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Supplementary information

Bioorthogonal information storage in L-DNA with a high-fidelity mirror-image *Pfu* DNA polymerase

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Supplementary Information

Bioorthogonal information storage in L-DNA with a high-fidelity mirror-image *Pfu* DNA polymerase

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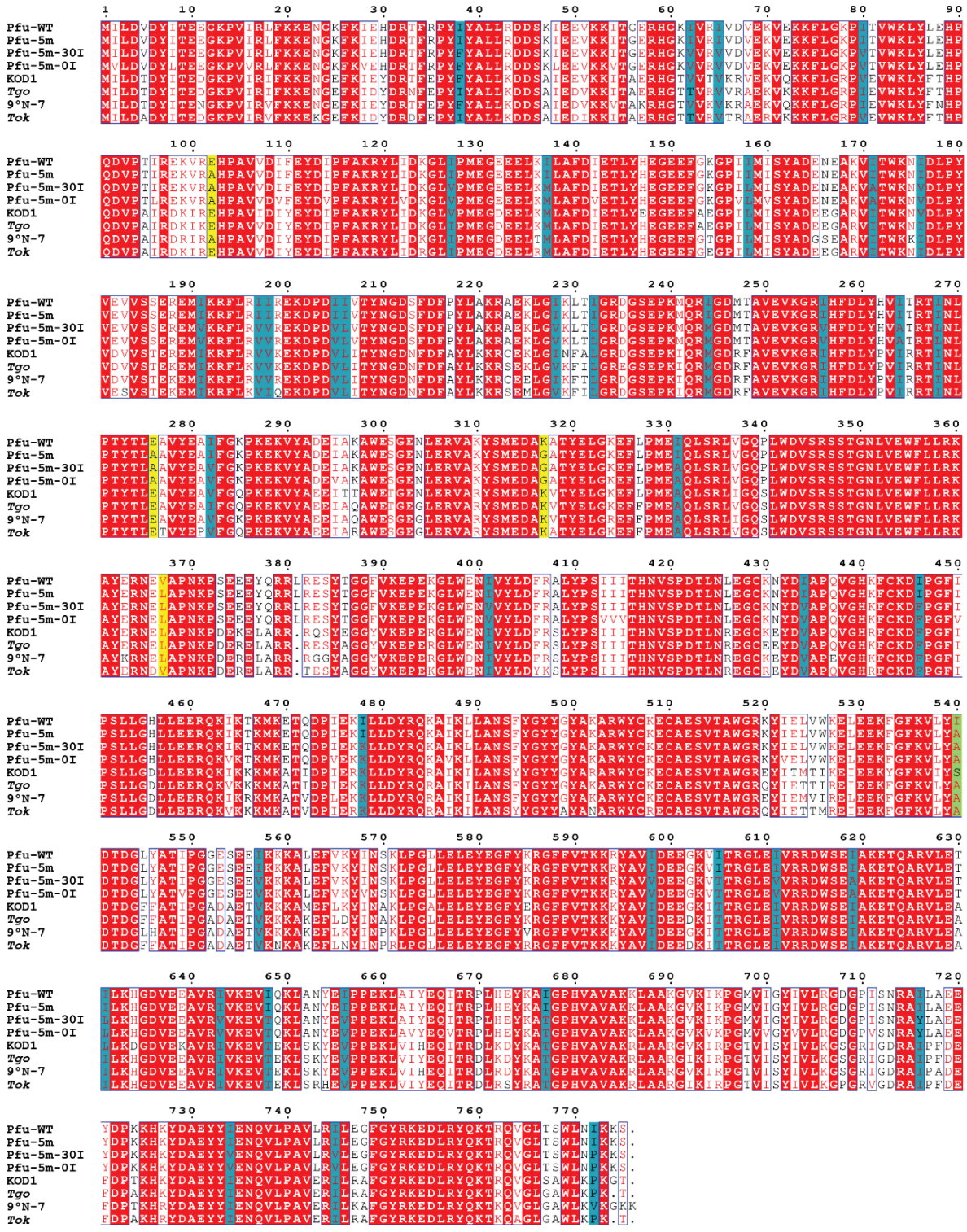
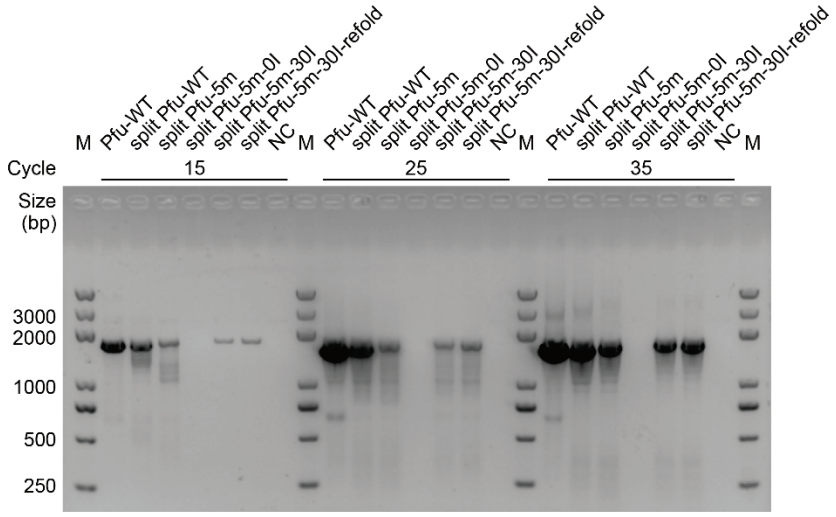


Supplementary Figure 1 | Multiple sequence alignment and design of mutant *Pfu* DNA polymerases. Multiple sequence alignment of Pfu-WT, Pfu-5m, Pfu-5m-30I, Pfu-5m-0I, KOD1, *Tgo*, 9°N-7, and *Tok* polymerases. Conserved amino acids are highlighted in red, mutations for introducing additional NCL sites are highlighted in yellow, substituted isoleucines are highlighted in blue, and I540A is highlighted in green for serving both purposes.

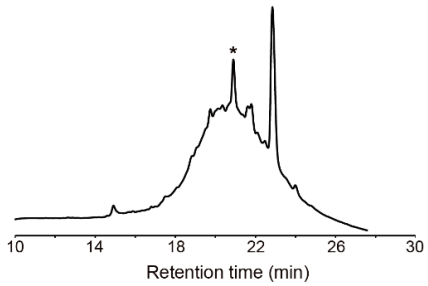


Supplementary Figure 2 | PCR activity of different mutants of *Pfu* DNA polymerase. PCR amplification of a 1.5-kb D-DNA template coding for the bacterial 16S rRNA gene by WT *Pfu* DNA polymerase (Pfu-WT), split WT *Pfu* DNA polymerase (split Pfu-WT), split *Pfu* DNA polymerase with 5 point mutations (split Pfu-5m), split Pfu-5m with all isoleucines substituted (split Pfu-5m-0I), split Pfu-5m with 30 isoleucines left (split Pfu-5m-30I), expressed and *in vitro* refolded split Pfu-5m-30I (split Pfu-5m-30I-refold), and negative control without polymerase (NC), analyzed by 1.2% agarose gel electrophoresis and stained by ExRed, with cycle numbers from which they were sampled indicated above the lanes. M, DNA marker. The experiment was performed twice with similar results.

a

Peptide sequence:

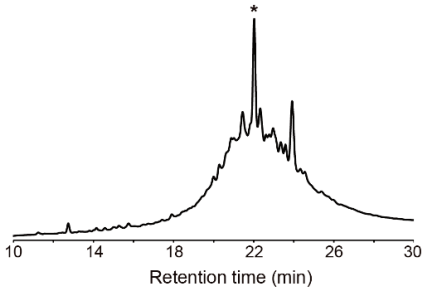
TfaThz-VIDEEGKVITRGLEIVRRDWSEIAKETQARVLETILKHGDVEEAVRIVKEVIQKL



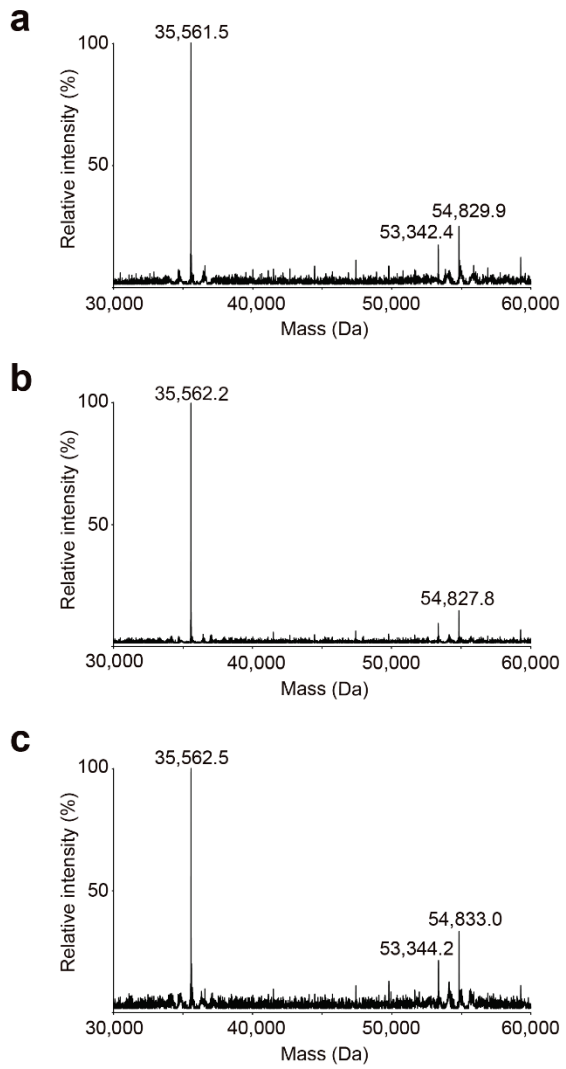
b

Peptide sequence:

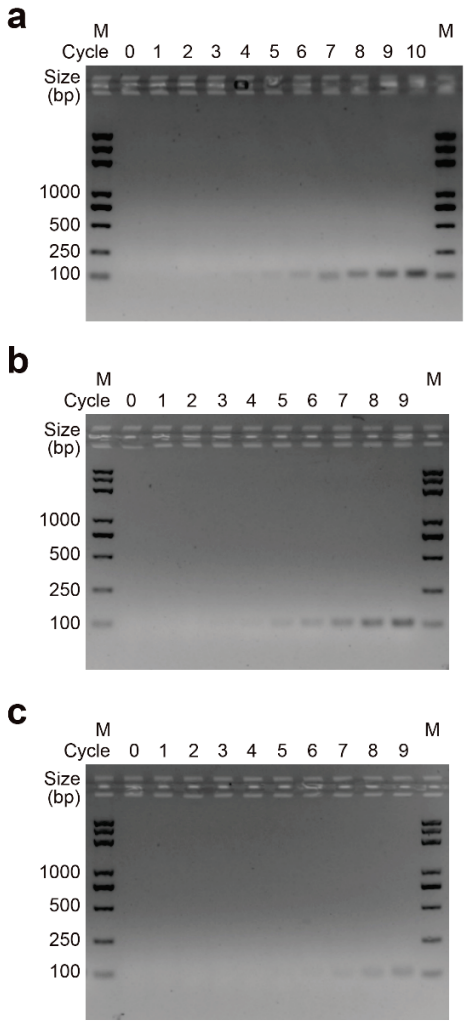
TfaThz-VVDEEGKVTTRGLEVVRRDWSEAAKETQARVLETLLKHGDVEEAVRVVKEVTQKL



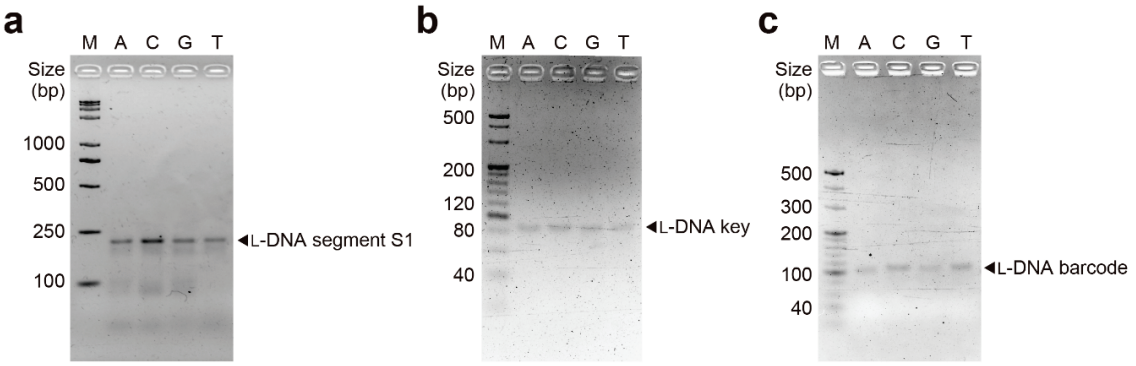
Supplementary Figure 3 | Isoleucine substitution facilitates the synthesis of the Pfu-C-4 peptide segment. a, RP-HPLC analysis of crude Pfu-C-4 peptide segment with WT amino acid sequence. **b,** RP-HPLC analysis of crude Pfu-C-4 peptide segment with all 7 isoleucine residues substituted (underlined). Asterisk, target peptide peak.



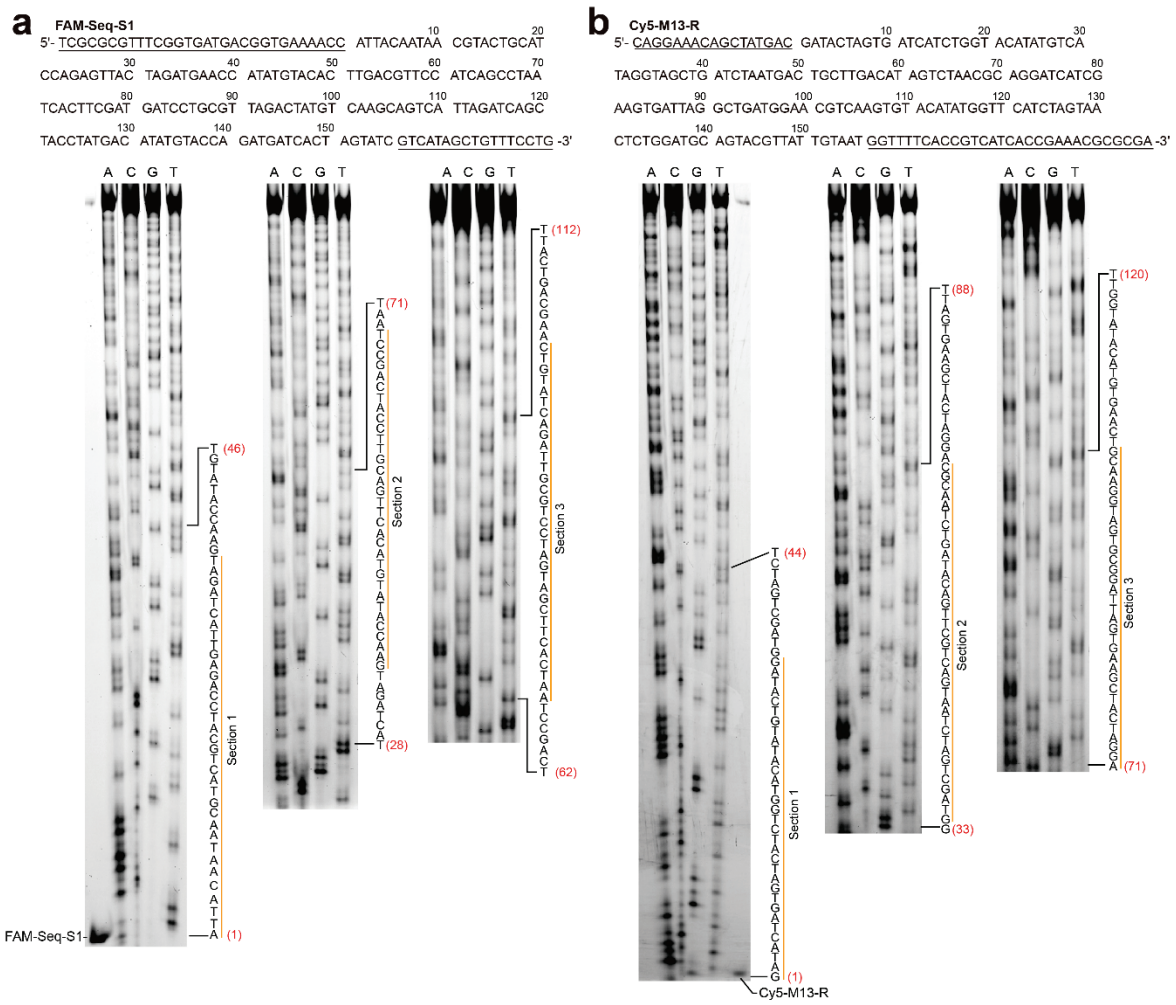
Supplementary Figure 4 | ESI-MS analysis of folded synthetic *Pfu* DNA polymerase before and after heat-precipitation. a, Deconvoluted ESI-MS spectrum of folded synthetic L- split Pfu-5m-30I before heat-precipitation. **b,** Deconvoluted ESI-MS spectrum of folded synthetic L- split Pfu-5m-30I after heat-precipitation. **c,** Deconvoluted ESI-MS spectrum of heat-precipitated pellet of synthetic L- split Pfu-5m-30I after *in vitro* folding. Calculated molecular mass of 54827.9 Da and 35561.0 Da for the folded Pfu-N and Pfu-C fragments, respectively, reflecting disulfide bond formation.



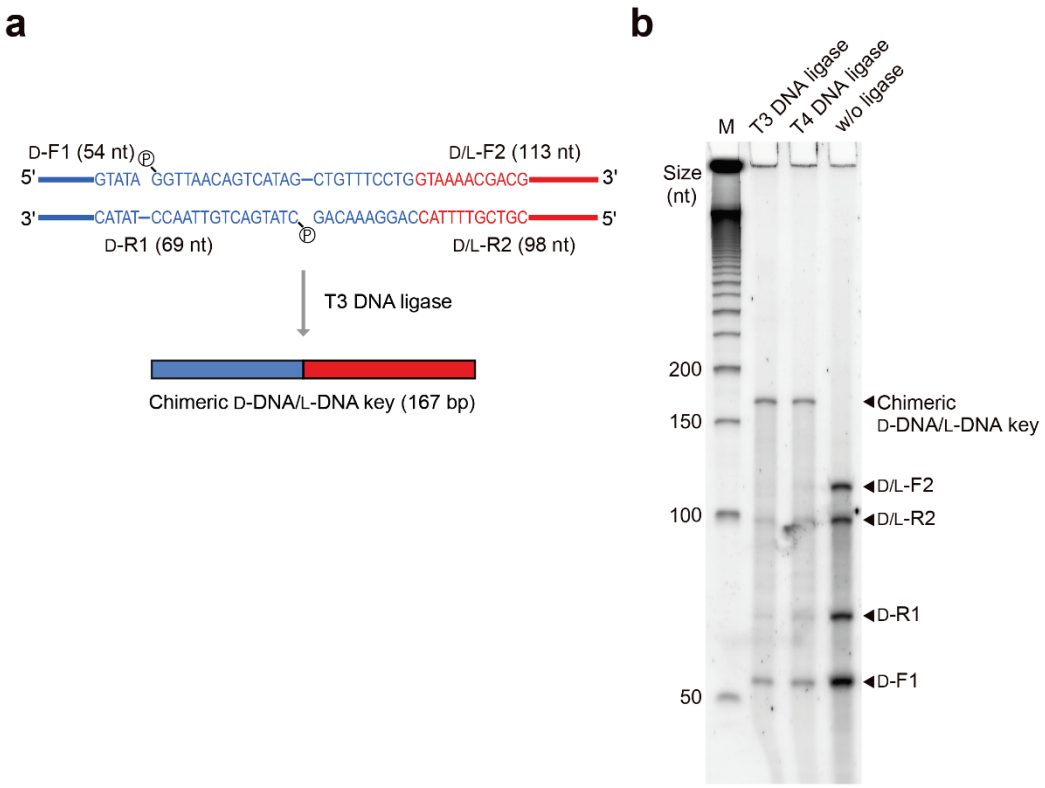
Supplementary Figure 5 | PCR amplification of a 100-bp sequence by WT, and synthetic L- and D- *Pfu* DNA polymerases. PCR amplification by **a,** WT, **b,** synthetic L- *Pfu* DNA polymerase (split L-Pfu-5m-30I), and **c,** synthetic D- *Pfu* DNA polymerase (split D-Pfu-5m-30I) were analyzed by 3% sieving agarose gel electrophoresis and stained by ExRed, with cycle numbers from which they were sampled indicated above the lanes. The PCR amplification efficiency of the synthetic D- *Pfu* DNA polymerase measured ~1.5, estimated based on the intensity of the product bands, analyzed by the ImageLab software (Bio-Rad Laboratories). M, DNA marker. The PCR amplifications by WT *Pfu* DNA polymerases were performed twice with similar results. The PCR amplifications by synthetic L- and D- *Pfu* DNA polymerase were performed once.



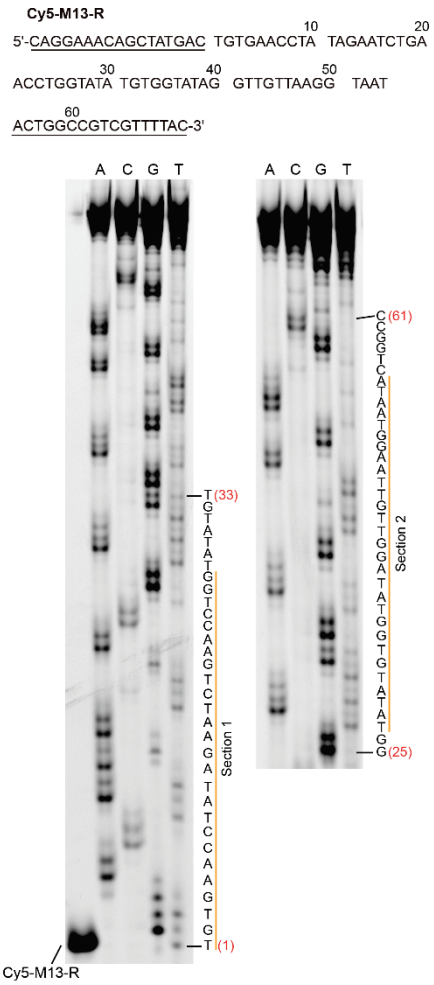
Supplementary Figure 6 | Mirror-image PCR with L-dNTPαSs for L-DNA phosphorothioate sequencing. a, L-DNA segment S1 of 203 bp from L-DNA storage library amplified by D-Dpo4-5m with L-dNTPαSs, analyzed by 2.5% agarose gel and stained by ExRed. The experiment was performed twice with similar results. **b,** The L-DNA part of the chimeric DNA key of 88 bp amplified by D-Dpo4-5m with L-dNTPαSs, analyzed by 4% sieving agarose gel and stained by ExRed. The experiment was performed twice with similar results. **c,** L-DNA barcode of 100 bp amplified after one year storage by D-Dpo4-5m with L-dNTPαSs, analyzed by 3% agarose gel and stained by ExRed. The experiment was performed twice with similar results. A, L-dATP replaced by L-dATPαS; C, L-dCTP replaced by L-dCTPαS; G, L-dGTP replaced by L-dGTPαS; T, L-dTTP replaced by L-dTTPαS. M, DNA marker.



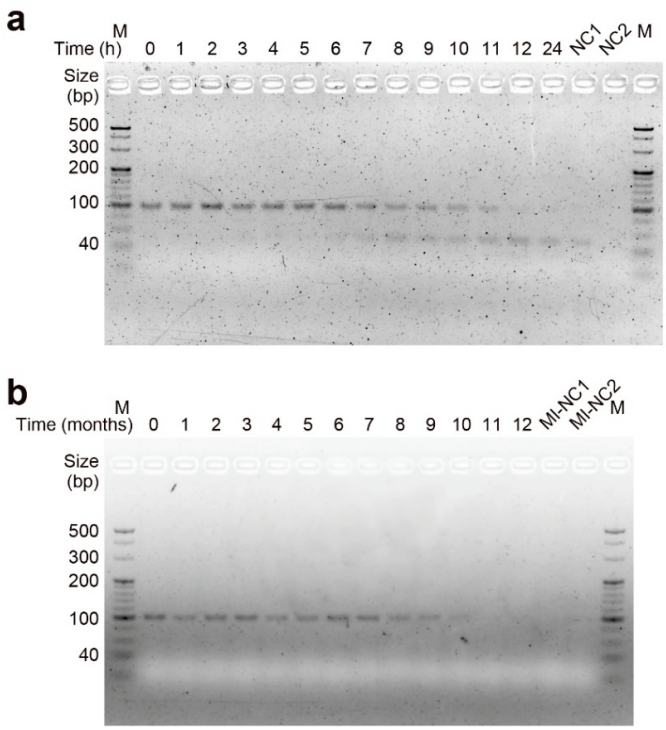
Supplementary Figure 7 | Sequencing of L-DNA segment S1 using D-Dpo4-5m by the phosphorothioate approach. a, Amplification products of L-DNA segment S1 by D-Dpo4-5m with L-dNTPαSs and 5'-FAM-labelled forward sequencing primer were each cleaved by 2-iodoethanol, and analyzed by 10% denaturing PAGE and scanned by a Typhoon Trio^{+} system operated under Cy2 mode. **b,** Amplification products of L-DNA segment S1 by D-Dpo4-5m with L-dNTPαSs and 5'-Cy5-labelled reverse sequencing primer were each cleaved by 2-iodoethanol, and analyzed by 10% denaturing PAGE and scanned by a Typhoon Trio^{+} system operated under Cy5 mode. The corresponding sequencing chromatograms are shown in Fig. 3f,g with each section (1 to 3) analyzed independently. The experiment was performed twice with similar results.



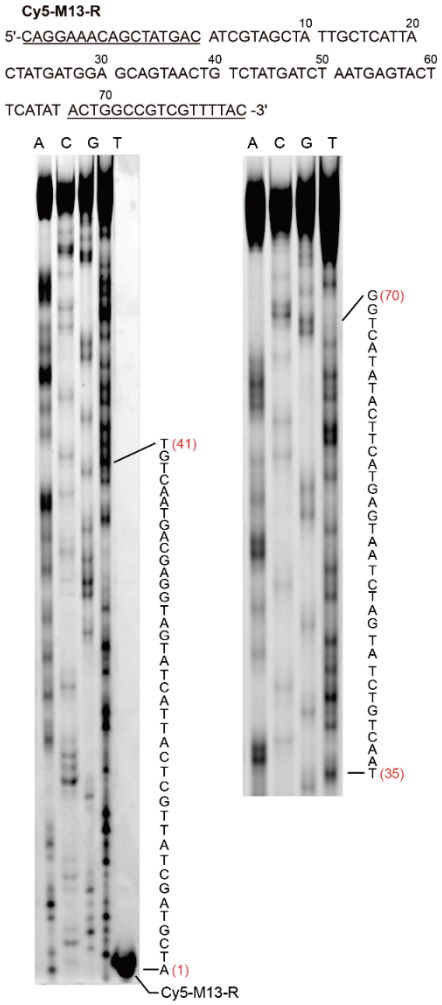
Supplementary Figure 8 | Chimeric D-DNA/L-DNA key for chiral steganography. a, Design and preparation of a chimeric double-stranded D-DNA/L-DNA key molecule by D-DNA/L-DNA oligo synthesis and enzymatic ligation. The D-DNA (blue) and L-DNA (red) parts of the key molecule are distinguished by color. **b,** Double-stranded chimeric D-DNA/L-DNA key molecule prepared using T3 or T4 DNA ligase, analyzed by 10% denaturing PAGE and stained by ExRed. M, ssDNA marker. The experiment was performed three times with similar results.



