CHAPTER III

RESEARCH METHODOLOGY

General Procedure

1. Measurement

All reactions were performed in oven-dried glasswares. The weight of all chemical substances was determined on a Sartorius electronic analytical balance. Evaporation of solvents was carried out on Büchi Rotavapor R-124 with a water aspirator model B-490 or a Refco Vacuubrand pump or a diaphragm pump. The magnetic stirrers and heater were of Heidolph and HARMONY. 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. Visualization was accomplished using either a UV light at 254 nm, potassium permanganate stain (1.5 g of $KMnO_4$, 10 g of K_2CO_3, 1.25 mL of 10% (w/w) NaOH in 200 mL of water) and ninhydrin stain (1.5 g of ninhydrin in 100 mL of *n*-butanol and 3 mL of AcOH. Column chromatography was performed on silica gel 70-230 mesh for column chromatography. Solvent mixtures used for TLC and column chromatography are reported in v/v ratios. Reverse phase HPLC experiments were performed on Water 600™ system equipped with gradient pump and Water 996™ photodiode array detector; optionally alternate to Rheodyne 7725 manual sample loop (100 µL sample size for analytical scale). An ACE 5 C18-AR HPLC column, 3 µm particle size 150 x 4.6 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 chromatography monitoring. The combined fractions were speed vaporized under reduced pressure using Heto Vacuum Centrifuge and MAXI dry-plus. Melting points were determined on a capillary melting point apparatus model 9100. ^1H and ^{13}C spectra were reported in ppm and recorded at Chulalongkorn University on Bruker Merury-400 plus spectrometer and on Bruker Avance 400 NMR spectrometer at Naresuan University operating at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. FT-IR spectra were recorded on Perkin Elmer

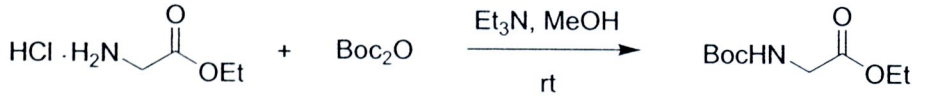
Spectrum GX FT-IR spectrometer. MALDI-TOF mass spectra of all *trans*-(3*R*,4*S*)- and *trans*-(3*S*,4*R*)-*apc*PNA were obtained at Chulalongkorn University on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics) using doubly recrystallized α-cyano-4-hydroxy cinnamic acid (CCA) as matrix. 0.1 % Trifluoroacetic acid in acetonitrile:water (1:2) was used as the diluents for preparation of MALDI-TOF samples.

2. Materials

All chemicals were purchased from Fluka, Merck, Acros Organics 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. Methanol for HPLC experiment was HPLC grade, obtained from BDH and was filtered through a membrane filter (13 mmϕ, 0.45 μm Nylon Lida) before use. Toluene was dried with calcium hydride under reflux. Anhydrous *N*,*N*-dimethylformamide ($H_2O \leq 0.01\%$) for solid phase peptide coupling reaction was obtained from Labscan 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-Lys(Mtt)-OH) was obtained from Calbiochem Novabiochem Co., Ltd. Acetic anhydride was synthesized from acetyl chloride and anhydrous sodium acetate according to the standard method. High purity up 99.99% of nitrogen and hydrogen gas were obtained from Thai Industrial Gas and Phitsanulok Oxygen Ltd. 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). Pyrrolidinyl PNA monomers namely (*N*-fluoren-9-ylmethoxycarbonylamino)-*cis*-4-(thymin-1-yl)-D-proline pentafluoro- phenyl ester, (*N*-fluoren-9-ylmethoxycarbonylamino)-*cis*-4-(N^2-isobutyrylguanin-9-yl)-D-proline pentafluorophenyl ester, (*N*-fluoren-9-ylmethoxycarbonylamino)-*cis*-4-(N^6-benzoyladenin-9-yl)-D-proline pentafluorophenyl ester, (*N*-fluoren-9-ylmethoxy carbonylamino)-*cis*-4-(N^4-benzoylcytosine-9-yl)-D-proline pentafluorophenyl ester and ACPC spacer namely (1*S*,2*S*)-2-(*N*-fluoren-9-ylmethoxycarbonyl)-aminocyclo- pentanecarboxylic acid pentafluorophenyl ester were synthesized and purified according to Vilaivan and co-worker [45, 46].

Synthesis of both enantiomeric 3-aminopyrrolidine-4-carboxylic (*apc*) spacers

1. *N*-Boc glycine ethyl ester (2)



(1) **(2)**

To a stirring solution of glycine ethyl ester salt (**1**) (4.19 g, 30 mmol) in absolute methanol (50 mL) were added triethylamine (7.80 mL, 60 mmol) and slowly dropped Boc_2O (6.54 g, 30 mmol) over 20 min. The mixture was stirred at ambient temperature for 24 hours. The methanol was removed *via* rotary evaporation. Water (50 mL) and 5% HCl (aqueous) were added dropwise while stirring to pH~4. The mixture was extracted with ethyl acetate (3x100 mL). The combined organic extract was dried over Na_2SO_4 anhydrous and evaporated to give compound **2** without purification as colorless oil (6.08 g) in 99% yield.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.18 [3H, *t*, *J* = 7.1 Hz, C<u>H</u>$_3$ Ethyl] 1.35 [9H, *s*, C<u>H</u>$_3$x3, Boc] 3.78, 3.79 [2H, *d*, *J* = 5.6 Hz, α-C<u>H</u>$_2$ rotamers] 4.07 [2H, *q*, *J* = 7.2 Hz, C<u>H</u>$_2$ Ethyl]; ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C 14.1 [<u>C</u>H$_3$ Ethyl] 28.2 [<u>C</u>H$_3$ Boc] 42.4 [<u>C</u>H$_2$ Ethyl] 61.2 [α-<u>C</u>H$_2$] 79.7 [<u>C</u>CH$_3$ Boc] 155.8 [<u>C</u>=O carbamate] 170.4 [<u>C</u>=O ester].

2. Ethyl 1-*tert*-butoxycarbonyl-3-oxopyrrolidine-4-carboxylate (3)

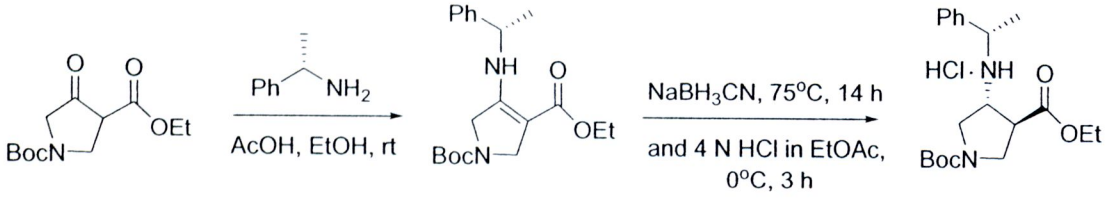
(2) **(3)**

To a stirring solution of potassium *t*-butoxide (2.83 g, 25 mmol) in dry toluene (20 mL), maintained at 0 °C under N_2 was added a solution of *N*-Boc glycine ethyl ester (**2**) (2.08 g, 10 mmol) and ethyl acrylate (1.08 mL, 10 mmol) in dry toluene (15 mL) by syringe. The solution was stirred at 0 °C for 2 hours. The mixture was allowed to warm to room temperature and stirred with a magnetic stirrer for 24 hours. Glacial acetic acid (1.60 mL) and a cold solution of 0.07 M sodium dihydrogen

phosphate (50 mL) were added. The mixture was extracted with dichloromethane (3x100 mL) and the combined organic extract was dried over Na_2SO_4 anh. and concentrated. The residue was dissolved in a cold toluene (20 mL) and the resulting solution extracted with pH 9.6 carbonate buffer (3x50 mL). The aqueous extracts were brought to pH~3 by phosphoric acid and re-extracted with dichloromethane (3x100 mL). The combined organic extract was dried over Na_2SO_4 anh. and evaporated. The crude product was purified by column chromatography eluting with hexane/ethyl acetate (6/1, v/v) on silica gel (R_f = 0.16) to give compound **3** as a pale-yellow oil (1.81 g) in 66% yield.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.23 [3H, *t*, *J* = 7.2 Hz, C$\underline{H}_3$ Ethyl] 1.42 [9H, *s*, C$\underline{H}_3$x3, Boc] 3.53 [1H, *t*, *J* = 8.2 Hz, COC$\underline{H}$CO] 3.79, 3.81 [1H, *d*, *J* = 9.2 Hz C$\underline{H}_2$N, $\underline{C}_5$] 3.96 [1H, *dt*, *J* = 11.6, 7.5 Hz, C$\underline{H}_2$N, $\underline{C}_5$] 3.93-4.01 [2H, *m*, C$\underline{H}_2$CO, $\underline{C}_2$] 4.18 [2H, *q*, *J* = 7.5 Hz, C$\underline{H}_2$ Ethyl]; ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C 14.1 [$\underline{C}H_3$ Ethyl] 28.4, 28.5 [$\underline{C}H_3$x3, Boc rotamers] 48.5, 48.8 [$\underline{C}H_2$ Ethyl rotamers] 51.1, 51.4 [$\underline{C}H_2NCH_2$, $\underline{C}_2$ rotamers] 60.6 [CO$\underline{C}$HCO] 62.2 [CH_2 N$\underline{C}H_2$, $\underline{C}_5$] 80.3, 80.9 [$\underline{C}CH_3$ Boc rotamers] 154.2 [$\underline{C}$=O carbamate] 166.8 [$\underline{C}$=O ester] 168.0, 168.2 [$\underline{C}$=O ketone rotamers].

