Supplementary Information

for

Late-Stage Peptide Nitroarylation: Stereoelectronic Tuning and Molecular Recognition

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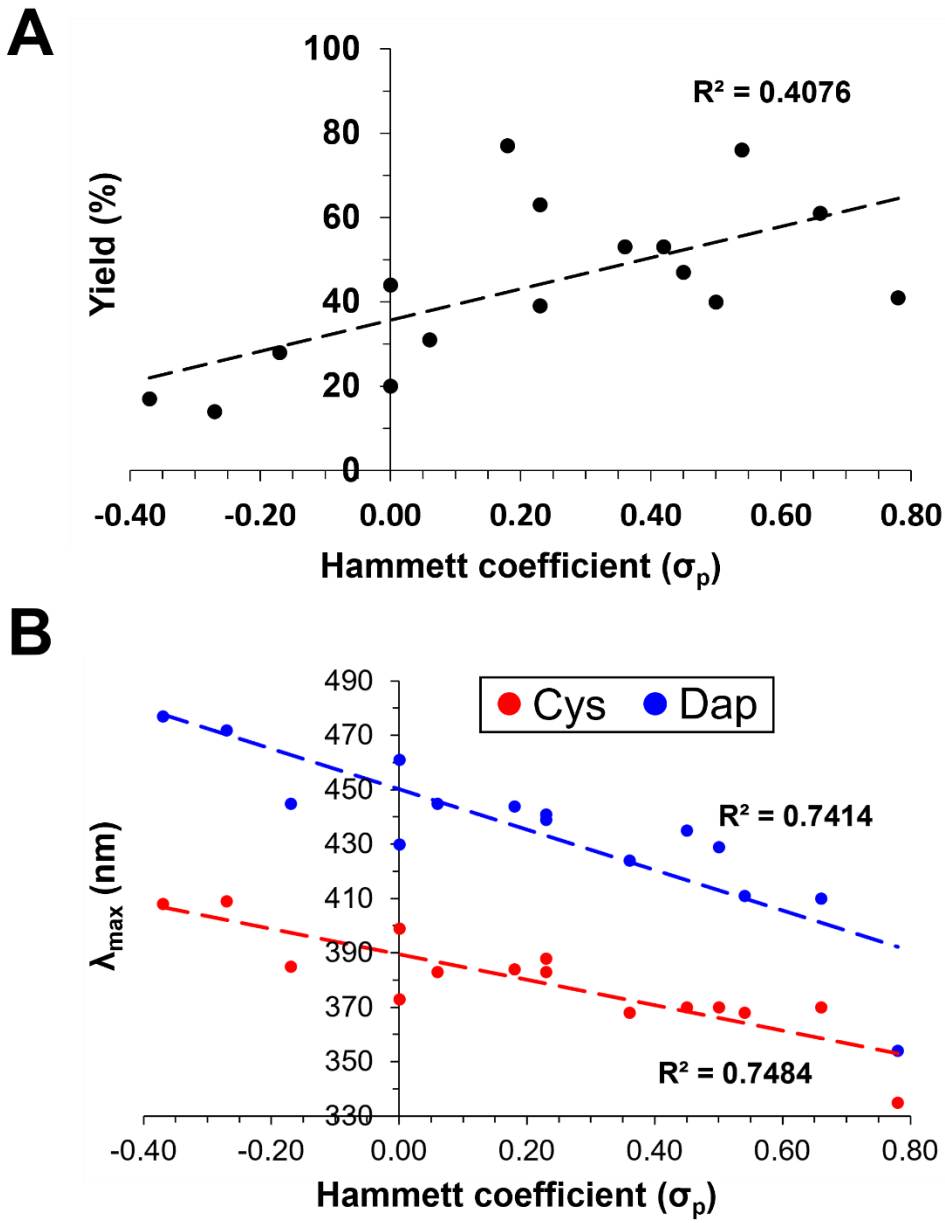


Figure S1. Correlation of Hammett coefficients with nitroaryl amino acid properties. A) Isolated yields for Dap nitroarylation and B) near UV/visible absorption maxima ($\lambda_{max,2}$ > 320 nm) for model Cys (red) and Dap (blue) peptides. Hammett coefficient values (σ_p): H = 0.00, F = 0.06, Cl = 0.23, Br = 0.23, I = 0.18, CF_3 = 0.54, CH_3 = -0.17, CN = 0.66, $COCH_3$ = 0.50, $CONH_2$ = 0.36, COOH = 0.45, NHAc = 0.00, NO_2 = 0.78, OCH_3 = -0.27, OH = -0.37.

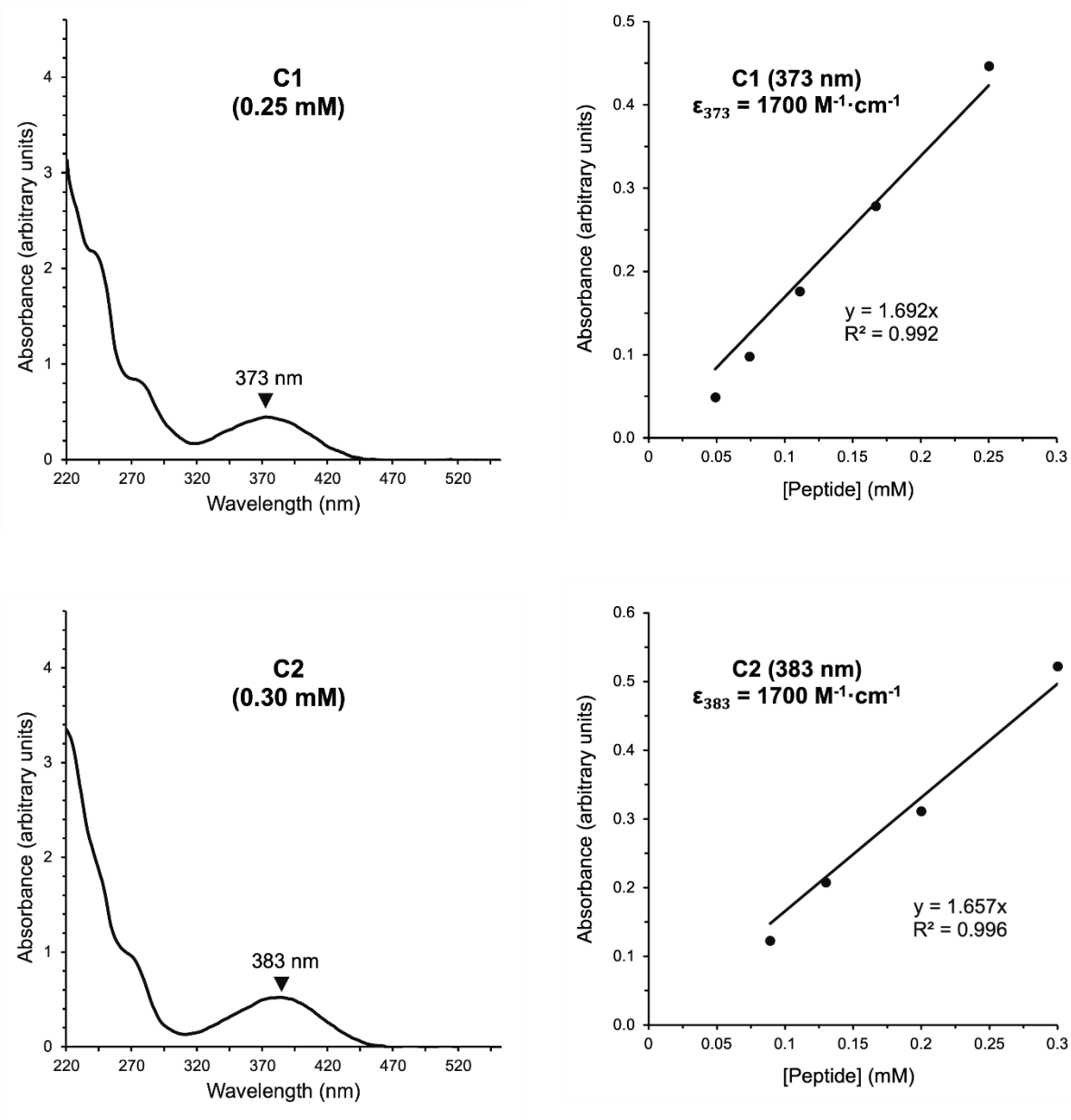


Figure S2. UV-visible spectra for nitroarylated Cys/Dap model peptides

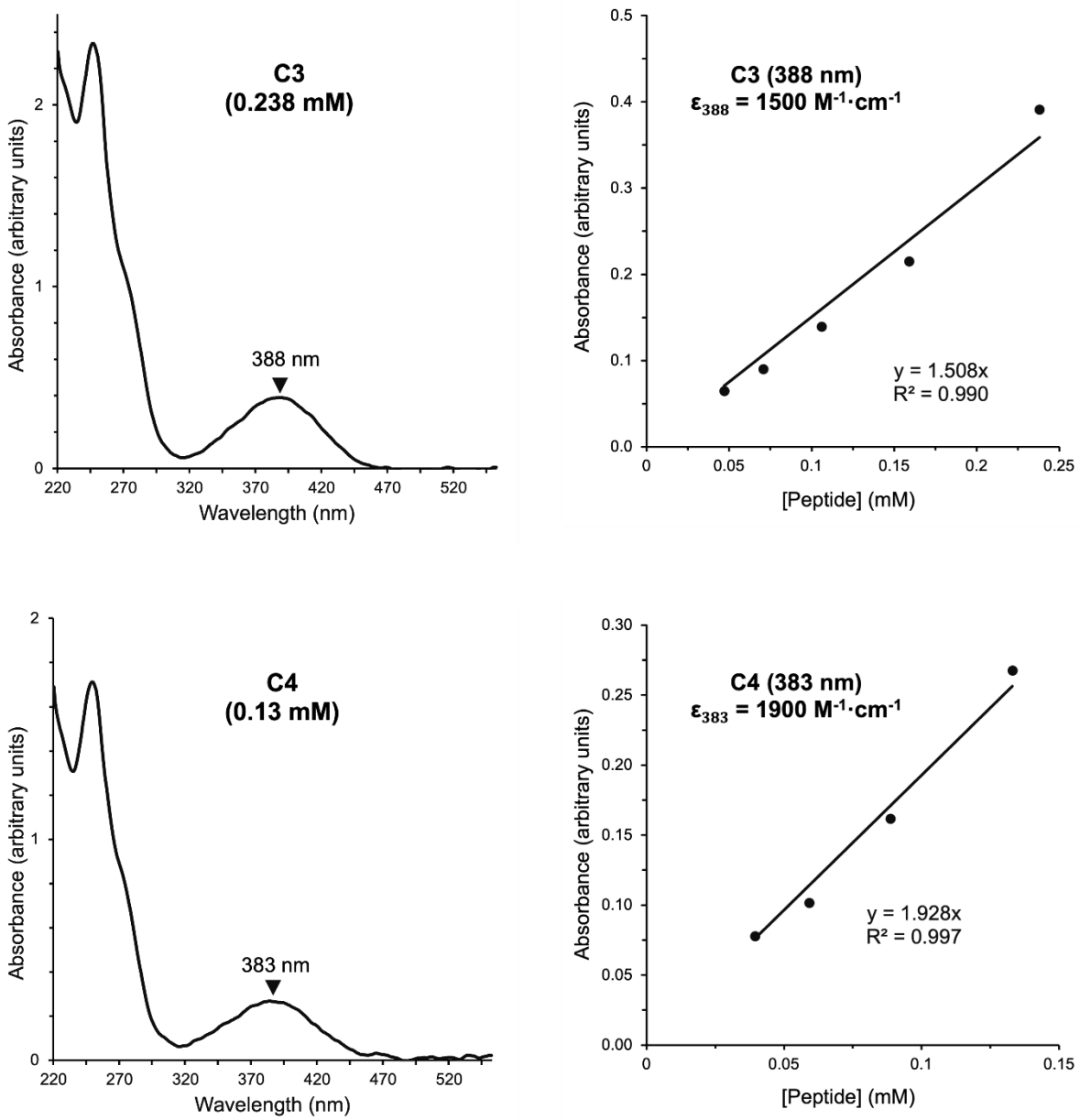
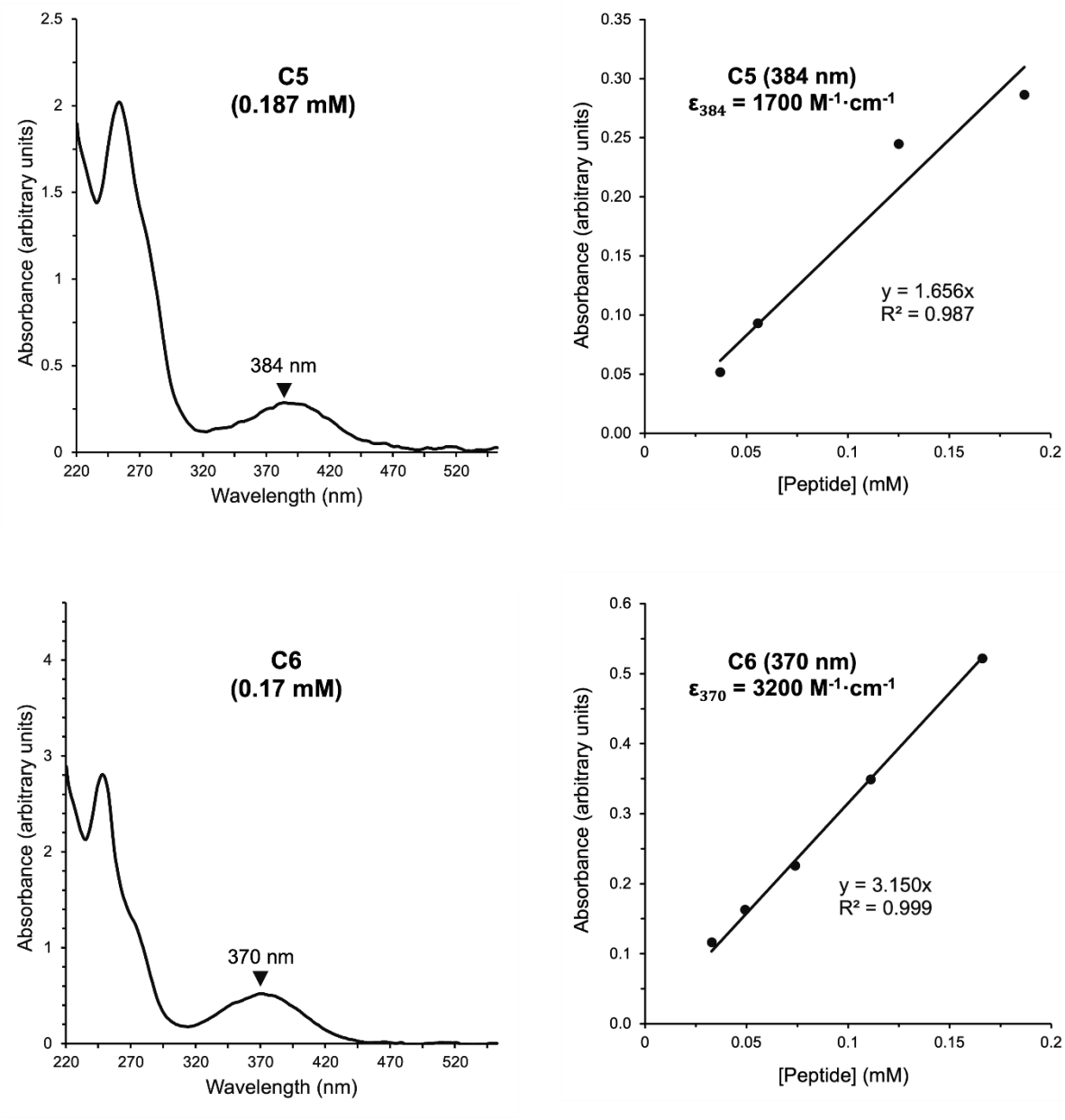


Figure S2. UV-visible spectra for nitroarylated Cys/Dap model peptides (continued)



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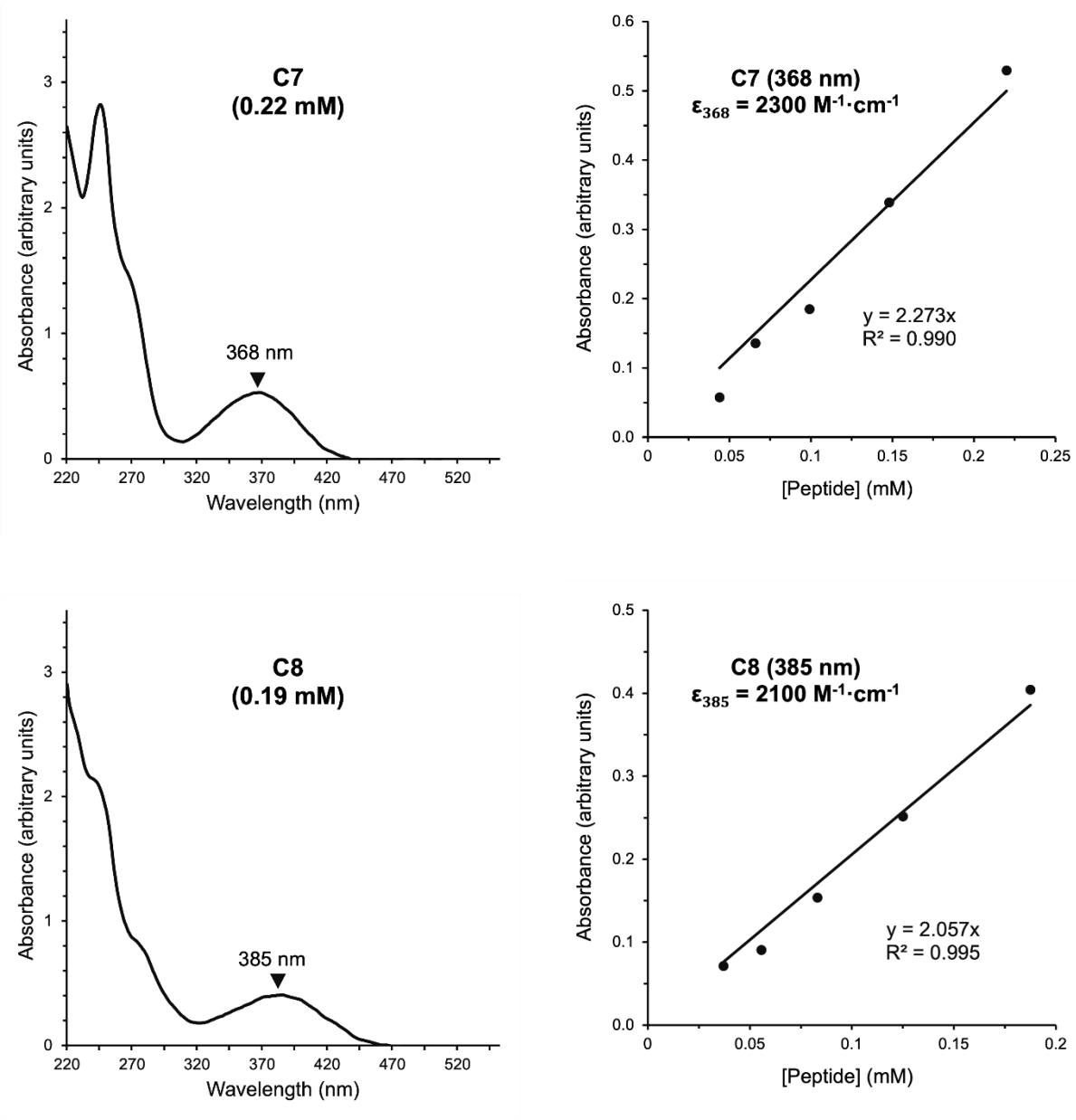

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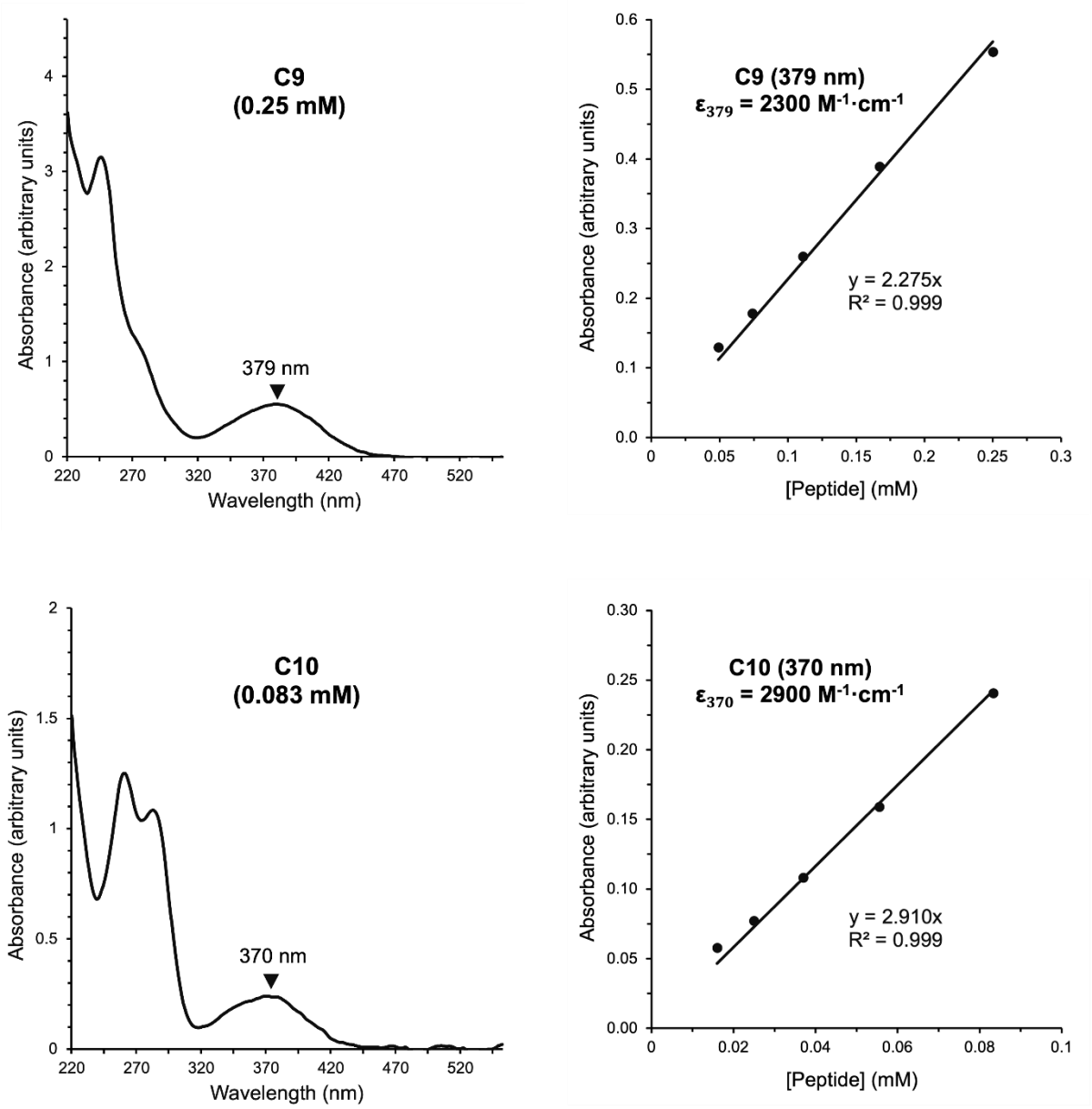

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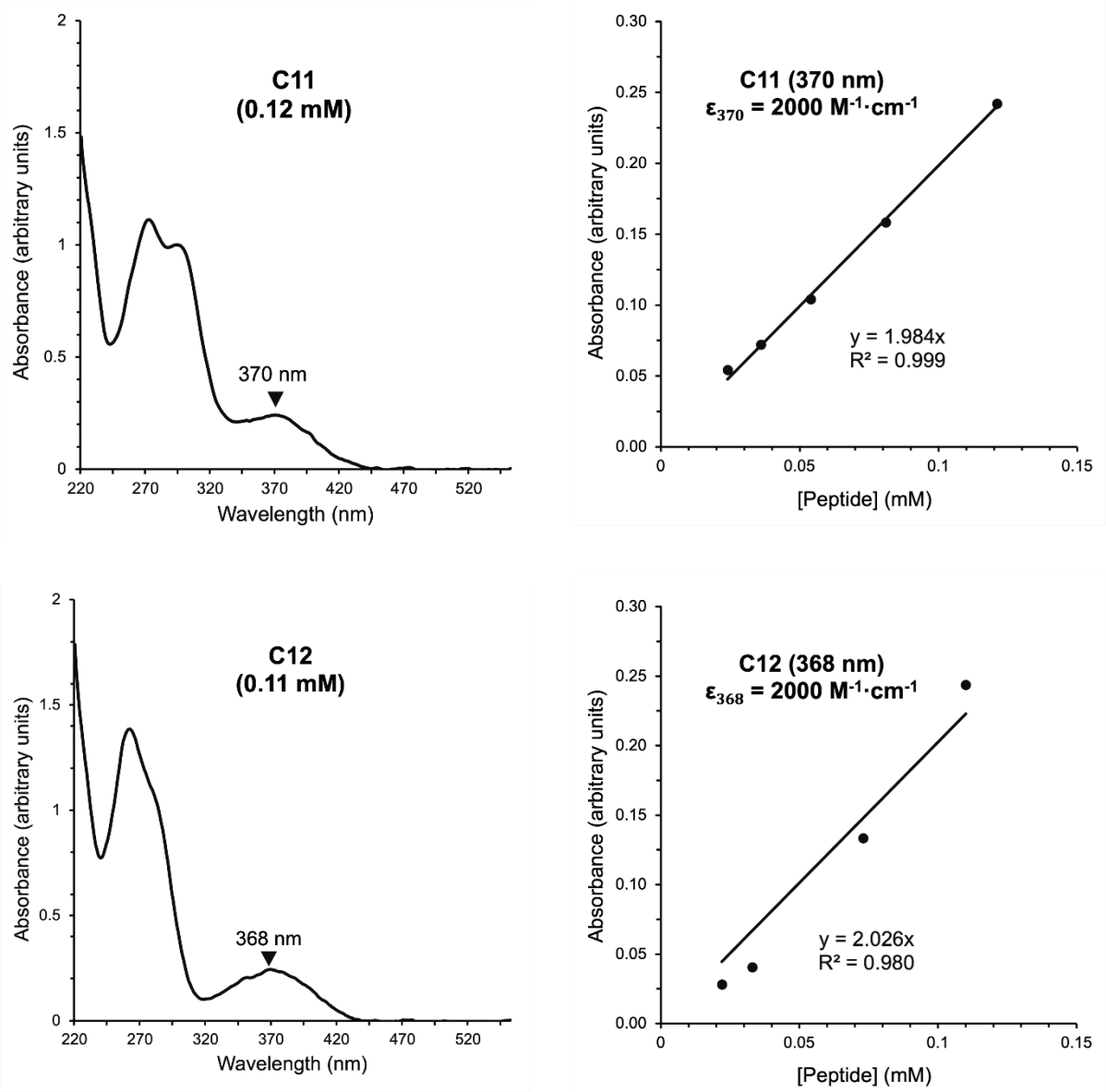

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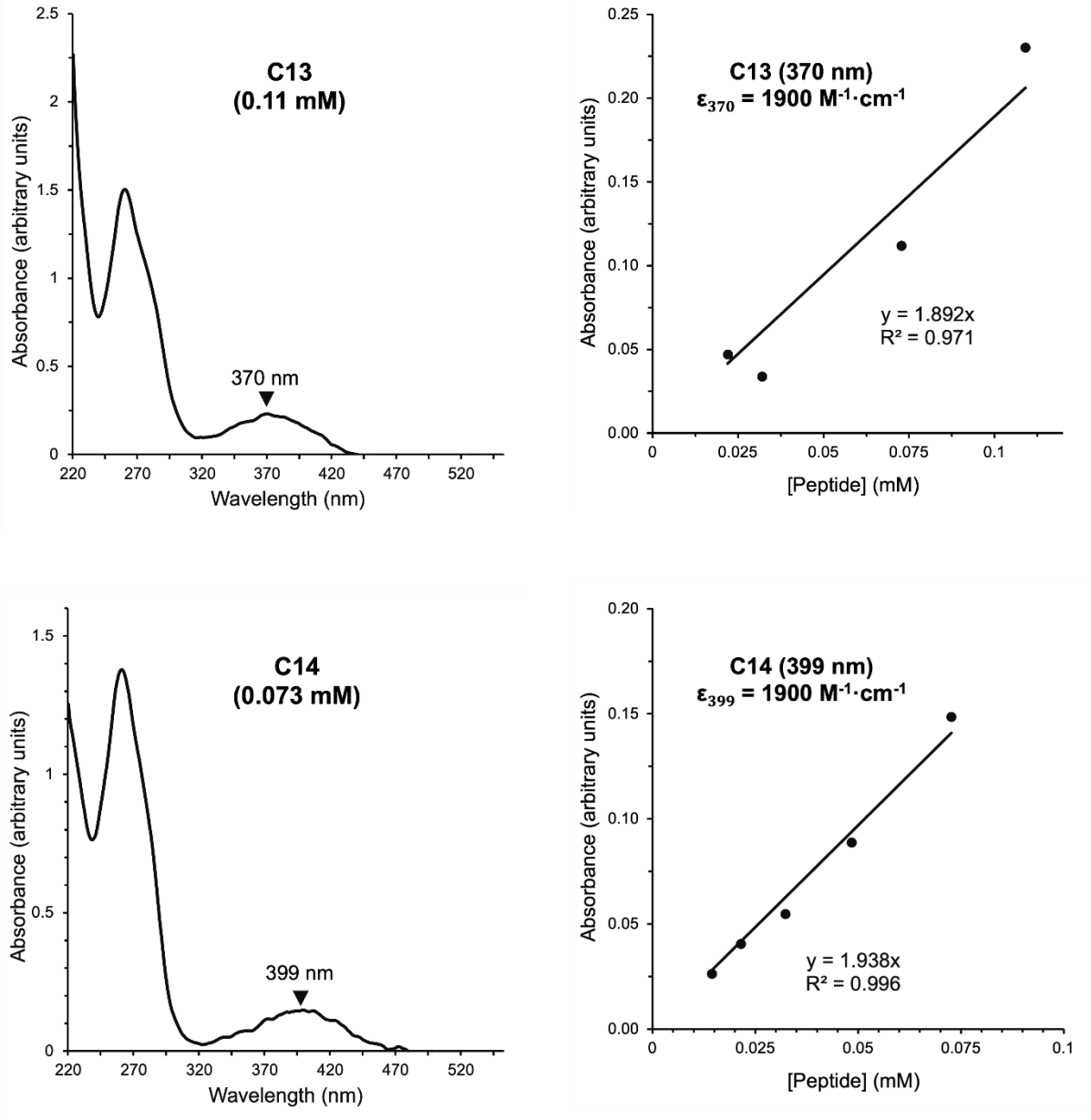

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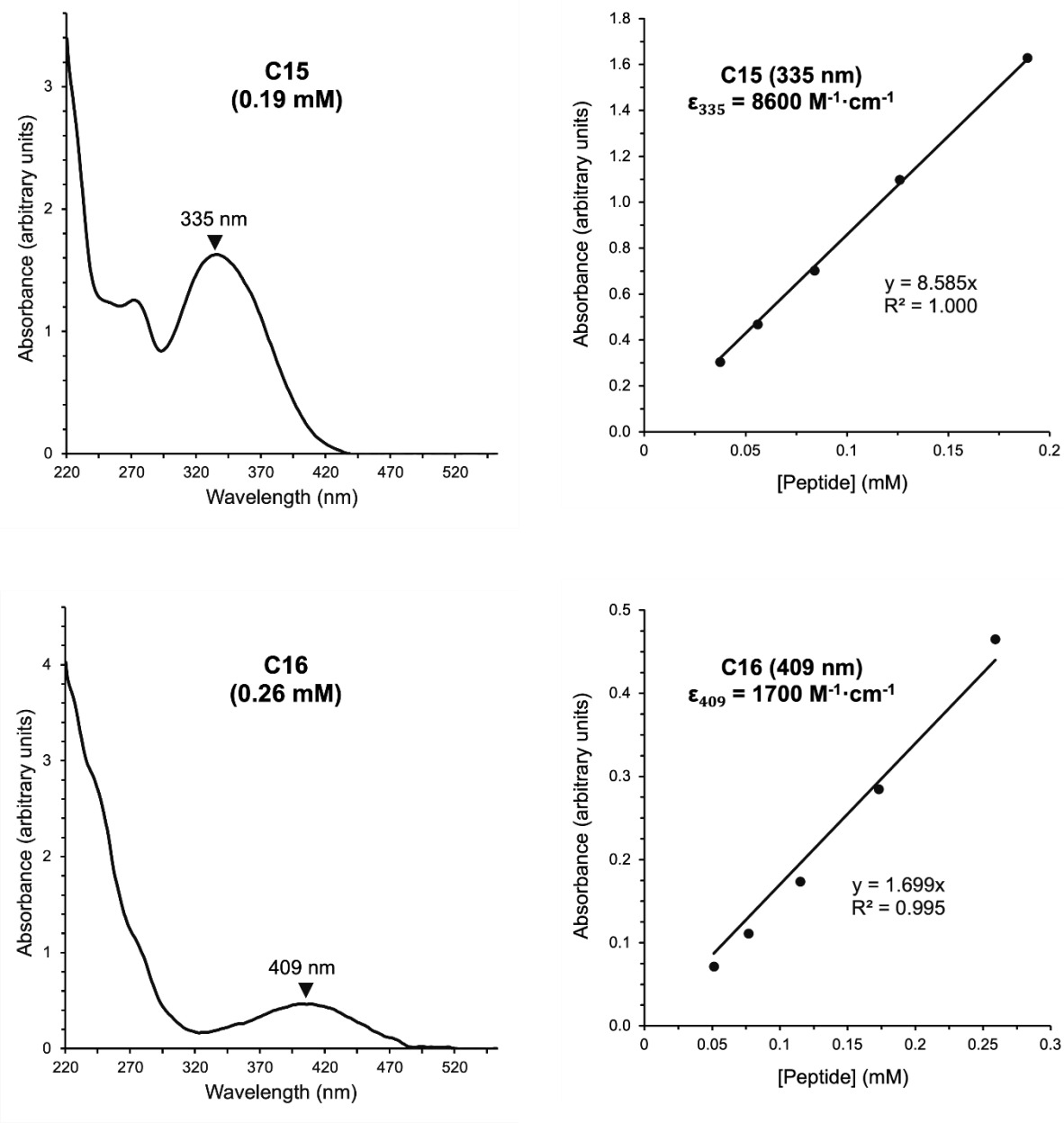

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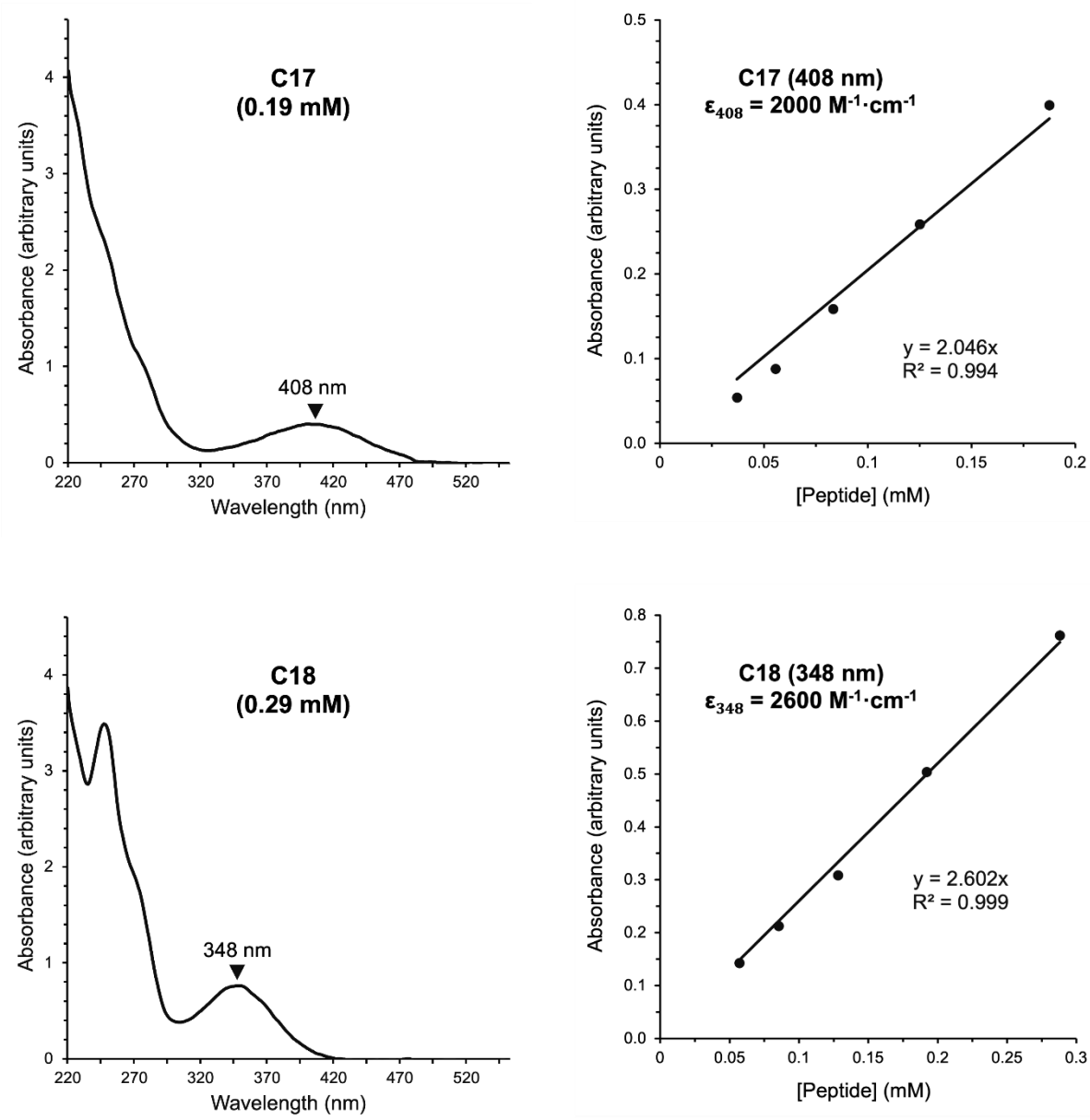

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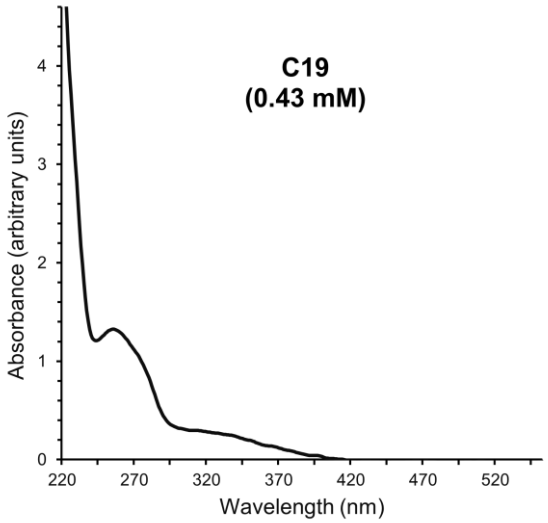

No local maximum > 300 nm

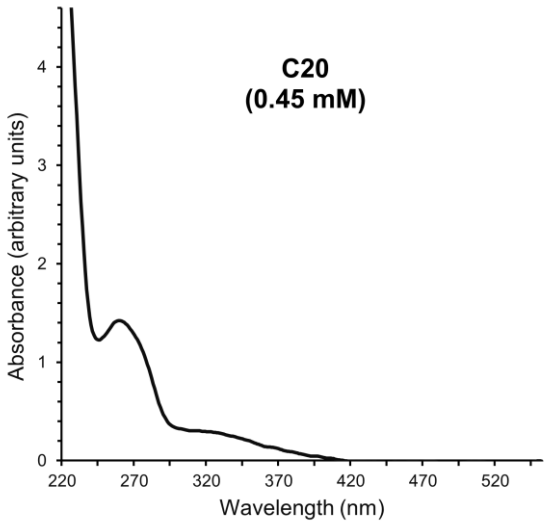

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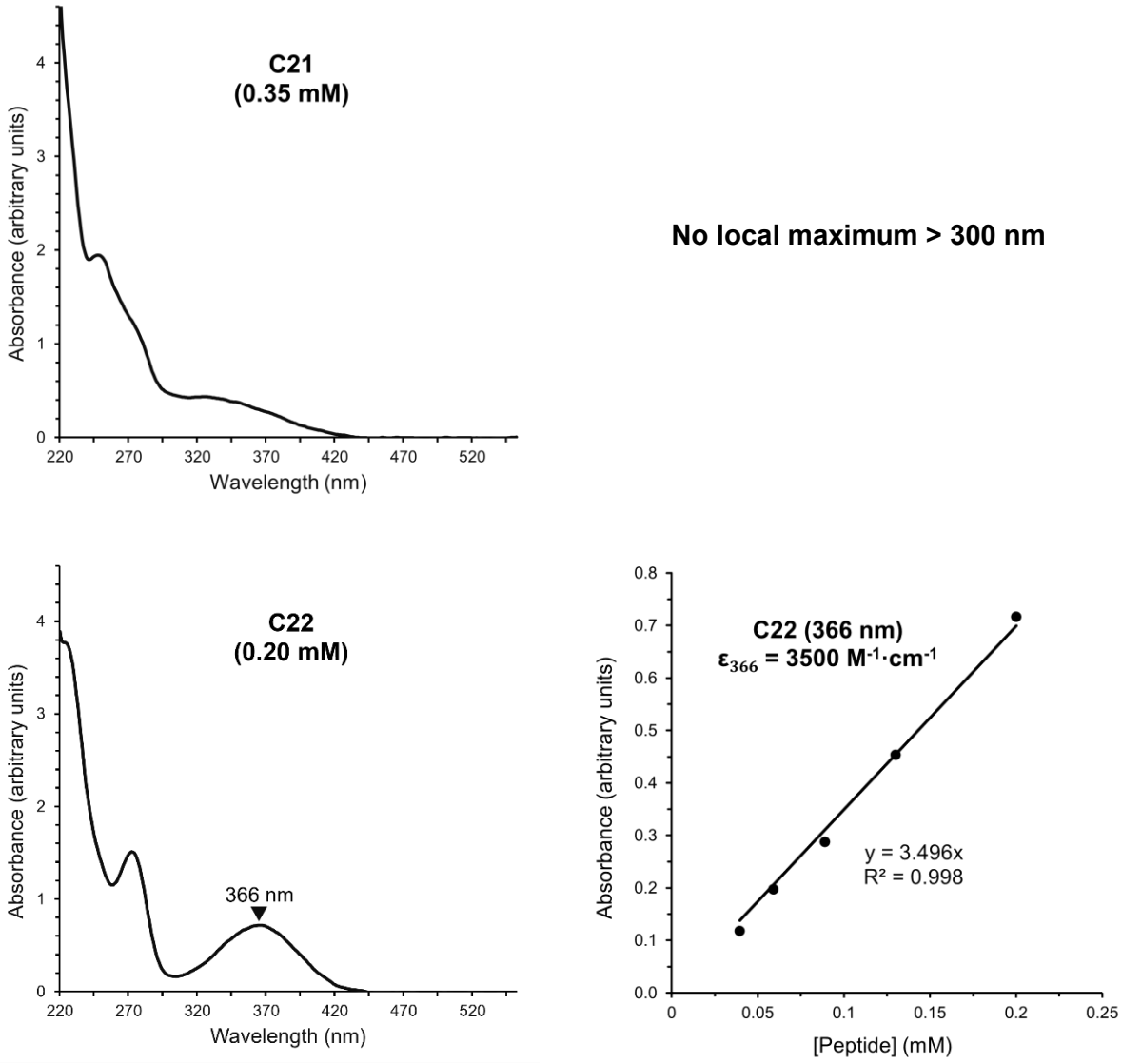
Figure S2. UV-visible spectra for nitroarylated Cys/Dap model peptides (continued)



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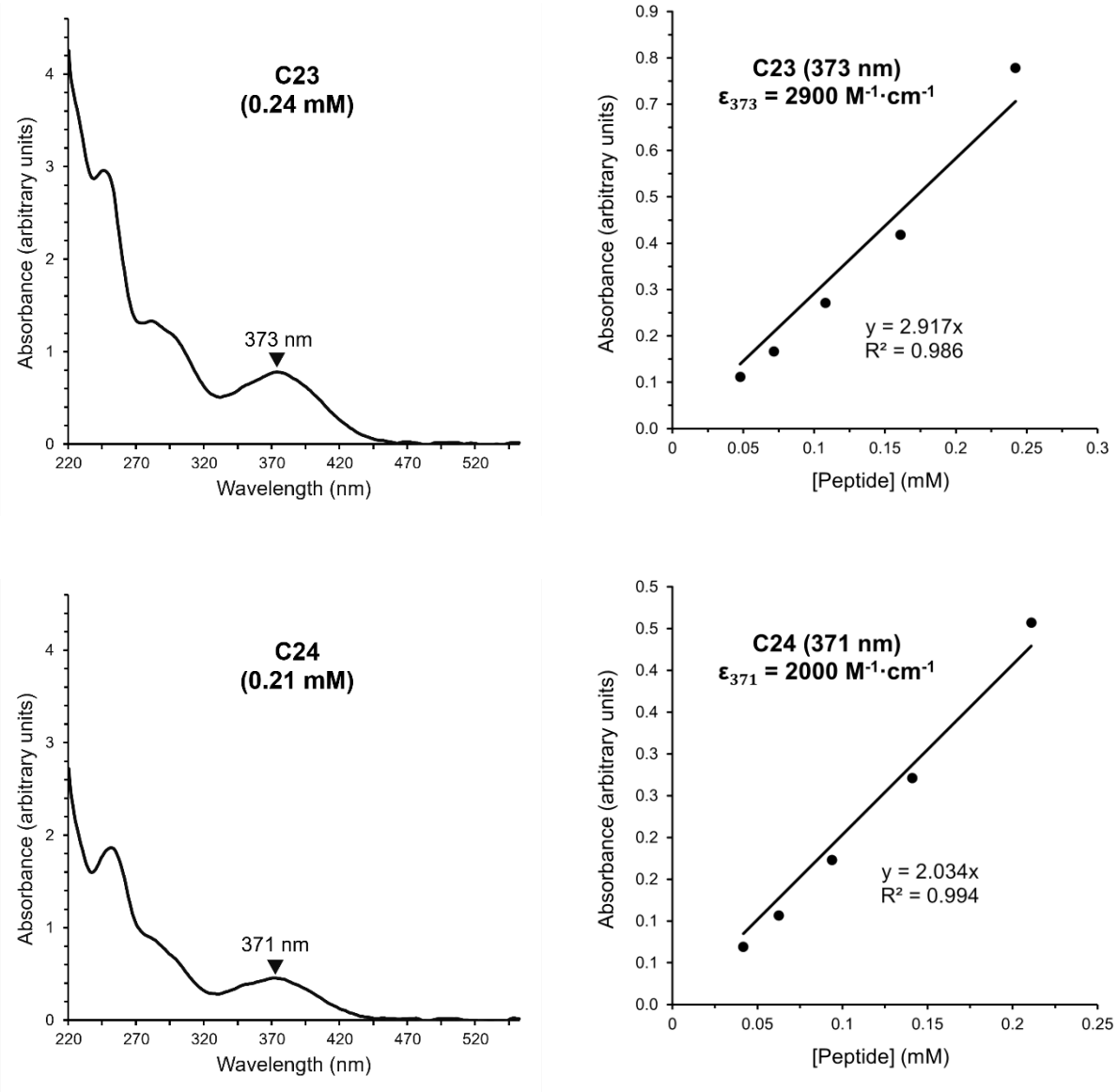

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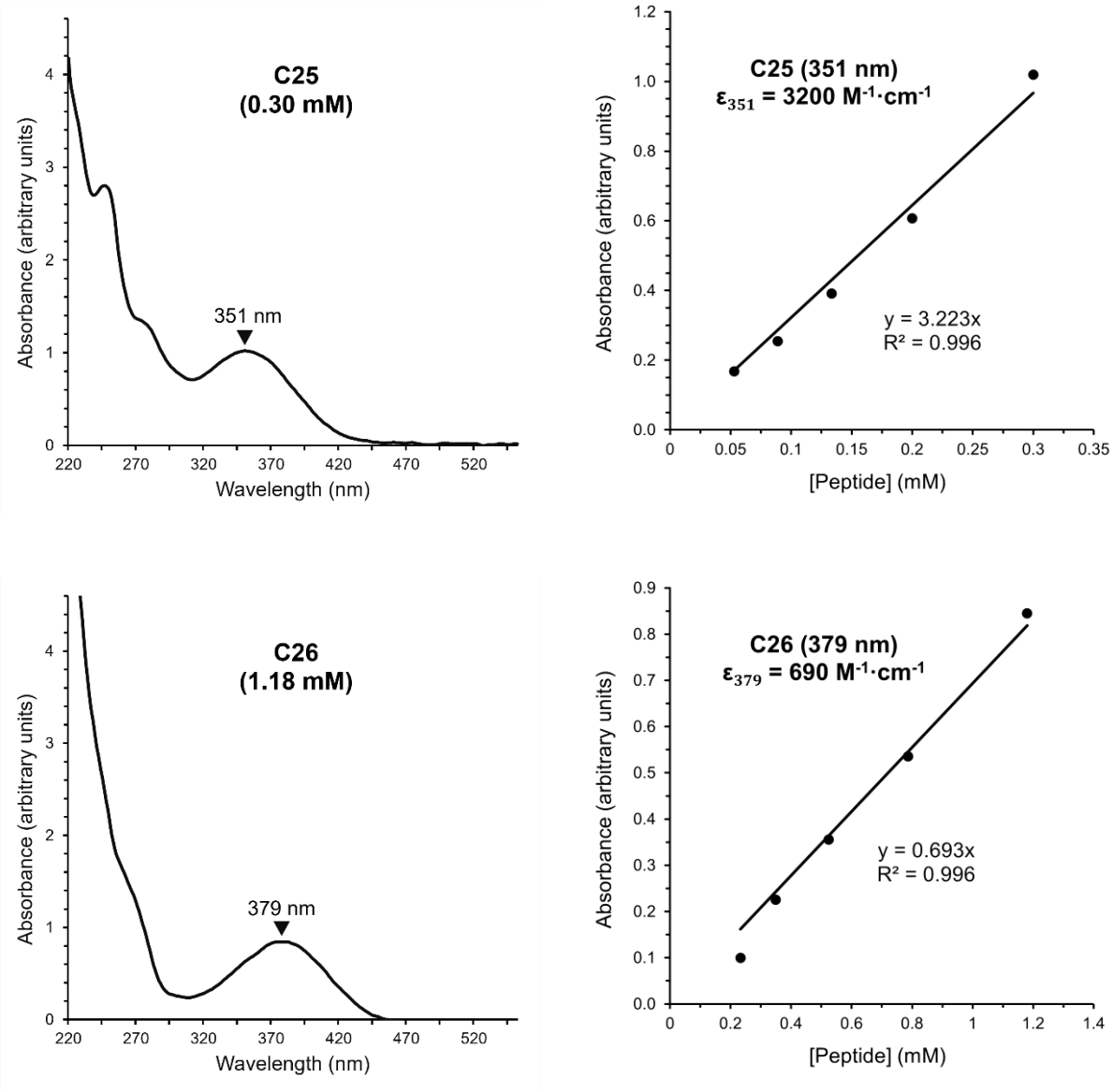

