Electronic Supplementary Information

A Copper(II) Peptide Helicate Selectively Cleaves DNA Replication Foci in Mammalian Cells

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Author attributions

The following table describes the contribution of each author according to the CRediT taxonomy as described in A. Brand, L. Allen, M. Altman, M. Hlava, J. Scott, *Learn. Publ.* **2015**, *28*, 151–155. This taxonomy provides a detailed classification of the various roles performed by each author. The degree of contribution is coded as *lead* (black), *equal* (dark grey), or *supporting* (light grey).

	AAO	NBP	BM	JGG	DB	FR	MEV	AK	JMC	MVL
Conceptualization. Ideas, formulation of overarching research goals and aims.										
Methodology. Development or design of methodology; creation of models.										
Validation. Verification of the overall reproducibility of results and research outputs.										
Formal Analysis. Application of formal techniques to analyze the data.										
Investigation: helicate synthesis, metal binding, in vitro DNA binding studies.										
Investigation: EPR studies.										
Investigation: In vitro nuclease activity studies.										
Investigation: In vivo fluorescence microscopy.										
Resources. Provision of reagents, materials, instrumentation, and analysis tools.										
Data Curation. Management to annotate, scrub and maintain research data.										
Writing: Original Draft. Preparation of the paper, specifically writing the initial draft.										
Writing: Review & Editing. Critical review, commentary, or revision.										
Visualization. Preparation, of the paper, specifically visualization &/or presentation.										
Supervision. Oversight responsibility for the research planning & execution.										
Project Administration. Management and coordination of the research.										
Funding Acquisition. Acquisition of the financial support leading to this publication.										

1. Reagents

The solvents and reagents used in the synthesis of the Fmoc-βAla5Bpy-OH were obtained from *Fisher Chemical* (acetonitrile for HPLC, chloroform, toluene, DMF, methanol, NaOH), *Scharlau* (absolute ethanol), *Sigma Aldrich* (5,5'-dimethyl-2,2'-bipyridine, $KMnO_4$, $SOCl_2$, hydrazine monohydrate, xylene, DIPEA), *Panreac* (celite and $NaNO_2$). For the synthesis of the peptide ligands, all the amino acids were provided by *Sigma Aldrich* and *IRIS Biotech GmbH* All the amino acids were used as their Fmoc protected derivatives. The resin employed for the SPPS was H-Rink-Amide *ChemMatrix* 35-100 mesh particle size from *Sigma Aldrich*. For the Alloc deprotection, $Pd(PPh_3)_4$, $PhSiH_3$ were obtained from *Sigma Aldrich*. $CuCl_2.2H_2O$ employed in the obtention of the Cu^{II} metallopeptides was acquired to *Acros Organics.* Mohr's salt, $(NH_4)_2Fe(SO_4)_2.6H_2O$*,* employed in the obtention of the Fe^{II} metallopeptides as a source of Fe^{II} ions, was provided by *Sigma Aldrich*. 6-Carboxytetramethylrhodamine succinimidyl ester (TAMRA) was provided by *Carbosynth.*

2. Experimental Methods

2.1. DNA sequences used in the fluorescent DNA binding studies and (fluorophore) non-labelled PAGE binding studies.

Fluorescein-labelled 3WJ (Flu-3WJ):

Y1: 5′-FAM-TTT TCA CCG CTC TGG TCC TC-3′
Y2: 5′-CAG GCT GTG AGC GGT G-3′
Y3: 5′-GAG GAC CAA CAG CCT G-3′

3WJ:

Y1: 5′-CAC CGC TCT GGT CCT C-3′
Y2: 5′-CAG GCT GTG AGC GGT G-3′
Y3: 5′-GAG GAC CAA CAG CCT G-3′

Fluorescein-labelled GC-3WJ (Flu-GC-3WJ):

Y1: 5′-FAM-TTTT CAC CGG GCC CCG GGG CCC CCT C-3′
Y2: 5′-CAG GGG GCC CCG GGG CCC GGT G-3′
Y3: 5′-GAG GGG GCC CCG GGG CCC CCT G-3′

Fluorescein-labelled dsDNA (Flu-dsDNA):

Z1: 5′-FAM-TTT TAA CAC ATG CAG GAC GGC GCT T-3′
Z2: 5′-AAG CGC CGT CCT GCA TGT GTT-3′

dsDNA:

Z1: 5′-AAC ACA TGC AGG ACG GCG CTT-3′
Z2: 5′-AAG CGC CGT CCT GCA TGT GTT-3′

2.2. Example of Dynafit script

```
[task]                              ;semicolons indicate comments
 task = fit                         ;nature of the calculation to be performed
 data = equilibria

[mechanism]                         ;Free-form 1:1 binding model with Kd
 R + L <==> RL : Kd dissoc          ;to be calculated as dissociation constant

[constants]                         ;Initial Kd value for iteration
 Kd = 1.0 ?                         ;the "?" indicates that this will be optimized

[concentrations]
 R = 2.0                            ;Fixed conc. of the DNA during the peptide titration

[responses]                         ;contribution to the spectroscopic signal of each
 R = 0.1 ?                          ;of the different components of the equilibrium
 RL = 1.5 ?                         ;these will be optimized ("?" after the values).

[data]                              ;location of files and information about the data
 variable  L                        ;the species that changes conc. during the titration
 offset auto ?
 directory ./exp/brHis              ;file path (relative to DynaFit program location)
 extension txt
 file f1                            ;name of the experimental data file

[output]
 directory ./exp/brHis/out          ;path indicating location of DynaFit output files

[settings]                          ;cosmetic settings that control DynaFit graphics
 {Output}                           ;fits were exported & finally plotted with
 XAxisUnit = uM                     ;GraphPad Prism 7.0c. GraphPad Software,
 BlackBackground = n                ;La Jolla California, www.graphpad.com
 XAxisLabel = [peptide]
 YAxisLabel = anisotropy
 WriteTXT = y
```

2.3. DNA sequences used in fluorophore labelled 3WJ and dsDNA PAGE binding studies.

3WJ:

Y1: 5′-FAM-TTT TCA CCG CTC TGG TCC TC-3′
Y2: 5′-ROX-TTT TCA GGC TGT GAG CGG TG-3′
Y3: 5′-Cy5-TTT TGA GGA CCA ACA GCC TG-3′

dsDNA:

Z1: 5′-FAM-TTT TAA CAC ATG CAG GAC GGC GCT T-3′
Z2: 5′-Cy5-TTT TAA GCG CCG TCC TGC ATG TGT T-3′

2.4 Mass spectrometry

Matrix-assisted laser desorption/ ionization mass spectrometry (MALDI-MS) was performed with a Bruker Autoflex MALDI-TOF model in positive scan mode by direct irradiation of the matrix-adsorbed peptide. 4-HCCA (α-cyano-4-hydroxycinnamic acid) was the selected matrix for all these experiments.

2.5 UV-Vis spectroscopy

UV measurements were made in a Jasco V-630 spectrophotometer coupled to a Jasco ETC-717 temperature controller, using a standard Hellma semi-micro cuvette (108.002-QS) with a light path of 10 mm. Measurements were made at 20 °C. Acquisition parameters were: 220-700 nm range, scan speed of 200 nm/min, resolution of 0.2 nm.

2.6. IR spectroscopy

FT-IR spectra were recorded on a Perkin-Elmer Spectrum Two equipped with a UATR accessory. Measurements were made at 20 °C. The acquisition parameters were in the 4000-400 cm^{-1} range.

2.7. Circular Dichroism

Circular dichroism measurements were made with a Jasco J-715 coupled to a Neslab RTE-111 thermostated water bath, using a Hellma 100-QS cuvette (2 mm light pass). Scan speed was 200 nm/min and the obtained spectra are the mean of three accumulations.

2.8. EPR studies

EPR measurements were made in a Bruker EMX spectrometer.

2.9. HPLC and UHPLC

a) *UHPLC-MS*

Peptide analysis was performed by analytical UHPLC-MS with an Agilent 1200 series LC/MS using a SB C18 (1.8 μm, 2.1 × 50mm) analytical column from Phenomenex. Standard conditions for analytical UHPLC consisted of a linear gradient from 5% to 95% of solvent B in 12 min at a flow rate of 0.35 mL/min (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA). Compounds were detected by UV absorption at 222, 270 and 330 nm. Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an Agilent 6120 Quadrupole LC/MS model in positive scan mode using direct injection of the purified peptide solution into the MS detector.

b) *Preparative HPLC*

Peptide purification was performed by preparative RP- HPLC with a Waters 1500 series Liquid Chromatograph using a Sunfire Prep C18 OBD (5 µm, 19 × 150 mm) reverse-phase column from Waters. Standard conditions for preparative RP- HPLC consisted of an isocratic regime during the first 5 min, followed by different linear gradients of solvent B during 30 min (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA). The gradients were adjusted for each peptide.

2.10. DNA hybridization

Oligonucleotides were obtained from Biomers. Concentration of the oligonucleotides was measured by UV-VIS by using the extinction coefficient given by the Commercial House Biomers.

For the hybridization process, a stochiometric mixture of the DNA strands was prepared in H_2O MQ and heated at 90°C for 10 minutes. After this time the mixture was slowly cooled down until room temperature, obtaining the resulting 3WJ/ds hybridized DNA.

The DNA sequences used can be found in the Supporting Information (Section 2.1).

2.11. Solid phase peptide synthesis

*a) **LLD** and **DDL** peptide ligand synthesis*

C-terminal amide **LLD** and **DDL** ligands were synthesized following standard Fmoc-peptide synthesis protocols on a 0.1 mmol scale using a 0.5 mmol/g loading H-Rink amide ChemMatrix resin (35–100 mesh size particle). The peptides were synthesized by hand, using HBTU as activating agent for the natural amino acids and HATU for the Fmoc-βAla5Bpy-OH (**1**) couplings. Each amino acid was activated for 1 min in DIEA/DMF 0.2 M (4 mL) before being added onto the resin. These manual couplings were conducted for 60 min. Deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF for 20 min. All the couplings were carried out in a 5-fold excess of the corresponding amino acid for the natural ones and in a 3.5-fold excess for the Fmoc-βAla5Bpy-OH (**1**).

