Supplementary Materials

Unveiling the Oxazolidine Character of Pseudoproline Derivatives by Automated Flow Peptide Chemistry

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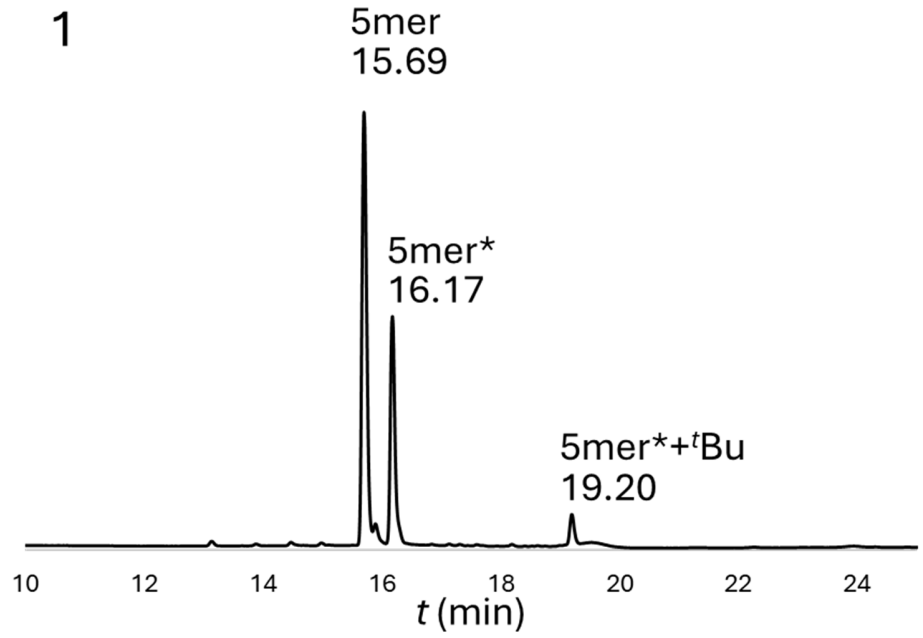


Figure S1: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #1 (see Table 2 for more details).

Table S1. Calculated and measured monoisotopic masses of molecules from synthesis #1.

Name	Retention time (min)	Calculated monoisotopic mass	Measured monoisotopic mass	Mass error (ppm)
5mer	15.69	576.314	576.304	17.352
5mer*	16.17	616.345	616.338	11.357
5mer*+ ^{t}Bu	19.20	672.407	672.287	178.463

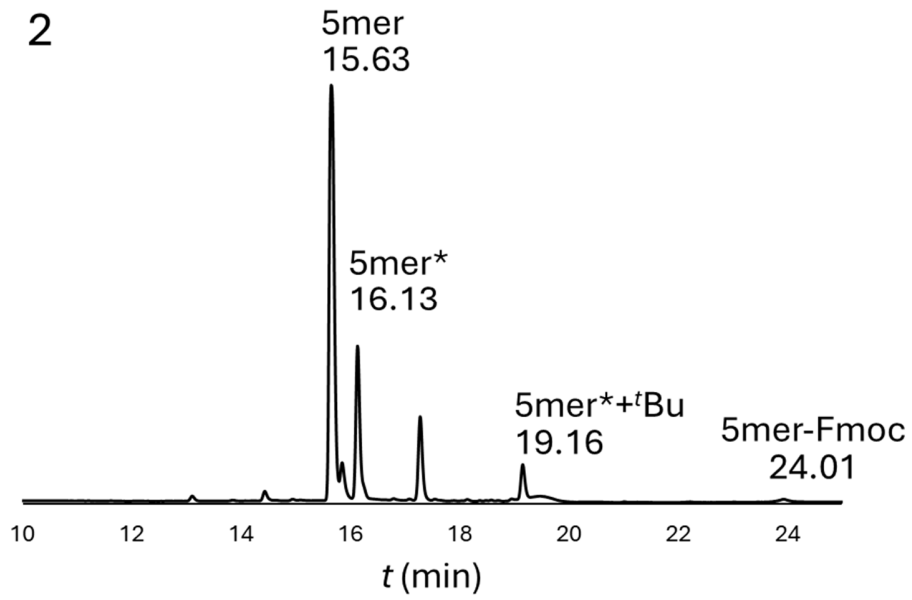


Figure S2: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #2 (see Table 2 for more details).

Table S2. Calculated and measured monoisotopic masses of molecules from synthesis #2.

Name	Retention time (min)	Calculated monoisotopic mass	Measured monoisotopic mass	Mass error (ppm)
5mer	15.63	576.314	576.304	17.352
5mer*	16.13	616.345	616.338	11.357
5mer*+ ^{t}Bu	19.16	672.407	672.287	178.463
5mer-Fmoc	24.01	798.382	798.378	5.010

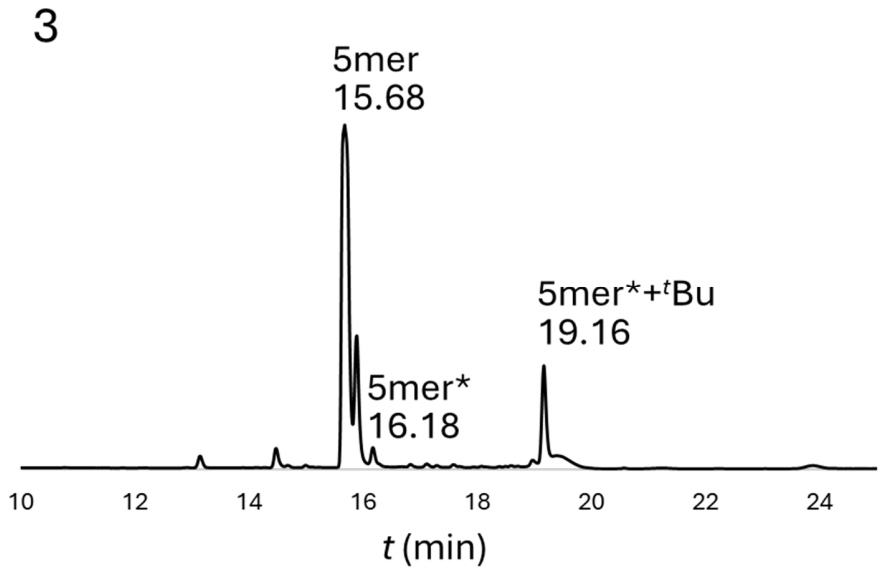


Figure S3: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #3 (see Table 2 for more details).

Table S3. Calculated and measured monoisotopic masses of molecules from synthesis #3.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer	15.68	576.314	576.304	17.352
5mer*	16.18	616.345	616.320	40.562
5mer*+^{t}Bu	19.16	672.407	672.268	206.720

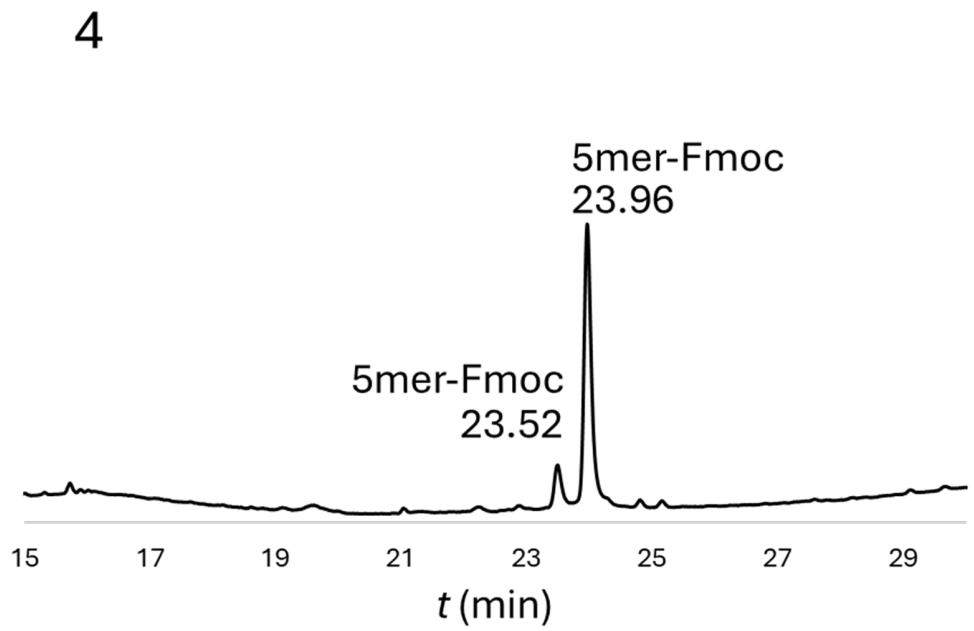


Figure S4: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #4 (see Table 2 for more details).. The two different peaks refer to the *cis* or *trans* orientation of the Fmoc group.

