Supplemental Files

Title: Differentiation of isobaric cross-linked peptides prepared via maleimide chemistry by MALDI-MS and MS/MS.

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Figure S1. The retro Michael reaction. The structure shown at the left represents a cysteine and a lysine residue cross-linked at their side chains by a *N*-propionyl thiosuccinimide ring (referred to herein as a thiosuccinimide linker). In vivo, molecules containing free thiols (R-SH) such as glutathione react readily with the thiosuccinimide ring and the original peptide is released from the conjugate. This reaction (known as a retro-Michael addition) not only impairs the biological activity of the conjugate, but also increases the cytotoxicity of the antibody-drug conjugate (ADC).

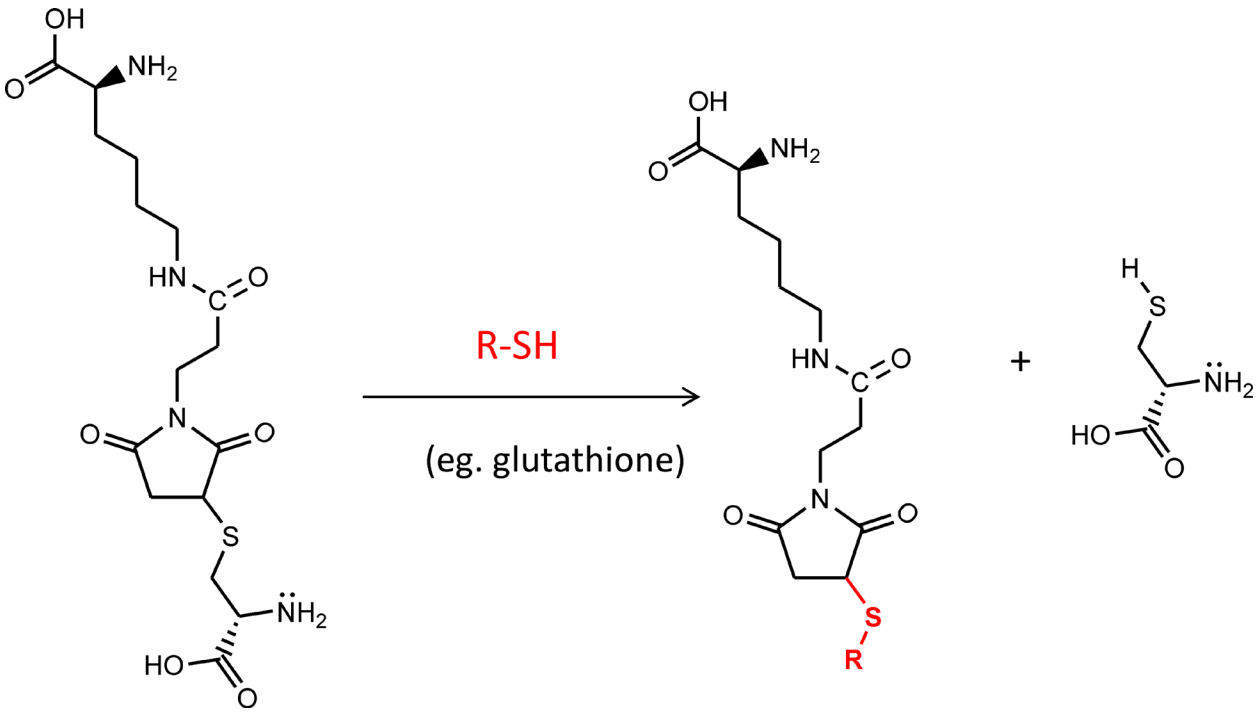

Figure S2. Reverse-phase liquid chromatograms of P3-P4 cross-linked peptide obtained after synthesis (b) and incubation in 100 mM Tris/HCl buffer, pH 8.0 for 6 hours at 37°C (a). The amino acid sequences of P3 and P4 are N**C**AGHK and Ac-GANAP**K***EPQR, respectively. Peaks in the upper chromatogram eluting at 4.74 and 4.86 min correspond to P3-P4 with the hydrolyzed and intact linker, respectively, which have been identified by MALDI-MS. The chromatographic conditions are as described in "**Materials and Methods**".

