Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Selenocysteine Containing Analogues of Atx1-based Peptides Protect Cells from Copper Ions Toxicity

Michal S. Shoshan[a#], Yonat Lehman[a#], Wojciech Goch[b], Wojciech Bal[b], Edit Y. Tshuva[a]* and Norman Metanis[a]*

[#]Equal contribution

[a] The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat Ram, Jerusalem 91904, Israel.

[b] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa 02106, Poland

Table of Content

Experimental Details

MTC was purchased from JPT Peptide Technologies GmbH (Berlin, Germany) with a purity of >95% by HPLC. H_2DCF-DA, D-Pen, TETA, TTM, NaH_2PO_4, Na_2HPO_4, 2,2'-dithiobis(5-nitropyridine) (DTNP), *DL*-dithiothreitol (DTT), ethanedithiol (EDT) and triisopropylsilane (TIS) were purchased from Sigma-Aldrich (Rehovot, Israel). Buffers were prepared using MilliQ water. All Fmoc-amino acids were obtained from CS Bio Co. (Menlo Park, CA) or Matrix Innovation (Quebec City, Canada), with the following side chain protecting groups: Arg(Pbf), Cys(Trt), Sec(4-MeOBzl), Ser(tBu), Thr(tBu), pro, Met, Gly (4-MeOBzl= 4-methoxybenzyl; Pbf = 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl). Fmoc-*L*-Gly-WANG resin was obtained from Iris Biotech GmbH. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) and Ethyl cyano(hydroxyimino)acetate (OxymaPure) were purchased from Luxembourg Biotechnologies Ltd. (Rehovot, Israel). All solvents: *N,N*-dimethylformamide (DMF), dichloromethane, acetonitrile (ACN), *N,N*-diisopropylethyl amine (DIEA), and piperidine (Pip) were purchased from Bio-Lab (Jerusalem, Israel) and were peptide synthesis, HPLC or ULC-grade. Trifluoroacetic acid (TFA) was a generous gift from Halocarbon Products (River Edge, NJ).

Solid-phase peptide synthesis

Peptides were prepared manually by Fmoc-SPPS on Fmoc-*L*-Gly-WANG resin, typically on a 0.25 mmol scale. Fmoc deprotection was carried out with 20% Pip in DMF (5 min × 2). Fmoc-amino acids (2 mmol in 5 mL DMF) activated with HATU (2 mmol in 5 mL DMF) and DIEA (4 mmol in 5 mL DMF) for 5 min and allowed to couple for 30 min, with constant shaking. Sec coupling was performed by DIC/oxyma procedure using 2 equiv. of Fmoc-Sec(Mob)-OH.[1] The resulting resins were washed with DMF (x3) and DCM (x3) and methanol (x3) and dried.

For cleavage of 200 mg resin, 7 mL cleavage cocktail was prepared (TFA: H2O: EDT: TIS, 94%:2.5%:2.5%:1%). If Sec was present, 2 equiv. of DTNP were added.[2] The mixture was added to the peptide-resin and shaken for 4 h. The resin was removed by filtration, and washed twice with neat TFA. TFA was removed by N_2 bubbling, followed by addition of cold ether to precipitate the peptide. After centrifugation (5000 rpm, 5

min) and decanting the ether, peptides were dissolved in 1:1 0.1% TFA in H_2O and 0.1% TFA in ACN and lyophilized. The resulting crude peptide was dissolved in aqueous ACN or phosphate buffer pH 7 and treated with DTT, and purified by preparative RP-HPLC.

High Performance Liquid Chromatography (HPLC): Analytical reversed-phase (RP) HPLC was performed on a Waters UPLC H-Class or Waters Alliance HPLC system with 220 nm UV detection using a XSelect CSH 130 C18 column (3.5 μm, 130 Å, 4.6 × 150 mm). The column was heated to 30 °C, and the following gradient was used: 99% A for 3 min, then to 70% A in 17 min, followed by washing. Preparative RP-HPLC was performed on a Waters 150LC system using a XSelect C18 column (5 μm, 130 Å, 30 × 250 mm). Linear gradients of ACN in water with 0.1% TFA were used for all systems to elute bound peptides. The flow rates were 1 mL/min (analytical) and 20 mL/min (preparative). Solvent A (0.1% TFA in water), solvent B (0.1% TFA in ACN)

Mass Spectrometry: Electrospray ionization MS (ESI-MS) was performed on LCQ Fleet Ion Trap mass spectrometer instrument (Thermo Scientific). Peptide masses were calculated from the experimental mass to charge (*m/z*) ratios from all of the observed multiply charged species of a peptide.