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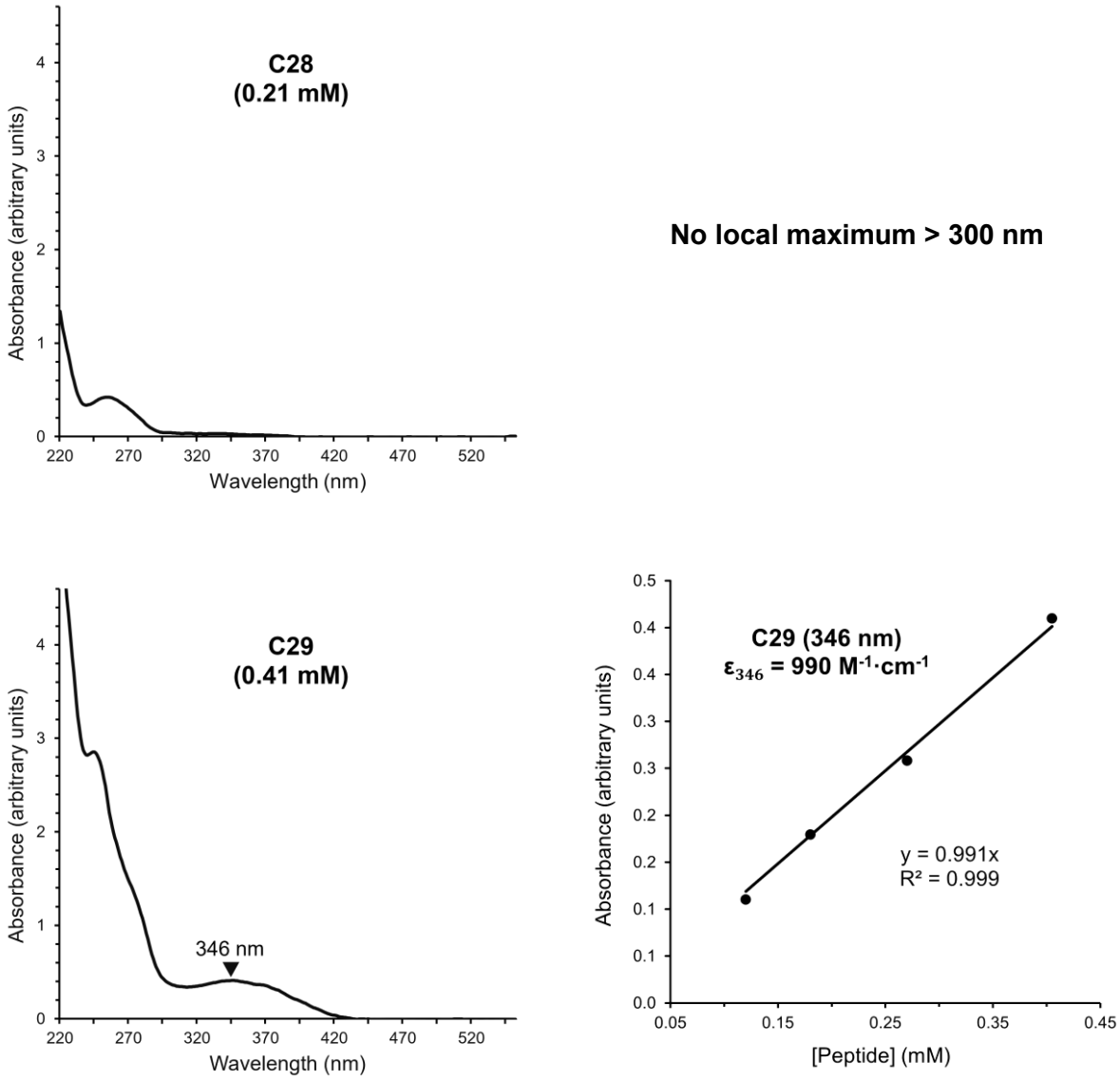

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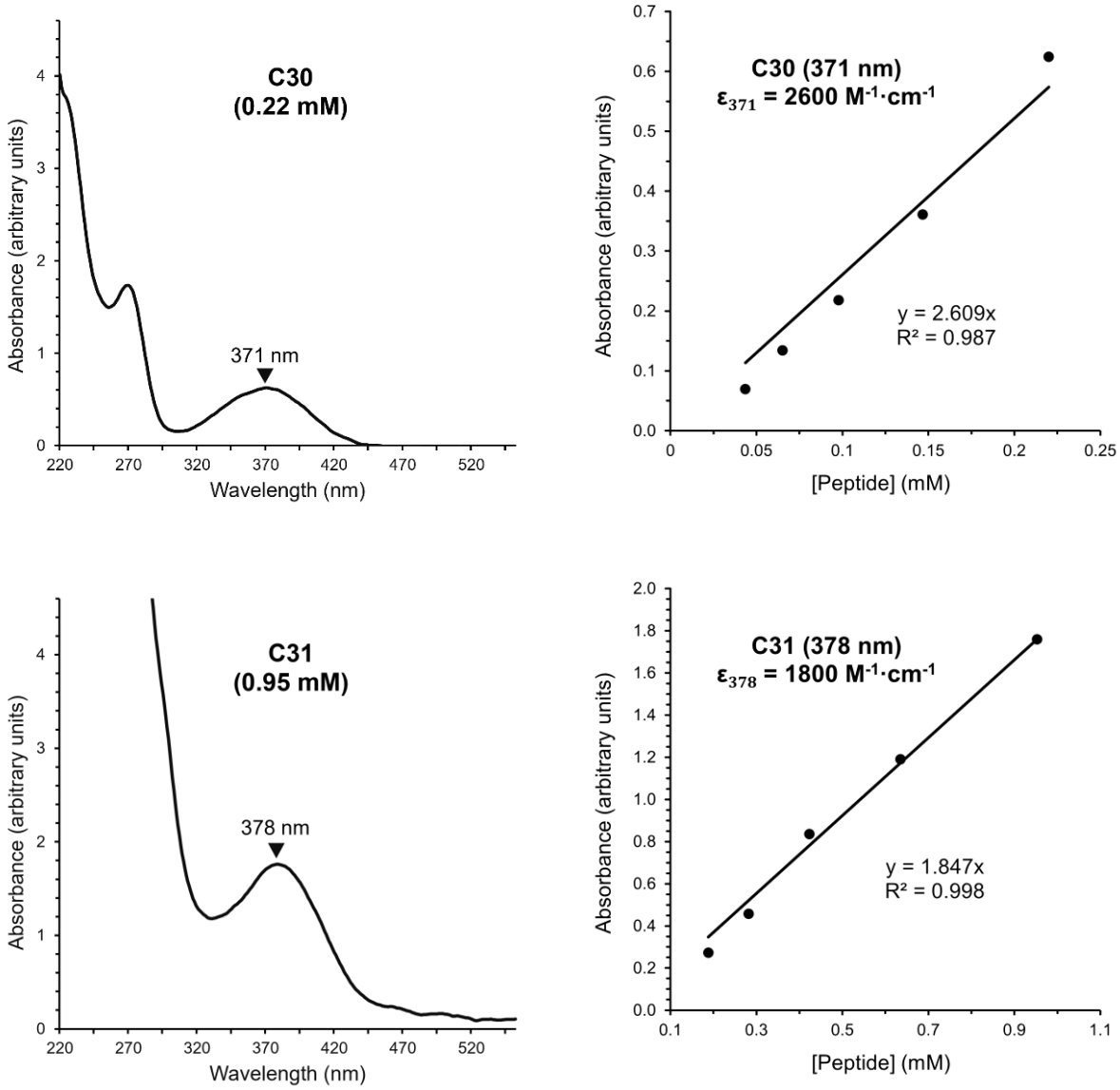

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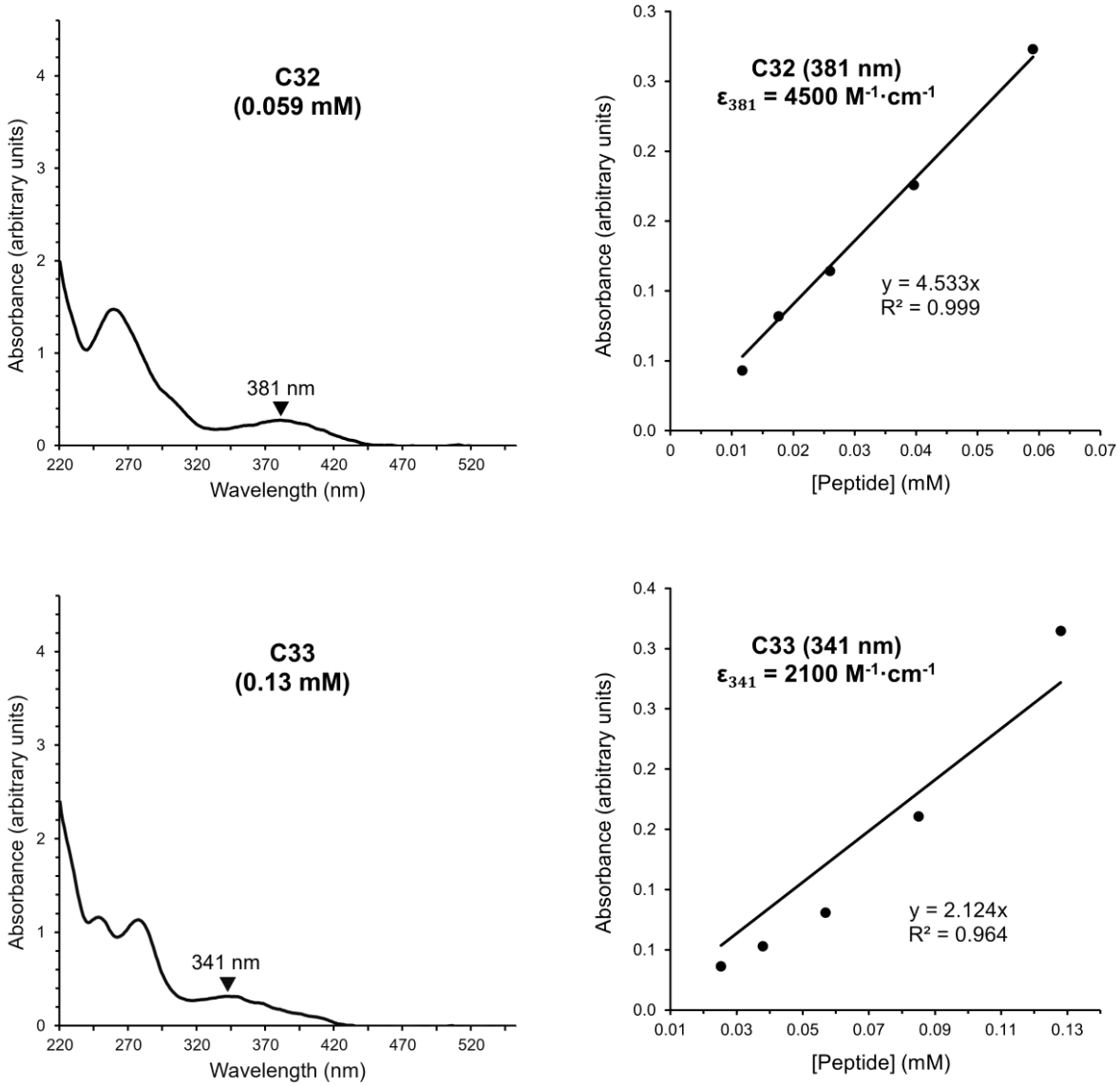

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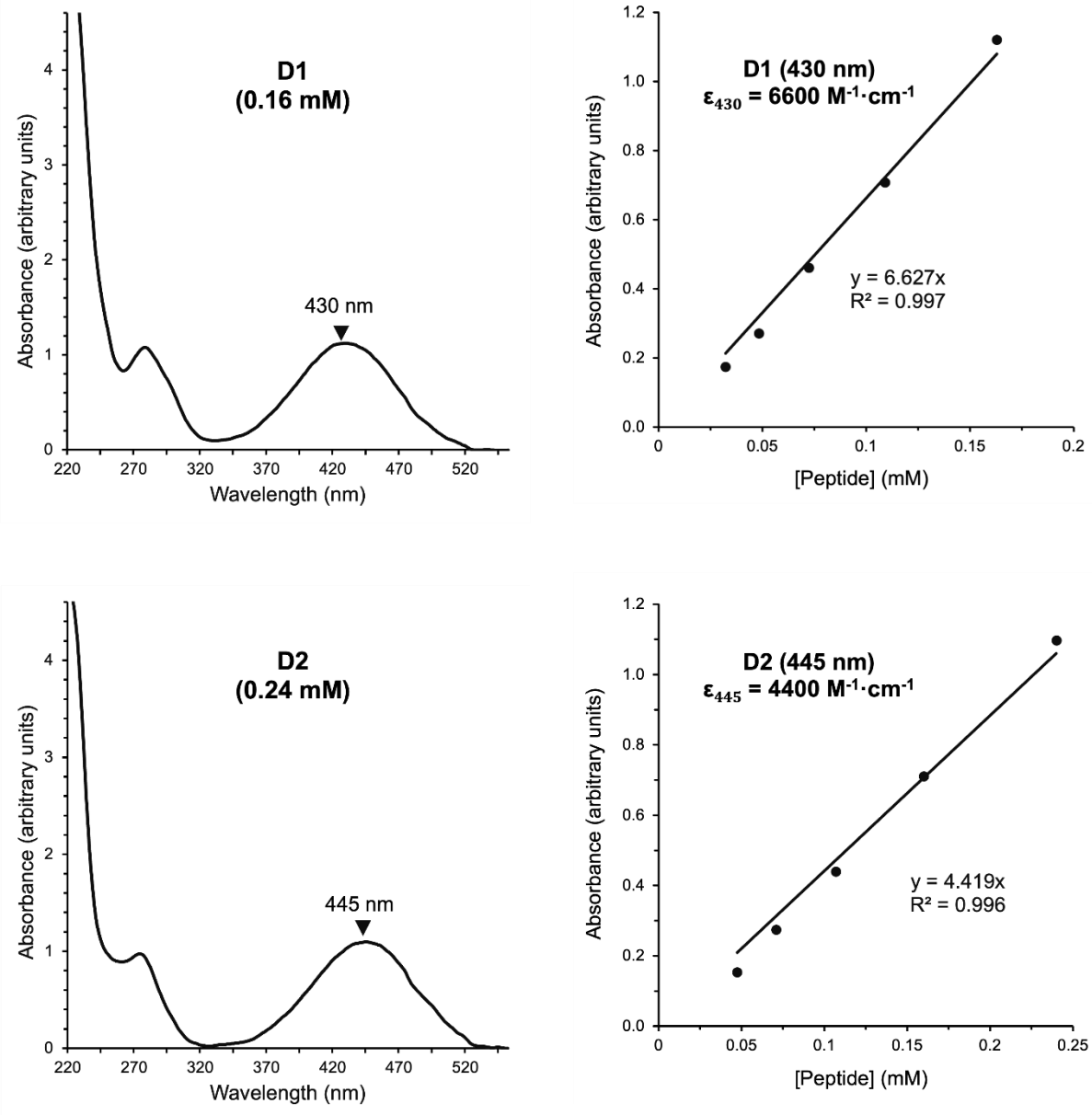

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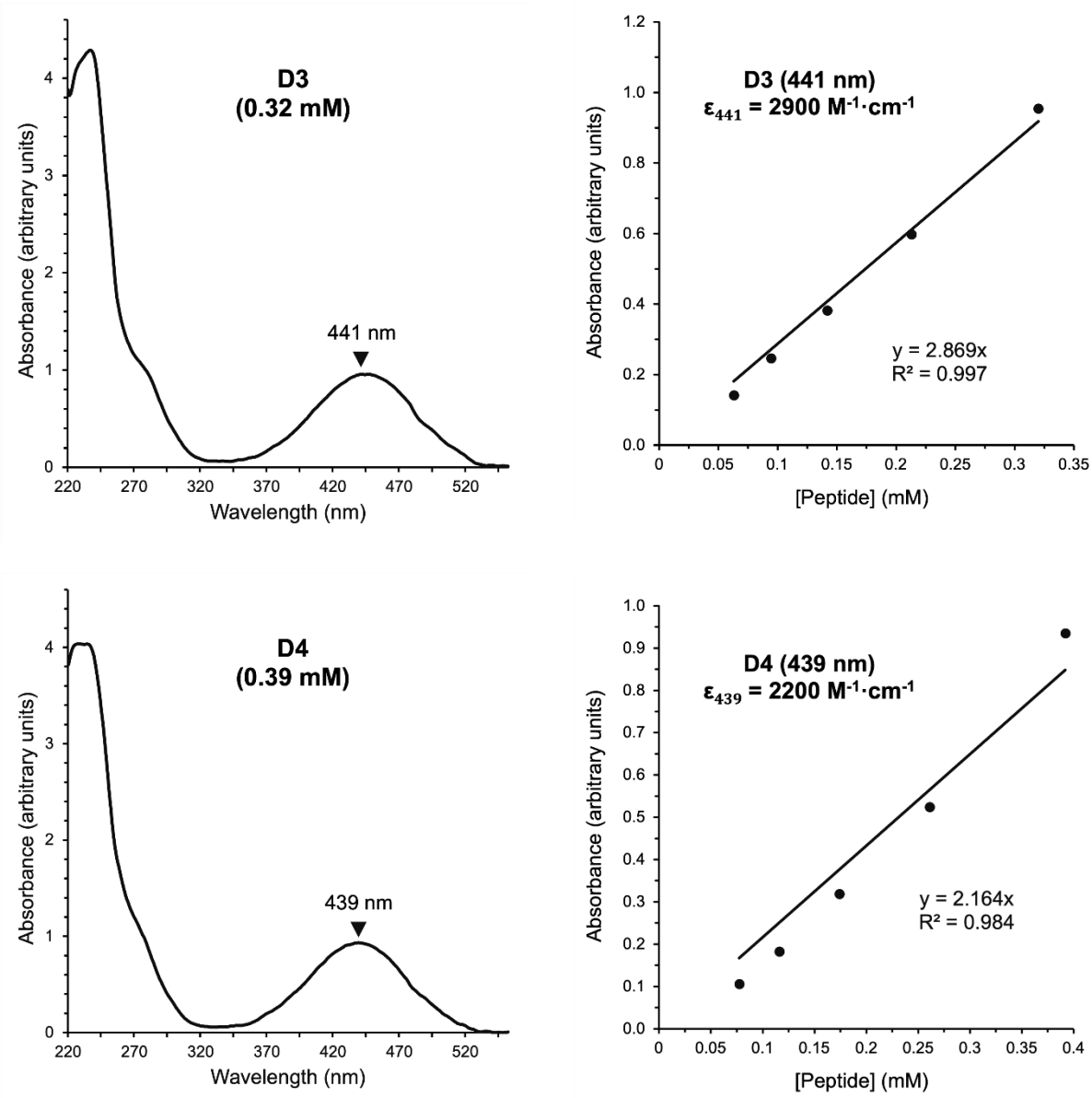

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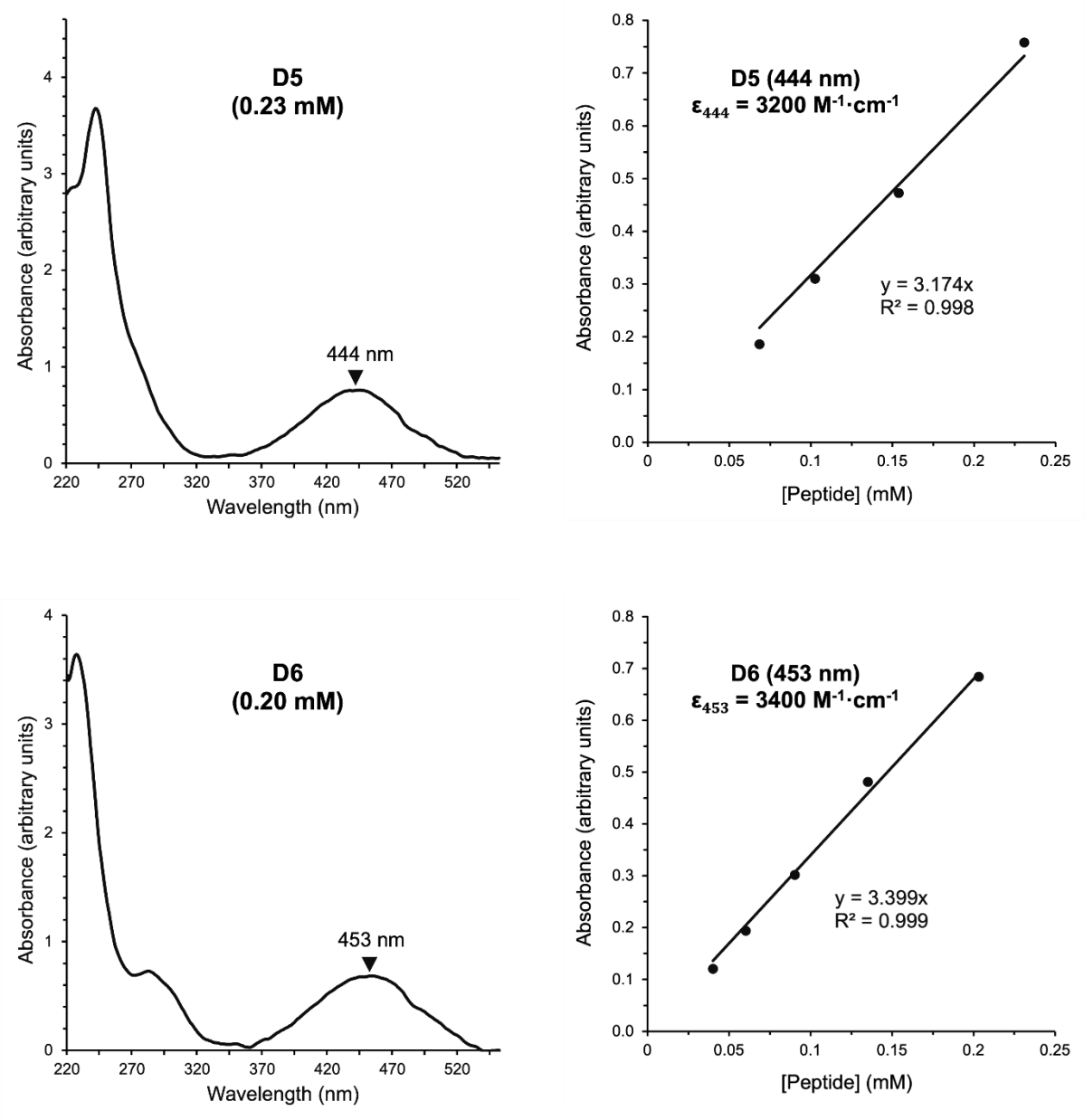

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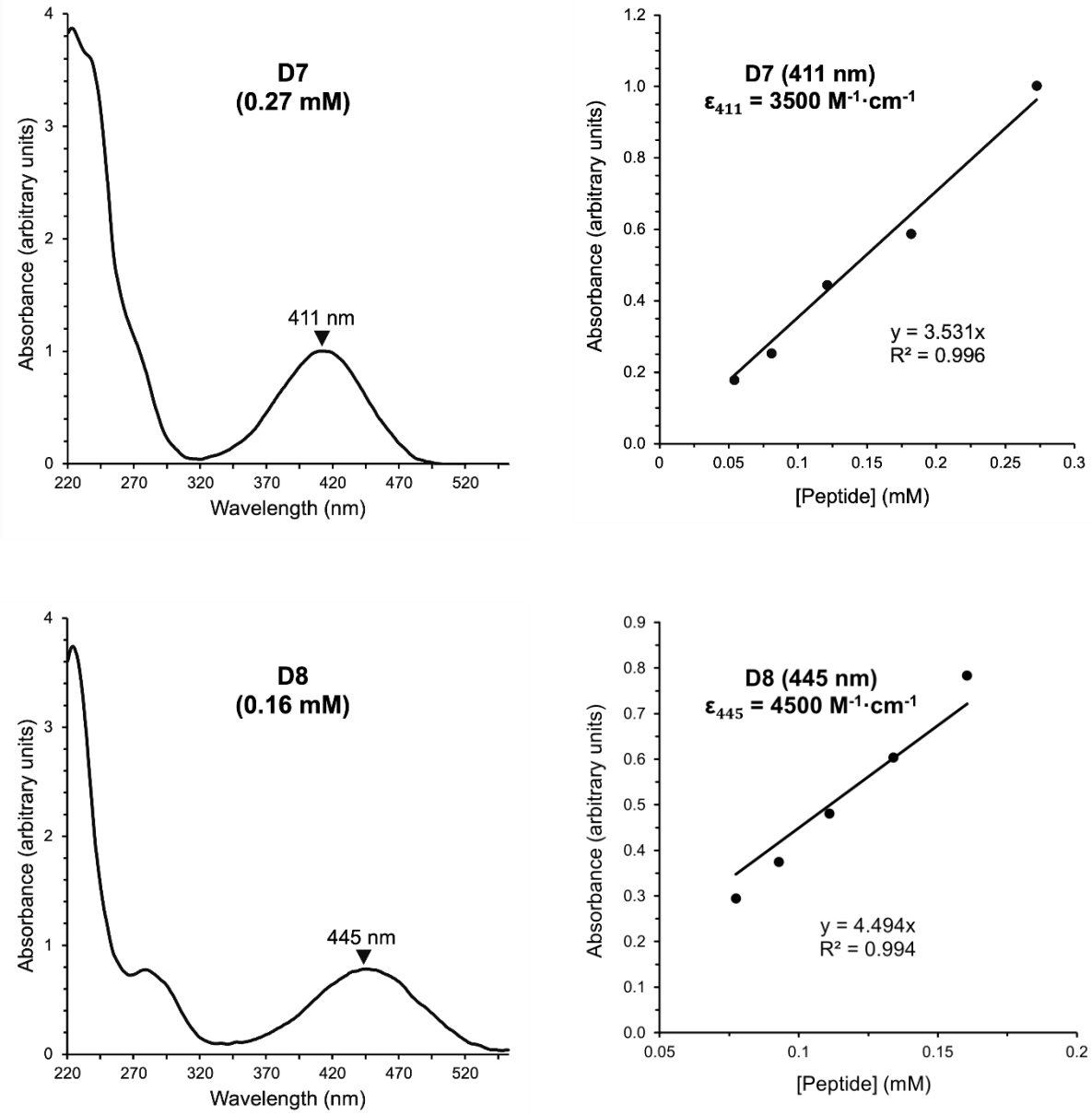

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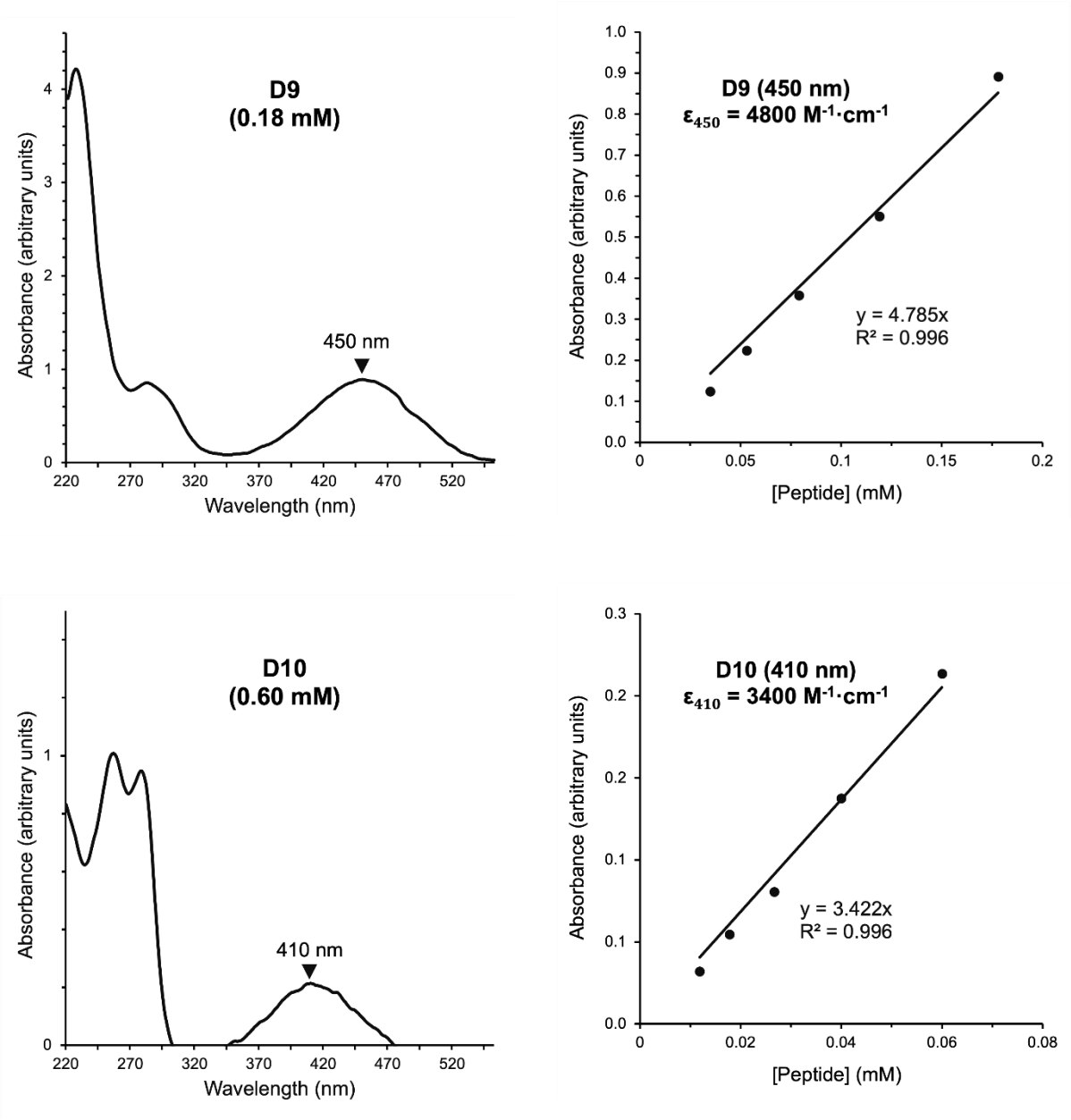

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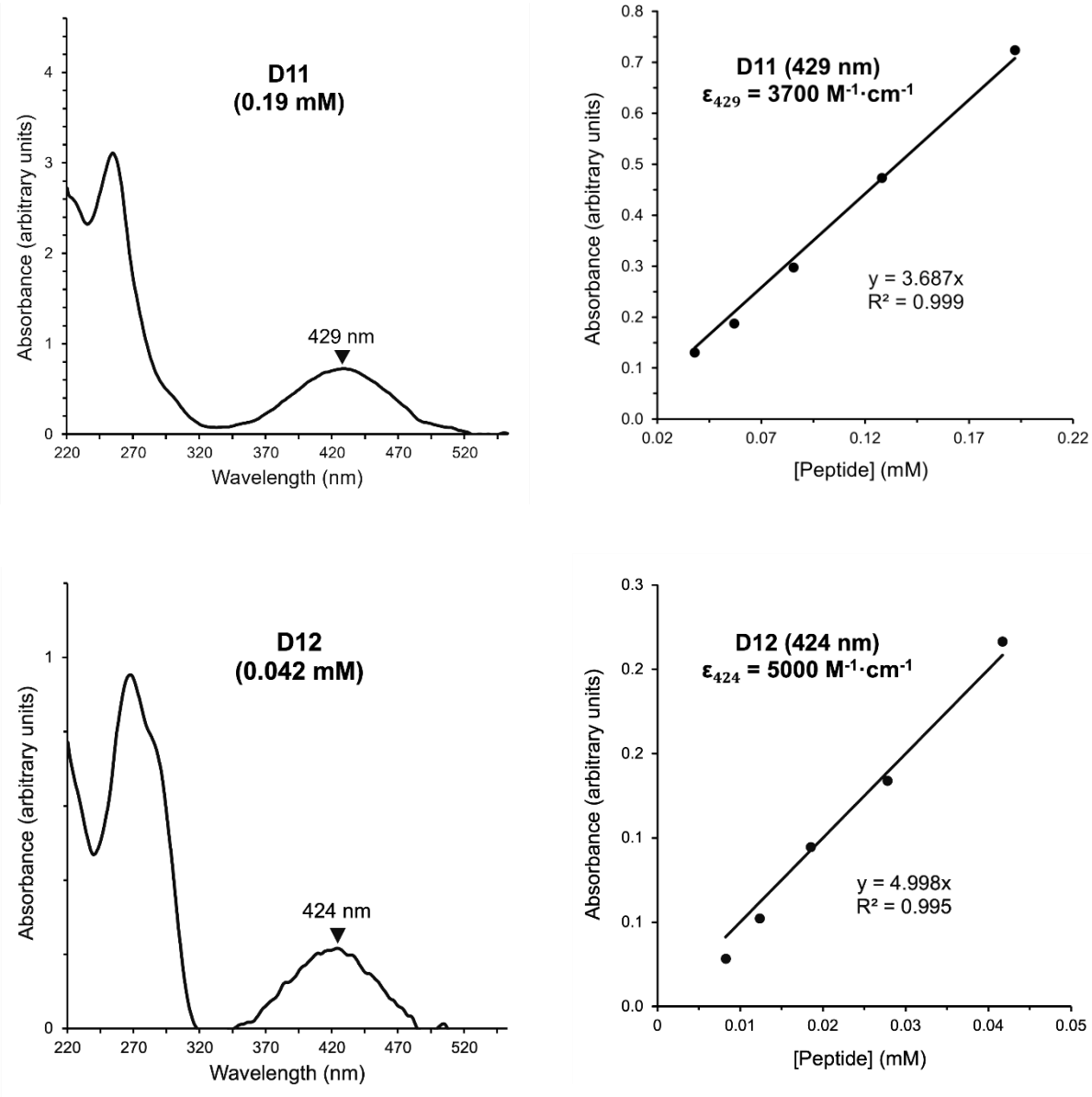

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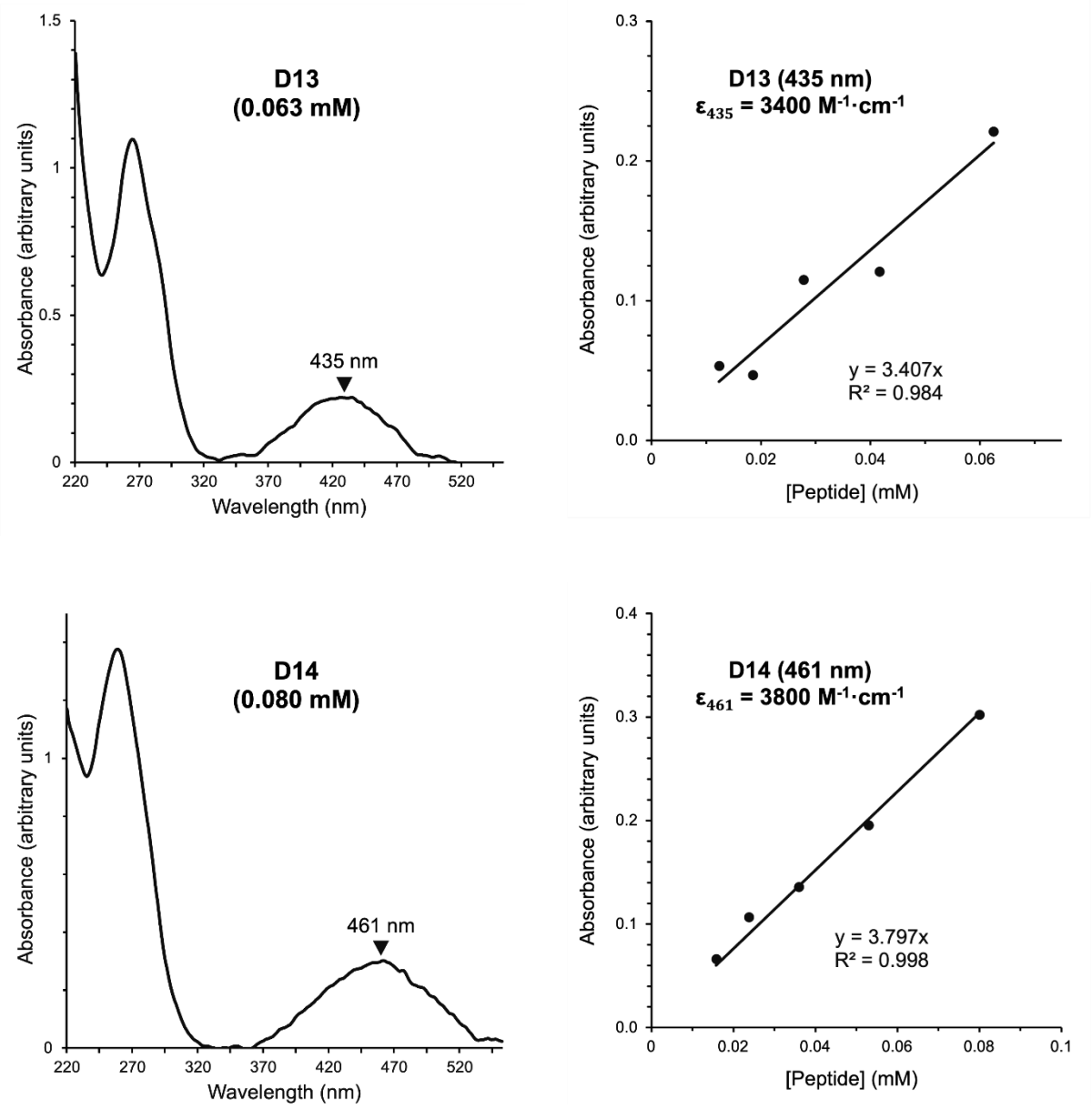

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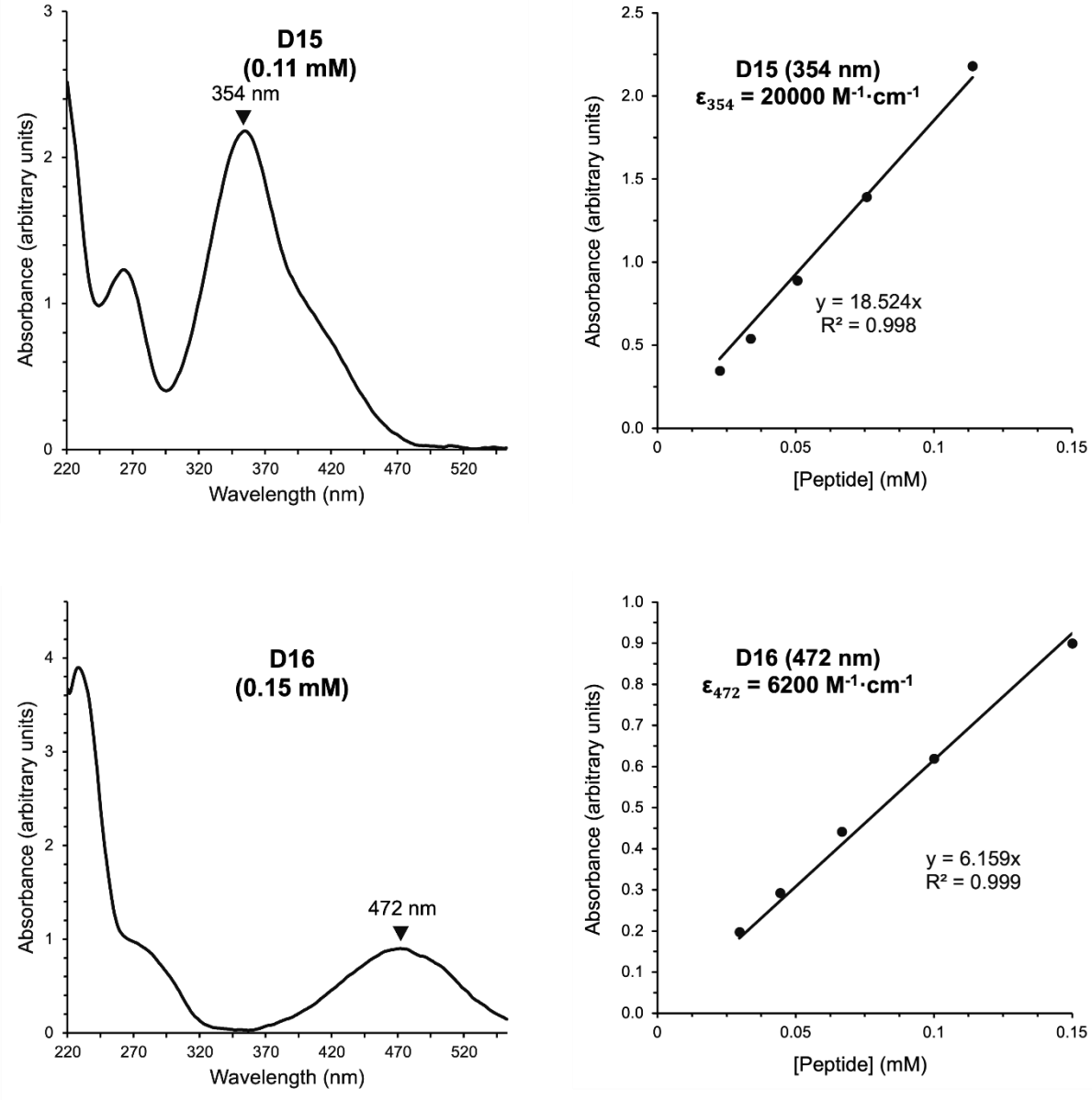

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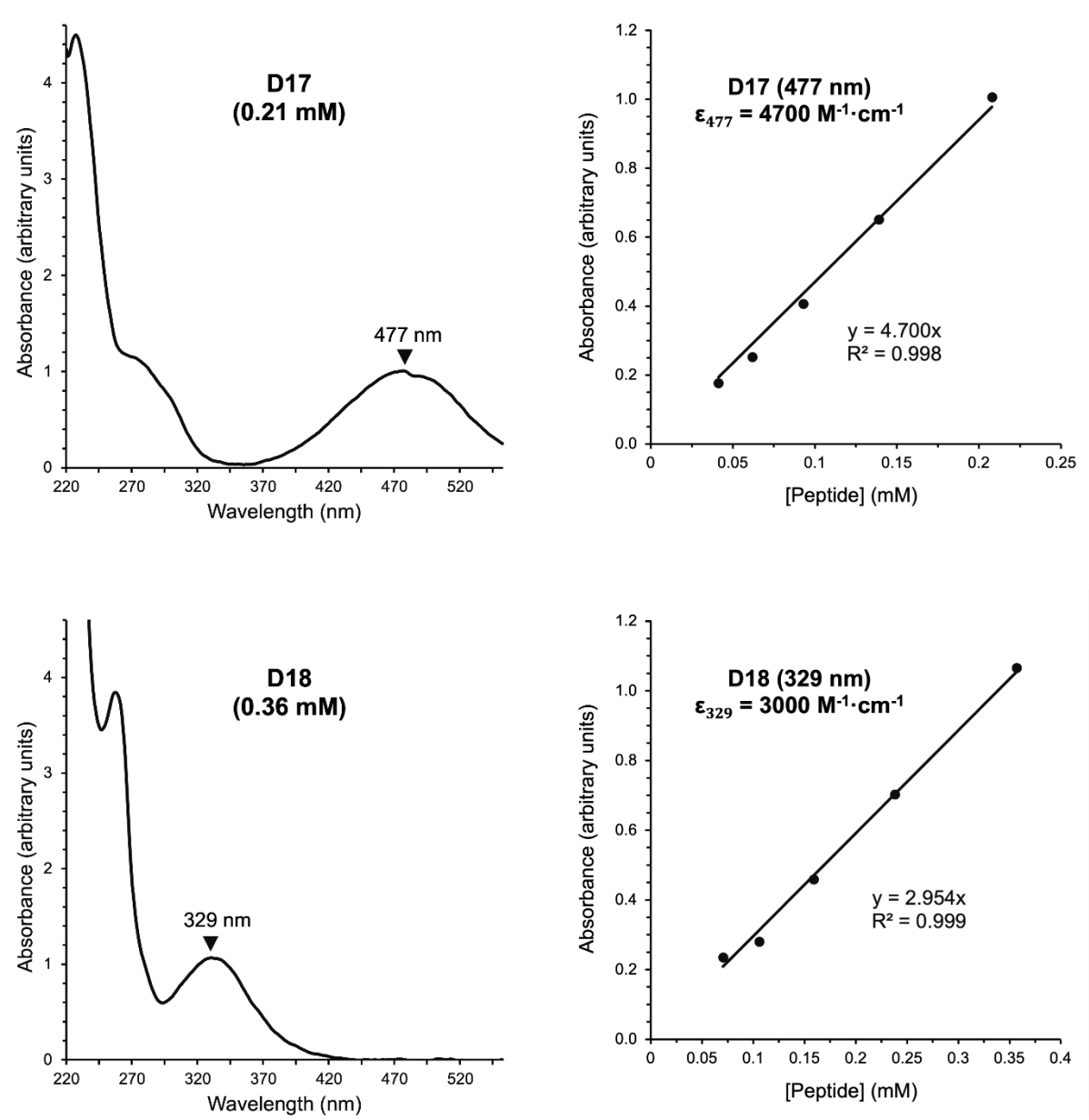

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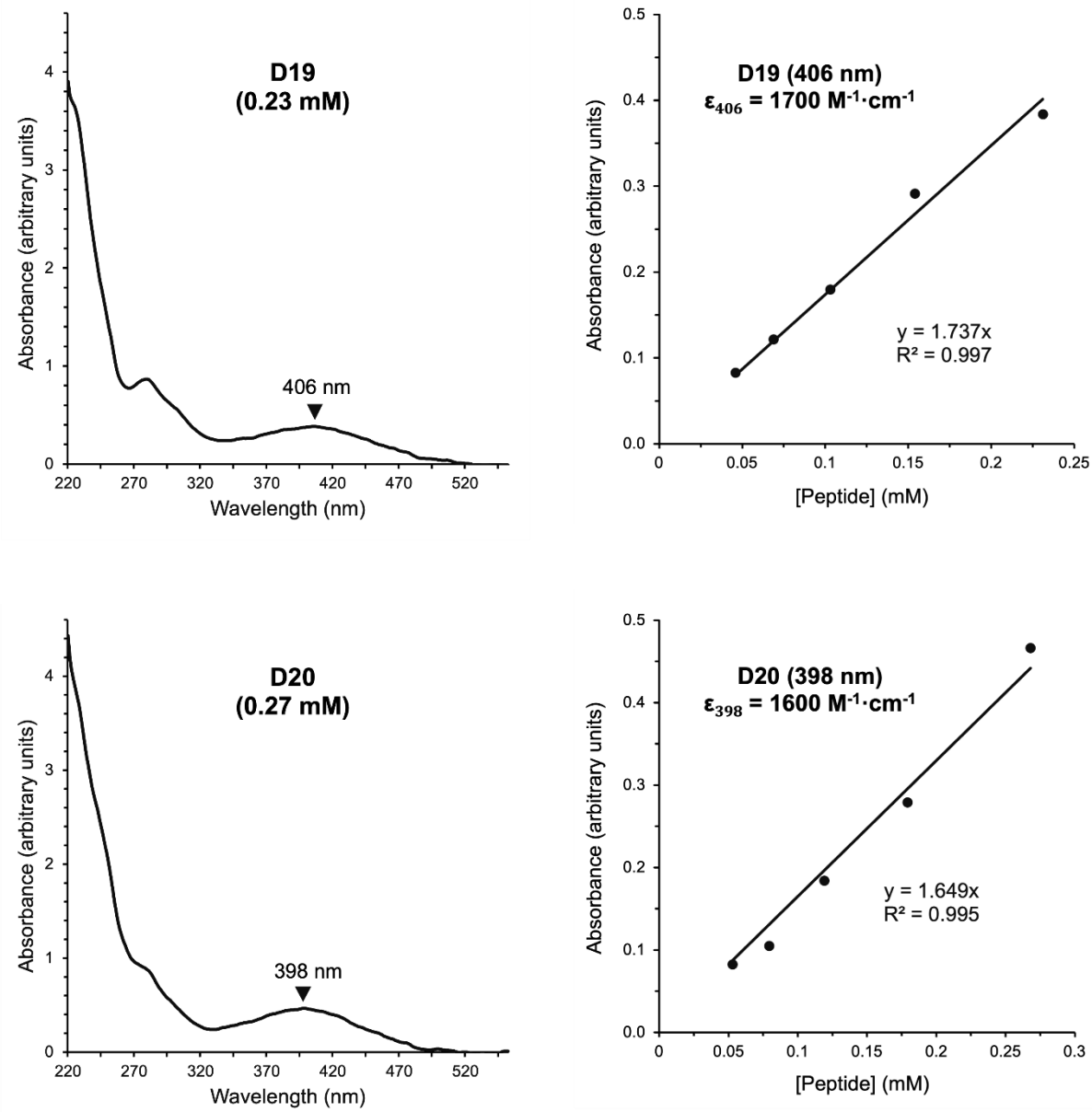

