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Supporting Information:

Broad-Spectrum Catalytic Metallopeptide Inactivators of Zika and West Nile Virus NS2B/NS3 Proteases

Andrew M. Pinkham,[†] *Zhen Yu,*[†] *and J. A. Cowan*[†]

[†]Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH, 43210, USA.

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MATERIALS AND METHODS

Materials. The pET28(b+)-ZKV-NS2B$_{47}$(G_4SG_4)NS3pro$_{184}$ construct was made by GenScript Inc. All Fmoc-protected amino acids, and other solid-phase peptide synthesis materials, were purchased from Chem-Impex Int'l. Inc. or Sigma. Trypsin and Chymotrypsin were purchased from Thermo-Fischer.

pNA substrate synthesis. pNA substrates were synthesized per the general method of Abbenante *et al.*,[1] on a Protein Technologies, Inc. PS3 peptide synthesizer. Substrates were characterized by analytical HPLC and ESI mass spectrometry.

Enzyme preparation and activity assay. The described protease plasmid pET28(b+)-ZKV-NS2B$_{47}$(G_4SG_4)NS3pro$_{184}$ was transformed into *E. coli* BL21(DE3) using standard molecular biology practices. Cultures of BL21(DE3)- pET28(b+)-ZKV-NS2B$_{47}$(G4SG4)NS3pro$_{184}$ were grown in 20 mL 2xYT medium overnight with 50 µg/mL kanamycin at 37 °C overnight in 50 mL sterile falcon tubes with shaking at 210 rpm. The R47A mutation was achieved using the Agilent quick change methodology, and the resulting mutant protease is referred to as ZKVP. The overnight culture was transferred to 2 L of fresh 2xYT medium with 50 µg/mL of kanamycin and incubated at 37 °C until $A_{600\ nm}$ reached ~0.6. Expression of the recombinant protease was induced by addition of isopropyl-β-D-thiogalactopyranose (IPTG) to a final concentration 0.5 mM and incubated for an additional 3 h at 22 °C. The cells were then harvested by centrifugation at 5000 g at 4.0 ºC and stored at -80.0 °C. Cell pellets were thawed on ice and resuspended in 10 mL of lysis buffer (50 mM HEPES free base, 300 mM NaCl, 10 mM imidazole, 5% glycerol (v/v), and pH adjusted to pH 7.5 with 6 M HCl). Protease inhibitors were added to the lysis buffer to give final concentrations of 1 µg/mL benzamide and 1 mM PMSF. The resuspended cells were lysed by sonication and the insoluble fractions separated by

pelleting via centrifugation at 20,000 g for 30 min at 4 °C. The cleared lysate was added to a 5 mL Ni^{2+} NTA column preequilibrated with 25 mL of column buffer (50 mM HEPES free base, 300 mM NaCl, 10 mM imidazole, 5% glycerol (v/v) and pH adjusted to pH 7.5 with 6 M HCl). The column was washed with 50 mL of wash buffer containing 40 mM imidazole and eluted with 50mL of wash buffer containing 500 mM imidazole and collected in 1 mL fractions. Fractions were analyzed by 12% (w/v) SDS-PAGE and protein-containing fractions were pooled and concentrated on a 10 kDa amicon membrane. The protease was then aliquoted, snap frozen in liquid N_2, and stored at -80 °C until needed.

The purified recombinant protease was assayed against Ac-TSGKR-pNA. Assays were conducted in a 96-well plate with a final reaction volume of 300 µL containing 0.5 µM of recombinant protease. Conditions were optimized following similar protocol for WNVP and DNVP. Final conditions used for all ZKVP assays were as follows; 50 mM Tris-HCl, pH 9.5, 30% glycerol (v/v) and 1 mM CHAPS. Substrate cleavage was monitored by the increase at 405 nM.

Following optimization of reaction conditions, Michaelis-Menten parameters were determined for both ZKV-NS2B$_{47}$(G4SG4)NS3pro$_{184}$ and ZKV-NS2B$_{R47A}$(G4SG4)NS3pro$_{184}$.[2,3] The measurements were conducted in a clear 96-well plate using the optimized conditions for proteolytic processing. Michaelis-Menten parameters were measured for all known ZKV polyprotein cleavage sequences. Measurements were made in triplicate and fitted to a Michaelis-Menten equation using Origin 7 software. All values are reported as measurement ± standard error.

Preparation of Metallopeptides. Peptide synthesis was performed on a Protein Technologies, Inc. PS3 peptide synthesizer by standard automated methods. Peptides were cleaved in 95% TFA (v/v), 1.25% thioanisole (v/v), 1.25% ethane dithiole (v/v), and 2.5% H_2O (v/v) at room temperature, with shaking, for 4 h. The cleavage mixture was triturated three times with ice cold diethyl ether and dried overnight in a vacuum desiccator. The crude peptide was resuspended in H_2O with 0.1% TFA (v/v) and purified on a prepscale Gemini C18 column from 100% A (100% H_2O, 0.1% TFA (v/v)) to 100% B

(90% acetonitrile (v/v), 10% H_2O (v/v), 0.1% TFA (v/v)) over the course of 100 min with the UV/Vis detector set to 220 nm. Fractions were taken every 1.0 min and analyzed by high resolution ESI, with fractions being >95% pure pooled together and lyophilized. All peptides were dissolved in 50 mM HEPES free base and pH adjusted to pH 7.5 with 6 M HCl. Peptide purity was determined by analytical HPLC and ESI. Stocks of each peptide were run on a Sunfire™ C18 5µm column with a UV detector set to 220 nm. The gradient for the HPLC assessment was developed from 100% buffer A (100% H_2O, 0.1% TFA (v/v)) to 100% buffer B (90% acetonitrile (v/v), 10% H_2O (v/v), 0.1% TFA (v/v)) over 30 min (Figure S6). The single peak was collected and assessed by ESI for purity. The sum of the mass intensities was determined and used to determine purity (Figures S10).

The concentration of each peptide was determined by UV/vis titration ($\varepsilon 525 = 110 \pm 6\ M^{-1}\ cm^{-1}$) with a solution of known concentration of copper(II) chloride. Concentrations of Cu-metallopeptide were prepared with a 1.1:1.0 ratio of peptide to Cu^{2+} in 50 mM Tris-HCl (pH 7.4) and incubated at room temperature for 30 min prior to further use.

The stability of parent metallopeptide Cu-**1** was determined by incubation of 50 µM Cu-**1** in 50 mM Tris-HCl pH 7.5 with 1 mM H_2O_2 1 mM and sodium ascorbate at 37 ºC. Time points were taken at 0, .5, 1, 3, 6, 12, and 24 h and separated using a reverse phase Sunfire™ C18 5µm column with a UV detector set to 220 nm. The gradient for the HPLC assessment was developed from 100% buffer A (100% H_2O, 0.1% TFA (v/v)) to 100% buffer B (90% Acetonitrile (v/v), 10% H_2O (v/v), 0.1% TFA (v/v)) over 60 min.

Binding affinity of metallopeptides to proteases and inhibition of protease activity. Measurements on protein solutions of 3 µM recombinant protease with and without a range of inhibitors (0 to 1000 µM) were made in triplicate in a 96-well plate in ZKVP activity buffer. After 1 h incubation at room temperature, fluorescence was measured at 25°C on a Spectra Mx GEMINI XS with λ_{ex}= 280 nm and λ_{em} = 340 nm.[31] Slit widths were set to 10 and 20 mm for excitation and emission. Similarly, the

measurements for binding affinity to WNVP were performed with WNVP in triplicate in activity buffer. Trp-50 fluorescence was fit to the corresponding quadratic one site binding equation Eq (1) that has been used in our previous work to determine K_D's of metallopeptides.[4,5]

$$F = F_0 + \frac{(K_D + R_0 + C) - \sqrt{(K_D + R_0 + C)^2 - (4R_0 \times C)}}{2R_0} \times (F_1 - F_0) + m \times C \quad (1)$$

This equation is based upon two lines intersecting where F_0 is the y intercept of the first line and F_1 is the y-intercept of the second line. K_D is the equilibrium dissociation constant and R_0 is the corresponding inflection point. To account for any additional phases (i.e. a non-zero slope of the second line) an m term, or slope, is used to define this. C is the independent variable in concentration. This equation was used to analyze binding plots and was fitted in Origin 7. All values are reported as measurement ± standard error.

The IC_{50} values measured for ZKVP in this work were obtained at several different concentrations of Ac-TKSGKR-pNA concentrations using methodologies described previously for WNVP and DNVP.[6] Briefly, 0.5 µM of ZKVP was tested with various concentrations of Cu-metallopeptides ranging from 0.1 to 50 µM in 50 mM Tris-HCl, pH 9.5, 30% glycerol (v/v) and 1 mM CHAPS and allowed to sit at 37 °C for 10 min before the reaction was initiated with the addition of 100, 250, and 500 µM Ac-TKSGKR-pNA. The initial velocity was collected by following the absorbance at 405 nm every 5 s for 2 min. These measurements were done under none oxidative conditions to obtain a better idea of binding kinetics to ZKVP and WNVP. Data was plotted to % activity vs. log[Cu-Metallopeptide]. All measurements were obtained in triplicate and are reported as measurement ± standard error. Data was fitted in Origin 7 using the dose response function. Measurements of IC_{50} was repeated for 0.5 µM WNVP under identical conditions, but with 12.5, 25, and 50 µM Np-KKR-pNA used to start the reaction.

Off-target binding analysis against Chymotrypsin and Trypsin. For percent inhibition experiments 25 μM of select copper bound metallopeptides were tested against 0.1 μM of Trypsin or Chymotrypsin. Briefly, 0.1 μM of each protease was allowed to equilibrate for 10 min in 300 μL of activity buffer (50 mM Tris-HCl pH 8.0 for Trypsin and 50 mM Tris-HCl pH 8.0 with 10 mM $CaCl_2$ for Chymotrypsin) with 25 μM copper bound metallopeptide. Reactions were started by the addition of 500 μM NH_2-AAVA-pNA for Trypsin or Bz-IEGR-pNA for Chymotrypsin and quenched by the addition of 100 μL 12 M aqueous NaOH.[6] Assays were conducted in a 96-well plate and absorbance at 405 nm was monitored and repeated in triplicate. Initial rates were compared to proteases without metallopeptides present and reported as an average ± S.E.

Time-dependent inactivation of Trypsin was conducted in a similar manner as ZKVP and WNVP. Reactions containing 1 μM of Cu-peptide were incubated with 5 μM Trypsin in 50 mM Tris-HCl pH 9.0 with 1 mM ascorbate and 1 mM H_2O_2 at room temperature. At times 0, 2, 5, 7, 10, 30 and 60 min, 0.1 μM of Trypsin was transferred to the previously described activity assay and allowed to equilibrate at 37 °C for 10 min. The initial rate was collected by the addition of 500 μM NH_2-AAVA-*p*NA with each data point was done in triplicate and reported as measurement ± standard error. Data was plotted as decrease in activity of Trypsin percent activity vs time.

Determination of metallopeptide kinetic parameters. For Michaelis-Menten studies of Cu-peptides, reactions containing 1 μM of Cu-peptide were incubated with concentrations of ZKVP or WNVP ranging from 5 to 60 μM and/or coreactants (1 mM ascorbate and or 1 mM H_2O_2 or no coreactants) in 50 mM Tris-HCl pH 7.5 with 1 mM H_2O_2 and 1 mM ascorbate. At times 0, 1, 2, 5, and 10 min an aliquot was taken and diluted to 1 μM protease concentration in 300 μL activity buffer (50 mM Tris-HCl pH 9.5, 30% glycerol (v/v), 1 mM CHAPS) at room temperature with 75 μM Np-Lys-Lys-Arg-*p*Na for WNVP or 200 μM Np-Lys-Lys-Arg-*p*Na for ZKVP. At each time point, 0.5 μM of ZKVP or WNVP was transferred to the previously described activity assay and allowed to equilibrate at

37 °C for 10 min. The initial rate was collected by the addition of 250 µM Ac-TSGKR-pNA or 50 µM Np-KKR-pNA. Each data point was done in triplicate and reported as measurement ± standard error. Plots of Cu-peptide initial velocity vs ZKVP or WNVP concentration were fitted to a standard Michaelis-Menten equation using Origin 7 software to obtain Cu-metallopeptide catalytic parameters.

