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Influence of the amino acid side chain on peptide bond hydrolysis catalyzed by a dimeric Zr(IV)-substituted Keggin type polyoxometalate†

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Peptide bond hydrolysis of 18 different dipeptides, divided into four groups depending on the nature of the amino acid side chain, by the dimeric Zr(IV)-substituted Keggin type polyoxometalate (POM) $(Et_2NH_2)_8[\{\alpha\text{-}PW_{11}O_{39}Zr\text{-}(\mu\text{-}OH)(H_2O)\}_2]\cdot 7H_2O$ (**1**) was studied by means of kinetic experiments and $^1H/^{13}C$ NMR spectroscopy. The observed rate constants highly depend on the bulkiness and chemical nature of the X amino acid side chain. X-Ser and X-Thr dipeptides showed increased reactivity due to intramolecular nucleophilic attack of the hydroxyl group in the side chain on the amide carbon, resulting in a reactive ester intermediate. A similar effect in which the amino acid side chain acted as an internal nucleophile was observed for the hydrolysis of Gly-Asp. Interestingly, in the presence of **1** deamidation of Gly-Asn and Gly-Gln into Gly-Asp and Gly-Glu was observed. Dipeptides containing positively charged amino acid side chains were hydrolyzed at higher rates due to electrostatic interactions between the negatively charged POM surface and positive amino acid side chains.

Introduction

Protein hydrolysis is an important procedure in analytical biochemistry. Unfortunately, unactivated peptide bonds are extremely stable under physiological conditions as demonstrated by half-lifes for dipeptide hydrolysis up to several hundreds of years.[1]

Proteolytic enzymes and chemical cleavage agents are currently used for this purpose. Although proteolytic enzymes are characterized by a high catalytic power, they frequently suffer from non-specific digestion of the target protein and cannot be used on a large-scale because of their high cost. Therefore, chemical reagents have been considered as an inexpensive alternative. The most commonly used chemical reagent is cyanogens bromide, however, it also has several shortcomings as it is toxic, must be used in great excess over the substrate, requires harsh conditions (70% formic acid as the solvent), and causes side reactions.[2] Clearly, new chemical reagents for efficient and selective hydrolysis of peptides are needed.

Transition-metal or lanthanide ions and their complexes are promising as new reagents for peptide bond cleavage since Lewis acid metal centers can coordinate to the amide carbonyl oxygen atom thereby activating the peptide bond towards hydrolysis.[3] However, a prominent limitation often observed for many transition metal and lanthanide ions when using them as catalysts for peptide bond hydrolysis is the formation of gels at neutral and basic pH conditions.[4] To avoid heterogeneous reaction conditions and to induce selectivity, stabilizing complexation ligands were used.[4a,5] Several metal complexes of Co(III),[6] Cu(II),[7] Zn(II),[3,8] Pd(II),[9] Ni(II),[10] Ce(IV),[11] and Zr(IV)[4a,5] have been reported to promote the hydrolysis of amide bonds in dipeptides, oligopeptides, and proteins. Although many attempts have been made, the number of metal complexes which are efficient catalysts for peptide bond hydrolysis at near physiological conditions is still very limited. For example, selective hydrolysis at Met and His residues was observed for Pt(II) and Pd(II) complexes, but only at low pH values.[12] Moreover, complexes of Cu(II) were shown to hydrolyze the X-Ser(Thr) bond in X-Ser(Thr)-His sequences, but lose their reactivity at physiological conditions due to the formation of strong metal–amide nitrogen bonds resulting in catalytically inactive complexes.[13]

In our goal towards developing novel artificial peptidases, we have recently focused on the use of polyoxometalates (POMs) as catalysts for hydrolytic processes. POMs are generally described as clusters of early transition metal ions in their highest oxidation state and oxo ligands. They are characterized by a wide range of molecular structures as well as physical/chemical properties.[14] As a result, they are currently studied in various research domains including medicine,[15] magnetism,[16] material science,[14a,17] and catalysis.[18] For example, the use of POMs as catalysts for

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† Electronic supplementary information (ESI) available: Additional kinetic profiles, ^1H and ^{13}C NMR data, grouping of dipeptides, and dipeptide structures. See DOI: 10.1039/c5nj00561b

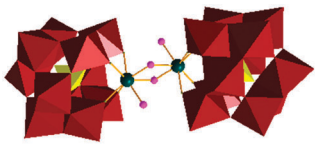


Fig. 1 Schematical representation of the complex **1**.

the hydrolysis of biologically relevant molecules such as phosphoesters was recently developed in our lab.[19] Many metal ions can be incorporated into the POM skeleton, forming metal-substituted POMs which are both kinetically and thermodynamically more stable than their parent POM structures at physiological pH conditions.[20] Moreover, the incorporated metal ion(s) can coordinate H_2O or other ligands at their free coordination site(s), making them attractive candidates for catalysis applications. As a result, the use of metal-substituted POMs as catalysts for a broad range of chemical reactions, such as Diels–Alder reactions and aldol reactions of imines,[21] H_2O_2-based oxidation of organic compounds,[22] and the oximation of aldehydes and ketones[23] was reported.

In our quest to further investigate POMs as catalysts for hydrolytic processes, metal-functionalized POMs containing the hydrolytically active Lewis acid metal cations Zr(IV) and Ce(IV) were studied as potential catalysts for peptide bond hydrolysis. Zr(IV) and Ce(IV) are both characterized by a high Lewis acidity, oxophilicity, kinetic lability, and they tend to form complexes with high coordination numbers. Moreover, their incorporation into POM structures prevents the formation of insoluble gels. The superior reactivity of Zr(IV) was confirmed when evaluating the reactivity of several metal-substituted Wells–Dawson type POMs containing Mn(III), Fe(III), Co(II), Ni(II), Cu(III), Y(III), La(III), Eu(III), Yb(III) towards glycylglycine hydrolysis.[24]

We recently showed that a series of Zr(IV)-substituted Keggin, Lindqvist and Wells–Dawson POMs and a Ce(IV)-substituted Keggin POM selectively hydrolyzed albumins and hen-egg-white lysozyme respectively under physiological conditions.[25] In addition, the hydrolytic activity of these Zr(IV)-substituted POMs was further examined towards phosphoesters and dipeptides.[4b,26] All complexes acted as homogeneous catalysts and significantly accelerated the hydrolysis reaction rates, demonstrating the potential of Zr(IV)-substituted POMs as novel artificial peptidases and phosphatases. Reactivity studies towards Gly-Ser hydrolysis with different Zr(IV)-substituted POMs revealed that the dimeric Zr(IV) Keggin $(Et_2NH_2)_8[\{\alpha\text{-}PW_{11}O_{39}Zr(\mu\text{-}OH)(H_2O)\}_2]\cdot 7H_2O$ POM (**1**) (Fig. 1) demonstrated a particular high reactivity as compared to other POMs.[4b,26b,27] In order to investigate the selectivity of **1** towards different amino acid sequences, in this study the effects of the side chain on the hydrolysis of dipeptides containing a Ser residue at the C-terminus (X-Ser) and dipeptides containing a Gly residue (Gly-X and X-Gly) are being investigated.

Experimental section

Chemicals

$(Et_2NH_2)_8[\{\alpha\text{-}PW_{11}O_{39}Zr\text{-}(\mu\text{-}OH)(H_2O)\}_2]\cdot 7H_2O$ (**1**) was synthesized according to the procedure published in literature[26b,28] and presented in ESI.† D_2O, DCl, NaOD, $H_3PW_{12}O_{40}\cdot 23H_2O$, and the dipeptides used in this study were purchased from Sigma-Aldrich and Bachem.

