Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Optimizing CSP1 Analogs for Modulating Quorum Sensing in *Streptococcus pneumoniae* with Bulky, Hydrophobic Nonproteogenic Amino Acid Substitutions

Tahmina A. Milly,[†] Alec R. Buttner,[§] Naomi Rieth,[§] Elizabeth Hutnick,[§] Emilee R. Engler,[§] Alexandra R. Campanella,[‡] Muralikrishna Lella,[†] Michael A. Bertucci*,[‡] and Yftah Tal-Gan,*,[†]

[†] Department of Chemistry, University of Nevada, Reno, 1664 North Virginia Street, Reno, Nevada, 89557, United States

[‡] Department of Chemistry, Lafayette College, 701 Sullivan Rd., Easton, PA 18042, United States

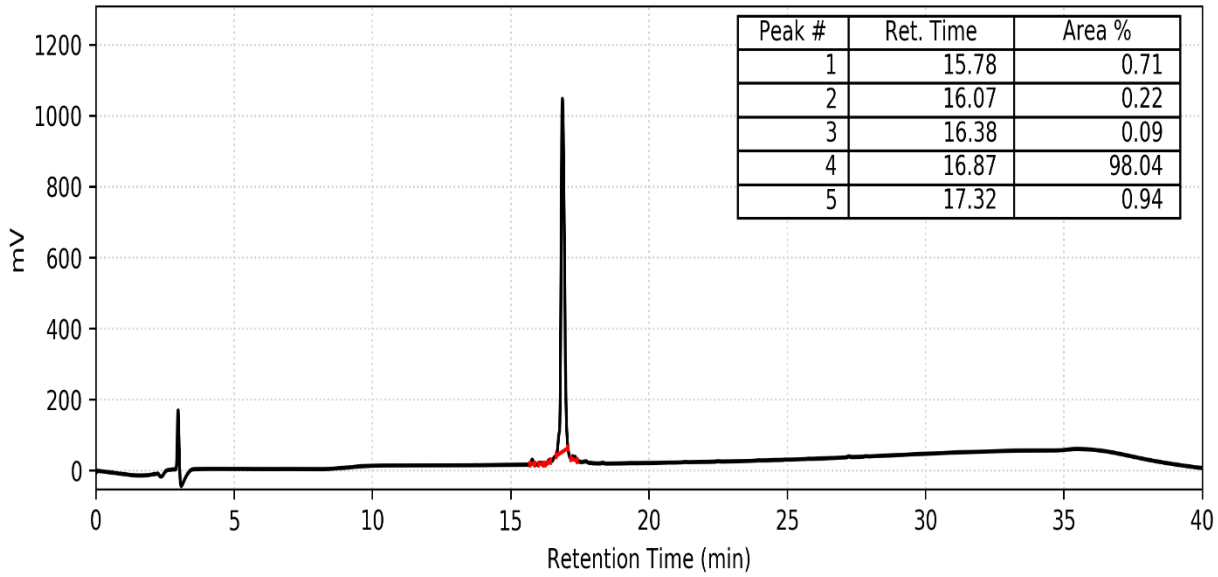
[§] Department of Chemistry, Moravian University, 1200 Main St., Bethlehem, PA 18018, United States

* To whom correspondence should be addressed. ytalgan@unr.edu, bertuccm@lafayette.edu

Supporting Information

HPLC Traces for CSP1 Analogs

CSP1-L4Cha



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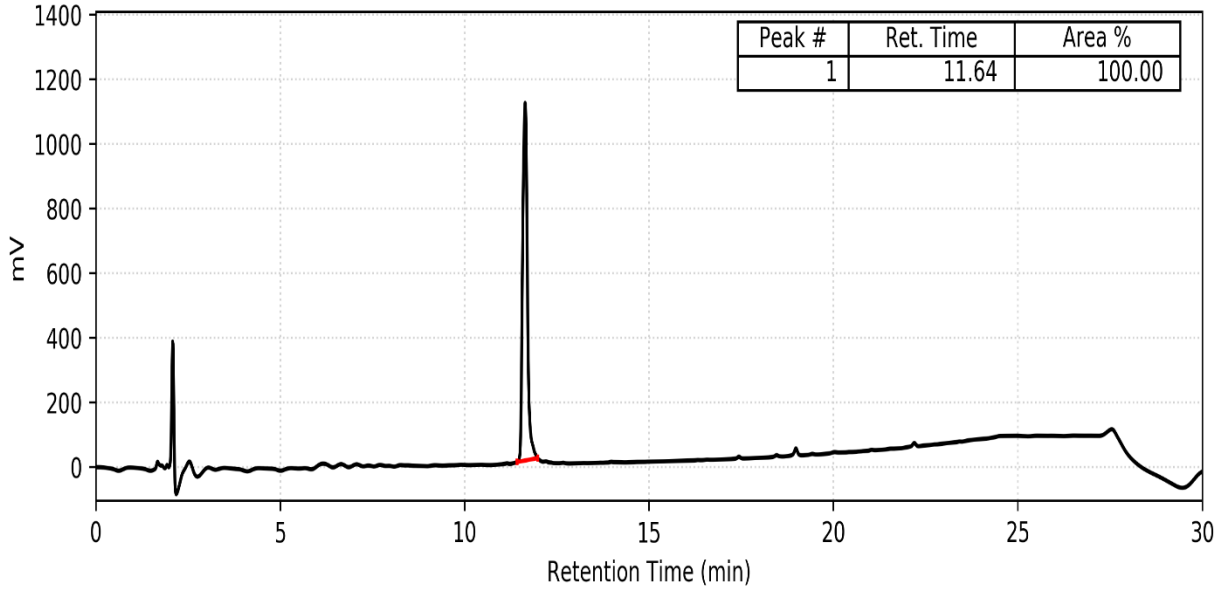

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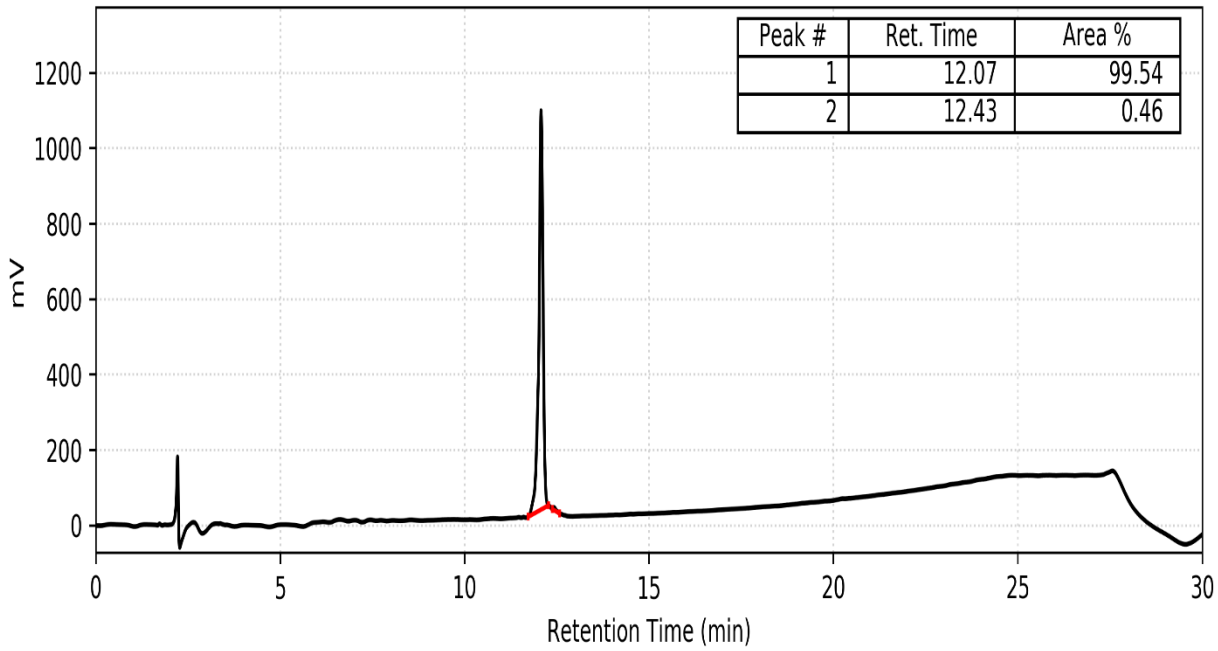

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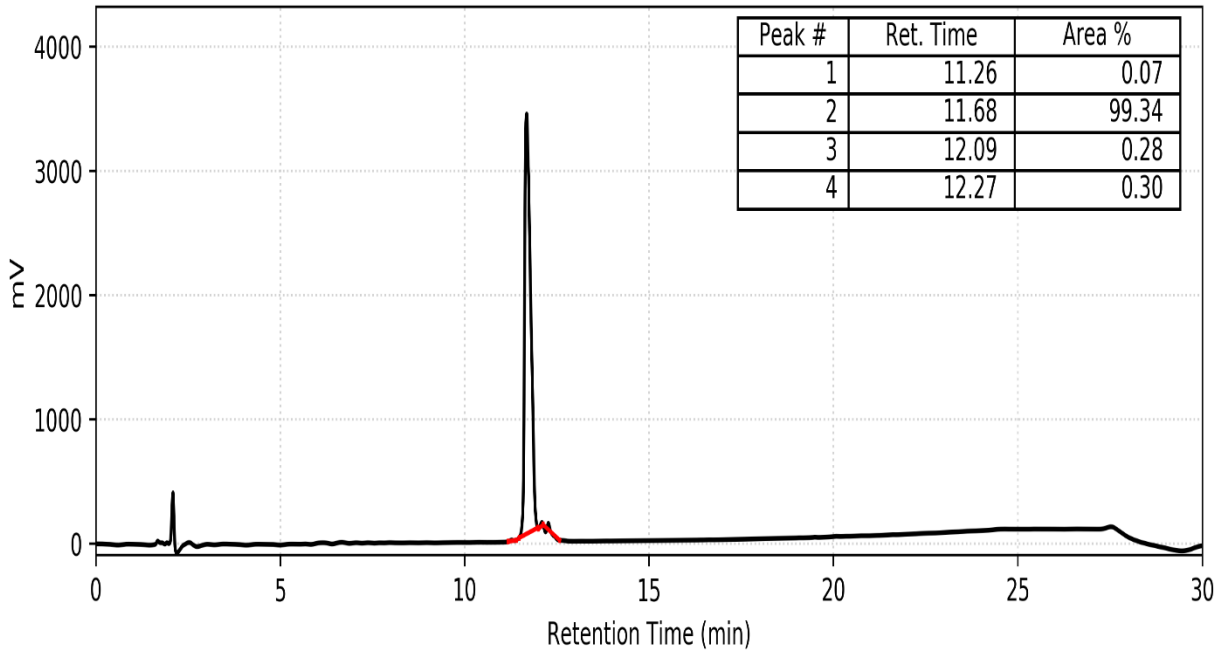

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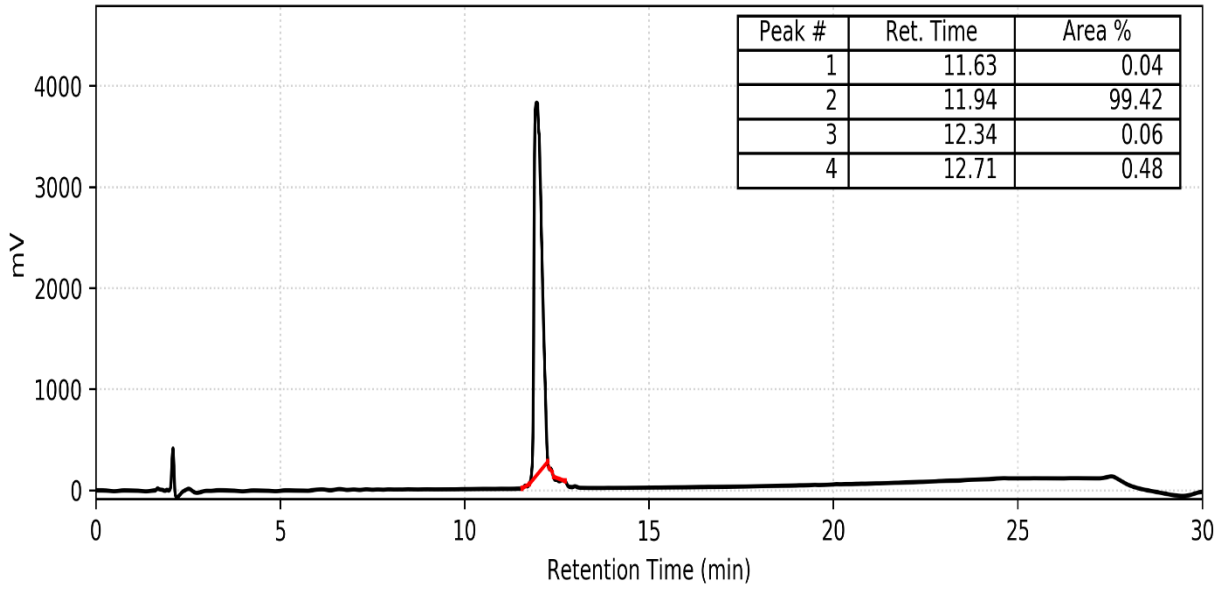

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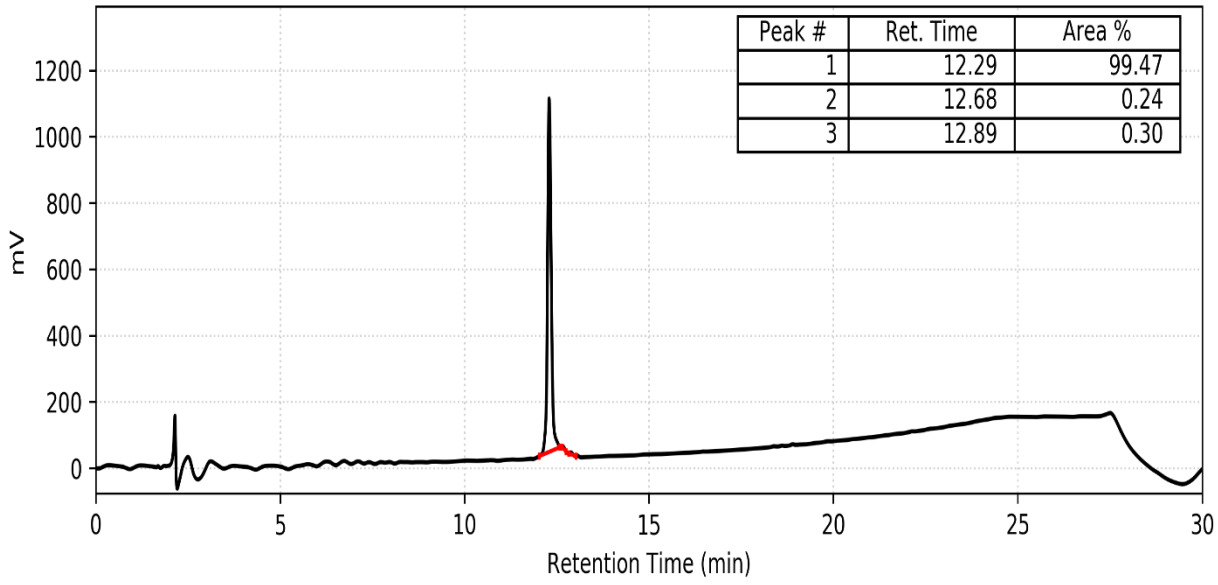

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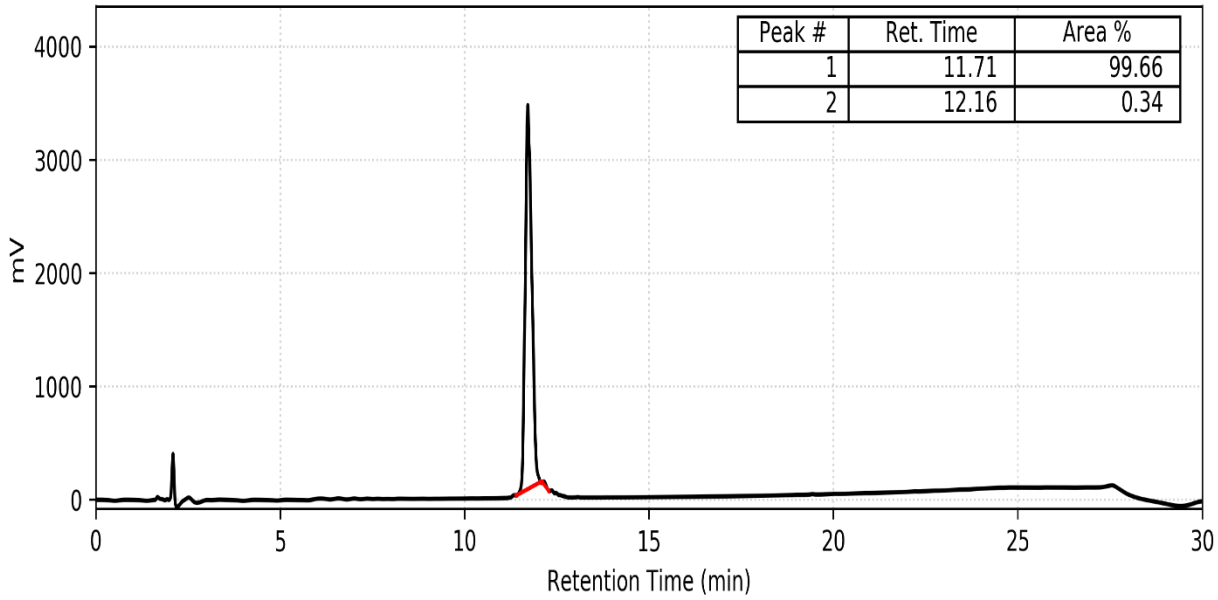

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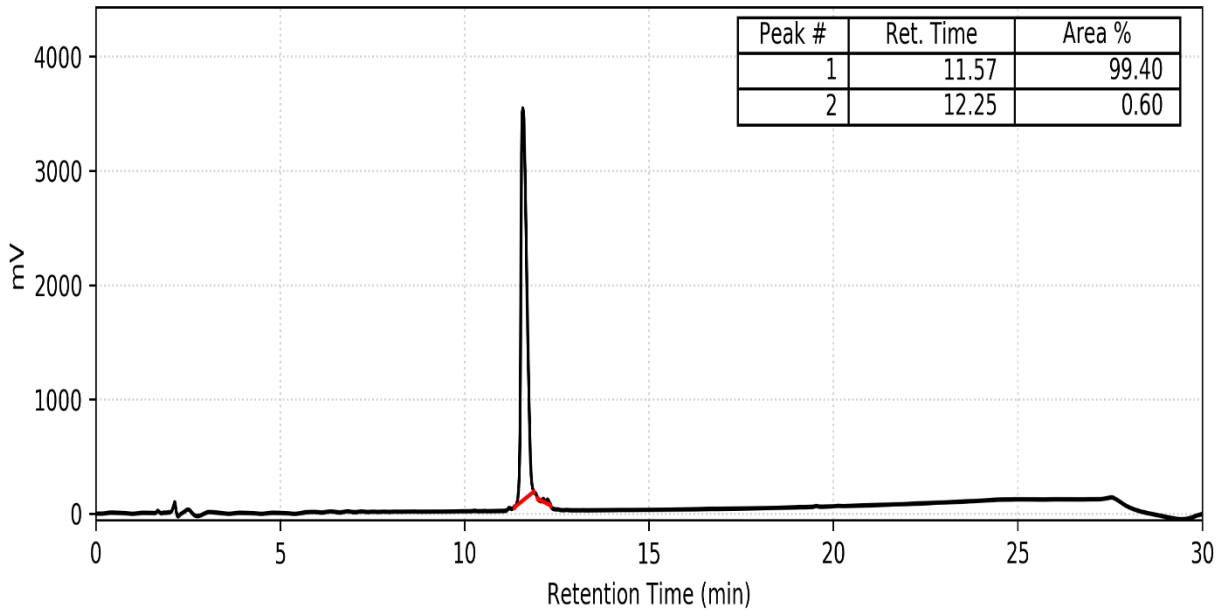

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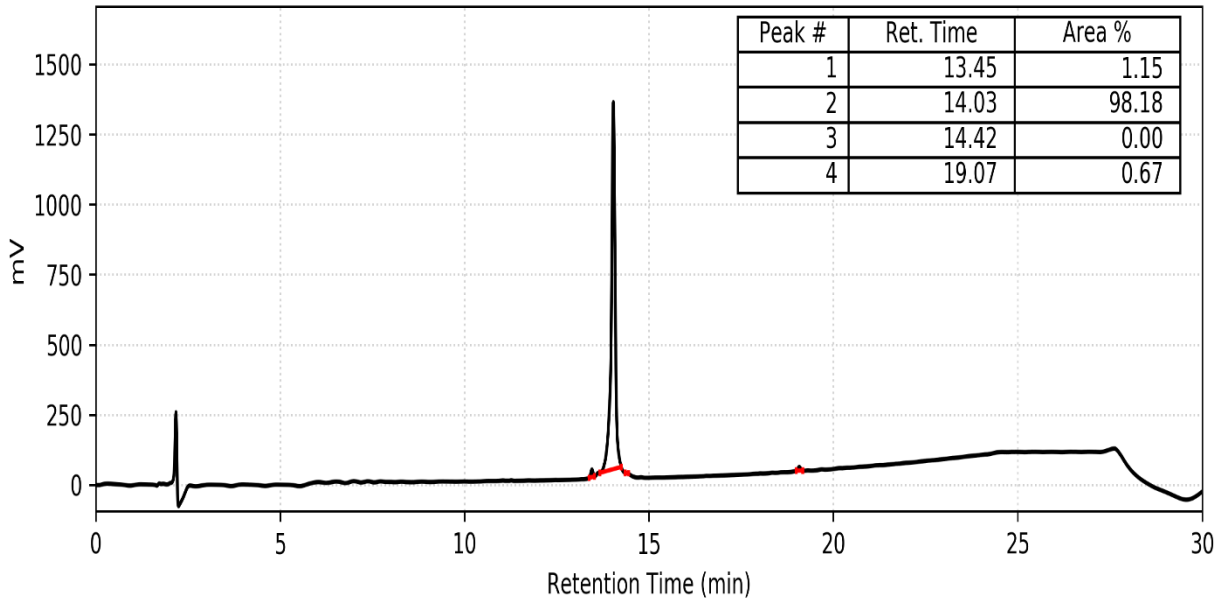