Table S4. Calculated and measured monoisotopic masses of molecules from synthesis #4.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer-Fmoc	23.52	798.382	798.378	5.010
5mer-Fmoc	23.96	798.382	798.378	5.010

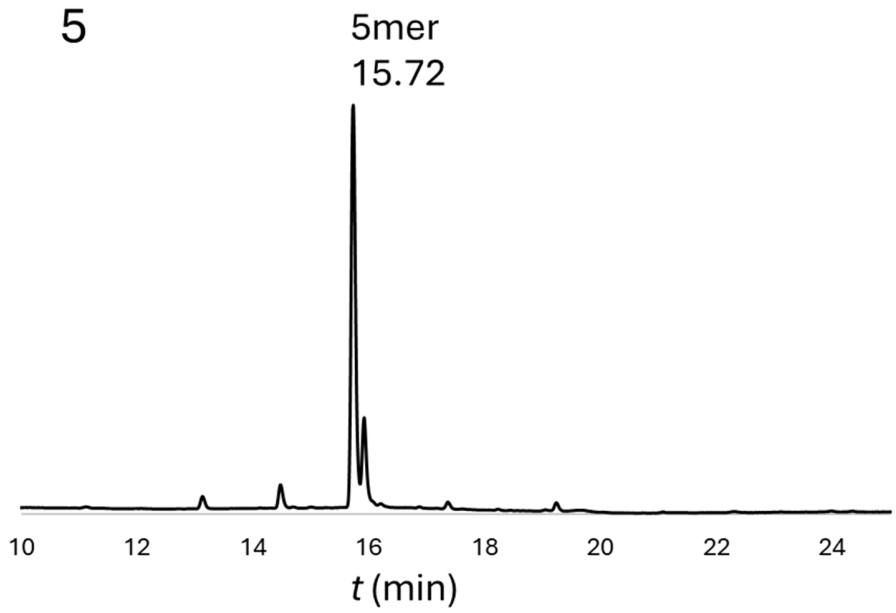


Figure S5: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #5 (see Table 2 for more details).

Table S5. Calculated and measured monoisotopic masses of molecules from synthesis #5.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer	15.72	576.3140	576.375	-105.845

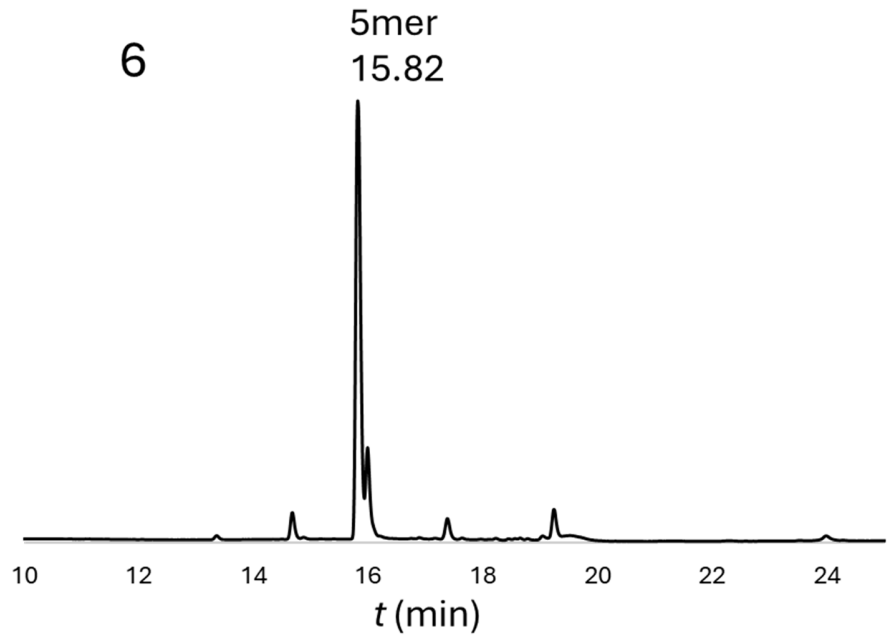


Figure S6: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #6 (see Table 2 for more details).

Table S6. Calculated and measured monoisotopic masses of molecules from synthesis #6.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer	15.82	576.3140	576.393	-137.078

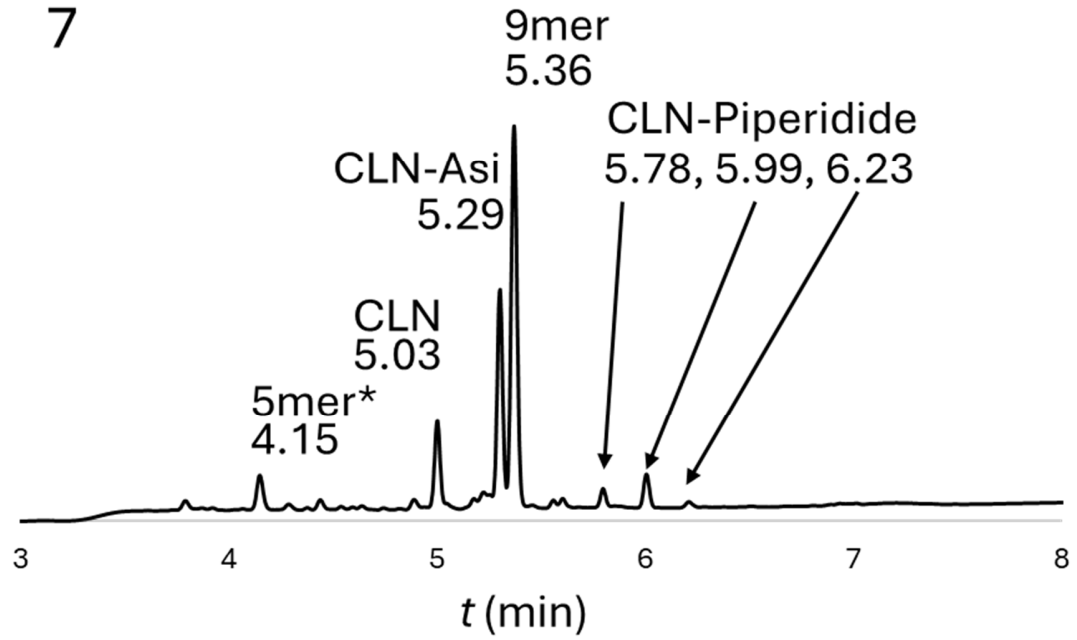


Figure S7: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #7 (see Table 3 for more details).

