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Chemical Pyrophosphorylation of Functionally Diverse Peptides

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Abstract

A highly selective and convenient method for the synthesis of pyrophosphopeptides in solution is reported. The remarkable compatibility with functional groups (alcohol, thiol, amine, carboxylic acid) in the peptide substrates suggests that the intrinsic nucleophilicity of the phosphoserine residue is much higher than previously appreciated. Because the methodology operates in polar solvents, including water, a broad range of pyrophosphopeptides can be accessed. We envision these peptides will find widespread applications in the development of mass spectrometry and antibody-based detection methods for pyrophosphoproteins.

To diversify the proteome, eukaryotic cells rely on an array of posttranslational modifications (PTMs).[1] These chemical alterations can control the enzymatic activity of proteins, modulate their association with other biomolecules, or determine their location in the cell. Among the extensive repertoire of PTMs, protein phosphorylation is one of the most common, and it is universally regarded that reversible protein phosphorylation is a signaling mechanism involved in essentially all cellular processes.[1,2] The availability of phospho-specific antibodies have made the detection of distinct phosphoproteins possible, and modern phosphoproteomic methods can provide detailed snapshots of phosphorylation-dependent signaling pathways.[3]

Protein pyrophosphorylation, a more recently described modification, is poorly understood by comparison. Snyder and co-workers discovered that a group of second messengers, the inositol pyrophosphates,[4] are able to transfer the high-energy β-phosphate group to protein substrates in an enzyme-independent fashion that requires only Mg^{2+} as a co-factor.[5] In fact, the β-phosphate group of the inositol pyrophosphate is added onto a pre-existing phosphoserine residue, yielding a pyrophosphorylated protein (Figure 1).[6] Genetic perturbation of the enzymes involved in inositol pyrophosphate biosynthesis results in a number of interesting phenotypes.[7] Most notable is the intimate involvement of the pyrophosphate messengers in insulin signaling and body weight regulation in mice and humans.[8] To what extent protein pyrophosphorylation contributes to these phenotypes has not been determined and remains an area of intense investigation.

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Supporting Information: Supporting Schemes, Tables, and Figures as well as experimental procedures and spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

While *in vitro* pyrophosphorylation has been well characterized, many questions surrounding this modification still linger. What is the detailed mechanism for this unique phosphoryl-transfer reaction? Is a phosphoserine residue sufficiently nucleophilic? Furthermore, direct evidence that protein pyrophosphorylation plays a regulatory role *in vivo* is missing to date. A dearth of suitable methods has precluded the identification of pyrophosphorylated proteins in complex cell lysates. Neither an antibody-based approach nor proteomic platforms are currently available. Evidently, new tools for the analysis of protein pyrophosphorylation in different cellular contexts are much needed.

Our interest in protein pyrophosphorylation led us to engage in the chemical synthesis of pyrophosphopeptides. To determine the optimal strategy, we synthesized pyrophosphoserine model compound **1** and evaluated its chemical stability (Table 1, Scheme S1). The pyrophosphate moiety exhibited considerable stability over a wide pH range (pH 4.5-9.5) and in the presence of various Lewis acidic metal cations (Table S1). Basic conditions resulted in complete loss of the pyrophosphate group via β-elimination (Table 1, entry 7), while treatment with 0.1M HCl (Table 1, entry 1) caused a substantial amount of hydrolysis. The acid sensitivity prompted us to investigate the compatibility of the pyrophosphate functional group with solid phase peptide synthesis (SPPS) conditions.[9] When **1** was exposed to TFA cleavage cocktails frequently used in SPPS, a significant quantity of hydrolyzed product was observed (Table 1, entries 8-9).[10] Consequently, an SPPS-based method did not appear feasible. Instead, we chose to install the pyrophosphate group in solution.[11] With this approach, we can take full advantage of the well-established procedures to obtain phosphopeptides[12] and incorporate the relatively labile pyrophosphate functional group in the last synthetic step.

