Supporting Information for

Human Defensin 5 Disulfide Array Mutants: Disulfide Bond Deletion Attenuates Antibacterial Activity Against *Staphylococcus aureus*

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This Supporting Information section includes:

Syntheses of HD5[Ser^{5,20}]$_{ox}$ Regioisomers.

The syntheses of HD5$_{ox}$ and related peptides have been reported.[S1,S2] These solid-phase syntheses have employed Boc chemistry, which requires the use of hydrogen fluoride (HF). In order to avoid using HF, we employed Fmoc solid-phase peptide synthesis for the preparation of the HD5 double mutants HD5[Ser^{5,20}]$_{ox}$ (3-31)(10-30) and (3-30)(10-31).

Materials. All Fmoc-protected amino acids (Fmoc-aa-OH) were purchased from Aapptec (Louisville, KY). The following Fmoc-protected amino acids were employed to afford full orthogonal protection: Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, and Fmoc-Tyr(^{t}Bu)-OH. Preloaded Fmoc-Arg(Pbf)-NovaSyn TGA resin was obtained from EMD Chemicals (San Diego, CA). DIPEA and piperidine were purchased from Sigma Aldrich (St. Louis, MO). The coupling reagents HOAt and HATU were purchased from AK Scientific, Inc. (Mountain View, CA). Organic solvents, including anhydrous DMF, DCM and NMP, and 1-acetylimidazole (Alfa Aesar) were purchased from commercial suppliers and used as received.

Solid-Phase Peptide Synthesis. Scheme S1 details the strategy employed for the solid-phase peptide syntheses of HD5[Ser^{5,20}]$_{ox}$ (3-31)(10-30) and (3-30)(10-31). The syntheses of both HD5[Ser^{5,20}] derivatives were performed manually by using a custom-made 25-mL fritted glass reaction vessel (Chemglass) on a 0.2 mmol scale. Standard solid-phase methodology for Fmoc chemistry was employed and preloaded Fmoc-Arg(Pbf)-NovaSyn TGA resin was utilized. After the 22-residue fragment Fmoc-ATRESLSGVSEISGRLYRL-Cys(Acm)-Cys(Trt)-R-NovaSyn TGA segment was assembled on the resin, the Fmoc group was removed (*vide infra*), and the resin was dried and split into two equal batches (by mass). Subsequently, two independent peptide syntheses were continued each on a 0.1 mmol scale. One portion of the resin was used to synthesized the HD5[Ser^{5,20}] peptide with Cys(Acm) in positions 10 and 30, and the peptide with Cys(Acm) in positions 3 and 30 was prepared on the second portion of the resin.

With the exceptions of Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH, Fmoc-protected amino acids were coupled using a coupling mixture containing Fmoc-aa-OH/HATU/HOAt/DIPEA

(1/1/1/2 mol ratio) in anhydrous DMF. The typical coupling time was 30 min and at least two coupling reactions were performed for each amino acid. For most amino acids, double coupling was performed. Triple coupling was employed for Fmoc-Arg(Pbf)-OH and each β-branch amino acid adjacent to another β-branch amino acid in the peptide sequence. In every case, 10 equiv of Fmoc-aa-OH was employed for the first coupling and 5 equiv of Fmoc-aa-OH was used for each repeated coupling.

Coupling of Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH to the peptide was accomplished by using 4 equiv of HATU, HOAt, and TMP (relative to the Fmoc-aa-OH) in a 1:1 mixture of anhydrous DMF/anhydrous DCM in order to minimize racemization.[S3]

Two pseudoproline dipeptides, Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-OH and Fmoc-Ile-Ser($\Psi^{Me,Me}$pro)-OH, were employed for Ala^{11}-Thr^{12} and Ile^{22}-Ser^{23}, respectively, to reduce peptide aggregation. A mixture of aa/HATU/HOAt/DIPEA in anhydrous DMF was used for the coupling of pseudoproline dipeptides. The molar ratio was 1/1/1/2 with three equiv of the pseudoproline dipeptide used in the first coupling and 1.5 equiv of pseudoproline dipeptide for subsequent couplings.

To prevent the formation of truncated peptides resulting from incomplete coupling reactions, a DCM solution containing 10 equiv of 1-acetylimidazole was added to the resin following each coupling step (this step includes repeated couplings of the same Fmoc-aa-OH) to cap any remaining free amine of the N-terminal amino acid. The resin was agitated with stream of N_2 for 10 min and the reaction vessel was drained and washed with NMR or DMF 3x before performing Fmoc removal. No capping was performed after coupling of the last amino acid.

Fmoc deprotection was performed by using 20% piperidine in NMP. Three cycles of deprotection were performed with reaction times of 5 min, 20 min and 20 min.

After the N-terminal Ala residue of HD5 was coupled to the peptide chain, the resin loading level was determined as described below and the N-terminal Fmoc group removed as described above. The resin was washed with DCM extensively to completely remove traces of other solvents used during the synthesis. The washed resin was then incubated with ~10 mL of

ice-cold cleavage mixture containing TFA/H_2O/TIS/EDT (94/2.5/1/2.5, volume ratio) for 3h. The TFA solution was collected and the resin was washed with the cleavage mixture 3 times. All TFA fractions were combined and concentrated to a volume of ~5 mL by using a gentle stream of N_2. Ice-cold diethyl ether was added to precipitate the crude peptide, and the resulting suspension was centrifuged (~3500 rpm x 20 min, 4 ^oC). The supernatant was removed and the pelleted precipitate was re-dissolved in a small volume of the cleavage mixture. The peptide was precipitated a second time by using ice-cold diethyl ether. After centrifugation, the supernatant was removed and the crude peptide was dissolved in a mixture of H_2O/MeCN/AcOH, frozen in liquid N_2, and lyophilized to yield ~250 mg of an off-white solid, which was stored at -20 ^oC. The cleavage reaction resulted in deprotection of all amino acid side-chains except for the two Acm-protected Cys residues.

Oxidative Folding. A two-step folding strategy was employed to prepare the HD5[$Ser^{5,20}$]$_{ox}$ (3-31)(10-30) and (3-30)(10-31) regioisomers. In the first step, 20 mg of crude HD5[$Cys(Acm)^{3,30}$, $Ser^{5,20}$] or HD5[$Cys(Acm)^{10,30}$, $Ser^{5,20}$] was dissolved in 1 mL of 8 M GuHCl and 3 mL of 0.25 M $NaHCO_3$ in 30% DMF was added. The reaction mixture was incubated overnight at room temperature and was dialyzed against 0.1% AcOH (3 x 4 L, 4 ^oC, 30 min per round of dialysis) by using a Spectrapor 7 1000 MWCO dialysis membrane and was subsequently lyophilized.

Removal of the Acm protecting groups and cysteine oxidation by I_2 was employed for the second folding step following a literature protocol.[S4] A portion of crude material from the first folding step was dissolved in 4:1 AcOH/0.1 M HCl to provide a peptide concentration of 0.5 mg/mL. An aliquot of 50 mM I_2 in AcOH was added to afford a 1:20 peptide/I_2 ratio and the reaction was stirred for 1 h at room temperature. The reaction was quenched by addition of 0.2 M ascorbic acid, and the resulting solution was dialyzed against 0.1% AcOH as described above, flash frozen in liquid N_2, and lyophilized to dryness. Semi-preparative HPLC was employed to isolate and purify the desired products (gradient: t=0 min, 22% B; t=2 min, 22% B; linear gradient to 27% B at t=25 min;4 mL/min flow rate; 30 ^oC column temperature). Each pure

regioisomer was obtained as a white powder following lyophilization. HD5[$Ser^{5,20}$]$_{ox}$ (3-31)(10-30): analytical HPLC retention time, 16.0 min (10-60% B in 30 min); MALDI-TOF calc $[M+H]^+$, 3553.0; obs, 3553.1. HD5[$Ser^{5,20}$]$_{ox}$ (3-30)(10-31): analytical HPLC retention time, 16.8 min (10-60% B in 30 min); MALDI-TOF calc $[M+H]^+$, 3553.0; obs, 3552.9.

Determination of Resin Loading. The progress of selected coupling steps was followed by using the Fmoc-release-based protocol, which provides an estimate of the amount of the Fmoc-protected peptide attached to the resin (hereafter referred to as "loading").[S5] Briefly, to estimate the loading level, the resin was thoroughly washed with NMP (3x), DCM (3x), and MeOH (3x), and subsequently dried on a high-vacuum line for ~20 min. An aliquot of the dry resin (1-3 mg) was suspended and 3 mL of 20% piperidine in DMF and this suspension was incubated at room temperature for 20 min with occasional shaking. The resulting solution was analyzed by optical absorption spectroscopy using 20% piperidine in DMF solution as a blank and the absorbance at 290 nm was monitored. The loading (mmol/g) was calculated using the equation 1:

$$\text{Loading (mmol/g)} = (\text{Abs}_{\text{sample}}) / (\text{mg of sample} \times 1.75) \qquad \text{(eq. 1)}$$

where $\text{Abs}_{\text{sample}}$ is the sample absorbance at 290 nm (1-cm pathlength cuvette). This value was determined before and after coupling of each Fmoc-Cys(Trt)-OH, Fmoc-Cys(Acm)-OH, and pseudoproline dipeptide. If the loading value was smaller than expected or desired, the coupling reaction was repeated and the loading analysis repeated.

Storage of Resin-Bound Peptide. During the course of the solid-phase peptide synthesis, the resin and growing peptide chain were routinely stored at -20 ^{o}C in the reaction vessel. Following removal from the freezer, the resin was dried on a high-vacuum line for ~20 min and subsequently swollen with DCM for ~20 min prior to continuing with the peptide synthesis.

References.

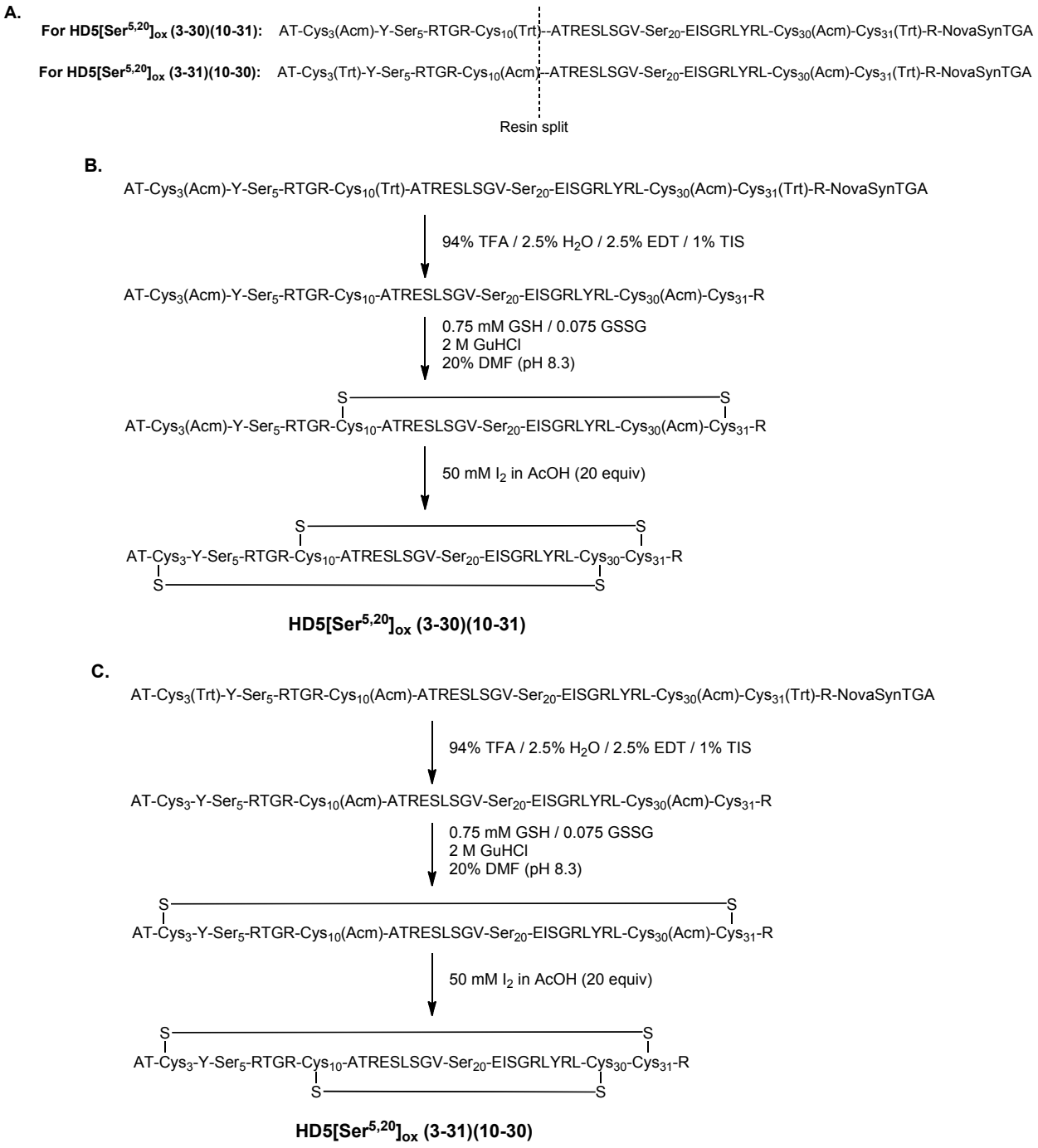
(S1) Wu, Z., Ericksen, B., Tucker, K., Lubkowski, J., and Lu, W. (2004) *J. Peptide Res. 64*, 118-125.

(S2) Rajabi, M., de Leeuw, E., Pazgier, M., Li, J., Lubkowski, J., and Lu, W. (2008) *J. Biol. Chem. 283***,** 21509-21518.

(S3) Han, Y., Albericio, F., and Barany, G. (1997) *J. Org. Chem. 62*, 4307-4312.

(S4) Klüver, E., Schulz-Maronde, S. Scheid, S., Meyer, B., Forssmann, W.G., and Adermann, K. (2005) *Biochemistry 44,* 9804-9816.

(S5) Chan, W. C. and White, P. D. *Fmoc solid phase peptide synthesis: a practical approach*. Oxford University Press: New York, 2000; p. 346.



Scheme S1. Strategy for the solid-phase peptide synthesis of the (3-31)(10-30) and (3-30)(10-31) regioisomers of HD5[$Ser^{5,20}$]$_{ox}$. (A) Following the synthesis of a 22-residue fragment and coupling of Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-OH, the resin was dried and split into two equal portions. The dotted line indicates the split. (B) Peptide cleavage, deprotection, and oxidation steps for the (3-30)(10-31) regioisomer. (C) Peptide cleavage, deprotection, and oxidation steps for the (3-31)(10-30) regioisomer.

Nucleotide sequence for the *E. coli*-optimized synthetic gene for HD5 and corresponding amino acid sequence:

TEV-HD5:
ENLYFQGATCYCRTGRCATRESLSGVCEISGRLYRLCCR

The synthetic gene for HD5 includes the following restriction sites:

N-terminus:
*Kpn*I: GGTACC (yellow)
*Spe*I: ACTAGT (purple)
*Nde*I: CATATG (underline)
C-terminus:
*Xho*I: CTCGAG (blue)

And also:
N-terminal TEV protease cleavage site (italics)
C-terminal STOP codon (bold)

Kpn1 – Spe1 – Nde1 – *TEV*-HD5 – stop – Xhol (*E. coli*) optimized sequence:

GGTACCACTAGTCATATG*GAGAACTTGTATTTCCAAGGT*GCGACTTGCTATTGTCGTACCG
GTCGTTGTGCAACCCGTGAGAGCCTGAGCGGTGTGTGTGAAATCAGCGGCCGTCTGTATC
GCCTGTGCTGCCGT**TAA**CTCGAG

Table S1. Molecular Weights and Extinction Coefficients for HD5 and Mutant Peptides.