Synthesis of protected Fmoc-Sec(Mob)-OH: The synthesis was performed as described elsewhere.[3]

Cu(I)-DTT complex preparation

The Cu(I)-DTT complex was prepared and characterized as previously described,[4-6] from $Cu(OAc)_2$ (25 μL, 0.1 M) and DTT (75 μL, 0.1 M) through *in situ* reduction, diluted with 4 mL of the appropriate ammonium acetate buffer of pH 7.4, as measured by GLP21 CRISON pH meter; final concentration of the complex was 0.61 mM.

Affinity determination

Affinity was measured as previously described.[4-6] Each reduced peptide (by one or two equiv of DTT) was dissolved in the Cu(I)-DTT buffer solution to achieve a final

concentration of 0.62 mM of the Cu(I)-peptide complex. Finally, the solution was diluted with buffer solution to a concentration of 0.16 mM.

The solutions were injected into electrospray ion source of an API2000 instrument (Applied Biosystems) by a syringe pump at 10 $\mu L \cdot min^{-1}$. The spectra were recorded for 5 min at the m/z range of 500-1800 Da with the following instrument parameters: ion spray voltage 5500 V; curtain gas 10 $L \cdot min^{-1}$; declustering potential 40 V; focusing potential 350 V. First all solutions were tested in the absence of free DTT in order to determine the complexation percentage. Increasing concentrations of free DTT were then added separately and the solutions were incubated for 2 min at room temperature. Each sample was analyzed by ESI-MS and the intensities of the apo- and holo-peptide peaks were measured.

K_d Calculation - considering oligomeric products of metal-DTT

In order to quantify dissociation constants of Cu(I)–protein complexes, the general reaction model was used, which takes into account different protonation states of DTT and all possible complexes of Cu(I) and DTT. DTT can be found in three protonation states, characterized by two protonation reactions (reactions 1-2) and Cu(I) and DTT can form the following complexes (reactions 3-6):[5]

(1) $HDTT^- \rightleftharpoons DTT^{2-} + H^+$ $\quad -\log K_{11} = 10.00$

(2) $H_2DTT \rightleftharpoons DTT^{2-} + 2H^+$ $\quad -\log K_{12} = 18.99$

(3) $Cu^+ + DTT^{2-} \rightleftharpoons CuDTT^-$ $\quad \log \beta_{11} = 15.3$

(4) $Cu^+ + 2\ DTT^{2-} \rightleftharpoons CuDTT_2^{3-}$ $\quad \log \beta_{12} = 24.64$

(5) $2\ Cu^+ + 3\ DTT^{2-} \rightleftharpoons Cu_2DTT_3^{4-}$ $\quad \log \beta_{23} = 48.9$

(6) $3\ Cu^+ + 4\ DTT^{2-} \rightleftharpoons Cu_3DTT_4^{5-}$ $\quad \log \beta_{34} = 70.26$

The strict 1:1 Cu(I):P (P is peptide) stoichiometry was assumed for all ligands studied, and was supported by ESI-MS (reaction 7):

(7) $Cu(I) + P \rightleftharpoons Cu(I)\text{-}P$

The dissociation constants for reactions 1-6 were calculated by applying the Levenberg – Marquardt algorithm[7,8] to nonlinear curve fitting, implemented in the Mathematica 9 environment. During this iterative procedure at every step the concentrations of all reagents were calculated and the minimized function was $\chi 2$ error of fitting predicted concentration of Cu(I)-P concentration to fractional content of the complex, calculated from experimental data, where intensities were obtained from mass spectrometry experiments. The cumulative stability constants (log β values),[5] which characterize reactions 1-6 were used for calculations.

In addition, for each sample Y, the fractional content of the complex was calculated according to Eq. 8:

$$(8) \qquad Y = \frac{Intensity_{(holo)}}{Intesity_{(holo+apo)}}$$

Free Cu(I) concentration was plotted against Y and fitted to a hyperbolic curve (9) using KaleidaGraph software. The K_D value of a monomeric complexation mode equals the free metal concentration where Y = 0.5.

$$(9) \qquad Y = \frac{[Cu(I)]}{K_D + [Cu(I)]}$$

Cell growth

In vitro experiments were measured on HT-29 colon cells obtained from ATCC Inc or mice embryonic fibroblasts (MEF) with and without knock-out in Atox1 gene (a kind gift from Prof. Svetlana Lutsenko from Johns-Hopkins University). Cells ($3.5x10^5$) in medium (88% RPMI-1640 for HT-29 or DMEM for MEF, purchased from Sigma Inc.,1% penicillin/streptomycin antibiotics, 1% *L*-glutamine, 10% fetal bovine serum (FBS), all purchased from Biological Industries Inc.) were seeded into 66 wells in a 96-well plate and allowed to attach for 24 hours at 37 ºC in 5% CO_2 atmosphere.

