Supplementary Information

Bioinorganic chemistry of calcitermin – the picklock of its antimicrobial activity

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Table S1. Potentiometric and spectroscopic data for Cu(II) and Zn(II) complexes with ligand H9A, T=298 K, I=0.1 mol dm^{-3} ($NaClO_4$) and M:L molar ratio = 0.9:1. Values in parentheses are standard deviations on the last significant figure.

			UV-Vis		CD		pH
Species	logβ	pK_a	λ (nm)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	λ (nm)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	
$[CuH_6L]^{5+}$	56.72 (6)	4.51	800		232	-1.67	4.1
$[CuH_5L]^{4+}$	52.21 (3)	5.66	670	33	231	-3.50	5.0
$[CuH_4L]^{3+}$	46.55 (4)	7.25	632	50	231	-4.84	6.1
$[CuH_3L]^{2+}$	39.30 (5)	7.62	613	53	339 270 231	-0.29 0.44 -2.78	7.0
$[CuH_2L]^{+}$	31.68 (5)	8.87	568	66	339 253	-0.69 3.09	8.0
[CuHL]	22.81 (6)	9.64	518	71	650 530 339 253	0.38 -0.45 -0.69 4.66	9.0
$[CuL]^{-}$	13.18 (6)	9.84	515	82	649 515 346 312 253	0.64 -0.88 -0.34 -0.03 5.42	9.9
$[CuH_{-1}L]^{2-}$	3.33 (5)						
$[CuH_{-3}L]^{4-}$	-18.01 (6)		512	89	649 515 355 318 253	0.78 -1.20 -0.31 0.25 6.43	11.0
$[ZnH_4L]^{3+}$	42.80 (6)	7.82					
$[ZnH_3L]^{2+}$	34.88 (8)	8.78					
$[ZnH_2L]^{+}$	26.20 (1)	8.81					
$[ZnH_1L]$	17.39 (8)	-					
$[ZnH_{-1}L]^{2-}$	-2.6 (1)	-					

Table S2. Potentiometric and spectroscopic data for Cu(II) and Zn(II) complexes with ligand H11A, *T*=298 K, *I*=0.1 mol dm^{-3} ($NaClO_4$) and M:L molar ratio = 0.9:1. Values in parentheses are standard deviations on the last significant figure.

			UV-Vis		CD		pH
Species	log*β*	pK_a	λ (nm)	Δε (M^{-1} cm^{-1})	λ (nm)	Δε (M^{-1} cm^{-1})	
$[CuH_6L]^{5+}$	55.71 (6)	5.35	662	128	655	-0.05	5.0
					242	-1.67	
$[CuH_5L]^{4+}$	50.37 (5)	6.55	630	214	655	-0.23	6.1
					316	0.10	
					248	-0.90	
$[CuH_4L]^{3+}$	43.82 (6)	7.53	618	256	655	-0.35	7.1
					316	0.22	
					246	-0.73	
$[CuH_3L]^{2+}$	36.28 (7)	8.71	596	243	609	-0.21	8.2
					350	-0.15	
					316	0.16	
					293	-0.05	
					261	1.49	
$[CuH_2L]^{+}$	27.57 (7)	9.17	518	282	658	0.12	9.0
					535	-0.57	
					350	-0.21	
					316	0.17	
					293	-0.31	
					262	2.15	
[CuHL]	18.40 (7)	10.18					
$[CuL]^{-}$	8.22 (9)	10.23	512	319	658	0.16	10.1
					535	-0.79	
					350	-0.14	
					316	0.30	
					293	-0.21	
					267	1.70	
$[CuH_{-1}L]^{2-}$	-2.01 (7)						
$[CuH_{-3}L]^{4-}$	-24.19 (8)		512	319	658	0.16	11.1
					535	-0.79	
					350	-0.14	
					316	0.30	
					293	-0.21	
					267	1.70	
$[ZnH_5L]^{4+}$	48.4 (1)	6.24					
$[ZnH_4L]^{3+}$	42.14 (4)	7.56					
$[ZnH_3L]^{2+}$	34.58 (5)	8.25					
$[ZnH_2L]^{+}$	26.32 (6)	8.70					
$[ZnH_1L]$	17.62 (6)	9.70					
$[ZnL]^{-}$	7.9 (1)	9.94					
$[ZnH_{-1}L]^{2-}$	-2.02 (9)	-					

Table S3. Potentiometric and spectroscopic data for Cu(II) and Zn(II) complexes with ligand H13A, *T*=298 K, *I*=0.1 mol dm^{-3} ($NaClO_4$) and M:L molar ratio = 0.9:1. Values in parentheses are standard deviations on the last significant figure.

			UV-Vis		CD		pH
Species	log*β*	pK_a	λ (nm)	Δε (M^{-1} cm^{-1})	λ (nm)	Δε (M^{-1} cm^{-1})	
$[CuH_5L]^{4+}$	51.82 (1)	5.74	653	47	241	-2.32	5.2
$[CuH_4L]^{3+}$	46.08 (2)	6.70	620	63	642	0.15	6.1
					547	-0.05	
					337	-0.27	
					290	0.15	
					241	-1.60	
$[CuH_3L]^{2+}$	39.38 (2)	7.97	570	81	642	0.39	6.9
					547	-0.37	
					340	-0.98	
					258	2.10	
$[CuH_2L]^{+}$	31.41 (3)	9.75	543	92	642	0.45	8.0
					547	-0.47	
					340	-1.26	
					255	3.39	
[CuHL]	21.66 (4)	10.21	540	82	642	0.45	9.1
					547	-0.47	
					340	-1.26	
					252	3.80	
$[CuL]^{-}$	11.45 (4)	10.93	525	96	642	0.45	10.1
					547	-0.47	
					340	-1.26	
					247	5.77	
$[CuH_{-1}L]^{2-}$	0.52 (7)						
$[CuH_{-3}L]^{4-}$	-21.8 (1)		520	110	642	0.45	11.0
					528	-0.57	
					340	-0.81	
					250	7.06	
$[ZnH_5L]^{4+}$	49.44 (4)	6.22					
$[ZnH_4L]^{3+}$	43.23 (3)	-					
$[ZnH_2L]^{+}$	27.24 (5)	-					

Table S4. Stoichiometry, molecular formula and average *m/z* value for the species present in ESI-MS spectra of Cu(II) and Zn(II) complexes with calcitermin, H9A, H11A and H13A; L:M molar ratio = 1:0.9 in water/methanol 50:50 solution.

	Species	Formula	Average *m/z*
calcitermin/Cu(II)	H_6L^{3+}	C77H124N23O20	563.6
	$([H_5L] \cdot Na)^{3+}$	C77H123N23O20Na	571.0
	$([H_4L] \cdot Na_2)^{3+}$	C77H122N23O20Na2	578.3
	$[CuH_4L]^{3+}$	C77H122N23O20Cu	583.9
	$([CuH_3L] \cdot Na)^{3+}$	C77H121N23O20CuNa	591.3
calcitermin/Zn(II)	H_6L^{3+}	C77H124N23O20	563.6
	$([H_5L] \cdot Na)^{3+}$	C77H123N23O20Na	571.0
	$([H_4L] \cdot Na_2)^{3+}$	C77H122N23O20Na2	578.3
	$[ZnH_4L]^{3+}$	C77H122N23O20Zn	584.3
	$([ZnH_3L] \cdot Na)^{3+}$	C77H121N23O20ZnNa	591.6
	$([ZnH_2L] \cdot NaK)^{3+}$	C77H120N23O20ZnNaK	604.3
	$([ZnHL] \cdot Na_2K)^{3+}$	C77H119N23O20ZnNa2K	611.9
H9A/Cu(II)	H_6L^{3+}	C74H122N21O20	541.7
	$([H_5L] \cdot Na)^{3+}$	C74H121N21O20Na	549.0
	$([H_4L] \cdot Na_2)^{3+}$	C74H120N21O20Na2	556.3
	$[CuH_4L]^{3+}$	C74H120N21O20Cu	563.7
	$([CuH_3L] \cdot Na)^{3+}$	C74H119N21O20CuNa	571.0
H9A/Zn(II)	H_6L^{3+}	C74H122N21O20	541.7
	$([H_5L] \cdot Na)^{3+}$	C74H121N21O20Na	549.0
	$([H_4L] \cdot Na_2)^{3+}$	C74H120N21O20Na2	556.3
	$[ZnH_4L]^{3+}$	C74H120N21O20Zn	563.6
H11A/Cu(II)	H_6L^{3+}	C74H122N21O20	541.7
	$([H_5L] \cdot Na)^{3+}$	C74H121N21O20Na	549.0
	$([H_4L] \cdot Na_2)^{3+}$	C74H120N21O20Na2	556.3
	$[CuH_4L]^{3+}$	C74H120N21O20Cu	562.0
	$([CuH_3L] \cdot Na)^{3+}$	C74H119N21O20CuNa	569.3
	$([CuH_2L] \cdot Na_2)^{3+}$	C74H118N21O20CuNa2	576.6
H11A/Zn(II)	H_6L^{3+}	C74H122N21O20	541.7
	$([H_5L] \cdot Na)^{3+}$	C74H121N21O20Na	549.0
	$([H_4L] \cdot Na_2)^{3+}$	C74H120N21O20Na2	556.3
	$[ZnH_4L]^{3+}$	C74H120N21O20Zn	562.3
	$([ZnH_3L] \cdot Na)^{3+}$	C74H119N21O20ZnNa	569.6
H13A/Cu(II)	H_6L^{3+}	C74H122N21O20	541.7
	$([H_5L] \cdot Na)^{3+}$	C74H121N21O20Na	549.0
	$([H_4L] \cdot Na_2)^{3+}$	C74H120N21O20Na2	556.3
	$[CuH_4L]^{3+}$	C74H120N21O20Cu	562.0
	$([CuH_3L] \cdot Na)^{3+}$	C74H119N21O20CuNa	569.3
	$([CuH_2L] \cdot Na_2)^{3+}$	C74H118N21O20CuNa2	576.6
H13A/Zn(II)	H_6L^{3+}	C74H122N21O20	541.7
	$([H_5L] \cdot Na)^{3+}$	C74H121N21O20Na	549.0
	$([H_4L] \cdot Na_2)^{3+}$	C74H120N21O20Na2	556.3
	$[ZnH_4L]^{3+}$	C74H120N21O20Zn	562.3
	$([ZnH_3L] \cdot Na)^{3+}$	C74H119N21O20ZnNa	569.6
	$([ZnH_2L] \cdot Na_2)^{3+}$	C74H118N21O20ZnNa2	577.0

Table S5. Hydrolysis constants for Cu(II)[1] and Zn(II).[2]

Species	log*β*
$[ZnH_{-1}]^{+}$	-8.96
$[ZnH_{-2}]$	-16.9
$[ZnH_{-3}]^{-}$	-28.4
$[ZnH_{-4}]^{2-}$	-41.2
$[CuH_{-1}]^{+}$	-7.7
$[Cu_2H_{-2}]^{2+}$	-10.75
$[Cu_3H_{-4}]^{2+}$	-21.36
$[CuH_{-4}]^{2-}$	-39.08

Table S6. Potential cytotoxic effect of Cu(II) and Zn(II) determined by an NR assay*.

concentration	24H		48H	
Cu(II) salt	Survival rate	Cytotoxicity	Survival rate	Cytotoxicity
0.06M	26%	74%	21%	79%
0.03M	98%	2%	88%	12%
0.015M	100%	0%	89%	11%
0.0075M	100%	0%	100%	0%
Zn(II) salt	Survival rate	Cytotoxicity	Survival rate	Cytotoxicity
0.06M	19%	81%	30%	70%
0.03M	76%	24%	59%	41%
0.015M	100%	0%	93%	7%

Table S7. Calculated dissociation constants (K_d/M), pM values and coordination hypotheses for Cu(II) and Zn(II) complexes at pH 5.4 and 7.4.