NMR measurements

^1H NMR spectra were recorded on a Bruker Avance 400 spectrometer and TMSP-d_4 was used as an internal reference. ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer. As a reference TMS in an internal reference tube was used. ^{31}P NMR spectra were recorded on a Bruker Avance 400 spectrometer and 25% H_3PO_4 in H_2O in an internal reference tube was used as a reference.

Hydrolysis studies

The hydrolysis reaction mixtures typically contain 2.0 mM dipeptide, 2.0 mM **1**, and 0.5 mM TMSP-2,2,3,3-d_4 (sodium-3-trimethylsilylpropionate) in D_2O. The pD of the reaction mixtures was adjusted to 5.4 with minor amounts of 1.0 M DCl or 1.0 M NaOD. The pH-meter reading was corrected by the equation: pD = pH + 0.41.[29] The reaction mixtures were kept at 60 °C and ^1H NMR spectra were taken after mixing and after different time increments. The hydrolysis products were identified by comparison of the ^1H NMR chemical shifts with those of the pure amino acids at the same pD value. The rate constants (k_{obs}) were calculated by fitting the decrease in dipeptide concentration to a first-order mono-exponential decay function.

Result and discussion

Aqueous solution stability of 1

Since the stability of metal-substituted POMs in aqueous solution is highly dependent on pH, temperature, time, concentration, and the presence of ligands,[24,26b,30] their stability under different conditions needs to be investigated before applying them as catalysts. The stability of **1** in aqueous solution was fully investigated by ^{31}P NMR spectroscopy and reported in our previous work.[26b,31] After pD adjustment of a 2.0 mM solution of **1** in the pD range 2.0–7.4 only complex **1** was detected, while the sandwich 1 : 2 complex (Fig. S1a, ESI†) simultaneously appeared at pD 9.0. The conversion of **1** into the 1 : 2 complex is facilitated by temperature and time. After one day at 60 °C about 25% of the 1 : 2 species was observed in a solution containing 2.0 mM **1** at pD 7.4, while at pD 5.4 complex **1** was retained its structure after 20 days at 60 °C. The single crystal X-ray structure of **1** shows that each Zr(IV) ion has one coordinated water molecule in the first coordination sphere,[28a] whereas the coordination sphere of Zr(IV) in the 1 : 2 type complex is saturated due to binding of two lacunary Keggin units.[28b] Previous studies have shown that lack of free coordination sites around Zr(IV) results in a low reactivity towards peptide bond hydrolysis.[26b] In that respect the dimeric structure of **1** should show superior catalytic activity as compared to the 1 : 2 Keggin POM complex. Moreover, our recent studies in which we combined advanced NMR techniques (DOSY and EXSY NMR) with DFT calculations have indicated that the 2 : 2

species is in fast equilibrium with the monomeric form and identified this 1 : 1 Zr(IV)–Keggin POM complex (Fig. S1b, ESI†) as the active species in solution.[31]

Hydrolysis of dipeptides

In this study the hydrolysis of 18 different dipeptides in the presence of **1** was examined. The dipeptides were divided into four groups depending on the nature of their amino acid side chains (Table S1, ESI†), and their hydrolysis in the presence of **1** was studied by mixing equimolar amounts (2.0 mM) of the dipeptide and **1** at pD 5.4 and 60 °C. The hydrolysis reaction was followed by ^{1}H NMR spectroscopy at different reaction times and the hydrolysis products were identified by comparison of the ^{1}H NMR chemical shifts with those of pure amino acids at the same pD value. The amount of the dipeptide and the hydrolysis fragments was determined by integration of their respective ^{1}H NMR spectroscopic resonances and the rate constants (k_{obs}) were calculated by fitting the decrease in dipeptide concentration to a first-order mono-exponential decay function ($\ln[A] = -k_{obs}t + \ln[A]_0$) and presented in Table 1. For comparison, control experiments were also performed in which the hydrolysis of all studied dipeptides was studied in the absence of **1** under the same experimental conditions. It is important to note that fully homogeneous reaction conditions were obtained during the hydrolysis of all dipeptides, while precipitation was observed when the Zr(IV) salts $ZrOCl_2$ or $ZrCl_4$ were used. In addition to these heterogeneous reaction conditions, $ZrOCl_2$ was shown to be 15 times less active than a Zr(IV)–POM towards peptide bond hydrolysis.[4b]

Dipeptides containing a hydroxyl group in the side chain

Dipeptides containing a hydroxyl group such as Gly-Ser, Ser-Gly, Leu-Ser, Ile-Ser, Tyr-Ser, Gly-Thr, and Gly-Tyr were examined (Table 1 – entries 1–7). The hydrolysis of Gly-Ser in the presence of **1** was fully investigated in our previous study.[26b] The Ser hydroxyl group makes this dipeptide more susceptible to hydrolysis due to the intramolecular attack of the hydroxyl group on the amide carbon, forming an oxazolidine intermediate that further rearranges to an ester intermediate (Scheme S1, ESI†).[27,32] Consequently, C-terminal Ser dipeptides can also be autocatalytically hydrolyzed (Table 1). Rate enhancements with a factor of 45, 37, and 20 were observed for the hydrolysis of Gly-Ser, Leu-Ser, and Gly-Thr in the presence of **1**, respectively. The increased hydrolysis rates of these dipeptides in the presence of **1** are the result of their binding to the Zr(IV) center *via* their amide oxygen and amine nitrogen atoms.[26b] As a result, the amide carbon becomes polarized and therefore more susceptible towards hydrolysis by intramolecular attack of the hydroxyl group. Interestingly, the ^{13}C NMR spectrum of Gly-Ser in the presence of Et_2NH_2Cl shows no shifts as compared to the ^{13}C NMR spectrum of pure Gly-Ser (Fig. S2, ESI†), supporting that the ammonium counter ions do not influence the ^{13}C NMR chemical shifts of Gly-Ser and that the observed shifts are a result of interaction between **1** and Gly-Ser.

The presence of an extra methyl group in the side chain of Thr hinders the intramolecular attack of the OH group on the amide carbonyl carbon, leading to a 6-fold reaction rate decrease in comparison to Gly-Ser. For Gly-Tyr, such intramolecular attack is unlikely to happen since it is located in the *para* position on a rigid aromatic ring, resulting in a 24-fold decrease in rate constant as compared to Gly-Ser. The steric effect can also clearly be seen in several dipeptides containing a Ser residue at the C-terminus. Fig. 2 shows the relationship between the hydrolysis rate constant of a series of X-Ser dipeptides and the volume of the X amino acid side chain.[33] The rate constant for the hydrolysis of dipeptides with an X-Ser sequence decreases as the volume of the X amino acid side chain increases. This is in accordance with previous studies[3,4b,12b,27,34] and implies that steric hindrance induced by the side chain plays a role in preventing the intramolecular attack of the hydroxyl group. Despite their identical volume, Ile more strongly hinders the intramolecular attack than Leu due to the presence of a β-methyl group more proximal to the backbone (Fig. S3, ESI†).

