A New Practical Strategy for the Synthesis of Long-Chain Phosphopeptide

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A new practical strategy has been developed for the synthesis of long-chain phosphopeptide. Both the 2-chlorobenzoyloxy carbonyl (CIZ) group for Lys and methyl (Me) for phosphoamino acids remained intact, while other commonly used side-chain protecting groups were cleaved quantitatively, during the reaction using a highly acidic trifluoromethanesulfonic acid (TFMSA)-based reagent system (High TFMSA: TFMSA-TFA-m-cresol=1:9:1, v/v). Selective deprotection of the CIZ and Me group-containing protected phosphopeptide resin with the High TFMSA gave a partially protected phosphopeptide fragment suitable for thioester-mediated fragment condensation. A deprotection protocol of the 9-fluorenylmethoxycarbonyl (Fmoc) group, which evades significant side reaction toward the protected phosphoamino acid, was also developed. These two new findings enabled us to synthesize long-chain phosphopeptide via thioester-mediated fragment condensation.

Key words phosphopeptide; trifluoromethanesulfonic acid (TFMSA); thioester-mediated fragment condensation

Protein phosphorylation plays an essential role in intracellular signal transduction. Phosphorylation events induce structural change in the protein as well as formation of a recognition motif by signaling protein. Structure analysis of long-chain phosphopeptide or phosphoprotein provides much information for investigation of signal transduction. Therefore, synthesis of long-chain phosphopeptide has received much attention for the elucidation of various cellular processes.

Thioester fragment condensation strategy serves as potential synthetic methodology for synthesis of large peptides. Protection of the amino- and thiol-functionality is needed to achieve this thioester fragment condensation. We have developed a selective deprotection condition for phosphopeptides, where treatment of a protected peptide resin including both O,O'-dimethylphosphoserine (pSer(OMe)) and $N^+\cdot2\cdot$chlorobenzoyloxy carbonyl lysine (Lys(CIZ)) with trifluoromethanesulfonic acid (TFMSA) in trifluoroacetic acid (TFA) was found to afford a protected peptide in which CIZ- and Me-protection remained intact. Based on the finding that the CIZ group on Lys resists the action of TFMSA in TFA, we explored the applicability of TFMSA-mediated deprotection reagent to preparation of a phosphopeptide fragment suitable for synthesis of large phosphopeptides using the thioester method.

Result and Discussion

Initially, in order to refine deprotection conditions using TFMSA which result in formation of a Lys(CIZ)-peptide fragment, 9-fluorenylmethoxycarbonyl (Fmoc)-Lys(CIZ)-OH was treated with several TFMSA-mediated deprotective systems. As shown in Table 1, only a slight amount of cleavage of the CIZ group was observed when using a highly acidic TFMSA system in the absence of sulfide (TFMSA-TFA-m-cresol=1:9:1, v/v, (High TFMSA)). Next, susceptibility of other protecting groups as well as peptide-resin linkers to the High TFMSA was examined. Protected amino acid derivatives including amino acid-linked resins were treated with the High TFMSA system (TFMSA-TFA-m-cresol=1:9:1, v/v), and deprotected amino acids were quantified by an amino acid analyzer. Use of the High TFMSA system resulted in regeneration of the parent amino acids from several protected amino acids and Merrifield resin (Table 2), while two methyl (Me) groups on pSer as well as the CIZ group remained almost intact.

These results indicated that application of the High TFMSA treatment to CIZ (for Lys)- and Me (for phosphoamino acid)-protected peptide Merrifield resin should afford a protected phosphopeptide fragment, which can be used for fragment condensation via thioester methodology. To examine this possibility, we attempted deprotection of a protected model peptide resin with the High TFMSA, and obtained a protected phosphopeptide without significant accompanying side reactions (Fig. 1). Based on our finding, we proposed a new synthetic strategy for large phosphopeptides as conceptually outlined in Fig. 2. Our strategy features the preparation of CIZ- and Me-protected phosphopeptide fragment using the High TFMSA system followed by thioester-mediated fragment coupling. Here it should be noted that Fmoc deprotection must be achieved in the presence of a dimethylprotected phosphoamino acid residue, which usually leads to formation of a dehydroamino acid residue due to β-elimination when Fmoc removal is performed by usual 20% piperidine in N,N-dimethylformamide (DMF). This fact necessitated the development of an Fmoc-deprotection protocol avoiding β-elimination. Wakamiya et al. reported that phosphoric diester (one protecting group on phosphoamino acid) is stable under piperidine in DMF treatment. Therefore, we investigated a practical conversion method of a phosphoric triester (two protecting groups) to the corresponding diester with concomitant removal of the Fmoc group. Consequently, treatment with 5% piperidine–25% 1,4-diazabicyclo[2,2,2]octane (Dabco™) in DMF at room temperature for 1 h was found to be applicable to the new strategy (Fig. 3).

