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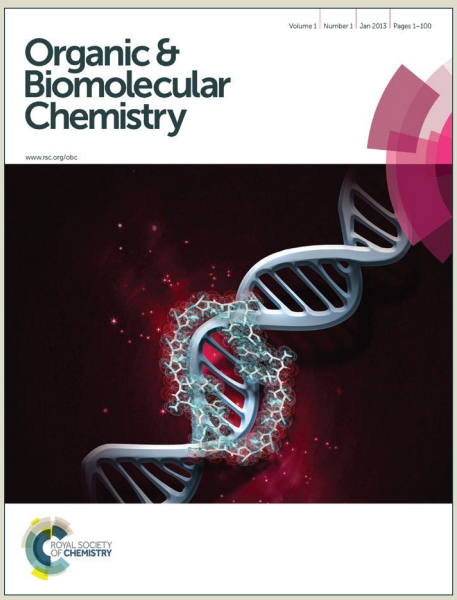





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# Development of Stable Phosphoarginine Analog for Producing Phosphoarginine Antibodies

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Protein phosphorylation is one of the most common and extensively studied posttranslational modifications (PTMs). Compared to the O-phosphorylation on Ser, Thr and Tyr residues, our understanding of arginine phosphorylation is relatively limited, both in prokaryote and eukaryotes, due to the intrinsic instability of phosphoarginine (pArg) and lack of feasible method to produce anti-pArg antibodies. We report the design and synthesis of a stable pArg analog, in which the labile N-P bond is replaced with non-hydrolyzable C-P bond. Significantly, this analog was successfully used as a hapten to raise immune response and the first mouse polyclonal antibody that specifically recognizes pArg-containing peptide and proteins was produced using analog-KLH conjugated as the immunogen. The generated antibody shows excellent specificity towards pArg-containing peptide and proteins, and could be used in variety of biological detection. This provide us a invaluble tool to unravel the mystery biological function of pArg.

## Introduction

Protein phosphorylation is well recognized as the primary post-translational modifications (PTMs) of proteins and extentsively studied.[1] Phosphorylation and dephosphorylation of protein takes determinative effect in the process of various physiological process including cell signaling, protein-protein interactions and epigenetics, and their misregulation has been linked to numerous human diseases. Thus far, 9 of 20 amino acids composed of natural proteins have been recognized as phosphorylation site in prokaryote and eukaryotes, the current focus are mainly on O-phosphorylation including serine (Ser), threonine (Thr) and tyrosine (Tyr).[2-4]

As one of three basic amino acids, arginine residues in protein can also be phosphorylated at its guanidine nitrogen.[5] The existence and biological role of arginine phosphorylation (pArg) has long been underestimated, despite it having been first identified over 40 years ago.[6] Recently, the discovery of a bacterial arginine kinase which phosphorylates a heat-shock regulator provided the first example of a signaling mechanism based on pArg.[7] Meanwhile, hundreds of pArg sites in numerous proteins were identified in *Bacillus subtilis in vivo*,[8] involved in the regulation of many critical cellular processes (e.g. protein degradation, competence and stress responses), which complements previous reports on pArg in mammalian tissues and proteins.[9-13] Remarkably, eukaryotic histone H3 was identified as a pArg-containing protein, implying that Arg phosphorylation activity might be relevant for epigenetic regulation.[13] Encouraged by the breakthrough on phosphohistidine (pHis),[14] another N-phosphorylation PTM, we have reasons to believe that pArg also plays important role in the signaling processes of eukaryotes.

Despite these discoveries, the intrinsic chemical properties of pArg and the dearth of adequate biology and chemistry research tools have hindered further understanding of this PTM. In contrast to the stable O-P bond in O-phosphorylated amino acids, the high-energy N-P bond in pArg is extremely acid-labile, unstable in hot alkali, and sensitive to heat,[5] making its detection and isolation from biological sources difficult. Since immunogens with pArg dephosphorylated rapidly in serum and can't survive long enough even in the mildly acidic environment of antigen-presenting compartments (APC), it is impossible to directly produce corresponding antibody (Ab) by using immunological method.[15] Thus, radioactive amino acid analysis and mass spectrometry are currently the main options, if not the only, for the detection of pArg.[13, 16, 17] To further complicate matters, producing of pArg-Ab is also hindered by the availability of pArg containing peptides and protein,[18] which is a critical requirement for antibody screening. As a result of this conundrum, Clausen and co-workers reported using *in vitro* phage-display as a alternative.[19] Recently, Fuhrmann *et al*. reported the first example on generating pArg-Ab in rabbit using a stable analog, which work is a back-to-back study with ours.[20] However, there is still urgent need to synthesize valid analogues in large scale with a convenient method.