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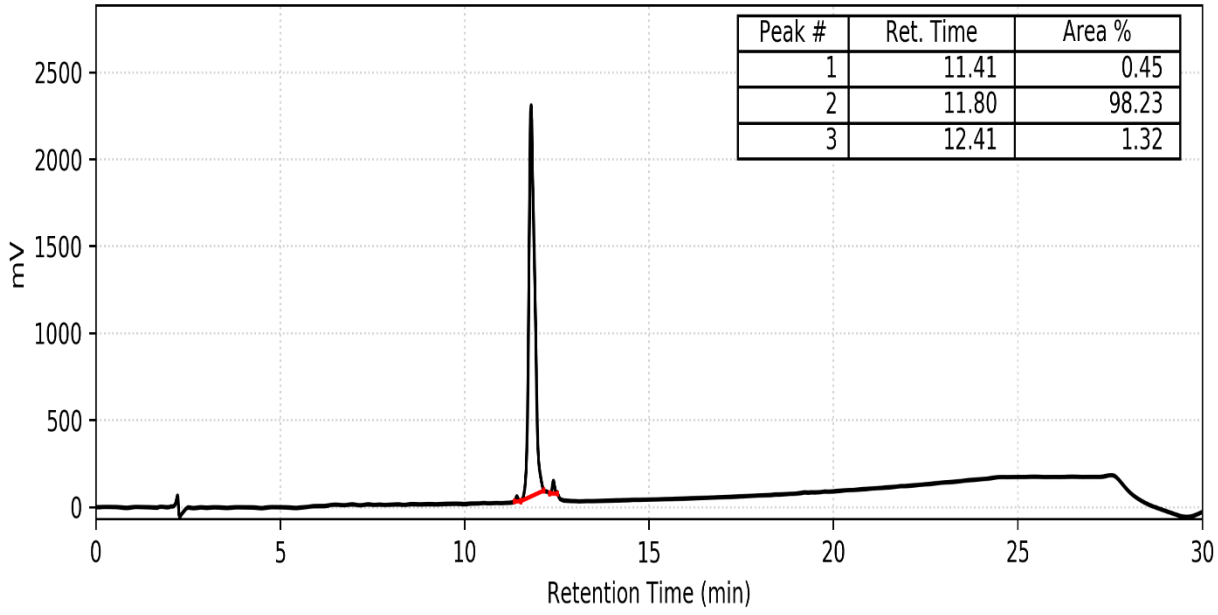

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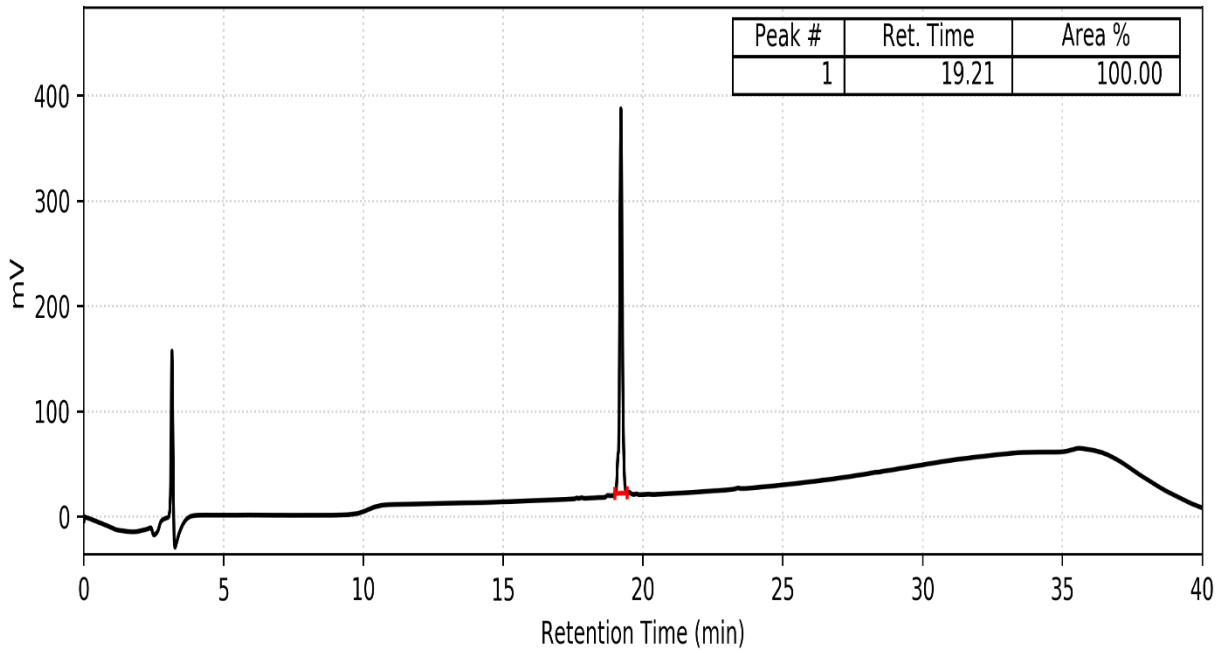

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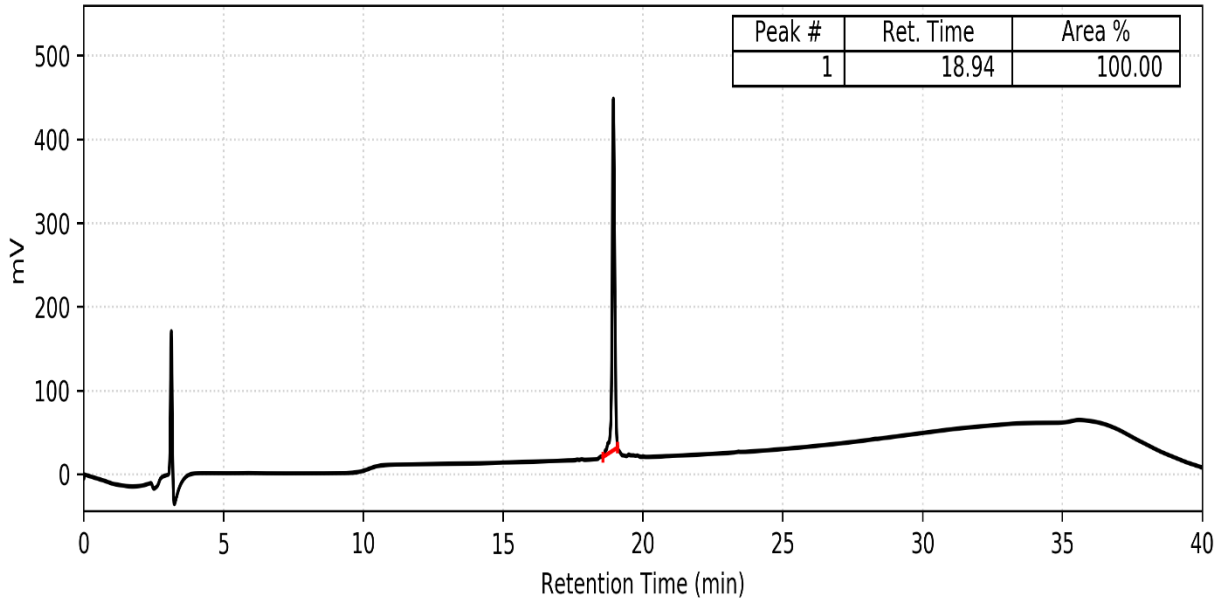

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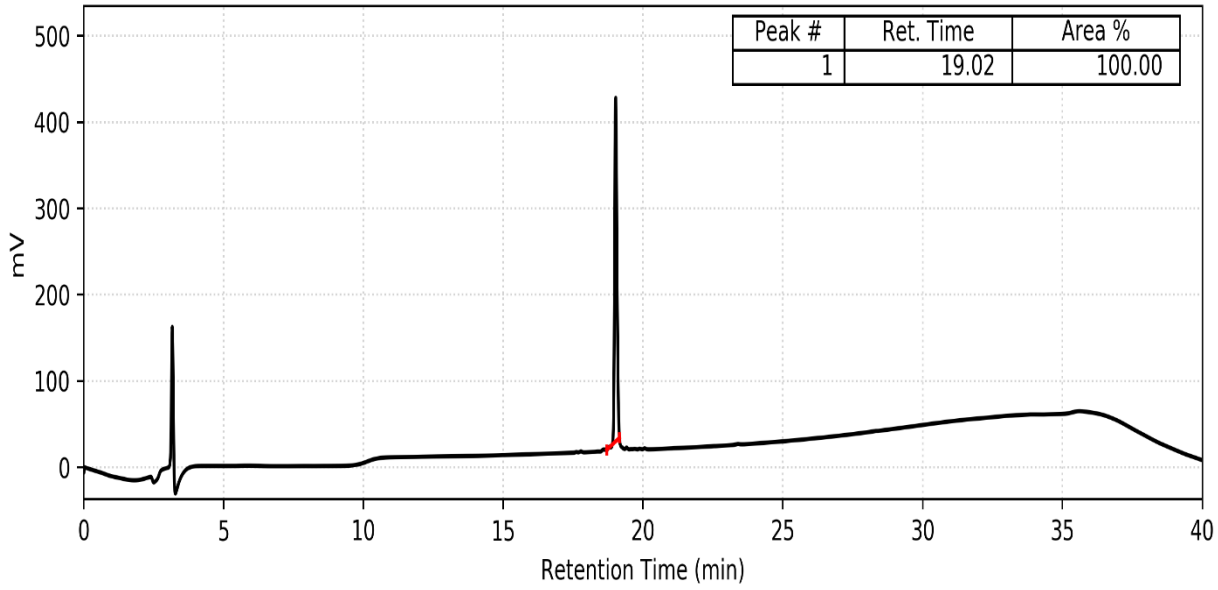

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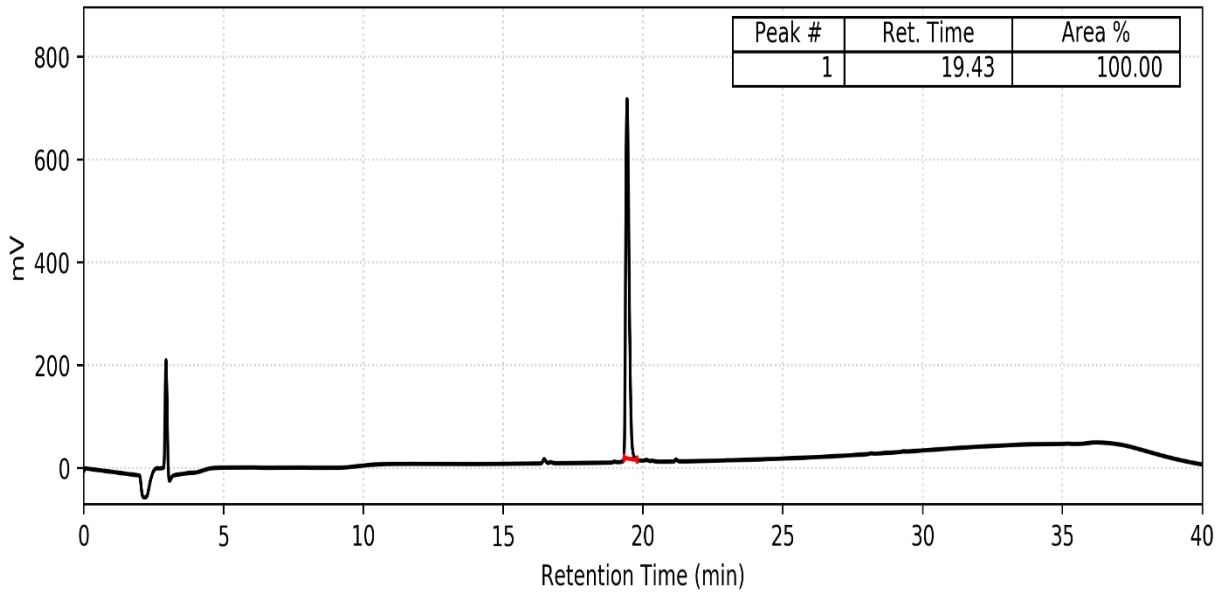

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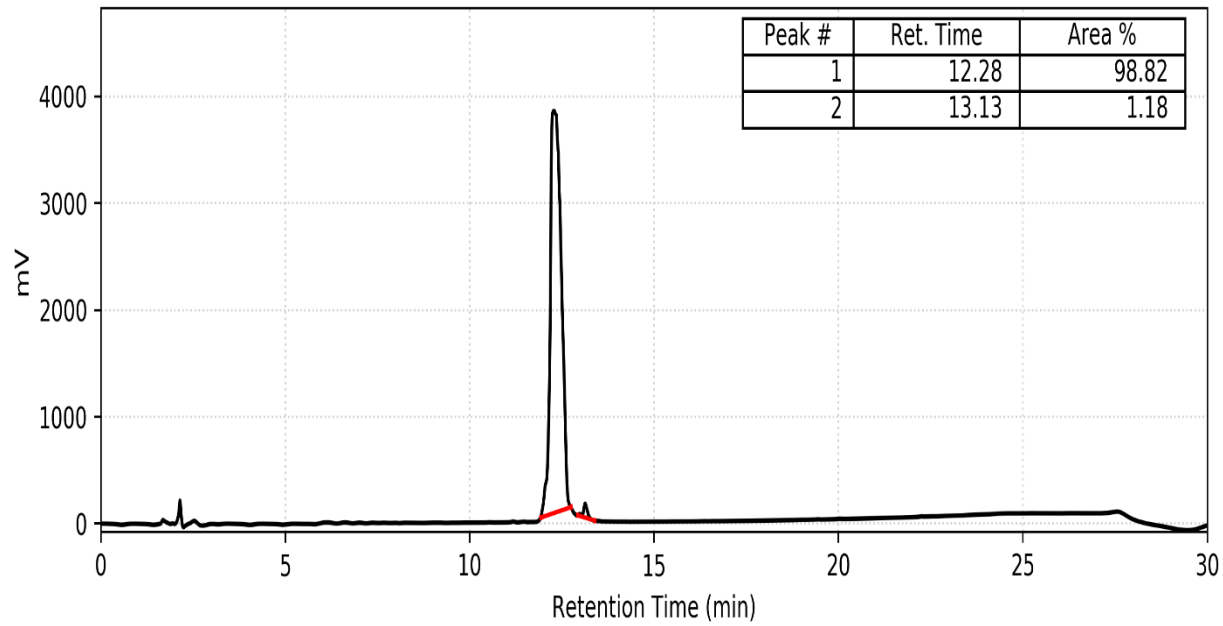

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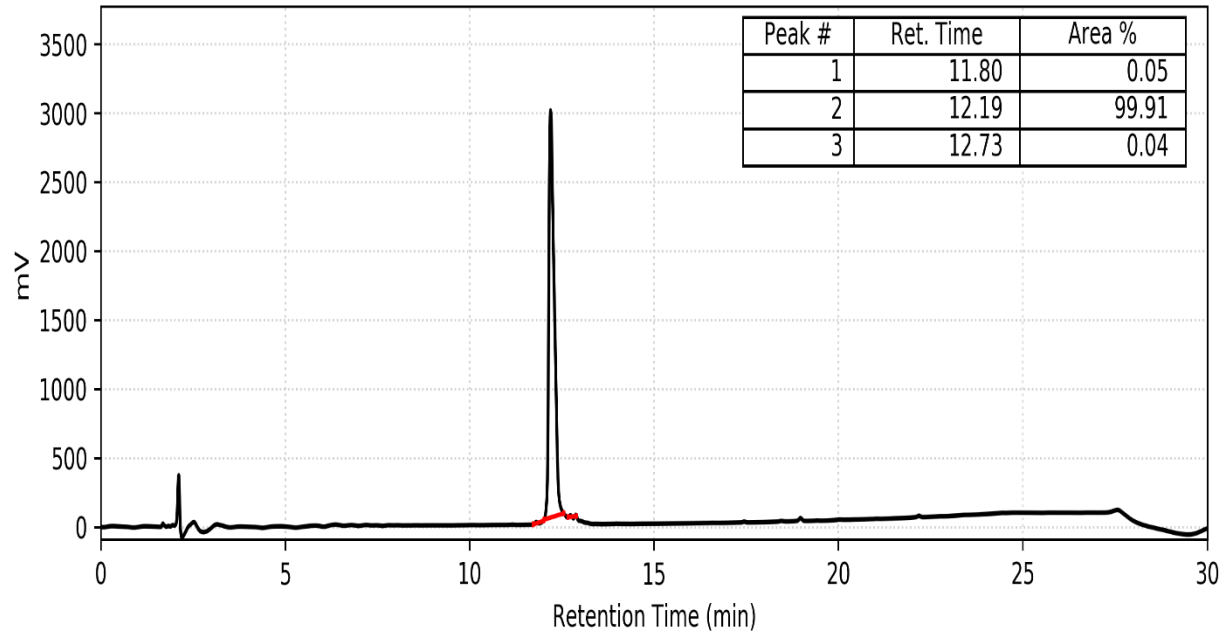

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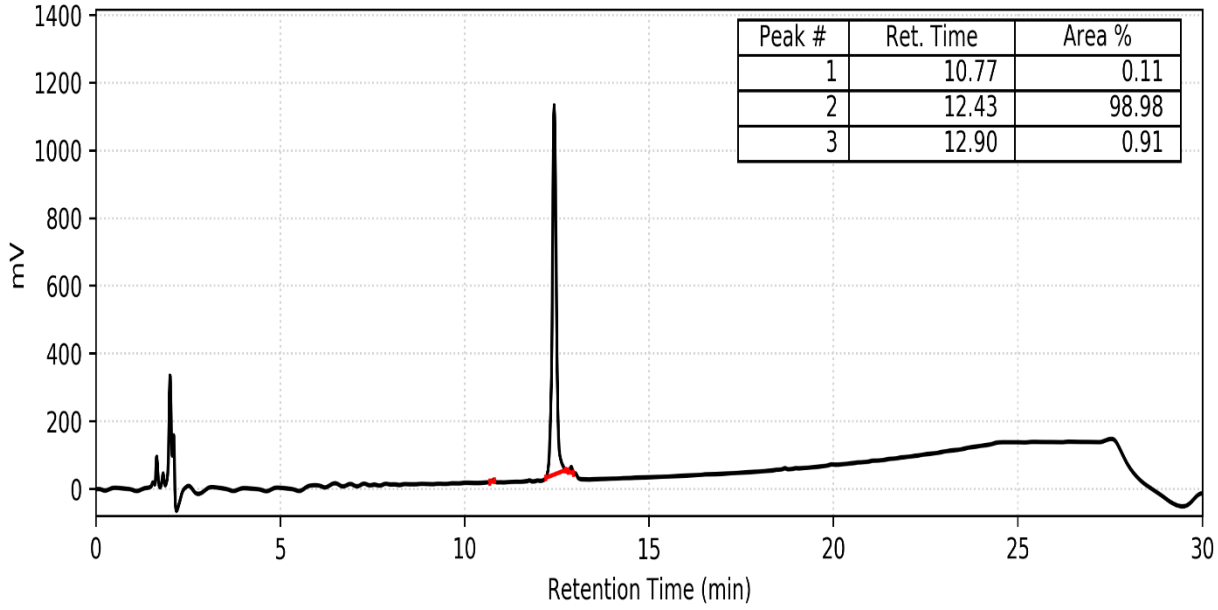

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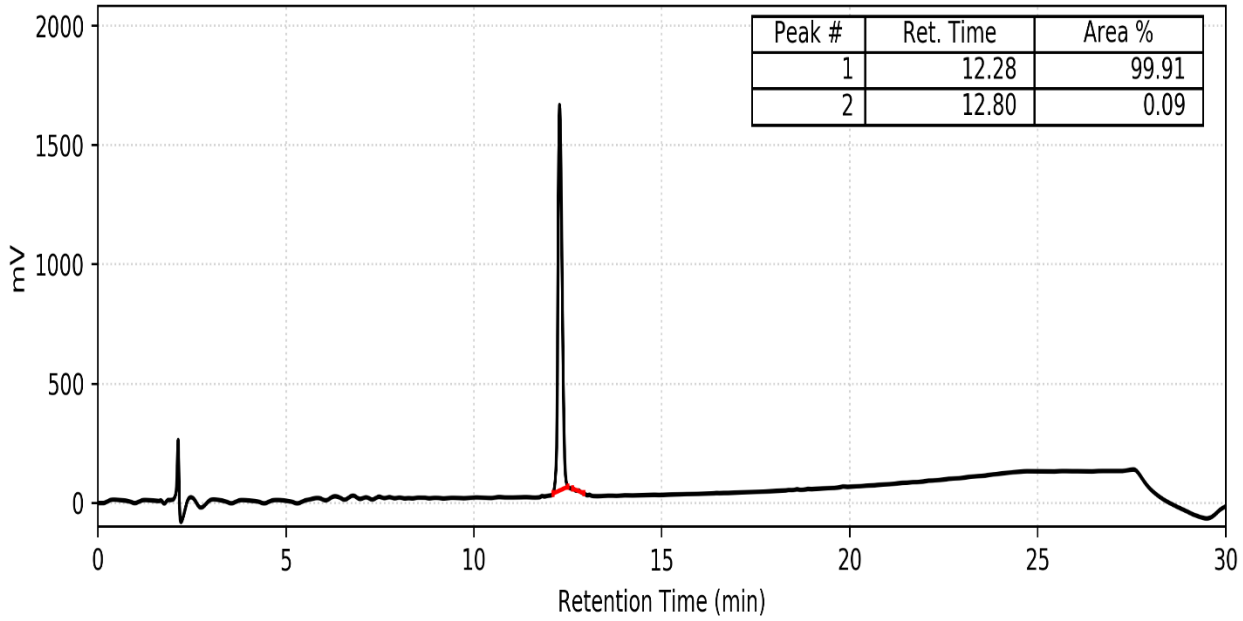

