SYNTHESIS AND DNA BINDING PROPERTIES OF PYRROLIDINYL PEPTIDE NUCLEIC ACID WITH CARBAZOLE DERIVATIVES

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ABSTRACT

Pyrrolidinyl peptide nucleic acid (acpcPNA) consists of D-prolyl-2-amino-cyclopentanecarboxylic acid (ACPC) repeating subunit which is a DNA mimic. ACPC subunit is replaced instead of deoxyribose-phosphate in the original DNA backbone. The acpcPNA can form more stable hybrid together with higher sequence specificity with its complementary DNA via Watson-Crick base pairing in a comparison to DNA·DNA hybrid. Although Watson-Crick base pairing between natural nucleobases work very well in biological system. Several instances of successful universal base incorporations with PNA systems have been reported. Following its success with other PNA systems, the aromatic compound 3,6-dinitrocarbazole (DNC) was purposed as a universal base in this study. DNC-containing acpcPNA sequences were synthesized and determined its DNA binding properties by thermal denaturation experiments. The results showed the difference in T_m between the most stable and least stable base pairs (ΔT_m) equal to 2.0°C, indicated a universal base property of DNC in mix-sequence acpcPNA. In addition, synthesis of the acpcPNA carrying a 3,6-diaminocarbazole (DAC) was carried out by a reduction of the acpcPNA containing DNC with Raney-Ni and was preliminary studied its fluorescence property. Unfortunately, the DAC-containing acpcPNA showed poor DNA binding and virtually no fluorescence change.

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LIST OF ABBREVIATIONS

δ	=	Chemical shift
μL	=	Microliter
μmol	=	Micromole
A	=	Adenine
Ac	=	Acetyl
Boc	=	*tert*-Butoxycarbonyl
calcd	=	Calculated
CCA	=	α-Cyano-4-hydroxy cinnamic acid
$CDCl_3$	=	Deuterated chloroform
D_2O	=	Deuterium oxide
DIAD	=	Diisopropylazodicarboxylate
DIEA	=	Diisopropylethylamine
DMF	=	*N,N'*-Dimethylformamide
DMSO-d_6	=	Deuterated dimethylsulfoxide
DNC	=	3,6-Dinitrocarbazole
DAC	=	3,6-Diaminocarbazole
DNA	=	Deoxyribonucleic acid
Dpm	=	Diphenylmethyl
Fmoc	=	9-Fluorenylmethoxycarbonyl
g	=	Gram
G	=	Guanine
HATU	=	O-(7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium haxafluorophosphate
HOAt	=	1-Hydroxy-7-azabenzotriazole
Lys	=	Lysine
J	=	Coupling constant
m	=	Multiplet

LIST OF ABBREVIATIONS (CONT.)

MeOTs	=	Methyl tosylate
MALDI-TOF	=	Matrix-assisted Laser Desorption Ionization-Time of Flight
MeCN	=	Acetonitrile
mg	=	Milligram
MHz	=	Megahertz
mL	=	Milliliter
mM	=	Millimolar
mmol	=	Millimol
MS	=	Mass spectrometry
m/z	=	Mass to charge ratio
NMR	=	Nuclear magnetic resonance
°C	=	Degree Celsius
OD_{xxx}	=	Optical density at xxx nm (= A_{xxx})
PCR	=	Polymerase Chain Reaction
pfp	=	Pentaflurophenyl
PNA	=	Peptide Nucleic Acid
ppm	=	Parts per million
R_f	=	Retention factor
s	=	Singlet
t	=	Triplet
T	=	Thymine
TFA	=	Tetrahydrofuran
TLC	=	Thin Layer Chromatography
T_m	=	Melting temperature
UV	=	ultraviolet

CHAPTER I

INTRODUCTION

Rationale for the study

In medical science, the nucleic acid manipulation is useful in analysis and diagnosis of some diseases in advance for treatment forecast and gene therapy treatment. To detect the specific gene of disease, a sequence of complementary oligonucleotide probe (short sequence of DNA or RNA) will be designed and synthesized to bind with its sense region via base pairing rules of Watson-Crick which nucleobase A and C are complementary to T and G, respectively. [1, 2] However, a major problem of DNA detection by using the oligonucleotide probe is an observation of single nucleotide polymorphism (SNPs) in the target sequences. SNPs is a genetic diversity of each individual person in term of a variation in at least one position on DNA sequence which differs from the original sequence causing an ambiguous DNA sequence and a difference human genotype and phenotype. Although a highly specific base pairing between two strands of DNA probe and DNA target is important, but this high specificity is undesirable in certain purposes especially in SNPs phenomena. So, universal bases has been designed and applied to use in the case of determination of DNA sequences that are closely related, but not identical (SNPs). [3] Universal base is a nucleobase analogue which can non-specific bound to the four DNA bases (A, T, C, G). The use of universal probe will reduce the number of probes to be designed and synthesized. Many research works have been demonstrated that universal base in DNA hybrid could fix at the position of the SNPs itself. [4]

In 1991, PNA (Peptide Nucleic acid or polyamide nucleic acid) was firstly introduced by Nielsen and coworkers, who synthesized and studied molecules which could imitate DNA and their properties (also known as aegPNA) (Figure 1). [5] PNA was designed by replacing sugar and phosphate backbone in DNA with *N*-2-aminoethyl glycine skeleton (resulting to uncharged molecule) which natural nucleobase attached to the core structure. Interestingly, PNA can form stable hybrid with DNA via Watson-Crick base paring similar to DNA duplex. In addition,

PNA·DNA complexes are more stable than the DNA·DNA complex because there is no electrostatic repulsion in the hybrid of PNA·DNA. Moreover, PNA is remarkably resistant to enzymatic and chemical degradation.

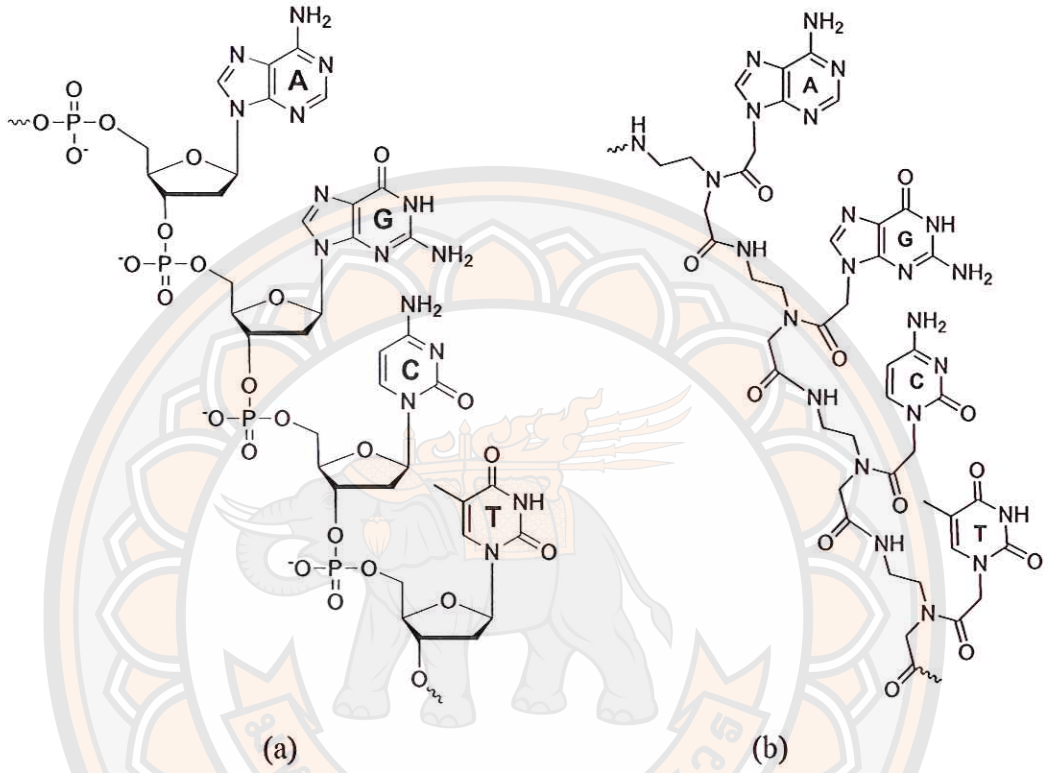


Figure 1 Structures of (a) DNA and (b) Nielsen's PNA (aegPNA)

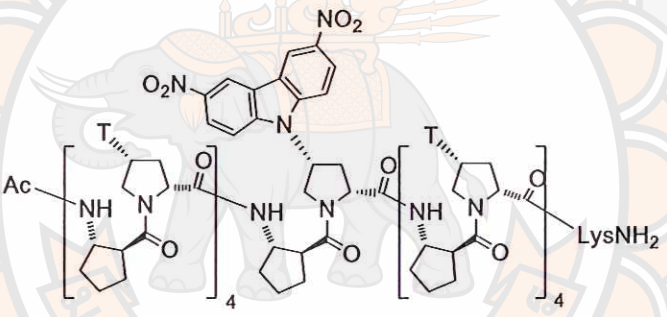
However, PNA also has some disadvantages that need to be further developed, such as the relatively poor water solubility and cellular uptake. [6] Other problems include unspecific binding to either DNA or RNA, direction of binding, non-specific binding due to hydrophobicity of molecule. [7] To improve the desired properties, PNA systems have been continuously developed by many researchers over the decades. In 2001, Vilaivan research group synthesized a new pyrrolidinyl PNA called acpcPNA (Figure 2). [8, 9, 10, 11] The novel acpcPNA consists of D-prolyl-2-aminocyclo-pentane carboxylic acid (ACPC) subunits which resulting to more rigid backbone than the original aegPNA. The acpcPNA showed a higher affinity and sequence specificity than DNA duplex and also aegPNA·DNA when bound to its complementary DNA while the stability of aegPNA·DNA complexes increased with

high %GC content, DNA hybrid of acpcPNA is relatively sensitive to base sequence. The hypothesis is that rigidity of acpcPNA structure can affect to the Watson-Crick hydrogen bond especially in G·C pair.

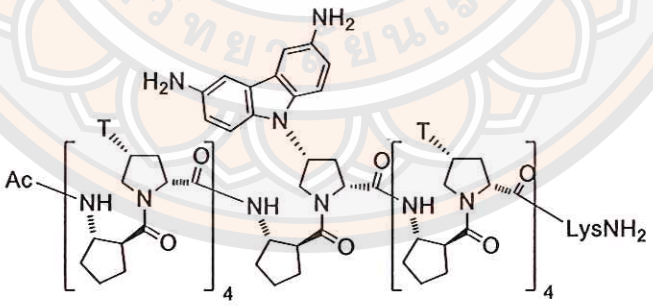
Therefore, the aim of this research is to focus on the study in the effect of nucleobase toward the stability of acpcPNA. Some potential universal base such as 3,6-dinitrocarbazole and 3,6-diaminocarbazole will replace the natural nucleobase in acpcPNA and the binding properties of these novel PNA with DNA will be investigated.

DNA 5′-AAAA**X**AAAA-3 X = ambiguous base (X= A, T, C, G)

PNA N- TTTT**B**TTTT -C B = carbazole derivatives



(a)



(b)

Figure 2 Structure of acpcPNA attached with (a) 3,6-dinitrocarbazole and (b) 3,6-diaminocarbazole)

Objectives of the study

1. Synthesis of pyrrolidinyl PNA containing universal base including 3,6-dinitrocarbazole and 3,6-diaminocarbazole.

2. Study of the stability and selectivity of the binding between the carbazole-containing synthesized PNA and DNA target.

Scope of this Research

1. Synthesis of 3,6-dinitrocarbazole

2. Synthesis of pyrrolidinyl PNA containing universal base such as 3,6-dini trocarbazole and 3,6-diaminocarbazole

3. Evaluation the stability and selectivity by T_m measurement from the base pairing between PNA and DNA target. The base pairing between PNA and DNA target will be evaluated the stability and selectivity by T_m measurement

4. Evaluation the fluorescence properties of PNA containing universal base as 3,6-diaminocarbazole

CHAPTER II

LITERATURE REVIEWS

Peptide nucleic acid (PNA)

In 1991, the original Peptide Nucleic Acid or polyamide acid (PNA) was first reported by Nielsen and coworkers. [12] PNA is DNA analogue in which deoxyribose phosphate backbone was replaced by 2-aminoethyl glycine (aeg) as shown in Figure 3. Owing to the absence of negatively charge phosphate groups, PNA not only forms a stable hybrid with DNA according to the Watson-Crick base but also retains excellent binding affinity and specificity to DNA target. Furthermore, the aegPNA can bind with DNA in both antiparallel and parallel directions, although the antiparallel binding is preferred. PNA·DNA hybrids are also proves to be more stable than the DNA·DNA hybrids. However, some disadvantages of aegPNA such as relative poor water solubility causing rather difficult in a cellular uptake to the cell target, need to be further developed. [13, 14]

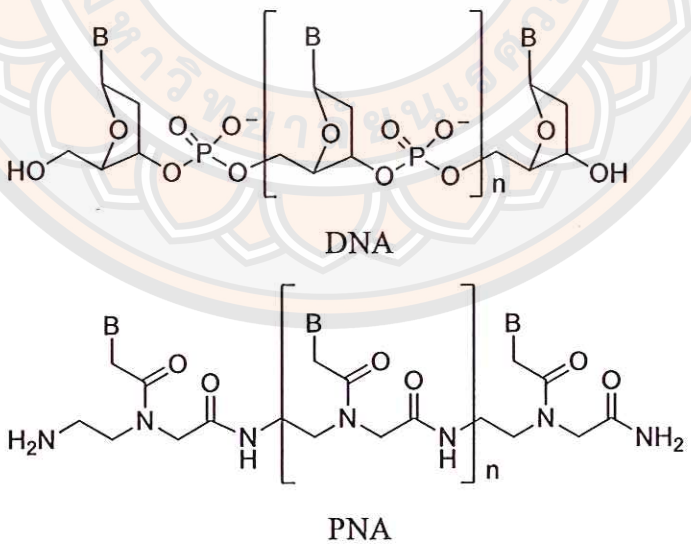
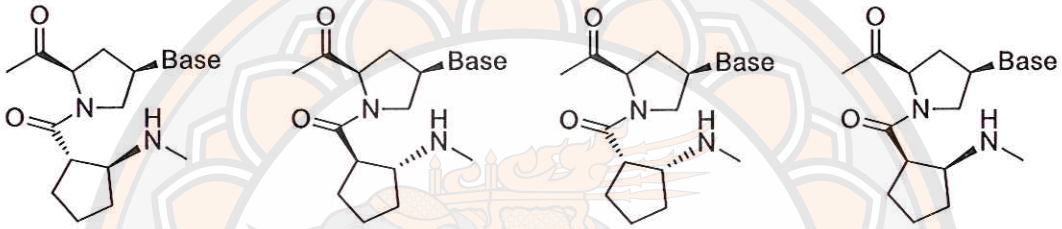


Figure 3 Chemical structures of a DNA molecule and an aegPNA molecule

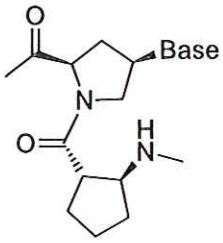
Many research groups had attempted to develop PNA system during the past 20 years. [15,16] One of the most interesting modification is pyrrolidinyl PNAs which is designed and studied by Vilaivan and co-worker. [8] Study to four derivatives of pyrrolidinyl PNA bearing 2-aminocyclopentane carboxylic acid (ACPC) backbone (Figure 4) revealed that incorporation of (1*S*,2*S*)-2-aminocyclopentane carboxylic acid provided strong binding affinity and high sequence specificity towards its complementary DNA target than aegPNA. [10] Furthermore, the acpcPNA also showed stronger preference for antiparallel binding mode. [11]



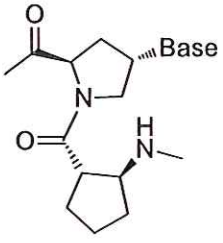
(1*S*,2*S*)-acpcPNA (1*R*,2*R*)-acpcPNA (1*S*,2*R*)-acpcPNA (1*R*,2*S*)-acpcPNA

Figure 4 Structure of pyrroidinyl PNA carrying 2-amiocyclopentane carboxylic acid (ACPC) spacers

A modification at a proline group in pyrrolodinyl PNA structure with the nucleobase at the 4-position with (2'*R*,4'*S*) configuration and a (1'*S*,2'*S*)-2-amino cyclopentanecarboxylic acid ((2'*R*,4'*S*)-*acpc*PNA) backbone is shown in Figure 5. [17, 18] It was found that the (2'*R*,4'*S*)-*acpc*PNA could substitute (2'*R*,4'*R*)-acpcPNA as probes for nucleic acid detection because of their similar DNA and RNA binding properties. Surprisingly, (2'*R*,4'*S*)-*acpc*PNA forms a more stable antiparallel self-hybrid than (2'*R*,4'*R*)-acpcPNA, which could not form self-hybrid at all.



