4-Phosphothiophen-2-yl alanine: a new 5-membered analogue of phosphotyrosine†

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Polyclonal antibodies raised against 4-phosphothiophen-2-yl alanine 2a, a novel five-membered ring analogue of phosphotyrosine, showed high selectivity for phosphotyrosine and no cross-reactivity with other phosphorylated amino acids. Western blots showed that the polyclonal was similarly effective, but different in selectivity, to a commercially available monoclonal antibody.

Protein tyrosine phosphatases (PTPs) catalyse the dephosphorylation of phosphotyrosine residues and together with protein tyrosine kinases (PTKs) regulate the tyrosine phosphoproteome in cells.1 Several PTPs have been identified as attractive therapeutic targets in human diseases such as cancer, diabetes, obesity, neurodegenerative disorders and inflammation.2–4 Since phosphotyrosine 1 is unstable under typical biological conditions, interest in the biological roles of PTPs concomitantly led to the development of stable analogues of phosphotyrosine.4,5 These analogues, azobenzene phosphonate (ABP),6 phosphotyramine,7 phosphonomethylphenylalanine (Pmp),8 and difluorophosphono-methylphenylalanine (F2Pmp)9,10 have been invaluable tools in unraveling the importance of phosphotyrosine in cellular processes. These benzenoid analogues have been useful not only as immunogens to generate antibodies that recognise phosphotyrosine with high specificity without cross-reactivity to other phosphoamino acids, but also as inhibitors and affinity ligands for PTPs and other pTyr binding proteins.

Significantly, incorporation of Pmp and F2Pmp into peptide sequences, has provided phospho-site specific antibodies and more potent or specific phosphatase inhibitors. These peptides have also been used as affinity ligands and in structural studies where they have provided invaluable information about protein–protein interactions.8–11 A major limitation is the absence of specific inhibitors against many PTPs. Consequently, PTP research has lagged behind PTK studies and the related field of serine/threonine protein phosphatases.12,13

Motivated by a report on the corresponding furan 2b that suggested it might be a non-hydrolysable and non-isomerisable analogue of phosphohistidine 3,14 we synthesised 4-phosphothiophen-2-yl alanine, 2a, with the aim of establishing whether the thiophene would better mimic the imidazole in 3 or the benzene ring in 1. Hirao cross coupling 15 between diethyl phosphite and 3-bromothiophene gave 3-diethoxylphosphothio-phene 4. Iodination of 4 gave, in addition to the 2,5-diodo-derivative (18%), the iodothiophene 5, which was subjected to Negishi cross-coupling with zinc reagent 6 using our recently optimised conditions 16 to give the protected amino acid 7. Sequential deprotection then gave 4-phosphothiophen-2-yl alanine 2a, via the corresponding acid 8 (Scheme 1).

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4-Phosphothiophen-2-yl alanine 2a and the protected form 4 were subjected to Western blot analysis to evaluate the specificity of the polyclonal antibody. The polyclonal antibody was shown to cross-react with the recombinant phosphotyrosine phosphatase (rPTPase) 1A (Scheme 2). A commercial mouse monoclonal antibody (clone 4G10) that was raised against 4-phosphothreonine was used to compare the selectivity of the polyclonal antibody. The results of the Western blot analysis are shown in Figure 1. The polyclonal antibody showed a single band at the expected molecular weight of recombinant phosphotyrosine phosphatase 1A (Scheme 2).

Scheme 1 Synthesis of 4-phosphothiophen-2-yl alanine 2a.

