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Amide bond hydrolysis in peptides and cyclic peptides catalyzed by a dimeric Zr(IV)-substituted Keggin type polyoxometalate†

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Detailed kinetic studies on the hydrolysis of glycylserine (Gly-Ser) and glycylglycine (Gly-Gly) in the presence of the dimeric zirconium(IV)-substituted Keggin type polyoxometalate $(Et_2NH_2)_8[\{\alpha\text{-}PW_{11}O_{39}Zr(\mu\text{-}OH)(H_2O)\}_2]\cdot 7H_2O$ (**1**) were performed by a combination of ^1H, ^{13}C and ^{31}P NMR spectroscopy. The observed rate constants for the hydrolysis of Gly-Ser and Gly-Gly at pD 5.4 and 60 °C were 63.3×10^{-7} s^{-1} and 4.44×10^{-7} s^{-1} respectively, representing a significant acceleration as compared to the uncatalyzed reactions. The pD dependence of the rate constant for both reactions exhibited a bell-shaped profile with the fastest hydrolysis observed in the pD range of 5.5–6.0. Interaction of **1** with Gly-Ser and Gly-Gly *via* their amine nitrogen and amide oxygen was proven by ^{13}C NMR spectroscopy. The effective hydrolysis of Gly-Ser in the presence of **1** is most likely a combination of the polarization of the amide oxygen due to its binding to the Zr(IV) ion in **1** and the intramolecular attack of the Ser hydroxyl group on the amide carbonyl carbon. The effect of temperature, inhibitors, and ionic strength on the hydrolysis rate constant was also examined. The solution structure of **1** was investigated by means of ^{31}P NMR spectroscopy, revealing that its stability is highly dependent on pH, concentration and temperature. A 2.0 mM solution of **1** was found to be fully stable under hydrolytic conditions (pD 5.4 and 60 °C) both in the presence and in the absence of the dipeptides.

Introduction

Polyoxometalates (POMs) are composed of early transition metal ions in their highest oxidation state bridged by oxo ligands. They are characterized by a wide range of dynamic molecular structures, and physical and chemical properties.[1] As a result POMs are attractive for applications in many different areas, for example, in catalysis,[2] medicine,[3] material science,[4] magnetism,[5] and nanotechnology.[6] Their molecular, chemical, and physical properties can be easily modified by changing the synthesis conditions.[7] It has been known that POMs undergo hydrolysis to form lacunary structures, which can readily react with a variety of transition metal and lanthanide ions that refill the vacant sites resulting in metal-substituted POMs.[8] In metal-substituted POMs, the substituted metal ion can coordinate to H_2O or other ligands due to its unshared coordination sites. This feature makes them attractive as catalysts for a wide variety of reactions. The majority of the applications of metal-substituted POMs are found in the area of catalysis for C–H hydroxylation and double-bond epoxidation,[9] H_2O_2-based oxidation,[10] and Lewis acid mediated organic reactions.[11] Examples of catalytic studies with lanthanide and transition metal-substituted POMs for peptide hydrolysis are rare.[12]

Protein hydrolysis is an important process in biotechnology and in modern proteomics. However, the rate of peptide bond hydrolysis under physiological conditions is extremely slow. At neutral pH and 25 °C, the half-life for the hydrolysis of the peptide bond in Gly-Gly is estimated to be around 350 years.[13] Several proteolytic enzymes are available for the hydrolysis of proteins. However, they often cleave proteins into too many fragments and are extremely sensitive to temperature and pH. To promote peptide bond cleavage, several metal-containing catalysts that facilitate this process have been developed. The hydrolysis of peptide bonds has mostly been studied in the presence of lanthanide and transition metal salts.[14] Particularly, they promote the hydrolysis of peptides containing Ser or Thr at the C-terminus by polarizing the peptide bond *via* coordination and intramolecular attack of the hydroxyl group on the amide carbon resulting in an ester intermediate. However, a drawback of using metal ions in this kind of reaction is the formation of insoluble gels and precipitates under neutral pH conditions.[15] In addition, several metal complexes

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of Pt(II),[16] Pd(II),[17] Cu(II),[18] Co(III),[19] Ni(II),[20] Zn(II),[14b,21] Ce(IV),[22] and Zr(IV) were studied for peptide bond hydrolysis.[12a,23] Pt(II) and Pd(II) complexes were shown to promote selective hydrolysis at Met and His residues, but only under low pH conditions.[24] Complexes of Cu(II), Co(III) and Ni(II) were able to cleave unactivated peptide bonds in peptides and proteins,[18a,c,20b,25] but Cu(II) and Ni(II) form strong metal–amide nitrogen bonds at pH 4 and pH 8 respectively[26] forming catalytically inactive complexes.

In our quest to understand the biological role of POMs on a molecular level, we recently discovered the unprecedented reactivity of polyoxomolybdates and polyoxovanadates towards phosphoester bonds in DNA and RNA model systems as well as ester bonds in a series of carboxyesters.[27] Furthermore, we have also shown that peptides with an X-Ser sequence were effectively hydrolyzed in oxomolybdate and oxovanadate solutions.[28] Although amide bond hydrolysis occurred in solutions containing a mixture of oxoanions, the kinetic experiments and DFT calculations revealed that the actual active species are monomeric oxoanions.[28b] The effective hydrolysis of phosphoester bonds in DNA and RNA model compounds was also achieved in the presence of the Zr(IV)-substituted Wells–Dawson POM[29] and the incorporation of Hf(IV) into this POM structure resulted in higher Lewis acidity for catalysis of Mukaiyama aldol and Mannich-type reactions,[11a] demonstrating the use of the Lewis acid activity of metal-substituted POMs for catalysis of organic reactions.[11b] Moreover, Zr(IV)-substituted POMs have been used for H_2O_2 based oxidation reactions,[10f,30] sulfide oxidation,[31] and the cyclization of citronellal.[11c] Our recent efforts to develop new catalysts for amide bond hydrolysis have been focused on the use of metal-functionalized POMs.[12] The incorporation of highly Lewis acid metal ions into the POM framework prevents the formation of inactive gels, which typically occurs at neutral and basic pH values. We have recently shown that the sandwich type 1 : 2 Zr(IV)-substituted Wells–Dawson POM, $K_{15}H[Zr(\alpha_2\text{-}P_2W_{17}O_{61})_2]\cdot 25H_2O$, efficiently hydrolyzed unactivated amide bond in glycylglycine in a fully homogeneous manner.[12a] The stability of this POM was shown to be highly dependent on the pH, concentration, temperature and reaction time. When the 1 : 2 dimeric species is dissolved in water, multiple equilibria between the dimeric 2 : 2, monomeric 1 : 1, and sandwich type 1 : 2 Zr(IV)–POM complexes are observed.[8i,32] Among all these species, the monomeric 1 : 1 form is expected to have the highest catalytic activity, due to the presence of several water molecules in the first coordination sphere of Zr(IV). Although the highest catalytic activity of this POM towards Gly-Gly hydrolysis was obtained at pD 5.0, multiple equilibria still exist in solution, preventing a detailed understanding of the catalytic mechanism.

