Arch. Pharm. Res. Vol.21, No.3, pp. 330-337, 1998

Reactivity and Suitability of *t*-Boc-protected Thiophosphotyrosine Intermediate Analogs for the Solid or Solution Phase Peptide Synthesis

Eun-kyung Kim, Heesung Choi[1] and Eung-Seok Lee*

College of Pharmacy, Yeungnam University, Kyongsan 712-749, Korea and [1]Dong Kook Pharm. Co., Central Research Institute, Chungbuk 365-830, Korea

(Received January 8, 1998)

N-(*tert*-Butoxycarbonyl)-*O*-(dimethylthiophosphono)-L-tyrosine (**6**) and *N*-(*tert*-butoxycarbony)-*O*-(dicyanoethylthiophosphono)-L-tyrosine (**15**) were prepared as intermediates for the synthesis of thiophosphotyrosine-containing peptides. The reactivity and suitability of two compounds for the solid phase or solution phase peptide synthesis utilizing *t*-Boc chemistry were examined.

Key words : Thiophosphotyrosine, Phosphotyrosine, Protein tyrosine kinase(PTK), Peptide synthesis, Cyanoethyl protection

INTRODUCTION

Phosphorylation or dephosphorylation of proteins on tyrosine residues in signal transduction pathways plays an important role in the regulation of cell growth and transformation (Cantly *et al.*, 1991). The protein tyrosine kinases (PTK's) specifically catalyze tyrosine phosphorylations in substrate proteins or peptides (Hunter *et al.*, 1985). Tyrosine phosphorylation regulates the activities of the *c-src* family PTK's and the associations of PTK's with cytosolic proteins such as phosphatidylinositol 3-kinase or phospholipase C-γ, which meditate further signal transduction. Such regulations are thought to be performed by binding of phosphotyrosine residues to the src homology region 2 (SH-2) domain in proteins (Koch *et al.*, 1991). The activities of the *c-src* family PTK's can also be regulated by the specific protein tyrosine phosphatases (PTPases) which specifically catalyze the dephosphorylation of phosphotyrosine residues in the regulatory domains of the *c-src* family PTK's (Hunter *et al.*, 1989; Tonks *et al.*, 1989).

The design and synthesis of analogs of thiophosphotyrosine-containing peptides as PTK inhibitors, as blocking agents of the association of PTK's with cytosolic proteins, or as PTPase inhibitors offers a strategy for the development of potential anticancer agents as well as a tool for investigation of mechanism of signal transduction pathways. Also the thiophosphotyrosine-containing peptides are expected to be stable to the action of non-specific cellular phosphatases.

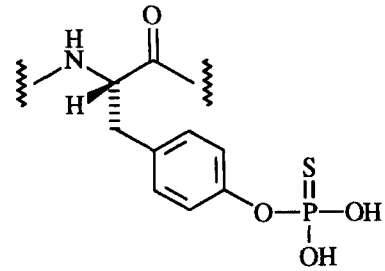


Fig. 1. Thiophosphotyrosine-containing peptide.

In this research, we designed and prepared *t*-Boc dimethyl thiophosphonotyrosine and *t*-Boc dicyanoethyl thiophosphonotyrosine as key intermediates for the peptide synthesis, and we examined the reactivity and/or suitability of each compounds for the solid or solution phase peptide synthesis.

MATERIALS AND METHODS

All reactions were performed under nitrogen or argon, unless stated otherwise, or unless an aqueous reaction medium was employed. Melting points were determined on a Thomas-Hoover Unimelt or Mel-Temp apparatus and are uncorrected. Analytical thin-layer chromatography was performed on Merck Kieselgel 60 F_{254} plates, 0.25 mm thickness. Column chromatography was performed on silica gel, 230~400 mesh. ^{1}H NMR spectra were recorded on a Brucker ARX 300 spectrometer. ^{13}C NMR spectra were record-

Correspondence to: Eung-Seok Lee, College of Pharmacy, Yeungnam University, Kyongsan 712-749, Korea

ed on a Brucker ARX 300 spectrometer operating at 75 MHz. IR spectra were recorded on a Perkin Elmer 1600 FTIR spectrometer. Fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS25 spectrometer, using a glycerol/thioglycerol or dithiothreitol/dithioerythritol (DTT/DTE) matrix. HPLC works were performed on a Beckman HPLC system.

Tetrahydrofuran was distilled from sodium metal and benzophenone or from calcium hydride. Diethyl ether was distilled from lithium aluminum hydride. Methylene chloride and *N,N*-dimethylformamide were distilled from calcium hydride. For all analytical and semi-preparative HPLC works, double distilled water and HPLC grade acetonitrile were used.

N,N-Diethylphosphoroamidous dichloride (1)

Diethylamine (36.5 g, 51.6 ml, 0.50 mol) was added dropwise to a vigorously stirred solution of phosphorus trichloride (34.4 g, 21.9 ml, 0.25 mol) in dry diethyl ether (150 ml) such that the reaction temperature was kept below 0°C. On completion of addition (ca. 1.5 h) the mixture was allowed to warm to 20°C and stirred for 3 h (white precipitation was formed during reaction). The mixture was then filtered through a large, sealable sintered funnel and the diethylamine·HCl sludge was rigorously washed with dry diethyl ether (4×50 ml). Evaporation of the combined ethereal solution at reduced pressure, followed by vacuum distillation of the crude liquid residue gave the product as a clear liquid (33.41 g, 76.9%): bp 72~75°C/14 mmHg (lit. (Perich *et al.*, 1988) 62°C/7 torr).

