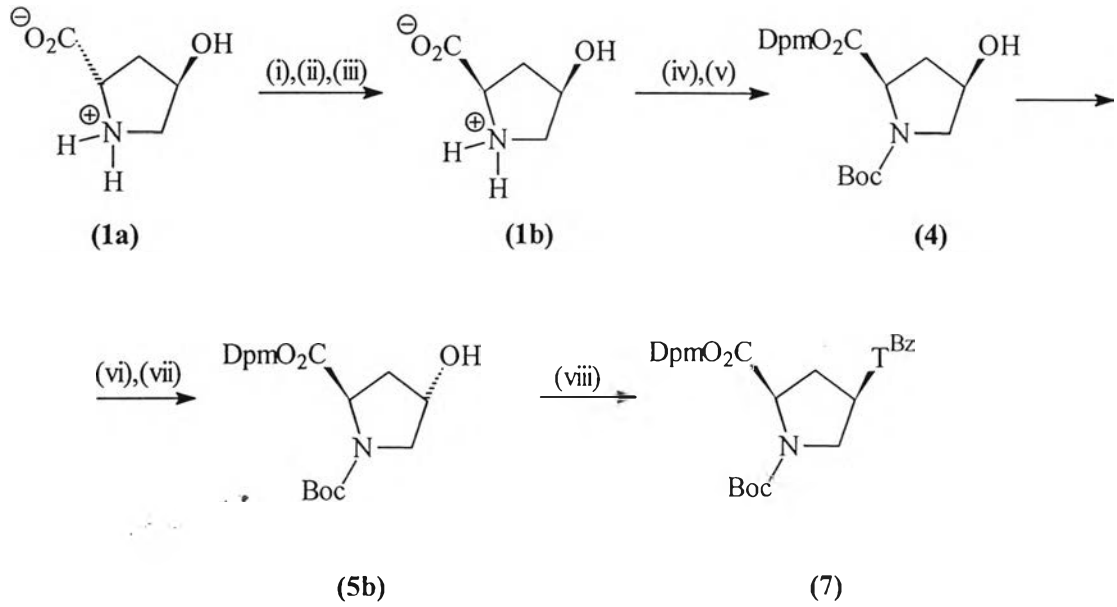
CHAPTER III

RESULTS AND DISCUSSION

Syntheses and properties of novel cPNA will be discussed in each part as follows.

3.1 Synthesis of cPNA monomers

3.1.1 Synthesis of intermediate, *N-tert*-butoxycarbonyl-*cis*-4-(N^3-benzoylthymin-1-yl)-D-proline diphenylmethyl ester (7)



i) Ac_2O / AcOH reflux 5.5 hr
ii) aq HCl reflux 3 hr
iii) Et_3N / EtOH then fractional crystallization
iv) Boc_2O/aq NaOH / ^tBuOH rt O/N
v) $Ph_2C{=}N^+{=}N^-$, EtOAc rt O/N
vi) HCO_2H / PPh_3 / DEAD in THF -20 °C rt O/N
vii) aq NH_3 / MeOH rt 1 hr
viii) T^{Bz} / PPh_3 / DEAD in THF -20 °C rt O/N

Figure 3.1 Synthesis of intermediate [Boc-D-Pro(*cis*- 4-T^{Bz})-ODpm, (7)]

Synthesis of building blocks required for both serine-containing cPNA and deoxyglycylproline cPNA requires the same common intermediate, ie. protected proline bearing thymine [Boc-D-Pro(*cis*-4-T^{Bz})-ODpm, (**7**)].[63] Synthesis of compound (**7**) have been reported and was summarized in figure 3.1. Since the starting material, *cis*-4-hydroxy-D-proline or (*2R,4R*)-4-hydroxy proline **(1b)** was more expensive than naturally occurring *trans*-L-isomer **(1a)** about 30 times, we have therefore attempted to find out possible methods which could provide the *cis*-D-isomer with reasonable cost and without too much difficulty. An effective method to epimerize *trans*-4-hydroxy-L-proline into *cis*-4-hydroxy-D-proline involving the inversion of configuration at the α-carbon has been reported by Greenstein.[64] Refluxing *trans*-4-hydroxy-L-proline in acetic anhydride/acetic acid mixture gave an equilibrium mixture of *N,O*-diacetylated of *cis*-D-epimer and *trans*-L-epimer at a ratio of 2:1, presumably *via* enolization at the α-carbon of the mixed anhydride intermediate (figure 3.2)

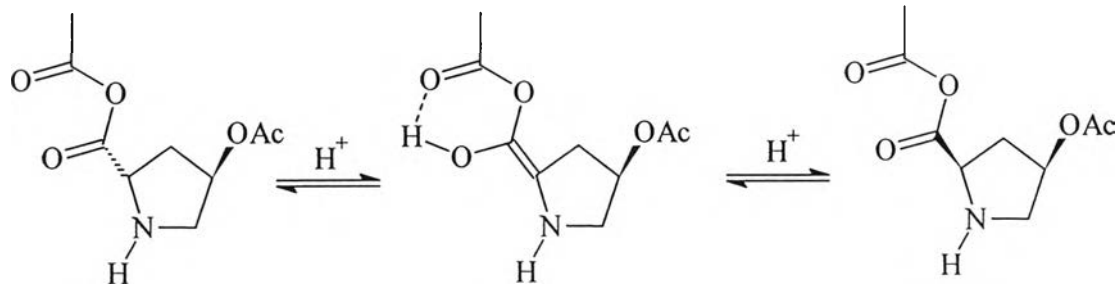


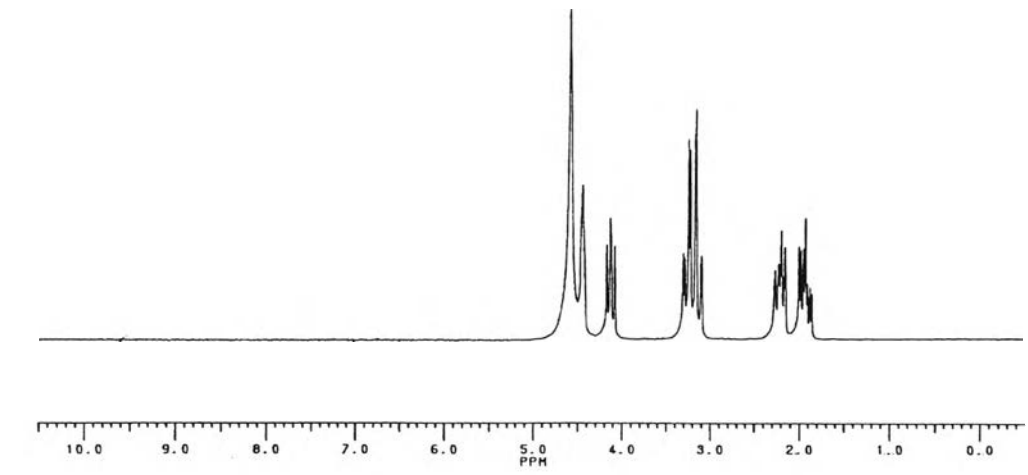
Figure 3.2 Epimerizaion of *trans*-4-hyroxy-L-proline into *cis*-4-hydroxy-D-proline *via* enolization mechanism

Acid hydrolysis of the initially formed *N,O* diacetylated adducts with dilute HCl gave a mixture of the *cis*-D and *trans*-L hydroxy proline hydrochloride salt. Stille reported a successful to separation of the *cis*-D-epimer from the mixture of hydroxyproline hydrochloride salt by recrystallization from ethanol-water,[65] but the result was not reproducible in our hand. An alternative method has been reported by Greenstein[64] whereby the chloride ion was first eliminated from the mixture of hydroxyproline hydrochloride epimers by treatment with silver carbonate followed by filtration to remove the silver chloride precipitate. Excess silver ion was subsequently removed by hydrogen sulfide treatment and filtration. However this method was considered unsuitable since it was too complicated and involved the use of expensive reagents. Vilaivan reported a more

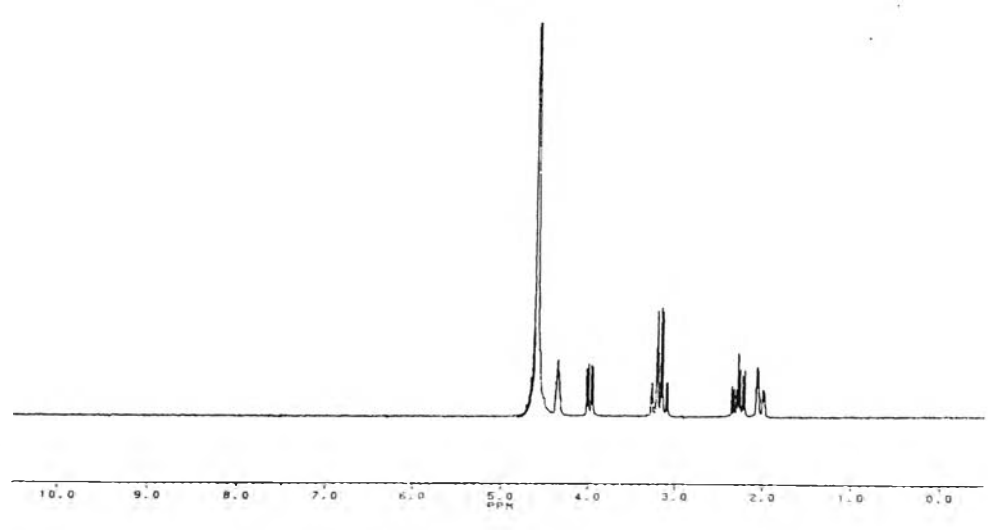
convenient method by using some organic bases such as pyridine, morpholine or triethylamine instead of silver carbonate and found to gave identical results to Greenstein method.[63] In the present study, triethylamine was chosen first due to its availability, low toxicity and cost. Hence it was decided to start our research with this acetic anhydride-acetic acid epimerization method and after hydrolysis with dilute HCl, the reaction mixture was adjusted to pH 8 by triethylamine followed by addition of absolute ethanol (9 fold volume). Under this condition, excess triethylamine and triethylamine hydrochloride salt were readily soluble while the free hydroxyproline epimers precipitated off. Notwithstandingly, parts of hydroxyproline was remained dissolved in the filtrate, therefore attempts to recover this material have been made. Firstly, sulfuric acid was used instead of hydrochloric acid in hydrolysis step with the hope that the residual sulfate ion would be removed as barium sulfate by treatment with barium hydroxide, but the result was unsuccessful due to the low solubility of both barium hydroxide and barium sulfate by-product which interfered the observation of the equivalent point. Secondly, it was thought that triethylamine and amphoteric hydroxyproline should have different affinity towards strongly basic anion exchange resin (hydroxyl form). A preliminary experiment showed that triethylamine could not be absorbed on the resin but chloride ion and hydroxy proline as zwitter ion did. The hydroxyproline was subsequently eluted from the resin with dilute HCl which converted the proline into free acid form, hence not further absorbed on the resin and was eluted from the resin as hydrochloride salt. According to the results, this ion chromatography method was seemed to be the best choice to recover the starting material.

We turned to consider the method to purify the hydroxyproline epimers free from triethylamine and chloride ion. Indeed, hydroxyproline epimers might be considered as a diastereomeric pair, hence it should be possible to separate the *cis*-D-and *trans*-L-isomer depending on the difference in some physical properties. Fractional crystallization using ethanol-water system have been reported to give good results.[63] However, we found that methanol containing a few drops of water was a better solvent system and gave exclusive *cis*-D-epimer **(1b)** up to 36 % which was higher than any previous reports. In addition, after removal of methanol-water, a mixture containing mainly *trans*-4-hydroxy-L-proline could be recovered from the mother liquor and may be recycled. The identity and purity of the *cis*-D epimer product was proved by comparison of its ^{1}H NMR spectrum with

authentic *cis*-4-hydroxy-D-proline. There was no trace peak of *trans*-4-hydroxy-L-proline (figure 3.3 a) appeared in the spectrum of *cis*-D-proline (figure 3.3 b) so obtained.



(a)



(b)

Figure 3.3 Comparison of ^{1}H NMR spectra (D_2O, 200 MHz) between *trans*-4-hydroxy-L-proline (a) and *cis*-4-hydroxy-D-proline (b)

This method requires a large amount of acetic anhydride which could not be easily obtained due to its strict regulation in Thailand. This obstacle hampered scaling up the synthesis therefore an alternative epimerization method was saught. Other attempts have been made in order to find the alternative epimerization methods without the need for acetic anhydride. Yamada has reported an effective method involving aldehyde series acted

as catalyst for epimerization of many amino acids, including proline.[66] Our first attempt was made with salicylaldehyde, but it was not seem to be worthwhile because analysis of ^{1}H NMR spectrum of the crude product after heating in glacial acetic acid at 100 °C for 3 hr showed less than 50 % of the *cis*-D isomer. Extending the reaction time did not change the ratio of the product but some decomposition was observed as suggested by formation of dark viscous impurities. Moreover, it was difficult to recover the starting material from the dirty reaction mixture. Other aldehyde including propionaldehyde, butyraldehyde or isobutyraldehyde gave similar poor results. Synthesis of *N,O*-diacetyl *trans*-4-hydroxy-L-proline by reaction with acetyl chloride in dried pyridine before epimerization in acetic acid was also attempted, but the result was not successful. According to the results above, no improvement could be made for this step, therefore we still chose the acetic anhydride/acetic acid as epimerizing reagent with minor modification for fractional crystallization by using methanol-water and method for recovery of the starting material by ion exchange chromatography. Luckily, acetic anhydride might be conveniently prepared *via* reaction between acetyl chloride and sodium acetate anhydrous followed by distillation according to the literature.[60] Not surprisingly, the resulting acetic anhydride could be used for epimerization hydroxyproline to give the same result as the reagent grade.

In the next step, the amino group of *cis*-4-hydroxy-D-proline **(1b)** was protected with a temporary protecting group in order to avoid the undesired nucleophilic displacement by nitrogen of the pyrrolidine ring. Two most popular urethane-type protecting groups, namely *tert*-butoxycarbonyl (Boc) and fluoren-9-ylmethoxycarbonyl (Fmoc) were considered for this purpose. Boc protection has considerable advantages because it is inexpensive and remarkably stable towards both nucleophiles and bases which is expected to be the condition of nucleobase substitution in the next above step while the Fmoc group is too base-labile. Reaction between *cis*-4-hydroxy-D-proline **(1b)** and di-*tert*-butyl dicarbonate (Boc_2O) in aqueous sodium hydroxide/*tert*-butanol gave *N*-*tert*-butoxy carbonyl-*cis*-4-hydroxy-D-proline sodium salt and carbon dioxide as a by-product (figure 3.4).

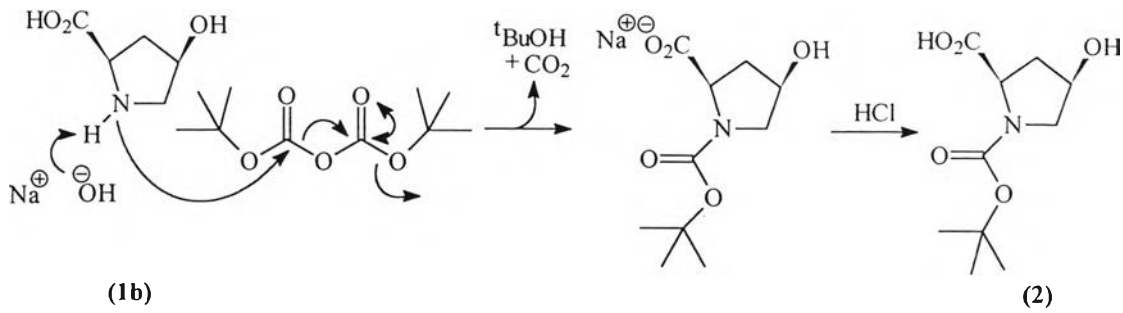


Figure 3.4 Reaction mechanism for synthesis of *N-tert*-butoxycarbonyl-*cis*-4-hydroxy-D-proline **(2)**

The reaction mixture was worked up by acidification followed by extraction and evaporation to afford Boc-D-Pro(*cis*-4-OH)-OH **(2)** (97% yield). ^{1}H NMR analysis showed two sharp singlet peaks at 1.32 and 1.37 ppm due to rotamers of the Boc group, resulted from the presence of two sets of non-equivalent *tert*-butyl protons due to the restricted rotation around the C-N bond. The two rotamers coexist in equilibrium and gave rise to two different sets of signals for protons in each of them. This phenomenon is common for amides which possess two resonance forms (figure 3.5 a) and give rise to a high rotational barrier (in the order of 75-90 kJ/mol) for interconversion between the *cis*- and *trans*-amide bond (figure 3.5 b).[67] This restricted rotation resulted in a slow rotation on the NMR time scale at room temperature therefore two sets of peaks due to each rotamer were observed.