H_2DCF-DA assay

The cells were treated according to Table S1, with incubation of H_2DCF-DA at 37 ºC in 5% CO_2 atmosphere. The fluorescence of each well was measured (Ex. 485, Em. 535) for

200 µl of the aforementioned solution by an Appliskan with one dispenser, 240 V, 50 Hz fluorimeter (Thermo Fisher Scientific). The control measurements included cells treated according to the procedure but without tested reagent. Each analysis is a representative of three different measurements at three different days. The ROS production was calculated using Origin Pro8 according to the following equation where error values are based on standard deviations.

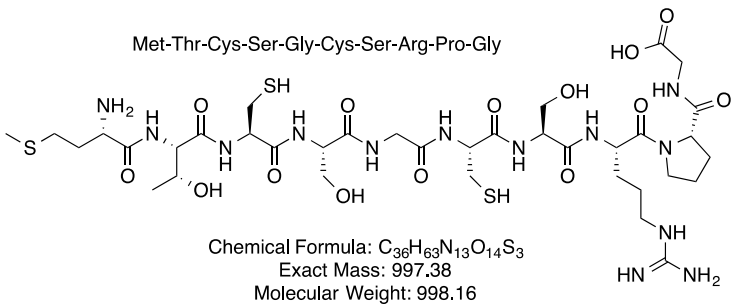
$$\%\ \text{ROS Production} = \frac{F_X - F_{blank}}{F_{control} - F_{blank}} \cdot 100\%$$

Table S1 Experimental steps for two procedures of ROS production detection by the H_2DCF-DA assay

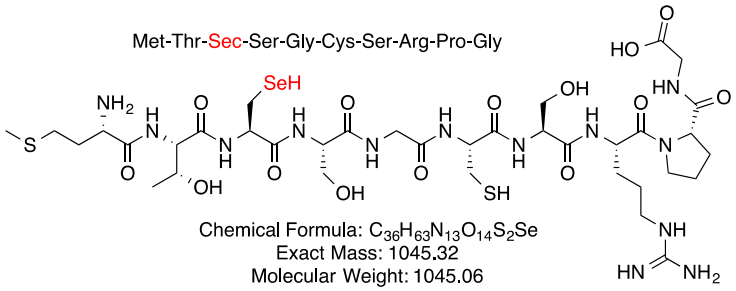
Procedure a	**Procedure b**
-	CuCl[a]
-	washing[b]
apo-compounds[c]	apo-compounds[c]
washing[d]	washing[d]
H_2DCF-DA[e]	H_2DCF-DA[e]
washing[d]	washing[d]
H_2O_2[f]	H_2O_2[f]
OH· detection	OH·detection

[a]0.1 mM in medium for D-Pen, TETA, and TTM; 0.2 mM in medium for all peptides; 60 min incubation
[b]washing twice with medium (RPMI-1640 for HT-29 or DMEM for MEF)
[c]0.2 mM tested compound in medium; 24 h incubation
[d]washing twice with PBS supplemented with 10% D-glucose
[e]1 mM H_2DCF-DA in DMSO; 60 min incubation
[f]10 mM H_2O_2 in medium; 30 min incubation

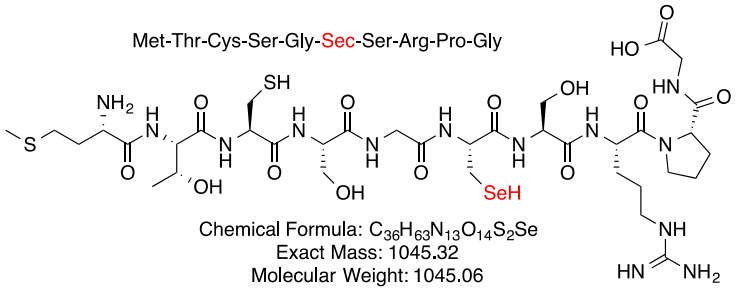
MTC



C3U



C6U



C3U/C6U

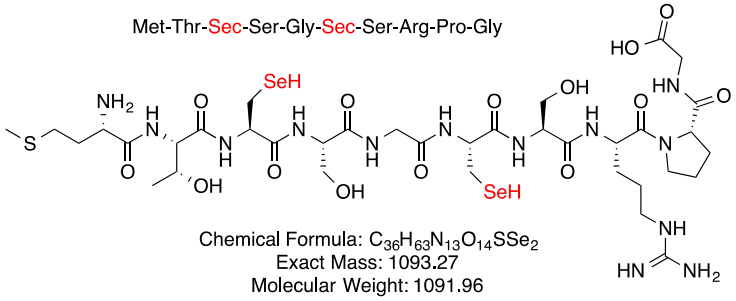


Figure S1. The structures of MTC peptide and selenocysteine-containing analogues: C3U, C6U and C3U/C6U.