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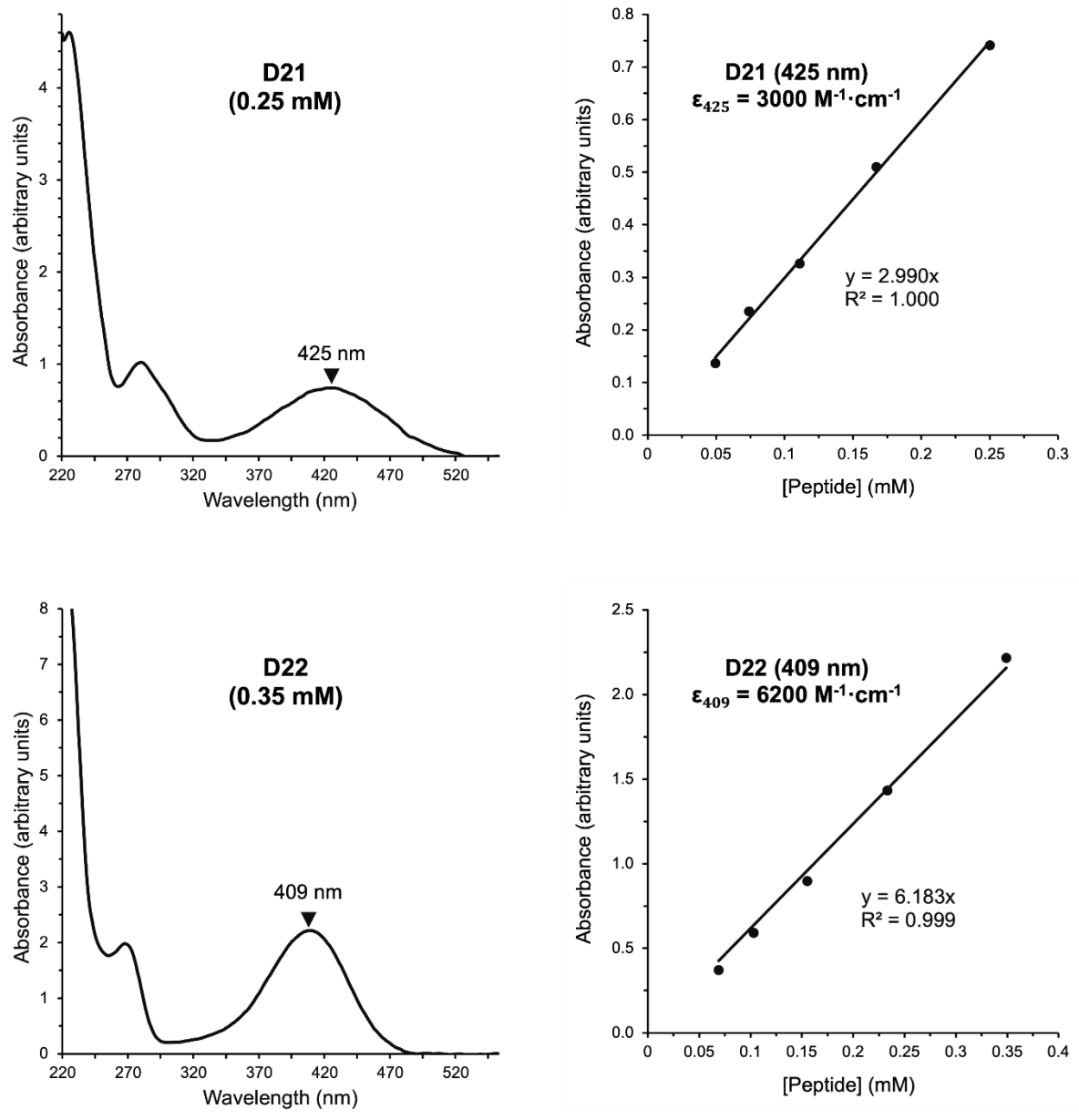

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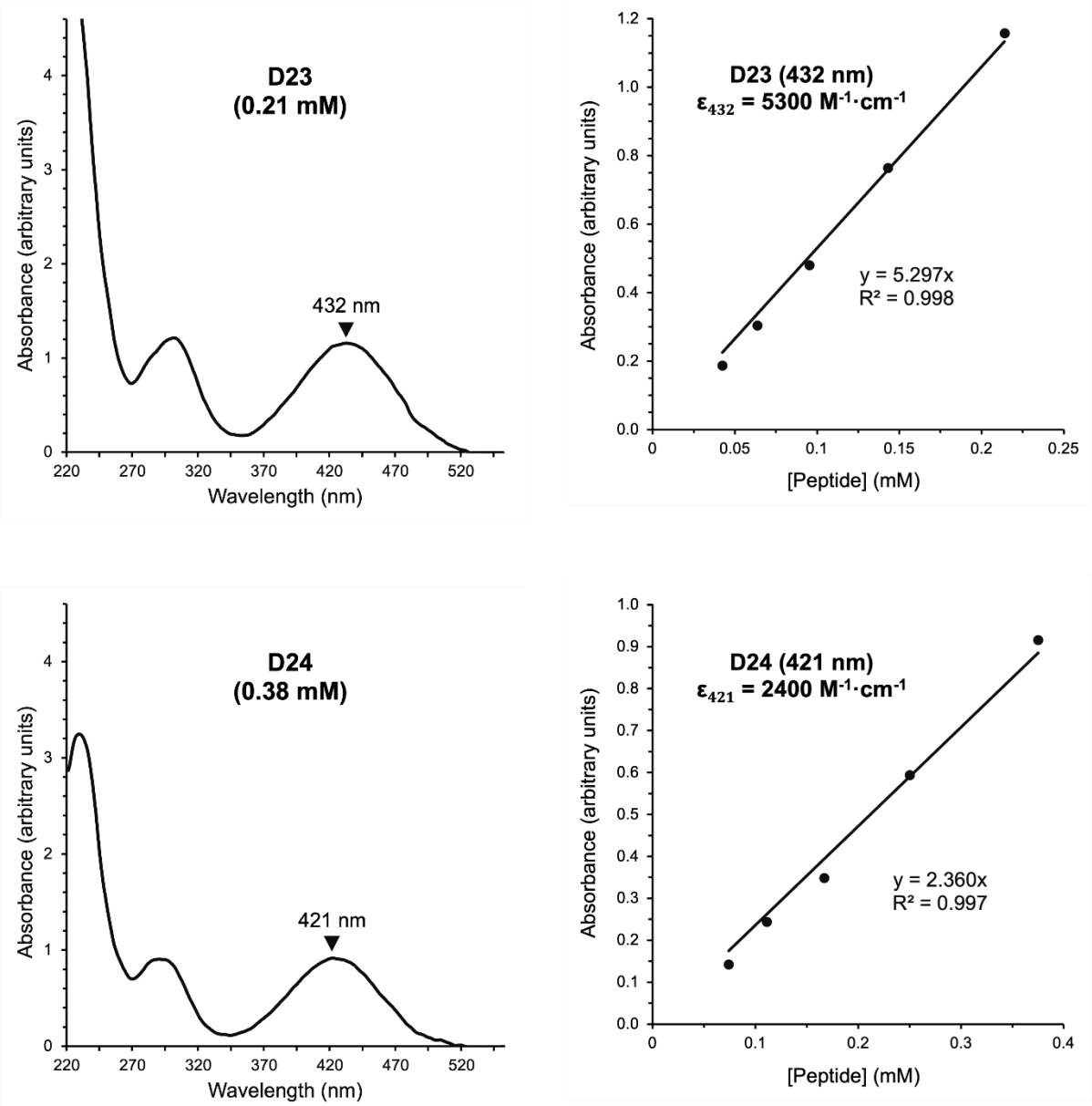

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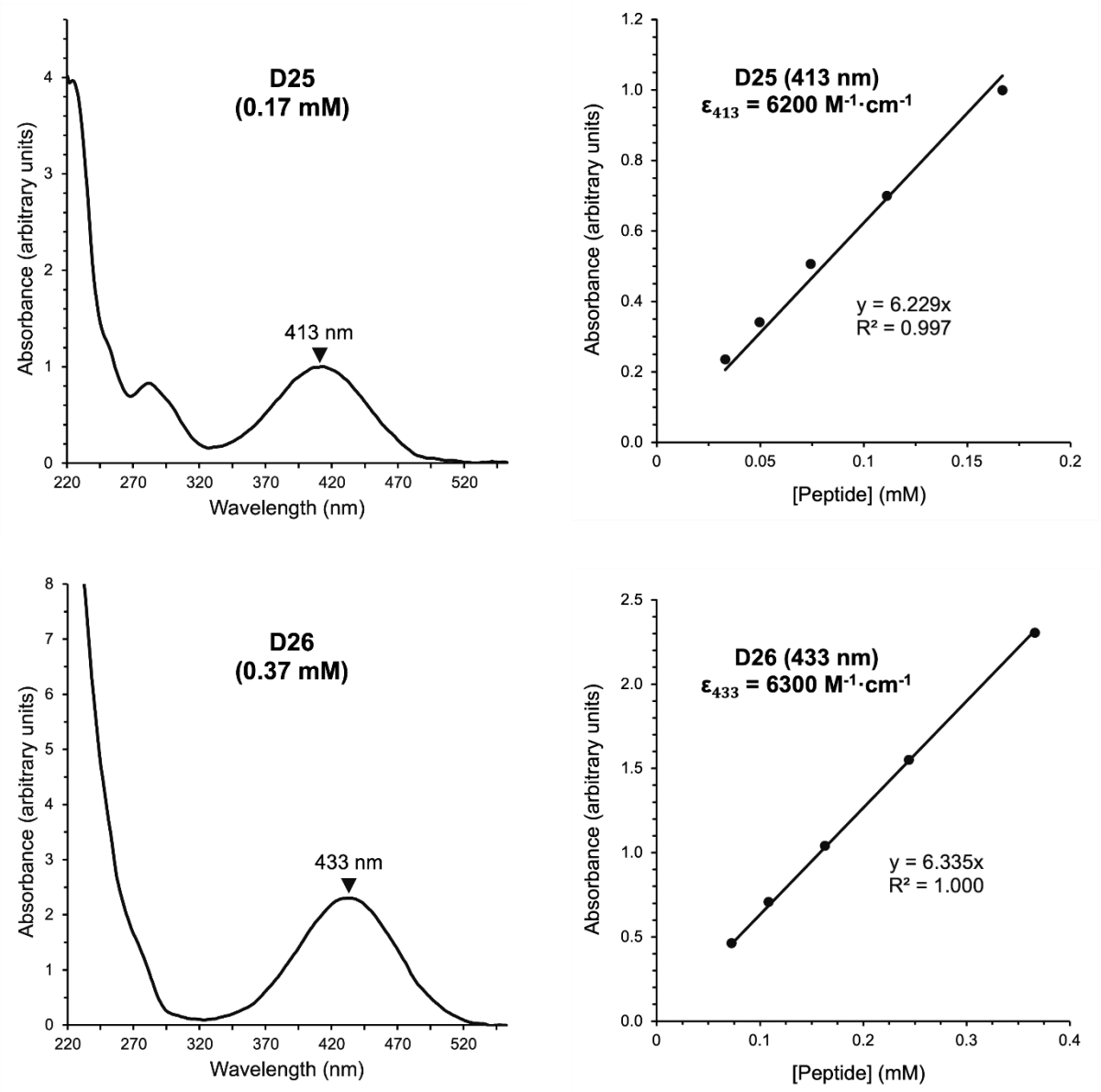
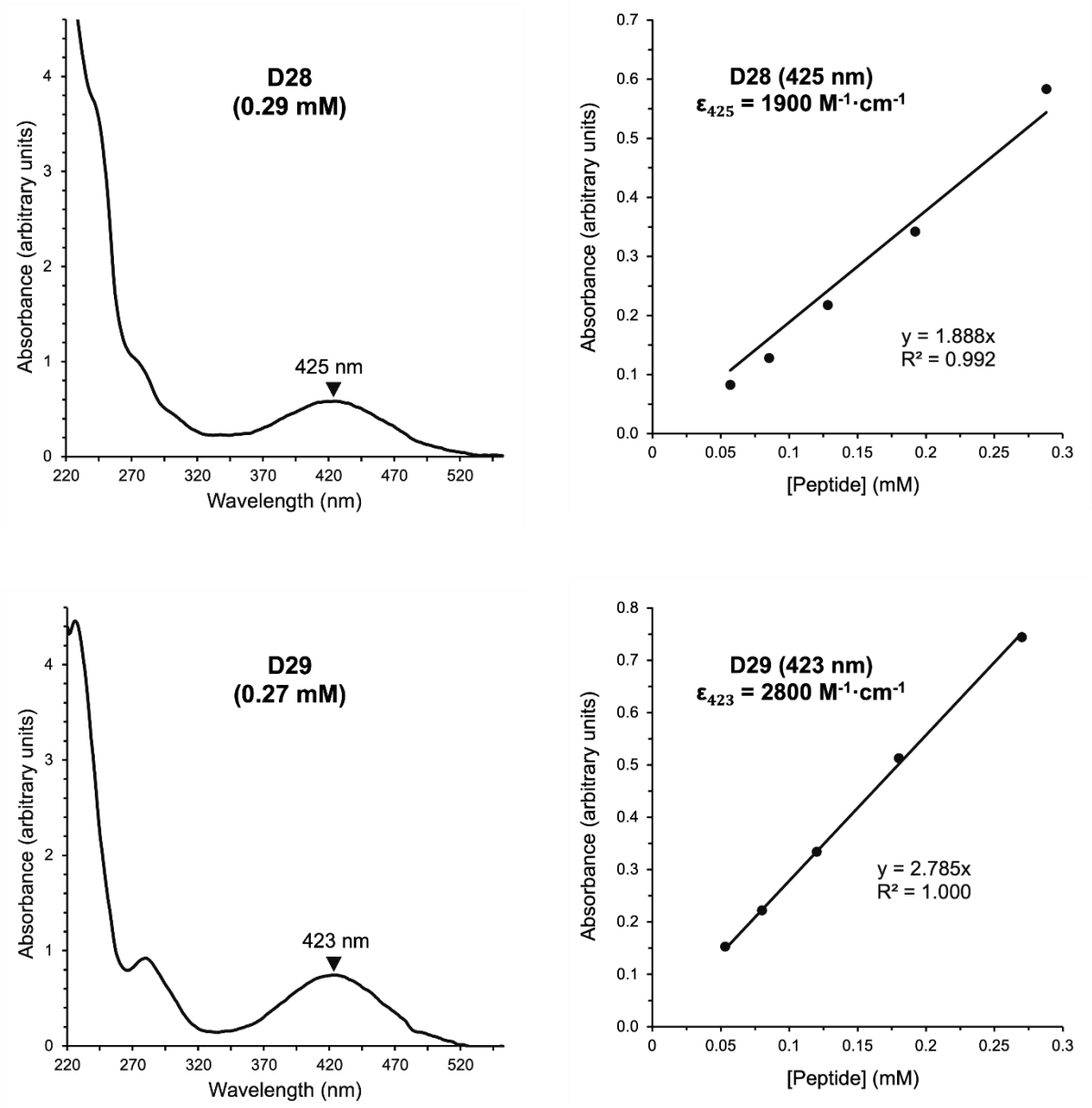


Figure S2. UV-visible spectra for nitroarylated Cys/Dap model peptides (continued)



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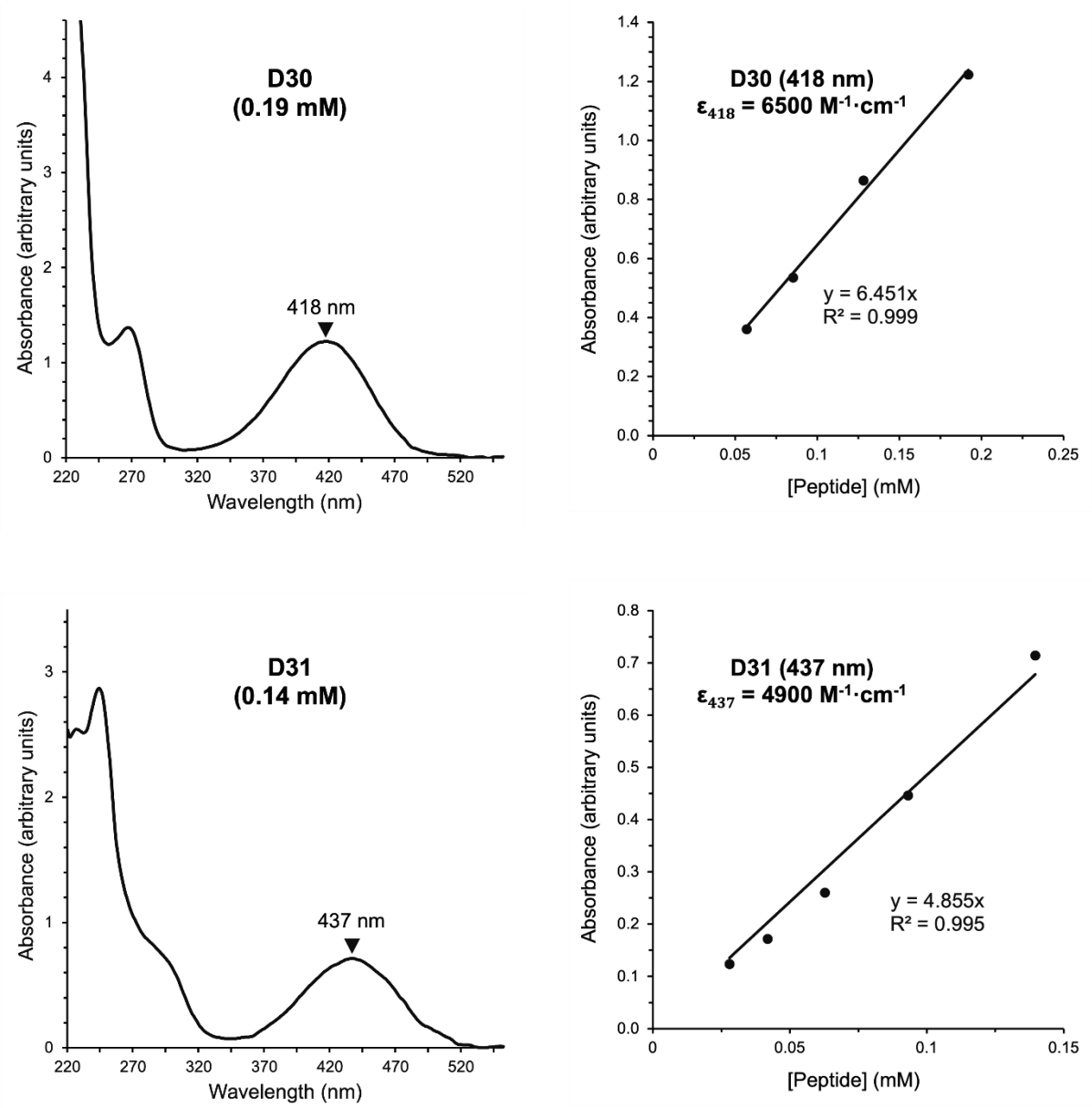

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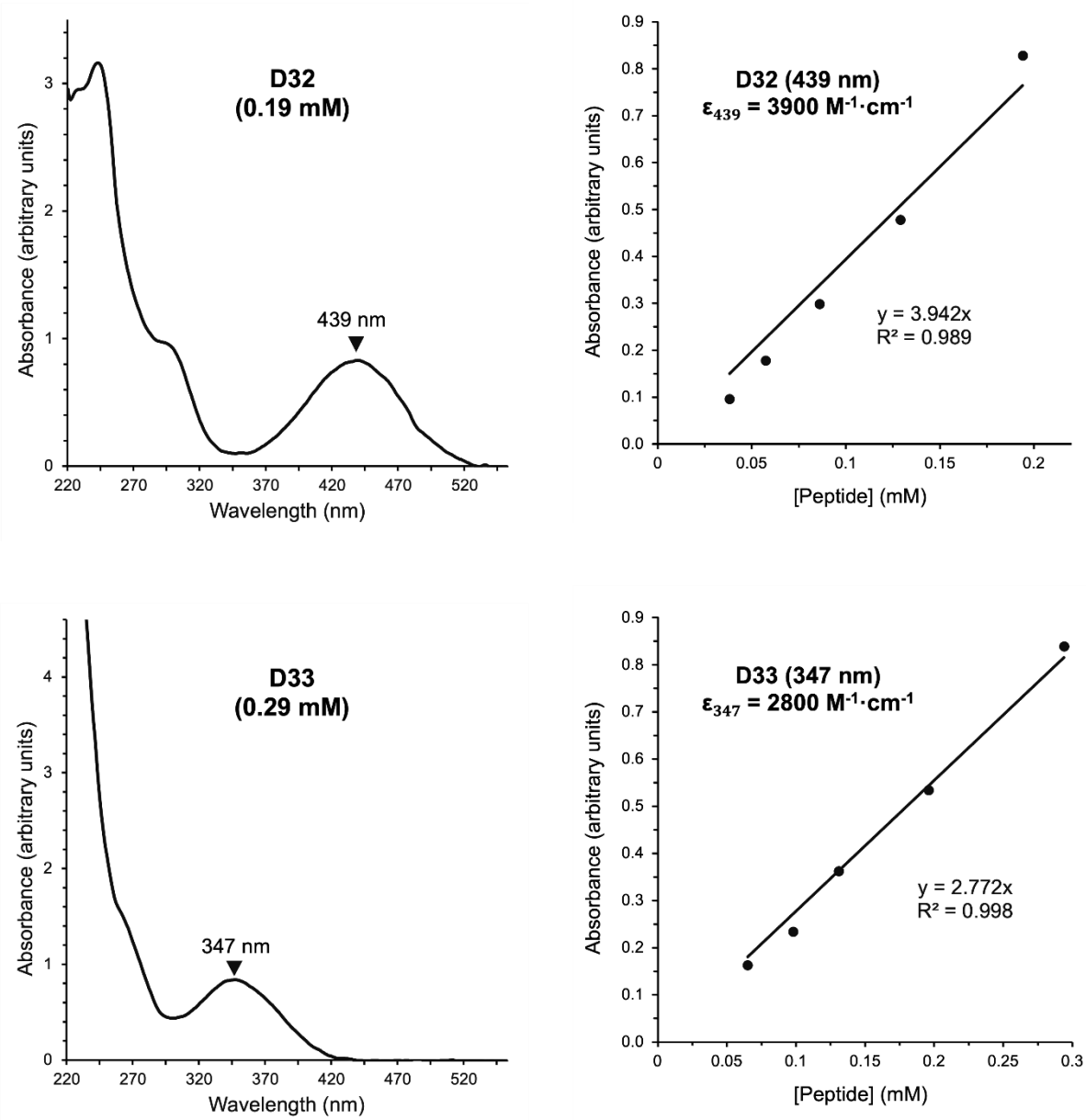

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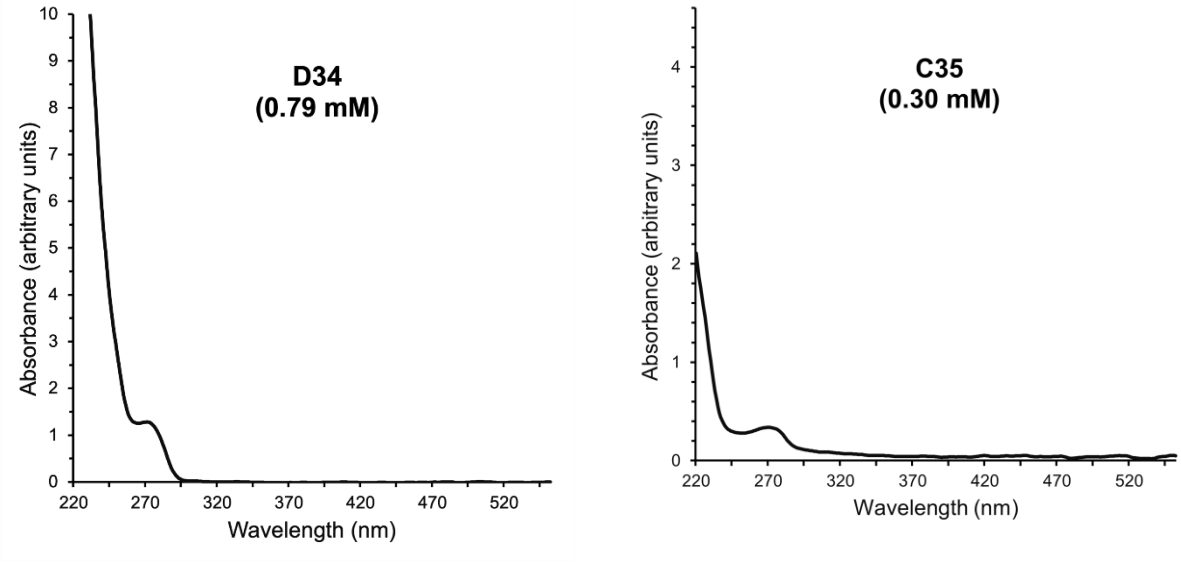

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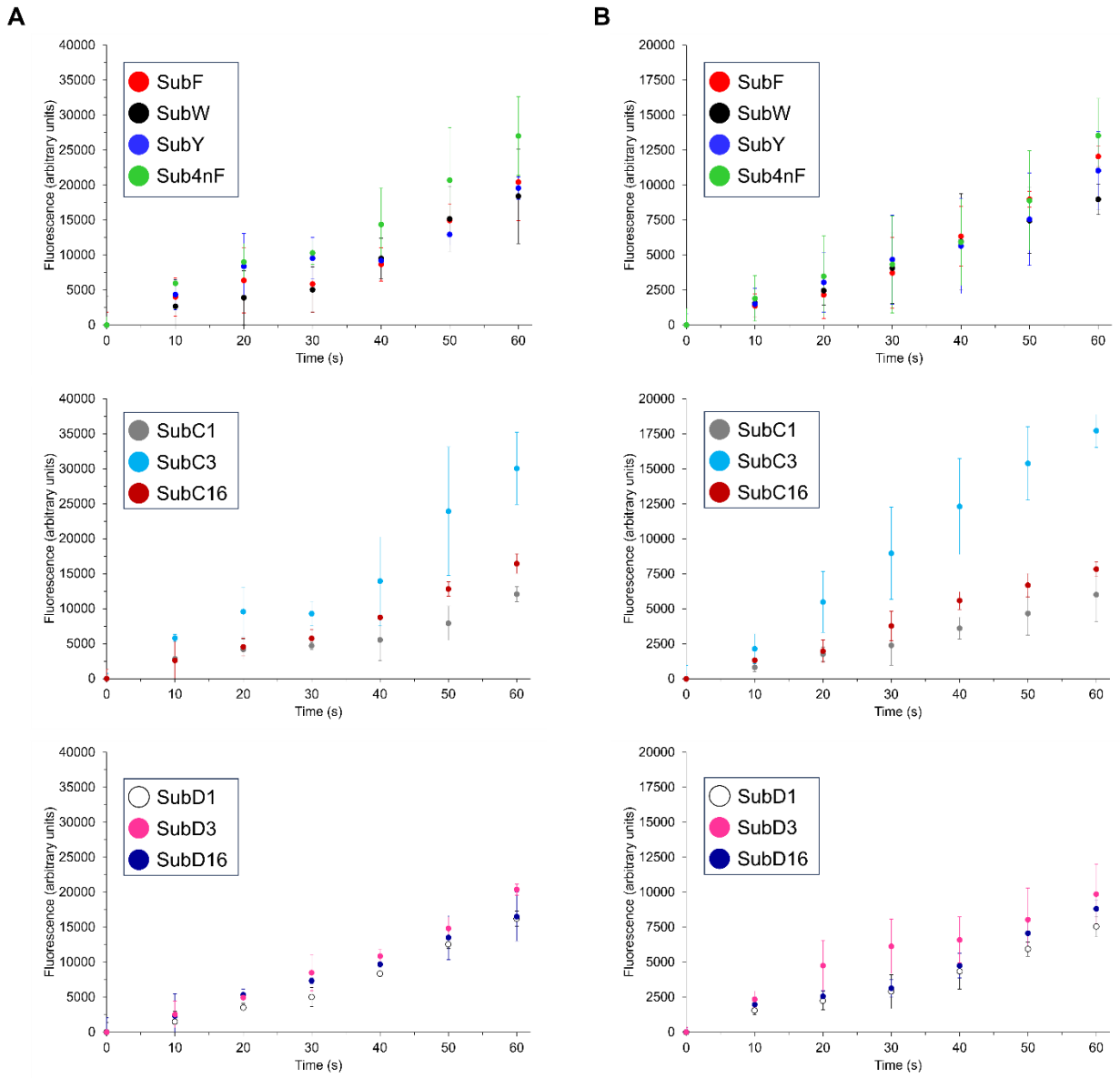


Figure S3. Plasmin assay data with model substrates. Fluorescence was monitored every 10 s for 1 min for samples containing 4.5 μM substrate (A) and 2.25 μM substrate (B) using 380 nm and 450 nm as the excitation and emission wavelengths, respectively. All substrates follow the sequence motif Ac-KQΦK-ACC-NH_2, where Ac = acetyl, NH_2 = amidated C-terminus, and ACC = 7-amino-4-carbamoylmethylcoumarin. The Φ residue for each substrate is as follows: SubF = phenylalanine, SubW = tryptophan, SubY = tyrosine, Sub4nF = 4-nitrophenylalanine, SubC1 = cysteine + **A1**, SubC3 = cysteine + **A3**, SubC16 = cysteine + **A16**, SubD1 = 2,3-diaminopropionic acid (Dap) + **A1**, SubD3 = Dap + **A3**, SubD16 = Dap + **A16** (see Tables S1 and S2 for additional information on **A1**, **A3**, and **A16**).

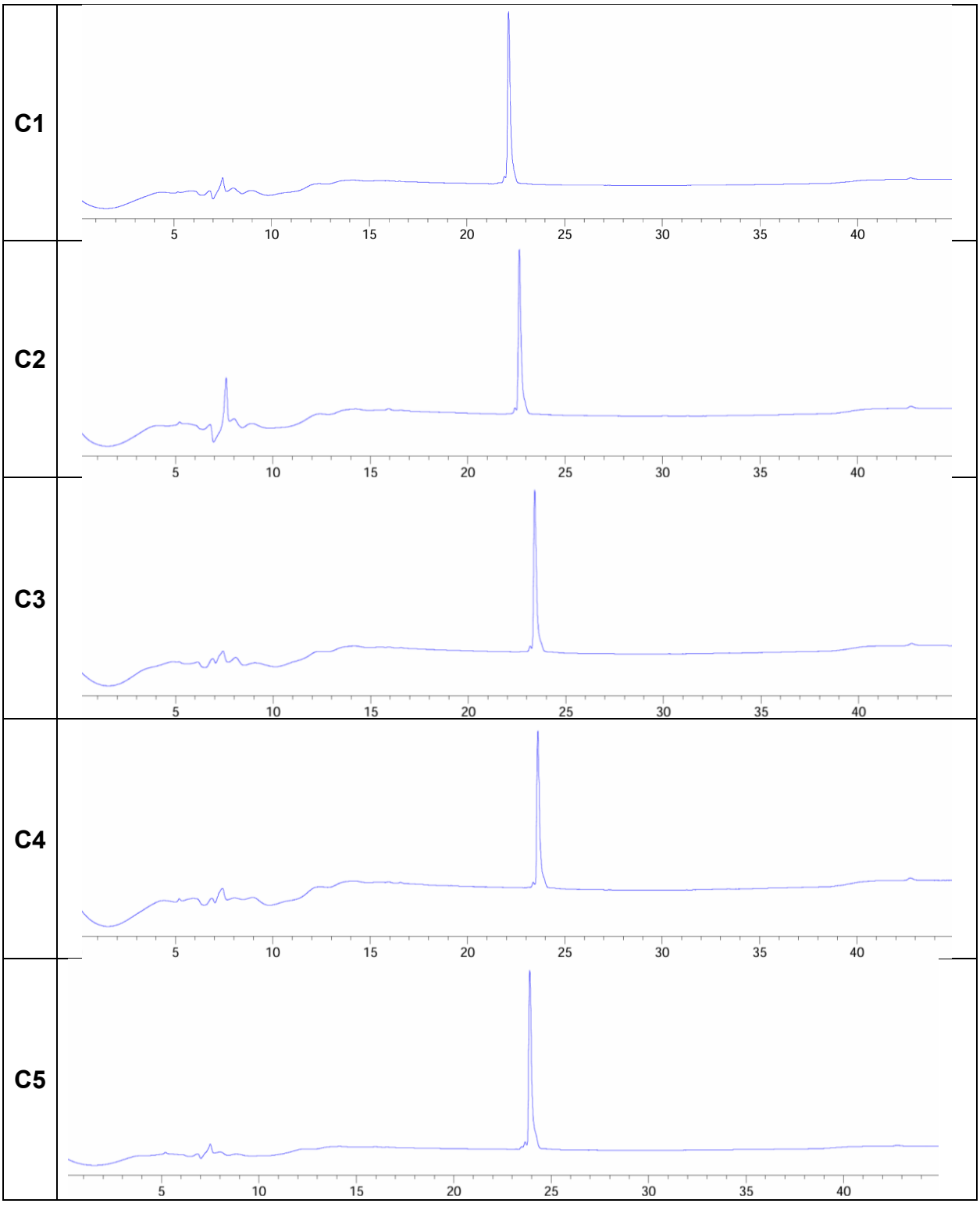


Figure S4. Analytical HPLC chromatograms for all peptides

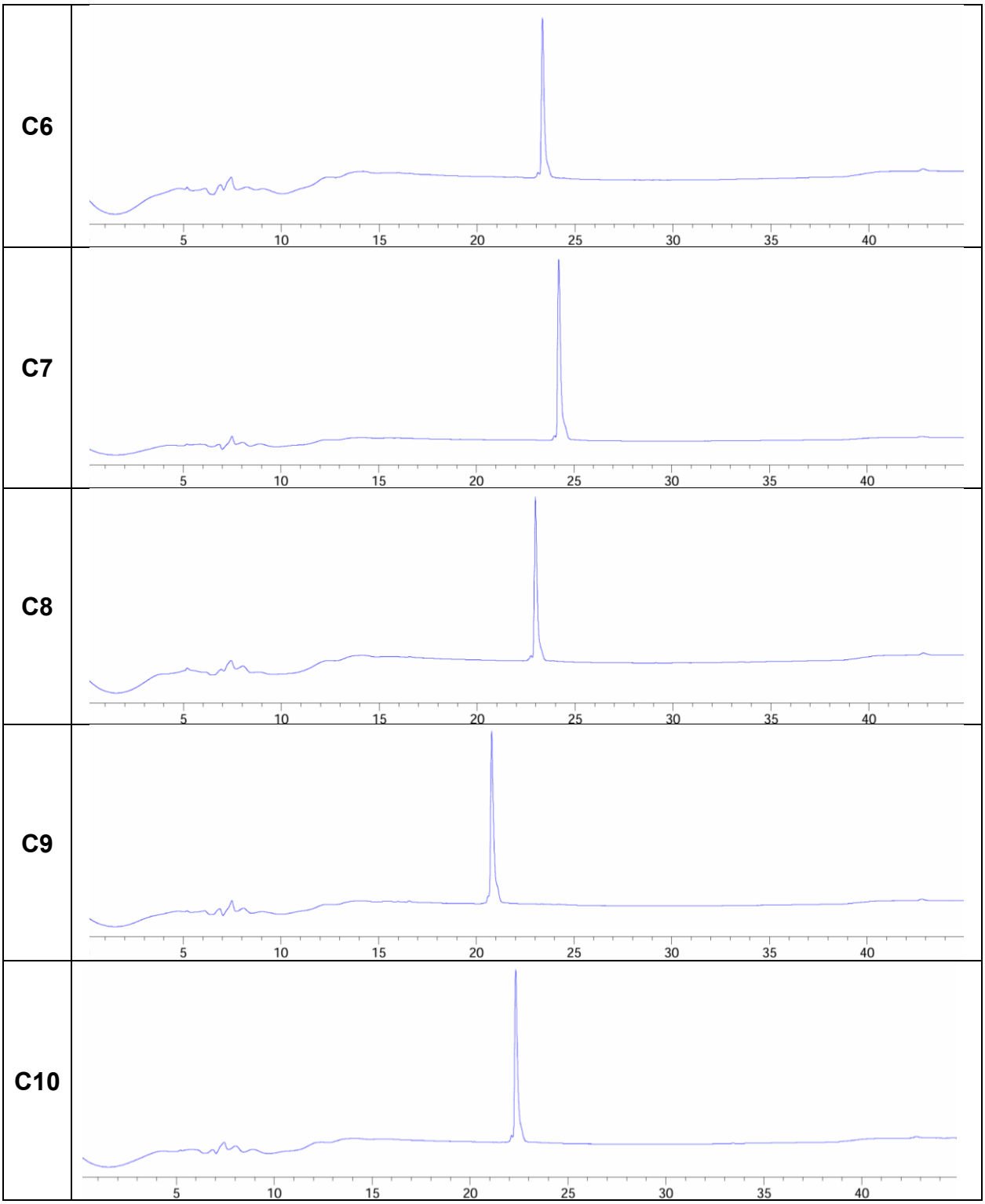


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

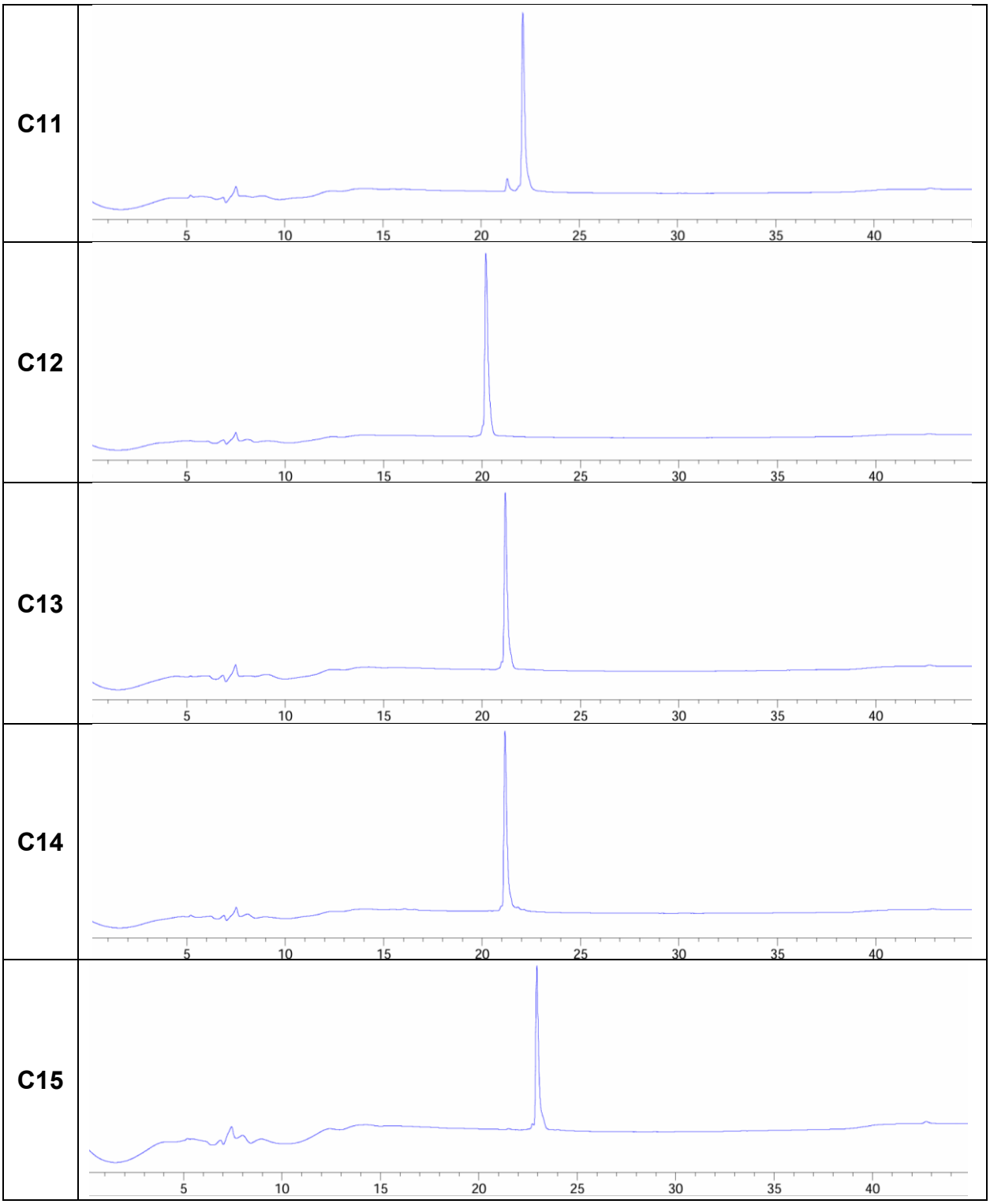
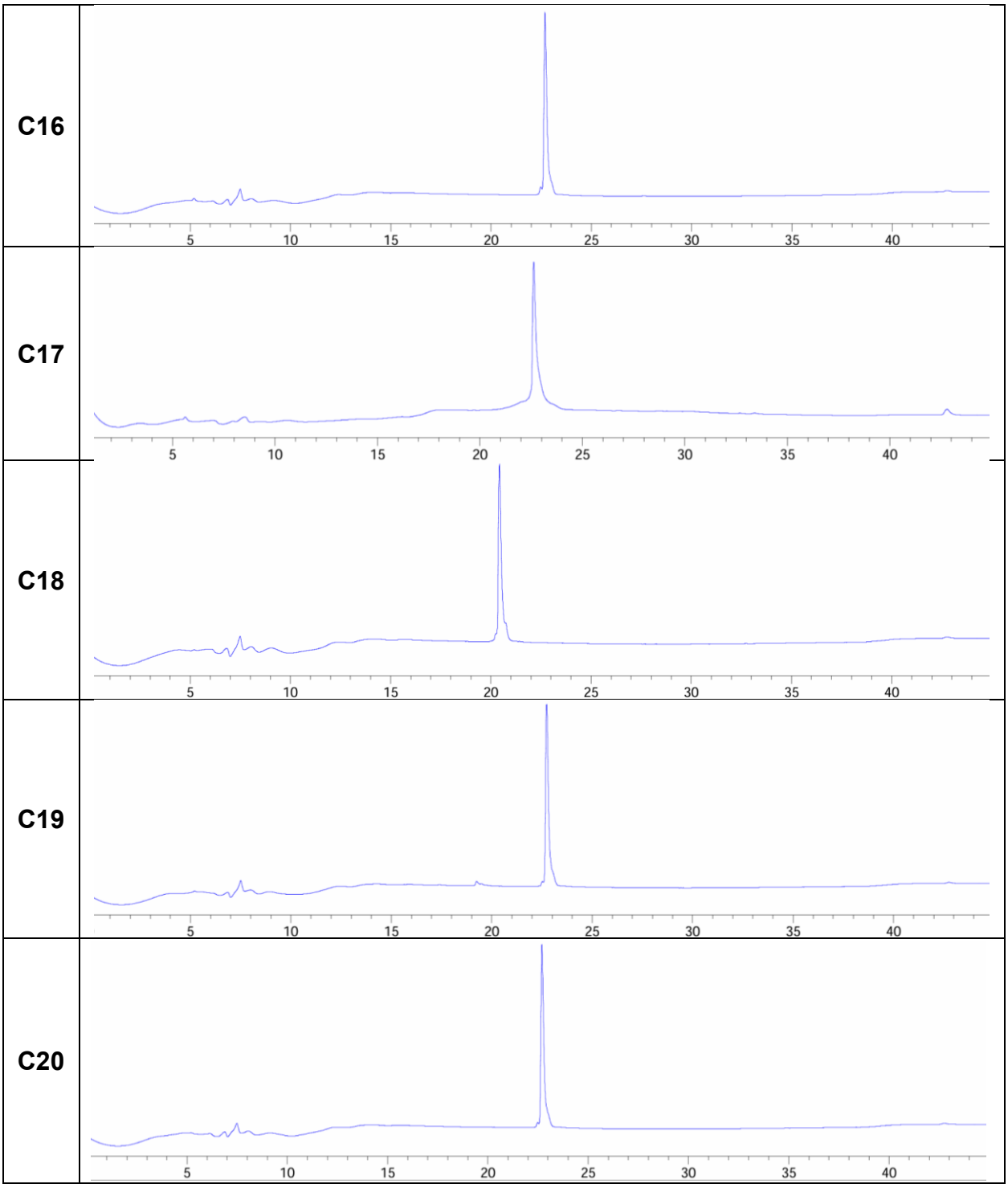


Figure S4. Analytical HPLC chromatograms for all peptides (continued)



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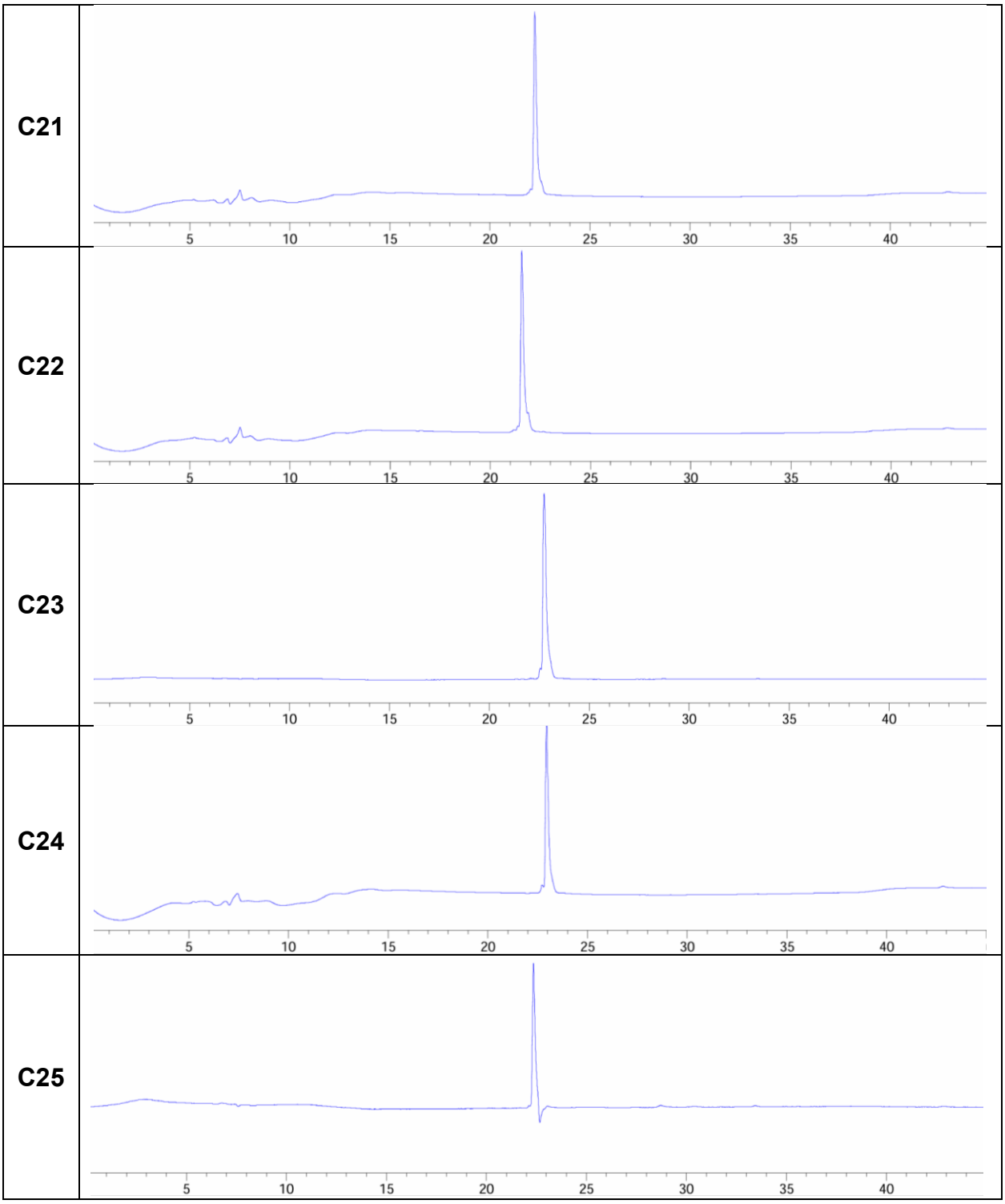
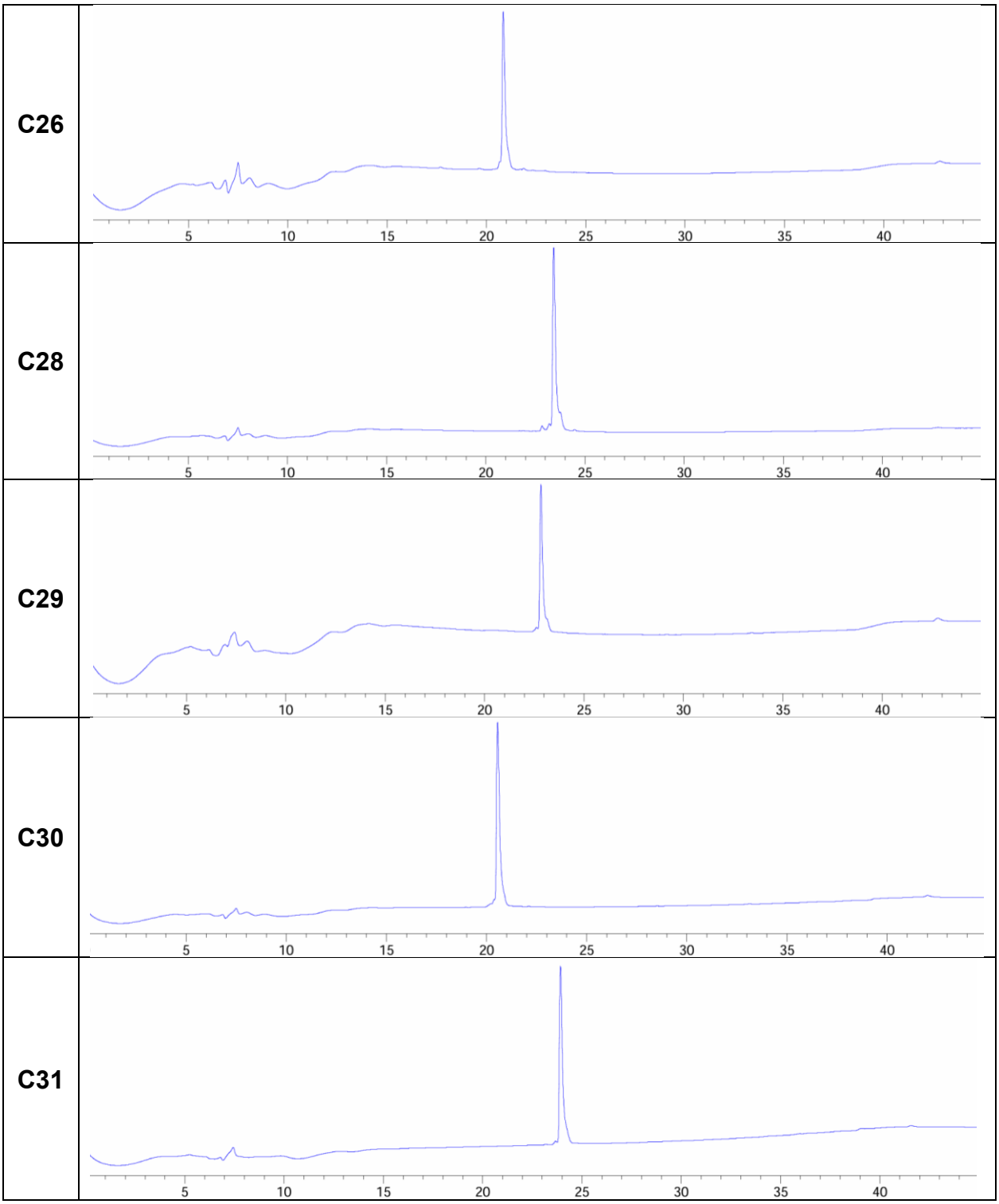


Figure S4. Analytical HPLC chromatograms for all peptides (continued)



