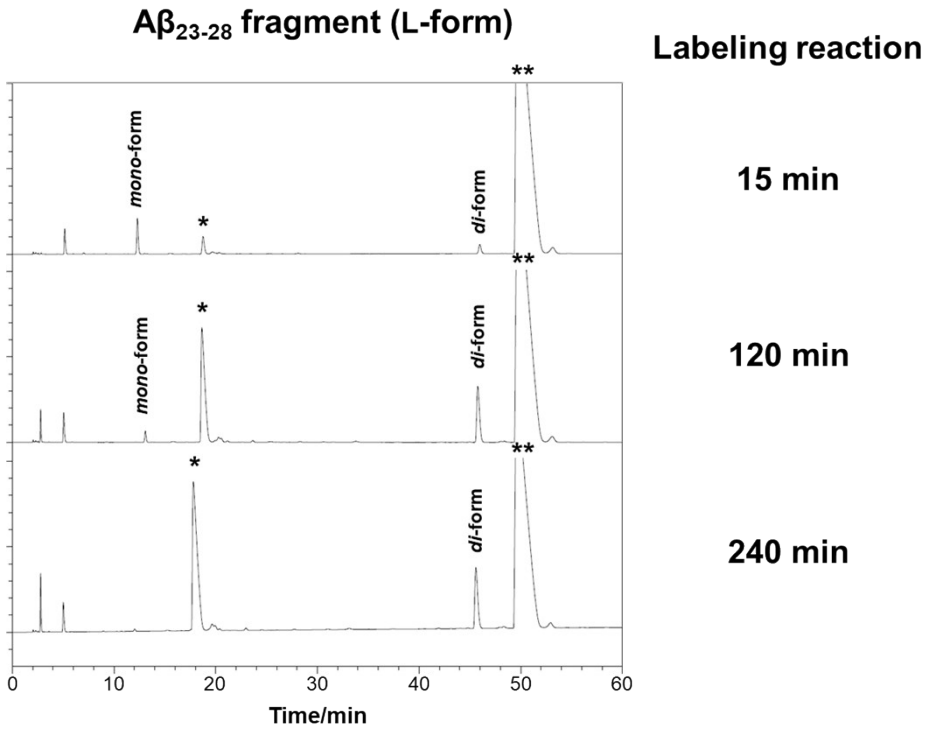
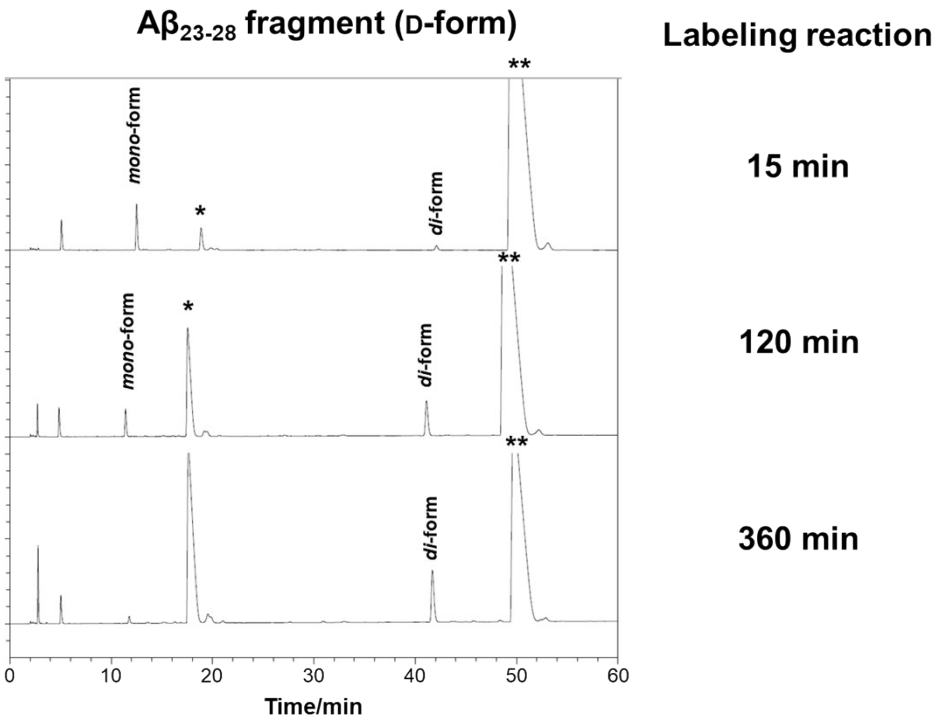
