Rapid access to glycopeptide antibiotic precursor peptides coupled with Cytochrome P450-mediated catalysis: towards a biomimetic synthesis of glycopeptide antibiotics

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Supplementary Information

Content

1. Characterization of the effect of MPAA oxidation on peptide thioester yields

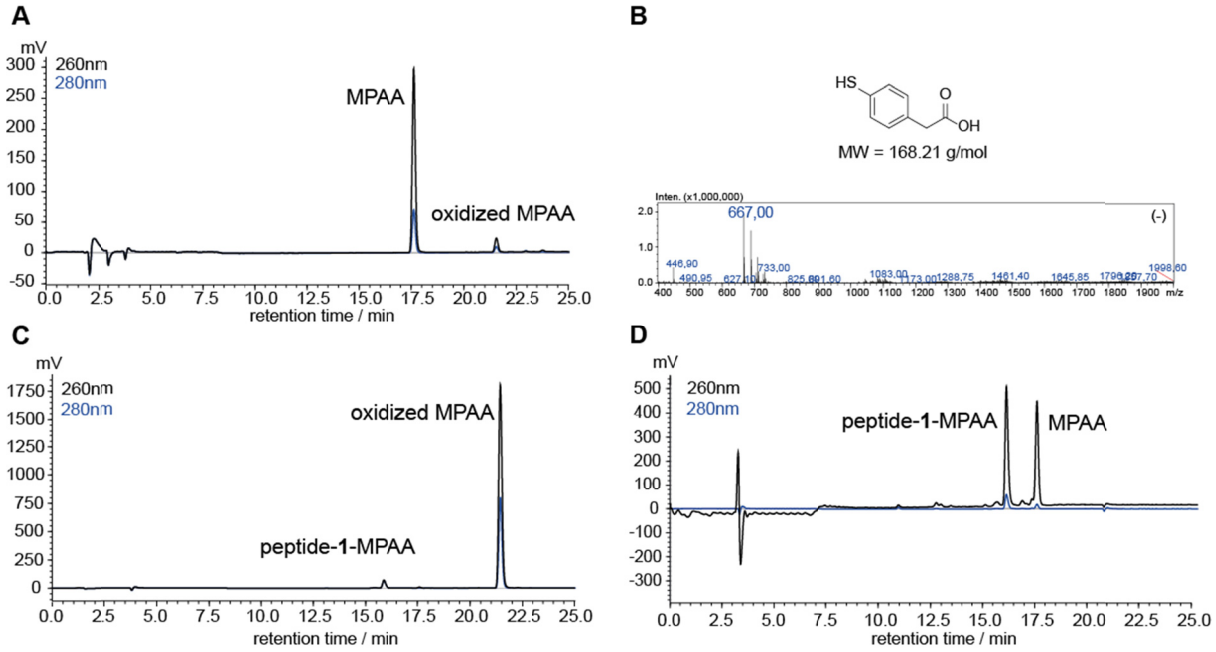

Figure S1. A) Analytical reverse-phased HPLC traces of commercially available MPAA. B) Mass spectra of oxidized MPAA in negative mode: 667 corresponds to a dimer of oxidized MPAA-disulfide (MW = 334.4). C) HPLC traces of crude synthesis mixture of peptide-**1**-MPAA after MPAA displacement under oxidizing conditions. D) HPLC trace of crude synthesis mixture of peptide-**1**-MPAA after MPAA displacement under reducing conditions (Argon, PBu_3). HPLC-gradient: 0 – 2 min 95 % solvent A, 2 – 25 min up to 25 % solvent B, flow rate 1 mL/min.

2. Characterization of peptides 1 - 5

Table S1. Summary of analytical data for peptides **1 – 5**

Peptide	Mol. Weight [g/mol]	Mass found [g/mol]	retention time t_R*
Peptide-1-MPAA	1020.11	1020.25 $(M+H)^+$, 510.95 $(M+2H)^{2+}$	16.01 min
Peptide-1-CoA	1619.43	1618.60 $(M-H)^-$, 808.50 $(M-2H)^{2-}$	13.37 min
Peptide-2-MPAA	993.04	993.35 $(M+H)^+$	15.67 min
Peptide-2-CoA	1592.37	1591.50 $(M-H)^-$	12.74 min
Peptide-3-MPAA	1089.17	1089.65 $(M+H)^+$	17.42 min
Peptide-3-CoA	1688.49	1687.70 $(M-H)^-$, 842.60 $(M-2H)^{2-}$	16.06 min
Peptide-4-MPAA	1091.15	1091.50 $(M+H)^+$	16.53 min
Peptide-4-CoA	1690.47	1689.25 $(M-H)^-$, 844.00 $(M-2H)^{2-}$	13.98 min
Peptide-5-MPAA	1128.21	1128.70 $(M+H)^+$	17.39 min
Peptide-5-CoA	1727.53	1726.60 $(M-H)^-$, 862.50 $(M-2H)^{2-}$	15.37 min

* Analytical reversed-phase HPLC; Gradient: 0 – 2 min 95 % solvent A, 2 – 25 min up to 25 % solvent B, flow rate 1 mL/min.

Peptide-1-MPAA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): δ = 8.81 (m, 2H, Hpg^{NH}, Tyr_6^{NH}), 8.40 (d, 1H, ^{3}J = 7.0 Hz, Leu_1^{NH}), 8.18 (d, 1H, ^{3}J = 6.9 Hz, Asn^{NH}), 7.64 (d, 1H, ^{3}J = 6.7 Hz, Hpg^{NH}), 7.47 – 7.37 (m, 3H, H_{ar}, Tyr_2^{NH}), 7.32 – 7.22 (m, 4H, H_{ar},), 7.13 – 7.04 (m, 6H, H_{ar}), 6.97 – 6.83 (m, 8H, H_{ar}), 5.36 (d, 1H, ^{3}J = 6.8 Hz, Hpg^{α}), 5.08 (d, 1H, ^{3}J = 7.3 Hz, Hpg^{α}), 4.59 (m, 1H, Asn^{α}), 4.37 (m, 1H, Tyr_2^{α}), 4.24 (m, 1H, Tyr_6^{α}), 3.66 (m, under water peak, Leu_1^{α}), 3.49 (s, under water peak, CH_2-MPAA), 3.09 – 3.04 (m, 1H, $Tyr_2^{\beta a}$), 2.89 – 2.82 (m, 1H, $Tyr_2^{\beta b}$), 2.70 – 2.62 (m, 2H, Tyr6 $^{\beta}$), 2.47 – 2.43 (m, 1H, $Asn_3^{\beta a}$), 2.35 – 2.27 (m, 1H, $Asn_3^{\beta b}$), 1.50 – 1.36 (m, 1H, Leu_1^{γ}), 1.35 – 1.21 (m, 2H, Leu_1^{β}), 0.78 (d, 3H, ^{3}J = 6.3 Hz, $Leu_1^{\delta 1}$), 0.66 (m, 3H, ^{3}J = 6.4 Hz, $Leu_1^{\delta 2}$) ppm.

HRMS (ESI): *m/z* calc. for $C_{52}H_{58}N_7O_{13}S$ $(M+H)^+$ 1020.38078, found: 1020.38015, Δ = 0.6 ppm; calc. mass for $C_{52}H_{57}N_7NaO_{13}S$ $(M+Na)^+$ 1042.36273, found: 1042.36194, Δ = 0.8 ppm.

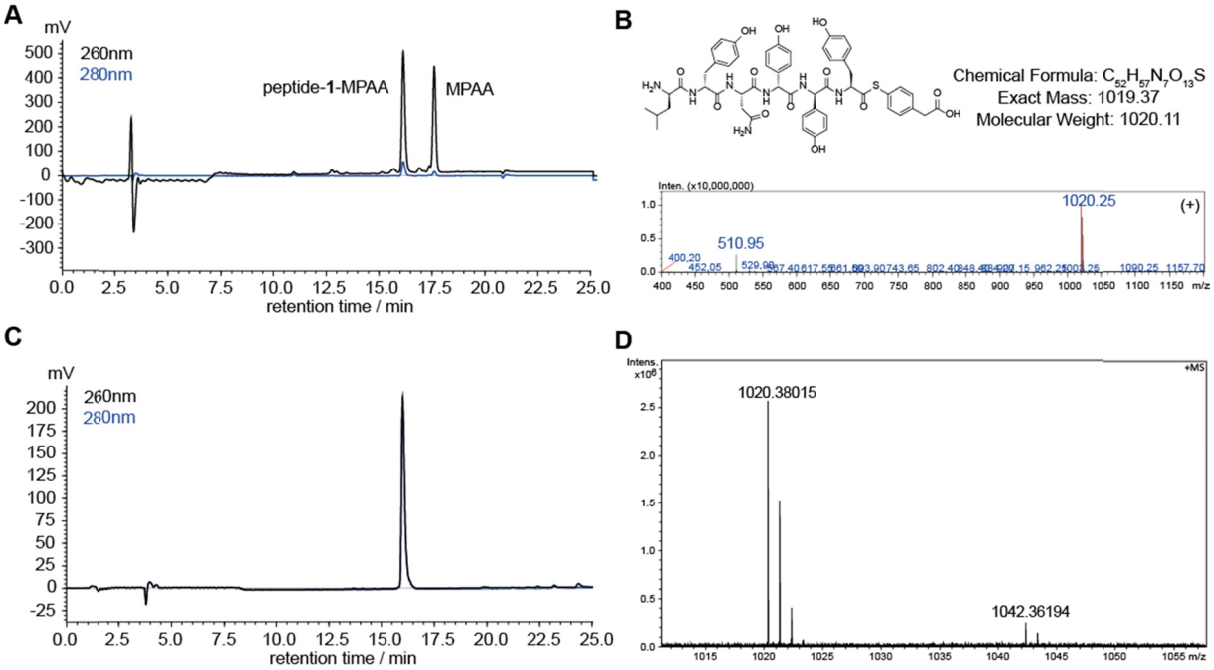

Figure S2. A) Analytical reversed-phase HPLC trace and (B) MS spectra **o**f crude peptide-**1**-MPAA. C) Analytical reversed-phase HPLC trace and (D) HRMS spectra of HPLC-purified peptide-**1**-MPAA.

Peptide-1-CoA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): δ = 8.84 (m, 1H, Tyr_6^{NH}), 8.78 (d, 1H, ^{3}J = 8.3 Hz, Hpg^{NH}), 8.65 (m, 1H, Tyr_2^{NH}), 8.43 – 8.35 (m, 2H, H8-CoA, Asn_3^{NH}), 8.24 – 8.09 (m, 4H, H2-CoA Glu^{NH}, N***H***CH_2CH_2S-CoA, NH_2), 7.93 (d, 1H, ^{3}J = 7.5 Hz, Hpg^{NH}), 7.79 (m, 1H, N***H***CH_2CH_2CO-CoA), 7.29 (bs, 2H, NH_2), 7.20 – 7.15 (m, 2H, H_{ar}), 7.05 – 6.96 (m, 4H, H_{ar}), 6.89 – 6.83 (m, 2H, H_{ar}), 6.71 – 6.52 (m, 8H, H_{ar}), 5.94 (d, 1H, ^{3}J = 5.3 Hz, H1'-CoA), 5.55 – 5.46 (m, 2H, 2 x Hpg^{α}), 4.89 (m, 1H, H3'-CoA), 4.71 (m, 1H, H2'-CoA), 4.62 (m, 1H, Asn_3^{α}), 4.48 (m, Tyr_2^{α}), 4.39 – 4.31 (m, 2H, H4'-CoA, Tyr_6^{α}), 4.11 (m, 2H, H5'-CoA), 3.86 – 3.79 (m, under water peak, {3.80} OC***H***$_2^{a}$C$(CH_3)_2$-CoA, Leu_1^{α}), 3.62 (m, under water peak, C***H***(OH)CO-CoA), 3.49 – 3.47 (under water peak, OC***H***$_2^{b}$C$(CH_3)_2$-CoA), 3.22 (under water peak, NHC***H***$_2$$CH_2$CO-CoA), 3.15 – 3.02 (m, NHC***H***$_2$$CH_2$S-CoA), 2.93 – 2.60 (m, 6H, $NHCH_2$C***H***$_2$S-CoA, Tyr_6 $^{\beta}$, Tyr_2^{β}), 2.51 - 2.27 (m, under DMSO signal, Asn_3^{β}, $NHCH_2$C***H***$_2$CO-CoA), 1.65 – 1.54 (m, 1H, Leu_1 $^{\gamma}$), 1.54 – 1.46 (m, 2H, Leu_1^{β}), 0.94, 0.75 (s, 2 x 3H, 2 x gem-CH_3-CoA), 0.87 – 0.81 (m, 6H, 2 x Leu_1^{δ}) ppm.

Numbering of Coenzyme A accords to reference [1].

HRMS (ESI): *m/z* calc. for $C_{65}H_{83}N_{14}O_{27}P_3S$ $(M-2H)^{2-}$ 808.22483, found: 808.22371, Δ = 1.4 ppm; calc. mass for $C_{65}H_{82}N_{14}NaO_{27}P_3S$ $(M-3H+Na)^{2-}$ 819.21580, found: 819.21462, Δ = 1.4 ppm; calc. mass for $C_{65}H_{82}KN_{14}O_{27}P_3S$ $(M-3H+K)^{2-}$ 827.20277, found: 827.19704, Δ = 6.9 ppm.

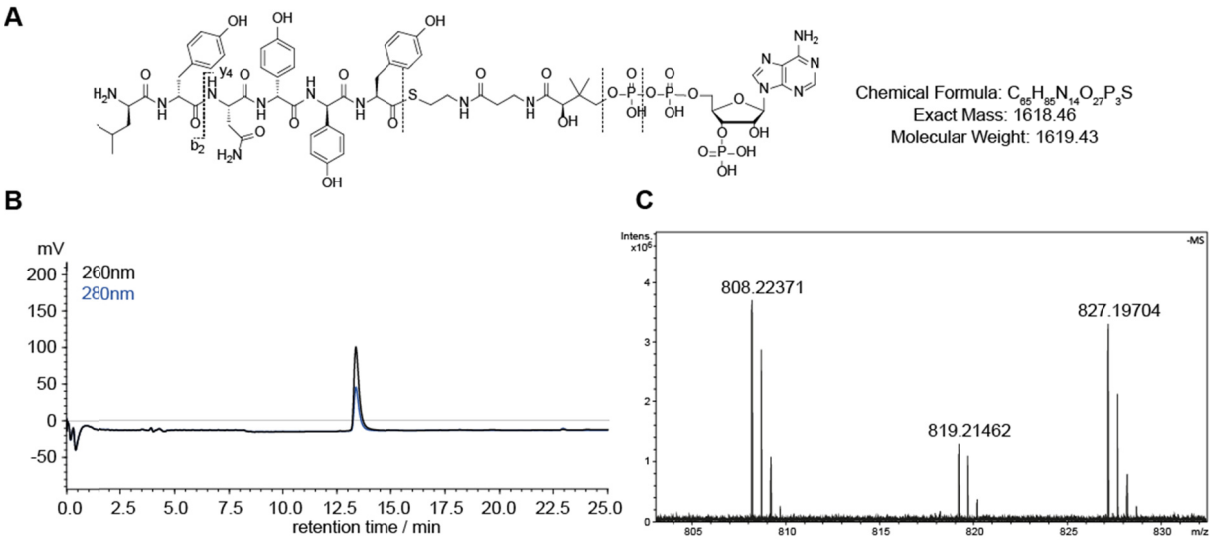

Figure S3. A) Structure of peptide-**1**-CoA, the observed MS fragmentation pattern is marked. B) Analytical reversed-phase HPLC trace, (C) HRMS spectra of HPLC-purified peptide-**1**-CoA (ions are doubly charged and correspond to the protonated, sodiated and potasiated species).