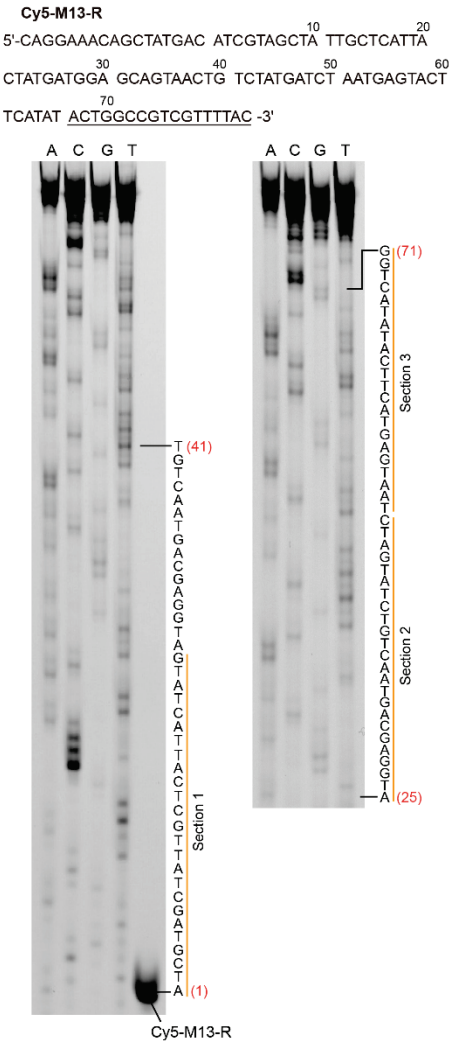
Supplementary Figure 9 | Sequencing of the L-DNA part of the chimeric DNA key using D-Dpo4-5m by the phosphorothioate approach. The L-DNA part of the chimeric DNA key was amplified by MI-PCR using D-Dpo4-5m, and further amplified by D-Dpo4-5m with L-dNTPαSs and 5'-Cy5-labelled reverse sequencing primer, cleaved by 2-iodoethanol, and analyzed by 10% denaturing PAGE and scanned by a Typhoon Trio^{+} system operated under Cy5 mode. The corresponding sequencing chromatograms are shown in Fig. 4f with each section (1 and 2) analyzed independently. The experiment was performed once.



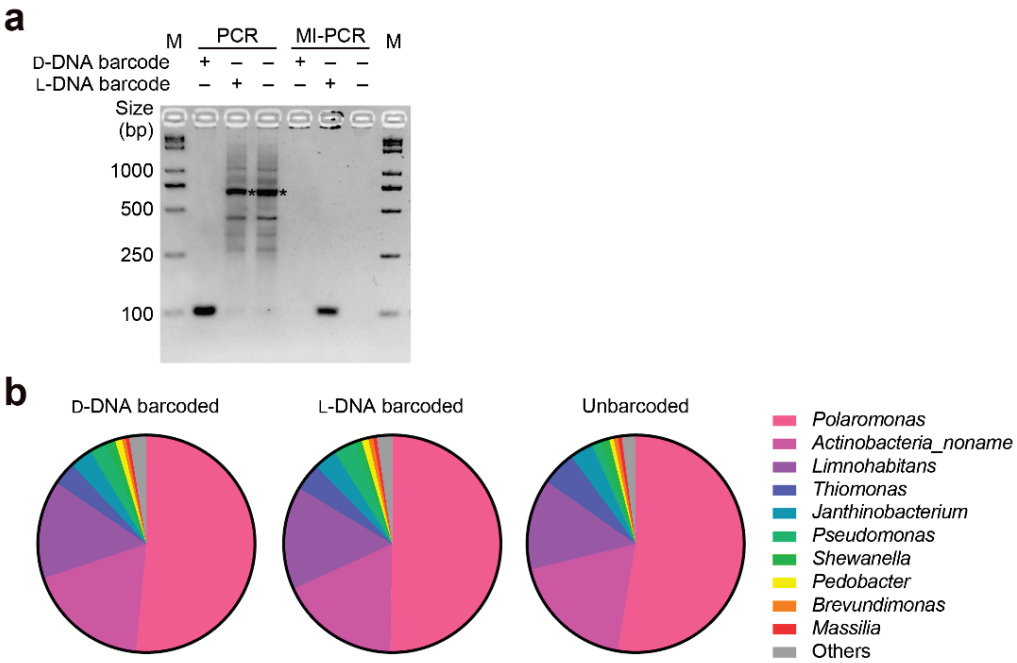
Supplementary Figure 10 | Degradation of D-DNA or L-DNA barcode in pond water samples stored at 4 °C. a, Time course (from 0 to 24 h) of degradation of the D-DNA barcode in pond water samples, extracted from 2-ml pond water samples stored at 4 °C, amplified by PCR using L-Dpo4-5m, analyzed by 3% sieving agarose gel electrophoresis and stained by ExRed. NC1, negative control without D-DNA barcode. NC2, negative control without L-Dpo4-5m. The experiment was performed twice with similar results. **b,** Time course (from 0 to 12 months) of degradation of the L-DNA barcode in pond water samples, extracted from 2-ml pond water samples stored at 4 °C, amplified by MI-PCR using D-Dpo4-5m, analyzed by 3% sieving agarose gel electrophoresis and stained by ExRed. The amplified L-DNA bands were very faint after 11 and 12 months, and thus an improved method using 40-ml pond water samples for amplification was applied to retrieve the information stored in the L-DNA barcode (Fig. 5c). MI-NC1, negative control without L-DNA barcode. MI-NC2, negative control without D-Dpo4-5m. M, DNA marker. The experiment was performed once.



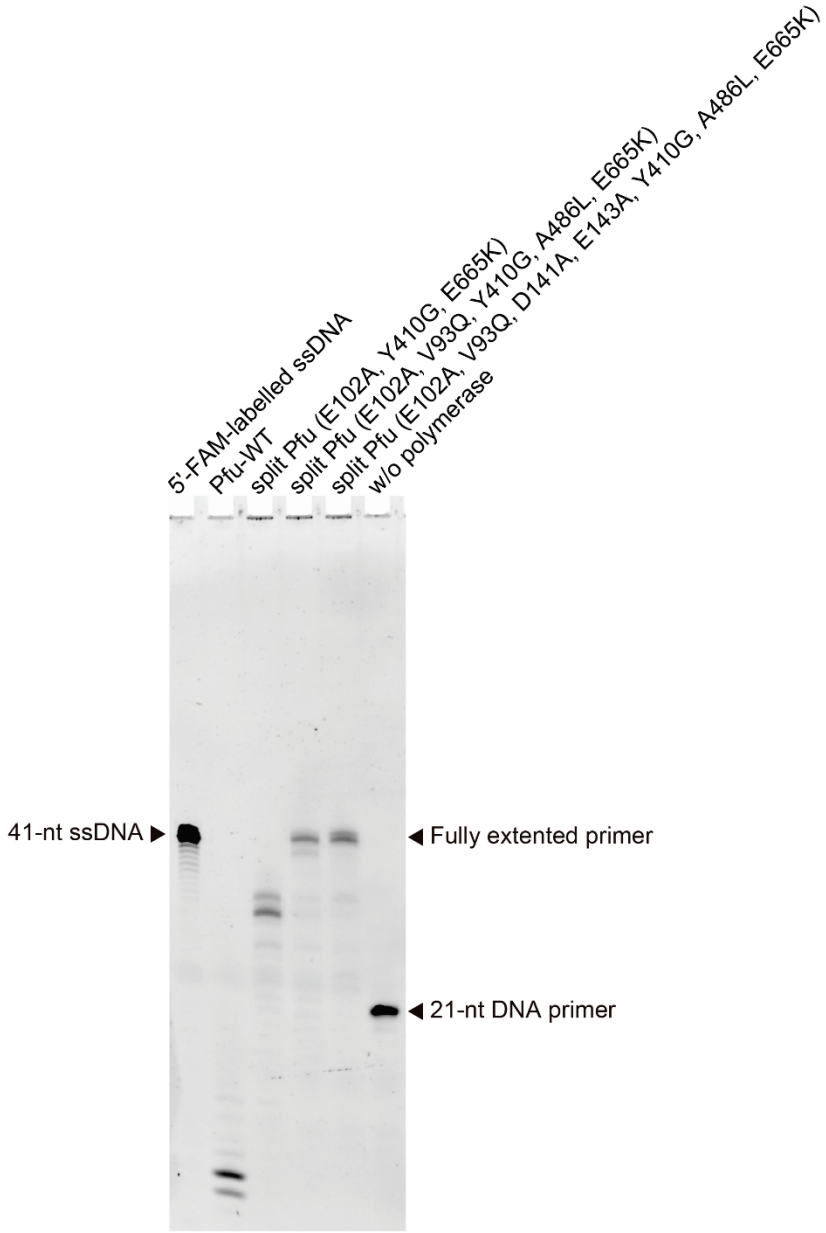
Supplementary Figure 11 | Sequencing of L-DNA barcode in pond water samples after 8 months using D-Dpo4-5m by the phosphorothioate approach. The L-DNA barcode in pond water samples was amplified after 8 months by D-Dpo4-5m with L-dNTPαSs and 5'-Cy5-labelled reverse sequencing primer, cleaved by 2-iodoethanol, and analyzed by 10% denaturing PAGE and scanned by a Typhoon Trio^{+} system operated under Cy5 mode. The experiment was performed once.



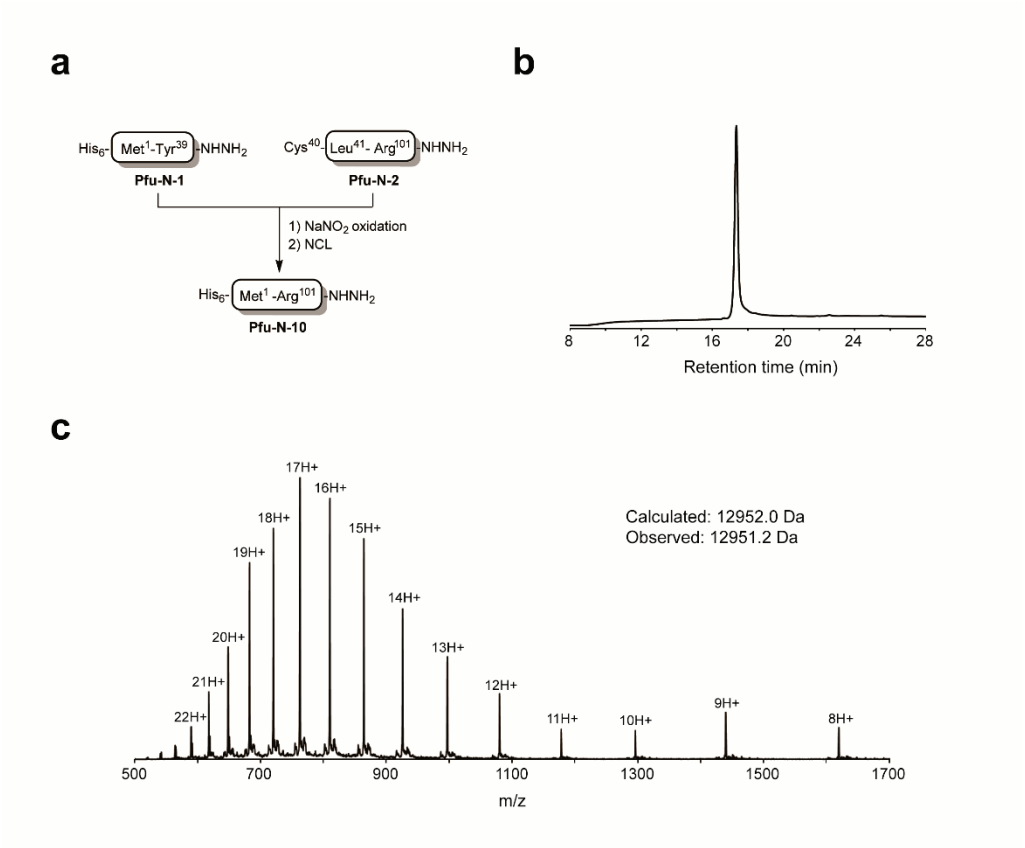
Supplementary Figure 12 | Sequencing of L-DNA barcode in pond water samples after 1 year using D-Dpo4-5m by the phosphorothioate approach. The L-DNA barcode in pond water samples was amplified after 1 year by D-Dpo4-5m with L-dNTPαSs and 5'-Cy5-labelled reverse sequencing primer, cleaved by 2-iodoethanol, and analyzed by 10% denaturing PAGE and scanned by a Typhoon Trio^{+} system operated under Cy5 mode. The corresponding sequencing chromatograms are shown in Fig. 5d with each section (1 to 3) analyzed independently. The experiment was performed three times with similar results.



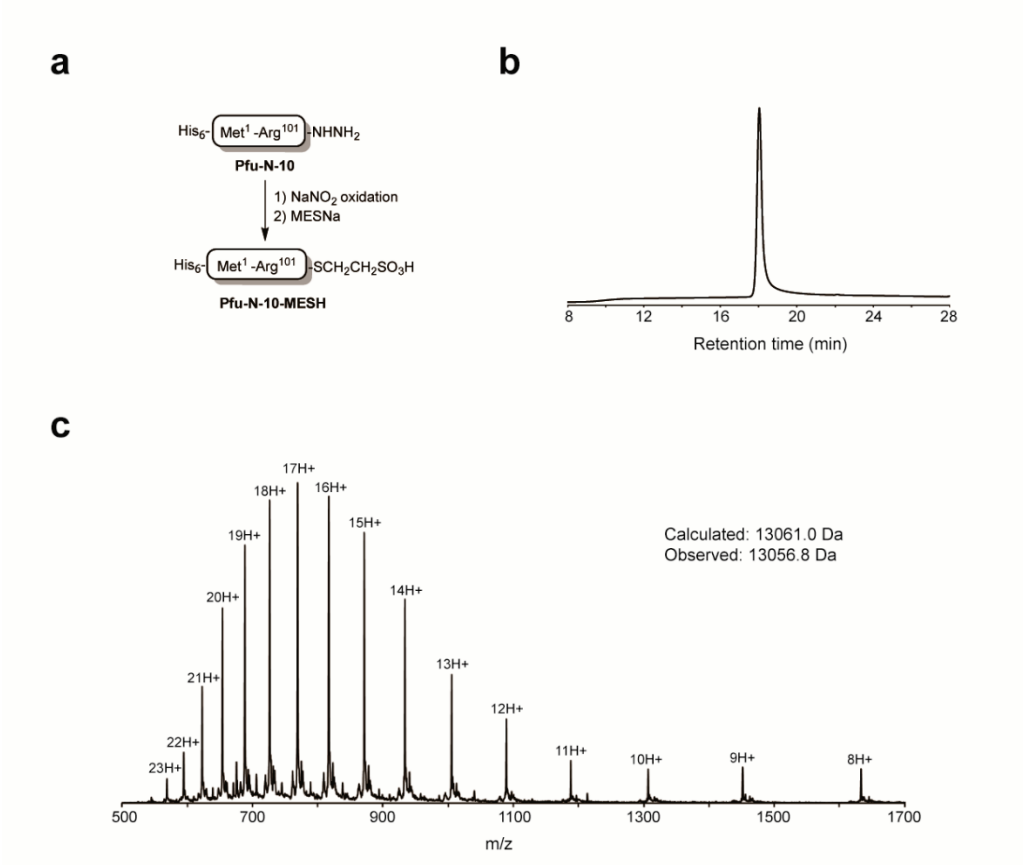
Supplementary Figure 13 | Spiking D- or L-DNA barcode into microbial DNA extracted from the pond water samples. a, The 100-bp double-stranded information-storing D- or L-DNA barcode was spiked into the microbial (D-) DNA extracted from the pond water samples, and amplified by synthetic natural and mirror-image *Pfu* DNA polymerases, analyzed by 3% sieving agarose gel electrophoresis and stained by ExRed. M, DNA marker. Asterisk, non-specific amplification bands appeared when the D-DNA primers annealed to the microbial DNA in the absence of D-DNA barcode. The experiment was performed twice with similar results. **b,** Metagenomic analysis of the top 10 genera of microbial organisms present in the pond water samples by MetaPhlAn 2.7.7, using D- or L-DNA barcoded, or unbarcoded microbial DNA samples.



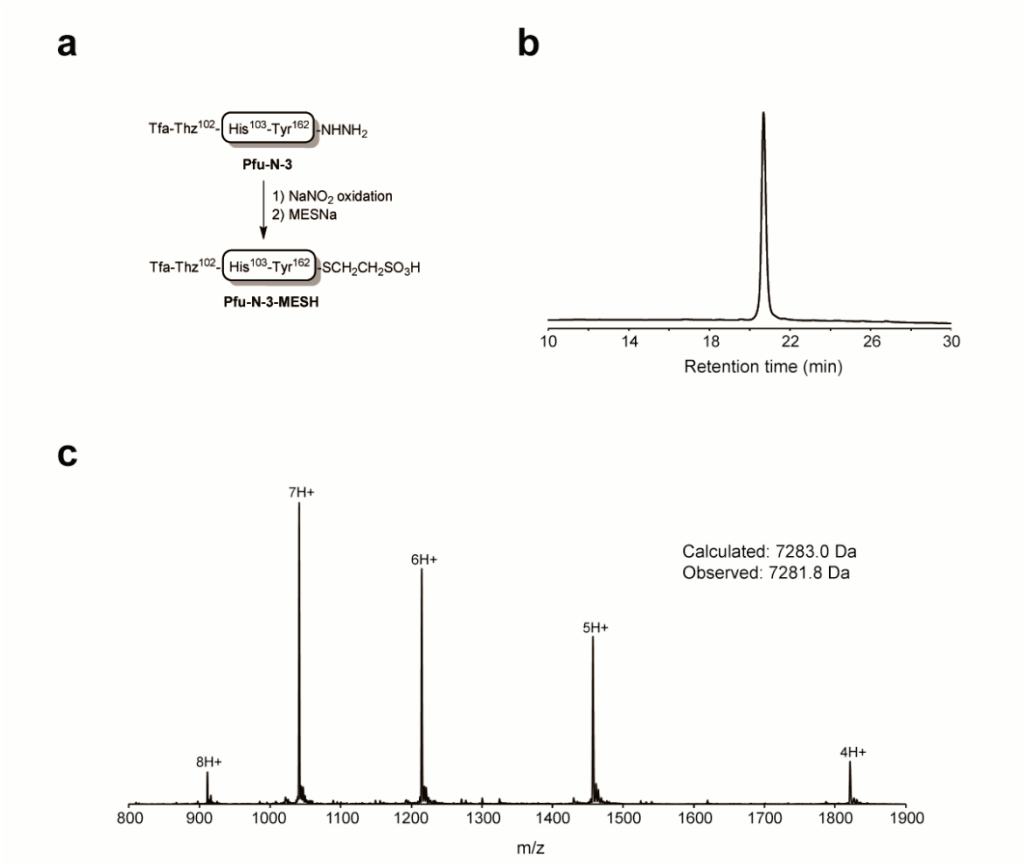
Supplementary Figure 14 | DNA-templated RNA polymerization by mutant *Pfu* DNA polymerases. DNA-template-directed primer extension by different *Pfu* DNA polymerase mutants with 41-nt single-stranded DNA template, 5'-FAM-labelled 21-nt DNA primer, and NTPs, incubated at 65 °C for 10 min, and analyzed by 20% denaturing PAGE and scanned by a Typhoon Trio+ system operated under Cy2 mode. Synthetic 5'-FAM-labelled 41-nt ssDNA and 21-nt DNA primer served as markers. The experiment was performed twice with similar results.



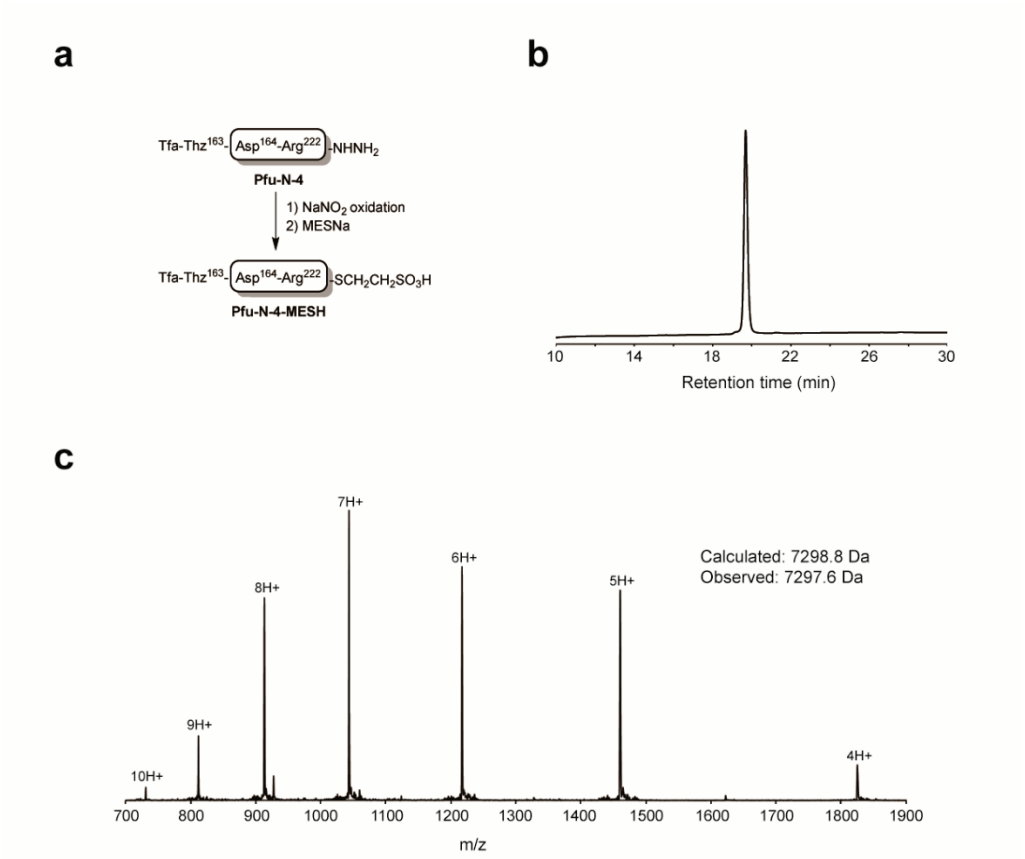
Supplementary Figure 15 | Preparation of L-Pfu-N-10. a, L-Pfu-N-1 (64.6 mg) was dissolved in 2.3 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 228 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 2.3 ml 0.2 M MPAA (in 6 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. After the addition of L-Pfu-N-2 (62.5 mg), the pH of the reaction mixture was adjusted to 6.6 with NaOH solution at room temperature. After 14 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 25-60% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). The ligation product was obtained with a yield of 34% (68.2 mg). **b,** Analytical HPLC chromatogram of the ligation product L-Pfu-N-10 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-10.



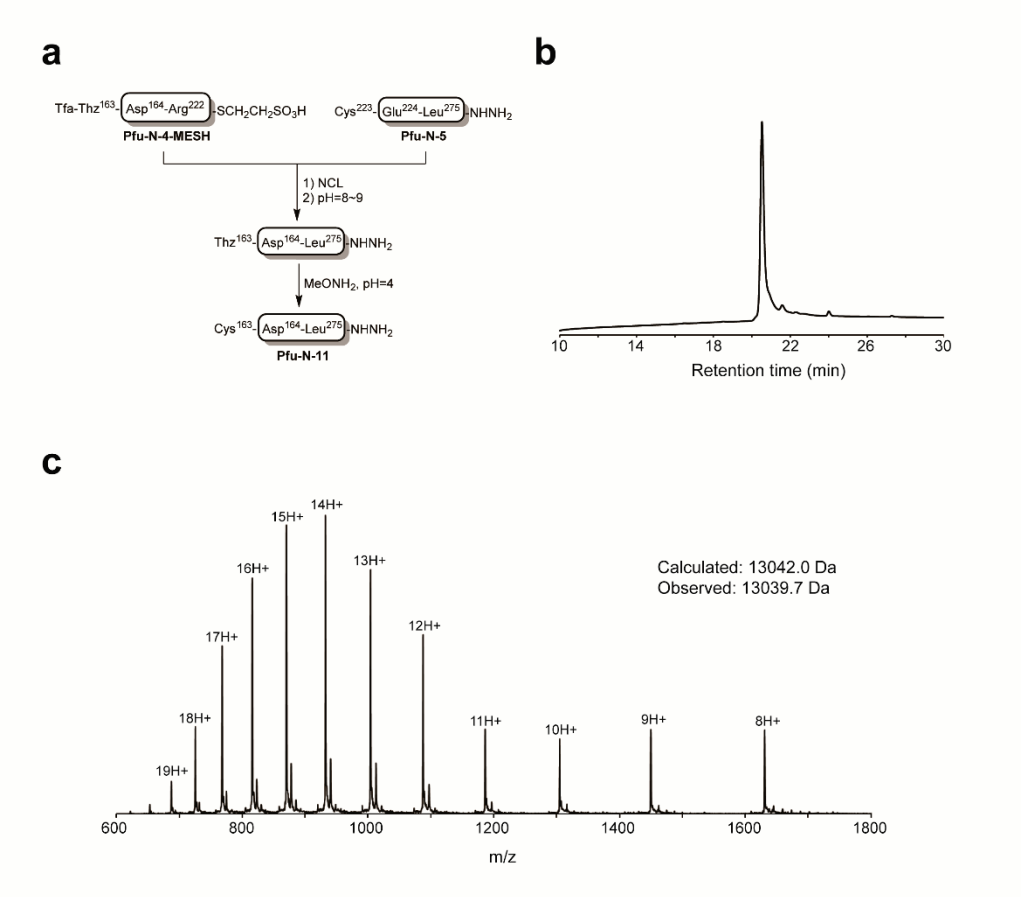
Supplementary Figure 16 | Preparation of L-Pfu-N-10 MESH. a, L-Pfu-N-10 (35.5 mg) was dissolved in 0.55 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 55 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 0.25 ml 0.4 M MESNa (in acidified ligation buffer) was added. The pH of the reaction mixture was adjusted to 4.8 with NaOH solution at room temperature. After 1 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 25-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-10 MESH was obtained with a yield of 67% (24.2 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-10 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-10 MESH.



