Supplementary Information for

Zn^{2+} triazamacrocyclic chelators with methylpyridine pendants arms for B-cells apoptosis: a structure-activity study

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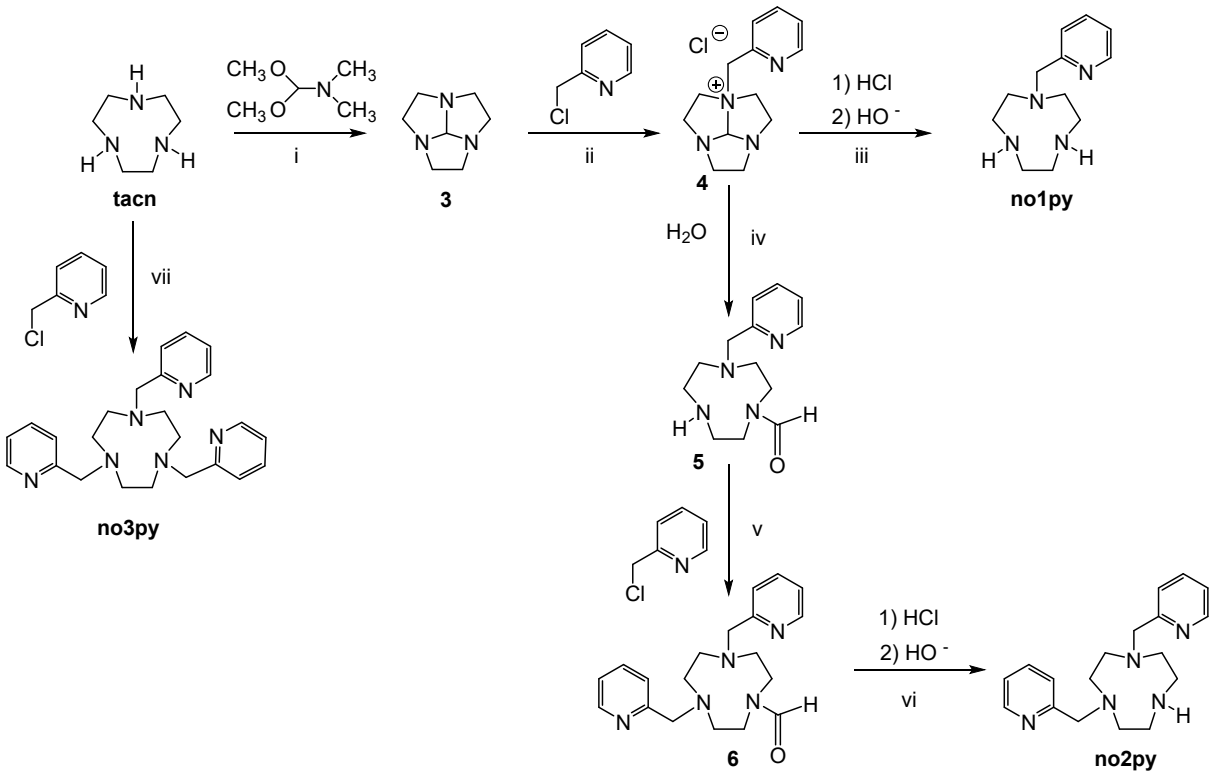
1. Chemical synthesis and characterization

1.1. Material and Methods

1,4,7-triazacyclononane was purchased from CheMatech (Dijon, France) and the reagents from Fisher, ACROS Organics and from Aldrich Chemical Co and used without further purification. All solvents were dried and distilled prior to use according to standard methods. Analytic HPLC was performed on a Prominence Shimadzu HPLC/LCMS-2020 equipped with a UV SPD-20 A detector. The chromatographic system employs HPLC (Vision HT C18 HL 5 µ 250× 4.6 mm) with H_2O and CH_3CN as eluents at a flow rate of 1 mL/min and UV detection at 254 and 350 nm. NMR spectra were recorded at the "Service commun" of the University of Brest. ^{1}H and ^{13}C NMR spectra were recorded using Bruker Avance 400 (400 MHz), or Bruker AMX-3 300 (300 MHz) spectrometers at 298K. High-resolution mass spectra were performed on a Bruker maXis mass spectrometer by the SALSA platform from ICOA laboratory (Orléans, France).

1.2. Synthesis procedure



Scheme S1 Chelators synthesis i) $CHCl_3$/toluene (2/8), rt, 12h; ii) THF, rt, 7 d; iii) 12M HCl/CH_3OH (1/1), reflux, 12h; NaOH (pH 12), CH_2Cl_2; iv) H_2O, 50°C, 12h; v) K_2CO_3, CH_3CN, rt, 3 d; vi) 12M HCl/CH_3OH (1/1), reflux, 12h; NaOH (pH 12), CH_2Cl_2; vii) K_2CO_3, CH_3CN, rt, 5 d.

1.2.1. 1-methylpyridinyl-1,4,7-triazacyclononane (no1py)

Dimethoxymethyl-*N*,*N*-dimethylamine (1.1g, 9.28 mmol) was added to a solution of tacn (1.2 g, 9.28 mmol) in a mixture of $CHCl_3$/toluene 2/8 v/v (10 mL). The reaction mixture was stirred at room temperature for 12 h, and the solvent was then evaporated under reduced pressure yielding **3** as a clear oil in quantitative yield (1.28 g, 99%). The protected tacn **3** (1.10 g, 7.90 mmol) was dissolved in THF (5 mL) and a solution of 2-(chloromethyl)pyridine (1.10 g, 8.69 mmol, 1.1 equivalent) in THF (5 mL) was added. The reaction mixture was stirred at room temperature for 7 days. The precipitate was filtered to yield **4** as a yellow powder (1.87 g, 89 %). The ammonium salt **4** (1.87 g, 7.02 mmol) was dissolved in 12M HCl/MeOH 1/1 v/v (20 mL) and stirred at reflux for 12 hours. After evaporation of the solvent, the crude product was dissolved in water and the pH of the aqueous solution was adjusted to pH > 12 with NaOH pellets. The aqueous solution was extracted with CH_2Cl_2 (3 × 30 mL), the combined organic layers were dried over $MgSO_4$, and evaporated to yield **no1py** as an orange oil (1.58 g, 99 %). ^{1}H NMR (300 MHz, D_2O, 298 K) δ (ppm) : 8.71 (d, 1H, J = 6 Hz, CH_{ar}) ; 8.54 (t, 1H, J = 9 Hz, CH_{ar}) ; 8.04 (d, 1H, J = 6 Hz , CH_{ar}) ; 7.96 (m, 1H, CH_{ar}); 4.25 (s, 2H, CH_2 py); 3.64 (m, 4H, CH_2 tacn); 3.34 (m, 4H, CH_2 tacn); 3.06 (m, 4H, CH_2 tacn). ^{13}C NMR (75 MHz, D_2O, 298 K) δ (ppm): 152.8 (C) ; 150.3, 144.6, 131.1, 129.4 (CH_{ar}) ; 58.2 (CH_2 py) ; 50.8, 46.5, 45.1 (CH_2 tacn). Retention time: 7.472 min (purity peak area: ≥95%).

1.2.2. 1,4-bis(methylpyridinyl)-1,4,7-triazacyclononane (no2py)

Compound **4** (1.44 g, 6.22 mmol) was dissolved in 20 mL of water and stirred at 50°C overnight to give compound **5** in quantitative yield as yellow oil after evaporation of water. ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) (presence of 2 rotamers) δ (ppm) : 8.48 (d, 2H, ^{3}J = 3 Hz, CH_{ar}); 7.97 (s, 1H, CHO) ; 7.75-7.85 (m, 2H, CH_{ar}); 7.34-7.41 (m, 4H, CH_{ar}); 3.96 (s, 2H, CH_2); 3.83 (s, 2H, CH_2); 3.65-3.76 (m, 4H, CH_2 tacn); 3.49-3.57 (m, 6H, CH_2 tacn); 3.23-3.27 (m, 4H, CH_2 tacn); 2.83-3.17 (m, 14H, CH_2 tacn). ^{13}C NMR (75 MHz, $CDCl_3$, 298 K) (presence of 2 rotamers) δ (ppm): 169.9 (C=O), 168.9, 160.4 (C); 151.2, 141.2, 141.1, 127.0, 126.9, 126.2 (CH_{ar}) ; 62.7, 62.4 (CH_2) ; 53.9, 53.7, 52.7, 51.8, 50.3, 49.2, 47.4, 47.0, 46.5, 46.0, 45.6, 44.2 (CH_2 tacn). Compound **5** (1.5 g ; 6.04 mmol) and K_2CO_3 (3.3 g ; 24.16 mmol) were dissolved in CH_3CN (37 mL). 2- (chloromethyl)pyridine (847 mg ; 6.64 mmol) was added and the reaction mixture was stirred at room temperature for 3 days. After filtration over celite, the filtrate was dried under reduced pressure. The obtained oil was purified through silica column chromatography (CH_2Cl_2 to CH_2Cl_2/MeOH 90/10). Compound **6** (1.3 g, 63%) was isolated as brown oil. ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) (presence of 2 rotamers) δ (ppm) :