N-capping with TAMRA was performed by reacting the N-terminal deprotected amine with the activated fluorophore —6-Carboxytetramethylrhodamine succinimidyl ester (TAMRA-OSu) in 1 mL DIEA/DMF 0.2 M during 2 h (approximately 7.5 mg of the reactive fluorophore/40 mg of resin).

b) Cleavage from the resin and final deprotection

Cleavage and deprotection of the peptides were simultaneously performed using standard conditions by incubating the resin for 2.5 h with an acidic mixture containing 50 µL CH_2Cl_2, 25 µL of H_2O, 25 µL of TIS (triisopropylsilane), and 900 µL of TFA. The resin was filtered, and the TFA filtrate was concentrated under a nitrogen stream to an approximate volume of 1 mL, and then added onto ice-cold diethyl ether (20 mL). After 10–30 min, the precipitate was centrifuged and washed again with 5 mL of ice-cold ether. The obtained residue was then dissolved in a mixture of H_2O and CH_3CN and purified by preparative HPLC for the obtention of the pure compounds.

2.12. Fluorescence spectroscopy

Luminescence experiments were made with a Varian Cary Eclipse Fluorescence Spectrophotometer coupled to a Cary Single Cell Peltier accessory (Agilent Technologies) temperature controller. All measurements were made with a Hellma semi-micro cuvette (108F-QS) at 20 °C. The settings for these measurements were adapted depending on the studied system.

a) Metal binding studies

A fluorescence titration of a 2 μM solution of **LLD** or **DDL** peptide in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0) with increasing concentrations of $CuCl_2 \cdot 2H_2O$ resulted in a series of emission spectra with decreasing emission intensity due to the quenching of the bipyridine residues upon coordination of the Cu^{II} ions. The resulting titration profiles at 420 nm could be fitted to a 1:2 binding mode using Dynafit 4.0 software.

b) DNA binding studies

The fluorescence emission of a 2 µM solution of the fluorescein-labelled 3WJ/dsDNA in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0) was monitored after the addition of increasing amounts of the preformed **ΔΔ-Cu^{II}_2LLD** peptide helicate. The quenching effect in the fluorescein emission band at 515 nm could be fitted to a 1:1 binding mode (Flu-DNA/helicate) plus nonspecific interactions using Dynafit 4.0 software.

c) Studies on the kinetic inertness of the Cu^{II} peptide helicate

The fluorescence emission at 420 nm of a 2 μM solution of **ΔΔ-Cu^{II}_2LLD** and 4 μM of EDTA in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0) was monitored during 60 min. The resulting complex half time was $t_{1/2}$ = 5.815 minutes and was calculated using Prism 9 software (by Dotmatics). The same experiment was repeated in the presence of 0.267 μM of 3WJ. The resulting complex half time was $t_{1/2}$ = 23.12 minutes and was also calculated using Prism 9 software (by Dotmatics).

d) Data analysis in fluorescence titrations

Fluorescence titration data were analyzed using the DynaFit 4.0 software, which characterizes the reacting system in terms of stoichiometric equations instead of mathematical notation; the chemical equations are then translated into the underlying mathematical equations using matrix theory.

DynaFit is available at http://www.biokin.com/dynafit/. Dynafit requires plain text files (scripts) that contain the chemical model underlying the experimental data, the values of model parameters, such as starting concentrations of reactants, as well as information about location of the files. A typical script used in the analysis titrations is included in Supporting Information (Section 2.2.)

2.13. Polyacrylamide gel electrophoretic analysis (PAGE)

PAGE experiments with fluorophore-labelled DNA were performed using an Invitrogen mini gel tank. Gels were cast using a SureCastTM gel casting system (Invitrogen) and powered by a BioRad basic power

pacTM. PAGE gels were visualised using a G:Box 9 mini gel documentation system and analysed with GeneSys software (Syngene). The multiplex assay was performed on GeneSys software (Syngene); an image with the excitation and emission for each fluorophore was captured, the images were overlayed, and a false colour applied for each fluorescent signal. Band densitometry was performed in triplicate on gels post-stained with SYBR gold (1X, Invitrogen) using GeneTools software (Syngene). Statistical analysis was performed using GraphPad Prism.

PAGE experiments with non-labelled DNA were performed with a BioRad Mini Protean gel system, powered by an electrophoresis power supplies PowerPac Basic model, maximum power 150 V, frequency 50–60 Hz at 130 V (constant V).

a) Non-labelled 3WJ and dsDNA binding studies

Non-labelled DNA sequences used in these studies can be found in ESI (Section 2.1). Binding reactions were performed over 30 min in 18 mM Tris-HCl (pH 7.5), 90 mM KCl, 1.8 mM $MgCl_2$, 9% glycerol, 0.11 mg/mL BSA, and 2.2% NP-40. For the experiments we used 200 nM of the DNAs (3WJ and dsDNA), different quantities of **ΔΔ-Cu^{II}_2LLD** (200, 400 or 600 mM) and a total incubation volume of 20 µL. After incubation for 30 min at room temperature, products were resolved by PAGE using a 10% non-denaturing polyacrylamide gel and 0.5 × TBE buffer (0.445 M Tris, 0.445 M Boric acid) for 35 min at 25 °C and analyzed by staining with SyBrGold (Molecular Probes: 5 µL in 50 mL of 1 × TBE) for 10 min and visualized by fluorescence (BioRad GelDoc XR+ molecular imager). The area of each band in the gel was measured using the Fiji ImageJ implementation using the Gel Analysis method outlined in the ImageJ documentation (https://imagej.nih.gov/ij/docs/menus/analyze.html#gels).

b) Fluorophore labelled 3WJ and dsDNA binding studies

Fluorescently labelled 3WJ (5 pmol) and dsDNA (5 pmol) sequences (ESI, Section 2.3) were incubated with 1, 2 or 3 eq. of **ΔΔ-Cu^{II}_2LLD** (5, 10 or 30 pmol) in HEPES buffer (10 mM HEPES, 750 mM NaCl, 50 mM $MgCl_2$, pH 7) at 37 ºC for 2 hours prior to 20% native PAGE analysis. The gel was run in 50 mM Tris-acetate buffer at 70 V for 240 min and visualised with a multiplex assay. FAM fluorescence was observed with blue light and 525 nm filter, ROX was excited by UV fluorescence and Cy5 was visualised with red light and a 705 nm filter.

c) In vitro nuclease activity studies

Nuclease activity was analysed by PAGE. Equal concentrations of each strand of the 3WJ were mixed in HEPES buffer (10 mM HEPES, 750 mM NaCl, 50 mM $MgCl_2$, pH 7) and heated to 95 ºC before being cooled in 5 □C increments to 4 ºC. The annealed 3WJ (5 pmol), and an off-target DNA duplex (5 pmol), were incubated at 37 ºC for 24 hours with **ΔΔ-Cu^{II}_2LLD** (1, 2, 5, 7.5 or 10 equivalents, that is, 5, 10, 25, 37.5 or 50 pmol) and Na-L-ascorbate (10 eq. to **ΔΔ-Cu^{II}_2LLD**, that is, 50, 125, 250, 375 or 500 pmol). The reaction was quenched with loading dye (6X, 10 mM Tris-HCL, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol, 60 mM EDTA) and loaded onto a 20% native PAGE gel (50 mM Tris-acetate, 75 mM NaCl, 5 mM $MgCl_2$, pH 6.1). The gel was run in 50 mM Tris-acetate buffer at 70 V for 240 min, and post-stained with SYBR gold (1X, Invitrogen). Gel was visualized under UV fluorescence. Band densitometry was performed in triplicate, and the significance of the data was analysed by Two-way ANOVA with Tukey's multiple comparisons.

Cleavage activity on fluorescently labelled DNA. Samples were prepared as described above, but fluorophore labelled 3WJ and dsDNA strands were utilised. Cleavage assay was quenched and loaded onto a 20% native PAGE gel (50 mM Tris-acetate, 75 mM NaCl, 5 mM $MgCl_2$, pH 6.1). The gel was run in 50 mM Tris-acetate buffer at 70 V for 240 min and visualised using the multiplex assay described above.

ΛΛ-Fe^{II}_2LLD cleavage (negative control). Samples containing 3WJ and dsDNA were prepared as previously described but **ΔΔ-Cu^{II}_2LLD** was replaced with **ΛΛ-Fe^{II}_2LLD**. All other reaction components were kept consistent with the Cu^{II} helicate cleavage assay.

Time-point study. Samples containing 3WJ, dsDNA, **ΔΔ-Cu^{II}_2LLD** (1, 5 and 10 eq. to DNA) and Na-L-ascorbate were prepared as described above, and incubated a various time points (1 hour, 3 hours and 12 hours) at 37 ºC before being quenched with loading dye (6X, 10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol, 60 mM EDTA) and loaded onto a 20% native PAGE gel (50 mM Tris-acetate, 75 mM NaCl, 5 mM $MgCl_2$, pH 6.1). Gel was run at 70 V for 240 min, post-stained with SYBR gold (1X, Invitrogen) and visualised by UV fluorescence.

Reactive oxygen species (ROS) analysis. The 3WJ and **ΔΔ-Cu^{II}_2LLD** samples were prepared as previously described, but the dsDNA off-target was replaced with a reactive oxygen species scavenger (50 nmol); L-histidine, D-mannitol, L-methionine, tiron (4,5-dihydroxy-1,3-benzendisulfonic acid), sodium azide (NaN_3), deuterium oxide (D_2O) or 4-hyrdoxy-TEMPO and incubated at 37 ºC for 24 hours. Products were analysed by 20% Tris-acetate PAGE (50 mM Tris-acetate, 75 mM NaCl, 5 mM $MgCl_2$, pH 6.1).

2.14. Cell internalization studies

Vero or HeLa cells were grown in DMEM (Dulbecco modified Eagle Medium) with 10% of fetal bovine serum in glass-bottom 35 mm tissue culture plates (IBIDI). One day before the internalization experiment, the cells were transfected with plasmid pc653-pENeGFPCNAL2mut (H. Leonhardt, 2000) using PEI Polyethylenimine HCl MAX (PEImax®, obtained from Polysciences) following the instructions from the manufacturer. That plasmid drives the expression of GFP-tagged Proliferating Cellular Nuclear Antigen (PCNA) to label DNA replication factories. The next day the cells were washed twice with transfer buffer (25 mM HEPES, 125 mM KOAc, 2 mM $Mg(AcO)_2$, 1 mM EDTA). Then, cells were treated with a digitonin solution (25 μg/mL) in the same previous buffer at 0 °C for 3 minutes. After this time cells were washed again twice with transfer buffer containing 10 mg/mL of BSA. Cells were then incubated in transfer buffer with 5 µM of **ΔΔ-Cu^{II}_2TAMRA-LLD** for 15 minutes. After this time cells were washed three times with transfer buffer containing 10 mg/mL of BSA and finally covered with DMEM supplemented with 10% of fetal bovine serum.

