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Short Communication

Evaluating the coupling efficiency of phosphorylated amino acids for SPOT synthesis

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Abstract: A high demand of interest concerning binding assays to study the consequences of posttranscriptional phosphorylation may be addressed by peptide array-based methods. A crucial factor for *de novo* chemical approaches to generate such arrays is the possibility to rationally permutate phosphorylation events along a huge number of sequences. The simple principle behind this advantage is the stepwise synthesis of peptides, which allows the incorporation of either phosphorylated or nonphosphorylated derivates at serine, threonine, and tyrosine positions. In spite of several reported applications of phosphopeptide arrays, there is, to our best knowledge, no reported analysis of the efficiency of the involved techniques. Here, we analyze different coupling conditions to introduce phosphoamino acids in standard SPOT synthesis. Our results clearly indicate that EEDQ is the preferable activator and can also be used in fully automated SPOT synthesis. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: phosphoserine; phosphothreonine; phosphotyrosine; coupling methods; EEDQ; SPOT synthesis; peptide array

INTRODUCTION

Posttranslational protein modifications such as phosphorylation, acetylation, methylation, or ubiquitinylation are important mechanisms of cellular regulation. Phosphorylation of proteins at serine, threonine, and tyrosine residues plays a role in eukaryotic cell cycle control, cell differentiation, apoptosis, and cytoskeletal rearrangement [1,2]. A basic principle behind these diverse mechanisms is a recently postulated three-part molecular kit composed of 'writer' molecules (kinases), 'eraser' molecules (phosphatases), and 'reader' molecules (recognition partners of the modified protein) [3]. The molecular recognition involved in reading phosphorylation events is mediated by devoted protein interaction domains (PIDs). For example, positive regulation (switch on) by phosphorylation is known for PIDs such as PTB, SH2, and 14-3-3 domains [4,5] that require phosphorylated ligands for binding. Negative regulation (switch off) of PID-mediated interactions has been reported for WW domains, where the domain–ligand interaction is disrupted by phosphorylated amino acids within the ligand [6,7]. Aberrations in the phosphorylation state of proteins are associated with diseases such as diabetes [8–10] and cancer [11–13].

Phosphopeptides have been widely used to determine the sequence specificity of protein phosphatases and PIDs [14–16]. However, the methods described in these reports were established for solid-phase peptide synthesis (SPPS) on resins.

To reveal positive as well as negative regulation of protein function, *in vitro* comparison of phosphorylated *versus* unphosphorylated peptide arrays could be a successful approach. The SPOT technology represents a valuable tool for such a screening process [17–20]. Owing to the fact that SPOT technology is now established in several laboratories, there is a demand for a robust synthesis process for cellulose membrane-bound phosphopeptides. Unfortunately, to the best of our knowledge, no analytical investigation has been reported for the activation and subsequent coupling of phosphorylated amino acids on the cellulose membranes. Here, we compare different coupling conditions for incorporating phosphorylated amino acids during SPOT synthesis. Purities and coupling efficiencies were revealed by HPLC and MS. Furthermore, we confirmed introduction of the three phosphorylated amino acids in peptide arrays with the corresponding antiphosphoamino acid antibodies.

MATERIALS AND METHODS

Synthesis of Cleavable Peptides

Peptides were synthesized on an N-modified cellulose-aminohydroxypropyl ether (N-CAPE) membrane [18] and prepared by a MultiPep SPOT-robot (INTAVIS Bioanalytical Instruments AG). Array design was performed with the aid of the in-house software LISA 1.82. Synthesis started with the definition of