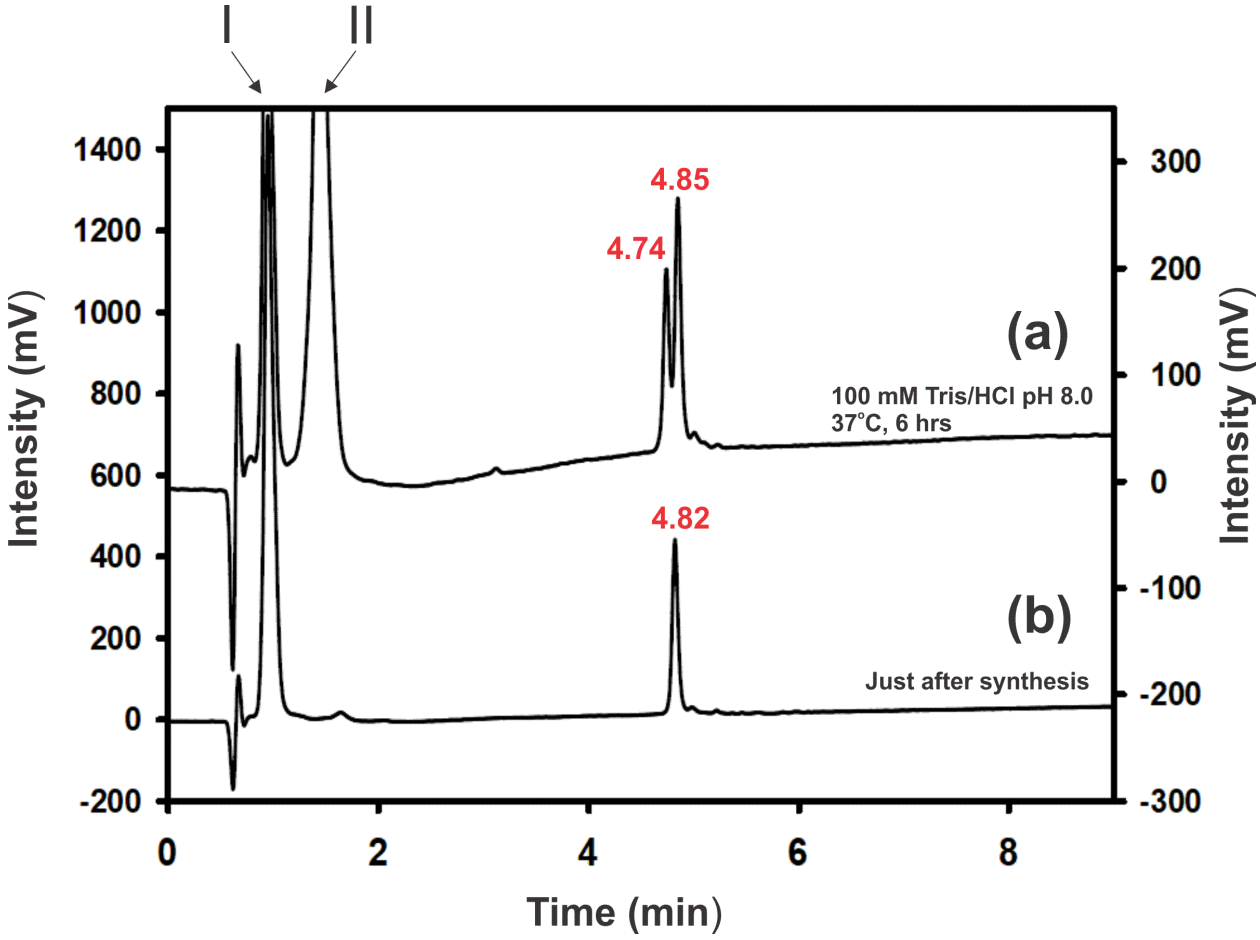

Figure S3. Reverse-phase liquid chromatograms of P1-P3 cross-linked peptide obtained after the synthesis (b) and incubation in 100 mM Tris/HCl buffer, pH 8.0 for 6 hours at 37°C (a). The amino acid sequences of P1 and P3 are (**A**AAGGGAAAAK) and (N**C**AGHK), respectively. Peaks in the upper chromatogram eluting at 4.16 and 4.34 min correspond to P1-P3 with the hydrolyzed and intact linker, respectively, which have been identified by MALDI-MS. The chromatographic conditions are as described in "**Materials and Methods**".

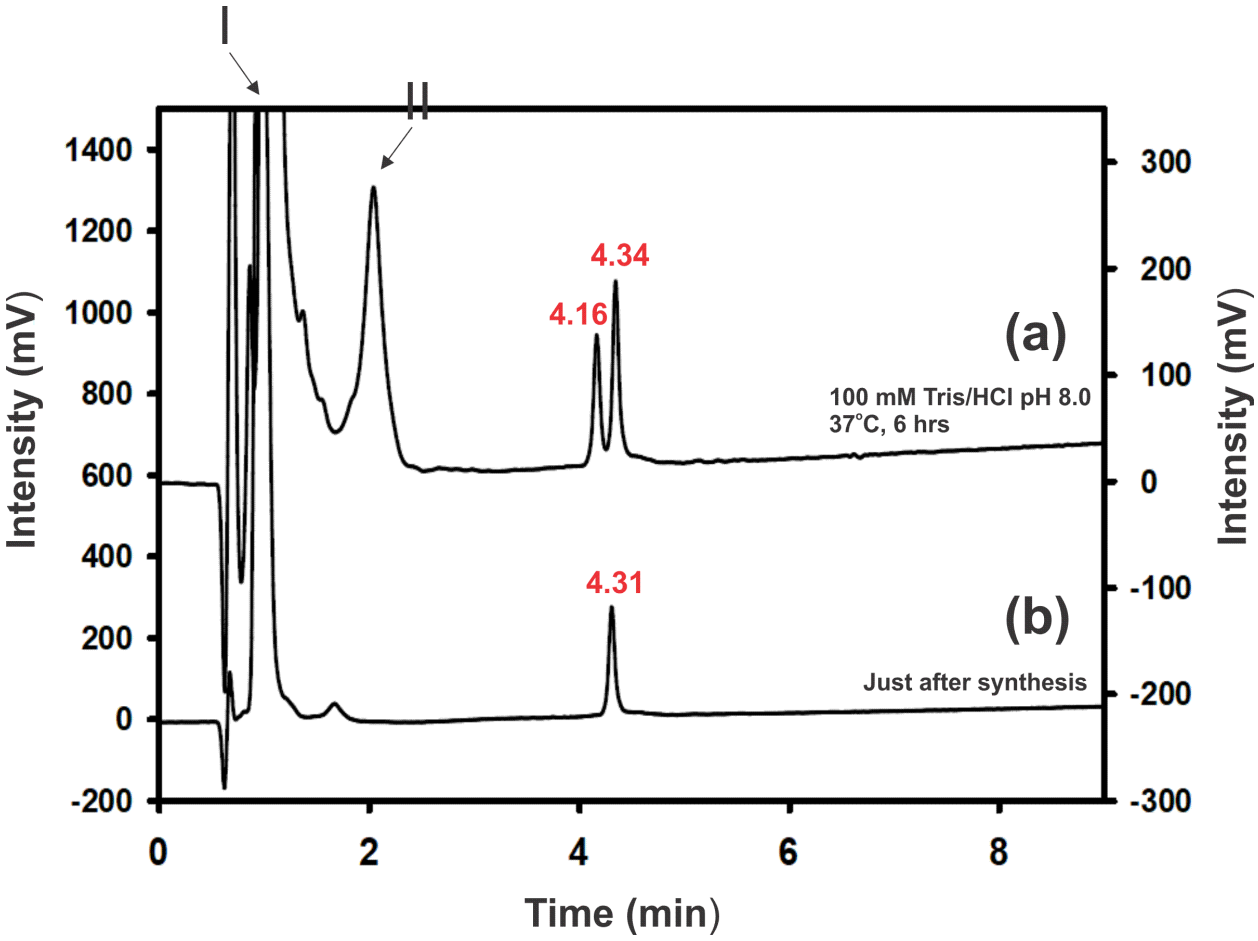

Figure S4. Reverse-phase liquid chromatograms of P2-P4 cross-linked peptide obtained after the synthesis (b) and incubation in 100 mM Tris/HCl buffer, pH 8.0 for 6 hours at 37°C (a). The amino acid sequences of P2 and P4 are (**C**AAGGGAAAAK) and (Ac-GANAP**K**EPQR), respectively. Peaks in the upper chromatogram eluting at 5.16, 5.28 and 5.45 min correspond to P2-P4 with the hydrolyzed thiosuccinimide, thiosuccinimide and thiazine linker, respectively, which have been identified by MALDI-MS. Peaks marked with asterisks indicate P2-P2 dimers linked by disulfide bonds. The chromatographic conditions are as described in "**Materials and Methods**".

