Electronic Supplementary Material (ESI) for Dalton Transactions. This journal is © The Royal Society of Chemistry 2016

Electronic Supplementary Information

Accompanying the manuscript

Significantly enhanced proteolytic activity of cyclen complexes by monoalkylation

Chrischani Perera-Bobusch, Jan Hormann, Christoph Weise, Stefanie Wedepohl, Jens Dernedde, Nora Kulak*

List of contents:

- **S-1** Experimental section
- S-2 Protein cleavage experiments
- S-3 Cytotoxicity studies
- S-4 "Pyrene 1:3 ratio" method for cmc determination

S-1 Experimental section

• S-1.1 Synthesis of monoalkylated cyclen derivatives

1-Decyl-, 1-dodecyl- and 1-hexadecyl-1,4,7,10-tetraazacyclododecane were synthesized according to a previously described procedure.¹ Precipitation as hydrochloride salts with subsequent recrystallization from ethanol, dissolution in KOH (20%) and extraction with $CHCl_3$ according to another procedure were necessary to completely purify the products.²



Scheme S-1 Synthesis of the ligands L1, L2, and L3.

The yields from the alkylation reactions can be taken from Table S-1.

Ligand	Yield
1-Decylcyclen (L1)	59%
1-Dodecylcyclen (L2)	66%
1-Hexadecylcyclen (L3)	69%

 Table S-1 Yields of the ligand syntheses.

S-1.2 Complex formation

In situ complex formation

For the cleavage activity studies complexes were generated *in situ* in aqueous solution. $Cu(NO_3)_2 \cdot 3 H_2O$ and $CoCl_2 \cdot 6 H_2O$ were applied as metal sources for the formation of the corresponding complexes. For the Co(III) complexes the corresponding Co(II) complexes were oxidized under air overnight. The oxidation was confirmed *via* UV/vis spectroscopy by comparison with similar established Co(III) complexes.^{3,4} For the cleavage experiments, the ligand was applied in an excess of 5% to ensure that the protein cleavage monitored *via* SDS-PAGE did not originate from free metal ions.

Precipitation of the complexes was necessary to ensure the complexes actually acting as cleavage agents. With the metal salts applied for *in situ* complex formation all attempts to precipitate the complexes failed. Thus salts with different counter ions than the ones used before were applied for precipitation, e.g. $Na_3[Co(CO_3)_3] \cdot 3 H_2O$ was used as a Co(III) precursor because this complex was published multiple times before as a reliable Co(III) source for the generation of cyclen complexes.^{5,6,7}

Co(III)-1-hexadecyl-1,4,7,10-tetraazacyclododecane

1-Hexadecyl-1,4,7,10-tetraazacyclododecane (0.39 mmol) was dissolved in 1 mL of 1.2 M HCl and Na₃[Co(CO₃)₃]·3 H₂O (0.44 mmol) was added in small portions. The purple mixture was heated to 66 °C for 5 min, cooled to room temperature and filtered. 90 mL of acetone were added to the filtrate yielding a purple precipitate. The mixture was kept at -18 °C overnight and the precipitate was filtered off and dried under vacuum.

Yield: 0.16 mmol (40%)

CHN analysis ($C_{25}H_{52}N_4O_3$ ClCo·7 H₂O·4 HCl): calcd. C 36.48, H 8.57 and N 6.81%; found C 36.67, H 8.58 and N 6.92%.

MS (ESI⁺) (m/z): calcd. for [M-Cl]⁺ 515.3366; found 515.3429.

¹H-NMR (700 MHz, D_2O): δ = 0.79 (t, 3H, CH₃), 1.21 (s, 26 H, alkyl-CH₂), 1.65 (m, 2 H, CH₃-CH₂), 2.55–3.34 (m, 18 H, cyclen-H, N-CH₂).

¹³C-NMR (500 MHz, D₂O): δ 13.9, 21.5, 22.7, 27.5, 29.6, 29.8, 30.0, 30.1, 30.2, 32.1, 47.5, 48.9, 49.6, 53.2, 54.5, 57.5, 166.7.