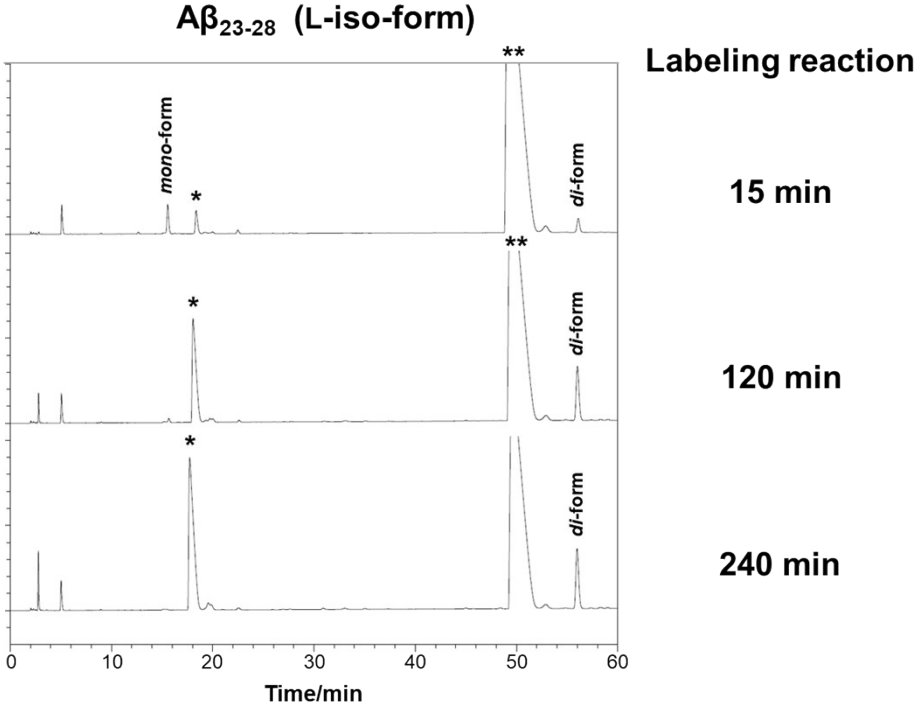
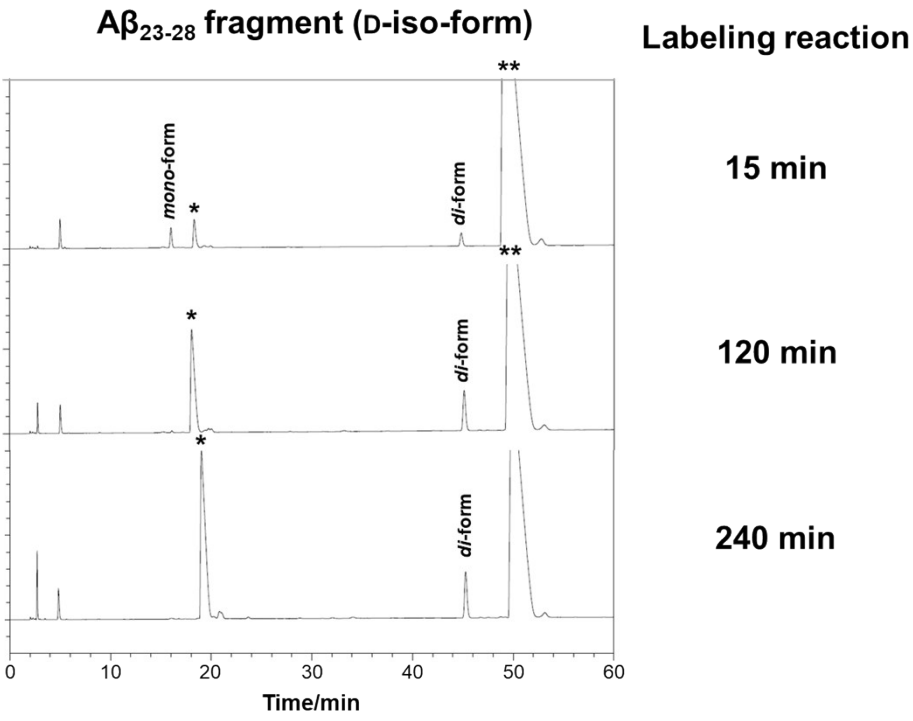