3. (3*R*,4*S*)-1-(*N*-*tert*-butoxycarbonyl)-3-[(1'*S*)-phenylethylamino]-4-ethoxy carbonylpyrrolidine hydrochloride (5)



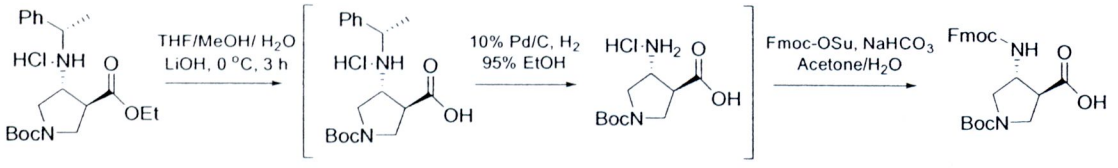
(3) **(4)** **(5)**

To a stirring solution of ethyl 1-*tert*-butoxycarbonyl-3-oxopyrrolidine-4-carboxylate (**3**) (3.86 g, 15 mmol) was dissolved in ethanol under N_2. *S*-(-)-α-methylbenzylamine (3.46 mL, 30 mmol) and glacial acetic acid (1.72 mL, 30 mmol) were added. The mixture was stirred at room temperature for 3 hours. Sodium cyanoborohydride (3.97 g, 60 mmol) was added to the reaction mixture and heated up to 75 °C for 14 hours. Then, ethanol was removed *via* rotary evaporation and water (20 mL) was added to the residue. The resulting mixture was extracted with diethyl ether (3x50 mL) and the combined organic extract was dried over Na_2SO_4 anh. and

evaporated to give colorless oil. The oil was applied to a plug of silica gel and washed with ethyl acetate/hexane (1/2, v/v). The filtrate was concentrated to give colorless oil (3.63 g) which was dissolved in ethyl acetate (10 mL) maintained at 0 °C. The solution was added dropwise a solution of 4 N HCl in ethyl acetate (3.75 mL) and cooled at 0 °C for 3 hours which precipitation was occurred in this step. The solid was filtered and washed with ethyl acetate (2x100 mL). This crude product could be purified by recrystallization from acetonitrile by suspending in acetonitrile (5 mL) and heating to reflux for 1 hour. The mixture was then cooled to 0 °C for 3 hours. The resulting precipitate was isolated by filtration and washed with acetonitrile (2x100 mL) to give compound **5** as white crystalline solid (1.20 g) in 21% yield, $[\alpha]_D^{27}$ = –4.6 (20 mg/ 2 mL) in methanol, mp 215-217 °C.

^{1}H-NMR (400 MHz, DMSO-*d*6): δ_H 1.14 [3H, *t*, *J* = 7.2 Hz, C<u>H</u>$_3$ Ethyl] 1.38 [9H, *s*, C<u>H</u>$_3$x3, Boc] 1.64 [1H, *d*, *J* = 9.8 Hz, C<u>H</u>$_3$CHNH] 3.43-3.81 [6H, *m*, C<u>H</u>$_2$N<u>H</u>C<u>H</u>C<u>H</u>$_2$] 4.03 [2H, *q*, *J* = 6.8 Hz and 7.2 Hz, C<u>H</u>$_2$ Ethyl] 4.45 [1H, *s*, BnC<u>H</u>NH] 7.40-7.65 [5H, *m*, C<u>H</u>, Benzene] 10.03-10.11 [2H, *m*, $^+$N<u>H</u>$_2$]; ^{13}C NMR (100 MHz, DMSO-*d*6): δ_C 13.8 [<u>C</u>H$_3$, Ethyl] 19.9 [<u>C</u>H$_3$CHPh] 28.0 [<u>C</u>H$_3$, Boc] 44.2, 45.0 [<u>C</u>H$_2$, <u>C</u>$_5$] 46.6, 48.0 [<u>C</u>H, <u>C</u>$_4$] 55.6 [CH$_3$<u>C</u>HPh] 56.5 [<u>C</u>HNH, <u>C</u>$_3$] 61.2 [<u>C</u>H$_2$, Ethyl] 79.2 [<u>C</u>CH$_3$, Boc] 128.1, 128.9, 129.0 [<u>C</u>H, Benzene] 136.6 [<u>C</u>, Benzene] 152.9 [<u>C</u>=O, Boc] 170.3 [<u>C</u>OOEt]

4. (3*R*,4*S*)-1-(*N-tert*-Bitpxucarbonyl)-3-(*9H*-fluoren-9-yl-methoxycarbonyl amino)-4-carboxypyrrolidine (8)



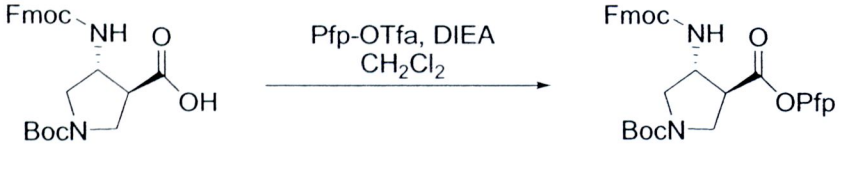
(5) **(6)** **(7)** **(8)**

To a stirring solution of (3*R*,4*S*)-1-(*N-tert*-butoxycarbonyl)-3-[(1'S)-phenyl ethylamino]-4-ethoxycarbonylpyrrolidine hydrochloride (**5**) (0.19 g, 0.5 mmol) in THF/methanol/water (6/3/1, v/v, 5 mL) and cooled in the ice-bath to 0 °C. Then, LiOH·H$_2$O (0.10 g, 2.5 mmol) was added and the mixture was stirred at 0 °C for 3 hours. Aqueous 1 N HCl (2.60 mL) was added at 0 °C and the solvent was removed

under reduced pressure to give a white solid which was dissolved in 95% ethanol (5 mL) under H_2. 10% Pd on activate carbon (0.02 g) was added and the mixture was stirred at room temperature for 24 hours. The Pd/C was removed by filtration and washed with methanol and the filtrate was concentrated to give colorless oil. The oil was dissolved in acetone/water (2/1, v/v, 5 mL) and cooled to 0 °C. $NaHCO_3$ (0.25 g) was added, followed by Fmoc-OSu (0.17 g, 0.5 mmol). The mixture was stirred at 0 °C for 2 hours, then at room temperature overnight. In the next step, water (10 mL) was added and the remain organic layer was removed under reduced pressure, the aqueous layer was extracted with diethyl ether (3x50 mL). The aqueous residue was acidified with $NaHSO_4$ and extracted with ethyl acetate (3x50 mL). The combined organic extracts were washed with dilute brine (50 mL), dried over Na_2SO_4 anh. and concentrated to give a foamy solid. The crude product was purified by crystallization from *n*-heptane/ethyl acetate to give compound **8** as white solid (0.18 g) in 78% yield, $[\alpha_D^{27}] = +21.9$ (20 mg/ 2 mL) in methanol, mp 106-108 °C.

