Supporting Information

Catalytic peptide/hemin complex from ester-amide exchange reaction mediated by deep eutectic solvents

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1. Experimental procedures

Materials

Hemin from bovine (≥90%), L-lactic acid (a, ≥98%), L-histidine (H, ≥99%), and L-aspartic acid (D, ≥98%) were purchased from Sigma Aldrich, USA. Glycine (G, 99%) was obtained from Across Organics, Belgium. Tetraethylammonium chloride (TEACl, >98%), L-leucine (L, >99%), L-arginine (R, >99%), and L-valine (V, >98%) were purchased from TCI, Japan. Dimethyl sulfoxide (DMSO, 99%), sodium phosphate monobasic (99%), and sodium phosphate dibasic (99%) were purchased from VETEC, USA. L-Phenylalanine (F, 99%), L-serine (S, 99%), and horseradish peroxidase were from Alfa Aesar, USA. 3,3',5,5'-tetramethylbenzidine (TMB) (99+%), L-methionine (M, 98%), L-cysteine (C, 98%), LC-MS grade water, and LC-MS grade acetonitrile were purchased from Thermo Fisher Scientific, USA. Hydrogen peroxide (H_2O_2, 35%) was obtained from SHOWA, Japan.

Peptide synthesis

Before the reaction started, 100 μL aqueous solution of 100 mM TEACl was added in a 1.5 mL microtube, and then dried in a 95 °C oven for 1 day. Three 100 μL aqueous solutions, each containing 100 mM lactic acid, histidine, and phenylalanine, respectively, were added to the dry TEACl to form a reaction mixture with a molar ratio of TEACl:a:H:F=100:100:100:100. The reaction mixture was placed into a temperature-controlled oven and reacted at 120 °C for 3 days. During the reaction process, 100 μL of water was added every day to homogenize the reaction mixture.

Formation of peptide/hemin complex

The peptide mixtures were dissolved in 40 μL of 12.5 mM hemin DMSO solution. Then, 960 μL of 100 mM pH 7.0 phosphate buffer solution was added to induce assembly. The pH value of the buffer was measured by using an OHAUS ST3100M pH meter.

Characterization

Analysis of hemin binding capability

The peptide/hemin assemblies were diluted 30-fold with 100 mM pH 7.0 phosphate buffer solution to a concentration equivalent to 10 μM hemin. The samples were then transferred into a quartz cuvette with a path length of 1 cm and analyzed using a Shimadzu UV-1900i spectrophotometer. The scan range was set from 250 nm to 700 nm with a data interval of 0.5 nm. The peptide samples without hemin were used as the background to avoid possible interference of the peptides with the absorbance of hemin.

Liquid chromatography-mass spectrometry (LC-MS) and tandem MS analysis

The as-synthesized products were dissolved in 1 mL of 50 vol% acetonitrile/water solution and then diluted 100-fold. The diluted samples were then filtrated through a hydrophilic PTFE filter with pore sizes of 0.22 μm. The analysis was performed using a Thermo Scientific UltiMate 3000 UHPLC system coupled to an electrospray ionization mass spectrometer (Q Exactive Plus Orbitrap) in negative ion mode. The mobile phase consisted of water (A) containing 0.02 vol% formic acid and acetonitrile (B), and the flow rate was 0.5 mL/min. The gradient separation started at 2 vol% B for the first 2 minutes, then gradually increased to 100 vol% B over 25 minutes, maintained for 10 minutes, and finally returned to 2 vol% B. The injection volume was 5 μL. The column was an Agilent InfinityLab Poroshell HPH-C18 (2.1×150mm, 2.7 μm particle size), and the column temperature was 40 °C. The capillary voltage was 3.5 kV. For the tandem MS analysis, the normalized collision energy was 30 %.

Atomic force microscopy (AFM) analysis

AFM analysis was performed using a Bruker Dimension Icon system. The tip resonant frequency was 150 kHz, and the spring constant was 5 N/m. The samples were prepared by dropping a suspension

in phosphate buffer (20 μM hemin, amino acids to hemin ratio was 80:1) onto a mica sheet, allowing it to stand for 1 minute, removing excess liquid, and drying at room temperature for 3 days.

Dynamic light scattering (DLS) analysis

The same suspensions of HF and HF/Hemin for AFM analysis were tested using a Zetasizer (Nano ZS, Malvern) to determine their particle size distribution.

Peroxidase-mimicking activity analysis

To assess the peroxidase-mimicking activity of the peptide/hemin assemblies under neutral pH conditions, TMB was selected as the model substrate. The experimental setup involved a reaction mixture comprising 100 μL of the peptide/hemin assemblies (equivalent to 0.5 mM hemin), 100 μL of TMB solution (15 mM), 100 μL of H_2O_2 solution (15 mM), and 2.7 mL of phosphate buffer (0.1 M, pH 7.0). Control groups included free hemin (dissolved in DMSO prior to addition due to its water insolubility) and HF peptide assemblies alone. For the kinetics analysis, the absorbance changes of the mixture at 652 nm were monitored using a Shimadzu UV-1900i spectrophotometer. The concentration of the oxidized TMB was determined using the absorbance at 652 nm with an extinction coefficient of 39000 $M^{-1}\cdot cm^{-1}$.[1] The reaction rate was calculated as the slope of the linear region of absorbance versus time in the initial phase of the reaction (0~30 s). Additionally, a steady-state kinetic analysis of the peptide/hemin assemblies was conducted using different TMB concentrations. The apparent kinetic parameters of the TMB oxidation were obtained by using the Michaelis-Menten model (Equ. S1) and Lineweaver Burk plot (Equ. S2) with Origin 2021 software.

Equ. S1:
$$v = \frac{k_{cat} \times [Hemin] \times [S]}{K_M + [S]}$$

Equ. S2:
$$\frac{1}{v} = \frac{K_M}{k_{cat} \times [Hemin][S]} + \frac{1}{k_{cat} \times [Hemin]}$$

v is the initial reaction rate of the enzyme-catalyzed reaction. V_{max} is the maximum reaction rate. K_M is The Michaelis constant. [S] is the substrate concentration ([TMB]). k_{cat} is the apparent turnover number.

2. Hemin-binding survey of peptides from different amino acids

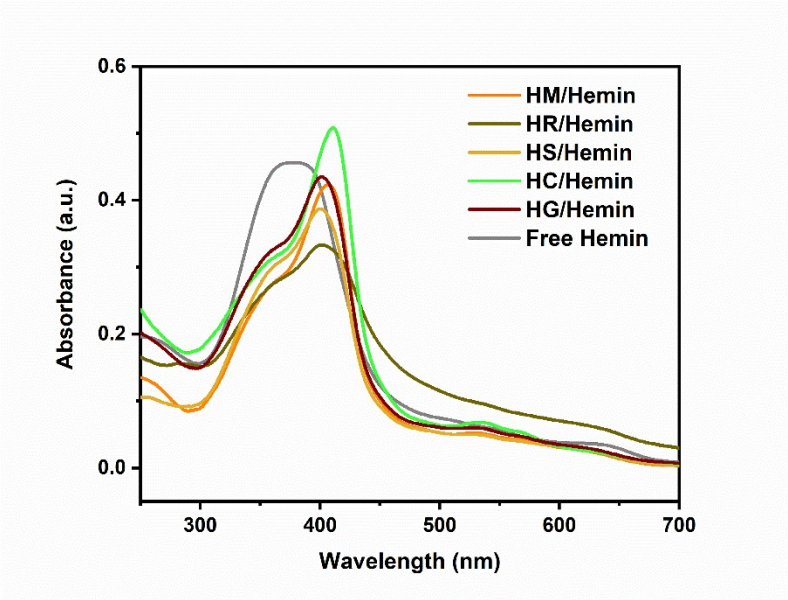

Fig. S1 UV-Vis spectra of hemin with peptides generated from different amino acids. The ratio of histidine to other amino acids was 100:100. The molar ratio of amino acids to hemin was 80:1.

3. LC-MS analysis of products synthesized in DES by different amino acids

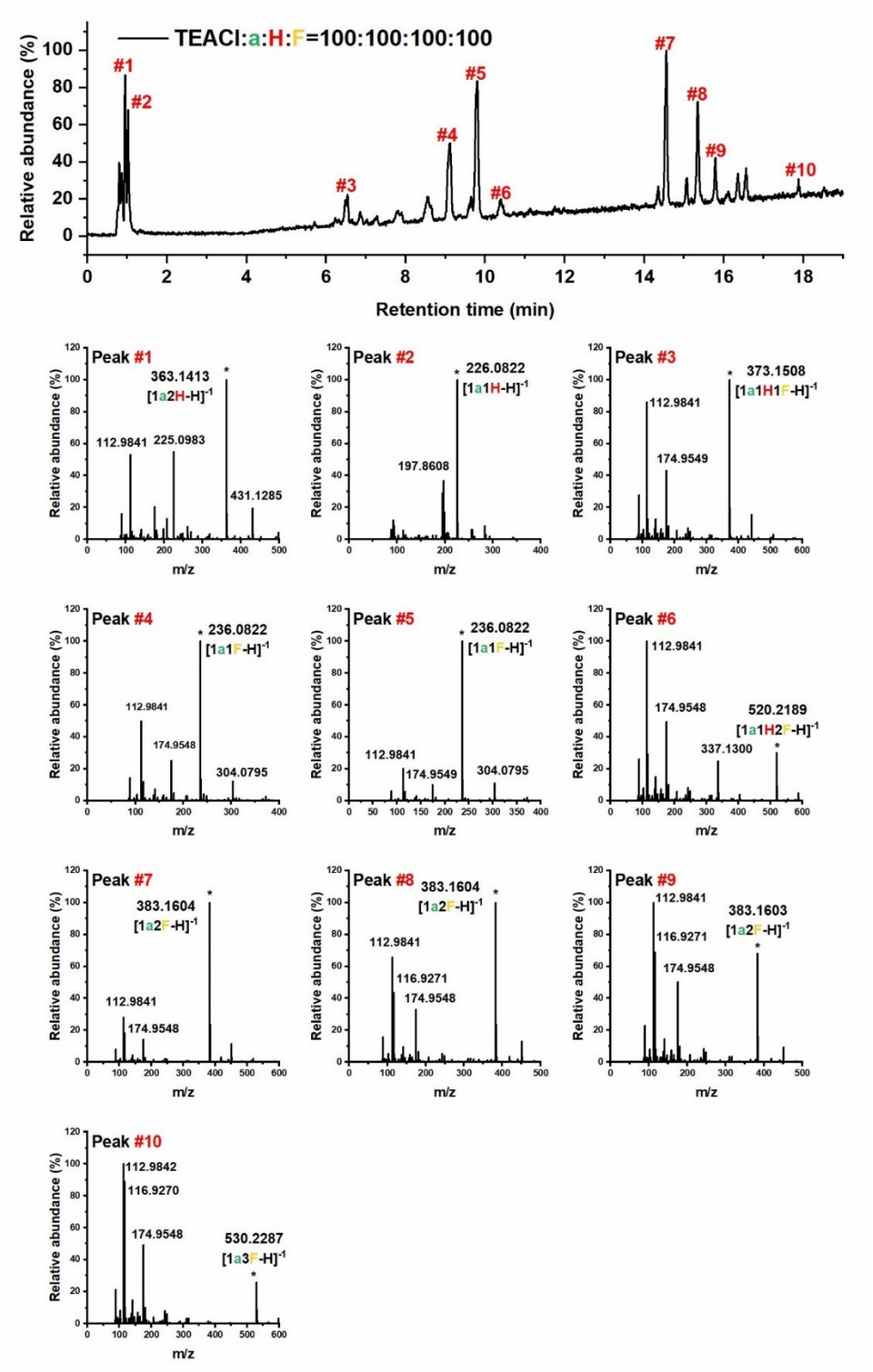

Fig. S2 LC-MS analysis of peptides from L-histidine (H)/L-phenylalanine (F) combination. The molar ratio of H to F was 100:100. The sample was prepared by heating at 120 °C for 3 days. Racemization occurred as two 1a1F oligomers eluted at different times (peak #4 and peak #5). These results are consistent with our previous study.[2] Oxazolone intermediates may form during the reaction, leading to diastereomers of peptides.