Peptide	Sequence	Molecular Weight (Da)[a]	ε_{278} ($M^{-1}cm^{-1}$)[b]
$HD5_{red}$	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	3588.2	2800
$HD5_{ox}$	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	3582.1	3181
$HD5[Ser^{3,31}]_{red}$	ATSYCRTGRCATRESLSGVCEISGRLYRLCSR	3556.1	2800
$HD5[Ser^{3,31}]_{ox}$	ATSYCRTGRCATRESLSGVCEISGRLYRLCSR	3552.0	3054
$HD5[Ser^{5,20}]_{red}$	ATCYSRTGRCATRESLSGVSEISGRLYRLCCR	3556.1	2800
$HD5[Ser^{5,20}]_{ox}$	ATCYSRTGRCATRESLSGVSEISGRLYRLCCR	3552.0	3054
$HD5[Ser^{10,30}]_{red}$	ATCYCRTGRSATRESLSGVCEISGRLYRLSCR	3556.1	2800
$HD5[Ser^{10,30}]_{ox}$	ATCYCRTGRSATRESLSGVCEISGRLYRLSCR	3552.0	3054
$HD5[Ala^{3,31}]_{red}$	ATAYCRTGRCATRESLSGVCEISGRLYRLCAR	3524.1	2800
$HD5[Ala^{3,31}]_{ox}$	ATAYCRTGRCATRESLSGVCEISGRLYRLCAR	3520.0	3054
$HD5[Ala^{10,30}]_{red}$	ATCYCRTGRAATRESLSGVCEISGRLYRLACR	3524.1	2800
$HD5[Ala^{10,30}]_{ox}$	ATCYCRTGRAATRESLSGVCEISGRLYRLACR	3520.0	3054
$HD5[Ser^{hexa}]$	ATSYSRTGRSATRESLSGVSEISGRLYRLSSR	3491.8	2800

[a] Molecular weights were calculated by using PROTEIN CALCULATOR v3.3 available at http://www.scripps.edu/~cdputnam/protcalc.html. [b] Extinction coefficients (278 nm) were calculated by using PROTEIN CALCULATOR v3.3. The peptides were dissolved in 0.01 N HCl (reduced forms) or water (oxidized forms) for extinction coefficient determination.

Table S2. Primers Employed for Site-Directed Mutagenesis.[a]

Primer	Sequence
M-1	5'-GAACTTGTATTTCCAAATGGCGACTTGCTATTGTCG-3'
M-2	5'-CTTGAACATAAAGGTTTACCGCTGAACGATAACAGC-3'
C3S-1	5' GTATTTCCAAATGGCGACTAGCTATTGTCGTACCGGTC 3'
C3S-2	5' GACCGGTACGACAATAGCTAGTCGCCATTTGGAAATAC 3'
C5S-1	5' CAAATGGCGACTTGCTATAGTCGTACCGGTCGTTGTG 3'
C5S-2	5' CACAACGACCGGTACGACTATAGCAAGTCGCCATTTG 3'
C10S-1	5' GTCGTACCGGTCGTAGTGCAACCCGTGAGAG 3'
C10S-2	5' CTCTCACGGGTTGCACTACGACCGGTACGAC 3'
C20S-1	5' GCCTGAGCGGTGTGAGTGAAATCAGCGGCCGTCTG 3'
C20S-2	5' CAGACGGCCGCTGATTTCACTCACACCGCTCAGGC 3'
C30S-1	5' GTCTGTATCGCCTGAGCTGCCGTTAACTCGAG 3'
C30S-2	5' CTCGAGTTAACGGCAGCTCAGGCGATACAGAC 3'
C31S-1	5' CTGTATCGCCTGTGCAGCCGTTAACTCGAG 3'
C31S-2	5' CTCGAGTTAACGGCTGCACAGGCGATACAG 3'
C3S-C5S-1	5' GTATTTCCAAATGGCGACTAGCTATAGTCGTACCGGTC 3'
C3S-C5S-2	5' GACCGGTACGACTATAGCTAGTCGCCATTTGGAAATAC 3'
C30S-C31S-1	5' CTGTATCGCCTGAGCAGCCGTTAACTCGAG 3'
C30S-C31S-2	5' CTCGAGTTAACGGCTGCTCAGGCGATACAG 3'
C3A-1	5' GTATTTCCAAATGGCGACTGCCTATTGTCGTACCGGTC 3'
C3A-2	5' GACCGGTACGACAATAGGCAGTCGCCATTTGGAAATAC 3'
C5A-1	5' CAAATGGCGACTTGCTATGCTCGTACCGGTCGTTGTG 3'
C5A-2	5' CACAACGACCGGTACGAGCATAGCAAGTCGCCATTTG 3'
C10A-1	5' GTCGTACCGGTCGTGCTGCAACCCGTGAGAG 3'
C10A-2	5' CTCTCACGGGTTGCAGCACGACCGGTACGAC 3'
C20A-1	5' GCCTGAGCGGTGTGGCTGAAATCAGCGGCCGTCTG 3'
C20A-2	5' CAGACGGCCGCTGATTTCAGCCACACCGCTCAGGC 3'
C30A-1	5' GTCTGTATCGCCTGGCCTGCCGTTAACTCGAG 3'

C30A-2	5’ CTCGAGTTAACGGCAGGCCAGGCGATACAGAC 3’
C31A-1	5’ CTGTATCGCCTGTGCGCCCGTTAACTCGAG 3’
C31A-2	5’ CTCGAGTTAACGGGCGCACAGGCGATACAG 3’

[a] The codons containing the mutations are underlined and highlighted in red.

Table S3. Templates and Primer Pairings Employed for Site-Directed Mutagenesis.

Template	**Product**	**Primer Pairing**
pET28b-TEV-HD5	pET28b-Met-HD5	M-1, M-2
pET28b-Met-HD5	pET28b-Met-HD5(C3S)	C3S-1, C3S-2
pET28b-Met-HD5	pET28b-Met-HD5(C5S)	C5S-1, C5S-2
pET28b-Met-HD5	pET28b-Met-HD5(C10S)	C10S-1, C10S-2
pET28b-Met-HD5(C3S)	pET28b-Met-HD5(C3S)(C31S)	C31S-1, C31S-2
pET28b-Met-HD5(C5S)	pET28b-Met-HD5(C5S)(C20S)	C20S-1, C20S-2
pET28b-Met-HD5(C10S)	pET28b-Met-HD5(C10S)(C30S)	C30S-1, C30S-2
pET28b-Met-HD5(C5S)(C20S)	pET28b-Met-HD5(C5S)(C10S)(C20S)	C10S-1, C10S-2
pET28b-Met-HD5(C5S)(C10S)(C20S)	pET28b-Met-HD5(C3S)(C5S)(C10S)(C20S)(C30S)	C3S-C5S-1, C3S-C5S-2
pET28b-Met-HD5(C3S)(C5S)(C10S)(C20S)(C30S)	pET28b-Met-HD5(C3S)(C5S)(C10S)(C20S)(C30S)(C31S)	C30S-C31S-1, C30S-C31S-2
pET28b-Met-HD5	pET28b-Met-HD5(C3A)	C3A-1, C3A-2
pET28b-Met-HD5	pET28b-Met-HD5(C5A)	C5A-1, C5A-2
pET28b-Met-HD5	pET28b-Met-HD5(C10A)	C10A-1, C10A-2
pET28b-Met-HD5(C3S)	pET28b-Met-HD5(C3A)(C31A)	C31A-1, C31A-2
pET28b-Met-HD5(C5S)	pET28b-Met-HD5(C5A)(C20A)	C20A-1, C20A-2
pET28b-Met-HD5(C10S)	pET28b-Met-HD5(C10A)(C30A)	C30A-1, C30A-2

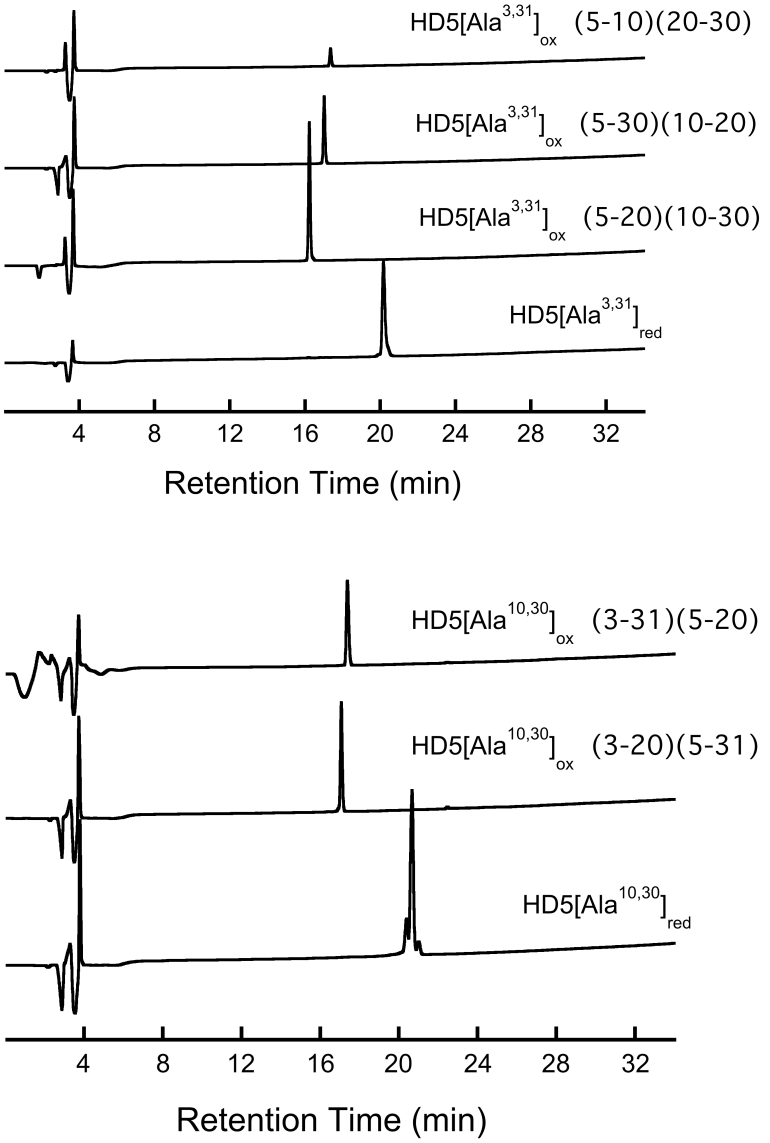


Figure S1. Analytical HPLC traces (220 nm absorption; 10-60% B in 30 min) of the purified reduced and oxidized Cys→Ala mutant peptides. Top plot: deletion of the Cys^{3}-Cys^{31} disulfide bond. Bottom plot: deletion of the Cys^{10}-Cys^{30} disulfide bond.

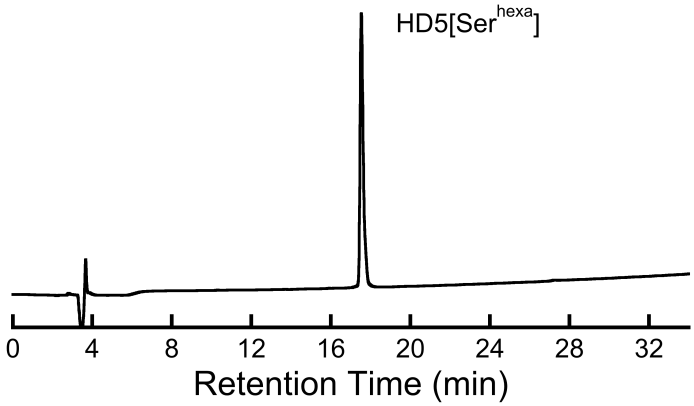


Figure S2. Analytical HPLC trace (220 nm absorption; 10-60% B in 30 min, 1 mL/min) of purified HD5[Ser^{hexa}].

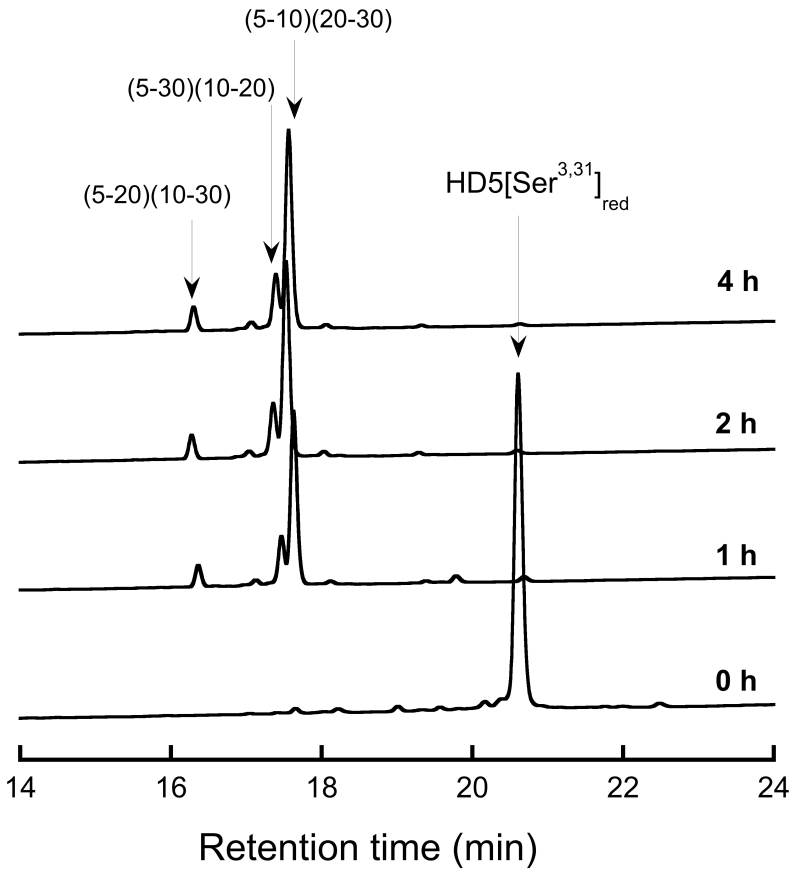


Figure S3. Peptide folding experiments with HD5[$Ser^{3,31}$]$_{red}$ monitored by analytical HPLC (220 nm absorption; 10-60% B in 30 min). The HD5[$Ser^{3,31}$]$_{ox}$ peaks are labeled according to S—S connectivity.

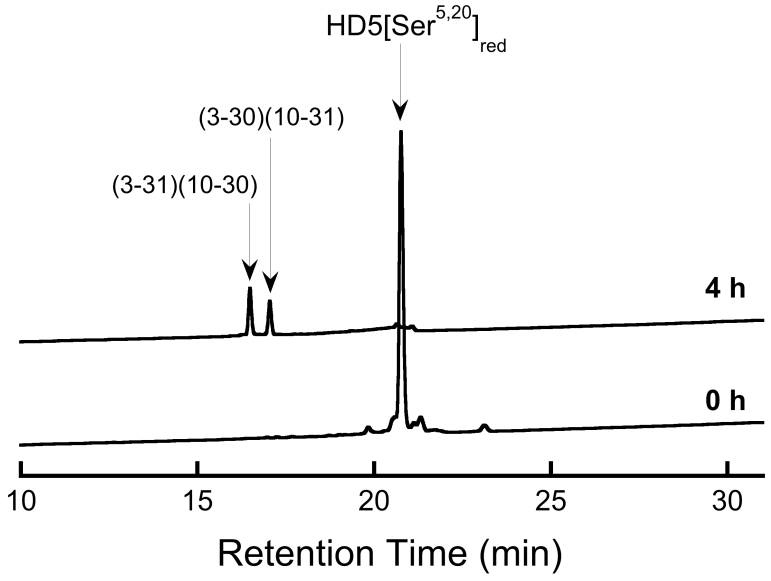


Figure S4. Peptide folding experiments with HD5[$Ser^{5,20}$]$_{red}$ monitored by analytical HPLC (220 nm absorption; 10-60% B in 30 min). The HD5[$Ser^{5,20}$]$_{ox}$ peaks are labeled according to S—S connectivity.