^{1}H-NMR (400 MHz, CD_3OD): δ_H 1.46 [9H, *s*, C<u>H</u>$_3$x3, Boc] 3.00[1H, *t*, *J* = 6.4 Hz, CH_2C<u>H</u>, Fmoc] 3.16 [1H, *dd*, *J* = 5.6 Hz and 6.0 Hz, COC<u>H</u>C] 3.55-3.70 [4H, *m*, C<u>H</u>$_2$NC<u>H</u>$_2$, <u>C</u>$_{2,5}$] 4.17 [1H, *dd*, *J* = 6.0 Hz and 6.4, C<u>H</u>NH] 4.35-4.39 [2H, *m*, C<u>H</u>$_2$CH, Fmoc] 7.28-7.39 [4H, *m*, 4xC<u>H</u>, Fmoc] 7.62 [2H, *d*, *J* = 6.8 Hz, 2xC<u>H</u>, Fmoc] 7.77 [2H, *d*, *J* = 7.6 Hz, 2xC<u>H</u>, Fmoc]; ^{13}C NMR (100 MHz, CD_3OD): δ_C 28.7 [<u>C</u>H$_3$, Boc] 48.0 [<u>C</u>H, <u>C</u>$_4$] 48.5 [CH_2<u>C</u>H, Fmoc] 50.1 [<u>C</u>HNH, <u>C</u>$_3$] 51.4, 51.9 [<u>C</u>H$_2$, <u>C</u>$_5$] 54.3, 54.9 [<u>C</u>H$_2$N, <u>C</u>$_2$] 67.8 [<u>C</u>H$_2$CH, Fmoc] 81.3 [<u>C</u>CH$_3$, Boc] 121.0 [2x<u>C</u>H, Fmoc] 126.2 [2x<u>C</u>H, Fmoc] 128.2 [2x<u>C</u>H, Fmoc] 128.8 [2x<u>C</u>H, Fmoc] 142.6 [2x<u>C</u>, Fmoc] 145.2 [2x<u>C</u>, Fmoc] 156.1 [<u>C</u>=O, Boc] 158.4 [<u>C</u>=O, Fmoc] 175.4 [<u>C</u>OOH]

5. (3*R*,4*S*)-1-(*N*-*tert*-Butoxycarbonyl)-3-(9*H*-fluoren-9-yl-methoxycarbonyl amino)-4-(pentafluorophenoxy)carbonyl)pyrrolidine (9)

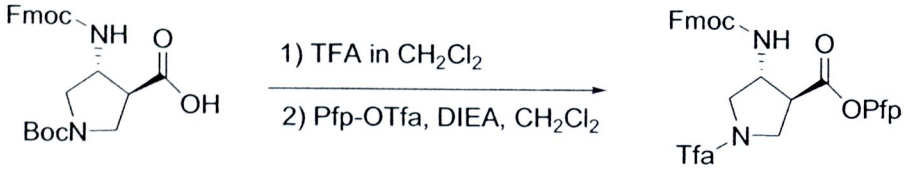


(8) **(9)**

To a stirring solution of (3*R*,4*S*)-1-(*N*-*tert*-butoxycarbonyl)-3-(9*H*-fluoren-9-yl-methoxycarbonylamino)-4-carboxypyrrolidine (**8**) (0.10 g, 0.22 mmol), pentafluorophenyl trifluoroacetate (PfpOTfa) (76.3 μL, 0.44 mmol) and *N*,*N*-diisopropylethylamine (DIEA) (75.7 μL, 0.44 mmol) in dichloromethane (1 mL) were stirred at room temperature for 30 min. The reaction was completed as indicated by TLC analysis. Dichloromethane (10 mL) was added and extracted with 10% HCl (2x20 mL). The organic extract was washed with water (20 mL) and extracted with excess saturated $NaHCO_3$ solution (2x20mL). The organic extracts dried over Na_2SO_4 anh. and concentrated *via* rotary evaporation. The crude product was purified by crystallization from hexane to give compound **9** as white solid (0.08 g) in 60% yield, $[\alpha_D^{27}] = +17.8$ (20 mg/ 2 mL) in chloroform, mp 97-98 °C.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.50 [9H, *s*, C$\underline{H}_3$x3, Boc] 3.37 [1H, *s*, COC$\underline{H}$C] 3.49 [1H, *s*, C$\underline{H}_2$NCH$_2$, C_5] 3.82 [3H, *s*, C$\underline{H}_2$NC$\underline{H}_2$, $C_{2,5}$] 4.23 [1H, *t*, *J* = 5.8 Hz, CH$_2$C$\underline{H}$, Fmoc] 4.51 [2H, *s*, C$\underline{H}_2$CH, Fmoc] 4.58 [1H, *s*, C$\underline{H}$NH] 5.13 [1H, *s*, CHN$\underline{H}$] 7.33 [2H, *t*, *J* = 7.3 Hz, 2xC$\underline{H}$, Fmoc] 7.42 [2H, *t*, *J* = 7.2 Hz, 2xC$\underline{H}$, Fmoc] 7.58 [2H, *d*, *J* = 7.4 Hz, 2xC$\underline{H}$, Fmoc] 7.78 [2H, *d*, *J* = 7.4 Hz, 2xC$\underline{H}$, Fmoc]; ^{13}C NMR (100 MHz, CD_3OD): δ_C 28.4 [$\underline{C}H_3$, Boc] 46.3, 47.2 [$\underline{C}H_2$, $\underline{C}_5$] 47.2 [CH$_2\underline{C}$H, Fmoc] 48.2 [$\underline{C}H_2$N, $\underline{C}_2$] 49.9, 50.3 [CH$_2\underline{C}$HC, $\underline{C}_4$] 53.4, 53.9 [$\underline{C}$HNH, $\underline{C}_3$] 66.9 [$\underline{C}H_2$CH, Fmoc] 80.5 [$\underline{C}CH_3$, Boc] 120.0 [2x$\underline{C}$H, Fmoc] 124.9 [2x$\underline{C}$H, Fmoc] 127.1 [2x$\underline{C}$H, Fmoc] 127.8 [2x$\underline{C}$H, Fmoc] 136.6 [$\underline{C}$F, *p*, Pfp] 138.5 [2x$\underline{C}$F, *o*, Pfp] 139.2 [2x$\underline{C}$F, *m*, Pfp] 141.1 [$\underline{C}$, Pfp] 141.4 [2x$\underline{C}$, Fmoc] 143.7 [2x$\underline{C}$, Fmoc] 154.0 [$\underline{C}$=O, Boc] 155.5 [$\underline{C}$=O, Fmoc] 167.7 [$\underline{C}$OOH]; IR (KBr disc): 3337 cm^{-1} [N-H stretch] 3069 cm^{-1} [C-H stretch, aromatic, Fmoc] 2979 cm^{-1} [ν_{as} C-H stretch, aliphatic, CH_3 (Boc) and CH_2] 2893 cm^{-1} [ν_sC-H stretch, aliphatic, CH_3 (Boc) and CH_2] 1783 cm^{-1} [C=O stretch, ester] 1695 cm^{-1} [C=O stretch, carbamate] 1521 cm^{-1} [N-H bend, secondary amide band, carbamate] 1411[C-H bend, CH_2] 1385 [C-H bend, CH_3 (Boc)] 1275 [C-N stretch] 1173 [C-O stretch] 1123 [C-F stretch, Pfp]

6. (3*R*,4*S*)-1-(2,2,2-trifluoroacetyl)-3-(9*H*-fluoren-9-yl-methoxycarbonyl amino)-4-((pentafluorophenoxy)carbonyl)pyrrolidine (10)



