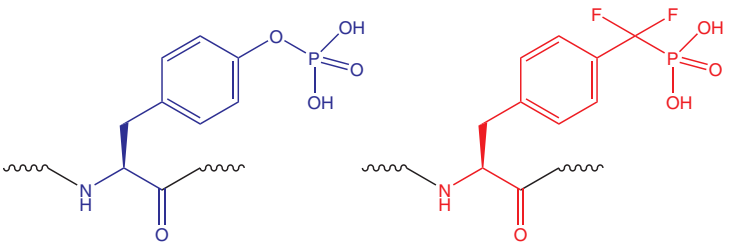
Novabiochem®

Innovations 1/11

Guide to the synthesis of phosphotyrosine related peptides by Fmoc SPPS



pTyr F_2Pmp

Phosphorylation and de-phosphorylation of key tyrosine residues within cytoplasmic proteins by protein-tyrosine kinases (PTKs) and phosphatases (PTPs), respectively, are key events in the cell signaling pathway. Phosphotyrosine-containing peptides are thus important tools for studying PTPs [1] and the phosphotyrosine-binding SH2 and PTB domains [2] of signaling proteins. However, the transient nature of the tyrosine phosphate group in biological systems has led to the development of hydrolytically stable pTyr analogs such by difluorophosphonomethylphenylalanine (F_2Pmp) [3], in which the oxygen of the phenyl phosphate has been replaced by a difluoromethylene group.

In this innovation, we explore the chemistry of the different derivatives available of the synthesis of phosphotyrosine and F2Pmp-containing peptides. Each approach has its own merits, and this discussion is intended to help you make an informed choice as to the best strategy to use for a particular application. The procedures and applications given are a compilation of methods developed at Novabiochem® and tested literature procedures.

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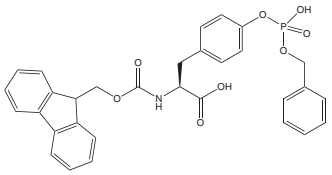
Introduction of phospho-tyrosine derivatives in Fmoc SPPS

Fmoc-Tyr(PO(OBzl)OH)-OH



Fmoc-Tyr(PO(OBzl)OH)-OH is the most frequently used derivative of introduction of phosphotyrosine in Fmoc SPPS [4]. However, the partially protected phosphate can cause issues during coupling and chain extension. Poor incorporations were observed using PyBOP® (or BOP) and DIPCDI, which have been ascribed to involvement of the phosphate hydroxyl in the activation process; fortunately, uronium-based coupling reagents appear to be effective [5]. Increasing the excess of DIPEA used with the uronium reagent from the typical two to three-fold excess has been found to be beneficial (Method 1). For example, the yield in the sterically challenging coupling of Fmoc-Thr(PO(OBzl)OH)-OH to H-Val-resin increased from 80% to 100% [6].

The partially protected phosphate can form salts with piperidine during Fmoc removal [7]. Acylation of this piperidine results in a reduction in the excess of activated amino acid present in the coupling reaction. Whilst this does not pose a significant problem when the peptide contains only one phosphoamino acid, it can lead to serious difficulties with incomplete coupling reactions with peptides containing numerous phosphorylated residues. Fortunately, this problem can be overcome by simply increasing the excess of coupling reagents used or by exchanging the piperidine counterion for a tertiary amine, as described in Method 2.

The benzyl side-chain protecting groups are normally removed in 1-2 hours during the course of the standard 95% TFA. Benzylation of sensitive residues such as Tyr, Cys during the cleavage reaction has occasionally been observed. In such cases, the use of EDT in the cleavage mixture should minimize this side reaction.

Method 1: Coupling protocol for phosphoamino acid building blocks

1. Dissolve Fmoc-Aaa(PO(OBzl)OH)-OH (5 eq.[a]), HCTU or HATU (5 eq.[a]) in the minimum volume of DMF.
2. Add DIPEA (15 eq.[a]) to mixture, mix and add immediately to Fmoc-deblocked peptide resin.
3. Allow to couple for 1-2h.
4. Check completeness of reaction with the Kaiser or TNBS test. Wash resin and repeat reaction if necessary.

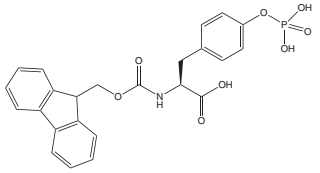
[a]Relative to resin substitution.

Method 2: Phosphate counterion exchange

1. Wash resin with DMF (2 x).
2. Wash resin twice with DMF containing DIPEA (20 eq.)[a] and TFA (18 eq.)[a].
3. Wash resin with DMF (2 x).

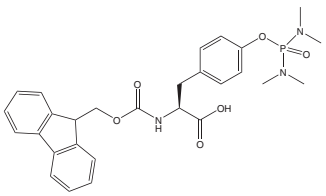
[a]Relative to phosphate content of resin.

