Supporting Information

Design and Evolution of a Macrocyclic Peptide Inhibitor of the Sonic Hedgehog/Patched Interaction

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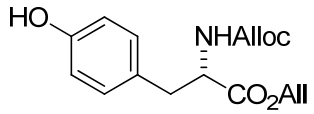
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Materials and Methods

General Information

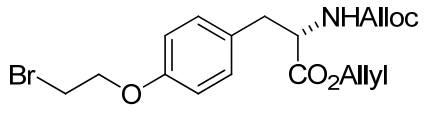
Chemical reagents and solvents were purchased from Sigma–Aldrich, Acros Organics, and Fluka and used without further purification unless stated otherwise. Rink Amide MBHA resin, activating reagents (COMU, PyBop and HOBt), Fmoc-protected amino acids, L-Tyrosine allyl ester (*p*Toluene sulfonate salt) and L-Cystine *tert*-butyl ester were purchased from Chemimpex. Fmoc-Asp(OEpe)-OH was purchased from Novabiochem. Silica gel chromatography purifications were carried out by using AMD Silica Gel 60 230–4nd00 mesh. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance spectrometers by using solvent peaks as reference. LC-MS analyses were performed on a Thermo Scientific LTQ Velos ESI/ion-trap mass spectrometer coupled to an Accela U-HPLC system. MALDI-TOF spectra were acquired on a Bruker Autoflex III MALDI-TOF spectrometer by using a stainless steel MALDI plate and sinapinic acid or alpha-cyano-4-hydroxycinnamic acid (CHCA) as matrix.

Synthetic Procedures

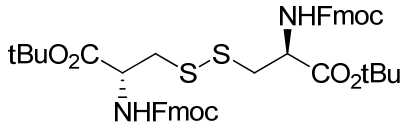


Synthesis of N-Alloc-L-Tyrosine allyl ester (2). L-Tyrosine allyl ester (*p*Toluene sulfonate salt) (**1**) (1.7 g, 4.32 mmol) was dissolved in 15 mL of water. Sodium carbonate was added to the solution (1,361 g, 12.96 mmol, 3 equiv), then allyl chloroformate (6.48 mmol, 0.68 mL, 1.5 equiv) was added dropwise to the reaction at 0°C. The reaction was stirred for 15 hours, after which it was quenched by addition of 1 M HCl (15 mL) and extracted with ethyl acetate (2 x 40 mL). The combined organic layers were washed with water (70 mL) and dried over Na_2SO_4. After removal of the solvent by rotary evaporation, the crude product was purified on a silica gel column using hexanes/ethyl acetate from 9:1 to 8:2 as eluent to yield **2** as a colorless oil (0.92 g, 70%). ^{1}H-NMR (400MHz, MeOD) δ 6.99-6.97 (d, 2H, J = 8.4 Hz), 6.67-6.65 (d, 2H, J = 8.4 Hz), 5.88-5.79 (m, 2H, J = 6.4 Hz),

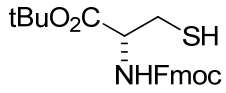
5.26-5.09 (m, 4H, J = 9.4 Hz), 4.54-4.53 (d, 2H, J= 5.6 Hz), 4.45-4.44 (d, 2H, J= 4.8 Hz), 4.34 (t, 1H, J= 6.0 Hz), 3.01-2.79 (m, 2H, J = 8.8 Hz). ^{13}C-NMR (100 MHz, MeOD) δ 171.7, 171.4, 156.7, 155.8, 132.7, 131.7, 129.7, 127.3, 117.1, 116.0, 114.7, 65.2, 64.9, 59.9, 55.7, 36.3, 19.4, 12.9 MS-ESI: Calc. Mass for $C_{16}H_{19}NO_5$: 305.3 Da. Obs. Mass for $[M-H]^-$: 304.3 Da.



Synthesis of N-Alloc O-(2-bromoethyl)-L-Tyrosine allyl ester (3). N-Alloc-L-tyrosine allyl ester **2** (0.92 g, 3.02 mmol) was dissolved in 15 mL dry DMF under argon flow. K_2CO_3 (1.25 g, 9.06 mmol, 3 equiv) was added to the reaction and stirred vigorously for 10 minutes. Then 1,2-dibromoethane (0.8 mL, 9.06 mmol, 3 equiv) was added to the suspension dropwise. The reaction was stirred overnight and then quenched with HCl 1M (15 mL). The crude product was extracted using ethyl acetate (2 x 40 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. After removal of the solvent by rotary evaporation, the crude product was purified on silica gel column using hexanes/ethyl acetate from 9:1 to 7:3 to yield **3** as a colorless oil (0.43 mg, 35%) and recovered starting material (0.55 g, 60%). ^{1}H-NMR (400MHz, MeOD) δ 7.02-7.00 (d, 2H, J= 8.4 Hz) 6.80-6.78 (d, 2H, J= 8.8 Hz), 5.89-5.78 (m, 2H, J= 6.0 Hz), 5.29-5.15 (m, 4H, J= 10.0 Hz), 4.57-4.56 (d, 2H, J= 5.6 Hz), 4.52-4.50 (d, 2H, J= 5.2 Hz), 4.57-4.51 (m, 1H,), 4.23-4.20 (t, 2H, J= 6.4 Hz), 3.59-3.56 (t, 2H, J= 6.4 Hz), 3.08-2.97 (m, 2H, J= 6.4 Hz) ^{13}C-NMR (100MHz, MeOD) δ 171.1, 157.1, 155.3, 132.4, 131.3, 130.3, 128.4, 118.9, 117.6, 114.7, 67.7, 65.9, 65.6, 54.7, 37.2, 28.9. ESI-MS: Calc. Mass for $C_{18}H_{22}BrNO_5$: 412.28 Da. Obs. Mass for $[M+Na]^+$: 434.3 Da.

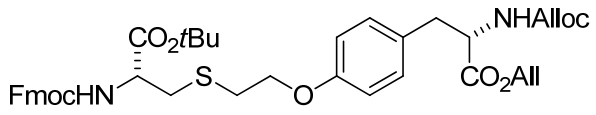


Synthesis of (2R,2'R)-di-tert-butyl 3,3'-disulfanediylbis(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)propanoate) (5). L-Cystine *tert*-butyl ester **4** (2 mmol, 704 mg) was suspended in 10 mL of THF and N-methyl morpholine (4 mmol, 0.520 mL, 2 equiv) was added to the suspension. The solution was chilled to 0°C in an ice bath and then 9-fluorenylmethyl-N-succinimidyl carbonate (Fmoc-OSu) (2 mmol, 675 mg) was added slowly portion-wise. The reaction was stirred for 18 hours allowing to return at room temperature . The solvent was removed under reduced pressure and the crude product was dissolved in 25 mL of ethyl acetate. The organic layer was washed with 20 mL of HCl 0.1 M and then with 20 ml of brine. The organic layer was dried over Na_2SO_4 filtered and evaporated. The crude product was purified by silica gel column using hexanes/diethyl ether (7:3) to yield **5** as a white solid (1.2 g, 75%). ^{1}H-NMR (400MHz, CDCl3) 7.76-7.74 (d, 4H, J= 7.6 Hz), 7.62-7.60 (d, 4H, J= 7.2 Hz), 7.41-7.37 (t, 2H, J= 7.2 Hz), 7.32-7.29 (t, 2H, J= 7.2 Hz), 4.48-4.46 (m, 2H), 4.39-4.38 (t, 4H, J= 7.2 Hz), 4.25-4.23 (t, 2H, J= 7.2 Hz), 3.24-3.15 (m, 4H), 1.47 (s, 9H). ^{13}C-NMR (100MHz, CDCl3) 169.4, 155.5, 143.6, 127.5, 126.9, 125.0, 125.0, 119.8, 82.9, 79.8, 67.1, 60.2, 46.9, 28.6, 28.2, 27.8, 27.3. ESI-MS. Calc. Mass for $C_{44}H_{48}N_2O_8S_2$: 796.29 Da Obs. Mass: 819.4 [M+Na].

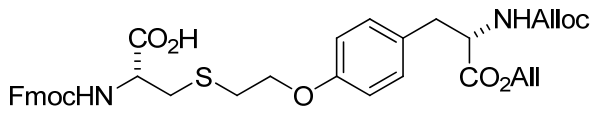


Synthesis of N-Fmoc-L-Cysteine *t*-butyl ester (6). 1.2 g of N,N'-Fmoc-Cystine *t*-butyl ester (**5**) (1.72 mmol) was dissolved in 20 mL of THF. Triphenylphosphine (0.9 g, 3.44 mmol, 2 equiv) was added to the solution and the reaction mixture was stirred for 2 hours at room temperature. Water (2 mL) was then added and the reaction mixture was stirred for 10 hours. The solvent was removed by rotary evaporation and the residue was taken up in EtOAc, washed with 10% citric acid and brine, dried over sodium sulfate and concentrated. The crude product was purified on silica gel column using hexanes/ethyl acetate from 95:5 to 8:2 ratio to yield **6** as a colorless oil (0.4 g, 60%). ^{1}H-NMR (400MHz,

CDCl3) 7.78-7.76 (d, 2H, J= 7.6 Hz), 7.62-7.60 (d, 2H, J= 7.2 Da), 7.42-7.39 (t, 2H, J= 7.6 Da), 7.34-7.30 (t, 2H, J= 7.6 Hz), 4.54 (m, 1H), 4.43-4.39 (t, 2H, J= 7.2 Hz), 4.25-4.21 (t, 1H, J= 6.8 Hz), 3.00-2.98 (m, 2H, J= 7.6 Hz), 1.43 (s, 9H). ^{13}C-NMR (100MHz, CDCl3) 171.0, 159.9, 141.1, 127.5, 127.4, 126.9, 125.0, 124.6, 119.8, 82.5, 68.2, 67.1, 60.2, 46.9, 27.9, 27.7, 20.8. MS-ESI$^-$: Calc. Mass for $C_{22}H_{25}NO_4S$: 399.51 Da Obs. Mass: 422.3 [M+Na].



