Proteomics

International Edition: DOI: 10.1002/anie.201411232
German Edition: DOI: 10.1002/ange.201411232

An Affinity Reagent for the Recognition of Pyrophosphorylated Peptides**

*John H. Conway and Dorothea Fiedler**

Abstract: *A resin-bound dinuclear zinc(II) complex for the selective capture of pyrophosphopeptides is reported. The metal complex binds diphosphate esters over other anionic groups, such as monophosphate esters, sulfate esters, and carboxylic acids, with high specificity. Immobilization of the compound provided a reagent capable of binding and retaining nanomolar quantities of pyrophosphopeptide in the presence of cell lysate. The high affinity and specificity of the reagent makes it an attractive tool for the study of in vivo pyrophosphorylation.*

Proteins are functionalized with a plethora of post-translational modifications (PTMs), which generate tremendous diversity, complexity, and heterogeneity among gene products.[1] Complete characterization of post-translationally modified proteins presents a formidable analytical challenge because many PTMs occur in substoichiometric amounts. Targeted approaches can help to reduce the complexity of the proteome by focusing on distinct subsets of proteins that harbor the same PTM.[2] To this end, chemical receptors for the recognition of trimethyllysine,[3] dimethylarginine,[4] or phosphoserine residues[5] have been developed. More traditional approaches for the enrichment of phosphopeptides and/or phosphoproteins from cell lysates include immobilized-metal affinity chromatography (IMAC),[6] metal-oxide affinity chromatography (MOAC),[6a] immunoprecipitation,[6a,7] and affinity tagging.[6a,8] These technologies have had a remarkable impact on the decryption of phosphorylation dependent signaling pathways.

For protein pyrophosphorylation, a modification mediated by the inositol pyrophosphate messengers,[9] no analogous methods exist. Pyrophosphorylation is thought to proceed through the non-enzymatic reaction of a phosphoserine residue with the β-phosphoryl group of an inositol pyrophosphate (Figure 1).[10] Genetic perturbation of inositol pyrophosphate biosynthesis has revealed a wide range of functions for these messengers,[9,11] the most notable being their roles in insulin homeostasis and body-weight regulation.[12] Nevertheless, the regulatory effect of protein pyrophosphorylation in controlling insulin signaling and central energy metabolism has not been elucidated. Because the identification of pyrophosphorylation substrates has exclusively relied on in vitro labeling strategies,[10] the in vivo relevance of these targets remains controversial. We therefore decided to develop a reagent that would enable the capture, and ultimately the comprehensive annotation, of in vivo pyrophosphorylated proteins.

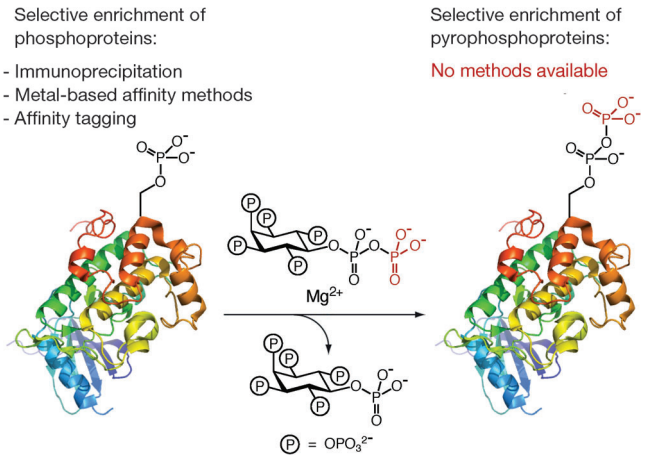


Figure 1. Phosphoproteins are pyrophosphorylated by inositol pyrophosphate messengers in the presence of magnesium ions. To date, no methods exist for the enrichment and characterization of in vivo pyrophosphorylated proteins.