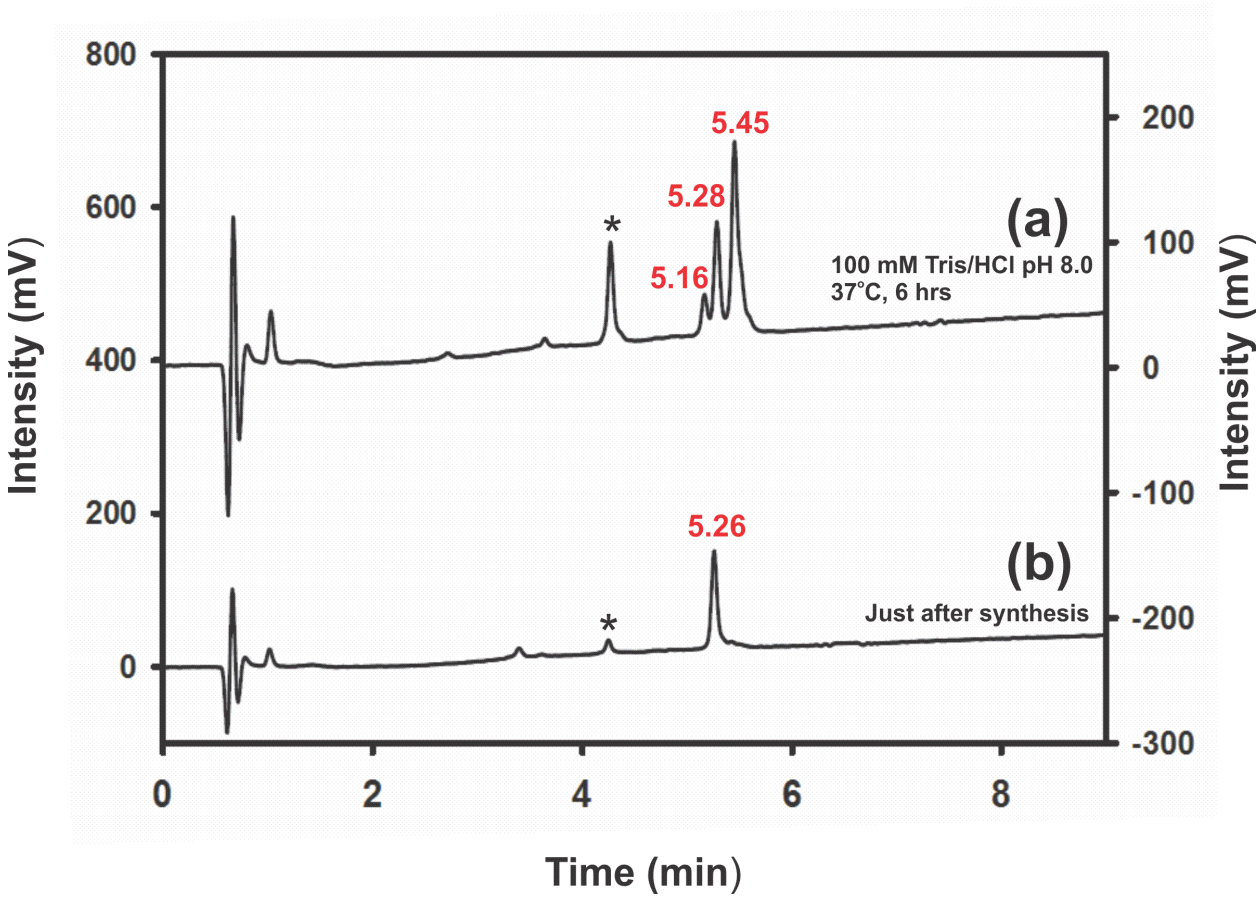

Figure S5. MALDI-MS/MS spectrum of the P2 peptide with a free Cys residue (*m/z*=847.43).

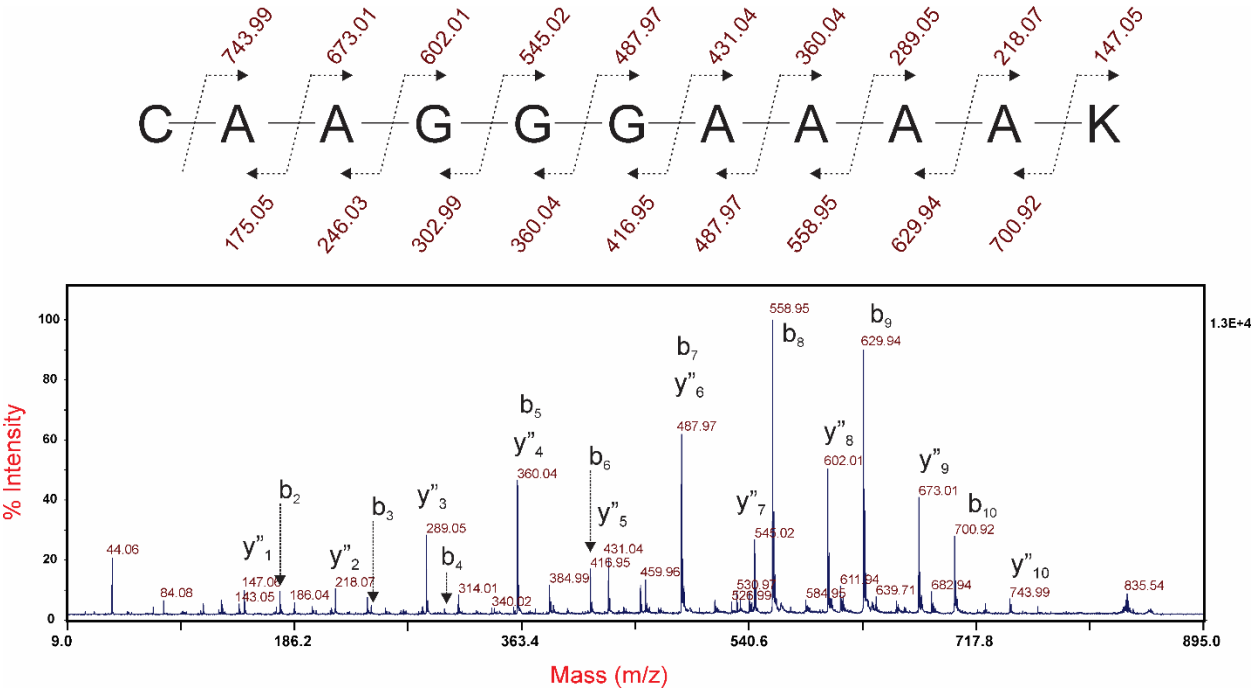

Figure S6. MALDI-MS/MS spectra from 968.50 (a) and 966.49 (b) observed in Figure 3a, which correspond to the structures (II) and (I) depicted in Figure 3d, respectively.