Peptide-2-MPAA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d$_6$): δ = 8.81 (d, 1H, ^{3}J = 7.4 Hz, Hpg^{NH}), 8.76 (d, 1H, ^{3}J = 7.0 Hz, Tyr$_6^{NH}$), 8.48 (d, 1H, ^{3}J = 6.7 Hz, Ala$_1^{NH}$), 8.29 (d, 1H, ^{3}J = 6.6 Hz, Glu^{NH}), 7.64 (d, 1H, ^{3}J = 7.0 Hz, Hpg^{NH}), 7.48 – 7.44 (m, 2H, H$_{ar}$), 7.30 – 7.24 (m, 3H, H$_{ar}$, Tyr$_2^{NH}$), 7.10 – 6.95 (m, 6H, H$_{ar}$,), 6.89 – 6.84 (m, 2H, H$_{ar}$), 6.67 – 6.57 (m, 8H, H$_{ar}$), 5.36 (d, 1H, ^{3}J = 6.8 Hz, Hpg$^{\alpha}$), 5.11 (d, 1H, ^{3}J = 7.41 Hz, Hpg$^{\alpha}$), 4.40 (m, 1H, Tyr$_2^{\alpha}$), 4.22 – 4.11 (m, 2H, Glu$_3^{\alpha}$, Tyr$_6^{\alpha}$), 3.94 (m, 1H, Ala$_1^{\alpha}$), 3.55 (s, 2H, CH$_2$-MPAA), 3.01 – 2.91 (m, 1H, Tyr$_2^{\beta a}$), 2.88 – 2.80 (m, 1H, Tyr$_2^{\beta b}$), 2.74 – 2.63 (m, 2H, Tyr6$^{\beta}$), 2.23 – 2.02 (m, 2H, Glu$_3$ $^{\gamma}$), 1.81 – 1.69 (m, 2H, Glu$_3^{\beta}$), (d, 3H, ^{3}J = 7.3 Hz, Ala$_1^{\beta}$) ppm.

HRMS (ESI): *m/z* calc. for $C_{50}H_{53}N_6O_{14}S$ $(M+H)^+$ 993.33350, found: 993.33359, Δ = 0.1 ppm; calc. mass for $C_{50}H_{52}N_6NaO_{14}S$ $(M+Na)^+$ 1015.31544, found: 1015.31681, Δ = 1.3 ppm.

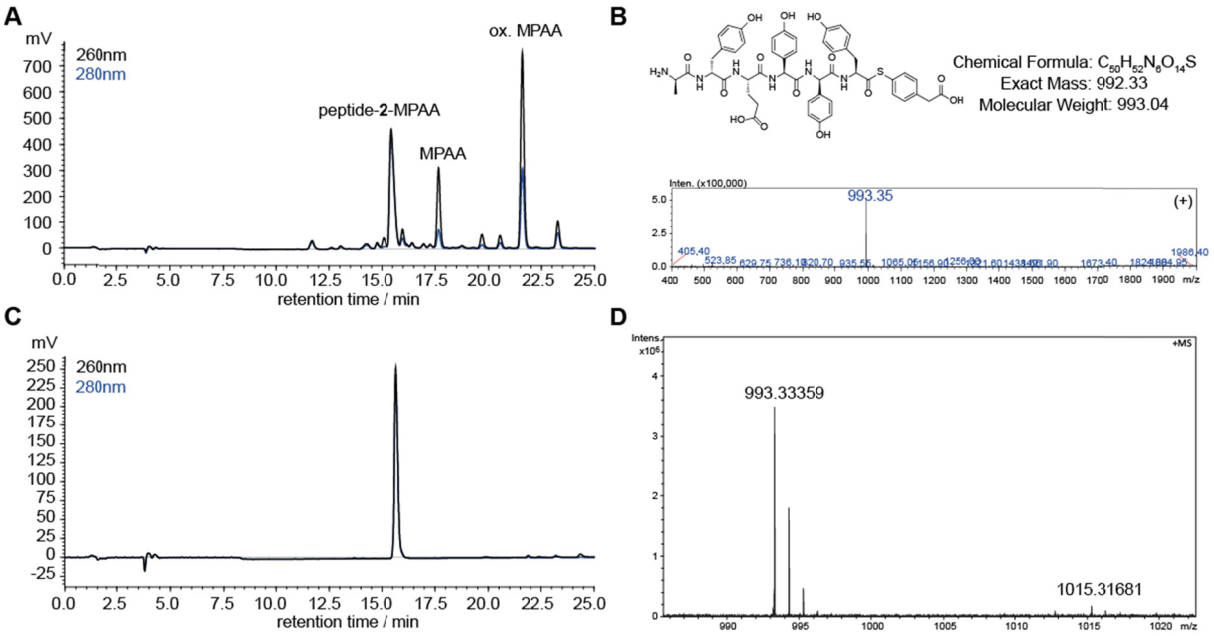

Figure S4. A) Analytical reversed-phase HPLC trace and (B) MS spectra of crude peptide-**2**-MPAA. C) Analytical reversed-phase HPLC trace and (D) HRMS spectra of HPLC-purified peptide-**2**-MPAA.

Peptide-2-CoA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): δ = 8.88 (d, 1H, ^{3}J = 7.8 Hz, Tyr_6^{NH}), 8.75 (d, 1H, ^{3}J = 8.3 Hz, Hpg^{NH}), 8.54 (d, 1H, ^{3}J = 7.4 Hz, Tyr_2^{NH}), 8.41 (s, 1H, H8-CoA), 8.28 – 8.19 (m, 3H, Glu^{NH}, Hpg^{NH}, N***H***CH_2CH_2S-CoA), 8.17 (s, 1H, H2-CoA), 8.09 (bs, 2H, NH_2), 7.79 (m, 1H, N***H***CH_2CH_2CO-CoA), 7.32 (bs, 2H, NH_2), 7.22 – 7.14 (m, 2H, H_{ar}), 7.10 – 6.93 (m, 4H, H_{ar}), 6.91 – 6.83 (m, 2H, H_{ar}), 6.73 – 6.53 (m, 8H, H_{ar}), 5.94 (d, 1H, ^{3}J = 5.3 Hz, H1'-CoA), 5.55 (d, 1H, ^{3}J = 7.8 Hz, Hpg^{α}), 5.50 (d, 1H, ^{3}J = 8.6 Hz, Hpg^{α}), 4.88 (m, 1H, H3'-CoA), 4.71 (m, 1H, H2'-CoA), 4.52 (m, Tyr_2^{α}), 4.39 – 4.32 (m, H4'-CoA, Glu_3^{α}, Tyr_6^{α}), 4.15 – 4.09 (m, H5'-CoA), 3.86 – 3.76 (m, under water peak, OC***H***$_2^{a}$C$(CH_3)_2$-CoA, {3.77} Ala_1^{α}), 3.62 (m, under water peak, C***H***(OH)CO-CoA), 3.50 – 3.48 (m, under water peak, OC***H***$_2^{b}$C$(CH_3)_2$-CoA), 3.34 – 3.26 (m, under water peak, NHC***H***$_2$$CH_2$CO-CoA), 3.16 – 3.07 (m, 2H, NHC***H***$_2$$CH_2$S-CoA), 2.94 – 2.61 (m, 6H, $NHCH_2$C***H***$_2$S-CoA, Tyr_6^{β}, Tyr_2^{β}), 2.30 (m, 2H, $NHCH_2$C***H***$_2$CO-CoA), 2.10 – 2.01 (m, 2H, Glu_3^{γ}), 1.85 – 1.76 (m, 1H, $Glu_3^{\beta a}$), 1.71 – 1.62 (m, 1H, $Glu_3^{\beta b}$), 1.33 (d, 3H, ^{3}J = 6.7 Hz, Ala_1^{β}), 0.94, 0.75 (s, 2 x 3H, 2 x gem-CH_3-CoA) ppm.

Numbering of Coenzyme A accords to reference [1].

HRMS (ESI): *m/z* calc. for $C_{63}H_{78}N_{13}O_{28}P_3S$ $(M-2H)^{2-}$ 794.70119, found: 794.69846, Δ = 3.4 ppm; calc. mass for $C_{63}H_{77}N_{13}NaO_{28}P_3S$ $(M-3H+Na)^{2-}$ 805.69216, found: 805.69386, Δ = 2.1 ppm; calc. mass for $C_{63}H_{77}KN_{13}O_{28}P_3S$ $(M-3H+K)^{2-}$ 813.67913, found: 813.67634, Δ = 3.4 ppm.

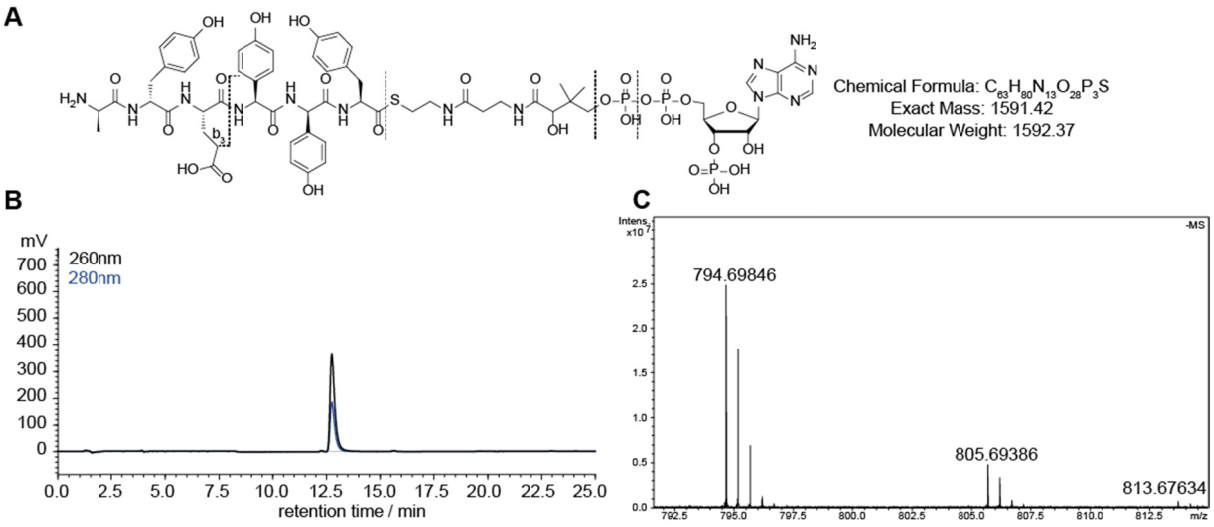

Figure S5. A) Structure of peptide-**2**-CoA, the observed MS fragmentation pattern is marked. B) Analytical reversed-phase HPLC trace, (C) HRMS spectra of HPLC-purified peptide-**2**-CoA (ions are doubly charged and correspond to the protonated, sodiated and potasiated species).

Peptide-3-MPAA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): 8.95 (d, 1H, ^{3}J = 7.0 Hz, Phe_3^{NH}), 8.86 (d, 1H, ^{3}J = 8.0 Hz, Hpg^{NH}), 8.73 (d, 1H, ^{3}J = 6.1 Hz, Tyr_6^{NH}), 8.58 (d, 1H, ^{3}J = 7.3 Hz, Hpg^{NH}), 8.38 (d, 1H, ^{3}J = 6.4 Tyr_2^{NH}), 7.39 – 7.09 (m, 8H, H_{ar},), 7.06 – 6.82 (m, 6H, H_{ar}), 6.70 – 6.39 (m, 11H, H_{ar}), 6.63(d, 1H, ^{3}J = 7.3 Hz, Hpg^{α}), 5.55 (d, 1H, ^{3}J = 8.0 Hz, Hpg^{α}), 4.46 (m, 2H, Phe^{α}, Tyr_2^{α}), 4.32 (m, 1H, Tyr_6^{α}), 3.61 (s, under water peak, CH_2-MPAA), 2.98 – 2.86 (m, 2H, 2 x $Tyr^{\beta a}$), 2.79 – 2.62 (m, 2H, 2 x $Tyr^{\beta b}$), 2.53 – 2.37 (m, under DMSO peak, Phe_3^{β}) ppm.

HRMS (ESI): *m/z* calc. for $C_{59}H_{57}N_6O_{13}S$ $(M+H)^+$ 1089.36988, found: 1089.36925, Δ = 0.6 ppm; calc. mass for $C_{59}H_{56}N_6NaO_{13}S$ $(M+Na)^+$ 1111.35183, found: 1111.35144, Δ = 0.3 ppm.

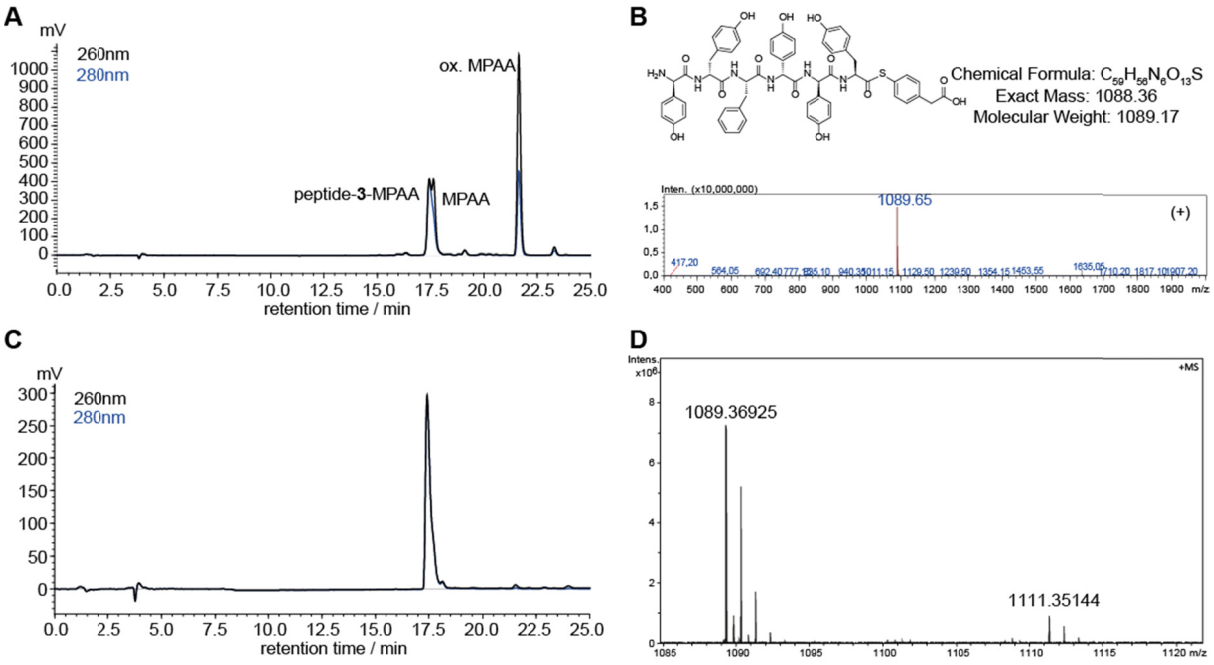

Figure S6. A) Analytical reversed-phase HPLC trace and (B) MS spectra **o**f crude peptide-**3**-MPAA. C) Analytical reversed-phase HPLC trace and (D) HRMS spectra of HPLC-purified peptide-**3**-MPAA.