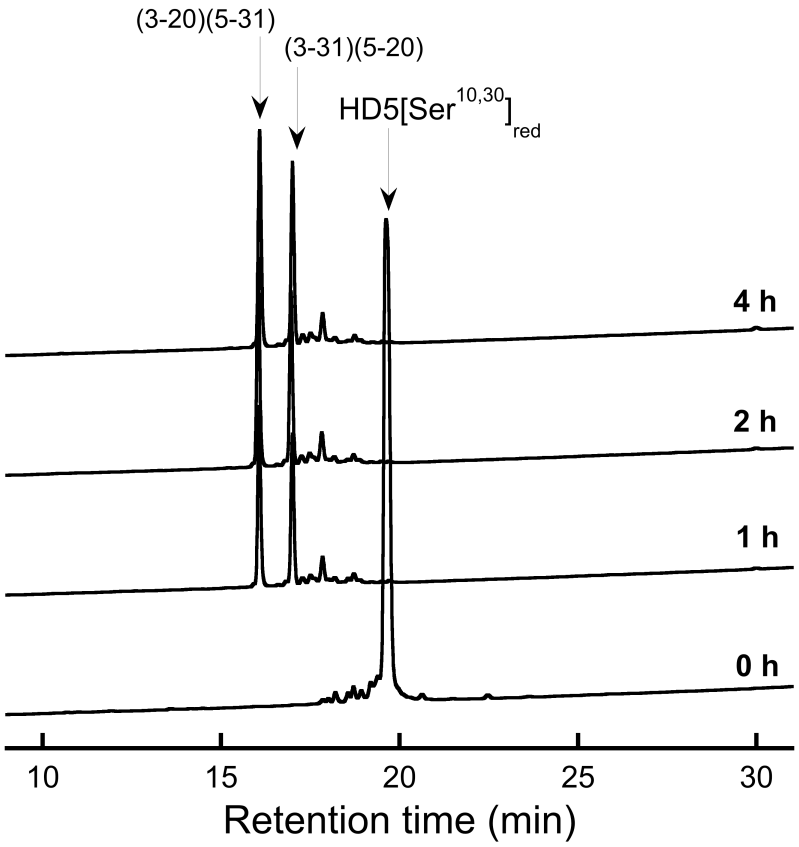


Figure S5. Peptide folding experiments with HD5[$Ser^{10,30}$]$_{red}$ monitored by analytical HPLC (220 nm absorption; 10-60% B in 30 min). The HD5[$Ser^{10,30}$]$_{ox}$ peaks are labeled according to S—S connectivity.

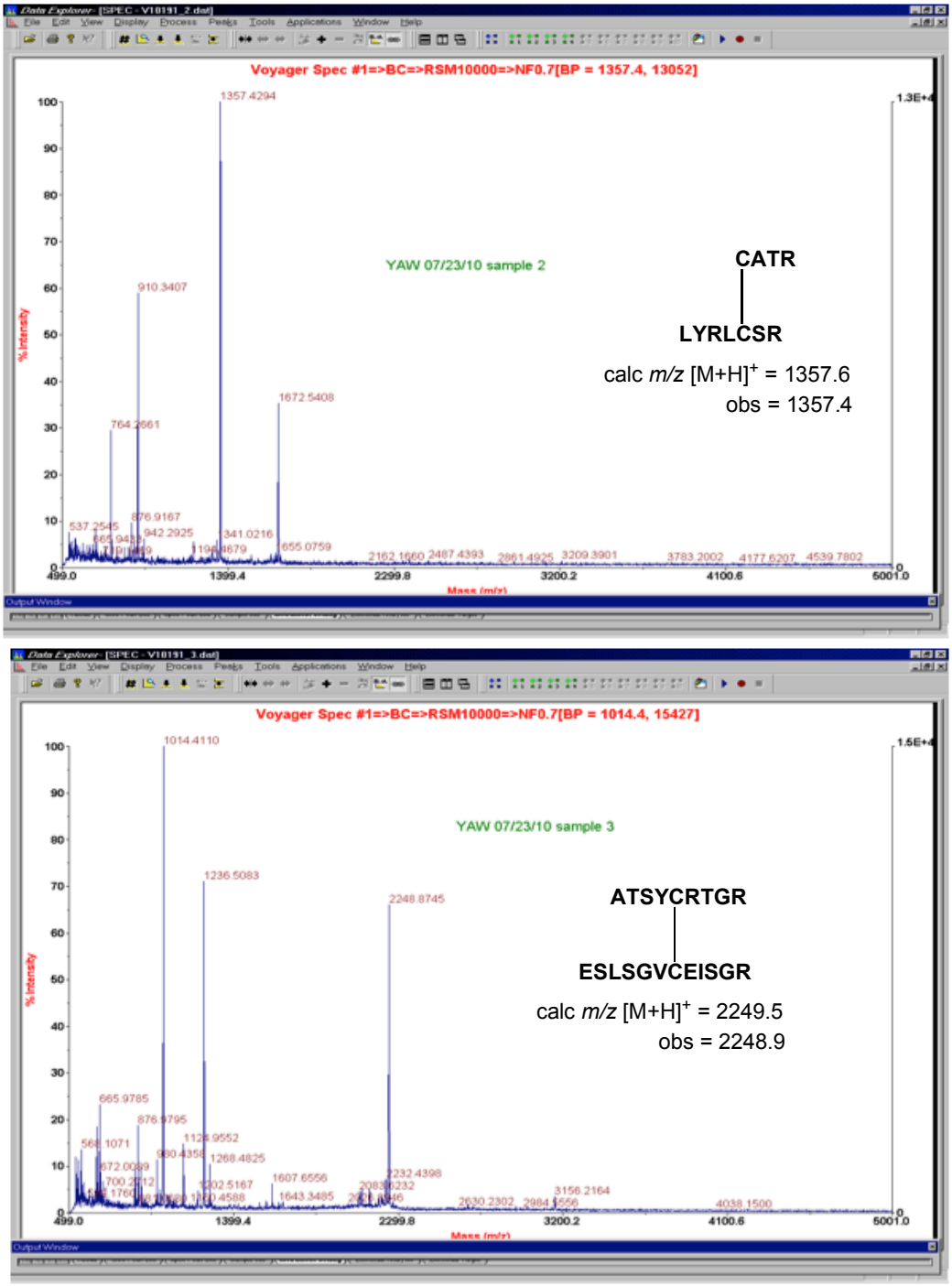


Figure S6. MALDI-TOF spectra for trypsin digest of HD5[$Ser^{3,31}$]$_{ox}$ (5-20)(10-30) and partial peak assignment. The trypsin digest was partially purified by HPLC prior to MALDI-TOF analysis.

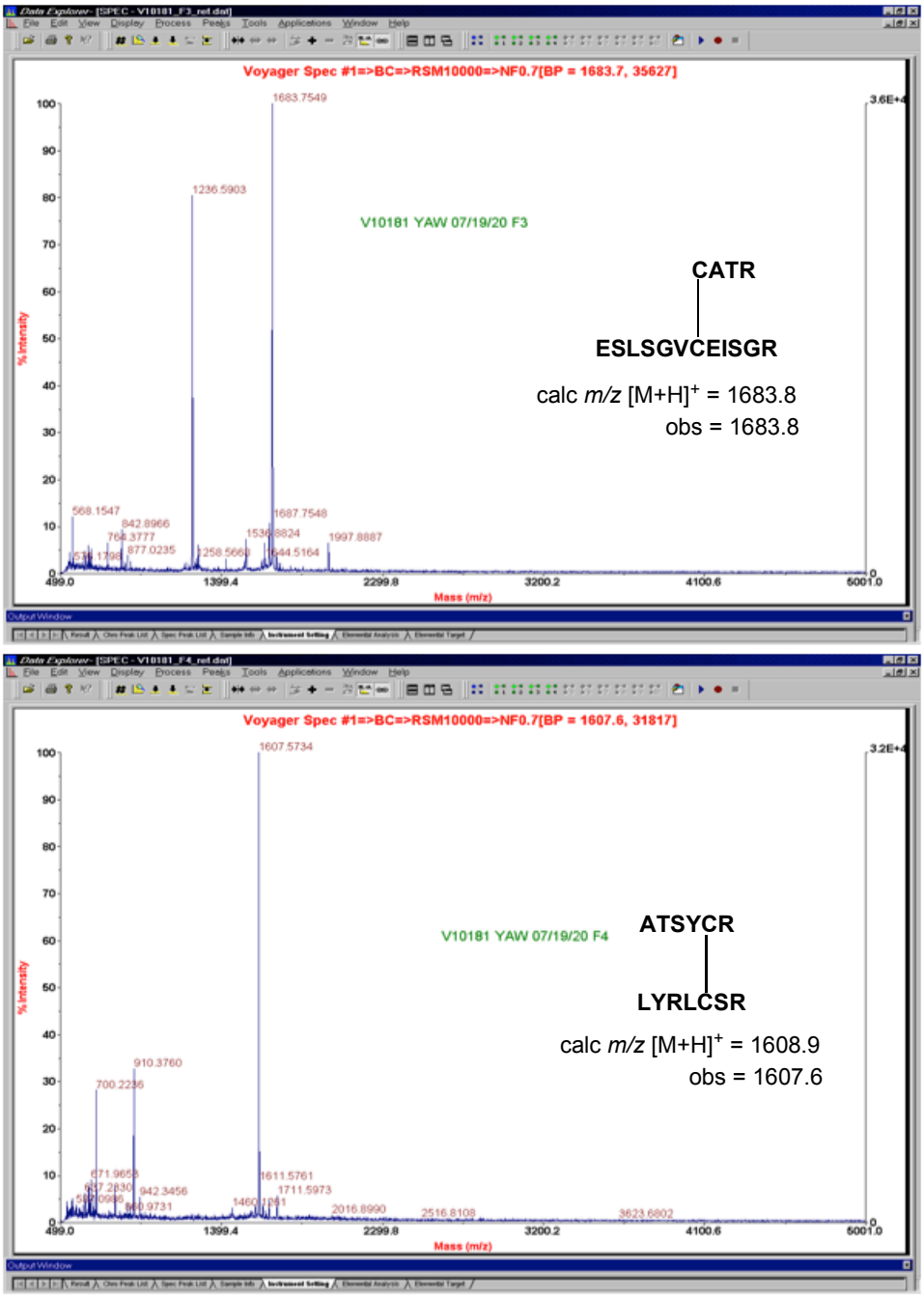


Figure S7. MALDI-TOF spectra for trypsin digest of HD5[$Ser^{3,31}$]$_{ox}$ (5-30)(10-20) and partial peak assignment. The trypsin digest was partially purified by HPLC prior to MALDI-TOF analysis.

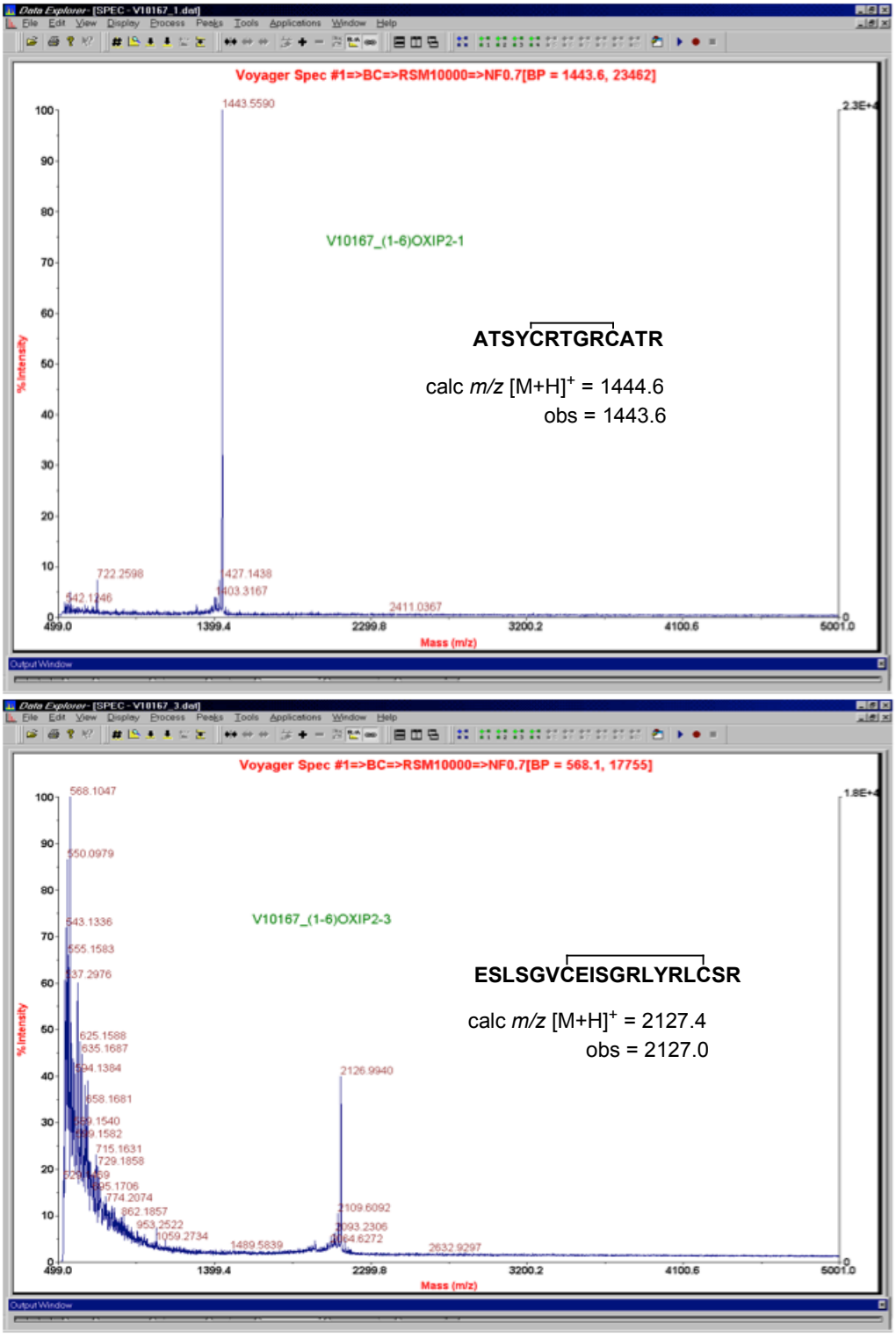


Figure S8. MALDI-TOF spectra for trypsin digest of HD5[$Ser^{3,31}$]$_{ox}$ (5-10)(20-30) and partial peak assignment. The trypsin digest was partially purified by HPLC prior to MALDI-TOF analysis.

