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Combined Fmoc-Alloc Strategy for a General Solid Phase Synthesis of Phosphoserine Peptides

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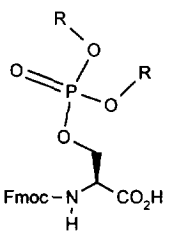
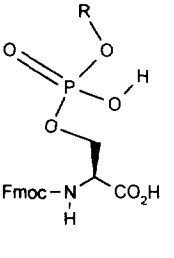
Summary: A building block method for the SPPS of serine phosphopeptides has been developed using a combination of Fmoc and Alloc strategies. Alloc-Ser[PO($OCH_2CH{=}CH_2$)$_2$]-OH, prepared in a one-pot procedure from Alloc-Ser-OH, was introduced at the N-terminus of a sequence prepared by standard Fmoc-SPPS. Global cleavage of the allyl ester protecting groups followed by coupling of a tripeptide fragment led to tau phosphopeptide, **1**, which was not available by post assembly phosphorylation strategies and is an important epitope of tau phosphoprotein in Alzheimer's Disease.

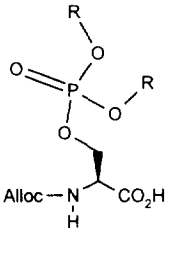
There has been a significant amount of effort directed to the development of methodology for the synthesis of phosphopeptides[1] due to the important role of protein phosphorylation in biology. We have been particularly interested in the synthesis of serine-threonine phosphopeptides.[2] The majority of solid phase peptide synthesis (SPPS) methods for serine phosphopeptides involve on resin phosphorylation[3] after a standard Fmoc[4] based peptide chain assembly. With these protocols the serine residue to be phosphorylated is either incorporated unprotected or in a selectively protected fashion. Although, in our hands, this method has generally been acceptable for synthesis of serine phosphopeptides, it has also failed for some particularly important sequences. This led us to develop a novel general building block method, based on a combination of Alloc[5] and Fmoc peptide synthesis strategies, which has been used to prepare an important epitope of the tau phosphoprotein present in Alzheimer's Disease: phosphotau (259-268), H-Lys-Ile-Gly-Ser(PO_3H)-Thr-Glu-Asn-Leu-Lys-His-OH, **1**.[6]



2 R = alkyl

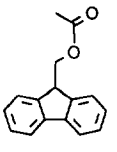


3 R = CH_2Ph
4 R = H

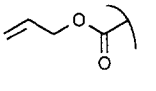


5 R = t-Bu
6 R = allyl
7 R = CH_2Ph

Fmoc=

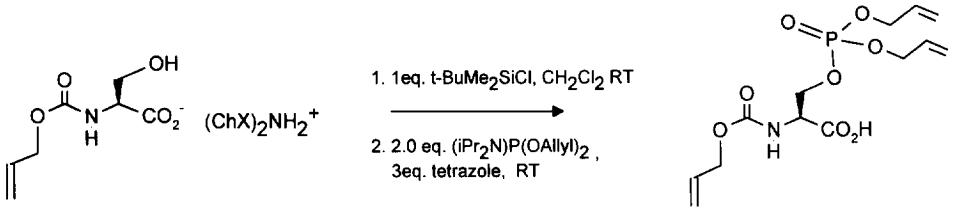


Alloc=



At the time we began this work there were two reports of building block based SPPS serine phosphopeptide synthesis. One method involved t-Boc (N-t-butyloxycarbonyl) SPPS[7] which required the use of an optimized o-toluoyl phosphate ester protection strategy and liquid HF mediated deprotection-resin cleavage.[8] The other method made use of α-N-allyloxycarbonyl (Alloc) amino acids and t-butyl phosphate ester building block **5** with Alloc deprotection on the solid phase requiring the efficient method developed by Guibé ($Bu_3SnH\backslash Pd(PPh_3)_4$) and which proceeds under neutral conditions.[9] No facile, Fmoc based SPPS methods employing a protected serine phosphate Fmoc-Ser[$PO(OR)_2$]-OH, **2**, had been described due to the high susceptibility of such phosphate ester functionality to β-elimination[1] under the basic conditions required for Fmoc deprotection. However, in the course of our work, Wakamiya reported a breakthrough method for synthesizing serine phosphopeptides using the monobenzyl protected Fmoc-Ser[$PO(OH)(OCH_2Ph)$]-OH, **3**.[10] Unlike diester derivatives, **3** is no longer sensitive to β-elimination under the conditions of Fmoc removal (20%piperidine-dimethylformamide) and this allowed SPPS of several serine phosphopeptides under standard Fmoc conditions.