Peptide-3-CoA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): δ = 8.86 (d, 1H, ^{3}J = 7.6 Hz, Tyr_6^{NH}), 8.76 (d, 1H, ^{3}J = 8.3 Hz, Hpg^{NH}), 8.50 (d, 1H, ^{3}J = 7.8 Hz, Hpg^{NH}), 8.40 (s, 1H, H8-CoA), 8.38 – 8.20 (m, 5H, Tyr_2^{NH}, Phe_3^{NH}, NH_2, N***H***CH_2CH_2S-CoA), 8.17 (s, 1H, H2-CoA), 7.77 (m, 1H, N***H***CH_2CH_2CO-CoA), 7.27 – 7.04 (m, 10H, H_{ar}), 7.02 – 6.83 (m, 3H, H_{ar}), 6.81 – 6.72 (m, 3H, H_{ar}), 6.73 – 6.59 (m, 5H, H_{ar}), 6.58 – 6.51 (m, 4H, H_{ar}), 5.94 (d, 1H, ^{3}J = 5.4 Hz, H1'-CoA), 5.57 - 5.52 (m, 2H, 2 x Hpg^{α}), 4.89 (m, 1H, H3'-CoA), 4.74 – 4.63 (m, 2H, H2'-CoA, Tyr_2^{α}), 4.46 (m, 1H, Phe_3^{α}), 4.41 – 4.31 (m, 2H, H4'-CoA, Tyr_6^{α}), 4.15 – 4.07 (m, 2H, H5'-CoA), 3.89 – 3.77 (m, under water peak, OC***H***$_2^{a}$C$(CH_3)_2$-CoA, Hpg_1^{α}), under water peak (C***H***(OH)CO-CoA, OC***H***$_2^{b}$C$(CH_3)_2$-CoA), 3.34 – 3.27 (m, under water peak, NHC***H***$_2CH_2$CO-CoA), 3.10 – 3.08 (m, 2H, NHC***H***$_2CH_2$S-CoA), 2.94 – 2.59 (m, 6H, $NHCH_2$C***H***$_2$S-CoA, Tyr_6 $^{\beta}$, Tyr_2^{β}), 2.36 – 2.23 (m, 2H, $NHCH_2$C***H***$_2$CO-CoA), 0.94, 0.75 (s, 2 x 3H, 2 x gem-CH_3-CoA) ppm.

Numbering of Coenzyme A accords to reference [1].

HRMS (ESI): *m/z* calc. for $C_{72}H_{82}N_{13}O_{27}P_3S$ $(M-2H)^{2-}$ 842.71938, found: 842.719581, Δ = 0.2 ppm; calc. mass for $C_{72}H_{82}N_{13}NaO_{27}P_3S$ $(M-3H+Na)^{2-}$ 853.71035, found: 853.70514, Δ = 6.1 ppm; calc. mass for $C_{72}H_{82}KN_{13}O_{27}P_3S$ $(M-3H+K)^{2-}$ 861.69732, found: 861.69375, Δ = 4.1 ppm.

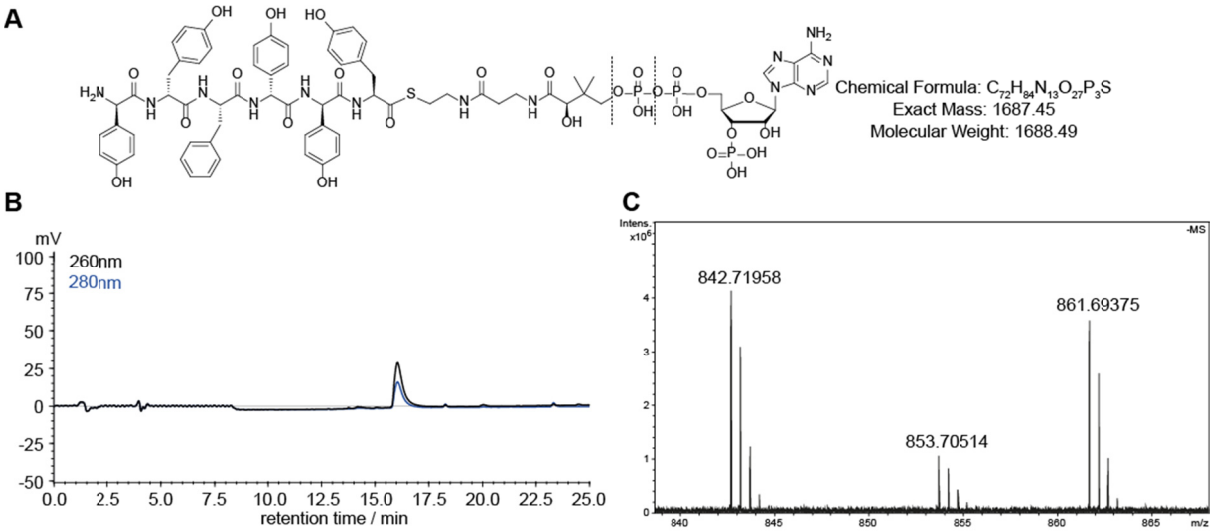

Figure S7. A) Structure of peptide-**3**-CoA, the observed MS fragmentation pattern is marked. B) Analytical reversed-phase HPLC trace, (C) HRMS spectra of HPLC-purified peptide-**3**-CoA (ions are doubly charged and correspond to the protonated, sodiated and potasiated species).

Peptide-4-MPAA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): 8.96 (d, 1H, ^{3}J = 6.7 Hz, Hpg^{NH}), 8.68 (d, 1H, ^{3}J = 7.0 Hz, Tyr$_6^{NH}$), 8.64 (d, 1H, ^{3}J = 6.9 Hz, Hpg^{NH}), 8.47 (d, 1H, ^{3}J = 6.7 Hz, Hpg^{NH}), 7.77 (d, 1H, ^{3}J = 7.0 Hz, Hpg^{NH}), 7.48 – 7.44 (m, 2H, H$_{ar}$, Tyr$_2^{NH}$), 7.33 – 7.14 (m, 3H, H$_{ar}$,), 7.10 – 6.94 (m, 8H, H$_{ar}$), 6.90 – 6.78 (m, 4H, H$_{ar}$), 6.72 – 6.53(m, 13H, H$_{ar}$), 5.39 (d, 1H, ^{3}J = 7.0 Hz, Hpg$^{\alpha}$), 5.35 (d, 1H, ^{3}J = 6.8 Hz, Hpg$^{\alpha}$), 5.05 (d, 1H, ^{3}J = 7.0 Hz, Hpg$^{\alpha}$), 4.99 (d, 1H, ^{3}J = 6.8 Hz, Hpg$^{\alpha}$), 4.65 (m, 1H, Tyr$_2^{\alpha}$), 4.32 (m, 1H, Tyr$_6^{\alpha}$), 3.54 (s, under water peak, CH_2-MPAA), 2.92 – 2.80 (m, 2H, Tyr$_2^{\beta}$), 2.78 – 2.60 (m, 2H, Tyr$_6^{\beta}$), ppm.

HRMS (ESI): *m/z* calc. for $C_{58}H_{55}N_6O_{14}S$ $(M+H)^+$ 1091.34915, found: 1091.34881, Δ = 0.3 ppm; calc. mass for $C_{58}H_{55}N_6NaO_{14}S$ $(M+Na)^+$ 1113.33109, found: 1113.33073, Δ = 0.3 ppm; calc. mass for $C_{58}H_{55}KN_6O_{14}S$ $(M+K)^+$ 1129.30503, found: 1129.30455, Δ = 0.4 ppm.

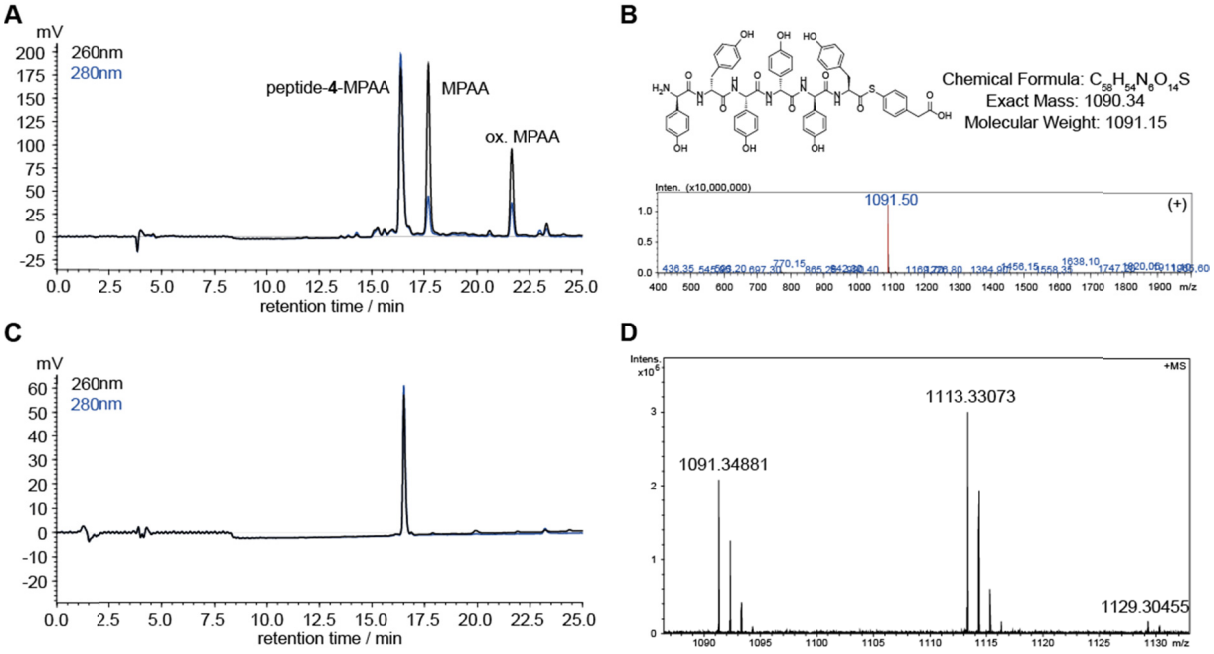

Figure S8. A) Analytical reversed-phase HPLC trace and (B) MS spectra **o**f crude peptide-**4**-MPAA. C) Analytical reversed-phase HPLC trace and (D) HRMS spectra of HPLC-purified peptide-**4**-MPAA.

Peptide-4-CoA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): δ = 8.86 (d, 1H, ^{3}J = 7.8 Hz, Tyr_6^{NH}), 8.74 (d, 1H, ^{3}J = 8.3 Hz, Hpg^{NH}), 8.57 (d, 1H, ^{3}J = 7.5 Hz, Hpg^{NH}), 8.47 – 8.32 (m, 5H, {8.44} Tyr_2^{NH}, {8.40} H8-CoA, Hpg^{NH}, NH_2), 8.23 (m, 1H, N***H***CH_2CH_2S-CoA), 8.17 (s, 1H, H2-CoA), 7.81 (m, 1H, N***H***CH_2CH_2CO-CoA), 7.34 – 7.20 (m, 4H, H_{ar}), 7.07 – 6.94 (m, 7H, H_{ar}), 6.87 (m, 2H, H_{ar}), 6.79 (m, 2H, H_{ar}), 6.66 – 6.53 (m, 9H, H_{ar}), 5.94 (d, 1H, ^{3}J = 5.3 Hz, H1'-CoA), 5.56 – 5.48 (m, 3H, 3 x Hpg^{α}), 4.89 (m, 1H, H3'-CoA), 4.70 (m, 1H, H2'-CoA), 4.65 (m, Tyr_2^{α}), 4.37 – 4.29 (m, 2H, H4'-CoA, Tyr_6^{α}), 4.11 (m, 2H, H5'-CoA), 3.87 – 3.78 (m, under water peak, {3.85} OC***H***$_2^{a}$C$(CH_3)_2$-CoA, H**p**g_1^{α}), 3.59 (m, under water peak, C***H***(OH)CO-CoA), 3.46 (m, under water peak, OC***H***$_2^{b}$C$(CH_3)_2$-CoA), 3.35 – 3.28 (m, under water peak, NHC***H***$_2$$CH_2$CO-CoA), 3.15 – 3.07 (m, 2H, NHC***H***$_2$$CH_2$S-CoA), 2.94 – 2.62 (m, 6H, $NHCH_2$C***H***$_2$S-CoA, Tyr_6^{β}, Tyr_2^{β}), 2.34 – 2.26 (m, 2H, $NHCH_2$C***H***$_2$CO-CoA), 0.94, 0.75 (s, 2 x 3H, 2 x gem-CH_3-CoA) ppm.

Numbering of Coenzyme A accords to reference [1].

HRMS (ESI): *m/z* calc. for $C_{71}H_{80}N_{13}O_{28}P_3S$ $(M-2H)^{2-}$ 843.70901, found: 843.70823, Δ = 0.9 ppm; calc. mass for $C_{71}H_{79}N_{13}NaO_{28}P_3S$ $(M-3H+Na)^{2-}$ 854.69999, found: 854.69979, Δ = 0.2 ppm; calc. mass for $C_{71}H_{78}N_{13}Na_2O_{28}P_3S$ $(M-3H+K)^{2-}$ 865.69096, found: 865.69083, Δ = 0.1 ppm.

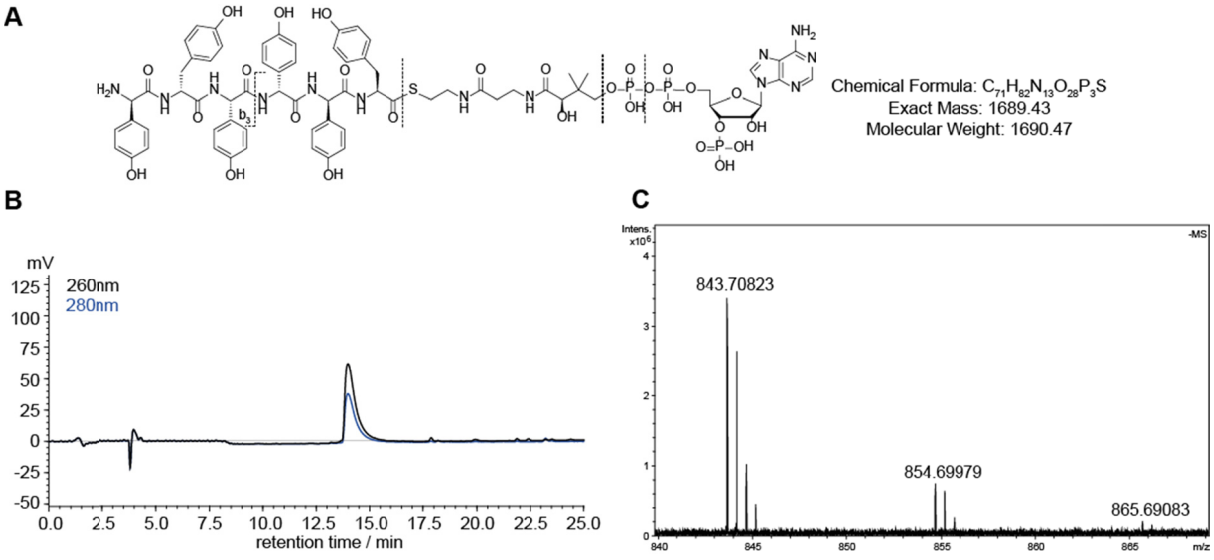

Figure S9. A) Structure of peptide-**4**-CoA, the observed MS fragmentation pattern is marked. B) Analytical reversed-phase HPLC trace, (C) HRMS spectra of HPLC-purified peptide-**4**-CoA (ions are doubly charged and correspond to the protonated, sodiated and potasiated species).

