Inorganic Chemistry

Article

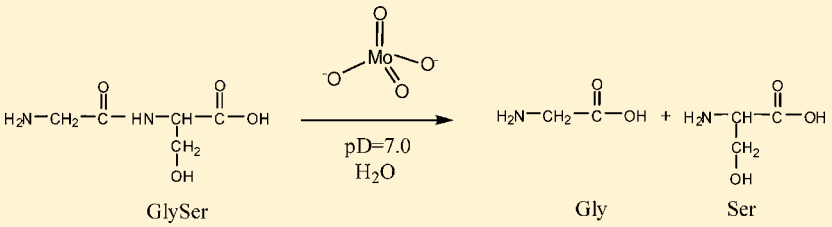
pubs.acs.org/IC

Hydrolysis of Serine-Containing Peptides at Neutral pH Promoted by $[MoO_4]^{2-}$ Oxyanion

Phuong Hien Ho, Karen Stroobants, and Tatjana N. Parac-Vogt*

Katholieke Universiteit Leuven, Department of Chemistry, Celestijnenlaan 200F, B-3001, Leuven, Belgium

Supporting Information



ABSTRACT: Hydrolysis of the dipeptides glycylserine (GlySer), leucylserine (LeuSer), histidylserine (HisSer), glycylalanine (GlyAla), and serylglycine (SerGly) was examined in oxomolybdate solutions by means of ^{1}H, ^{13}C, and ^{95}Mo NMR spectroscopy. In the presence of a mixture of oxomolybdates, the hydrolysis of the peptide bond in GlySer proceeded under neutral pD conditions (pD = 7.0, 60 °C) with a rate constant of $k_{obs} = 5.9 \times 10^{-6}$ s^{-1}. NMR spectra did not show evidence of the formation of paramagnetic species, excluding the possibility of Mo(VI) reduction to Mo(V), indicating that the cleavage of the peptide bond is purely hydrolytic. The pD dependence of k_{obs} exhibits a bell-shaped profile, with the fastest cleavage observed at pD 7.0. Comparison of the rate profile with the concentration profile of oxomolybdate species implicated monomolybdate MoO_4^{2-} as the kinetically active complex. Kinetics experiments at pD 7.0 using a fixed amount of GlySer and increasing amounts of MoO_4^{2-} allowed for calculation of the catalytic rate constant ($k_2 = 9.25 \times 10^{-6}$ s^{-1}) and the formation constant for the GlySer−MoO_4^{2-} complex ($K_f = 15.25$ M^{-1}). The origin of the hydrolytic activity of molybdate is most likely a combination of the polarization of amide oxygen in GlySer due to the binding to molybdate, followed by the intramolecular attack of the Ser hydroxyl group.

INTRODUCTION

Reagents that promote selective cleavage of peptides and proteins are needed for a number of applications, such as the study of protein function and solution structure,[1−6] mapping of enzyme active sites,[7−15] ligand-induced conformational changes,[16−18] and elucidation of metal binding sites.[8,9,11,13,14] Recently, the use of peptide-cleaving agents as catalytic drugs has been proposed.[19−22] However, the remarkable inertness of the peptide bond, with an estimated half-life of up to 600 years under physiological conditions, makes its hydrolysis a challenging task.[23] Cyanogen bromide, the most common chemical reagent for fragmentation of proteins, has several shortcomings: it is volatile and toxic, is applied in a 100-fold excess over methionine residues, requires 70% formic acid as a solvent, and gives several side reactions.[24]

The potential of transition metal complexes to promote peptide bond hydrolysis was recognized long ago. A large number of studies on the metal-promoted hydrolysis of amides have been performed with activated amides or amino acid esters as peptide models.[25] In recent years, a number of metal complexes including zinc(II),[26] copper(II),[27] nickel(II),[28] palladium(II),[29] cerium(IV),[30] and zirconium(IV)[31] have been found to be effective at promoting the hydrolysis of unactivated amide bonds in peptides and proteins.

Besides the basic requirement that the complex should be able to promote amide bond cleavage, the selectivity of the cleavage remains a big challenge. Currently, there is great interest in the development of reagents that target diverse amino acids and promote cleavage under mild conditions.[32] Selective hydrolysis at cysteine, methionine, and histidine residues has been achieved with Pt and Pd complexes.[33] Molybdocene dichloride has been shown to selectively hydrolyze cysteine-containing peptides.[34] Selective hydrolysis of serine-containing peptides by zinc salts has been reported by the group of Komiyama,[26] and more recently Ni(II) ions have also been used to selectively hydrolyze the X−Ser sequence in a series of oligopeptides.[28]

The chemistry of molybdenum(VI) oxo-complexes has been intensively investigated because of their importance in biochemical and chemical applications. In neutral and alkaline solutions, molybdate is present as the monomeric anion MoO_4^{2-}. However, acidification results in condensation and the formation of larger polyoxomolybdate species. Depending on the pH, concentration, and temperature, a range of different complexes, among which $[Mo_7O_{24}]^{6-}$ and $[Mo_8O_{26}]^{6-}$ have been fully structurally characterized, are formed.[35] The

Received: July 14, 2011
Published: October 31, 2011

   dx.doi.org/10.1021/ic2015034 | Inorg. Chem. 2011, 50, 12025−12033

biological chemistry of oxomolybdates has been marked with reports which revealed that their *in vivo* antitumoral activity on several types of tumor cells is comparable to that of some commercial drugs.[36] More recent studies have shown that $[Mo_7O_{24}]^{6-}$ exhibits potent antitumor activity against pancreatic cancer cells, which are extremely resistant toward most of the known therapeutic agents.[37] Several studies have revealed that molybdate binds to nucleotides, resulting in slight hydrolysis at high temperatures.[38] Oxomolybdates have also been shown to catalyze the hydrolysis of the labile "high energy" phosphoanhydride bonds in ATP.[39,40]

In our recent studies, we examined the reactivity of oxomolybdates toward 4-nitrophenyl phosphate (NPP), bis-(*p*-nitrophenyl)phosphate (BNPP), and 2-hydroxypropyl-4-nitrophenyl phosphate (HPNP), commonly used DNA and RNA model phosphodiesters.[41−44] The interaction between these model systems and oxomolybdate complexes led to purely hydrolytic cleavage of a phosphodiester bond, demonstrating the first example of a phosphodiester bond cleavage promoted by a negatively charged oxyanion. The efficient hydrolysis of a range of carboxyesters in which monomeric molybdate was identified as the effective nucleophilic catalyst has been also reported.[45−47]

In this study, we explore the reactivity of oxomolybdates toward a range of peptides and discover the efficient hydrolysis under neutral pH of the peptides containing the X−Ser sequence. By combining kinetic experiments and NMR measurements, we give a full account on the mechanism of this novel reaction.

RESULTS AND DISCUSSION

The reactivity of molybdate (VI) oxyanions toward a range of peptides was initially examined in aqueous solutions at pD 7.0 and 60 °C. The extent of hydrolysis after 60 h was determined from the ^{1}H NMR spectra by integration of the NMR resonances of the peptide and free amino acid products at different time increments. As the results in Table 1 show, the conversion was mainly dependent on the sequence of the peptide. The conversion ranged from 2% for Gly−Ala and Gly−Tyr to 91% for His−Ser. Among all of the peptides examined, peptides with the sequence X−Ser and X−Thr were most readily hydrolyzed. These dipeptides have a serine or threonine residue at their C terminus, indicating the importance of the hydroxyl group in the side chain for efficient hydrolysis.

