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An Efficient Procedure for Solid-Phase Synthesis of Phosphopeptides by the Fmoc Strategy [1)]

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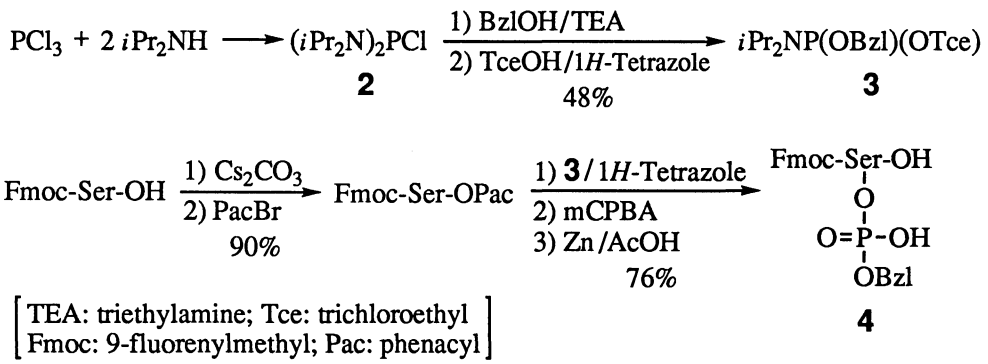
The Fmoc-mode solid-phase method was successfully applied to establish a practical procedure for the synthesis of phosphopeptides. Both Fmoc-phosphoserine with free phosphoric moiety and its monobenzyl phosphate derivative were examined as one of starting materials for the synthesis of peptides. The employment of the latter gave better result in respects of the yield and purity of the product than that obtained with the former.

Synthesis of phosphopeptides related to phosphoproteins is strongly required for the biochemical study on protein phosphorylation. Recently, we reported a practical procedure for solid-phase synthesis of phosphopeptides based on the pre-phosphorylation strategy using Boc-phosphoamino acid derivatives whose phosphoric moiety was protected with the acid stable 4-nitrobenzyl or cyclohexyl group.[2)] In particular, the usefulness of the cyclohexyl protection was confirmed by the synthesis of phosphopeptides related to several biologically important phosphoproteins such as small heat shock protein HSP27[3)] and phosphorylated human tau protein.[4)] Furthermore, the Boc-mode solid-phase phosphopeptide synthesis based on the pre-phosphorylation strategy was extended to the use of the acid labile benzyl protection for the phosphoric moiety.[3, 5)]

So far the synthesis of phosphopeptides was generally carried out by the Boc strategy to avoid the ready β-elimination of phosphate under basic conditions in the Fmoc strategy.[6,7)] However, we assumed that the stability of phosphate may increase when the phosphoric diester is replaced with the monoester. In order to confirm this assumption, we examined the Fmoc-mode solid-phase synthesis of phosphoserine-containing peptide related to heat shock protein, i.e., HSP27-(87-92) (**1**). At first, N^{α}-Fmoc-*O*-(monobenzylphosphono)serine (**4**)[8)] was prepared by conventional amidite method[9)] as shown in Scheme 1.[10)] For this purpose, iPr$_2$NP(OBzl)(OTce) (**3**) was newly prepared by stepwise reaction of the benzyl and trichloroethyl (Tce) alcohols with (iPr$_2$N)$_2$PCl (**2**).[8,11,12)] The synthesis of **1** was also carried out by employing N^{α}-Fmoc-phosphoserine (Fmoc-PSer) (**5**), which seems to be more stable to base, and base labile Fmoc-PSer dibenzyl phosphate (**6**) for comparison.

Although we had already known that the acidic structure such as **4** in peptides did not disturb the peptide coupling reaction,[3,5,13)] the reactivity of **4** or **5**[14)] has not been examined yet. As a result of the synthesis of model peptides as shown in Scheme 2, we confirmed that only the