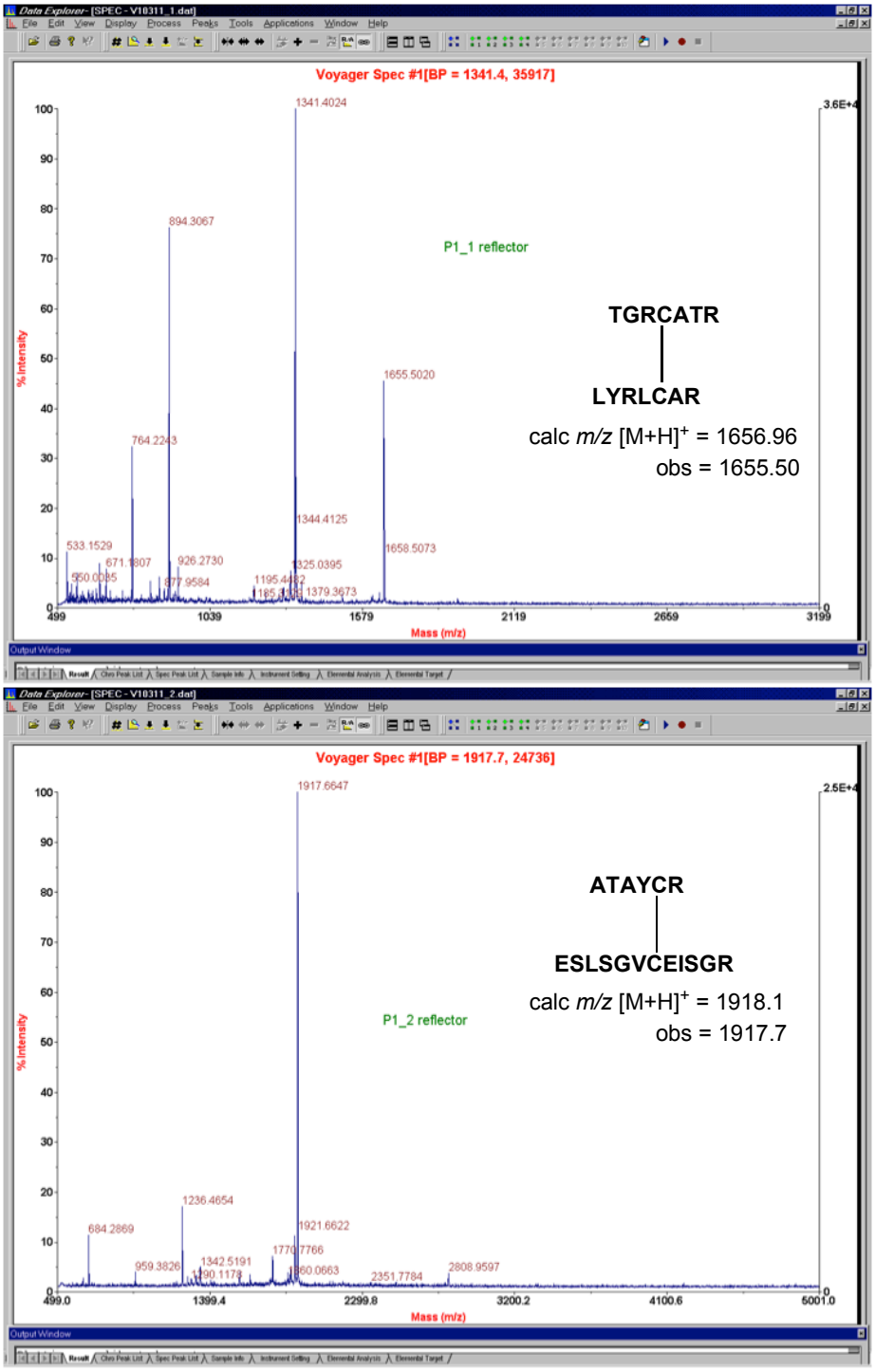


Figure S9. MALDI-TOF spectra for trypsin digest of HD5[Ala^{3,31}]$_{ox}$ (5-20)(10-30) and partial peak assignment. The trypsin digest was partially purified by HPLC prior to MALDI-TOF analysis.

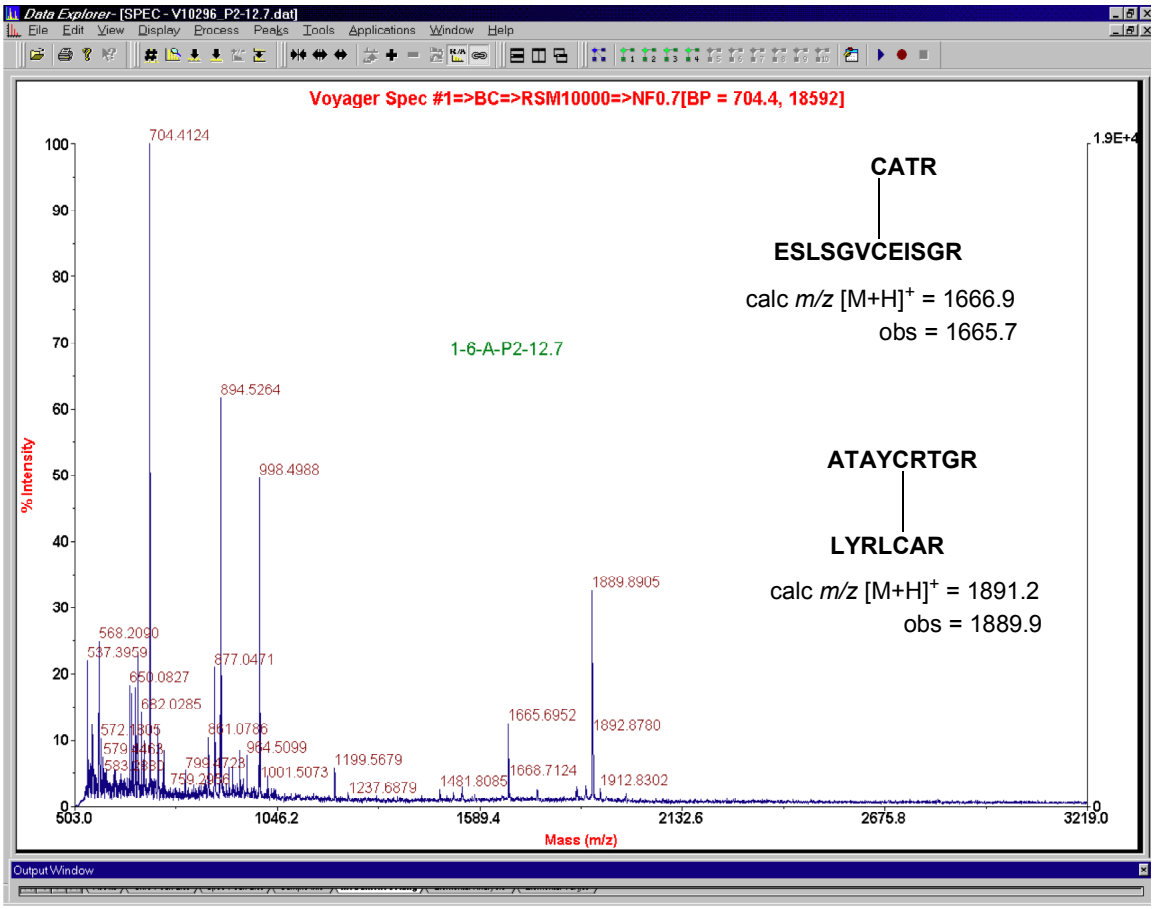


Figure S10. MALDI-TOF spectra for trypsin digest of HD5[Ala^{3,31}]$_{ox}$ (5-30)(10-20) and partial peak assignment. The trypsin digest was partially purified by HPLC prior to MALDI-TOF analysis.

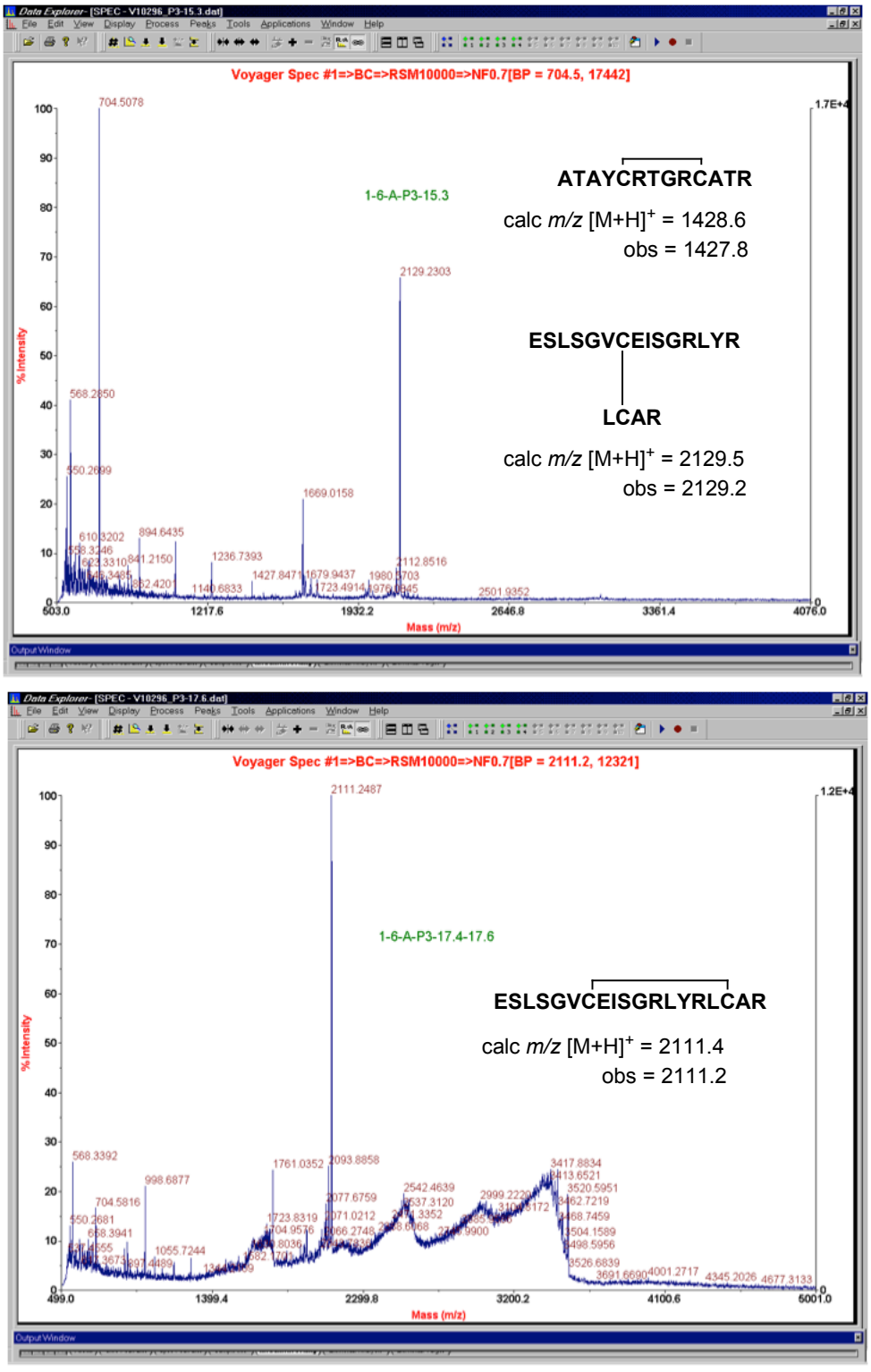


Figure S11. MALDI-TOF spectra for trypsin digest of HD5[Ala^{3,31}]$_{ox}$ (5-10)(20-30) and partial peak assignment. The trypsin digest was partially purified by HPLC prior to MALDI-TOF analysis.

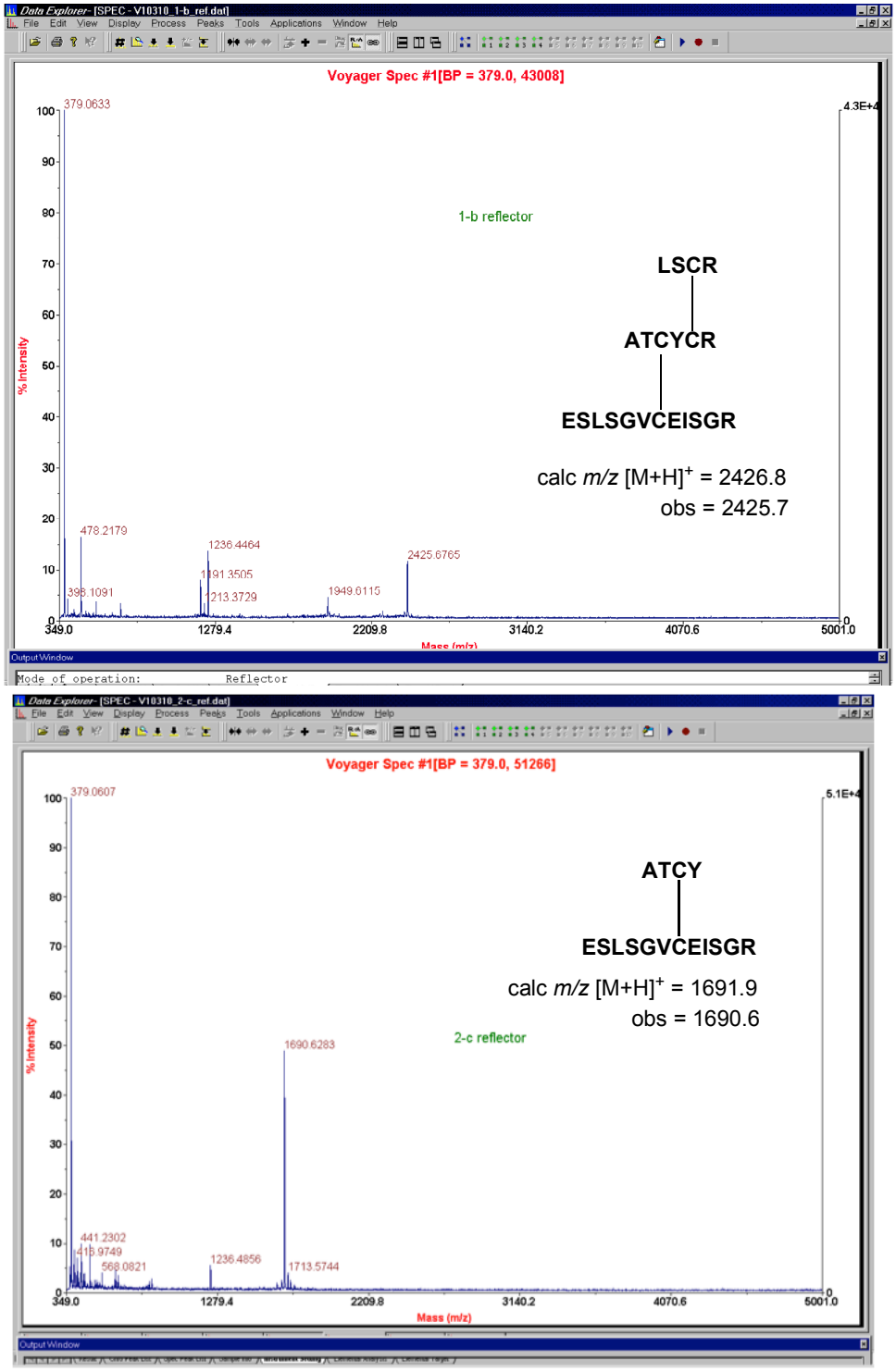


Figure S12. MALDI-TOF spectra for trypsin/chymotrypsin double digest of HD5[$Ser^{10,30}$]$_{ox}$ (3-20)(5-31) and partial peak assignment. The double digest was partially purified by HPLC prior to MALDI-TOF analysis.