(R)-tert-butyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-((2-(4-((S)-2-(((allyloxy)carbonyl)amino)-3-oxo-3-(prop-1-en-1-yloxy)propyl)phenoxy)ethyl)thio) propanoate (6b). N-alloc-O-(2-bromoethyl)-L-Tyrosine allyl ester **3** (0.43 mg, 1.04 mmol) and N-Fmoc-L-cysteine *t*-butyl ester **6** (0.41 mg, 1.04 mmol) were dissolved in 5 mL of dry ethyl acetate. Tetrabutylammonium bromide (1.29 g, 4.0 mmol) was dissolved in 5 mL of nitrogen-sparged $NaHCO_3$ solution (0.5 M), which was added to the reaction mixture dropwise under argon. The reaction was stirred vigorously for 16 hours, then diluted with ethyl acetate. The organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated. The crude product was purified on silica gel column using hexanes/ethyl acetate from 9:1 to 7:3 to yield **7** as a colorless oil (0.29 g, 40%).^{1}H-NMR (400MHz, $CDCl_3$) δ 7.73-7.71 (d, 2H, J= 7.2 Hz), 7.57-7.55 (d, 2H, J= 7.6 Hz), 7.37-7.34 (t, 2H, J= 7.6 Hz), 7.28-7.24 (t, 2H, J= 8.0 Hz), 6.99-6.96 (d, 2H, J= 8.4 Hz), 6.78-6.75 (d, 2H, J= 8.4 Hz), 5.82 (m, 2H, J= 6.4 Hz), 5.29-5.15 (m, 4H, J= 10.8 Hz), 4.57-4.55 (d, 2H, J= 5.6 Hz), 4.52-4.50 (d, 2H, J= 5.2 Hz), 4.35-4.34 (t, 2H, J= 3.6 Hz), 4.18 (t, 1H, J= 6.8 Hz), 4.07 (t, 2H, J= 7.2 Hz), 3.10-2.99 (m, 4H, J= 5.2 Hz), 2.90-2.87 (t, 2H, J= 6.0 Hz), 1.45 (s, 9H). ^{13}C-NMR (100MHz, $CDCl_3$) δ 171.1, 169.5, 157.39, 155.5, 155.3, 143.6, 141.1, 131.2, 130.2, 127.9, 127.5, 126.9, 124.9, 119.8, 118.9, 117.7, 114.5, 82.8, 67.6, 66.9, 65.8, 65.6, 60.2, 54., 54.2, 46.9, 37.1, 35.0, 31.7, 27.8, 14.0.



Synthesis of (R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-((2-(4-((S)-2-(((allyloxy)carbonyl)amino)-3-oxo-3-(prop-1-en-1-yloxy)propyl)phenoxy)ethyl)thio) propanoic acid (7). To a solution of **6b** (0.29 g, 0.4 mmol) in CH_2Cl_2 (6 mL) was added 4 mL of trifluoroacetic acid (TFA) at 0°C. The reaction was stirred at 0°C for 2 hours. The product was concentrated in vacuo, then washed extensively with diethyl ether. The final product was yielded as a white crystalline powder. ^{1}H-NMR (400MHz, MeOD) δ 7.77-7.75 (d, 2H, J= 7.2 Hz), 7.65-7.63 (d, 2H, J= 6.8 Hz), 7.37-7.34 m (t, 2H, J= 7.6 Hz), 7.29-7.25 (t, 2H, J= 7.6 Hz), 7.08-7.057 (d, 2H, J= 8.4 Hz), 6.82-6.79 (d, 2H, J= 8.8 Hz), 5.85 (m, 2H), 5.29-5.11 (m, 4H), 4.57-4.30 (m, 8H, J= 5.6 Hz), 4.19 (t,1H), 4.11-4.08 (t, 2H, J= 6.0 Hz), 2.99-2.84 (m, 6H). ^{13}C-NMR (100MHz, MeOD) δ 173.5, 171.5, 171.4, 169.8, 157.6, 157.4, 156.1, 156.0, 155.8, 143.7, 141.3, 132.4, 131.7, 131.3, 130.4, 128.1, 128.0, 127.7, 127.1, 125.1, 124.8, 120.0, 119.2, 119.1, 118.3, 114.8, 114.7, 83.2, 67.9, 67.3, 66.2, 55.9, 55.0, 47.1, 37.4, 35.9, 31.9.

Figure S1. Close-up view of HHIP L2 loop interaction with Shh (pdb 3HO5). The Shh protein is shown as a surface model (green), whereas the L2 loop region of HHIP is shown as a stick model (pink). The remainder of the HHIP protein as shown in Figure 2 is omitted for clarity. The residues selected for the installation of the thioether bridge along with the zinc ion binding aspartate residue are labeled. The zinc ion is shown as sphere model (blue).

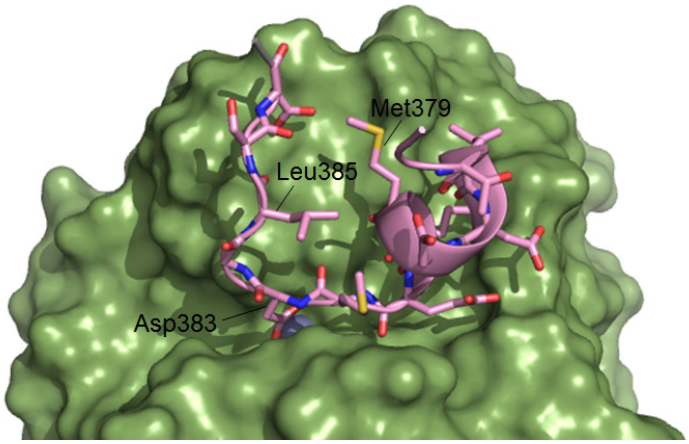


Figure S2. Model of evolved macrocyclic peptide HL2-m5 in complex with Shh. Shh protein is shown as a surface model (green), whereas the macrocyclic peptide is shown as a stick model (blue). The mutated residues with respect to HL2-m1 are color coded as shown in **Figure 3**. The N-terminal and C-terminal residues along with Trp4, Ala7, and Met10 are labeled.

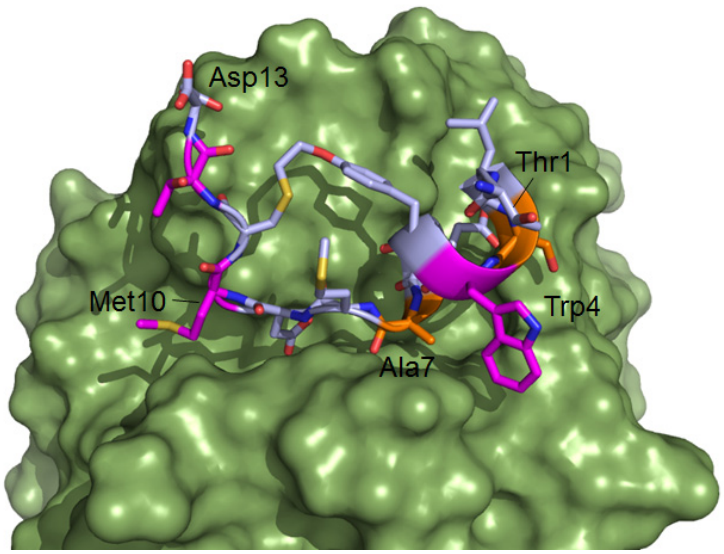


Figure S3. Relative Shh binding activity for representative hits from the single-site site-saturation libraries. Absorbance values (X axis) are normalized to that of HL2-m1. Indicated mutations (Y axis) are relative to the HL2-m1 sequence. The mean values and error bars were derived from rescreening of the hits identified during the library screening in triplicate.

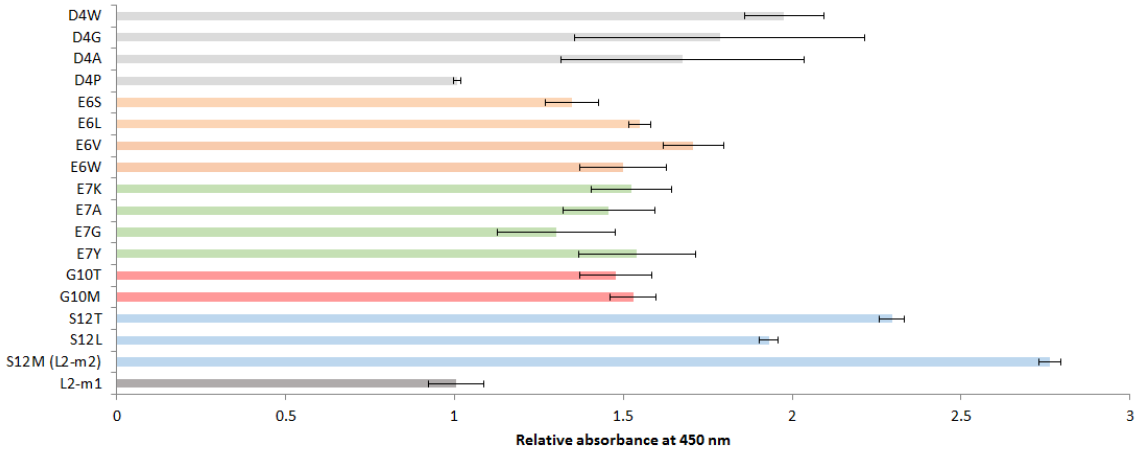


Figure S4. Relative Shh binding activity for representative hits from the multi-site recombinant libraries. Absorbance values (X axis) are normalized to that of HL2-m1. Indicated mutations (Y axis) are relative to the HL2-m1 sequence. The mean values and error bars were derived from rescreening of the hits identified during the library screening in triplicate.

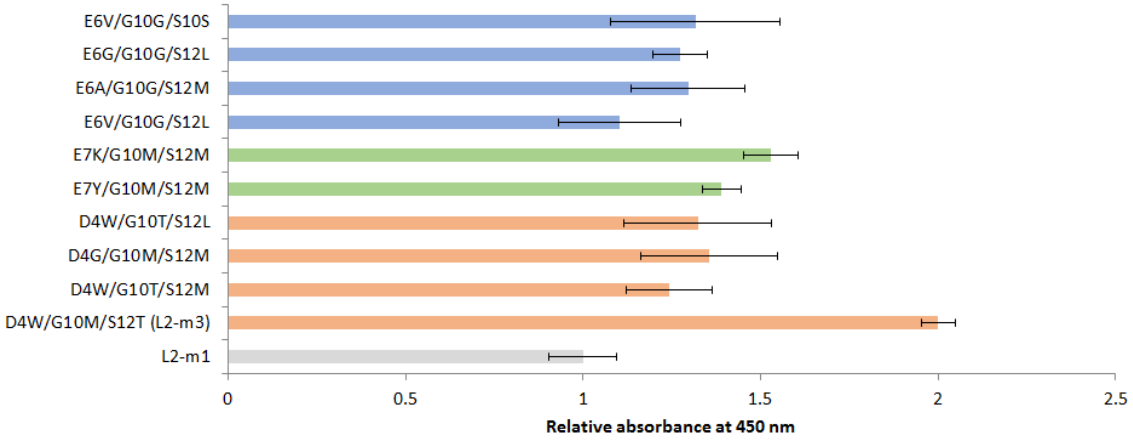


Figure S5. SDS-PAGE gel of recombinantly expressed GST-Shh, GST-Ihh, and GST-Dhh after purification by Ni-affinity chromatography.