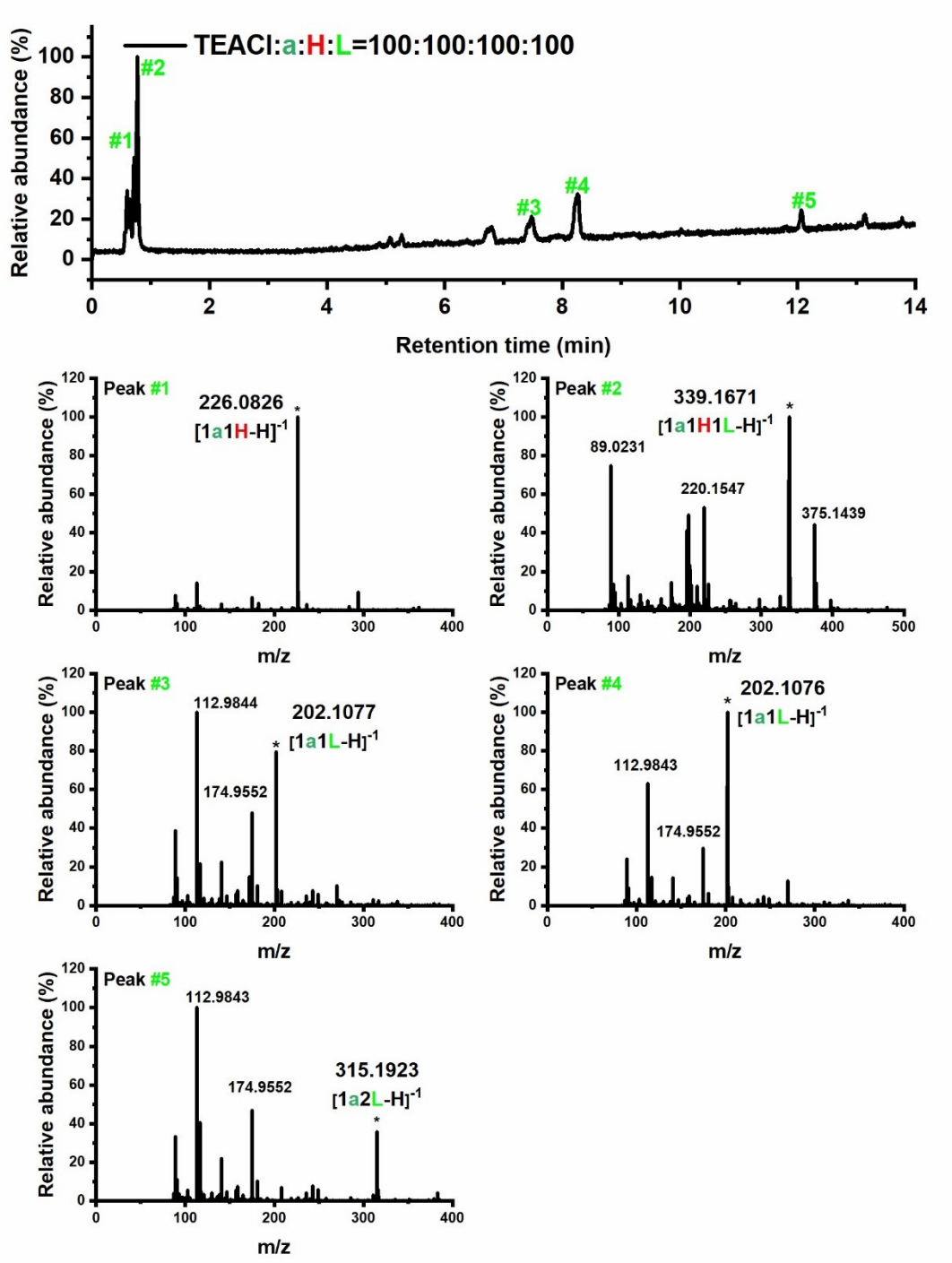

Fig. S3 LC-MS analysis of peptides from L-histidine (H)/L-leucine (L) combination. The molar ratio of H to L was 100:100. The sample was prepared by heating at 120 °C for 3 days.

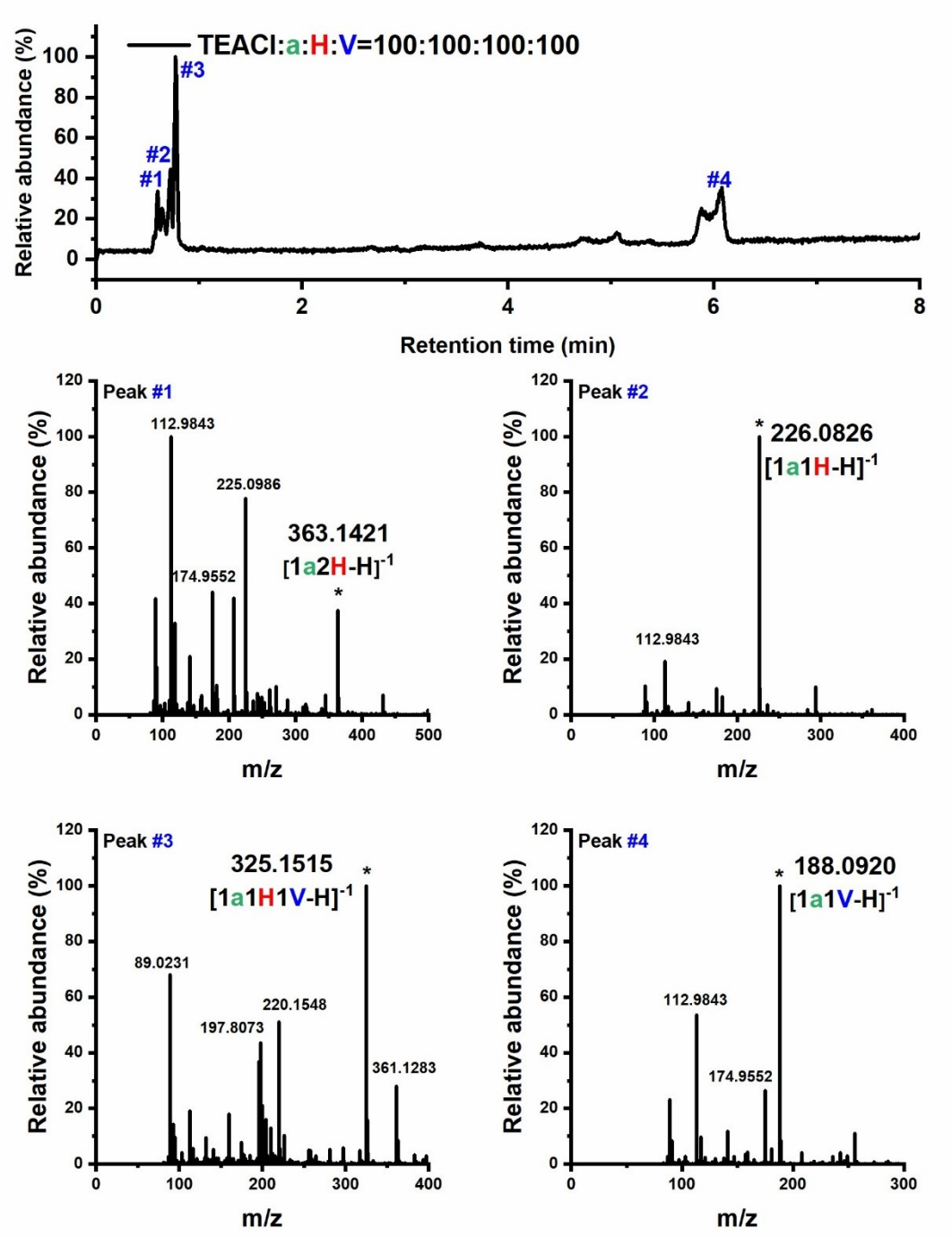

Fig. S4 LC-MS analysis of peptides from L-histidine (H)/L-valine (V) combination. The molar ratio of H to V was 100:100. The sample was prepared by heating at 120 °C for 3 days.

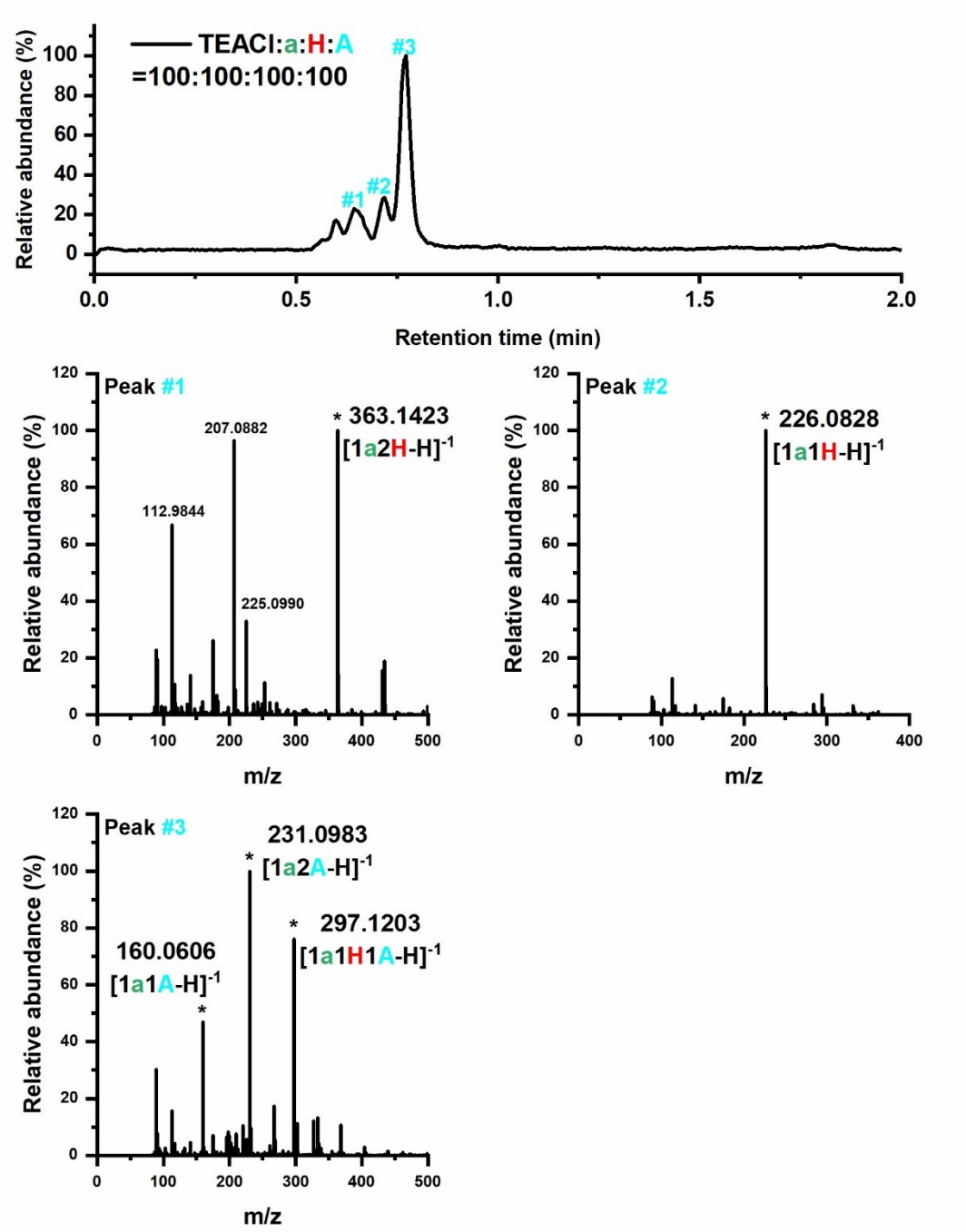

Fig. S5 LC-MS analysis of peptides from L-histidine (H)/L-alanine (A) combination. The molar ratio of H to A was 100:100. The sample was prepared by heating at 120 °C for 3 days.

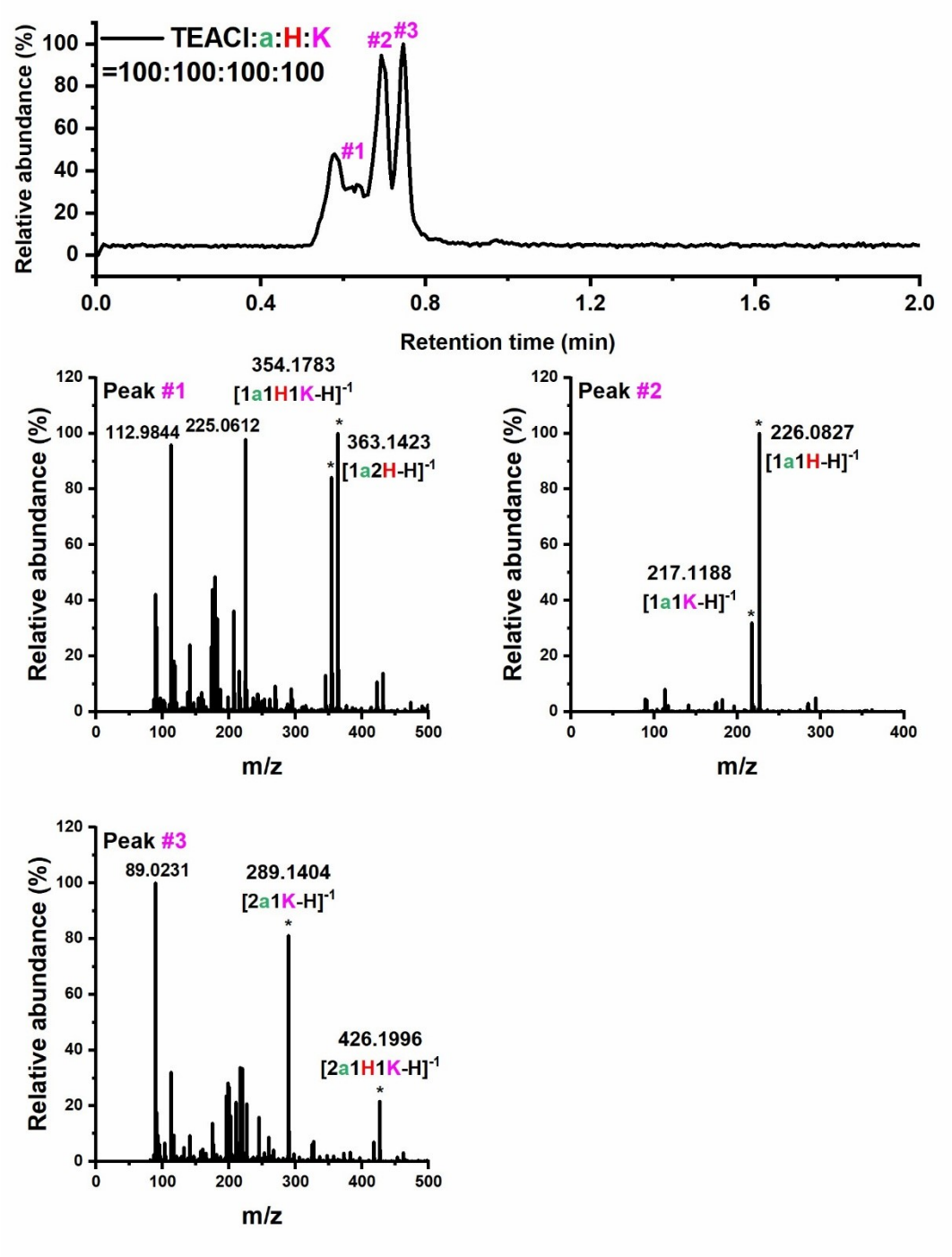

Fig. S6 LC-MS analysis of peptides from L-histidine (H)/L-lysine (K) combination. The molar ratio of H to K was 100:100. The sample was prepared by heating at 120 °C for 3 days.

