CHAPTER II

EXPERIMENTAL SECTION

2.1 General Procedure

2.1.1 Measurement

The weight of all substances chemical was determined on a Metler Toledo electrical balance. Evaporation of solvents was carried out on Büchi Rotavapor R-200 with a water aspirator model B-490 or a Refco Vacubrand pump. The magnetic stirrers were of Corning. The progress of the reaction was followed by thin layer chromatography (TLC) performed on Merck D.C. silica gel 60 F_{254} 0.2 mm. precoated aluminium plates cat. no. 1.05554 and visualized using UV light (254 nm). Column chromatography was performed on silica gel 230-400 mesh for flash column chromatography. Reverse phase HPLC experiments were performed on Water 600^{TM} system equipped with gradient pump and Water 996^{TM} photodiode array detector; optionally alternate to Rheodyne 7725 manual sample loop (100 μL sample size for analytical scale). A Varian^{TM} C_{18} HPLC column 3 μm particle size 4.6 × 50 mm was used for both analytical purposes. Peak monitoring and data processing were performed on the base Empower software. Fractions from HPLC were collected manually which was assisted by real-time HPLC chromatogram monitoring. The combined fractions were speed vaporized under reduced pressure using Heto Vacuum Centrifuge and MAXI dry-plus. Melting points were recorded on an electrothermal melting point apparatus model 9100. ^{1}H and ^{13}C spectra were recorded on Varian Mercury-400 plus operating at 400 MHz by Dr. Tirayut Vilaivan and Miss Woraluk Mansawat. MALDI-TOF mass spectra of all *cis*-D-*ss*ACPC PNAs were obtained on a Microflex MALDI-TOF mass spectrometry (Bruker Daltonics) using doubly recrystallized α-cyano-4-hydroxy cinnamic acid (CCA) as matrix. 0.1 % Trifluoroacetic acid in acetonitrile:water (1:2) was used as the diluents for preparation of MALDI-TOF samples.

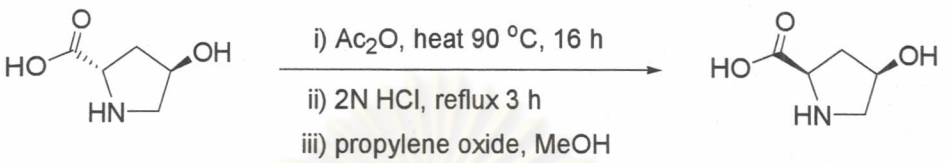
2.1.2 Materials

All chemicals were purchased from Fluka, Merck or Aldrich Chemical Co., Ltd., and were used as received without further purification. Commercial grade solvents were distilled before use for column chromatography. Solvents for reactions and crystallization were reagent grade and used without purification. Acetonitrile for HPLC experiment was HPLC grade, obtained for BDH and was filtered through a membrane filter (13 mm ϕ, 0.45 μm Nylon Lida) before use. Tetrahydrofuran for Mitsunobu reaction was dried with fresh thin-cut sodium metal and benzophenone under reflux. Anhydrous *N,N*-dimethylformamide ($H_2O \leq 0.01\%$) for solid phase peptide coupling reaction was obtained from Fluka and dried with activated 3Å molecular sieves. The solid support for peptide synthesis (TentaGel S RAM Fmoc resin) and trifluoroacetic acid were obtained from Fluka. The protected amino acids (Fmoc-L-Lys(Boc)-OPfp) was obtained form Calbiochem Novabiochem Co., Ltd. Acetic anhydride was synthesized from acetyl chloride and anhydrous sodium acetate according to the standard method.[48] Nitrogen gas was obtained from TIG with high purity up to 99.5 %. MilliQ water was obtained from ultrapure water system with Millipak® 40 filter unit 0.22 μm, Millipore (USA). Oligonucleotides were purchased from Bioservice Unit, National Science and Technology Development Agency (Thailand). *N-tert*-butoxycarbonyl-*trans*-4-tosyloxy-D-proline diphenylmethyl ester (**21**), N^3-benzoylthymine, N^4-benzoyladenine, N^4-benzoylcytosine and *N-tert*-butyloxycarbonyl-*cis*-4-(N^2-isobutylguanin-9-yl)-D-proline diphenylmethyl ester (**24**) was prepared by Miss Patcharee Ngamviriyavong and Mr. Chaturong Suparpprom.

2.2 Synthesis of PNA monomers

2.2.1 Synthesis of intermediate

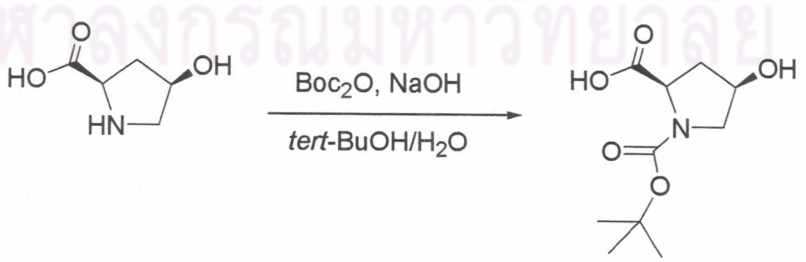
***cis*-4-Hydroxy-D-proline (13)** [61]



(13) **(14)**

A stirred mixture of *trans*-4-hydroxy-D-proline (**14**) (6.55g, 50 mol), and acetic anhydride (40 mL) was heated to 90 °C for 16 h under the nitrogen. Then the mixture was allowed to cool at 30 °C. The solvent was removed under reduced pressure to give dark thick oil that was dissolved in 50 mL of 2 M hydrochloric acid and was then refluxed for another 3 h. The solvent was removed again by rotary evaporation to give dark thick oil which was redissolved in absolute methanol. Then propylene oxide was slowly added and stirred at 30 °C for 8 h. The solvent was removed by rotary evaporation and residue was stirred with absolute ethanol. The precipitate was collected by suction filtration and recrystallized from ethanol containing a little water to give the desired product (**14**) as colorless needle (4.01 g, 61 %).

***N*-*tert*-Butoxycarbonyl-*cis*-4-hydroxy-D-proline (15)** [40]

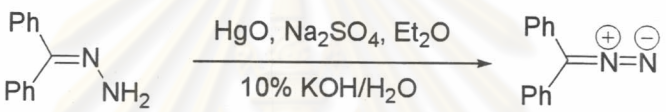


(14) **(15)**

To a stirred mixture of *cis*-4-hydroxy-D-proline (**14**) (4.01 g, 30.5 mmol) in 4% sodium hydroxide (50 mL) was added the solution of di-*tert*-butyl dicarbonate

(8.06 g, 37 mmol) in *tert*-butanol (15 mL) dropwise to avoid a sudden increase of temperature. While the resulting emulsion became homogeneous, vigorous evolution of carbon dioxide was observed. The solution was allowed to stir at 30 °C for 8 h. The solvent was removed by rotary evaporation and the residue was dissolved in 10 mL of water and acidified to pH 2 with 10% hydrochloric acid. The acidified solution was extracted with ethyl acetate (3 × 20 mL). The combined organic extract was dried over magnesium sulfate and evaporated under reduced pressure to give clear thick oil. Scratching the oil with ice-cold hexane afford the product (**15**) as white solid (5.12 g, 70 %).

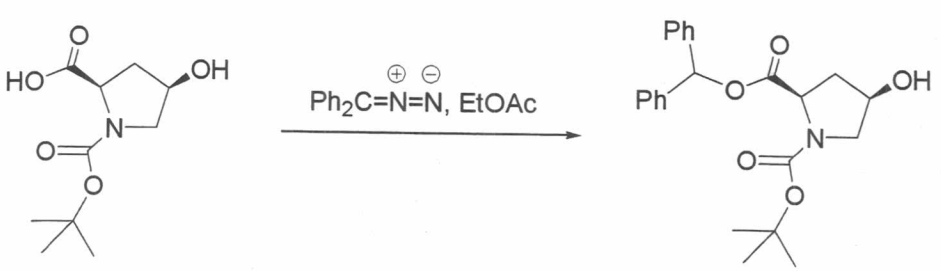
Diphenyldiazomethane (16) [62]



(16)

In a 1000 mL round bottle flask, wrapped with aluminium foil to protect from light, benzophenone hydrazone (13.74 g, 70.0 mmol), mercuric oxide (15.16 g, 70.0 mmol) and anhydrous sodium sulfate (9.94 g, 70.0 mmol) were suspended in 150 mL diethyl ether with stirring. Then 15 mL of 10% potassium hydroxide in ethanol was added until the solution turned to purple. The reaction was stirred in the dark for 6 h. The used mercuric oxide and sodium sulfate mixture were filtered off and washed with diethyl ether. Diethyl ether was removed by rotary evaporation from the purple filtrate to obtain product (**16**) as a purple liquid which was used for the next step without characterization and purification.

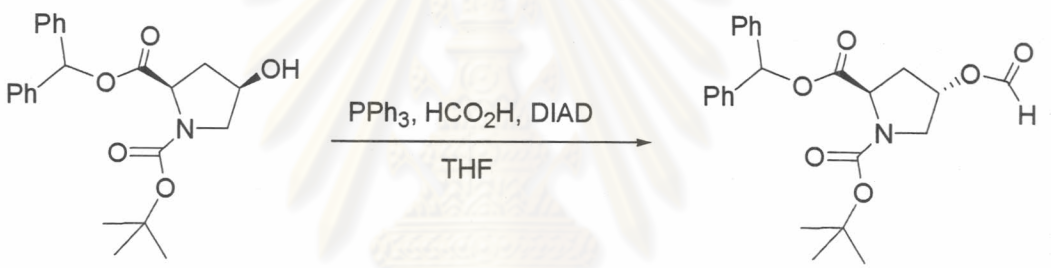
***N-tert*-Butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester (17)** [41]



(15) **(17)**

The solution of *N-tert*-Butoxycarbonyl-*cis*-4-hydroxy-D-proline (**15**) (12.05 g, 52.1 mmol) in ethyl acetate (30 mL) was slowly added freshly prepared diphenyldiazomethane (**16**) until the solution was permanently purple in color. The reaction was allowed to stir in the dark for 8 h. When the solution became colorless, a further portion of freshly prepared diphenyldiazomethane (**16**) was added and the stirring was continued until the reaction was completed as indicated by TLC analysis. The solvent was evaporated by rotary evaporation to obtain the crude product as a pink sticky oil which solidified after scratching with hexane. The white precipitate formed was collected by suction filtration, washed with hexane and dried under vacuum to give product (**17**) as a white fluffy solid (17.10 g, 82 %).