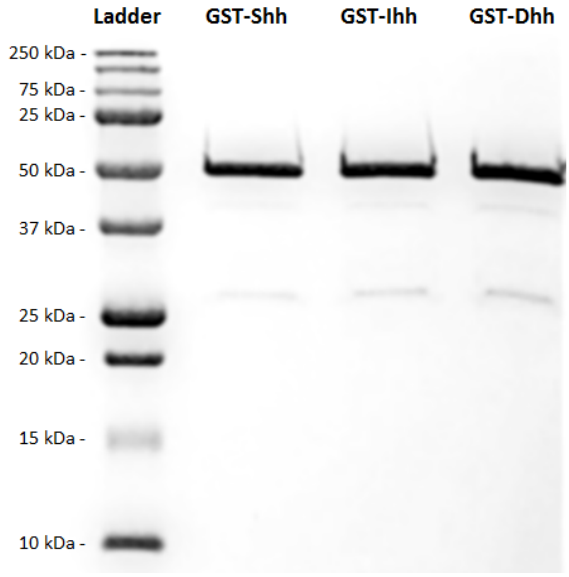


Figure S6. Thiol-induced intein cleavage reactions. MALDI-TOF MS spectra corresponding to the GyrA cleavage reactions for FLAG-(HL2-m1)-GyrA (a), FLAG-(HL2-m3)-GyrA (b), and FLAG-(HL2-m5)-GyrA (c) after incubation with thiophenol. Calculated and observed *m/z* values corresponding to the proton adducts of the macrocycles are indicated. The species labeled with the star (*) corresponds to thiophenol thioester. The absence of acyclic or hydrolysis byproducts indicates that the constructs have undergone quantitative cyclization upon expression in *E. coli* cells.

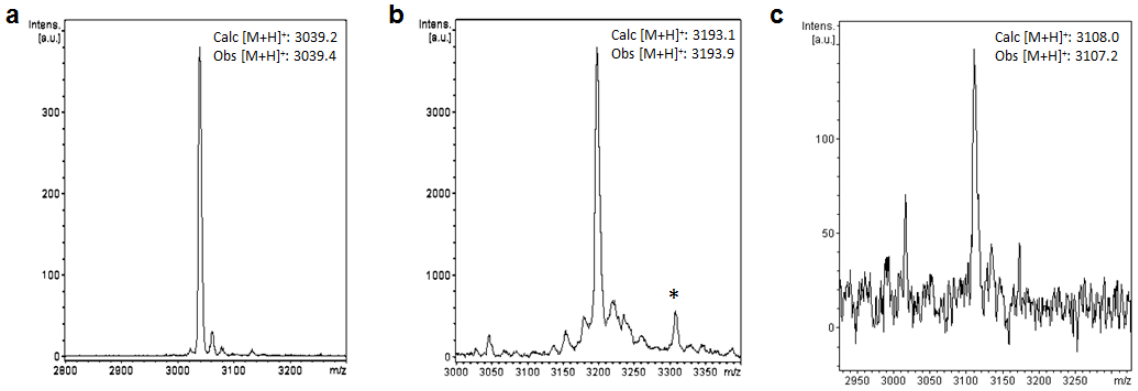


Figure S7. MALDI-TOF MS spectra corresponding to purified FLAG-tagged linear and cyclic L2 mimics obtained via recombinant expression. Calculated and observed *m/z* values corresponding to the proton adduct of the macrocycles are indicated.

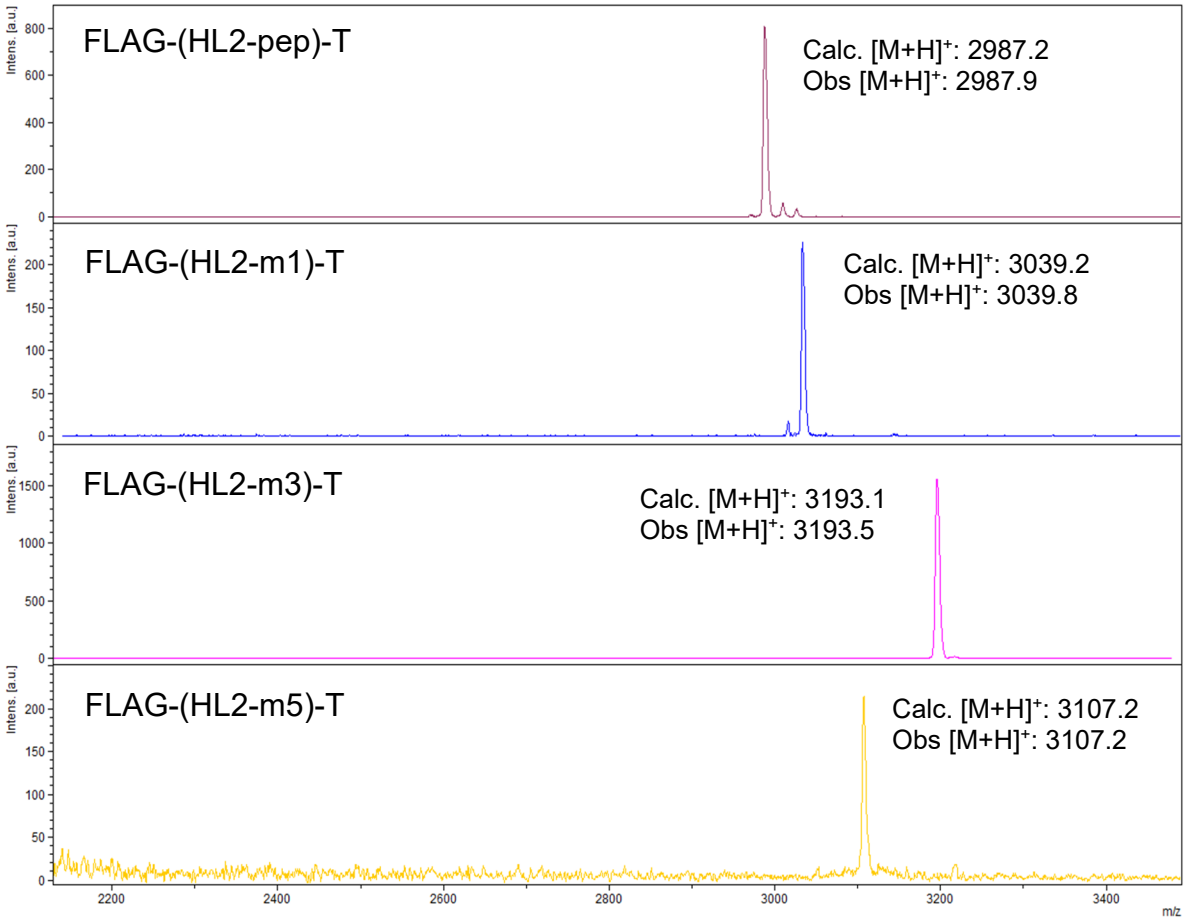
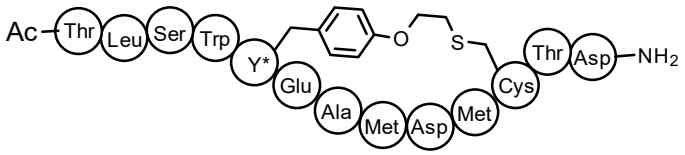
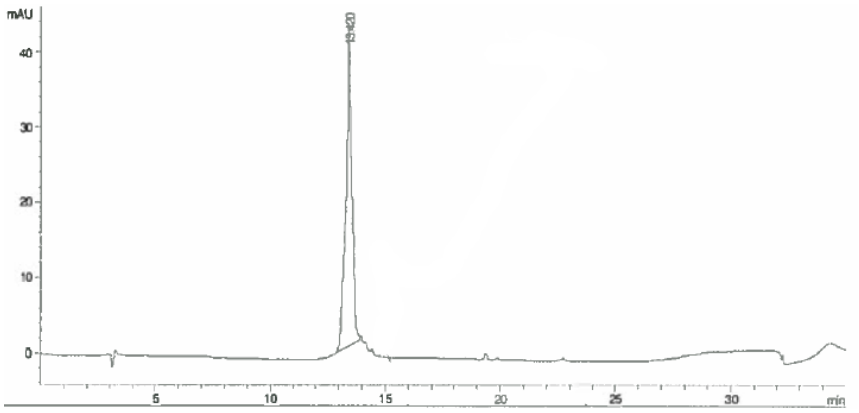


Figure S8. Analytical HPLC chromatogram (A) and ESI-MS spectra in positive (B) and negative mode (C) corresponding to synthetic HL2-m5. Y* = alkylated O2beY. See Table S2 for further details.