The utility of IMAC and TiO_2-MOAC in enriching phosphopeptides from complex mixtures[6] inspired us to pursue an immobilized metal complex for the recognition of pyrophosphorylated peptides and proteins. Specifically, we chose to investigate dinuclear zinc(II)–dipicolylamine [Zn^{II}dpa] complexes because these reagents have found widespread use in anion sensing and recognition and show a high propensity for binding to phosphate-containing anions in aqueous solution.[13,14] Furthermore, the immobilized Zn^{II}dpa complex "Phos-tag" has been employed for affinity chromatography of phosphorylated peptides and proteins.[6b] Because phosphoproteins are presumably more abundant than pyrophosphoproteins, the pyrophosphopeptide capture reagent must exhibit high affinity and selectivity for diphosphate esters over monophosphate esters. We therefore initiated our studies with complex **1** (Figure 2), since this

[*] J. H. Conway, Prof. D. Fiedler
Department of Chemistry, Princeton University
Washington Rd, Princeton, NJ 08544 (USA)
E-mail: dfiedler@princeton.edu

[**] We gratefully acknowledge Dr. P. Jeffrey and the Hughson group for access to their isothermal titration calorimeter, Dr. I. Pelczer for assistance with NMR analysis, and Dr. J. Eng for support with mass spectrometry. We are grateful to Dr. V. Dekaris, L. Yates, and Dr. Z. Brown for aid with chemical synthesis. Furthermore, we thank Dr. F. Williams, A. Marmelstein, and C. Orillac, as well as the Yang, Muir, MacMillan, and Doyle groups for providing access to instruments and reagents. Financial support from the NIH (DP2 CA186753) and Princeton University is gratefully acknowledged. D.F. is a Kimmel Scholar and a Rita Allen Scholar.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201411232.

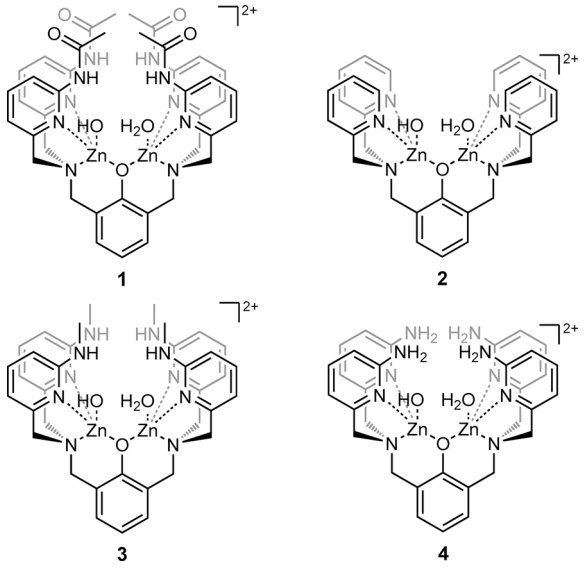


Figure 2. Dinuclear zinc complexes investigated for binding affinity and selectivity toward diphosphate esters. For synthetic details, see the Supporting Information and Schemes S1–S4.

compound possesses very high affinity for inorganic pyrophosphate (PP_i) in aqueous solution.[14d] However, no binding data for complex **1** with mono- and diphosphate esters were reported.

For detailed characterization of **1**, we used isothermal titration calorimetry (ITC) to determine the binding constants of PP_i and simple nucleotides, which served as model mono- and diphosphate esters (Table 1, entries 1, 2, and 3). In line with the published report,[14d] the association of **1** with PP_i was so strong that it could not be determined directly by ITC. By contrast, the association constant of UDP was much smaller ($K_a = 2.4 \times 10^5$, Table 1, entry 3). The thermodynamic data revealed that binding of the diphosphate ester was enthalpically unfavorable and entirely entropy-driven. Interestingly, with the related complex **2**, which lacks hydrogen-bond donors, the binding of ADP was enthalpically favorable (Table 1, entry 4). We hypothesized that steric repulsion between the acyl groups of **1** and the nucleoside contributed to the positive enthalpy of binding. We consequently designed complexes **3** and **4**, which incorporate smaller hydrogen-bond donors (Figure 2). Indeed, the endothermicity of binding was abrogated for **3** and **4**, and we obtained complexes with high affinity and selectivity toward ADP. In both cases, the association constants for ADP were in the $10^7\,M^{-1}$ range, and the selectivity for ADP over AMP was about 100-fold (Table 1, entries 6, 7, 9 and 10).[15]

The observed selectivity for the diphosphate ester moiety was encouraging, but in complex samples such as cell lysates, several other protein functional groups could compete for affinity enrichment. We therefore investigated the binding properties of **4** with additional substrates. ADP ribose, which is present in ADP-ribosylated proteins, displayed low affinity toward **4**, as did a sulfate ester, a group encountered in sulfated proteins (Table 1, entries 12 and 14). Previous work showed that [Zn^{II}dpa] complexes interact with imidazole in organic solvent,[16] however no binding of L-His was observed

Table 1: Association constants for the zinc complexes with various anions.