Traditional phosphorylation methods rely on electrophilic phosphorus (III) and phosphorus (V) reagents.[13] Therefore, as illustrated in Scheme 1, three distinct pyrophosphorylation methods were tested and optimized on an N- and C-terminally protected phosphopeptide (5), which was initially devoid of reactive amino acid side chains. Reaction of **5** with phosphoramidite **2**, followed by oxidation, proceeded cleanly to intermediate **9a** (Method A, Table 2, entry 1). Subsequent hydrogenolysis afforded the desired pyrophosphopeptide **13**. Negative ion mode electron spray mass spectrometry confirmed the product *via* the detection of the $[M-H]^-$ ion (918.2561), and ^{31}P NMR spectroscopy unequivocally corroborated the formation of the pyrophosphate group, as can be seen by two diagnostic doublets at −6.5 and −10.5 ppm (Figure S1). In comparison, much shorter reaction times were required when the more electrophilic phosphoryl chloride **3** was used to produce **9a** in high conversion (Method B, Table 2, entry 2). Finally, we tested phosphorimidazolide **4** as a source of electrophilic phosphorus (V) with restrained reactivity (Method C).[14] Using the imidazolide, peptide **5** was completely converted to intermediate **9b** in 90 minutes at 45 °C with ZnCl2 as an additive (Table 2, entry 3).[15, 16]

Given the functional diversity of peptides, we next determined the side chain functional group compatibility of the pyrophosphorylation methods. The phosphoramidite chemistry (Method A) was tolerant of lysine (Table 2, entry 4), but exhibited significant cross-reactivity with the thiol group of cysteine (Table 2, entry 5). A dehydrated product was observed with aspartic acid-containing substrate **8**, presumably due to competing cyclization

of an activated acyl-phosphate intermediate (Table 2, entry 6). Dehydrated by-products of peptide **8** were also observed with the phosphoryl chloride reagent (Method B) (Table 2, entry 9). Method B, however, did not exhibit cross-reactivity with cysteine (Table 2, entry 8). By far the best functional group compatibility was achieved using phosphorimidazolide **4** for the pyrophosphorylation reaction (Method C). The presence of an amine, thiol, or carboxylic acid did not significantly interfere with formation of the desired product in *N*,*N*-dimethylacetamide (DMA) (Table 2, entries 10-12).[17] Analysis of the crude reaction mixtures by ^{31}P NMR revealed that the formation of P-N and P-S bond-containing species was negligible. Also, primary alcohols did not impair pyrophosphorylation, as the reaction proceeded smoothly in methanol.[18] The high tolerance towards numerous functional groups was unexpected and suggests that, when presented with the appropriate electrophile, the intrinsic nucleophilicity of phosphoserine is higher than typically presumed.[19]

In contrast to phosphoramidites and phosphoryl chlorides, phosphorimidazolide reagents are stable to aqueous environments.[20] We therefore tested whether the pyrophosphorylation reaction with phosphorimidazolide **4** could be carried out in water. Since **4** can tolerate both amine and carboxylic acid functionalities (as discussed in the previous section) the N- and C-terminally deprotected phosphopeptide **17** was prepared and used as a substrate. The standard conditions of Method C yielded 17% of pyrophosphorylated peptide **17** when water was used as the sole solvent. By increasing the amount of phosphoimidazolide reagent and zinc additive in combination with lengthening the reaction time, 85% conversion to the pyrophosphopeptide **17** was obtained (Scheme 2, Table S5). To maintain constant pH, the aqueous reactions were buffered near pH 7. Notably, imidazole buffer did not slow down pyrophosphorylation, indicating that the imidazole moiety, as encountered in a histidine side chain, is also well compatible with our method.[21] With DMA as the solvent, peptide **16** was fully converted to benzyl-protected intermediate **17** and subsequent hydrogenolysis provided pyrophosphopeptide **18**.

To demonstrate the general utility of this pyrophosphorylation strategy, we applied our method to a highly challenging substrate, a Nopp140 peptide fragment (amino acid 76-100). Nopp140 is a mammalian protein, and the peptide fragment (aa76-100) is known to undergo inositol pyrophosphate mediated pyrophosphorylation *in vitro*.[2] The peptide contains 25 amino acids and is densely functionalized (5 lysine, 7 serine/threonine, and 8 glutamic/aspartic acid residues, Figure 2). Remarkably, when treated with phosphorimidazolide **4**, the Nopp140 peptide **19** was selectively phosphorylated on the phosphoserine residue. Both mass spectrometry and ^{31}P NMR clearly confirmed the formation of the desired pyrophosphopeptide **20** (Figure 2).

