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Development of Stable Phosphohistidine Analogues

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Abstract: Protein phosphorylation is one of the most common and extensively studied posttranslational modifications (PTMs). Compared to the *O*-phosphorylation of Ser, Thr, and Tyr residues, our understanding of histidine phosphorylation is relatively limited, particularly in higher eukaryotes, due to technical difficulties stemming from the intrinsic instability and isomerism of phosphohistidine (pHis). We report the design and synthesis of stable and nonisomerizable pHis analogues. These pHis analogues were successfully utilized in solid-phase peptide synthesis and semi-synthesis of histone H4. Significantly, the first antibody that specifically recognizes pHis was obtained using the synthetic peptide as the immunogen.

Protein phosphorylation is one of the most common and extensively studied posttranslational modifications (PTMs).[1] Phosphorylation and dephosphorylation of proteins control the function of the target protein, and their misregulation has been linked to numerous human diseases. Accordingly, protein kinases and phosphatases have emerged as important drug targets, boosted by the prominent success of Gleevec, a tyrosine kinase inhibitor used to treat leukemia and other malignancies.[2,3]

While most research on protein phosphorylation has focused on the *O*-phosphorylation of Ser, Thr, and Tyr residues, histidine can also be phosphorylated at its imidazole nitrogens. The role of histidine phosphorylation is well documented in bacterial two-component signaling pathways;[4] however, proteins with phosphohistidine (pHis) residues are also found in eukaryotic cells.[5,6] In the case of *Physarum polycephalum*, for instance, pHis accounts for 6% of the total phosphoamino acids in its basic nuclear proteins.[7] The prevalence of pHis is strikingly high among these proteins, considering that phosphotyrosine (pTyr) is found in less than 1% of eukaryotic cellular phosphoproteins.[8] We are just beginning to appreciate that histidine phosphorylation plays an important role in the signaling processes of higher eukaryotes.[9] The modification has been implicated in several processes in recent years, including G-protein signaling,[10] ion conduction,[11] secondary metabolism,[12] and chromatin biology.[13]

Despite these discoveries, the intrinsic chemical properties of pHis and the dearth of adequate research tools have hindered further understanding of this PTM. In contrast to the phosphate ester in *O*-phosphorylated amino acids, the high-energy phosphoramide in pHis undergoes facile hydrolysis under acidic conditions (Figure 1a), making its detection and isolation from biological sources difficult. For example, there are no specific antibodies against pHis-containing proteins since immunogens with pHis will be dephosphorylated in the serum. Thus, amino acid analysis and mass spectrometry are currently the only options for the detection of pHis.[14]

To further complicate matters, pHis has two isomeric forms, 3-phosphohistidine (3-pHis, **2**) and 1-phosphohistidine (1-pHis, **3**),[15] both of which have been found *in vivo*.[14] While chemical phosphorylation of proteins and peptides can be selectively carried out on histidine residues, it is very difficult to obtain the 1-pHis form regioselectively since 1-pHis isomerizes to the more thermodynamically stable 3-pHis under the reaction conditions.[14]

We envisioned that nonhydrolyzable and nonisomerizable analogues of both pHis isomers would provide a solution to the aforementioned problems associated with this elusive PTM. Such analogues should be useful in the development of pHis antibodies and the preparation of semi-synthetic pHis-containing proteins to further investigate the physiological functions of histidine phosphorylation.

With these goals in mind, we designed phosphoryltriazolylalanine (pTza) isomers **4** and **7** (Figure 1b) as stable analogues of 3-pHis and 1-pHis, respectively. On the basis of molecular modeling studies (see Supporting Information [SI]), these analogues are expected to mimic the geometry and electronics of pHis. Importantly, the hydrolytically labile N−P bond is replaced with a nonhydrolyzable C−P bond.[16] In addition, we designed both 1- and 3-pHis analogues as tools to differentiate the biological functions of each isomer.

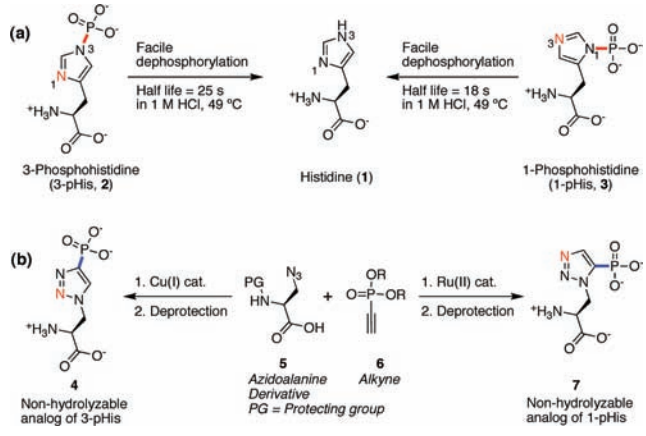

Figure 1. (a) Structures of 3- and 1-pHis. (b) Design of pHis analogues. Nitrogen atoms in red (H-bond acceptors) are conserved in the pHis analogues. Hydrolytically labile N−P bonds (red) in pHis are replaced with stable C−P bonds (blue). Both analogues can be prepared from the same building blocks by using different catalysts.