In order to verify the usefulness of the new strategy for the synthesis of long-chain phosphopeptide, we attempted the convergent synthesis of HIV-1 Rev (34—116) peptide, which was divided into three peptide parts for thioester-mediated fragment coupling (Fig. 4). A C-terminal fragment (F-1), possessing two pSer and Lys residues, corresponding to Rev (91—116) was constructed on tert-butylloxycarbonyl (Boc)-Glu(Obz)-O-Merrifield resin using Boc-based solid-
phase protocols. Deprotection of the protected F-1 resin with the High TFMSA yielded pSer(OMe)₂ and Lys(CIZ)-containing peptide fragment. After HPLC purification, F-1 was obtained in 19% yield based on the protected peptide resin. Peptide thioester fragments corresponding to both the N-terminal (34–65, F-3), and the middle part (66–90, F-2) were prepared from Boc-Gly-S-(CH₂)₄-CO-Leu-O-Merrifield resin. The Fmoc group was used for N-terminal protection to directly obtain N-terminal protected F-2 and F-3 from acidic deprotection reaction. Exposure of Lys(CIZ)-containing protected peptide assembled on the thioester resin to the High TFMSA should give Lys(CIZ)-peptide thioester, however, the protected thioester resins were subjected to 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)-thioanisole in TFA to obtain the desired thioester fragment (F-2 and F-3) since both fragments possessed no Lys residue. The thus-obtained crude F-2 and F-3 were subjected to HPLC purification to yield purified thioester fragments in 17% and 15% yield, respectively. Fragment condensations were conducted with the aid of AgNO₃, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt) and 4-N,N-dimethylmorpholine (NMM) in dimethyl sulfoxide (DMSO) as conceptually shown in Fig. 2. Coupling of F-1 and F-2 was completed within 4 h to afford Fmoc-protected (F-1+F-2) fragment. After HPLC purification (62% yield), Fmoc and one Me group were removed by using 5% piperidine–25% Dabco in DMF (room temperature, 2 h), followed by HPLC purification (81%), to yield N-terminal free fragment which, upon coupling with the protected thioester F-3 fragment, yielded the protected peptide corresponding to HIV-1 Rev (34–116) in 58% yield after HPLC purification. Fmoc removal, followed by HPLC, gave S-acetamidomethyl (Cys(Acm)), Lys(CIZ), and O-methylphosphoserine (pSer(OMe)(OH))-containing Rev (34–116) in 81% yield. The purified protected peptide was

Table 1. Treatment of Fmoc-Lys(CIZ)-OH with Several TFMSA Systems

<table>
<thead>
<tr>
<th>Run</th>
<th>Conditions (at 4 °C, for 1 h, v/v))</th>
<th>Formation of Fmoc-Lys-OH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TFMSA: TFA : m-cresol (1 : 9 : 1)</td>
<td>N.D. 13</td>
</tr>
<tr>
<td>2</td>
<td>TFMSA: TFA : p-cresol (0.5 : 8 : 5)</td>
<td>5.5</td>
</tr>
<tr>
<td>3</td>
<td>TFMSA: TFA : anisol (1 : 9 : 1)</td>
<td>N.D. 9.9</td>
</tr>
<tr>
<td>4</td>
<td>TFMSA: TFA : dimethyl sulfoxide : m-cresol (1 : 8 : 1 : 1)</td>
<td>11.5 31.5</td>
</tr>
<tr>
<td>5</td>
<td>TFMSA: TFA : thioanisol : m-cresol (1 : 8 : 1 : 1)</td>
<td>41.9 83.6</td>
</tr>
<tr>
<td>6</td>
<td>TFMSA: TFA : m-cresol : N-Me (1 : 9 : 1)</td>
<td>5.5 5.8</td>
</tr>
<tr>
<td>7</td>
<td>TFMSA: TFA : m-cresol (1 : 9 : 1)</td>
<td>64.6 68.6</td>
</tr>
</tbody>
</table>

a) One equivalent to Fmoc-Lys-CIZ-OH; b) Z=benzylimido-carbonyl. c) N.D. = not detected.