The observed specificity of the pyrophosphorylation reaction with a functionally complex peptide substrate underlines the unique reactivity of the phosphoserine residue towards the phosphorus (V) electrophile. Considering phosphoserine's high intrinsic reactivity provides a first clue as to how Nature may accomplish the formation of pyrophosphoproteins without the involvement of an enzyme.

In summary, we have described a method that provides easy access to pyrophosphopeptides. The procedure operates in polar protic and aprotic solvents and can thus be applied to a wide

range of peptides. These peptides will be essential reagents for elucidating the cellular functions of protein pyrophosphorylation. For example, they can be used to optimize mass spectrometry techniques for the detection of pyrophosphopeptides in complex cell lysates. Likewise, to raise antibodies, the pyrophosphoserine residue needs to be incorporated into various amino acid sequences, and availability of pyrophosphopeptides will be an integral part of the process.

Since peptide pyrophosphorylation can be carried out in water, we envision that further reaction development can provide a unique bioconjugation method. It is now possible to site-specifically incorporate phosphoserine residues into proteins using amber codon suppression.[22] The phosphoproteins could then be pyrophosphorylated with our approach to characterize the *in vitro* biochemical and biophysical effects of protein pyrophosphorylation. In all, the accessibility of pyrophosphopeptides by the reported methodology provides the necessary gateway for entering this emerging area of signal transduction research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Walsh, CT. Posttranslational Modifications of Proteins: Expanding Nature's Inventory. 1. Roberts and Co. Publishers; 2005.
2. (a) Hunter T. Philos T R Soc B. 2012; 367:2513.(b) Brognard J, Hunter T. Curr Opin Genet Dev. 2011; 21:4. [PubMed: 21123047] (c) Derouiche A, Cousin C, Mijakovic I. Curr Opin Biotech. 2012; 23:585. [PubMed: 22119098]
3. (a) Paradela A, Albar JP. J Proteome Res. 2008; 7:1809. [PubMed: 18327898] (b) Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Trends Biotechnol. 2002; 20:261. [PubMed: 12007495]
4. For recent reviews on diphosphoinositol polyphosphate signaling see:(a) Wilson MSC, Livermore TM, Saiardi A. Biochem J. 2013; 452:369. [PubMed: 23725456] (b) Wundenberg T, Mayr GW. Biol Chem. 2012; 393:979. [PubMed: 22944697] (c) Chakraborty A, Kim S, Snyder SH. Sci Signal. 2011; 4(d) Shears SB, Gokhale NA, Wang H, Zaremba A. Adv Enzyme Regul. 2011; 51:13. [PubMed: 21035493]
5. Saiardi A, Bhandari R, Resnick AC, Snowman AM, Snyder SH. Science. 2004; 306:2101. [PubMed: 15604408]
6. Bhandari R, Saiardi A, Ahmadibeni Y, Snowman AM, Resnick AC, Kristiansen TZ, Molina H, Pandey A, Werner JK, Juluri KR, Xu Y, Prestwich GD, Parang K, Snyder SH. Proc Natl Acad Sci USA. 2007; 104:15305. [PubMed: 17873058]
7. (a) Prasad A, Jia YH, Chakraborty A, Li YT, Jain SK, Zhong J, Roy SG, Loison F, Mondal S, Sakai J, Blanchard C, Snyder SH, Luo HR. Nat Immunol. 2011; 12:752. [PubMed: 21685907] (b) Koldobskiy MA, Chakraborty A, Werner JK, Snowman AM, Juluri KR, Vandiver MS, Kim S, Heletz S, Snyder SH. Proc Natl Acad Sci USA. 2010; 107:20947. [PubMed: 21078964] (c) Lachance J, Vernot B, Elbers CC, Ferwerda B, Froment A, Bodo JM, Lema G, Fu WQ, Nyambo