Images were obtained with an Andor Dragonfly spinning disk confocal system mounted on a Nikon TiE microscope equipped with a Zyla 4.2 PLUS camera (Andor).

2.15. Nuclease activity assays in cells

a) Cell synchronization

4x106 Vero cells were grown in T175 flasks. After 24 hours of incubation at 37 ºC with 5% CO_2, the medium was removed, and cells were washed three times with PBS 1X and incubated in DMEM-HEPES with 1% antibiotics and without Glutamine and FBS. After 42 hours, the medium was replaced by DMEM + 10% FBS and 1% glutamine + antibiotics. After 16 hours the cells were trypsinized and counted.

b) In cellulo nuclease assay

Vero cells were pelleted (500 x g, 5 min 4 ºC) and washed three times with Transfer Buffer (25 mM HEPES pH 7.9, 125 mM KOAc, 2 mM $Mg(AcO)_2$, 1 mM EDTA). Subsequently, cells were treated with 25 μg/ml digitonin in transfer buffer for 3 min on ice. After this time cells were centrifuged, and the supernatant were removed. Cells were resuspended according to the treatment in: transfer buffer, transfer buffer + 2.5 mM H_2O_2, transfer buffer + 2.5 mM H_2O_2 + 5 µM **ΛΛ-Fe^{II}_2LLD** and transfer buffer + 2.5 mM H_2O_2 + 5 µM **ΔΔ-Cu^{II}_2LLD** and incubated 2 hours at 37 ºC shaking every 15 minutes. After this time, cells were centrifuged and washed twice with Transfer buffer + 10 mg/ml BSA and twice with PBS 1X. Finally, cells were fixed for the TUNEL Assay.

c) TUNEL Assay

Vero cells were fixed with 5 mL of 1% PFA (paraformaldehyde) in PBS for 15 min at 4 ºC. Subsequently, cells were centrifuged (500 x g, 5 min, 4 °C) and washed twice with PBS 1X. Then, the cells were resuspended in 5 mL of ice-cold 70% ethanol and incubate 5 days at -20 ºC. The cells were then collected by centrifugation and subjected to TUNEL assay following the manufacturer´s instructions (APO-BrdUTM TUNEL Assay Kit, Thermo Fisher Scientific). Briefly, they were washed with 1 mL of Wash buffer and then incubated with DNA-labelling solution overnight at RT on a nutator mixer. Next, the cells were washed twice with Rinse Buffer and incubated with Alexa Fluor 488TM dye-labelled anti-BrdU antibody for 30 min at RT on a nutator and analysed by flow cytometry using a Guava easyCyte HT Millipore flow cytometer equipped with a 488 nm blue laser coupled with a 525/30 nm filter. One-way ANOVA with Dunnett's multiple comparisons was performed to determine significance of data collected.

2.16. Cytotoxicity studies

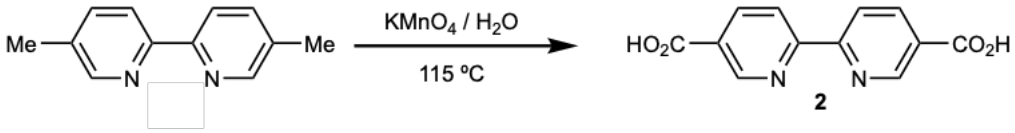
The effect of **ΔΔ-Cu^{II}_2LLD** on Vero cell viability was determined with the Cell Counting Kit-8 (CCK-8, TargetMol) (E. Oner, 2023).

3. Experimental data

3.1. Synthesis of Fmoc-βAla5Bpy-OH (8)

The synthesis of this chelating residue was carried out following the methodology recently published by our group.[1] All the obtained products were analyzed by HPLC-MS to test their purity.

3.1.1. Synthesis of 2,2'-bipyridine-5,5'-dicarboxilic acid (2)



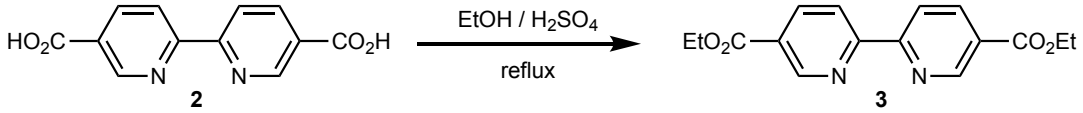
A mixture of 39 g of potassium permanganate and 7 g of 5,5'-dimethyl-2,2' -bipyridine in 250 mL of H_2O was heated for 2 h (115 ºC), cooled at room temperature and filtrated trough celite. The filtrate was cooled to 4 ºC and acidified with HCl until precipitation of a white solid, which was filtrated, washed with water and lyophilized to afford the desired product in a 93% yield (8.7 g)

^{1}H-NMR (500 MHz, DMSO-d_6, δ): 13.50 (br); 9.18 (dd, ^4J = 2.15, 5J = 0.8 Hz 2H); 8.55 (dd, ^3J= 8.3; 5J = 0.8 Hz, 2H); 8.44 (dd, ^3J = 8.3; 4J= 2.15 Hz, 2H).

^{13}C-NMR (125 MHz, DMSO-d_6, δ): 165.49, 156.84, 149.82, 137.97, 126.65, 120.62.

MALDI-TOF (m/z) $[M+H]^+$ calculated for $[C_{12}H_8N_2O_4]$ 245.0; found 245.0.

3.1.2. Synthesis of diethyl [2,2'-bipyridine]-5,5'-dicarboxylate (3)



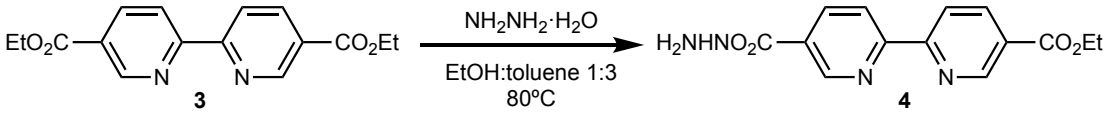
2,2'-bipyridine-5,5'-dicarboxylic acid (10.0 g, 41 mmol) was suspended in 150 mL of absolute ethanol. Concentrated sulfuric acid (20.0 mL) was slowly added, and the resulting mixture was refluxed for 18 h. The solution was cooled at room temperature and added over 400 mL of water at 4 ºC causing the precipitation of a white solid, which was filtered, washed with water and lyophilized. 11.5 g (93.0 %)

^{1}H-NMR (500 MHz, DMSO-d_6, δ): 9.20 (dd, ^4J = 2.15, 5J = 0.8 Hz 2H); 8.57 (dd, ^3J= 8.3; ^5J = 0.8 Hz, 2H); 8.46 (dd, ^3J = 8.3; ^4J = 2.15 Hz, 2H); 4.0 (q, ^3J = 7.1 Hz, 4H); 1.37 (t, ^3J = 7.1 Hz, 6H).

^{13}C-NMR (125 MHz, DMSO-d_6, δ): 164.2, 157.2, 149.8, 138.0, 126.2, 121.0, 61.1, 13.9.

MALDI-TOF (m/z) $[M+H]^+$ calculated for $[C_{16}H_{16}N_2O_4]$ 301.1; found 301.1.

3.1.3. Synthesis of Ethyl 5'-Carbohydrazido-2,2'-bipyridine-5-carboxylate (4)



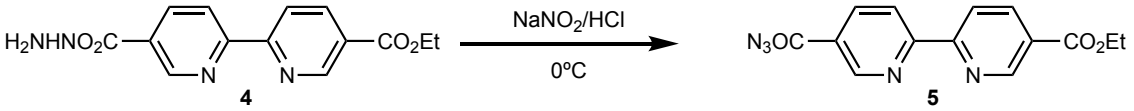
A mixture of diethyl 2,2'-bipyridine-5,5'-dicarboxylate (15.00 g, 50 mmol) and hydrazine hydrate (3.75 mL, 55 mmol) in a solution of EtOH (42 mL) and toluene (128 mL) was heated at 80 °C for 48 h. The precipitate was filtered, washed with $CHCl_3$ and dried under vacuum 11.4 g (80%). The unreacted diethyl ester was concentrated and mixed again to obtain a global yield of (90%).

^{1}H-NMR (500 MHz, DMSO-d_6, δ): 1.35 (t, 3H), 4.37 (q, 2H), 8.35 (d, ^3J = 8.4 Hz, 1H), 8.45 (dd, ^3J = 8.4 Hz, ^4J) 2 Hz, 1H), 8.52 (d, ^3J = 8.4 Hz, 1H), 8.57 (d, ^3J = 8.4 Hz, 1H), 9.1 (d, ^4J = 2 Hz, 1H), 9.2 (d, ^4J = 2 Hz, 1H), 10.1 (br, 1H).

^{13}C-NMR (125 MHz, DMSO-d_6,δ): 14.1, 61.3, 120.9, 120.9, 126.1, 129.6, 136.2, 138.2, 148.1, 150.0, 155.8, 157.8, 163.8, 164.5.

MALDI-TOF (m/z) $[M+H]^+$ calculated for $[C_{14}H_{14}N_4O_3]$ 287.1; found 287.1.

3.1.4. Synthesis of Ethyl 5'-Carbohydrazido-2,2'-bipyridine-5-carboxylate (5)



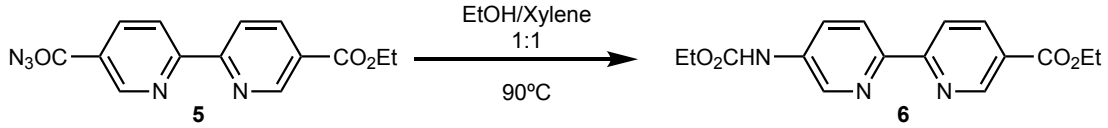
A stirred solution of 5-(ethoxycarbonyl)-5'-carbohydrazido-2,2'-bipyridine (5.7 g, 20 mmol) in concentrated HCl (100 mL) was cooled to 0 °C, and then an aqueous solution of $NaNO_2$ (1.73 g, 25 mmol; 15 mL) was added dropwise, maintaining the temperature at 0 °C. After 60 min, the yellow solution was diluted with water (300 mL) to precipitate the monoester 5 as a white powder, which was filtered, washed with water, and lyophilized 5.5 g (92 %)

^{1}H-NMR (500 MHz, Acetone-d^6,δ): 9.13 (d, ^4J = 1.91 Hz, 1H), 9.10 (d, ^4J = 1.91 Hz, 1H), 8.71 (d, ^3J = 12.5 Hz, 1H), 8.69 (d, ^3J = 12.5 Hz, 1H), 8.517 (m, 2H; 4.31 (q, ^3J = 7.15 Hz, 2H); 1.29 (t, ^3J= 7.15, 3H).