Peptide-5-MPAA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): δ = 10.76 (s, 1H, $Trp_2^{IndolNH}$), 8.96 (d, 1H, ^{3}J = 8.0 Hz, Tyr_6^{NH}), 8.72 (d, 1H, ^{3}J = 8.2 Hz, Hpg^{NH}), 8.60 (d, 1H, ^{3}J = 8.0 Hz, Hpg^{NH}), 8.56 (d, 1H, ^{3}J = 7.7 Hz, Hpg^{NH}), 8.50 (m, 1H, Trp_2^{NH}), 7.64 (d, 1H, ^{3}J = 7.7 Hz, $Trp_2^{IndolH4}$), 7.36 – 7.30 (m, 3H, H_{ar}, $Trp_2^{IndolH7}$), 7.27 – 7.18 (m, 6H, H_{ar}), 7.14 (s, 1H, $Trp_2^{IndolH2}$), 7.06 – 6.89 (m, 8H, H_{ar}, $Trp_2^{IndolH6}$, $Trp_2^{IndolH5}$), 6.70 – 6.55 (m, 10H, H_{ar}), 5.64 (d, 1H, ^{3}J = 7.7 Hz, Hpg^{α}), 5.55 (d, 1H, ^{3}J = 8.0 Hz, Hpg^{α}), 5.52 (d, 1H, ^{3}J = 8.5 Hz, Hpg^{α}), 4.76 (m, 1H, Trp_2^{α}), 4.48 (m, 1H, Tyr_6^{α}), 3.68 (bs, 1H, Tyr_6^{α}), 3.63 (s, 2H, CH_2-MPAA), 3.19 – 3.12 (m, 1H, $Trp_2^{\beta a}$), 3.03 – 2.89 (m, 3H, 2 x $Tyr^{\beta a}$, $Trp_2^{\beta b}$), 2.78 – 2.61 (m, 2H, 2 x $Tyr^{\beta b}$) ppm.

HRMS (ESI): *m/z* calc. for $C_{61}H_{58}N_7O_{13}S$ $(M+H)^+$ 1128.38078, found: 1128.38111, Δ = 0.3 ppm; calc. mass for $C_{61}H_{57}N_7NaO_{13}S$ $(M+Na)^+$ 1150.36273, found: 1150.36331, Δ = 0.5 ppm.

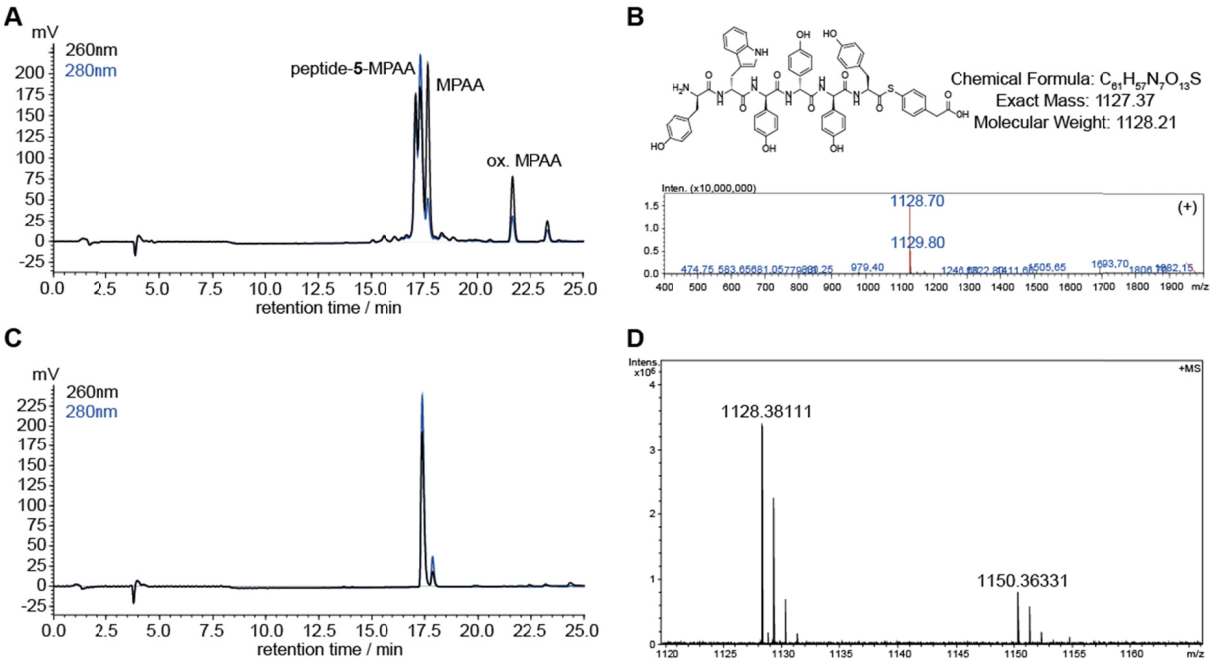

Figure S10. A) Analytical reversed-phase HPLC trace and (B) MS spectra of crude peptide-**5**-MPAA. C) Analytical reversed-phase HPLC trace and (D) HRMS spectra of HPLC-purified peptide-**5**-MPAA.

Peptide-5-CoA

^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): δ = 10.82 (s, 1H, $Trp_2^{IndolNH}$), 8.86 (d, 1H, ^{3}J = 8.0 Hz, Tyr_6^{NH}), 8.76 (d, 1H, ^{3}J = 8.2 Hz, Hpg^{NH}), 8.66 (d, 1H, ^{3}J = 7.5 Hz, Trp_2^{NH}), 8.60 (d, 1H, ^{3}J = 8.0 Hz, Hpg^{NH}), 8.56 (d, 1H, ^{3}J = 7.7 Hz, Hpg^{NH}), 8.39 (s, 1H, H8-CoA), 8.26 – 8.21 (m, 1H, N***H***CH_2CH_2S-CoA), 8.16 (s, 1H, H2-CoA), 8.00 (bs, 2H, NH_2), 7.84 – 7.79 (m, 1H, N***H***CH_2CH_2CO-CoA), 7.60 (d, 1H, ^{3}J = 8.0 Hz, $Trp_2^{IndolH4}$), 7.32 (d, 1H, ^{3}J = 8.1 Hz, $Trp_2^{IndolH7}$), 7.26 (bs, 2H, NH_2), 7.12 (s, 1H, $Trp_2^{IndolH2}$), 7.07 – 6.94 (m, 10 H, H_{ar}, {6.94} $Trp_2^{IndolH5}$, {7.04} $Trp_2^{IndolH6}$), 6.89 – 6.85 (m, 2H, H_{ar}), 6.72 – 6.68 (m, 2H, H_{ar}), 6.64 – 6.58 (m, 6H, H_{ar}), 6.57 – 6.52 (m, 2H, H_{ar}), 5.93 (d, 1H, ^{3}J = 5.4 Hz, H1'-CoA), 5.54 – 5.49 (m, 3H, 3 x Hpg^{α}), 4.91 (m, 1H, H3'-CoA), 4.82 (m, 1H, Trp_2^{α}), 4.70 (m, 1H, H2'-CoA), 4.37 – 4.31 (m, 3H, H4'-CoA, Tyr_6^{α}), 4.15 – 4.08 (m, 2H, H5'-CoA), 3.87 – 3.79 (m, under water peak, OC***H***$_2^{a}$C$(CH_3)_2$-CoA, Tyr_1^{α}), 3.45 (m, under water peak, OC***H***$_2^{b}$C$(CH_3)_2$-CoA) 3.34 – 3.31 (m, under water peak, C***H***(OH)CO-CoA, NHC***H***$_2CH_2$CO-CoA), 3.12 – 3.00 (m, under water peak, NHC***H***$_2CH_2$S-CoA, $Trp_2^{\beta a}$), 2.98 – 2.63 (m, $NHCH_2$C***H***$_2$S-CoA, $Tyr^{\beta a,b}$, $Trp_2^{\beta b}$), 2.33 – 2.78 (m, 2H, $NHCH_2$C***H***$_2$CO-CoA), 0.94, 0.74 (s, 2 x 3H, 2 x gem-CH_3-CoA) ppm.

Numbering of Coenzyme A accords to reference [1].

HRMS (ESI): *m/z* calc. for $C_{74}H_{83}N_{13}O_{27}P_3S$ $(M\text{-}2H)^{2-}$ 862.22483, found: 862.22339, Δ = 1.7 ppm; calc. mass for $C_{74}H_{82}N_{13}NaO_{27}P_3S$ $(M\text{-}3H+Na)^{2-}$ 873.21580, found: 873.20838, Δ = 8.5 ppm; calc. mass for $C_{74}H_{82}KN_{13}O_{27}P_3S$ $(M\text{-}3H+K)^{2-}$ 881.20277, found: 881.19707, Δ = 6.5 ppm.

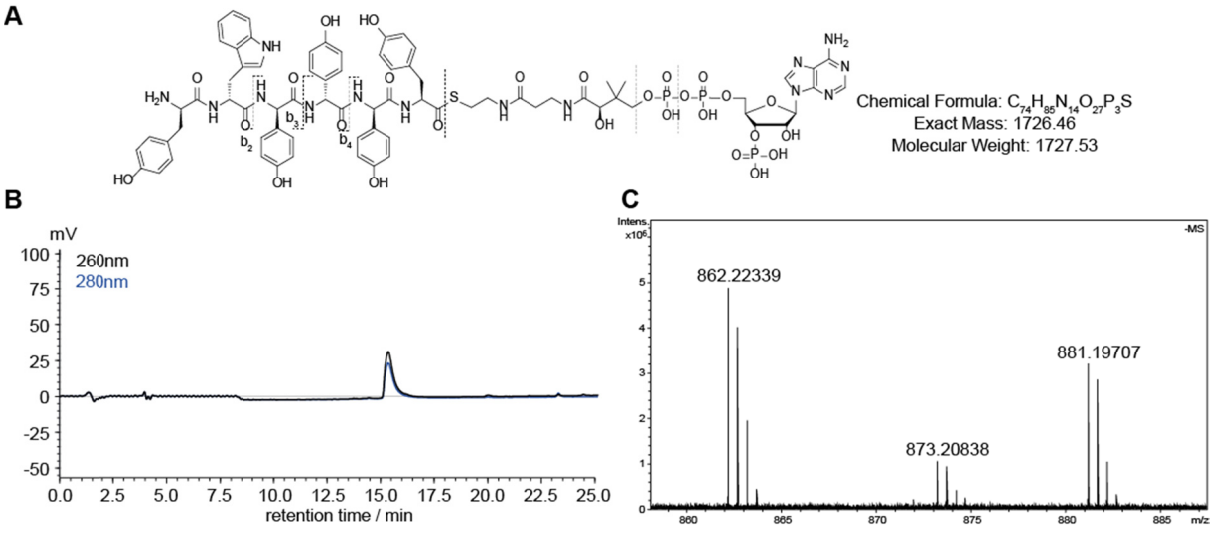

Figure S11. A) Structure of peptide-**5**-CoA, the observed MS fragmentation pattern is marked. B) Analytical reversed-phase HPLC trace, (C) HRMS spectra of HPLC-purified peptide-**5**-CoA (ions are doubly charged and correspond to the protonated, sodiated and potasiated species).

3. Primers used in the cloning of $OxyB_{van}$ and PCP domains

Table S2. Overview of primers used for cloning.

Protein	Protein ID	Gene name	Selected sequence range	MW [kDa]	Primer 5' to 3' (restriction sites underlined)	
$OxyB_{van}$	Q8RN04	*oxyB*	1-398	47.7	for	CGGCAGCCATATGAGCGAGGACGACCCG
					rev	CAGTCGCTAGCCCAAGCAACCATCAGCTCGGTC
GB1-$PCP7_{cep}$	O52821	-	963-1041	18.9	for	TATCCATGGCGAGCGAGAAAGCGCCCGAGAACG
					rev	ATAACTCGAGGGATTTGGCGGCCAGAGCAC
Trx-$PCP7_{sta}$	Q8KLL6	-	966-1041	22.9	for	GCTACCATGGCAACCAGCGAAAAAGCAC
					rev	GCTACTCGAGGCTTGCTTTTGCTGCCAGTGCACG
Trx-$PCP7_{com}$	Q93N86	-	954-1029	23.5	for	ATATTCCATGGTTGCAGGTCGTGCACCG
					rev	AATTACTCGAGACCAGTCTCGGGCAGGCTTGCTTTTGCTGCCAGAAC

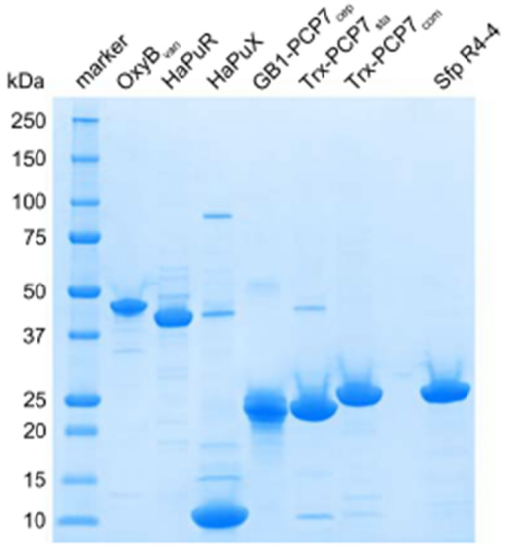

Figure S12. Electrophoretic analysis of proteins used in this study resolved using a 4-20% gradient SDS-PAGE gel (4–20% Mini-PROTEAN® TGX™ Gel (Bio-Rad)). Conditions: 2.5 µg of $OxyB_{van}$ and HaPuR, 5 µg of HaPuX, GB1-$PCP7_{cep}$, Trx-$PCP7_{sta}$, Trx-$PCP7_{com}$, Sfp R4-4. As marker "Precision Plus Protein™ All Blue Standards" (Bio-Rad) was used; staining was performed using Instant Blue (Expedeon).

4. CD spectra and melting temperature determination of GB1-PCP7$_{cep}$, Trx-PCP7$_{sta}$ and Trx-PCP7$_{com}$

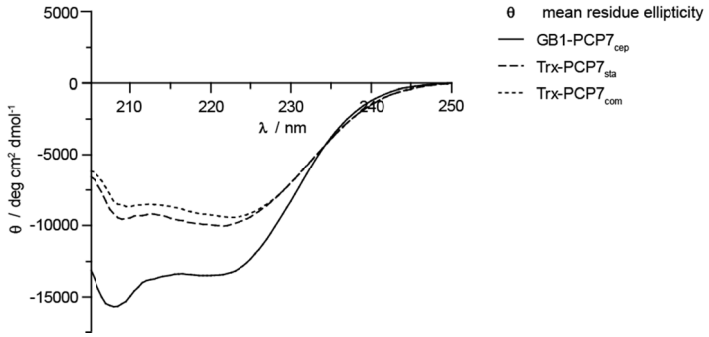

Figure S13. CD spectra of GB1-PCP7$_{cep}$, Trx-PCP7$_{sta}$ and Trx-PCP7$_{com}$. Data was recorded from λ=205 – 260 nm.