TB, Rebbeck TR, Zhang K, Akey JM, Tishkoff SA. Cell. 2012; 150:457. [PubMed: 22840920] (d) Lee YS, Huang K, Quiocho FA, O'Shea EK. Nat Chem Biol. 2008; 4:25. [PubMed: 18059263]

8. (a) Bhandari R, Juluri KR, Resnick AC, Snyder SH. Proc Natl Acad Sci USA. 2008; 105:2349. [PubMed: 18268345] (b) Chakraborty A, Koldobskiy MA, Bello NT, Maxwell M, Potter JJ, Juluri KR, Maag D, Kim S, Huang AS, Dailey MJ, Saleh M, Snowman AM, Moran TH, Mezey E, Snyder SH. Cell. 2010; 143:897. [PubMed: 21145457] (c) Chakraborty A, Koldobskiy MA, Bello NT, Maxwell M, Potter JJ, Juluri KR, Maag D, Kim S, Huang AS, Dailey MJ, Saleh M, Snowman AM, Moran TH, Mezey E, Snyder SH. Cell. 2010; 143:897. [PubMed: 21145457] (d) Kamimura J, Wakui K, Kadowaki H, Watanabe Y, Miyake K, Harada N, Sakamoto M, Kinoshita A, Yoshiura K, Ohta T, Kishino T, Ishikawa M, Kasuga M, Fukushima Y, Niikawa N, Matsumoto N. J Hum Genet. 2004; 49:360. [PubMed: 15221640]
9. (a) Amblard M, Fehrentz JA, Martinez J, Subra G. Mol Biotechnol. 2006; 33:239. [PubMed: 16946453] (b) Sabatino G, Papini AM. Curr Opin Drug Discovery Dev. 2008; 11:762.
10. Exposure of the benzyl-protected precursor of 1 to traditional piperidine Fmoc-deprotection conditions also resulted in complete decomposition of the pyrophosphate moiety. See Supp. Scheme S2.
11. A previous method (ref 6) utilized a convergent peptide synthesis approach in which the pyrophosphoserine monomer was coupled to N- and C-terminal peptide fragments. Given the results obtained in our study, we presume this strategy can yield the desired products, although with low yield due to a significant amount of cross-reactivity and decomposition.
12. Attard TJ, O'Brien-Simpson N, Reynolds EC. Int J Pept Res Ther. 2007; 13:447.
13. (a) McMurray JS, Coleman DRT, Wang W, Campbell ML. Biopolymers. 2001; 60:3. [PubMed: 11376430] (b) Beaucage SL, Caruthers MH. Tetrahedron Lett. 1981; 22:1859.(c) Cremosnik GS, Hofer A, Jessen HJ. Angew Chem Int Ed. 201310.1002/anie.201306265
14. (a) Lohrmann R, Orgel LE. Nature. 1973; 244:418. [PubMed: 4355060] (b) Strenkowska M, Wanat P, Ziemniak M, Jemielity J, Kowalska J. Org Lett. 2012; 14:4782. [PubMed: 22966945]
15. For complete method optimization, see Supp. Tables S2, S3, S4.
16. To assess the generality of Method C, we also subjected phosphothreonine and phosphotyrosine-containing peptides (**21** and **22**) to the reaction conditions. In both cases, high conversion to pyrophosphopeptides **23** and **24**, respectively, was observed. (See Supp. Info.)
17. Peptides **10a**, **11a**, and **12a** were subjected to standard Pd black hydrogenolysis conditions. While hydrogenolysis of **10a** and **12a** proceeded as expected to peptides **14** and **15**, repeated attempts to hydrogenate the cysteine-containing peptide **11a** were unsuccessful presumably due to catalyst poisioning. Future work will focus on developing alternative hydrogenolysis procedures or changing the benzyl protecting group on the phosphorimidazolide reagent.
18. To further validate the compatibility of Methods A, B, and C with alcohols, competition experiments with serine-containing peptide **25** and N-/C-protected phosphopeptide **5** were performed. We observed 80-100% conversion of **5** to pyrophosphopeptide **13** for all methods, while serine-containing peptide **25** showed no reactivity. See Supp. Info. for experimental set-up and HPLC assay.
19. For examples of phosphate as a non-innocent, nucleophilic buffer see:(a) Westwood NJ, Schofield CJ, Claridge TDW. J Chem Soc Perk T 1. 1997; 2725(b) Gill MS, Neverov AA, Brown RS. J Org Chem. 1997; 62:7351. [PubMed: 11671851] For an example of phosphate as a nucleophilic catalyst see:(c) Prakash GKS, Vaghoo H, Panja C, Surampudi V, Kultyshev R, Mathew T, Olah GA. Proc Natl Acad Sci USA. 2007; 104:3026. [PubMed: 17360603]
20. Sawai H, Wakai H, Shimazu M. Tetrahedron Lett. 1991; 32:6905.
21. To further validate compatibility with an imidazole functional group, histidine-containing peptide **26** was synthesized. Subjecting **26** to Method C yielded the desired pyrophosphopeptide **27** in 100% conversion. (See Supp. Info.)
22. (a) Park HS, Hohn MJ, Umehara T, Guo LT, Osborne EM, Benner J, Noren CJ, Rinehart J, Soll D. Science. 2011; 333:1151. [PubMed: 21868676] (b) Heinemann IU, Rovner AJ, Aerni HR, Rogulina S, Cheng L, Olds W, Fischer JT, Soll D, Isaacs FJ, Rinehart J. Febs Lett. 2012; 586:3716. [PubMed: 22982858]

