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PHOSPHO-SERINE AND PHOSPHO-THREONINE BUILDING BLOCKS FOR THE SYNTHESIS OF PHOSPHORYLATED PEPTIDES BY THE FMOC SOLID PHASE STRATEGY

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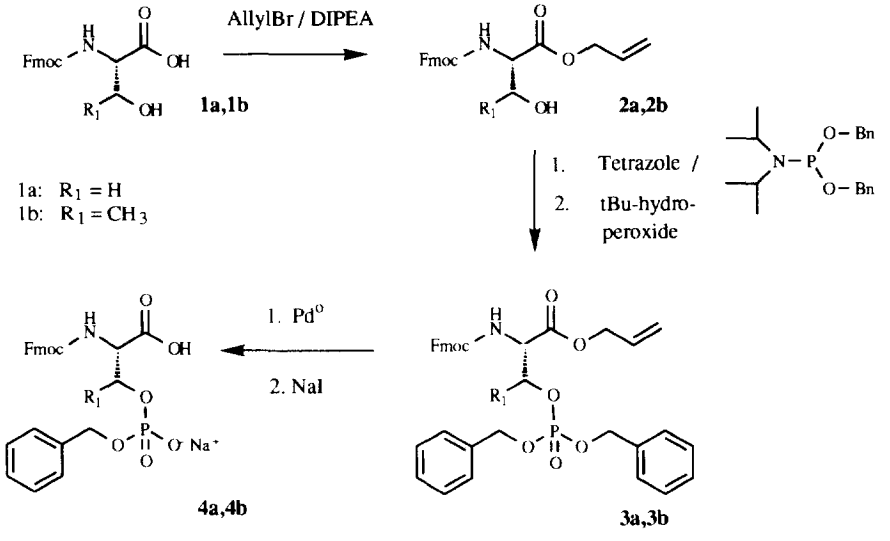
Abstract: The synthesis of phospho-Ser and phospho-Thr-containing peptides based on Nα-Fmoc-O-(monobenzylphosphono)-Ser and -Thr building blocks is described. The building blocks were obtained by phosphorylation of the Fmoc-protected allyl ester derivatives following oxidation, removal of the allyl-group and selective cleavage of one phospho benzyl ester function with NaI.

Phosphorylations and dephosphorylations of defined Ser, Thr, and/or Tyr residues play an important role in signal transduction in the regulation of various cellular processes. Phosphorylated peptides serve as adequate tools to study these phenomena on a structural and functional basis, especially as substrates for phosphatases. Several methods for the synthesis of phosphopeptides have been described. They can be divided into procedures for phosphorylation of the corresponding peptide after incorporation of the residue to be phosphorylated[1,2] dubbed as global phosphorylation and the application of appropriate building blocks for stepwise solid phase synthesis. Recently, we have reported on a comparison of the global phosphorylation and the building block approach for the Fmoc-based synthesis of phosphotyrosine-containing peptides applying different phosphate-protecting groups.[3,4] Whereas the global approach could be extended to certain Ser- and Thr-containing peptides[5], the phosphotriester building blocks for Ser and Thr show a tendency to eliminate to the corresponding dehydroamino acid residues. Larsson et al[6] reported the successful synthesis of a phospho-Thr-containing peptide using a O-dibenzyl-phosphono Fmoc derivative. These authors claim Fmoc deprotection in 20% morpholine in DMF is sufficiently mild to prevent ß-elimination. We aspired to overcome this inherent problem by synthesizing the phosphodiester building blocks **4a** and **4b** (Scheme). The phosphodiester function of these derivatives should result in a reduced tendency for ß-elimination during the basic steps employed for the removal of the Fmoc group. Furthermore, we had observed that phosphodiester functions are not activatied by uronium-type coupling reagents like HBTU or TPTU (unpublished results). While this work was in progress, a Fmoc protected Ser synthon and its application was reported using (benzyloxy)(trichloroethyloxy)(diisopropylamino)phosphine for phosphorylation of Fmoc-Ser-phenacyl ester.[7] This communication prompted us to present our results on phosphodiester approach for the synthesis of phospho-Ser and phospho-Thr-containing peptides.