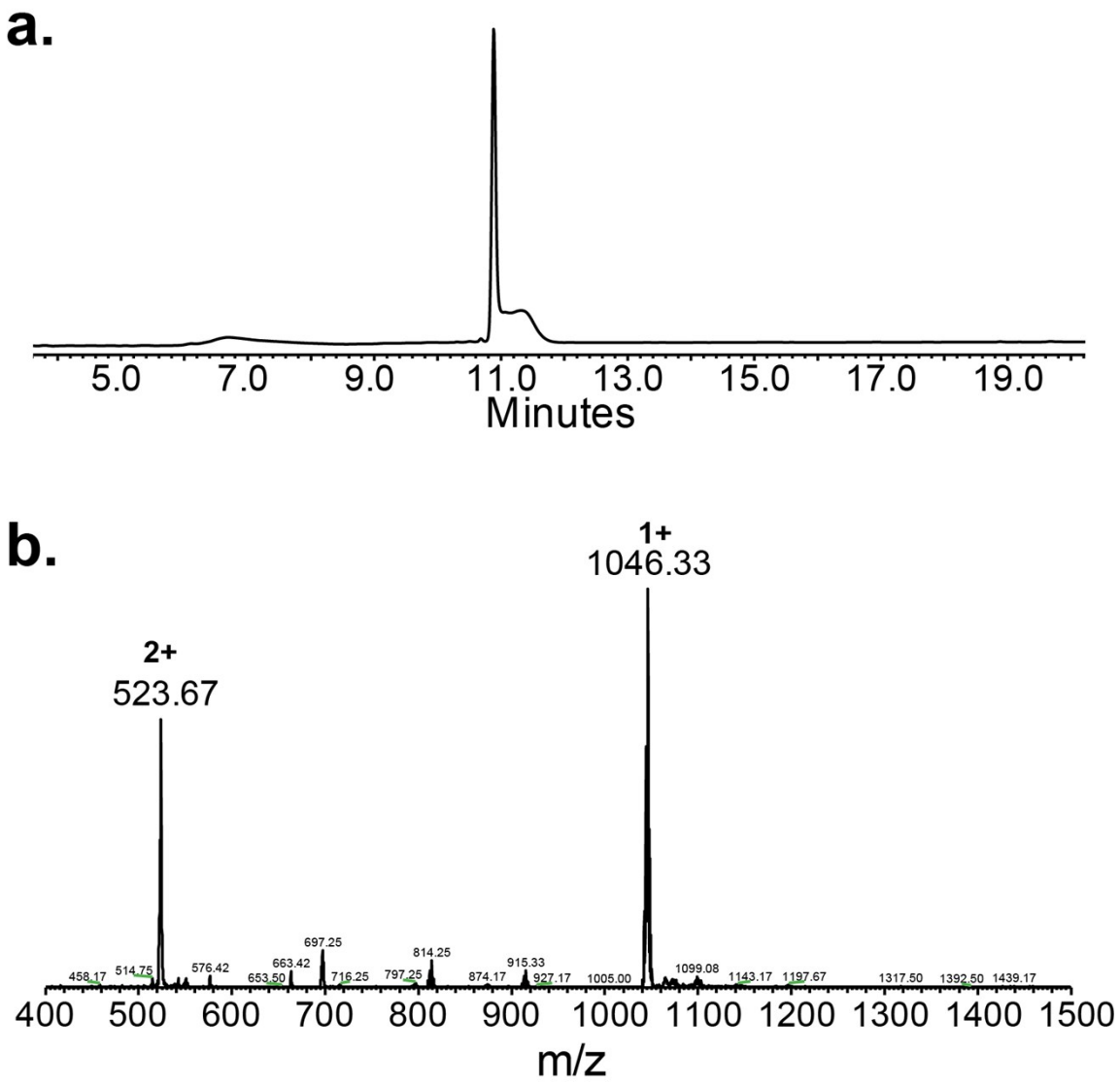


Figure S2. Analytical HPLC (**a**) and ESI-MS (**b**) of the purified peptide C3U. Prior to HPLC analysis, the peptide was reduced with TCEP in the presence of sodium ascorbate to prevent possible deselenization side-reaction.[9] Under these conditions, the peptide is mainly in the reduced form. Calc. mass 1045.32, observed 1045.33. The shoulder on the right of the main peak showed a mass for oxidized dimeric peptide.