Table S7. Calculated and measured monoisotopic masses of molecules from synthesis #7.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.15	616.345	616.338	11.357
CLN	5.03	1163.573	1163.563	8.594
CLN-Asi	5.29	1145.562	1145.552	8.729
9mer	5.36	1048.546	1048.538	7.630
Piperidide	5.78, 5.99, 6.23	1230.651	1230.640	8.938

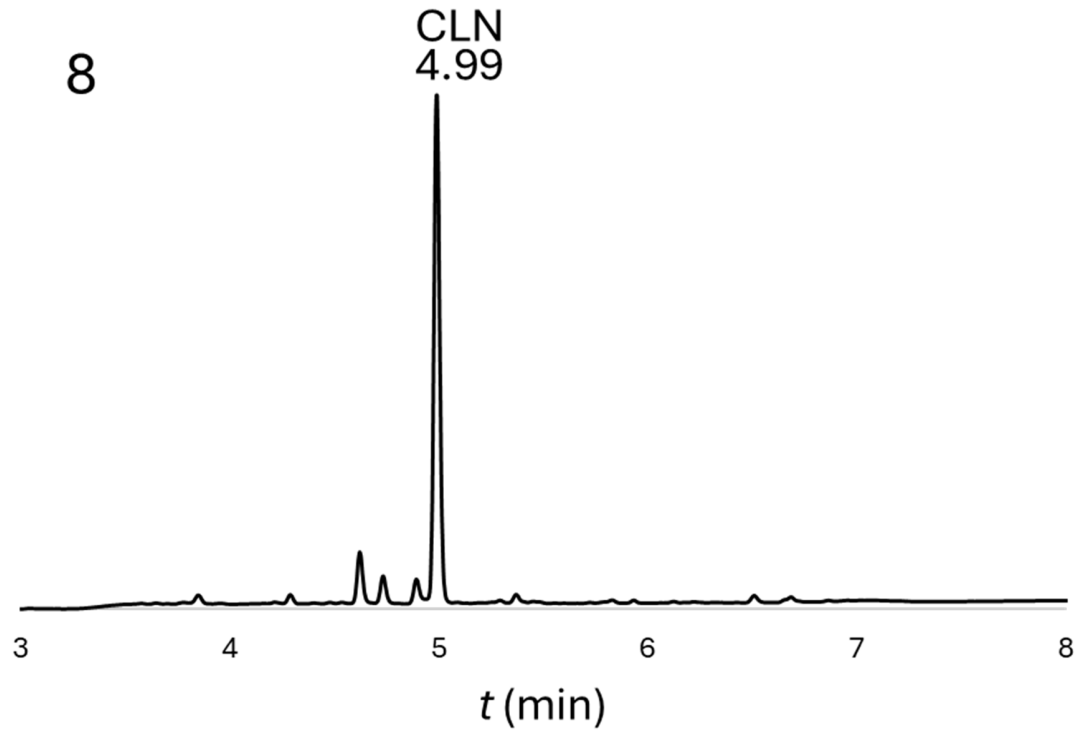


Figure S8: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #8 (see Table 3 for more details).

Table S8. Calculated and measured monoisotopic masses of molecules from synthesis #8.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	-	616.345	-	-
CLN	4.99	1163.573	1163.541	27.501
CLN-Asi	-	1145.562	-	-
9mer	-	1048.546	-	-
Piperidide	-	1230.651	-	-

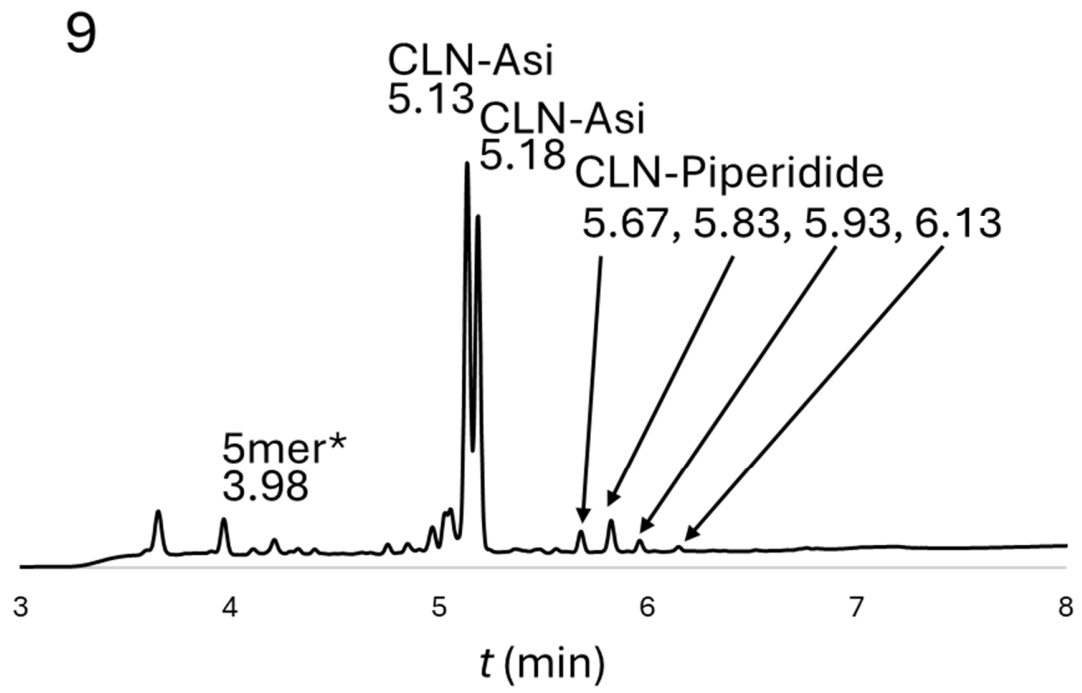


Figure S9: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #9. In this instance, it is important to note that the peptide contains Ser rather than Thr at the 6th position. (see Table 3 for more details).

Table S9. Calculated and measured monoisotopic masses of molecules from synthesis #9.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	3.98	602.329	602.314	24.903
CLN	-	1149.557	-	-
CLN-Asi	5.13, 5.18	1131.546	1131.518	24.745
9mer	-	1034.530	-	-
Piperidide	5.67, 5.83, 5.93, 6.13	1216.636	1216.605	25.480

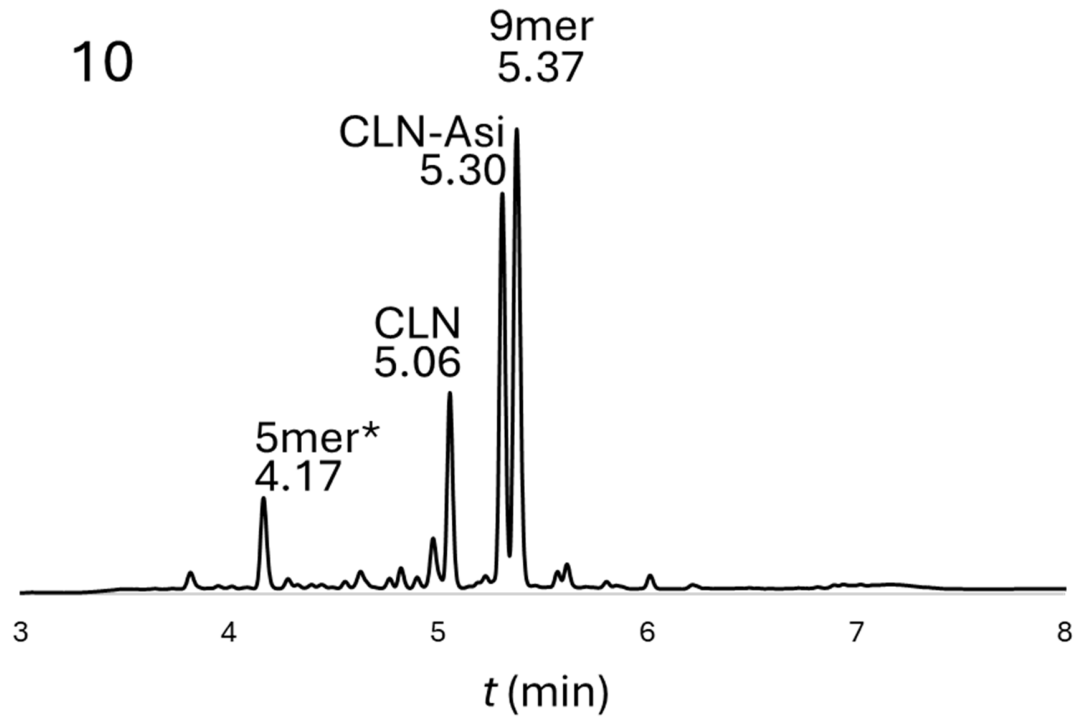


Figure S10: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #10 (see Table 3 for more details).

