Dioxygen Oxidation Cu(II)→Cu(III) in the Copper Complex of *cyclo*(Lys-DHis-*β*Ala-His): a Case Study by EXAFS and XANES Approach.

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Supporting Information

Experimental Part

***Fmoc-His(Trt)-OAl* (5)**

A solution of DCC (400 mg, 1.94 mmol) in allyl alcohol (1.0 mL, 14.7 mmol) was slowly added at 0 °C to a stirred suspension of Fmoc-His(Trt)-OH (**4**) (1.0 g, 1.6 mmol) in dry DCM (20 mL). The mixture was stirred at room temperature for 16 h, and the reaction monitored by TLC (DCM/MeOH 10:1; R_f = 0.90). The white precipitate was filtered off and the excess of DCC was eliminated with AcOH (0.5 mL). The filtered solution was concentrated to dryness, and the residue crystallized from EtOAc/hexane (780 mg, 74% yield). ^{1}H-NMR ($CDCl_3$, 200 MHz), δ (ppm): 2.86–3.16 (2 H, m, β-H_2), 4.20 (1 H, t, Fmoc 9-H), 4.3–4.7 (3 H, α-H and Fmoc 10-H_2), 4.65 (2 H, d, allyl O-CH_2), 5.20–5.34 (2 H, CH_2 = CH), 5.8–6.0 (2 H, NH and CH_2 = CH), 7.32 (4 H, m, Fmoc 2-H, 3-H, 6-H and 7-H), 7.57 (2 H, d, Fmoc 1-H and 8-H), 7.73 (2 H, d, Fmoc 4-H and 5-H).

***Fmoc-His-OAl* (6)**

The product (**5**) was treated with TFA (7 mL) in 20 mL of DCM at 0 °C and stirred for 2 h, monitoring deprotection by TLC (DCM/MeOH 10:1; R_f = 0.35). The solution was evaporated to dryness and at the residue was added water (2 mL) and lyophilized. The crude oil was treated with Et_2O to obtain a white powder (450 mg, 93% yield). ^{1}H-NMR ($CDCl_3$, 200 MHz), δ (ppm): 3.49-3.09 (2 H, m, β-H_2), 4.26 (2 H, m, Fmoc CH_2), 4.50 (1 H, His α-H), 4.58 (2 H, m, All 1-H_2), 5.29 (2 H, m, All 3-H), 5.79 (1 H, All 2-H), 6.34 (1 H, d, His NH), 6.97 (1 H, s, His 5-H), 7.71-7.19 (8 H, d, Fmoc Ar), 7.71 (2 H, m, Fmoc CH_2).

***Fmoc-His(trityl-resin)-OAl* (7)**

The chloro-trityl resin (2.57 g, 1.35 mmol/g) was dried under vacuum and then swollen with dry DCM. A solution of Fmoc-His-OAl (0.868 g, 0.41 mmol) and DIPEA (1.54 mL, 8.34 mmol) in dry DCM (20 mL) was added to the resin. After 2 h, the resin was washed with DMF (3 times × 2 min) and DCM (2 times × 2 min), and then endcapped with DCM/MeOH/DIPEA (17:2:1) (2 times × 2 min). After washing with DCM (2 times × 2 min), DMF (2 times × 2 min) and DCM (2 times × 2 min), the resin was dried under vacuum and the resin loading was determined from the Fmoc release, monitored by UV absorbance.

***Fmoc-Lys(Boc)-OAl* (9)**

Product (**9**) was prepared starting from Fmoc-Lys(Boc)-OH (**8**) (2.01 g, 4.29 mmol), following the same steps and ratios described for (**5**). The reaction was monitored by TLC (DCM/MeOH 10:1; R_f