M(II)/L system	pH 5.4			pH 7.4		
	Coordination hypothesis (% of M complexation)	K_d	**pM**	**Coordination hypothesis**	K_d	**pM**
WT-Cu(II)	$3N_{Im}$ (100%)	$1.16 \cdot 10^{-6}$	6.95	$3N_{Im}$, NH_2	$1.31 \cdot 10^{-10}$	10.83
H9A-Cu(II)	$2N_{Im}$ (97%)	$3.95 \cdot 10^{-6}$	6.53	$2N_{Im}$, NH_2	$3.07 \cdot 10^{-9}$	9.47
H11A-Cu(II)	N_{Im} (84%)	$5.40 \cdot 10^{-5}$	6.07	N_{Im}, NH_2, N^-	$8.15 \cdot 10^{-7}$	7.08
H13A-Cu(II)	$2N_{Im}$ or N_{Im}, NH_2 (94%)	$4.03 \cdot 10^{-6}$	6.52	$2N_{Im}$, NH_2	$2.50 \cdot 10^{-9}$	9.56
WT-Zn(II)	$2N_{Im}$, COO^- (36%)	$1.02 \cdot 10^{-3}$	6.00	$3N_{Im}$, COO^-	$3.07 \cdot 10^{-7}$	7.48
H9A-Zn(II)	$2N_{Im}$ (4%)	$6.08 \cdot 10^{-2}$	6.00	$2N_{Im}$	$4.32 \cdot 10^{-5}$	6.09
H11A-Zn(II)	$1N_{Im}$, COO^- (10%)	$8.60 \cdot 10^{-3}$	6.00	$2N_{Im}$, COO^-	$4.35 \cdot 10^{-5}$	6.10
H13A-Zn(II)	$1N_{Im}$ (35%)	$1.24 \cdot 10^{-3}$	6.00	$2N_{Im}$	$1.16 \cdot 10^{-6}$	6.26

* Neutral Red (NR) cytotoxicity *in vitro* assay was performed in regular fibroblast (L929) cells from ECACC collection. The experiment was performed according to ISO:10993 guidelines (Biological evaluation of medical devices; Part 5: Tests for in vitro cytotoxicity; Part 12: Biological evaluation of medical devices, sample preparation and reference materials (ISO 10993-5:2009 and ISO/IEC 17025:2005). A standard protocol for the NR assay was used from *Nature Protocols* [7] Media: DMEM and a suitable amount of antibiotics (amphotericin B, penicillin-streptomycin) were used. Following concentrations of metal ions were studied: 0.06 M, 0.03 M, 0.015 M and 0.0075 M of Cu(II) salt solution and 0.06 M, 0.03 M and 0.015 M for Zn(II) salt solution. Each salt solution was dissolved in medium. Incubation with proper mixture for 24/48h in 5% CO_2 at 37^{o}C was proceeded. After incubation, the medium was removed and 100 µl of NR solution (40 ug/mL) was added to wells of the plate. Cells were incubated with NR for 2 h at 37^{o}C. After incubation, a dye was removed and wells were rinsed with PBS (Sigma Aldrich) and left to dry. At the same time destain solution (1% glacial acetic acid, 50% of 96% ethanol and 49% of deionized water; v/v) was added to each well. The plate was shaken (30 min) until NR was extracted from the cells and formed a homogenous solution. Furthermore, an absorbance was measured using microplate reader at 540 nm. As a positive control, cells not treated with salt solution was considered with 100% of potential cellular growth.

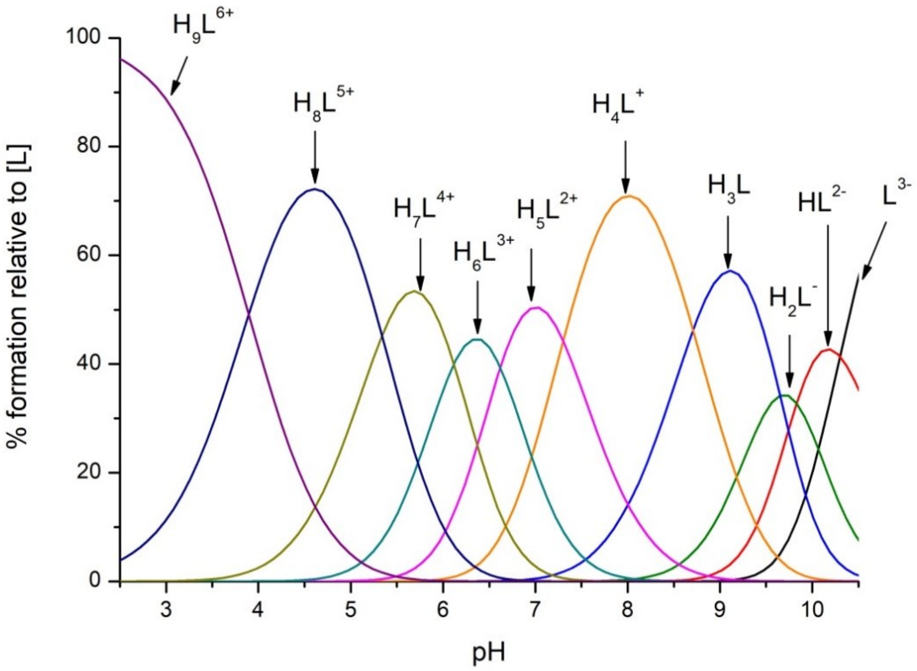


Figure S1. Species distribution diagram for protonation equilibria of ligand calcitermin (VAIALKAAHYHTHKE).

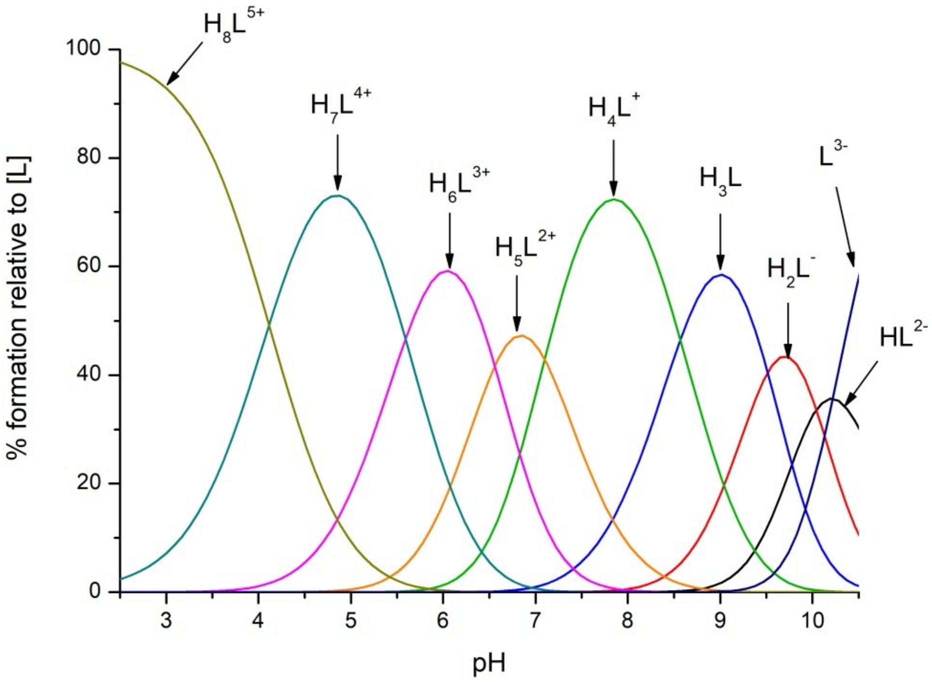


Figure S2. Species distribution diagram for protonation equilibria of ligand H9A (VAIALKAAAYHTHKE).

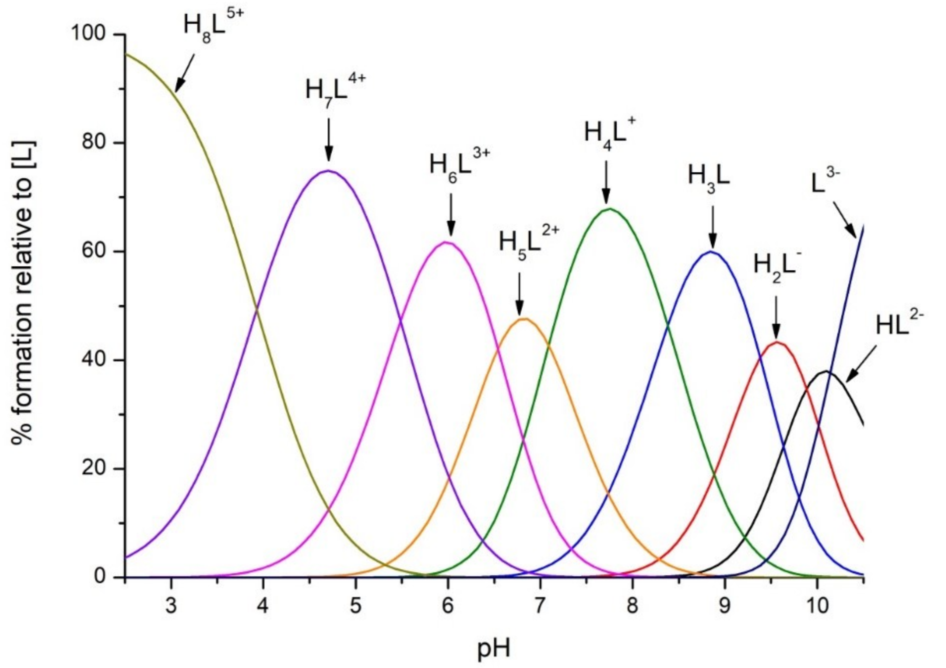


Figure S3. Species distribution diagram for protonation equilibria of ligand H11A (VAIALKAAHYATHKE).

