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An Efficient Procedure for Synthesis of Phosphopeptides through the Benzyl Phosphate-Protection by the Boc Mode Solid-Phase Method

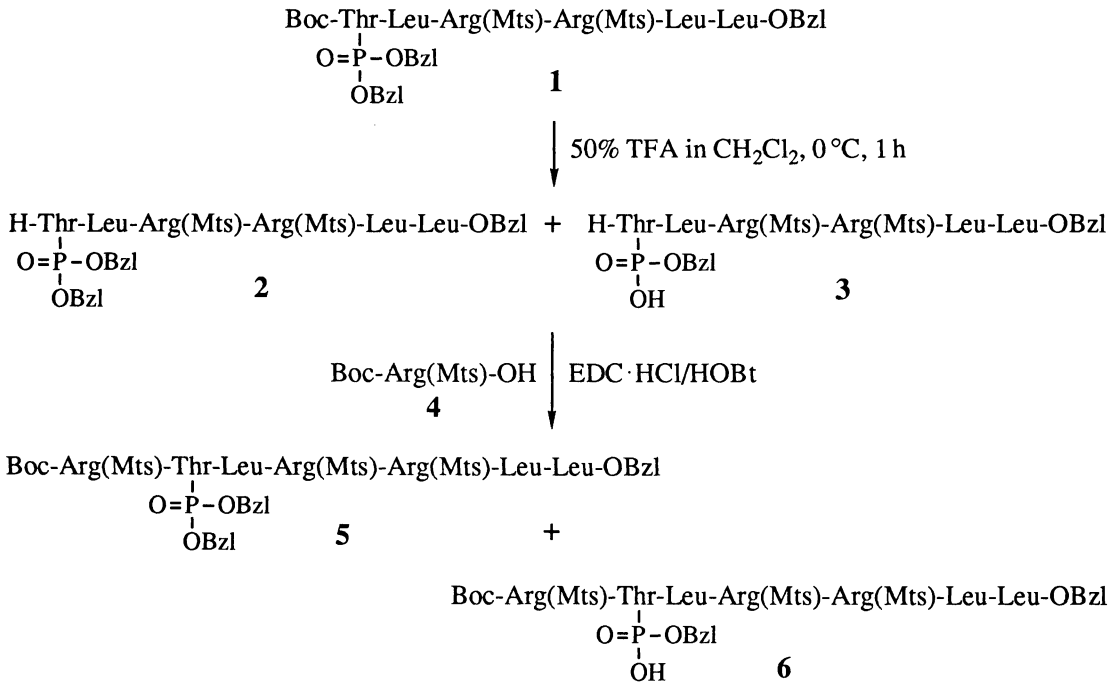
Tateaki WAKAMIYA,* Kunio SARUTA, Shoichi KUSUMOTO, Kiichiro NAKAJIMA,†
Kumiko YOSHIZAWA-KUMAGAYE,† Shinobu IMAJOH-OHMI,†† and Shiro KANEGASAKI††
Department of Chemistry, Faculty of Science, Osaka University, Toyonaka, Osaka 560
†Peptide Institute, Protein Research Foundation, Minoh, Osaka 562
††The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108

The benzyl phosphate-protection for phosphoamino acid is very useful for Boc mode solid-phase synthesis of phosphopeptides. A phosphothreonine-containing peptide related to the EGF receptor protein was synthesized by use of this methodology.

