CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200800432

Synthetic Receptors for the Differentiation of Phosphorylated Peptides with Nanomolar Affinities

Andreas Grauer, Alexander Riechers, Stefan Ritter, and Burkhard König*[a]

Abstract: Artificial ditopic receptors for the differentiation of phosphorylated peptides varying in $i+3$ amino acid side chains were synthesized, and their binding affinities and selectivities were determined. The synthetic receptors show the highest binding affinities to phosphorylated peptides under physiological conditions (HEPES, pH 7.5, 154 mM NaCl) reported thus far for artificial systems. The tight and selective binding was achieved by high cooperativity of the two binding moieties in the receptor molecules. All receptors interact with phosphorylated serine by bis(Zn^{II}–cyclen) complex coordination and a second binding site recognizing a carboxylate or imidazole amino acid side chain functionality.

Keywords: chelates · cooperativity · emission spectroscopy · molecular recognition · peptides

Introduction

Phosphorylation of proteins is a ubiquitous regulation mechanism in biology and plays a central role in controlling intracellular signaling networks.[1] Protein phosphorylation regulates enzyme activity and the reversible formation of signaling complexes by specific recognition of phosphorylated proteins by phosphoprotein binding domains.[2] To investigate, monitor or specifically inhibit such phosphorylation-dependent processes, analytical tools allowing a specific recognition of phosphorylated peptides and proteins are desirable. Historically, phosphorylation detection methods relied on radioisotopes or phosphoamino acid selective antibodies. Chromatographic, staining and surface device techniques extended the available methods.[3] Several biosensors for protein kinase activity based on GFP-FRET probes[4–6] or synthetic fluorophores[7] have been reported, which typically signal their own phosphorylation or dephosphorylation. However, the number of artificial systems for a specific recognition of phosphorylated peptides is still limited. Hamachi et al.[8,9] recently reported fluorescent dinuclear zinc complexes for the detection of peptide phosphorylation and extended the concept to a hybrid receptor[10] consisting of a natural WW binding site (a protein module that binds proline-rich ligands)[11] for phosphorylated peptides and a fluorescent metal complex. Their hybrid receptor[12] shows enhanced micromolar binding affinity and selectivity towards diphosphorylated peptides derived from sequences of the C-terminal domain of the RNA polymerase II.

We report herein the design, synthesis and binding properties of synthetic ditopic receptors with nanomolar affinity to phosphorylated peptides in buffered aqueous solution under physiological pH. Their affinity depends on a second amino acid residue present in the peptide, namely carboxylate or histidine.

As target peptides Flu-GpSAAEV-NH_2 (**1**) and Flu-GpSAAHV-NH_2 (**2**) were selected from sequences of human STAT (signal transducer and activator of transcription) proteins[13] and prepared by standard solid-phase synthesis methods. The peptides were N-terminally labeled by fluorescein (Flu) to facilitate the binding studies. A bis(Zn^{II}–cyclen) triazine complex was used in the receptor design as the binding site for phosphoserine. The high affinity of this complex to phosphates was previously shown on modified surfaces.[14] Peptide **1** presents, beside the phosphate ester, as an additional functional group for specific molecular recognition a carboxylate in the sidechain of the $i+3$ glutamic acid. Guanidinium moieties have been used as binding sites for carboxylate binding.[15] Thus, to achieve selective affinity for target peptide **1**, both binding sites for phosphoserine and the carboxylate side chain were connected by a peptidic linker giving compounds **3** and **4**. Receptors **5** and **6** consist of two bis(Zn^{II}–cyclen) triazine complexes

[a] Dipl.-Chem. A. Grauer, Dipl.-Chem. A. Riechers, Dr. S. Ritter, Prof. Dr. B. König
Institute for Organic Chemistry
University of Regensburg, Universitätsstr. 31
93040 Regensburg (Germany)
Fax: (+49) 941-943-1717
E-mail: burkhard.koenig@chemie.uni-regensburg.de

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200800432.