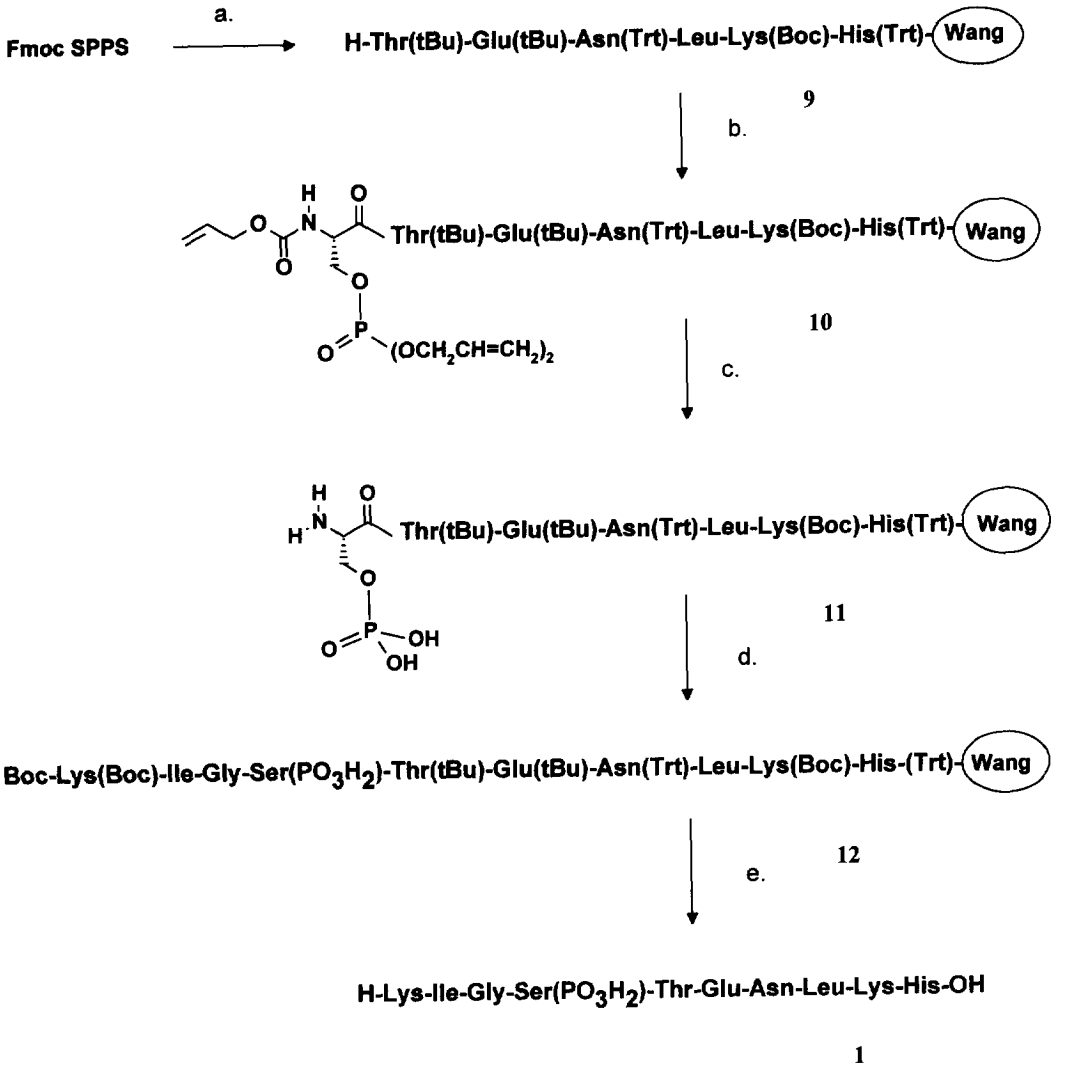
We desired to establish a building block method based primarily on standard Fmoc-SPPS which would be general, easy to perform and allow the preparation of longer sequences with no amino acid restrictions.[11] However, unsure of the reliability of the coupling of an unprotected Fmoc phosphoserine such as **3** or **4**, we devised a method which would allow the phosphoserine to be introduced into the peptide chain with the phosphate moiety fully protected. To this end, Alloc-Ser[$PO(OCH_2CH{=}CH_2)_2$]-OH, **6** was chosen as the key building block with the following strategy. Standard Fmoc-SPPS should be performed up to the phosphoserine residue at which point **6** is introduced. After incorporation of **6**, the α-N-alloc group should be cleaved concommitantly with both phosphate allyl ester groups using the efficient deprotection method we had developed for solid phase applications.[12] With the so generated free N-terminal serine phosphate on the solid phase stable to β-elimination, extension of the peptide chain might then again be possible via individual amino acid couplings and Fmoc-SPPS or by fragment condensation(s). The realization of this strategy is detailed below.