Dimethyl *N,N*-diethylphosphoramidite (2)

A solution of methanol (12.36 g, 15.63 ml, 0.39 mol) and triethylamine (42.87 g, 59.05 ml, 0.42 mol) in dry diethyl ether (140 ml) was added to a stirred solution of *N,N*-diethylphosphoroamidous dichloride (**1**, 33.41 g, 0.19 mol) in dry diethyl ether (60 ml) such that the reaction temperature was kept below 0°C. On completion of addition (ca. 40 min), the mixture was allowed to warm to 20°C and was stirred for 3 h. A solution of 5% aqueous $NaHCO_3$ (70 ml) was then added, the mixture was transferred to a separating funnel, and the aqueous phase was discarded. The ethereal phase was washed with 5% aqueous $NaHCO_3$ solution (2×60 ml), saturated sodium chloride solution (60 ml) and dried with magnesium sulfate. Evaporation of solvent at reduced pressure, followed by distillation of the crude residue gave a clear liquid (20.83 g, 66.4%): bp 38°C/5 mmHg; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.12 (t, 6 H, $-CH_3$), 3.13 (d of q, 4 H, $J_{P\text{-}N\text{-}C\text{-}H}$= 12 Hz, $-CH_2$-), 3.45 (d, 6 H, $J_{P\text{-}O\text{-}C\text{-}H}$=12 Hz, $-OCH_3$).

N-(*tert*-Butoxycarbonyl)-*O*-(dimethylthiophosphono)-L-tyrosyl *p*-nitrobenzyl ester (5)

1*H*-tetrazole (2.3 g, 33 mmol) was added in one portion to a stirred solution of *t*-Boc-L-tyrosyl *p*-nitrobenzyl ester (**3**, 5.0 g, 12 mmol) and dimethyl *N,N*-diethylphosphoramidite (**2**, 2.13 g, 13 mmol) in dry THF (40 ml) and the mixture was stirred for 15 min at 20°C. The mixture was then cooled to 0°C and a solution of sulfur (1.4 g, 44 mmol) in CS_2 (40 ml) was added. After stirring for 2 h at 20°C, 10% aqueous $NaHSO_3$ (70 ml) was added and the mixture was stirred for a further 10 min. The mixture was then transferred to a separatory funnel and the organic phase was decanted, evaporated at reduced pressure and dissolved in ethyl acetate (20 ml). The aqueous phase was extracted with ethyl acetate (3×30 ml). The combined ethyl acetate solution was washed with 10% aqueous $NaHSO_3$ solution (2×20 ml), 5% aqueous $NaHCO_3$ solution (2×20 ml), saturated sodium chloride solution and dried with magnesium sulfate. Evaporation of solvent at reduced pressure gave a mixture of sulfur and the product. The sulfur was removed by washing with hexane several times (3×20 ml) to give a yellow oil (5.78 g, 89.1%) which was purified by column chromatography, eluting with EtOAc/hexane (1:4) to yield a light yellow oil (5.38 g, 83.0%): TLC (silica gel, EtOAc/hexane=1:1, v:v), R_f 0.48; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 9 H, CH_3), 3.03 (d, 2 H, CH_2), 3.83 (d, 6 H, $J_{P\text{-}O\text{-}C\text{-}H}$=13.7 Hz, $-OCH_3$), 4.57 (m, 1 H, CH), 4.96 (d, 1 H, NH), 5.16 (s, 2 H, CH_2), 7.04 (s, 4 H, aromatic), 7.37 (d, 2 H, aromatic), 8.18 (d, 2 H, aromatic).

N-(*tert*-Butoxycarbonyl)-*O*-(dimethylthiophosphono)-L-tyrosine (6)

N-(*tert*-Butoxycarbonyl)-*O*-(dimethylthiophosphono)-L-tyrosyl *p*-nitrobenzyl ester (**5**, 1.15 g, 2.1 mmol) was dissolved in methanol (40 ml) containing acetic acid (3.0 ml) and hydrogenolyzed at room temperature using 10% Pd/C (400 mg) at atmospheric pressure for 5 h. After filtration of the Pd/C residue and removal of solvent by evaporation at reduced pressure, the crude product was dissolved in ethyl acetate (30 ml) and washed with 1 N hydrochloric acid (2×15 ml), water (2×15 ml) and then extracted with 0.5 N aqueous NaOH solution (3×10 ml). The aqueous extract was washed with diethyl ether (15 ml) and ethyl acetate (20 ml) and acidified to pH 3 at 0°C with 1 N hydrochloric acid and extracted with ethyl acetate (3×15 ml). The combined ethyl acetate solution was washed with saturated sodium chloride solution and dried with magnesium sulfate. Evaporation of solvent at reduced pressure afforded a light yellow oil (0.64 g, 73.9%): TLC (silica gel, EtOAc/hexane=1:1, v:v), R_f 0.28; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 9 H, CH_3), 3.14 (m, 2 H, CH_2), 3.83 (d, 6 H, $J_{P\text{-}O\text{-}C\text{-}H}$=13.7 Hz, $-OCH_3$), 4.53 (m, 1 H, CH), 4.92 (d, 1 H, NH), 7.12 (q, 4 H, aromatic).

N^{α}-(*tert*-Butoxycarbonyl)-*O*-(dimethylthiophosphono)-L-tyrosine-*N*-methylamide (7)

A solution of *N*-(*tert*-butoxycarbonyl)-*O*-(dimethylthiophosphono)-L-tyrosine (**6**, 1.25 g, 3.07 mmol) in dry tetrahydrofuran (8 ml) was cooled to -20°C and *N*-methylmorpholine (0.34 ml, 0.31 g, 3.07 mmol) was added. After 1 min, isobutylchloroformate (0.40 ml, 0.42 g, 3.07 mmol) was added and after 2 min at -20°C, methylamine (2.3 ml, 40% w/w aqueous solution) was added and stirring was continued at - 20°C for 90 min. A 2 M aqueous solution of potassium bicarbonate (2.0 ml) was added and the mixture was stirred at 0°C for 30 min. After evaporation of solvent at reduced pressure, the residue was partitioned between ethyl acetate (60 ml) and water (20 ml). The organic layer was washed with 1 N hydrochloric acid (2×10 ml), water (10 ml), saturated sodium chloride solution (10 ml) and dried with magnesium sulfate. Evaporation of solvent at reduced pressure afforded a pale yellow solid (1.01 g, 78.7%) which was homogeneous in TLC and ^{1}H NMR: mp 97°C; TLC (silica gel, EtOAc/hexane=1:1, v:v), R_f 0.17; FABMS m/e 419 (MH^+); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (s, 9 H, CH_3), 2.68 (d, 3 H, N-CH_3), 3.00 (d, 2 H, CH_2), 3.82 (d, 6 H, $J_{H\text{-}C\text{-}O\text{-}P}$ =13.7 Hz, -OCH_3), 4.24 (q, 1 H, C-H), 5.02 (bs, 1 H, NH), 5.80 (bs, 1 H, NH), 7.11 (q, 4 H, aromatic).