For select metallopeptides second order rate kinetics were determined. This was achieved by incubating 25 µM protease with 1, 2, 5, 10, 25, 50, 100, or 200 µM select metallopeptides in 50 mM Tris-HCl pH 7.5 with 1 mM H_2O_2 and 1 mM ascorbate. At times 0, 1, 2, 5, and 10 min an aliquot was taken and diluted to 1 µM protease concentration in 300 µL activity buffer (50 mM Tris-HCl pH 9.5, 30% glycerol (v/v), 1 mM CHAPS) with 75 µM Np-Lys-Lys-Arg-*p*Na for WNVP or 200 µM Np-Lys-Lys-Arg-*p*Na for ZKVP. Each data point was compared to the time zero and plotted as $\ln(A/A_0)$ v time to get rates of deactivation and fit to a linear regression. This data was also used to determine the turnover number for select metallopeptides. The rates of deactivation were then plotted against metallopeptide concentration to obtain second-order rate constants for each reaction. All measurements were performed in a 96-well plate with each point as a set of triplicate measurements reported as ± S.E.

To determine the effects of buffer composition on the overall reaction progress 10 µM WNVP was incubated with 1 µM Cu-**1** or with Cu-**C** in 50 mM NaH_2PO_4 pH 7.5 or 50 mM Tris-HCl pH 7.5 with 1 mM H_2O_2 and 1 mM ascorbate. An aliquot was withdrawn every 10 min for 1 h and diluted to a final protease concentration of 1 µM in 300 µL of activity buffer with 75 µM of Np-Lys-Lys-Arg-*p*Na. All measurements were done in a 96 well plate with each point as a set of triplicate measurements reported as ± S.E. The overall time dependent inactivation was then compared.

Turnover numbers for select metallopeptides were determined in a similar manner, by incubating 25 µM protease with 1 µM select metallopeptides in 50 mM Tris-HCl pH 7.5 with 1 mM H_2O_2 and 1 mM ascorbate. At times 0, 1, 2, 5, 10, and 15 min an aliquot was taken and diluted to 1 µM protease concentration in 300 µL activity buffer (50 mM Tris-HCl pH 9.5, 30% glycerol (v/v), 1 mM CHAPS)

with 75 µM Np-Lys-Lys-Arg-*p*Na for WNVP or 200 µM Np-Lys-Lys-Arg-*p*Na for ZKVP. Each data point was compared to the time zero and plotted as % activity v time. The turnover number was calculated by dividing the amount of deactivated protease by the metallopeptide concentration in solution. This provides a lower limit on turnover number.

Enzyme digestion and LC-MS/MS studies. A 10 µM ZKVP or WNVP sample in 50 mM Tris-HCl (pH 7.4) was incubated with 50 µM Cu-peptide with and without 1 mM coreagents for 3 h followed by three rounds of dialysis with 50 mM Tris-HCl pH 8.0 to remove any metallopeptides or co-reagents. Samples were heated at 95 °C for 15 min to denature. Samples were cooled to room temperature and incubated with chymotrypsin in a ratio of 1:20 overnight at 37 °C. The resulting solution was subsequently injected to a LC-MS/MS instrument. Samples were prepared in triplicate. Liquid chromatography was performed by use of an Agilent 1260 Series LC system equipped with a Zorbax SB-C18 column (1.8 µM, 2.1 × 50 mm). The mobile phase was a mixture of acetonitrile/water with 0.1% formic acid (v/v), and linear gradient was applied. The flow rate was 0.5 mL/min, and the temperature of the column oven was 30 °C. The LC system was directly connected to an Agilent 6460 Triple Quad mass spectrometer. The mass spectrometer was operated under the product ion mode with drying gas (N_2, 350 °C) at a gas flow rate of 6 L/min and a nebulizing pressure of 30 psi. Collision energy of 10-70 V and fragmentor of 135 V were applied to promote CID (collision-induced dissociation) of the indicated protein fragments from trypsin digestion.

Time-dependent attenuation of ZKVP and WNVP with metallopeptides. To determine the increase of K_M or the decrease in k_{cat} following protease treatment with Cu-peptides the following experiment was performed. Recombinant protease (10 µM) was incubated with 1 µM of metallopeptide in 50 mM Tris-HCl pH 7.5, 1 mM ascorbate, and 1 mM H_2O_2 at room temperature. At times 2, 7, 12, 30, and 60 min, aliquots of the reaction mixture were taken to give a final recombinant protease concentration of 0.5 µM and assayed using 20, 40, 80, 100, 250, 500, 1000, and 2000 µM Np-KKR-

pNA for WNVP and 100, 200, 400, 800, 1000, 1500, 2000, 3000 µM Ac-TSKGKR-pNA for ZKVP in 50 mM Tris-HCl, pH 9.5, 30% Glycerol (v/v), and 1 mM CHAPS. Michaelis-Menten plots for each time point where repeated in triplicate used to determine the change in K_M and k_{cat}. Values are reported as value ± standard error.

Table S1. Metallopeptides sequences. Unnatural amino acids D-2,3-diaminioproponic acid ($_D$Dap), L-Phenylglycine and D-Phenylglycine (Phg and $_D$Phg respectively) were used. Systematic change of D-Phenylglycine to 4-hydroxy-D-Phenylglycine (4-OH-$_D$Phg), 4-Benzyloxy-D-Phenyglycine (4-BnO-$_D$Phg), and [4-(3-Methyl)benzyloxy]-D-enyglycine (4-(-3MeO)-BnO-$_D$Phg) was used to probe effects of hydrophobicity at the S1 position. End cap optimization achieved by use of 2-aminobenzoic acid (2Abz), Phg, $_D$Phg, and 2,2'-bithiophene-5-carboxylic acid (2,2'Btp) in addition to benzoylation (Bz) and naphthoylation (Np).

	Sequence
C	Cu-Gly-Gly-His-OH
Cu	Cu^{2+}
1	Bz-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Gly-His-NH_2
2	Bz-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Val-His-NH_2
3	Bz-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Phg-His-NH_2
4	Bz-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Phe-His-NH_2
5	Bz-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Tyr-His-NH_2
6	Bz-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Trp-His-NH_2
7	2,2'Btp-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Gly-His-NH_2
8	2Abz-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Gly-His-NH_2
9	Phg-Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Gly-His-NH_2
10	$_D$Phg- Arg-Lys-$_D$Phg-Gly-Gly-($_D$Dap)-Gly-His-NH_2
11	Bz-Arg-Lys-(4-OH-$_D$Phg)-Gly-Gly-($_D$Dap)-Gly-His-NH_2
12	Bz-Arg-Lys-(4-BnO-$_D$Phg)-Gly-Gly-($_D$Dap)-Gly-His-NH_2
13	Bz-Arg-Lys-(4-(-3MeO)-BnO-$_D$Phg)-Gly-Gly-($_D$Dap)-Gly-His-NH_2
14	Np-Lys-Lys-Arg-Gly-Gly-($_D$Dap)-Gly-His-NH_2

Table S2. K_D and IC_{50} values of metallopeptides against ZKVP and WNVP (N.D. = Not Determined)

Metallopeptide	ZKVP		WNVP	
	IC_{50} (μM)	K_D (μM)	IC_{50} (μM)	K_D (μM)
Cu-**C**	>200	>200	>500	>200
Cu	>500	>200	>500	>200
Cu-**1**	1.9 (1.7, 2.1)	2.0 ± 0.8	2.0 (1.7, 2.3)	0.3 ± 0.2
Cu-**2**	6.7 (5.9, 7.5)	1.0 ± 0.4	8.1 (7.0, 9.3)	10.0 ± 0.1
Cu-**3**	3.8 (3.1, 4.5)	0.3 ± 0.1	5.0 (4.7, 5.3)	2.8 ± 0.5
Cu-**4**	4.0 (3.4, 4.6)	0.4 ± 0.3	N.D.	N.D.
Cu-**5**	4.5 (5.2, 3.8)	3.3 ± 0.4	2.4 (2.3, 2.5)	4.9 ± 0.7
Cu-**6**	4.8 (4.0, 5.6)	N.D.	2.8 (2.2, 3.4,)	N.D.
Cu-**7**	3.3 (2.7, 3.9)	1.0 ± 0.1	9.0 (8.1, 9.9)	0.5 ± 0.03
Cu-**8**	15.0 (14.4, 15.6)	N.D.	11.7 (11.0, 12.4)	N.D.
Cu-**9**	50.7 (50.0, 51.4)	30.0 ± 2.3	N.D.	N.D.
Cu-**10**	4.2 (3.6, 4.8)	0.50 ± 0.02	1.5 (1.2, 1.8)	0.50 ± 0.05
Cu-**11**	4.7 (4.0, 5.4)	1.0 ± 0.3	6.1 (5.8, 6.4)	0.5 ± 0.1
Cu-**12**	36.5 (30.7, 42.3)	30.8 ± 0.4	N.D.	N.D.
Cu-**13**	13.3 (12.7, 13.9)	8.0 ± 0.5	12.5 (11.8, 13.2)	1.0 ± 0.7
Cu-**14**	5.0 (4.3, 5.7)	N.D.	6.3 (5.4, 7.2)	N.D.

Table S3. Inhibition of 0.1 µM Chymotrypsin with 25 µM of select copper metallopeptides in 300 µL of 50 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$ with 500 µM Bz-IEGR-*p*NA.

Peptide	% Inhibition
1 mM H_2O_2 and Ascorbate	N/A
Cu-**C**	N/A
Cu-**1**	0.5 ± 0.1
Cu-**2**	0.9 ± 0.2
Cu-**8**	2.0 ± 0.5
Cu-**11**	4.5 ± 1.0
Cu-**14**	N/A

Table S4. Inhibition of 0.1 µM Trypsin with 25 µM of select copper metallopeptides in 300 µL of 50 mM Tris-HCl pH 8.0 with 500 µM NH_2-AAVA-*p*NA.

Peptide	% Inhibition
1 mM H_2O_2 and Ascorbate	N/A
Cu-**C**	N/A
Cu-**1**	9.7 ± 2.0
Cu-**2**	16.0 ± 3.0
Cu-**8**	15.2 ± 4.3
Cu-**11**	1.2 ± 0.6
Cu-**14**	8.7 ± 1.2

Table S5. Cu-Metallopeptide Michaelis-Menten parameters against ZKVP. Note change from h^{-1} to s^{-1} for the last column.

Metallopeptide	K_M (μM)	k_{cat} (h^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Cu-**C**	16.0 ± 14.0	0.5 ± 0.2	8.7 ± 2.8
Cu	12.0 ± 20.0	1.8 ± 1.0	42 ± 30
Cu-**1**	22.0 ± 10.0	117.0 ± 22.0	1477 ± 726
Cu-**2**	10.0 ± 3.7	40.0 ± 6.7	1111 ± 451
Cu-**3**	6.2 ± 2.8	30.0 ± 3.7	1344 ± 629
Cu-**4**	3.3 ± 1.6	23.3 ± 2.8	1961 ± 980
Cu-**5**	12.1 ± 6.3	17.9 ± 2.6	411 ± 222
Cu-**6**	15.1 ± 8.7	58.6 ± 9.8	1077 ± 646
Cu-**7**	25.0 ± 10.0	104.0 ± 19.0	1156 ± 508
Cu-**8**	24.8 ± 15.6	108.1 ± 22.4	1210 ± 801
Cu-**9**	27.4 ± 19.0	140.4 ± 33.8	1423 ± 1000
Cu-**10**	19.8 ± 15.2	102.8 ± 24.2	1442 ± 386
Cu-**11**	27.3 ± 15.2	63.0 ± 12.1	641 ± 378
Cu-**12**	50.0 ± 15.6	25.0 ± 5.0	139 ± 51
Cu-**13**	30.5 ± 5.6	85.0 ± 12.8	774 ± 183
Cu-**14**	6.0 ± 2.5	60.0 ± 10.0	2778 ± 711

Table S6. Metallopeptide Michaelis-Menten kinetic parameters against WNVP. Note change from h^{-1} to s^{-1} for the last column.