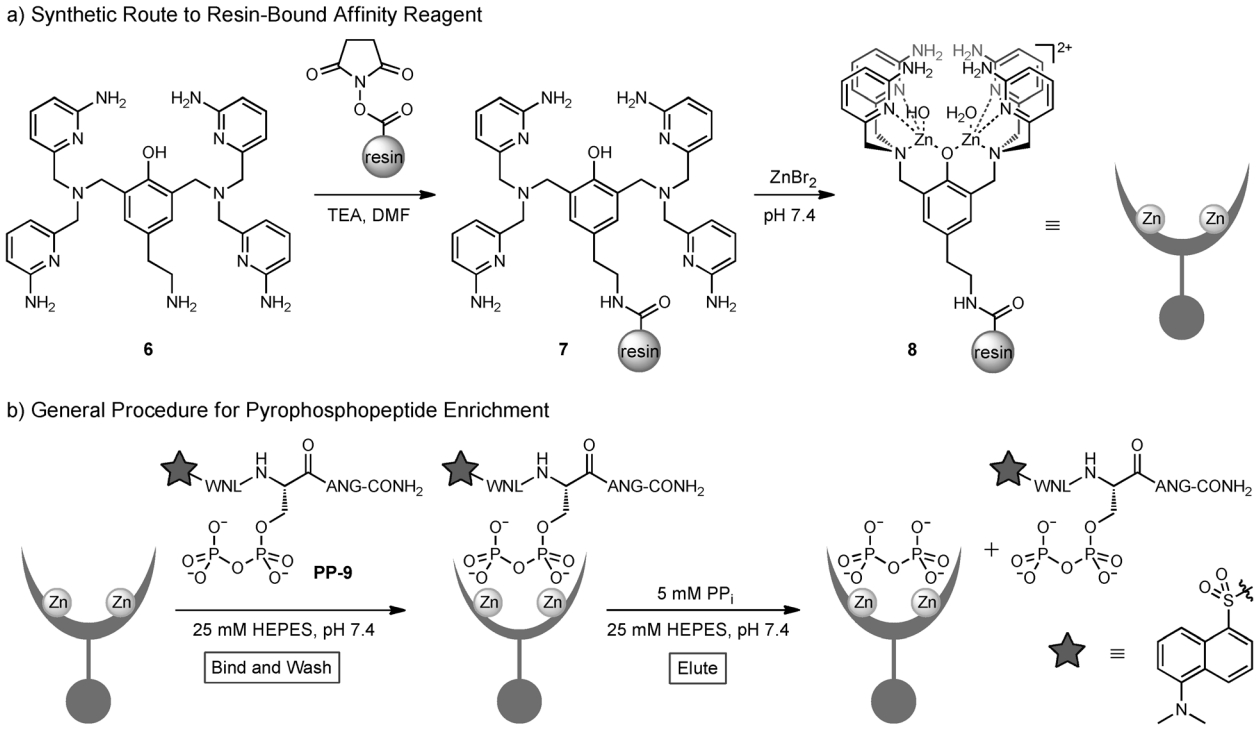
Entry	Complex	Substrate	K_a [M^{-1}]	ΔH [kcal mol^{-1}]	$T\Delta S$ [kcal mol^{-1}]
1	**1**	PP_i	$>10^8$[a]		
2	**1**	AMP	$(1.8\pm0.2)\times10^3$	1.84 ± 0.09	6.22
3	**1**	UDP[b]	$(2.4\pm0.3)\times10^5$	2.39 ± 0.03	9.65
4	**2**	ADP	$(2.1\pm0.1)\times10^5$	-3.37 ± 0.02	3.84
5	**2**	AMP	$(3.5\pm0.5)\times10^4$	-3.00 ± 0.04	3.13
6	**3**	ADP	$(1.9\pm0.4)\times10^7$	-6.82 ± 0.07	3.10
7	**3**	AMP	$(1.9\pm0.3)\times10^5$	-1.70 ± 0.03	5.43
8	**3**	PP_i	$(5.9\pm1.0)\times10^6$	-14.7 ± 0.2	-5.58
9	**4**	ADP	$(1.1\pm0.5)\times10^7$	-4.41 ± 0.14	5.07
10	**4**	AMP	$(1.1\pm0.2)\times10^5$	-2.24 ± 0.03	4.57
11	**4**	PP_i	$(1.8\pm0.3)\times10^6$	-13.4 ± 0.2	-4.93
12	**4**	ADP ribose	$(9.2\pm0.7)\times10^3$	-1.90 ± 0.06	3.45
13	**4**	SO_4^{2-}	n.d.[c]	n.d.[c]	n.d.[c]
14	**4**	$MeOSO_3^-$	n.d.[c]	n.d.[c]	n.d.[c]
15	**4**	L-His	n.d.[c]	n.d.[c]	n.d.[c]
16	**4**	Citrate	$(1.6\pm0.9)\times10^4$	-1.75 ± 0.12	3.92
17	**4**	UDP	$(1.4\pm0.4)\times10^7$	-8.07 ± 0.14	1.60
18	**4**	UMP	$(9.4\pm2.4)\times10^4$	-1.82 ± 0.12	4.90
19	**4**	**PP-5**[d]	$(5.2\pm1.6)\times10^6$	-7.52 ± 0.10	1.55
20	**4**	**P-5**[d]	$(1.6\pm0.5)\times10^5$	-2.41 ± 0.13	15.6