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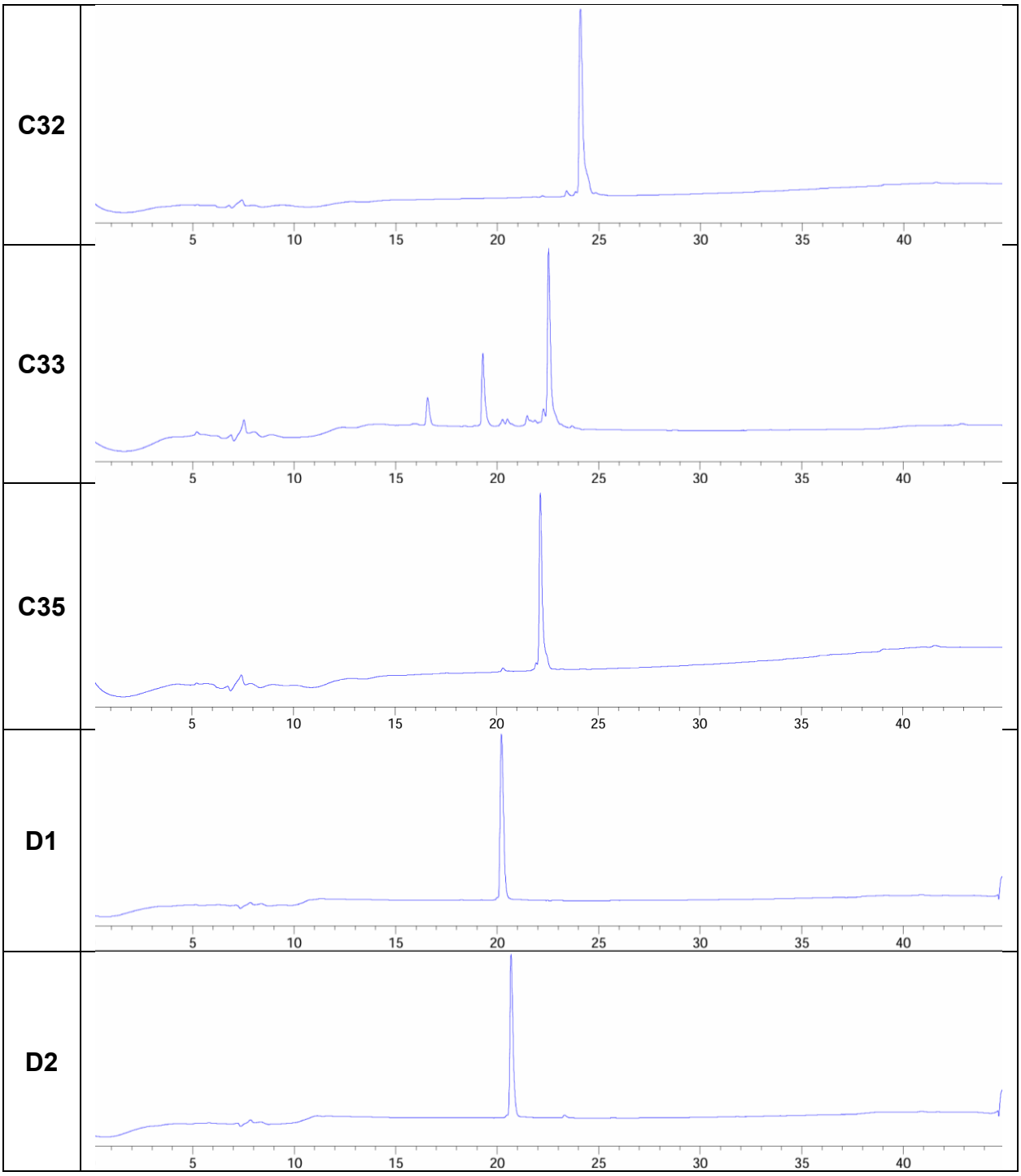

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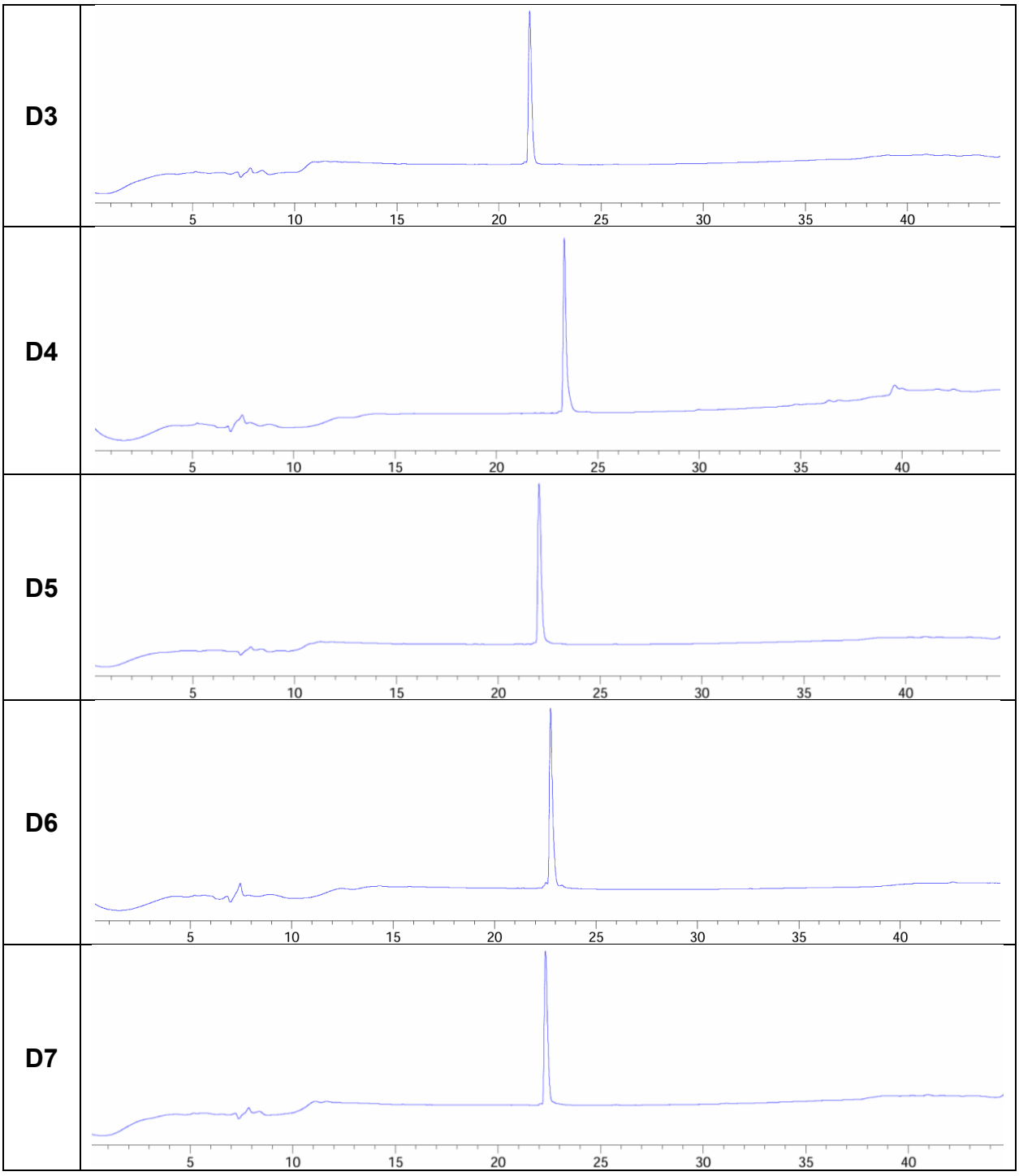

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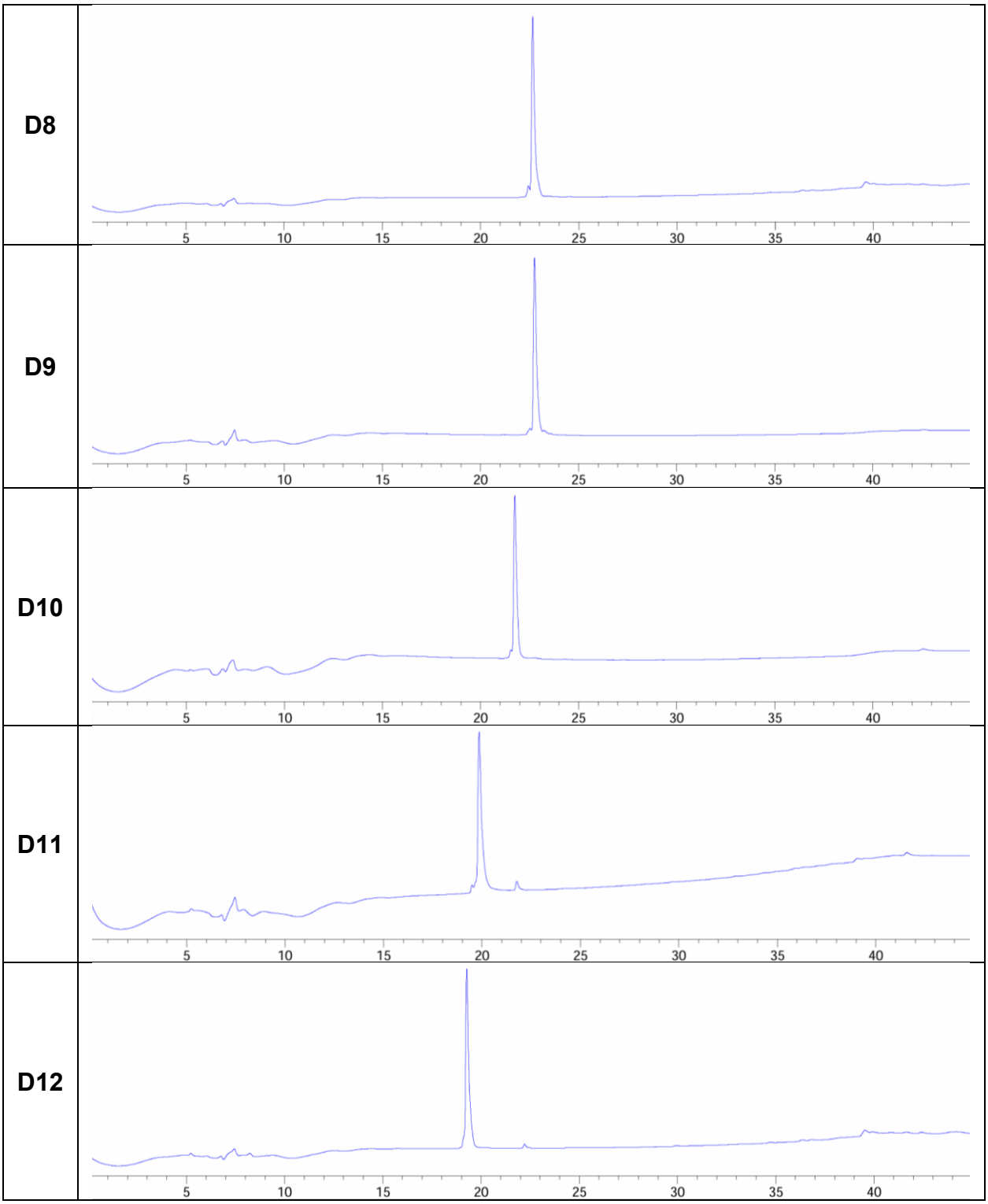
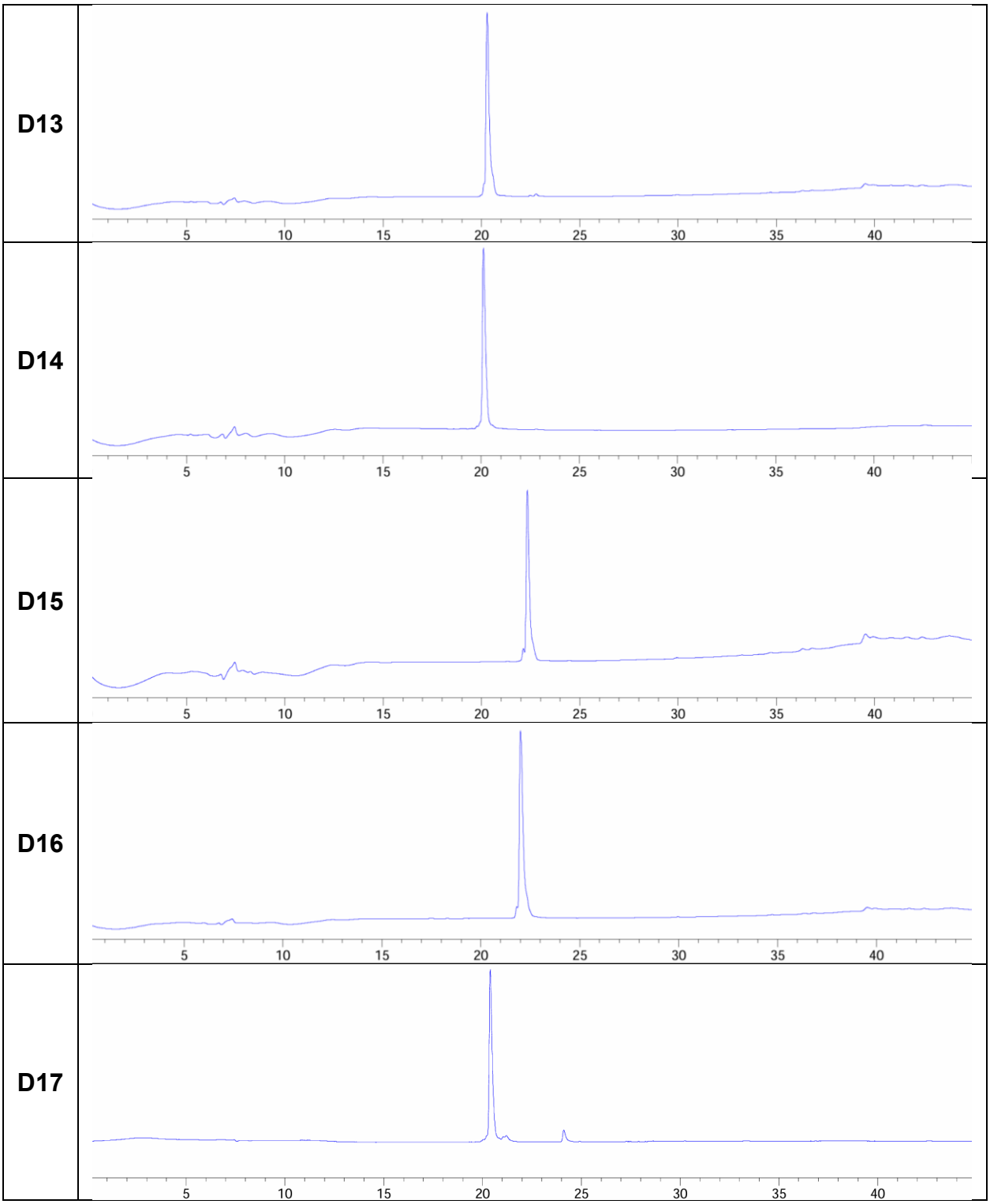


Figure S4. Analytical HPLC chromatograms for all peptides (continued)



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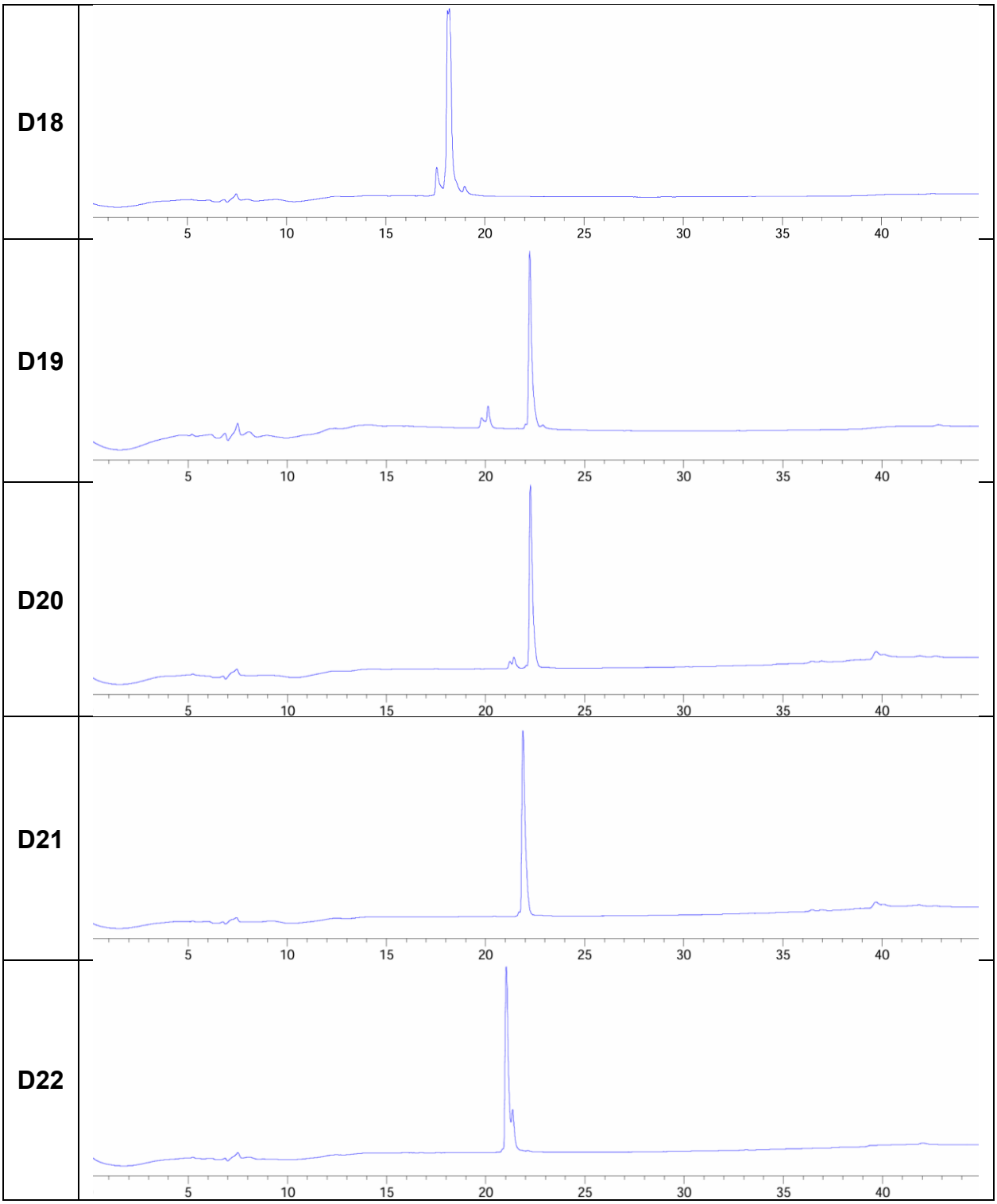

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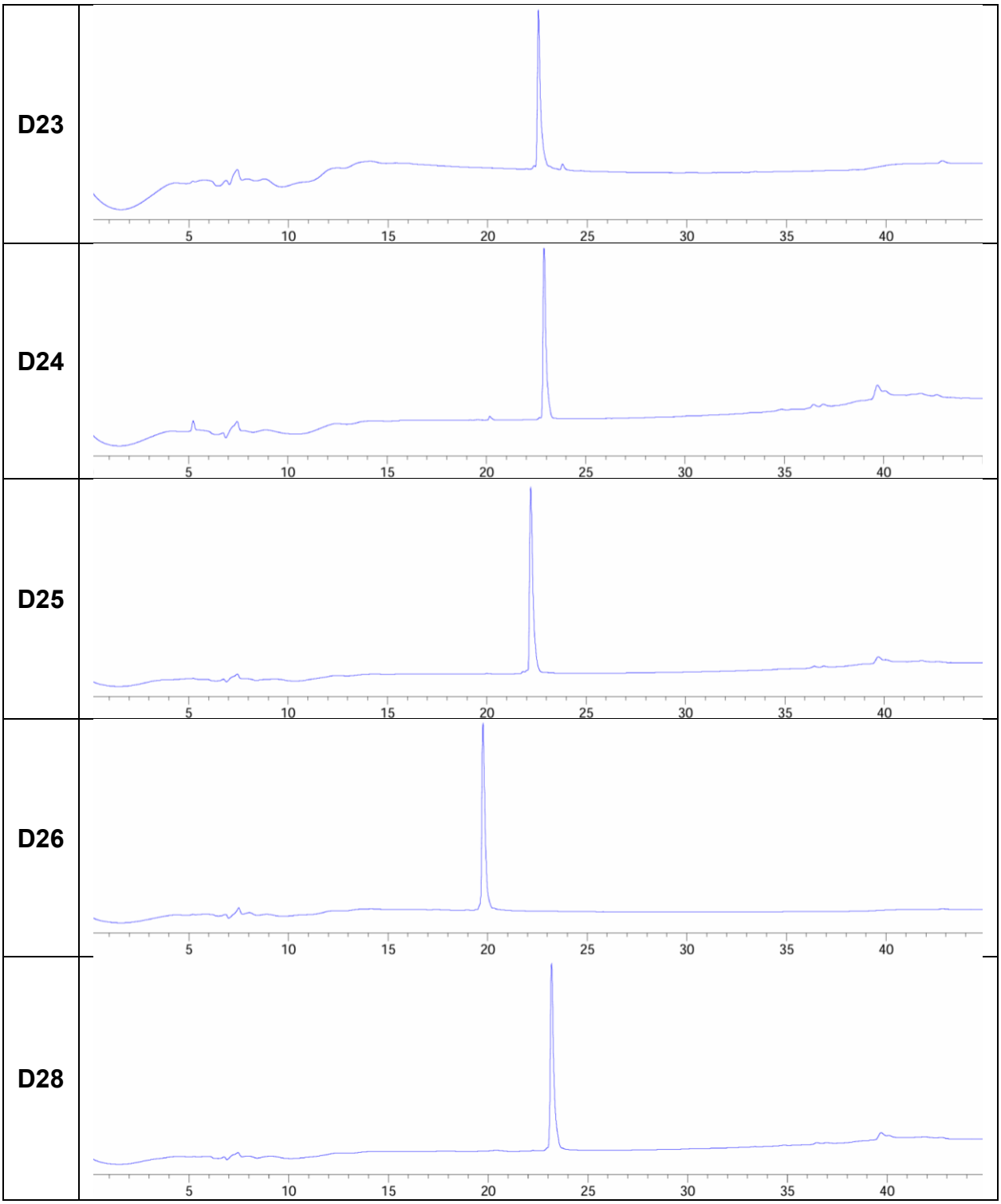

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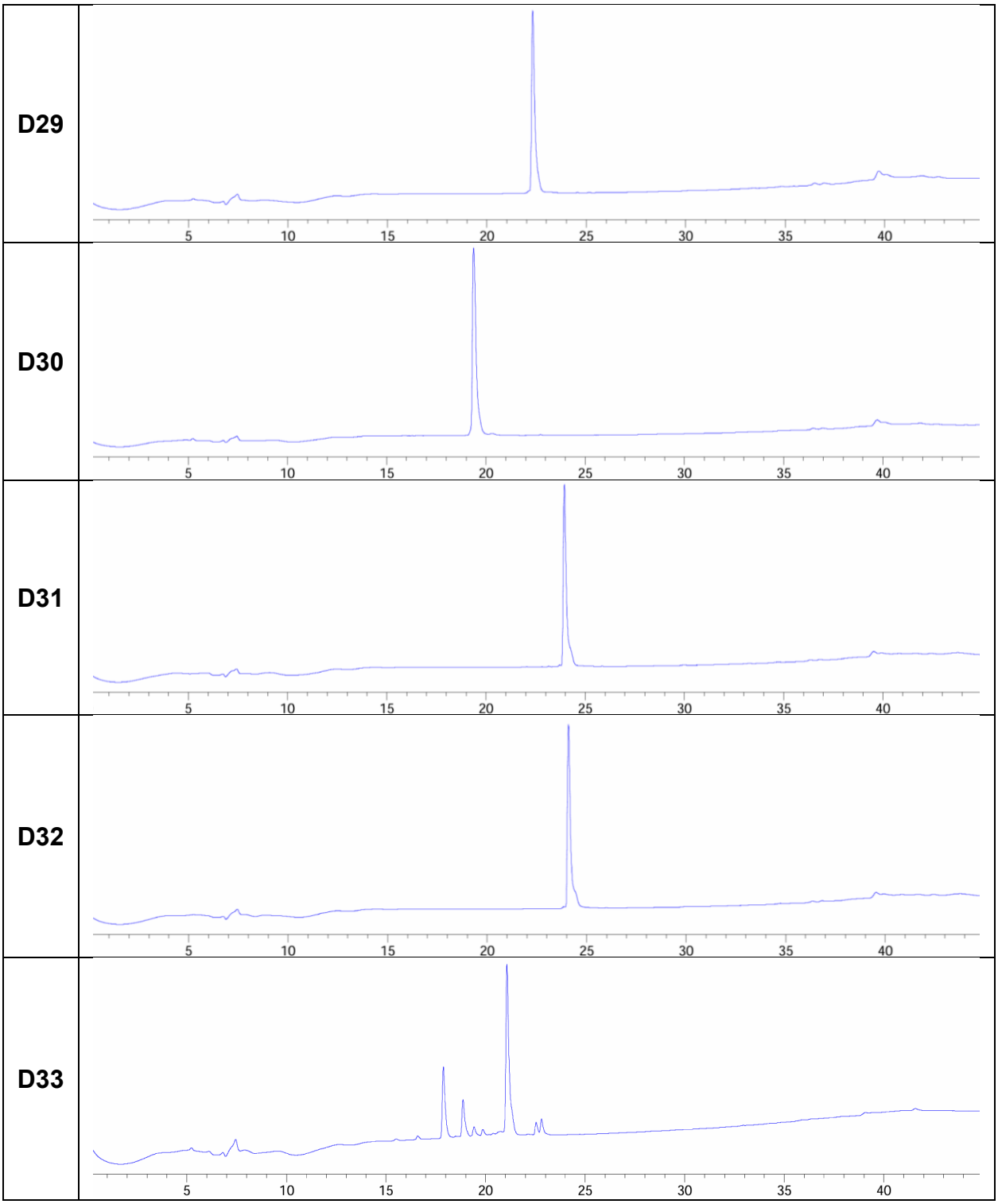

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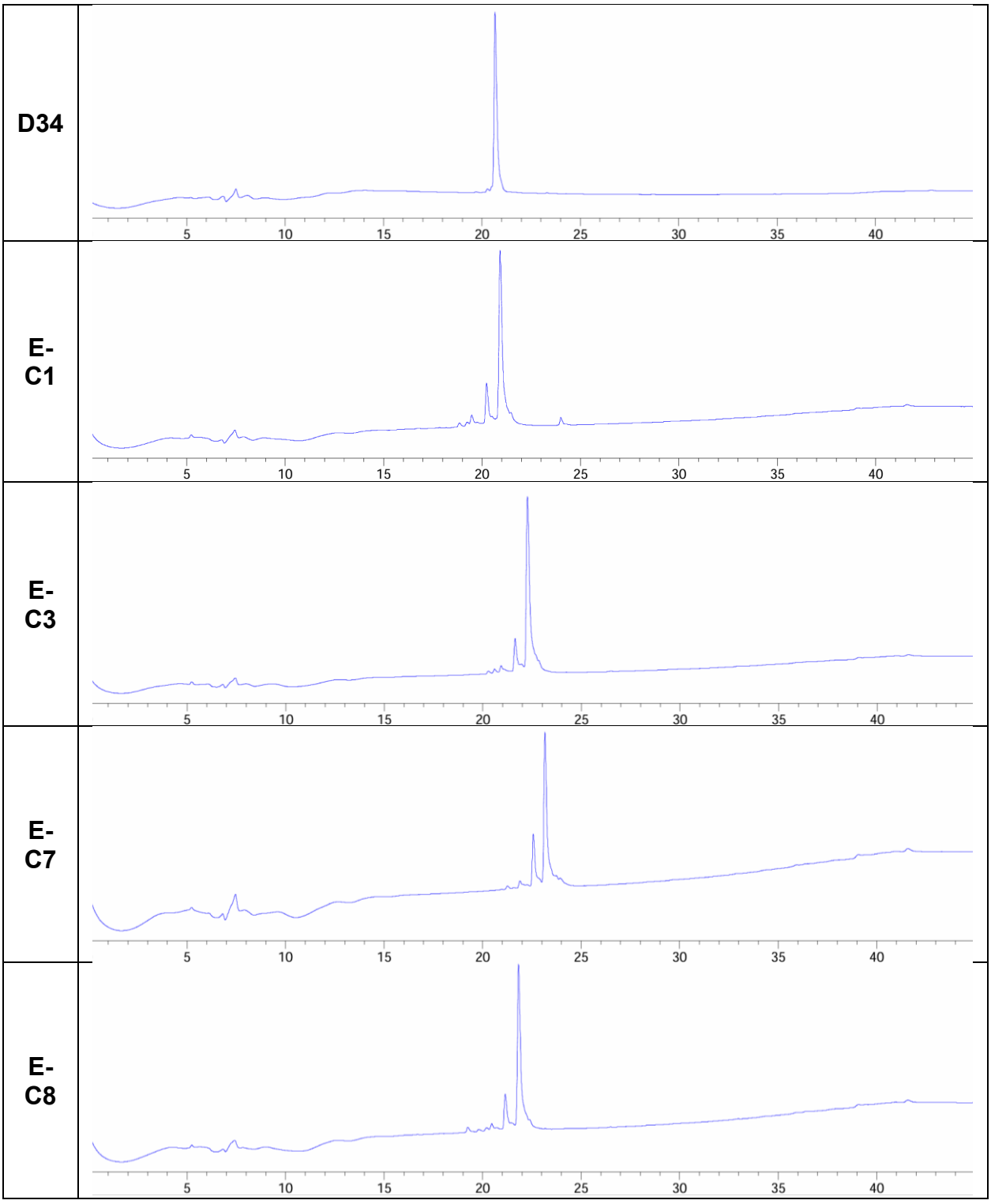