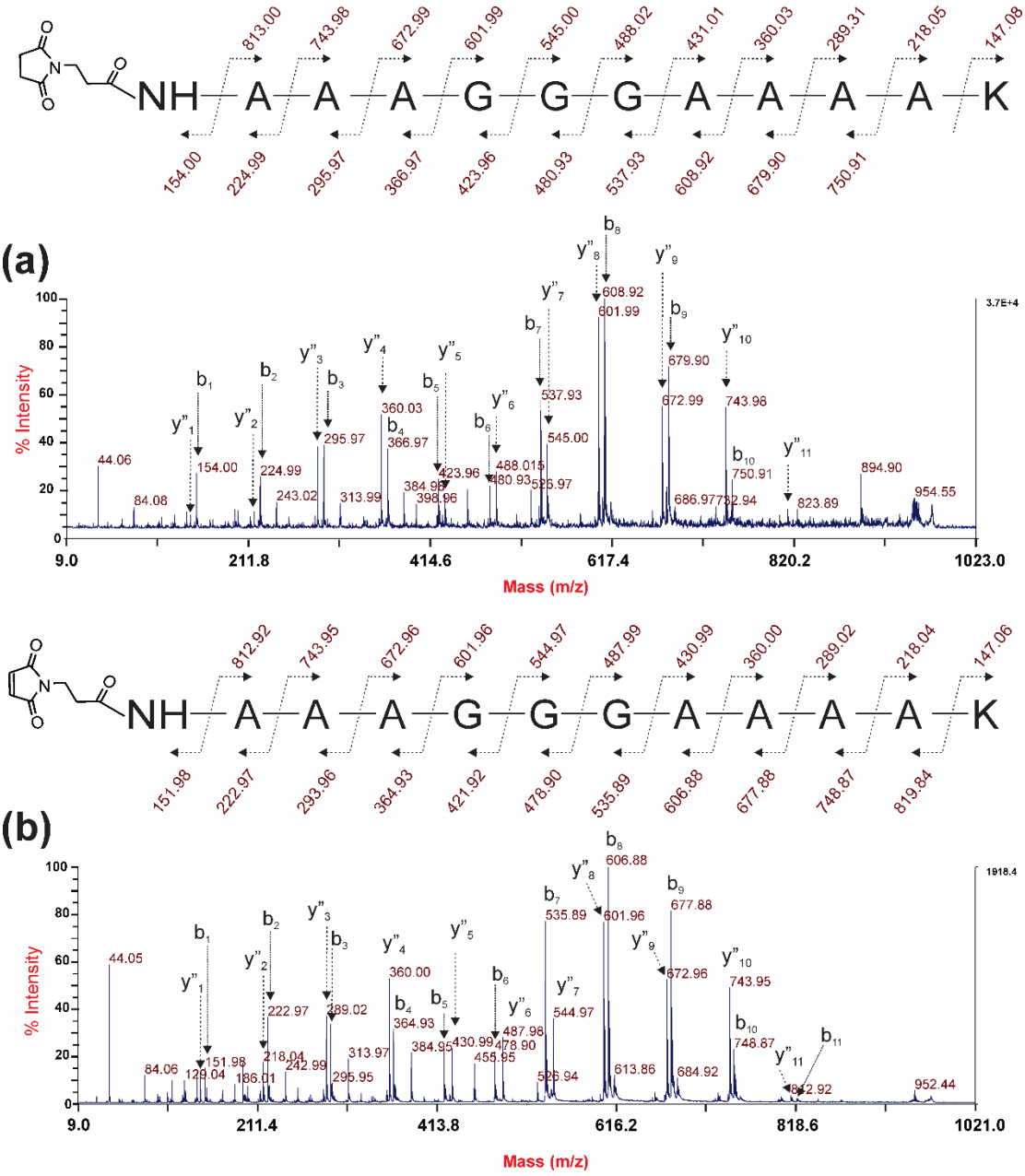

Figure S7. MALDI-MS spectra of P1-P3 (*m/z* 1594.64) (a) and $(P1\text{-}P3)_H$ (*m/z* 1612.30) (b). The insets in (a) and (b) are expanded views of the red dashed squares. The signals marked with I, II, III, and IV in the insets are assigned to the structures in (c). P3-SH indicates the P3 peptide with a free Cys.

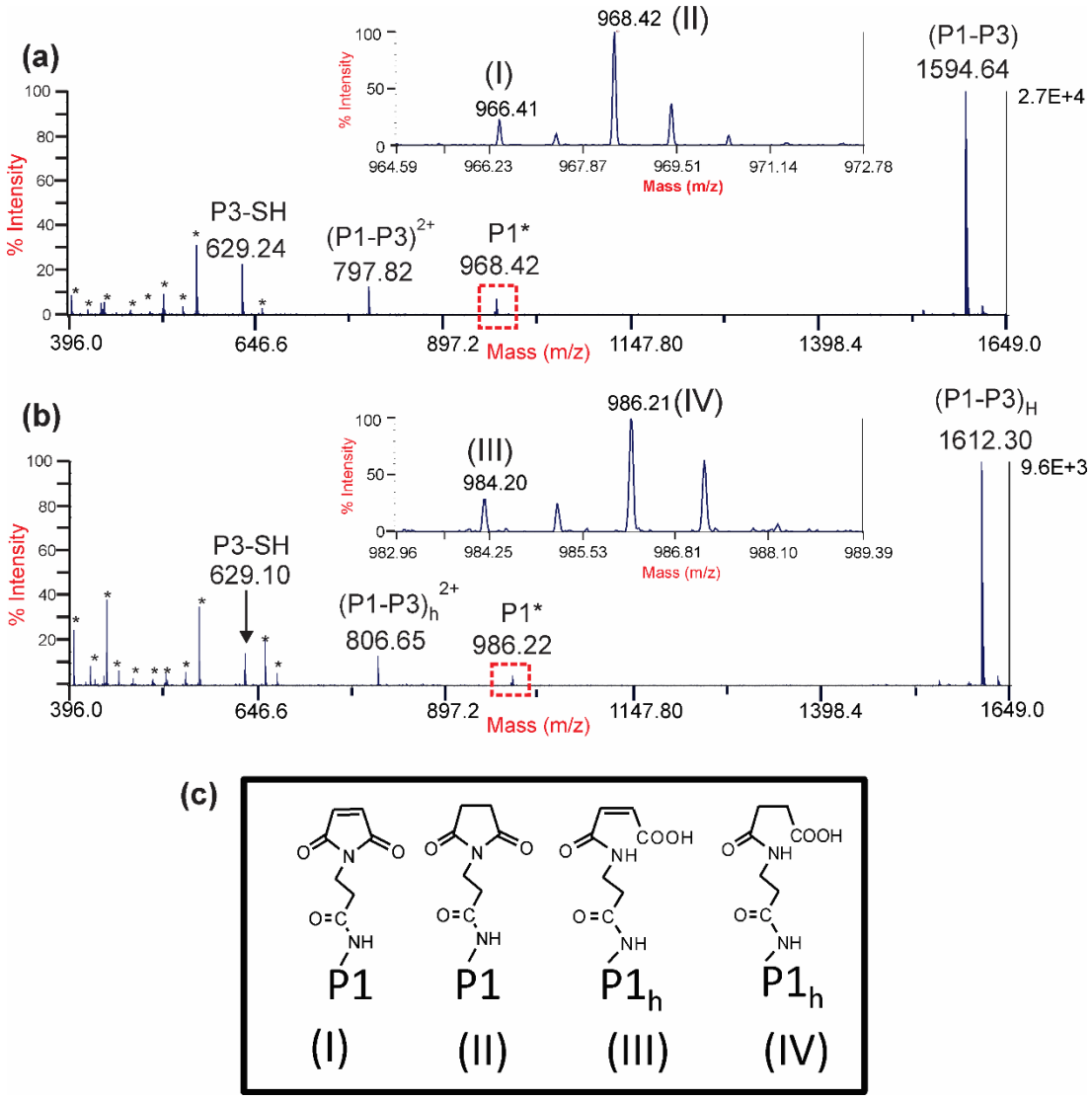

Figure S8. MALDI-MS spectra of P2-P4 (*m/z* 2106.79) (a) and $(P2\text{-}P4)_H$ (*m/z* 2124.80) (b). The insets in (a) and (b) are expanded views of the red dashed squares. The signals marked with I, II, III, and IV in the insets are assigned to the structures in (c). P2-SH indicates the P2 peptide with a free Cys.