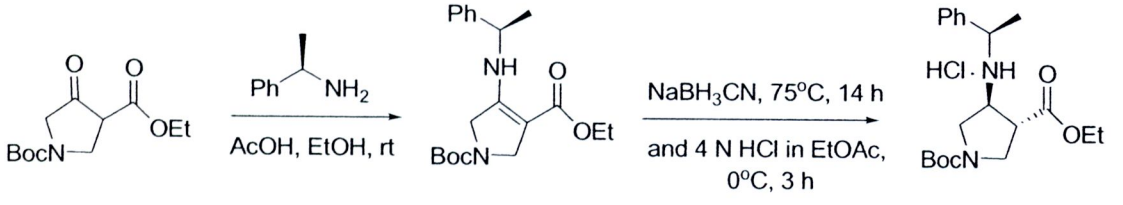
(8) **(10)**

To a stirring solution of (3*R*,4*S*)-1-(*N*-*tert*-butoxycarbonyl)-3-(9*H*-fluoren-9-yl-methoxycarbonylamino)-4-carboxypyrrolidine **(8)** (0.17 g, 0.35 mmol) and trifluoroacetic acid (0.5 mL) in dichloromethane (1 mL) were stirred at room temperature for 30 min. The mixture was concentrated *via* rotary evaporation followed by flushing with N_2 to eradicate excess TFA. The residue was dissolved in dichloromethane (1 mL). *N*,*N*-diisopropylethylamine (DIEA) (180 µL, 1.05 mmol) and pentafluorophenyl trifluoroacetate (121 µL, 0.70 mmol) were added. The resulted mixture was stirred at room temperature for 30 mins. The reaction was completed as indicated by TLC analysis and purified by column chromatography eluting with ethyl acetate/hexane (1/1, v/v) on silica gel and crystallization from hexane to give compound 10 as white solid (0.13 g) in 60% yield, $[\alpha_D^{27}]$ = +22.1 (20 mg/ 2 mL) in chloroform, mp 97-98 °C.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 3.59 [1H, *s*, COC<u>H</u>C] 3.72 [1H, *s*, C<u>H</u>$_2$NCH$_2$, C_5] 4.08 [3H, *b*, C<u>H</u>$_2$NC<u>H</u>$_2$, $C_{2,5}$] 4.23 [1H, *t*, *J* = 5.2 Hz, CH$_2$C<u>H</u>, Fmoc] 4.52 [2H, *s*, C<u>H</u>$_2$CH, Fmoc] 4.58 [1H, *s*, C<u>H</u>NH] 5.12 [1H, *b*, CHN<u>H</u>] 7.33 [2H, *t*, *J* = 7.2 Hz, 2xC<u>H</u>, Fmoc] 7.42 [2H, *t*, *J* = 7.2 Hz, 2xC<u>H</u>, Fmoc] 7.58 [2H, *d*, *J* = 7.2 Hz, 2xC<u>H</u>, Fmoc] 7.78 [2H, *d*, *J* = 7.2 Hz, 2xC<u>H</u>, Fmoc]; ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 45.3, 46.6 [<u>C</u>H$_2$, <u>C</u>$_5$] 47.2 [CH$_2$<u>C</u>H, Fmoc] 47.54, 48.22 [<u>C</u>H$_2$N, <u>C</u>$_2$] 50.17, 50.9 [CH$_2$<u>C</u>HCO, <u>C</u>$_4$] 52.0 [<u>C</u>HNH, <u>C</u>$_3$] 67.0 [<u>C</u>H$_2$CH, Fmoc] 116.6 [<u>C</u>F$_3$, Tfa] 120.1 [2x<u>C</u>H, Fmoc] 124.8 [2x<u>C</u>H, Fmoc] 127.1 [2x<u>C</u>H, Fmoc] 127.9 [2x<u>C</u>H, Fmoc] 136.7 [<u>C</u>F, *p*, Pfp] 139.2 [2x<u>C</u>F, *o*, Pfp] 139.6 [2x<u>C</u>F, *m*, Pfp] 141.4 [<u>C</u>, Pfp] 142.0 [4x<u>C</u>, Fmoc] 143.4 [2x<u>C</u>, Fmoc] 155.5 [<u>C</u>=O, Fmoc] 166.5 [<u>C</u>=O, Tfa] 167.0 [<u>C</u>=O, Pfp]; IR (KBr disc): 3340 cm^{-1} [N-H stretch] 3068 cm^{-1} [C-H stretch, aromatic, Fmoc] 2970 cm^{-1} [ν_{as}C-H stretch, aliphatic, CH$_2$] 2897 cm^{-1} [ν_sC-H stretch, aliphatic, CH$_2$] 1783 cm^{-1}

[C=O stretch, ester] 1694 cm^{-1} [C=O stretch, Tfa] 1522 cm^{-1} [N-H bend, secondary amide band, carbamate] 1392 [C-H bend, CH_2] 1280 [C-N stretch] 1259 [C-F stretch, CF_3] 1211 [C-O stretch] 1128 [C-F stretch, Pfp]

7. (3*S*,4*R*)-1-(*N*-*tert*-butoxycarbonyl)-3-[(1'*S*)-phenylethylamino]-4-ethoxy carbonylpyrrolidine hydrochloride (12)

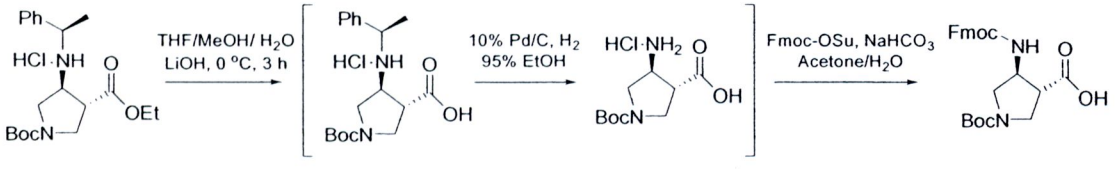


(3) **(11)** **(12)**

To a stirring solution of ethyl 1-*tert*-butoxycarbonyl-3-oxopyrrolidine-4-carboxylate (**3**) (3.15 g, 12.25 mmol) was dissolved in ethanol (30 mL) under N_2. *R*-(+)-α-methylbenzylamine (3.12 mL, 24.5 mmol) and glacial acetic acid (1.72 mL, 24.5 mmol) were added. The mixture was stirred at room temperature for 3 hours and sodium cyanoborohydride (3.08 g, 49 mmol) was added to the reaction mixture and heated to 75 °C for 14 hours under N_2. The ethanol was removed *via* rotary evaporation, and then water (20 mL) was added. The resulting mixture was extracted with diethyl ether (3x50 mL) and the combined organic extract was dried over Na_2SO_4 anh. and evaporated to give colorless oil. The oil was applied to a plug of silica gel and washed with ethyl acetate/hexane (1/2, v/v). The filtrate was concentrated to give colorless oil (3.42 g) which was dissolved in ethyl acetate (10 mL) maintained at 0°C and added dropwise a solution of 4 N HCl in ethyl acetate (3.10 mL). The solution was cooled at 0 °C for 3 hours which precipitation was occurred in this step. The solid was filtered and washed with ethyl acetate (2x100 mL). This crude product could be purified by recrystallization from acetonitrile by suspended in acetonitrile (5 mL) and heated to reflux for 1 hour. The mixture was then cooled to 0 °C for 3 hours. The resulting precipitate was isolated by filtration and washed with acetonitrile (2x100 mL) to give compound **15** as white crystalline solid (0.80 g) in 17% yield, $[\alpha_D^{27}] = +4.6$ (20 mg/ 2 mL) in methanol, mp 215-217 °C.

^{1}H-NMR (400 MHz, DMSO-*d*6): δ_H 1.14 [*t*, 3H, *J* = 7.2 Hz, C$\underline{H}_3$ Ethyl] 1.38 [*s*, 9H, C$\underline{H}_3$x3, Boc] 1.64 [*d*, 1H, *J* = 9.8 Hz, C$\underline{H}_3$CHNH] 3.43-3.81[*m*, 6H, C$\underline{H}_2$N$\underline{H}$C$\underline{H}$C$\underline{H}_2$] 4.03 [*q*, 2H, *J* = 7.2 Hz and 6.8 Hz, C$\underline{H}_2$ Ethyl] 4.45 [*s*, 1H, BnC$\underline{H}$NH] 7.40-7.65 [*m*, 5H, C$\underline{H}$, Benzene] 10.03-10.11 [*m*, 2H, $^+$N$\underline{H}_2$]; ^{13}C NMR (100 MHz, DMSO-*d*6): δ_C 13.8 [$\underline{C}$H$_3$, Ethyl] 20.0 [$\underline{C}$H$_3$CHPh] 28.0 [$\underline{C}$H$_3$, Boc] 44.2, 45.0 [$\underline{C}$H$_2$, $\underline{C}_5$] 46.7, 48.0 [$\underline{C}$H, $\underline{C}_4$] 55.6 [CH$_3\underline{C}$HPh] 56.5 [$\underline{C}$H, $\underline{C}_3$] 61.2 [$\underline{C}$H$_2$, Ethyl] 79.1 [$\underline{C}$CH$_3$, Boc] 128.1, 128.9, 129.0 [5C, $\underline{C}$H, Benzene] 136.7 [$\underline{C}$, Benzene] 152.9 [$\underline{C}$=O, Boc] 170.4 [$\underline{C}$OOEt]

8. (3*S*,4*R*)-1-(*N*-*tert*-Butoxycarbonyl)-3-(9*H*-fluoren-9-yl-methoxycarbonyl amino)-4-carboxypyrrolidine (15)



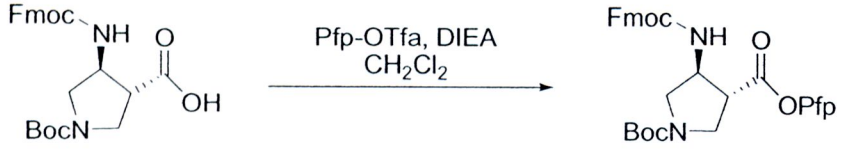
(12) **(13)** **(14)** **(15)**

To a stirred solution of (3*S*,4*R*)-1-(*N*-*tert*-butoxycarbonyl)-3-[(1'*R*)-phenyl ethylamino]-4-ethoxycarbonylpyrrolidine hydrochloride (**12**) (0.38 g, 1 mmol) in THF/methanol/water (6/3/1, v/v, 5 mL) and cooled in the ice-bath to 0 °C. Then, LiOH·H$_2$O (0.21 g, 5 mmol) was added and the mixture was stirred at 0 °C for 3 hours. Aqueous 1 N HCl (5.20 mL) was added at 0 °C and the solvent was removed under reduced pressure to give a white solid which was dissolved in 95% ethanol (5 mL) under H$_2$. 10% Pd on activate carbon (0.04 g) was added and the mixture was stirred at room temperature for 24 hours. The Pd/C was removed by filtration and washed with methanol and the filtrate was concentrated to give colorless oil. The oil was dissolved in acetone/water (2/1, v/v, 5 mL) and cooled to 0 °C. NaHCO$_3$ (0.50 g) was added, followed by Fmoc-OSu (0.34 g, 1.0 mmol). The mixture was stirred at 0 °C for 2 hours, then at room temperature overnight. In the next step, water (10 mL) was added and the remain organic layer was removed under reduced pressure, the aqueous layer was extracted with diethyl ether (3x50 mL). The aqueous residue was acidified with NaHSO$_4$ and extracted with ethyl acetate (3x50 mL). The combined organic extracts were washed with dilute brine (50 mL), dried over Na$_2$SO$_4$ anh. and concentrated to give a foamy solid. The crude product was purified by crystallization

from *n*-heptane/ethyl acetate to give compound **15** as white solid (0.29 g) in 66% yield, $[\alpha_D^{27}] = -22.0$ (20 mg/ 2 mL) in methanol, mp 104-105 °C.