To further exploit POMs as catalysts for the hydrolysis of peptide bonds, herein we report the use of dinuclear Zr(IV)-substituted Keggin POM as an artificial peptidase. By using the monolacunary α-Keggin anion $[\alpha\text{-}PW_{11}O_{39}]^{7-}$ as a ligand for Zr(IV), the dinuclear zirconium(IV) α-Keggin polyoxometalate $(Et_2NH_2)_8[\{\alpha\text{-}PW_{11}O_{39}Zr(\mu\text{-}OH)(H_2O)\}_2]\cdot 7H_2O$ (**1**) was formed.[8j] The single crystal X-ray structure of **1** shows that two water molecules, which can be potentially substituted with peptide substrate, are coordinated in the first coordination sphere of the Zr(IV) ion.[8j] In this study we examine the reactivity of **1** towards the hydrolysis of Gly-Gly, which has a non-coordinating side chain, and Gly-Ser, a dipeptide containing a hydroxyl group in the side chain. We reason that the use of the Keggin POM as a chelating agent for Zr(IV) will not only result in a homogeneous reaction mixture, but may also benefit from the protein binding properties of the POM in future experiments involving the hydrolysis of proteins, as we recently demonstrated in the first example of protein hydrolysis promoted by a Ce(IV)-substituted Keggin polyoxometalate.[12b]

Experimental procedures

Chemicals

D_2O, DCl, NaOD, $H_3PW_{12}O_{40}\cdot 23H_2O$, $NaHCO_3$, $ZrOCl_2$, HCl, Et_2NH_2Cl, EtOH, Et_2O, LiCl, Gly-Ser, Gly-Gly, oxalic, malonic, succinic, adipic, citric and malic acid were purchased from Sigma-Aldrich. The 2 : 2 complex **1** was synthesized according to the procedure described in the literature[8j,33] with some modifications as described below. The 1 : 2 complex $(Et_2NH_2)_{10}[Zr(\alpha\text{-}PW_{11}O_{39})_2]\cdot 7H_2O$ (**2**) was synthesized according to the procedure described in the literature.[33]

Synthesis of 1

The synthesis of **1** was accomplished by a 1 : 1 molar ratio reaction of the *in situ*-generated $[Zr(\alpha\text{-}PW_{11}O_{39})_2]^{10-}$ with $ZrOCl_2$ in an aqueous HCl solution, followed by the addition of an excess amount of solid Et_2NH_2Cl. $H_3PW_{12}O_{40}\cdot 23H_2O$ (4.94 g) was dissolved in 25 mL of H_2O, followed by addition of 1.0 M $NaHCO_3$ until a pH value of 5.25 was obtained. The volume was adjusted to 50 mL by adding H_2O. $ZrOCl_2$ (0.256 g) was added and the solution was stirred for 5 minutes at room temperature. HCl (1 mL of 1.0 M) was added dropwise under vigorous stirring. The solution was stirred for an additional 30 minutes at room temperature. To this solution $ZrOCl_2$ (0.256 g dissolved in 4 mL of 1 M HCl) was added dropwise. The solution was stirred for 30 minutes and filtered with a folded filter paper. The colourless filtrate was evaporated at 40 °C to 20 mL. After stirring for 1 minute at 95 °C, 2.5 g of Et_2NH_2Cl was added and the mixture was stirred for 5 minutes. The white suspension was cooled to room temperature and stirred for 1 hour. White powder was collected and washed with EtOH (30 mL) and Et_2O (3 × 50 mL), and dried *in vacuo* for 2 hours. A water-soluble white powder (4.18 g – 88%) was obtained. The ^{31}P NMR spectrum of complex **1** in D_2O shows one signal at −13.49 ppm.

Measurements

^1H NMR spectra were recorded on a Bruker Advance 400 spectrometer and TMSP-d_4 was used as an internal reference. ^{13}C NMR spectra were recorded on a Bruker Advance 400 spectrometer. As a reference TMS in an internal reference tube

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was used. ^{31}P NMR spectra were recorded on a Bruker Advance 400 spectrometer and 25% H_3PO_4 in H_2O in an internal reference tube was used.

Stability studies

To study the effect of pD on the stability of **1**, 2.0 mM solutions of **1** in D_2O were adjusted to pD 2.0, 3.0, 4.9, 7.4, 9.0, and 10.4 by using 1.0 M DCl or 1.0 M NaOD. To study the effect of time and temperature on the stability, 2.0 mM solutions of **1** were adjusted to pD 5.4 and 7.4 and kept at 60 °C. To study the effect of POM concentration on the stability of **1**, 2.0 mM and 6.0 mM solutions of **1** were adjusted to pD 5.4 and kept at 60 °C. The pH-meter reading was corrected by the equation pD = pH + 0.41.[34]

Hydrolysis studies

The hydrolysis of 2.0 mM peptide in the presence of a 2.0 mM solution of **1** was followed by ^{1}H NMR spectroscopy. Typically, 800 μL of a 2.5 mM solution of **1** in D_2O, 100 μL of a 20.0 mM solution of the dipeptide in D_2O, and 100 μL of a 5.0 mM solution of TMSP-2,2,3,3-d_4 (sodium-3-trimethylsilylpropionate) in D_2O were mixed. The pD of the final solution was adjusted to 5.40 with DCl or NaOD and was measured at the beginning and at the end of each experiment. The pD difference was less than 0.10 unit. The reaction samples were kept at 60 °C. ^{1}H NMR spectra were taken after mixing and after different time increments.

