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**Supplementary Information**

# Synthesis of the copper chelator TGTA and evaluation of its ability to protect biomolecules from copper induced degradation during copper catalyzed azide-alkyne bioconjugation reactions

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**Experimental section**

Reagents and solvents were purchased from commercial sources. Reactions solvents were dried and distilled prior to use when necessary. All reactions containing moisture- and/or air sensitive reagents were carried out under an argon atmosphere. TLC was performed on aluminium sheets pre-coated with silica gel 60 F254 (Merck). Flash chromatography was carried out on silica gel 60 (0.040 – 0.060 mm). Spots were visualized by UV followed by charring with 1:5 $H_2SO_4$:MeOH and heating. Mass spectra were obtained with a Bruker Ultraflex III mass spectrometer operated in positive mode. NMR was recorded with Bruker Avance spectrometers operating at 600 MHz ($^{1}$H: 600 MHz, $^{13}$C: 150 MHz) or 500 MHz ($^{1}$H: 500 MHz, $^{13}$C: 125 MHz). The probe temperature was kept at 22 °C unless otherwise stated. Compound **4** was analyzed by $^{1}$H NMR spectroscopy and compound **2** was fully characterized by use of 1D $^{1}$H and $^{13}$C NMR spectroscopy in combination with DQF-COSY, ROESY, HSQC, TOCSY and HMBC 2D NMR techniques with pulse sequences provided by the instrument manufacturer. Chemical shifts are expressed on the $\delta$-scale (in ppm) using TMS (tetramethylsilane), residual chloroform, $H_2O$ or methanol as internal standards. Coupling constants are given in Hz and provided only once when first encountered. Coupling patterns are given as s, singlet, d, doublet, t, triplet etc. The computational analysis of the $^{1}$H NMR spectra of both compounds was performed by the use of the PERCH NMR software with starting values and spectral parameters obtained from the various NMR techniques used.

**Synthesis of TGTA.**

**TGTA (2)**. To a solution containing 43 mg of **3** (0.15 mmol, 5 equiv.) and 4.3 µl tripropargylamine (0.03 mmol, 1 equiv.) in 2 ml of DMF:$H_2O$ (3:1) was added 2.4 mg $CuSO_4$ (0.015 mmol, 0.5 equiv.) and 6.4 mg sodium L-ascorbate (0.03 mmol, 1 equiv.). The resulting mixture was stirred at RT for 40 h (during this time a white solid precipitated from the reaction mixture). After 40 h, the reaction mixture was diluted with 20 ml EtOAc transferred to a separatory funnel and washed with 5 ml $NH_4Cl$-solution (prepared by dissolving a saturated $NH_4Cl$-solution with equal amount of water 1:1 v/v) and 15 ml brine. The organic phase was dried over $Na_2SO_4$, filtered and concentrated to give the crude product. The crude product was purified by column chromatography (EtOAc $\rightarrow$ EtOAc:MeOH 3:1) to give **4** as a colorless oil (30 mg, quant.). TLC: $R_f$ = 0.22 (EtOAc). $^{1}$H NMR (600 MHz, $CDCl_3$, 25°C): δ = 8.56 (s, 3 H, triazole-C*H*), 5.48 (d, 3 H, $J_{1,2}$ = 5.0 Hz, H-1), 4.67 (dd, 3 H, $J_{6a,5}$ = 3.1, $J_{6a,6b}$ = –14.1 Hz, H-6a), 4.65 (dd, 3 H, $J_{3,2}$ = 2.5, $J_{3,4}$ = 8.1 Hz, H-3), 4.58 (dd, 3 H, $J_{6b,5}$ = 9.0 Hz, H-

6b), 4.41 and 4.33 (each d, each 3 H, $J_{NCH2a,NCH2b}$ = –14.1 Hz, N(C*H*$_2$)$_3$), 4.32 (dd, 3 H, H-2), 4.25 (dd, 3 H, $J_{4,5}$ = 1.4 Hz, H-4), 4.17 (ddd, 3 H, H-5), 1.50, 1.39, 1.37 and 1.25 (each s, each 9 H, $O_2C(CH_3)_2$) ppm. MALDI-TOF: calcd. for $C_{45}H_{66}N_{10}O_{15}Na$ [M+Na]$^+$ 1009.46; found 1009.40.