8.58 (d, 2H, ^{3}J = 3 Hz, CHar) ; 8.13 (s, 1H, CHO) ; 7.66-7.77 (m, 2H, CH_{ar}) ; 7.42-7.50 (m, 2H, CH_{ar}) ; 7.20-7.26 (m, 2H, CH_{ar}) ; 3.92 (s, 4H, CH_2) ; 3.54-3.56 (m, 2H, CH_2 tacn) ; 3.26-3.41 (m, 4H, CH_2 tacn) ; 3.01-3.13 (m, 2H, CH_2 tacn) ; 2.56-2.70 (m, 2H, CH_2 tacn) ; 2.79-2.92 (m, 2H, CH_2 tacn). ^{13}C NMR (75 MHz, $CDCl_3$, 298 K) δ (ppm): 162.8 (C=O) ; 159.2, 158.9 (C) ; 148.4, 148.2, 121.3, 135.8, 135.6, 122.3, 122.1, 121.3 (CH_{ar}) ; 63.1, 62.4 (CH_2) ; 57.3, 53.8, 46.5, 53.6, 52.6, 49.7 (CH_2 tacn).

Compound **6** (1.30 g; 3.83 mmol) was dissolved in 40 mL of 12 M HCl/MeOH (1/1, v/v). The reaction mixture was refluxed overnight. After evaporation of the solvent, the residue was taken in H_2O and NaOH pellets were added at 0°C until pH > 12. Then, the product was extracted twice with $CHCl_3$, organic fraction was dried with $MgSO_4$ and dried under reduced pressure. **no2py** was obtained as orange oil without purification (1.01 g, 84 %). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ (ppm): 8.49 (d, 2H, ^{3}J = 6 Hz, CH_{ar}) 7.55-7.61 (m, 2H, CH_{ar}) 7.44 (d, 2H, ^{3}J = 9 Hz, CH_{ar}) 7.09-7.24 (m, 2H, CH_{ar}) 4.18 (s, 1H, NH) 3.85 (s, 4H, CH_2) 2.73- 2.78 (m, 4H, CH_2 tacn) 2.66-2.71 (m, 4H, CH_2 tacn) 2.64 (s, 4H, CH_2 tacn). ^{13}C NMR (75 MHz, $CDCl_3$, 298 K) δ (ppm): 160.0 (C) 148.9, 136.4, 123.1, 121.9 (CH_{ar}); 63.1 (CH_2) 52.8, 51.7, 46.5 (CH_2 tacn). Retention time: 14.441 min (purity peak area: ≥95%).

1.2.3. 1,4,7-tris(pyridin-2-ylmethyl)-1,4,7-triazonane (no3py)

A solution of 2-(chloromethyl)pyridine (1.629 g; 12.77 mmol; 3.3 equiv.) in acetonitrile (6 mL) was added to a solution of **tacn** (500 mg; 3.87 mmol) in acetonitrile (30 mL) with K_2CO_3 (2.14 g ; 15.48 mmol ; 4 equiv.). The reaction mixture was stirred at room temperature for 5 days. K_2CO_3 was removed by filtration and the filtrate was dried under reduced pressure. The red residue was purified via neutral alumina column chromatography (CH_2Cl_2 to CH_2Cl_2/CH_3OH 98/2). **no3py** was obtained as brown oil (91.3 mg ; 59 %). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ (ppm): 8.35-8.34 (d, 3H, CHar) 7.53-7.49 (m, 3H, CHar) 7.44-7.42 (d, 3H, CHar) 7.09-7.06 (m, 3H, CHar) 3.99 (s, 6H, CH_2N) 2.96 (s, 12H, CH_2 tacn). ^{13}C NMR (75 MHz, $CDCl_3$, 298 K) δ (ppm): 160.2 (C) 148.9, 136.3, 123.3, 121.9 (CH_{ar}); 64.6 (CH_2) 55.9 (CH_2 tacn). Analytical HPLC: Retention time: 16.9 min (purity peak area: ≥95%).

1.3. ^{1}H and ^{13}C NMR spectra

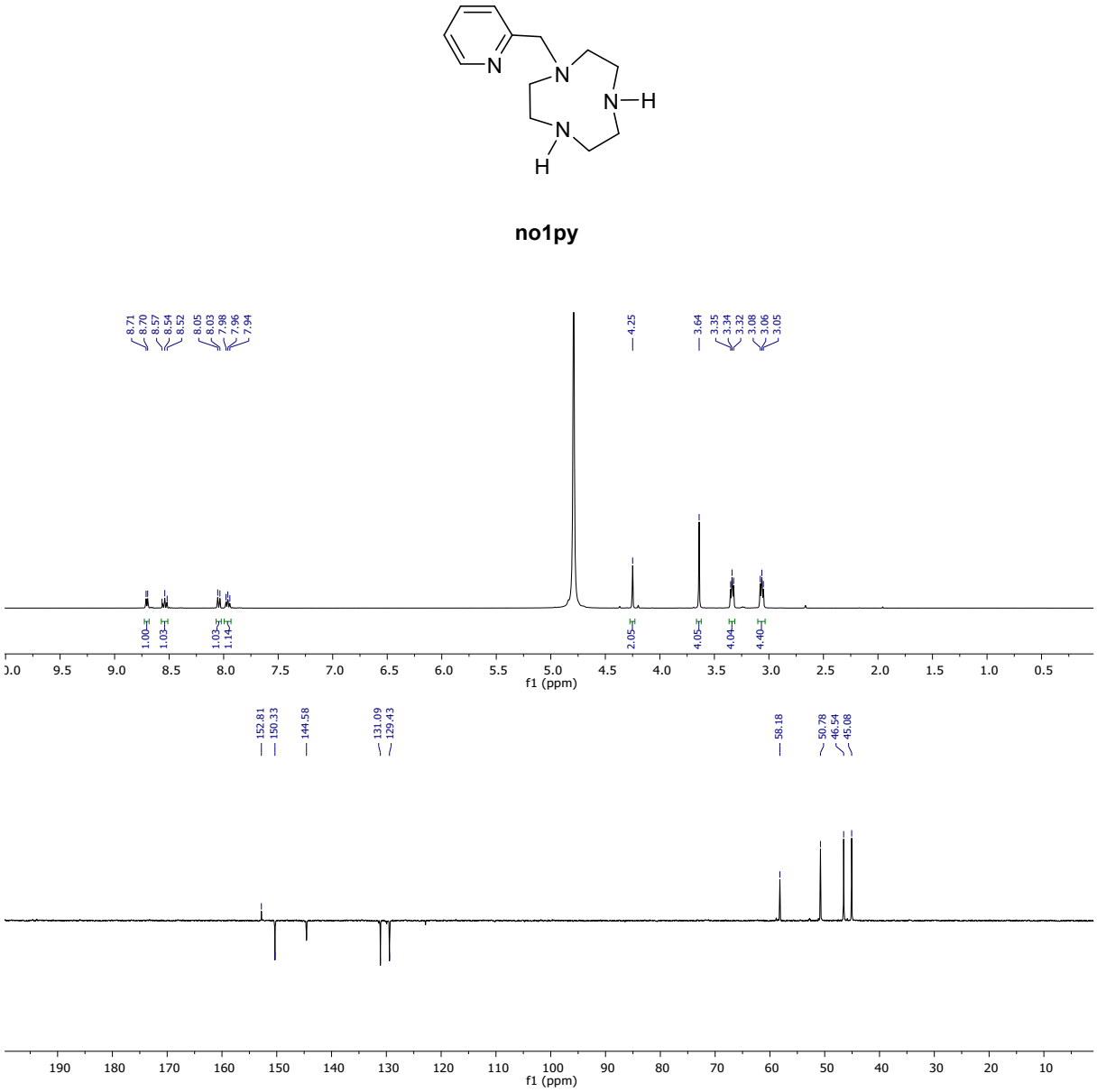


Figure S1. ^{1}H (300 MHz, 298 K, $CDCl_3$) and ^{13}C (75 MHz, 298 K, $CDCl_3$) NMR spectra of **no1py**

