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Supporting Information

P450 monooxygenase ComJ catalyses side chain phenolic cross-coupling during complestatin biosynthesis

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Design of Gibson Assembly primers and Gibson Assembly protocol

All pANK constructs were designed to have the open reading frame (ORF) inserted into a pET28a expression vector. Thus, a pET28a vector was linearized through digestion with NdeI and EcoRI for 4 hr at 37 °C and purified by gel electrophoresis and gel extraction (GenCatch Advanced Gel Extraction Kit, Epoch Life Science). In the case of pAM2, the starting vector was pET24b-MBP-PCP7-X_{van},[1] which was digested overnight with NcoI and XhoI, and again purified by gel electrophoresis and gel extraction.

Separately, primer pairs were designed to amplify the ORFs of interest from *S. lavendulae* gDNA (see next page). Each primer was composed of the following two elements: (i) 19 base pairs of homology to the desired insert sequence, allowing for base pairing during PCR amplification; and, (ii) 25 base pairs of homology with the linearized pET28a/pET24b vector, containing the restriction site. The primers were ordered from Eurofins Genomics and used to amplify the gene of interest *via* PCR (see next page), and the amplicon purified by gel electrophoresis and gel extraction.

Once the insert and vector had been obtained as described above, each at a concentration of > 20 ng/µL after gel extraction, the two were combined in a 3:1 insert:vector **molar** ratio in a volume of 5 µL and added to 15 µL of Gibson Assembly® Master Mix (New England BioLabs), consisting of T5 Exonuclease, Phusion Polymerase, Taq Ligase, dNTPs, and $MgCl_2$ in Tris-HCl buffer. The resulting mixture was incubated for 1 hr at 50 °C and then transformed into DH5α competent cells *via* a 90-second, 42 °C heat shock. Resulting colonies were used to inoculate 10 mL LB/kan cultures (50 µg/mL) and grown overnight at 37 °C, 225 RPM. Plasmid DNA was isolated using a Qiagen QIAprep Spin Miniprep Kit and sequenced using standard T7 + T7term primers (Eurofins Genomics).

[1] J. Bogomolovas, B. Simon, M. Sattler and G. Stier, *Protein Expr. Purif.*, 2009, **64**, 16–23.

Gibson Assembly primer list and thermal cycling conditions

Below are the primers used to amplify *S. lavendulae* gDNA for the construction of plasmids encoding ComI, ComJ, ComK, and MBP-PCP7-X_{com}. Restriction sides are **bolded** (restriction enzyme written on the right of the primer sequence), while the sequence complementary to the insert is underlined.

Primers for plasmid encoding ComI (pANK00, pET28a):

For:	CCTGGTGCCGCGCGGCAGC**CATATG**GCGTCCCGTGACGTCCCCG	NdeI
Rev:	AAGCTTGTCGACGGAGCTC**GAATTC**CTACCAGGCCACCGGCAGC	EcoRI

Primers for plasmid encoding ComJ (pANK01, PET28a):

For:	CCTGGTGCCGCGCGGCAGC**CATATG**CCGCAGCAAGCCCAGCGAC	NdeI
Rev:	AAGCTTGTCGACGGAGCTC**GAATTC**TCACCAGGCGACCGGAAGG	EcoRI

Primers for plasmid encoding ComK (pANK12, PET28a):

For:	CCTGGTGCCGCGCGGCAGC**CATATG**GAGATCCGGATCGACCGCG	NdeI
Rev:	AAGCTTGTCGACGGAGCTC**GAATTC**TCAGCGGCGCCCTCCCCGC	EcoRI

Primers for plasmid encoding MBP-PCP7-X_{com} (pAM2, pET24b):

For:	AATCTTTATTTTCAGGGCG**CCATGG**CCCGGGTCGCCGGACGCGCCC	NcoI
Rev:	AGTGGTGGTGGTGGTGGTG**CTCGAG**GGTCACCTGCCGCTCGGTG	XhoI

Thermal cycling conditions for pANK00 and pANK01 (5% DMSO, 1.1 µg gDNA, Taq Polymerase):

1. 94 °C 3:00
2. 94 °C 1:00
3. 62 °C 1:00
4. 72 °C 2:00
5. GO TO 2, 34 times
6. 72 °C 10:00
7. 4 °C HOLD

Thermal cycling conditions for pANK12 (3% DMSO, 67 ng gDNA, MiProof Polymerase):

1. 98 °C 0:30
2. 98 °C 0:10
3. 75 °C 0:30
4. GO TO 2, 34 times
5. 72 °C 10:00
6. 4 °C HOLD

Thermal cycling conditions for pAM2 (67 ng gDNA, Q5® High-Fidelity DNA Polymerase, 20% Q5 High GC Enhancer):

1. 98 °C 1:00
2. 98 °C 0:10
3. 90 °C (- 0.5 °C/CYC) 0:30
4. 72 °C 1:00
5. GO TO 2, 29 times
6. 98 °C 0:10
7. 74 °C 0:30
8. 72 °C 1:00
9. GO TO 6, 19 times
10. 72 °C 10:00
11. 4 °C HOLD

Standard cloning primer list

Below are the primers used to amplify the E. coli optimized gene *comD* (Eurofins MWG) for the construction of plasmids encoding MBP-PCP7$_{com}$ and MBP-X$_{com}$. Restriction sides are **bolded** (restriction enzyme written on the right of the primer sequence), while the sequence complementary to the insert is underlined.

Primers for plasmid encoding MBP-PCP7$_{com}$:

For:	TATTA**CCATGG**GCGTTGCGGGTCGTGCCCCGGAATC	NcoI
Rev:	TATAA**CTCGAG**CGGACGCTCGTCGATCTGACGCAGTG	XhoI

Primers for plasmid encoding MBP-X$_{com}$:

For:	TATTAC**CATGG**GCGTACGGCCAGCACTGCGTCAGATCG	NcoI

Rev: TATAA**CTCGAG**TGTTACCTGGCGCTCGGTGTC XhoI

LC/MS analysis of turnover mixtures

Following elution from SPE columns, the turnover mixtures were analyzed on an Agilent 6545 Q-TOF LC/MS. The column used was an Agilent Eclipse plus C18 with a pore size of 5 µm, and the flow rate was kept constant at 0.2 mL/min. Solvents used were: A = HPLC-grade H_2O incl. 0.1% formic acid (FA), B = HPLC-grade acetonitrile (ACN) incl. 0.1% FA. Gradient: 0 min 5% B; 30 min up to 40% B, 31 min up to 95% B, 31-43 min 95% B, 44 min down to 5% B, 44-56 min 5% B. The UV detector was set to 205 nm, and species detected in negative mode in the mass spectrometer.