Metallopeptide	K_M (μM)	k_{cat} (h^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Cu-**C**	46.0 ± 20.6	0.04 ± 0.01	0.24 ± 0.11
Cu	-	-	-
Cu-**1**	8.2 ± 4.1	20.8 ± 3.0	705 ± 352
Cu-**2**	14.0 ± 4.5	69.1 ± 7.5	1370 ± 441
Cu-**7**	10.0 ± 2.5	30.2 ± 6.0	839 ± 210
Cu-**8**	32.0 ± 6.0	107.1 ± 1.5	930 ± 175
Cu-**10**	10.8 ± 3.8	50.1 ± 1.4	1289 ± 454
Cu-**11**	14.9 ± 2.5	47.5 ± 6.0	886 ± 149
Cu-**14**	10.0 ± 9.0	4.1 ± 1.2	114 ± 102

Table S7. Select metallopeptide 2nd order rates against 25 μM WNVP and ZKVP over the course of 10 min.

WNVP			
Metallopeptide	K_I (μM)	$k_{inactivation}$ (min^{-1})	$k_{inactivation}/K_I$ ($M^{-1}s^{-1}$)
Cu-**C**	46.0 ± 20.6	0.04 ± 0.01	0.24 ± 0.11
Cu	-	-	-
Cu-**1**	8.2 ± 4.1	20.8 ± 3.0	705 ± 352
Cu-**2**	14.0 ± 4.5	69.1 ± 7.5	1370 ± 441
Cu-**7**	10.0 ± 2.5	30.2 ± 6.0	839 ± 210
ZKVP			
Metallopeptide	K_I (μM)	$k_{inactivation}$ (min^{-1})	$k_{inactivation}/K_I$ ($M^{-1}s^{-1}$)
Cu-**C**	100 ± 10	0.06 ± 0.01	10 ± 1.0
Cu-**1**	1.3 ± 0.3	0.20 ± 0.10	2830 ± 1167
Cu-**2**	2.0 ± 0.8	0.30 ± 0.05	2500 ± 1167
Cu-**8**	3.0 ± 0.5	0.40 ± 0.12	22167 ± 1330
Cu-**11**	3.7 ± 1.0	0.90 ± 0.10	4167 ± 167

Table S8. Turn over numbers of 1 μM select metallopeptides against 25 μM WNVP and ZKVP.

Metallopeptide	ZKVP	WNVP
Cu-**C**	5 ± 1	6 ± 1
Cu-**1**	17 ± 4	19 ± 4
Cu-**2**	21 ± 9	22 ± 1
Cu-**8**	21 ± 11	21 ± 3
Cu-**11**	16 ± 3	23 ± 1

Table S9. Time dependent change in Michaelis-Menten parameters of ZKVP and WNVP with selected metallopeptides. Note change from h^{-1} to s^{-1} for the k_{cat}/K_M columns.

Compound	ZKVP			WNVP		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
N/A	283 ± 55	0.8 ± 0.01	2900 ± 150	52 ± 34	1.2 ± 0.3	23070 ± 6000
1 mM Co-reagents	330 ± 50	0.7 ± 0.1	2100 ± 440	66 ± 11	1.1 ± 0.1	16000 ± 3000
Cu-**C**	830 ± 100	0.7 ± 0.1	840 ± 160	45 ± 10	1.0 ± 0.1	22000 ± 1000
Cu-**1**	920.0 ± 280	0.06 ± 0.01	65.2 ± 20	760 ± 90	0.3 ± 0.1	400 ± 140
Cu-**2**	475 ± 200	0.04 ± 0.01	85 ± 38	440 ± 80	0.016 ± 0.001	37 ± 1
Cu-**8**	320 ± 120	0.05 ± 0.004	150 ± 60	100 ± 30	0.011 ± 0.002	130 ± 50
Cu-**11**	270 ± 50	0.05 ± 0.004	185 ± 37	120 ± 20	0.013 ± 0.001	120 ± 40

Table S10. Raw sequencing data and assignments for oxidized ZKVP 432.2 peak modified by Cu-**1** with modified amino acids highlighted in red.

Observed *m/z*	Control *m/z*	Calculated *m/z*	Ion type	Sequence	Δ amu
86.3	79.1	78.5	2+ C	NH_2-**H**	+16
136.0	127.6	127.5	2+ C	NH_2-**H**V	+16
193.6	178.0	177.5	2+ C	NH_2-**H**V**T**	+48
257.3	233.1	233.6	2+ B	NH_2-**H**V**T**K	+48
237.4	236.6	237.0	2+ Y	KGSAL-COOH	
346.9	346.2	346.5	1+ Y	GSAL-COOH	
288.5	292.1	289.8	1+ Y	SAL-COOH	
203.2	202.0	202.1	1+ Y	AL-COOH	
130.7	132.0	131.4	1+ Y	L-COOH	

Table S11. Raw sequencing data and assignments for oxidized 431.9 peak in ZKVP, modified by Cu-**2** with oxidized amino acids highlighted in red

Observed *m/z*	Control *m/z*	Calculated *m/z*	Ion type	Sequence	Δ amu
86	79.1	78.5	2+ C	NH_2-**H**	+16
134.6	127.6	127.5	2+ C	NH_2-**H**V	+16
201.5	178.0	177.5	2+ C	NH_2-**H**V**T**	+48
256.8	233.1	233.6	2+ B	NH_2-**H**V**T**K	+48
237.6	236.6	237.0	2+ Y	KGSAL-COOH	
345.8	346.2	346.5	1+ Y	GSAL-COOH	
289.1	292.1	289.8	1+ Y	SAL-COOH	
202.0	202.0	202.1	1+ Y	AL-COOH	
131.0	132.0	131.4	1+ Y	L-COOH	

Table S12. Raw sequencing data and assignments for oxidized ZKVP 432.0 peak modified by Cu-**11** with modified amino acids highlighted in red.

Observed *m/z*	Control *m/z*	Calculated *m/z*	Ion type	Sequence	Δ amu
86.7	79.1	78.5	2+ C	NH_2-**H**	+16
134.5	127.6	127.5	2+ C	NH_2-**H**V	+16
199.9	178.0	177.5	2+ C	NH_2-**H**V**T**	+48
258.1	233.1	233.6	2+ B	NH_2-**H**V**T**K	+48
236.0	236.6	237.0	2+ Y	KGSAL-COOH	
346.8	346.2	346.5	1+ Y	GSAL-COOH	
292.5	292.1	289.8	1+ Y	SAL-COOH	
202.7	202.0	202.1	1+ Y	AL-COOH	
133.0	132.0	131.4	1+ Y	L-COOH	

Table S13. Raw sequencing data and assignments for oxidized 423.0 peak in ZKVP, modified by Cu-**8** with oxidized amino acids highlighted in red

Observed *m/z*	Control *m/z*	Calculated *m/z*	Ion type	Sequence	Δ amu
80.0	79.1	78.5	2+ C	NH_2-H	+16
128.5	127.6	127.5	2+ C	NH_2-HV	+16
194.2	178.0	177.5	2+ C	NH_2-HV**T**	+48
249.9	233.1	233.6	2+ B	NH_2-HV**T**K	+48
236.0	236.6	237.0	2+ Y	KGSAL-COOH	
346.8	346.2	346.5	1+ Y	GSAL-COOH	
292.5	292.1	289.8	1+ Y	SAL-COOH	
201.7	202.0	202.1	1+ Y	AL-COOH	
130.6	132.0	131.4	1+ Y	L-COOH	

Table S14. Raw sequencing data and assignments for oxidized WNVP 429.0 peak modified by Cu-**2** with modified amino acids highlighted in red.

Observed *m/z*	Control *m/z*	Calculated *m/z*	Ion type	Sequence	Δ amu
68.0	59.9	59.0	2+ A2	NH_2-G**S**	16
139.5	131	131.1	2+ C3	NH_2-G**S**V	16
185.1	178.8	178.1	2+ A4	NH_2-G**S**V**K**	15
425.0	439.6	439.2	1+ A5	NH_2-G**S**V**KE**	-15
295.9	317.6	317.2	2+ C6	NH_2-G**S**V**KED**	-45
227.0	258.0	258.2	2+Z4	**ED**RL-OH	-60
186.1	202.0	201.6	2+ Y3	**D**RL-OH	-30
127.9	127.1	127.1	2+ Z2*	RL-OH	
114.8	115.2	115.0	1+ Z1.	L-OH	

Table S15. Raw sequencing data and assignments for oxidized WNVP 429.0 peak modified by Cu-**8** with modified amino acids highlighted in red.

Observed *m/z*	Control *m/z*	Calculated *m/z*	Ion type	Sequence	Δ amu
74.0	74.5	75.1	1+ C1	NH_2-G	
69.1	59.9	59.0	2+ A2	NH_2-G**S**	+16
138.5	131.0	131.1	2+ C3	NH_2-G**S**V	+16
185.1	178.8	178.1	2+ A4	NH_2-G**S**V**K**	+15
243.9	251.5	251.1	2+ B5	NH_2-G**S**V**KE**	-15
284.0	308.0	308.6	2+ B6	NH_2-G**S**V**KED**	-45
244.1	258.5	258.1	2+ Z4	**D**RL-OH	-30
127.9	127.1	127.1	2+ Z2	RL-OH	
114.8	115.2	115.0	1+ Z1.	L-OH	

Table S16. Raw sequencing data and assignments for oxidized WNVP 429.0 peak modified by Cu-**11** with modified amino acids highlighted in red.

Observed *m/z*	Control *m/z*	Calculated *m/z*	Ion type	Sequence	Δ amu
69.1	59.9	59.0	2+ A2	NH_2-G**S**	+16
260.2	244.5	244.1	1+ B3	NH_2-G**S**V	+16
241.1	251.5	251.1	2+ B5	NH_2-G**S**V**KE**	-15
284.0	308.0	308.6	2+ B6	NH_2-G**S**V**KED**	-45
348.8	380.0	379.7	2+ Y5	**KED**RL-OH	-61
300.3	330.7	330.2	2+ Y2	**ED**RL-OH	-60
185.8	201.5	201.6	2+ Y3	**D**RL-OH	-30
128.7	127.1	127.1	2+ Z2	RL-OH	
132.0	131.5	131.1	1+ Y1.	L-OH	