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spots by a standard protocol (β-alanine, two cycles, double coupling, 15 min reaction each), followed by the coupling of a solution of Fmoc-Rink linker (0.3 M) in DMF activated with 1.1 eq TBTU and 2 eq DIPEA (fourfold coupling, 15 min reaction each). The model peptide LKW was subsequently synthesized by standard SPOT synthesis [20]. Solutions of Fmoc-Leu-OPfp, Fmoc-Lys(Boc)-OPfp, and Fmoc-Trp(Boc)-OPfp in NMP (0.3 M) were used. Furthermore, Fmoc-Ser(PO(OBzl)OH)-OH, Fmoc-Thr(PO(OBzl)OH)-OH (Novabiochem) and Fmoc-Tyr(PO_3(MDPSE)$_2$)-OH (Bachem) each as a 0.3 M solution in DMF were activated under different conditions, as described in Table 1, and a fourfold coupling procedure (15 min reaction each) was applied. After the final Fmoc-deprotection step and washing procedure [20], the spots were punched out and transferred into Eppendorf tubes. The peptides were released from the support and side-chains were deprotected by the following protocol: (i) Peptides were cleaved from the support by treatment with a mixture of TFA (60%), TIBS (3%), and H_2O (2%) in DCM for 2.5 h. (ii) Afterward, the cellulose spots were separated from the solution, which was subsequently evaporated in a vacuum concentrator. (iii) The remaining residuum was once again treated with a mixture of TFA (90%), TIBS (3%), and H_2O (2%) in DCM for 1.0 h and the solution was again removed in a vacuum. (iv) TFA-salt complexes were removed with H_2O by azeotropic distillation. (v) The residue was then washed three times with *tert*-butylmethylether and finally dried. For optimal yields, the exact order of the TFA cleavage steps is important. This TFA procedure represents our standard cleavage protocol for peptides synthesized on cellulose-bound linkers as reported in [19,21]. The caution is that although the material safety sheet does not contain specific warnings, EEDQ is a neurotoxic alkylating agent [22–24].

Peptide Synthesis on Resin

Standard solid-phase peptide synthesis was performed according to standard Fmoc-chemistry on TentaGel S Ram resin (Rapp Polymere) and PyBOP activation for all amino acids (twofold coupling) using a multiple peptide synthesizer (Syro II, MultiSynTech). Peptides were purified to >95% by preparative HPLC and their identity was determined by ESI MS (Q-TOFmicro, Micromass).

Table 1 Coupling conditions (cc) for the phosphoamino acid derivates

cc	Coupling reagent	Additional reagents
1	0.3 M HATU	0.6 M DIPEA
2	0.3 M HBTU	0.6 M DIPEA
3	0.3 M TBTU	0.3 M HOBt, 1.5 M DIPEA
4	0.9 M EEDQ	−/−
5	0.9 M EEDQ	0.6 M BF_3* Ether[a]
6	0.9 M CDI[b]	−/−
7	0.3 M DCC	0.3 M NHS

In all cases, the activated phosphoamino acids (0.3 M in DMF) were coupled four times.
[a] Addition after 15 min preactivation.
[b] Freshly prepared solution.

Analysis of Soluble Peptides

HPLC analysis (Waters) was carried out using a linear gradient of solvents: A, 0.05% TFA in water and B, 0.05% TFA in acetonitrile; gradient 5–60% B over 30 min. HPLC conditions: UV detector 214 nm, RP-18 column. SPOT-synthesized peptides were dissolved in 60 µl H_2O/acetonitrile (1 : 1). The identity of peptides was validated by ESI MS (Q-TOFmicro™, Micromass).

Synthesis of a Membrane-bound Phosphopeptide Array

Replicas of a peptide array containing 100 peptide sequences and their phosphorylated versions were automatically synthesized as 11 mer according to Boisguerin *et al.* [19] using the MultiPep SPOT-robot. Sequences represent the 100 best binders of the AF6 PDZ domain and are given in Boisguerin *et al.* [25]. For standard SPOT synthesis, Fmoc-aa-OPfp (0.3 M in NMP) were used with the following side-chain protections: E-, D-(OtBu); C,- S-, T-, Y-(tBu); K-, W-(Boc); N-, Q-, H-(Trt); R-(Pbf). Incorporation of the phosphoamino acid derivates, Fmoc-Ser(PO(OBzl)OH))-OH, Fmoc-Thr(PO(OBzl)OH))-OH and Fmoc-Tyr(PO_3(MDPSE)$_2$)-OH (0.3 M DMF solutions) was achieved with 3 eq EEDQ and a fourfold coupling procedure.