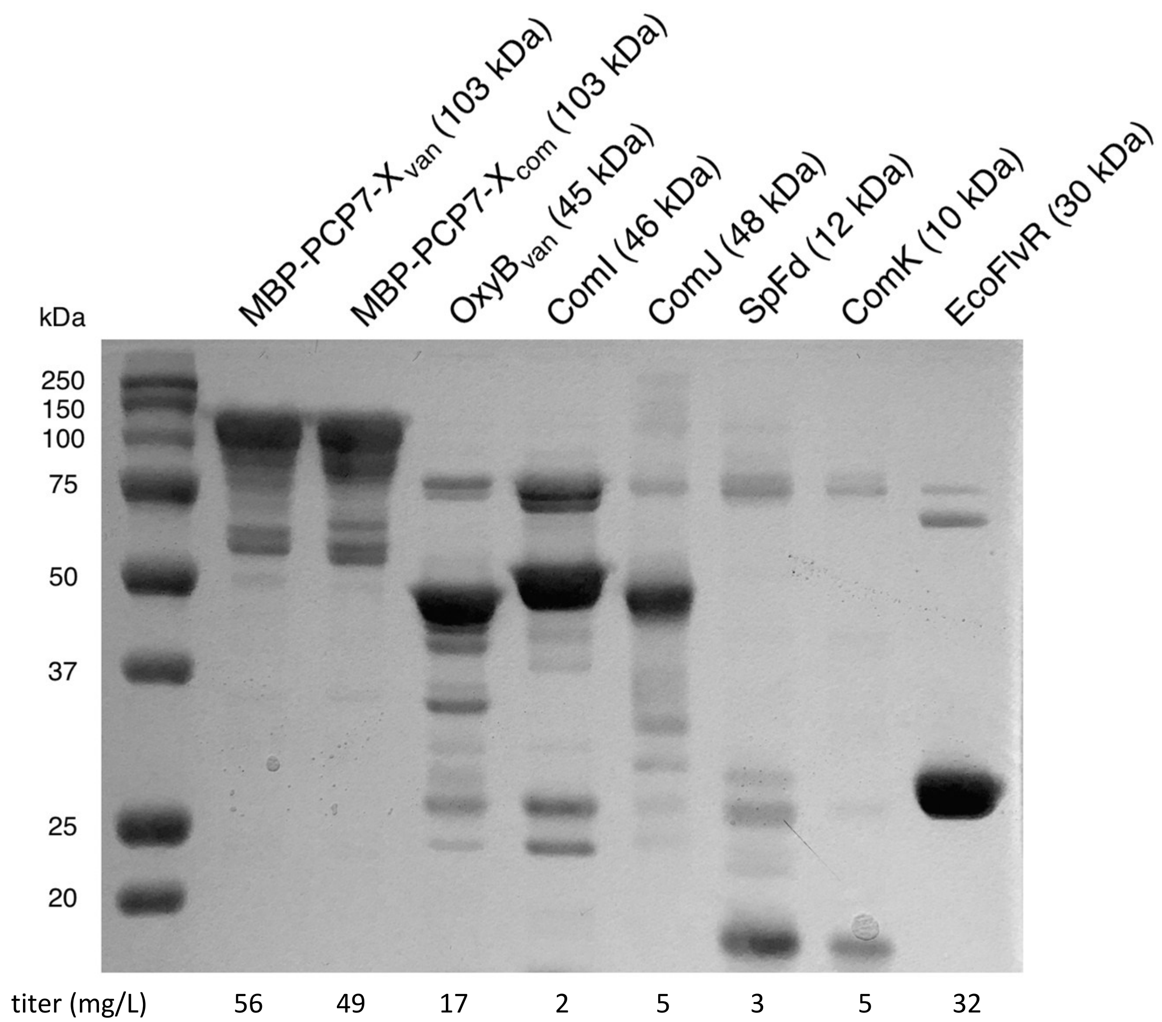


Fig. S1 SDS-PAGE gel and titers of all proteins used. Titers (to the nearest mg/L) are indicated beneath each lane. Expected molecular weights are indicated in parentheses following the name of the protein. Ladder used: Precision Plus Protein™ All Blue Standards (Bio-Rad)

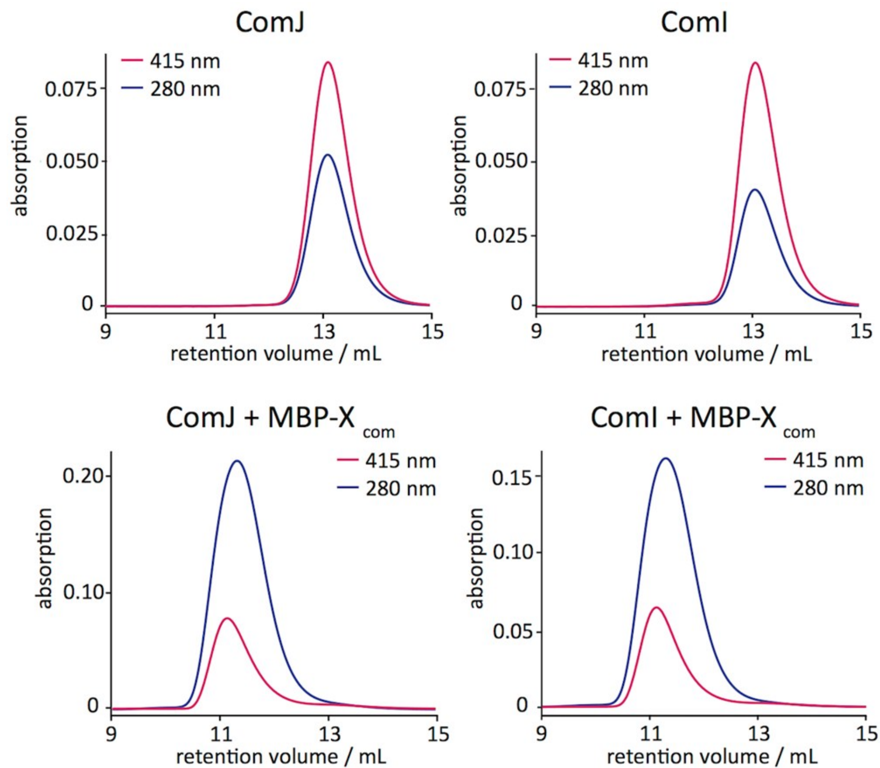


Fig. S2 SEC interaction analyses of ComJ (left) and ComI (right) with X_{com}. A clear shift in retention volume is evident upon introduction of MBP-X_{com} (bottom) compared to the P450 alone (top), indicating a tight interaction between the P450 and X_{com} domain. The absorptions at λ = 280 nm (blue; protein-specific) and at λ = 415 nm (magenta; heme-specific) were monitored simultaneously. [P450] 33.3 μM, [X] 100 μM.

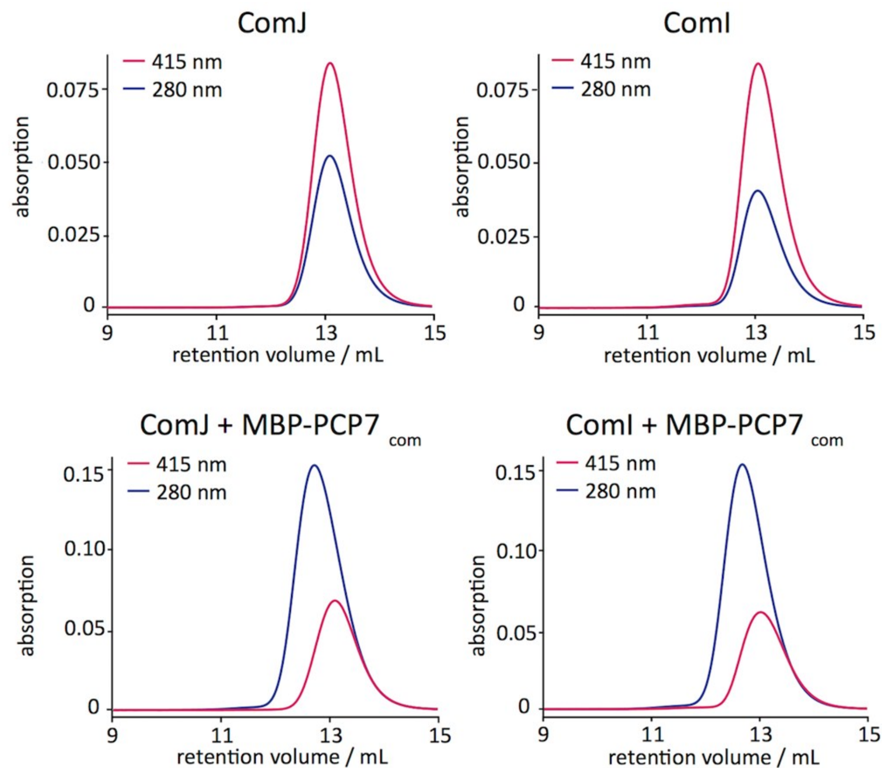


Fig. S3 SEC interaction analyses of ComJ (left) and ComI (right) with $PCP7_{com}$. No significant shift in retention volume is evident upon introduction of MBP-$PCP7_{com}$ (bottom) compared to the P450 alone (top), indicating a lack of any strong interaction between the two proteins. The absorptions at λ = 280 nm (blue; protein-specific) and at λ = 415 nm (magenta; heme-specific) were monitored simultaneously. [P450] 33.3 μM, [PCP]: 100 μM.