Binding studies

The binding of **1** to the dipeptides was studied by ^{1}H and ^{13}C NMR spectroscopy, by examining changes in the NMR spectra of the peptide upon addition of **1**. The solutions for NMR contained 20.0 mM to which 5.0 mM of **1** in D_2O was added. The pH of the solution was adjusted to pD 6.4 or pD 9.0 by using 1.0 M DCl or 1.0 M NaOD.

Results and discussion

Stability of 1 in aqueous solution

Depending on the synthesis conditions, Zr(IV) can be incorporated into the lacunary Keggin POM, forming dimeric 2 : 2 ($[\{\alpha\text{-}PW_{11}O_{39}Zr(\mu\text{-}OH)(H_2O)\}_2]^{8-}$ (**1**) or sandwich 1 : 2 ($[Zr(\alpha\text{-}PW_{11}O_{39})_2]^{10-}$ (**2**) structures.[8j,33] Single crystal X-ray structures show that the Zr(IV) ion in **1** has two coordinated water molecules in the first coordination sphere, whereas the Zr(IV) ion in **2** is saturated with two lacunary Keggin units (Fig. 1).

Fig. 1 Schematic representation of $(Et_2NH_2)_8[\{\alpha\text{-}PW_{11}O_{39}Zr(\mu\text{-}OH)(H_2O)\}_2]\cdot 7H_2O$ (**1**) and $(Et_2NH_2)_{10}[Zr(\alpha\text{-}PW_{11}O_{39})_2]\cdot 7H_2O$ (**2**).

It is expected that **1** will display a higher catalytic activity since the Zr(IV) center in **1** has free coordination sites available for the interaction with substrate molecules. In aqueous solution the conversion between both complexes is a reversible process that is dependent on the pH, temperature, time and concentration. The stability of **1** and **2** in aqueous solution can be monitored by ^{31}P NMR spectroscopy since both complexes are characterized by a specific ^{31}P NMR resonance. The ^{31}P NMR spectra of 2.0 mM **1** at different pD values are shown in Fig. 2. While at pD 7.4 the only observable signal at −13.49 ppm is the dimeric complex **1**,[8j] at pD 9.0 a new set of resonances at −14.60 and −14.69 ppm, corresponding to the sandwich complex **2**,[33,35] simultaneously appeared. The complete conversion of **1** into **2** and the lacunary $[\alpha\text{-}PW_{11}O_{39}]^{7-}$ anion (δ −10.67 ppm)[8b,36] occurred at pD 10.4. However, in the pD range 2.0–7.4 only the dimeric complex **1** was present in solution. A slight shift of 0.13 ppm was seen in acidic media, which might be due to the protonation of coordinated water molecules.[8j] As the hydrolysis of peptide bonds was studied at 60 °C in the course of several days, the effect of time on the species distribution at 60 °C was further investigated. Solutions containing 2.0 mM **1** at pD 5.4 and 7.4 were kept at 60 °C and their ^{31}P NMR spectra at different time increments are presented in Fig. S1†. After 24 h at pD 7.4 and 60 °C 25% of **2** was detected in solution. Equilibrium between these two complexes was obtained after 2 days representing a ratio of *ca.* 3 : 2 between **1** and **2**. Interestingly, no products derived from the decomposition of **1** were detected after 20 days at 60 °C and pD 5.4. Next, the effect of the initial concentration of **1** on the conversion at pD 5.4 and 60 °C was investigated. The sandwich type complex **2** was formed in the 6.0 mM solution of **1** after one day (Fig. S2†), while the 2.0 mM solution of **1** remained unchanged after 20 days.

The pH dependence of the reversible conversion process of complex **2** in aqueous solution was also investigated. The ^{31}P NMR spectra of the 2.0 mM solution of **2** were recorded after pD adjustment (Fig. S3†). Only complex **2** was observed in the

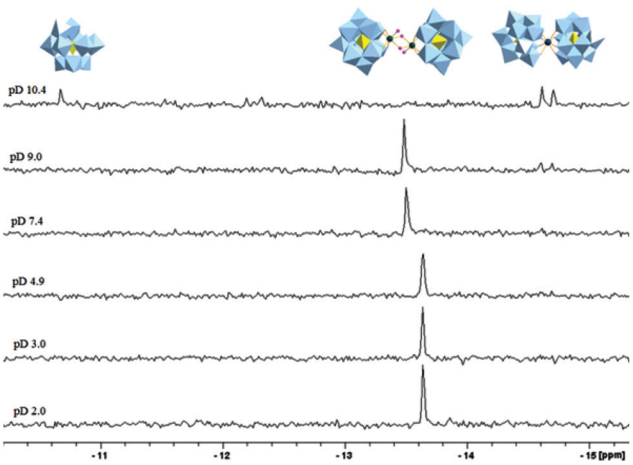

Fig. 2 ^{31}P NMR spectra of 2.0 mM solutions of **1** at different pD values recorded immediately after pD adjustment.

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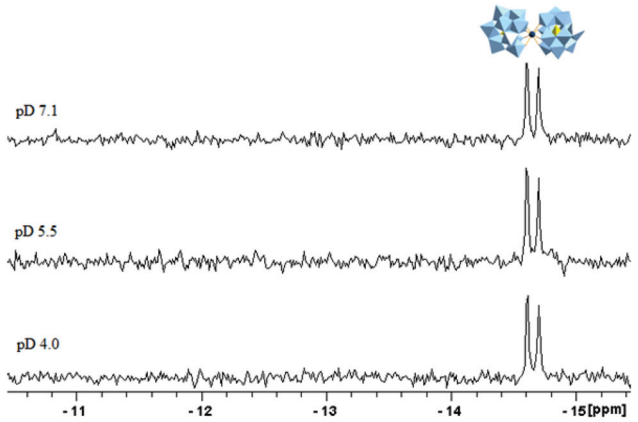

Fig. 3 ^{31}P NMR spectra of a 2.0 mM solution of **2** after 10 days at 60 °C and in the pD range 4.0–7.1.

pD range 4.0–7.1, but the monolacunary species simultaneously appeared at pD 8.1. The sandwich complex **2** was stable after 10 days at 60 °C and in the pD range 4.0–7.1 (Fig. 3).

