Fmoc-chemistry of a stable phosphohistidine analogue†

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We report the synthesis of the phosphohistidine analogue, Fmoc-4-diethylphosphonotriazolylalanine 5 and its incorporation into peptides. Our synthesis of 5 has enabled us to demonstrate that the analogue is compatible with Fmoc-solid phase peptide synthesis (SPPS) conditions. Standard cleavage conditions yield the diethyl phosphonate-protected peptide, however this can be subsequently deprotected using trimethylsilyl bromide to yield the free phosphonic acid-containing peptides.

Phosphorylation of amino acids is central to the post-translational regulation of proteins[1] and the role of the acid-stable phosphate monoesters, phosphoserine, phosphothreonine and phosphotyrosine in cell physiology and biochemistry is well established. Phosphorylation of several other amino acids including histidine, arginine, lysine and aspartate is also observed.[2,3] In these cases the phosphorylated amino acid residues are less stable due to the inherent instability of the phosphoramidate and mixed phospho-anhydride bond. Because of this relative lability, phosphorylated histidine and aspartate both act to mediate phosphate transfer in pathways of bacterial two-component signal transduction[4] and phosphohistidine is observed as a reactive intermediate in numerous enzymatic reaction mechanisms. Histidine phosphorylation has also been observed in mammalian systems, including on histones I and IV,[5] on heterotrimeric G-proteins,[6] on P-selectin[7] and on annexin I.[8] Corresponding histidine kinases[9] and histidine phosphatases[10] have been described. This suggests that regulated phosphorylation of histidine may be important for eukaryotic cell biochemistry. Further study of the importance of histidine phosphorylation is currently hampered by the lack of available biochemical and chemical tools. Mass spectrometric methods to detect the modification have recently been described[11] but these are not currently readily applicable to whole proteome studies and until the recent publication by Kee *et al.*[12] there were no available methods to detect the modification selectively using antibodies.

The *tele*-regioisomer (τ-pHis, **1**) of phosphohistidine is the dominant form observed in proteins.[2] The chemical instability of both this and the pros-regioisomer (π-pHis) is due to protonation of the non-phosphorylated imidazole nitrogen ($pK_a \approx 6.4$ in τ-pHis).[2] Methods to chemically phosphorylate[13] and thiophosphorylate[14] histidine in a protein context have been described, however we wished to develop an effective stable mimic of the residue amenable to Fmoc-SPPS. Two stable analogues of phosphohistidine have previously been reported: the furanyl and pyrrolylalanine derivatives **2** & **3** (Fig. 1).[2] The synthesis of the furanyl derivative was reported by Schenkels *et al.*[15] and takes ten steps leading to a compound still requiring further derivatisation before being suitable for incorporation into peptides using Fmoc chemistry. We therefore chose to synthesize the triazolylalanine phosphonates **4** & **5**. In these molecules the labile P–N bond is replaced with a hydrolytically stable P–C bond; dephosphonylation of these molecules would first require protonation at the carbon adjacent to the phosphorus atom leading to loss of aromaticity suggesting that these molecules will not be readily hydrolysed in acid. This work complements that of Kee *et al.*[12] who recently reported the synthesis of Boc-4-diethylphosphonotriazolylalanine **4** and its application to the biochemistry of phosphohistidine.

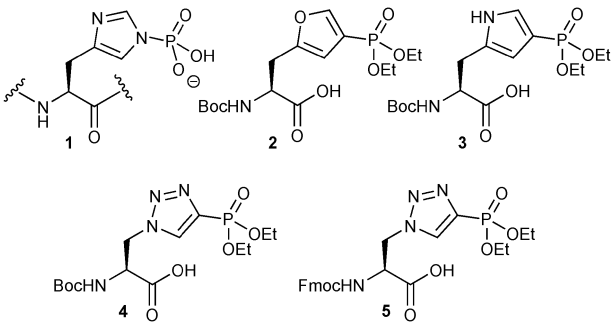

Fig. 1 Structure of *tele*-phosphohistidine **1** and analogues designed to mimic it including those of Schenkels *et al.* **2** and Kee *et al.* **4**. We report the use of the Fmoc-protected derivative **5**.

Our synthetic route (Scheme 1) to the triazole derivative is based upon the cycloaddition of diethyl acetylenylphosphonate **6** with azidoalanine derivatives. Several routes to **6** are described in the literature,[16] however we prepared it by direct addition of acetylenyl magnesium bromide to diethyl chlorophosphate in THF to give the desired alkyne **6** in 24% yield.† *N*-Boc-azidoalanine **8** was generated from commercially available *N*-α-Boc-diaminopropionic acid **7** *via* Cu-catalysed diazo-transfer using the method of Link *et al.*,[17] Cu(I)-catalysed [3 + 2] cycloaddition of **8** with the alkyne **6** then generated the Boc-protected triazolylalanine **4** in 88% yield. This could be converted to the corresponding Fmoc-protected triazolylalanine **5** in 60% overall yield. Alternatively, this compound has subsequently been prepared by direct addition of commercially available Fmoc-azidoalanine **10** to the alkyne **6** to generate **5** in 96% yield.

