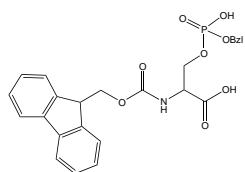


# Optimized protocols for the introduction of phosphoamino acids

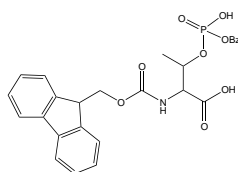
No. 4/01

## Fmoc-Aaa(PO(OBzl)OH)-OH

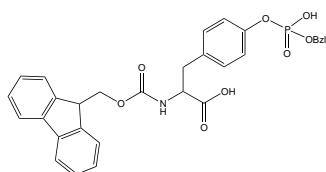
### Fmoc-Ser(PO(OBzl)OH)-OH



### Fmoc-Thr(PO(OBzl)OH)-OH



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



Since their commercial introduction by Novabiochem® in 1995, Fmoc-Ser(PO(OBzl)OH)-OH [1], Fmoc-Thr(PO(OBzl)OH)-OH [2, 3] and Fmoc-Tyr(PO(OBzl)OH)-OH [2] have become the reagents of choice for the preparation of phosphopeptides by Fmoc chemistry because their use permits the direct step-wise solid phase synthesis of such peptides without recourse to difficult and cumbersome postsynthetic phosphorylation procedures.

In contrast to triester derivatives of phosphoserine and -threonine which undergo piperidine-mediated  $\beta$ -elimination, Ser(PO(OBzl)OH) and Thr(PO(OBzl)OH) residues are fully compatible with the standard conditions employed for Fmoc group removal. This stability can be attributed to ionization of the phosphate groups inhibiting elimination under basic conditions (Figure 1).

In recent years, however, a number of researchers have observed difficulties with coupling and chain extension that appear to be directly attributable to the partially protected phosphate group.

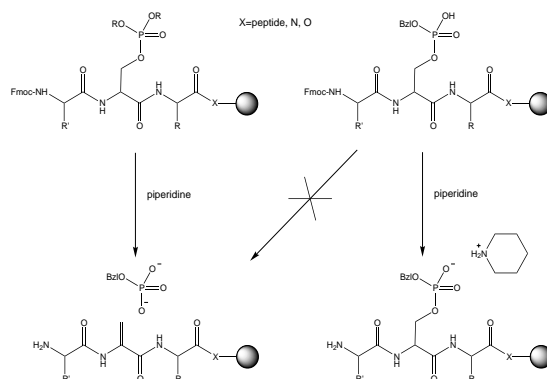


Fig. 1: Stability of triester versus diester phosphoamino acid residues by piperidine.

Pascal, *et al.* [4] have found that acylation of the piperidine associated with partially protected phosphate groups can lead to a reduction in the excess of activated amino acid and tertiary amine present in the coupling reaction (Figure 1). Whilst this may not pose a significant problem when the peptide contains only one phosphoresidue, this phenomenon could in principle lead to serious difficulties with incomplete coupling reactions in the synthesis of multiphosphorylated sequences. 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 (Method 1).

Perich, *et al.* [5] have observed poor incorporation of monobenzyl protected phosphoamino acids when using PyBOP® (or BOP) and DIPCDI coupling methods, which they ascribed to involvement of the phosphate hydroxyl in the activation process. They found uronium-based coupling reagents in conjunction with HOBT/HOAt and DIPEA to be the most effective of the reagents tested.

The problem of poor coupling of mono-benzyl protected phosphoamino acids was further addressed in a recent paper [6]. Using the challenging coupling of Fmoc-Thr(PO(OBzl)OH)-OH to a resin-bound Val as a test system, it was found that, by simply employing a 3-fold excess of DIPEA in the standard TBTU/HOBT/DIPEA coupling regime advocated by Perich, *et al.*, incorporation of the phosphothreonine residue could be increased from 80% to 100% (Method 2). The rationale for this observation was that under standard conditions the quantity of base used is insufficient for efficient activation as 1 eq. of base will be neutralized by the acidic partially protected phosphate group. The efficacy of this approach is exemplified in Applications 1 and 2.



