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# A Stable Pyrophosphoserine Analog for Incorporation into Peptides and Proteins

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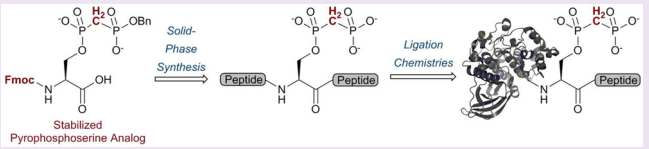
Supporting Information

**ABSTRACT:** Protein pyrophosphorylation is a covalent modification of proteins, mediated by the inositol pyrophosphate messengers. Although the inositol pyrophosphates have been linked to a range of cellular processes, the role of protein pyrophosphorylation remains minimally characterized *in vivo*. The inherent instability of the phosphoanhydride bond has hampered the development of useful bioanalytical techniques to interrogate this novel signaling mechanism. Here, we describe the preparation of a pyrophosphoserine analog containing a stable methylene-bisphosphonate group that is compatible with solid-phase peptide synthesis. The resulting peptides demonstrate enhanced stability in Eukaryotic cell lysates and mammalian plasma and display resistance toward chemical degradation, when compared to the corresponding pyrophosphopeptides. In addition, the peptides containing the stable pyrophosphoserine analog are highly compatible with common ligation methods, such as native chemical ligation, maleimide conjugation, and glutaraldehyde ligation. The bisphosphonate-containing peptides will, therefore, be well-suited for future pyrophosphoserine antibody generation and affinity capture of pyrophosphoprotein binding partners and provide a key entry point to study the regulatory role of protein pyrophosphorylation.




Protein phosphorylation is one of the most common post-translational modifications (PTMs) and is critical to almost all cellular processes.[1] In eukaryotic cells, phosphorylation occurs primarily on serine, threonine, and tyrosine residues, resulting in the formation of acid-stable phosphoesters. These phosphoesters are compatible with the generation of phospho-specific antibodies[2,3] and the development of phosphoproteomics methods,[4,5] which, in turn, has led to the discovery of thousands of phosphorylation sites.

In contrast to protein phosphorylation, protein pyrophosphorylation is a less explored modification, and tools to study this more labile PTM are lacking. Protein pyrophosphorylation is mediated by the inositol pyrophosphate messengers,[6−8] a group of molecules that can transfer the $\beta$-phosphoryl group onto prephosphorylated serine side chains, forming a diphosphate group.[9,10] Defects in the biosynthesis of inositol pyrophosphates result in impaired insulin secretion and resistance to weight gain in mice,[11,12] as well as reduced motility of cancer cells.[13] However, direct evidence for the *in vivo* involvement of protein pyrophosphorylation is missing because the intrinsic chemical properties of this PTM have impeded the development of suitable detection methods.

To enable the study of protein pyrophosphorylation, our group previously developed a two-step solution-phase method for the chemical synthesis of pyrophosphopeptides (Figure 1a).[14] These peptides have attested to the enzymatic reversibility of pyrophosphorylation[15] and have proved useful in the development of selective enrichment methods for pyrophosphopeptides.[16] The lability of the diphosphate group in a cellular environment though has made the generation of

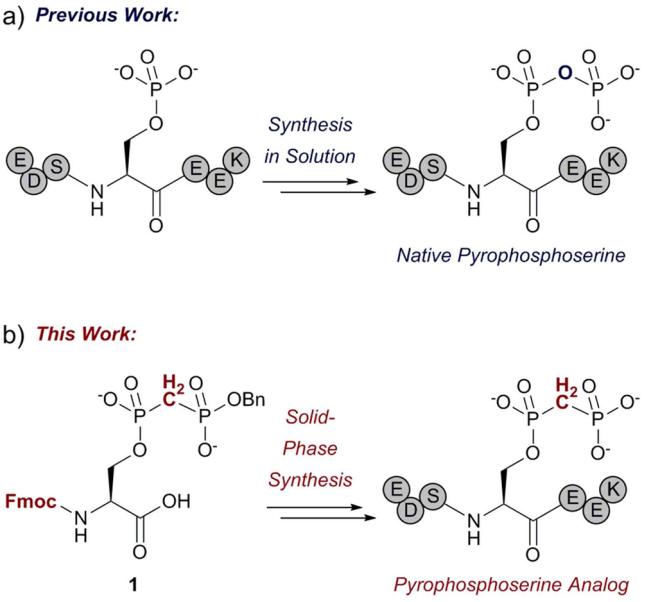



**Figure 1.** (a) Pyrophosphopeptides can be obtained in solution, using a 2-step protocol. (b) Pyrophosphoserine analog **1** can be installed during solid-phase synthesis to prepare peptides incorporating the stabilized bisphosphonate moiety.