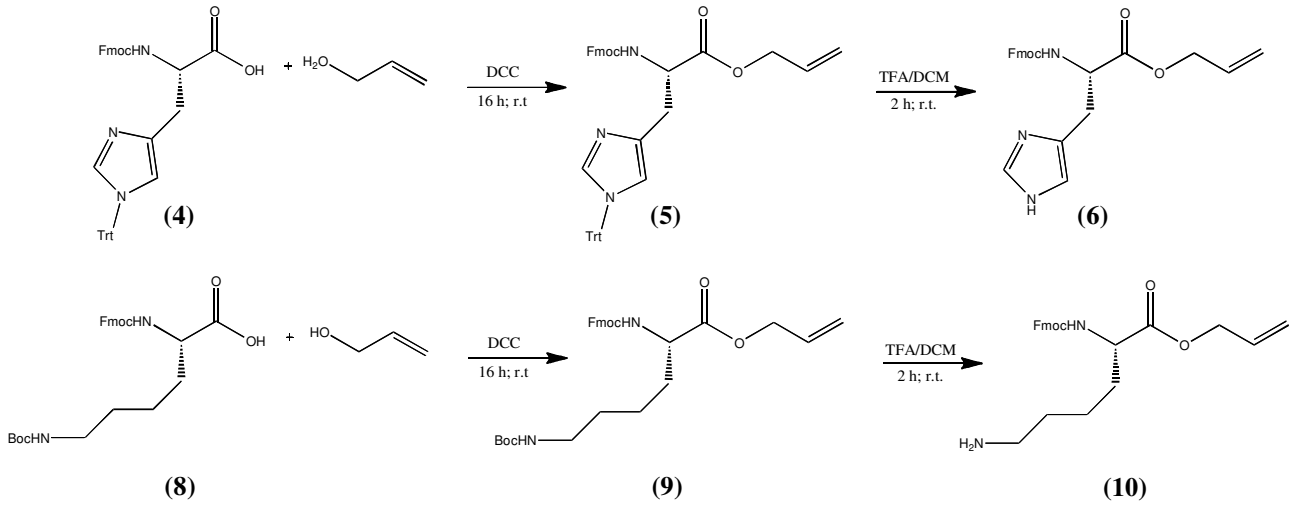
= 0.8), and the product (1.9 g, 87% yeld) analysed by ^{1}H-NMR ($CDCl_3$, 200 MHz), δ (ppm): 1.18-1.94 (15 H, m, 3×CH_2 e *t*Bu) 3.18-2.85 (2 H, m, CH_2NH) 4.40-4.0 (4 H, m, α-CH, 9-H Fmoc, CH_2 Fmoc) 4.62-4.43 (2 H, m, allyl O-CH_2), 5.14–5.30 (2 H, m, CH=CH_2), 5.94-5.70 (1 H, m, CH_2=CH), 6.70 (1 H, s br, α-NH) 7.30 (4 H, m, Fmoc 2-H, 3-H, 6-H e 7-H), 7.59 (2 H, d, J=7.4, Fmoc 1-H e 8-H), 7.69 (2 H, d, J=7.4, Fmoc 4-H e 5-H).

***Fmoc-Lys-OAl* (10)**

Compound (**10**) was obtained by Boc removal from Lys ester **9**, following the same steps and ratios described for derivative **6**. The complete Boc removal was monitored by TLC (DCM/MeOH 10:1; R_f = 0.59), and the final product (1.506 g, 96% yield) analysed by ^{1}H-NMR ($CDCl_3$, 200 MHz), δ (ppm): 1.18-1.94 (6 H, m, 3×CH_2) 3.14-2.81 (2 H, m, CH_2NH) 4.40-4.0 (4 H, m, α-CH, 9-H Fmoc, CH_2 Fmoc) 4.62-4.43 (2 H, m, allil O-CH_2), 5.14–5.30 (2 H, m, CH=CH_2), 5.94-5.70 (1 H, m, CH_2=CH), 6.70 (1 H, s br, 1α-NH), 7.30 (4 H, m, Fmoc 2-H, 3-H, 6-H e 7-H), 7.59 (2 H, d, J=7.4, Fmoc 1-H e 8- H), 7.69 (2 H, d, J=7.4, Fmoc 4-H e 5-H).

***Fmoc-Lys(trityl-resin)-OAl* (11)**

The building-block was prepared as described for **7**, and the resin loading was determined from the Fmoc release, monitored by UV absorbance.



Scheme 1S. Synthesis of allyl esters.