WILEY InterScience DISCOVER SOMETHING GREAT
© 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

tethered by a peptidic linker. X-ray structure analysis of imidazole–Zn^{II}–cyclen cocrystals (see Supporting Information)[16] and potentiometric titrations[17] have shown that the complex can well accommodate imidazole as a guest. Therefore binding specificity for peptide **2** was expected. The third group of synthetic receptors **7** and **8** contains a Zn^{II}–NTA (NTA = nitrilotriacetic acid) complex beside the bis(Zn^{II}–cyclen) triazine complex. Unlike Cu^{II}- and Ni^{II}–NTA complexes which bind imidazole and are widely used for purification of His-tagged proteins by immobilized metal affinity chromatography (IMAC),[18,19] the binding affinity of Zn^{II}–NTA complexes for imidazole is significantly reduced.[20] However, the NTA ligand also represents a truncated EDTA motif, known to bind Zn^{II} with an affinity of $\log K = 16.5$.[21] Accordingly, it can be expected that a carboxylate can coordinate to the unoccupied coordination sites in the NTA complex intramolecularly, thus completing a mimic of an EDTA coordination sphere.[22] Intermolecularly, this interaction has already been described.[23] For receptors **7** and **8** (Figure 1), the two Zn^{II} complexes were connected by an alkyne azide cycloaddition[24] giving a triazole linker. All details of the synthesis and the spectroscopic characterization of receptors **3–8** are provided in the Supporting Information of this article.