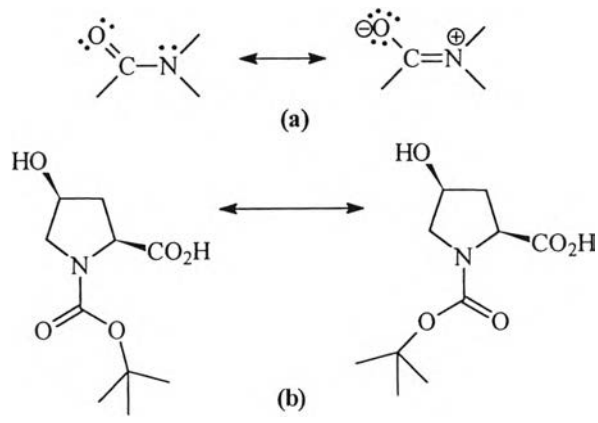


Figure 3.5 (a) The two resonance forms of amide bond; (b) restricted rotation around C-N bond in Boc-D-Pro(*cis*-4-OH)-OH **(2)**

In the second step, the carboxyl function of Boc-derivative **(2)** in figure 3.6 was then protected to avoid side reactions during introduction of the nucleobase. Simple methyl ester was not suitable due to its too high stability, thus very difficult to remove without affecting other parts of the molecule. In this respect, acid labile protecting groups such as *tert*-butyl (^{t}Bu), diphenylmethyl (Dpm) or benzyl esters carrying electron donating group which must also have a high degree of orthogonality to the Boc protecting should be considered. Dpm group was found to be the best candidate due to its good stability under nucleophilic condition yet its deprotection can be achieved under different conditions such as acidic, basic or hydrogenolysis condition. Tozuka has reported protection of *trans*-4-hydroxy-L-proline as Boc/Dpm derivative.[68] Similar protection of *cis*-D isomer has also been recently reported by Vilaivan.[63] Boc-D-Pro(*cis*-4-OH)-OH **(2)** was treated with freshly prepared diphenyldiazomethane **(3)** which gave product as *N*-*tert*-butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester **(4)** with a good yield (87 %) together with nitrogen gas as a by-product (figure 3.6). Diphenyldiazomethane was prepared from oxidation of benzophenone hydrazone with yellow mercury (II) oxide in the presence of potassium hydroxide as catalyst and anhydrous sodium sulfate to remove any water in the reaction.[61] Because diphenyldiazomethane might decomposed under photolytic or thermolytic condition, the preparation condition should free from these risk and the product must be used immediately. ^{1}H NMR analysis of **(4)** revealed two singlet peaks of C<u>H</u>Ph$_2$ rotamers at 6.86 and 6.94 ppm and broad multiplet with 10 H of integration at 7.31-7.62 ppm of phenyl aromatic C<u>H</u>, which confirmed the success of Dpm protection.

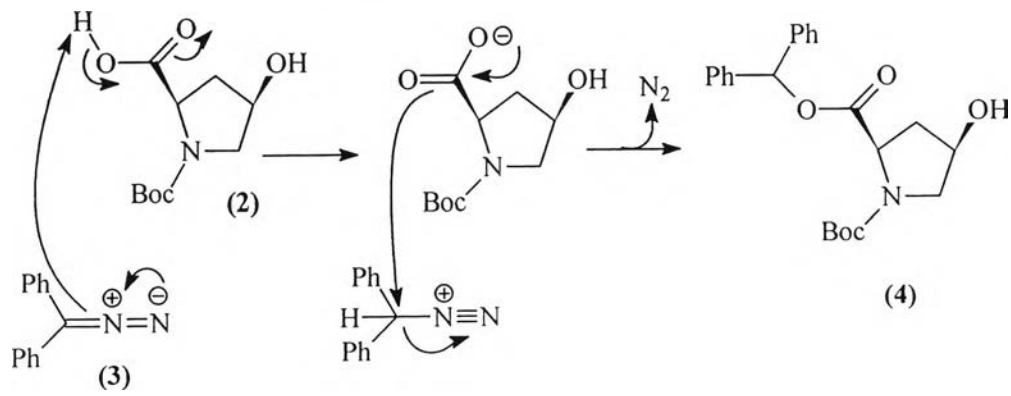


Figure 3.6 Reaction mechanism of Boc-D-Pro(*cis*-4-OH)-OH **(2)** reacted with diphenyl-diazomethane **(3)**

Many alkylation methods have been reported in order to introduce nucleobases into the proline ring at C-4 position.[63] Attempts to inverse the configuration of the 4-OH group of **(4)** by tosylation first followed by nucleobase displacement *via* S_N^2 reaction was only successful with adenine or cytosine (figure 3.7). Other methods, for example, ring construction of the pyrimidine or purine ring from protected 4-aminoproline and 4-amino-prolinol has been reported but it requires a large number of steps and harsh conditions.[69,70,71,72,73] Direct coupling of protected hydroxyproline with unprotected thymine by Mitsunobu reaction was also reported to gave O^2, O^4-disubstituted derivative as undesired product.[63]

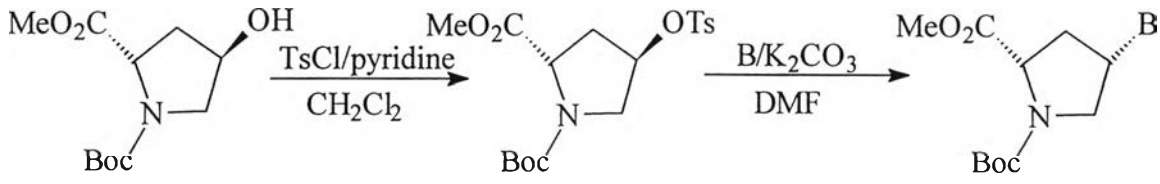


Figure 3.7 Introducing nucleobase into proline ring *via* tosylation and S_N^2 displacement where B is adenine or cytosine

In 1997, regioselective alkylation of fully protected hydroxyproline with N^3-benzoylthymine under Mitsunobu condition which gave only N^1-substituted producted with inversion of configuration at C-4 have been reported based on the pioneer work by Benner et. al.[74] This method offers two advantages over the previous method: mild condition for synthesis and no prior conversion of hydroxyl group into a good leaving group is needed. With our requirement that after the nucleobase alkylation, the product would have to retain the *cis*-D-configuration, thus the fully protected *cis*-D-proline must be converted into *trans*-D-isomers (first inversion) before alkylation with nucleobase (second inversion). Although the first inversion step might be accomplished *via* tosylation followed by S_N^2 displacement with acetate ion and hydrolysis respectively.[64] Mitsunobu reaction with acetic or formic acid seemed to be more attractive since it was a one step procedure. The success of this methods depended on the quality of the starting material which had to be free from water and other nucleophiles in order to prevent the side reactions. The mechanism is proposed to involve the 4-OH group of compound **(4)** was deprotonation by

PPh_3-DEAD complex and become to anion which re-attack the phosphorus center to give the phosphonium compound. Subsequent S_N^2 displacement of the phosphonium salt by formate ion gave *trans*-4-formate ester **(5a)** and by products (triphenylphosphine oxide and diethylhydrazinedicarboxylate) which was separated by column chromatography to give **(5a)** as the exclusive product (95 %) (figure 3.8). Diisopropylazodicarboxylate (DIAD) have also been successfully used in place of DEAD because it is cheaper than DEAD (about 2 times). ^{1}H NMR analysis showed a diagnostic signal at 7.97 ppm (HCO), ^{13}C NMR analysis at 160.2 ppm (HCO) and IR signal at 1751 cm^{-1}, all indicated the presence of formate group. After methanolysis of this formate ester using ammonia/methanol, Boc-D-Pro(*trans*-4-OH)-ODpm **(5b)** was obtained in excellent yield (92%). Comparison of ^{1}H NMR spectrum between *trans*-D-derivative **(5b)** and *cis*-D-derivative **(4)** revealed that they are not quite identical due to the influence of different stereochemistry at C-4 position.

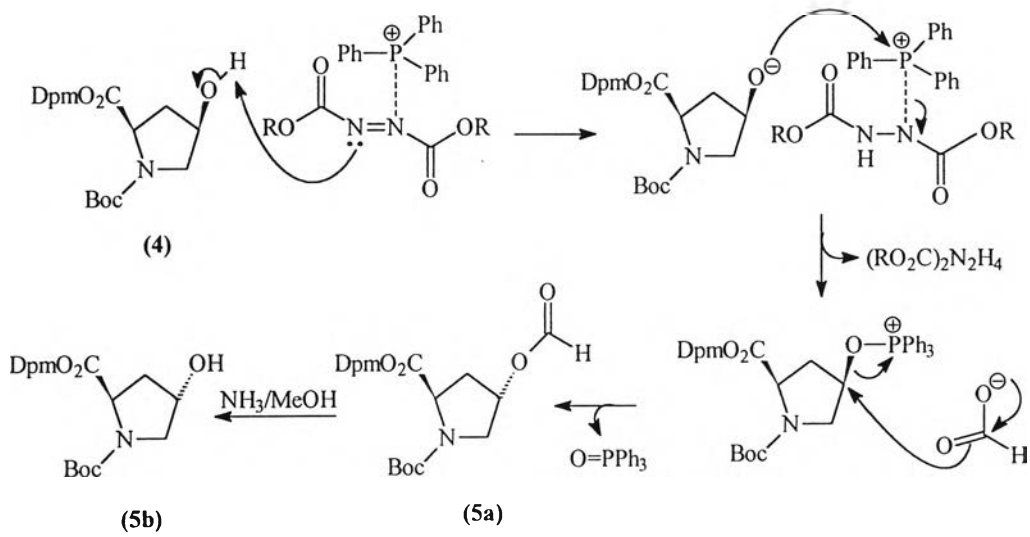


Figure 3.8 Synthesis of *trans*-4-formate ester **(5a)** and Boc-D-Pro(*trans*-4-OH)-ODpm **(5b)** where R= Et is DIEA; R= iPr is DIAD

Incorporation of thymine into the proline ring have also been achieved by Mitsunobu reaction. In order to avoid the regioselectivity problem of N^1/N^3 alkylation, thymine was protected as N^3-benzoyl derivative before the reaction. Preparation of N^3-benzoylthymine was achieved according to the literature.[75] Reaction between thymine with excess benzoyl chloride in anhydrous pyridine/acetonitrile for 16 hr gave a mixture of

N^3-monobenzoylate and N^1,N^3-dibenzoylate adduct as indicated by TLC analysis. The dibenzoylate adduct lose the N^1-benzoyl group easily under alkaline hydrolysis using 0.25 M potassium carbonate in aqueous dioxane at 70 °C for 45 min. After removal of benzoic acid by-product by recrystallization from absolute ethanol, the N^3-benzoylthymine **(6)** was obtained in moderate yield (74%). It should be noted that this might be one reason that why regioselective alkylation *via* S_N2 displacement of tosylate with N^3-benzoylthymine under potassium carbonate condition failed, if debenzoylation has occurred prematurely and hence led to non-regioselective alkylation. Synthesis of intermediate compound **(7)** was successfully achieved by Mitsunobu reaction as described in the literature.[63] Reaction of *trans*-D-derivative **(5b)**, DEAD or DIAD, triphenylphosphine and N^3-benzoylthymine **(6)** in THF gave Boc-D-Pro-(*cis*-4-T^{Bz})-ODpm **(7)** with acceptable yield (47 %) according to the mechanism shown in figure 3.9.

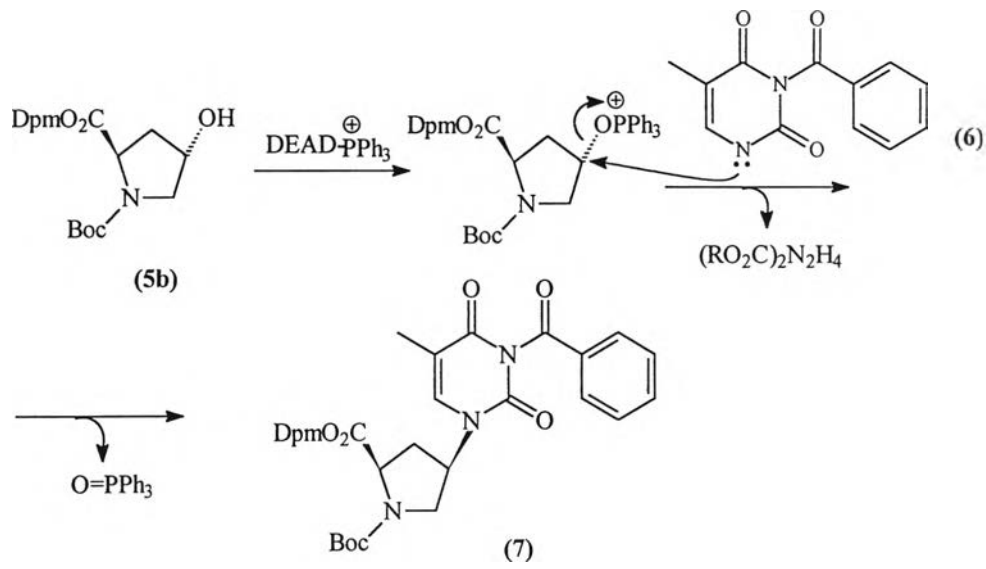


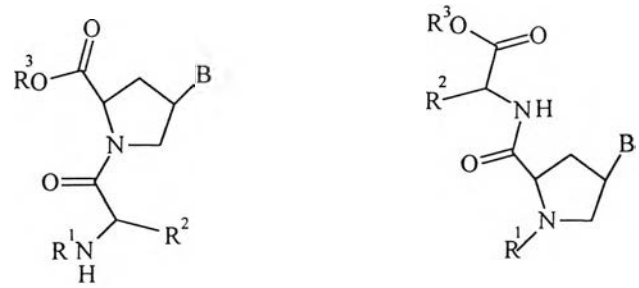
Figure 3.9 Synthesis of intermediate **(7)** *via* Mitsunobu reaction

According to the literature,[63] the product have been purified by column chromatography but it was discovered that the product is only slightly soluble in cold methanol while other by-products (triphenylphosphine oxide and diethyl hydrazinedicarboxylate) are readily soluble Hence, purification of product (7) could be simplified by recrystallization of the crude reaction mixture from cold methanol which gave a better results without the need for extensive chromatography.

3.1.2 Synthesis of serylproline cPNA monomers

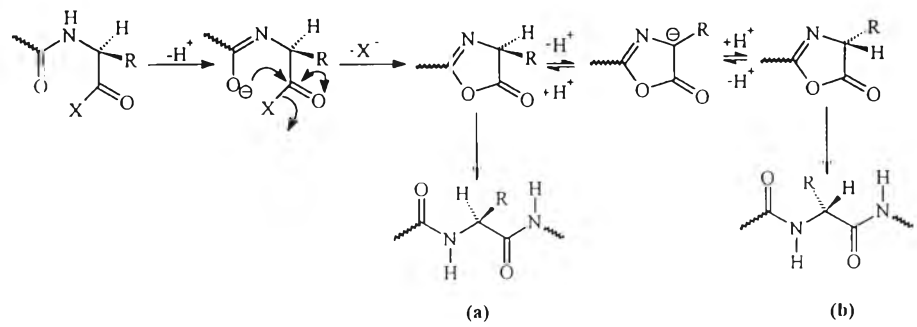
It has been noted that combination of two amino acids to form dipeptide repeating unit prior to oligomerization on solid phase synthesis resulted in more efficient peptide synthesis. This technique was known as "dipeptide fragment coupling" and provide more advantages compared to the conventional stepwise coupling, for example reduction the number of solid phase coupling by a half, reduction the waste of the starting materials and reaction time. Furthermore it was also believed that this method might serve to increase the overall yield and minimize the risk of failure in peptide synthesis.[63]

Although there are two possible ways to join the modified proline derivative **(7)** with Fmoc-spacer amino acid as shown in figure 3.10, successful synthesis of the analogous glycylproline cPNA *via* dipeptide of type (a) warrants its use in this case too.[63] Although dipeptide (a) needs more synthesis steps and involves difficult peptide bond formation process, it helps avoiding racemization by mean of oxazolone mechanism since the C-terminal amino acid was proline which could not participate in such racemization mechanism (figure 3.11)



(a) (b)

Figure 3.10 Two possible ways to join intermediate **(7)** and a spacer amino acid together



(a) (b)

Figure 3.11 Racemization of a peptide by oxazolone mechanism **(a)** retention;**(b)**inversion

The protected dipeptide Fmoc-L-Ser(O^{t}Bu)-D-Pro(*cis*-4-T^{Bz})-ODpm **(8)** was synthesized in an analogous manner to the glycylproline dipeptide monomer.[63] Intermediate **(7)** was treated with 2.5 equivalents of *p*-toluenesulfonic acid in acetonitrile in order to remove the Boc-protecting group without cleavage of Dpm ester which provided the free amine in form of tosylate salt. Without extra-purification the solution was then neutralized with excess diisopropylethylamine (DIEA). The reason for choosing this amine was not only because DIEA possesses low nucleophilicity and basicity due to steric effect therefore it did not destroy Fmoc protecting group even at high concentration.[76] The resulting free amine was immediately coupled with Fmoc-L-Ser(O^{t}Bu)-OH in the presense of dicyclohexylcarbodiimide (DCC) as coupling agent and 1*H*-hydroxybenzotriazole (HOBt) as catalyst in DMF to give the protected dipeptide product according to the mechanism proposed in figure 3.12

After column chromatography, Fmoc-L-Ser(O^{t}Bu)-D-Pro(*cis*-4-T^{Bz})-ODpm **(8)** was obtained in acceptable yield (60%). A 65% yield was also realized in the case of D-series **(9)** under identical condition. Identification of product was performed by elemental analysis, ^{1}H and ^{13}C NMR.