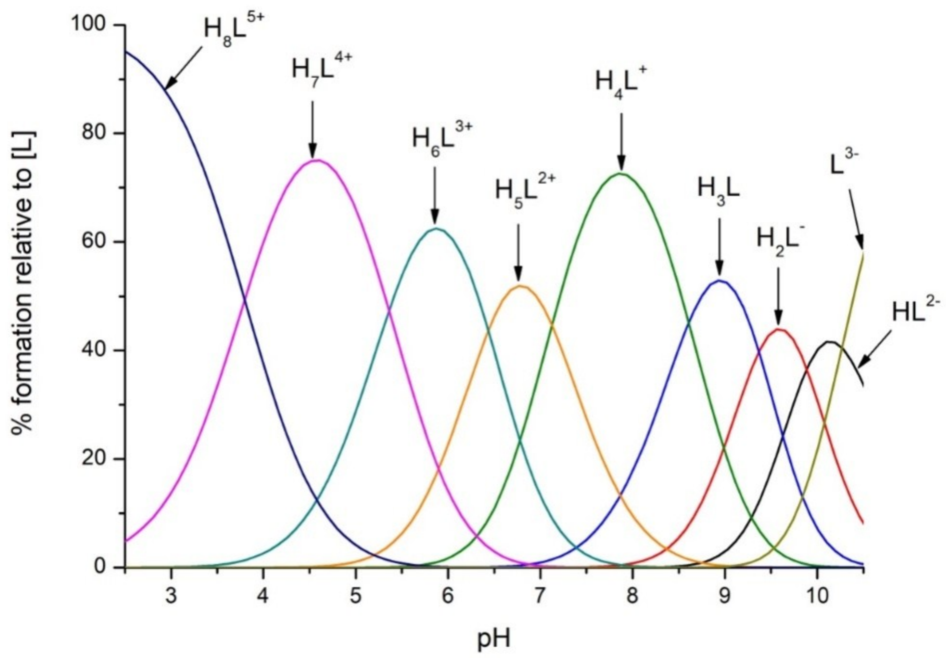


Figure S4. Species distribution diagram for protonation equilibria of ligand H13A (VAIALKAAHYATAKE).

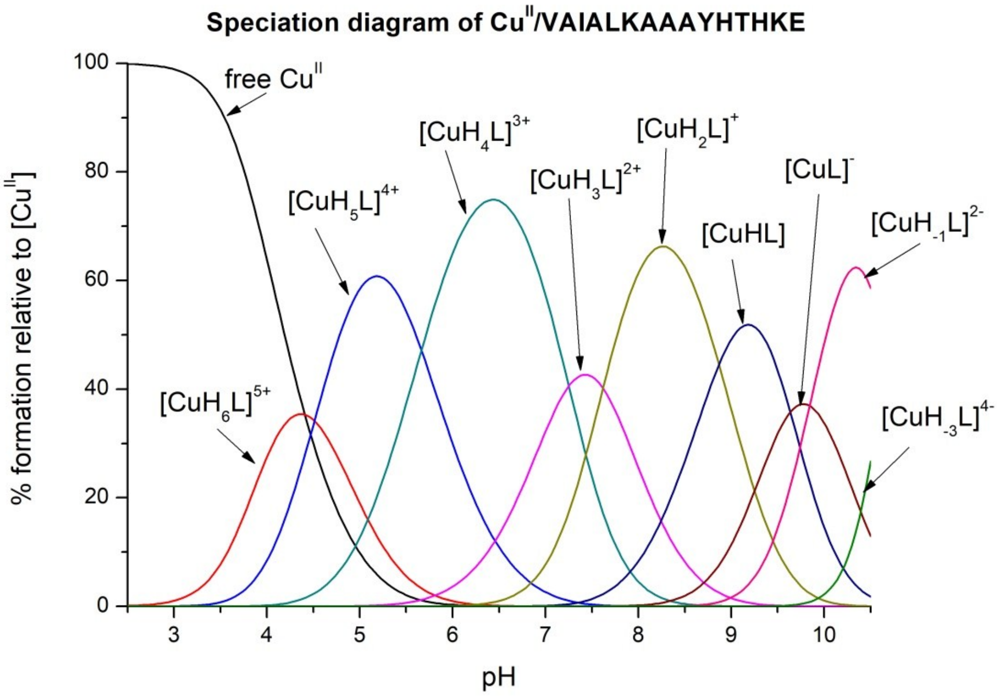


Figure S5. Exemplificative species distribution diagram relative to Cu(II)/H9A complexes; M:L molar ratio = 0.9:1.

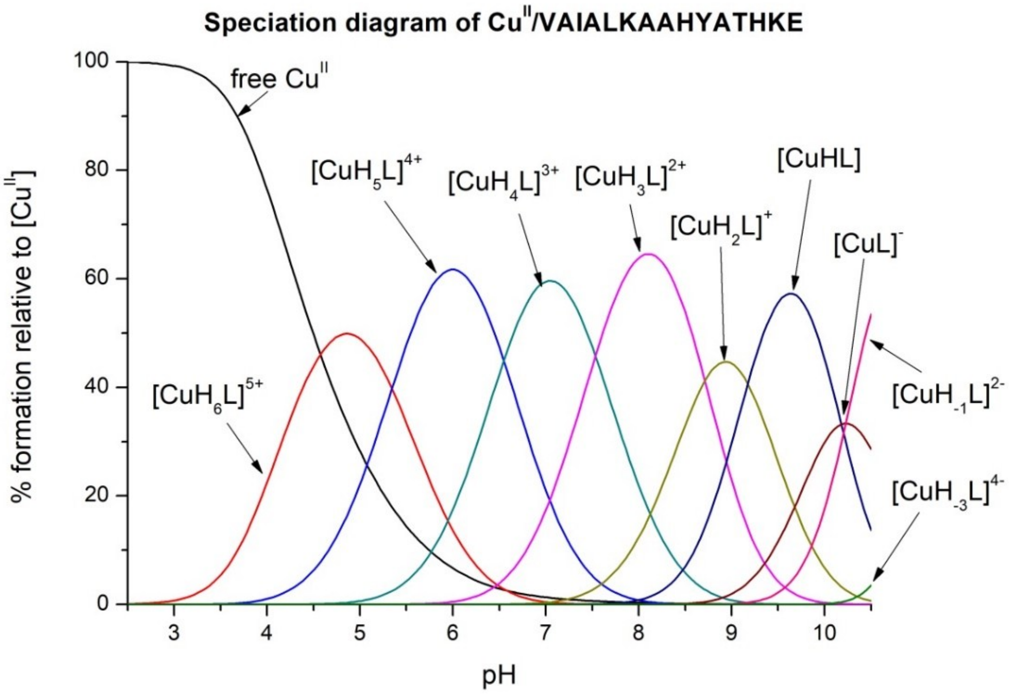


Figure S6. Exemplificative species distribution diagram relative to Cu(II)/H11A complexes; M:L molar ratio = 0.9:1.

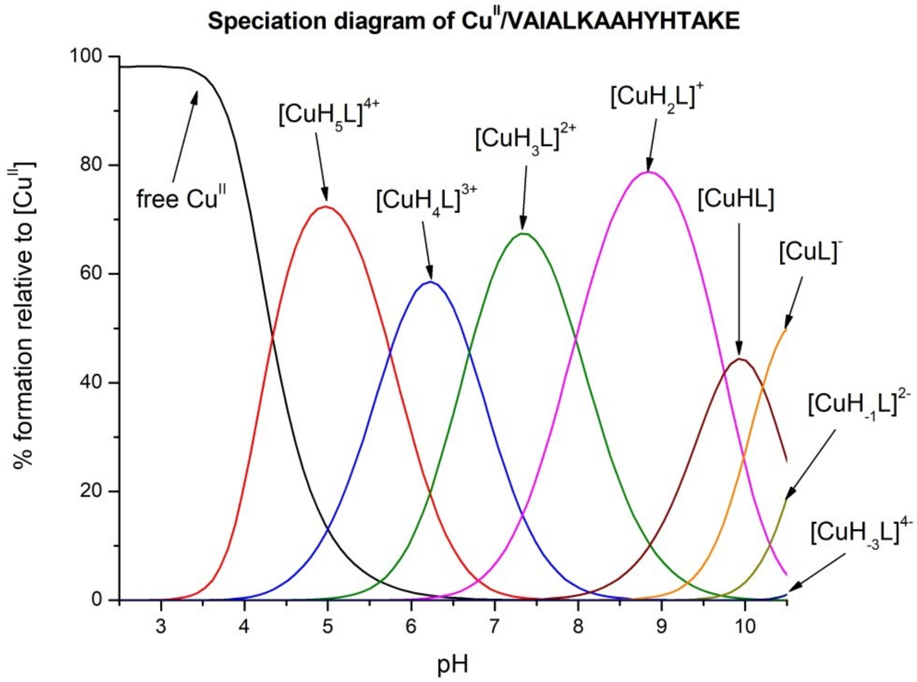


Figure S7. Exemplificative species distribution diagram relative to Cu(II)/H13A complexes; M:L molar ratio = 0.9:1.

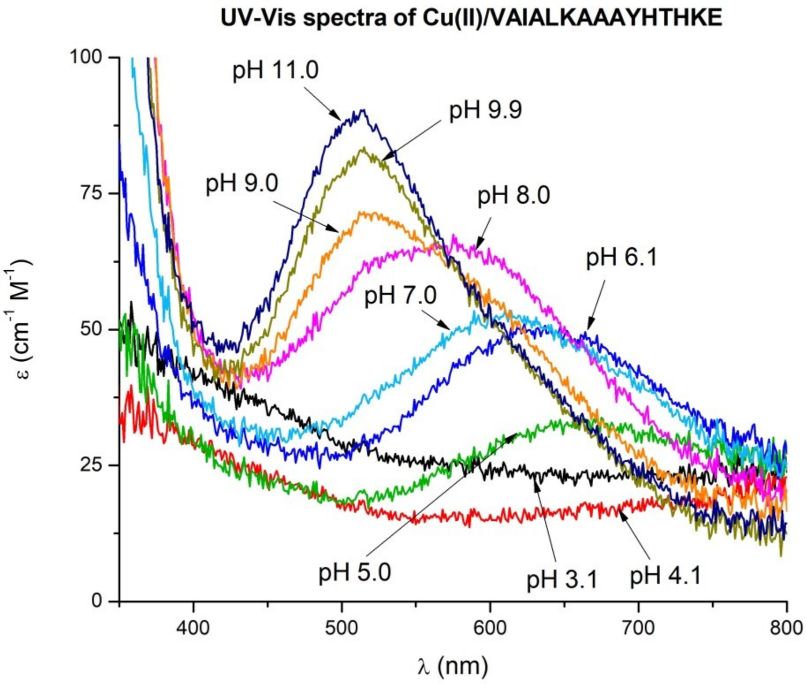


Figure S8. Vis absorption spectra [350–800 nm; optical path 1 cm] for Cu(II) complexes with H9A; M:L ratio = 0.9:1.

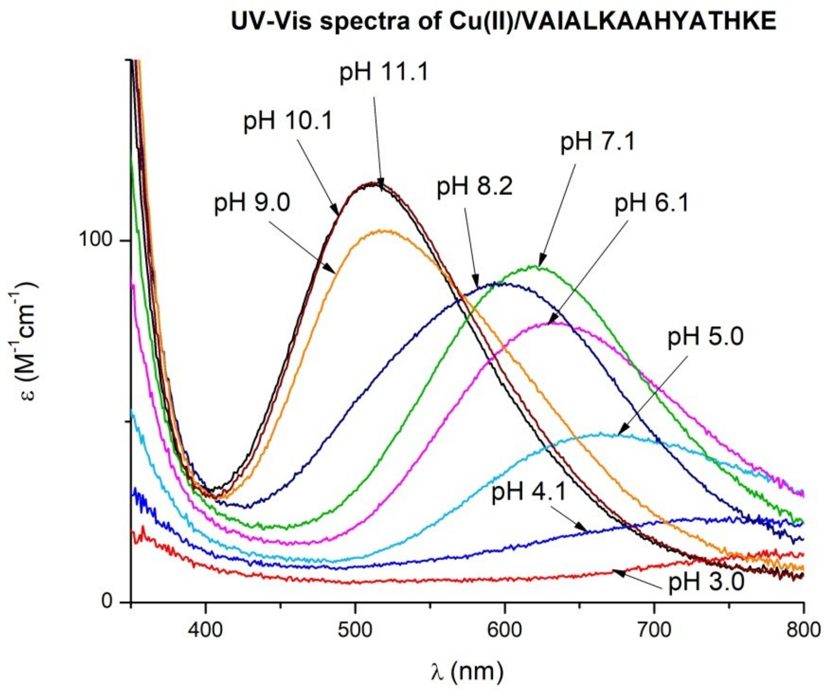


Figure S9. Vis absorption spectra [350–800 nm; optical path 1 cm] for Cu(II) complexes with H11A; M:L ratio = 0.9:1.