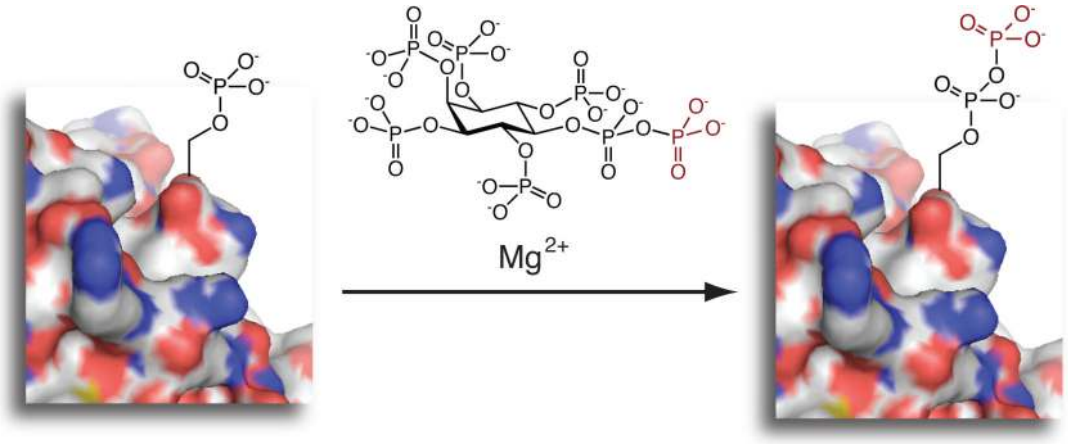


Figure 1.
Proteins are pyrophosphorylated by inositol pyrophosphate messengers in the presence of magnesium.