Table 1 Observed rate constants for the hydrolysis of X-Ser, Gly-X, and X-Gly in the absence and presence of **1** ([**1**] = [dipeptide] = 2.0 mM, pD 5.4, 60 °C)

		In the absence of **1**		In the presence of **1**
Entry	Peptide	$10^7 \times k_{obs}$ (s^{-1})	Conversion after 7 months (%)	$10^7 \times k_{obs}$ (s^{-1})
1	Gly-Ser	1.39 ± 0.21		63.33 ± 0.11
2	Leu-Ser	1.22 ± 0.19		45.33 ± 0.13
3	Ile-Ser		34	9.42 ± 0.21
4	Tyr-Ser		18	4.71 ± 0.40
5	Gly-Thr	0.56 ± 0.15		11.4 ± 0.15
6	Gly-Tyr		0	2.63 ± 0.24
7	Ser-Gly		20	8.13 ± 0.12
8	Cys-Gly		20	3.61 ± 0.13
9	Gly-Met		0	1.11 ± 0.20
10	Gly-Asp		50, 21[a]	5.31 ± 0.21 1.67 ± 0.28[b]
11	Asp-Gly		6[a]	0.72 ± 0.11
12	Gly-Glu		10	3.18 ± 0.19
13	Gly-Asn		8	4.42 ± 0.32
14	Gly-Gln		15	3.05 ± 0.21
15	Gly-His		10	3.20 ± 0.31
16	His-Gly		28	5.75 ± 0.22
17	Gly-Lys		0	2.26 ± 0.42
18	Gly-Arg		0	1.94 ± 0.21

[a] Conversion after 3 months. [b] pD 7.4, 60 °C.

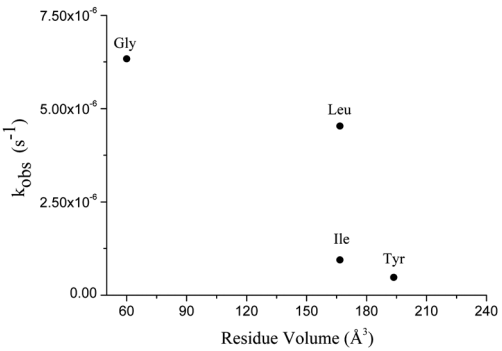


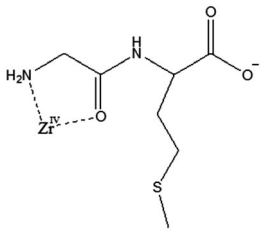
Fig. 2 Reaction rate constants for the hydrolysis of 2.0 mM X-Ser dipeptides in the presence of 2.0 mM **1** at pD 5.4 and 60 °C as a function of the X amino acid volume.

As a result a 5-fold reduction in the rate constant of Ile-Ser as compared to Leu-Ser was observed. For Ser-Gly a less favourable four-membered ring transition state is formed by the attack of the OH group on the amide carbon resulting in a lower hydrolysis rate constant in comparison to Gly-Ser.

In order to test the role of the Brønsted acidity of the $Et_2NH_2^+$ ammonium counter ions, the hydrolysis of 2.0 mM Gly-Ser in the presence of 16.0 mM Et_2NH_2Cl under the same experimental conditions (pD 5.4 and 60 °C) was studied. The results show that the rate constant for the hydrolysis of 2.0 mM Gly-Ser in the presence of 16.0 mM Et_2NH_2Cl is exactly the same as the one observed in the absence of any catalyst. This result clearly supports that the ammonium counter ions have no role in facilitating the hydrolysis of Gly-Ser.

Dipeptides containing a sulfur atom in the side chain

Methionine-containing peptides have previously selectively been hydrolyzed by Pd(II) and Pt(II) complexes.[9a,35] The sulfur atom in the side chain of Met was shown to bind to the metal ions in these complexes and facilitate hydrolysis of the second amide bond upstream from the anchor.[9a,35b,36] Ni(II) and Zn(II) complexes also promote the hydrolysis of cysteine-containing peptides.[10b,37] The rate constants of Gly-Met and Cys-Gly as well as those of Gly-X dipeptides in the presence of **1** as a function of the amino acid residue volume[33] are presented in Fig. 3. As can be seen, the hydrolysis rate of Gly-Met and Cys-Gly was slower compared to other peptides with similar volume but having a carbonyl group or positively charged group in their side chain. This suggests that there is no coordination between the sulfur atom to Zr(IV) or that this binding is not strong enough to significantly enhance hydrolysis. To confirm this, ^{13}C NMR spectra of Gly-Met in the absence and in the presence of **1** were recorded (Fig. S4 and Table S2, ESI†). Upon addition of **1**, shifts of 0.19 and 0.08 ppm at C-3 and C-4 carbons were respectively observed, indicative for coordination *via* the amide oxygen and the amine nitrogen atoms, resulting in a hydrolytically active complex (Scheme 1) which is similar to the complex as obtained for Gly-Gly.[26b] There are almost no changes in the chemical shifts at C-6 and C-7 carbons, indicating that the sulfur atom is not involved in binding with Zr(IV). Although the hydrolysis of Gly-Met in the presence of **1** was slow ($k_{obs} = 1.11 \times 10^{-7}$ s^{-1}), this represents a significant acceleration compared to the uncatalyzed reaction in which Gly-Met was not hydrolyzed after seven months under the same experimental conditions.



Scheme 1 Proposed coordination of Gly-Met to Zr(IV) in **1** to form the hydrolytically active complex.

^{13}C NMR spectra of Cys-Gly in the absence and presence of **1** were also measured (Fig. S5, ESI†). The amide carbon and the C_α of Cys residue were respectively shifted 0.51 and 0.21 ppm upon addition of complex **1** (Table S3, ESI†), pointing out that coordination occurred at the amide oxygen and the amine nitrogen (pK_a 6.94)[38] atoms. Additionally, the C-5 carbon was also shifted 0.33 ppm. Although binding of the sulfur atom (pK_a 9.37)[38] to Zr(IV) cannot be excluded, the observed shift most likely results from hydrogen bonding to terminal oxygen atoms of the POM framework. The hydrolysis of Gly-Gly, Gly-Met, and Cys-Gly induced by **1** is mainly due to the binding of Zr(IV) to the amide carbonyl oxygen and amine nitrogen atoms, resulting in a decrease in reaction rates observed when the volume of side chain increases (Fig. 3).

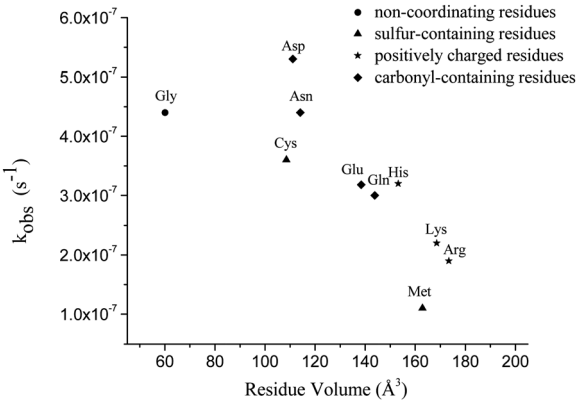


Fig. 3 Reaction rate constants for the hydrolysis of 2.0 mM Gly-X in the presence of 2.0 mM **1** at pD 5.4 and 60 °C as a function of the volume of amino acid X. For comparison, the rate constant of Gly-Gly in the presence of 2.0 mM **1** at pD 5.4 and 60 °C (4.44×10^{-7} s^{-1}) is also plotted.

Dipeptides containing a carbonyl group in the side chain

Gly-Asp, Asp-Gly, Gly-Asn, Gly-Glu and Gly-Gln peptides all contain carbonyl groups in the side chain, either in the form of a carboxyl group (Gly-Asp, Asp-Gly, Gly-Glu) or amide group (Gly-Asn, Gly-Gln). The relation between the rate constants of Gly-Asp, Gly-Asn, Gly-Glu and Gly-Gln and the bulkiness of side chain are presented in Fig. 3. As can be seen from Fig. 3, the hydrolysis of carbonyl-containing dipeptides in the presence of **1** is characterized by higher rates in comparison to the sulfur-containing dipeptides with a similar volume. Among the carbonyl-containing dipeptides, the highest hydrolysis rate was observed for Gly-Asp.