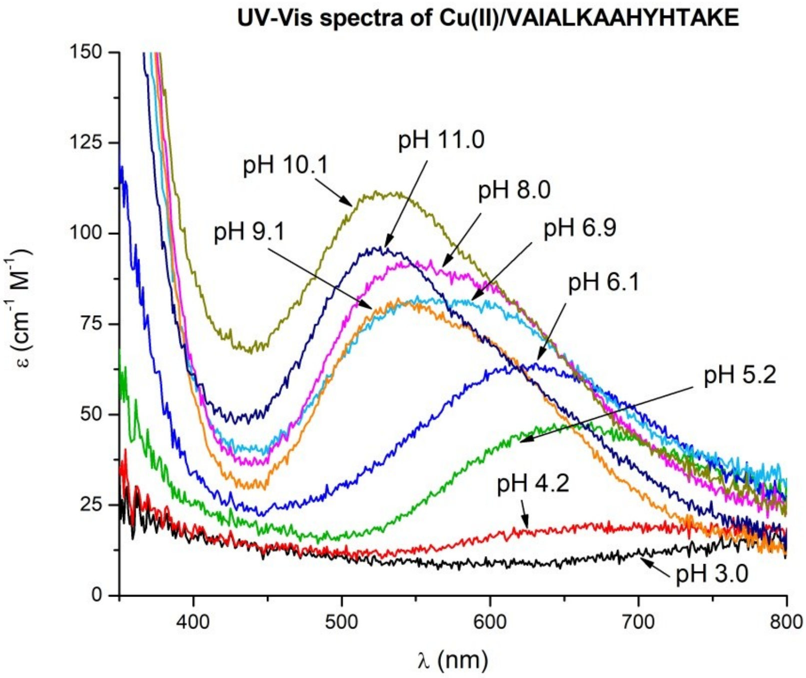


Figure S10. Vis absorption spectra [350–800 nm; optical path 1 cm] for Cu(II) complexes with H13A; M:L ratio = 0.9:1.

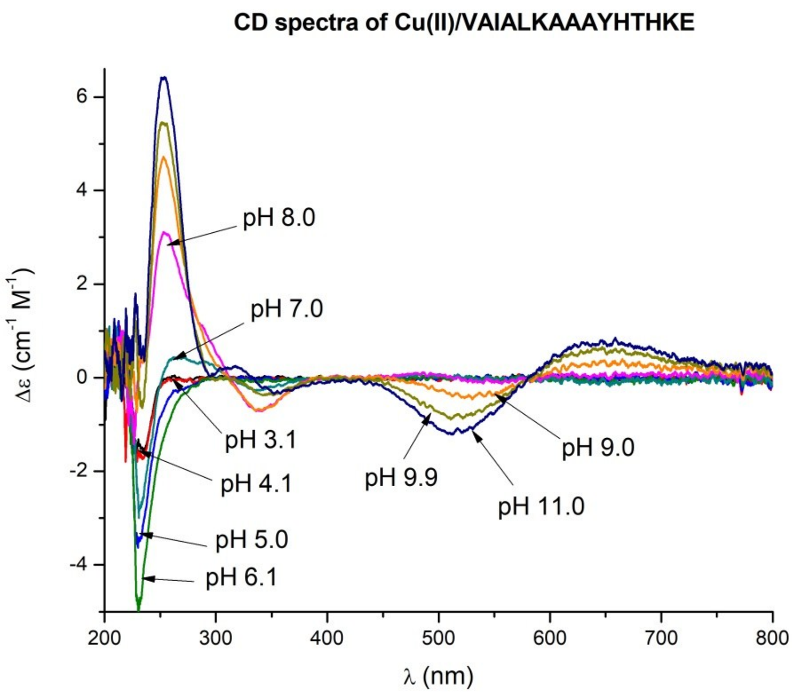


Figure S11. CD spectra [220–800 nm; optical path 1 cm] for Cu(II) complexes with H9A; M:L ratio = 0.9:1.

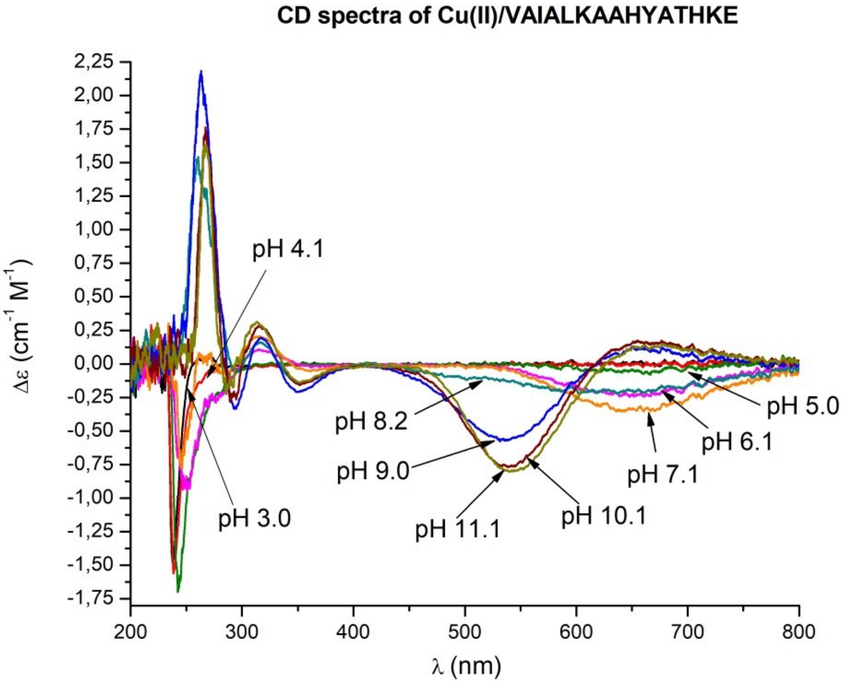


Figure S12. CD spectra [220–800 nm; optical path 1 cm] for Cu(II) complexes with H11A; M:L ratio = 0.9:1.

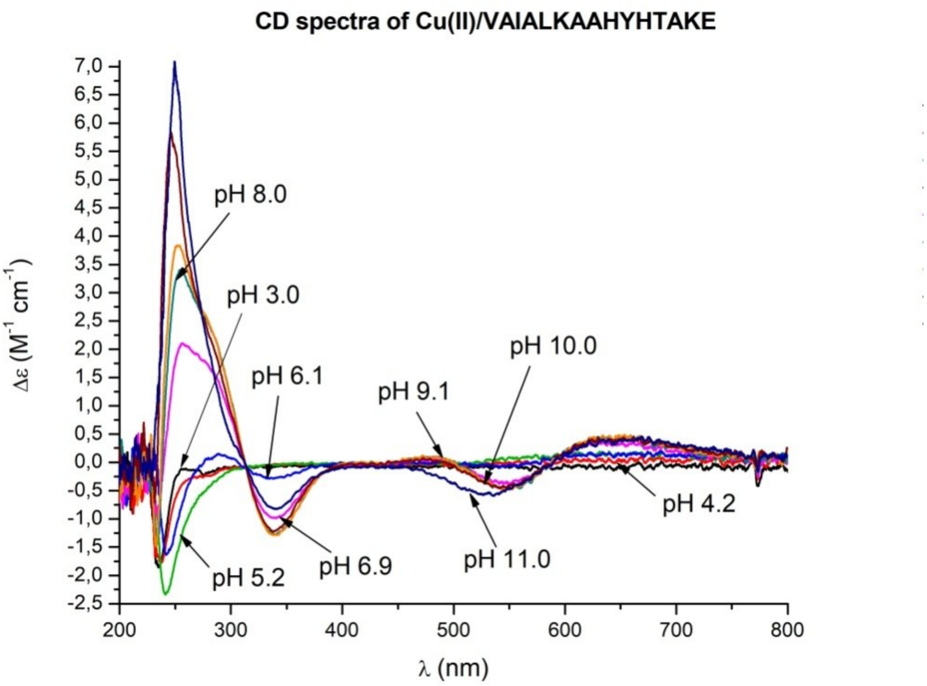
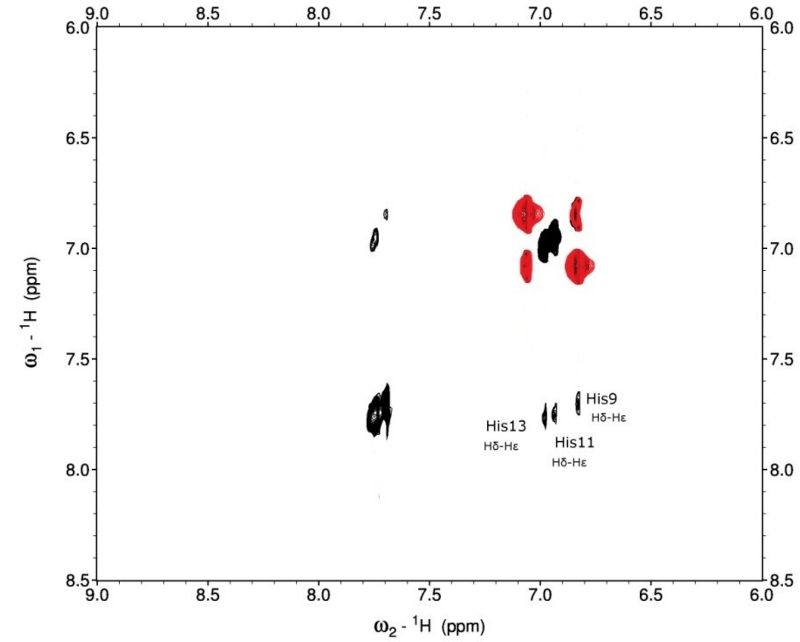
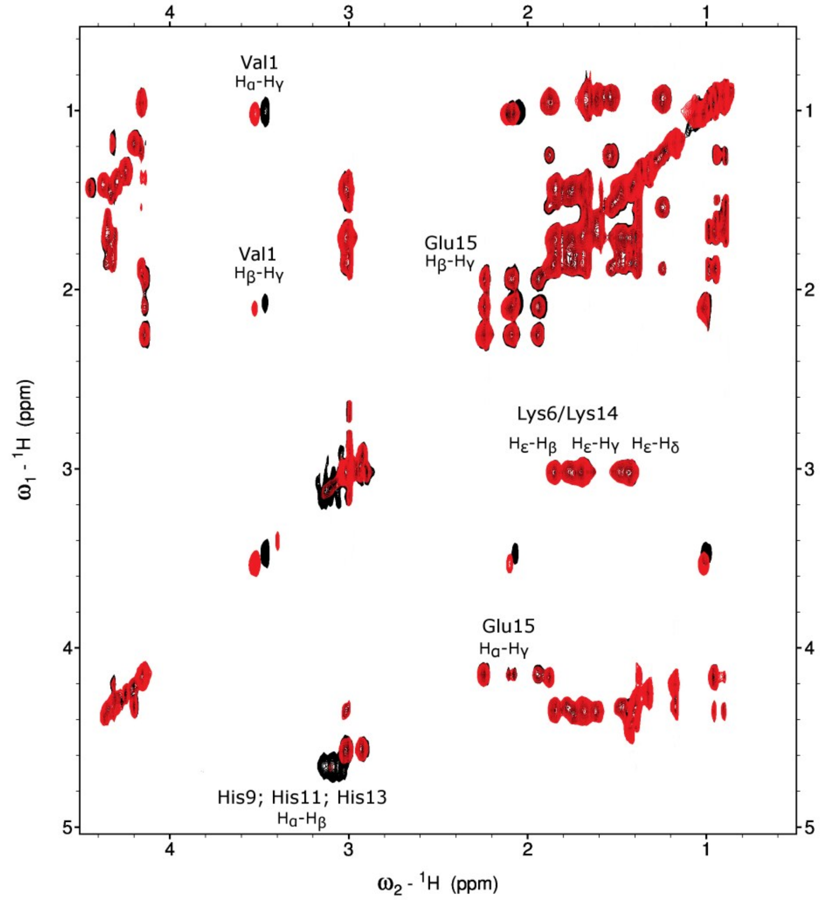