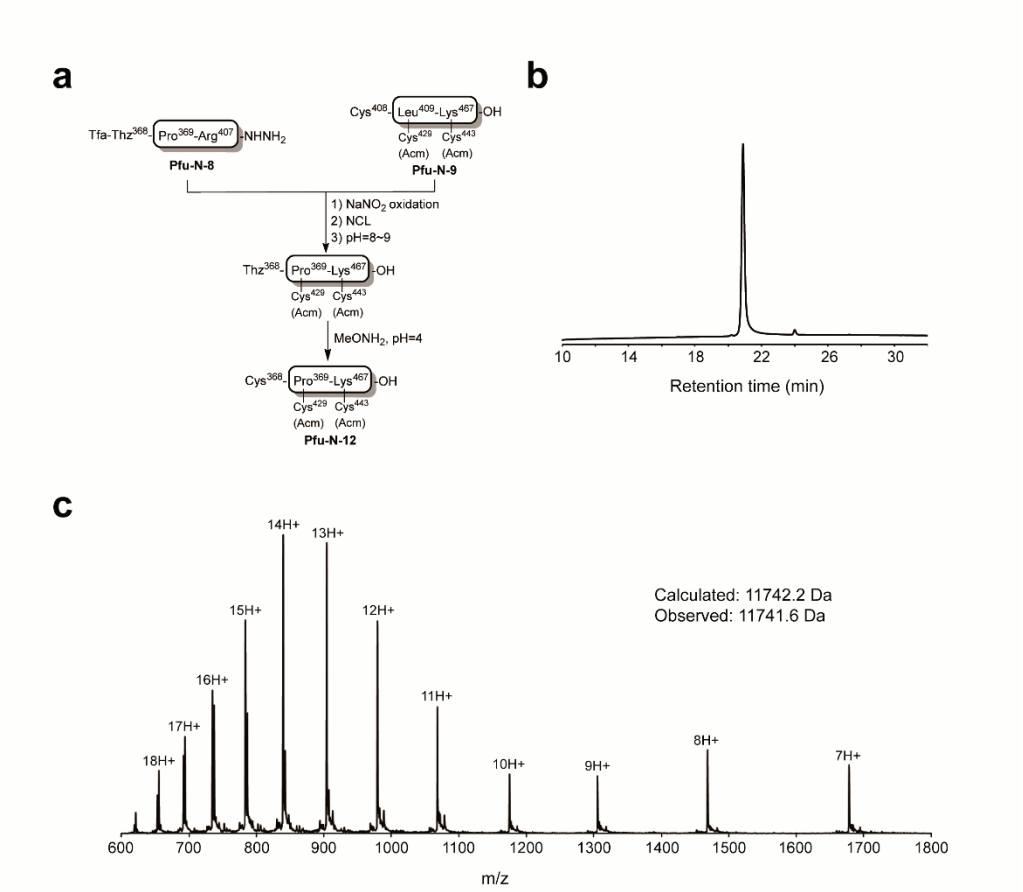
Supplementary Figure 17 | Preparation of L-Pfu-N-3 MESH. a, L-Pfu-N-3 (41.7 mg) was dissolved in 1.5 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath, and 120 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath (-10 °C) under stirring for 25 min, after which 0.6 ml 0.4 M MESNa (in acidified ligation buffer) was added. The pH of the reaction mixture was adjusted to 4.8 with NaOH solution at room temperature. After 1 h, the products were purified by HPLC (purification conditions: 35-75% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-3 MESH was obtained with a yield of 56% (23.6 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-3 MESH (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-3 MESH.



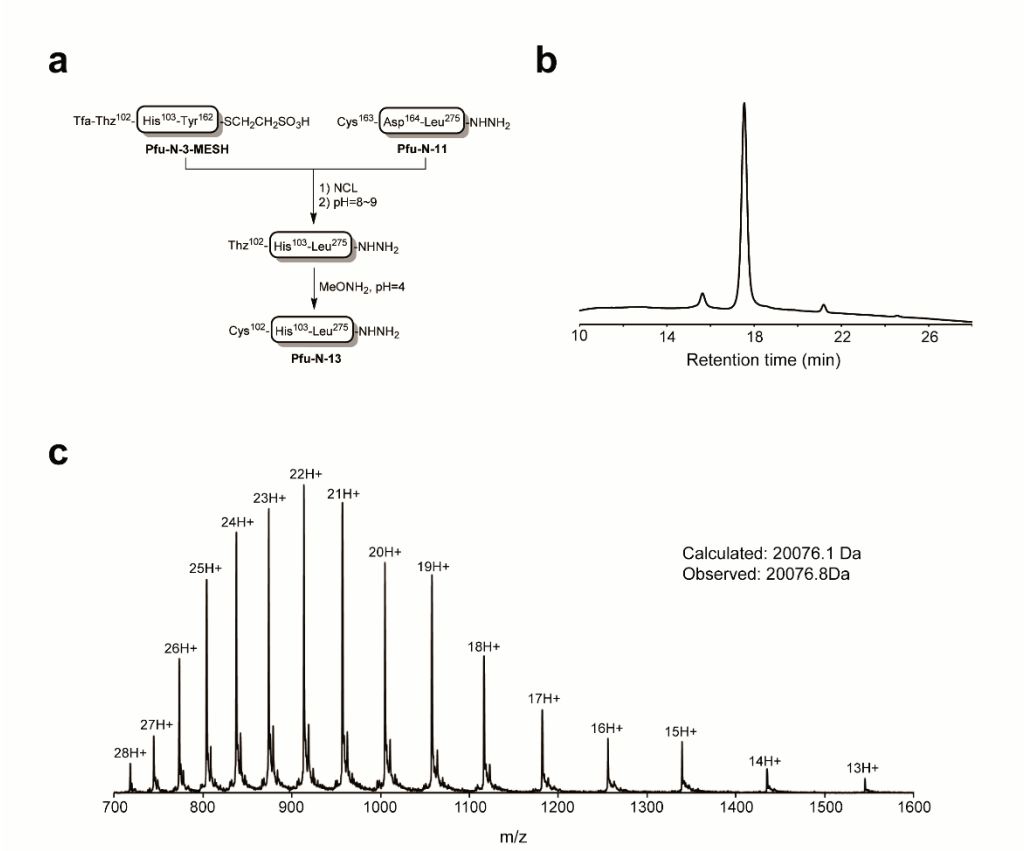
Supplementary Figure 18 | Preparation of L-Pfu-N-4 MESH. a, The preparation of L-Pfu-N-4 MESH (46.7 mg) was carried out following a procedure similar to the preparation of L-Pfu-N-3 MESH (Supplementary Fig. 17). Purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. L-Pfu-N-4 MESH was obtained with a yield of 69% (32.5 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-4 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-4 MESH.



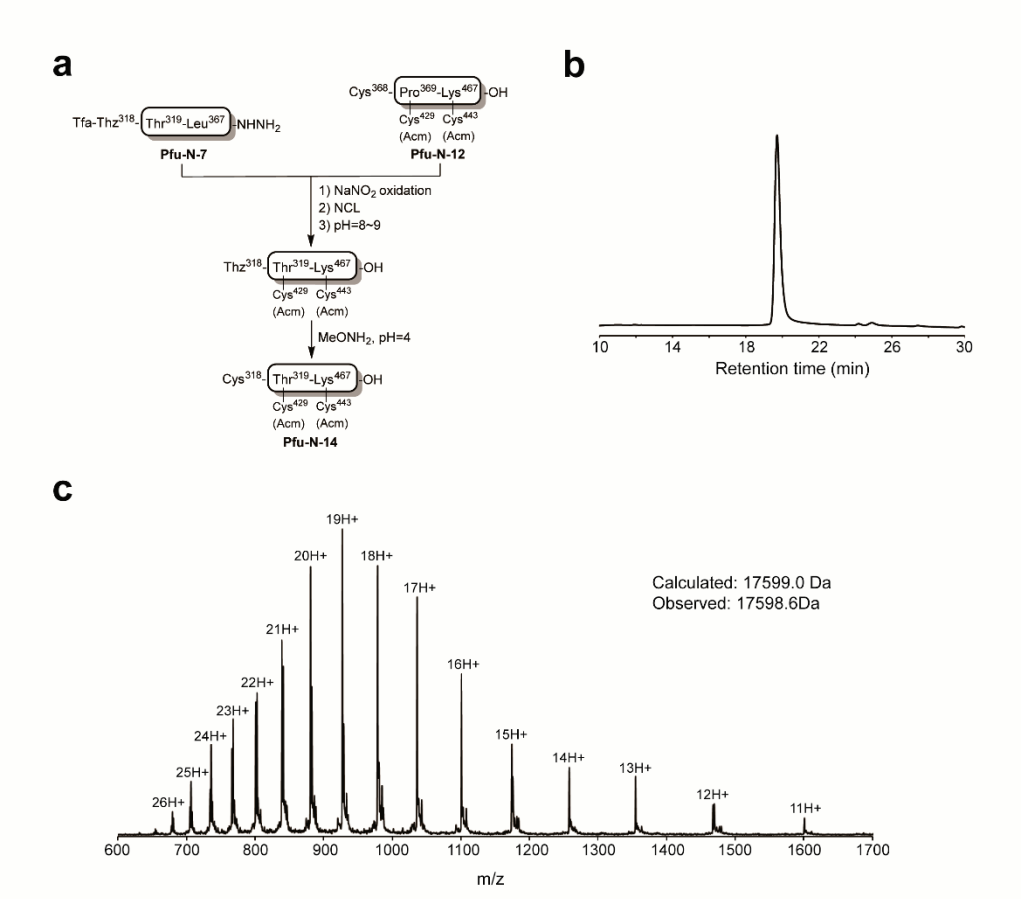
Supplementary Figure 19 | Preparation of L-Pfu-N-11. a, L-Pfu-N-4 MESH (32.7 mg) and L-Pfu-N-5 (33.1 mg) were dissolved in a 2.2 ml aqueous solution of 6 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP and 100 mM MPAA. The pH of the reaction mixture was adjusted to 6.5. After 15 h, the products were analyzed by HPLC, the pH was adjusted to 9.0 to promote the complete removal of the Tfa group. After 1 h, the products were analyzed, and $MeONH_2$·HCl (26.9 mg) was added to carry out the conversion of Thz into Cys. TCEP·HCl was added until the pH of the reaction mixture reached 4.0. After 3 h, the products were analyzed and purified by HPLC (purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-11 was obtained with a yield of 57% (34.1 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-11 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-11.



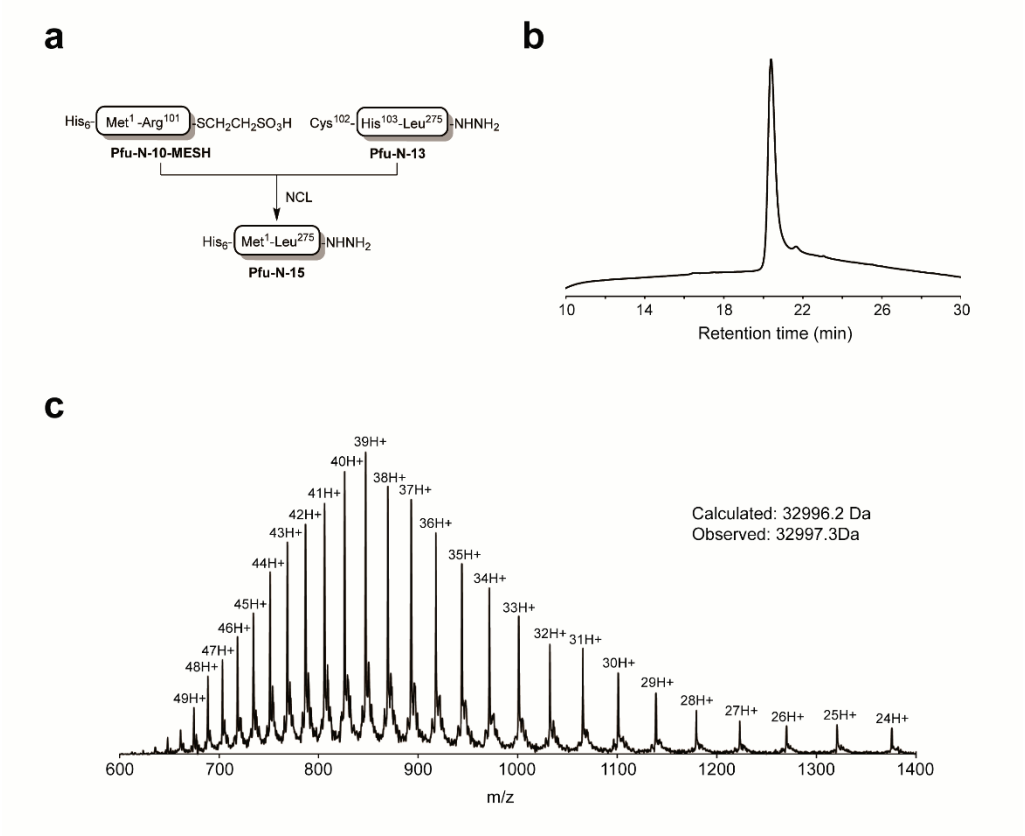
Supplementary Figure 20 | Preparation of L-Pfu-N-12. a, L-Pfu-N-8 (62.4 mg) was dissolved in 2.6 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 260 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 2.5 ml 0.2 M MPAA (in 6 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. The pH of the reaction mixture was adjusted to 5.3 with NaOH solution at room temperature. After the addition of L-Pfu-N-9 (71.6 mg), the pH of the reaction mixture was further adjusted to 6.5. After 14 h, the products were analyzed and the pH was adjusted to 9.0 to promote the complete removal of the Tfa group. After 1 h, the products were analyzed, and $MeONH_2$·HCl (76 mg) was added to carry out the conversion of Thz into Cys. TCEP·HCl was added until the pH of the reaction mixture reached 4.0. After 3 h, the products were analyzed and purified by HPLC (purification conditions: 25-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-12 was obtained with a yield of 46% (55.7 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-12 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-12.



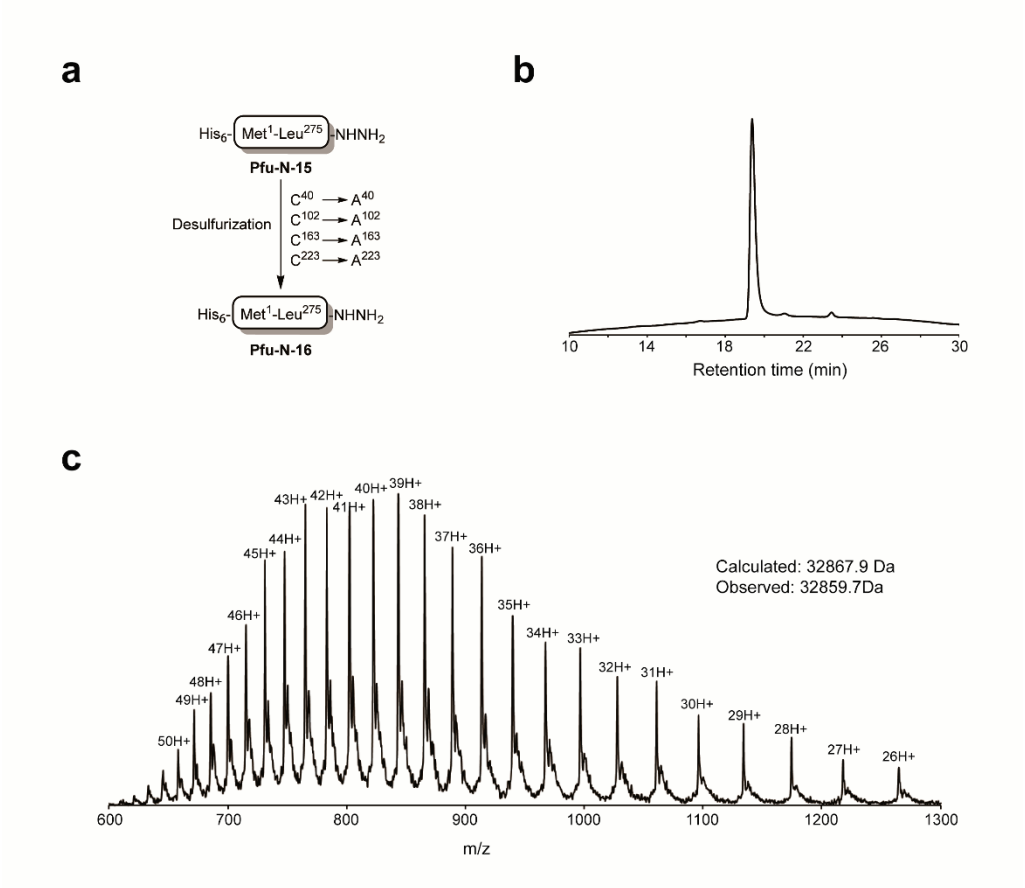
Supplementary Figure 21 | Preparation of L-Pfu-N-13. a, The ligation of L-Pfu-N-3 MESH (42.3 mg) and L-Pfu-N-11 (63.7 mg) was carried out following a procedure similar to the ligation of L-Pfu-N-4 MESH and L-Pfu-N-5 (Supplementary Fig. 19). Purification conditions: 35-75% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. L-Pfu-N-13 was obtained with a yield of 48% (46.8 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-13 (λ=214 nm). Column: Welch C4. Gradient: 30-80% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-13.



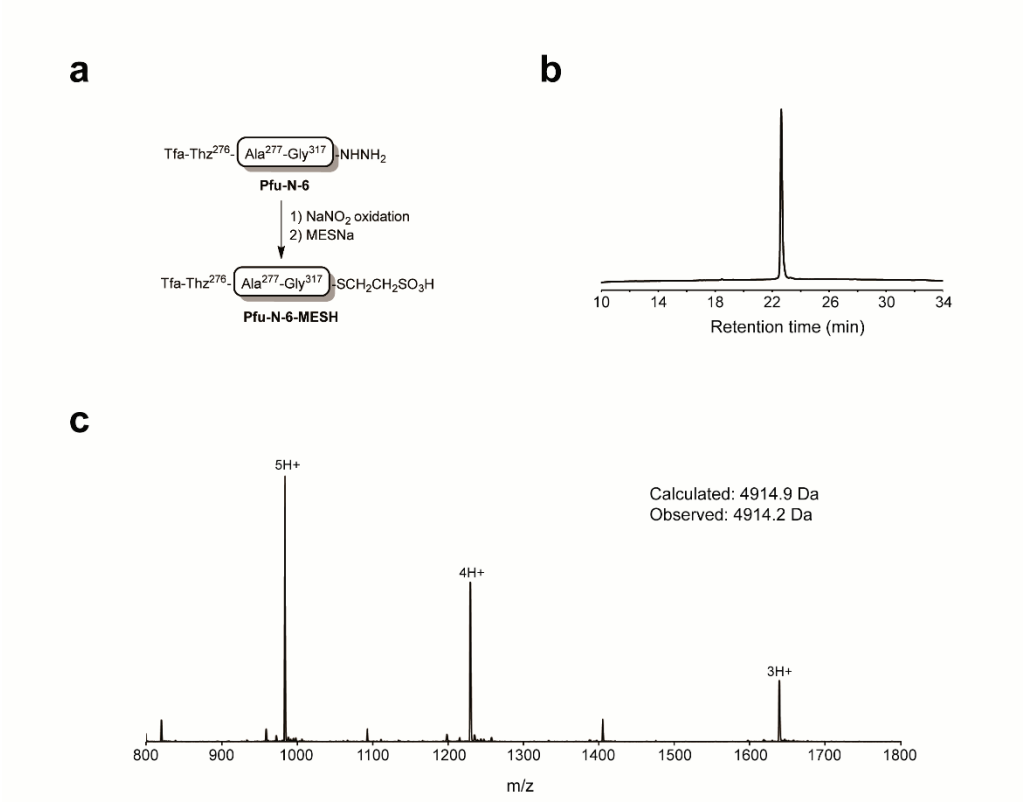
Supplementary Figure 22 | Preparation of L-Pfu-N-14. a, The ligation of L-Pfu-N-7 (42.3 mg) and L-Pfu-N-12 (62.8 mg) was carried out following a procedure similar to the ligation of L-Pfu-N-8 and L-Pfu-N-9 (Supplementary Fig. 20). Purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. L-Pfu-N-14 was obtained with a yield of 48% (46 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-14 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-14.



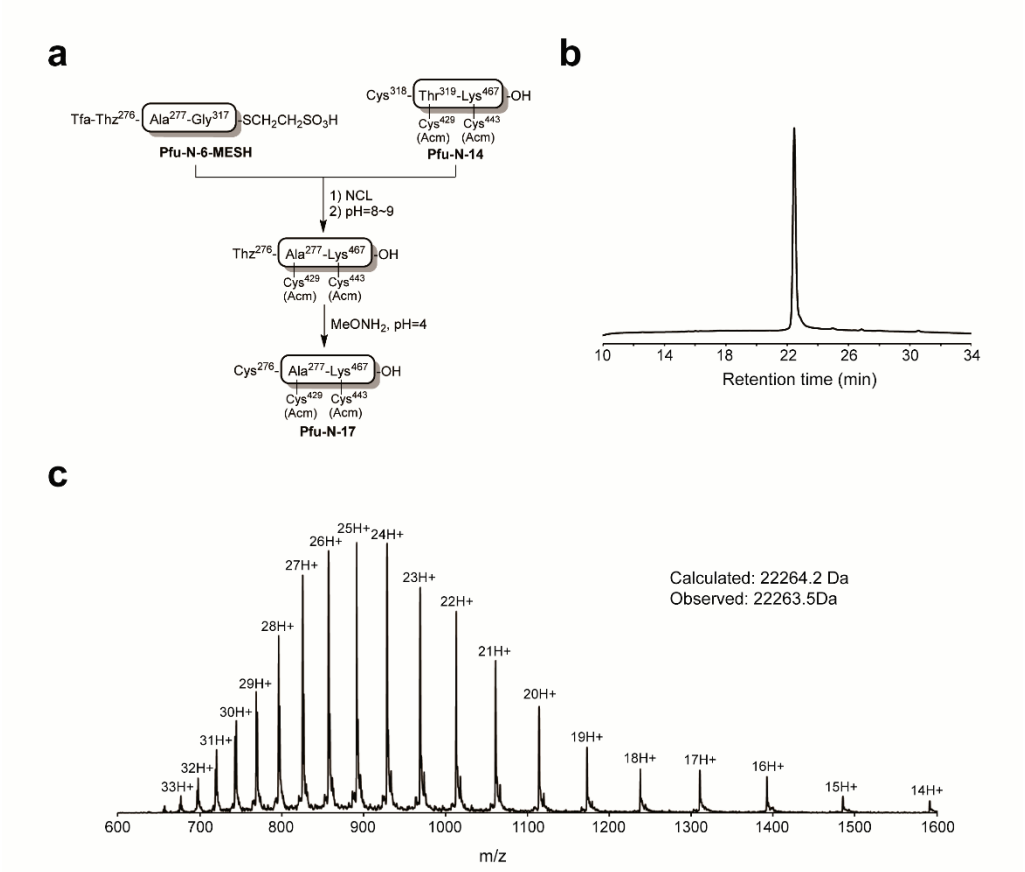
Supplementary Figure 23 | Preparation of L-Pfu-N-15. a, L-Pfu-N-10 MESH (15.8 mg) and L-Pfu-N-13 (22.1 mg) were dissolved in a 1.5 ml aqueous solution of 6 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP and 100 mM MPAA. The pH of the reaction mixture was adjusted to 6.5. After 14 h, the products were analyzed by HPLC (purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-15 was obtained with a yield of 65% (23.7 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-15 (λ=214 nm). Column: Welch C4. Gradient: 20-80% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-15.



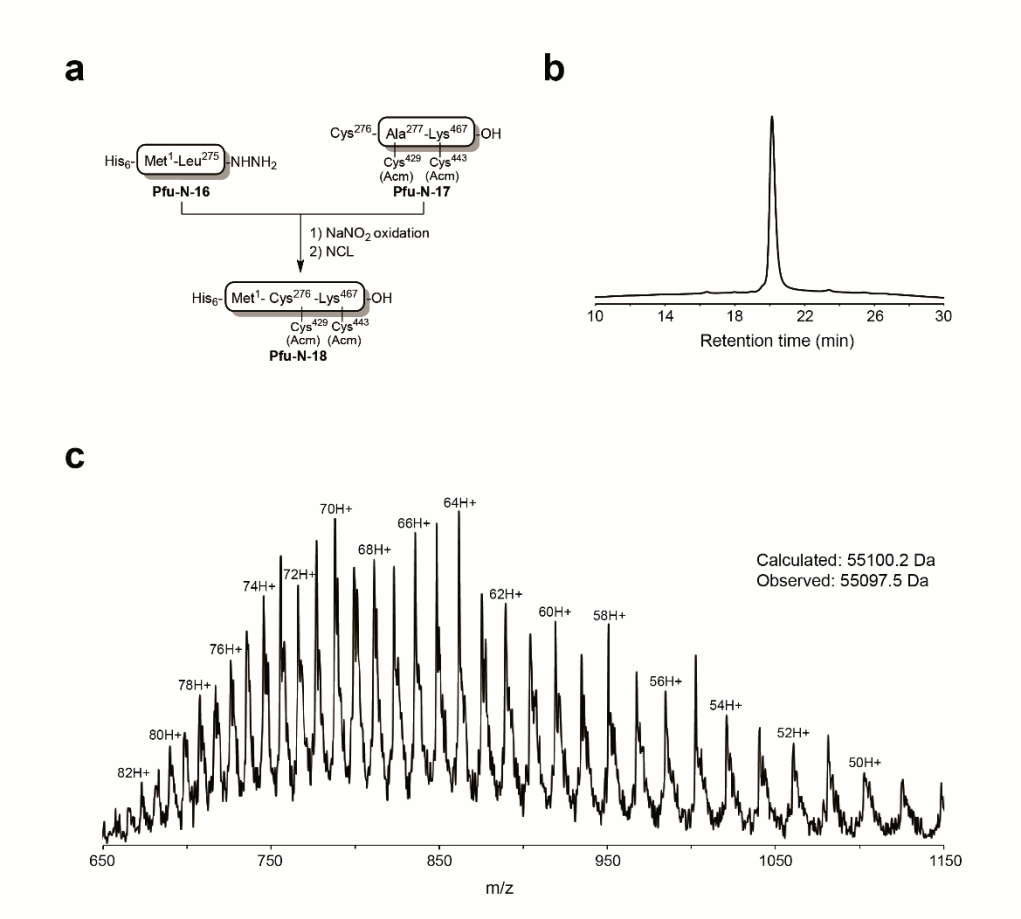
Supplementary Figure 24 | Preparation of L-Pfu-N-16. a, L-Pfu-N-15 (23.7 mg) was dissolved in 8 ml 200 mM TCEP solution (6 M Gn·HCl and 0.1 Na_2HPO_4, pH 7.0), containing 20 mM VA-044 and 40 mM reduced L-glutathione. The reaction was under stirring overnight at 37 °C. The desulfurization product L-Pfu-N-16 was analyzed by HPLC and ESI-MS and purified by semi-preparative HPLC (purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-16 was obtained with a yield of 75% (17.8 mg). **b,** Analytical HPLC chromatogram of the desulfurization product L-Pfu-N-16 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-16.



