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Zinc(II) Complexes of Tripodal Peptides Mimicking the Zinc(II)-Coordination Structure of Carbonic Anhydrase

Ulrike Herr,[a] Werner Spahl,[a] Gunter Trojandt,[a] Wolfgang Steglich,[a,*] Florian Thaler[b] and Rudi van Eldik[b]

[a]*Institut für Organische Chemie, Universität München, Karlstraße 23, D-80333 München, Germany*
[b]*Institut für Anorganische Chemie, Universität Erlangen-Nürnberg, Egerlandstraße 1, D-91058 Erlangen, Germany*

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Abstract—Two new tripodal peptide ligands with histidine side chains have been synthesized and were shown to form stable zinc(II) complexes. Their NMR and mass spectra indicate a structure that is analogous to the active center of carbonic anhydrase. Both the ligands and the zinc complexes were titrated potentiometrically in order to obtain the pK_a values for the coordinated water of the zinc complexes; due to the low solubility of the complexes only estimates could be obtained.

Introduction

The enzyme carbonic anhydrase (CAII) catalyzes the reversible hydration of carbon dioxide with an impressively high turnover number ($10^6\ s^{-1}$, eq (1)).[1,2]

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \qquad (1)$$

This crucial and rather simple reaction has fascinated numerous research groups. Extensive studies have been carried out to elucidate the chemical and structural aspects of its mechanism.[2–8] The results of these investigations help to shed light upon the evolution of CAII into biochemically important noncatalytical functions, such as signal transduction.[9–13]

The structures of CAII in the zinc–water, zinc–hydroxide, and zinc–bicarbonate forms that are involved in the reaction, are well characterized.[2,5,14,15] The active site of CAII consists of a zinc(II) ion pseudotetrahedrally coordinated by three histidine imidazoles (His-96, -94, and -119) and either a water molecule or an OH^- ion in the fourth position as depicted in Figure 1.[14]

The zinc(II)-bound water molecule is deprotonated to form a zinc(II)-bound hydroxide species, which is widely accepted to be the catalytically active complex.[13,16,17] Thus, the ability of CAII to hydrate CO_2 is characterized by a pK_a value (~7) of this deprotonation.[18]

To mimic the zinc(II)-coordination structure or the function of the zinc(II) ion at the active site, several mononuclear model zinc(II) complexes have been designed and investigated (Fig. 2).[19–37]

Up to now, the best functional models of CAII are the zinc(II) complexes of 1,5,9-triazacyclododecane ([12]aneN$_3$) (**1**)[24–27] and 1,4,7,10-tetraazacyclododecane([12]aneN$_4$) (**2**)[28–30] in the forms $ZnL(H_2O)^{2+}$ and $ZnL(OH^-)^{2+}$. Woolley's enzyme model **3**[31] was also successful in reproducing catalysis of carbon dioxide hydrolysis. The pK_a values of complexes **1a**–**3a** were reported to approximate that of CAII very closely. The Zn(II)–OH^- complex **4**[32–34] was the first structural model of the active site of CAII to be isolated. Recently, Parkin et al.[37] synthesized and characterized the mononuclear zinc(II)-complex **5**, in which the zinc(II) ion is coordinated by three imidazole moieties.

However, there have been relatively few attempts to design and prepare zinc(II)-binding peptides.[38] Here, we report the synthesis of two peptide ligands with three histidine side chains featuring an environment analogous to the active center of CAII.

Results and Discussion

Synthesis of the tripodal histidine ligands

C_3-Symmetrical molecules are of particular interest as model compounds for natural ionophores and as ligands for metal complexation.[39,40] The optically pure C_3-symmetrical triacid (*SSS*)-N(BzGly*ValOH)$_3$ (**6**)

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*Corresponding author. Tel.: +49-89-5902-528; fax: +49-89-5902-604; e-mail: wos@org.chemie.uni-muenchen.de

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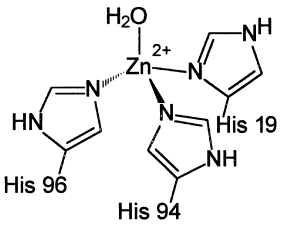


Figure 1. Active center of carbonic anhydrase.

proved to be an ideally preorganized template for building up longer tripodal peptides.[41,42] We therefore chose **6** as starting material for the synthesis of zinc(II)-binding pseudo-peptides. To introduce the histidine moiety we used a π-Bom protected histidine ester since its derivatives have been successfully applied in suppressing racemization during coupling of histidine.[43] The side chain protected histidine ester **7** was obtained by coupling the template (*SSS*)-**6** with H-His(π-Bom)OMe·2HCl, which could be easily obtained by deprotection of commercially available Boc-His(π-Bom)OMe·HCl with HCl in ethyl acetate.[44] Subsequent cleavage of the Bom groups by hydrogenolysis in 80% acetic acid[45,46] yielded the chiral tripodal histidine ligand **8** (Scheme 1).

In order to increase the water solubility of the histidine ligand, we elongated the three peptide chains by a serine residue (Scheme 2). Saponification[47] of the pseudo-nonapeptide **7** with lithium hydroxide yielded the pseudo-nonapeptide **9**. The synthesis of the π-Bom protected ligand **10** could be achieved by coupling of **9** with L-serine methyl ester. In contrast to the synthesis of **7**, the reaction had to be performed in DMF. Furthermore, the workup of **10** was only possible by using chloroform for extraction, which indicates a higher water solubility of the serine derivative. In order to cleave the Bom groups by hydrogenolysis to generate the more polar histidine ligand **11**, pressure (4 bar) had to be applied.

Synthesis of the zinc(II) complexes

The reaction of the histidine ligands **8** and **11** with equimolar amounts of $Zn(ClO_4)_2 \cdot 6H_2O$ in ethanol afforded the zinc(II) complexes **12** and **13**, respectively, as white precipitates that could be separated by centrifugation. Structural assignment for the zinc(II) complexes was achieved by FAB/ESI HRMS and NMR spectra.