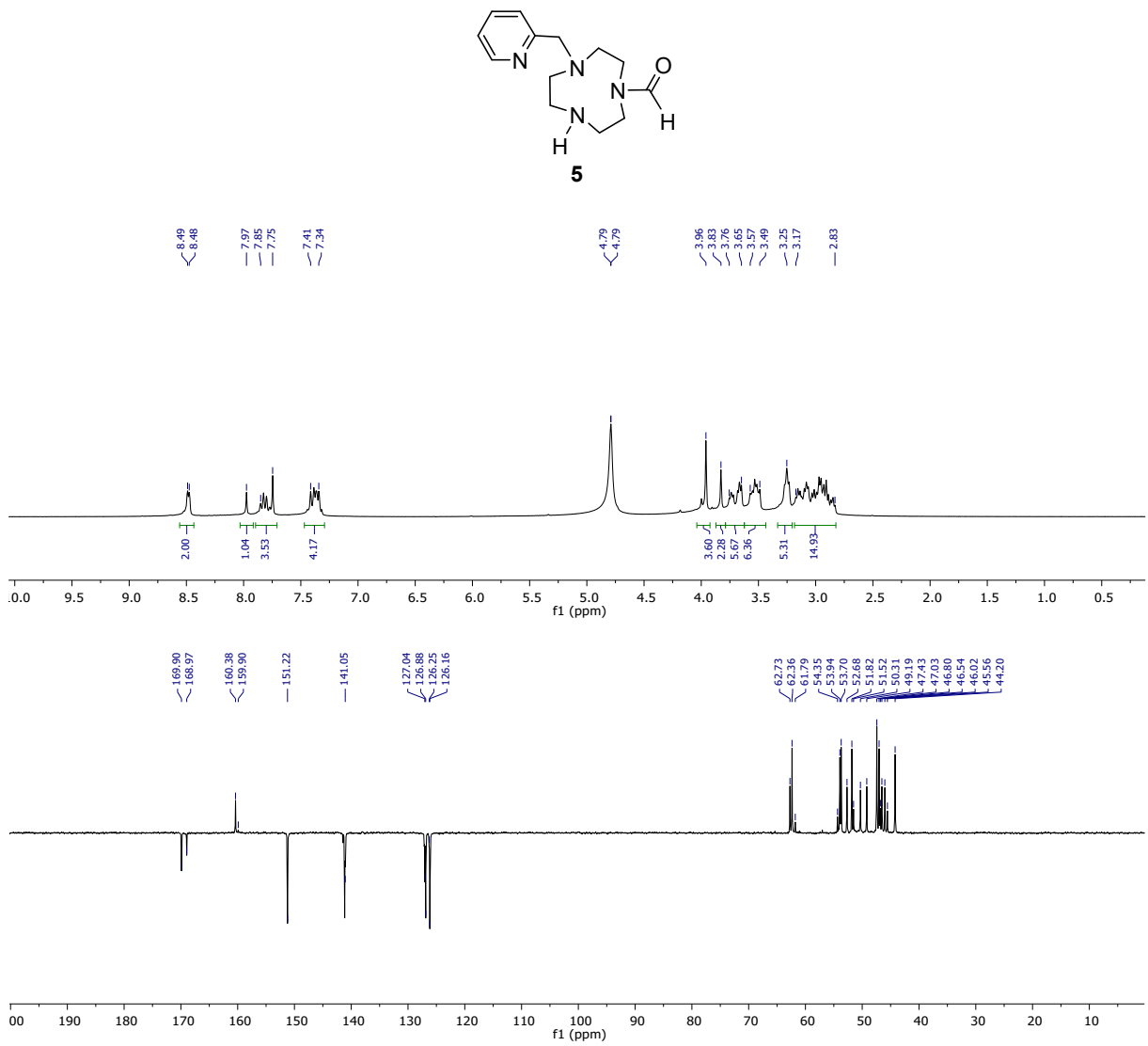


Figure S2. ^{1}H (300 MHz, 298 K, $CDCl_3$) and ^{13}C (75 MHz, 298 K, $CDCl_3$) NMR spectra of compound **(5)**

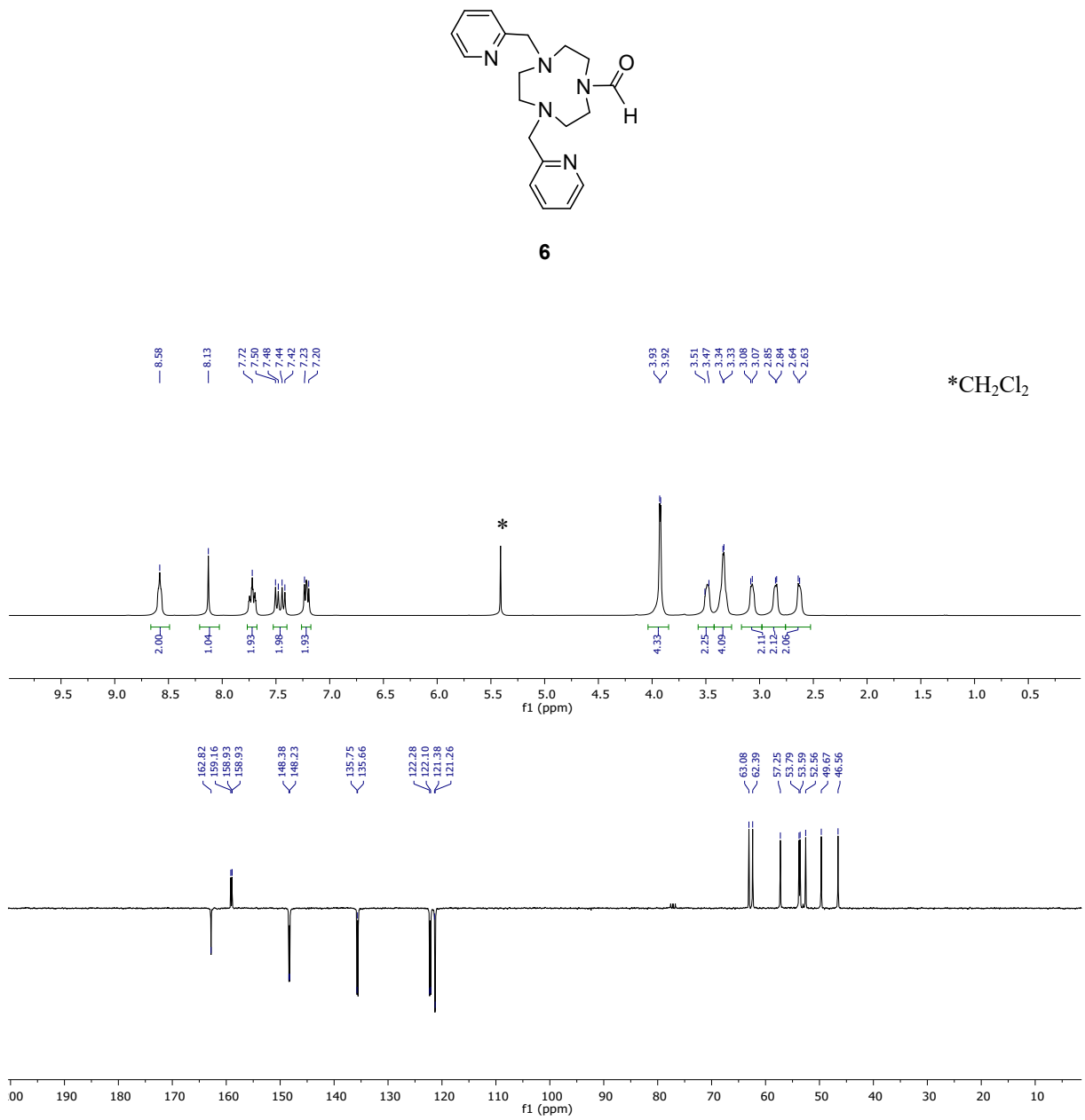


Figure S3. ^{1}H (300 MHz, 298 K, $CDCl_3$) and ^{13}C (75 MHz, 298 K, $CDCl_3$) NMR spectra of compound **(6)**