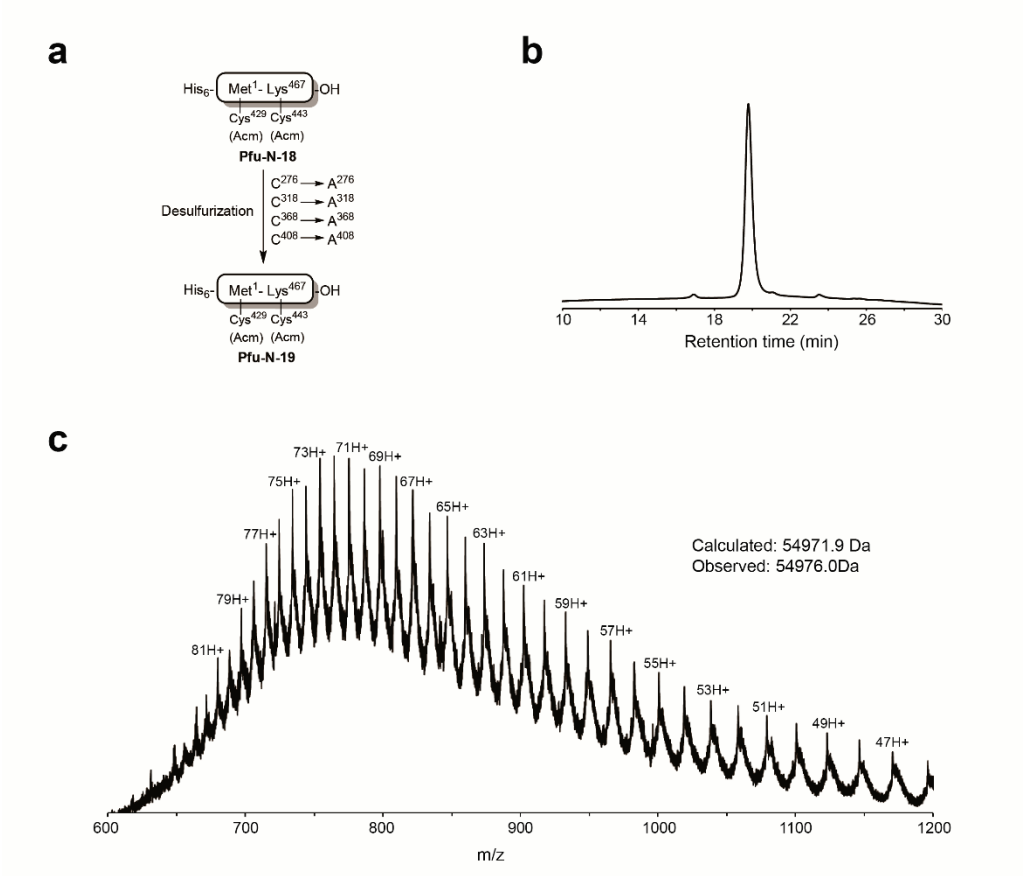
Supplementary Figure 25 | Preparation of L-Pfu-N-6 MESH. a, The preparation of L-Pfu-N-6 MESH (24 mg) was carried out following a procedure similar to the preparation of L-Pfu-N-3 MESH (Supplementary Fig. 17). Purification conditions: 25-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. L-Pfu-N-6 MESH was obtained with a yield of 68% (16.8 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-6 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-6 MESH.



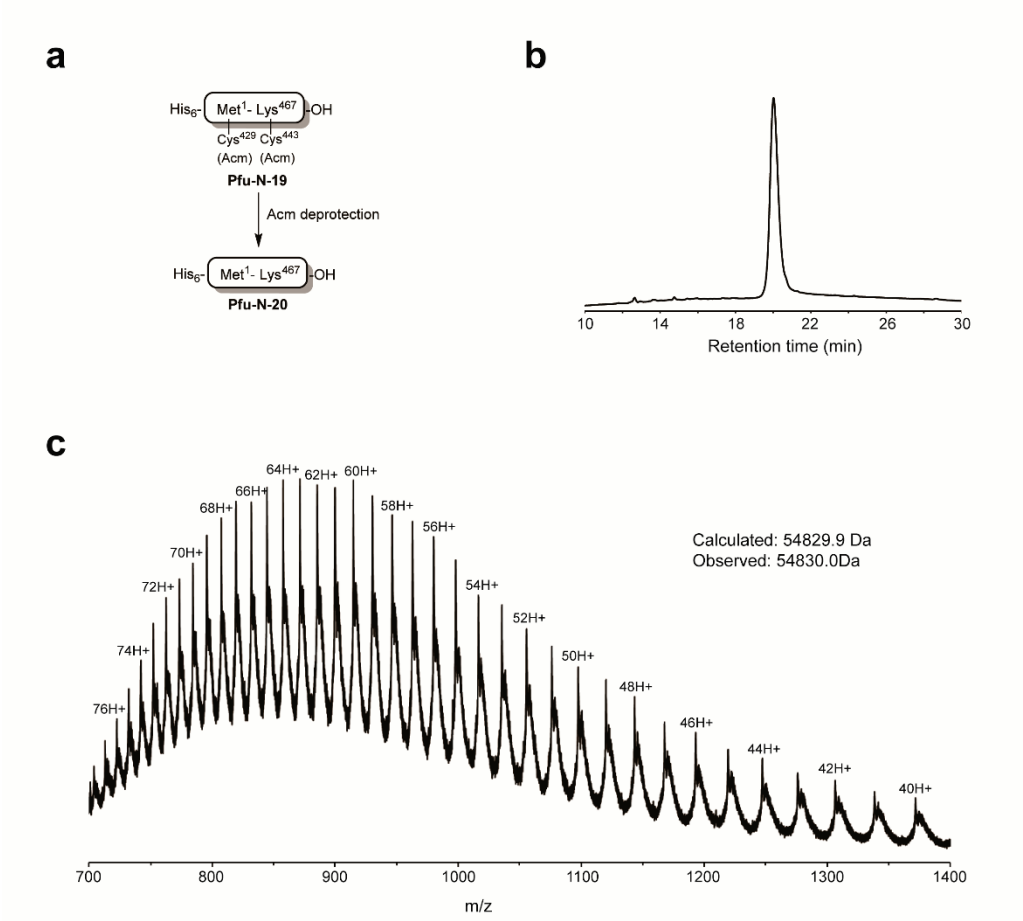
Supplementary Figure 26 | Preparation of L-Pfu-N-17. a, The ligation of L-Pfu-N-6 MESH (14.7 mg) and L-Pfu-N-14 (44.4 mg) was carried out following a procedure similar to the ligation of L-Pfu-N-4 MESH and L-Pfu-N-5 (Supplementary Fig. 19). Purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. L-Pfu-N-17 was obtained with a yield of 73% (41.2 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-17 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-17.



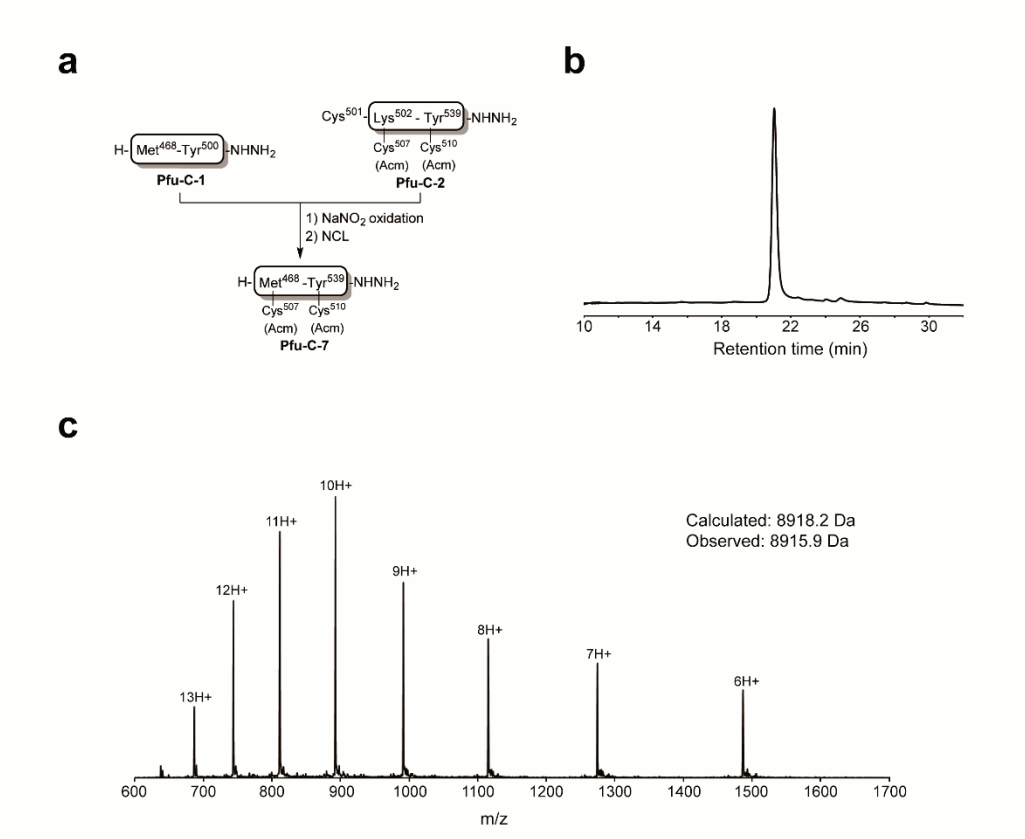
Supplementary Figure 27 | Preparation of L-Pfu-N-18. a, L-Pfu-N-16 (17.8 mg) was dissolved in 0.2 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 11 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 23 min, after which 3.7 mg MPAA was added. After the addition of L-Pfu-N-17 (13.8 mg), the pH of the reaction mixture was adjusted to 6.5 with NaOH solution at room temperature. After 14 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). The ligation product L-Pfu-N-18 was obtained with a yield of 52% (15.6 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-18 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-18.



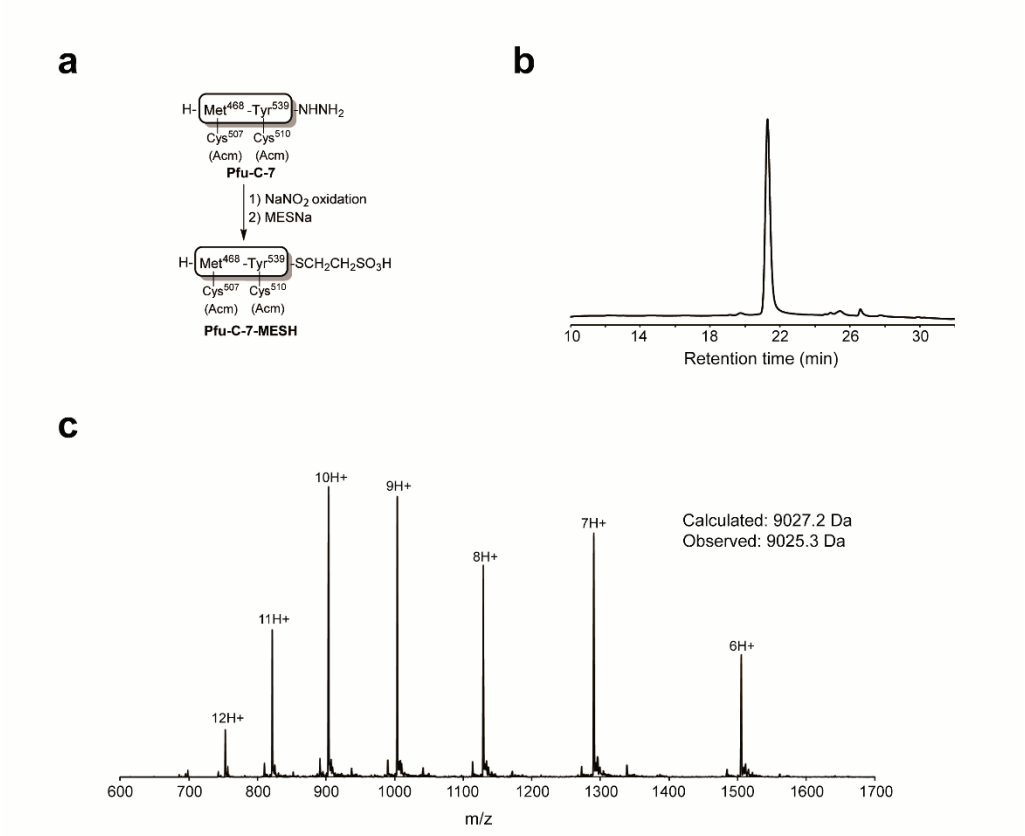
Supplementary Figure 28 | Preparation of L-Pfu-N-19. a, L-Pfu-N-18 (15.6 mg) was dissolved in 3.5 ml 200 mM TCEP solution (6 M Gn·HCl and 0.1 Na_2HPO_4, pH 7.0), containing 20 mM VA-044 and 40 mM reduced L-glutathione. The reaction was under stirring overnight at 37 °C. The desulfurization product L-Pfu-N-18 was analyzed by HPLC and ESI-MS and purified by semi-preparative HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-18 was obtained with a yield of 49% (7.5 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-19 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-19.



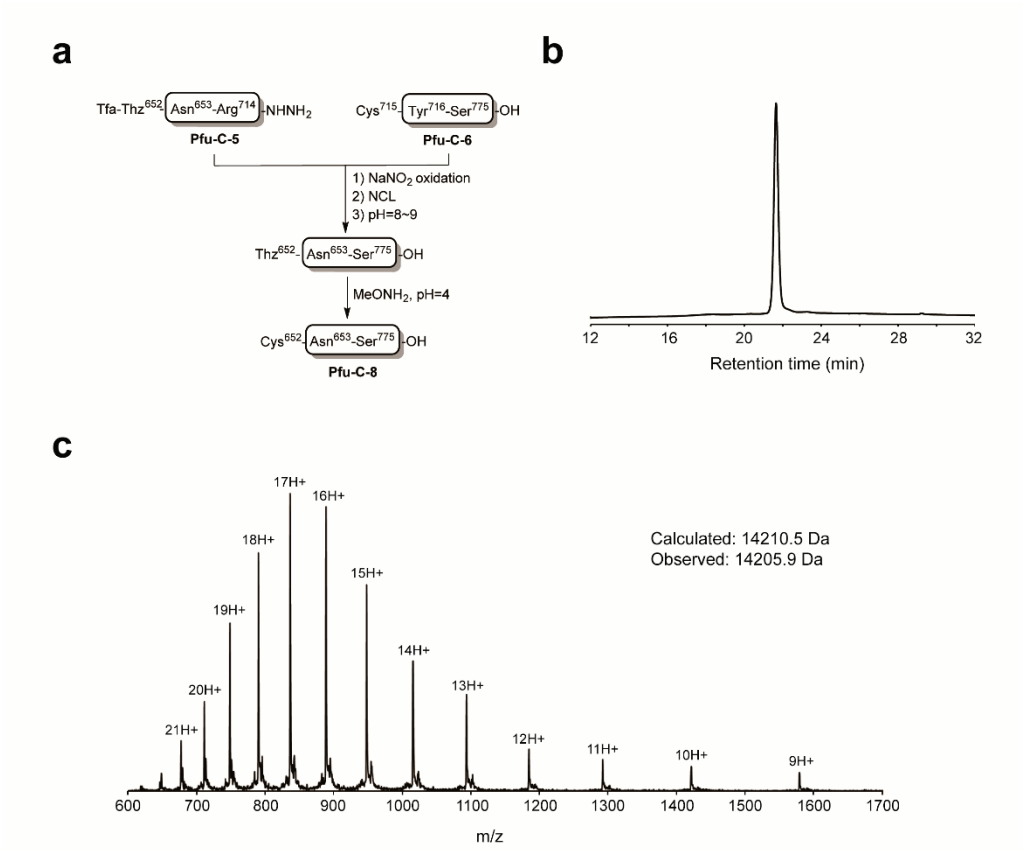
Supplementary Figure 29 | Preparation of L-Pfu-N-20. a, L-Pfu-N-19 (7.5 mg) was dissolved in a 0.3 ml aqueous solution of 8 M Gn·HCl, 0.1 M Na_2HPO_4 and 40 mM TCEP, pH 7.0. $PdCl_2$ (2 mg) was dissolved in a 0.07 ml aqueous solution of 6 M Gn·HCl and added to the peptide solution. The reaction mixture was kept under stirring at 30 °C. After 2.5 h, 1 ml 2 M DTT (in an aqueous solution of 6 M Gn·HCl) was added. The reaction mixture was under stirring for 30 min and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-N-20 was obtained with a yield of 58% (4.3 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-20 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-N-20. The synthesis was performed once.



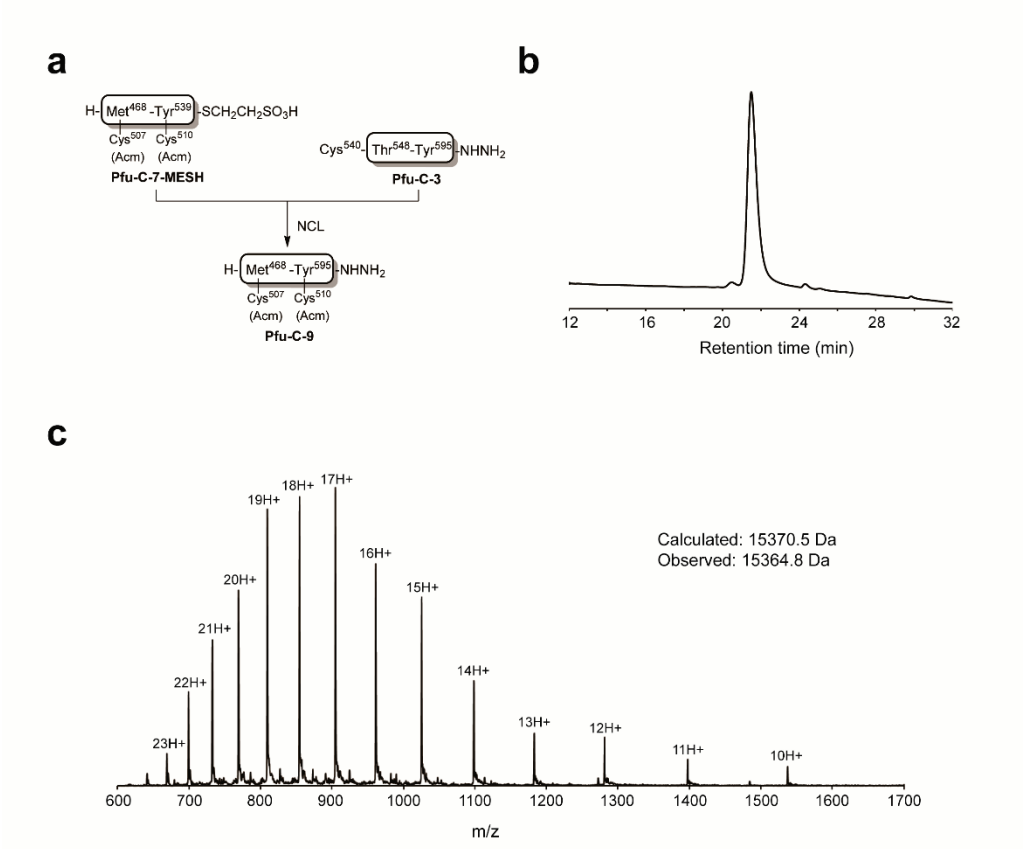
Supplementary Figure 30 | Preparation of L-Pfu-C-7. a, L-Pfu-C-1 (33 mg) was dissolved in 1.8 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 165 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 0.9 ml 0.4 M MPAA (in 8 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. After the addition of L-Pfu-C-2 (35.14 mg), the pH of the reaction mixture was adjusted to 6.5 with NaOH solution at room temperature. After 16 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-C-7 was obtained with a yield of 38% (24.3 mg). **b,** Analytical HPLC chromatogram of L-Pfu-C-7 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-7.



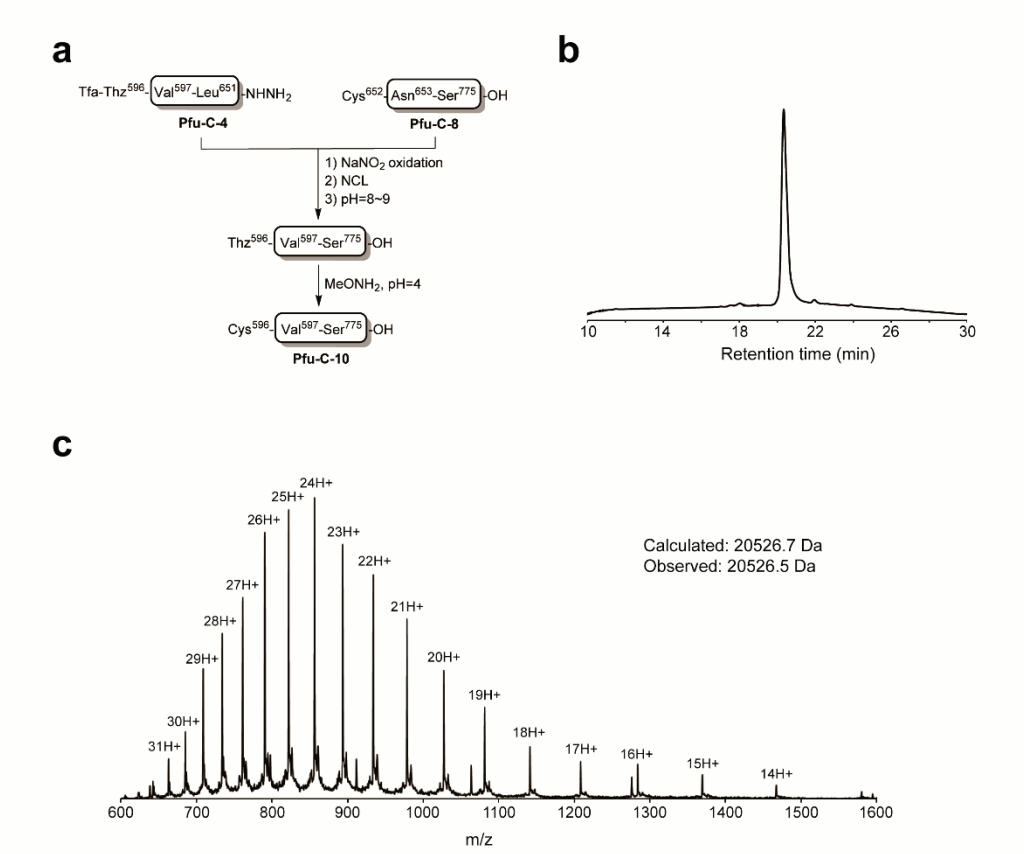
Supplementary Figure 31 | Preparation of L-Pfu-C-7 MESH. a, L-Pfu-C-7 (17.3 mg) was dissolved in 0.38 ml acidified ligation buffer (aqueous solution of 8 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0), and then 0.12 ml 0.1M NaH_2PO_4, pH 3.0 was added to dilute the concentration of Gn·HCl to 6 M. The mixture was cooled in ice-salt bath (-10 °C), and 39 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 12.8 mg MESNa powder was added. The pH of the reaction mixture was adjusted to 4.7 with NaOH solution at room temperature. After 1 h, the products were purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-C-7 MESH was obtained with a yield of 43% (7.5 mg). **b,** Analytical HPLC chromatogram of L-Pfu-C-7 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-7 MESH.



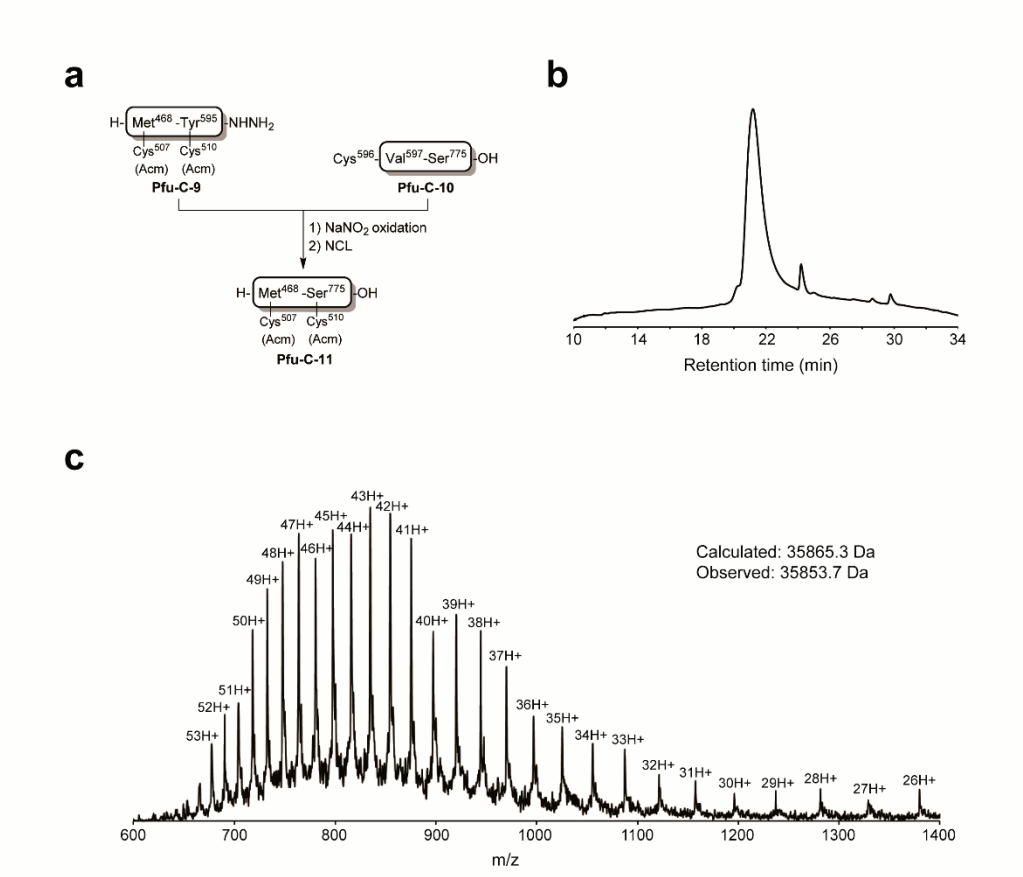
Supplementary Figure 32 | Preparation of L-Pfu-C-8. a, L-Pfu-C-5 (72.5 mg) was dissolved in 2.2 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 206 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 2.1 ml 0.2 M MPAA (in 6 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. The pH of the reaction mixture was adjusted to 5.3 with NaOH solution at room temperature. After the addition of L-Pfu-C 6 (66.1 mg), the pH of the reaction mixture was further adjusted to 6.5. After 14 h, the products were analyzed and the pH was adjusted to 9.0 to promote the complete removal of the Tfa group. After 1 h, the products were analyzed, and $MeONH_2$·HCl (61.8 mg) was added to carry out the conversion of Thz into Cys. TCEP·HCl was added until the pH of the reaction mixture reached 4.0. After 3 h, the products were analyzed and purified by HPLC (purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-C-8 was obtained with a yield of 46% (59.7 mg). **b,** Analytical HPLC chromatogram of L-Pfu-N-12 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-8.