Fig. 4 shows ^1H NMR spectra of Gly-Asp in the presence of **1** recorded at different time increments. The intensity of the ^1H NMR resonances at 3.89 ppm and 4.53 ppm, attributed to H_α of the Gly and Asp residues in Gly-Asp respectively, decreased, while the intensity of the resonances at 3.56 ppm and 3.94 ppm, attributed to H_α of free Gly and free Asp, respectively, increased over time, indicating that hydrolysis of the amide bond in Gly-Asp occurred. Similar to Gly-Gly and Gly-Ser hydrolysis,[24,26b] cyclization was observed. The cyclic Gly-Asp, c(Gly-Asp), identified by the resonances at 4.01–4.08 ppm and 4.29 ppm, is reopened to form both Gly-Asp and Asp-Gly (identified by the resonance

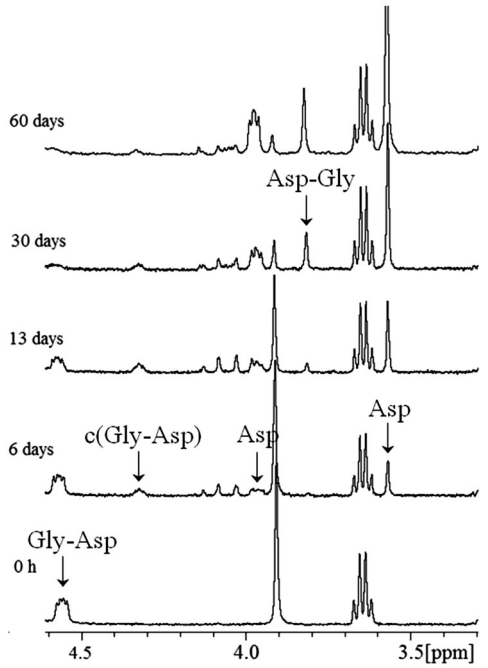


Fig. 4 ^{1}H NMR spectra of Gly-Asp recorded at different time increments for the reaction between 2.0 mM Gly-Asp and 2.0 mM **1** at pD 5.4 and 60 °C.

at 3.78 ppm). The percentage of Gly-Asp, Gly, c(Gly-Asp), and Asp-Gly is shown in Fig. 5. The data show that cGly-Asp is gradually hydrolyzed after reaching a maximum of 22%, while the amount of Asp-Gly gradually increased. Since the hydrolytic rate of Asp-Gly is ca 7 times slower than that of Gly-Asp (Table 1), the amount of Asp-Gly exceeded the amount of Gly-Asp and c(Gly-Asp) after 800 h. Due to its low hydrolysis reaction rate at 60 °C, the hydrolysis of 2.0 mM Gly-Asp in the presence of 2.0 mM **1** was studied at 80 °C to observe 100% cleavage of Asp-Gly. Fig. S6 (ESI†) clearly shows that only Gly and Asp were detected at the end of the reaction.

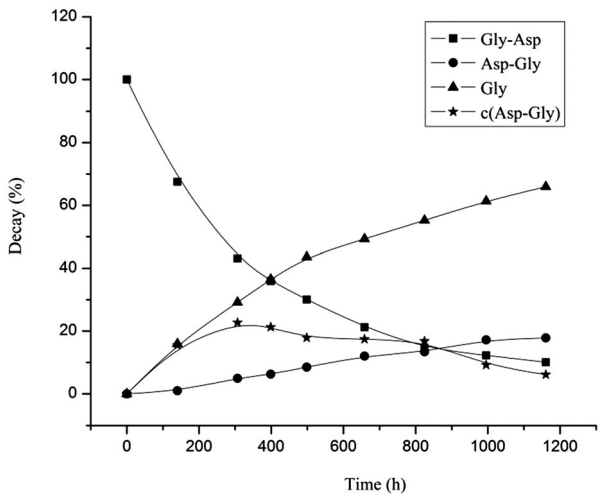
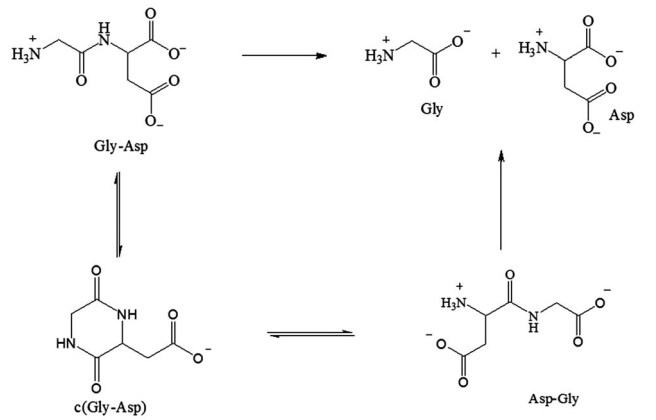


Fig. 5 Fraction of Gly-Asp, Gly, cGly-Asp, and Asp-Gly as a function of time for the reaction between 2.0 mM Gly-Asp and 2.0 mM **1** at pD 5.4 and 60 °C.

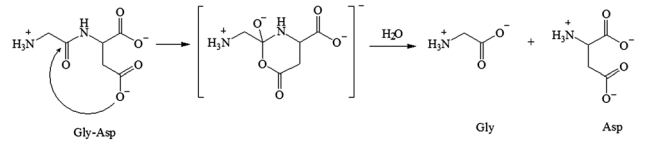


Scheme 2 Hydrolysis of Gly-Asp by **1**.

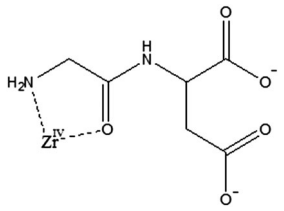
To confirm ring opening to give both Gly-Asp and Asp-Gly, 2.0 mM c(Gly-Asp) was hydrolyzed in the presence of 2.0 mM **1** at pD 5.4 and 80 °C. The ^{1}H NMR spectra recorded at different time increments are shown in Fig. S7 (ESI†). Both Gly-Asp and Asp-Gly were found during the ring opening of c(Gly-Asp). Fig. S8 (ESI†) presents the percentage of c(Gly-Asp), Gly-Asp, Asp-Gly, and Gly. It is noteworthy that after 7 h no Gly and Asp were observed, while 3% and 0.5% of Gly-Asp and Asp-Gly were obtained respectively. Free Gly and Asp could only be seen after sufficient amounts of Gly-Asp were formed. This finding suggests that simultaneous breaking of two amide bonds in c(Gly-Asp) is unfavorable. Since the hydrolysis rate of Gly-Asp is about 7 times faster than that of Asp-Gly (Table 1), the maximal percentage of Gly-Asp (9%) is lower than that of Asp-Gly (30%). Based on these observations, the reaction scheme shown in Scheme 2 is proposed.

The faster hydrolysis rate of Gly-Asp compared to other carbonyl-containing dipeptides can be attributed to the presence of the side chain carboxyl group which assists hydrolysis by nucleophilic attack on the amide carbonyl carbon to form a tetrahedral intermediate under mildly acidic solutions (Scheme 3).[39] The intermediate is then broken down at the ester bond and the C(sp^3)–N bond to form Gly and Asp. As can be seen in Table 1, the highest conversion in the absence of **1** was obtained for Gly-Asp, indicating the important role of the side chain carboxyl group at the C-terminus.