Fmoc-Tyr(PO_3H_2)-OH



Fmoc-Tyr(PO_3H_2)-OH is the simplest and most cost effective derivative for the introduction of phosphotyrosine by Fmoc synthesis [8]. As the phosphate group is not protected, there are no issues with protecting group removal as can be the case with other derivatives. However, like the situation with Fmoc-Tyr(PO(OBzl)OH)-OH, the lack of phosphate protection does lead to problems during its introduction and that of subsequent residues. Coupling is generally very sluggish and is best achieved using HATU in conjunction with at least 3 equivalents of DIPEA (Method 1). In peptides containing multiple phosphotyrosine residues, piperidine counterion exchange should be carried out as described in Method 2. Furthermore, pyrophosphate formation has been observed in peptides containing adjacent Tyr(PO_3H_2) residues [9, 10].

Fmoc-Tyr(PO(NMe_2)$_2$)-OH



In contrast to Fmoc-Tyr(PO_3H_2)-OH and Fmoc-Tyr(PO(OBzl)OH)-OH, which have potentially reactive acid functionalities in their side-chains, the phosphate group in Fmoc-Tyr(PO(NMe_2)$_2$)-OH [11] is fully protected. This confers not only improved solubility properties on the compound but, more importantly, eliminates pyrophosphate formation [9, 10], incompatibility with PyBOP® and carbodiimide coupling reagents [5], and quenching of activated amino acid derivatives [7] associated with the use of the previously mentioned derivatives.

Fmoc-Tyr(PO(NMe_2)$_2$)-OH can be introduced using any of the standard coupling methods, such as PyBOP®/DIPEA, TBTU/DIPEA and DIPCDI/HOBt, and in peptides containing multiple phosphotyosine residues, it is not necessary to increase the excess of reagents to compensate for the consumption of activated amino acid derivatives through piperidide formation.

Application 1: Synthesis of H-Gly-Asp-Phe-Glu-Ile-Pro-Glu-Glu-Tyr(PO_3H_2)-Leu-NH_2 using Fmoc-Tyr($PO(NMe_2)_2$)-OH

H-Gly-Asp(OtBu)-Phe-Glu(OtBu)-Ile-Pro-Glu(OtBu)-Glu(OtBu)-Tyr($PO(NMe_2)_2$)-Leu-Rink Amide MBHA resin was prepared automatically using a NovaSyn®Crystal peptide synthesizer on Rink Amide MBHA resin. All acylation reactions were carried out using a 5-fold excess of Fmoc-amino acid activated with 1 eq. of PyBOP®in the presence of 1 eq. of HOBt and 2 eq. of DIPEA. A coupling time of 1 h was used throughout. The peptidyl resin was treated with TFA/TIS/water (95:2.5:2.5) (5 ml) for 3 h, after which time a sample was removed for HPLC analysis (Figure 1). Water (0.5 ml) was then added and the mixture was left to stand for 22 h. The peptide was isolated in the usual manner by evaporation and ether precipitation. The crude peptide was analyzed by HPLC (Figure 2) and ES-MS [expected M+H$^+$ 1291.1, found 1290.3].

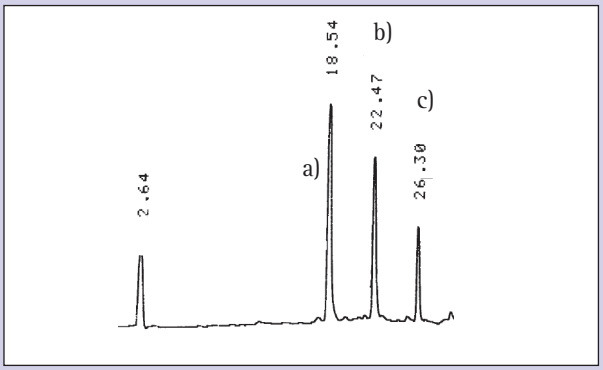


Fig. 1: HPLC elution profile of cleavage reaction after 3 h. a) Product; b) partially protected phosphate product; c) fully protected phosphate product.

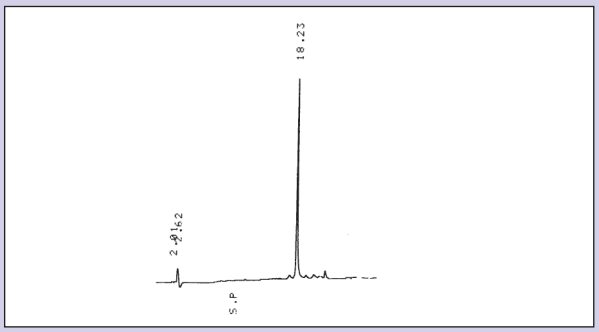
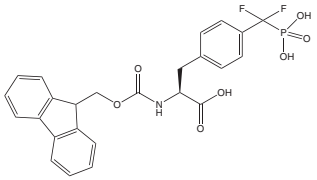


Fig. 2: HPLC elution profile of cleavage reaction after 22 h.