N^{α}-Acetyl-*O*-(dimethylthiophosphono)-L-tyrosine-*N*-methylamide (9)

To N^{α}-(*tert*-butoxycarbonyl)-*O*-(dimethylthiophosphono)-L-tyrosine-*N*-methylamide (**7**, 0.21 g, 0.50 mmol), 4 M HCl solution in dioxane (0.5 ml, 2 mmol) and *m*-cresol (0.05 ml) were added and the mixture was stirred for 30 min at room temperature. Diethyl ether (5 ml) was added to induce precipitation of the deprotected compound **8**. The precipitate was filtered and washed with diethyl ether (3×10 ml). After the residue was dried under vacuum, compound **8** was treated with *N*-methylmorpholine (3.0 ml) for 30 min at room temperature. After *N*-methylmorpholine was evaporated at reduced pressure, the free amine of compound **8** was acylated by mixed anhydride generated from acetic acid and isobutylchloroformate. To a stirred solution of acetic acid (30 mg, 32 ml, 0.50 mmol) in dry tetrahydrofuran (1 ml) at -20°C, *N*-methylmorpholine (62 ml, 0.5 mmol) followed by isobutylchloroformate (65 ml, 0.5 mmol) were added. After 2 min at -20°C, a solution of free amine-containing compound **8** in tetrahydrofuran (2 ml) was added and the mixture was stirred for 90 min at -20°C. 2 M Aqueous potassium bicarbonate solution (0.5 ml) was added and the mixture was stirred for a further 30 min at 0°C. After evaporation of solvent at reduced pressure, the residue was partitioned between ethyl acetate (15 ml) and water (5 ml). The organic phase was washed with 1 N hydrochloric acid (2×5 ml), water (5 ml), saturated sodium chloride solution (5 ml) and dried with magnesium sulfate. Evaporation of solvent at reduced pressure afforded product **9** as a pale yellow oil (56 mg, 31.1%) which was homogeneous in TLC and ^{1}H NMR: TLC (silica gel, EtOAc/MeOH=2:1, v:v), R_f 0.74; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.99 (s, 3 H, C-CH_3), 2.71 (d, 3 H, N-CH_3), 3.04 (m, 2 H, CH_2), 3.86 (d, 6 H, $J_{H\text{-}C\text{-}O\text{-}P}$=13.8 Hz, O-$CH_3$), 4.58 (q, 1 H, CH), 6.01 (bs, 1 H, NH), 6.53 (bs, 1 H, NH), 7.09 (d, 2 H, aromatic), 7.18 (d, 2 H, aromatic).

Treatment of compound 9 with TFMSA/TFA/DMS/*m*-cresol (10)

To N^{α}-acetyl-*O*-(dimethylthiophosphono)-L-tyrosine-*N*-methylamide (**9**, 0.391 g, 1.08 mmol) was added a mixture of TFMSA/TFA/DMS/*m*-cresol (2.5 ml, 10:50:30:10, v:v) and the mixture was stirred for 8 h at room temperature. Diethyl ether (10 ml) was added to induce formation of precipitate, which was filtered and washed with diethyl ether several times (3×10 ml). The filtrate was dissolved in 5% aqueous acetonitrile (2 ml) and purified by semi-preparative HPLC on a Beckman column (Dynamax-300A, C-18, 12 μ, 10×250 mm) with a gradient elution of 10% to 70% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 3 ml/min. The fractions corresponding to the peak (UV detector 254 nm) of retention time 8.9 min were collected and lyophilized to yield a white solid (0.15 g, 59.8%): ^{1}H NMR (300 MHz, acetone-d_6) δ 1.86 (s, 3 H, C-CH_3), 2.65 (d, 3 H, N-CH_3), 2.98 (m, 2 H, CH_2), 4.52 (q, 1 H, CH), 6.72 (d, 2 H, aromatic), 7.03 (d, 2 H, aromatic), 7.20 (bs, 1 H, NH), 7.30 (d, 1 H, NH).

Treatment of compound 9 with 10% TMSBr in acetonitrile (11)

To N^{α}-acetyl-*O*-(dimethylthiophosphono)-L-tyrosine-*N*-methylamide (**9**, 43.5 mg, 0.12 mmol) was added 10% TMSBr in acetonitrile (5.0 ml) and the mixture was stirred for 5.5 h at room temperature. After evaporation of solvent at reduced pressure, water (5 ml) was added and the mixture was stirred for 30 min at room temperature. Evaporation of solvent at reduced pressure afforded a light yellow oil which was purified by column chromatography with a gradient elution of EtOAc/MeOH/acetic acid (80:20:1) to EtOAc/MeOH/acetic acid (66:33:1) to yield a colorless oil (8.5 mg, 20.8%): TLC (silica gel, EtOAc/MeOH/acetic acid=66:33:1, v:v), R_f 0.33; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.752 (s, 3 H, $COCH_3$), 2.540 (d, 3 H, N-CH_3), 2.641 (q, 1 H, CH), 2.850 (q, 1 H, CH), 3.410 (d, 3 H, $J_{P\text{-}O\text{-}C\text{-}H}$ =12.91 Hz, $POCH_3$), 4.320 (m, 1 H, CH), 7.015 (q, 4 H, aromatic), 7.925 (q, 1 H, NH), 8.122 (d, 1 H, NH).