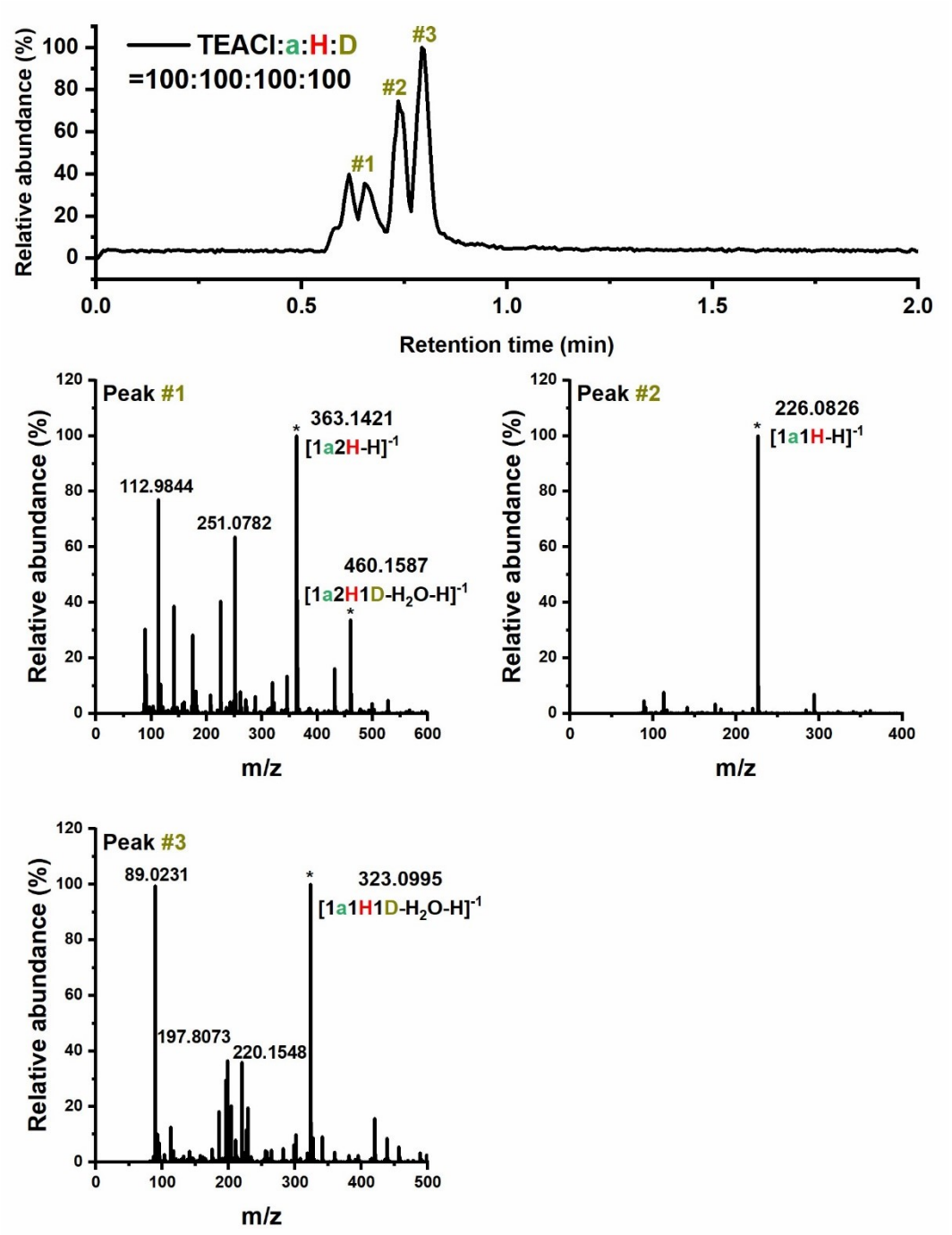

Fig. S7 LC-MS analysis of peptides from L-histidine (H)/L-aspartic acid (D) combination. The molar ratio of H to D was 100:100. The sample was prepared by heating at 120 °C for 3 days. Oligomers with loss of water molecules were also observed, possibly due to the cyclization of aspartic acid residues through the side chains.

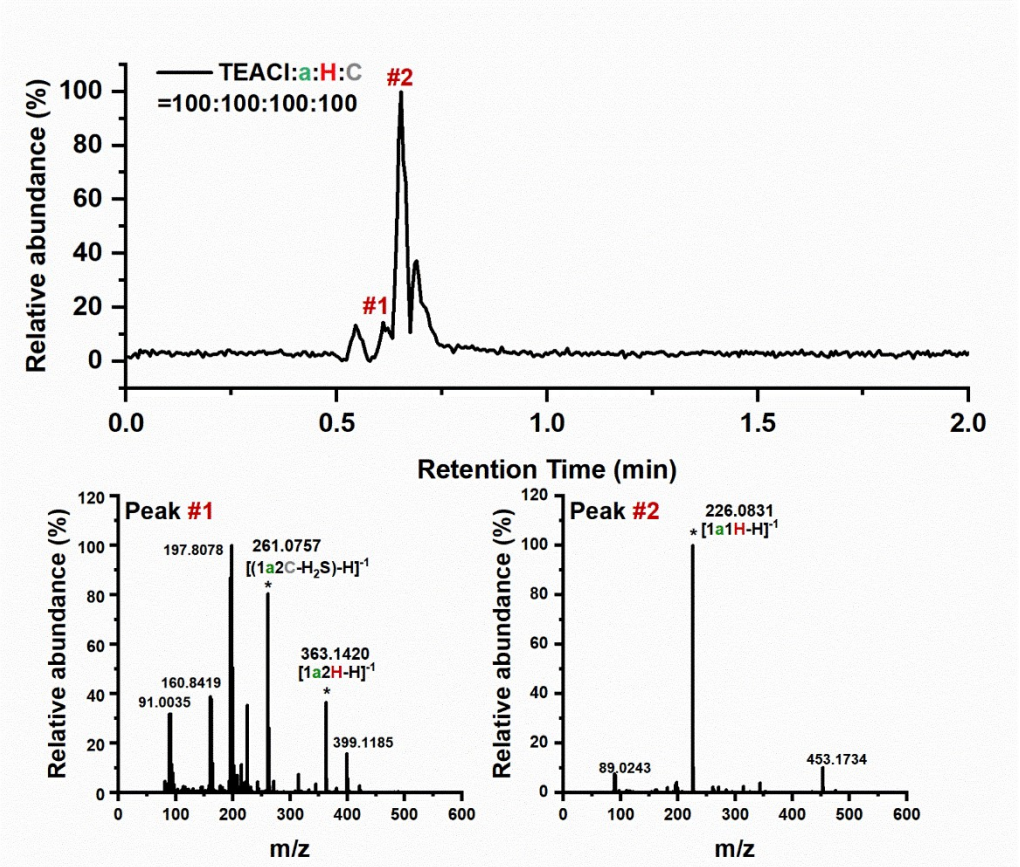

Fig. S8 LC-MS analysis of peptides from L-histidine (H)/L-cysteine (C) combination. The molar ratio of H to C was 100:100. The sample was prepared by heating at 120 °C for 3 days.

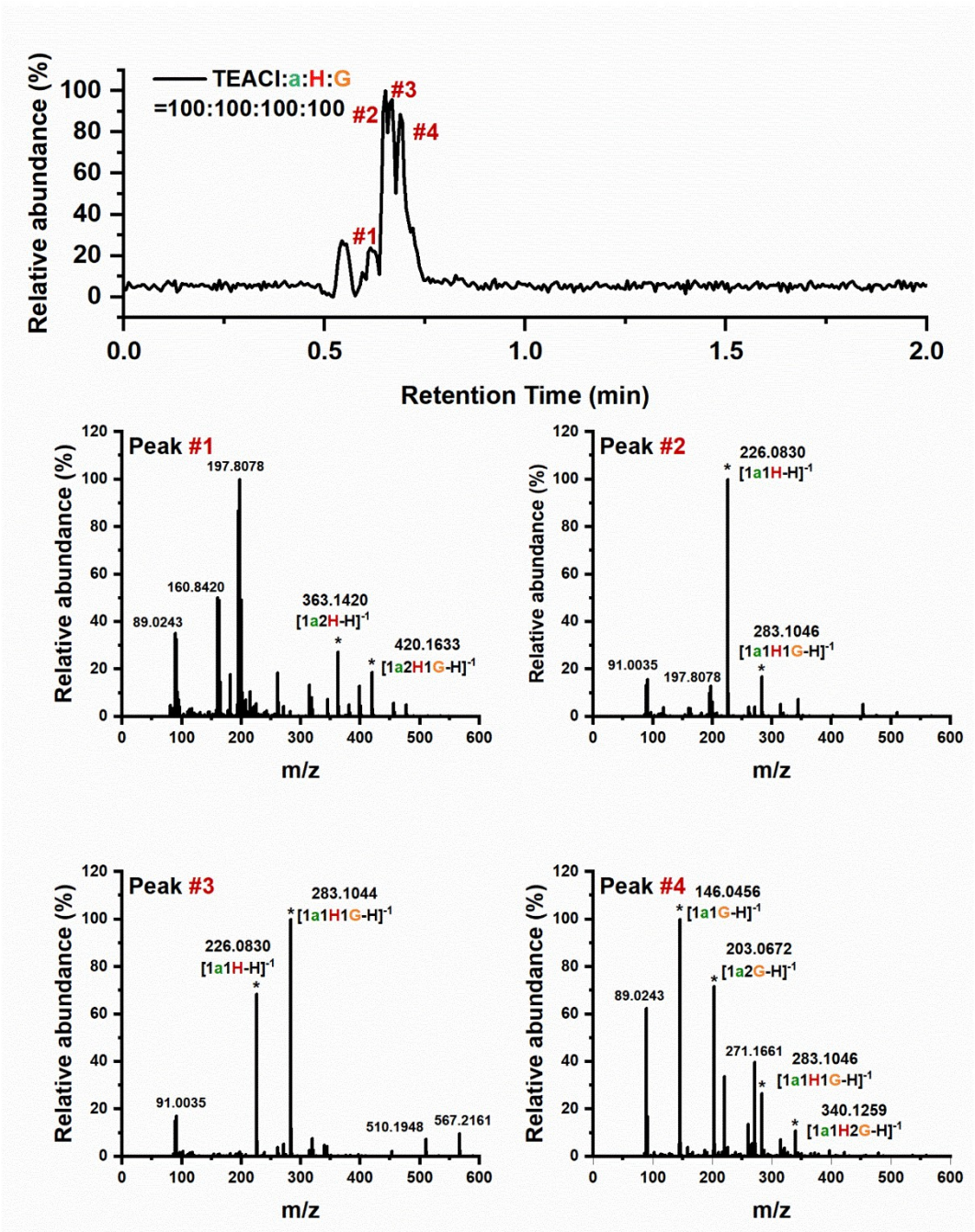

Fig. S9 LC-MS analysis of peptides from L-histidine (H)/glycine (G) combination. The molar ratio of H to G was 100:100. The sample was prepared by heating at 120 °C for 3 days.

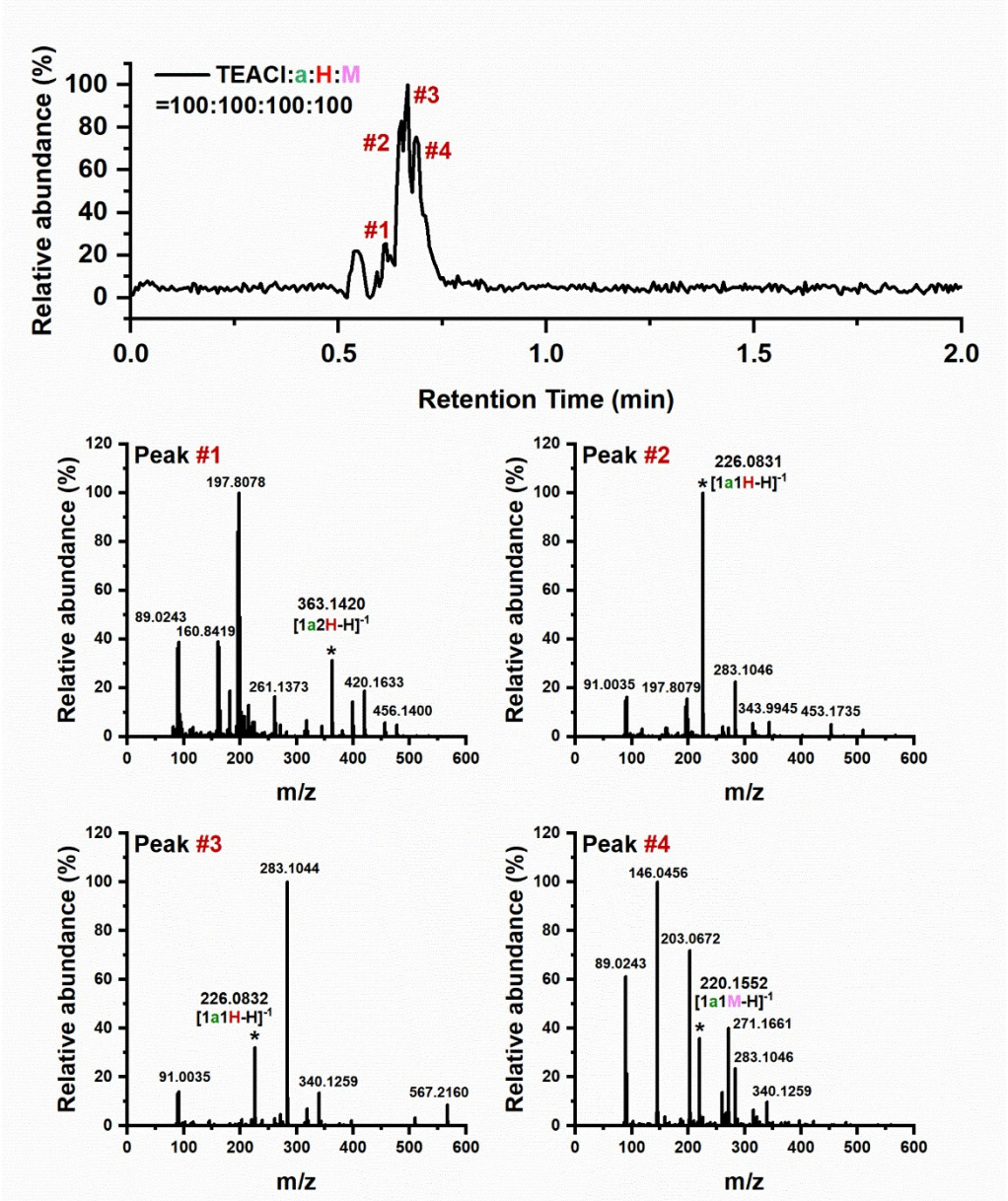

Fig. S10 LC-MS analysis of peptides from L-histidine (H)/L-methionine (M) combination. The molar ratio of H to M was 100:100. The sample was prepared by heating at 120 °C for 3 days.

