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Fmoc-based Chemical Synthesis and Selective Binding to Supercoiled DNA of the p53 *C*-terminal Segment and its Phosphorylated and Acetylated Derivatives‡

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Abstract: The *C*-terminal domain of p53 comprises a linker, the tetramerization domain and the regulatory domain, and contains at least seven sites of potential post-translational modification. An improved strategy was developed for the synthesis of large peptides that contain phosphorylated amino acids and p53(303–393), a 91-amino acid peptide, and three post-translationally modified derivatives were synthesized through the sequential condensation of three partially protected segments. Peptide thiolesters were prepared using the sulfonamide-based 'safety-catch' resin approach and employing Fmoc-based solid-phase peptide synthesis. At the *N*-terminus of the middle building block, a photolabile protecting group, 3,4-dimethoxy-6-nitrobenzyloxycarbonyl, was incorporated to differentiate the *N*-terminal amino group from the side-chain amino groups. Two sequential couplings were accomplished following this protection strategy. The synthetic products, p53(303–393) and its phosphorylated or acetylated derivatives, exhibited the ability to bind specifically to supercoiled DNA, which is one of the characteristics of this domain. Published in 2004 by the European Peptide Society and John Wiley & Sons, Ltd.

Keywords: p53 *C*-terminal domain; peptide thiolester condensation; photolabile protecting group; safety-catch resin approach; selective binding to supercoiled DNA

INTRODUCTION

Specifically modified proteins are of great use for biochemical and biophysical studies. For example, the specific post-translational modifications that are induced by signal transduction networks may differ in normal and disease states. Although biological expression methods are widely utilized and are of great power for the preparation of proteins, they have limited utility for the preparation of site-specifically modified products. Chemical synthesis, however, is quite suitable for this purpose. Since 1991, peptide thiolesters have been utilized extensively as building blocks for constructing polypeptides [1–3]. Several methods have been developed both for segment coupling [1–8] and the preparation of peptide α-thiolesters [1, 9–13]. Each method has advantages and disadvantages

Abbreviations: Ac, acetyl; AM, aminomethyl; Boc, *tert*-butyloxycarbonyl; Boc-ON, 2-(*tert*-butyloxycarbonyloxyimino)-2-phenylacetonitrile; BSA, bovine serum albumin; Bzl, benzyl; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(benzotriazol-1-yl-)1,1,3,3-tetramethyluronium hexafluorophosphate; HMP, 4-hydroxymethylphenoxy; HOBt, 1-hydroxy-1,2,3-benzotriazole; HOOBt, 3,4-dihydroxy-4-oxo-1,2,3-benzotriazine; MALDI-TOF MS, matrix assisted laser desorption ionization/time-of-flight mass spectrometry; *NVOC*, 3,4-dimethoxy-6-nitrobenzyloxycarbonyl; PBS, phosphate buffered saline; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; SPPS, solid-phase peptide synthesis; ^{t}Bu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl (triphenylmethyl).

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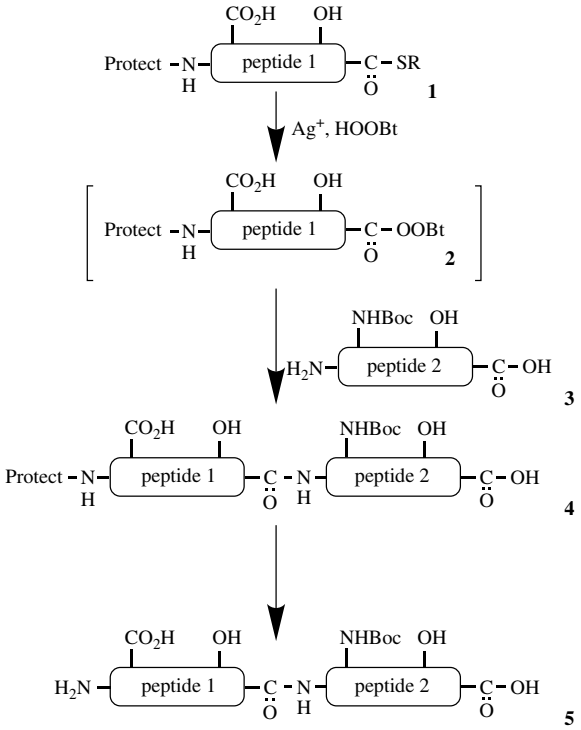
with respect to the selection of amino acid(s) at the coupling site and requirements for side-chain protection and deprotection [14]. In the thiolester method [1], the *C*-terminal thiolester group of one segment is selectively activated by silver ions, and then condensed with the *N*-terminus of the second segment (Scheme 1). Previously, peptide thiolesters have been prepared by Boc-based peptide elongation on 2-mercaptopropionic acid loaded onto a resin support [3], and have been used for the synthesis of peptides containing phosphorylated [15] or glycosylated [16] residues. Recent investigations, however, have shown that some side reactions occur during the Boc-based preparation of phosphorylated peptide thiolesters, primarily during the deprotection step [17]. The Fmoc solid-phase method requires neither the repetitive use of TFA nor strong acid treatment during the cleavage step. These features are advantageous in the preparation of post-translationally modified peptide thiolesters. Several distinct improvements in Fmoc-SPPS based peptide thiolester preparation methodology have been reported recently [9–13]. For the construction of large peptides, however, a multiple segment condensation strategy, in which more than two segments are condensed sequentially, should be adopted. This requires an orthogonal protection strategy between the *N*-terminal and side-chain amino groups.



Scheme 1 Schematic drawing of segment condensation via the thiolester method.

The tumor suppressor protein p53 plays a central role as guardian of the genome [18] through enhancing DNA repair, regulating the cell cycle or inducing apoptosis [19]. In response to DNA damage or other stresses, a complex signal transduction network results in the phosphorylation or acetylation of several among at least 18 distinct sites on p53, resulting in its stabilization and activation [20]. p53 is regarded as a modular protein, consisting of transactivation, SH3-like, DNA binding, tetramerization and regulatory domains [21] (Figure 1). Phosphorylation or acetylation of the transactivation and regulatory domains leads to transcriptional activation through binding by the DNA binding domain to specific sequences in the promotor regions of critical genes [22]. Recent developments in the functional analysis of this protein, however, have revealed that the *C*-terminal domain, which consists of a linker region to the DNA binding domain, the tetramerization domain and the regulatory domain, has the potential to bind to specific DNA structures, such as supercoiled DNA [23–25]. The effects of post-translational modifications and functional truncation of the p53 *C*-terminal domain on this selective binding

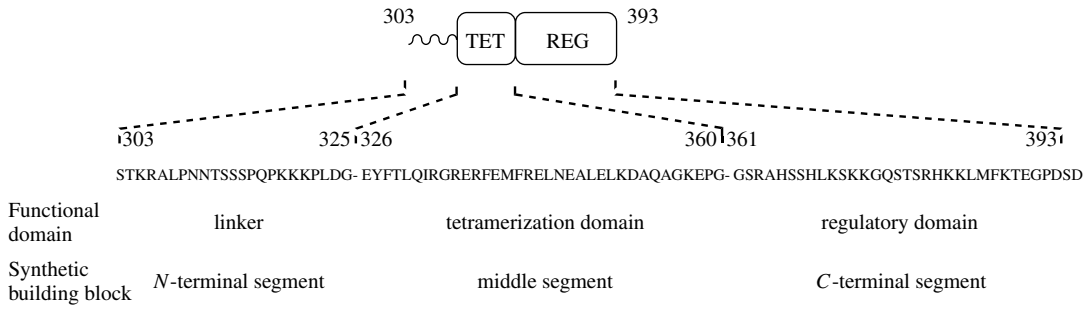


Figure 1 Schemes of the domain structure of the p53 *C*-terminal region and the amino acid sequence.

ability, however, have not been investigated. For this purpose, a chemical preparation of the p53 *C*-terminal domain is necessary.

A chemical synthesis of the p53 *C*-terminal domain (91 amino acid residues) employing Boc-based SPPS was reported previously [26]. Here the synthesis is reported of p53(303–393) and its phosphorylated and acetylated derivatives, [Ser^{315}(PO_3H_2)]-, [Lys^{320}(Ac)]- and [Ser^{378}(PO_3H_2)]-p53(303–393), in which Fmoc-SPPS was used for the preparation of three building blocks and the thiolester condensation method for coupling. To make the multi-segment condensation strategy feasible, the NVOC group [27] was utilized as the *N*-terminal protecting group for the middle building block in order to provide for protection orthogonal to the side-chain protection. Finally, the synthetic products were used to investigate the effects of modifications or truncation of the p53 *C*-terminal domain upon selective binding to supercoiled DNA.