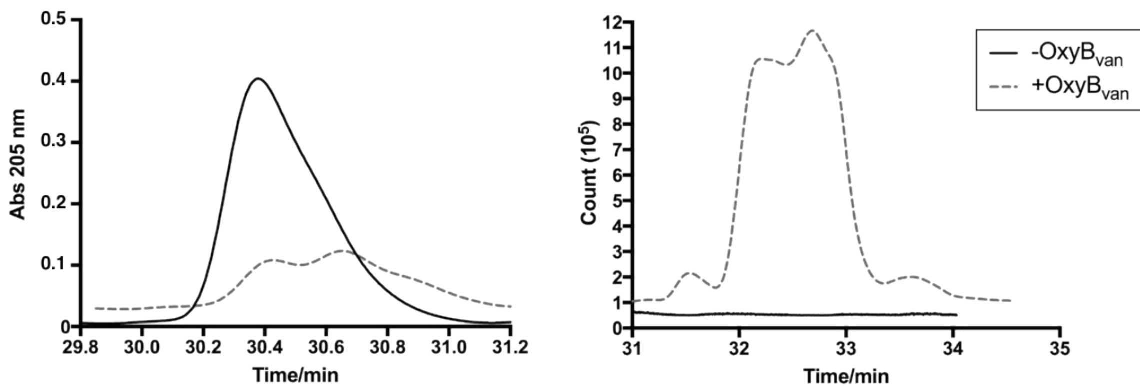


Fig. S4 Turnover of D-Hpg-D-Hpg-L-Tyr (**1**) on PCP7-X_{van} by $OxyB_{van}$. Left: UV spectrum (205 nm) showing the decrease in reactant concentration upon addition of $OxyB_{van}$ (grey dashed line) compared to the no-Oxy negative control (thick black line). Right: Extracted ion count (EIC) spectrum showing the increase in cyclized product (*m/z* 531.2) upon addition of $OxyB_{van}$ (grey dashed line) compared to the no-Oxy negative control (thick black line).

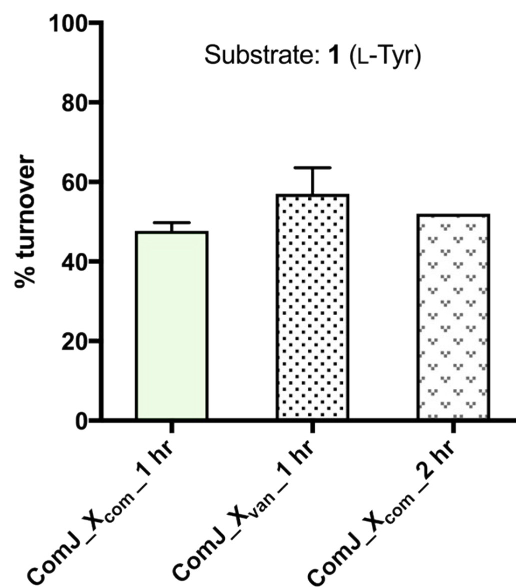


Fig. S5 Turnover of **1** in the presence of ComJ and the following conditions: MBP-PCP7-X_{com}, 1 hr (left, reproduced from Fig. 5E in the main text); MBP-PCP7-X_{van}, 1 hr (center); MBP-PCP7-X_{com}, 2 hr (right). For all three, the reduction system consisted of SpFd and EcoFlvR, with NADPH as the electron donor and G6P and G6P-DH to regenerate depleted NADPH.

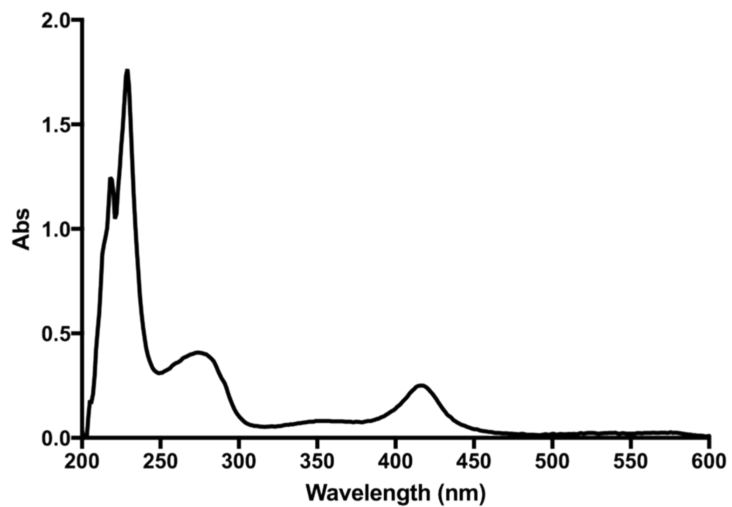


Fig. S6 UV-vis spectrum of purified ComK. The maximum at ~422 nm is characteristic of ferredoxin [2Fe-2S] clusters.