Dicyanoethyl *N,N*-diethylphosphoramidite (12)

A solution of 3-hydroxypropionitrile (25.58 g, 24.60 ml, 0.360 mol) and triethylamine (38.64 g, 53.22 ml, 0.382 mol) in dry diethyl ether (150 ml) was added to *N,N*-diethylphosphoroamidous dichloride **1** (30.51 g, 0.175 mol) in dry diethyl ether (100 ml) such that the reaction temperature was kept below 0°C. On completion of addition (ca. 40 min), the mixture was allowed to warm to room temperature and stirred for 3 h. 5% Aqueous sodium bicarbonate solution (70 ml) was added, then the mixture was transferred to a separatory funnel and the aqueous layer was discarded. The ethereal phase was washed with 5% aqueous sodium bicarbonate (2×60 ml), saturated sodium chloride solution (60 ml) and dried with magnesium sulfate. Evaporation of solvent at reduced pressure afforded a colorless oil (37.93 g, 89.1%) which was used in the next step without further purification: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.08 (t, 6 H, J_H=7.5 Hz), 2.67 (t, 4 H, J_H=6.5 Hz, -CH_2-CN), 3.09 (m, 4 H, J_H=7.5 Hz, $J_{P\text{-}N\text{-}C\text{-}H}$=10.0 Hz, N-$CH_2$), 3.87 (m, 4 H, J_H=6.5 Hz, J_H=7.5 Hz, $J_{P\text{-}O\text{-}C\text{-}H}$=10.5 Hz, O-$CH_2$).

***N*-(*tert*-Butoxycarbonyl)-*O*-(dicyanoethylthiophosphono)-L-tyrosyl *p*-nitrobenzyl ester (14)**

1*H*-tetrazole (2.30 g, 32.83 mmol) was added to a stirred solution of *t*-Boc-L-tyrosyl *p*-nitrobenzyl ester (**3**, 5.00 g, 12.01 mmol) and dicyanoethyl *N,N*-diethylphosphoramidite (**12**, 3.14 g, 12.91 mmol) in dry tetrahydrofuran (40 ml) and the mixture was stirred for 15 min at room temperature. The mixture was cooled to 0°C and a solution of sulfur (0.42 g, 13.13 mmol) in carbon disulfide (40 ml) was added. After stirring for 2 h at room temperature, 10% aqueous $NaHSO_3$ (70 ml) was added and the mixture was stirred for further 10 min. The mixture was transferred to a separatory funnel and the organic phase was evaporated at reduced pressure and dissolved in ethyl acetate (20 ml). The aqueous phase was extracted with ethyl acetate (3×20 ml). The combined ethyl acetate solution was successively washed with 10% aqueous $NaHSO_3$ (2×20 ml), 5% aqueous $NaHCO_3$ (2×20 ml), water (20 ml), saturated sodium chloride solution (20 ml) and dried with magnesium sulfate. Evaporation of solvent at reduced pressure gave an oil (6.56 g) which was purified by column chromatography with an elution of EtOAc/hexane (1:2, v:v) to afford a light yellow oil (4.87 g, 65.6%): TLC (silica gel, EtOAc/hexane=1:1, v:v), R_f 0.27; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9 H), 2.81 (m, 4 H, CH_2-CN), 3.08 (d, 2 H, CH_2-C), 4.38 (m, 4 H, CH_2-O), 4.62 (q, 1 H, CH), 5.07 (d, 1 H, NH), 5.20 (q, 2 H, -CH_2-O), 7.09 (s, 4 H, aromatic), 7.40 (d, 2 H, aromatic), 8.21 (d, 2 H, aromatic).

***N*-(*tert*-Butoxycarbonyl)-*O*-(dicyanoethylthiophosphono)-L-tyrosine (15)**

N-(*tert*-Butoxycarbonyl)-*O*-(dicyanoethylthiophosphono)-L-tyrosyl *p*-nitrobenzyl ester (**14**, 1.26 g, 2.04 mmol) was dissolved in methanol (40 ml) containing acetic acid (3.0 ml) and the mixture was hydrogenolyzed at atmospheric pressure with 10% Pd/C (0.57 g) for 12 h at room temperature. After filtration of the Pd/C residue and evaporation of solvent at reduced pressure, the residue was dissolved in ethyl acetate (50 ml). The solution was washed with 1 N hydrochloric acid (2×15 ml), water (15 ml) and extracted with 5% aqueous sodium bicarbonate (3×15 ml). The aqueous extract was washed with diethyl ether (15 ml), ethyl acetate (15 ml) and acidified to pH 2 at 0°C with 1 N hydrochloric acid and extracted with ethyl acetate (3×15 ml). The combined organic phase was washed with 1 N hydrochloric acid (15 ml), water (15 ml), saturated sodium chloride solution (10 ml) and dried with magnesium sulfate. Evaporation of solvent at reduced pressure afforded an oil which was purified by column chromatography with an elution of EtOAc/hexane/AcOH (60:30:5) to yield a colorless oil (0.50 g, 50.7%): TLC (silica gel, EtOAc/hexane/acetic acid=60:30:5, v:v), R_f 0.35; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 9 H), 2.79 (q, 4 H), 3.11 (m, 2 H), 4.39 (m, 4 H), 4.59 (q, 1 H), 5.08 (d, 1 H), 7.18 (m, 4 H).

***N*-(*tert*-Butoxycarbonyl)-thiophosphono-L-tyrosine ammonium salt (16)**

To a stirred solution of concentrated ammonium hydroxide (10 ml) was added *N*-(*tert*-butoxycarbonyl)-*O*-(dicyanoethylthiophosphono)-L-tyrosyl *p*-nitrobenzyl ester (**14**, 0.1 g, 0.16 mmol) at room temperature. The temperature was increased to 55°C and stirred for 12 h. The reaction mixture was washed with diethyl ether (2×10 ml) and concentrated at reduced pressure. The residue was washed with diethyl ether (2×10 ml) and dried at high vacuum. The crude product was analyzed by ^{1}H NMR without further purification: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30 (s, 9 H), 2.60 (m, 2 H), 4.01 (m, 1 H), 6.04 (bs, 12 H), 6.78 (d, 1 H), 7.04 (m, 4 H).

