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GLOBAL PHOSPHORYLATION OF PEPTIDES CONTAINING OXIDATION-SENSITIVE AMINO ACIDS.

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Abstract: We report on an extension of the global phosphorylation procedure for the preparation of phosphopeptides containing oxidation-sensitive amino acids such as tryptophan, methionine or cysteine.

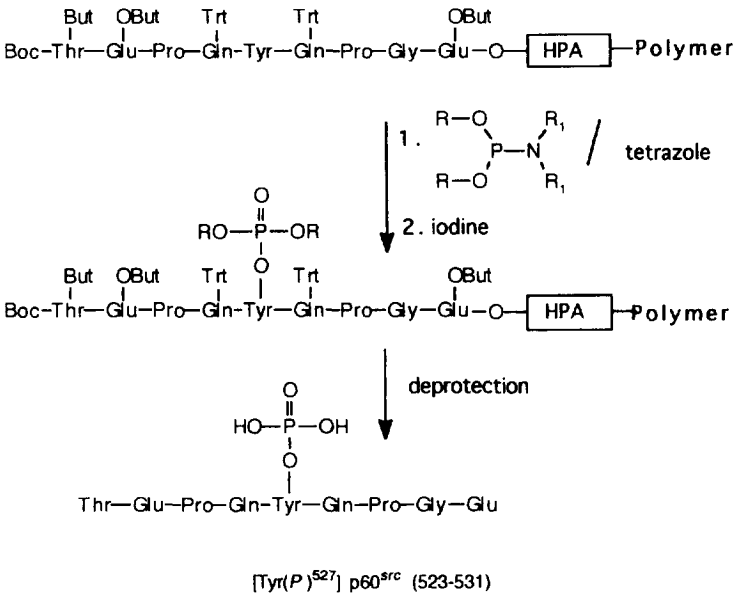
Protein phosphorylation is an important post-translational modification which is involved in the regulation of various cellular processes and in signal transduction pathways. It has been observed that proteins are most frequently phosphorylated on tyrosine, serine or threonine residues. The level of phosphorylation is regulated by two classes of enzymes: protein kinases and protein phosphatases.[1,2] Phosphorylated peptides have been shown to serve as appropriate tools in signal transduction research especially as substrates to study the specificities of phosphatases.[3]

Several methods for the synthesis of phosphopeptides have been described. Principally, they can be divided into two strategies. One can prepare the corresponding phosphorylated building blocks of amino acids and incorporate them into the stepwise mode of solid phase synthesis. Alternatively, the peptide can be phosphorylated after solid phase synthesis if the residue to be phosphorylated is incorporated as the side-chain unprotected form. This latter approach is referred to as global phosphorylation.

Recently we have compared the two approaches for the synthesis of phospho-tyrosine-containing peptides.[4,5]

The phosphorylation step for the synthesis of the phosphotyrosine building blocks as well as the global phosphorylation are based on P^{III} chemistry as outlined in Scheme 1 for the phosphorylation of the p60 src-(523-531) nonapeptide on solid support.[6]

Since the global phosphorylation is carried out at the very end of the solid phase synthesis the approach offers the use of different phosphoryl protecting groups, even those which are not stable to the conditions of the elongation cycles. If both, the phosphorylated as well as the unphosphorylated peptide are needed, they can be obtained from the same peptide synthesis by phosphorylating part of the resin-bound peptide.



[Tyr(*P*)^{527}] p60^{src} (523-531)

Scheme1

A disadvantage of the global approach is the application of an oxidation step to transfer the initially formed phosphoric acid triester after phosphinylation into the corresponding phosphotriester which might cause side reactions in those peptides which contain oxidation-sensitive amino acids like cysteine, tryptophan or methionine. Oxidation reaction is normally carried out with *m*-chloroperbenzoic acid or a mixture of iodine/lutidine/THF/water.[4]

Here we would like to report ways to avoid side reactions resulting from oxidation steps during the global phosphorylation procedure on Cys, Trp and Met residues.