Figure S13. CD spectra [220–800 nm; optical path 1 cm] for Cu(II) complexes with H13A; M:L ratio = 0.9:1.

A)



B)



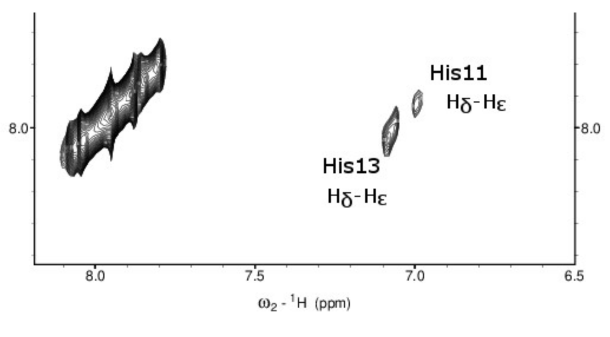
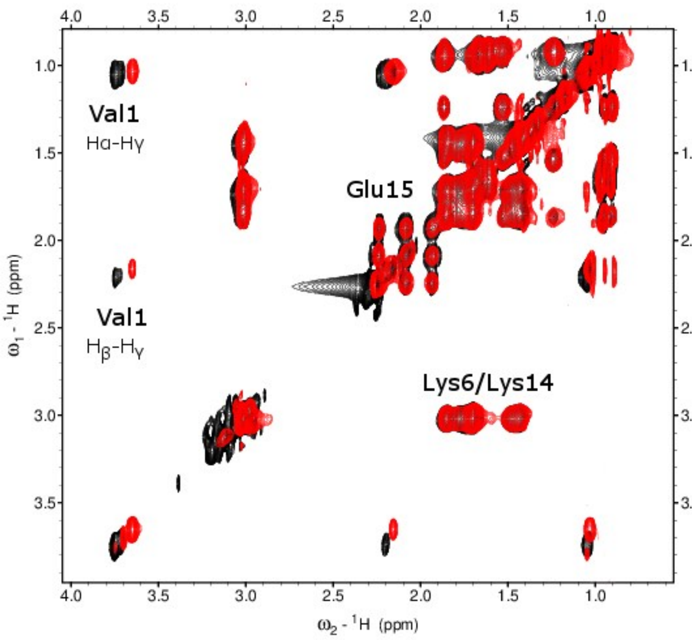


Figure S14. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM wild type calcitermin, *T* = 298 K, in the absence (black) and in the presence (red) of 0.1 Cu(II) eq., pH 7.4

A)

B)



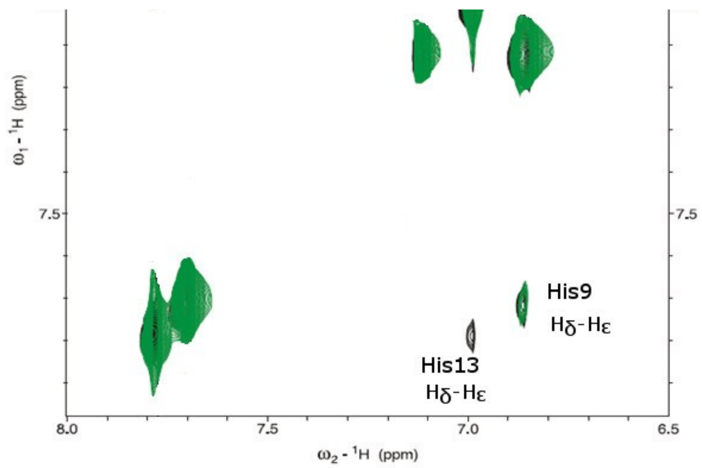
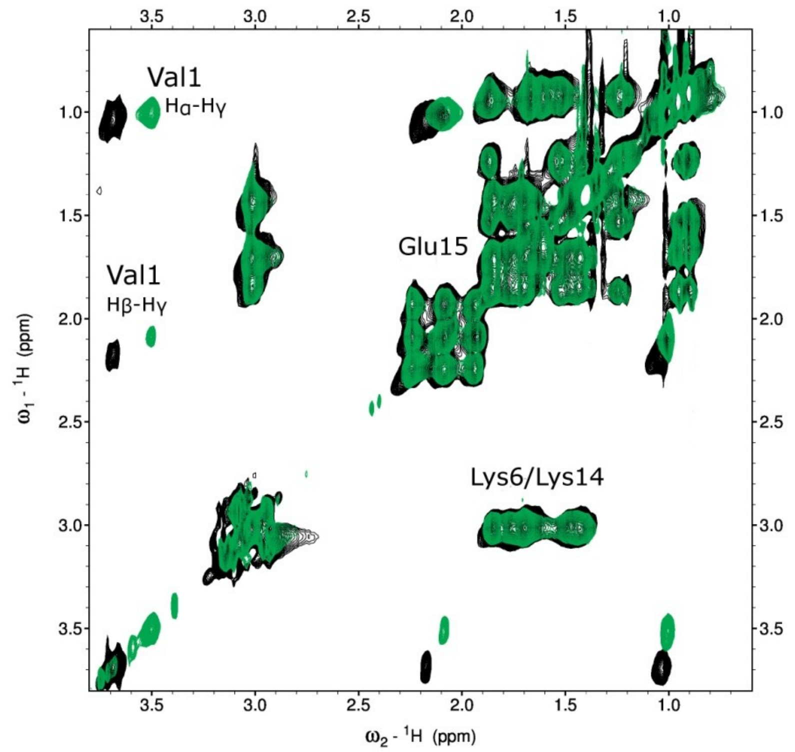


Figure S15. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM H9A mutant, *T* = 298 K, in the absence (black) and in the presence (red) of 0.1 Cu(II) eq., pH 7.4

A)

B)



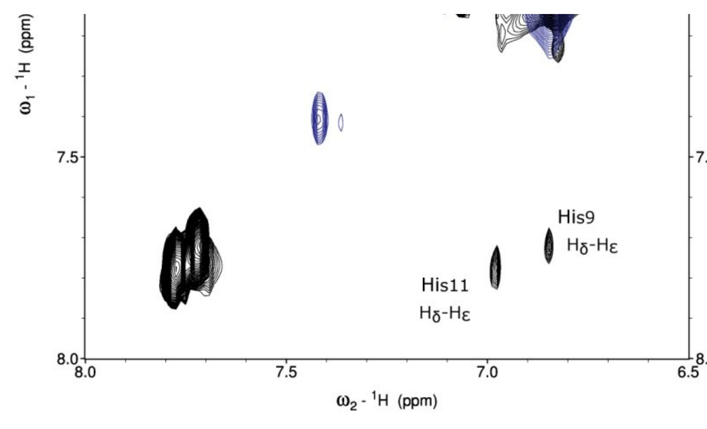


Figure S16. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM H11A mutant, *T* = 298 K, in the absence (black) and in the presence (green) of 0.1 Cu(II) eq., pH 7.4

A)

B)

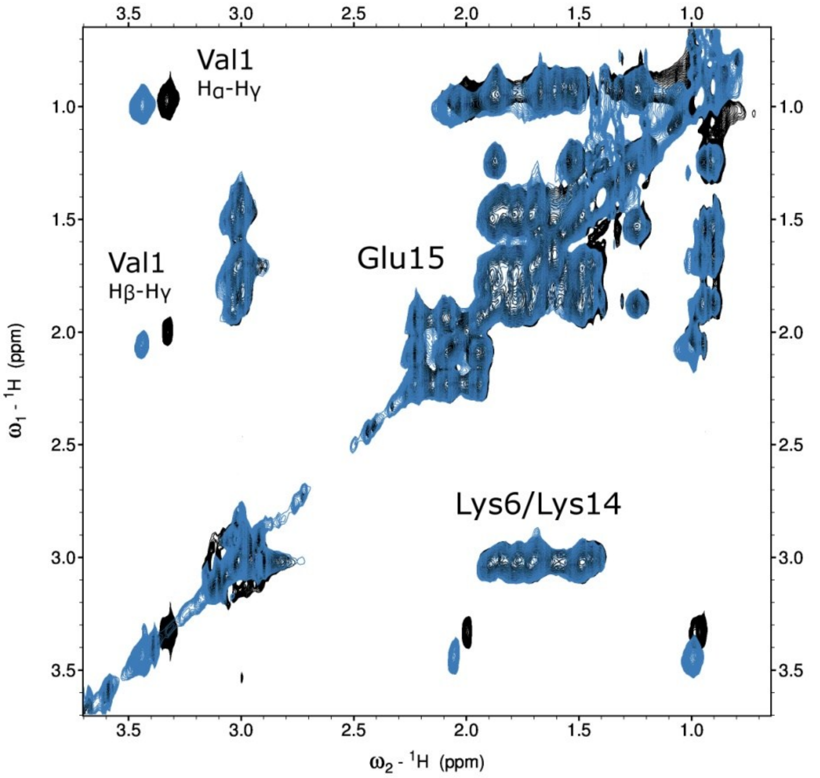


Figure S17. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM H13A mutant, T = 298 K, in the absence (black) and in the presence (blue) of 0.1 Cu(II) eq., pH 7.4