General synthesis for cyclopeptides:

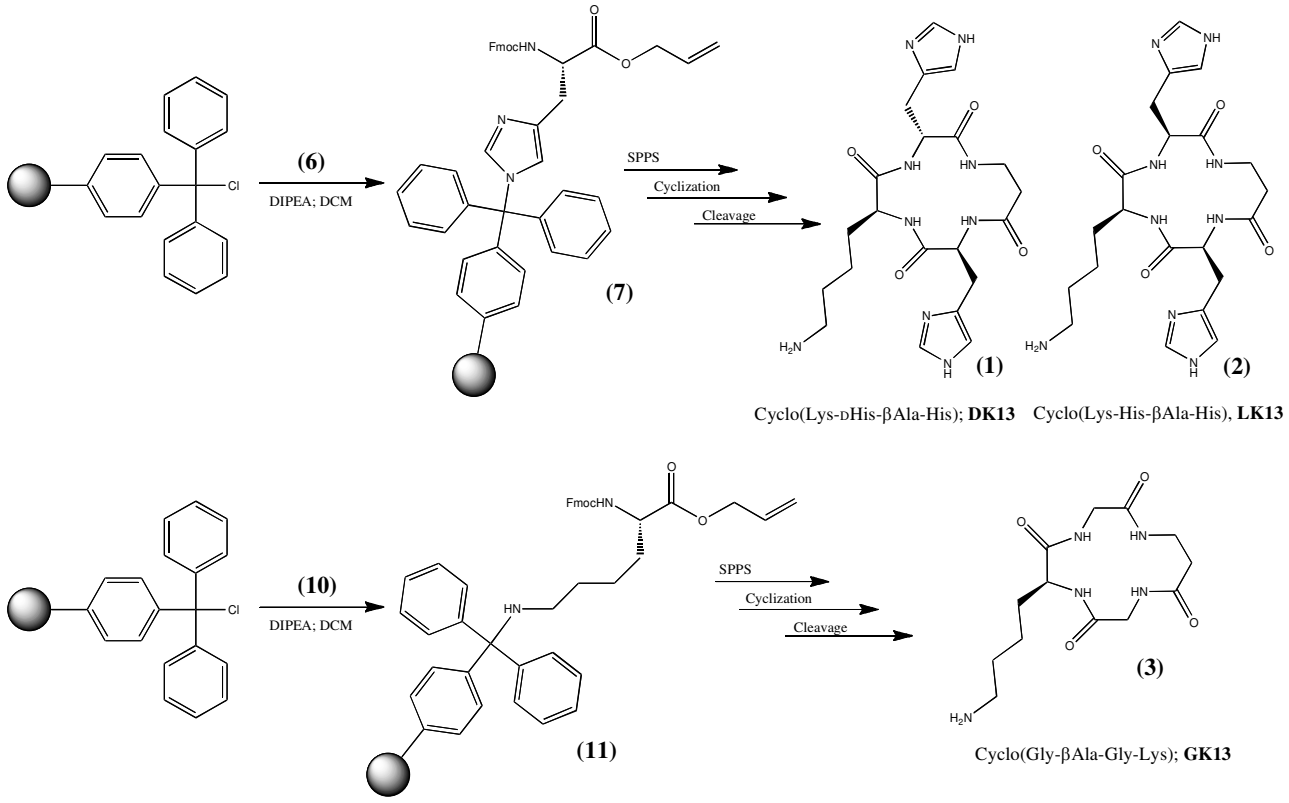
Precursor tetrapeptides were synthesized on a semi automatic synthesizer (MultiSyn Tech - Germany) following the SPPS method and using the orthogonal Fmoc/Trt/allyl protection scheme. The synthesis started from Fmoc-His(trityl-resin)-OAl (**7**) (500 mg, 0.46 mmol/g) for **DK13** (**1**) and **LK13** (**2**), and from Fmoc-Lys(trityl-resin)-OAl (**11**) for **GK13** (**3**). Deprotections of amino acids were performed by 20% piperidine in DMF (2 × 5 mL) for 15 min. The amino acids residues were introduced according to the TBTU/HOBt/NMM method with formation of active esters. The activated Fmoc-aminoacids were introduced in this order: β-Ala, D-His, Lys for **DK13**; β-Ala, L-His, Lys for **LK13**; and Gly, β-Ala Gly for **GK13**. The coupling reactions were performed by using excess of the amino acids (2.5 eq), HOBt and TBTU (5 eq) and of NMM (10 eq) in DMF, under vortexing for 40 min. After each coupling, the resin was washed with DMF (3 times × 5 mL) and DCM (2 times × 5 mL). Coupling reactions were controlled by the Kaiser test.[1]