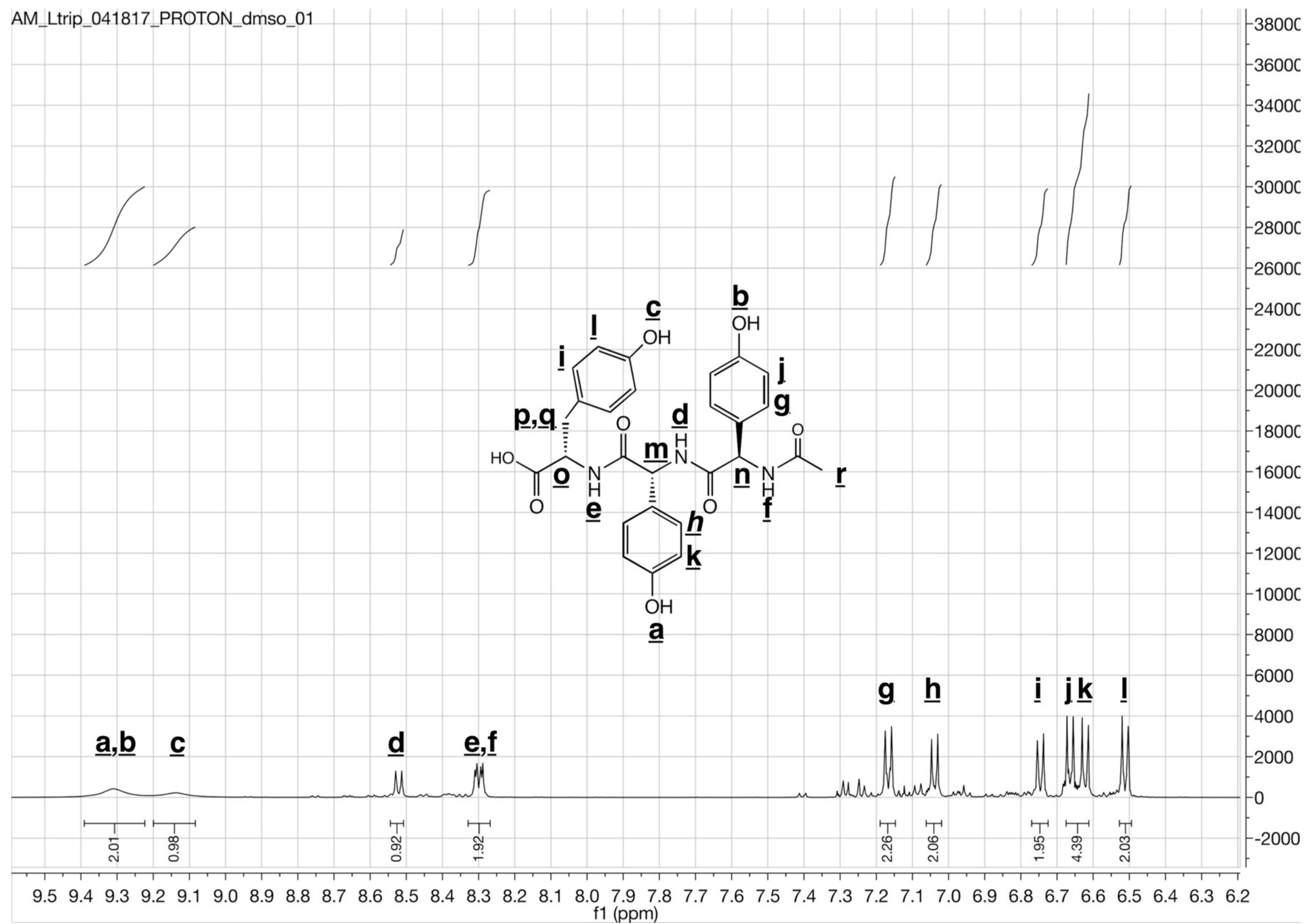
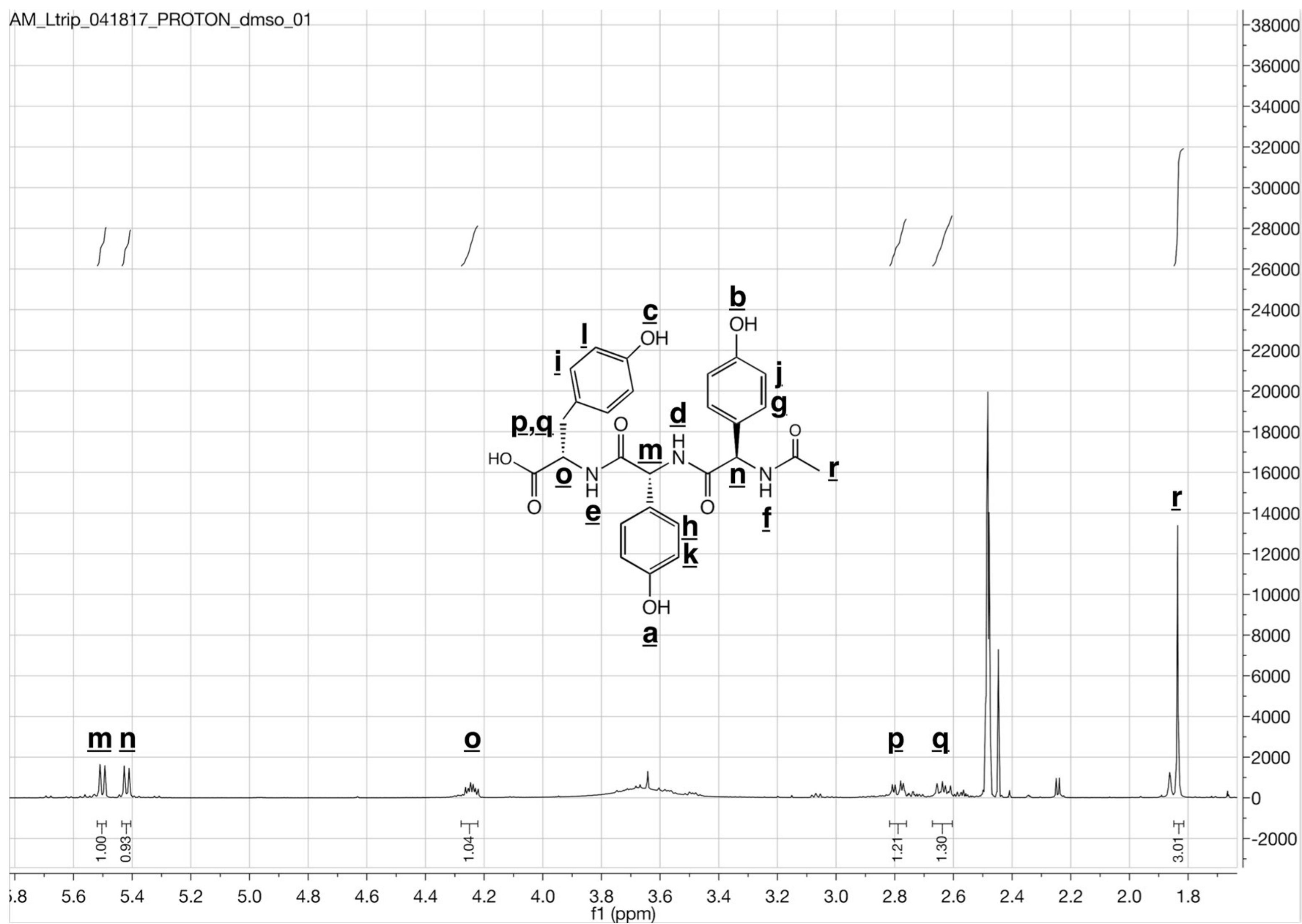
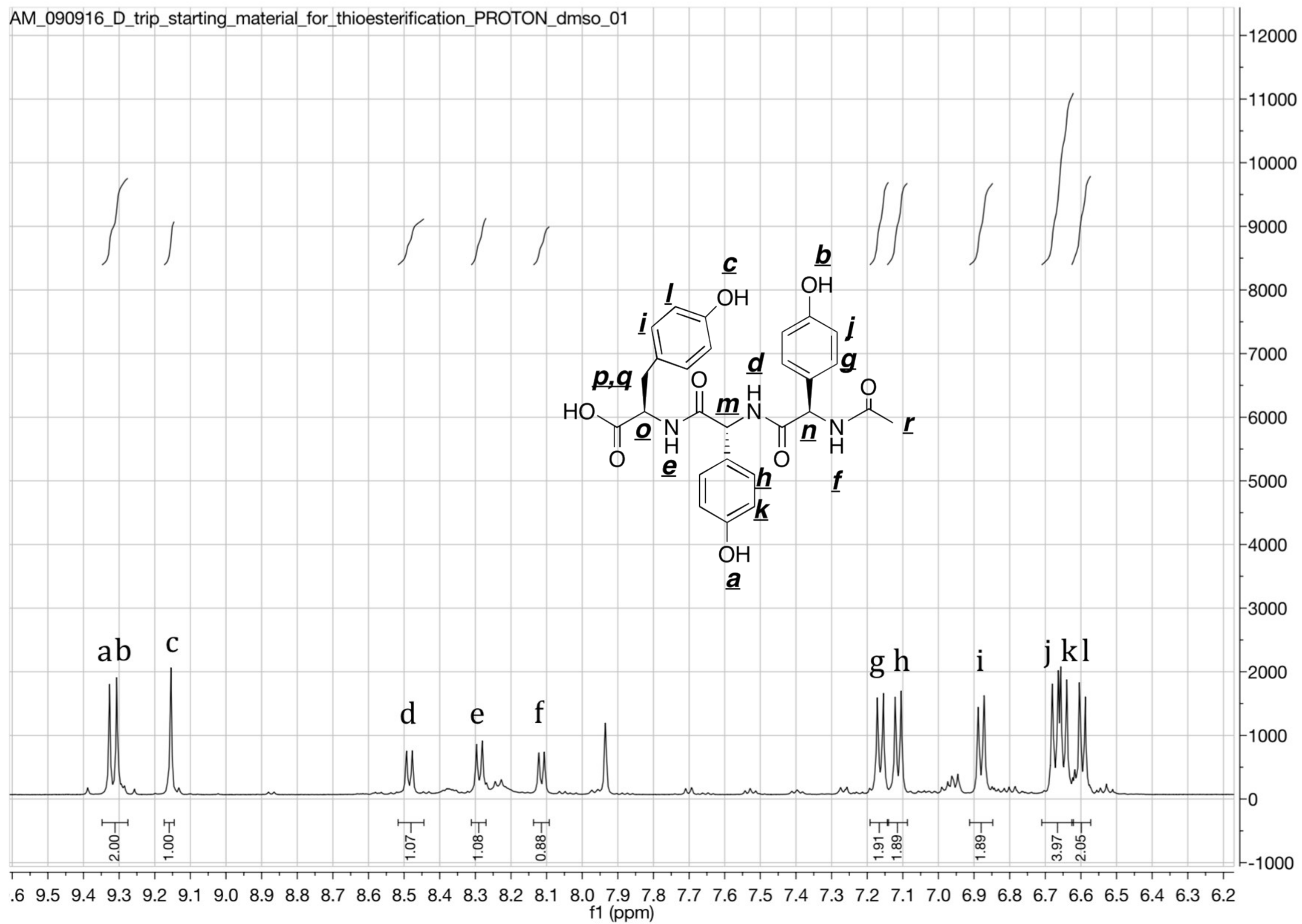


Fig. S7 (part 1 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-L-Tyr-OH (DMSO-D_6).