Covalent modification of the receptor proteins in plasma membrane by phosphorylation plays a key role in the regulation of receptor functions.[1,2] Synthetic study of the phosphopeptides related to the phosphoproteins is very important for understanding the biological role of protein phosphorylation. The synthesis of phosphopeptides was generally carried out by the Boc strategy, since β-elimination of *O*-phosphono moiety occurs readily under basic condtions in the Fmoc strategy. Therefore, many efforts were devoted to find acid stable phosphate-protecting groups such as phenyl,[3-5] trichloroethyl,[6] and allyl.[7,8] Unfortunately, these protecting groups were not so helpful for the practical synthesis of phosphopeptides because of the following reasons, i.e., i) two step reactions are required for final deprotection; ii) the use of the phenyl group which must be removed by catalytic hydrogenation over PtO_2 is restricted to peptides devoid of aromatic or sulfur-containing amino acids. On the other hand, the 4-nitrobenzyl and cyclohexyl groups had been proposed as desirable candidates for acid stable phosphate-protection, since they are stable under the acidic conditions for de-*t*-butoxycarbonylation, but are removable together with all other protecting groups by one step reactions with trifluoromethanesulfonic acid (TFMSA) in TFA.[9] In particular, the usefulness of the cyclohexyl phosphate-protection was confirmed by the Boc mode solid-phase synthesis of phosphoserine-containing peptides related to some biologically important phosphoproteins such as small heat shock protein HSP27 and phosphorylated human tau protein.[10] However, since the removal of the cyclohexyl group requires longer period of hard acid treatment than usual,[11] a little more acid labile protecting group is desirable for the synthesis of phosphopeptides including acid sensitive sequence such as -Asp-Gly- or -Asp-Ser- in the molecule.

It is known that the benzyl group for phosphate-protection is considerably labile in TFA.[12-15] Indeed, the de-*t*-butoxycarbonylation of phosphothreonine (PThr)-containing peptide **1**, a synthetic intermediate of the phosphopeptide related to the EGF receptor protein (EGFRP), produced undesirable monobenzylphosphono derivative **3** (10 - 30% yield) together with the dibenzylphosphono derivative **2** as major

product.[15] (Scheme 1) It is interesting that when the mixture of the amine components thus formed was directly subjected to the next coupling reaction, both **2** and **3** reacted with the carboxyl component **4** to give corresponding products **5** and **6**, respectively. This result indicated that the acidic group of monobenzylphosphate did not interfere the peptide coupling reaction and thus the compound **6** may be adoptable for subsequent reactions. In the case of the solution peptide synthesis, the product of each step is generally separated for characterization and then subjected to the next reaction. Therefore, partial cleavage of the benzyl group during de-*t*-butoxycarbonylation causes a serious problem. On the other hand, such separation procedure can not be performed in the solid-phase method, resulting in the utilization of both dibenzylphosphono and monobenzylphoshono derivatives for further reactions.



Scheme 1.

According to the considerations mentioned above, we had previously pointed out the applicability of the benzyl phosphate-protection to solid-phase synthesis of phosphopeptide.[15] This concept was confirmed by the synthesis of Cys-[EGFRP-(649-659)] based on the Boc mode solid-phase method as summarized in Scheme 2. The cleavage of the synthetic peptide from the resin and the removal of all protecting groups were carried out by the hard acid deprotection procedure using 1 M TFMSA in TFA and thioanisole as additive.[11] As shown in Fig. 1, the HPLC analysis of crude precipitate from ether showed the formation of desired Cys-[EGFRP-(649-659)] as a major product which was purified by preparative HPLC.[17]

We established a very efficient procedure for the synthesis of phosphopeptide by the Boc mode solid-phase method through the benzyl phosphate-protection for phosphoamino acids. Syntheses of other phosphopeptides related to biologically important phosphoproteins are currently undertaken and the results will be reported soon elsewhere.

Synthesizer : Peptide Synthesizer 430A, Applied Biosystems Inc., Foster City, CA, USA
Resin : Boc-Leu-OCH_2-phenylacetamidomethyl (PAM) resin (Boc-Leu: 0.73 mmol/g)
Amino acid derivatives : Boc-Arg(Mts)-OH (x 4); Boc-Cys(MBzl)-OH; Boc-Ile-OH; Boc-Leu-OH (x 2); Boc-Lys(ClZ)-OH; Boc-Thr[$PO(OBzl)_2$]-OH;[16] Boc-Val-OH
(Mts: mesitylenesulfonyl; MBzl: *p*-methoxybenzyl; ClZ: 2-chlorobenzyloxycarbonyl)
Procedure : The 0.5 mmol scale standard protocol of the benzotriazole active ester method in the system of software Ver. 1.40 NMP/HOBt/*t*-Boc was employed.