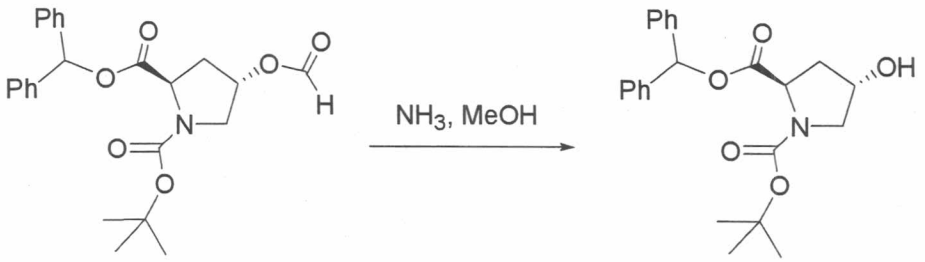
***N-tert*-Butoxycarbonyl-*trans*-4-formyl-D-proline diphenylmethyl ester (18)** [41]



(**17**) (**18**)

In a dried 100 mL round bottom flask equipped with a magnetic bar, *N-tert*-butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester (**17**) (2.98 g, 7.5 mmol), formic acid (0.35 µL, 8.7 mmol) and triphenylphosphine (2.35 g, 9.0 mmol) were dissolved in dry THF (10 mL) and cooled down to 0 °C in an ice bath. The mixture was stirred under nitrogen balloon and DIAD (1.6 µL, 9.0 mmol) was added dropwise during 15 min. The solution was stirred at 30 °C for 8 h. The solvent was evaporated and the residue was chromatographed on silica gel using hexane:ethylacetate (3:1) as eluent to give the compound (**18**) as a colorless oil which was used directly for the next step.

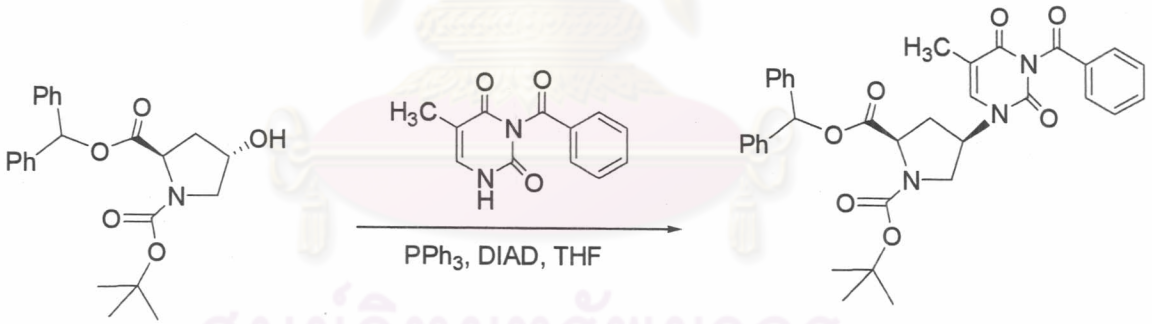
***N-tert*-Butoxycarbonyl-*trans*-4-hydroxy-D-proline diphenylmethyl ester (19)** [41]



(18) **(19)**

The *trans*-4-formate ester (**18**) was dissolved in methanol and concentrated aqueous ammonia (1 mL) was added. After 1 h, the aminolysis was completed as indicated by TLC analysis. The solvent was removed by rotary evaporation to obtain compound (**19**) which was dried under vacuum to give a colorless solid (2.30 g), 78 % from (**17**).

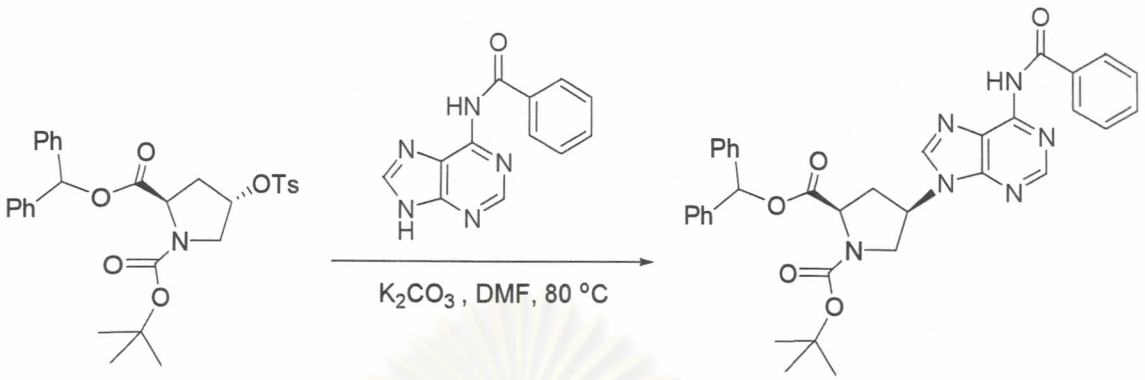
***N-tert*-Butoxycarbonyl-*cis*-4-(N^3-benzoylthymin-1-yl)-D-proline diphenylmethyl ester (20)** [41]



(19) **(20)**

In a dried 100 mL round bottom flask equipped with a magnetic bar, *N-tert*-butoxycarbonyl-*trans*-4-hydroxy-D-proline diphenylmethyl ester (**19**) (0.79 g, 2.0 mmol), triphenylphosphine (0.63 g, 2.4 mmol), and N^3-benzoylthymine (0.42 g, 2.0 mmol) were dissolved in dry THF (15 mL) and cooled down to 0 °C in an ice bath. The solution was stirred under nitrogen balloon and DIAD (0.49 µL, 2.4 mmol) was added dropwise during 15 min. The mixture was stirred at 30 °C for 8 h. The solvent was evaporated under reduce pressure to complete dryness. The residue was recrystallized from ethanol to afford compound (**20**) as a white fluffy solid (0.79 g, 63%).

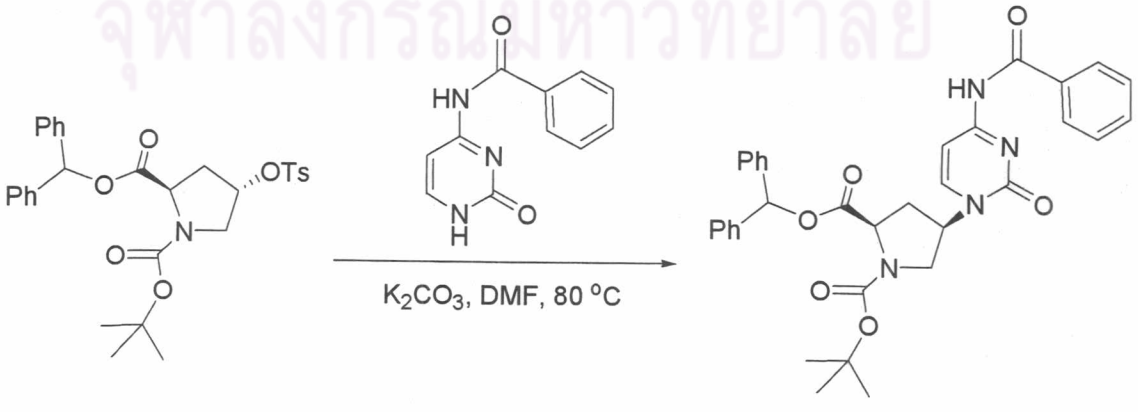
***N-tert*-Butoxycarbony-*cis*-4-(N^4-benzoyladenin-9-yl)-D-proline diphenylmethyl ester (22)** [41]



(21) **(22)**

In a dried 100 mL round bottom flask, *N-tert*-butoxycarbonyl-*trans*-4-tosyl-D-proline diphenylmethyl ester (**21**) (5.50 g, 9.97 mmol) and N^4-benzoyladenine (2.87 g, 12.0 mmol) was dissolved in anhydrous *N,N'*-dimethylformamide (20 mL) and anhydrous potassium carbonate (3.45 g, 25.0 mmol) was added. The mixture was heated to 80 °C with stirring for 8 h. After the reaction was completed as indicated by TLC analysis, the solvent was removed by rotary evaporation. The residue was diluted with dichloromethane (20 mL) and extracted with water (3 × 30 mL) for removing the residual *N,N'*-dimethylformamide. The organic layer was concentrated and purified by column chromatography eluting with hexane:ethyl acetate (1:3) on silica gel to afford the compound (**22**) as a white solid (1.85g, 30%).

***N-tert*-Butoxycarbonyl-*cis*-4-(N^4-benzoylcytosin-1-yl)-D-proline diphenylmethyl ester (23)** [41]

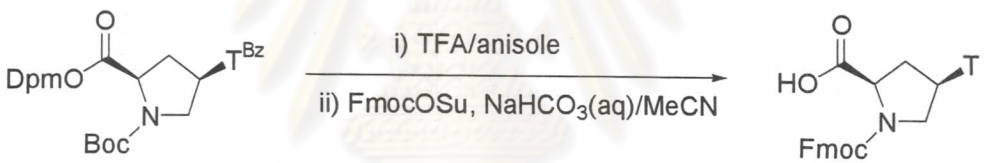


(21) **(23)**

In a dried 100 mL round bottom flask, *N*-*tert*-butoxycarbonyl-*trans*-4-tosyl-D-proline diphenylmethyl ester (**8**) (2.02 g, 3.6 mmol) and N^4-benzoylcytosine (0.93 g, 4.3 mmol) was dissolved in anhydrous *N*,*N*'-dimethylformamide (20 mL) and anhydrous potassium carbonate (1.24 g, 4.3 mmol) was added. The mixture was heated to 80 °C with stirring for 8 h. After the reaction was completed as indicated by TLC analysis, the solvent was removed by rotary evaporation. The residue was diluted with dichloromethane (20 mL) and extracted with water (3 × 30 mL) for removing the residual *N*,*N*'-dimethylformamide. The organic layer was concentrated and purified by column chromatography eluting with hexane:ethyl acetate (1:3) on silica gel to afford the compound (**23**) as a white solid (0.41 g, 19 %).