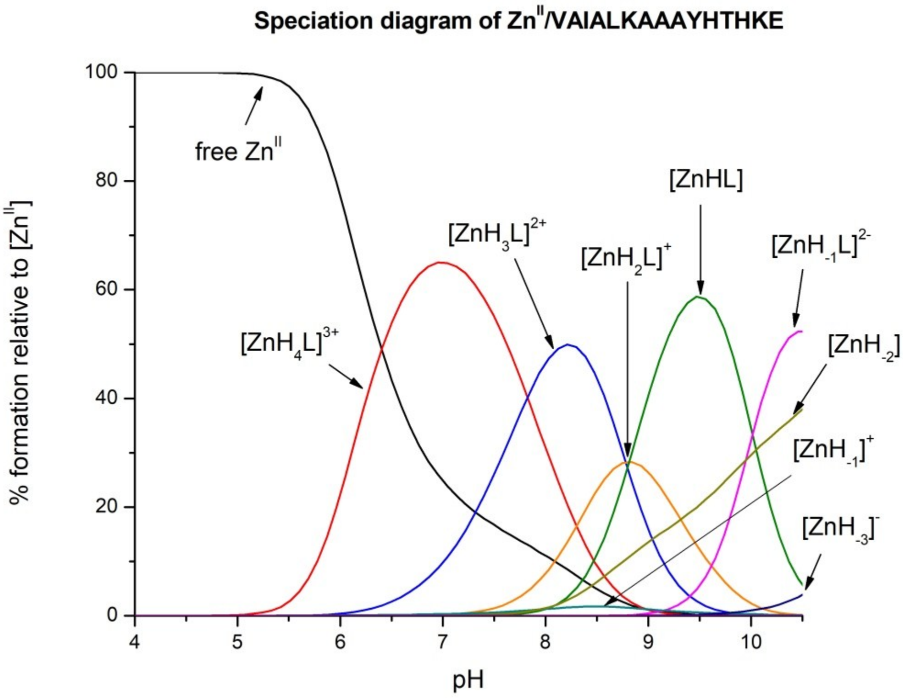


Figure S18. Exemplificative species distribution diagram relative to Zn(II)/H9A complexes; M:L molar ratio = 0.9:1.

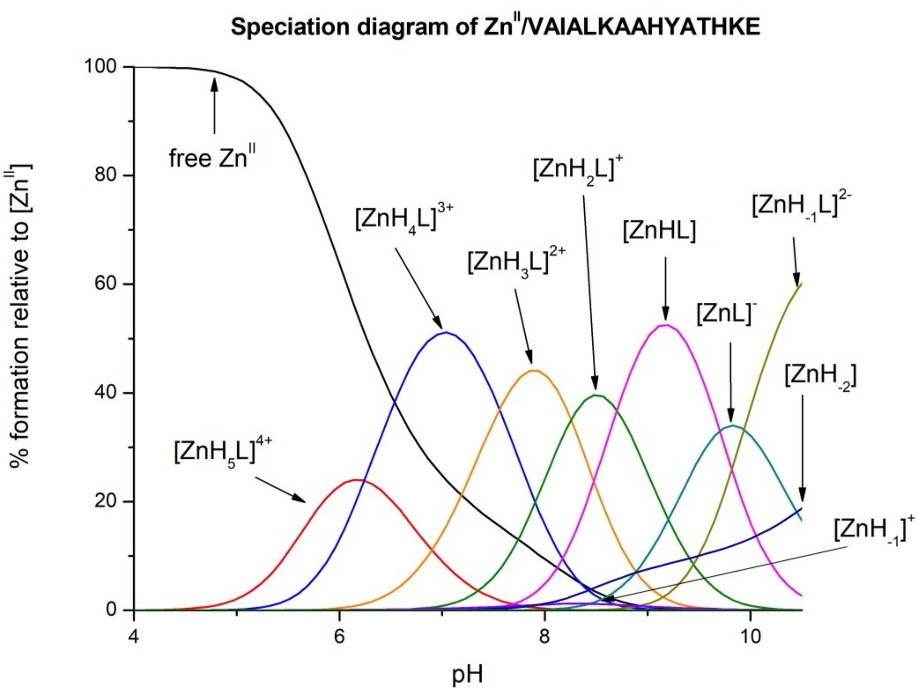


Figure S19. Exemplificative species distribution diagram relative to Zn(II)/H11A complexes; M:L molar ratio = 0.9:1.

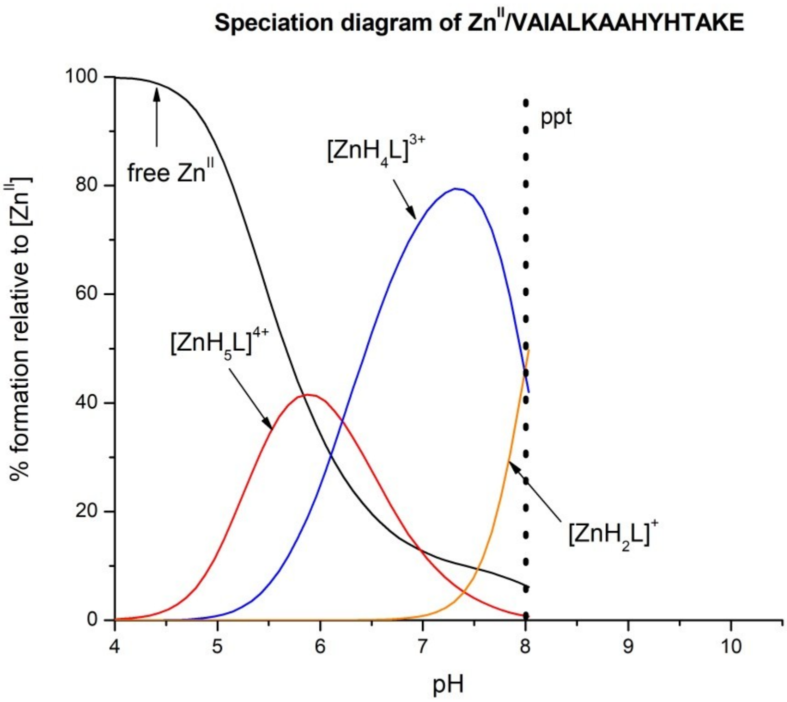
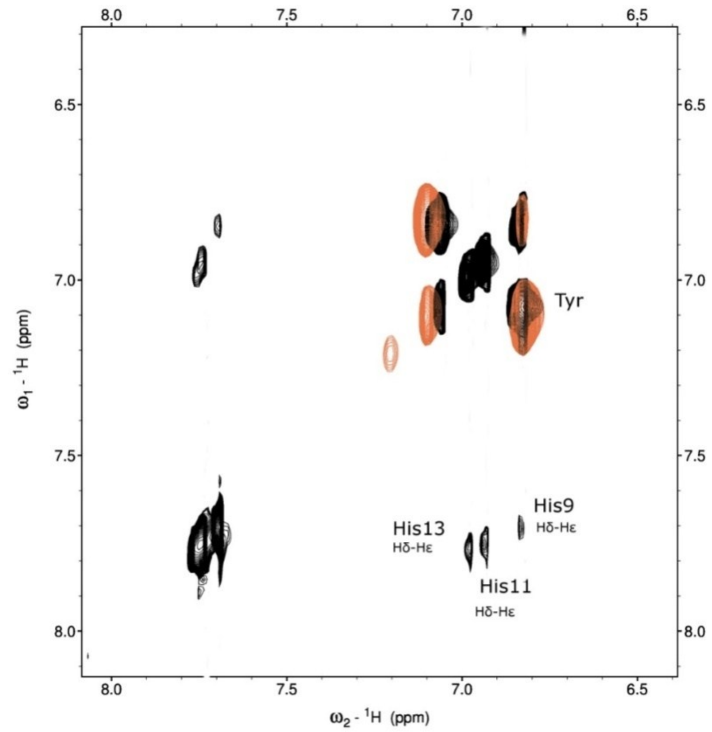


Figure S20. Exemplificative species distribution diagram relative to Zn(II)/H13A complexes; M:L molar ratio = 0.9:1.

A)



B)

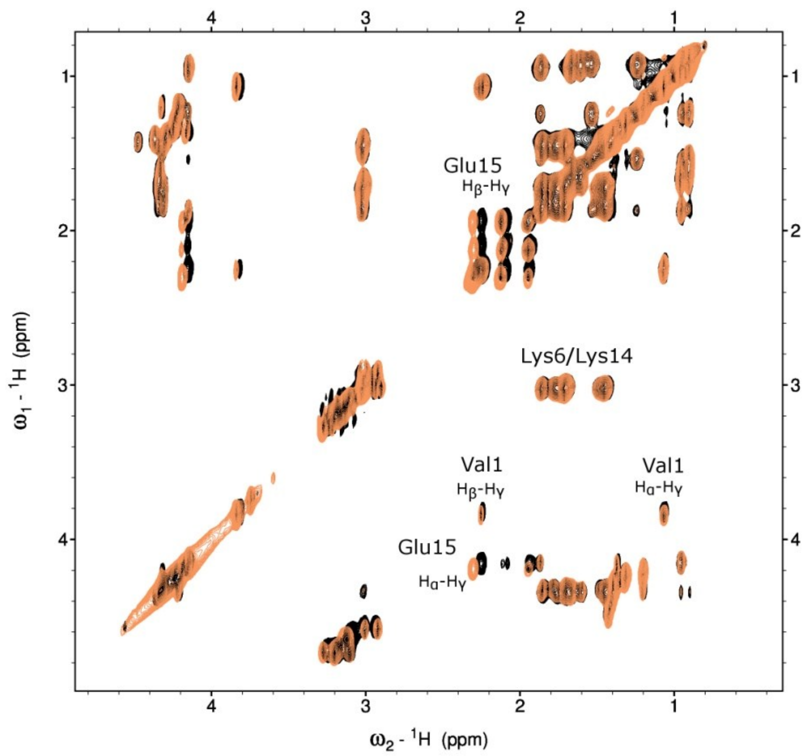
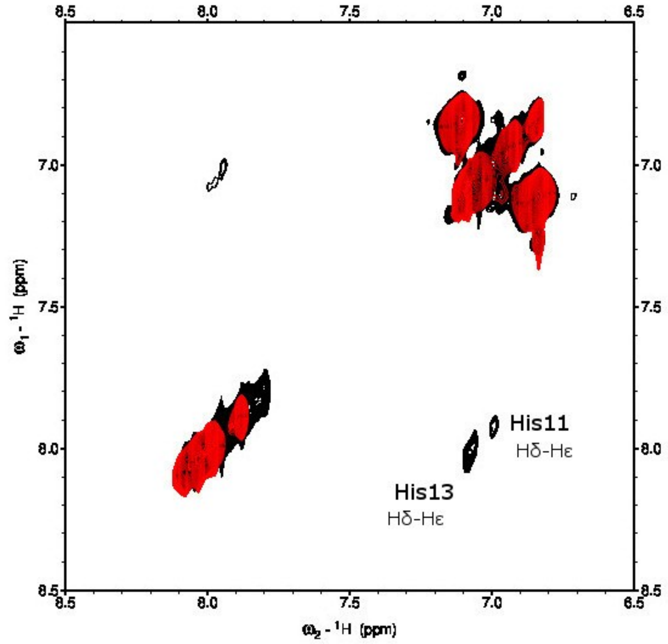


Figure S21. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM wild type calcitermin, *T* = 298 K, in the absence (black) and in the presence (orange) of 0.9 Zn(II) eq., pH 7.4

A)



B)

