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SOLID PHASE SYNTHESIS OF PHOSPHONOSERINE ISOSTERES OF PHOSPHOSERINE PEPTIDES

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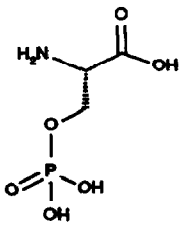
Summary: Using the recently synthesized building block L-Fmoc-Abu[PO(OCH_2-$CH{=}CH_2$)$_2$]-OH, (5) phosphonopeptide isosteres of serine phosphopeptides are readily accessible by Fmoc-solid phase peptide synthesis.

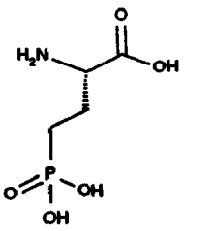
The synthesis of phosphoserine peptides and their analogs is currently a subject of considerable interest due to the importance of protein phosphorylation in biology. While there have been numerous reports on the synthesis of phosphoserine, *Ser(P)*, 1, containing peptides there is, to our knowledge, only one report on the synthesis of peptides containing the corresponding phosphonate analog, *Abu(P)*, 2.[1] Such phosphonoserine-peptides are clearly of interest since they would be stable to metabolism



1

L-O-phosphoserine

Ser(P)



2

L-2-amino-4-phosphonobutanoic acid

Abu(P)

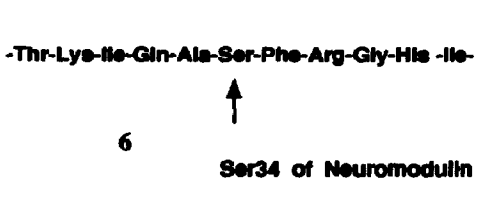
H-Leu-Abu(P)-Glu-OH

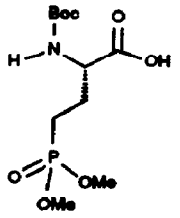
3

Figure 1

by protein phosphatases and could be used to delineate biological mechanisms. Perich et al. have reported (Figure 1) the solution phase synthesis of the tripeptide 3, H-Leu-Abu(P)-Glu-OH, using t-Boc strategy and the building block 4 (Figure 2).[1] For the synthesis of peptides of significant complexity or length, solid phase peptide synthesis (SPPS) methodology is clearly to be preferred, in particular the facile Fmoc strategy.[2] To this end we have recently reported the asymmetric synthesis of both D and L isomers of Fmoc-Abu[PO($OCH_2CH{=}CH_2$)$_2$]-OH, (5) (Figure 2) the allyl protected Fmoc derivative of 2.[3] Herein, the successful incorporation of 2 into diverse peptide sequences is described.

Sequences derived from known phosphoserine proteins were chosen for preparing the phosphonoserine analogs. Neuromodulin an important synaptic protein appears to be regulated by phosphorylation at serine or threonine.[4] One of the candidates is Ser34 which is embedded in the partial





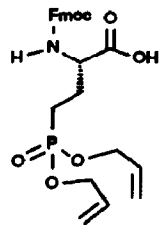


Figure 2

4 5

peptide sequence 6 (Figure 2). Thus, we set out to prepare phosphonopeptide analogs derived from this sequence. The RaMPs multiple peptide synthesis system[5] was used for preparing these analogs. In all cases Wang resin was employed in which the first amino acid was already attached on a scale of 0.10mmol.[6] Fmoc-L-amino acids were used with standard t-butyl protection strategy (t-butyl ester for Asp, Boc for Lys and Orn, t-butyl ether for Thr) while Arg was used with pentamethylchromansulfonyl (PMC)[7] protection; His, Cys and Gln with trityl. The L-isomer of Fmoc-Abu[PO($OCH_2CH{=}CH_2$)$_2$]-OH was always used for the introduction of Abu(P). Couplings were performed in standard fashion in dimethylformamide (DMF) using 2.5 equivalents of Fmoc-amino acid and diisopropylcarbodiimide (DIC)/hydroxybenztriazole (HOBt)/Fmoc-amino acid in 1:1:1 ratio. Fmoc cleavage was performed with piperidine/dimethylformamide 1:1 and resin washes with DMF-methanol-DMF.[8] After peptide chain