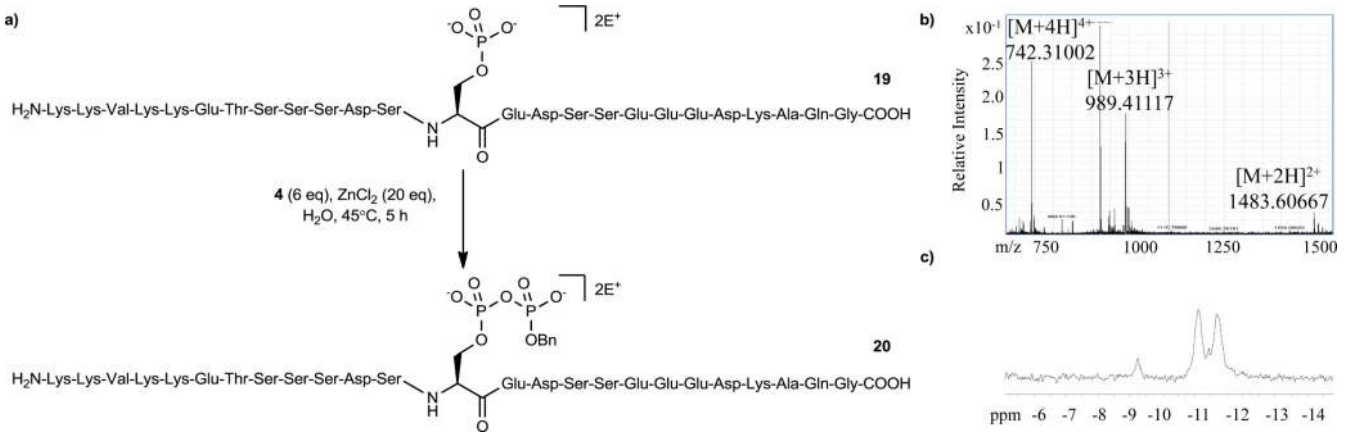
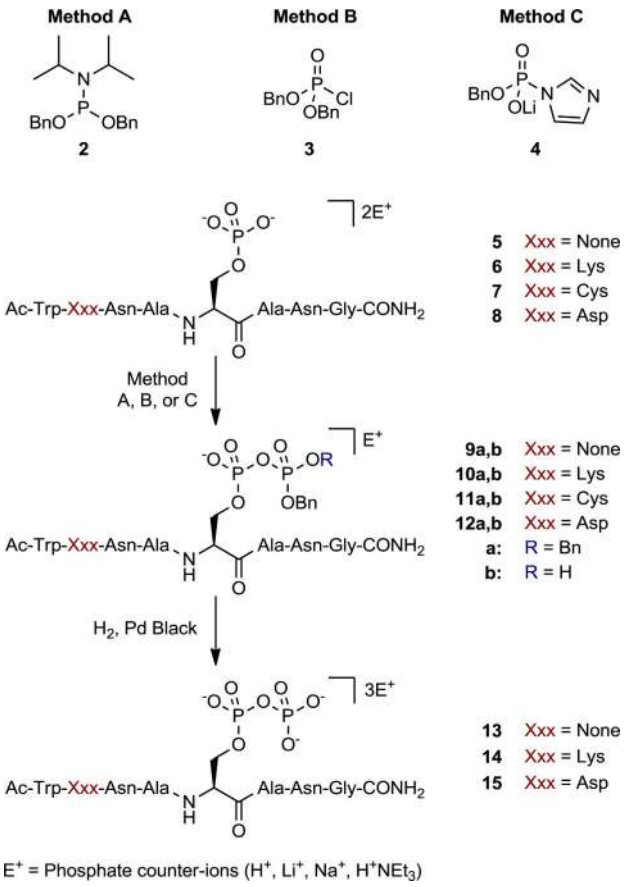


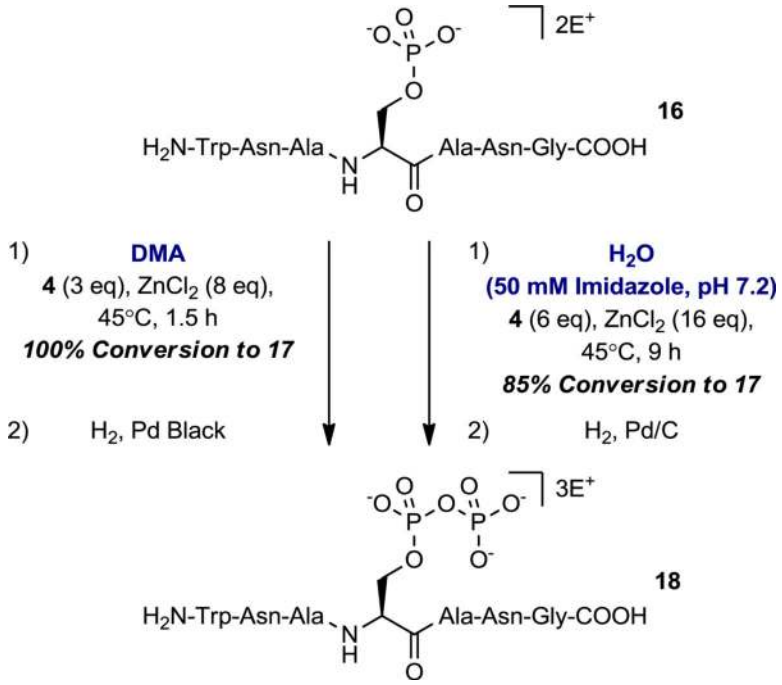
Figure 2. Chemical Pyrophosphorylation and Characterization of Highly Functionalized Nopp140 Peptide Fragment