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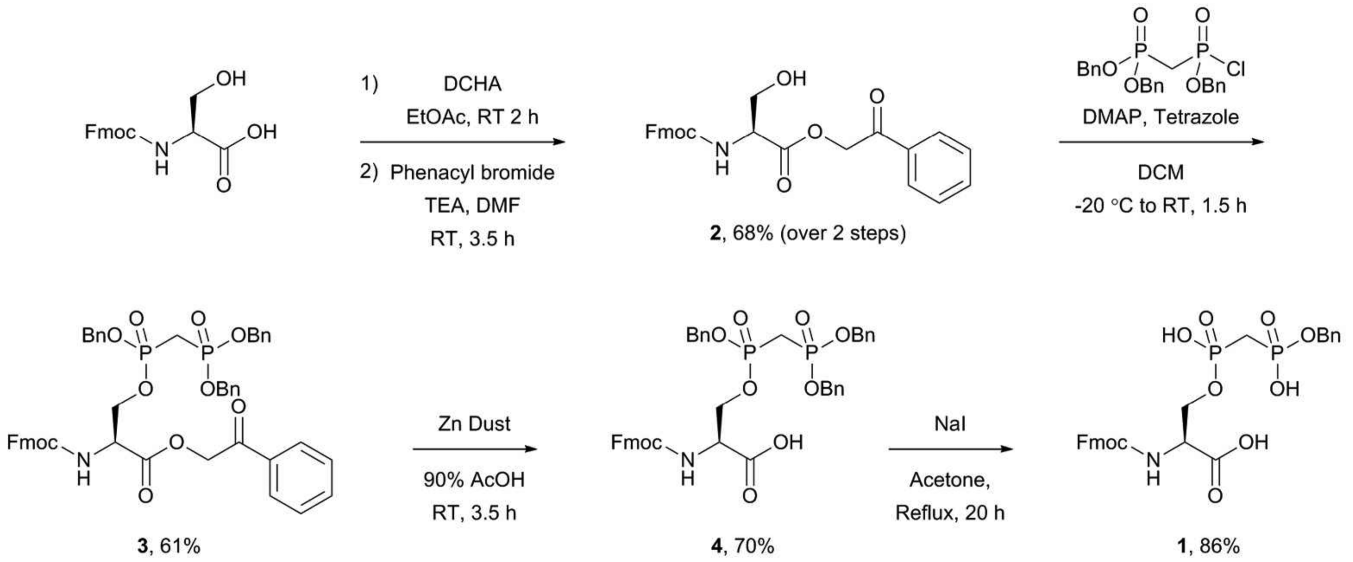
**Scheme 1. Synthesis of Pyrophosphoserine Analog 1**




Fmoc = Fluorenylmethyloxycarbonyl, DCHA = dicyclohexylamine, TEA = triethylamine, DMF = dimethylformamide, DMAP = 4-dimethylaminopyridine, Bn = benzyl.

antibodies and the identification of protein binding partners of this modification a challenging endeavor.

Historically, stable analogs of phosphorylated amino acids have facilitated the elucidation of phosphorylation-based signaling pathways. Burke and co-workers developed non-hydrolyzable phosphotyrosine mimics, phosphonomethylene-phenylalanine and phosphono-(difluoromethyl)phenylalanine, which were incorporated into peptides sequences via solid-phase synthesis.[17] These analogs, for example, then served to expose the role of tyrosine phosphorylation in the phosphatase SHP-2.[18] Additionally, a stable phosphoserine analog, phosphonomethylene alanine, was used to uncover the phosphorylation-dependent interactions of histone H3.[19] More recently, stable analogs have greatly aided the study of more labile PTMs, such as phosphoarginine (P-Arg)[20] and phosphohistidine (P-His),[21−23] by enabling the generation of antibodies against these modifications.

To expand the set of chemical tools for the study of protein pyrophosphorylation, we have developed nonhydrolyzable pyrophosphoserine analog **1** (Figure 1b). The stabilized analog can be incorporated into peptides using standard fluorenylmethyloxy-carbonyl (Fmoc)-solid phase synthesis conditions, and we subsequently demonstrate the enhanced chemical and biochemical stability of these peptides. Furthermore, the pyrophosphoserine analog proves to be compatible with traditional protein ligation chemistries, including native chemical ligation, maleimide conjugation, and glutaraldehyde ligation. The bisphosphonate-containing peptides are thus likely to find numerous applications, such as the generation of antibodies, the identification of binding partners and reader domains of pyrophosphorylated peptides, or the elucidation of the effect of pyrophosphorylation on protein structure and function.

## RESULTS AND DISCUSSION

**Synthesis of a Pyrophosphoserine Analog.** Among the stable analogs for the diphosphate group, the methylene-bisphosphonate moiety is widely used.[24,25] Replacement of the oxygen atom between the phosphorus atoms with a methylene group provides increased stability toward chemical and enzymatic hydrolysis. Despite the slightly different properties of the bisphosphonate isosters,[25,26] bisphosphonate-containing analogs have found numerous applications. For example, a nonhydrolyzable version of adenosine 5′- triphosphate has provided structural insight into the inhibitor selectivity of RIP2,[27] and methylene bisphosphonate analogs of the inositol pyrophosphates have been employed to probe the signaling mechanisms of these messenger molecules.[28,29]

Inspired by these examples, we sought to develop a scalable synthesis of a pyrophosphoserine analog incorporating a bisphosphonate group for enhanced stability (Scheme 1). To generate a variety of peptides containing the bisphosphonate functionality, the analog should be compatible with Fmoc-solid-phase peptide synthesis (SPPS). We thus pursued the synthesis of an Fmoc-protected monomer, modifying commercial Fmoc-serine on the C-terminus with phenacyl bromide to form compound **2**.[30] The bisphosphonate group was then installed using benzyl ((bis(benzyloxy)phosphoryl)methyl) phosphonochloridate and a mild base (4-dimethylaminopyridine [DMAP]/tetrazole) to afford intermediate **3**. The choice of the DMAP/tetrazole system proved to be critical, as the use of strong bases such as potassium tert-butoxide and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) resulted in significant Fmoc-deprotection,[31] while milder bases, including *N*,*N*-diisopropylethylamine (DIPEA) and 2,6-lutidine, provided poor conversion to the desired product.

