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Electronic Supplementary Information for

Stabilization of Peptides against Proteolysis through Disulfide-Bridged Conjugation with Synthetic Aromatics

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HPLC chromatograms and mass spectra of NDI-peptide conjugates

1=NDI:

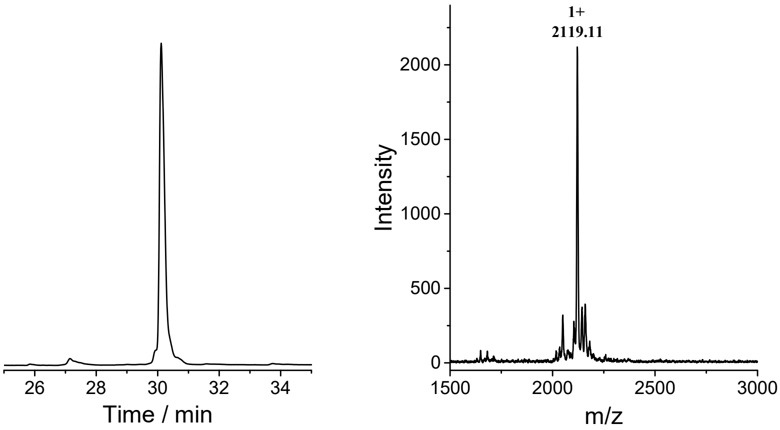


Figure S1. Analytical HPLC chromatogram and mass spectrum data of the purified **1**=NDI; m/z calculated: 2117.37, m/z found: 2119.11 $[M+H]^+$.

2=NDI:

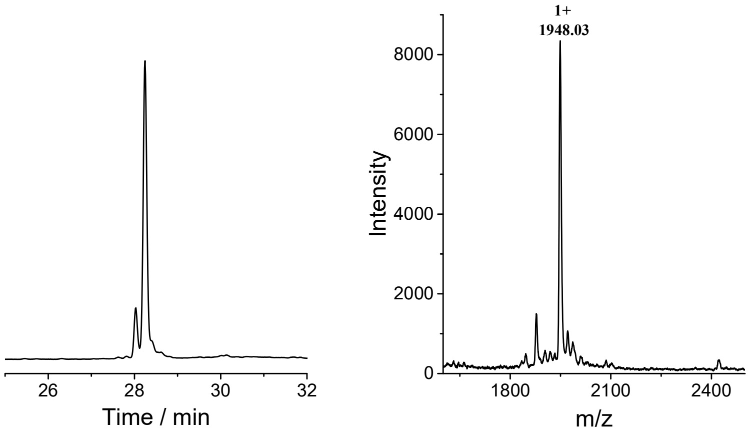


Figure S2. Analytical HPLC chromatogram and mass spectrum data of the purified **2**=NDI; m/z calculated: 1946.17, m/z found: 1948.03 $[M+H]^+$.

3=NDI:

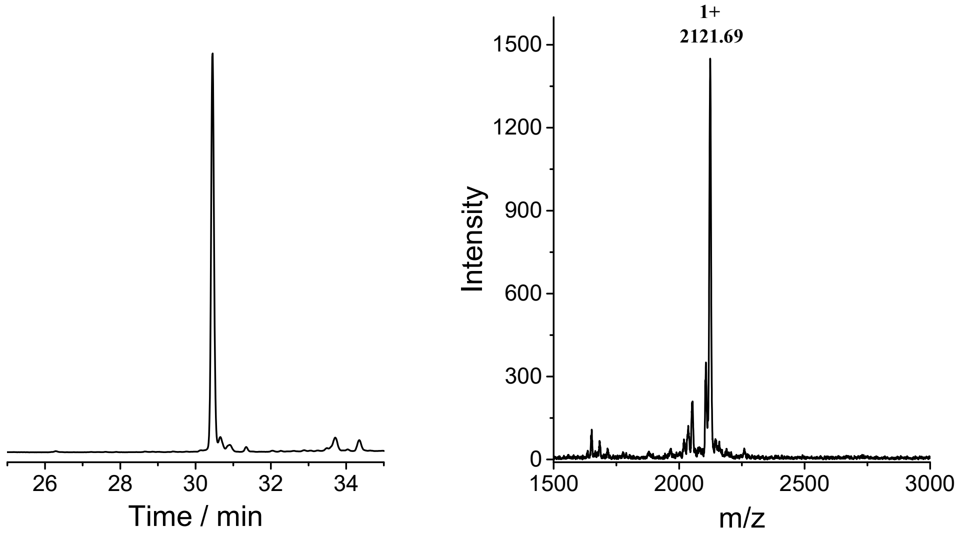


Figure S3. Analytical HPLC chromatogram and mass spectrum data of the purified **3**=NDI; m/z calculated: 2118.35, m/z found: 2121.69 $[M+H]^+$.

4=NDI:

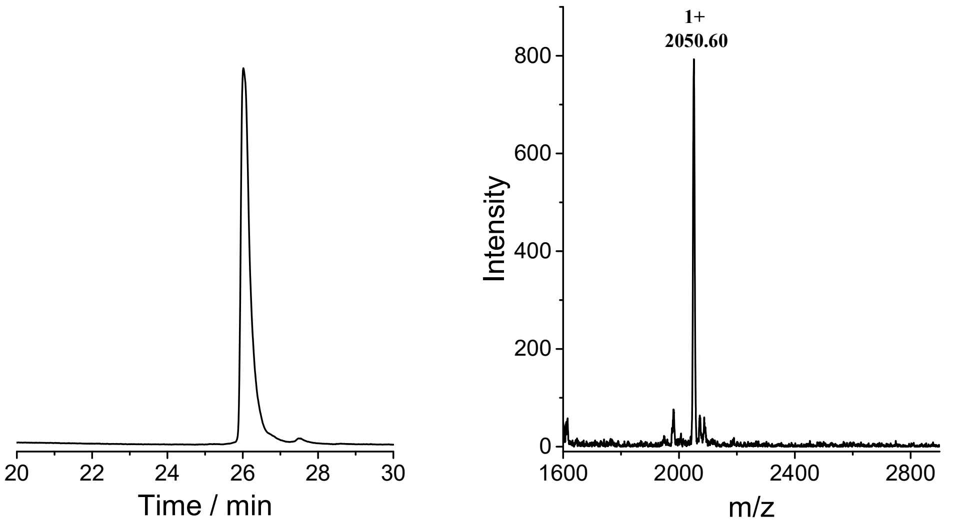


Figure S4. Analytical HPLC chromatogram and mass spectrum data of the purified **4**=NDI; m/z calculated: 2047.31, m/z found: 2050.60 $[M+H]^+$.

5-NDI:

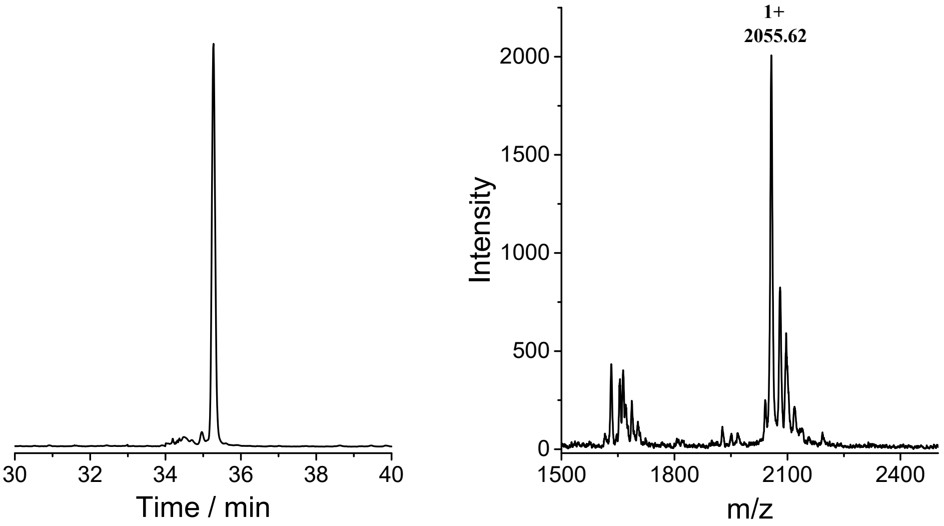


Figure S5. Analytical HPLC chromatogram and mass spectrum data of the purified **5**-NDI; m/z calculated: 2055.23, m/z found: 2055.62 $[M+H]^+$.

5-NDI-**5**:

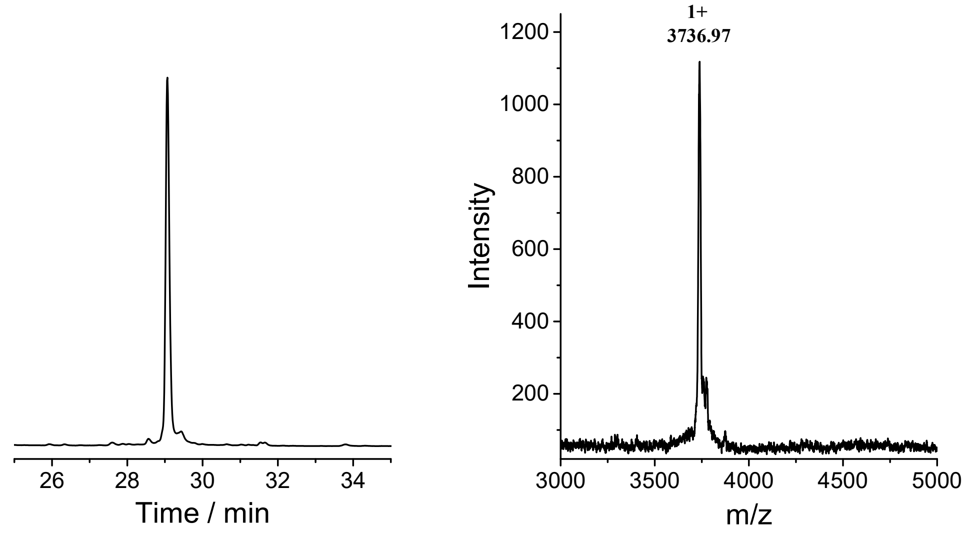


Figure S6. Analytical HPLC chromatogram and mass spectrum data of the purified **5**-NDI-**5**; m/z calculated: 3732.04, m/z found: 3736.97 $[M+H]^+$.

6-NDI:

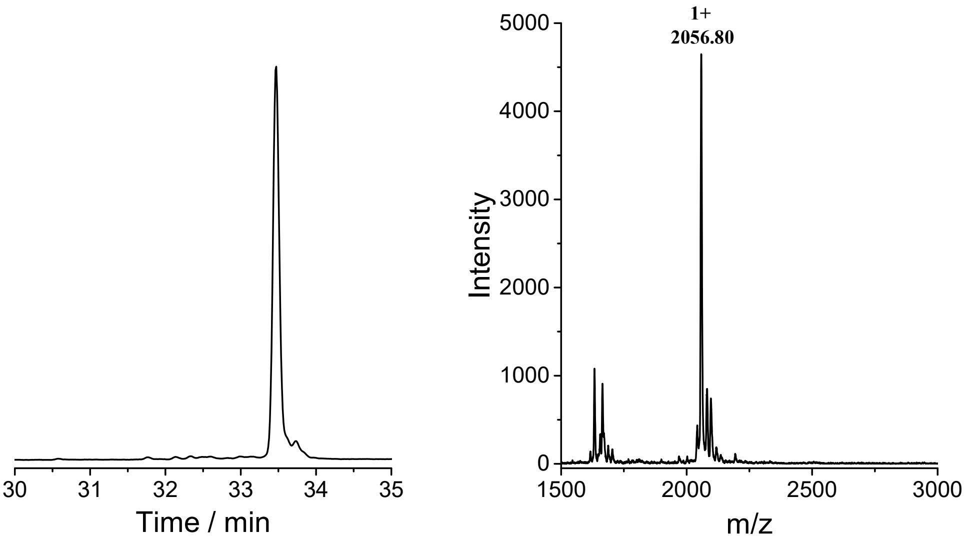


Figure S7. Analytical HPLC chromatogram and mass spectrum data of the purified **6**-NDI; m/z calculated: 2055.23, m/z found: 2056.80 $[M+H]^+$.