8 **6**

Alloc-Ser[$PO(OCH_2CH{=}CH_2)_2$]-OH, **6** was readily prepared in multigram quantity[13] using the four step procedure described[9] for preparing Alloc-Ser[$PO(OtBu)_2$)]-OH, **5** which we had previously used to prepare the dibenzyl analog **7**.[12] In addition, we have developed an even simpler two step preparation of **6** by adapting Bannwarth's method for the synthesis of tyrosine phosphate esters.[14] In this protocol[14] the acid function of Fmoc-Tyr-OH is transiently protected "in situ" as the t-butyldimethylsilylester, which allows phosphoramidate mediated phosphitylation followed by oxidation to the phosphate stage with aqueous work up yielding the Fmoc-Tyr(PO_3R_2)-OH derivatives. In our hands, although this method was satisfactory for the preparation of

Scheme 1



a. Fmoc-Amino Acids Trt=trityl, tBoc=t-butyloxycarbonyl, tBu= t-butyl

Amino acid couplings 3eq. DIC-HOBt-FmocAA (1:1:1) preactivated in DMF

Fmoc cleavage: Piperidine-DMF (20%)

b. 3eq. Alloc-Ser[PO($OCH_2CH=CH_2$)$_2$)]-OH\DIC\HOBt (1:1:1) DMF

c. Me_3SiN_3\$Bu_4NF(3H_2O)$\(8:3), 20mol% $Pd(PPh_3)_4$, CH_2Cl_2 RT 30min

d. 3eq Boc-Lys(Boc)-Ile-Gly-OH\DIC\HOBt (1:1:1), double coupling 3hrs

e. CF_3COOH, 5% ethanedithiol

Boc and Fmoc serine phosphate esters, the results with Alloc-Ser-OH were poor. Reasoning that a great part of the difficulty with Alloc-Ser-OH was its low solubility, we prepared the corresponding dicyclohexylamine salt Alloc-Ser-OH:DCHA, **8**[15] which was soluble in dichloromethane. Indeed, **8** could be silylated, *in situ*, with no additional base in dichloromethane followed by the standard phosphorylation protocoll, workup and chromatography to give **6** in moderate yield.[16] Thus, an expedient route to **6** is available which is preferable for smaller scale application.[17]