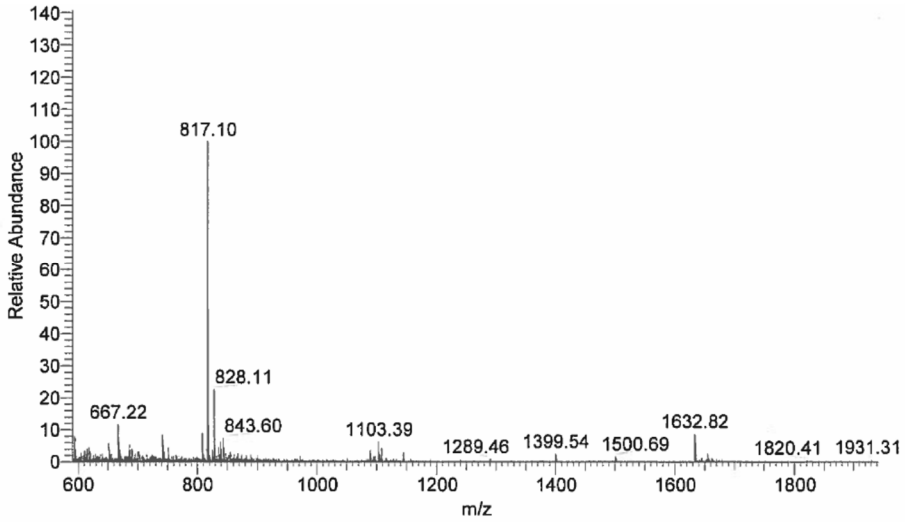


HL2-m5

A)



B)



C)

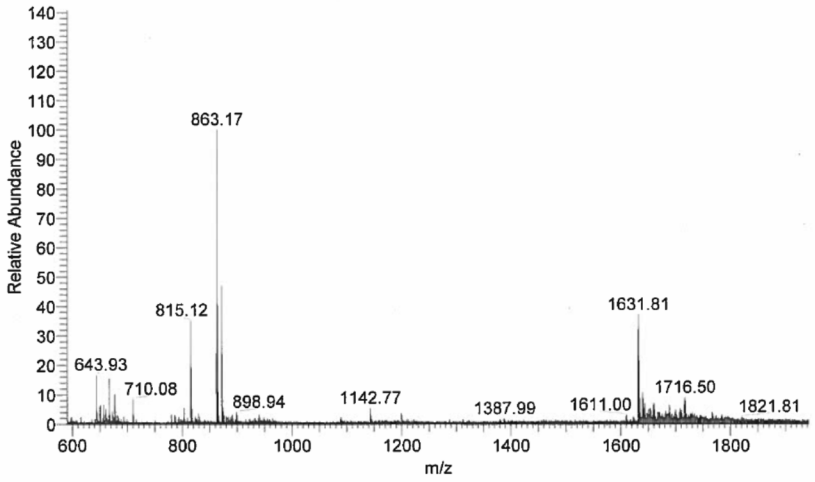
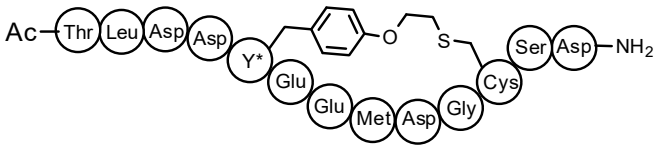
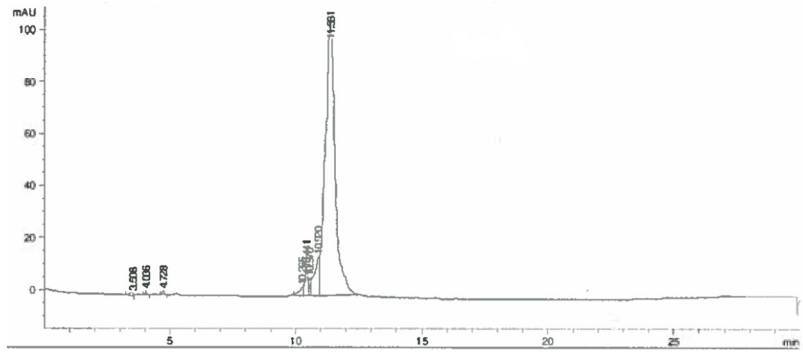

Relative Abundance
m/z
863.17
815.12
1631.81
643.93
710.08
898.94
1142.77
1387.99
1611.00
1716.50
1821.81

Figure S9. Analytical HPLC chromatogram (A) and ESI-MS spectra in positive (B) and negative mode (C) corresponding to synthetic HL2-m1. Y* = alkylated O2beY. See Table S2 for further details.

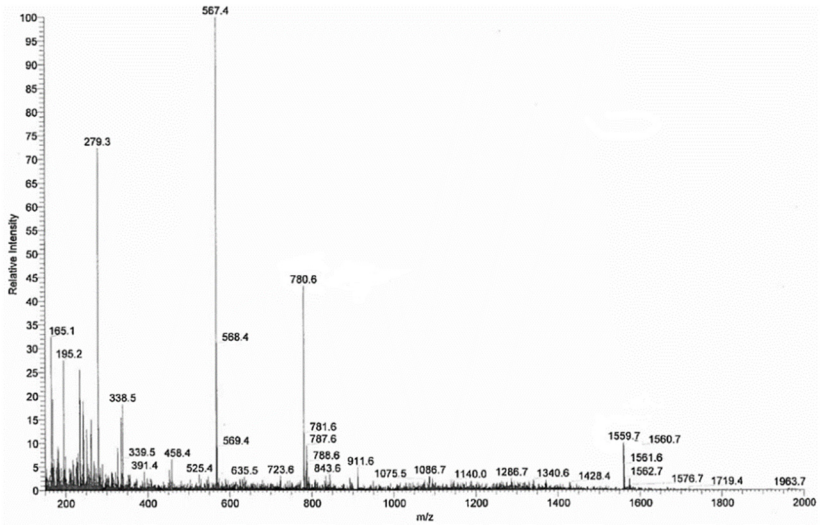


HL2-m1

A)



B)



C)

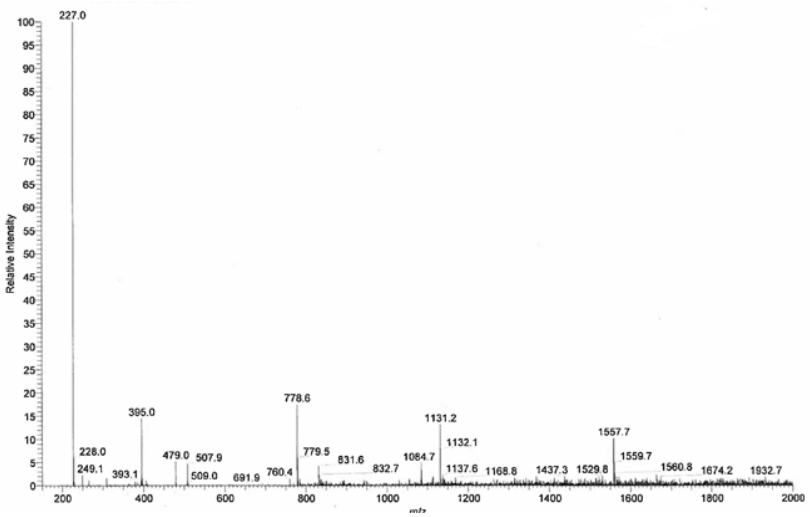

Relative Intensity
227.0
395.0
778.6
1131.2
1132.1
1557.7
228.0
249.1
393.1
479.0
507.9
509.0
691.9
760.4
779.5
831.6
832.7
1064.7
1137.6
1168.8
1437.3
1529.8
1559.7
1560.8
1674.2
1932.7

Figure S10. Inhibition curve corresponding to HL2-m5 induced inhibition of FLAG-HL2-m5 binding to plate-immobilized GST-Shh. The data were fitted to a four-parameter equation, from which a IC_{50} of 280 ± 50 nM was calculated. The mean values and standard deviations were obtained from experiments performed in triplicate. The similarity between the IC_{50} value determined in this assay and the K_D value measured for FLAG-HL2-m5 in the direct Shh binding assay (**Figure 3**) indicates the FLAG tag does not significantly affect the Shh binding affinity of the cyclic peptide.

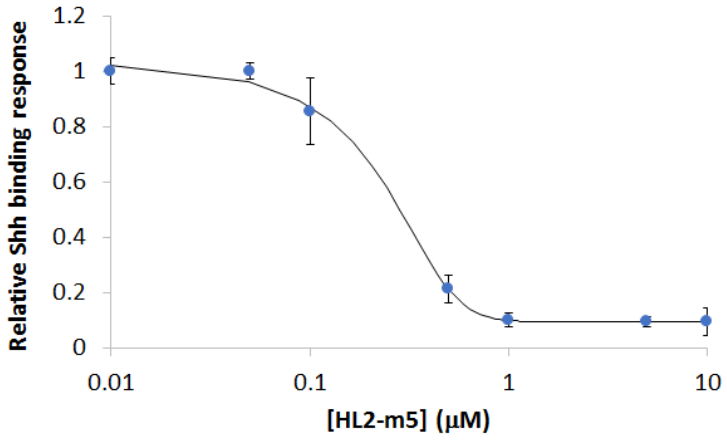


Figure S11. Dose-response curves for direct binding of FLAG-HL2-m5 to plate-immobilized GST-Shh, GST-Ihh, or GST-Dhh as determined using the colorimetric assay with HRP-conjugated anti-FLAG antibody.

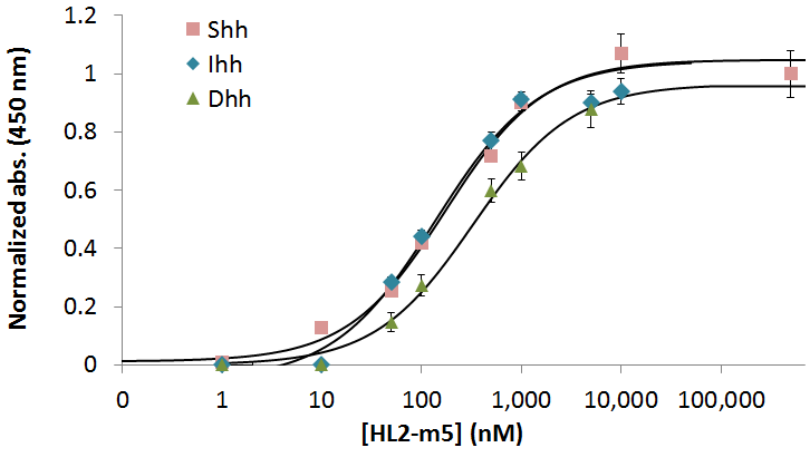


Figure S12. Proteolytic stability of linear and cyclic L2 mimics. The graph indicates the residual amount of HL2-pep, HL2-m1, and HL-m5 peptides related after incubation in human blood serum (37°C) at different time points as determined by analytical HPLC. Values are normalized to peak areas corresponding to the same peptide in buffer only. Under identical assay conditions, an unrelated linear peptide ($p53_{15–29}$ peptide in Smith *et al.*, *Chemical Commun.* 2014, 50, 5027) exhibited a half-time ($t_{1/2}$) < 1 hour, indicating the HHIP L2-derived sequence is inherently resistant to proteolytic degradation.

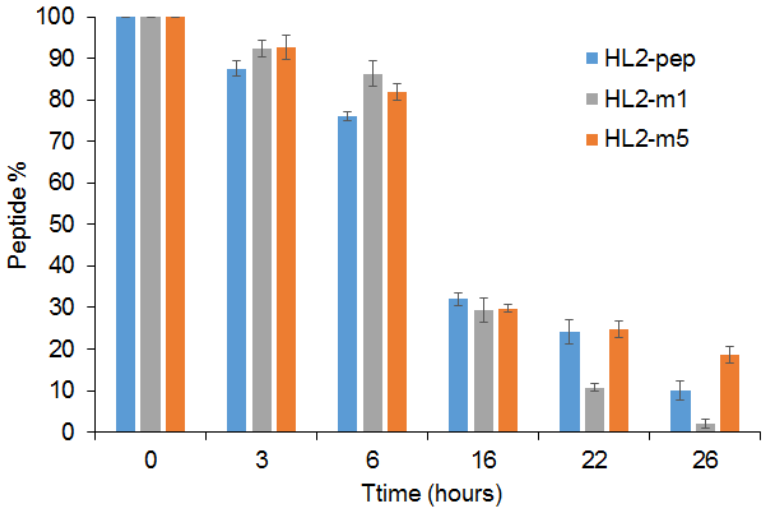


Table S1. Oligonucleotide sequences.