6-NDI-**6**:

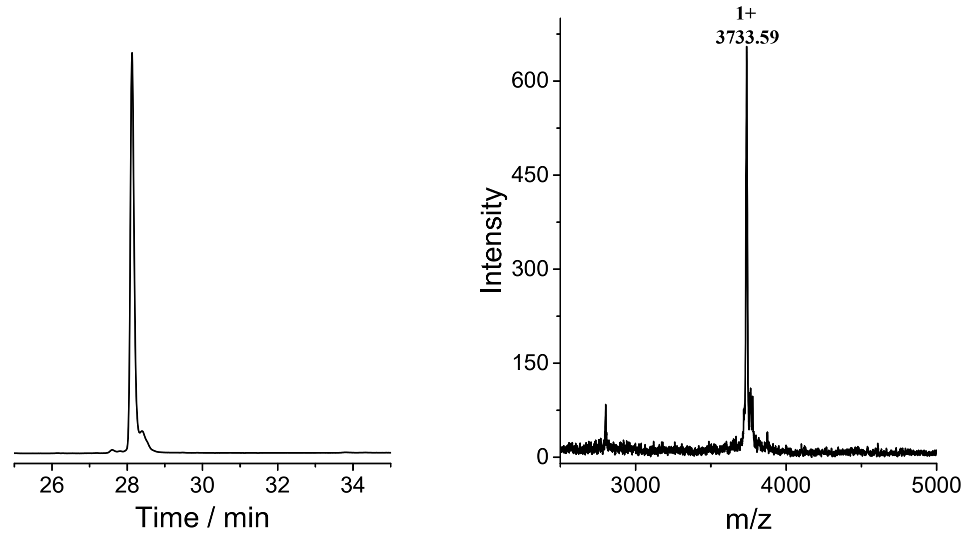


Figure S8. Analytical HPLC chromatogram and mass spectrum data of the purified **6**-NDI-**6**; m/z calculated: 3732.04, m/z found: 3733.59 $[M+H]^+$.

7-NDI:

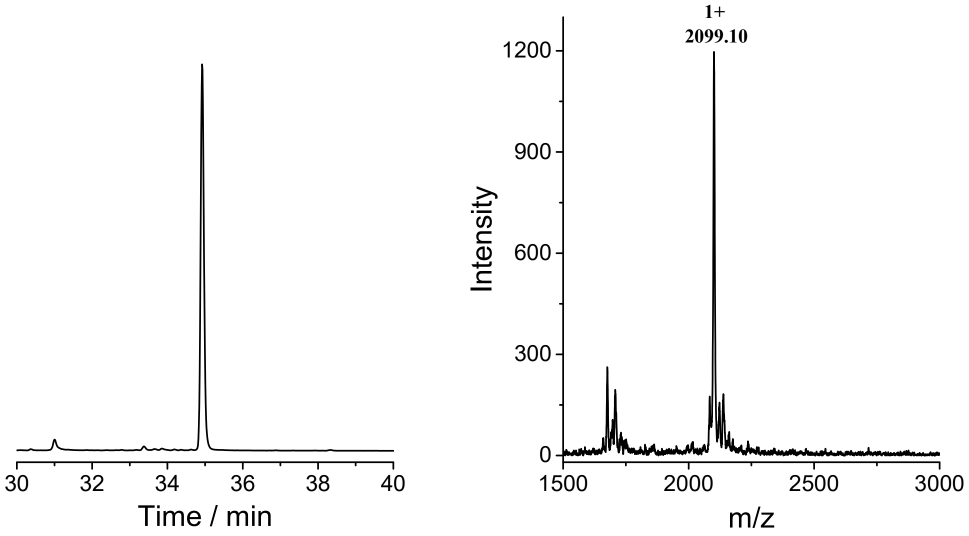


Figure S9. Analytical HPLC chromatogram and mass spectrum data of the purified **7**-NDI; m/z calculated: 2097.28, m/z found: 2099.10 $[M+H]^+$.

7-NDI-**7**:

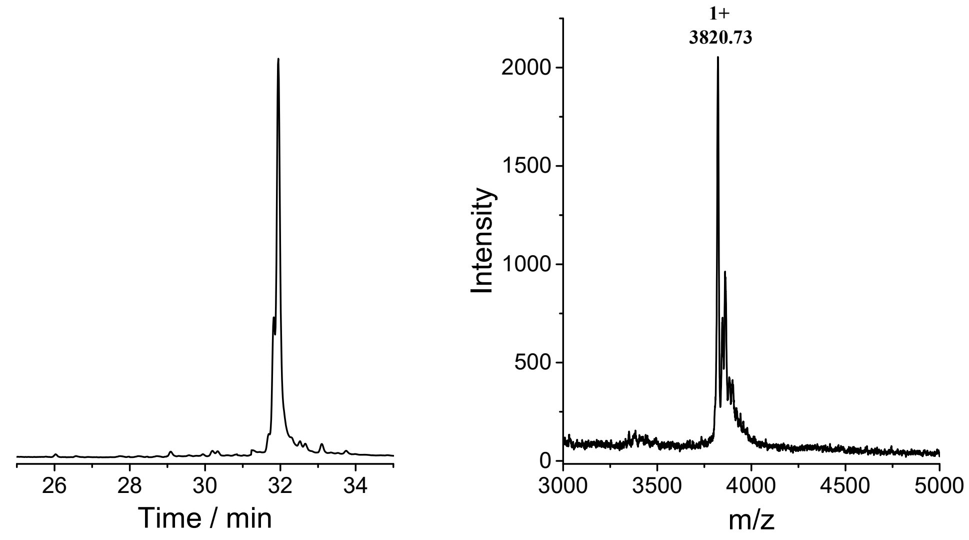


Figure S10. Analytical HPLC chromatogram and mass spectrum data of the purified **7**-NDI-**7**; m/z calculated: 3816.14, m/z found: 3820.73 $[M+H]^+$.

8-NDI:

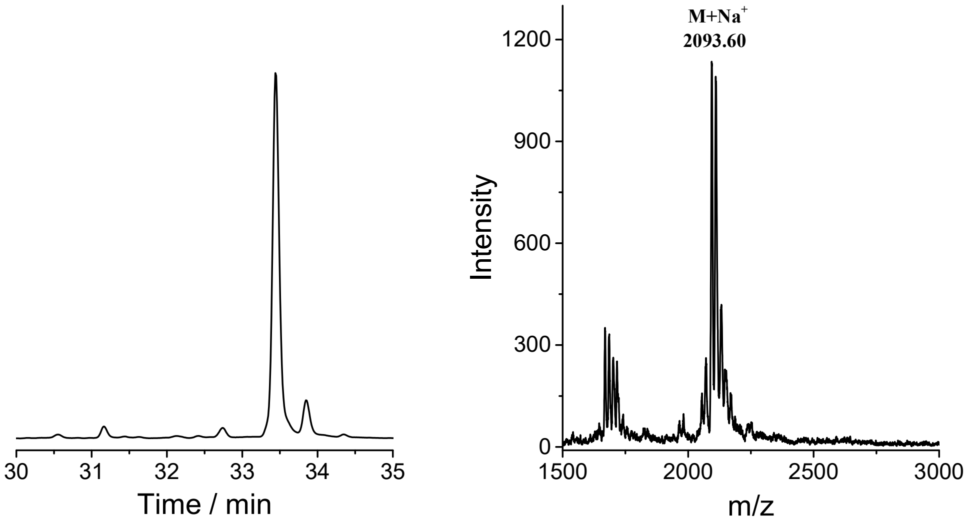


Figure S11. Analytical HPLC chromatogram and mass spectrum data of the purified **8**-NDI; m/z calculated: 2070.25, m/z found: 2093.60 $[M+Na]^+$.

8-NDI-**8**:

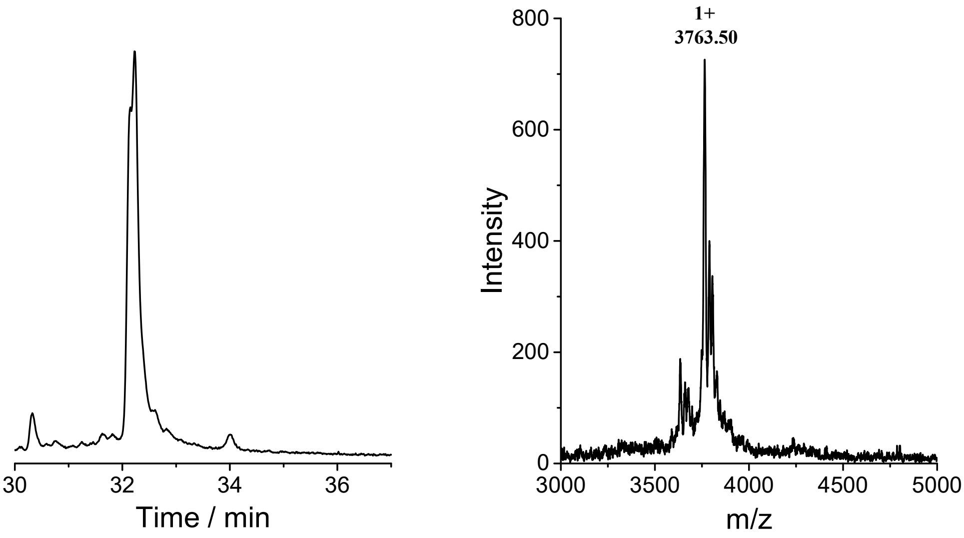


Figure S12. Analytical HPLC chromatogram and mass spectrum data of the purified **8**-NDI-**8**; m/z calculated: 3762.08, m/z found: 3763.50 $[M+H]^+$.

6-6

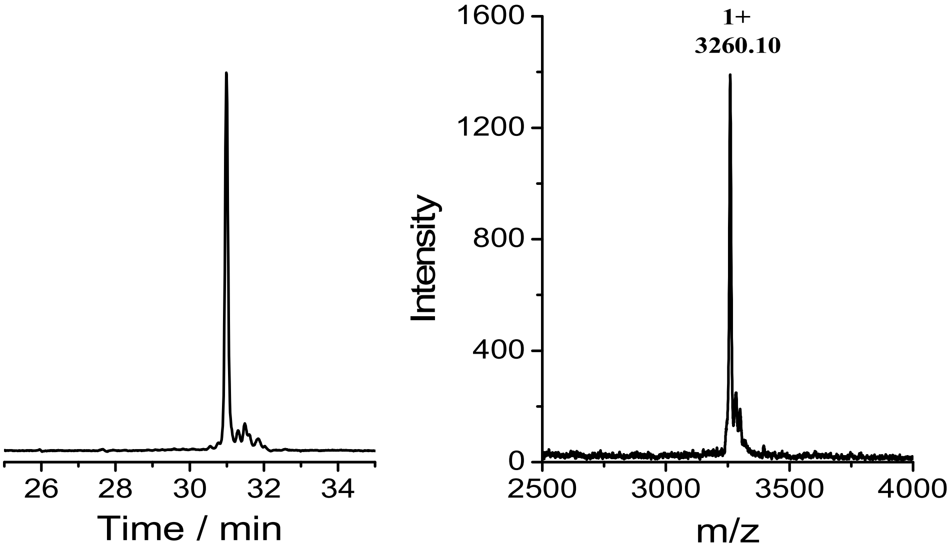


Figure S13. Analytical HPLC chromatogram and mass spectrum data of the purified **6-6**; m/z calculated: 3259.60, m/z found: 3260.10 $[M+H]^+$.

6-Bme-**6**

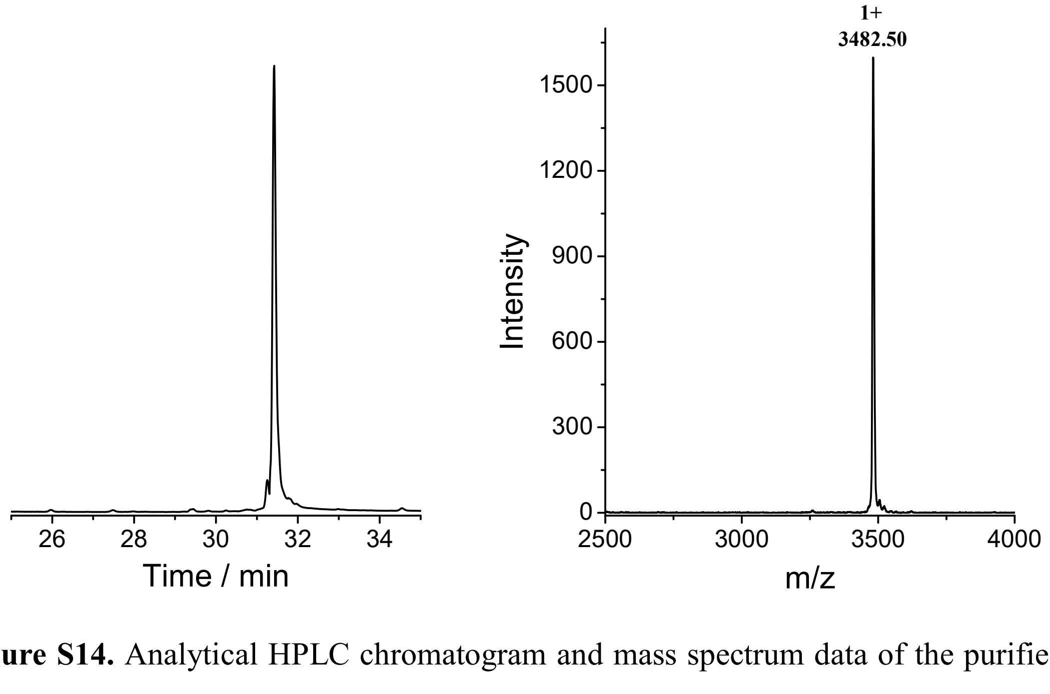


Figure S14. Analytical HPLC chromatogram and mass spectrum data of the purified **6**-Bme-**6**; m/z calculated: 3481.65, m/z found: 3482.50 $[M+H]^+$.