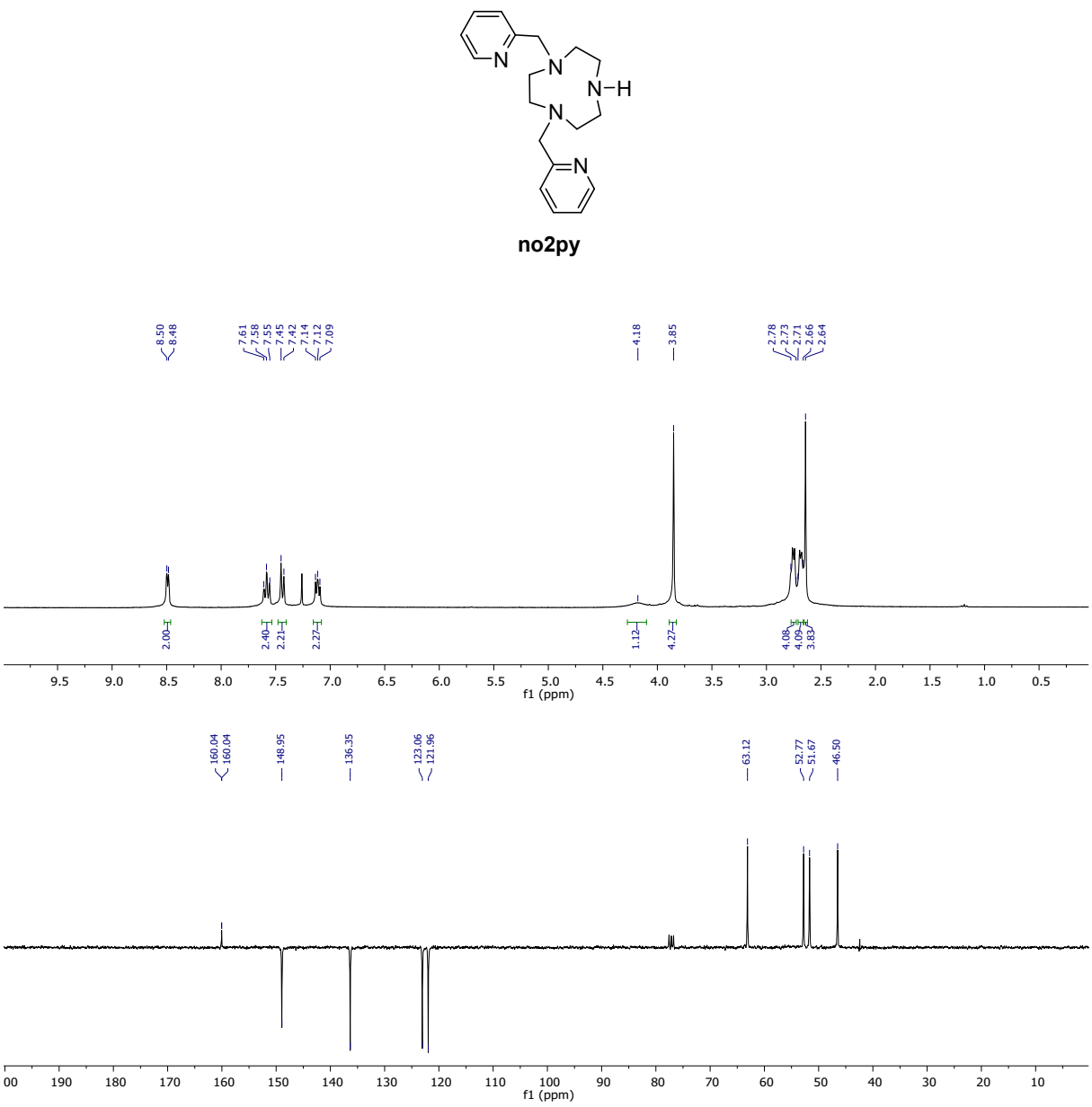


Figure S4. ^{1}H (300 MHz, 298 K, $CDCl_3$) and ^{13}C (75 MHz, 298 K, $CDCl_3$) NMR spectra of **no2py**

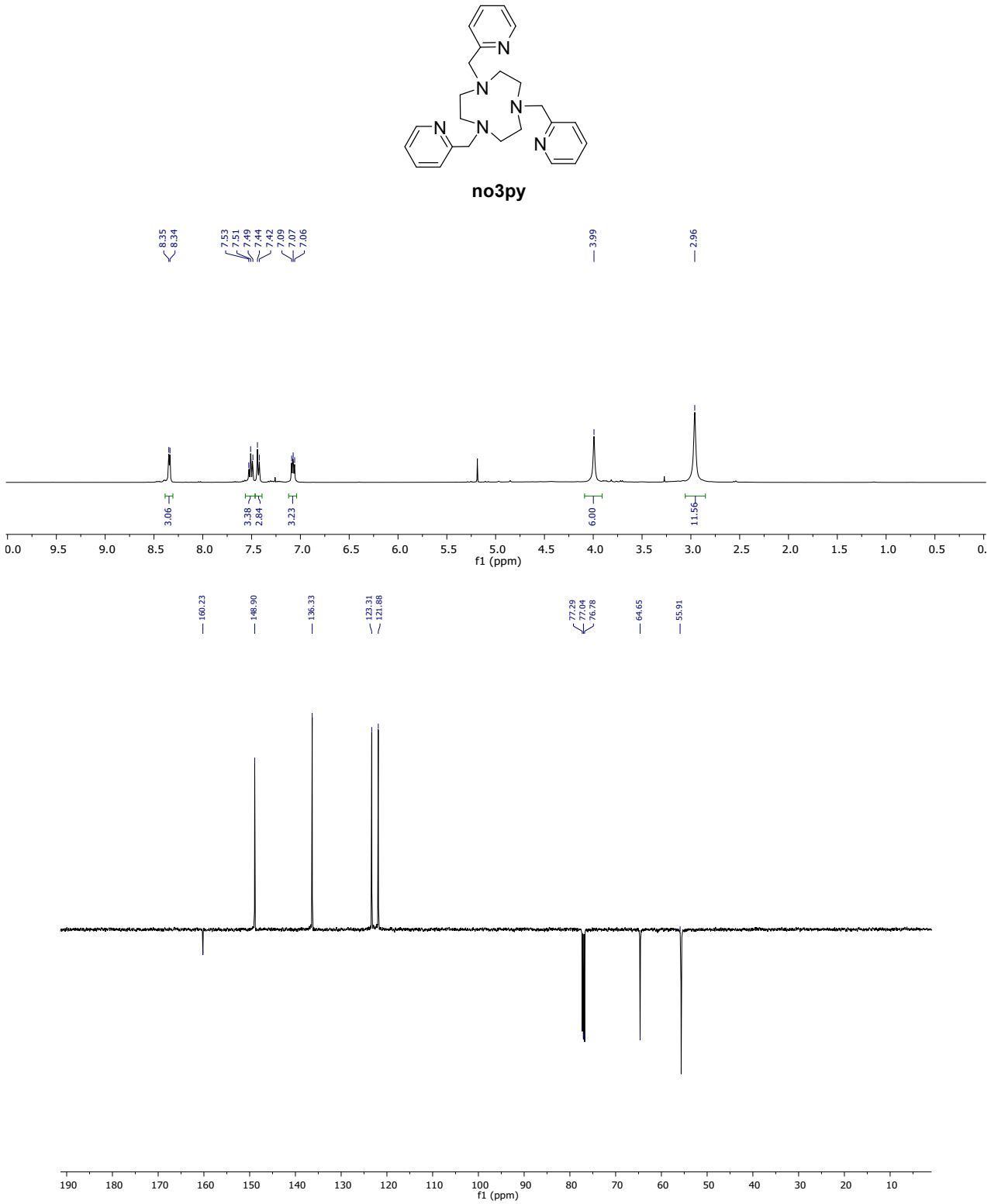


Figure S5. ^{1}H (300 MHz, 298 K, $CDCl_3$) and ^{13}C (75 MHz, 298 K, $CDCl_3$) NMR spectra of **no3py**

1.4. HPLC traces

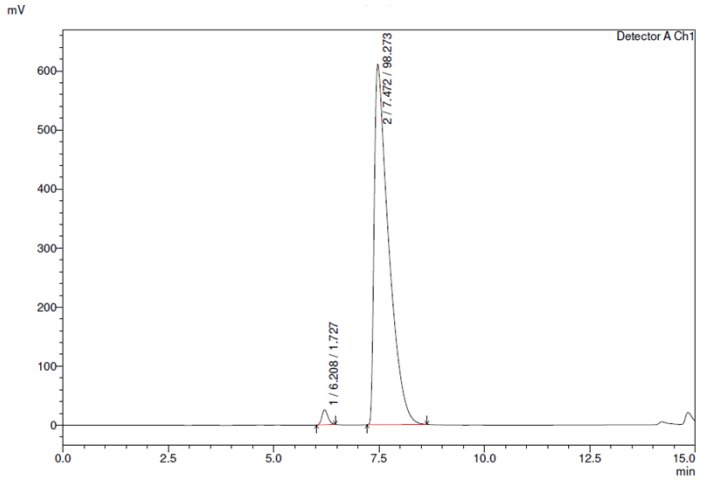


Figure S6. Analytical HPLC of **no1py** Column: Vision HT C18 HL 5 µ 250× 4.6 mm. Gradient: 95% CH_3CN 0-6 min, 5→90% CH_3CN 6-16 min, 90% CH_3CN 16-21 min, 90→5% CH_3CN 21-27 min. Flow: 1mL/min. Retention time: 7.472 min (purity peak area: ≥95%).