Values measured by isothermal titration calorimetry (ITC). Titration conditions: HEPES buffer (25 mM), pH 7.4, KCl (100 mM), 22 °C. [a] Binding was too strong for direct measurement of the association constant by ITC. [b] ADP caused precipitation of the complex, thus precluding measurement. [c] ΔH was too low to generate a measurable signal. [d] Single-letter peptide sequence: ANPSNWK. The serine residue is either monophosphorylated (**P-5**) or pyrophosphorylated (**PP-5**).

in aqueous solution (Table 1, entry 15). Polyacidic stretches within proteins could also interfere with pyrophosphoprotein capture by **4**,[17] but the K_a for citrate was quite low, thus alleviating this concern (Table 1, entry 16). Furthermore, the binding preference of **4** for diphosphate esters over monophosphate esters was independent of the nucleobase, as evidenced by the selectivity for UDP over UMP (Table 1, entries 17 and 18). Importantly, this selectivity also extended to peptide substrates since we observed a much tighter association of pyrophosphopeptide **PP-5** with **4** compared to monophosphopeptide **P-5** (Table 1, entries 19 and 20).[18]

Given that **4** exhibited desired features for the affinity enrichment of pyrophosphorylated peptides and proteins, we selected this complex for attachment to a solid-phase resin. Consequently, we synthesized a modified version of the bis(dipicolylamino)phenol ligand of **4** that contains a primary amine (**6**, Scheme S5). Compound **6** was subsequently linked to an *N*-hydroxysuccinimide (NHS)-activated resin to provide **7** (Scheme 1a).[19] The immobilized ligand was then treated with a solution of $ZnBr_2$ in 25 mM HEPES (pH 7.4) to yield affinity reagent **8**.

To establish the binding properties of **8** in this heterogeneous system, a short, dansylated pyrophosphopeptide (**PP-9**) was synthesized, along with its phosphopeptide precursor (**P-9**). The concentrations of these peptides can be quantified in solution by fluorescence spectroscopy.[20] When a suspension of affinity reagent **8** (43 nmol in 500 μL total volume) was treated with **PP-9** (2.00 μM, 1.00 nmol) in 25 mM HEPES at pH 7.4, only a small quantity of peptide remained in the supernatant (Scheme 1b and Figure 3a).[21] The binding of



Scheme 1. Preparation and use of the resin-bound affinity reagent.

PP-9 to **8** was due to a specific interaction with the capture reagent since the zinc-free immobilized ligand (**7**) was not able to retain **PP-9** (Figure 3a). Affinity reagent **8** also captured a large fraction of the phosphopeptide **P-9**. Elution of the bound peptides was accomplished by subsequent treatment with a 5 mM solution of PP_i (Figure 3b). Importantly, we were able to elute **P-9**, but not **PP-9**, with 200 μM inorganic phosphate (P_i) (Figure 3b). This differential sensitivity to the elution conditions will prove useful for experiments in cell lysates, where phosphoproteins most likely outnumber pyrophosphoproteins. The ability to remove a large fraction of the phosphoproteins with the phosphate wash will greatly decrease the background signal and improve the sensitivity and efficiency of our enrichment procedure.