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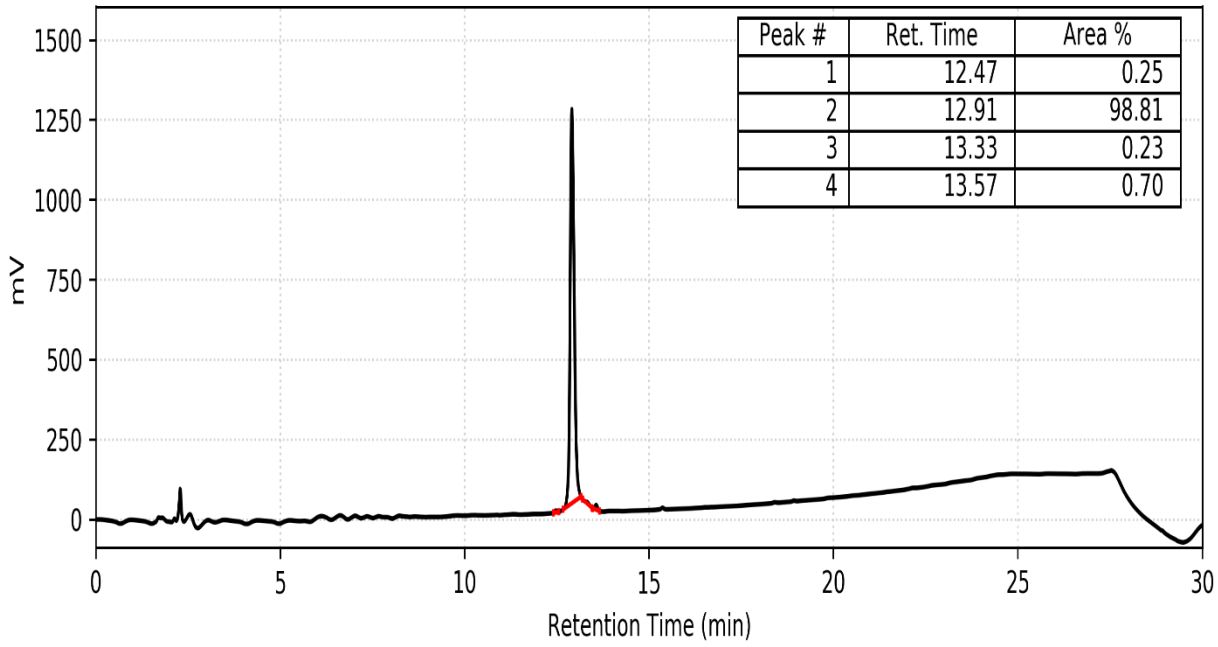

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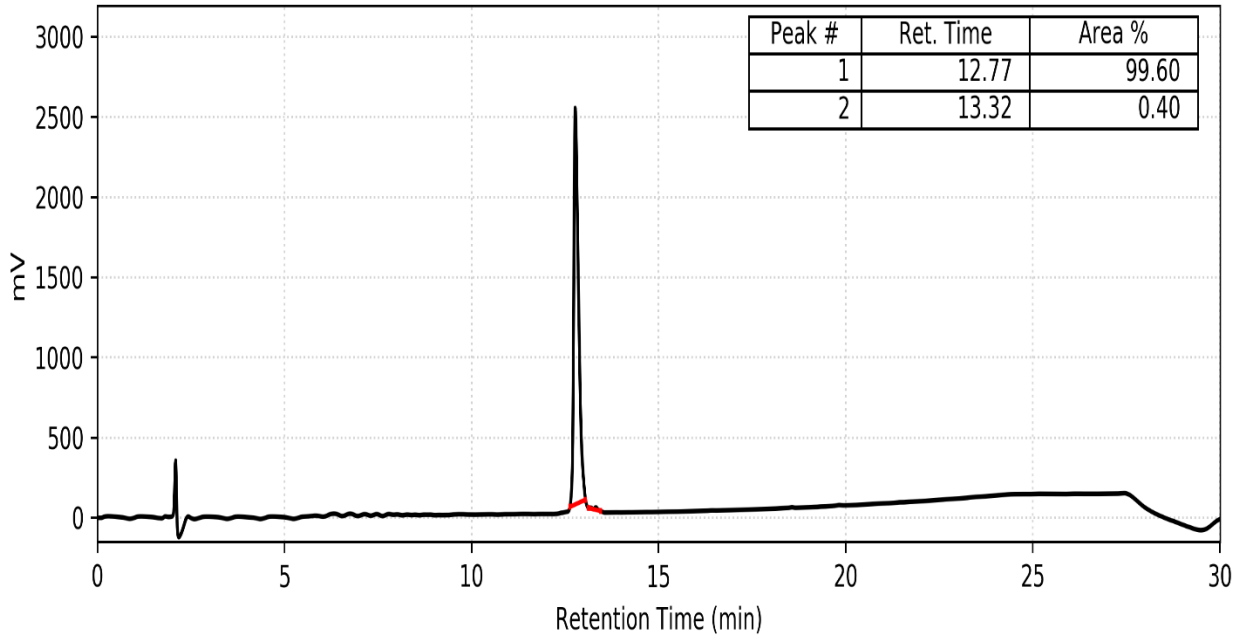

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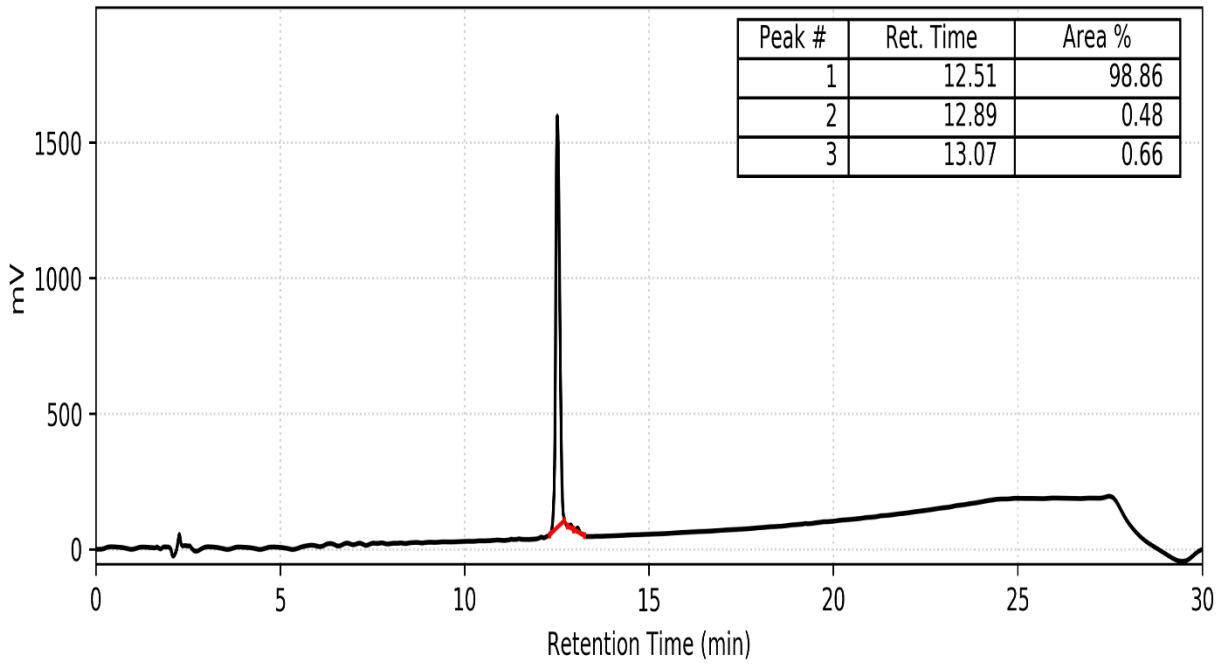

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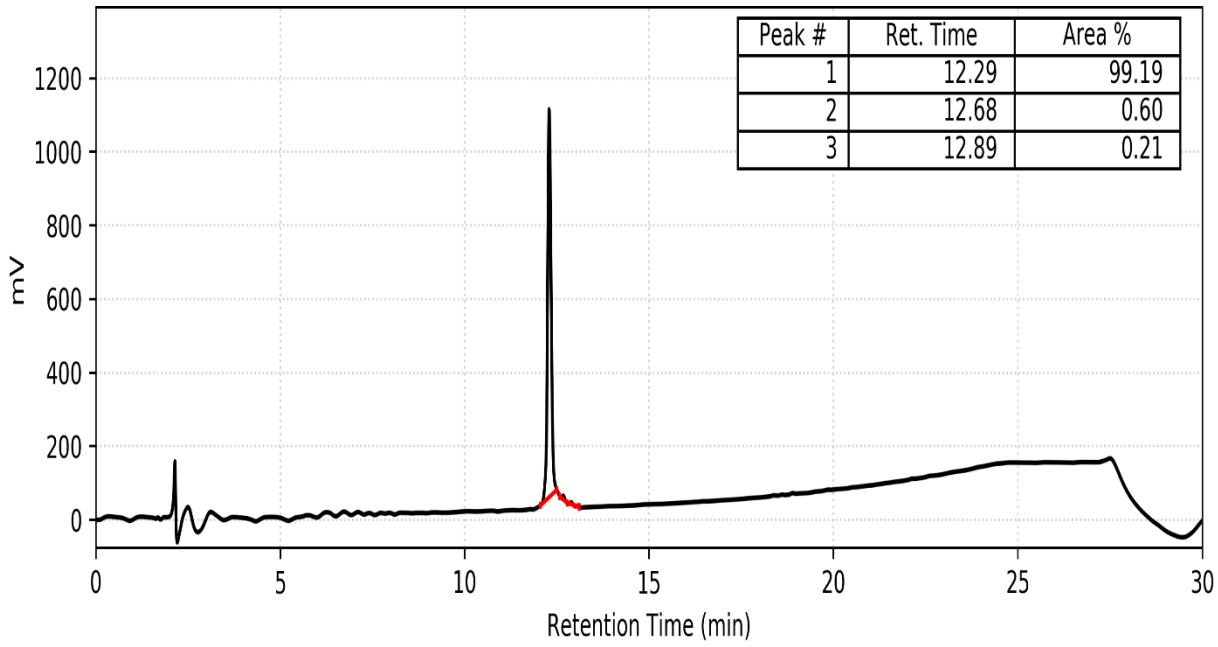

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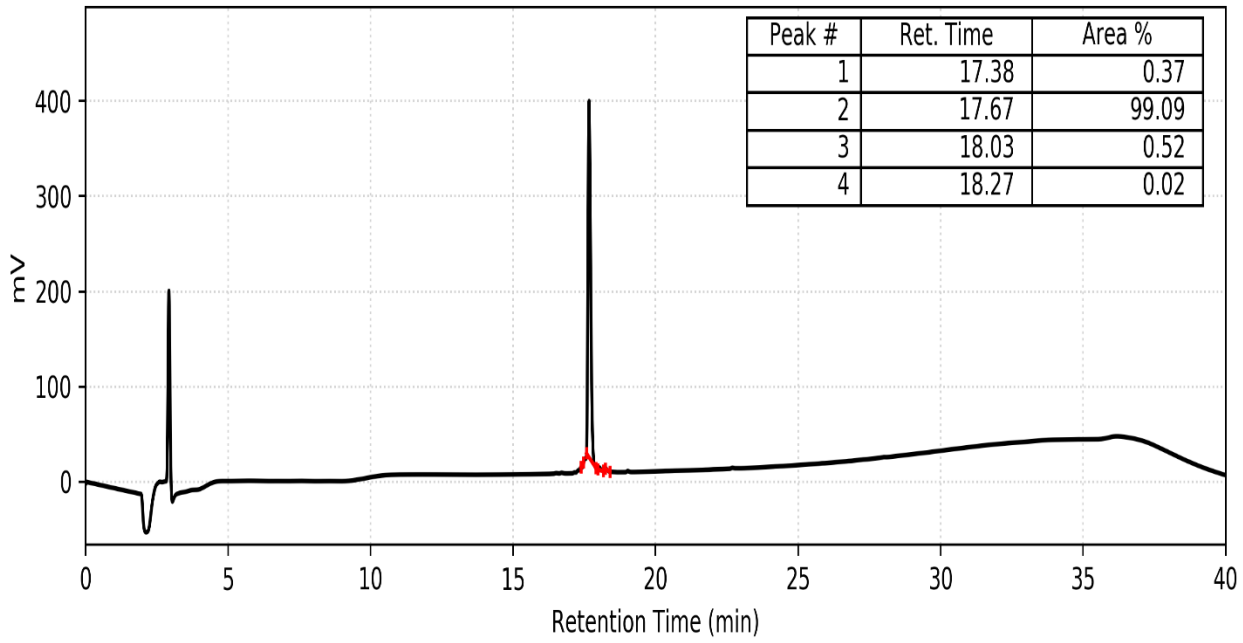

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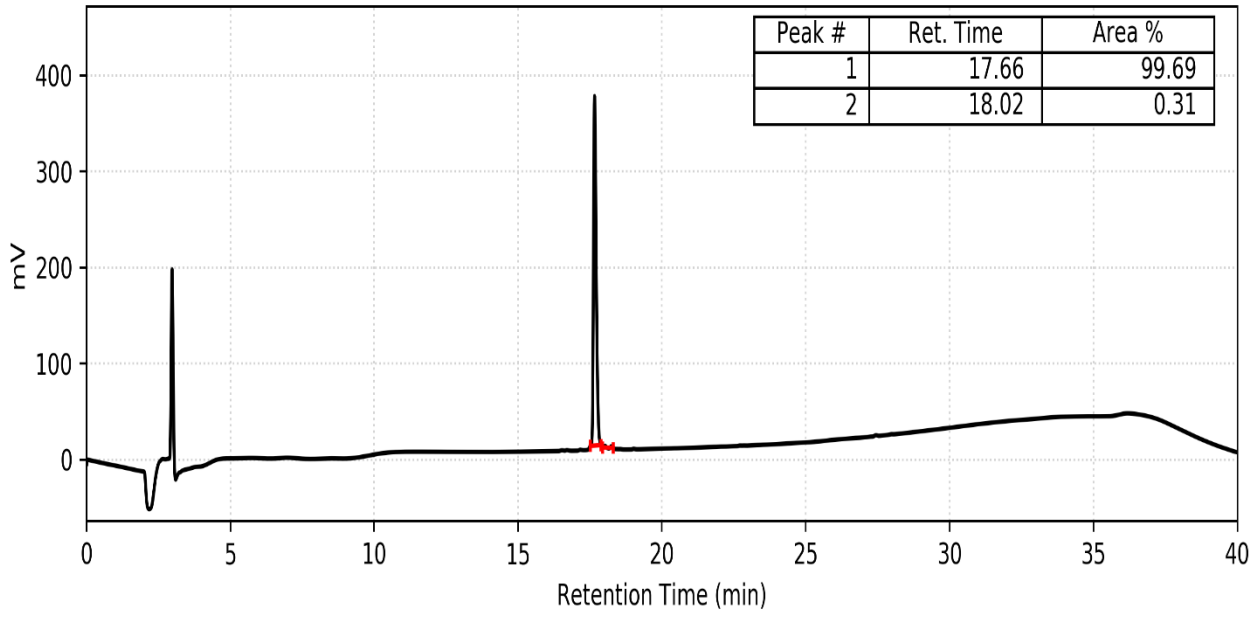

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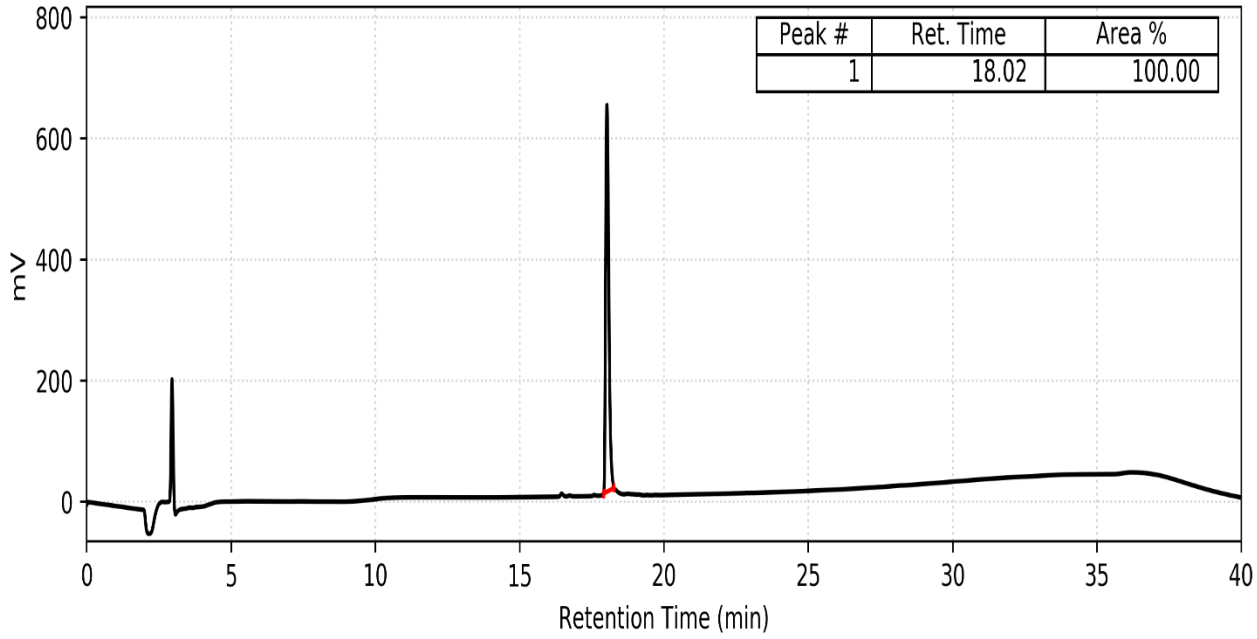

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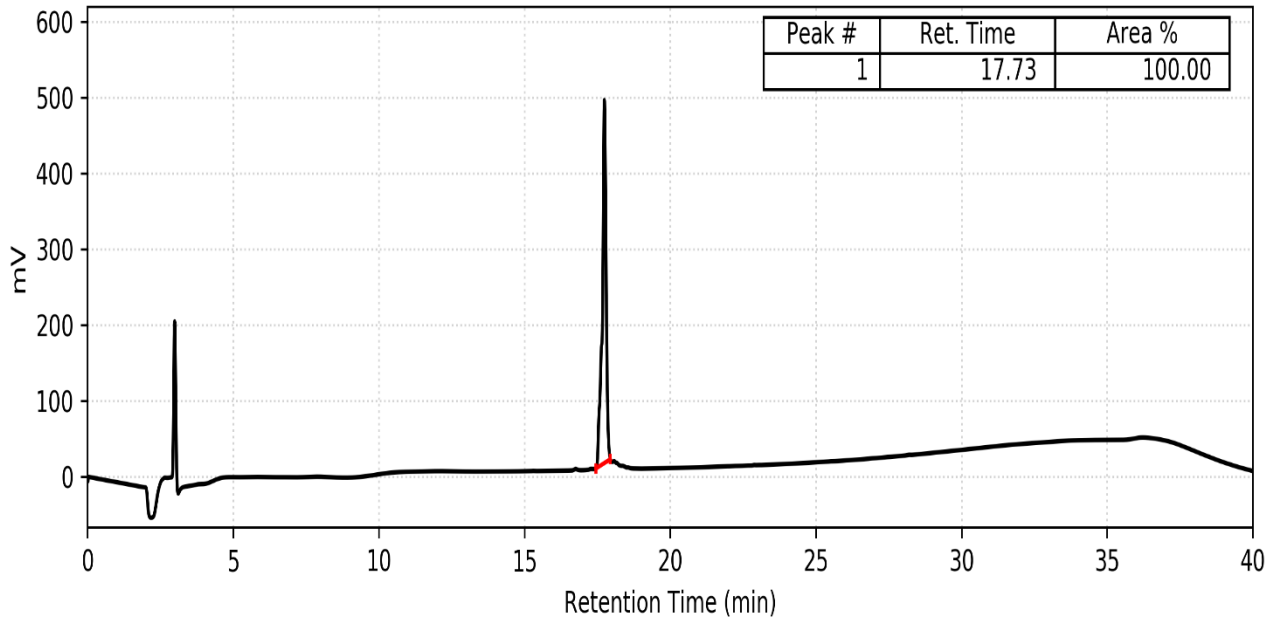

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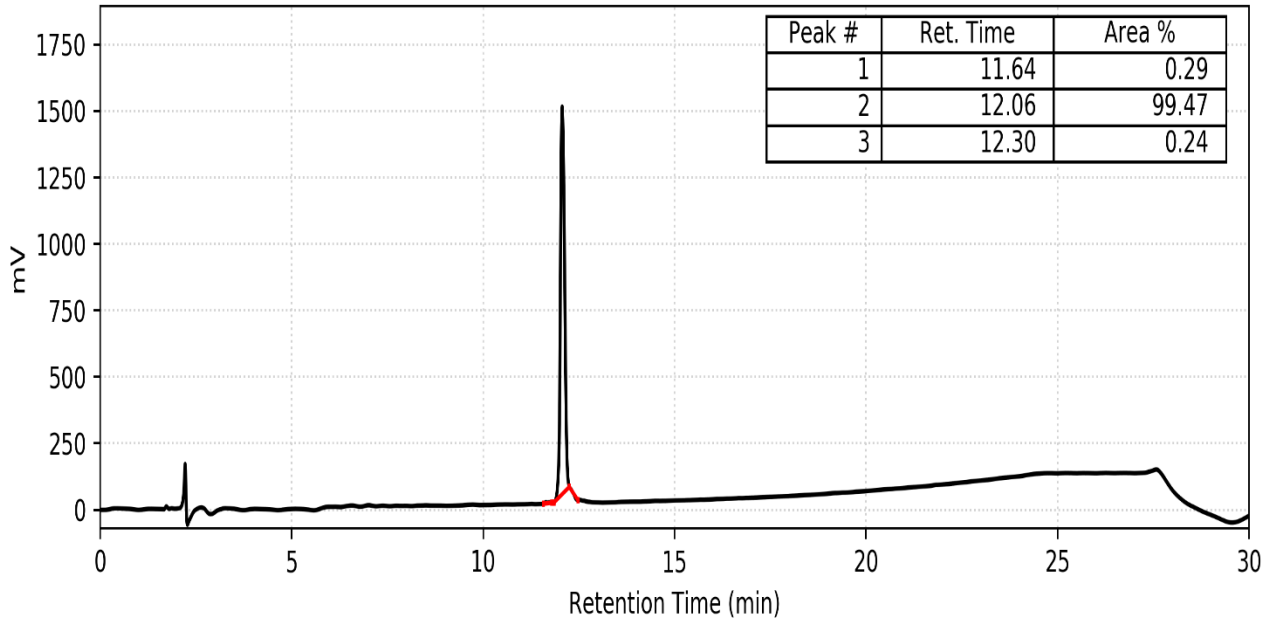