After chain elongation, the allyl protecting group must be removed. The peptide-resin was dried under vacuum and swollen in dry DCM (2 times × 20 min) under Ar. The resin was shaken for 5 min with a solution of $PhSiH_3$ (24 eq) in dry DCM under Ar, and then a solution of $Pd(PPh_3)_4$ (0.25 eq) in dry DCM was added. After 40 min the resin was washed with dry DCM for 5 min. The treatment with $PhSiH_3$/$Pd(PPh_3)_4$ was repeated once again. The resin was washed with DCM for 5 min, a solution of 0.5% sodium diethyldithiocarbamate in DMF (2 times × 5 min), DMF (3 times × 2 min), and DCM (3 times × 2 min). The Fmoc group was then removed with 20% piperidine in DMF (2 times × 10 min) and the resin washed with DMF (5 times × 2 min).

To obtain the cyclopeptides, the on-resin cyclization was performed in pseudo-dilution conditions, vortexing at r.t. for 2 h with a solution of coupling reagents TBTU/DIPEA (1:2 equiv) in DMF (5 mL). After cyclization, the resin was washed with DMF (3 times × 2 min), and DCM (2 times × 2 min). The reactions were checked by the Kaiser test.

Peptide cleavage from the resin and deprotection of the amino-acids side chains were carried out with TFA/H_2O/TIS cleavage cocktail (95:2.5:2:5, 2.5h). The resin was washed with TFA and the filtrate partially evaporated under N_2 flow. The crude product was precipitated with diethyl ether, collected by centrifugation, dissolved in H_2O and lyophilized. The peptide was then pre-purified by solid phase extraction (SPE) with silica C_{18}-E Strata™ (Phenomenex) and using H_2O/CH_3CN as eluent. The purification of the peptide was performed by semi-preparative RP-HPLC on a Varian Aqua C_{18} column (5 µm, 250 mm × 10 mm; eluents: A 0.1% TFA in H_2O; B 0.1% TFA in CH_3CN;

flow 4 mL/min; gradient: 0% to 20% of B in 30 min.). The products (60 mg, 65 mg, 58 mg) were obtained with overall yields of 15%, 16% and 14% respectively, based on the resin loading.



Scheme 2S. Schematic synthesis of cyclic peptides.

Characterization of the products by analytical HPLC, ESI-MS and NMR spectroscopy.

Analytical HPLCs were performed on a Waters instrument (2996 Alliance) using a Phenomenex Jupiter column C_{18} 300Å (150 × 2.6 mm). The solvent systems used were: A (0.1% TFA in H_2O) and B (0.1 % TFA in CH_3CN); gradient: 3% to 20% of B in 5 min at 600 µl/min.
Mass spectra were registered on the ESI LCQ Advantage mass spectrometer (ThermoFinnigan).