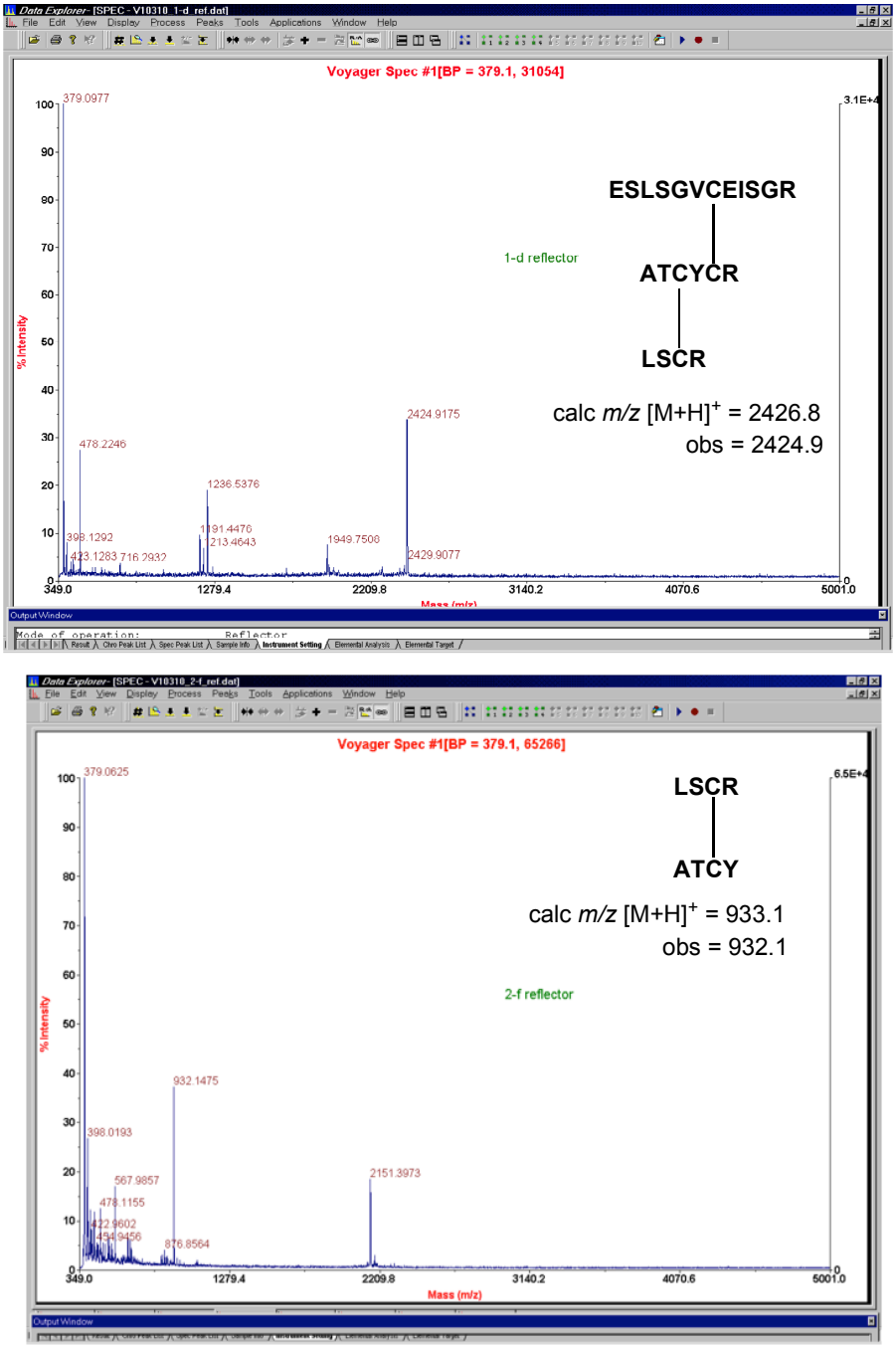


Figure S13. MALDI-TOF spectra for trypsin/chymotrypsin double digest of HD5[$Ser^{10,30}$]$_{ox}$ (3-31)(5-20) and partial peak assignment. The double digest was partially purified by HPLC prior to MALDI-TOF analysis.

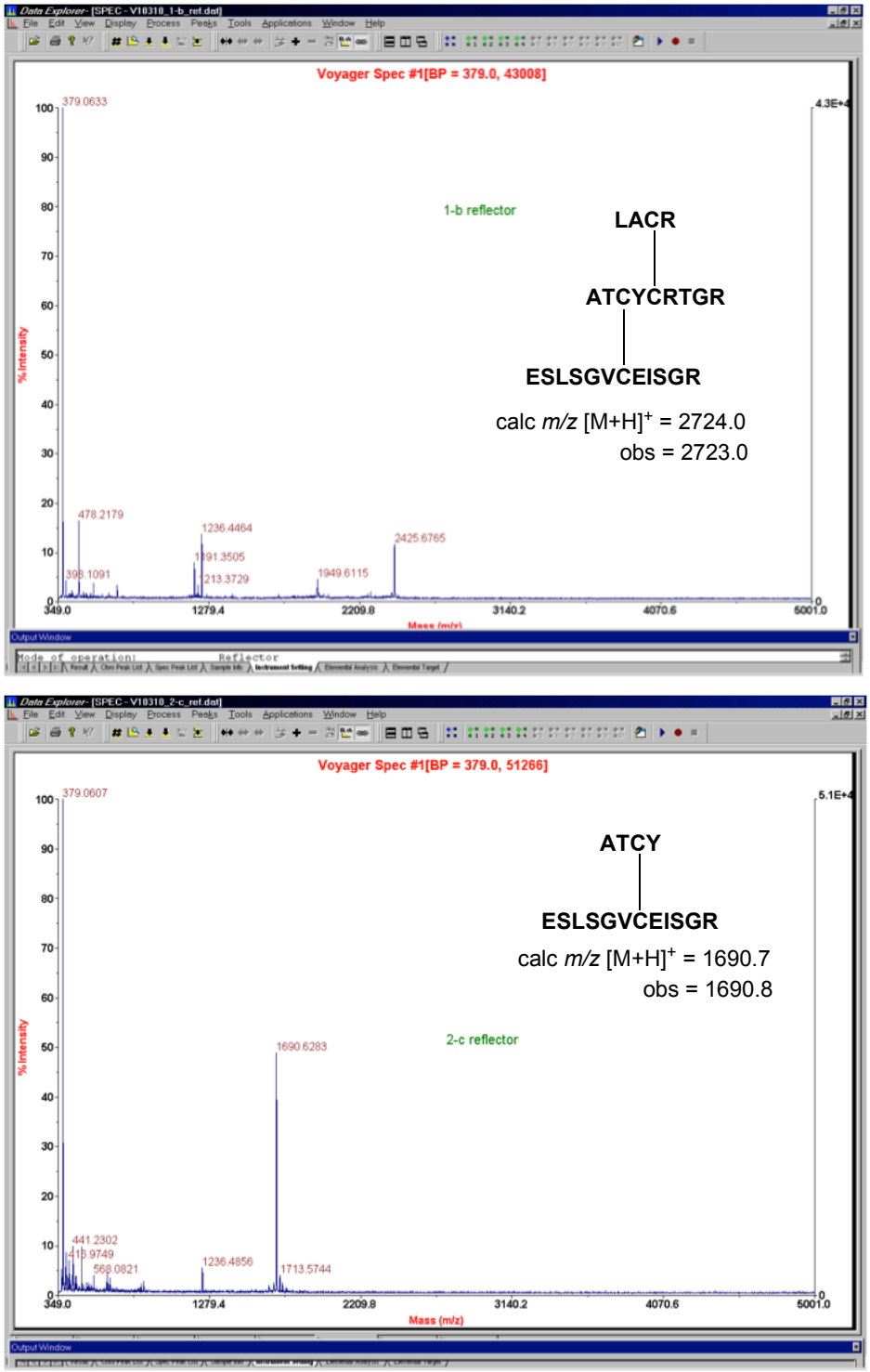


Figure S14. MALDI-TOF spectra for trypsin/chymotrypsin double digest of HD5[Ala^{10,30}]$_{ox}$ (3-20)(5-31) and partial peak assignment. The double digest was partially purified by HPLC prior to MALDI-TOF analysis.

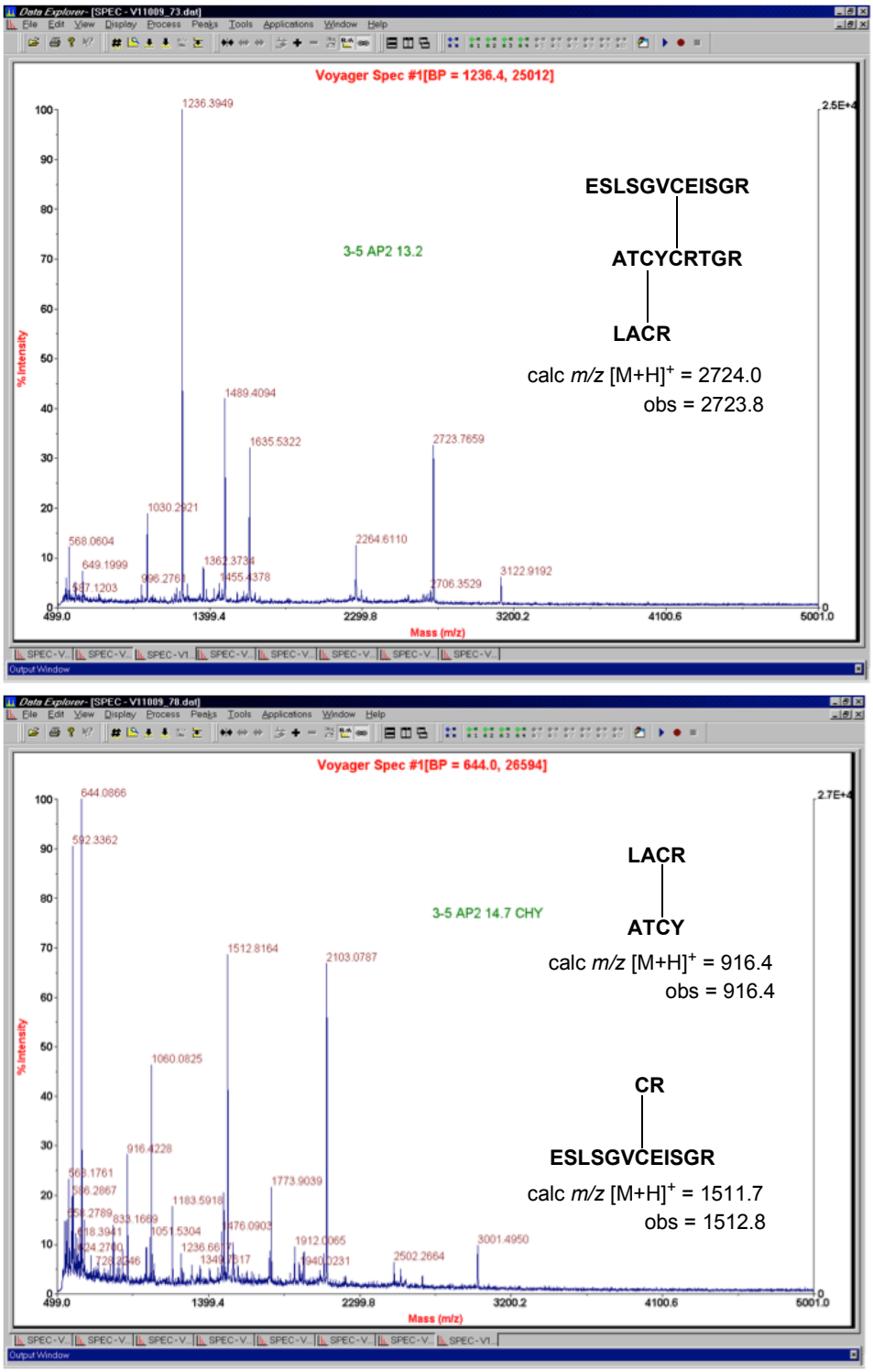


Figure S15. MALDI-TOF spectra for trypsin/chymotrypsin double digest of HD5[Ala^{10,30}]$_{ox}$ (3-31)(5-20) and partial peak assignment. The double digest was partially purified by HPLC prior to MALDI-TOF analysis.

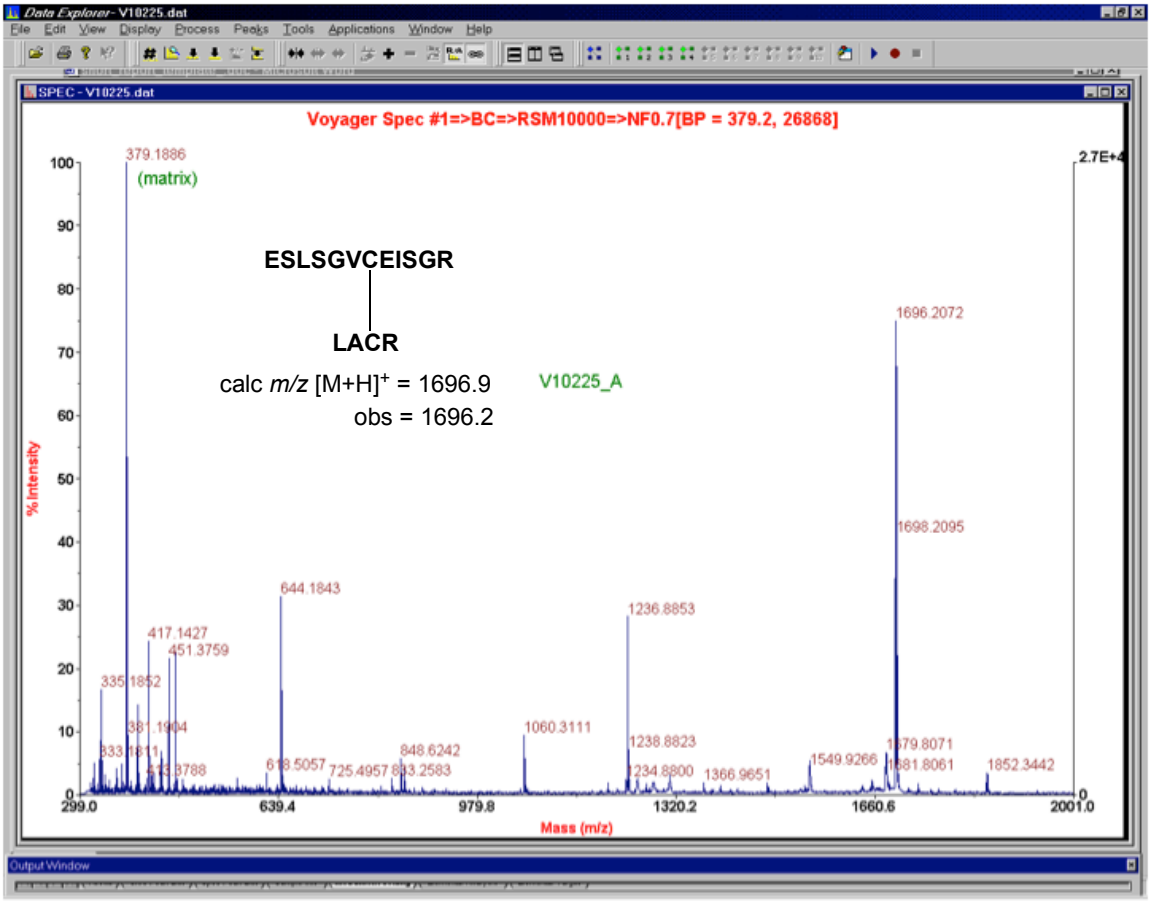


Figure S16. MALDI-TOF spectra for trypsin/chymotrypsin double digest of HD5[Ala^{10,30}]$_{ox}$ (3-5)(20-31) and partial peak assignment. The double digest was partially purified by HPLC prior to MALDI-TOF analysis.

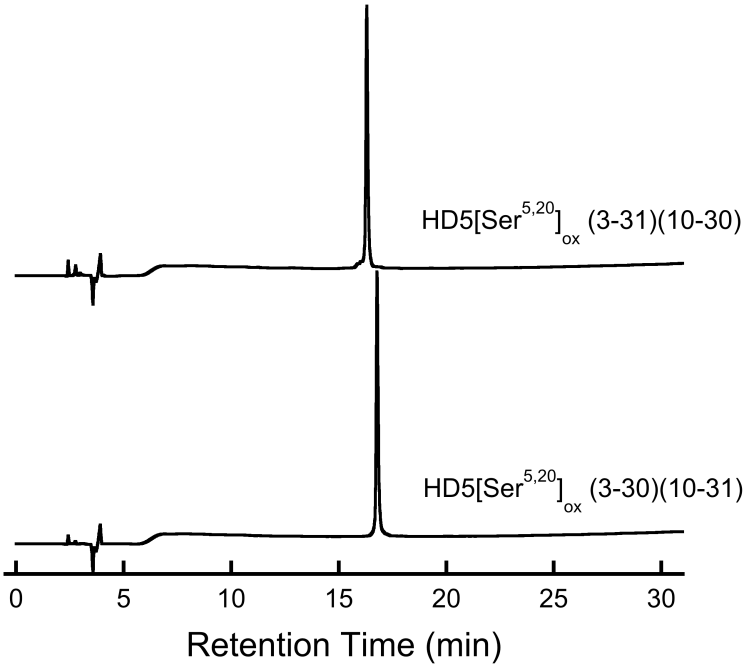


Figure S17. Analytical HPLC traces (220 nm absorption; 10-60% B in 30 min) of purified synthetic HD5[$Ser^{5,20}$]$_{ox}$ (3-31)(10-30) and HD5[$Ser^{5,20}$]$_{ox}$ (3-30)(10-31).

