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Synthesis of an NDPK Phosphocarrier Domain Peptide Containing a Novel Triazolylalanine Analogue of Phosphohistidine Using Click Chemistry

Sung Hyun Yang, Dong Jun Lee, and Margaret A. Brimble*

School of Chemical Sciences, The University of Auckland, 23 Symonds Street, Auckland, New Zealand

m.brimble@auckland.ac.nz

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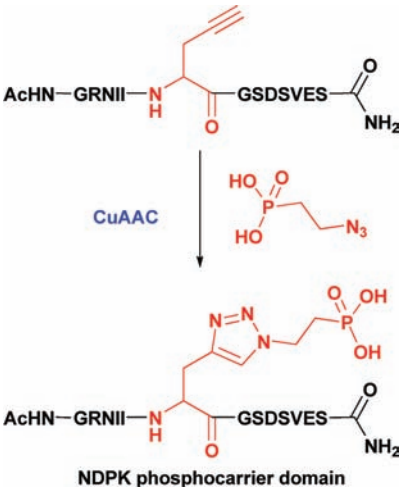
ABSTRACT



NDPK phosphocarrier domain

Click phosphorylation of a propargylated unprotected peptide and phosphoryl azide using chaotrope-assisted Cu(I)-catalyzed 1,3-dipolar cycloaddition enabled a high-yielding and rapid synthesis of a nucleoside diphosphate kinase (NDPK) phosphocarrier domain. The synthesis showcases a valuable synthetic platform for the synthesis of biologically relevant phosphopeptide analogues.

Protein phosphorylation represents one of the key post-translational modifications which plays an essential role in the regulation of protein function (termed "phosphoregulation").[1] In most cases of phosphoregulation, a functional protein is activated/deactivated by reversible enzymatic phosphorylation/dephosphorylation of up to nine naturally occurring amino acids; the most extensively reported being serine, threonine, and tyrosine.[2,3] A less well-studied phosphorylated amino acid, phosphohistidine (pHis), has attracted an exponential growth in interest in the past decade for its involvement in a number of important biological systems including the bacterial phosphoenol pyruvate:sugar phosphotransferase system; two-component sytems that are involved in sensing variations in biological environments such as pH, temperature, chemoattractants, and osmolarity; G-protein signaling; ion conduction; and a number of kinase enzymes such as the nucleoside diphosphate kinase (NDPK).[4] However, the biochemical study of pHis has

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been hampered by the intrinsic instability of the P−N phosphoramidate bond.[2] Unlike the phosphoester derivatives in which the P−O bonds are chemically stable even in an acidic environment, the higher free energy of the P−N bond and its chemical instability at low pH meant the investigation and characterization of protein histidine phosphorylation and the associated protein kinases have been slower to progress.[2] In addition, the existence of two regioisomeric forms of pHis (the 1- and 3-regioisomers) further complicates the study.[5] Nevertheless, this unique chemical instability of the P−N bond is attributed to the ability of pHis to undergo rapid transfer of the phosphoryl group, reflecting its wide role in protein regulation.

In order to investigate pHis in biological systems, effective stable mimics of the pHis are required to be designed and synthesized. To date, three different types of stable analogues of pHis have been reported: the furanylalanine **2**;[6] the pyrrolylalanine **3**;[6] and the triazolylalanine derivatives **4**[7] and **5**[8] (Figure 1). The difficulties in the syntheses of the furanyl and pyrrolylalanine derivatives[6,7] led to the discovery of triazoylalanine mimics **4** and **5** using a very efficient and chemoselective Cu(I)-catalyzed 1,3-dipolar cycloaddition (so-called 'click chemistry')[9] between protected azidoalanine and alkynyl diethylphosphonate to synthesize the fully protected building blocks.[7,8] The P−C bond linked to the unnatural triazole moiety is very stable under both basic and acidic conditions and is resistant to hydrolysis; thus these phosphonotriazole mimics were deemed suitable for use in studies involving phosphohistidines in proteins.

The first synthesis and application of the triazolylalanine mimic was carried out by Muir et al.,[7] who reported the synthesis of Boc-4-diethylphosphonotriazolylalanine **4** and its incorporation into Boc SPPS for the synthesis of the N-terminal tail of histone H4. Their study demonstrated that these click mimics could be used as haptens to produce antibodies which recognize phosphohistidines in a sequence-dependent manner. Complementary work was carried out independently by Webb et al.,[8] who synthesized a test peptide sequence from a phosphocarrier domain of pyruvate orthophosphate dikinase by incorporating Fmoc-4-diethylphosphonotriazolylalanine **5** into Fmoc SPPS. Their computational modeling suggested that although the phosphonotriazoles were effective for the generation of sequence-specific antibodies, further synthetic analogues of pHis would be required in order