Cysteine-containing peptides:

In the Fmoc/But strategy of solid phase peptide synthesis the thiol group of cysteine is commonly protected as the trityl thioether which can be cleaved under acidic conditions. In order to test optimal conditions for the oxidation step we have treated the resin-bound peptide Boc-Cys(Trt)-Thr(But)-Glu(OBut)-Pro-Gln(Trt)-Tyr-Gln(Trt)-Pro-Gly-Glu(OBut)-O-HPA-Support under different oxidation conditions and investigated the effect on the peptide by comparing the HPLC data of the crude peptide H-Cys-Thr-Glu-Pro-Gln-Tyr-Gln-Pro-Gly-Glu-OH (1) after acidic deprotection and peptide-resin cleavage. Performing the oxidation with *m*-chloroperbenzoic acid (Figure 1a) or peracetic acid resulted in the formation of major byproducts whereas oxidation with tetrabutylammonium periodate, *t*-butylhydroperoxide or iodine/lutidine/THF/water (Figure 1b) gave no significant side product formation.

To demonstrate the compatibility with the global approach the above peptide was phosphinylated with bis (benzyloxy) (diisopropylamino) phosphine followed by oxidation with iodine/lutidine/THF/water to yield the phosphorylated analogue H-Cys-Thr-Glu-Pro-Gln-Tyr(phospho)-Gln-Pro-Gly-Glu-OH (**2**).[4] The HPLC of the crude product is shown in Figure 1c. From our experiments it became clear that oxidation procedures which involve acidic conditions, and therefore lead to a partial deprotection of the trityl protecting group of the cysteine, are not suited for the global approach. In contrast, the oxidation under non-acidic conditions does not cause problems in the global phosphorylation of peptides containing cysteine residues of which the thiol function is protected by the trityl group.

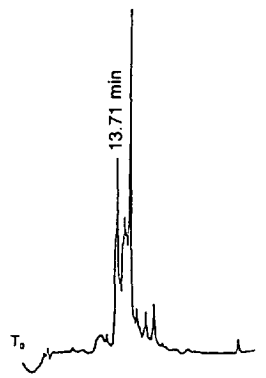


Fig.1a: RP-HPLC of crude **1** after *m*-chloroperbenzoic acid oxidation

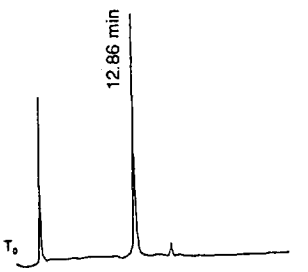


Fig.1b: RP-HPLC of crude **1** after I_2/THF//lutidine/H_2O oxidation

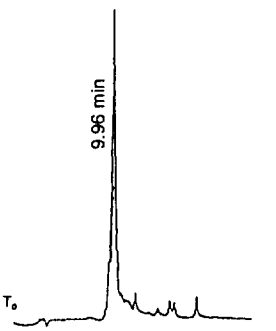


Fig.1c: RP-HPLC of crude **2**

Tryptophan-containing peptides:

The indole heterocycle of tryptophan is prone to two major side reactions: alkylation and oxidation. To study the global phosphorylation approach in Trp-containing peptides we have chosen the enkephalin derivative Tyr-Gly-Gly-Trp-Leu-NH_2 (**3**). The peptide was synthesized on an amide resin incorporating the side chain-free Fmoc-Trp-OH and Boc-Tyr-OH at the end of the synthesis. The peptide was submitted to the global phosphorylation approach with bis (benzyloxy) (diisopropylamino) phosphine and the oxidation step was carried out using either *m*-chloro perbenzoic acid or iodine/lutidine/THF/water. When the oxidation was performed with *m*-chloroperbenzoic acid the desired product was not obtained according to MS analysis. A unique product with 32 mass units greater than expected corresponding to the oxidation product was produced. In contrast, oxidation with iodine/lutidine/THF/water yielded the desired Tyr(phospho)-Gly-Gly-Trp-Leu-NH_2 (**4**) as the major product (Figure 2). This result indicates that indole unprotected tryptophan is not oxidized during the global phosphorylation if the oxidation is performed with iodine/lutidine/THF/water.