The next step was selective deprotection of Dpm ester without cleavage of both Fmoc and *tert*-butyl ether group. The presence of *tert*-butyl ether has troublesome effects since it is unstable under acidic conditions similar to Dpm ester while the Fmoc group was base labile. Selective deprotection under acidic conditions was first attempted with some common acids, eg, *p*-toluenesulfonic acid/acetonitrile or HCl/dioxane. Disappointingly all results revealed that the complete deprotection of both Dpm and *tert*-butyl ether according to TLC analysis after many variations of the acid quantity to use. Selective deprotection under basic condition was considered next even if it would almost certainly has negative effect to the Fmoc protecting group. Pascal and co-worker reported that selective deprotection of some amino acid methyl ester carrying Fmoc group as N-terminus protecting group could be accomplished by using alkaline hydrolysis (NaOH/water) modified with calcium chloride/ isopropanol at room temperature.[77] It was believed that calcium ion could coordinate with the C=O group of the ester, and therefore stabilize the negatively charged tetrahedron transition state and also reduced the hydroxide ion

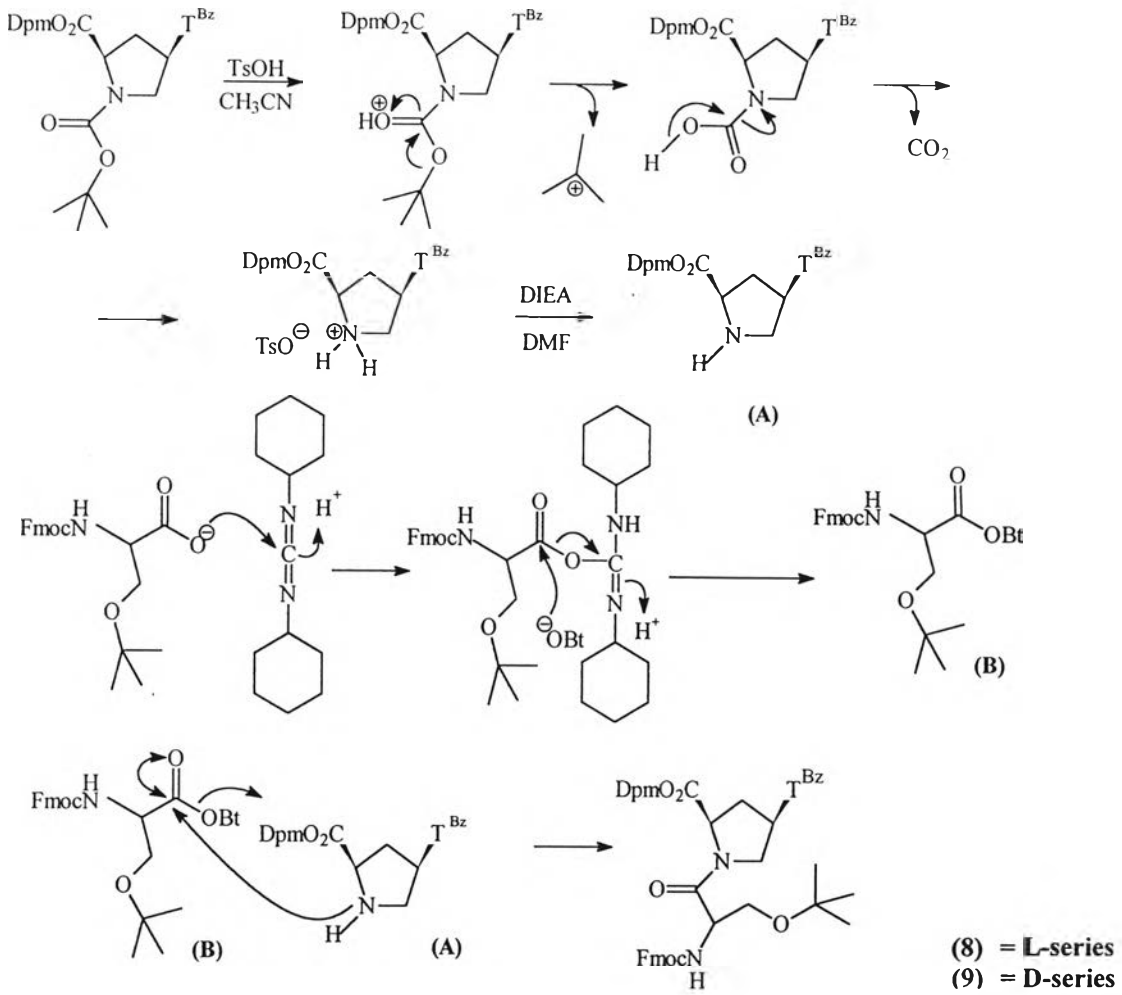
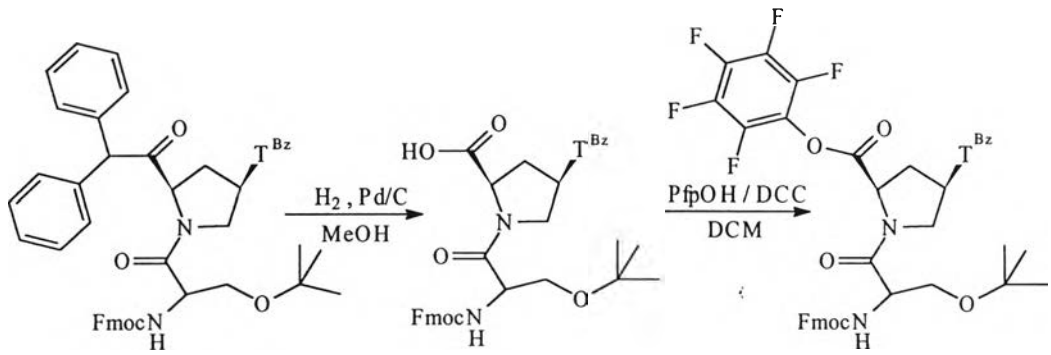


Figure 3.12 Coupling mechanism for synthesis of serylproline cPNA monomers [**(8)** and **(9)**]

concentration due to the formation of incompletely dissociated calcium hydroxide. This deprotection condition was tried with our dipeptide **(8)**, but no deprotection was observed at room temperature which might be due to the low solubility of the starting material. The temperature of this mixture was gradually increased in order to improve its solubility. Unfortunately, at high temperature, both Dpm and Fmoc groups were found to be cleaved simultaneously although the rate of cleavage of Fmoc group was somewhat slower. In the light of these results, high temperature must be avoided and isopropanol have been replaced with other suitable solvents such as acetonitrile or dioxane so that the starting material remained dissolved at room temperature. However the results were the same as

before. It was further thought to improve this method by using sodium carbonate in acetonitrile. At this point, the reaction was attempted with Fmoc-L-Ser(O^{t}Bu)-ODpm as a model, although the Dpm group was deprotected faster than Fmoc group initially, the rate was still slow and cleavage the Fmoc group was observed upon prolonging the reaction time. These disappointing results prompted us to search for more effective method. Catalytic hydrogenolysis seemed to be most hopeful since it gave clean reaction when hydrogen balloon and Pd/C was used (figure 3.13). In our experiment the deprotection was completely occurred within 1 hr. The free acid product was easily separated from the impurity (diphenylmethane) by washing with hexane/diethyl ether which gave the free acid product **(10)** for L-series and **(11)** for D-series as white solids. Although this method could not give exclusively pure product because the Fmoc group was still partially removed, they are adequately pure for the use as starting material for the next step.



(8) = L-series
(9) = D-series

(10) = L-series
(11) = D-series

(12) = L-series
(13) = D-series

Figure 3.13 Selective deprotection of Dpm ester [**(8)** or **(9)**] into free acid derivative [**(10)** or **(11)**] and synthesis of pentafluorophenyl ester [**(12)** or **(13)**]

In the next step the dipeptide free acid **(10)** was reacted with pentafluorophenol/ DCC to give 30 % yield of Fmoc-L-Ser(O^{t}Bu)-D-Pro(*cis*-4-T^{Bz})-OPfp **(12)** as the final product in the L-series. In the same manner, starting from dipeptide free acid **(11)**, 18 % of D-series, Fmoc-D-Ser(O^{t}Bu)-D-Pro(*cis*-4-T^{Bz})-OPfp **(13)** was obtained. After column chromatography the products were identified by elemental analysis, ^{1}H and ^{13}C NMR. Both isomeric products were obtained in practical pure state and were stable enough to keep as solid at 8 °C or below for over three months.

Since the selective deprotection and activation steps become the greatest problem which led to unsatisfactory yield, perhaps the stepwise coupling might give a better result although this will increase a double the number of step involve in solid phase synthesis. It was, however, decided to synthesize its monomer which is compatible with Fmoc/O^{t}Bu chemistry. The intermediate **(7)** was deprotected with *p*-toluenesulfonic acid/acetonitrile, neutralized with DIEA and coupled with FmocCl to afford Fmoc-D-Pro(*cis*-4-T^{Bz})-ODpm **(14)** as the only product (70 %, 2 steps). The next step was deprotection of Dpm ester with HCl/dioxane to give Fmoc-D-Pro(*cis*-4-T^{Bz})-OH **(15)** (94%). At this point, selective deprotection of Dpm ester was possible because the substrate possessed no acid-labile *tert*-butyl ether. The free acid **(15)** was activated as Pfp ester by reacting with pentafluorophenol and DCC which gave the Fmoc-D-Pro(*cis*-4-T^{Bz})-OPfp **(16)** as first monomer product (55%). The second monomer, Fmoc-L-Ser(O^{t}Bu)-OPfp, could be synthesized from Fmoc-L-Ser(O^{t}Bu)-OH, pentafluorophenol and DCC. It was disappointing to find that Fmoc-L-Ser(O^{t}Bu)-OPfp was obtained as viscous oil which made it difficult to accurately weight in order to use for each step coupling during solid phase synthesis. Hence we looked back to the free acid **(15)** which might coupled with Fmoc-L-Ser(O^{t}Bu)-OH under HTBU activation on solid phase peptide synthesis but the result was unsuccessful due to the poor coupling efficiency (overall 9 %) was obtained within after 9 cycles.

Finally, It was decided to insist on the synthesis of the serylproline cPNA by dipeptide fragment strategy even though the monomers were obtained in poor yield which probably due to the decomposition of the Pfp esters **(12)** and **(13)** during column chromatography. In this respect, cleaner method of making Pfp esters should be investigated so as to give pure products without the need for chromatographic purification.

3.1.3 Synthesis of deoxyglycylproline cPNA monomer

Despite the fact that the structure of deoxyglycylproline monomer was not quite the same as the serylproline dipeptide due to the lack of the tertiary amide bond, it might still be appropriate to call it as "pseudodipeptide" system. Hence it was possible to consider its synthetic route based on the previous dipeptide system starting from the same intermediate (7) so that *cis*-D-configuration was still preserved without much more synthetic steps. With

retrosynthetic analysis, three most important routes might be feasible which includes i) reduction of tertiary amides with borane derivative ii) reductive alkylation of peptides carrying free amino terminus with an amino aldehyde and iii) nucleophilic displacement of suitably protected aminoethyl synthon such as N-protected aminoethyl halide or activated aziridine according to figure 3.14

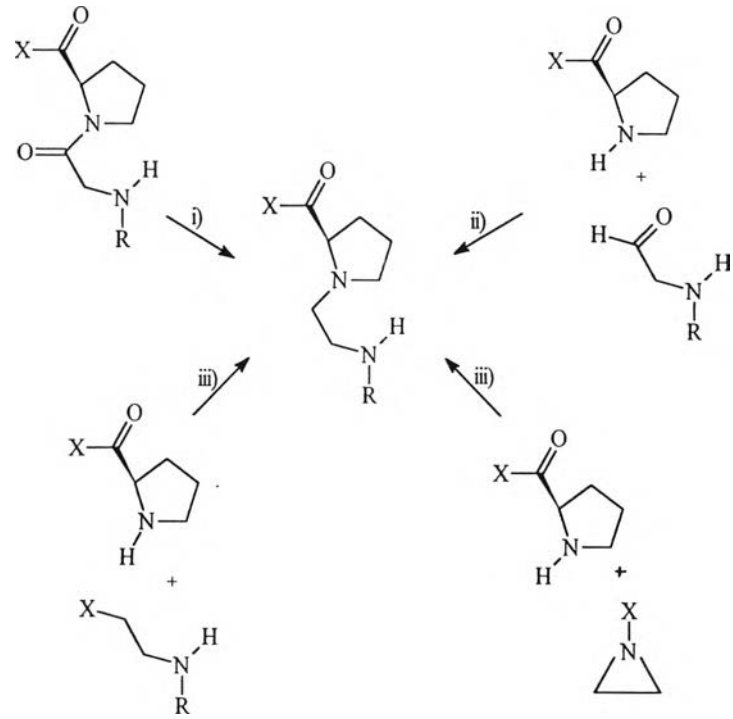


Figure 3.14 Possible routes to synthesize dipeptide carrying reduced amide bond

However, the route i) was seem to be difficult to find out the appropriate reducing agent in order to selectively reduce the tertiary amide bond into amine and route ii) was usually made under conditions which presented risk of racemization to the dipeptide monomer therefore it was decided to consider route iii) in order to obtain the aminoethyl synthon, one of two candidates between alkylation with aminoethyl halide and aziridine ring opening *via* S_N^2 displacement were considered. Due to the secondary amino group in proline was probably not adequately strong enough nucleophile to attack simple alkylating agents due to steric effect. Hence aziridine route was seem to be more effective due to the great ring strain of the aziridine ring system which could be relieved upon ring opening.

Ring opening of aziridine requires activation by electron withdrawing substituent on the nitrogen atom such as N-acyl or N-sulphonyl group in order to enhance leaving ability of the nitrogen atom and to prevent polymerization by the liberated nucleophilic free amino group. Even though *N*-toluenesulfonylaziridine have been synthesized from *N,O*-ditosylethanolamine under basic conditions and have been widely used in organic synthesis,[78] its utility was somewhat limited since desulfonylation of the *N*-tosylamino-ethyl product after ring opening would be difficult. Many methods to remove the *N*-tosyl group available including HBr/HOAc, alkali metal/NH_3,[79] Mg/MeOH, Na/naphthalene, samarium (II) iodide or photocleavage method[80] would not be compatible with the substrate in this case. Synthesis of N-acyl or N-alkoxycarbonyl aziridine were also attempted by the thermal dehydration of *β*-aminoethyl sulfuric acid to give aziridine and then N-acetylated with $(Boc)_2O$. But it was considered unsuitable since synthesis of such components involving free aziridine which is highly toxic. It was therefore a great delight to find that Maligres have reported the new aminoethyl synthon by activating aziridine with 4-nitrobenzenesulfonyl (nosyl) group.[78] Unlike tosylaziridine, the nosyl group in nosylaziridine could be easily removed by thiolysis under mild conditions especially when the hydrogen on the nitrogen atom is masked by acylation with *tert*-butoxycarbonyl group. Therefore it was decided to synthesize *N*-nosylaziridine and use as an aminoethyl synthon for our purpose. The synthesis was started from a reaction of ethanolamine and nosyl chloride (2eq) in the presence of excess triethylamine with or without DMAP as catalyst in order to generated *N,O*-dinosylate product followed by ring closure with strong base such as sodium hydroxide and 18-crown-6 as catalyst. But this attempt was unsuccessful because the main product obtained was the N-mononosylate product **(17)** and no cyclization was observed. Attempt to convert *N*-nosylaminoethanol **(17)** into *N*-nosyl, *O*-tosyl disubstituted form with tosyl chloride and triethylamine gave just only small of the required products and was not suitable for large scale synthesis of *N*-nosylaziridine. Attempts to convert hydroxyl group of N-mononosylate **(17)** into better leaving group by PPh_3/CCl_4 and $SOCl_2$/Pyridine were also unsuccessful. Alonso reported the synthesis of *N*-nosyl-(2*S*)-benzyl aziridine by nosylation of the (*S*)-phenylalaninol, mesylation and cyclization at low temperature.[80] The same reaction was tried with *N*-nosylethanolamine with expectation to cyclize spontaneously after mesylation in the presence of excess triethylamine (2.2 eq) in two steps. Regretfully, the required product was decomposed

rapidly under this condition even on cooling down the reaction to 0 °C or below. One pot reaction was thus attempted with the hope to obtain a better result, *N*-nosylaminoethanol (**17**) reacted with tosyl chloride, sodium hydroxide (2.2 eq) in the presence of tetra-n-butyl ammonium hydrogen sulfate as catalyst was not successful. Coy and co-workers reported the direct synthesis of *N*-nosylaziridine starting from *N*-(2-bromoethyl)-4-nitroarene sulphonamide and sodium ethoxide/dry ethanol at 25 °C but this method always provide piperazine as by-product and the product was somewhat unstable in this strong basic condition.[81] Alonso has also recently presented the synthesis of 2,3-disubstituted tosylaziridine *via* Mitsunobu reaction.[80] This method has advantage over previous methods discussed above since there is no need to convert OH group into good leaving group, it is a one step cyclization and its condition should be compatible with the sensitive aziridine product. Firstly, *N*-nosylaminoethanol (**17**) was synthesized prior to cyclization into *N*-nosylaziridine by starting from 1 eq of ethanolamine, 1.1 eq nosyl chloride and triethylamine in dichloromethane and the reaction was then acidified to remove the residual base during work up step to give the product (**17**) as yellow solid in 82 % yield. The structure of the product was confirmed by its ^{1}H NMR spectrum which showed the signals at 8.02 and 8.41 ppm belonged to *para*-substituted aromatic protons of the nosyl group in addition to two triplets of the CH_2CH_2 group in the aliphatic region. ^{13}C NMR data also revealed the signal at 124.5, 128.0, 146.4 and 149.5 ppm was aromatic carbon in nosyl group. Further characterization included DEPT, IR, ^{1}H-^{1}H COSY and elemental analysis, all of which confirmed the identity of the product. The next step involved cyclization of (**17**) *via* Mitsunobu reaction. The *N*-nosylethanolamine (**17**) was reacted with triphenylphosphine-diisopropylazodicarboxylate complex in dry THF at -10 °C to give the desired *N*-nosylaziridine (**18**) (figure 3.15). The product seemed to be unstable under Mitsunobu condition as evidenced from the formation of brown precipitate after prolonged reaction time. This impurity brought about difficulty in chromatographic purification of the product and must be separated as soon as possible. In addition it was also found that the product was completely destroyed if the reaction mixture was evaporated at too high temperature. For this reason the dry dichloromethane have been used instead of THF in order to reduce the risk of decomposition because removal of dichloromethane could be done at room temperature under reduced pressure. According to

this modification, the reaction mixture became clear after addition of DIAD and only small quantity of unknown brown precipitate was slowly formed. The *N*-nosylaziridine was easily purified by flash column chromatography using dichloromethane:hexane (3:1) with dry-loading technique to gave the product **(18)** in 68% yield.