Treatment of compound 14 with 10% DIEA in DMF

To a stirred solution of 10% *N,N*-diisopropylethylamine (DIEA) in DMF (5 ml) was added *N*-(*tert*-butoxycarbonyl)-*O*-(dicyanoethylthiophosphono)-L-tyrosyl *p*-nitrobenzyl ester (**14**, 80 mg, 0.13 mmol) and the mixture was stirred for 1 h at room temperature. After evaporation of solvent at high vacuum, the crude residue was analyzed by ^{1}H NMR without further purification: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9 H), 2.81 (m, 4 H), 3.08 (d, 2 H), 4.38 (m, 4 H), 4.62 (q, 1 H), 5.07 (d, 1 H), 5.20 (q, 2 H), 7.09 (s, 4 H), 7.40 (d, 2 H), 8.21 (d, 2 H).

N-(*tert*-Butoxycarbonyl)-*O*-(dicyanoethylthiophosphono)-L-tyrosyl-L-leucyl-Pam resin (17)

The dipeptide resin **17** was prepared by solid phase synthesis. *t*-Boc-L-leucine-Pam resin (0.23 g, 0.11 mmol) was treated with 10 mL of 33% trifluoroacetic acid (TFA) in dichloromethane (DCM) containing 5% *m*-cresol for 30 min at room temperature. After washing with dichloromethane (15 ml) and DMF (15 ml), the resin was treated with 10% *N,N*-diisopropylethylamine (DIEA) in DMF (10 ml) for 3 min at room temperature. The resin was washed with DMF (15 ml), DCM (15 ml) and treated with a solution of *N*-(*tert*-butoxycarbonyl)-*O*-(dicyanoethylthiophosphono)-L-tyrosine (**15**, 0.16 g, 0.33 mmol) in DCM (2 ml) and 0.5 M DCC solution in DCM (0.66 ml, 0.33 mmol) for 2 h at room temperature. The resin (0.22 g) was washed with DCM (15 ml) and dried at high vacuum. The ninhydrin test indicated that more than 99% of coupling was completed: IR (KBr) 2255 cm^{-1} (-CN). The solid phase synthesis was performed on the same method reported in the literature (Stewart *et al.*, 1984).

Treatment of resin 17 with 10% DIEA in DMF (18)

The resin **17** (0.22 g) was treated with 10% *N,N*-diisopropylethylamine (DIEA) in DMF (5 mL) 30 times. The treatment was performed on the same method reported in the literature (Stewart *et al.*, 1984): IR (KBr) 2255 cm^{-1} (-CN).

Conversion of peptide-resin 18 to L-tyrosyl-L-leucine (19)

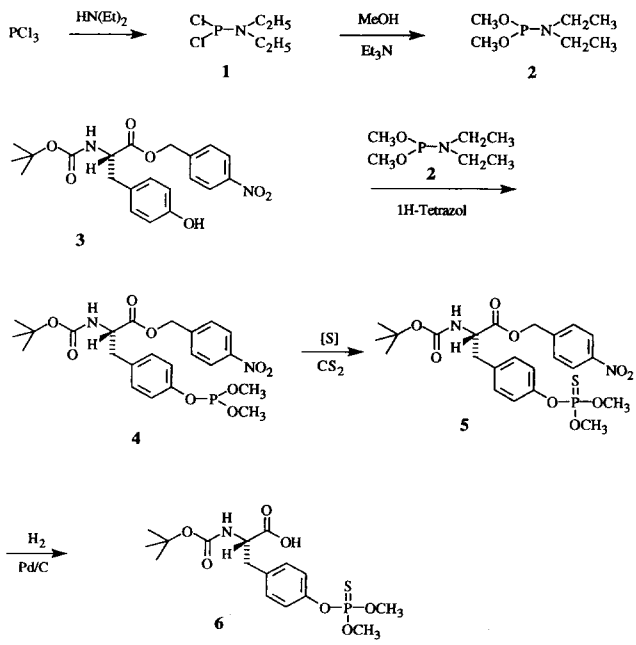
The crude dipeptide **19** (16 mg) obtained from HF cleavage of the peptide-resin **18** was purified by HPLC on a Beckman column (Dynamax-300A, C-18, 12 μ, 10×250 mm). A gradient elution of 10% to 40% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 4.5 ml/min was used. Fractions corresponding to the peak (UV detector at 230 nm) of retention time 9.10 min were collected. The combined fractions were lyophilized to yield a white solid (13.2 mg, 82.5%): FABMS *m/e* 295 (MH^+); ^1H NMR (300 MHz, D_2O) δ 0.667 (d, 3 H, $-CH_3$), 0.710 (d, 3 H, $-CH_3$), 1.313 (p, 1 H), 1.428 (m, 2 H), 2.923 (q, 1 H), 2.990 (q, 1 H), 4.020 (t, 1 H), 4.097 (d of d, 1 H), 6.687 (d, 2 H), 6.982 (d, 2 H).

RESULTS AND DISCUSSION

The general synthetic strategy for the preparation of thiophosphonotyrosine-containing peptides involved the utilization of *t*-Boc chemistry and the thiophosphorylation to tyrosine derivatives and the utilization of the dialkyl protecting group on the thiophosphate moiety (Stewart *et al.*, 1984).