Fig. S7. HPLC chromatograms of $A\beta_{23–28}$ fragment labeled with D-FDLDA. Analysis was performed using a COSMOSIL $3C_{18}$-AR-II column (3.0 mm I.D. × 150 mm; particle size, 3 μm) for analysis with 20% acetonitrile in ultrapure water (containing 0.1% formic acid) and a linear gradient from 0% to 40% with 50% acetonitrile in ultrapure water (containing 0.1% formic acid) over 60 min at a flow rate of 0.4 mL/min at 40 °C. UV detection was performed at 340 nm. *Peak derived from hydrolysis. **Peak derived from D-DLDA-S-$C_6H_{12}OH$.


Aβ23-28 fragment (D-form)
Labeling reaction
mono-form
di-form
*
**
15 min
120 min
360 min
0
10
20
30
40
50
60
Time/min


Aβ23-28 (L-iso-form)
Labeling reaction
mono-form
di-form
*
**
15 min
120 min
240 min
0
10
20
30
40
50
60
Time/min


Aβ23-28 fragment (D-iso-form)
Labeling reaction
mono-form
di-form
*
**
15 min
120 min
240 min
0
10
20
30
40
50
60
Time/min

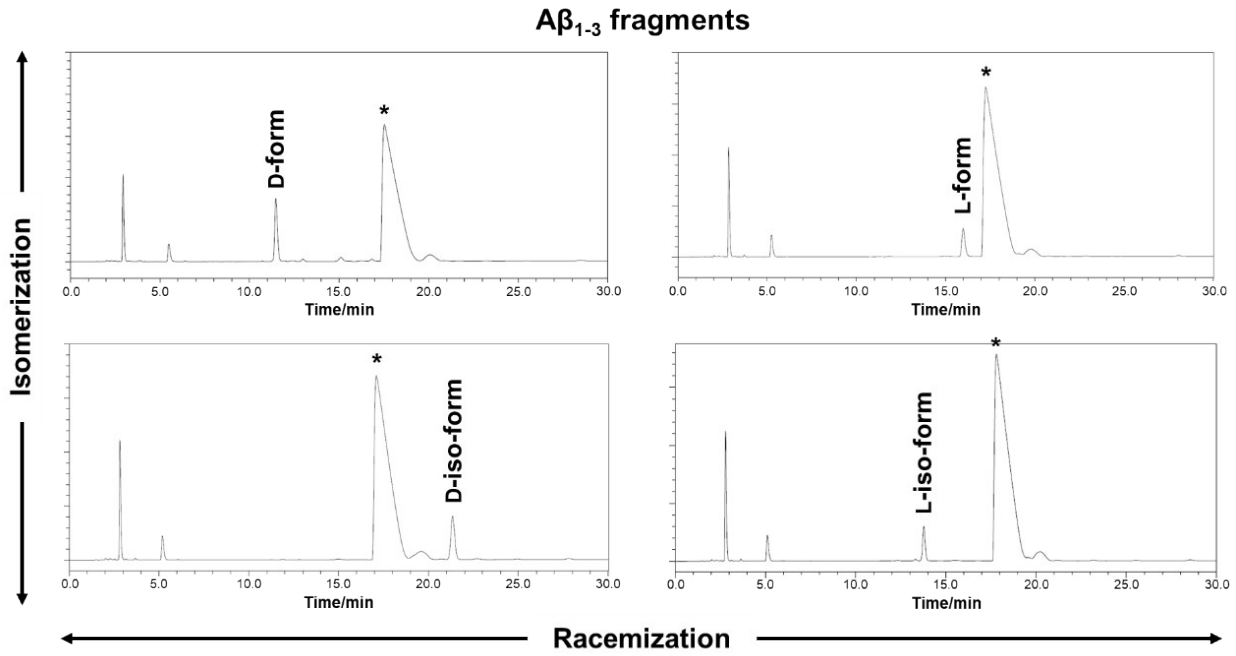
