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A Second-Generation Phosphohistidine Analog for Production of Phosphohistidine Antibodies

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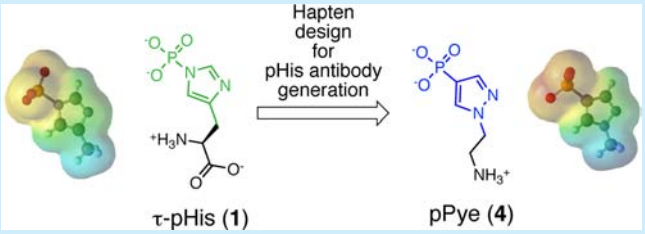
Supporting Information

ABSTRACT: Protein histidine phosphorylation plays a crucial role in cell signaling and central metabolism. However, its detailed functions remain elusive due to technical challenges in detecting and isolating proteins bearing phosphohistidine (pHis), a labile posttranslational modification (PTM). To address this issue, we previously developed the first pHis-specific antibodies using stable, synthetic triazole-based pHis analogs. A second-generation, pyrazole-based pHis analog that enabled the development of a pan-pHis antibody with much improved pHis specificity is now reported.



Protein phosphorylation is an extensively studied post-translational modification (PTM). Aberrant cell signaling involving this PTM has been linked to many human diseases including cancer.[1] While phosphorylation can occur on various nucleophilic amino acid side chains, the spotlight has primarily focused on the phosphoesters of serine (pSer), threonine (pThr), and tyrosine (pTyr).

In contrast to the vast knowledge accrued on pSer, pThr, and pTyr, the chemistry and biology of *N*-phosphorylated amino acids such as phosphohistidine (pHis), phosphoarginine (pArg), and phospholysine (pLys) have remained underexplored.[2] Of these N-phosphorylated PTMs, a clear biological role for pHis in cellular signal transduction and central metabolism has been established,[3] and evidence for its involvement in epigenetics,[4] G protein signaling,[5] and ion conduction[6] is also emerging.

Despite a growing appreciation for the various roles of pHis in biology, there remain significant roadblocks that hinder studies in this area, namely (1) the intrinsic instability[7] of the pHis bond under the acidic conditions that are often employed in biochemical and phosphoproteomic workflows[8] and (2) a lack of research tools geared toward detecting and analyzing pHis in biological samples.

In an effort to address these analytical deficiencies, we have recently developed the first pan- and sequence-specific pHis antibodies.[9] Our success hinged upon the use of non-hydrolyzable synthetic analogs of pHis as haptens, a strategy that overcomes the in vivo decomposition problems that preclude use of the native PTM in antibody generation.[2a] Triazole-based analogs such as pTza (**2**) and pTze (**3**) are designed to mimic the structure and electronics of pHis (**1**) while replacing the chemically labile phosphoramidate linkage with a more stable phosphonate (Figure 1). Consequently,

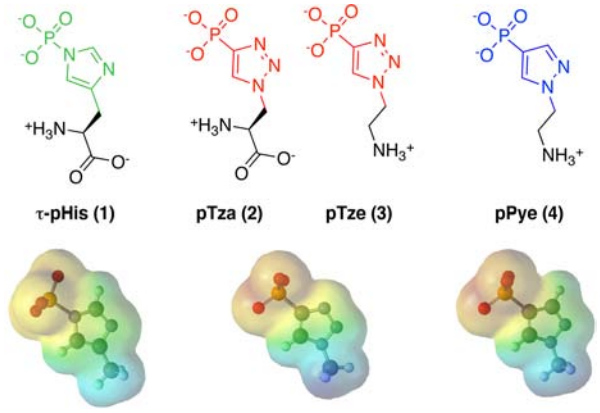


Figure 1. Phosphohistidine (pHis, **1**) and its stable analogs. The structure and calculated electrostatic map of the head groups (colored) are presented.

these synthetic haptens successfully elicited immune responses in host animals for the generation of antibodies, which also cross-reacted with native pHis proteins.

While the triazole-based analogs have afforded useful pHis-specific antibodies, these reagents do have a few shortcomings. First, the antibodies obtained using these first-generation pHis analogs have slightly lower affinity for native pHis compared to the analog itself. Second, and perhaps more importantly, the antibodies have mild cross-reactivity to pTyr,[9b] potentially limiting applications in mammalian biology where pTyr-bearing proteins could give rise to strong background signals.

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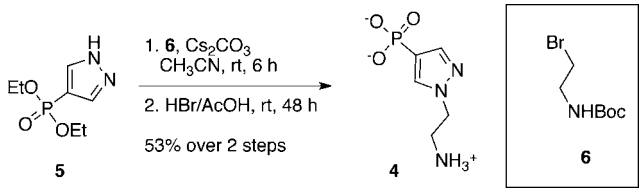
These observations suggest that our original triazole-based pHis analog is not a perfect mimic of native pHis, leading us to consider the use of other analogs as potential haptens. Accordingly, we turned to a pyrazole-based pHis analog, pPye (phosphono-pyrazolyl ethylamine, **4** (Figure 1)).[10] The pyrazole core of pPye retains the C−H group found at the C2 position in pHis, whereas a nitrogen atom is present at this position in pTza and pTze. DFT calculations[11] (Figure 1) indicate that the extra nitrogen in the triazole analogs leads to subtle differences in shape (C−H vs N lone pair) and electronics (more polarization in the triazole). In addition, pyrazolyl phosphonates are known to have pK_a values of 1.8 and 6.7[12] and, hence, should be primarily in the dianionic form, like pHis, at physiological pH.[7] Based on these considerations, we anticipated that pPye would be a good mimic of pHis.