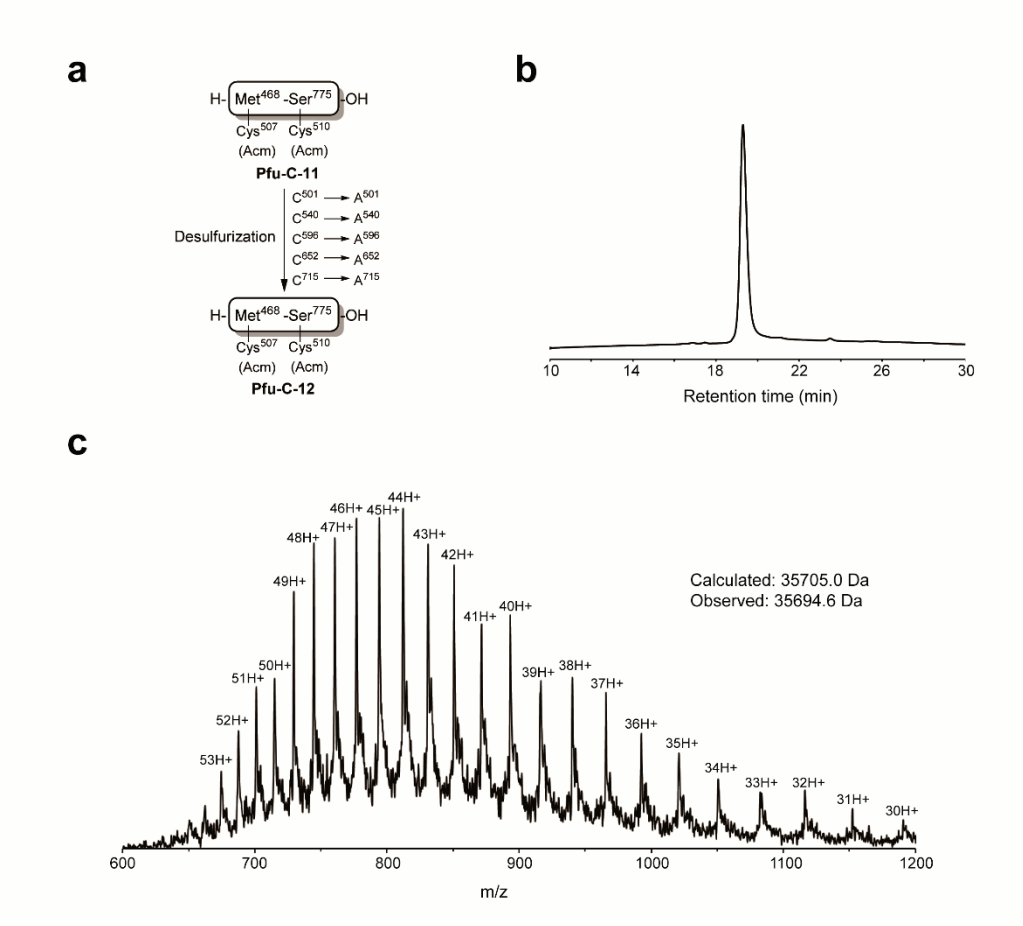
Supplementary Figure 33 | Preparation of L-Pfu-C-9. a, L-Pfu-C-7 MESH (7.5 mg) and L-Pfu-C-3 (6.4 mg) were dissolved in a 0.4 ml aqueous solution of 7 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP and 100 mM MPAA. The pH of the reaction mixture was adjusted to 6.5. After 14 h, the products were purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-C-9 was obtained with a yield of 67% (8.6 mg). **b,** Analytical HPLC chromatogram of L-Pfu-C-9 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-9.



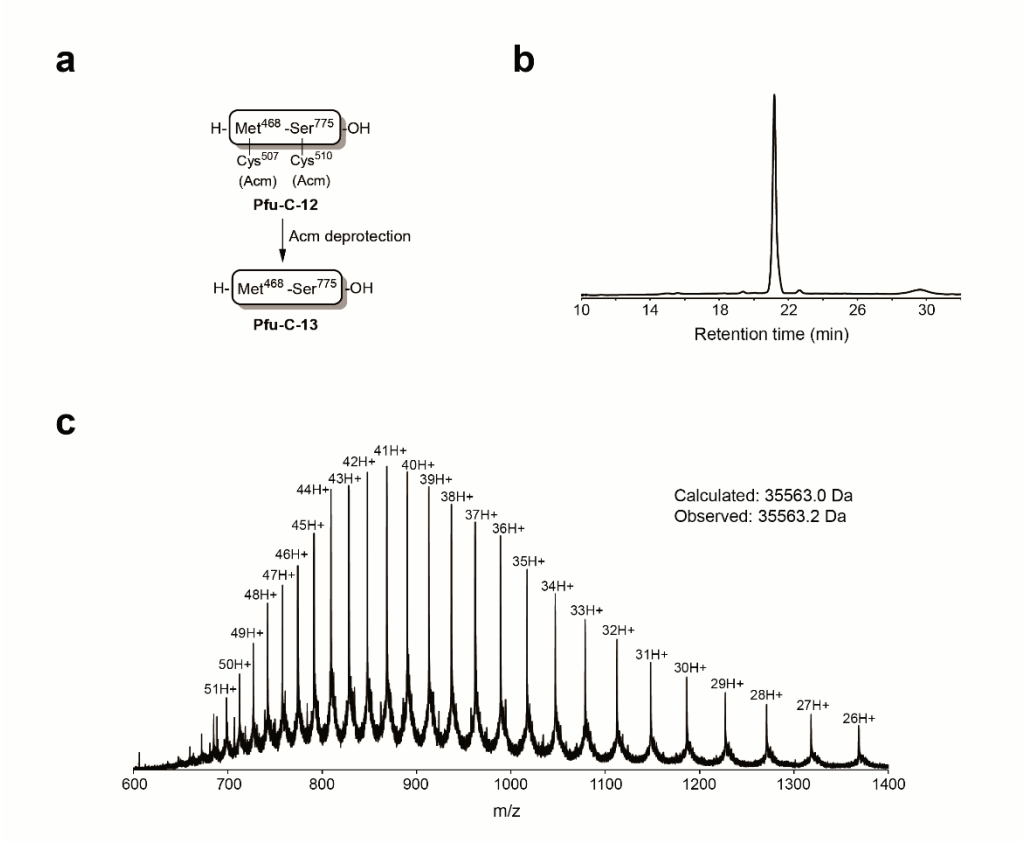
Supplementary Figure 34 | Preparation of L-Pfu-C-10. a, The ligation of L-Pfu-C-4 (16.2 mg) and L-Pfu-C-8 (30.5 mg) was carried out following a procedure similar to the ligation of L-Pfu-C-5 and L-Pfu-C-6 (Supplementary Fig. 32). Purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. L-Pfu-C-10 was obtained with a yield of 46% (20.4 mg). **b,** Analytical HPLC chromatogram of L-Pfu-C-10 (λ=214 nm). Column: Welch C4. Gradient: 20-80% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-10.



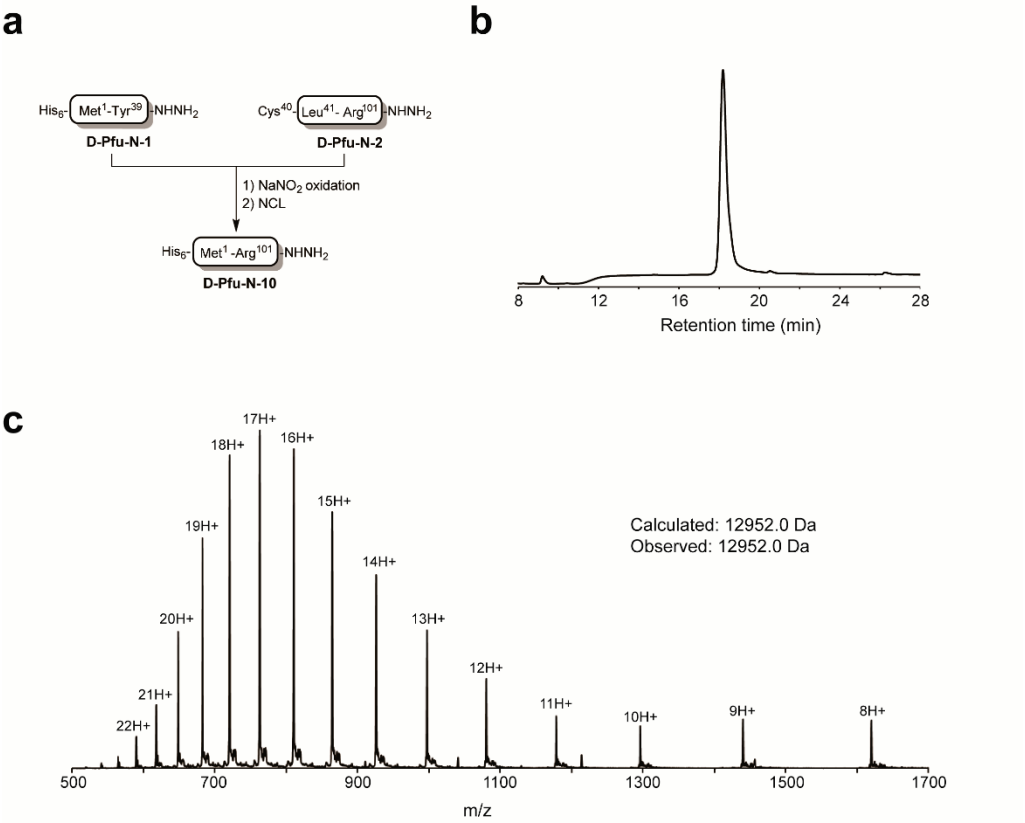
Supplementary Figure 35 | Preparation of L-Pfu-C-11. a, L-Pfu-C-9 (14 mg) was dissolved in 0.27 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 18 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 0.15 ml 0.25 M MPAA (in 8 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. After the addition of L-Pfu-C-10 (16.5 mg), the pH of the reaction mixture was adjusted to 6.6 with NaOH solution at room temperature. After 14 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-C-11 was obtained with a yield of 40% (11.4 mg). **b,** Analytical HPLC chromatogram of L-Pfu-C-11 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-11.



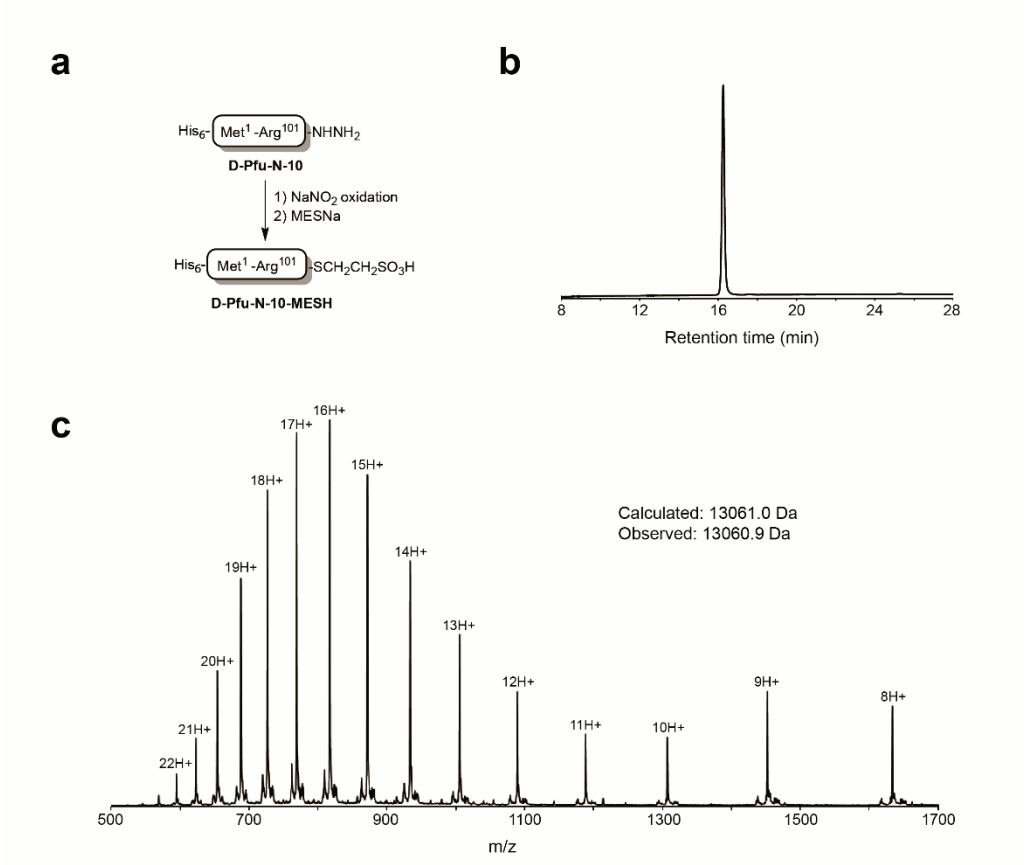
Supplementary Figure 36 | Preparation of L-Pfu-C-12. a, L-Pfu-C-11 (6.8 mg) was dissolved in 2.3 ml 200 mM TCEP solution (6 M Gn·HCl and 0.1 Na_2HPO_4, pH 7.0), containing 20 mM VA-044 and 40 mM reduced L-glutathione. The reaction was under stirring overnight at 37 °C. The desulfurization product L-Pfu-C-12 was analyzed by HPLC and ESI-MS and purified by semi-preparative HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-C-12 was obtained with a yield of 43% (2.9 mg). **b,** Analytical HPLC chromatogram of the desulfurization product L-Pfu-C-12 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-12.



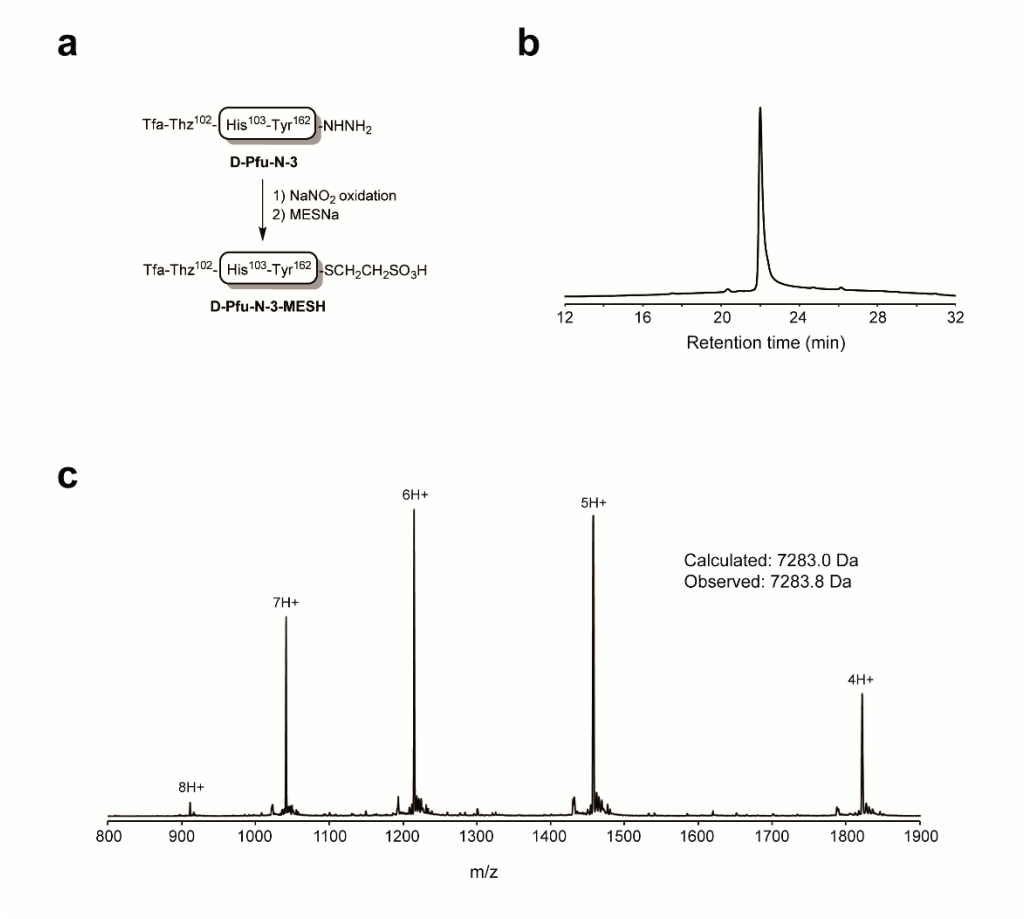
Supplementary Figure 37 | Preparation of L-Pfu-C-13. a, L-Pfu-C-12 (3.6 mg) was dissolved in a 0.25 ml aqueous solution of 8 M Gn·HCl, 0.1 M Na_2HPO_4 and 40 mM TCEP, pH 7.0. $PdCl_2$ (1 mg) was dissolved in a 0.05 ml aqueous solution of 6 M Gn·HCl and added to the peptide solution. The reaction mixture was kept under stirring at 30 °C. After 3 h, 1 ml 2 M DTT (in an aqueous solution of 6 M Gn·HCl) was added. The reaction mixture was under stirring for 30 min and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). L-Pfu-C-13 was obtained with a yield of 62% (2.2 mg). **b,** Analytical HPLC chromatogram of L-Pfu-C-13 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of L-Pfu-C-13. The synthesis was performed twice.



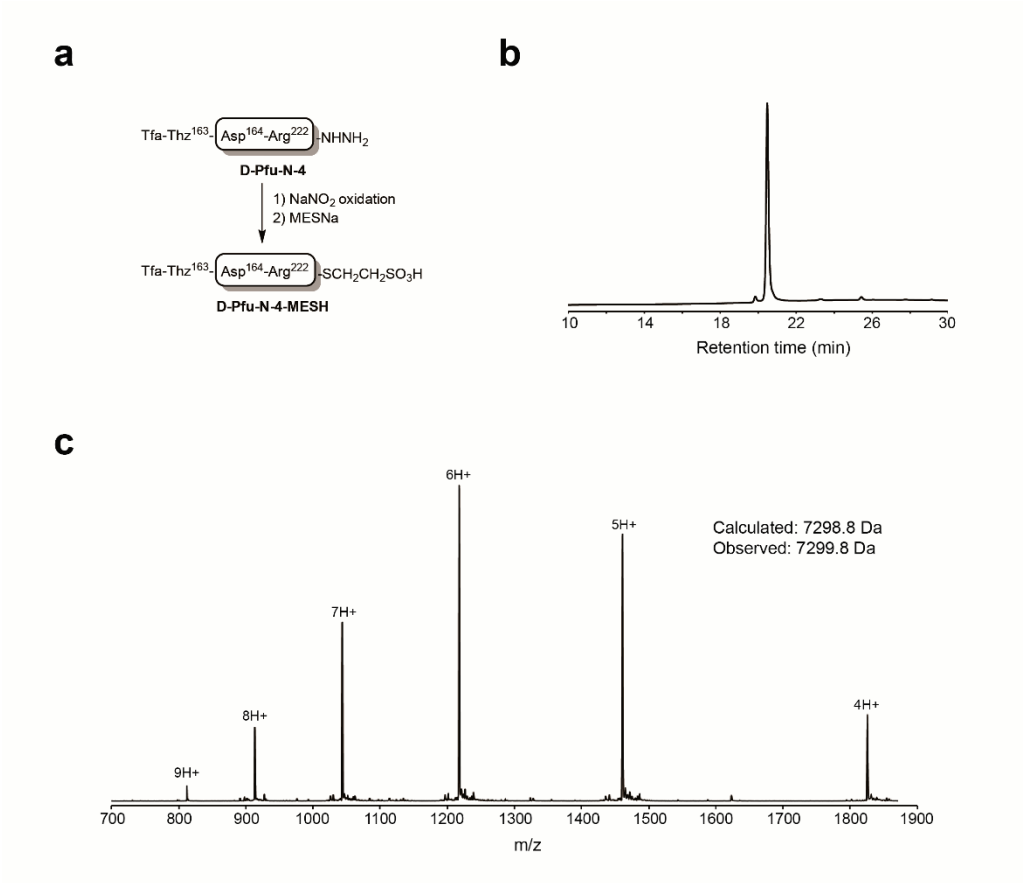
Supplementary Figure 38 | Preparation of D-Pfu-N-10. a, D-Pfu-N-1 (63 mg) was dissolved in 2.3 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 222 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 2.2 ml 0.2 M MPAA (in 6 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. After the addition of D-Pfu-N-2 (76.7 mg), the pH of the reaction mixture was adjusted to 6.6 with NaOH solution at room temperature. After 14 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 25-60% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). The ligation product was obtained with a yield of 38% (52 mg). **b,** Analytical HPLC chromatogram of the ligation product D-Pfu-N-10 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-10.



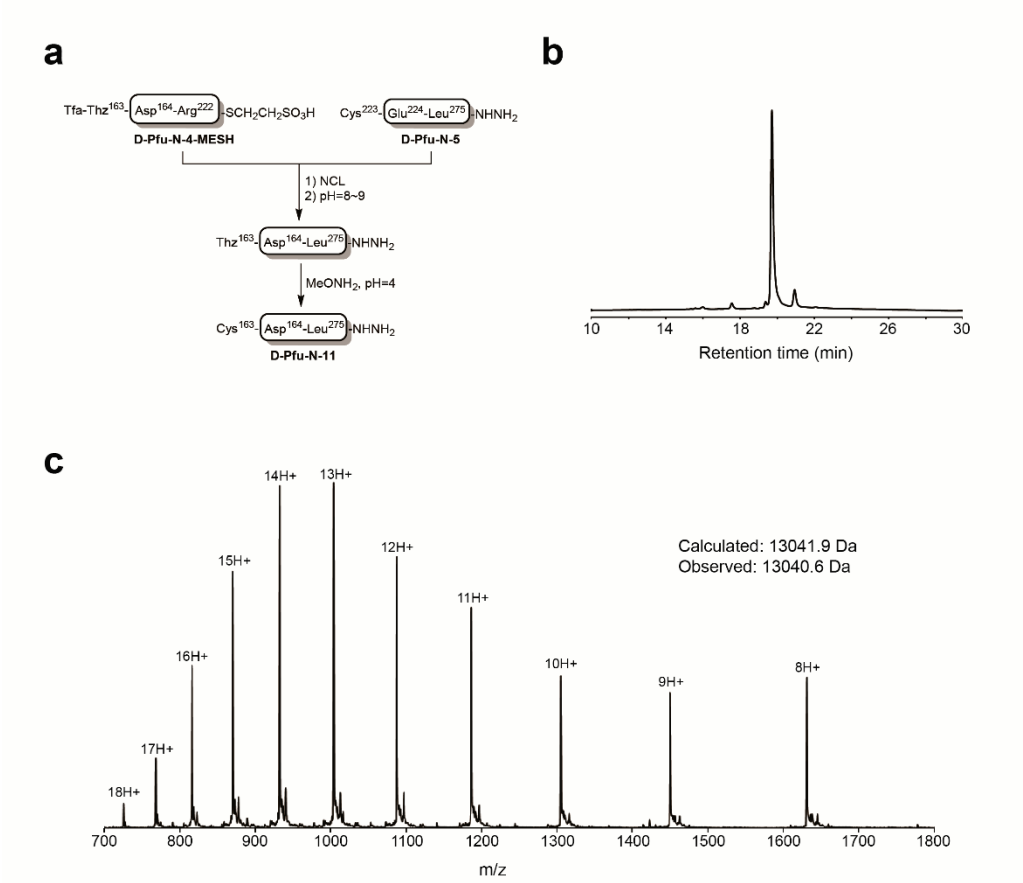
Supplementary Figure 39 | Preparation of D-Pfu-N-10 MESH. a, D-Pfu-N-10 (52 mg) was dissolved in 0.8 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 55 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 23 min, after which 0.4 ml 0.4 M MESNa (in acidified ligation buffer) was added. The pH of the reaction mixture was adjusted to 4.6 with NaOH solution at room temperature. After 1 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 25-60% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-10 MESH was obtained with a yield of 68% (35.6 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-10 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-10 MESH.



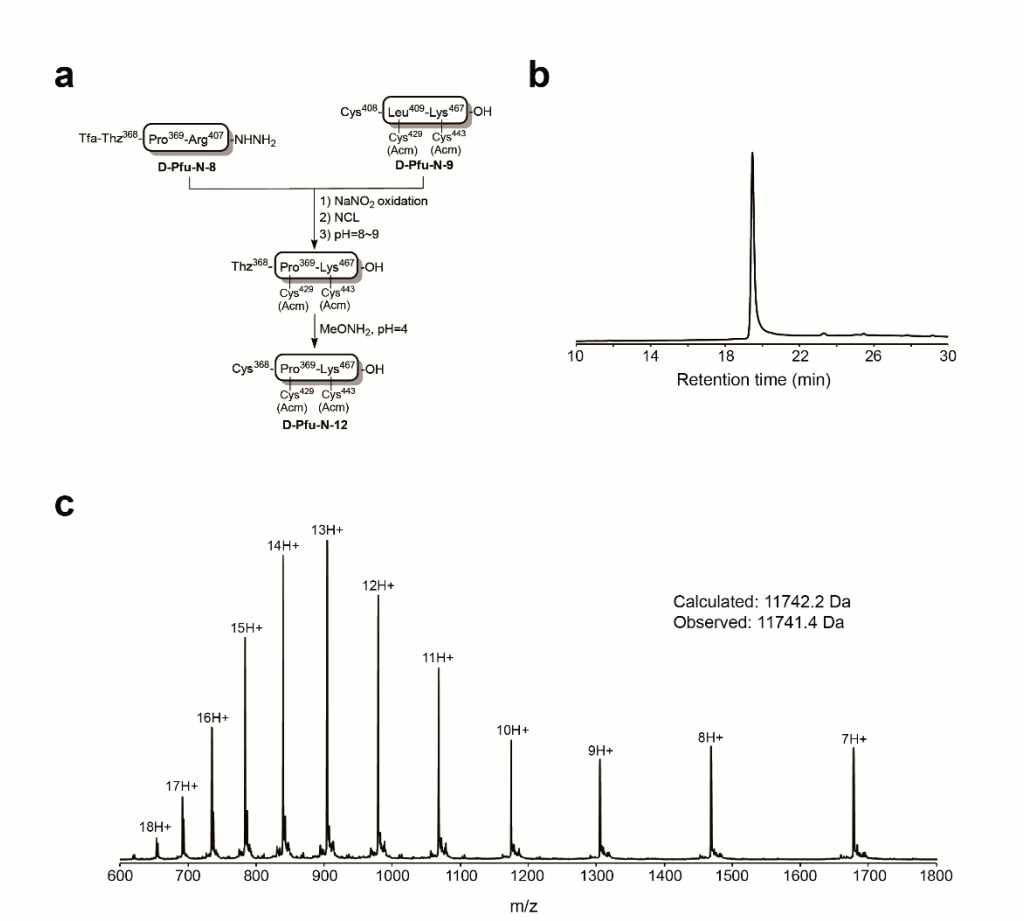
Supplementary Figure 40 | Preparation of D-Pfu-N-3 MESH. a, D-Pfu-N-3 (46.8 mg) was dissolved in 1.3 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath, and 130 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath (-10 °C) under stirring for 25 min, after which 0.7 ml 0.4 M MESNa (in acidified ligation buffer) was added. The pH of the reaction mixture was adjusted to 4.5 with NaOH solution at room temperature. After 1 h, the products were purified by HPLC (purification conditions: 35-75% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-3 MESH was obtained with a yield of 58% (27.8 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-3 MESH (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-3 MESH.