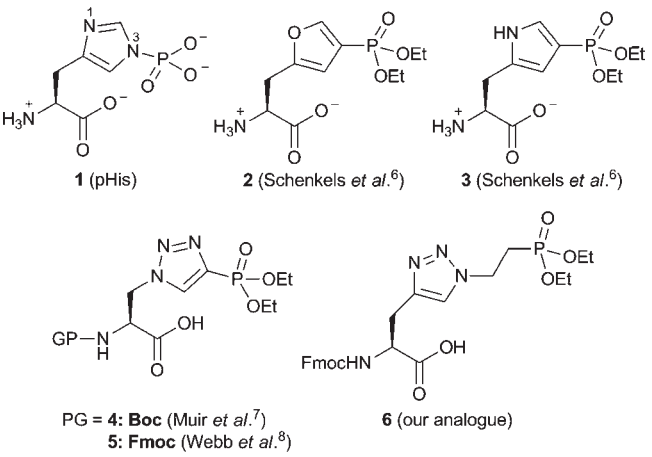
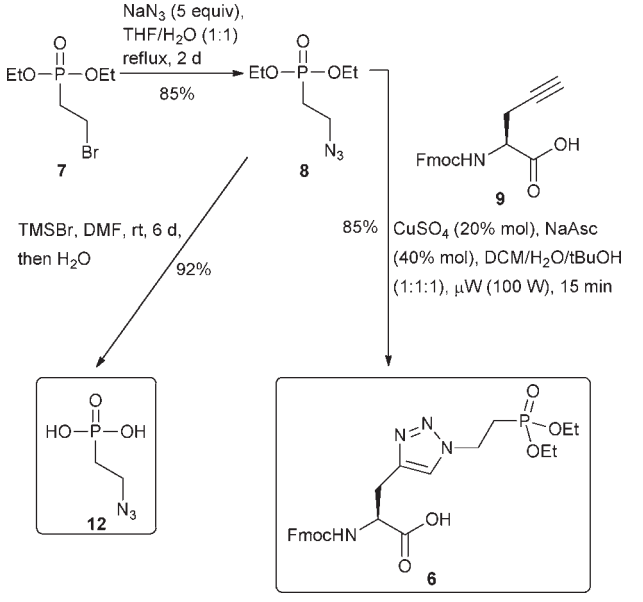


Figure 1. Chemical structures of phosphohistidine (pHis) **1** and its analogues **2**–**6**.

to induce sequence-independent antibodies. Studies by both groups were elegantly carried out; however, the low-yielding addition reaction of ethynyl magnesium bromide to diethyl chlorophosphate utilized by both groups led us to design an alternative route toward the synthesis of a complementary triazolylalanine derivative **6** that could also be used as an effective stable pHis mimic, thereby supplementing the work of Muir et al.[7] and Webb et al.[8]

We therefore herein report the synthesis and application of a novel triazolylalanine analogue of pHis. The biologically relevant phosphocarrier domain of nucleoside diphosphate kinase (NDPK), Ac-G^{113}RNIIHGSDSVES^{125}-$^{\alpha}$CONH$_2$, was chosen as our primary synthetic target for the purpose of this investigation.[10,11]