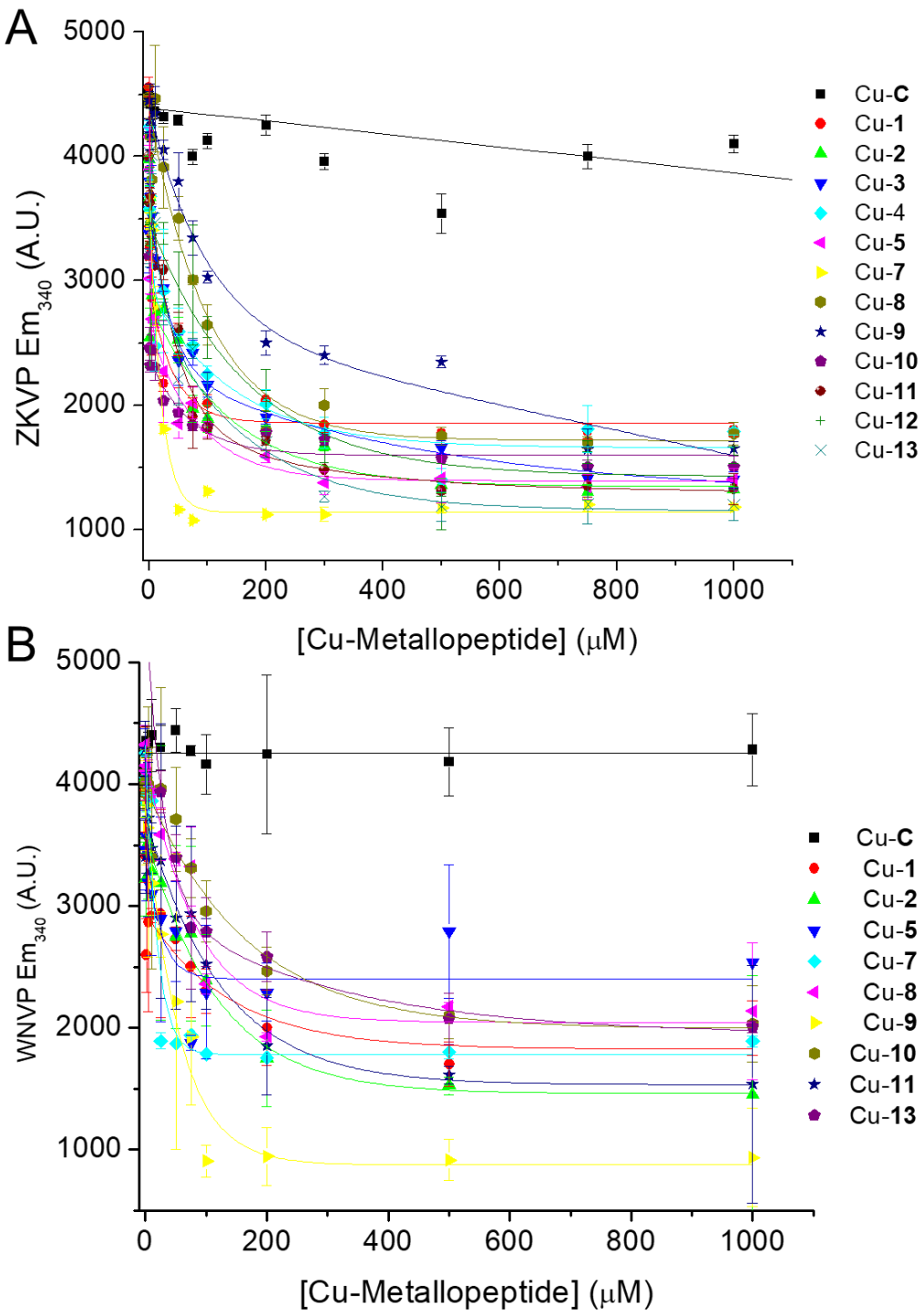


Figure S1. (**A**) K_D determinations of Cu-metallopeptides against 3 μM ZKVP (**B**) and K_D determinations Cu-metallopeptides against 3 μM WNVP in 50 mM Tris-HCl pH 9.5, 30% Glycerol (v/v) and 1 mM CHAPS.

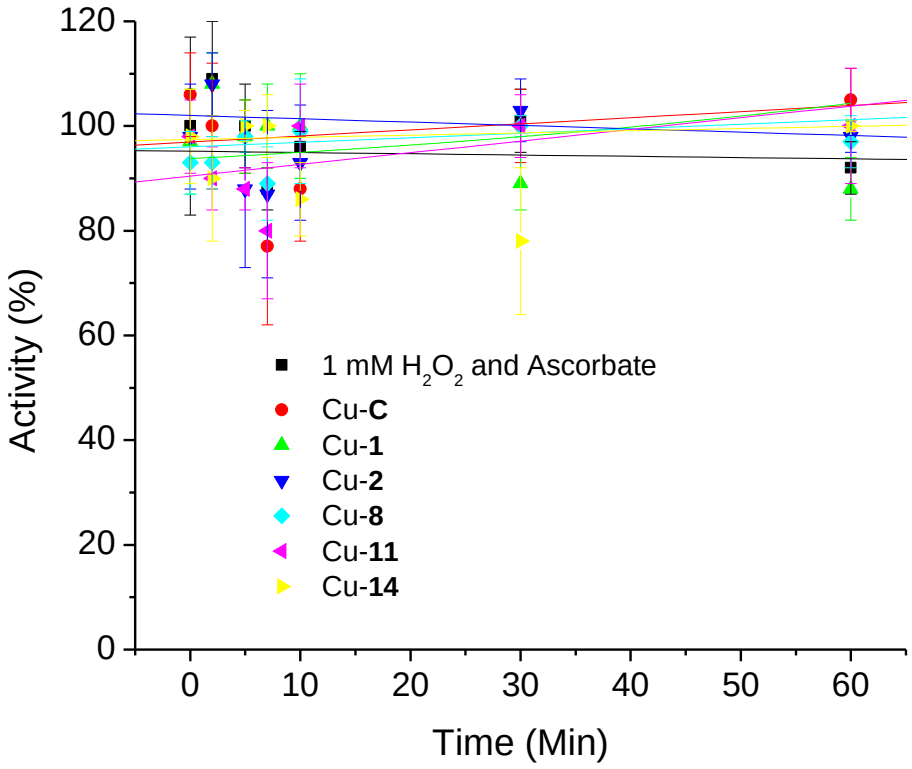


Figure S2. Time-dependent inactivation of 5 µM Trypsin with 1 µM of select copper bound metallopeptides in 50 mM Tris-HCl pH 9.0 with 1 mM H_2O_2 and ascorbate.

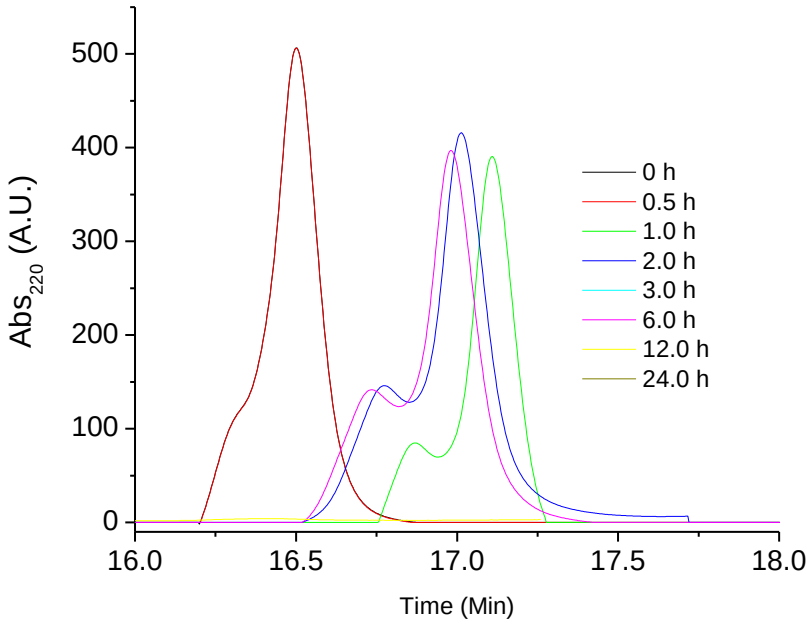
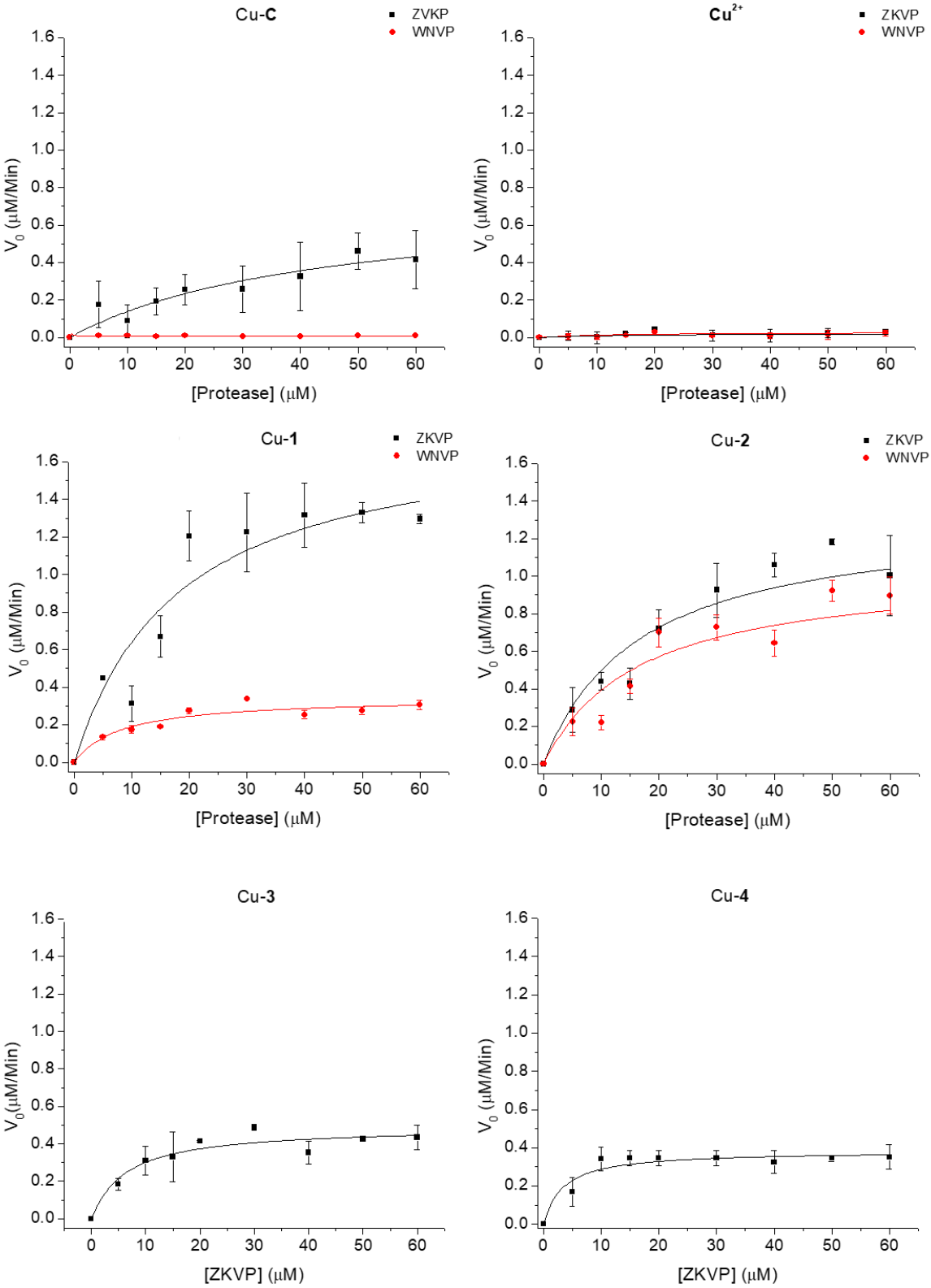
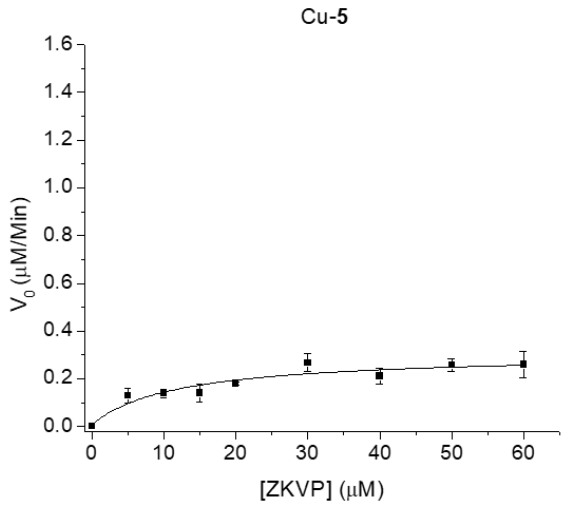
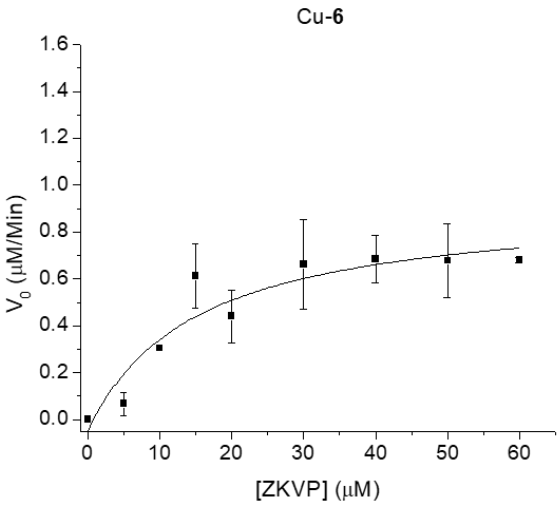
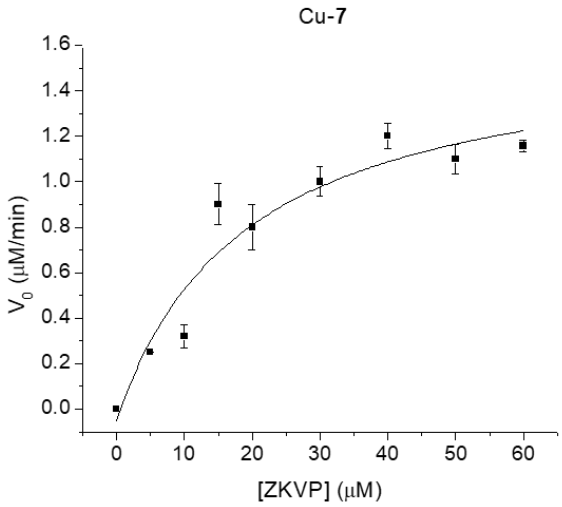