The FAB mass spectrum of the zinc(II) complex of $N(BzGly^*ValHisOMe)_3$ (**12**) exhibited peaks at *m/z* 1400 $[M-H+K]^+$, 1362 $[M-H]^+$ and 1299 $[L]^+$. The peak at *m/z* 1362 has been confirmed by FAB HRMS. In the ESI mass spectrum peaks at *m/z* 1400 $[M-H+K]^+$, 1299 $[L]^+$ and 650 (100) $[L]^{2+}$ were found. The ^1H NMR spectra of the tripodal histidine ligand **8** and its resulting zinc(II) complex **12** (Fig. 3) show close similarities except for the imidazole 2-H and 4-H protons. For the peptide ligand **8**, a broad signal at $\delta = 6.90$ for the His 4-H protons and a sharp singlet at $\delta = 7.53$ for the His 2-H protons were obtained. These data are comparable with ^1H NMR data for histidine derivatives reported in the literature.[48,49] In the ^1H NMR spectrum of the zinc(II) complex **12**, the resonances of these protons are significantly shifted downfield ($\delta = 7.0$ and $\delta = 8.08$, respectively) and broadened. Thus, these data support coordination of the zinc(II) ion by the three histidine ligands and a close structural analogy to the active center of carbonic anhydrase.

The ESI mass spectrum of the zinc(II) complex of $N(BzGly^*ValHisSerOMe)_3$ (**13**) showed peaks at *m/z* 1625 $[M+H]^+$, 1623 $[M-H]^+$, 1583 $[L+Na]^+$, 1561 $[L+H]^+$, and 812 $[M]^{2+}$. The base peak at *m/z* 812 strongly supports the complex formation. The molecular composition $C_{72}H_{93}N_{19}O_{21}Zn$ at *m/z* 1625 has been confirmed by ESI HRMS. In the ^1H NMR spectrum of the zinc(II) complex **13**, we also observed a remarkable

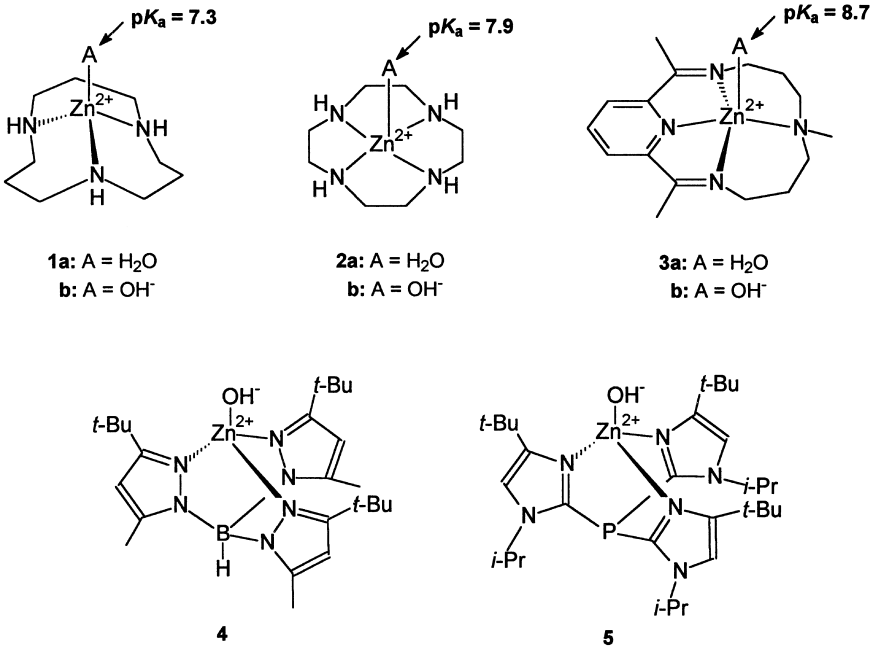
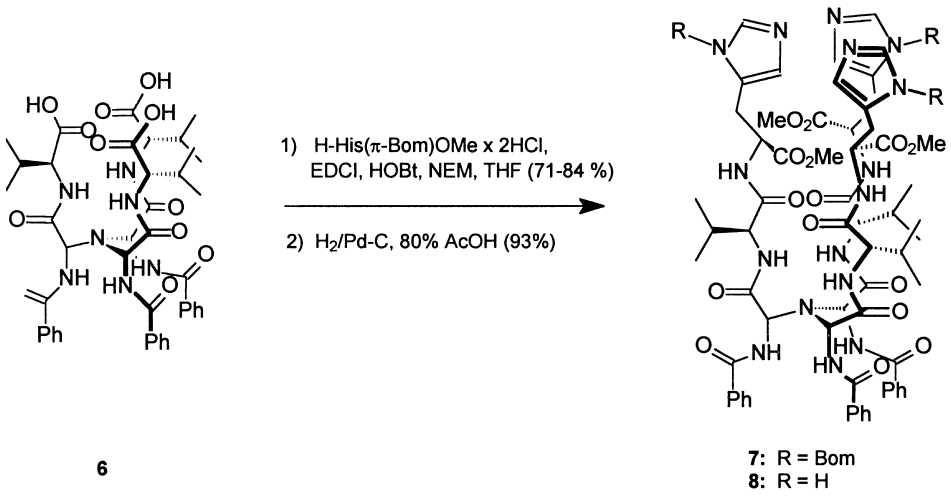
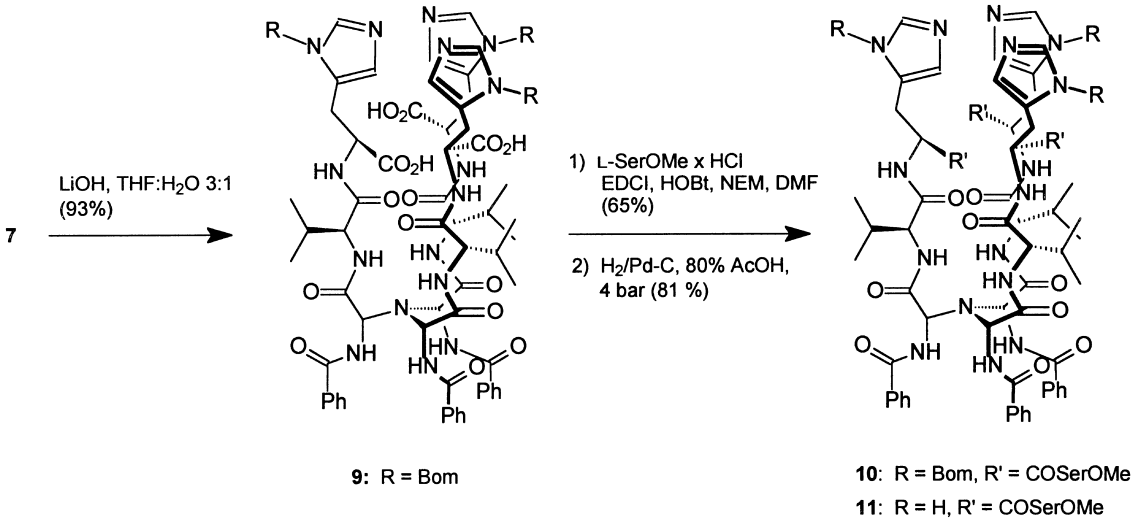