Primer	Sequence (5' to 3')
01_Shh_Forward	CTGCGCCATGGGTGGACCGGGCAGGGGGT
02_Shh-Reverse	GAAGACTCGAGTCAGCCTCCCGATTTGGCCG
03_Dhh_Forward	ACTATACCATGGGTGGGCCGGGCCG
04_Dhh_Reverse	ACTATACTCGAGTCAGCCCGCCCGGAC
05_Ihh_Forward	ACTATACCATGGGTGGGCCGGGTCGGGTGGT
06_Ihh_Reverse	ACTATACTCGAGTCAGCCCGTCTTGGCTGCGG
07_L2(T)_Forward	TAGAGGATCCACCCTGGACGATATGGAAGAGATGGACGGCCTGAGTGA TACCTGCATCACGG
08_GyrA reverse	CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTA
09_L2(D)_Forward	TAGAGGATCCACCCTGGACGATATGGAAGAGATGGACGGCCTGAGTGA TGATTGCATCACGG
10_L2-m1(T)_Forward	TAGAGGATCCACCCTGGACGATTAGGAAGAGATGGACGGCTGCAGTGA TACCTGCATCACGG
11_L2-m1(D)_Forward	TAGAGGATCCACCCTGGACGATTAGGAAGAGATGGACGGCTGCAGTGA TGATTGCATCACGG
12_D4(NNK)_Forward	TAGAGGATCCACCCTGGACNNKTAGGAAGAGATGGACGGCTGCAGTGA TTGCATCACGGG
13_E6(NNK)_Forward	TAGAGGATCCACCCTGGACGATTAGNNKGAGATGGACGGCTGCAGTGA TTGCATCACGGG
14_E7(NNK) Forward	TAGAGGATCCACCCTGGACGATTAGGAANNKATGGACGGCTGCAGTGA TTGCATCACGGG
15_G10(NNK)_Forward	TAGAGGATCCACCCTGGACGATTAGGAAGAGATGGACNNKTGCAGTGA TTGCATCACGGG
16_S12(NNK)_Forward	TAGAGGATCCACCCTGGACGATTAGGAAGAGATGGACGGCTGCNNKGA TTGCATCACGGG
17_Recombination1_F1	TAGAGGATCCACCCTGGACGATTAGKBGGAGATGGACAYGTGCWYGGA TTGCATCACGGG
18_ Recombination1_F2	TAGAGGATCCACCCTGGACGATTAGKBGGAGATGGACGGCTGCWYGGA TTGCATCACGGG
19_ Recombination2_F1	TAGAGGATCCACCCTGGACGATTAGGAAWAMATGGACAYGTGCWYGG ATTGCATCACGGG
20_ Recombination2_F2	TAGAGGATCCACCCTGGACGATTAGGAAWAMATGGACGGCTGCWYGG ATTGCATCACGGG
21_ Recombination3_F1	TAGAGGATCCACCCTGGACKGGTAGGAAGAGATGGACAYGTGCWYGG ATTGCATCACGGG
22_ Recombination3_F2	TAGAGGATCCACCCTGGACGATTAGGAAGAGATGGACGGCTGCWYGGA TTGCATCACGGG
23_L2-m3_D3(NNK)_Forward	TAGAGGATCCACCCTGNNKTGGTAGGAAGAGATGGACATGTGCACCGA TACCTGCATCAC
24_L2-m3_E7(NNK)_Forward	TAGAGGATCCACCCTGGATTGGTAGGAANNKATGGACATGTGCACCGA TACCTGCATCAC
25_L2-m3(T)	TAGAGGATCCACCCTGGATTGGTAGGAAGAGATGGACATGTGCACCGA TACCTGCATCAC
26_L2-m5(T)	TAGAGGATCCACCCTGTCCTGGTAGGAAGCCATGGACATGTGCACCGAT ACCTGCATCAC
27_Cyclophilin F*	TATAAGGGTTCCTCCTTTCACAGAA
28_Cyclophilin R*	GGACCTGTATGCTTTAGGATGAAGT
29_Gli1F*	AAGGAATTCGTGTGCCATTGGG
30_Gli1R*	ACATGTAAGGCTTCTCACCCGT

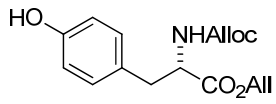
31_Gli2F*	TCCAGTCAATGGTTCTGTCC
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33_Ptch1F*	CATAGCTGCCCAGTTCAAGT
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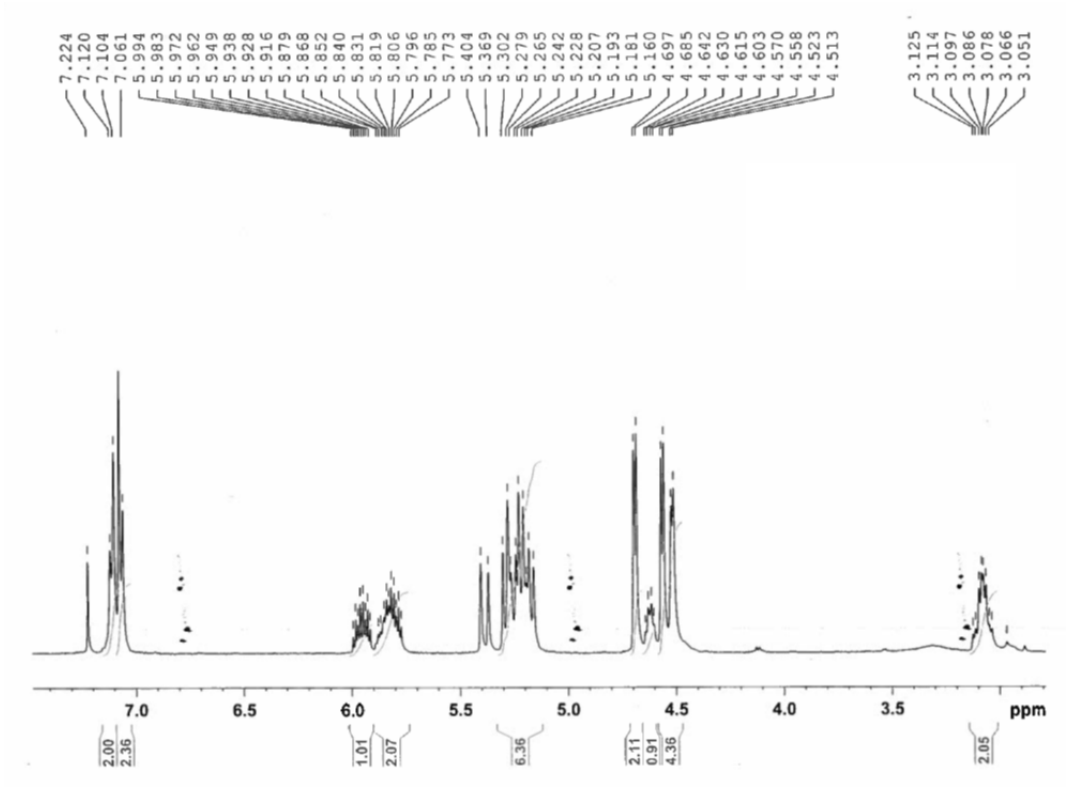
(*) Denotes real-time PCR primer.

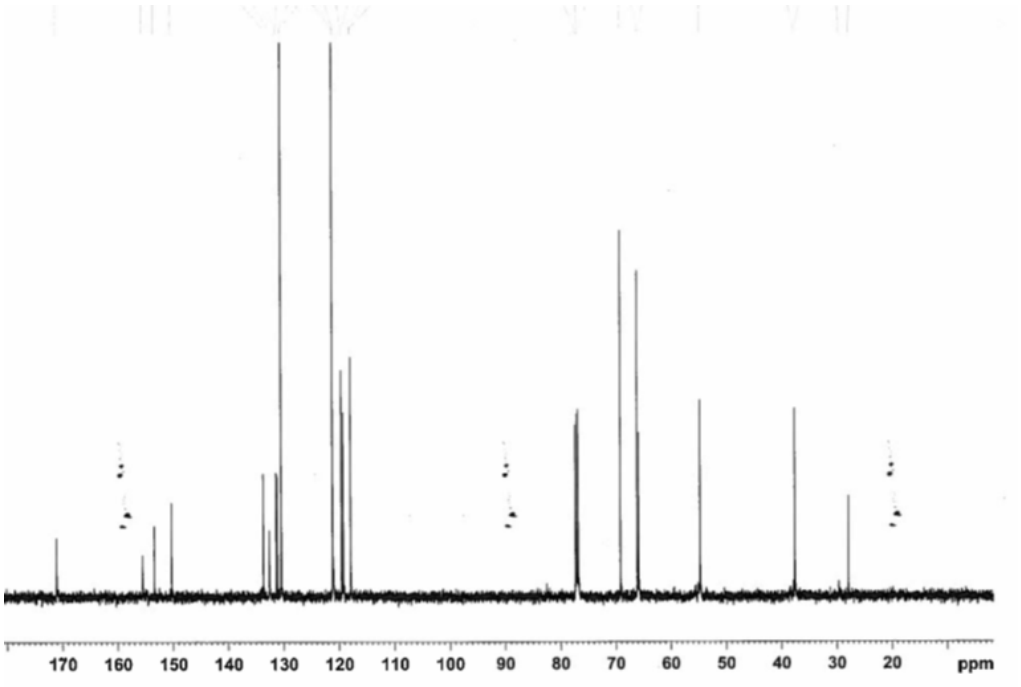
Table S2. MS data and retention times for linear and cyclic L2 mimics. HPLC analyses were performed using an Agilent 1200 series HPLC system equipped with a Grace Vision HT C18 HL column (21.2 x 250 mm; 5μ) and multidiode array detector. Method: linear gradient of H_2O (0.1% TFA)/CH_3CN (0.1% TFA) from 20 to 75% of CH_3CN (0.1% TFA) in 17 min at a flow rate of 1 mL/min.