^{1}H-NMR (400 MHz, CD_3OD): δ_H 1.46 [*s*, 9H, C$\underline{H}_3$ Boc] 2.92 [*t*, 1H, *J* = 6.4 Hz, $CH_2C\underline{H}$, Fmoc] 3.14 [*dd*, 1H, *J* = 5.6 Hz and 4.8 Hz, COC$\underline{H}$C] 3.54-3.71 [*m*, 4H, $C\underline{H}_2NC\underline{H}_2$, $\underline{C}_{2,5}$] 4.18 [*dd*, 1H, *J* = 5.6 Hz and *J* = 6.0 Hz, C$\underline{H}$NH] 4.34-4.36 [*m*, 2H, $C\underline{H}_2CH$, Fmoc] 7.28-7.40 [*m*, 4H, 4xC$\underline{H}$, Fmoc] 7.63 [*d*, 2H, *J* = 7.6 Hz, 2xC$\underline{H}$, Fmoc] 7.77 [*d*, 2H, *J* = 7.2 Hz, 2xC$\underline{H}$, Fmoc]; ^{13}C NMR (100 MHz, CD_3OD): δ_C 28.8 [$\underline{C}H_3$, Boc] 48.5 [$\underline{C}H$, $\underline{C}_4$] 50.5 [$CH_2\underline{C}H$, Fmoc] 51.3 [$\underline{C}HNH$, $\underline{C}_3$] 51.7, 52.2 [$\underline{C}H_2$, $\underline{C}_5$] 54.5, 55.2 [$\underline{C}H_2N$, $\underline{C}_2$] 67.8 [$\underline{C}H_2CH$, Fmoc] 81.1 [$\underline{C}CH_3$, Boc] 120.9 [2x$\underline{C}H$, Fmoc] 126.2 [2x$\underline{C}H$, Fmoc] 128.2 [2x$\underline{C}H$, Fmoc] 128.8 [2x$\underline{C}H$, Fmoc] 142.6 [2x$\underline{C}$, Fmoc] 145.3 [2x$\underline{C}$, Fmoc] 156.2 [$\underline{C}$=O, Boc] 158.3[$\underline{C}$=O, Fmoc] 177.0 [$\underline{C}$OOH]

9. (3*S*,4*R*)-1-(*N*-*tert*-Butoxycarbonyl)-3-(9*H*-fluoren-9-yl-methoxycarbonyl amino)-4-((pentafluorophenoxy)carbonyl)pyrrolidine (17)



(15) **(16)**

To a stirred solution of (3*S*,4*R*)-1-(*N*-*tert*-Butoxycarbonyl)-3-(9*H*-fluoren-9-yl-methoxycarbonylamino)-4-carboxypyrrolidine **(15)** (0.10 g, 0.22 mmol), pentafluorophenyl trifluoroacetate (PfpOTfa) (76.3 µL, 0.44 mmol) and *N*,*N*-diisopropylethylamine (DIEA) (75.7 µL, 0.44 mmol) in dichloromethane (1 mL) were stirred at room temperature for 30 min. The reaction was completed as indicated by TLC analysis. Dichloromethane (10 mL) was added and extracted with 10% HCl (2x20 mL). The organic extract was washed with water (20 mL) and extracted with excess saturated $NaHCO_3$ solution (2x20mL). The organic extracts dried over Na_2SO_4 anh. and concentrated *via* rotary evaporation. The crude product was purified by crystallization from hexane to give compound **16** as white solid (0.09 g) in 66% yield, $[\alpha_D^{27}] = -17.3$ (20 mg/ 2 mL) in chloroform, mp 95-97 °C.

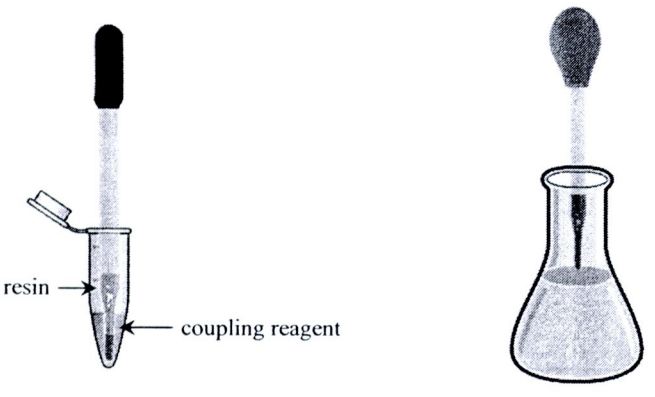
^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.51 [9H, *s*, C$\underline{H}_3$ Boc] 3.38 [1H, *s*, COC$\underline{H}$C] 3.49 [1H, *s*, $C\underline{H}_2NCH_2$, C_5] 3.81 [3H, *s*, $C\underline{H}_2NC\underline{H}_2$, $C_{2,5}$] 4.23 [1H, *t*, *J* = 5.2 Hz, $CH_2C\underline{H}$,

Fmoc] 4.52 [2H, *s*, C$\underline{H}_2$CH, Fmoc] 4.58 [1H, *s*, C$\underline{H}$NH] 5.12 [1H, *b*, CHN$\underline{H}$] 7.33 [*t*, 2H, *J* = 7.2 Hz, 2xC$\underline{H}$, Fmoc] 7.42 [2H, *t*, *J* = 7.2 Hz, 2xC$\underline{H}$, Fmoc] 7.58 [2H, *d*, *J* = 7.2 Hz, 2xC$\underline{H}$, Fmoc] 7.78 [2H, *d*, *J* = 7.2 Hz, 2xC$\underline{H}$, Fmoc]; ^{13}C NMR (100 MHz, CD_3OD): δ_C 28.4 [$\underline{C}H_3$, Boc] 46.2, 46.4 [$\underline{C}H_2$, $\underline{C}_5$] 47.2 [$CH_2\underline{C}H$, Fmoc] 48.3 [$\underline{C}H_2N$, $\underline{C}_2$] 49.9, 50.3 [$CH_2\underline{C}HC$, $\underline{C}_4$] 53.4, 53.9 [$\underline{C}HNH$, $\underline{C}_3$] 66.9 [$\underline{C}H_2CH$, Fmoc] 80.5 [$\underline{C}CH_3$, Boc] 120.0 [2x$\underline{C}$H, Fmoc] 124.9 [2x$\underline{C}$H, Fmoc] 127.1 [2x$\underline{C}$H, Fmoc] 127.8 [2x$\underline{C}$H, Fmoc] 136.6 [$\underline{C}$F, *p*, Pfp] 138.5 [2x$\underline{C}$F, *o*, Pfp] 139.2 [2x$\underline{C}$F, *m*, Pfp] 141.1 [$\underline{C}$, Pfp] 141.4 [2x$\underline{C}$, Fmoc] 143.7 [2x$\underline{C}$, Fmoc] 154.0 [$\underline{C}$=O, Boc] 155.5 [$\underline{C}$=O, Fmoc] 167.7 [$\underline{C}$OOH]; IR (KBr disc): 3331 cm^{-1} [N-H stretch] 3069 cm^{-1} [C-H stretch, aromatic, Fmoc] 2979 cm^{-1} [ν_{as}C-H stretch, aliphatic, CH_3 (Boc) and CH_2] 2894 cm^{-1} [ν_sC-H stretch, aliphatic, CH_3 (Boc) and CH_2] 1782 cm^{-1} [C=O stretch, ester] 1695 cm^{-1} [C=O stretch, carbamate] 1520 cm^{-1} [N-H bend, secondary amide band, carbamate] 1411[C-H bend, CH_2] 1385 [C-H bend, CH_3 (Boc)] 1275 [C-N stretch] 1171 [OC-O stretch] 1123 [C-F stretch, Pfp]

The procedure for solid phase synthesis of PNA

1. Preparation of reaction pipette and apparatus for solid phase synthesis

All peptide syntheses were carried out using a custom-made peptide synthesis columns equipped with sintered glass as previously described [45]. The resin was weighed into the peptide synthesis column and first swelled in DMF at least 1 hour before use. For each reactions, the reagent was directly sucked in, ejected out or hold on using rubber teat attached on the top of the column. All washings were performed by filling the solvent *via* the top of column. The excess solvent was ejected out by squeezing the rubber teat as shown in Figure 22.