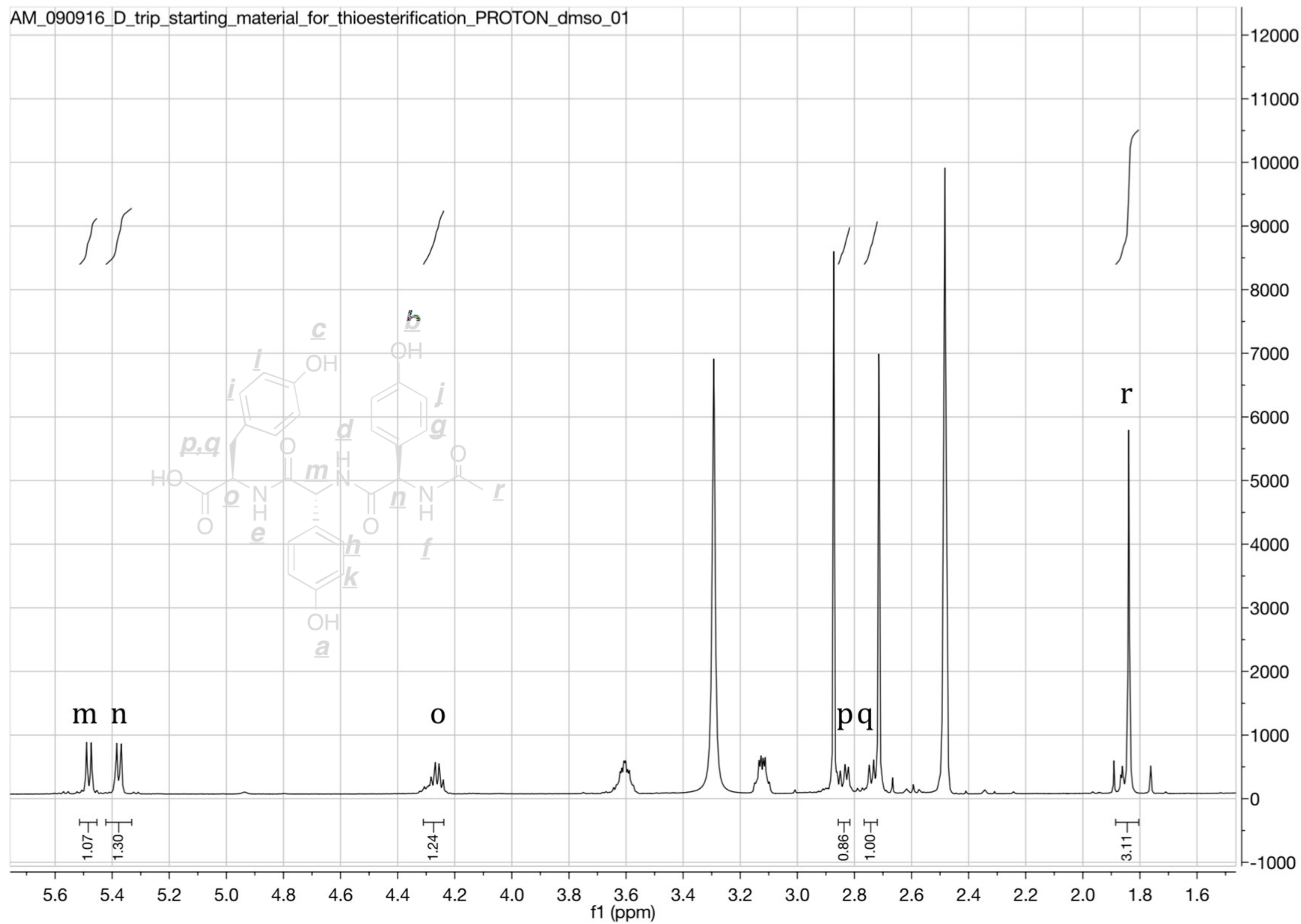
* Peak at 2.50 ppm is a residual solvent peak.

Fig. S7 (part 2 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-L-Tyr-OH (DMSO-D_6).



* Peak at 7.95 ppm is a residual DMF peak.

Fig. S8 (part 1 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-D-Tyr-OH (DMSO-D_6).



* Peak at 3.33 ppm is a residual water peak; peak at 2.50 ppm is a residual solvent peak.

Fig. S8 (part 2 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-D-Tyr-OH (DMSO-D_6).

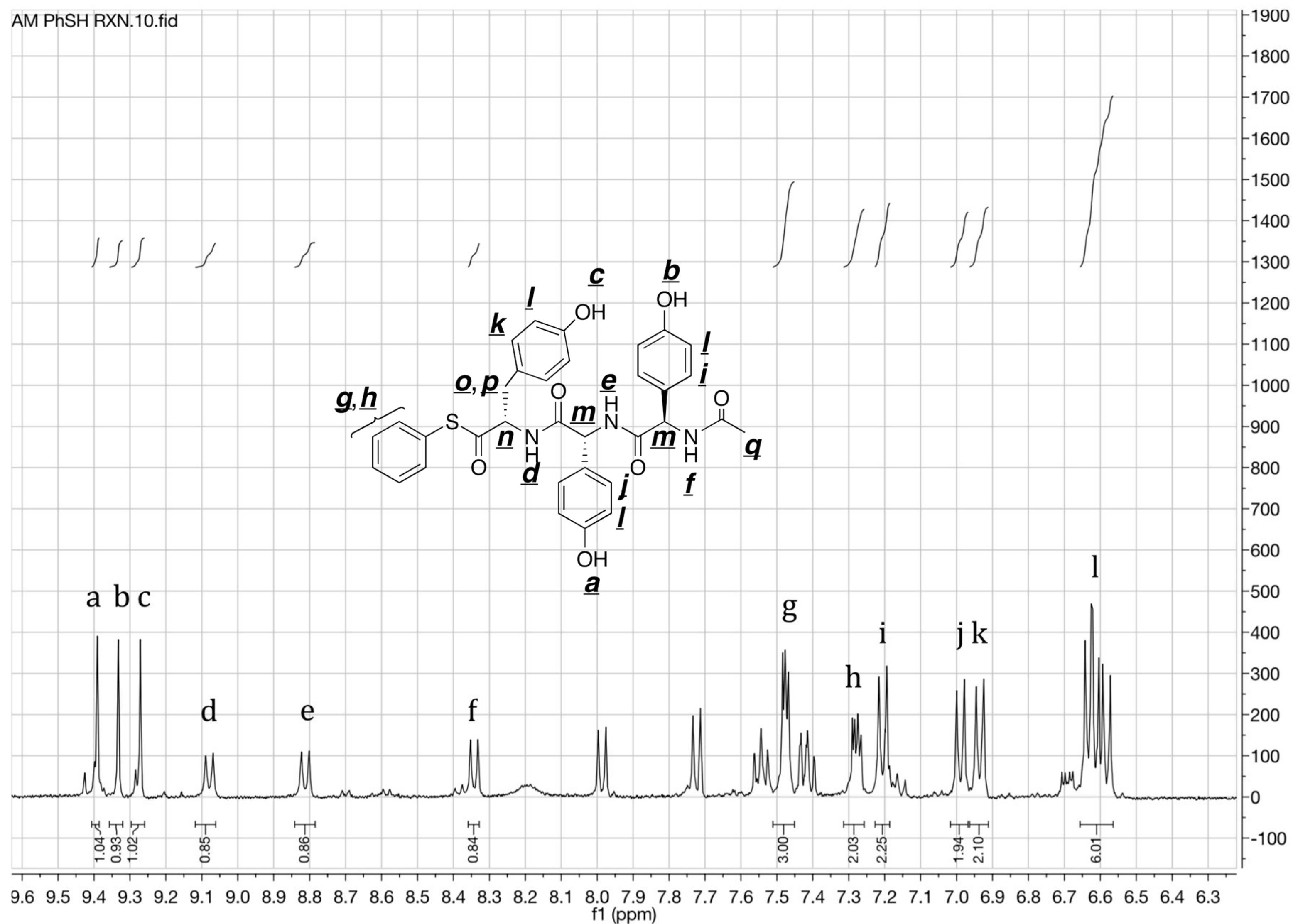
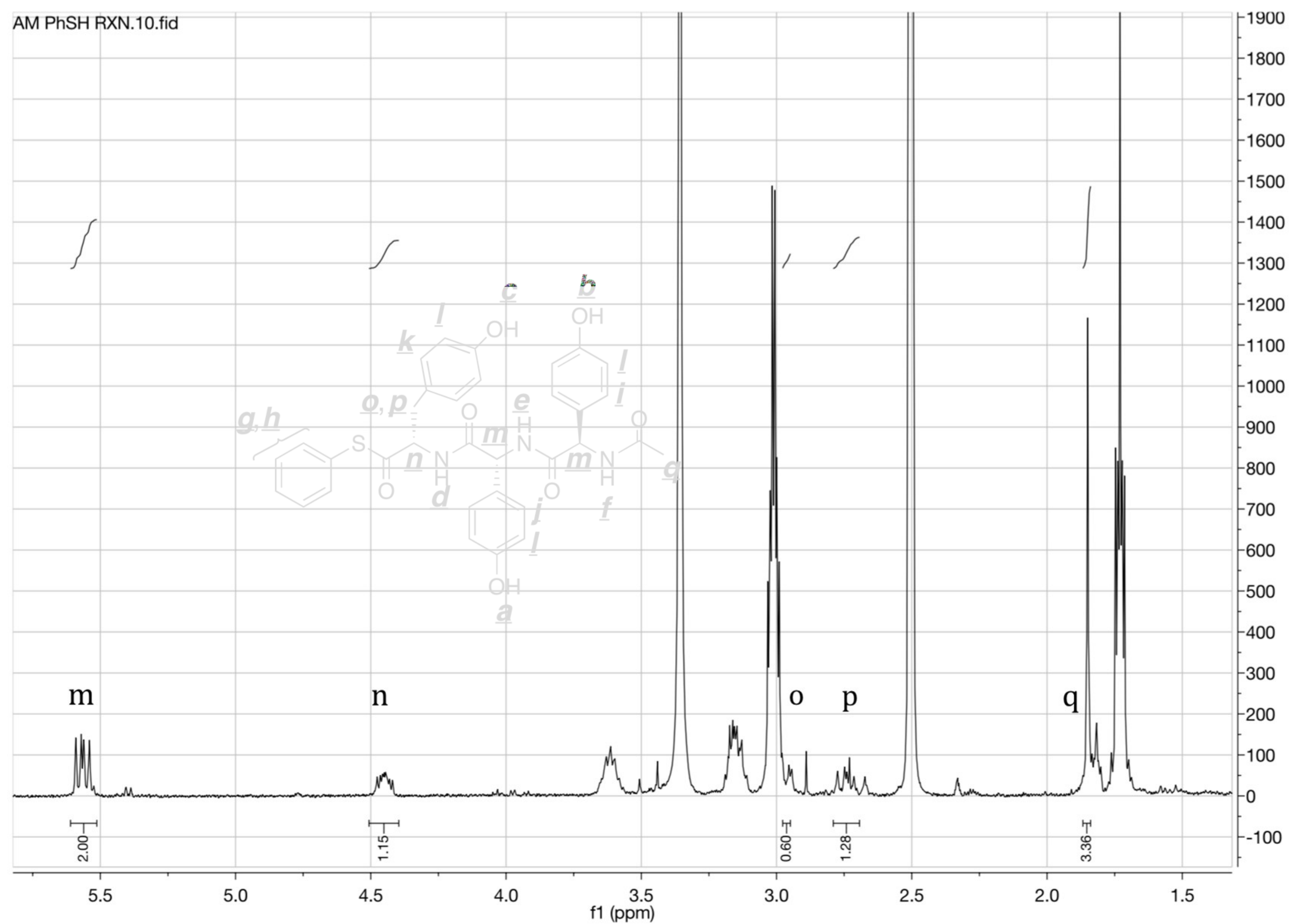