Table 1. The Hydrolysis of a Series of Dipeptides (2 mM) Measured in the Absence and in the Presence of 120 mM Na_2MoO_4 (pD = 7.0, 60°C) after 60 h

entry	dipeptide	conversion (%) with Na_2MoO_4	conversion (%) without Na_2MoO_4
1	GlySer	68	7
2	HisSer	91	10
3	LeuSer	43	6
4	GlyThr	60	6
5	SerGly	3	0
6	CysGly	17	0
7	GlySerPhe	25	0
8	AspAla	3	0
9	GlyAla	2	0
10	GluCysGly	4	0
11	GlyTyr	2	0
12	GlyGly	4	0
13	AlaHis	5	0
14	GlyAsn	3	0
15	GlyMet	2	0
16	LysGly	2	0

On the basis of the results given in Table 1, we focused our attention on the hydrolysis of a series of dipeptides (Scheme 1) containing a serine residue, such as glycylserine (GlySer), leucylserine (LeuSer), histidylserine (HisSer), and serylglycine (SerGly). For a comparison, glycylalanine (GlyAla) was studied as well.

The Hydrolysis of Dipeptide GlySer by Molybdate(VI) Oxyanions. The model reaction between GlySer and molybdate(VI) oxyanions has been performed in solutions containing 2 mM GlySer and 120 mM Na_2MoO_4. During the course of reaction, ^{1}H NMR spectroscopy showed a gradual intensity decrease of the GlySer resonances at 3.95 ppm (CH_2 of glycine residue), 3.82−3.98 ppm (CH_2 of serine residue), and 4.35 ppm (CH of serine residue) and the appearance of free glycine resonance at 3.58 ppm and free serine resonances at 3.83−3.90 and 3.92−4.06 ppm, indicating that the cleavage of the peptide bond in GlySer occurred (Figure 1).

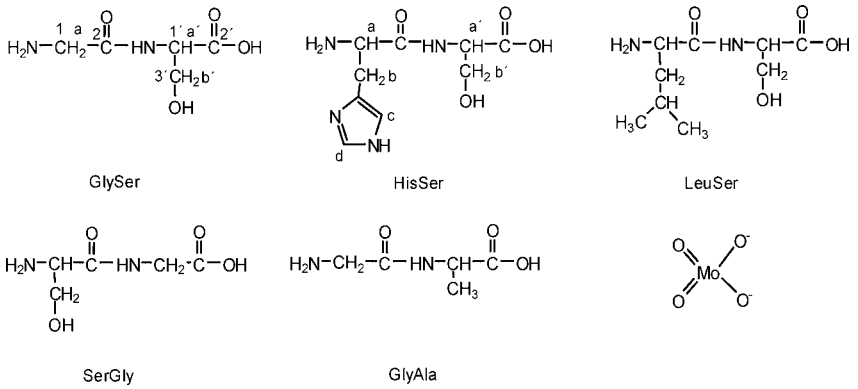
Integration of the proton NMR resonances at different time increments allowed for the calculation of the hydrolysis rate constant for GlySer (k_{obs} = 5.9 × 10^{-6} s^{-1} at 60 °C, pD = 7.0; Figure 2). This represents an acceleration of nearly 20 times compared to the hydrolysis measured under the same conditions but in the absence of molybdate. It is worth mentioning that the reaction also occurred under physiological pH and temperature (37 °C, pD = 7.0) with a rate constant of 2.5 × 10^{-7} s^{-1}.

It is important to note that the NMR spectra did not show evidence of any paramagnetic species, which excludes the possibility of oxidative cleavage and the reduction of Mo(VI) to Mo(V). The appearance of "blue species" was not observed during any point of the reaction, indicating that the hydroxyl group in the Ser side-chain did not cause oxidation, as has been previously observed in reactions of oxomolybdates with some alcohols.[48] The "molybdenum blue" species are obtained by the reduction of Mo(VI) in the presence of reducing agents, including organic acids and sugars, and are characterized by a typical electronic absorption spectrum with λ_{max} at 748 nm.[49,50] Although the general term "molybdenum blue" is often used, not always completely identical solutions are formed; however, the common feature for all of the "molybdenum blues" is their characteristic blue color that originates from the electronic transition of the intervalence ($Mo^V \rightarrow Mo^{IV}$) charge transfer type.[51] The absence of such blue species suggests that the reaction is purely hydrolytic in nature and can be presented as shown in Scheme S1 (Supporting Information).

Effect of pH on the Hydrolysis of GlySer. The pH plays an important role in the speciation of oxomolybdate anions, and molybdate solutions often result in a complex mixture of different species. Acidification of $[MoO_4]^{2-}$ to a pH below 7.0 results in the formation of the heptamolybdate $[Mo_7O_{24}]^{6-}$ cluster and its protonated forms. ^{95}Mo NMR and Raman spectroscopy studies have shown that $[MoO_4]^{2-}$ and $[Mo_7O_{24}]^{6-}$ anions coexist in the pH range 4.5−6.5. Further acidification produces $[Mo_8O_{26}]^{4-}$ and different protonated forms of this polyoxoanion. The effect of pD on the cleavage reaction was examined in order to correlate the rate-pH profile with the species distribution diagram. The fact that the

dx.doi.org/10.1021/ic2015034 | *Inorg. Chem.* 2011, 50, 12025−12033

Scheme 1. Structures of the Dipeptides and MoO_4^{2-} Used in the Study



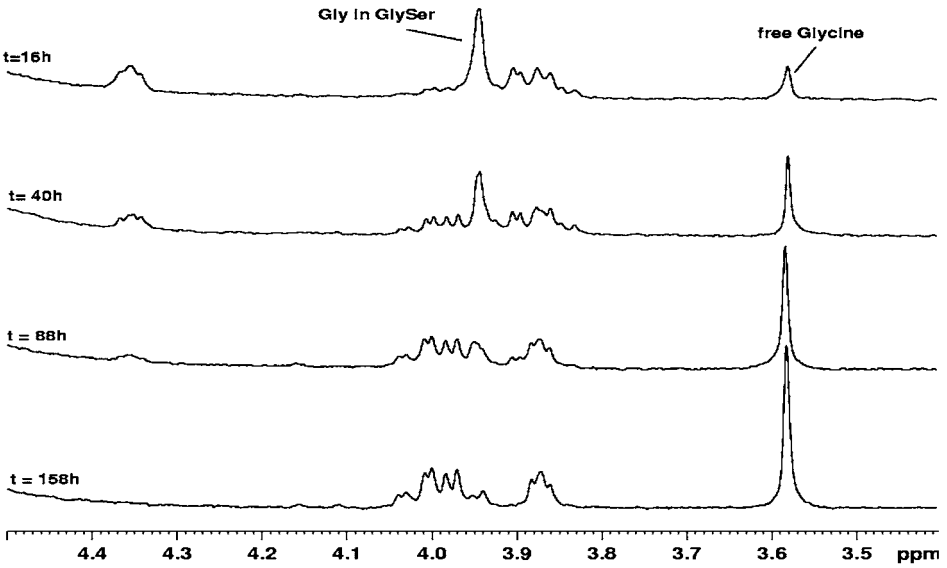


Figure 1. ^{1}H NMR spectra of the reaction between 2 mM GlySer and 120 mM Na_2MoO_4 at pD 7.0 and 60 °C measured at different time increments.