The tau phosphopeptide **1**, which we were unable to prepare by post synthetic, "global" phosphorylation[18] of the unprotected, but apparently inaccessible serine hydroxyl function, was the target of the combined Fmoc-Alloc building block strategy as conceived above. Hexapeptide H-Thr(tBu)-Glu(tBu)-Asn(Trt)-Leu-Lys(t-Boc)-His(Trt)-Wang,[19] **9**, was prepared by Fmoc-SPPS (Scheme 1) either manually with standard diisopropylcarbodiimide-hydroxybenzotriazole (DIC-HOBt) mediated couplings and piperidine Fmoc cleavages or on the ABI-430A automated peptide synthesizer using the Fast-Fmoc cycles routine.[20] The simple DIC-HOBt coupling of **6** gave the best results proceeding smoothly to give **10**.[21] The following key concomitant deprotection of the Alloc and phosphate allyl ester groups using our method[12] (Me_3SiN_3\\$Bu_4NF(3H_2O)$\20mol% $Pd(PPh_3)_4$ in CH_2Cl_2, 30min room temperature) followed by DMF resin washing gave excellent results. The reaction was analyzed by standard cleavage of the product from the resin with trifluoroacetic acid containing ethanedithiol as a cation scavenger (Scheme 1). HPLC analysis of this crude cleavage product showed virtually a single peak which corresponded to H-Ser($PO(OH)_2$)-Thr-Glu-Asn-Leu-Lys-His-OH from the FAB-MS spectrum. In the chain elongation with the next amino acid, Fmoc-Gly-OH, ninhydrin monitoring of the coupling reaction proved unreliable and HPLC analysis after cleavage from a resin sample was required for this purpose. Although the coupling at the N-terminal of **11** was sluggish, the reaction could be driven to virtual completion without significant side product formation (HPLC analysis) with a single repetition of the coupling procedure (double coupling). After a subsequent synthesis cycle (Fmoc-cleavage, coupling) HPLC analysis indicated increased side product formation. Thus, we encountered problems of an undetermined nature with the free phosphate which compromise chain extension *via* the standard Fmoc method. Indeed Wakamiya has reported use of the free serine phosphate building block **4** in standard Fmoc SPPS to be accompanied by more side reactions. This stands in marked contrast to the unproblematic sequential chain extension using the benzyl monophosphate protection strategy. It was apparent that the best strategy for phosphopeptide synthesis using our method would involve a single convergent fragment coupling onto the free phosphoserine N-terminal followed by liberation of the product directly from the resin. To this end Boc-Lys(Boc)-Ile-Gly-OH prepared on Sasrin resin[22] was appended to **11** via double DIC-HOBt coupling. Cleavage of the product **12** from the resin gave a crude product which demonstrated a single major peak of ca. 40% by HPLC.[23] Purification to analytical HPLC homogeneity by semipreparative HPLC[24] showed the major peak to be the desired phosphopeptide **1** (ca.30% yield, FAB-MS). For more complete characterization, 1D-WATERGATE[25] and 2D-HMBC[26] (heteronuclear multiple bond phosphorus-proton correlated) NMR experiments on **1** were performed at 400MHz in water. These NMR analyses indicated **1** to be very pure aside from a minor component (ca 8%) which is presumed to be the isomer having the corresponding D-histidine stereochemistry.[27] The HMBC experiment allowed the unambiguous assignment of the phosphate moiety to the serine residue. For purposes of comparison we have independently prepared **1** via Wakamiya's Fmoc method using Fmoc-Ser[PO(OH)(OCH_2Ph)]-OH, **3**. We found the method to be satisfactory and suitable for use in

automated Fmoc-synthesis using the HBTU coupling reagent.[20] The yield of **1** after purification was comparable to that obtained with our method.