Fig. S9 (part 1 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-L-Tyr-SPh (DMSO-D_6).



* Peak at 3.33 ppm is a residual water peak; peak at 2.50 ppm is a residual solvent peak.

Fig. S9 (part 2 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-L-Tyr-SPh (DMSO-D_6).

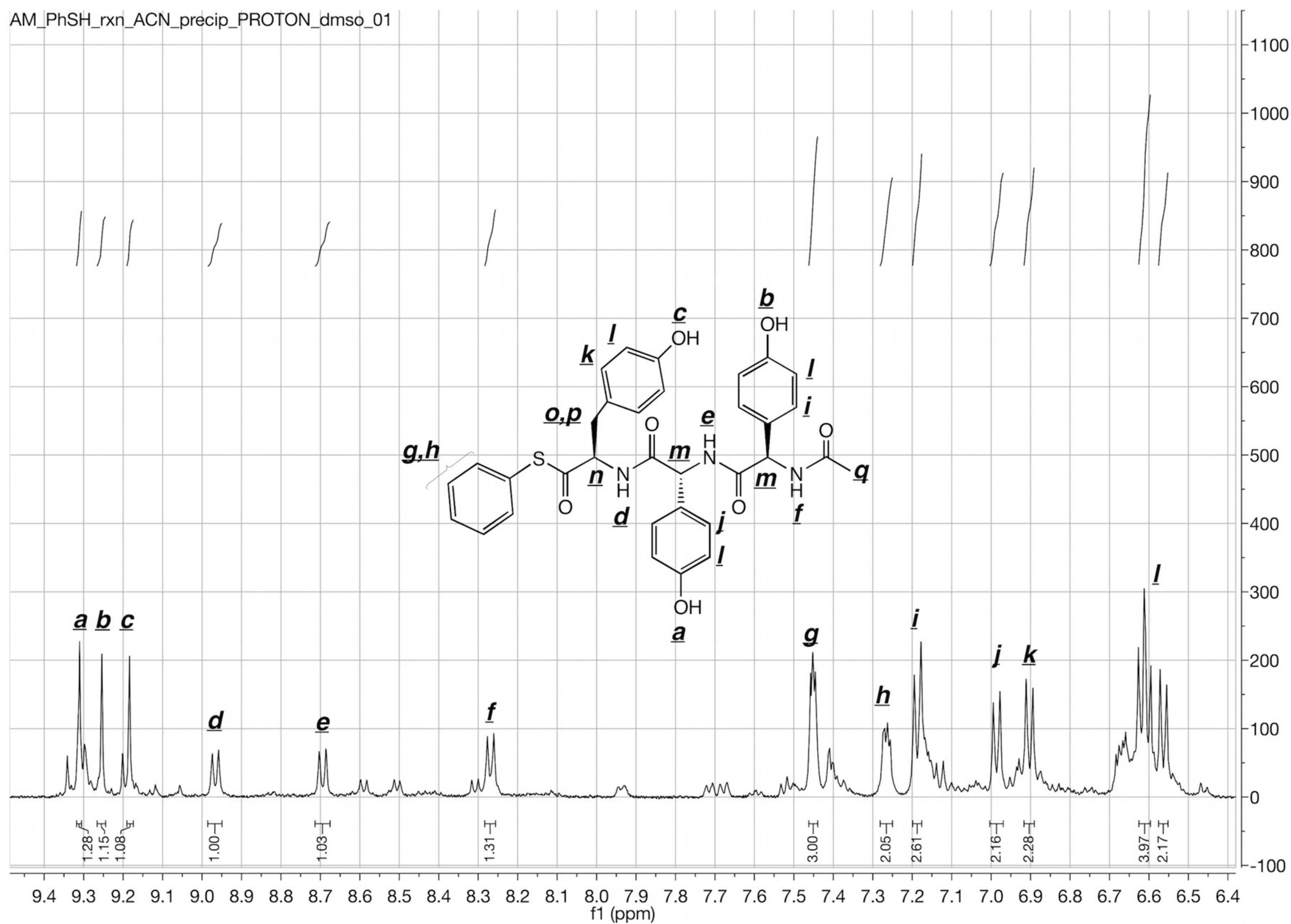
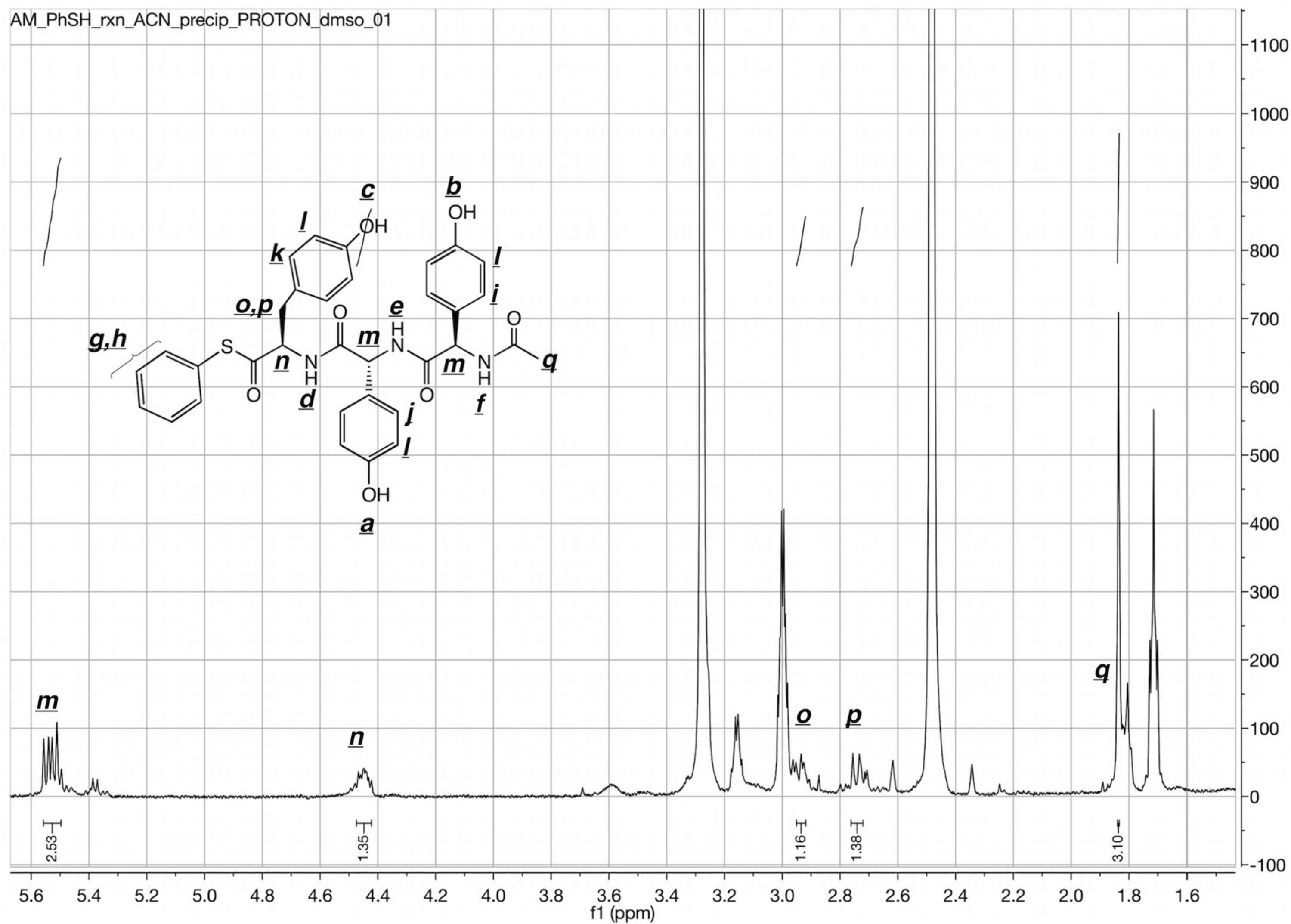
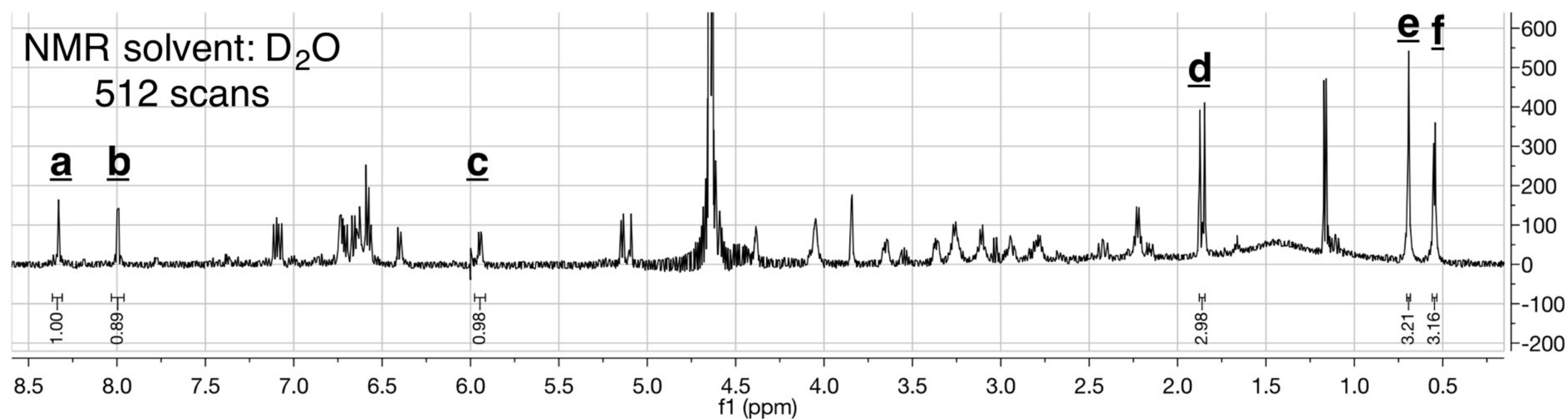