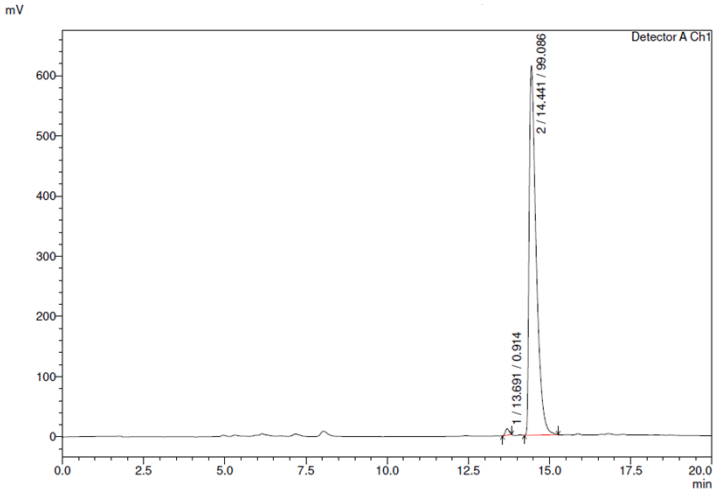


Figure S7. Analytical HPLC of **no2py** Column: Vision HT C18 HL 5 µ 250× 4.6 mm. Gradient: 95% CH_3CN 0-6 min, 5→90% CH_3CN 6-16 min, 90% CH_3CN 16-21 min, 90→5% CH_3CN 21-27 min. Flow: 1mL/min. Retention time: 14.441 min (purity peak area: ≥95%).

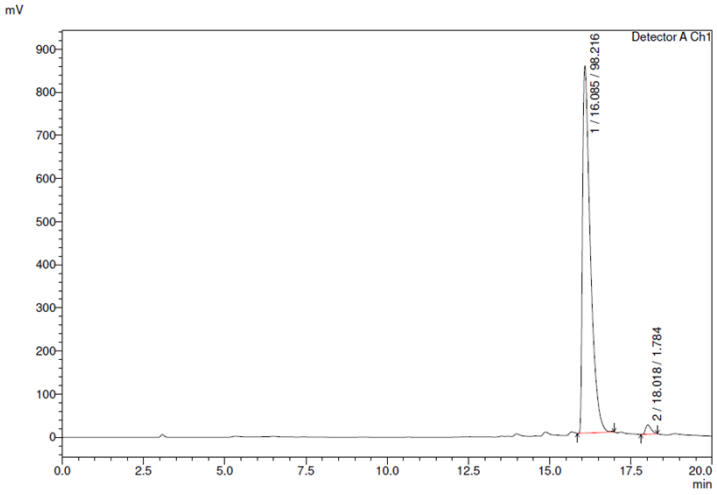


Figure S8. Analytical HPLC of **no3py** Column: Vision HT C18 HL 5 µ 250× 4.6 mm. Gradient: 95% CH_3CN 0-6 min, 5→90% CH_3CN 6-16 min, 90% CH_3OH 16-21 min, 90→5% CH_3CN 21-27 min. Flow: 1mL/min. Retention time: 16.085 min (purity peak area: ≥95%).

2. Zn^{2+} complexation monitored by ^1H NMR

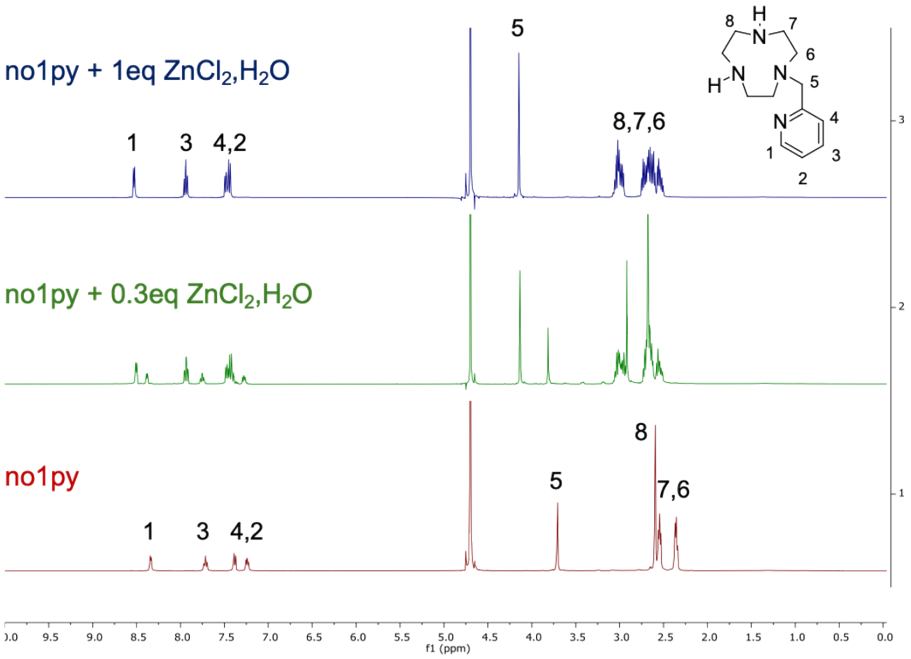


Figure **S9**. Zn^{2+} complexation with no1py monitored by ^1H NMR (400 MHz, 298 K, D_2O)

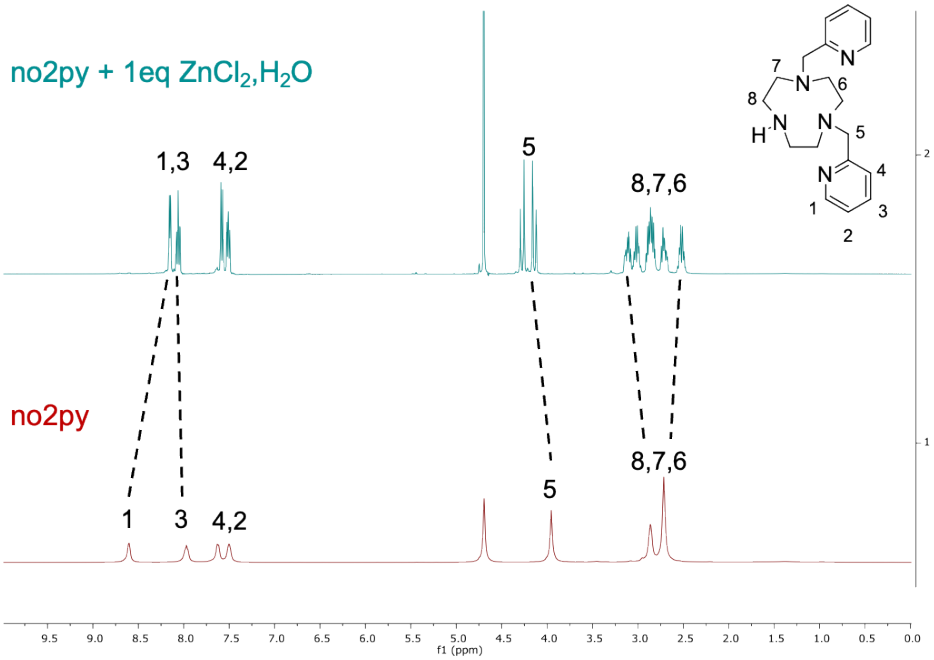


Figure **S10**. Zn^{2+} complexation with no2py monitored by ^1H NMR (400 MHz, 298 K, D_2O/CD_3CN 2/1)