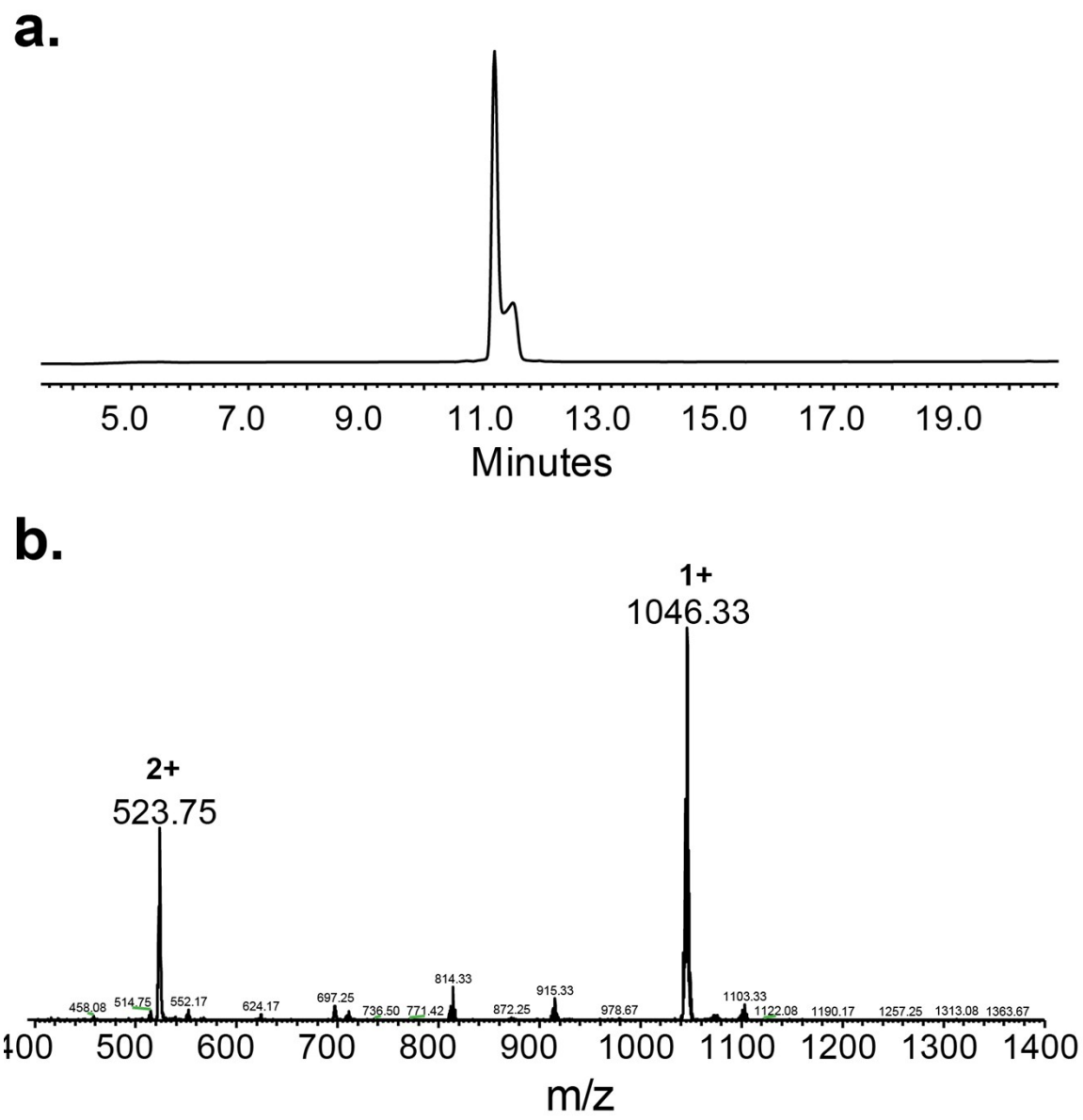


Figure S3. Analytical HPLC (**a**) and ESI-MS (**b**) of the purified peptide C6U. Similar conditions for Figure S2 were used. Calc. mass 1045.32, observed 1045.41.