^{13}C-NMR (125 MHz, Acetone-d_6,δ): 170.8, 164.5, 159.2, 157.7, 150.3, 150.0, 138.0, 137.9, 127.0, 121.3, 61.2, 13.6.

MALDI-TOF (m/z) $[M+H]^+$ calculated for $[C_{14}H_{11}N_5O_3]$ 298.1; found 298.1

3.1.5. Synthesis of Ethyl 5'-(ethoxycarbonyl)amino-2,2'-bipyridine-5-carboxylate (6)



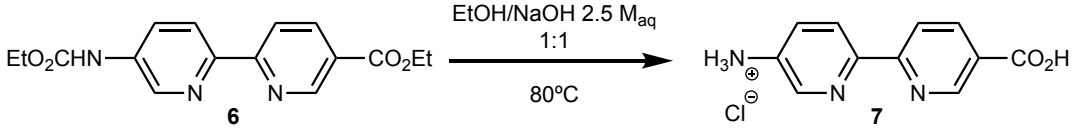
A solution of ethyl 5'-carbazido-2,2'-bipyridine-5-carboxylate (8.6 g, 29 mmol) in a mixture of EtOH (100 mL) and xylene (100 mL) was heated at 90 ºC for 4 h. The solvent was evaporated under reduced presure, and the yellow residue was washed with EtOH and dried in vacuo. 8.21 g (90%).

^{1}H-NMR (500 MHz, DMSO-d_6, δ): 1.27 (t, J = 7.2 Hz, 3H), 1.35 (t, J = 7.2 Hz, 3H), 4.17 (q, J = 7.2 Hz, 2H), 4.36 (q, J = 7.2Hz, 2H), 8.08 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H), 8.35 (dd, ^3J = 8.3 Hz, ^4J = 2 Hz, 1H), 8.40 (dd, ^3J = 8.3Hz, ^5J = 1 Hz, 1H), 8.45 (d, ^3J = 8 Hz, 1H), 8.70 (d, ^4J = 2 Hz, 1H), 9.12 (d, ^4J = 2 Hz, ^5J = 1 Hz, 1H), 10.13 (s, 1H).

^{13}C-NMR (125 MHz, DMSO-d_6, δ): 13.6, 13.9, 60.3, 60.6, 119.1, 121.2, 124.46, 124.9, 136.7, 137.4, 139.0, 147.4, 149.4, 153.1, 158.1, 164.2.

MALDI-TOF (m/z) $[M+H]^+$ calculated for $[C_{16}H_{17}N_3O_4]$ 316.1; found 316.1.

3.1.6. Synthesis of 5'-Amino-2,2'-bipyridine-5-carboxylic acid hydrochloride (7)



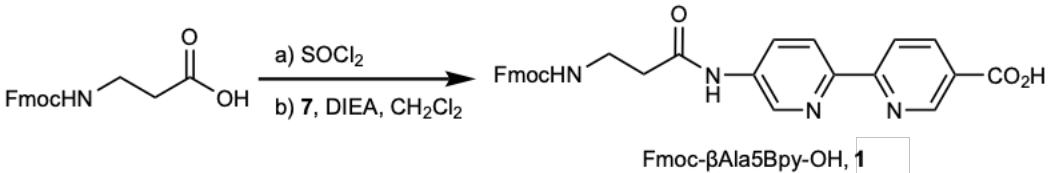
A stirred solution of ethyl 5'-[(ethoxycarbonyl)amino]-2,2'-bipyridine-5-carboxylate (14.55 g, 45.9 mmol) in a mixture of EtOH (50 mL) and 2.5 M aqueous NaOH (50 mL) was heated at 75 ºC for 14 h. The EtOH was concentrated in vacuum, and the aqueous solution was acidified with HCl to afford a bright yellow precipitate, which was filtered, washed with cold water, and lyophilized 9.80 g (85%).

^{1}H-NMR (500 MHz, D_2O, δ): 7.00 (dd, ^3J = 8.5 Hz, ^4J = 2.8 Hz, 1H), 7.55, 7.61 (d, ^3J = 8.5 Hz, 2H), 7.81 (d, ^4J = 2.9 Hz, 1H), 7.98 (dd, ^3J = 8.5 Hz, ^4J = 2.9 Hz, 1H), 8.65 (d, ^4J = 2.8 Hz, 1H)

^{13}C-NMR (125 MHz, D_2O, δ): 123.3d, 126.2, 126.7, 133.8, 139.8, 141.34, 147.5, 147.4, 152.4, 159.6, 176.04

MALDI-TOF (m/z) $[M+H]^+$ calculated for $[C_{11}H_9N_3O_2]$ 216.1; found 216.1

3.1.7. Synthesis of Fmoc-βAla5Bpy-OH (1)



Over 4.58 g (14.72 mmol) of Fmoc-βAla 10 mL of $SOCl_2$ was added carefully at room temperature. The solution was stirred magnetically for 30 min, the thionyl chloride was evaporated in vacuum and the yellow solid was washed with CH_2Cl_2 (3x10 mL), and dried under reduced pressure. The compound (**7**) 3.7 g (14.8

mmol), 20 mL of CH_2Cl_2 and 5 mL of DIEA were added. The suspension was stirred at room temperature overnight. The solvent was evaporated, the solid was suspended in acetonitrile 0.1 %TFA/H2O (2:1), centrifuged and washed with acetonitrile and H_2O and lyophilized 6.70 g (89 %).

Regarding the nomenclature of the coordinating residue **1**, Fmoc is the amine protecting group necessary to introduce the 2,2'-bipyridine residue into a peptide sequence by SPPS methodology. βAla is the amino acid attached to the amino group of the 2,2'-bipyridine amino acid. This extra amino acid is key to being able to integrate the 2,2'-bipyridine amino acid into the peptide sequence, as its amino group is not basic enough to form peptide bonds by a SPPS methodology. The number 5 indicates that the substitutions of the 2,2'-bipyridine molecule to transform it into an artificial amino acid have been made at their 5 and 5' positions.

^{1}H–NMR (300 MHz, DMSO-d_6, δ): 10.44 (s, 1H); 9.13 (d, 4J = 1.36 Hz, 1H); 8.89 (d, ^4J = 2.12 Hz, 1H); 8.42 (m, 3H); 8.25(dd, ^3J = 8.48 Hz, ^4J = 2.12 Hz, 1H); 7.88 (d, ^3J = 7.46 Hz, 2H); 7.67 (d, ^3J = 7.38 Hz, 2H); 7.49 (t, ^3J = 5.34 Hz, NH); 7.40 (t, ^3J = 7.21 Hz, 2H); 7.30 (t, ^3J = 7.38 Hz, 2H); 4.30 (d, ^3J = 6.95 Hz, 2H); 4.21 (t, ^3J = 6.95 Hz, 1H) 3.32 (t, ^3J = 6.70 Hz, 2H); 2.60 (t, ^3J = 6.70 Hz, 2H).

^{13}C-NMR (75 MHz, DMSO-d_6, δ): 169.7 (C), 168.6 (C), 157.2 (C), 150.9 (C), 149.2 (C), 144.7 (C), 143.4 (C), 142.2 (C), 141.6 (C), 140.3 (CH), 138.3 (CH), 138.1 (CH), 129.7 (CH), 128.1 (CH), 125.9 (CH), 122.1 (CH), 120.9 (CH), 120.8 (CH), 118.7 (CH), 110.3 (CH), 67.4 (CH_2), 63.8 (CH_2), 47.6 (CH), 41.7 (CH_2).

MALDI-TOF (m/z) $[M+H]^+$ calculated for $[C_{29}H_{25}N_4O_5]$ 509.1; found 509.1.

3.2. UHPLC-MS of the purified peptide ligands

3.2.1. LLD: *H-(βAla5Bpy)$_2$RPr(βAla5Bpy)$_2$RPr(βAla5Bpy)$_2$-NH$_2$*

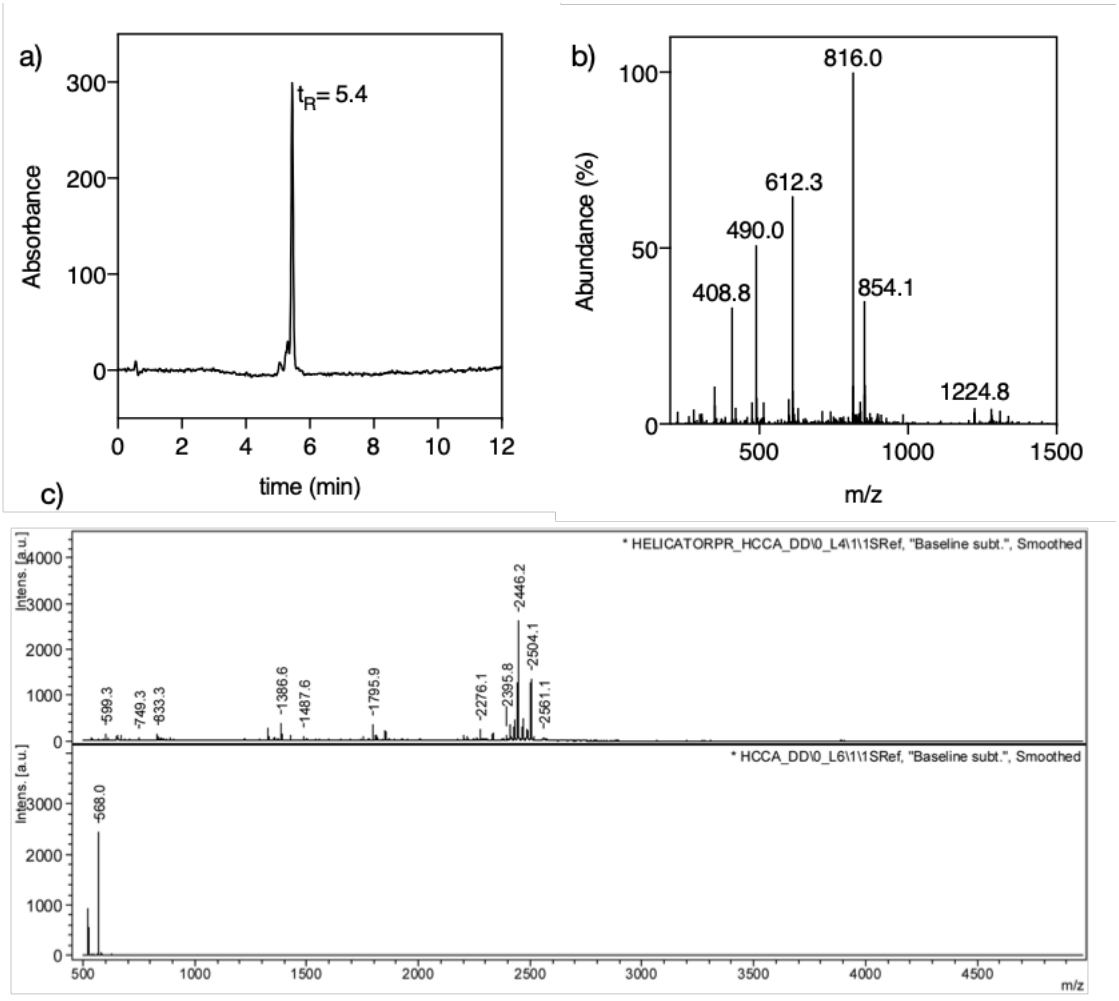


Figure S1. *a) Chromatogram of the purified **LLD** peptide ligand. b) ESI mass spectra corresponding to the peak with t_R of 5.4 min. c) MALDI mass spectra of **LLD**.*

HPLC-MS (ESI): (5-95% B, t_R= 5.4 min) Calculated for $C_{118}H_{137}N_{43}O_{18}$ = 2444.11; found $[M+2H+TFAH]^{2+}$ = 1289.7; $[M+3H]^{3+}$ = 816.0; $[M+3H+TFAH]^{3+}$ = 854.1; $[M+4H]^{4+}$ = 612.3; $[M+5H]^{5+}$ = 490.0; $[M+6H]^{6+}$ = 408.8.