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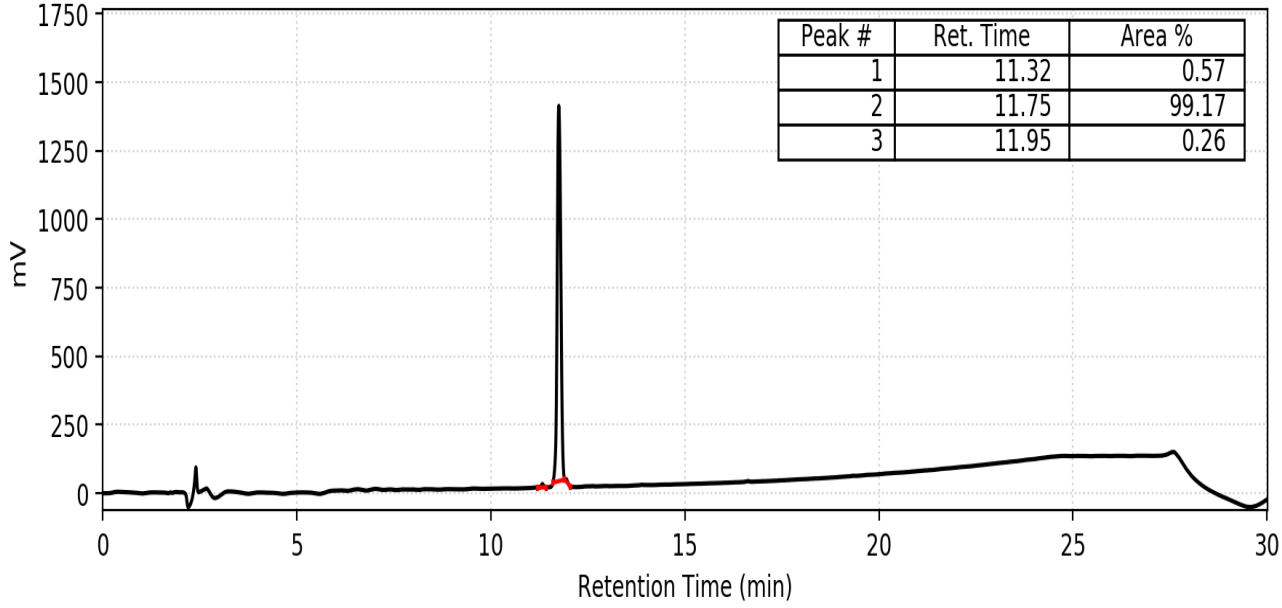

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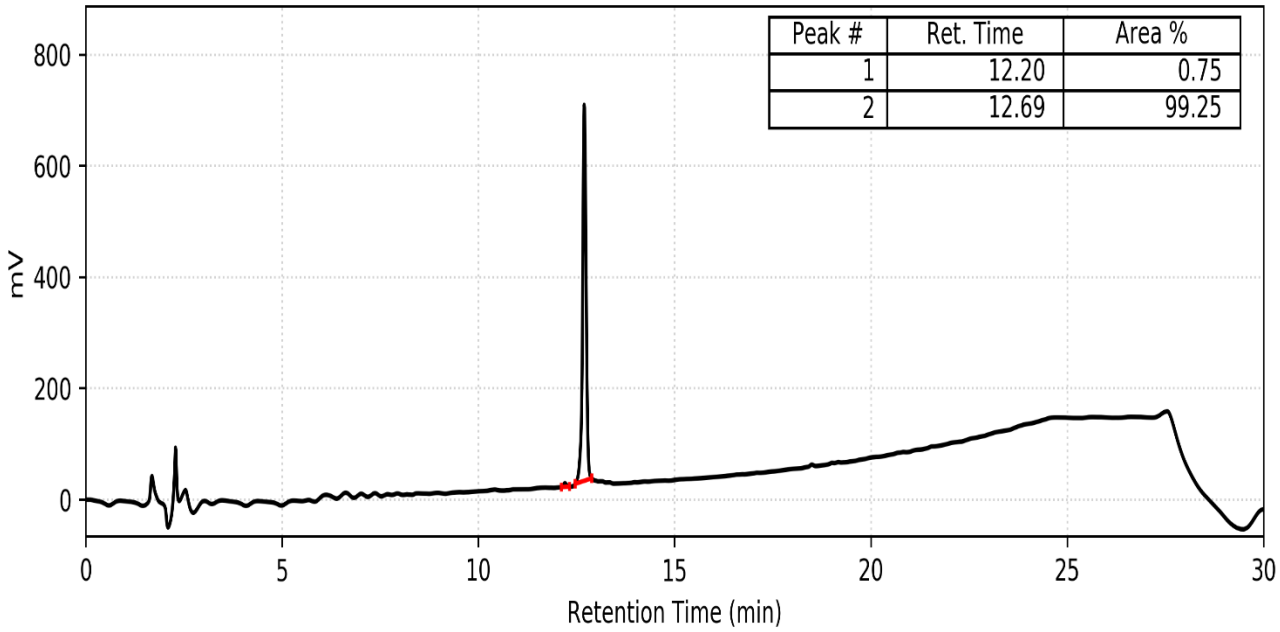

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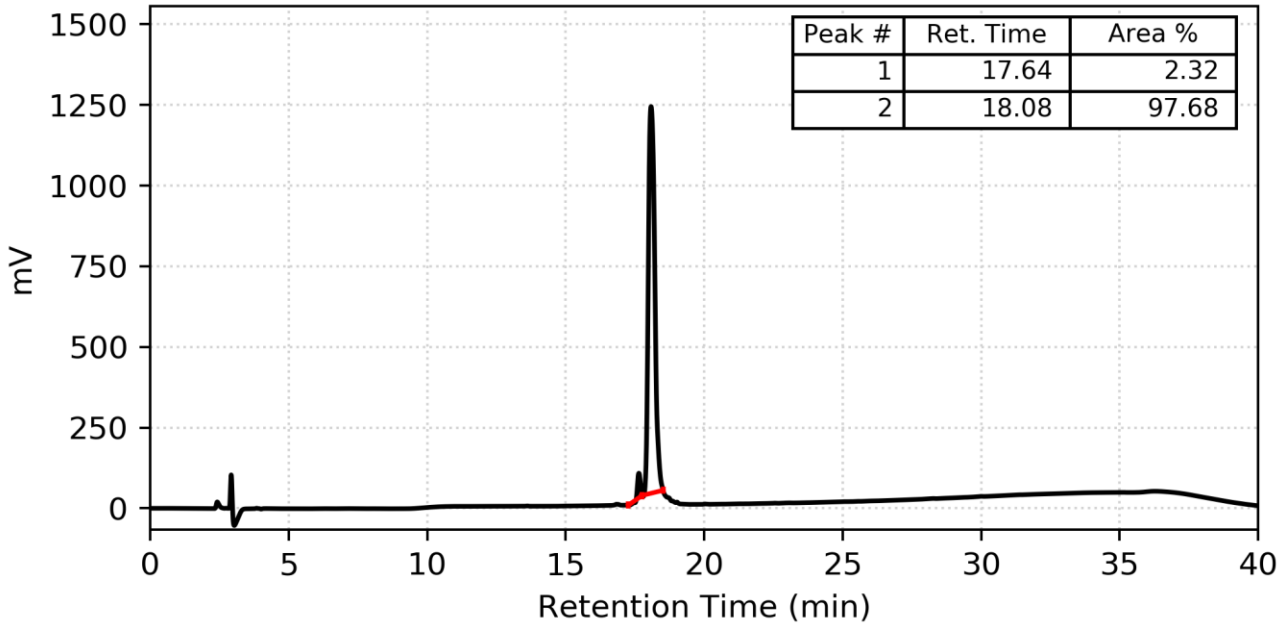


MS and HPLC Data for CSP1 Analogs

Table S1. MS and HPLC data for CSP1 single and double mutant analogs.

Compound Name	Calc. EM MH_2^{2+}	Obs. EM MH_2^{2+}	Purity (%)
CSP1-L4Cha	1142.1528	1142.1529	≥98
CSP1-L4HLeu	1129.1449	1129.1451	≥99
CSP1-F7Cha	1125.1606	1125.1561	≥99
CSP1-F7HLeu	1112.1528	1112.1537	≥99
CSP1-F8Cha	1125.1606	1125.1616	≥99
CSP1-F8HLeu	1112.1528	1112.1535	≥99
CSP1-F11Cha	1125.1606	1125.1614	≥99
CSP1-F11HLeu	1112.1528	1112.1532	≥99
CSP1-I12Cha	761.7709*	761.7678*	≥98
CSP1-I12HLeu	1129.1449	1129.1437	≥98
CSP1-F7Cha/F8Cha	1128.1841	1128.1838	≥99
CSP1-F7Cha/F8HLeu	1115.1762	1115.1743	≥99
CSP1-F7HLeu/F8Cha	1115.1762	1115.1754	≥99
CSP1-F7HLeu/F8HLeu	1102.1684	1102.1718	≥99
CSP1-F7Cha/I12Cha	1145.1762	1145.1771	≥98
CSP1-F7Cha/I12HLeu	1132.1684	1132.1691	≥99
CSP1-F7HLeu/I12Cha	1132.1684	1132.1725	≥98
CSP1-F7HLeu/I12HLeu	1119.1606	1119.1620	≥99
CSP1-F8Cha/I12Cha	1145.1762	1145.1775	≥98
CSP1-F8Cha/I12HLeu	1132.1684	1132.1720	≥99
CSP1-F8HLeu/I12Cha	1132.1684	1132.1711	≥98
CSP1-F8HLeu/I12HLeu	1119.1606	1119.1622	≥99

EM = Exact Mass. See methods above, *MH_3^{3+}.

Table S2. MS and HPLC data for CSP1-E1A mutant analogs.

Compound Name	Calc. EM MH_2^{2+}	Obs. EM MH_2^{2+}	Purity (%)
CSP1-E1A/F7Cha	1096.1578	1096.1562	≥99
CSP1-E1A/F7HLeu	1083.1500	1083.1493	≥99
CSP1-E1A/F8Cha	1096.1578	1096.1598	≥99
CSP1-E1A/F8HLeu	1083.1500	1083.1488	≥99
CSP1-E1A/I12Cha	1113.1500	1113.1506	≥99
CSP1-E1A/I12HLeu	1100.1422	1100.1461	≥99
CSP1-E1A/F7Cha/I12Cha	1116.1735	1116.1759	≥99
CSP1-E1A/F7Cha/I12HLeu	1103.1657	1103.1672	≥97

EM = Exact Mass. See methods above.

Primary Reporter Gene Assay Data

S. pneumoniae D39pcomX::lacZ (ComD1)

Agonism assays were performed at 10 µM concentration of synthetic CSP1 analogs. CSP1 was used as the positive control (100%) while DMSO as the negative control (0%). Percent (%) ComD1 activation was measured by normalizing the Miller units obtained for each peptide to that of the native CSP1. All peptides were screened in triplicate over three separate trials. Error bars indicate standard error of the mean of nine values.

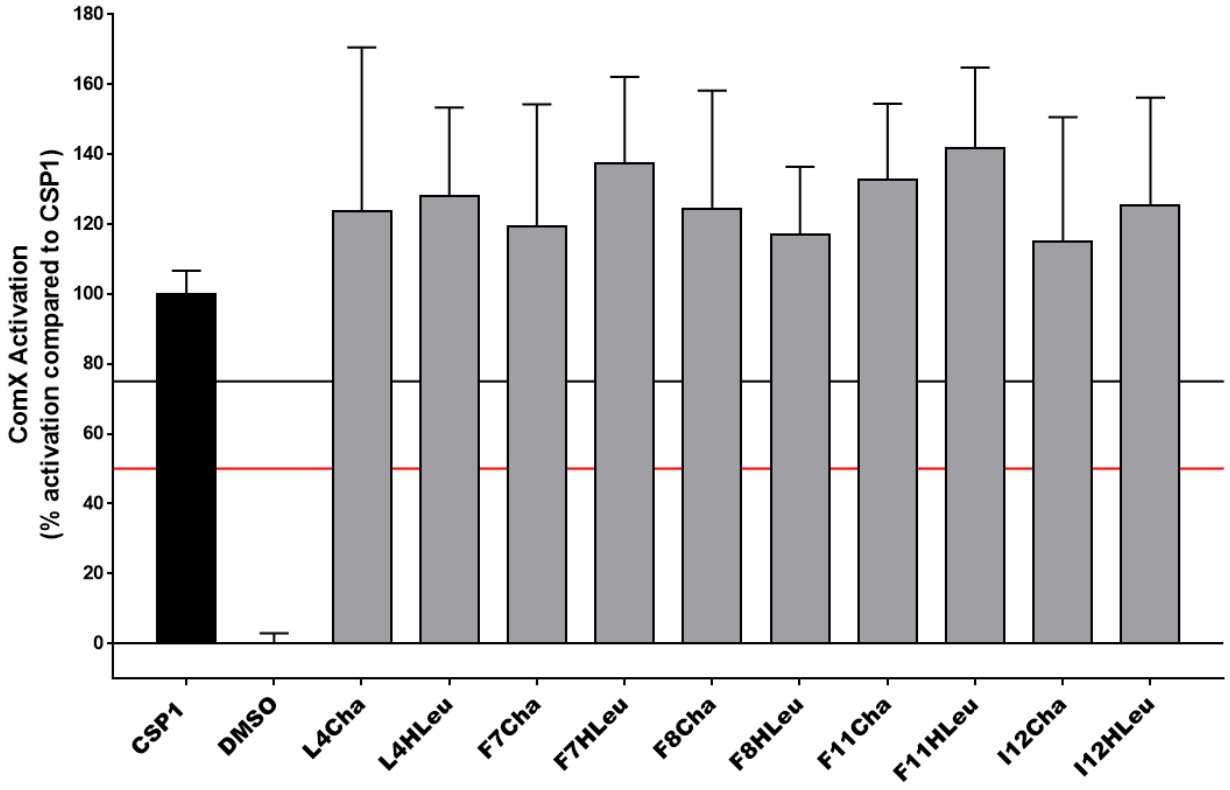


Figure S-1. Primary agonism screening assay data for the CSP1 single mutant analogs. Peptides that exhibited over 75% activation were further evaluated to determine their EC_{50} values.

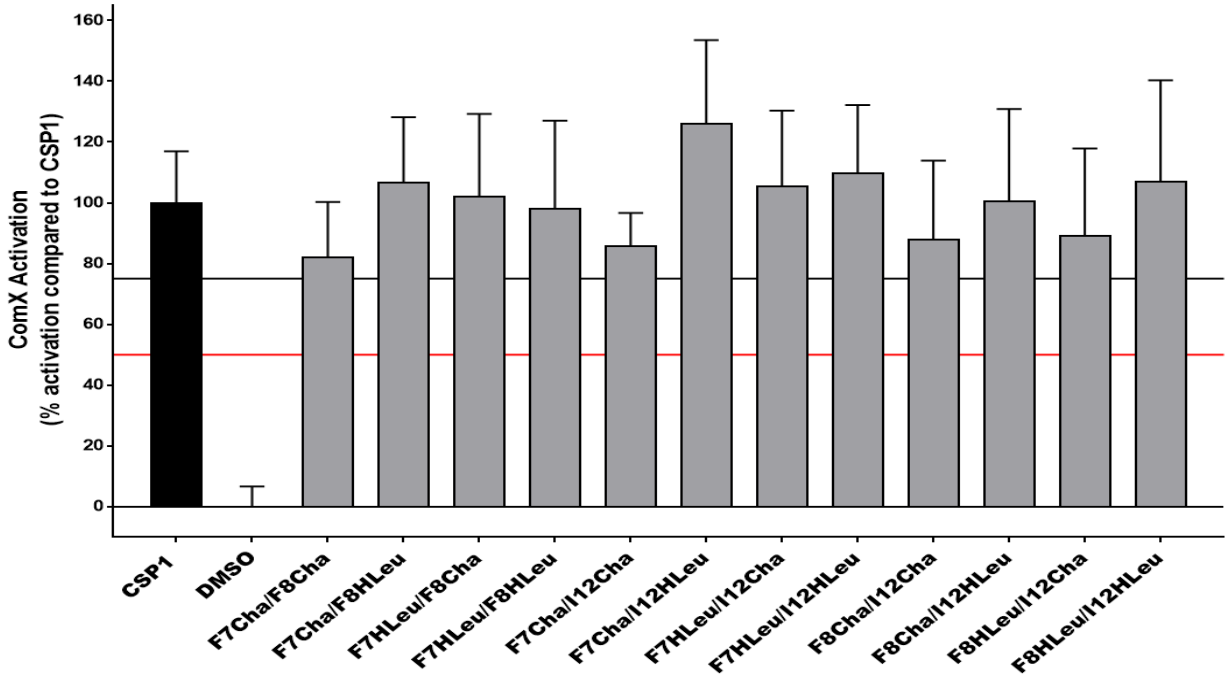


Figure S-2. Primary agonism screening assay data for the CSP1 double mutant analogs. Peptides that exhibited over 75% activation were further evaluated to determine their EC_{50} values.

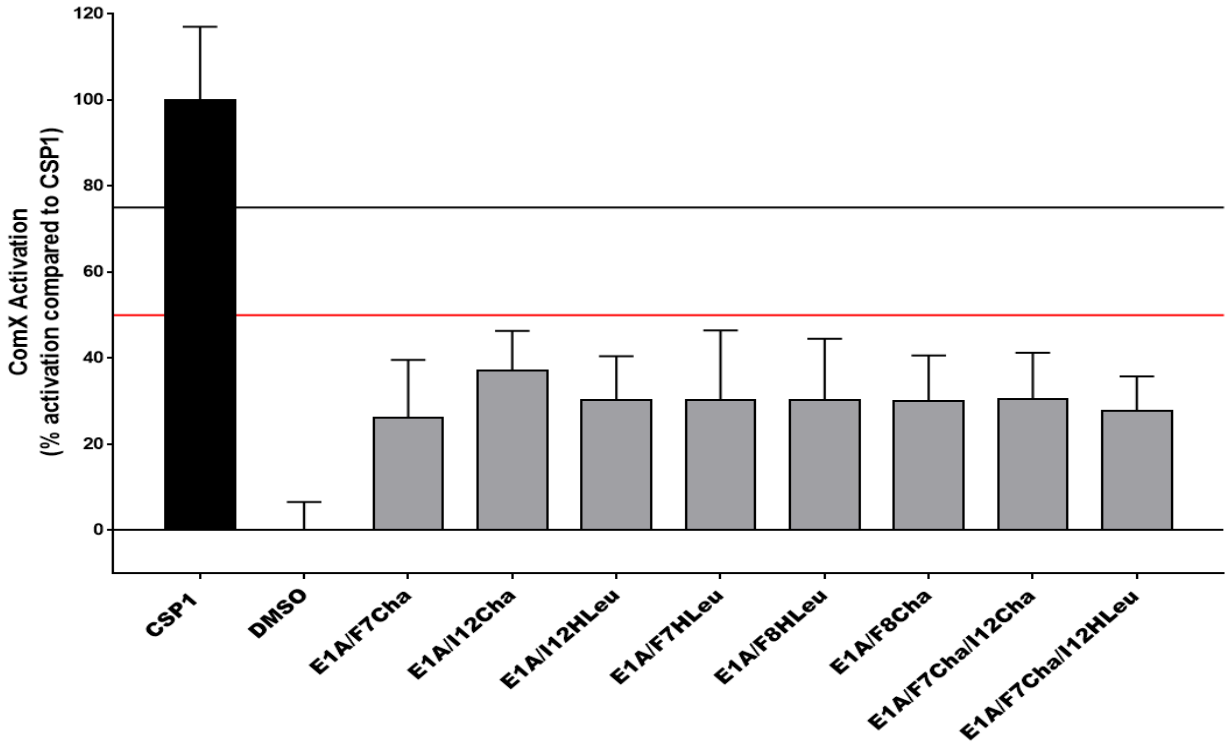


Figure S-3. Primary agonism screening assay data for the CSP1-E1A mutant analogs. None of the peptides exhibited activation of the ComD1 receptor and peptides that exhibited less than 50% activation were evaluated as potential competitive inhibitors.

Antagonism assays were performed at 10 μM concentration of peptides against 50 nM concentration of CSP1. CSP1 (50 nM) was used as the positive control (100%) while DMSO as the negative control (0%). Percent (%) *comX* activation was measured by normalizing the Miller units obtained for each peptide to that of CSP1. All peptides were screened in triplicate over three separate trials. Error bars indicate standard error of the mean of nine values.

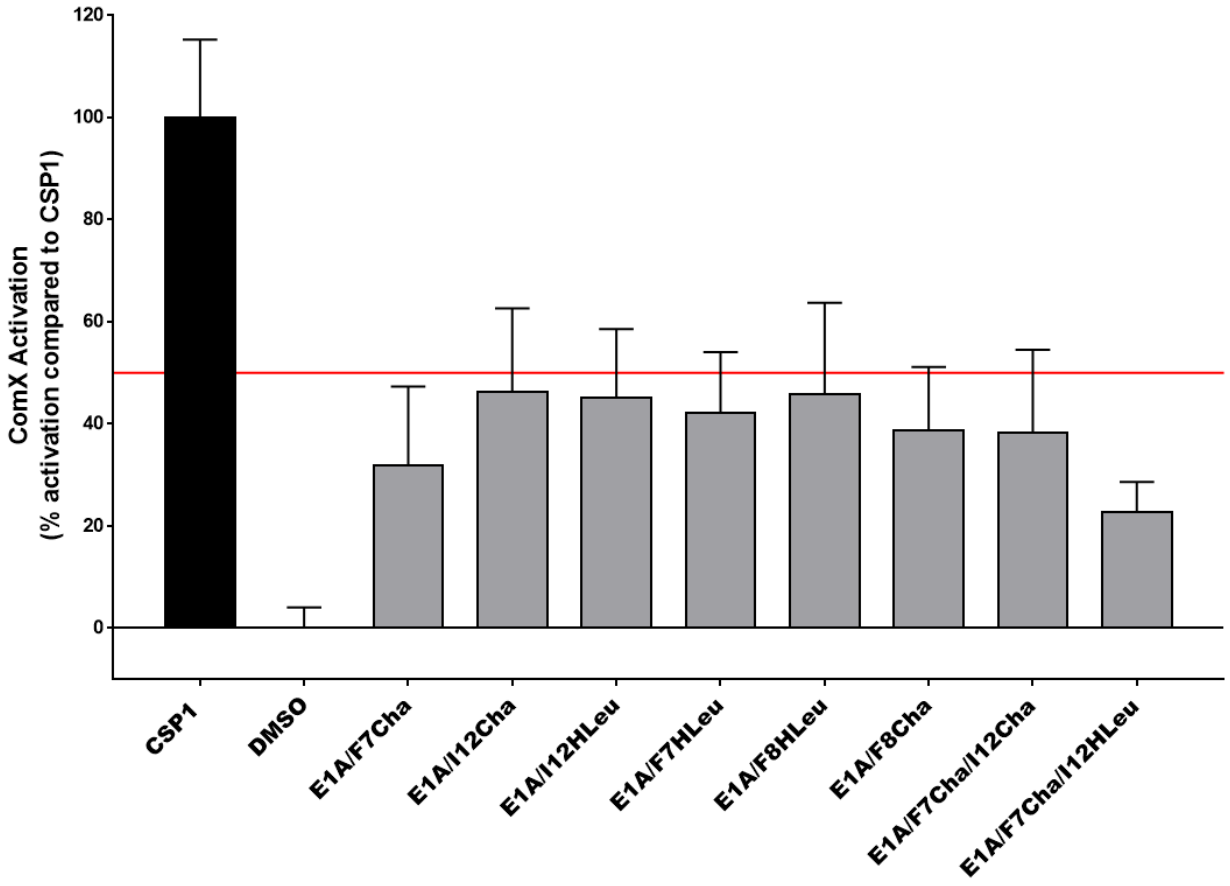


Figure S-4. Primary antagonism screening assay data for the CSP1-E1A mutant analogs. Peptides that exhibited less than 50% activation were further evaluated to determine their IC_{50} values.