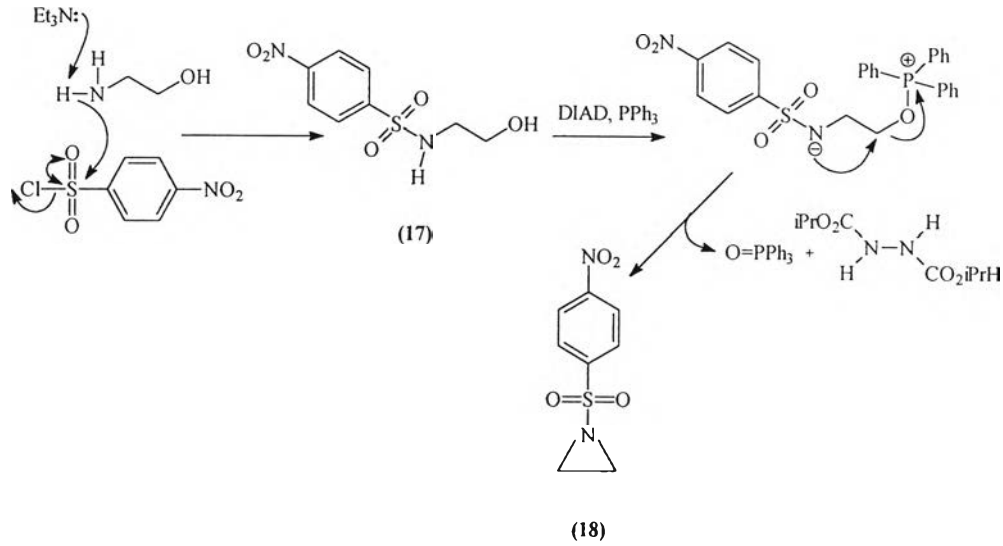


Figure 3.15 Synthesis of *N*-nosylethanolamine **(17)** and *N*-nosylaziridine **(18)**

^{1}H NMR of this product revealed a singlet peak at 2.45 ppm due to symmetric aziridine aliphatic proton (2x$\underline{C}H_2$) and an AA′XX′ quartet at 8.12 and 8.39 ppm of nosyl aromatic protons. In addition, ^{13}C NMR spectrum revealed signals of aziridine $\underline{C}H_2$ at 28.1 ppm, nosyl $\underline{C}$H at 124.3 and 129.2 ppm and nosyl $\underline{C}$ at 143.7 ppm and 150.6 ppm. Other techniques include DEPT, IR, elemental analysis and ^{1}H-^{1}H COSY also confirmed the expected structure of *N*-nosylaziridine. Unsubstituted *N*-nosylaziridine should have been well-known substance in organic synthesis, but surprisingly synthesis of this particular compound especially by Mitsunobu reaction have never been reported in the literature.

S_N^2 ring opening of *N*-nosylaziridine with the intermediate **(7)** after removal of the Boc group by treatment with *p*-toluenesulfonic acid/acetonitrile took place in the presence of DIEA and finally gave the desired ring opening product **(19)** (figure 3.16). ^{1}H NMR of the product showed the important signals at 2.60-2.67 and 2.68-2.75 ppm (NsNHCH$_2$C$\underline{H}_2$N), 2.76-2.80 and 3.18-3.23 ppm (NsNHC$\underline{H}_2$CH$_2$N), and *para*-substituted aromatic proton of nosyl group at 7.98 ppm and 8.25 ppm. The structure was further confirmed by ^{13}C NMR. DEPT, ^{1}H-^{1}H COSY, IR and elemental analysis.

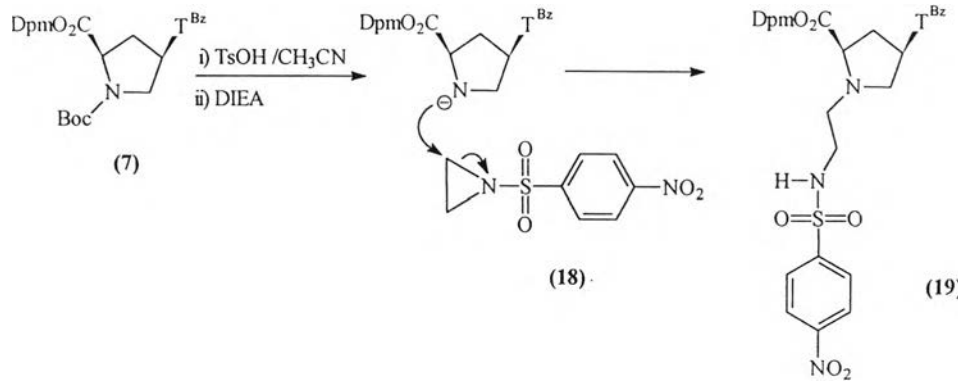


Figure 3.16 Synthesis of Ns-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(19)**

The next step was N-acylation of derivative **(19)** with Boc_2O in order to drain the electron from the nitrogen atom through π-bond (amide) which could serve to enhance the leaving ability of the nosyl group toward the nucleophilic cleavage by thiophenoxide ion. Reaction of **(19)** with Boc_2O in the presence of dimethylaminopyridine (DMAP) as catalyst overnight gave the Boc/Ns-(ψ-CH_2)Gly-D-Pro-(*cis*-4-T^{Bz})-ODpm **(20)** as yellow crystalline solids in 92 % (figure 3.17). ^1H NMR spectrum showed a characteristic singlet at 1.25 ppm with integration of 9H due to the Boc group and ^{13}C NMR signal at 27.7 ppm and 85.6 ppm of Boc $\underline{C}H_3$ and Boc $\underline{C}$ respectively. The structure was further confirmed by DEPT, ^1H-^1H COSY, IR spectrum and elemental analysis.

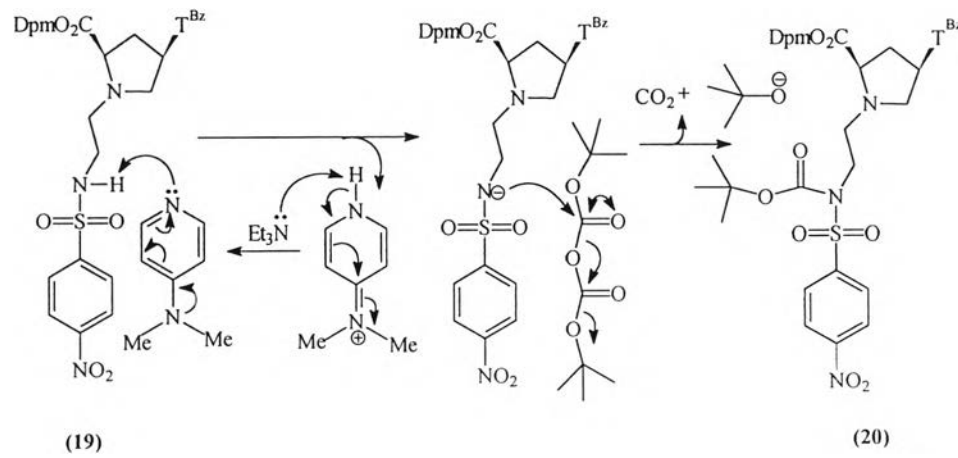


Figure 3.17 Synthesis of Boc/Ns-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(20)**

The nosyl group in the Boc/Ns derivative **(20)** could be easily eliminated by treatment with thiophenol/K_2CO_3 in DMF for 2 hr at room temperature which gave Boc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(21)** as the only product in good yield (63%). This mild condition for cleavage of nosyl group made use of thiophenoxide ion to serve the function of both nucleophile and scavenger for the liberated nosyl group. This method was somewhat inconvenient because thiophenol possesses awful smell hence its residue should be destroyed with sodium hypochlorite solution after use. The required product was diluted with dichloromethane and extracted with water until free from thiophenol before chromatographic purification. After column chromatography with hexane:ethyl acetate, the product was obtained as white amorphous solid. The structure of the product was interpreted according to ^1H NMR which revealed the significant signal at 1.43 ppm with integration of 9H of Boc group and peaks due to *para*-substituted aromatic proton of nosyl group was no longer observed. The ^{13}C NMR spectrum showed signals at 28.4 ppm of Boc $\underline{C}H_3$ and 155.9 ppm of Boc $\underline{C}O$. The mechanism for this step was demonstrated in figure 3.18.

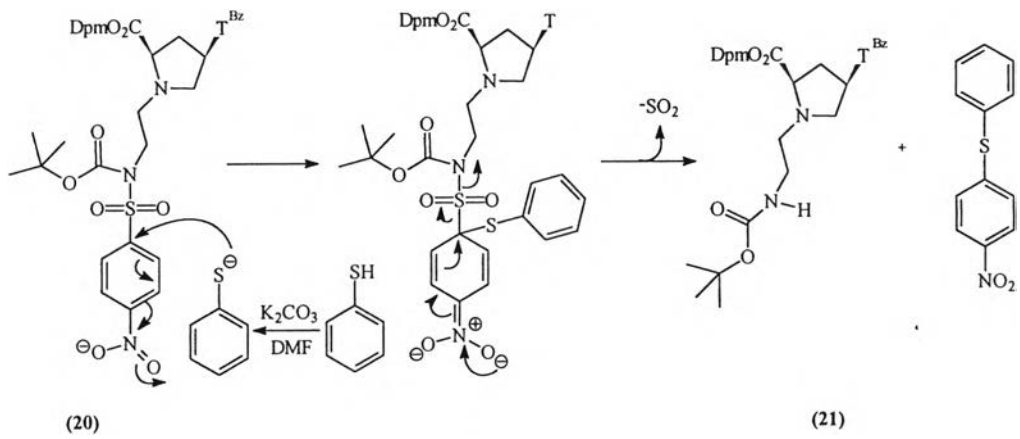


Figure 3.18 Synthesis of Boc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(21)**

The Boc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(21)** might be easily converted into the Boc protected free acid derivative by deprotection of Dpm ester. Although the resulting product should be useful as protected building block for oligomerization on solid phase synthesis according to the classical Boc chemistry, it was considered that Fmoc chemistry had a number of advantages over classical Boc method in term of mild conditions and

possibility of monitoring the coupling efficiency by quantitative determination of an chromophore with UV-spectrophotometry after Fmoc deprotection. It was decided to replace the Boc group with Fmoc group as follows.

The Boc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(21)** was converted into Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(22)** in the usual manner by selective deprotection of the Boc group from Boc/Dpm in derivative **(21)** using *p*-toluenesulfonic acid in acetonitrile. In this case the deprotection took place more slowly and was completed within 2.5 hr probably because primary amine product was a poorer leaving group than secondary amine generated from the intermediate **(7)** thus requiring longer deprotection time. The ammonium tosylate adduct was neutralized with excess DIEA and then reacted with 9-fluorenylmethylchloroformate (FmocCl) for 6 hr at room temperature (figure 3.19). The reaction mixture was worked up by normal acid-base extraction and chromatographically purified with hexane:ethyl acetate as eluent to afford a 75% yield of **(22)** as white solid.

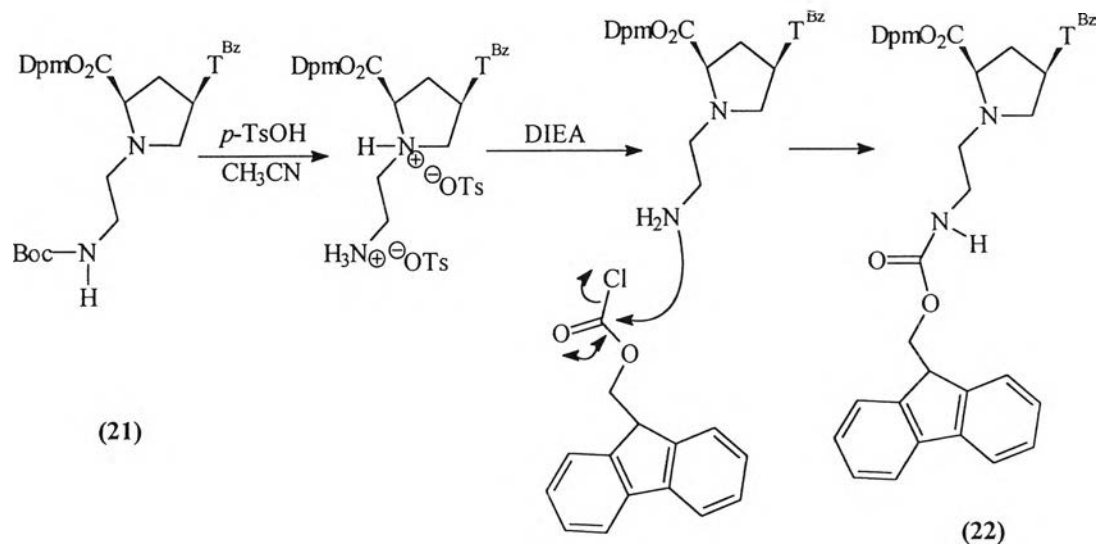
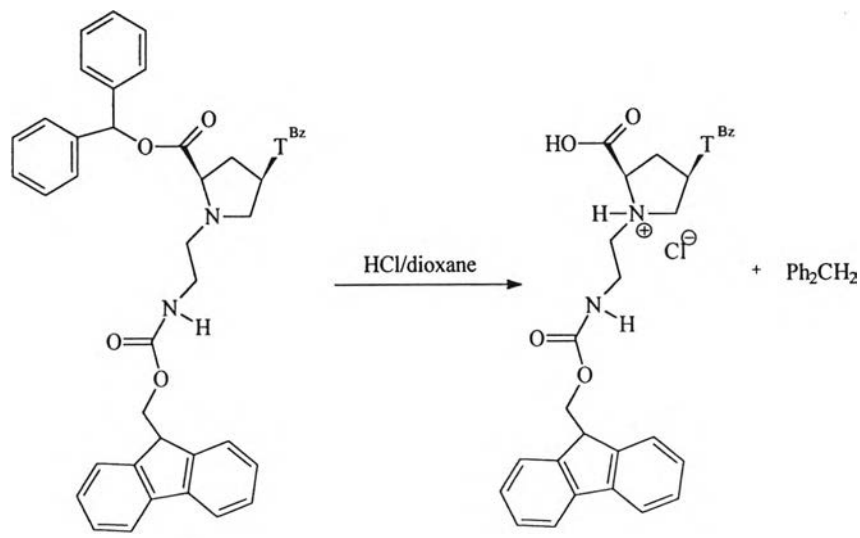


Figure 3.19 Synthesis of Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(22)**

Analysis of ^{1}H NMR data of Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(22)** revealed significant signals at 4.14 and 4.24 ppm which belonged to Fmoc aliphatic CH_2 and Fmoc aliphatic CH respectively. Fmoc aromatic CH signals were found at 7.30-7.36, 7.52-7.58 and 7.81-7.87 ppm but was partially overlapped with some peaks of benzoyl

thymine. The signals at 7.18-7.30 ppm confirmed the presence of Dpm ester. The structure was further confirmed by ^{13}C NMR signals at 47.2 ppm (Fmoc aliphatic CH), 65.0 ppm (Fmoc CH_2), 120.0 ppm (Fmoc aromatic CH), 141.3, 143.8 and 143.9 ppm (Fmoc aromatic C), 156.4 ppm (Fmoc CO) and the signals of Dpm at 125.1-130.4 ppm (Dpm aromatic CH), 139.2 and 139.3 (Dpm aromatic C). Other structural details were confirmed by DEPT, ^1H-^1H COSY, IR and elemental analysis.