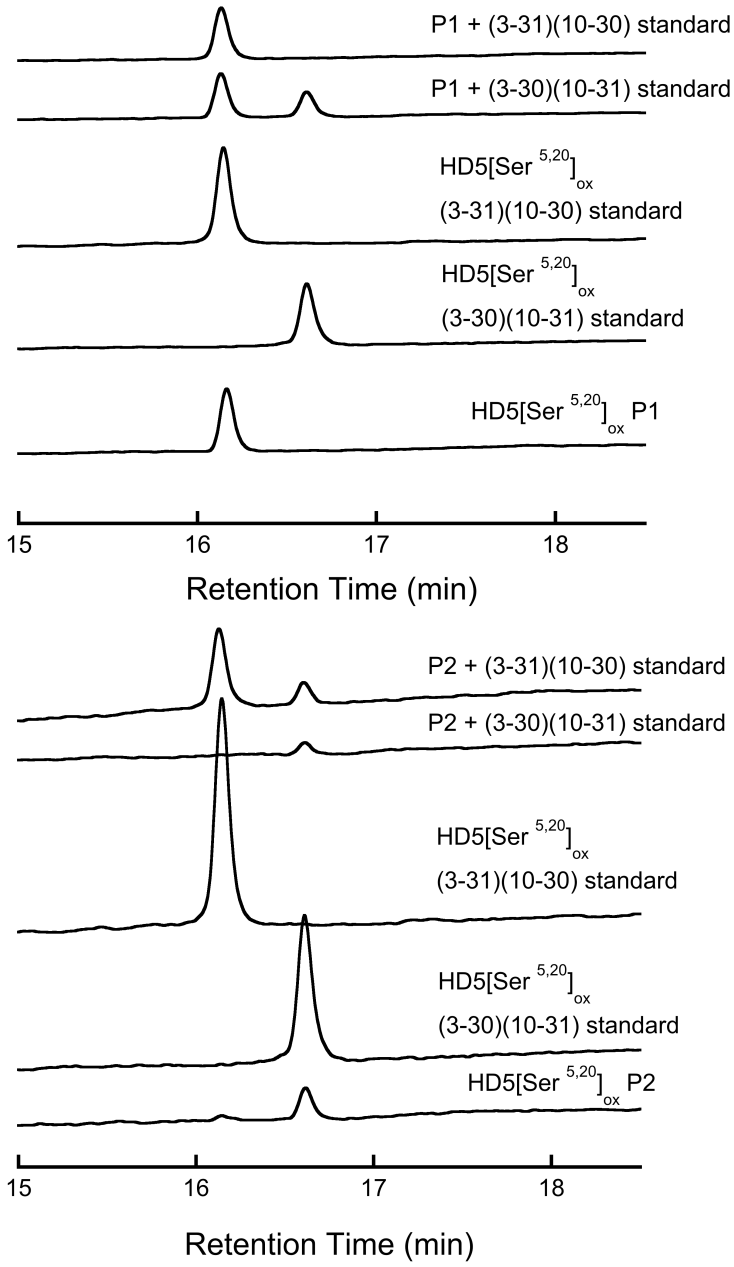


Figure S18. Regioisomer assignment for HD5[Ser^{5,20}]$_{ox}$. Analytical HPLC traces (220 nm absorption) of the two oxidized products obtained by folding HD5[Ser^{5,20}]$_{red}$, the synthetic standards, and spiked samples. P1 (top plot) and P2 (bottom plot) refer to the HD5[Ser^{5,20}]$_{ox}$ samples obtained by oxidative folding of HD5[Ser^{5,20}]$_{red}$ with the lower and higher HPLC retention times, respectively. The standards are the HD5[Ser^{5,20}]$_{ox}$ (3-30)(10-31) and (3-31)(10-30) peptides prepared by solid-phase peptide synthesis. The intensities of the HD5[Ser^{5,20}] P2 peaks are low because of limited sample availability.

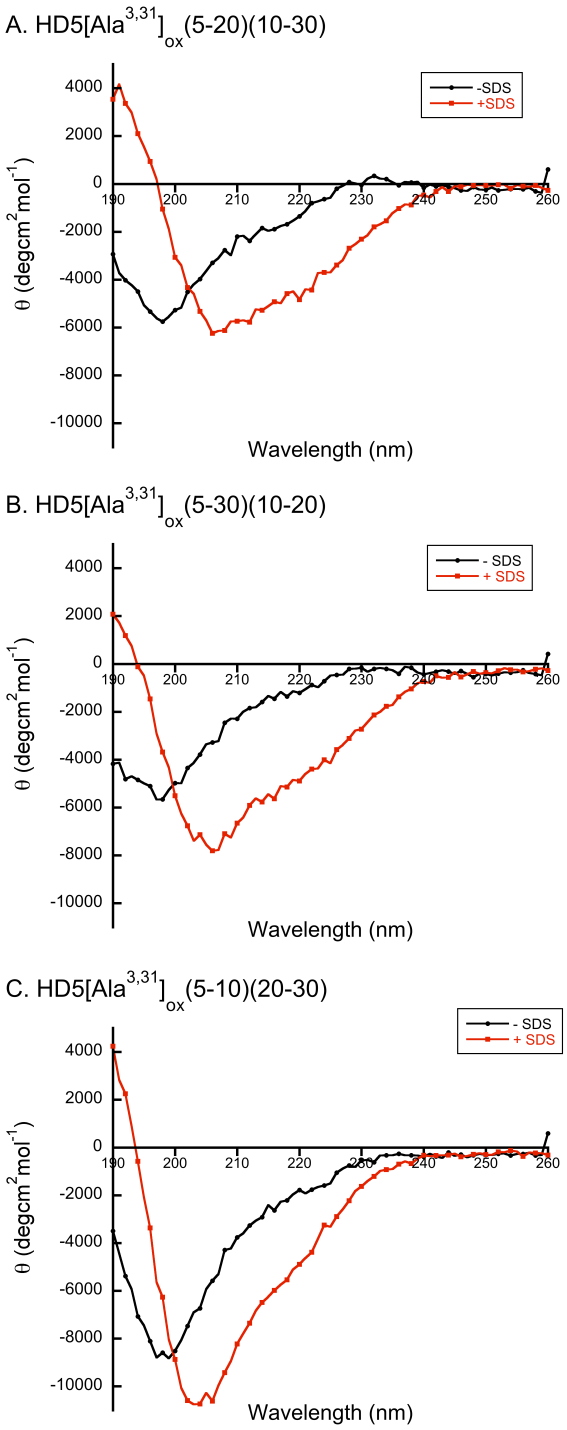


Figure S19. CD spectra for the HD5[Ala^{3,31}]$_{ox}$ regioisomers in the absence and presence of 10 mM SDS (5 mM sodium phosphate buffer, pH 7.0).

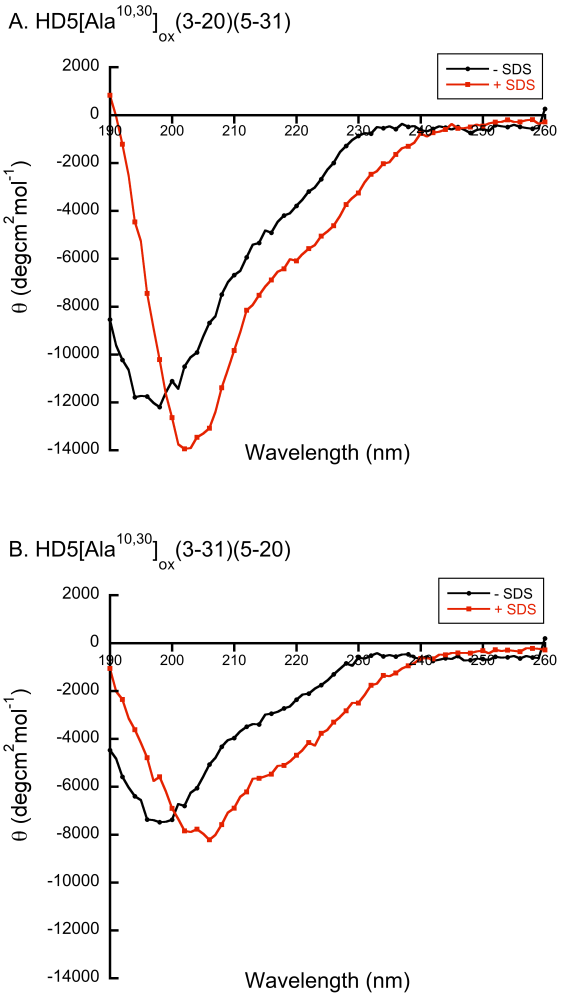


Figure S20. CD spectra for the HD5[Ala^{10,30}]$_{ox}$ regioisomers in the absence and presence of 10 mM SDS (5 mM sodium phosphate buffer, pH 7.0).

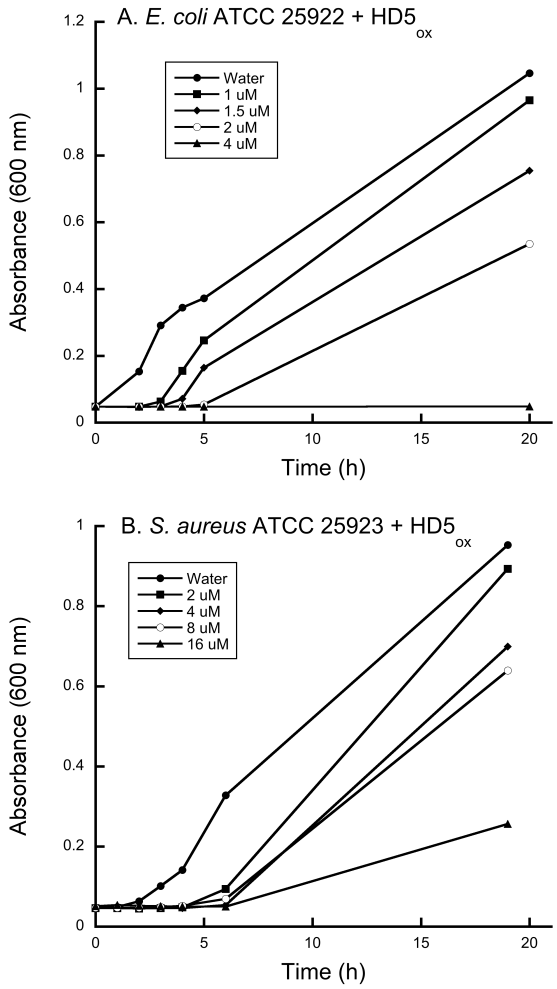


Figure S21. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of $HD5_{ox}$. (A) *E. coli* ATCC 25922; (B) *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of $HD5_{ox}$ in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

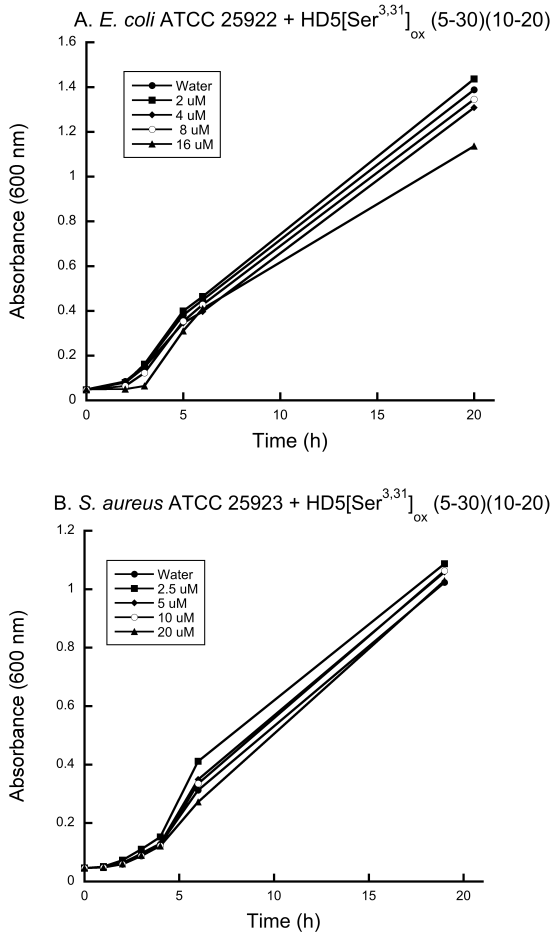


Figure S22. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of HD5[Ser^{3,31}] (5-30)(10-20). (A) *E. coli* ATCC 25922; (B) *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of HD5[Ser^{3,31}] (5-30)(10-20) in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

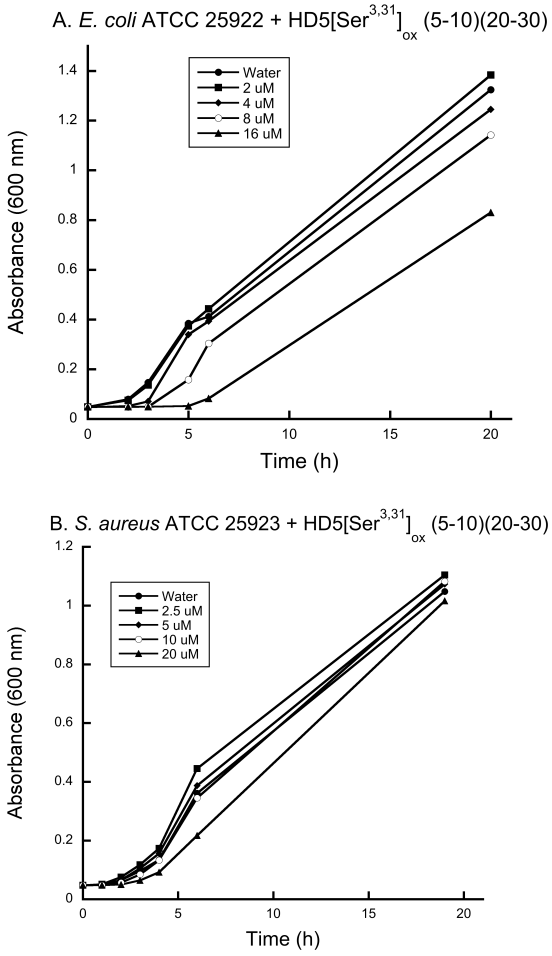


Figure S23. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of HD5[Ser^{3,31}] (5-10)(20-30). (A) *E. coli* ATCC 25922; (B) *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of HD5[Ser^{3,31}] (5-10)(20-30) in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

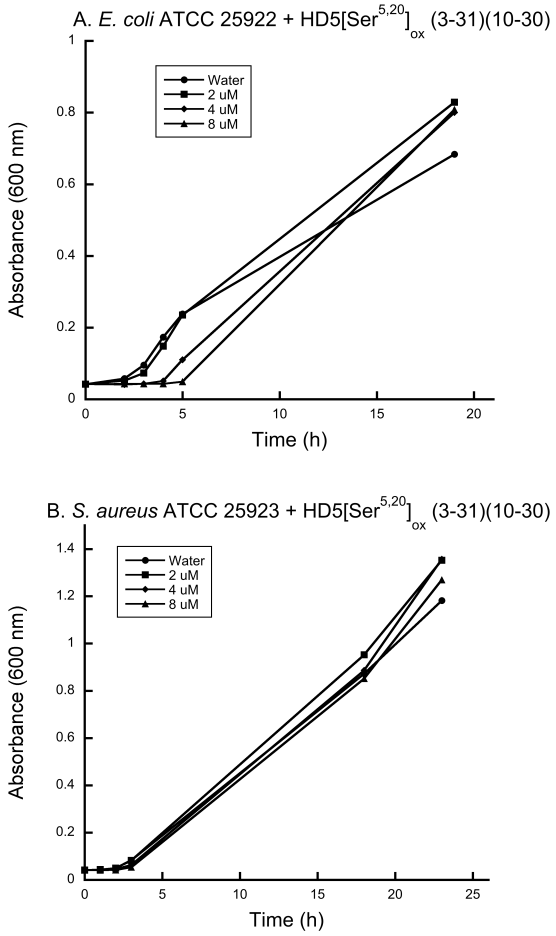


Figure S24. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of HD5[$Ser^{5,20}$] (3-31)(10-30). (A) *E. coli* ATCC 25922; (B) *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of HD5[$Ser^{5,20}$]$_{ox}$ (3-31)(10-30) in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