Subsequently, the phenacyl group of intermediate **3** was removed in the presence of zinc dust and acid to furnish the C-terminally deprotected compound **4**. While **4** could theoretically be used in SPPS, the tribenzylated methylene bisphosphonate is likely to undergo a $\beta$-elimination during the iterative piperidine Fmoc-deprotection steps.[32] For SPPS incorporation of phosphoserine and phosphothreonine monomers, removal of one of the protecting groups increased the base stability of the phosphate ester toward $\beta$-elimination;[30,33,34] consequently, we aimed to minimize this decomposition pathway by removing at least one of the benzyl groups from compound **4**. Fortuitously, upon sodium iodide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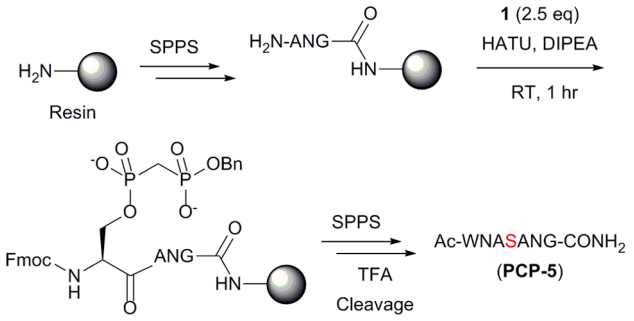
treatment, a clean precipitate of analog **1** was formed as the sole product of the reaction.[35] $^{31}P-^{1}H$ correlation NMR spectroscopy indicated that one benzyl group remained at the $\beta$-position. The presence of this benzyl protecting group should also help to decrease the nucleophilicity of the bisphosphonate moiety and thereby minimize the formation of branched side-products during solid-phase synthesis.

**Pyrophosphoserine Analog Can Be Incorporated into Peptide Sequences *via* Solid-phase Synthesis.** Next, the suitability of monomers **4** and **1** for SPPS was evaluated. Initial attempts to incorporate compound **4** on the solid phase to yield peptide **PCP-5** proved difficult, as significant byproducts were observed (Table 1, Figure S1). As anticipated, the primary side

**Table 1. Fmoc Solid-Phase Synthesis of Peptides Using Pyrophosphoserine Monomer 1**[a]




| Entry | Peptide Sequence[b] | Sequence Origin | % Isolated Yield[d] |
|---|---|---|---|
| 1 | Ac-WNASANG-CONH$_2$ (**PCP-5**) | Random | 29 |
| 2 | LIAPFSLAWA (**PCP-6**) | Random | 28 |
| 3 | Ac-KEEDSSEDSS-CONH$_2$ (**PCP-7**) | CK2 Cons. | 45 |
| 4 | Ac-CEEDSSEDSS-CONH$_2$ (**PCP-8**) | CK2 Cons. | 33 |
| 5 | SHHDDEEESSEKKK (**PCP-9**) | Rpa34 | 94[e] |
| 6 | S[P-S]EDSSEEEDK (**PCP-10**)[c] | Nopp140 | 31 |

[a]Abbreviations: HATU = 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate. DIPEA = *N*,*N*-diisopropylethylamine. [b]Unless otherwise noted, peptides are *N*-/C-terminally deprotected. Product number listed in parentheses. Red amino acid contains the methylene bisphosphonate moiety. [c][P−S] = phosphoserine. [d]Based on initial resin loading. [e]Significant amount of basic amount acids increases TFA-salt adduct formation thus inflating yield based on initial resin loading.

product was the peptidic alkene, most likely resulting from the $\beta$-elimination pathway. In contrast, when analog **1** was incorporated using standard SPPS conditions,[36] no significant byproducts were observed during the synthesis of model peptide **PCP-5** (Table 1, entry 1, Figure S2). Therefore, we selected compound **1** for subsequent peptide synthesis efforts.

While synthesizing peptide **PCP-5**, we evaluated the nature of the coupling reagent, the length of reaction time, and the fold-excess of compound **1** required for the most efficient integration. Using HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3- triazolo[4,5b] pyridinium 3-oxid hexafluorophosphate) as the coupling reagent, with 2.5 equiv of analog **1**, installation of the pyrophosphoserine mimic into **PCP-5** went to full conversion after an hour of incubation at RT. Following incorporation of monomer **1**, the synthesis of peptide **PCP-5** was accomplished using standard Fmoc-SPPS methods for phosphopeptide synthesis.[36] The peptide was then cleaved from the resin with a standard trifluoroacetic acid cocktail (95% TFA, 2.5% triisopropylsilane, 2.5% water). Importantly, TFA cleavage also removed the benzyl protecting group from the bisphosphonate moiety. Peptide **PCP-5** was then purified using reverse-phase HPLC, and the peptide's identity was confirmed by high-resolution mass spectrometry (HRMS, Table 1, entry 1). In addition, $^{31}P$ NMR spectroscopy provided a diagnostic set of signals in the 12 to 20 ppm range, corroborating the presence of the methylene bisphosphonate group.