MATERIALS AND METHODS

General Methods

Fmoc-amino acid derivatives, reagents for peptide synthesis and 4-sulfamylbutyryl AM resin were purchased from Novabiochem (Läufelfingen, Switzerland). Boc-ON [28], NVOC-Cl, HOOBt and cyanuric fluoride were purchased from Sigma Aldrich (St Louis, MO, USA). Peptide chain elongations were performed on a model 433A peptide synthesizer (Applied Biosystems, Foster City, CA, USA) using the 0.1 mmol scale standard protocol employing HBTU/HOBt activation with slight modifications. Analytical HPLC (Hewlett Packard series 1050, Agilent Technologies, Palo Alto, CA, USA) was carried out on a reverse-phase column (Vydac C_{18}, 4.6 × 250 mm) that was eluted with a linear gradient of CH_3CN (5%–60%, 55 min) in 0.05% aqueous TFA at a flow rate of 0.5 ml/min. Yields of peptides were determined by quantitative amino acid analysis after hydrolysis with constant boiling hydrochloric acid (Pierce, Rockford, IL, USA) at 110 °C for 24 h in an evacuated sealed tube. Except as noted below, product yields were based on the determination of the Gly residue. Mass numbers were determined by MALDI-TOF MS using a TofSpec E instrument (Micromass, Beverly, MA, USA) with α-cyano-4-hydroxycinnamic acid as the matrix. Concentrations of peptides dissolved in PBS buffer were determined using NanoOrange® (Molecular Probes, Eugene, OR, USA) referred to BSA as a standard. The fluorescence (485 nm exc./590 nm em.) was recorded by a microplate fluorometer (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA). All peptide concentrations are given as the monomer.

Preparation of Building Blocks

An Fmoc-Gly-NH-SO_2-$(CH_2)_3$-AM resin (1.36 g, 0.80 mmol g^{-1}) was prepared from 4-sulfaminobutyryl AM resin (1.00 g, 1.1 mmol) using Fmoc-Gly-F (2.6 g, 8.8 mmol) [29]. Fmoc-Gly-F was prepared according to the previously reported protocol [30].

Boc-(Lys(Boc)^{305,319,320,321})-p53(303–325)-SBzl (12). Starting from the Fmoc-Gly-NH-SO_2-$(CH_2)_3$-AM resin (127 mg, 0.10 mmol), a protected peptide resin corresponding to the sequence of p53(303–325), Ser(^{t}Bu)-Thr(^{t}Bu)-Lys(Boc)-Arg(Pmc)-Ala-Leu-Pro-Asn(Trt)-Asn(Trt)-Thr(^{t}Bu)-Ser(^{t}Bu)-Ser(^{t}Bu)-Ser(^{t}Bu)-Pro-Gln(Trt)-Pro-Lys(Boc)-Lys(Boc)-Lys(Boc)-Pro-Leu-Asp(O^{t}Bu)-Gly-NHSO_2-$(CH_2)_3$-AM resin (472 mg) was prepared on the peptide synthesizer. The *N*-terminal amino group of an aliquot of the resin (171 mg, 36 μmol) of the side chain-protected resin was protected with the Boc group by using Boc-ON (89 mg, 0.36 mmol) in DMF (2.0 ml) containing DIEA (10 μl, 58 μmol). The resulting resin was treated with iodoacetonitrile (174 μl, 2.4 mmol) and DIEA (83 μl, 0.48 mmol) in DMF (3.0 ml). After mixing for 16 h, the reagent was filtered, and then the resin was washed with DMF (3 ml × 5), DCM (3 ml × 3) and DMF (3 ml × 5). To the resin, benzyl mercaptan (248 μl, 2.0 mmol) and DIEA (83 μl, 0.48 mmol) in DMF (3.0 ml) were added and the mixture stirred for 16 h at room temperature. The resin was separated from the solution and washed with DMF (3 ml × 2). The combined filtrates were concentrated, yielding a dark solid. To the residue Reagent K+ (TFA: phenol: H_2O: thioanisole: 1,2-ethanedithiol: triisopropylsilane = 80 : 5 : 5 : 5 : 2.5 : 2.5 (v/v) [31], 15 ml) was added and stirred for 4 h at room temperature. The crude peptide thiolester mixture was precipitated, washed with diethyl ether and dissolved with aqueous acetonitrile containing 0.05% TFA, then lyophilized. The crude peptide thiolester (66.3 mg) was purified by RP-HPLC (Vydac C_8, 20 × 250 mm). The purified peptide thiolester (45 mg) was dissolved with DMSO (1.0 ml). To the solution, Boc-ON (50 mg, 0.20 mmol) and DIEA (40 μl, 0.23 mmol) were added. After the reaction solution was stirred for 30 min, diethyl ether (20 ml) was added to the

reaction solution and the precipitate was washed (5 ml × 3) to obtain the partially protected peptide thiolester Boc-[Lys(Boc)^{305,319,320,321}]-p53(303–325)-SBzl (**12**) (54 mg). The precipitate was dissolved with aqueous CH_3CN containing 0.05% TFA, then lyophilized. The yield was 43% based on the first Gly residue of the resin. The peptide thiolester, p53(303–325)-SBzl, was subjected to analytical HPLC and MS analysis. MALDI-TOF MS: observed $[M + H]^+ = 2559.4$, calculated for p53(303–325)-SBzl $[M + H]^+ = 2558.9$.

Boc-(Ser^{315}(PO$_3$H$_2$), Lys(Boc)^{305,319,320,321})-p53 (303–325)-SBzl (13) and Boc-(Lys(Ac)^{320}, Lys(Boc)^{305,319,321})-p53(303–325)-SBzl (14). Starting from aliquots of the Fmoc-Gly-NH-SO_2-$(CH_2)_3$-AM resin (128 mg, 0.10 mmol), the protected peptide resins that correspond to **13** (424 mg) and **14** (436 mg) were prepared on the peptide synthesizer using the same procedure described in the preparation of **12** except that Fmoc-Ser[PO(OBzl)OH]-OH [32] was incorporated in the position of Ser^{315} for the preparation of **13** and the commercially available Fmoc-Lys(Ac)-OH was incorporated in the position of Lys^{320} for the preparation of **14**. The *N*-terminal amino groups of aliquots of the respective resins were treated as described above to give the crude products. The purified peptide thiolesters similarly were protected to give Boc-[Ser^{315}(PO$_3$H$_2$), Lys(Boc)^{305,319,320,321}]-p53(303–325)-SBzl (**13**) and Boc-[Lys^{320}(Ac), Lys(Boc)^{305,319,321}]-p53(303–325)-SBzl (**14**) in 36% and 63% yield, respectively, based on the first Gly residue of the resin. The peptide thiolesters were subjected to analytical HPLC and MS analysis. MALDI-TOF MS: observed $[M + H]^+ = 2638.6$, calculated for [Ser^{315}(PO$_3$H$_2$)]-p53(303–325)-SBzl $[M + H]^+ = 2638.9$. MALDI-TOF MS: observed $[M + H]^+ = 2601.0$, calculated for [Lys(Ac)^{320}]-p53(303–325)-SBzl $[M + H]^+ = 2600.2$.

NVOC-(Lys(Boc)^{351,357})-p53(326–360)-SBzl (15). Starting from the Fmoc-Gly-$NHSO_2$-$(CH_2)_3$-AM resin (124 mg, 0.10 mmol), the protected peptide resin, Glu(O^{t}Bu)-Tyr(^{t}Bu)-Phe-Thr(^{t}Bu)-Leu-Gln(Trt)-Ile-Arg(Pmc)-Gly-Arg(Pmc)-Glu(O^{t}Bu)-Arg(Pmc)-Phe-Glu(O^{t}Bu)-Met-Phe-Arg(Pmc)-Glu(O^{t}Bu)-Leu-Asn(Trt)-Glu(O^{t}Bu)-Ala-Leu-Glu(O^{t}Bu)-Leu-Lys(Boc)-Asp(O^{t}Bu)-Ala-Gln(Trt)-Ala-Gly-Lys(Boc)-Pro-Gly-$NHSO_2$-$(CH_2)_3$-AM resin, was obtained in the same manner as the preparation of **12**. The side-chain protected peptide resin was mixed with NVOC-Cl (0.20 g, 0.70 mmol) and DIEA (87 µl, 0.50 mmol) in 1,4-dioxane (5.0 ml). After mixing for 6 h, the resin was washed with 1,4-dioxane (3 ml × 3) and DMF (3 ml × 5), then DCM (3 ml × 5). From a portion (151 mg, 29 µmol) of the protected resin (524 mg), crude peptide thiolester (57 mg) was obtained. To the side-chain amino groups of the purified peptide thiolester (6.8 mg), Boc groups were incorporated with Boc-ON (2.4 mg, 9.7 µmol) in the presence of DIEA (9.8 µmol, 1.7 µl). The residual solid resulting from diethyl ether precipitation was dissolved, frozen and lyophilized. The NVOC-[Lys(Boc)^{351,357}]-p53(326–360)-SBzl (**15**) (1.4 µmol) was obtained in 5% yield based on the first Gly residue of the resin. The peptide thiolester, NVOC-p53(326–360)-SBzl, was subjected to analytical HPLC and MS analysis. MALDI-TOF MS: observed $[M + H]^+ = 4519.2$, calculated for NVOC-p53(326–360)-SBzl $[M + H]^+ = 4519.0$.