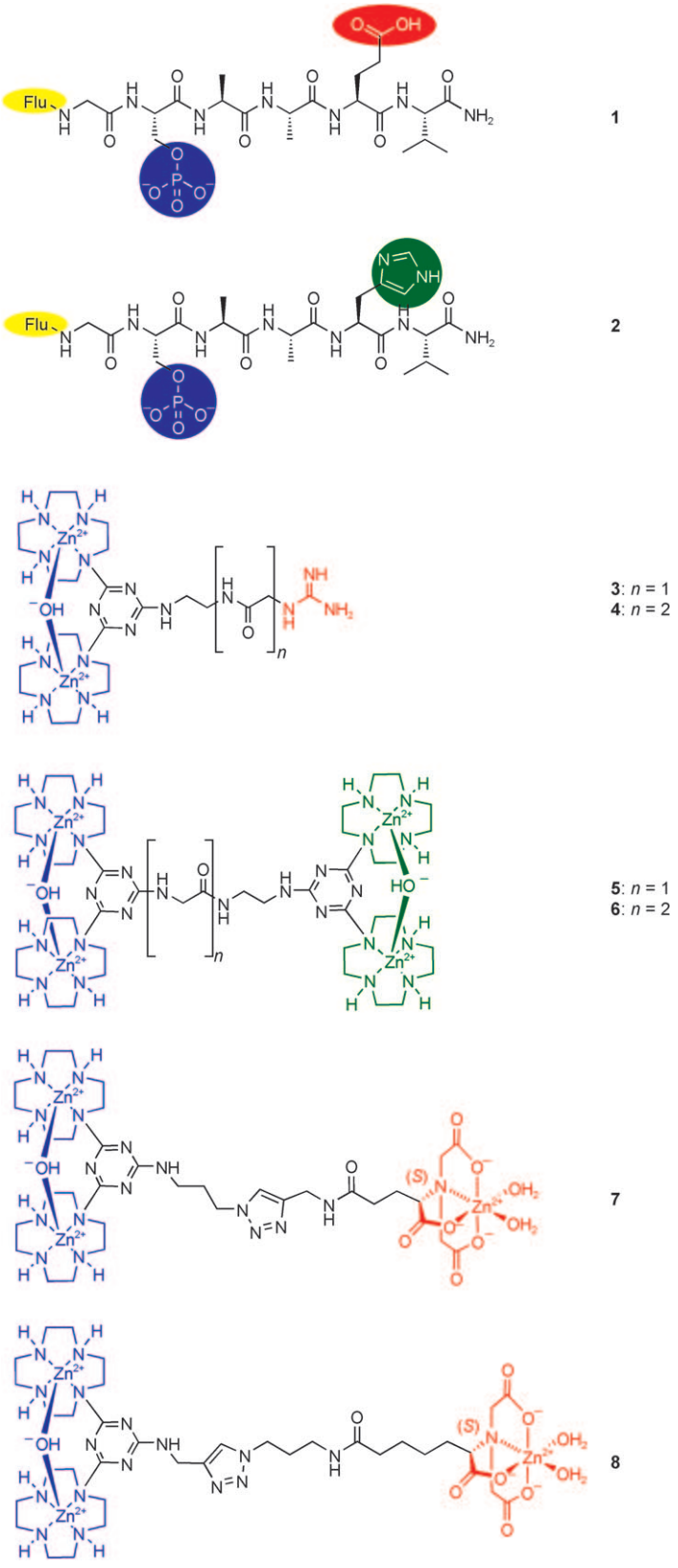


Figure 1. Phosphorylated peptides **1** and **2**, and synthetic receptors **3–8** for their binding.

Materials and methods

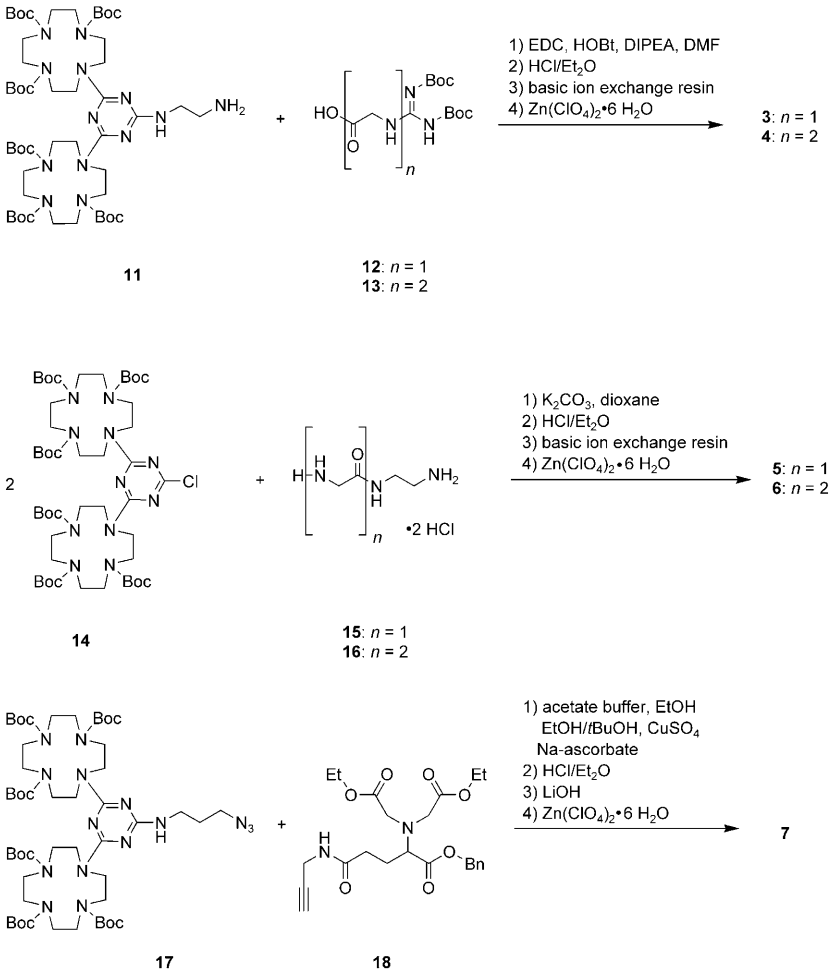
Synthesis of receptors 3–8: For the synthesis of the complexes **3** and **4** the cyclen building block precursor **11** and the two guanidine building block precursors **12** and **13** were coupled using standard peptide coupling chemistry with EDC, HOBt and DIPEA as reagents. Compound **11** was synthesized by a substitution on the literature known triazene **14**[13a] using benzyl-2-aminoethylcarbamate (**35**). The building blocks **12** and **13** were prepared by a substitution reaction on 1,3-bis(*tert*-butyloxycarbonyl)-2-methyl-2-thiourea (**38**) with H-Gly-OMe·HCl (**37**) for the first and H-Gly-Gly-OMe·HCl (**41**) for the latter. The resulting methyl esters were cleaved with LiOH and after workup with an aqueous $KHSO_4$ solution the free acids were obtained and used for the amide bond formation. The Boc-protecting groups of the protected precursors of **3** and **4** were cleaved with a saturated solution of HCl in diethyl ether. The ammonium salts precipitated from solution quantitatively and were deprotonated using a strongly basic anion exchanger in its hydroxide ion form. After lyophilization, the ligands were complexed with Zn^{II} perchlorate and the receptors **3** and **4** were obtained after recrystallization from a methanol water mixture.