Scheme 1. Synthesis of Building Block **6**



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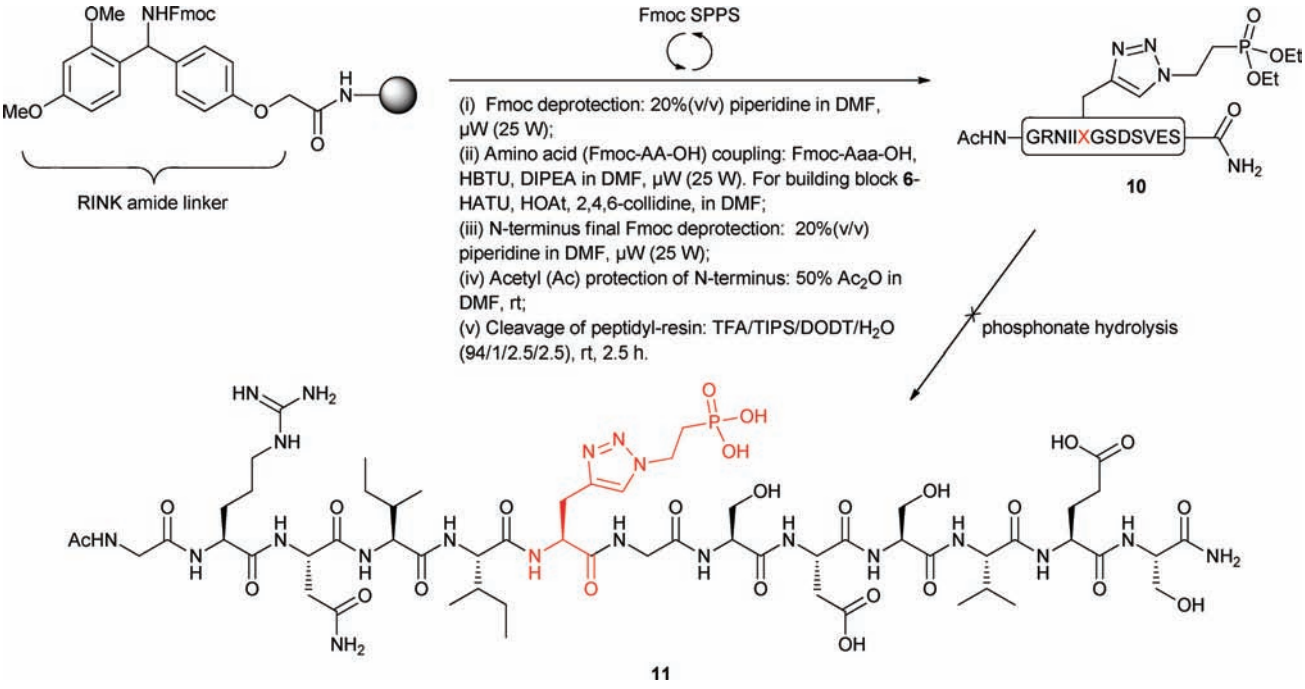
Scheme 2. Synthesis of Phosphonotriazolyl Peptide **11**



NDPK is a protein consisting of 152 amino acid residues and catalyzes the phosphorylation of nucleoside 5′-diphosphates to the corresponding triphosphates via phosphate transfer between GDP/GTP and ADP/ATP. Due to its paramount importance in the regulation of biological function it was deemed suitable for use in our study.[10,11]

Our initial approach involved the synthesis of triazolylalanine diethylphosphonate building block **6**, incorporation of this into Fmoc SPPS, and then final hydrolysis of the diethylphosphonate to afford the desired phosphopeptide **11** (Scheme 2). The synthesis of the triazolylalanine building block **6** is summarized in Scheme 1. Commercially available bromophosphonate **7** was heated under reflux with NaN_3 in THF/H_2O (1:1) to afford the azide **8** in 85% yield.[12] CuAAC of **8** with Fmoc-Pra-OH **9** (synthesized using published methods[12]) using $CuSO_4$ (20% mol) and NaAsc (40% mol) in $DCM/H_2O/t$BuOH (1:1:1) with microwave irradiation (100 W, 70 °C)[13] afforded the desired building block **6** in 85% yield after purification. The test peptide **10** was synthesized using microwave enhanced Fmoc SPPS on amino-functionalized polystyrene resin prepared 'in-house' that contained the RINK amide linker. Building block **6** was incorporated into the peptide chain using the conditions shown in Scheme 2, replacing the phosphohistidine at the selected position. Following cleavage of the peptide **10** and HPLC purification, hydrolysis of the diethyl phosphonate to the corresponding phosphonic acid was next attempted. A survey of the literature revealed that phosphonate esters can readily be hydrolyzed using TMS-halide reagents.[15–18] Webb et al.[8] reported the use of TMSBr for this purpose; however only partial hydrolysis was achieved thus necessitating careful HPLC purification.[8] However, in the present case various attempts using TMSBr,[16] TMSI,[17] and HBr/AcOH[18] in different organic solvents including DMF and MeCN failed to yield the desired phosphopeptide **11**.

Disappointed by these results, we next devised a more convergent approach for the synthesis of the desired phosphonotriazolyl peptide **11**. Recently, our group reported an optimized protocol to carry out CuAAC between unprotected peptides containing propargyl groups and unprotected sugar azides for the synthesis of a library

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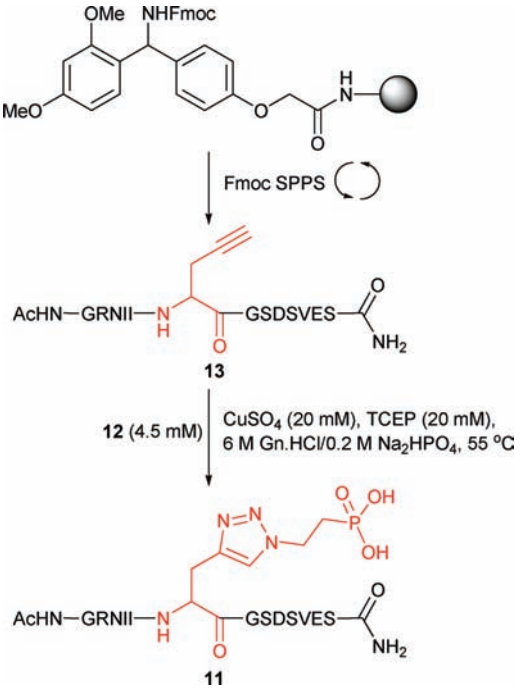
of click MUC1 *neo*glycopeptides.[19] The procedure involved an important chaotrope in peptide chemistry, guanidine hydrochloride, to assist and enhance the cycloaddition reaction between the two unprotected click partners. We envisaged that this procedure could be applied for the convergent synthesis of our desired phosphopeptide **11**.