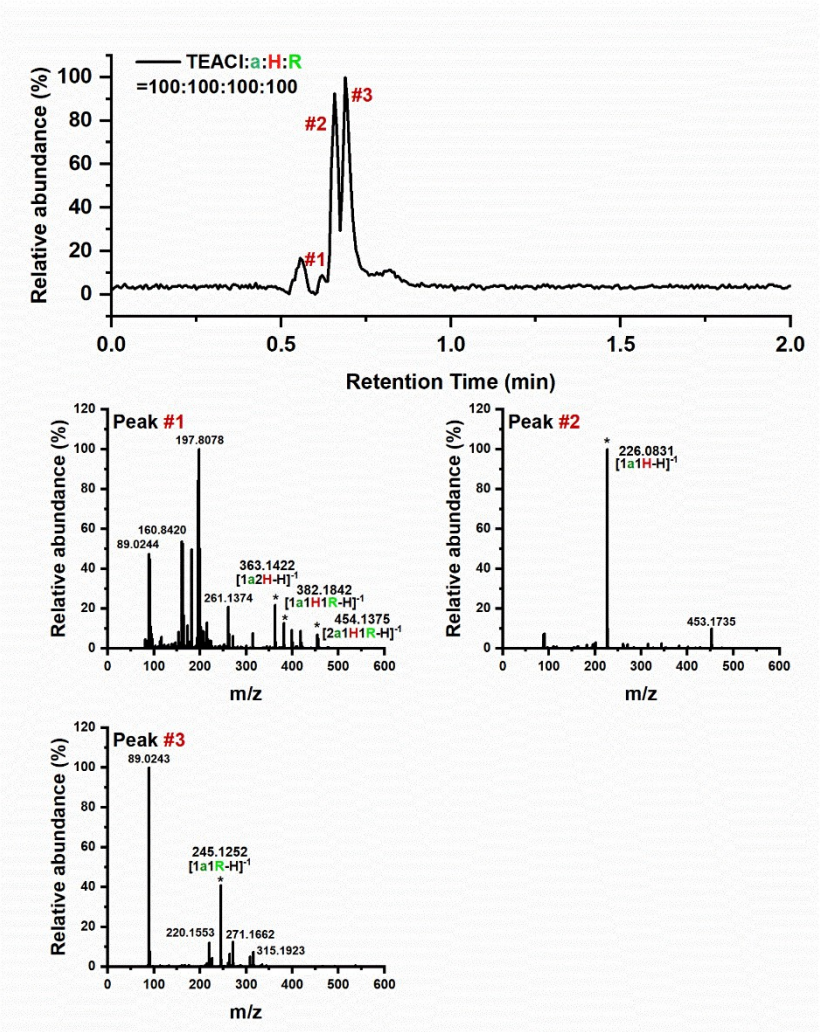

Fig. S11 LC-MS analysis of peptides from L-histidine (H)/L-arginine (R) combination. The molar ratio of H to R was 100:100. The sample was prepared by heating at 120 °C for 3 days.

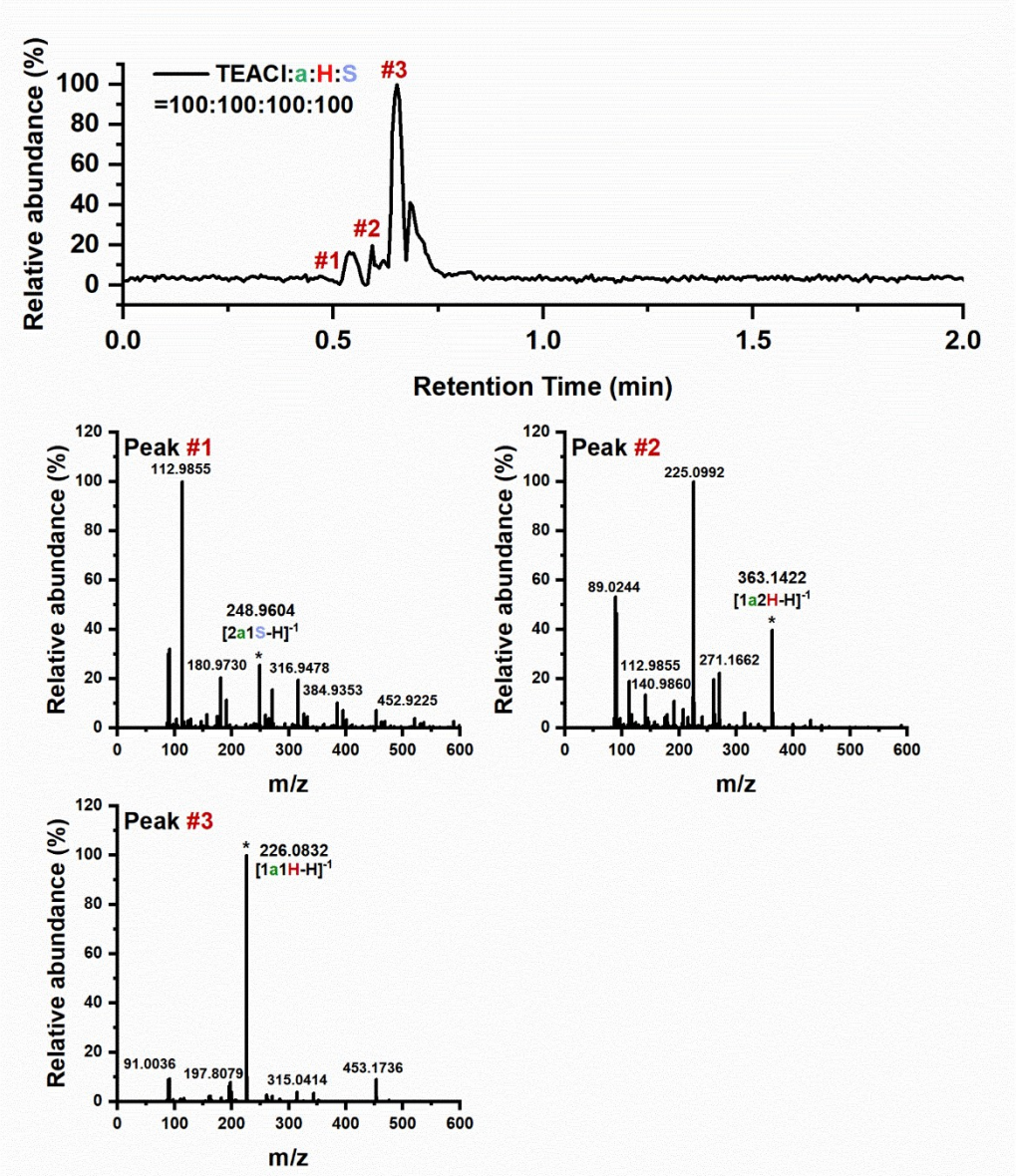

Fig. S12 LC-MS analysis of peptides from L-histidine (H)/L-serine (S) combination. The molar ratio of H to S was 100:100. The sample was prepared by heating at 120 °C for 3 days.

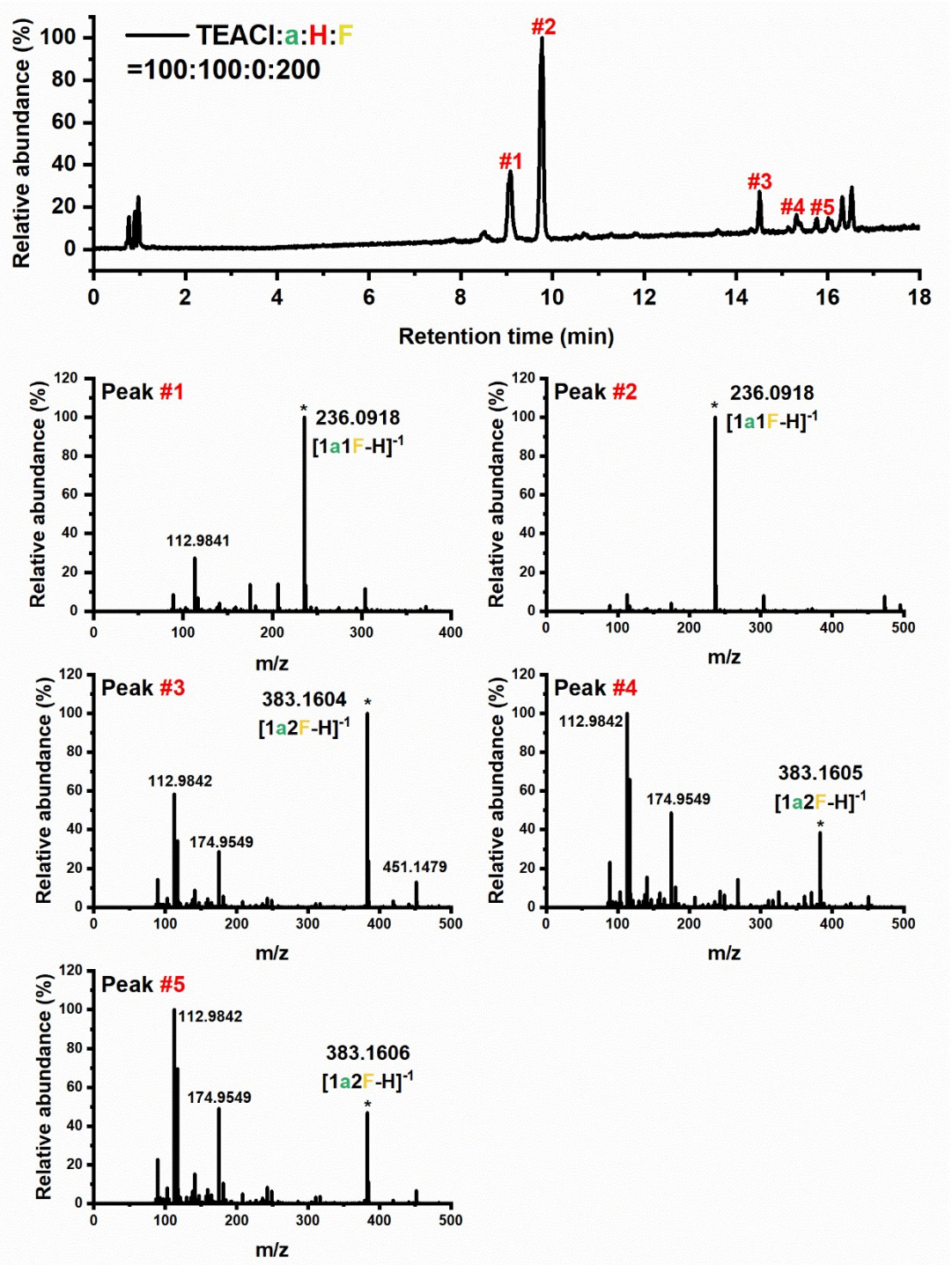

Fig. S13 LC-MS analysis of H/F combination. The molar ratio of H to F was 0:200. The sample was prepared by heating at 120 °C for 3 days.

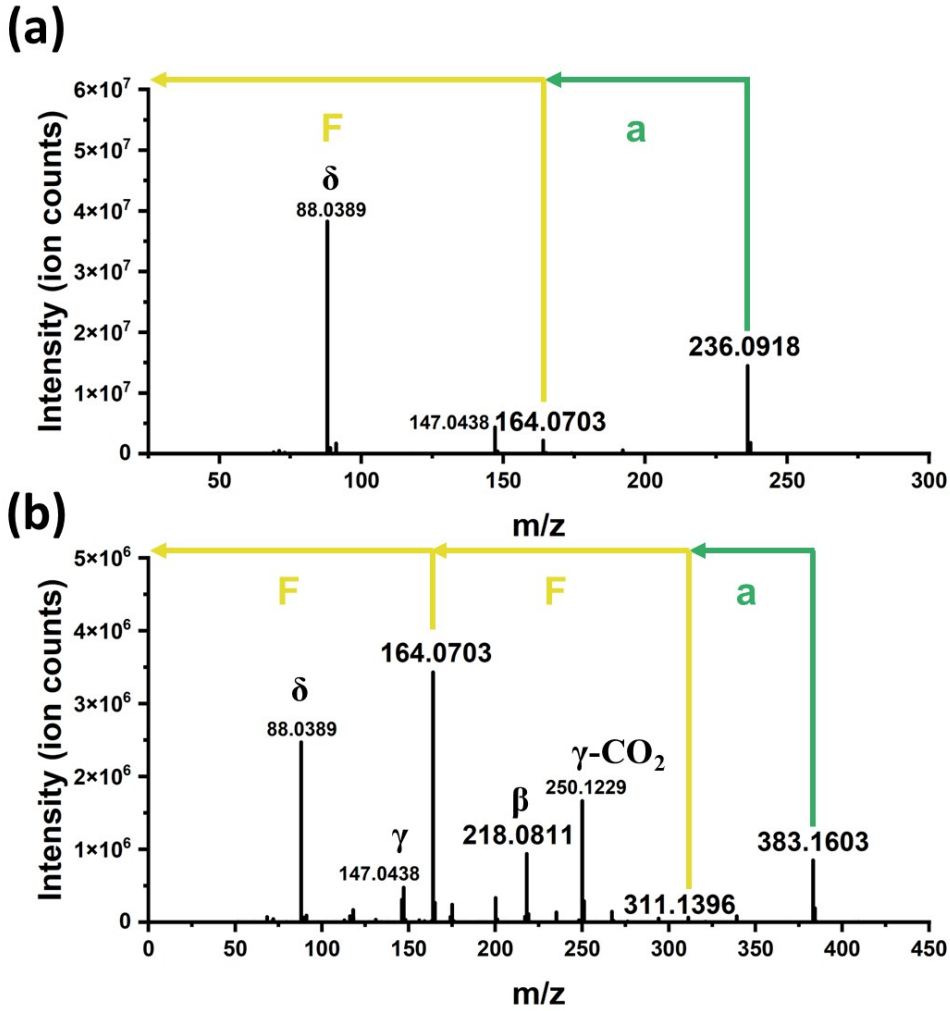

Fig. S14 Tandem MS fragmentation analysis of the products from TEACl:a:H:F=100:100:0:200. The sequence of each oligomer was determined by α-cleavages. Signals from β-cleavages (**β**), γ-cleavages (**γ**), δ-cleavages (**δ**), and losses of CO_2 were also found. The assignment of fragmentation patterns was shown in previous literature.[3] The sample was prepared by heating at 120 °C for 3 days.