Complex **1** having two labile water molecules coordinated to Zr(IV) is expected to be a better catalyst for peptide bond hydrolysis since Zr(IV) can coordinate to the amide carbonyl oxygen, polarizing the carbonyl bond, resulting in an amide carbon that is more susceptible to nucleophilic attack. Therefore, the hydrolysis of Gly-Gly and Gly-Ser dipeptides in the presence of **1** was explored at pD 5.4 and 60 °C, as under these experimental conditions only **1** was present in solution making the solution species distribution more controllable when compared to the Zr(IV)-substituted Wells–Dawson type POM and therefore allowing a more detailed mechanistic study. In order to compare the catalytic reactivity of complexes **1** and **2**, the hydrolysis of Gly-Ser and Gly-Gly by **2** was also investigated under the same experimental conditions.

Hydrolysis of Gly-Ser

The cleavage of 2.0 mM Gly-Ser by 2.0 mM **1** was examined at pD 5.4 and 60 °C. ^{1}H NMR spectra of the reaction recorded at different time increments are presented in Fig. 4. The signal at 4.41 ppm corresponds to the CH proton of serine residue in Gly-Ser. The signal of the CH_2 proton of the glycyl residue (3.90 ppm) overlaps with that of the serine residue (3.85–3.96 ppm) in Gly-Ser. The intensity of these signals decreased during the course of the reaction, while an increase in intensity of free glycine (3.57 ppm) and free serine (3.85–3.87 and 3.96–4.00 ppm) was observed. These changes indicate that the hydrolysis of the amide bond in Gly-Ser occurred. Additionally, the cyclic Gly-Ser (cGly-Ser), identified by comparison of the chemical shifts with those of pure cGly-Ser, was also detected with a maximum concentration of 10% in total. The percentage of Gly-Ser, Gly, Ser and cGly-Ser is shown in Fig. 5. As can be seen from Fig. 5, the intensity of the cGly-Ser resonances remained relatively constant during the reaction, suggesting that the cyclization to cGly-Ser is

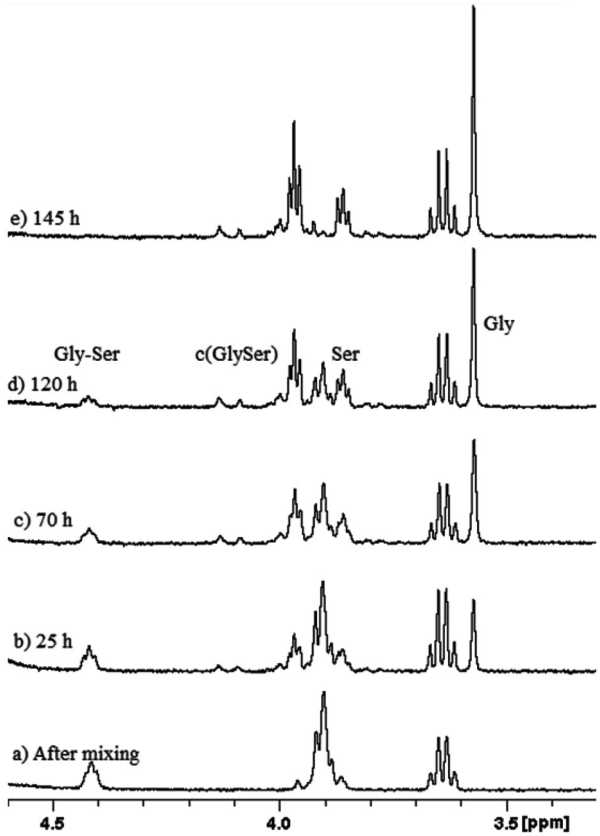

Fig. 4 ^{1}H NMR spectra recorded at different reaction times during the hydrolysis of 2.0 mM Gly-Ser by 2.0 mM **1** at pD 5.4 and 60 °C.

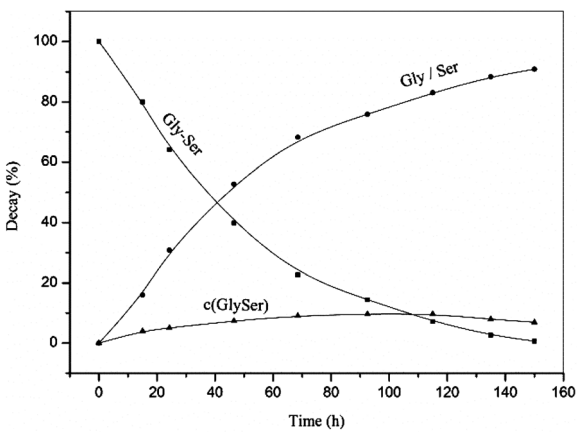

Fig. 5 Fraction of Gly-Ser, Gly, Ser and cGly-Ser as a function of time in the reaction between 2.0 mM Gly-Ser and 2.0 mM **1** at pD 5.4 and 60 °C.

irreversible or the cleavage of cGly-Ser is too slow compared to Gly-Ser hydrolysis. To clarify this issue, the hydrolysis of 2.0 mM cGly-Ser in the presence of 2.0 mM **1** at pD 5.4 and 60 °C was explored. Ring-opening of cGly-Ser occurred to form Gly-Ser and Ser-Gly (identified by spiking the authentic sample), which further hydrolyzed forming Gly and Ser (Fig. S4† and Fig. 6). This finding proves that the cyclization of Gly-Ser is a reversible process. Remarkably, no gel or

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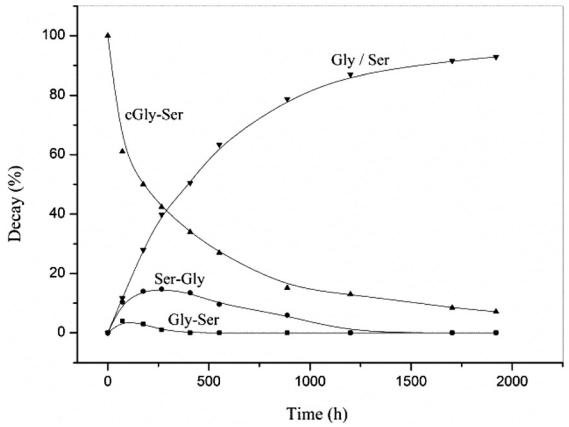

Fig. 6 Fraction of cGly-Ser, Gly-Ser, Ser-Gly and Gly as a function of time in the reaction between 2.0 mM cGly-Ser and 2.0 mM **1** at pD 5.4 and 60 °C.

precipitation was observed in any of the reactions in the presence of **1**, indicating that hydrolysis of the peptide bond occurs under fully homogenous conditions. Notably, the presence of **1** does not only accelerate the peptide bond cleavage, but also promotes the cyclization of Gly-Ser which did not occur in the control reactions.