Cu(II)-1-hexadecyl-1,4,7,10-tetraazacyclododecane

1-Hexadecyl-1,4,7,10-tetraazacyclododecane (0.85 mmol) was added to 15 mL of methanol and $Cu(ClO_4)_2 \cdot 6 H_2O$ (0.81 mmol) was added. The mixture was heated to 70 °C and was kept at -18 °C overnight. A blue precipitate was filtered off and dried under vacuum.

Yield: 0.54 mmol (63%)

CHN analysis (C $_{24}H_{52}N_4O_8Cl_2Cu\cdot 2$ CH $_3OH$): calcd. C 43.18, H 8.36 and N 7.75%; found C 43.31, H 8.05 and N 7.44%.

MS (ESI⁺) (m/z): calcd. for [M-H]⁺ 458.3404; found 458.3433.

S-2 Protein cleavage experiments

S-2.1 SDS-PAGE

In a typical experiment, to Tris-HCl buffer (50 mM final concentration, pH 7.4 or 9, in deionized water) the protein of interest was added from a stock solution (75 μ M for BSA, 225 μ M for myoglobin). The final protein concentration was 0.75 μ M and 2.25 μ M (22.5 μ M for tricine gels), respectively. The metal complexes were added in the respective concentrations from an *in situ* formed stock solution if not stated otherwise.

The samples were incubated for given time and temperature. After incubation 10 μ L of the 500 μ L incubation solution were added to 3.3 μ L of reducing loading buffer (Rotiload 1, Carl Roth) and incubated for 5 min at 85 °C. 10 μ L of this solution were loaded onto the gel (Any kD^M Mini-PROTEAN® TGX Stain-Free^M Gels, Bio-Rad). Electrophoresis was carried out at 200 V for 35 (BSA) or 30 (myoglobin) min in SDS buffer (Laemmli Buffer, Carl Roth; Rotiphorese[®] 10X SDS-PAGE, Carl Roth)⁸ using a vertical electrophoresis unit (Mini-PROTEAN Tetra cell, Bio-Rad). The gels were activated (UV light-induced reaction of compounds within the gels with tryptophan residues of the protein) to generate fluorescence and recorded with a Bio-Rad Gel Doc^M EZ system.

For the tricine gel a Mini-PROTEAN[®] Tris-tricine Precast Gel (10-20%), 10X Tris/Tricine/SDS, Tricine sample buffer and β -mercaptoethanol from Bio-Rad were applied. 10 µL of the sample were mixed with 9.5 µL of the sample buffer and 0.5 µL of β -mercaptoethanol. The solution was heated to 70 °C for 10 min and 10 µL were loaded onto the gel. Electrophoresis was carried out for 85 min at 100 V. The gels were stained with RotiBlue[®] (Carl Roth) according to the company's manual and recorded with a Bio-Rad Gel Doc[™] EZ system.

konsta . *		2.4	· · · · ·						0	01	0.05	0.1	0.5	1	1 5	2
0	0.01	0.05	0.1	0.5	1	1.5	2	0.	0	.01	0.05	0.1	0.5	1	1.5	2
-		· ,							-							
0	0.01	0.05	0.1	0.5	1	1.5	2	C	,	0.01	0.05	0.1	0.5	1	1.5	2
-								1-1-1-1		Anna A	(and					
0	0.01	0.05	0.1	0.5	1	1.5	2	0	0	.01	0.05	0.1	0.5	1	1.5	2

Fig. S-1 SDS-PAGE, concentration-dependent cleavage of BSA with the complexes of 1-hexadecylcyclen, 1dodecylcyclen and 1-decylcyclen (**L3**, **L2** and **L1** from top to bottom, Co(III) on the left, Cu(II) on the right). Incubation at 50 °C and pH 9 for 48 h. Complex concentrations in the figure are given in mM.