(2'*R*,4'*R*)-*SS*-acpcPNA



(2'*R*,4'*S*)-*SS*-acpcPNA

Figure 5 Structure of two configuration of acpcPNA

Universal base

Universal base can be any compounds which can form base pairing with the natural DNA/RNA bases without the discrimination between the four natural nucleobases (A, T, C, G).

Many compounds were designed and studied to find out the effective properties. Universal bases can be categorized into 3 classes by their structures and modes of binding. [19] The first category is universal base using hydrogen bonding interaction to form stable pairing with natural nucleobase. [20, 21] These universal bases are designed to improve and mimic the natural structure of a nitrogen base to retain the formation of a hydrogen bond. The principle of design and development of this class of universal base has been studied and revealed that the main drawbacks are difficulty in the design and to synthesize any molecule as a good universal base. Some of these classes of molecules are shown in Figure 6.

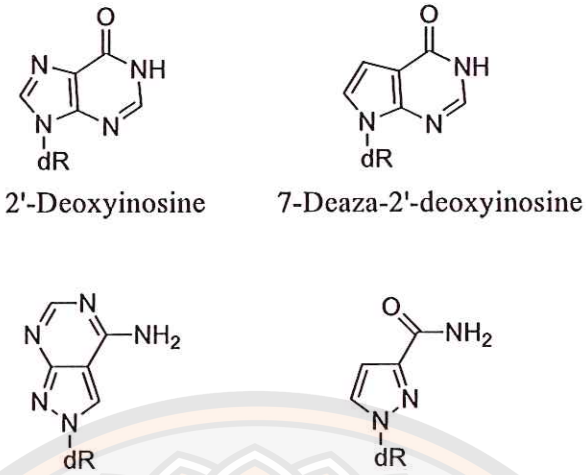


Figure 6 Structure of universal bases with hydrogen bonding interaction

The second class is universal base using non-hydrogen bonding interaction. In this case, the nitrogen base is replaced by a carbocyclic aromatic ring such as phenyl, naphthalene, biphenyl and pyrene (Figure 7). [22, 23, 24] This group of universal base was designed to interact with other natural bases using π-π stacking interaction between planar aromatic ring and nucleobase. Unfortunately, the interactions between these aromatic groups and nucleobase in DNA are not very strong due to the lack of electron withdrawing group on their aromatic ring in order to encourage π-π stacking.

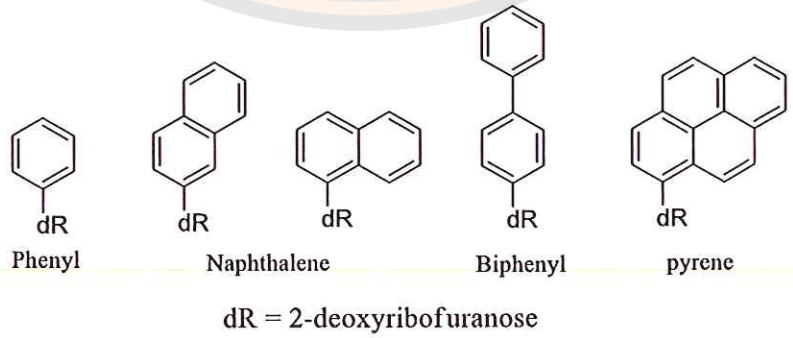
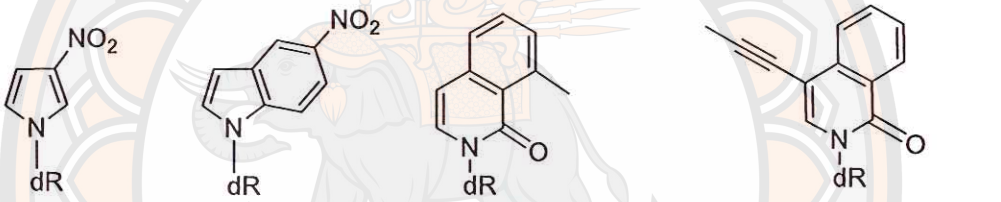


Figure 7 Structure of universal bases with non-hydrogen bonding interaction

The last class of universal base is heterocyclic aromatic compounds containing one or more nitrogen atoms. There were many reports about the use of heterocyclic in a replacement of nitrogen base such as 3-nitropyrrole, [25] 5-nitroindole, [26] C_3-methylisocarbostyril and propynylisocarbostyril (Figure 8). [27] The study of 3-nitropyrrole found that a stability in the pairing of 3-nitropyrrole and DNA base in the duplex was in an acceptable range but the less stable base pairing was observed in case of DNA triplex. [25] In a comparison, 5-nitroindole could form more stable duplex than that of 3-nitropyrrole. In addition, C_3-methylisocarbostyril and propynylisocarbo-styril were investigated for their universal base properties and results showed that both compounds are efficient universal base. Moreover, this kind of these compound are easy to design and can be synthesized in a few step.



3-nitropyrrole 5-nitroindole C_3-Methylisocarbostyril Propynylisocarbostyril

dR = deoxyribofuranose

Figure 8 Structure of universal bases with heterocyclic structures

Factors for being a Universal base

In general, the double helix of the DNA·DNA hybrid is bound by hydrogen bonds between a pair of complementary nucleobases. Specific pairing between G-C pair and A-T pair was reported by Watson and Crick in 1953. In an investigation of universal base molecules, many factors must be considered for examples.

1. The π–π stacking interaction is the interaction for compound that containing π-orbital. (Figure 9) It is a strong interaction in the fused aromatic ring system which has less than three rings. [28, 29] This interaction does not differ from the van der Waals force that occurs in the saturated hydrocarbon molecules. The π–π stacking in the system containing one or two aromatic rings is one interaction which stabilized nucleobases binding during DNA double helix formation. But sometimes, if

there are more than two aromatic rings, the π-π stacking which is too large may cause a repulsion in the system.

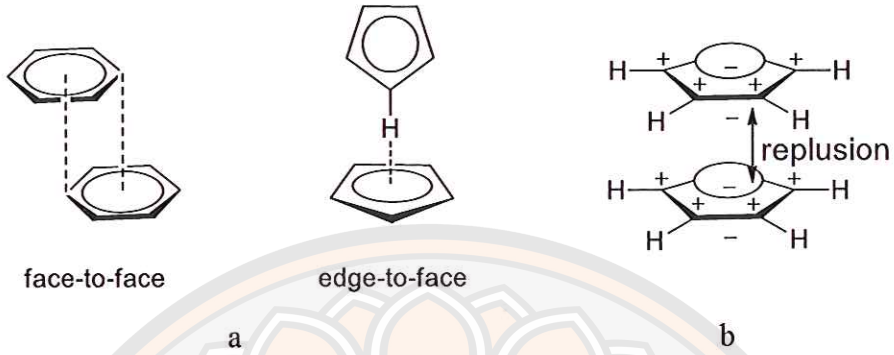


Figure 9 Intermolecular interaction in type π-π interaction (a) face-to-face and edge-to-face π stacking formation and (b) repulsion between π-π effect

2. The van der Waals force is the interaction between the atoms, molecules and the surface of the molecule. This force is an intermolecular force occurring from anisotropic effect which is dependent on the relationship between two molecules. [30]

3. Electrostatic effect is a force which receiving an influence from structure, reaction reactivity or molecular property but it does not relate to the steric effect. Electrostatic force affects molecules in various ways for example an inductive effect which comes from a distribution of the electron density via σ-bond between two atoms (having different EN) in the molecule. [31]

4. The solvation effect, or sometimes called dissolution, is a combination between molecule with solvent or ions of the solute while soluble ions are separated from molecules of solvent. In a polar solvent, the structure of the molecule has a dipole which induces the solvation by polar solvent which is called solvate ions. The solvation is a combination of different effects from intramolecular interactions that are hydrogen bonding, ion-dipole, dipole-dipole and van der Waals force.

At present time, natural DNA nucleobases and fourteen non-natural base analogs were studied a correlation of base structure and stacking formation in term of stacking free energy (ΔΔG). [32] As shown in Figure 10, the ability of seven non-natural bases in stacking formation with DNA was compared with natural DNA bases.

It was found that the stacking formation by large non polar aromatic compound is better than by natural DNA base, observing from a higher ΔΔG value.

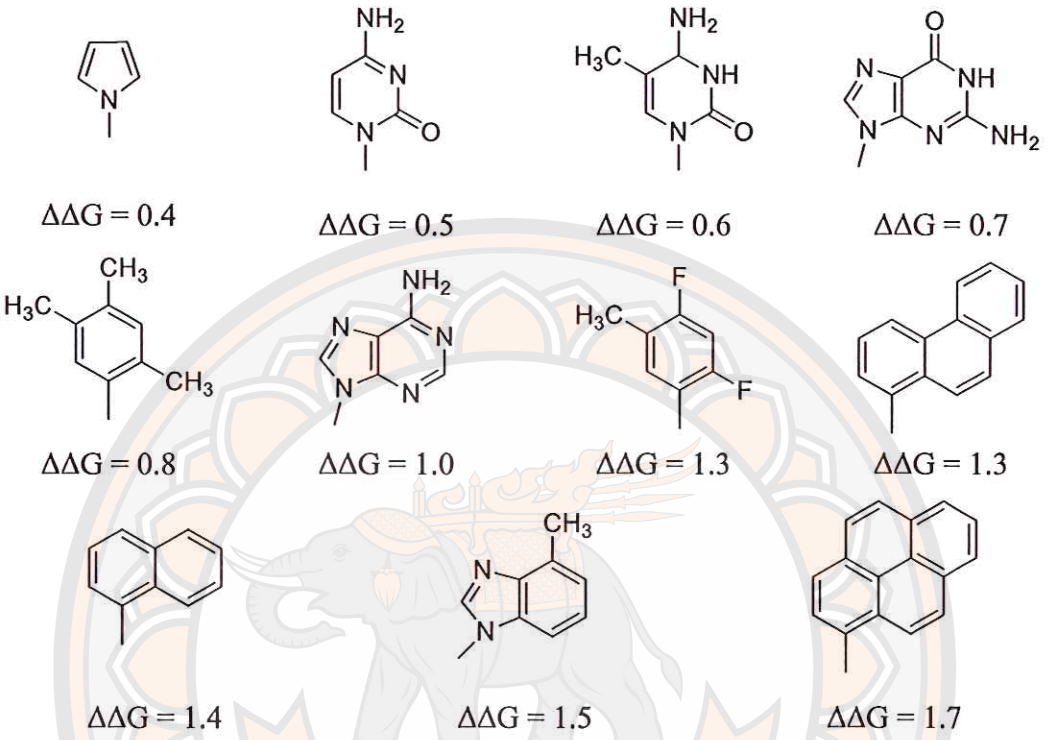


Figure 10 Structure free energy, ΔΔG (kcal/mol) of natural base and aromatic compounds in DNA

Synthesis and study properties of universal bases

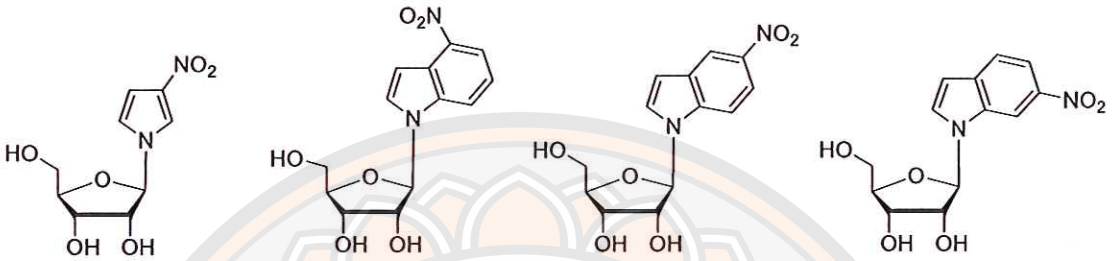
The research study of various universal bases in DNA oligomer with complementary DNA base will be referred in this section. The first universal base analogue which described by Bergstrom and received prominent attention was 3-nitropyrrole. An electronic charge distribution encourage this compound as a good candidate for stacking. [33, 34] When 3-nitropyrrole was incorporated with each four natural nucleobases in DNA, T_m variant range was reported to be only 3 °C. This result revealed that this system is general lowering of the T_m over the fully complementary duplexes as shown in Table 1

Table 1 T_m Data for hybridization of the sequence 5'-d($C_2T_5XT_5G_2$)-3' with 5'-d($C_2A_5YA_5G_2$)-3' X= 3-nitropyrrole

Base-pair (X-Y)	T_m (°C)
C-G	59
C-A	43
C-T	49
A-G	50
A-C	45
A-T	57
3NP-G	43
3NP-C	45
3NP-A	46
3NP-T	45

New series of nitroindole derivatives which are 4-, 5- and 6-nitroindole were further investigated (Figure 11). [26] All nitroindoles derivatives were compared the binding properties with 3-nitropyrrole as universal base in oligodeoxy nucleotides. Each nitroindole derivatives were incorporated into the heptadecamer sequence at various sites (at the end and in the middle of the sequence). The melting curve of the duplex with the complementary strand was determined for each duplex as shown in Table 2. The fully complementary duplex of this heptadecamer without universal base showed T_m at 72 °C. In the case of 5-nitroindole, it showed the less destabilising of the duplex by decreasing in T_m value. In addition, it was found a generally less destabilising with only 2 °C (entry 3) decreasing in T_m value when incorporated towards near the ends of 17-mer duplex and 5 °C in the middle incorporated (entry 2). In a comparison in T_m result with 3-nitropyrrole, it was found that 3-nitropyrrole reduced the T_m by 6 °C (entry 5). These results indicated that 5-nitroindole showed a better potential in stacking within the duplex over the 3-nitropyrrole because the smaller size of aromatic ring in 3-nitropyrrole affects a less stacking with DNA nucleobase. The result of 6-nitro and 4-nitroindole in a comparison of ΔH (or stacking enthalpy) with 3-nitropyrrole was studied and revealed that when adding more

universal base in the sequence, T_m values of nitroindole remained constant whilst ΔH value are decreased. In contrast with 3-nitropyrrole, the stacking enthalpy is not increased. These results indicated that 4- and 6-nitroindole are more destabilising duplex than 3-nitropyrrole.



3-nitropyrrole 4-nitroindole 5-nitroindole 6-nitroindole

Figure 11 Structure of 3-nitropyrole and nitroindole derivatives

Table 2 T_m **and thermodynamic parameters data for heptadeca deoxynucleotide Duplex and PNA**

Entry	Sequence DNA																	T_m (°C)	ΔH (kJ/mol)
DNA	T	C	T	T	G	G	C	C	A	C	C	A	T	T	T	T	G		
1	A	G	A	A	C	C	G	G	T	G	G	T	A	A	A	A	C	72	514.4
2	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	67	424.0
3	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	70	374.9
4	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	70	431.0
5	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	64	565.0
6	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	66	404.7
7	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	66	421.4
8	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-	65	459.6
9	-	-	-	6	-	-	-	-	-	-	-	-	6	-	-	-	-	64	274.8
10	-	-	-	4	-	-	-	-	-	-	-	-	4	-	-	-	-	61	307.3
11	-	-	-	4	-	-	-	-	-	-	-	-	4	4	-	-	-	61	290.5

5 = 5-nitroindole, 3 = 3-nitropyrrole, 6 = 6-nitroindole, 4 = 4-nitroindole

In 1994, a comparison of the base pairing properties of nitroazole nucleobase analogs in oligodeoxy nucleotide sequence was investigated (Figure 12). [35] Several nitroazole derivatives were modified into dodecamer DNA.

Bergstrom's research group was focused on 6 universal bases; 3-nitropyrrole, 5-nitroindole, hypoxanthine, 4-nitropyrazole, 4-nitroimidazole and imidazole. The binding stability of duplex considering from ΔG value was initially study. More negative ΔG value means more stable duplex formation. After that, indiscrimination in base pairing with the four nucleobase in DNA strands was investigated by considering from ΔT_m range. T_m value indicates the stability and selectivity of binding with natural nucleobase in double stranded DNA. High T_m means the strong binding between nucleobase and universal base and thus universal base which provided less ΔT_m range is an efficient universal base.