After successful incorporation of analog **1** into peptide **PCP-5**, we investigated the compatibility of the procedure for the synthesis of alternative peptide sequences. Monomer **1** was efficiently installed into another hydrophobic peptide (**PCP-6**, Table 1, entry 2) as well as more acidic peptide sequences containing the casein-kinase 2 (CK2) consensus sequence (**PCP-7** and **PCP-8**, Table 1, entries 3−4) since pyrophosphorylation is believed to require a priming phosphorylation event by CK2.[10] Peptides **PCP-7** and **PCP-8** include a singular lysine or cysteine residue, respectively, for future bioconjugation applications. Analog **1** was also incorporated into two peptides, **PCP-9** and **PCP-10**, derived from the *in vitro* targets of protein pyrophosphorylation, Rpa34 (yeast RNA polymerase I subunit A34.5)[37] and Nopp140 (mammalian nucleolar and coiled-body phosphoprotein 1),[10] in good yields (Table 1, entries 5−6).[38,39] The identities of peptides **PCP-6−10** were confirmed by high-resolution mass spectrometry and $^{31}P$ NMR (Figure S3). Notably, the Nopp140 peptide fragment **PCP-10** also highlights the ability to access peptides containing both monophosphorylated and pyrophosphorylated (in the stabilized form) serine residues at specific sites. Since analog **1** can be installed during SPPS, it is compatible with all Fmoc-protected amino acids, thus enabling the investigation of the effect of pyrophosphorylation on peptide structure and function in the context of other PTMs, including phosphorylation.

**Bisphosphonate-containing Peptides Exhibit Superior Chemical Stability Compared to Pyrophosphopeptides.** With a set of bisphosphonate-containing peptides (PCP-peptides) in hand, we wanted to compare the chemical stability of these peptides to those bearing the native pyrophosphate moiety. To do so, a subset of PCP-peptides was treated with commonly used buffers for 24 h at 37 °C, and the levels of hydrolysis were monitored by HPLC. While the diphosphate group in peptide **PP-7** was hydrolyzed to a significant extent (10−68% hydrolysis), no hydrolysis of peptide **PCP-7** was observed under the same conditions, even in the presence of strong acid (Table 2, entries 1−5).[40] Only the addition of a strong base (0.1 M NaOH) caused decomposition of peptide **PCP-7** (Table 2, entry 6). To demonstrate that the stability of the bisphosphonate analogs was sequence independent, PCP-peptides **PCP-5** and **PCP-9** and their corresponding pyrophosphopeptides **PP-5** and **PP-9** were incubated with the buffers listed in Table 2. No hydrolysis of the methylene bisphosphonate moiety was observed (Table S3) for these two peptides, while significant hydrolysis of the diphosphate group occurred for both **PP-5** and **PP-9**, especially in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES, Table S3, 26% and 21% hydrolysis, respectively).

Given the high prevalence of metal cations in biological systems, we also investigated the stability of the pyrophosphoserine analog in the presence of biologically relevant Lewis-acidic metal cations. Since pyrophosphorylation sites tend to localize to highly acidic stretches, we were most interested in


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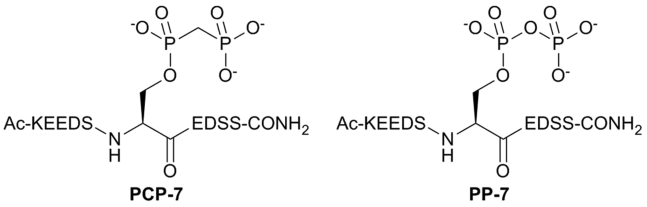
**Table 2. Stability of PCP-7 Compared to PP-7 in Various Buffer Conditions and in the Presence of Divalent Metal Cations**[a]




| Entry | Buffer/Metal | pH | % Hydrolysis[a] PCP-7 | % Hydrolysis[a] PP-7 |
|---|---|---|---|---|
| 1 | 0.1 M HCl | 0.89 | 0 | 14 |
| 2 | 50 mM HEPES | 7.10 | 0 | 68 |
| 3 | 50 mM Imidazole | 7.23 | 0 | 10 |
| 4 | 50 mM Tris | 7.52 | 0 | 29 |
| 5 | 50 mM MOPS | 7.89 | 0 | 11 |
| 6 | 0.1 M NaOH | 13.1 | decomp.[b] | decomp.[b] |
| 7 | 200 μM $Mg^{2+}$ | 7.89 | 0 | 1.0 |
| 8 | 200 μM $Zn^{2+}$ | 7.89 | 0 | 10 |
| 9 | 200 μM $Fe^{2+}$ | 7.89 | 0 | 13 |
| 10 | 200 μM $Mn^{2+}$ | 7.89 | 0 | 0 |
| 11 | 200 μM $Ca^{2+}$ | 7.89 | 0 | 8.0 |
| 12 | 200 μM $Co^{2+}$ | 7.89 | 0 | 0 |
| 13 | 200 μM $Cu^{2+}$ | 7.89 | 0 | 0 |

[a]Abbreviations: HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, MOPS = 3-(*N*-morpholino) propanesulfonic acid. Conditions: Entries 1−6:50−100 mM buffer, 100 μM peptide, 37 °C, 24 h. Entries 7−13:50 mM MOPS, pH 7.89, 100 μM peptide, 200 μM metal chloride salt, 37 °C, 24 h. [b]Percent hydrolysis measured by analytical HPLC, normalized to time *t* = 0 min. [c]decomp. = decomposition through a proposed β-elimination pathway.

determining the stability of the acidic peptides **PCP-7** and **PP-7** in the presence of a number of divalent metal cations (Table 2). None of the metals investigated caused hydrolysis of **PCP-7** (Table 2, entries 6−13) while the corresponding pyrophosphopeptide **PP-7** was hydrolyzed [$Ca^{2+}$ (8.4%), $Zn^{2+}$ (10%), and $Fe^{2+}$ (13%)]. Other bisphosphonate-containing peptides, **PCP-9** and **PCP-5**, also exhibited high stability when incubated with divalent metal cations, while some hydrolysis of the diphosphate moiety in **PP-9** and **PP-5** was observed once again in the presence of $Ca^{2+}$ and $Fe^{2+}$ ions (Table S3, 4.5% and 15% hydrolysis for **PP-9** and 16% and 6.4% for **PP-5**, respectively). Overall, the pyrophosphoserine analog showed improved stability in most buffer conditions and in the presence of divalent metal cations when compared to the corresponding pyrophosphoserine-containing peptides, regardless of the surrounding peptide sequence.