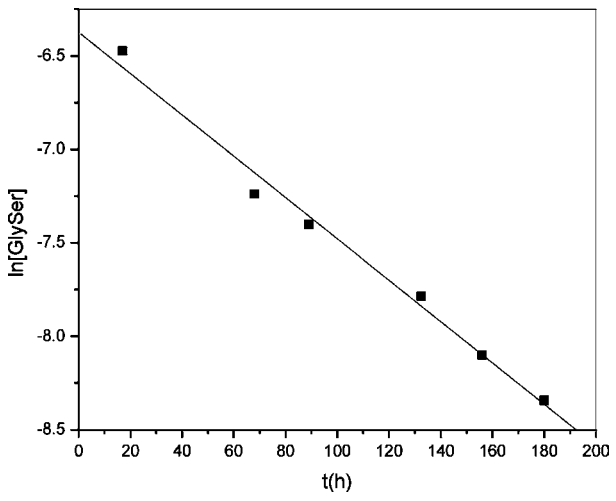


Figure 2. Kinetic profile for the hydrolysis of 2 mM GlySer in the presence of 120 mM Na_2MoO_4 in a D_2O solution at pD 7.0 and 60 °C.

reactivity follows the pD profile of a certain species suggests that k_{obs} is proportional to the concentration of that species. Since the kinetic measurements were performed in D_2O solutions, the rates were plotted as a function of pD, which was obtained by adding 0.41 pH units to the recorded pH value.[52] The hydrolysis of GlySer was measured at 60 °C in the pD range 4.0–8.8, both in the presence and in the absence of molybdate. The data (Table S1, Supporting Information) clearly indicated that the acceleration of the hydrolysis was due to the presence of oxomolybdate and not due to the acid or base catalysis. This is illustrated in Figure 3, where the values of k_{obs} and the fraction of polyoxometalate species are plotted as a function of pD. The concentration of molybdate under a given pD, ionic strength, and temperature was calculated by using a thermodynamic model previously described by our group.[43] The distribution of Mo species calculated by this model agrees very well with the distribution that was determined by several experimental methods.

As can be seen in Figure 3, the pD dependence of k_{obs} exhibits a bell-shaped profile, with the fastest cleavage observed at pD 7.0. The data points were fitted to the Michaelis function (eq 1) describing a bell-shaped dependence of the rate constant on pH.[53]

$$k_{obs} = k/(1 + h/K_1 + K_2/h) \quad (1)$$

In eq 1, k_{obs} is the measured kinetic constant; $h = 10^{-pH}$; and k, K_1, and K_2 are parameters to be estimated. Comparison of the

dx.doi.org/10.1021/ic2015034 | Inorg. Chem. 2011, 50, 12025–12033

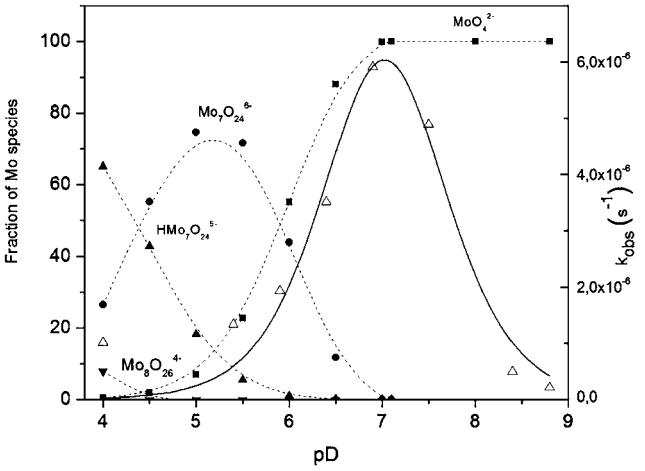


Figure 3. The pD dependence of k_{obs} (solid line) for the cleavage of GlySer (2 mM) in the presence of Na_2MoO_4 (120 mM) at 60 °C. The fractions of various oxo-molybdenum species as a function of pD have been added for comparison (dashed lines).

rate constant profile with the concentration profile of oxomolybdate species shows that, up to pD 7.0, the best overlap of the k_{obs} profile is observed with the concentration of MoO_4^{2-}. Although in the acidic pD range, other molybdenum(VI) species such as $[Mo_7O_{24}]^{6-}$ and $[Mo_8O_{26}]^{4-}$ are present in solution; they seem to be catalytically much less active. Slow hydrolysis at lower pD values, where $[Mo_7O_{24}]^{6-}$ and $[Mo_8O_{26}]^{4-}$ predominate, implies low catalytic activity of these species. The hypothesis that monomeric MoO_4^{2-} is the hydrolytically active species was further strengthened by the fact that full hydrolysis of GlySer was observed at pD 7.5 (k_{obs} = 4.9 × 10^{-6} s^{-1}, 60 °C) where MoO_4^{2-} was the only molybdenum(VI) species present in solution.

Binding of GlySer to MoO_4^{2-}. The rate increase of GlySer hydrolysis in the presence of MoO_4^{2-} suggests that interaction between molybdate and the peptide must take place in solution. The binding constant between molybdate and GlySer was determined by kinetic experiments using a fixed amount of GlySer and increasing amounts of molybdate at pD 7.0 and 60 °C. Assuming that the monomeric molybdate is the hydrolytically active species, the binding constant between GlySer and molybdate could be determined by fitting the data shown in Figure 4 to a general catalytic scheme presented in eq 1:

$$\text{GlySer} + [MoO_4^{2-}] \rightleftharpoons [\text{GlySer}\cdots MoO_4^{2}] \rightarrow \text{Gly} + \text{Ser} \qquad (1a)$$

The values for the catalytic (k_2 = 9.3 × 10^{-6} s^{-1}) and formation constants for the $[\text{GlySer}/MoO_4^{2-}]$ complex (K_f = k_{-1}/k_1 = 15.25 M^{-1}) were obtained by a computer generated least-squares fit of k_{obs} to eq 2.

$$k_{obs} = \frac{k_2[MoO_4^{2-}]_0}{\frac{k_{-1}}{k_1} + [MoO_4^{2-}]_0} \qquad (2)$$

Interestingly, the value of K_f is similar to the formation constant previously determined for the binding between GlySer and mononuclear complexes formed from a *cis,cis*-1,3,5-triaminocyclohexane ligand L, $[CuL]^{2+}$, which has a binding constant of 15.97 (M^{-1}).[27a]

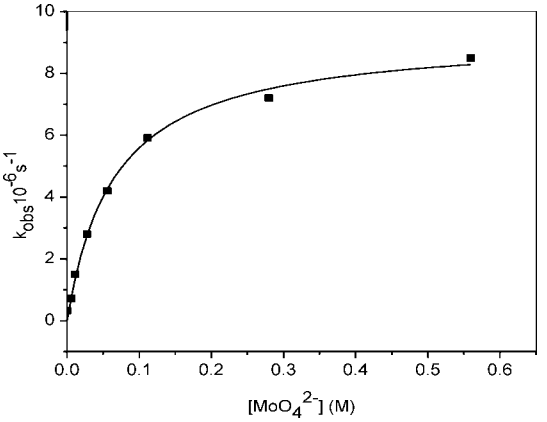


Figure 4. Influence of Na_2MoO_4 concentration on k_{obs} for the cleavage of 2 mM GlySer at T = 60 °C and pD 7.0.