Addition of **1** enhanced the hydrolytic rate of Gly-Asp up to 14 times, implying that interaction between Gly-Asp and **1** must occur. ^{13}C NMR spectra of Gly-Asp in the absence and presence of **1** at different pD values were recorded after pD adjustment (Fig. S9 and Table S4, ESI†). The ^{13}C NMR data show that at pD values between 5.4 and 8.1 several coordination modes can be



Scheme 3 Aspartic acid residue facilitates the hydrolytic cleavage of Gly-Asp.



Scheme 4 Proposed coordination of Gly-Asp to Zr(IV) in **1** to form the hydrolytically active complex.

found. While at pD 5.4 chemical shift changes are observed for carbons 1, 2, 5 and 6 upon addition of **1**, which are indicative for binding of Gly-Asp *via* its C-terminal and side chain carbonyl groups to Zr(IV), at higher pD values coordination mainly occurs *via* the amine nitrogen and amide oxygen atoms as evidenced by chemical shift changes for carbons 3 and 4 (Scheme 4).

Asp-Gly hydrolysis in the presence of **1** was about 7 times slower than that of Gly-Asp (Table 1). ^{13}C NMR spectra of Asp-Gly in the presence of **1** were further examined (Fig. S10 and Table S5, ESI†). While at pD 7.4, a clear preference for the formation of a hydrolytically active species was seen in the case of Gly-Asp, evidenced by chemical shift changes at carbons 3 and 4, in the case of Asp-Gly chemical shifts at carbons 1, 3, 4, 5 and 6 are observed, indicative for the presence of multiple coordination modes. This may explain the lower hydrolysis rate of Asp-Gly compared to Gly-Asp.

The hydrolysis of Gly-Glu in the presence of **1** was approximately 2 times slower than that of Gly-Asp. The ^{13}C NMR spectra of Gly-Glu in the absence and presence of **1** at pD 5.4 show shifts of all carbons in the Glu side chain, but the shifts are smaller compared to those observed in the side chain of Asp (Fig. S11 and Table S6, ESI†). The binding mode of Gly-Glu to **1** at pD 7.8 was analogous to that of Gly-Asp, resulting in larger shifts of the amide carbonyl carbon and the C_α of Gly in Gly-Glu, and almost no shifts at the C-terminal and side chain carboxyl groups (Fig. S11 and Table S6, ESI†). The weak interaction *via* C-terminal carbons facilitates hydrolysis of Gly-Asp and Gly-Glu as their hydrolysis in the presence of **1** was up to 4 times faster than in the presence of a Zr(IV)-substituted Wells–Dawson POM in which binding mainly occurs *via* the carboxylate groups of the side chain and the C-terminus.[27]

The hydrolysis of Gly-Asn was also fully investigated. Fig. 6 shows ^{1}H NMR spectra of Gly-Asn at different time increments. The resonances at 3.86–3.89 ppm correspond to the CH_2 protons of Gly in Gly-Asn, while the resonances at 3.56 ppm and 4.03–4.05 ppm belong to H_α of free Gly and free Asn, respectively. The intensity of the resonance at 3.86 ppm gradually decreased, while those of free Gly and free Asn steadily increased, indicating that the hydrolysis of the amide bond in Gly-Asn occurred to form Gly and Asn. Interestingly, the intensity of the signal at 3.89 ppm, corresponding to the CH_2 protons of Gly in Gly-Asp, increased during the course of hydrolytic reaction, suggesting deamidation of Gly-Asn. The deamidation of the single amino acid Asn to Asp was also observed in the presence of **1** (Fig. S12, ESI†). Asn derived from Gly-Asn hydrolysis

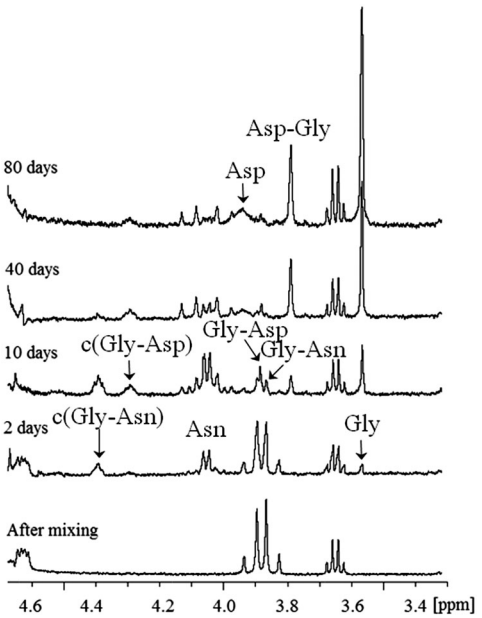


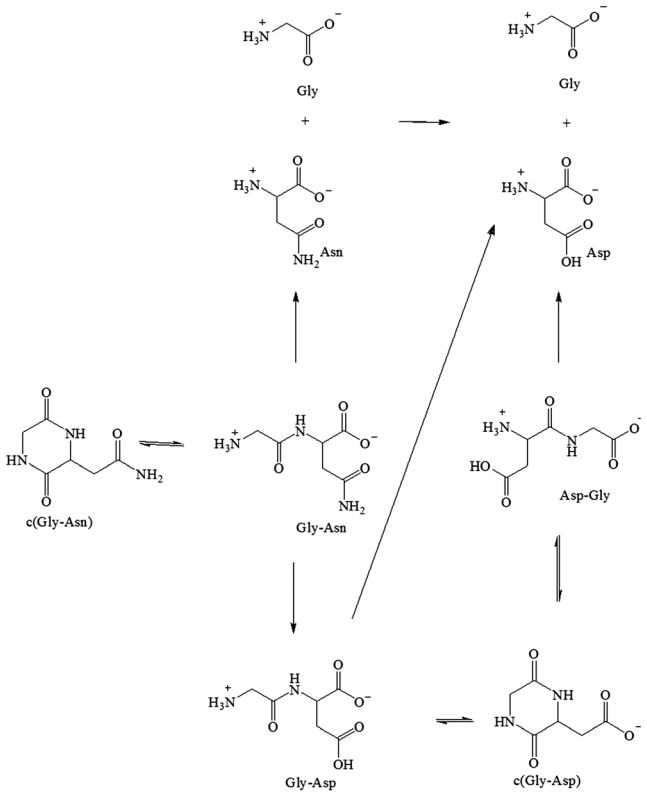
Fig. 6 ^{1}H NMR spectra of Gly-Asn recorded at different time increments for the reaction between 2.0 mM Gly-Asn and 2.0 mM **1** at pD 5.4 and 60 °C.

was completely converted into Asp since it disappeared at the end of the reaction. Cyclization of Gly-Asn and Gly-Asp also occurred during Gly-Asn hydrolysis. The percentages of Gly-Asn, Gly, Asp, Gly-Asp, c(Gly-Asn), c(Gly-Asp), and Asp-Gly are shown in Fig. S13 (ESI†). From these findings we can conclude that there are two different Gly-Asn degradation routes, *i.e.* the cleavage of the peptide bond and the deamidation of the side chain amide group, as shown in Scheme 5.

The same tendency in degradation of Gly-Gln in the presence of **1** was obtained. The main intermediate products (Gly-Glu and Glu-Gly) and the final product (Gly) are clearly seen and assigned in Fig. S14 (ESI†). This finding is consistent with a published study,[40] in which Gly-Gln was hydrolyzed to Gly and Glu in buffer solution at 100 °C.