(a) (b)

Figure 22 A diagram showing the manual technique for solid phase peptide synthesis; (a) coupling, deprotection and cleaving process; (b) washing process. [45]

2. Solid phase peptide synthesis of *apc*PNA

All PNA contains alternating *cis*-D-pyrrolidine monomers, APC and ACPC spacer. PNA **P1-P3** were homothymine nonamer carrying only ACPC, (3*R*,4*S*)-APC and (3*S*,4*R*)-APC spacer, respectively. PNA **P4-P8** were modified with a single APC unit in the middle of the ACPC strand. The PNA have fluorescent labels attached at the nitrogen atom in the pyrrolidine ring of the (3*R*,4*S*)-APC spacer. The PNA sequences synthesized in this work are shown in Table 1.

Table 1 Sequences of PNA oligomers used in this work

Code of PNA	Base Sequences (N-terminus to C-terminus)[a]
P1	Ac-TTTTTTTTT-Lys-NH_2
P2	Bz-TTTTTTTTT-Lys-NH_2
P3	Bz-TTTTTTTTT -Lys-NH_2
P4	Bz-TTTTTTTTT-Lys-NH_2
P5	Bz-TTTTTTTTT-Lys-NH_2

Table 1 (cont.)

Code of PNA	Base Sequences (N-terminus to C-terminus)[a]
P6	Bz-GTAGA$\underline{T}$CAC T-Lys-NH_2
P7	Ac-TTTT$^{(Py)}\underline{T}$TTTT-Lys-NH_2
P8	Ac-TTTT$^{(PyBu)}\underline{T}$TTTT-Lys-NH_2
P9	Ac-GTAGA$^{(Py)}\underline{T}$CAC T-Lys-NH_2
P10	Bz-GTAGA$^{(PyBu)}\underline{T}$CAC T-Lys-NH_2

[a] T = ssACPC-T; $\underline{T}$ = (3*R*,4*S*)-APC-T; $\underline{T}$ = (3*S*,4*R*)-APC-T, G = (2*S*,2*R*)-ACPC-G; C = (2*S*,2*R*)-ACPC-C; A = (2*S*,2*R*)-ACPC-A

P1 were synthesized by Miss Chalotorn Boonlua

Solid phase synthesis is a process by which chemical transformations can be carried out on solid support. Synthesized of PNA oligomers consist of three major steps as follows:

1. Removal of the *N*-terminal Fmoc group from the growing PNA oligomer

2. Coupling of the activated monomers onto the N-terminus of the growing PNA chain

3. Capping of the unreacted amino group

Each of the individual steps in the synthesis cycle has been previously optimized for the preparation of PNA oligomers. After coupling of last monomer, the resin-bound PNA was cleaved from the resin by the appropriate reagent [42,43].

The **P1-8** were synthesized according to the procedure outlined in Figure 23 as described previously.

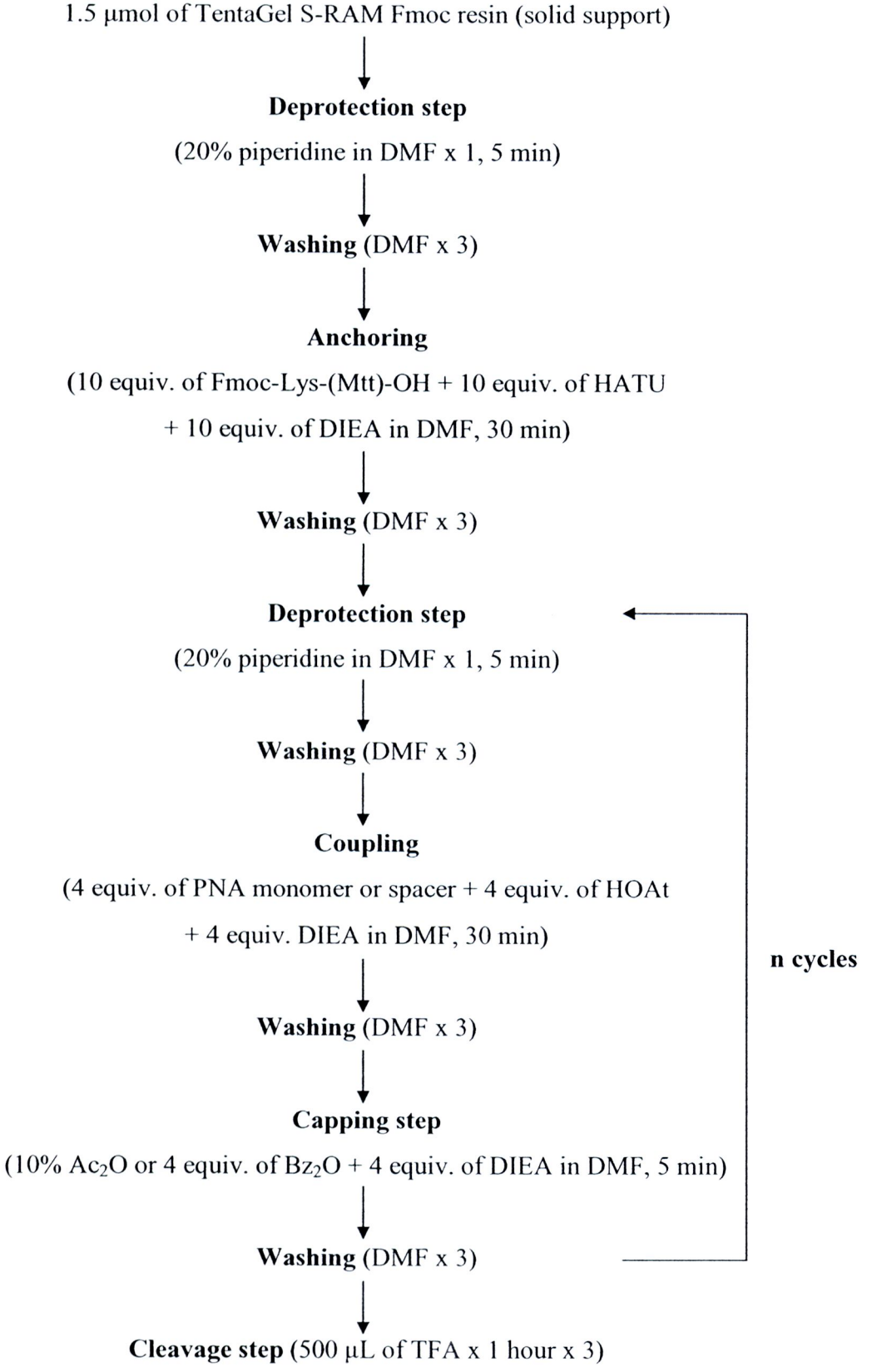


Figure 23 The procedure for solid phase synthesis of PNA

Syntheses of PNAs were typically carried out on 1.5 μmol scale. Before commencing the synthesis, the following three stocks solutions were prepared.

Stock 1: Deprotection solution consists of piperidine (200 μL), DBU (20 μL) in anhydrous DMF (780 μL)

Stock 2: Coupling and capping solution that consists of DIEA (70 μL) in anhydrous DMF (930 μL)

Stock 3: HOAt stock solution consists of HOAt (5.5 mg) in anhydrous DMF (100 μL)

2.1 Removal of the N-terminal Fmoc protection group of solid support

The reaction pipette prepared as described above was loaded with containing TentaGel S-RAM Fmoc resin (7.1 mg, 1.5 μmol). The resin was treated 100 μL of deprotection solution (stock 1) in a 1.5 mL eppendorf tube at room temperature for 5 min with occasional agitation. After the specified period of time, the reagent was squeezed off and the resin was extensively washed with DMF.

2.2 Anchoring with the first amino acid (Lys) residue

Fmoc-Lys-(Mtt)-OH (3.75 mg, 6 μmol), HATU (2.2 mg, 6 μmol) and 15 μL of DIEA stock 2 were mixed in a 1.5 mL eppendorf tube to give a yellow solution. The prepared resin was treated with this solution at room temperature for 30 min with occasional agitation. After the specified period of time, the reagent was squeezed off and the resin was extensively washed with DMF.

2.3 Deprotection of the Fmoc protection group at *N*-terminus

After the coupling and capping were completed, the resin was treated 100 μL of deprotection solution (stock 1) in a 1.5 mL eppendorf tube at room temperature for 5 min with occasional agitation. After the specified period of time, the reagent was squeezed off and the resin was extensively washed with DMF.