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Furthermore, generation of a monoclonal Ab based on mouse hybridoma technology, is also highly desirable.

Herein, we report the design and synthesis of a non-hydrolyzable pArg analog, 2-((2-ammonioethyl)amino)-2-iminoethyl phosphonic acid (pAIE) with a convenient method. We envisioned that this stable pArg analog could mimic the structure and electrostatic status of pArg, used as a hapten to produce specific pArg-Ab, and would provide a way to the above mentioned problems associated with this elusive PTM. In addition, we hope that the proposed synthetic route is robust, while the key intermediate could be easily adopted for the preparation of its relevant derivatives, and facilitate the investigation on the physiological functions of arginine phosphorylation. Significantly, pAIE was successfully raised immune response and the first mouse polyclonal pArg-Ab that specifically recognizes pArg-containing peptide and proteins was produced using pAIE-KLH conjugated as the immunogen. The specificity, practicality and effectiveness of pArg-Ab for conventional bio-analysis including ELISA, Dot blot and Western blot, were carefully evaluated and proven. Notably, all the intermediate we synthesized or used are in solid form, making them easily purified by using recrystallization rather than flash column. Moreover, the proposed strategy on producing mouse pArg-Ab could be further developed to generate monoclonal Ab, with which the riddle of pArg as a novel PTM could be unraveled.

## Results and discussion

### Analogue design and synthesis

Inspired by the development of stable pHis analog and reported pHis antibodies,[21-23] we designed 2-((2-ammonioethyl)amino)-2-iminoethyl phosphonic acid (pAIE) as a stable analogues to the side chain of pArg. On the basis of molecular modeling studies, the iminoethyl phosphonic acid moiety in pAIE is expected to mimic the geometry and electronics of the head groups of pArg side-chain (Figure 1). Importantly, the hydrolytically labile N-P bond is replaced with a non-hydrolyzable C-P bond according to "Grimm's hydride displacement law",[24] while retaining the imino group (-C=N-H) and adjacent secondary amine group (-NH-) in guanidine group. This minimal substitution is hope to create an acid-stable analog to pArg, and also be in compliance with isosterism theory. Furthermore, the newly introduced methylene group (-$CH_2$-) could also work as a potential hydrogen bond donor, mimicking the substitued "–NH-" in N-P bond, in protein-protein interaction.

Our pArg analogue design also took future application and synthetic convenience into consideration. (Figure 1) A free primary amine group (-$NH_2$) together with a short linker (-$CH_2CH_2$-) is introduced to facilitate protein conjugation. In addition, if the similarity between the core of pAIE to the side chain of pArg is firstly proved in immune response, other derivatives (e.g. amino acid typed pAIE derivatives used in solid-phase peptide synthesis), can be readily synthesize from the same building block, intermediate **2**.[25]

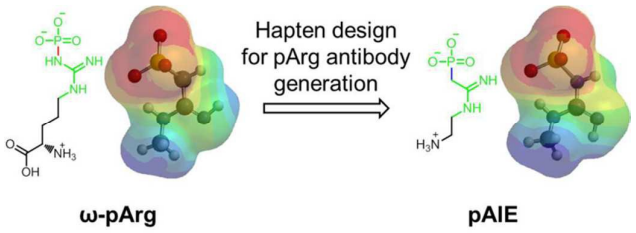
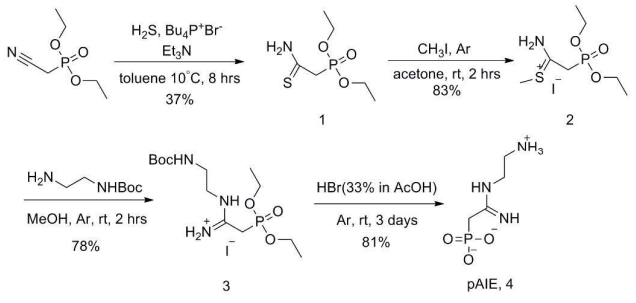



Fig. 1 $N_\omega$-Phospho-L-arginine (left) and its stable mimic pAIE (right). The structure and calculated electrostatic map of the head groups (colored) are presented. Hydrolytically labile N-P bond (red) in pArg is replaced with stable C-P bond (blue).