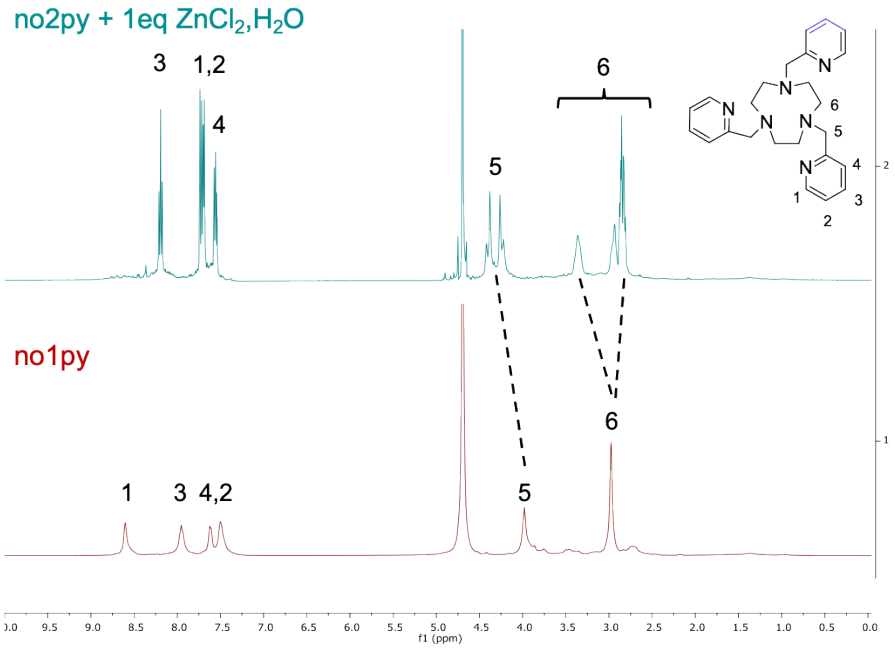


Figure S11. Zn^{2+} complexation with **no3py** monitored by ^{1}H NMR (400 MHz, 298 K, D_2O/CD_3CN 2/1)

3. Overall (β) protonation constants used for thermodynamic stability constants determination by potentiometric titrations

	no1py	**no2py**	**no3py**[1]	**tpen**[2]
Equilibrium		$\log\beta_{LHn}$		
$L + H^+ \rightleftharpoons LH^+$	10.55 (2)	11.27 (2)	11.07 (2)	7.19
$L + 2H^+ \rightleftharpoons LH_2$	17.00 (2)	15.73 (2)	16.14 (3)	12.05
$L + 3H^+ \rightleftharpoons LH_3$	19.92 (3)	17.07 (5)	19.69 (3)	15.40
$L + 4H^+ \rightleftharpoons LH_4$	nd	nd	21.47 (4)	18.35

nd: not detected. ([L] = 2 mM; 25 °C; I = 0.1 M KNO_3)

Table S1. Overall (β) protonation constants in log units of ligands **noXpy** (X = 1, 2 or 3) at 298 K in KNO_3 (I = 0.1M).

4. Biological assays

4.1. Kinetic of cell death and cell proliferation

4.1.2. Procedure

a. On B-cell lines

Cells were cultured into 96-well plates 50,000/well in 200µl of complemented RPMI culture medium in the presence of increasing concentrations compounds (1-25 µM) for 3 days. Real time Incucyte® Cytotox Green fluorescence assay was performed in 96-well plate using the Incucyte® Live-Cell Analysis System (20 objective, 3 images per well) with a scan interval of 2 hours. Time-dependent increase of Incucyte® Cytotox Green Dye staining was used to calculate cell death as the cytotoxicity index, *i.e.* the green object count per well normalized to T_0 for Ramos and Raji cells cultured with **no1py** (a), **no2py** (c), **no3py** (e) and **tpen** (g) and cell proliferation index as the phase area per well normalized to T_0 for cells cultured with **no1py** (b), **no2py** (d), **no3py** (f) and **tpen** (h). Data represents mean values and standard deviations based on 3 wells per condition.

b. On CLL PBMCs

Cells were cultured into 96-well plates 200,000/well in 200µl of complemented RPMI culture medium in the presence of increasing concentrations of compounds (2,5 - 20 µM) for 1 day. Real time Incucyte® Cytotox Green fluorescence assay was performed in 96-well plate using the Incucyte® Live-Cell Analysis System (20 objective, 3 images per well) with a scan interval of 1 hour. Time-dependent increase of Incucyte® Cytotox Green Dye staining was used to calculate cell death as the green area normalized to phase area (surface confluence) for Daudi cells cultured with chelator compounds. Data represents mean values and standard deviations based on 3 wells per condition. This CLL is representative of 3 different experiments.

4.1.3. Results

a. On Ramos cells

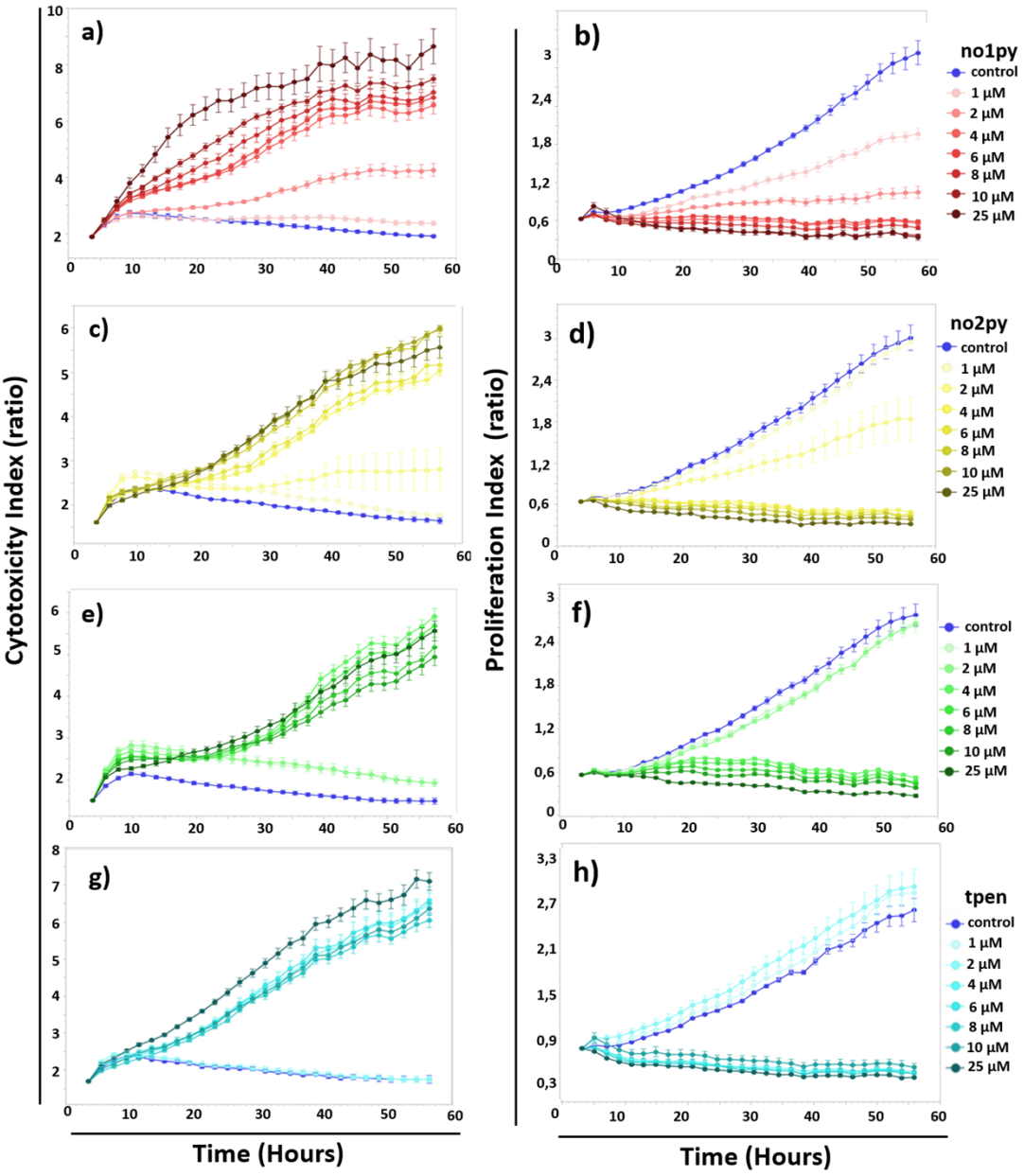


Figure S12. Kinetics of cell death and proliferation after **no1py**, **no2py**, **no3py** and **tpen** exposure of Ramos cells.