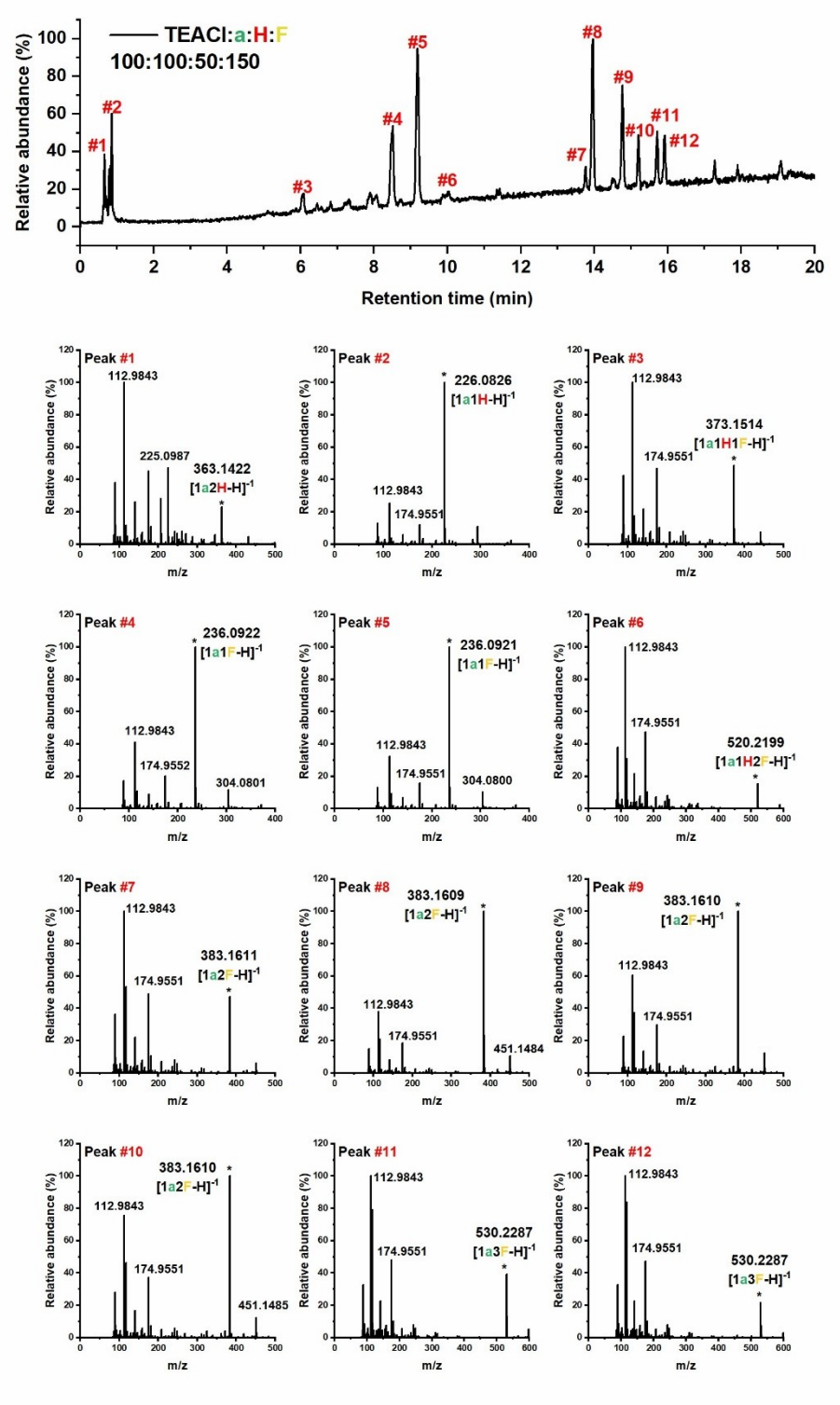

Fig. S15 LC-MS analysis of H/F combination. The molar ratio of H to F was 50:150. The sample was prepared by heating at 120 °C for 3 days.

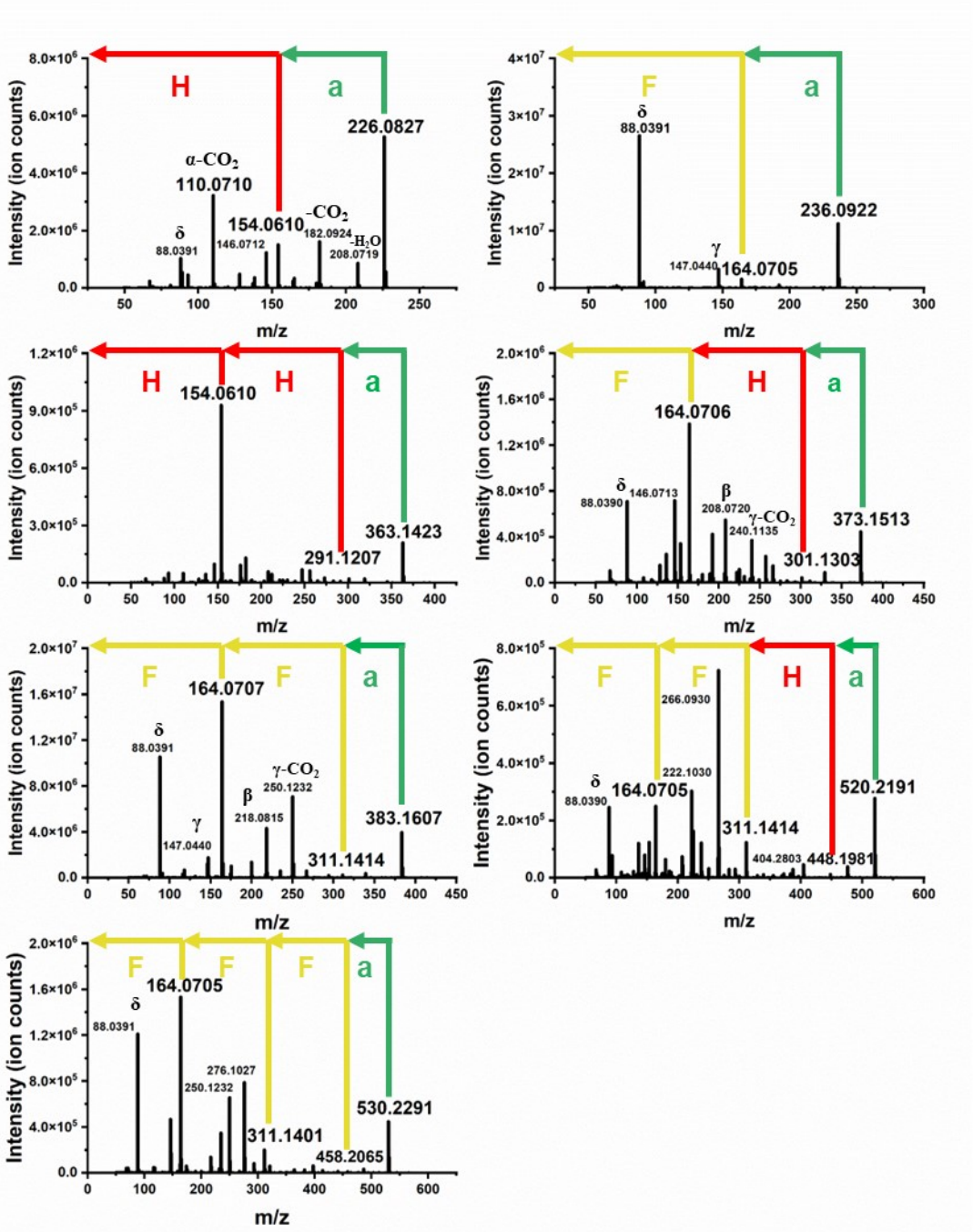

Fig. S16 Tandem MS fragmentation analysis of the products from TEACl:a:H:F=100:100:50:150. The sequence of each oligomer was determined by α-cleavages. Signals from β-cleavages (**β**), γ-cleavages (**γ**), δ-cleavages (**δ**), losses of H_2O, and losses of CO_2 were also found. The assignment of fragmentation patterns was shown in previous literature.[3] The sample was prepared by heating at 120 °C for 3 days.

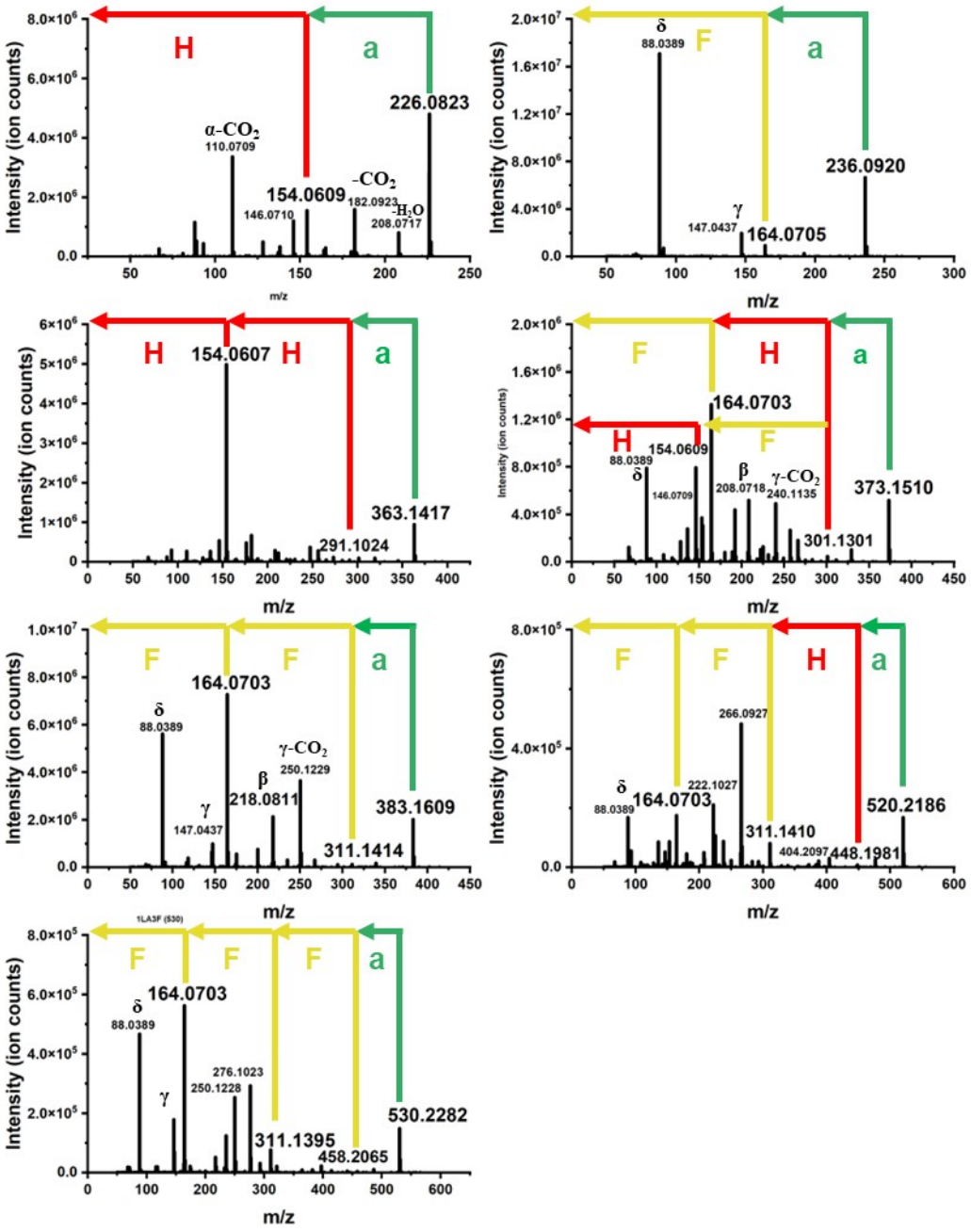

Fig. S17 Tandem MS fragmentation analysis of the products from TEACl:a:H:F=100:100:100:100. The sequence of each oligomer was determined by α-cleavages. Signals from β-cleavages (**β**), γ-cleavages (**γ**), δ-cleavages (**δ**), losses of H_2O, and losses of CO_2 were also found. The assignment of fragmentation patterns was shown in previous literature.[3] The sample was prepared by heating at 120 °C for 3 days.