2.4 Coupling with PNA monomer

2.4.1 Pyrrolidine nucleobase monomer

After deprotect Fmoc group, the free amino groups were further coupled with the nucleobase monomer. The coupling solution consisted of the activated nucleobase monomer (6 μmol), DIEA stock 2 (15 μL) and HOAt stock 3 (15 μL) in a 1.5 mL eppendorf tube at room temperature for 5 min with occasional

agitation. After the specified period of time, the reagent was squeezed off and the resin was extensively washed with DMF.

2.4.2 ACPC or APC spacer

After coupling of nucleobase monomer, capping and removing of Fmoc group were completed. The resin was treated with the activated ACPC spacer (3.10 mg, 6 μmol) or APC spacer (3.71 mg, 6 μmol), DIEA stock 2 (15 μL) and HOAt stock 3 (15 μL) in a 1.5 mL eppendorf tube at room temperature for 5 min with occasional agitation. After the specified period of time, the reagent was squeezed off and the resin was extensively washed with DMF.

2.5 End capping

After anchoring or coupling step, the free amino residue was capped with 5 μL of acetic anhydride and 30 μL of DIEA stock 2 in a 1.5 mL eppendorf tube to prevent formation of deletion sequences. The reaction pipette was occasional agitated with this solution at room temperature for 5 min. After the specified period of time, the reagent was squeezed off and the resin was extensively washed 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 nonamer.

2.6 Acetylation and benzylation of nonamer PNA

The synthesis cycle was repeated until the growing peptide chain was extended up to nonamer. After final removal of Fmoc, the PNAs was treated with 5 μL of acetic anhydride and 30 μL of DIEA stock 2 in a 1.5 mL eppendorf tube for acetylation or 2 mg of benzoic anhydride and 30 μL of DIEA stock 2 in a 1.5 mL eppendorf tube for benzoylation. The reaction pipette was occasional agitated with this solution at room temperature for 5 min. After the specified period of time, the reagent was squeezed off and the resin was extensively washed with DMF.

2.7 Deprotection of nucleobase and APC protecting group

For PNA oligomers bearing adenine, cytosine or guanine bases and APC in the sequence, the protecting group (Bz for A and C, Ibu for G, Tfa for APC) must be removed before the cleavage of PNAs from the resin. The resin was treated with aqueous ammonia/dioxane (1/1, v/v) in a sealed test tube at 60 °C for 6 hours.

After the specified period of time, the reagent was squeezed off and the resin was extensively washed with methanol.

2.8 Attachment of pyrene fluorescent labels

Coupling of the pyrenecarbonyl and pyrenebutyryl labeling group at the nitrogen atom in the pyrrolidine ring of the (3*R*,4*S*)-*apc* spacer was carried out after removal of the protecting groups by ammonia treatment (step vii). The washed resin was re-swollen in DMF. The resin (0.5 μmol) treated with a mixture of pyrenecarboxylic acid (1.5 mg, 6 μmol, 12 equiv.) or pyrenebutyric acid (1.6 mg, 6 μmol, 12 equiv.), HATU (2.2 mg, 6 μmol) and 15 μL of DIEA stock 2 in a 1.5 mL eppendorf tube. The reaction pipette was treated with this solution at room temperature for 1 hour with occasional agitation. After the specified period of time, the reagent was squeezed off and the resin was extensively washed with DMF.

2.9 Cleavage of PNA oligomers from the resin

The resin-bound PNA was cleaved from the resin by treatment with trifluoroacetic acid (3 x 0.5 mL x 1 hour) in a 1.5 mL eppendorf tube at room temperature with occasional agitation. The resin became red during this time. The removal of trifluoroacetic acid was achieved by gentle nitrogen stream in fume hood. The resulting residue was treated with diethyl ether (1 mL), centrifuged and decanted to obtain the crude PNA as a white solid. Finally the crude PNA was air-dried at room temperature and stored at -20 °C until further used.

3. Purification of PNA oligomers

The crude PNA was prepared for HPLC analysis by dissolving the solid in 120 μL of MilliQ water. Analysis and purification were performed by reverse phase HPLC, monitoring by UV-absorbance at 254 nm and eluting with gradient system of 0.1% TFA in methanol/water. The following conditions were used for HPLC gradient system; Solvent A = 0.1% trifluoroacetic acid in methanol and Solvent B = 0.1% trifluoroacetic acid in MilliQ water. The gradient set-up is as shown in Table 2.

Table 2 Gradient system for HPLC purification of PNA

Time (min)	Flow Rate (mL/min)	Solvent A (%)	Solvent B (%)
0	0.5	10	90
5	0.5	10	90
90	0.5	90	10
100	0.5	90	10
110	0.5	10	90
120	0.5	10	90

4. Characterization

After purified by HPLC method, the PNA was collected and confirmed by MALDI-TOF mass spectrometry. The mass spectra of all PNA samples were on Microflex MALDI-TOF mass spectrometry and recorded in linear positive ion mode with accelerating voltage of 25 kV. All samples used matrix solution containing CCA in 0.1% trifluoroacetic acid in acetonitrile:water (1:2) solution. External mass calibration was performed using PNA known molecular weight.

Solid phase peptide synthesis of *apc*PNA

1. Solid phase peptide synthesis of T_9 (P2)

Synthesis of Bz-TTTTTTTTT-LysNH_2 (**P2**) was accomplished in the same way as described above. Starting from TentaGel S RAM Fmoc resin (7.1 mg, 1.5 μmol), Fmoc-Lys(Mtt)-OH (3.75 mg, 6 μmol) and monomer, Fmoc-T-Pfp (3.77 mg, 6 μmol), Fmoc-(3*R*,4*S*)-APC-Pfp (**9**) (3.71 mg, 6 μmol) were alternated in coupling step until the peptide had been extended up to nonamer. Final Fmoc removed and benzoylation was performing as usual. After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P2** showed $M{\cdot}H^+_{obs}$ = 3250.121, $M{\cdot}H^+_{calcd}$ = 3250.465

2. Solid phase peptide synthesis of T_9 (P3)

Synthesis of Bz-TTTTTTTTT-LysNH_2 (**P3**) was accomplished in the same way as described above. Starting from TentaGel S RAM Fmoc resin (7.1 mg, 1.5 μmol), Fmoc-Lys(Mtt)-OH (3.75 mg, 6 μmol) and monomer, Fmoc-T-Pfp (3.77 mg, 6

μmol), Fmoc-(3*S*,4*R*)- APC-Pfp (**17**) (3.71 mg, 6 μmol) were alternated in coupling step until the peptide had been extended up to nonamer. Final Fmoc removed and benzoylation was performing as usual. After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P3** showed $M{\cdot}H^{+}_{obs}$ = 3251.157, $M{\cdot}H^{+}_{calcd}$ = 3250.465

3. Solid phase peptide synthesis of T_9 (P4)

Synthesis of Bz-TTTTTTTTT-LysNH$_2$ (**P4**) was accomplished in the same way as described above. Starting from TentaGel S RAM Fmoc resin (7.1 mg, 1.5 μmol), Fmoc-Lys(Mtt)-OH (3.75 mg, 6 μmol) and monomer, Fmoc-T-Pfp (3.77 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol) were used in each coupling cycle respectively until a T_4 sequence was obtained. The resin was further coupled with Fmoc-T-Pfp (3.77 mg, 6 μmol) and Fmoc-Tfa-(3*R*,4*S*)-APC-Pfp (**10**) (3.68 mg, 6 μmol), respectively. Then the coupling was continued with Fmoc-T-Pfp (3.77 mg, 6 μmol) and Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol) alternately until the peptide had been extended up to nonamer. Final Fmoc removed and benzoylation was perform as usual. In case of Fmoc-Tfa-(3*R*,4*S*)-APC-Pfp (**10**) spacer, before cleavage form resin, the Tfa protecting group must be removed by treatment of the resin with ammonia/dioxane 1:1 at 60 °C for 6 hours. After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P4** showed $M{\cdot}H^{+}_{obs}$ = 3242.638, $M{\cdot}H^{+}_{calcd}$ = 3242.500

4. Solid phase peptide synthesis of T_9 (P5)

Synthesis of Bz-TTTTTTTTT-LysNH$_2$ (**P5**) was accomplished in the same way as described above. Starting from TentaGel S RAM Fmoc resin (7.1 mg, 1.5 μmol), Fmoc-Lys(Mtt)-OH (3.75 mg, 6 μmol) and monomer, Fmoc-T-Pfp (3.77 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol) were used in each coupling cycle respectively until a T_4 sequence was obtained. The resin was further coupled with Fmoc-T-Pfp (3.77 mg, 6 μmol) and Fmoc-(3*S*,4*R*)-APC-Pfp (**17**) (3.71 mg, 6 μmol), respectively. Then the coupling was continued with Fmoc-T-Pfp (3.77 mg, 6 μmol) and Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol) alternately until the peptide had been extended up to nonamer. Final Fmoc removed and benzoylation was perform as usual. After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P5** showed $M{\cdot}H^{+}_{obs}$ = 3243.692, $M{\cdot}H^{+}_{calcd}$ = 3242.500