The next step was deprotection of Dpm ester, unlike serylproline system, this selective deprotection of Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(22)** could be easily achieved since it has no side chain which required protection and thus there was no need to consider for orthogonality. Acid condition appeared to be suitable since it has no effects towards the Fmoc protecting group. The Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(22)** was treated with excess saturated hydrogen chloride in dioxane under anhydrous condition for 2 hr at room temperature to give the desired product, Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})OH.HCl **(23)**, as a white solid in 68% yield (figure 3.20). The volatile hydrogen chloride residue was eliminated by coevaporation with dichloromethane a few times and the diphenylmethane by-product was easily removed by washing with hexane/diethyl ether. The tertiary amine group on the proline ring could be protonated as ammonium chloride salt and no attempt to obtain the free amine has been made.



(22) (23)

Figure 3.20 Synthesis of Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-OH.HCl **(23)**

Preliminary identification was performed by ^{1}H NMR with DMSO-d_6 as a solvent but the some significant signals were obscured with the broad OH peak of the acid function as shown in figure 3.21 (lower). In order to avoid this problem, a drop of D_2O was added to examine that the broad signal was due to exchangeable proton (figure 3.21 upper).

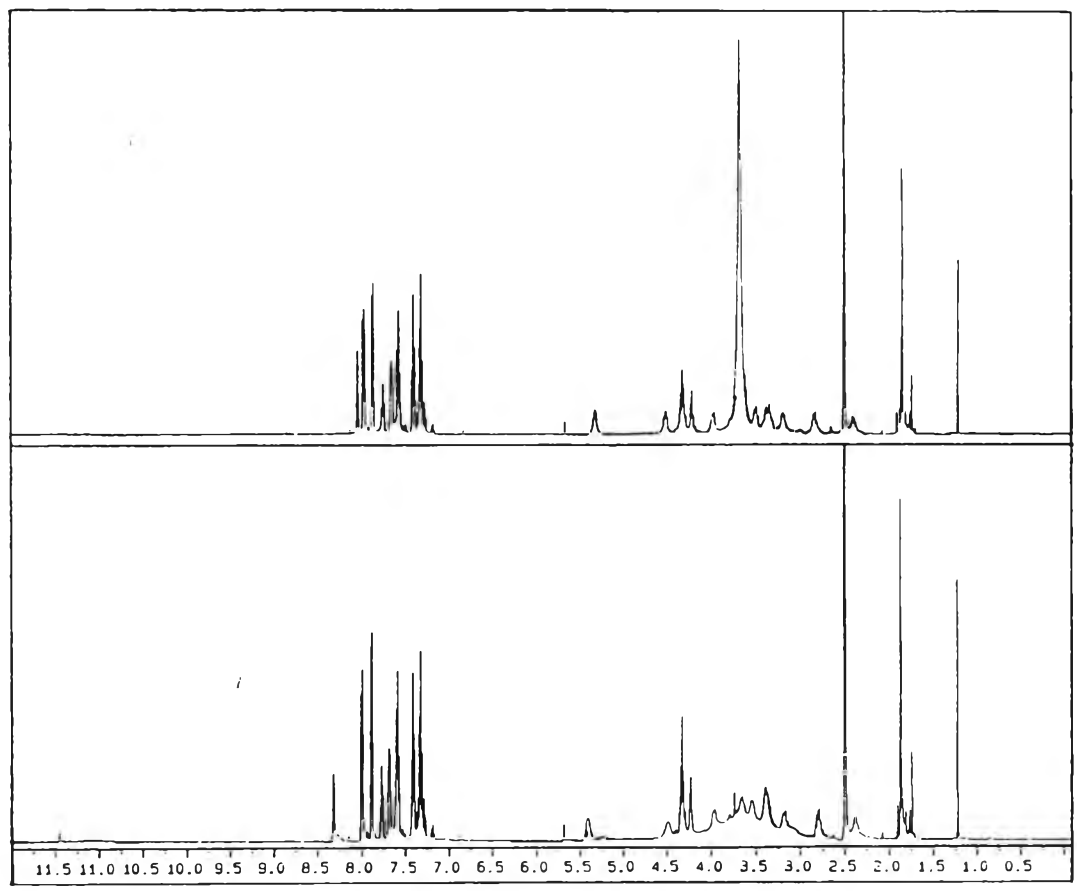


Figure 3.21 ^{1}H NMR spectrum of Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-OH.HCl **(23)** in DMSO-d_6+1 drop of D_2O (upper) and in DMSO-d_6 (lower)

From the result, it was clear that the product was Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-OH.HCl **(23)** since the signals of Dpm which should be appeared as multiplet at higher than 7 ppm was not observed in the spectrum. Other signals were consistent with the Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-ODpm **(22)** as well as data from ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY and IR spectrum. However, as evidenced from the elemental analysis result, it was expected to obtain C 63.3 %, H 5.2 % and N 8.7 % which calculated from the

formula $C_{34}H_{33}N_4O_7Cl$ but the first analysis result was: C 60.9 %, H 5.0 % and N 8.2 %. In order to check the purity of product **(23)**, the HPLC experiment was performed and reveal the result was as shown in figure 3.22 which revealed only one predominent peak of product **(23)** without other significant impurity by monitoring at 264 nm. Since it was possible that the product might still has residual solvent or moisture which was necessary to remove these impurity under high reduced pressure for 48 hr before resubmitted for elemental analysis. It was surprising that the second analysis result was still nearly identical equal the previous result. It was expected that the product should be stable as ammonium chloride salt but the elemental analysis results led to re-calculation for alternative formula of the product which might be responsible for the discrepancy observed. It was finally found that calculation based on derivative **(23)** plus another molecule of HCl ($C_{34}H_{33}N_4O_7Cl.HCl$; C 59.9 %, H 5.0 %, and N 8.2 %) or two molecules of water ($C_{34}H_{33}N_4O_7Cl.2H_2O$; C 59.9 %, H 5.5 %, and N 8.2 %) could provide closely percent of mass in each item. However the presence of two molecule of water was seem to be more reasonable considering there is only one basic nitrogen atom in **(23)**.

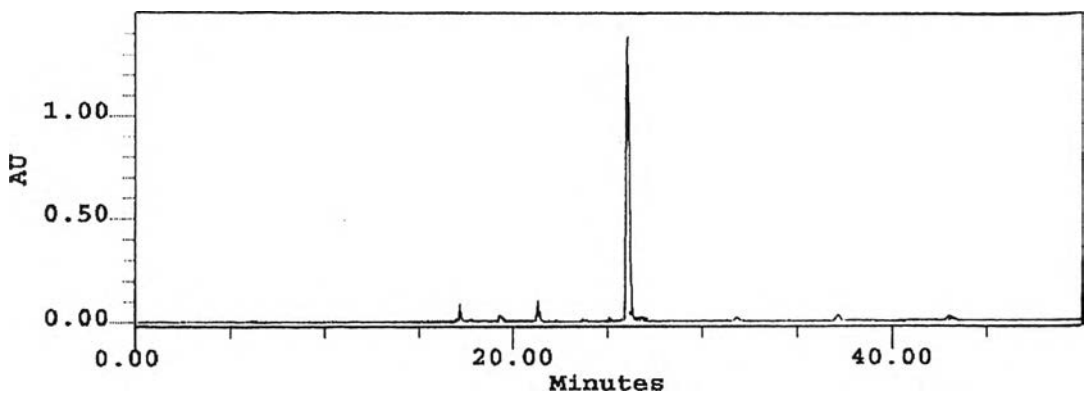


Figure 3.22 HPLC chromatogram of Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-OH.HCl **(23)** HPLC gradient system: solvent A = 0.1 % trifluoroacetic acid in acetonitrile; solvent B = 0.1 % trifluoroacetic acid in deionized water; First A:B (10:90) for 5 min then linear gradient to A:B (90:10) over a period of 50 min after that hold on for 5 min and suddenly release to A:B (10:90)

Even though we would like to covert the free acid derivative **(23)** into its Pfp ester which could be oligomerized by Fmoc and Pfp ester chemistry following glycylproline cPNA as in previous reports,[63] the presence of flexible aminoethyl part in the molecule could probably cyclized into ketopiperazine derivative (figure 3.23) even when the N-terminus was protected as soon as the C-terminus was activated as pentafluorophenyl ester due to the self-catalysis by tertiary proline nitrogen in the same molecule. Therefore, as described above, we decided not to attempt the synthesis of Pfp ester and the free acid derivative **(23)** was used as monomer in oligomerization which will be described in the next section.

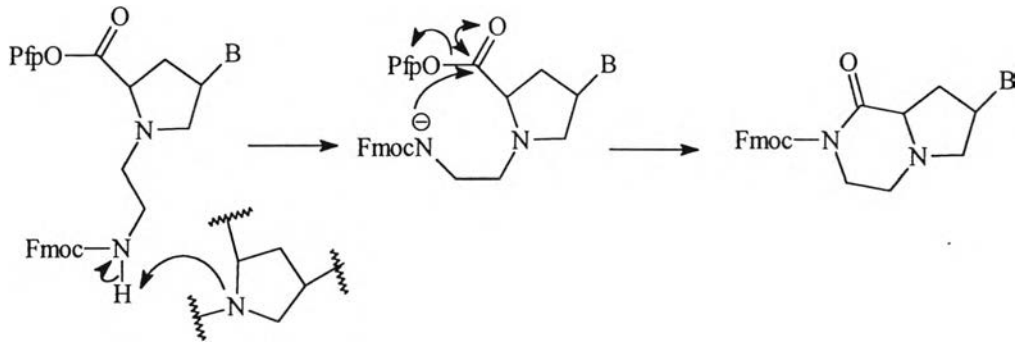


Figure 3.23 A possible mechanism of cyclization of Pfp ester of Fmoc-(ψ-CH_2)Gly-D-Pro (*cis*-4-T^{Bz})-OH.HCl **(23)**

After synthesis of compound **(21)** and Fmoc derivative **(23)** have been completed in our hand another synthesis of stereomeric and enantiomeric derivatives of compound **(21)** have been reported by Costa and co-worker.[82] Their synthesis was started from (*2S,4R*) hydroxyproline and then converted into methyl ester prior to first alkylation with *N*-Boc-aminoethyl bromide and then with N^3-benzoylthymine. It was noted that overall synthesis have been made in only 4 steps and aminoethyl spacer was introduced into proline before nucleobase alkylation to give *cis*-L-isomer (*2S,4S*). In addition (*2R,4S*) derivative was also synthesized by epimerizing the hydroxyproline intermediate with acetic anhydride and acetic acid before alkylation. However our theoretical model has suggested that the *cis*-D-configuration of proline should have stronger binding to complementary natural oligonucleotide due to having the analogous configuration.[63] Moreover we use a different

strategy in oligomerization of the peptide therefore the two studies are different and should complement each other.

3.2 Oligomerization of cPNA

It has recently been demonstrated that synthesis of peptides from amino acids could effectively be accomplished by growing the peptide chain on a heterogeneous solid support such as polystyrene resin.[83] With this major development, purification of the product has become much easier than the classical solution phase synthesis by simple washing the excess reagent from the resin-bound peptide. This technique was named as "Solid Phase Peptide Synthesis" or in short "SPPS ". Oligomerization of serylproline monomers **(12)**, **(13)** and deoxyglycylproline monomer **(23)** upto 10 repeating units were thus performed by solid phase synthesis methodology as shown in Table 3.1 in order to study their binding stability toward the complementary natural oligonucleotide and the results compared with the glycylproline cPNA.[63] The results will be presented and discussed in each parts as follows.

Table 3.1 Structures of 10-mer cPNAs

cPNA	Monomer No.	10-mer No.	Structure of 10-mer cPNA
Deoxyglycylproline	**(23)**	**(24)**	H-[(ψ-CH_2)Gly-D-Pro(T)]$_{10}$-LysNH_2
Serylproline (L-series)	**(12)**	**(25)**	H-[L-Ser-D-Pro(T)]$_{10}$-LysNH_2
Serylproline (D-series)	**(13)**	**(26)**	H-[D-Ser-D-Pro(T)]$_{10}$-LysNH_2

3.2.1 The decamers of deoxyglycylproline cPNA (24)

In previous reports,[63] 10-mer glycylproline cPNA was synthesized by oligomerization of Fmoc-Gly-D-Pro(*cis*-4-T^{Bz})-OPfp ester monomer by using Fmoc/O^{t}Bu protection chemistry. It was suggested that this strategy offer a number of advantages over the classical Boc chemistry; for example the use of milder condition, applicable for small

scale synthesis, reduced reaction time and most importantly, the ability to quantitatively investigate the coupling efficiency of each step by simple spectrophotometric method. The peptide synthesis procedure was summarized according to the protocol as shown in figure 3.24.

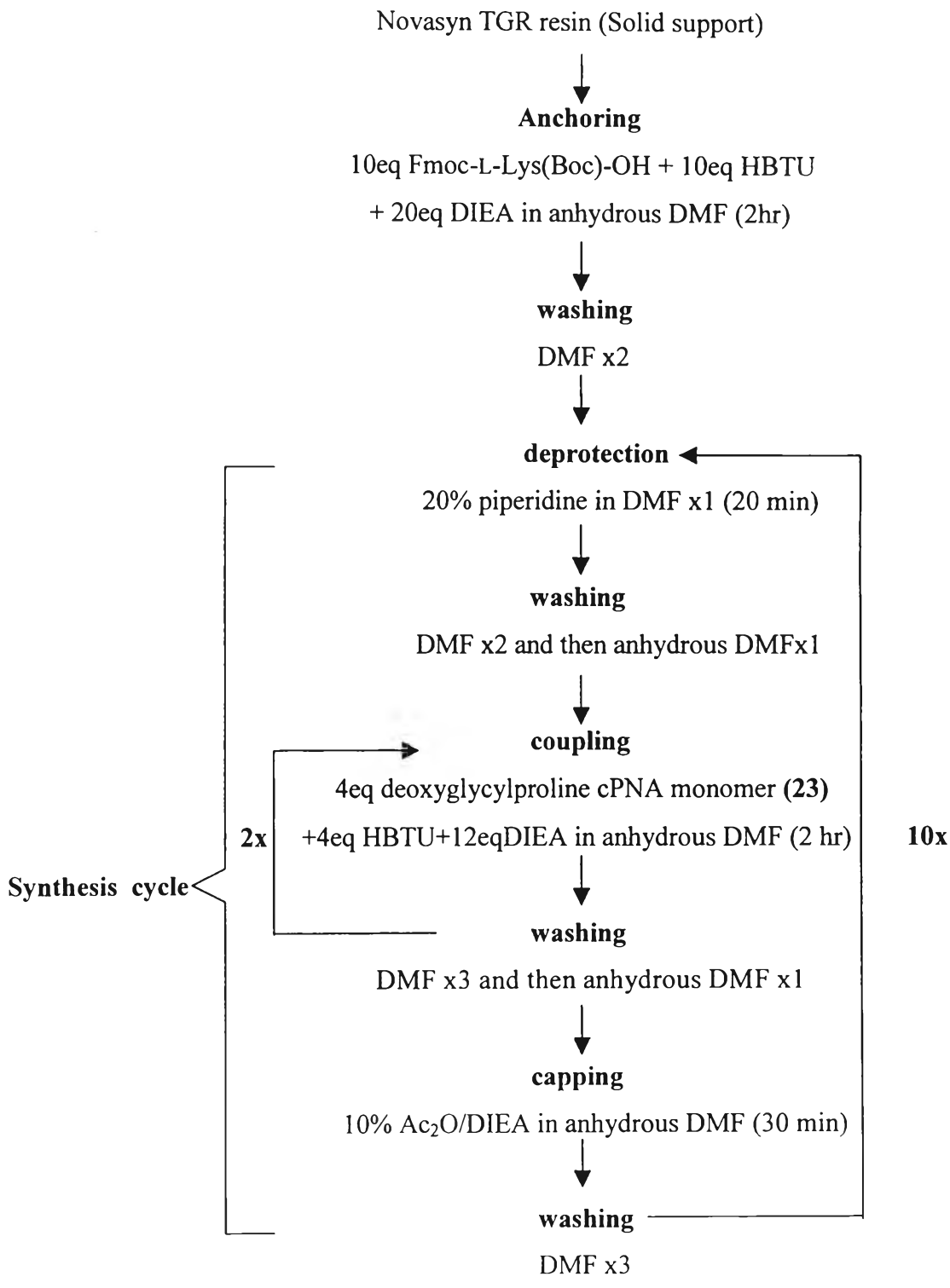


Figure 3.24 The protocol for solid phase synthesis of deoxyglycylproline cPNA **(24)**

In practice, the microscale synthesis was carried out in pipette as described in section 2.4.1. Novasyn TGR resin which carries an acid-labile dimethoxybenzhydrylamine linker was chosen as the solid support so that after cleavage with trifluoroacetic acid, a peptide amide would be obtained. First of all, the resin was swollen in anhydrous DMF and then anchored with Fmoc-Lys(Boc)-OH as the first amino acid residue in the presence of HBTU as coupling agent and DIEA according to the mechanism in figure 3.25. Due to the heterogeneous nature of the reaction, it would not be completed simply with 1:1 stoichiometric proportion of reactant and would require a very long reaction time. Bearing this in mind, excess and high concentration of coupling reagents were generally used in order to enhance the coupling efficiency within a reasonable time scale.