## Ordering Information

<a href="#">04-12-1154</a> Fmoc-Ser(PO(OBzl)OH)-OH	1 g
	5 g
<a href="#">04-12-1155</a> Fmoc-Thr(PO(OBzl)OH)-OH	1 g
	5 g
<a href="#">04-12-1156</a> Fmoc-Tyr(PO(OBzl)OH)-OH	1 g
	5 g
<b>Novabiochem's other phosphoamino acids:</b>	
<a href="#">04-12-1125</a> Fmoc-Tyr(PO <sub>3</sub> H <sub>2</sub> )-OH	1 g
	5 g
<a href="#">04-12-1205</a> Fmoc-Tyr(PO(NMe <sub>2</sub> ) <sub>2</sub> )-OH	1 g
NEW	5 g

## Practical procedures

To overcome quenching of activated amino acid by resin-bound piperidinium salts, the procedure described in Method 1 should be inserted into the synthetic cycle between the Fmoc deprotection reaction and coupling reaction. Alternatively, the excess of activated amino acid derivative can be increased by one equivalent for every phosphoamino acid present in the peptide chain.

### Method 1: Phosphate counterion exchange

1. Wash resin with DMF (2 x).
  2. Wash resin twice with DMF containing DIPEA (20 eq.<sup>a</sup>) and TFA (1.8 eq.)<sup>a</sup>.
  3. Wash resin with DMF (2 x).
- <sup>a</sup>Relative to phosphate content of resin.

### Method 2: TBTU activation of Fmoc-Aaa(PO(OBzl)OH)-OH

1. Dissolve Fmoc-Aaa(PO(OBzl)OH)-OH (5 eq.<sup>a</sup>), TBTU (5 eq.<sup>a</sup>) and HOBt (5 eq.<sup>a</sup>) in the minimum volume of DMF.
2. Add DIPEA (15 eq.<sup>a</sup>) 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.

<sup>a</sup>Relative to resin substitution.

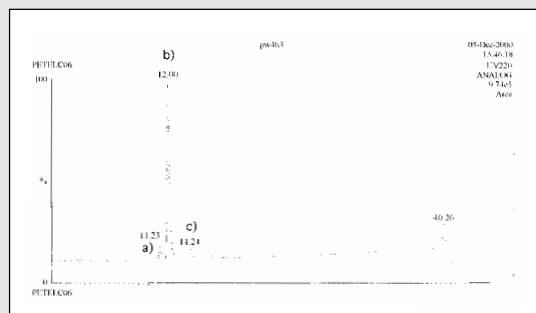
## Applications

To test the efficacy of Method 2, the following model peptides were prepared and the crude products subjected to detailed analysis by LC-MS:

### Application 1: Synthesis of H-Gly-Phe-Glu-Thr(PO<sub>3</sub>H<sub>2</sub>)-Val-Pro-Glu-Thr(PO<sub>3</sub>H<sub>2</sub>)-Gly-NH<sub>2</sub> (1)

H-Gly-Phe-Glu(OtBu)-Thr(PO(OBzl)OH)-Val-Pro-Glu(OtBu)-Thr(PO(OBzl)OH)-Gly-NovaSyn® TGR resin was prepared automatically using a NovaSyn® Crystal peptide synthesizer on NovaSyn® TGR 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, with the exception of Fmoc-Thr(PO(OBzl)OH)-OH which