Boc-(Lys(Boc)^{351,357})-p53(326–360)-SBzl (16). Starting from Fmoc-Gly-$NHSO_2$-$(CH_2)_3$-AM resin (122 mg, 0.10 mmol), the protected peptide resin (545 mg) was obtained as described in preparation of **15**. The side-chain protected peptide resin (322 mg, 59 µmol) was mixed with Boc-ON (0.18 g, 0.73 mmol) and DIEA (20 µl, 0.11 mmol) in DMF (5.0 ml). After mixing for 1 h, the resin was washed with DMF (3 ml × 5) and DCM (3 ml × 5). The resin was treated as described above to produce the protected thiolester. Boc-[Lys(Boc)^{351,357}]-p53(326–360)-SBzl (**16**) (1.4 µmol) was obtained in 7% yield based on the first Gly residue of the resin. The peptide thiolester, p53(326–360)-SBzl, was subjected to analytical HPLC and MS analysis. MALDI-TOF MS: observed $[M + H]^+ = 4280.1$, calculated for p53(326–360)-SBzl $[M + H]^+ = 4279.8$.

(Lys(Boc)^{370,372,373,381,382,386})-p53(361–393) (17) and (Ser^{378}(PO$_3$H$_2$), Lys(Boc)^{370,372,373,381,382,386})-p53(361–393) (18). Starting from Fmoc-Asp(O^{t}Bu)-HMP resin (Applied Biosystems, 0.64 mmol g^{-1}, 215 mg, 0.14 mmol), the protected peptide resin corresponding to **17**, Fmoc-Gly-Ser(^{t}Bu)-Arg(Pmc)-Ala-His(Trt)-Ser(^{t}Bu)-Ser(^{t}Bu)-His(Trt)-Leu-Lys(Boc)-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Gly-Gln(Trt)-Ser(^{t}Bu)-Thr(^{t}Bu)-Ser(^{t}Bu)-Arg(Pmc)-His(Trt)-Lys(Boc)-Lys(Boc)-Leu-Met-Phe-Lys(Boc)-Thr(^{t}Bu)-Glu(O^{t}Bu)-Gly-Pro-Asp(O^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-HMP resin, (834 mg) was prepared on the peptide synthesizer. A protected peptide resin corresponding to **18** was prepared similarly except that Fmoc-Ser[PO(OBzl)OH]-OH was incorporated in the position of Ser^{378}. For each sample, a portion of the protected resin was

treated with Reagent K+ to obtain the crude peptide. Following purification of the peptides, a portion of each was treated with Boc-ON and DIEA. After diethyl ether precipitation and washing, the residual solids were treated with 20% piperidine in DMSO for 30 min. The peptides were precipitated from the reaction solution by diethyl ether then washed, dissolved and lyophilized. The products [Lys(Boc)^{370,372,373,381,382,386}]-p53(361–393) (**17**) and [Ser^{378}(PO_3H_2), Lys(Boc)^{370,372,373,381,382,386}]-p53 (361–393) (**18**) were obtained in 34% and 33% yield, respectively, based on the first Asp residue of the resin. The peptides were subjected to analytical HPLC and MS analysis. For Fmoc-p53(361–393) MALDI-TOF MS: observed $[M+H]^+ = 3876.3$, calculated for Fmoc-p53(361–393) $[M+H]^+ = 3876.3$. For Fmoc-[Ser^{378}(PO_3H_2)]-p53(361–393), MALDI-TOF MS: observed $[M+H]^+ = 3956.4$, calculated for Fmoc-[Ser^{378}(PO_3H_2)]-p53(361–393) $[M+H]^+ = 3956.3$.

Estimation of NVOC Deprotection Efficiency and Stability of the Phosphate Group under Deprotection Conditions

The protected resin (640 mg, 86 μmol) described in the preparation of **18** was treated with 20% (v/v) piperidine in DMF (5 ml) and washed with DMF (5 ml × 3). The protected peptide resin was mixed with NVOC-Cl (0.14 g, 0.50 mmol), DIEA(87 μl, 0.50 mmol) in 1,4-dioxane (3.0 ml). After mixing for 6 h, the resin was washed with 1,4-dioxane (3 ml × 3), DMF (3 ml × 5) and DCM (3 ml × 5), then dried. From a portion (300 mg, 39 μmol) of the protected resin (661 mg), crude product (186 mg) was obtained. A portion of the crude peptide (71 mg) was purified to give the purified NVOC-[Ser^{378}(PO_3H_2)]-p53(361–393), **23**, (21 mg). Fmoc-[Ser^{378}(PO_3H_2)]-p53(361–393), **24**, (16.5 mg) was prepared as described in the preparation of **18.** **23** (2.0 mg), **24** (1.6 mg) and DTT (7.0 mg) were dissolved in DMSO (300 μl). The solution was irradiated by UV lamp (365 nm, 1.3 mW/cm^2) and 10 μl of the solution were sampled periodically. The samples were diluted with water containing 1% TFA (360 μl). Aliquots (40 μl) of the samples were analysed on RP-HPLC. The concentrations of the peptide components were determined from a combination of amino acid analysis and peak area. The product, [Ser^{378}(PO_3H_2)]-p53(361–393), **25**, was identified by MS analysis. MALDI-TOF MS: observed $[M+H]^+ = 3973.6$ for **23**, calculated for NVOC-[Ser^{378}(PO_3H_2)]-p53(361–393) $[M+H]^+ = 3974.2$. MALDI-TOF MS: observed $[M+H]^+ = 3735.6$ for **25**, calculated for [Ser^{378}(PO_3H_2)]-p53(361–393) $[M+H]^+ = 3735.1$.

Segment Condensation

Synthesis of p53(326–393) (10) and (Ser^{378}(PO_3H_2))-p53(326–393) (11). Aliquots of **16** (0.60, μmol), **17** (0.30 μmol) or **18** (0.30 μmol), HOOBt (15 μmol, 2.4 mg), DIEA (10 μmol, 2.1 μl) and $AgNO_3$ (1.5 μmol, 0.26 mg) were dissolved in DMSO (500 μl) and the resulting solutions were stirred for 16 h at 37 °C. The reactions were quenched by 4 mg of DTT. Then, the reaction mixtures were precipitated by diethyl ether and washed. The residual solids were treated with Reagent K + (300 μl) for 90 min. To the reaction mixtures, diethyl ether was added to obtain the crude products, p53(326–393) (**10**), and [Ser^{378}(PO_3H_2)]-p53(326–393) (**11**), respectively. The residual solids were dissolved with aqueous acetonitrile containing 0.05% TFA, then lyophilized. After isolating the coupling products, **10** and **11**, by RP-HPLC (Vydac C_{18}, 10 × 250 mm, flow rate 2.2 ml/min) using a linear gradient of 18%–34% B over 35 min, the fractions were combined and lyophilized. The yields of **10** and **11** were 33% and 32% based on **17** and **18**, respectively. MALDI-TOF MS: observed $[M+H]^+ = 7809.5$ for **17**, calculated for p53(326–393) $[M+H]^+ = 7809.7$. MALDI-TOF MS: observed $[M+H]^+ = 7889.1$ for **18**, calculated for [Ser^{378}(PO_3H_2)]-p53(326–393) $[M+H]^+ = 7889.7$.