2.2.2 Synthesis of pyrrolidinyl PNA monomer

(*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-4-(thymin-1-yl)-D-proline (25)

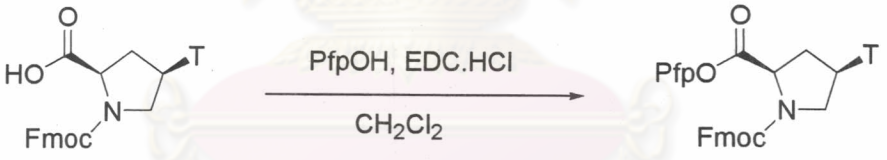


(**20**) (**25**)

(*N*-*tert*-butyloxycarbonyl)-*cis*-4-(thymin-1-yl)-D-proline diphenylmethyl ester (**20**) (0.61 g, 1.00 mmol) was treated with trifluoroacetic acid containing 10% anisole (2mL) and left at 30 °C for 8 h. The solvent was removed by a gentle stream of nitrogen. The residue was triturated and washed with diethyl ether. After drying under vacuum, it was dissolved in 1:1 H_2O:MeCN (5mL/mmol) and treated with solid $NaHCO_3$ until pH = 8. FmocOSu (0.39 g, 1.16 mmol) was then added in small portions with stirring. After stirring at 30 °C for 8 h, the solvent was removed by rotary evaporation. The residue was diluted with water (20 mL) and extracted with diethyl ether (3 × 20 mL). After purging the extracted aqueous layer to remove the dissolved ether with N_2, the pH was adjusted to 2 with concentrated HCl in aqueous phase. The Fmoc-amino acid precipitate was collected by filtration, washed with water, diethyl ether and dried under vacuum to afford the title compound (**25**) as white solid (0.31, 76%).

^{1}H NMR (400 MHz; $CDCl_3$): δ 1.76 (s, 3H; T CH_3), 2.14 and 2.22 (2×m, 1H; 1×CH_2 3′ rotamers), 2.60 and2.70 (2×m, 1H; 1×CH_2 3′ rotamers), 3.47 (m, 1H; 1×CH_2 5′ rotamers), 3.86 (m, 1H; 1×CH_2 5′ rotamers), 4.26 (m, 1H; Fmoc CH), 4.30 (m, 2H; Fmoc CH_2), 4.44 (m, 1H; CH 2′ rotamers), 4.97 and 5.31 (2×m, 1H; CH 4′ rotamers), 7.15 (s, 1H; T H6), 7.31 (m, 2H; Fmoc CH), 7.39 (m, 2H; Fmoc CH), 7.59 (d, ^3J(H,H) = 7.0 Hz, 2H; Fmoc CH), 7.85 (d, ^3J(H,H) = 7.0 Hz, 2H; Fmoc CH), 11.30 (s, 1H; T NH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 12.6 (T CH_3), 33.5 and 34.9 (CH_2 3′), 47.1 (Fmoc CH), 48.8 and 49.4 (CH 5′), 52.7 and 53.3 (CH 4′), 57.4 and 57.8 (CH 2′), 67.4 and 67.7 (Fmoc CH_2), 109.5 and 109.6 (T C5), 120.6 (Fmoc Ar CH), 125.6 (Fmoc Ar CH), 127.7 (Fmoc Ar CH), 128.2 (Fmoc Ar CH), 138.3 (T C6H), 141.2 (Fmoc Ar C), 144.1 (Fmoc Ar C), 151.4 (T C2), 154.4 (Fmoc CO), 164.2 (T C4), 173.1 and 173.7 (Pro CO); HRMS (FAB$^+$) Calcd for $C_{25}H_{23}O_6N_3$ (M·H$^+$) 462.1666, Found 462.1656, $[\alpha]^{25}_D$ = -3.85 (c = 1.0 g/100 mL, DMF), mp = 201 °C (dec.).

(*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-4-(thymin-1-yl)-D-proline pentafluorophenyl ester (26)



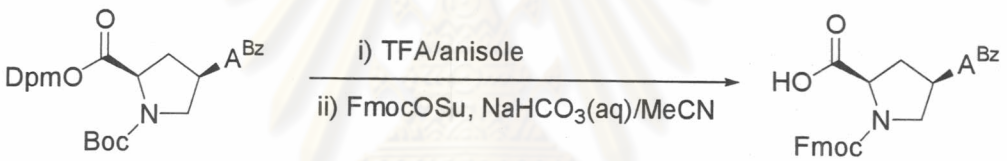
(25) **(26)**

To a suspension of (*N*-fluoren-9-ylmethoxycarbonyl)-*cis*-4-(thymin-1-yl)-D-proline (**25**) (0.22 g, 0.48 mmol) and pentafluorophenol (1.5 equiv) in dichloromethane (2 mL) was added EDC.HCl (1.5 equiv). The resulted mixture was stirred at 30 °C for one hour. The reaction was completed as indicated by TLC analysis and purified by flash column chromatography eluting with hexane: ethyl acetate (1:1) on silica gel to obtain the title compound (**26**) as white solid (0.26 g, 85 %).

^{1}H NMR (400 MHz; $CDCl_3$): δ 1.93 (s, 3H; T CH_3), 2.33 and 2.39 (2×m, 1H; 1×CH_2 3′ rotamers), 2.97 (m, 1H; 1×CH_2 3′ rotamers), 3.60 and 3.75 (2×m, 1H; 1×CH_2 5′ rotamers), 3.95 and 4.04 (2×m, 1H; 1×CH_2 5′ rotamers), 4.23 (m, 1H; Fmoc CH),

4.52 (m, 2H; Fmoc CH_2), 4.68 and 4.76 (2×m, 1H; CH 2′ rotamers), 5.22 and 5.31 (2×m, 1H; CH 4′ rotamers), 7.15 (s, 1H; T H6), 7.30 (m, 2H; Fmoc CH), 7.39 (m, 2H; Fmoc CH), 7.56 (d, ^3J(H,H) = 7.0 Hz, 2H; Fmoc CH), 7.76 (d, ^3J(H,H) = 7.0 Hz, 2H; Fmoc CH), 10.16 (s, 1H; T NH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 12.5 (T CH_3), 34.2 and 35.7 (CH_2 3′), 47.1 (Fmoc CH), 48.9 and 49.3 (CH 5′), 52.4 and 52.8 (CH 4′), 56.9 and 57.3 (CH 2′), 68.2 and 68.5 (Fmoc CH_2), 111.9 and 112.3 (T C5), 120.1 (Fmoc Ar CH), 124.8 (Fmoc Ar CH), 127.1 (Fmoc Ar CH), 127.9 (Fmoc Ar CH), 136.6-142.2 (Pfp C), 135.7 and 135.9 (T C6H), 141.3 (Fmoc Ar C), 143.5 (Fmoc Ar C), 151.3 (T C2), 154.0 and 154.5 (Fmoc CO), 163.9 (T C4), 168.1 (Pro CO); HRMS (FAB^+) Calcd for $C_{31}H_{23}O_6N_3F_5$ ($M{\cdot}H^+$) 628.1508, Found 628.1505, $[\alpha]^{25}_D$ = -2.45 (c = 3.8 g/100 mL, $CHCl_3$), mp = 107-110 °C.

(*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^4-benzoyladenin-9-yl)-D-proline (27)



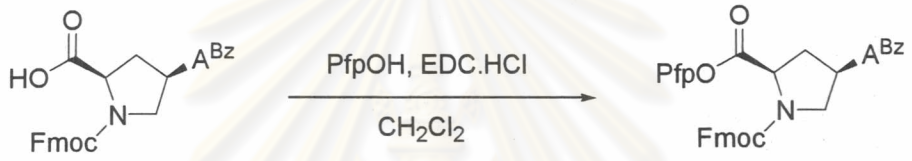
(**22**) (**27**)

Synthesis of the title compound (**27**) was accomplished in the same way as described for compound (**25**) above. Starting from (*N*-*tert*-butyloxycarbonyl)-*cis*-4-(N^4-benzoyladenin-9-yl)-D-proline diphenylmethyl ester (**22**) (0.54 g, 0.87 mmol), and trifluoroacetic acid containing 10% anisole (2 mL) followed by FmocOSu (0.32 g, 0.96 mmol) and $NaHCO_3$ (3 equiv excess) in 1:1 H_2O:MeCN (5mL/mmol) afforded (**27**) (0.41, 82%).

^{1}H NMR (400 MHz; $CDCl_3$): δ 2.68 and 2.73 (2×m, 1H; 1×CH_2 3′ rotamers), 2.95 and 3.06 (2×m, 1H; 1×CH_2 3′ rotamers), 3.95 (m, 1H; 1×CH_2 5′ rotamers), 4.20-4.26 (m, 2H; Fmoc CH and 1×CH_2 5′ rotamers), 4.34 (m, 2H; Fmoc CH2 rotamers), 4.60 and 4.86 (2×m, 1H; CH 2′ rotamers), 5.29 (m, 1H; CH 4′), 7.32 (2H, m, Fmoc CH), 7.42 (m, 2H; Fmoc CH), 7.54 (m, 2H; Bz CH), 7.68 (m, 3H; Fmoc and Bz CH), 7.88 (d, ^3J(H,H) = 7.0 Hz, 2H; Fmoc CH), 8.04 (m, 2H; Bz CH), 8.54 (2×s, 1H; A CH), 8.76 (s, 1H; A CH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 34.0 and 35.2 (CH_2 3′), 47.1

(Fmoc CH), 50.0 and 50.5 (CH 5′), 52.4 and 53.0 (CH 4′), 57.7 and 58.0 (CH 2′), 67.5 and 67.8 (Fmoc CH_2), 120.6 (Fmoc Ar CH), 126.2 (A C5), 125.8 (Fmoc Ar CH), 126.2 (Bz CH),127.6 (Bz CH), 128.2 (Fmoc Ar CH), 128.9 (Fmoc Ar CH) 132.9 (Bz C) 133.6 (Bz CH), 141.1 (A C8H and Fmoc Ar C), 143.7 (Fmoc Ar C), 150.7 (A C4), 151.8 (A C6), 152.9 (A C2H), 154.3 (Fmoc CO), 166.0 (Bz CO), 173.0 and 173.5 (Pro CO); HRMS (FAB^+) Calcd for $C_{32}H_{26}O_5N_6$ ($M{\cdot}H^+$) 575.2043, Found 575.2041, $[\alpha]^{25}_D$ = -8.52 (c = 1.0 g/100 mL, DMF), mp = 200 °C (dec.).

(*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^4-benzoyladenin-9-yl)-D-proline pentafluorophenyl ester (28)



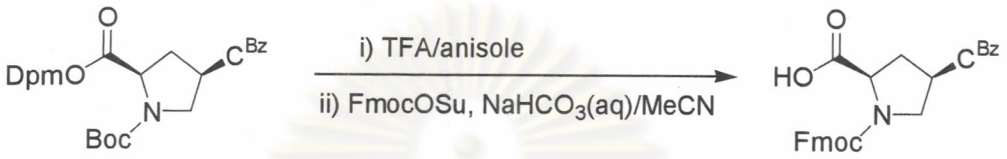
(**27**) (**28**)

Synthesis of the title compound (**28**) was accomplished in the same way as described for compound (**26**) above. Starting from (*N*-fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^4-benzoyladenin-9-yl)-D-proline (**27**) (0.25 g, 0.43 mmol), pentafluorophenol (1.5 equiv) and EDC.HCl (1.5 equiv) in dichloromethane (2 mL) afforded (**28**) (0.26 g, 81 %), as a white solid.