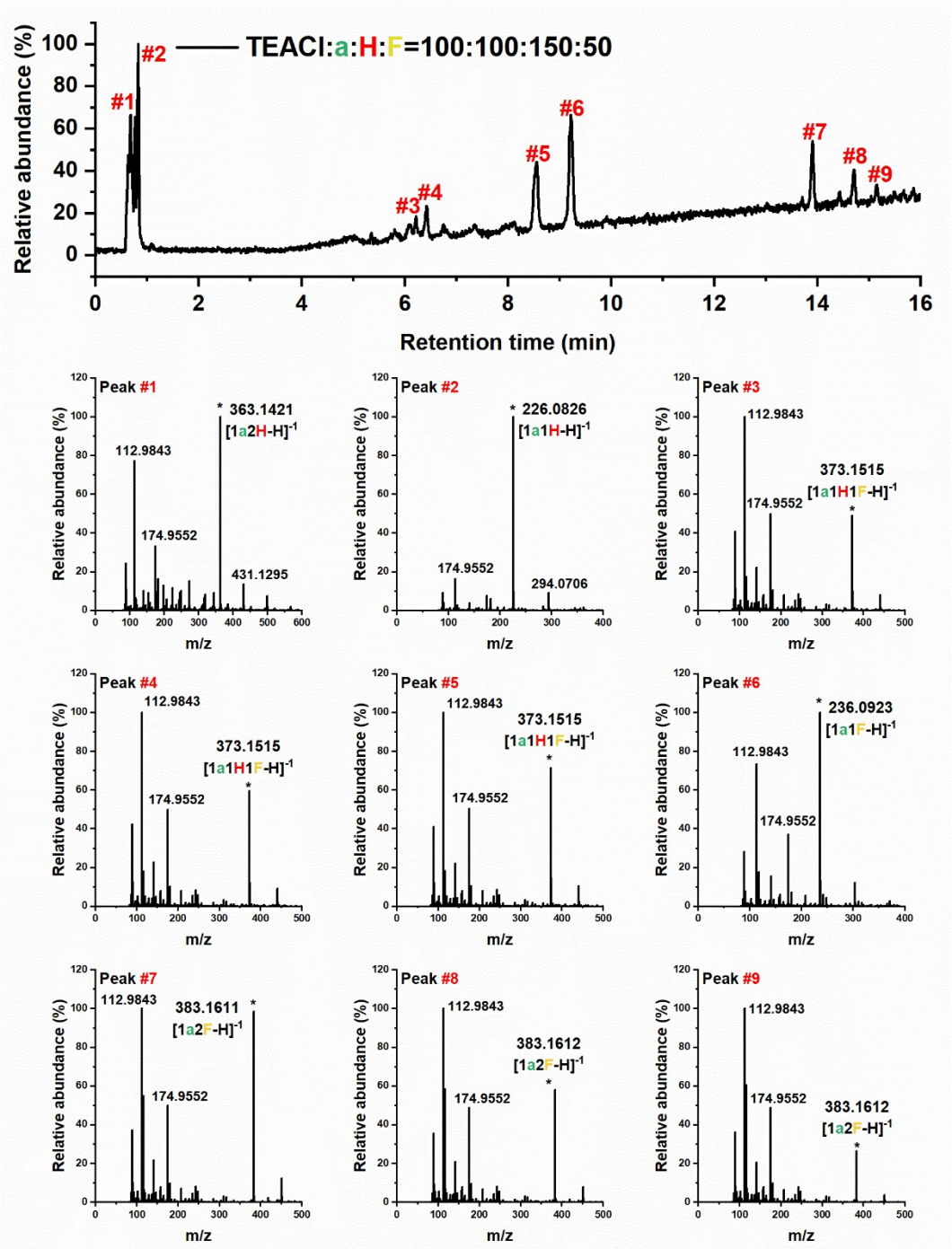

Fig. S18 LC-MS analysis of H/F combination. The molar ratio of H to F was 150:50. The sample was prepared by heating at 120 °C for 3 days.

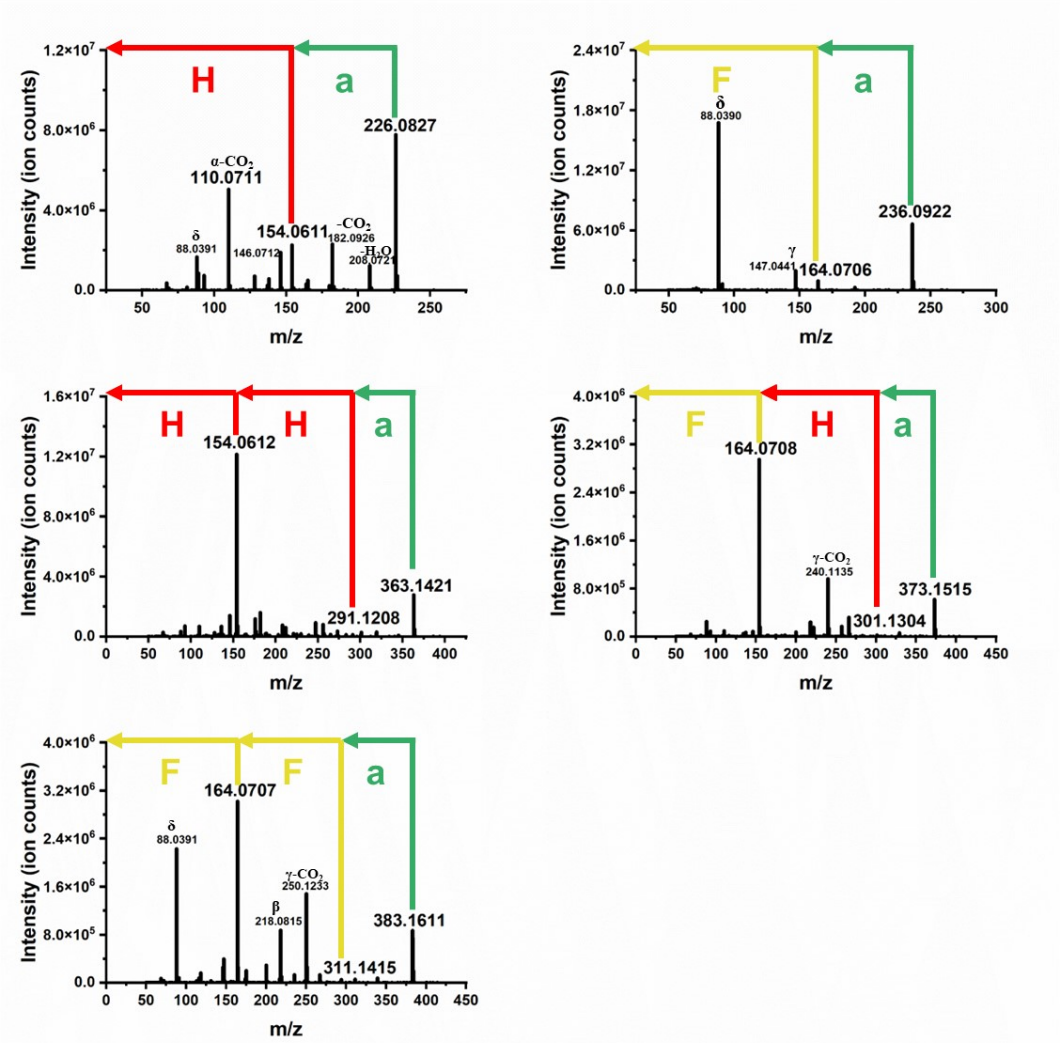

Fig. S19 Tandem MS fragmentation analysis of the products from TEACl:a:H:F=100:100:150:50. The sequence of each oligomer was determined by α-cleavages. Signals from β-cleavages (**β**), γ-cleavages (**γ**), δ-cleavages (**δ**), losses of H_2O, and losses of CO_2 were also found. The assignment of fragmentation patterns was shown in previous literature.[3] The sample was prepared by heating at 120 °C for 3 days.

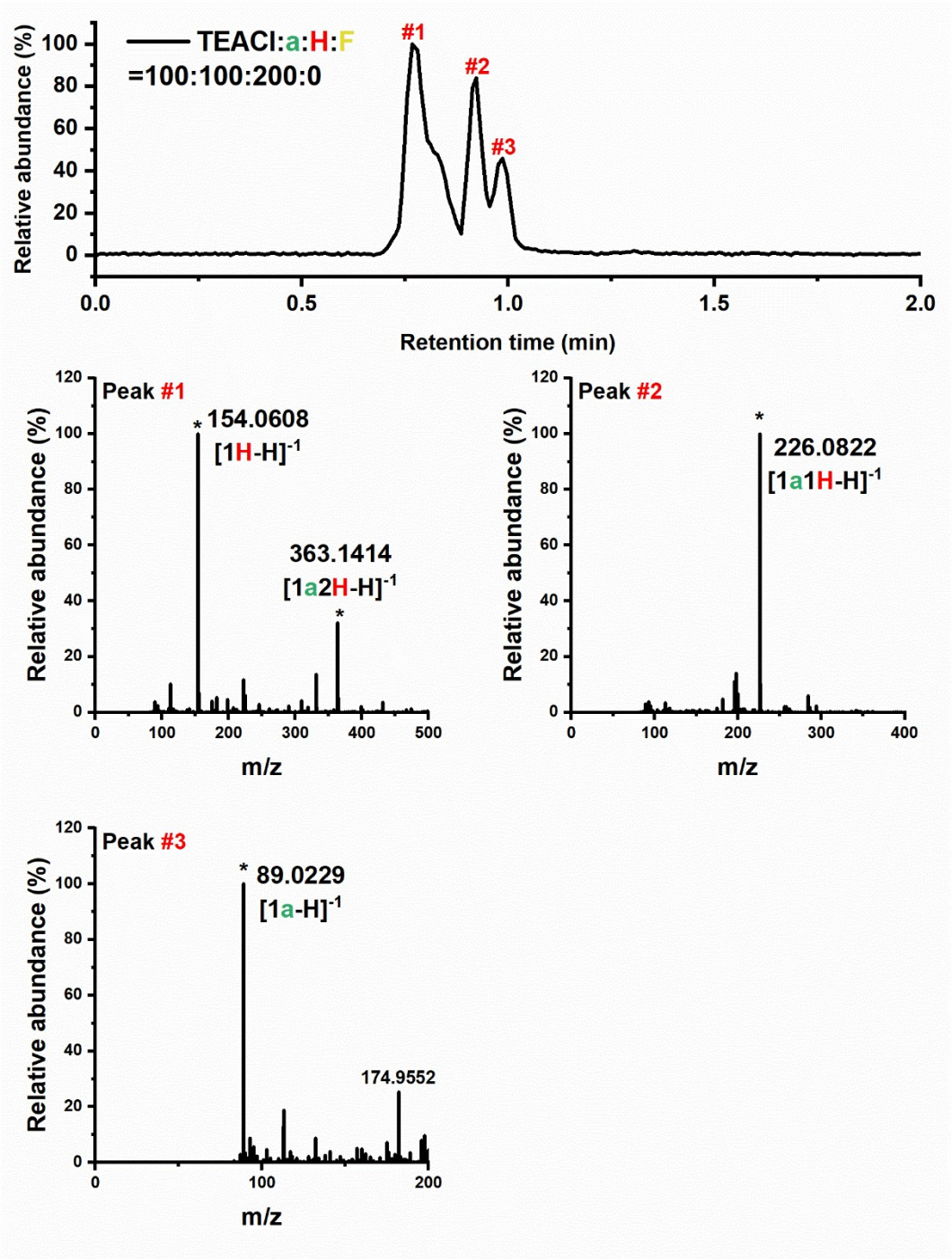

Fig. S20 LC-MS analysis of H/F combination. The molar ratio of H to F was 200:0. The sample was prepared by heating at 120 °C for 3 days.

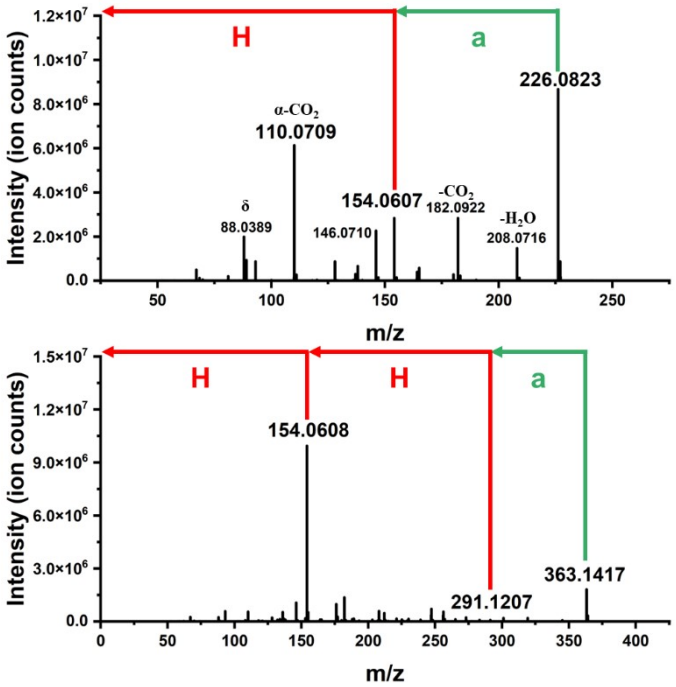

Fig. S21 Tandem MS fragmentation analysis of the products from TEACl:a:H:F=100:100:200:0. The sequence of each oligomer was determined by α-cleavages. Signals from δ-cleavages (**δ**), losses of H_2O, and losses of CO_2 were also found. The assignment of fragmentation patterns was shown in previous literature.[3] The sample was prepared by heating at 120 °C for 3 days.

4. Hemin-binding effect of histidine monomer

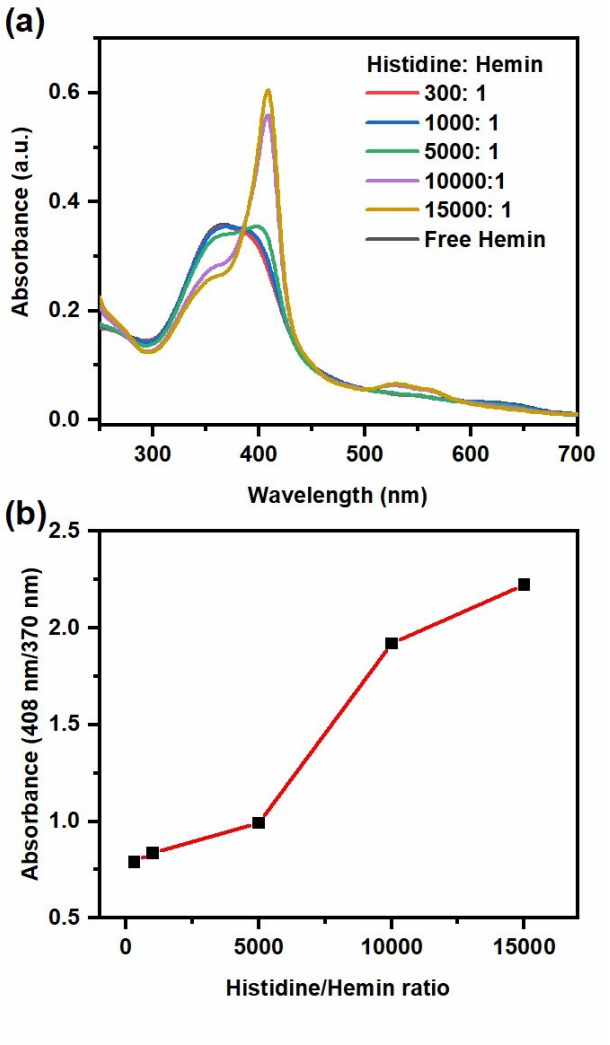

Fig. S22 (a) UV-vis spectra of hemin using different histidine/hemin ratios. (b) Absorbance change *vs.* histidine/hemin ratio. The solution was a 100 mM phosphate buffer solution with a pH of 7.0. The concentration of hemin was 10 μM.