Complete cleavage of Gly-Ser was obtained, resulting in 5% of cGly-Ser and 95% of Gly and Ser in the mixture (Fig. 5). By fitting the decrease in the concentration of Gly-Ser to a monoexponential function, the rate constant $k_{obs} = 63.3 \times 10^{-7}$ s^{-1} ($t_{\frac{1}{2}}$ = 30 h) for the hydrolysis was calculated. It is important to note that despite the fact that the reaction rate appears slow, it represents a significant acceleration of nearly 50 times compared to the uncatalyzed reaction. In Gly-Ser samples without the catalyst no hydrolysis of the peptide bond was observed in the time period during which full peptide bond hydrolysis occurred in the presence of **1** (Table S2†). Similarly, the observed rate constant of 3.33×10^{-7} s^{-1} ($t_{\frac{1}{2}}$ = 577 h) for the hydrolysis of cGly-Ser was obtained, which is about 20 times slower compared to Gly-Ser cleavage. Under the same experimental conditions the hydrolysis of 2.0 mM Gly-Ser in the presence of 2.0 mM **2** occurred with a rate constant of 3.05×10^{-7} s^{-1}, which is only about two times larger than the uncatalyzed reaction (Table S2†), but about 20 times smaller than the observed rate constant in the presence of **1** under the same reaction conditions. This indicates that the dimeric complex **1** with free coordinated water molecules and unshared coordination sites available for interaction with the peptide is far more active than the sandwich type complex **2**. Despite the fact that no water molecules are coordinated to the Zr(IV) ion in **2**, the limited reactivity can be explained by the fact that binding of the substrate can displace the equilibrium towards the catalytically active 1 : 1 species.[37]

Hydrolysis of Gly-Gly

A mixture containing 2.0 mM Gly-Gly and 2.0 mM solution of **1** was reacted at 60 °C and pD 5.4. The ^{1}H NMR spectra (Fig. S5†) measured at different time increments showed a gradual decrease of the two Gly-Gly resonances (3.86 and 3.89 ppm) and a steady increase of the free glycine resonance at 3.56 ppm. In addition, the cyclic Gly-Gly (cGly-Gly) at 4.04 ppm was observed during the course of the hydrolytic reaction. The percentage of Gly-Gly, Gly and cGly-Gly is shown in Fig. 7. The data show that the fraction of cGly-Gly gradually decreased to zero after reaching a maximum of 11%. The degradation of cGly-Gly can directly form Gly by breaking two chemical bonds at the same time or indirectly form Gly *via* Gly-Gly. To gain more insights into the disappearance of cGly-Gly, we examined the hydrolysis of 2.0 mM cGly-Gly in the presence of 2.0 mM **1** at pD 5.4 and 60 °C. Fig. S6† shows the ^{1}H NMR spectra recorded at various reaction times of the cGly-Gly cleavage. The percentage of cGly-Gly, Gly-Gly and Gly is presented in Fig. 8. As can be seen, 27% of cGly-Gly is converted to Gly-Gly after one day. Notably, no free glycine was detected after one day and it could only be seen after sufficient amounts of Gly-Gly were formed, which were subsequently hydrolyzed to free glycine. This finding suggests that simultaneously breaking two amide bonds in cGly-Gly is unfavourable and that the cyclization of Gly-Gly to cGly-Gly by **1** is a reversible process. The hydrolysis of cGly-Gly by **1** is presented in Scheme 1.

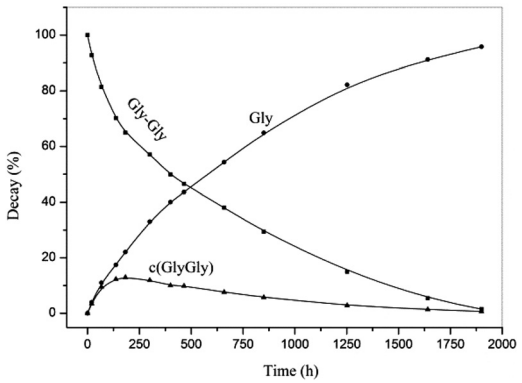

Fig. 7 Fraction of Gly-Gly, Gly, and cGly-Gly as a function of time in the reaction between 2.0 mM Gly-Gly and 2.0 mM **1** at pD 5.4 and 60 °C.

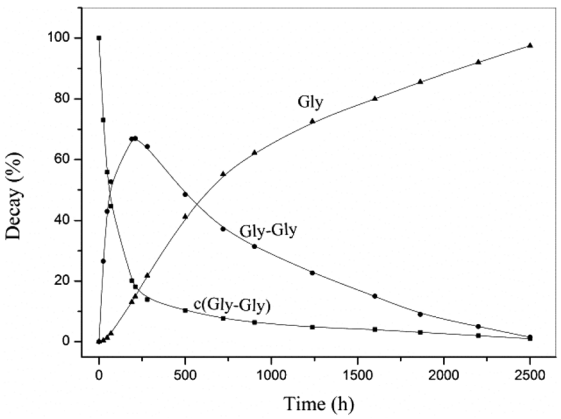

Fig. 8 Fraction of cGly-Gly, Gly-Gly and Gly as a function of time in the reaction between 2.0 mM cGly-Gly and 2.0 mM **1** at pD 5.4 and 60 °C.

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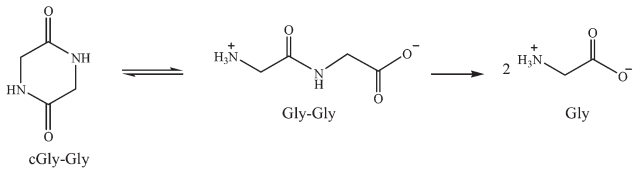

Scheme 1 Hydrolysis of cGly-Gly by **1**.