Synthesis of (Lys(Boc)^{351,357,370,372,373,381,382,386})-p53(326–393) (21) and (Ser^{378}(PO_3H_2), Lys(Boc)^{351,357,370,372,373,381,382,386})-p53(326–393) (22). Peptide thiolester**15** (0.70 μmol), and peptide **17** (0.25μmol) or **18** (0.25 μmol), HOOBt (15 μmol, 2.4 mg), DIEA (10 μmol, 2.1 μl) and $AgNO_3$ (0.20 mg, 1.2 μmol) were dissolved in DMSO (700 μl) and the resulting solutions were stirred for 48 h at 37 °C. The reactions were quenched with DTT (3.0 mg). Then, the peptide mixtures were precipitated by diethyl ether and washed. The residual solids were treated with Reagent K + (200 μl) for 60 min. To the reaction mixtures, diethyl ether was added to obtain the NVOC-p53(326–393) (**19**), and NVOC-[Ser^{378}(PO_3H_2)]-p53(326–393) (**20**) crude products, respectively. The residual solids were dissolved with CH_3CN/H_2O and lyophilized. After isolating the coupling products, **19** and **20** by RP-HPLC (Vydac C_{18}, 10 × 250 mm, flow rate 2.5 ml/min) using a linear gradient of 18%–38% B over 32 min, the fractions

were combined and lyophilized. The lyophilized powder, **19** or **20**, was dissolved with 200 µl of DMSO containing Boc-ON (1.0 mg, 4.0 µmol) and DIEA (1.5 µl, 8.6 µmol) and stirred for 30 min. To the reaction solution, diethyl ether was added and the residual solids were washed. The residual solids were dissolved with DMSO (200 µl) containing 2.5 mg of DTT. The solution was irradiated by UV lamp (365 nm, 1.3 mW/cm^2, 90 min). To each resulting solution, diethyl ether was added to precipitate [Lys(Boc)^{351,357,370,372,373,381,382,386}]-p53(326–393) (**21**) and [Ser^{378}(PO_3H_2), Lys(Boc)^{351,357,370,372,373,381,382,386}]-p53(326–393) (**22**). The yields of **21** and **22** were 22% and 26% based on **17** and **18**, respectively. MALDI-TOF MS: observed $[M + H]^+$ = 8049.0 for **19**, calculated for NVOC-p53(326–393) $[M + H]^+$ = 8048.9. MALDI-TOF MS: observed $[M + H]^+$ = 8127.6 for **20**, calculated for NVOC-[Ser^{378}(PO_3H_2)]-p53(326–393) $[M + H]^+$ = 8128.9.

Synthesis of p53(303–393) (6). Peptide thiolester**12** (300 nmol), peptide **21** (48 nmol), HOOBt (1.5 mg, 9.0 µmol), DIEA (5.8 µmol, 1.0 µl) and $AgNO_3$ (0.15 mg, 0.90 µmol) were dissolved in DMSO (500 µl) and the reaction solutions were stirred for 16 h at 37 °C. The reaction was quenched with DTT (2.0 mg). Then, each peptide mixture was precipitated by diethyl ether and washed. The residual solids were treated with Reagent K + (200 µl) for 60 min. To the reaction mixture, diethyl ether was added to obtain the p53(303–393) crude product. The residual solid was dissolved with aqueous CH_3CN and lyophilized. Then, p53(303–393) was isolated on RP-HPLC (Vydac C_{18}, 10 × 250 mm, flow rate 2.5 ml/min) using a linear gradient of 10%–35% B over 32 min. The free peptide, p53(303–393) (**6**), was obtained in 52% yield based on **21**. MALDI-TOF MS: observed $[M + H]^+$ = 10 246 for **6**, calculated for p53(303–393) $[M + H]^+$ = 10 243.

Synthesis of (Ser^{315}(PO_3H_2))-p53(303–393) (7), and (Lys^{320}(Ac))-p53(303–393) (8). Peptide thiolester **13** (150 nmol) or **14** (150 nmol), peptide **21** (22 nmol), HOOBt (0.75 mg, 4.5 µmol), DIEA (3.0 µmol, 0.5 µl) and $AgNO_3$ (0.075 mg, 0.45 µmol) were dissolved in DMSO (250 µl) and the reaction solutions were stirred for 36 h at 37 °C. The reaction was quenched with DTT (2.0 mg). Then, the peptide mixtures were precipitated by diethyl ether and washed. The residual solids were treated with Reagent K + (200 µl) for 60 min. To the reaction mixtures, diethyl ether was added to precipitate the coupling products. The residual solids were dissolved with aqueous CH_3CN and lyophilized. Then, [Ser^{315}(PO_3H_2)]-p53(303–393) and [Lys^{320}(Ac)]-p53(303–393) were isolated on RP-HPLC under the same conditions for the purification of **6**. The coupling products, [Ser^{315}(PO_3H_2)]-p53(303–393) (**7**) and [Lys^{320}(Ac)]-p53(303–393) (**8**), were obtained in 52% and 57% yields, respectively, based on **21**. MALDI-TOF MS: observed $[M + H]^+$ = 10 322 for **7**, calculated for [Ser^{315}(PO_3H_2)]-p53(303–393) $[M + H]^+$ = 10 323. MALDI-TOF MS: observed $[M + H]^+$ = 10 286 for **8**, calculated for [Lys^{320}(Ac)]-p53(303–393) $[M + H]^+$ = 10 285.

(Ser^{378}(PO_3H_2))-p53(303–393) (9). Peptide thiolester **12** (150 nmol), peptide **22** (22 nmol), HOOBt (0.75 mg, 4.5 µmol), DIEA (3.0 µmol, 0.5 µl) and $AgNO_3$ (0.075 mg, 0.45 µmol) were dissolved in DMSO (250 µl) and the reaction solution was stirred for 36 h at 37 °C. The reaction was quenched with DTT (2.0 mg). Then, the peptide mixture was precipitated by diethyl ether and washed. The residual solid was treated with Reagent K + (200 µl) for 60 min. To the reaction mixture, diethyl ether was added to precipitate the coupling product. The residual solid was dissolved with aqueous CH_3CN and lyophilized. Then, [Ser^{378}(PO_3H_2)]-p53(303–393) was isolated by RP-HPLC under the same conditions for purification of **6**. The coupling product, [Ser^{378}(PO_3H_2)]-p53(303–393) (**9**), was obtained in 66% yield based on **22**. MALDI-TOF MS: observed $[M + H]^+$ = 10 323 for **9**, calculated for [Ser^{315}(PO_3H_2)]-p53(303–393) $[M + H]^+$ = 10 323.

Competition Assay

Solutions of p53 *C*-terminal domain peptides were incubated with equimolar amounts of supercoiled DNA [pBlueScript II KS(+), Stratagene] and linearized DNA (*SmaI* digested) in binding buffer containing 5 mM Tris-HCl, pH 7.6, 0.5 mM EDTA, 50 mM KCl and 0.01% Triton X-100 for 1 h at 0 °C. Samples were loaded onto a 1.3% agarose gel containing 0.33× Tris-borate-EDTA (TBE) buffer [23]. The total amount of DNA was in the range 0.3–0.6 µg. After 3.5 h electrophoresis (at 5 V/cm), the DNA bands were visualized by Sybr Green I (Molecular Probes) staining. Gels were blotted onto a nitrocellulose transfer membrane (HighBond™ ECL™, Amersham Biosciences, Piscataway, USA) by the capillary transfer with 10× SSC buffer. The membrane was blocked with 5% nonfat milk in PBS buffer

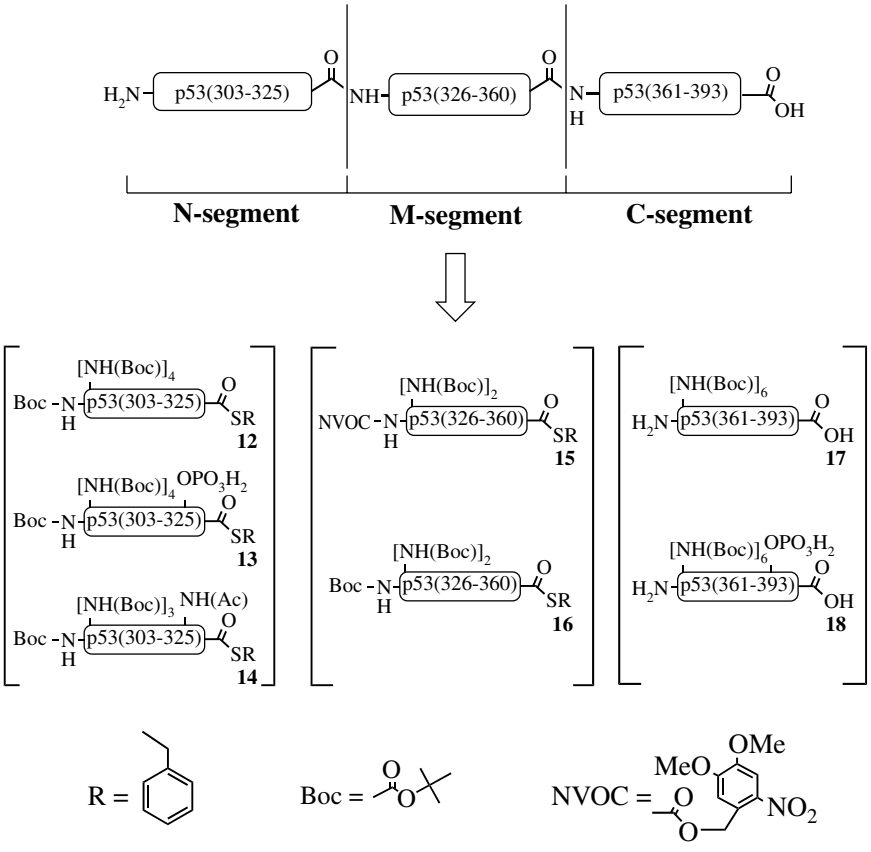
containing 0.1% Tween-20. The primary antibody, mAbC19 (Santa Cruz Biotechnology, Santa Cruz, USA), the epitope of which is contained in the *C*-terminal 19 amino acids of p53, was used to detect p53(326–393), p53(303–393), [Ser^{315}(PO_3H_2)]- and [Lys^{320}(Ac)]-p53(303–393). The primary antibody mAbP378, which is specific for phosphorylated Ser^{378}, was used for the detection of [Ser^{378}(PO_3H_2)]-p53(326–393) and [Ser^{378}(PO_3H_2)]-p53(303–393). The antibody, mAbP378, is an affinity purified rabbit polyclonal antibody raised against a peptide, Ac-[Ser^{378}(PO_3H_2)]-p53(373–383)-Cys-OH. Then, the blotting membrane was reacted with the appropriate peroxidase-conjugated secondary antibody, and visualized with the ECL plus™ detection system (Amersham Biosciences).