Binding Studies of Cellulose-bound Peptides

The peptide arrays were incubated with aa(PO_3H_2) detection-antibody systems. Tyr(PO_3H_2) was screened with anti-pY (1 : 2500, P3300, Sigma–Aldrich) and detected with anti-mouse-HRP (1 : 500, A5906, Sigma–Aldrich); Ser(PO_3H_2) was screened with anti-pS (1 : 5000, P5747, Sigma–Aldrich) and detected with anti-mouse-HRP (1 : 500, A5906, Sigma–Aldrich); and finally, Thr(PO_3H_2) was screened with anti-pT (1 : 500, KAP-ST211E, Biomol) and detected with anti-rabbit-HRP (1 : 5000, A1949, Sigma–Aldrich). The signal intensities were detected with the LumiImager (Roche) and recorded as Boehringer Light Units (BLU) using the LumiAnalyst software.

RESULTS AND DISCUSSION

An N-CAPE cellulose membrane [18] was used instead of standard β-alanine membrane [20] to avoid additional ester-bound formation, which could be labile under TFA cleavage conditions. On this N-CAPE membrane, we first coupled the Fmoc-Rink linker and thereafter, our in-house model peptide LKW was synthesized stepwise according to the standard SPOT synthesis protocol [20]. The resulting construct [LKW-Rink linker] was then used to test seven coupling conditions for activating the phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) derivates. The correct choice of phosphorylated derivatives is a crucial point due to the side reactions of the phosphate groups. For example, bis-protected pS shows a phosphate group elimination under basic conditions (e.g. piperidine) [26,27]. To avoid such β-elimination, we used only mono-protected phosphoamino acids for serine and threonine phosphoderivates.

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We examined the common coupling reagents for SPPS such as HBTU, TBTU, HATU, and DCC/NHS, as well as EEDQ and CDI (Table 1). Previous reports have shown that EEDQ and CDI are valuable coupling reagents in SPOT synthesis [18,21,28,29]. Each solution was pre-activated for 15 min and a fourfold coupling procedure was applied to attach the activated phosphoamino acid derivates to the cellulose membrane-bound LKW-Rink linker block moiety.

Finally, the spots were punched out and the phosphopeptides (pX-LKW) were released as amides from the cellulose membrane using a two-step cleavage procedure (details in Materials and Methods). During the 60% TFA solution procedure (2.5 h), the linkage between Rink linker and the peptide is cleaved, but without releasing the Rink linker moiety from the cellulose membrane. After removing the cellulose spots, complete peptide side-chain deprotection was assumed by treatment with 90% TFA for an additional 1 h.

Additionally, as a control and for comparison, the peptides pS-LKW, pT-LKW, pY-LKW, and LKW were synthesized as amides on a Tentagel SRam resin (Rapp polymer) and purified by HPLC (Table 2).

All SPOT-synthesized peptides were analyzed by HPLC and MS. Measured and expected peptide masses, ratio of phosphorylated *versus* unphosphorylated peptides as well as peptide purities are presented in Table 2. With one exception (DCC/NHS for pS), the applied coupling conditions resulted in the phosphopeptides pS-LKW, pT-LKW, and pY-LKW, but with different efficiencies. The highest coupling efficiency was achieved with 3 eq of EEDQ, resulting in a ratio of >95 : 5 (pX-LKW : LKW) for all three phosphoamino acids. The peptide purities determined by HPLC correspond to 78% for pS-LKW, 77% for pT-LKW, and 85% for pY-LKW (Figure 1). The main peak in the panels corresponds either to the retention times of the model peptide LKW (Figure 1(a)) or to the corresponding

Table 2 Quantification of the coupling efficiencies of phosphoamino acid derivates under different conditions