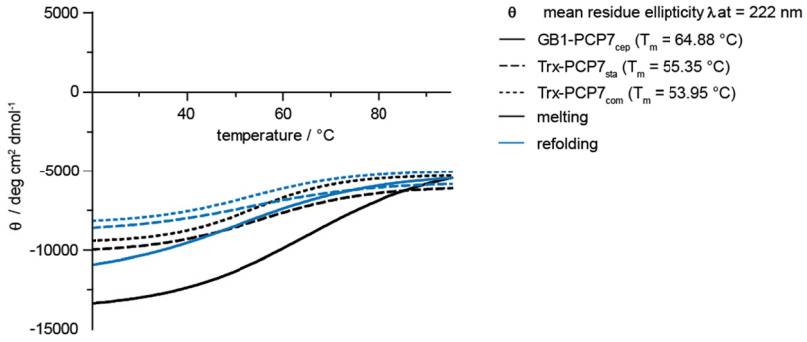

Figure S14. Melting curves of GB1-PCP7$_{cep}$, Trx-PCP7$_{sta}$ and Trx-PCP7$_{com}$. Data was recorded at λ=222 nm from 20 – 95 °C with a rate of 1 °C/min. Data was analyzed using Boltzmann sigmoidal fit and melting temperatures were obtained (GB1-PCP7$_{cep}$: T_m = 64.88 °C, Trx-PCP7$_{sta}$: T_m = 55.35 °C, Trx-PCP7$_{com}$: T_m = 53.95 °C). Melting curves are shown in black, refolding curves in blue.

5. Alignments of the PCP domains used in this study

A

```
PCP7cep -SEKAPENET EKVLCALFGE ILSVDQVGVD DTFNDLGGSS ALAMRLIARI REELGADLPI 59
PCP7sta TSEKAPESET EKVLCALYAE ILGVERVGVD DAFHDLGGSS ALAMRLIARI REEIGADLPI 60
PCP7com -AGRAPESET EQQMCTLFAE VLALDEVGAE DNFFELGGDS GLGMRLARRI REELGVDLPI 59

          : :***.** *: :*:*:.* :*.::.**.: * * :***.* .*.***  ** ***:*.****

PCP7cep RQLFSSPTPA GLARALAAKS - 79   } 83.95%      } 59.26%
PCP7sta RQLFSSPTPA GIARALAAKA S 81   }  identity   }  identity
PCP7com RQLFSAPTPA GVARVLAAKA S 80

        *****:**** *:**.****:
```

B

```
PCP7van SEKAPENETEKVLCALFAEILSVDQVGVDDAFQDLGGSSALAMRLVARIREELGADLPIR 60
PCP7cep SEKAPENETEKVLCALFGEILSVDQVGVDDTFNDLGGSSALAMRLIARIREELGADLPIR 60
        *****************.************:*:************:**************

PCP7van QLFSSPTPAGLARALAAKS 79
PCP7cep QLFSSPTPAGLARALAAKS 79
        ******************
```

Figure S15. A) Sequence alignment of carrier protein domains used in this study (without fusion partners). The Ser residue which bears the posttranslational phosphopantetheine-derived modification is highlighted in yellow. Predicted helical segments (from Jpred) are underlined. B) Sequence alignment of $PCP7_{van}$ (VpsC protein, Uniprot: G4V4R2) and $PCP7_{cep}$ exhibiting 94.94% sequence identity; differing amino acids are highlighted in red.

6. *In vitro* studies

6.1 Sfp-catalyzed loading of PCP domains

Table S3. MALDI results of peptide-loaded PCPs.

	GB1-PCP7$_{cep}$			**Trx-PCP7$_{sta}$**			**Trx-PCP7$_{com}$**		
	MH^+ calc.	mass observed	Δ[a]	MH^+ calc.	mass observed	Δ[a]	MH^+ calc.	mass observed	Δ[a]
***apo*-PCP**	18854	18827	-27	22804	22782	-22	23354	23334	-20
Peptide-1	20046	20021	-25	23996	23987	-9	24547	24536	-11
Peptide-2	20019	20008	-11	23969	23962	-7	24520	24512	-8
Peptide-3	20115	20093	-24	24065	24047	-18	24616	24597	-19
Peptide-4	20117	20096	-21	24067	24044	-23	24618	24593	-25
Peptide-5	20154	20134	-20	24104	24120	+16	24655	24667	+12

[a] Mass differences can be attributed to the presence of salt in each sample (50 mM Hepes, 50 mM NaCl, 10 mM $MgCl_2$) and to deviations due to the 3-point calibration of the mass spectrometer.

6.2 Results of turnover assays

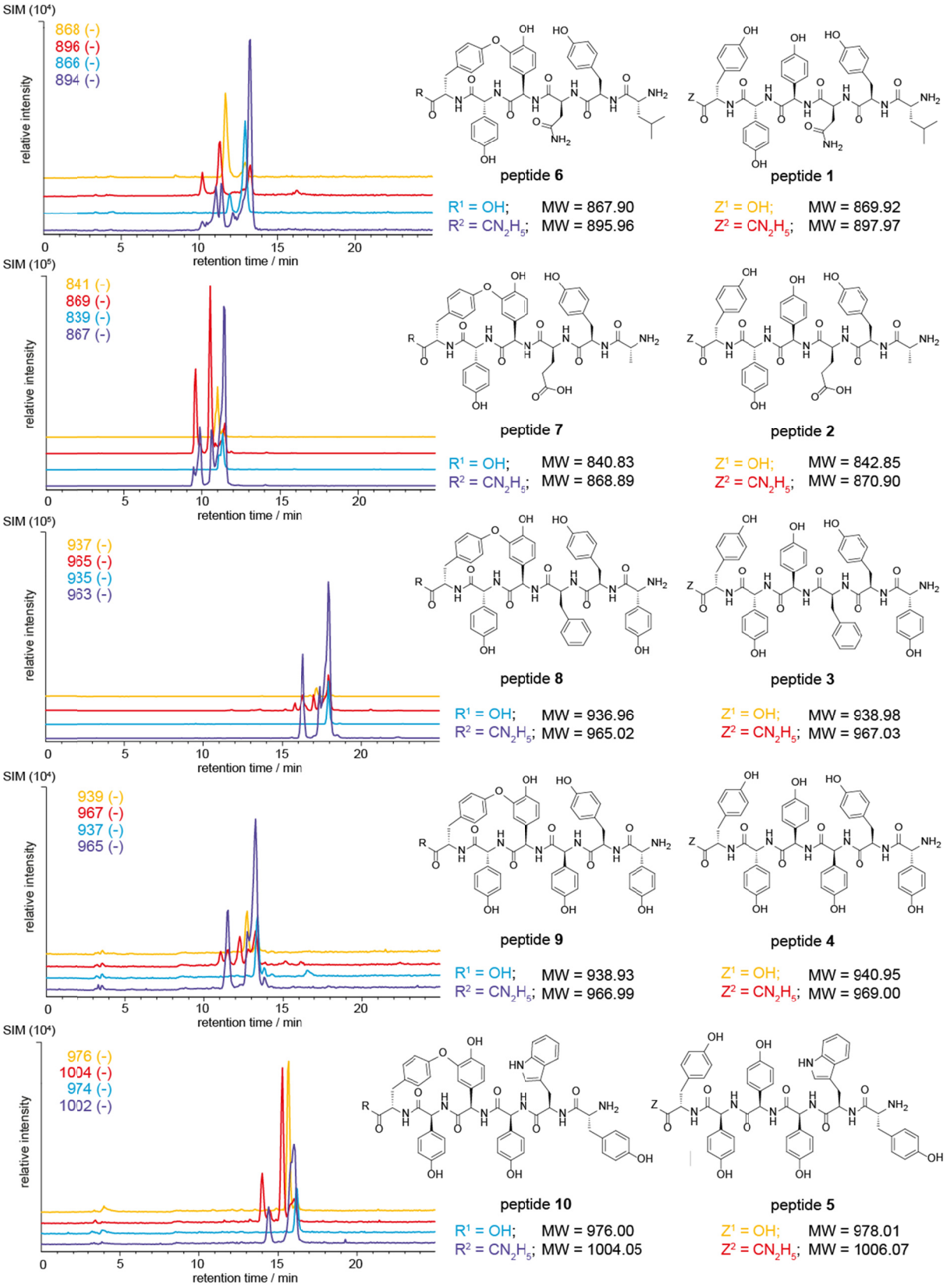

Figure S16. Representative HPLC-MS traces of linear (peptides **1**-**5**-Z) and crosslinked peptides (peptides **6**-**10**-R) isolated from turnover assays with peptides **1**-**5**. Through methylhydrazine

cleavage peptide-methylhydrazides (R^2, Z^2) as well as hydrolyzed peptides (R^1, Z^1) are generated; the later due to a concomitant rise in pH on methylhydrazine addition. For the peptide methylhydrazides two peaks are detected due to the generation of two regioisomers of the methylhydrazides. For the crosslinked peptides additional peaks can be detected, which can be ascribed to diastereomer formation due to enhanced racemization of crosslinked C-terminal tyrosine (such an observation has been previously reported). [2,3]

6.3 ESI-MS/MS characterization of peptides from turnover assays

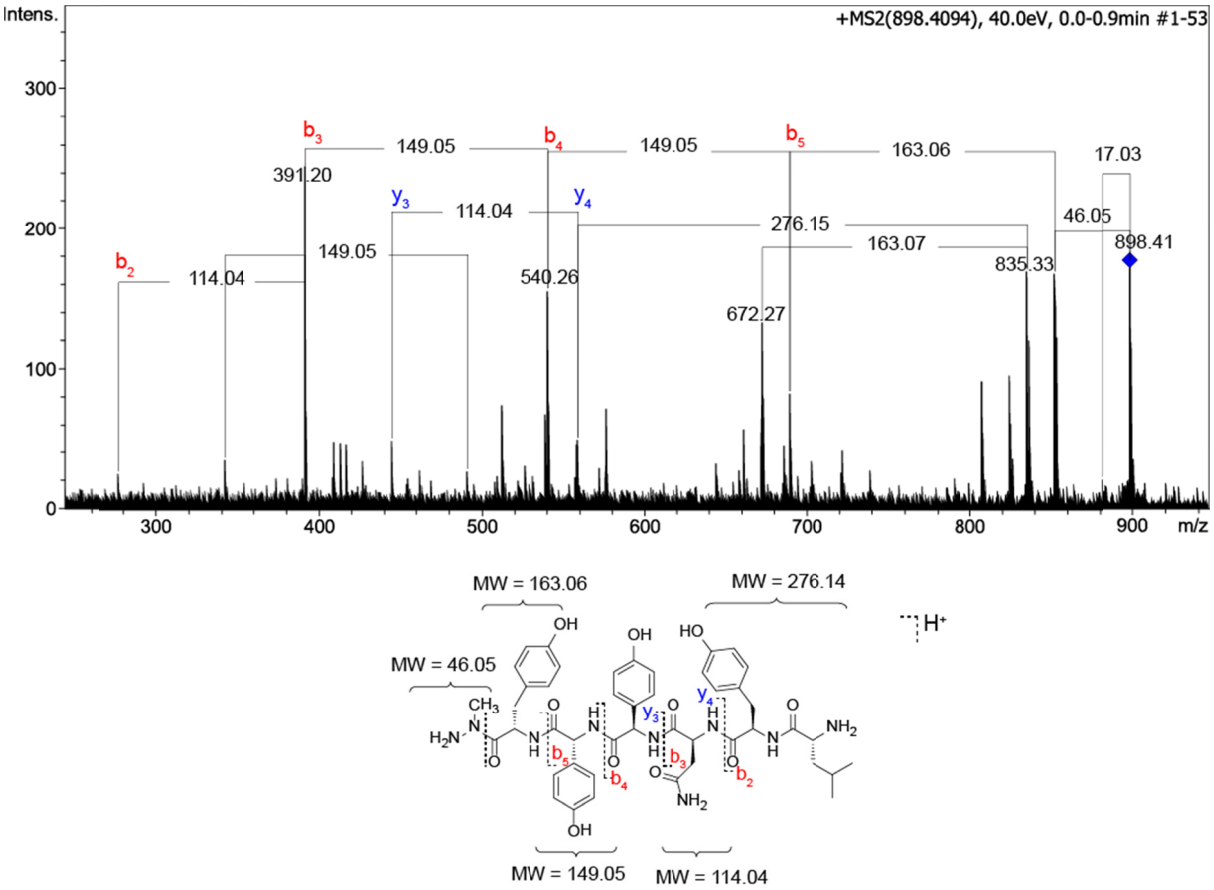

Figure S17. ESI-MS/MS of peptide **1**-Z^{2}: *m*/*z* 898.41, calc. mass ($C_{45}H_{56}N_9O_{11}$) 898.41 $(M+H)^+$.

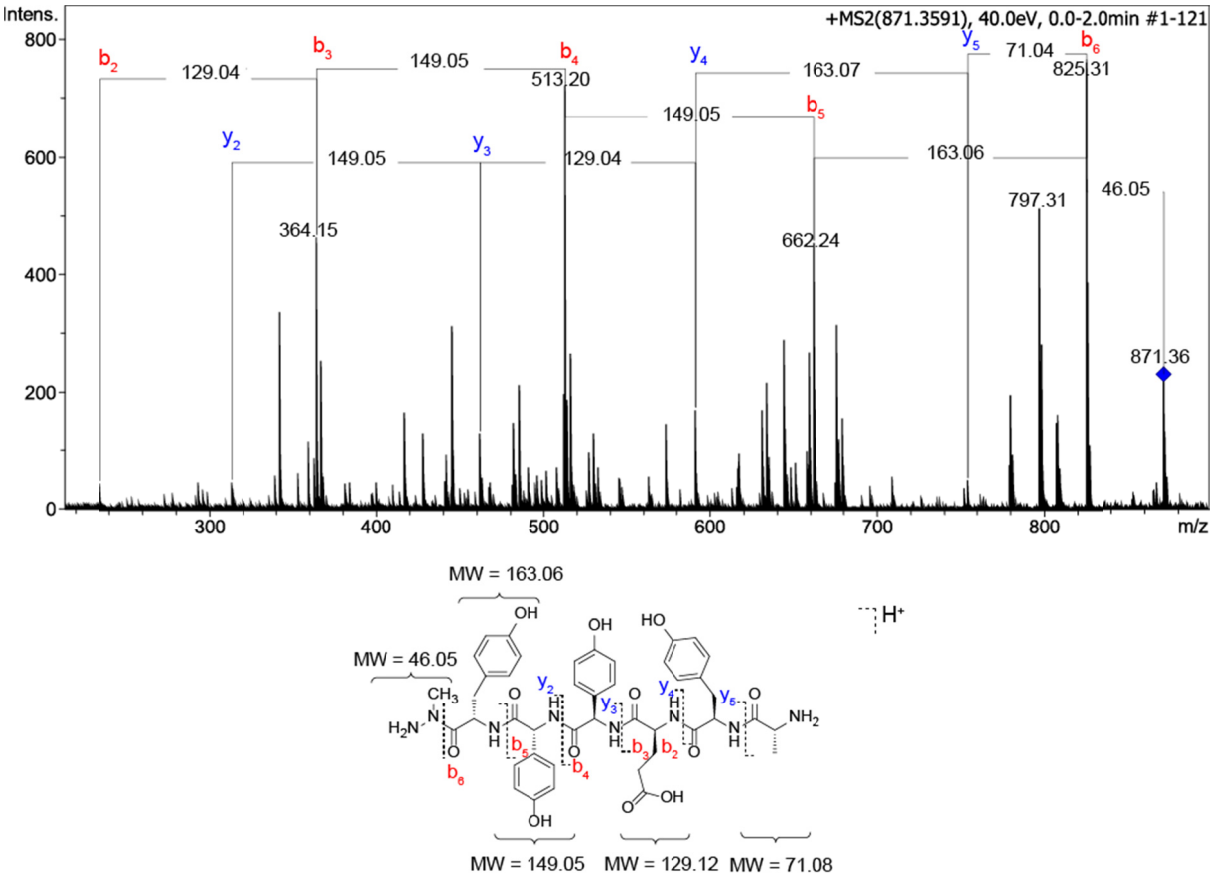

Figure S18. ESI-MS/MS of peptide **2**-Z^2: m/z 871.36, calc. mass ($C_{43}H_{51}N_8O_{12}$) 871.36 $(M+H)^+$.