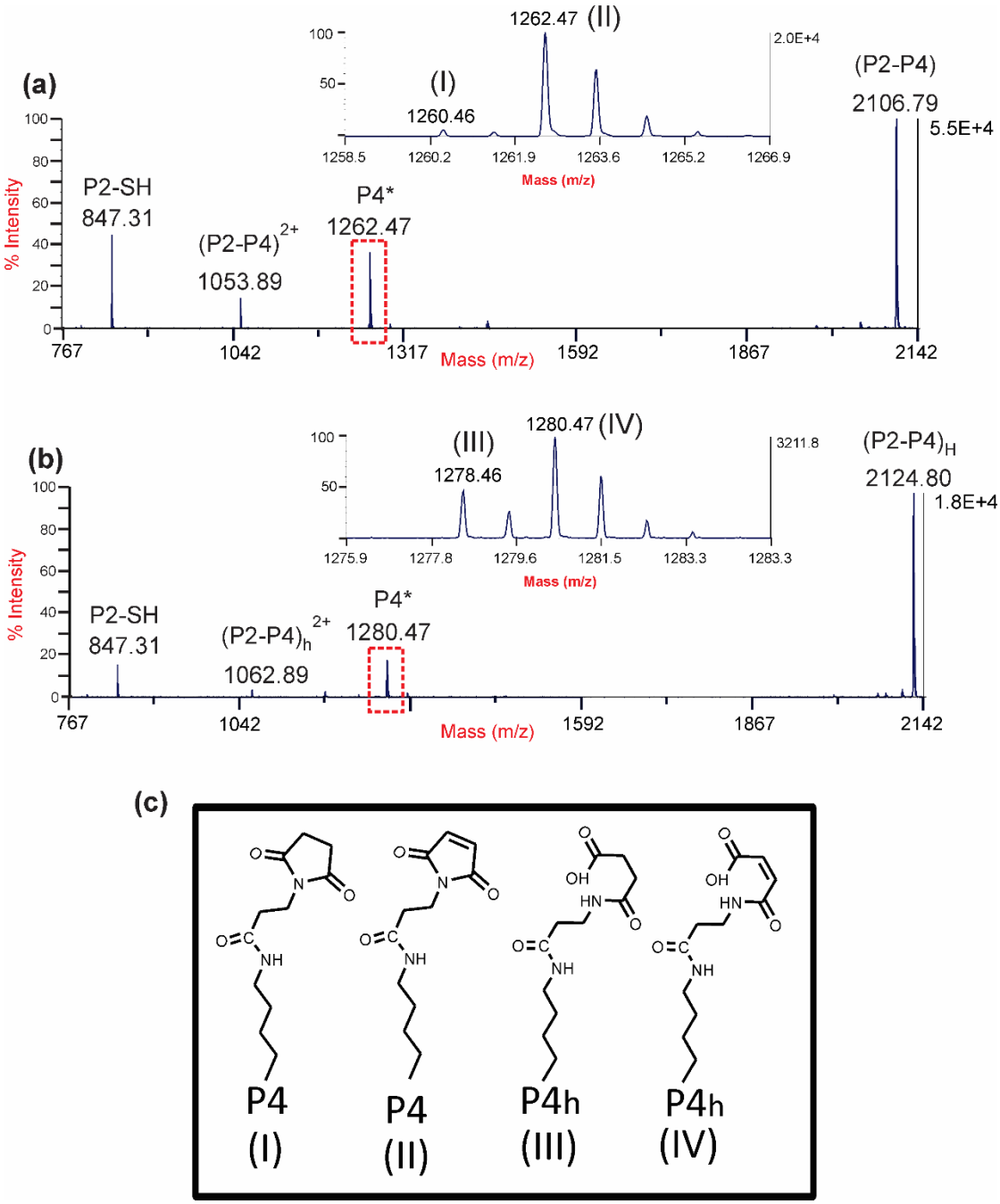

Figure S9. MALDI-MS spectra of P3-P4 (*m/z* 1888.42) (a) and $(P3\text{-}P4)_H$ (*m/z* 1906.46) (b). The insets in (a) and (b) are expanded views of the red dashed squares. The signals marked with I, II, III, and IV in the insets are assigned to the structures in (c). P3-SH indicates the P3 peptide with a free Cys.

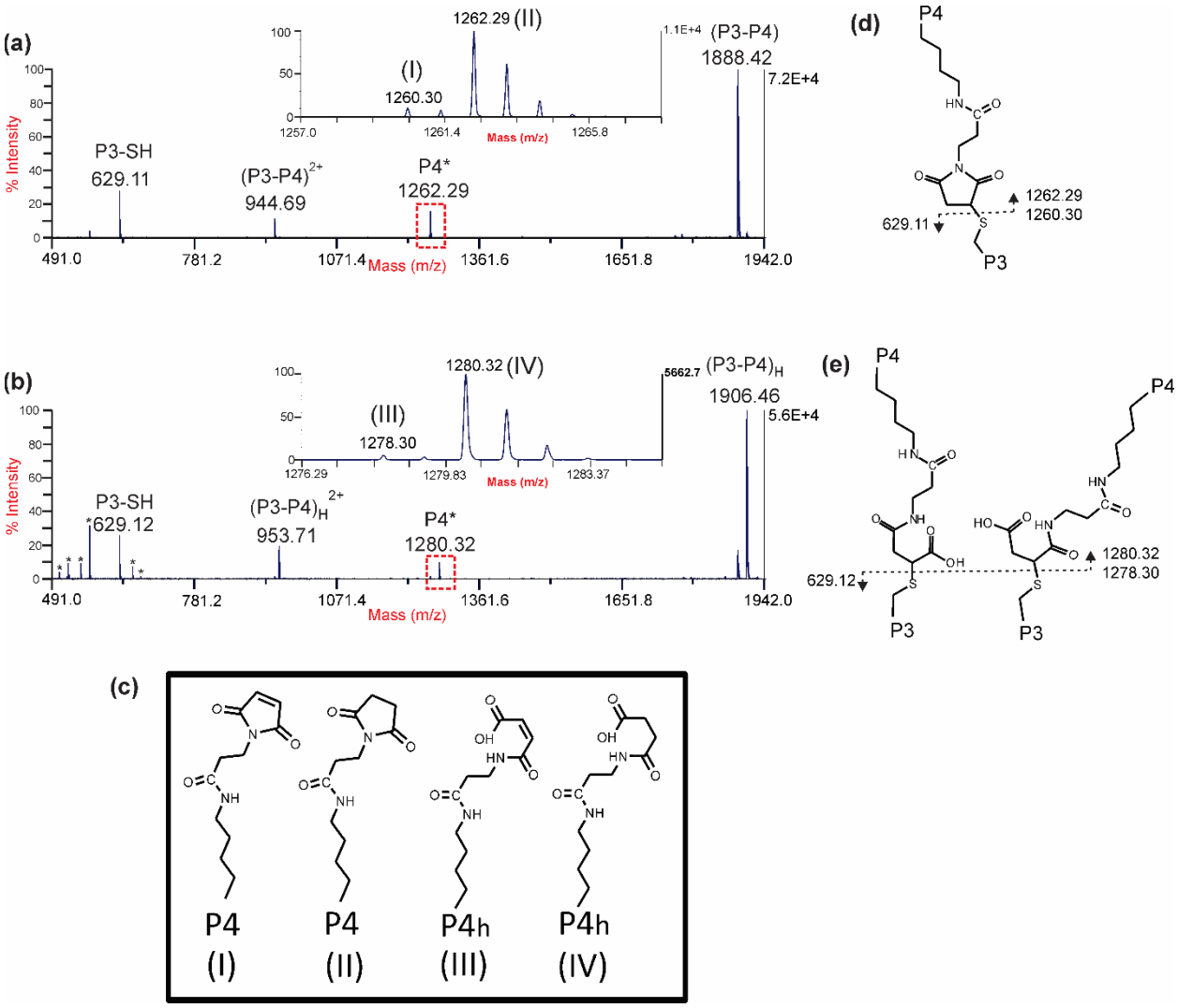

Figure S10. Summary of the backbone fragment ions ($a_{n\alpha}$, $b_{n\beta}$, $y''_{n\alpha}$, and $y''_{n\beta}$) observed for $(P1\text{-}P2)_T$ cross-linked peptide in Figure 6d. The nomenclature for the backbone fragment ions for the cross-linked peptides is as was used in a previous report [25].