b. On Raji cells

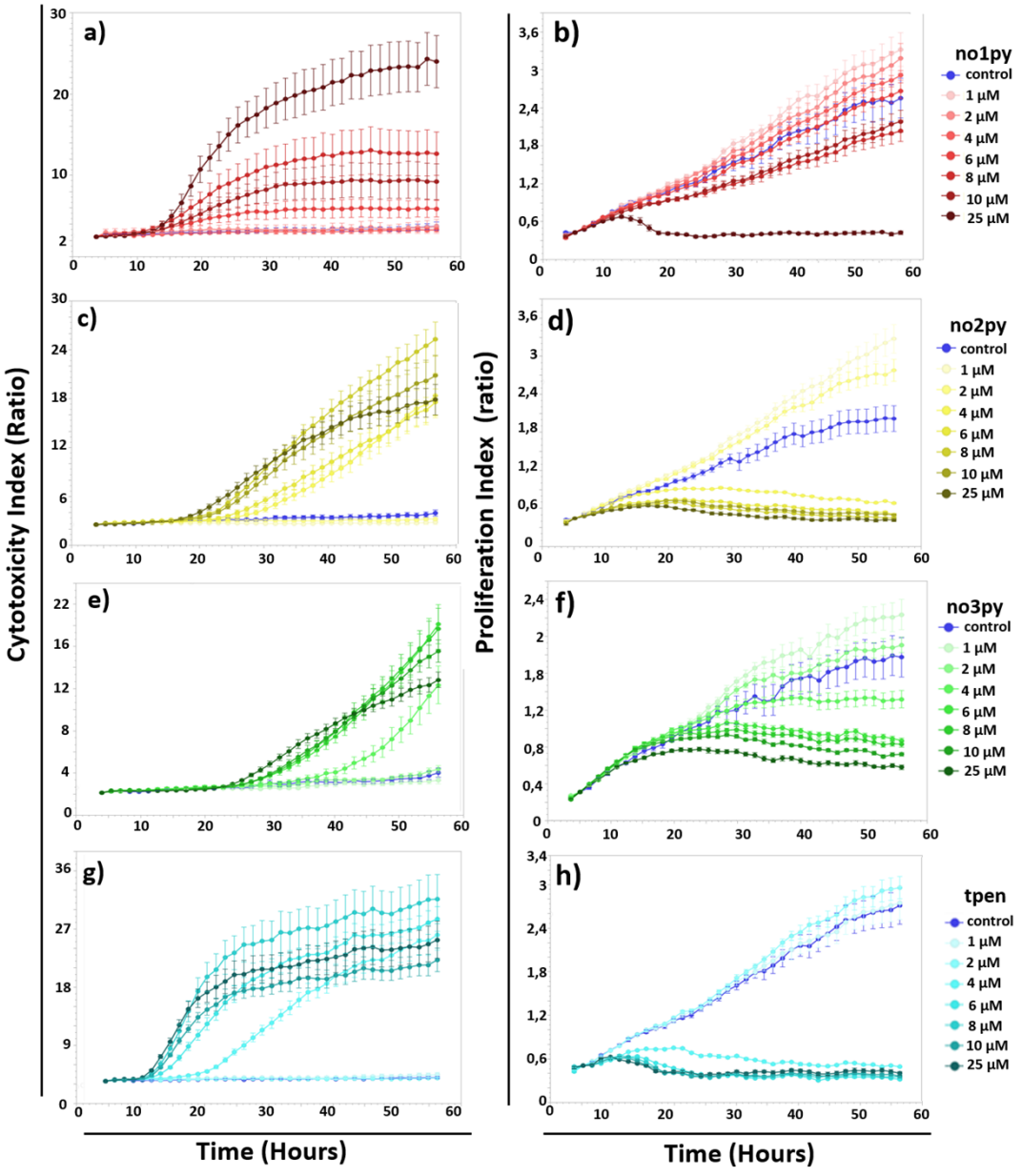


Figure S13. Kinetics cell death and proliferation after **no1py**, **no2py**, **no3py** and **tpen** exposure of Raji cells.

*c. EC50 and anti-proliferative effect of **noXpy** derivatives*

		Daudi	Raji	Ramos
EC50 after 24 h (µM)	no1py	15-20	13-18	10-15
	no2py	25-30	20-23	13-18
	no3py	30-40	26-30	20-25
Anti-proliferative dose (µM)	no1py	> 8	> 10	> 4
	no2py	> 4	> 4	> 6
	no3py	> 6	> 6	> 4

Table S2. EC50 and anti-proliferative effect of noXpy derivatives were evaluated after 24 hours of **no1py**, **no2py**, **no3py** and **tpen** exposure. The range of values represent cumulative results for experiments conducted using flow cytometry and real-time monitoring (Incucyte®).

d. On CLL PBMCs

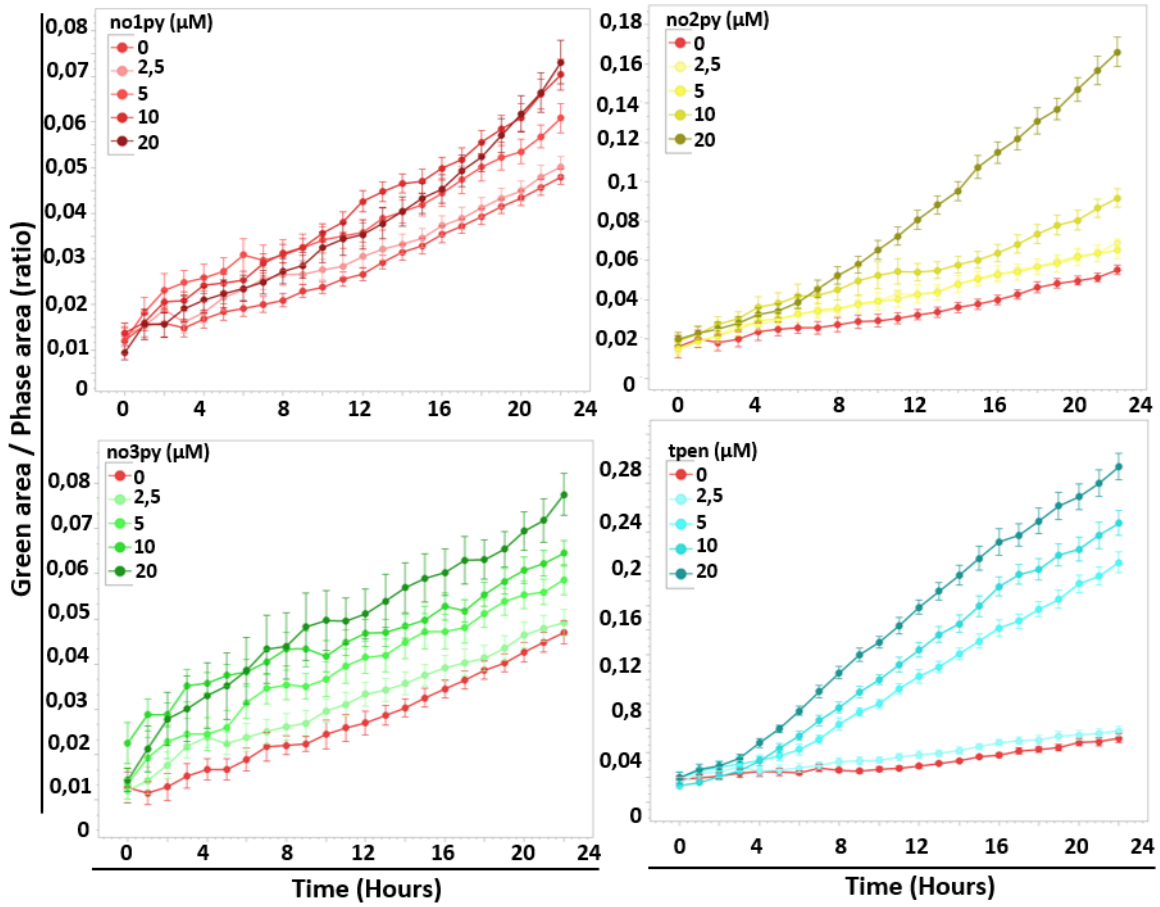


Figure S14. Kinetics of cell death after CLL PBMCs exposure to **no1py**, **no2py**, **no3py** and **tpen**.