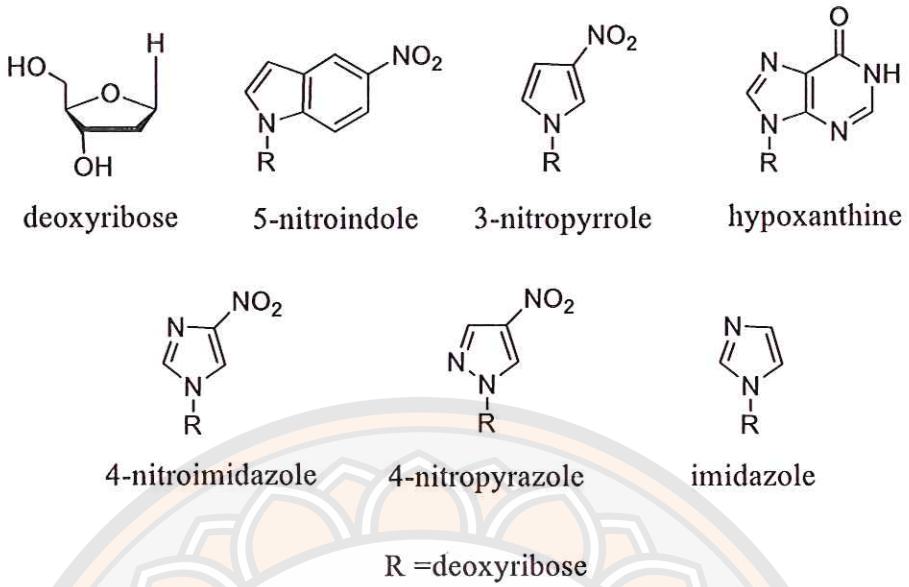


Figure 12 Structures of nitroazole nucleobases

A comparison of ΔG values among all universal bases, showed that T_m values of 4-nitropyazole and 4-nitroimidazole showed high selectivity in pairing to base A and G respectively. The highest T_m values which are 29 °C and 40 °C obtained from 4-nitropyrazole and 4-nitroimidazole, respectively. This result can be explained from the position of hetero N atom in the ring. The N3 nitrogen could assume a position that is nearly ideal for formation of a hydrogen bond with the opposite of N1 deoxyguanine (Figure 13) From all results, it can be concluded that 3-nitroindole and 5-nitroindole behaves less discriminately toward other natural base in DNA and still form duplex with more stable.

Table 3 T_m and thermodynamic parameters of the sequence 5'-d(CGCXATTYGCG)-3'

X	Y	T_m(°C)	ΔT_m	ΔG 25 °C (kcal/mol)
A	T	65.7		15.4
C	G	70.5		16.5
3-nitropyrrole	A	19.4	5.1	6.1
	C	24.5		6.5
	G	21.0		6.2
	T	20.7		6.2
5-nitroindole	A	39.0	7.4	7.7
	C	44.5		8.3
	G	40.1		8.0
	T	46.4		8.5
H-R deoxyribose	A	7.0	6.8	3.8
	C	6.6		4.0
	G	11.1		5.5
	T	13.4		5.6
hypoxanthine	A	52.1	26.8	11.2
	C	62.2		15.1
	G	35.4		8.0
	T	47.1		10.6
4-nitropyrazole	A	29.0	10.5	7.2
	C	19.3		6.1
	G	18.5		6.2
	T	21.4		6.4
4-nitroimidazole	A	19.5	23.0	6.3
	C	21.1		6.3
	G	40.9		9.3
	T	17.9		28.8
imidazole	A	8.5	8.5	5.0
	C	<0		4.5
	G	7.2		5.1
	T	7.7		4.7

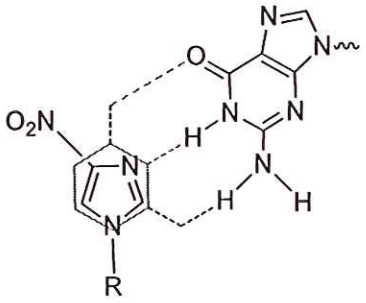


Figure 13 Proposed hydrogen bonding of the universal base with guanine

In 1999, Chulla and co-workers firstly reported the study of nitroindole and nitropyrrole in aegPNA system (Figure 14). [36] The investigation in structure characteristic on PNA·DNA duplex containing 3-nitropyrrole and 5-nitroindole was found that there is no significantly difference in ΔT_m between the system of nitropyrrole and nitroindole which were 1.5 °C and 1.3 °C, respectively. Therefore, these results can be concluded that 3-nitropyrrole and 5-nitroindole can replace and act as universal base in aegPNA system.

3-nitropyrrole PNA monomer 5-nitroindole PNA monomer

(A) (B)

Figure 14 Structures of (A) 3-nitropyrrole (B) 5-nitroindole PNA monomer

Table 4 T_m Data for hybridization of the sequence DNA: 3'-ACATGCYGTGTTG AT-5' PNA: H-TGTACGXCACAACTA-NH_2

	Y	T_m	ΔT_m
X=3-nitropyrrole	A	56.2	1.5
	G	54.7	
	C	55.9	
	T	55.1	
X=5-nitroindole	A	58.4	1.3
	G	58.9	
	C	59.7	
	T	59.3	
X=T	A	68.5	

A comparison with PNA·DNA duplex containing 3-nitropyrrole was described by Zhang and co-workers in 2001. [37] The binding property of PNA·DNA duplex was studied comparatively with the binding of DNA·DNA duplex using the same universal base. In the PNA·DNA duplexes, 3-nitropyrrole was nearly as non-discriminating universal base. The range in ΔT_m values was 2.9 °C but T_m values of the PNA containing nitropyrrole relative to the unmodified PNA·DNA duplex was decreased. Nevertheless, the PNA·DNA duplexes containing 3-nitropyrrole were more stable than their DNA·DNA counterparts by an average of 11.5 °C.

Table 5 T_m Data for hybridization of the following sequence DNA: 3'-ACATGCAYTGT TGAT-5', DNA: 5'-TGTACGTXACAA CTA 3', PNA: C'-TGTACGTXACAACTA-N', X=C, 3-nitropyrrole (Q); Y=A, C, G, T

X·Y	T_m (°C)			
	DNA·DNA	ΔT_m	PNA·PNA	ΔT_m
C·G	55.0±0.1		69.7±0.2	
Q·A	40.3±0.1	1.6	52.7±0.2	2.9
Q·C	38.7±0.2		50.7±0.3	
Q·G	39.3±0.1		49.8±0.2	
Q·T	40.0±0.1		51.3±0.2	

In 2007, PNA containing nitrocarbazole derivatives was studies by Muktung (Figure 15). [38, 39] DNA binding study indicated that nitrocarbazole derivatives still exhibited a low binding interaction even the ΔT_m value was quite good. In order to improve the binding property, the molecular structures of new universal bases need to be investigated. Therefore, several factors such as hydrophobicity and surface area have been extensively studied in order to design the best universal base that exhibits both high binding interaction and less selectivity against all natural bases. Except for to hydrophobicity and surface area, dipole moment is also another important factor that contributes the binding property and selectivity of universal base. It was found that 3-nitrocarbazole and 3,6-dinitrocarbazole showed average ΔT_m around 1.5 °C and 2.9 °C, respectively. Whereas ΔG value of 3-nitrocarbazole and 3,6-dinitrocarbazole was approximately 16.0 kcal/mol and 18.0 kcal/mol. From these results, it displayed that nitrocarbazole derivatives exhibited less discrimination against all natural bases and still maintained strong binding interaction during duplex formation

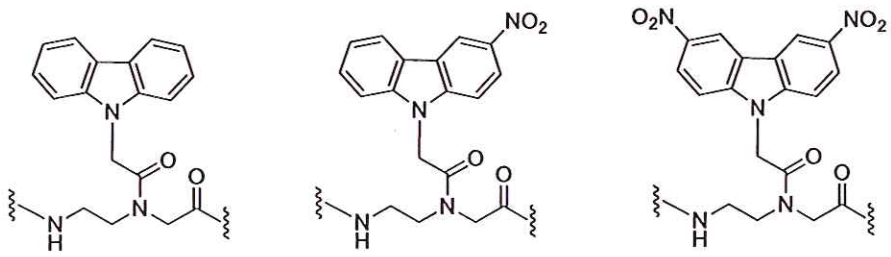


Figure 15 Structure of PNA with nitrocarbazole derivative

CHAPTER III

RESEARCH METHODOLOGY

This chapter indicates the methodology of this research including general procedure and synthetic method of PNA and evaluation method of binding properties between PNA and DNA.

General Procedure

1. Measurement

All reactions were performed in clean and oven dried glassware. The progress of the reaction was checked by thin layer chromatography (TLC) using Merck D.C. silica gel 60 F_{254} 0.2 mm pre-coated aluminium plates aluminum plate and visualized using UV light (254). Column chromatography was performed on silica gel 70-230 mesh. Solvent mixtures using in TLC and column chromatography are reported in v/v ratios. NMR spectra were recorded in ppm and recorded on a Varian Mercury 400 plus or a Bruker Avance 400 at Chulalongkorn University operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C and on a Bruker Avance 400 NMR spectrometer at Naresuan University operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Reverse phase HPLC experiments were performed on Water 600™ system equipped with a gradient pump and Water 2996 photodiode array detector; Alternatively, Rheodyne 7725 manual sample loop (100 μL sample size for analytical scale). An ACE 5 A71197, C18-AR HPLC column, 3 μm particle size, 150 x 4.6 mm was used for analytical purification. Peak monitoring and data processing were performed on the base Empower software. Fraction 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 recorded on a capillary melting point apparatus Model 9100. FT-IR spectra were recorded on Perkin Elmer Spectra GX FT-IR spectrometer. MALDI-TOF mass spectra of acpcPNA were obtained on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics) at Chulalongkorn

University 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, Sigma Aldrich Chemical Co., Ltd., and were used as received without further purification. Commercial grade solvents were distilled for column chromatography. Solvents for reactions and crystallization were reagent grade and used without purification. Tetrahydrofuran was dried with sodium metal, benzophenone as an indicator and then distilled under nitrogen. TentaGel S-RAM Fmoc resin, 0.24 mmol/g was used as solid support for peptide synthesis and was purchased from Fluka. Fmoc-L-Lys(Boc)-OPfp were obtained from supplier Calbiochem Novabiochem Co., Ltd. All Oligonucleotides were purchased from Pacific Science (Bangkok, Thailand) or BioDesign Co., Ltd. (Bangkok, Thailand). Anhydrous *N*,*N*-dimethylformamide (DMF) for solid phase peptide synthesis was obtained from RCI Labscan (Thailand) and stored over 4A molecular sieves. Trifluoroacetic acid (98%) was purchased from Fluka. High purity (99.99%) of nitrogen gas was obtained from Thai Industrial Gas and Phitsanulok Gas. MilliQ water was obtained from ultrapure water system with Millipak® 40 filter unit 0.22 µm, Millipore (USA). Pfp-activated pyrrolidinyl PNA monomers (A^{Bz}-Pfp, Fmoc-C^{Bz}-OPfp, Fmoc-G^{Ibu}-OH and Fmoc-T-OPfp) and ACPC spacer were synthesized by Ms. Boonsong Ditmangklo, Ms. Duangrat Nimanussornkul and Mr. Chayan Charoenpakdee using the previously published protocols.[9]

Synthesis of pyrrolidinyl monomer modified with dinitrocarbazole

A synthetic scheme for dinitrocarbazole-modified pyrrolidinyl PNA monomers in this research are displayed in Figure 16. This synthetic route employed *trans*-4-hydroxy-L-proline as a starting material and was prepared using previously published procedures to get compound (**5**) as intermediate. [17] After that, 3,6-dinitrocarbazole was attached to the intermediate (**5**) at position C-4 via standard substitution reaction using K_2CO_3 in DMF at 80°C, which afforded the pyrrolidine monomer containing 3,6-dinitrocarbazole (**6**). Protecting group was changed from *N*-Boc to *N*-Fmoc and the product was treated with 1:4:4 anisole/TFA/CH_2Cl_2 to get (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline carboxylic acid (**9**) as pyrrolidine monomer for PNA peptide synthesis.

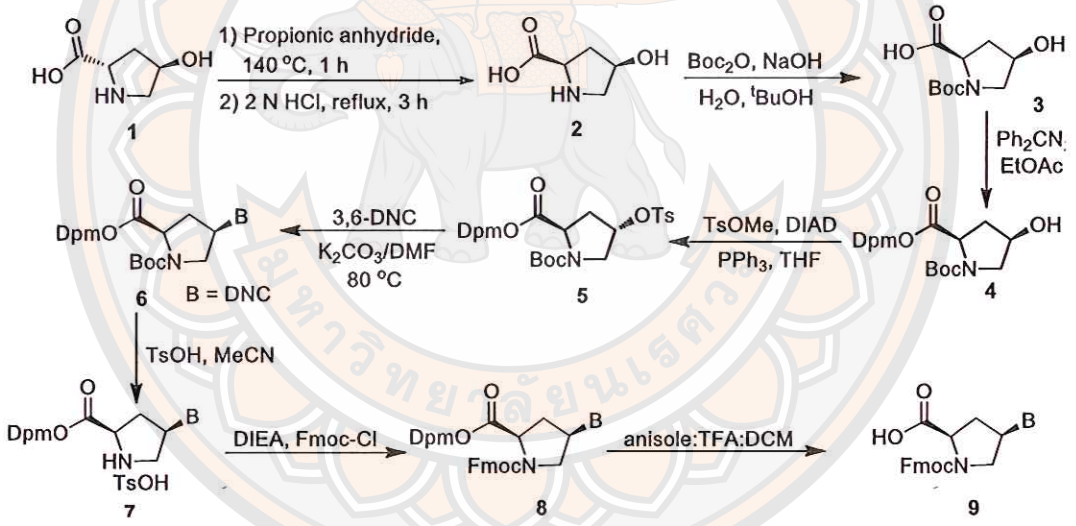
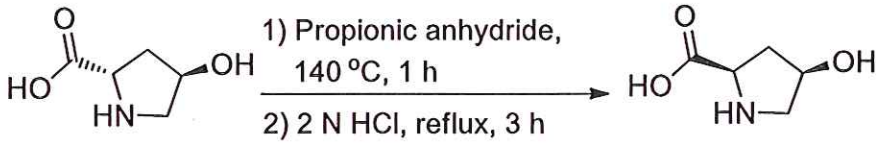


Figure 16 Synthetic routes for acpc-PNA monomer carrying 3,6-dinitrocarbazole

The Synthesis of Pyrrolidinyl monomer

1. Synthesis of *cis*-4-hydroxy-D-proline (2)

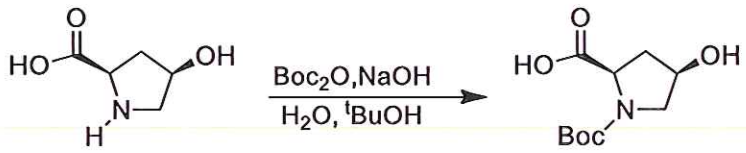


(1) **(2)**

Cis-4-hydroxy-D-proline (**2**) can be prepared using previously published procedures as following. [17] 4-Hydroxy-D-proline (**1**) (10.0450 g, 76.60 mmol) was dissolved in propionic anhydride (50 mL) into round bottom flask. The solution was heated and stirred at 140 °C for 1 hr. Then the solvent was evaporated. 2N HCl was added to dissolve the oil crude. The obtained solution was refluxed at 100 °C for 3 hr. The solution was decolorized by adding of activated charcoal and boiling the mixture for a while. After that, the charcoal was filtered. The organic layer was evaporated off the solvent under reduced pressure. The crude was obtained as white powder (14.2868 g) in 70% yield. Finally the crude was dissolved in a mixture of H_2O (42 mL) and Et_3N (12 mL) and crystalized the product by adding EtOH 389 mL. The desired product was obtained as white powder in 4.932 g (48% yields).