5. Hemin-binding products prepared from different reaction conditions

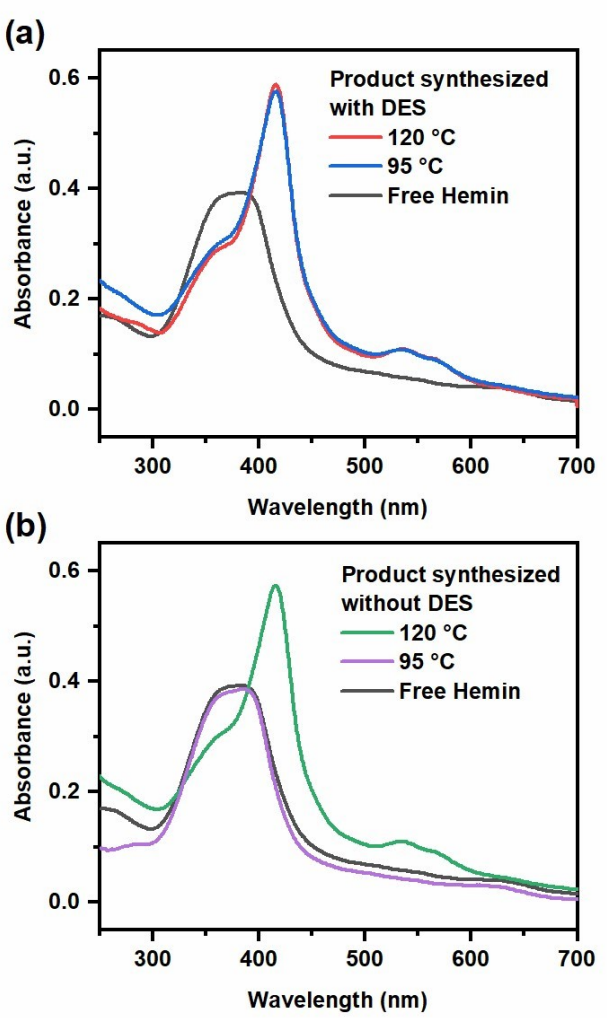

Fig. S23 (a) UV-Vis spectra of hemin with HF peptides synthesized in DES at different temperatures. (b) UV-Vis spectra of hemin with HF peptides synthesized without DES at different temperatures. The solution was a 100 mM phosphate buffer solution with a pH of 7.0. The concentration of hemin was 10 μM. The molar ratio of H to F was 100:100.

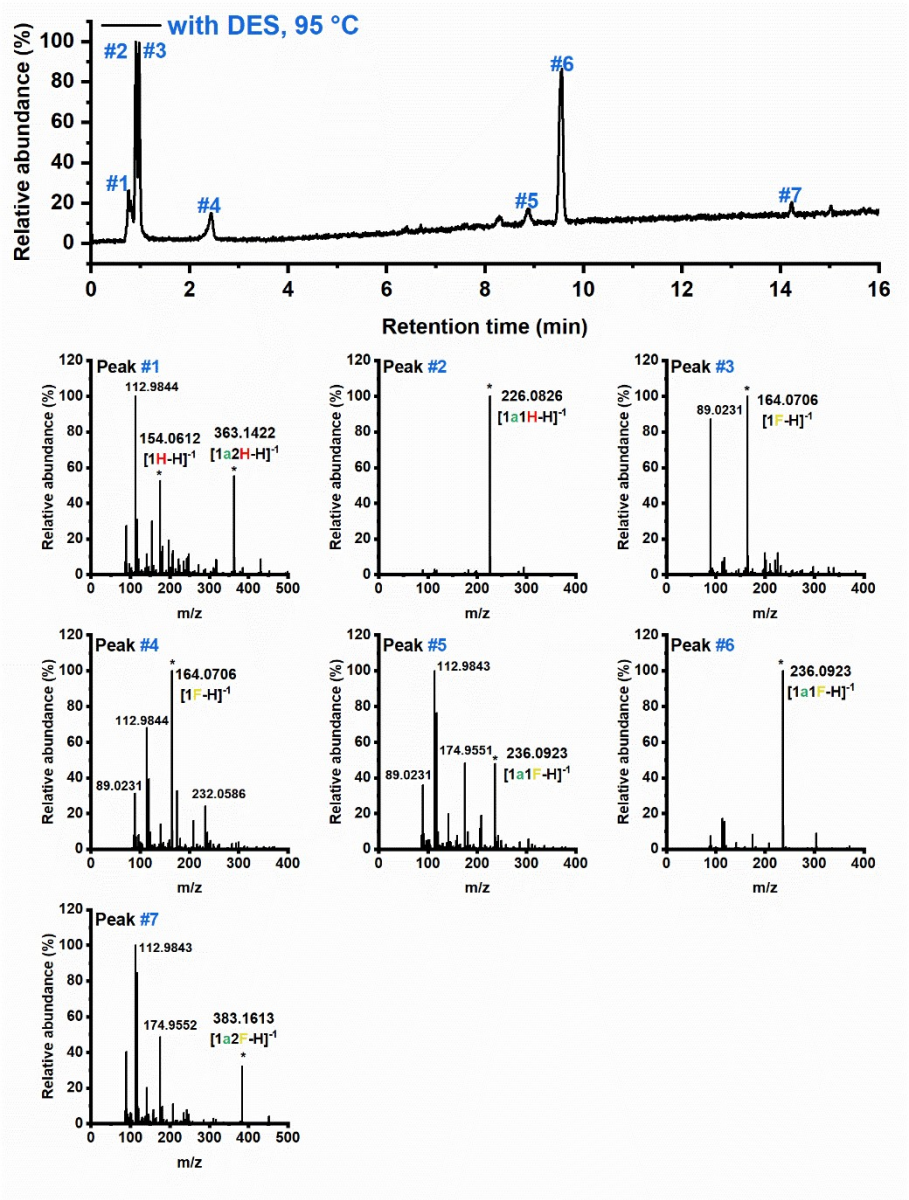

Fig. S24 LC-MS analysis of H/F combination (TEACl:a:H:F=100:100:100:100) reacted at 95 °C in DES for 3 days.

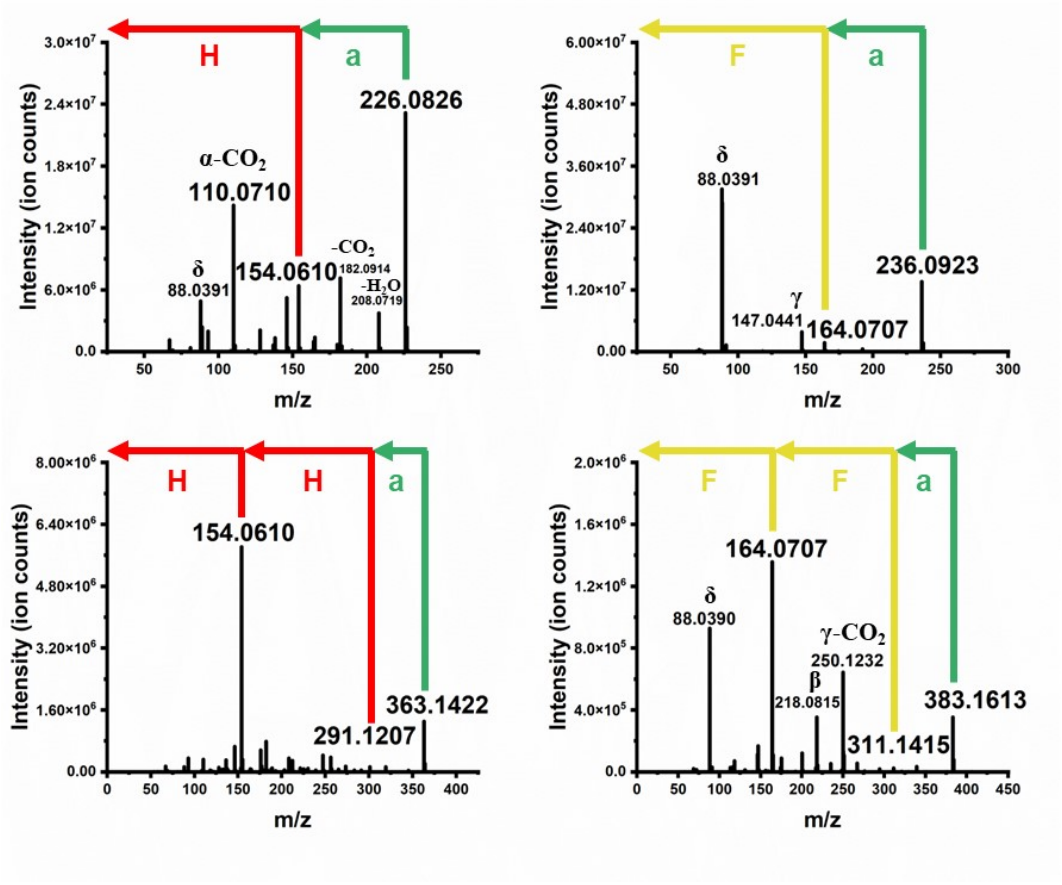

Fig. S25 Tandem MS fragmentation analysis of the products from TEACl:a:H:F=100:100:100:100 reacted at 95 °C in DES for 3 days. The sequence of each oligomer was determined by α-cleavages. Signals from β-cleavages (**β**), γ-cleavages (**γ**), δ-cleavages (**δ**), losses of H_2O, and losses of CO_2 were also found. The assignment of fragmentation patterns was shown in previous literature.[3]

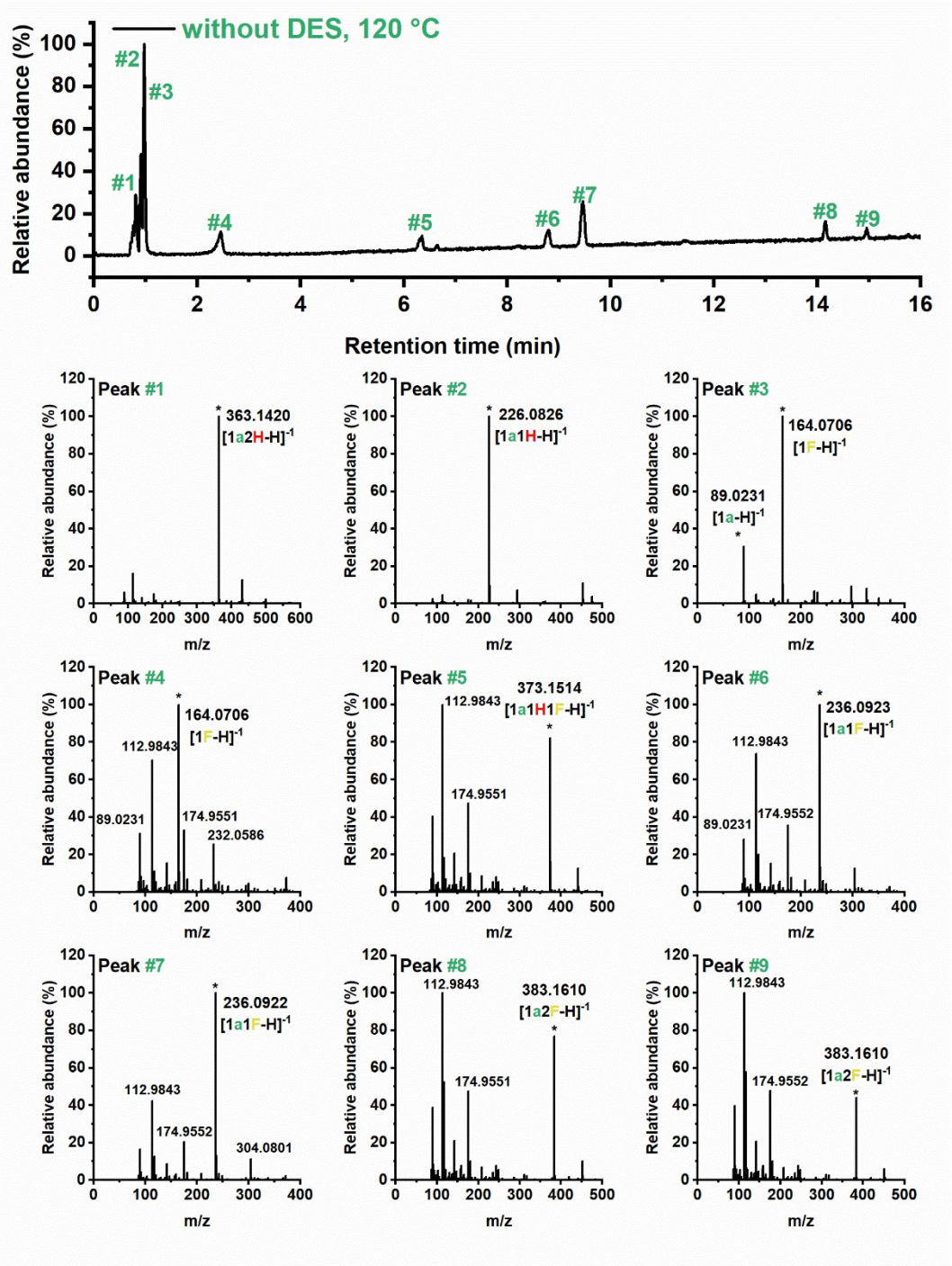

Fig. S26 LC-MS analysis of H/F combination reacted at 120 °C for 3 days without DES (a:H:F=100:100:100).