6-Bph-**6**

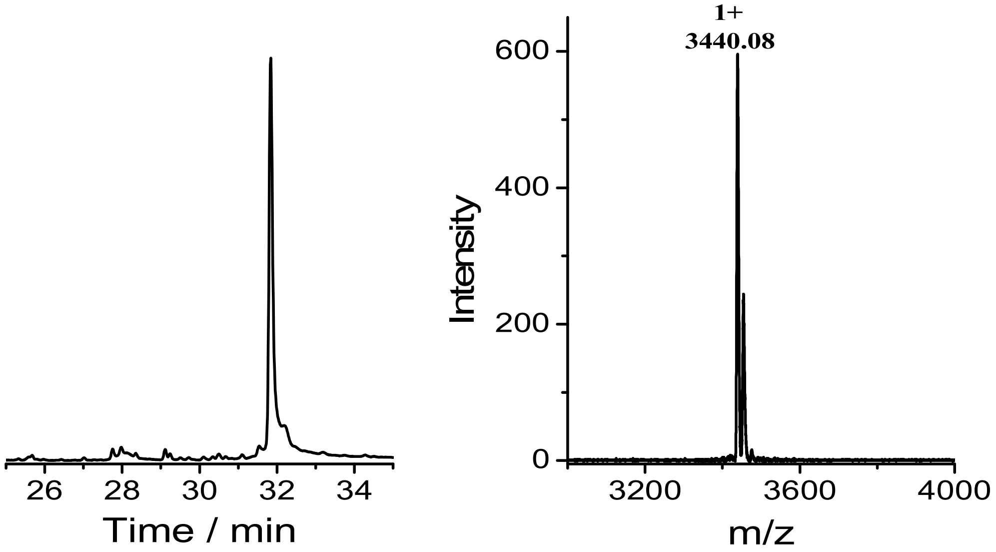


Figure S15. Analytical HPLC chromatogram and mass spectrum data of the purified **6**-Bph-**6**; m/z calculated: 3439.65, m/z found: 3440.08 $[M+H]^+$.

Enzymatic digestion of free peptides (1–4) and their NDI conjugates

1:

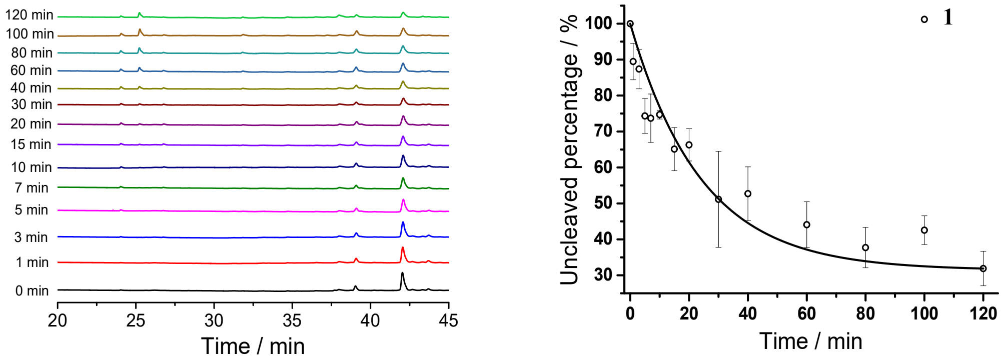


Figure S16. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 μM, peptide concentration (**1**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

2:

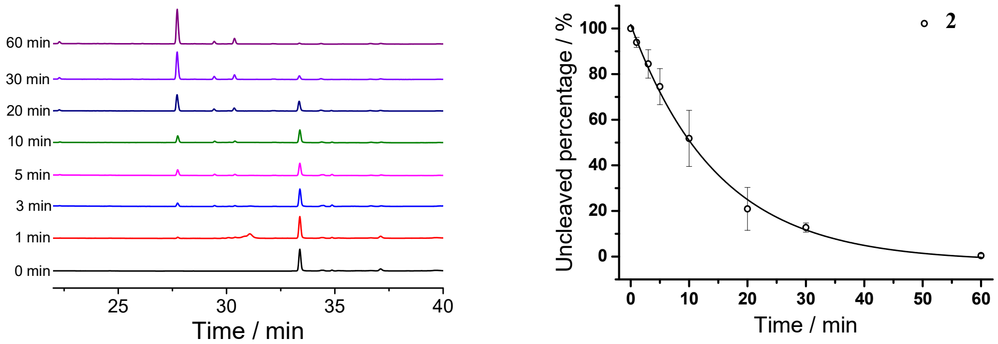


Figure S17. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 μM, peptide concentration (**2**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

1=NDI:

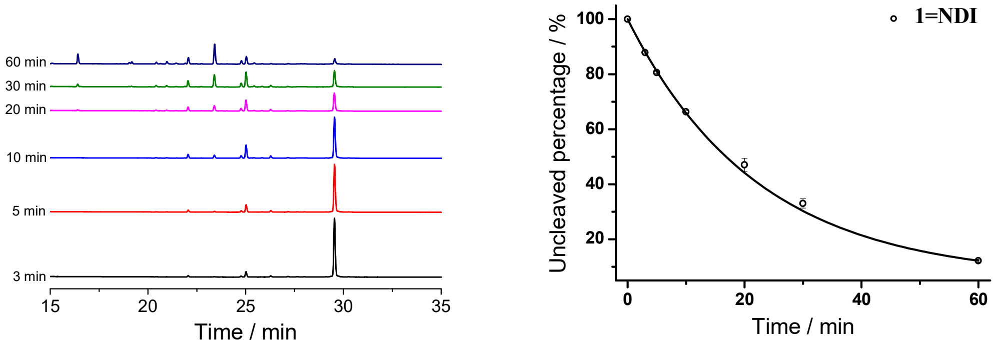


Figure S18. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 µM, peptide concentration (**1**=NDI): 50 µM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

2=NDI:

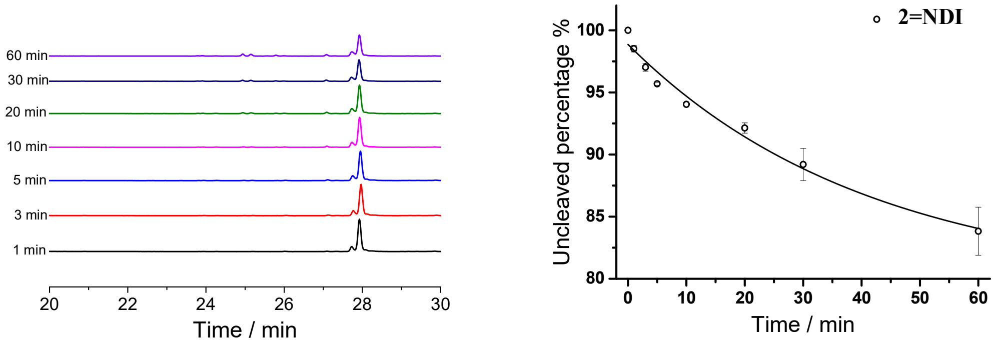


Figure S19. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 µM, peptide concentration (**2**=NDI): 50 µM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 2).

3:

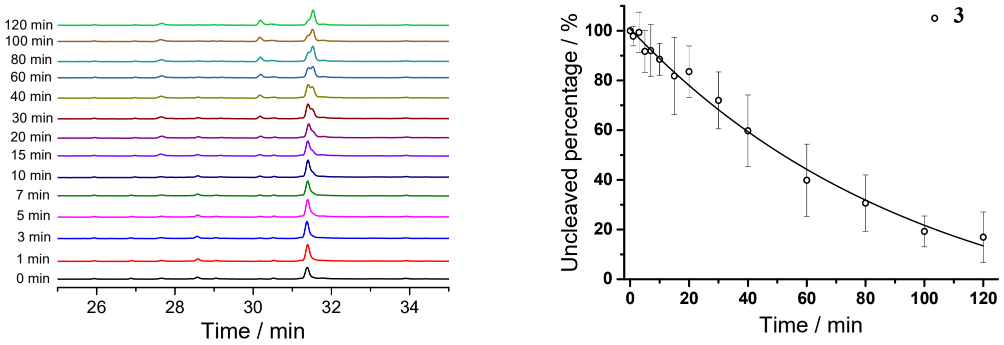


Figure S20. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 μM, peptide concentration (**3**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

4:

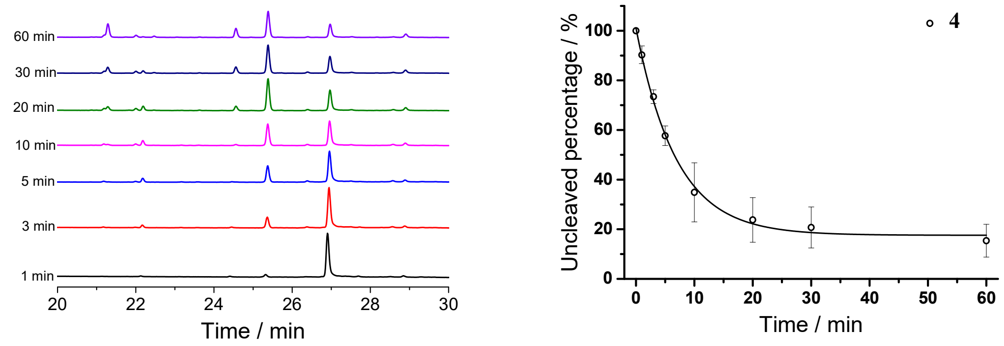


Figure S21. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 μM, peptide concentration (**4**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

3=NDI:

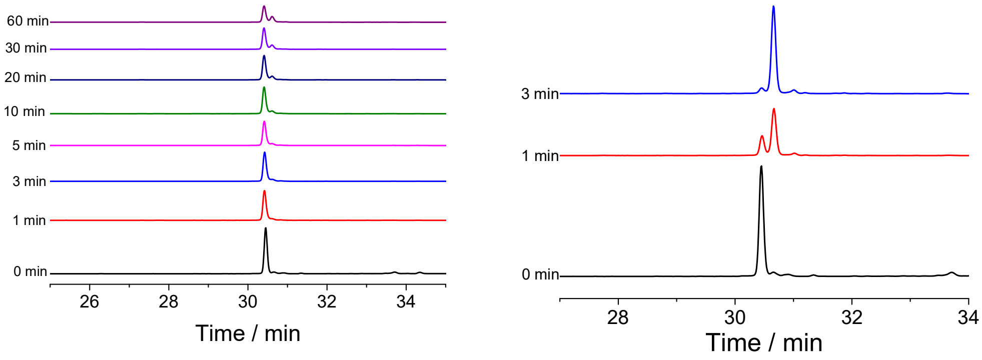


Figure S22. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.1 μM (left) or 10 μM (right), peptide concentration (**3**=NDI): 50 μM. Chromatograms were recorded under 363 nm.