The protected precursors of the receptors **5** and **6** were obtained by a two-fold substitution of the previously prepared bisamines **15** and **16** with the cyclen building block **14**. The amines were synthesized starting from *tert*-butyl 2-aminoethylcarbamate (**45**), which was coupled with Boc-Gly-OH (**44**) in the first and Boc-Gly-Gly-OH (**48**) in the latter case by standard peptide coupling conditions using EDC, HOBt and DIPEA as reagents. After the deprotection with

 © 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

a saturated solution of HCl in diethyl ether the precipitated ammonium salts were used for the substitution reaction without further purification. The resulting precursors of **5** and **6** were deprotected using a saturated solution of HCl in diethyl ether. The ammonium salts were deprotonated by the use of a strongly basic ion exchanger column in its hydroxide ion form. After lyophilisation the ligands were complexed with Zn^{II} perchlorate and the receptors **5** and **6** were obtained after recrystallization from a methanol water mixture.

Scheme 1 shows exemplary the synthesis of the protected precursor of **7**. For the receptor **7** the cyclen building block precursor **17** and the NTA-building block precursor **18** were used in a Cu^{I}-mediated Huisgen azide alkyne cycloaddition with sodium ascorbate and $CuSO_4$. The copper- and ascorbate salt were not used in catalytic amounts as this proved to give higher yields after shorter reaction times. As the reduction of Cu^{II} by sodium ascorbate caused a lowering of the pH value, the reaction was carried out in buffered solution (acetate buffer, pH 5, $c = 0.5$ M). Subsequently, the Boc-protecting groups were cleaved with a saturated solution of HCl in diethyl ether. The ammonium salts precipitated from solution quantitatively and were deprotonated using a strongly basic anion exchanger in its hydroxide ion form. Cleavage of the ester groups during this procedure was not observed. The obtained aqueous solutions of the free amines were treated with a stoichiometric amount of LiOH to cleave the benzyl, methyl and ethyl ester functionalities. Lyophilisation of these solutions gave the polydentate ligands in quantitative yield, ready for complexation with Zn^{II} perchlorate.



Scheme 1. Synthesis of the synthetic phosphopeptide receptors.

Binding studies

All binding studies were conducted in buffered aqueous solution (50 mM HEPES, pH 7.5, 154 mM NaCl). A Varian Cary Eclipse Fluorometer was used for the emission titrations. The cuvette with 800 μL of peptide in HEPES buffer was titrated stepwise with small amounts (beginning with 0.13 equiv) of the receptor solution. After each addition the solution was allowed to equilibrate for 2 min before the fluorescence intensity and the UV spectrum (where permitted by the concentration range) were recorded. The stoichiometries were determined by Job's plot analysis. To determine the binding constants, the obtained fluorescence intensities at 520 nm were volume corrected, plotted against the receptor concentration and evaluated by nonlinear fitting. Fluorescence polarization titrations were conducted under identical conditions using an ISS K2 Multifrequency Phase Fluorometer.