Next, we assessed the ability of **8** to bind **PP-9** in the presence of high concentrations of phosphopeptide **10** (Figure 3c). To our delight, affinity reagent **8** suffered no loss of enrichment of **PP-9** even in the presence of 100 equiv of **10**, thus demonstrating that the selectivity on resin is similar, if not better, than in solution. By contrast, a similar experiment with an IMAC resin revealed a notable decrease in the binding of **PP-9** at high concentrations of **10** (Figure S5).[22]

To confirm that the recognition of the pyrophosphate ester was independent of the surrounding amino acid sequence, we prepared three additional model peptides as both the mono- and pyrophosphorylated species. These peptides were designed to contain positively charged (**P-11** and **PP-11**), hydrophobic (**P-12** and **PP-12**), or negatively charged (**P-13** and **PP-13**) side chains (for full sequences, see Figure S6). Notably, affinity reagent **8** exhibited a strong preference for retaining the pyrophosphopeptides in all three

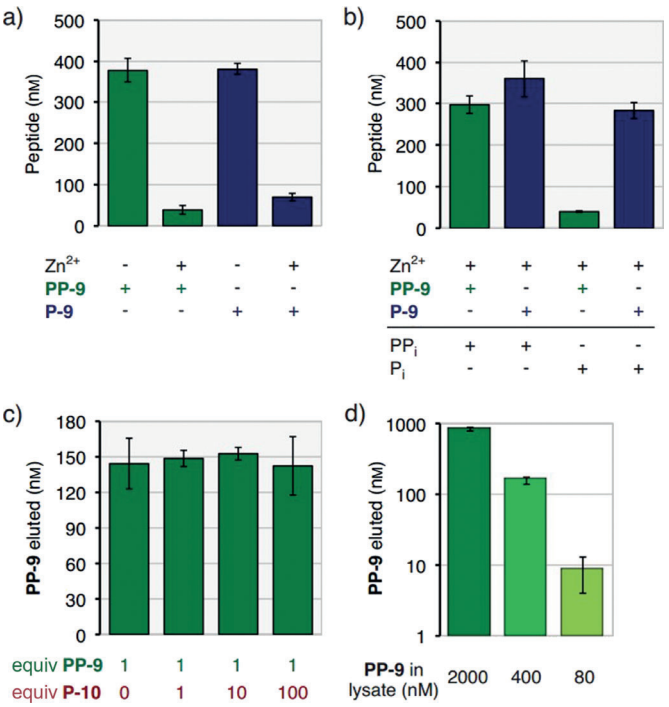


Figure 3. Binding of fluorescently labeled peptides to affinity reagent **8**. a) Amount of peptide in the supernatant after incubation with either **7** ($-Zn^{2+}$) or **8** ($+Zn^{2+}$). b) Amount of peptide eluted from the resin with 5 mM PP_i or 200 μM P_i. c) Enrichment of **PP-9** in the presence of increasing concentrations of phosphopeptide **10** (Ac-WNLpSANG-$CONH_2$). See Figure S3 for representative fluorescence spectra. d) Capture of **PP-9** (various concentrations) from a cell lysate (5.0 mg mL^{-1}), as measured after PP_i elution.

cases, thus suggesting that **8** is indeed compatible with complex peptides of varying sequence (Figure S7).[23]

Finally, we tested the capacity of **8** to remove **PP-9** from a cell lysate. To avoid interference from nucleotides and other small molecules, the protein fraction of an *S. cerevisiae* cell lysate was separated from the metabolites through acetone precipitation.[24] We then combined this protein fraction (at 5.0 mg mL^{-1}) with varying concentrations of **PP-9** (2 μM to 80 nM) and exposed the mixtures to reagent **8**. Upon elution with PP_i, we were able to detect **PP-9** at all concentrations tested (Figure 3d), thus illustrating that affinity reagent **8** is able to bind and retain the pyrophosphopeptide from the sea of functional groups in the *S. cerevisiae* proteome.[25] The capability of **8** to capture pyrophosphopeptide **PP-9** at such low concentrations in the presence of a large excess of cell lysate holds great promise for its application in proteome-wide studies.