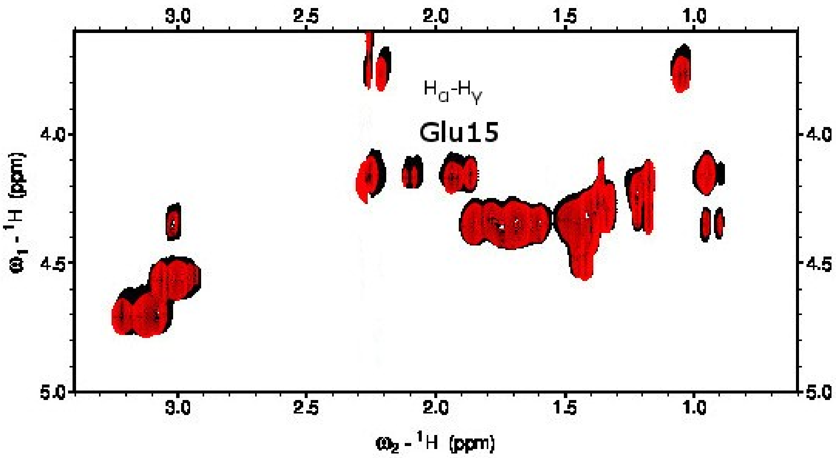
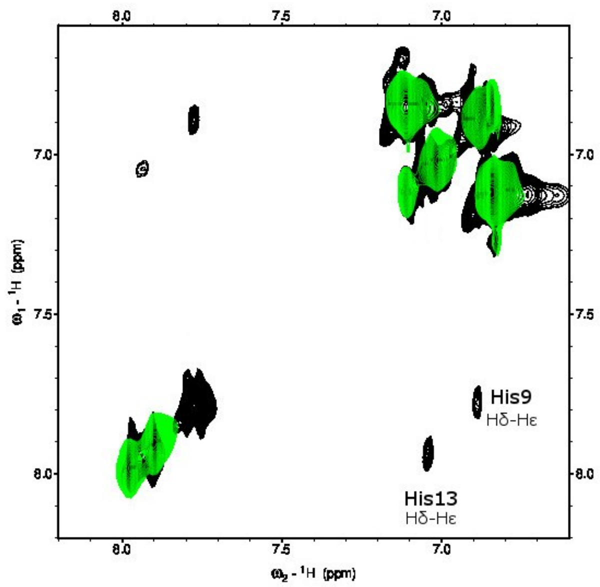


Figure S22. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM H9A mutant, T = 298 K, in the absence (black) and in the presence (red) of 0.9 Zn(II) eq., pH 7.4



A)

B)

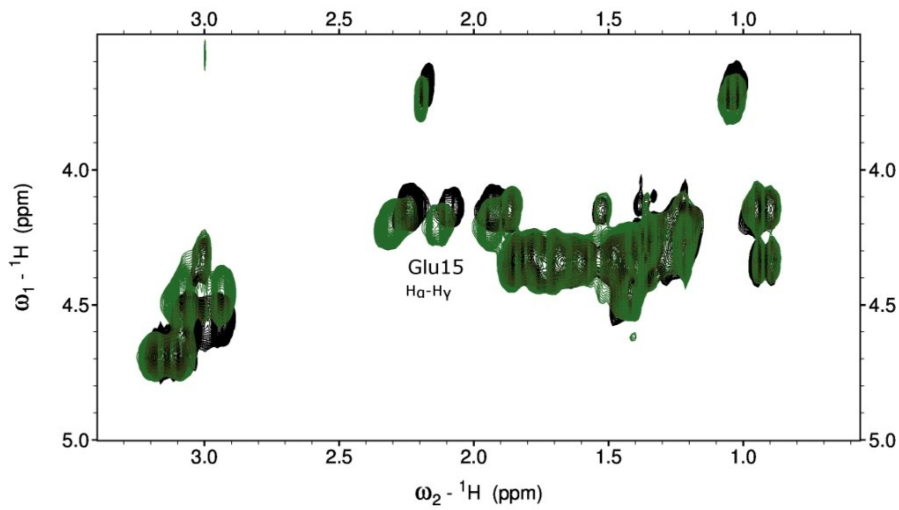
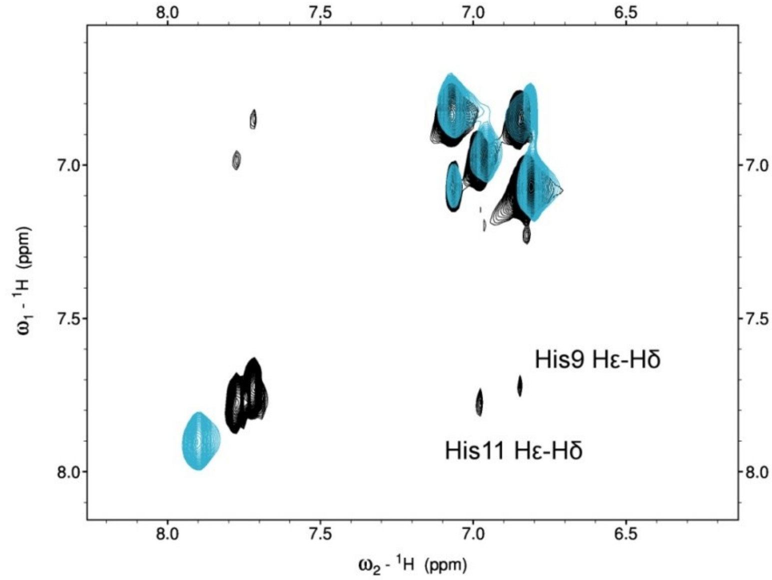


Figure S23. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM H11A mutant, T = 298 K, in the absence (black) and in the presence (green) of 0.9 Zn(II) eq., pH 7.4

A)



B)

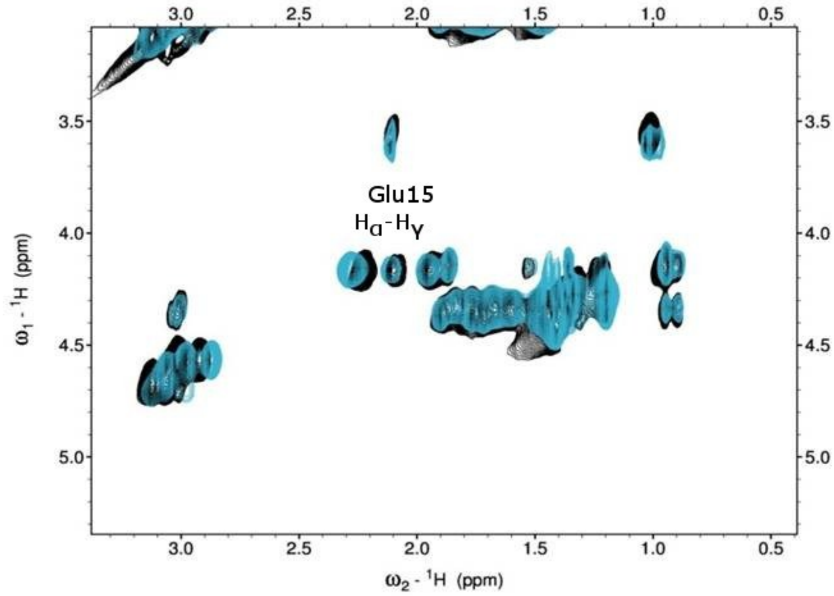


Figure S24. (A) Aromatic and (B) aliphatic region of ^{1}H-^{1}H TOCSY NMR spectra of 3.0 mM H13A mutant, *T* = 298 K, in the absence (black) and in the presence (blue) of 0.9 Zn(II) eq., pH 7.4

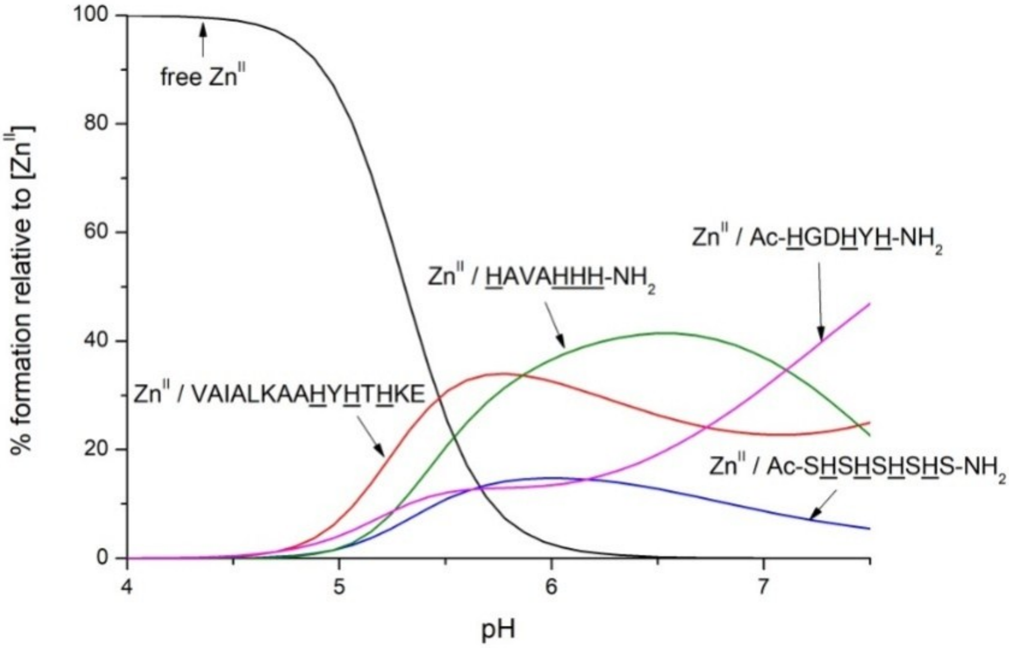


Figure S25. Competition plot for a solution containing equimolar concentration (1mM) of Zn(II), wild-type calcitermin VAIALKAAHYHTHKE, Ac-SHSHSHSHS-NH_2,[3] HAVAHHH-NH_2[4] and Ac-HGDHYH-NH_2.[5]

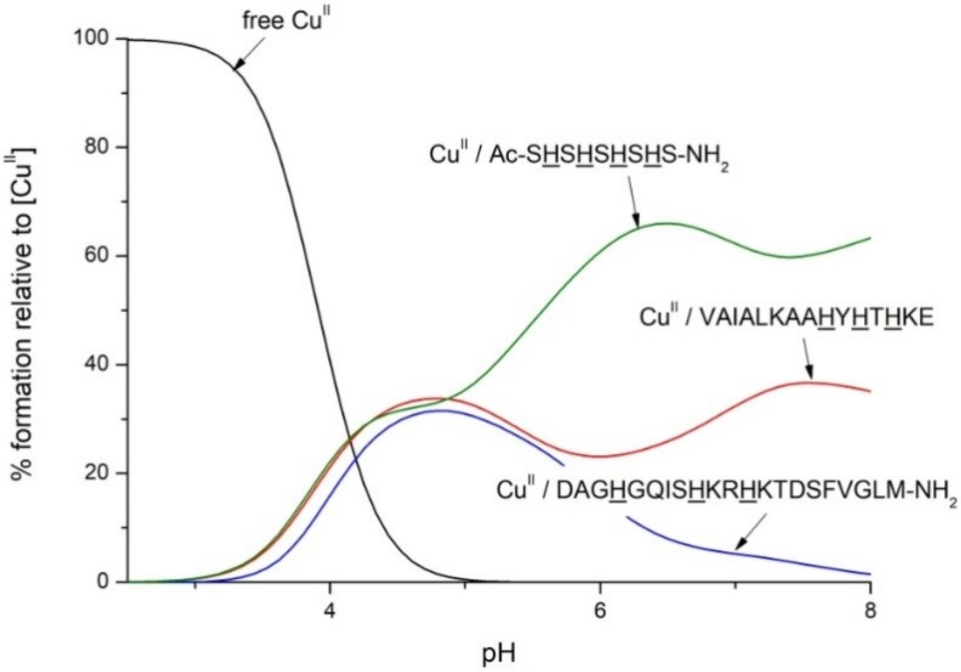


Figure S26. Competition plot for a solution containing equimolar concentration (1mM) of Cu(II), wild-type calcitermin VAIALKAAHYHTHKE, Ac-SHSHSHSHS-NH_2[3] and DAGHGQISHKRHKTDSFVGLM-NH_2.[6]