^{1}H NMR (400 MHz, D_2O): δ_H 2.283 [d, *J*=15.34 Hz, 1H, C$\underline{H}_2$ (3') 2.535 [t, *J*=12.23 Hz, 1H, C$\underline{H}_2$(3)] 4.23 [dd, *J*= 13.23, 40.12 Hz, 2H, C$\underline{H}_2$(5)] 45.32 [d, *J*= 8.02 Hz, 1H, C$\underline{H}_2$(2)], 4.523 [br, 1H, C$\underline{H}$(4)]

2. Synthesis *of N-tert*-Butoxycarbonyl-*cis*-4-hydroxy-D-proline (3)

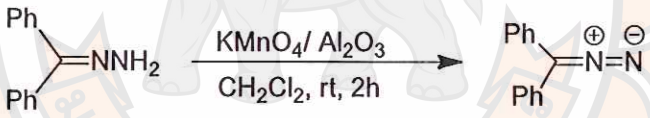


(2) **(3)**

N-tert-Butoxycarbonyl-*cis*-4-hydroxy-D-proline (**3**) can be prepared using previously published procedures as following. [17] The solution of NaOH was mixed with *cis*-4-hydroxy-D-proline **(2)** (4.8736 g, 0.021 mole) into round bottom flask. Di-*tert*-butyl-bicarbonate (9.97 g, 0.0446 mole) which dissolved in *tert*-butanol 25 mL was slowly dropped to the solution of *cis*-4-hydroxy-D-proline at room temperature overnight. Then the reaction mixture was evaporated off the solvent and adjusted pH to approximately 2 by adding $NaHSO_3$. The organic layer was extracted using 40 mL EtOAc. The product in organic layer was recrystallized by adding EtOAc 30 mL. The product was obtained as white powder in 9.632 g (90% yields).

^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 1.32, 1.37 [2×s, 9H, C<u>H</u>$_3$ Boc rotamers] 1.73-1.81 [m, 1H, C<u>H</u>$_2$(3')] 2.22-2.35 [m, 1H, C<u>H</u>$_2$(3')] 3.02-3.11 [m, 1H, C<u>H</u>$_2$(5')] 3.41-3.50 [m, 1H, C<u>H</u>$_2$(5')] 4.02-4.10 [m, 2H, C<u>H</u>(2')] and 4.15-4.19 [m, 1H, C<u>H</u>(4')]

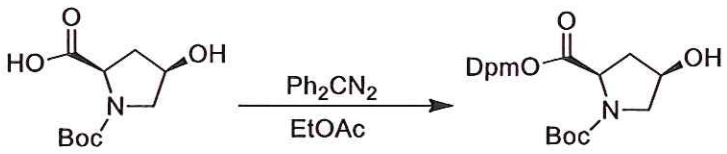
3. Synthesis of Diphenyldiazomethane (3b)



(3a) **(3b)**

Diphenyldiazomethane can be prepared using the previously reported method of Ms. Penthip Muangkaew. Benzophenone hydrazone (5.0251 g, 25 mmol) was dissolved in CH_2Cl_2 into round bottom flask which wrapped with aluminium foil to protect from light. The mixture of $KMnO_4$ (7.9 g, 50 mmol) and Al_2O_3 (25.0 g, 50 mmol) was slowly added to the solution. After 2 hr, the residue was filtered and the solvent was evaporated. The desired product was obtained in violet oil and was freshly used in the further reaction without purification and characterization.

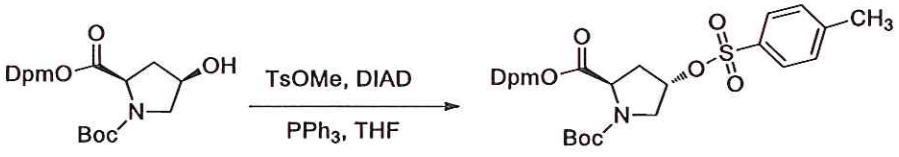
4. Synthesis of *N*-tert-Butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenyl methyl ester (4)



(3) **(4)**

N-tert-Butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester (4) can be prepared using previously published procedures as following. [17] The solution of *N-tert*-butoxycarbonyl-*cis*-4-hydroxy-D-proline(3) (9.504 g, 41 mmol) was dissolved in EtOAc. The solution was cooled at 2 °C and Ph_2CN_2 was slowly added until the solution going to be stuffed pink solution. The reaction mixture was evaporated off the solvent. Finally, the crude product was crystallized by hexane addition. The desired product was obtained as white powder in 12.563 g (95% yields). ^{1}H-NMR (400 MHz, DMSO): δ_H 1.24,1.47 [2×s, 9H, C$\underline{H}_3$ Boc rotamers] 2.18-2.42 [m, 1H, C$\underline{H}_2$(3')] 3.49-3.70 [m, 2H, C$\underline{H}_2$(5')] 4.29-4.36 [m, 1H, C$\underline{H}$(4')] 4.42-4.54 [dd, J = 44.0, 12.0 Hz, 1H, C$\underline{H}$(2')] 6.89, 6.96 [2×s, 1H, C$\underline{H}$Ph$_2$ rotamers] 7.28-7.39 [m, 10H, phenyl C$\underline{H}$]

5. Synthesis of *N-tert*-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenyl methyl ester (5)



(4) **(5)**

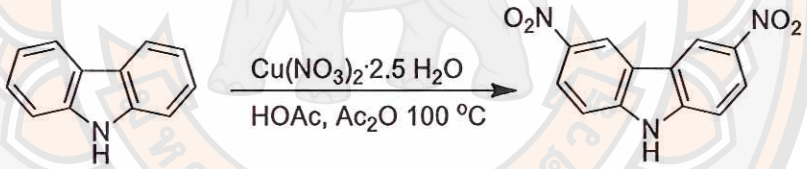
N-tert-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenyl methyl ester (5) can be prepared using previously published procedures as following. [17] *N*-Boc-4-hydroxy-D-proline diphenylmethyl ester (0.500 g, 12 mmol, and triphenyl phosphine

(0.495 g, 15 mmol) were mixed and dissolved in dry THF. The solution was stirred at 0°C under nitrogen atmosphere. Then methyltoluenesulfonate (2.27 mL, 15 mmol) was slowly added to the reaction mixture at room temperature, followed by a slow addition of diisopropyl azodicarboxylate (3 mL, 15 mmol) and solution mixture was allowed to stir from 0°C to rt. After 8 h, the solvent was evaporated. The crude product was recrystallized by EtOH. The desired product was obtained as white powder in 4.642 g (60% yields).

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.582-1.222 [m, 9H, 3x$C\underline{H}_3$ Boc] 3.680-3.7726 [m, 2H, C<u>H</u>(4') proline] 2.414 [s, $C\underline{H}_3$ OTs] 4.525, 4.401 [dd, 1H $C\underline{H}_2$(1') proline] 5.053-5.066 [m, 1H, C<u>H</u>(3') proline] 7.556-7.616 [m, 14H, C<u>H</u> Benzene] 6.831 [s, 1H, C<u>H</u>ODpm]

Synthesis of 3,6-dinitrocarbazole

1. Synthesis of 3,6-dinitrocarbazole using copper(II)nitrate

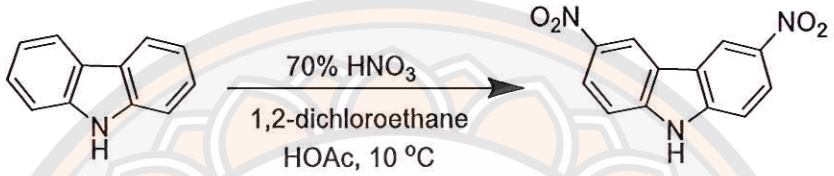


(5a) **(5b)**

3,6-Dinitrocarbazole was prepared using the previously published procedures as following. [40] A homogeneous mixture of $Cu(NO_3)_2{\cdot}2.5H_2O$ (0.341g, 1.4 mmol), acetic acid (1 mL), and acetic anhydride (1.5 mL) was prepared at room temperature. To this solution was added carbazole (0.201g, 1.2 mmol) in small portions over 10 min by maintaining reaction temperature at 15-20 °C. Temperature was allowed to rise to room temperature (27 °C) over a period of 30 min. Then reaction was stirred for 30 min at 90 °C. The mixture was diluted with an additional 0.4 mL of acetic acid, and poured into 10 mL of distilled water with constant stirring. The precipitate was collected by filtration, and washed with 10 mL distilled water five times each. The wet residue was dissolved in a cold solution of 0.8 g of KOH, 10 mL of ethanol, and 10 mL of water to obtain a red solution which was stirred for 30 min,

and filtered. The filtrate was then acidified with concentrated hydrochloric acid, and allowed to settle for 30 min. A yellow precipitate was collected by filtration, washed several times with cold water. In the last step, the yellow solid was recrystallized in nitrobenzene but the crystal was not obtained.

2. Synthesis of 3,6-dinitrocarbazole using nitric acid



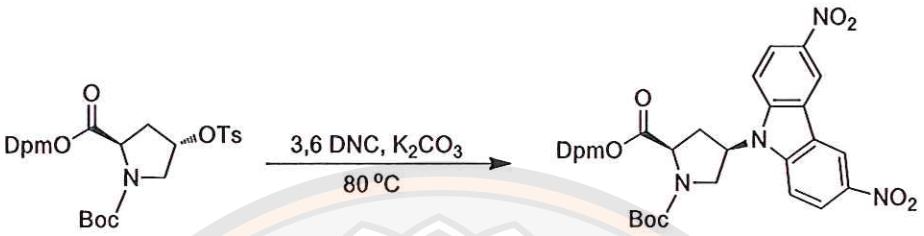
(5a) **(5b)**

3,6-Dinitrocarbazole **(5b)** can be prepared using previously published procedures. [38] A stirred slurry of carbazole (0.201 g, 1.2 mmol) in 1,2-dichloroethane was cooled to 10°C and 70% HNO_3 (0.4 mL) diluted with an equal volume of AcOH was added dropwise. The system was then stirred for 3 hours. After warming to room temperature, the occurred precipitate was collected and then stirred with 4 M KOH for 1 hour. The pH of the solution was adjusted to pH 2.0 with 2 M HCl. The crude product was purified by silica gel column chromatography using Hexane: Ethyl acetate 2:1 as mobile phase. The desired product obtained in 0.090 g (54% yield).

^{1}H-NMR (400 MHz, DMSO): δ_H 7.74-7.76 [d, 2H, C<u>H</u>(1,8) DNC] 8.36-8.39 [dd, 2H, C<u>H</u>(2,7) DNC] 9.48-9.48 [d, H, C<u>H</u>(4,5) DNC]

The Synthesis of Pyrrolidinyl monomer carrying 3,6-dinitrocarbazole

1. Synthesis of *N-tert*-Butoxycarbonyl-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethyl ester using potassium carbonate

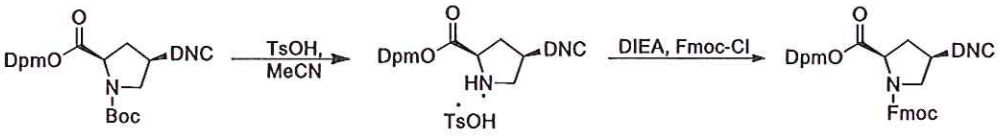


(5) **(6)**

A solution of 3,6-dinitrocarbazole in DMF which cooled to 0°C was added with potassium carbonate (0.691 g, 5 mmol) under nitrogen atmosphere. Next, the solution of *N-tert*-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenylmethyl ester (0.550 g, 1 mmol) in dry DMF was slowly added. The mixture was heated at 80°C for 12 hr. Then the reaction mixture was evaporated off the solvent and adjusted pH to approximately 7 by adding HCl. Finally, the crude product was purified by silica gel column chromatography using hexane:ethyl acetate (2:1) as mobile phase. The desired product was obtained in 0.076 g (12% yield).

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 9.00 [d, J = 2 Hz, 2H] 8.22 [d, J = 8.4 Hz, 2H] 7.51 [d, J = 9.2 Hz, 2H], 7.32 [m, 10H] 7.051 [m, H] 5.49 [br m, 1H] 4.79, 4.70 [br m, 1H] 4.17 [br m, 2H] 2.92, 2.71[br m, 2H] 1.31, 1.25 [2×s, 9H]; ^{13}C NMR (100 Hz, $CDCl_3$) δ 28.1, 28.4 [$\underline{C}H_3$ Boc rotamers] 46.9, 47.2 [$\underline{C}H_2$ (3') rotamers] 52.6, 53.2 [$\underline{C}H_2$ (5') rotamers] 57.8,58.1 (4') rotamers 78.4,78.7 [$\underline{C}CH_3$ Boc rotamers] 81.7 [CH Dpm rotamers] 110.6 [$\underline{C}$H(3) carbazole] 117.7 [$\underline{C}$H(4) carbazole] 123.3, 123.1 [$\underline{C}$H(7) carbazoe] 127.3 [$\underline{C}$H(6) carbazole] 127.1-131.0 [$\underline{C}$H Ar Dpm rotamers] [139.6 $\underline{C}$ Dpm rotamers] 142.2 $\underline{C}$CH(5) carbazole]145.5 [$\underline{C}$CH(2) carbazole] 153.5, 154.1 [$\underline{C}$O Boc rotemers] 171.5 [$\underline{C}$O rotamers]

2. Synthesis of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethyl ester



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

DNC = 3,6-dinitrocarbazole

N-Fluoren-9-ylmethoxycarbonyl-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethyl ester (**8**) was prepared using previously published procedures. [41] *N-tert*-Butoxycarbonyl-*cis*-(3,6-dinitrocarbazole-9-yl)-D-proline diphenylmethyl ester (**6**) (0.2018 g, 0.3166 mmol) was dissolved in MeCN and then *p*-toluenesulfonic acid monohydrate (150 mg, 0.7915 mmol) was slowly added to the solution. After 5 hr, diisopropylethylamine (DIEA) (0.2 mL, 1.1 mmol) was added to the reaction mixture at room temperature followed by FmocCl (0.0819 g, 0.31 mmol) for 1 hr. The solvent was removed by evaporation. Finally, the crude product was purified by silica gel column chromatography using gradient hexane/EtOAc. The desired product was obtained as pale yellow solid (0.0731 g) 31% yield.

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 9.04 (s, 1H, CH DNC), 8.25-8.78 (m, 1H,CH DNC) 7.82 (s, 1HCH DNC) 7.22-7.72 [m, 18H,CH Fmoc, Dpm] 5.41 [s, 1H, CH proline] 4.72, 4.61 [2×dd 1H CH_2 proline] 2.680-2.7926 [m, 2H, CH proline] ^{13}C NMR (100 Hz, $CDCl_3$) 29.6 [CH_2 Fmoc] 31.1, 32.1 [CH_2(3') rotamers 46.9, 47.1 CH_2(5') rotamers] 52.4, 53.2 [CH_2(4') rotamers] 57.7, 58.1[CH_2(2') rotamers] 68.1[CH Fmoc] 77.3, 78.5 [CH Dpm rotamers] 110.4 [CH(3) carbazole] 117.6 [CH(4) carbazole] 120.0 [CH (7) cabazoole] 123.2 [CH(3) Ar Fmoc] 124.8 [CH(4) Ar Fmoc] 127.1 [CH(4) Ar Fmoc] 127.8 [CH(6) carbazole]128.4-128.7 [CH Ar Dpm rotamers] 128.7 [CCH(5) carbazole] 139.0 [CCH(2) carbazole 141.3 [CCH Ar Dpm] 142.2 [CH(7) Ar Fmoc] 143.2 [CH(2) Ar Fmoc]

3. Synthesis of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline carboxylic acid

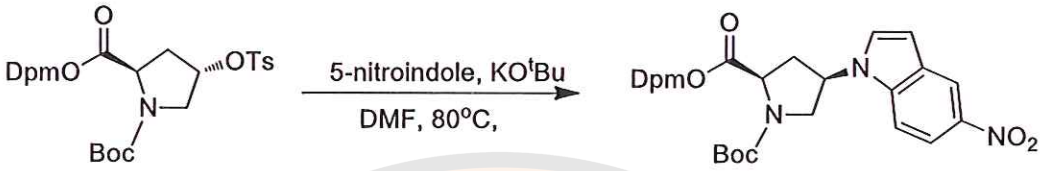
(8) **(9)**

N-Fluoren-9-ylmethoxycarbonyl-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethyl ester (**8**) (0.061 g, 8.0 mmol) was treated with 1:4:4 anisole/TFA/CH_2Cl_2 for 15 min at room temperature. After the solvent were removed using a stream of nitrogen gas, the residue was washed with diethyl ether to obtain a yellow solid as the final product. The desired product was obtained as pale yellow solid (0.033 g) 70% yield.