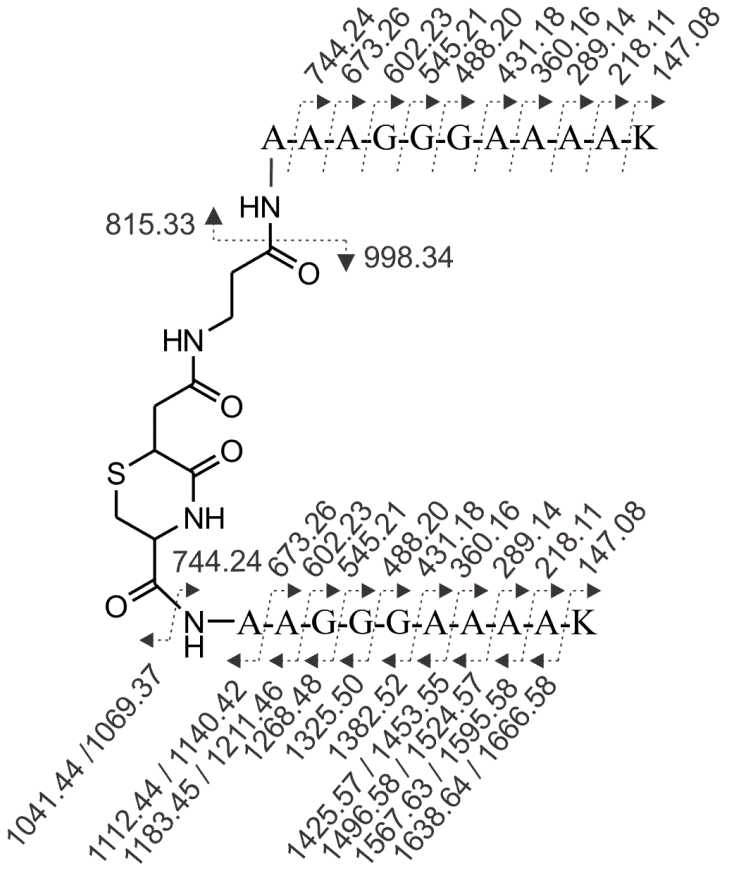

Figure S11. Summary of the backbone fragment ions ($a_{n\alpha}$, $b_{n\beta}$, $y''_{n\alpha}$, and $y''_{n\beta}$) observed for P1-P2 cross-linked peptide in Figure 6a.

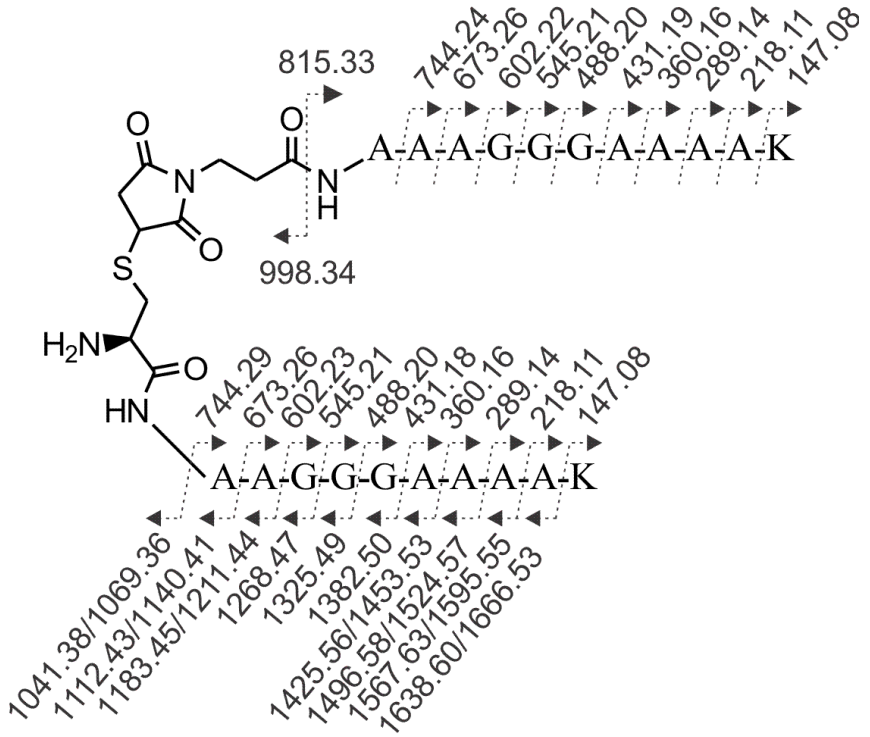

Figure S12. MALDI-MS/MS spectrum of P1-P3. The area enclosed by the dashed squares in (a) is enlarged in (b). The *m/z* values in green indicate the fragment ions that were specifically observed for the cross-linked peptide with a thiosuccinimide linker (c). The assignments of backbone-derived fragment ions are depicted in (d).