Regeneration of phosphotyrosine from the phosphodiamidate is effected by acid catalyzed hydrolysis. Detachment of the peptide from the resin and deprotection of side-chains is first carried out in the usual manner with TFA containing the appropriate scavengers. 10 % by volume of water is then added to the cleavage solution and the mixture left to stand overnight, during which time hydrolysis of the phosphodiamidate takes place.

The reaction can be monitored by HPLC by following the disappearance of the more hydrophobic phosphodiamidate and phosphoamidates, as illustrated in Application 1. Since the reaction does not liberate any reactive cationic species, there is no risk of formation of alkylated by-products, as can be the case with Fmoc-Tyr(PO(OBzl)OH)-OH.

Fmoc–Phe($CF_2PO_3H_2$)–OH



Fmoc-Phe($CF_2PO_3H_2$)-OH (Fmoc-F_2Pmp-OH) was developed by Burke and coworkers [3] to overcome the limitations of the original phosphoTyr isostere phosphonophenylalanine (Pmp) [12]. The former isostere, whilst overcoming the inherent instability of the nature Tyr phosphate moiety, appears to be a poor substitute for pTyr, as substitution of pTyr by Pmp often leads to a significant reduction in biological activity [13]. It was postulated this effect was due to the lack of the H-bond acceptor phenyl oxygen and incomplete ionization of the phosphonic acid at neutral pH (Pmp, pKa2 7.72 vs pTyr, pKa2 6.22). F_2Pmp on the otherhand has a pKa2 of 5.71 and is therefore fully ionized at neutral pH, and the methylene fluorine atoms can undergo H-bonding. Peptides substituted with F_2Pmp exhibit higher binding affinities to SH2 domains than Pmp analogs [14]. 1000-Fold enhancements in affinities of F_2Pmp-containing peptide compared with those containing Pmp have been observed in assays against PTPs [15].

Fmoc-F_2Pmp-OH is the preferred derivative for the introduction of F_2Pmp residues as its use avoids the harsh conditions required to remove the ethyl protecting group usually employed for phosphonate protection [16]. However, lack of phosphate protection does result in sluggish coupling of Fmoc-F_2Pmp-OH and the subsequent amino acid derivative. Overnight coupling with HATU and 3 equivalents of DIPEA appears to be effective (Application 2).

Application 2: Synthesis of H-Trp-Ser-Lys-Asp-Thr-Ser-Phe($CF_2PO_3H_2$)-Ala-OH using Fmoc-Phe($CF_2PO_3H_2$)-OH

H-Trp(Boc)-Ser(tBu)-Lys(Boc)-Asp(OtBu)-Thr(tBu)-Ser(tBu)-Phe($CF_2PO_3H_2$)-Ala-Wang resin was prepared automatically using an ABI 433 peptide synthesizer on Rink Amide MBHA resin. Phe($CF_2PO_3H_2$) was introduced by treatment of the peptidyl resin with Fmoc-Phe($CF_2PO_3H_2$)-OH/HATU/DIPEA 3:3:9 for 18 h. All other acylation reactions were carried out using a 5-fold excess of Fmoc-amino acids activated with 1 eq. of HBTU in the presence of 1 eq. of HOBt and 2 eq. of DIPEA for 1 h. The product was isolated by treating the peptidyl resin with TFA/TIS/water (95:2.5:2.5) (5 ml) for 3 h. The crude peptide was analyzed by HPLC (Figure 3) and ES-MS [expected M+H$^+$ 1071.4, found 1071.5].

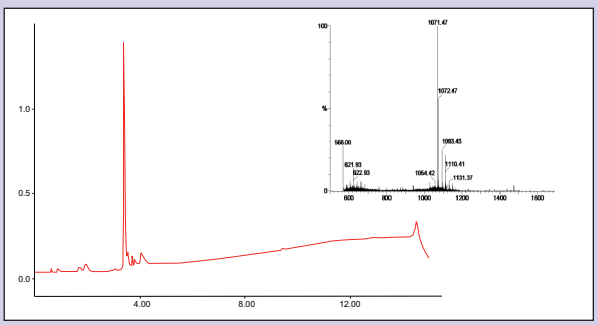


Fig. 3: HPLC elution profile and ESI-MS of crude phosphonopeptide.

Ordering information

852069	Fmoc-Ser(PO(OBzl)OH)-OH	1 g
		5 g
852244	Fmoc-D-Ser(PO(OBzl)OH)-OH	1 g
		5 g
852070	Fmoc-Thr(PO(OBzl)OH)-OH	1 g
		5 g
852245	Fmoc-D-Thr(PO(OBzl)OH)-OH	1 g
		5 g
852071	Fmoc-Tyr(PO(OBzl)OH)-OH	1 g
		5 g
852246	Fmoc-D-Tyr(PO(OBzl)OH)-OH	1 g
		5 g
852058	Fmoc-Tyr(PO_3H_2)-OH	1 g
		5 g
852090	Fmoc-Tyr($PO(NMe_2)_2$)-OH	1 g
		5 g
852288	Fmoc-Phe($CF_2PO_3H_2$)-OH	100 mg

Please enquire for bulk quantities

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