RESULTS AND DISCUSSION

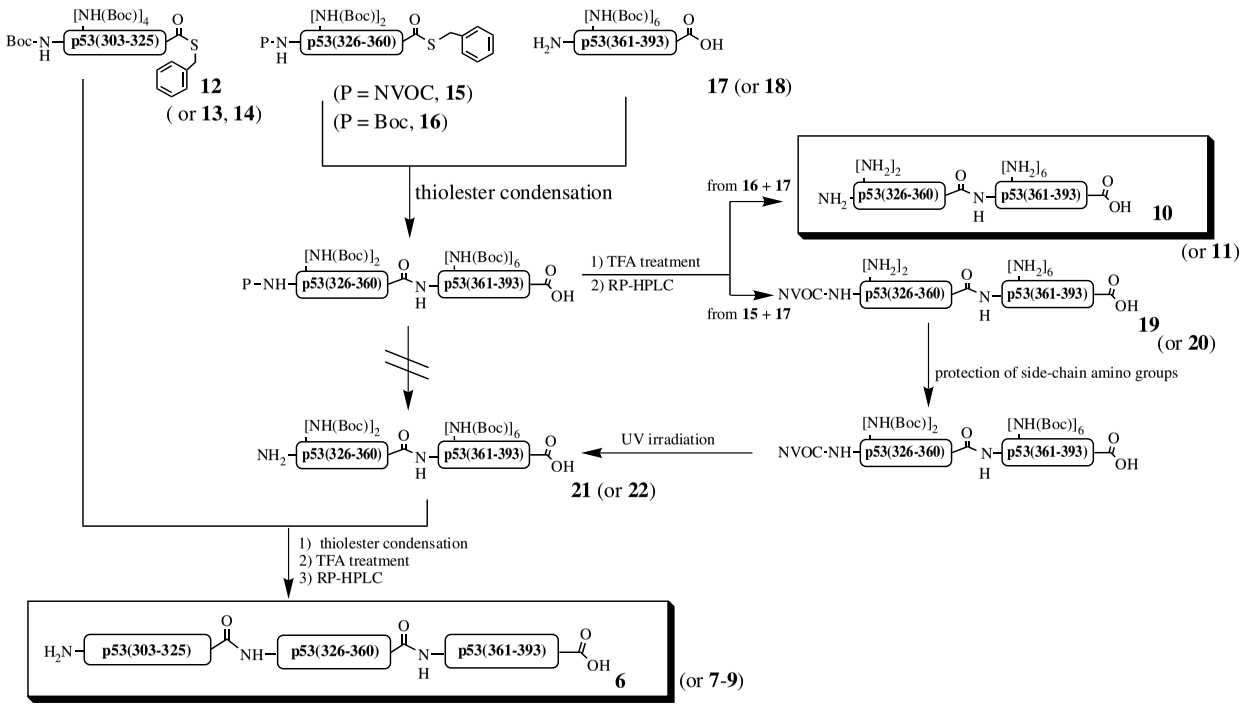
Construction Strategy

The domain structure [11] and amino acid sequence of the *C*-terminal region of human p53 are shown in Figure 1. In this study p53(303–393) and its derivatives, [Ser^{315}(PO_3H_2)]-, [Lys^{320}(Ac),]- and [Ser^{378}(PO_3H_2)]-p53(303–393), were synthesized. The synthetic process involved in the thiolester method is shown in Scheme 1. The peptide thiolester (**1**) is activated to the corresponding peptide active ester in the presence of silver ions and HOOBt [33, 34]. The active ester (**2**) is reacted with another partially protected peptide (**3**), to give a peptide (**4**). The amino groups on the side chains, however, must be protected to provide discrimination between the *N*-terminal amino group and amino groups of the side chains. The Boc group was used for this purpose. The product (**5**) can be utilized for subsequent couplings as a *C*-terminal building block.

The thiolester method possesses an advantage over other methods of condensation in that the choice of the *N*-terminal amino acid of the *C*-terminal segment is unconstrained [1–3]. The main drawback of the method, however, is the requirement that nucleophilic side chains, *i.e.* the



Scheme 2 Building blocks for constructing p53(303–393) (**6**), [Ser^{315}(PO_3H_2)]-p53(303–393) (**7**), [Lys^{320}(Ac)]-p53 (303–393) (**8**), [Ser^{378}(PO_3H_2)]-p53(303–393) (**9**), p53(326–393) (**10**) and [Ser^{378}(PO_3H_2)]-p53(326–393) (**11**).



Scheme 3 Representative synthetic scheme [synthesis of p53(303–393) and p53(326–393)]. Starting from three partially protected building blocks described in Scheme 2, p53(303–393), its derivatives and functionally truncated peptides were constructed *via* the thiolester condensation method. The numbers **7**–**9**, **13**, **14**, **18**, **11**, **20**, **22**, represent specifically phosphorylated or acetylated derivatives corresponding to the building block or product.

amino group of Lys residue and the thiol group of Cys, must be protected. In order to make chemoselective ligation feasible without side-chain protecting groups, some condensation strategies require a Cys residue at the coupling site(s) [35, 36], which may result in the synthesis of analogs or mimetics of the target protein. An alternative strategy requires strong deprotecting reagents to remove auxiliaries [35, 37, 38] that makes it unsuitable for the synthesis of phosphorylated peptides [39].

The building blocks and the synthetic strategy for p53(303–393) and its derivatives are outlined in Schemes 2 and 3, respectively. To construct the unmodified *C*-terminal domain of p53, the 91 amino acid peptide was divided into three segments, containing 23, 35 and 33 amino acid residues. The two coupling sites were chosen based on the following considerations. (i) To avoid epimerization that occurs during the preparation of peptide thiolesters [29] and during thiolester condensation [40], the *C*-terminus of the peptide thiolesters must be a Gly residue. (ii) To facilitate the investigation of the properties of the p53 *C*-terminal segment, the building blocks should correspond to functional units: the linker to the DNA binding domain, the tetramerization domain and the regulatory domain. The linker and the regulatory domain contain the phosphorylation and acetylation sites, whereas the middle segment alone is able to form tetramers [41–43] (Figure 1). (iii) The length of each segment should be within applicable SPPS [44] and RP-HPLC limitations. Furthermore, for a multiple segment condensation strategy, the *N*-terminus of internal segments must be protected, while retaining discrimination between the *N*-terminal and side-chain amino groups in order to generate the building block for the next coupling reaction. Other condensation strategies share this requirement to prevent an internal segment from reacting with itself.

Preparation of Building Blocks and *N*-terminal Protection Group for the Middle Segment

The partially protected peptide thiolesters (**12**–**16**) and peptides **17** and **18** (Scheme 2) were synthesized by Fmoc-based SPPS. For the preparation of peptide thiolesters **12**–**16**, the 'safety-catch' resin approach was employed, in which the peptide segments were elongated on a 4-sulfamyl linker attached to a resin, followed by activation by alkylation and nucleophilic thiolysis. As mentioned in the Construction Strategy section above, the *N*-terminus of the middle segment (**15**) must be protected to provide discrimination between the main-chain and side-chain amino groups of the building block. Previously, the Fmoc group has been utilized in Boc-based peptide thiolester preparation to meet this requirement. However, it was observed that the Fmoc group was eliminated during the thiolysis step. The elimination was confirmed by subjecting Fmoc-Ala-NH-resin to the conditions used for thiolester preparation, either with or without iodoacetonitrile treatment, and testing for the presence of a free amino group [45]. To avoid this problem, NVOC [27], a photolabile amino protecting group, was used to provide the necessary orthogonal protection. The NVOC group satisfied the two requirements for an *N*-terminal amino protecting group: (i) it is stable to the alkylation and nucleophilic thiolysis steps and to TFA treatment for side-chain deprotection, and (ii) it is removable without the use of strong deblocking reagents. Following this approach, the *N*-protected peptide α-thiolester corresponding to p53(326–360) was prepared successfully. Specifically, this protection was stable to alkylation with ICH_2CN, thiolysis with Bzl-SH in the presence of DIEA and side-chain deprotection with Reagent K+.