Table S10. Calculated and measured monoisotopic masses of molecules from synthesis #10.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.17	616.345	616.345	0.000
CLN	5.06	1163.573	1163.572	0.859
CLN-Asi	5.30	1145.562	1145.566	-3.492
9mer	5.37	1048.546	1048.547	-0.954
Piperidide		1230.651	-	-

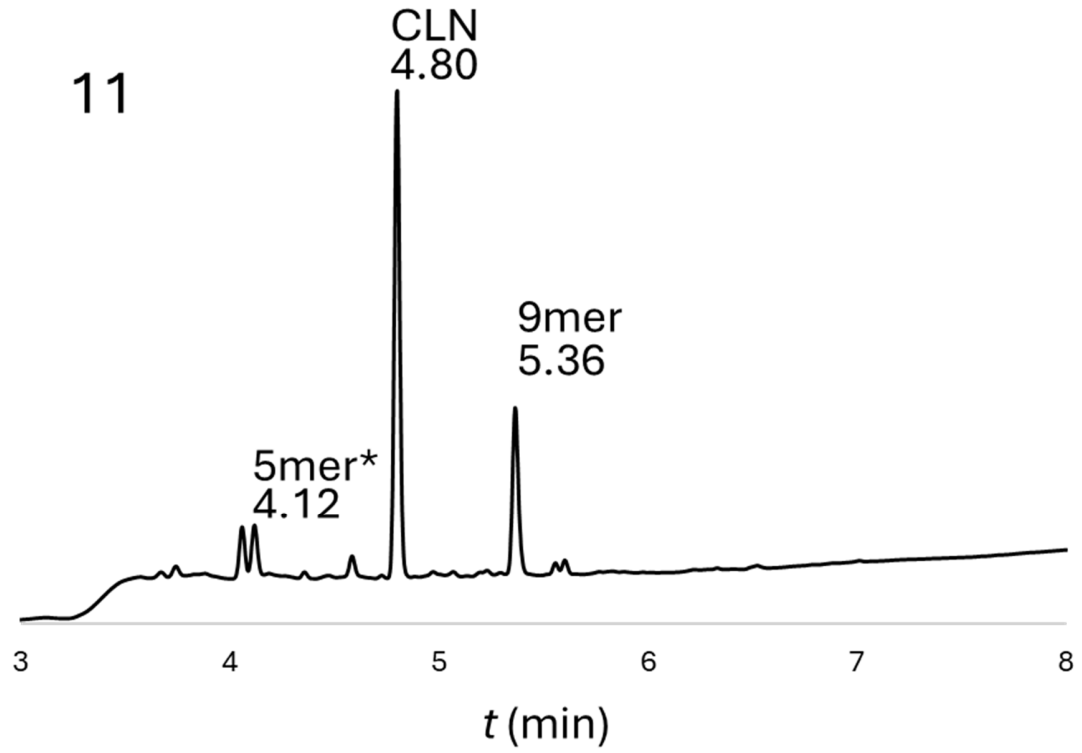


Figure S11: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #11 (see Table 3 for more details).

Table S11. Calculated and measured monoisotopic masses of molecules from synthesis #11.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.12	616.345	616.341	6.490
CLN	4.80	1162.589	1162.584	4.301
CLN-Asi	-	1144.578	-	-
9mer	5.36	1048.546	1048.543	2.861
Piperidide	-	1230.651	-	-

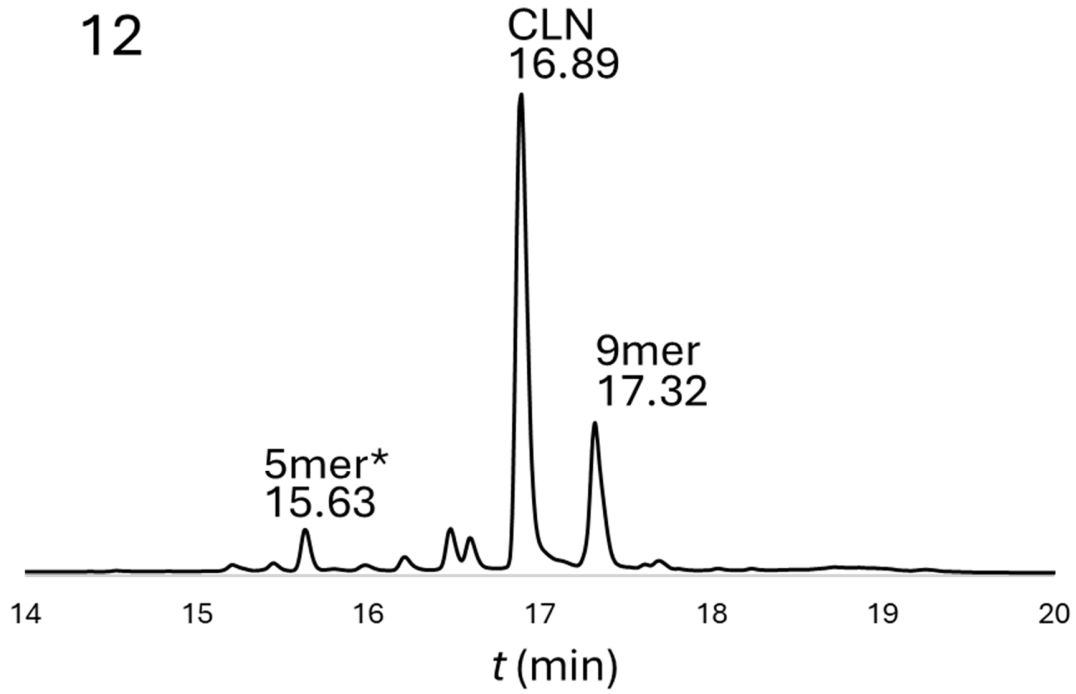


Figure S12: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #12 (see Table 3 for more details).. Different UPLC gradient was used (molecules are identified with MS)

Table S12. Calculated and measured monoisotopic masses of molecules from synthesis #12.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	15.63	616.345	616.407	-100.593
CLN	16.89	1163.573	1163.686	-97.115
CLN-Asi	-	1145.562	-	-
9mer	17.32	1048.546	1048.658	-106.815
Piperidide	-	1230.651	-	-