Methyl protecting group approach

The preparation of protected thiophosphonotyrosine compound **5** was performed by the phosphorylation of protected tyrosine utilizing a phosphoramidite as the phosphorylating agent (Perich *et al.*, 1988a; Perich *et al.*, 1988b), followed by the oxidation of phosphite with sulfur (Agrawal *et al.*, 1989) (Scheme 1). The key reagent, dimethyl *N,N*-diethylphosphoramidite (**2**) as a phosphorylating agent was prepared in 66.4% yield by addition of methanol and triethylamine to *N,N*-diethylphosphoroamidous dichloride (**1**), which was prepared from diethylamine and phosphorus trichloride (Perich *et al.*, 1987). *t*-Boc-L-Tyrosyl *p*-nitrobenzyl ester **3** which was previously reported in the literature for the synthesis, was phosphorylated by treatment of phosphoamidite **2** and 1*H*-tetrazol to form intermediate **4**, followed by the oxidation of the phosphite moiety with sulfur in carbon disulfide (Agrawal *et al.*, 1989) to afford *t*-Boc-dimethylthiophosphono-L-tyrosyl *p*-nitrobenzyl ester **5** in 83.0% yield after purification by gel chromatography. The ^1H NMR spectrum displayed a new resonance at 3.83 ppm with coupling constant of 13.7 Hz (P-O-C-H) corresponding to the methyl protecting group on the thiophosphate moiety thereby conforming its structure. *t*-Boc-Dimethylthiophosphono-L-tyrosine **6** was prepared by hydrogenolysis of compound **5** in 73.9% yield. The ^1H NMR spectrum of compound **6** displayed a doublet peak at 3.83 ppm with coupling constant of 13.7 Hz. Because the thiophosphate moiety possibly acted as a catalyst poison (House *et al.*, 1972), prolonged reaction time was required and the product yield was relatively low

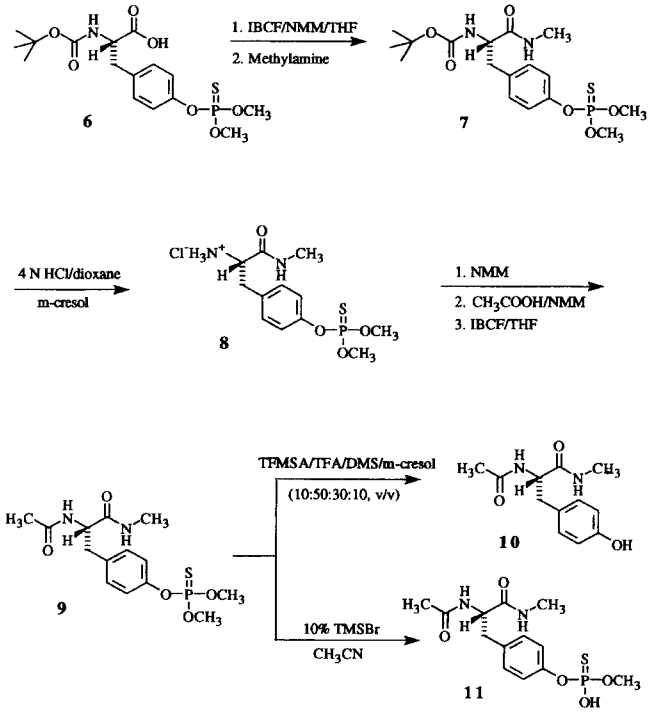


Scheme 1. Synthesis of compound **6**

compared to that of common hydrogenolysis reaction.

To determine the suitability of the methyl protected *O*-thiophosphonotyrosine derivative **6** for use in standard solid phase peptide synthesis methodology, it was necessary to investigate its facile removal and stability of the thiophosphate moiety to reagents commonly employed in deprotection. To facilitate an investigation of the removal of the methyl protecting groups, the model derivative, N^{α}-acetyl-*O*-(dimethylthiophosphono)-L-tyrosine-*N*-methylamide **9** was prepared (Valerio *et al.*, 1989) (Scheme 2). It was conceived that the mode of deprotection of the model derivative should be representative of that of deprotection of methyl protected *O*-thiophosphonotyrosine residues in peptide sequences. The protected derivative **6** was converted to *N*-methyl amide **7** by reaction of its corresponding mixed anhydride intermediate with *N*-methylamine. After selective deprotection of the *t*-Boc group of compound **7** by treatment with 4 N HCl in dioxane, the desired compound **9** was prepared by the mixed anhydride method using acetic acid and isobutylchloroformate in the presence of *N*-methylmorpholine. The acetyl derivative **9** was recovered in overall yield 24.5% as a light yellow oil after purification by gel chromatography. The ^{1}H NMR spectrum of compound **9** indicated the doublet peak of P-O-CH_3 at 3.86 ppm with coupling constant of 13.8 Hz.

In order to investigate facile removal of the methyl protecting group on the thiophosphate moiety, the model compound **9** was treated with a mixture of TFMSA/TFA/DMS/*m*-cresol (10:50:30:10, v:v) for 8 h at room temperature (Cushman *et al.*, 1992; Lee *et al.*, 1994). Although the common reaction time for this reagent was within 2 h, the deprotection did not proceed well for compound **9** according to monitoring of the reaction by HPLC. So it was necessary to extend the reaction time. Crude product was analyzed by HPLC and the major product was separated by semi-preparative HPLC with a gradient elution of 10% to 70% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 3 mL/min. The ^{1}H NMR analysis of the isolated product determined that the product was the dephosphorylated compound **10**. Deprotection of the model compound **9** with an alternative reagent, 10% TMSBr in acetonitrile (Valerio *et al.*, 1989), did not proceed well either. With prolonged reaction time (5.5 h) at room temperature, only a small amount of product was detected by TLC. After separation of a major product by gel chromatography, it turned out to be monomethyl-containing compound **11** in 20.8% yield, while most of the starting material was remained unreacted. It was concluded that the methyl group was not suitable for the protection of thiophosphonotyrosine derivative.