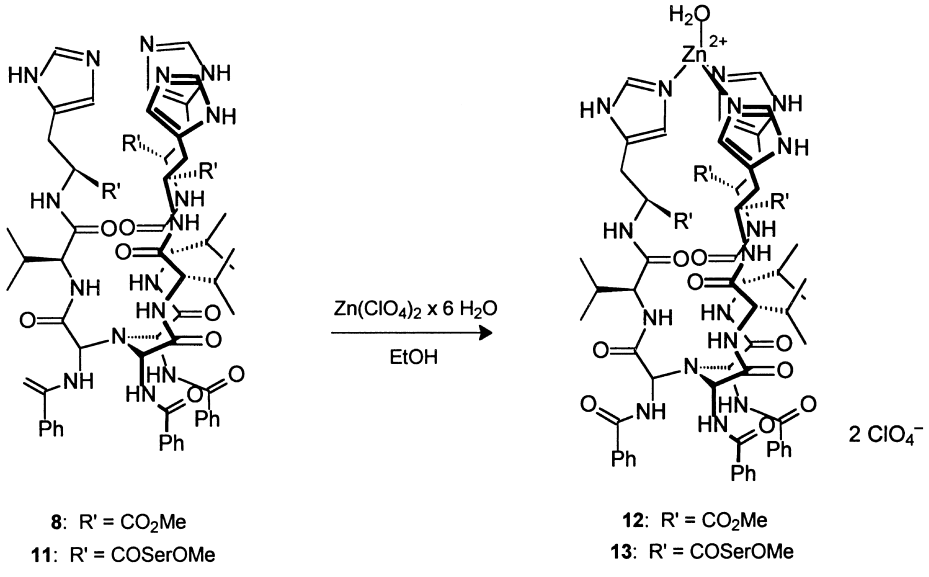
Figure 2. Mononuclear model complexes for carbonic anhydrase.



Scheme 1. Synthesis of the tripodal histidine ligand **8**.



Scheme 2. Preparation of the tripodal histidine ligand **11**.



Scheme 3. Preparation of the zinc(II) complexes **12** and **13**.

downfield shift of the imidazole 2-H and 4-H protons (Fig. 4). For the peptide ligand **11**, the resonances of the His 4-H and the His 2-H protons were found at $\delta = 6.88$ and 7.55, respectively. The corresponding signals of the zinc(II) complex **13** were significantly broadened and appeared at $\delta = 6.94$ and $\delta = 8.13$, respectively. Interestingly, the resonance of the NH-imidazole protons of **13** was shifted significantly to lower field ($\Delta\delta = 0.83$).

Protonation and complex-formation constants

The protonation constants (K_n) of the ligand N(BzGly*ValHisOMe)$_3$ (**8**) were determined by potentiometric pH-titration of **8**·3H$^+$ using 0.10 M NaOH at an ionic strength of $I = 0.10$ ($NaClO_4$) at 25.0 °C. The titrations were performed in 33% aqueous ethanol due to the insolubility of the ligand in pure water. The titration curve of **8** is depicted in Figure 5(a).

The titration curve shows an inflection at 3 equiv of base. Titration data were analyzed for equilibria 1–3 (eqs (2–4)). The calculated protonation constants (log K_n) are summarized in Table 1. The protonation constants are defined as follows:

$$H^+ + L \rightleftharpoons HL^+ K_1 = [HL^+]/[H^+][L] \quad (2)$$

$$H^+ + HL^+ \rightleftharpoons H_2L^{2+} K_2 = [H_2L^{2+}]/[H^+][HL^+] \quad (3)$$

$$H^+ + H_2L^{2+} \rightleftharpoons H_3L^{3+} K_3 = [H_3L^{3+}]/[H^+][H_2L^{2+}] \quad (4)$$

For determination of the complex stability constant and the pK_a of the coordinated water molecule, **8**·3H$^+$ was

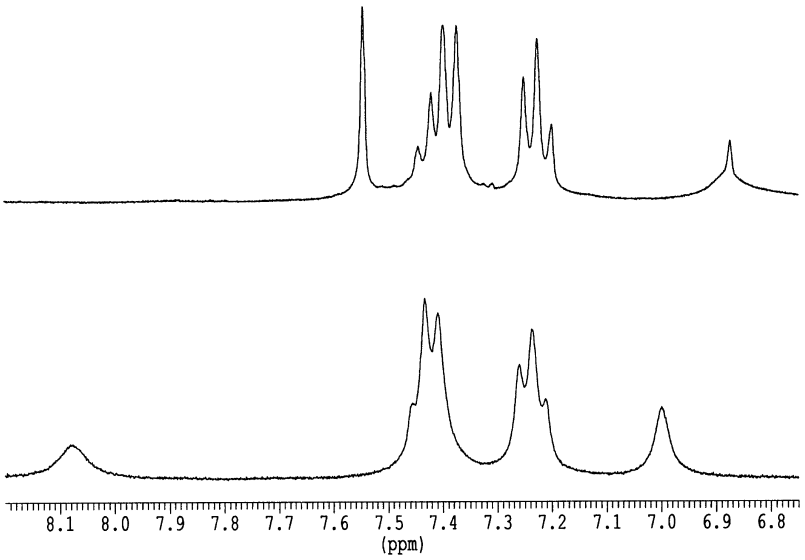


Figure 3. Aromatic region of the 300 MHz ^{1}H NMR spectra (DMSO-d_6) of histidine ligand **8** and its zinc(II) complex **12**.