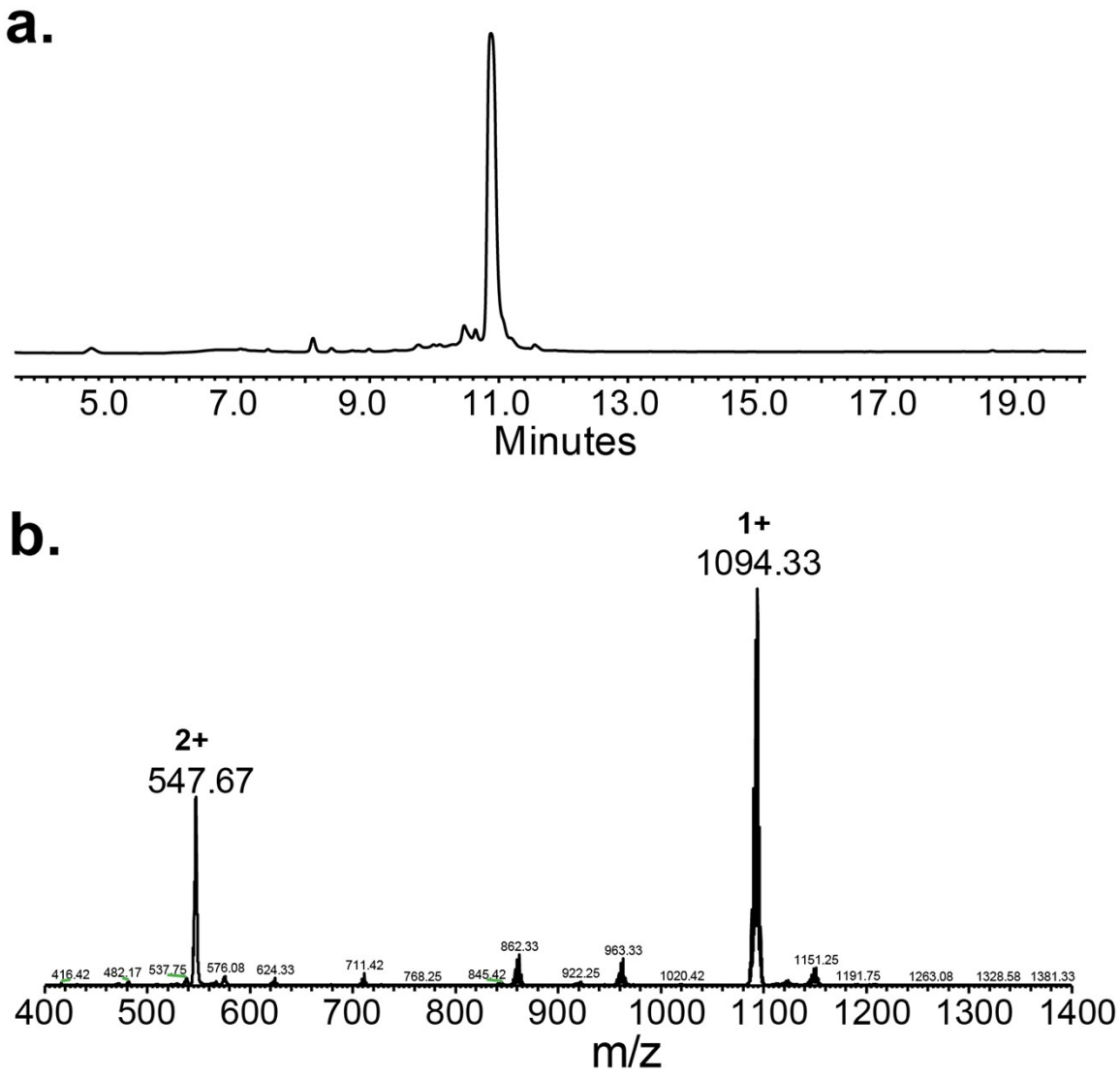


Figure S4. Analytical HPLC (**a**) and ESI-MS (**b**) of the purified peptide C3U/C6U. Similar conditions for Figure S2 and S3 were used. Calc. mass 1093.27, observed 1093.33.