33 mg of **4** (0.034 mmol) was dissolved in 3 ml 60% TFA (in $H_2O$) and stirred at 50 °C for 1.5 hours. The reaction mixture was then diluted with water, concentrated and dried under vacuum to give **2** as a white solid (25 mg, quant., α:β = 2:3).

Data for the β-anomer:

$^1$H NMR (500 MHz, $D_2O$): δ = 8.29 (s, 3 H, triazole-C*H*), 4.74 (dd, 3 H, $J_{6a,5}$ = 3.6, $J_{6a,6b}$ = – 14.4 Hz, H-6a), 4.68 (dd, 3 H, $J_{6b,5}$ = 8.9 Hz, H-6b), 4.56 (s, 6 H, N(C*H*$_2$)$_3$), 4.48 (d, 3 H, $J_{1,2}$ = 8.0 Hz, H-1), 4.11 (ddd, 3 H, $J_{5,4}$ = 0.8 Hz, H-5), 3.96 (dd, 3 H, $J_{4,3}$ = 3.5 Hz, H-4), 3.64 (dd, 3 H, $J_{3,2}$ = 9.9 Hz, H-3) and 3.50 (dd, 3 H, H-2) ppm.

$^{13}$C NMR (125 MHz, $D_2O$): δ = 136.9 (triazole-*C*), 129.6 (triazole-*C*H), 97.4 (C-1), 74.0 (C-5), 73.5 (C-3), 72.6 (C-2), 69.9 (C-4), 52.0 (C-6) and 47.7 (N(*C*H$_2$)$_3$) ppm.

Data for the α-anomer:

$^1$H NMR (500 MHz, $D_2O$): δ = 8.29 (s, 3 H, triazole-C*H*), 5.19 (d, 3 H, $J_{1,2}$ = 3.9 Hz, H-1), 4.71 (dd, 3 H, $J_{6a,5}$ = 4.1 Hz, $J_{6a,6b}$ = – 14.4 Hz, H-6a), 4.66 (dd, 3 H, $J_{6b,5}$ = 9.4 Hz), 4.56 (s, 6 H, N(C*H*$_2$)$_3$), 4.47 (ddd, 3 H, $J_{5,4}$ = 1.0 Hz, H-5), 4.02 (dd, 3 H, $J_{4,3}$ = 3.3 Hz, H-4), 3.85 (dd, 3 H, $J_{3,2}$ = 10.3 Hz, H-3), and 3.80 (dd, 3 H, H-2) ppm.

$^{13}$C NMR (125 MHz, $D_2O$): δ = 136.9 (triazole-*C*), 129.4 (triazole-*C*H), 93.2 (C-1), 70.4 (C-4), 69.9 (C-3), 69.7 (C-5), 69.0 (C-2), 52.1 (C-6) and 47.7 (N(*C*H$_2$)$_3$) ppm.

MALDI-TOF: calcd. for $C_{27}H_{42}N_{10}O_{15}Na$ [M+Na]$^+$ 769.27; found 769.23.