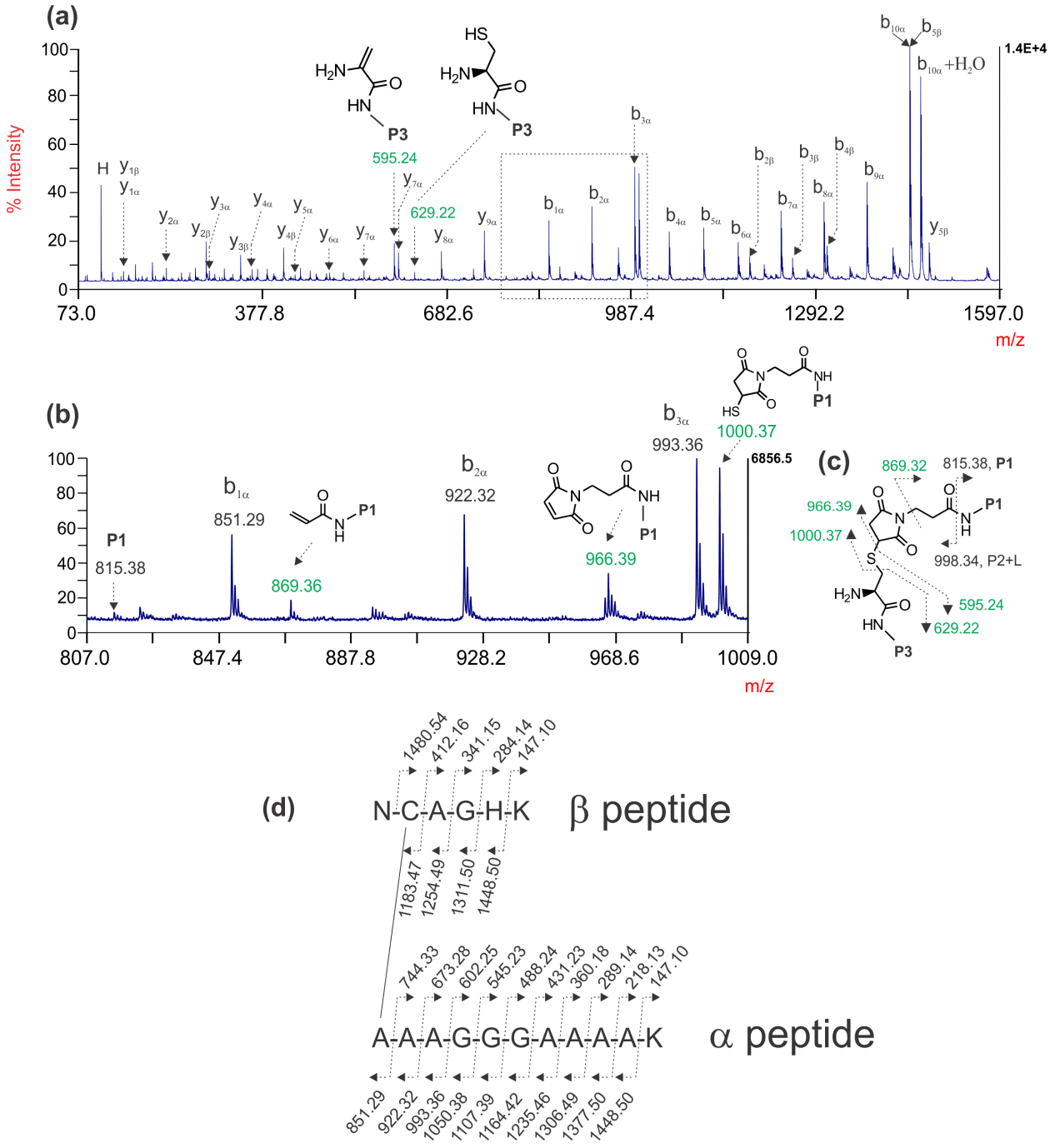

Figure S13. MALDI-MS/MS spectrum of P3-P4 (a). The *m/z* values in green indicate the fragment ions specifically observed for the cross-linked peptide with a thiosuccinimide linker (b).

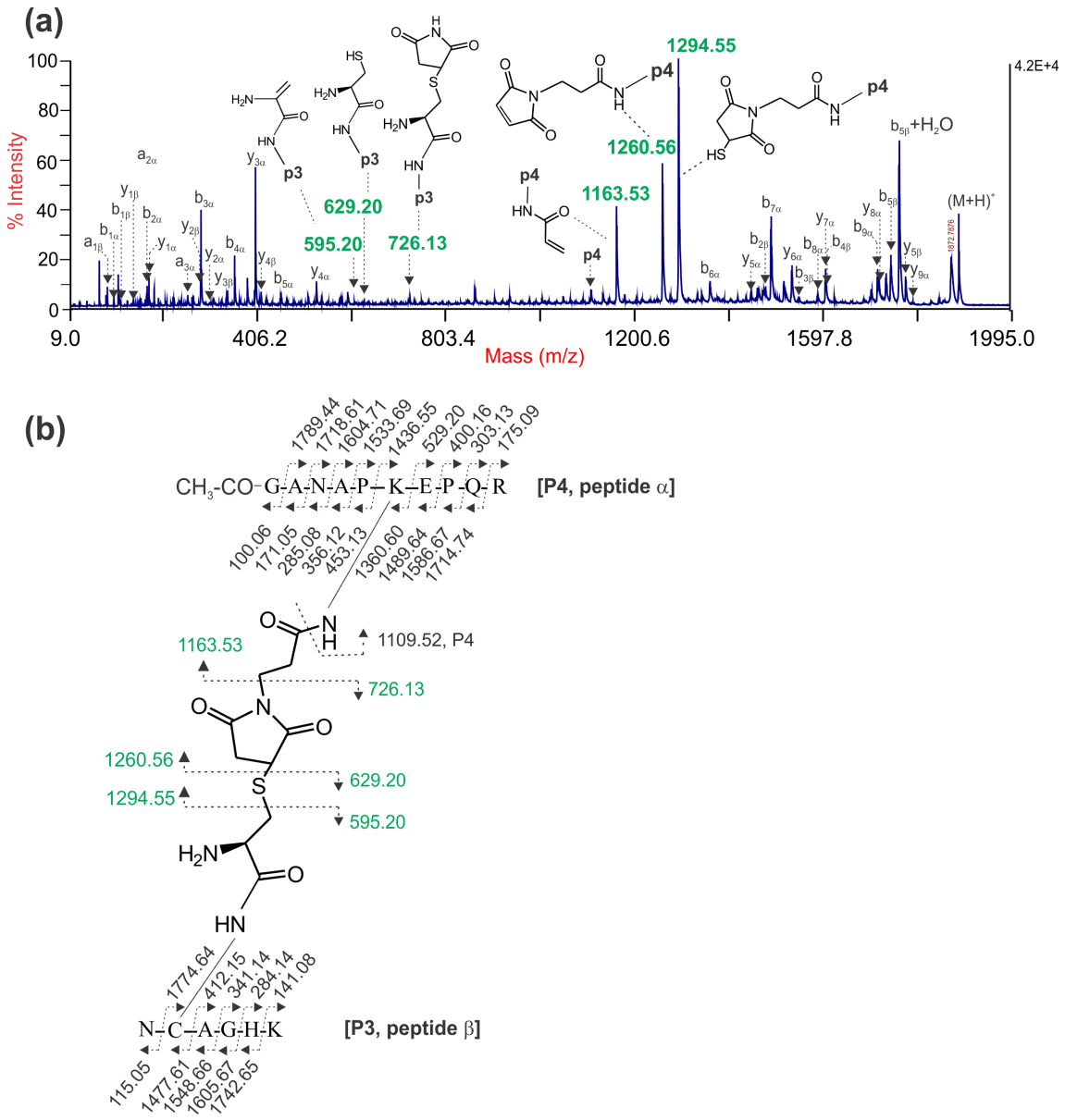

Figure S14. MALDI-MS/MS spectra of the cross-linked peptides, $(P2\text{-}P4)_T$ (a) and P2-P4 (f). The areas enclosed by the dashed squares in (a) and (f) are enlarged in (b) and (d), respectively. The *m/z* values in green and red indicate fragment ions specifically observed for the thiosuccinimide (e) and thiazine (c) forms, respectively. “L” indicates the *N*-propionyl maleimide linker.