**Bisphosphonate-containing Peptides Display Enhanced Stability in Cell Lysates and Plasma.** Ultimately, we envision the utilization of the pyrophosphopeptide analogs for raising antibodies against this novel modification, for identifying "reader domains" of specific pyrophosphopeptide sequences, and for isolating enzymes involved in the dynamic regulation of this signaling mark. It was therefore important to establish the stability of the bisphosphonate-containing peptides in the presence of cell lysate. **PCP-5** and the corresponding pyrophosphopeptide **PP-5** were treated with a *S. cerevisiae* lysate (50 mM Tris, pH 7.5, 37 °C; Figure 2a), and time points were taken over the course of 24 h to monitor the hydrolysis to peptide **5** by analytical HPLC. Consistent with our previous work,[14] we observed significant enzyme-mediated hydrolysis (54%) of the pyrophosphate moiety of peptide **PP-5** in a *S. cerevisiae* lysate after 24 h of incubation. In comparison, the level of hydrolysis of **PCP-5** was substantially decreased (24% hydrolysis after 24 h incubation Figure 2b). When **PCP-5** was incubated with a heat denatured lysate, no hydrolysis of the bisphosphonate moiety occurred (Figure S4), demonstrating

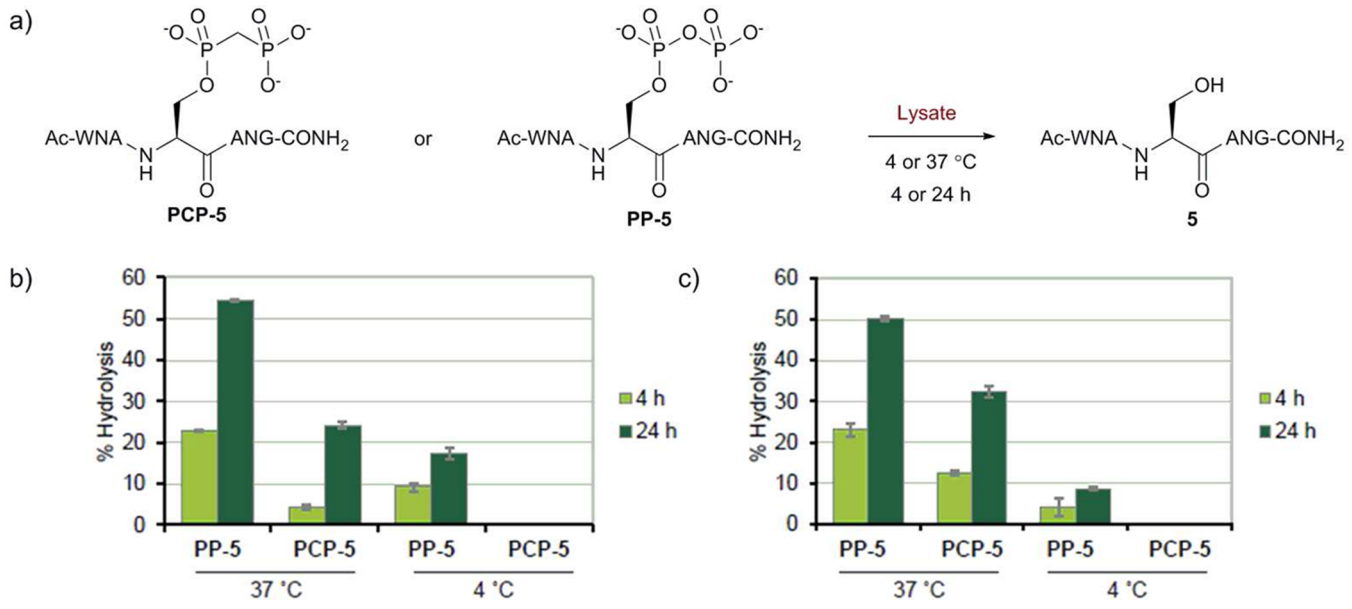



**Figure 2.** (a) General reaction scheme for lysate stability studies of **PCP-5** and **PP-5**. (b) Stability of peptides **PCP-5** and **PP-5** in a yeast cell lysate at 37 and 4 °C, illustrating the high degree of stability of **PCP-5** under these conditions. (c) The levels of hydrolysis of **PCP-5** are reduced compared to pyrophosphopeptide **PP-5** at 37 and 4 °C in the presence of a HeLa cell lysate. Error bars represent the standard deviation for triplicate experiments.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that the removal of the bisphosphonate group, similar to the pyrophosphate moiety, occurred enzymatically.