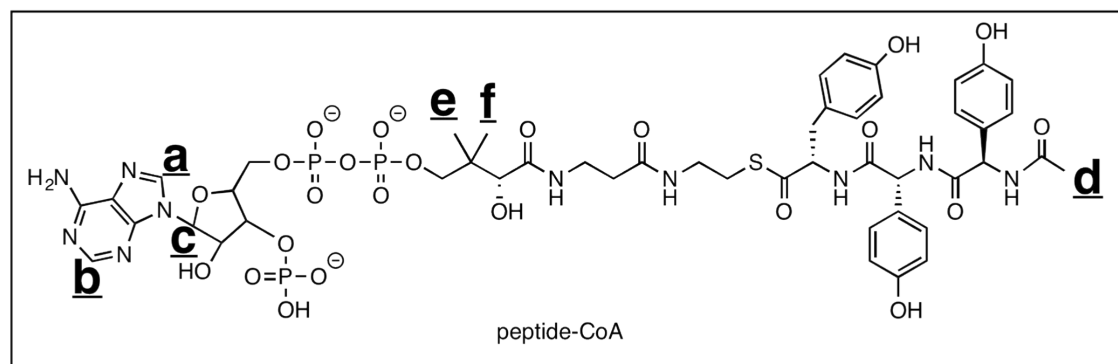
Fig. S10 (part 1 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-D-Tyr-SPh (DMSO-D_6).



* Peak at 3.33 ppm is a residual water peak; peak at 2.50 ppm is a residual solvent peak.

Fig. S10 (part 2 of 2) ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-D-Tyr-SPh (DMSO-D_6).

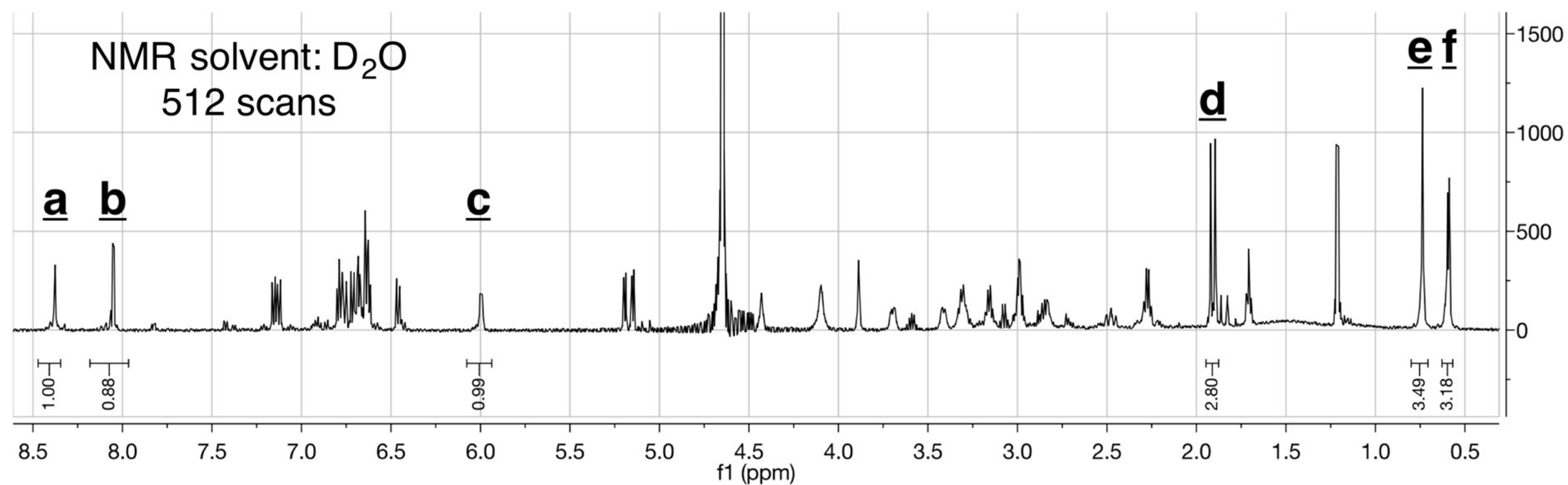


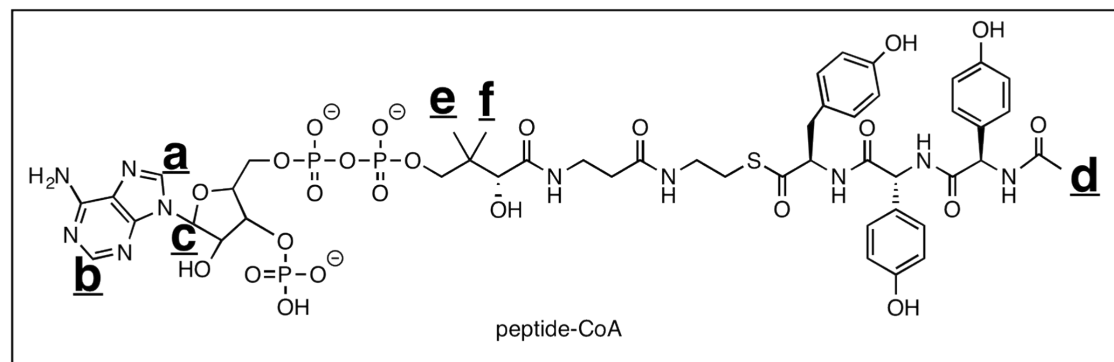


	Pred. shift* (ppm)	Obs. shift (ppm)
a	8.58	8.33
b	8.35	7.99
c	6.16	5.95
d	1.84	1.86
e	0.89	0.69
f	0.89	0.55

*ChemDraw ^{1}H shift predictions in DMSO-D_6

Fig. S11 Partially assigned ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-L-Tyr-SCoA. Peaks “a” through “f” represent signature peaks that highlight the successful formation of the CoA conjugate.