Table 2. Deprotection of Protected Amino Acid Derivatives with High TFMSA System

<table>
<thead>
<tr>
<th>Amino acid derivatives</th>
<th>Regeneration (% of free amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>Boc-Asp(OBz)-OH</td>
<td>100</td>
</tr>
<tr>
<td>Boc-Ser(Bz)-OH</td>
<td>86</td>
</tr>
<tr>
<td>Boc-Thr(Bz)-OH</td>
<td>95</td>
</tr>
<tr>
<td>Boc-Glu(OBz)-OH</td>
<td>95</td>
</tr>
<tr>
<td>Boc-Tyr(Cl,CZ)-OH</td>
<td>83</td>
</tr>
<tr>
<td>Boc-His(Bzm)-OH</td>
<td>100</td>
</tr>
<tr>
<td>Boc-Trp(Mts)-OH</td>
<td>78</td>
</tr>
<tr>
<td>Boc-Arg(Mts)-OH</td>
<td>92</td>
</tr>
<tr>
<td>Boc-Glu(OBz)-O-Merrifield resin</td>
<td>70 76 84</td>
</tr>
<tr>
<td>Boc-Leu-PAM resin</td>
<td>17</td>
</tr>
</tbody>
</table>

a) TFMSA : TFA : m-cresol (1 : 9 : 1, v/v) at 4 °C.

Fig. 1. Deprotection of Protected Phosphopeptide Resin Using High TFMSA and HPLC Examination of the Crude Deprotected Peptides

Column: μBondaprep SFC+100 Å (3.9×150 mm); buffer A: 0.1% aqueous TFA; B: CH₃CN (0.1% TFA); linear gradient, 25–50% B in A over 30 min; flow rate, 1.0 ml/min, detection at 220 nm.

Fig. 2. New Synthetic Strategy for Long-Chain Phosphopeptide

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subjected to one-pot, two-step deprotection protocol\(^1\) (first step: 1 M TMSOTf-thioanisole in TFA; second step: addition of dimethyl sulfide-TMSOTf), which is critical for removal of the Me group from the protected phosphoamino acid, to yield purified Cys(Ascm)\(^{85,89}\) -Rev (34–116) in 60% yield after HPLC purification.

Extensive application of the present protocol, consisting of High TMPSA deprotection and thioster-mediated fragment condensation, to several phosphopeptides should provide a practical methodology for the synthesis of long chain phosphopeptide.

**Experimental**

**General Procedure**

HPLC separations were carried out using either a µBondapack 5 µC\(_{18}\)-100 Å analytical (3.9 x 150 mm) or a Cosmosil 5C\(_{18}\)-AR semipreparative column (10 x 250 mm). Eluting products were detected by UV at 220 nm. Solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. A flow rate of 1 ml/min was used for all analytical separations and a 2.5 ml/min flow rate for preparative runs. In all cases, linear gradient programs were utilized. Ion spray mass spectra (IS-MS) were obtained with a ScieX API LIE triple quadrupole mass spectrometer.

**Synthesis of Protected Peptide Resin**

Protected peptide resins were manually constructed using Boc-based solid-phase method on appropriate Boc-amino acid linked Merrifield resins (0.2 mmol scale each, Boc-Gly-O-Merrifield resin (0.67 mmol Glu/g) for model peptide shown in Fig. 1, Boc-Glu(OBzl)-O-Merrifield resin (0.20 mmol Glu/g) for F-1 (Rev (91–116)), and Boc-Leu-O-Merrifield resin (0.40 mmol Leu/g) for F-2 (Rev (66–90)) and F-3 (Rev (34–65)). Boc deprotection was achieved using 50% TFA–2% anisole in toluene (1:x:1 min, 1:x:15 min). 5% N,N-disopropylethylamine (DIPEA) in toluene (2:x:1 min) was used for neutralization of the TFA salts. Except for derivation of the Leu-O-Merrifield resin for F-2 and F-3, Boc-protected amino acids (2.5-fold molar excess, 0.5 mmol) were sequentially condensed using diisopropylcarbodiimide (DIPCDI) (0.5 mmol)-1-hydroxybenzotriazole (HOBr) (0.5 mmol) in DMF. For introduction of a thioster unit, Boc-Gly-S(CHO)-COH was coupled on TFA-treated Boc-Leu-O-Merrifield resin to afford thioster-functionalized resin, which was subsequently subjected to solid-phase protocol described above.