We describe here an alternative synthesis for Nα-Fmoc-O-(monobenzyl phosphono)-Ser and -Thr building blocks[11] and their application in solid phase peptide synthesis.[12,13] Following the strategy outlined in the Scheme, we were able to obtain the phospho-Ser and phospho-Thr building blocks **4a** and **4b** in an overall yield of 37% and 38%, respectively.[11] When the carboxyl group was protected as the TBDMS-ester, we obtained also the desired derivatives **4a** and **4b** , albeit in a lower yield (23-25% and 13% respectively). The purity of the building blocks was checked by TLC and RP-HPLC and they were characterized by ^{1}H-NMR and ^{31}P-NMR as well as by Ion Spray Mass Spectrometry.

The sodium salts of **4a** and **4b** could be directly applied to solid phase peptide synthesis with TPTU activation in the presence of collidine or DIPEA[8], since the resulting activated ester dissolved in a 3:1 DMEU/acetonitrile mixture. The peptides **5-8** were synthesized using automated couplings for the standard Fmoc amino acid building blocks. Derivatives **4a** and **4b** were introduced manually and the reactions were assessed by the Kaiser test[9] (Table).

Scheme



After introduction of the phospho-Ser or the phospho-Thr building block, synthesis was completed by automated peptide synthesis and crude peptides (Table) were obtained by acidic cleavage.[12,13] HPLC analyses of crude peptides **5-8** are presented in Figure 1.[14]

Table. Sequences and MS data of nonphosphorylated and phosphorylated peptides

		mass (theor.)	mass (found)
5	T-G-F-L-T-E-Y-V-A-T-NH_2	1100.24	1099.6
6	T-G-F-L-T(phospho)-E-Y-V-A-T-NH_2	1180.22	1179.5
7	C-N-N-T-S-S-P-Q-P-K-K-NH_2	1202.37	1202.6
8	C-N-N-T-S-S(phospho)-P-Q-P-K-K-NH_2	1282.35	1282.0
9	T-E-P-Q-S(phospho)-Q-P-G-E	1051.96	1052.5
10	T-E-P-Q-T(phospho)-Q-P-G-E	1065.98	1066.5
11	T(tBu)-E(tBu)-P-Q(Trt)-T(PO_2-OBn)-Q(Trt)-P-G-E(tBu)	1809.07	1808.6

The peptides showed the characteristic ion series when analyzed by Ion Spray MS (data not shown). HPLC traces of the crude peptides and their phosphorylated derivatives indicate an optimal incorporation of both phospho building blocks resulting in a purity of the crude material similar to that observed for the unphosphorylated peptides (Figure 1).

Furthermore, we tested whether protected fragments could be prepared including a monobenzyl protected phospho diester derivative. Thus, the Fmoc group of fully protected resin bound peptide **11** was

removed on the solid support and the acid sensitive HMPB-linker was cleaved with 80% acetic acid at 80° C for 1h.

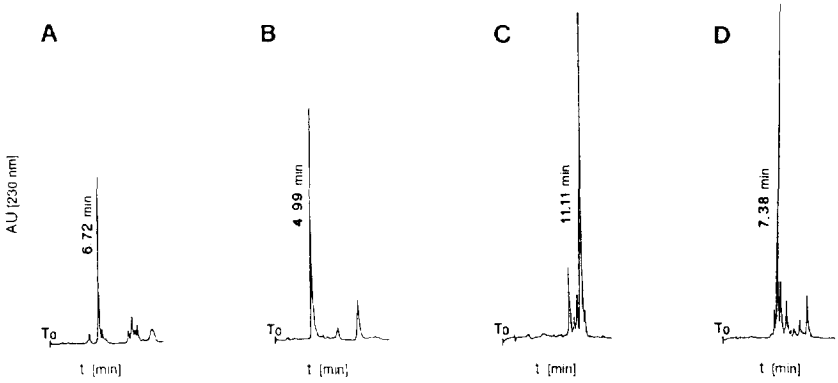


Figure 1: RP18-HPLC of crude peptides. [14] A: peptide **5**. B: peptide **6**. C: peptide **7**. D: peptide **8.**