	Pred. shift* (ppm)	Obs. shift (ppm)
a	8.58	8.38
b	8.35	8.05
c	6.16	6.00
d	1.84	1.91
e	0.89	0.75
f	0.89	0.59

*ChemDraw ^{1}H shift predictions in DMSO-D_6

Fig. S12 Partially assigned ^{1}H-NMR spectrum of Ac-NH-D-Hpg-D-Hpg-D-Tyr-SCoA. Peaks “a” through “f” represent signature peaks that highlight the successful formation of the CoA conjugate.

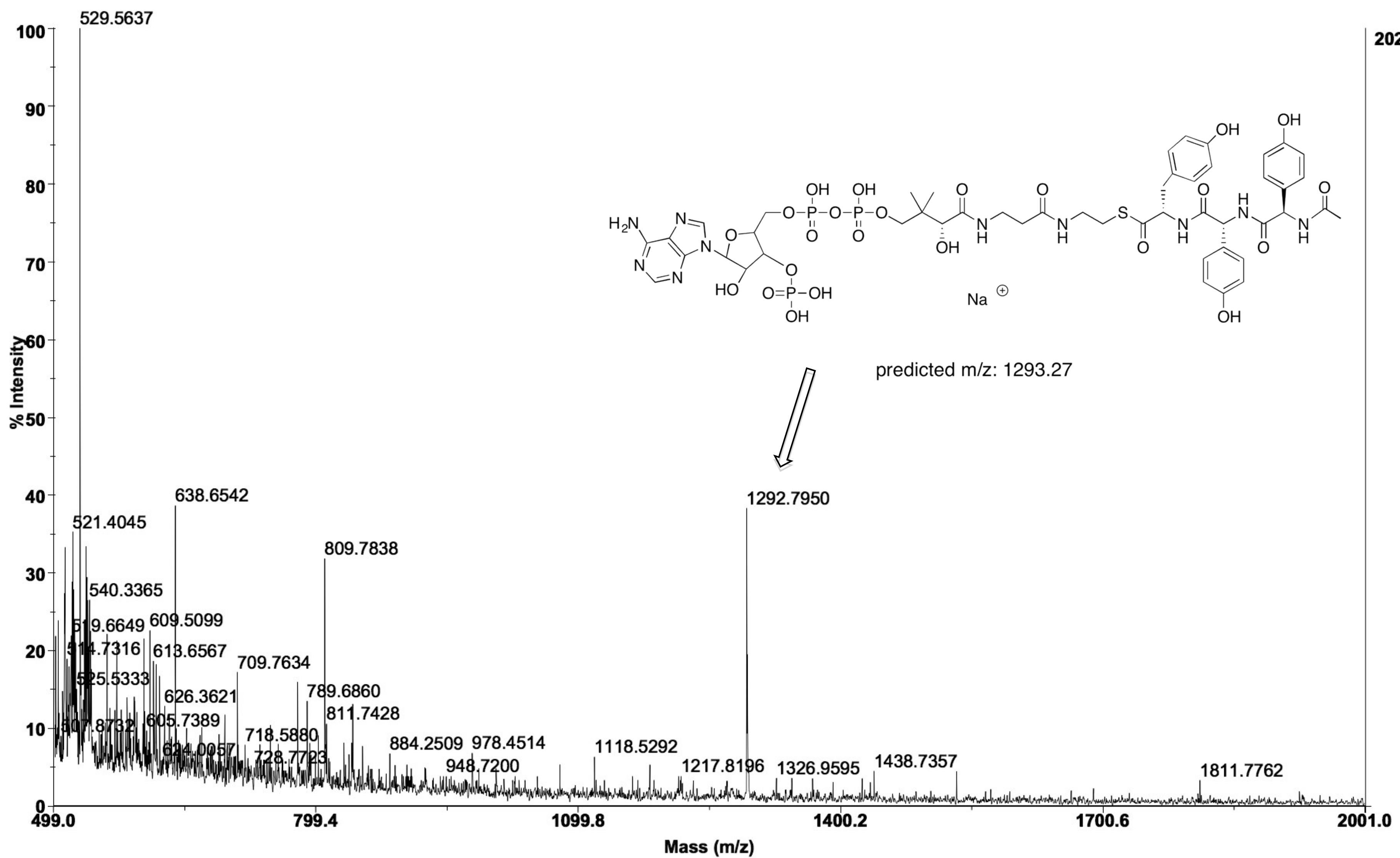


Fig. S13 MALDI-TOF-MS spectrum of Ac-NH-D-Hpg-D-Hpg-L-Tyr-SCoA.

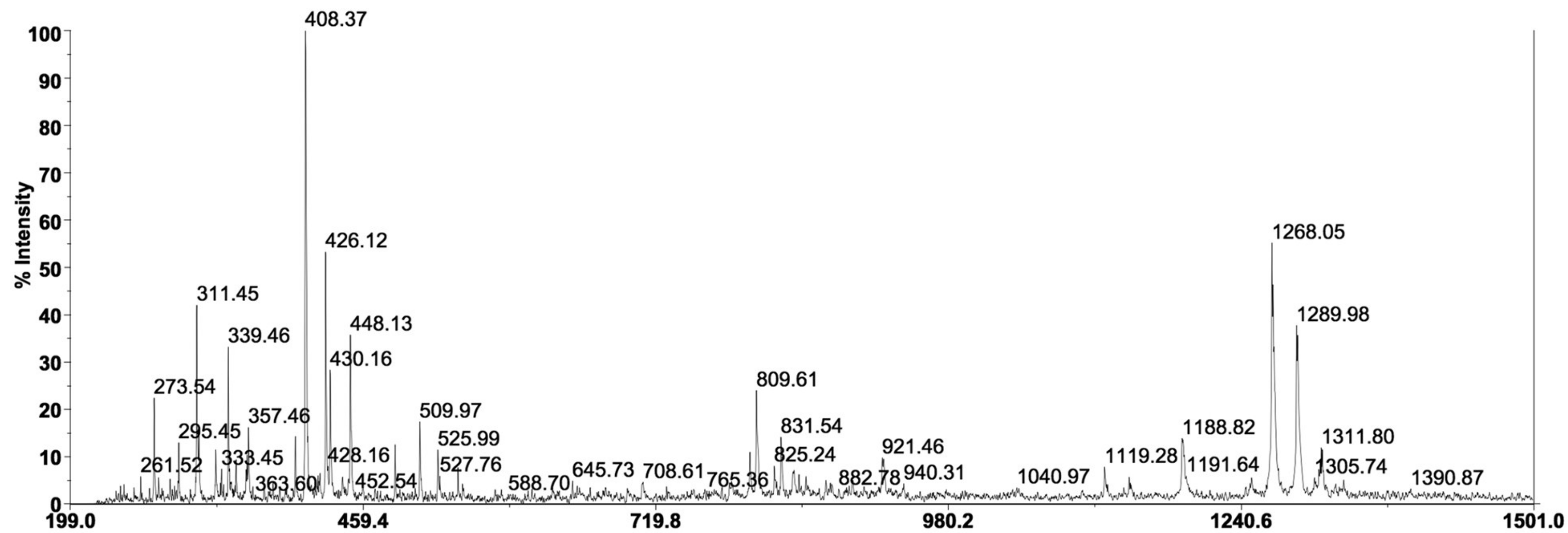


Fig. S14 MALDI-TOF-MS spectrum of Ac-NH-D-Hpg-D-Hpg-D-Tyr-SCoA.