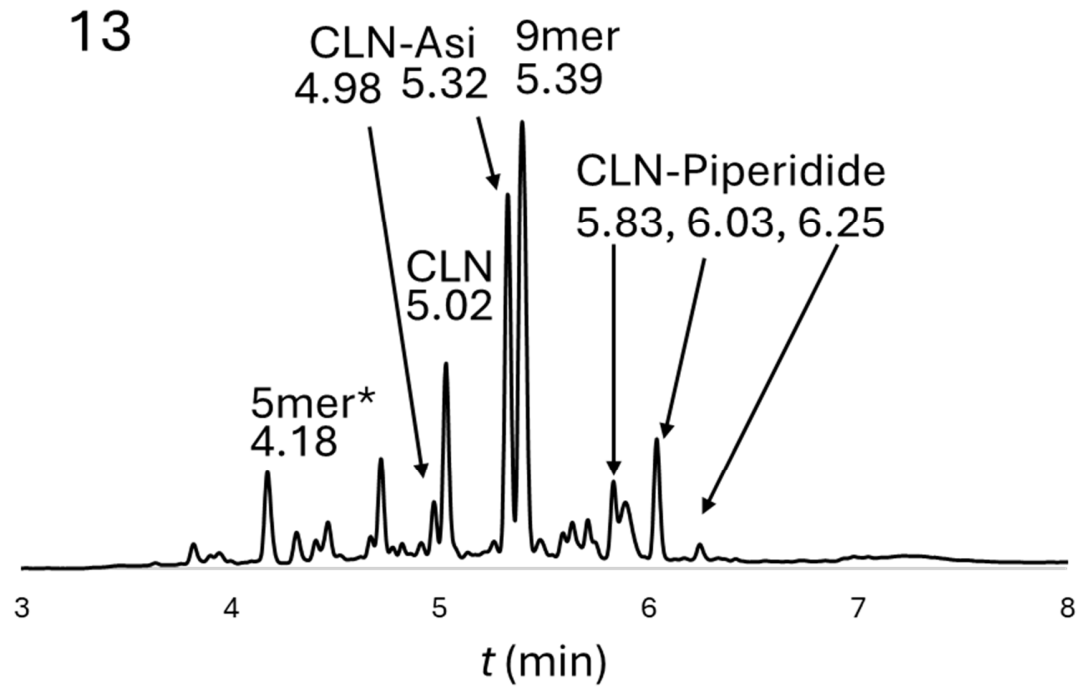


Figure S13: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #13 (see Table 3 for more details)..

Table S13. Calculated and measured monoisotopic masses of molecules from synthesis #13.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.18	616.345	616.351	-9.735
CLN	5.02	1163.573	1163.586	-11.172
CLN-Asi	4.98, 5.32	1145.562	1145.579	-14.840
9mer	5.39	1048.546	1048.560	-13.352
Piperidide	5.83, 603, 6.25	1230.651	1230.668	-13.814

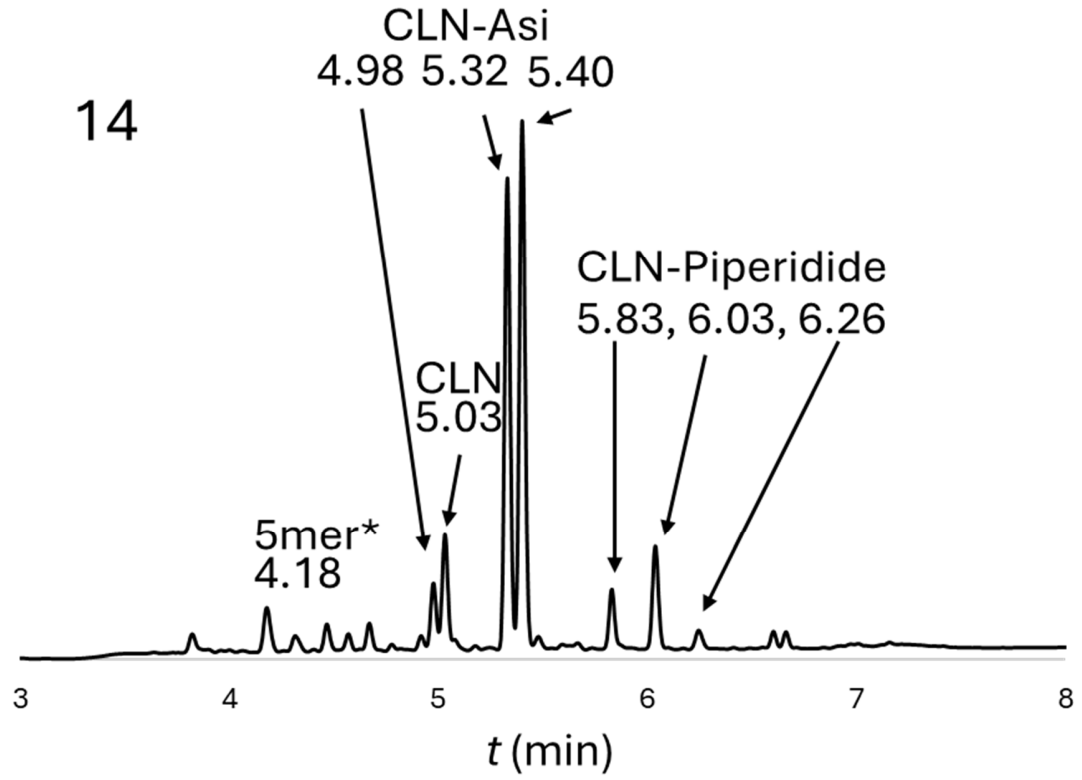


Figure S14: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #14 (see Table 3 for more details).

Table S14. Calculated and measured monoisotopic masses of molecules from synthesis #14.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.18	616.345	616.351	-9.735
CLN	5.03	1163.573	1163.591	-15.470
CLN-Asi	4.98, 5.32, 5.4	1145.562	1145.579	-14.840
9mer	-	1048.546	-	-
Piperidide	5.83, 6.03, 6.26	1230.651	1230.668	-13.814

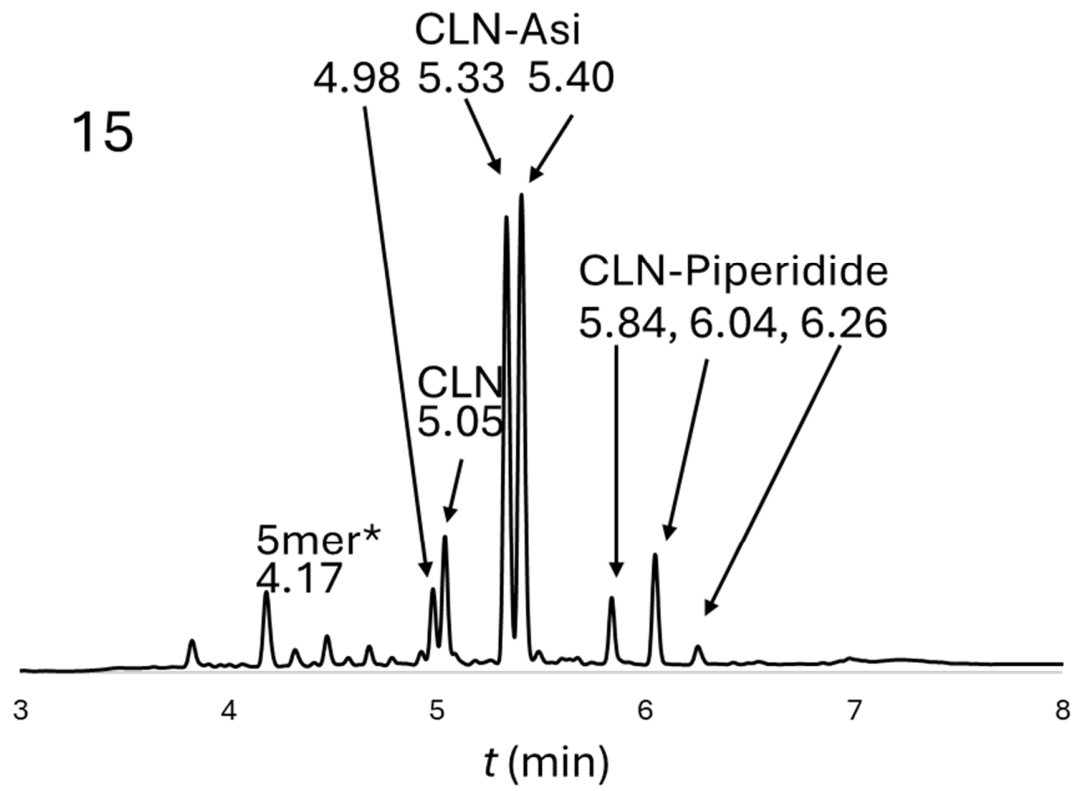


Figure S15: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #15 (see Table 3 for more details).