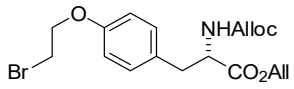
Peptide	Calc. Mass	Observed Mass $[M+H]^+$	Observed Mass $[M+Na]^+$	Observed Mass $[M-H]^-$	Retention Time
HL2-pep	1511.59 Da	1512.44 Da	Not obs.	n/a	9.9 min
HL2-m1	1559.59 Da	1559.70 Da	Not obs.	1557.70 Da	11.3 min
HL2-m5	1632.84 Da	1633.82 Da	1656.40 Da	1631.81 Da	13.42 min

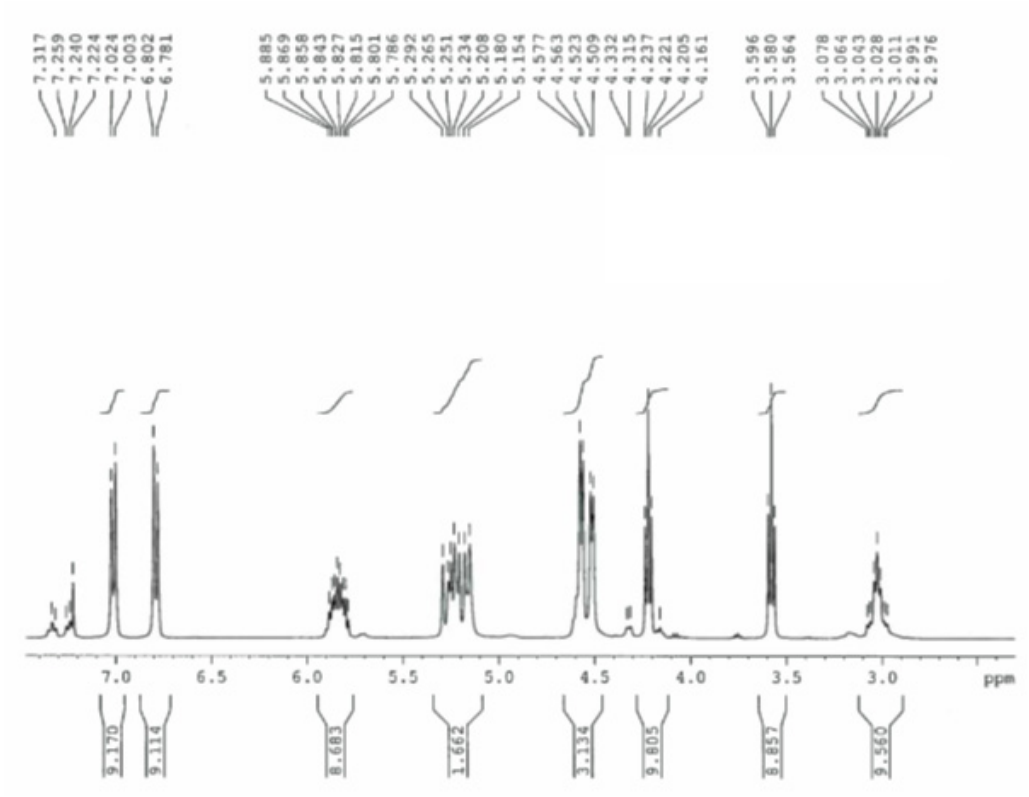
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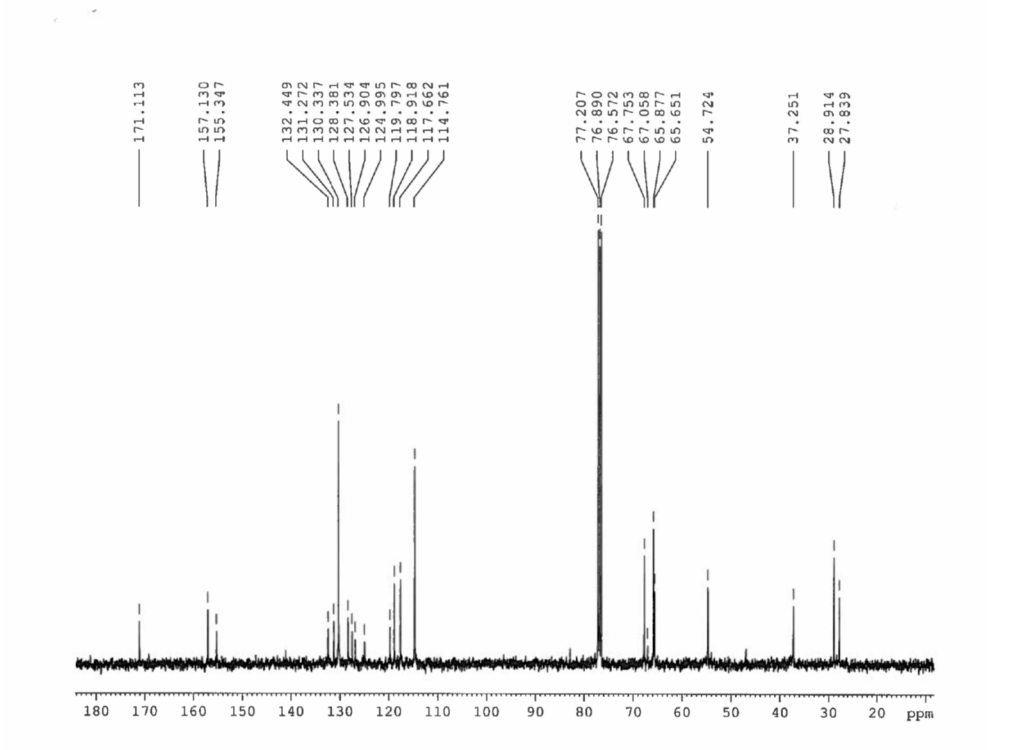

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CO2All

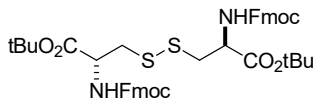

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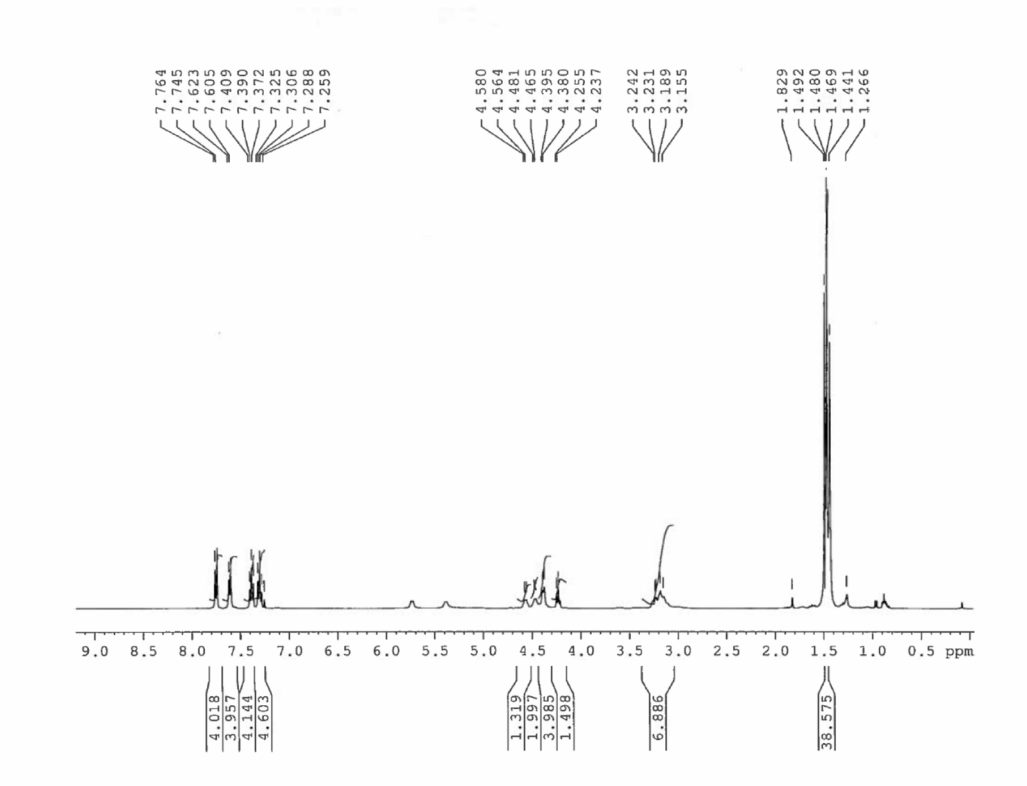

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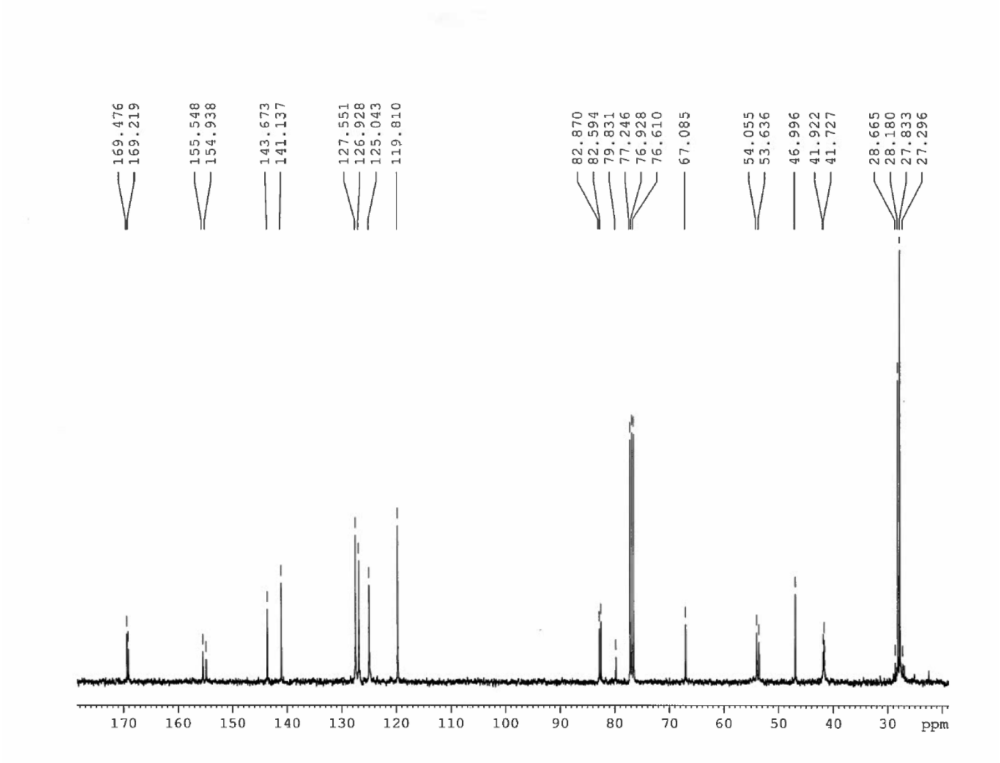