Sequence	Conditions	Calculated $[M+H]^+$	Measured $[M+H]^+$	HPLC t [min][b]	Ratio	Purity [(%)area]
pS-LKW	HATU	612.29	612.22	11.39	6 : 1	75
	HBTU	612.29	612.24	11.43	12 : 1	75
	TBTU	612.29	612.25	11.43	3 : 1	56
	EEDQ	**612.29**	**612.25**	**11.38**	**>95 : 5**	**80**
	EEDQ/BF_3	612.29	612.30	11.40	1 : 2	26
	CDI	612.29	612.32	11.33	1 : 2	28
	DCC	612.29	612.31	11.19[a]	<5 : 95	0
	SPPS	612.29	612.31	11.47	−/−	100
pT-LKW	HATU	626.30	626.25	11.38	3 : 1	48
	HBTU	626.30	626.29	11.36	3 : 2	40
	TBTU	626.30	626.28	11.35	3 : 2	43
	EEDQ	**626.30**	**626.29**	**11.22**	**>95 : 5**	**78**
	EEDQ/BF_3	626.30	626.31	11.33	1 : 4	18
	CDI	626.30	626.31	11.37	1 : 3	24
	DCC	626.30	626.32	11.34	1 : 5	15
	SPPS	626.30	626.27	11.39	−/−	100
pY-LKW	HATU	688.32	688.26	11.39	6 : 1	57
	HBTU	688.32	688.27	11.34	30 : 1	57
	TBTU	688.32	688.31	11.37	2 : 1	69
	EEDQ	**688.32**	**688.31**	**11.38**	**>95 : 5**	**85**
	EEDQ/BF_3	688.32	688.32	11.34	1 : 1	45
	CDI	688.32	688.33	11.40	1 : 2	20
	DCC	**688.32**	**688.33**	**11.36**	**>95 : 5**	**80**
	SPPS	688.32	688.28	11.38	−/−	100
LKW	Spot	445.25	445.26	11.18	−/−	>95
	SPPS	445.25	445.26	11.11	−/−	100

SPPS are the resin-synthesized peptides; Ratio depicts the relation between the pX-LKW : LKW peak area in %.

[a] Corresponds to LKW.

[b] Measurement errors of uncooled HPLC column correspond to 0.09 min; the best coupling conditions are highlighted in bold.