Table 1 Replacements of Abu(P) for Serine34 in Neuromodulin

Natural Sequences	One Additional Mutation
H-Thr-Lys-Ile-Gln-Ala-Abu(P)-Phe-Arg-Gly-OH	H-Gln-Ala-Abu(P)-His-Arg-Gly-OH
H-Lys-Ile-Gln-Ala-Abu(P)-Phe-Arg-Gly-OH	H-Gln-Ala-Abu(P)-Trp-Arg-Gly-OH
H-Ile-Gln-Ala-Abu(P)-Phe-Arg-Gly-OH	H-Gln-Ala-Abu(P)-Ala-Arg-Gly-OH
Ac-Gln-Ala-Abu(P)-Phe-Arg-Gly-OH	H-Gln-Ala-Abu(P)-Asp-Arg-Gly-OH
H-Gln-Ala-Abu(P)-Phe-Arg-Gly-OH	H-Gln-Ala-Abu(P)-Phe-Lys-Gly-OH
H-Gln-Ala-Abu(P)-Phe-Arg-Gly-His-Ile-OH	H-Gln-Ala-Abu(P)-Phe-His-Gly-OH
	H-Gln-Ala-Abu(P)-Phe-Phe-Gly-OH
	H-Gln-Ala-Abu(P)-Phe-Asp-Gly-OH
	H-Gln-Ala-Abu(P)-Phe-Orn-Gly-OH
	H-Cys-Ala-Abu(P)-Phe-Arg-Gly-OH

assembly was completed the N-terminal Fmoc group was left intact and the allyl phosphonate ester

protecting groups were removed first on the resin using Noyori's protocol.[9] Thus, the resin was stirred with 20mol% $Pd(PPh_3)_4$ and butylamine(4eq):formic acid(16eq) in tetrahydrofuran for 5 hours at 50°C to ensure complete deprotection.[10] After cleavage of the N-terminal Fmoc group in standard fashion, the resin was treated with trifluoroacetic acid and a mixture of cation scavengers[11] to afford the deprotected resin cleaved crude phosphonopeptide. All crude phosphonopeptides were purified by semipreparative reverse phase (RP-18) HPLC which was followed by desalting ion exchange chromatography.[12] In this manner the 16 phosphonopeptides listed in Table 1 were readily prepared giving satisfactory FAB-MS, HPLC (>95%) and amino acid analysis.[13] Aside from the additional step for cleaving the allyl phosphonate protecting groups the method corresponds to standard Fmoc-SPPS protocol for peptide synthesis. Until now no sequences have encountered which have presented difficulties and such beyond the realm of the corresponding peptide are not anticipated.

Finally, in relation to the synthesis of inhibitors of the protein phosphatase 2B (calcineurin) which has been identified as a target for the immunosuppresive drugs cyclosporin and FK-506,[14] phosphonopeptide 7 (Figure 3) became of interest to us. In an assay for calcineurin activity the corresponding 19-residue phosphopeptide 8, a partial sequence of the cAMP dependent protein kinase regulatory subunit, displays a K_m of 26μmol.[15] Furthermore, it has been demonstrated that N-terminal truncation of 4 residues from 8 yields a much poorer substrate for calcineurin.[16] Phosphonopeptide 7 was

H-Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Abu(P)-Val-Ala-Ala-Glu-OH

7

H-Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser(P)-Val-Ala-Ala-Glu-OH

8 Substrate Phosphopeptide used for Calcineurin Assay K_m= 26 micromolar

Figure 3

synthesized on an Applied Biosystems(ABI) 430A automated peptide synthesizer using the standard Fmoc cycles program provided by ABI. In this protocol 0.50mmol of Wang resin is used and couplings are mediated with DIC-HOBt and Fmoc cleavages with piperidine. Glu and Asp were protected as t-butyl esters and Arg as PMC. The deprotection, resin cleavage and purification of 7 was performed as described above for the other phosphonopeptides. This gave 7 >95% HPLC pure with satisfactory FAB-MS, and amino acid analysis.[17] Suprisingly, in our newly described HPLC assay for calcineurin activity,[18] 7 did not show inhibitory activity (IC_{50}> 100umol). It may be that the phosphate of phosphopeptide 8 binds to the calcineurin as a dianion, while the corresponding dianion of 7 is present to a much lesser extent at physiological pH.[19]

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10. Compromising these conditions led to incomplete deprotection.
11. A cleavage system recommended by Applied Biosystems was routinely used: 10mL trifluoroacetic acid, 0.75g phenol, 0.25mL ethanedithiol, 0.5mL thioanisole, and 0.5mL water.
12. Semipreparative HPLC chromatography was performed using an RP-18 and eluting with an A/B gradient: A= (1000 H_2O/ 20 H_3PO_4), B= (800 CH_3CN/ 200 H_2O/20 H_3PO_4), which was customized for each peptide depending on the analytical HPLC result (detection λ=205nm, Vydac or Stagroma columns, semipreparative 250/10mm, analytical 125/4mm). For the ion exchange chromatography (desalting) Biorad AG 4X-4, 100-200 mesh was used with 2NAcOH eluent.
13. Isolated yields ranged from 7-13% with the average being 10%. Peptide content derived from the amino acid analyses ranged typically from 75-90%. Abu(P) eluted just after the solvent front peak but was not quantitated.
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16. For 8 K_m=26μmol and V_{max}= 1700, (k_{cat}= 65), for the N-terminal truncated 15mer K_m=130μmol and V_{max}= 240, (k_{cat}= 1.8).[15]
17. The isolated yield of 7 was 11%. From the amino acid analysis the peptide content of 7 was ca. 75%.
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