4.2. Apoptotic cells imaging

4.2.1. Procedure

Daudi cells were cultured (1.10^6 cells / ml) in RPMI1640-complete medium in the Incucyte® Live-Cell Analysis System (Essen Bioscience, Ann Arbor, MI, USA) containing the Incucyte® Cytotox Green dye for counting dead cells (5µM). Representative phase/green images of Daudi cells after 24 hours of exposure to no1py, no2py, no3py and tpen at 10 µM were captured during the Incucyte® Cytotox Green fluorescence assay. Scale bars represent 200 µm.

4.2.2. Results

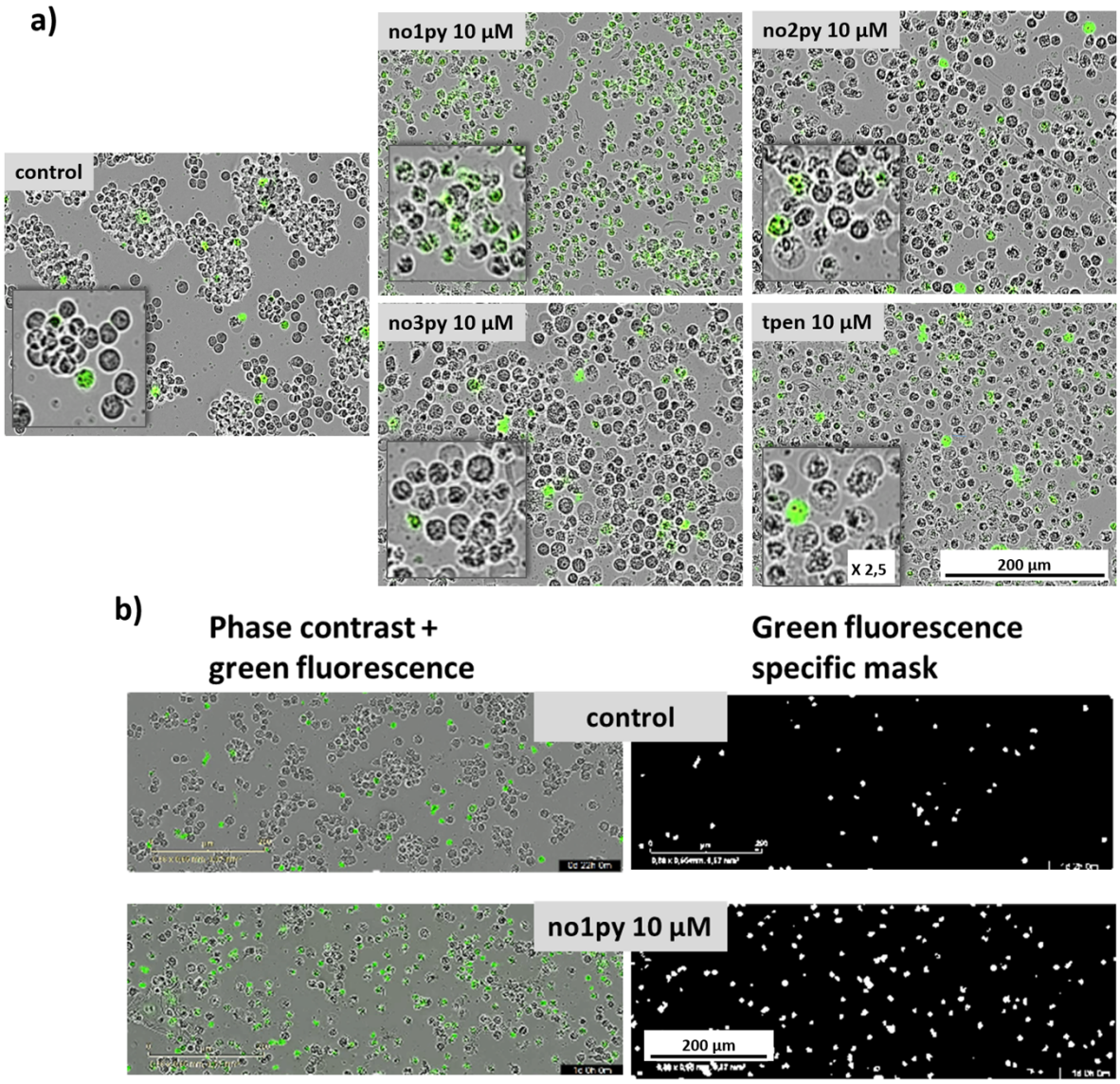


Figure S15. Chelators (10 µM) induced apoptotic cell morphology. (a) Representative phase/green images of Daudi cells after 24 hours; (b) Example of green fluorescence mask used for green area calculation.

4.3. Caspase 3/7 activation

4.3.1. Procedure

Caspase 3/7 activity was analyzed after culture of Ramos/Raji cells with 10 µM of **no1py**, **no2py**, **no3py** and **tpen**. Real time acquisition was performed during 2 days using the Incucyte® Live-Cell Analysis System (x 20 objective, 3 images per well). Caspase 3/7 activity was analyzed with a scan interval of 1 hour using the Caspase 3/7® Green dye (n=2). Time-dependent increase of Caspase3/7® Green positive cells was calculated as the green area per well normalized to phase area (surface confluence) at T0 and presented as the mean ± SD.

4.3.2. Results

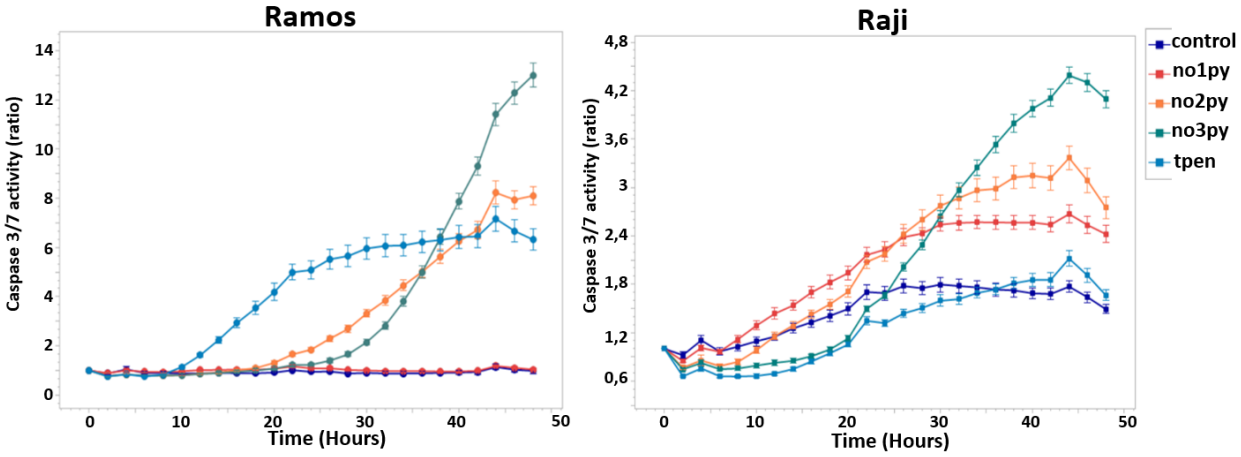


Figure S16. Level of caspase 3/7 activation in Ramos/Raji cells after chelators exposure. Cells were exposed to 10 µM of chelateurs.

5. References

[1] Guillou A., Lima L.M.P., Roger M., Esteban-Gómez D., Delgado R., Platas-Iglesias C., Patinec V., Tripier R.. Tacn-based Bifunctional Picolinate Ligands for an Efficient Copper Complexation. *Eur. J. Inorg. Chem*, **2017**, 2435 doi : 10.1002/ejic.201700176

[2] Anderegg, G., Hubmann E., Podder N. G., Wenk F., Pyridine derivatives as complexing agents XI. Thermodynamics of metal complex formation with bis-, tris- and tetrakis[(2-pyridyl)methyl]-amines, *Helvetica Chimica acta*, **1977**, 60, 1, 123-140. https://doi.org/10.1002/hlca.19770600115