Similarly to Gly-Ser hydrolysis, no gel formation or precipitation was observed in any of the reactions in the presence of **1**. The observed rate constant of 4.44×10^{-7} s^{-1} for the cleavage of Gly-Gly was calculated by fitting the decrease in Gly-Gly concentration to a monoexponential function. Although the hydrolysis of Gly-Gly is relatively slow and has a half-life of approximately 18 days, this represents a significant acceleration compared to the uncatalyzed reaction in which only 25% of Gly-Gly was hydrolyzed after seven months under identical conditions. Additionally, under the same experimental conditions the hydrolysis of 2.0 mM Gly-Gly in the presence of 2.0 mM **2** was also studied. After 15 days at pD 5.4 and 60 °C only 3% of Gly-Gly was hydrolyzed. The very low activity of **2** compared to **1** is most likely due to the lack of free coordination sites at Zr(IV) as discussed earlier in the text. Significantly, the ^{31}P NMR spectra of 2.0 mM **1** in the presence of 2.0 mM Gly-Ser or in the presence of 2.0 mM Gly-Gly at pD 5.4 recorded at the end of the hydrolytic reactions showed only one peak corresponding to **1**, indicating its stability in the presence of the dipeptides (Fig. S7†).

Rate constants for the hydrolysis of Gly-Ser and Gly-Gly by other metal-based catalysts are presented in Table S3.† A direct comparison is difficult due to the different reaction conditions (pH, temperature, and concentration) used in these studies. While the hydrolysis of Gly-Ser catalyzed by **1** was approximately two times faster than the one in the presence of the Zr(IV)–Wells–Dawson POM, hydrolysis of Gly-Gly was three times faster when the Zr(IV)–Wells–Dawson POM was used instead of **1**. The Zr(IV)/4,13-Diaza-18-crown-6 system displayed a higher reactivity both for Gly-Ser and Gly-Gly hydrolysis when compared to **1**. However, the use of **1** resulted in homogeneous reaction conditions, while the Zr(IV)/4,13-Diaza-18-crown-6 system led to heterogeneous reaction conditions due to the formation of Zr(IV) gels.

Binding modes of 1 with dipeptides

The binding mode of **1** was constructed on the basis of the shifts in ^{13}C NMR spectra of dipeptides upon addition of **1**. At pD 6.4 the highest shift was obtained for the amide carbonyl carbon in Gly-Ser dipeptide (Fig. 9 and Table 1), indicating that the interaction occurs at the amide oxygen. This interaction is important for the hydrolysis of peptide bonds since it increases the partial positive charge on the amide carbon and renders it more susceptible to nucleophilic attack by water.[38] It has been known that the amine group plays a key role in the coordination since the hydrolysis was not obtained for N-terminal blocked peptides.[12a,22,28a] As a consequence, a shift of

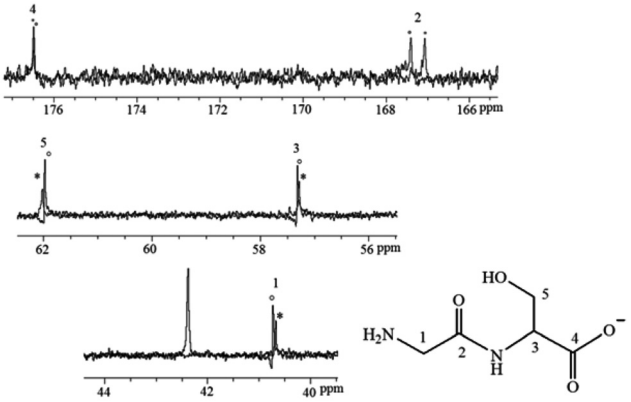

Fig. 9 ^{13}C NMR spectrum of Gly-Ser in the presence (*) and absence (°) of **1** at pD 6.4 (the signal at 42.4 ppm belongs to the CH_2 carbon of the ethyl group of the diethyl ammonium counter ions of **1**).

Table 1 ^{13}C NMR chemical shift values (ppm) of 20.0 mM Gly-Ser in the presence and in the absence of 5.0 mM **1**

	pD 6.4			pD 9.0		
^{13}C NMR	Gly-Ser	Gly-Ser + **1**	$\Delta\delta$ (ppm)	Gly-Ser	Gly-Ser + **1**	$\Delta\delta$ (ppm)
δ_1	40.72	40.64	0.08	42.06	40.95	1.11
δ_2	167.11	166.84	0.27	170.49	167.34	3.15
δ_3	57.31	57.29	0.02	57.14	57.33	0.19
δ_4	176.00	175.98	0.01	176.11	176.05	0.06
δ_5	61.97	61.98	0.01	62.06	62.05	0.01

0.08 ppm at the C-1 carbon was observed. Additionally, the ^{13}C NMR chemical shift of the C-terminal carboxylate group was unaffected by the addition of **1**, confirming the non-involvement of the carboxylate group in coordination. Such a coordination is stronger at pD 9.0 since large shifts of 3.15 and 1.11 ppm were observed at the amide carbonyl carbon and the Cα of Gly residue respectively (Table 1 and Fig. S8†). In addition, the C-3 atom experienced a shift of 0.19 ppm. This could be a result of binding to the amide nitrogen forming a hydrolytically inactive complex in which Zr(IV) binds to Gly-Ser *via* both amine and amide nitrogens. The tendency for forming such an inactive peptide complex in basic media was also observed for Cu^{2+} and Ni^{2+}.[39]

Similarly, the binding of Gly-Gly to **1** *via* the amine nitrogen and amide oxygen at pD 6.4 causes changes in ^{13}C NMR chemical shifts of 0.10 and 0.34 ppm at the C-1 and amide carbons respectively (Table S1 and Fig. S9†). Stronger interaction was also obtained at pD 9.0 and the formation of a hydrolytically inactive complex caused a shift of 0.19 ppm at the C-3 carbon (Fig. S10†). Thus hydrolytically active and inactive complexes of Gly-Ser and Gly-Gly to **1** were proposed in Scheme 2.

Effect of pD on the hydrolysis of Gly-Ser and Gly-Gly

The influence of pD on the rate constant for the reaction between 2.0 mM Gly-Ser and 2.0 mM **1** at 60 °C was further

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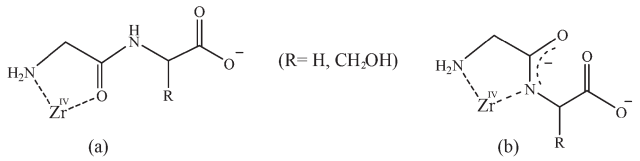

Scheme 2 Hydrolytically active (a) and inactive (b) complexes.