***c*(His-βAla-ᴅHis-Lys) (DK13) (1)**

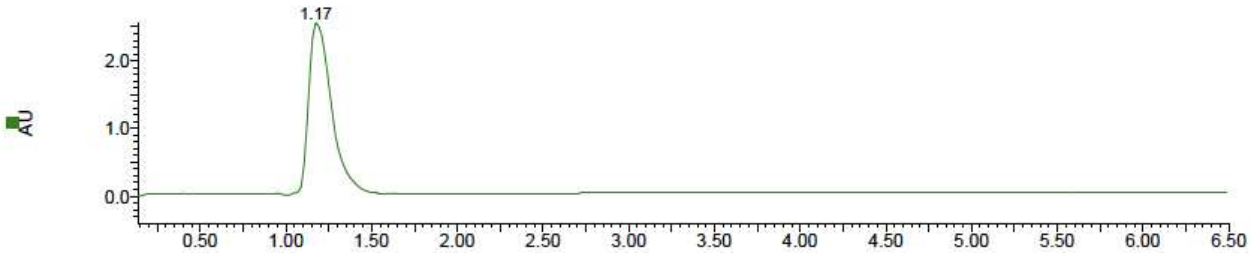


Figure 1S. HPLC chromatogram

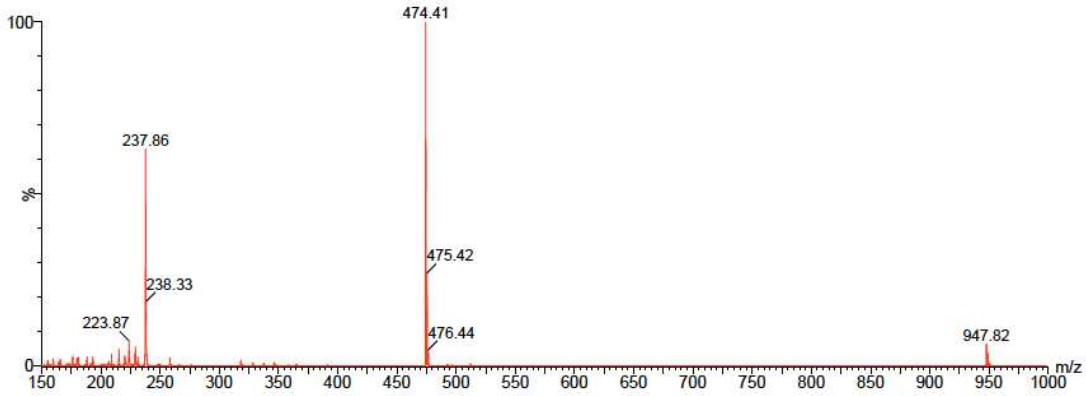


Figure 2S. ESI-MS spectra

^{1}H NMR (DMSO/D_2O, 300 MHz), δ(ppm): 8.43-8.35 (d, 2H), 7.11 (s, 1H), 7.04 (s, 1H), 4.50-4.35 (m, 2H), 3.95-3.90 (t, 1H), 3.21-3.06 (m, 4H), 2.92-2.79 (m, 4H), 2.35-2.29 (m, 2H), 1.54-1.41 (m, 4H), 1.14-0.95 (m, 2H).