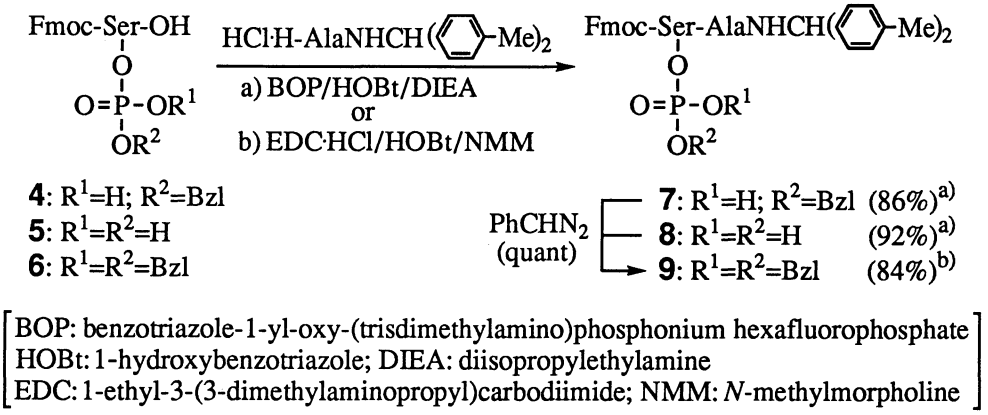


Scheme 1.

carboxyl group reacted with the amine component, since both coupling products **7** and **8** were converted into the compound **9** by treatment with phenyldiazomethane (Scheme 2).[11]

The Fmoc-mode solid-phase synthesis of the target peptide was carried out by means of manual procedure as summarized in Scheme 3. HPLC analysis of each crude product obtained by ether precipitation after deprotection procedure clearly shows that Fmoc-PSer(OBzl)(OH)-OH (**4**) is the most suitable derivative for the Fmoc strategy, while the use of dibenzyl phosphate derivative is obviously inadequate as we expected (Fig. 1).[15] Fmoc-PSer-OH (**5**) was also confirmed to be usable, but the yield was not so good because of the formation of by-products whose structures were not determined yet. Recently, Barany and his coworkers reported the successful application of Fmoc-phosphotyrosine with free phosphoric moiety to the Fmoc-mode solid-phase synthesis,[16] which suggests that phosphoserine and phosphotyrosine may possess different chemical character in their phosphoric groups.

As mentioned above, we established a very efficient procedure for the synthesis of phosphopeptides by the Fmoc mode solid-phase method through the monobenzyl phosphate-protection for phosphoamino acids. Syntheses of other phosphopeptides related to biologically important phosphoproteins containing not only phosphoserine but also phosphothreonine or phosphotyrosine are currently undertaken and the results will be reported soon elsewhere.



Scheme 2.

Fmoc-Ile-$OCH_2C_6H_4OCH_2C_6H_4$-Resin (Fmoc-Ile-Alko resin; Fmoc-Ile: 0.52 mmol/g)

[Reaction scale: 0.2 mmol]

i) 20% piperidine/DMF (10 ml: 2 min x 1; 8 min x 1)

ii) washing (DMF: 10 ml x 2 min x 5; CH_2Cl_2: 10 ml x 2 min x 5)

iii) preactivation: Fmoc-amino acid (4 eq)/BOP/HOBt/DIEA/DMF (2 ml; 10 min at r.t.)

iv) coupling (1 h) (*double coupling)

v) washing (DMF: 10 ml x 2 min x 5; CH_2Cl_2: 10 ml x 2 min x 5)

Fmoc-Glu(O*t*Bu)-OH
Fmoc-Ser[PO(OR^1)(OR^2)]-OH*
a) R^1=Bzl; R^2=H (**4**)
b) R^1=R^2=H (**5**)
c) R^1=R^2=Bzl (**6**)
Fmoc-Val-OH*
Fmoc-Gly-OH
Fmoc-Ser(*t*Bu)-OH

vi) elongation of peptide chain

vii) 20% piperidine/DMF (10 ml: 2 min x 1; 8 min x 1)

H-Ser(*t*Bu)-Gly-Val-Ser[PO(OR^1)(OR^2)]-Glu(O*t*Bu)-Ile-Alko resin

viii) deprotection: thioanisole : *m*-crezol : TFA = 12 : 10 : 78 (200 eq; r.t., 2h)