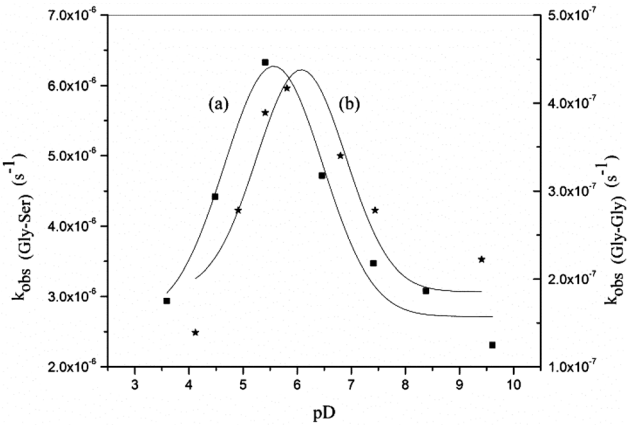

Fig. 10 pD dependence of the rate constant for the hydrolysis of 2.0 mM Gly-Ser (a) and 2.0 mM Gly-Gly (b) in the presence of 2.0 mM **1** at 60 °C.

investigated. Fig. 10a shows a bell-shape profile with a maximum at pD 5.5. The highest rate of the reaction at this pD value is related to the species distribution in aqueous solution. Only the active dimeric complex **1** containing coordination sites that can anchor Gly-Ser exists in aqueous solution under identical conditions (pD 5.4 and 60 °C). The rate constant decreases at higher pD values due to the formation of an inactive complex at high pD values, as discussed above. Furthermore, higher pH values also favour the conversion of **1** into **2**, which is proven to have a lower catalytic activity. In acidic media, although only the dimeric complex **1** is detectable, a decrease in reaction rate is observed. This is most likely due to the fact that coordination of Zr(IV) to the amine nitrogen is hindered due to protonation of this group in acidic solutions.

The hydrolysis of 2.0 mM Gly-Ser in the presence of 2.0 mM $ZrOCl_2$ salt at pD 5.4 or pD 8.4 and 60 °C was also investigated, and gel formation in both reaction mixtures was observed. Rate constants of 10.83×10^{-7} s^{-1} and 8.05×10^{-7} s^{-1} at pD 5.4 and pD 8.4, respectively, were determined, which are approximately 6 times slower compared to the hydrolysis in the presence of **1**.

The effect of pD on the observed rate constant of Gly-Gly shows a similar trend as obtained in Gly-Ser cleavage (Fig. 10b). The fasted hydrolytic rate occurs at pD 6.0 where complex **1** is dominant in solution. Similarly to Gly-Ser, the coordination of **1** to Gly-Gly was studied by ^{13}C NMR spectroscopy and the results (Table S1 and Fig. S9†) are analogous to those obtained for Gly-Ser. At pD 6.4, the coordination of Gly-Gly to **1** occurs *via* the amide carbonyl oxygen and amine nitrogen, while at higher pH values the formation of inactive complexes is also observed.

Effect of concentration of 1 on the hydrolysis of Gly-Gly and Gly-Ser

The influence of the POM concentration on the observed rate constant for the hydrolysis of 2.0 mM Gly-Gly by **1** at pD 5.4 and 60 °C was further studied (Fig. 11). This study reveals that 2.0 mM is the optimal concentration of **1** for the cleavage of the peptide bond in Gly-Gly. A 30% decrease of the rate constant was observed when a 6.0 mM solution of **1** was used. The conversion of **1** into the much less active **2** is considered to be the main reason leading to a decrease in reaction rate at high concentrations of **1**. Similar results were obtained for the hydrolysis of 2.0 mM Gly-Ser by **1** at pD 5.4 and 60 °C (Fig. S11†). Noticeably, **1** is not only able to promote Gly-Gly and Gly-Ser hydrolysis, but also capable of hydrolyzing an excess amount of the substrate. In both reactions, a 10-fold excess of dipeptides was completely hydrolyzed by 0.2 mM **1**, indicating that a catalytic turnover is possible.

Effect of experimental conditions and inhibitors on the hydrolysis of Gly-Gly and Gly-Ser

The effect of temperature on the hydrolysis of the amide bond in the two dipeptides at pD 5.4 was determined by measuring the observed reaction rate constant at different temperatures ranging from 37 °C to 70 °C. The experimental activation energy parameter (E_a) was obtained from the Arrhenius plot (Fig. S12a and S13a†). The activation values of these two reactions are similar and in good agreement with previously published results.[12a] For comparison, the activation energy (109.93 kJ mol^{-1}) of Gly-Ser in the absence of **1** at pD 5.4 was calculated (Fig. S14†). Apparently, the presence of the catalyst lowers the activation energy for the hydrolysis of Gly-Ser by 20 kJ mol^{-1}. Linear fitting of $\ln(k_{obs}/T)$ as a function of $1/T$ (Fig. S12b and S13b†) allows for the calculation of the enthalpy and the entropy of activation (Table 2). The Gibbs energies of activation ($\Delta G^{\#}$) for the hydrolysis of Gly-Gly and Gly-Ser at

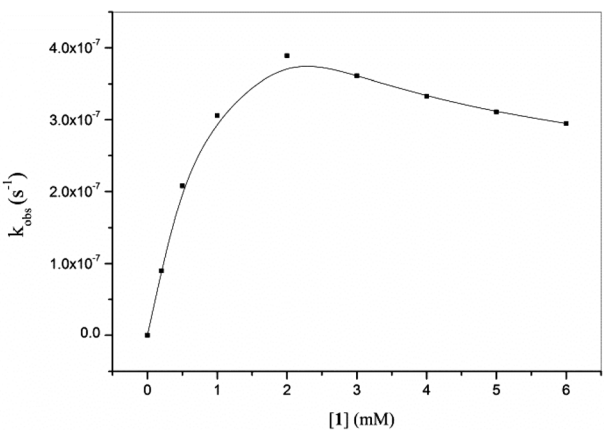

Fig. 11 Influence of the concentration of **1** on the observed rate constant for the hydrolysis of 2.0 mM Gly-Gly at pD 5.4 and 60 °C.