^{1}H NMR (400 MHz; $CDCl_3$): δ 2.86 and 2.92 (2×m, 1H; 1×CH_2 3′ rotamers), 3.14 (2×m, 1H; 1×CH_2 3′ rotamers), 4.04 and 4.15 (2×m, 1H; 1×CH_2 5′ rotamers), 4.20-4.35 (m, 2H; Fmoc CH and 1×CH_2 5′ rotamers), 4.50 and 4.60 (m, 2H; Fmoc CH2 rotamers), 4.77 and 4.86 (2×m, 1H; CH 2′ rotamers), 5.29 (m, 1H; CH 4′), 7.28 (2H, m, Fmoc CH), 7.37 (m, 2H; Fmoc CH), 7.46 (m, 2H; Bz CH), 7.56 (m, 3H; Fmoc and Bz CH), 7.73 (d, ^3J(H,H) = 7.0 Hz, 2H; Fmoc CH), 8.00 (m, 2H; Bz CH), 8.23 and 8.31 (2×s, 1H; A CH), 8.76 (s, 1H; A CH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 34.6 and 35.9 (CH_2 3′), 47.1 (Fmoc CH), 49.8 and 50.2 (CH 5′), 52.6 and 53.3 (CH 4′), 56.9 and 57.2 (CH 2′), 68.1 and 68.6 (Fmoc CH_2), 120.0 (Fmoc Ar CH), 122.3 (A C5), 124.8 (Fmoc Ar CH), 127.1 (Bz CH), 127.8 (Bz CH), 128.0 (Fmoc Ar CH), 128.8 (Fmoc Ar CH) 133.0 and 133.1 (Bz C/CH), 136.5-142.0 (Pfp C), 141.3 (A C8H and

Fmoc Ar C), 143.4 (Fmoc Ar C), 149.5 (A C4), 151.7 (A C6), 152.4 (A C2H), 153.9 and 154.4 (Fmoc CO), 165.1 (Bz CO), 167.5 and 167.7 (Pro CO); HRMS (FAB^+) Calcd for $C_{38}H_{26}O_5N_6F_5$ ($M{\cdot}H^+$) 741.1885, Found 741.1898, $[\alpha]^{25}_D$ = -18.76 (c = 1.0 g/100 mL, $CHCl_3$), mp = 110 °C (dec.).

(*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^4-benzoylcytosin-1-yl)-D-proline (29)

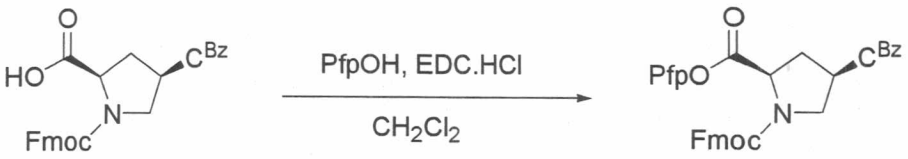


(23) **(29)**

Synthesis of the title compound (**29**) was accomplished in the same way as described for compound (**25**) above. Starting from (*N-tert*-butyloxycarbonyl)-*cis*-4-(N^4-benzoylcytosin-9-yl)-D-proline diphenylmethyl ester (**23**) (0.29 g, 0.49 mmol), and trifluoroacetic acid containing 10% anisole (2 mL) followed by FmocOSu (0.19 g, 0.54 mmol) and $NaHCO_3$ (3 equiv excess) in 1:1 H_2O:MeCN (5 mL/mmol) afforded (**29**) (0.22 g, 76 %), as a white solid.

1H NMR (400 MHz; $CDCl_3$): δ 2.28 and 2.34 (m, 1H; 1×CH_2 3′ rotamers), 2.75 and 2.84 (m, 1H; 1×CH_2 3′ rotamers), 3.67 (m, 3H; Fmoc CH and CH_2 5′ rotamers), 4.40-4.60 (m, 2H; Fmoc CH), 4.78 (m, 1H; CH 2′), 5.26 (m, 1H; CH 4′), 736 (m, 3H; C C5H and Fmoc CH), 7.42 (m, 2H; Fmoc CH), 7.53 (m, 2H; Bz CH), 7.66 (m, 3H; Fmoc Ar CH and Bz CH), 8.03 (m, 2H; Fmoc CH), 8.14 (m, 3H; Bz CH and C C6H); ^{13}C NMR (100 MHz; $CDCl_3$): δ 33.5 and 33.6 (CH_2 3′), 47.1 (Fmoc CH), 49.2 and 49.8 (CH 5′), 55.5 and 56.1 (CH 4′), 57.7 and 58.0 (CH 2′), 67.4 and 67.7 (Fmoc CH_2), 96.6 (C C5H), 120.6 (Fmoc Ar CH), 125.7 (Fmoc Ar CH), 128.2 (Bz CH), 128.8 (Bz CH), 125.7 (Fmoc Ar CH), 127.6 (Fmoc Ar CH), 133.1 (Bz C), 133.6 (Bz CH), 141.1 (Fmoc Ar C), 144.2 (Fmoc Ar C), 147.9 (C C6H), 154.4 (Fmoc CO), 155.4 (C C2), 163.1 (C C4), 167.9 (Bz CO), 173.2 and 173.8 (Pro CO); HRMS (FAB^+) Calcd for $C_{31}H_{26}O_6N_4$ ($M{\cdot}H^+$) 551.1910, Found 551.1938, $[\alpha]^{25}_D$ = -14.51 (c = 1.0 g/100 mL, DMF), mp = 159 °C (dec)

(*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^4-benzoylcytosin-1-yl)-D-proline pentafluorophenyl ester (30)

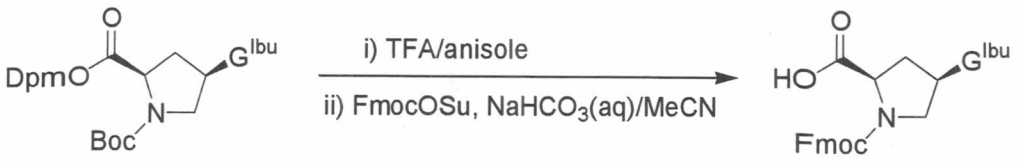


(29) **(30)**

Synthesis of the title compound (**30**) was accomplished in the same way as described for compound (**26**) above. Starting from (*N*-fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^4-benzoylcytosin-9-yl)-D-proline (**29**) (0.25 g, 0.45 mmol), pentafluorophenol (1.5 equiv) and EDC.HCl (1.5 equiv) in dichloromethane (2 mL) afforded (**30**) (0.24 g, 76 %), as a white solid.

1H NMR (400 MHz; $CDCl_3$): δ 2.49 and 2.54 (m, 1H; 1×CH_2 3′ rotamers), 2.99 and 3.08 (m, 1H; 1×CH_2 3′ rotamers), 3.69-4.22 (m, 3H; Fmoc CH and CH_2 5′ rotamers), 4.40-4.60 (m, 2H; Fmoc CH), 4.78 (m, 1H; CH 2′), 5.26 (m, 1H; CH 4′), 7.29 (m, 3H; C C5H and Fmoc CH), 7.38 (m, 2H; Fmoc CH), 7.45 (m, 2H; Bz CH), 7.56 (m, 3H; Fmoc Ar CH and Bz CH), 7.73 (m, 2H; Fmoc CH), 7.90 (m, 3H; Bz CH and C C6H); ^{13}C NMR (100 MHz; $CDCl_3$): δ 34.8 and 36.2 (CH_2 3′), 47.1 (Fmoc CH), 49.3 and 49.6 (CH 5′), 55.2 and 55.8 (CH 4′), 56.9 and 57.3 (CH 2′), 67.9 and 68.5 (Fmoc CH_2), 97.1 and 97.3 (C C5H), 120.0 (Fmoc Ar CH), 124.8 (Fmoc Ar CH), 127.2 (Bz CH), 127.8 (Bz CH), 127.9 (Fmoc Ar CH), 128.9 (Fmoc Ar CH), 132.7 (Bz C), 133.3 (Bz CH), 136.6-142.0 (Pfp C), 141.3 (Fmoc Ar C), 143.5 (Fmoc Ar C), 145.7 and 145.7 (C C6H), 154.2 and 154.4 (Fmoc CO), 155.2 (C C2), 162.4 (C C4), 166.8 (Bz CO), 167.9 and 168.0 (Pro CO); HRMS (FAB^+) Calcd for $C_{37}H_{26}O_6N_4F_5$ ($M{\cdot}H^+$) 717.1773, Found 717.1758, $[\alpha]^{25}_D$ = -2.13 (c = 0.5 g/100 mL, $CHCl_3$), mp = 114-115 °C.

(*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^2-isobutyrylguanin-9-yl)-D-proline (31)

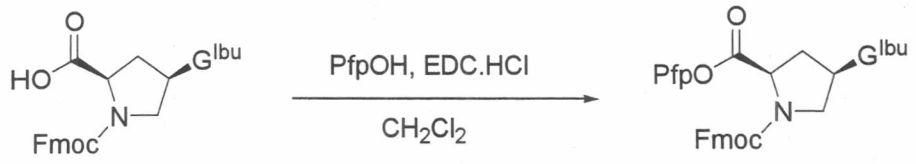


(24) **(31)**

Synthesis of the title compound (**31**) was accomplished in the same way as described for compound (**25**) above. Starting from (*N*-*tert*-butyloxycarbonyl)-*cis*-4-(N^2-isobutylguanin-9-yl)-D-proline diphenylmethyl ester (**24**) (0.30 g, 0.49 mmol) and trifluoroacetic acid containing 10% anisole (2 mL) followed by FmocOSu (1.19 g, 0.54 mmol) and $NaHCO_3$ (3 equiv excess) in 1:1 H_2O:MeCN (5 mL/mmol) afforded (**31**) (0.13 g, 49 %) as a white solid.