^{1}H-NMR (400 MHz, DMSO): δ_H 2.54-2.70, 2.82-3.3 [m, 2H, C$\underline{H}_2$(3') rotamers] 4.07-4.25 [m, 2H, C$\underline{H}_2$ (5') rotamers] 4.27-4.35 [m, 3H, C$\underline{H}_2$ Fmoc and C$\underline{H}$ Fmoc rotamers] 4.39-4.48, 4.58-4.67 [m, 1H, C$\underline{H}$(2') rotamers] 5.95 [m,1H, C$\underline{H}$ (4')] 7.24-7.52 [m, 4H, C$\underline{H}$ Fmoc] 7.62-7.78 [m, 2H, C$\underline{H}$ Fmoc] 7.80-7.99 [m, 2H, C$\underline{H}$ Fmoc and [m, 2H, C$\underline{H}$ DNC] 8.408.51 [d, 2H, C$\underline{H}$ DNC] 9.51 [s, 2H, C$\underline{H}$ DNC] 13.1 [br, s, 1H, N$\underline{H}$ DNC]. ^{13}C NMR (100 Hz, DMSO) 31.4, 32.5 [$\underline{C}H_2$(3') rotamer] 46.5, 47.1[$\underline{C}$H Fmoc rotamers] 51.6-51.8 [$\underline{C}H_2$(5') rotamers] 52.5 [$\underline{C}$H(4') rotamers] 57.5, 58.1 [$\underline{C}$H(2') rotamers] 67.1, 67.5 [CH_2 Fmoc rotamers] 111.5 [$\underline{C}$H DNC(3)] 118.6 [$\underline{C}$H, DNC](4)] 120.0 $\underline{C}$H DNC(8)] 122.5 [$\underline{C}$H Fmoc] 122.8 [$\underline{C}$H Fmoc] 125.1 [$\underline{C}$H Fmoc] 127.1 [$\underline{C}$H Fmoc] 127.7 [$\underline{C}$H DNC (5,10)] 140.6 [$\underline{C}$H DNC(2,13)] 141.5 [$\underline{C}$ Fmoc] 143.6 [$\underline{C}$ Fmoc] 154.1 [$\underline{C}$O Fmoc] 173.3, 173.8 [$\underline{C}$O Proline rotamers] IR (KBr disc) ν_{max} 3068, 1763, 1675, 1604, 1517, 1288 cm^{-1}; HRMS (ESI+) calcd for $C_{32}H_{24}N_4O_8$ ($M+Na^+$) 615.1486, found 615.1480

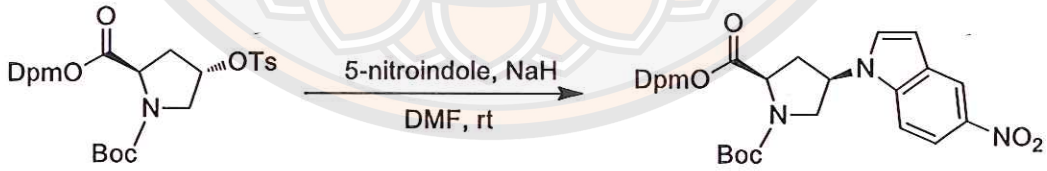
Unsuccessfull synthetic method

1. Synthesis of *N-tert*-Butoxycarbonyl-*cis*-5-nitroindole-D-proline diphenylmethylester using potassium-*t*-butoxide



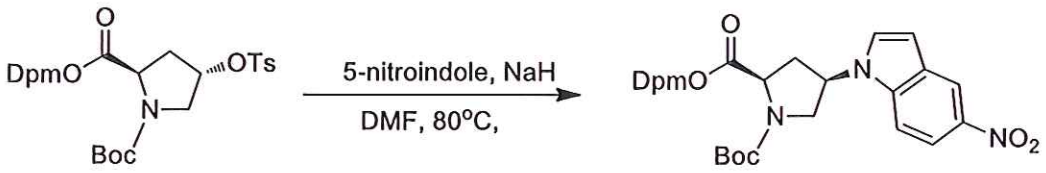
N-Boc-4-hydroxy-D-proline diphenylmethyl ester (0.502 g, 0.9 mmol) and commercially available 5-nitroindole (0.106 g, 0.9 mmol) were dissolved in dried DMF into a 250 mL round bottom flask under nitrogen atmosphere. The solution of KO^{t}Bu (0.132 g, 1.8 mmol) was freshly prepared by disperse KO^{t}Bu into dried DMF and cooled down to 0 °C. To this solution was slowly added the solution of 5-nitroindole and stirred at room temperature for 12 hours under nitrogen atmosphere. The reaction was followed by TLC using ethyl acetate:hexane (1:4) as eluent but the new product could not be observed by this condition.

2. Synthesis of *N-tert*-Butoxycarbonyl-*cis*-5-nitroindole-D-proline diphenylmethyl ester using sodium hydride at 0 °C



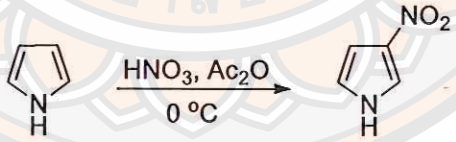
5-Nitroindole (0.064 g, 0.4 mmol) and sodium hydride (0.0173 g, 0.4 mmol) which already washed with hexane, were dissolved in DMF and stirred this mixture in ice bath for 15 mins. A solution of *N-tert*-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenyl methyl ester (0.200 g, 0.36 mmol) in DMF was slowly dropped into the mixture of 5-nitroindole under nitrogen atmosphere (as monitored by TLC). It was found that the desired product was not obtained from this reaction.

3. Synthesis of *N-tert*-Butoxycarbonyl-*cis*-5-nitroindole-D-proline diphenylmethyl ester using sodium hydride at 80 °C



A solution of 5-nitroindole (0.1927 g, 1.1 mmol) in DMF was adjusted the temperature to 0°C and then added NaH (0.0363 g, 0.9 mmol). The mixture was stirred at 0 °C for 30 min and then continued stir at room temperature for 15 min under nitrogen atmosphere. A freshly prepared solution of *N-tert*-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenyl methyl ester (0.5020 g, 0.9 mmol) in DMF was added dropwise into the 5-nitroindole solution at room temperature. The mixture was stirred for 30 min at room temperature and then heated the reaction mixture up to 80 °C overnight. After the reaction completed, the desired product could not be separated by silica gel column chromatography.

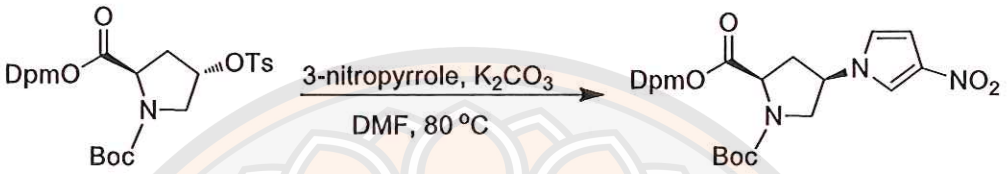
4. Synthesis of 3-nitropyrrole



Pyrrole (0.25 mL, 3.6 mmol) was dissolved in acetic anhydride at 0 °C. A solution of nitric acid (2.84 mL, 44.4 mmol) in acetic anhydride was slowly added. After the reaction completed, an aqueous solution of $NaHCO_3$ was added to stop the reaction and the product was extracted from the reaction using dichloromethane. The organic phase was evaporated off the solvent and the crude product was purified by silica gel column chromatography using hexane:ethyl acetate (10:1) as eluent. The desired product was obtained as a black powder (0.075 g, 18% yield).

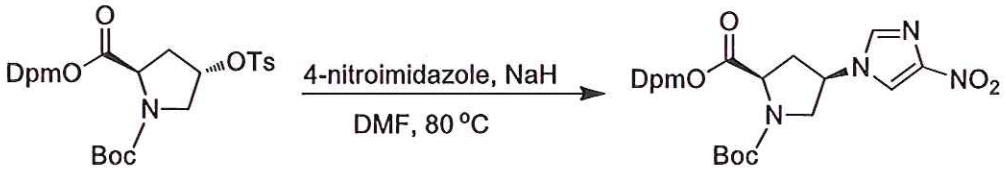
^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 6.29 [q, J = 6.3 Hz, 1H, CH(5)] 7.00 [q, J = 7.0 Hz, 1H, CH(4)] 7.12 [quin, J = 7.13 Hz,1H, CH(2)]

5. Synthesis of *N-tert*-Butoxycarbonyl-*cis*-3-nitropyrrole-D-proline diphenylmethyl ester using potassium carbonate



A solution of 3-nitropyrrole (0.061 g, 2.72 mmol) was added K_2CO_3 (0.376 g, 0.54 mmol) under nitrogen atmosphere and stirred at room temperature for 15 min. A solution of *N-tert*-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenylmethyl ester (0.305 g, 0.50 mmol) in DMF was slowly added into the reaction mixture and stirred for 30 min. The reaction temperature was increased to 80 °C and kept at this temperature overnight. The reaction was stopped by adding 5% hydrochloric acid and then extracted the product using ethyl acetate. The crude product was purified by silica gel column chromatography using a gradient solvent system (hexane and ethyl acetate). It was found that the desired product could not be separated from *N*-Boc-4-hydroxy-D-proline diphenylmethyl ester.

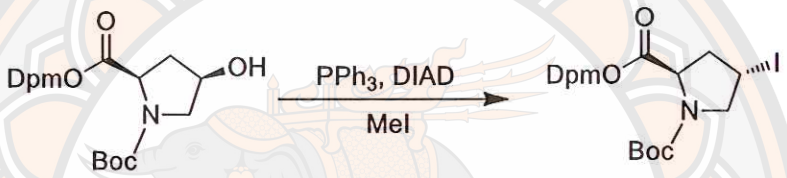
6. Synthesis of *N-tert*-Butoxycarbonyl-*cis*-4-nitroimidazole-D-proline diphenylmethylester using sodium hydride



At the temperature 0 °C, a solution of 4-nitroimidazole (0.102 g, 0.907 mmol) in DMF was added NaH (0.046 g, 1.1 mmol) and stirred for 15 min. Then, the mixture was continued stir at room temperature for 30 min under nitrogen

atmosphere. A freshly prepared solution of *N-tert*-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenyl methyl ester (0.503 g, 0.907 mmol) in DMF was slowly dropped into the 4-nitroimidazole solution at room temperature and the mixture was stirred for 30 min. The reaction temperature was adjusted to 80 °C overnight. After the reaction completed, the desired product could not be separated by silica gel column chromatography.

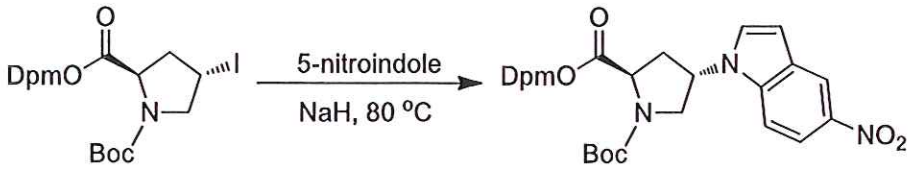
7. Synthesis of *N-tert*-Butoxycarbonyl-*cis*-4-nitroimidazole-D-proline diphenylmethyl ester using methyliodide



N-tert-Butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester (1.004 g, 2.5 mmol), triphenylphosphine (PPh_3) (0.787 g, 3 mmol) were dissolved in 10 mL of THF into a 250 mL round bottom flask under nitrogen atmosphere. The solution was stirred with magnetic stirrer and cooled down to 0 °C. Then MeI (0.426 g, 3 mmol) and diisopropyl azodicarboxylate (DIAD) (0.609 g, 3 mmol) were slowly added to the solution and the reaction mixture was stirred at room temperature for 8 hours. The reaction was followed by TLC using ethyl acetate: hexane (1:4) as eluent. After the reaction complete, the reaction was removed off the solvent and purified by a silica gel column chromatography using gradient hexane and ethyl acetate as eluent. The desired product was obtained as brown powder in 150 mg (10% yields).

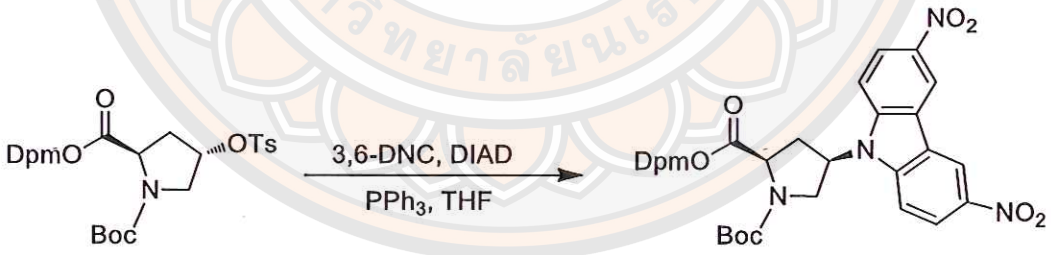
^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 1.513,1.237 [m, 9H, 3x$C\underline{H}_3$ Boc] 2.43-2.21 [m, 1H, $C\underline{H}_2$(3')] 3.61-4.32 [m, 4H, proline] 6.92, 6.87 [2×s, 1H, $C\underline{H}Ph_2$ rotamers] 7.26-7.37 [m, 10H, phenyl C$\underline{H}$]

8. Synthesis of *N-tert*-Butoxycarbonyl-*tran*-5-nitroindole-D-proline diphenylmethylester



A solution of 5-nitroindole (48 mg, 0.297 mmol) in dry DMF was mixed with NaH (0.015 g, 0.354 mmol) under nitrogen atmosphere. Then, the solution of *N-tert*-butoxycarbonyl-*cis*-4-iodo-D-proline diphenylmethyl ester (150 mg, 0.297 mmol) in dry DMF was slowly added. The mixture was heated at 80 °C for 12 hr. After the reaction complete, the crude product was purified by silica gel column chromatography. It was found that the desired product could not be afforded by this procedure.

9. Synthesis of *N-tert*-Butoxycarbonyl--3,6-dinitrocarbazole-D-proline diphenylmethylester using Mitsunobu condition



(5) **(6)**

N-tert-Butoxycarbonyl-*trans*-4-tosyl-D-proline diphenylmethyl ester (0.189 g, 0.5 mmol), triphenylphosphine (PPh_3) (0.273 g, 0.75 mmol) and 3,6-dinitrocarbazole (0.08 g, 0.75 mmol) were dissolved in 10 mL of THF into a 250 mL round bottom flask under nitrogen atmosphere. The solution was stirred with magnetic stirrer and cooled down to 0°C. Then diisopropyl azodicarboxylate (DIAD) (0.45 mL, 0.75 mmol) were slowly added to the solution and the reaction mixture was

stirred at room temperature for 8 hours. The reaction was followed by TLC using ethyl acetate: hexane (1 : 4) as eluent. After the reaction complete, the reaction was removed off the solvent and purified by a silica gel column chromatography using gradient hexane and ethyl acetate as eluent. However, it was found that the desired product could not be afforded by this procedure.

Synthesis of acpcPNA carrying 3,6-dinitrocarbazole as a base replacement

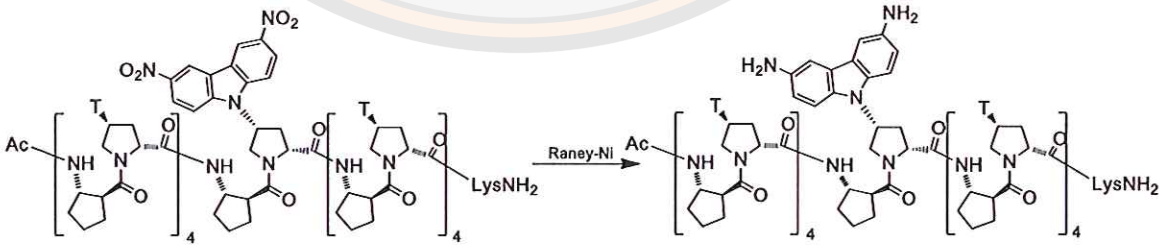
Fmoc-solid phase peptide synthesis method according to our previously published protocol was used for a preparation of the acpcPNA. [17] The preparation started from a deprotection of the Fmoc protecting group on the solid support (1.5 μmol) using 100 μL solution of 2% DBU and 20% piperidine in DMF (100 μL). Then, the deprotected product was coupled with the PNA monomer or ACPC spacer which was previously activated by treatment with the solution of DIEA (8 equiv) and HOAt (4.0 equiv.) (for Pfp-activated monomers) or HATU (4 equiv.) (for free acid monomers) in DMF (30 μL) for 40 min. Next, the unreacted amino group was capped with the solution of DIEA (8 equiv.) and acetic anhydride (34.5 equiv.) in DMF (30 μL) for 5 min. This synthesized cycle was repeated until the desired PNA sequence was obtained.