***S. pneumoniae* TIGR4 pcomX::lacZ (ComD2)**

Agonism assays were performed at 10 μM concentration. CSP2 was used as the positive control (100%) while DMSO as the negative control (0%). Percent (%) ComD2 activation was measured by normalizing the Miller units obtained for each peptide to that of the native CSP2. All peptides were screened in triplicate over three separate trials. Error bars indicate standard error of the mean of nine values.

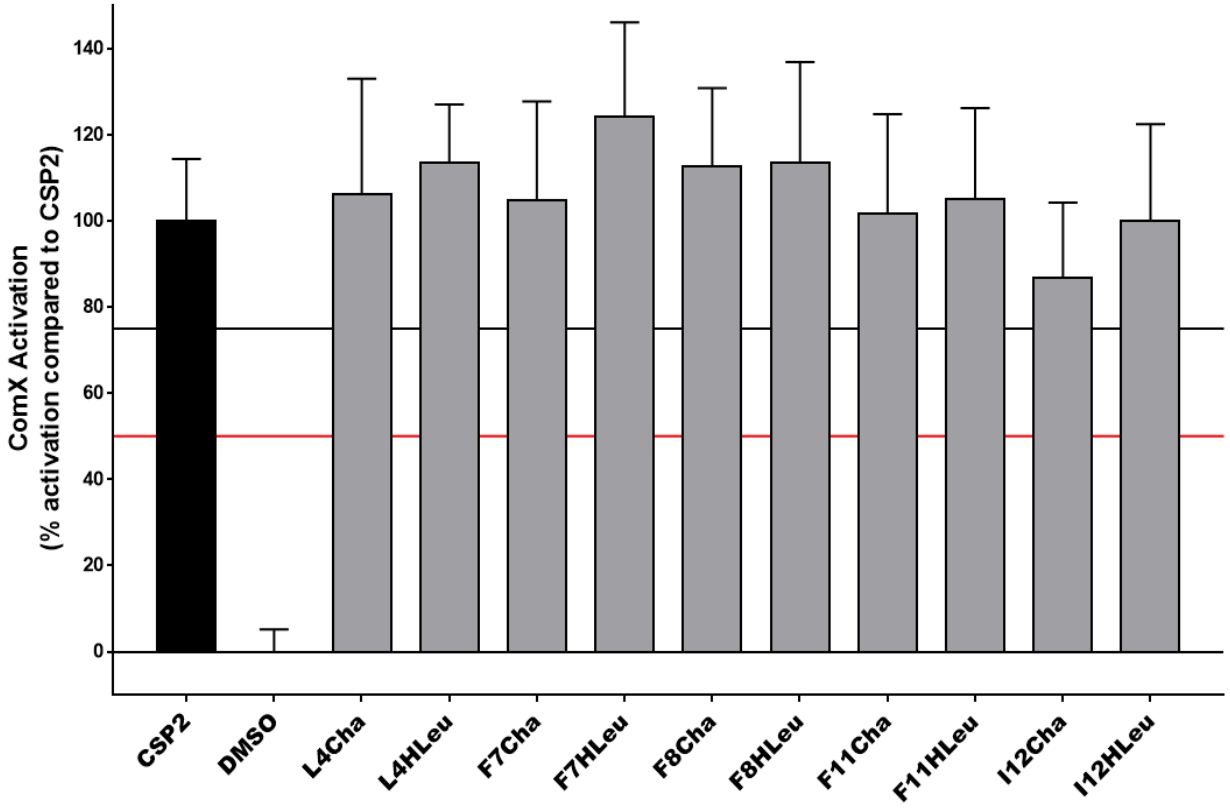


Figure S-5. Primary agonism screening assay data for the CSP1 single mutant analogs. Peptides that exhibited over 75% activation were further evaluated to determine their EC_{50} values.

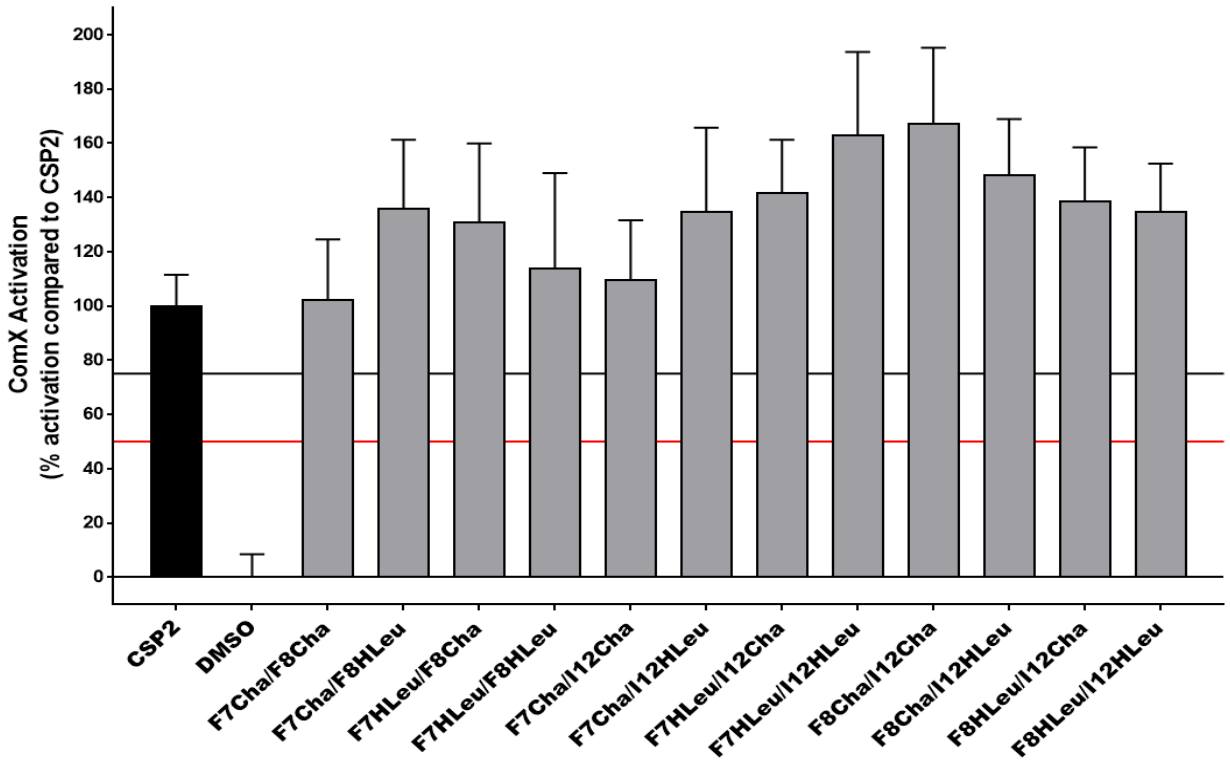


Figure S-6. Primary agonism screening assay data for the CSP1 double mutant analogs. Peptides that exhibited over 75% activation were further evaluated to determine their EC_{50} values.

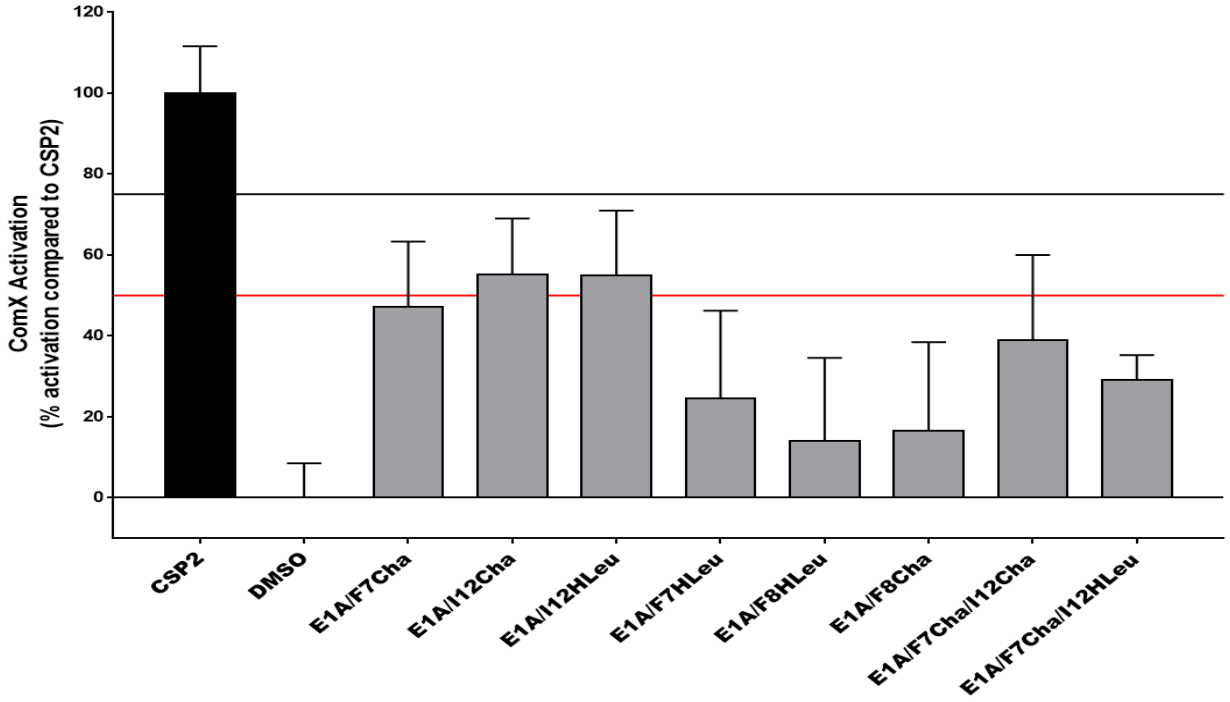


Figure S-7. Primary agonism screening assay data for the CSP1-E1A mutant analogs. None of the peptides exhibited activation of the ComD2 receptor and peptides that exhibited less than 50% activation were evaluated as potential competitive inhibitors.

Antagonism assays were performed at 10 μM concentration of peptides against 250 nM concentration of CSP2. CSP2 (250 nM) was used as the positive control (100%) while DMSO as the negative control (0%). Percent (%) *comX* activation was measured by normalizing the Miller units obtained for each peptide to that of CSP2. All peptides were screened in triplicate over three separate trials. Error bars indicate standard error of the mean of nine values.

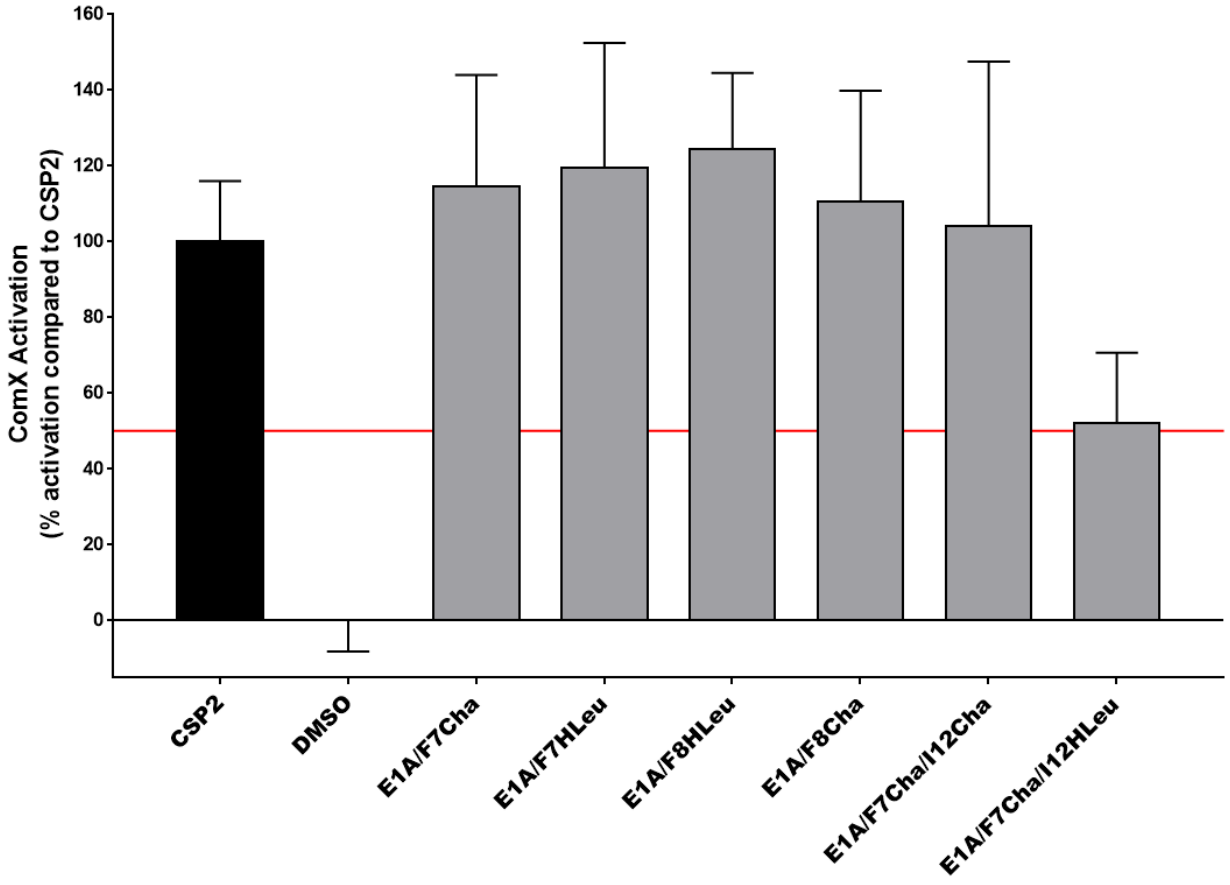


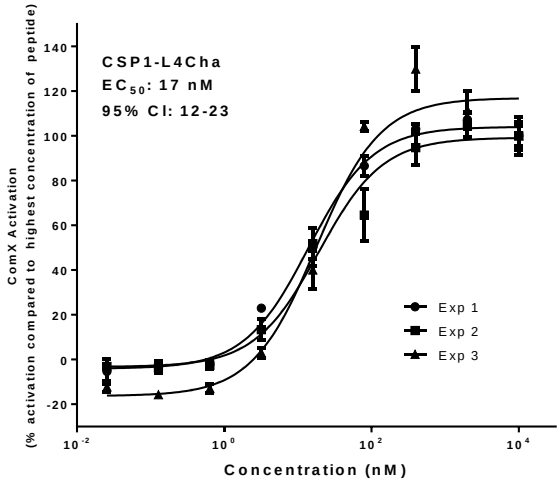
Figure S-8. Primary antagonism screening assay data for the CSP1-E1A mutant analogs. None of the peptides exhibited inhibition of the ComD2 receptor.

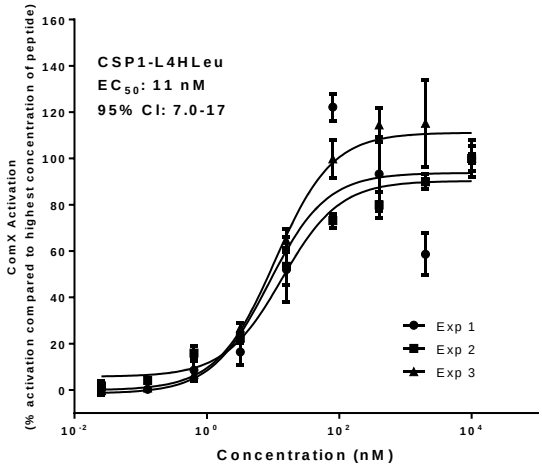
Agonism and Antagonism Dose Response Curves

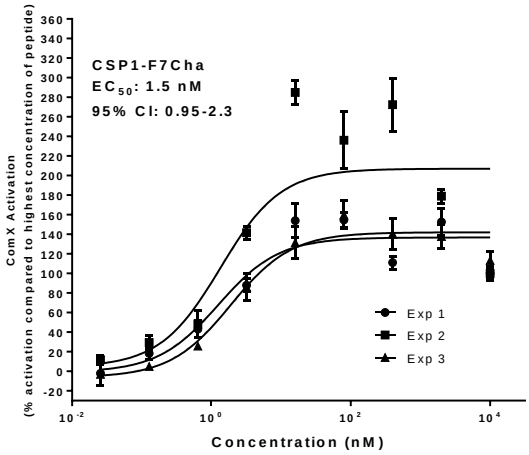
CSP1 analogs were tested to determine their EC_{50} or IC_{50} values over varying concentrations in the two indicated *S. pneumoniae* β-galactosidase reporter strains. Each dose response curve is representative of three independent experiments performed on three separate occasions (i.e., experiments (Exp.) #1-3; shown for each peptide below). Error bars represent standard error of the mean of triplicate values. In each plot, the peptide, as well as its EC_{50} or IC_{50} value (in nM) and 95% confidence interval (95% CI) values (in nM), are indicated at top left or top right.

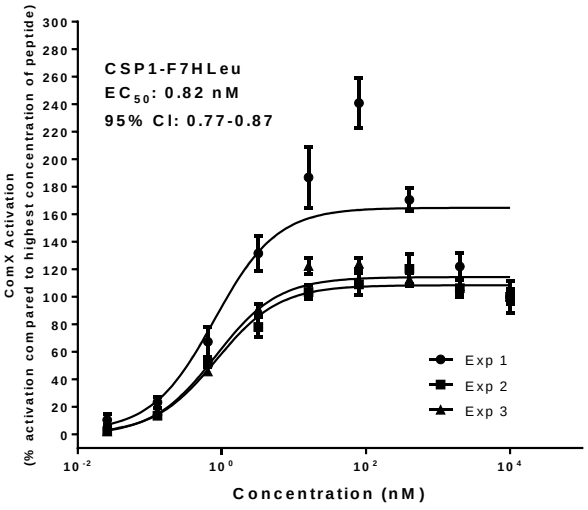
S. pneumoniae D39 pcomX::lacZ (ComD1)

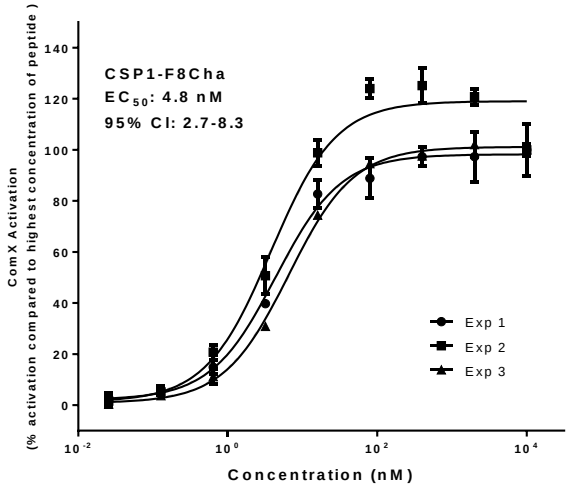
Activation dose response curves

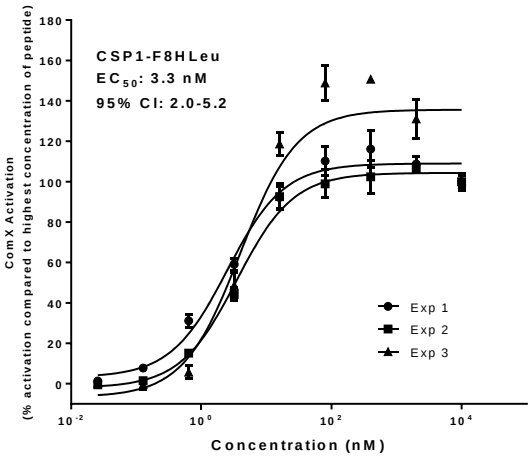


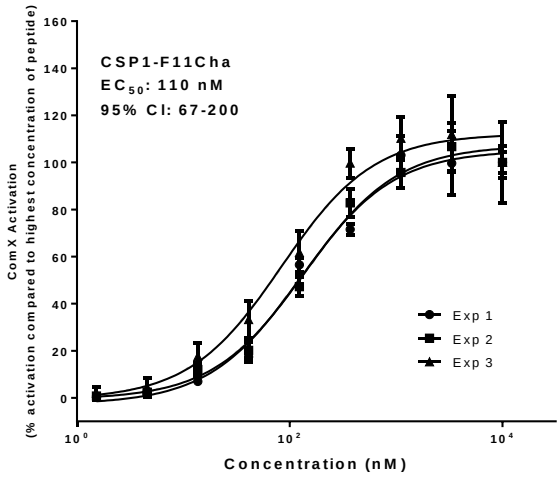


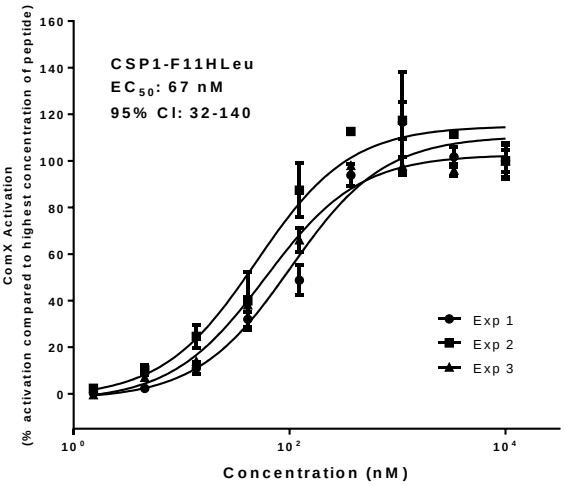


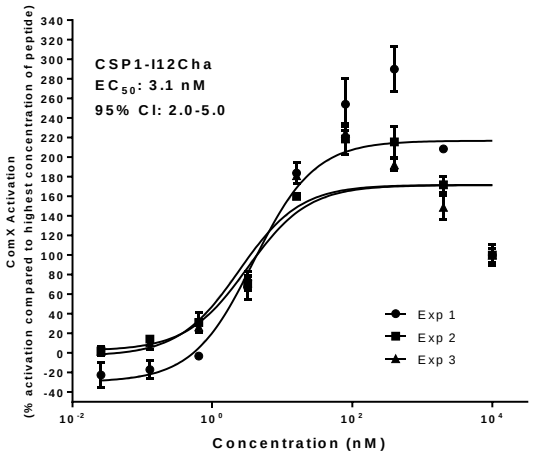


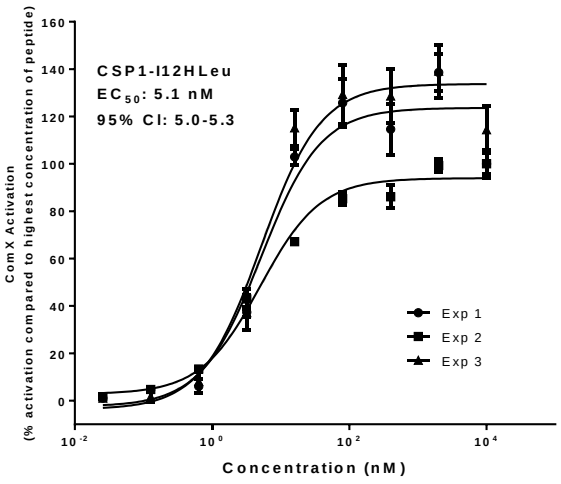

CSP1-F8Cha
EC50: 4.8 nM
95% CI: 2.7-8.3
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