O
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Br
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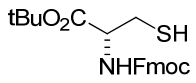

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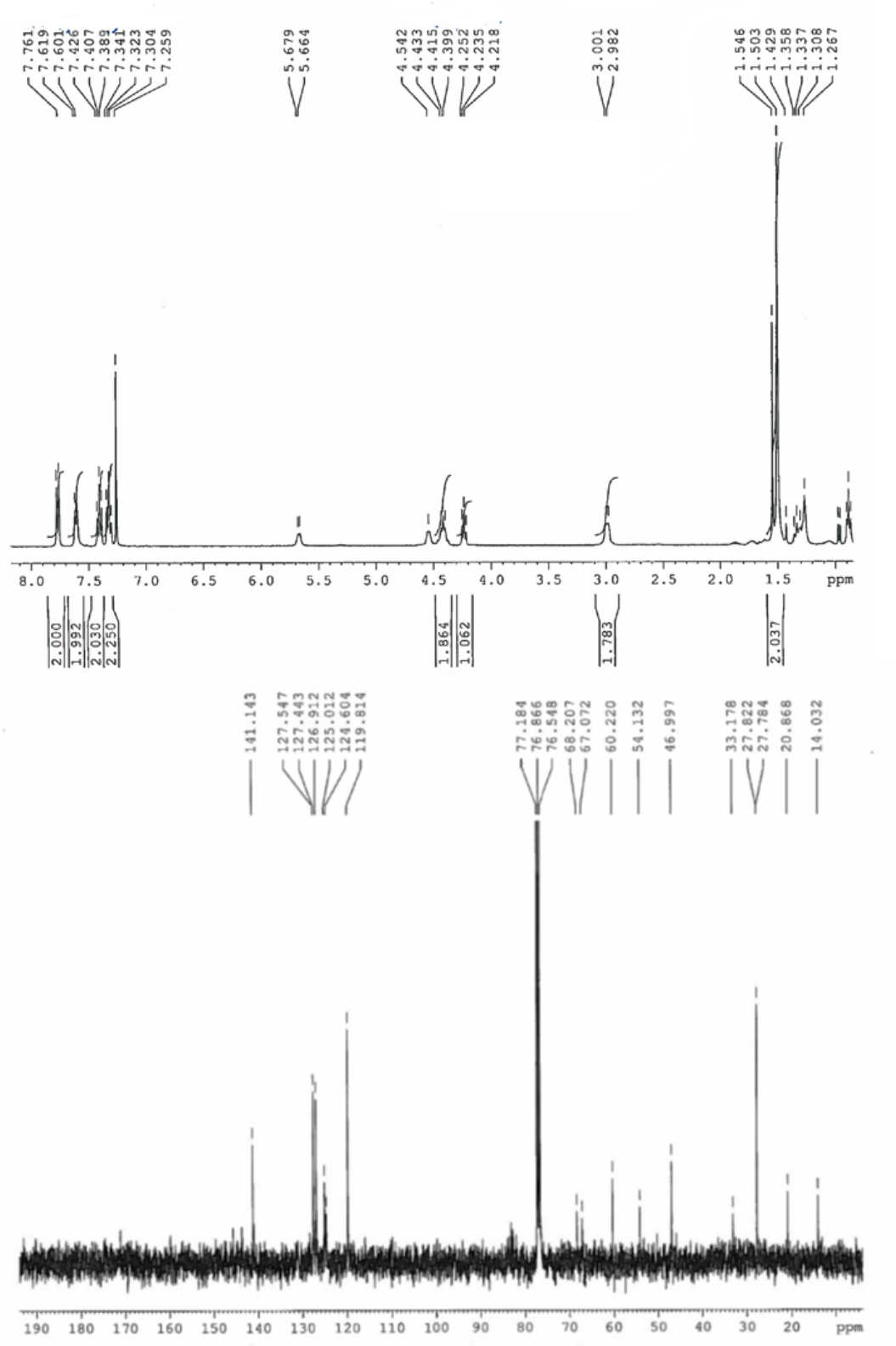

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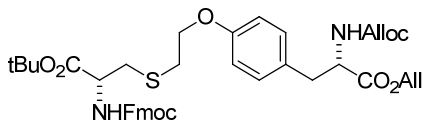

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S
S
CO2tBu
NHFmoc

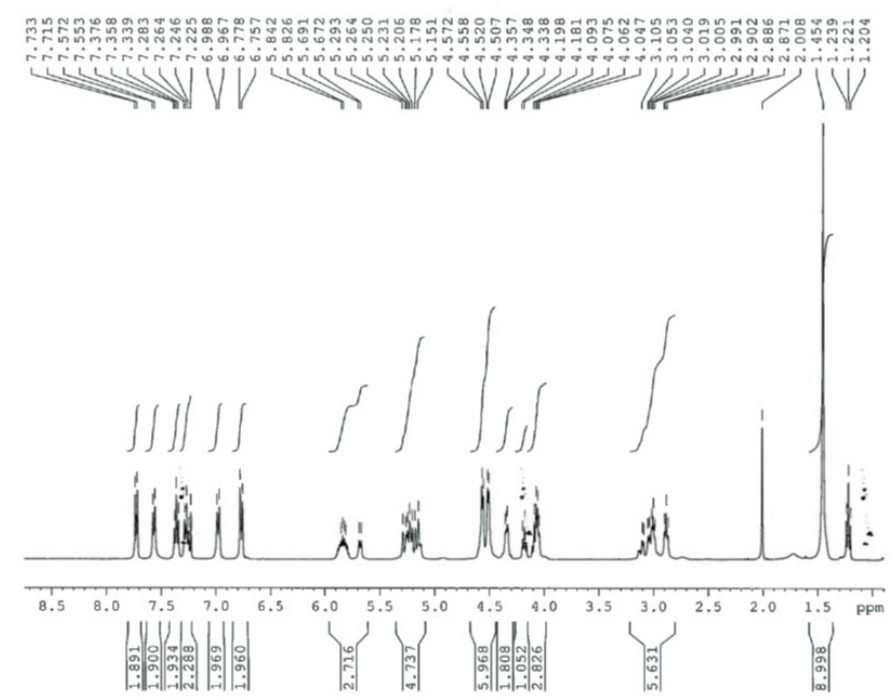

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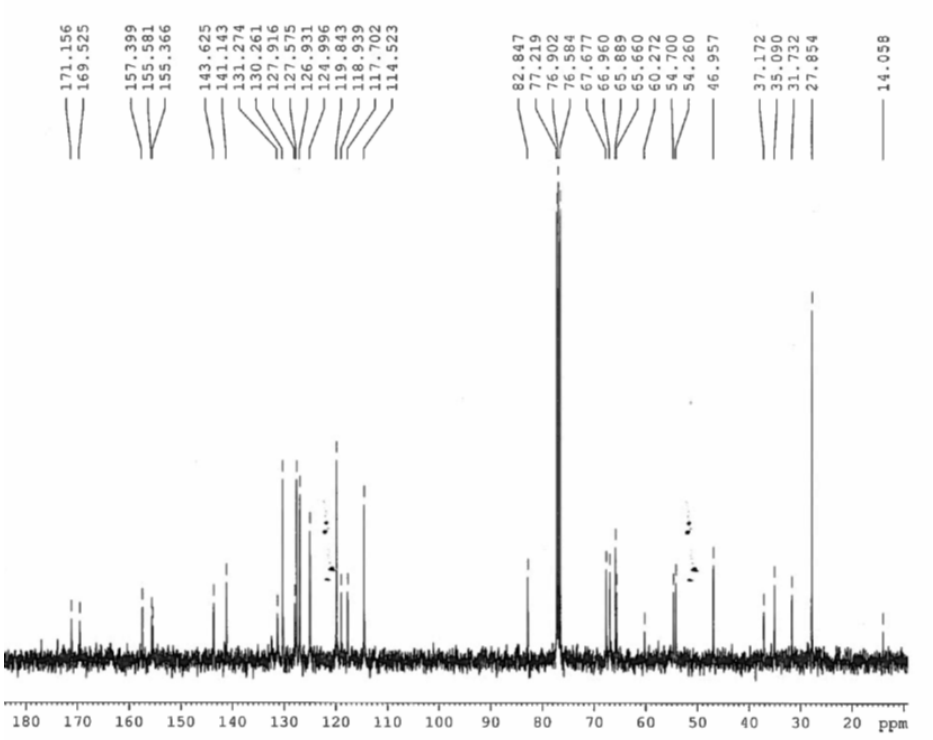

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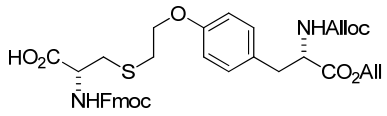