Three acpcPNA sequences, Ac-GTAGCXGCACT-LysNH_2, Ac-GTAGAX TCACT-LysNH_2 and Ac-TTTTXTTTT-LysNH_2 where X = 3,6-dinitrocarbazole was synthesized manually via Fmoc-solid phase peptide synthesis using the same method as described above. This acpcPNA can be improved its solubility in water by adding a lysine residue at the C-terminus. After completing the PNA oligomer synthesis, the free amino group was capped by acetylation. Finally, cleavage the obtained PNA from the resin and purification by reverse phase HPLC afforded the DNC-PNA in 4.4% isolated yield. The identity of the Ac-GTAGCXGCACT-LysNH_2, Ac-GTAGAXTCACT-LysNH_2 and Ac-TTTTXTTTT-LysNH_2 was confirmed by MALDI-TOF mass spectrometry which revealed the expected mass signals (calcd. m/z 4023.1; found 4023.6 $[M+H]^+$, 4007.3 $[M-O+H]^+$, 3991.8 $[M-2O+H]^+$), (calcd m/z 4022.2; found 4022.4 [M+H]+, 4007.0 [M-O+H]+, 3990.5 and (calcd. m/z 3310.44; found 3309.26 $[M+H]^+$, 3293.14 $[M-O+H]^+$, respectively. Deoxygenation of the nitro group probably occurred during the ionization process.

Synthesis of acpcPNA carrying 3,6 diaminocarbazole (DAC-acpcPNA) by reduction of DNC-acpcPNA

AcpcPNA with a sequence Ac-TXT-LysNH$_2$ where T = thymine and X = 3,6-dinitrocarbazole was first attempted on the solid support for investigating the best reducing agent. Two reducing agents were tried in this reaction, $SnCl_2 \cdot 2H_2O$ and $Na_2S_2O_4/K_2CO_3$/TBAHS. The reduction started from dipping Ac-TXT-LysNH$_2$ on resin into a solution of reducing agent in DMF (30 microliter). The temperature for reducing with $SnCl_2 \cdot 2H_2O$ was started at room temperature for 2 hours but the reduction did not happen. The temperature was raised to 80°C overnight. The product from the reduction which was monitored using MALDI-TOF mass spectrometry found that only one nitro group was reduced. In case of $Na_2S_2O_4/K_2CO_3$/TBAHS, the reducing agent was dissolved in water and then dichloromethane was added to obtain two liquid layers. After that, Ac-TXT-LysNH$_2$ on resin was dipped into the mixture solution for 6 hours. Result from MALDI-TOF mass spectrometry revealed that no reduction product was obtained.

AcpcPNA with a sequence Ac-T$_4$XT$_4$-LysNH$_2$ where T = thymine and X = 3,6-dinitrocarbazole was dissolved in milliQ water 100 µL. After that, the reduction of the nitro group was performed by treatment with Raney-Ni in an aqueous solution at room temperature. The progress of the reaction was monitored by MALDI-TOF mass spectrometry. After the reduction was completed, the reduction product was purified by reverse phase HPLC to obtain DAC-acpcPNA in 0.2% yield.



Purification by reverse phase HPLC and characterization of acpcPNA linked with 3,6-dinitrocarbazole

The crude PNA product was dissolved in 120 µL MilliQ water. The solution was filtered through a nylon membrane filter (0.45 µm) before being purified by reverse phase HPLC performing on Water Delta 600™ system on an *ACE 5 C18-AR* (*150 x 4.6 mm*) HPLC column, eluting with a gradient system of 0.1% TFA in methanol/water. The HPLC gradient system consisted of solvent A (0.1% TFA in MilliQ water) and solvent C (0.1% TFA in methanol). The elution started with A:C (90:10) at flow rate 0.5 mL/min for 5 min followed by a linear gradient to A:C (10:90) over a period of 70 min, then holding for 10 min before reverting back to A:C (90:10). The collected fractions (monitored with UV detection at 260 nm) were analyzed by MALDI-TOF MS. Fractions containing only the desired products were combined and removed all solvents by freeze-drying to afford the purified PNA.

PNA-DNA binding properties by UV-melting experiment

Hybridization property of the acpcPNA with DNA was investigated by the T_m measurements. The T_m values were obtained on 260 nm on a CARY 100 Bio UV-Visible spectrophotometer (Varian Ltd.) equipped with a thermal melt system by scanning and recording A260 in heating from 20°C to 90°C (heating block temperature) with a temperature ramp of 1 °C/min and collect absorbance of solution at 260 nm. The sample for the T_m measurement was prepared by mixing oligonucleotide and PNA solution to give the final concentration of 1 µM in 10 mM sodium phosphate buffer (pH 7.0) in 1000 µL Milli-Q water in a 10 mm quartz cell. The samples were equilibrated at the starting temperature for 10 min and cooled down for 10 min after finished collect data. Melting temperature (T_m) was calculated from the maximum of the first derivative of normalized absorbance at 260 nm as a function of corrected temperature after smoothing using KaliedaGraph 3.6 (Synergy Software). T_m values obtained from independent experiments were accurate to within ± 0.5°C. Correct temperature and normalized absorbance are defined as follows.

$$\text{Correct Temp.} = (16.910 \times T_{block}) - 0.0018$$

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

The equation for temperature correction was previously obtained by measuring the actual temperature in the cuvette using a temperature probe and plotting against the set temperature (T_{block}) from 20-90 °C. A linear relationship $Y = 16.910X - 0.0018$ and $R^2 > 0.99$ was obtained [Ngamwiriyawong et al., 2004].

Fluorescence experiments of DAC-acpcPNA

The fluorescence emission of DAC-PNA sample was firstly measured at 1 μM of PNA in 10 mM sodium phosphate buffer pH 7.0 (1000 μL). The fluorescence spectra were recorded at 25°C with excitation wavelength = 315 nm and PMP voltage was set to high. After that DNA was added into the PNA sample (1.2 μM final concentration) and the mixture was recorded the fluorescence spectrum again.

CD spectroscopy

CD spectra of DNC-PNA and its DNA hybrid were recorded at 20°C in a 10 mm path length cuvette from 400 to 200 nm at the rate of 100 nm/min and averaged 4 times. The sample was prepared as same as method in title 8, chapter 3, except for the concentrations of PNA and DNA (2.5 μM each). All spectra were processed using Microsoft Excel and OriginPro7G (OriginLab Corporation) and were baseline subtracted.

CHAPTER IV

RESULTS AND DISCUSSION

In this research, new pyrrolidine monomers attached 3,6-dinitrocarbazole as a universal bases at C-4 position of pyrrolidine ring for pyrrolidinyl PNA were synthesized and investigated the binding properties between those universal base and four natural nucleobases (A, T, C and G). Then, pyrrolidinyl PNA containing 3,6-dinitrocarbazole will be reduced 3,6-dinitrocarbazole moiety to 3,6-diaminocarbazole and examine the fluorescence properties as a hybridization responsive DNA probe. The structure of the novel acpcPNA containing 3,6-dinitrocarbazole and 3,6-diaminocarbazole in this study is shown in Figure 17.

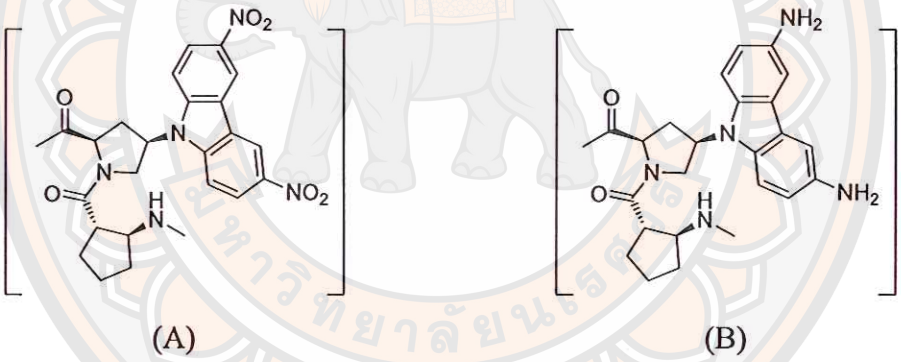


Figure 17 (A) structure of acpcPNA containing 3,6-dinitrocarbazole (B) structure of acpcPNA containing 3,6-diaminocarbazole

The research plan for this research was divided into 2 parts. The first part is synthetic of universal base pyrrolidine PNA monomers for use in PNA peptide synthesis. For 4 natural base monomers and spacer; T-monomer, A-monomer, C-monomer, G-monomer and acpc-monomer were kindly received from Professor Dr. Tirayut Vilaivan's Laboratory. Oligomerization by solid phase peptide synthesis of these pyrrolidine monomers gave the desired 3 PNA sequences; TTTTXTTTT, GTAGC X GCACT and GTAGA X TCACT (X = 3,6-dinitrocarbazole). The second

part is the evaluation the binding properties of PNA·DNA hybrid by spectroscopic methods such as UV-melting temperature, CD spectroscopy and fluorescence spectrophotometry.

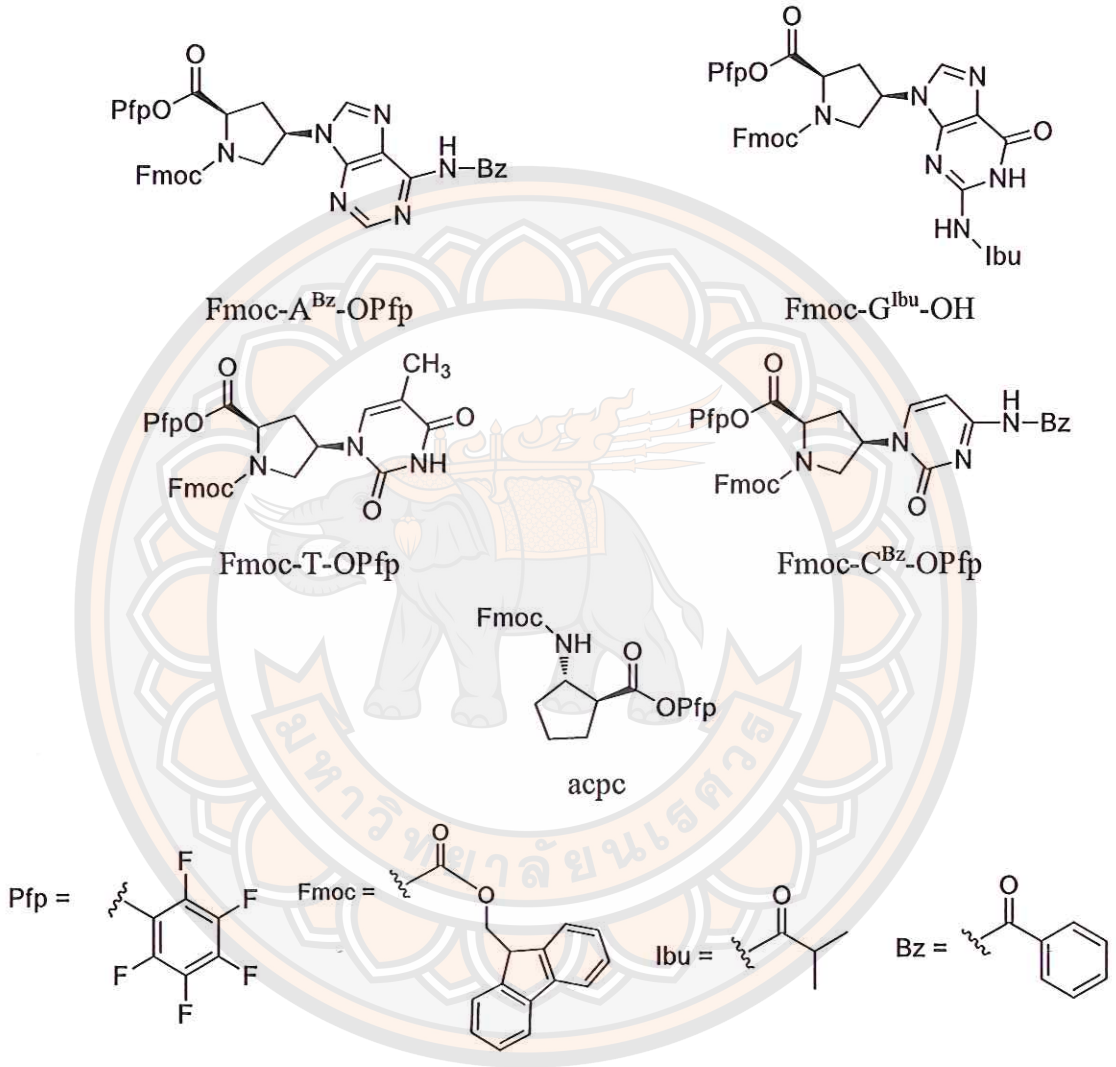


Figure 18 Structure of pyrrolidinyl PNA monomers used for the solid phase peptide synthesis

Synthesis of pyrrolidine monomers attached universal bases

The synthesis of pyrrolidine monomers attached 3,6-dinitrocarbazole started from epimerization at α-position of *trans*-4-hydroxy-L-proline (**1**) to obtain *cis*-4-hydroxy-D-proline (**2**). The synthetic procedure of compound **2-5** were performed according to previously published procedures. [17]

For universal base; 3,6-dinitrocarbazole, it can be prepared *via* nitration reaction of commercially available carbazole. The mixture of $Cu(NO_3)_2 \cdot 2.5H_2O$ in acetic acid and acetic anhydride was used as nitrating reagent. [40] After the several attempts, there was no product was obtained under in this condition. So, other method using 70% HNO_3 in 1,2-dichloroethane at 45°C was carried out. [38] It was found that two nitro groups were successfully substituted at 3- and 6-position on carbazole ring to obtain the desired 3,6-dinitrocarbazole in 54% yield.

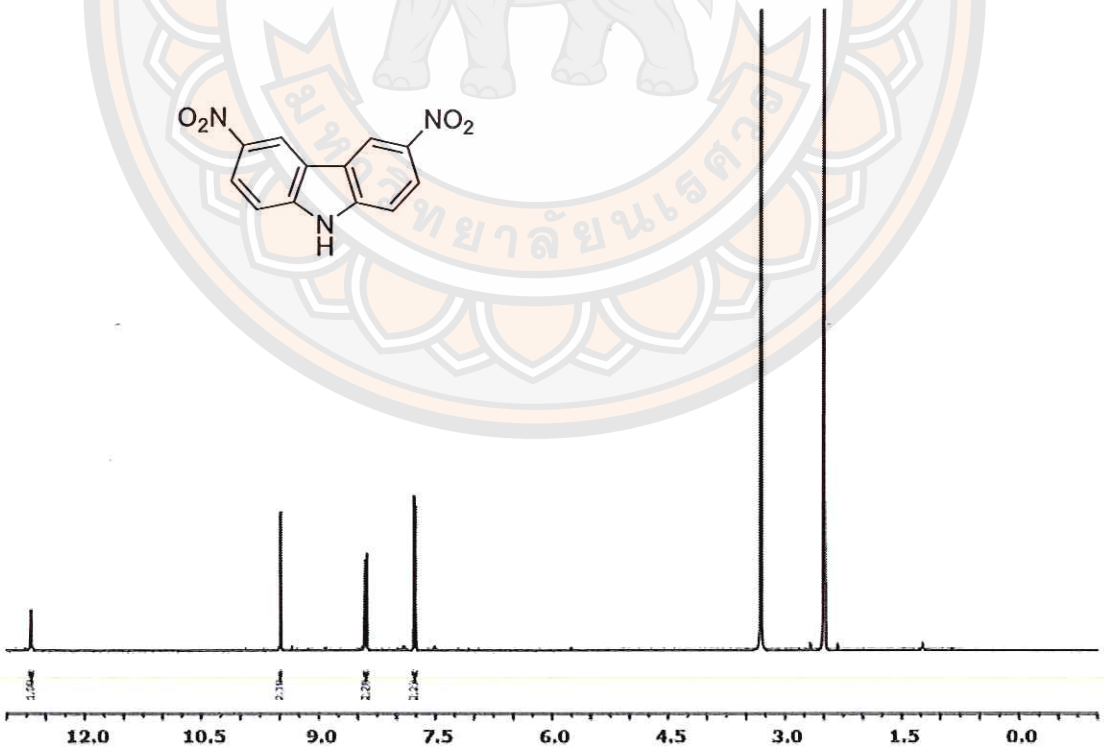


Figure 19 ^{1}H NMR (DMSO, 400 MHz) of 3,6-dinitrocarbazole

From the ^{1}H-NMR spectrum as shown in Figure 19, this spectrum confirmed a formation of 3,6-dinitrocarbazole by showing a doublet signal of C<u>H</u>(1,8) Aromatic at 7.68 ppm, a doublet of doublet of C<u>H</u>(2,7) Aromatic at 8.35 ppm and a broad singlet of C<u>H</u>(4,5) Aromatic at 9.54 ppm.