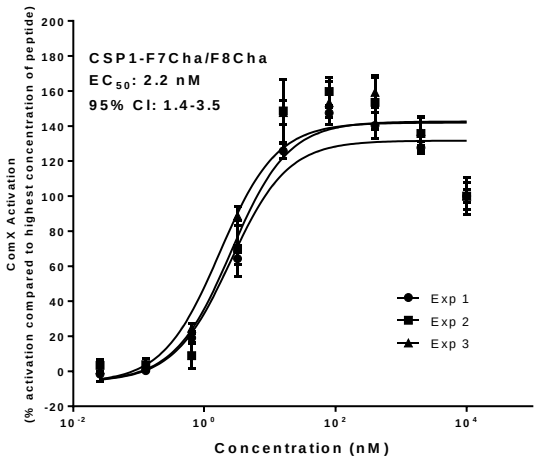

CSP1-F8HLeu
EC50: 3.3 nM
95% CI: 2.0-5.2
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

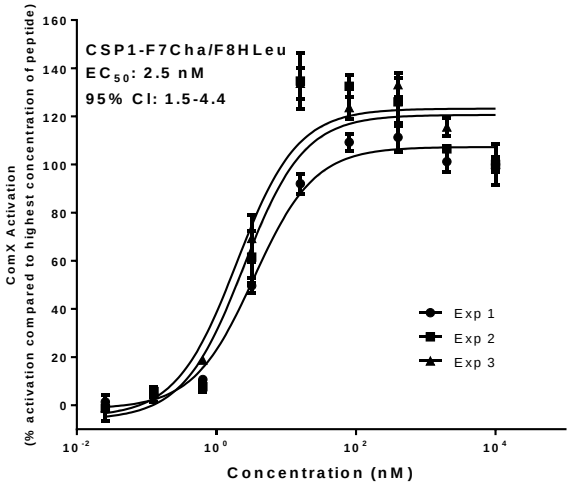

CSP1-F11Cha
EC50: 110 nM
95% CI: 67-200
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

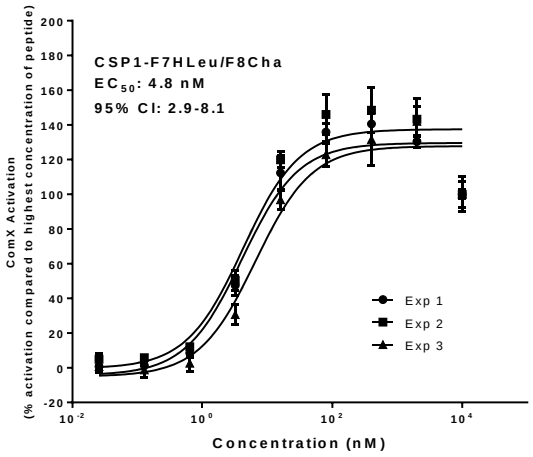

CSP1-F11HLeu
EC50: 67 nM
95% CI: 32-140
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

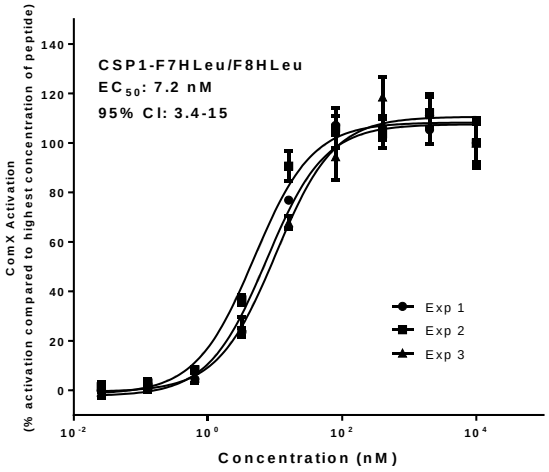

CSP1-I12Cha
EC50: 3.1 nM
95% CI: 2.0-5.0
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

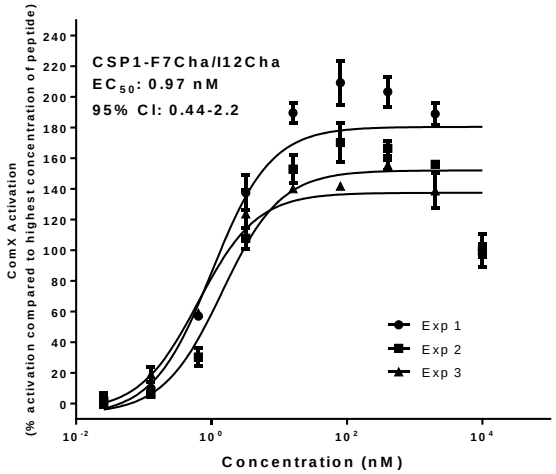

CSP1-I12HLeu
EC50: 5.1 nM
95% CI: 5.0-5.3
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

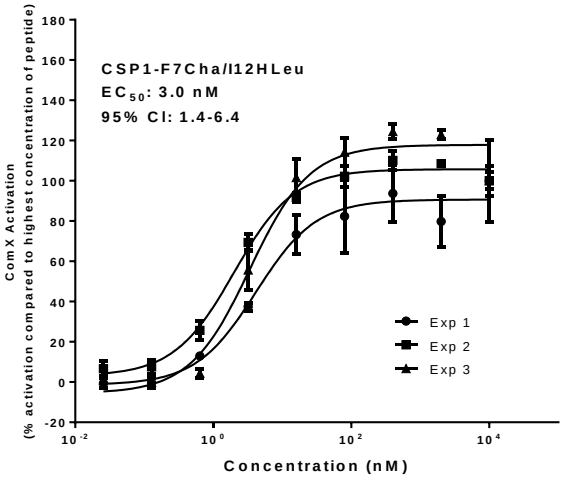

CSP1-F7Cha/F8Cha
EC50: 2.2 nM
95% CI: 1.4-3.5
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

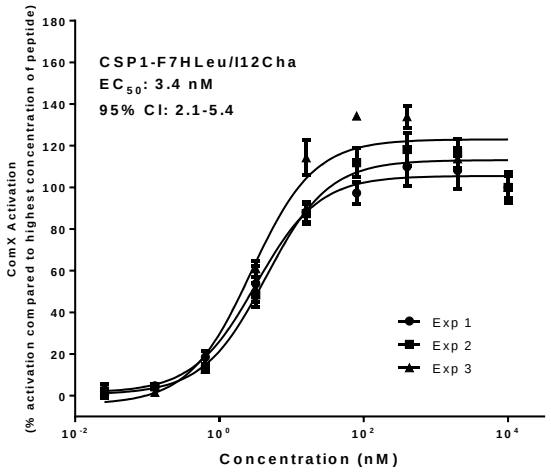

CSP1-F7Cha/F8HLeu
EC50: 2.5 nM
95% CI: 1.5-4.4
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

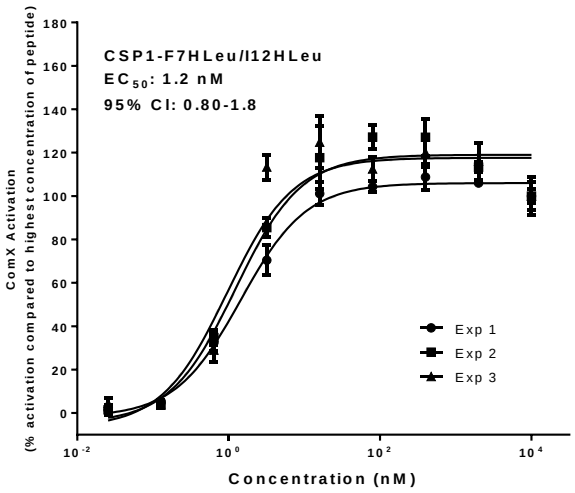

CSP1-F7HLeu/F8Cha
EC50: 4.8 nM
95% CI: 2.9-8.1
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

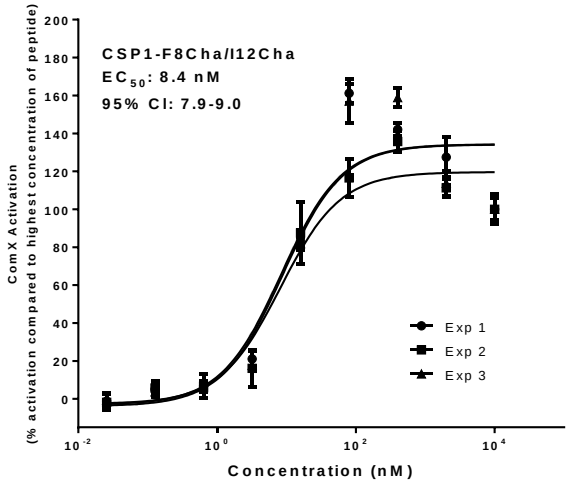

CSP1-F7HLeu/F8HLeu
EC50: 7.2 nM
95% CI: 3.4-15
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

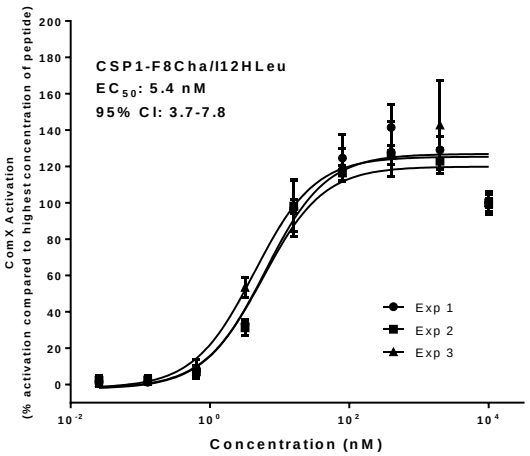

CSP1-F7Cha/I12Cha
EC50: 0.97 nM
95% CI: 0.44-2.2
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

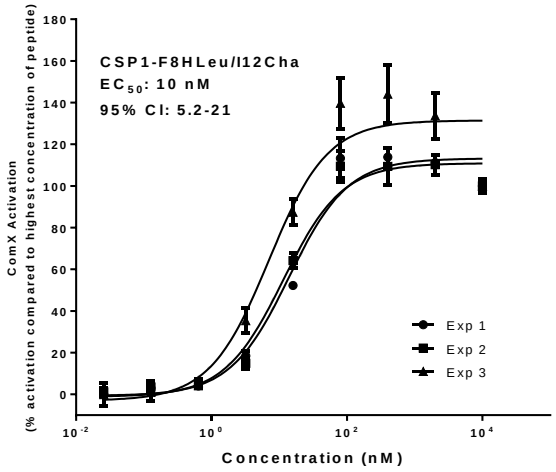

CSP1-F7Cha/I12HLeu
EC50: 3.0 nM
95% CI: 1.4-6.4
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

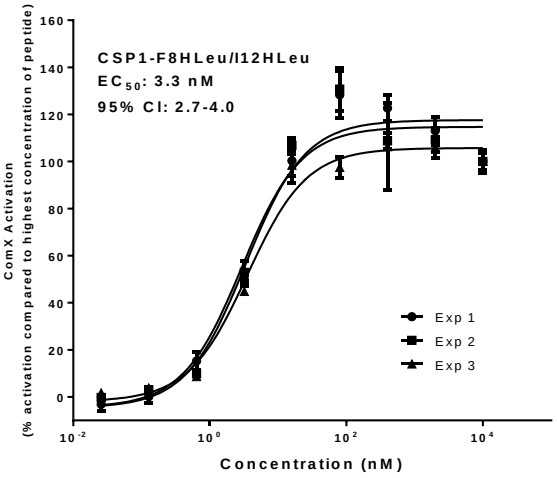

CSP1-F7HLeu/I12Cha
EC50: 3.4 nM
95% CI: 2.1-5.4
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3


CSP1-F7HLeu/I12HLeu
EC50: 1.2 nM
95% CI: 0.80-1.8
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

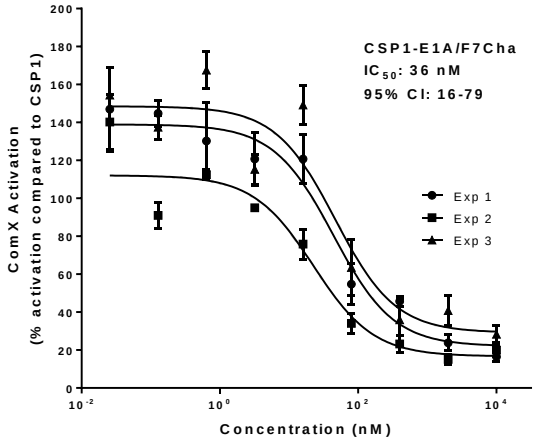

CSP1-F8Cha/I12Cha
EC50: 8.4 nM
95% CI: 7.9-9.0
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

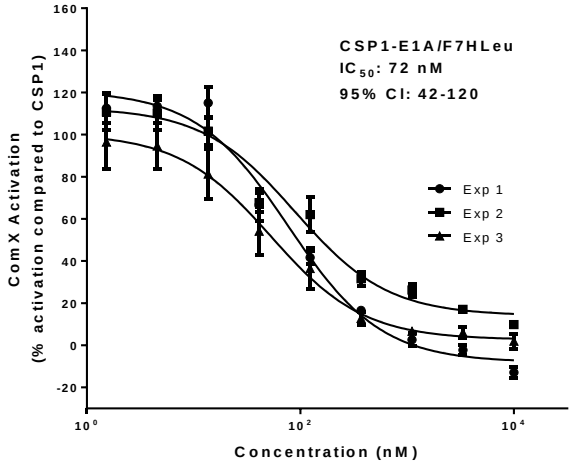

CSP1-F8Cha/I12HLeu
EC50: 5.4 nM
95% CI: 3.7-7.8
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

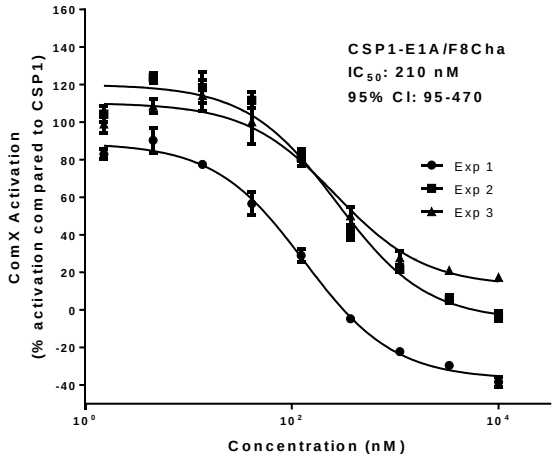

CSP1-F8HLeu/I12Cha
EC50: 10 nM
95% CI: 5.2-21
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

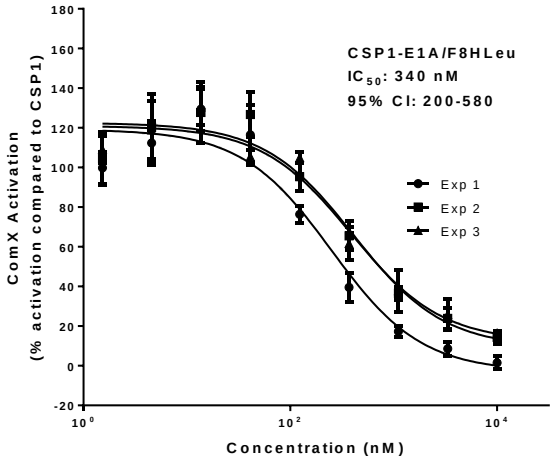

CSP1-F8HLeu/I12HLeu
EC50: 3.3 nM
95% CI: 2.7-4.0
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

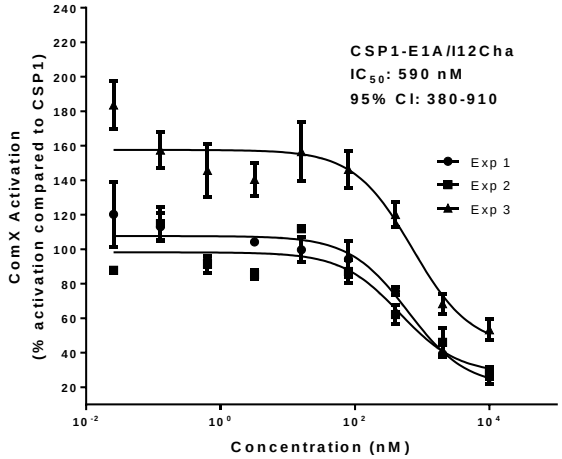
Inhibition dose response curves

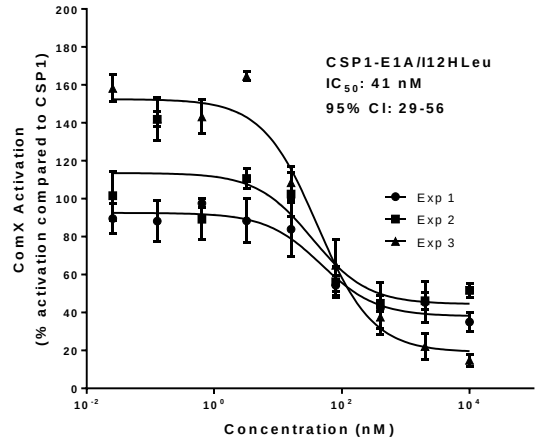


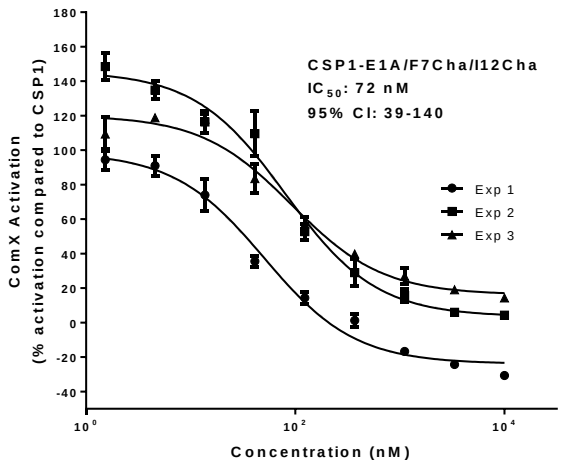


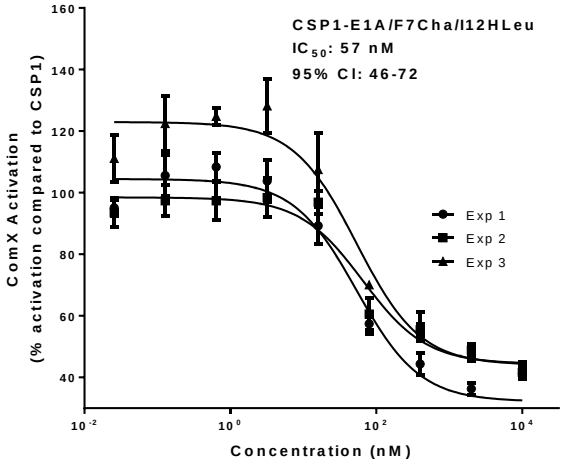






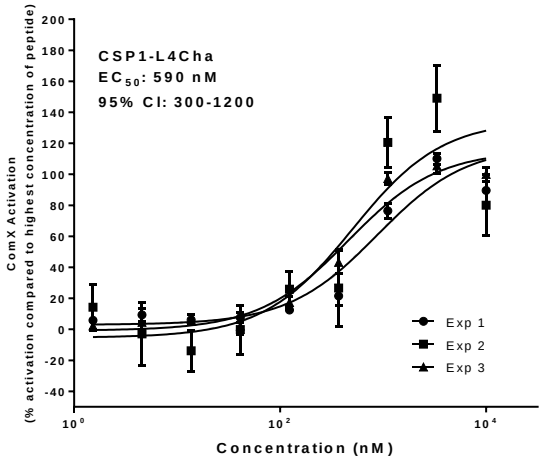


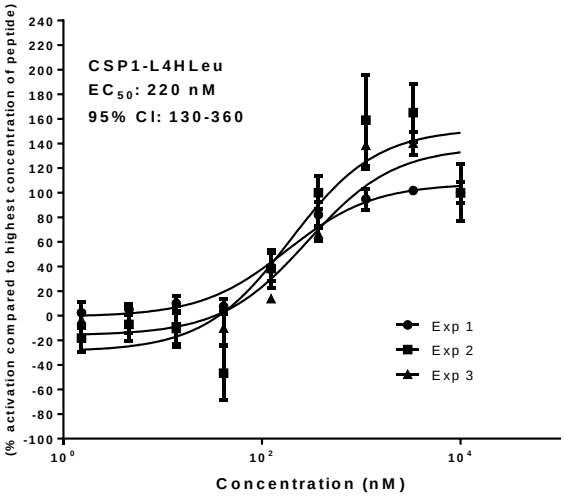


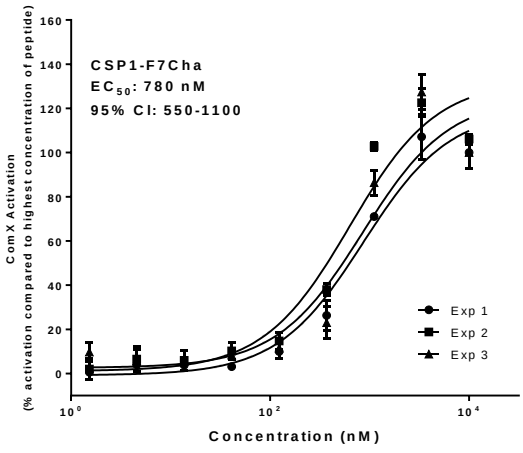


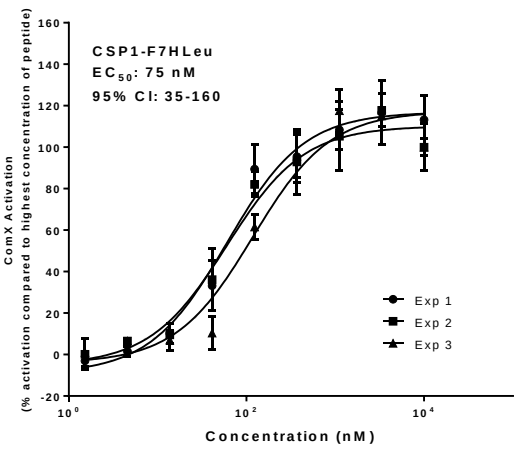
S. pneumoniae TIGR4 pcomX::lacZ (ComD2)