In summary, we have developed a reagent for the selective recognition of the diphosphate ester functional group. Attachment of the zinc complex to a solid support generated a capture reagent that can bind and retain pyrophosphopeptides in the presence of cell lysate. The tunable nature of the affinity reagent allows for the optimization of additional parameters, such as metal ion or pH, in the future. We envision that our optimized synthetic receptor, much like a pan-specific antibody, will enable the characterization of dynamic changes in protein pyrophosphorylation when combined with modern mass spectrometry methods. This annotation could then provide the long awaited understanding of the molecular mechanisms by which the inositol pyrophosphates orchestrate cellular energy metabolism.

Keywords: affinity reagents · proteins · proteomics · pyrophosphorylation · signal transduction

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 3941–3945
Angew. Chem. **2015**, *127*, 4013–4017

[1] C. T. Walsh, *Posttranslational Modification of Proteins: Expanding Nature's Inventory*, 1st ed., Roberts & Co., **2005**.

[2] a) H. C. Hang, M. E. Linder, *Chem. Rev.* **2011**, *111*, 6341; b) Y. H. Seo, K. S. Carroll, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 16163.

[3] a) C. S. Beshara, C. E. Jones, K. D. Daze, B. J. Lilgert, F. Hof, *ChemBioChem* **2010**, *11*, 63; b) S. A. Minaker, K. D. Daze, M. C. F. Ma, F. Hof, *J. Am. Chem. Soc.* **2012**, *134*, 11674; c) S. Tabet, S. F. Douglas, K. D. Daze, G. A. E. Garnett, K. J. H. Allen, E. M. M. Abrioux, T. T. H. Quon, J. E. Wulff, F. Hof, *Bioorg. Med. Chem.* **2013**, *21*, 7004.

[4] L. I. James, J. E. Beaver, N. W. Rice, M. L. Waters, *J. Am. Chem. Soc.* **2013**, *135*, 6450.

[5] a) A. Späth, C. Koch, B. König, *Eur. J. Inorg. Chem.* **2010**, 2926; b) T. Zhang, N. Y. Edwards, M. Bonizzoni, E. V. Anslyn, *J. Am. Chem. Soc.* **2009**, *131*, 11976.

[6] a) P. A. Grimsrud, D. L. Swaney, C. D. Wenger, N. A. Beauchene, J. J. Coon, *ACS Chem. Biol.* **2010**, *5*, 105; b) E. Kinoshita, A. Yamada, H. Takeda, E. Kinoshita-Kikuta, T. Koike, *J. Sep. Sci.* **2005**, *28*, 155; c) S. Li, C. Dass, *Anal. Biochem.* **1999**, *270*, 9; d) T. S. Nühse, A. Stensballe, O. N. Jensen, S. C. Peck, *Mol. Cell. Proteomics* **2003**, *2*, 1234.

[7] S. B. Breitkopf, M. Yuan, G. A. Pihan, J. M. Asara, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 16190.

[8] "Chemical Tagging Strategies for Mass Spectrometry-Based Phospho-proteomics": A. Leitner, W. Lindner, in *Phospho-Proteomics*, *Vol. 527* (Ed.: M. Graauw), Humana Press, Totowa, NJ, **2009**, pp. 229–243.

[9] For recent reviews, see: a) M. S. C. Wilson, T. M. Livermore, A. Saiardi, *Biochem. J.* **2013**, *452*, 369; b) T. Wundenberg, W. Mayr Georg, *Biol. Chem.* **2012**, *393*, 979; c) A. Chakraborty, S. Kim, S. H. Snyder, *Sci. Signaling* **2011**, *4*, re1.

[10] a) R. Bhandari, A. Saiardi, Y. Ahmadibeni, A. M. Snowman, A. C. Resnick, T. Z. Kristiansen, H. Molina, A. Pandey, J. K. Werner, K. R. Juluri, Y. Xu, G. D. Prestwich, K. Parang, S. H. Snyder, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 15305; b) A. Saiardi, R. Bhandari, A. C. Resnick, A. M. Snowman, S. H. Snyder, *Science* **2004**, *306*, 2101; c) C. Azevedo, A. Burton, E. Ruiz-Mateos, M. Marsh, A Saiardi, *Proc. Nat. Acad. Sci.* **2009**, *106*, 21161; d) Z. Szijgyarto, A. Garedew, C. Azevedo, A. Saiardi, *Science* **2011**, *334*, 802.