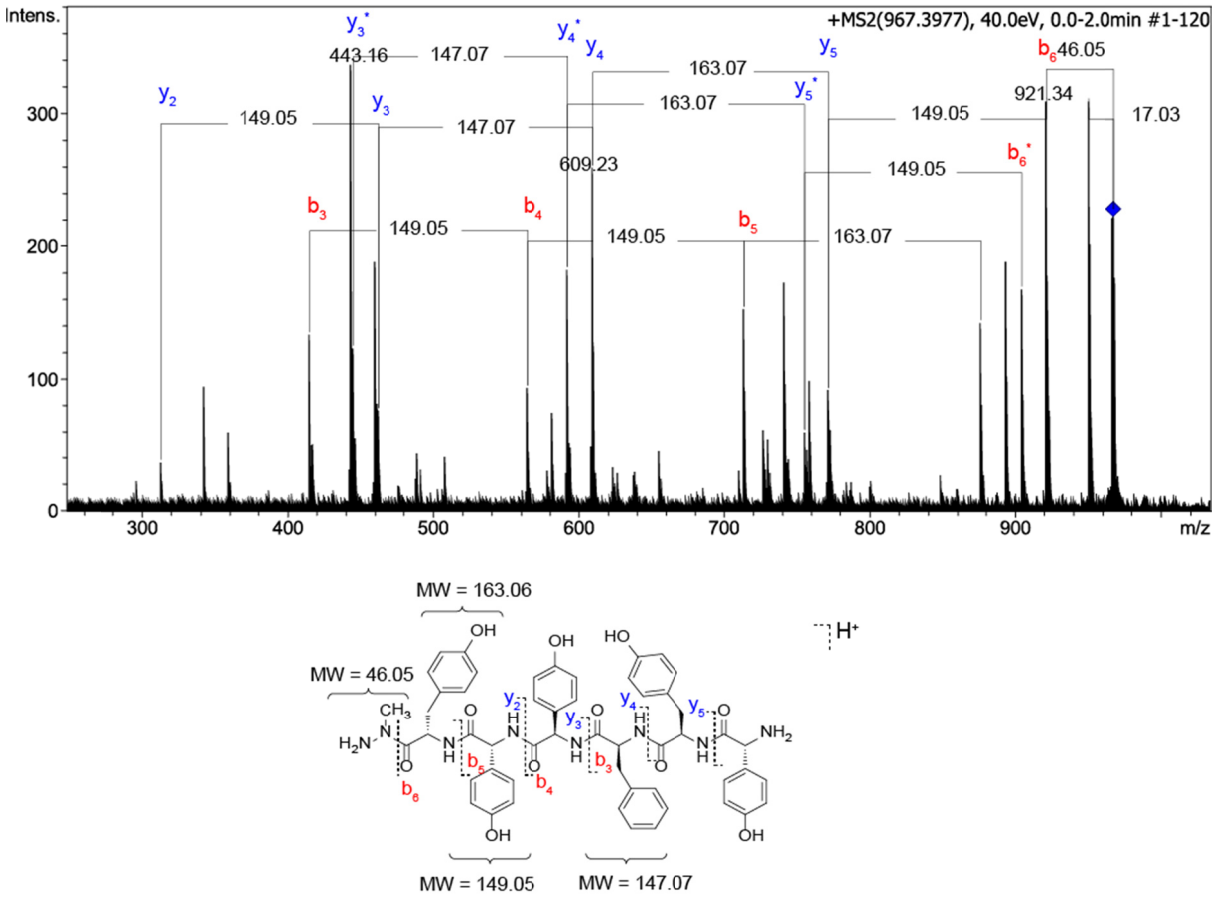

Figure S19. ESI-MS/MS of peptide **3**-Z^2: ***m/z*** 967.40, calc. mass ($C_{52}H_{55}N_8O_{11}$) 967.40 $(M+H)^+$.

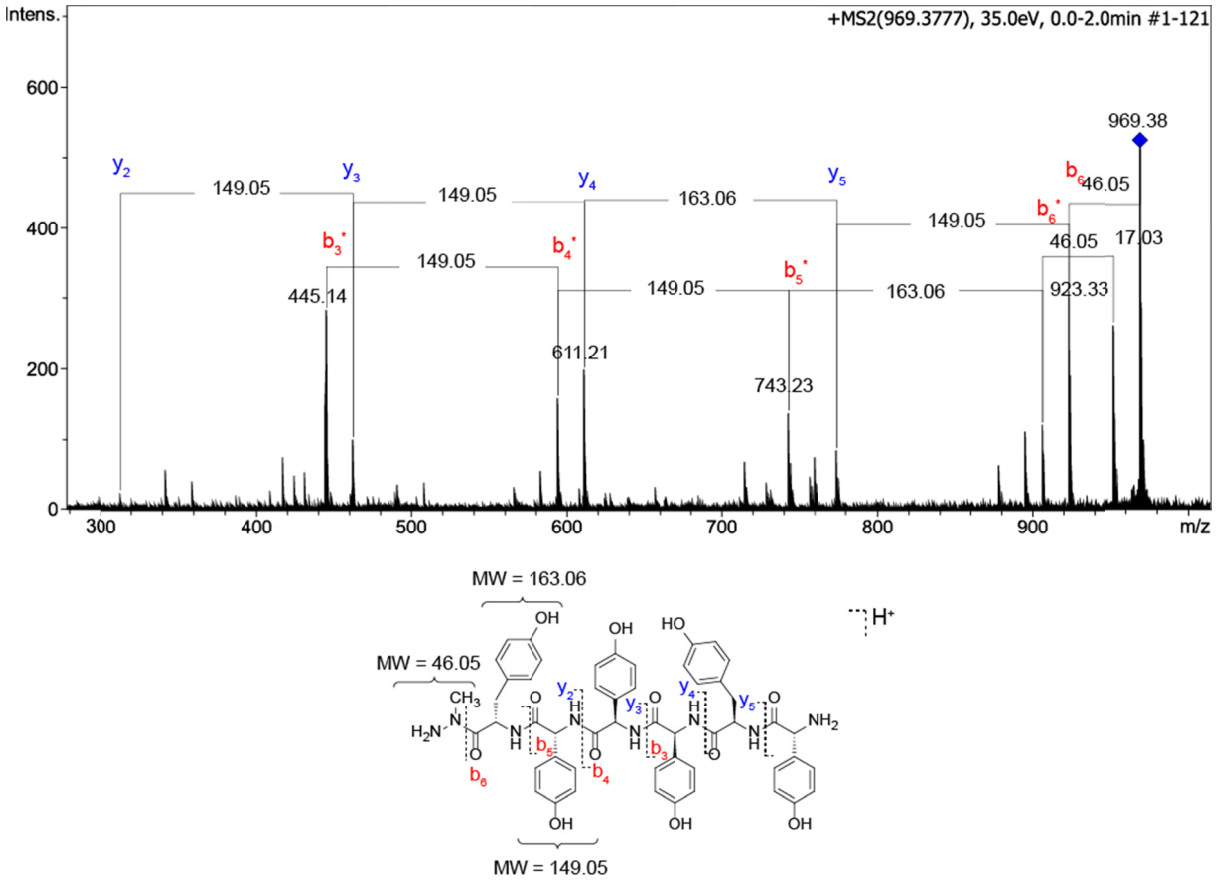

Figure S20. ESI-MS/MS of peptide **4**-Z^2: m/z 969.38, calc. mass ($C_{51}H_{53}N_8O_{12}$) 969.38 $(M+H)^+$.

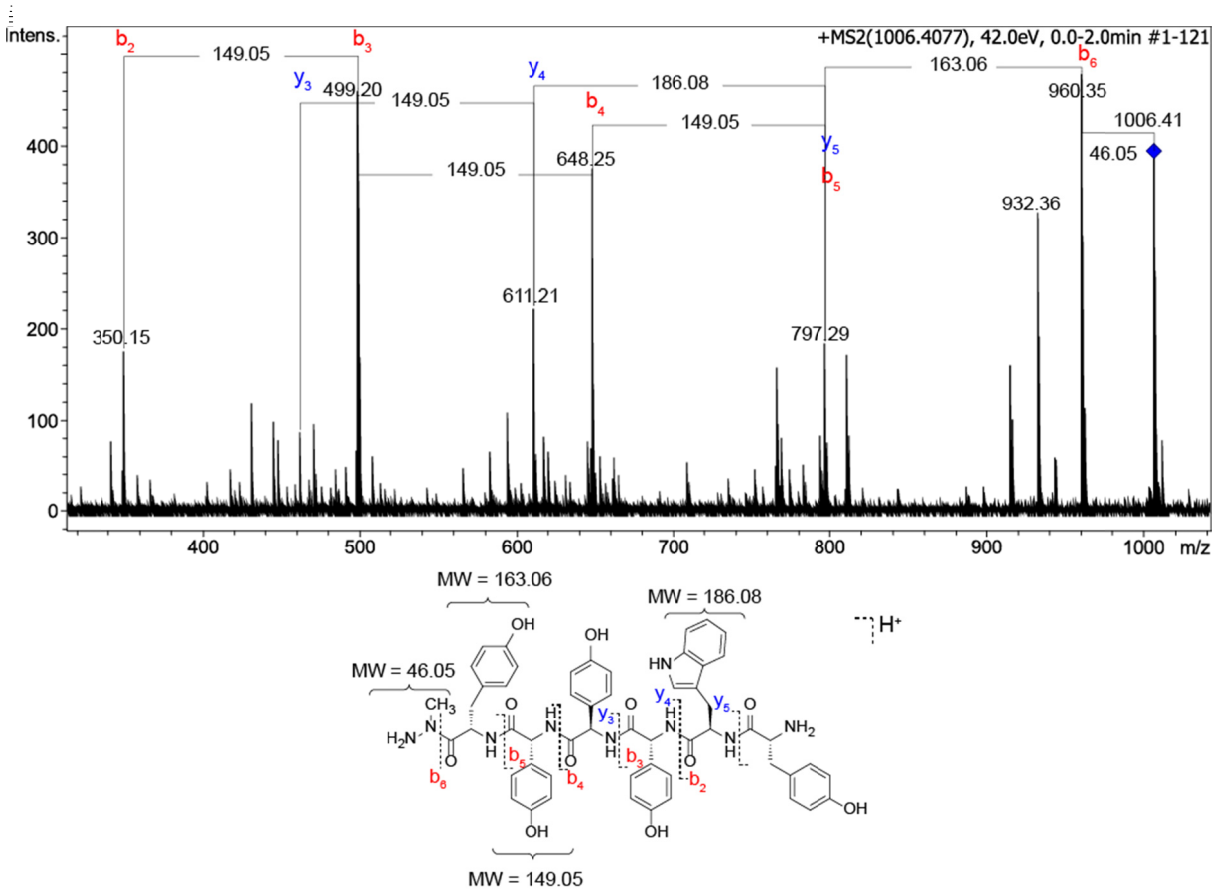

Figure S21. ESI-MS/MS of peptide **5**-Z²: *m/z* 1006.41, calc. mass ($C_{54}H_{56}N_9O_{11}$) 1006.41 $(M+H)^+$.

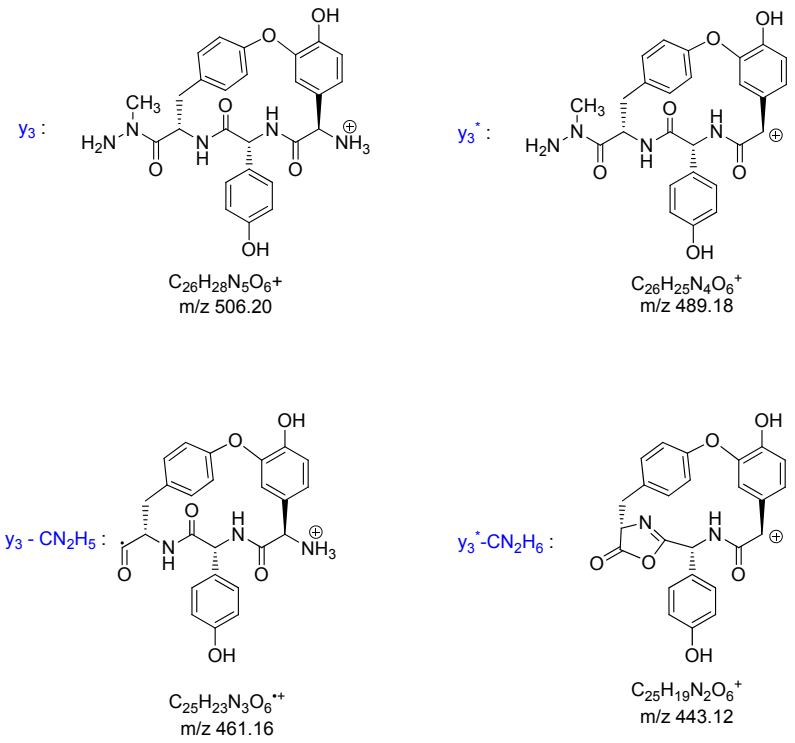

Figure S22. Typical fragments observed for the C-*O*-D part of all crosslinked peptides.

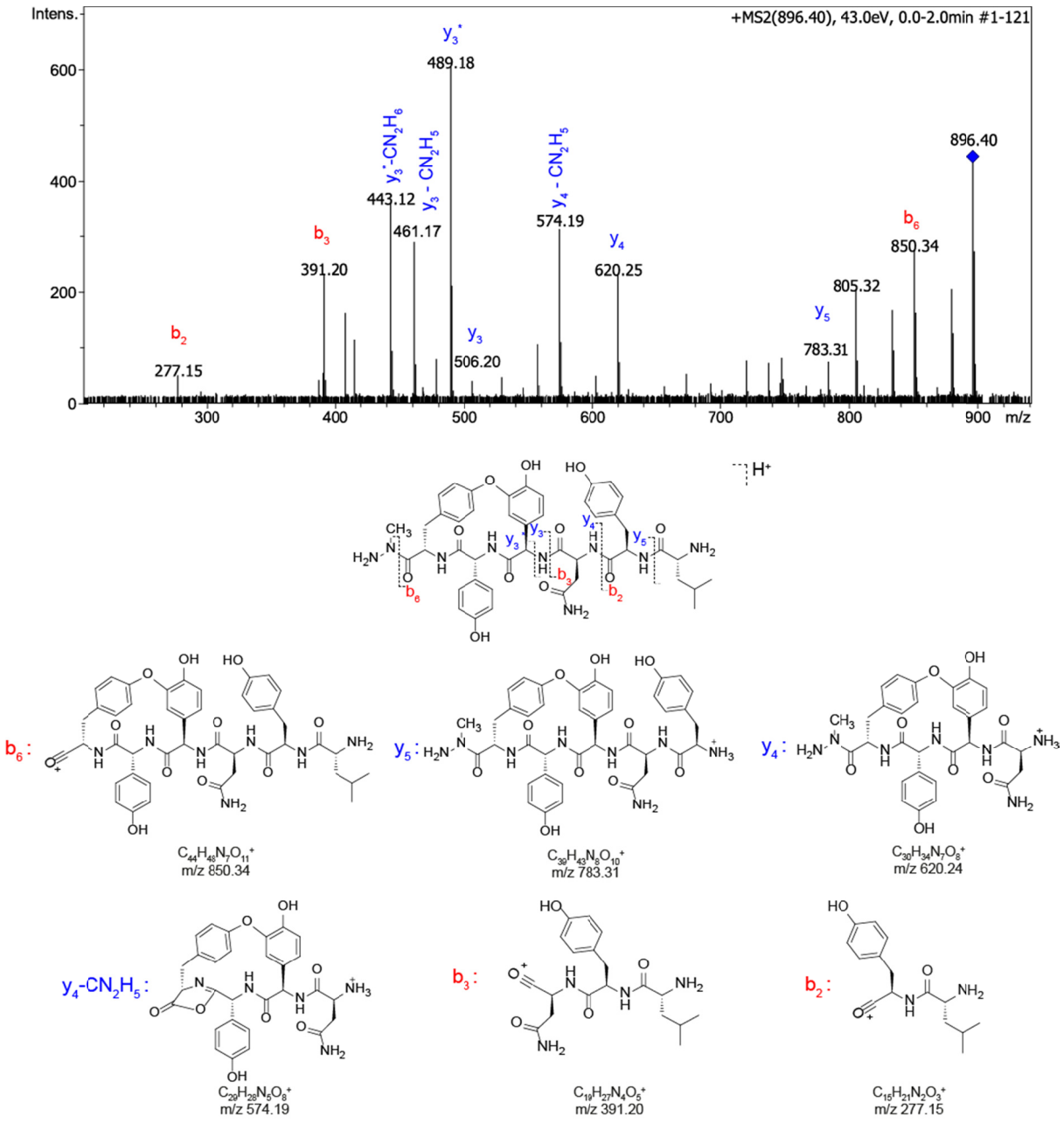

Figure S23. ESI-MS/MS of peptide **6**-Z^2: *m/z* 896.40, calc. mass ($C_{45}H_{54}N_9O_{11}$) 896.39 $(M+H)^+$.