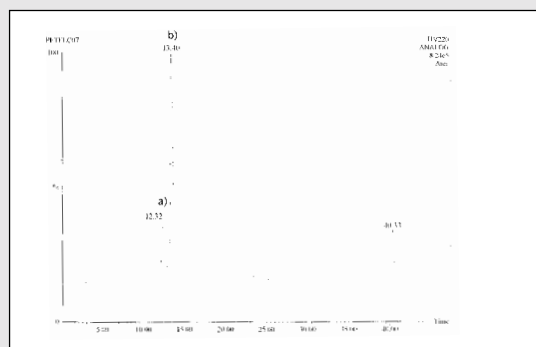
was introduced using Method 1. A coupling time of 1 h was used throughout. The peptidyl resin was treated with TFA/ TIS /water 95:2.5:2.5 (5 ml) for 3 h, and then the peptide was isolated in the usual manner by evaporation and ether precipitation. The crude peptide was analyzed by HPLC (Figure 2) and ES-MS.



**Fig. 2:** HPLC elution of total crude peptide 1. Peak a: des-Val-1 [expected M+1 996.3, found M+1 996.3]; peak b: 1 [expected M+1 1095.4, found 1095.3]; peak c: 1 + benzyl [expected M+1 1185.4, found M+1 1185.4].

### Application 2: Synthesis of H-Ala-Asp-Phe-Glu-Ser(PO<sub>3</sub>H<sub>2</sub>)-Ile-Pro-Ser(PO<sub>3</sub>H<sub>2</sub>)-Glu-Ser(PO<sub>3</sub>H<sub>2</sub>)-Leu-NH<sub>2</sub> (2)

H-Ala-Asp(OtBu)-Phe-Glu(OtBu)-Ser(PO(OBzl)OH)-Ile-Pro-Ser(PO(OBzl)OH)-Glu-Ser(PO(OBzl)OH)-Leu-NovaSyn® TGR resin using a NovaSyn® Crystal peptide synthesizer on NovaSyn® TGR resin as described in Application 1, except a 10-fold excess of coupling reagents was used. The peptidyl resin was treated with TFA/ TIS /water 95:2.5:2.5 (5 ml) for 3 h, and then the peptide was isolated in the usual manner by evaporation and ether precipitation. The crude peptide was analyzed by HPLC (Figure 3) and ES-MS.



**Fig. 3:** HPLC elution profile of total crude peptide 2. Peak a: des-Ile-2 [expected M+1 1320.4, found M+1 1320.3]; peak b: 2 [expected M+1 1433.5, found 1433.4].

## References

1. T. Wakamiya, et al. (1994) *Chem. Lett.*, 1099.
2. P. White & J. Beythien in "Innovations & Perspectives in Solid Phase Synthesis & Combinatorial Libraries, 4th International Symposium", R. Epton (Ed.), Mayflower Scientific Ltd., Birmingham, 1996, pp. 557.
3. T. Vorherr, et al. (1995) *Bioorg. Med. Chem. Lett.*, 5, 2661.
4. R. Pascal, et al. in "Peptides 2000, Proc. 26th European Peptide Symposium", J. Martinez & J.-A. Fehrentz (Eds), Éditions EDK, Paris, 2001, pp. 263.
5. J. Perich, et al. (1999) *Lett. Pept. Sci.*, 6, 91.
6. P. White, poster 159 presented at the 17th American Peptide Symposium, San Diego, June 2001.

Novabiochem, NovaSyn and PyBOP are registered trademarks of Calbiochem-Novabiochem AG. Created 11/7/01 (P.W)

**Australia**  
Calbiochem-  
Novabiochem  
Tel 800 023 956  
Fax 800 678 508

**Germany**  
Calbiochem-  
Novabiochem GmbH  
Tel 0800 6931 000  
Fax 0800 62361 00

**Great Britain**  
CN Biosciences  
(UK) Ltd.  
Tel 0800 622935  
Fax (0115) 9430951

**Japan**  
Merck  
Japan Ltd.  
Tel 0120 189390  
Fax 0120 189350

**Switzerland**  
Calbiochem-  
Novabiochem AG  
Tel (062) 285 25 25  
Fax (062) 285 25 20

**USA & Canada**  
Calbiochem-  
Novabiochem Corp.  
Tel 800 228-9622  
Fax 800 432-9622