4=NDI:

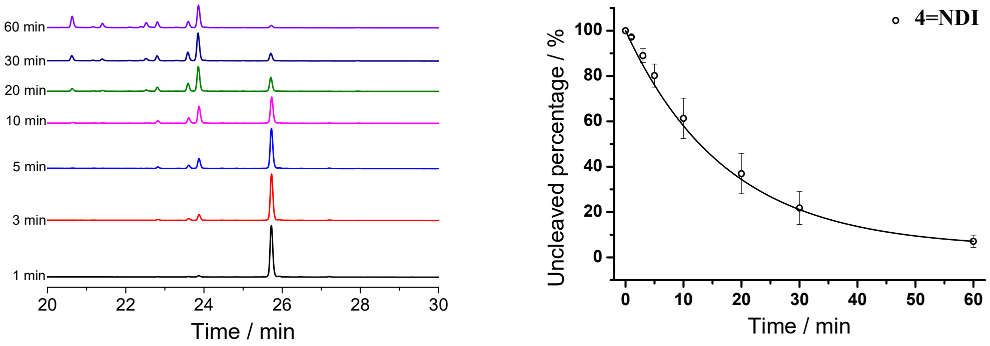


Figure S23. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.1 μM, peptide concentration (**4**=NDI): 50 μM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

CD Spectra of free peptides (1–4) and their NDI conjugates

1 and **1**=NDI:

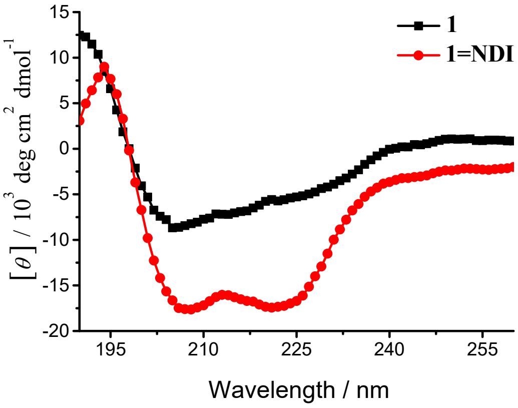


Figure S24. CD spectra of **1** and **1**=NDI in phosphate buffer (50 mM, pH 7.4) containing ~12 vol% ACN; concentration: 30 μM.

2 and **2**=NDI:

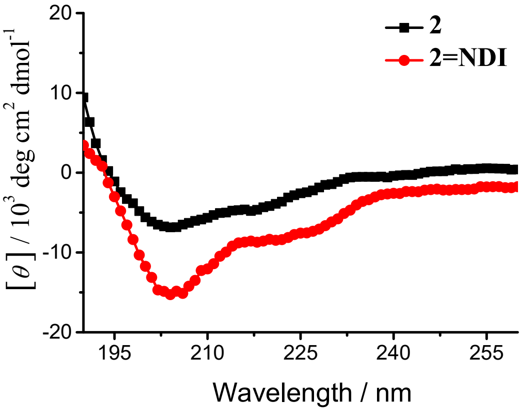


Figure S25. CD spectra of **2** and **2**=NDI in phosphate buffer (50 mM, pH 7.4); concentration: 30 μM.

3 and **3**=NDI:

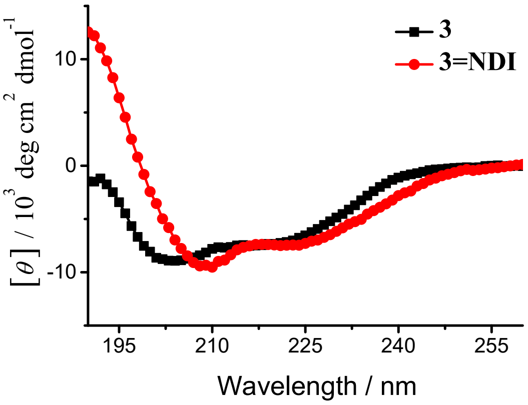


Figure S26. CD spectra of **3** and **3**=NDI in phosphate buffer (50 mM, pH 7.4); concentration: 30 μM.

4 and **4**=NDI:

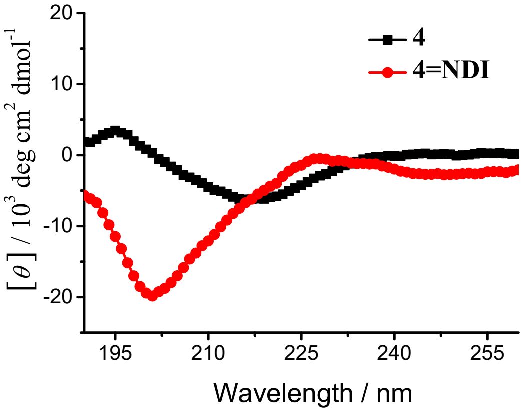


Figure S27. CD spectra of **4** and **4**=NDI in phosphate buffer (50 mM, pH 7.4); concentration: 30 μM.

Enzymatic digestion of free peptides (5–8) and their NDI conjugates

5:

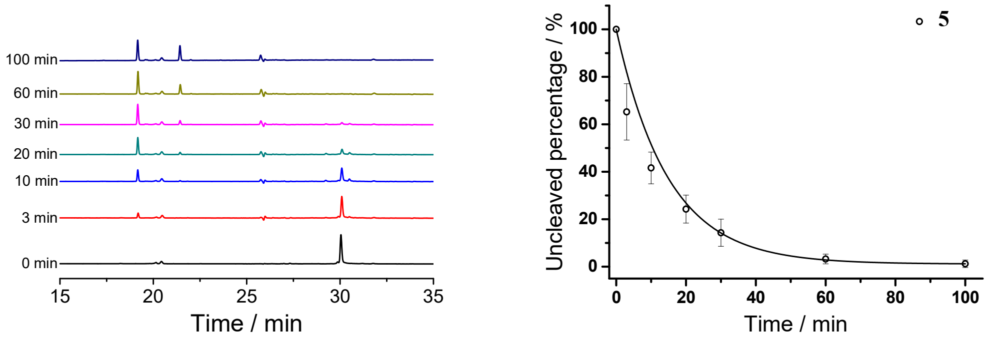


Figure S28. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 µM, peptide concentration (**5**): 50 µM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

5-NDI:

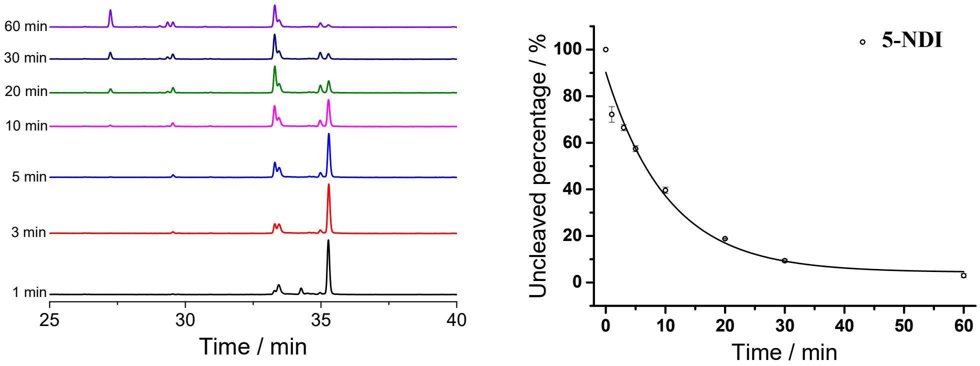


Figure S29. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 µM, peptide concentration (**5**-NDI): 50 µM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

5-NDI-**5**:

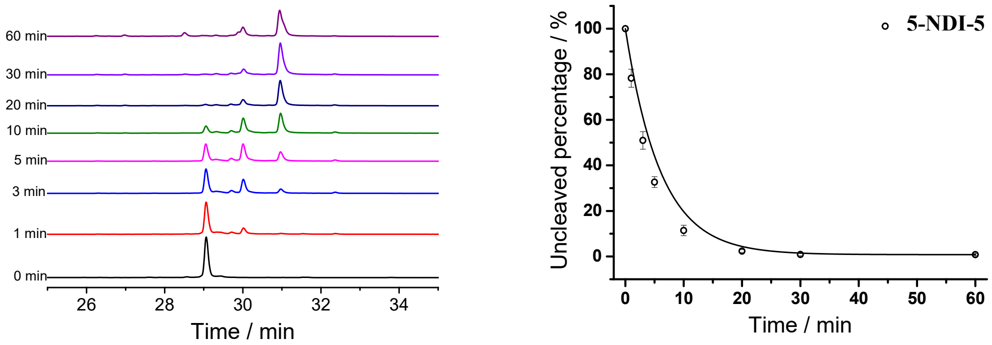


Figure S30. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 µM, peptide concentration (**5**-NDI-**5**): 50 µM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

6:

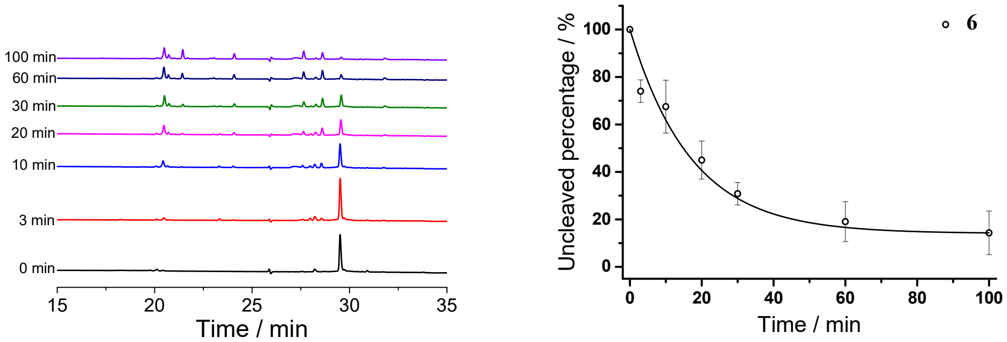


Figure S31. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 µM, peptide concentration (**6**): 50 µM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

6-NDI:

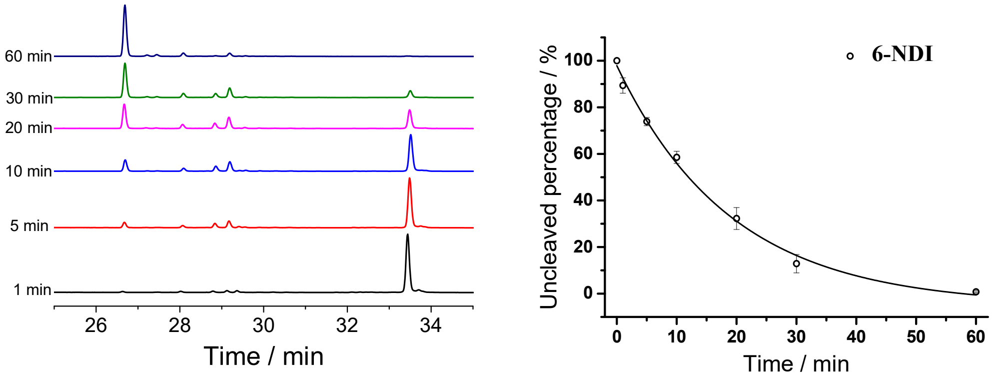


Figure S32. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 µM, peptide concentration (**6**-NDI): 50 µM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

6-NDI-**6**:

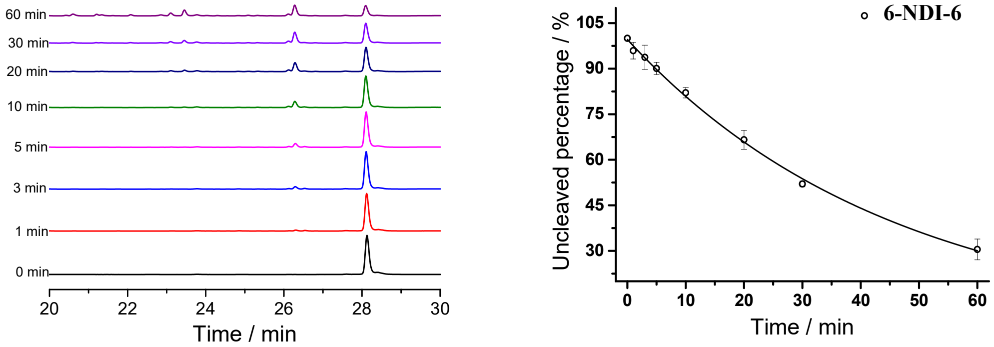


Figure S33. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 µM, peptide concentration (**6**-NDI-**6**): 50 µM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

7:

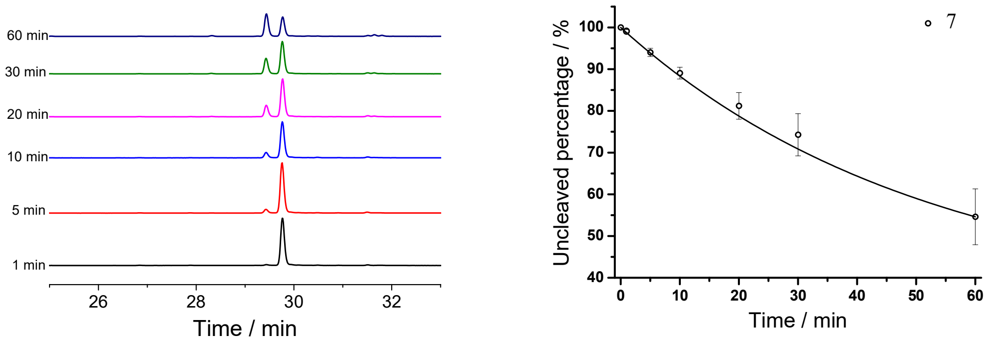


Figure S34. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 μM, peptide concentration (**7**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