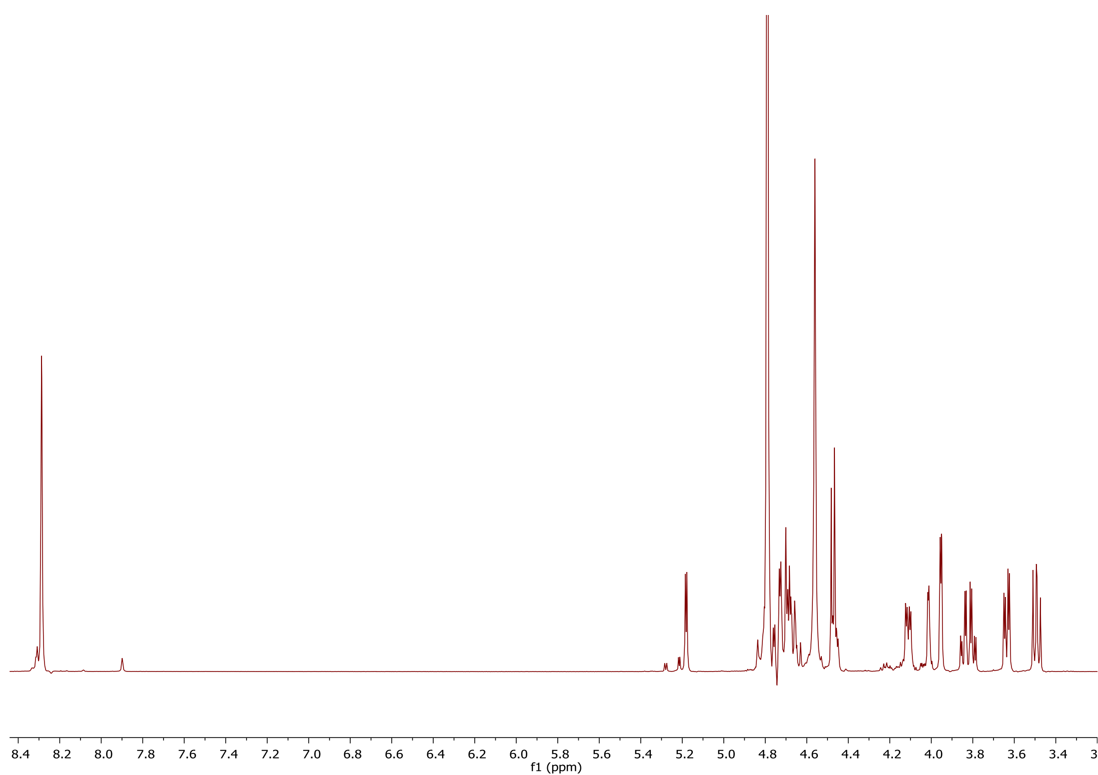


Figure 1. $^{1}H$ NMR spectrum of **2** (500 MHz, 22 deg., $D_2O$).

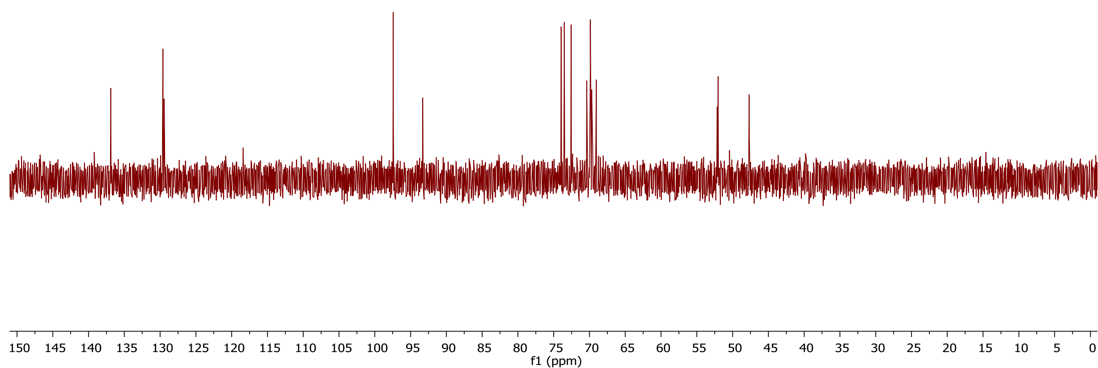


Figure 2. $^{13}C$ NMR spectrum of **2** (500 MHz, 22 deg., $D_2O$).