First, the azide **8** was hydrolyzed using TMSBr in DMF to afford the azido-phosphonic acid **12** in 92% yield that did not require further purification.[20] In parallel, the propargylated peptide **13** was synthesized using Fmoc SPPS and purified by HPLC (Scheme 3). With these two click partners in hand, CuAAC was next attempted using the conditions from our previous work on MUC1 *neo*glycopeptides.[19] Peptide **13** (3 mM) was dissolved in 6 M GnHCl/0.2 M Na_2HPO_4 containing $CuSO_4$ (20 mM) and tris(2-carboxyethyl)phosphine (20 mM). After the addition of **12** (4.5 mM) the reaction was monitored by analytical HPLC. Initial attempts at ambient temperature revealed sluggish conversion into the desired click phosphopeptide **11**. However, to our delight, quantitative conversion of the starting peptide into the click phosphopeptide **11** was observed at an elevated temperature (55 °C) in 5 h without noticeable byproduct or decomposition (Figure 2). Subsequent reversed-phase HPLC purification afforded our desired phosphopeptide **11** in high purity and isolated yield (29%). The identity of the product was confirmed by ESI-MS (Figure 2).

Scheme 3. Synthesis of **11** by Direct Click Method on Propargyl-Peptide **13** Using Unprotected Phosphoryl Azide **12**



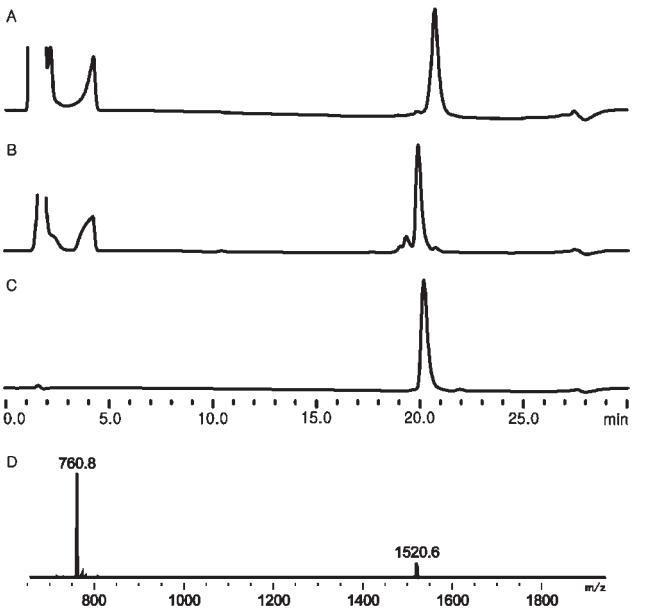


Figure 2. HPLC and MS profile of click reaction depicted in Scheme 3. (A) $t = 5$ min, peptide **13**; (B) $t = 5$ h, product peptide **11**; (C) purified **11**; (D) ESI-MS spectra of **11**.

In conclusion, we have demonstrated the robust synthesis of a stable triazolylalanine analogue of phosphohistidine using click chemistry and successfully incorporated it into Fmoc SPPS. Although the final hydrolysis of the diethyl phosphonate ester containing peptide was unsuccessful, we were able to obtain our desired phosphonotriazole-containing peptide **11** via a more convergent approach by carrying out independent syntheses of the unprotected propargyl-peptide **13** and the azido-phosphate **12** and then subsequently effecting their union using click chemistry, performed in a chaotropic aqueous buffer. Despite the fact that our devised pHis analogue has a slightly larger pseudimidazole–phosphorus distance and a higher configurational flexibility than the native pHis, the realistic effects of this on antibody recognition and enzymatic activity have yet to be revealed and biological studies are currently underway. Nevertheless, this new synthetic strategy using post-SPPS click chemistry which utilizes unprotected building blocks (i.e., peptide and phosphoryl azide) enables the rapid synthesis of synthetic phosphopeptide analogues for biological screening and further biochemical evaluation.

Supporting Information Available. Synthetic details for compounds **6**, **8**, and **12**, and a procedure for the click reaction between **12** and **13**. This material is available free of charge via the Internet at http://pubs.acs.org.