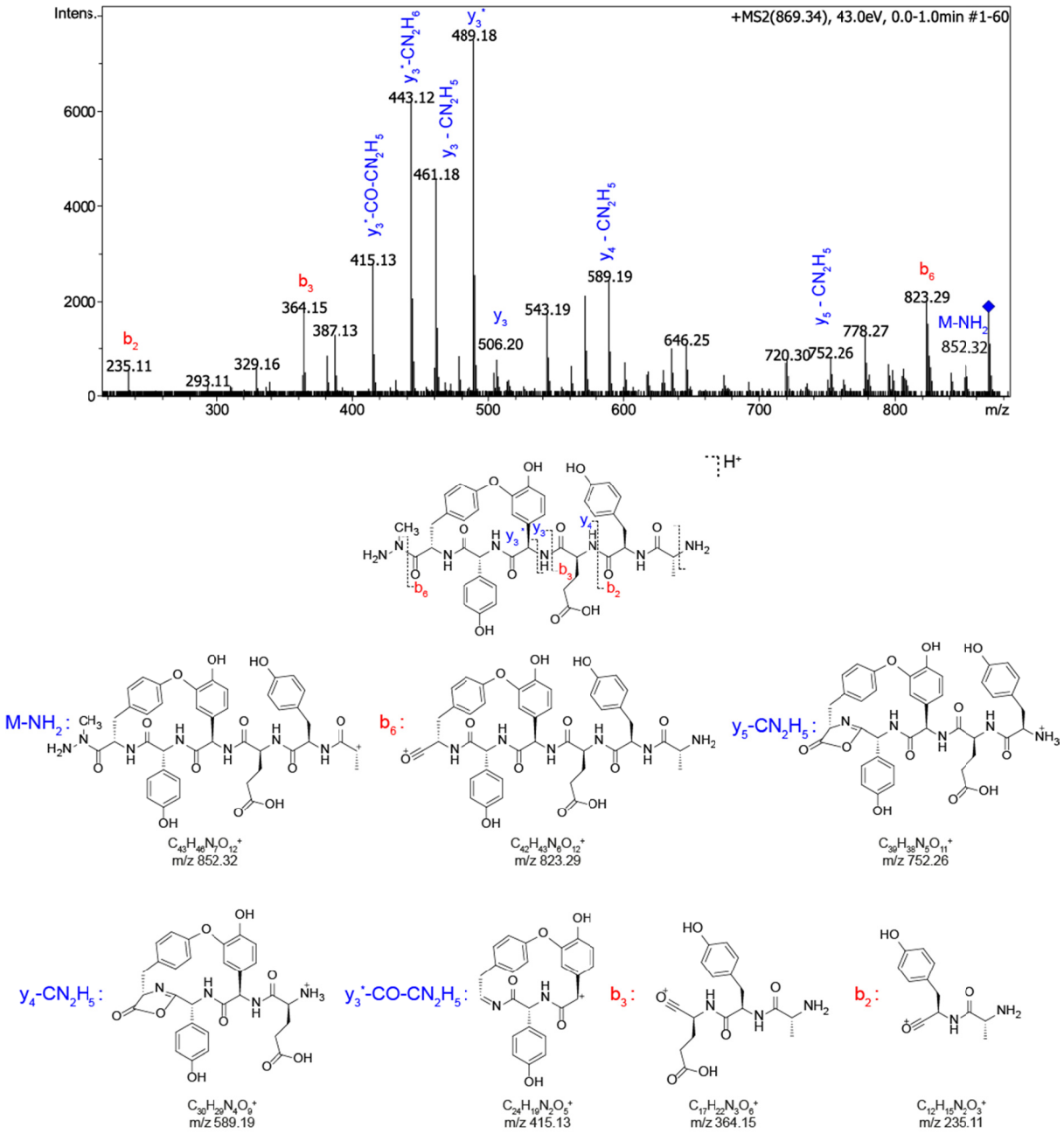

Figure S24. ESI-MS/MS of peptide **7**-Z^2: m/z 869.40, calc. mass ($C_{45}H_{49}N_8O_{11}$) 869.35 $(M+H)^+$.

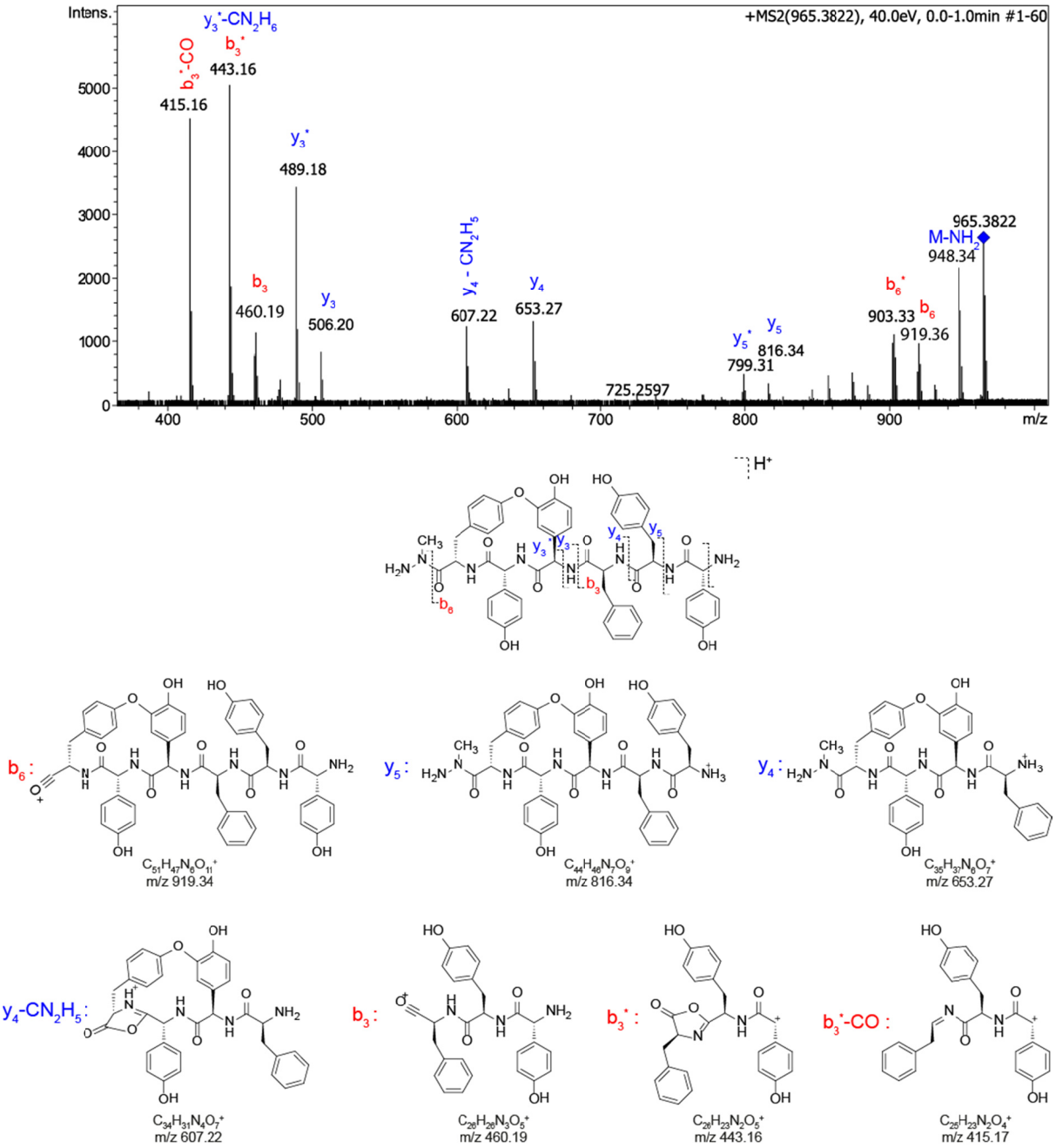

Figure S25. ESI-MS/MS of peptide **8**-Z^2: m/z 965.38, calc. mass ($C_{52}H_{53}N_8O_{11}$) 965.38 $(M+H)^+$.

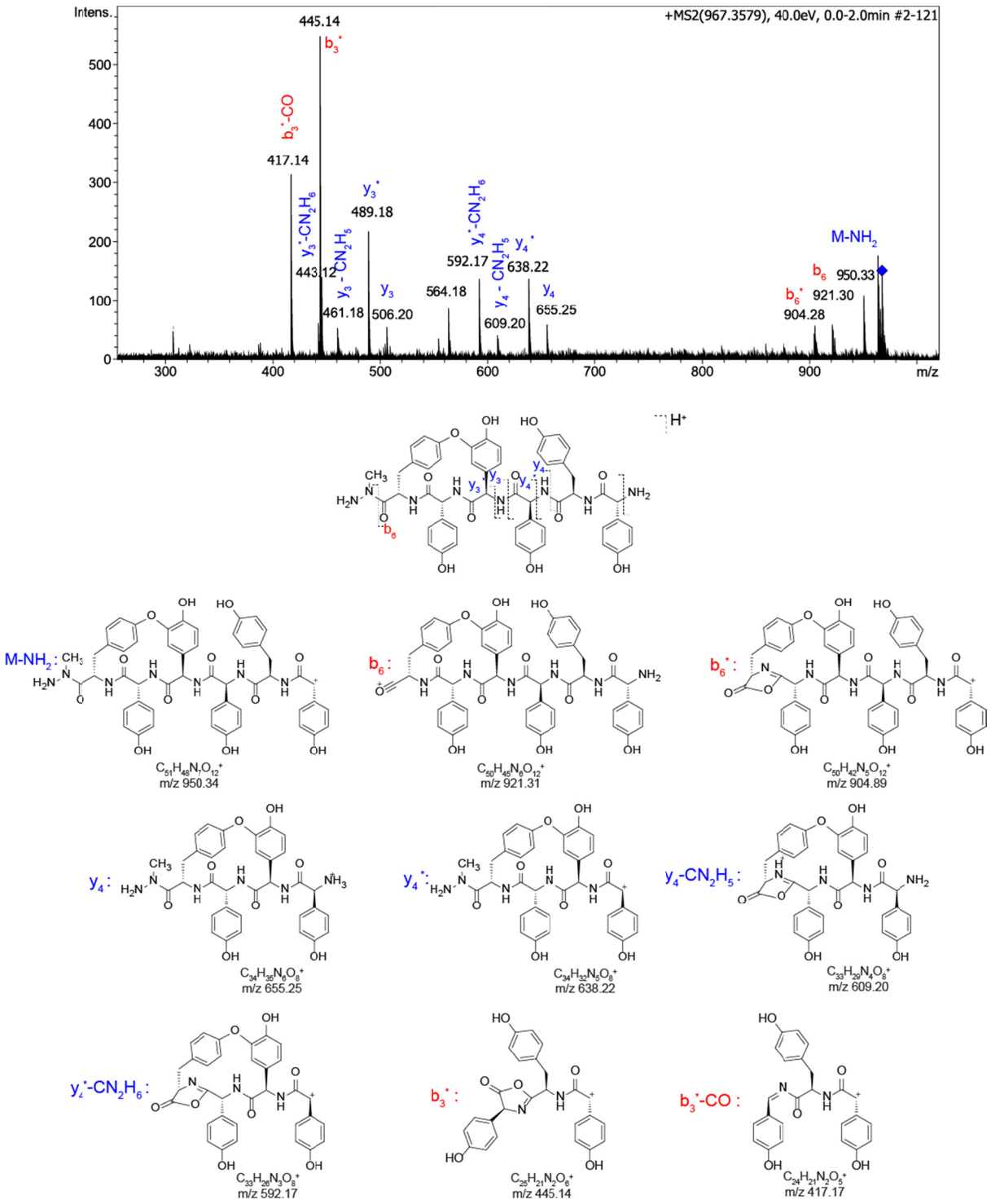

Figure S26. ESI-MS/MS of peptide **9**-Z^2: *m/z* 967.36, calc. mass ($C_{51}H_{51}N_8O_{12}$) 967.36 $(M+H)^+$.

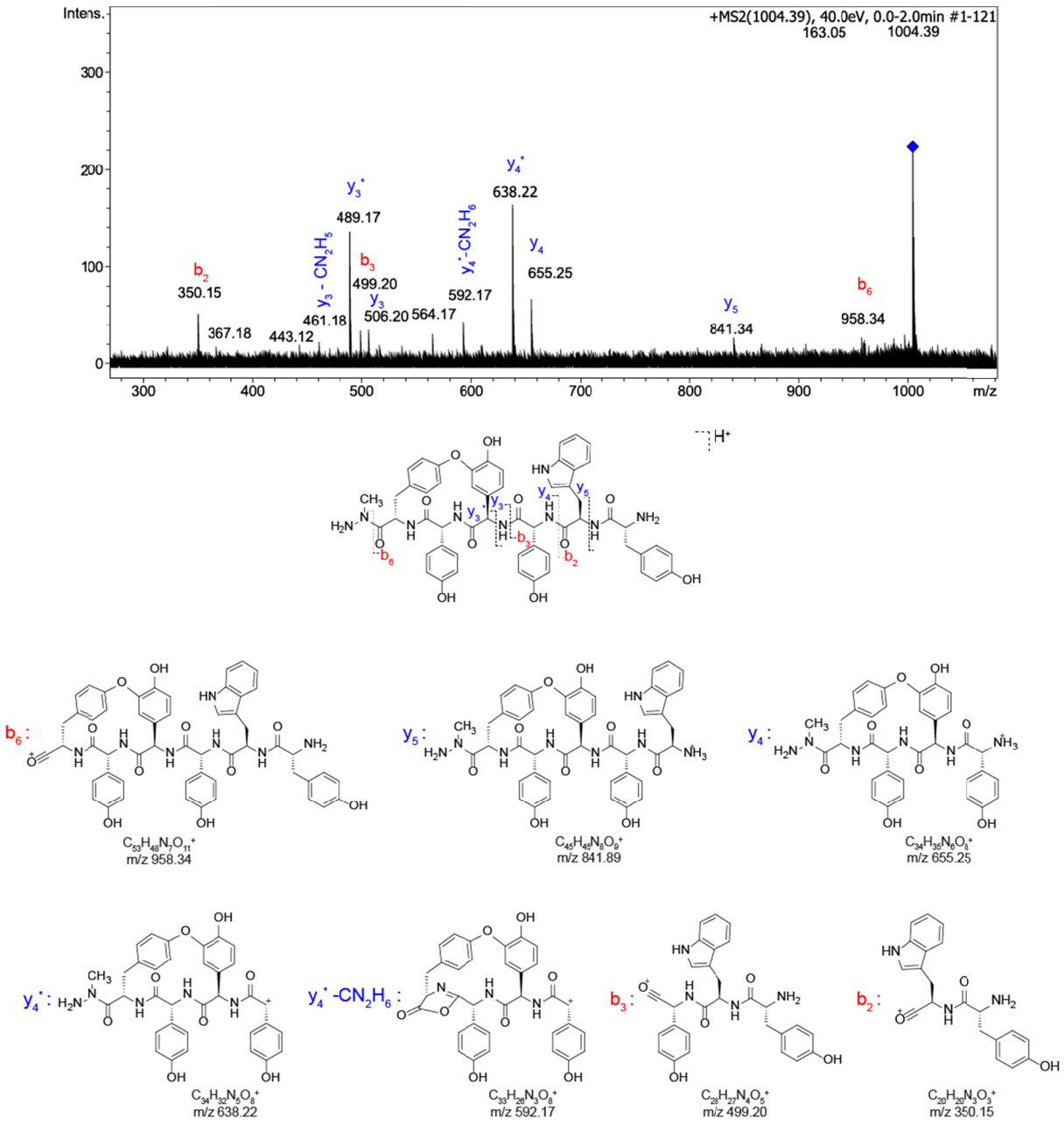

Figure S27. ESI-MS/MS of peptide **10**-Z^2: m/z 1004.39, calc. mass ($C_{54}H_{54}N_9O_{11}$) 1004.39 $(M+H)^+$.