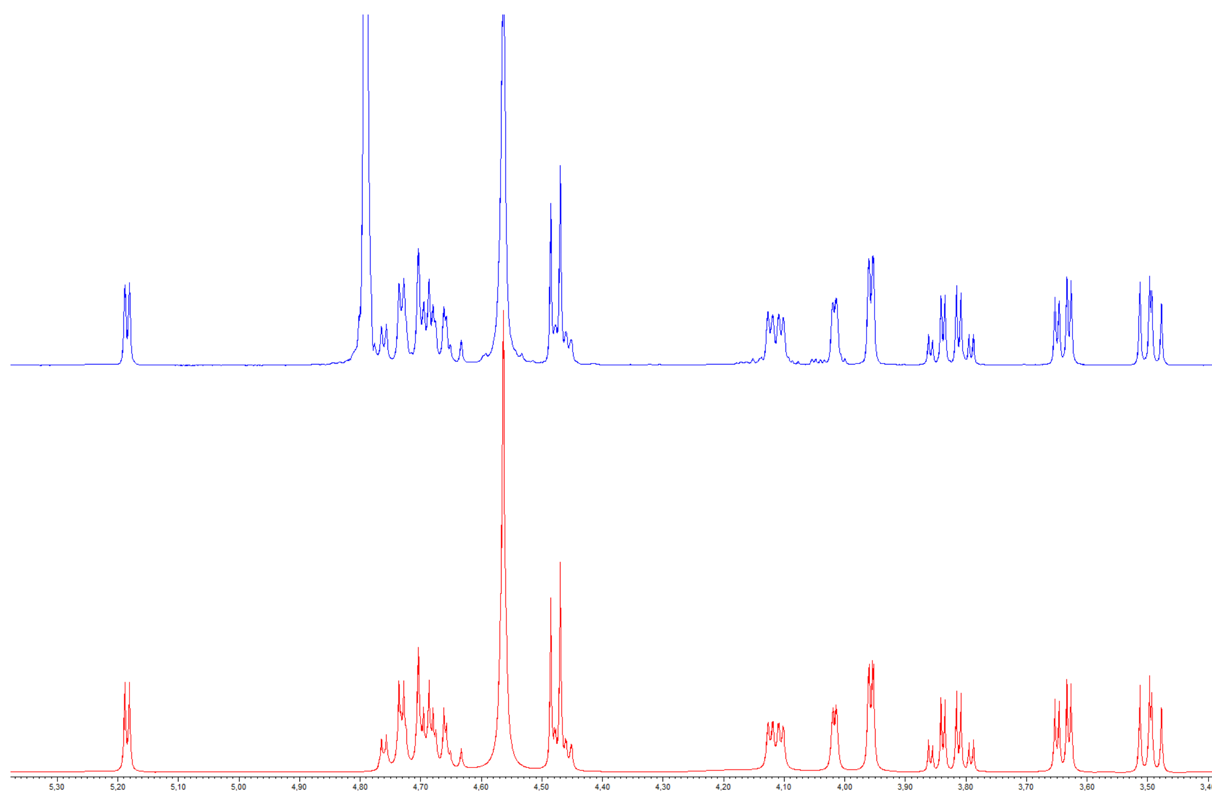


Figure 3. Spectral simulations of the $^1$H NMR spectrum of **2** (5.40-3.40 ppm region) with the PERCH-software. Top: measured spectrum (furanose signals partially removed). Bottom: simulated spectrum of the major components (furanose signals and water peak neglected).

**Synthesis of fibroblast growth factor conjugates.**

50µg (3 nmol) of recombinant human basic fibroblast growth factor (Peprotech; FGF) in 25mM sodium phosphate buffer pH 7.3 (80 µl) was incubated with 4 molar excess (12 nmol) of NHS-$PEG_4$-$N_3$ (Life Technologies; delivered in 3 µl dimethylsulphoxide (DMSO)) for 3 h at RT. Non-reacted NHS-$PEG_4$-$N_3$ was removed by Amicon centrifugal filter unit (Millipore; 10K cut-off). According to MALDI-TOF mass spectrometric analysis, on average 5.1 $PEG_4$-$N_3$ units (mass increment 273 Da) were attached to one FGF molecule (average molecular weights: FGF intact 17239 Da; product 18621 Da).

**CuAAC bioconjugation reactions.**

FGF-galactose conjugates were synthesized by a CuAAC reaction. Three parallel reactions were performed in order to compare the performance of the copper(I) ion chelators TGTA and TBTA to the control sample ($H_2O$): TGTA (A), TBTA (B) and $H_2O$ (C). Reactions A, B and C were incubated at room temperature for 5 hours, and the reactions were stopped and the FGF-galactose conjugates were purified by Amicon centrifugal filter units (10K).