The influence of the ionic strength on the rate constant was studied by following the hydrolytic reaction in the presence of different amounts of $NaClO_4$ salt. An increase in the $NaClO_4$ concentration resulted in the increase of the GlySer hydrolysis rate constant, resulting in a positive salt effect. Nonlinearity of the plot in which $\log(k/k_o)$ was plotted as a function of the ionic strength implied that the salt concentration not only influenced the hydrolysis rate but that $NaClO_4$ may also have exhibited a secondary effect on the composition and stability of species in solution (Figure S1, Supporting Information).

The effect of temperature on the hydrolytic reaction was determined by measuring the temperature dependence of k_{obs}. As expected, at higher temperatures, increased rates have been observed. Activation Gibbs function ($\Delta G^{\ddagger}$ = 114.20 kJ mol^{-1} at 37 °C), enthalpy ($\Delta H^{\ddagger}$ = 90.6 kJ mol^{-1}), and entropy ($\Delta S^{\ddagger}$ = −76.34 J mol^{-1} K^{-1}) were obtained from the Arrhenius and Eyring plots (Figure S2, Supporting Information). The negative value of the activation entropy is in agreement with the formation of the complex $[\text{GlySer}/MoO_4^{2-}]$. However, the activation parameters need to be interpreted with caution, as they are usually derived from the composite rate constants that also include contribution from the binding of the substrate to the catalyst.

Coordination of GlySer to Molybdate. The binding between GlySer and molybdate was further examined by ^{95}Mo, ^{13}C, and ^{1}H NMR spectroscopy. The addition of GlySer to MoO_4^{2-} solution did not cause detectable changes in the ^{95}Mo NMR spectrum, which is probably due to very low NMR sensitivity of this nucleus. However, the ^{1}H spectrum was more informative, as a number of ^{1}H NMR GlySer resonances were shifted upon mixing with MoO_4^{2-} (Figure S3, Supporting Information). The binding was also examined by ^{13}C NMR at pD 7.0 and pD 8.4, and the results (Table S2, Supporting Information) indicated that, at pD 7.0, the largest shifts were observed for the CH_2 (C1) and carbonyl C═O group (C2) in the glycyl residue and the carboxylate COO^- group (C2′) in the serine residue, while the CH group (C′1) of the serine residue was not much affected by addition of MoO_4^{2-} (see Scheme 1 for atom designation). The larger shifts of carbon C1, C2, and C2′ resonances in ^{13}C NMR spectra suggest that MoO_4^{2-} binds to GlySer via the amino nitrogen, the amide oxygen, and the carboxylate oxygen. The NMR chemical shifts observed at pD 8.4 also implicate coordination of the amino nitrogen and the carboxylate oxygen atoms; however, significant shifting of the α-carbon of Ser residue suggests

dx.doi.org/10.1021/ic2015034 | *Inorg. Chem.* 2011, 50, 12025–12033

that at higher pD values not the amide oxygen but the amide nitrogen coordinates to MoO_4^{2-}. Furthermore, while at pD 7.0 a weak interaction between the C3′ hydroxyl group of serine and MoO_4^{2-} could be plausible; at pD 8.4, this interaction is very unlikely. While the carbon atoms adjacent to the coordinating groups experience shifts from 0.2 to 0.75 ppm, the shift of the C3 carbon adjacent to the hydroxyl group is only 0.05 ppm.

The proposed coordination environment of molybdenum(VI) is analogous to that previously reported for molybdate complexes with malate and citrate ligands in which a MoO_3 unit is bound to three heteroatoms originating from the ligand.[54] While this requires cleavage of one bond between molybdenum(VI) and oxygen in MoO_4^{2-} and the increase of the coordination number of molybdenum(VI) from four to six, this is not unusual, as previous studies between vanadate, VO_4^{3-}, and dipeptides have shown that coordination of peptides also leads to the cleavage of a V−O bond and expansion of the coordination number of vanadium(V) from four to six.[55]

Since molybdate is known to interact with amino acids and organic acids,[35] the interaction of molybdate with glycine and serine, which are the products of GlySer hydrolysis, was also examined. ^{13}C NMR spectra of glycine and serine recorded in the absence and presence of molybdate were considerably different, indicating that interaction took place in solution (Table S3, Supporting Information). The binding of glycine and serine to MoO_4^{2-} most likely occurs via the amine nitrogen and carboxylate oxygen, which was confirmed by large chemical shifts (0.45 and 1.25 ppm) of the carbonyl carbon atoms of glycine and serine, respectively. Although present in solution as products of amide bond cleavage, glycine and serine do not seem to significantly inhibit the GlySer hydrolysis. The addition of a 25 M excess of Gly or Ser to the reaction mixture had no significant effect on the hydrolysis rate, most likely due to the preferential binding of molybdate to the GlySer.

Effect of Inhibitors on the Hydrolysis Rate Constant of GlySer. The inhibitory effect of several nonreactive substrate analogues was examined. In the screening experiments, the rate constant for the hydrolysis of GlySer (2 mM) by MoO_4^{2-} (60 mM) was determined in the presence of an excess of several organic acids that are expected to bind to molybdate (Table 2). According to the results in Table 2, among all of the examined substrates only malic and citric acid reduced the effectiveness of MoO_4^{2-} toward the hydrolysis of GlySer. However, the binding of malic acid to MoO_4^{2-} appears to be rather weak since a 25 molar excess of the inhibitor resulted only in a 26% decrease of the rate constant. Due to the presence of an additional carboxylic group, the binding of citric acid to MoO_4^{2-} is stronger compared to malic acid, and consequently, its presence resulted in a 52% decrease of the reaction rate constant. The inhibitory effect of aliphatic dicarboxylic acids was rather small. Their bidentate mode of binding to molybdate is less effective compared to the tridentate binding of GlySer that occurs via amino nitrogen, the amide oxygen and the carboxylate oxygen.

Table 2. Rate Constants of GlySer (2 mM) Peptide Bond Hydrolysis Measured in the Presence of Na_2MoO_4 (120 mM) and Different Inhibitors (50 mM) at pD = 7.0 and 60 °C

entry	inhibitor	formula	k_{obs} (10^{-6} s^{-1})
1	no inhibitor		3.86
2	alanine	H_2NCH_2COOH	3.64
3	malonic acid	$HOOCCH_2COOH$	3.72
4	succinic acid	$HOOC(CH_2)_2COOH$	3.87
5	glutaric acid	$HOOC(CH_2)_3COOH$	3.80
6	adipic acid	$HOOC(CH_2)_4COOH$	3.84
7	malic acid	$HOOCCH_2CH(OH)COOH$	2.85
8	citric acid	$HOOCCH_2C(COOH)OHCH_2COOH$	1.67

Hydrolysis of Ac−GlySer and GlySer−OEt. The amino group in Ac−GlySer is acetylated and mimics an internal amide bond. Interestingly, the hydrolysis of Ac−GlySer was about 20 times slower (k_{obs} = 1.5 × 10^{-7} s^{-1}, pD = 7.0, 60 °C) than the hydrolysis of GlySer measured under the same conditions. This implies that the presence of a free amino group is essential for efficient hydrolysis. According to ^{13}C and ^1H NMR data, the amino group in GlySer coordinates to MoO_4^{2-}, resulting in effective tridentate coordination for peptide hydrolysis to occur. Due to the much higher pK_a value of the proton on the amide nitrogen, the coordination of this nitrogen atom to MoO_4^{2-} is unlikely to occur at pD = 7.0. Further indication that the attachment of the amino group to MoO_4^{2-} is essential for effective hydrolysis comes from the pD profile of the hydrolysis rate constant of GlySer. The decrease of the hydrolysis rate under acidic conditions (Figure 3) may also be explained by the protonation of the amino group, which hinders coordination to MoO_4^{2-}.