Dipeptides containing a positively charged side chain residue

Under the experimental conditions used in this study (pD 5.4), the residues His, Lys and Arg are positively charged. Interestingly, the hydrolysis of Gly-His, Gly-Lys, and Gly-Arg in the presence of **1** occurred faster than for the sulfur-containing dipeptides with similar volumes (Fig. 3). This could be the result of electrostatic interactions between the positively charged side chain and negatively charged POM surface, making the complex between **1** and these substrates more stable. In order to validate this hypothesis, ^{13}C NMR spectra of the substrates in the presence and in the absence of **1** were recorded. Upon addition of **1**, shifts of 0.24 and 0.19 ppm at the amide carbon and C_α of the Gly residue in Gly-Lys were observed respectively (Fig. 7 and Table 2), indicating that binding occurred at the amide carbonyl oxygen and the amine nitrogen. Remarkably, all carbons at the side chain (from C-5 to C-8 carbons) were also shifted. It is worth to mention that the side chain amino group is



Scheme 5 Degradation routes of Gly-Asn by **1** at pD 5.4 and 60 °C.

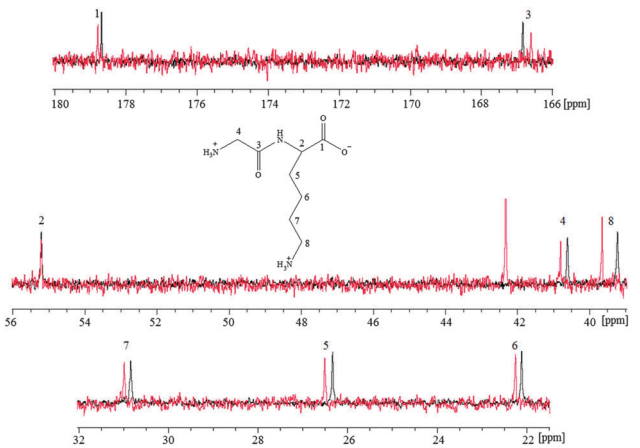


Fig. 7 ^{13}C NMR spectra of Gly-Lys in the presence (red) and absence (black) of **1** at pD 7.4 (the signal at 42.4 ppm belongs to the CH_2 carbon of the ethyl group in the counter ion).

mostly protonated at pD 7.4 (pK_a = 10.50),[41] excluding binding of the side chain to the Zr(IV) center.

As the presence of salt influences electrostatic interactions, the influence of the ionic strength on the interaction between Gly-Lys and **1** was studied by adding LiCl to a reaction mixture containing 2.0 mM Gly-Lys and 2.0 mM **1** at pD 5.4 and 60 °C (Fig. S15, ESI†). The results indicated that LiCl has a negative effect on the hydrolysis of Gly-Lys catalyzed by **1** since adding increasing amounts of LiCl resulted in decreased

Table 2 ^{13}C NMR chemical shift values (ppm) of 15.0 mM Gly-Lys in the absence and presence of 2.0 mM **1** at pD 7.4

^{13}C NMR	15.0 mM Gly-Lys	15.0 mM Gly-Lys + 2.0 mM **1**	$\Delta\delta$ (ppm)
δ_1	178.67	178.75	0.08
δ_2	55.20	55.20	0.00
δ_3	166.83	166.59	0.24
δ_4	40.60	40.79	0.19
δ_5	26.33	26.50	0.17
δ_6	22.10	22.24	0.14
δ_7	30.84	30.99	0.15
δ_8	39.22	39.64	0.42

Gly-Lys rate constants. ^{13}C NMR spectra of Gly-Lys in 1.2 M LiCl solutions with and without **1** were also recorded (Fig. S16 and Table S7, ESI†). It can clearly be seen that while in the presence of LiCl coordination of the amide oxygen to Zr(IV) still occurred, the proposed electrostatic interaction between the positively charged Lys side chain and the negatively charged POM surface was reduced since only small shifts for the side chain carbons were observed. These results further support the hypothesis that the interaction between Gly-Lys and **1** is partially electrostatic in nature.

Upon addition of **1** the ^{13}C NMR spectra of Gly-Arg also show shifts at the carbons of the Arg side chain, besides the expected shifts at the amide carbon and the C_α of the Gly residue (Fig. S17 and Table S8, ESI†). Due to strong basicity of the Arg side chain (pK_a = 12.5),[27] it cannot bind to the Zr(IV) center at pD 7.4. Therefore, it is plausible that the shifts at the side chain carbons are also related to the electrostatic interaction, as observed for Gly-Lys.

Binding of Gly-His to **1** was also investigated. However, solubility problems of Gly-His in the presence of **1** prevented the study in mildly acidic solutions. Therefore, ^{13}C NMR spectra of Gly-His in the absence and presence of **1** at pD 7.4 were recorded (Fig. S18 and Table S9, ESI†). As can be seen, all carbons of Gly-His were shifted upon addition of **1**, indicative for multiple and/or complicated coordination modes between Gly-His and **1**. As reported by Garribba *et al.* oxovanadium can bind Gly-His *via* [(NH_2, N^-, N_{im}); (CO, COO^-)] and [(NH_2, N^-, N_{im}); (CO, NH_2)] donor sets under neutral conditions.[38] The hydrolysis of His-Gly catalyzed by **1** was about 2 times faster than that of Gly-His. In addition, as compared to Gly-His, this peptide is more soluble in the presence of **1** at mildly acidic conditions. The interaction between His-Gly and **1** was studied by ^{1}H NMR spectroscopy, by recording spectra of 2.0 mM His-Gly in the absence and presence of 2.0 mM **1** at different pD values (Fig. S19, ESI†). The proton chemical shifts induced by **1** at various pD values are summarized in Table S10 (ESI†). The change in chemical shift of H-4 significantly increased with increasing pD, indicating that the amine nitrogen is involved in the coordination of His-Gly to **1**. ^{13}C NMR spectra of His-Gly (Fig. S20 and Table S11, ESI†) show that shifts of 0.47 and 0.09 ppm at the C-4 carbon were observed at pD 7.4 and 5.8 respectively, supporting the coordination of **1** to the amine nitrogen and indicating that the coordination is largely suppressed when the amine group (pK_a 7.69)[38] is protonated. In addition, a large difference in ^{13}C NMR chemical shift for the amide carbonyl

carbon was observed due to the coordination of **1** to the amide oxygen. Moreover, large chemical shift changes for the carbons in the side chain were observed upon addition of **1** at pD 7.4. This could be a consequence of binding between **1** and the N_{im} nitrogen (pK_a 5.94).[38] Binding between His-Gly and Zr(IV) *via* this (NH_2, CO, N_{im}) coordination mode was also observed in the presence of a Zr(IV)-substituted Lindqvist type POM[4b] and reported for the binding of oxovanadium to His-Gly.[38] The ^{13}C NMR spectra of His-Gly recorded at pD 5.8 (Table S11, ESI†) indicates that at this pD the shifts still remain. In addition, significant shifts for the protons in the ring were observed even in acidic solution (pD 4.5) (Table S10, ESI†). These findings indicate that electrostatic interactions between the negatively charged POM surface and the positively charged dipeptide His-Gly side chain are present, since the pK_a of the side chain is 5.94,[38] explaining the effective hydrolysis of His-Gly in the presence of **1**. Similar to His-Gly, ^1H NMR spectra of 2.0 mM Gly-His in the absence and presence of 2.0 mM **1** at different pD values display significant shifts (Table S12 and Fig. S21, ESI†). Gly-His is characterized by three pK_a values: 2.51 (acidic group), 6.78 (imidazole nitrogen), and 8.24 (amino group).[38] Extreme shifts at the ring protons were obtained in neutral and basic solutions as a consequence of coordination of imidazole nitrogen to the Zr(IV) center, while in acidic solutions considerable shifts of these protons were observed, probably due to electrostatic interactions. These results indicate that the faster hydrolysis rate observed for positively charged Gly-His dipeptide in the presence of **1** at pD 5.4 is also due to favorable electrostatic interaction.