^{1}H NMR (400 MHz; $CDCl_3$): δ 1.14 (d ^3J(H,H) = 6.2 Hz, 6H; Ibu CH_3), 2.40 and 2.50 (m, 2H; CH_2 3′), 2.77 (m, 1H; Ibu CH), 2.86 and 2.96 (m, 2H; CH_2 3′), 3.74 and 3.86 (m, 2H; CH_2 5′), 4.06 (m, 1H; Fmoc CH), 4.46 (m, 2H; Fmoc CH_2), 4.50 (m, 1H; CH 2′),.4.93 (m, 1H; CH 4′), 7.23 (m, 2H; Fmoc Ar CH), 7.33 (m, 2H; Fmoc Ar CH), 7.52 (m, 2H; Fmoc Ar CH), 7.68 (m, 2H; Fmoc Ar CH), 8.00 and 8.07 (2×s, 1H; G H8), 10.01 and 10.25 (2×s, 1H; G NH), 12.27 (s, 1H; G NH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 19.3 (Ibu CH_3), 34.8 and 35.6 (CH_2 3′), 35.1 (CH Ibu), 47.0 (Fmoc CH), 50.5 and 51.1 (CH 5'), 52.1 and 52.9 (CH 4′), 57.7 and 58.2 (CH 2′), 67.4 and 67.7 (Fmoc CH_2), 120.5 (G C5), 120.2 (Fmoc Ar CH), 125.8 (Fmoc Ar CH), 127.6 (Fmoc Ar CH), 128.1 (Fmoc Ar CH), 138.3 (G C8), 141.0 and 144.1 (Fmoc Ar C), 148.2 (G C6), 149.1 (G C2), 154.4 (Fmoc CO), 155.3 (G C4), 173.0 and 173.6 (Pro CO), 180.6 (Ibu CO); HRMS (FAB$^+$) Calcd for $C_{29}H_{28}O_6N_6$ (M·H$^+$) 557.2149, Found 557.2149, $[\alpha]^{25}_D$ = -65.96 (c = 0.6 g/100 mL, DMF), mp = 184 °C (dec.).

(*N*-Fluoren-9-ylmethoxycarbonylamino)-*cis*-4-(N^2-isobutyrylguanin-9-yl)-D-proline pentafluorophenyl ester (32)



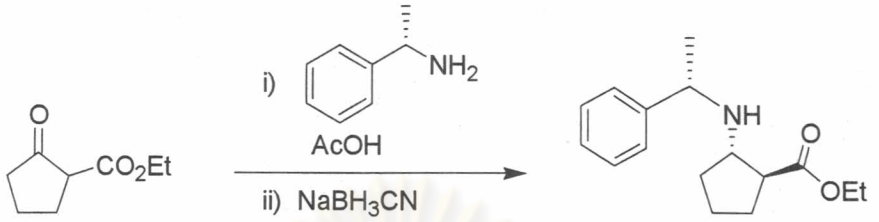
(**31**) (**32**)

Synthesis of the title compound (**32**) was accomplished in the same way as described for compound (**26**) above. Starting from (*N*-fluoren-9-ylmethoxycarbonyl)-*cis*-4-(N^2-isobutyrylguanin-9-yl)-D-proline (**31**) (0.09 g, 0.17 mmol), pentafluorophenol (1.5 equiv) and EDC.HCl (1.5 equiv) in dichloromethane (2 mL) afforded (**32**) (0.08 g, 66 %), as a white solid.

^{1}H NMR (400 MHz; $CDCl_3$): δ 1.20 (d ^3J(H,H) = 6.2 Hz, 6H; Ibu CH_3), 2.80 and 3.12 (m, 2H; CH_2 3′), 2.81 (m, 1H; Ibu CH), 4.00 and 4.20 (m, 2H; CH_2 5′), 4.20 (m, 1H; Fmoc CH), 4.40 (m, 2H; Fmoc CH_2), 4.79 (m, 1H; CH 2′), 5.15 (m, 1H; CH 4′), 7.23 (m, 2H; Fmoc Ar CH), 7.33 (m, 2H; Fmoc Ar CH), 7.52 (m, 2H; Fmoc Ar CH), 7.68 (m, 2H; Fmoc Ar CH), 8.00 and 8.07 (2×s, 1H; G H8), 10.01 and 10.25 (2×s, 1H; G NH), 12.27 (s, 1H; G NH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 18.9 (Ibu CH_3), 34.5 and 35.5 (CH_2 3′), 36.1 (CH Ibu), 47.0 (Fmoc CH), 49.9 (CH 5′), 52.8 and 53.3 (CH 4′), 56.9 and 57.4 (CH 2′), 68.2 and 68.5 (Fmoc CH_2), 119.7 (G C5), 120.0 (Fmoc Ar CH), 124.9 (Fmoc Ar CH), 127.1 (Fmoc Ar CH), 127.8 (Fmoc Ar CH), 136.4-142.1 (Pfp C), 137.5 and 137.8 (G C8), 141.2 (Fmoc Ar C), 143.5 (Fmoc Ar C), 148.2 and 148.3 (G C6), 148.5 (G C2), 153.8 and 154.4 (Fmoc CO), 155.0 and 155.1 (G C4), 167.8 and 168.0 (Pro CO), 179.7 and 179.9 (Ibu CO); HRMS (FAB$^+$) Calcd for $C_{35}H_{28}O_6N_6F_5$ ($M{\cdot}H^+$) 723.1991, Found 723.2001, $[\alpha]^{25}_D$ = -10.81 (c = 0.5 g/100 mL, $CHCl_3$), mp = 140 °C (dec.).

2.2.3 Synthesis of *trans*-(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid (ACPC) spacer

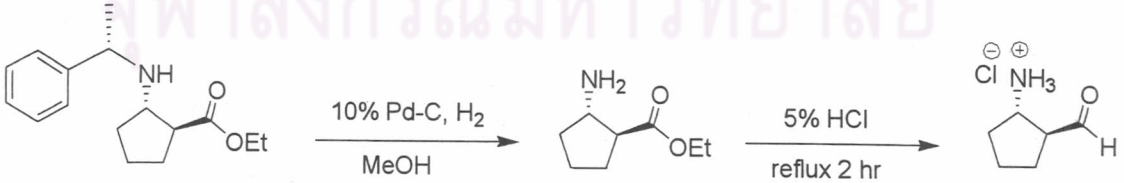
Ethyl (1*S*,2*S*)-2-[(1′*S*)-phenylethyl]-aminocyclopentane carboxylate (34) [62]



(33) **(34)**

To a stirred solution of ethyl cyclopentanone-2-carboxylate (**33**) (3.75 mL, 25.8 mmol) in absolute ethanol (30 mL) was added (*S*)-(-)-α-methylbenzylamine (3.82 mL, 30.0 mmol) and glacial acetic acid (3 mL, 32.0 mmol). The reaction mixture was stirred at 30 °C until the formation of enamine was complete (2 h, monitored by TLC, 3:1 hexane/EtOAc, R_f = 0.60) The reaction mixture was heated to 72 °C and sodium cyanoborohydride (3.24 g, 52.0 mmol) was then added to the reaction mixture in five portions over a 5 h period. The disapprarance of enamine was monitored by TLC. When the reaction was complete, water (40 mL) was added, and the ethanol was removed by rotary evaporation. The resulting mixture was extracted with diethyl ether. The diethyl ether was removed by rotary evaporation. The crude product was purified by flash column chromatography eluting with 10:1 hexane:EtOAc on silica gel to obtain the title compound (**34**) as colorless liquid (1.40 g, 21 %).

(1*S*,2*S*)-2-Aminocyclopentane carboxylic acid hydrochloride (36) [62]

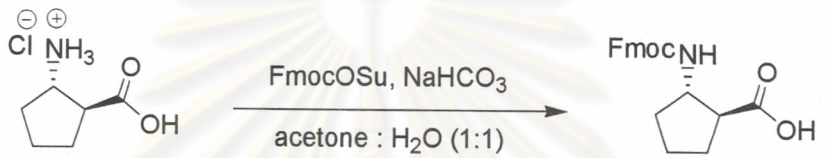


(34) **(35)** **(36)**

Ethyl (1*S*,2*S*)-2-[(1′*S*)-phenylethyl]-aminocyclopentane carboxylate (**34**) (1.03 g, 3.95 mmol) was dissolved in methanol (5 mL) and palladium on charcoal (0.10 g) was added with stirring at 30 °C under H_2. TLC monitoring indicated

disappearance of compound (**34**) and formation of ethyl (1*S*,2*S*)-2-aminocyclopentane carboxylate (**35**) were completed. The palladium on charcoal was filtered off with the aid of celite and washed with methanol. The filtrate was evaporated by rotary evaporation to obtain compound (**35**). Next, a mixture of compound (**35**) and 5% HCl (20 mL) was refluxed for 2 h. Then the mixture was allowed to cool at 30 °C. The solvent was removed by rotary evaporation to obtain the title compound (**36**) as a white solid (0.39 g, 64% from **34**).

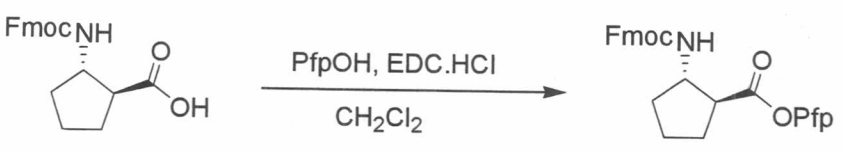
(1*S*,2*S*)-2-(*N*-Fluoren-9-ylmethoxycarbonyl)-aminocyclopentanecarboxylic acid (37) [62]



(**36**) (**37**)

The (1*S*,2*S*)-2-aminocyclopentane carboxylic acid hydrochloride (**36**) (0.39 g, 2.54 mmol) was dissolved in acetone: water (1:1, 6 mL) and $NaHCO_3$ (3 equiv excess) was added until the solution was basic (pH = 8). FmocOSu (0.91 g, 2.54 mmol) was slowly added with stirring at 30 °C for 8 h. The acetone was removed by rotary evaporation under reduced pressure. The residue was dilute with water (20 mL) and extracted with diethyl ether (3 × 20 mL). The pH was adjusted to 2 with concentrated HCl in aqueous phase. The white solid precipitate was extracted with dichloromethane (3 × 20 mL). Solvent was removed by rotary evaporation and dried in vacuum to afford the title compound (**37**) as a white solid (0.56 g, 63 %), $[\alpha]^{25}_{D}$ = +36.40 (c = 1.0 g/100 mL, MeOH).

(1*S*,2*S*)-2-(*N*-Fluoren-9-ylmethoxycarbonyl)-aminocyclopentane pentafluorophenyl ester (38)



(**37**) (**38**)

A suspension of (1*S*,2*S*)-2-(*N*-fluoren-9-ylmethoxycarbonyl)-aminocyclo pentanecarboxylic acid (**37**) (0.30 mg, 0.85 mmol) and pentafluorophenol (1.5 equiv) in dichloromethane (3 mL) was added EDC.HCl (1.5 equiv). The resulted mixture was stirred at 30 °C for one hour. The reaction was completed as indicated by TLC analysis and purified by flash column chromatography eluting with hexane: ethyl acetate (5:1) on silica gel to obtain the title compound (**38**) as a white solid (0.34 g, 78 %).