In summary, a new combined Fmoc-Alloc general building block SPPS strategy has been successfully applied to the synthesis of the tau phosphopeptide **1** which was inaccessible by the standard post synthetic phosphorylation strategy. Experiments to raise antibodies to **1** which may cross react with the corresponding epitope of the tau phosphoprotein relevant to Alzheimer's Disease are currently in progress and will be reported in due course. At this point, the Fmoc procedure using **3** appears to be the most practical method for synthesizing serine phosphopeptides. Nevertheless, there may be cases where the coupling of **3** onto a given N-terminus is problematic while the coupling with fully protected **6** may be achieved and provide an alternative. Finally, our method provides the possiblity for further modification of a resin bound free phosphate group as found in **11** and **12**. Such a resin bound free serine phosphate has been crucial to the synthesis of peptide serine adenosine phosphates which are bisubstrate inhibitors of protein kinases.[28] These compounds have been prepared by the coupling of adenosine phosphates to the resin bound phosphopeptide, Boc-Leu-Arg(PMC)-Arg(PMC)-Ala-Ser(PO_3H_2)-Leu-Gly-Wang, in which the free serine phosphate was introduced by a postsynthetic phosphorylation-allyl phosphate deprotection protocoll. For this class of compounds an attractive alternative building block method now exists for sequences which are not amenable to the postsynthetic phosphorylation methodology.

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9. Lacombe, J. M.; Andriamanampisoa, F. and Pavia, A. A. *Int. J. Peptide Protein Res.*, **1990**, *36*, 275.
10. Wakamiya, T.; Saruta, K.; Yasuoka, J. and Kusumoto, S. *Chemistry Lett.* **1994**, 1099. Compound **3** is now commercially available from Novabiochem, Switzerland.
11. The most commonly employed post assembly solid phase phosphorylation procedure involves phosphitylation with a large excess of phosphoramidite, followed by an oxidation step (eg. t-BuOOH, I_2). [3b] Methionine, cysteine and tryptophan residues are also oxidized under these conditions, thus also limiting this strategy. Phosphorylation with phosphoryl chlorides (Otvos, L., Elekes, I. and Lee, *Int. J. Peptide Protein Res.*, **1989**, *34*, 129.) obviates an oxidation step, but difficulties have also been encountered with this method.[3b]
12. Shapiro, G. and Büchler, D. *Tetrahedron Lett.* **1994**, *35*, 5421. This procedure is particularly intended for solid phase applications where the allyl azide formed may simply and safely be disposed of in solution. Extreme caution should be exercised in applying the method to standard solution phase chemistry.