In the case of GlySer−OEt, in which the carboxylic group is converted into an ester, ^1H NMR spectra showed the presence of ethanol resonances and indicated that full hydrolysis of the ester group occurred within 24 h. The hydrolysis of the peptide bond occurred only after GlySer−OEt was converted into GlySer. These results suggest that MoO_4^{2-} also effectively promotes the hydrolysis of esters, which may not be surprising considering the much higher kinetic lability of the ester bond compared to the peptide bond.

Hydrolysis of HisSer. Among all examined peptides, HisSer was the most readily hydrolyzed (Table 1). At pD 7.0 and 60 °C, the rate constant for hydrolysis was 1.1 × 10^{-5} s^{-1}, meaning that complete hydrolysis was achieved in less than four days. Similarly to GlySer hydrolysis, the pD dependence of k_{obs} exhibits a bell-shaped profile, with the fastest cleavage observed at pD 7.0 (Figure 5).

The ^1H NMR spectrum of HisSer in the presence of MoO_4^{2-} indicated that the H_6 proton in the imidazole ring experienced the largest shift (Table S4, Supporting Information), suggesting that HisSer coordinates to Mo(VI) via its imidazole nitrogen. ^{13}C NMR measurements (Table S5, Supporting Information) confirmed that besides the imidazole nitrogen atom, the amine nitrogen, amide oxygen, and carboxylate oxygen atoms are also involved in the binding to molybdate (Scheme 2). However, at pD values below 5.3, the coordination of the imidazole nitrogen becomes unfavorable, and the coordination mode becomes the same as observed for GlySer. The slow hydrolysis of HisSer at low pD values may be the combination of this change in coordination and the decrease of MoO_4^{2-} concentration in the acidic media.

The Mechanism of X−Ser Peptide Hydrolysis. From the screening experiments of a range of different dipeptides, it was evident that the presence of the hydroxide group in the side chain of X−Ser-type dipeptides was essential for efficient

dx.doi.org/10.1021/ic2015034 | Inorg. Chem. 2011, 50, 12025−12033

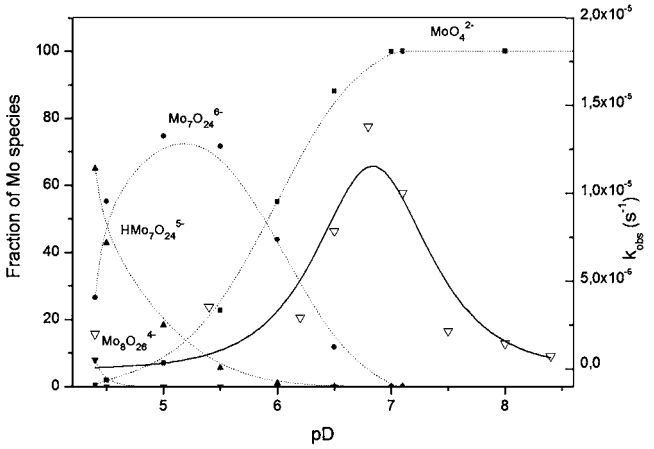
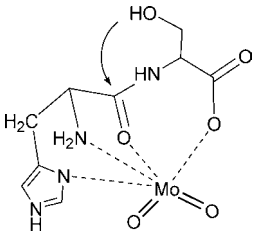


Figure 5. The pD dependence of k_{obs} (solid line) for the cleavage of HisSer (2 mM) in the presence of Na_2MoO_4 (120 mM) at 60 °C. Fractions of oxo-molybdenum species as a function of pD have been added for comparison (dashed lines).

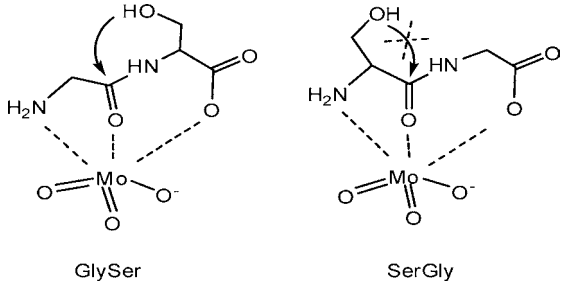
Scheme 2. Possible Mechanism of HisSer Hydrolysis Promoted by MoO_4^{2-}



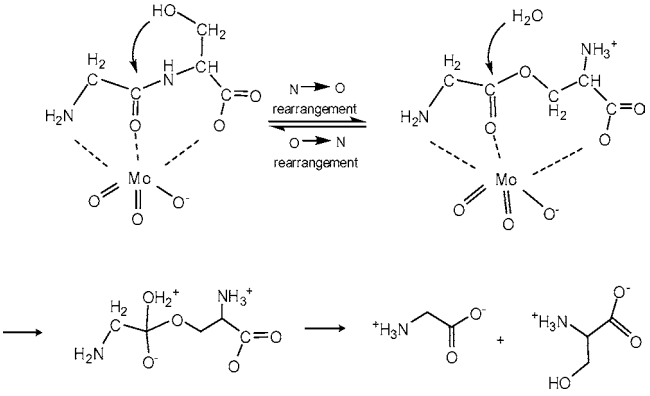
hydrolysis. For example, in comparison to GlySer, the hydrolysis of GlyAla, which has a similar bulkiness of the side chain but lacks a hydroxyl group, was approximately two orders of magnitude slower. As shown in Table 1, all dipeptides that were readily hydrolyzed have a serine residue at the C-terminus, suggesting the important role of the hydroxyl group in the Ser side chain as an intramolecular nucelophile. The hypothesis that the hydrolysis of X−Ser dipeptides is due to a N→O acyl rearrangement is strongly supported by the fact that the hydrolysis of GlySer was about 2 orders of magnitude faster than the hydrolysis of SerGly. While the intramolecular attack of the Ser hydroxyl group on the amide carbonyl carbon is possible in GlySer, it is impossible in SerGly. In the case of SerGly, the intramolecular attack of the Ser hydroxyl group results in a five-membered ring transition state, while in the case of SerGly the intramolecular attack should form an unfavorable four-membered ring transition state (Scheme 3).[26b]

The acceleration of GlySer hydrolysis in the presence of MoO_4^{2-} suggested that the oxyanion, in addition to the hydroxyl group within the peptide, plays an important role in the hydrolysis of the dipeptide. The role of MoO_4^{2-} is most likely to polarize the carbonyl group by coordinating to it and to facilitate the attack of nucleophile. The coordination of molybdate to the NH_2 group seems to play an important role since the rate constant for the cleavage of GlySer is much faster than that of Ac−GlySer. This coordination seems to assist the effective interaction of the carbonyl group with MoO_4^{2-}. All of the data above allow one to postulate a possible mechanism for the hydrolysis of GlySer, as shown in Scheme 4. Slow hydrolysis of LysGly, which is positively charged at pD = 7.0, suggests that a favorable electrostatic interaction between the

Scheme 3. Intramolecular Attack of Ser Hydroxyl Group on Amide Carbonyl Carbon in GlySer and SerGly



Scheme 4. Possible Mechanism of GlySer Hydrolysis Promoted by MoO_4^{2}



peptide and MoO_4^{2-} is not sufficient to effect the hydrolysis; this further supports the hypothesis that the intramolecular attack of the Ser hydroxyl group is essential for the cleavage of the amide bond.