MS (MALDI-TOF): Calculated for $C_{118}H_{137}N_{43}O_{18}$ = 2444.11; found: $[M+H]^+$ = 2446.21.

3.2.2. DDL: *H-(βAla5Bpy)$_2$rpR(βAla5Bpy)$_2$rpR(βAla5Bpy)$_2$-NH$_2$*

HPLC-MS (ESI): (5-95% B, t_R= 5.4 min) Calculated for $C_{118}H_{137}N_{43}O_{18}$ = 2444.11; found $[M+2H+TFAH]^{2+}$ = 1289.7; $[M+3H]^{3+}$ = 816.0; $[M+3H+TFAH]^{3+}$ = 854.1; $[M+4H]^{4+}$ = 612.3; $[M+5H]^{5+}$ = 490.0; $[M+6H]^{6+}$ = 408.8.

MS (MALDI-TOF): Calculated for $C_{118}H_{137}N_{43}O_{18}$ = 2444.11; found: $[M+H]^+$ = 2446.21.***3.2.3. TAMRA-LLD*** *TAMRA-O1Pen(βAla5Bpy)$_2$RPr(βAla5Bpy)$_2$RPr(βAla5Bpy)$_2$-NH$_2$*

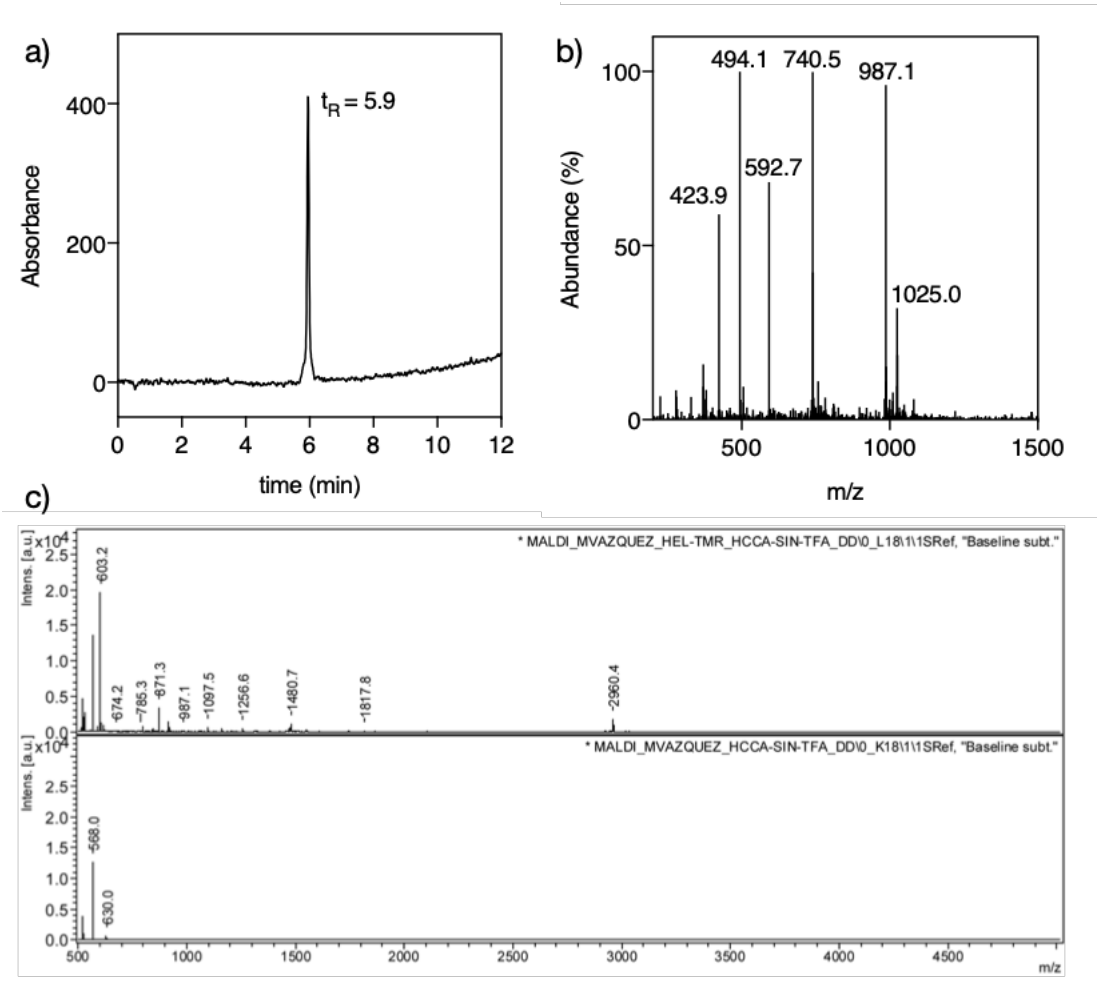


Figure S2. *a) Chromatogram of the purified **TAMRA-LLD** peptide ligand. b) ESI mass spectra corresponding to the peak with t_R of 5.9 min. c) MALDI mass spectra of **TAMRA-LLD**.*

HPLC-MS (ESI): (5-95% B, t_R= 5.9 min) Calculated for $C_{147}H_{166}N_{46}O_{24}$ = 2959.31; found $[M+3H+TFAH]^{3+}$ = 1025.0; $[M+3H]^{3+}$ = 987.1; $[M+4H]^{4+}$ = 740.5; $[M+5H]^{5+}$ = 592.7; $[M+6H]^{6+}$ = 494.1; $[M+7H]^{7+}$ = 423.9.

MS (MALDI-TOF): Calculated for $C_{147}H_{166}N_{46}O_{24}$ = 2959.31; found: $[M+H]^+$ = 2960.43.

3.3. Synthesis of the peptide helicates

3.3.1. Synthesis of the Fe^{II} peptide helicates

The synthesis of **ΛΛ-Fe$^{II}_2$LLD** and **ΛΛ-Fe$^{II}_2$TAMRA-LLD** metallopeptides were made following the procedure reported in our previous paper (J. Gómez-González, 2021).

3.3.2. Synthesis of the Cu^{II} peptide helicates

Onto a solution of **LLD**, **DDL** or **TAMRA-LLD** in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0), $CuCl_2.2H_2O$ was added as source of Cu^{II} ions until saturating conditions (5 eq).

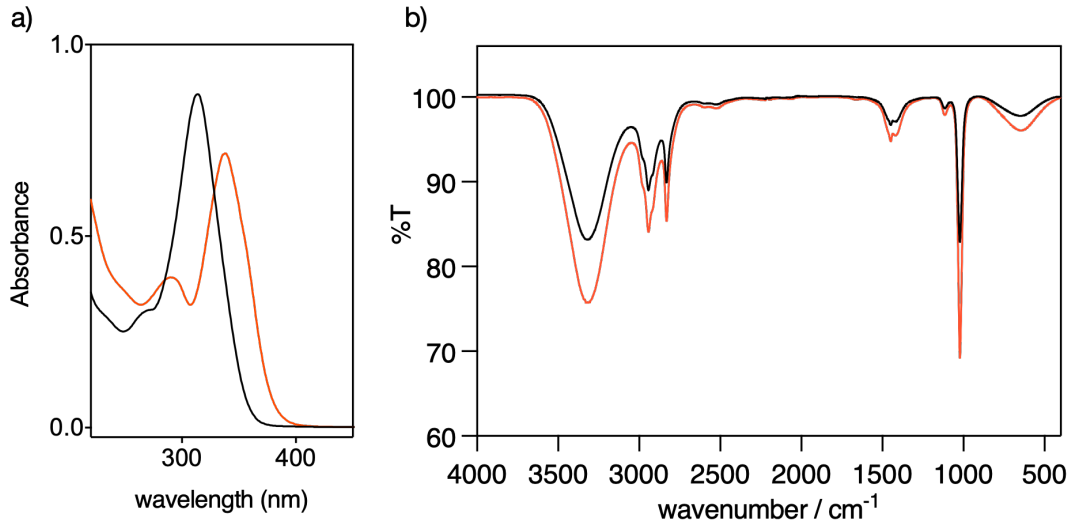


Figure S3. *a) UV-Vis spectrum of a 5 μM solution of* ***LLD*** *peptide ligand (1 mM phosphate buffer, 10 mM NaCl, pH 7.0) before (black solid line) and after the addition of 5 equivalents of $CuCl_2.2H_2O$ (orange line); b) IR spectrum of a 5 μM solution of* ***LLD*** *peptide ligand in MeOH before (black line) and after the addition of 5 equivalents of $CuCl_2.2H_2O$ (orange line).*