Table S15. Calculated and measured monoisotopic masses of molecules from synthesis #15.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.17	616.345	616.348	-4.867
CLN	5.05	1163.573	1163.582	-7.735
CLN-Asi	4.98, 5.33, 5.4	1145.562	1145.570	-6.983
9mer	-	1048.546	-	-
Piperidide	5.84, 6.04, 6.26	1230.651	1230.664	-10.564

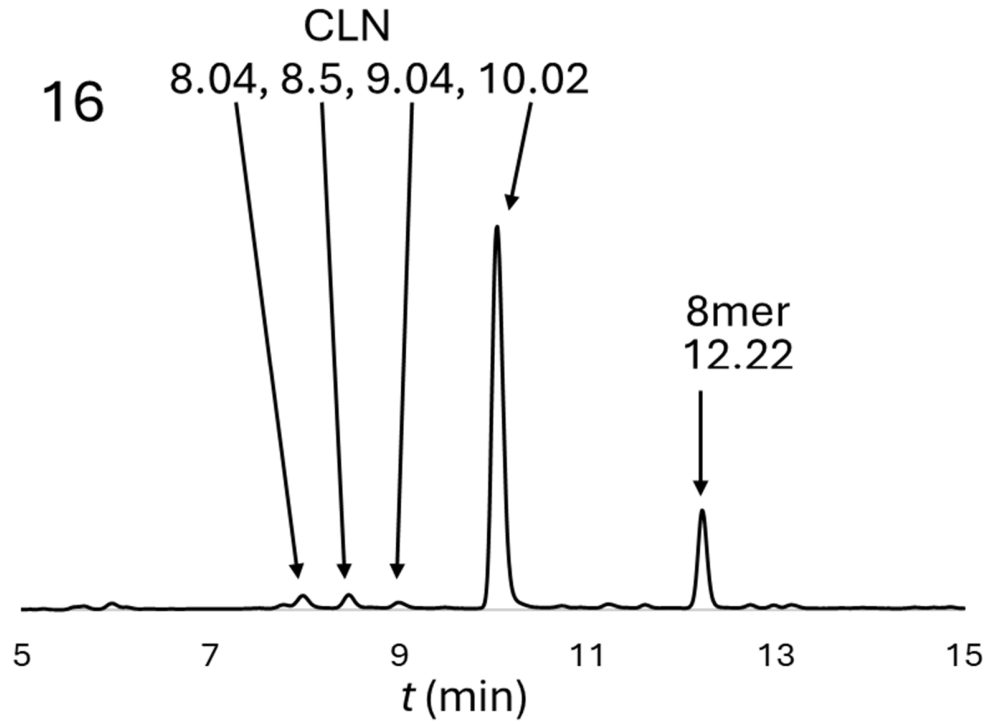


Figure S16: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #16 (see Table 3 for more details).

Table S16. Calculated and measured monoisotopic masses of molecules from synthesis #16.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	-	616.345	-	-
CLN	8.04, 8.5, 9.04, 10.02	1163.573	1163.473	85.942
CLN-Asi	-	1145.562	-	-
9mer	-	1048.546	-	-
Piperidide	-	1230.651	-	-
8mer	12.22	947.498	947.417	85.488

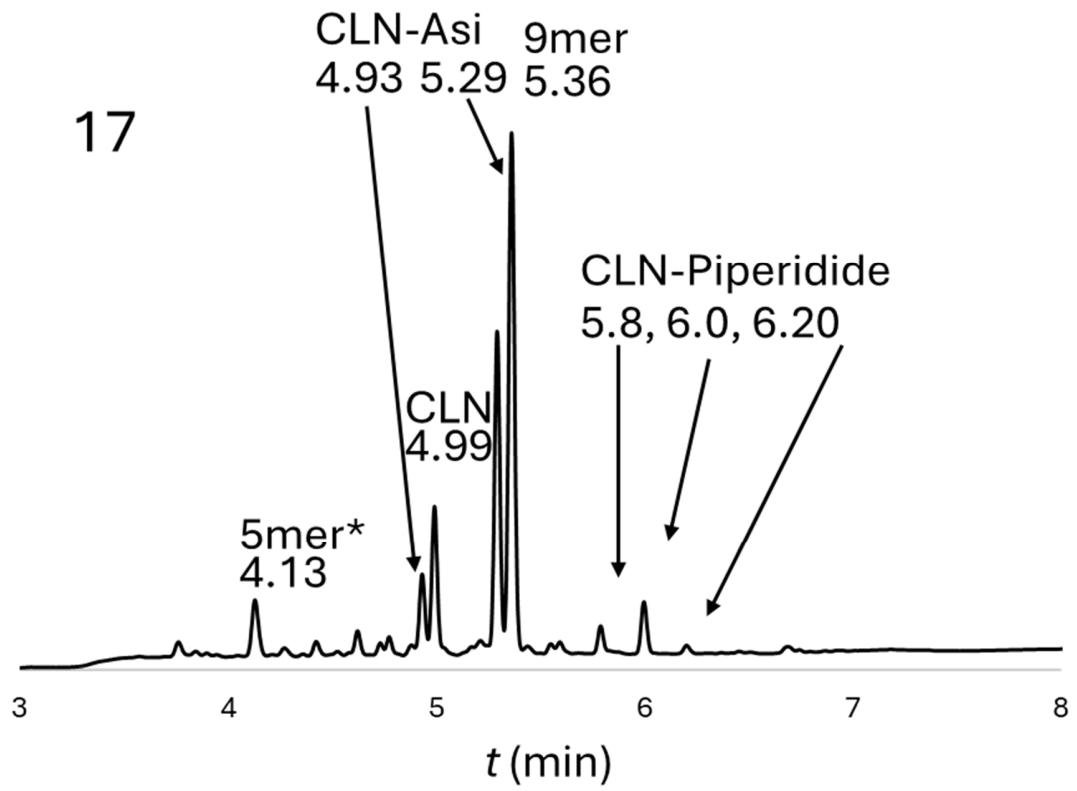


Figure S17: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #17 (see Table 3 for more details).

Table S17. Calculated and measured monoisotopic masses of molecules from synthesis #17.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.13	616.345	616.325	32.449
CLN	4.99	1163.573	1163.535	32.658
CLN-Asi	4.93, 5.2	1145.562	1145.525	32.299
9mer	5.36	1048.546	1048.512	32.426
Piperidide	5.8, 6.0, 6.2	1230.651	1230.612	31.691

