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A Simple and Effective Procedure for the Synthesis of the 'Difficult' Phosphotyrosine-containing Peptide Stat 91 (695-708)

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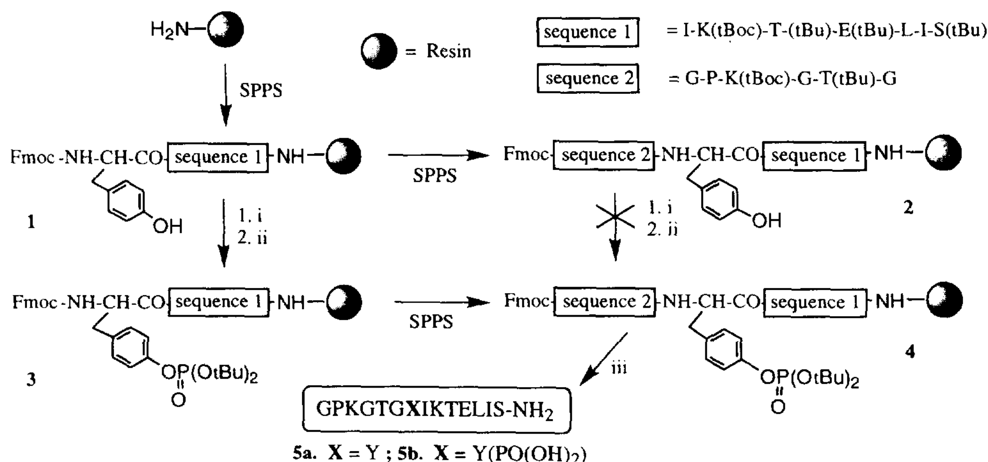
Abstract: A phosphotyrosine peptide (Stat 91$^{695-708}$) that proved inaccessible via the conventional SPPS procedures was synthesised using an inter-assembly phosphorylation strategy. Introduction of the phosphate group via phosphitylation and oxidation was successfully achieved immediately after assembly of the side chain unprotected tyrosine residue. Copyright © 1996 Elsevier Science Ltd

It is well recognised that tyrosine phosphorylated proteins play a primary role in many cell regulatory processes[1]. The recent identification of polypeptide signalling via tyrosine phosphorylation of Jak and Stat proteins prompted us to investigate the synthesis of the relevant phospho-containing peptides[2]. Two generic solid phase strategies have been developed in recent years that allow access to phosphorylated peptides. In one approach a protected phosphorylated residue is used as a building block in conventional solid phase assembly[3]. In the second method[4] (Scheme 1: 2 to 4) the full length protected peptide is first assembled on resin, leaving side chains unprotected where phosphorylation is required. These unprotected sites are typically phosphitylated by treating the resin-bound peptide with a dialkyl phosphoramidite and 1H-tetrazole and the resulting phosphites oxidised to the corresponding phosphates[4].

In the Fmoc solid phase synthesis (Scheme 1) of the non-phosphorylated Stat peptide **5a** (Stat 91$^{695-708}$)[2] we encountered sequence-related difficulties from Glu^{11} to Gly^{6}. In particular, coupling Lys^{9} to Thr^{10} and side-chain unprotected Tyr^{7} to Ile^{8} required relatively forcing conditions[5]. Similar difficulties incorporating Fmoc-Tyr(PO(OR)$_2$)OH (R= H, tBu) were encountered ; hence effective coupling to Ile^{8} could not be achieved. Rather than further experimenting and optimising yields with expensive phosphotyrosine building blocks, we decided to evaluate the post-assembly phosphorylation procedure[4].



Scheme 1: (i) $(Et)_2NP(OtBu)_2$ (10 eq.) in DCM; 1H-tetrazole (30 eq.) 1h (argon); (ii) 2 eq MCPBA in DCM, 10 min; (iii) a) piperidine/DMF (1/1), b) TFA/triisopropylsilane (9/1),15 minutes.

The partially protected Stat $91^{695-708}$ sequence containing an unprotected phenolic moiety was successfully assembled on Ramage resin[6] using modified coupling protocols[7]. Several attempts to phosphorylate the resin bound peptide **2** (Scheme 1) under a variety of conditions failed. TFA cleavage yielded predominantly the non-phosphorylated peptide **5a** with only traces of the target phosphopeptide **5b** present. Apparently the tyrosine phenolic functionality is unavailable for phosphorylation[8].

We subsequently considered an attractive alternative where phosphorylation is carried out immediately after introduction of the unprotected tyrosine residue thus reducing the potential for steric hindrance. Sequence 1 (Scheme 1) was assembled[7] on Ramage resin and Fmoc-Tyr-OH introduced. The phenolic-containing resin **1** was then dried under high vacuum and phosphorylation successfully performed via the conventional procedures[4]. Resin **3** was subsequently washed with DMF and chain assembly continued to give the full length peptide-resin **4**. The HPLC profile and ISMS spectrum of the crude cleavage product (Figure 1) indicated high yields of phosphorylation (yield of purified **5b** from theoretical s.v. of the resin = 36%). This is in stark contrast with the very low yields of phosphopeptide obtained from the post-assembly phosphorylation approach. Thus it appears likely that this inter-assembly phosphorylation strategy will produce purer phosphotyrosine peptides in higher yields when compared to the conventional post-assembly phosphorylation strategy.

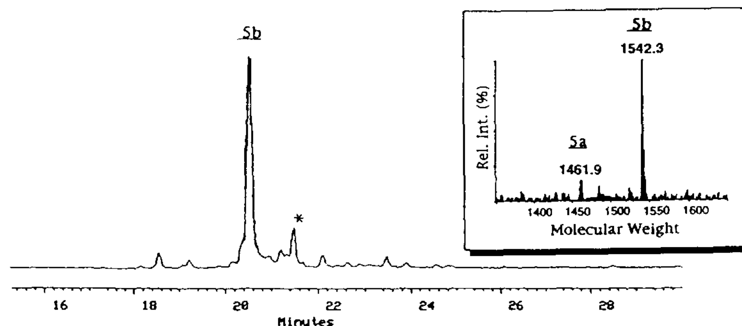


Figure 1 : HPLC profile (reverse phase C18) and ISMS reconstructed spectrum of crude product 5b (*corresponds to Acetyl-IKTELIS-NH_2).

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5. Using our standard coupling protocols (Alewood, P.F., Croft, M.; Schnolzer, M.; Kent, S., *Peptides* , Giralt, E., Andreu, D. Eds., ESCOM,1990, 174) >99.5% acylation is generally achieved within 10 minutes ; Here double coupling yields (ie. yields after twice that treatment) were 64% for Lys^9 and 88% for Tyr^7; replacing HBTU by HATU did not significantly improve the yield. Yield of these couplings were increased to >99% by 15h treatment at 30°C.
6. Ramage, R., Irving, S.L.; McInnes, C., *Tetrahedron Lett.* **1993**, 34, 6599.
7. The solid phase synthesis of this difficult sequence via modified procedures will be published elsewhere.
8 There is literature precedence suggesting that steric hindrance can cause difficulties in post-assembly phosphorylation; see eg: Bannwarth, W.; Kitas, E.A., *Helv. Chim. Acta* **1992**, 75, 707.

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