To our delight, target molecule pAIE (**4**) was achieved in a 4-step reaction with a total yield of 19% (scheme 1, calcuated based on the staring material). Practically, diethyl (2-amino-2-thioxoethyl)phosphonate (**1**) and 2-(Diethylphosphono)-S-methylthioacetamidium Iodide (**2**) were synthesized according to literature with a modification.[25] Due to the inherent difficulty in gas-liquid reaction involved in getting **1**, we improved the method by adding $Bu^4P^+Br^-$ as a phase-transfer catalyst to increase the yield of **2**, from literature reported 23% to 37%. Air-sensitive **2** was purified rapidly and directly used for the next-step reaction. Compound **3** was easily synthesized by the addition of N-Boc-ethylenediamine to freshly prepared **2**, followed by global deprotection with HBr in acetic acid afforded pAIE (**4**). All intermediates and the final product were characterized by using NMR and MS. (Supporting information, Figure S2-S12) As a convenient a robust synthetic route, all starting materials are commercial available, and the key intermediate **2** is accessible in gram scale in two steps.

### Charge status of pAIE

With pAIE (**4**) in hand, we first measured its pH-dependent $^{31}$P-NMR chemical shift. Since the $^{31}$P chemical shift is sensitive to adjacent functional group and its surrounding chemical environment, it could be used as an indicator, reflecting the charge status of phosphate. As showed in Figure 2, two jump points have been observed in $^{31}$P-NMR spectrum, subsequently have been identified to the second oxygen of iminoethyl phosphonic acid (pKa ≈6.5) and imino group (pKa ≈ 12.3), respectively. Hence the iminoethyl phosphonic acid in pAIE should be primarily in dianionic form, like pArg, at physiological pH (pH=7–8).[5] Based on this result, we anticipated that pAIE would probably be a good mimic of pArg.




**Scheme 1** Synthesis of pAIE.

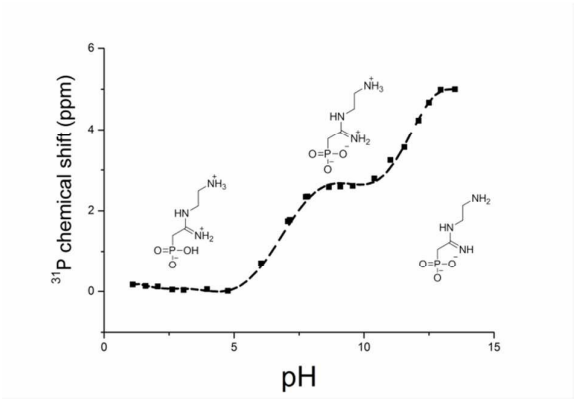



Fig. 2 pH-dependent $^{31}$P-chemical shift of pAIE. The curve is fitting with Oringin Software. The identified charge status of pAIE is indicated in the figure.

### Antibody producing

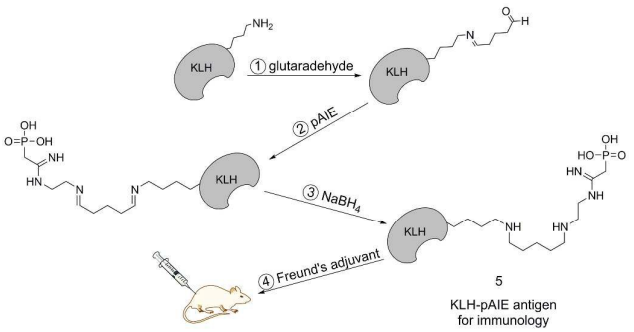
To produce mouse polyclonal antibodies that can selectively recognize pArg in phosphoproteins (pArg-Ab), pAIE (**4**) was firstly conjugated via its primary amine group to carrier protein, keyhole limpet hemocyanin (KLH) through its ε-amino group on Lys residue, and served as the immunogen (**5**). (Scheme 2) Glutaraldehyde was employed for bio-conjugation, and to introduce a flexible linker at the meanwhile.[26] It was hoped that such antibodies raised against pAIE-KLH could cross-react with pArg moiety in both proteins and peptides, thereby validating pAIE as a valid analogue of pArg. Four mice were subcutaneously injected with pAIE-KLH conjugates using a standard immunization protocol. The individual bleeds obtained from inoculated animals were screened by ELISA, using pArg-BSA and BSA alone as positive and negative controls, respectively. (a complete protocol is given in Supplementary Note 3)

### Antibody purification

After immunization, the most promising serum was purified over Protein A agarose column to eliminate serum albumin and other impurities. As showed in Figure 3, all fractions in the purification process were monitored by using ELISA and SDS-PAGE, and the most pure fraction E1 with the highest cross-react to pArg-BSA relative to BSA, was used as a purified mouse polyclonal pArg-Ab for further assessment.