Results and Discussion

Binding constants of receptors **3**–**8** to peptides **1** and **2** were determined by fluorescence emission titrations in aqueous solution (HEPES, pH 7.5, 50 mM, 154 mM NaCl) and nonlinear fitting of the data (see Supporting Information). Job's plots were used to determine the binding stoichiometry, which was found to be 1:1 for all experiments. For comparison the affinity of complexes **9** and **10** (Figure 2), representing the single binding sites, to peptides **1** and **2** were measured. Table 1 summarizes the results, while Figures 3 and 4 illustrate the binding selectivity and show exemplary emission titrations.

 © 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Binding affinities of complexes **3**–**10** to peptides **1** and **2**.

receptor	Binding affinity [log$K$] peptide **2**	peptide **1**
3	4.5	8.0/8.0[a]
4	5.8	7.8
5	7.5	5.0
6	6.5	4.9
7	4.8	8.0/8.4[a]
8	4.6	7.9
9	n.d.	< 3
10	4.8	4.8

[a] Reference values from fluorescence polarization titrations.

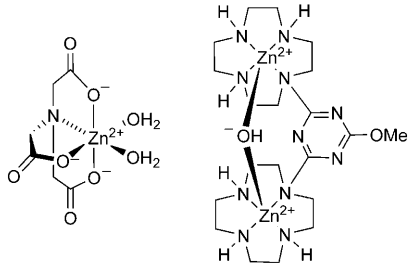


Figure 2. Complexes **9** and **10** representing receptor substructures.

Receptors **3** and **4** show affinities for Flu-GpSAAEV-NH_2 (**1**) of log$K$ = 8.0 and 7.8, respectively, while the binding to peptide **2** is two to three orders of magnitude less (log$K$ = 4.5 and 5.8, respectively). This is in accordance with our expectations, as the interaction of the guanidine binding site with the glutamate carboxylate of peptide **1** is significantly stronger than with the imidazole side chain of peptide **2** due to electrostatic attraction. The reverse binding selectivity is observed for tetra(Zn^{II}–cyclen) receptors **5** and **6**: The histidine-containing peptide **2** is bound one to two orders of magnitude stronger (log$K$ = 7.5 and 6.5, respectively) than peptide **1** (log$K$ = 5.0 and 4.9, respectively). The interaction of the second bis(Zn^{II}–cyclen) triazine with the imidazole side chain contributes to the aggregate's stability, although the selectivity is not as pronounced as in the case of complexes **3** and **4**. Additional entropic stabilization comes from the bivalent structure of **5** and **6** with two identical binding sites, in our eyes also an example of positive cooperativity in enthalpy.[25] Bis(Zn^{II}–cyclen)–Zn^{II}–NTA receptors **7** and **8** again show a pronounced selectivity towards peptide **1** with log$K$ = 8.0 and 7.9, respectively. The binding to peptide **2** is about one thousand fold weaker (log$K$ = 4.8 and 4.6, respectively) and we attribute the selectivity to the interaction of Zn^{II}–NTA with the glutamate carboxylate. Generally, we find a strong cooperativity of binding in the "matched" cases, a behavior which has been described before for artificial receptors.[26]

A comparison of the peptide binding affinities of bis(Zn^{II}–cyclen) triazine complex **10** to receptors **3**–**8** reveals the contribution of the second binding site to the overall affinity. Complex **10** binds to both peptides with identical