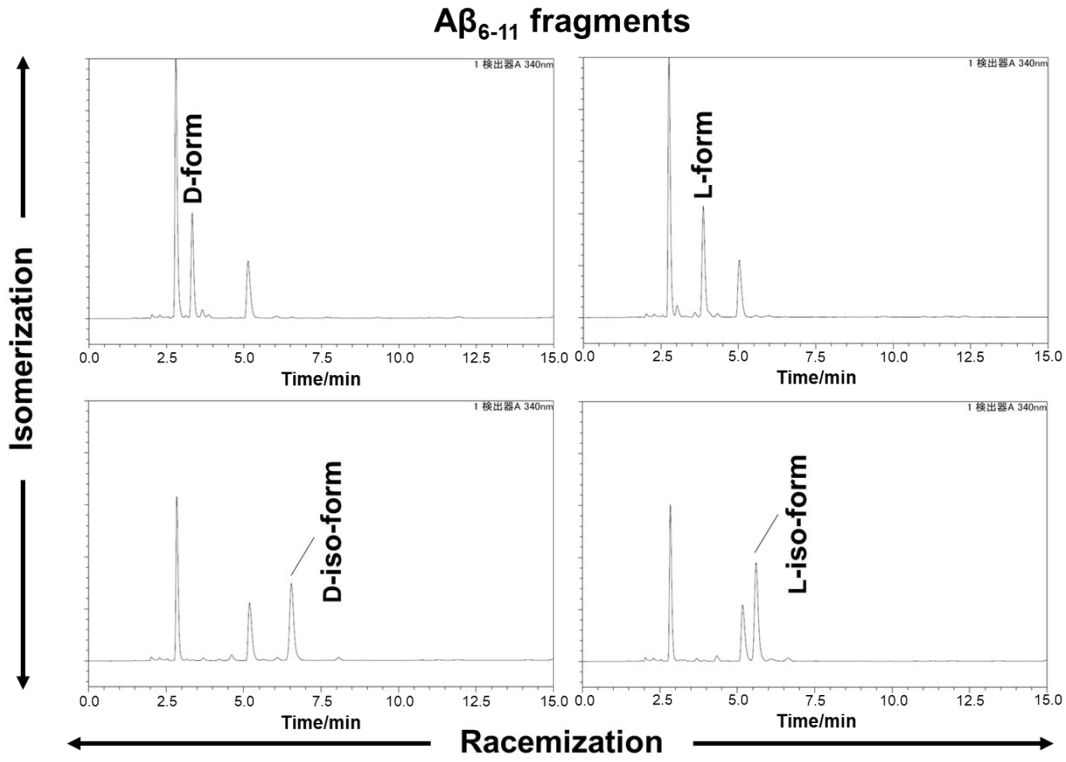
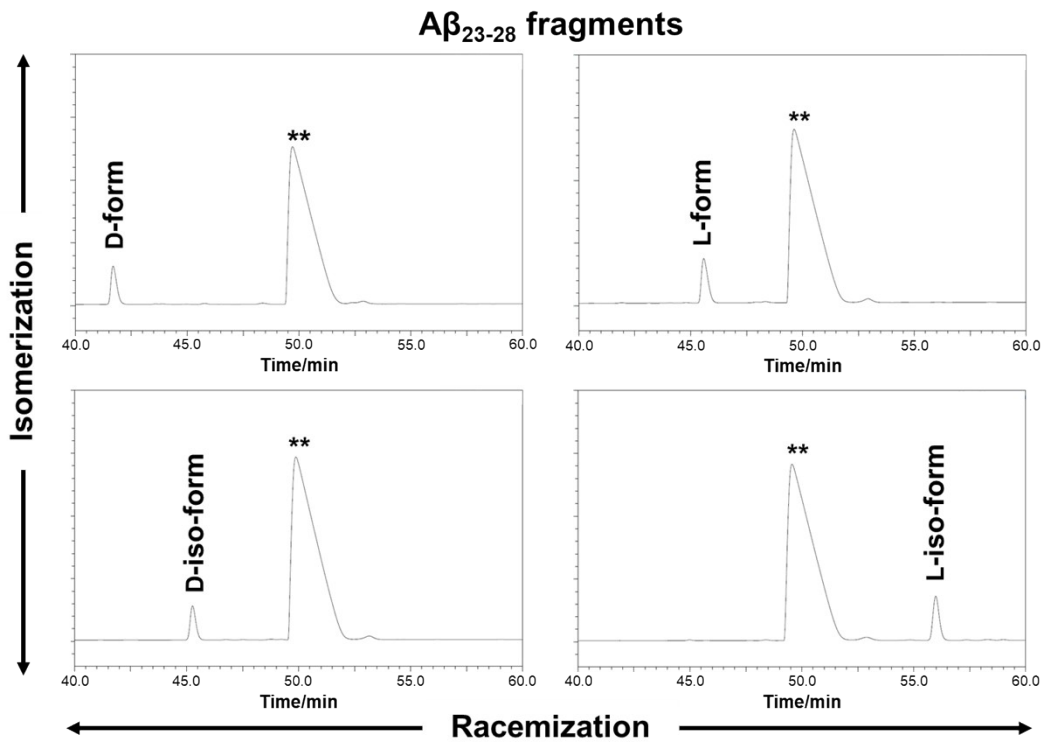


Fig. S8. HPLC chromatograms of $A\beta_{1–3}$, $A\beta_{6–11}$, and $A\beta_{23–28}$ fragments labeled with D-FDLDA. Analysis was performed using a COSMOSIL $3C_{18}$-AR-II column (3.0 mm I.D. × 150 mm; particle size, 3 μm) for analysis with 20% acetonitrile in ultrapure water (containing 0.1% formic acid) and a linear gradient from 0% to 40% with 50% acetonitrile in ultrapure water (containing 0.1% formic acid) over 60 min at a flow rate of 0.4 mL/min at 40 °C. UV detection was performed at 340 nm. *Peak derived from hydrolysis. **Peak derived from D-DLDA-S-$C_6H_{12}OH$.


Aβ6-11 fragments
Isomerization
D-form
L-form
D-iso-form
L-iso-form
1 検出器A 340nm
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Time/min
Racemization


Aβ23-28 fragments
Isomerization
D-form
**
L-form
**
D-iso-form
**
L-iso-form
**
40.0
45.0
50.0
55.0
60.0
Time/min
Racemization