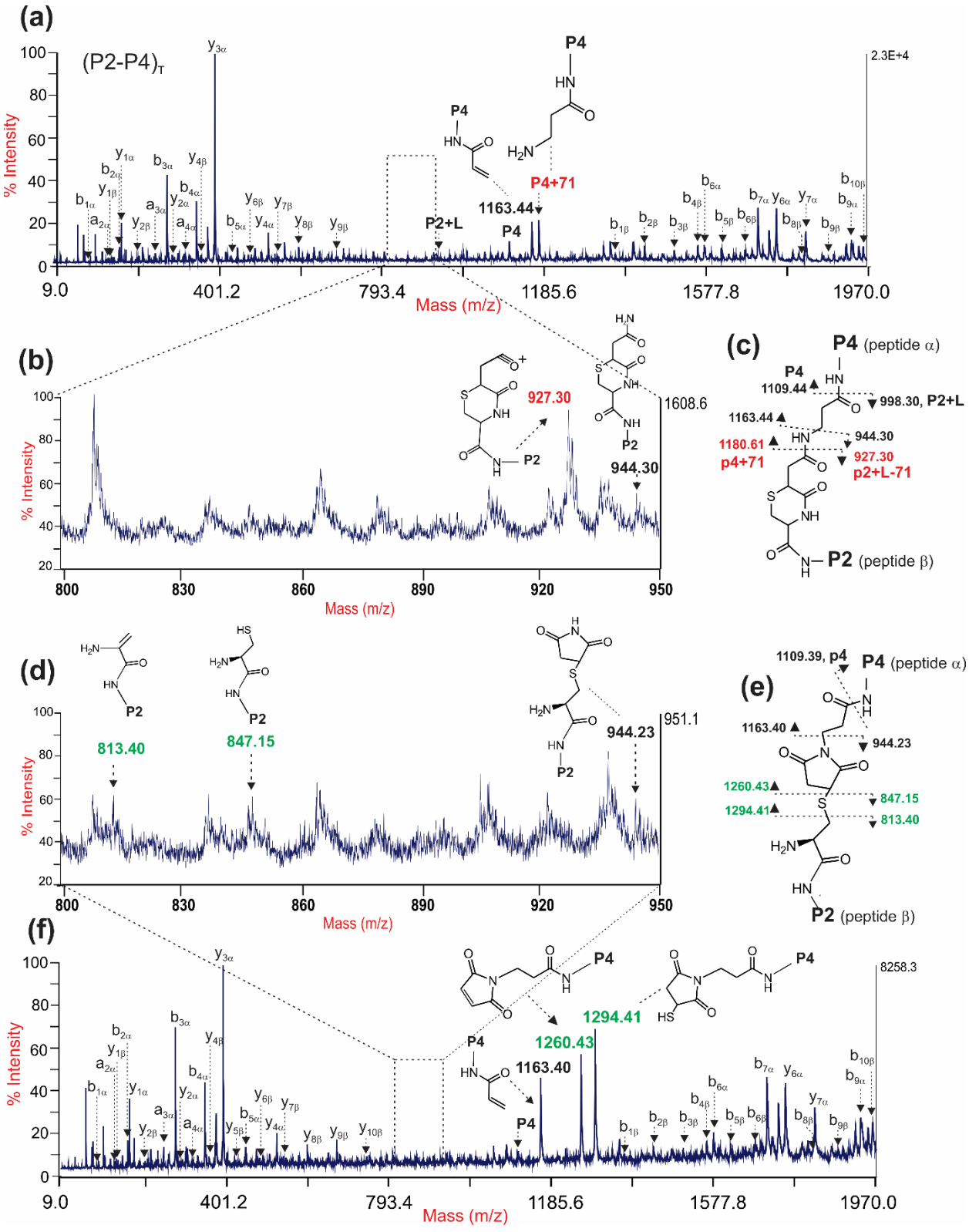
(a)
(P2-P4)T
% Intensity
Mass (m/z)
2.3E+4
P4+71
1163.44
P2+L
(b)
927.30
944.30
1608.6
(c)
P4 (peptide α)
1109.44
998.30, P2+L
1163.44
944.30
1180.61
p4+71
927.30
p2+L-71
P2 (peptide β)
(d)
813.40
847.15
944.23
951.1
(e)
1109.39, p4
1163.40
944.23
1260.43
847.15
1294.41
813.40
(f)
1294.41
1260.43
1163.40
8258.3

Figure S15. Summary of the fragment ions observed for $(P2\text{-}P4)_T$ (a) and P2-P4 (b) in Figure S14. The *N*-terminal end of P4 is blocked with an acetyl group.

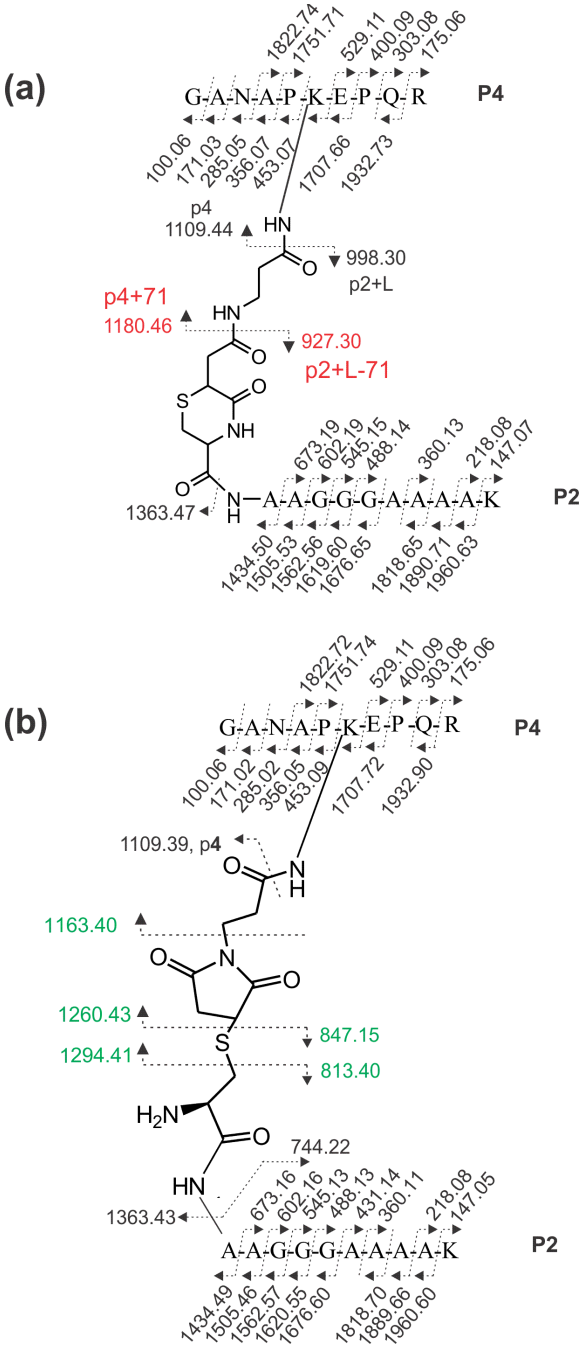

Figure S16. UVPD MS/MS spectra of P2-P4 (*m/z* = 703.338, 3+) (a) and $(P2\text{-}P4)_T$ (*m/z* = 703.339, 3+) (c). The inset in (a) shows the expanded region of the ion corresponding to P2-SH (*m/z* 847.41, 1+). The *m/z* values in green indicate the fragment ions specifically observed for the thiosuccinimide form (b). Linker-derived fragment ions were not observed for the thiazine form (d). The measurement conditions are described in "**Materials and Methods**".