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

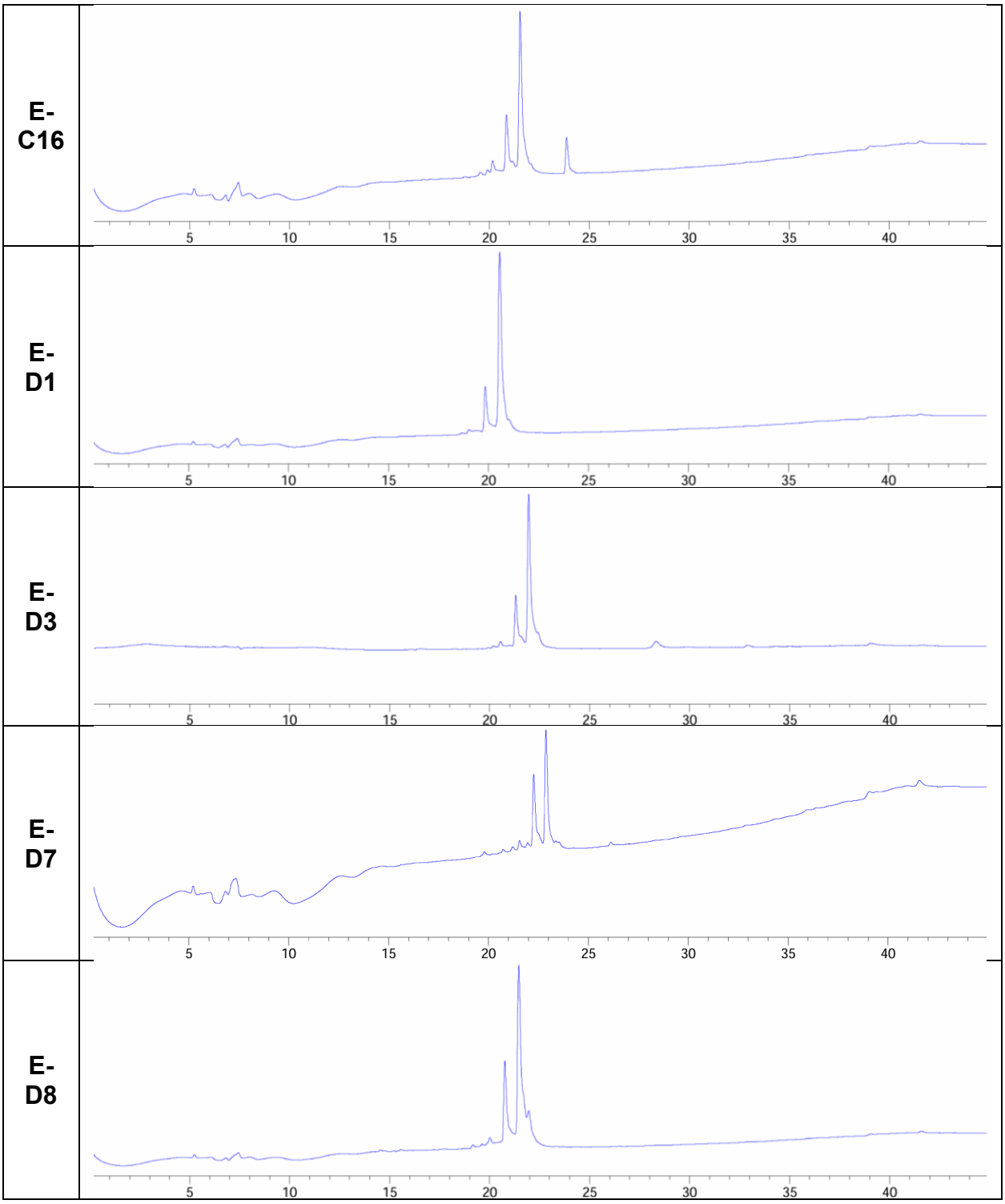


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

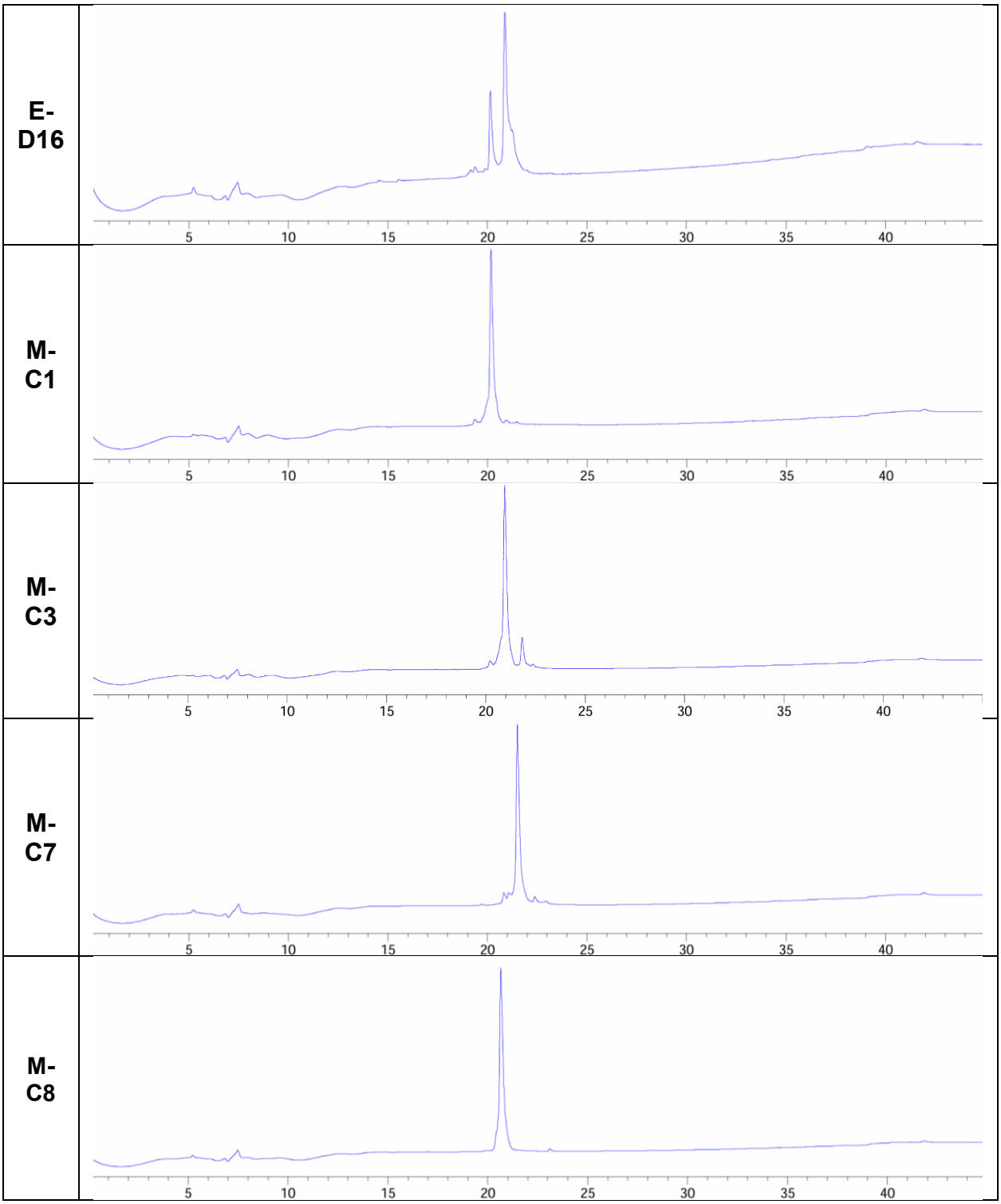


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

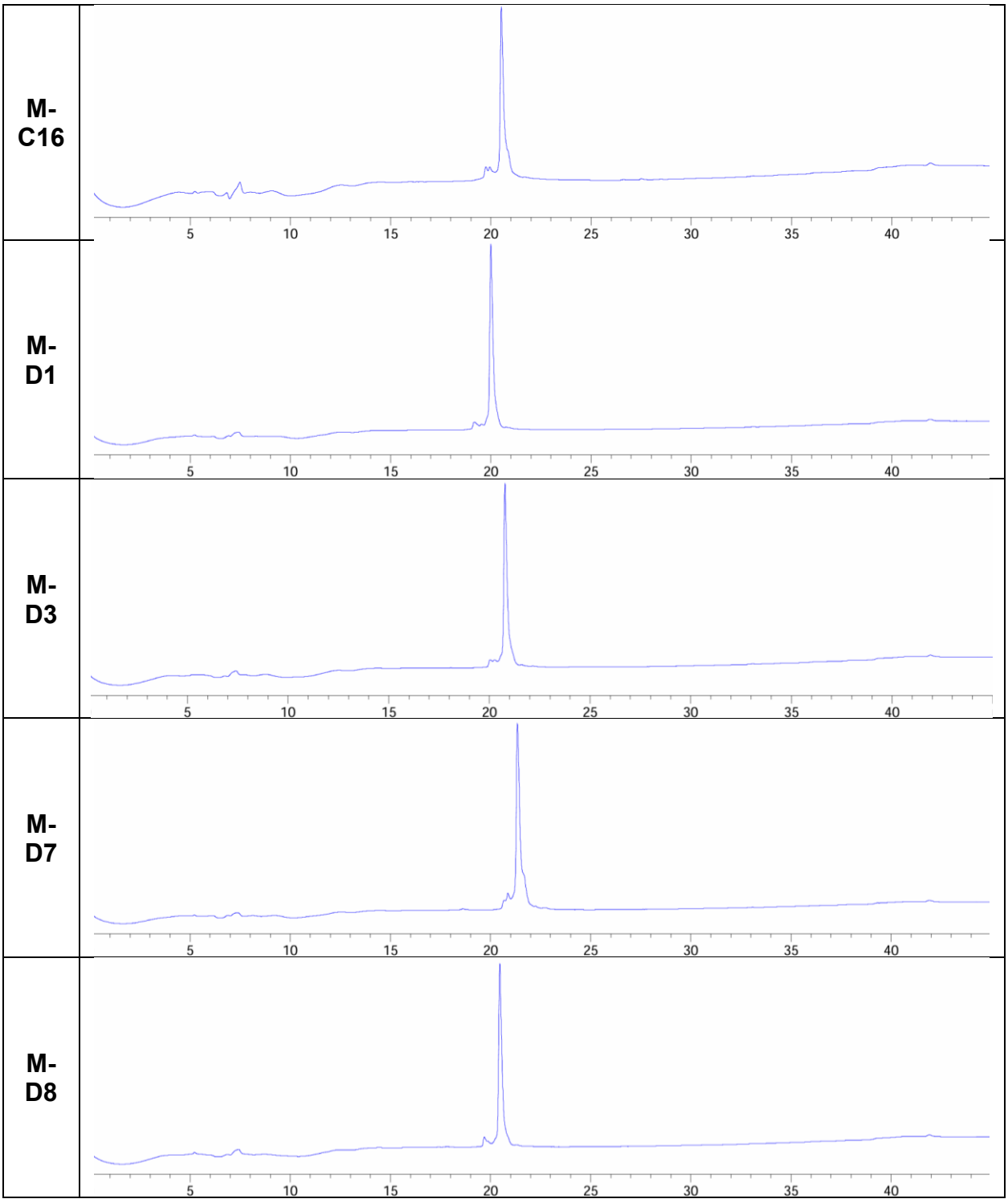


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

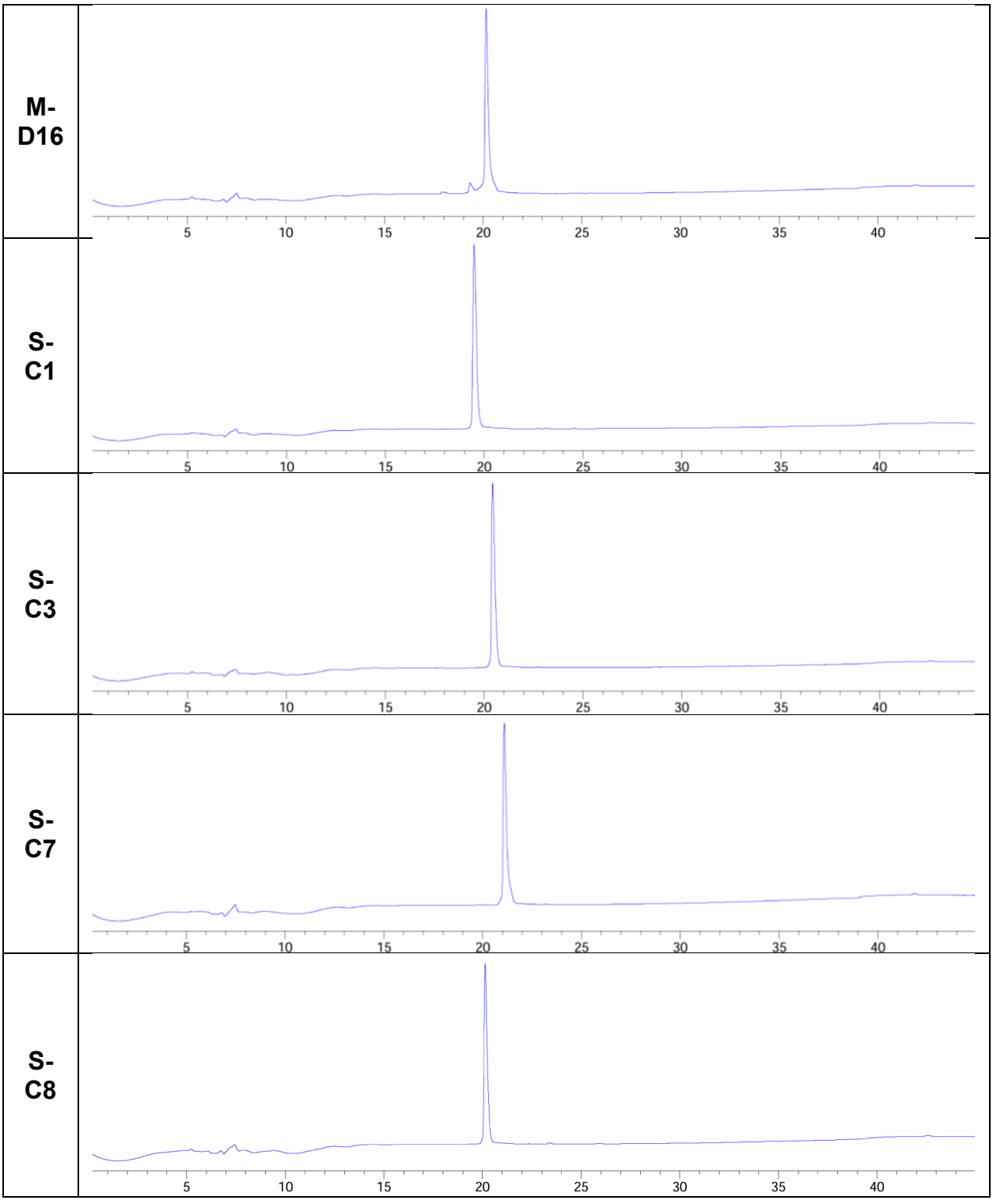


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

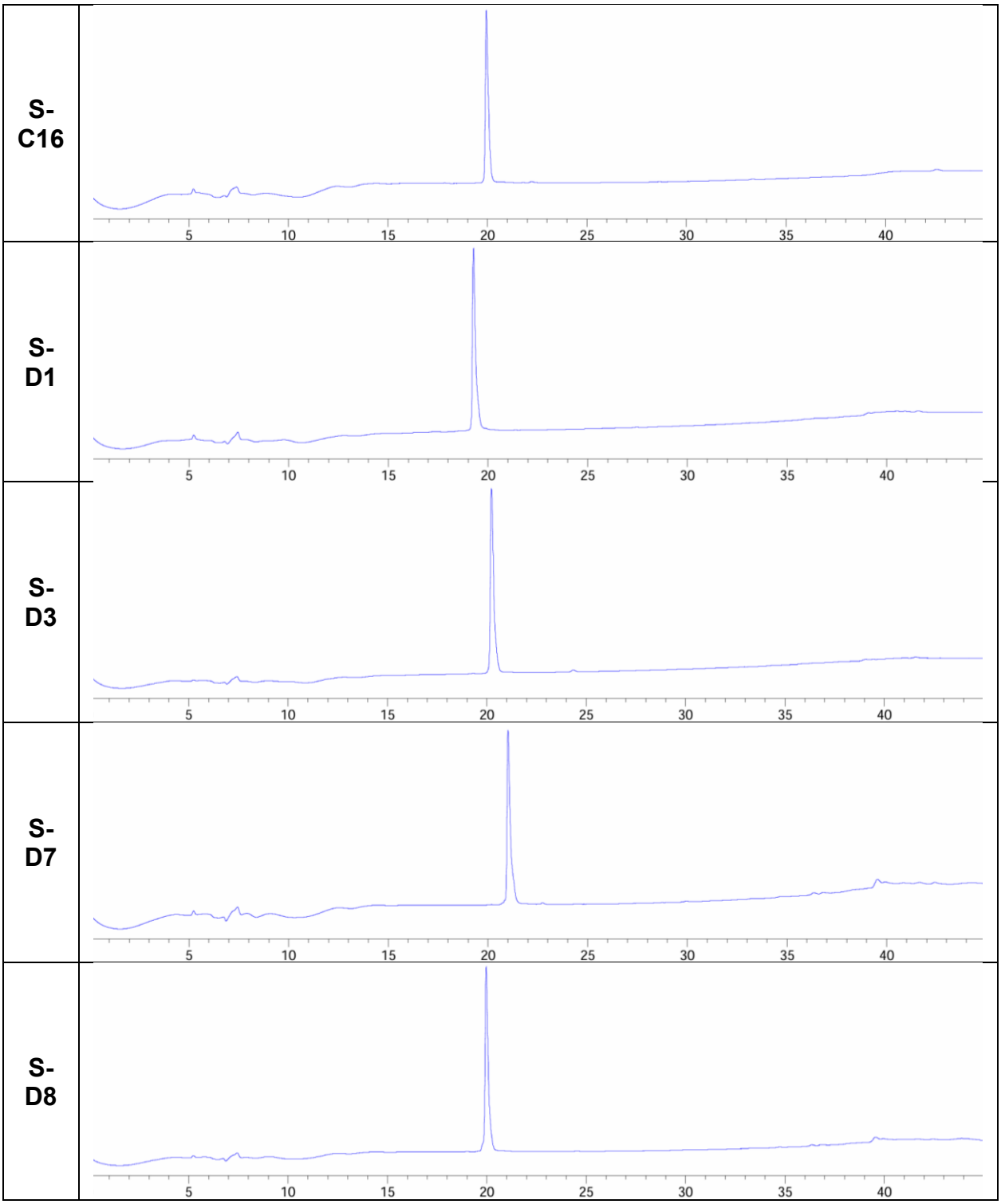


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

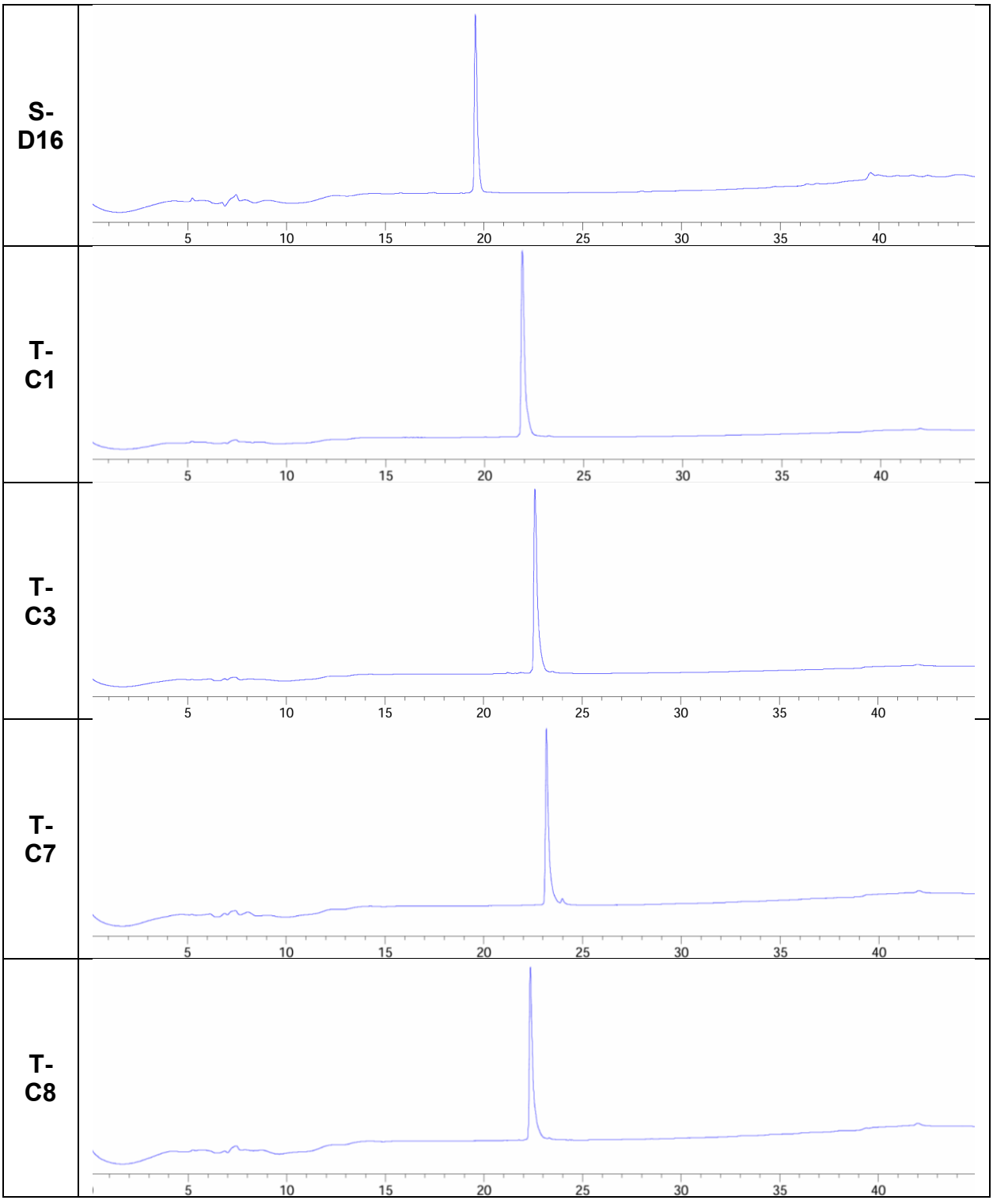


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

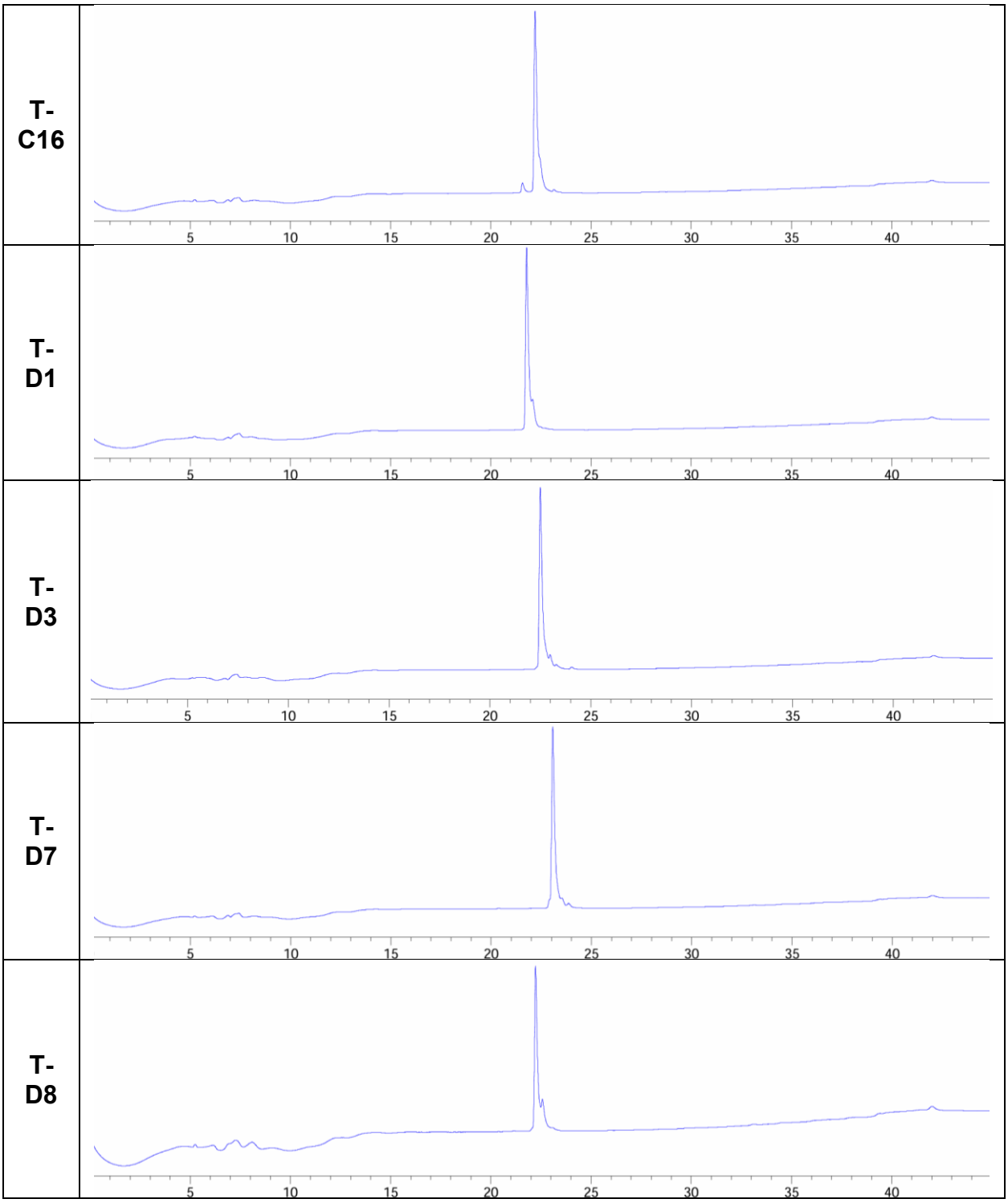


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

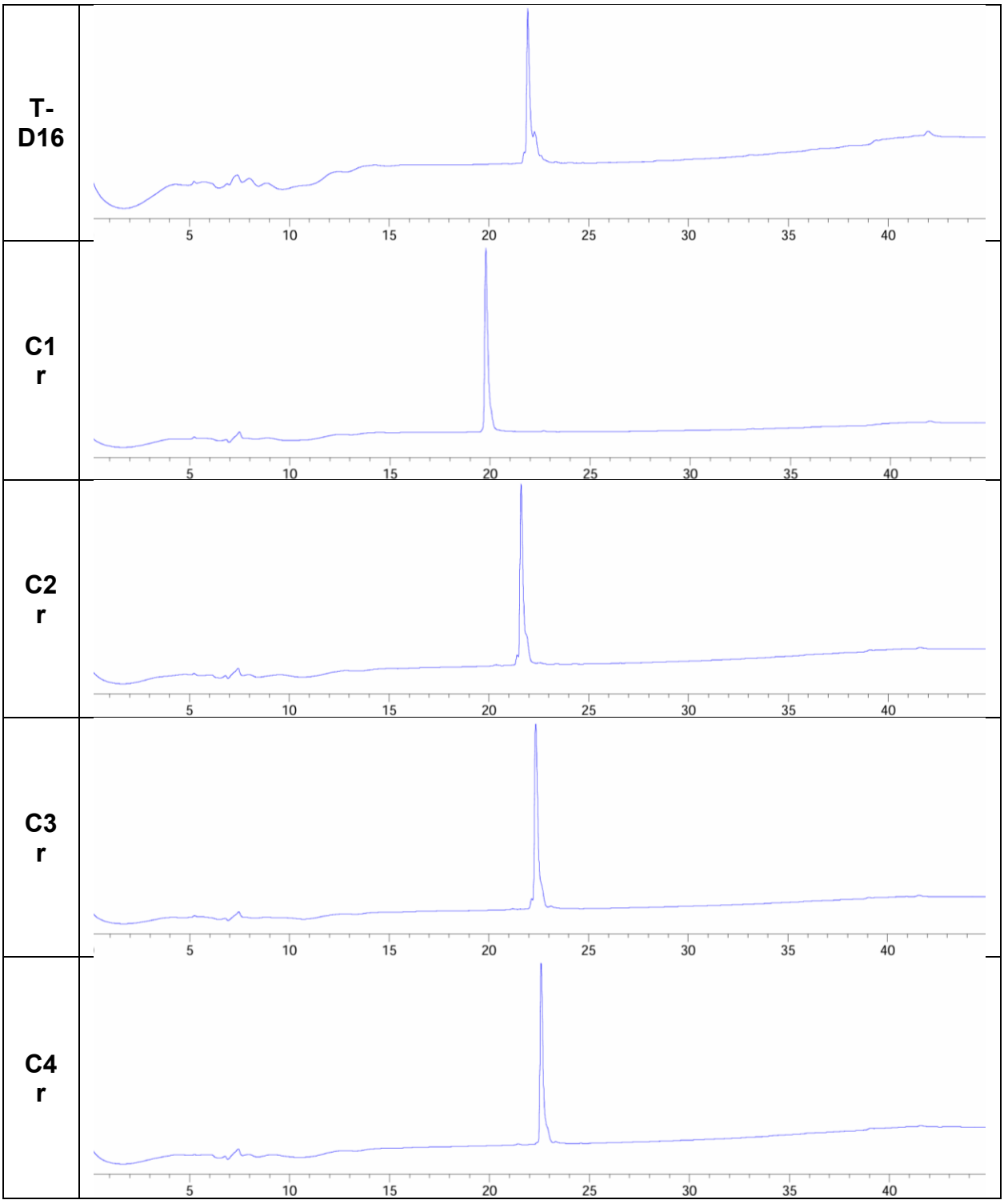


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

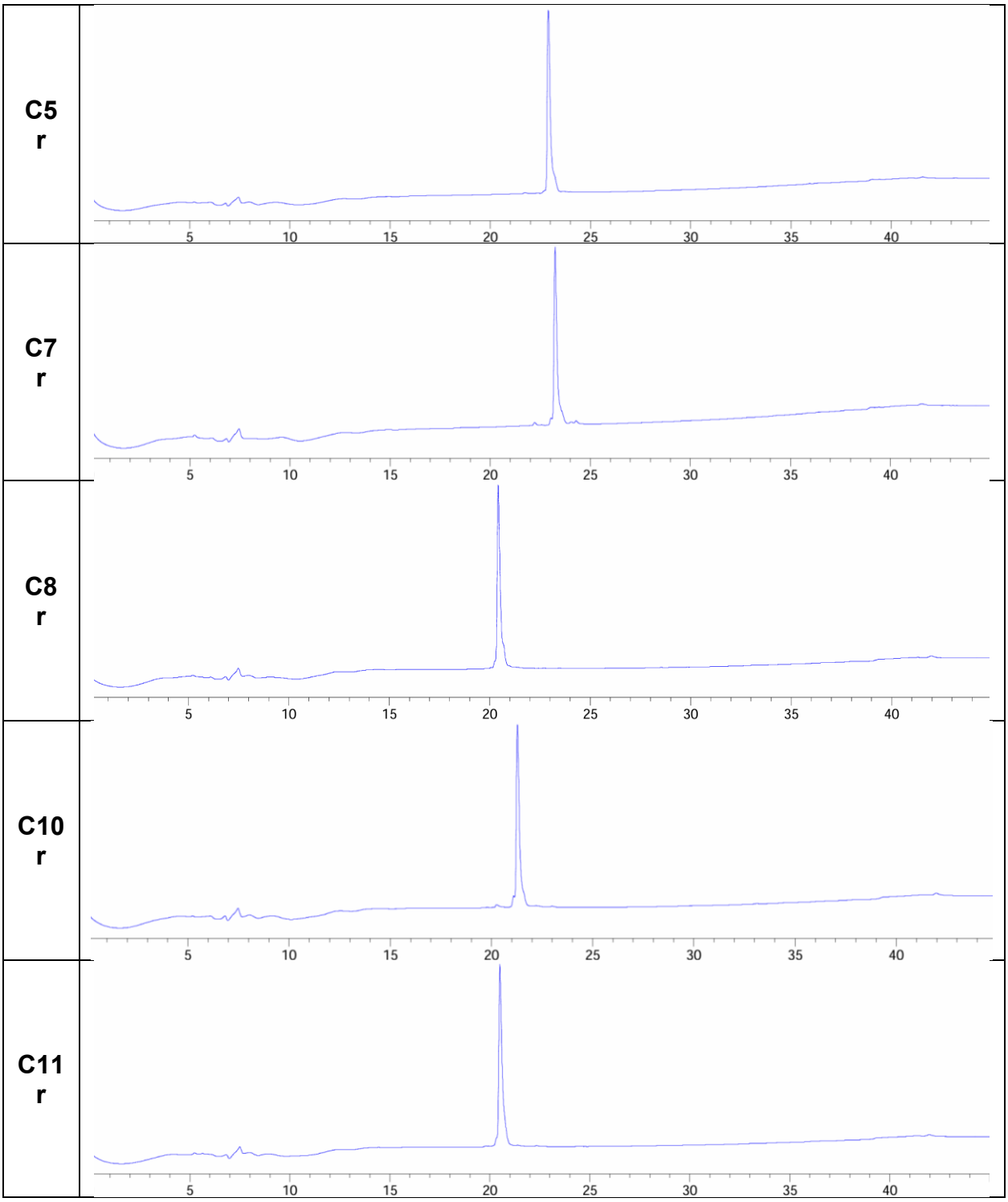


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

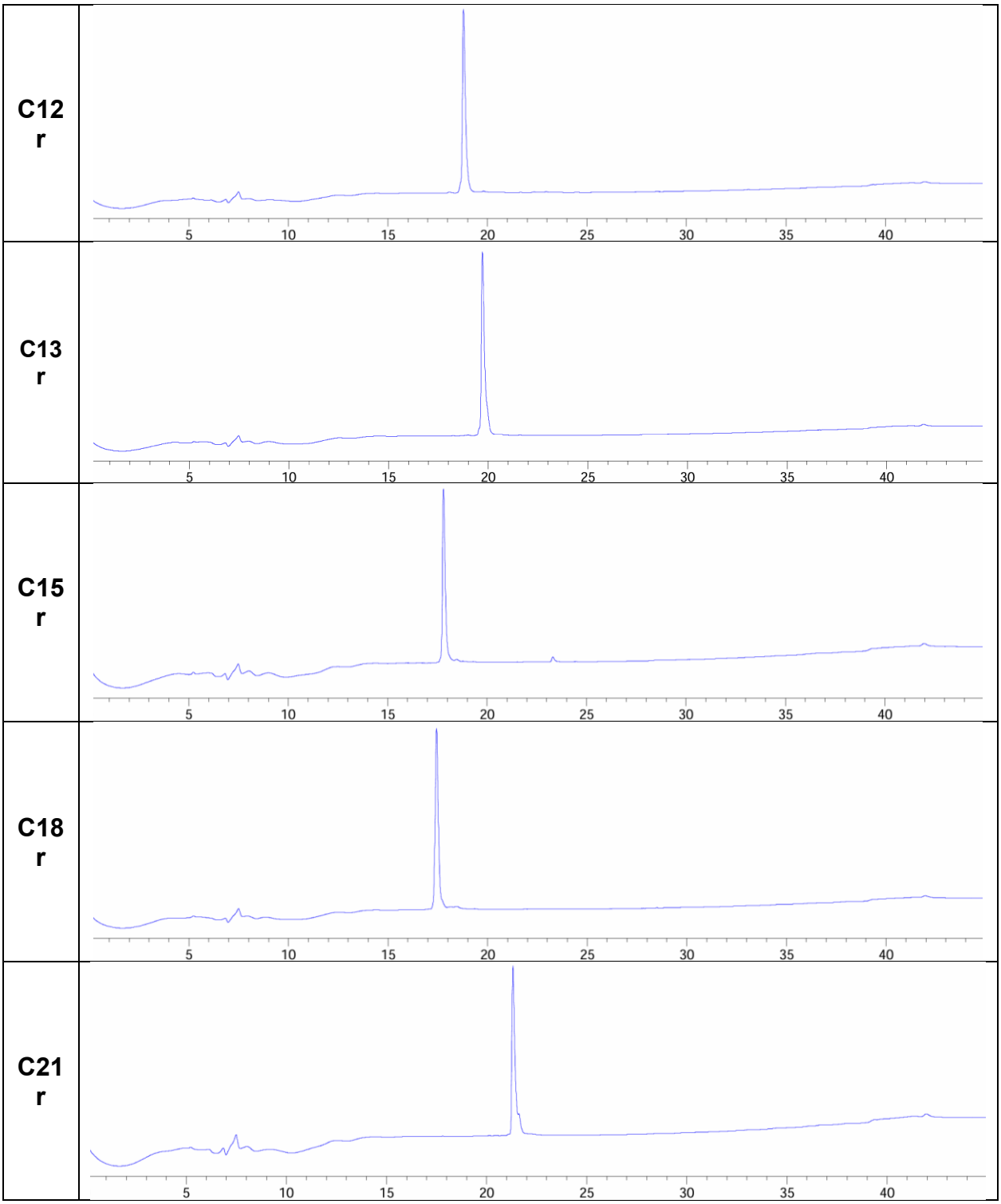


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

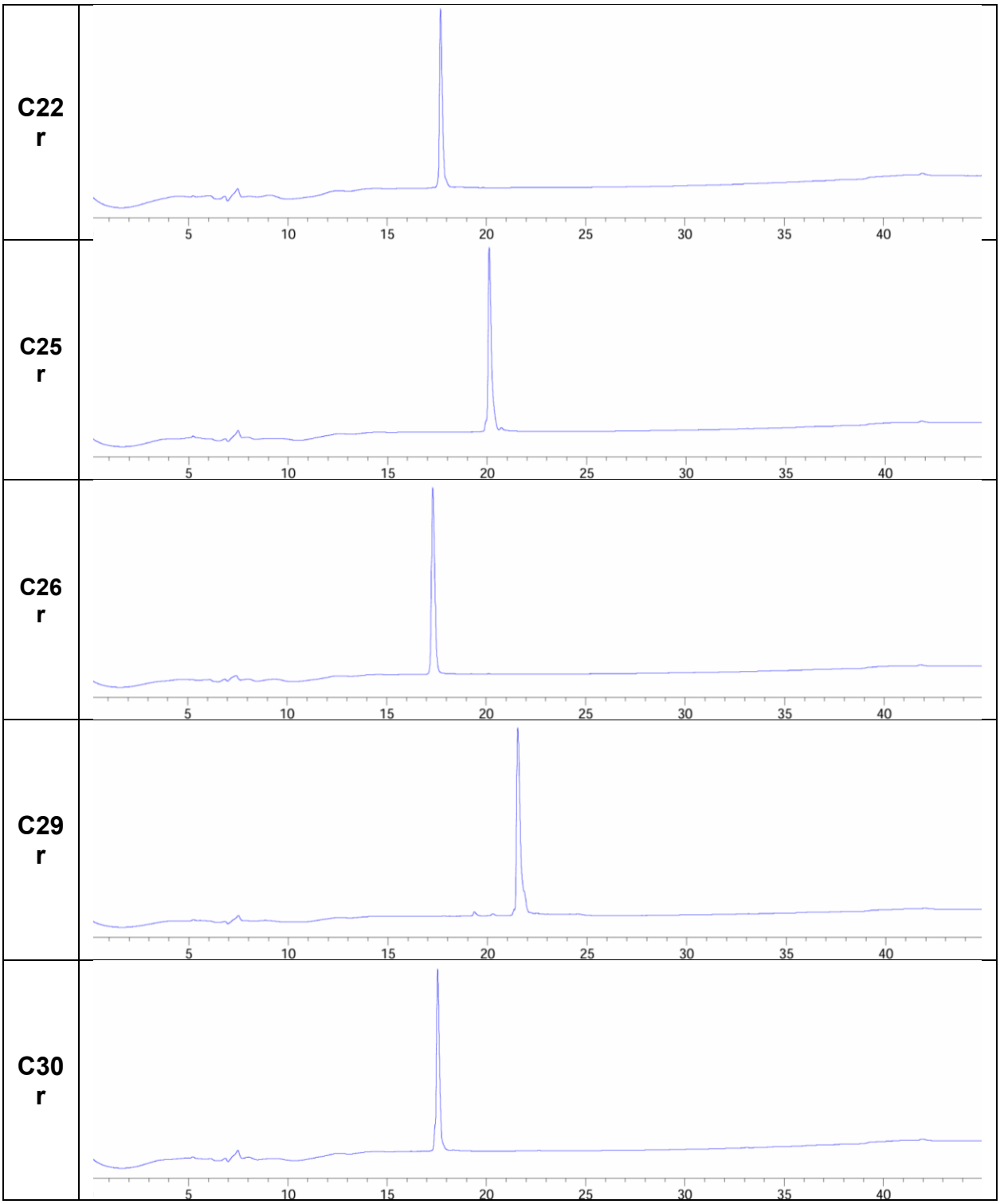


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

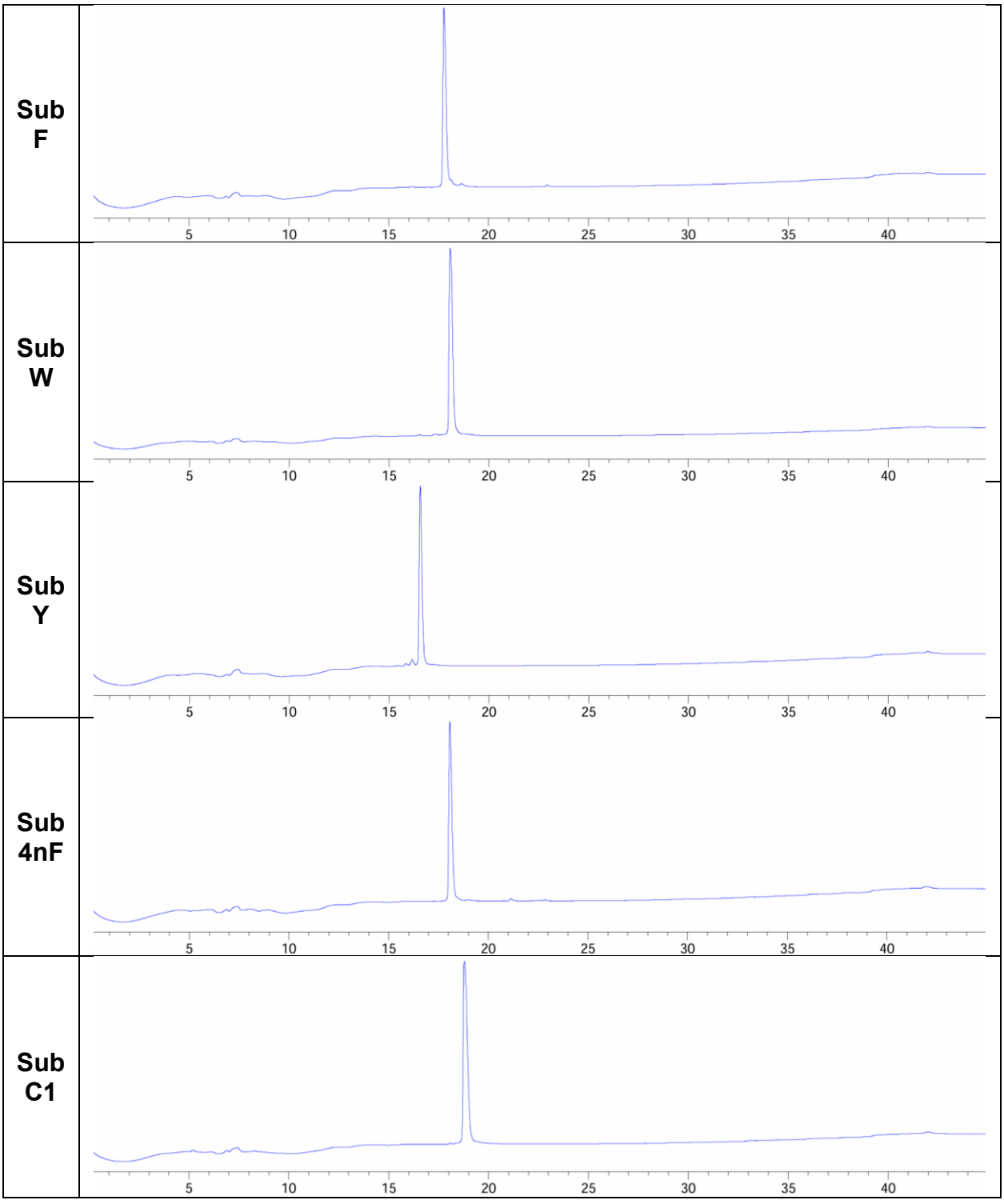


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

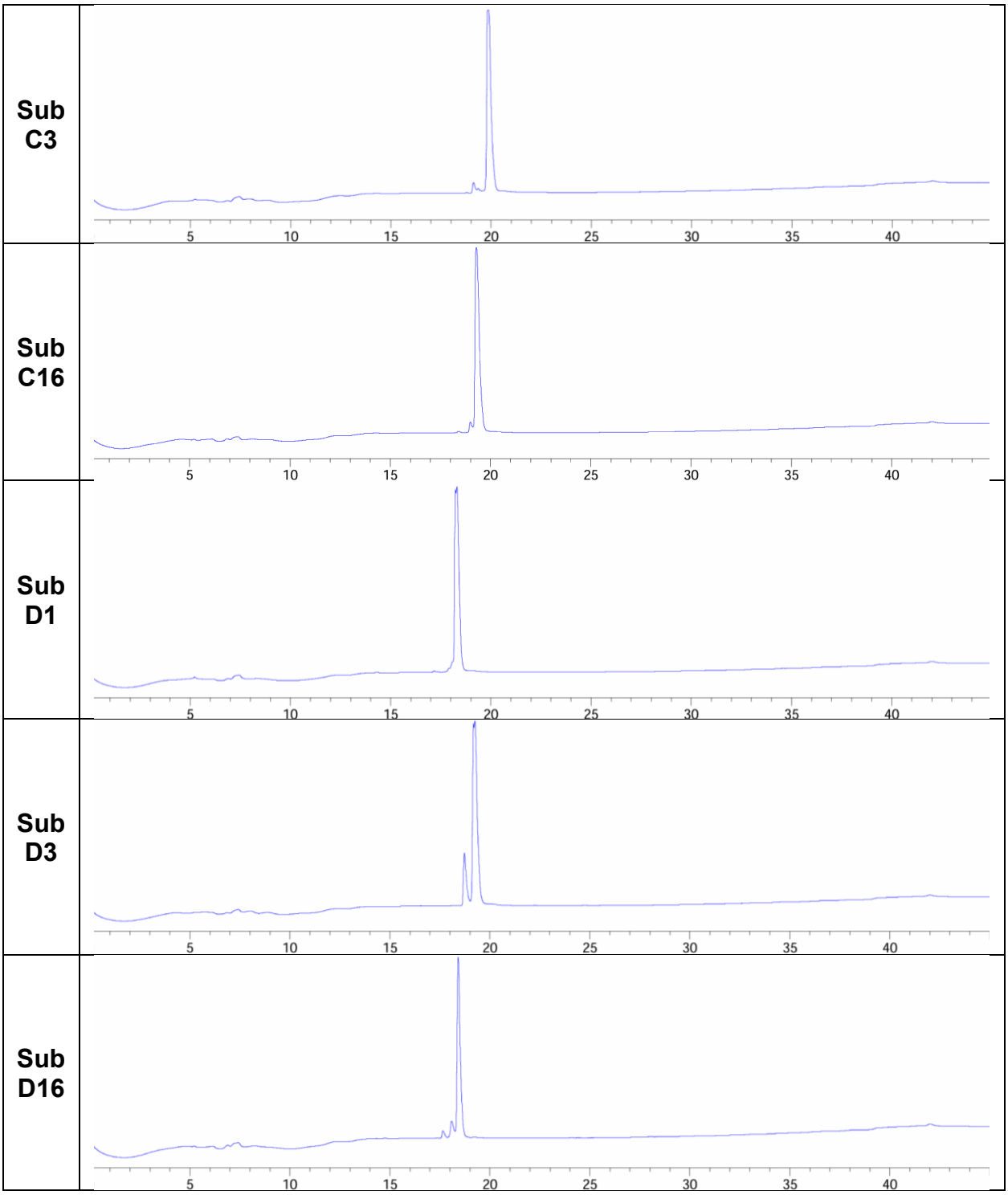
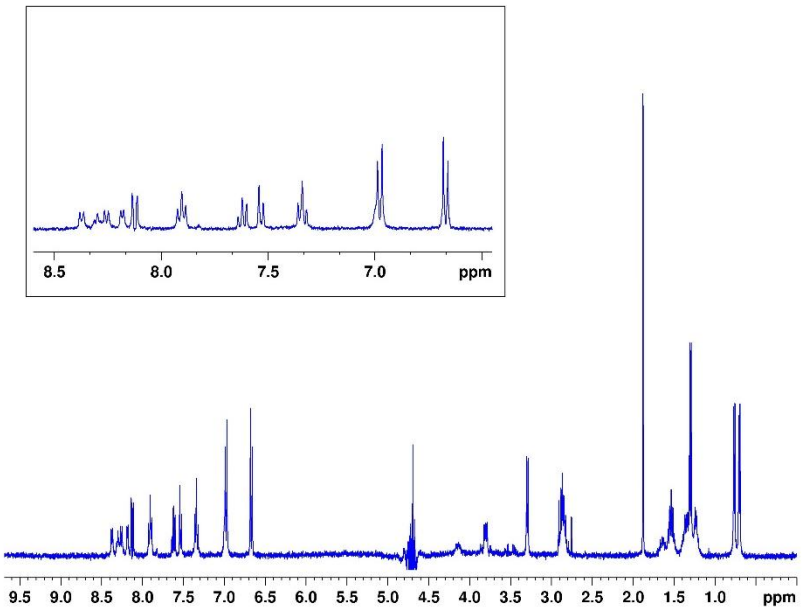


Figure S4. Analytical HPLC chromatograms for all peptides (continued)

C1



C2

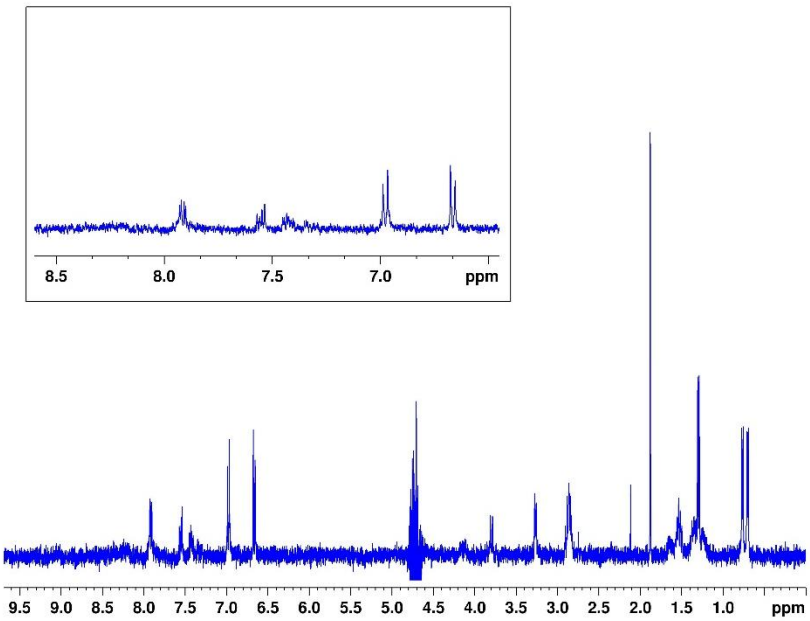


Figure S5. NMR spectra for nitroarylated model peptides

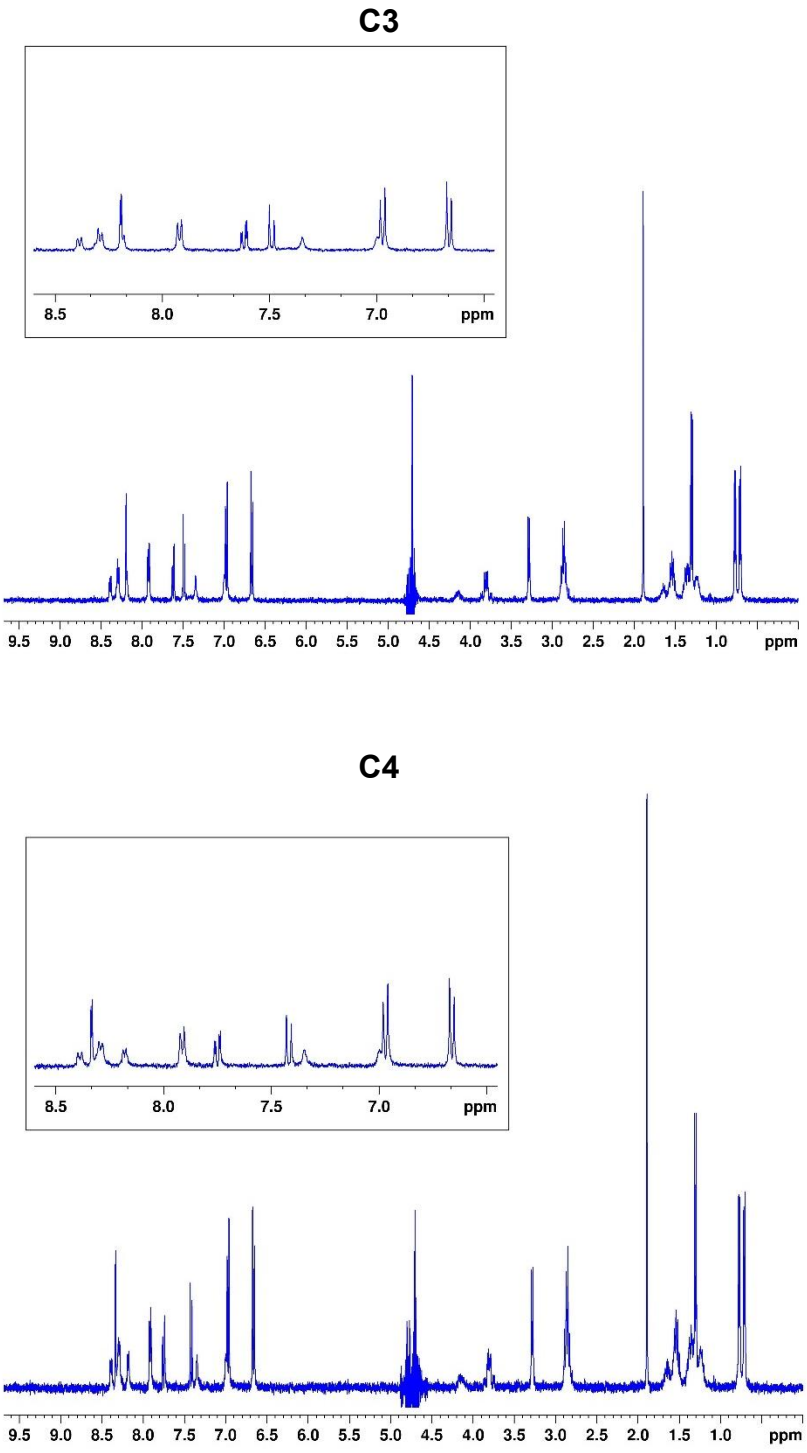


Figure S5. NMR spectra for nitroarylated model peptides (continued)

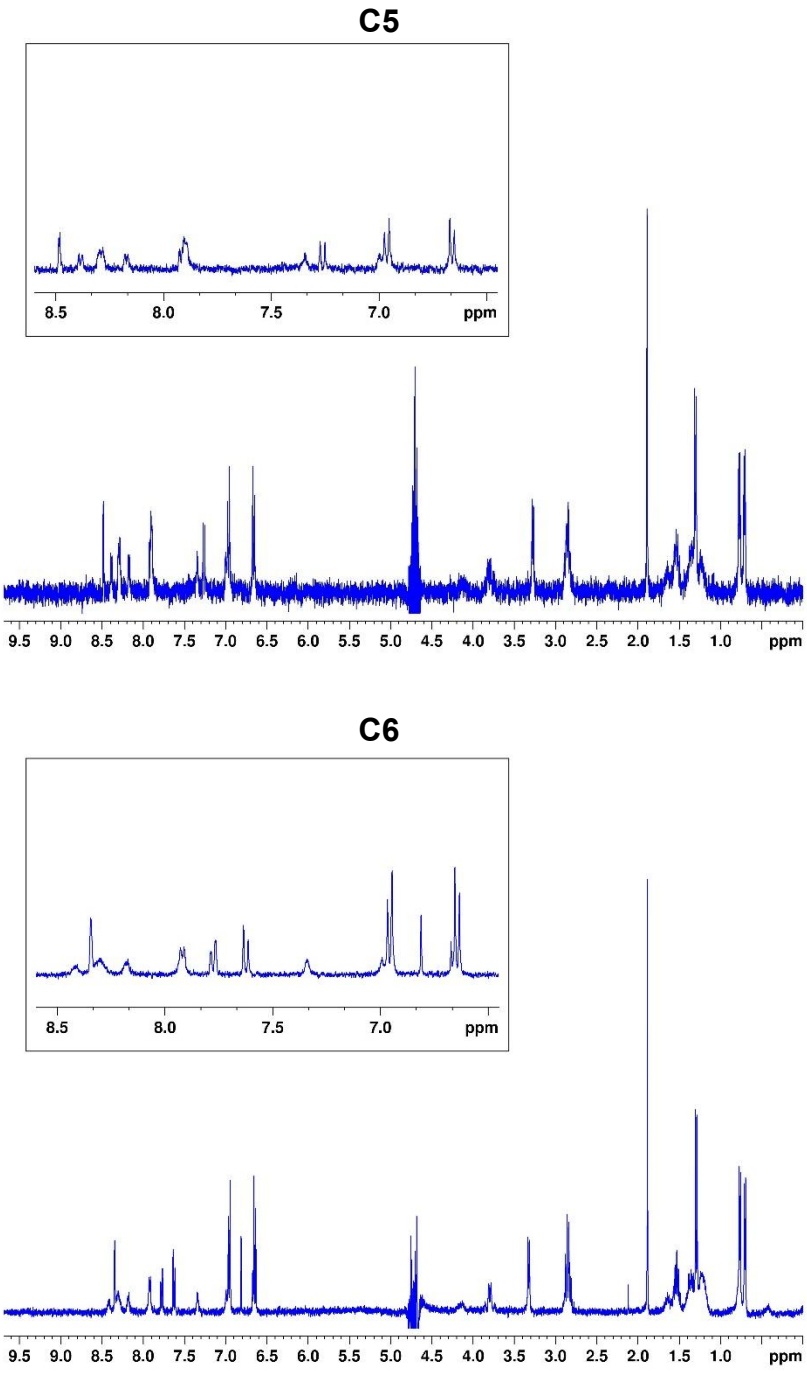
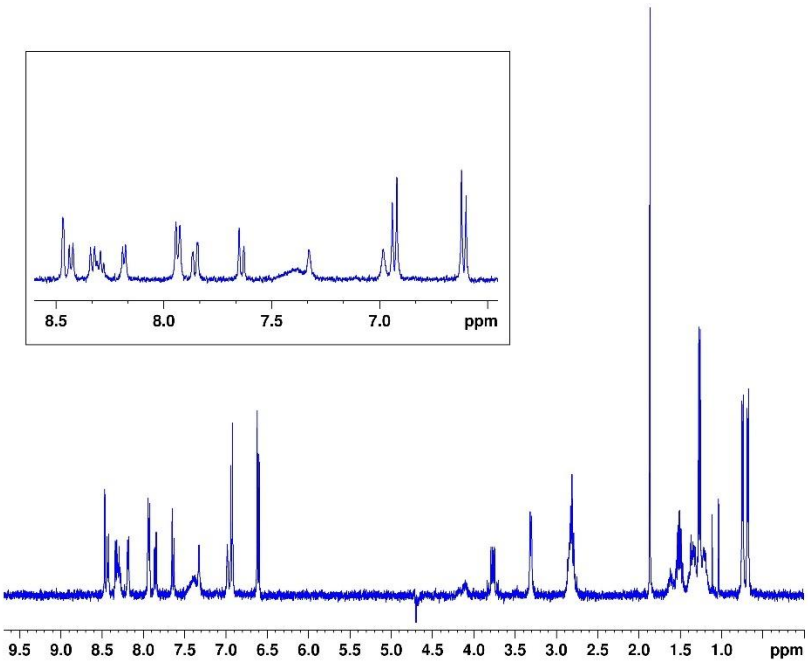


Figure S5. NMR spectra for nitroarylated model peptides (continued)

C7



C8

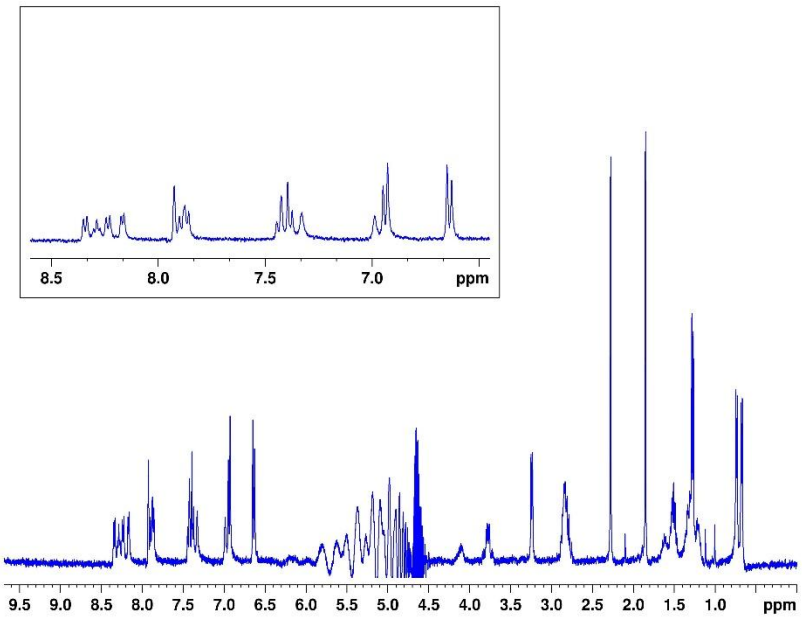


Figure S5. NMR spectra for nitroarylated model peptides (continued)

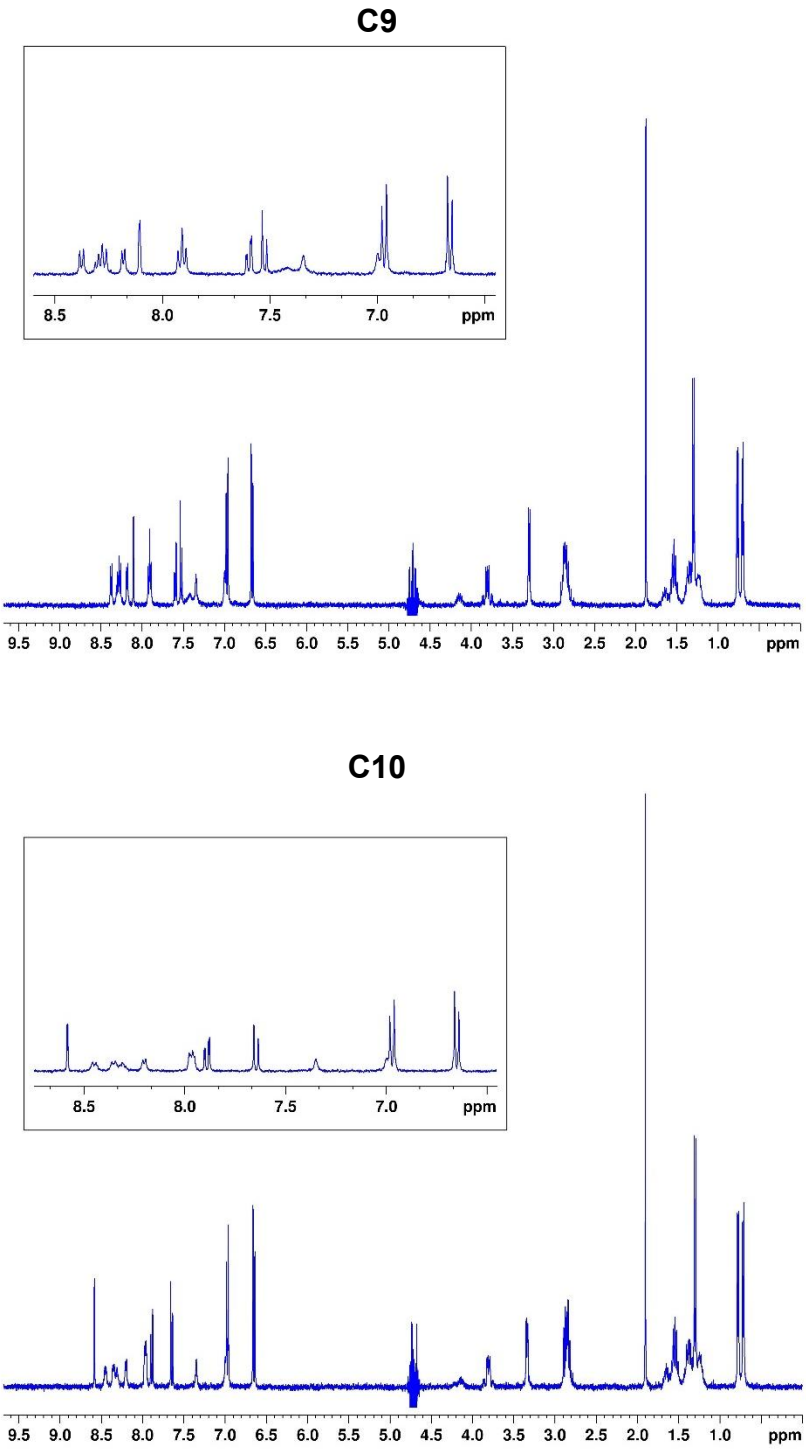


Figure S5. NMR spectra for nitroarylated model peptides (continued)

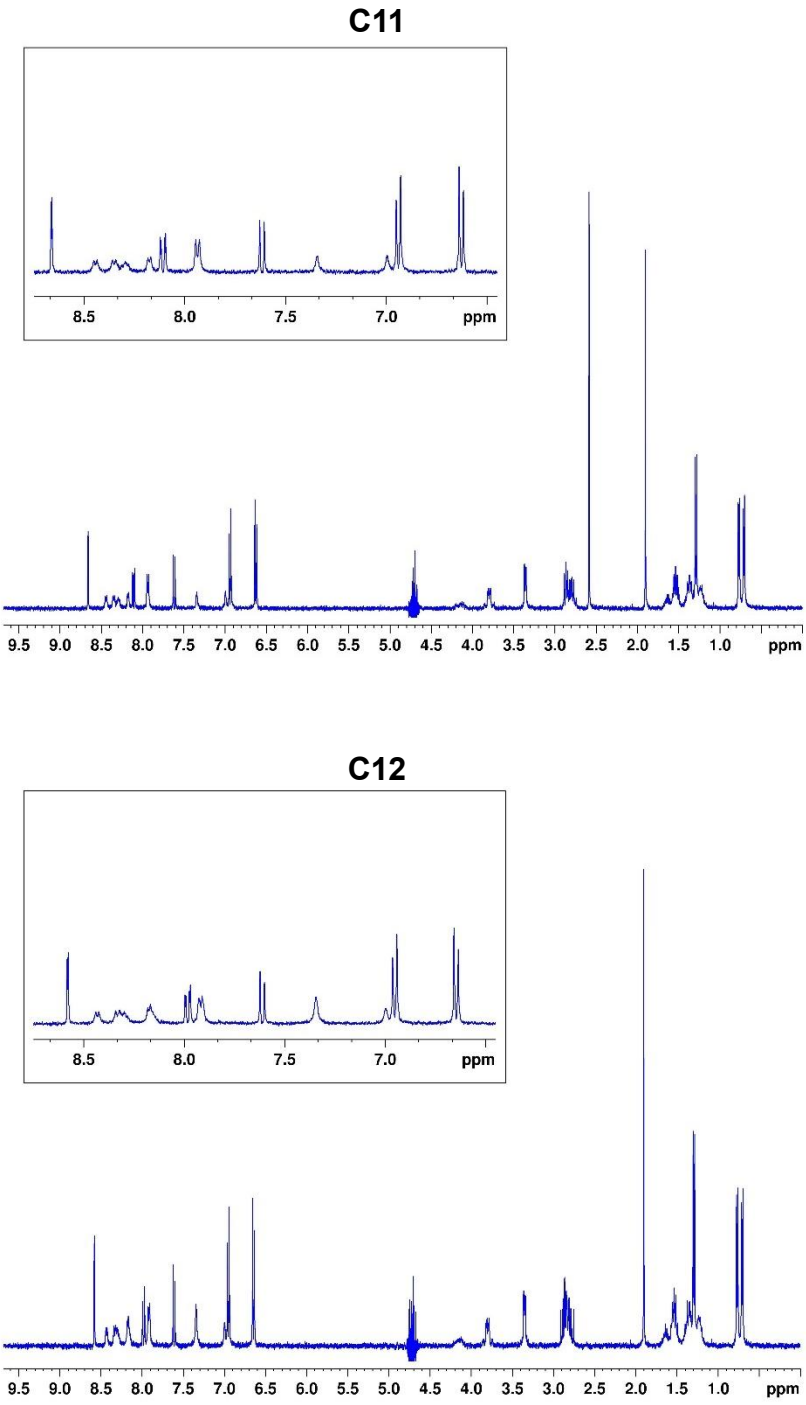


Figure S5. NMR spectra for nitroarylated model peptides (continued)

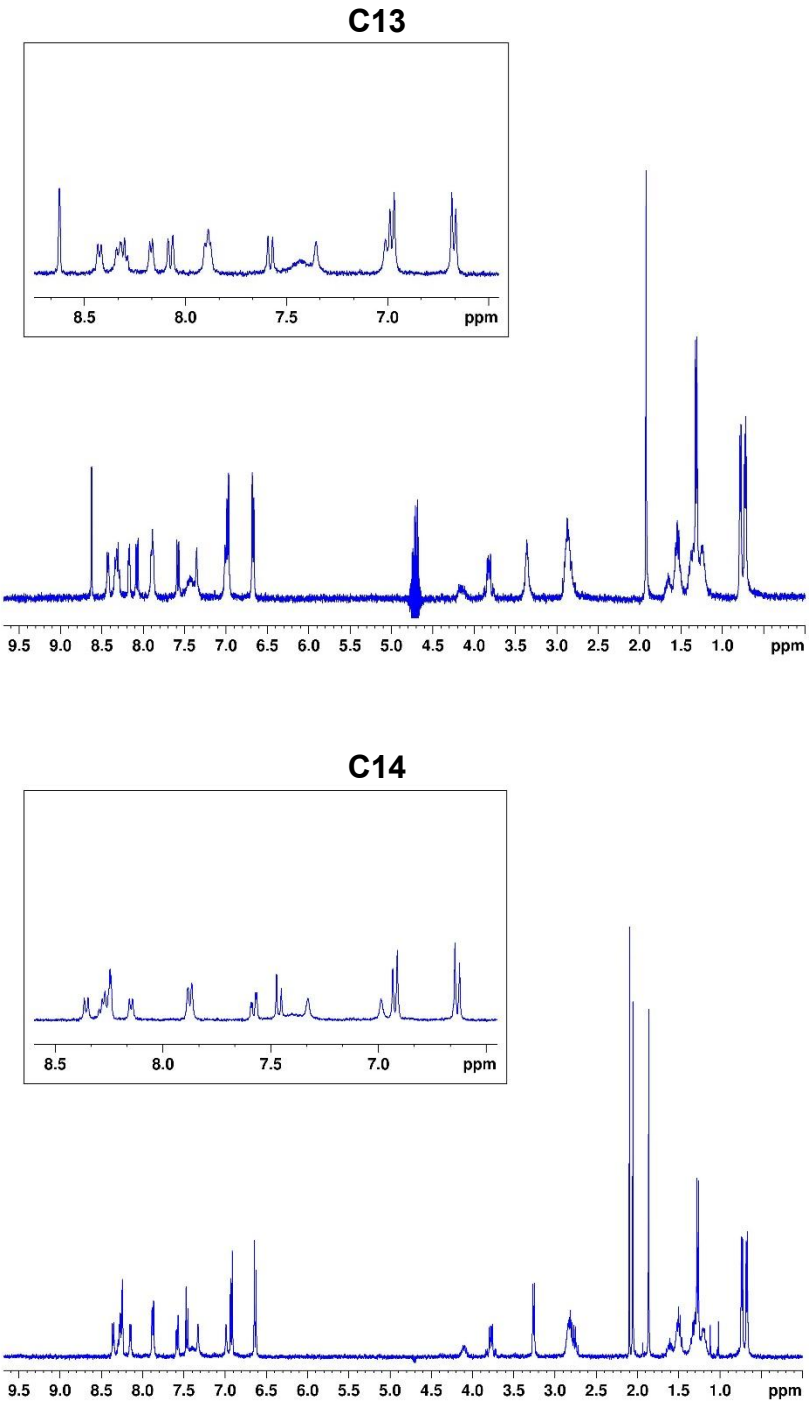


Figure S5. NMR spectra for nitroarylated model peptides (continued)