^{1}H NMR (400 MHz; $CDCl_3$): δ 1.68 (m, 1H; ring CH), 1.87 (m, 2H; ring CH), 2.10 (m, 1H; ring CH), 2.23 (m, 2H; ring CH), 3.11 (m, 1H; CHCO), 4.26 (t, ^3J(H,H) = 6.8 Hz, 1H; Fmoc CH), 4.38 (m, 1H; C<u>H</u>NH), 4.48 (d, ^3J(H,H) = 6.8 Hz, 2H; Fmoc CH_2), 4.98 (d ^3J(H,H) = 5.6 Hz, 1H; NH), 7.34 (m, 2H; Fmoc Ar CH), 7.42 (m, 2H; Fmoc Ar CH), 7.62 (d, ^3J(H,H) = 7.2 Hz, 2H; Fmoc Ar CH), 7.80 (d ^3J(H,H) = 7.2 Hz, 2H; Fmoc Ar CH); ^{13}C NMR (100 MHz; $CDCl_3$): δ 23.2 (ring CH_2), 28.9 (ring CH_2), 32.8 (ring CH_2), 47.2 (<u>C</u>HCO), 49.8 (Fmoc CH), 56.4 (CHNH), 66.6 (Fmoc CH_2), 120.0 (Fmoc Ar CH), 125.0 (Fmoc Ar CH), 127.1 (Fmoc Ar CH), 127.7 (Fmoc Ar CH), 136.0-141.2 (Pfp C), 141.3 (Fmoc Ar C), 143.8 (Fmoc Ar C), 155.7 (Fmoc CO), 170.8 (Pro CO); Anal Calcd. for $C_{27}H_{10}NO_4F_5$ C, 62.67; H, 3.90; N, 2.71 %, Found C, 62.93; H, 3.86; N, 2.75 %, $[\alpha]^{25}_D$ = +51.44 (c = 1.0 g/100 mL, $CHCl_3$).

2.3 Oligomerization of *cis*-D-*ss*ACPC PNA (12)

2.3.1 Preparation of the reaction pipette and apparatus for solid phase synthesis

All peptide syntheses were carried out using a custom-made peptide synthesis column from Pasteur pipette with fritted glass as described below. A new glass Pasteur pipette was plugged with a small amount of glass powder and sintered on a small flame. The length of the sintered glass should be about 3-5 mm. The resin was weighed accurately into the pipette and the pipette was equipped with a rubber teat. The resin in the pipette should be swollen in the required solvent al least 1 h before use. For each reactions, the reagent was directly sucked in, ejected out or hold on by manual control for the specified period of time. Occasional agitation may be performed using this device under manual control. All washing could be done by

filling the solvent via the top of the pipette. The excess solvent was ejected out by squeezing the rubber teat as shown in **Figure 2.1**.

Figure 2.1 A diagram showing the manual technique for solid phase peptide synthesis; (**a**) coupling, deprotection and cleaving process; (**b**) washing process

2.3.2 Solid phase peptide synthesis of T_5 (12c)

Synthesis of this PNA T_5 (**12c**) was carried out on 1.0 μmol scale. The synthesis was divided step as follows.

i Removing Fmoc protecting group form the resin

A reaction pipette prepared as described above was loaded with containing TentaGel S RAM Fmoc resin (4.2 mg, 1.0 μmol). The resin was treated 20% piperidine in DMF (piperidine 50 μL and DMF 200 μL) in a 1.5 mL eppendorf tube at 30 °C for 15 min occasional agitation. After the specified period of time, the reagent was squeezed off and the reaction column was washed exhaustively with DMF.

ii Anchoring with the first amino acid (Lys) residue

Fmoc-L-Lys(Boc)-OPfp (6.3 mg, 10 μmol) and HOAt (2.0 mg, 10 μmol) were dissolved in anhydrous DMF (30 μL) in a 1.5 mL eppendorf tube. Then DIEA (3.4 μL, 20 μmol) was added in this mixture. The prepared resin was soaked in this solution with occasional agitation at 30 °C for 1 h. After the specified period of time, the reagent was squeezed off and the reaction column was washed exhaustively with DMF.

iii Deprotection of the Fmoc protection group at N-terminal

After the coupling was completed, the resin was treated with 20% piperidine in DMF (piperidine 50 μL and DMF 200 μL) in a 1.5 mL eppendorf tube at 30 °C for 15 min occasional agitation. After the specified period of time, the reagent was squeezed off and the reaction column was washed exhaustively with DMF. The used deprotecting reagent can be used to determine the coupling efficiency by diluting with an appropriate volume of methanol and then the UV-absorbance of dibenzofulvene-piperidine adduct at 264 nm measured. The first UV-absorbance of the adduct, released from preloaded Fmoc-L-Lys(Boc)-resin, was assumed to be 100%. Such determination of coupling efficiency was advantageous in terms of determining how the solid phase reaction progress. The efficiency should be >95 % for each step in order to give acceptable yield of the pentamer PNA (**12**) from the synthesis. If the overall efficiency had dropped bellows 50 %, the coupling must be stopped to save the valuable monomers.

iv Coulping with pyrrolidinyl monomer

The free amino group, generated from the deprotection step (**iii**) above, was further coupled with pyrrolidine T monomer. Fmoc-D-Pro-(*cis*-4-T)-OPfp (**26**) (2.5 mg, 4.0 μmol), HOAt (0.5 mg, 4.0 μmol) and DIEA (1.4 μL, 8 μmol) were dissolved in 30 μL anhydrous DMF. The reaction pipette was treated with this solution at 30 °C for 30 min with occasional agitation. After the specified period of time, the reagent was squeezed off and the reaction column was washed exhaustively with DMF.

v Coulping with *SS*-ACPC spacer monomer

In the same way as described for **iv**, *ss*ACPC-spacer monomer (**38**) (2.0 mg, 4 μmol) was coupled next to pyrrolidinyl T monomer. This constituted one unit of *cis*-D-*ss*ACPC PNA. Alternate couplings of the pyrrolidinyl monomer and *ss*ACPC spacer were performed in the subsequent steps until the complete sequence was obtained.

vi End capping

After anchoring or coupling step, the free amino residue was capped with 10% lauroyl chloride/DIEA in anhydrous DMF (lauroyl chloride 5 μL, DIEA 5 μL and DMF 40 μL) in a 1.5 mL eppendorf tube to prevent formation of deletion sequences. The reaction pipette was occasionally agitated with this solution at 30 °C for 10 min. After the specified period of time, the reagent was squeezed off and the reaction column was washed exhaustively with DMF.

After that, the next cycle (deprotection, coupling and capping) were carried out with the same method until the resin bound peptide had been extended up to pentamer.

vii Acetylation at N-terminal of pentamer PNA (12c)

The synthesis cycle was repeated until the growing peptide chain was extended up to pentamer. After final cleavage of Fmoc, the pentamer PNA (**12c**) was treated with 10% Ac_2O/DIEA in anhydrous DMF (Ac_2O 5 μL, DIEA 5 μL and DMF 40 μL) in a 1.5 mL eppendorf tube. The reaction pipette was occasionally agitated with this solution at 30 °C for 15 min. After the specified period of time, the reagent was squeezed off and the reaction column was washed exhaustively with DMF.

viii Method for cleavage the pentamer PNA (12c) from the resin

The resin bound PNA pentamer was released from the resin by treatment with trifluoroacetic acid (0.5 mL) at 30 °C for 2 h with occasional agitation. During the

time, the resin becomes red. After the specified period of time, the trifluoroacetic acid was removed by a nitrogen stream (fume hood).The cleavage was repeated again with another 0.5 mL of fresh TFA to ensure a complete cleavage of the peptide from the resin. The sticky residue was treated with diethyl ether to precipitate the crude PNA. The suspension was the centrifuged and decanted. The crude peptide was centrifugally washed with diethyl ether 3 times. Finally the crude peptide was air dried at 30 °C and stored dried at
-20 °C until used.

ix Purification and Identification

The crude peptide was prepared for HPLC analysis by dissolving a mixture in 200 μL deionized water. The solution was filtered through a nylon membrane filter (0.45 μm). Analysis and purification was performed by reverse phase HPLC, monitoring by UV-absorbance at 260 nm and eluting with a gradient system of 0.01% TFA in acetonitrile/water. Conditions for HPLC gradient system;
solvent A = 0.01% trifluoroacetic acid in acetonitrile
solvent B = 0.01% trifluoroacetic acid in milliQ water
First A:B (10:90) for 5 min then linear gradient to A:B (90:10) over a period of 25 min then hold on for 5 min before revert back to A:B (10:90).

After purification by HPLC, the peak of T_5(**12c**) appeared at t_R = 18.8 min. The major product was collected and confirmed by MALDI-TOF mass spectrometry. MALDI-TOF $M{\cdot}H^+_{obs}$ = 1848.306; $M{\cdot}H^+_{calcd}$ = 1848.480.

2.3.3 Solid phase peptide synthesis of T_9 (12f)

Synthesis of T_9 (**12f**) was accomplished in the same way as described for T_5 (**12c**) above. After cleavage from resin and purification by reverse phase HPLC, the chromatogram of appeared at t_R = 27.24 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ = 1848.306; $M{\cdot}H^+_{calcd}$ = 1848.480.

2.3.4 Solid phase peptide synthesis of T_4AT_4 (12g), T_4GT_4 (12h), T_4CT_4 (12i)

Synthesis of T_4AT_4 (**12g**), T_4GT_4 (**12h**), T_4CT_4 (**12i**) were accomplished in the same way as described for T_5 (**12c**) above. Starting from TentaGel S RAM Fmoc resin (8.4 mg, 2 μmol) and monomers, Fmoc-T-Pfp (**26**) (5.1 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2 μmol), Fmoc-T-Pfp (**26**) (5.1 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2 μmol), Fmoc-T-Pfp (**26**) (5.1 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2 μmol), Fmoc-T-Pfp (**26**) (5.1 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2 μmol) were used in each coupling cycle respectively until a T_4 sequence was obtained. The resin was split to three parts and then further coupled with Fmoc-A-Pfp (**28**) (2.0mg, 0.7 μmol), Fmoc-C-Pfp (**30**) (2.0 mg, 0.7 μmol) and Fmoc-G-Pfp (**32**) (2.0 mg, 0.7 μmol) in each column separately. Then the coupling was continued with monomer Fmoc ACPC before Fmoc-T-Pfp (**26**) (1.7 mg, 0.7 μmol), alternately until the peptide had been extended up to nonamer. Final Fmoc removed and acetylation was perform as usual. In case of mixed-base, before cleavage T_4AT_4 (**12g**), T_4GT_4 (**12h**) and T_4CT_4 (**12i**) from resin, the nucleobase protecting groups (Bz for A and C, Ibu for G) must be removed by treatment of the resin with aqueous ammonia/dioxane 1:1 at 55 °C for 6 h.