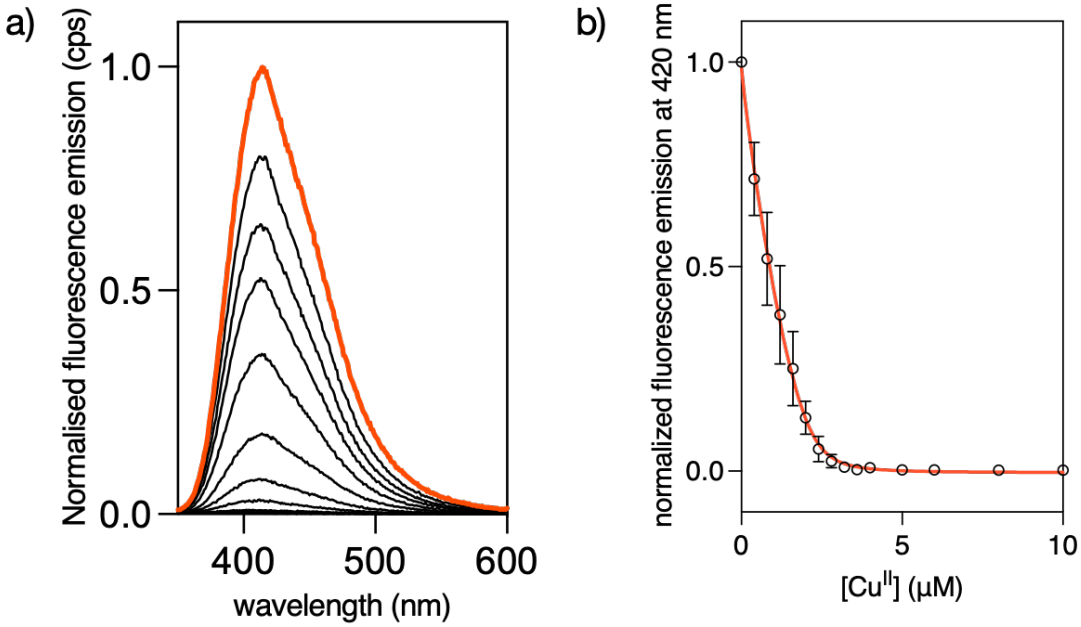


Figure S4. *a) Normalized emission spectrum of a 2 μM solution of* ***DDL*** *in phosphate buffer (1 mM, NaCl 10 mM, pH 7.0, orange line), and spectra of the same solution in the presence of increasing concentrations of Cu^{II} ions (black lines). b) Titration profile of three independent fluorometric titration experiments at 420 nm and best fit according to a 1:2 model in DynaFit (same range as main plot) with stepwise dissociation constants of approximately 0.3 and 0.15 μM for the first and the second Cu^{II} ions. λ_{exc} = 308 nm.*

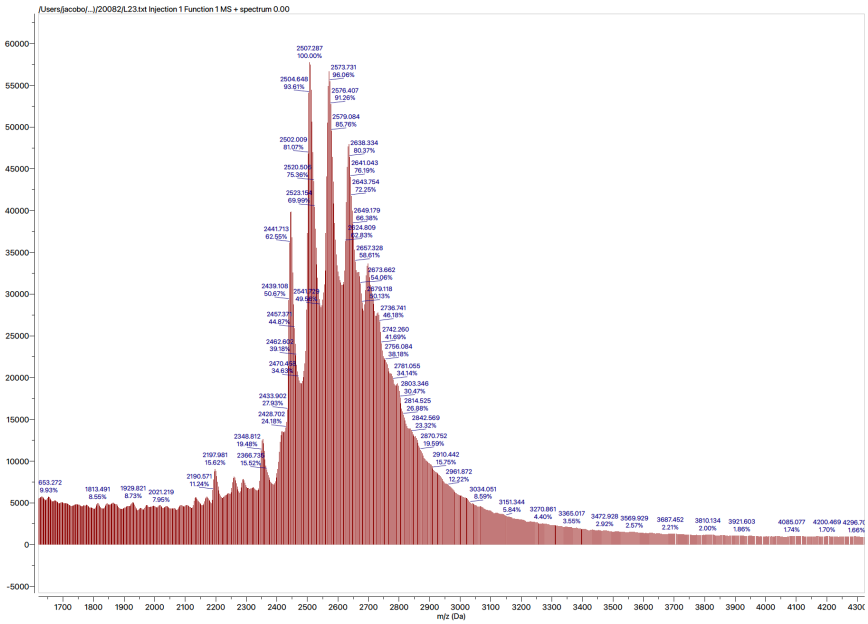


Figure S5. *MALDI spectra of* ***ΔΔ-Cu$_2^{II}$LLD***

MS (MALDI-TOF): Calculated for **ΔΔ-Cu$^{II}_2$LLD** $C_{118}Cu_2H_{137}N_{43}O_{18}$ = 2572.77; found: $[M+2Cu+H]^+$ = 2573.73.

MS (MALDI-TOF): Calculated for **ΛΛ-Cu$^{II}_2$DDL** $C_{118}Cu_2H_{137}N_{43}O_{18}$ = 2572.77; found: $[M+2Cu+H]^+$ = 2573.73.

MS (MALDI-TOF): Calculated for **ΔΔ-Cu$^{II}_2$TAMRA-LLD** $C_{147}Cu_2H_{166}N_{46}O_{24}$ = 3086.32; found: [M+2Cu] = 3086.75.

3.4. EPR studies

X-band EPR spectra of the **ΔΔ-Cu$_2^{II}$LLD** metallopeptide were obtained in solution [200 μM of metallopeptide in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0)] at 300 K and 120 K.

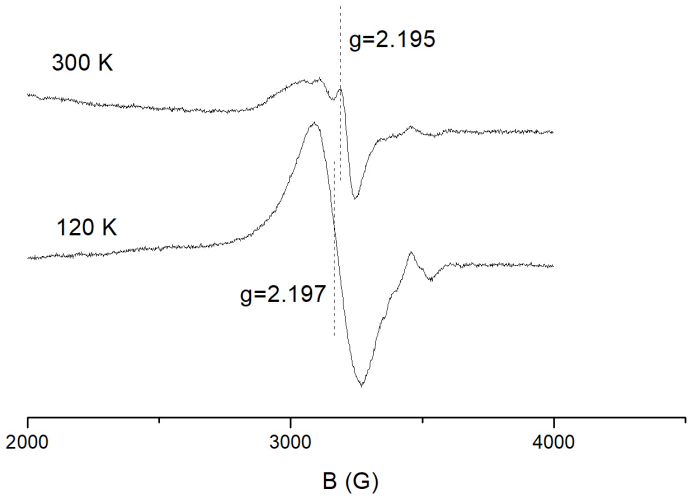


Figure S6. *X-band Cu^{II} EPR spectra of the* ***ΔΔ-Cu$^{II}_2$LLD*** *peptide helicate measured in solution (200 μM of metallopeptide in phosphate buffer 1mM, 10 mM NaCl, pH 7.0) at 120 K (bottom) and 300 K (top).*

3.5. Fluorescence titrations experiments with GC-3WJ DNA

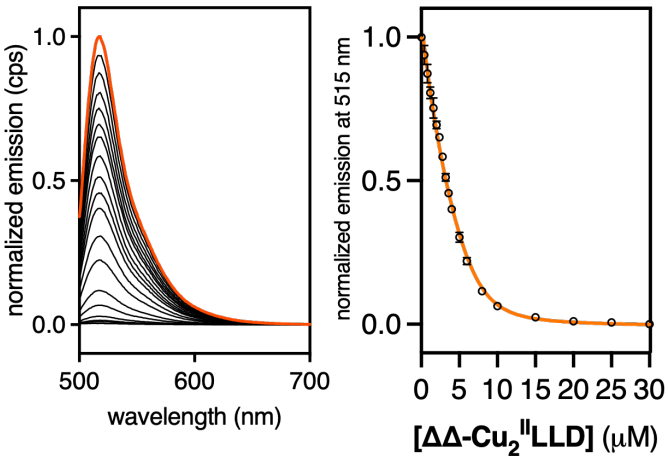


***Figure S7**. Left: full emission spectra of a 2 μM solution of fluorescein-labelled GC-3WJ (Flu-GC-3WJ) in absence and in presence of increasing concentrations of **ΔΔ-Cu$^{II}_2$LLD** (decreasing intensity). Right: profile of the full titration with the best fit (orange line) according to the 1:1 model plus unspecific interactions in DynaFit, with an apparent dissociation constant K_D = 300 nM. Conditions: phosphate buffer 1 mM, NaCl 10 mM, pH 7.0.*

3.6. Studies on the kinetic inertness of ΔΔ-Cu$^{II}_2$LLD in physiological media

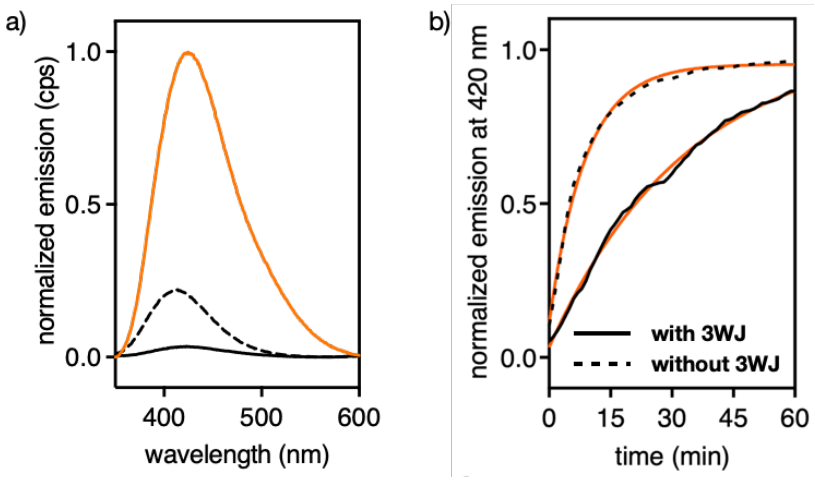


***Figure S8**. a) Full emission spectra of a 2 μM solution of **LLD** (orange line), and spectra of the same solution with 10 μM of Cu^{II} ions (black line), and with 4 μM of EDTA (S. Chaberek Jr, 1955) after 60 min of stabilization (black dashed line); b) fluorescence emission at 420 nm of the 2 μM solution of **ΔΔ-Cu$^{II}_2$LLD** and 4 μM of EDTA without (black continuous line) and with (black dashed line) the presence of 0.267 μM of 3WJ during 60 minutes, together with their respective best fits (orange lines) resulting in a complex half time $t_{1/2}$ = 5.815 and 23.12 minutes, respectively. Conditions: phosphate buffer 1 mM, NaCl 10 mM, pH 7.0.*

3.7. 3WJ-binding properties of the free peptide ligand LLD

The key role played by metal ions in the recognition of 3WJ by the helicates derived from the peptide ligand **LLD** was demonstrated in our previous work in ACIE (J. Gómez-González, 2021) through a fluorescence anisotropy titration of a 2 μM solution of the fluorescein-labelled **LLD** peptide ligand with increasing concentrations of 3WJ. The descending and oscillating pattern of the spots obtained in the titration demonstrated the absence of interaction between the free peptide ligand **LLD** and 3WJ.