After that, 3,6-dinitrocarbazole were attached to the pyrrolidine monomer (**5**) at position C-4 *via* S_N2 substitution reaction. In the first attempt, Mitsunobu reaction between *N*-tert-butoxycarbonyl-*trans*-4-hydroxy-D-proline diphenylmethyl ester and 3,6-dinitrocarbazole was tried and any new spot on TLC plate was not observed. Thus, the tosylate intermediate (**5**) was reacted with 3,6-dinitrocarbazole in DMF using K_2CO_3 as base catalyst to give compound (**6**) in 12% yield, whose structure confirmed by ^{1}H NMR. The NMR spectrum showed both peaks of nitrocarbazole moiety at 9.00, 8.22 and 7.51 ppm and also pyrrolidine ring peaks including Boc and Dpm protective groups.

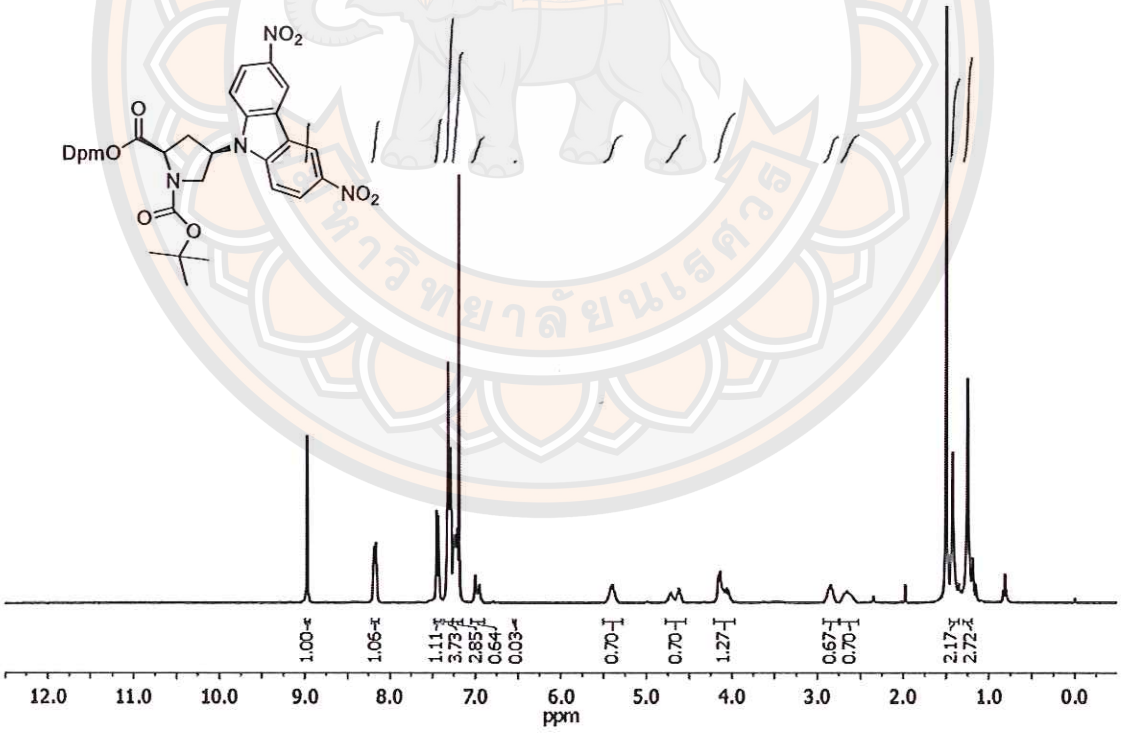


Figure 20 **^{1}H NMR ($CDCl_3$, 400 MHz) of *N-tert*-Butoxycarbonyl-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethylester using potassium carbonate (6)**

The low percentage yield in this step was obtained because of a high acidity of 3,6-dinitrocarbazole resulting in a poor nucleophilicity which decreased its ability to replace a leaving group. The equilibrium acidity of 9*H*-carbazole and their nitro derivatives have been measured in dimethyl sulfoxide solution as part of a study to determine the relative acidities of carbon and nitrogen acids. The measured pK_a of 9*H*-carbazole is 19.9. The acidities of 3-nitro-9*H*-carbazole and 3,6-dinitro-9*H*-carbazole are reported to have pK_a = 14.16 and 13.07, respectively. [42]

After that the pyrrolidine intermediate **(8)** was obtained in 31% yield from protecting group exchange from *N*-Boc to *N*-Fmoc in one pot using TsOH in acetonitrile followed by FmocCl/DIEA. Finally, the diphenylmethyl ester (ODpm) protecting group was deprotected using 1:4:4 anisole/TFA/CH_2Cl_2. The protecting group *N*-Fmoc was selected in accordance with standard oligopeptide synthesis in literature. [41] The identity of compound **(9)** was confirmed by an observation of ^{1}H-NMR peaks of Fmoc and a disappearance of ODpm in range of 7.22-7.72 ppm.

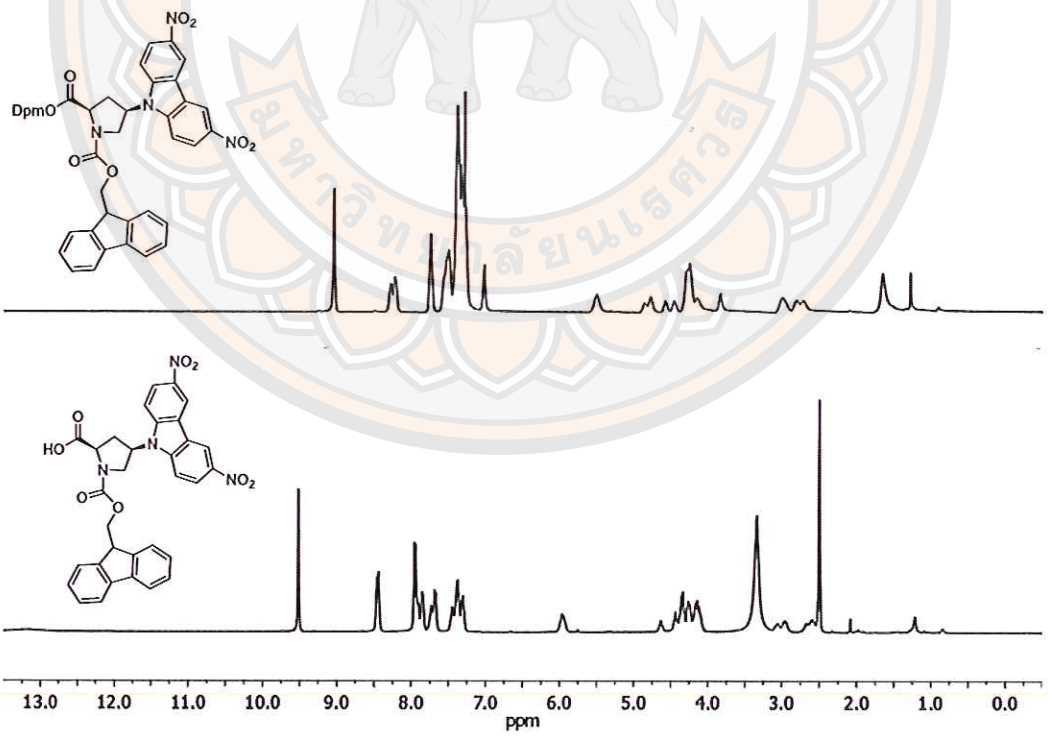


Figure 21 ^{1}H NMR spectrum of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethylester (8)

Preparation of 3,6-dinitrocarbazole (acpc PNA)

The desire PNA sequence prepared using alternatively coupling of Fmoc-protected PNA monomer (A, T, C and G) and acpc-spacer onto solid support according to a known protocol. [11] There are three step in the synthesis of PNA. First step is deprotecting step. Second step is coupling step. Third step is capping step.

After all synthetic steps was finished, the successfully three synthesized PNA sequence in the length of monomer (TTTTXTTT): (**P1**), (GTAGCXGCACT): (**P2**) and (GTAGAXTCACT): (**P3**), X=3,6-dinitrocarbazole were obtained in 24% , 4.4% and 4.2% yield respectively. PNA were purified by C-18 reverse-phase HPLC with UV detection 260 nm. The identities of the obtained PNA sequence before and after purification were confirmed by MALDI-TOF mass spectrometry. It was found that deoxygenation of the nitro group probably occurred during the ionization process. [43] HPLC chromatogram and mass spectrum of PNA are illustrated in table 6 figure 22.

Table 6 Sequence and characterization data of 3,6-dinitrocarbazole-acpcPNA (P1), (P2) and (P3)

Sequence[a] (N→C)	m/z (calcd)[b]	m/z (found)[c]
P1: TTTTXTTTT	3310.4	3309.2, 3293.1
P2: GTAGCXGCACT	4023.1	4023.6 , 4007.3, 3991.8
P3: GTAGAXTCACT	4022.2	4022.4, 4007.0, 3990.5

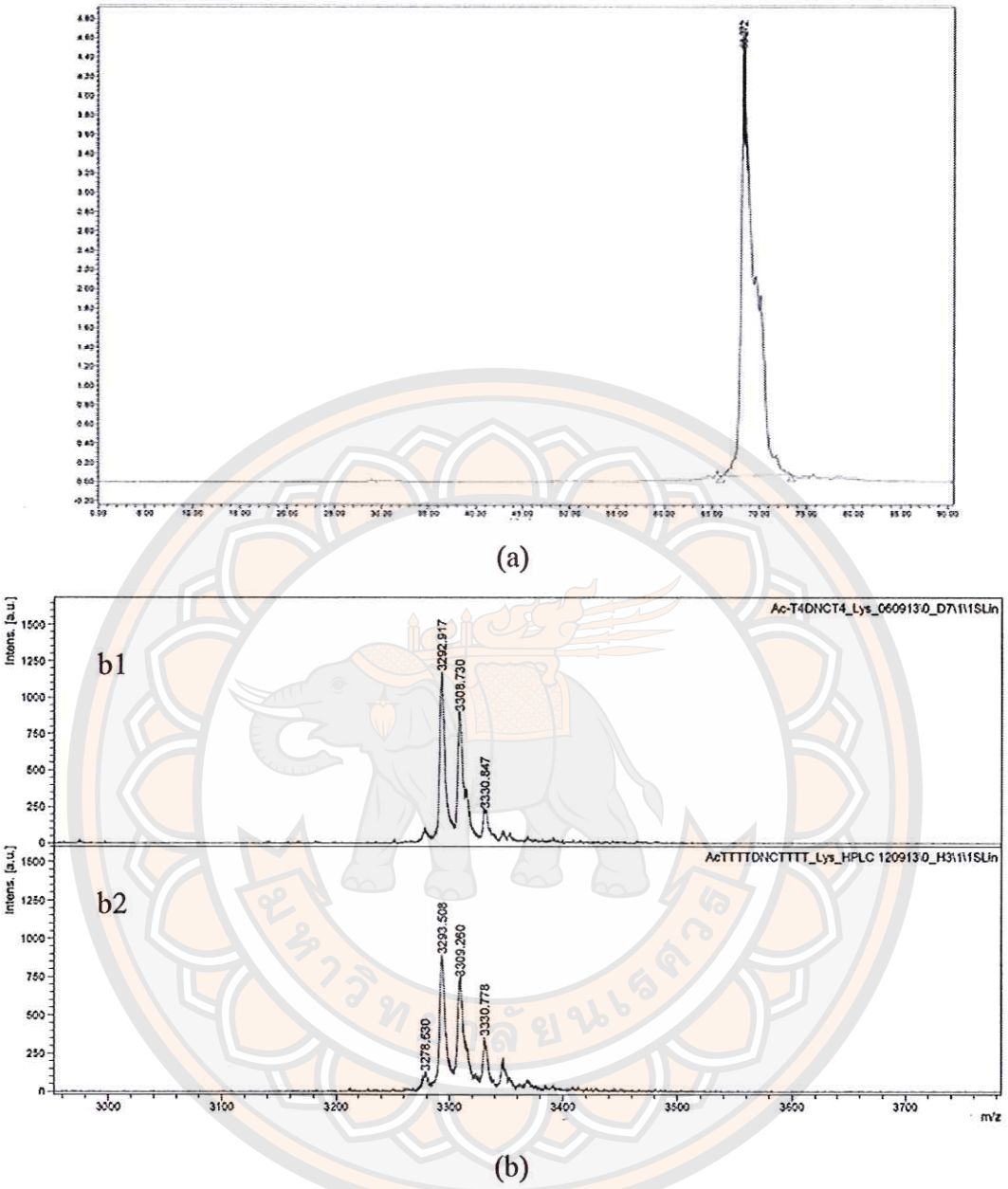


Figure 22 (a) HPLC chromatogram of purified PNA (P1) and (b1) MALDI-TOF MS before purified PNA (P1) (b2) MALDI-TOF MS after purified PNA (P1)

PNA·DNA binding properties

The ability of 3,6-dinitrocarbazole as the universal base in acpcPNA was investigated the base-pairing specificity between acpcPNA with four DNA sequences by UV-melting temperature experiment. T_m experiment were performed under the condition as following; ratio of PNA:DNA= 1:1, concentration of PNA strand = 2 mM, 10 mM sodium phosphate buffer pH 7.0, heating rate 0.5 °C/mM. Monitoring absorbance at 260 nm, recording OD_{260} from 20-90 °C. In a preliminary experiment, the PNA **P1** (T_4XT_4) where T = thymine and X = 3,6-dinitrocarbazole hybrid with complementary four DNA: A_4YA_4, where Y = A, T, C and G were measured and all T_m data is shown in Table 7 and T_m curves and first derivative is shown in Figure 24.

Table 7 T_m values of hybrids between P1 and four DNA sequences compared with original acpcPNA[a]

PNA(X)	DNA(Y)	T_m (°C)	ΔT_m	ΔT_m [b]
DNC	A	55.4	17.7	21.4
	T	45.5		21.3
	C	47.5		16.7
	G	37.7		15.8
T	A	76.8[a]	23.3	
A	T	66.8[a]		
G	C	64.2[a]		
C	G	53.5[a]		

[a]Data from literature; *Org. Lett.* 2006, 8, 9, 1897-1900.[9] [b]ΔT_m calculated by compared with original acpcPNA.

When base Y in DNA sequence was varied; the values of T_m results of **P1** were different, the highest and the lowest T_m value was obtained in Y=A (55.4) and Y=G (37.7) respectively. The T_m of remaining hybrid (Y=T and C) was approximately similar, the value was 45.5 to 47.5°C respectively. In a comparison to the T_m of original acpcPNA, these T_m values of modified base acpcPNA were varied in the same trend as the original acpcPNA which T·A pair shows the highest stability (76.8°C)

while C·G pair shows the lowest stabilities (53.5°C). The moderate stabilities at 64.2-66.8°C were found in the case of A·T and G·C pairs. The ΔT_m calculation (T_m highest – T_m lowest) which exhibited a discrimination of **P1** and original acpcPNA was 17.7 and 23.3°C respectively. The obtained ΔT_m from **P1** indicated that, the attachment of 3,6-dinitrocarbazole to acpcPNA bring about the lowering of base discrimination from 23.3 to 17.7 °C. This means that the lack of hydrogen bond donors of 3,6-dinitrocarbazole provides a possibility of this compound to be a candidate for universal base, but it also leads to an unstable PNA·DNA hybridization. The reason can be explained from planar aromatic structure of DNC could be stacked in the duplex structure but its large flat molecule might be hampered with opposite nucleobase in the space between two strands simultaneously. From all T_m results above which showed a large discrimination between natural bases (A, T, C, G), it can be concluded that 3,6-dinitrocarbazole-acpcPNA could not behave as a good universal base in this sequence. We assumed that the large discrimination of the 3,6-dinitrocarbazole-acpcPNA come from the steric hindered of nitro groups and a large flat molecule of carbazole ring which is too hindered and not appropriately fit in the space between PNA·DNA strands.