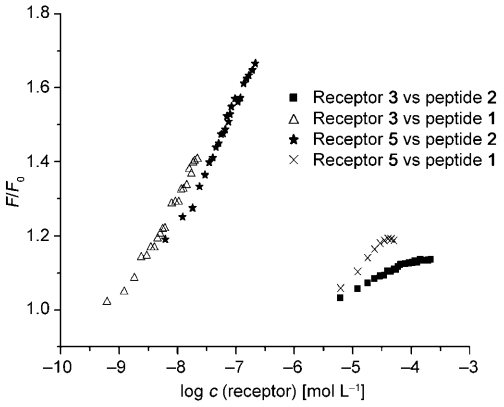


Figure 3. Emission response of peptides **1** and **2** in the presence of receptors **3** and **5**. The logarithmic x axis allows the depiction of the binding selectivity in orders of magnitude of the concentration range.

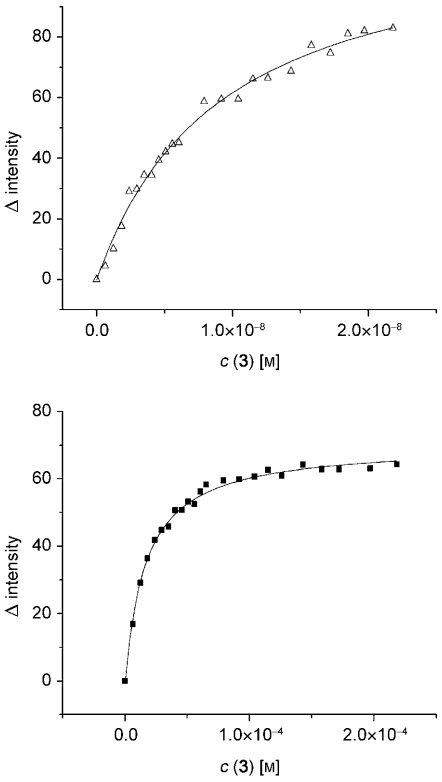


Figure 4. Emission titrations of receptor **3** against peptides **1** (top) and **2** (bottom). The continuous line represents the Hill equation fit.

strength (log$K$ = 4.8), which shows that the interaction of the phosphate ester with the bis(Zn^{II}–cyclen) binding site is not affected by the peptide sequence. The binding affinities of receptors **3** and **4** to peptide **2** are similar to this value. This leads to the conclusion that interactions of the guanidinium moiety to peptide **2** are negligible. The same applies to the interaction of receptors **7** and **8** with peptide **1**: The Zn^{II}–NTA–imidazole interaction does not contribute to the receptor affinity as the Zn^{II}–NTA–carboxylate binding does. This is confirmed by the binding data of Zn^{II}–NTA complex

 © 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

9 to peptides **1** and **2**. A small, but significant interaction is observed with peptide **1**, while no interaction is determined with peptide **2**.

To verify the results from the emission titrations by an independent method, fluorescence polarization titrations were conducted under identical conditions as the emission titrations. The two values determined for receptors **3** and **7** are in good agreement with the results from the emission titrations. Fluorescence labels may falsify the binding results by contributing to the peptide affinity. To exclude interactions of the peptide label with the receptors, fluorescein sodium was titrated with a receptor of each binding motif showing no emission changes even at large receptor excess.

The length or type of the linkers connecting the two receptor binding sites has little or no influence on the binding affinity. This is not surprising as the short peptides display no stable secondary structure in solution and the receptors linkers are flexible. The binding selectivity and affinity of the receptor molecules rely on the presence of complementary functional groups for non-covalent interaction in reach and not on their exact spatial position.

Conclusion

The combination of bis(Zn^{II}–cyclen)–triazine metal complex binding sites with guanidinium moieties or Zn^{II}–NTA complexes leads to artificial receptors for the differentiation of phosphorylated small peptides. Using the right combination of binding moieties, nanomolar peptide binding affinities at physiological conditions are achieved. To the best of our knowledge these are the highest affinities of phosphopeptide binding by artificial receptors reported thus far. Depending on a second functional group beside the phosphate ester selectivities of up to three orders of magnitude are observed. To better resolve the affinity of artificial receptors and peptides in the distance of the involved functional groups, the binding properties of more rigidified receptors and cyclopeptides are currently investigated and results will be reported in due course. Furthermore, projects employing these recognition moieties in molecular biology are under way.