(A) To the solution of 0.59 nmol of FGF-$PEG_4$-$N_3$ in 25 mM sodium phosphate buffer pH 7.3 (22 µl) and 15 molar excess of 6-*O*-propargyl-D-galactose in $H_2O$ (0.5 µl), 3 molar excess of $CuSO_4$ in $H_2O$ (0.5 µl), 15 molar excess of sodium ascorbate in $H_2O$ (0.5 µl), 7.5x molar excess of TGTA in $H_2O$ (0.5 µl) and 1 µl of 0.5 M sodium phosphate buffer pH 7.3 were added. Total volume of the reaction was 25 µl.

(B) To the solution of 0.59 nmol of FGF-$PEG_4$-$N_3$ in 25 mM sodium phosphate buffer pH 7.3 (22 µl) and 15 molar excess of 6-*O*-propargyl-D-galactose in $H_2O$ (0.5 µl), 3 molar excess of $CuSO_4$ in $H_2O$ (0.5 µl), 15 molar excess of sodium ascorbate in $H_2O$ (0.5 µl), 7.5x molar excess of TBTA in DMSO (0.5µl) and 1µl of 0.5 M sodium phosphate buffer pH 7.3 were added. Total volume of the reaction was 25µl.

(C) To the solution of 0.59 nmol of FGF-$PEG_4$-$N_3$ in 25 mM sodium phosphate buffer pH 7.3 (22 µl) and 15 molar excess of 6-*O*-propargyl-D-galactose in $H_2O$ (0.5 µl), 3 molar excess of $CuSO_4$ in $H_2O$ (0.5 µl), 15 molar excess of sodium ascorbate in $H_2O$ (0.5 µl), 0.5 µl of $H_2O$ and 1µl of 0.5M sodium phosphate buffer pH 7.3 were added. Total volume of the reaction was 25µl.

MALDI-TOF MS analysis of the reaction products were carried out as follows: Aliquots corresponding to 50 pmol of protein were purified using ZipTip C18 pipette tips (Millipore) according to manufacturer's instructions. In brief, tips were preconditioned by acetonitrile and equilibrated with 0.1 % aqueous trifluoroacetic acid (TFA), followed by sample adsorption. Purified protein was eluted with 5 µl of 50% acetonitrile in 0.1 % aqueous TFA, and for MS analysis 1 µl of eluate was mixed with sinapic acid matrix prepared as saturated solution in 50% acetonitrile in 0.1 % aqueous TFA. Mass spectra were collected in the linear positive-ion mode. The spectra obtained are shown in Figure 2B-D (in the paper). The reaction containing TGTA gave a protein product with average mass of 19668 Da, implying that on average 4.8 propargylgalactose units (mass increment 218 Da) were conjugated to $N_3$-$PEG_4$ FGF. In marked contrast, no clear protein product was obtained with TBTA or without chelator.

SDS-PAGE analysis of the reaction products was performed as follows: 10 µl of 5 x Laemmli sample buffer was added to all three Amicon-purified reactions (A, B and C, 40 µl each) and the samples were boiled for 5 min. A FGF-$PEG_4$-$N_3$ sample (µg) was prepared similarly. The samples were run on 4-15 % gradient SDS-PAGE gel (MiniProtean TGX gels, BioRad) using Spectra Broad Range Protein Ladder (Fermentas) as a molecular weight standard. The gel was stained with Imperial Protein Stain (Thermo Scientific) according to manufacturer's instructions. In the stained SDS-PAGE gel the FGF-

$PEG_4$-$N_3$ appeared as 18.4 kDa size band. The FGF-galactose conjugate from Sample A appeared as 19.1 kDa size band, indicating that the reaction had proceeded as expected. No smaller size bands were detected in these two samples, thus no degradation products were detectable in Sample A. No protein bands were detected in sample C, showing that the protein was degraded in a CuAAC reaction performed without a chelator molecule.