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† Electronic supplementary information (ESI) available: Experimental procedures and NMR spectra for 2a, 4, 5, 7 and 8. See DOI: 10.1039/c4cc03393k
To explore its potential as a phosphoamino acid analogue, compound 2a was used as a hapten to produce antibodies. Polyclonal antibodies were raised in rats using KLH-conjugated analogue 2a as the immunogen. In one of the initial reports of a phosphotyrosine antibody generated using ABP as immunogen, Frackelton et al. observed cross-reactivity towards phosphohistidine 3,6 In the course of this study, several non-hydrolysable analogues of phosphohistidine have been reported including phosphopyrrole,17 phosphoryltriazolylalanine isomers,18,19 a sulfonamide-based transition-state analogue of enzymatic phosphohistidine dephosphorylation20 and phosphoryltriazyloethylamine.21 Use of phosphoryltriazolylalanine as a mimic of one phosphohistidine in a histone H4 peptide allowed the generation of antibodies that did not universally recognise phosphohistidine in proteins.18 Antibodies subsequently generated to phosphoryltriazyloethylamine showed cross-reactivity towards phosphohistidine and phosphotyrosine.21 The reactivity of the antibodies generated to 2a was initially tested using dot-botts spotted with BSA-conjugated histidine, phosphoserine, phosphothreonine, phosphotyrosine and τ-phosphohistidine. Antibodies showed strong reactivity towards phosphotyrosine without any cross-reactivity for τ-phosphohistidine, histidine, tyrosine or other phosphohydroxy amino acids (results not shown). This suggested that 2a was a potential analogue for phosphotyrosine. The antibodies were therefore rescreened against tyrosine, phosphotyrosine, phosphoserine, phosphothreonine and τ-phosphohistidine (Fig. 1, entry A). The antibodies showed high selectivity for phosphotyrosine with minimal side reactivity towards BSA-conjugated τ-phosphohistidine (Fig. 1, A lane v). However, this apparent τ-phosphohistidine cross-reactivity was simply removed by affinity depletion using a histidine--KLH glutaraldehyde conjugated Sepharose column (Fig. 1, entry B) suggesting that the cross linker itself produced a reasonable immune response.

The thiophene in analogue 2a is evidently a poor mimic of the corresponding imidazole present in τ-phosphohistidine, but a good mimic of the benzene ring present in phosphotyrosine, which has resulted in a polyclonal serum that detects phosphotyrosine in preference to τ-phosphohistidine. Competition studies, performed by ELISA on the purified polyclonal antibody, showed no binding or inhibition of the antisera by any of the amino acids apart from phosphotyrosine (Fig. 2). This indicates that while analogue 2a may not be a suitable analogue for τ-phosphohistidine it was likely to be a useful analogue for phosphotyrosine. Competitive inhibition ELISA analysis using O-phospho-L-tyrosine (pTyr) or 2a, demonstrated that the antibody generated from 2a has a higher affinity towards 2a compared to phosphotyrosine (Fig. 3, see inset), as expected.

In Western blot analysis, using cytosolic fractions obtained from sheep tracheal epithelia, the antibody generated from 2a gave a staining profile comparable to that of a commercially available anti-phosphotyrosine monoclonal antibody (pY99) – albeit with varying degrees of staining or detection intensity for various protein bands by either antibody (Fig. 4A). This indicates that the antibody generated by 2a is able to detect phosphotyrosine containing proteins and recognises many protein bands that are also detected by the anti-phosphotyrosine pY99. Interestingly, the antibody generated from 2a also detected proteins that were not detected by the pY99 (and vice versa). This is consistent with previous studies where different types of antibodies provided varied results in the same systems.22 Furthermore, in total lysates of human bronchial epithelial cell lines (16HBE140-) treated with Na3VO4 (a broad spectrum PTP inhibitor) for 45 min, both the antibody generated from 2a and pY99 detect enhanced phosphorylation of a high molecular

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weight protein (Fig. 4B, filled arrow). However, pY99 additionally detects enhanced phosphorylation of three other proteins (approx. 75, 100, 180 kDa) in Na3VO4 treated cells (Fig. 4B, left panel, unfilled arrow heads). Interestingly, both antibodies also detect several other proteins that were insensitive to Na3VO4 and in this regard, pY99 antibodies detected two, whereas the antibody generated from 2a detected six, of these proteins. In lysates from cells stimulated with EGF (1 nM for 4 hours), both the antibody detected six, of these proteins. In lysates from cells stimulated with 2a

In conclusion, we have synthesized 4-phosphothiophen-2-yl alanine, 2a and demonstrated its potential as a novel 5-membered ring analogue of phosphotyrosine. Our data suggests that analogue 2a, and resulting polyclonal antibody, may potentially be a useful tool to both characterize the biological effects of phosphotyrosine and the role of specific PTP(s) in cells.

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Notes and references