Conclusions

In this paper we have performed a detailed examination of the effect of the amino acid side chain nature on the hydrolysis rate of a series of dipeptides in the presence of a dimeric Zr(IV)-substituted Keggin polyoxometalate. The rates of peptide bond hydrolysis were significantly accelerated by formation of hydrolytically active complexes in which the amine nitrogen and amide oxygen atoms coordinate to Zr(IV) which is highly dependent on the bulkiness and the chemical nature of the side chain. An increase in the volume of the side chain resulted in a decrease in the hydrolysis rate in each dipeptide group. The hydrolysis of peptides containing positively charged side chains is assisted by the secondary interactions with the negatively charged surface of polyoxometalate framework. Remarkably, the presence of the hydroxyl or carboxyl groups at the C-terminus enhanced the hydrolysis rate *via* the intramolecular attack of the functional groups on the amide carbon. Most reaction rates in this paper are comparable to those in the presence of Zr(IV)-substituted Wells–Dawson polyoxometalate.[27] These results are of great importance in thorough understanding of the selectivity of Zr(IV)-substituted polyoxometalates as artificial peptidases.

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Notes and references

1 (*a*) R. A. R. Bryant and D. E. Hansen, *J. Am. Chem. Soc.*, 1996, **118**, 5498–5499; (*b*) A. Radzicka and R. Wolfenden, *J. Am. Chem. Soc.*, 1996, **118**, 6105–6109; (*c*) R. M. Smith and D. E. Hansen, *J. Am. Chem. Soc.*, 1998, **120**, 8910–8913.
2 L. R. Croft, *Handbook of Protein Sequence Analysis*, Wiley, Chichester, 1980.
3 M. Yashiro, Y. Sonobe, A. Yamamura, T. Takarada, M. Komiyama and Y. Fujii, *Org. Biomol. Chem.*, 2003, **1**, 629–632.
4 (*a*) M. Kassai, R. G. Ravi, S. J. Shealy and K. B. Grant, *Inorg. Chem.*, 2004, **43**, 6130–6132; (*b*) H. G. T. Ly, G. Absillis, S. R. Bajpe, J. A. Martens and T. N. Parac-Vogt, *Eur. J. Inorg. Chem.*, 2013, 4601–4611; (*c*) A. Singhal, L. M. Toth, J. S. Lin and K. Affholter, *J. Am. Chem. Soc.*, 1996, **118**, 11529–11534.
5 M. Kassai and K. B. Grant, *Inorg. Chem. Commun.*, 2008, **11**, 521–525.
6 M.-J. Rhee and C. B. Storm, *J. Inorg. Biochem.*, 1979, **11**, 17–24.
7 (*a*) J. Suh, *Acc. Chem. Res.*, 2003, **36**, 562–570; (*b*) M. C. B. de Oliveira, M. Scarpellini, A. Neves, H. Terenzi, A. J. Bortoluzzi, B. Szpoganics, A. Greatti, A. S. Mangrich, E. M. de Souza, P. M. Fernandez and M. R. Soares, *Inorg. Chem.*, 2005, **44**, 921–929; (*c*) E. L. Hegg and J. N. Burstyn, *J. Am. Chem. Soc.*, 1995, **117**, 7015–7016; (*d*) Y. Fujii, T. Kiss, T. Gajda, X. S. Tan, T. Sato, Y. Nakano, Y. Hayashi and M. Yashiro, *JBIC, J. Biol. Inorg. Chem.*, 2002, **7**, 843–851.
8 M. T. Bordignon Luiz, B. Szpoganicz, M. Rizzoto, M. G. Basallote and A. E. Martell, *Inorg. Chim. Acta*, 1999, **287**, 134–141.
9 (*a*) N. M. Milović and N. M. Kostić, *J. Am. Chem. Soc.*, 2002, **124**, 4759–4769; (*b*) D. P. Ašanin, S. Rajković, D. Molnar-Gabor and M. I. Djuran, *Monatsh. Chem.*, 2004, **135**, 1445–1453; (*c*) L. Zhu and N. M. Kostic, *Inorg. Chim. Acta*, 2002, **339**, 104–110; (*d*) T. N. Parac and N. M. Kostic, *J. Am. Chem. Soc.*, 1996, **118**, 5946–5951; (*e*) L. G. Zhu, L. Qin, T. N. Parac and N. M. Kostic, *J. Am. Chem. Soc.*, 1994, **116**, 5218–5224.
10 (*a*) E. Kopera, A. Krezel, A. M. Protas, A. Belczyk, A. Bonna, A. Wyslouch-Cieszynska, J. Poznanski and W. Bal, *Inorg. Chem.*, 2010, **49**, 6636–6645; (*b*) A. M. Protas, A. Bonna, E. Kopera and W. Bal, *J. Inorg. Biochem.*, 2011, **105**, 10–16; (*c*) A. Krezel, M. Mylonas, E. Kopera and W. Bal, *Acta Biochim. Pol.*, 2006, **53**, 721–727.
11 T. Takarada, M. Yashiro and M. Komiyama, *Chem. – Eur. J.*, 2000, **6**, 3906–3913.
12 (*a*) T. N. Parac, G. M. Ullmann and N. M. Kostic, *J. Am. Chem. Soc.*, 1999, **121**, 3127–3135; (*b*) T. N. Parac and N. M. Kostic, *J. Am. Chem. Soc.*, 1996, **118**, 51–58; (*c*) T. N. Parac and N. M. Kostić, *Inorg. Chem.*, 1998, **37**, 2141–2144.
13 R. B. Martin, *Met. Ions Biol. Syst.*, 2001, **38**, 1.

14 (*a*) D.-L. Long, E. Burkholder and L. Cronin, *Chem. Soc. Rev.*, 2007, **36**, 105–121; (*b*) N. Mizuno and K. Kamata, *Coord. Chem. Rev.*, 2011, **255**, 2358–2370; (*c*) A. Proust, R. Thouvenot and P. Gouzerh, *Chem. Commun.*, 2008, 1837–1852; (*d*) D.-L. Long, R. Tsunashima and L. Cronin, *Angew. Chem., Int. Ed.*, 2010, **49**, 1736–1758.

15 (*a*) H. Stephan, M. Kubeil, F. Emmerling and C. E. Müller, *Eur. J. Inorg. Chem.*, 2013, 1585–1594; (*b*) G. Fiorani, O. Saoncella, P. Kaner, S. A. Altinkaya, A. Figoli, M. Bonchio and M. Carraro, *J. Cluster Sci.*, 2014, **25**, 839–854.

16 (*a*) J. M. Clemente-Juan, E. Coronado and A. Gaita-Arino, *Chem. Soc. Rev.*, 2012, **41**, 7464–7478; (*b*) K. M. Seemann, A. Bauer, J. Kindervater, M. Meyer, C. Besson, M. Luysberg, P. Durkin, W. Pyckhout-Hintzen, N. Budisa, R. Georgii, C. M. Schneider and P. Koegerler, *Nanoscale*, 2013, **5**, 2511–2519.