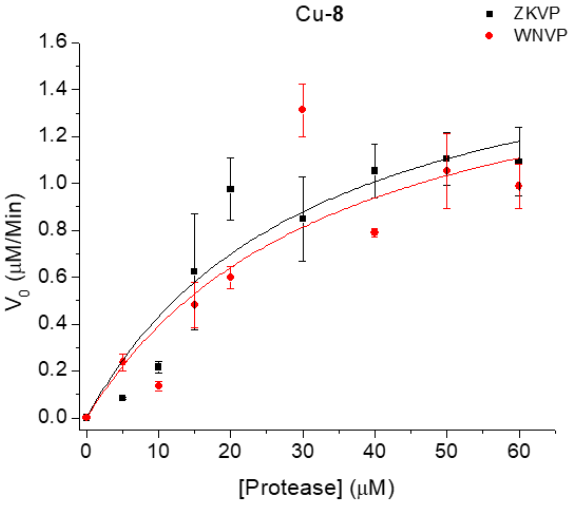
Figure S3. The time dependent stability of Cu-**1** when incubated at 37 ºC in 50 mM Tris-HCl pH 7.5 with 1 mM H_2O_2 and 1 mM ascorbate. The degradation of the metallopeptide was followed by using an analytical reverse phase HPLC with UV/Vis detector set to 220 nm.

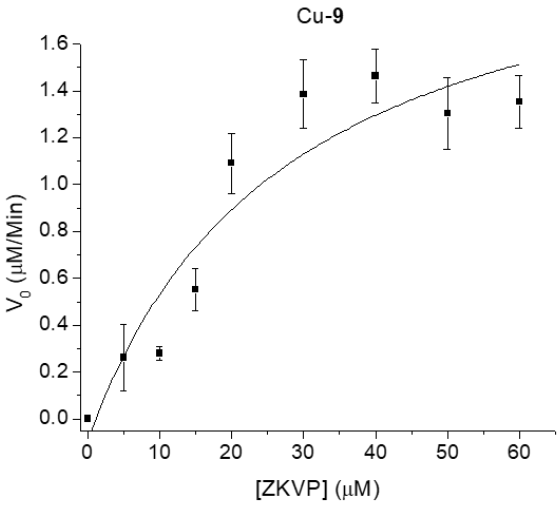

Cu-C
ZVKP
WNVP
V_0 (μM/Min)
[Protease] (μM)
Cu^{2+}
ZKVP
WNVP
V_0 (μM/Min)
[Protease] (μM)
Cu-1
ZKVP
WNVP
V_0 (μM/Min)
[Protease] (μM)
Cu-2
ZKVP
WNVP
V_0 (μM/Min)
[Protease] (μM)
Cu-3
V_0(μM/Min)
[ZKVP] (μM)
Cu-4
V_0 (μM/Min)
[ZKVP] (μM)

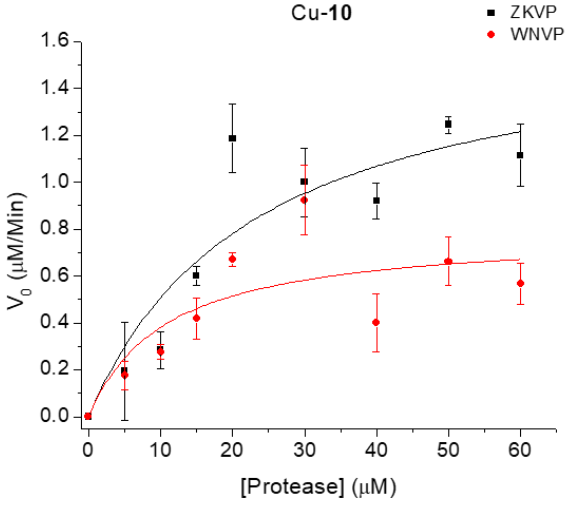

Cu-5
V_0 (μM/Min)
[ZKVP] (μM)


Cu-6
V_0 (μM/Min)
[ZKVP] (μM)


Cu-7
V_0 (μM/min)
[ZKVP] (μM)


Cu-8
ZKVP
WNVP
V_0 (μM/Min)
[Protease] (μM)


Cu-9
V_0 (μM/Min)
[ZKVP] (μM)


Cu-10
ZKVP
WNVP
V_0 (μM/Min)
[Protease] (μM)

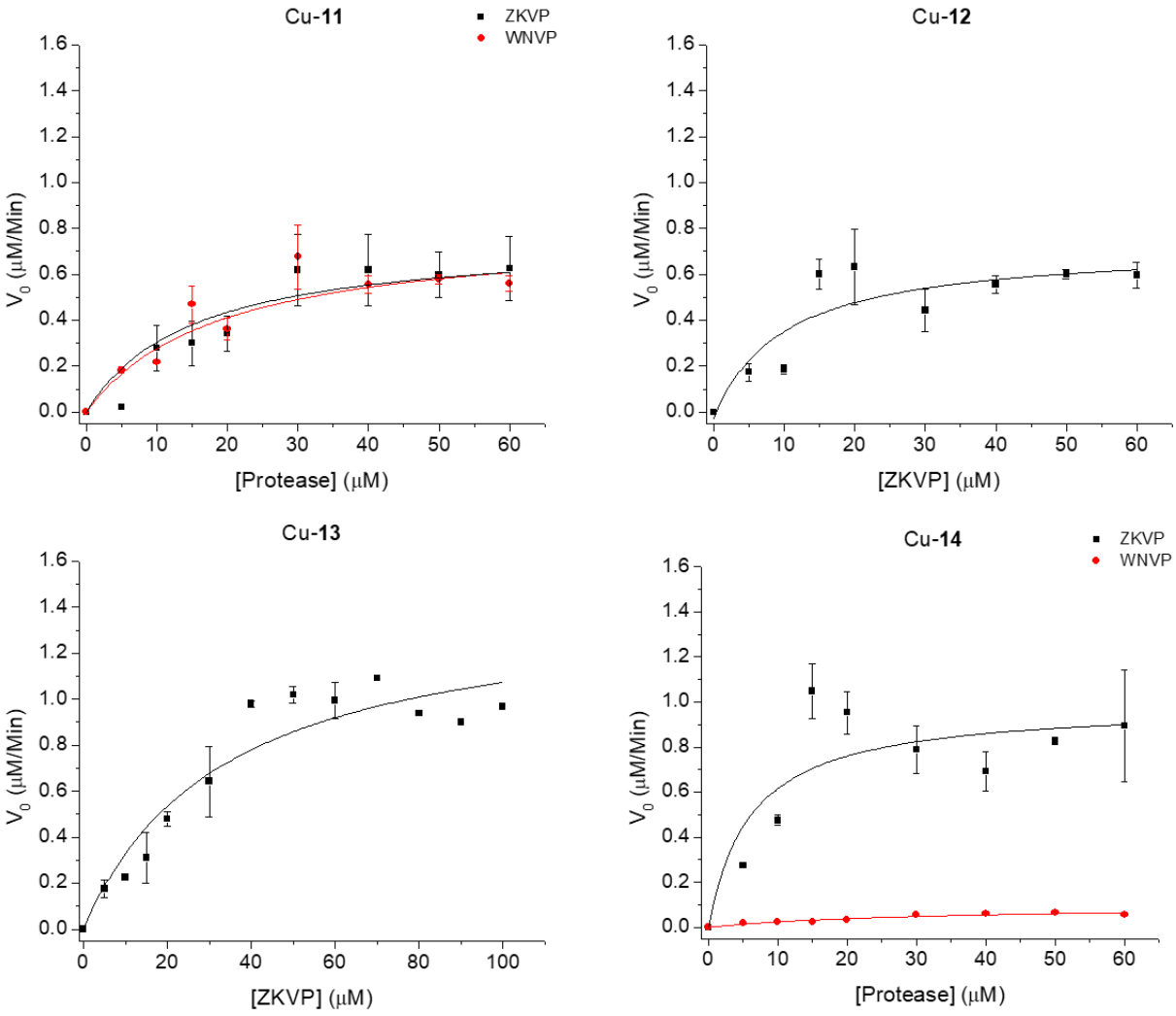
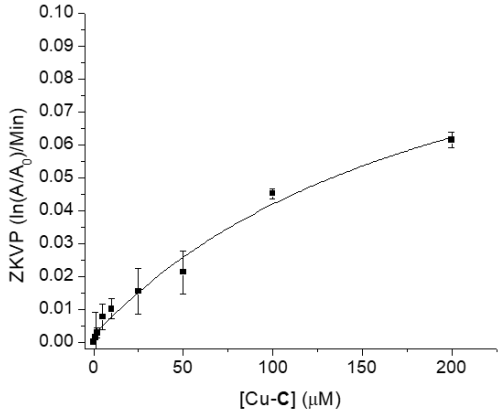
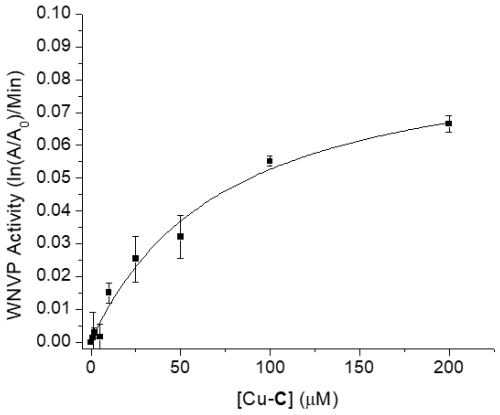
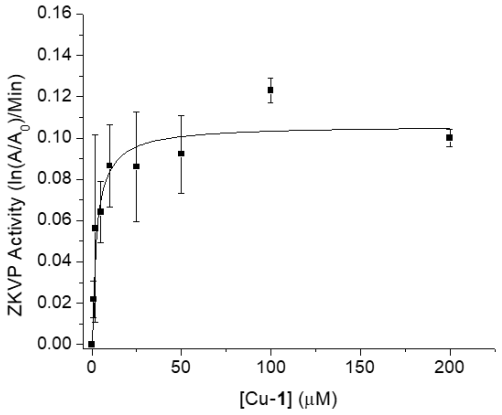
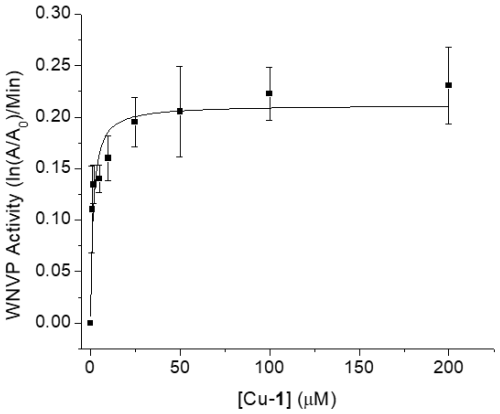