Our pHis analogue design also took synthetic practicality into consideration since these analogues were to be used in solid-phase peptide synthesis (SPPS), which generally requires excess amounts of reagents. Lengthy and inefficient syntheses of pHis analogues

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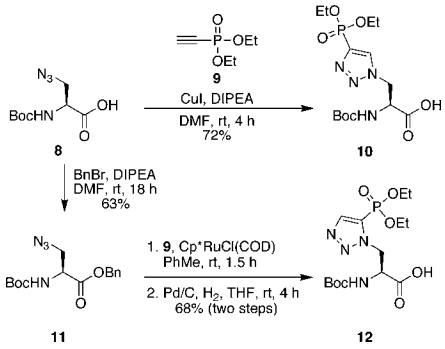
would render the subsequent SPPS impractical. In principle, pTza isomers **4** and **7** can be readily prepared by metal-catalyzed cycloaddition reactions between an azidoalanine derivative (**5**) and an alkyne (**6**). Due to the mild reaction conditions and functional group tolerance, the "click" reaction has found a variety of applications in bioconjugation and amino acid chemistry.[17] Significantly, either analogue can be obtained from the same building blocks simply by employing copper or ruthenium catalysts, allowing divergent syntheses of both isomeric pHis analogues (Figure 1b).[18]

To our delight, the Cu-catalyzed cycloaddition between Boc-azidoalanine (**8**)[19] and diethyl ethynylphosphonate (**9**)[20] provided the desired pTza derivative **10** in good yield (Scheme 1).[21] For the Ru-catalyzed cycloaddition,[18,22] it was necessary to protect the carboxylic acid functionality as a benzyl ester. Simple debenzylation after the cycloaddition provided the pTza derivative **12**. Both starting materials **8** and **9** are accessible in gram scale in one or two steps from commercial materials, and this concise route allowed preparative-scale synthesis of the protected pTza isomers **10** and **12** to be used in the subsequent SPPS.

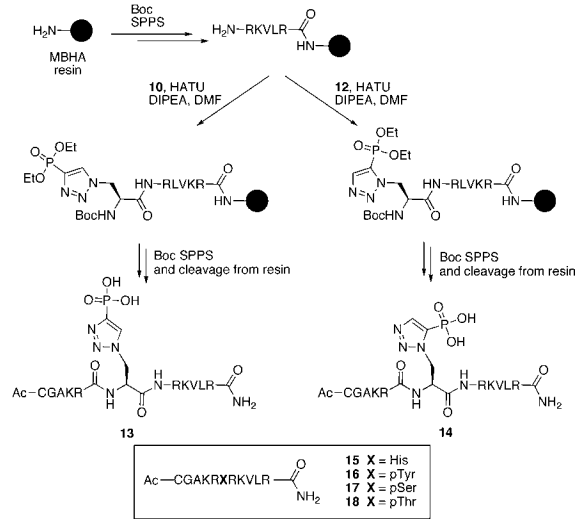
Scheme 1. Synthesis of Protected pTza Isomers



With building blocks **10** and **12** in hand, the synthesis of phospho-peptides containing these amino acids was the next goal. The N-terminal tail of histone H4 was chosen as our initial synthetic target. Phosphorylation of histidine residues 18 and 75 in histone H4 has been correlated with increased DNA synthesis *in vivo*, and both 1-pHis and 3-pHis have been detected.[13a−c] Significantly, histone H4 histidine kinase (HHK) activity is upregulated by 200-fold in human hepatocellular carcinomas compared to normal liver tissues.[13d] Peptides incorporating stable pHis analogues could be used as tools to study histone histidine phosphorylation. Accordingly, peptides **13** and **14** were designed that correspond to the N-terminal tail of human histone H4 (residues 14−23) and contain either the stable 1-pHis (**14**) or 3-pHis (**13**) analogue in place of His18. Both peptides were synthesized according to standard Boc-SPPS methods (Scheme 2).[23] Following the cleavage from the resin with HF and RP-HPLC purification, both peptides were successfully obtained in multimilligram amounts, demonstrating the compatibility of our pHis analogues **10** and **12** with SPPS.

We reasoned that one of the most important applications of our pHis analogues would be in the generation of antibodies that can selectively recognize pHis in phosphoproteins. Therefore, peptide **13** was employed as the immunogen to obtain rabbit polyclonal antibodies.[24] It was hoped that such antibodies would cross-react with a native 3-pHis moiety in the H4 tail, thereby validating pTza as a mimetic of pHis. The crude sera obtained from inoculated animals were screened using peptide dot blot assays and the most promising serum was purified via affinity depletion using a nonmodified H4 peptide (**15**) to afford the polyclonal antibody **Ab-3pHis**.

Scheme 2. SPPS of Histone H4 Tail Peptides with the pHis Analogues[a]



[a] Inset: control peptides used in dot blot assays.