7-NDI:

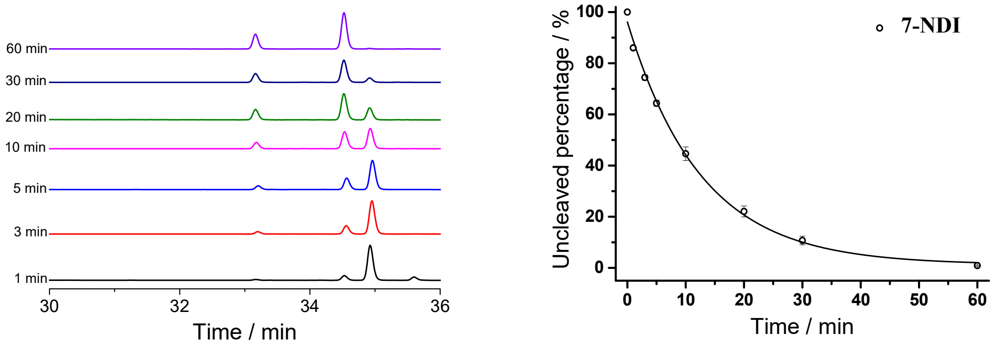


Figure S35. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 μM, peptide concentration (**7**-NDI): 50 μM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

7-NDI-**7**:

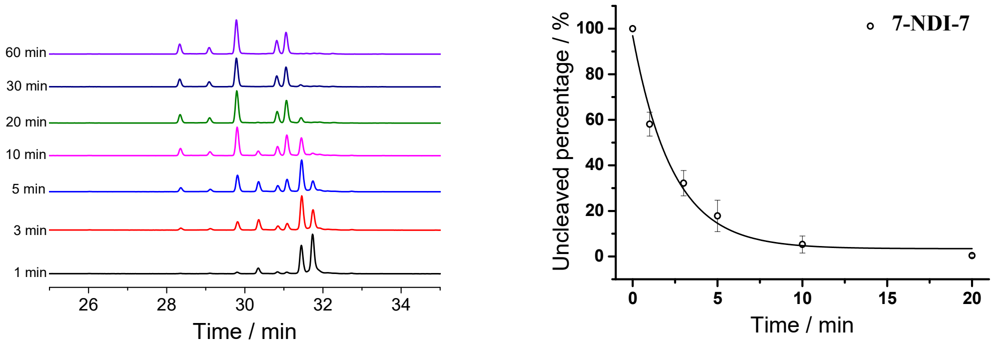


Figure S36. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 µM, peptide concentration (**7**-NDI-**7**): 50 µM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

8:

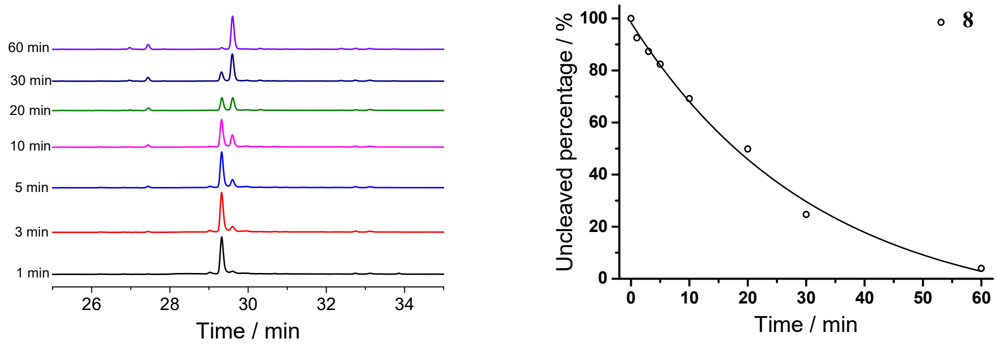


Figure S37. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.02 μM, peptide concentration (**8**): 50 μM. Chromatograms were recorded under 280 nm.

8-NDI:

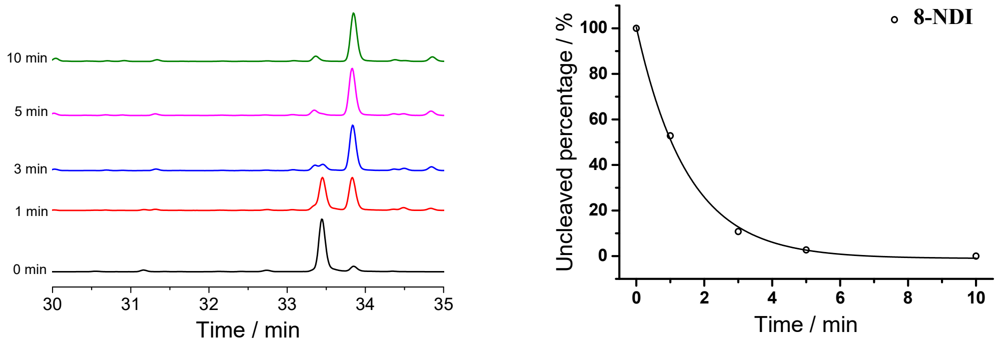


Figure S38. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 μM, peptide concentration (**8**-NDI): 50 μM. Chromatograms were recorded under 363 nm.

8-NDI-**8**:

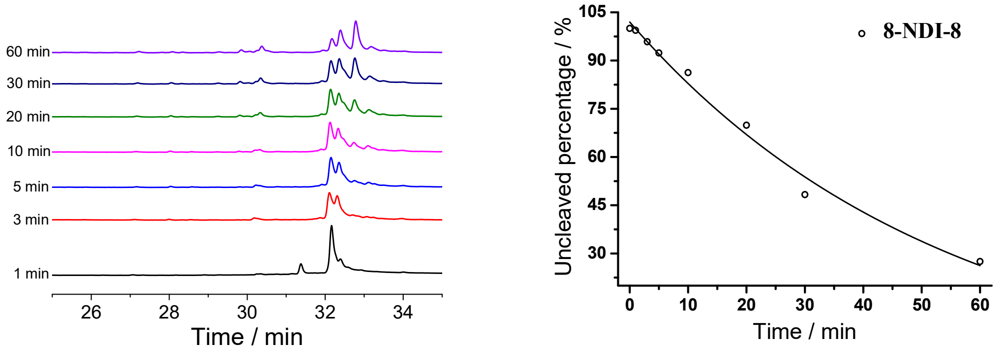


Figure S39. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 μM, peptide concentration (**8**-NDI-**8**): 50 μM. Chromatograms were recorded under 363 nm.

6:

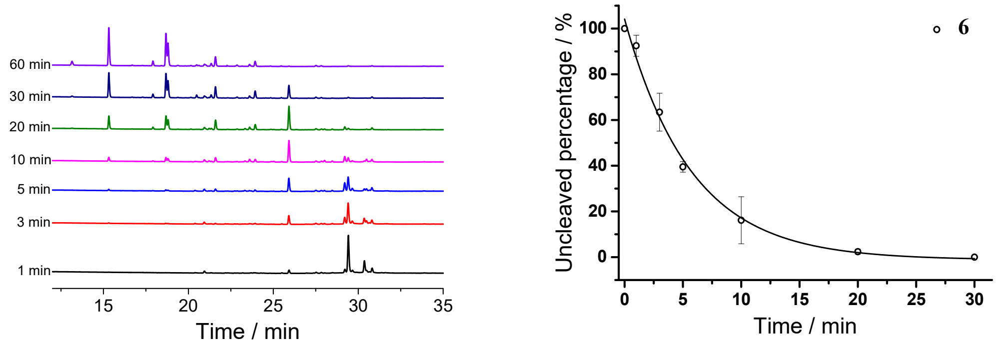


Figure S40. Kinetics of peptide digestion by proteinase K in 100 mM phosphate buffer, pH 7.4; proteinase K concentration: 0.01 μM, peptide concentration (**6**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

6-NDI-**6**:

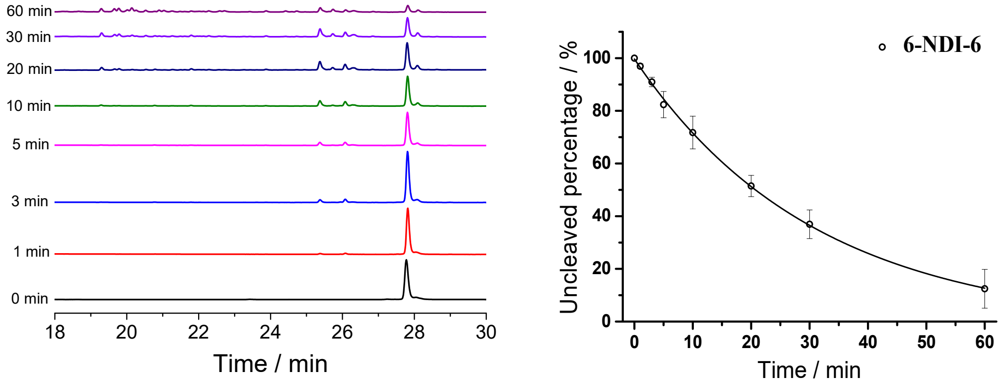


Figure S41. Kinetics of peptide digestion by proteinase K in 100 mM phosphate buffer, pH 7.4; proteinase K concentration: 0.1 μM, peptide concentration (**6**-NDI-**6**): 50 μM. Chromatograms were recorded under 363 nm. Data were presented as mean ± s.d. (n = 3).

6-6

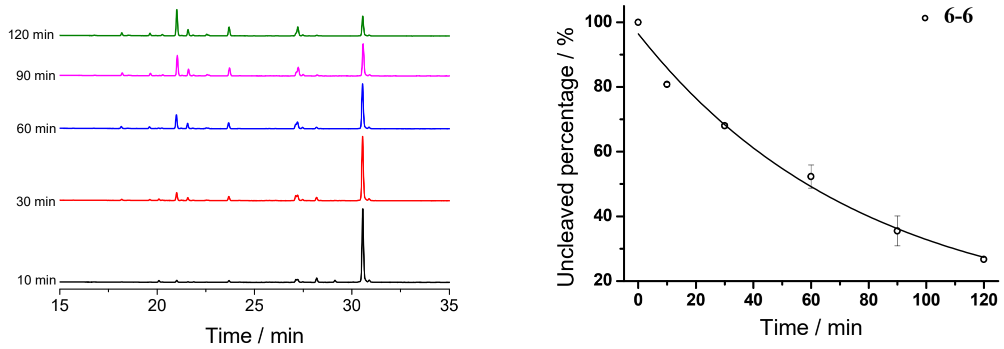


Figure S42. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.1 μM, peptide concentration (**6-6**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

6-Bme-**6**

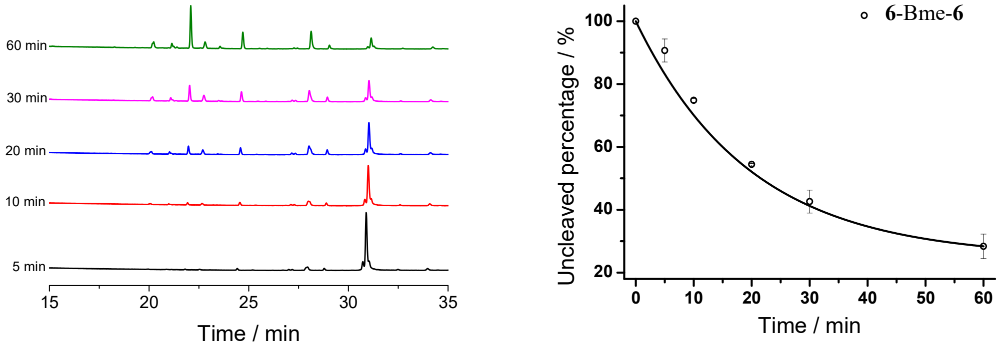


Figure S43. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 0.1 μM, peptide concentration (**6**-Bme-**6**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