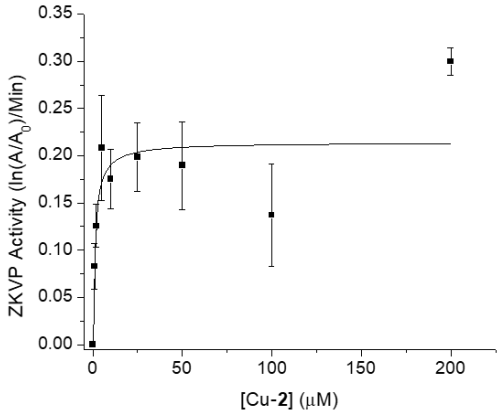
Figure S4. Michaelis-Menten kinetics of metallopeptides against ZKVP (black square) and select Cu-metallopeptides against WNVP (red circle).

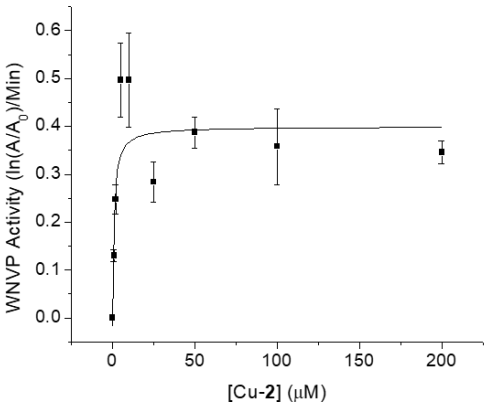

ZKVP (ln(A/A0)/Min)
[Cu-C] (μM)


WNVP Activity (ln(A/A0)/Min)
[Cu-C] (μM)


ZKVP Activity (ln(A/A0)/Min)
[Cu-1] (μM)


WNVP Activity (ln(A/A0)/Min)
[Cu-1] (μM)


ZKVP Activity (ln(A/A0)/Min)
[Cu-2] (μM)


WNVP Activity (ln(A/A0)/Min)
[Cu-2] (μM)

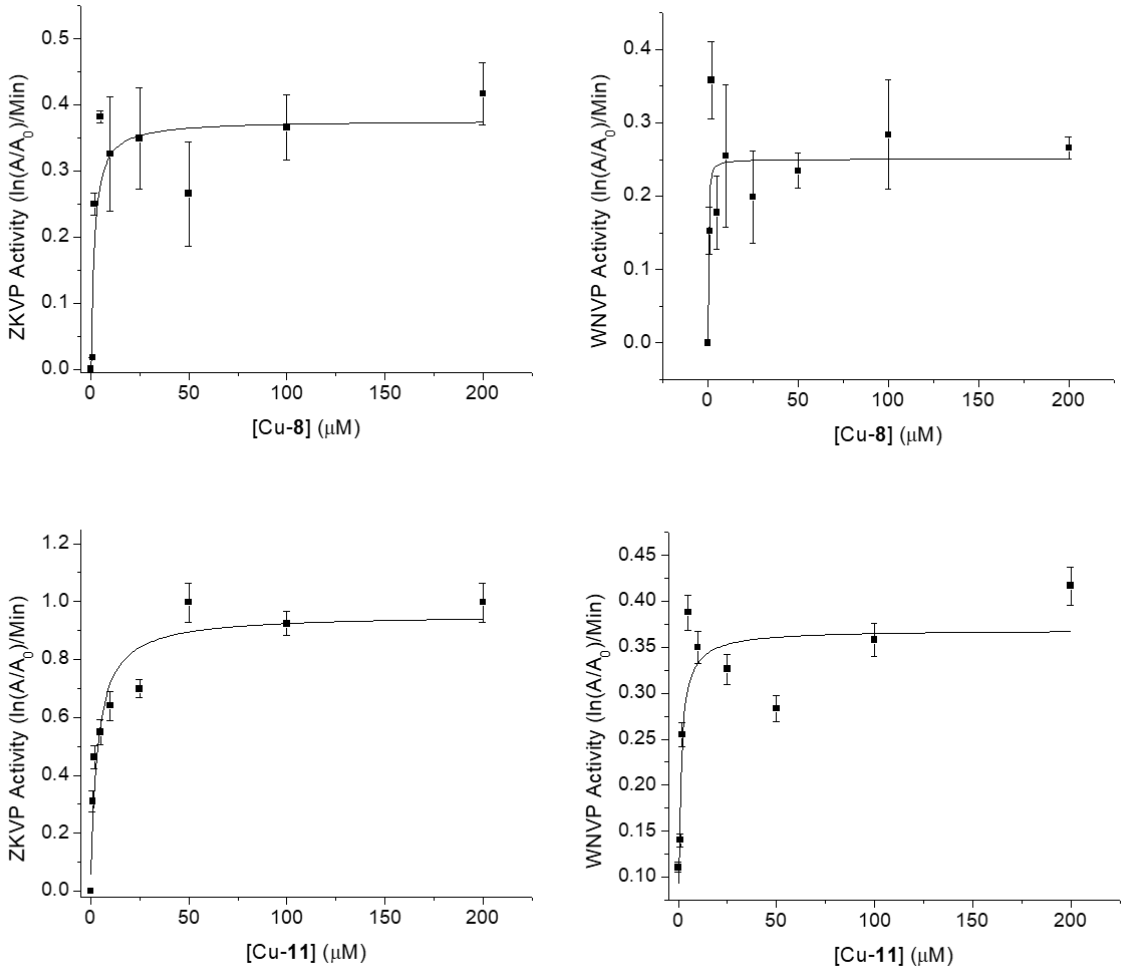


Figure S5. Second order rate kinetics of select metallopeptides against ZKVP and WNVP.

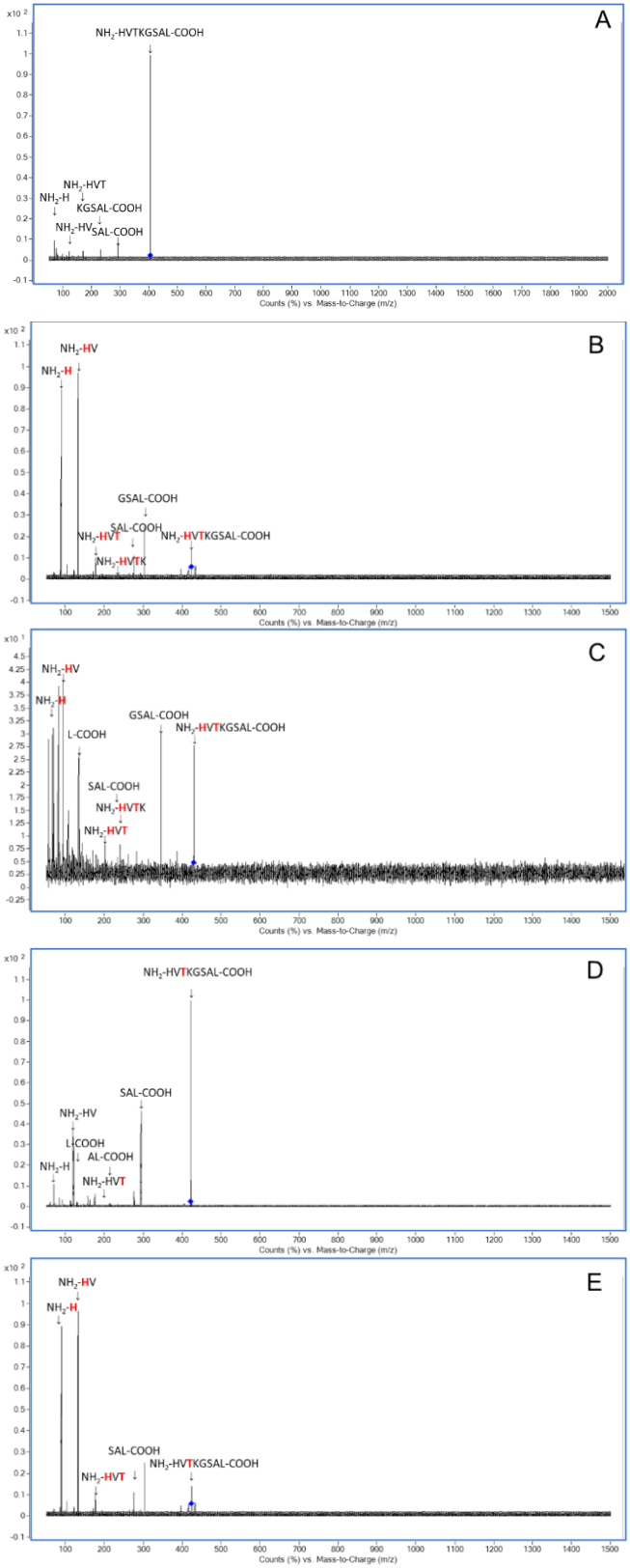


Figure S6. Raw LC-MS/MS data for oxidized ZKVP fragment HVTKGSAL caused by (A) Cu-**C**, (B) Cu-**1**, (C) Cu-**2**, (D) Cu-**8**, and (E) Cu-**11**.