		-			Respi	and the second	eres a	passed (point.
0 0.5	1	2.5	5	7.5	10	15	0	0.5	1	2.5	5	7.5	10	15

Fig. S-2 SDS-PAGE, concentration-dependent cleavage of BSA with Cu(II) cyclen (left) and cyclen (right). Incubation at 50 °C and pH 9 for 48 h. Concentrations of complex and ligand in the figure are given in mM.





Fig. S-3 SDS-PAGE, concentration-dependent cleavage of BSA with the precipitated and re-dissolved complexes of L3 (Co(III) on the left, Cu(II) on the right). Incubation at 50 °C and pH 9 for 48 h. Complex concentrations in the figure are given in mM.



Fig. S-4 SDS-PAGE, concentration-dependent cleavage of myoglobin with the complexes of L3 (Co(III) on the left, Cu(II) on the right). Incubation at 50 °C and pH 9 for 48 h. Complex concentrations in the figure are given in mM.

Rectored		And the second					
0	0.01	0.05	0.1	0.5	1	1.5	2

Fig. S-5 SDS-PAGE, concentration-dependent cleavage of BSA with Cu(II) **L3**. Incubation at 37 °C and pH 7.4 for 48 h. Complex concentrations in the figure are given in mM.

Second .					. •		Sec	• 1000 A	•					
R	4	6 8	16	24	39	48 h	R	4	6	8	16	24	39	48 h

Fig. S-6 SDS-PAGE, time-dependent cleavage of BSA with the complexes of **L3** (Co(III) on the left, Cu(II) on the right, 0.05 mM). Incubation at 50 °C and pH 9 for 48 h. Complex concentrations in the figure are given in mM.

-							-	_
3							•	
-	Cu(II) L3	Cu(II) L3	Co(III) L3	Co(III)	L3 $Cu(NO_3)_2$	$Cu(NO_3)_2$	CoCl ₂	CoCl ₂
0	0.01	0.05	0.01	0.05	0.01	0.05	0.01	0.05

Fig. S-7 SDS-PAGE, BSA cleavage with Cu(II) **L3**, Co(III) **L3**, Cu(NO₃)₂·3 H₂O and CoCl₂·6 H₂O, respectively. Incubation at 50 °C and pH 9 for 48 h. Cleavage agent concentrations in the figure are given in mM.



Fig. S-8 SDS-PAGE (Tricine), myoglobin cleavage with Cu(II) **L3** (0.05 mM), pH given in the figure. Incubation at 50 °C for 48 h. Myoglobin concentration: 22.5 μ M.

	(· .				•	~
O	0.01	0.05	0.1 0.5	1	1.5	2

Fig. S-9 SDS-PAGE, BSA cleavage with Zn(II) **L3**. Incubation at 50 °C and pH 9 for 48 h. Complex concentrations in the figure are given in mM.



Fig. S-10 SDS-PAGE, comparison of the proteolytic activity of Cu(II) **L3** towards BSA incubated with Tris-HCl buffer and MOPS-NaOH (50 mM, pH 7.4). Incubation at 50 °C and pH 9 for 48 h. Complex concentrations in the figure are given in mM.

• S-2.2 MALDI-ToF MS

For MS measurements the myoglobin samples were prepared in the same way as for the SDS-PAGE experiments with the same concentrations of all compounds but at a pH of 7.4 (Tris-HCl, 50 mM).

Protein masses were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS) using an Ultraflex-II ToF/ToF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam[™]

laser. The mass spectrometer was operated in the positive linear mode. MS spectra were acquired over an m/z range of 3,000–20,000, and data were analyzed using FlexAnalysis 2.4 software provided with the instrument. Sinapinic acid was used as the matrix and the samples were spotted using the dried-droplet technique (undiluted sample directly from the incubation sample and saturated matrix solution 1:1).