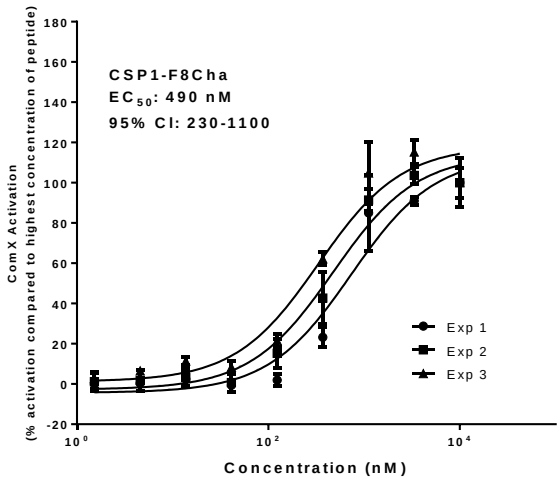
Activation dose response curves

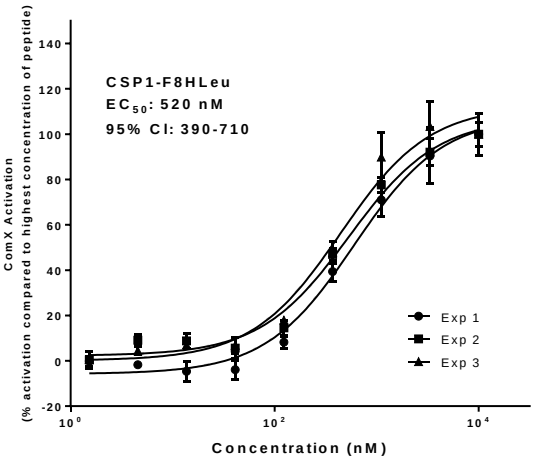


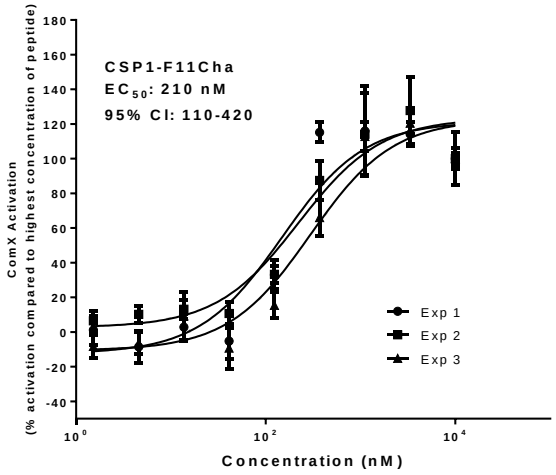


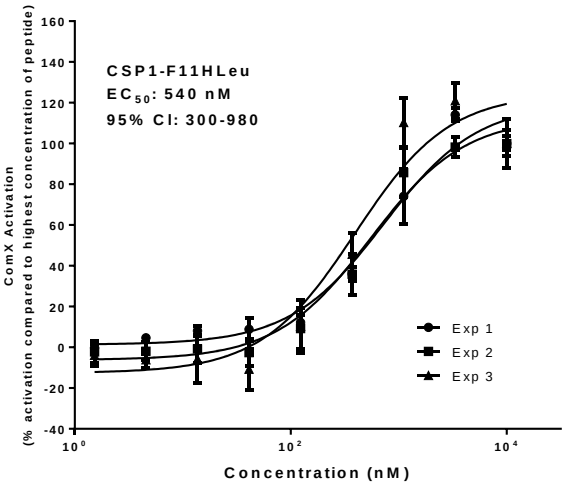


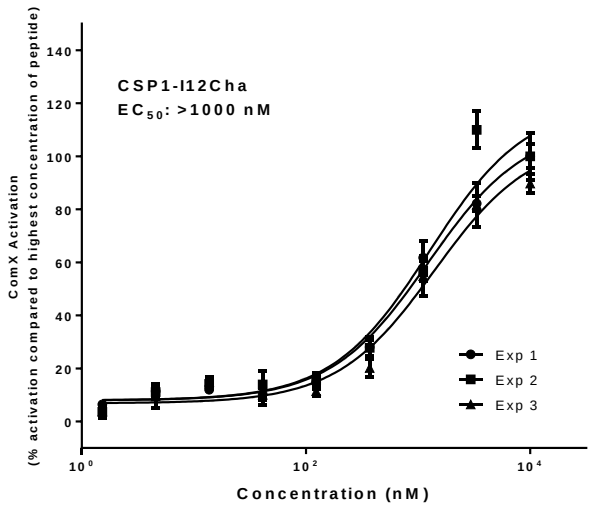


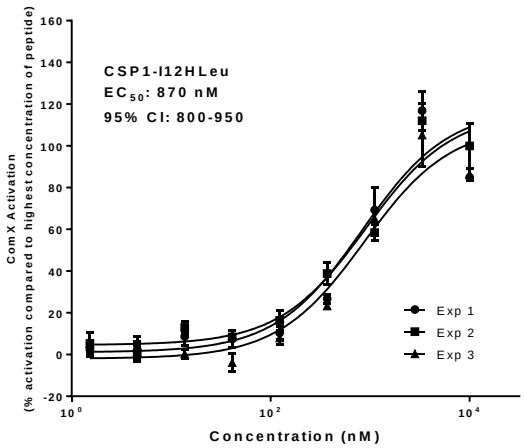

CSP1-F8Cha
EC50: 490 nM
95% CI: 230-1100
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

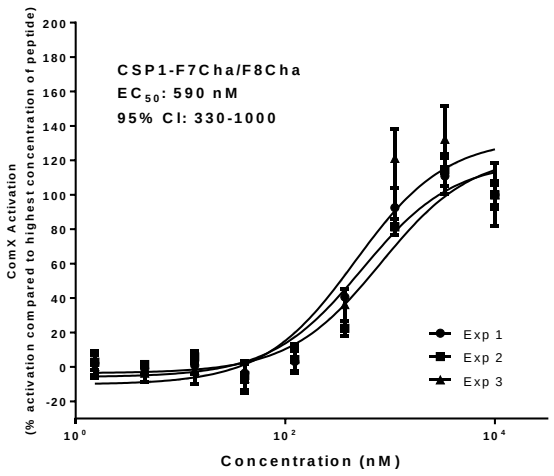

CSP1-F8HLeu
EC50: 520 nM
95% CI: 390-710
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

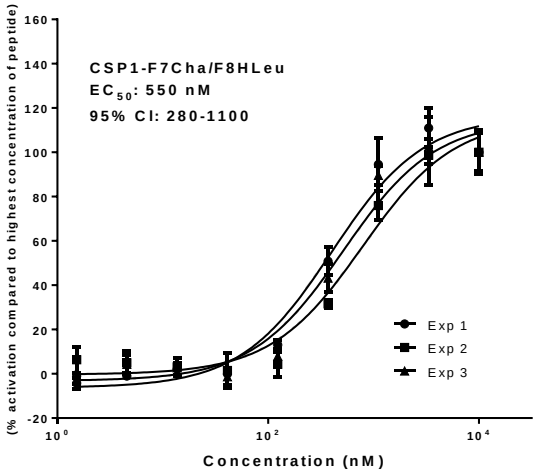

CSP1-F11Cha
EC50: 210 nM
95% CI: 110-420
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

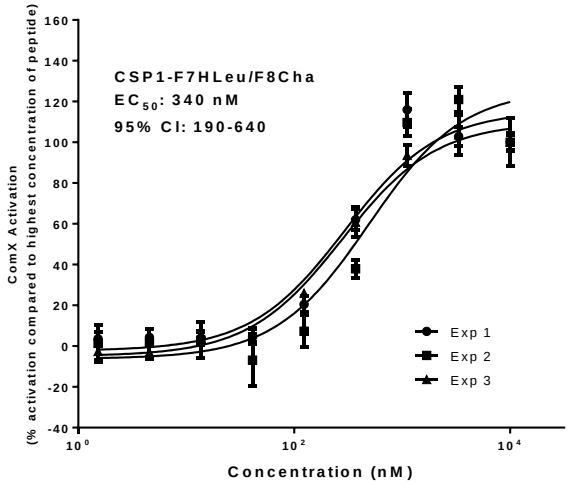

CSP1-F11HLeu
EC50: 540 nM
95% CI: 300-980
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

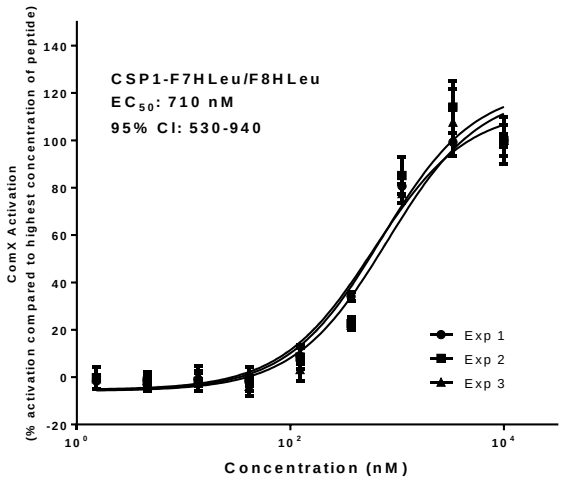

CSP1-I12Cha
EC50: >1000 nM
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

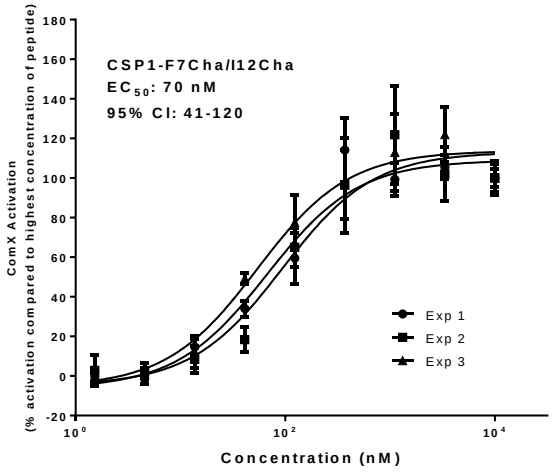

CSP1-I12HLeu
EC50: 870 nM
95% CI: 800-950
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

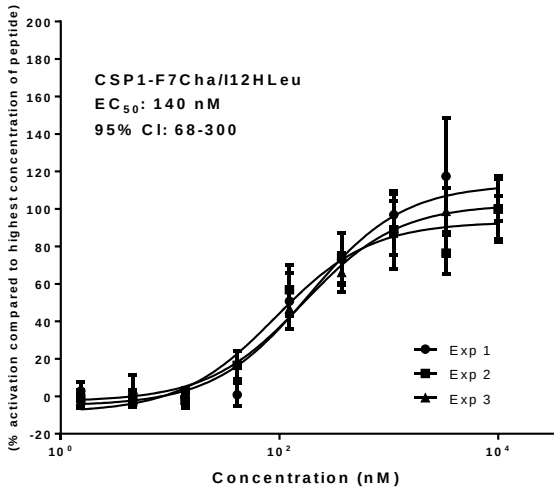

CSP1-F7Cha/F8Cha
EC50: 590 nM
95% CI: 330-1000
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

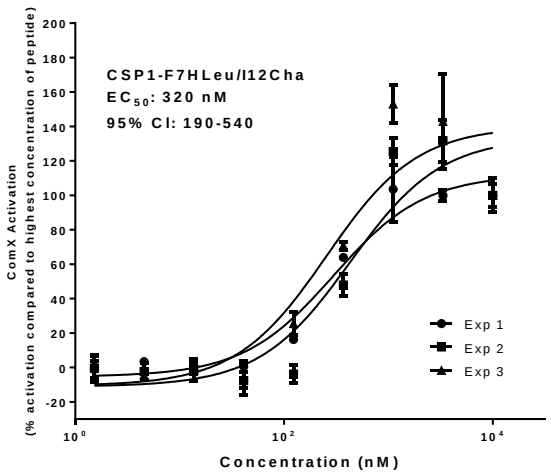

CSP1-F7Cha/F8HLeu
EC50: 550 nM
95% CI: 280-1100
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

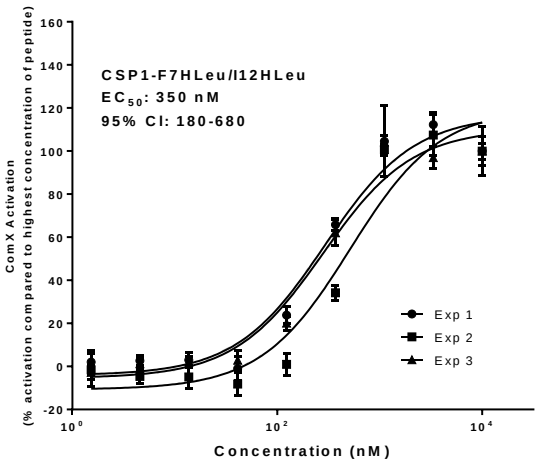

CSP1-F7HLeu/F8Cha
EC50: 340 nM
95% CI: 190-640
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

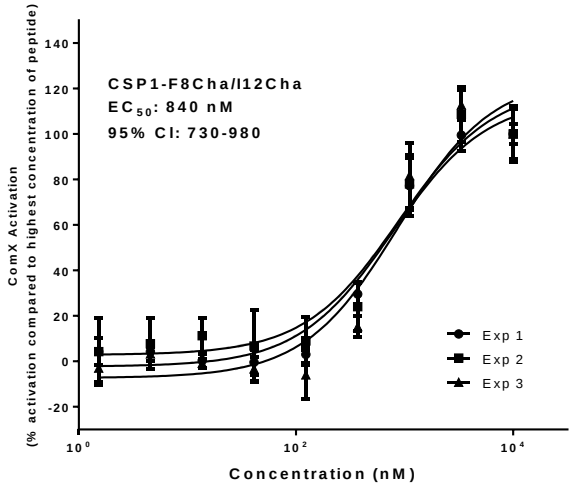

CSP1-F7HLeu/F8HLeu
EC50: 710 nM
95% CI: 530-940
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

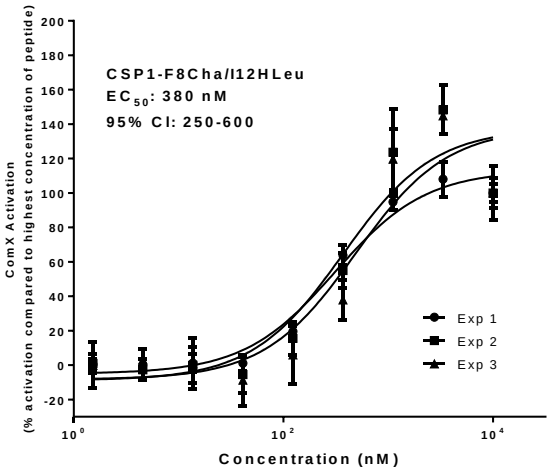

CSP1-F7Cha/I12Cha
EC50: 70 nM
95% CI: 41-120
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

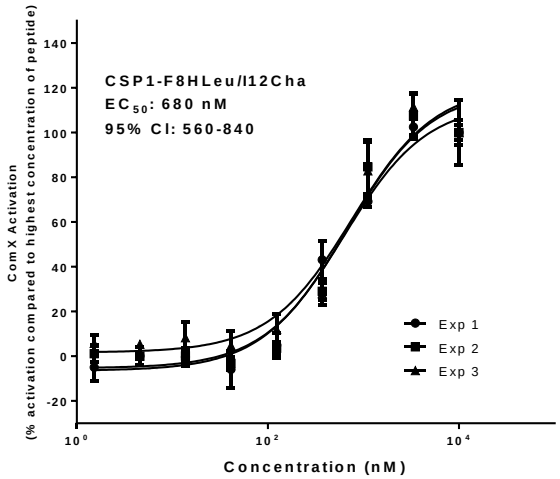

CSP1-F7Cha/I12HLeu
EC50: 140 nM
95% CI: 68-300
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

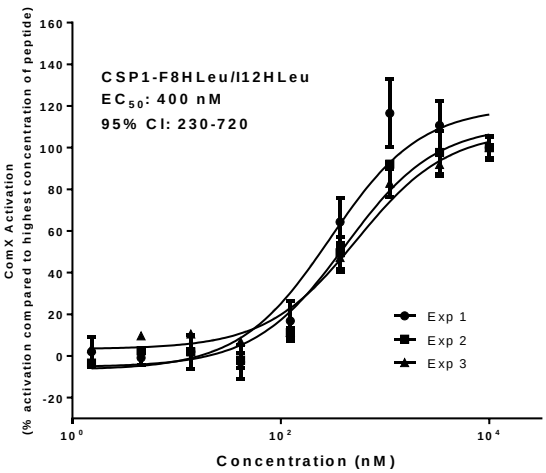

CSP1-F7HLeu/I12Cha
EC50: 320 nM
95% CI: 190-540
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3


CSP1-F7HLeu/I12HLeu
EC50: 350 nM
95% CI: 180-680
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3


CSP1-F8Cha/I12Cha
EC50: 840 nM
95% CI: 730-980
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3


CSP1-F8Cha/I12HLeu
EC50: 380 nM
95% CI: 250-600
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3


CSP1-F8HLeu/I12Cha
EC50: 680 nM
95% CI: 560-840
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3


CSP1-F8HLeu/I12HLeu
EC50: 400 nM
95% CI: 230-720
ComX Activation
(% activation compared to highest concentration of peptide)
Concentration (nM)
Exp 1
Exp 2
Exp 3

Metabolic Stability Analysis of Lead CSP1 Analogs

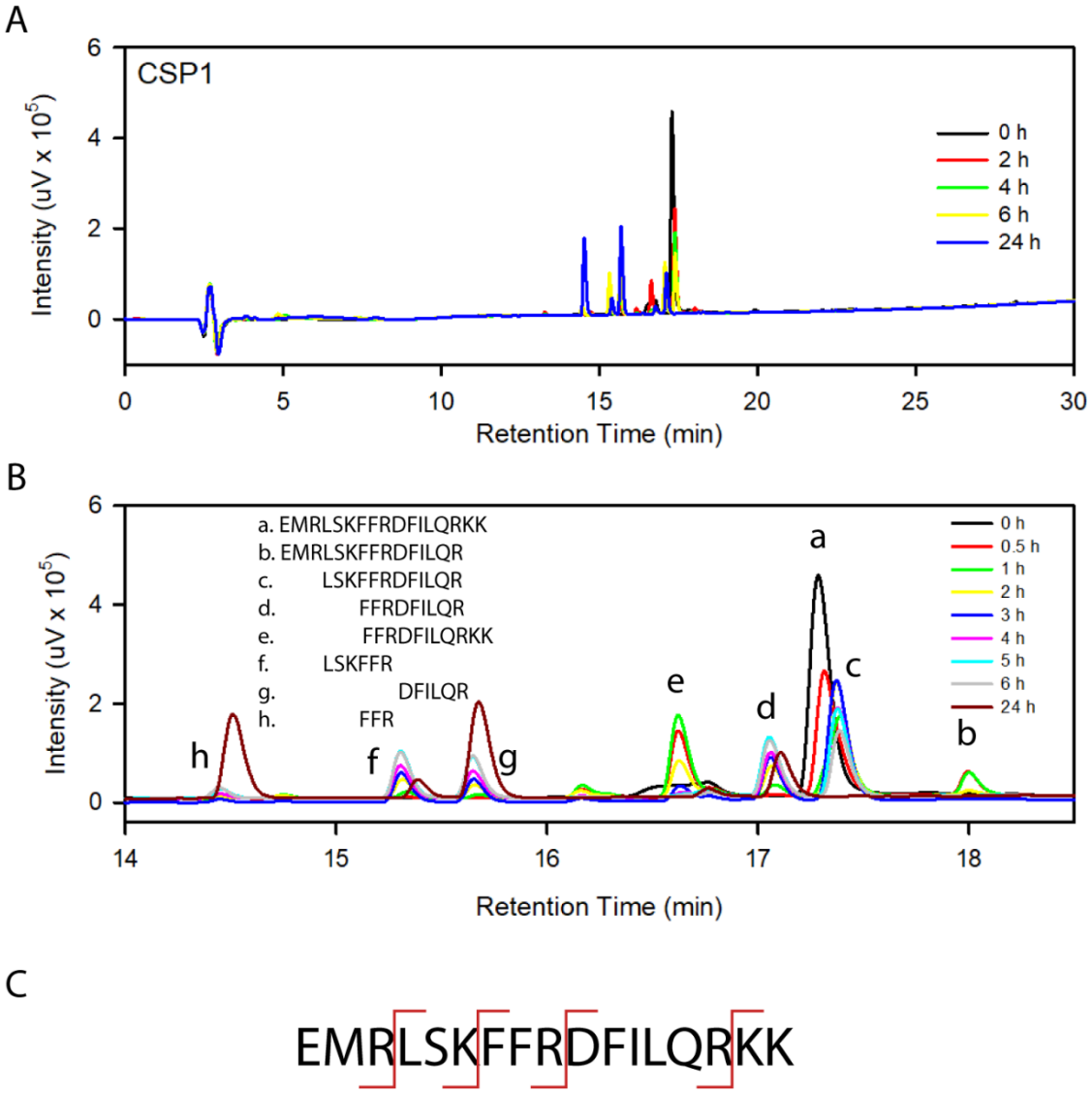


Figure S-9. Degradation pattern analysis of CSP1 in the presence of trypsin. A) Comparison of analytical HPLC chromatograms (220 nm) across the time points taken at 0, 2, 4, 6 and 24 h. B) Zoomed view of panel A with all the time points (0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h). All peaks were collected and analyzed via mass spectrometry (MALDI-TOF MS and ESI-TOF LC-MS). The degradation products corresponding to each peak are listed on the left. C) A summary of observed trypsin cleavage sites annotated on the CSP1 sequence.