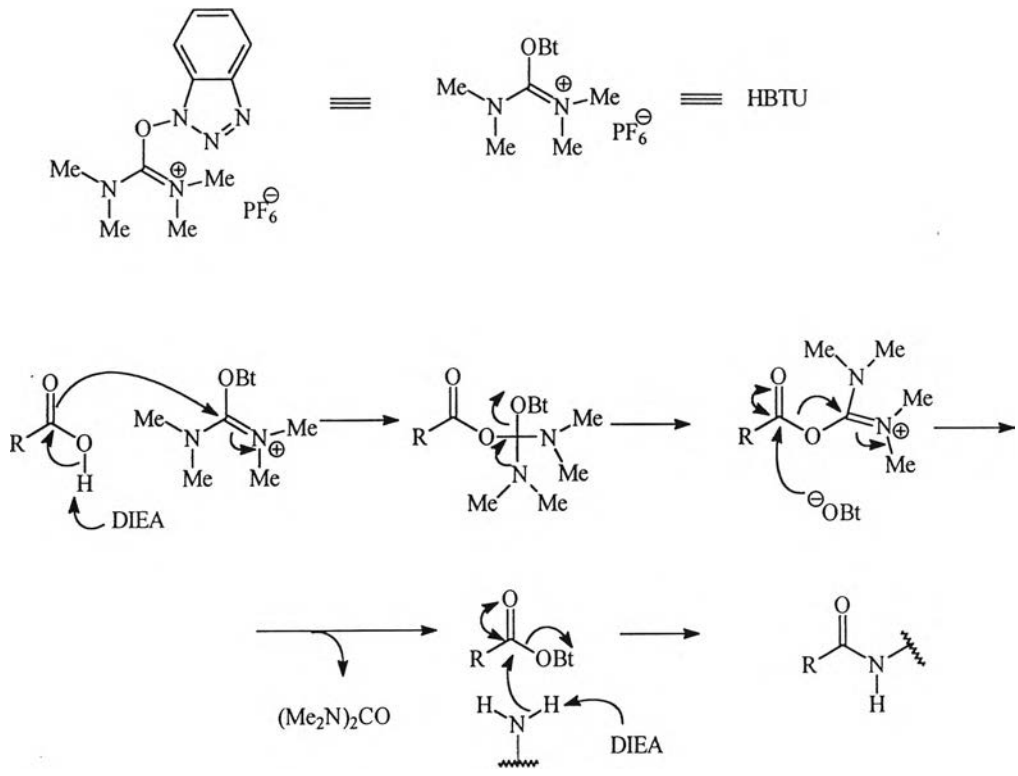


Figure 3.25 The mechanism for coupling or anchoring *via* HBTU activation

It has also been suggested that introducing a lysine at C-terminus of peptide chain could serve to prevent the self aggregation of the peptide chain due to the repulsion of the positively charged side chain and also increase the water solubility of the peptide.[12,13,14,15] Although it was later recommended to use alanine instead of lysine because the methyl

side chain of alanine has no charge and would not affect the binding ability of the resulting peptide containing alanine to complementary natural oligonucleotide and could still induce the handedness of peptide chain similar to lysine.[84] It was still decided to use lysine as the first amino residue in order to compare the result with previous studies of glyclyproline cPNA. To ensure that the coupling efficiency was as high as possible, double coupling employing a fresh coupling reagent was performed before capping with a mixture of acetic anhydride-DIEA to prevent undesired growing of the deletion sequence. The next step of the synthesis cycle consisted of deprotection of the Fmoc group, coupling with a monomer **(23)** and capping , washing with DMF was performed after each step. The deprotection was carried out by treatment of the resin with 20 % piperidine in DMF for 20 min in order to remove the terminal Fmoc protecting group which was released from the resin-bound peptide as the piperidine-dibenzofulvene adduct according to the mechanism type of E1cb *via* the stabilized dibenzocyclopentadienyl anion as shown in figure 3.26

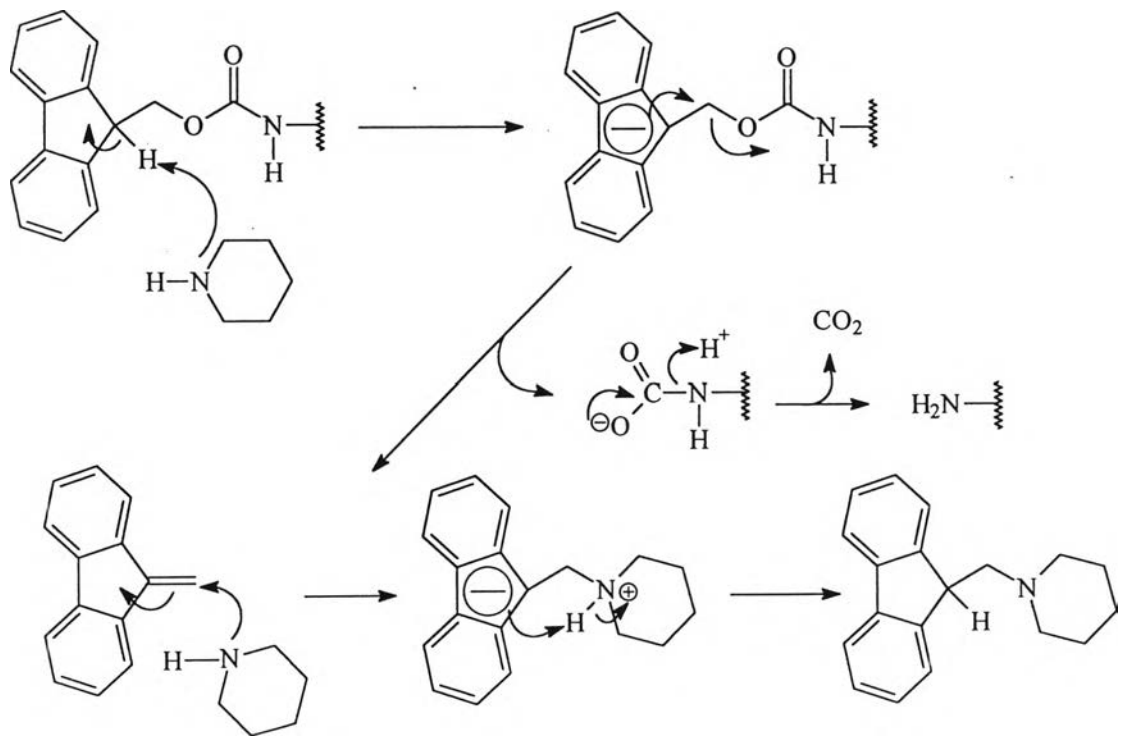


Figure 3.26 Mechanism for deprotection of Fmoc protecting group from resin bound peptide

Estimation of the coupling efficiency could be performed by diluting the resulting deprotecting solution with an appropriate volume of absolute methanol and then the UV-absorption of the piperidine-dibenzofulvene adduct measured at 264 nm. This number is related to the total amounts Fmoc group remained on the resin-bound peptide and indirectly indicated the efficiency of previous coupling cycle. For convenience, it was proposed that the first absorption value of which obtained after deprotection of Fmoc-L-Lys-peptide-resin corresponded to 100 % coupling efficiency. Therefore comparison of the next absorption with this initial value could be used to calculate the percent of coupling efficiency after each coupling step and the overall efficiency of the synthesis could be evaluated. It was noticed that this method is a very sensitive technique even the synthesis was performed just only microscale. After deprotection, the resin was washed with excess DMF in order to remove the residual piperidine. In the next step, the Fmoc-(ψ-CH_2)Gly-D-Pro(*cis*-4-T^{Bz})-OH.HCl **(23)** was coupled to the free amino group at N-terminus of the resin-bound peptide using HBTU as coupling reagent in the presence of DIEA according to the figure 3.25. High concentration of coupling reagent was used to enhance the coupling efficiency and double coupling was also be performed according to the reason discussed above. DIEA should be used approximately two folds of the monomer equivalent, one for deprotonation of acid function on monomer and the another for terminal N-amino group on resin-bound peptide. But as the monomer **(23)** was obtained as a hydrochloride salt, it was therefore necessary to add a further equivalent of DIEA into the coupling mixture in order to neutralize the hydrochloride salt. In practice even after double coupling with excess reagent, the coupling efficiency was not always approaching 100 %. Some of the residual free amino groups on the resin have not been coupled and was still reactive enough to react with the monomer in the next coupling cycle. This situation was therefore resulted in skipping of one coupling cycle which brought about missing of a repeating unit in peptide chain. In fact, this might happen more than once and might occur anywhere in peptide chain. The resulting "incomplete peptide" is generally called "deletion sequences" and would contaminate the peptide product. Worse still, those truncated peptides generally possess similar polarity to the desired peptide product which led to difficulty in purification. However it was possible to solve this problem by acetylation the unreacted amino group residue at N-terminus on resin bound peptide into acetamide derivative with 10% acetic anhydride/DIEA in DMF in order to change the polarity of truncated peptide

chain and also to diminish the nucleophilicity of the nitrogen atom at N-terminus as an acetamide which would not be able to grow in the next coupling cycles. This acetylation procedure is normally called "Capping" and is sometime considered as optional for solid phase synthesis. However, we decided to perform this ended capping every time after coupling step was finished for easier purification.

The synthesis cycle was repeated until the growing peptide chain was extended up to 10-mer (10 cycles). The coupling efficiency in each step was calculated from UV-absorption and summarized in Table 3.2

As shown in Table 3.2, the coupling efficiency seemed to decrease gradually throughout the 10 synthesis cycles and the final product was obtained in only 17.3 %. It was believed that the major causes came from the residual moisture in the starting material, probably in the form of dihydrate, and could not be removed even after prolonged drying under vacuum before use. An another possible cause was that the activated monomer might cyclize into a ketopiperazine derivative due to HBTU activation of C-terminus on proline moiety under excess basic condition lead to self cyclization. In addition, the aminoethyl spacer was flexible enough to flip its conformation to be "*cis*-like" and thus assisted the cyclization as shown in figure 3.27. This situation brought about the lost of monomer during the coupling step and might, at least partially, account for the low overall yield obtained.

Table 3.2 The UV-absorption data and percent coupling efficiency of 10-mer deoxyglycylproline cPNA **(24)**

Item	Cycle No.										
	1	2	3	4	5	6	7	8	9	10	Overall
OD_{264}	0.885	0.865	0.617	0.465	0.388	0.304	0.224	0.213	0.176	0.153	N/A
%efficiency	100.0	98.2	71.0	75.4	83.5	78.4	74.3	94.3	82.6	86.9	17.3

Remark All OD_{264} were received from 200 fold dilution with absolute methanol.

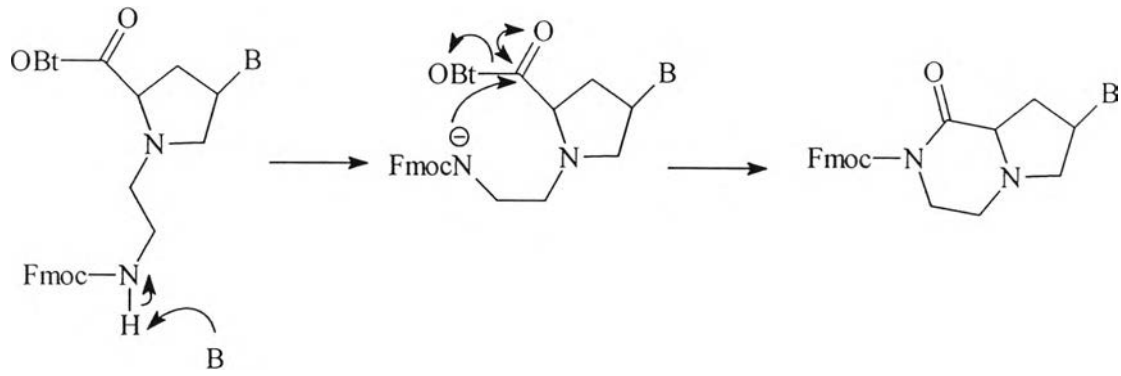
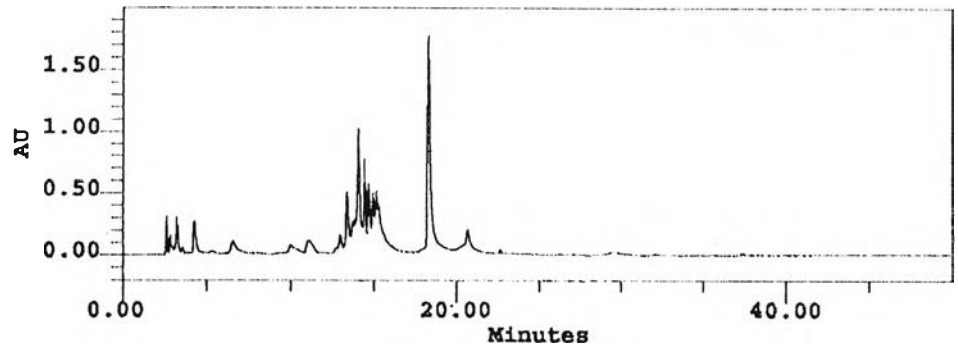


Figure 3.27 A possible mechanism for cyclizing of deoxyglycylproline cPNA monomer **(23)** into ketopiperazine

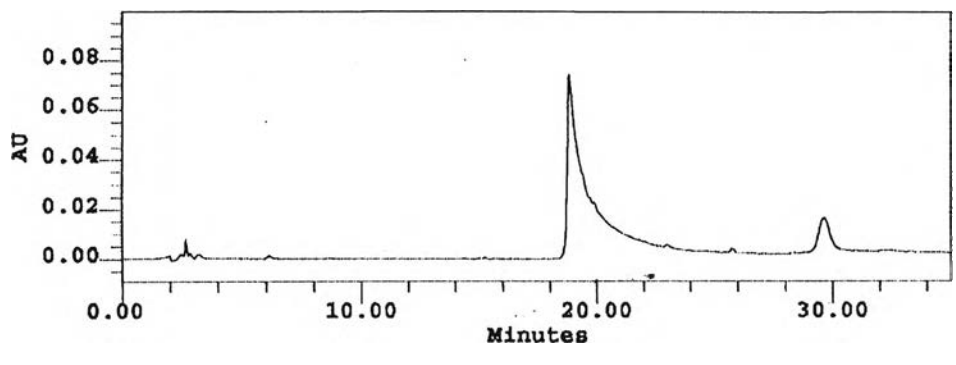
After the synthesis of **(24)** was successful, our first attention was to remove the N-terminal Fmoc protecting group before purification. Hence after final capping, the resin was treated with 20 % piperidine/DMF for 20 min (final deprotection), then washed with a large excess DMF and methanol and dried in air. The total peptide was released from the resin by treatment with trifluoroacetic acid/thioanisole for 3 hr and resin became gradually red. The volatile trifluoroacetic acid was removed by a stream of nitrogen which gave sticky residue which after treatment with diethyl ether gave precipitate of the product. After centrifugation, washed with more diethyl ether and air dried, the crude peptide was subjected to reverse phase HPLC analysis but the result was not satisfactory due to all peaks in the chromatogram appeared closely and impossible to collect them separately. This separation problem has conducted us to recall the reason of capping step which could inhibit the deletion sequence growing and change the polarity of this impurity. It was therefore possible to preserve the terminal Fmoc group by omitting the final deprotection with the expectation that the complete peptide having Fmoc group would have different in polarity enough to permit its separation from the acetylated deletion sequences.

As expected, separation of Fmoc-ON **(24)** was successful as shown in figure 3.28 (a) One broad complicated peak ($t_R \approx$ 12-15 min), a high sharp peak (t_R = 18.3 min) and a small peak (t_R = 20.7 min) were observed both of analytical and preparative scale. The two conspicuous peaks were collected on preparative scale, lyophilized and then identified by MALDI-TOF mass spectrometry. It was clear that the first higher peak (t_R = 18.3 min) was the desired Fmoc-ON **(24)** (M_r calcd. for M_r = 3010.4 found 3013.3) and the second peak

(t_R = 20.7 min) was the same Fmoc-ON **(24)** having an extra benzoyl group (M_r calcd. for M_r = 3115.5 found 3118.6) and the complicated peaks between 12 min and 15 min should be the mixture of acetylated deletion sequence impurity. For convenience we shall call the two major products as Fmoc-ON and Fmoc/Bz-ON cPNA respectively.