6-Bph-**6**

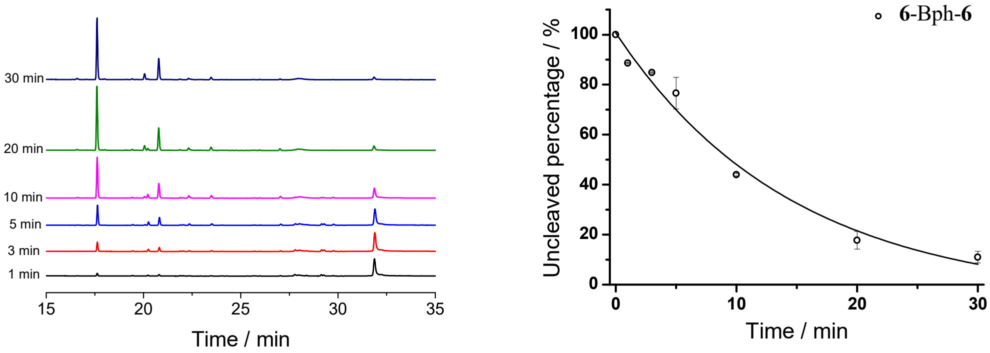
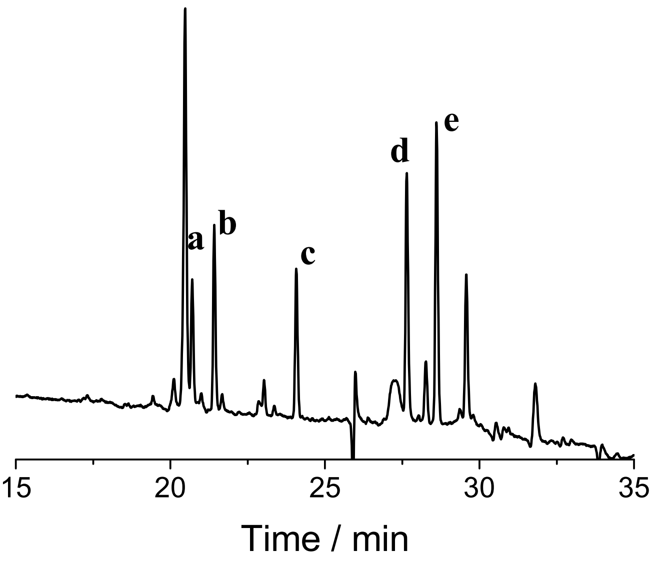
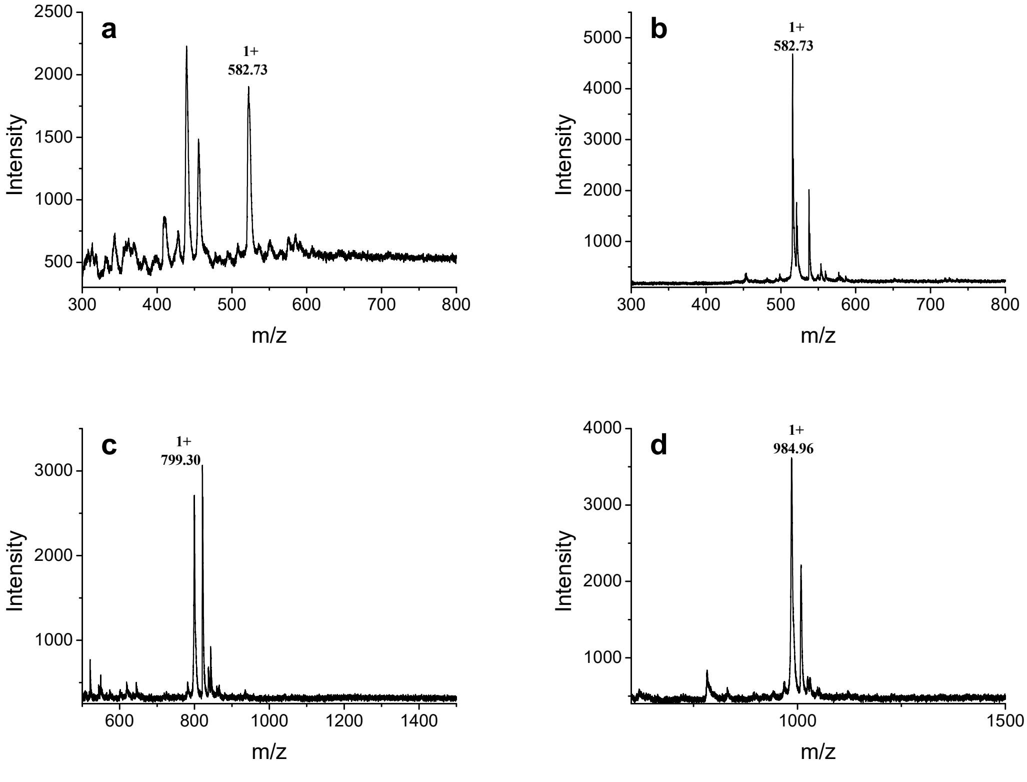


Figure S44. Kinetics of peptide digestion by chymotrypsin in 100 mM phosphate buffer, pH 7.4; chymotrypsin concentration: 10 μM, peptide concentration (**6**-Bph-**6**): 50 μM. Chromatograms were recorded under 280 nm. Data were presented as mean ± s.d. (n = 3).

HPLC and mass spectrometry (MS) analysis of cleavage sites

6





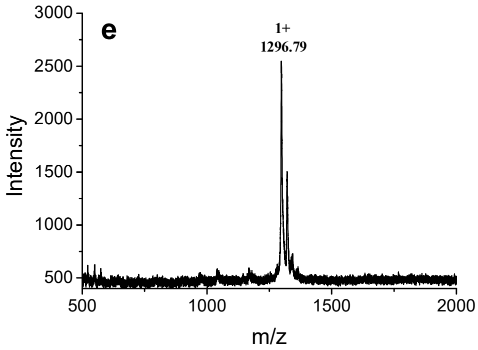
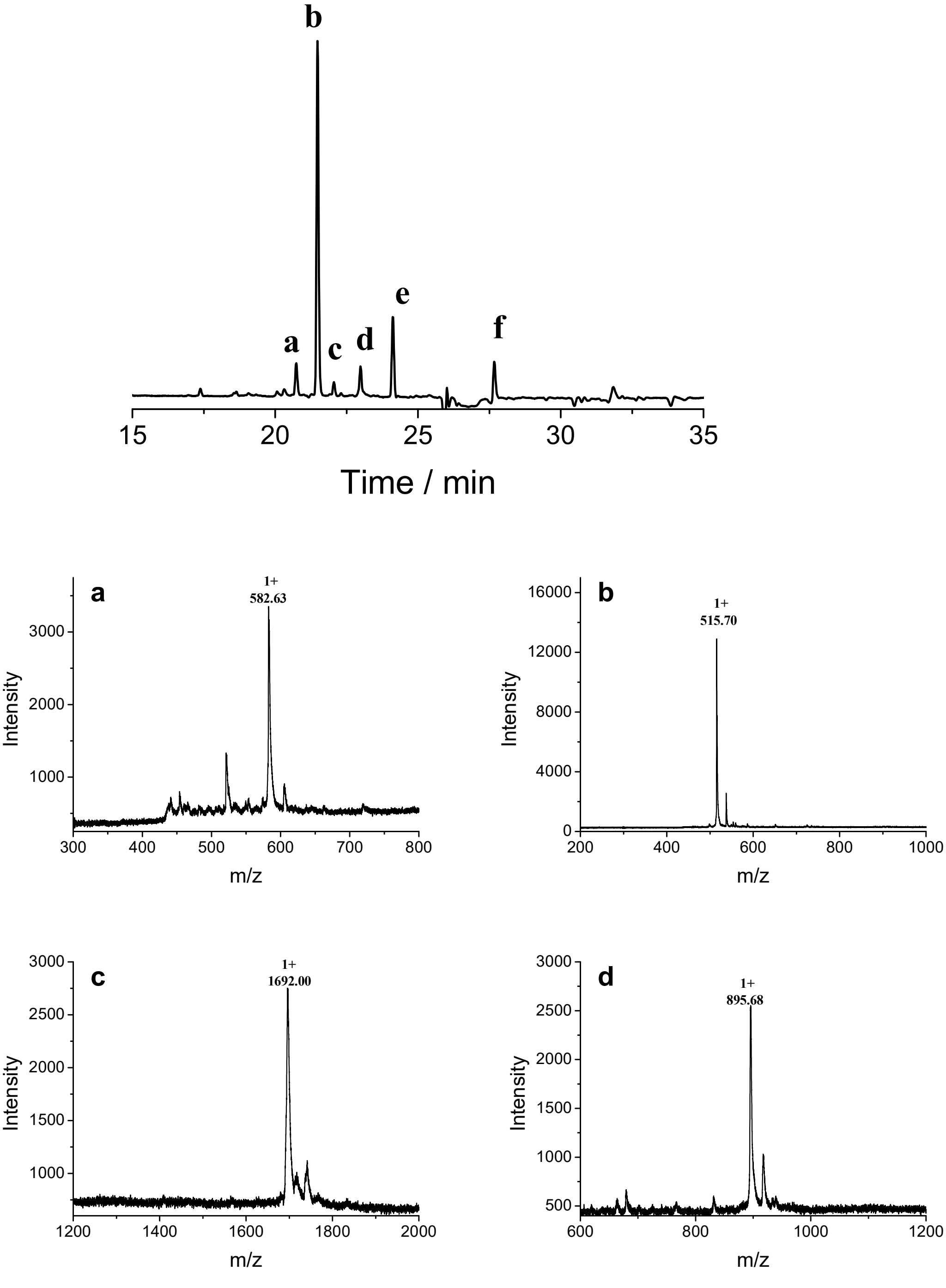


Figure S45. Chymotrypsin digestion HPLC and mass spectrometry analysis of **6**. **6** was digested with chymotrypsin and purified by HPLC. Then, the isolated fragments were analyzed by mass spectrometry. From top to bottom: Chromatogram of digested sample in UV channel (280 nm), mass spectra of peaks a-e labeled in the chromatograms.

Assignment of the peptide fragments:

Peak No.	**Sequence**	**m/z expected**	**m/z ($M+H^+$) found**
a	SEYW	583.6	582.73
b	WAQL	516.6	515.57
c	Ac-LTFSEY	800.86	799.30
d	Ac-LTFSEYW	987.08	984.96
e	Ac-LTFSEYWAQL	1299.45	1296.79

6-6


b
a
c
d
e
f
15
20
25
30
35
Time / min
a
1+
582.63
Intensity
1000
2000
3000
300
400
500
600
700
800
m/z
b
1+
515.70
Intensity
0
4000
8000
12000
16000
200
400
600
800
1000
m/z
c
1+
1692.00
Intensity
1000
1500
2000
2500
3000
1200
1400
1600
1800
2000
m/z
d
1+
895.68
Intensity
500
1000
1500
2000
2500
3000
600
800
1000
1200
m/z

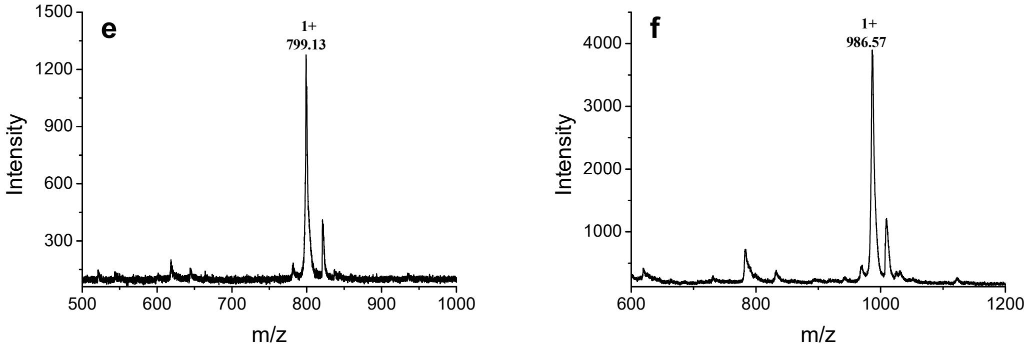


Figure S46. Chymotrypsin digestion HPLC and mass spectrometry analysis of **6-6**. **6-6** was digested with chymotrypsin and purified by HPLC. Then, the isolated fragments were analyzed by mass spectrometry. From top to bottom: Chromatogram of digested sample in UV channel (280 nm), mass spectra of peaks a-f labeled in the chromatograms.