### Specificity of pArg-Ab

Encouraged by above ELISA results, we next determined the specificity of pArg-Ab. We carried out peptide dot blot assays with a series of model peptides harboring pArg (**8**) as well as Arg (**7**), pSer (**9**), pThr (**10**), or pTyr (**11**) which are derived from CtsR, the natural substrate of protein arginine kinase McsB.[7] Particularly necessary to point out that, pArg-containing peptide **8** was enzymatically synthesized from **7**, and separated by HPLC (Figure 4a). Notably, pArg-Ab only recognizes **8** but not **7**, and **9**-**11**, demonstrating no cross-reactivity towards Arg or other phosphoamino acid residues. The specificity of pArg-Ab was further evaluated using diverse phospho-amino acids conjugated BSA as analytes, as shown in Figure 4b. To our exciting, pArg-Ab not only shows excellent performance in specificity, but also is sensitive enough to detect as low as 0.1 ng of pArg-BSA, implying its abnormal affinity and potential application in bio-analysis. Compared to the reported pArg-Ab generated from phage-display or using rabbit as a host, our mouse pArg-Ab shows the best detection limitation (<0.1 ng vs. 25 ng and 2.5 ng, respectively).[20]




**Scheme 2** Preparation of pAIE-KLH conjugates for producing antibody.

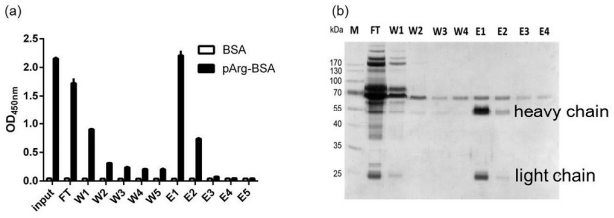



Fig. 3 Purification of mouse polyclonal antibodies raised against pAIE-KLH. (a) ELISA analysis of input, flow through (FT), wash fractions (W1-W5) and elution fractions (E1-E5) from the Protein A agarose purification for pArg-BSA binding affinity. Fraction E1 had the highest affinity for pArg-BSA relative to the BSA control (n=3; mean ± s.d.). (b) SDS-PAGE analysis of above mentioned fractions. The bands were visualized by using silver staining. The heavy chain and light chain of antibody are indicated in the picture.

### Application of pArg-Ab

To validate the usability of our mouse pArg-Ab toward the full-length protein in western blotting (WB) experiments, recombinant $His_6$-McsB and $His_6$-CtsR were expressed in *E.coli* and purified (Figure S15 and S16). CtsR controls the expression of genes encoding the HSP100/Clp chaperones and the protease ClpP.[27, 28] Phosphorylation of arginine residues 62 in the DNA binding domain of CtsR by McsB, is proved critical for the release of this repressor from DNA in *bacillus subtilis*, which process participates in the stress response and protein quality control systems.[7, 29] Meanwhile, McsB is believed to undergo autophosphorylation on its arginine residues.[30] Accordingly, McsB/CtsR system is a good model for our study. WB analysis clearly shows that the autophosphorylation naturally exited on *E.coli* recombinant McsB, and slightly increased when performing *in vitro* incubation with ATP (Figure 4c), implying that recombinant McsB derived from *E.coli* is heterogenous and composed of both phosphorylated and unphosphorylated forms. In addition, pArg-Ab selectively recognizes arginine-phosphorylated CtsR, but not the nonphosphorylated counterpart (Figure 4c). Acid treatment of the phosphorylated McsB and CtsR, abolishes this antibody

recognition, showing that phosphorylation is indeed at arginine. Detailed identification of phosphorylation site was also carried out and confirmed by using mass spectrometry. (Figure S19). To the best of our knowledge, this constitutes the first example of producing mouse polyclonal antibody that selectively recognizes pArg-containing protein through animal immunization.