The yields of the building blocks (**12**–**18**) were 43%, 36%, 63%, 5%, 7%, 34% and 33%, respectively, based on determination of the Fmoc group of the first amino acid of the starting resins. A good yield was obtained for all the segments except the middle segments (**15** and **16**). The low yield of the middle segments is due to addition of the methylcyano of iodoacetonitrile to the sulfur of methionine, a previously reported side reaction [46]. Near co-elution of the desired and modified-methionine products on HPLC prevented good recovery.

Efficiency of NVOC Deprotection and Stability of the Phosphate Group under Deprotection Conditions

To estimate the rate of photo-cleavage of the NVOC group and to confirm the stability of the phosphate group under photo-cleavage conditions, a solution of NVOC-[$Ser^{378}(PO_3H_2)$]-p53(361–393) (**23**) and Fmoc-[$Ser^{378}(PO_3H_2)$]-p53(361–393) (**24**) was irradiated with a UV lamp (365 nm, 1.3 mW cm^{-2}) in the presence of DTT to obtain the reaction product, [$Ser^{378}(PO_3H_2)$]-p53(361–393) (**25**). The reaction mixture was sampled periodically, and aliquots were analysed by RP-HPLC. The

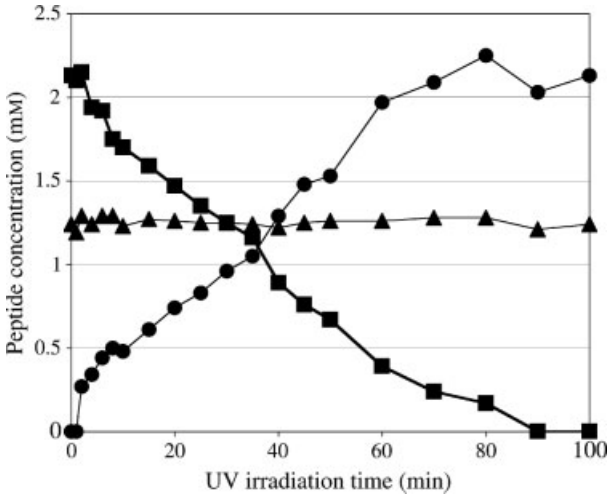


Figure 2 Time course of NVOC deprotection and stability of phosphate group under the deprotection conditions. NVOC-[Ser^{378}(PO_3H_2)]-p53(361–393) (**23**, ■) and Fmoc-[Ser^{378}(PO_3H_2)]-p53(361–393) (**24**, ▲) were dissolved with DMSO containing DTT. The mixture was irradiated by 365 nm UV light at a fluence of 1.3 mW cm^{-2} to give the product, [Ser^{378}(PO_3H_2)]-p53(361–393) (**25**, ●). The peptides were separated by RP-HPLC. The concentrations were determined from amino acid analysis and HPLC peak area.

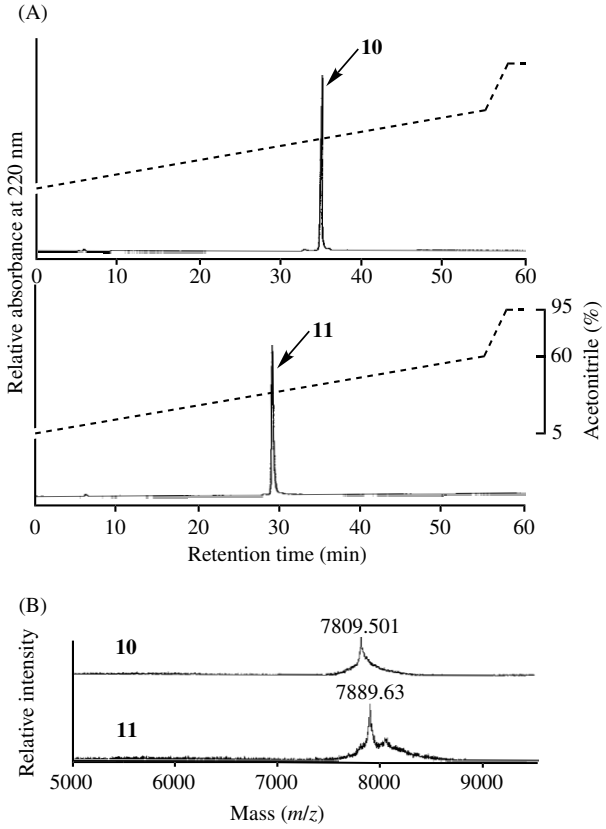


Figure 3 Purified p53(326–393) (**10**), and [Ser^{378}(PO_3H_2)]-p53(326–393) (**11**). (A) RP-HPLC elution profile. Column: Vydac C_{18}(4.6 × 250 mm), eluent: 0.05% TFA in aqueous acetonitrile, 0.5 ml/min. (B) MALDI-TOF MS.

results (Figure 2) show the time course of the decrease of the NVOC-derivative, the production of [Ser^{378}(PO_3H_2)]-p53(361–393) and the lack of any significant change in Fmoc-[Ser^{378}(PO_3H_2)]-p53(361–393). The results show that the NVOC group is useful for the synthesis of peptides containing phospho-Ser by multiple segment condensation.

Thiolester Condensations

The peptide thiolester (**16**) and peptide **17** or **18** were reacted to give the condensation product, Boc-[Lys(Boc)^{351,357,370,372,373,381,382,386}]-p53(326–393) or Boc-[Lys(Boc)^{351,357,370,372,373,381,382,386}, Ser^{378}(PO_3H_2)]-p53(326–393), respectively, as described above. After processing, p53(326–393) (**10**) and [Ser^{378}(PO_3H_2)]-p53(326–393) (**11**) were obtained with yields of 33% and 32% based on **17** and **18**, respectively. The RP-HPLC elution profiles and the mass spectra of the purified peptides (**10** and **11**) are shown in Figure 3.

In contrast to the preparation of the deprotected product **10** or **11** from the completely Boc-protected condensation product, the preparation of the building block for the last coupling (**21** or **22**) from the NVOC *N*-terminal-protected and Boc side chain-protected condensation product required several intermediate steps. The peptide thiolester (**15**) and peptide **17** or **18**, were reacted as described above to give the condensation product, NVOC-[Lys(Boc)^{351,357,370,372,373,381,382,386}]-p53(326–393) or NVOC-[Lys(Boc)^{351,357,370,372,373,381,382,386}, Ser^{378}(PO_3H_2)]-p53(326–393), respectively. Following the usual protocol, the crude products were precipitated by diethyl ether, but direct conversion from the ether precipitate to the next building block, [Lys(Boc)^{351,357,370,372,373,381,382,386}]-p53(326–393) (**21**) (Scheme 3), or [Lys(Boc)^{351,357,370,372,373,381,382,386}, Ser^{378}(PO_3H_2)]-p53(326–393) (**22**) was unsuccessful. UV irradiation of the precipitate gave a complex mixture, and furthermore, since the Lys(Boc) residue is abundant in the sequence, the product was so hydrophobic that efficient separation by RP-HPLC was not possible. Therefore, the NVOC- and Boc-protected residual solid was treated with scavenger-containing

TFA, then the *N*-protected coupling product, NVOC-p53(326–393) (**19**) or NVOC-[Ser^{378}(PO_3H_2)]-p53(326–393) (**20**) were purified. The RP-HPLC elution profiles and the mass spectra of the purified *N*-terminally protected peptides (**19** and **20**) are shown in Figure 4. After re-protecting the side chain amino groups of the purified product with Boc groups, the *N*-terminal protecting group, NVOC, was removed in the presence of DTT. Thus, the building blocks for the last coupling, [Lys(Boc)^{351,357,370,372,373,381,382,386}]-p53(326–393) (**21**) and [Lys(Boc)^{351,357,370,372,373,381,382,386}, Ser^{378}(PO_3H_2)]-p53(326–393) (**22**) whose *N*-termini are free and side-chain amino groups are protected, were prepared successfully with yields of 22% and 26% based on **17** and **18**, respectively.