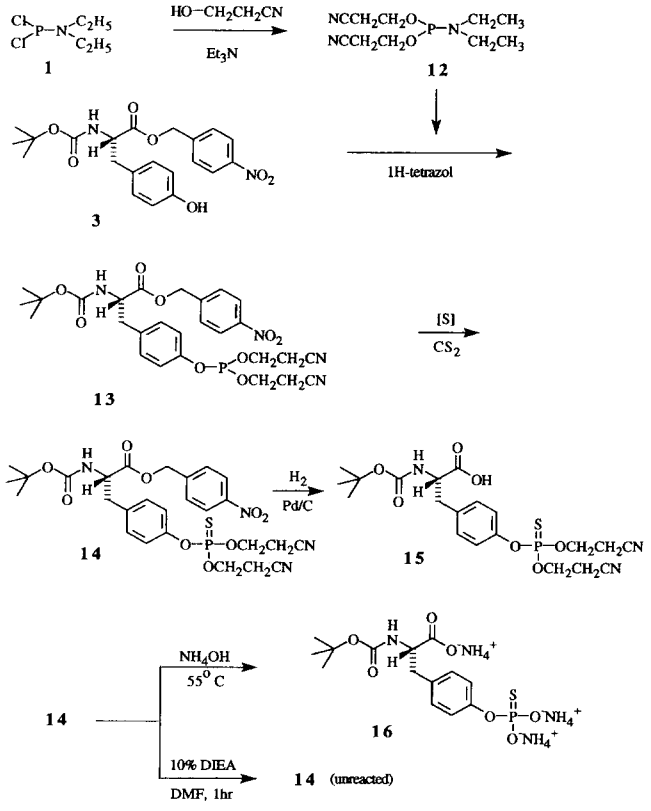
Scheme 2. Synthesis of model compound **9**

Cyanoethyl protecting group approach

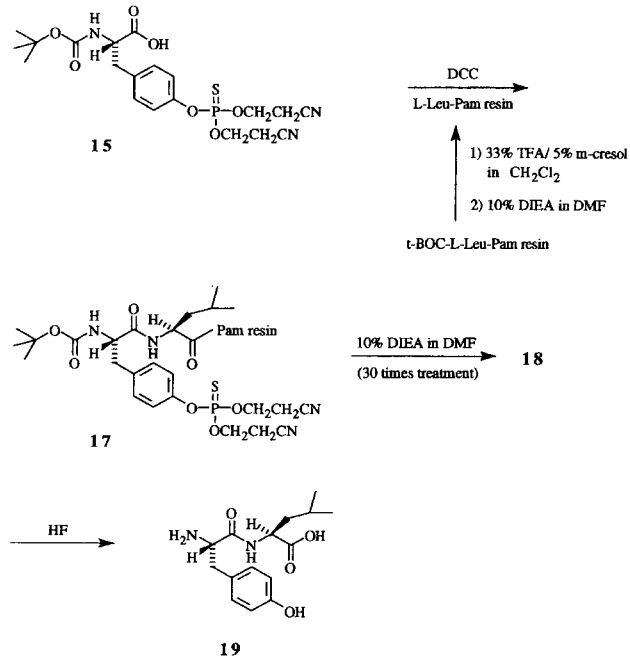
We next considered the application of the cyanoethyl protecting group (Reese *et al.*, 1987; Tener *et al.*, 1961) for the protection of the *O*-thiophosphonotyrosine derivative in peptide synthesis. The cyanoethyl group has been widely utilized for the protection of the phosphate group in DNA synthesis (Hsiung *et al.*, 1983; Sinha *et al.*, 1984). The facile removal of the cyanoethyl group under mild basic conditions and reliable stability in acidic conditions encouraged its adoption for thiophosphonotyrosine-containing peptide synthesis. The cyanoethyl protected compound **15** was prepared as reported in the literature (Kim *et al.*, 1997).

To determine the suitability of the protected *O*-thiophosphonotyrosine derivative **15** for utilization in existing peptide synthesis procedures, it was necessary to determine its stability to a variety of reagents commonly employed in peptide synthesis and to develop suitable conditions for removal of the protecting groups on the thiophosphate moiety. The investigation for the facile removal of the cyanoethyl protecting group was performed by treatment of compound **14** with a concentrated ammonium hydroxide solution at 55°C for 12 h to yield the deprotected compound **16** in satisfactory result (Scheme 3).

It was necessary to determine the stability of the cyanoethyl protecting group to 10% diisopropylethylamine (DIEA), which served as a neutralizing agent in standard solid phase peptide synthesis, because the cyanoethyl group was reported to be labile under basic conditions (UhlMann *et al.*, 1986). The initial in-



Scheme 3. Synthesis of compound **15**



Scheme 4. Synthesis and model study of dipeptide-resin **17**

vestigation was conducted by treatment of compound **14** with 10% DIEA in DMF at room temperature for 1 h which was equivalent to 20 cycles of coupling steps in solid phase peptide synthesis. The product was analyzed by ^{1}H NMR, which indicated compound **14** remained stable under these conditions. To confirm the stability of the protecting group and other functional groups in thiophosphonotyrosine derivatives, dipeptide-Pam resin containing the dicyanoethylthiophosphonotyrosine residue was synthesized by the solid phase method (Stewart *et al.*, 1984) and treated with reagents employed in peptide synthesis, including HF treatment for peptide-resin cleavage (Scheme 4).