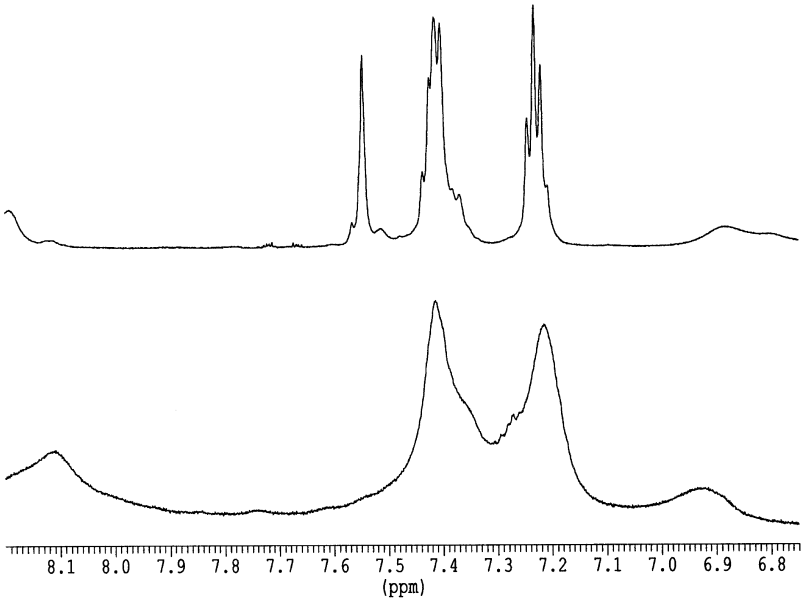


Figure 4. Aromatic region of the 600 MHz ^{1}H NMR spectra (DMSO-d_6) of histidine ligand **11** and its zinc(II) complex **13**.

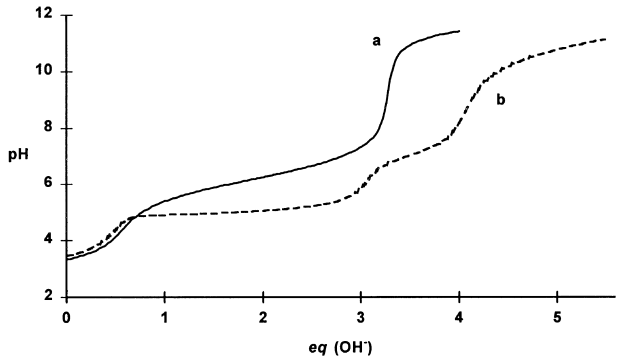


Figure 5. pH-Titration curves for N(BzGly*ValHisOMe)$_3$ (**8**) at $I = 0.10$ ($NaClO_4$) and 25.0 °C in 33% EtOH: (a) 1.00 mM $[L{\cdot}3H^+]^{3+}$; (b) solution **a** + 1.00 mM $Zn^{2+}SO_4$. *eq*(OH^-) is the moles of base (0.10 M NaOH) per mole of ligand.

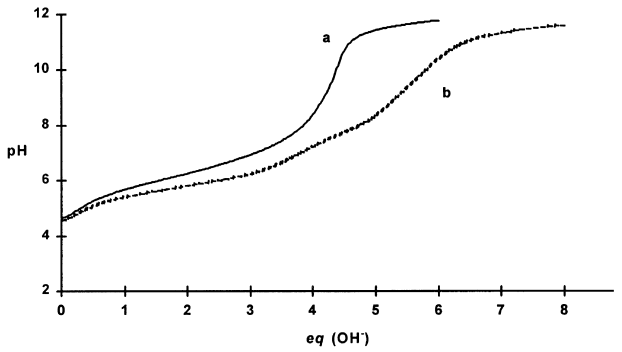


Figure 6. pH-Titration curves for N(BzGly*ValHisSerOMe)$_3$ (**11**) at $I = 0.10$ ($NaClO_4$) and 25.0 °C in 52% EtOH: (a) 1.00 mM $[L{\cdot}3H^+]^{3+}$; (b) solution **a** + 1.00 mM $Zn^{2+}SO_4$. *eq*(OH) is the moles of base (0.10 M NaOH) per mole of ligand.

Table 1. Comparison of the protonation constants of histidine ligands **8** (33% EtOH) and **11** (52% EtOH) and zinc(II) complexation constants of **11** (25.0 °C, $I = 0.10$ M, $NaClO_4$)

	Ligand **8**	Ligand **11**
log K_1	7.32 ± 0.03	6.73 ± 0.01
log K_2	6.78 ± 0.03	6.13 ± 0.01
log K_3	5.04 ± 0.02	5.80 ± 0.01
log K_{L*}		−7.58 ± 0.01
log K_{ZnL}		5.59 ± 0.05
log K_a		−6.19 ± 0.05

titrated in the presence of an equimolar amount of zinc(II) using 0.10 N NaOH. The equilibria are defined as follows (eqs (5) and (6)):

$$Zn(H_2O)^{2+} + L \rightleftharpoons ZnL(H_2O)^{2+}$$
$$K_{ZnL} = [ZnL(H_2O)^{2+}]/[Zn(H_2O)^{2+}][L] \quad (5)$$

$$ZnL(H_2O)^{2+} \rightleftharpoons ZnL(OH)^{+} + H^{+}$$
$$K_a = [ZnL(OH)^{+}][H^{+}]/[ZnL(H_2O)^{2+}] \quad (6)$$

Since during the titration the complex precipitated at a pH of about 5, the constants could not be calculated. The titration curve is shown in Figure 5(b).

The titration curves of **11** are depicted in Figure 6.

The protonation constants (K_n) of the histidine ligand **11** were determined by potentiometric pH-titration of **11**·3H$^+$ using 0.10 M NaOH at an ionic strength of $I = 0.10$ ($NaClO_4$) at 25.0 °C (Fig. 6(a)). In contrast to the titration curve of **8**, an inflection occurred at 4 equiv of base. Consequently, an additional consumption of 1 equiv of base is taking place. We assume therefore that the fourth proton could possibly be released from one of the serine hydroxy groups. The pK_a value of the hydroxy group in serine is about 12–13, but in the case of the protease chymotrypsin a significant perturbation of the pK_a value of serine 195 can be observed. The pK_a value is shifted dramatically to a value of about 7 due to the coordination to histidine 57.[50–52] The large perturbation in the pK_a of amino acids can also be found in other enzyme systems.[53]

Titration data were analyzed for equilibria 1–4 (eqs (2)–(4), and (7)). The calculated protonation constants (log K_{1-3}) and the deprotonation constant log K_{L*} are summarized in Table 1. The mixed protonation constants K_{1-3} are defined in the eqs (2)–(4). In addition, the deprotonation of the serine hydroxy group has to be considered (eq (7)).

$$L \rightleftharpoons L^* + H^+ K_{L^*} = [L^*][H^+]/[L] \quad (7)$$

when L* = deprotonated ligand.