Affinity-enrichment procedures, such as the isolation of phosphoprotein interacting partners are typically carried out at lower temperatures.[41,42] To emulate such experiments, **PCP-5** and **PP-5** were incubated with a *S. cerevisiae* lysate at 4 °C (50 mM Tris, pH 7.5), and the reactions were monitored over the course of 24 h. Throughout the full time course, no degradation of **PCP-5** was observed, while the pyrophosphate moiety of **PP-5** was noticeably hydrolyzed (17% after 24 h) under these conditions (Figure 2b). These results emphasize the stability of the pyrophosphoserine analog under standard enrichment conditions and support its use in generating affinity reagents to identify new pyrophosphatases as well as protein binding partners of peptides containing this modification.

Next, we wanted to establish the stability of PCP-peptides in mammalian cell lysate and in plasma, which is an important determinant in their applicability as antigens. **PCP-5** and **PP-5** were treated with a HeLa cell lysate (50 mM Tris, pH 7.5, 37 °C; Figure 2c), and time points were taken at 4 and 24 h to monitor hydrolysis. Prominent hydrolysis of the pyrophosphate moiety of peptide **PP-5** (50% hydrolysis after 24 h incubation) was observed, while the level of hydrolysis of **PCP-5** was moderately decreased (32% hydrolysis after 24 h incubation). Although some hydrolysis of **PCP-5** occurred at 37 °C in a HeLa cell lysate, the bisphosphonate group was stable when it was exposed for 24 h to the mammalian lysate at 4 °C (Figure 2c), mirroring the results from the yeast lysate experiments. Finally, **PCP-5** was treated with human plasma serum at 37 °C for 4 to 48 h, and hydrolysis was monitored by $^{31}$P NMR. Remarkably, no degradation of the methylene-bisphosphate moiety of **PCP-5** was detected even after 48 h of incubation (Figure S5). These results support the future use of the stabilized analogs for preparation of antibodies against pyrophosphoserine from mammalian hosts.

**PCP-Peptides Tolerate Common Ligation Procedures.** A particularly useful application of stabilized analogs is to produce antibodies against a specific peptide sequence which incorporates the labile modification. While peptides serve as good recognition sites for the antibody, they themselves cannot elicit an immune response, and the ability to conjugate synthetic peptides to carrier proteins is vital. The most common methods[43] for generating hapten-carrier conjugates include native chemical ligation,[44,45] maleimide conjugation,[46] and glutaraldehyde ligation.[46] We therefore evaluated the behavior of peptides containing the bisphosphonate moiety under these ligation conditions motivated by our long-term goal to obtain pan-specific antibodies against pyrophosphoserine.

Native chemical ligation (NCL) enables a site-selective ligation of peptides to proteins between a C-terminal thioester and an *N*-terminal cysteine to yield a native peptide bond. As proof of principle, we targeted a stretch of Nopp140 (amino acids 89−106), which contains one of the proposed pyrophosphorylation sites (Ser-91)[10] for preparation by NCL. The sequence was split into two 9-mers at Ala-98, which was changed to a cysteine residue required for the ligation reaction. Both 9-mer peptides (**11** and **12**) were prepared by solid-phase synthesis, and pyrophosphoserine analog **1** was incorporated into peptide **11**, which can be converted into the requisite thioester *in situ*.[47] Peptides **11** and **12** were ligated following standard NCL conditions to provide 18-mer product **13** (89−106, A98C) in good yield over two steps (29%, Figure 3a), illustrating that the stable analog is well compatible with the NCL conditions (Figure S6). To remove the "unnatural" cysteine residue in the peptide sequence, a free radical desulfurization was conducted using a water-soluble radical initiator (V-50) with tris(2-carboxyethyl)phosphine (TCEP) and glutathione as the reducing agent and hydrogen source, respectively.[48] We were pleased to isolate the desired native Nopp140 sequence (product **14**, AA 89−106) with high conversion (Figure 3a). Characterization of product **14** by mass spectrometry and $^{31}$P NMR spectroscopy showed no signs of decomposition of the bisphosphonate group after exposure to both the NCL and the desulfurization reaction conditions (Figure 3b). Given the success of the NCL/desulfurization sequence at the peptide level, one could imagine utilizing this strategy to prepare fusion proteins containing this modification to study the effect on protein structure and function.

Next, we wanted to explore the conjugation of PCP-peptides onto full-length proteins, specifically known carrier proteins, using conventional conjugation techniques. We chose commercially available maleimide-activated bovine serum albumin (M.A.-BSA)[49] as our model carrier protein to ligate the PCP-peptides, relying on an *N*-terminal cysteine residue (**PCP-8**) for maleimide conjugation or an *N*-terminal lysine residue (**PCP-7**) for glutaraldehyde ligation. To monitor these ligation reactions, we needed to establish an assay to confirm the integrity of the bisphosphonate group upon ligation. Our lab recently developed a ratiometric fluorescent sensor, which could be used as an SDS-PAGE gel stain to detect pyrophosphorylated peptides and proteins.[50] We reasoned that this sensor could also recognize the bisphosphonate moiety, considering its structural similarity to the native pyrophosphoserine. To confirm this hypothesis, **PCP-5**, **PCP-7**, and **PCP-9** and the corresponding pyrophosphopeptides **PP-5**, **PP-7**, and **PP-9** were treated with zinc complex **15** in solution (Figure S8). The fluorescence ratios of the PCP-peptides ($F_{370}/F_{355}$ = 0.76, 0.80, 0.65, for **PCP-5**, **PCP-7**, and **PCP-9**, respectively) were comparable to those of the analogous pyrophosphopeptides ($F_{370}/F_{355}$ = 0.67, 0.62, 0.55, for **PP-5**, **PP-7**, and **PP-9**, respectively),[51] illustrating that complex **15** could indeed be utilized to detect the bisphosphonate moiety after ligation to a protein.