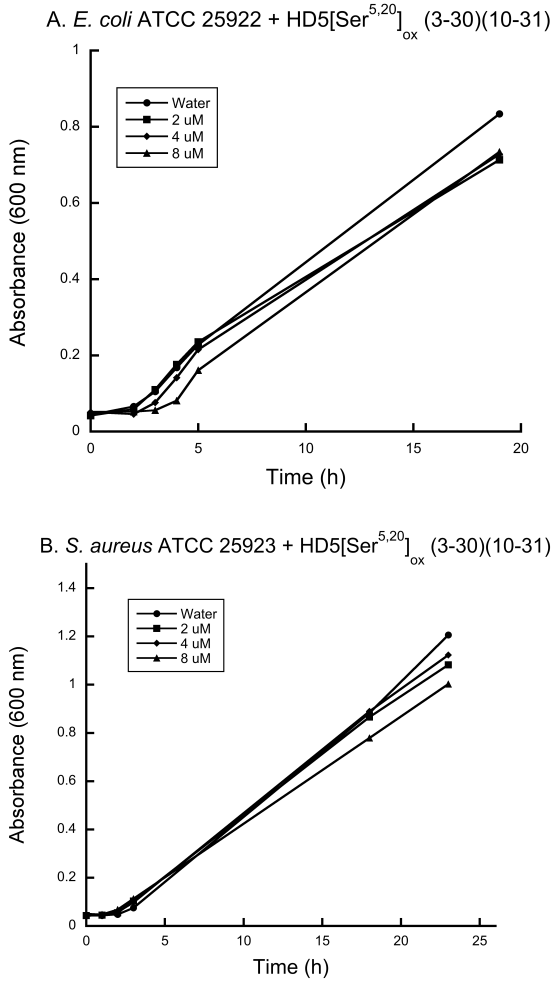


Figure S25. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of HD5[$Ser^{5,20}$] (3-30)(10-31). (A) *E. coli* ATCC 25922; (B) *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of $HD5[Ser^{5,20}]_{ox}$ (3-30)(10-31) in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

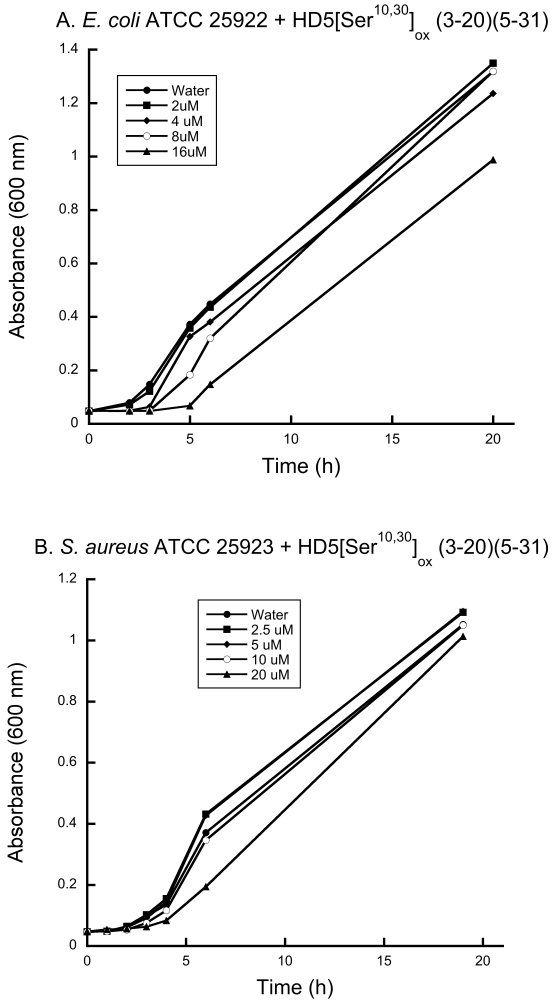


Figure S26. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of HD5[$Ser^{10,30}$] (3-20)(5-31). (A) *E. coli* ATCC 25922; (B) *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of HD5[$Ser^{10,30}$]$_{ox}$ (3-20)(5-31) in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

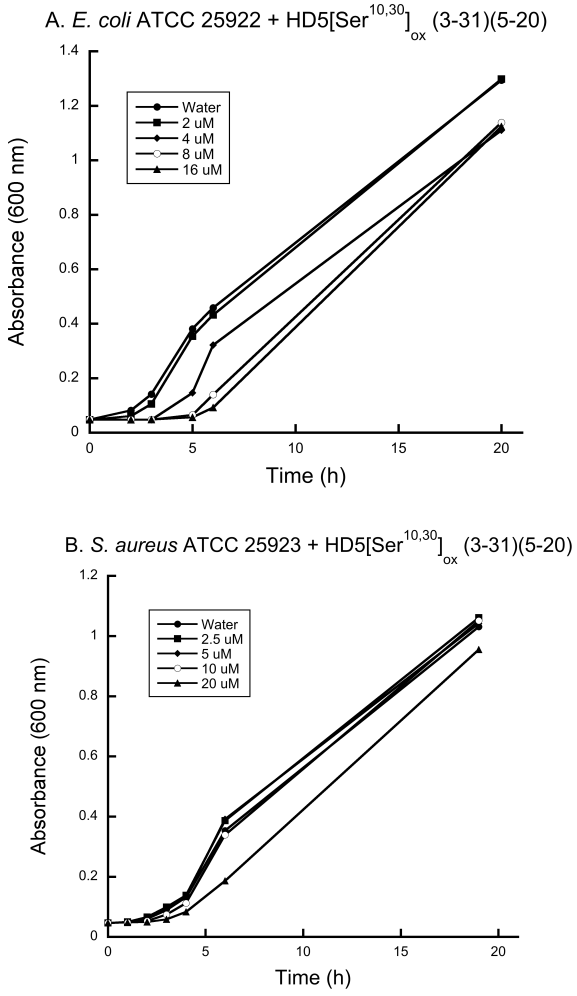


Figure S27. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of HD5[$Ser^{10,30}$] (3-31)(5-20). (A) *E. coli* ATCC 25922; (B) *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of HD5[$Ser^{10,30}$]$_{ox}$ (3-31)(5-20) in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

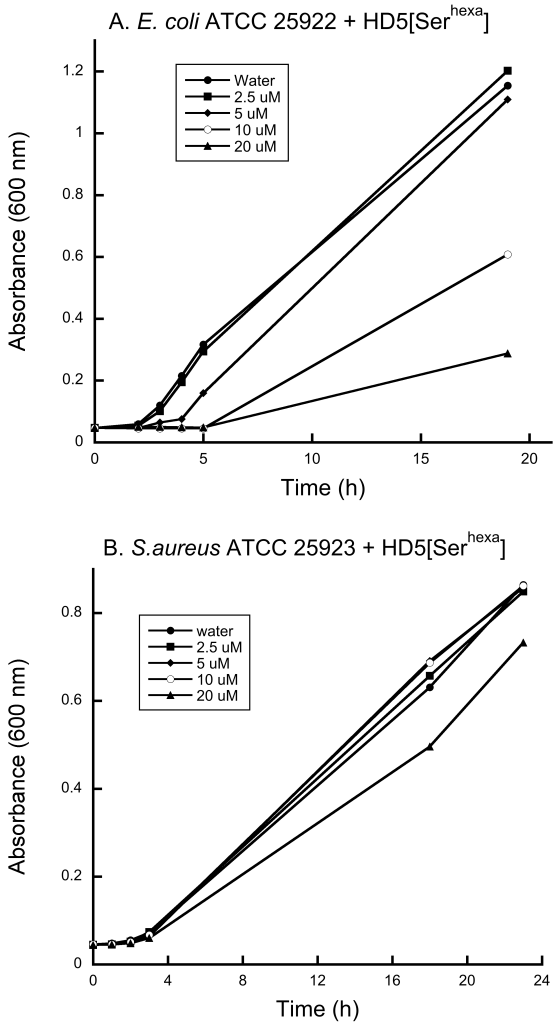


Figure S28. Turbidity assay for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 treated with varying concentrations of HD5[Ser^{hexa}]. (A) *E. coli* ATCC 25922; (B), *S. aureus* ATCC 25923. In this assay, the bacteria were treated with varying concentrations of HD5[Ser^{hexa}] in 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB (1 h, 37 ^{o}C). The cultures were then diluted with an equal volume of 2x MHB and incubated at 37 ^{o}C with shaking. The OD_{600} values were monitored at varying time points to provide a measurement of bacterial cell growth. Water refers to the no-peptide control.

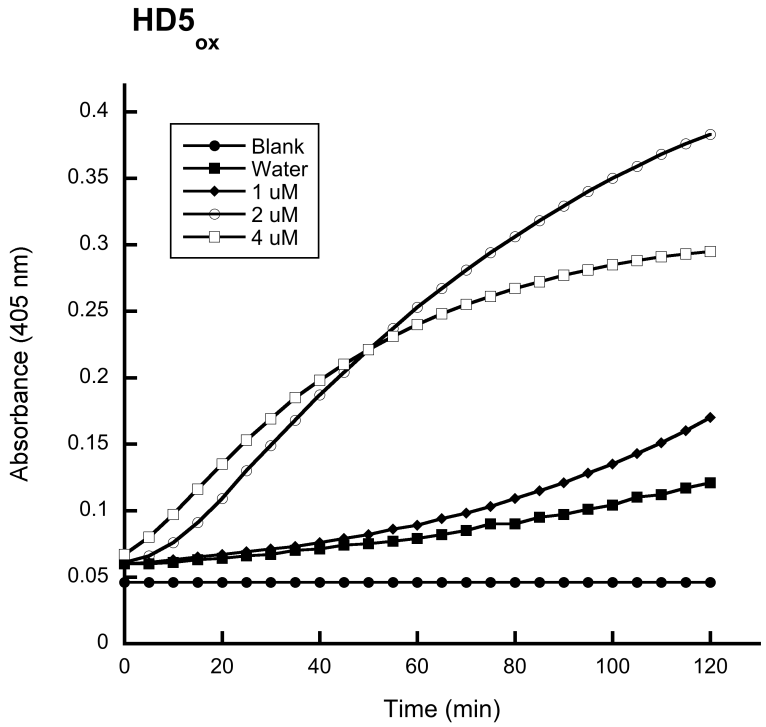


Figure S29. Inner-membrane permeabilization assay employing *E. coli* ML35 (1 x 10^6 CFU), 2.5 mM ONPG and 0, 1, 2, 4 μM of $HD5_{ox}$ (10 mM sodium phosphate buffer containing 1% TSB, pH 7.4, 37 ^oC). Blank refers to a well containing only 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB. Water refers to a no-peptide control. Absorbance at 405 nm was monitored at 5 min intervals.

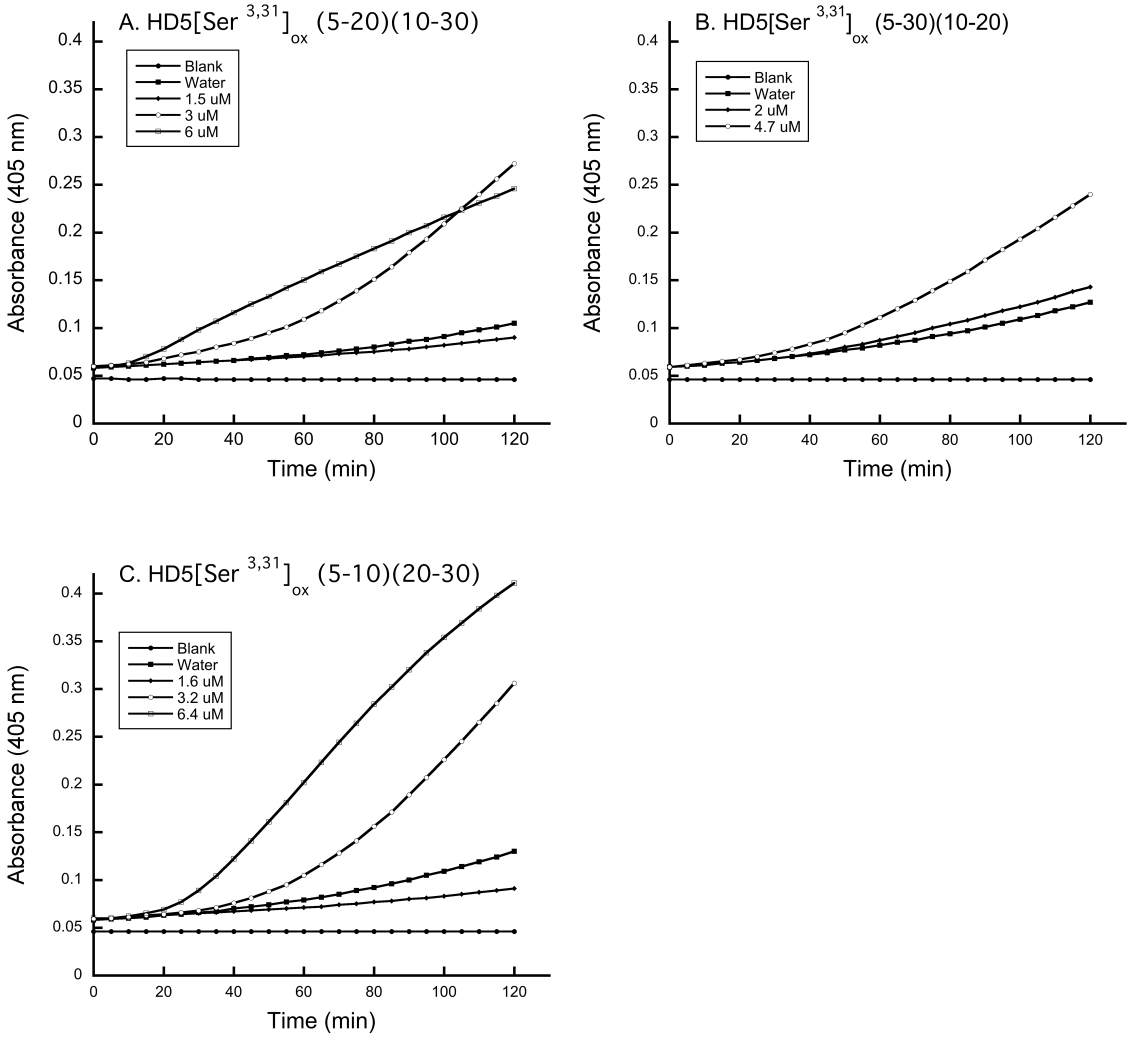


Figure S30. Inner-membrane permeabilization assay employing *E. coli* ML35 (1 x 10^6 CFU), 2.5 mM ONPG and variable concentrations of the HD5[$Ser^{3,31}$]$_{ox}$ regioisomers (10 mM sodium phosphate buffer containing 1% TSB, pH 7.4, 37 ^{o}C). **A**, HD5[$Ser^{3,31}$]$_{ox}$ (5-20)(10-30) **B**, HD5[$Ser^{3,31}$]$_{ox}$ (5-30)(10-20); **C**, HD5[$Ser^{3,31}$]$_{ox}$ (5-10)(20-30). Blank refers to a well containing only 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB. Water refers to a no-peptide control. Absorbance at 405 nm was monitored at 5 min intervals.