With this design in hand, pPye was prepared from readily available materials. The known phosphonopyrazole[13] **5** was alkylated with commercially available bromide **6**. Subsequent global deprotection with HBr in acetic acid afforded pPye (**4**) in good yield (Scheme 1). Using pH-dependent chemical shift

Scheme 1. Synthesis of pPye



measurements, we confirmed that the phosphoryl group in **4** is in the dianionic state at physiological pH, similar to native pHis (Supplementary Figure 1).

pPye was conjugated via its primary amine group to the carrier protein, keyhole limpet hemocyanin (KLH), and used as the immunogen for antibody generation in rabbits. The pHis binding antibodies from the rabbit antiserum were then affinity purified over immobilized pHis-bovine serum albumin (BSA-pHis) (Supplementary Figure 2). These affinity-purified antibodies were tested for pHis specificity over other phospho-amino acid types by enzyme-linked immunosorbent assay (ELISA) (Figure 2). The pPye-derived antibodies displayed much higher binding affinity to BSA-pHis over BSA or BSA conjugates of other phosphoamino acids. Notably, the new antibodies exhibited much improved specificity for pHis over pTyr compared to our first generation, pTze-derived antibody (Ab 1.0) (Figure 2). Moreover, our pPye-derived

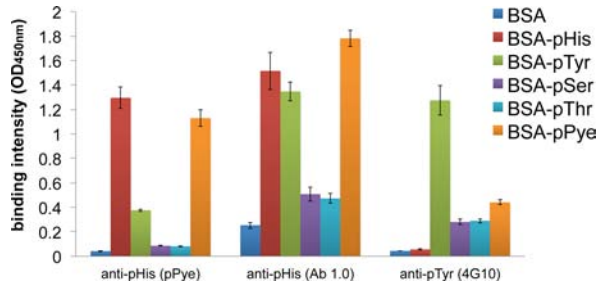
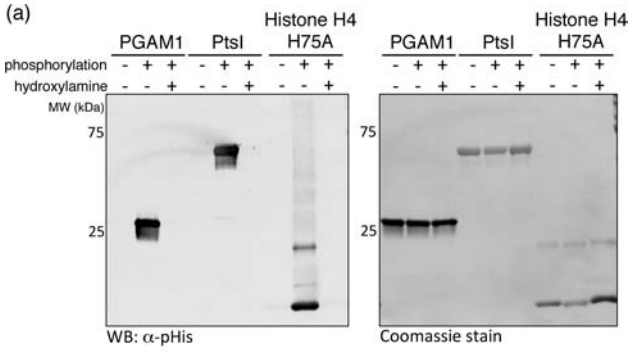


Figure 2. ELISA analysis of the pPye-derived pHis antibody, first generation pTze-derived pHis antibody (Ab 1.0), and pTyr antibody (4G10) against BSA, BSA-pHis, BSA-pTyr, BSA-pSer, BSA-pThr, and BSA-pPye substrates (n = 4; ±s.d).

antibody exhibited even better selectivity over pSer or pThr than a commercial pan-pTyr antibody (4G10) (Figure 2). Dot blot assays using synthetic phosphopeptides also indicated an improved selectivity of the pPye-derived antibodies compared to Ab 1.0 (Supplementary Figure 3).

To test whether the pPye-derived pHis antibodies are sequence-independent, we carried out Western blot analysis on a series of known pHis proteins (Figure 3). These proteins



(b)

protein (*species*)	sequence near pHis
PGAM1 (*H. sapiens*)	LVLIRpH_{18}GESAW
PtsI (*E. coli*)	GGRTSpH_{189}TSIMA
Histone H4 H75A (*H. sapiens*)	GGAKRpH_{18}RKVLR

Figure 3. (a) Western blot analysis of *in vitro* phosphorylated proteins. Left panel shows the Western blot using the pPye-derived antibody, and the right panel is a Coomassie stain of the membrane to serve as the loading control. Note that the dimer band (~22 kDa) observed for Histone H4 is likely due to aggregation (see Supporting Information). (b) Amino acid sequences around the pHis sites.

were either enzymatically phosphorylated (phosphoglycerate mutase 1 (PGAM1) and phosphoenolpyruvate-protein phosphotransferase (PtsI)) or chemically phosphorylated (histone H4) selectively on histidine. Since there are two histidine sites on native histone H4 that could be chemically phosphorylated (His-18 and His-75), thus confounding our ability to investigate sequence specificity of the antibody, we used a single histidine containing histone H4 where the His-75 site was mutated to alanine (histone H4 H75A). Gratifyingly, all of the proteins we tested showed robust signals when they were phosphorylated on the histidine whereas the unphosphorylated proteins did not (Figure 3a). Furthermore, the addition of hydroxylamine to the phosphorylated proteins, which leads to selective dephosphorylation of pHis, resulted in loss of detection by Western blot. The peptide sequences around the pHis sites show diversity in terms of charge and hydrophobicity, supporting the idea that the antibody is indeed sequence-independent (Figure 3b).

In summary, we have successfully developed a second-generation pan-pHis antibody. This was enabled by the molecular design and synthesis of pPye, a pyrazole-based stable pHis analog, which we show serves as an effective hapten for antibody generation. Importantly, this new antibody features an improved pHis specificity over pTyr and high affinity for pHis proteins in a sequence-independent fashion. Given the growing interest in the role of pHis-bearing proteins in mammalian biology, including cancer metabolism,[14] we expect this antibody will be a valuable addition to the arsenal of research tools for histidine phosphorylation. Efforts to explore the role of histidine phosphorylation in mammalian biology are underway.

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■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures, experimental procedures, and characterization data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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