5. Solid phase peptide synthesis of GTAGATCACT (P6)

Synthesis of Bz-GTAGATCACT-LysNH$_2$ (**P6**) was accomplished in the same way as described above. Starting from TentaGel S RAM Fmoc resin (7.1 mg, 1.5 μmol), Fmoc-Lys(Mtt)-OH (3.75 mg, 6 μmol) and monomer, Fmoc-T-Pfp (3.77 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-C-Pfp (4.30 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-A-Pfp (4.44 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-C-Pfp (4.30 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-T-Pfp (3.77 mg, 6 μmol), Fmoc-(3*R*,4*S*)-APC-Pfp (**10**) (3.68 mg, 6 μmol), Fmoc-A-Pfp (4.44 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-G-Pfp (4.34 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-A-Pfp (4.44 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-T-Pfp (3.77 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), Fmoc-G-Pfp (4.34 mg, 6 μmol), Fmoc-(1*S*,2*S*)-ACPC-Pfp (3.10 mg, 6 μmol), were used in each coupling cycle respectively until respectively. Final Fmoc removed and benzoylation was perform as usual. In case of mix-base and Fmoc-Tfa-(3*R*,4*S*)-APC-Pfp (**10**) spacer, before cleavage form resin, the protecting group must be removed by treatment of the resin with ammonia/dioxane 1:1 at 60 °C for 6 hours. After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P6** showed $M{\cdot}H^+_{obs}$ = 3622.180, $M{\cdot}H^+_{calcd}$ = 3620.910

6. Solid phase peptide synthesis of TTTT$^{(Py)}$TTTTT (P7)

Synthesis of Ac-TTTT$^{(Py)}$TTTTT-LysNH$_2$ (**P7**) was accomplished in the same way as described for T_9 (**P5**) above. Final Fmoc removed, acetylation and removed the Tfa protecting group of Fmoc-Tfa-(3*R*,4*S*)-APC-Pfp (**10**) spacer was perform as usual, the resin was further coupled with pyrenecarboxylic acid (1.5 mg, 6 μmol). After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P7** showed $M{\cdot}H^+_{obs}$ = 3411.023, $M{\cdot}H^+_{calcd}$ = 3409.500

7. Solid phase peptide synthesis of TTTT$^{(PyBu)}$TTTTT (P8)

Synthesis of Ac-TTTT$^{(PyBu)}$TTTTT-LysNH$_2$ (**P8**) was accomplished in the same way as described for T_9 (**P5**) above. Final Fmoc removed, acetylation and removed the Tfa protecting group of Fmoc-Tfa-(3*R*,4*S*)-APC-Pfp (**10**) spacer was perform as usual, the resin was further coupled with pyrenebutyric acid (1.7 mg, 6

μmol). After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P8** showed $M{\cdot}H^{+}_{obs}$ = 3452.030, $M{\cdot}H^{+}_{calcd}$ = 3451.550

8. Solid phase peptide synthesis of GTAGA$^{(Py)}$TCACT (P9)

Synthesis of Ac-GTAGA$^{(Py)}$TCACT-LysNH$_2$ (**P9**) was accomplished in the same way as described for GTAGATCACT (**P6**) above. Final Fmoc removed, acetylation and removed the Tfa protecting group of Fmoc-Tfa-(3*R*,4*S*)-APC-Pfp (**10**) spacer was perform as usual, the resin was further coupled with pyrenecarboxylic acid (1.5 mg, 6 μmol). After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P9** showed $M{\cdot}H^{+}_{obs}$ = 3789.445, $M{\cdot}H^{+}_{calcd}$ = 3788.900

9. Solid phase peptide synthesis of GTAGA$^{(PyBu)}$TCACT (P10)

Synthesis of Bz-GTAGA$^{(PyBu)}$TCACT-LysNH$_2$ (**P10**) was accomplished in the same way as described for GTAGATCACT (**P6**) above. Final Fmoc removed, benzoylation and removed the Tfa protecting group of Fmoc-Tfa-(3*R*,4*S*)-APC-Pfp (**10**) spacer was perform as usual, the resin was further coupled with pyrenebutyric acid (1.7 mg, 6 μmol). After cleavage PNAs from resin and purification, MALDI-TOF mass spectrum of **P10** showed $M{\cdot}H^{+}_{obs}$ = 3893.667, $M{\cdot}H^{+}_{calcd}$ = 3893.010

Biophysical studies

1. T_m experiment

The T_m curves were measured on 260 nm with a CARY 100 Bio UV-Visible spectrophotometer (Varian Ltd.) equipped with a thermal melt system (Organic Synthesis Research Unit, Faculty of Science, Chulalongkorn University). The sample for T_m experiment was prepared by mixing calculated amount of stock oligonucleotide and PNA solution together to give final concentration of nucleotides, 10 mM sodium phosphate buffer (pH 7.0), 100 mM sodium chloride and final volumes were adjusted to 1000 μL in a 10 mm quartz cell with a Teflon stopper and equilibrated at the starting temperature for 10 min. The A_{260} was recorded in heating from 20-90 °C (block temperature) with a temperature ramp of 1 °C/min. The temperature recorded was the block temperature and was corrected by a linear equation obtained from a built-in temperature probe.

Correct temperature and normalized absorbance are defined as follows.

$$\text{Correct Temp.} = (0.9696 \times T_{block}) - 0.8396$$

$$\text{Normalized Abs.} = Abs_{obs}/Abs_{init}$$

The melting temperature was determined from the maximum of the first derivative after smoothing using KaliedaGraph 3.6 (Synergy Software). Data analysis was performed on a PC compatible computer using Microsoft Excel XP (Microsoft Corp.). T_m values obtained from independent experiments were accurate.

2. UV-titration experiment

The UV-titration experiment was performed on a MALTON ROY spectronic 3000 array UV spectrophotometer in 25 °C (Organic Synthesis Research Unit, Faculty of Science, Chulalongkorn University). To a solution containing the T_9 **(P2)** (2 μM), 10 mM sodium phosphate buffer pH 7.0 (8 μL) and 100 mM sodium chloride (80 μL) was added a 5-10 μL aliquot of a concentrated stock solution of dA_9 (40 μM). After the absorbance is stabilized (10-15 min) the absorbance was read against a blank (10 mM sodium phosphate) and more dA_9 aliquot were added until a total volume of 200 μL (corresponds to 1:4 PNA:DNA ratio) had been added. The ratio of the observed A_{260} and the calculated A_{260} were plotted against the mole ratio T:A nucleotide and the stoichiometry was determined from the inflection point.

$$\text{Calcd. } OD_{260} = \frac{OD_{260(PNA2)} \times V_{PNA2} + OD_{260(DNA)} \times V_{DNA}}{V_{PNA2} + V_{DNA}}$$

$$= \frac{0.16 \times 800\mu L + 3.60 \times V_{DNA}(\mu L)}{800\ \mu L + V_{DNA}(\mu L)}$$

$$\text{Ratio of T:A} = \frac{\varepsilon_{(DNA)} \times OD_{260(PNA2)} \times V_{PNA2}}{\varepsilon_{(PNA2)} \times OD_{260(DNA)} \times V_{DNA}}$$

$$= \frac{97.2 \times 0.16 \times 800\mu L}{79.2 \times 3.60 \times V_{DNA}(\mu L)}$$

3. Circular dichroism spectroscopy

CD experiments were performed on JASCO Model J-715 spectropolarimeter (Pharmaceutical Research Equipment Center, Faculty of Pharmaceutical Science, Chulalongkorn University). The sample for CD experiment was prepared by mixing calculated amount of stock oligonucleotide and PNA solution together to give final concentration of nucleotides, 10 mM sodium phosphate buffer (pH 7.0), 100 mM sodium chloride and final volumes were adjusted to 1000 μL in a 10 mm quartz cell with a Teflon stopper. The spectra were measured at 25 °C from 200-400 nm.

4. Fluorescence experiments

Fluorescence experiments were performed on a fluorescence spectrophoto-meter at Department of chemistry, Faculty of Science, Chulalongkorn University. The sample for fluorescence experiment was prepared by mixing calculated amount of stock oligonucleotide and PNA solution together to give final concentration of nucleotides, 10 mM sodium phosphate buffer (pH 7.0) and final volumes were adjusted to 1000 μL in a 10 mm quartz cell with a Teflon stopper. Excitation wavelength was 345 nm at 25 °C.