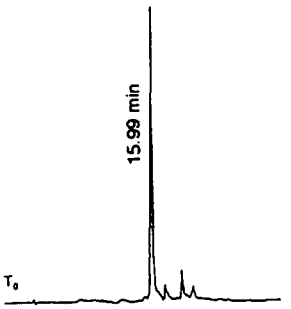


Fig.2: RP-HPLC of crude **4** after oxidation with iodine/lutidine/THF/water

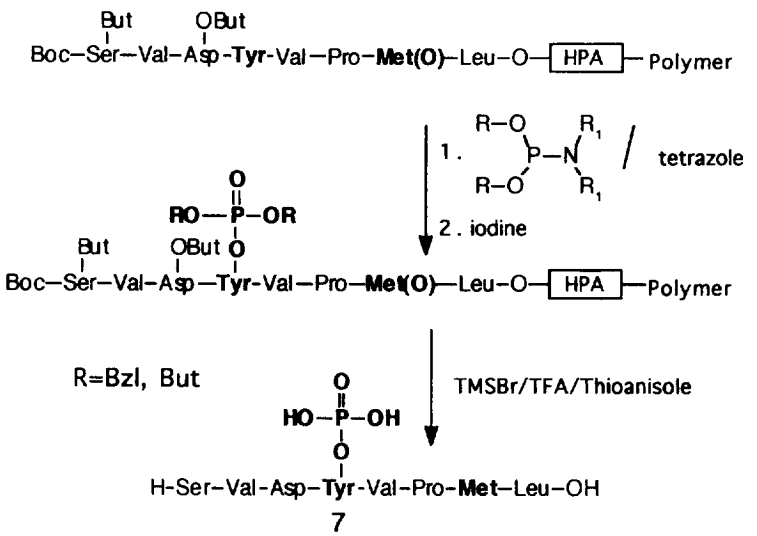
Alternatively, we have recently demonstrated that side reactions on tryptophan can be minimized by protecting the indole moiety with allyloxycarbonyl (Aloc).[7]

Thus, Tyr(phospho)-Gly-Gly-Trp(Aloc)-Leu-NH_2 (**5**) was synthesized by stepwise synthesis on solid support followed by global phosphorylation and the oxidation was performed again either with *m*-chloroperbenzoic acid or iodine/lutidine/THF/water. Final cleavage of the Aloc group yielded then the desired product (**4**). In both cases the desired product was obtained in high yield. Therefore, Aloc protection of Trp is able to prevent indole oxidation when oxidation is performed under acidic conditions.

Methionine-containing peptides:

The sulfur of methionine can be easily oxidized to either the corresponding sulfoxide or the sulfone. This might be a potential side reaction during global phosphorylations of peptides containing methionine residues. To evaluate this side reaction we have chosen the target peptide Tyr(phospho)-Gly-Gly-Met-Leu-NH_2 (**6**) which was synthesized on solid support equiped with an amide linker and incorporating Boc-Tyr-OH as the last amino acid building block. Global phosphorylation was then performed using bis (benzyloxy) (diisopropylamino) phosphine followed by oxidation with either *m*-chloroperbenzoic acid or iodine/lutidine/THF/water. A comparison of the HPLC and MS data of the crude products showed that oxidation with the former oxidizing reagent led to the formation of the corresponding sulfone whereas the latter reagent resulted in the formation of the desired phosphorylated peptide (**6**) and no oxidation of methionine.

Alternatively, we have evaluated another approach for the global phosphorylation of methionine-containing peptides. Yajima et al. have described a procedure for the reduction of sulfoxides of methionine with TMSBr.[8,9]



Scheme 2

Thus, we have synthesized a phosphorylated PDGF-β sequence (**7**) by incorporating directly the methionine building block as the sulfoxide (Scheme 2). Global phosphorylation of the resin-bound peptide was carried out with bis (benzyloxy) (diisopropylamino) phosphine or with bis (*tert.*–butoxy) (diisopropylamino) phosphine. Oxidation was performed with iodine/lutidine/THF/water. The final deprotection was carried out in both cases with TMSBr/TFA/thioanisole which should remove all the protecting groups, cleave the peptide from the support and reduce at the same time the sulfoxide of the methionine residue. Comparison of the HPLC and MS data show that this is indeed the case but the bis (benzyloxy) (diisopropylamino) phosphine leads also to a partial benzyl alkylation on the sulfur. With bis (*tert.*-butoxy) (diisopropylamino) phosphine for the phosphinylation only the desired product **7** is obtained. Fig.3 shows the HPLC data of the crude unphosphorylated **8** and the phosphorylated PDGF-β peptide **7**.