(a)



(b)

Figure 3.28 Reverse phase HPLC chromatogram of 10-mer deoxyglycylproline cPNA **(24)** **(a)** Fmoc–ON or Fmoc-[(ψ-CH_2)Gly-D-Pro(T)]$_{10}$-LysNH_2 and Fmoc/Bz-ON or Fmoc-[(ψ-CH_2)Gly-D-Pro(T^{Bz})]-[(ψ-CH_2)Gly-D-Pro(T)]$_9$-LysNH_2 **(b)** Fmoc-OFF crude peptide after treatment with 20% piperidine; see HPLC gradient system in section 2.4.3 (f)

Indeed, it has been suggested that the debenzoylation was completely occurred in each cycle when the Fmoc deprotection with piperidine was performed *via* the mechanism shown in figure 3.29.[63]

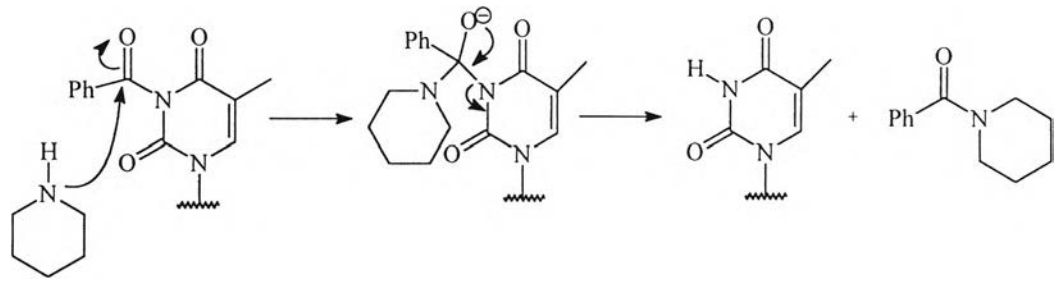


Figure 3.29 A possible mechanism for debenzoylation of N^3-benzoylthymine by piperidine.

For this reason, there should be only one benzoyl group remained on the peptide **(24)** because the final deprotection was omitted before releasing the peptide from the resin. Although the N^3-benzoyl group is susceptible to both hydrolytic and non-hydrolytic cleavage by strong bases and strong acids, it was still not completely deprotected within a few hours in the presence of trifluoroacetic acid alone. It was, however, found that addition of thioanisole to the cleavage mixture before treatment with the peptide on resin gave clean Fmoc-ON **(24)** and very small amounts of the Fmoc/Bz-ON **(24)** was observed in the HPLC chromatogram.

In the next step, the Fmoc-ON **(24)** and Fmoc/Bz-ON **(24)** were mixed together by vortexing followed by addition of 20 µl of piperidine for 20 min in order to remove both the Fmoc group and benzoyl group. The reaction mixture was then diluted with excess diethyl ether to precipitate the crude peptide and eliminate the used piperidine, this process was repeated twice and the product air dried before HPLC separation. The reverse phase HPLC was carried out by using the same gradient elution both of analytical and preparative condition. The chromatogram revealed only one broad peak at t_R = 18.8 min (figure 3.28 b) which was identified as Fmoc-OFF **(24)** (M_r calcd.for M_r =2787.1 found 2788.4) by MALDI-TOF mass spectrometry). Interestingly, all products including Fmoc-ON, Fmoc/Bz-ON and Fmoc-OFF **(24)** tends to form adducts with alkali metal ions especially sodium and potassium as evidenced in their mass spectra which was summarized in Table 3.3

The Fmoc-ON, Fmoc/Bz-ON and Fmoc-OFF **(24)** were obtained in high purity enough to study on the biophysical properties in the next section.

Table 3.3 Mass spectral data of Fmoc-ON, Fmoc/Bz-ON and Fmoc-OFF of 10-mer deoxyglycylproline cPNA **(24)**

Structure	N-terminal protecting groups	M_r (found)	M_r (cald)
Fmoc-[(ψ-CH_2)-Gly-D-Pro(T)]$_{10}$-$LysNH_2$	Fmoc-ON	3013.3	3010.4 (M)
		3035.3	3033.4 (M+Na)
		3053.6	3049.5 (M+K)
		3075.0	3072.5 (M+K+Na)
		3095.2	3095.4 (M+K+Na)
Fmoc-[(ψ-CH_2)-Gly-D-Pro(T^{Bz})]-[(ψ-CH_2)-Gly-D-Pro(T)]$_9$-$LysNH_2$	Fmoc/Bz-ON	3118.6	3115.5 (M)
		3140.0	3138.4 (M+Na)
		3158.5	3154.6 (M+K)
		3180.4	3177.6 (M+K+Na)
		3201.8	3200.5 (M+K+2Na)
		3223.5	3223.5 (M+K+3Na)
H-[(ψ-CH_2)-Gly-D-Pro(T)]$_{10}$-$LysNH_2$	Fmoc-OFF	2788.4	2787.1 (M)
		2810.7	2811.1 (M+Na)
		2826.9	2826.9 (M+K)

3.2.2 The decamers of serylproline cPNAs (25) and (26)

Oligomerization of both diastereomers of serylproline cPNA monomer were carried out by Fmoc chemistry. Since this monomer has been synthesized in the form of pentafluorophenyl ester carrying *tert*-butyl ether as the protecting group of serine alcohol side chain, it was necessary to adjust the protocol to be compatible with Fmoc/O^{t}Bu chemistry which was summarized in figure 3.30

The synthesis was accomplished with 4.0 μmol in case of L-series **(25)** and 1.8 μ mol for D-series **(26)** due to the limited amount of the D-series monomer obtained section 2.3.2. The oligomerization was started from anchoring the resin with Fmoc-L-lysine as first

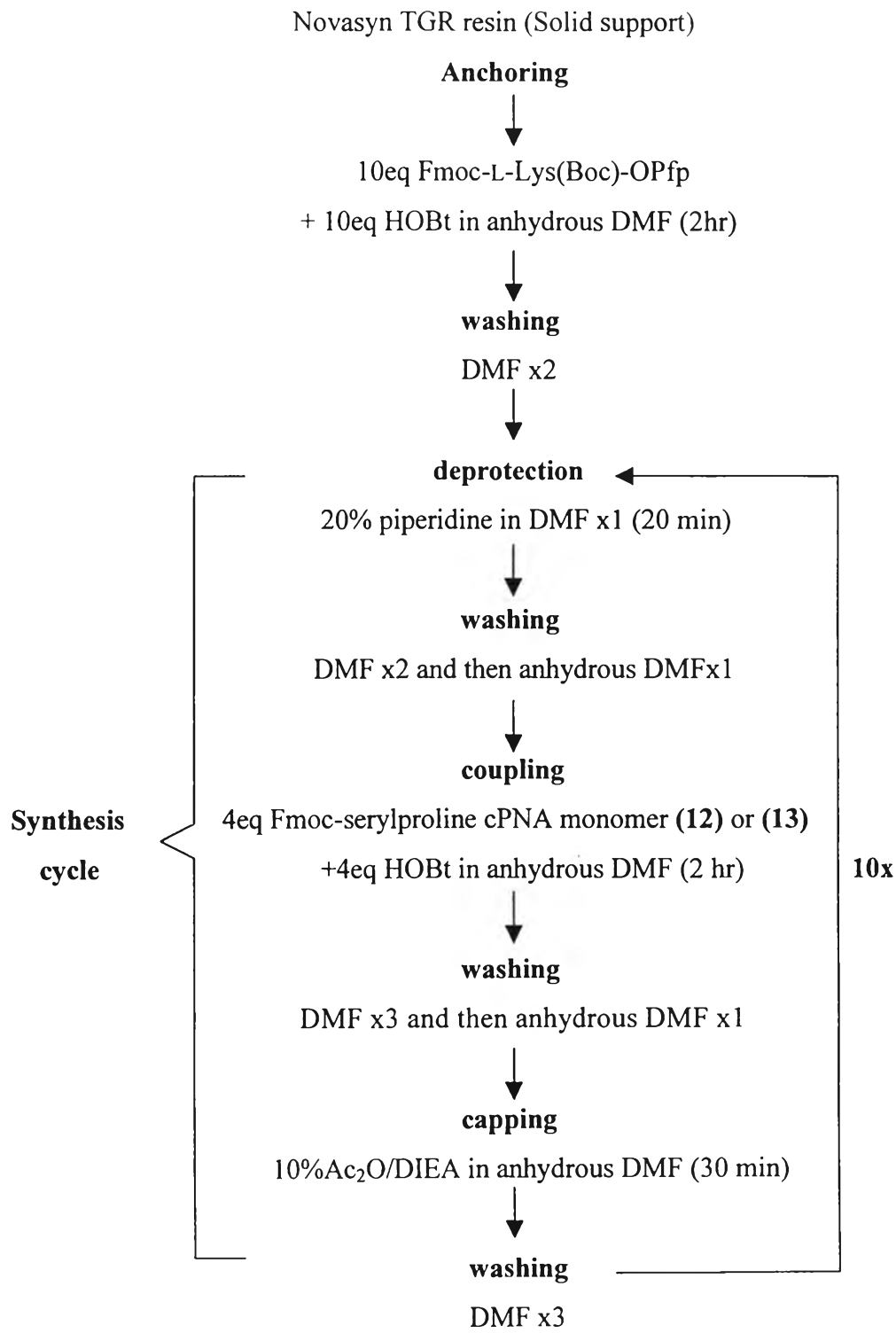


Figure 3.30 The protocol for solid phase synthesis of serylproline cPNA **(25)** and **(26)**

amino acid residue. Unlike the deoxyglycylproline cPNA oligomerization, Fmoc-L-Lys (Boc)-OPfp was used instead of Fmoc-L-Lys(Boc)-OH in order to parallelize with subsequent coupling steps employing Pfp ester monomers. Pfp esters have been well-known as an active ester which was used to form peptide bond smoothly *via* ester aminolysis mechanism which was facilitated by the good leaving ability of the pentafluorophenyl moiety. The peptide bond formation was accelerated in the presence of HOBt as shown in figure 3.31.

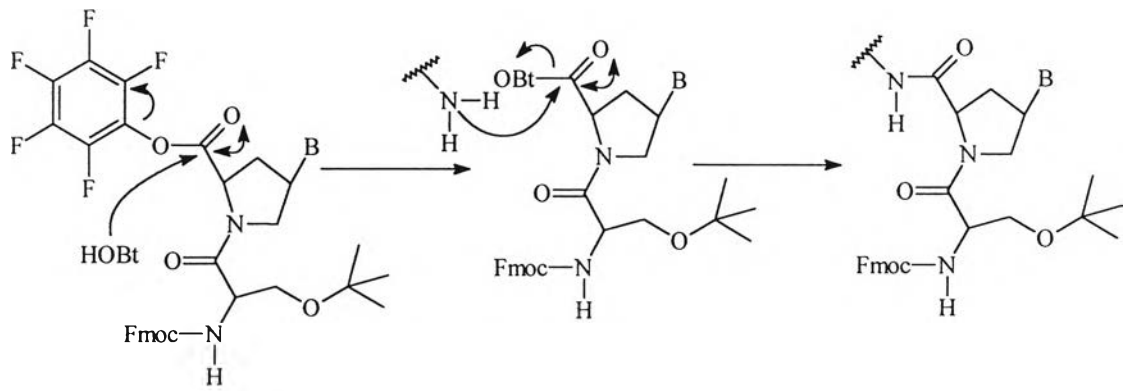


Figure 3.31 Coupling mechanism of Pfp active ester **(12)** and **(13)**

In practice, the oligomerization was still carried out in pasteur pipette similar to previous experiments. After the anchoring step was finished, the resin was then subjected to the next synthesis cycle. The Fmoc group was deprotected with piperidine and the UV-absorbance measured in order to calculate the coupling efficiency and compare with the next step. The coupling proceeded with L-serylproline cPNA monomer **(12)** or **(13)** for D-series and HOBt in equal equivalent according to the mechanism in figure 3.28. Double coupling have not been performed since it was limited by total amounts of the monomers available in our hand. Luckily, the use of Pfp ester did not require basic condition which could reduce the risk of racemization and lost of the monomer by base-catalyzed cyclization during the coupling step. Although it was also reasonable that *trans*-amide bond could interconvert easily into *cis*-configuration in order to assist the cyclization process which becomes diketopiperazine derivative, this incident would not occur without basic catalyst. Certainly, there still remain the unreacted free amino group on resin after

coupling step which need to be acetylated by acetic anhydride/DIEA into acetamide derivative in capping step.

The coupling efficiency of each cycle was determined and calculated to be percentage as shown in Table 3.4.

Table 3.4 The UV-absorption data and percent coupling efficiency of 10-mer L-serylproline cPNA **(25)** and D-serylproline cPNA **(26)**

cPNA	Item	Cycle No.										
		1	2	3	4	5	6	7	8	9	10	overall
(25)	OD_{264}	0.848	0.836	0.790	0.714	0.629	0.511	0.496	0.436	0.351	0.339	N/A
	%efficiency	100.0	98.6	94.5	90.4	88.1	81.2	97.1	87.9	80.5	96.6	40.0
(26)	OD_{264}	0.943	0.937	0.818	0.759	0.647	0.599	0.591	0.564	0.483	0.421	N/A
	%efficiency	100	99.4	87.3	92.8	85.2	92.6	98.7	95.4	85.6	87.2	44.6

Remark All OD_{264} were obtained from 200 fold dilution with absolute methanol

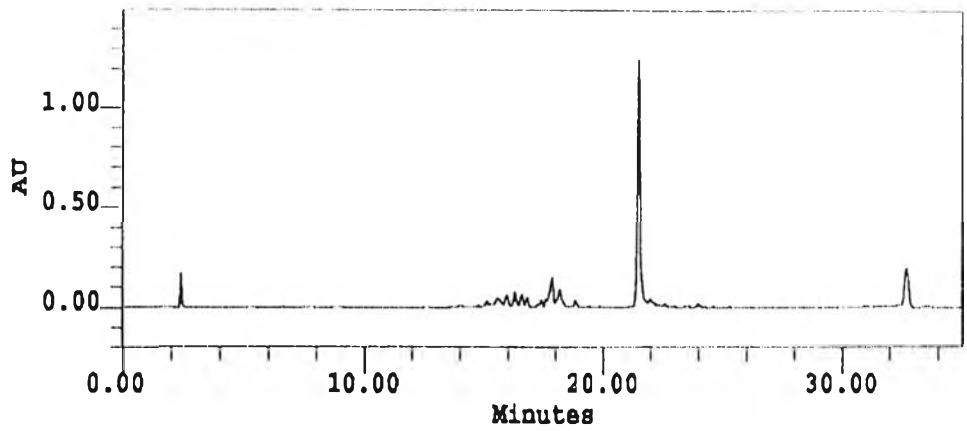
As the experimental results were satisfactory with overall yield 40 % for L-series and 44.6 % for D-series even only single coupling protocol were employed. It was believed that this was due to the non-basic condition required for Pfp ester coupling which would reduce the risk to cyclization into diketopiperazine. Another important reason is that the Pfp ester could be purified before use which serve to reduce side reactions or other competitive reactions.

In case L-series **(25)**, after the resin-bound peptide was extended upto 10 synthesis cycle, it was decided to follow the purification route of deoxyglycylproline cPNA by preserving the N-terminal Fmoc group after cleavage of the peptide from resin. Hence the synthesis was stopped after the final capping cycle and then treated with trifluoroacetic acid for 6 hr. After removal of the trifluoroacetic acid and precipitating of the crude peptide by diethyl ether. The crude peptide was separated by reverse phase HPLC using the gradient system as described in section 2.4.2.1 (f). The separating result was shown in

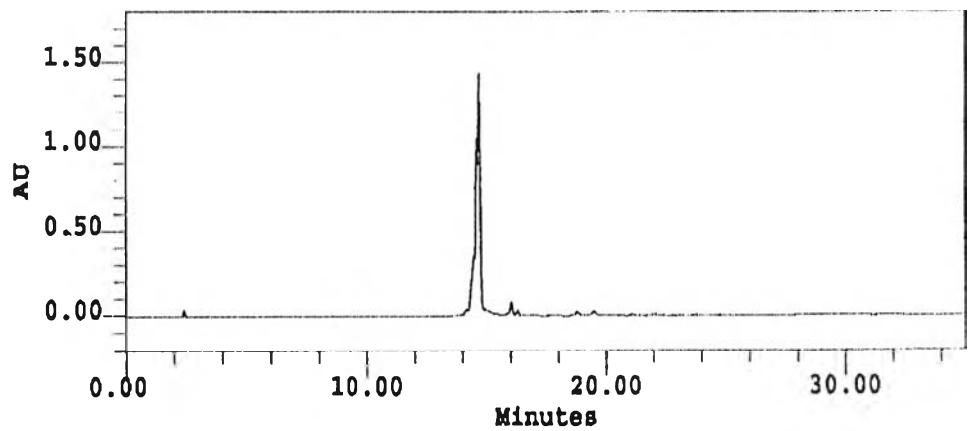
figure 3.32 (a).Only one major peak was observed at t_R = 21.5 min and was subsequently identified as Fmoc-ON **(25)**. The complicated peaks at $t_R \approx$ 15-18 min was believed to be the acetylated deletion peptides. Interestingly, no peak of Fmoc/Bz-ON **(25)** was observed in the HPLC chromatogram in this case which indicated that the benzoyl group of the peptide was completely removed during peptide cleavage within 6 hr. The Fmoc-ON product was collected from preparative reverse phase HPLC and then treated with 20 μL piperidine to remove the Fmoc group prior to precipitation with diethyl ether. The Fmoc-OFF **(25)** was obtained after reverse phase HPLC separation at t_R = 14.7 min as a single sharp peak in figure 3.32 (b) and was identified by MALDI-TOF mass spectrometry (Table 3.5).