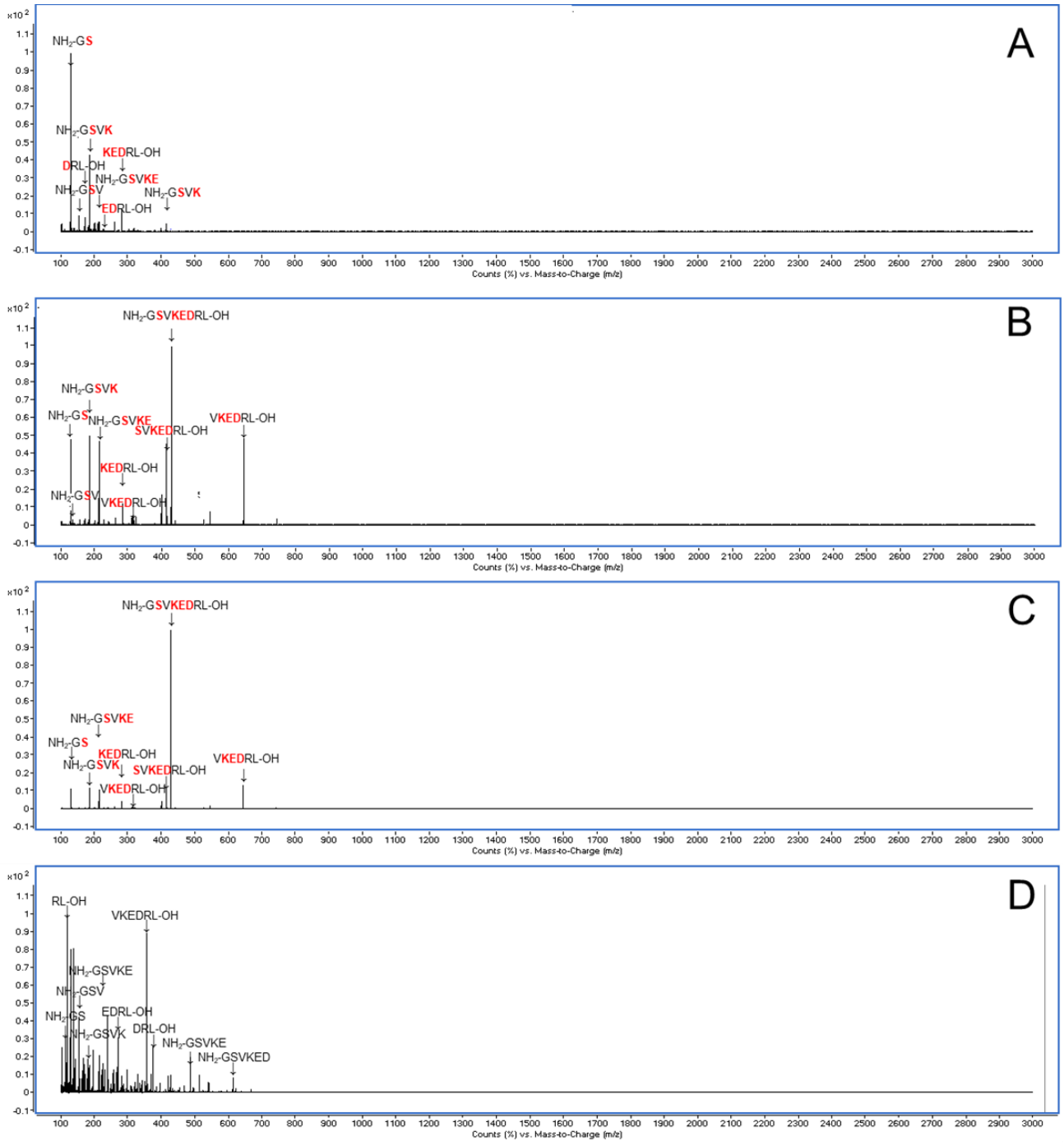


Figure S7. Raw LC-MS/MS data for oxidized WNVP fragment GSVKEDRL caused by (A) Cu-**2**, (B) Cu-**8**, (C) Cu-**11**, and (D) Cu-**C**.

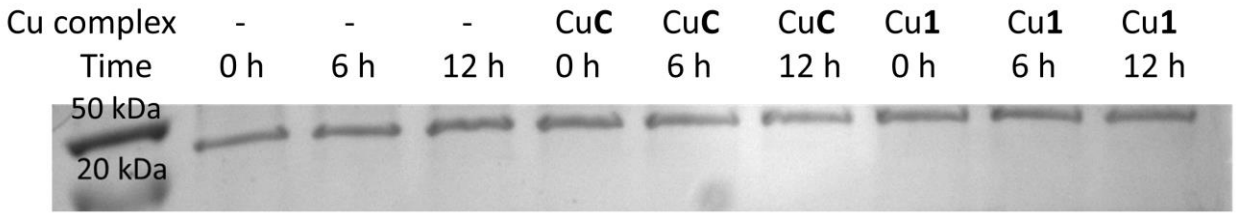


Figure S8. Analysis of time dependent inactivation of WNVP by Cu-**1** in 50 mM Tris-HCl pH 7.5 to check for peptide backbone cleavage at 0, 6, and 12 h using 12% (w/v) SDS-PAGE. Reactions contain 100 µM WNVP with 1 mM H_2O_2 and 1 mM ascorbate in the absence of metallopeptide or presence of 5 µM Cu-**C** or 5 µM Cu-**1**.

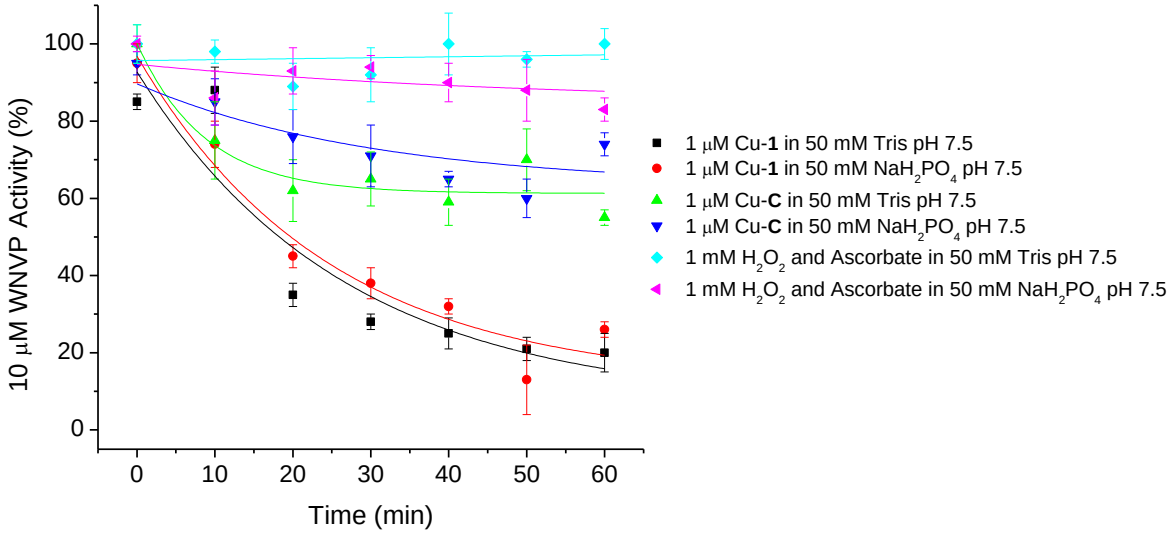


Figure S9. Comparison of buffer effects on time dependent inactivation of 10 µM WNVP with 1 µM Cu-**1** with 1 mM H_2O_2 and 1 mM ascorbate, Cu-**C** with 1 mM H_2O_2 and 1 mM ascorbate, and the effects of 1 mM H_2O_2 and 1 mM ascorbate in 50 mM Tris-HCl, pH 7.5, or 50 mM NaH_2PO_4, pH 7.5.

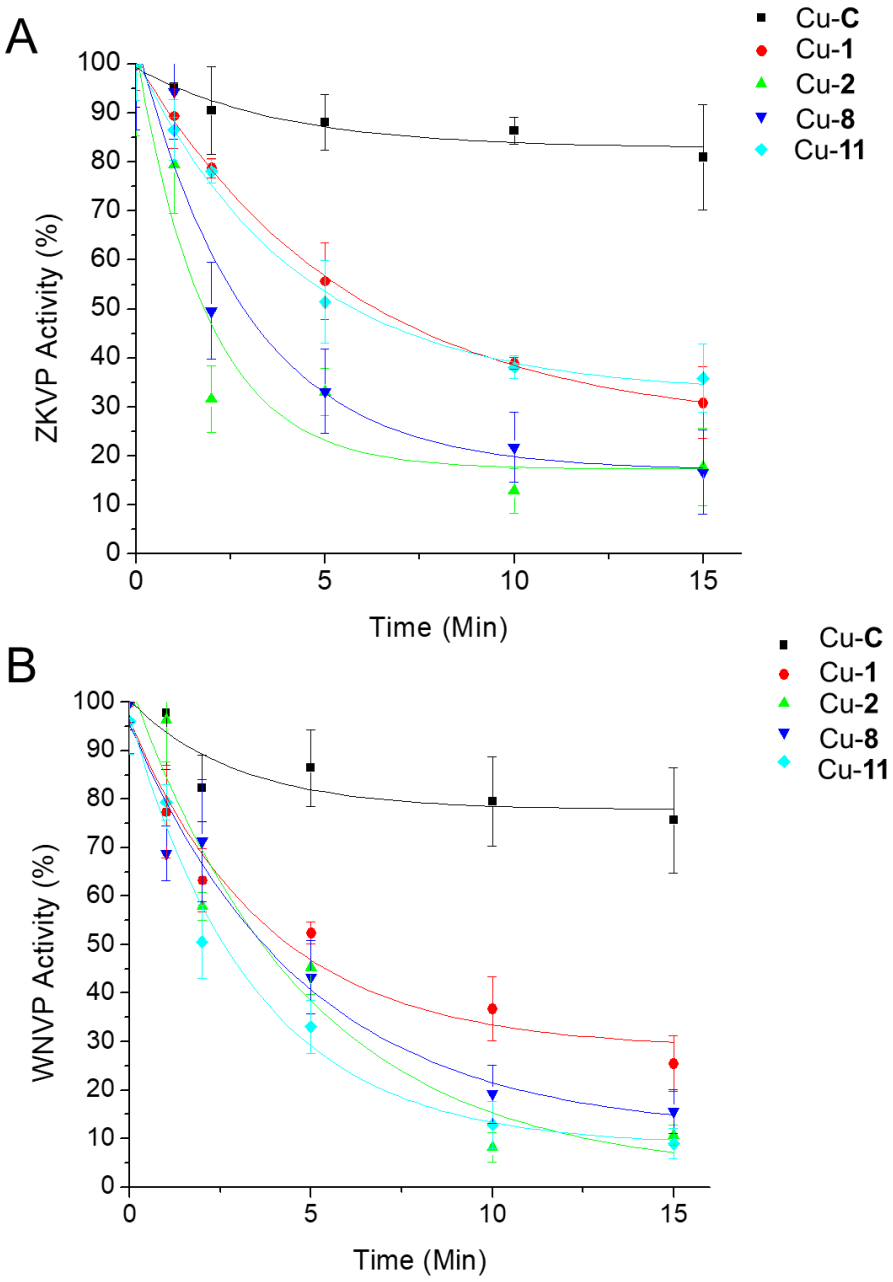
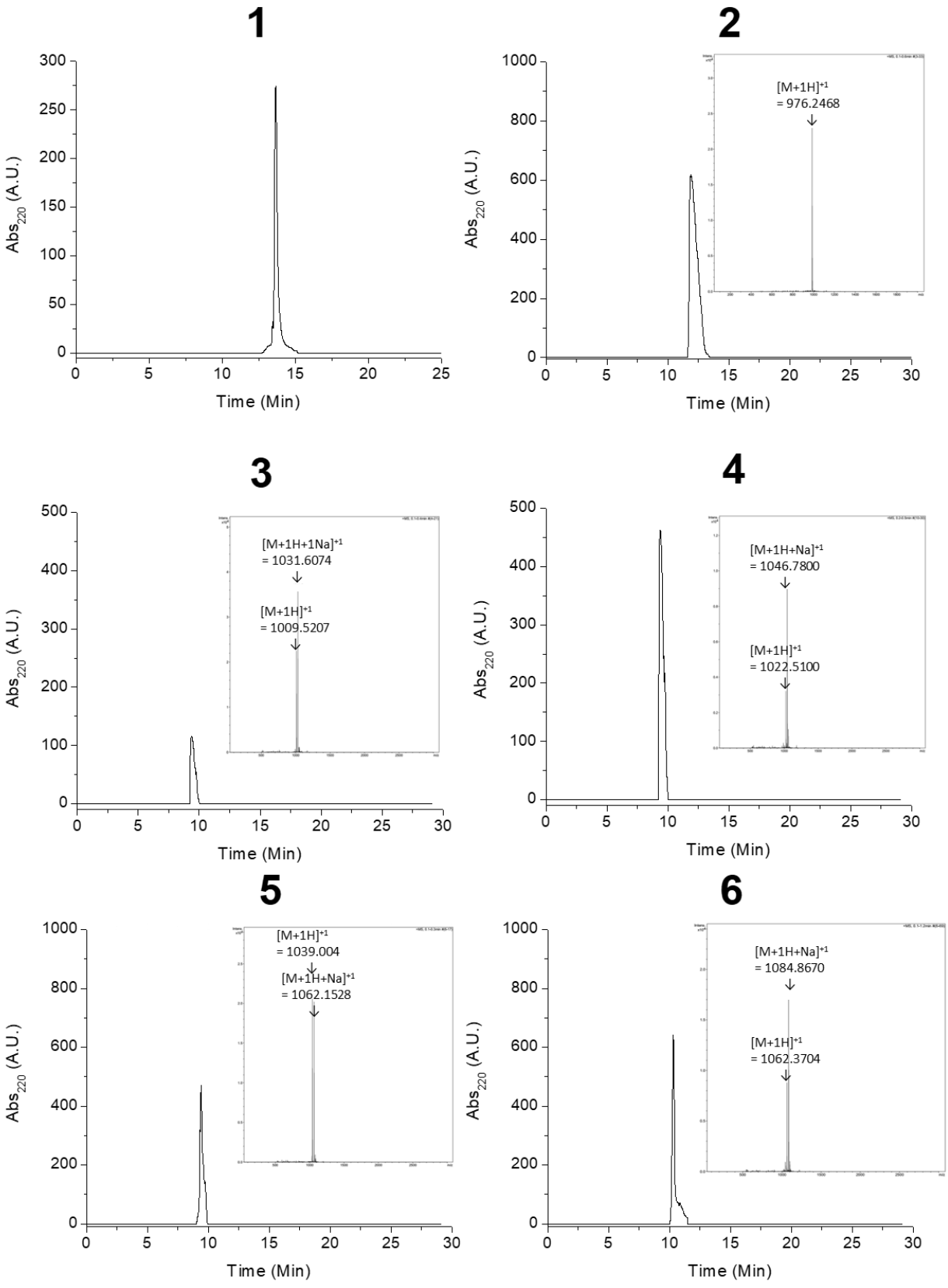
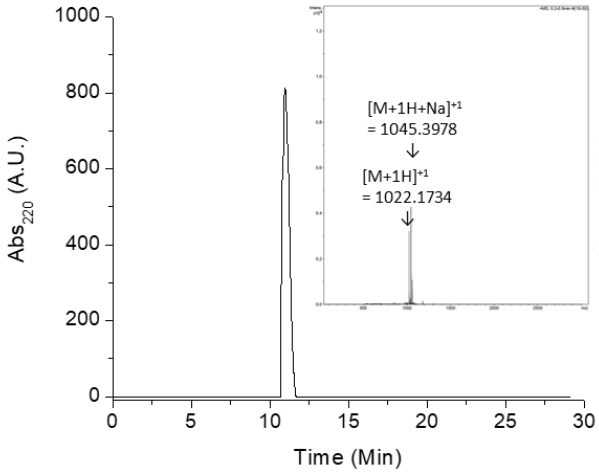