17 (*a*) M. Carraro and S. Gross, *Materials*, 2014, **7**, 3956–3989; (*b*) A. Proust, B. Matt, R. Villanneau, G. Guillemot, P. Gouzerh and G. Izzet, *Chem. Soc. Rev.*, 2012, **41**, 7605–7622.

18 (*a*) N. V. Izarova, M. T. Pope and U. Kortz, *Angew. Chem., Int. Ed.*, 2012, **51**, 9492–9510; (*b*) A. Sartorel, M. Bonchio, S. Campagna and F. Scandola, *Chem. Soc. Rev.*, 2013, **42**, 2262–2280; (*c*) J. J. Stracke and R. G. Finke, *J. Am. Chem. Soc.*, 2011, **133**, 14872–14875.

19 (*a*) N. Steens, A. M. Ramadan and T. N. Parac-Vogt, *Chem. Commun.*, 2009, 965–967; (*b*) E. Cartuyvels, G. Absillis and T. N. Parac-Vogt, *Chem. Commun.*, 2008, 85–87; (*c*) N. Steens, A. M. Ramadan, G. Absillis and T. N. Parac-Vogt, *Dalton Trans.*, 2010, **39**, 585–592; (*d*) G. Absillis, E. Cartuyvels, R. Van Deun and T. N. Parac-Vogt, *J. Am. Chem. Soc.*, 2008, **130**, 17400–17408; (*e*) G. Absillis, R. Van Deun and T. N. Parac-Vogt, *Inorg. Chem.*, 2011, **50**, 11552–11560; (*f*) L. Thi Kim Nga, G. Absillis, P. Shestakova and T. N. Parac-Vogt, *Eur. J. Inorg. Chem.*, 2014, 5276–5284.

20 (*a*) T. Meißner, R. Bergmann, J. Oswald, K. Rode, H. Stephan, W. Richter, H. Zänker, W. Kraus, F. Emmerling and G. Reck, *Transition Met. Chem.*, 2006, **31**, 603–610; (*b*) C. E. Müller, J. Iqbal, Y. Baqi, H. Zimmermann, A. Röllich and H. Stephan, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 5943–5947.

21 C. Boglio, G. Lemiere, B. Hasenknopf, S. Thorimbert, E. Lacote and M. Malacria, *Angew. Chem., Int. Ed.*, 2006, **45**, 3324–3327.

22 (*a*) N. Mizuno, K. Yamaguchi and K. Kamata, *Coord. Chem. Rev.*, 2005, **249**, 1944–1956; (*b*) N. Mizuno, K. Kamata and K. Yamaguchi, *Top. Catal.*, 2010, **53**, 876–893; (*c*) C. N. Kato, K. Hayashi, S. Negishi and K. Nomiya, *J. Mol. Catal. A: Chem.*, 2007, **262**, 25–29; (*d*) A. C. Estrada, I. C. M. S. Santos, M. M. Q. Simoes, M. G. P. M. S. Neves, J. A. S. Cavaleiro and A. M. V. Cavaleiro, *Appl. Catal., A*, 2011, **392**, 28–35.

23 S. Zhao, L. Huang and Y.-F. Song, *Eur. J. Inorg. Chem.*, 2013, 1659–1663.

24 G. Absillis and T. N. Parac-Vogt, *Inorg. Chem.*, 2012, **51**, 9902–9910.

25 (*a*) K. Stroobants, E. Moelants, H. G. T. Ly, P. Proost, K. Bartik and T. N. Parac-Vogt, *Chem. – Eur. J.*, 2013, **19**, 2848–2858; (*b*) K. Stroobants, V. Goovaerts, G. Absillis, G. Bruylants, E. Moelants, P. Proost and T. N. Parac-Vogt, *Chem. – Eur. J.*, 2014, **20**, 9567–9577; (*c*) K. Stroobants, G. Absillis, E. Moelants, P. Proost and T. N. Parac-Vogt, *Chem. – Eur. J.*, 2014, **20**, 3894–3897.

26 (*a*) S. Vanhaecht, G. Absillis and T. N. Parac-Vogt, *Dalton Trans.*, 2012, **41**, 10028–10034; (*b*) H. G. T. Ly, G. Absillis and T. N. Parac-Vogt, *Dalton Trans.*, 2013, **42**, 10929–10938.

27 S. Vanhaecht, G. Absillis and T. N. Parac-Vogt, *Dalton Trans.*, 2013, **42**, 15437–15446.

28 (*a*) K. Nomiya, Y. Saku, S. Yamada, W. Takahashi, H. Sekiya, A. Shinohara, M. Ishimaru and Y. Sakai, *Dalton Trans.*, 2009, 5504–5511; (*b*) C. N. Kato, A. Shinohara, K. Hayashi and K. Nomiya, *Inorg. Chem.*, 2006, **45**, 8108–8119.

29 P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, 1960, **64**, 188.

30 (*a*) C. Boglio, B. Hasenknopf, G. Lenoble, P. Rémy, P. Gouzerh, S. Thorimbert, E. Lacôte, M. Malacria and R. Thouvenot, *Chem. – Eur. J.*, 2008, **14**, 1532–1540; (*b*) N. Dupré, P. Rémy, K. Micoine, C. Boglio, S. Thorimbert, E. Lacôte, B. Hasenknopf and M. Malacria, *Chem. – Eur. J.*, 2010, **16**, 7256–7264.

31 T. K. N. Luong, P. Shestakova, T. T. Mihaylov, G. Absillis, K. Pierloot and T. N. Parac-Vogt, *Chem. – Eur. J.*, 2015, **21**, 4428–4439.

32 P. H. Ho, K. Stroobants and T. N. Parac-Vogt, *Inorg. Chem.*, 2011, **50**, 12025–12033.

33 A. A. Zamyatnin, *Prog. Biophys. Mol. Biol.*, 1972, **24**, 107.

34 T. W. Johnson and N. M. Kostic, *J. Serb. Chem. Soc.*, 2004, **11**, 887–899.

35 (*a*) O. Hohage and W. S. Sheldrick, *J. Inorg. Biochem.*, 2006, **100**, 1506–1513; (*b*) S. Rajković, B. Đ. Glišić, M. D. Živković and M. I. Djuran, *Bioorg. Chem.*, 2009, **37**, 173–179; (*c*) N. M. Milović and N. M. Kostić, *J. Am. Chem. Soc.*, 2002, **125**, 781–788.

36 N. M. Milović and N. M. Kostic, *J. Am. Chem. Soc.*, 2003, **125**, 781–788.

37 I. Sóvágó, T. Kiss, K. Várnagy and B. D.-L. Révérend, *Polyhedron*, 1988, **7**, 1089–1093.

38 E. Garribba, E. Lodyga-Chruscinska, G. Micera, A. Panzanelli and D. Sanna, *Eur. J. Inorg. Chem.*, 2005, 1369–1382.

39 (*a*) J. Vlasak and R. Ionescu, *mAbs*, 2011, **3**, 253–263; (*b*) B. Testa and J. M. Mayer, *Hydrolysis in Drug and Prodrug Metabolism*, Wiley-VCH, Weinheim, 2003; (*c*) C. Oliyai and R. T. Borchardt, *Pharm. Res.*, 1993, **10**, 95–102.

40 K. Arii, T. Kai and Y. Kokuba, *Eur. J. Pharm. Sci.*, 1999, **7**, 107–112.

41 G. Kottra, A. Stamfort and H. Daniel, *J. Biol. Chem.*, 2002, **277**, 32683–32691.