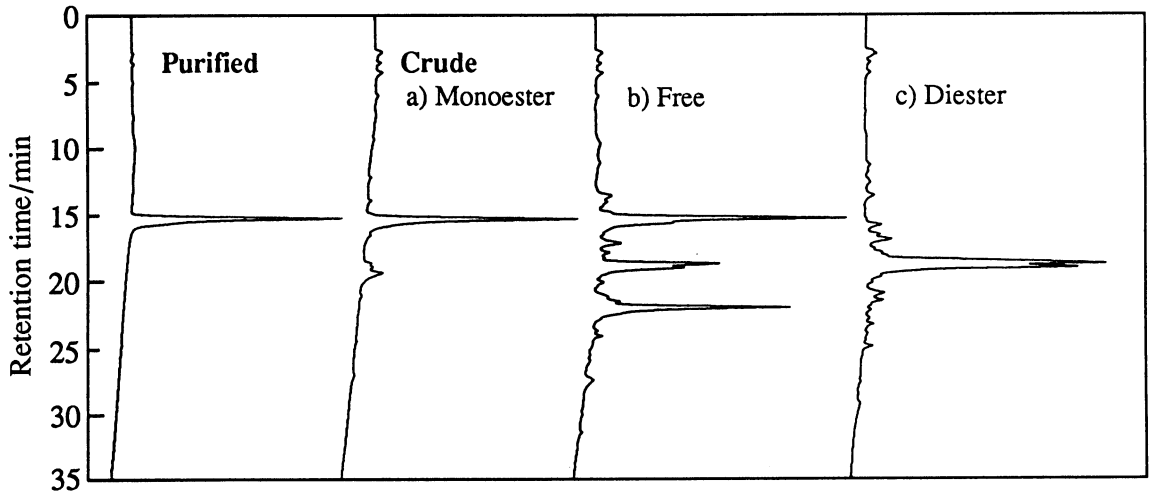
ix) precipitation from ether

x) extaction with 0.1% TFA

xi) HPLC purification: YMC Pac ODS-AM (20 x 250 mm); CH_3CN/0.1% TFA aq (gradient: 0-50%; 1%/min)

H-Ser-Gly-Val-PSer-Glu-Ile-OH
HSP27-(87-92) (**1**)

Scheme 3.



Column: Cosmosil 5C_{18}-AR (4.6 x 250 mm)
Elution: CH_3CN - 0.1% TFA aq (10 - 35%, 1%/min)
Flow rate: 1 ml/min; Detection: UV 220 nm

Fig. 1. HPLC profiles of purified **1** and crude products obtained by the use of a) **4**, b) **5** , and c) **6**.

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References

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9) Details of the preparation will be reported soon elsewhere.
10) Compound **4** was obtained as crystalline substance: mp 110 - 111 °C; $[\alpha]_D^{24}$ + 5.5° (*c* 1.0, C_2H_5OH).
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12) Compound **3** was purified by silica-gel column chromatography (Merck silica gel # 9385; pre-washed and eluted with hexane : triethylamine = 20 : 1). FAB-MS = m/z 386.1 [(M + H)$^+$].
13) T. Wakamiya, M. Kawahara, S. Kusumoto, S. Imajoh-Ohmi, and S. Kanegasaki, "Peptide Chemistry 1991," ed by A. Suzuki, Protein Research Foundation, Osaka (1992), p. 383.
14) Fmoc-Ser[PO(OBzl)$_2$]-OH (**6**) was prepared by the amidite method using iPr$_2$N(OBzl)$_2$,[8] while Fmoc-Ser[PO(OH)$_2$]-OH (**5**) was prepared by the acylation of commercially available phosphoserine with Fmoc-OSu. Details will be reported soon elsewhere.
15) The yield of HSP27-(87-92) after HPLC purification was tentatively calculated as TFA salt: 74% in the use of **4** and 24% in the use of **5**. The structure of purified product **1** was confirmed by amino acid analysis and measurement of the molecular weight with PD-MS using a Bio-Ion 20, Applied Biosystems Inc.,: m/z 671.6 [(M+H)$^+$] (calcd: m/z 671.3 [(M+H)$^+$]). Positive color reaction with Dittmer-Lester reagent (J. D. Dittmer and R. L. Lester, *J. Lipid Res.*, **5**, 126 (1964)) shows the presence of phosphorus in the molecule. On the other hand, the structures of two main products obtained in the use of **6** are not determined yet.
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