**Treatment of Fmoc-Lys(CIZ)-OH with Several TFMSA Systems**

To a powder of Fmoc-Lys(CIZ)-OH (10 µmol) each was added TFMSA-based reagent mixture (550 µl) each) as listed in Table 1. After being stirred at 4°C, at specified intervals (20 and 60 min), aliquots (10 µl each) were sampled and diluted with 70% CH\(_3\)CN in H\(_2\)O (1 ml). The sample solution was subjected to HPLC analysis (B: 10–60% over 30 min). Each component, corresponding to Fmoc-Lys(CIZ)-OH and Fmoc-Lys-OMe, was identified using IS-MS analysis. The ratio of components was calculated from the HPLC peak areas.

**Examination of Susceptibility of Other Protected Amino Acid Derivatives toward High TFMSA System**

Mixture of Boc-Arg(Mts)-OH (9.07 µmol), Boc-Ser(Bzl)-OH (11.5 µmol), Boc-Glu(OBzl)-OH (9.6 µmol), Boc-Thr(Bzl)-OH (10.7 µmol), Boc-His(Bom)-OH (11.5 µmol), Boc-Trp(Mts)-OH (7.11 µmol), Boc-Tyr(Clz)-OH (9.15 µmol), Boc-Asp(OBzl)-OH (10.3 µmol), and H-Ala-OMe (16.9 µmol, internal standard) was treated with High TFMSA system (TFMSA (200 µl)-TEA (1.8 ml)-m-cresol (200 µl)) at 4°C. At specified intervals (30 min, 1 h, and 2 h), aliquots (20 µl each) were sampled and diluted with H\(_2\)O (1 ml). The amount of each regenerated amino acid in the sample solution was quantified using amino acid analyzer. Each protected Boc amino acid linked resin (Boc-Glu(OBzl)-O-Merrifield resin (9.50 µmol) and Boc-Leu-PAM resin (10.4 µmol)) was also treated with the High TFMSA in the presence of H-Ala-OMe, and subjected to amino acid analysis.

**Deprotection of Model Peptide Resin [Fmoc-Lys(CIZ)]-Gln-Hle-pSer(OMe)\(_{2}\)-Val-Arg(Mts)-OH-Gly-O-Merrifield Resin and Treatment of the Obtained Peptide with the 5% Piperidine–25% Dabclo in DMF**

Protected model peptide resin (2 mg, 0.66 µmol) was treated with TFMSA (10 µl)–TFA (90 µl)–m-cresol (10 ml) at 4°C. After 0.5 h, the solution was added to the precipitate and triturated. The precipitate was collected by centrifugation and washed with ether (3 times) to remove the scavenge, and was dissolved with H\(_2\)O (0.5 ml). After filtering the solution, the filtrate was subjected to HPLC analysis (B: 25–50% over 30 min). A component, corresponding to Fmoc-Lys(CIZ)-Gln-Hle-pSer(OMe)\(_{2}\)-Val-Arg-Gly-OH (a), was identified using an IS-MS analysis (Fig. 1). IS-MS (reconstructed) 1285.74 (Caled for C\(_{39}\)H\(_{49}\)N\(_{2}\)O\(_{12}\)PcI \(\text{Fmoc-Lys(CIZ)}\)-Gln-Hle-pSer(OMe)\(_{2}\)-Val-Arg-Gly-OH (a), identified using an IS-MS analysis (Fig. 1). IS-MS (reconstructed) 1285.74 (Caled for C\(_{39}\)H\(_{49}\)N\(_{2}\)O\(_{12}\)PcI)

**Crude deprotected peptide obtained from the model peptide resin (2 mg, 0.66 µmol) according to the same procedure described above was treated with piperidine (2.5 µl–Dabclo (12.5 mg) in DMF (47.5 µl). After being stirred for 1 h at room temperature, the reaction mixture was diluted with 50% CH\(_3\)CN in H\(_2\)O (1 ml). The solution was subjected to HPLC analysis (B: 0–50% over 30 min). Components, corresponding to pSer(OMe)-OH-peptide (b), dehydroala-peptide (c), and pSer(OMe)\(_{2}\)-peptide (d), were identified using IS-MS analyses (Fig. 3). IS-MS (reconstructed) 1048.74 (Caled for C\(_{39}\)H\(_{49}\)N\(_{2}\)O\(_{12}\)PcI \(\text{Fmoc-Lys(CIZ)}\)-Gln-Hle-pSer(OMe)\(_{2}\)-Val-Arg-Gly-OH (a), identified using an IS-MS analysis (Fig. 1). IS-MS (reconstructed) 1285.74 (Caled for C\(_{39}\)H\(_{49}\)N\(_{2}\)O\(_{12}\)PcI)

**Fig. 3.** Treatment of Fmoc-Protected pSer(OMe)\(_{2}\)-Containing Peptide (a) with 5% Piperidine–25% Dabclo in DMF and HPLC Examination of the Reaction Mixture

An asterisk denotes non-peptidic impurities: column, µBondapack 5 µC\(_{18}\)-100 Å (3.9 x 150 mm); buffer A, 0.1% aqueous TFA; B, CH\(_3\)CN (0.1% TFA); linear gradient, 0–50% B in A over 30 min; flow rate, 1.0 ml/min; detection at 220 nm.

**Fig. 4.** Peptide Sequence of Rev (34–116)

Synthesis of [pSerpOMe]^{29} \text{Lys(CIZ)}^{11} \text{Rev (91–116): Protected F-1}

Protective peptide resin (0 mg, 0.10 mmol) was treated with TFA/CH₃CN/H₂O (1:9:1) for 1 h. After removal of the resin by filtration, the filtrate (40 ml) was added to the filtrate to precipitate the crude product. The precipitate was collected by centrifugation and washed with ether (40 ml, each, three times) to remove the scavenger. HPLC purification (B: 29–33% over 30 min) of the crude peptide gave the pure F-1 fragment as a white powder (yield: 4.1 mg, 19% based on the protected peptide resin), IS-MS (reconstructed) 2969.98 (Calcd for C₁₁₅H₁₇₆N₅₀O₆₅S₁₂P₁₂Cl₁₂: 2970.23).

Synthesis of Fmoc-[CyC(Acm)]^{30} \text{S-(CH₂)}₃\text{CO-Leu-OMe}^{31} \text{Rev (66–90): Protected F-2}

Protective peptide resin (0 mg, 0.09 mmol) corresponding to Rev (66–90) was treated with 1M TMSOTf-thioanisole in TFA (2 ml) in the presence of m-cresol (100 μl) and ethanedithiol (EDT) (100 μl) at 4°C for 1.5 h. To obtain the crude peptide, procedure identical to that described above was utilized. HPLC purification (B: 30–45% over 30 min) of the crude peptide gave the pure title peptide as a white powder: 6.0 mg (17% based on the protected peptide resin), IS-MS (reconstructed) 3331.23 (Calcd for C₁₆₈H₂₂₉N₆₀O₆₆S₁₂: 3331.77).

Synthesis of Fmoc-[CyC(Acm)]^{32} \text{S-(CH₂)}₃\text{CO-Leu-OMe}^{33} \text{Rev (91–116): Protected F-1+F-2}

Fmoc-[CyC(Acm)]^{32} \text{S-(CH₂)}₃\text{CO-Leu-OMe}^{33} \text{Rev (66–90): S-(CH₂)}₃\text{CO-Leu-OMe}^{33} \text{Rev (91–116): pSrpOMe}_{32,33} \text{Lys(CIZ)}^{11} \text{Rev (91–116): Protected F-1+F-2}

Fmoc-[CyC(Acm)]^{32} \text{S-(CH₂)}₃\text{CO-Leu-OMe}^{33} \text{Rev (66–90): S-(CH₂)}₃\text{CO-Leu-OMe}^{33} \text{Rev (91–116): pSrpOMe}_{32,33} \text{Lys(CIZ)}^{11} \text{Rev (91–116): Protected F-1+F-2}

Fmoc-[CyC(Acm)]^{32} \text{S-(CH₂)}₃\text{CO-Leu-OMe}^{33} \text{Rev (66–90): S-(CH₂)}₃\text{CO-Leu-OMe}^{33} \text{Rev (91–116): pSrpOMe}_{32,33} \text{Lys(CIZ)}^{11} \text{Rev (91–116): Protected F-1+F-2}

References and Notes

1) The following abbreviations, except for those appeared in text, are used: Rev=regulator of virion protein expression; Bz=benzyl; C₆H₁₂=2,6-dichlorobenzyl, Bm=benzoylmethyl, Mt=2-mesitylenesulfonyl, PAM=4-(oxymethyl)phenylaceticimidomethyl