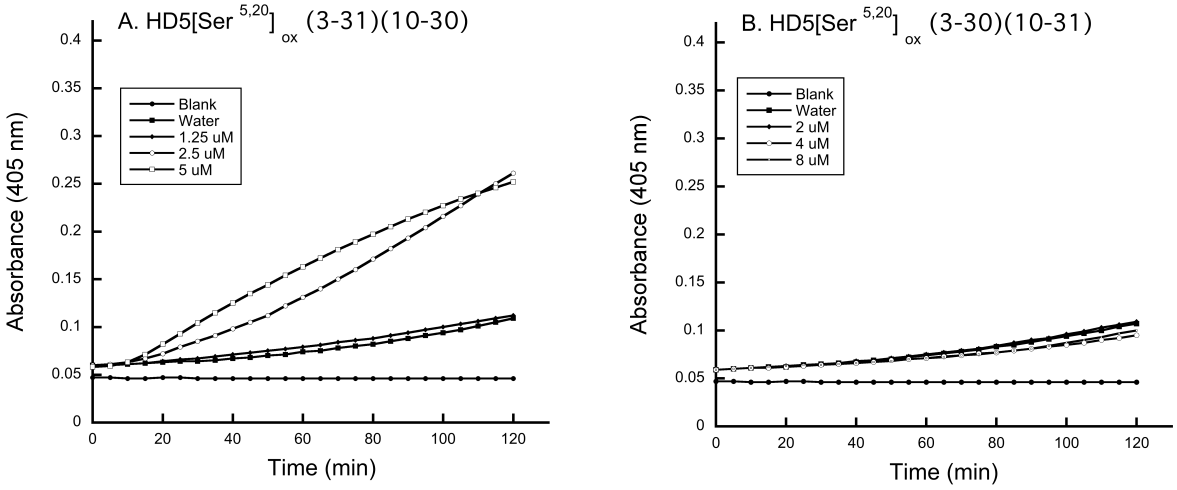


Figure S31. Inner-membrane permeabilization assay employing *E. coli* ML35 (1 x 10^6 CFU), 2.5 mM ONPG and variable concentrations of the HD5[$Ser^{5,20}$]$_{ox}$ regioisomers (10 mM sodium phosphate buffer containing 1% TSB, pH 7.4, 37 ^{o}C). **A**, HD5[$Ser^{5,20}$]$_{ox}$ (3-31)(10-30); **B**, HD5[$Ser^{5,20}$]$_{ox}$ (3-30)(10-31). Blank refers to a well containing only 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB. Water refers to a no-peptide control. Absorbance at 405 nm was monitored at 5 min intervals.

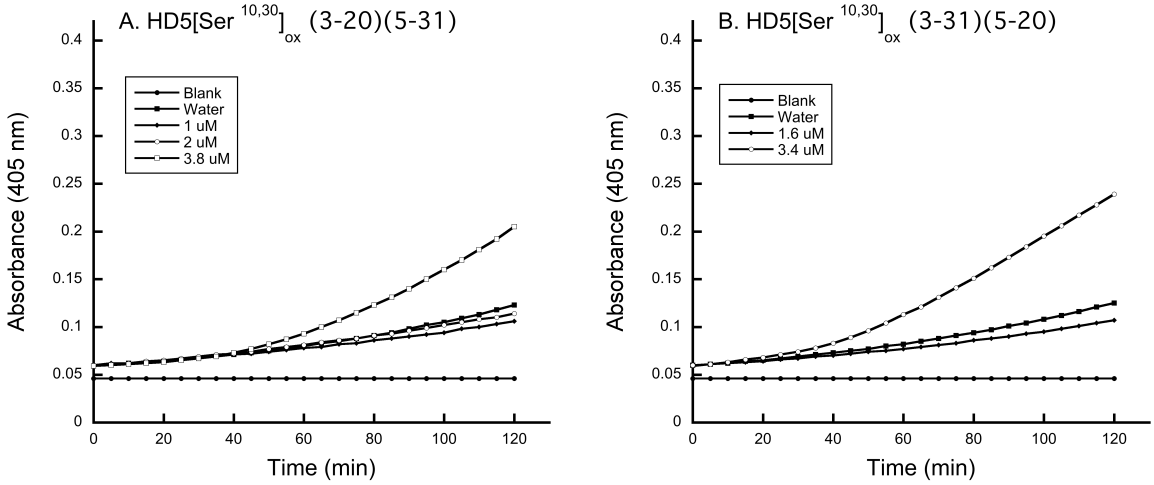


Figure S32. Inner-membrane permeabilization assay employing *E. col* ML35 (1 x 10^6 CFU), 2.5 mM ONPG and variable concentrations of the HD5[$Ser^{10,30}$]$_{ox}$ regioisomers (10 mM sodium phosphate buffer containing 1% TSB, pH 7.4, 37 ^{o}C). (A) HD5[$Ser^{10,30}$]$_{ox}$ (3-20)(5-31); (B) HD5[$Ser^{10,30}$]$_{ox}$ (3-31)(5-20). Blank refers to a well containing only 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB. Water refers to a no-peptide control. Absorbance at 405 nm was monitored every 5 min.

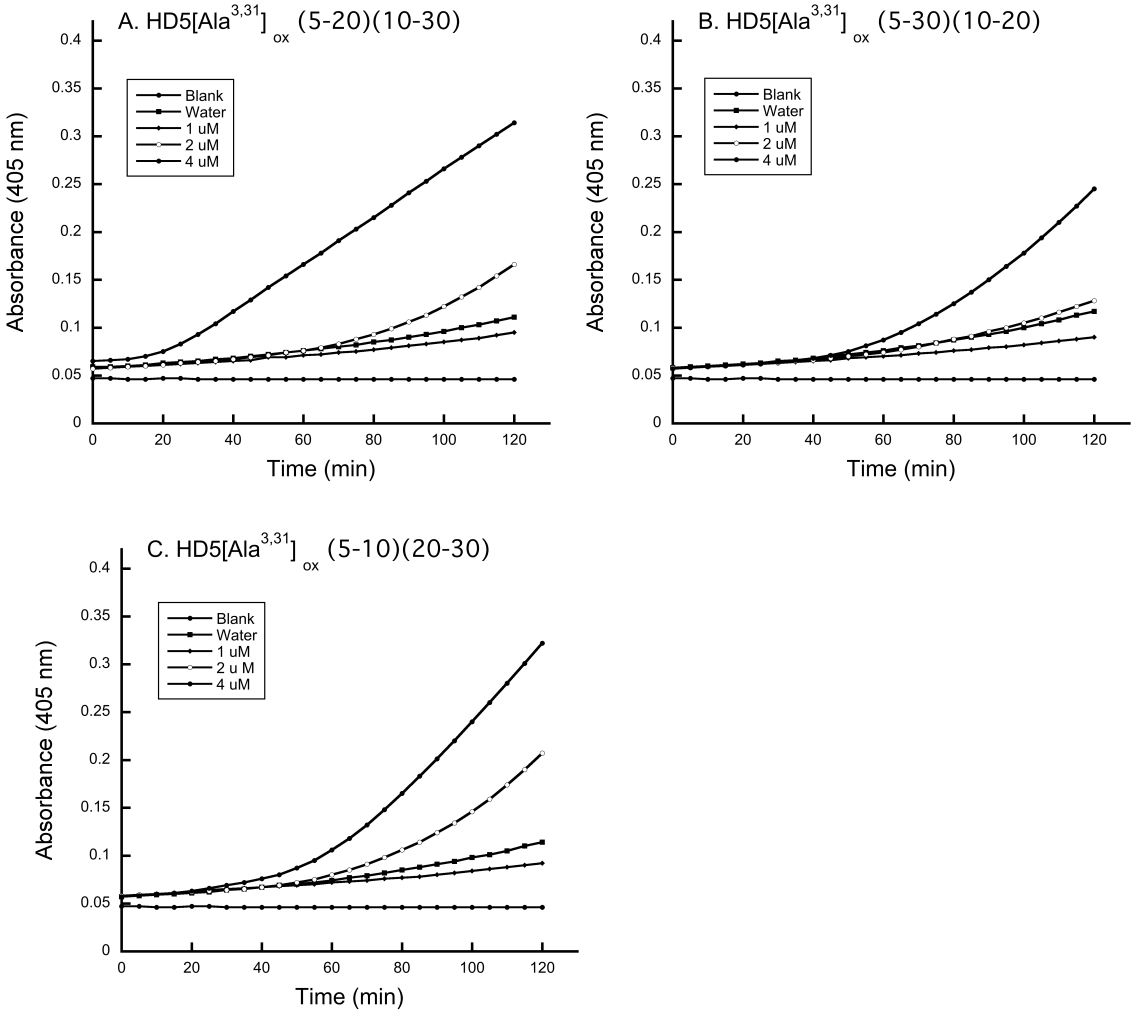


Figure S33. Inner-membrane permeabilization assay employing *E. coli* ML35 (1 x 10^6 CFU), 2.5 mM ONPG and variable concentrations of the HD5[$Ala^{3,31}$]$_{ox}$ regioisomers (10 mM sodium phosphate buffer containing 1% TSB, pH 7.4, 37 ^{o}C). (A) HD5[$Ala^{3,31}$]$_{ox}$ (5-20)(10-30); (B) HD5[$Ala^{3,31}$]$_{ox}$ (5-30)(10-20); **C**, HD5[$Ala^{3,31}$]$_{ox}$ (5-10)(20-30). Blank refers to a well containing only 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB. Water refers to a no-peptide control. Absorbance at 405 nm was monitored at 5 min intervals.

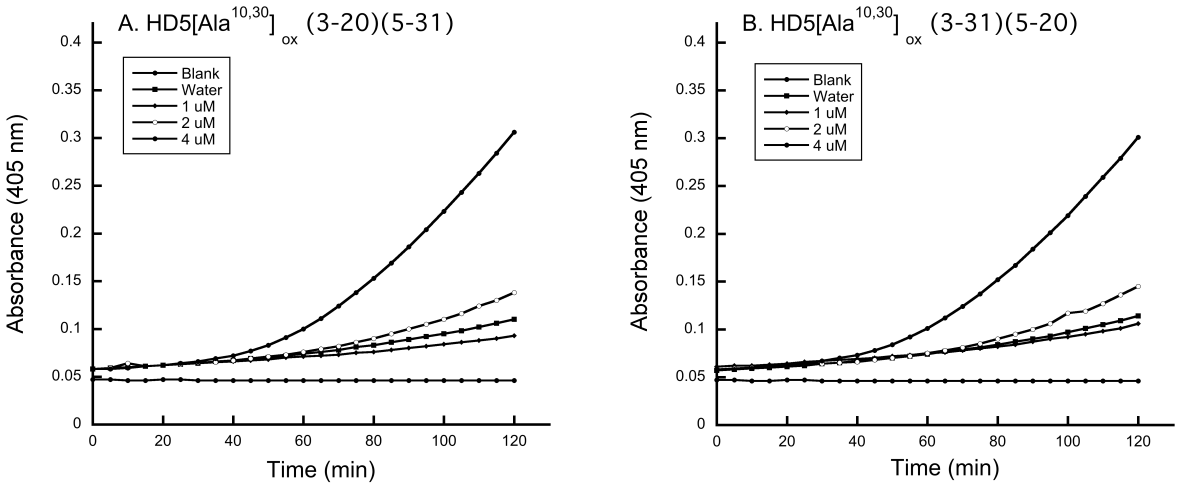


Figure S34. Inner-membrane permeabilization assay employing *E. coli* ML35 (1 x 10^6 CFU), 2.5 mM ONPG and variable concentrations of the HD5[$Ala^{10,30}$]$_{ox}$ regioisomers (10 mM sodium phosphate buffer containing 1% TSB, pH 7.4, 37 ^{o}C). (A) HD5[$Ala^{10,30}$]$_{ox}$ (3-20)(5-31); (B) HD5[$Ala^{10,30}$]$_{ox}$ (3-31)(5-20). Blank refers to a well containing only 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB. Water refers to a no-peptide control. Absorbance at 405 nm was monitored at 5 min intervals.

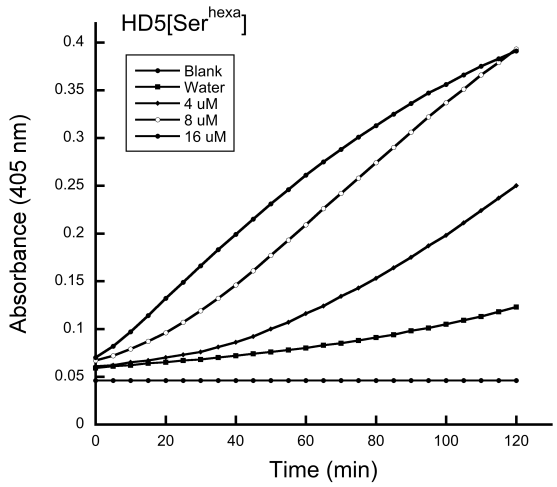


Figure S35. Inner-membrane permeabilization assay employing *E. coli* ML35 (1 x 10^6 CFU), 2.5 mM ONPG and variable concentrations of the HD5[Ser^{hexa}] (10 mM sodium phosphate buffer containing 1% TSB, pH 7.4, 37 ^{o}C). Blank refers to a well containing only 10 mM sodium phosphate buffer adjusted to pH 7.4 and containing 1% TSB. Water refers to a no-peptide control. Absorbance at 405 nm was monitored at 5 min intervals.

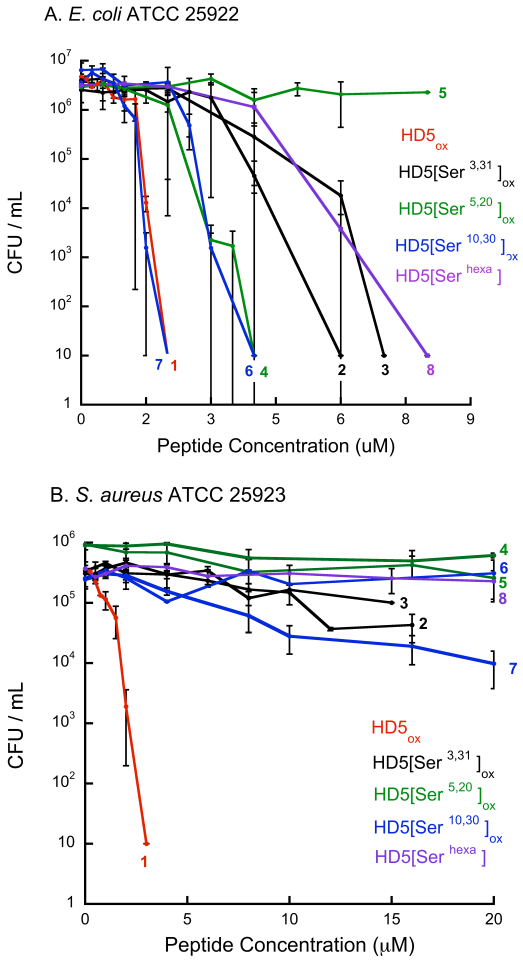


Figure S36. Antibacterial activity assays with the $HD5_{ox}$ mutant family (CFU method). The error bars represent the standard error of the mean (sem) for three independent trials conducted on different days. 1, $HD5_{ox}$; 2, $HD5[Ser^{3,31}]_{ox}$ (5-30)(10-20); 3, $HD5[Ser^{3,31}]_{ox}$ (5-10)(20-30); 4, $HD5[Ser^{5,20}]_{ox}$ (3-31)(10-30); 5, $HD5[Ser^{5,20}]_{ox}$ (3-30)(10-31); 6, $HD5[Ser^{10,30}]_{ox}$ (3-31)(5-20); 7, $HD5[Ser^{10,30}]_{ox}$ (3-20)(5-31). (A) Activity of $HD5_{ox}$ and the Ser mutants against *E. coli* ATCC 25922. (B) Activity of $HD5_{ox}$ and the Ser mutants against *S. aureus* ATCC 25923. In both instances a CFU of 10 indicates no colony formation. The data represent the averages of three independent trials and the error bars are omitted for clarity.