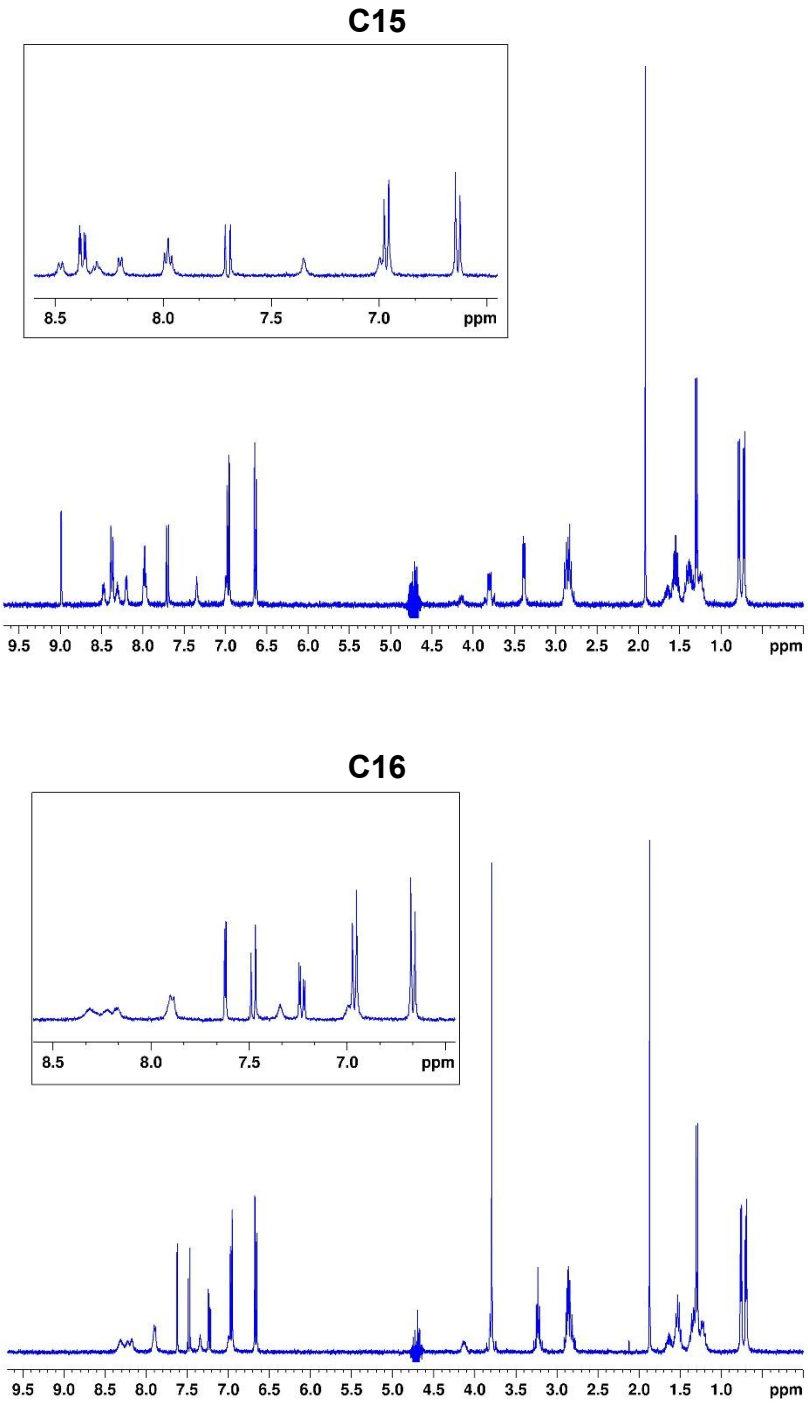


Figure S5. NMR spectra for nitroarylated model peptides (continued)

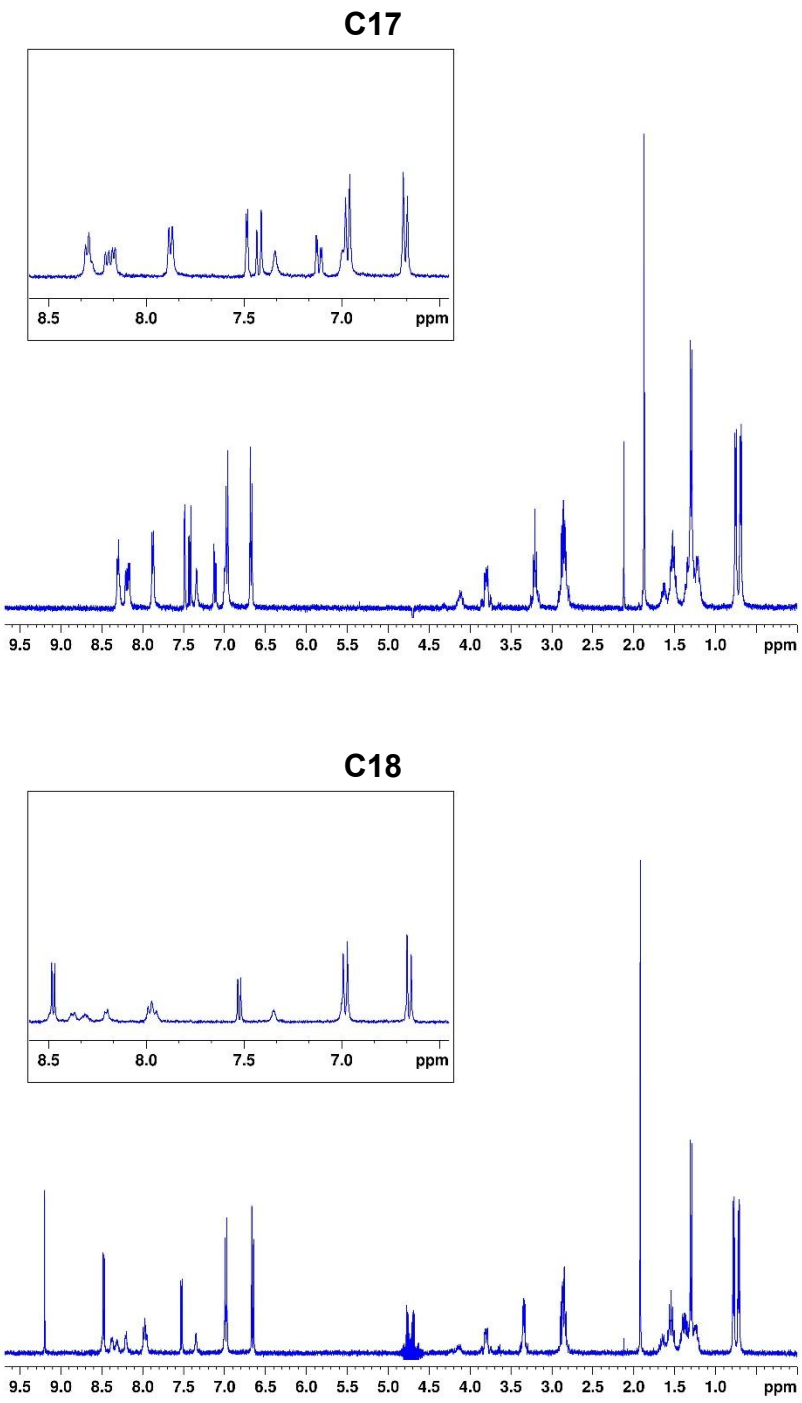


Figure S5. NMR spectra for nitroarylated model peptides (continued)

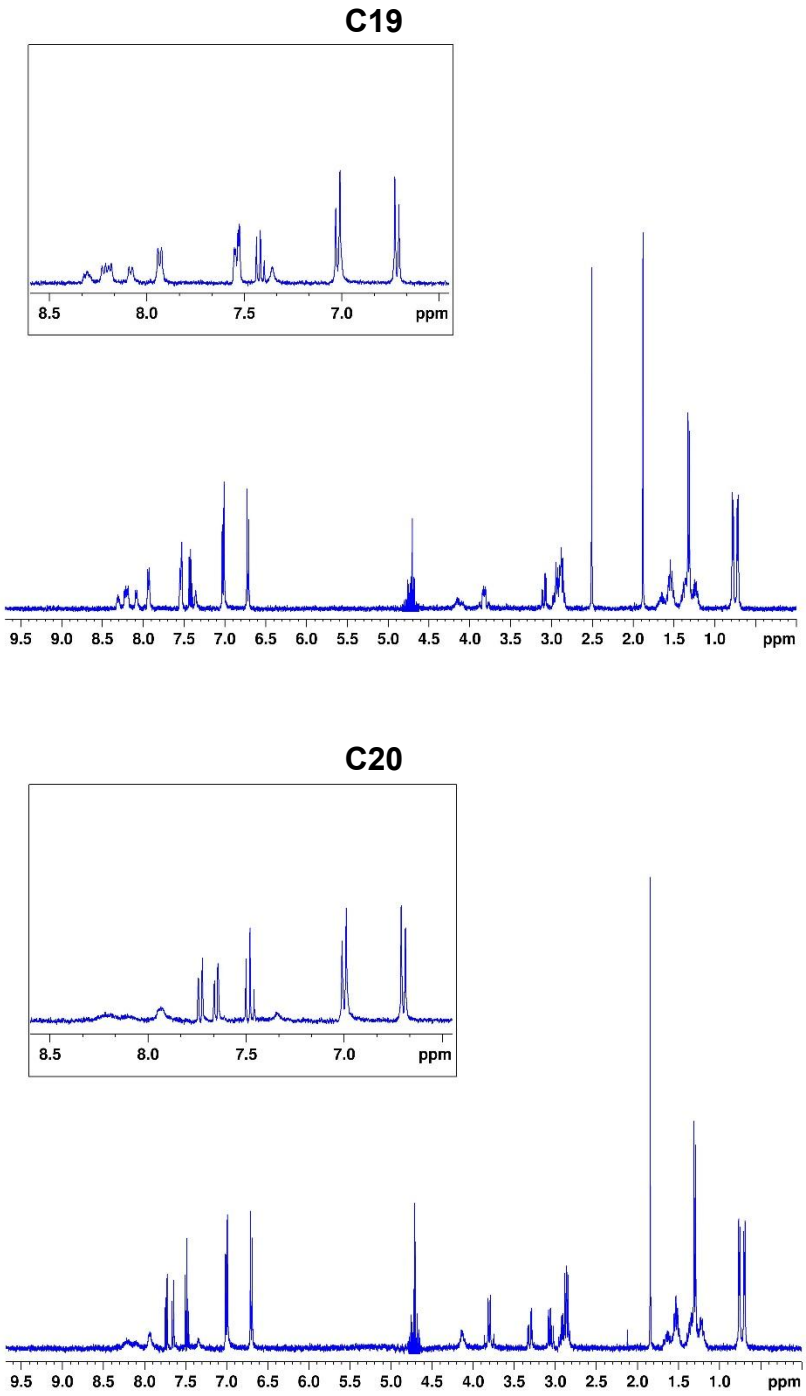


Figure S5. NMR spectra for nitroarylated model peptides (continued)

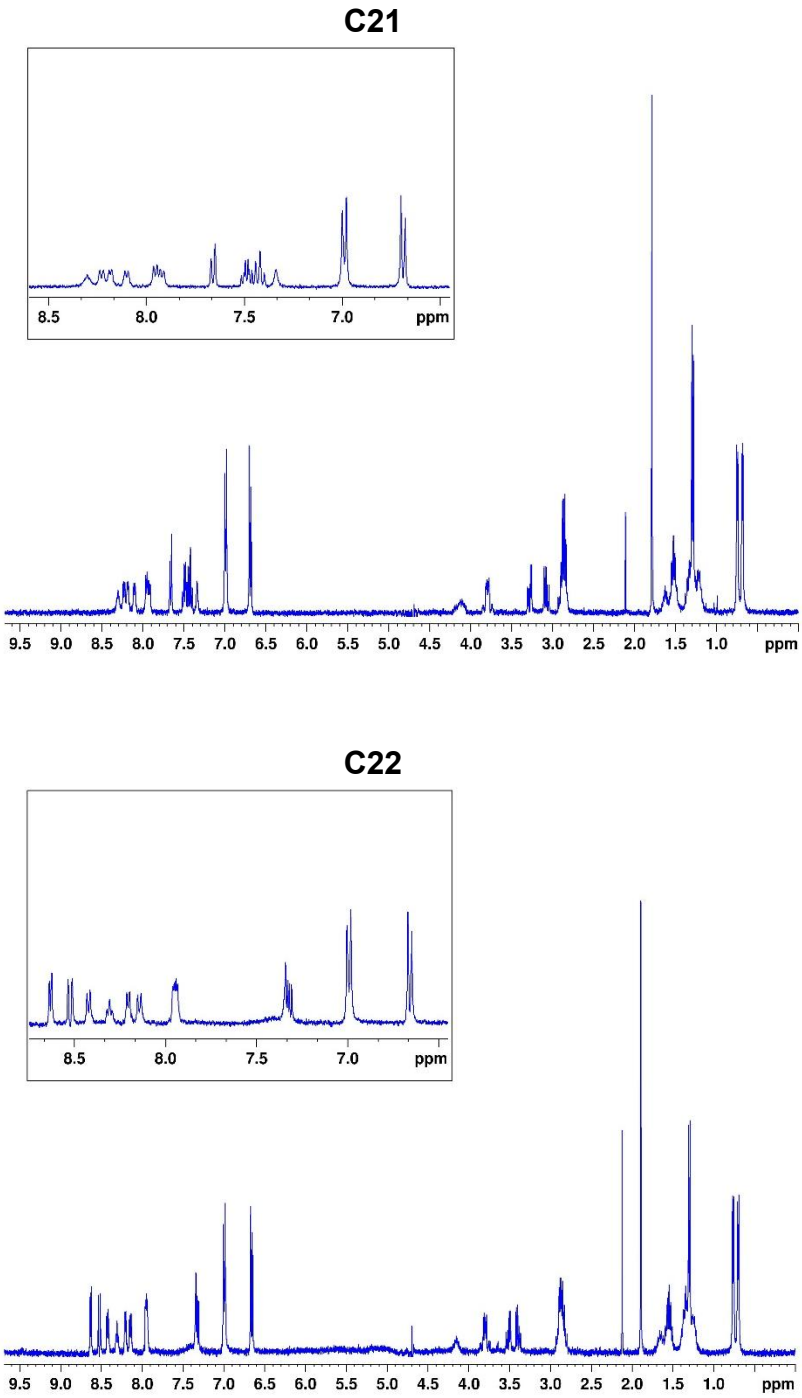


Figure S5. NMR spectra for nitroarylated model peptides (continued)

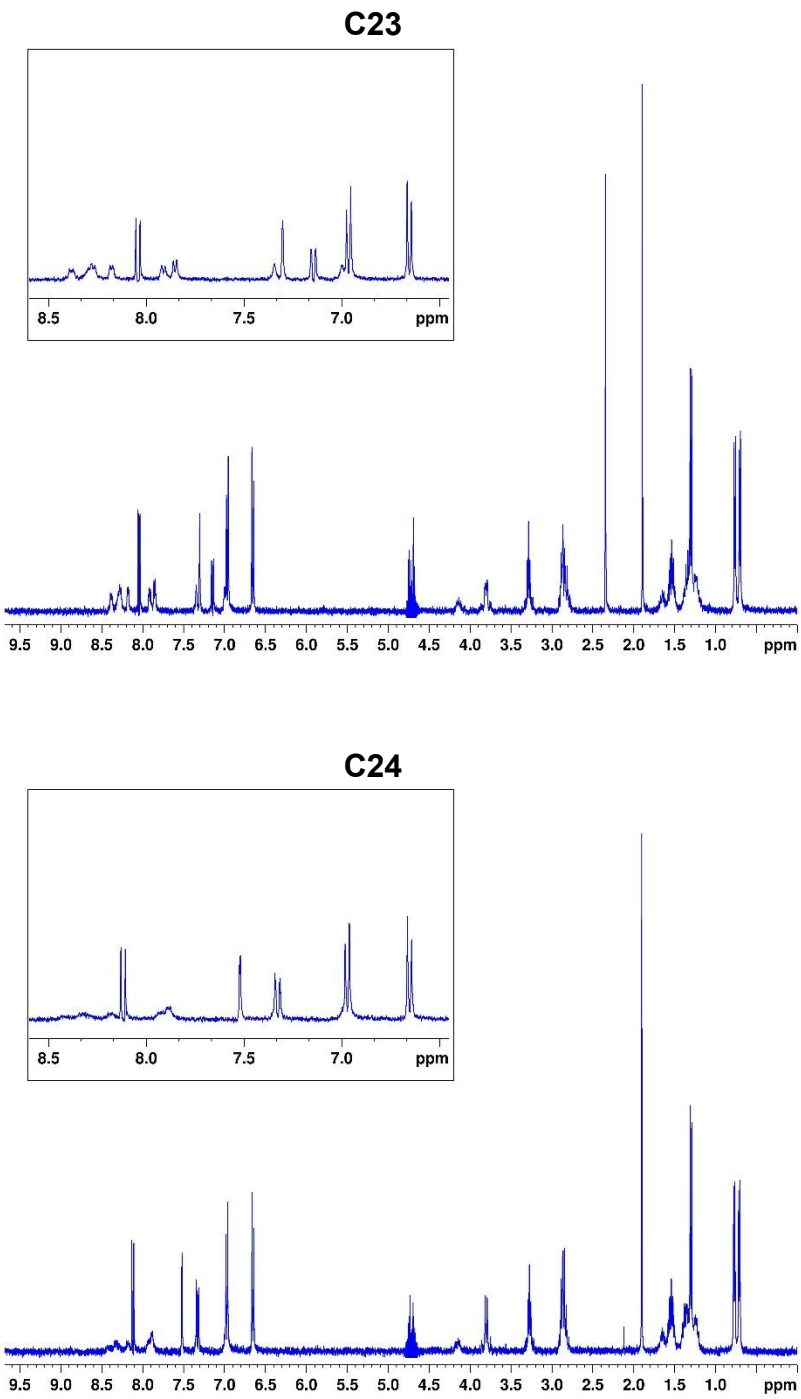


Figure S5. NMR spectra for nitroarylated model peptides (continued)

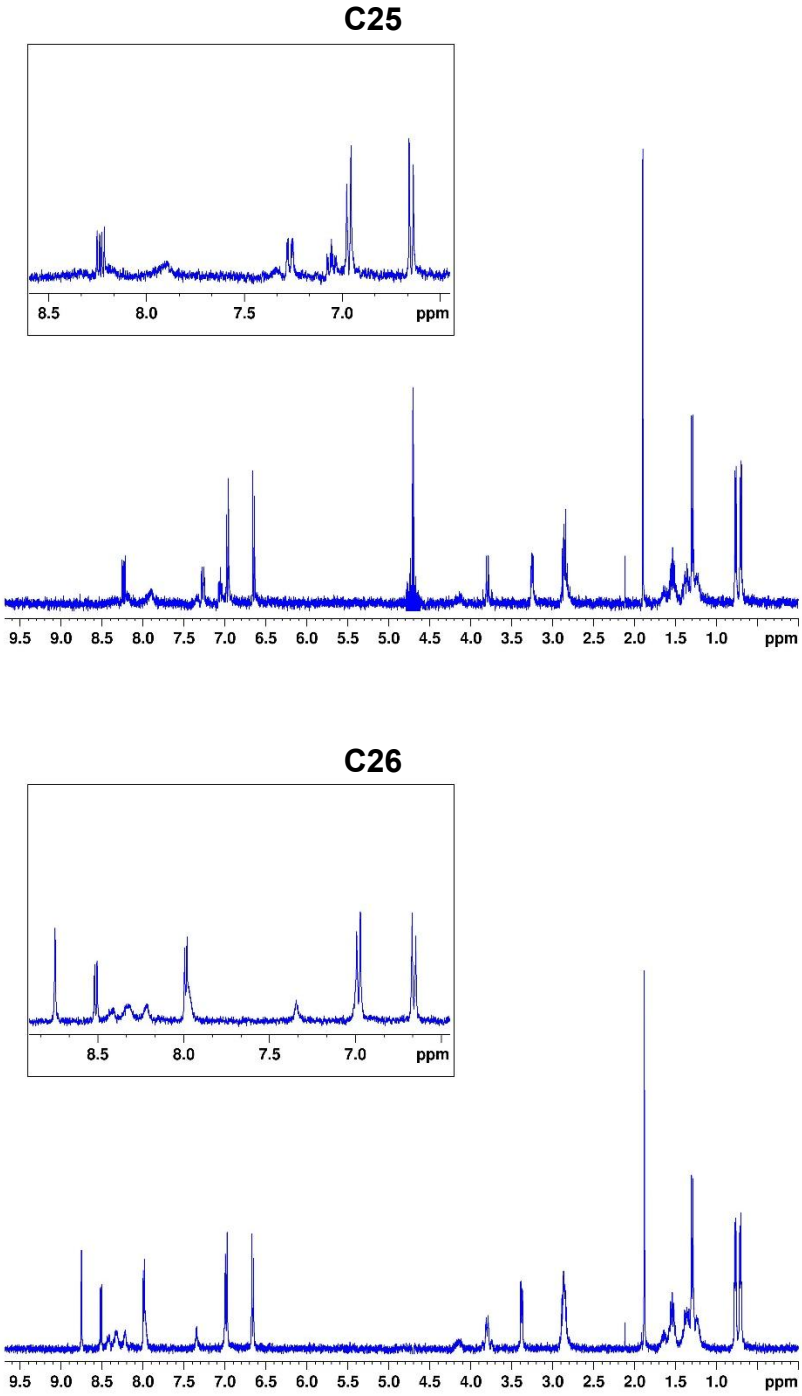


Figure S5. NMR spectra for nitroarylated model peptides (continued)

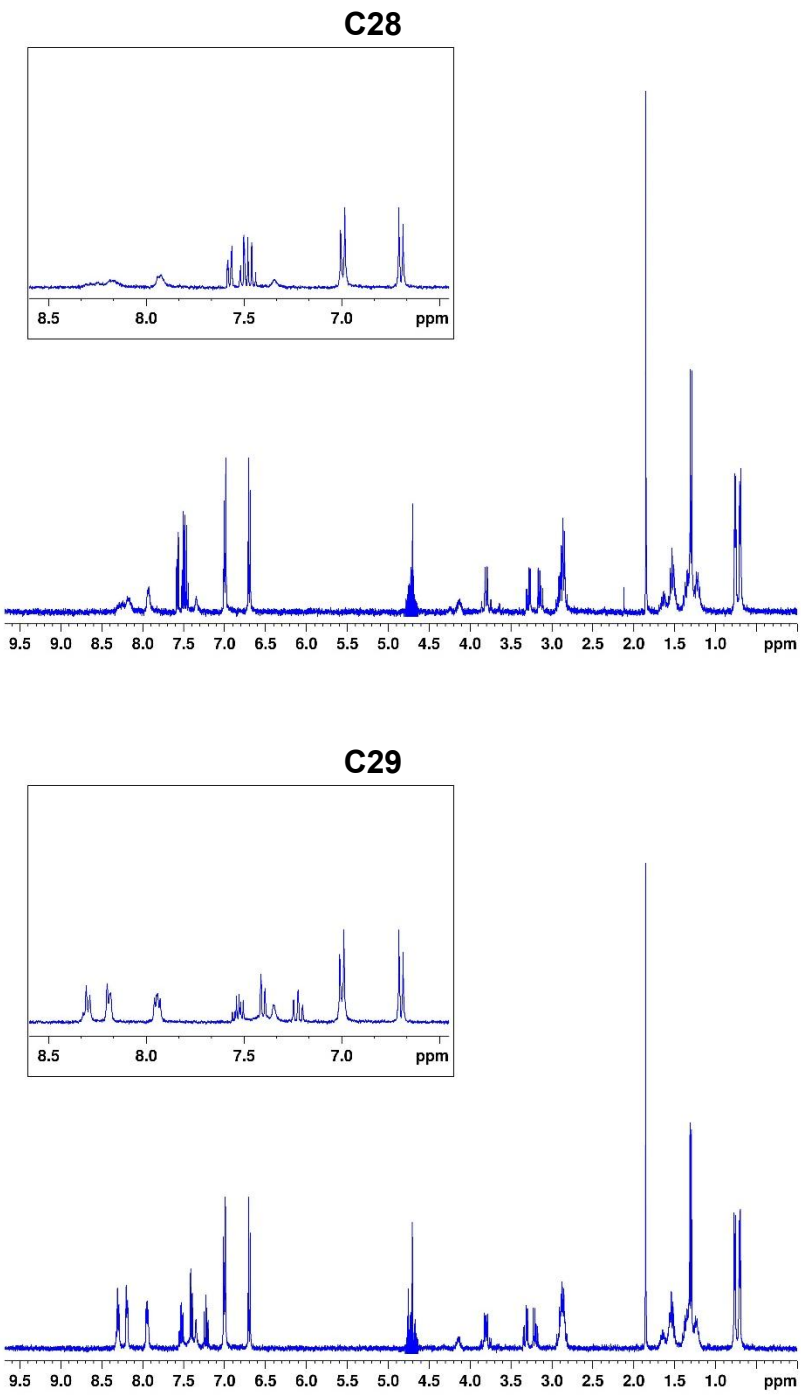


Figure S5. NMR spectra for nitroarylated model peptides (continued)

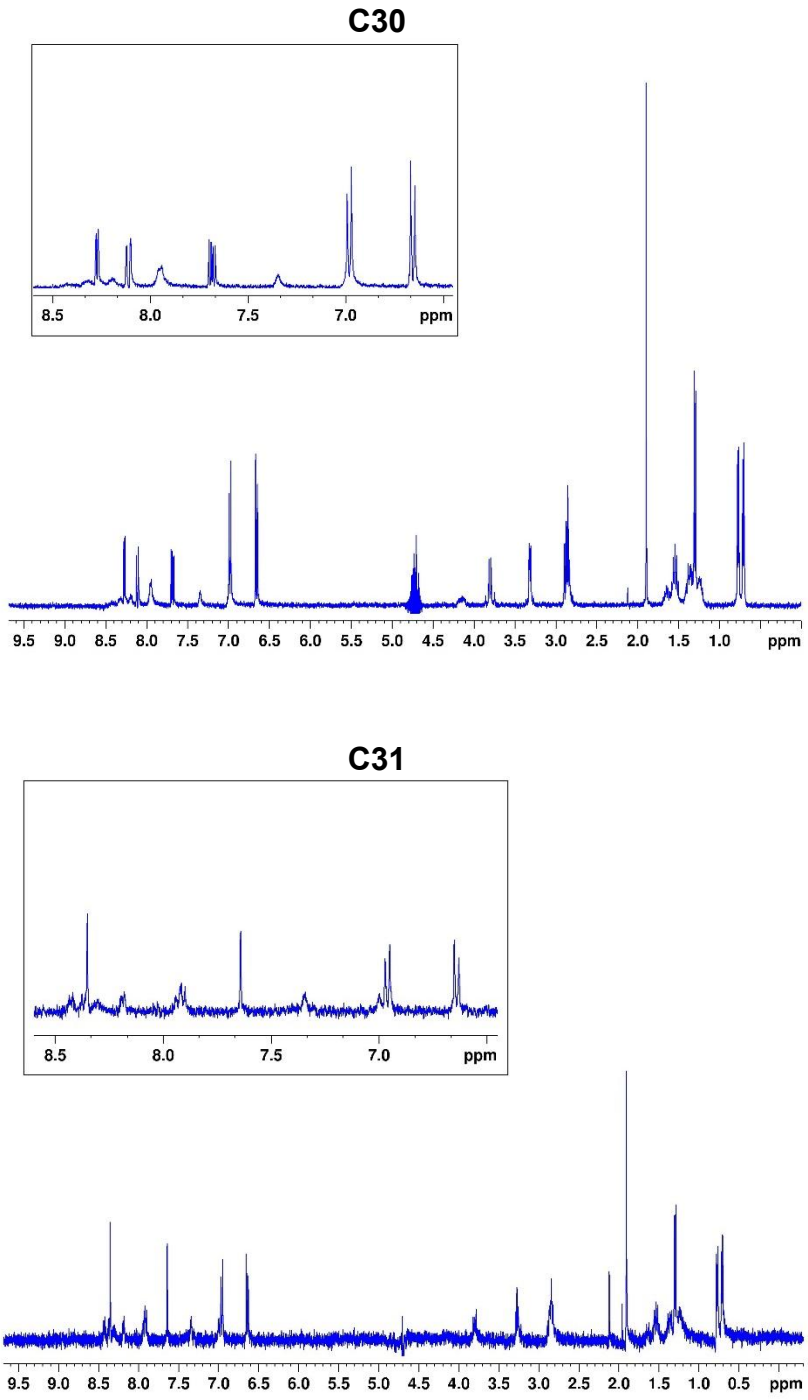
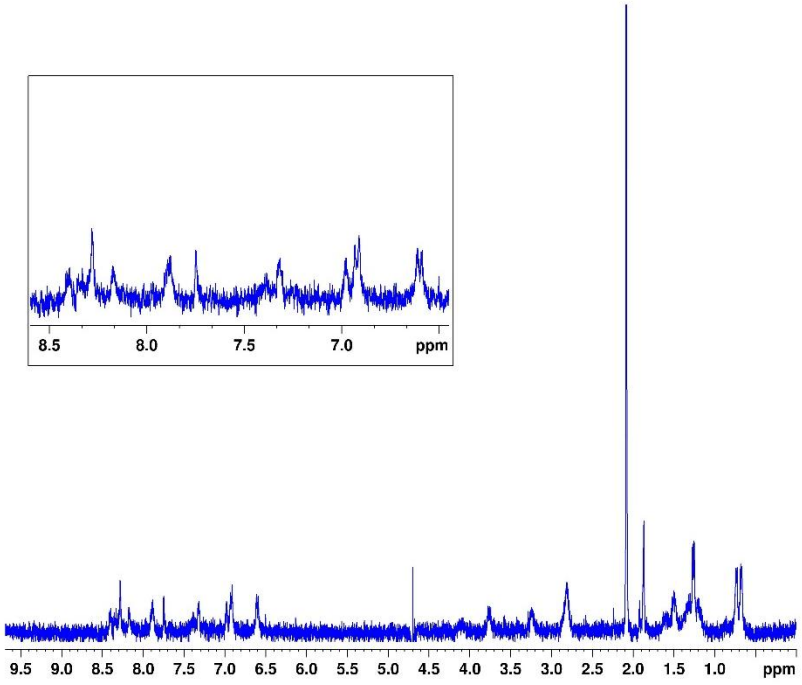


Figure S5. NMR spectra for nitroarylated model peptides (continued)

C32



C33

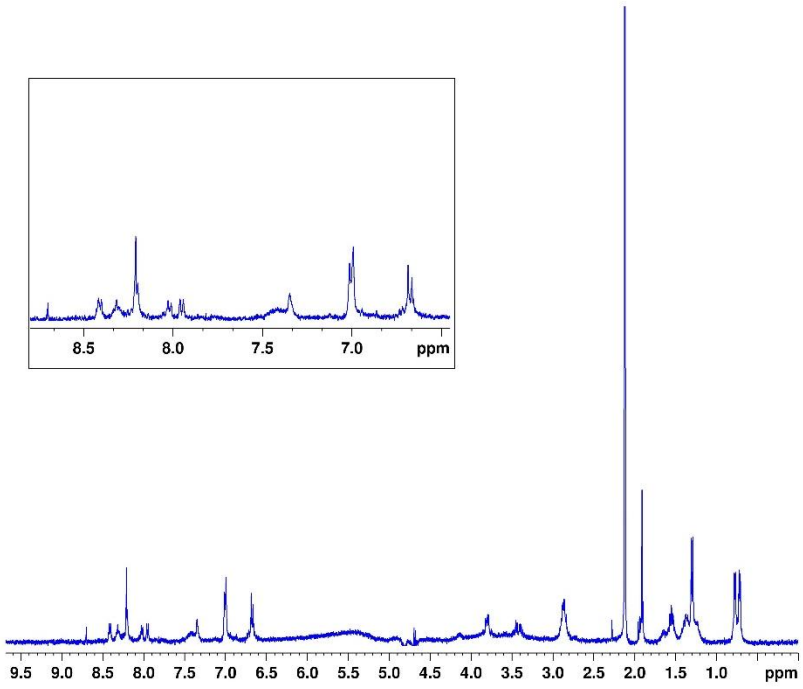


Figure S5. NMR spectra for nitroarylated model peptides (continued)

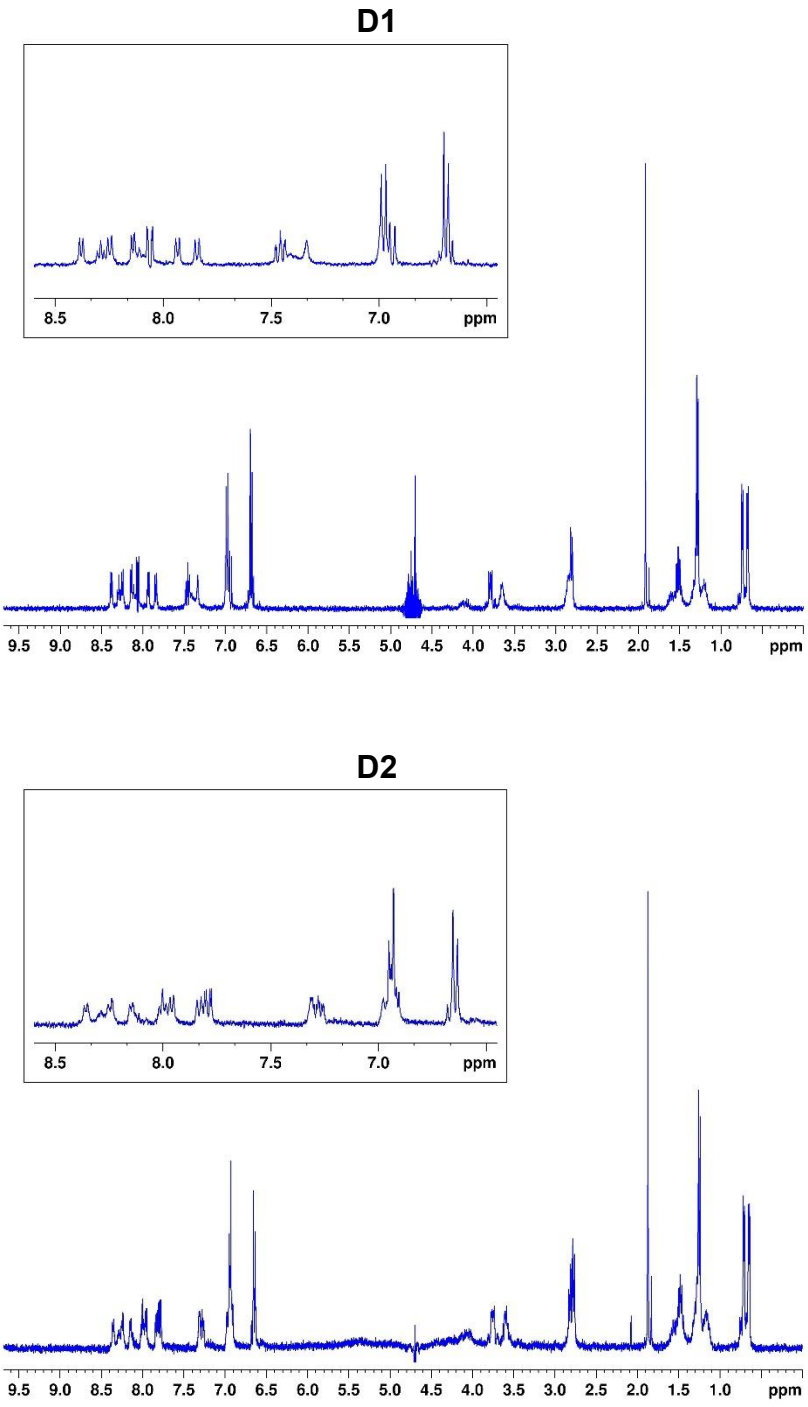
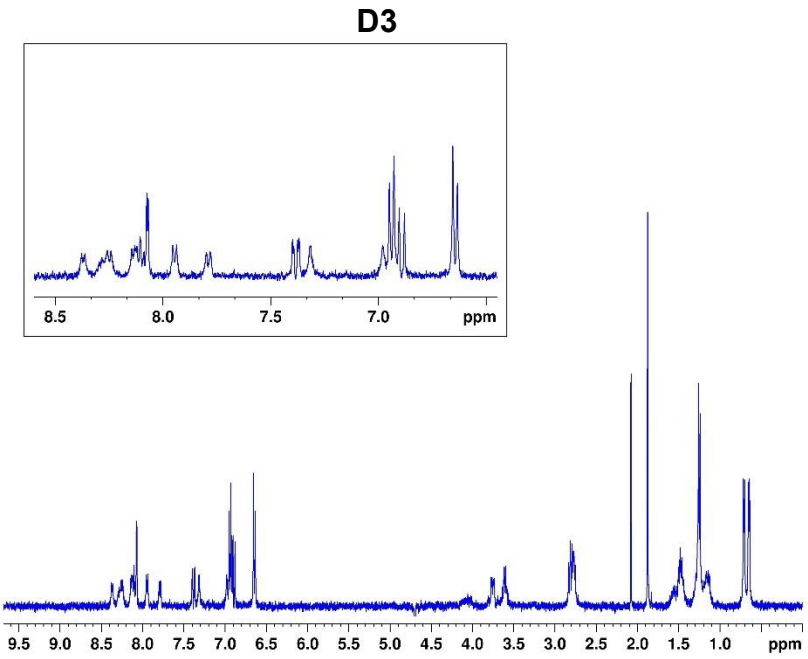


Figure S5. NMR spectra for nitroarylated model peptides (continued)



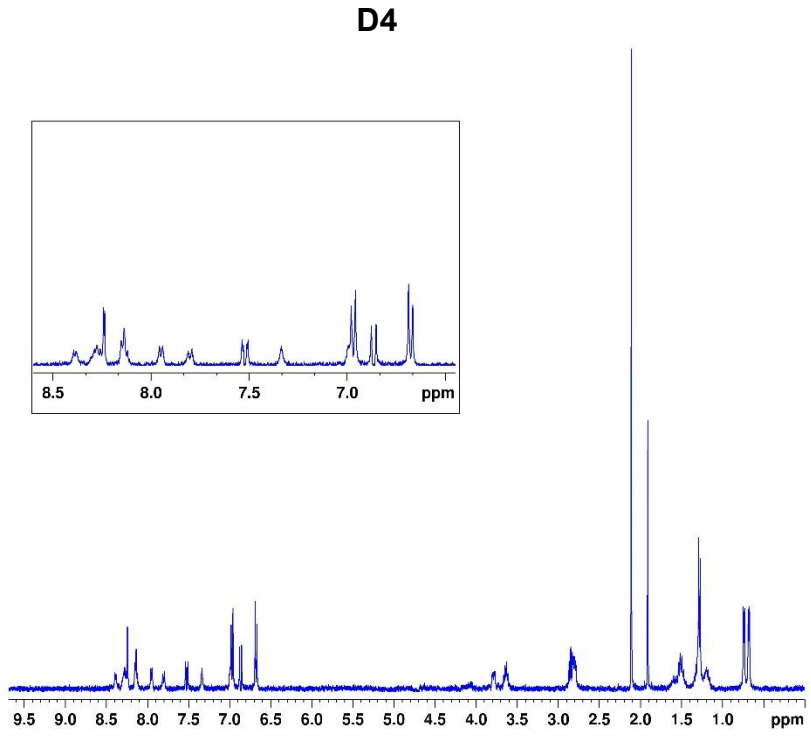


Figure S5. NMR spectra for nitroarylated model peptides (continued)

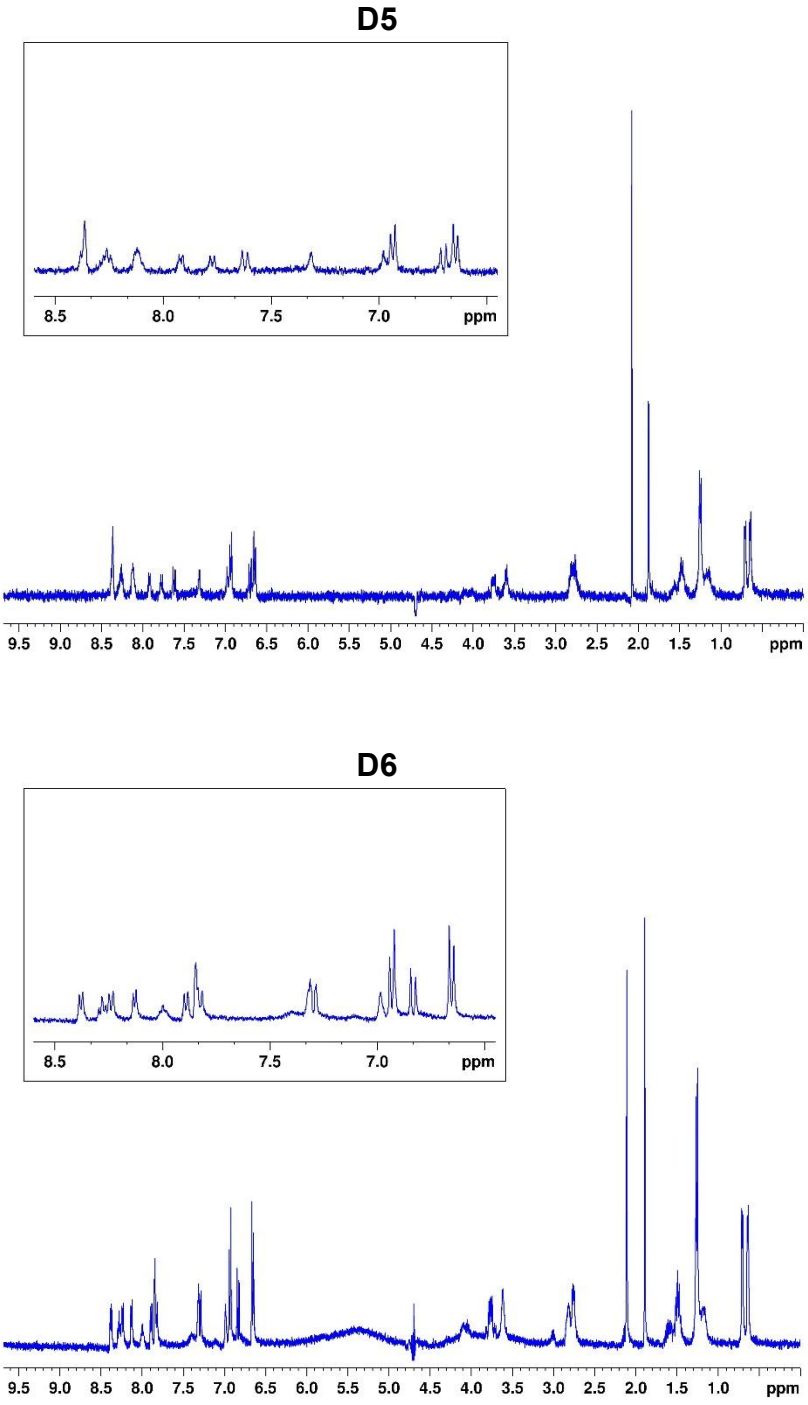


Figure S5. NMR spectra for nitroarylated model peptides (continued)

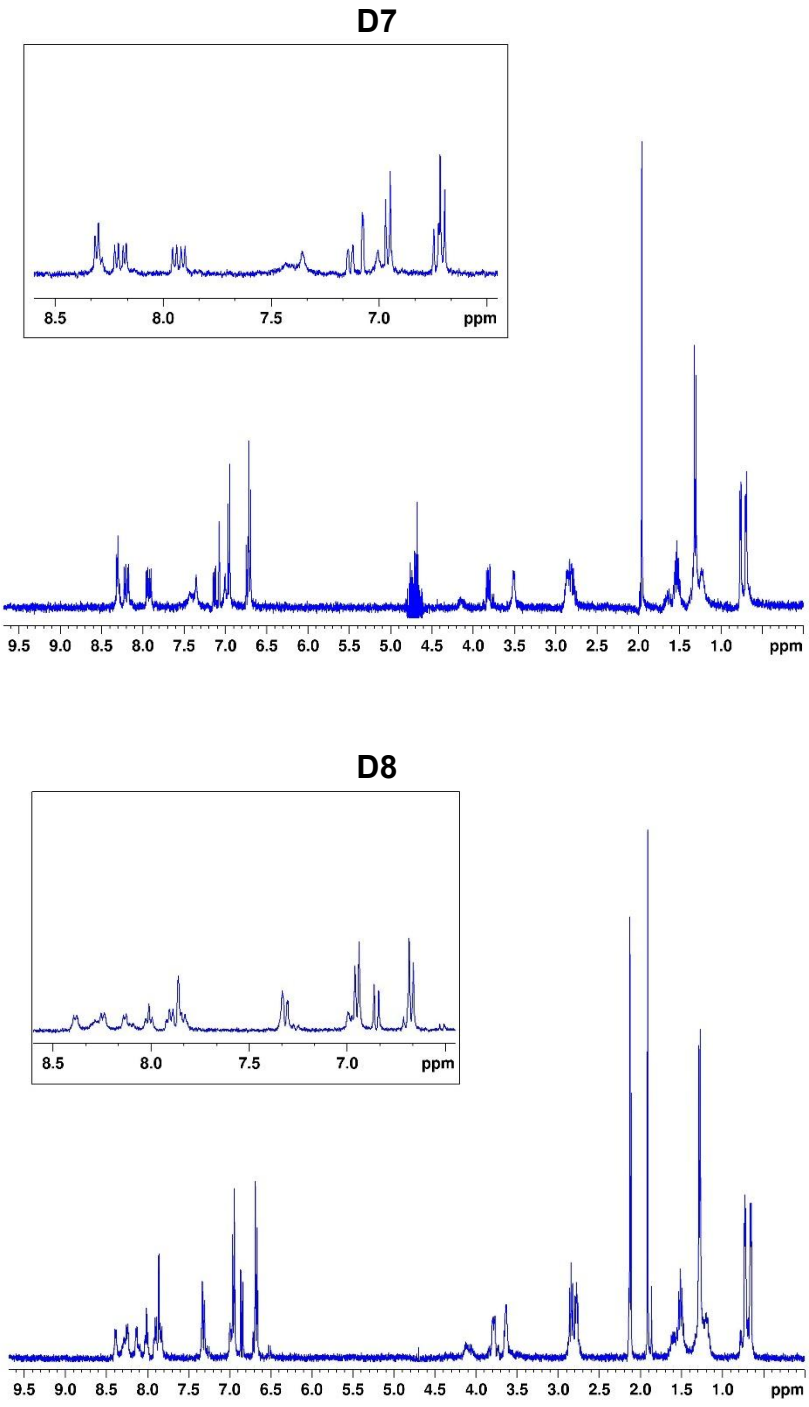
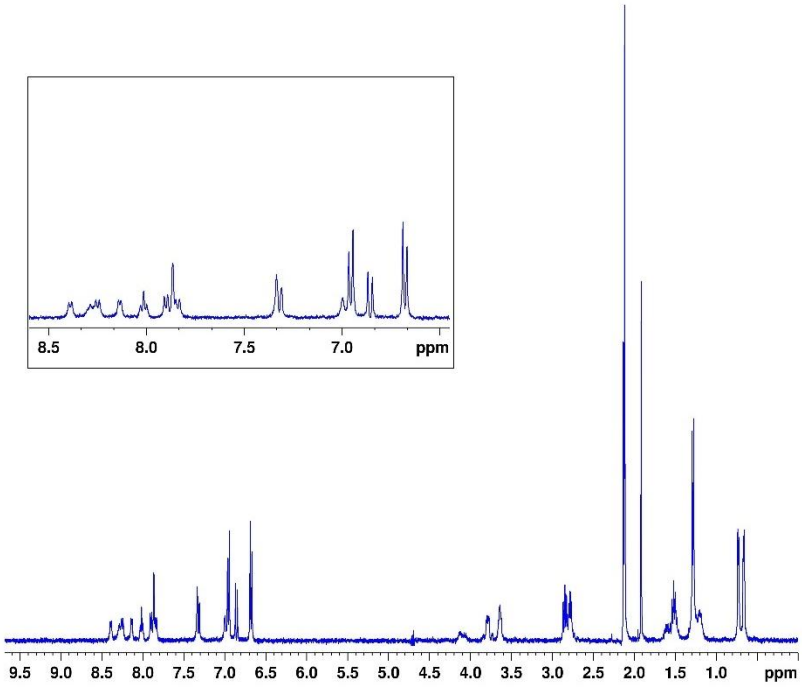


Figure S5. NMR spectra for nitroarylated model peptides (continued)

D9



D10

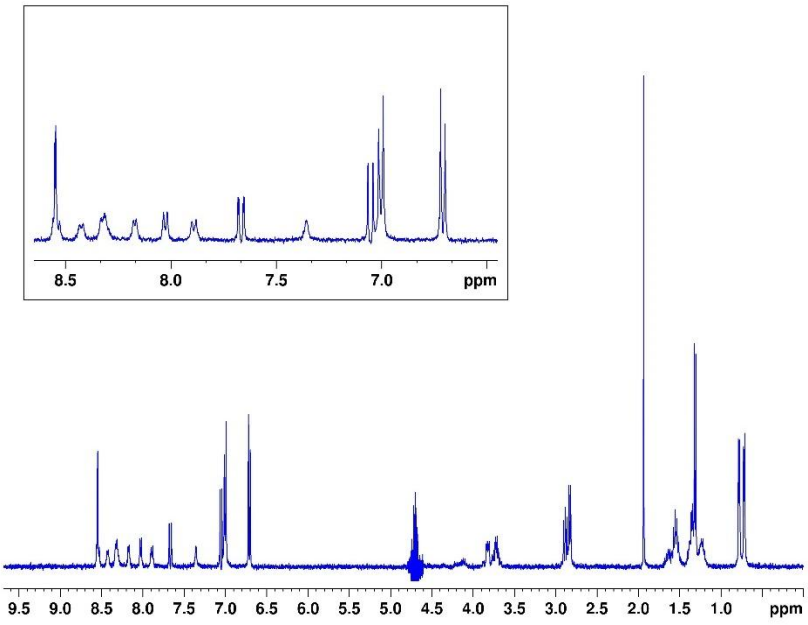
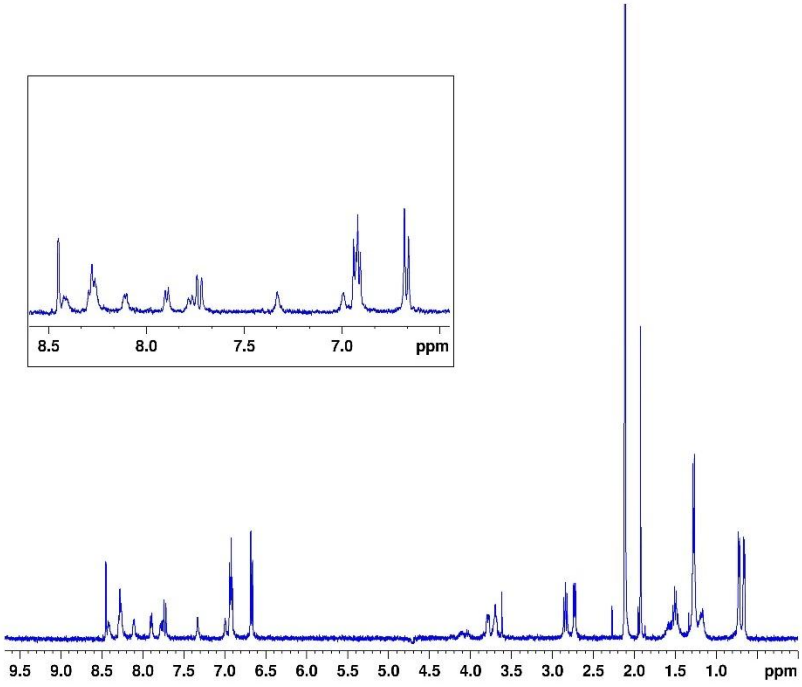