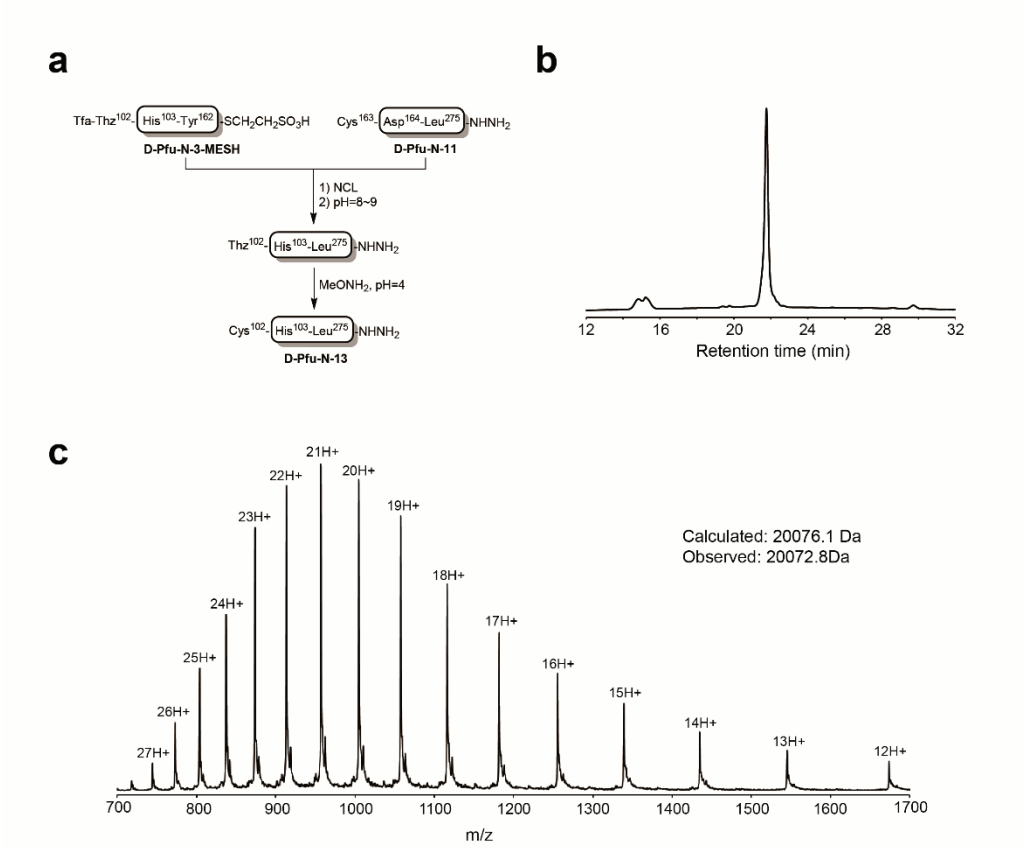
Supplementary Figure 41 | Preparation of D-Pfu-N-4 MESH. a, The preparation of D-Pfu-N-4 MESH (41.5 mg) was carried out following a procedure similar to the preparation of D-Pfu-N-3 MESH (Supplementary Fig. 40). Purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. D-Pfu-N-4 MESH was obtained with a yield of 75% (31.5 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-4 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-4 MESH.



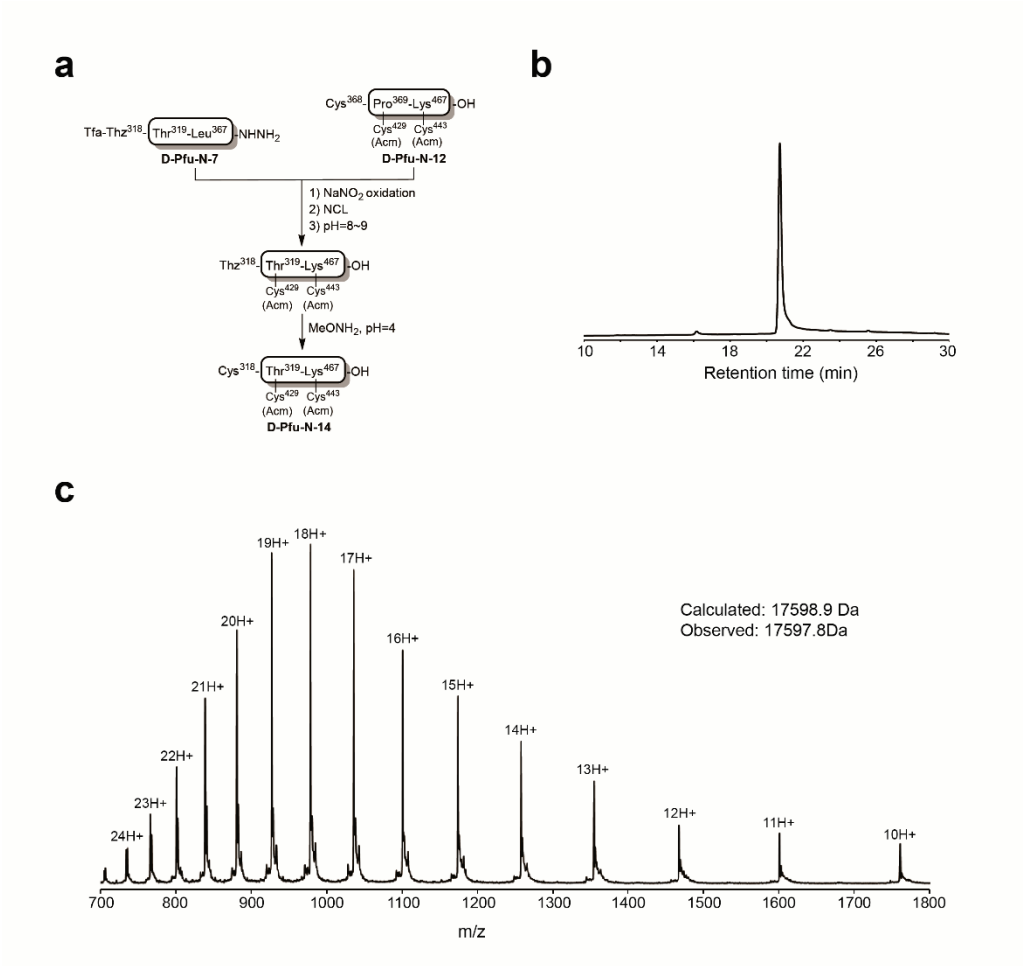
Supplementary Figure 42 | Preparation of D-Pfu-N-11. a, D-Pfu-N-4 MESH (43.3 mg) and D-Pfu-N-5 (41.1 mg) were dissolved in a 2.5 ml aqueous solution of 6 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP and 100 mM MPAA. The pH of the reaction mixture was adjusted to 6.5. After 15 h, the products were analyzed by HPLC, the pH was adjusted to 9.0 to promote the complete removal of the Tfa group. After 1 h, the products were analyzed, and $MeONH_2$·HCl (35.4 mg) was added to carry out the conversion of Thz into Cys. TCEP·HCl was added until the pH of the reaction mixture reached 4.0. After 3 h, the products were analyzed and purified by HPLC (purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-11 was obtained with a yield of 59% (45.6 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-11 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-11.



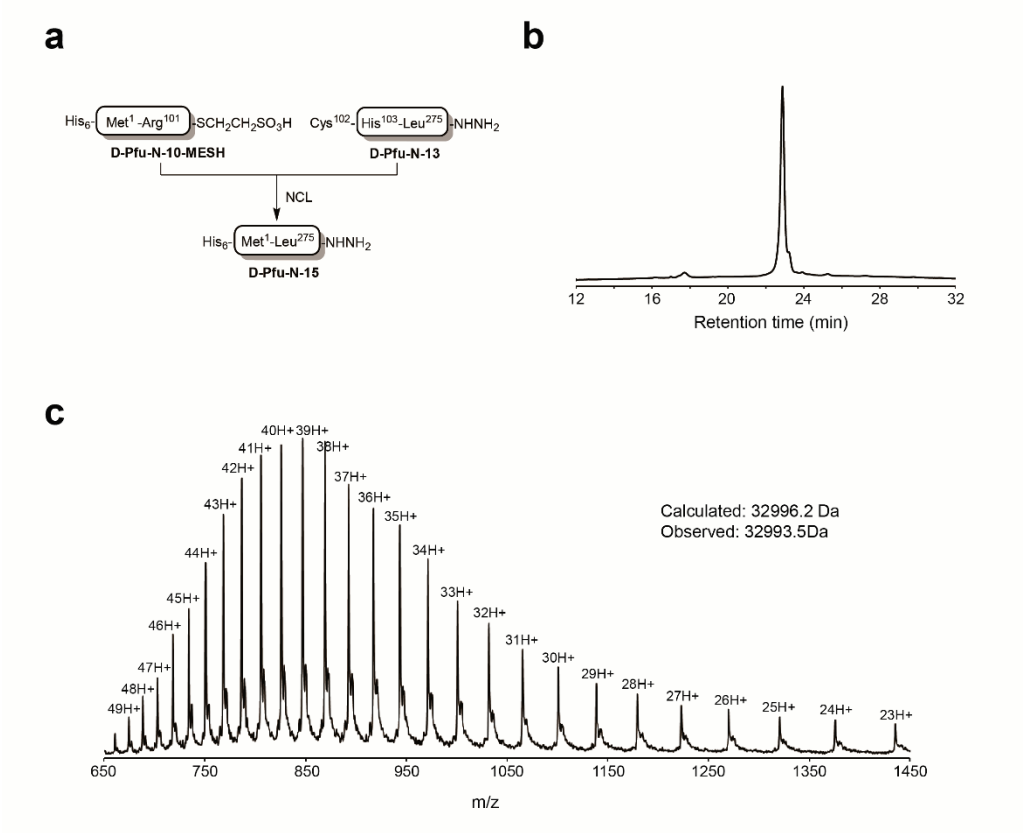
Supplementary Figure 43 | Preparation of D-Pfu-N-12. a, D-Pfu-N-8 (52.4 mg) was dissolved in 2.1 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 210 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 2.1 ml 0.2 M MPAA (in 6 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. The pH of the reaction mixture was adjusted to 5.3 with NaOH solution at room temperature. After the addition of Pfu-N-9 (61.5 mg), the pH of the reaction mixture was further adjusted to 6.5. After 14 h, the products were analyzed and the pH was adjusted to 9.0 to promote the complete removal of the Tfa group. After 1 h, the products were analyzed, and $MeONH_2$·HCl (63.3 mg) was added to carry out the conversion of Thz into Cys. TCEP·HCl was added until the pH of the reaction mixture reached 4.0. After 3 h, the products were analyzed and purified by HPLC (purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-12 was obtained with a yield of 50% (52.1 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-12 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-12.



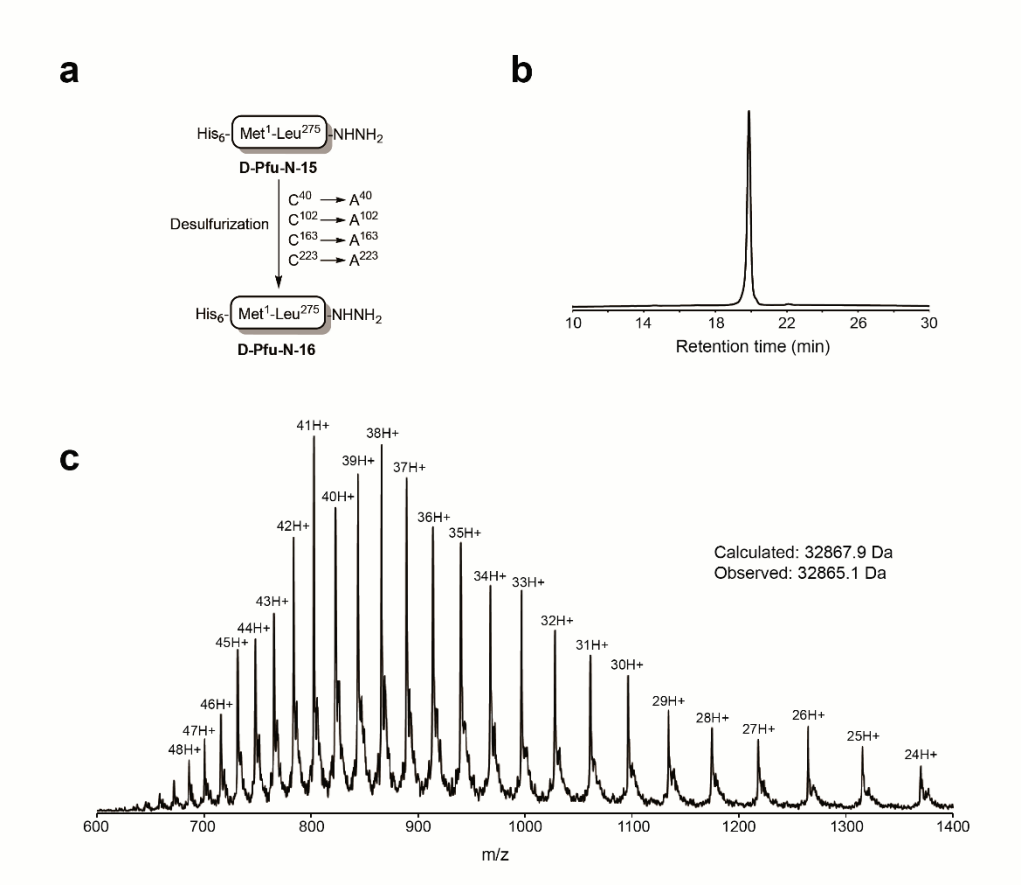
Supplementary Figure 44 | Preparation of D-Pfu-N-13. a, The ligation of D Pfu-N-3 MESH (29.2 mg) and D-Pfu-N-11 (45.6 mg) was carried out following a procedure similar to the ligation of D-Pfu-N-4 MESH and D-Pfu-N-5 (Supplementary Fig. 42). Purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. D-Pfu-N-13 was obtained with a yield of 58% (40.1 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-13 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-13.



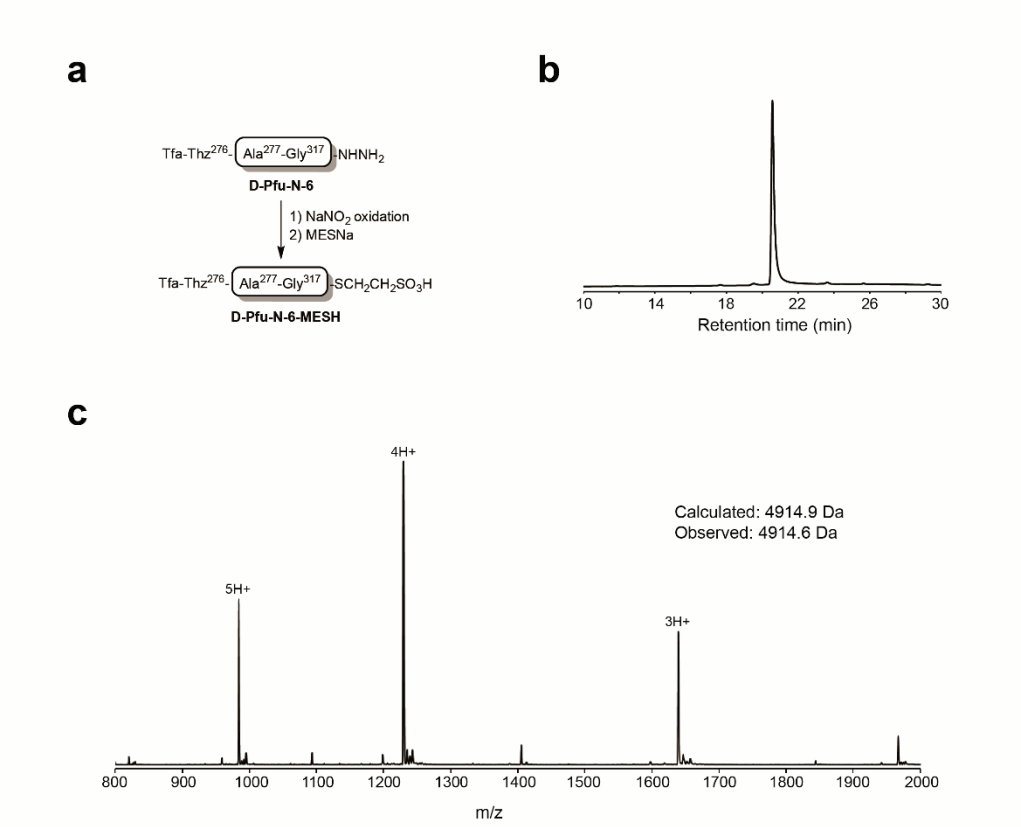
Supplementary Figure 45 | Preparation of D-Pfu-N-14. a, The ligation of D-Pfu-N-7 (31.9 mg) and D-Pfu-N-12 (52.1 mg) was carried out following a procedure similar to the ligation of D-Pfu-N-8 and D-Pfu-N-9 (Supplementary Fig. 43). Purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. D-Pfu-N-14 was obtained with a yield of 59% (45.7 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-14 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-14.



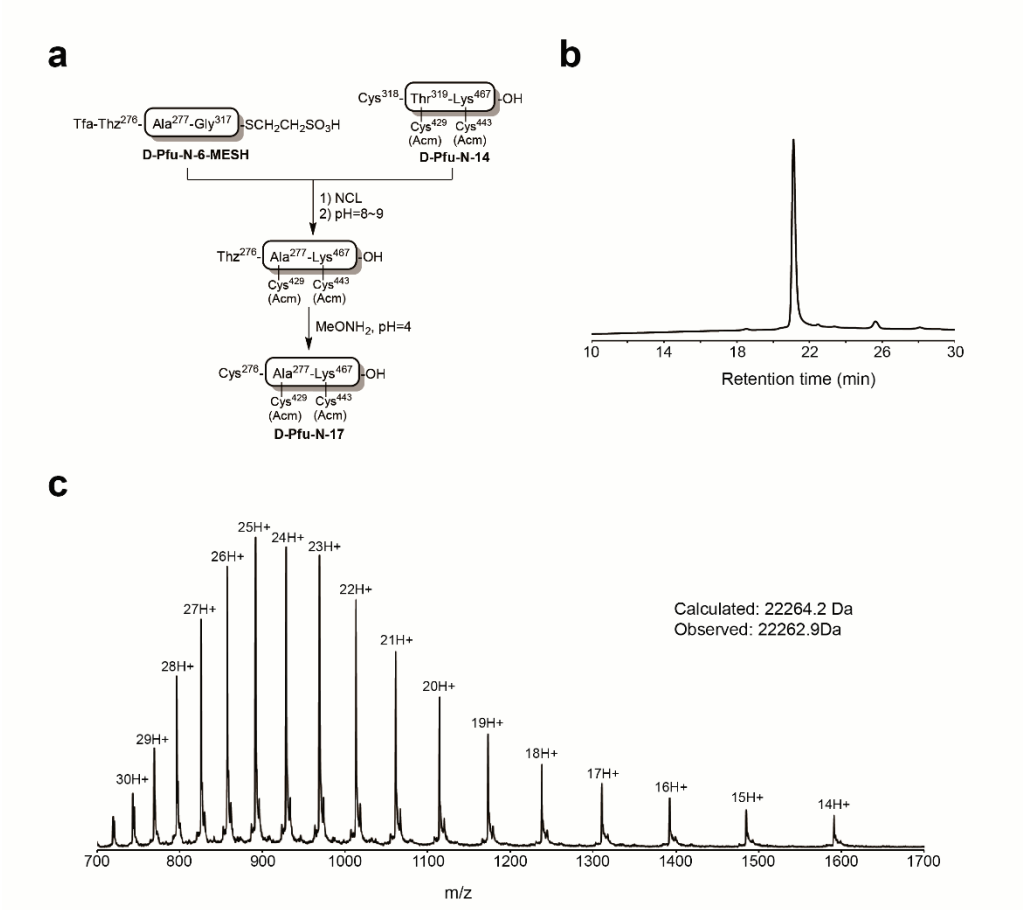
Supplementary Figure 46 | Preparation of D-Pfu-N-15. a, D-Pfu-N-10 MESH (30.1 mg) and D-Pfu-N-13 (40.1 mg) were dissolved in a 1.0 ml aqueous solution of 6 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP and 100 mM MPAA. The pH of the reaction mixture was adjusted to 6.5. After 14 h, the products were purified by HPLC (purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-15 was obtained with a yield of 47% (31.2 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-15 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-15.



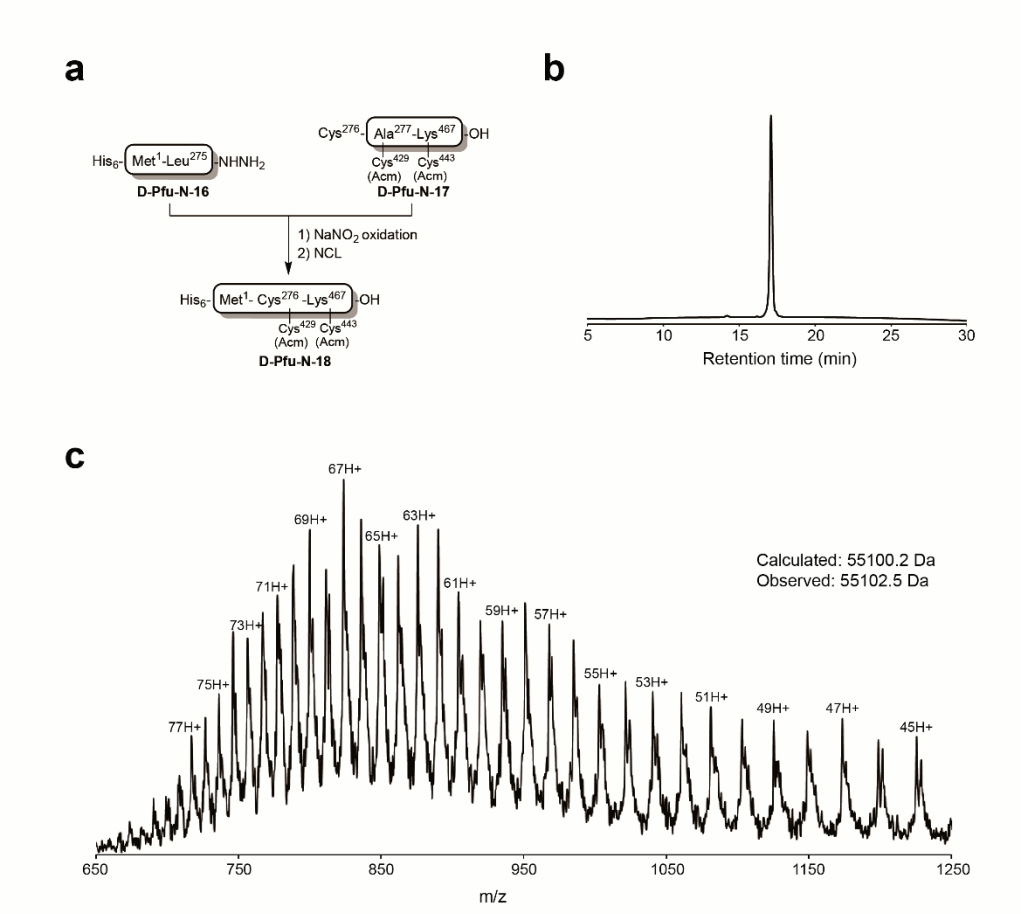
Supplementary Figure 47 | Preparation of D-Pfu-N-16. a, D-Pfu-N-15 (15.9 mg) was dissolved in 5.5 ml 200 mM TCEP solution (6 M Gn·HCl and 0.1 Na_2HPO_4, pH 7.0), containing 20 mM VA-044 and 40 mM reduced L-glutathione. The reaction was under stirring overnight at 37 °C. The desulfurization product D-Pfu-N-16 was analyzed by HPLC and ESI-MS and purified by semi-preparative HPLC (purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-16 was obtained with a yield of 69% (10.9 mg). **b,** Analytical HPLC chromatogram of the desulfurization product D-Pfu-N-16 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-16.



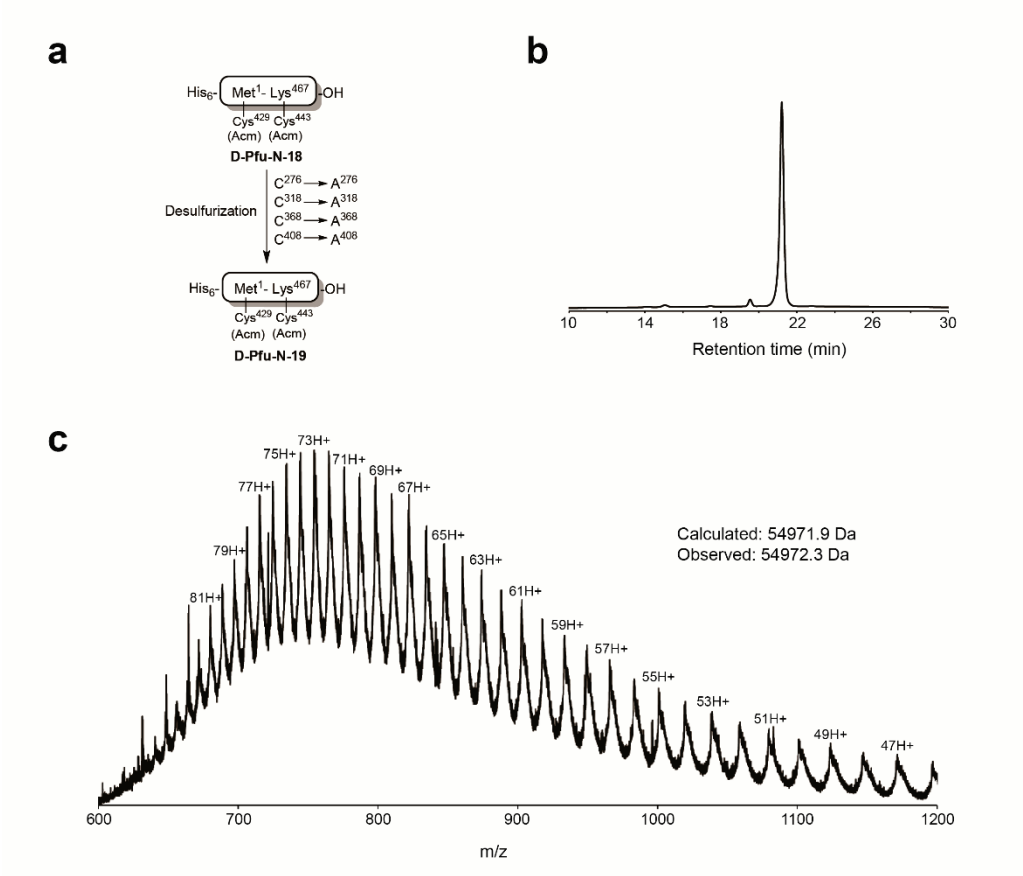
Supplementary Figure 48 | Preparation of D-Pfu-N-6 MESH. a, The preparation of D-Pfu-N-6 MESH (30.4 mg) was carried out following a procedure similar to the preparation of D-Pfu-N-3 MESH (Supplementary Fig. 40). Purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. D-Pfu-N-6 MESH was obtained with a yield of 63% (18.6 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-6 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-6 MESH.