The crude peptide of D-series **(26)** was similarly released from the resin by treatment with trifluoroacetic acid for 3 hr. HPLC analysis showed a major peak at t_R = 18.9 min and minor peak at t_R = 21.8 min in the chromatogram which were identified as Fmoc-ON **(26)** and Fmoc/Bz-ON **(26)** respectively according to MALDI-TOF mass spectra. The two peaks were collected, recombined and then treated with 20 μL piperidine to deprotect the Fmoc and benzoyl group simultaneously. The Fmoc-OFF **(26)** was further purified by reverse phase HPLC with the same gradient system and showed only one major sharp peak at t_R = 13.9 min which was identified as Fmoc-OFF **(26)** as expected by MALDI-TOF mass spectrometry. The HPLC chromatogram and mass data of Fmoc-ON, Fmoc/Bz-ON and Fmoc-OFF of D-serylproline cPNA **(26)** were shown in figure 3.33 and Table 3.6.

The final 10-mer serine-containing cPNA of both diastereomers possessed high enough purity to use for studying on their binding affinity towards the complementary natural oligonucleotide which will be discussed in the next section.

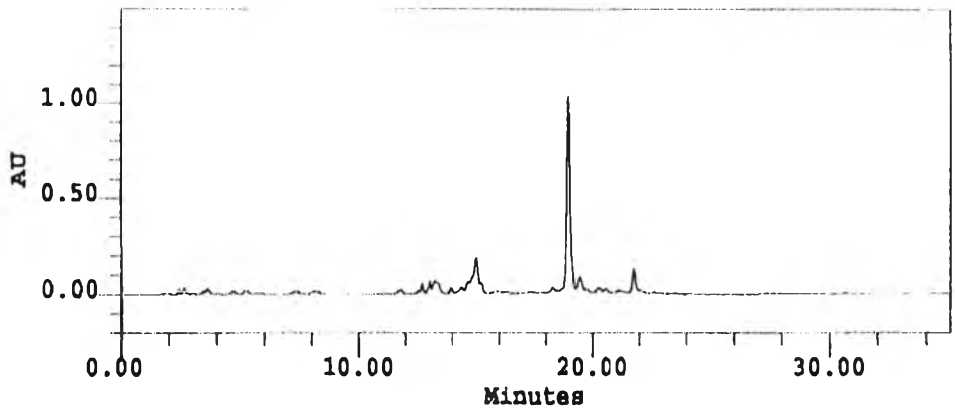


(a)

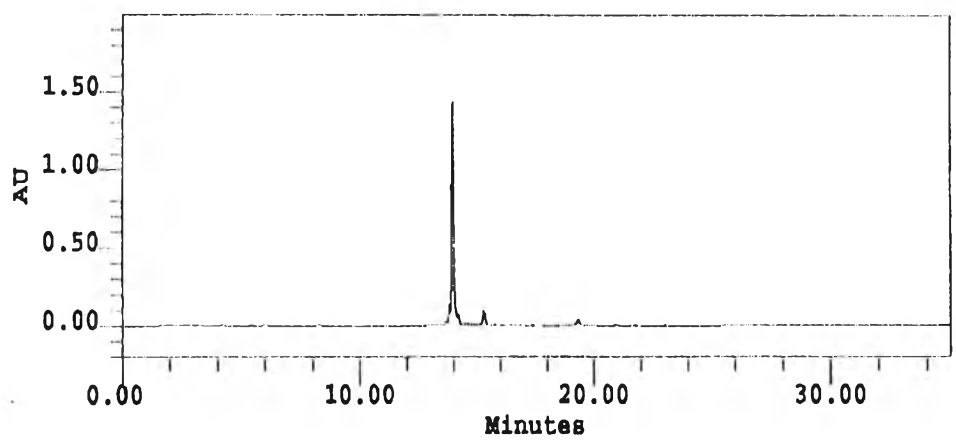


(b)

Figure 3.32 Reverse phase HPLC chromatogram of 10-mer L-serylproline cPNA **(25)** (a) Fmoc-ON (b) Fmoc-OFF



(a)



(b)

Figure 3.33 Reverse phase HPLC chromatogram of 10-mer D-serylproline cPNA **(26)** (a) Fmoc-ON and Fmoc/Bz-ON (b) Fmoc-OFF

Table 3.5 Mass spectral data of Fmoc-ON, Fmoc-OFF of 10-mer L-serylproline cPNA(**25**)

Structure	N-terminal protecting groups	M_r (found)	M_r (cald)
Fmoc-[L-Ser-D-Pro(T)]$_{10}$ -LysNH$_2$	Fmoc-ON	3450.8	3450.5 (M)
		3472.4	3473.5 (M+Na)
		3489.6	3489.6 (M+K)
		3511.6	3512.6 (M+K+Na)
		3531.0	3528.7 (M+2K)
H-[L-Ser-D-Pro(T)]$_{10}$ -LysNH$_2$	Fmoc-OFF	3227.5	3228.2 (M)
		3248.8	3251.2 (M+Na)
		3266.0	3267.3 (M+K)

Table 3.6 Mass spectral data of Fmoc-ON, Fmoc/Bz-ON and Fmoc-OFF of 10-mer D-serylproline cPNA **(26)**

Structure	N-terminal protecting groups	M_r (found)	M_r (cald)
Fmoc-[D-Ser-D-Pro(T)]$_{10}$ -LysNH$_2$	Fmoc-ON	3456.6	3450.5 (M)
		3478.2	3473.4 (M+Na)
		3495.3	3489.6 (M+K)
		3515.4	3512.6 (M+K+Na)
		3535.4	3528.7 (M+2K)
Fmoc-[D-Ser-D-Pro(T^{Bz})]-[D-ser-D-Pro(T)]$_9$-LysNH$_2$	Fmoc/Bz-ON	3557.4	3555.6 (M)
		3578.1	3578.6 (M+Na)
		3595.3	3594.7 (M+K)
		3614.3	3617.7 (M+K+Na)
		3635.2	3633.8 (M+2K)
H-[D-Ser-D-Pro(T)]$_{10}$-LysNH$_2$	Fmoc-OFF	3227.9	3228.2 (M)
		3249.5	3251.2 (M+Na)
		3265.7	3267.3 (M+K)
		3286.8	3290.3 (M+K+Na)

3.3 Biophysical studies

After the three novel cPNAs have been successfully synthesized as described in section 3.1 and 3.2, we were delighted to find that all cPNA could easily dissolve in water to a concentration of more than 5 mg/mL. Preliminary studies of binding stability of our cPNAs towards the complementary natural oligonucleotide were carried out with gel electrophoresis experiment. In principle, this technique was not only separating the molecule based on size, but also based on charge difference. Hence it was considered suitable technique to be used for identify the new hybrid formed between these cPNAs and their complementary unmodified oligonucleotides. The oligonucleotide (dA_{10}) labelled with fluorescent tag at 5'-terminal [abbreviated F(dA_{10})] was used as a probe to investigate binding to cPNA by gel-shift binding assay.[63] The F(dA_{10}) was diluted to 1.2 nmol/μL for use as stock solution. The concentration of our cPNA were calculated from the UV-absorbance at 264 nm and molar extinction coefficients (ε) of thymine was 8.8 mL/μmol.cm.[63] But for convenience, all of cPNA were shortened into symbolic codes as represented in Table 3.7.

Table 3.7 Representative code for 10-mer cPNA

cPNA	N-teminal protecting groups	Code
deoxyglycylproline **(24)**	Fmoc-ON	Fm-ψG-CDT_{10}
	Fmoc-OFF	H-ψG-CDT_{10}
L-serylproline **(25)**	Fmoc-ON	Fm-LS-CDT_{10}
	Fmoc-OFF	H-LS-CDT_{10}
D-serylproline **(26)**	Fmoc-ON	Fm-DS-CDT_{10}
	Fmoc/Bz-ON	Fm/Bz-DS-CDT_{10}
	Fmoc-OFF	H-DS-CDT_{10}

Each cPNA was mixed with F(dA_{10}) at 1:1 stoichiometric ratio before being introduced into 20 % polyacrylamide gel. The idea is that any new species, generated from

their binding, would move slower than F(dA_{10}) alone in the gel due to the increased mass and size of the hybrid while the same or decrease number of negative charges remained. Disappointingly, no new band was observed on the resulting gel which indicated the lack of binding of all cPNA synthesized in this study as shown in figure 3.34.

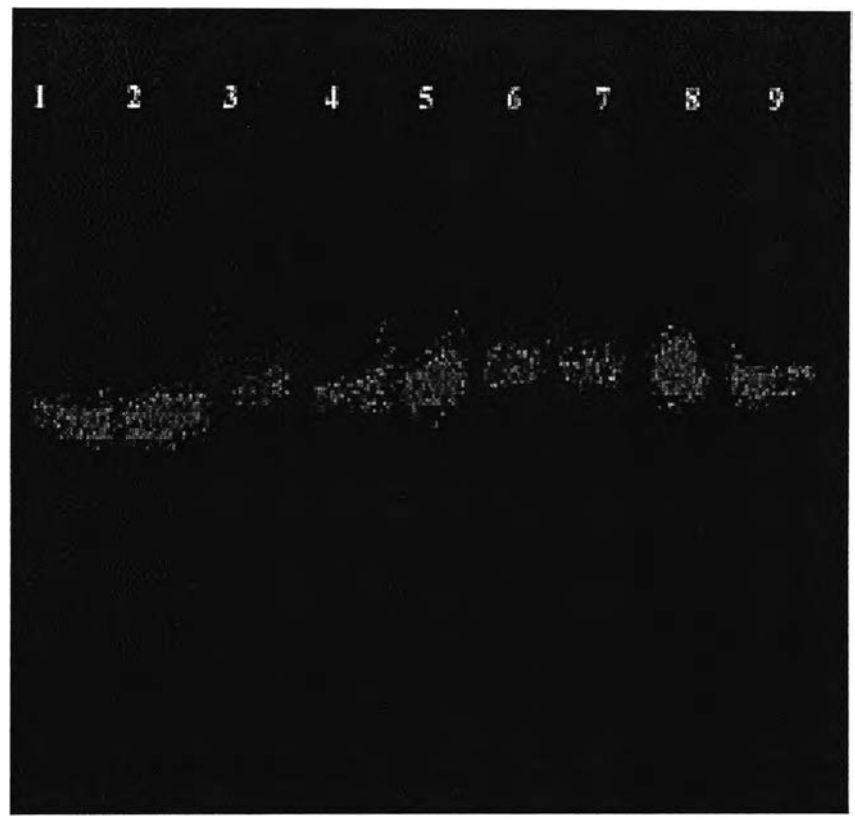


Figure 3.34 Gel electrophoresis results of 1:1 stoichiometric ratio binding: Lane 1 and Lane 9 was F(dA_{10}) (negative control), Lane 2; F(dA_{10}): Fm-LS-CDT_{10}, Lane3; F(dA_{10}): H-LS-CDT_{10}, Lane 4; F(dA_{10}): Fm-DS-CDT_{10}, Lane 5; F(dA_{10}): Fm/Bz-DS-CDT_{10}, Lane 6; F(dA_{10}): H-DS-CDT_{10}, Lane 7; F(dA_{10}): H-ψG-CDT_{10}, Lane 8; F(dA_{10}): Fm-ψG-CDT_{10}

These inability of both H-LS-CDT_{10} and H-DS-CDT_{10} to bind with F(dA_{10}) were also supported by the melting temperature experiments and ^{1}H NMR studies which have been independently investigated by Vilaivan.[85] Hence it was therefore concluded that both diastereomers of serylproline cPNA could not bind to F(dA_{10}) no matter with or without Fmoc at N-terminus. The result that deoxyglycylproline cPNA did not show the new band

on the gel was more surprising since the analogous cPNA with *cis*-L and *trans*-D stereochemistry around the proline ring were very recently reported to bind strongly with complementary DNA.[82] To test that this was not due to the unsuitable stoichiometric ratio of F(dA_{10}):cPNA, even though photometric titration of glycylproline cPNA to (dA_{10}) revealed a 1:1 stoichiometry,[63] it was decided to re-perform the gel electrophoresis experiment of deoxyglycylproline cPNA to F(dA_{10}) by variation of stoichiometric ratio of F(dA_{10}):cPNA as follows: 1:1, 1:2, 1:3, 1:5 and 1:10. The results were as shown in figure 3.35.

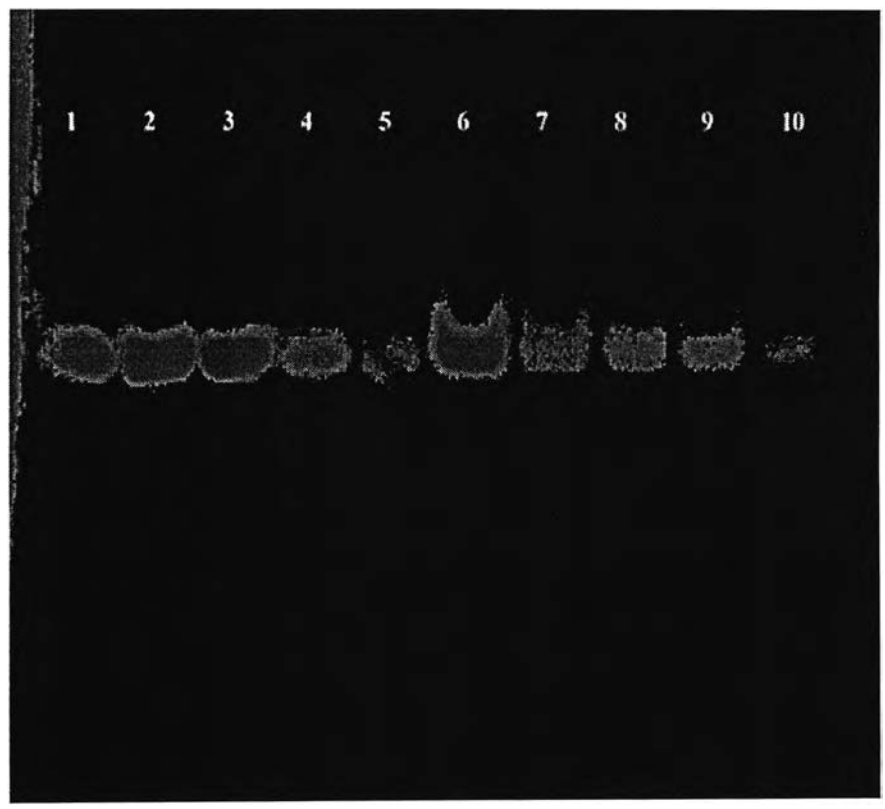


Figure 3.35 Gel electrophoresis result with stoichiometric variation: Lane 1-5 were F(dA_{10}): Fm-ψG-CDT_{10} with stoichiometric ratio 1:1, 1:2, 1:3, 1:5 and 1:10 respectively, Land 6-10 were F(dA_{10}): H-ψG-CDT_{10} with the same stoichiometric ratio.

As evidenced from the gel, there was no new band appeared on the gel which confirmed our previous results. The failure of all cPNAs to interact with complementary

oligonucleotide is quite unexpected. It is not clear why incorporation of a hydrophilic group of serine or removal of the glycyl amide carbonyl group would have such dramatic effect against the binding stability. Nevertheless, we presevere to explain this negative result which include: i) formation of some intramolecular hydrogen bonding at hydroxylic side chain of serine led to stabilization of the single strand cPNA ii) steric effect of serine side chain towards the oligonucleotide backbone to destabilize the complex.

It was expected that the positively charge on deoxyglycylproline cPNA would increase the binding stability due to electrostatic attraction to negatively phosphate oxygen. It was possible that destabilizing effect might came from the enforcement of applied voltage which could induce the positively charge on Fm-ψG-CDT_{10} or H-ψG-CDT_{10} moved apart from F(dA_{10}) to cathode (upperward in figure 3.34 and 3.35). Unfortunately, no suitable method to investigate such movement of cPNA was available since they do not possess fluorescent tag and simple staining with coomassic brillant blue was not successful. These unexpected results certainly need more experiments to fully understand the explanation.