The protected phosphonotriazole **5** is readily incorporated into peptides using standard Fmoc-SPPS methodology. We synthesized the test peptide sequence, Ac-GMTSTzAA-NH_2 **11**, corresponding to the phosphocarrier domain of pyruvate,

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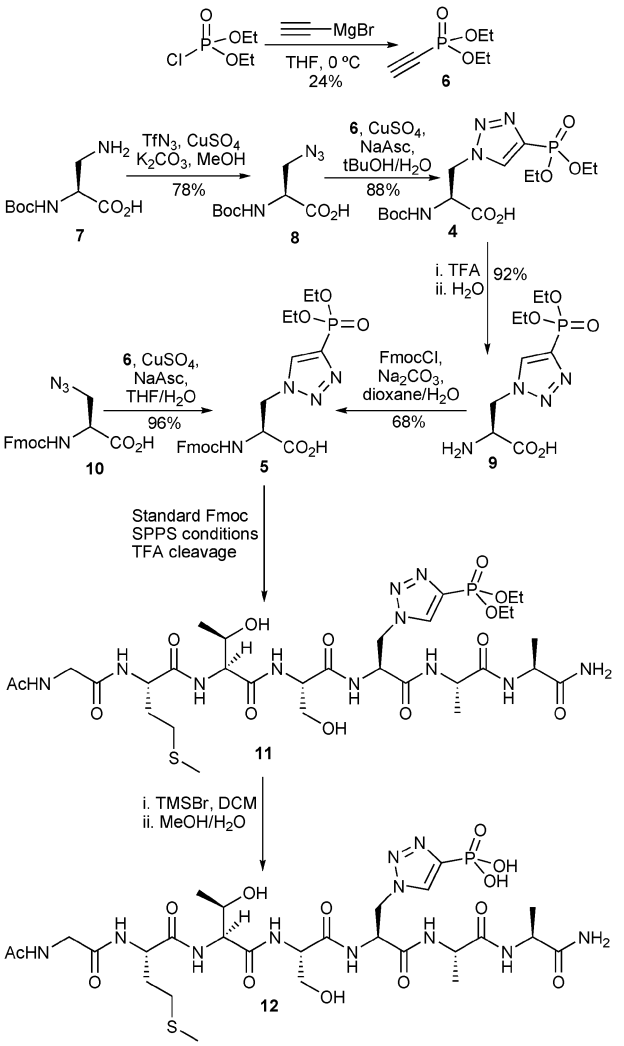

Scheme 1 Synthesis of **5**, incorporation into peptide and deprotection (NaAsc—sodium L-ascorbate).

orthophosphate dikinase[18] in 48% yield overall. Deprotection of the diethylphosphonate did not occur using the standard Fmoc-cleavage cocktail employed. After lyophilisation of the crude peptide, the diethyl phosphonate ester could be hydrolysed by stirring with TMSBr in anhydrous DCM followed by quenching with $MeOH/H_2O$.[19] This procedure yielded the deprotected peptide **12** as a mixture with both the mono- and di-protected esters. Isolation of the phosphonic acid-containing peptide at this stage therefore requires preparative HPLC. Kee *et al.* showed that deprotection of the diethyl esters occurs during cleavage of the peptide from resin using HF[12]; the milder TMSBr cleavage is however more readily accessible.

Kee *et al.* have also demonstrated that this class of phosphonotriazoles can be used as haptens to produce an antibody which specifically recognises phosphohistidine.[12] This antibody was specific to phosphorylation of His18 of histone IV and did not recognise phosphorylation of His75. This indicates that interactions with the neighbouring amino acids are occurring in addition to recognition of the phosphate of phosphohistidine. Our initial computational modelling (Fig. 2) suggests that hydrogen-bonding interactions to the

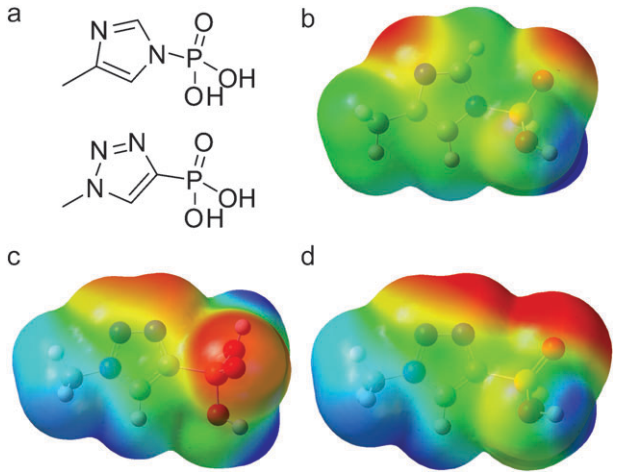

Fig. 2 Density functional calculation of the electrostatic potential for phosphohistidine and triazole analogues.† (a) Model structures used for evaluation of electrostatic potential (ESP) surfaces of τ-phosphohistidine (**13**) and 4-phosphonotriazolylalanine (**14**); (b–d) ESP plotted on an isosurface at $\rho = 4 \times 10^{-4}$ e bohr^{-3}. (b) **13**, (c) **14**, (d) **14** with torsional restraint applied upon rotation around C–P bond.

heterocycle of the triazole will be dominated by interactions to N^3 of the triazole ring rather than N^2 as shown by the higher electrostatic potential at this position. If an antibody was raised to the isolated triazole, the antibody would be expected to preferentially interact with N^3 over N^2 and therefore may not also recognise an isolated phosphohistidine residue. This suggests that while this class of amino acids is clearly effective for the development of sequence-specific antibodies, further synthetic analogues of phosphohistidine may be needed if a sequence-independent antibody to phosphohistidine is to be found.

In conclusion, we have demonstrated the synthesis and use of stable analogues of phosphohistidine using Fmoc-chemistry. Although these analogues are readily incorporated into peptides, side-chain deprotection does not occur during cleavage of the peptide from resin, though deprotection can be readily achieved using mild reaction conditions.

Notes and references

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