Slower hydrolysis of GlySerPhe compared to GlySer is most likely due to the less effective coordination of this tripeptide to molybdate. While the carboxylate group in GlySer is available for coordination, resulting in the formation of a chelating complex shown in Scheme 3, this carboxylate group is transformed into an amide group in the tripeptide, which is less effective as a ligand for molybdate at neutral pH. However, it is also possible that the presence of the bulky phenyl group in the side chain of the GlySerPhe tripeptide sterically impedes the intramolecular attack of the Ser hydroxyl group on the amide carbonyl carbon.

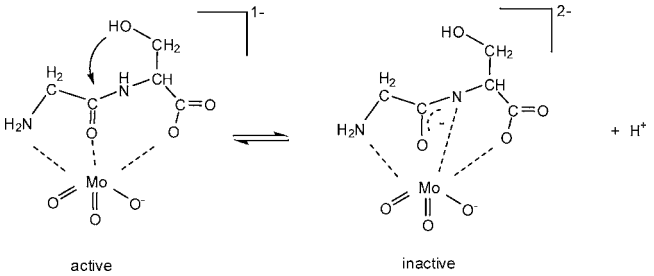
In general, the best metal ions for the hydrolysis of the amide bond are strong Lewis acids characterized by high positive charge and small size.[31,32] A similar mechanism to that shown in Scheme 4 has been previously proposed for Zn(II)-assisted hydrolysis of GlySer.[26b] While oxomolybdate and Zn(II) have rather distinct coordination chemistry, they both have the tendency to coordinate to the carbonyl group and cause its polarization. Oxomolybdate has been shown to hydrolyze carbon and phosphorus esters,[45−47] and it was suggested that the origin of this hydrolytic activity lies in the ability of Mo(VI) in oxomolybdate to serve as an electrophilic catalyst that interacts with the oxygen atom of the carbonyl and the phosphoester group. It is therefore plausible that a similar interaction can occur with the carbonyl group of the amide bond. The rate of peptide hydrolysis in serine-containing peptides promoted by oxomolybdate compares roughly to those previously reported for Zn(II) and Ni(II) salts. While the exact comparison is difficult to make due to the different

dx.doi.org/10.1021/ic2015034 | Inorg. Chem. 2011, 50, 12025−12033

conditions (pH, temperature, concentration, type of serine peptides) used in our study and the previous study, Table S6 (Supporting Information) gives the summery of available kinetic data.

The gradual decrease in hydrolysis rate constant at pD > 7 can be explained by the formation of a hydrolytically inactive complex between an oxyanion and X−Ser peptides observed in basic solutions.[56] Many studies showed that the formation of high stability peptide complexes is linked to the involvement of the deprotonated amide function. A large number of other metal ions were also reported to form M−N amide bonds in peptides, including cobalt(II) and cobalt(III), zinc(II), platinum(II) and platinum(IV), vanadium(V), and chromium-(III).[57] In this case, the pK_a of the peptide amide nitrogen is lowered by the positive charge of metal ions. Metals bind strongly to deprotonated amide nitrogens and are much less polarizing than protons. As a result, substitution of the amide hydrogen atom for a metal greatly reduces the susceptibility of the amide carbonyl carbon atom toward nucleophilic attack.[58] ^{13}C NMR measurements of a GlySer solution at pD 8.4 (Table S2, Supporting Information) have shown that in the presence of MoO_4^{2-}, the carbon atoms in the CH_2 and carbonyl C═O group of the glycyl residue show large shifts. This is consistent with the deprotonation of the amide group and its coordination to MoO_4^{2-} (Scheme 5).

Scheme 5. Active and Inactive Complexes [Mo−GlySer]



The faster hydrolysis of HisSer compared to other X−Ser dipeptides containing noncoordinating side chains is most likely due to the effective coordination of the imidazole nitrogen to MoO_4^{2-} and the formation of a more stable complex. However, it is also plausible that imadozole nitrogen acts as a base, accepting the hydroxyl proton from Ser residue and facilitating the intramolecular attack. The slower hydrolysis rate constant for LeuSer can be explained by the steric hindrance caused by the Leu side chain, which inhibits nucleophilic attack and results in slower peptide hydrolysis.

Since our recent studies on the reactivity of oxomolybdates toward phosphodiesters have shown that the fastest hydrolysis occurred under mildly acidic conditions and implicated $[Mo_7O_{24}]^{6-}$ as the hydrolytically active complex,[41−44] it is interesting to address the reasons that might account for the different pD and speciation trends exhibited by peptides versus activated phosphodiesters. The interaction between a phosphodiester and $[Mo_7O_{24}]^{6-}$ leads to an intramolecular exchange process which results in partial detachment of one MoO_4 tetrahedron and allows for the attachment of the structurally related phosphodiester tetrahedron into the polyoxometalate structure. The incorporation of the phosphodiester group into the polyoxomolybdate skeleton results in the sharing of its oxygen atoms with the Mo(VI) center that can cause polarization and strain of the P−O ester bond, which in turn makes it more susceptible toward hydrolysis. This implies that water from the solvent acts as a nucleophile and that the catalyst acts through destabilization of the phosphodiester bond through its incorporation into the polyoxometalate skeleton. While the structural analogy between phosphate and molybdate forms the basis of this interaction, it is easy to understand that such interaction between structurally distinct molybdate and peptides is less plausible. Indeed, to the best of our knowledge, there are no studies reporting on complexation between $[Mo_7O_{24}]^{6-}$, which is a dominant species under mildly acidic solutions, and peptides. On the other hand, monomeric molybdate MoO_4^{2-}, which predominates at neutral and basic pH, has a tendency to coordinate to polydentate ligands. Therefore, the different coordination affinities of monomeric and polyoxo forms of molybdate toward phosphoesters and peptides are most likely the reason for their different reactivaties exhibited toward these classes of compounds.

Hydrolysis of GlySer by Other MO_4^{2-} (M = Cr, W) Oxyanions. Besides MoO_4^{2-}, two other iso-structural MO_4^{2-} oxyanions, CrO_4^{2-} and WO_4^{2-}, were examined for their efficiency toward the hydrolysis of GlySer. The rate constants are compared in Table 3.

Table 3. Rate Constants for the Hydrolysis of 2 mM GlySer in the Presence of 120 mM Concentration of Different Oxyanions (pD = 7.0 and 60 °C)

oxyanion	k_{obs} (s^{-1})
MoO_4^{2-}	5.9×10^{-6}
CrO_4^{2-}	4.4×10^{-7}
WO_4^{2-}	6.5×10^{-7}

As can be seen from Table 3, compared to MoO_4^{2-}, both CrO_4^{2-} and WO_4^{2-} were less effective toward the hydrolysis of the peptide bond in GlySer. The size of MO_4^{2-} increases in the order $CrO_4^{2-} < MoO_4^{2-} \approx WO_4^{2-}$. Therefore, solvation of the MO_4^{2-} species in H_2O is the most significant for CrO_4^{2-}, which hinders effective coordination to GlySer and results in low reactivity. Previous studies have shown that tungsten chelates are significantly more labile with respect to individual metal−ligand bonds than molybdenum chelates.[59] ^{13}C NMR studies of GlySer performed in the presence and the absence of WO_4^{2-} showed smaller shifts of all ^{13}C NMR resonances as compared to MoO_4^{2-} (Table S7, Supporting Information). The lability of the $[WO_4^{2-}$/GlySer] complex most likely results in lower reactivity toward GlySer peptide bond hydrolysis.