After purification by HPLC, the peak of T_4AT_4 (**12g**), T_4GT_4 (**12h**) and T_4CT_4 (**12i**) appeared at t_R = 27.31, 27.48 and 27.33 min, respectively. . MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ = 3186.709, 3205.015and 3161.131; $M{\cdot}H^+_{calcd}$ = 3186.483., 3202.478 and 3162.472 respectively.

2.3.5 Solid phase peptide synthesis of A_5 (12d) and A_9 (12k)

The synthesis of A_5 (**12d**) and A_9 (**12k**) was accomplished in the same way as described for T_5 (**12c**) above. Starting from TentaGel S RAM Fmoc resin (4.2 mg, 1 μmol) and monomer, Fmoc-A^{Bz}-Pfp (**28**) (3.0 mg, 1 μmol) and Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol) was alternated in coupling step until the peptide had been extended up to pentamer. The resin was split to two pipettes. The first pipette was further coupling until the peptide had been extended up to nonamer. The second pipette was already the pentamer. Before cleavage both A_5 (**12d**) and A_9 (**12k**) from resin, the nucleobase protecting group was removed as described above.

In case of purification, the HPLC peak of A_5 (**12d**) and A_9 (**12k**) appeared at t_R = 21.63 and 22.28 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ = 1893.414, and 3258.678; $M{\cdot}H^+_{calcd}$ = 1893.938, and 3258.577 respectively.

2.3.6 Solid phase peptide synthesis of A_4TA_4 (12j), A_4GA_4 (12l) and A_4CA_4 (12m)

The synthesis of A_4TA_4 (**12j**), A_4GA_4 (**12l**) and A_4CA_4 (**12m**) were accomplished in the same way as described for T_5 (**12c**) above. Starting from TentaGel S RAM Fmoc resin (8.4 mg, 2 μmol) and monomer, Fmoc-A-Pfp (**28**) (6.0 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2μmol) was alternated in coupling step until the peptide had been extended up to tetramer. The resin was split to three pipette and then further coupled with Fmoc-T-Pfp (**26**) (1.7 mg, 0.7 μmol), Fmoc-C-Pfp (**30**) (2.0 mg, 0.7 μmol) and Fmoc-G-Pfp (**32**) (2.0 mg, 0.7 μmol) in each column separately. Then the coupling was continued with monomer Fmoc-A-Pfp (**28**) (2.0 mg, 0.7 μmol), Fmoc-ACPC-Pfp (**38**) (1.4 mg, 0.7 μmol) was alternately until the peptide had been extended up to nonamer. Final Fmoc removal and acetylation was perform as usual.

After purification by HPLC, the peak of A_4TA_4 (**12j**), A_4GA_4 (**12l**) and A_4CA_4 (**12m**) appeared at t_R = 22.35, 22.22, and 22.17 min respectively. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ = 3249.666, 3274.822 and 3234.840; $M{\cdot}H^+_{calcd}$ = 3249.565, 3274.572 and 3234.566 respectively.

2.3.7 Solid phase peptide synthesis of TCACTACTA (12n), TCACAACTA (12o), TCACGACTA (12p) and TCACCACTA (12q)

The synthesis of TCACTACTA (**12n**), TCACAACTA (**12o**), TCACGACTA (**12p**) and TCACCACTA (**12q**) were accomplished in the same way as described for T_5 (**12c**) above. Starting from TentaGel S RAM Fmoc resin (8.4 mg, 2 μmol) and monomer, Fmoc-A-Pfp (**28**) (6.0 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2μmol), Fmoc-T-Pfp (**26**) (5.1 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2 μmol), Fmoc-C-Pfp (**30**) (6.0 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2 μmol), Fmoc-A-Pfp (**28**) (5.1 mg, 2 μmol), Fmoc-ACPC-Pfp (**38**) (4.2 mg, 2 μmol) were used in each coupling cycle respectively. The resin was split to four and then further coupled with

Fmoc-T-Pfp (**26**) (1.3 mg, 0.5 μmol), Fmoc-A-Pfp (**28**) (1.5 mg, 0.5 μmol), Fmoc-C-Pfp (**30**) (1.5 mg, 0.5 μmol) and Fmoc-G-Pfp (**32**) (1.5 mg, 0.5 μmol) in each column separately. Then the coupling was continued with monomer Fmoc-C-Pfp (**26**) (1.5 mg, 0.5 μmol), Fmoc-ACPC-Pfp (**38**) (1.1 mg, 0.5 μmol), Fmoc-A-Pfp (**28**) (1.5 mg, 0.5 μmol), Fmoc-ACPC-Pfp (**38**) (1.1 mg, 0.5 μmol), Fmoc-C-Pfp (**30**) (1.5 mg, 0.5 μmol), Fmoc-ACPC-Pfp (**38**) (1.1 mg, 0.5 μmol), Fmoc-T-Pfp (**26**) (1.5 mg, 0.5 μmol) and Fmoc-ACPC-Pfp (**38**) (1.1 mg, 0.5 μmol) were used in each coupling cycle respectively until the peptide had been extended up to nonamer in column separately.

After purification by HPLC, the peak of TCACTACTA (**12n**), TCACAACTA (**12o**), TCACGACTA (**12p**) and TCACCACTA (**12q**) appeared at t_R = 19.27, 18.03, 17.91, and 17.14 min respectively. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ = 3159.468, 3167.392, 3184.388 and 3143.355; $M{\cdot}H^+_{calcd}$ = 3159.508, 3168.520, 3184.515 and 3144.508 respectively.

2.3.8 Solid phase peptide synthesis of GTAGATCACT (12r)

The synthesis of GTAGATCACT (**12r**) was accomplished in the same way as described for T_5 (**12d**) above. Starting from TentaGel S RAM Fmoc resin (4.2 mg, 1 μmol) and monomer, Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp **(38)** (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol) were used in each coupling cycle respectively

After purification by HPLC, the peak of GTAGATCACT (**12r**) appeared at t_R = 19.71 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ = 3555.940; $M{\cdot}H^+_{calcd}$ = 3556.669.

2.3.9 Solid phase peptide synthesis of AGTGATCTAC (12s)

The synthesis of AGTGATCTAC (**12s**) was accomplished in the same way as described for T_5 (**12d**) above. Starting from TentaGel S RAM Fmoc resin (4.2 mg, 1 μmol) and monomer, Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp **(38)** (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol) were used in each coupling cycle respectively.

After purification by HPLC, the HPLC peak of AGTGATCTAC (**12s**) appeared at t_R = 22.34 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ = 3556.003; $M{\cdot}H^+_{calcd}$ = 3556.669.

2.3.10 Solid phase peptide synthesis of CATCTAGTGA (12t)

The synthesis of CATCTAGTGA (**12t**) was accomplished in the same way as described for T_5 (**12d**) above. Starting from TentaGel S RAM Fmoc resin (4.2 mg, 1 μmol) and monomer, Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol) were used in each coupling cycle respectively.

After purification by HPLC, the HPLC peak of CATCTAGTGA (**12t**) appeared at t_R = 19.04 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ 3556.463; $M{\cdot}H^+_{calcd}$ = 3565.669.

2.3.11 Solid phase peptide synthesis of GACATGACAT (12u)

The synthesis of GACATGACAT (**12u**) was accomplished in the same way as described for T_5 (**12d**) above. Starting from TentaGel S RAM Fmoc resin (4.2 mg, 1 μmol) and monomer, Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**28**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol) were used in each coupling cycle respectively.

After purification by HPLC, the HPLC peak of GACATGACAT (**12u**) appeared at t_R = 17.81 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ 3565.172; $M{\cdot}H^+_{calcd}$ = 3565.680.

2.3.12 Solid phase peptide synthesis of TATGTACTAT (12v)

The synthesis of TATGTACTAT (**12v**) was accomplished in the same way as described for T_5 (**12d**) above. Starting from TentaGel S RAM Fmoc resin (4.2 mg, 1 μmol) and monomer, Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-

ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol) were added in each coupling cycle respectively. Before cleavage TATGTACTAT (**12v**) from resin, It must be deprotected nucleobase protecting group (Bz, Ibu) treatment of the resin with aqueous ammonia/dioxane 1:1 at 55 °C for 6 h. In case of purification, the HPLC peak of TATGTACTAT (**12v**) appeared at t_R = 27.37 min.

After purification by HPLC, the HPLC peak of TATGTACTAT (**12v**) appeared at t_R = 27.37 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ 3546.949; $M{\cdot}H^+_{calcd}$ = 3546.662.

2.3.13 Solid phase peptide synthesis of GCTACGTCGC (12w)

The synthesis of GCTACGTCGC (**12w**) was accomplished in the same way as described for T_5 (**12d**) above but the first lysine attachment was skipped. Starting from TentaGel S RAM Fmoc resin (4.2 mg, 1 μmol) and monomer, Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**29**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol) were added in each coupling cycle respectively. Fmoc-L-Lysine was coupled after the last spacer was introduced followed by Fmoc removal and acetylation.

After purification by HPLC, the HPLC peak of GCTACGTCGC (**12w**) appeared at t_R = 17.81 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ 3534.654; $M{\cdot}H^+_{calcd}$ = 3533.653.

2.3.14 Solid phase peptide synthesis of TGTACGTCACAACTA (12x)

The synthesis of TGTACGTCACAACTA (**12x**) was accomplished in the same way as described for T_5 (**12d**) above. Starting from TentaGel S RAM Fmoc

resin (4.2 mg, 1 μmol) and monomer, Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**30**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-C-Pfp (**30**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-A-Pfp (**28**) (3.0 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-G-Pfp (**32**) (2.9 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), Fmoc-T-Pfp (**26**) (2.5 mg, 1 μmol), Fmoc-ACPC-Pfp (**38**) (2.1 mg, 1 μmol), were used in each coupling cycle respectively.

After purification by HPLC, the HPLC peak of TGTACGTCACAACTA (**12x**) appeared at t_R = 15.71 min. MALDI-TOF mass spectrum showed $M{\cdot}H^+_{obs}$ 5200.304.; $M{\cdot}H^+_{calcd}$ = 5205.433.