For determination of the complex-formation constant and the pK_a of the coordinated water molecule, **11**·3H$^+$ was titrated in the presence of an equimolar amount of zinc(II) using 0.10 N NaOH. Even though the serine groups have increased the polarity of **11** compared to the histidine ligand **8** the complex started to precipitate at pH 6, (i.e., after 4 equiv of base had been consumed). Three equivalents of protons are released from the histidine units when the complex is formed and the fourth equivalent of base is neutralized either by the coordinated water of the zinc complex or is due to the OH group of serine. In the latter case this would mean that the pK_a value of −7.58 ± 0.01 for the free ligand decreases towards a pK_a value of −6.19 ± 0.05 for the complex. In contrast to **8**, the complex was dissolved again at a pH of about 11.5. The equilibria are defined as follows (eqs (8) and (9)):

$$Zn(H_2O)^{2+} + L \rightleftharpoons ZnL(H_2O)^{2+}$$
$$K_{ZnL} = [ZnL(H_2O)^{2+}]/[Zn(H_2O)^{2+}][L] \quad (8)$$

$$ZnL(H_2O)^{2+} \rightleftharpoons ZnL(OH)^{+} + H^{+}$$
$$K_a = [ZnL(OH)^{+}][H^{+}]/[ZnL(H_2O)^{2+}] \quad (9)$$

or

$$ZnL(H_2O)^{2+} \rightleftharpoons ZnL^*(H_2O)^{+} + H^{+}$$
$$K_a = [ZnL^*(H_2O)^{+}][H^{+}]/[ZnL(H_2O)^{2+}]$$

when L* = deprotonated ligand

The calculated complex formation constant (log K(ZnL)) and the deprotonation constant (log K_a) are summarized in Table 1.

Conclusion

The structural features of the zinc(II) complexes **12** and **13** that can be concluded from the ^{1}H NMR and mass spectra indicate that both peptide ligands **8** and **11** feature an environment comparable to that of the active center of CAII. The potentiometric titrations of the ligands **8** and **11** could be carried out only in a water–ethanol mixture since both ligands have a low solubility in pure water. An unexpected observation was made in the case of the pseudo-peptide **11**, where there is possible evidence for the perturbation of the pK_a of the hydroxy group of one serine. While the zinc(II) complex of **8** has a low solubility at the given experimental conditions and no complex stability constant could be obtained, the zinc(II) complex has a higher solubility and a first deprotonation constant of the complex could be obtained. The pK_a value of 6.19 ± 0.05 is somewhat lower than for CAII (pK_a of about 7), but it is not completely certain that the proton is released from the coordinated water on the zinc or from the ligand itself (i.e., the hydroxy groups of serine).

Experimental

Instrumental analyses

THF was distilled from potassium under an atmosphere of dry argon. All common reagents and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. TLC was performed on aluminum-backed plates coated with silica gel 60 with F_{254} indicator. Optical rotations were determined with a Perkin–Elmer 241 polarimeter using a Na lamp; data are reported as follows: $[\alpha]_D^{25}$ (concentration in g per 100 mL, solvent). Melting points were determined with a Reichert thermovar apparatus and a Büchi 350 and are uncorrected. IR spectra were recorded on a Perkin–Elmer 1420 spectrometer and a Bruker IFS 45 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX-300 or a Bruker ARM-600 instrument. All chemical shifts were recorded in ppm downfield from TMS on the δ scale. Mass spectra were recorded on a Finnigan MAT90 and a Finnigan MAT95Q (high resolution mass spectra). Elemental analyses were performed by the University of Munich Microanalytical Laboratory.

Synthesis of the histidine peptide ligands

***N,N′,N″*-[(*SSS*)-Nitrilotris[2-benzoylamino)-1-oxo-2,1-ethandiyl]]tris-[L-valyl-*N*(π)-benzyloxymethyl-L-histidine] trimethylester (7).** Compound (*SSS*)-**6** (499 mg, 0.59 mmol) and *N*(π)-benzyloxymethyl-L-histidine methyl ester dihydrochloride (851 mg, 2.35 mmol)[42] were suspended in THF (70 mL) and treated with NEM (0.74 mL, 5.90 mmol). After cooling to 0 °C, HOBt (478 mg, 3.54 mmol) and EDCI (566 mg, 2.95 mmol) were added. Stirring was continued for 1 h at 0 °C, then the reaction mixture was allowed to warm to room temperature (23 h). Ethyl acetate (75 mL) was added, and the organic layer was washed with saturated $NaHCO_3$ (2×70 mL) and 10% citric acid (5×70 mL). The combined citric acid extracts were neutralized with satd $NaHCO_3$ and extracted with ethyl acetate (5×70 mL). The ethyl acetate extracts were combined and washed with brine. Drying ($MgSO_4$) and evaporation gave a colorless solid, which was recrystallized from ethyl acetate/petroleum ether to afford 694–823 mg **7** (71–84%); mp 104–106 °C; R_f 0.70 (H_2O/methanol/$CHCl_3$, 2/20/80); $[\alpha]_D^{25}$ +10.5 (*c* 1.00, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (d, J=7 Hz, 9H, $CH(CH_3)_2$), 0.93 (d, J=7 Hz, 9H, $CH(CH_3)_2$), 2.12 (m, 3H, $CH(CH_3)_2$), 3.24 (m, 6H, CH_2-His), 3.69 (s, 9H, OCH_3), 4.26 (dd, J=7 Hz, 3H, CH-Val), 4.41 (d, J=12 Hz, 3H, OCH_2), 4.48 (d, J=12 Hz, 3H, OCH_2), 4.98 (m, 3H, CH-His), 5.34 (d, J=11 Hz, 3H, NCH_2), 5.49 (d, J=11 Hz, 3 H, NCH_2), 5.82 (d, J=9 Hz, 3H, NHC*H*N), 6.81 (s, 3 H, His4H), 7.11 (dd, J=8 Hz, 6H, *m*Ph), 7.28–7.35, 7.40 (m, 18H, d, J=7 Hz, 6H, Ph), 7.49 (s, 3H, His2H), 8.08 (d, J=9 Hz, 3H, NH-Val), 8.33 (d, J=7 Hz, 3H, NH-His), 8.62 (d, J=9 Hz, 3H, N*H*CHN); ^{13}C NMR (75.44 MHz, $CDCl_3$): δ 18.59, 19.31, 26.36, 30.73, 51.62, 52.44, 60.30, 63.49, 70.12, 73.49, 126.65, 127.42, 128.13, 128.27, 128.67, 129.94, 131.72, 132.89, 136.20, 138.69, 167.81, 169.00, 171.30, 171.36; IR (KBr): ν̃ 3300, 3020, 2960, 2860, 1740, 1660, 1600, 1580, 1510, 1480, 1340, 1280, 1210, 1090, 1070, 930, 750, 700, 660; FAB MS (NBA); *m*/*z* (%): 1600 (96) $[M]^+$; $C_{87}H_{102}N_{16}O_{18}$ (1659.87), calcd C 62.95, H 6.19, N 13.50; found, C 62.81, H 6.08, N 13.58.