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Table 2 Experimental activation parameters of the hydrolysis of Gly-Gly and Gly-Ser by **1** at pD 5.4

Dipeptide	E_a (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ at 310 K (kJ mol^{-1})
Gly-Gly	90.03	87.33	−104.18	119.63
Gly-Ser	89.97	87.22	−83.71	113.17

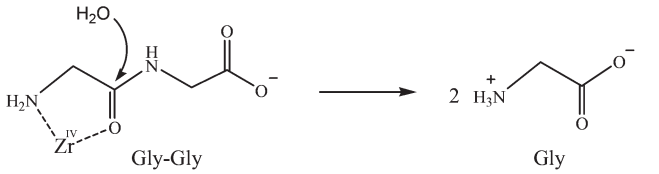
310 K are 119.63 and 113.17 kJ mol^{-1} respectively. The negative activation entropy obtained is most likely the result of the coordination of the dipeptides to the Zr(IV) center.

The influence of ionic strength on the rate constant of Gly-Gly and Gly-Ser hydrolysis was investigated by adding LiCl to a reaction mixture containing 2.0 mM Gly-Gly or 2.0 mM Gly-Ser and 2.0 mM **1** at pD 5.4 and 60 °C. LiCl has a negative effect on the hydrolysis of both dipeptides (Fig. S15†) as high LiCl concentrations cause a decrease in the reaction rate. The presence of 0.6 M LiCl causes a 3-fold decrease in the rate constant. Complex **1** is stable in the presence of the LiCl salt since the ^{31}P NMR spectroscopy of **1** in the presence of 1.0 M LiCl at pD 5.4 and 60 °C after 5 days does not show any traces of its conversion into **2** (Fig. S16†). Therefore the decrease in k_{obs} caused by the salt is most likely due to hindering of the electrostatic interaction between the dipeptide and **1**.

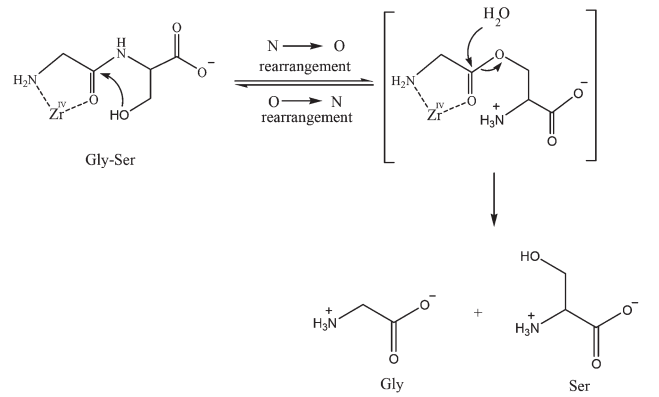
Several non-reactive substrate analogues (Fig. S17†) were chosen to study the inhibitive effect on the binding between dipeptides and **1**. Due to the oxophilicity of Zr(IV), dicarboxylic acids with multiple donor groups are expected to coordinate to the Zr(IV) center. As can be seen from Table 3, the presence of these dicarboxylic acids causes a decrease in the overall rate constant for the hydrolysis of the examined dipeptides. Particularly, the strongest inhibition effect was obtained in the presence of oxalic acid as well as the carboxylic acids containing an alcohol donor group adjacent to one of the carboxylate groups (malic and citric acid), whereas dicarboxylic acids with a longer aliphatic chain length caused less inhibition. The competitive inhibition effect of the former on the hydrolysis of Gly-Gly and Gly-Ser can be explained by the formation of their strong complexes with five- and six-membered chelate rings with Zr(IV). For dicarboxylic acids with a long aliphatic chain length, their bidentate mode of binding to Zr(IV) is less effective, causing a weaker inhibitor effect as observed in both hydrolytic reactions.

Table 3 The observed rate constants of the hydrolysis of 2.0 mM Gly-Gly or 2.0 mM Gly-Ser by 2.0 mM **1** in the presence of 5.0 mM inhibitor at pD 5.4 and 60 °C

	$10^7 \times k_{obs}$ (s^{-1})	
Inhibitor (5.0 mM)	2.0 mM Gly-Gly + 2.0 mM **1**	2.0 mM Gly-Ser + 2.0 mM **1**
No inhibitor	4.44	63.33
Oxalic acid	0.28	3.33
Citric acid	0.56	6.39
Malic acid	0.83	4.17
Malonic acid	1.94	23.89
Succinic acid	2.78	42.50
Adipic acid	3.61	55.28



Scheme 3 Proposed mechanism for Gly-Gly hydrolysis in the presence of **1**.



Scheme 4 Proposed mechanism for Gly-Ser hydrolysis in the presence of **1**.

Hydrolysis mechanism

The binding studies of **1** to the dipeptides and inhibition and kinetic experiments showed the important role of the amide carbonyl oxygen in metal coordination. This coordination polarizes the amide carbon and makes it more susceptible to nucleophilic attack by water, resulting in the hydrolysis of Gly-Ser and Gly-Gly. In addition, the coordination to the N-terminus is indispensable for effective hydrolysis.[12a,28a] The mechanism for the hydrolysis of Gly-Gly by **1** is proposed in Scheme 3, assuming that the peptide coordinates to one Zr(IV). It is, however, also plausible that the peptide bridges two Zr(IV) ions in **1**, but the available experimental evidence is not sufficient to distinguish between these two possibilities. The more effective hydrolysis of Gly-Ser can be a result of the polarized amide carbon due to the binding to the Zr(IV) center, followed by the intramolecular attack of the Ser hydroxyl group (Scheme 4) on the amide carbonyl carbon producing a five-membered cyclic transition state which is rearranged to an ester intermediate.

Conclusion

In conclusion, we report on the detailed mechanism of amide bond hydrolysis catalyzed by the dimeric Zr(IV)-substituted Keggin type polyoxometalate. Homogeneous hydrolysis of the peptide bond in Gly-Ser, a hydroxyl-containing side chain dipeptide, and Gly-Gly with a non-coordinating side chain was

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achieved in the presence of the POM. The highest catalytic activity of this POM towards peptide bond hydrolysis was obtained at pD 5.5–6.0, at which no traces of dissociation of complex **1** were observed resulting in a more controllable species distribution and thereby allowing a more profound mechanistic study when compared to the Zr(IV)-substituted Wells–Dawson type polyoxometalate typically characterized by multiple equilibria at pD 5.5–6.0. Coordination of **1** to dipeptides *via* the amine nitrogen and amide carbonyl oxygen is proposed, resulting in polarization of the peptide bond and making it more susceptible to hydrolysis. This and our previous findings reporting on the peptidase activity of POMs provide new and growing opportunities for metal-substituted POM as a novel class of catalyst for peptide bond hydrolysis. The hydrolysis of different proteins by **1** is currently being investigated.

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