2.4 Biophysical studies

2.4.1 T_m experiments [60]

T_m experiments were performed on a CARY 100 Bio UV-Visible spectrophotometer (Varian Ltd.) equipped with a thermal melt system. The sample for T_m measurement was prepared by mixing calculated amounts of stock oligonucleotide and PNA solutions together to give final concentration of nucleotides and sodium phosphate buffer (pH 7.0) and the final volumes were adjusted to 3.0 mL by addition of deionized water. The samples were transferred to a 10 mm quartz cell with a Teflon stopper and equilibrated at the starting temperature for 10 min. The A_{260} was recorded in steps heating from 20-90 °C, cooling 90-20 °C and reheating 20-90 °C (block temperature) with a temperature ramp of 1 °C /min. The temperature recorded was the actual temperature measured by a built-in temperature probe. Only the result taken from the last heating cycle was used and was normalized by dividing the absorbance

at each temperature by the initial absorbance (see **Table 2.2**). T_m was obtain from derivative plot after smoothing using KaliedaGraph 3.6 (Synergy Software) and analysis of the data was performed on a PC compatible computer using Microsoft Excel XP (Microsoft Corp.) (see **Figure 2.2**). The independent experiments were accurate within ±0.5 °C.

Table 2.1 Data examples from T_m analysis of GTAGATCACT (**12r**) with d(AGTGATCTAC) by UV spectrophotometry

entry	GTAGATCACT (**12r**) with d(AGTGATCTAC) at 20.00-90.00 °C			
	Temperature (°C)	**Absorbance**	**Correct temp* (°C)**	**Normalized Abs**
1	20.62	0.1586	19.56	1.0000
2	40.52	0.1633	39.022	1.0300
3	45.52	0.1656	43.91	1.0442
4	50.52	0.1691	48.80	1.0663
5	55.47	0.1747	53.64	1.1020
6	60.52	0.1826	58.58	1.1516
7	65.52	0.1901	63.47	1.1987
8	70.47	0.1943	68.31	1.2253
9	75.52	0.1959	73.25	1.2354
10	80.52	0.1964	78.14	1.2383
11	85.52	0.1965	83.03	1.2920
12	90.52	0.1964	87.92	1.2385

*The equation for determining the corrected temp was obtained by measuring the actual temp in the cuvette using a temperature probe and plotting against the set temperature (T_{block}) from 20-90 °C. The linear equation and relationship were obtained with Y = 0.978X-0.6068 and $r^2 > 0.99$.

Correct temperature and normalized absorbance are defined as follows.

Correct. Temp. = $(0.978 \times T_{block}) - 0.6068$

Normalized Abs. = Abs_{obs}/Abs_{init}

In entry 1; T_{obs} = 20.62 °C, Abs_{init} = 0.1586

Abs_{obs} = 0.1586

Correct. Temp. = $(0.978 \times T_{obs}) - 0.6068$

= $(0.978 \times 20.62) - 0.6068$

= 19.56 °C

Normalized Abs. = Abs_{obs}/Abs_{init}

= 0.1586/0.1586

= 1.0000

In entry 2; T_{obs} = 40.52 °C, Abs_{init} = 0.1586

Abs_{obs} = 0.1633

Correct. Temp. = $(0.978 \times T_{obs}) - 0.6068$

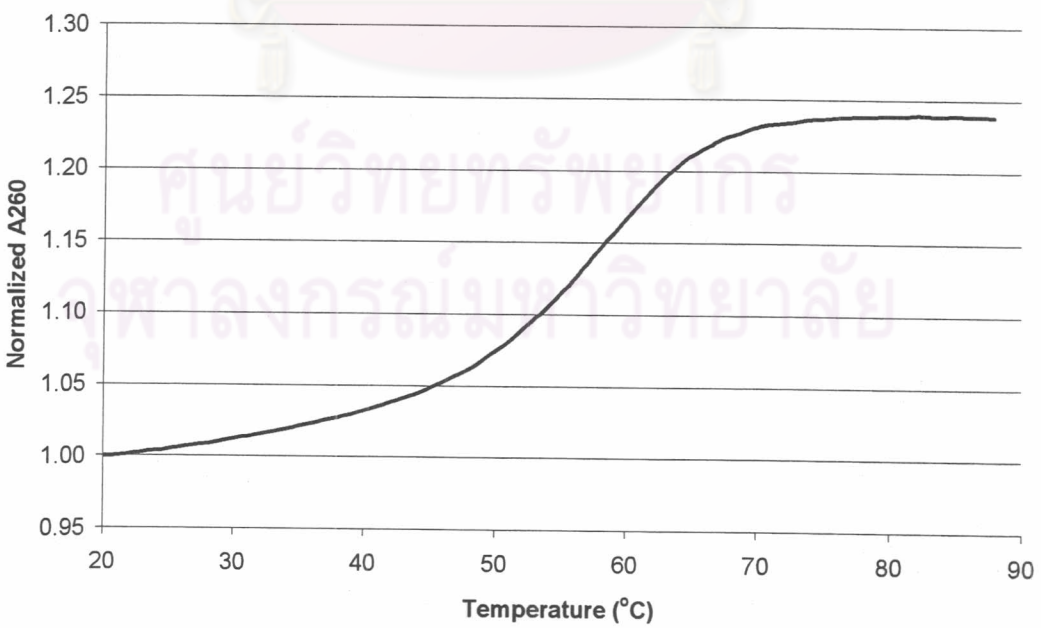
Correct. Temp. = $(0.978 \times 40.52) - 0.6068$

= 39.02 °C

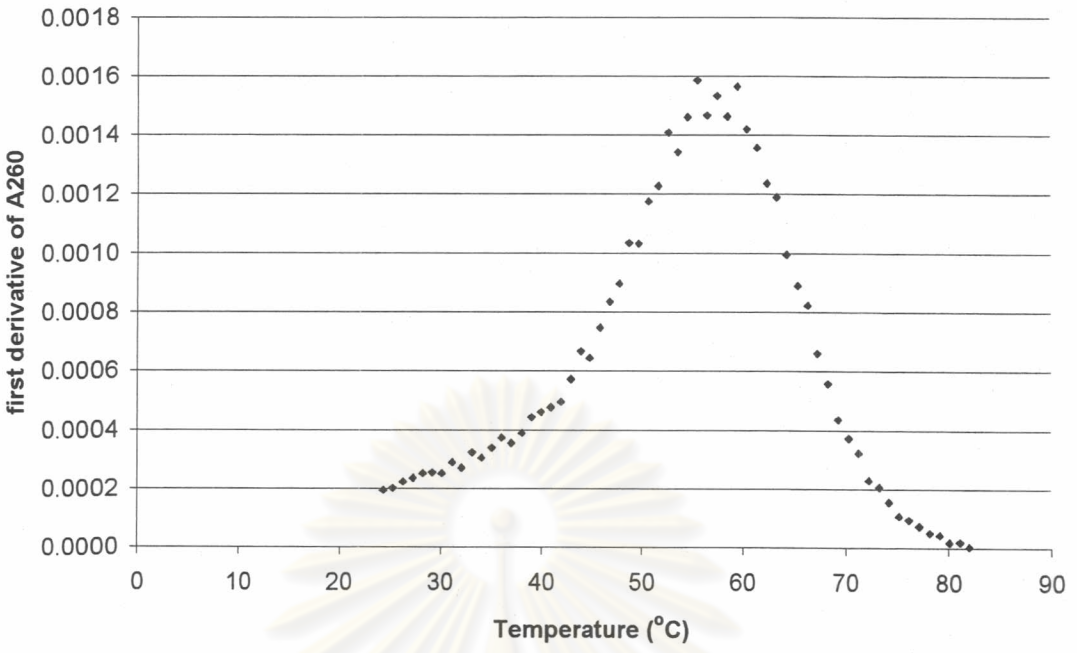
Normalized Abs. = Abs_{obs}/Abs_{init}

= 0.1633/0.1586

= 1.0300



(a)



(b)

Figure 2.2 (a) Melting curve and (b) UV-T_m first derivative curves of GTAGATCACT (**12r**) with d(AGTGATCTAC). Conditions: 10 mM sodium phosphate buffer pH 7.0 1.0 μM ratio of PNA:DNA = 1:1

2.4.2 UV-titration experiments [60,64]

The UV titration experiment was performed on a MILTON ROY spectronic 3000 array UV spectrophotometer at 25 °C. To a solution containing the TGTACGTCACAACTA (**12x**) (8.5 μM) and 10 mM sodium phosphate buffer (2500 μL) was added a 5-20 μL aliquot of a concentrated stock solution of d(TAGTTGTGACGTACA) (830.9 μM). After the absorbance is stabilized (10-15 min) the absorbance was read against a blank (10 mM sodium phosphate) and more d(TAGTTGTGACGTACA) aliquots were added until a total volume of 120 μL (corresponds to 1:5 PNA (**12x**):DNA) had been added. The ratio of the observed A_{260} and the calculated A_{260} were plotted against the mole ratio of PNA (**12x**):DNA nucleotide and the stoichiometry was determined form the inflection point.

$$\text{Calcd. } OD_{260} = \frac{OD_{260(\mathbf{12x})} \times V_{(\mathbf{12x})} + OD_{260(DNA)} \times V_{(DNA)}}{V_{(\mathbf{12x})} + V_{(DNA)}}$$

$$= \frac{0.084 \times 2500 + 8.4 \times V_{(DNA)}\,(\mu L)}{2500 + V_{(DNA)}\,(\mu L)}$$

$$\text{ratio of T:A} = \frac{\varepsilon_{(DNA)} \times OD_{260(\mathbf{12x})} \times V_{(\mathbf{12x})}}{\varepsilon_{(\mathbf{12x})} \times OD_{260(DNA)} \times V_{(DNA)}}$$

$$= \frac{151.6 \times 0.084 \times 2500}{148.3 \times 8.4 \times V_{(DNA)}\,(\mu L)}$$

2.4.3 Circular dichroism spectroscopy (CD)

CD experiments were performed on a JASCO Model J-715 spectropolarimeter (Pharmaceutical Research Equipment Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The sample were prepared by mixing calculated amounts of stock oligonucleotide and PNA solutions together in a 10 mm quartz cell and the final volumes were adjusted to 2.5 mL by addition of deionized water containing an appropriate amount of sodium phosphate buffer pH 7.0 to give the appropriate concentration of each component as described in the text. The spectra were measured at 25 °C from 200 to 300 nm and averaged 4 times then subtracted from a spectrum of 10 mM sodium phosphate buffer pH 7.0 under the same condition.

2.4.4 CD-titration experiment

To a solution containing the DNA (1.0 μM) and 10 mM sodium phosphate buffer pH 7.0 (2.5 mL) was added 5 μL aliquot of a concentrated stock solution of PNA (concentration = 83 μM). The spectra were measured at 25 °C from 200 to 300 nm and averaged 4 times then subtracted from a spectrum of pure water under the same conditions. More PNA stocks were added until a total volume of 150 μL (5 equiv) has been added.