***N,N′,N″*-[(*SSS*)-Nitrilotris[2-benzoylamino)-1-oxo-2,1-ethandiyl]]tris-[L-valyl-L-histidine] trimethyl ester (8).** The protected histidine ligand **7** (500 mg, 0.30 mmol) was dissolved in 80% aqueous acetic acid (40 mL) and treated with palladium on carbon (10%, 500 mg). The suspension was hydrogenated for 24 h (TLC control). The palladium on carbon was removed by filtration and the solvent was evaporated. The residue was neutralized with saturated $NaHCO_3$ and extracted with ethyl acetate (5×70 mL). The combined ethyl acetate extracts were washed with brine, dried ($MgSO_4$) and evaporated to yield **8** as a colorless solid (365 mg, 93 %); mp 145–147 °C; $[\alpha]_D^{25}$ −3.2 (*c* 1.00, methanol); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.92 (d, J=6 Hz, 18H, $CH(CH_3)_2$), 2.11–2.18 (m, 3H, $CH(CH_3)_2$), 2.94 (s, br, 6H, CH_2-His), 3.52 (s, 9H, OCH_3), 4.24 (dd, J=6 Hz, 3H, CH-Val), 4.51 (m, 3H, CH-His), 5.65 (d, J=9 Hz, 3H, NHC*H*N), 6.90 (s, br, 3H, His4H), 7.22 (dd, J=8 Hz, 6H, *m*Ph), 7.37–7.44 (m, 9H, Ph), 7.54 (s, br, His2H), 8.50 (m, br, 9H, N*H*CHN, NH-His, NH-Val), 11.81 (s, br, NH-Im); ^{13}C NMR (75.44 MHz, DMSO-d_6): δ 17.97, 19.06, 28.78, 29.98, 51.53, 52.62, 58.83, 59.65, 63.23, 126.81, 128.05, 131.75, 132.57, 166.41, 167.75, 170.76, 171.44; IR (KBr): ν̃ 3260, 3060, 2950, 1740, 1660, 1600, 1580, 1520, 1490, 1440, 1340, 1320, 1210, 1170, 1080, 980, 930, 800, 710, 690, 620; FAB MS (NBA); *m*/*z* (%): 1322 (9) $[M+Na]^+$, 1300 (53) $[M+H]^+$, 1299 (71) $[M]^+$, 105 (100) [Ph-C≡O]$^+$; FAB

HRMS (NBA) calcd for $C_{63}H_{79}N_{16}O_{15}$ $[M+H]^+$: 1299.5911, found (m/z) 1299.5987.

***N,N′,N″*-[(*SSS*)-Nitrilotris[2-benzoylamino)-1-oxo-2,1-ethandiyl-]]tris-[L-valyl-*N*(π)-benzyloxymethyl-L-histidine] trimethyl ester (9).** To a cooled solution of the triester **7** (400 mg, 0.241 mmol) in THF (9 mL) and water (3 mL), lithium hydroxide (24 mg, 0.963 mmol, 4 equiv) was added. After 2.5 h at 0 °C the solution was adjusted to pH 4.5 by addition of 1 N HCl. The volatile components (THF) were evaporated and the water removed by lyophilization. The resulting yellow solid was purified on a column of Sephadex LH 20, swollen and eluted with methanol to afford a colorless solid (361 mg, 93%); mp 168–170 °C.; $[\alpha]_D^{25}$ + 29.92° (c 1.00, methanol); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.87 (d, J = 7 Hz, 18H, $CH(CH_3)_2$), 2.14 (m, 3H, $CH(CH_3)_2$), 2.94–3.19 (m, 6H, CH_2-His), 4.26 (dd, J = 7 Hz, 3H, CH-Val), 4.45 (s, 6H, OCH_2), 5.44 (dd, J = 17, 11 Hz, 9H, CH-His, N-CH_2), 5.69 (d, J = 9 Hz, 3H, NHC*H*N), 6.75 (s, 3H, His4H), 7.20 (t, J = 8 Hz, 6H, *m*Ph), 7.24–7.43 (m, 24H, Ph), 7.70 (s, 3H, His2H), 8.50 (m, br, 9H, N*H*CHN, NH-His, NH-Val); ^{13}C NMR (75.44 MHz, DMSO-d_6): δ 17.95, 19.22, 25.83, 28.08, 29.99, 53.06, 59.33, 63.62, 69.15, 73.36, 126.90, 127.24, 127.48, 128.12, 128.51, 131.73, 132.54, 132.66, 137.25, 137.79, 166.57, 166.19, 170.34, 172.77; IR (KBr): ν̃ 3429, 3063, 2965, 2932, 1969, 1654, 1579, 1522, 1488, 1392, 1221, 1100, 1028, 930, 747, 698, 628; FAB MS (NBA); m/z (%): 1641 (0.81) $[M+Na]^+$; ESI MS; m/z (%): 1618 (5.12) $[M+H]^+$, 810 (100) $[M+2\ H]^{2+}$; ESI HRMS: calcd for $C_{84}H_{97}N_{16}O_{18}$ $[M+H]^+$ 1617.7167, found (m/z) 1617.7138.