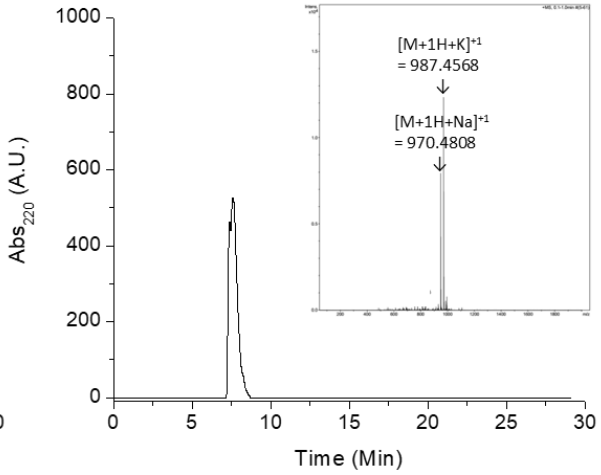
Figure S10. (A) Deactivation of ZKVP with select metallopeptides used to determine turnover number. (B) Deactivation of WNVP activity with select metallopeptides used to determine turnover number. Turnover numbers for select metallopeptides were determined by incubating 20 µM protease ZKVP or WNVP with 1 µM select metallopeptides in 50 mM Tris-HCl pH 7.5 with 1 mM H_2O_2 and 1 mM ascorbate. At times 0, 2, 7, 12, 20, 30, 40, and 50 min an aliquot was taken and diluted to 1 µM protease concentration in 300 µL activity buffer (50 mM Tris-HCl pH 9.5, 30% glycerol (v/v), 1 mM CHAPS) with 75 µM Np-Lys-Lys-Arg-*p*Na for WNVP or 200 µM Np-Lys-Lys-Arg-*p*Na for ZKVP.


1
Abs220 (A.U.)
Time (Min)
2
[M+1H]+1
= 976.2468
Abs220 (A.U.)
Time (Min)
3
[M+1H+1Na]+1
= 1031.6074
[M+1H]+1
= 1009.5207
Abs220 (A.U.)
Time (Min)
4
[M+1H+Na]+1
= 1046.7800
[M+1H]+1
= 1022.5100
Abs220 (A.U.)
Time (Min)
5
[M+1H]+1
= 1039.004
[M+1H+Na]+1
= 1062.1528
Abs220 (A.U.)
Time (Min)
6
[M+1H+Na]+1
= 1084.8670
[M+1H]+1
= 1062.3704
Abs220 (A.U.)
Time (Min)

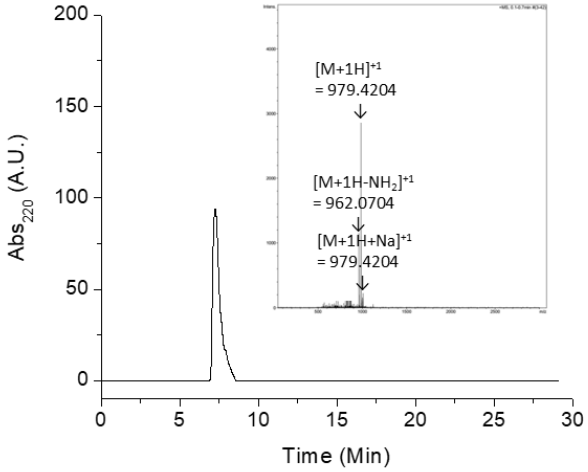
7


[M+1H+Na]+1
= 1045.3978
[M+1H]+1
= 1022.1734
Abs220 (A.U.)
Time (Min)

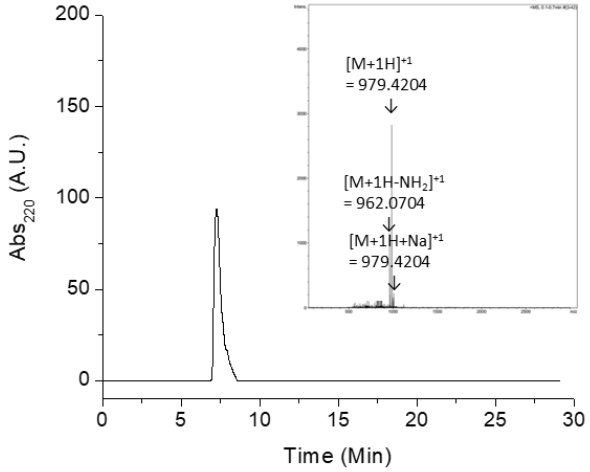
8


[M+1H+K]+1
= 987.4568
[M+1H+Na]+1
= 970.4808
Abs220 (A.U.)
Time (Min)

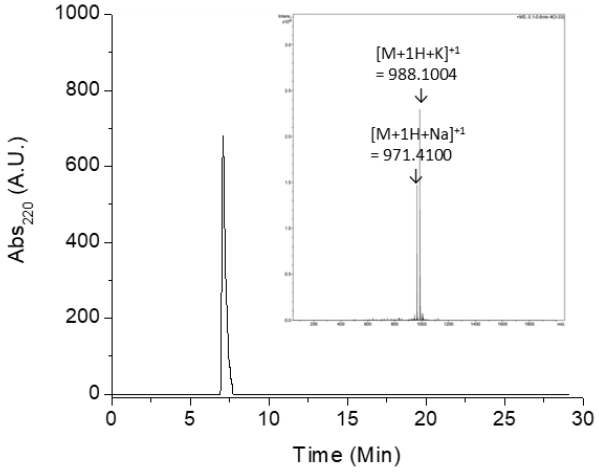
9


[M+1H]+1
= 979.4204
[M+1H-NH2]+1
= 962.0704
[M+1H+Na]+1
= 979.4204
Abs220 (A.U.)
Time (Min)

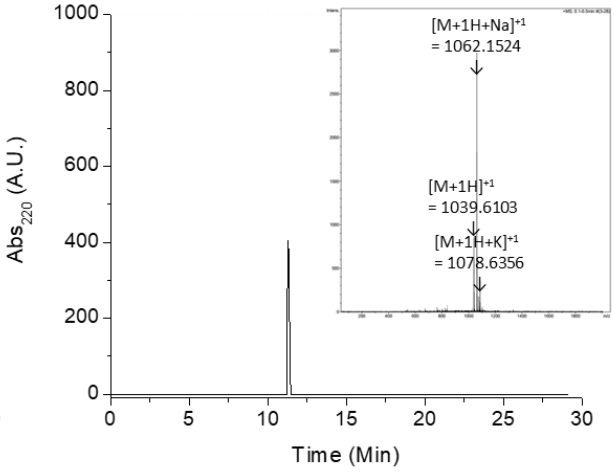
10


[M+1H]+1
= 979.4204
[M+1H-NH2]+1
= 962.0704
[M+1H+Na]+1
= 979.4204
Abs220 (A.U.)
Time (Min)

11


[M+1H+K]+1
= 988.1004
[M+1H+Na]+1
= 971.4100
Abs220 (A.U.)
Time (Min)

12


[M+1H+Na]+1
= 1062.1524
[M+1H]+1
= 1039.6103
[M+1H+K]+1
= 1078.6356
Abs220 (A.U.)
Time (Min)

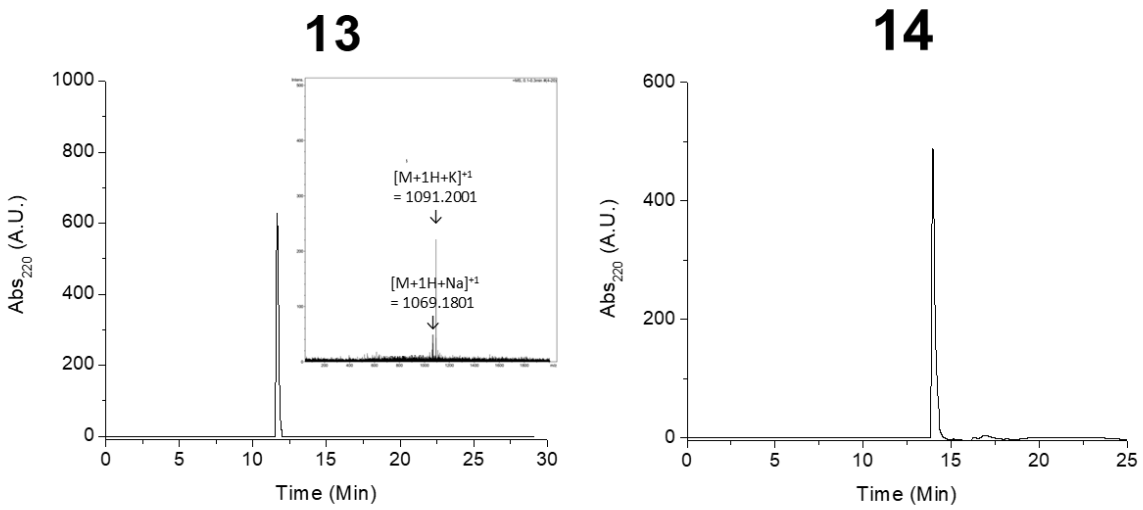


Figure S11. HPLC traces with UV/Vis detector set to 220 nm of metallopeptides made in this work, with inserts of HRMS of metallopeptide stocks. We have described the characterization of **1** and **14** elsewhere.[4]

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