3.8. Gel electrophoresis: *DNA binding studies*

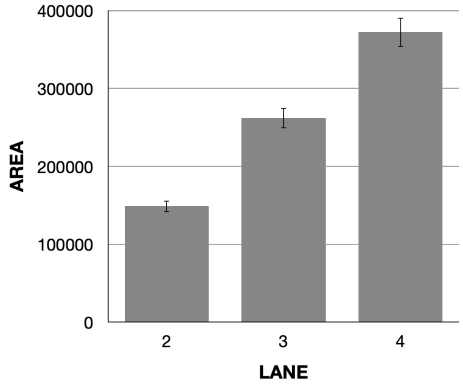


Figure S9. *The area of each band in the gel of Figure 3b of the manuscript was measured using the Fiji ImageJ implementation (C. A. Schneider, 2012; J. Schindelin, 2012) using the Gel Analysis method outlined in the ImageJ documentation (https://imagej.nih.gov/ij/docs/menus/analyze.html#gels). The area of each band clearly shows a saturation profile and concentration dependent signal, consistent with the progressive saturation of 3WJ binding with the Cu^{II} helicate.*

3.9. Gel electrophoresis: *In vitro* nuclease activity studies

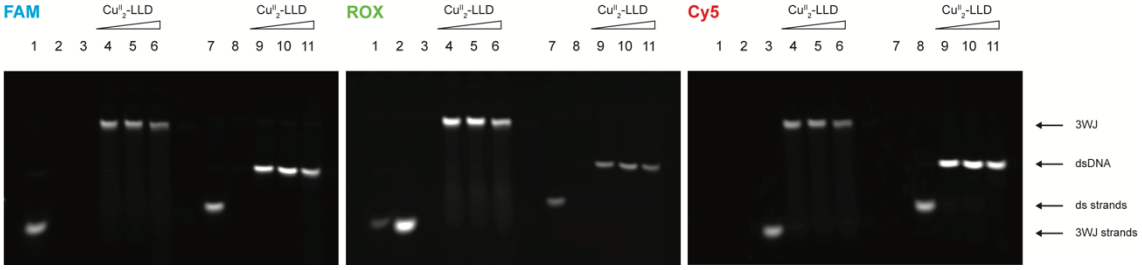


Figure S10. *Images obtained during multiplex assay with FAM (left), ROX (middle) and Cy5 (right) excitation and emission filters.*

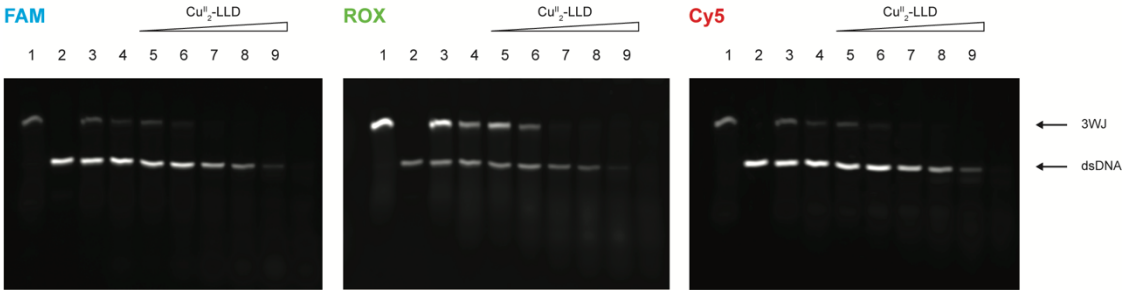


Figure S11. *FAM (left), ROX (middle) and Cy5 (right) images for fluorophore cleavage assay.*

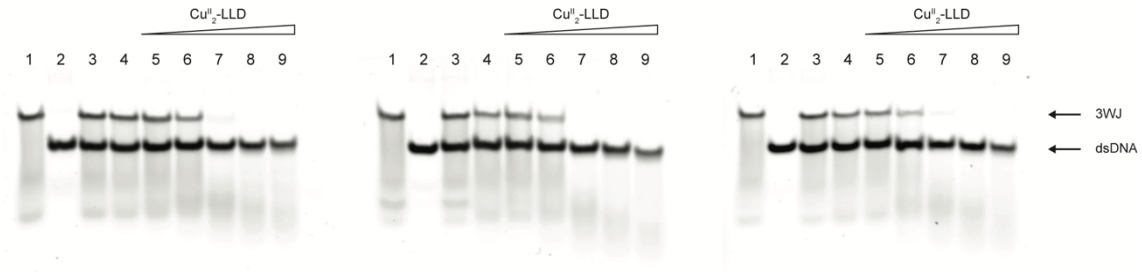


Figure S12. *20% native page of cleavage inflicted by **ΔΔ-Cu$^{II}_2$LLD** in triplicate for densitometry. Lane 1; 3WJ. Lane 2; dsDNA. Lane 3; 3WJ and dsDNA incubated with **ΔΔ-Cu$^{II}_2$LLD**. Lane 4: Target and off-target with **LLD** (no Cu^{II} present). Lanes 5-9; 3WJ and dsDNA incubated with 1, 2, 5, 7.5 or 10 eq. of **ΔΔ-Cu$^{II}_2$LLD** and Na-L-ascorbate (10 eq. to **ΔΔ-Cu$^{II}_2$LLD**).*

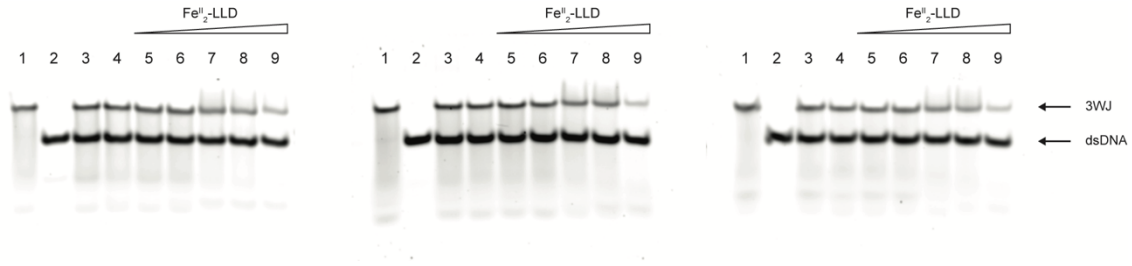


Figure S13. *$\Lambda\Lambda$-Fe$^{II}_2$LLD cleavage for densitometry. $\Lambda\Lambda$-Fe$^{II}_2$LLD cleavage assay was performed in triplicate for band densitometry analysis. Lane 1; 3WJ. Lane 2; dsDNA. Lane 3; Target 3WJ and off-target dsDNA incubated with $\Lambda\Lambda$-Fe$^{II}_2$LLD. Lane 4; 3WJ and dsDNA with LLD (no Fe^{II} present). Lanes 5-9; Target 3WJ and off-target dsDNA with 1, 2, 5, 7.5 or 10 eq. of $\Lambda\Lambda$-Fe$^{II}_2$LLD and Na-L-ascorbate (10 eq. to $\Lambda\Lambda$-Fe$^{II}_2$LLD). Gel on the left is also shown in Figure 4c.*

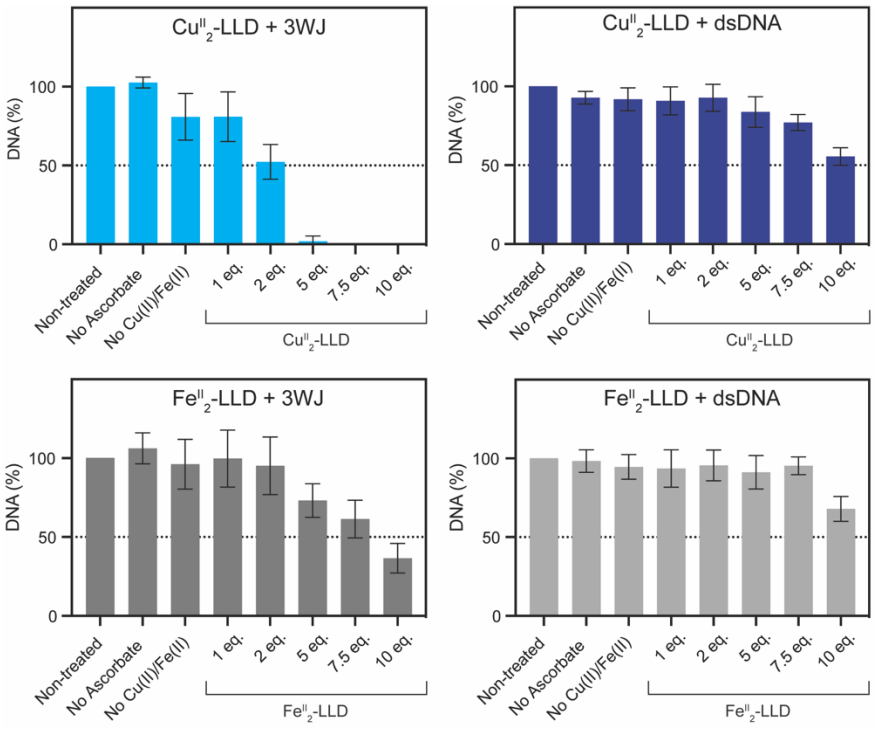


Figure S14. *Band densitometry analysis of cleavage inflicted by $\Delta\Delta$-Cu$^{II}_2$LLD and $\Lambda\Lambda$-Fe$^{II}_2$LLD on target 3WJ and off-target dsDNA. Densitometry was performed in triplicate, average DNA remaining, and standard deviation were calculated and plotted. This data is also shown in Figure 4d of the manuscript.*