CONCLUSIONS

In conclusion, we report the first example of hydrolytic peptide bond cleavage promoted by a negatively charged oxyanion. To the best of our knowledge, the hydrolytic cleavage of the peptide bond has been so far achieved only by metal cations or positively charged metal complexes. The hydrolytic effect of MoO_4^{2-} can be attributed to its ability to efficiently coordinate X−Ser peptides and polarize their carbonyl group toward the internal nucleophilic attack by the hydroxyl group of the Ser residue. Although the hydrolysis occurred in solutions containing polyoxo forms of molybdate, the kinetic studies indentified the monomeric molybdate as the hydrolytically active complex. We are currently investigating the reactivity of MoO_4^{2-} toward the hydrolysis of different oligopeptides and proteins. This and future studies focusing on the reactivity of oxomolybdates toward relevant biomolecules are important, as

dx.doi.org/10.1021/ic2015034 | Inorg. Chem. 2011, 50, 12025−12033

they may shed more light on the molecular origin of their biological activity.

EXPERIMENTAL SECTION

Materials. Glycylserine (GlySer), leucylserine (LeuSer), histidylserine (HisSer), glycylalanine (GlyAla), serylglycine (SerGly), and sodium molydate were purchased from Acros and used without further purification. The pD of the solutions for the ^{1}H NMR studies was adjusted with D_2SO_4 and NaOD, both from Acros. Ac−GlySer and GlySer−OEt were synthesized using a standard procedure.[60]

NMR Spectroscopy. ^{1}H and ^{13}C spectra were recorded on Bruker Advance 400 and Bruker Advance 600 spectrometers. D_2O with 0.05 wt % 3-(trimethylsilyl) propionic acid as an internal standard was used as a solvent. A solution of TMS in $CDCl_3$ was employed as an external ^{13}C NMR reference.

Kinetics. In a typical kinetic experiment, the hydrolysis of a 2 mM peptide in the presence of 120 mM molybdate was followed by ^{1}H NMR spectroscopy. For example, the typical reaction mixture was prepared by dissolving 0.32 mg of GlySer in 1 mL of D_2O to which 92 mg of $Na_2MoO_4 \times 2\ H_2O$ was added. The pH of the final mixture was adjusted by adding small amounts (typically 1−5 μL) of 2 M D_2SO_4 or 1.5 M NaOD. The pH of the solution was measured in the beginning and at the end of the hydrolytic reaction, and the difference was typically less than 0.1 unit. The pD value of the solution was obtained by adding 0.41 to the pH reading, according to formula pD = pH + 0.41.[48] The reaction samples were kept at constant temperature (typically 60 °C), and the rate constants for the hydrolysis were determined by following the appearance of the free glycine resonance in the ^{1}H NMR spectra at different time intervals. The observed first order rate constants (k_{obs}) were calculated by the integral method from at least 90% conversion. This included integrating proton NMR resonances of free glycine and plotting them as a function of time. The linear fitting method ($\ln[A] = k_{obs} \times t + C$), where A is the concentration of the substrate and t is the time at which the concentration measured was used. The R values were generally higher than 0.98. The Michaelis−Menten kinetic measurements were performed on samples which contain a fixed concentration of GlySer (2 mM), while the concentration of molybdate was increased from 0 to 625 mM.

The influence of the ionic strength on the reaction rate was studied by following the reaction between 2 mM GlySer and 120 mM Na_2MoO_4 (pD = 7.0, 60 °C) in the presence of different amounts of $NaClO_4$. As before, the rate constants were determined by the integration of the ^{1}H NMR signal intensities of the CH_2 group of free glycine.

ASSOCIATED CONTENT

Supporting Information

Additional tables, figures, and schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: Tatjana.Vogt@chem.kuleuven.be.

ACKNOWLEDGMENTS

T.N.PV. thanks K. U. Leuven for the financial support (START1/09/028). P.H.H. thanks the Vietnamese Government and K. U. Leuven for a doctoral fellowship. K.S. thanks F.W.O. Flanders for the doctoral fellowship.