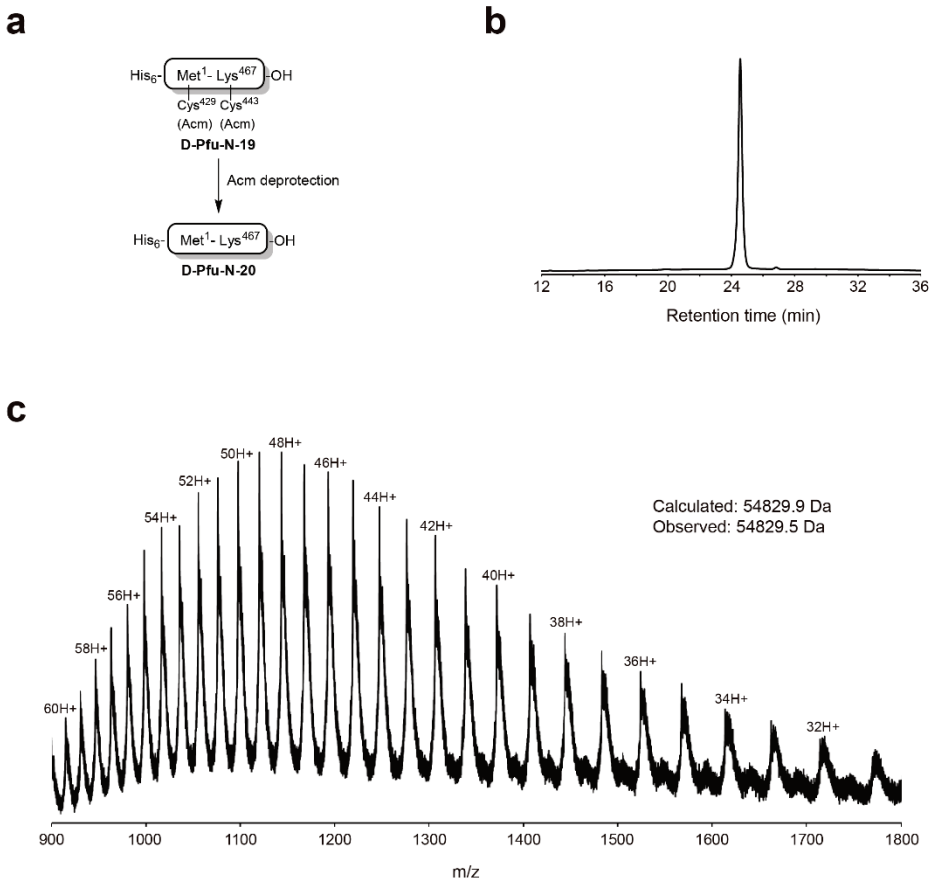
Supplementary Figure 49 | Preparation of D-Pfu-N-17. a, The ligation of D-Pfu-N-6 MESH (15.4 mg) and D-Pfu-N-14 (46.9 mg) was carried out following a procedure similar to the ligation of D-Pfu-N-4 MESH and D-Pfu-N-5 (Supplementary Fig. 42). Purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. D-Pfu-N-17 was obtained with a yield of 69% (41.2 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-17 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-17.



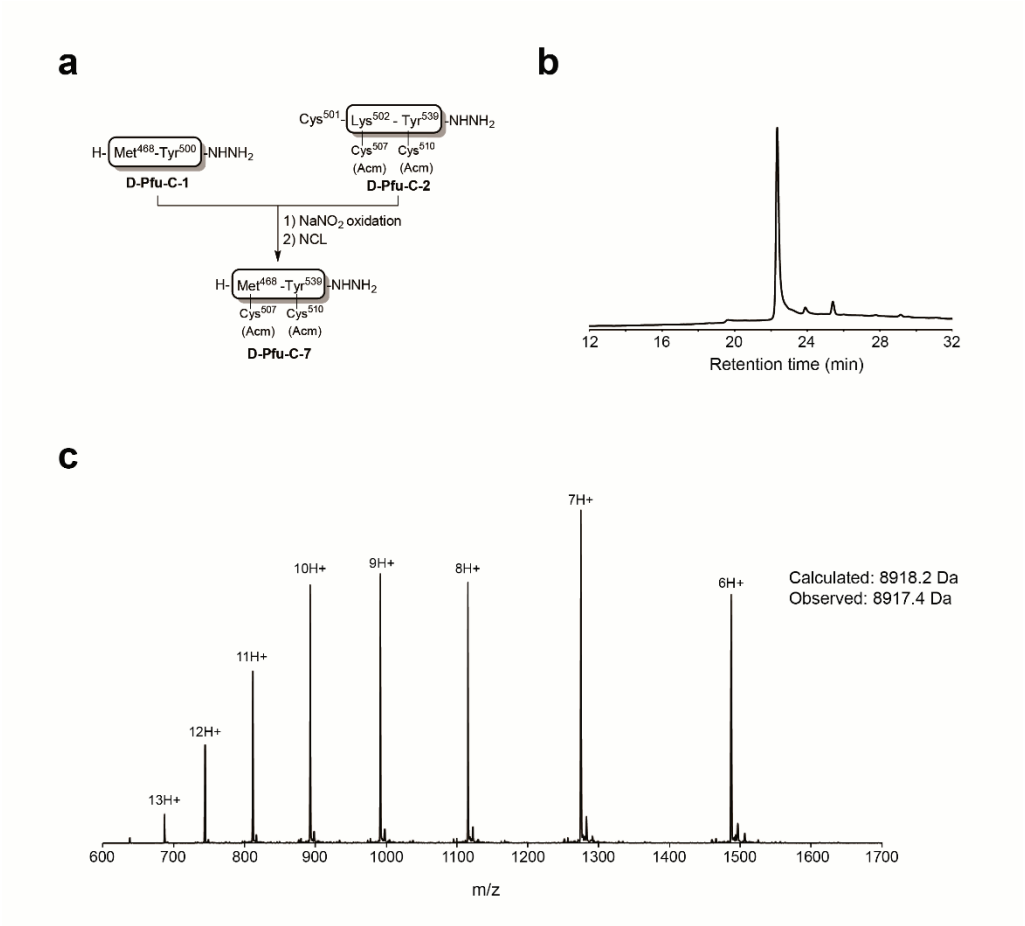
Supplementary Figure 50 | Preparation of D-Pfu-N-18. a, D-Pfu-N-16 (30.4 mg) was dissolved in 0.26 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 18.5 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 23 min, after which 6.3 mg MPAA was added. After the addition of D-Pfu-N-17 (23.8 mg), the pH of the reaction mixture was adjusted to 6.6 with NaOH solution at room temperature. After 14 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 30-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). The ligation product D-Pfu-N-18 was obtained with a yield of 47% (24 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-18 (λ=214 nm). Column: Welch C4. Gradient: 25-100% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-18.



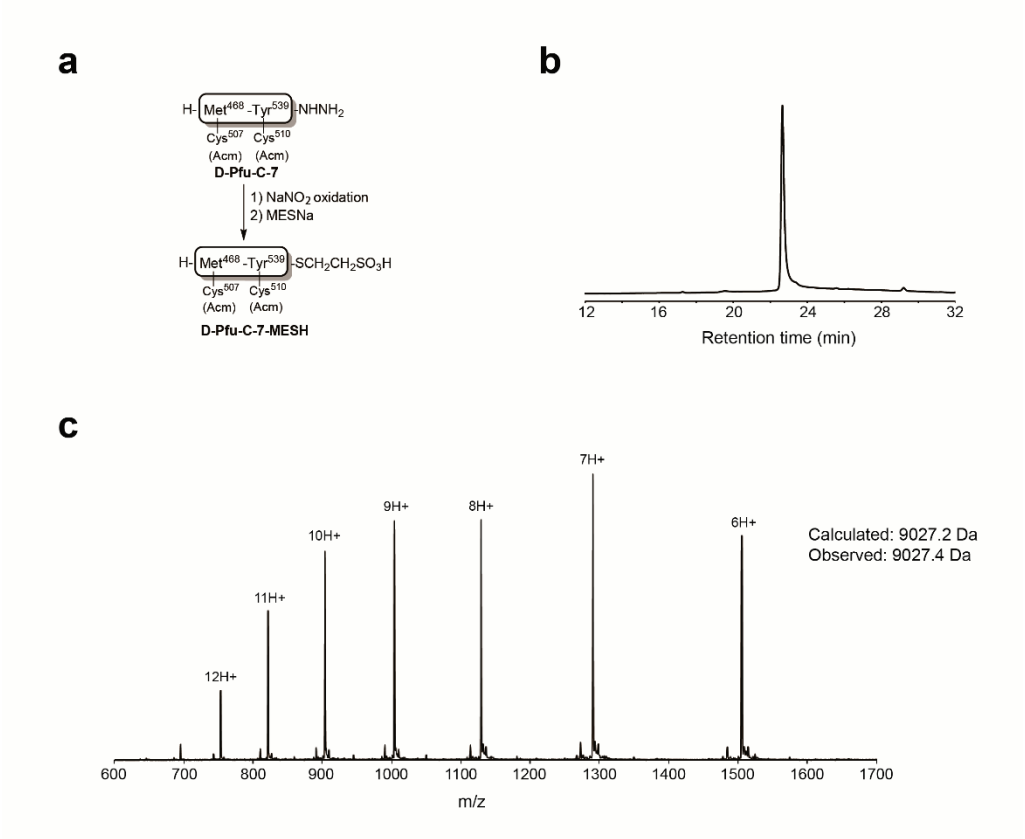
Supplementary Figure 51 | Preparation of D-Pfu-N-19. a, D-Pfu-N-18 (24 mg) was dissolved in 8 ml 200 mM TCEP solution (6 M Gn·HCl and 0.1 Na_2HPO_4, pH 7.0), containing 20 mM VA-044 and 40 mM reduced L-glutathione. The reaction was under stirring overnight at 37 °C. The desulfurization product D-Pfu-N-18 was analyzed by HPLC and ESI-MS and purified by semi-preparative HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-19 was obtained with a yield of 47% (11.3 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-19 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-19.



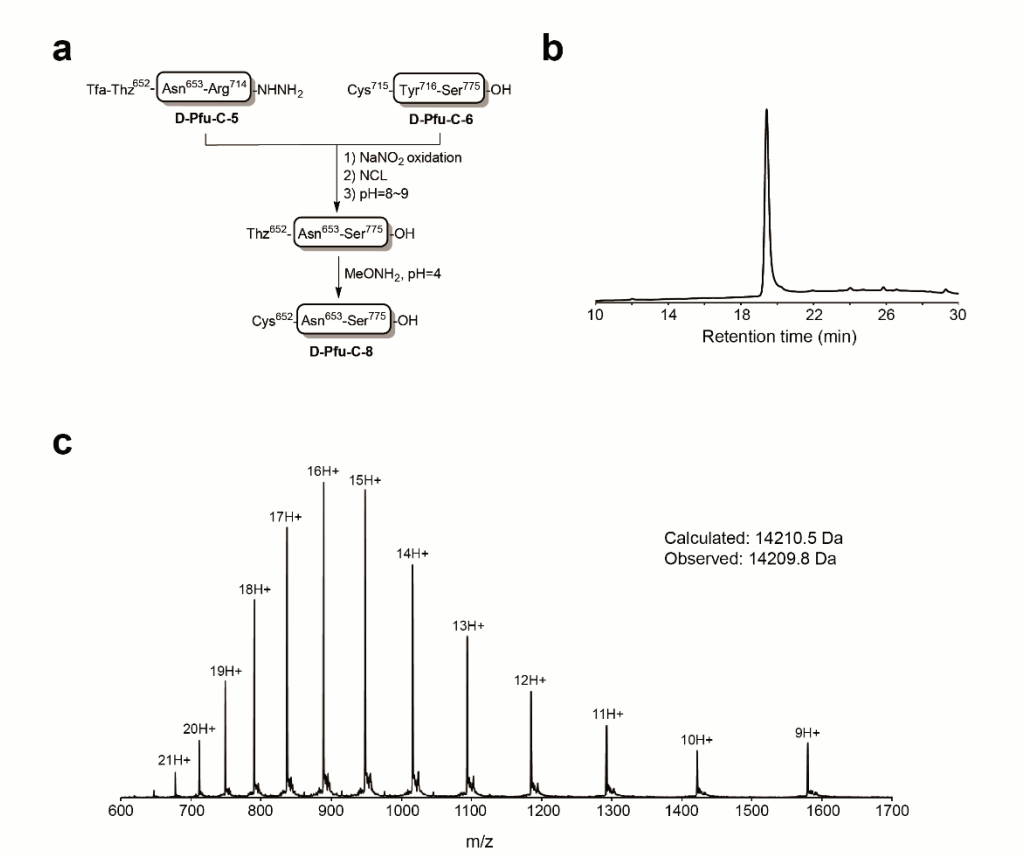
Supplementary Figure 52 | Preparation of D-Pfu-N-20. a, D-Pfu-N-19 (26.9 mg) was dissolved in 1 ml aqueous solution of 8 M Gn·HCl, 0.1 M Na_2HPO_4 and 40 mM TCEP, pH 7.0. $PdCl_2$ (4 mg) was dissolved in a 0.15 ml aqueous solution of 6 M Gn·HCl and added to the peptide solution. The reaction mixture was kept under stirring at 30 °C. After 3 h, 1.5 ml 2 M DTT (in an aqueous solution of 6 M Gn·HCl) was added. The reaction mixture was under stirring for 30 min and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-N-20 was obtained with a yield of 61% (16.5 mg). **b,** Analytical HPLC chromatogram of D-Pfu-N-20 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-N-20. The synthesis was performed three times.



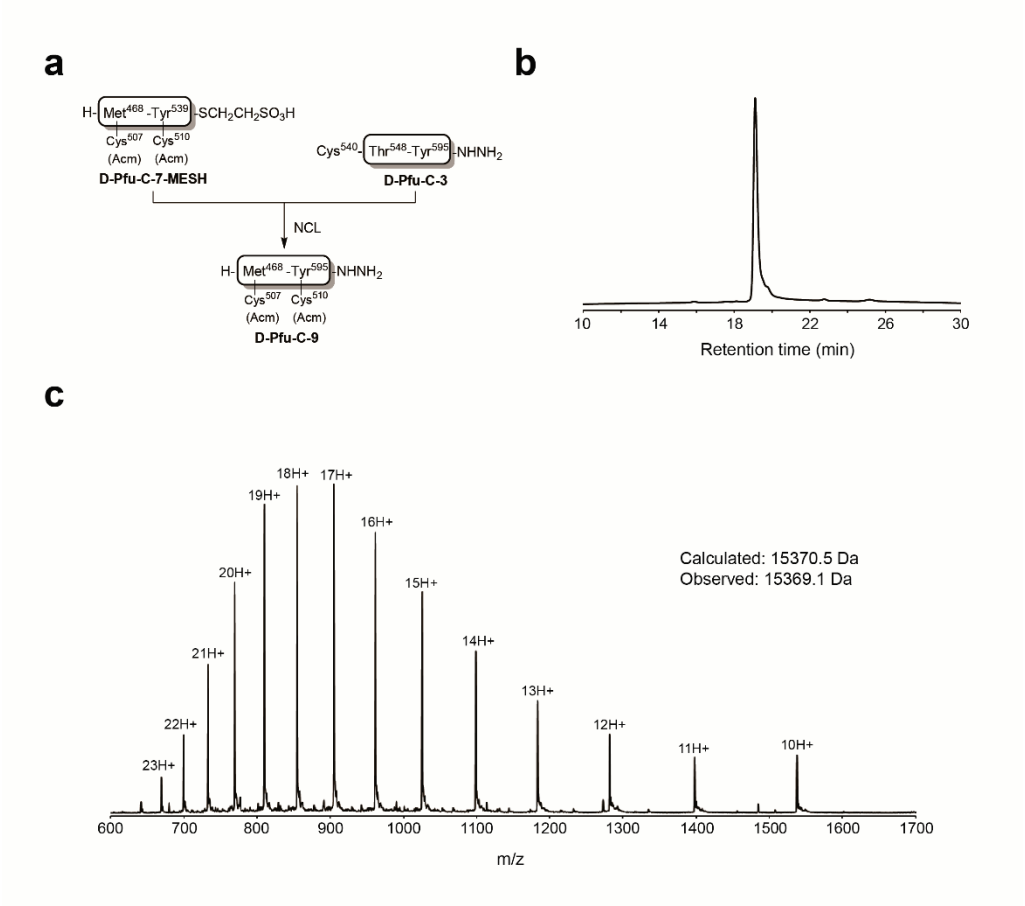
Supplementary Figure 53 | Preparation of D-Pfu-C-7. a, D-Pfu-C-1 (27 mg) was dissolved in 1.35 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 135 μl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 23 min, after which 0.7 ml 0.4 M MPAA (in 8 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.7) was added. After the addition of D-Pfu-C-2 (29 mg), the pH of the reaction mixture was adjusted to 6.5 with NaOH solution at room temperature. After 14 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 35-75% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-C-7 was obtained with a yield of 36% (18.6 mg). **b,** Analytical HPLC chromatogram of D-Pfu-C-7 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-7.



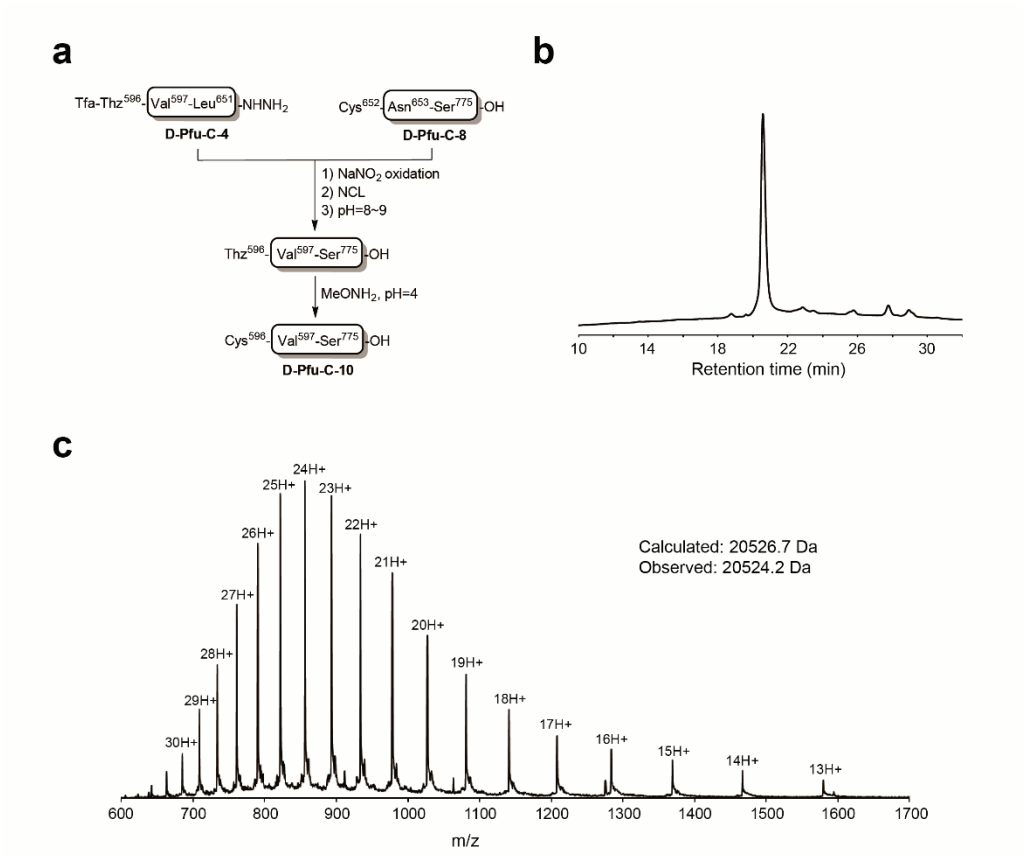
Supplementary Figure 54 | Preparation of D-Pfu-C-7 MESH. a, D-Pfu-C-7 (36.1 mg) was dissolved in 0.8 ml acidified ligation buffer (aqueous solution of 8 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0), and then 0.25 ml 0.1M NaH_2PO_4, pH 3.0 was added to dilute the concentration of Gn·HCl to 6 M. The mixture was cooled in ice-salt bath (-10 °C), and 85 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 27 mg MESNa powder was added. The pH of the reaction mixture was adjusted to 4.6 with NaOH solution at room temperature. After 1 h, the products were purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-C-7 MESH was obtained with a yield of 46% (16.8 mg). **b,** Analytical HPLC chromatogram of D-Pfu-C-7 MESH (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-7 MESH.



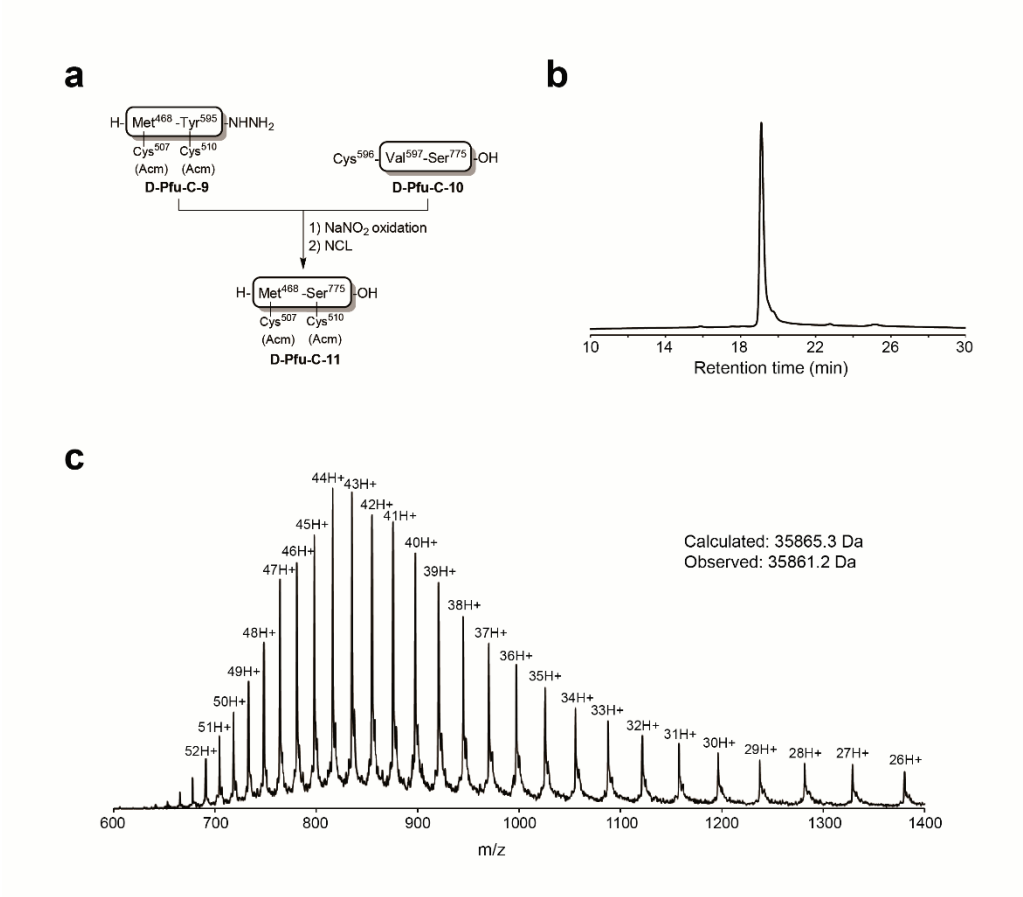
Supplementary Figure 55 | Preparation of D-Pfu-C-8. a, D-Pfu-C-5 (.37.1 mg) was dissolved in 1.1 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 106 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 1.0 ml 0.2 M MPAA (in 6 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.5) was added. The pH of the reaction mixture was adjusted to 5.3 with NaOH solution at room temperature. After the addition of D-Pfu-C 6 (36.6 mg), the pH of the reaction mixture was further adjusted to 6.5. After 14 h, the products were analyzed and the pH was adjusted to 9.0 to promote the complete removal of the Tfa group. After 1 h, the products were analyzed, and $MeONH_2$·HCl (32 mg) was added to carry out the conversion of Thz into Cys. TCEP·HCl was added until the pH of the reaction mixture reached 4.0. After 3 h, the products were analyzed and purified by HPLC (purification conditions: 30-65% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-C-8 was obtained with a yield of 40% (28.1 mg). **b,** Analytical HPLC chromatogram of D-Pfu-C-8 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-8.