Subsequently, maleimide-activated BSA was treated with excess **PCP-8** in phosphate buffer (pH 7.3) at RT for 2 h (Figure 3c). To monitor for potential degradation of the analog during conjugation, the corresponding dephosphorylated peptide **8** was prepared by solid-phase synthesis and ligated onto M.A.-BSA following the same procedure. The resulting peptide-carrier conjugates were visualized on an SDS-PAGE gel using Coomassie-G blue stain to confirm successful ligations based on the upward mobility shift of the ligation products (Figure 3d). Staining of the gel with compound **15** revealed strong fluorescence for BSA-**PCP-8**, but not for BSA-**8**, which indicated that the bisphosphonate moiety withstood the ligation conditions (Figure 3d).

To confirm that the PCP-peptides were also compatible with other ligation procedures, **PCP-7** and an alternative protein, glutathione S-transferase (GST), were incubated with glutaraldehyde for an amine-to-amine cross-linking reaction. As with the previous ligation reactions, staining with Coomassie-G blue and fluorescent dye **15** of the SDS-PAGE gel showed that the ligation reactions were successful and that the bisphosphonate group tolerated the glutaraldehyde conjugation conditions (Figure S9).[52]


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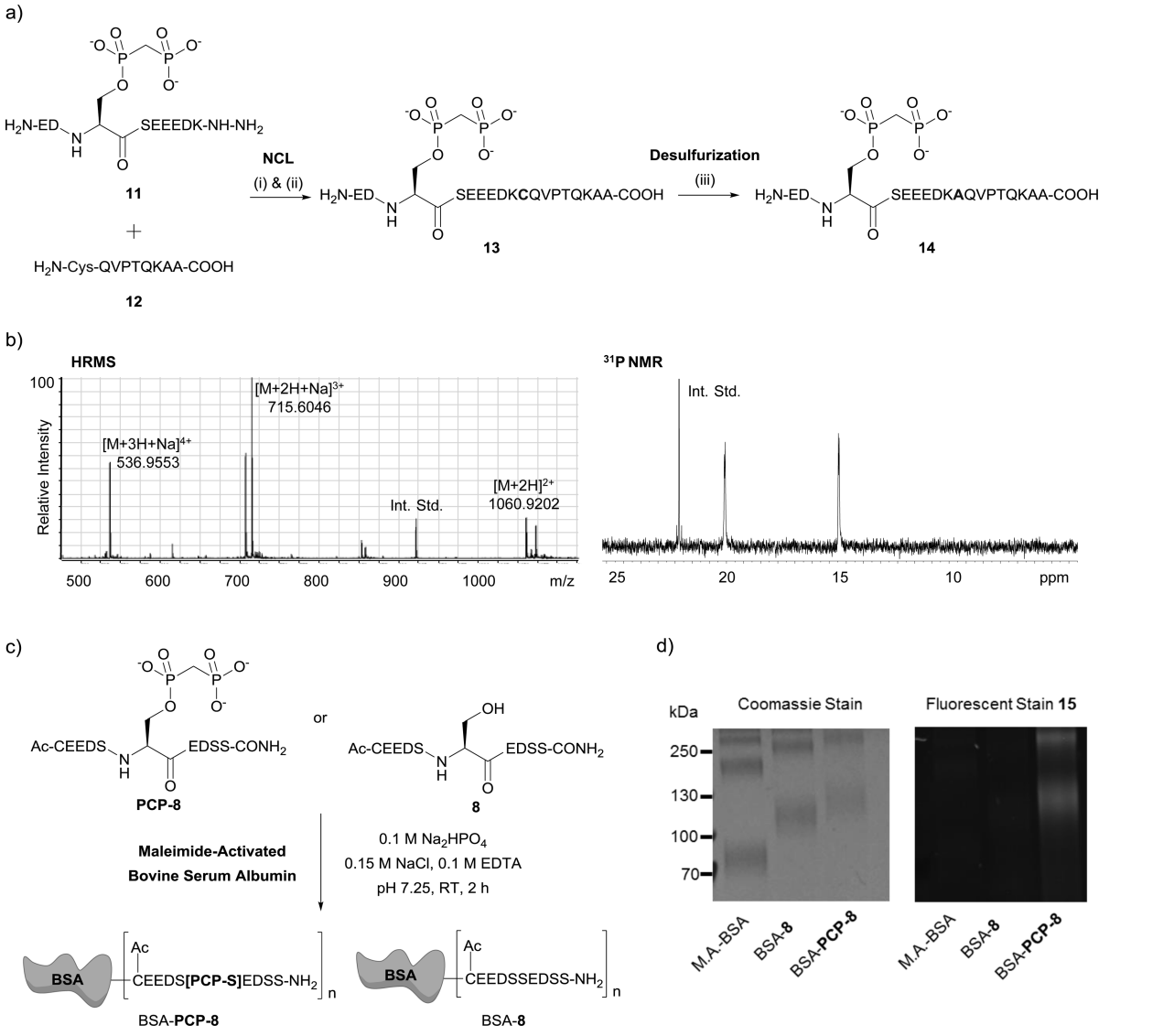