The peptide thiolester (**12**–**14**) was condensed with peptide **21** and, separately, peptide thiolester (**12**), was condensed with peptide **22**, as described above. After condensation, the peptides were precipitated by diethyl ether, then treated with TFA to remove the Boc groups. The crude products were subjected to RP-HPLC to give p53(303–393) (**6**), [Ser^{315}(PO_3H_2)]-p53(303–393) (**7**), [Lys^{320}(Ac)]-p53(303–393) (**8**), and [Ser^{378}(PO_3H_2)]-p53(303–393) (**9**) with yields of 52%, 52%, 57% and 66% based on **21** for **6**–**8**, and **22** for **9**, respectively. RP-HPLC elution profiles and the mass spectra of the purified peptides, **6**–**9**, are shown in Figure 5.

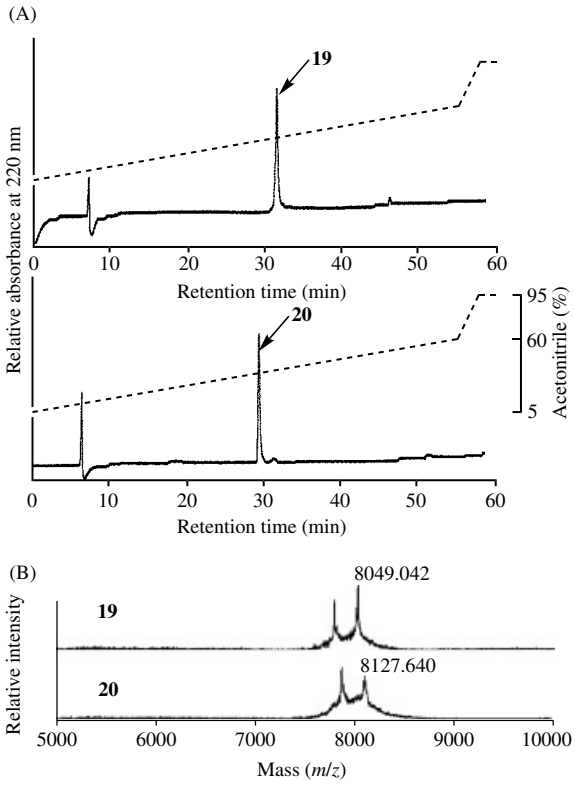


Figure 4 Purified NVOC-p53(326–393) (**19**) and NVOC-[Ser^{378}(PO_3H_2)]-p53(326–393) (**20**). (A) RP-HPLC elution profile. Column: Vydac C_{18}(4.6 × 250 mm), eluent: 0.05% TFA in aqueous acetonitrile, 0.5 ml/min. (B) MALDI-TOF MS.

Thus, multiple segment couplings were accomplished successfully utilizing the NVOC group to provide the protection orthogonal to the side-chain protection that is necessary for combining thiolester condensation with the 'safety-catch' linker method of peptide thiolester preparation. The NVOC group may also be useful for multiple component syntheses using other ligation strategies. Currently, the acetoamidomethyl group, which requires heavy metal for deprotection, is used for sulfur protection of the *N*-terminal cysteine residue in the native chemical ligation method [47]. Alternatively, harsh acidic conditions, such as trifluoromethanesulfonic acid or HF, are required for recovery of the product following ligation with removable auxiliaries [6,7,37,38].

Preferential Binding of the p53 *C*-Terminal Segment Peptides to Supercoiled Compared with Linearized Plasmid DNA

In an earlier study of intact p53 and various p53 truncations, the *C*-terminal segment was identified as critical to highly selective binding to supercoiled DNA [25]. The synthetic products were used to investigate further the ability of the *C*-terminal subdomains to bind selectively to supercoiled DNA. The results of a gel shift assay are shown in Figure 6(A). At this peptide concentration, only p53(303–393) produced an obvious upward shift in the supercoiled DNA band. The absence of selective binding to supercoiled DNA by p53(326–393) contrasts to the selective binding by p53(319–393) and p53(320–393) reported previously [24,25] and suggests that lysines 319 to 321 are important for the stability of the tetramer or contribute to selective binding to supercoiled DNA. To better assess binding by truncated peptides that contain the regulatory sub-domain, amino acids 361–393, a competitive gel shift assay was carried out using a range of concentrations. For p53(361–393) concentrations from 1.8 to 7.3 μM and p53(326–393) concentrations from 0.90 to 3.6 μM, the assay showed concentration-dependent upward shifts for

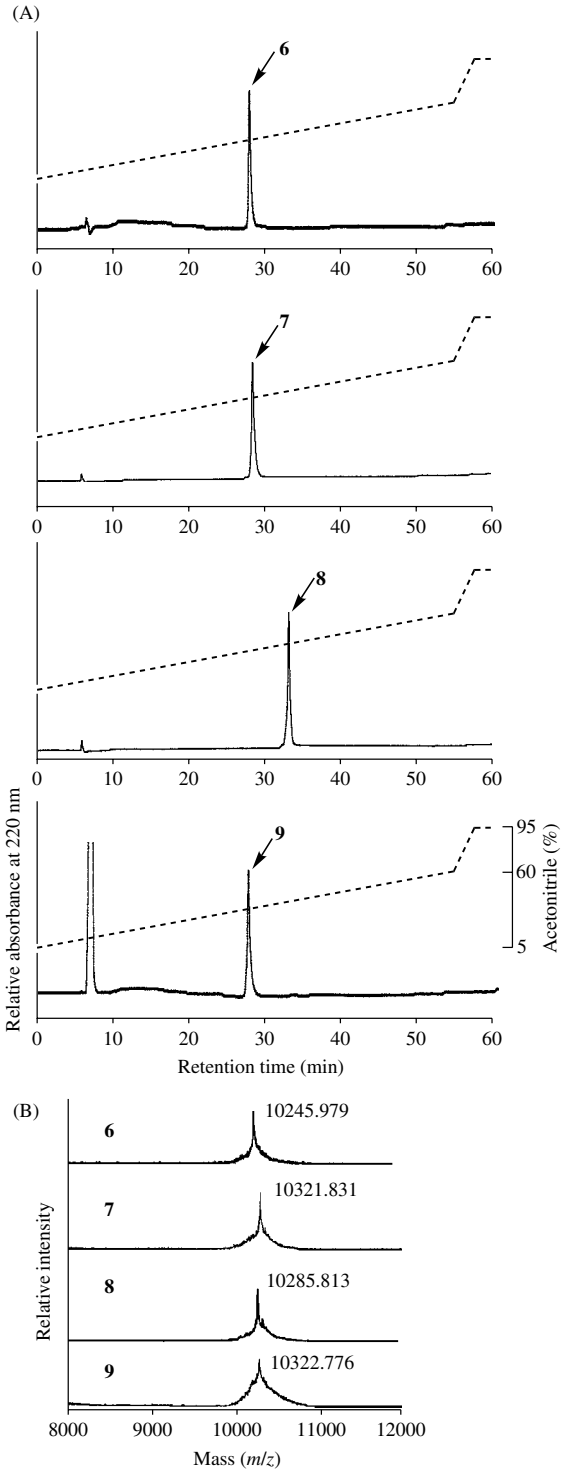


Figure 5 Purified p53(303–393) (**6**) [Ser^{315}(PO_3H_2)]-p53(303–393) (**7**) [Lys^{320}(Ac)]-p53(303–393) (**8**) and [Ser^{378}(PO_3H_2)]-p53(303–393) (**9**). (A) RP-HPLC elution profile. Column: Vydac C_{18}(4.6 × 250 mm), eluent: 0.05% TFA in aqueous acetonitrile, 0.5 ml/min. (B) MALDI-TOF MS.

