Enabling identification of phosphorylated cysteine: Novel chemoselective synthetic and analytical methods

Review Article

Protein phosphorylation plays a key role in the regulation of signaling pathways and many other cellular processes. Studies of phosphorylation have focused on serine, threonine and tyrosine amino-acid side chains, while the phosphorylation of other amino acids, such as phospho-histidine (pHis), -arginine (pArg), -lysine (pLys) and -cysteine (pCys), is less well understood. Studies have been hindered by technical limitations, primarily acid lability. To address this problem, Jordi Bertran-Vicente and colleagues have developed a novel chemoselective and stereochemically defined phosphorylation strategy for Cys residues. The method employs the nucleophilic reactivity of P(III)-reagents (phosphites) with electrophilic disulfides. The research team has also developed a mass spectrometry-based proteomic approach to identify and characterize pCys sites that naturally occur in peptides.

Synthesis strategy

pCys functions as an intermediate in the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) in the dephosphorylation of phosphotyrosine residues by protein tyrosine phosphatases, and in bacterial signaling and regulation. Earlier methods to prepare pCys required the preparation of Dha residues and also lacked stereoselectivity, and this stimulated the authors to develop a novel strategy for chemoselective and stereochemically defined phosphorylation of Cys residues.

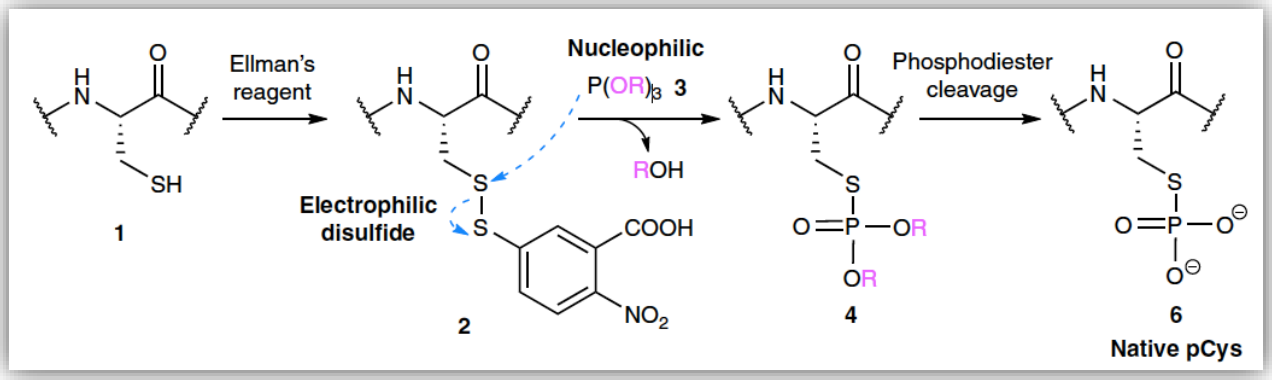

Figure 1. Synthetic strategy to insert pCys residues in unprotected peptides. A stereochemically defined pCys residue was prepared by site-specific addition of phosphite to an activated Elfman-disulfide. From Figure 1, Bertran-Vicente et al, 2016.

The synthesis strategy was based on the reactivity of an electron-deficient disulfide peptide (Figure 1). The Cys-containing peptides (1) were reacted with Ellman's reagent to generate electrophilic disulfides (2), which were then reacted with phosphites to prepare the corresponding phosphorothiolate ester peptides (4). These yield pCys-peptides after phosphodiester cleavage (6).

Peptide synthesis

Peptides were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl)-based solid phase peptide synthesis (SPPS) on Rink Amide or Wang resins. Peptides were synthesized either manually or with a Tribute® peptide synthesizer using standard Fmoc-based conditions with HOBt/HBTU/DIPEA activation and piperidine Fmoc deprotection in DMF. Peptides were cleaved and isolated by reversed-phase-HPLC and verified by electrospray ionization mass spectroscopy.

Synthesis optimization

The synthetic strategy was used to synthesize a small Cys-containing peptide (LYRCAK), including a number of phosphite/solvent combinations (see Figure 2). Phosphite 3d, for example, resulted in a 97% yield of peptide 4d.