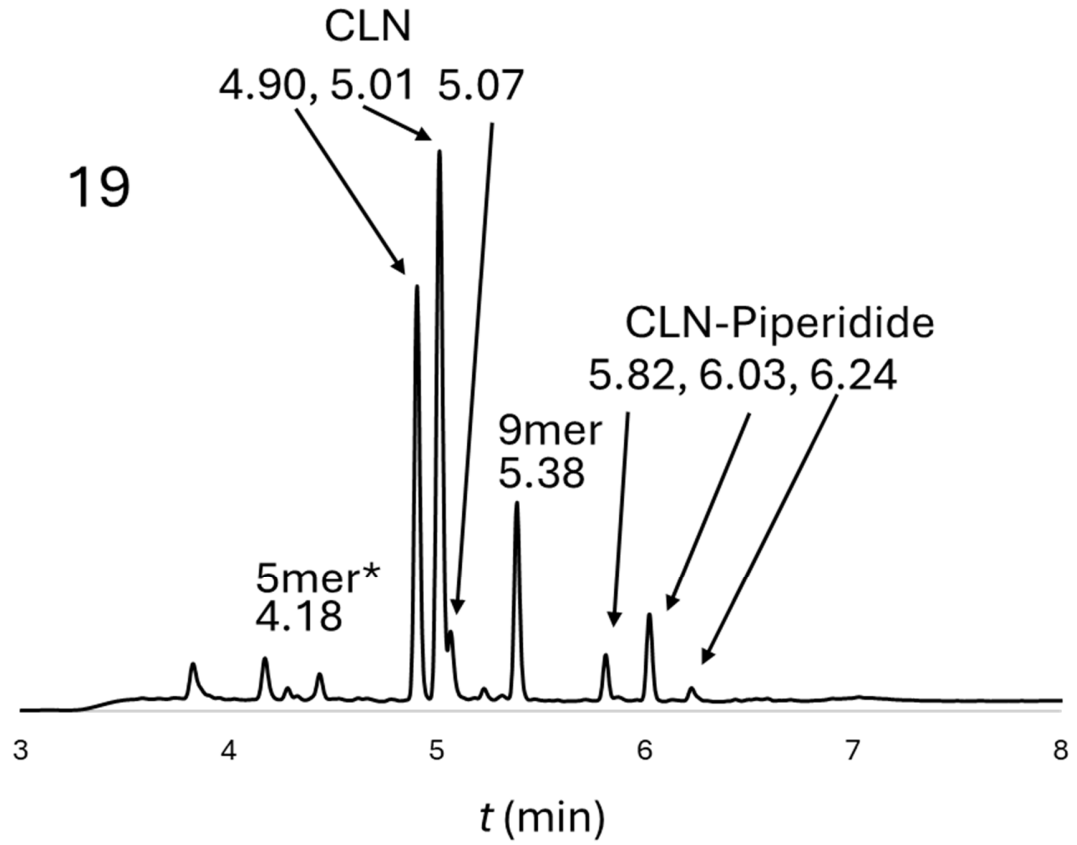


Figure S18: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #18 (see Table 3 for more details).

Table S18. Calculated and measured monoisotopic masses of molecules from synthesis #18.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	
5mer*	4.18	616.345	616.355	-16.225
CLN	4.63, 4,77, 5.02	1163.573	1163.591	-15.470
CLN-Asi	4.95, 5.32, 5.39	1145.562	1145.579	-14.840
9mer	-	1048.546	-	-
Piperidide	5.82, 6.03, 6.24	1230.651	1230.668	-13.814

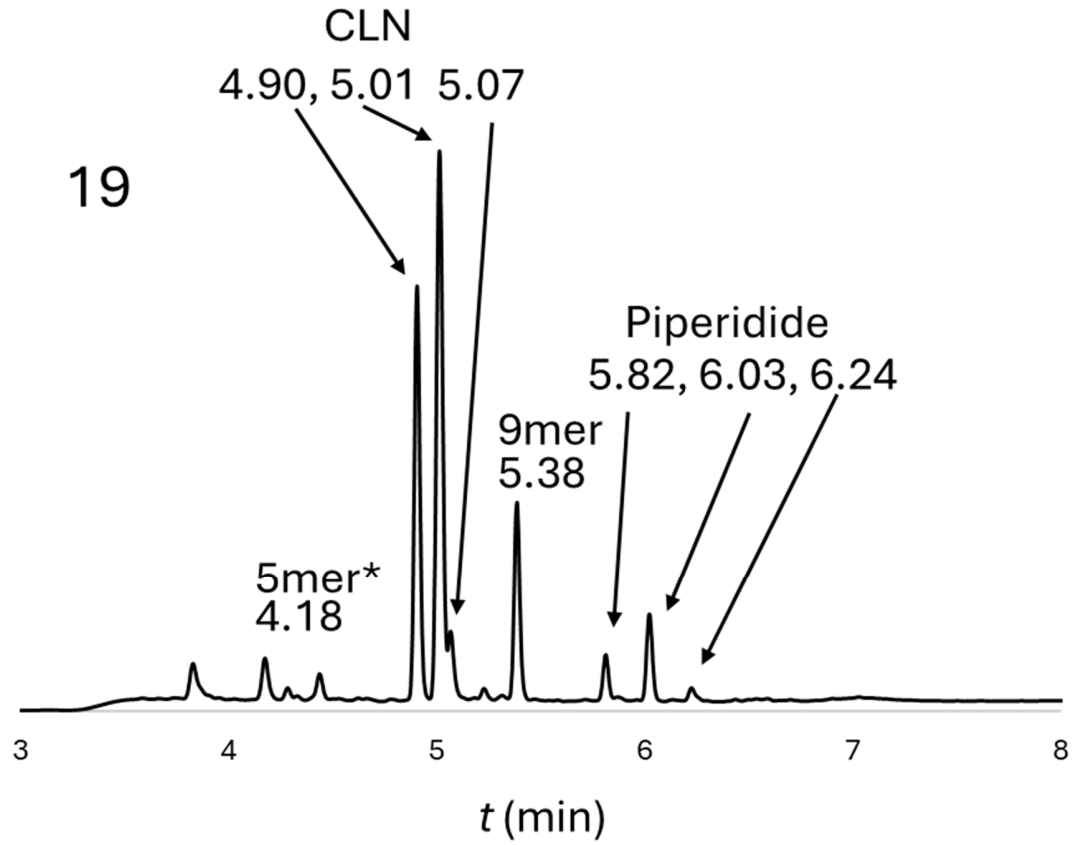


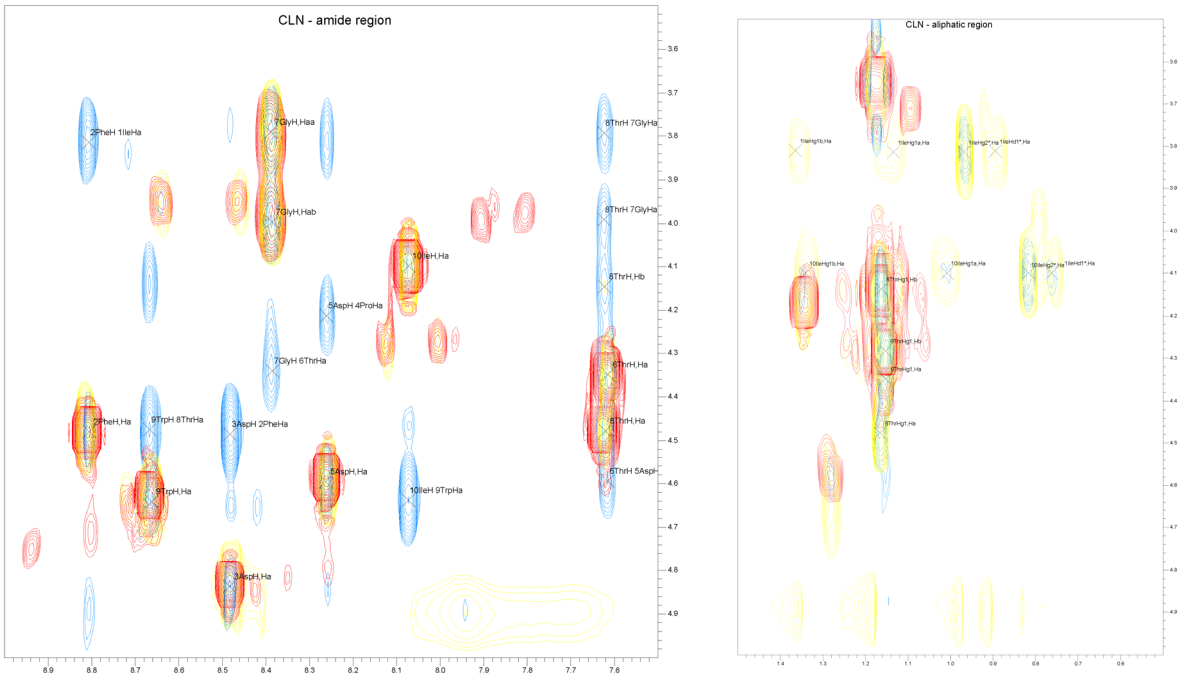
Figure S19: UPLC chromatogram of the crude peptide with key molecules assigned from synthesis #19 (see Table 3 for more details).