To determine the specificity of **Ab-3pHis**, we carried out peptide dot blot assays with a series of histone H4 tail peptides harboring the two pTza isomers (**13**, **14**) as well as His (**15**), pTyr (**16**), pSer (**17**), or pThr (**18**) (Figure 2a). Gratifyingly, **Ab-3pHis** only recognizes **13** but not **14**−**18**, demonstrating no cross-reactivity toward His or other phosphoamino acid residues.

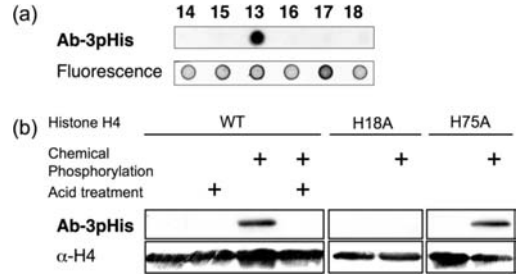


Figure 2. (a) Peptide dot blot using **Ab-3pHis**. The indicated peptides were labeled with 5-iodoacetamidofluorescein and spotted on a PVDF membrane (50 pmol each). To check for equal loading, the fluorescence of the same membrane was recorded at 520 nm upon excitation at 490 nm. (b) Western blots of recombinant histone H4 and mutants using **Ab-3pHis**. For the loading control, the initial blot with **Ab-3pHis** was stripped and reprobed with an α-H4 antibody.

To validate the cross-reactivity of **Ab-3pHis** toward the phosphorylated full-length histone H4, recombinant histone H4 protein was chemically phosphorylated on histidines in 3-pHis form, following well-established protocols.[25] Western blot analysis clearly shows that **Ab-3pHis** selectively recognizes the histidine-phosphorylated histone H4, but not the nonphosphorylated counterpart (Figure 2b). Acid treatment of the phosphorylated histone abolishes this antibody recognition, showing that phosphorylation is indeed at histidine. Since histone H4 has two histidines (His18 and His75), we also tested mutant histones lacking each histidine. Experiments using these mutants show that **Ab-3pHis** selectively recognizes the phosphorylation of His18 residue, as per our design. Notably, **Ab-3pHis** was also specific for histidine-phosphorylated histone H4 in the presence of a mammalian cell lysate (Figure S5, SI). To the best of our knowledge, this constitutes the first example of an antibody that selectively recognizes a pHis-containing protein.

We next utilized pHis analogue, **10**, in the SPPS of an α-thioester peptide, an essential component in preparing proteins *via* native

chemical ligation.[26] Proteins harboring our pHis analogues will be invaluable in investigating the functional role of this PTM. Peptide α-thioester **19**, corresponding to H4 residues 1−22 and containing pTza isomer **4**, was successfully synthesized following standard protocols.[27]

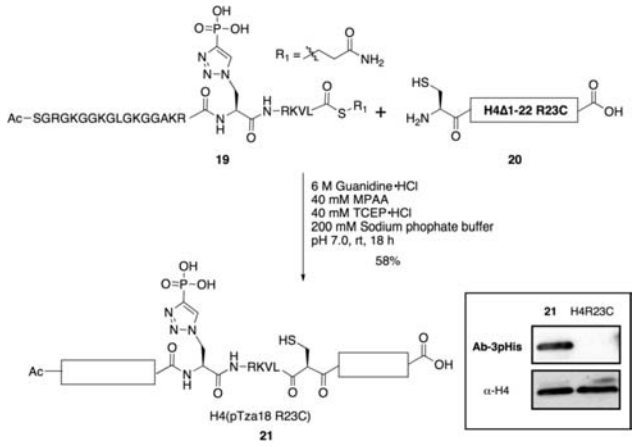


Figure 3. Semi-synthesis of full-length histone H4 containing the 3-pHis analogue. Inset: Western blot showing that **Ab-3pHis** recognizes pTza-containing H4 protein **21** but not unmodified H4 control.

With the synthetic α-thioester peptide **19** in hand, we proceeded to the semi-synthesis of full-length histone H4. Since histone H4 does not have a native Cys, a recombinant fragment of the protein with an N-terminal Cys (H4Δ1−22 R23C, **17**) was chosen as the ligation partner (Figure 3). This mutant H4 fragment has been successfully used in native chemical ligation to prepare histone H4−K16Ac, which was essential in elucidating the biological role of the lysine acetylation.[28] Native chemical ligation between thioester **19** and recombinant H4 fragment **20** proceeded smoothly, affording the desired full-length histone H4 (**21**) in milligram quantities following purification. Western blot analysis showed that **Ab-3pHis** selectively recognizes **21** over the H4R23C mutant, again corroborating the specificity of the antibody (Figure 3).

In summary, we reported herein the design and synthesis of pTza derivatives as stable pHis analogues. We also demonstrated the application of these analogues in solid-phase peptide synthesis and protein semi-synthesis. Significantly, the first antibodies specific to a pHis-containing protein were developed as a proof of concept. With these novel tools, we hope to deepen our understanding of histidine phosphorylation, particularly in eukaryotic systems. Further studies in this direction are under way in our laboratory.

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Supporting Information Available: Synthetic methods and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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