7. NMR spectra of peptides 1 – 5-CoA

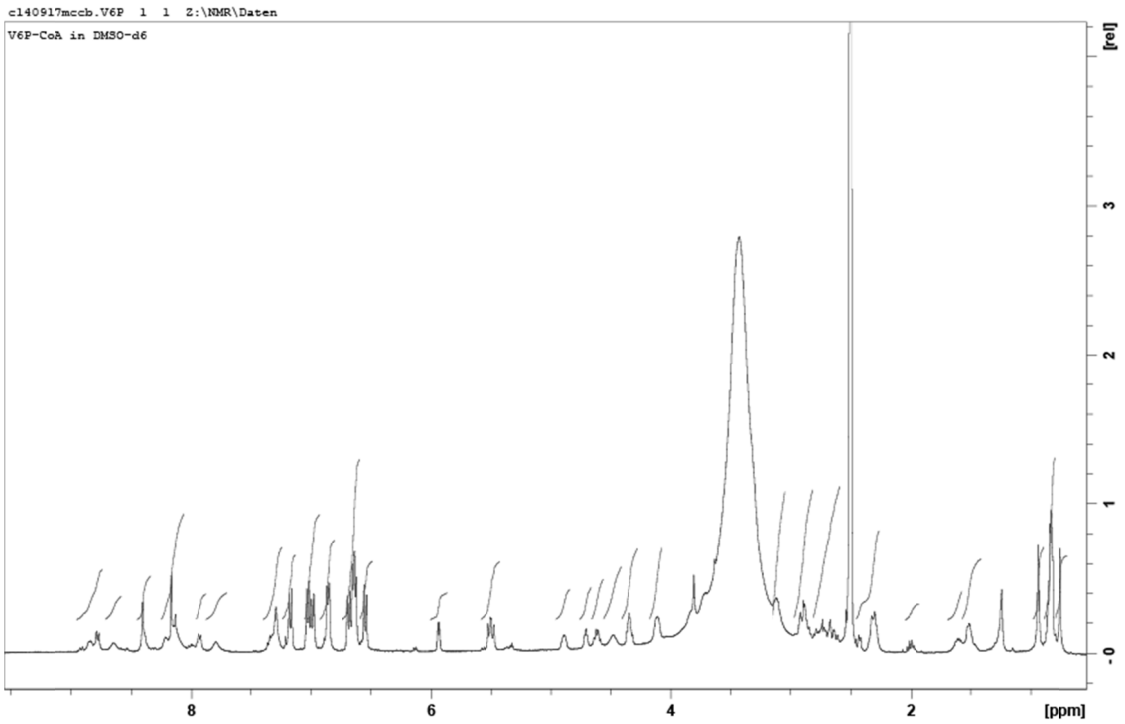

Figure S28. ^{1}H-NMR of peptide-**1**-CoA in DMSO-d_6 (400 M**H**z).

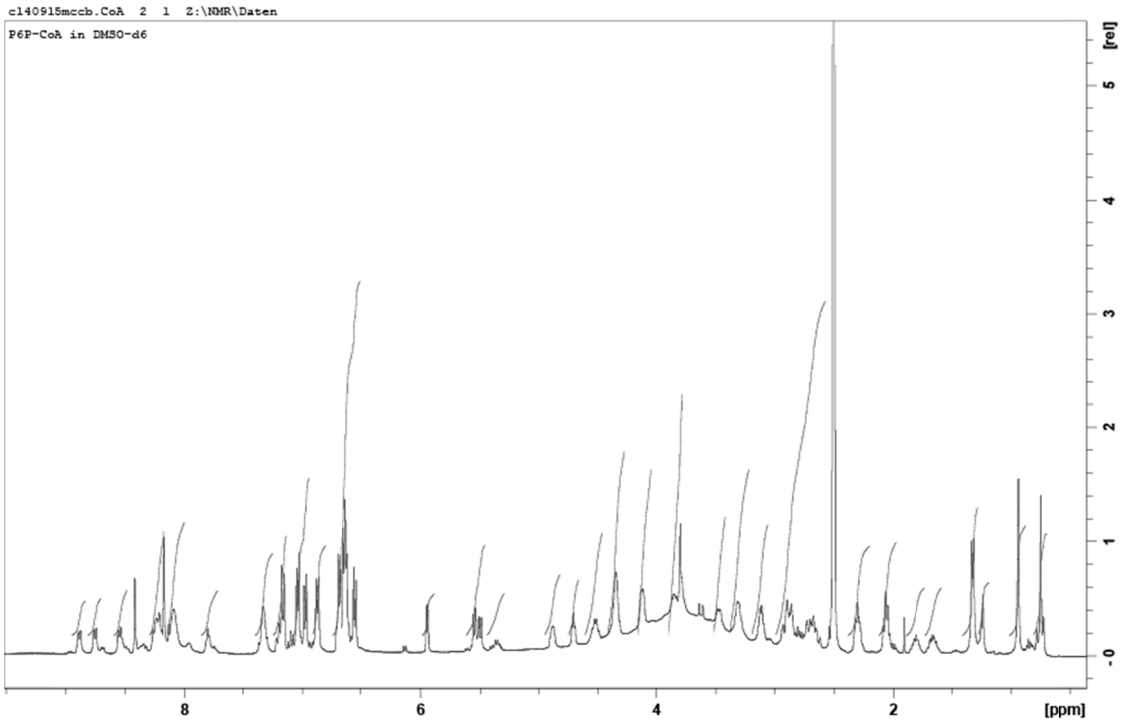

Figure S28. ^{1}H-NMR of peptide-**2**-CoA in DMSO-d_6 (400 M**H**z).

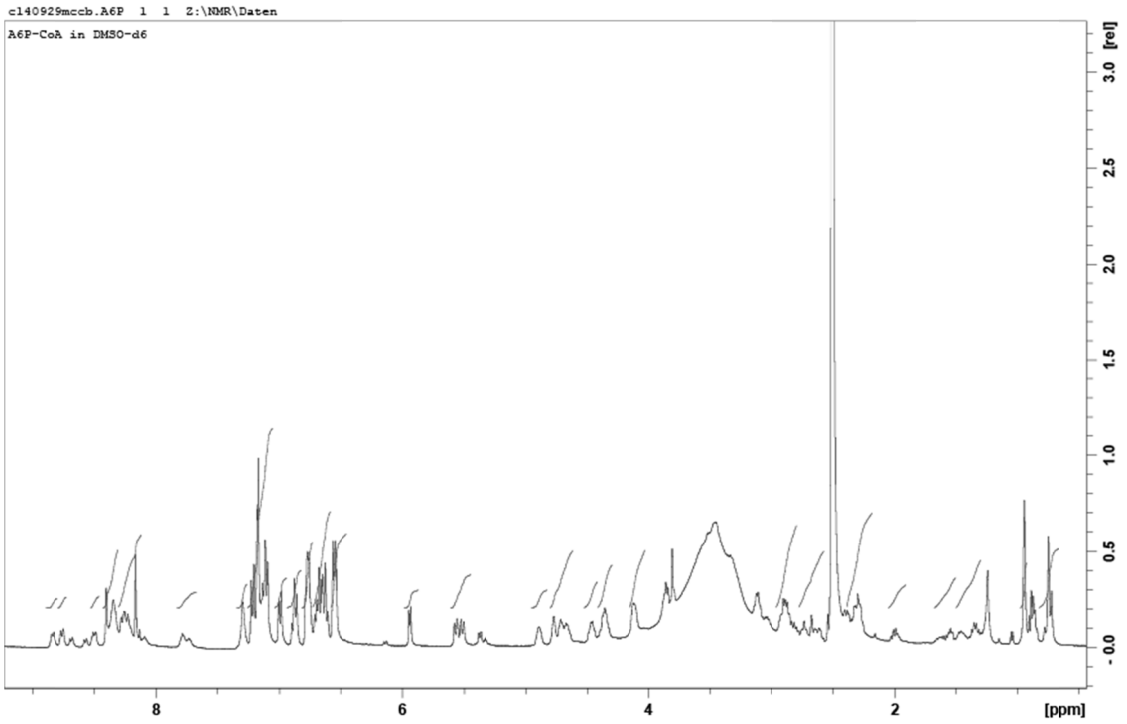

Figure S28. ^{1}H-NMR of peptide-**3**-CoA in DMSO-d_6 (400 MHz).

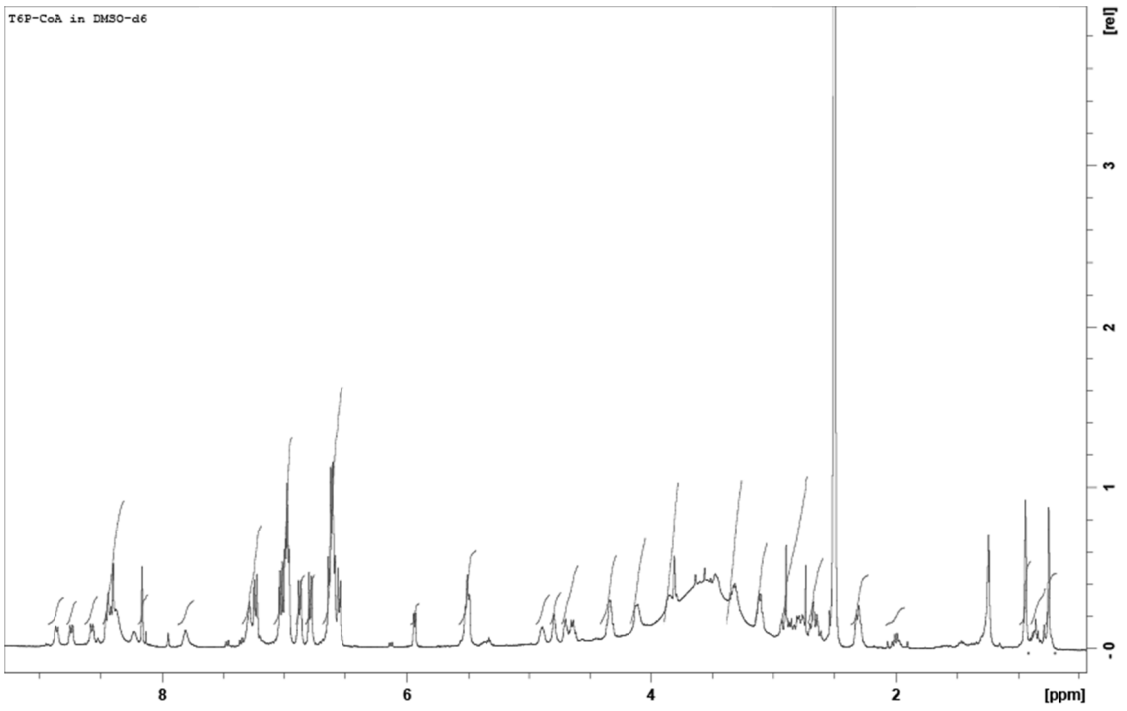

Figure S28. ^{1}H-NMR of peptide-**4**-CoA in DMSO-d_6 (400 MHz).

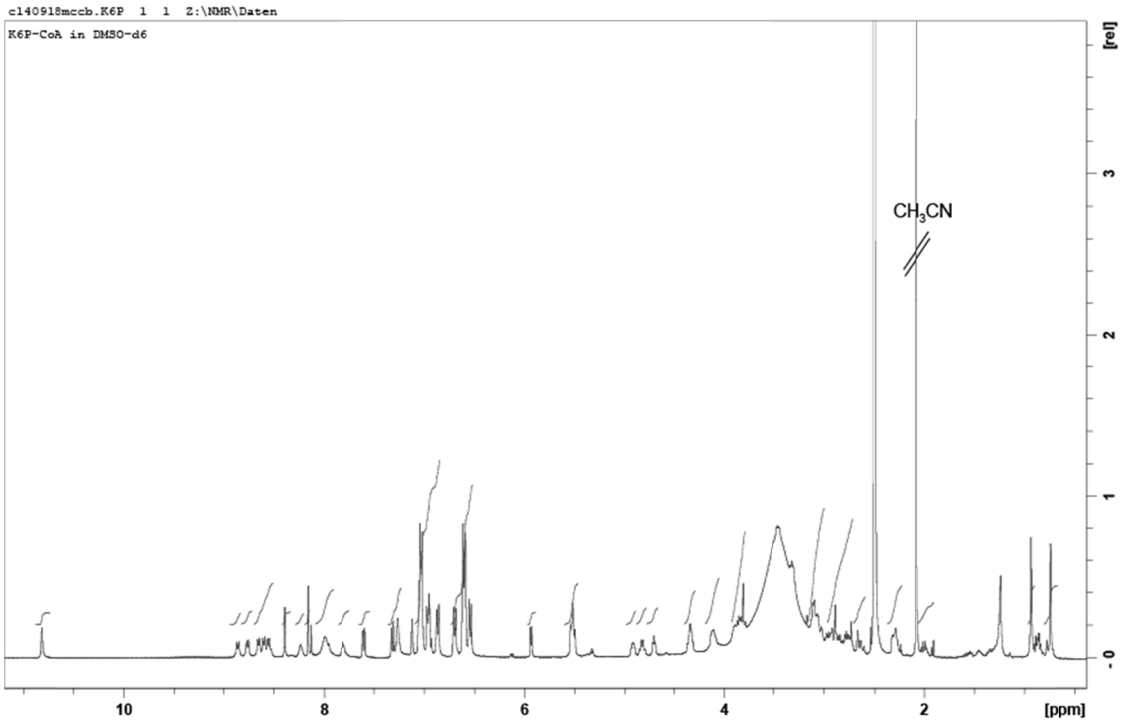

Figure S28. ^{1}H-NMR of peptide-**5**-CoA in DMSO-d_6 (400 MHz).

8. References

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