Table S19. Calculated and measured monoisotopic masses of molecules from synthesis #19.

Name	Retention time (min)	Calculated monoisotopic mass $[M+H]^+$	Measured monoisotopic mass $[M+H]^+$	Mass error (ppm)
5mer*	4.18	616.345	616.341	6.490
CLN	4.9, 5.01, 5.07	1163.573	1163.568	4.297
CLN-Asi	-	1145.562	-	-
9mer	5.38	1048.546	1048.54	5.722
Piperidide	5.82, 6.03, 6.24	1230.651		-

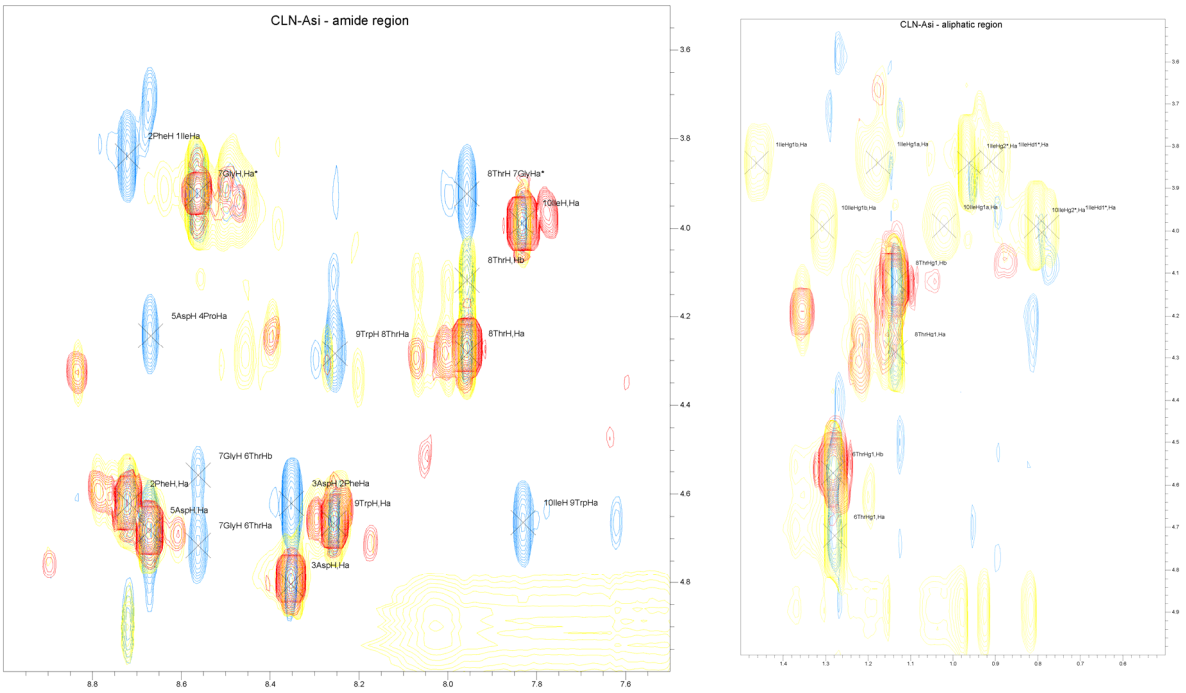
NMR Study of the CLN and CLN-Asi polypeptides

In **Figure S20 -A**, we can see the amide region of the CLN, where both threonine residues (Thr4 and Thr6) exhibit visible amide proton signals (**Figure S20-A** vertical column around 7.62 ppm, red square) with their respective sequential NOESY cross peaks designating the preceding and subsequent residues. In the suspected aspartimide peptide (Asi), the Thr6 amide proton signal was found to be missing, (**Figure S21-A**) however, the presence of a NOE peak (light green square at column of 8.58 ppm) between Thr6Hα/Hb (5.15 ppm / 5.60 ppm) and Gly7H (8.58 ppm) amide proton indicates the presence of Thr6 side chains. This is only possible if the Thr6 amide proton is missing due to succinimide ring formation of the Asp5 residue, as shown in **Figure S21-A**.



(**A**) (**B**)

Figure S20. (A) The amide region of the CLN proton's NMR resonances: TOCSY/yellow, COSY/red, and NOESY/blue. (B) The side chain region with the proton signals of Thr6 and Thr8 residues marked.



(**A**) (**B**)

Figure S21. (A) The amide region of the CLN-Asi derivative showing the missing Thr6H amide proton signal, however the NOE crosspeak of the Thr6Hα and Gly7H are presented. TOCSY/yellow, COSY/red, and NOESY/blue. (B) Sidechain region with the proton signals of Thr6 and Thr8 residues.

Table S20. NMR shift list of CLN peptide.

		H	HA	HB	HG	HD	HE	HZ
1	Ile	-	3.81	1.95	1.36, 0.97, 1.13	0.9	-	-
2	Phe	8.81	4.48	2.64, 2.74	-	6.88	-	-
3	Asp	8.48	4.84	2.92, 2.45	-	-	-	-
4	Pro	-	4.21	2.32, 2.32	2.00, 2.00	3.81, 3.75	-	-
5	Asp	8.26	4.59	2.83, 2.83	-	-	-	-
6	Thr	7.62	4.35	4.28	1.15	-	-	-
7	Gly	8.39	4.00, 3.79	-	-	-	-	-
8	Thr	7.63	4.48	4.14	1.16	-	-	-
9	Trp	8.66	4.64	3.24, 3.19	-	7.22	10.19, 7.25	
10	Ile	8.07	4.1	1.67	1.34, 1.00, 0.81	0.76	-	-

Table S21. NMR shift list of CLN-Asi peptide.

		H	HA	HB	HG	HD	HE	HZ
1	Ile	-	3.84	1.94	1.47, 1.18, 0.96	0.91	-	-
2	Phe	8.72	4.62	3.09, 2.96	-	7.22	7.48	-
3	Asp	8.35	4.8	2.45, 2.70	-	-	-	-
4	Pro	-	4.24	2.20, 2.20	1.94, 1.94	3.46, 3.72	-	-
5	Asp	8.67	4.68	3.17, 2.80	-	-	-	-
6	Thr	-	4.72	4.56	1.28	-	-	-
7	Gly	8.56	3.92, 3.92	-	-	-	-	-
8	Thr	7.96	4.29	4.12	1.14	-	-	-
9	Trp	8.26	4.66	3.26, 3.26	-	7.21	10.15	7.48
10	Ile	7.83	3.99	1.67	0.80, 1.30, 1.02	0.78	-	-

Table S22. Main parameter sets of the protocols described in Figure 1.

Optimized parameters		protocol s	protocol a	protocol b	protocol c
Fmoc-Xaa-OH	eq.	3	3	5	5
OxymaPure	eq.	3	3	5	5
DIC	eq.	6	6	6	10
Volume of injection	mL	1	1	0.8	0.8
Flow rate Coupling / washing (after Fmoc- deprotection)	mL/min.	0.3/1	0.3/1	0.3/1	0.15/1
Cycle time	min.	7.5	12	12	18
Temperature	°C	80	80	80	80