[11] a) M. A. Koldobskiy, A. Chakraborty, J. K. Werner, A. M. Snowman, K. R. Juluri, M. S. Vandiver, S. Kim, S. Heletz, S. H. Snyder, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 20947; b) Y.-S. Lee, S. Mulugu, J. D. York, E. K. O'Shea, *Science* **2007**, *316*, 109; c) A. Prasad, Y. Jia, A. Chakraborty, Y. Li, S. K. Jain, J. Zhong, S. G. Roy, F. Loison, S. Mondal, J. Sakai, C. Blanchard, S. H. Snyder, H. R. Luo, *Nat. Immunol.* **2011**, *12*, 752.

[12] a) R. Bhandari, K. R. Juluri, A. C. Resnick, S. H. Snyder, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2349; b) A. Chakraborty, M. A. Koldobskiy, N. T. Bello, M. Maxwell, J. J. Potter, K. R. Juluri, D. Maag, S. Kim, A. S. Huang, M. J. Dailey, M. Saleh, A. M. Snowman, T. H. Moran, E. Mezey, S. H. Snyder, *Cell* **2010**, *143*, 897; c) C. Illies, J. Gromada, R. Fiume, B. Leibiger, J. Yu, K. Juhl, S. N. Yang, D. K. Barma, J. R. Falck, A. Saiardi, C. J. Barker, P. O. Berggren, *Science* **2007**, *318*, 1299.

[13] For recent reviews, see: a) S. K. Kim, D. H. Lee, J.-I. Hong, J. Yoon, *Acc. Chem. Res.* **2009**, *42*, 23; b) H. T. Ngo, X. Liu, K. A. Jolliffe, *Chem. Soc. Rev.* **2012**, *41*, 4928; c) A. E. Hargrove, S. Nieto, T. Zhang, J. L. Sessler, E. V. Anslyn, *Chem. Rev.* **2011**, *111*, 6603.

[14] a) M. S. Han, D. H. Kim, *Angew. Chem. Int. Ed.* **2002**, *41*, 3809; *Angew. Chem.* **2002**, *114*, 3963; b) D. H. Lee, J. H. Im, S. U. Son, Y. K. Chung, J.-I. Hong, *J. Am. Chem. Soc.* **2003**, *125*, 7752; c) D. H. Lee, S. Y. Kim, J.-I. Hong, *Angew. Chem. Int. Ed.* **2004**, *43*, 4777; *Angew. Chem.* **2004**, *116*, 4881; d) J. H. Lee, J. Park, M. S. Lah, J. Chin, J.-I. Hong, *Org. Lett.* **2007**, *9*, 3729; e) E. Kinoshita, M. Takahashi, H. Takeda, M. Shiro, T. Koike, *Dalton Trans.* **2004**, 1189; f) J. A. Drewry, S. Fletcher, H. Hassan, P. T. Gunning, *Org. Biomol. Chem.* **2009**, *7*, 5074; g) S. Yang, G. Feng, N. H. Williams, *Org. Biomol. Chem.* **2012**, *10*, 5606; h) X. Liu, H. T. Ngo, Z. Ge, S. J. Butler, K. A. Jolliffe, *Chem. Sci.* **2013**, *4*, 1680; i) J. Wongkongkatep, Y. Miyahara, A. Ojida, I. Hamachi, *Angew. Chem. Int. Ed.* **2006**, *45*, 665; *Angew. Chem.* **2006**, *118*, 681; j) H.-W. Rhee, H.-Y. Choi, K. Han, J.-I. Hong, *J. Am. Chem. Soc.* **2007**, *129*, 4524; k) A. Ojida, I. Takashima, T. Kohira, H. Nonaka, I. Hamachi, *J. Am. Chem. Soc.* **2008**, *130*, 12095; l) Y. Kurishita, T. Kohira, A. Ojida, I. Hamachi, *J. Am. Chem. Soc.* **2010**, *132*, 13290; m) A. Ojida, Y. Mito-oka, M.-a. Inoue, I. Hamachi, *J. Am. Chem. Soc.* **2002**, *124*, 6256; n) H.-W. Rhee, S. H. Lee, I.-S. Shin, S. J. Choi, H. H. Park, K. Han, T. H. Park, J.-I. Hong, *Angew. Chem. Int. Ed.* **2010**, *49*, 4919; *Angew. Chem.* **2010**, *122*, 5039; o) A. Ojida, M.-a. Inoue, Y. Mito-oka, H. Tsutsumi, K. Sada, I. Hamachi, *J. Am. Chem. Soc.* **2006**, *128*, 2052; p) E. Kinoshita, E. Kinoshita-Kikuta, K. Takiyama, T. Koike, *Mol. Cell. Proteomics* **2006**, *5*, 749; q) B. Gruber, S. Balk, S. Stadlbauer, B. König, *Angew. Chem. Int. Ed.* **2012**, *51*, 10060; *Angew. Chem.* **2012**, *124*, 10207; r) W. M. Leevy, S. T. Gammon, H. Jiang, J. R. Johnson, D. J. Maxwell, E. N. Jackson, M. Marquez, D. Piwnica-Worms, B. D. Smith, *J. Am. Chem. Soc.* **2006**, *128*, 16476; s) B. A. Smith, W. J. Akers, W. M. Leevy, A. J.