REFERENCES

(1) Rana, T. M.; Meares, C. F. *J. Am. Chem. Soc.* **1990**, *112*, 2457.
(2) Xu, G. H.; Chance, M. R. *Chem. Rev.* **2007**, *107*, 3514.
(3) Wu, J.; Perrin, D. M.; Sigman, D. S.; Kaback, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9186.
(4) Qi, D. F.; Tann, C. M.; Haring, D; Distefano, M. D. *Chem. Rev.* **2001**, *101*, 3081.
(5) Goldshleger, R.; Karlish, S. J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9596.
(6) Chin, J. *Acc. Chem. Res.* **1991**, *24*, 145.
(7) Mocz, G.; Gibbons, I. R. *J. Biol. Chem.* **1990**, *265*, 2917.
(8) Duff, M. R; Kumar, C. V. *Metallomics* **2009**, *1*, 518.
(9) Wei, C. H.; Chou, W. Y.; Huang, S. M.; Lin, C. C.; Chang, G. G. *Biochemistry* **1994**, *33*, 7931.
(10) Luo, S.; Ishida, H.; Makino, A.; Mae, T. *J. Biol. Chem.* **2002**, *277*, 12382−12387.
(11) Zaychikov, E.; Martin, E.; Denissova, L.; Kozlov, M.; Markovtsov, V.; Kashlev, M.; Heumann, H.; Nikiforov, V.; Goldfarb, A.; Mustaev, A. *Science* **1996**, *273*, 107.
(12) Gallagher, J.; Zelenko, O.; Walts, A. D.; Sigman, D. S. *Biochemistry* **1998**, *37*, 2096.
(13) Grodsky, N. B.; Soundar, S.; Colman, R. F. *Biochemistry* **2000**, *39*, 2193.
(14) Hlavaty, J. J.; Benner, J. S.; Hornstra, L. J.; Schildkraut, I. *Biochemistry* **2000**, *39*, 3097.
(15) Hua, S.; Inesi, G.; Toyoshima, C. *J. Biol. Chem.* **2000**, *275*, 30546.
(16) Cheng, X.; Shaltiel, S.; Taylor, S. A. *Biochemistry* **1998**, *37*, 14005.
(17) Baichoo, N.; Heyduk, T. *Protein Sci.* **1999**, *8*, 518.
(18) Montigny, C.; Jaxel, C.; Shainskaya, A.; Vinh, J.; Labas, V.; Moller, J. V.; Karlish, S. J. D.; le Maire, M. *J. Biol. Chem.* **2004**, *279*, 43971.
(19) Lee, T. Y; Suh, J. H. *Chem. Soc. Rev.* **2009**, *38*, 1949.
(20) Chei, W. S; Suh, J. *Prog. Inorg. Chem.* **2007**, *55*, 79.
(21) Suh, J; Chei, W. S. *Curr. Opin. Chem. Biol.* **2008**, *12*, 207.
(22) Suh, J. *Acc. Chem. Res.* **2003**, *36*, 562.
(23) Radzicka, A; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105.
(24) Whitelegge, J. P.; Gomez, S. M.; Faull, K. F. *Adv. Protein Chem.* **2003**, *65*, 271.
(25) Chin, J *Acc. Chem. Res.* **1991**, *24* (4), 2005.
(26) (a) Bamann, E; Hass, J. G.; Trapmann, H. *Arch. Pharm.* **1961**, *294*, 569. (b) Yashiro, M.; Sonobe, Y.; Yamamura, A.; Takarada, T.; Komiyama, M.; Fujii, Y. *Org. Biomol. Chem.* **2003**, *1*, 629.
(27) (a) Fujii, Y.; Kiss, T.; Gajda, T.; Tan, X. S.; Sato, T.; Nakano, Y.; Hayashi, Y.; Yashiro, M. *J. Biol. Inorg. Chem.* **2002**, *7*, 843. (b) Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015. (c) Zhang, L.; Mei, Y.; Zhang, Y.; Li, S.; Sun, X.; Zhu, L. *Inorg. Chem.* **2003**, *42*, 492.
(28) (a) Krezel, A.; Kopera, E.; Protas, A. M.; Poznanski, J.; Wysłouch-Cieszynska, A.; Bal, W. *J. Am. Chem. Soc.* **2010**, *132* (10), 3355. (b) Kopera, E.; Krezel, A.; Protas, A. M.; Belczyk, A.; Bonna, A.; Wyslouch-Cieszynska, A.; Poznanski, J.; Bal, W. *Inorg. Chem.* **2010**, 6636−6645.
(29) (a) Milovic, N. M.; Kostic, N. M. *J. Am. Chem. Soc.* **2003**, *125*, 781. (b) Zhu, L. G.; Qin, L.; Parac, T. N.; et al. *J. Am. Chem. Soc.* **1994**, *116*, 5218.
(30) Takarada, T.; Yashiro, M.; Komiyama, M. *Chem.—Eur. J.* **2000**, *6*, 3906−3913.
(31) Kassai, M.; Grant, K. B. *Inorg. Chem. Commun.* **2008**, *11*, 521. (b) Kassai, M.; Ravi, R. G.; Shealy, S. J.; Grant, K. B. *Inorg. Chem.* **2004**, *43*, 6130.
(32) Grant, K. B.; Kassai, M. *Curr. Org. Chem.* **2006**, *10*, 1035.
(33) (a) Parac, T. N.; Ullmann, G. M.; Kostic, N. M. *J. Am. Chem. Soc.* **1999**, *121*, 3127−3135. (b) Parac, T. N.; Kostic, N. M. *Inorg. Chem.* **1998**, *37*, 2141. (c) Milinkovic, S. U.; Parac, T. N.; Djuran, M. I.; et al. *Dalton Trans.* **1997**, *16*, 2771. (d) Parac, T. N.; Kostic, N. M. *J. Am. Chem. Soc.* **1996**, *118*, 51. (e) Parac, T. N.; Kostic, N. M. *J. Am. Chem. Soc.* **1996**, *118*, 5946.
(34) Erxleben, A. *Inorg. Chem.* **2005**, *44*, 1082−1094.
(35) Cruywagen, J. J. *Adv. Inorg. Chem.* **2000**, *49*, 127 and references therein.
(36) Fujita, H.; Fujita, T.; Sakurai, T.; Seto, Y. *Chemotherapy* **1992**, *40*, 173.

(37) (a) Ogata, A.; Mitsui, S.; Yanagie, H.; Kasano, H.; Hisa, T.; Yamase, T.; Eriguchi, M. *Biomed. Pharmacother.* **2005**, *59*, 240. (b) Yanagie, H.; Ogata, A.; Mitsui, S.; Hisa, T.; Yamase, T.; Eriguchi, M. *Biomed. Pharmacother.* **2006**, *60*, 349. (c) Ogata, A.; Yanagie, H.; Ishikawa, E.; Morishita, Y; Mitsui, S.; Yamashita, A.; Hasumi, K.; Takamoto, S.; Yamase, T.; Eriguchi, M. *Br. J. Cancer* **2008**, *98*, 399.
(38) Weil-Malherbe, H.; Green, R. H. *Biochem. J.* **1951**, *49*, 286.
(39) Cartuyvels, E.; Van Hecke, K.; Van Meervelt, L.; Görller-Walrand, C.; Parac-Vogt, T. N. *J. Inorg. Biochem.* **2008**, *102*, 1589.
(40) Ishikawa, E.; Yamase, T. *J. Inorg. Biochem.* **2006**, *100*, 344.
(41) Cartuyvels, E.; Absillis, G.; Parac-Vogt, T. N. *Chem. Commun.* **2008**, 85.
(42) Van Lokeren, L.; Cartuyvels, E.; Absillis, G.; Willem, R.; Parac-Vogt, T. N. *Chem. Commun.* **2008**, 2774.
(43) Absillis, G.; Cartuyvels, E.; Van Deun, R.; Parac-Vogt, T. N. *J. Am. Chem. Soc.* **2008**, *130*, 17400.
(44) Absillis, G.; Van Deun, R.; Parac-Vogt, T. N. *Inorg. Chem.* In press. DOI: dx.doi.org/10.1021/ic201498u.
(45) Wikjord, B. R.; Byers, L. D. *J. Am. Chem. Soc.* **1992**, *114*, 5553.
(46) Wikjord, B. R; Byers., L. D *J. Org. Chem.* **1992**, *57*, 6814.
(47) Ahn, B-T; Park, H.-S.; Lee, E.-J.; Um, I.-H. *Bull. Korean Chem. Soc.* **2000**, *21*, 905.
(48) Yamase, T. *J. Chem. Soc., Dalton. Trans.* **1991**, *11*, 3055.
(49) Müller, A.; Meyer, J.; Krickemeyer, E.; Diemann, E. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1206.
(50) Buckley, R. I.; Clark, R. J. H. *Coord. Chem. Rev.* **1985**, *65*, 167.
(51) Müller, A.; Serain, C. *Acc. Chem. Res.* **2000**, *33*, 2.
(52) Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188.
(53) Cornish-Bowden, A.; Endrenyi, L. *Biochem. J.* **1986**, *234*, 21.
(54) (a) Cruywagen, J. J.; Rohwer, E. A.; Wessels, G. F. S. *Polyhedron* **1995**, *14*, 3481. (b) Cruywagen, J. J.; Rohwer, E. A.; van de Water, R. F. *Polyhedron* **1997**, *16*, 24.
(55) Jaswal, J. S.; Tracey, A. S. *Can. J. Chem.* **1991**, *69*, 1600.
(56) Gorzsas, A.; Andersson, I.; Schmidt, H.; Rehder, D.; Pettersson, L. *Dalton Trans.* **2003**, 1161.
(57) Sovago, I.; Osz, K. *Dalton Trans.* **2006**, 3841.
(58) Martin, R. B. *Metal Ions Biol. Syst.* **2001**, *38*, 1.
(59) Majlesi, K.; Zare, K. *Phys. Chem. Liq.* **2006**, *44* (3), 257.
(60) In Benoiton, N. L.; *Chemistry of Peptide Synthesis*; CRC Press: Boca Raton, FL, 2005.