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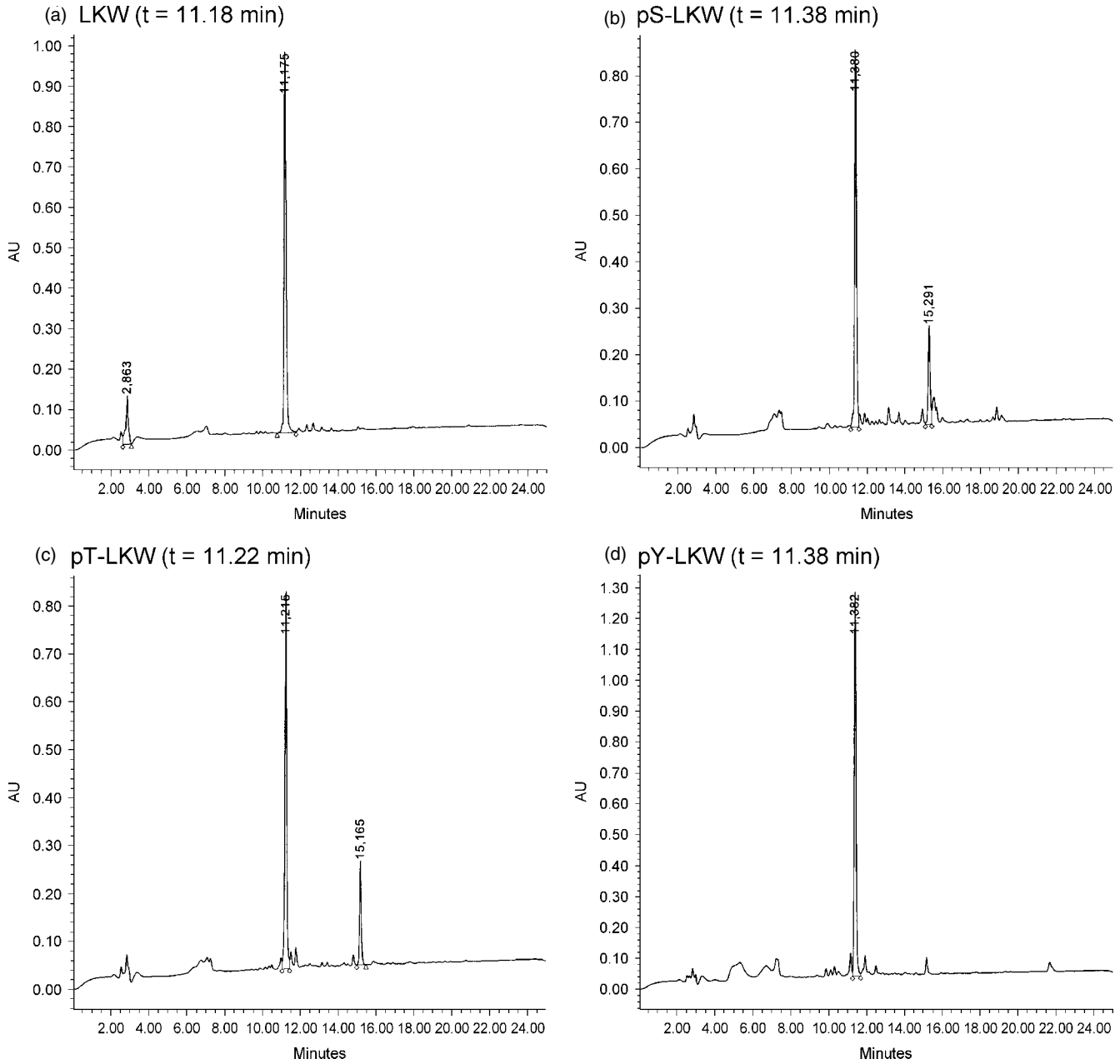

Figure 1 HPLC chromatograms of the crude model peptides obtained by EEDQ coupling of phosphoamino acid derivates. (a) Peptide LKW as a control (t = 11.18 min, peak area = 86.35%), (b) pS-LKW (t = 11.38 min, peak area = 78.20%), (c) pT-LKW (t = 11.22 min, peak area = 78.57%), (d) pY-LKW (t = 11.38 min, peak area = 100%). Measurement errors of uncooled HPLC column correspond to 0.09 min as shown in Table 2.

phosphorylated version (Figure 1(b)–(d)) as shown in Table 2. In some cases, we observed a peak at ~15 min, which represents the peptide with side-chain protection. The mass spectra revealed a mass difference of 90 Da corresponding to Bzl-group of the pS and pT derivates. Nevertheless, these impurities were always approximately 20% (peak area of pS 78.20% and of pT 78.57% as shown in Figure 1).

For the coupling of pY, the condition DCC/NHS also gave an excellent ratio of >95 : 5 and a purity of 80%. However, this condition is not suitable for fully automated spot robots, since the urea formed during activation may block the needle.

The best coupling procedure, the EEDQ approach, was used to synthesize three identical peptide arrays each containing 100 peptide sequences (11 mers) and their phosphorylated counterparts [19]. To confirm that phosphorylated amino acids were incorporated during the SPOT synthesis steps, the peptide arrays were incubated with anti-pY, anti-pT, and anti-pS monoclonal antibodies (Figure 2(a)–(c)). For each array, we could correctly detect over 80% of the