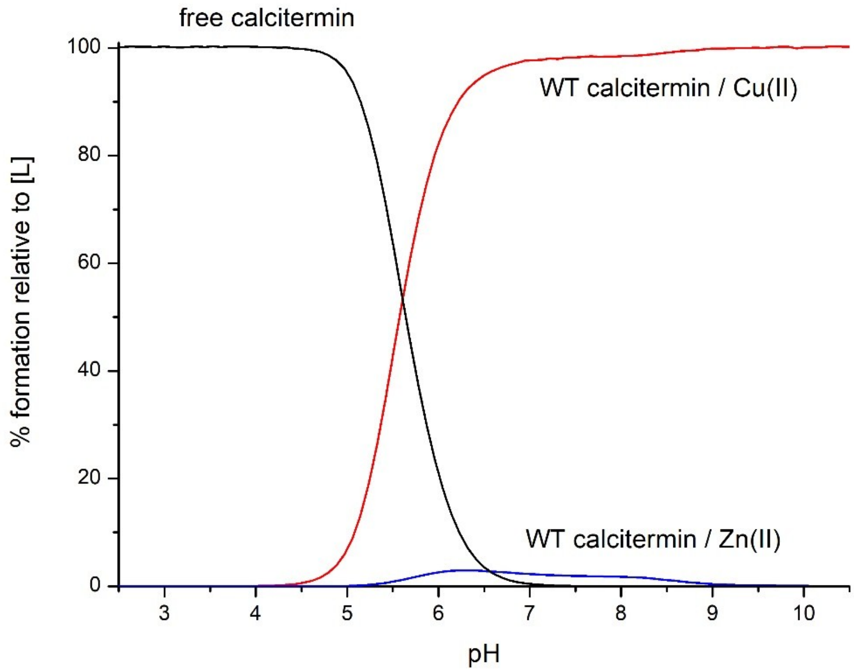


Figure S27. Competition plot for a solution containing Cu(II) (0.77 µM), Zn(II) (8.9 µM) and wild-type calcitermin VAIALKAAHYHTHKE (0.60 µM).

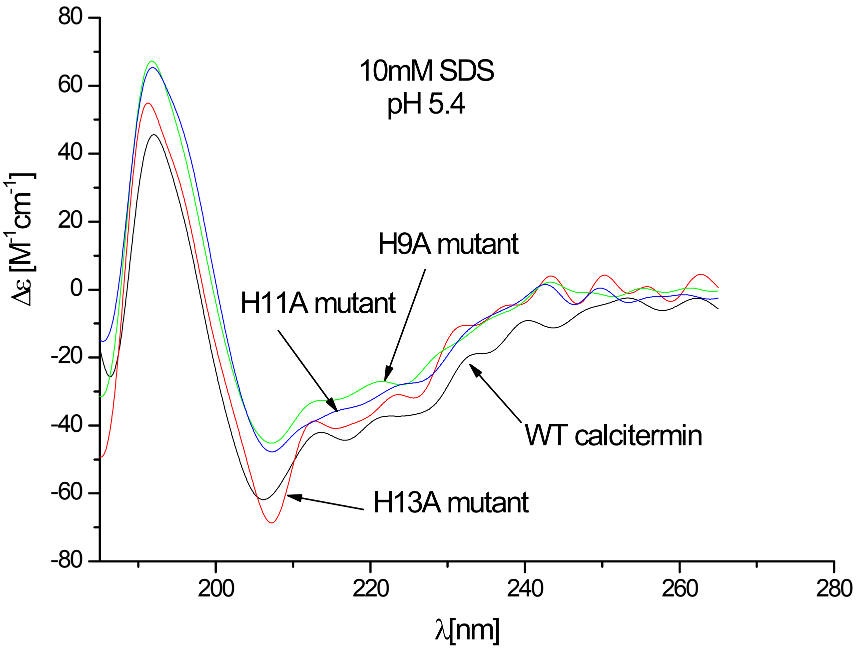


Figure S28. Comparison of CD spectra of WT calcitermin and its H9A, H11A and H13A mutants in 10mM SDS solution, pH 5.4.

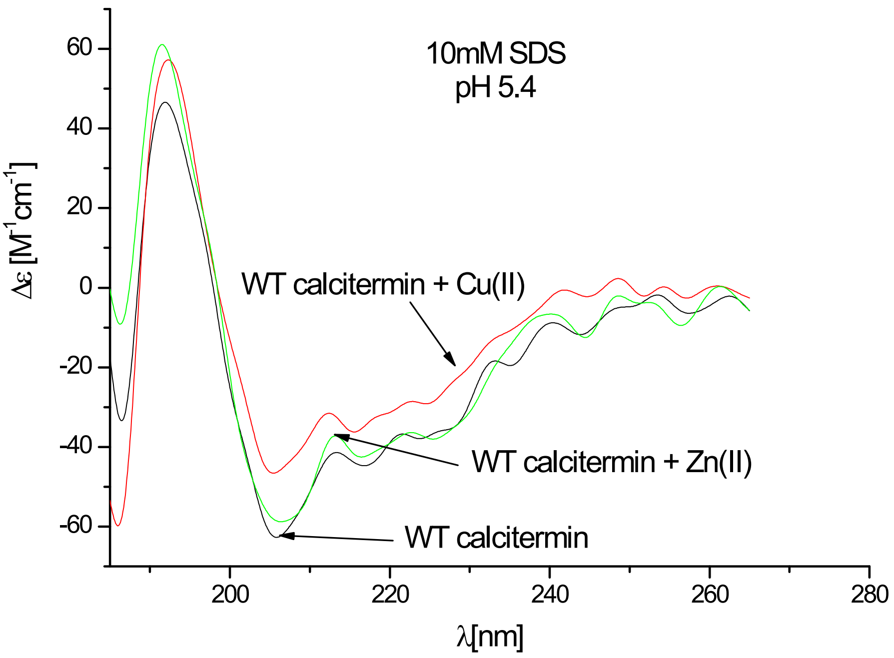


Figure S29. Comparison of CD spectra of WT calcitermin and its complexes with Zn(II) and Cu(II) in 10mM SDS solution, pH 5.4, M:L ratio = 1:1.

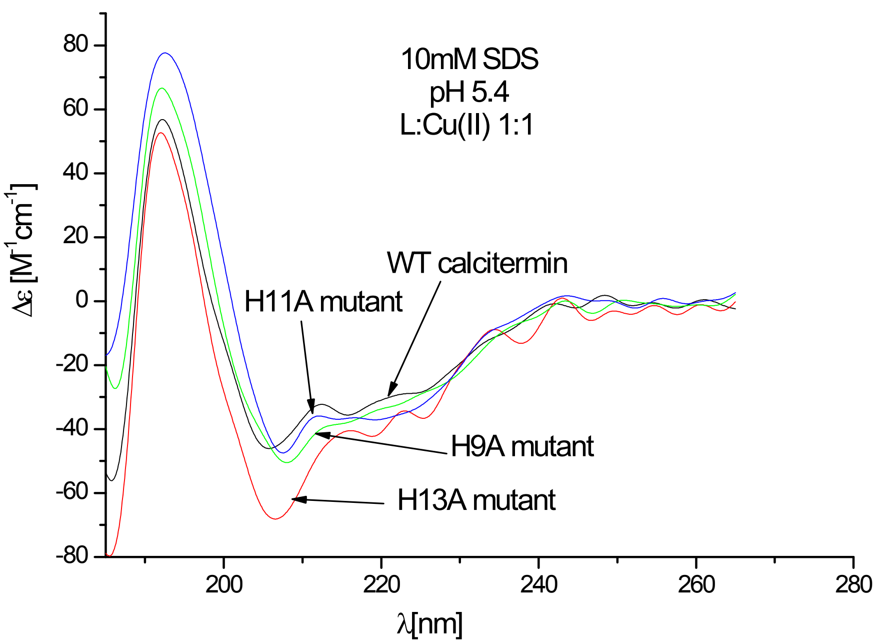


Figure S30. Comparison of CD spectra of Cu(II) complexes of WT calcitermin, H9A, H11A and H13A mutants in 10mM SDS solution, pH 5.4, M:L ratio = 1:1.

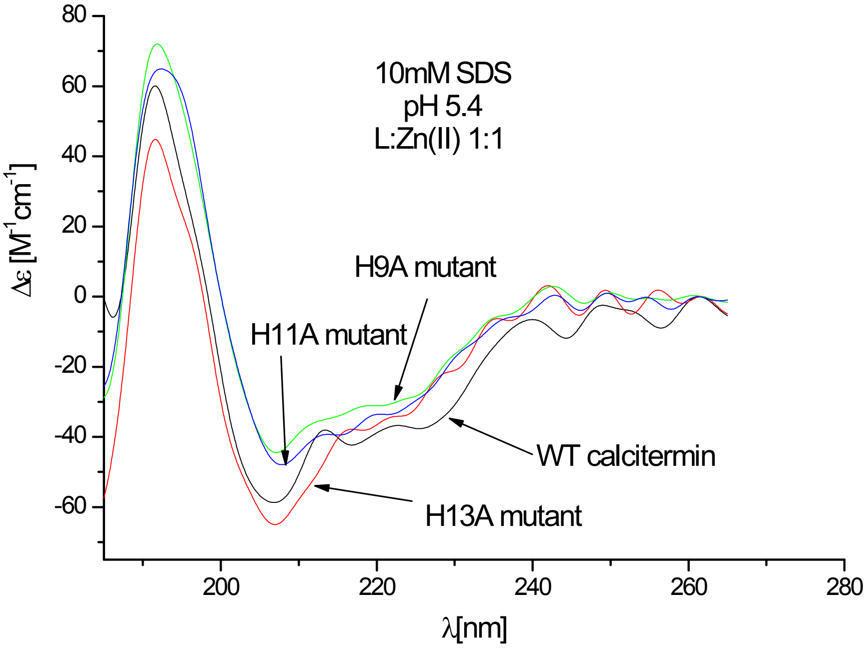


Figure S31. Comparison of CD spectra of Zn(II) complexes of WT calcitermin, H9A, H11A and H13A mutants in 10mM SDS solution, pH 5.4, M:L ratio = 1:1.

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