**Figure 3.** (a) Native chemical ligation (NCL) between PCP-peptide-hydrazide **11** and *N*-terminal cysteine peptide **12** to yield **13**, a cysteine-containing version of a Nopp140 fragment (amino acids 89−106, A98C) followed by free radical desulfurization to afford peptide **14**, the native Nopp140 fragment (AA 89−106). Conditions: (i) 45 mM $NaNO_2$, 6 M guanidine hydrochloride (GnHCl), 0.2 M sodium phosphate, pH 3.10, −10 °C, 20 min. (ii) 0.1 M 4-mercaptophenylacetic acid, 6 M GnHCl, 0.2 M sodium phosphate, pH 6.97, RT, 1.5 h. (iii) 50 mM 2,2′-azobis(2-methylpropionamidine)dihydrochloride (V-50), 40 mM glutathione, 250 mM tris(2-carboxyethyl)phosphine (TCEP), 6 M GnHCl, 0.2 M sodium phosphate, pH 7.40, 37 °C, 3.5 h. (b) High-resolution mass spectrometry and $^{31}P$ NMR characterization of ligated and desulfurized product **14**. HRMS Int. Std. = Hexakis(1H, 1H, 3H - tetrafluoropropoxy)phosphazine and $^{31}P$ NMR Int. Std = tetramethylphosphonium bromide. (c) Maleimide ligation of peptides **PCP-8** and **8** with maleimide-activated bovine serum albumin (M.A.-BSA) yielded BSA-peptide conjugates, BSA-**PCP-8** and BSA-**8**. (d) Commassie-G staining (left) and staining with fluorescent dye **15** (right) of SDS-PAGE gel (10% Bis Tris, XT-MES buffer) show successful ligation and integrity of bisphosphonate moiety, respectively. Gel was visualized at 365 nm excitation for fluorescence. The appearance of multiple bands and the broadness of the bands are observed for the maleimide-BSA (M.A.-BSA) starting material and the ligation products and can be attributed to the intra- and intermolecular reaction of cysteine residues on BSA with the maleimide functionality (for more information see Figure S7).

Overall, the peptides containing the pyrophosphoserine analog could undergo a range of ligation reactions. The compatibility of peptides with these different chemical conditions opens the door for numerous bioanalytical applications. These applications include conjugation of bisphosphonate-containing peptides to carrier proteins for pyrophosphoserine antibody generation, as well as biophysical investigation of this modification on full-length proteins via site-specific incorporation of the bisphosphonate group by NCL.

**Conclusions.** To date, protein pyrophosphorylation is a marginally characterized PTM. The *in vivo* relevance of protein pyrophosphorylation remains a precarious subject because the tools and reagents for the study of this labile modification are scarce. To promote the study of protein pyrophosphorylation, we have prepared a stabilized, nonhydrolyzable pyrophosphoserine analog. Analog **1** is readily incorporated into peptide sequences, using standard Fmoc-SPPS conditions. The bisphosphonate-containing peptides exhibit superior chemical, cell lysate, and plasma stability compared to the native diphosphate group. Consequently, the availability of the


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pyrophosphoserine analog will facilitate the affinity purification of endogenous pyrophosphatases and protein binding partners of this modification. Additionally, we demonstrated that the bisphosphonate-containing peptides are well compatible with common ligation strategies. These techniques enable the ligation of the pyrophosphoserine analogs onto carrier proteins, for the subsequent development of antibodies. Furthermore, the bisphosphonate-containing peptides could be activated as C-terminal thioesters, to participate in an NCL-desulfurization reaction sequence. The peptides can therefore be used in the future to site-specifically incorporate the stabilized pyrophosphoserine functionality into full-length proteins. These synthetic proteins, in turn, can then serve for the biophysical characterization of the effect of pyrophosphorylation on protein structure and function. Overall, the peptides containing stable pyrophosphoserine analog **1** described here will enable a range of bioanalytical applications to provide pivotal insights into the physiological role of protein pyrophosphorylation and its involvement in complex signaling networks.

## METHODS

Chemical syntheses of pyrophosphoserine analog **1**, intermediates **2**–**4**, phosphopeptides, pyrophosphopeptides, and PCP-peptides are described in detail in the Supporting Information. Assays for the chemical, lysate, and plasma stability studies of PCP-peptides and protocols for native chemical ligation, maleimide conjugation, glutaraldehyde ligation, in-solution fluorescence studies, and gel staining with dye **15** are also described in the Supporting Information.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00972.

Experimental procedures, spectroscopic data, and supporting figures and tables (PDF)

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

PTM, post-translational modification; SPPS, solid-phase peptide synthesis; Fmoc, fluorenylmethyloxy-carbonyl; TFA, trifluoroacetic acid; CK2 cons, casein kinase 2 consensus site; HPLC, high performance liquid chromatography; HRMS, high-resolution mass spectrometry; $^{31}$P NMR, phosphorus-31 nuclear magnetic resonance spectroscopy; PCP-peptide, bisphosphonate-containing-peptide; NCL, native chemical ligation; BSA, bovine serum albumin; GST, glutathione S-transferase

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(39) Isolated yields of PCP-peptides containing stable analog **1** are comparable to the yields of the corresponding phosphopeptides. See Table S1 for phosphopeptide **P-5**, **P-7**, and **P-9** isolated yields.
(40) The stability of the methylene-bisphosphonate moiety in trifluoroacetic acid (TFA) peptide cleavage solutions was also evaluated. See Table S2 for the stability comparison of peptides **PCP-7** and **PP-7** in various TFA cleavage solutions.
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(52) In addition, ligations of the dephosphorylated peptide **7** and **PP-7** were also successful. The similar staining intensities observed for ligated peptides **PCP-7** and **PP-7** demonstrated the similarities of the chemical properties of the pyrophospho and bisphosphonate moieties upon ligation (Figure S9).


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