Figure S5. NMR spectra for nitroarylated model peptides (continued)

D11



D12

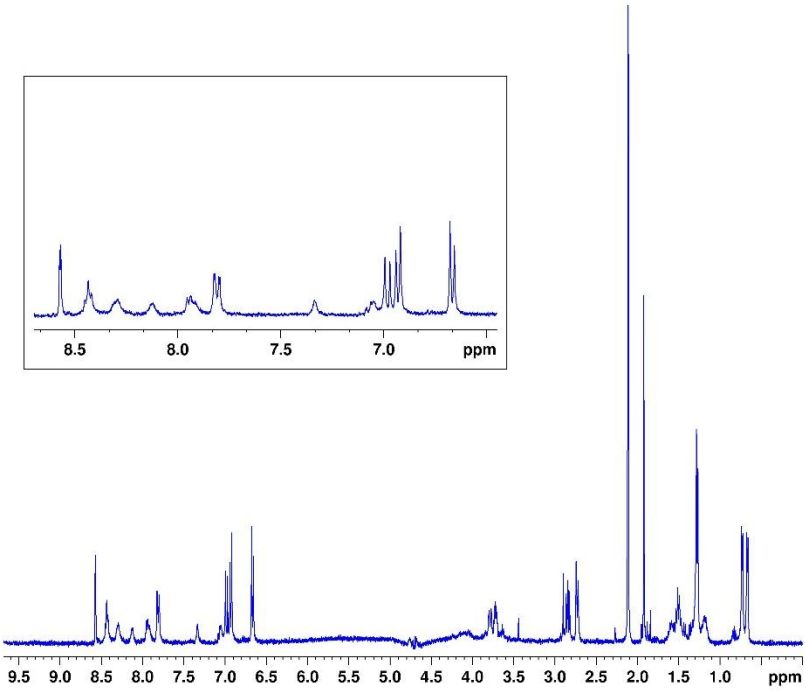
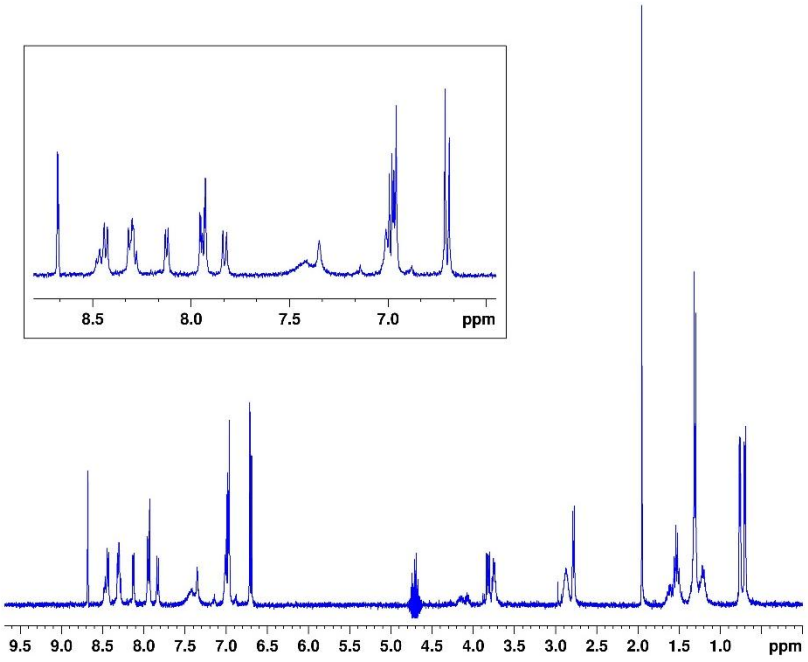


Figure S5. NMR spectra for nitroarylated model peptides (continued)

D13



D14

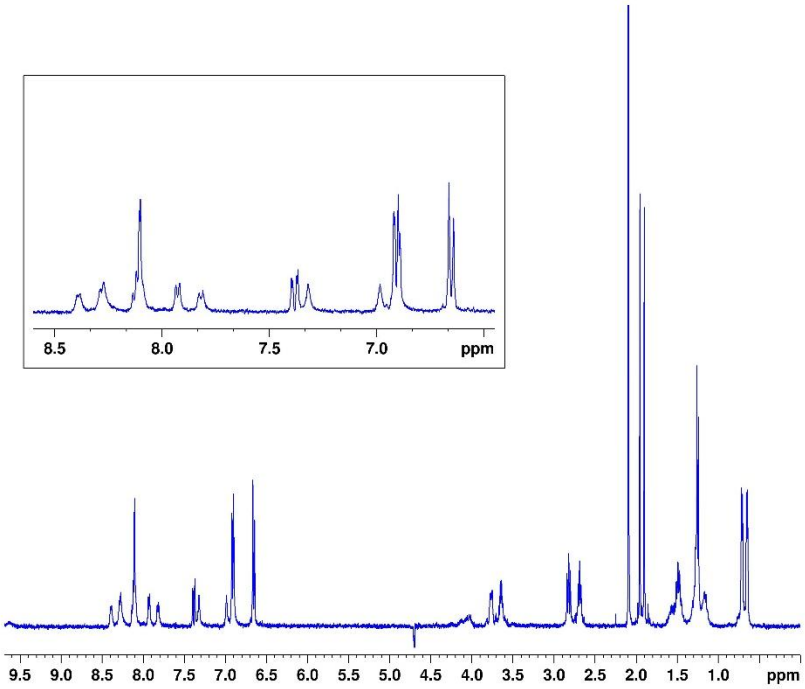
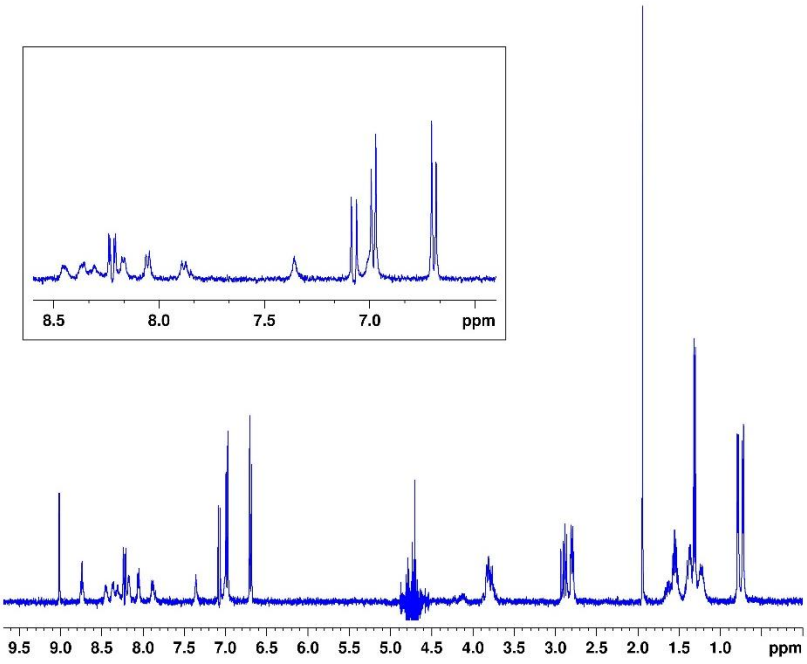


Figure S5. NMR spectra for nitroarylated model peptides (continued)

D15



D16

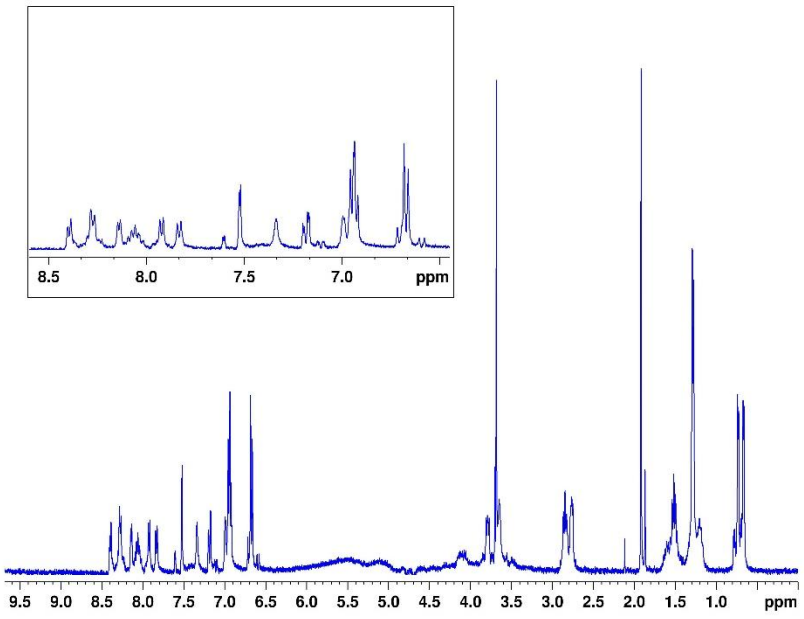
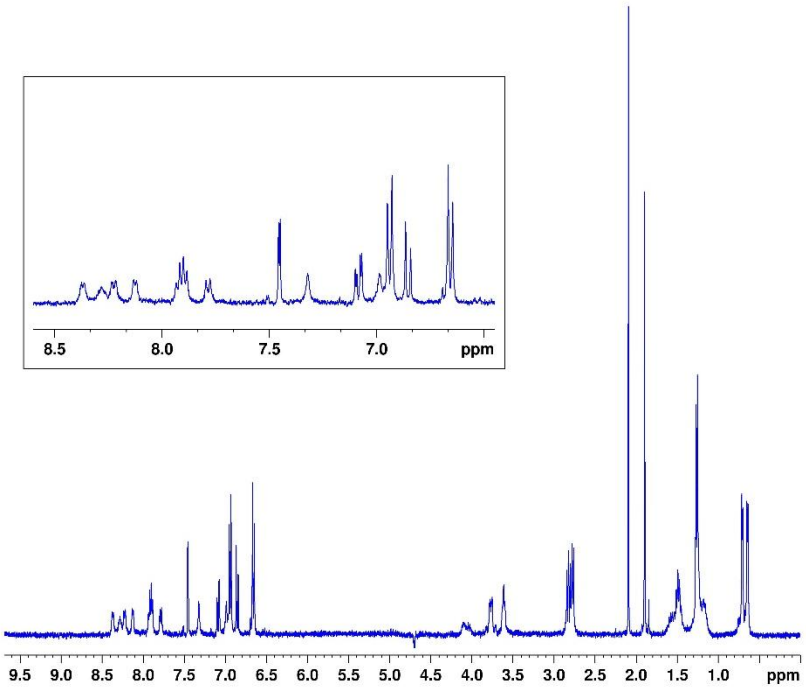


Figure S5. NMR spectra for nitroarylated model peptides (continued)

D17



D18

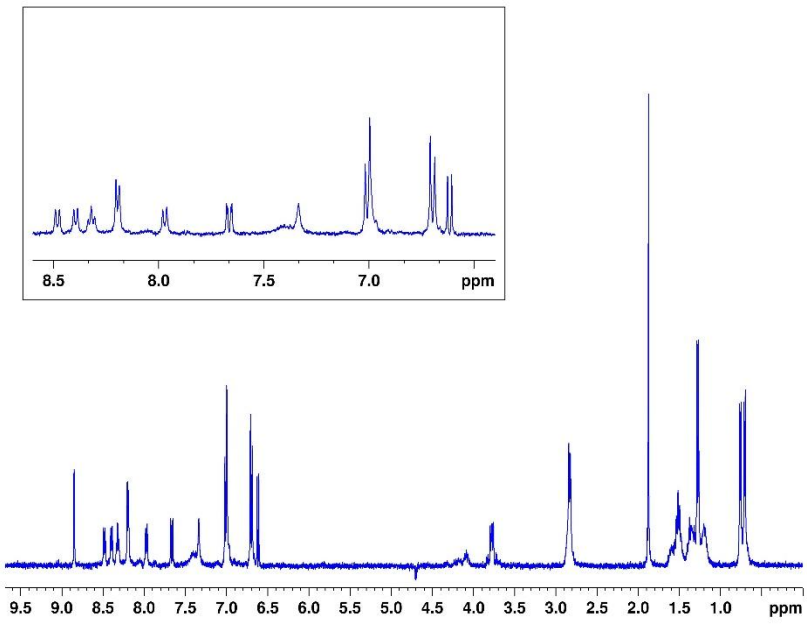


Figure S5. NMR spectra for nitroarylated model peptides (continued)

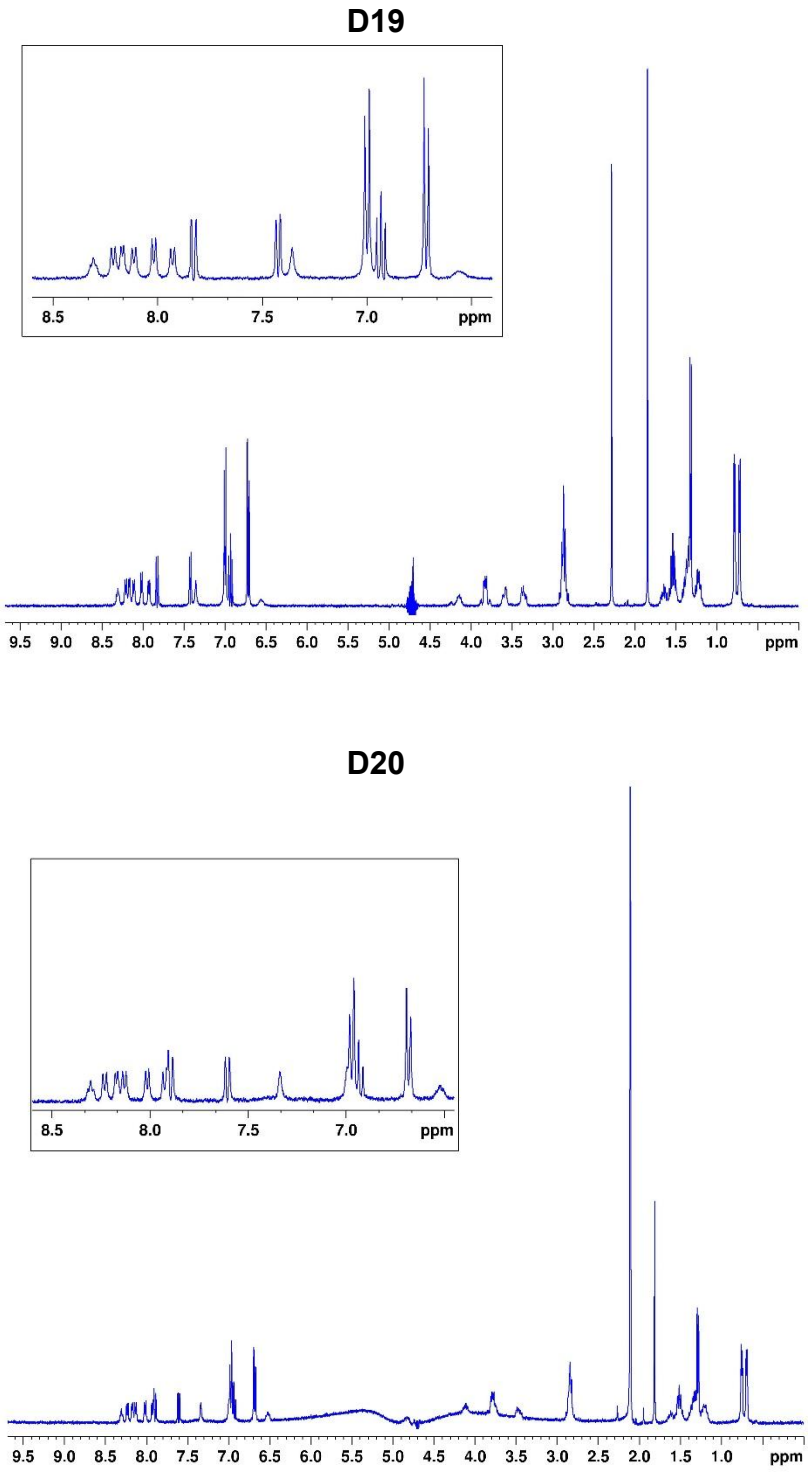


Figure S5. NMR spectra for nitroarylated model peptides (continued)

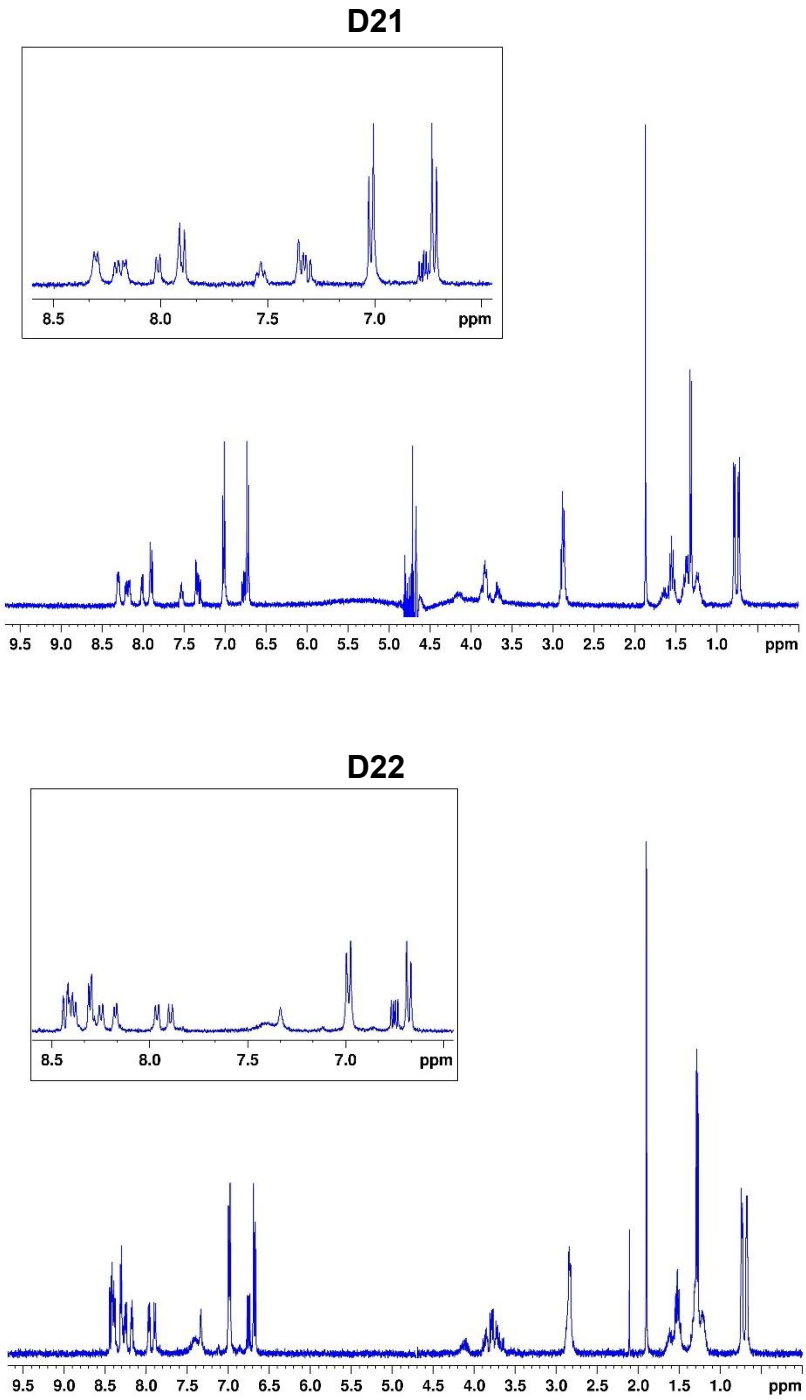
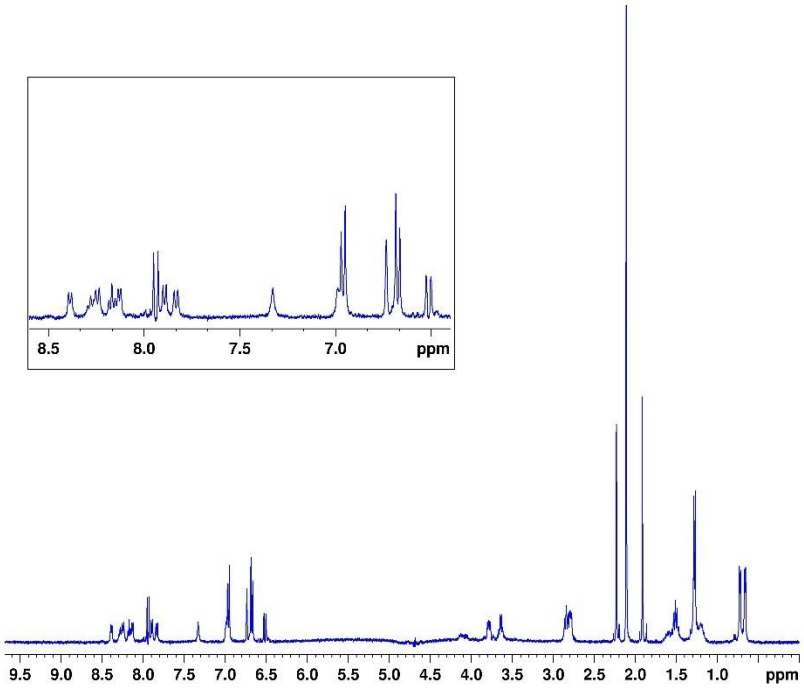


Figure S5. NMR spectra for nitroarylated model peptides (continued)

D23



D24

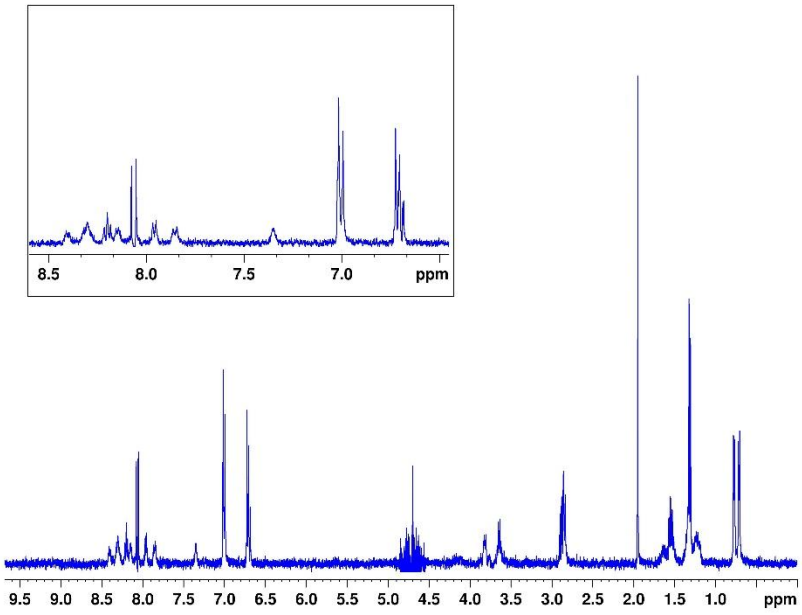


Figure S5. NMR spectra for nitroarylated model peptides (continued)

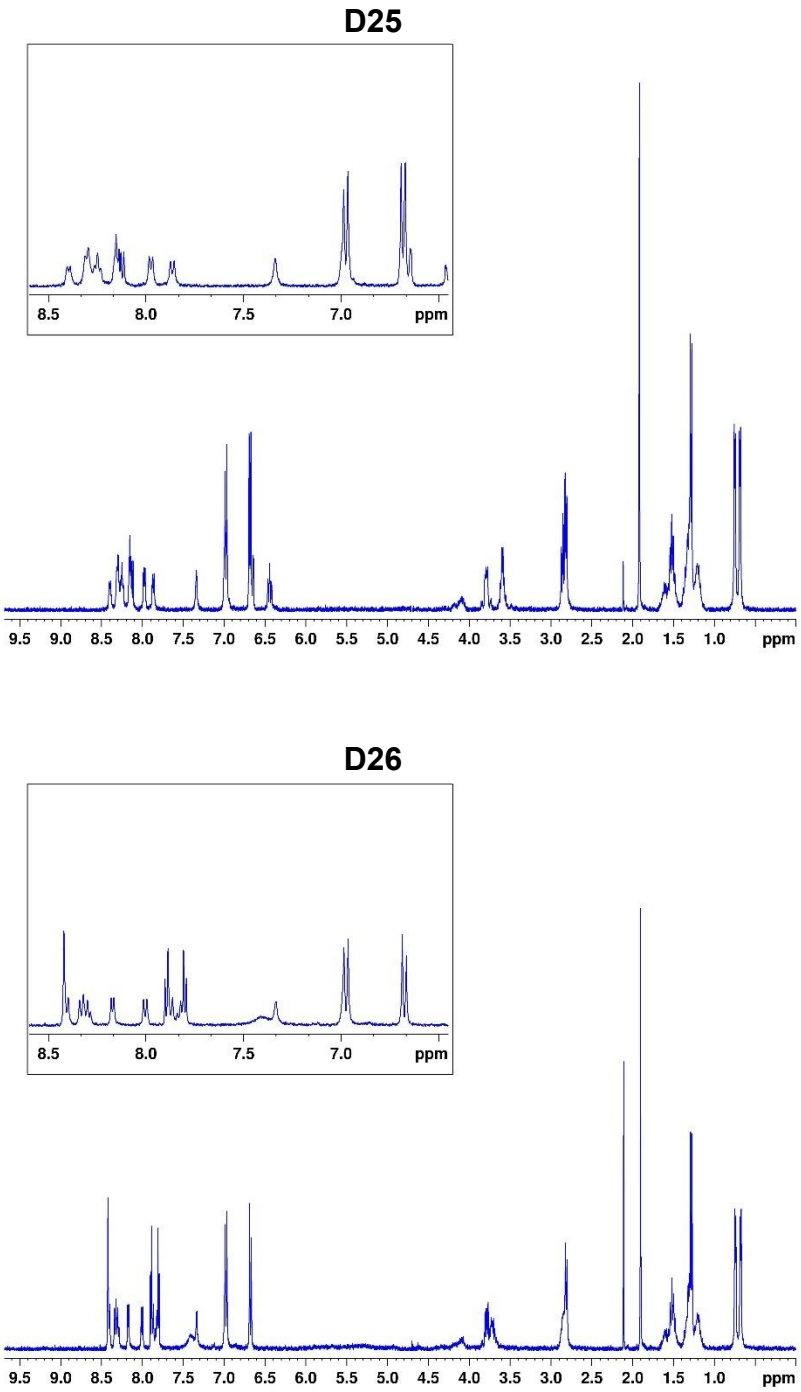


Figure S5. NMR spectra for nitroarylated model peptides (continued)

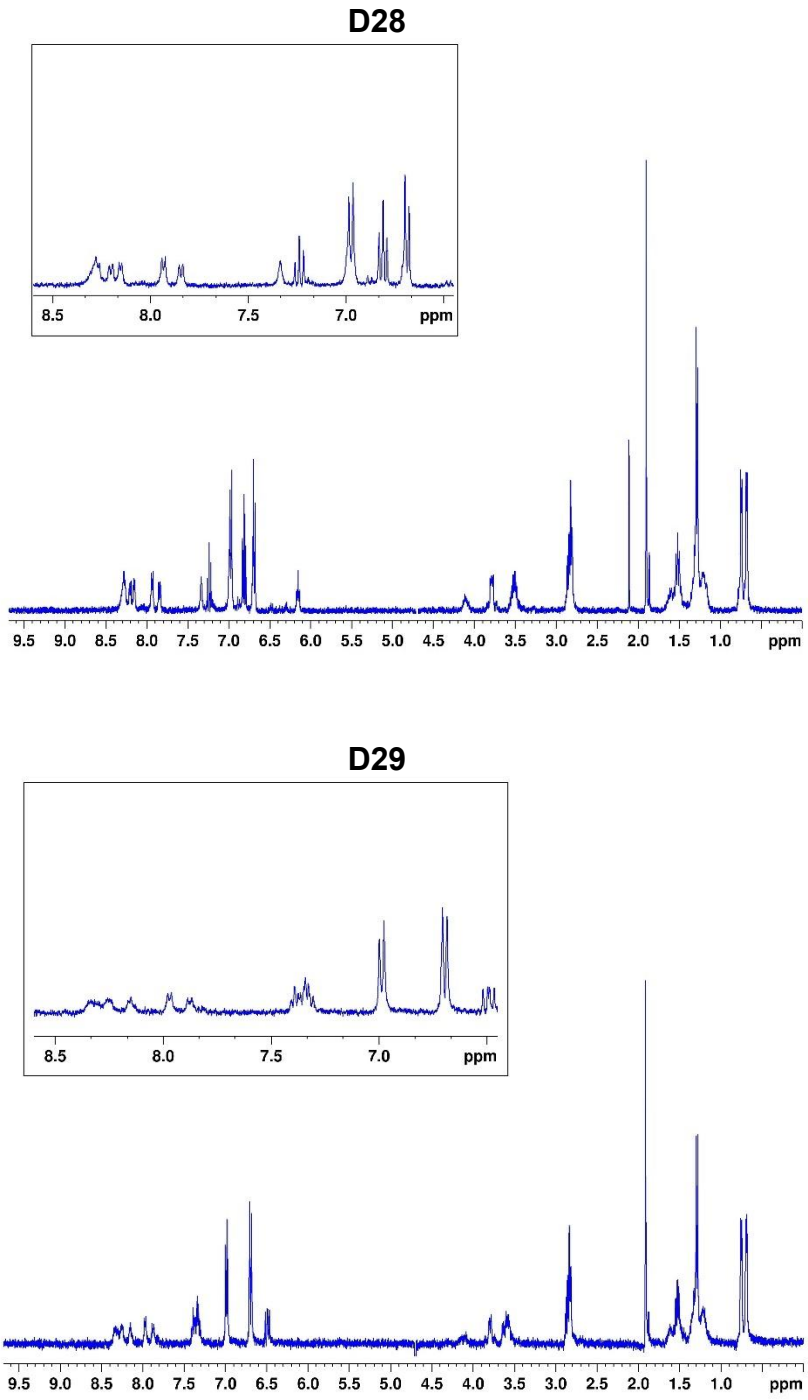
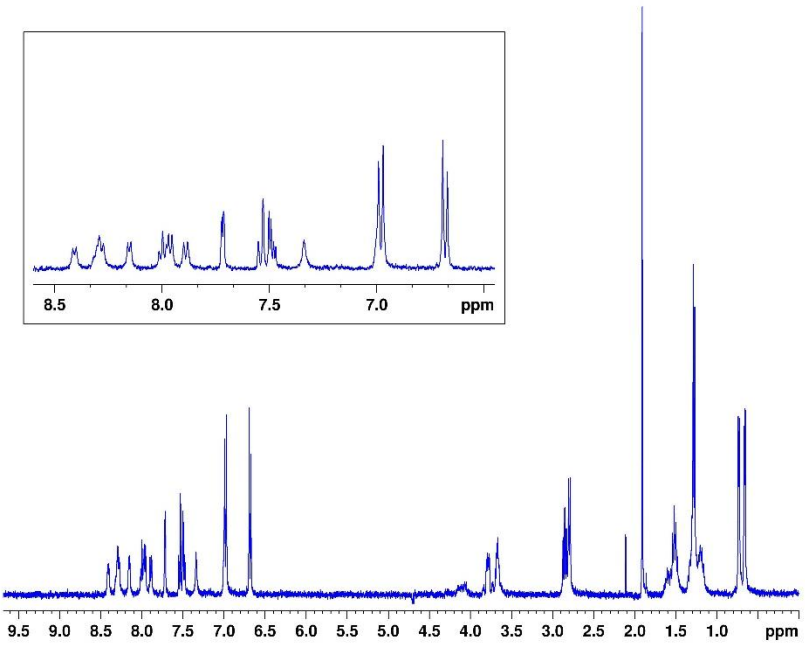


Figure S5. NMR spectra for nitroarylated model peptides (continued)

D30



D31

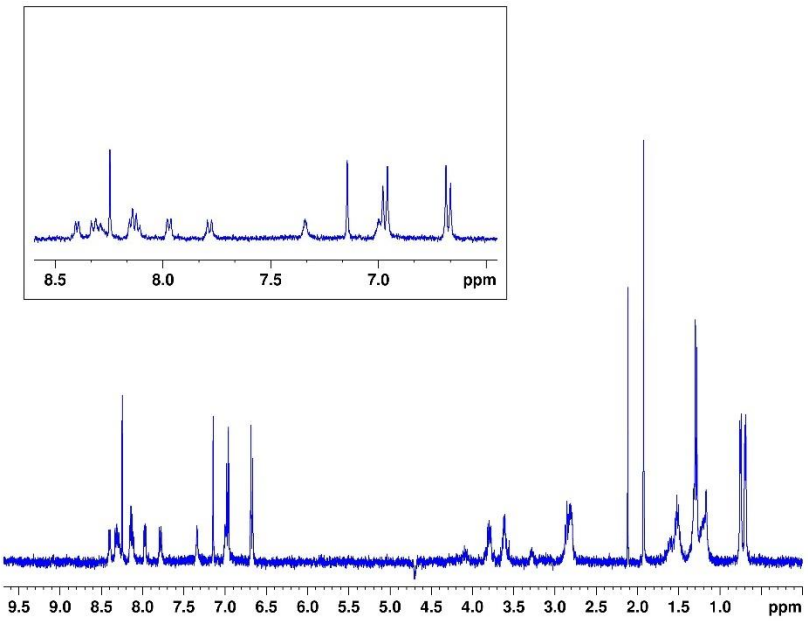


Figure S5. NMR spectra for nitroarylated model peptides (continued)

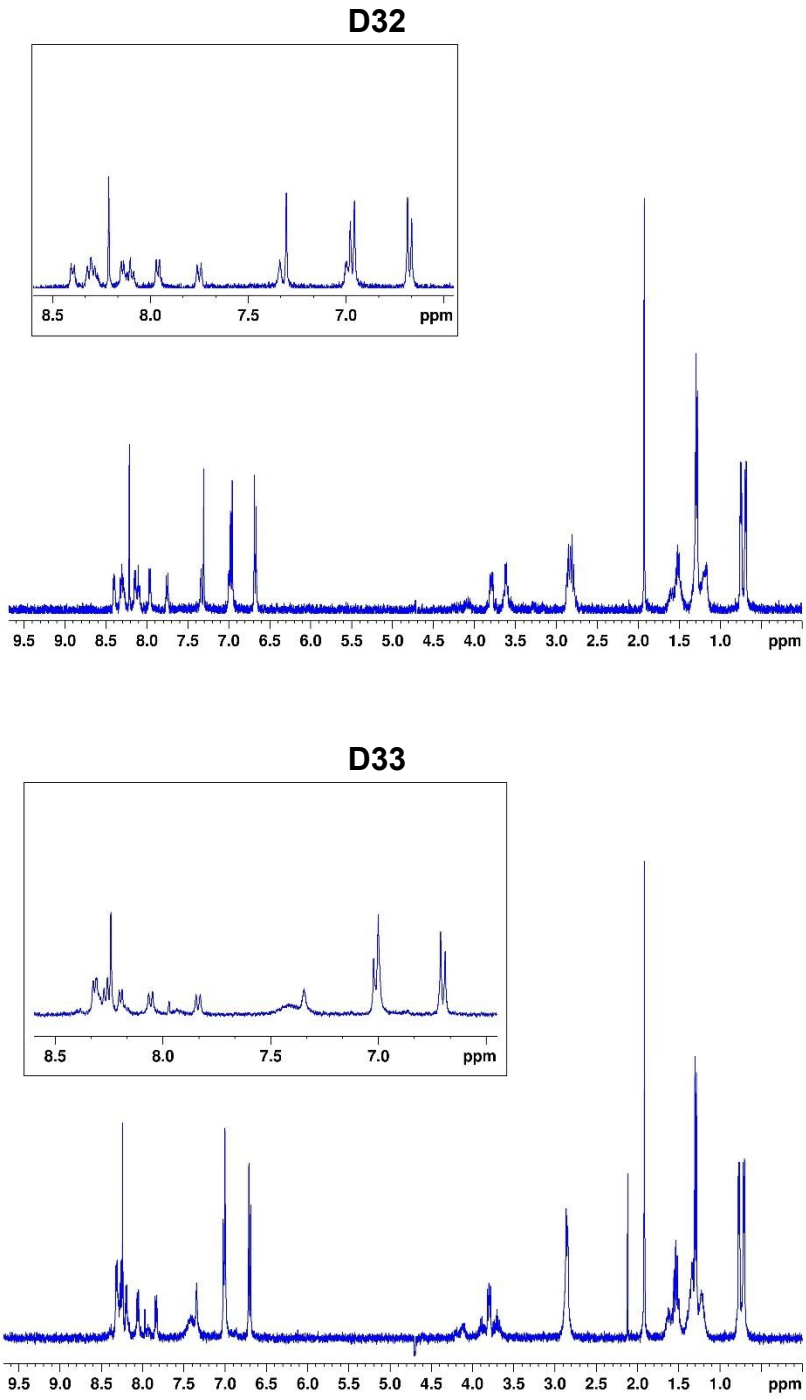


Figure S5. NMR spectra for nitroarylated model peptides (continued)

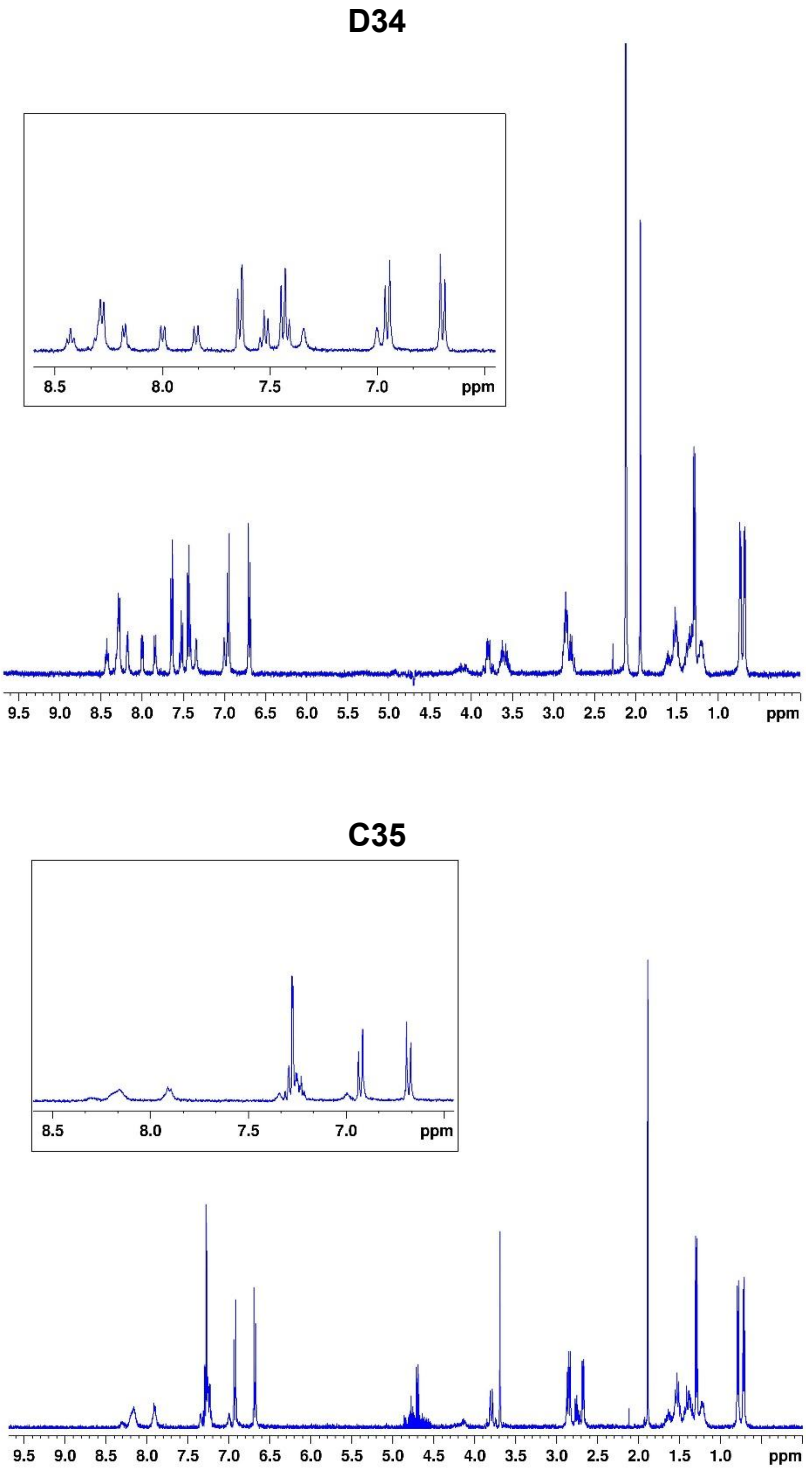


Figure S5. NMR spectra for nitroarylated model peptides (continued)

Table S1. Nitroarene reagents

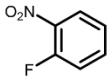
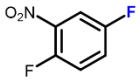
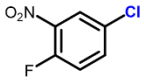
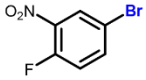
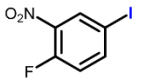
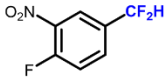
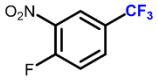
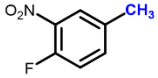
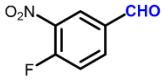
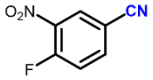
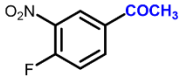
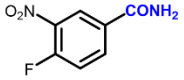
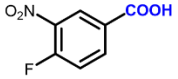
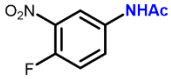
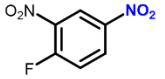
Arene	Chemical Name	CAS Number	Structure	Source
A1	1-Fluoro-2-nitrobenzene	1493-27-2		Oakwood Chemical
A2	2,5-Difluoronitrobenzene	364-74-9		Oakwood Chemical
A3	5-Chloro-2-fluoronitrobenzene	345-18-6		Oakwood Chemical
A4	5-Bromo-2-fluoronitrobenzene	364-73-8		Oakwood Chemical
A5	1-Fluoro-4-iodo-2-nitrobenzene	364-75-0		Oakwood Chemical
A6	4-(Difluoromethyl)-1-fluoro-2-nitrobenzene	61324-89-8		Ambeed
A7	4-Fluoro-3-nitrobenzotrifluoride	367-86-2		Oakwood Chemical
A8	4-Fluoro-3-nitrotoluene	446-11-7		Oakwood Chemical
A9	4-Fluoro-3-nitrobenzaldehyde	42564-51-2		Ambeed
A10	4-Fluoro-3-nitrobenzonitrile	1009-35-4		Ambeed
A11	4'-Fluoro-3'-nitroacetophenone	400-93-1		Ambeed
A12	4-Fluoro-3-nitrobenzamide	349-02-0		Ambeed
A13	4-Fluoro-3-nitrobenzoic acid	453-71-4		Ambeed
A14	N-(4-Fluoro-3-nitrophenyl)acetamide	351-32-6		Synthesized based on ref. 67
A15	1-Fluoro-2,4-dinitrobenzene	70-34-8		Oakwood Chemical

Table S1. Nitroarene reagents (continued)

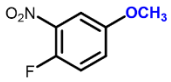
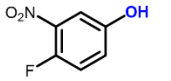
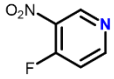
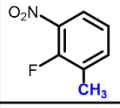
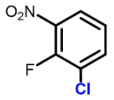
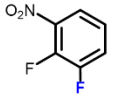
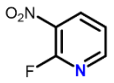
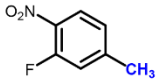
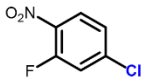
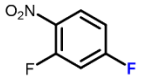
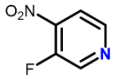
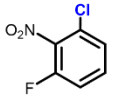
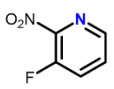
Arene	Chemical Name	CAS Number	Structure	Source
A16	4-Fluoro-3-nitroanisole	61324-93-4		Ambeed
A17	4-Fluoro-3-nitrophenol (from 4-(1,1-dimethylethoxy)-1-fluoro-2-nitrobenzene)	2105-96-6 (from CAS 1369848-20-3)		Ether prepared based on ref. 68
A18	4-Fluoro-3-nitropyridine	115812-96-9		Ambeed
A19	2-Fluoro-3-nitrotoluene	437-86-5		Ambeed
A20	3-Chloro-2-fluoronitrobenzene	2106-49-2		Ambeed
A21	2,3-Difluoronitrobenzene	6921-22-8		Ambeed
A22	2-Fluoro-3-nitropyridine	1480-87-1		Ambeed
A23	3-Fluoro-4-nitrotoluene	446-34-4		Ambeed
A24	4-Chloro-2-fluoronitrobenzene	700-37-8		Ambeed
A25	2,4-Difluoronitrobenzene	1009-35-4		Ambeed
A26	3-Fluoro-4-nitropyridine	13505-01-6		Ambeed
A27	3-Fluoro-2-nitrotoluene	3013-27-2		Ambeed
A28	2-Chloro-6-fluoronitrobenzene	453-71-4		Ambeed
A29	1,3-Difluoro-2-nitrobenzene	19064-24-5		Ambeed
A30	3-Fluoro-2-nitropyridine	54231-35-5		Ambeed

Table S1. Nitroarene reagents (continued)

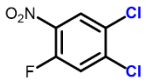
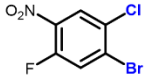
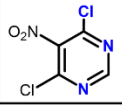
Arene	Chemical Name	CAS Number	Structure	Source
A31	1,2-Dichloro-4-fluoro-5-nitrobenzene	2339-78-8		Ambeed
A32	1-Bromo-2-chloro-5-fluoro-4-nitrobenzene	1027833-17-5		Ambeed
A33	4,6-Dichloro-5-nitropyrimidine	4316-93-2		Ambeed

Table S2. MALDI mass spectrometry characterization of peptides (Ac = acetyl, NH_2 = amidated C-terminus)

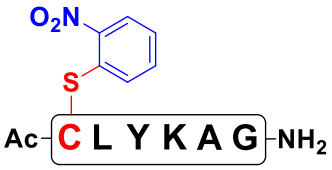
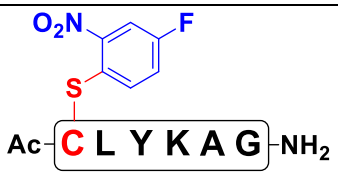
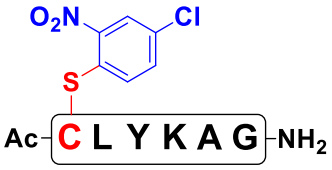
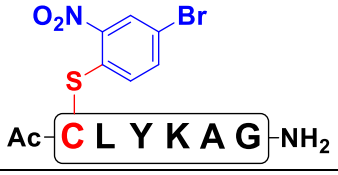
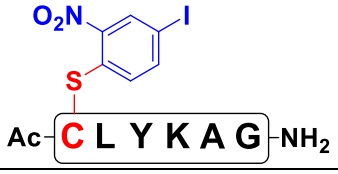
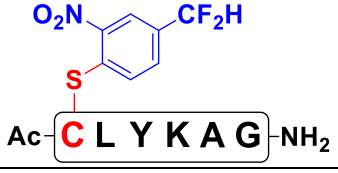
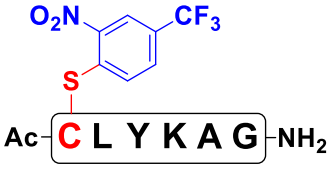
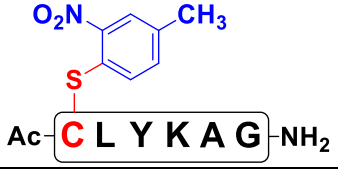
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
C1	Ac-C(**A1**)LYKAG-NH_2		816.4	816.6
C2	Ac-C(**A2**)LYKAG-NH_2		834.4	834.9
C3	Ac-C(**A3**)LYKAG-NH_2		850.3	849.7
C4	Ac-C(**A4**)LYKAG-NH_2		942.3	941.5
C5	Ac-C(**A5**)LYKAG-NH_2		942.3	941.5
C6	Ac-C(**A6**)LYKAG-NH_2		866.4	865.5
C7	Ac-C(**A7**)LYKAG-NH_2		884.4	883.7
C8	Ac-C(**A8**)LYKAG-NH_2		830.4	830.7

Table S2. MALDI mass spectrometry characterization of peptides (continued)
(Ac = acetyl, NH_2 = amidated C-terminus)

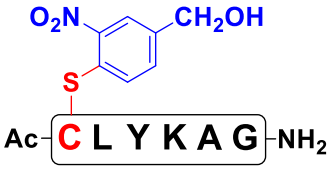
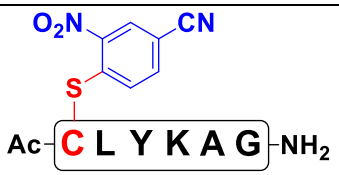
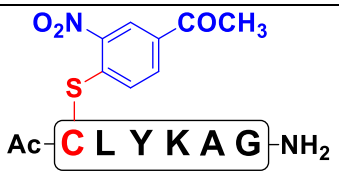
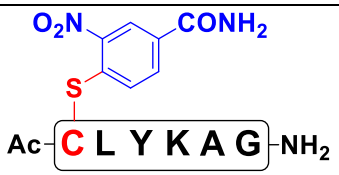
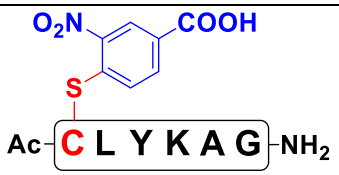
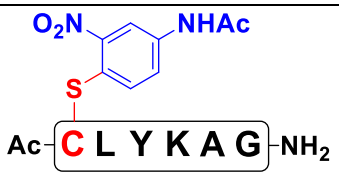
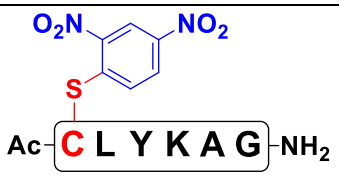
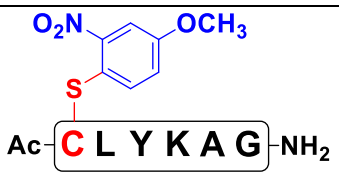
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
C9	Ac-C(**A9***)LYKAG-NH_2 * original aldehyde reduced to methyl alcohol		846.4	845.4
C10	Ac-C(**A10**)LYKAG-NH_2		841.4	840.6
C11	Ac-C(**A11**)LYKAG-NH_2		858.4	858.5
C12	Ac-C(**A12**)LYKAG-NH_2		859.4	858.8
C13	Ac-C(**A13**)LYKAG-NH_2		860.4	860.0
C14	Ac-C(**A14**)LYKAG-NH_2		873.4	873.5
C15	Ac-C(**A15**)LYKAG-NH_2		861.4	860.8
C16	Ac-C(**A16**)LYKAG-NH_2		846.4	845.6