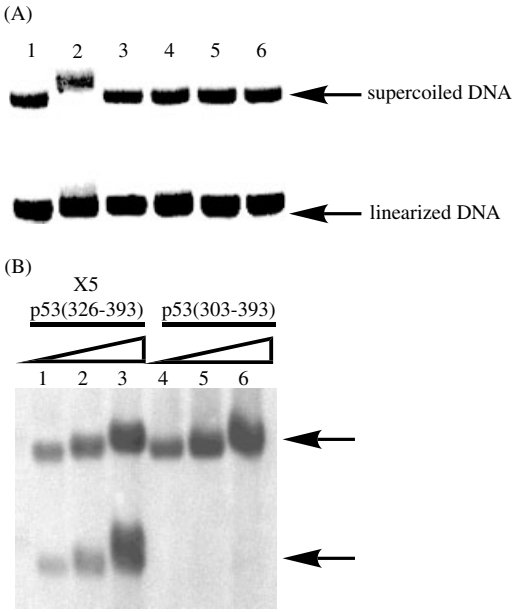


Figure 6 The intact *C*-terminal segment, p53(303–393), selectively binds supercoiled DNA. (A) Competition gel mobility shift assay. Supercoiled DNA (0.36 μg) and linear DNA (0.36 μg) were incubated without or with 273 nM (monomer) p53 peptides in binding buffer and were separated by agarose gel electrophoresis. DNA was visualized by Sybr Green I staining. Lane 1, no peptide; lane 2, p53(303–393); lane 3, p53(361–393); lane 4, p53(326–393); lane 5, p53(326–360); lane 6, p53(303–325). (B) Immunoblot of competition gel mobility shift assay. Supercoiled DNA (0.12 μg, 4.7 nM) and linear DNA (0.12 μg, 4.7 nM) were incubated with p53 *C*-terminal peptides, p53(326–393): lane 1, 0.90 μM; lane 2, 1.8 μM; lane 3, 3.7 μM; p53(303–393): lane 4, 0.18 μM; lane 5, 0.36 μM; lane 6, 0.73 μM. Following agarose gel electrophoresis, the gels were blotted onto nitrocellulose membranes. Bound peptides were detected with the anti-p53 antibody, mAbC19.

both supercoiled and linearized plasmid DNA (data not shown). The observation that p53(361–393) produces mobility shifts for both supercoiled and linearized DNA is in agreement with an earlier report in which the binding was detected by immunoblot [25]. To further characterize the binding of the tetrameric peptides, an immunoblot was performed on gels containing p53(303–393)-DNA and p53(326–393)-DNA complexes (Figure 6(B)). p53(303–393) binds to supercoiled DNA with high selectivity. To provide for adequate detection of the bands, the concentrations of p53(326–393) used were five times those of p53(303–393). At these concentrations, p53(326–393) binds to both supercoiled and linear DNA.

Both phosphorylation and acetylation of p53 affect its DNA binding and biological function [20]. To investigate the effects of specific modifications, the tetrameric peptides and their modified derivatives were tested in the competition gel mobility shift assay. The results for p53(303–393), its acetylated or phosphorylated derivatives, and p53(326–393) and its phosphorylated derivative are shown in Figure 7(A). p53(303–393) and its derivatives, [Ser^{315}(PO_3H_2)]-, [Lys^{320}(Ac)]- and [Ser^{378}(PO_3H_2)]-p53(303–393), exhibit preferential binding to supercoiled DNA as opposed to linearized plasmid DNA, with only subtle differences between the modified and unmodified forms. In contrast, although p53(326–393) does not markedly shift supercoiled or linear DNA, as observed above, the phosphorylated peptide, [Ser^{378}(PO_3H_2)]-p53(326–393), produces a large shift in the mobility of the supercoiled DNA. Selective binding to supercoiled DNA by p53(303–393), [Ser^{315}(PO_3H_2)]-p53(303–393) and [Lys^{320}(Ac)]- p53(303–393) was confirmed by immunoblot, as shown in Figure 7(B). At the higher peptide concentrations, some binding of the phospho-Ser^{315} and acetylated Lys^{320} derivatives to linear DNA was observed, suggesting that these modifications slightly reduce the specificity for supercoiled DNA. Immunoblot detection with a specific antibody to the phospho-Ser^{378} epitope, as shown in Figure 7(C), confirms the increased binding to supercoiled DNA by [Ser^{378}(PO_3H_2)]-p53(326–393) compared with [Ser^{378}(PO_3H_2)]-p53(303–393).

Brázdová *et al.* [25] inferred that amino acid residues 375–378 participate in the binding of p53 to supercoiled DNA because binding of an antibody to the 375–378 epitope prevents binding to supercoiled DNA. Our results show that phosphorylation of Ser^{378} enhances the supercoiled DNA-specific binding in comparison with p53(326–393) and reinforces the importance of that region. However, in the context of the larger peptide, p53(303–393), phosphorylation of Ser^{378} had only a small effect on the binding, suggesting that the linker may participate in the supercoiled DNA-specific binding through the presence of additional positively charged residues.

The p53 *C*-terminal segment contains at least two additional phosphorylation sites, two additional acetylation sites and one SUMO-ylation site and, moreover, is involved in various protein–protein interactions [20]. The peptides described in this report will be useful in investigating the effects of specific post-translational modifications on the interaction of p53 with other proteins.

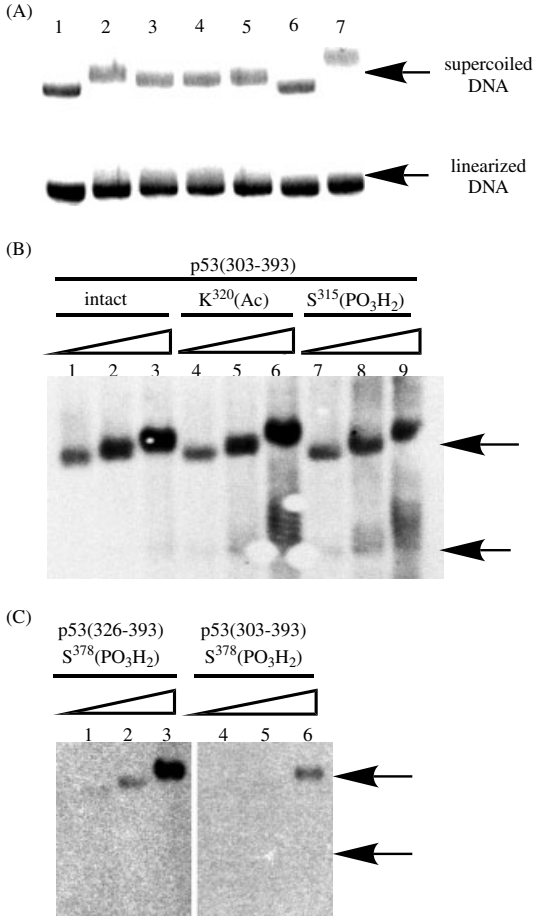


Figure 7 The derivatives of p53(303–393) selectively bind supercoiled DNA. (A) Competition gel mobility shift assay. Supercoiled DNA (0.36 μg) and linear DNA (0.36 μg) were incubated without or with 273 nM (monomer) p53 peptides in binding buffer and were separated by electrophoresis. DNA was visualized by Sybr Green I staining. Lane 1, no peptide; lane 2, p53(303–393); lane 3, [Lys^{320}(Ac)]-p53(303–393); lane 4, [Ser^{315}(PO_3H_2)]-p53(303–393); lane 5, [Ser^{378}(PO_3H_2)]-p53(303–393); lane 6, p53(326–393); lane 7, [Ser^{378}(PO_3H_2)]-p53(326–393). (B) Immunoblot of competition gel mobility shift assay. Supercoiled DNA (0.12 μg, 4.7 nM) and linear DNA (0.12 μg, 4.7 nM) were incubated p53(303–393) (lanes 1–3), [Lys^{320}(Ac)]-p53(303–393) (lanes 4–6) and [Ser^{315}(PO_3H_2)]-p53(303–393) (lanes 7–9). Peptide concentrations were 0.18 μM (lanes 1, 4 and 7), 0.36 μM (lanes 2, 5 and 8) and 0.73 μM (lanes 3, 6 and 9). Bound peptides were detected with the anti-p53 antibody mAbC-19. (C) Supercoiled DNA (0.11 μg, 4.3 nM) and linear DNA (0.11 μg, 4.3 nM) were incubated with [Ser^{378}(PO_3H_2)]-p53 *C*-terminal peptides: [Ser^{378}(PO_3H_2)]-p53(326–393): lane 1, 0.08 μM; lane 2, 0.17 μM; lane 3, 0.42 μM; [Ser^{378}(PO_3H_2)]-p53(303–393): lane 4, 0.08 μM; lane 5, 0.17 μM; lane 6, 0.42 μM. Bound peptides were detected with the antibody mAbP378.

CONCLUSIONS

In conclusion, the p53 *C*-terminal segment has been and synthesized successfully post-translationally modified or functionally truncated derivatives selected. The building blocks were prepared by Fmoc-based SPPS and subsequently coupled via successive thiolester condensations. To make two sequential condensations feasible, a photolabile protecting group, NVOC, was useful in providing a selective reaction site. It is likely that this protecting group will be applicable to other condensation strategies in addition to the strategy described here. Quite recently, Kawakami and Aimoto [48] reported a novel peptide ligation strategy, in which a photoremovable ligation auxiliary was employed to overcome the requirement for using strong reagents to remove conventional auxiliaries. Our results show that a photolabile group is also useful for the construction of a peptide that contains post-translational modifications.

The p53 *C*-terminal fragment, p53(303–393), and its phosphorylated and acetylated derivatives exhibited selective binding to supercoiled DNA compared with linearized plasmid DNA. Interestingly, phosphorylation of Ser^{378} dramatically enhances specific binding of p53(326–393) to supercoiled DNA. This result reinforces published evidence [25] that the site of interaction with supercoiled DNA is centered in the region of amino acids 375–378.

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