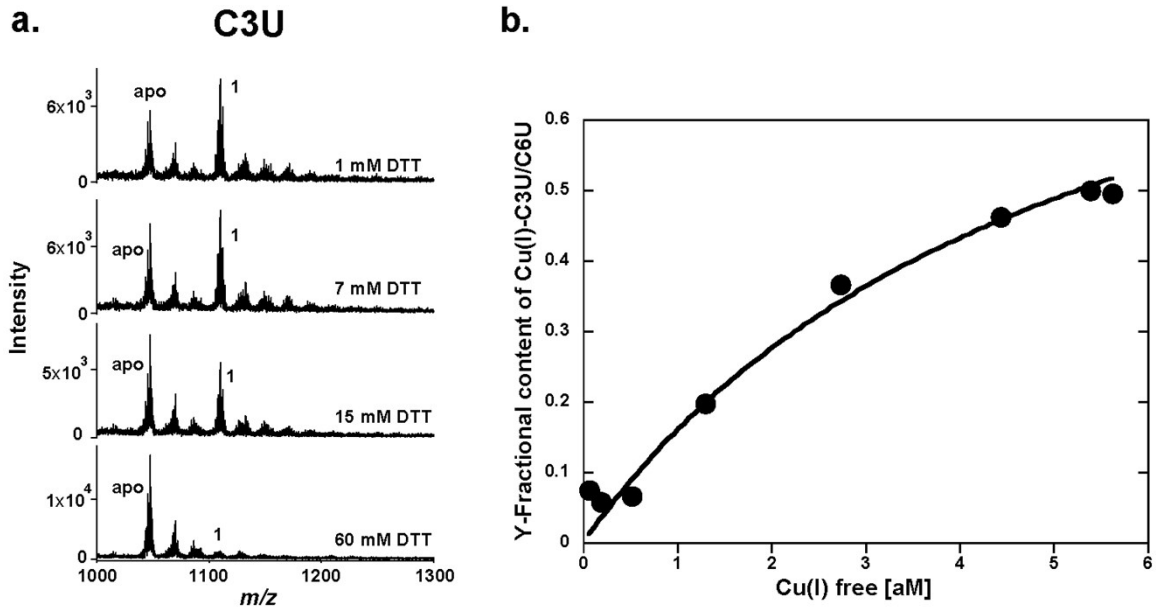


Figure S5. The apparent K_D determination of Cu(I)-C3U complex at pH 7.4, 25 °C.[4] **a**) ESI-MS spectra of Cu(I)-C3U in the presence of 0.93 mM - 60 mM DTT; **b**) Fractional content of Cu(I)-C3U dependence on concentration of free Cu(I) ions as calculated based on the consideration of oligomeric products of Cu-DTT.[5]

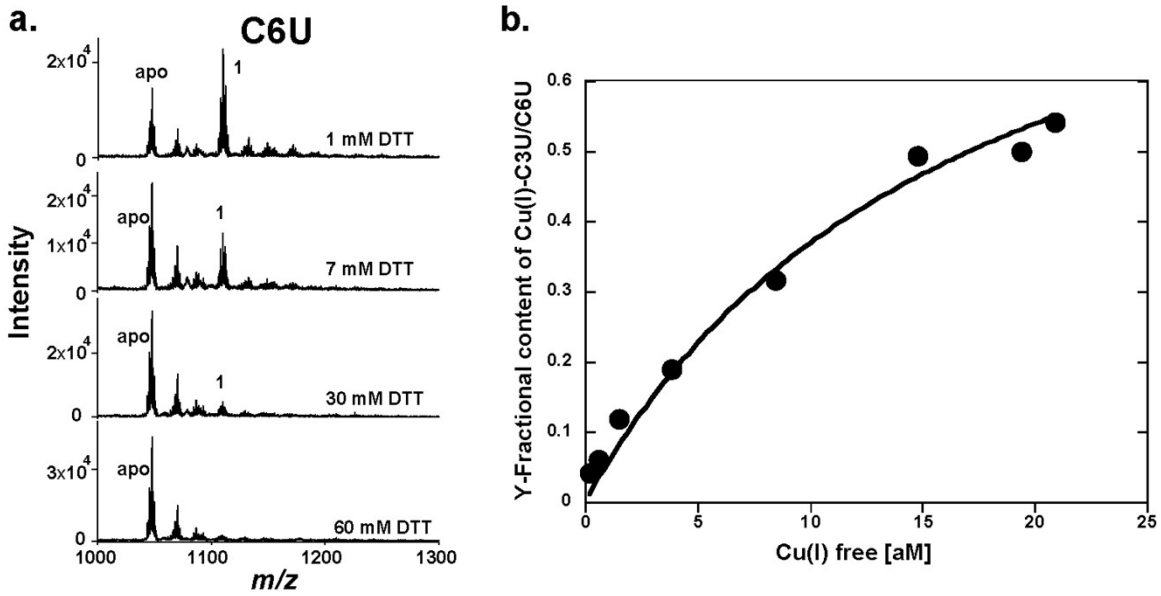


Figure S6. The apparent K_D determination of Cu(I)-C6U complex at pH 7.4, 25 °C.[4] **a**) ESI-MS spectra of Cu(I)-C6U in the presence of 0.93 mM - 60 mM DTT; **b**) Fractional content of Cu(I)-C6U dependence on concentration of free Cu(I) ions as calculated based on the consideration of oligomeric products of Cu-DTT.[5]