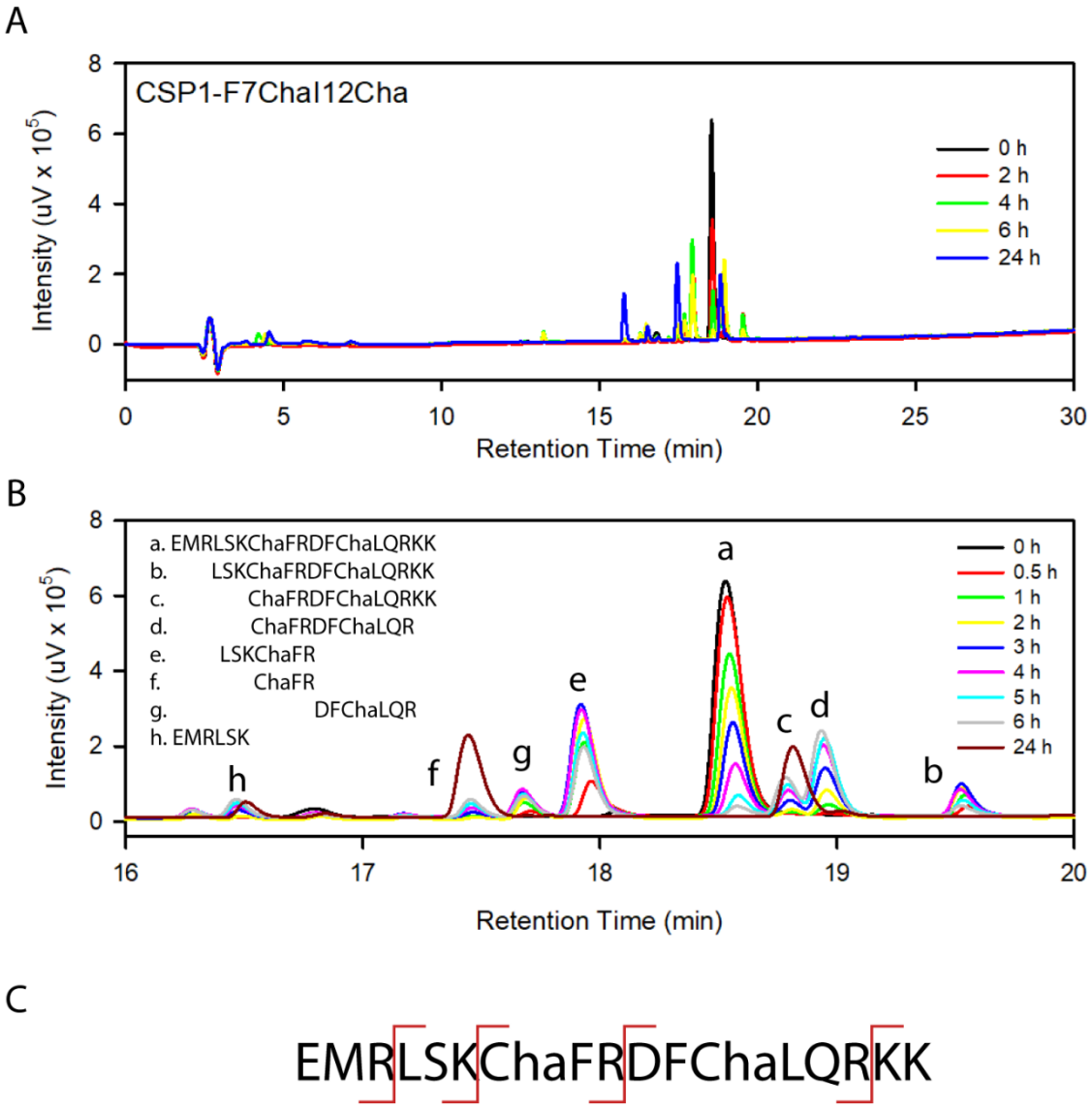


Figure S-10. Degradation pattern analysis of CSP1-F7ChaI12Cha in the presence of trypsin. A) Comparison of analytical HPLC chromatograms (220 nm) across the time points taken at 0, 2, 4, 6 and 24 h. B) Zoomed view of panel A with all the time points (0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h). All peaks were collected and analyzed via mass spectrometry (MALDI-TOF MS and ESI-TOF LC-MS). The degradation products corresponding to each peak are listed on the left. C) A summary of observed trypsin cleavage sites annotated on the CSP1-F7ChaI12Cha sequence.

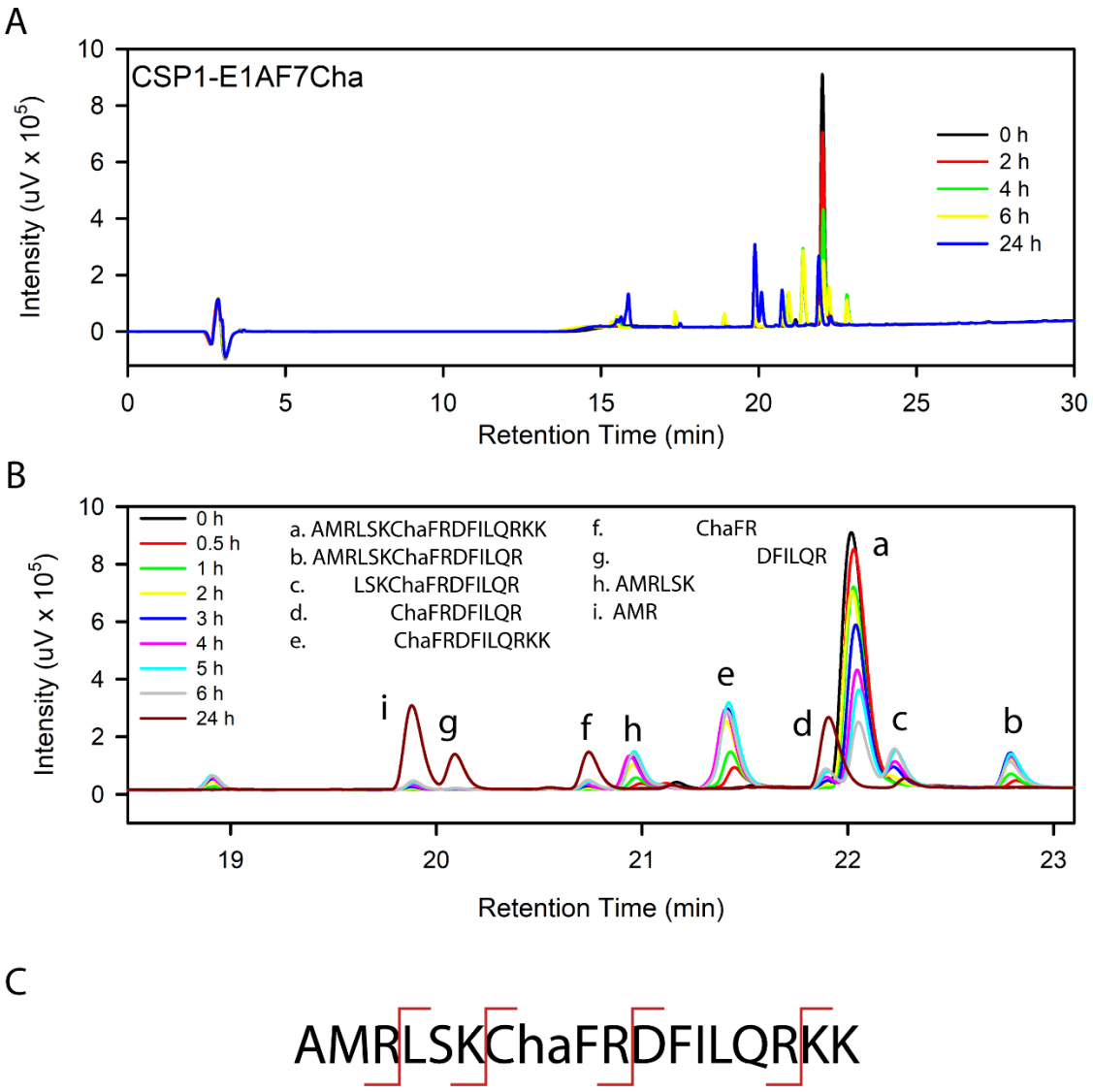


Figure S-11. Degradation pattern analysis of CSP1-E1AF7Cha in the presence of trypsin. A) Comparison of analytical HPLC chromatograms (220 nm) across the time points taken at 0, 2, 4, 6 and 24 h. B) Zoomed view of panel A with all the time points (0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h). All peaks were collected and analyzed via mass spectrometry (MALDI-TOF MS and ESI-TOF LC-MS). The degradation products corresponding to each peak are listed on the left. C) A summary of observed trypsin cleavage sites annotated on the CSP1-E1AF7Cha sequence.

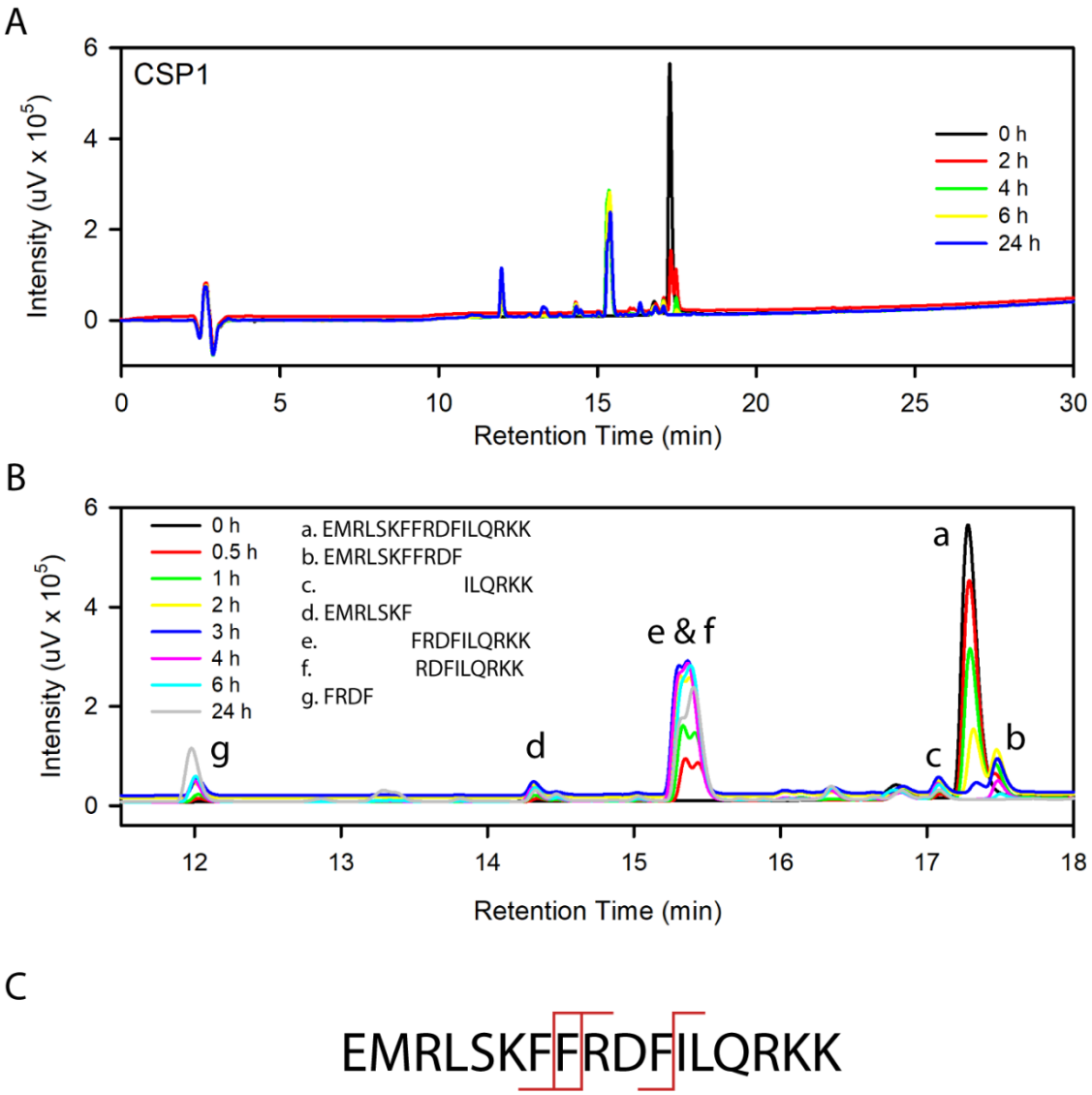


Figure S-12. Degradation pattern analysis of CSP1 in the presence of chymotrypsin. A) Comparison of analytical HPLC chromatograms (220 nm) across the time points taken at 0, 2, 4, 6 and 24 h. B) Zoomed view of panel A with all the time points (0, 0.5, 1, 2, 3, 4, 6 and 24 h). All peaks were collected and analyzed via mass spectrometry (MALDI-TOF MS and ESI-TOF LC-MS). The degradation products corresponding to each peak are listed on the left. C) A summary of observed chymotrypsin cleavage sites annotated on the CSP1 sequence.

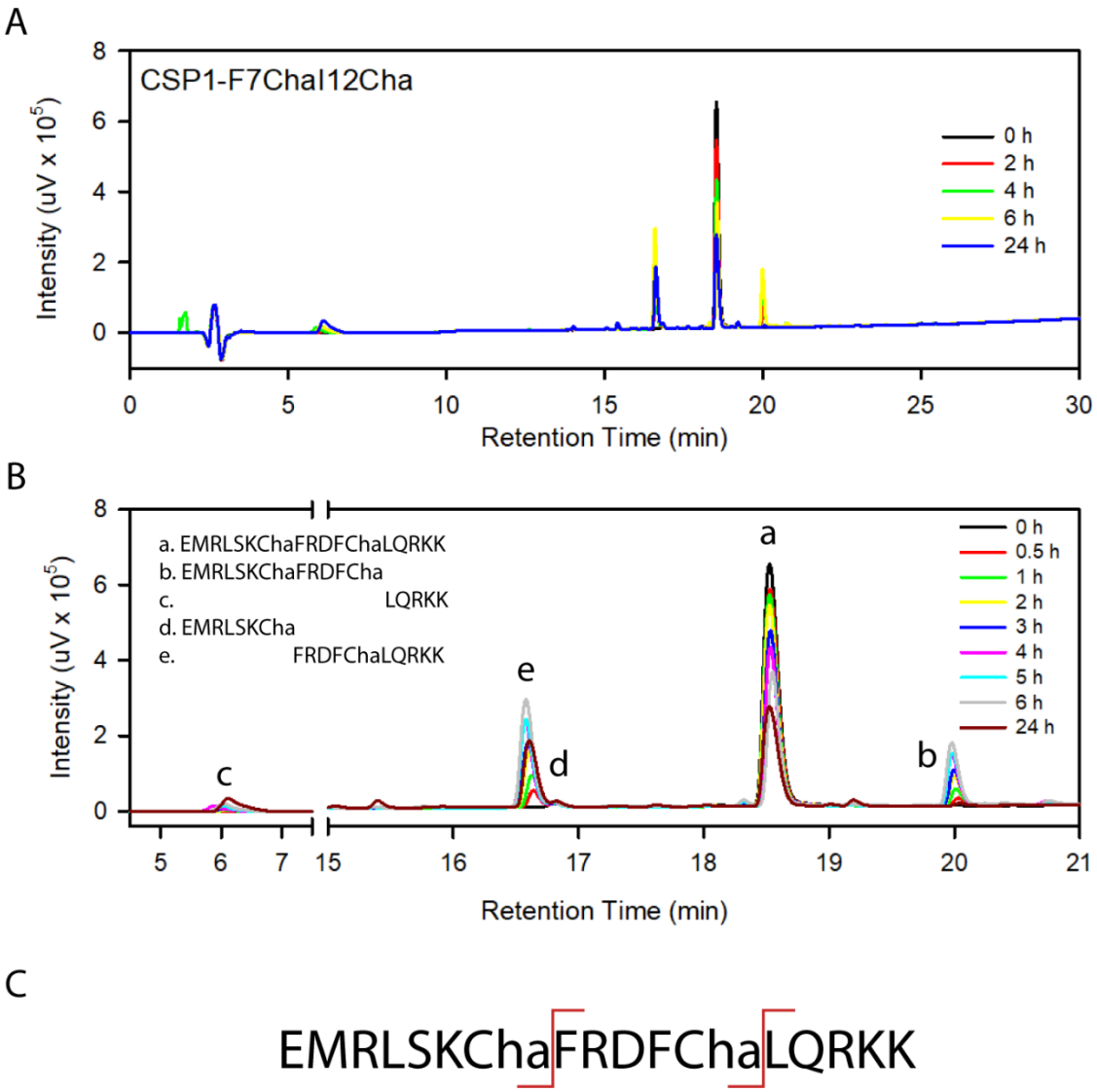


Figure S-13. Degradation pattern analysis of CSP1-F7ChaI12Cha in the presence of chymotrypsin. A) Comparison of analytical HPLC chromatograms (220 nm) across the time points taken at 0, 2, 4, 6 and 24 h. B) Zoomed view of panel A with all the time points (0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h). All peaks were collected and analyzed via mass spectrometry (MALDI-TOF MS and ESI-TOF LC-MS). The degradation products corresponding to each peak are listed on the left. C) A summary of observed chymotrypsin cleavage sites annotated on the CSP1-F7ChaI12Cha sequence.

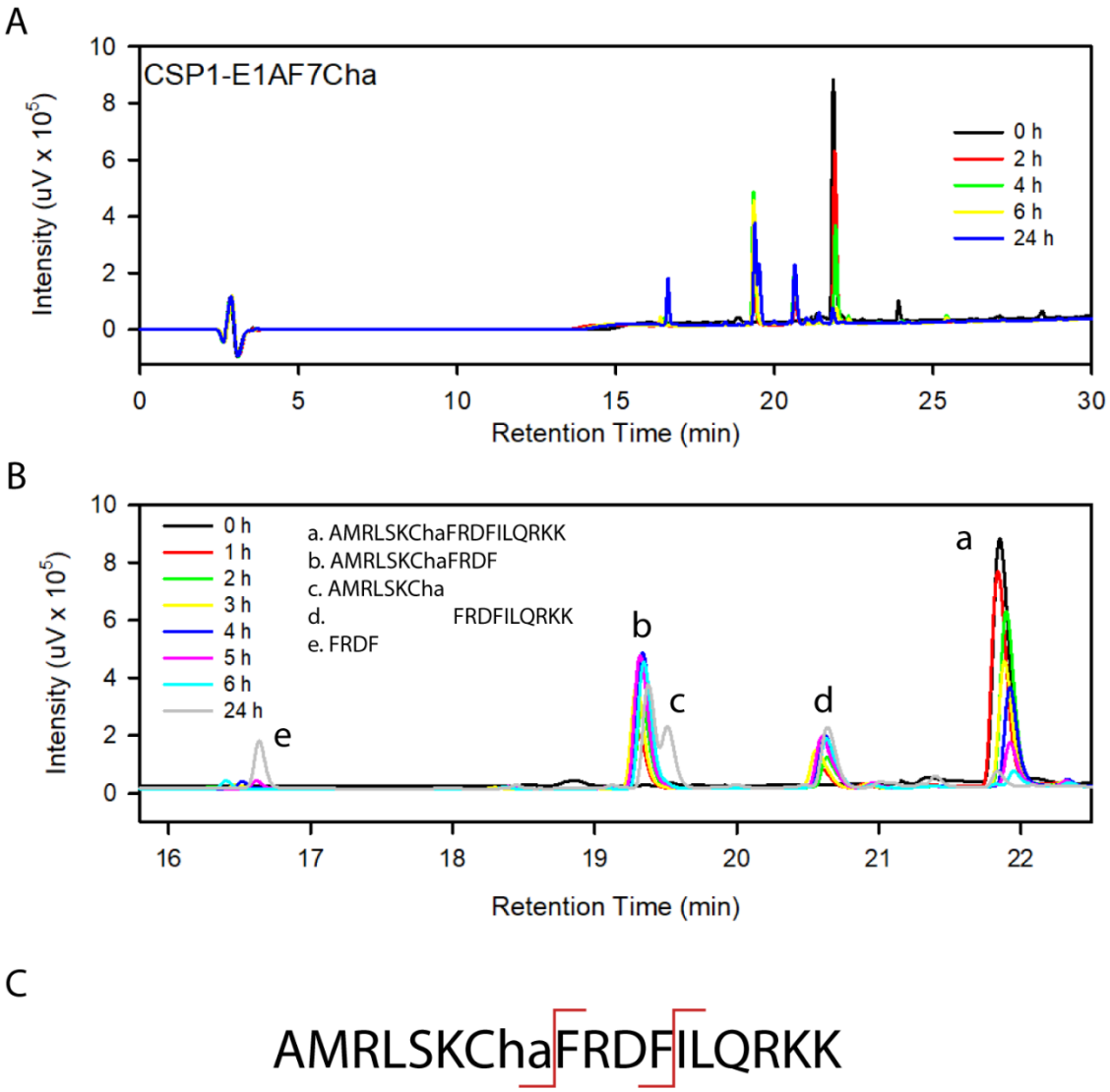


Figure S-14. Degradation pattern analysis of CSP1-E1AF7Cha in the presence of chymotrypsin. A) Comparison of analytical HPLC chromatograms (220 nm) across the time points taken at 0, 2, 4, 6 and 24 h. B) Zoomed view of panel A with all the time points (0, 1, 2, 3, 4, 5, 6 and 24 h). All peaks were collected and analyzed via mass spectrometry (MALDI-TOF MS and ESI-TOF LC-MS). The degradation products corresponding to each peak are listed on the left. C) A summary of observed chymotrypsin cleavage sites annotated on the CSP1-E1AF7Cha sequence.