Lampkins, S. Xiao, W. Wolter, M. A. Suckow, S. Achilefu, B. D. Smith, *J. Am. Chem. Soc.* **2010**, *132*, 67; t) V. Ganesh, K. Bodewits, S. J. Bartholdson, D. Natale, D. J. Campopiano, J. C. Mareque-Rivas, *Angew. Chem. Int. Ed.* **2009**, *48*, 356; *Angew. Chem.* **2009**, *121*, 362; u) T. Kohira, K. Honda, A. Ojida, I. Hamachi, *ChemBioChem* **2008**, *9*, 698; v) G. Liu, K. Y. Choi, A. Bhirde, M. Swierczewska, J. Yin, S. W. Lee, J. H. Park, J. I. Hong, J. Xie, G. Niu, D. O. Kiesewetter, S. Lee, X. Chen, *Angew. Chem. Int. Ed.* **2012**, *51*, 445; *Angew. Chem.* **2012**, *124*, 460.

[15] Through ^{31}P NMR spectroscopy of **4** in complex with thiamine pyrophosphate, we confirmed that both phosphate groups participate in binding to the zinc centers. A downfield shift of both phosphorus resonances was observed (Figure S1), which is consistent with the binding mode observed in related systems.

[16] T. Routasalo, J. Helaja, J. Kavakka, A. M. P. Koskinen, *Eur. J. Org. Chem.* **2008**, 3190.

[17] a) H. Nonaka, S. Tsukiji, A. Ojida, I. Hamachi, *J. Am. Chem. Soc.* **2007**, *129*, 15777; b) A. Ojida, K. Honda, D. Shinmi, S. Kiyonaka, Y. Mori, I. Hamachi, *J. Am. Chem. Soc.* **2006**, *128*, 10452.

[18] A. M. Marmelstein, L. M. Yates, J. H. Conway, D. Fiedler, *J. Am. Chem. Soc.* **2014**, *136*, 108.

[19] Studies with *N*-hydroxysuccinimide showed that acylation occurred exclusively on the primary amine, as determined by ^{1}H NMR.

[20] For concentration calibration curves, see Figure S2.

[21] After the P_i wash, an elution with 5 mM PP_i released the bound **PP-9** as expected, and confirmed that very little **P-9** remained on the resin (Figure S4). Note that the incubation volumes were 500 μL, whereas the supernatant and elution volumes were approximately 2 mL, thus resulting in a 4-fold dilution for the fluorescence measurements.

[22] A quantitative assessment of TiO_2-based MOAC is difficult because the number of binding sites for this reagent is not specified.

[23] For concentration calibration curves and regressions, see Figures S8–S10.

[24] We confirmed that acetone precipitation does not degrade pyrophosphopeptides (see Figure S11).

[25] In a related experiment, we enriched a synthetic pyrophosphopeptide added into a *S. cerevisiae* lysate, which enabled its detection by mass spectrometry (see Figure S12).

Received: November 19, 2014
Published online: February 4, 2015