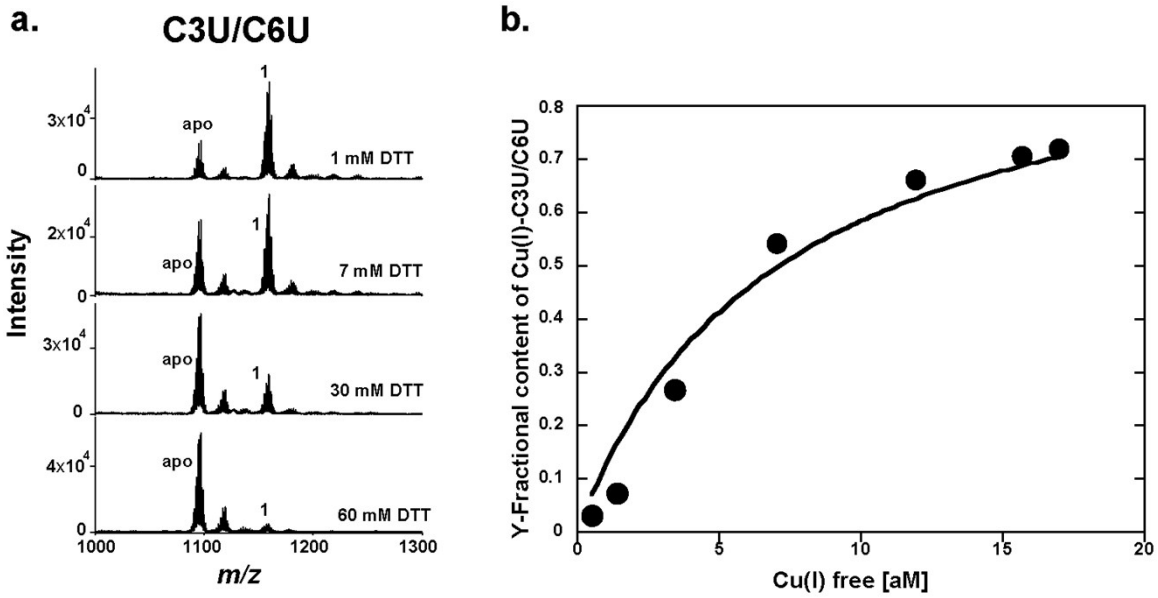


Figure S7. The apparent K_D determination of K_D of Cu(I)-C3U/C6U complex at pH 7.4, 25 °C.[4] **a**) ESI-MS spectra of Cu(I)-C3U/C6U in the presence of 0.93 mM - 60 mM DTT; **b**) Fractional content of Cu(I)-C3U/C6U dependence on concentration of free Cu(I) ions as calculated based on the consideration of oligomeric products of Cu-DTT.[5]

Table S2 Summary of all ROS production (%) in HT-29 cell line

Compound	Procedure a [%]	Procedure b [%]
MTC	61 ± 4	24 ± 1
C3U	51 ± 2	21 ± 2
C6U	70 ± 2	28 ± 2
C3U/C6U	46 ± 2	12 ± 2
TETA	81 ± 7	75 ± 5
TTM	79 ± 12	54 ± 2
D-Pen	92 ± 7	95 ± 5
CuCl	325 ± 52	

Table S3 Summary of all ROS production (%) in MEF cell lines

	Control MEF		MEF Atox1 -/-	
Compound	**Procedure a [%]**	**Procedure b [%]**	**Procedure a [%]**	**Procedure b [%]**
MTC	78 ± 2	13 ± 1	40± 3	9.0 ± 0.2
C3U	73 ± 1	8 ± 1	32 ± 3	6 ± 1
C6U	86 ± 2	14 ± 2	56 ± 3	16 ± 2
C3U/C6U	65 ± 6	7.4 ± 0.4	26 ± 3	5.3 ± 0.4
TETA	116 ± 6	43 ± 2	71 ± 1	35 ± 2
TTM	112 ± 5	34 ± 3	73 ± 3	25 ± 3
D-Pen	134 ± 5	75 ± 3	102 ± 9	106 ± 3
CuCl	958 ± 91		419 ± 23	

1 S. Dery, P. S. Reddy, L. Dery, R. Mousa, R. N. Dardashti, N. Metanis, *Chem. Sci.* 2015, 6, 6207-6212.
2 K. M. Harris, S. Flemer, R. J. Hondal, *J. Pept. Sci.* 2007, **13**, 81.
3 M. D. Gieselman, L. L. Xie, W. A. van der Donk, *Org. Lett.* 2001, **3**, 1331.
4 L. Banci, I. Bertini, S. Ciofi-Baffoni, T. Kozyreva, K. Zovo, P. Palumaa, *Nature* 2010, **465**, 645-648.
5 A. Krezel, W. Lesniak, M. Jezowska-Bojczuk, P. Mlynarz, J. Brasun, H. Kozlowski, W. Bal, *J. Inorg. Biochem.* 2001, **84**, 77.
6 M. S. Shoshan, N. Dekel, W. Goch, D. E. Shalev, T. Danieli, M. Lebendiker, W. Bal, E. Y. Thsuva, *J. Inorg. Biochem.* 2016, **159**, 29-36.
7 K. Levenberg, *Q. Appl. Math.* 1944, **2**, 164-168.
8 D.W. Marquardt, *J. Soc. Ind. Appl. Math.* 1963, **11**, 431–441.
9 P. S. Reddy, S. Dery, N. Metanis, *Angew. Chem. Int. Ed.* 2016, **55**, 992-995.