Table S2. MALDI mass spectrometry characterization of peptides (continued)
(Ac = acetyl, NH_2 = amidated C-terminus)

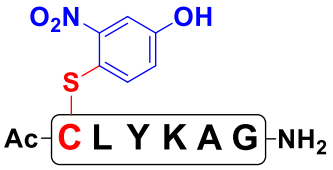
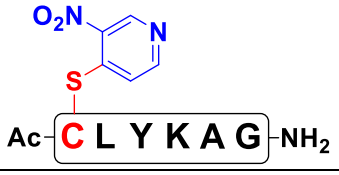
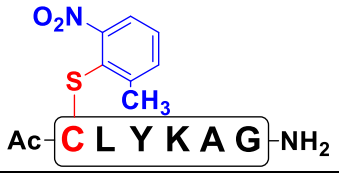
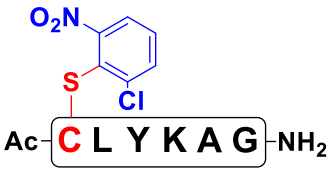
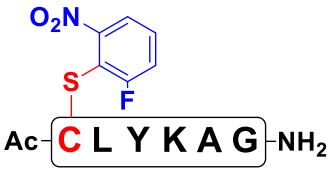
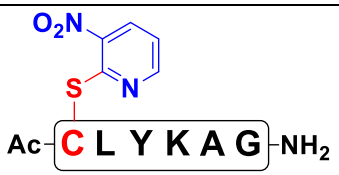
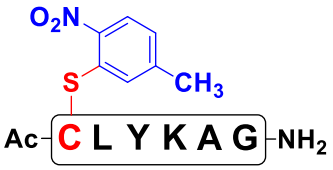
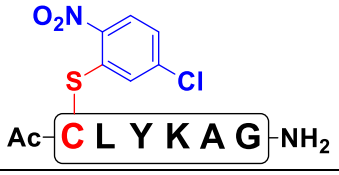
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
C17	Ac-C(**A17**)LYKAG-NH_2		832.9	832.7
C18	Ac-C(**A18**)LYKAG-NH_2		817.4	817.7
C19	Ac-C(**A19**)LYKAG-NH_2		830.4	829.7
C20	Ac-C(**A20**)LYKAG-NH_2		850.3	849.8
C21	Ac-C(**A21**)LYKAG-NH_2		834.4	833.8
C22	Ac-C(**A22**)LYKAG-NH_2		817.4	816.6
C23	Ac-C(**A23**)LYKAG-NH_2		830.4	829.8
C24	Ac-C(**A24**)LYKAG-NH_2		850.3	849.8

Table S2. MALDI mass spectrometry characterization of peptides (continued)
(Ac = acetyl, NH_2 = amidated C-terminus)

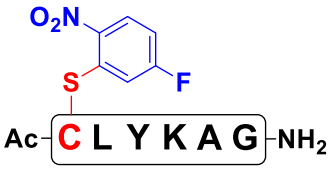
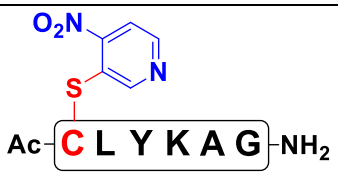
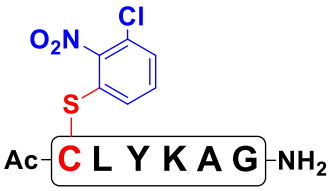
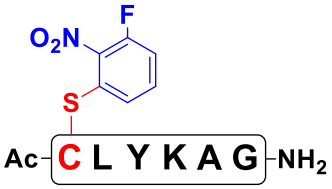
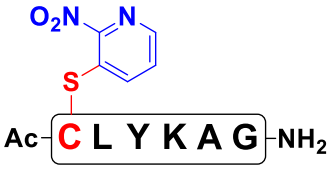
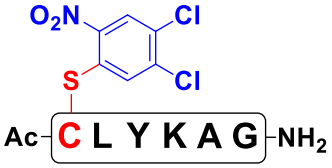
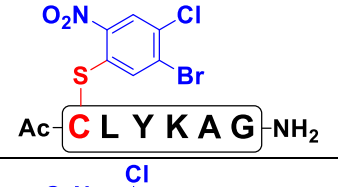
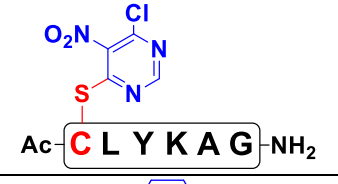
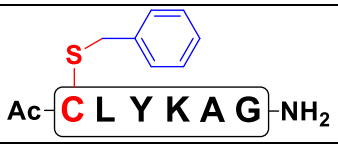
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
C25	Ac-C(**A25**)LYKAG-NH_2		834.4	834.8
C26	Ac-C(**A26**)LYKAG-NH_2		817.4	817.0
C28	Ac-C(**A28**)LYKAG-NH_2		850.3	850.5
C29	Ac-C(**A29**)LYKAG-NH_2		834.4	834.8
C30	Ac-C(**A30**)LYKAG-NH_2		817.4	817.9
C31	Ac-C(**A31**)LYKAG-NH_2		884.3	883.9
C32	Ac-C(**A32**)LYKAG-NH_2		928.2	928.6
C33	Ac-C(**A33**)LYKAG-NH_2		852.3	853.2
C35	Ac-C(**A35**)LYKAG-NH_2		785.4	785.9

Table S2. MALDI mass spectrometry characterization of peptides (Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue)

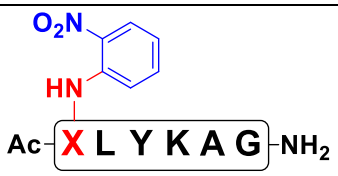
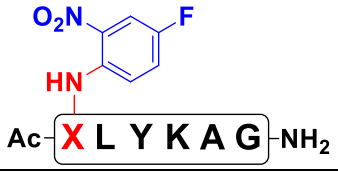
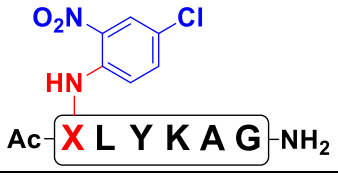
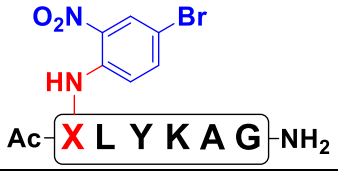
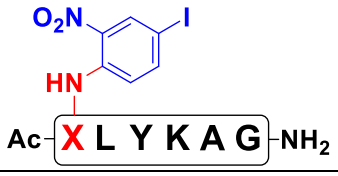
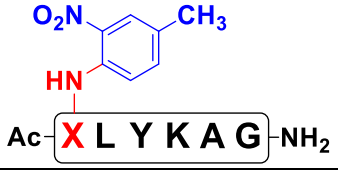
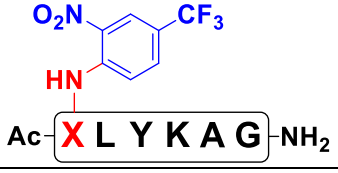
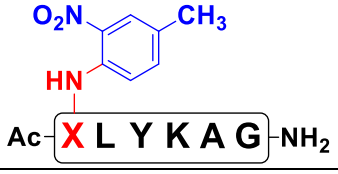
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
D1	Ac-Dap(**A1**)LYKAG-NH_2		799.4	798.7
D2	Ac-Dap(**A2**)LYKAG-NH_2		817.4	817.0
D3	Ac-Dap(**A3**)LYKAG-NH_2		833.3	833.0
D4	Ac-Dap(**A4**)LYKAG-NH_2		878.8	879.1
D5	Ac-Dap(**A5**)LYKAG-NH_2		925.3	926.1
D6	Ac-Dap(**A6***)LYKAG-NH_2 *original difluoromethyl group reduced to methyl group		813.4	812.8
D7	Ac-Dap(**A7**)LYKAG-NH_2		867.4	866.9
D8	Ac-Dap(**A8**)LYKAG-NH_2		813.4	813.1

Table S2. MALDI mass spectrometry characterization of peptides (continued) (Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue)

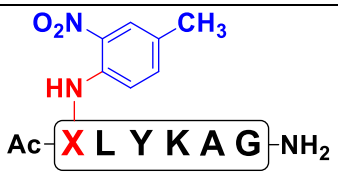
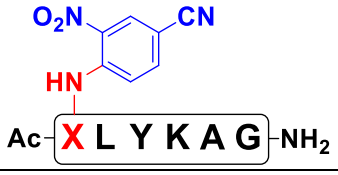
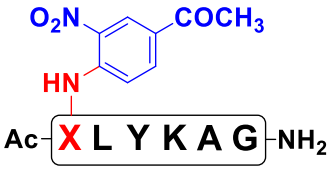
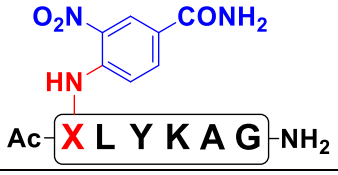
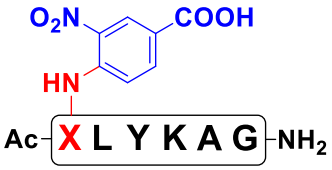
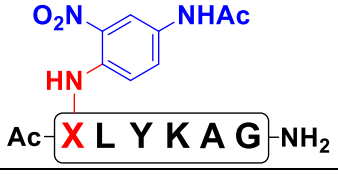
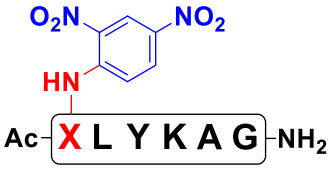
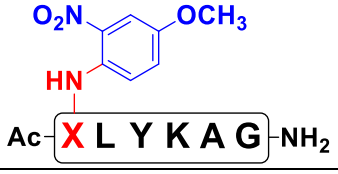
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
D9	Ac-Dap(**A9***)LYKAG-NH_2 *original aldehyde reduced to methyl group		813.4	813.7
D10	Ac-Dap(**A10**)LYKAG-NH_2		824.4	823.9
D11	Ac-Dap(**A11**)LYKAG-NH_2		842.4	842.6
D12	Ac-Dap(**A12**)LYKAG-NH_2		842.4	843.0
D13	Ac-Dap(**A13**)LYKAG-NH_2		843.4	844.2
D14	Ac-Dap(**A14**)LYKAG-NH_2		856.4	856.6
D15	Ac-Dap(**A15**)LYKAG-NH_2		844.4	845.3
D16	Ac-Dap(**A16**)LYKAG-NH_2		829.4	829.8

Table S2. MALDI mass spectrometry characterization of peptides (continued) (Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue)

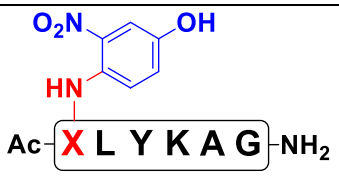
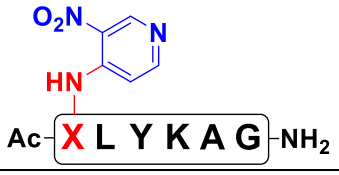
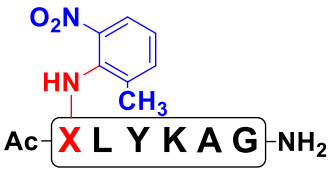
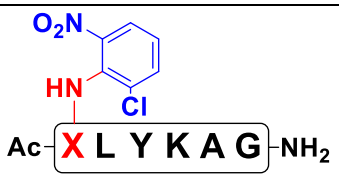
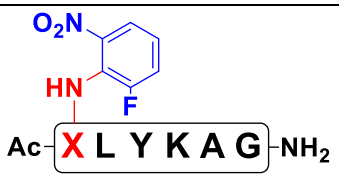
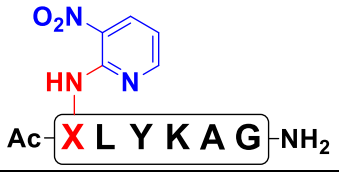
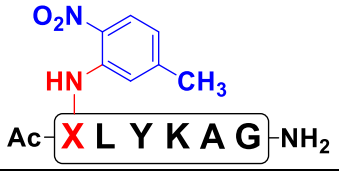
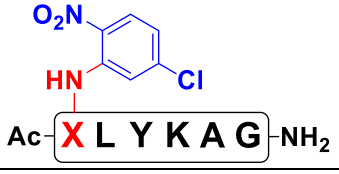
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
D17	Ac-Dap(**A17**)LYKAG-NH_2		815.9	815.7
D18	Ac-Dap(**A18**)LYKAG-NH_2		800.4	800.7
D19	Ac-Dap(**A19**)LYKAG-NH_2		813.4	813.1
D20	Ac-Dap(**A20**)LYKAG-NH_2		833.3	833.2
D21	Ac-Dap(**A21**)LYKAG-NH_2		817.4	816.9
D22	Ac-Dap(**A22**)LYKAG-NH_2		800.4	799.5
D23	Ac-Dap(**A23**)LYKAG-NH_2		813.4	814.0
D24	Ac-Dap(**A24**)LYKAG-NH_2		833.3	832.9

Table S2. MALDI mass spectrometry characterization of peptides (continued)
(Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue)

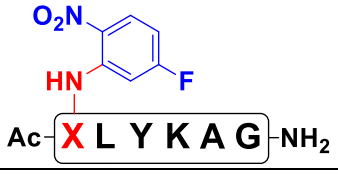
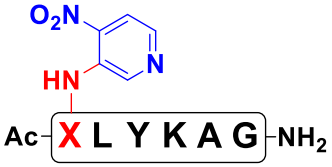
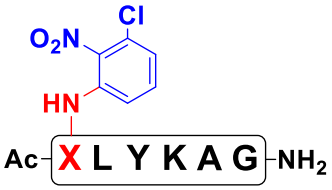
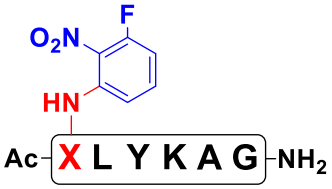
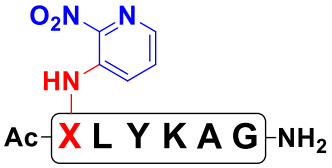
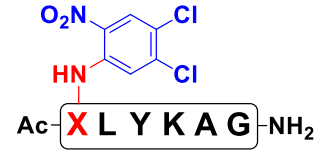
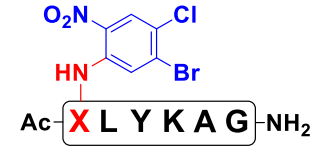
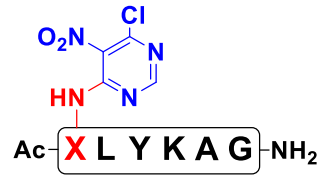
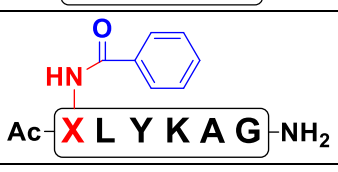
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
D25	Ac-Dap(**A25**)LYKAG-NH_2		817.4	817.0
D26	Ac-Dap(**A26**)LYKAG-NH_2		800.4	800.7
D28	Ac-Dap(**A28**)LYKAG-NH_2		833.3	833
D29	Ac-Dap(**A29**)LYKAG-NH_2		817.4	817.1
D30	Ac-Dap(**A30**)LYKAG-NH_2		800.4	799.8
D31	Ac-Dap(**A31**)LYKAG-NH_2		867.3	866.9
D32	Ac-Dap(**A32**)LYKAG-NH_2		911.2	911.0
D33	Ac-Dap(**A33**)LYKAG-NH_2		835.4	834.9
D34	Ac-Dap(**A34**)LYKAG-NH_2		782.4	782.8

Table S2. MALDI mass spectrometry characterization of peptides (continued)
(NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue, c = 3-mercaptopropionic acid; r = L-homoarginine)

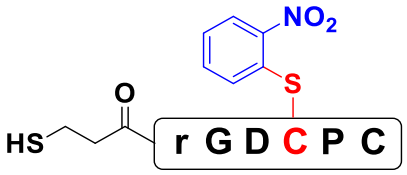
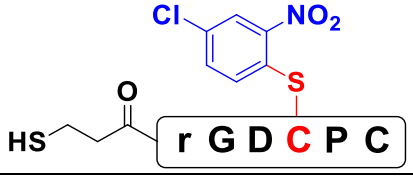
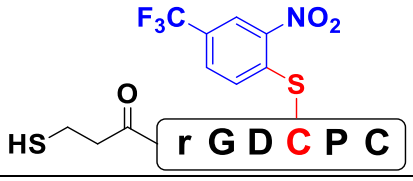
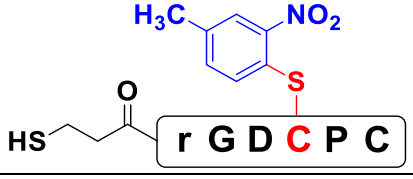
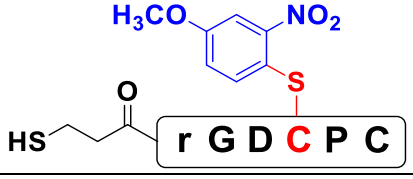
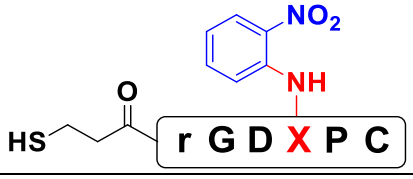
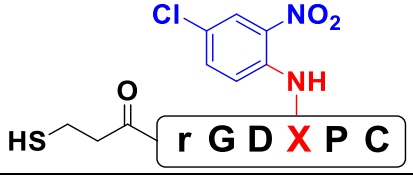
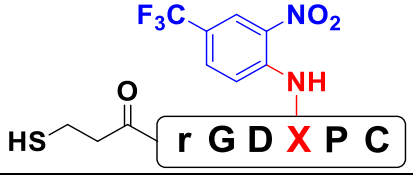
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
E-C1	crGDC(**A1**)PC-NH_2		872.3	872.7
E-C3	crGDC(**A3**)PC-NH_2		906.2	906.6
E-C7	crGDC(**A7**)PC-NH_2		940.2	940.6
E-C8	crGDC(**A8**)PC-NH_2		886.3	886.8
E-C16	crGDC(**A16**)PC-NH_2		902.3	902.8
E-D1	crGD-Dap(**A1**)PC-NH_2		855.3	855.3
E-D3	crGD-Dap(**A3**)PC-NH_2		889.3	889.9
E-D7	crGD-Dap(**A7**)PC-NH_2		923.3	923.6

Table S2. MALDI mass spectrometry characterization of peptides (continued) (Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue, c = 3-mercaptopropionic acid; r = L-homoarginine)

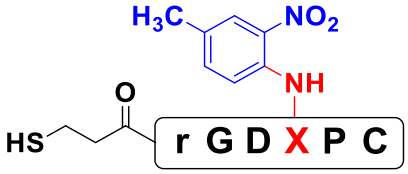
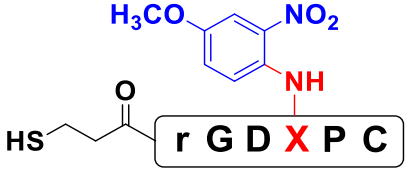
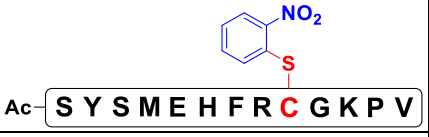
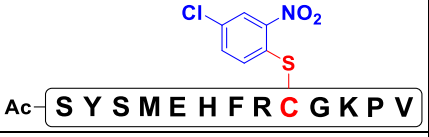
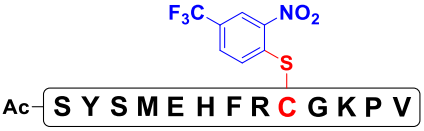
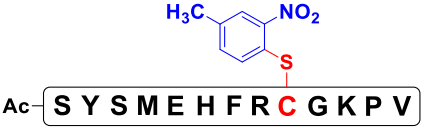
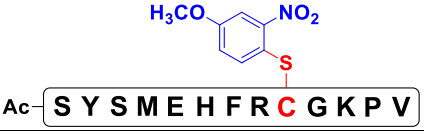
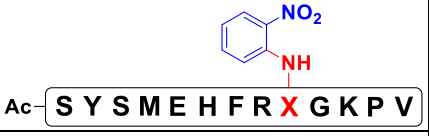
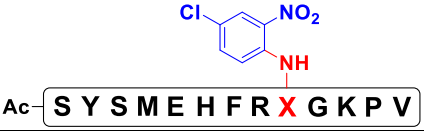
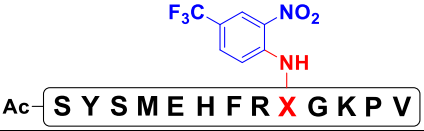
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
E-D8	crGD-Dap(**A8**)PC-NH_2		869.3	869.5
E-D16	crGD-Dap(**A16**)PC-NH_2		885.3	885.7
M-C1	Ac-SYSMEHFRC(**A1**)GKPV-NH_2		1702.7	1703.5
M-C3	Ac-SYSMEHFRC(**A3**)GKPV-NH_2		1736.7	1736.2
M-C7	Ac-SYSMEHFRC(**A7**)GKPV-NH_2		1770.7	1770.9
M-C8	Ac-SYSMEHFRC(**A8**)GKPV-NH_2		1716.8	1717.9
M-C16	Ac-SYSMEHFRC(**A16**)GKPV-NH_2		1732.8	1733.7
M-D1	Ac-SYSMEHFR-Dap(**A1**)GKPV-NH_2		1685.8	1686.4
M-D3	Ac-SYSMEHFR-Dap(**A3**)GKPV-NH_2		1719.7	1720.4
M-D7	Ac-SYSMEHFR-Dap(**A7**)GKPV-NH_2		1753.8	1753.1

Table S2. MALDI mass spectrometry characterization of peptides (continued)
(Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue)

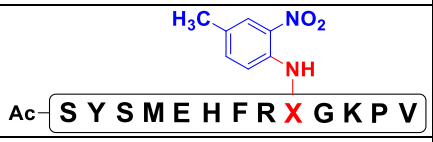
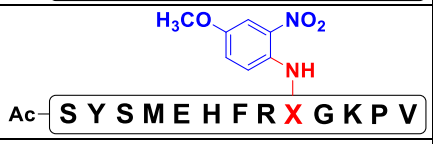
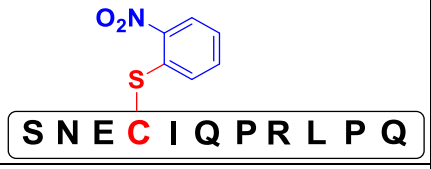
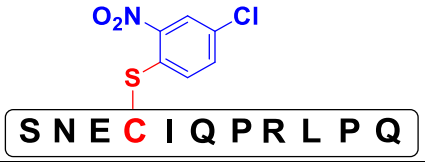
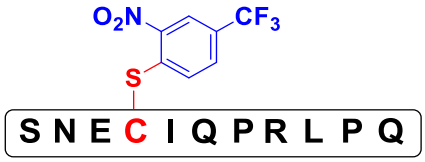
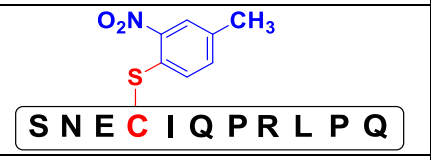
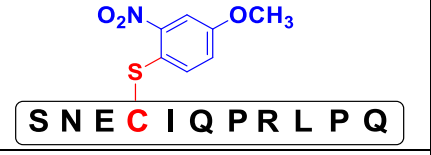
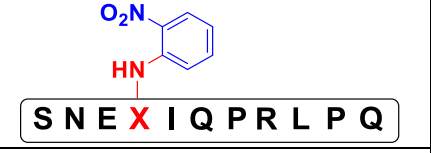
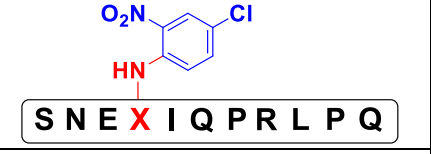
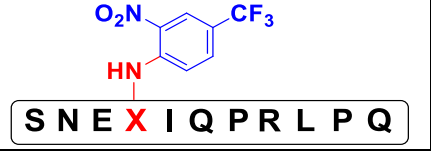
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
M-D8	Ac-SYSMEHFR-Dap(**A8**)GKPV-NH_2		1699.8	1700.5
M-D16	Ac-SYSMEHFR-Dap(**A16**)GKPV-NH_2		1715.8	1716.5
S-C1	SNEC(**A1**)IQPRLPQ-NH_2		1404.7	1404.3
S-C3	SNEC(**A3**)IQPRLPQ-NH_2		1418.7	1418.2
S-C7	SNEC(**A7**)IQPRLPQ-NH_2		1472.6	1472.0
S-C8	SNEC(**A8**)IQPRLPQ-NH_2		1418.3	1417.9
S-C16	SNEC(**A16**)IQPRLPQ-NH_2		1434.7	1434.3
S-D1	SNE-Dap(**A1**)IQPRLPQ-NH_2		1387.7	1387.3
S-D3	SNE-Dap(**A3**)IQPRLPQ-NH_2		1422.7	1421.9
S-D7	SNE-Dap(**A7**)IQPRLPQ-NH_2		1455.7	1456.1

Table S2. MALDI mass spectrometry characterization of peptides (continued) (Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue, e = L-pyroglutamic acid, w = D-tryptophan)

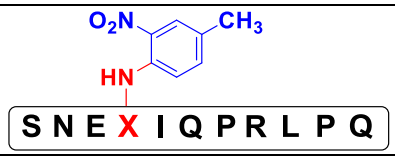
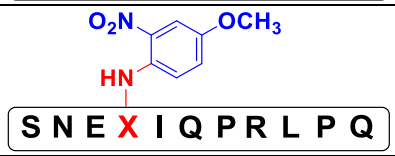
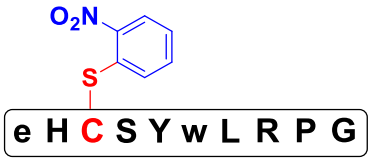
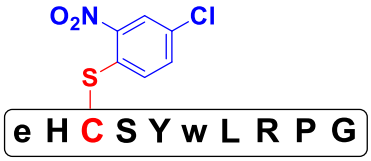
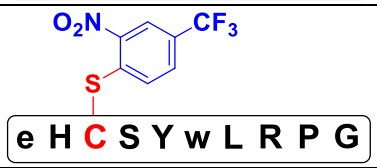
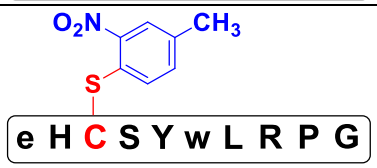
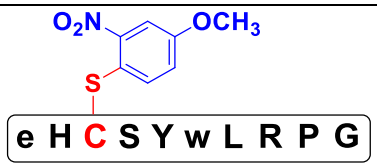
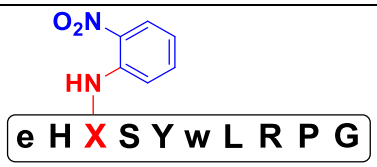
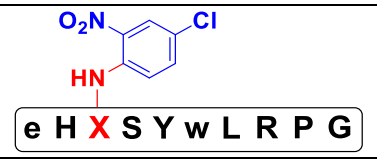
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
S-D8	SNE-Dap(**A8**)IQPRLPQ-NH_2		1401.7	1401.4
S-D16	SNE-Dap(**A16**)IQPRLPQ-NH_2		1417.7	1417.6
T-C1	eHC(**A1**)SYwLRPG-NH_2		1349.6	1349.8
T-C3	eHC(**A3**)SYwLRPG-NH_2		1383.5	1384.0
T-C7	eHC(**A3**)SYwLRPG-NH_2		1417.6	1417.9
T-C8	eHC(**A3**)SYwLRPG-NH_2		1364.0	1363.2
T-C16	eHC(**A3**)SYwLRPG-NH_2		1379.6	1379.9
T-D1	eH-Dap(**A1**)SYwLRPG-NH_2		1332.6	1333.4
T-D3	eH-Dap(**A3**)SYwLRPG-NH_2		1365.6	1366.9

Table S2. MALDI mass spectrometry characterization of peptides (continued) (Ac = acetyl, NH_2 = amidated C-terminus, Dap/X = 2,3-diaminopropionic acid residue, e = L-pyroglutamic acid, w = D-tryptophan, ACC = 7-amino-4-carbamoylmethylcoumarin)

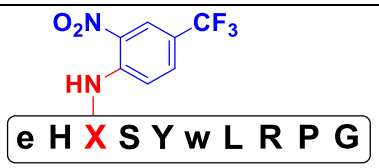
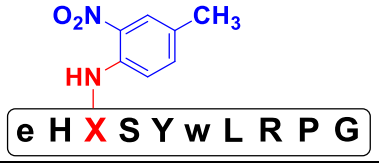
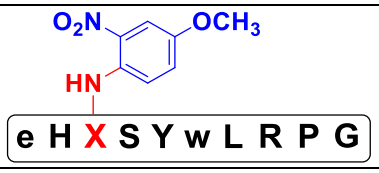
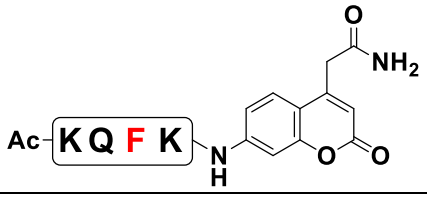
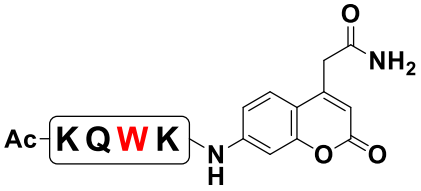
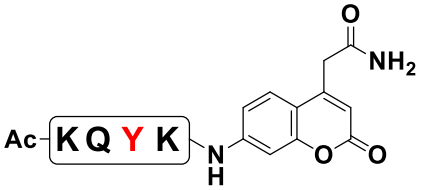
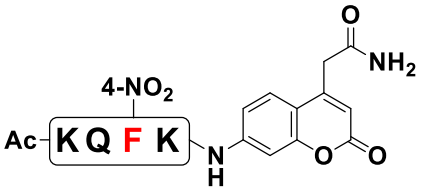
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
T-D7	eH-Dap(**A7**)SYwLRPG-NH_2		1400.6	1401.0
T-D8	eH-Dap(**A8**)SYwLRPG-NH_2		1346.6	1346.7
T-D16	eH-Dap(**A16**)SYwLRPG-NH_2		1362.6	1363.1
SubF	Ac-KQFK-ACC-NH_2		792.4	792.6
SubW	Ac-KQWK-ACC-NH_2		831.4	831.9
SubY	Ac-KQYK-ACC-NH_2		808.4	808.6
Sub4nF	Ac-KQ-4nF-K-ACC-NH_2		837.4	837.8

Table S2. MALDI mass spectrometry characterization of peptides (continued) (Ac = acetyl, NH_2 = amidated C-terminus, X = 2,3-diaminopropionic acid residue, ACC = 7-amino-4-carbamoylmethylcoumarin)

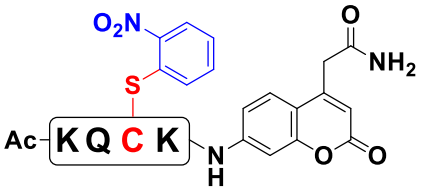
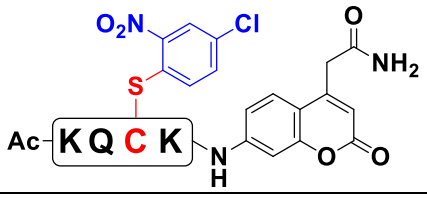
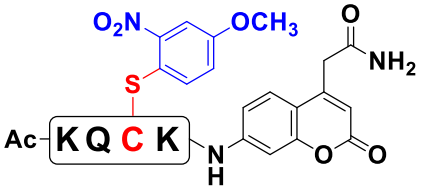
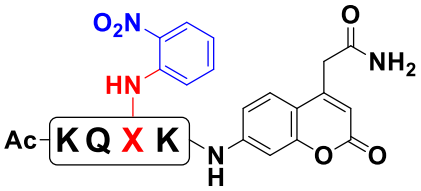
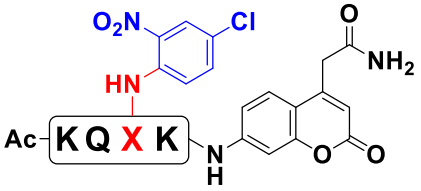
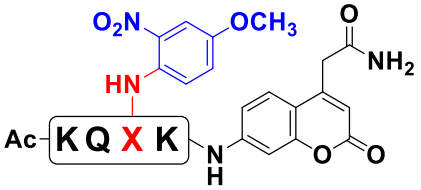
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
SubC1	Ac-KQC(**A1**)K-ACC-NH_2		869.4	869.7
SubC3	Ac-KQC(**A3**)K-ACC-NH2		903.3	903.5
SubC16	Ac-KQC(**A16**)K-ACC-NH_2		899.4	899.4
SubD1	Ac-KQ-Dap(**A1**)K-ACC-NH_2		852.4	852.8
SubD3	Ac-KQ-Dap(**A3**)K-ACC-NH_2		886.4	887.1
SubD16	Ac-KQ-Dap(**A16**)K-ACC-NH_2		882.4	882.7

Table S2. MALDI mass spectrometry characterization of peptides (Ac = acetyl, NH_2 = amidated C-terminus; r indicates nitro reduction to amine)

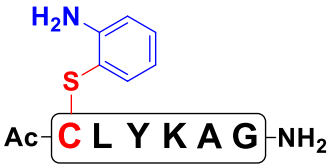
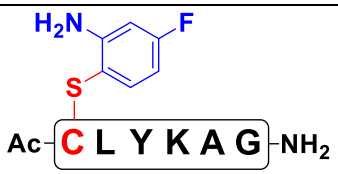
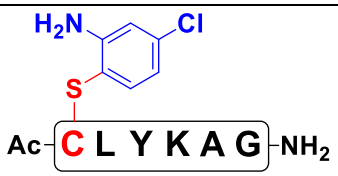
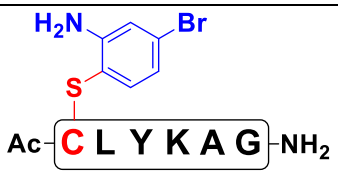
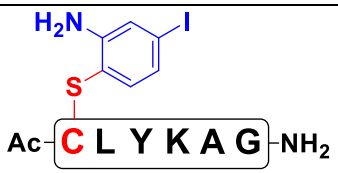
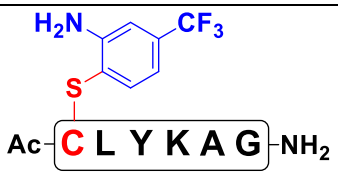
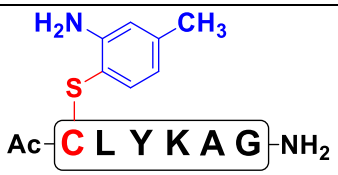
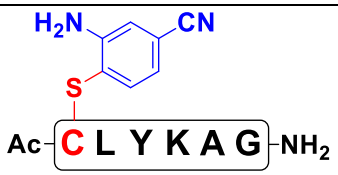
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
C1r	Ac-C(**A1r**)LYKAG-NH_2		786.4	785.8
C2r	Ac-C(**A2r**)LYKAG-NH_2		804.4	803.6
C3r	Ac-C(**A3r**)LYKAG-NH_2		820.4	819.9
C4r	Ac-C(**A4r**)LYKAG-NH_2		864.3	863.4
C5r	Ac-C(**A5r**)LYKAG-NH_2		912.3	911.3
C7r	Ac-C(**A7r**)LYKAG-NH_2		854.4	854.1
C8r	Ac-C(**A8r**)LYKAG-NH_2		800.4	799.6
C10r	Ac-C(**A10r**)LYKAG-NH_2		811.4	810.9

Table S2. MALDI mass spectrometry characterization of peptides (Ac = acetyl, NH_2 = amidated C-terminus; r indicates nitro reduction to amine)

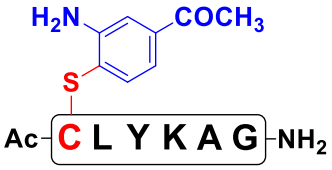
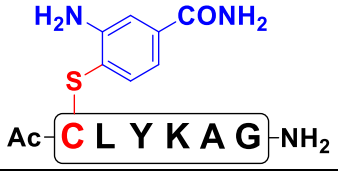
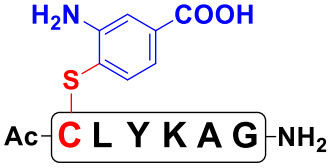
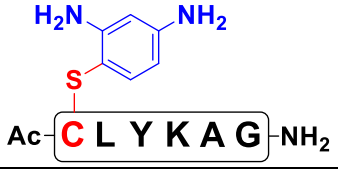
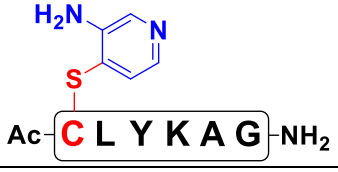
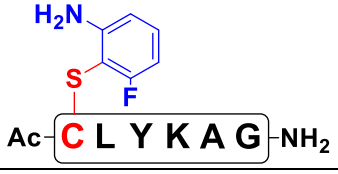
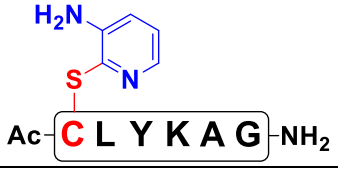
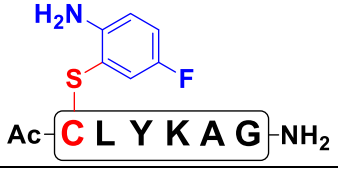
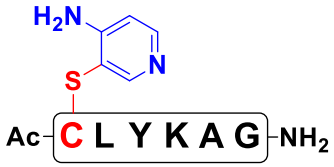
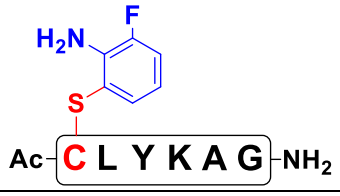
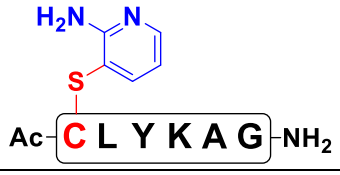
Peptide	Sequence	Structure	Expected m/z ($M+H^+$)	Observed m/z ($M+H^+$)
C11r	Ac-C(**A11r**)LYKAG-NH_2		828.4	827.6
C12r	Ac-C(**A12r**)LYKAG-NH_2		829.4	828.2
C13r	Ac-C(**A13r**)LYKAG-NH_2		830.4	829.4
C15r	Ac-C(**A15r**)LYKAG-NH_2		801.4	800.5
C18r	Ac-C(**A18r**)LYKAG-NH_2		787.4	786.5
C21r	Ac-C(**A21r**)LYKAG-NH_2		804.4	803.5
C22r	Ac-C(**A22r**)LYKAG-NH_2		787.4	786.5
C25r	Ac-C(**A25r**)LYKAG-NH_2		804.4	803.5

Table S2. MALDI mass spectrometry characterization of peptides (Ac = acetyl, NH_2 = amidated C-terminus; r indicates nitro reduction to amine)

C26r	Ac-C(**A26r**)LYKAG-NH_2		787.4	786.4
C29r	Ac-C(**A29r**)LYKAG-NH_2		804.4	803.7
C30r	Ac-C(**A30r**)LYKAG-NH_2		787.4	787.1

Materials and Methods

General Information. Commercially purchased solvents and reagents were used without further purification. Nα-Fmoc-protected amino acids and peptide synthesis reagents were purchased from Advanced ChemTech, ChemImpex International, Oakwood Chemical, Gyros Protein Technologies, and WuXi TIDES. Specialized amino acids are as follows: Boc-Ser(tBu)-OH (CAS 13734-38-8, Ambeed), Fmoc-Cys(Mmt)-OH (CAS 177582-21-7, WuXi), Fmoc-Dap(Mtt)-OH (CAS 654670-89-0, WuXi), pyroglutamic acid (CAS 98-79-3, Ambeed), Fmoc-D-Trp(Boc)-OH (CAS 163619-04-3, Oakwood), 3-(tritylthio)propionic acid (CAS 27144-18-9, Ambeed), Fmoc-L-homoarginine(Pbf)-OH (CAS 1159680-21-3, Ambeed). A complete list of nitroarenes (including CAS numbers and sources) is provided in the Supporting Information (Table S1). Plasmin was purchased from Hematologic Technologies as described previously.[1]

Peptides were purified on preparative C_{18} columns using reverse-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu Nexera HPLC system using gradients of water and acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). Peptide purity was evaluated by analytical HPLC using a Luna 5 µm C18(2) column (150 × 4.6 mm) on an Agilent 1100 HPLC system with a flow rate of 0.3 mL/min (gradient: 5–95% solvent B over 30 min, solvent A = 0.1% aqueous TFA, B = 95% ACN, 5% water, 0.1% TFA; Figure S4). High-resolution mass spectrometry data were collected on a Shimadzu MALDI-8020 Benchtop MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix (Table S2).

Synthesis of Compounds A14 and A17. Compound **A14** (N-(4-fluoro-3-nitrophenyl)acetamide, CAS# 351-32-6) was prepared according to the method of Cordeiro *et al*.[2] Compound **A17** (4-(1,1-dimethylethoxy)-1-fluoro-2-nitrobenzene, CAS# 1369848-20-3) was prepared according to the method of Bartoli *et al*.[3]

Peptide Synthesis. All peptides were synthesized using a Gyros Protein Technologies PurePep™ Chorus synthesizer with peptide coupling reactions performed at 55 °C following standard Fmoc solid-phase approaches on Rink MBHA resin. All peptides were synthesized on low-loading resin (0.2-0.4 mmol/g resin). Dried Rink MBHA resin was swollen for 10 min in N,N-dimethylformamide (DMF) before the first deprotection reaction. Coupling reactions were performed using 4 mol equiv each of Nα-Fmoc-protected amino acid, O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), and N-methylmorpholine (NMM) at 55 °C. Deprotection of the Fmoc protecting group was carried out at RT by two sequential reactions of 15 min each using 20% (v/v) piperidine in DMF. Between coupling and deprotection reactions, the resin was washed three times with DMF. At the end of peptide synthesis, the peptide was washed with DMF (3×) and dichloromethane (DCM) (4×).

For the model peptide Ac-**X**LYKAG-NH_2 (X = Cys(Mmt) or Dap(Mtt)), Cys/Dap substituted α-MSH variants, and Cys/Dap-substituted plasmin substrate peptides, the N-

terminal Fmoc group was removed as described above. After Fmoc removal, peptides were acetyl capped by reaction of 8 mol equiv acetic anhydride and 1 mol equiv N,N-diisopropylethylamine (DIEA) in 2-3 mL DMF for 30-60 min at RT. For variants of the other three peptides (SNEW, triptorelin, and eptifibatide), the N-terminal amino acids did not require further manipulation prior to arylation. For SNEW, the N-terminal serine was coupled as Boc-Ser(tBu)-OH. For triptorelin, the N-terminal pyroglutamic acid was coupled without protection. For eptifibatide, the N-terminal 3-mercaptopropionic acid was coupled as 3-(tritylthio)propionic acid.

At various points during synthesis, microcleavage reactions were performed by cleaving a small amount of resin with 1 mL of freshly prepared 95% TFA, 2.5% H_2O, and 2.5% triisopropylsilane (TIPS) for 30 min. After dilution with 3–4 mL acetonitrile, 1 µL of sample was mixed with 4 µL of saturated α-cyano-4-hydroxycinammic acid (CHCA, saturated solution prepared in 1:1 acetonitrile/water with 0.1% trifluoroacetic acid). A 1 µL volume of this mixture was added to a stainless steel MALDI plate and analyzed by MALDI.

Selective Deprotection of Cys(Mmt) and Dap(Mtt).

Mmt and Mtt deprotection were performed using 1% TFA and 5% TIPS in DCM (100 mL/mmol peptide). After 5 minutes, the deprotection solution was filtered out, and a fresh batch of deprotection solution was added. This procedure was repeated until no visible color was observed in the deprotection solution (typically 6-8 rounds). For Cys-containing peptides, deprotection was performed immediately before arylation to minimize competing oxidation/disulfide bond formation.

Selective Deprotection of Cys(STmp).

For Cys variants of dihydro-eptifibatide, which already has one cysteine residue and a 3-mercaptopropionyl group at the N-terminus in addition to the Cys/Dap residue to be modified, we incorporated Cys(STmp) at the site of modification instead of Cys(Mmt) because the standard trityl protecting group for cysteine is partially removed during Mmt deprotection.[4] For STmp deprotection on resin, we followed the protocol described by Postma *et al.*[4] Briefly, a solution containing 5% (w/v) dithiothreitol (DTT) and 0.1 M *N*-methylmorpholine was prepared in DMF. This solution was added to the resin, reacted for 5 min at RT, and filtered out. This procedure was repeated three times with freshly prepared solution each time.

Selective Deprotection of Dap(Alloc).

For dihydro-eptifibatide, we incorporated Dap(Alloc) at the site of modification instead of Dap(Mtt). For Alloc deprotection on resin, we followed the protocol described by Gomez-Martinez *et al* and others.[5,6] Briefly, a solution containing 0.1 mol equiv tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) and 5 mol equiv triphenylphosphine (PPh_3) was prepared in DCM, added to DCM-washed resin with 6 mol equiv ammonia-borane complex ($H_3N{\cdot}BH_3$), and shaken for 30 min at RT. This procedure was repeated a second time with 0.2 mol equiv $Pd(PPh_3)_4$ with filtration after each round of deprotection.

After deprotection, the resin was sequentially washed as follows: 4× DCM, 2× 0.2% TFA in DCM, 3× DCM, 4× 5% DIEA in DCM, 4× DCM, and 3× DMF. Once washed, a solution containing 5% diethyldithiocarbamate in DMF was prepared to remove residual Pd. The resin was shaken three times for 5 min at RT with portions of this solution with filtration after each round.

Cysteine Arylation. Immediately after Cys side-chain deprotection, the resin was washed 3x with DCM. A solution containing 5 mol equiv of fluoronitroarene and 5 mol equiv of DIEA was prepared in DMF (10 mL/mmol peptide). After washing the resin 3x with DMF, the fluoronitroarene solution was immediately and carefully added to completely cover the resin. Arylation reactions were shaken overnight (16-20 h) at RT. After arylation, the solution was filtered, and the resin was washed 3x DCM and 3x DMF then dried 2x with diethyl ether. For all peptides other than the model peptide, 10 mol equiv of fluoronitroarene and 10 mol equiv of DIEA were added instead of 5 mol equiv of each.

Dap Arylation. After Dap side-chain deprotection, the resin was washed 3x DCM and 3x DMF then dried 2x with diethyl ether. Dried resin was then added to a microcentrifuge tube. A solution containing 5 mol equiv fluoronitroarene and 10 mol equiv DIEA in DMF (10 mL/mmol peptide) was added to the resin. The tubes were sealed and added to an oil bath at 50-60 °C overnight (16-20 h). For all peptides other than the model peptide, 10 mol equiv of fluoronitroarene and 20 mol equiv of DIEA were added.

Peptide Cleavage. Peptides were globally deprotected and cleaved from resin by incubation with a cocktail of 95% TFA, 2.5% water, and 2.5% TIPS for 2 h. After filtration, rotary evaporation of TFA and precipitation with ice-cold diethyl ether yielded the crude peptide. Isolated yields indicate the mass recovered after a single round of reversed-phase HPLC.

Nitro Reduction. Peptides were dissolved to 150 mM in dimethylsulfoxide (DMSO) solution with 4 mol equiv hypodiboric acid, $B_2(OH)_4$, and 5 mol% 4,4'-bipyridine catalyst for 10 min. Solutions of reducing agent and catalyst in DMSO were prepared separately and then combined to form a dark blue solution. The dark blue reducing agent-catalyst solution was then added immediately to the peptide solution before the dark blue color faded, which occurs within one minute of combination.[7] Reactions were stopped after 10 min by dilution with water and frozen at -20°C until purified by reversed-phase HPLC.

Nuclear Magnetic Resonance (NMR) Spectroscopy. Purified peptides **C1**-**C33**, **C35**, and **D1**-**D34** were dissolved in water and diluted to 1 mM in a 90%H_2O/10% D_2O mixture. All ^1H NMR spectra were collected on a Bruker 400 MHz NMR spectrometer using the ZGESGP pulse sequence for water suppression (32-48 scans, SWH = 4K, TD = 32768, Figure S5).

UV-Visible Spectrophotometry. Purified peptides **C1**-**C33**, **C35**, and **D1**-**D34** were dissolved in water and diluted to 15 mM for acquisition of an initial absorbance spectrum

from 220 nm to 750 nm. Based on this measurement, a peptide dilution was prepared with a target A_{280} value of 1.0. This dilution was then used as the starting point for a 1.5-fold serial dilution to prepare 3-4 additional dilutions within the 0.1-1.0 range for A_{280}. Linear regression was applied to the absorbance values for the local maxima at wavelengths greater than 300 nm ($\lambda_{max,2}$) to estimate the extinction coefficients at those wavelengths for the nitroarylated residues.

Plasmin Activity Assays. Plasmin activity assays were performed as described by Harris *et al*.[1] Briefly, a stock solution for each plasmin substrate was prepared at 20 μM in assay buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.1% Pluronic acid). These solutions were further diluted to either 5 or 2.5 μM in assay buffer, and 45 μL volumes were added to triplicate wells of a black round-bottom 96-well plate. A solution containing 10 nM plasmin was prepared by diluting 1 μL of plasmin stock solution (5.0 mg/mL, 8.96 U/mg) in 6.023 mL assay buffer.

Before the assay, the 96-well plate was added to a Biotek Synergy H1 96-well plate reader pre-equilibrated to 25 °C, and fluorescence was measured using excitation and emission wavelengths of 380 nm and 450 nm, respectively, to establish the baseline fluorescence of each substrate/well. To start the assay, a volume of 5 μL of 10 nM plasmin was added to a single row (10 substrate-containing wells + 2 wells containing only assay buffer) using a multichannel pipette. The 96-well plate was then immediately added to the plate reader, and after 5 s of shaking, fluorescence was monitored every 10 s for 5 min using 380 nm and 450 nm as excitation and emission wavelengths, respectively. This procedure was repeated until all substrate-containing wells were read. Only 12 wells were read per addition of plasmin to ensure that all wells were read within the 10 s time interval.

For data analysis, the average buffer fluorescence was subtracted from each sample, and replicate samples were averaged. The rate of change in fluorescence over 60 s was then determined by subtracting the fluorescence value at 0 s from the fluorescence value at 60 s. The rate of change in fluorescence was normalized to the substrate concentrations used (4.5 or 2.25 μM), and these normalized rates were averaged as a measure of plasmin activity on a given substrate.

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