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SH
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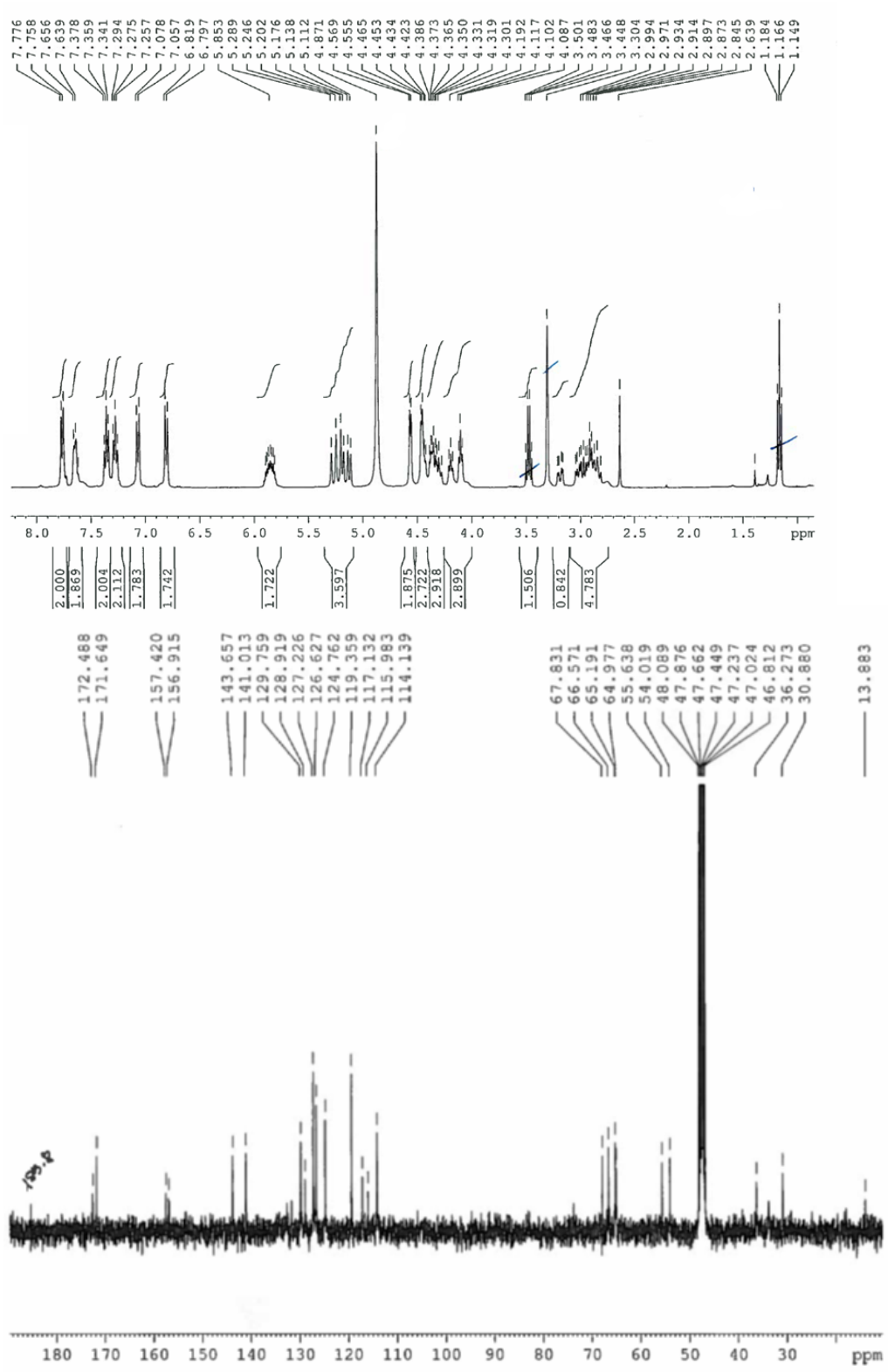

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tBuO2C
S
O
NHAlloc
CO2All
NHFmoc


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HO2C
S
O
NHAlloc
CO2All
NHFmoc


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Rosetta files

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```

```
      <OperateOnResidueSubset name=relaxRestWithCSTs selector=not_peptide >
         <RestrictToRepackingRLT/>
      </OperateOnResidueSubset>

   </TASKOPERATIONS>

   <FILTERS>
   </FILTERS>

   <MOVERS>

      <AddOrRemoveMatchCsts name=enzCST cst_instruction="add_new" cstfile="binding.cst" keep_covalent=1
/>
      <AtomCoordinateCstMover name=floppyPeptide coord_dev=0.2 bounded=true bound_width=0.1
sidechain=true native=false task_operations=relaxPeptide />
      <FastRelax name=fastrelax repeats=8 scorefxn=myscore
task_operations=keep_curr,init,relaxPeptide,relaxRestWithCSTs />
      <LoopOver name=fast5 mover_name=fastrelax iterations=5 drift=true/>

   </MOVERS>

   <APPLY_TO_POSE>
   </APPLY_TO_POSE>

   <PROTOCOLS>
      <Add mover=floppyPeptide />
      <Add mover=enzCST />
      <Add mover=fast5 />
   </PROTOCOLS>
</ROSETTASCRIPTS>
```

Binding.cst:

```
CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: C2 C1 S1
  TEMPLATE::  ATOM_MAP: 1 residue3: XLK

  TEMPLATE::  ATOM_MAP: 2 atom_type: OH  ,
  TEMPLATE::  ATOM_MAP: 2 residue3: TYZ

  CONSTRAINT::  distanceAB: 1.43      0.05   50.0    1
  CONSTRAINT::     angle_A: 109.4     5.0   15.0    360.0
  CONSTRAINT::     angle_B: 105.0     5.0   15.0    360.0
  CONSTRAINT::   torsion_A: 180.0     30.0  10.0    120.0
  CONSTRAINT::   torsion_B: 0.        10.0  10.0    180.0
  CONSTRAINT::  torsion_AB: 180.0     30.0  10.0    120.0
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: C1 C2 O1
  TEMPLATE::  ATOM_MAP: 1 residue3: XLK

  TEMPLATE::  ATOM_MAP: 2 atom_type: S   ,
  TEMPLATE::  ATOM_MAP: 2 residue3: CYZ

  CONSTRAINT::  distanceAB: 1.82     0.05   50.0     1
  CONSTRAINT::     angle_A: 109.4    5.0   10.0    360.0
  CONSTRAINT::     angle_B: 95.0     5.0   15.0    360.0
  CONSTRAINT::   torsion_A: 180.0    30.0   10.0    120.0
  CONSTRAINT::   torsion_B: 180.0    30.0   10.0    120.0
  CONSTRAINT::  torsion_AB: 180.0    30.0   10.0    120.0
CST::END
```

```
CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: ZN CA1 CA2
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: NE2 CD2 CG
  TEMPLATE::  ATOM_MAP: 2 residue3: HIS

  CONSTRAINT::  distanceAB: 2.069     0.05   50.0    1
  CONSTRAINT::     angle_A: 57.5      5.0   15.0    360.0
  CONSTRAINT::     angle_B: 122.7     5.0   15.0    360.0
  CONSTRAINT::   torsion_A: -55.4     10.0  10.0    360.0
  CONSTRAINT::   torsion_B: -173.0    10.0  10.0    360.0
  CONSTRAINT::  torsion_AB: 123.7     10.0  10.0    360.0
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: ZN CA1 CA2
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: ND1 CG CB
  TEMPLATE::  ATOM_MAP: 2 residue3: HIS

  CONSTRAINT::  distanceAB: 2.073     0.05   50.0    1
  CONSTRAINT::     angle_A: 150.4     5.0   15.0    360.0
  CONSTRAINT::     angle_B: 135.8     5.0   15.0    360.0
  CONSTRAINT::   torsion_A: -116.7    10.0  10.0    360.0
  CONSTRAINT::   torsion_B: 2.4       10.0  10.0    360.0
  CONSTRAINT::  torsion_AB: -102.1    10.0  10.0    360.0
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: ZN CA1 CA2
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: OD1 CG CB
  TEMPLATE::  ATOM_MAP: 2 residue3: ASP

  CONSTRAINT::  distanceAB: 1.997     0.05   50.0    1
  CONSTRAINT::     angle_A: 56.7      5.0   15.0    360.0
  CONSTRAINT::     angle_B: 120.5     5.0   15.0    360.0
  CONSTRAINT::   torsion_A: 174.4     10.0  10.0    360.0
  CONSTRAINT::   torsion_B: 171.2     10.0  10.0    360.0
  CONSTRAINT::  torsion_AB: -21.6     10.0  10.0    360.0
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: ZN CA1 CA2
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: OD2 CG CB
  TEMPLATE::  ATOM_MAP: 2 residue3: ASP

  CONSTRAINT::  distanceAB: 2.168     0.05   50.0    1
  CONSTRAINT::     angle_A: 109.0     5.0   15.0    360.0
  CONSTRAINT::     angle_B: 111.3     5.0   15.0    360.0
  CONSTRAINT::   torsion_A: 38.4      10.0  10.0    360.0
  CONSTRAINT::   torsion_B: -178.0    10.0  10.0    360.0
  CONSTRAINT::  torsion_AB: 64.5      10.0  10.0    360.0
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: ZN CA1 CA2
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: OD1 CG OD2
  TEMPLATE::  ATOM_MAP: 2 residue3: ASP
```

```
  CONSTRAINT::  distanceAB: 2.869     0.05   50.0    1
  CONSTRAINT::     angle_A: 85.4      5.0   15.0    360.0
  CONSTRAINT::     angle_B: 77.4      5.0   15.0    360.0
  CONSTRAINT::   torsion_A: 82.1      30.0  10.0    360.0
  CONSTRAINT::   torsion_B: -1.8      10.0  10.0    360.0
  CONSTRAINT::  torsion_AB: -119.0    30.0  10.0    360.0
CST::END

#Calcium csts with OOCs start here

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA2 ZN CA1
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_name: OD1 CG CB
  TEMPLATE::  ATOM_MAP: 2 residue3: ASP

  CONSTRAINT::  distanceAB: 2.321    0.01   50.0     1
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA2 ZN CA1
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_name: OD2 CG CB
  TEMPLATE::  ATOM_MAP: 2 residue3: ASP

  CONSTRAINT::  distanceAB: 2.335     0.01  50.0     1
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA1 CA2 ZN
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: OOC ,
  TEMPLATE::  ATOM_MAP: 2 residue3: ASP

  CONSTRAINT::  distanceAB: 2.331     0.01  50.0     1
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA1 CA2 ZN
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: OOC ,
  TEMPLATE::  ATOM_MAP: 2 residue3: GLU

  CONSTRAINT::  distanceAB: 2.335     0.01  50.0     1
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA1 CA2 ZN
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: OOC ,
  TEMPLATE::  ATOM_MAP: 2 residue3: GLU

  CONSTRAINT::  distanceAB: 2.343     0.01  50.0     1
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA1 CA2 ZN
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_name: OE2 CD CG
```

```
  TEMPLATE::  ATOM_MAP: 2 residue3: GLU

  CONSTRAINT::  distanceAB: 2.336     0.01  50.0     1
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA1 CA2 ZN
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_name: O CA N
  TEMPLATE::  ATOM_MAP: 2 residue3: THR

  CONSTRAINT::  distanceAB: 2.355     0.01  50.0     1
CST::END

CST::BEGIN
  TEMPLATE::  ATOM_MAP: 1 atom_name: CA2 CA1 ZN
  TEMPLATE::  ATOM_MAP: 1 residue3: CAZ

  TEMPLATE::  ATOM_MAP: 2 atom_type: OOC ,
  TEMPLATE::  ATOM_MAP: 2 residue3: GLU

  CONSTRAINT::  distanceAB: 2.327     0.01  50.0     1
CST::END
~
```

Run command:

```
~/Rosetta/main/source/bin/rosetta_scripts.linuxgccrelease @general.flags -s $1
```