Assignment of the peptide fragments:

Peak No.	**Sequence**	**m/z expected**	**m/z (M+H$^+$) found**
a	SEYW	583.6	582.63
b	WAQL	516.6	515.70
c	WAQLCSAA-NH_2 WAQLCSAA-NH_2	1693.98	1692.00
d	SEYWAQL	895.97	895.68
e	Ac-LTFSEY	800.86	799.13
f	Ac-LTFSEYW	987.08	986.57

6-NDI

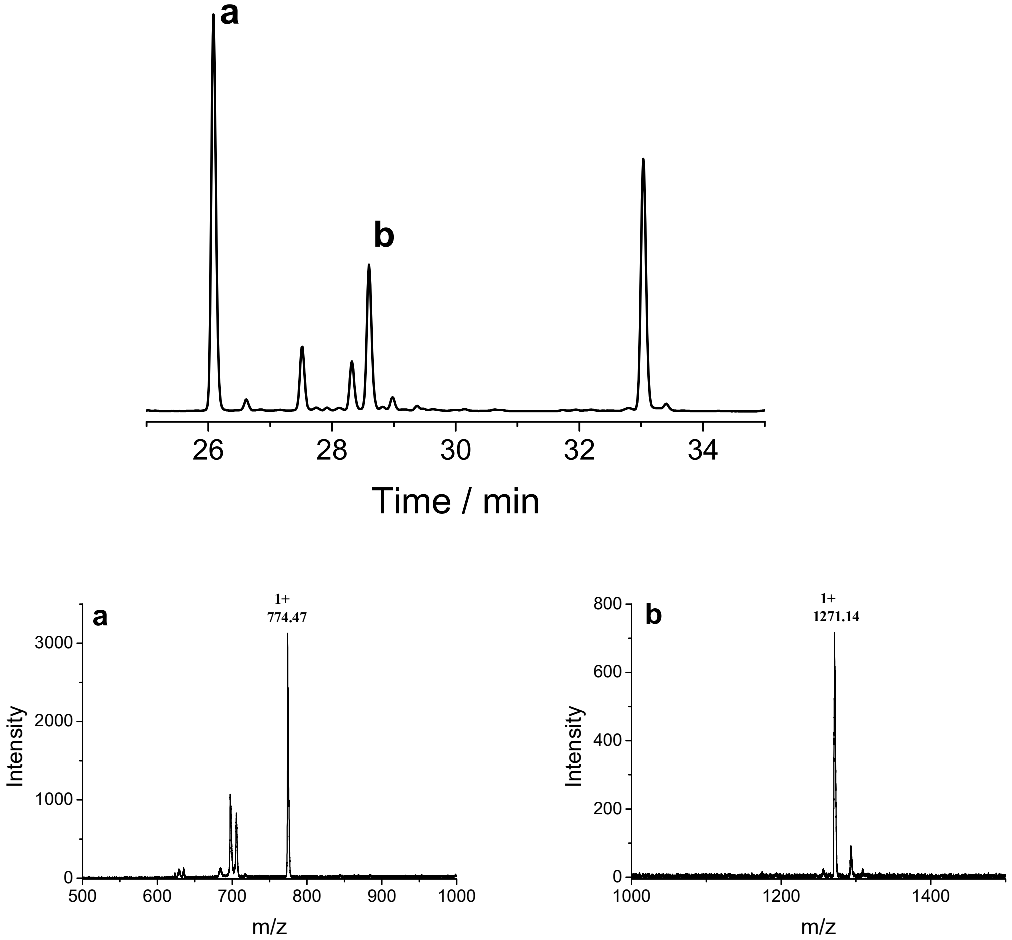


Figure S47. Chymotrypsin digestion HPLC and mass spectrometry analysis of **6**-NDI. **6**-NDI was digested with chymotrypsin and purified by HPLC. Then, the isolated fragments were analyzed by mass spectrometry. From top to bottom: Chromatogram of digested sample in UV channel (363 nm), mass spectra of peaks a and b labeled in the chromatograms.

Assignment of the peptide fragments:

Peak No.	**Sequence**	**m/z expected**	**m/z (M+H$^+$) found**
a	CSAA-NH$_2$ NDI	774.8	774.47
b	WAQLCSAA-NH$_2$ NDI	1272.43	1271.14

6-NDI-**6**

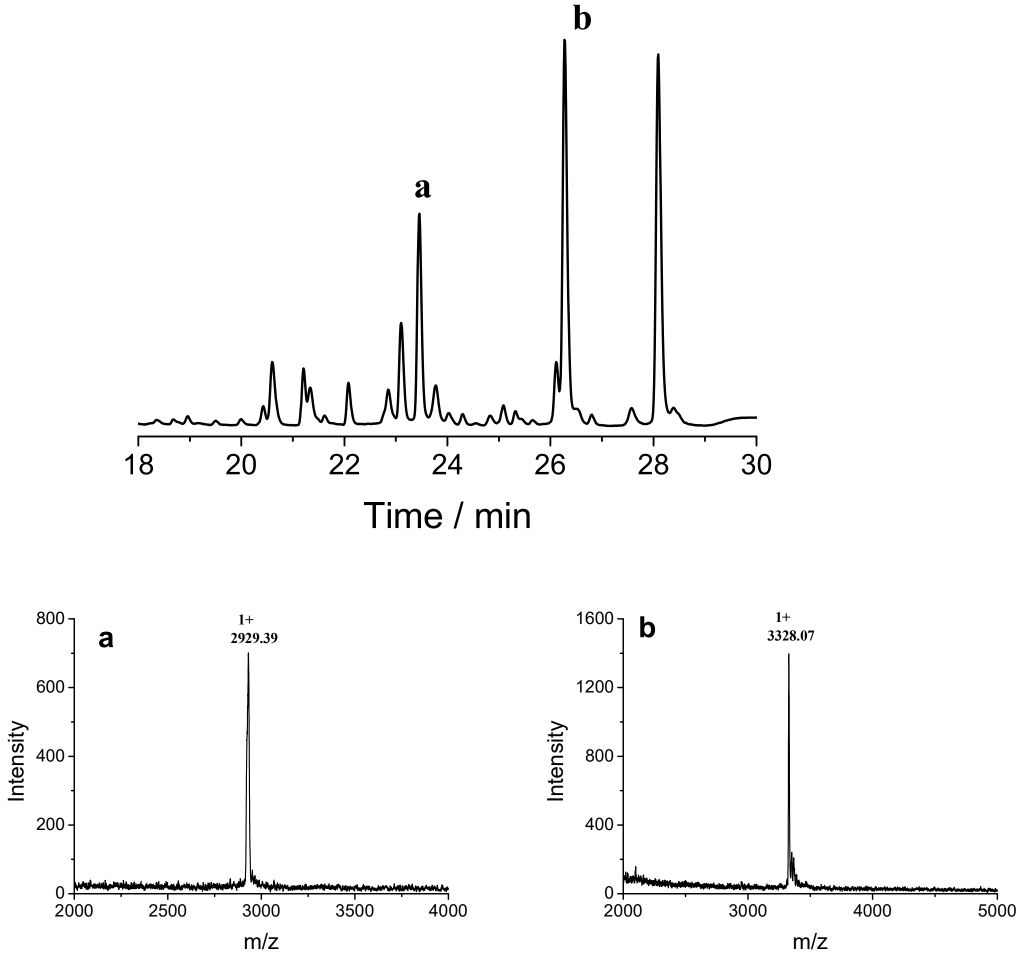


Figure S48. Chymotrypsin digestion HPLC and mass spectrometry analysis of **6**-NDI-**6**. **6**-NDI-**6** was digested with chymotrypsin and purified by HPLC. Then, the isolated fragments were analyzed by mass spectrometry. From top to bottom: Chromatogram of digested sample in UV channel (363 nm), mass spectra of peaks a and b labeled in the chromatograms.

Assignment of the peptide fragments:

Peak No.	**Sequence**	**m/z expected**	**m/z ($M+H^+$) found**
a	SEYWAQLCSAA-NH_2 NDI SEYWAQLCSAA-NH_2	2929.18	2929.39
b	Ac-LTFSEYWAQLCSAA-NH_2 NDI SEYWAQLCSAA-NH_2	3328.62	3328.07

Data on HREMD simulations

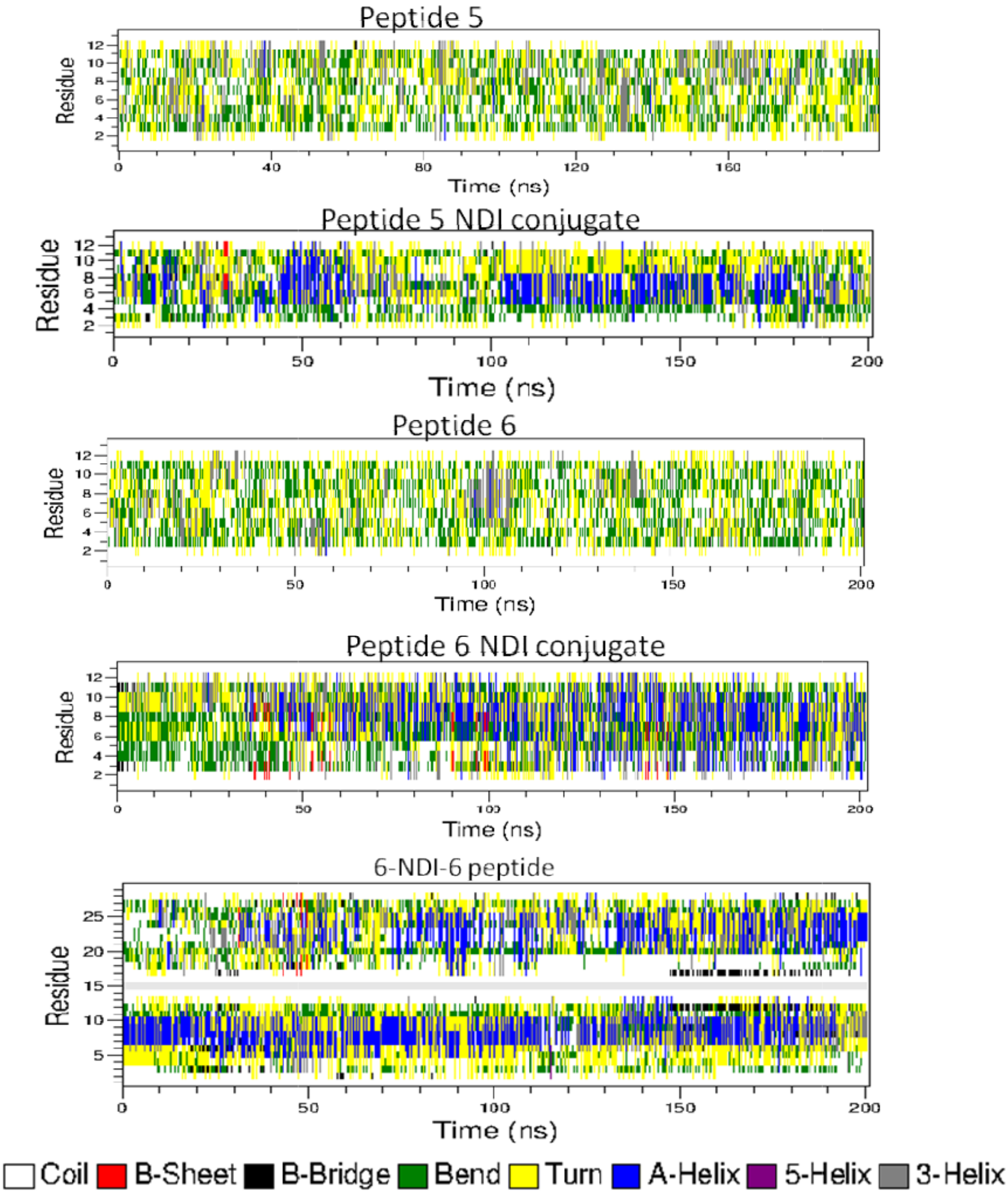


Figure S49. Evolution of secondary structures of peptides **5** and **6** and their NDI conjugates.

Data on HREMD simulations

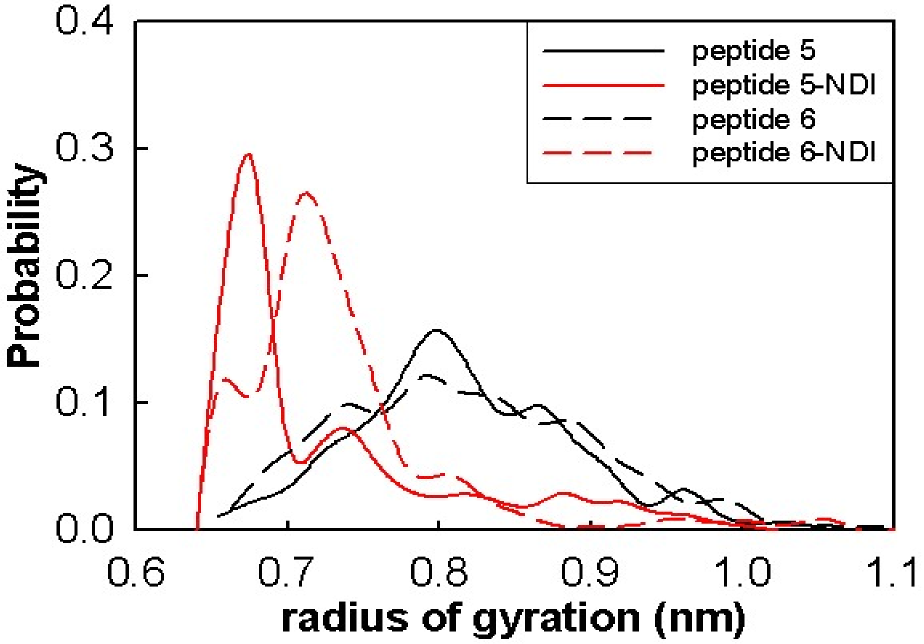


Figure S50. Probability distribution of the radius of gyration of peptide **5**, **6** and their NDI conjugates.