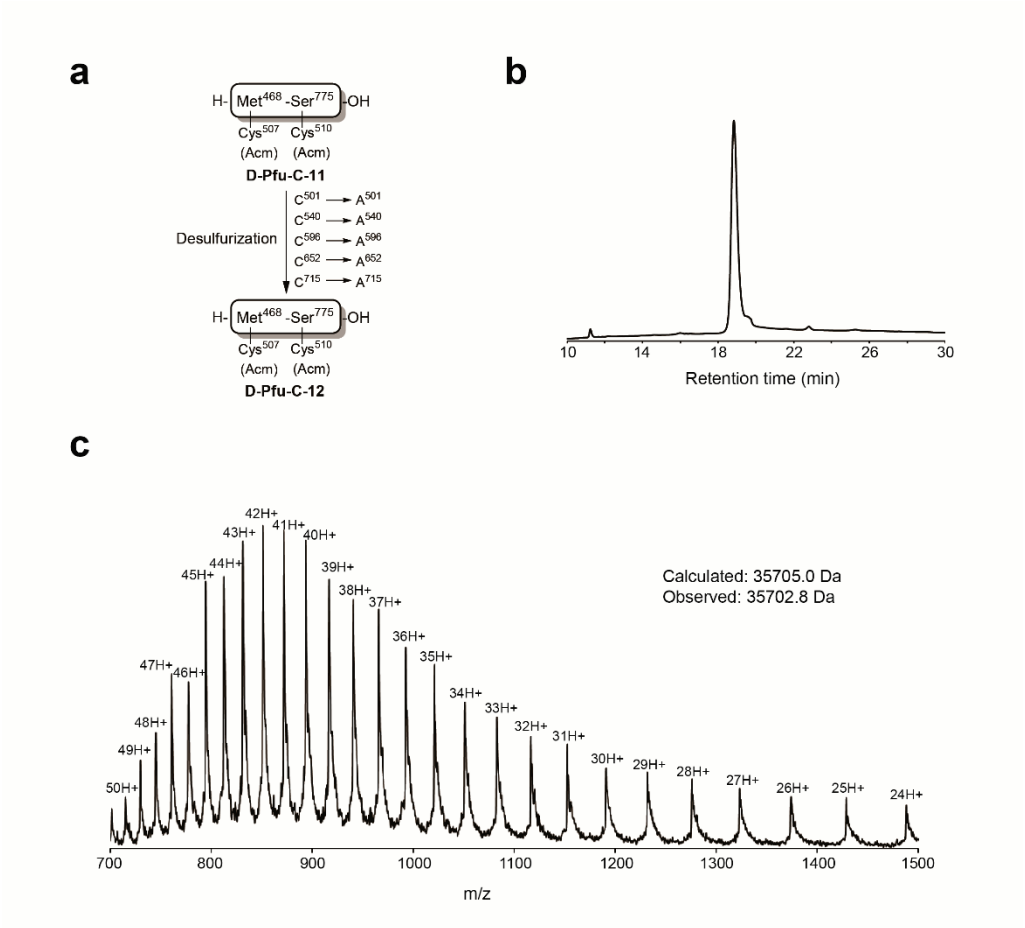
Supplementary Figure 56 | Preparation of D-Pfu-C-9. a, D-Pfu-C-7 MESH (27.3 mg) and D-Pfu-C-3 (23.5 mg) were dissolved in a 1.5 ml aqueous solution of 7 M Gn·HCl, 0.1 M Na_2HPO_4, 40 mM TCEP and 100 mM MPAA. The pH of the reaction mixture was adjusted to 6.5. After 14 h, the products were purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-C-9 was obtained with a yield of 70% (32.8 mg). **b,** Analytical HPLC chromatogram of D-Pfu-C-9 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-9.



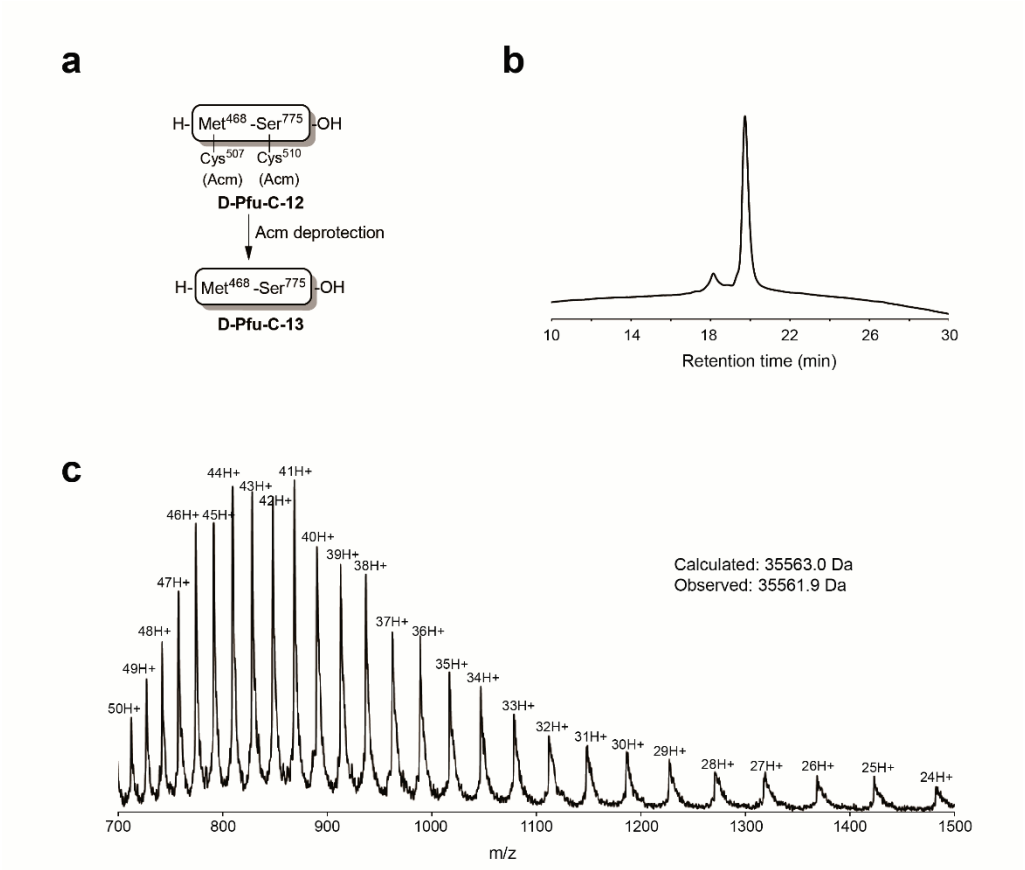
Supplementary Figure 57 | Preparation of D-Pfu-C-10. a, The ligation of D-Pfu-C-4 (15 mg) and D-Pfu-C-8 (28.1 mg) was carried out following a procedure similar to the ligation of D-Pfu-C-5 and D-Pfu-C-6 (Supplementary Fig. 55). Purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column. D-Pfu-C-10 was obtained with a yield of 28% (11.7 mg). **b,** Analytical HPLC chromatogram of D-Pfu-C-10 (λ=214 nm). Column: Welch C4. Gradient: 20-70% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-10.



Supplementary Figure 58 | Preparation of D-Pfu-C-11. a, D-Pfu-C-9 (19.3 mg) was dissolved in 0.3 ml acidified ligation buffer (aqueous solution of 6 M Gn·HCl and 0.1 M NaH_2PO_4, pH 3.0). The mixture was cooled in ice-salt bath (-10 °C), and 25 µl 0.5 M $NaNO_2$ (in acidified ligation buffer) was added. The reaction was kept in ice-salt bath under stirring for 25 min, after which 0.15 ml 0.25 M MPAA (in 8 M Gn·HCl and 0.1 M Na_2HPO_4, pH 5.6) was added. After the addition of D-Pfu-C-10 (22.9 mg), the pH of the reaction mixture was adjusted to 6.6 with NaOH solution at room temperature. After 14 h, the reaction mixture was reduced by TCEP and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-C-11 was obtained with a yield of 47% (18.9 mg). **b,** Analytical HPLC chromatogram of D-Pfu-C-11 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-11.



Supplementary Figure 59 | Preparation of D-Pfu-C-12. a, D-Pfu-C-11 (18.9 mg) was dissolved in 5.4 ml 200 mM TCEP solution (6 M Gn·HCl and 0.1 Na_2HPO_4, pH 7.0), containing 20 mM VA-044 and 40 mM reduced L-glutathione. The reaction was under stirring overnight at 37 °C. The desulfurization product D-Pfu-C-12 was analyzed by HPLC and ESI-MS and purified by semi-preparative HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-C-12 was obtained with a yield of 68% (12.8 mg). **b,** Analytical HPLC chromatogram of the desulfurization product D-Pfu-C-12 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-12.



Supplementary Figure 60 | Preparation of D-Pfu-C-13. a, D-Pfu-C-12 (23.8 mg) was dissolved in a 1.6 ml aqueous solution of 8 M Gn·HCl, 0.1 M Na_2HPO_4 and 40 mM TCEP, pH 7.0. $PdCl_2$ (6 mg) was dissolved in a 0.2 ml aqueous solution of 6 M Gn·HCl and added to the peptide solution. The reaction mixture was kept under stirring at 30 °C. After 2 h, 3 ml 2 M DTT (in an aqueous solution of 6 M Gn·HCl) was added. The reaction mixture was under stirring for 30 min and purified by HPLC (purification conditions: 35-70% CH_3CN (with 0.1% TFA) gradient in H_2O (with 0.1% TFA) over 30 min on a Welch C4 column). D-Pfu-C-13 was obtained with a yield of 50% (11.9 mg). **b,** Analytical HPLC chromatogram of D-Pfu-C-13 (λ=214 nm). Column: Welch C4. Gradient: 25-75% CH_3CN (with 0.1% TFA) in H_2O (with 0.1% TFA) over 30 min. **c,** ESI-MS spectrum of D-Pfu-C-13. The synthesis was performed three times.

Supplementary Table 1 | Error rate analysis of synthetic L-*Pfu* DNA polymerase in PCR and gene assembly

Procedure	Deletion	Insertion	Substitution	Total mutations	Total sequenced bases	Oligo purification method	Polymerase error rate
PCR (45 cycles)	0	0	4	4	91728	-	3.6×10^{-6}
Procedure	Deletion	Insertion	Substitution	Mutational clones	Total sequenced clones	Oligo purification method	Percentage of correct clones
Gene assembly	28	0	2	4	7	Oligonucleotide purification cartridge (OPC)	43%
Gene assembly	0	0	1	1	10	Denaturing PAGE with single-nucleotide resolution	90%

Supplementary Table 2 | Primer sequences

Primer	Sequence
16S-F1	5'-TTTGTTGGAGAGTTTGATCCTGGCTCAGGGTGAACGCTGGCGGCGTGCCTAAGACATGCAAGTCGTGCGGGCCGCGGGGTTTTACTCCGT-3'
16S-R1	5'-TTTCCCCGGGTTGTCCCCCTCTTCCGGGTAGGTCACCCACGCGTTACTCACCCGTCCGCCGCTGACCACGGAGTAAAACCCCGCGGCCCG-3'
16S-F2	5'-GGAAGAGGGGGACAACCCGGGGAAACTCGGGCTAATCCCCCATGTGGACCCGCCCCTTGGGGTGTGTCCAAAGGGCTTTGCCCGCTTCCG-3'
16S-R2	5'-CGGCTACCCGTCGTCGCCTTGGTGGGCCATTACCCCACCAACTAGCTGATGGGACGCGGGCCCATCCGGAAGCGGGCAAAGCCCTTTGGA-3'
16S-F3	5'-AAGGCGACGACGGGTAGCCGGTCTGAGAGGATGGCCGGCCACAGGGGCACTGAGACACGGGCCCCACTCCTACGGGAGGCAGCAGTTAGG-3'
16S-R3	5'-ACCCCGAAGGGCTTCTTCCTCCAAGCGGCGTCGCTCCGTCAGGCTTGCGCCCATTGCGGAAGATTCCTAACTGCTGCCTCCCGTAGGAGT-3'
16S-F4	5'-CTTGGAGGAAGAAGCCCTTCGGGGTGTAAACTCCTGAACCCGGGACGAAACCCCCGACGAGGGGACTGACGGTACCGGGGTAATAGCGCC-3'
16S-R4	5'-ACGCCCAGTGAATCCGGGTAACGCTCGCGCCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTGGCCGGCGCTATTACCCCGGTACCGTC-3'
16S-F5	5'-GCGTTACCCGGATTCACTGGGCGTAAAGGGCGTGTAGGCGGCCTGGGGCGTCCCATGTGAAAGACCACGGCTCAACCGTGGGGGAGCGTG-3'
16S-R5	5'-TATCTGCGCATTTCACCGCTACTCCGGGAATTCCACCACCCTCTCCCACCGTCTAGCCTGAGCGTATCCCACGCTCCCCCACGGTTGAGC-3'
16S-F6	5'-AATTCCCGGAGTAGCGGTGAAATGCGCAGATACCGGGAGGAACGCCGATGGCGAAGGCAGCCACCTGGTCCACCCGTGACGCTGAGGCGC-3'
16S-R6	5'-AGACCTAGCGCGCATCGTTTAGGGCGTGGACTACCCGGGTATCTAATCCGGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCACGGGTGG-3'
16S-F7	5'-CCCTAAACGATGCGCGCTAGGTCTCTGGGTCTCCTGGGGGCCGAAGCTAACGCGTTAAGCGCGCCGCCTGGGGAGTACGGCCGCAAGGCT-3'

16S-R7	5'-TTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCC-3'
16S-F8	5'-GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCTAGGGAACCCGGGTGAAAGCCTGGGGTGCCCCGC-3'
16S-R8	5'-GGACTTAACCCAACACCTCACGGCACGAGCTGACGACGGCCATGCAGCACCTGTGCTAGGGCTCCCCTCGCGGGGCACCCCAGGCTTTCA-3'
16S-F9	5'-CGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGCCGTTAGTTGCCAGCGGTTCGGCCGGGCACTCTAACGGGACTG-3'
16S-R9	5'-TGTGTCGCCCAGGCCGTAAGGGCCATGCTGACCAGACGTCGTCCCCTCCTTCCTCCCGCTTTCGCGGGCAGTCCCGTTAGAGTGCCCGGC-3'
16S-F10	5'-GGCCCTTACGGCCTGGGCGACACACGTGCTACAATGCCCACTACAAAGCGATGCCACCCGGCAACGGGGAGCTAATCGCAAAAAGGTGGG-3'
16S-R10	5'-GATCCGCGATTACTAGCGATTCCGGCTTCATGGGGTCGGGTTGCAGACCCCAATCCGAACTGGGCCCACCTTTTTGCGATTAGCTCCCCG-3'
16S-F11	5'-GCCGGAATCGCTAGTAATCGCGGATCAGCCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGCCATGGGAGCGG-3'
16S-R11	5'-CGACTTCGCCCCAGTCACGGGCCCTACCCTCGGCGCCTGCCCGTAGGCTCCCGGCGACTTCGGGTAGAGCCCGCTCCCATGGCGTGACGG-3'
16S-R12	5'-CCGCACCTTCCGGTACAGCTACCTTGTTACGACTTCGCCCCAGTCACGGGCCCT-3'
M13-F	5'-GTAAAACGACGGCCAGT-3'
M13-R	5'-CAGGAAACAGCTATGAC-3'

Supplementary Table 3 | Encoding table for L-DNA information storage

Character	Code	Character	Code
a	ACG	space	ATC
b	GTA	,	TCC
c	CAG	.	TCT
d	TGC	0	ATT
e	ATG	1	ACA
f	CTA	2	ACC
g	GAT	3	AGA
h	TCG	4	AGG
i	AGC	5	TAA
j	AAT	6	TAT
k	GCA	7	TTA
l	TGA	8	TTC
m	CTG	9	TTG
n	TAC	-	TGT
o	AGT	?	TGG
p	GAC	:	CAA
q	AAC	;	CAC
r	TCA	!	CTT
s	TAG	*	CTC
t	ACT	/	CCA
u	CAT	/n	CCT
v	GTC	°	CCG
w	CGA	'	CGC
x	GCT	"	CGG
y	CGT	(	GAA
z	AAG	)	GAG
^	ATA		

Supplementary Table 4 | Sequences for L-DNA information storage

Lowercase: M13-F and M13-R sequences for storage library amplification
Underlined: Unique sequences for segment-specific amplification and sequencing

Segment	Sequence
Storage S1	5'-gtaaaacgacggccagtTCGCGCGTTTCGGTGATGACGGTGAAAACCATTACAATAACGTACTGCATCCAGAGTTACTAGATGAACCATATGTACACTTGACGTTCCATCAGCCTAATCACTTCGATGATCCTGCGTTAGACTATGTCAAGCAGTCATTAGATCAGCTACCTATGACATATGTACCAGATGATCACTAGTATCgtcatagctgtttcctg-3'
Storage S2	5'-gtaaaacgacggccagtTCTGACACATGCAGCTCCCGGAGACGGTCAATTACCCGATCGAGCCAGTCGATCACTTCGATGATCACGTAGCGTCTGCTGATGACTTCACGTATCAGTCTAATCTACACGACTCATTCAACGTGAATCGACTCAAGTTGCCATCAGACTTAGATCAGCTAGATCTGCCATATGATCTAGTCGAGTgtcatagctgtttcctg-3'
Storage S3	5'-gtaaaacgacggccagtCAGCTTGTCTGTAAGCGGATGCCGGGAGCAATTAGACATTGATGCATCCAGTCGACGTACGATATGATCAGCACTTAGATCTAGATGTACTAGATGATCAGTTCAATCTGCAGCTCAATGCAGACTAGCAGTTACTCCATCACTTCGATGATCCAGAGTTACTAGACTAGCACTCATACTAGCGTCgtcatagctgtttcctg-3'
Storage S4	5'-gtaaaacgacggccagtGACAAGCCCGTCAGGGCGCGTCAGCGGGTCATTAGGATGATCATGTGAATGCTGATGTACACTTAGATCAGTCTAATCACGTGATGAATCTGAAGCGTCAGCTACGATATCGTAATGAGCTACGATTAGATCCGAAGTCATTGATGCATCACGTAGTAGCATCTGATGATCACTTCGATGATCAGTgtcatagctgtttcctg-3'
Storage S5	5'-gtaaaacgacggccagtTTGGCGGGTGTCGGGGCTGGCTTAACTATGATTTAAGACGACAGTTAGAGCACTATGATCACGTAGCGTCTGCTGATGACTTCACGTTCTATCATAGACATGTCATCGACGGACTAGATCACGATCTACATGCGAATCCGAAGTTCATGATGCATCCGAAGTCATTGATGCATCGACTCAATGTAGgtcatagctgtttcctg-3'
Storage S6	5'-gtaaaacgacggccagtCGGCATCAGAGCAGATTGTACTGAGAGTGCATTTATATGTACACTATCAGCACTTAGATGTGACTAATCACTAGTATCAGTCATTCAATCGTCAGCATGCGATCTATCATACGATCGAGTATCCAGAGTCATTGATGCATCCTAAGTTCAATGTAGATGATGATCACTTCGATGATCAGTTCAGATgtcatagctgtttcctg-3'

Storage S7	5'-gtaaaacgacggccagtACCATATGCGGTGTGAAATACCGCACAGATATTTTAACGTACAGCTAGACGACTAGCAGTTACATCAGTCTAATCTGAAGCGTCAGCTACGATATCACTTCGAGCTACGATTAGATCAGCCTAATCCAGATGTGATGACATTGAAGTTAGATGTCCATCTCAAGCGATTCGACTATCACGTAGATCgtcatagctgtttcctg-3'
Storage S8	5'-gtaaaacgacggccagtGCGTAAGGAGAAAATACCGCATCAGGCGTGATTTTCAGCACTATCAGCTAGTCCATCGTAATGCAGACGCTGATGATCTGAATGCTAACTCACATCAGCCTAATCACTTCGATGATCACGTGAGTACATCTGATGTACATCAGTCTAATCACTTCGATGATCGTATGAAGTAGTTGCTCCATCTACgtcatagctgtttcctg-3'
Storage S9	5'-gtaaaacgacggccagtATTCGCCATTCAGGCTGCGCAACTGTTGGGATTTTGAGTCGAATCTGAATGCTAACTTCCATCGTAATGCAGACGCTGATGATCTCAAGCGATTCGACTTGGATCATAACTTCGATGTAGATGATCACGTCAATGATCCTGCGTTAGACTATGTCAAGCATGTAGATCCGATCGAGCCAGTCGATCgtcatagctgtttcctg-3'
Storage S10	5'-gtaaaacgacggccagtAAGGGCGATCGGTGCGGGCCTCTTCGCTATACAATTCTACATTCATACAGCTAGTCGATCCTGCATCAGTCGATCCGAAGTTCAGCAATCCTAAGTTCAATCACTTCGATGATCCTACATACTCATTCAATGTCCATCACGTACTGCATCTGCATGCTGACGTACTGCATCTCGATGTACCAGATGgtcatagctgtttcctg-3'
Storage S11	5'-gtaaaacgacggccagtTACGCCAGCTGGCGAAAGGGGGATGTGCTGACAACACTAAGTTCAACTTCGATCACTTCGATGATCCTGAGTTAGACTATCTAGATGTCAAGCAGTCATTAGATCCAGAGTTACTAGAGCTGCATGTCAACGACTAGCAGTTACATCCTATCAAGTCTGATCTAGCAGAGCATGTACCAGATGTCTgtcatagctgtttcctg-3'
Pond water sample barcode	5'-gtaaaacgacggccagtATATGAAGTACTCATTAGATCATAGACAGTTACTGCTCCATCATAGTAATGAGCAATAGCTACGATgtcatagctgtttcctg-3'

Supplementary Table 5 | Sequences for chiral steganography

Lowercase: D-DNA
Uppercase: L-DNA
Underlined: Sequences for segment-specific amplification and sequencing

Oligo	Sequence
D-F1	5'-gtgctgcaaggcgattaattaggtatacaaccagaaccagattaagattgtata-3'
D-R1	5'-ctatgactgttaacctatacaatcttaatctggttctggttgtatacctaattaatcgccttgcagcac-3'
D/L-F2	5'-ggttaacagtcatagctgtttcctgGTAAAACGACGGCCAGTATTACCTTAACAACCTATACCACATATACCAGGTTCAGATTCTATAGGTTCACAGTCATAGCTGTTTCCTG-3'
D/L-R2	5'-CAGGAAACAGCTATGACTGTGAACCTATAGAATCTGAACCTGGTATATGTGGTATAGGTTGTTAAGGTAATACTGGCCGTCGTTTTACcaggaaacag-3'
D-DNA key-F	5'-gtgctgcaaggcgatta-3'
D-DNA key-R	5'-caggaaacagctatgac-3'
L-DNA key-F	5'-GTAAAACGACGGCCAGT-3'
L-DNA key-R	5'-CAGGAAACAGCTATGAC-3'
Chimeric D-DNA/L-DNA key	5'-gtgctgcaaggcgattaattaggtatacaaccagaaccagattaagattgtataggttaacagtcatagctgtttcctgGTAAAACGACGGCCAGTATTACCTTAACAACCTATACCACATATACCAGGTTCAGATTCTATAGGTTCACAGTCATAGCTGTTTCCTG-3'

Supplementary Table 6 | Sanger sequencing results of D-DNA segments in the storage library and the D-DNA part of the chimeric DNA key

Underlined: Sequences for segment-specific amplification and sequencing

DNA segment	Sanger sequencing result
D-S1	5'-TCGCGCGTTTCGGTGATGACGGTGAAAACCATTACAATAACGTACTGCATCCAGAGTTACTAGATGAACCATATGTACACTTGACGTTCCATCAGCCTAATCACTTCGATGATCCTGCGTTAGACTATGTCAAGCAGTCATTAGATCAGCTACCTATGACATATGTACCAGATGATCACTAGTATCGTCATAGCTGTTTCCTG-3'
D-S2	5'-TCTGACACATGCAGCTCCCGGAGACGGTCAATTACCCGATCGAGCCAGTCGATCACTTCGATGATCACGTAGCGTCTGCTGATGACTTCACGTATCAGTCTAATCTACACGACTCATTCAACGTGAATCGACTCAAGTTGCCATCAGACTTAGATCAGCTAGATCTGCCATATGATCTAGTCGAGTGTCATAGCTGTT–CCTG-3'
D-S3	5'-AGCTTGTCTGTAAGCGGATGCCGGGAGCAATTAGACATTGATGCATCCAGTCGACGTACGATATGATCAGCACTTAGATCTAGATGTACTAGATGATCAGTTCAATCTGCAGCTCAATGCAGACTAGCAGTTACTCCATCACtTcGaTGATCCAGAGTTACTAGACTAGCACTCATACTAGCGTCGTCATAGCTGTT–CCTG-3'
D-S4	5'-GACAAGCCCGTCAGGGCGCGTCAGCGGGTCATTAGGATGATCATGTGAATGCTGATGTACACTTAGATCAGTCTAATCACGTGATGAATCTGAAGCGTCAGCTACGATATCGTAATGAGCTACGATTAGATCCGAAGTCATTGATGCATCACGTAGTAGCATCTGATGATCACTTCGATGATCAGTGTCATAGCTGTTTCCTG-3'
D-S5	5'-TTGGCGGGTGTCGGGGCTGGCTTAACTATGATTTAAGACGACAGTTAGAGCACTATGATCACGTAGCGTCTGCTGATGACTTCACGTTCTATCATAGACATGTCATCGACGGACTAGATCACGATCTACATGCGAATCCGAAGttcaTGATGCATCCGAAGTCATTGATGCATCGACTCAATGTAGGTCATAGCTGTT–CCTG-3'
D-S6	5'-CGGCATCAGAGCAGATTGTACTGAGAGTGCATTTATATGTACACTATCAGCACTTAGATGTGACTAATCACTAGTATCAGTCATTCAATCGTCAGCATGCGATCTATCATACGATCGAGTATCCAGAGTCATTGATGCATCCTAAGTTCAATGTAGATGATGATCACTTCGATGATCAGTTCAGATGTCATAGCTGTTTCCTG-3'

D-S7	5'-ACCATATGCGGTGTGAA–TACCGCACAGATATTTTAACGTACAGCT AGACGACTAGCAGTTACATCAGTCTAATCTGAAGCGTCAGCTACGATA TCACTTCGAGCTACGATTAGATCAGCCTAATCCAGATGTGATGACATT GAAGTTAGATGTCCATCTCAAGCGATTCGACTATCACGTAGATCGTCA TAGCTGTTTCCTG-3'
D-S8	5'-GCGTAAGGAGAAAATACCGCATCAGGCGTGATTTTCAGCACTATC AGCTAGTCCATCGTAATGCAGACGCTGATGATCTGAATGCTAACTCAC ATCAGCCTAATCACTTCGATGATCACGTGAGTACATCTGATGTACATC AGTCTAATCACTTCGATGATCGTATGAAGTAGTTGCTCCATCTACGTCA TAGCTGTTCCTG-3'
D-S9	5'-–TTCGCCATTCAGGCTGCGCAACTGTTGGGATTTTGAGTCGAATCT GAATGCTAACTTCCATCGTAATGCAGACGCTGATGATCTCAAGCGATT CGACTTGGATCATAACTTCGATGTAGATGATCACGTCAATGATCCTGC GTTAGACTATGTCAAGCATGTAGATCCGATCGAGCCAGTCGATCGTCA TAGCTGTTTCCTG-3'
D-S10	5'-–AGGGCGATCGGTGCGGGCCTCTTCGCTATACAATTCTACATTCATA CAGCTAGTCGATCCTGCATCAGTCGATCCGAAGTTCAGCAATCCTAAG TTCAATCACTTCGATGATCCTACATACTCATTCAATGTCCATCACGTAC TGCATCTGCATGCTGACGTACTGCATCTCGATGTACCAGATGGTCATA GCTGTTTCCTG-3'
D-S11	5'-TACGCCAGCTGGCGAAAGGGGGATGTGCTGACAACACTAAGTTC AACTTCGATCACTTCGATGATCCTGAGTTAGACTATCTAGATGTCAAG CAGTCATTAGATCCAGAGTTACTAGAGCTGCATGTCAACGACTAGCA GTTACATCCTATCAAGTCTGATCTAGCAGAGCATGTACCAGATGTCTG TCATAGCTGTTTCCTG-3'
D-DNA key	5'-GTGCTGCAAGGCGATTAATTAGGTATACAACCAGAACCAGATTAAG ATTGTATAGGTTAACAGTCATAGCTGTTTCCTG-3'

Supplementary Table 7 | Metagenomic sequencing summary of D- or L-DNA barcoded, or unbarcoded microbial DNA samples

Sample	Total reads	Number of barcode sequences
D-DNA barcoded	117,760,298	20,315
L-DNA barcoded	113,056,024	0
Unbarcoded	128,710,640	0