The crude lyophilized material was applied to a Poros II R/H column[15] (Figure 2B). The main peak was isolated on a corresponding preparative Poros-HPLC column (100 x 25 mm) to afford the purified protected segment (Figure 2C).

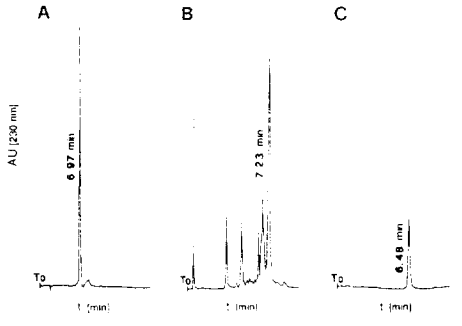


Figure 2: A: RP18-HPLC[14] of purified peptide **10**. B: Poros-HPLC[15] of crude peptide **11**. C: Poros-HPLC of purified peptide **11**.

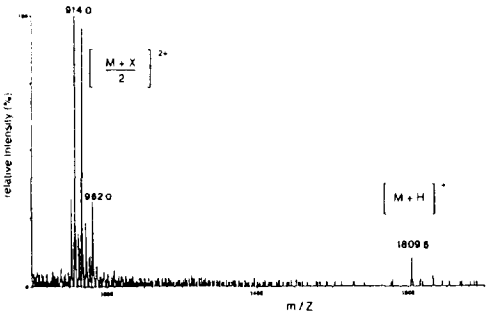


Figure 3: Ion spray MS of purified peptide **11**: 2^+ charged ions (X = Na, NH_3, K, and Ni adducts), monocharged $M+H^+$.

The HPLC profile of purified peptide **10** is shown for comparison (Figure 2A). The protected peptide was characterized by Ion Spray MS (Figure 3). This experiment demonstrated the potential for purification of protected peptide segments which would in principle allow for a reprotection at the N-terminus and application of the fragment in segment condensations.

In conclusion, we have outlined a synthetic procedure for monobenzyl protected Fmoc phospho-serine and Fmoc phospho-threonine derivatives by phosphinylation of the Fmoc-protected allyl esters followed by monodebenzylation with NaI. Furthermore, we have demonstrated application of these building blocks in solid phase synthesis of the corresponding phospho-serine and phospho-threonine-containing peptides. Couplings proceeded with high yield and after deprotection the phosphorylated peptides were obtained in high purity. In addition, the potential for the synthesis of phosphorylated protected peptide fragments was exemplified by synthesis and purification of protected peptide **11**.