***N,N′,N″*-[(*SSS*)-Nitrilotris[2-benzoylamino)-1-oxo-2,1-ethandiyl-]]tris-[L-valyl-*N*(π)-benzyloxymethyl-L-histidyl-L-serine] trimethyl ester (10).** To a stirred solution of **9** (700 mg, 0.44 mmol) in DMF (40 mL), H-L-Ser-OMe·HCl (275 mg, 1.77 mmol) and NEM (0.28 mL, 2.21 mmol) were added consecutively. After cooling to 0 °C, HOBt (358 mg, 2.65 mmol) and EDCI (423 mg 2.21 mmol) were added. Stirring was continued for 1h at 0 °C, and the reaction mixture was then allowed to warm to room temperature (23 h). The solvent was evaporated and the resulting yellow oil dissolved in $CHCl_3$ (40 mL). The solution was washed with saturated $NaHCO_3$ (2×40 mL) and 10% citric acid (6×40 mL). The combined citric acid extracts were neutralized with saturated $NaHCO_3$ and extracted with $CHCl_3$ (6×40 mL). The $CHCl_3$ extracts were combined and washed with brine. Drying ($MgSO_4$) and evaporation gave a colorless solid which was purified on a column of Sephadex LH 20, swollen and eluted with acetone/methanol (4/1) to afford a colorless solid (547 mg, 65%); mp 118–120 °C; R_f 0.70 (H_2O/methanol/$CHCl_3$, 2/20/80); $[\alpha]_D^{25}$ −2.94° (c 1.00, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.89 (d, J = 6 Hz, 9H, $CH(CH_3)_2$), 0.90 (d, J = 6 Hz, 9H, $CH(CH_3)_2$), 2.11 (m, 3H, $CH(CH_3)_2$), 2.96 (dd, J = 15 Hz, 8 Hz, 3H, CH_2-His), 3.11 (dd, J = 15 Hz, 5 Hz, 3H, CH_2-His), 3.58 (s, 9H, OCH_3), 3.58–3.61 (m, 3H, CH_2OH), 3.68–3.71 (m, 3H, CH_2OH), 4.26 (dd, J = 6 Hz, 3H, CH-Val), 4.34 (m, 3H, CH-Ser), 4.42 (s, 6H, OCH_2), 4.72 (m, 3H, CH-His), 5.11 (m, 3H, CH_2OH), 5.43 (s, 6H, N-CH_2), 5.70 (d, J = 9 Hz, 3H, NHCHN), 6.83 (s, 3H, His4H), 7.21 (t, J = 8 Hz, 6H, *m*Ph), 7.25–7.33 (m, 18H, Ph), 7.40–7.43 (m, 6H, Ph), 7.71 (s, 3H, His2H), 8.38 (d, J = 7 Hz, 3H, NH), 8.41 (d, J = 7 Hz, 3H, NH), 8.45 (d, J = 8 Hz, 3H, NH), 8.65 (d, J = 9 Hz, 3H, NH); ^{13}C NMR: (150.91 MHz, DMSO-d_6): δ 18.18, 19.21, 25.95, 30.40, 51.78, 51.86, 54.65, 58.90, 61.12, 63.81, 69.20, 73.40, 127.00, 127.30, 127.62, 127.86, 128.07, 128.25, 131.75, 132.80, 137.20, 138.18, 166.73, 167.91, 170.49, 170.58, 170.73; IR (KBr) ν̃ 3412, 3062, 2963, 1744, 1659, 1578, 1523, 1488, 1438, 1372, 1293, 1222, 1158, 1076, 1029, 931, 748, 698, 660, 607; ESI MS; m/z (%): 1943 (5) $[M+Na]^+$, 1921 (8) $[M+H]^+$, 961 (100) $[M+2H]^{2+}$; ESI HRMS: calcd for $C_{96}H_{118}N_{19}O_{24}$ $[M+H]^+$ 1920.8597, found (m/z) 1920.8623.

***N,N′,N″*-[(*SSS*)-Nitrilotris[2-benzoylamino)-1-oxo-2,1-ethandiyl-]]tris-[L-valyl-L-histidyl-L-serine] trimethyl ester (11).** The protected histidine ligand **10** (372 mg, 0.194 mmol) was dissolved in 80% aqueous acetic acid (50 mL) and treated with palladium on carbon (10%, 750 mg). The suspension was hydrogenated under pressure (4 bar) for 24 h (TLC control). The palladium on carbon was removed by filtration and the solvent was evaporated. The crude product was dissolved in water (5 mL) and methanol (5 mL), and the pH of the solution (initial pH 5) was adjusted to pH 9 by addition of aqueous ammonia. The volatile components (THF) were evaporated. Water and the resulting ammonium acetate were removed by lyophilization. The yellowish residue was purified on a column of Sephadex LH 20, swollen and eluted with acetone/methanol (4/1) to afford a colorless solid (245 mg, 81%); mp 161–163 °C; $[\alpha]_D^{25}$ −5.9° (c 1.00, MeOH); ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.89 (m, 18 H, $CH(CH_3)_2$), 2.13 (m, 3 H, $CH(CH_3)_2$), 2.88 (m, 3H, CH_2-His), 2.97 (m, 3H, CH_2-His), 3.59 (s, 9H, OCH_3), 3.57–3.60 (m, 3H, CH_2OH), 3.69–3.71 (m, 3H, CH_2OH), 4.22 (m, 3 H, CH-Val), 4.28 (m, 3H, CH-Ser), 4.57 (m, 3H, CH-His), 5.31 (s, br, 3 H, CH_2OH), 5.69 (d, J = 9 Hz, 3H, NHCHN), 6.88 (s, 3H, His4H), 7.23 (t, J = 8 Hz, 6H, *m*Ph), 7.37–7.44 (m, 9H, *o*,*p*-Ph), 7.55 (s, 3H, His2H), 8.19 (s, br, 3H, NH), 8.34 (s, br, 3H, NH), 8.49 (s, br, 3H, NH), 8.58 (s, br, 3H, NH), 11.82 (s, br, 3H, NH-Im); ^{13}C NMR: (150.91 MHz, DMSO-6_6): δ 18.10, 19.10, 30.05, 51.62, 52.62, 54.60, 58.99, 60.85, 63.58, 126.87, 128.04, 131.74, 132.65, 134.58, 166.66, 167.86, 170.22, 170.74; IR (KBr) ν̃ 3420, 2964, 1741, 1657, 1578, 1522, 1487, 1438, 1226, 1182, 1143, 1081, 935, 714, 694, 619; ESI MS; m/z (%):1583 (47) $[M+Na]^+$, 1561 (100) $[M+H]^+$, 781 (22) $[M+2H]^{2+}$; ESI HRMS: calcd for $C_{72}H_{94}N_{19}O_{21}[M+H]^+$ 1560.6871, found (m/z) 1560.6827.