***c*(His-βAla-LHis-Lys) (LK13) (2)**

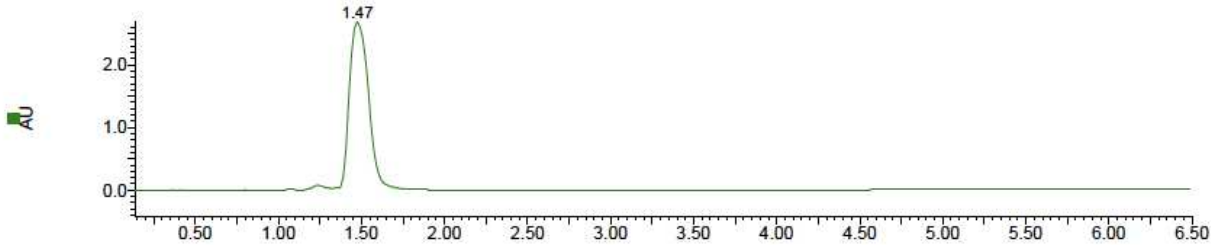


Figure 3S. HPLC chromatogram

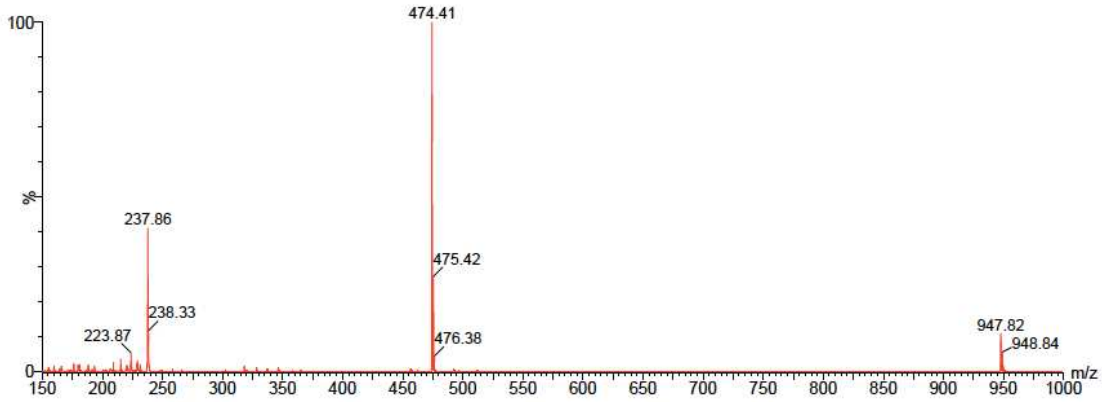


Figure 4S. ESI-MS spectra

^{1}H NMR (DMSO/D_2O, 300 MHz), δ(ppm): 8.94-8.92 (d, 2H), 7.39 (s, 1H), 7.21 (s, 1H), 4.49-4.39 (m, 2H), 4.07-4.02 (t, 1H), 3.13-3.00 (m, 4H), 2.90-2.68 (m, 4H), 2.47-2.17 (m, 2H), 1.53-1.38 (m, 4H), 1.23-0.97 (m, 2H).

***c*(Gly-βAla-Gly-Lys) (GK13) (3)**

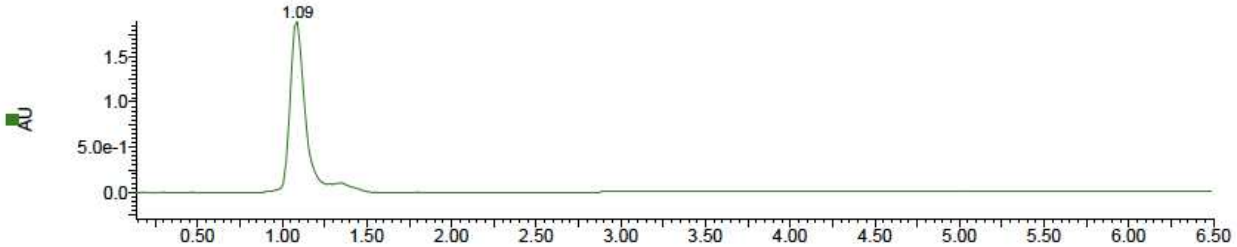


Figure 5S. HPLC chromatogram

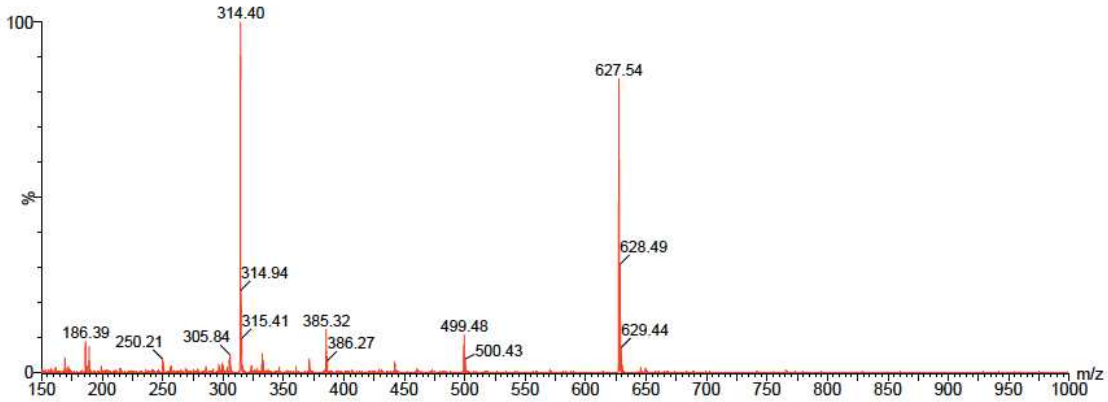


Figure 6S. ESI-MS spectra

^{1}H NMR (DMSO/D_2O, 300 MHz), δ(ppm): 4.39-4.35 (t, 1H), 4.10-4.07 (m, 4H), 3.35-2.99 (m, 2H), 2.99-2.96 (m, 2H), 2.39-2.32 (m, 2H), 1.74-1.31 (m, 6H).

Synthesis of Copper/Cyclopeptide complexes

The purified cyclopeptide (obtained as trifluoroacetate salt), DK13·3TFA (815.22 g/mol; 18.7 mg) was dissolved in 0.5 mL of water (MilliQ grade). In order to avoid the presence of free copper(II), the complex was prepared with DK13/Cu ratio of 1:0,8 respectively, so 2.94 mg of anhydrous $CuSO_4$ were added to the solution and the pH was adjusted to 12,0 with diluted NaOH. The resulting solution was lyophilized and the solid obtained stored under argon. The same methods and quantities were used also for LK13·3TFA (815.22 g/mol; 18.7 mg). Instead, for GK13·1TFA (427.17 g/mol; 18.7 mg) were used 1.54 mg of anhydrous $CuSO_4$, in order to maintain the same cyclopeptide/Copper ratio.