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11. Preparation of **4a,b**: Fmoc protected derivatives **1a,1b** (0.13 mol) were reacted with allyl bromide (0.13 mmol) in the presence of DIPEA (0.13 mmol) in 200 ml of DMF at rt. The solvent was evaporated and the product was taken up in ethyl acetate followed by basic and acidic extractions. Addition of n-hexane afforded **2a** (yield: 50%),**2b** (yield: 76%). Derivatives **2a,2b** (78 mmol) dissolved in 200 ml of CH_3CN and 250 ml of CH_2Cl_2 were reacted with $iPr_2NP(OBzl)_2$ (156 mmol) and tetrazole (390 mmol in 800 ml in CH_3CN) under an Ar atmosphere. Oxidation was performed by adding t-butyl hydroperoxide in n-octane (150 mmol). After evaporation of the solvent, silica gel chromatography (CH_2Cl_2 / MeOH, 19:1) yielded **3a,3b** as oils. The allyl ester deprotection of derivatives **3a,3b** (15 mmol) was achieved with $Pd(PPh_3)_4$ (0.6 mmol), acetic acid (37.5 mmol) and tributylstannane (30 mmol) in 50 ml CH_2Cl_2 to prevent oxidation of Pd^0 by residual traces of peroxide. After evaporation of the solvent, column chromatography on silica gel (CH_2Cl_2 / MeOH, 19:1) yielded the O-(dibenzyl- phosphono) derivatives. Selective deprotection of one benzyl ester was achieved in CH_3CN (10 ml for 3.7 mmol of O(dibenzylphosphono) derivatives) by adding 2 equivalents of NaI. The resulting mono sodium salt (**4a,b**) precipitated directly (Ser derivative) or was precipitated after addition of diethylether. 73% (**4a**) and 50% (**4b**) yield starting from compounds **2a,2b** were obtained.
12. Peptides **5-8** were synthesized in parallel on a Syro II robotic synthesizer (Fa. MultiSyn Tech) using double coupling cycles (10 fold excess of Fmoc-derivative) starting from 100 mg of Fmoc-TMBHA-tentagel resin. The first coupling reaction involved activation by DIC/HOBt and the second coupling step was performed by activation with TPTU in the reaction vessel using 2 equivalents of collidine. Fmoc was cleaved by 30% piperidine in DMF. Phospho building blocks were activated with TPTU in DMEU/CH_3CN 3:1 using 2 equivalents of collidine and a 5 fold excess of derivative. The coupling step was repeated after 20 min and automated synthesis was continued. Peptides **5** and **6** were cleaved for 1.5h in TFA containing 2.5% of water, 2.5% of DTT. Peptides **7** and **8** were cleaved with reagent K.[10] Yields of crude peptides were > 80%, with respect to the initial loading of the support.
13. Peptides **9**,**10** and **11** were obtained by solid phase peptide synthesis on a Milligen 9050 continous-flow synthesizer[6] starting from HMPB-tentagel resin esterified with Fmoc-Glu(tBu). Peptides **9** and **10** were obtained after cleavage with reagent K (2h). Yields of crude peptides **9** and **10** were 77% and 82%, respectively, based on the initial substitution of the resin. Cleavage with 80% acetic acid at 80 degrees for 1h afforded crude peptide **11** in 60 % yield.
14. The peptides were characterized by analytical HPLC (Merck RP-18, 5µm, 4 x 125 mm, Lichrospher-100Å) using an acetonitril gradient (0-80% acetonitril in 30 min, flow: 1ml/min) and UV detection at 230 nm.
15. Poros HPLC was carried out on a Poros II R/H colum (100x 4.6 mm) running a gradient from 10% B to 100% B in 10 min (A: 0.1 % TFA, B: 80% acetonitril / 20% water; flow rate : 4ml/min; UV detection: 230 nm). The partially protected peptide **11** was obtained in 16% yield, with respect to the crude material.
16. Abbreviations: Bn: Benzyl; DIC: 1,3-Diisopropylcarbodiimide; DIPEA: Diisopropyl-ethylamine; DTT: Dithiothreitol; Fmoc: 9-Fluorenylmethoxy-carbonyl; DMEU: 1,3-Dimethyl-2-imidazolinone; HMPB: (4-Hydroxymethyl-3-methoxy) phenoxy- butyric acid; HOBt: 1-Hydroxybenzotriazole; $iPr_2NP(OBzl)_2$: Bis(benzyloxy)(diisopropylamino) phosphine; RP18-HPLC: Reversed phase (C18) high performance liquid chromatography; TBDMS: t-Butyl-dimethylsilyl; tBu: t-butyl; TMBHA: p-(R,S-α-1-(9H-Fluoren-9-yl)methoxyformamido 2,4-dimethoxybenzyl)) phenoxyacetic acid; TPTU: 2-(2-Oxo-1(2H)-pyridyl) 1,1,3,3-tetramethyluronium tetrafluoroborate; Trt: trityl.

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