(**a**) General reaction scheme to synthesize pyrophosphopeptide **20**. (**b**) Mass spectrometry analysis of **20**. (**c**) ^{31}P NMR spectrum of **20.**



Scheme 1.

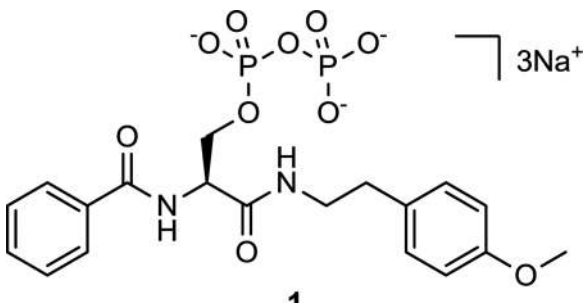
Peptide Pyrophosphorylation Using Different Electrophilic Phosphorus Reagents.



Scheme 2. Optimized Method on a Deprotected Peptide

Table 1

Stability of a Pyrophosphoserine Analog



1

Entry	Buffer	pH	*rt* % Hydrolysis[a]	*50°C* % Hydrolysis[a]
1	0.1M HCl	0.97	22.5	100.0
2	NH_4HCO_3	4.53	0.2	4.3
3	Imidazole	7.18	1.0	2.9
4	HEPES	7.22	1.2	2.6
5	TRIS HCl	7.50	2.4	3.0
6	CHES	9.30	1.0	1.7
7	0.1M NaOH	13.06	63.6[b]	100.0[b]
8	TFA Mix A[c]		25.0	N/D
9	TFA Mix B[d]		11.4	N/D

N/D = Not determined. *Standard Conditions*: 90 mM buffer, 1 mM substrate, 24 h.

[a] Normalized to time t = 0.

[b] % β-elimination reported for these entries.

[c] 95% TFA + 2.5% H_2O + 2.5% Triisopropylsilane; 3 h.

[d] 1% TFA in DCM; 3 h.

Table 2
Optimized Pyrophosphorylation Conditions on Diverse Peptide Substrates

Entry	Method	Xxx	Solvent	% Conversion (Yield)[a]
1	A	None	DMF	88 (40)[b]
2	B	None	DMA	88 (42)[b]
3	C	None	DMA	90 (54)[b]
4	A	Lys	DMF	93
5	A	Cys	DMF	18
6	A	Asp	DMF	38
7	B	Lys	DMA	0[c]
8	B	Cys	DMA	67
9	B	Asp	DMA	53
10	C	Lys	DMA	79
11	C	Cys	DMA	93
12	C	Asp	DMA	87

Reagents & Conditions: Method A: (1) **2**, NEt_3, 1-*H*-tetrazole, 16h, rt, (2) *t*BuOOH, 16h, 0°C to rt; Method B: **3**, NEt_3, 2h, 0°C to rt; Method C: **4**, $ZnCl_2$. 1.5h, 45°C.

[a] Conversion determined by HPLC assay to desired pyrophosphopeptide intermediates **9a**, **10a**, **11a**, **12a** (Method A & B) or **9b**, **10b**, **11b**, **12b** (Method C). Identification of significant byproducts discussed in Supp. Info.

[b] Two-step isolated yields of hydrogenated product **13** reported in parentheses.

[c] A presumed tri-fluoroacetylated peptide formed as the major product. See Supp. Info.