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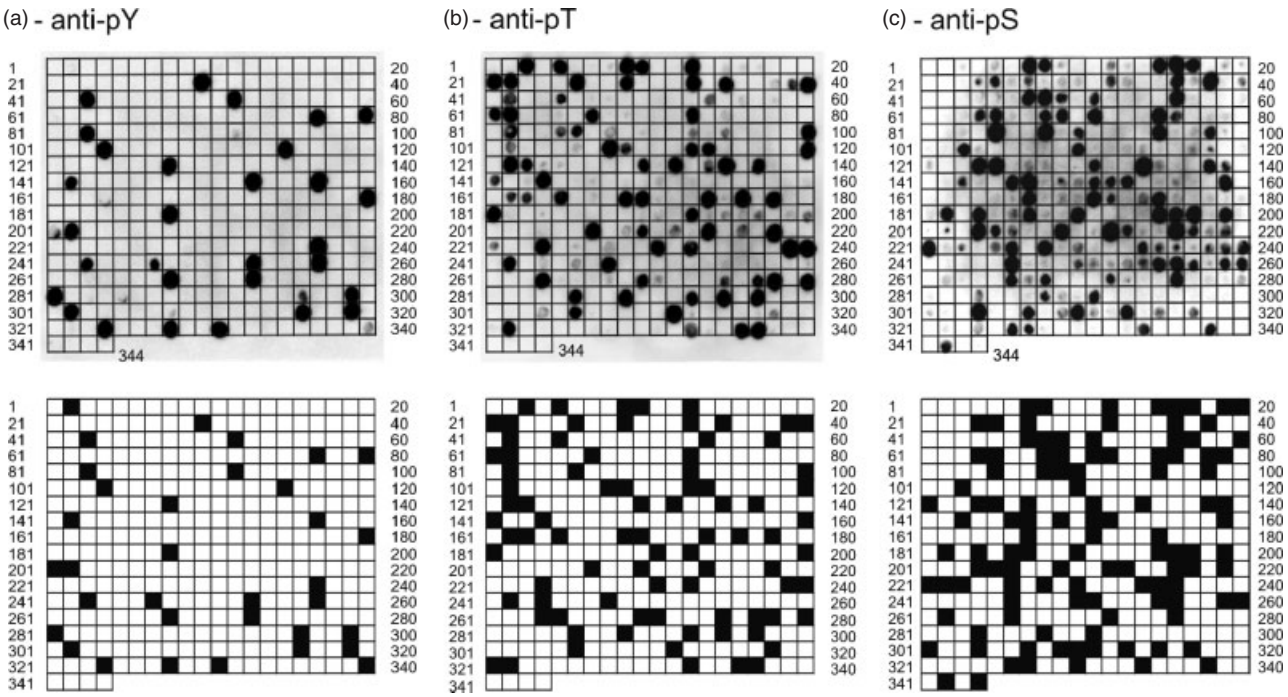

Figure 2 Determination of the phosphorylation pattern. Peptide arrays containing 100 peptide sequences and their phosphorylated versions were synthesized using the inverted peptide protocol [19]. To confirm that phosphorylated amino acids were preserved during synthesis steps, the peptide arrays were incubated with anti-pY (a), anti-pT (b) and anti-pS (c) antibodies, respectively. A pictogram with the corresponding position of the phosphorylated amino acids (black boxes) is shown under each panel. 41 pY-, 86 pT- and 121 pS-peptides are represented in each array. The amount of false negatives found on each assay are 19.5% (a), 12.7% (b), and 11.6% (c). Furthermore, we found 8.5% false positive in (c) due to cross-reactivity of pS-antibody.

phosphorylated sequences. The missing 20% is mainly due to the missing phosphoamino acid recognition or the cross-reactivity of the phosphoantibodies.

In summary, EEDQ proved to be an optimal coupling reagent to incorporate phosphorylated amino acids into peptides synthesized using the SPOT technology. Furthermore, several properties of EEDQ make it a suitable coupling reagent: the stability of the formed ester allows for semiautomization and full automatization of synthesis (unpublished data), it allows coupling in high yields, and it does not lead to racemization [30].

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