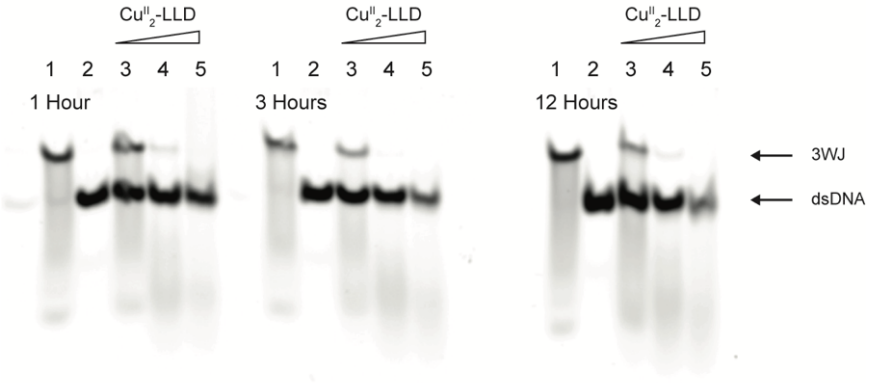


Figure S15. *PAGE analysis of cleavage profile at 1, 3 and 12 hours. Lane 1; Target 3WJ. Lane 2; off-target dsDNA. Lane 3-5; 3WJ and dsDNA with 1, 5 or 10 eq. $\Delta\Delta$-Cu$^{II}_2$LLD and Na-L-ascorbate ascorbate (10 eq. to $\Delta\Delta$-Cu$^{II}_2$LLD).*

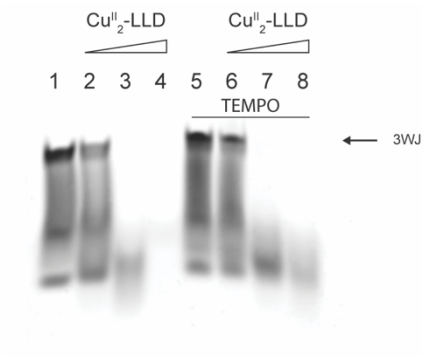


Figure S16. *PAGE analysis of cleavage profile of* ***ΔΔ-Cu$^{II}_2$LLD*** *in the presence of 4-hydroxy-TEMPO. Lane 1-4 3WJ incubated with 0, 1, 5 and 10 eq.* ***ΔΔ-Cu$^{II}_2$LLD*** *and Na-L-ascorbate. Lane 5-8 3WJ incubated with 0, 1, 5 and 10 eq.* ***ΔΔ-Cu$^{II}_2$LLD,*** *Na-L-ascorbate and 4-hydroxy-TEMPO, a diffusible superoxide scavenger.*

3.10. Cell-internalization studies

3.10.1. Cell-internalization of the peptide ligand TAMRA-LLD in Vero cells

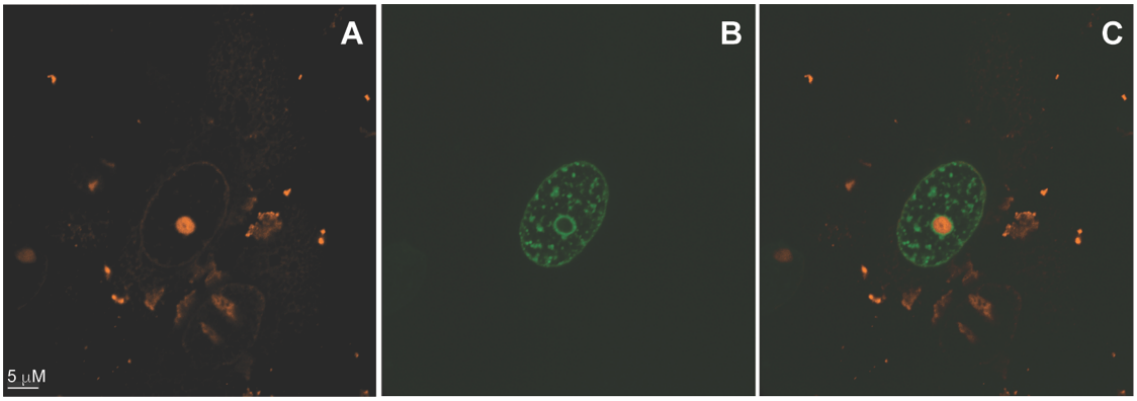


Figure S17. *Free peptide ligand* ***TAMRA-LLD*** *alone enters the cell nucleus but cannot recognize DNA replication sites. Vero cells expressing protein GFP-PCNAL2 were incubated in the presence of 25 μg mL^{-1} of Digitonin for 3 min and then in the presence of 5 μM of* ***TAMRA-LLD*** *for 15 min (these reported concentrations of digitonin, and peptide ligand refer to the final amount of these compounds in the cell culture transfer buffer after their addition from more concentrated stock solutions). A. Red channel emission showing the distribution of the rhodamine-labelled peptide ligand; B. green channel, corresponding to the emission of the GFP-PCNAL2 probe labelling the DNA replication foci; C. overlay of the green and red channels. Nucleoli are clearly labelled as big red spots inside the cell nuclei. The images show a representative confocal section of the Vero cells.*

3.10.2. Cell-internalization of the Cu^{II} peptide helicate in HeLa cells

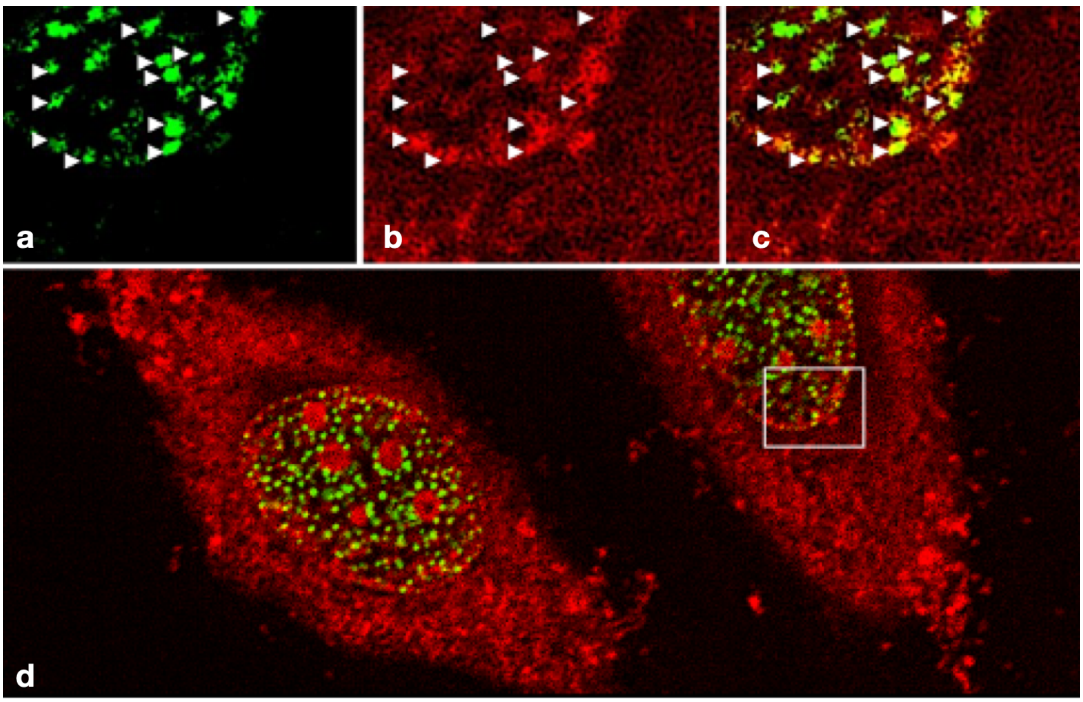


Figure S18. ΔΔ-Cu$^{II}_2$TAMRA-LLD *selectively stains DNA replication sites. HeLa cells expressing protein GFP-PCNAL2 were incubated in the presence of 25 μg mL^{-1} of digitonin for 3 min and then in the presence of 5 μM of* ***TAMRA-LLD*** *for 15 min (these reported concentrations of digitonin and helicate refer to the final amount of these compounds in the cell culture transfer buffer after their addition from more concentrated stock solutions). The Cu^{II} peptide helicate used in these studies was pre-formed in an independent Eppendorf vial just before the internalization experiment by adding 5 equivalents of $CuCl_2.2H_2O$ to an aqueous solution of* ***TAMRA-LLD*** *peptide ligand. a) Red channel emission showing the distribution of the rhodamine-labelled helicate; b) green channel, corresponding to the emission of the GFP-PCNAL2 probe labelling the DNA replication foci; c) overlay of the green and red channels of the square region shown in d). Arrows highlight some foci where the staining of GFP-PCNAL2 and* ***ΔΔ-Cu$^{II}_2$TAMRA-LLD*** *overlap. Nucleoli are clearly labelled as big red spots inside the cell nuclei. The images show a representative confocal section of the HeLa cells.*

3.11. Cytotoxicity studies

As can be seen from the data shown in Figure S19, at the concentrations and incubation time used in the cell viability assay (1.25, 2.5, 5.0 and 10.0 μM of helicate, and 20 hours of incubation), **ΔΔ-Cu$^{II}_2$LLD** presents considerable cytotoxicity. On the other hand, we have observed that at the incubation time used in the *in cellulo* nuclease studies reported in this work (2 hours), we were not able to detect the compound inside cells, which is the reason for using digitonin permeabilization. Although it might be interesting to determine the cause for the detected cytotoxicity, it is far beyond the scope of this study, as it might be related to the DNA damage caused by the Cu^{II} peptide helicate activity, or to any other still unknown effect on the integrity of the membrane, cell homeostasis, etc.

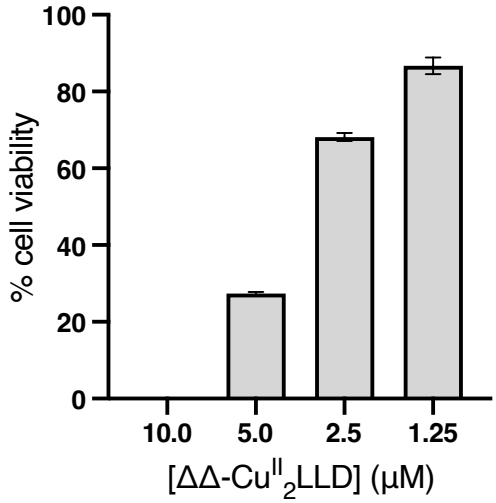


Figure S19. *Vero cells were plated at 4000 cells/well in a 96-well plate and incubated 24 hours in a humidified incubator at 37ºC, 5% CO_2. Then, different concentrations (10.0 μM, 5.0 μM, 2.5 μM and 1.25 μM) of* ***ΔΔ-Cu$^{II}_2$LLD*** *were added to the cells, that were incubated for additional 20 hours at 37ºC. 1% Triton X-100 was used as positive (killing) and medium as negative (100% viability) controls. After incubation, 10 μl/well of CCK-8 solution (Target Mol) was added and incubated for 2 hours at 37ºC, 5% CO_2. Absorbance at 450 nm was then measured in a microplate reader Tecan Infiniti 200 PRO and the percentage of cell viability was calculated by comparison with controls.*

4. References

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