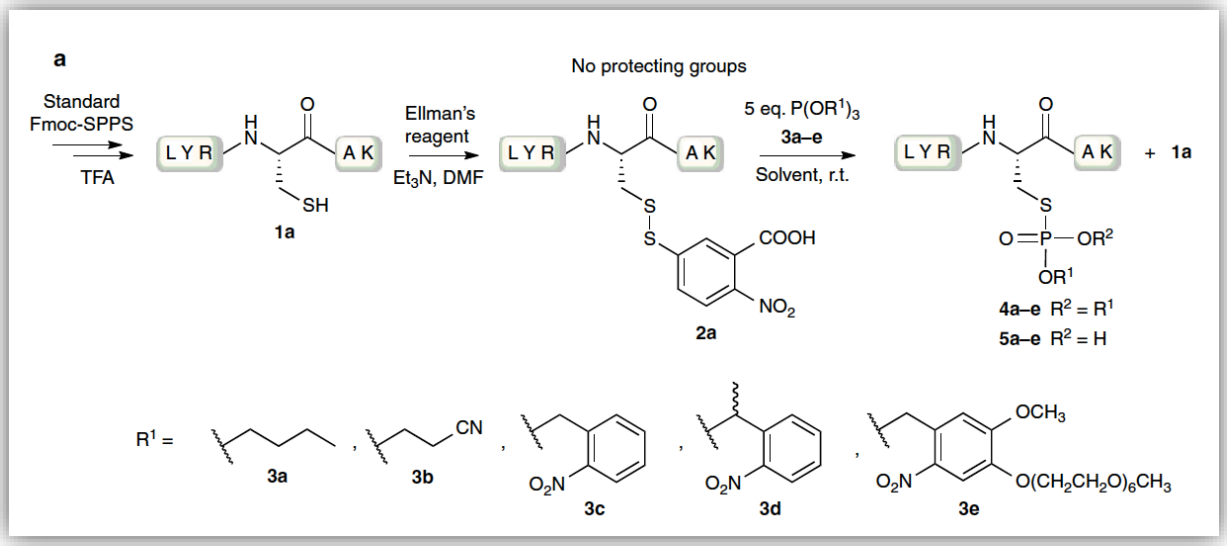

Figure 2. Chemoselective synthesis of phosphorothiolate esters peptides using Ellman's reagent and peptide and phosphite esters. From Figure 2a, Bertran-Vicente et al, 2016.

A test using a small peptide sequence (YCA) indicated that the synthetic strategy was free from epimerization. The team then applied ultraviolet radiation or alkaline deprotection to peptides 4b, 4d, and 4e to prepare native phosphorylated Cys peptides 6. Photodeprotection of peptide 4d lead to efficient conversion to the desired peptide 6a, which could be isolated in 65% yield at a purity of >90% (Figure 3).

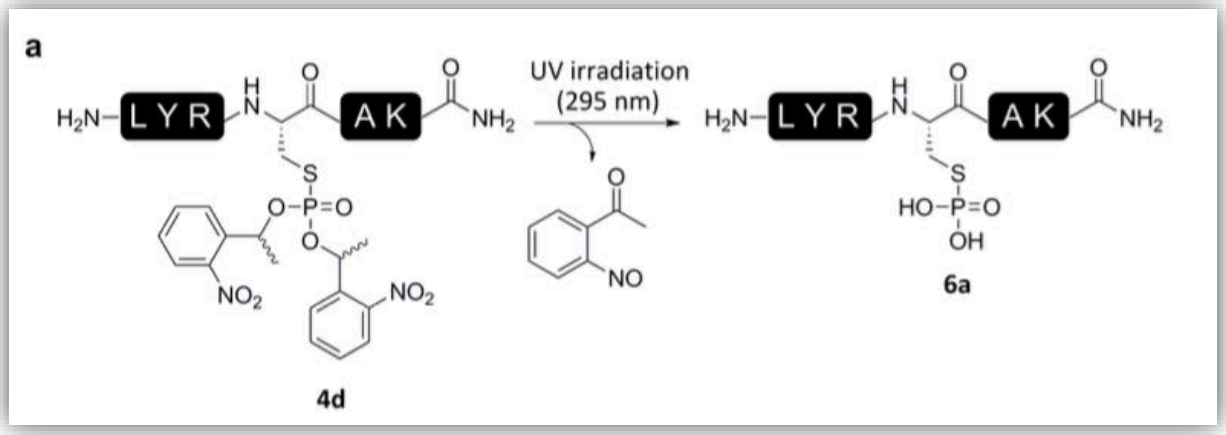

Figure 3. Efficient photodeprotection of peptide 4d to peptide 6a. From Supplementary Figure 17, Bertran-Vicente et al, 2016.

pCys characterization

While acid conditions decreased the stability of peptide 6a as expected, the LYRCAK peptide proved to be stable at pH 7.4 and 8.4 over 24 hours. The team used nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and EThcD (electron-transfer dissociation, ETD, with supplemental activation) to characterize the synthetic pCys peptides. This included confirming the site-specific modification of the peptide and that there had not been any phosphate transfer or migration during the synthesis.

MS characterization of an endogenous pCys peptide

The successful analysis of the synthetic peptides using EThcD tandem MS encouraged the research team to use this method to identify a pCys in the glucose-specific transporter $IICB^{Glc}$, which is known to be involved in the PTS. They overexpressed the four subunits required for the phosphorylation event in *Escherichia coli*, induced *in vitro* phosphorylation, and then prepared fragments using gel electrophoresis, tryptic digestion, and chromatography. They were then able to use EThcD tandem MS to identify the pCys tryptic peptide ENITNLDApCITR, together with the corresponding unphosphorylated peptide.

Valuable tools for Cys phosphorylation research

Together, these novel synthesis and analytical methods should help in identifying endogenous pCys peptides and provide new insights into the role Cys phosphorylation plays in crucial cellular processes.

Reference

Chemoselective synthesis and analysis of naturally occurring phosphorylated cysteine peptides. Bertran-Vicente J et al, Nat Commun. 2016 Sep 2;7:12703. doi: 10.1038/ncomms12703.

https://www.ncbi.nlm.nih.gov/pubmed/27586301