t-Boc-L-Leu-Pam resin was treated with 33% TFA in dichloromethane containing 5% *m*-cresol for 30 min to cleave the *t*-Boc group, followed by treatment of 10% DIEA in DMF to generate free amine, and coupled with compound **15** in the presence of DCC in dichloromethane for 2 h to yield dipeptide-Pam resin **17**. The coupling was monitored by ninhydrin test which indicated more than 99% of completion. The IR spectra of dipeptide-Pam resin **17** showed that a new absorption of the nitrile functional group appeared at 2255 cm^{-1} compared to that of uncoupled resin, *t*-Boc-L-Leu-Pam resin. Peptide-resin **17** was treated with 10 % DIEA in DMF 30 times, which was equivalent to 10 cycles of coupling steps, to confirm the stability of the cyanoethyl protecting group in solid phase synthesis. The crude resin **18** was analyzed by IR, which indicated that the absorption of the nitrile functional group at 2255 cm^{-1} existed with similar intensity. Resin **18** was treated with HF for peptide-resin cleavage. The crude dipeptide obtained from the resin was analyzed by HPLC with a gradient elution of 10% to 50% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 ml/min. The analytical HPLC tracing of crude product indicated the presence of one major product having retention time of 8.310 min. After separation of the product on the semi-preparative scale, structure of the product was determined by ^{1}H NMR to be the unexpected compound **19** whose thiophosphate group was completely dephosphorylated. This result was quite unexpected because the phosphate groups in dialkylphosphonotyrosine derivatives were quite stable to HF treatment. FABMS analysis confirmed the dephosphorylated structure. It was concluded that the cyanoethyl protecting group on the thiophosphate moiety was not suitable for solid phase peptide synthesis using *t*-Boc chemistry methodology. But it would be possible to utilize cyanoethyl protecting group for the solution phase peptide synthesis which was not required the strong condition such as HF treatment for the cleavage between residue and peptide bond. For the future studies, the application of Fmoc chemistry methodology in solid state peptide synthesis which required milder conditions for peptide-resin cleavage, and utilization of the *t*-butyl protecting group on the thiophosphate moiety could be considered (Fields *et al.*, 1990).

ACKNOWLEDGMENTS

This research was supported in part by the Yeungnam University Research Grants. And authors thank to Purdue Peptide Synthesis Lab. in Purdue University for the cleavage of peptide-resin bond by HF treatment.

REFERENCES CITED

Agrawal, S., Ikeuchi, T., Sun, D., Sarin, D. S., Konopka, A., Maizei, J. and Zamecnik, P. C., Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogs. *Proc. Natl. Acad. Sci. USA*, 86, 7790-7794 (1989).

Cantley, L. C., Auger, K. R., Carpenterm, C., Duckworth, B., Graziani, A. Kapeller, R and Soltoff, S., Oncogenes and signal transduction. *Cell*, 64, 281-302 (1991).

Cushman, M. and Lee, E. S., Preparation of an Angiotensin I Analog containing a *p*-phosphonomethyl-L-phenylalanine residue via asymmetric synthesis of *t*-boc-*p*-dimethyl(phosphonomethyl)-L-phenylalanine. *Tetrahedron Lett.*, 33, 1193-1196 (1992).

Fields, G. B. and Noble, R. L., Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Peptide Protein Res.*, 35, 161-214 (1990).

House, H. O. *Modern Synthetic Reactions. 2nd edition,* The Benjamin/Cummings Publishing Co., pp 15-18, 1972.

Hsiung, H., Inouye, S., West, J., Sturm, B. and Inouye, M., Further improvements on the phosphotriester synthesis of deoxyribooligonucleotides and the oligonucleotide directed site-specific mutagenesis of *E. coli* lipoprotein gene. *Nucleic Acid Res.*, 11, 3227-3239 (1983).

Hunter, T., Protein-tyrosine phosphatases: the other side of the coin. *Cell*, 58, 1013-1016 (1989).

Hunter, T. and Cooper, J. A., Protein-tyrosine kinases. *Ann. Rev. Biochem.*, 54, 897-930 (1985).

Kim, E. K., Choi, H., and Lee, E. S., Synthesis of the key intermediate for the preparation of thiophosphotyrosine-containing peptide derivatives. *Yakhak Hoeji*, 41, 588-594 (1997).

Koch, C. A., Anderson, D., Moran, M. F., Ellis, C. and Pawson, T., SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science*, 252, 668-674 (1991).

Lee, E. S. and Cushman, C., Synthesis of a Phosphotyrosine-containing peptide fragment of the regulatory domain of $pp60^{c\text{-}src}$. *J. Org. Chem.*, 59, 2086-2091 (1994).

Perich, J. W. and Johns, R. B., Di-*tert*-butyl *N,N*-diethylphosphoramidite. A new phosphorylating agent for the efficient phosphorylation of alcohols. *Synthesis*, 142-144 (1988a).

Perich, J. W. and Johns, R. B., Conversion of alcohols into their dibenzyl phosphotriesters using *N,N*-dibenzyl phosphoramidite. *Tetrahedron Lett.*, 28, 101-102 (1987).

Perich, J. W. and Johns, R. B., Di-*t*-butyl *N,N*-diethylphosphoramidite and dibenzyl *N,N*-diethylphosphoramidite. Highly reactive reagents for the phosphite-triester phosphorylation of serine-containing peptide. *Tetrahedron Lett.*, 29, 2369-2372 (1988b).

Reese, C. B.; and Ward, J. G., Synthesis of D-myoinositol 1,4,5-triphosphate. *Tetrahedron Lett.*, 28, 2309-2312 (1987).

Sinha, N. D., Biernat, J., McManus, J. and Koster, H., Polymer support oligonucloetide synthesis X VII: use of beta-cyanoethyl-*N,N*-dialkylamino-/*N*-morpholinophosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucleic Acid Res.*, 12, 4539-4557 (1984).

Stewart, J. M. and Young, J. D. *Solid Phase Peptide Synthesis. 2nd edition*, Pierce Chemical, Co., Rockford, Il., 1984.

Tener, G. M., 2-Cyanoethyl phosphate and its use in the synthesis of phosphate esters. *J. Am. Chem. Soc.*, 83, 159-168 (1961).

Tonks, N. K., and Charbonneau, H., Protein-tyrosine dephosphorylation and signal transduction. *Trends Biochem. Sci.*, 14, 497-500 (1989).

UhlMann, E. and Engels, J., Chemical 5'-phosphorylation of oligonucleotides valuable in automated DNA synthesis. *Tetrahedron Lett.*, 27, 1023-1026 (1986).

Valerio, R. M., Alewood, P. F., Johns, R. B. and Kemp, B. E. *Int. J. Peptide Protein Res.*, 33, 428-438 (1989).

Valerio, R. M., Perich, J. W., Kitas, E. A., Alewood, P. F. and Johns, R. B. *Aust. J. Chem.*, 42, 1519-1525 (1989).