Synthesis of the zinc(II)-complexes

General procedure. A solution of $Zn(ClO_4)_2{\cdot}6H_2O$ (29 mg, 0.077 mmol) in ethanol (2 mL) was added carefully to a stirred solution of the histidine ligand (0.077 mmol) in ethanol (5 mL). Upon the addition of one drop of the solution of $Zn(ClO_4)_2{\cdot}6H_2O$ in ethanol, a colorless precipitate was formed that was separated by centrifugation (3000 U/min, 10 min). The solution was

removed, the precipitate washed with ethanol (5 mL) and centrifugated (3000 U/min, 10 min, 3×). The product was dried by lyophilization.

Zinc(II) complex of 8 (12)

The title compound was obtained according to the general procedure starting from **8** (100 mg, 0.077 mmol) and $Zn(ClO_4)_2 \cdot 6H_2O$ (29 mg, 0.077 mmol). Colorless solid (85 mg, 70%); mp decp > 220 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.92 (s, br, 18 H, CH(CH_3)$_2$), 2.14 (s, br, 3H, C*H*(CH_3)$_2$), 2.90 (s, br, 6 H, CH_2-His), 3.57 (s, 9 H, OCH_3), 4.26 (s, br, 3 H, CH-Val), 4.50 (s, br, 3H, CH-His), 5.71 (s, br, 3H, NHC*H*N), 7.00 (s, br, 3H, His4H), 7.24 (dd, J = 8 Hz, 6H, *m*Ph), 7.43 (m, 9H, Ph), 8.08 (s, br, His2H), 8.50 (m, br, 9H, NHCHN, NH-His, NH-Val); ^{13}C NMR (75.44 MHz, DMSO-d_6): δ 17.56, 19.27, 30.53, 52.01, 63.82, 127.01, 128.18, 131.99, 132.57, 137.05, 167.04, 168.37, 170.83, 171.18; IR (KBr) ν̃ 3431, 2964, 1741, 1658, 1578, 1520, 1487, 1440, 1219, 1121, 1108, 693, 635; FAB MS (NBA); *m*/*z* (%): 1400 (0.15) $[M-H+K]^+$, 1362 (0.54) $[M-H]^+$, 1299 (3.31) $[L]^+$; ESI MS; *m*/*z* (%): 1400 (9) $[M-H+K]^+$, 1299 (31) $[L]^+$, 650 (100) $[L]^{2+}$; FAB HRMS: calcd for $C_{63}H_{77}N_{16}O_{15}Zn$ $[M-H]^+$ 1361.5046, found (*m*/*z*) 1361.5126.

Zinc(II) complex of 11 (13)

According to the general procedure starting from **11** (121 mg, 0.077 mmol) and $Zn(ClO_4)_2 \cdot 6H_2O$ (29 mg, 0.077 mmol). Colorless solid (89 mg, 58%); mp 218–220 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.88 (s, br, 18H, CH(CH_3)$_2$), 2.10 (s, br, 3H, C*H*(CH_3)$_2$), 2.89 (s, br, 3H, CH_2-His), 3.15 (s, br, 3H, CH_2-His), 3.62 (s, 9H, OCH_3, m, 3H, C*H*$_2$OH), 3.76 (s, br, 3H, C*H*$_2$OH), 4.23 (s, br, 3H, CH-Val), 4.36 (s, br, 3H, CH-Ser), 4.65 (s, br, 3H, CH-His), 5.41 (s, br, 3H, NHC*H*N), 6.94 (s, br, 3H, His4H), 7.23 (s, 6 H, *m*Ph), 7.43 (m, 9H, Ph), 8.13 (s, br, His2H), 8.40 (m, br, 9H, NHCHN, NH-His, NH-Val), 12.64 (s, br, 3H, NH-Im); ^{13}C NMR (150.91 MHz, DMSO-d_6): δ 17.95, 19.21, 26.98, 30.20, 51.85, 54.55, 58.54, 60.95, 63.84, 127.05, 127.57, 127.69, 127.99, 128.25, 131.73, 132.74, 136.82, 167.22, 168.87, 170.49, 170.68; IR (KBr) ν̃ 3430, 2963, 2929, 1741, 1655, 1579, 1518, 1487, 1439, 1439, 1224, 1122, 1109, 694, 623; ESI MS; *m*/*z* (%):1625 (14.04) $[M+H]^+$, 1623 (7.07) $[M-H]^+$, 1583 (4.69) $[L+Na]^+$, 1561 (7.06) $[L+H]^+$, 812 (100) $[M]^{2+}$; ESI HRMS: calcd for $C_{72}H_{93}N_{19}O_{21}Zn$ $[M+H]^+$ 1624.6161, found (*m*/*z*) 1624.6073.

Potentiometric pH-titration

The pH-titrations were performed with an automatic titrator (Metrohm 718 STAT Titrino) coupled to a Metrohm electrode. The temperature was maintained at 25.0 ± 0.1 °C and the solution was kept under an atmosphere of argon. The electrode was calibrated by titrating a known amount of $HClO_4$ employing the same conditions used for titration of the ligands and a correction of the measured pH values to those calculated was taken into consideration in the calculation to determine the equilibrium constants. The titrations of the ligands and its zinc complexes were performed as follows: a 33% EtOH aqueous solution (50 mL) of the ligand **8** (1.00 mM) with 3 equiv of $HClO_4$ (3.00 mM) in the absence or presence of equivalent amounts of $ZnSO_4$ (1.00 mM) was titrated with carbonate-free 0.100 M NaOH aqueous solutions. Before the titration, the ionic strength of the solution was adjusted to I = 0.10 ($NaClO_4$). Titration data for ligand **11** were obtained by the same procedure but in contrast to **8**, a 52% EtOH aqueous solution (50 mL) of the ligand **11** (1.00 mM) was chosen. At least three independent titrations were performed for the determination of the constants. The ligand protonation constants (K_{1-3}), the deprotonation constant of the ligand **11** (K_{L*}), the zinc(II) complexation constant (K_{ZnL}) and the deprotonation constant of the zinc(II) complex (K_a) were calculated with the PSEQUAD program.[54]

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