S-3 Cytotoxicity studies

A549 lung epithelial cancer cells (DSMZ – German collection of microorganisms and cell cultures, #ACC 107) were maintained in DMEM medium (Life Technologies) with 10% FBS (FBS Superior, Merck Millipore) and 1% penicillin/streptomycin (Life Technologies) at 37 °C and 5% CO₂. For each MTT assay, 10,000 cells/well were seeded into 96 well plates and grown overnight. The next day, medium was discarded and replaced with 50 µL fresh medium and 50 µL of serial dilutions of the test compounds in triplicates and put back in the incubator for 48 h. After this time, the medium was discarded and replaced with 100 μ L/well fresh medium and 10 μ L/well MTT (Sigma, 5 mg/mL stock solution in PBS) and incubated for another 4 h at 37 °C. The supernatant was discarded and 100 µL/well isopropanol with 0.04 M HCl was added to dissolve the formazan dye crystals formed. Absorbance was read at 570 nm in a microplate reader (Tecan Infinite M200Pro). Absorbance values of untreated cells were set as 100% and relative viabilities were calculated as percentage of 100% control. Standard errors of the mean (SEM) were calculated and nonlinear dose-response curves (log (inhibitor) vs. normalized response variable slope) were fitted to the data of three independent experiments using GraphPad Prism 5.



Fig. S-11 Cytotoxicity profiles of Cu(II) and Co(III) cyclen and L2 complexes as determined by MTT assay on A549 lung cancer cells. Error bars are +/- SEM.

S-4 "Pyrene 1:3 ratio" method for cmc determination

Pyrene was dissolved in Tris-HCl buffer (50 mM, pH 9) resulting in a 0.4 μ M solution of the fluorescent probe. The complex solution was titrated to the buffer and fluorescence spectra were recorded for various concentrations on a Hitachi F-4500 fluorescence spectrometer. For all complexes the fluorescence intensity decreased drastically throughout the experiment before the cmc was reached. This made a change of the slit

width from 5 to 10 nm necessary to detect a spectrum with a convenient signal-to-noiseratio. Since the third vibronic peak I_3 was not clearly detectable, values found in the literature (385 nm for I_3 and 375 nm for I_1) were utilized to calculate the cmc.⁹ A sigmoidal curve was obtained for all complexes as can be seen exemplarily in Figure S-12 for Co(III) L3, and the cmc was read off at the inflection point.



Fig. S-12 Plot of I_1/I_3 vs. logarithmic concentration of Co(III) L3.

The values for the cmc of all complexes can be found in Table S-2.

Гable	S-2	Critical	micellar	concentration

Complex	cmc [mM]
Cu(II) L1	0.47
Cu(II) L2	0.18
Cu(II) L3	0.08
Co(III) L3	0.12

References

- 1 J. Massue, S. E. Plush, C. S. Bonnet, D. A. Moore and T. Gunnlaugsson, *Tetrahedron Lett.*, 2007, **48**, 8052–8055.
- 2 W. C. Baker, M. J. Choi, D. C. Hill, J. L. Thompson and P. A. Petillo, *J. Org. Chem.*, 1999, **64**, 2683–2689.
- 3 R. W. Hay and B. Jeragh, *Transition Met. Chem.*, 1979, **4**, 288–291.
- 4 R. W. Hay and A. M. Danby, *Polyhedron*, 1998, **17**, 3795–3802.
- 5 H. F. Bauer and W. C. Drinkard, J. Am. Chem. Soc., 1960, **82**, 5031–5032.
- 6 E. U. Akkaya and A. W. Czarnik, *J. Phys. Org. Chem.*, 1992, **5**, 540–548.
- 7 Y. N. Belokon', V. I. Maleev, D. A. Kataev, T. F. Saveleva, T. V. Skrupskaya, Y. V. Nelyubina and M. North, *Tetrahedron: Asymm.*, 2009, **20**, 1746–1752.
- 8 U. K. Laemmli, *Nature*, 1970, **227**, 680–685.
- 9 A. I. Mitsionis and T. C. Vaimakis, *Chem. Phys. Lett.*, 2012, **547**, 110–113.