Boc-Leu-OCH_2-PAM resin

i) removal of Boc (50% TFA/CH_2Cl_2)
ii) neutralization (5% DIEA/CH_2Cl_2)
iii) washing
iv) coupling (DCC-HOBt/*N*-Methylpyrrolidone (NMP))
v) capping (10% Ac_2O)

vi) elongation of peptide chain

Protected Cys-[EGFRP-(649-659)]-OCH_2-PAM resin

(The synthesis was stopped after de-*t*-butoxycarbonylation of the *N*-terminal Cys residue to avoid undesirable addition of the *t*-butyl cation arising from the Boc group to the thiol group during the following hard acid deprotection procedure.)

vii) hard acid deprotection: TFMSA (180eq), thioanisole (180 eq) and *p*-cresol (180eq) in TFA [1 M TFMSA soln]/ -2 - -5 °C, 2 h

viii) precipitation from ether (0 °C)

ix) HPLC purification: YMC Pack ODS (30 x 250 mm) CH_3CN/0.1% TFA aq: gradient elution (11-31%; 0.17%/min; 20 ml/min)

H-Cys-Ile-Val-Arg-Lys-Arg-PThr-Leu-Arg-Arg-Leu-Leu-OH
Cys-[EGFRP-(649-659)]

Scheme 2.

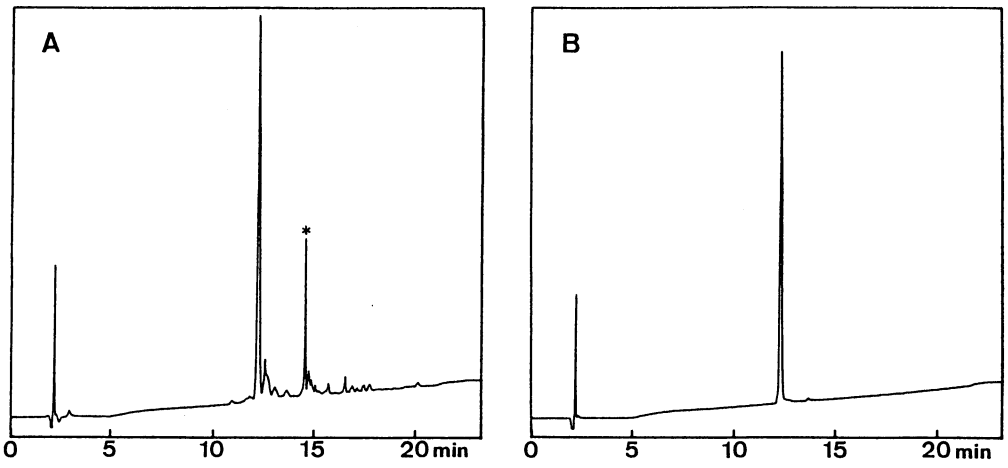


Fig. 1. HPLC profiles of (A) crude precipitate from ether and (B) purified Cys-[EGFRP-(649-659)]. Column: YMC Pack ODS-AM (4.6 x 150 mm); elution: CH_3CN/0.1% TFA aq (10-60% gradient elution, 2%/min, 1 ml/min); detection: UV 220 nm; temp: 50 °C. A peak asterisked was identified as a by-product containing the Cys(SPh) residue (yield: 8%), though the mechanism of its formation was not cleared yet.[18]

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16) Recently we obtained this compound as fine prisms: mp 65 - 70 °C; $[\alpha]_D^{24}$ +9.0° (*c* 0.37, MeOH).
17) The final pure product was obtained in a 61% yield. The structure was confirmed by amino acid analysis and measurement of the molecular weight with plasma desorption mass spectrometry (PD-MS) using a Bio-Ion 20, Applied Biosystems Inc., : m/z 1608.2 [$(M+H)^+$] (calcd: m/z 1606.9 [$(M+H)^+$]). Positive reaction with 5,5'-dithiobis(2-nitrobenzoic acid) [Ref: G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).] showed the presence of free mercapto group in the molecule.
18) The structure was estimated by measurement of PD-MS: m/z 1716.4 [$(M+H)^+$] (calcd: m/z 1715.1 [$(M+H)^+$]). The phenylthio group was readily removable by dithiothreitol reduction to give the thiol compound coinciding with the major product.

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