The preparation of the samples for the measurements was done in to a glove box under argon atmosphere, being the samples very hygroscopic and deliquescent because of the presence of small particles of solid NaOH. The solid samples were then milled in a mortar and applied in a thin layer between two Kapton films.

Synthesis of Sodium Ditelluratocuprate(III)

The synthesis of the internal standard compound Ditelluratocuprate(III) $Na_5[Cu\{TeO_4(OH)_2\}_2]\cdot 16H_2O$ (DTC) was performed in accordance with the method reported by Sen Gupta et al.[2]

Copper sulphate (0.78 g) was added to a mixture of telluric acid (1.43 g), potassium persulfate (2.11 g), and potassium hydroxide (4.0 g) in water (40 mL). The mixture was heated until the solution was an intense red. The boiling was continued for another 30 min to ensure the complete removal of persulfate. The ditelluratecuprate(III) was crystallized by the solution, cooled at r.t. and added of 10

mL of saturated $NaNO_3$ solution. Almost immediately deep red crystals started appearing and crystallisation is complete (ca. 12 h) when the supernatant liquid is colourless. The crystals were filtered and washed several times with demineralised water and dried under vacuum.
The final product was characterized by X-Ray structure analysis, and the results are in complete agreement with those reported in literature.[3,4]

Theoretical XANES calculations.

XAS calculations are carried out starting from the Fermi's golden rule to obtain the photoabsorption cross section within the dipole approximation. One important step is to calculate the final state of the photoelectron transition moment, which describes the physical process of scattering the electron photoemitted by the central atom by outer atoms. This is done in the framework of MS theory[5] using a MT approximation for the shape of the potential of the cluster of atoms included. The cluster size and the l_{max} value (i.e. the maximum l value of the spherical harmonic expansion of the scattering path operators) were chosen on the basis of a convergence criterion; MT radii were chosen on the basis of the Norman criterion[6] including an overlap between MT spheres that is optimized by fitting the *ovlp* parameter (only one parameters for the entire set of MT radii). The choice of MT radii fixes the constant MT potential V_0 and the charge densities within each MT sphere. Different values of *ovlp*, within reasonable values (between 1% and 10%), change the goodness of the fit but without relevant effects in the structural parameters determination.
V_{0imp} is the value of the Muffin-Tin potential (V_0) when it is refined to best fit the XANES spectra of copper compounds. The potential generator of MXAN (program VGEN) calculates this parameter automatically, however refining V_{0imp} provides an improvement both in □2 and thus in the accuracy of structural results. The value of (10 ± 2) eV was found to be optimal for the system under study.
The real part of the exchange term was calculated using the Hedin-Lundqvist energy-dependent potential, while all of the inelastic losses were taken into account by a phenomenological method previously described in detail.[7] According to this method, inelastic processes are taken into account by a convolution with a broadening function, with a width Γ given by $\Gamma = \Gamma_c + \Gamma(E)$. The constant part Γ_c includes contributions from the core-hole lifetime (1.5 eV, lorentzian convolution) and the experimental resolution (0.6 - 0.8 eV, gaussian convolution), while the energy dependent term Γ(E) (lorentzian convolution) represents the inelastic processes. Γ(E) is zero below an onset energy E_s, and begins to increase from a value A_s following the universal functional form related to the mean free path in a solid.[8] This method introduces three nonstructural parameters that are derived during the fit on the basis of a Monte Carlo search at each step of computation.

A constant experimental error of 0.012 normalized units (i.e. units for which the absorption jump is equal to 1) was chosen.

Starting model structure used for the calculations has been built according to DFT calculations. Preliminary calculations of theoretical spectra have been performed using Hedin-Lundquist real potential, calculating Muffin-Tin radii according to the Norman criterion, and allowing 10% sphere overlap. The minimization of the $\Box^2$ function was performed in the space of structural parameters**,** i.e. the first (4 nitrogens) and second (8 carbons) coordination shell.

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