CD Spectra of free peptides (5 and 6) and their NDI conjugates

5, **5**-NDI and **5**-NDI-**5**:

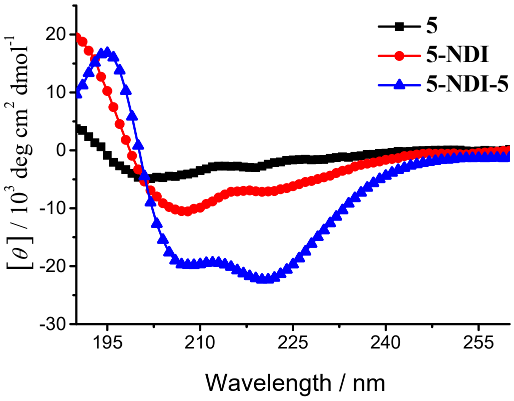


Figure S51. CD spectra of **5**, **5**-NDI and **5**-NDI-**5** in phosphate buffer (50 mM, pH 7.4) containing ~5 vol% ACN; concentration: 30 μM.

6, **6**-NDI and **6**-NDI-**6**:

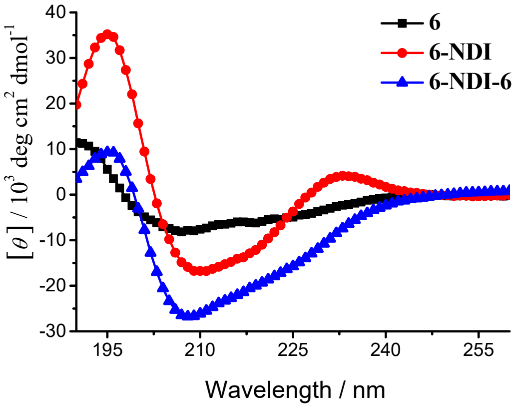


Figure S52. CD spectra of **6**, **6**-NDI and **6**-NDI-**6** in phosphate buffer (50 mM, pH 7.4) containing ~5 vol% ACN; concentration: 30 μM.

Fluorescence spectra of peptides

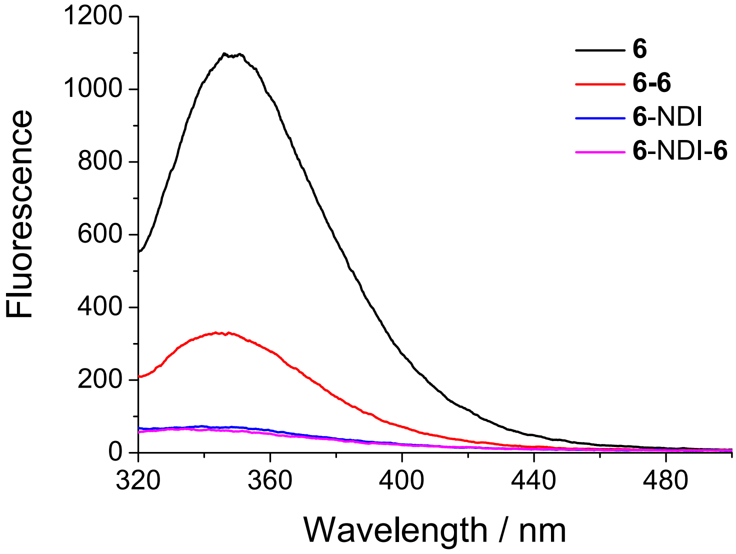


Figure S53. Fluorescence emission spectra of **6** (1.4 μM), **6-6** (0.7 μM), **6**-NDI (1.4 μM), and **6**-NDI-**6** (0.7 μM) in 10 mm phosphate buffer; excitation wavelength: 280 nm.

Reduction kinetics of NDI-peptide conjugates in redox buffers

5-NDI:

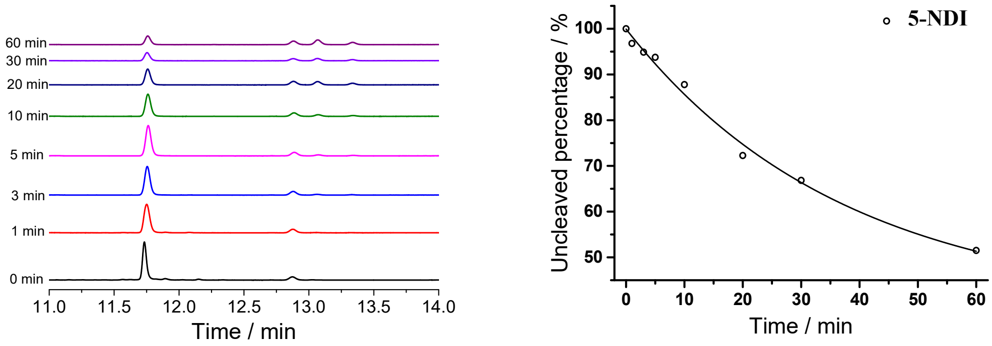


Figure S54. Kinetics of reduction of **5**-NDI in 10 mM DTT buffer (100 mM phosphate buffer, pH 7.4); concentration of **5**-NDI: 50 μM, chromatograms were recorded under 363 nm.

6-NDI-**6**:

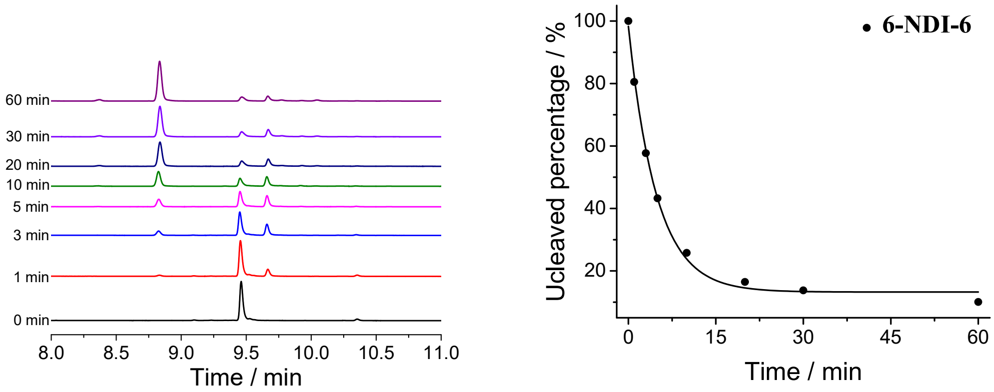


Figure S55. Kinetics of reduction of **6**-NDI-**6** in 10 mM DTT buffer (100 mM phosphate buffer, pH 7.4); concentration of **6**-NDI-**6**: 50 μM, chromatograms were recorded under 363 nm.

To confirm the reduction of disulfide bonds of 6-NDI-6 in cells:

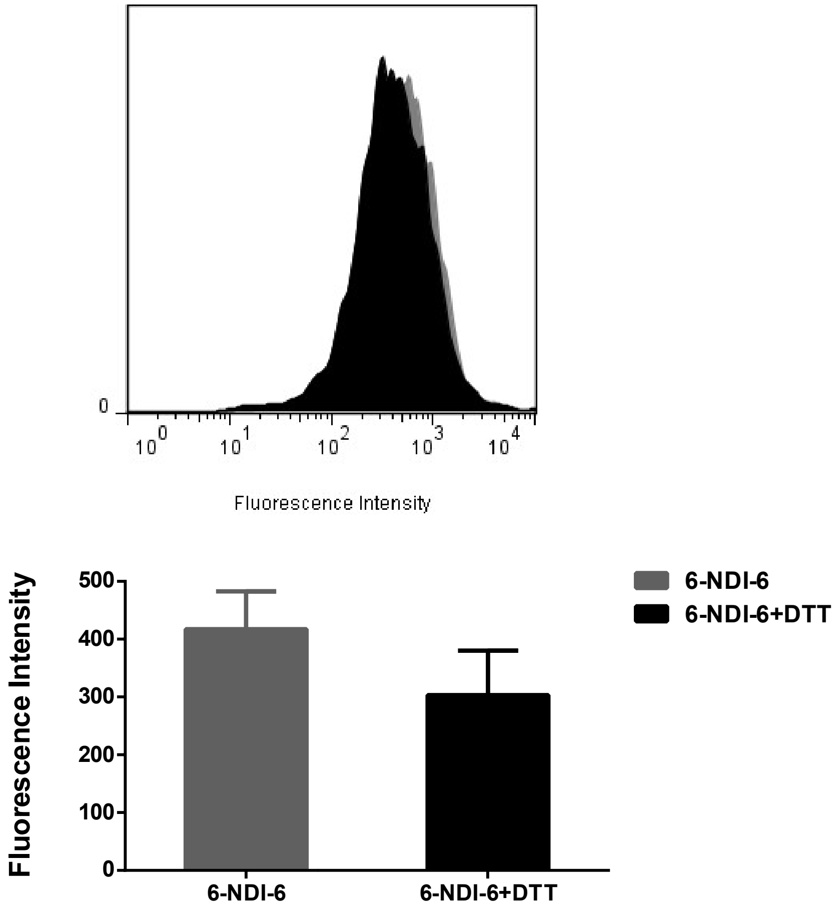


Figure S56. Flow cytometric analysis of MCF7 cells incubated with 1.0 μM **6**-NDI-**6**. Mean fluorescence intensities were presented as mean ± s.d. (n = 3). MCF7 cells were first incubated with 1.0 μM **6**-NDI-**6** for 24 h. After that, the medium was removed and 2 mM DTT was added. After ~10 min, the supernatant was removed and the cells were collected and analyzed by a Flow Cytometry.

Bioactivity of peptide and NDI-peptide conjugates in 10% serum

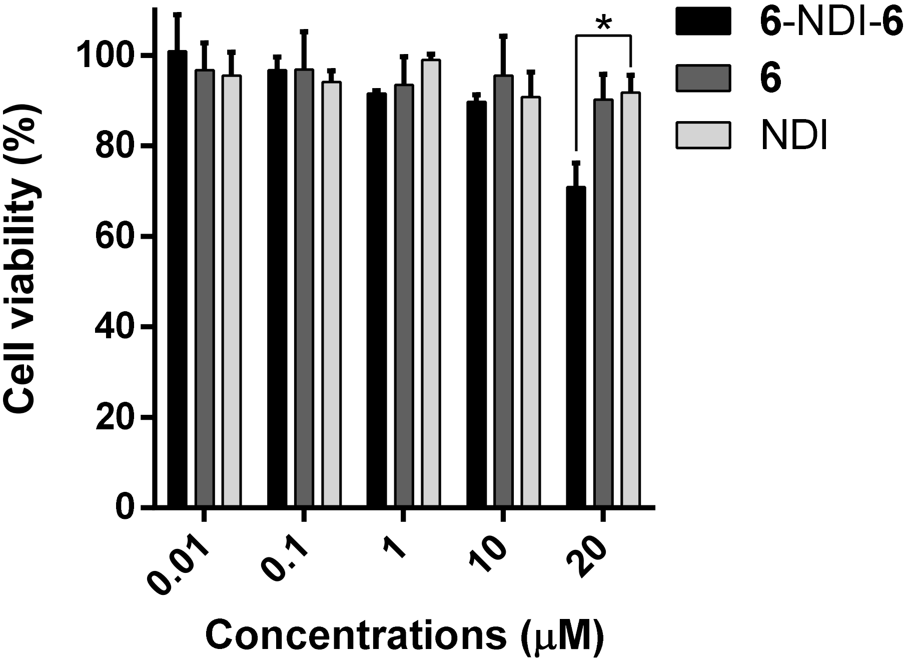


Figure S57. Viability of MCF7 cells determined using MTT assays. Cells were incubated with various concentrations of **6**-NDI-**6** (or **6** and NDI) in 10% FBS-containing medium for 24 h. Results are expressed as mean ± s.d. (n = 3). Single star denotes statistically significant differences in a t-test (P<0.05).