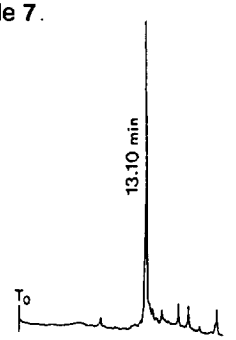


Fig.3a: RP-HPLC of crude **7**

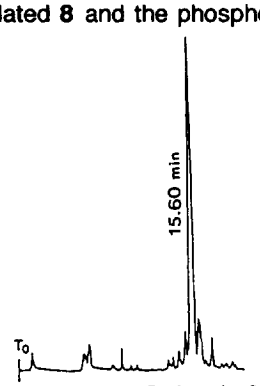


Fig.3b: RP-HPLC of crude **8**

In summary, we have demonstrated that peptides containing Cys, Trp or Met residues can be phosphorylated after solid phase assembly by the global phosphorylation approach if iodine/lutidine/THF/water is used for the oxidation of the phosphoric acid triester to the phosphotriester. No significant side reactions were observed for these oxidation-sensitive amino acids.

For Trp-containing peptides one can alternatively insert the corresponding Aloc-protected tryptophan building block. At the very end of the deprotection the Aloc group can be removed from the indole part with $Pd^{(0)}$ or a mixture containing piperidine/water (1:1).[10]

In Met-containing peptides we have also used the corresponding sulfoxide building block, which could be reduced in the final deprotection step with TMSBr/TFA/thioanisole.[11,12]

References and Notes:

1. Hunter,T; Cooper, J.A. *'Enzymes 1986'*, 3rd edn., Vol. 17, p.191-264.
2. Krüger, N.X.; Streuli, M.; Saito, H. *Eur. Mol. Biol. Organis. (EMBO) J.* **1990**, 9, 3241.
3. Cho, H.; Krishnaraj, R.; Itoh, M.; Kitas, E.; Bannwarth, W.; Saito, H.; Walsh, C.T. *Protein Science* **1993**, 2, 977.
4. Kitas, E.; Knorr, R.; Trzeciak, A.; Bannwarth, W. *Helv. Chim. Acta* **1991**, 74, 1314.
5. Bannwarth, W.; Kitas, E.; *Helv. Chim. Acta* **1992**, 75, 707.
6. Bannwarth, W.; Trzeciak, A. *Helv. Chim. Acta* **1987**, 770, 175.
7. Trzeciak, A.; Vorherr, T.; Bannwarth, W.; *Peptides 1992*, (Schneider, C.H., Eberle A.N., Eds.), 342.
8. Fujii, N.; Otaka, A.; Sugiyama, N.; Hatano, M.; Yajima, M. *Chem. Pharm. Bull.* **1987**, 35, 3880.
9. Iselin, B.; *Helv. Chim. Acta* **1961**, 44, 61.
10. Vorherr, T.; Trzeciak, A.; Bannwarth, W.; unpublished results.
11. Solid phase peptide synthesis and global phosphorylation was carried out on a HPA-modified solid support as described in Ref. 4. All peptides were characterized by MS.
12. Abbreviations: TFA: trifluoroacetic acid; TMSBr: trimethylsilyl bromide, PDGF: platelet-derived growth factor; HPA: (4-(hydroxymethyl)phenoxy) acetic acid.
RP-HPLC conditions: Fig 1and Fig 2: 0-80% CH_3CN in 0.14% TFA, 30 min, 230 nm;
Fig 3: 0-100% CH_3CN in 0.05% TFA, 30 min, 230 nm.

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