13. After chromatographic purification **6** was obtained as an oil which in pure form was sensitive to polymerization and as such is better stored in the cold in dilute solution. Alternatively **6** was converted to its analytically pure dicyclohexylamine (DCHA) salt in methyl-t-butyl ether (ethyl acetate/methyl-t-butyl ether recrystallization, mp. 99-104°C. The DCHA salt of **6** was stable to storage at room temperature and can be converted to the free acid by extracting from citric acid solution with methyl-t-butyl ether. **6** (free acid): ^{1}H-NMR (400MHz, $CDCl_3$) d= 8.45 (broad singlet, NH), 5.83-5.99 (m, 3H), 5.17-5.39 (m, 6H), 4.31-4.63 (m, 8H), 4.23-4.34 (m, 1H). FAB-MS MH^+= 350. 13C-NMR (100MHz, $CDCl_3$) d= 170.5, 155.8, 132.2, 131.7, 131.6, 118.8, 118.7, 117.7, 68.6, 67.4, 65.8, 53.9. $[\alpha_D]^{20}$= +25.2, c=1.0 in CH_2Cl_2.
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15. Alloc-Ser-OH:DCHA **8**, was recrystallized from methyl-t-butyl ether, mp 148-150°C, $[\alpha_D]^{20}$= +13.0, c=1.6 in CH_2Cl_2.
16. To a solution of the Alloc-Ser-OH:DCHA, **8**, (3.54 g, 9.5 mmol) in 100mL dry dichloromethane under argon was added a solution of *tert*-butyldimethyl chlorosilane (1.57 g, 10.4 mmol) in 10mL CH_2Cl_2. After stirring for 30 minutes at room temperature, *1H*-tetrazole (2.0 g, 28.4 mmol) and a solution of diallyl *N,N*-diisopropyl phosphoroamidite (4.64g, 18.9 mmol) in 10mL CH_2Cl_2 were added. After stirring for 3 hours at room temperature, the mixture was cooled to -40°C and *tert*-butylhydroperoxide (6.3 mL, 3M in toluene, 18.9 mmol) was added. The mixture was stirred for 30 minutes at this temperature and 1.5 hours at room temperature. After cooling the reaction to 0°C, 100mL of 10 % $Na_2S_2O_5$ were slowly added, the organic phase separated and washed again with 100 mL with 10 % $Na_2S_2O_5$. The solution was concentrated, and the residue dissolved in 50 mL of methyl *tert*-butylether and washed with 10% citric acid (2x100 mL) and brine (1x100 mL). The organic phase was dried over Na_2SO_4 and concentrated to a dark yellow oil which was purified by flash chromatograpy eluting with a gradient CH_2Cl_2/MeOH 20:1 to CH_2Cl_2/MeOH /AcOH 10:1:0.1 This gave 1.4 g (42%) of Alloc-Ser[P(O)(OAllyl)$_2$]-OH, **6**, as a light yellow oil.
17. The chromatographic purification of **6** prepared as above is an absolute requirement whereas crude material received from the clean last step as described in ref. 9 is of sufficient purity to use directly.
18. Use of either the method described in 3a or 3b resulted in insignificant if any phosphorylation product, **1**, but a good yield of the unphosphorylated material.
19. Fmoc-His(Trt)-Wang, (Wang, S. *J. Am. Chem. Soc.* **1973**, *95*, 1328) and Fmoc amino acids were purchased from Novabiochem, Switzerland.
20. The Fast-Fmoc cycles routine from Applied Biosystems Inc. (ABI) employs benztriazolyl-tetramethyluronium hexafluorophosphate salt (HBTU) as a coupling reagent: Knorr, R; Trzeciak, A.; Bannwarth, W. and Gillessen D. *Tetrahedron Lett.* **1989**, *30*, 1927.
21. Standard DIC-HOBt mediated coupling was found to be preferrable to the more rapid coupling methods such as HBTU which gave uncharacterized side products by RP-18 analytical HPLC analysis. HPLC and FAB-MS analysis after cleavage of a resin sample demonstrated that Alloc-Ser(PO($OCH_2CH{=}CH_2$)-Thr-Glu-Asn-Leu-Lys-His-OH was formed cleanly.
22. Mergler, M.; Tanner, R.; Gosteli, J. and Grogg, P. *Tetrahedron Lett.* **1988**, *29*, 4005. Sasrin resin was purchased from Bachem, Switzerland. Boc-Lys(Boc)-Ile-Gly-OH was purified by simple chromatography over silica gel eluting with ethyl acetate-hexane (1:1).
23. Analytical HPLC was performed using a 5μ C18-nucleosil 300 column (100mm X 4mm) from Macherey-Nagel (Switzerland) and a gradient 100A:0B, 40A:60B over 20min. flow=1.5mLmin^{-1} (A = H_2O/H_3PO_4 100: 0.1; B = CH_3CN/H_3PO_4 100:0.1) UV detection at 205nm. Retention time for **1** = 4.58min.
24. Semipreparative HPLC, Vydac 218TP1022 column (22 X 250mm), C18 10μ, water-acetonitrile 0.1% TFA gradient.
25. Working in water with the WATERGATE pulse sequence allows the amide signals to be observed, Piotto, M.; Saudek, V.and Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661.
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27. The distinctive, well resolved singlet for the C4-imidazole ring proton of **1** was accompanied by an almost baseline resolved upfield singlet (ca. 8% by peak height) which is best accounted for by the isomeric D-His-**1** species. The problem of racemization upon attaching histidine derivatives to Wang resin is well recognized, but this was not determined for the His(Trt)-Wang *a priori*.
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