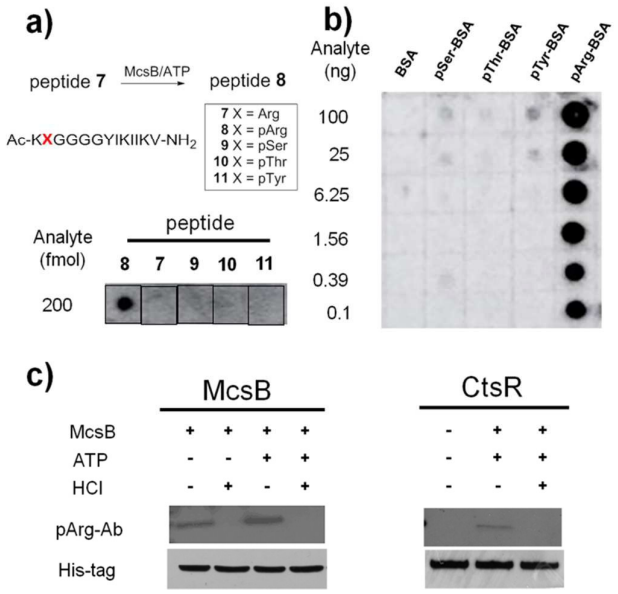



Fig. 4 (a) Peptide dot blot using pArg-Ab. pArg-containing peptide 8 was enzymatically synthesized from pep-tide 7. Peptides 9-11 were synthesized using standard solid-phase peptide synthesis (SPPS) method. (b) Protein dot blot using pArg-Ab. Various phospho-amino acids were chemically conjugated to BSA via glutaraldehyde. (c) Western blots of arginine phosphorylation in McsB and CtsR. Acid treatment (0.1 N HCl, 55 °C, 15 min) of the phosphorylated McsB and CtsR, abolished pArg-Ab recognition, showing that phosphorylation is indeed acid-labile and at arginine. For the loading control, all proteins were verified by using anti-His antibody.

#### Antibody subtype

So far, a practical draw-back of all polyclonal antibodies is its finite supply, unavoidable deviation among different batches and individuals, and limitation of synthesized hapten. Generation of monoclonal antibodies (mAb) will fundamentally address these issue, providing ample and homogenerous materials. Another question is, although IgG-type mAb is more preferred than IgM-type, the latter one being always dominated in hapten-protein raised serum.[31] We further detected the subtype of our mouse polyclonal pArg-Ab raised by pAIE. Consequently, mouse $IgG_{2a}$ and $IgG_{2b}$ are the most abundant subtype in the gained anti-serum (Figure S21), which provide us a chance to perform a mAb screening based on widely used mouse hybridoma technology, in the future, to obtain sustainable supply of monoclonal antibodies.

## Conclusions

In summary, we reported herein the design and synthesis of pAIE as stable pArg analogues. By adopting our convenient and robust method, pAIE could be obtained in gram-scale without using sophisticated purification procedures. We also demonstrated the application of this analogue as a valid hapten for producing of anti-pArg antibodies. Significantly, the first mouse polyclonal antibodies specific to pArg-containing proteins, were developed as a proof of concept. With this invaluable tool, we hope to deepen our understanding of arginine phosphorylation in both prokaryotic and eukaryotic system, and determine the importance of pArg as a novel PTM. Further studies in this direction are under way in our laboratory.

## Acknowledgements

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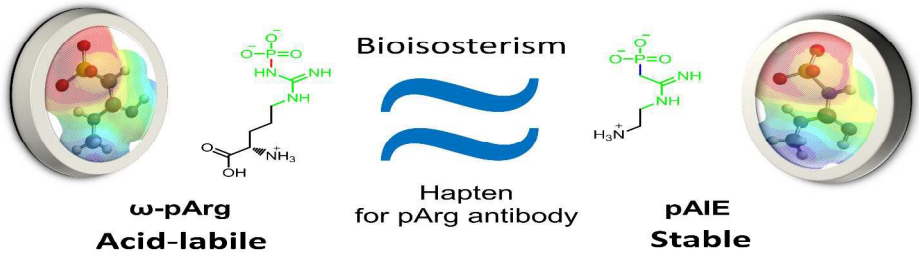


pAIE is designed and synthesized as a stable analog and bioisosterism of acid-labile pArg, to produce pArg specific antibodies, facilitating the detection of protein argininine phosphorylation.