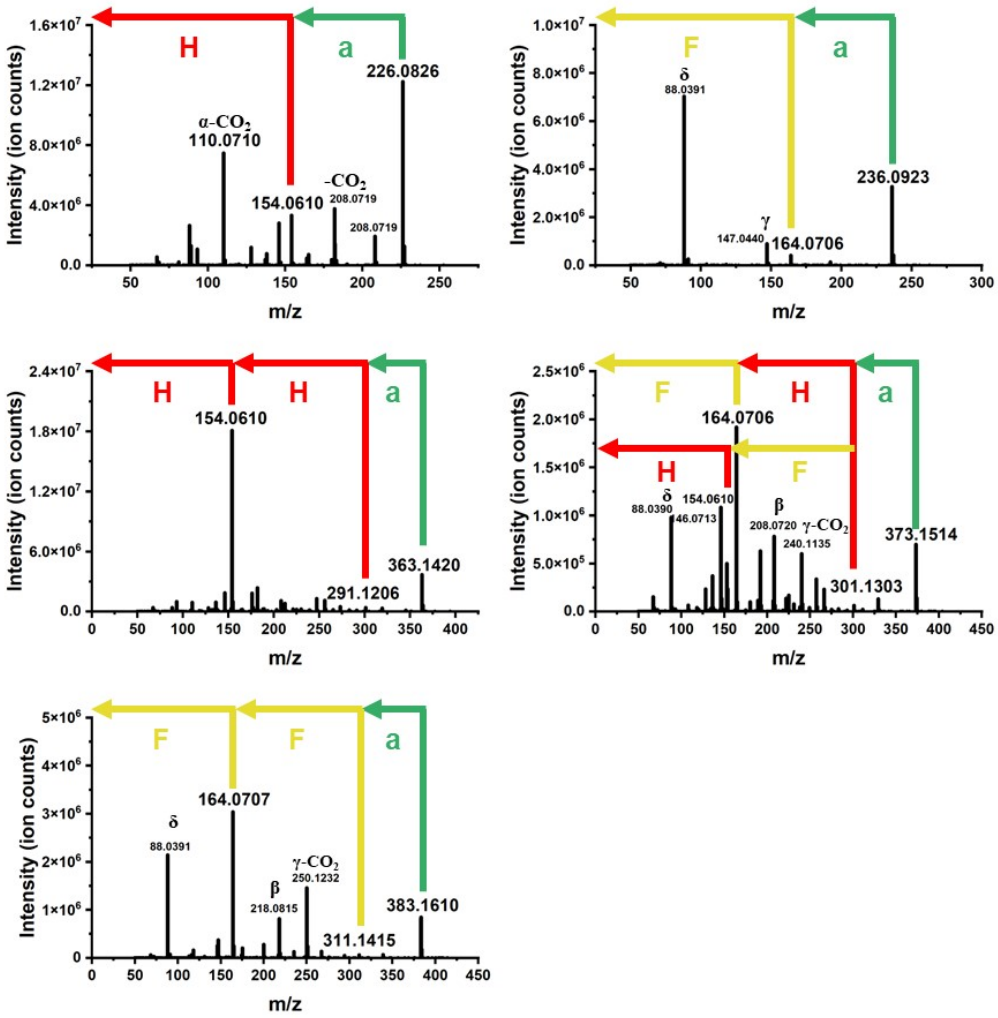

Fig. S27 Tandem MS fragmentation analysis of the products from a:H:F=100:100:100 reacted at 120 °C for 3 days. The sequence of each oligomer was determined by α-cleavages. Signals from β-cleavages (**β**), γ-cleavages (**γ**), δ-cleavages (**δ**), losses of H_2O, and losses of CO_2 were also found. The assignment of fragmentation patterns was shown in previous literature.[3]

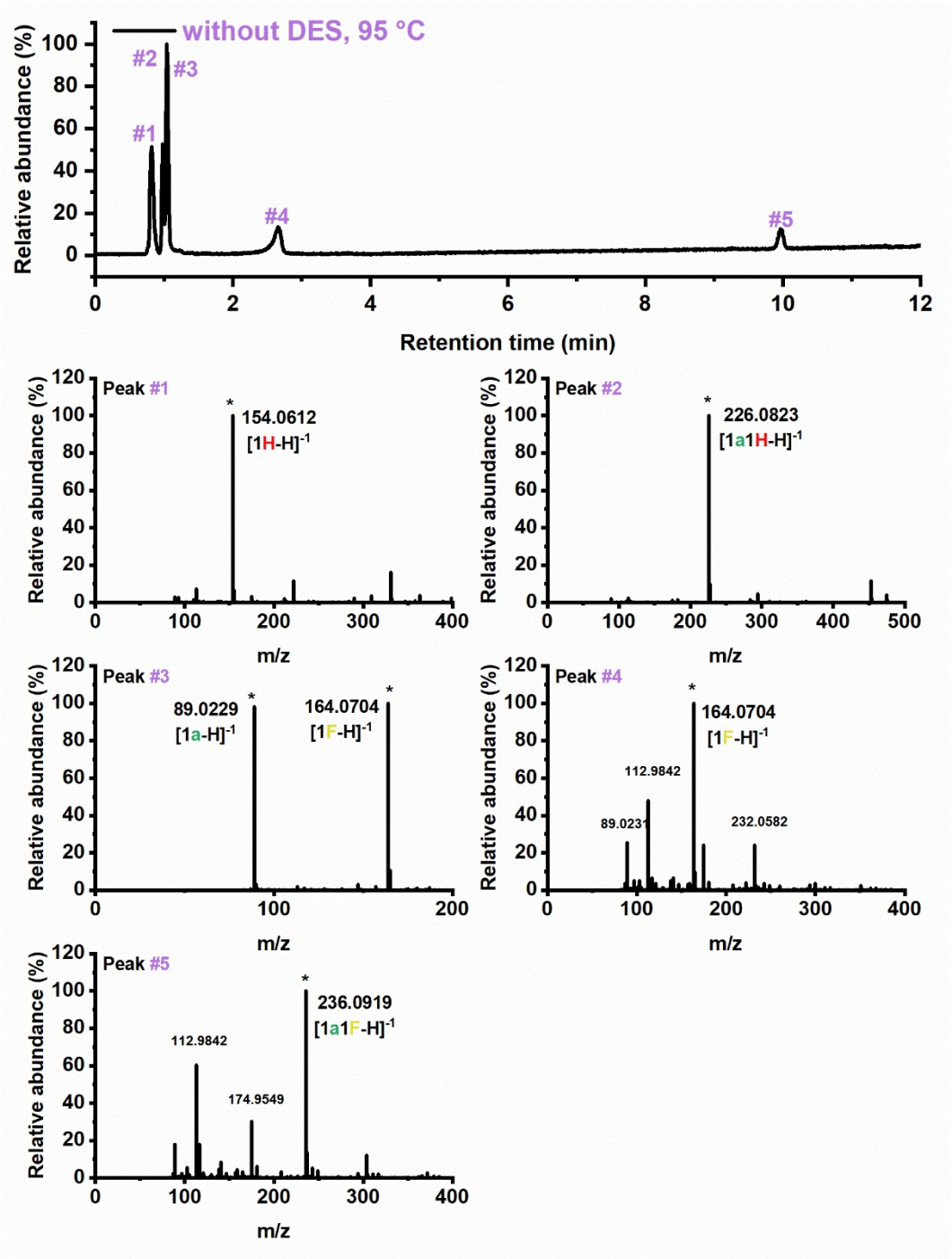

Fig. S28 LC-MS analysis of H/F combination reacted at 95 °C for 3 days without DES (a:H:F=100:100:100).

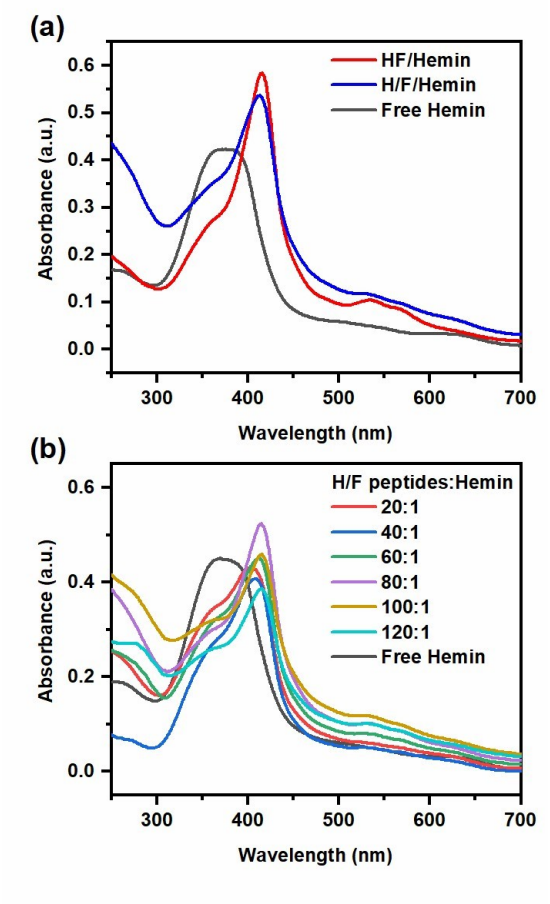

Fig. S29 (a) UV-Vis spectra of hemin with samples synthesized from different conditions. For HF/Hemin, histidine and phenylalanine monomers were reacted together in DES to form peptides. For H/F/Hemin, peptides were synthesized by only histidine or phenylalanine. The two batches of peptides were physically mixed with hemin for UV-Vis spectra analysis. The molar ratio of H and F was 100:100 in the mixed sample. The molar ratio of amino acids to hemin was 80:1. (b) UV-Vis spectra of hemin with the physically mixed peptides using different amino acids to hemin ratio. The solution was a 100 mM phosphate buffer solution with a pH of 7.0. The concentration of hemin was 10 μM.

6. Supplementary data for TMB oxidation

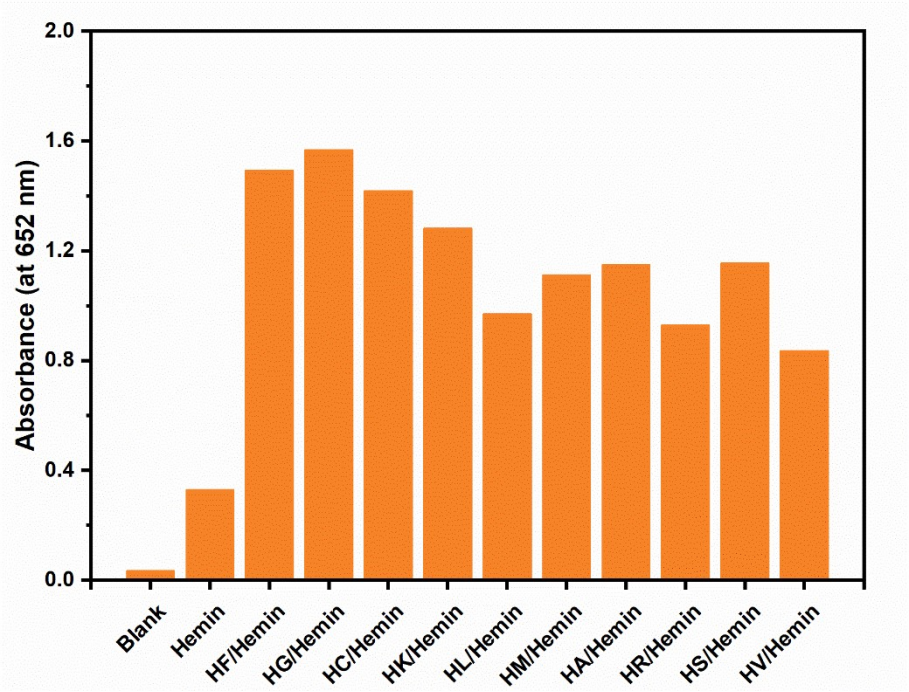

Fig. S30 Absorbance of different samples after 500 sec of TMB oxidation.

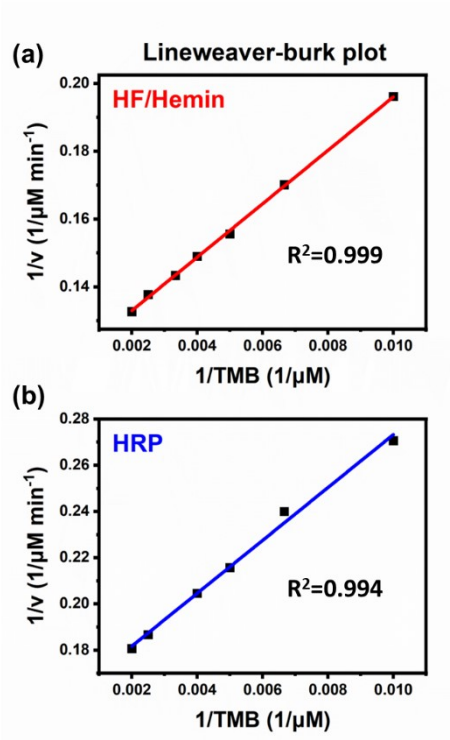

Fig. S31 (a) The Lineweaver-Burk plot for TMB oxidation catalyzed by HF/Hemin complex. HF peptides were prepared from the mixture of TEACl:a:H:F=100:100:100:100 at 120 °C in DES for 3 days. For TMB oxidation, the molar ratio of amino acids to hemin was 80:1, and the hemin concentration was 16.68 μM. 500 μM of H_2O_2 in 100 mM phosphate buffer at pH 7.0 were used. (b) The Lineweaver-Burk plot for TMB oxidation catalyzed by HRP. HRP concentration was 1.89×10^{-4} μM. 500 μM of H_2O_2 in 100 mM phosphate buffer at pH 7.0 were used.

Table S1 Comparison of the apparent Michaelis-Menten constants of HF/Hemin complex and HRP in catalyzing the oxidation of TMB.

	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1} \cdot min^{-1}$)
HF/Hemin	0.508	66.739	0.00761
HRP	33500	74.329	45100

7. References

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