Experimental Section

Synthetic details and titration plots are available in the Supporting Information.

Acknowledgements

We thank the Volkswagen Foundation, the Fonds der Chemischen Industrie, the University of Regensburg and the Graduiertenkolleg GRK 760 for support of this research. We further thank Axel Dürkop for help with the fluorescence polarization measurements and Chiara Cabrele for advice and valuable discussions.

[1] a) L. N. Johnson, R. J. Lewis, *Chem. Rev.* **2001**, *101*, 2209–2242; b) T. Hunter, *Cell* **2000**, *100*, 113–127.
[2] M. B. Yaffe, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 177–186.
[3] a) A. Ojida, T. Kohira, I. Hamachi, *Chem. Lett.* **2004**, 1024–1025; b) K. Martin, T. H. Steinberg, L. A. Cooley, K. R. Gee, J. M. Beecham, W. F. Patton, *Proteomics* **2003**, *3*, 1244–1255; c) E. Kinoshita, E. Kinoshita-Kikuta, K. Takiyama, T. Koike, *Mol. Cell. Proteomics* **2006**, *5*, 749–757; d) E. Kinoshita, E. Kinoshita-Kikuta, K. Takiyama, T. Koike, *Mol. Cell. Proteomics* **2006**, *5*, 749–757; e) S. Yamada, H. Nakamura, E. Kinoshita, E. Kinoshita-Kikuta, T. Koike, Y. Shiro, *Anal. Biochem.* **2007**, *360*, 160–162; f) E. Kinoshita-Kikuta, Y. Aoki, E. Kinoshita, T. Koike, *Mol. Cell. Proteomics* **2007**, *6*, 356–366; g) R. Freeman, R. Gill, I. Willner, *Chem. Commun.* **2007**, 3450–3452; h) H.-Y. Lin, C.-T. Chen, Y.-C. Chen, *Anal. Chem.* **2006**, *78*, 6873–6878.
[4] S. Moritoshi, O. Takeaki, I. Kouichi, A. Tomoichiro, U. Yoshio, *Nat. Biotechnol.* **2002**, *20*, 287–294.
[5] K. Kurokawa, N. Mochizuki, Y. Ohba, H. Mizuno, A. Miyawaki, M. Matsuda, *J. Biol. Chem.* **2001**, *276*, 31305–31310.
[6] a) A. Y. Ting, K. H. Kain, R. L. Klemke, R. Y. Tsien, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 15003–15008; b) J. Zhang, Y. Ma, S. S. Taylor, R. Y. Tsien, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 14997–15002; c) M. Sato, T. Ozawa, K. Inukai, T. Asano, Y. Umezawa, *Nat. Biotechnol.* **2002**, *20*, 287–294.
[7] a) M. D. Shults, B. Imperiali, *J. Am. Chem. Soc.* **2003**, *125*, 14248–14249; b) D. M. Rothman, M. D. Shults, B. Imperiali, *Trends Cell Biol.* **2005**, *15*, 502–510; c) B. R. Sculimbrene, B. Imperiali, *J. Am. Chem. Soc.* **2006**, *128*, 7346–7352.
[8] a) A. Ojida, Y. Mito-Oka, M. Inoue, I. Hamachi, *J. Am. Chem. Soc.* **2002**, *124*, 6256–6258; b) A. Ojida, M. Inoue, Y. Mito-Oka, I. Hamachi, *J. Am. Chem. Soc.* **2003**, *125*, 10184–10185.
[9] A. Ojida, Y. Mito-oka, K. Sada, I. Hamachi, *J. Am. Chem. Soc.* **2004**, *126*, 2454–2463.
[10] T. Anai, E. Nakata, Y. Koshi, A. Ojida, I. Hamachi, *J. Am. Chem. Soc.* **2007**, *129*, 6232–6239.
[11] P.-J. Lu, X. Z. Zhou, Y.-C. Liou, J. P. Noel, K. P. Lu, *J. Biol. Chem.* **2002**, *277*, 2381–2384.
[12] Other ditopic receptors recognizing non-phosphorylated peptides have been reported, for example: a) A. Hossain, H.-J. Schneider, *J. Am. Chem. Soc.* **1998**, *120*, 11208–11209; b) L. Hernandez-Folgado; C. Schmuck, *Org. Biomol. Chem.* **2007**, *5*, 2390–2394.
[13] C. P. Lim, X. Cao, *Mol. Biosyst.* **2006**, *2*, 536–550.
[14] a) D. S. Turygin, M. Subat, O. A. Raitman, V. V. Arslanov, B. Koenig, M. A. Kalinina, *Angew. Chem.* **2006**, *118*, 5466–5470; *Angew. Chem. Int. Ed.* **2006**, *45*, 5340–5344; b) D. S. Turygin, M. Subat, O. A. Raitman, S. L. Selector, V. V. Arslanov, B. König, M. A. Kalinina, *Langmuir*, **2007**, 23, 2517–2524; c) H. Fujioka, T. Koike, N. Yamada, E. Kimura, *Heterocycles* **1996**, *42*, 775–787.
[15] a) C. Schmuck, *Coord. Chem. Rev.* **2006**, 250, 3053–3067; b) R. J. Fitzmaurice, F. Gaggini, N. Srinivasan, K. Natarajan, D. Jeremy, *Org. Biomol. Chem.* **2007**, *5*, 1706–1714; c) C. Schmuck, *Chem. Eur. J.* **2000**, *6*, 709–718.
[16] C. Pariya, T.-Y. Chi, T. K. Mishra, C. S. Chung, *Inorg. Chem. Commun.* **2002**, *5*, 119–123.
[17] M. Kruppa, D. Frank, H. Leffler-Schuster, B. König, *Inorg. Chim. Acta* **2006**, *359*, 1159–1168.
[18] M. Y. Wang, Y. Y. Kau, M. S. Lee, S. R. Doong, J. Y. Ho, L. H. Lee, *Biotechnol. Bioeng.* **2000**, *67*, 104–111.
[19] M. Kruppa, B. König, *Chem. Rev.* **2006**, *106*, 3520–3560.
[20] J. Volz, *J. Chromatogr. A* **1998**, *800*, 29–37.
[21] R. Delgado, M. de Fátima Cabral, R. Castanheira, A. Zhang, R. Herrmann, *Polyhedron* **2002**, *21*, 2265–2276.
[22] Although NTA–Zn^{II} complexes are known to be chelated by His-tagged proteins, these interactions are based on the chelating effect of two imidazoles occupying both remaining coordination sites. A single histidine residue is not sufficient for a binding event. See also ref. [20].

 © 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[23] a) D. Hopgood, R. J. Angelici, *J. Am. Chem. Soc.* **1968**, *90*, 2508–2513; b) D. L. Rabenstein, G. Blakney, *Inorg. Chem.* **1973**, *12*, 128–132.
[24] J. E. Moses, A. D. Moorhouse, *Chem. Soc. Rev.* **2007**, *36*, 1249–1262.
[25] D. H. Williams, M. S. Westwell, *Chem. Soc. Rev.* **1998**, *27*, 57–63.
[26] G. J. Kirkovits, J. A. Shriver, P. A. Gale, J. L. Sessler, *J. Inclusion Phenom. Macrocyclic Chem.* **2001**, *41*, 69–75.

Received: March 10, 2008
Published online: August 11, 2008

 © 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim