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Polypeptide Synthesis Using an Expressed Peptide as a Building Block for Condensation with a Peptide Thioester: Application to the Synthesis of Phosphorylated p21Max Protein(1–101)

TORU KAWAKAMI[a], KOKI HASEGAWA[a], KENTA TERUYA[a], KENICHI AKAJI[a], MASATAKA HORIUCHI[b,c], FUYUHIKO INAGAKI[b,c], YASUYUKI KURIHARA[d], SEIICHI UESUGI[d] and SABURO AIMOTO[a,*]

[a] Institute for Protein Research, Osaka University, Osaka 565-0871, Japan
[b] Graduate School of Pharmaceutical Sciences, Hokkaido University, Hokkaido 060-0812, Japan
[c] CREST, Japan Science and Technology Corporation, Tokyo 170-0013, Japan
[d] Faculty of Engineering, Yokohama National University, Kanagawa 240-8051, Japan

Abstract: An expressed peptide proved to be useful as a building block for the synthesis of a polypeptide via the thioester method. A partially protected peptide segment, for use as a *C*-terminal building block, could be prepared from a recombinant protein; its *N*-terminal amino acid residue was transaminated to an α-oxoacyl group, the side-chain amino groups were then protected with *t*-butoxycarbonyl (Boc) groups, and, finally, the α-oxoacyl group was removed. On the other hand, an *O*-phosphoserine-containing peptide thioester was synthesized via a solid-phase method using Boc chemistry. These building blocks were then condensed in the presence of silver ions and an active ester component. During the condensation, epimerization at the condensation site could be suppressed by the use of *N*,*N*-dimthylformamide (DMF) as a solvent. Using this strategy, a phosphorylated partial peptide of the p21Max protein, [Ser(PO_3H_2)^{2,11}]-p21Max(1–101), was successfully synthesized. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: expressed peptide; Max protein; peptide synthesis; peptide building block; peptide thioester; phosphorylated peptide; transamination

INTRODUCTION

The availability of site-specific modified peptides is of importance for biochemical and biophysical studies. For example, the site-specific phosphorylation and dephosphorylation of proteins constitute important processes for cellular regulations, and, as a result, such materials are needed for further study of these reactions. Although biological methods such as expression using bacteria are useful, they are not always applied to the synthesis of peptides with site-specific modifications. Chemical methods constitute viable alternatives to those biological approaches. Furthermore, combinations of peptides obtained by chemical and biological means for use as building blocks offer great potential as a route to the synthesis of a wide variety of polypeptides.

In 1991, Hojo and Aimoto reported one method for polypeptide synthesis by using *S*-alkyl peptide thioesters (peptide thioesters) as building blocks [1,2]. Since then, improvements have been made to the thioester method [3–5], and it is now useful in the synthesis of various types of polypeptides, including phospho- [6] and glycopeptides [7]. Since

Abbreviations: Boc-OSu, *N*-*t*-butoxycarbonyloxysuccinimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fmoc-NH-SAL-resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-resin; *c*Hex, cyclohexyl; $HOCH_2$-Pam, 4-(hydroxymethyl) phenylacetamidomethyl; HOObt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; IEC, ion exchange chromatography; NMP, 1-methyl-2-pyrrolidinone; *c*Pen, cyclopentyl.

* Correspondence to: Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan; e-mail: aimoto@protein.osaka-u.ac.jp

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the original report, several methods of peptide syntheses using the peptide thioesters as building blocks have been developed [8,9], including the so-called native chemical ligation [10]. Expressed peptides have been also used as building blocks for peptide synthesis. The phenomenon of protein splicing [11,12] involves peptide thioesters as intermediates [13], which can be trapped by the other peptide segments [14,15]. In addition, a segmentally stable isotope-labeled protein for nuclear magnetic resonance (NMR) study has been prepared [16] It is also noteworthy that splicing elements have been applied to a purification method for recombinant proteins [17], and the peptide thioester intermediates have been used as building blocks for peptide synthesis [18–22]. In those, however, a cysteine residue is required at the coupling site, and in most cases some spacer residues are inserted. In contrast, the thioester method has no restrictions regarding residues at the condensation sites, in principle. This is of great advantage in the synthesis of any desired sequences by the use of expressed peptides as building blocks.

We wish to report herein, a method for polypeptide synthesis by using an expressed peptide as a *C*-terminal building block. A partially protected peptide segment for the thioester method was prepared from a recombinant peptide, and its condensation with a peptide thioester, which contained *O*-phosphoserine residues, was achieved. In this report, a synthesis of a partial sequence of phosphorylated p21Max protein [23–25] is described as a model [26]. Max protein is known to bind specifically to duplex DNA by forming homo- and hetero-complexes with the Myc family of proteins, and its binding activity is reduced by phosphorylation. The success of this synthesis would greatly contribute to our understanding of the structural basis of the regulation mechanism of Max protein, as the result of phosphorylation.

MATERIALS AND METHODS

General

Amino acid derivatives used were of the L-configuration, unless otherwise noted. Boc- and Fmoc-amino acid derivatives were purchased from the Peptide Institute (Osaka, Japan). Fmoc-Ser[PO(OBzl)OH]-OH was purchased from Novabiochem (Läufelfingen, Switzerland). Boc-Ser[PO_3(*c*Pen)$_2$]-OH was prepared using a previously reported procedure [27]. Fmoc-NH-SAL-resin, DIEA, and TFA were purchased from Watanabe Chemical (Hiroshima, Japan), and Boc-Leu-OCH_2-Pam resin was purchased from Applied Biosystems (Foster City, CA, USA). Commercially available 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOObt) was recrystallized from aqueous ethanol prior to use. Silver chloride was purchased from Wako Pure Chemical (Osaka, Japan). The DMF and DMSO used for the segment condensation reaction were silylation grade (Pierce, Rockford, IL, USA).

RP-HPLC was performed on Cosmosil 5C18ARII (10×250 mm), Cosmosil 5C18ARII (4.6×250 mm), Cosmosil 5C4AR-300 (4.6×150 mm) (Nacalai Tesque, Kyoto, Japan), YMC-Pack ODS-AM (20×250 mm) (YMC, Kyoto, Japan), or Poros R2/M (Applied Biosystems) using a linear increasing gradient of acetonitrile in 0.1% aqueous TFA, unless otherwise noted. Ion exchange chromatography (IEC) was performed on SP-sepharose Fast Flow, HiTrap™ SP (1 mL) or Resource™ S (1 mL) (Pharmacia, Piscataway, NJ, USA) using a linear increasing gradient of sodium chloride in 40 mM sodium phosphate buffer containing 8 M urea, unless otherwise noted. Yields of peptides were determined by quantitative amino acid analyses, which were performed on an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan) after hydrolysis with constant boiling point hydrochloric acid for amino acid analysis (Nacalai Tesque) at 110°C for 24–48 h in an evacuated sealed tube. NMR spectra were recorded on a Brucker AVANCE™ 400 NMR spectrometer. Mass numbers were determined by matrix assisted laser desorption ionization/time-of-flight mass spectroscopy (MALDI-TOF MS) using a Voyager™ DE instrument (PerSeptive Biosystems, Framingham, MA, USA) using α-cyano-4-hydroxycinnnamic acid or sinapinic acid as a matrix.

Preparation and Transamination of Model Peptides

A peptide, ADKRAHHNAL-NH_2 (**9**) was prepared starting from an Fmoc-NH-SAL-resin on a 433A peptide synthesizer (Applied Biosystems) by an Fmoc solid-phase method. A fully protected peptide resin, Ala-Asp(O^{t}Bu)-Lys(Boc)-Arg(Pmc)-Ala-His(Trt)-His-(Trt)-Asn(Trt)-Ala-Leu-NH-SAL-resin, was obtained, which was then treated with reagent K [28] to give a crude powder. Purification by RP-HPLC on an ODS column to give peptide **9**: MS (MALDI-TOF) found 1131.8 (MH^+), calcd 1131.6. Amino acid analysis: $Asp_{1.9}Ala_3Leu_{1.1}Lys_{0.95}His_{1.9}Arg_{0.96}$.

Peptide **9** (10 mg, 5.6 μmol) was stirred in 0.5 M glyoxylic acid, 5 mM nickel(II) sulfate, 2 M sodium

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acetate, 1 M acetic acid (5.0 mL). After 4 h, EDTA (45 mg) was added, followed by purification by RP-HPLC to give CH_3COCO-DKRAHHNAL-NH_2 (**10**) (6.8 mg, 3.8 μmol, 68% yield): MS (MALDI-TOF) found 1130.8 (MH^+), calcd 1130.6. Amino acid analysis: $Asp_{1.9}Ala_2Leu_{1.0}Lys_{0.93}His_{1.9}Arg_{0.93}$.

A peptide, ADKRVF-NH_2 (**11**), was prepared on a multiple organic synthesizer, 440Ω MOS (Advanced ChemTech, Louisville, KY, USA) by an Fmoc solid-phase method: ^{1}H-NMR (400 MHz, DMSO-d_6, 27°C) δ 0.74 (d, $J = 5.9$ Hz, 6H), 1.22–1.38 (m, 2H), 1.32 (d, $J = 6.9$ Hz, 3H), 1.41–1.58 (m, 6H), 1.62–1.70 (m, 2H), 1.87–1.97 (m, 1H), 2.46–2.55 (m, 1H), 2.66–2.76 (m, 3H), 2.79 (dd, $J = 9.2$, 13.9 Hz, 1H), 2.99 (dd, $J = 4.9$, 13.8 Hz, 1H), 3.04–3.13 (m, 2H), 3.82–3.91 (m, 1H), 4.06–4.11 (m, 1H), 4.18–2.29 (m, 2H), 4.41–4.48 (m, 1H), 4.57–4.61 (m, 1H), 7.09 (s, 1H), 7.14–7.26 (m, 5H), 7.40 (s, 1H), 7.69 (d, $J = 8.3$ Hz, 1H), 7.79 (s, 2H), 7.90 (d, $J = 7.9$ Hz, 1H), 8.0 (s, 1H), 8.08–8.19 (m, 3H), 8.69 (d, $J = 7.5$ Hz, 1H), 12.54 (s, 1H); MS (MALDI-TOF) found 734.6 (MH^+), calcd 734.4.

Peptide **11** was treated with glyoxylic acid (0.5 M) in the presence of copper(II) sulfate or nickel(II) sulfate (5 mM) in an acetate buffer (2 M sodium acetate and 1 M acetic acid) to give CH_3COCO-DKRVF-NH_2 (**12**) along with side reaction products. Peptide **12** was further treated with glyoxylic acid (0.5 M) in the presence of copper(II) sulfate (5 mM) in an acetate buffer (2 M sodium acetate and 1 M acetic acid) to give side reaction product **13**, the mass number of which was 74 Da larger than that of peptide **12**. **12**: ^{1}H-NMR (400 MHz, DMSO-d_6, 27°C) δ 0.72 (d, $J = 6.4$ Hz, 6H), 1.19–1.36 (m, 2H), 1.38–1.56 (m, 6H), 1.58–1.71 (m, 2H), 1.87–1.96 (m, 1H), 2.35 (s, 3H, CH_3CO), 2.66–2.76 (m, 4H), 2.79 (dd, $J = 9.1$, 13.9 Hz, 1H), 2.98 (dd, $J = 5.2$, 13.9 Hz, 1H), 3.04–3.11 (m, 2H), 4.10 (dd, $J = 6.6$, 8.7 Hz, 1H); 4.20–4.31 (m, 2H), 4.46 (dt, $J = 5.1$, 8.7 Hz, 1H), 4.55 (dt, $J = 5.4$, 7.8 Hz, 1H), 7.09 (s, 1H), 7.14–7.26 (m, 5H), 7.40 (s, 1H), 7.49 (s, 1H), 7.64–7.54 (m, 4H), 7.90 (d, $J = 8.2$ Hz, 1H), 7.99 (d, $J = 7.9$ Hz, 1H), 8.09 (d, $J = 7.7$ Hz, 1H), 8.70 (d, $J = 8.2$ Hz, 1H), 12.39 (s, 1H); MS (MALDI-TOF) found 734.1 (MH^+), calcd 733.9. **13**: MS (MALDI-TOF) found 807.4 (MH^+), calcd 807.4.

Introduction of a Boc Group and Removal of a Pyruvoyl Group

A solution of peptide **10** (8.3 mg, 4.0 μmol), *N-t*-butoxycarbonyloxysuccinimide (Boc-OSu) (5.9 mg, 28 μmol), and DIEA (15 μL) in DMSO (0.50 mL) was stirred for 1 h, and the mixture was then washed with ether (3 × 5 mL) to give CH_3COCO-DK(Boc)RAHHNAL-NH_2 (**14**): MS (MALDI-TOF) found 1231.9 (MH^+), calcd 1230.9.

The residue of peptide **14** was treated with *o*-phenylenediamine (40 mM) in an acetate buffer (2 M sodium acetate and 2 M acetic acid) under argon for 24 h. A product was purified by RP-HPLC to give DK(Boc)RAHHNAL-NH_2 (**15**) (4.0 mg, 1.9 μmol, 47%): MS (MALDI-TOF) found 1160.8 (MH^+), calcd 1160.6. Amino acid analysis: $Asp_{1.9}Ala_2Leu_{1.0}$-$Lys_{0.93}His_{1.9}Arg_{0.96}$.

Preparation of Boc-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (19)

Starting from Boc-Ala-SCH_2CH_2CO-Leu-OCH_2-Pam resin (**16**) (Ala: 0.58 mmol/g, 0.733 g, 0.425 mmol) [3–6], a protected peptide resin (1.58 g) corresponding to the sequence of p21Max(1–13), Boc-Met-Ser-(Bzl)-Asp(O*c*Hex)-Asn-Asp(O*c*Hex)-Asp(O*c*Hex)-Ile-Glu(OBzl)-Val-Glu(OBzl)-Ser(Bzl)-Asp(O*c*Hex)-Ala-SCH_2CH_2CO-Ala-OCH_2-Pam resin (**17**), was prepared on a 430A peptide synthesizer (Applied Biosystems). The 0.5-mmol standard protocol of system software version 1.40 NMP/HOBt *t*-Boc was employed, and end capping with acetic anhydride was performed after the introduction of each amino acid. An aliquot of resin **17** (0.472 g) was treated with a mixture of hydrogen fluoride (9.0 mL) and anisole (1.0 mL) on an ice bath for 1.5 h. After evaporation of the hydrogen fluoride, ether was added to the mixture, and the resulting precipitate was washed twice with ether and then dissolved in aqueous acetonitrile. The peptide solution was passed through a disposable ODS cartridge to give crude product (0.225 g) after freeze-drying. This preparation was purified by RP-HPLC on Cosmosil 5C18ARII (10 × 250 mm) to yield p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**18**) (81 mg): MS (MALDI-TOF) found 1642.1 (MH^+), calcd 1641.7 (average). Amino acid analysis: $Asp_{4.7}Ser_{1.7}Glu_{1.9}Ala_1Val_{0.87}Met_{0.95}$-$Ile_{1.0}Leu_{1.0}$.

To a solution of peptide **18** (20 mg) in DMSO (0.40 mL) were added Boc-OSu (7.2 mg, 33 μmol) and DIEA (16 μL). After stirring for 2.5 h at room temperature, the peptide was isolated by RP-HPLC on Cosmosil 5C18ARII (10 × 250 mm) to give Boc-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**19**) (8.6 mg, 3.2 μmol, 9.1% yield based on Ala in the starting resin **16**): MS (MALDI-TOF) found 1742.4 (MH^+), calcd 1741.8 (average). Amino acid analysis: $Asp_{4.7}Ser_{1.7}Glu_{1.9}Ala_1Val_{0.87}Met_{0.97}Ile_{1.0}Leu_{1.0}$.

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Preparation of Boc-$(Ser(PO_3H_2)^{2,11})$-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (22)

Starting from resin **16** (Ala: 0.61 mmol/g, 0.80 g, 0.49 mmol), a protected peptide resin (1.59 g) corresponding to the sequence p21Max(1–13), Boc-Met-Ser[PO_3(*c*Pen)$_2$] - Asp(OBzl) - Asn - Asp(OBzl) - Asp-(OBzl)-Ile-Glu(OBzl)-Val-Glu(OBzl)-Ser[PO_3(*c*Pen)$_2$]-Asp(OBzl)-Ala-SCH_2CH_2CO-Leu-OCH_2-Pam resin (**20**), was prepared on a 430A peptide synthesizer. The 0.5 mmol standard protocol of system software version 1.40 NMP/HOBt *t*-Boc was employed, and end capping with acetic anhydride was performed after each amino-acid was introduced. Each Ser[PO_3(*c*Pen)$_2$] residue was introduced manually by mixing for 3 h with a peptide resin, which had free α-amino groups, and Boc-Ser[PO_3(*c*Pen)$_2$]-OBt, which was prepared by mixing Boc-Ser[PO_3(*c*Pen)$_2$]-OH (0.28 g, 0.66 mmol), HOBt·H_2O (0.11 g, 0.73 mmol), and DCC (0.14 g, 0.66 mmol) in a 1-methyl-2-pyrrolidinone (NMP) solution (1.4 mL) for 40 min. An aliquot of the resin (50 mg) was stirred on an ice bath with a reagent that contained TFMSA (0.45 mL), thioanisole (0.60 mL), *m*-cresol (0.50 mL), 1,2-ethanedithiol (0.20 mL) and TFA (3.25 mL) for 2 h. The reaction mixture was added to cold ether, and the resulting precipitate was washed with ether, and collected by centrifugation. The precipitate was washed twice with ether, and then dissolved in aqueous acetonitrile. The peptide solution was passed through a disposable ODS cartridge, and purified by RP-HPLC on YMC-Pack ODS-AM (20 × 250 mm) to yield $[Ser(PO_3H_2)^{2,11}]$-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**21**) (6.5 mg), which included a dehydration product. **21**: MS (MALDI-TOF) found 1802.3 (MH^+), calcd 1801.7 (average). Amino acid analysis: $Asp_{4.6}Ser_{1.5}Glu_{2.0}Ala_1Val_{0.92}Met_{0.94}Ile_{0.99}$-$Leu_{1.0}$.

To a solution of peptide **21** (6.5 mg) in DMSO (0.30 mL), Boc-OSu (2.5 mg, 12 μmol) and DIEA (15 μL) were added. After stirring for 2.5 h at room temperature, the peptide was isolated by RP-HPLC on Cosmosil 5C18ARII (4.6 × 250 mm) to give Boc-$[Ser(PO_3H_2)^{2,11}]$ - p21Max(1–13) - SCH_2CH_2CO - Leu-OH (**22**) (4.2 mg, 1.7 μmol, 11% based on the Ala residue in the starting resin **16**): MS (MALDI-TOF) found 1898.9 $(M-H)^-$, calcd 1898.6. Amino acid analysis: $Asp_{5.2}Ser_{1.6}Glu_{2.1}Ala_1Val_{0.96}Met_{0.99}Ile_{0.93}$-$Leu_{1.0}$.

Expression and Purification of p21Max(13–101) (23)

Ala-p21Max(14–101) DNA fragments corresponding to amino acid residues 13–101 of p21Max were amplified by the polymerase chain reaction from p22Max cDNA using the primers 5′GCGCCCAT GGCTGACAAACGGGCTCATCATAATGC and 3′GG CCGGATCCATTATTGGGCACTTGACCTCGCC. The amplified DNA fragments were digested by restriction enzymes, Nco I and Bam HI, and cloned into pET-11d expression vectors (Novagen, Madison, WI, USA). *Escherichia coli* BLR (DE3) pLysS, containing Ala-p21Max(14–101) expression vectors, were grown in M9 minimal media at 37°C until the absorbance at 600 nm reached 0.5. Isopropyl-β-D-thiogalactopyranoside (1 mM) was then added to induce expression. Cells were harvested after 6 h of induction by centrifugation and lysed in 20 mM Bicine buffer (pH 8.5) by sonication. The supernatant of the lysate was recovered by centrifugation, and fractionated by the addition of ammonium sulfate to 50% saturation. The precipitate of the recombinant protein was separated by the addition of ammonium sulfate to 80% saturation. The precipitate was dissolved in 20 mM Bicine buffer (pH 8.5) and applied to a cation-exchange column, SP-sepharose Fast Flow. The protein was eluted with linear gradient of sodium chloride concentration (0–1 M) in 20 mM Bicine buffer (pH 8.5). The fractions containing the recombinant proteins were applied to a RP-column, Poros R2/M, and eluted with a linear gradient of acetonitrile (0–80%) in 0.1% TFA. The elution fractions of Ala-p21Max(14–101) (**23**) were lyophilized and stored at −20°C. The *N*-terminal amino acid sequences of the purified protein were confirmed with an automated protein sequencer (Model PPSQ-21, Shimadzu, Kyoto, Japan) based on the Edman degradation method, and were in full agreement with the designed Ala-p21Max(14–101) peptide, except for the first methionine. **23**: MS (MALDI-TOF) found 10486 (MH^+), calcd 10485 (average). Amino acid analysis: $Asp_{10.8}Thr_{2.0}Ser_{5.9}Glu_{13.8}Pro_{1.3}Gly_{1.3}Ala_{10.3}Val_{2.3}$-$Met_{0.69}Ile_{3.9}Leu_8Tyr_{1.7}Phe_{1.2}Lys_{7.8}His_{5.8}Arg_{11.3}$.

Preparation of $(Lys(Boc)^{15,25,31,48,57,68,80,95})$-p21Max(14–101) (26)

A solution of peptide **23** (0.70 μmol) was stirred in the presence of nickel(II) sulfate (5 mM) in 0.5 M glyoxylic acid, 1 M acetic acid, and 2 M sodium acetate. The reaction was monitored by IEC using HiTrap™ SP (1 mL) [50 mM sodium phosphate, 8 M urea buffer (pH 5.9), a linear gradient of sodium chloride concentration]. After 2 h, EDTA (25 mg) was added, and the product, CH_3COCO-p21Max(14–101) (**24**), was partially purified by

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RP-HPLC on Cosmosil 5C4AR-300 (4.6×150 mm) to give 8.9 mg of a powder, after freeze-drying: MS (MALDI-TOF) found 10483 (MH^+), calcd 10484 (average).

To a solution of peptide **24** (8.9 mg) in DMSO (1.0 mL), Boc-OSu (12 mg, 56 μmol) and DIEA (50 μL) were added. After stirring for 1 h, the reaction mixture was washed with ether (three times) to give CH_3COCO-[Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(14–101) (**25**): MS (MALDI-TOF) found 11283 (MH^+), calcd 11285 (average).

The obtained precipitate was dissolved in an acetate buffer (1 M acetic acid and 1 M sodium acetate) containing 25 mM *o*-phenylenediamine and 30% DMF under argon, and the mixture was gently stirred for 2 days. A product was isolated by RP-HPLC on Cosmosil 5C4AR-300 (4.6×150 mm) to give [Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(14–101) (**26**) (4.7 mg, 0.24 μmol, 34%): MS (MALDI-TOF) found 11216 (MH^+), calcd 11214 (average). Amino acid analysis: $Asp_{11}Thr_{2.1}Ser_{6.1}Glu_{15}Pro_{1.0}Gly_{1.3}$-$Ala_9Val_{2.2}Met_{0.20}Ile_{3.9}Leu_{8.0}Tyr_{1.2}Phe_{1.0}Lys_{7.4}His_{5.6}$-$Arg_{10}$.

Preparation of p21Max(1–101) (28)

To a solution of peptide thioester **19** (2.5 mg, 0.93 μmol), peptide **26** (3.5 mg, 0.14 μmol), HOObt (4.9 mg, 30 μmol) and DIEA (3.5 μL, 20 μmol) in DMF (0.20 mL) was added silver chloride (0.4 mg, 3 μmol). After stirring for 48 h, dithiothreitol (DTT) (3 mg) was added, and the mixture was then washed with ether (3×1 mL) to give a crude peptide, Boc-[Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(1–101) (**27**): MALDI-TOF MS found 12734 (MH^+), calcd 12736 (average).

Crude peptide **27** was treated with TFA containing 5% water (0.20 mL) for 0.5 h. The product was precipitated with ether (1 mL), and then lyophilized from aqueous acetonitrile. After purification by IEC on Resource™ S (1 mL), then RP-HPLC on Cosmosil 5C4AR-300 (4.6×150 mm), p21Max(1–101) (**28**) was obtained (1.4 mg, 63 nmol, 45% yield based on peptide **26**): MS (MALDI-TOF) found 11839 (MH^+), calcd 11835 (average). Amino acid analysis: $Asp_{15}Thr_{2.1}Ser_{7.9}Glu_{15}Pro_{1.0}Gly_{2.0}Ala_{9.4}Val_{2.9}Met_{1.3}$-$Ile_{5.0}Leu_8Tyr_{1.9}Phe_{1.1}Lys_{7.4}His_{5.5}Arg_{11}$.

Preparation of (Ser(PO_3H_2)^{2,11})-p21Max(1–101) (30)

To a solution of peptide thioester **22** (2.0 mg, 0.98 μmol), peptide **26** (2.9 mg, 0.20 μmol), HOObt (4.8 mg, 29 μmol) and DIEA (3.4 μL, 20 μmol) in DMF (0.20 mL) was added silver chloride (0.2 mg, 2 μmol). After stirring for 72 h, DTT (6.5 mg) was added, and the mixture was then washed with ether (3×0.5 mL) to give the crude peptide, Boc-[Lys-(Boc)^{15,25,31,48,57,68,80,95}, Ser(PO_3H_2)^{2,11}]-p21Max(1–101) (**29**): MS (MALDI-TOF) found 12891 (MH^+), calcd 12896 (average).

The crude peptide **29** was treated with TFA containing 5% water (0.20 mL) for 0.5 h. After the addition of aqueous acetonitrile (1 mL), the mixture was lyophilized. After purification by IEC on Resource™ S (1 mL) and RP-HPLC on Cosmosil 5C4AR-300 (4.6×150 mm), [Ser(PO_3H_2)^{2,11}]-p21Max(1–101) (**30**) was obtained (1 mg, 62 nmol, 31% yield based on peptide **26**): MS (MALDI-TOF) found 11993 (MH^+), calcd 11995 (average). Amino acid analysis: $Asp_{17}Thr_{2.2}Ser_{7.8}Glu_{18}Pro_{0.99}$-$Gly_{1.7}Ala_{10}Val_{3.8}Met_{0.48}Ile_{5.0}Leu_{8.3}Tyr_{1.1}Phe_{0.94}Lys_{7.8}$-$His_{5.9}Arg_{10}$.

Digestion of Synthetic p21Max

Synthetic phosphorylated peptide **30** (6 nmol), was treated with lysyl endopeptidase (8 mAU) (Wako Pure Chemical) in 50 mM ammonium hydrogencarbonate (0.10 mL) at 35°C under argon for 2 h. The reaction mixture was analysed by RP-HPLC on Cosmosil 5C18ARII (4.6×250 mm) (Figure 8(A)), and mass numbers [MALDI-TOF (MH^+)] of the fractions were as follows: **a**, 619.5; **b**, 824.9; **c**, 1232.2; **d**, 1844.6; **e**, 1002.2; **f**, 1464.2; **g**, 1459.3; **h**, 1903.9; **i**, 1797.4. These correspond to **a**, p21Max(96–101) (calcd 619.3); **b**, p21Max(26–31) (calcd 825.0); **c**, p21Max(16–25) (calcd 1232.4); **d**, [Ser(PO_3H_2)^{2,11}]-p21Max(1–15) (calcd 1843.7); **e**, p21Max(49–57) (calcd 1002.2); **f**, p21Max(69–80) (calcd 1464.5); **g**, p21Max(58–68) (calcd 1459.7); **h**, p21Max(32–48) (calcd 1903.0); **i**, p21Max(81–95) (calcd 1797.1), respectively. When non-phosphorylated peptide **28** was digested, a mass number [1684.1 (MH^+)] corresponding to p21Max(1–15) (calcd 1683.7) was observed between peaks **f** and **g**. The retention times of the peptide fragments, p21Max(1–15) and [Ser(PO_3H_2)^{2,11}]-p21Max(1–15), were compared with authentic peptides, which were prepared by an Fmoc solid-phase method on a multiple organic synthesizer, 440Ω MOS.

RESULTS AND DISCUSSION

The reaction process involved in the thioester method is shown in Scheme 1. The peptide thioester

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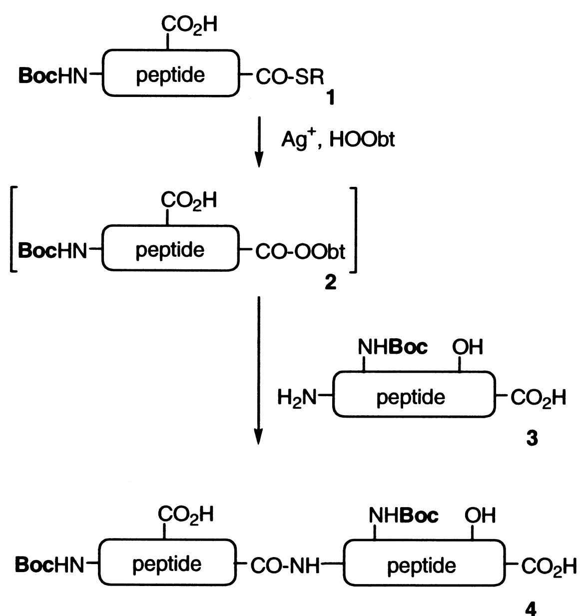


Scheme 1 Segment condensation by the thioester method.

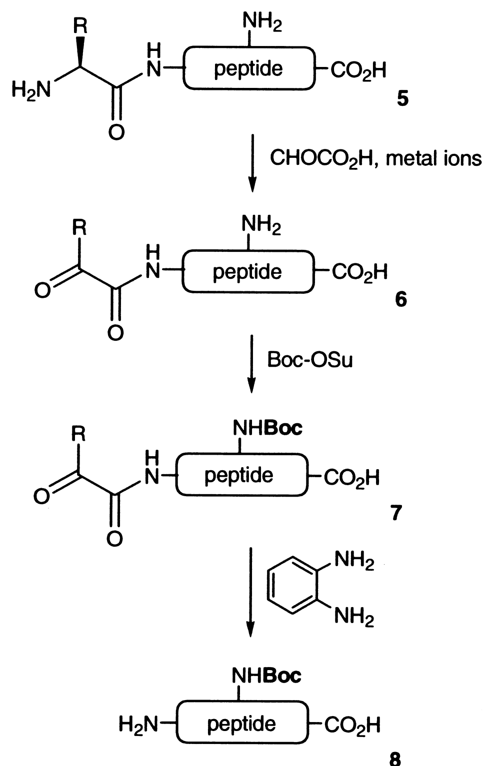
A strategy for the synthesis of *C*-terminal partially protected peptide segments is shown in Scheme 2. This is based on a method for the removal of the *N*-terminal residue of a protein via a transamination reaction [29]. That method involves two steps; first, peptide **5** is transaminated to give a stable intermediate, N^{α}-α-oxoacyl peptide **6**, the α-oxoacyl group is then removed by reaction with diamines. If it were possible to introduce Boc groups to the side chain amino groups of N^{α}-α-oxoacyl peptide **6** and then remove the α-oxoacyl group, a selectively protected *C*-terminal building block **8** would be obtained via peptide **7**.

Transamination conditions of an *N*-terminal amino group were initially examined using a model peptide, ADKRAHHNAL-NH_2 (**9**), which corresponds to p21Max(13–22)-NH_2. Peptide **9** was treated with glyoxylic acid in a pyridine buffer (10%) in the presence of copper(II) sulfate, elution profile by RP-HPLC after a 1-h reaction of which is shown in Figure 1(A). A transaminated product, CH_3COCO-p21Max(14–22)-NH_2 (**10**), was observed in the

1 is activated to the corresponding peptide active ester **2** in the presence of silver ions and an active ester component such as HOObt. The active ester **2** is reacted with another partially protected peptide **3** to give a polypeptide **4**. In this thioester method, selectively protected peptides on amino groups are required, in order to avoid the formation of branched peptides.

Strategy for the Synthesis of Partially Protected Peptides from Free Peptides

Transformation of *N*-terminal amino acid residues to α-oxoacyl groups, followed by removal of them were reported by Dixon *et al.* [29–31]. The N^{α}-α-oxoacyl peptides have been used in the synthesis of protein analogues using biologically prepared peptides [32]. For example, Gaertner *et al.* reported on hydrazone bond formation as the result of the reaction of peptide hydrazide and N^{α}-glyoxyl peptides, which were prepared from recombinant proteins [33]. Although these reactions were observed to proceed chemoselectively, they do not give rise to a native peptide bond. Although protecting groups are required on the side-chain nucleophilic functional groups, the thioester method can produce native peptide bonds with native sequences.



Scheme 2 Strategy for the preparation of partially protected peptide segments from free peptides.

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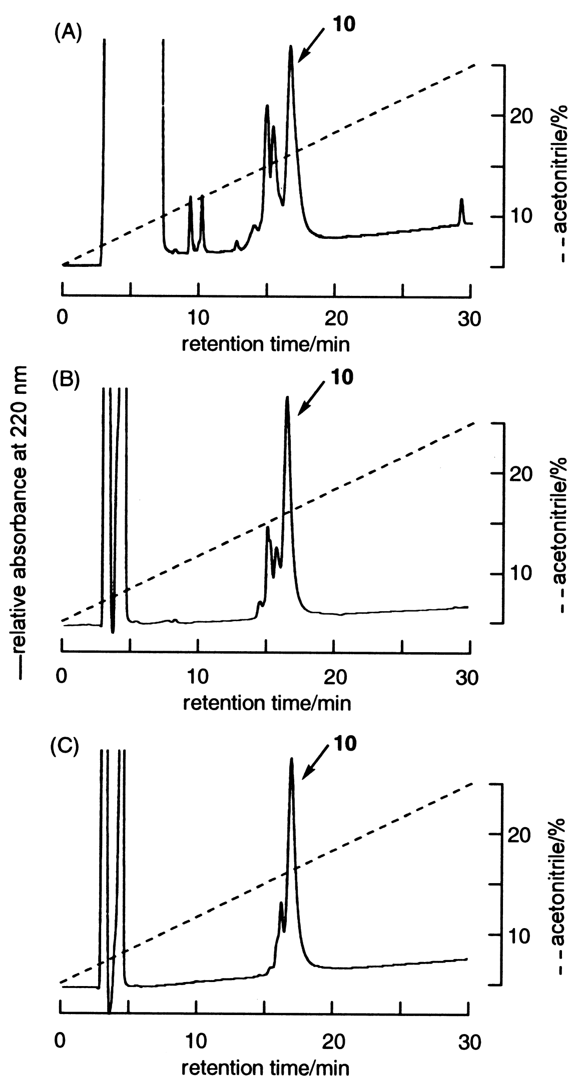

Figure 1 RP-HPLC elusion profiles of reaction mixtures of transaminated peptide **10**, which is indicated by an arrow. Peptide **9** was treated in the following conditions: (A) 0.5 M glyoxylic acid, 8 mM $CuSO_4$ in 10% pyridine, 1 h. (B) 0.5 M glyoxylic acid, 5 mM $CuSO_4$ in 1 M acetic acid and 2 M sodium acetate, 1 h. (C) 0.5 M glyoxylic acid, 5 mM $NiSO_4$ in 1 M acetic acid and 2 M sodium acetate, 4 h. Column: Cosmosil 5C18ARII (4.6 × 250 mm), eluent: 0.1% TFA in aq acetonitrile, 1.0 mL/min.

fraction, indicated by an arrow, which was confirmed by MALDI-TOF MS and amino acid analysis. However, the product gradually reacted further with glyoxylic acid under the same conditions as were used for its formation. When an acetate buffer (1 M acetic acid and 2 M sodium acetate) was used instead of a pyridine buffer, after a 1-h reaction, the desired product **10** was formed more selectively (Figure 1(B)). Side reaction products were also observed and these increased gradually with time. Nickel ions are also used as promoters for the transamination [34]. Nickel(II) sulfate was found to catalyse the reaction more slowly than copper(II) sulfate in an acetate buffer, and the reaction reached completion after 4 h (Figure 1(C)). Peptide **10** was stable in the reaction mixture for periods of up to 8 h. This indicates that the reaction can be easily controlled via the use of nickel ions.

Although the side reaction products could not be fully characterized, analyses of the reaction of another model peptide, ADKRVF-NH_2 (**11**), by MALDI-TOF MS and ^{1}H-NMR spectroscopy showed that the mass number of one of the side reaction products was 74 Da larger than that of the desired peptide, CH_3COCO-DKRVF-NH_2 (**12**), and the ^{1}H signal of the methyl group of the pyruvoyl group disappeared. These findings indicate that the pyruvoyl peptide **12** reacted further with glyoxylic acid to give an aldol adduct, HOCOCH(OH)CH_2COCO-DKRVF-NH_2 (**13**), under the reaction conditions, used.

An amino group of a lysine residue in the N^{α}-pyruvoyl peptide **10** was protected by a Boc group using Boc-OSu in DMSO in the presence of DIEA, to give CH_3COCO-[Lys(Boc)^{15}]-p21Max(14–22)-NH_2 (**14**). To remove the pyruvoyl group, peptide **14** was treated with *o*-phenylenediamine (40 mM) in an acetate buffer (2 M acetic acid and 2 M sodium acetate) [29–31] for 24 h to give a partially protected peptide, [Lys(Boc)^{15}]-p21Max(14–22)-NH_2 (**15**).

Strategy for the Synthesis of Phosphorylated p21Max Protein

It is now possible to prepare selectively protected peptide segments from free peptides. In order to evaluate this strategy, we synthesized a partial sequence of phosphorylated p21Max protein using a recombinant peptide, expressed in *E. coli*. A partial sequence of phosphorylated p21Max protein, [Ser(PO_3H_2)^{2,11}]-p21Max(1–101) (**30**), is as follows; M**pS**DNDDIEVE**pS**DA↓DKRAHHNALERKRRDHIKD SFHSLRDSVPSLQGEKASRAQILDKATEYIQYMRRK NHTHQQDIDDLKRQNALLEQQVRALEKARSSAQ, where **pS** represents an *O*-phosphoserine residue and an arrow, ↓, indicates the coupling site.

The strategy for the synthesis of peptide **30** is shown in Scheme 3. For synthetic purposes, two peptide segments corresponding to the sequences, p21Max(1–13) (**21**), whose *C*-terminal is the thioester, and p21Max(13–101) (**23**), are prepared via chemical and biological means, respectively.

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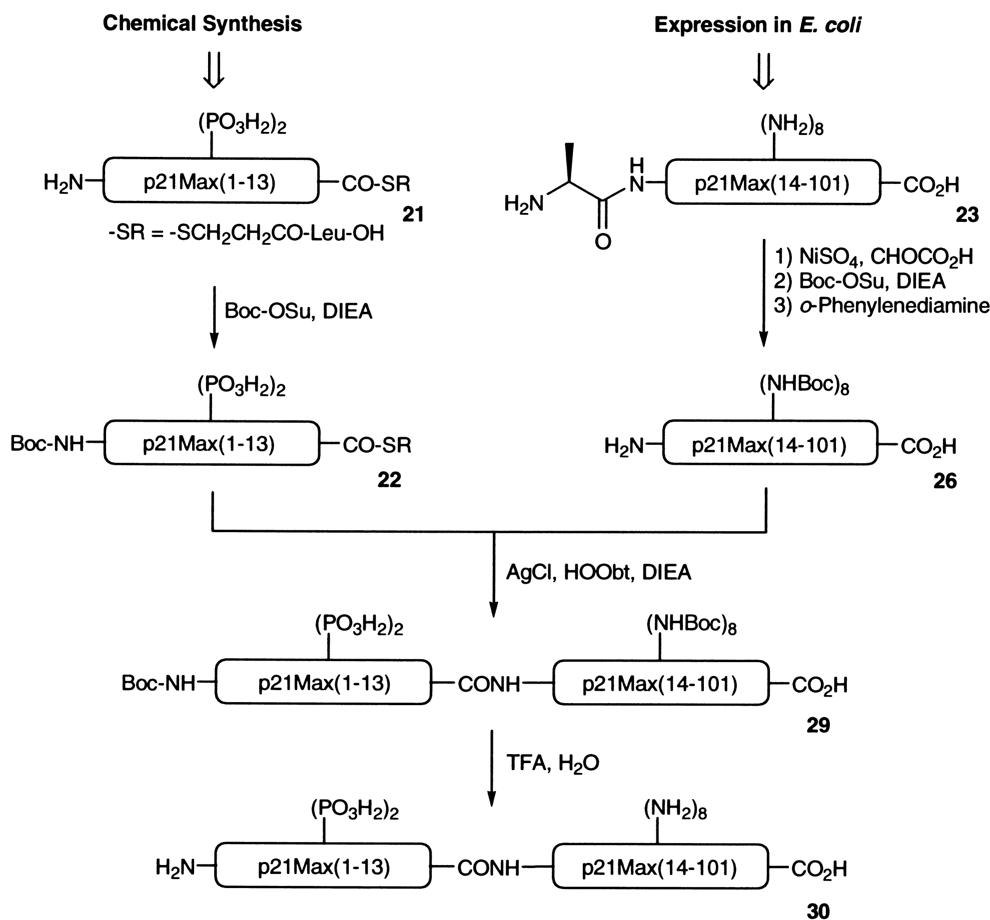

Scheme 3 Strategy for the synthesis of [Ser(PO_3H_2)^{2,11}]-p21Max(1–101) (**30**).

Each will be chemically converted to the partially protected forms, Boc-[Ser(PO_3H_2)^{2,11}]-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**22**) and [Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(14–101) (**26**), respectively. Condensation of these peptides would give Boc-[Ser(PO_3H_2)^{2,11}, Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(1–101) (**29**), which, after deprotection, gives peptide **30**.

Preparation of a Partially Protected *C*-terminal Peptide Segment

Ala-p21Max(14–101) (**23**), which was prepared by expression in *E. coli* [35] was transaminated by treatment with nickel(II) sulfate and glyoxylic acid in an acetate buffer (1 M acetic acid and 2 M sodium acetate). The reaction was monitored by IEC using a sulfopropyl (SP) column (Figure 2), and the elution profile after a 2-h reaction is shown in Figure 2(B). An N^{α}-pyruvoyl product, CH_3COCO-p21Max(14–101) (**24**), was efficiently formed. Transamination of peptide **23** using copper(II) sulfate also gave the desired product **24**, which gradually degraded under the reaction conditions, used, in the presence of copper ions. After the transamination of peptide **23**, EDTA was added to the reaction mixture, in order to inactivate nickel ions, and the resulting N^{α}-pyruvoyl product **24** was partially purified by RP-HPLC using a C4 column to remove excess reagents. Peptide **24**, thus obtained, was treated with Boc-OSu and DIEA in DMSO to give CH_3COCO-[Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(14–101) (**25**). The reaction proceeded without any problems.

Next, to remove the pyruvoyl group, peptide **25** was treated with *o*-phenylenediamine in an acetate buffer which contained 30% DMF. A reaction at room temperature for 48 h gave [Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(14–101) (**26**) in 34% yield based on peptide **23** after purification by RP-HPLC using a C4 column (Figure 3). The mass number and the amino acid composition of peptide

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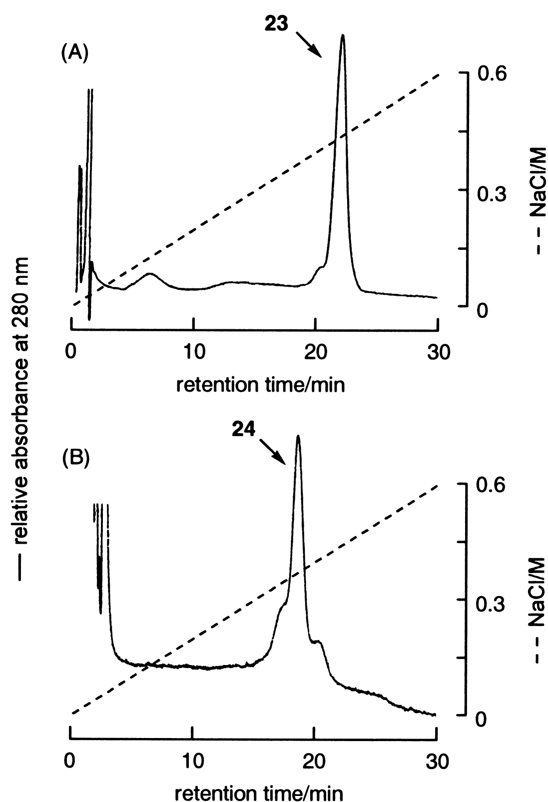


Figure 2 IEC elution profiles of (A) Ala-p21Max(14–101) (**23**) and (B) a reaction mixture of CH_3COCO-p21Max(14–101) (**24**). Column: HiTrap™ SP (1 mL). Eluent: 50 mM sodium phosphate buffer containing 8 M urea (pH 5.9) using a linear gradient of sodium chloride (1.0 mL/min).

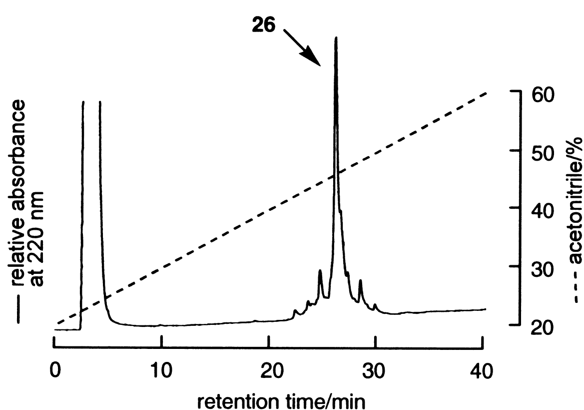
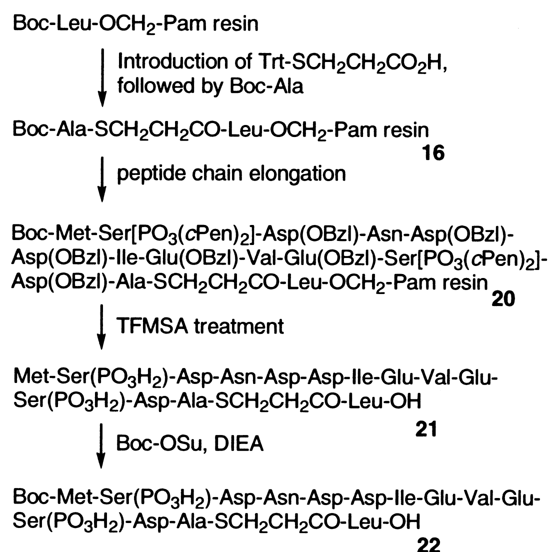


Figure 3 RP-HPLC elution profile of the reaction mixture of [Lys(Boc)^{15,25,31,48,57,68,80,95}]-p21Max(14–101) (**26**). Column: Cosmosil 5C4AR-300 (4.6 × 150 mm), eluent: 0.1% TFA in aq acetonitrile, 1.0 mL/min.

26 were in good agreement with the calculated values. Thus, the partially protected peptide segment for the thioester method was prepared from the expressed free peptide.



Scheme 4 Preparation of phosphorylated peptide thioester, Boc-[Ser(PO_3H_2)^{2,11}]-p21Max(1–13)-SCH_2CH_2-CO-Leu-OH (**22**).

Preparation of Phosphorylated Peptide Thioester

A peptide thioester, Boc-[Ser(PO_3H_2)^{2,11}]-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**22**), was synthesized via a solid-phase method using the Boc protocol (Scheme 4). Starting from a Boc-Leu-OCH_2-Pam resin, after removal of the Boc group, 3-(triphenylmethylthio)propionic acid was introduced by using a DCC–HOBt method, after which, the Trt group was removed by treatment with TFA containing 5% 1,4-butanedithiol. Boc-Ala-OH was then introduced by using a DCC–HOBt method in the presence of an equal amount of DIEA to give the Boc-Ala-SCH_2CH_2CO-Leu-OCH_2-Pam resin (**16**) [3–6]. Using this resin, the peptide chain was then elongated by an automated peptide synthesizer, to give Boc-Met-Ser[PO_3(*c*Pen)$_2$]-Asp(OBzl)-Asn-Asp(OBzl)-Asp-(OBzl)Ile-Glu(OBzl)-Val-Glu(OBzl)-Ser[PO_3(*c*Pen)$_2$]-Asp(OBzl)-Ala-SCH_2CH_2CO-Ala-OCH_2-Pam resin (**20**). In this synthesis, Boc-Ser[PO_3(*c*Pen)$_2$]-OH [27] was introduced via a manual operation. Resin **20** was treated with 1 M TFMSA in TFA containing *m*-cresol, thioanisole, and 1,2-ethanedithiol, to give a crude preparation of a peptide, [Ser(PO_3H_2)^{2,11}]-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**21**). The RP-HPLC of this preparation is shown in Figure 4(A),

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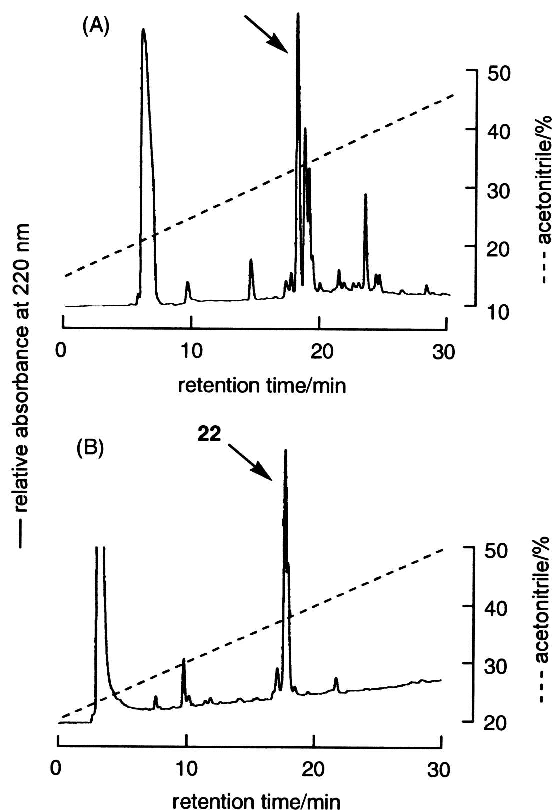

Figure 4 RP-HPLC elution profiles of the reaction mixtures of (A) $[Ser(PO_3H_2)^{2,11}]$-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**21**) [column: Cosmosil 5C18ARII (10 × 250 mm), eluent: 0.1% TFA in aq acetonitrile, 2.5 mL/min] and (B) Boc-$[Ser(PO_3H_2)^{2,11}]$-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**22**) [column: Cosmosil 5C18ARII (4.6 × 250 mm), eluent: 0.1% TFA in aq acetonitrile, 1.0 mL/min].

and the fraction corresponding to a major peak contained the desired peptide **21** as well as a side reaction product, which had a smaller mass number than peptide **21** by 18 Da. The latter would arise from the succinimide formation at the Asp residue. After partial purification by RP-HPLC, this peptide was treated with Boc-OSu to protect the *N*-terminal amino group. The desired product **22** was separated by RP-HPLC (Figure 4(B)) in 11% yield based on the Ala residue on the starting resin **16**.

We previously reported on the preparation of a phosphorylated peptide thioester in the synthesis of phosphorylated cAMP response element binding protein 1 (19–106) [6]. A 27-residue peptide thioester, which contains two *O*-phosphothreonine residues, was prepared. In that synthesis, a benzyl (Bzl) ester was used for the side-chain protection of the Asp residues and a small amount of succinimide formation was observed. A cycloheptyl ester has been reported as a protecting group, which can be removed by treatment with TFMSA [36], but its use failed to improve the synthesis of that peptide. Further difficulty was expected in the synthesis of phosphopeptide thioester **22**, because it contains four Asp and one Asn residues in 13 residues. Indeed, it was estimated from the elution profile of the reaction mixture of Boc-protected peptide **22** (Figure 4(B)) that one third had been damaged. In the synthesis of non-phosphorylated peptide thioester, Boc-p21Max(1–13)-SCH_2CH_2CO-Leu-OH (**19**), in which Boc-Asp(O*c*Hex)-OH was used to introduce Asp residues, followed by hydrogen fluoride cleavage, in fact, the same yield was obtained. This suggests that the difficulty of this synthesis resides in the sequence which contains multiple Asp residues, and not the phosphorylated residues.

Segment Condensation

The segment condensation of peptides **22** and **26** proceeded at a reasonable rate by treatment with silver chloride in the presence of HOObt and DIEA in DMF. The RP-HPLC elution profile after a 24-h reaction is shown in Figure 5(A). A condensation product, Boc-$[Lys(Boc)^{15,25,31,48,57,68,80,95}$, $Ser(PO_3H_2)^{2,11}]$-p21Max(1–101) (**29**), was observed in the fraction indicated by an arrow. After washing the reaction mixture with ether, peptide **29** was treated with TFA containing 5% water to afford the final product, $[Ser(PO_3H_2)^{2,11}]$-p21Max(1–101) (**30**). The RP-HPLC elution profile is shown in Figure 5(B). Peptide **30** was contained in the fraction indicated by an arrow, although the peptide, derived from the *C*-terminal segment, was also detected by MALDI-TOF MS. Therefore, the final product was purified by IEC (Figure 6), followed by RP-HPLC on a C4 column, to give a yield of 31% based on the *C*-terminal peptide **26**. The RP-HPLC elution profile and the mass spectrum of the purified peptide **30** are shown in Figure 7. The mass number and amino acid analysis of peptide **30** were in good agreement with the calculated values.

Peptide **30** was further digested with Lysyl Endopeptidase to estimate the amount of epimerization at the condensation site. The RP-HPLC elution profile of the digest is shown in Figure 8. All the fragments were observed by MALDI-TOF MS analysis. The amount of epimerization of the Ala residue at the condensation site was less than 5% (Figure 8(B)).

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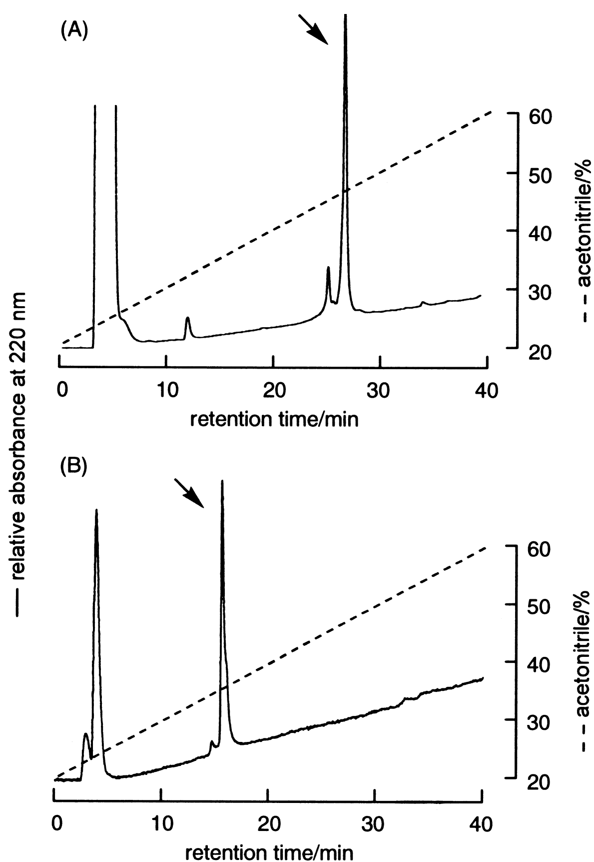

Figure 5 RP-HPLC elution profiles of the reaction mixtures of (A) Boc-[Lys(Boc)[15,25,31,48,57,68,80,95], Ser-$(PO_3H_2)^{2,11}$]-p21Max(1–101) (**29**) and (B) [Ser$(PO_3H_2)^{2,11}$]-p21Max(1–101) (**30**). Column: Cosmosil 5C4AR-300 (4.6 × 150 mm), eluent: 0.1% TFA in aq acetonitrile, 1.0 mL/min.

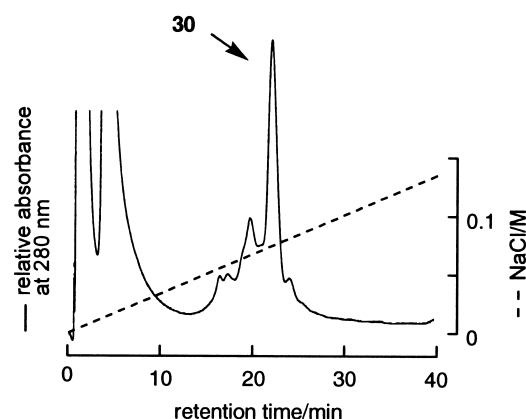

Figure 6 IEC elution profile of the reaction mixture of [Ser$(PO_3H_2)^{2,11}$]-p21Max(1–101) (**30**). Column: Resource™ S (1 mL), eluent: 40 mM sodium phosphate buffer containing 8 M urea (pH 5.0) using a linear gradient of sodium chloride concentration, 1.0 mL/min.

When DMSO was used as a solvent for the thioester condensation, the measured epimerization was 14% (Figure 8(C)). In a previous synthesis of the reaper protein, we also observed the epimerization at a comparable level (18%) [5]. Coupling conditions, which suppress the epimerization, were examined using model peptides, and our findings to date indicate that the use of DMF as a solvent suppresses epimerization to some extent (Teruya K *et al.*, unpublished data). In the condensation of peptides **22** and **26**, the epimerization could be actually suppressed to less than 5%. It was also possible to prepare the non-phosphorylated p21Max(1–101) (**28**) by the condensation of peptides **19** and **26** in about the same yield as for the phosphorylated sample with less than 3% epimerization.

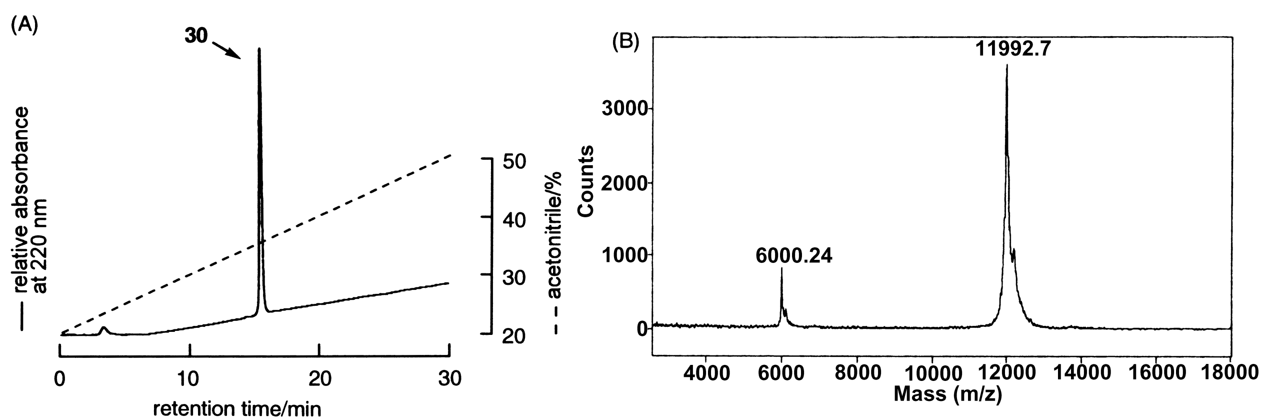

Figure 7 Purified [Ser$(PO_3H_2)^{2,11}$]-p21Max(1–101) (**30**). (A) RP-HPLC elution profile. Column: Cosmosil 5C4AR-300 (4.6 × 150 mm), eluent: 0.1% TFA in aq acetonitrile, 1.0 mL/min. (B) MALDI-TOF MS spectrum. Matrix: sinapinic acid.

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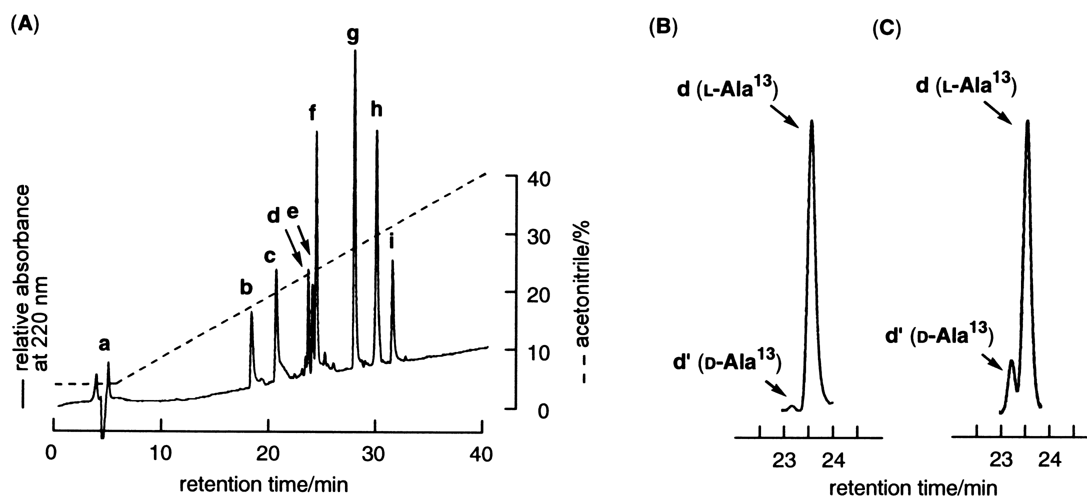

Figure 8 Digestion of [Ser(PO_3H_2)^{2,11}]-p21Max(1–101) (**30**) with lysyl endopeptidase. (A) RP-HPLC elution profile of the digest. Each peak corresponds to the fragment: **a**, (96–101); **b**, (26–31); **c**, (16–25); **d**, (1–15); **e**, (49–57); **f**, (69–80); **g**, (58–68); **h**, (32–48); **i**, (81–95), respectively. Column: Cosmosil 5C18ARII (4.6 × 250 mm), eluent: 0.1% TFA in aq acetonitrile, 1.0 mL/min. (B) A part of the RP-HPLC of the digest of peptide **30** obtained in DMF as a solvent for the condensation reaction. The D-Ala^{13} containing peptide was observed in less than 5%. (C) A part of the RP-HPLC of the digest of peptide **30** obtained in DMSO as a solvent for the condensation reaction. The D-Ala^{13} containing peptide was observed in 14%.

CONCLUSIONS

The above data clearly show that an expressed peptide can be used as a building block for the thioester method via transamination of the *N*-terminal amino acid residue. In the transamination, nickel ions can be used more safely than copper ions, and the subsequent protection of side chain amino groups with Boc group can be performed without any problems. Removal of α-oxoacyl group was carried out in the presence of DMF as a co-solvent because of the low solubility of a Boc-protected peptide in an aqueous-only buffer, and proceeded smoothly. Although some difficulty in the preparation of phosphorylated peptide thioesters, which contains multiple Asp residues, remains, once those can be prepared, it should be possible to perform segment condensation by the thioester method without any problems. Furthermore, epimerization at the condensation site can be suppressed by using DMF as a solvent, and this greatly simplifies the synthesis of proteins from recombinant proteins, because the any condensation sites can be selected. This methodology will, in the future, be applied to the synthesis of a wide variety of polypeptides including segmentaly isotope-labelled proteins.

Quite recently, Huse *et al.* also reported a synthesis of a phosphorylated polypeptide using a recombinant protein by native chemical ligation [37]. In that synthesis, a phosphorylated peptide thioester was synthesized in a low yield via the use of a 'safety catch' resin [38], and a mutation was required at the condensation site to expose a cysteine residue in the *C*-terminal building block. In contrast, any residues can be condensed using the thioester method. In addition, the synthesis of phosphorylated peptide thioesters is under investigation using a modified Fmoc strategy, which we previously reported for the synthesis of peptide thioesters [39].

Acknowledgements

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