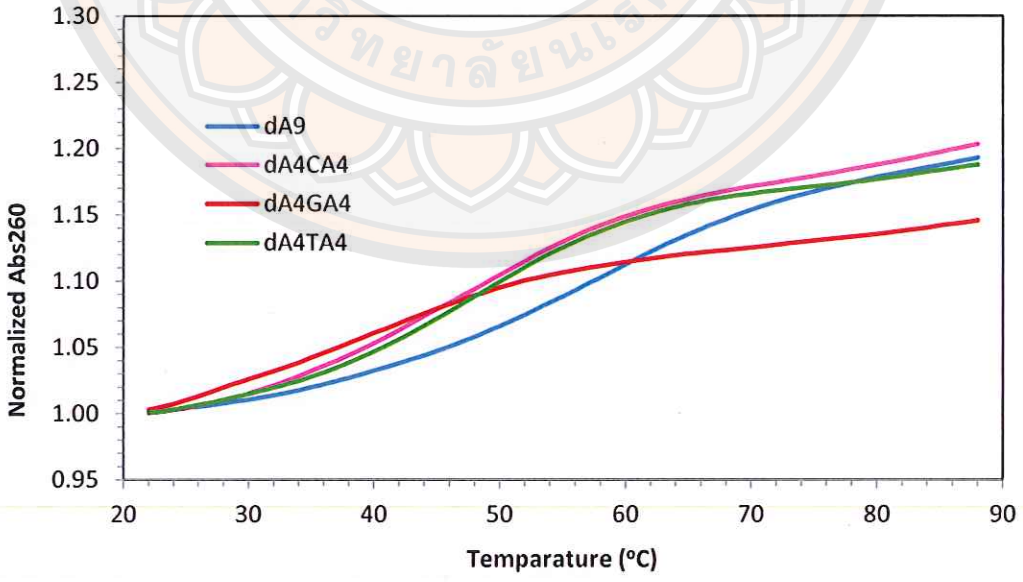


Figure 23 T_m curve of P1 hybrid with complementary DNA

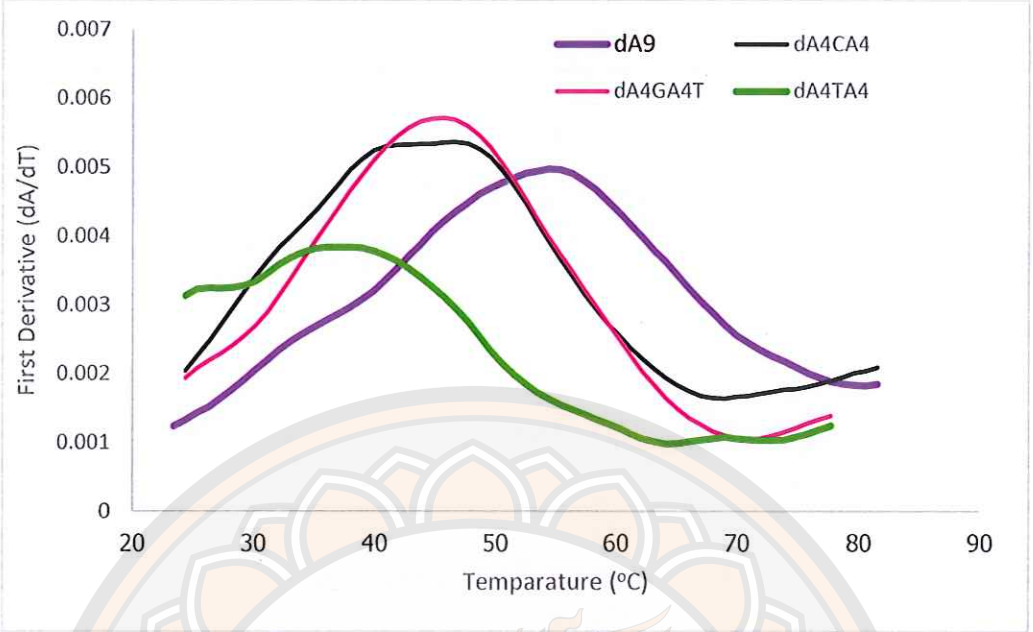


Figure 24 First derivative plots of P1 hybrid with complementary DNA

Although the result from the study of the hybrid between PNA (**P1**)·DNA revealed the unappropiation of 3,6-dinitrocarbazole in behaving as a universal base in acpcPNA because of the obtaining of the large discrimination between four natural bases. However, T-rich sequence like **P1** might not be the good model system because of the low stacking energy of A·T pairs compared to C·G pairs. Therefore, 3,6-dinitrocarbazole in mix base PNA sequence with G·C and A·T as neighboring base pairs GTAGCXGCACT, X = DNC; (**P2**) and GTAGAXTCACT, X = DNC; (**P3**) were also synthesized and investigated universal base property.

The ability of 3,6-dinitrocarbazole to act as a universal base in mix-base acpcPNA were investigated by thermal denaturation experiments of the hybrids between the **P2** and **P3** with four "complementary" DNA sequences (AGTGCYGCTAC, Y = A, T, C and G) and (AGTGAYTCTAC, Y = A, T, C and G) by monitoring UV absorbance at 260 nm. The T_m values of the PNA·DNA hybrids are summarized and compared with our previous results with the T_4XT_4 sequence (X = DNC) in Table 8.

Table 8 T_m data of the DNC-acpcPNA·(P1) and (P2) DNA hybrids

PNA	DNA(Y)	T_m	ΔT_m
P2 N-GTAGCXGCACT-C	A	63.0	2.0
	T	64.0	
	C	62.0	
	G	62.5	
P1 N-TTTTXTTTT-C	A	55.4	17.7
	T	45.5	
	C	47.5	
	G	37.7	

From Table 8, to identify the universal base properties and neighboring base participation of X in the mix-sequence PNA **P2** (GTAGCXGCACT) which has the 3,6-dinitrocarbazole placed between C·G pairs, the calculated ΔT_m values were performed and displayed much smaller discrimination among all four nucleobases (A, T, C and G). The ΔT_m in this case was only 2.0°C in a comparison with ΔT_m = 17.7°C from PNA **P1**. This result indicated that 3,6-dinitrocarbazole in mix-sequence acpcPNA with neighboring G·C pairs can potentially act as a universal base.

To confirm the hybrid formation between **P2** and its complementary DNA, CD spectroscopy was studied. As shown in Figure 25, the CD spectra of a mixture of PNA **P2** and DNA at 1:1 ratio and single-stranded PNA, single-stranded DNA as well as the sum of the two spectra were compared. The original positive band at 218 nm in the DNA (green line) was slightly red shifted to 212 with opposite Cotton effect upon hybrid formation. In addition, the increasing of intensity at 257 nm (negative band) and 284 nm (positive band) were observed in the PNA·DNA hybrid compared to DNA. The spectra in the aromatic region (250-300 nm) of the hybrid and of the single stranded PNA are very different. The difference between CD spectra of PNA·DNA hybrid and summed spectra of the two components suggests the change of hybrid conformation because of the specific interactions of **P2** with DNA.

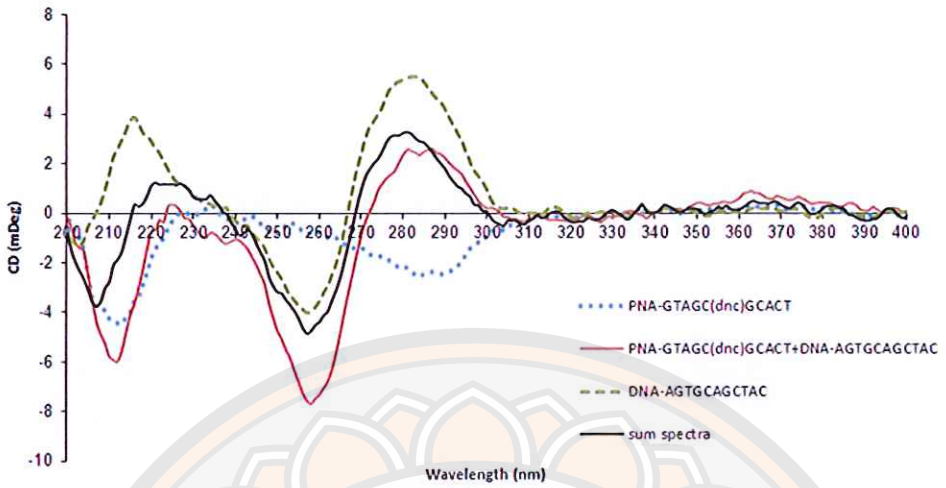


Figure 25 CD spectra of DNC-PNA [GTAGC(DNC)GCACT] (violet), DNC-PNA 1.2 equiv. dAGTGCAGCTAC (red), dAGTGCAGCTAC (green) and summation of PNA and DNA spectra (black). All CD spectra were measured in 10 mM sodium phosphate buffer, pH 7.0, at 25°C, [PNA] = 2.5 μM and [DNA] = 2.5 μM

In the case of the mix-sequence PNA **P3** (GTAGAXTGCACT) which has the X = 3,6-dinitrocarbazole placed between neighboring A·T pairs. The hybridization of **P3** with DNA was also studied by a comparison of the calculated ΔT_m values. As shown in Figure 26, T_m curves of each **P3** hybrids exhibited two transition curves around 48–50 and 67°C which mean that there are two T_m values in this system. This result exhibits a characteristic of triplex hybrid formation by first forming **P3**·DNA duplex and then the occurring duplex further bind with other DNA chain. The reasonable mechanism explanation is still unknown at this stage and supplementary experiments need to be performed to explore this phenomenon. However, it can conclude that 3,6-dinitrocarbazole is delicate to surrounding environment especially its neighboring base. This results is in good agreement with Tantirungrotechai's work that explain the significant difference between the cases of the purine and the pyrimidine bases at opposite position. [39] Purine bases can create the overlapping with 3,6-dinitrocarbazole and enhance the stacking energy.

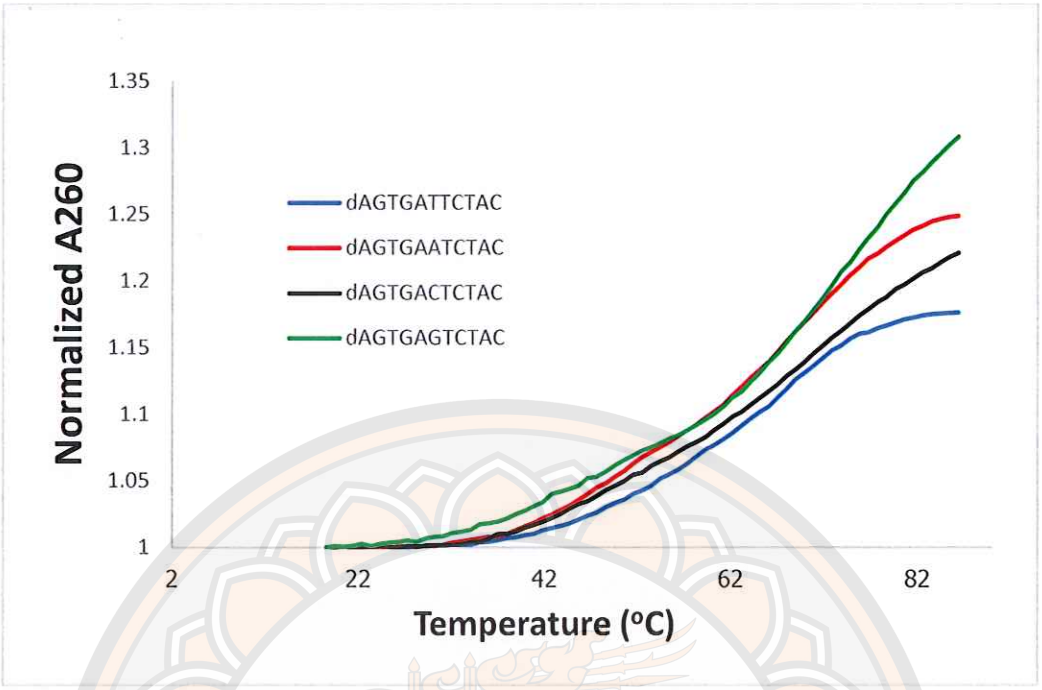


Figure 26 T_m **curve of P2 hybrid with complementary DNA**

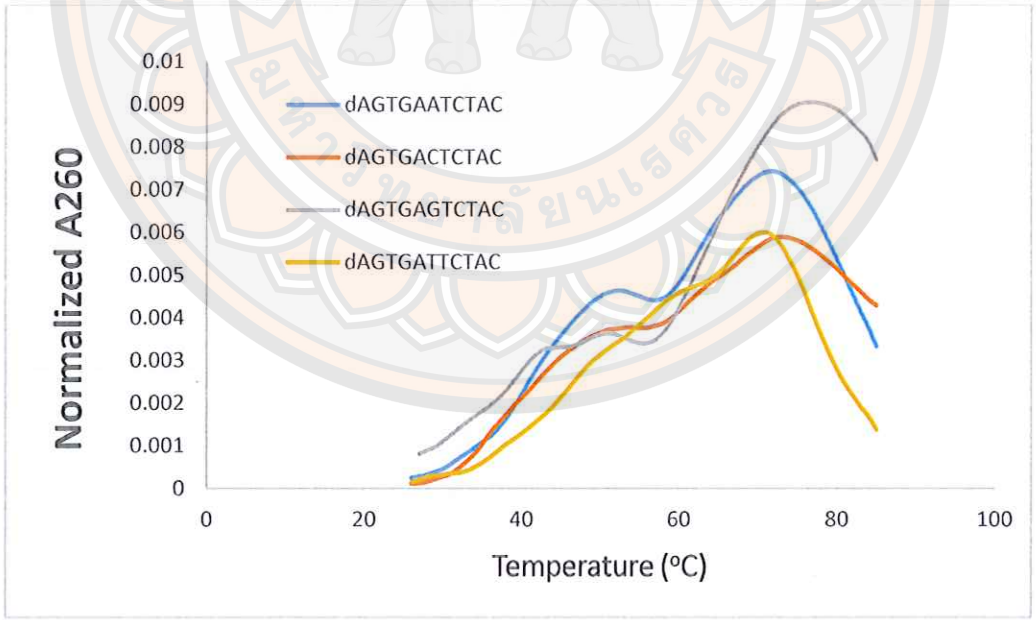


Figure 27 First derivative plots of P3 hybrid with complementary DNA

Reduction of 3,6-dinitrocarbazole-acpcPNA to 3,6-diaminocarbazole-acpcPNA

The study of carbazole derivatives was continuously attempted because of the discovery of a remarkable fluorescent property of 3,6-diaminocarbazole-modified acpcPNA. [44] It is interesting to study in the effects of 3,6-diaminocarbazole as a backbone modifier in literature and as a base replacement in this research. With the 3,6-dinitrocarbazole-acpcPNA at hands, the reduction of dinitrocarbazole on the acpcPNA without affecting the others functionality on the acpcPNA were challenged. A model study which is a short 3,6-dinitrocarbazole-acpcPNA sequence TXT where T = thymine and X = 3,6-dinitrocarbazole was firstly prepared. The reduction of the TXT PNA oligomer (0.25 μmol) using $SnCl_2 \cdot 2H_2O$ (0.5 mg, 10 equiv) in DMF (100 μL) was first attempted on the solid support at room temperature overnight. The progress of the reaction was monitored by MALDI-TOF mass spectrometry. Unfortunately, the reduction did not complete under this condition, even after prolonged heating at 100°C. MALDI-TOF analysis suggested the reduction of only one nitro group at best. An attempt in using sodium dithionite as an alternative reducing agent under phase transfer conditions [$Na_2S_2O_4$ (2.18 mg)/K_2CO_3 (1.7 mg)/tetrabutylammonium hydrogen sulfate 10% mol in a 9:1 mixture of DCM and water] was also failed to give the complete reduction. [45]

Fortunately, a reduction with Raney-Ni was successful and provided a clean and complete reduction of the model 3,6-diamino carbazole-acpcPNA sequence. [45] This condition was the first attempt to reduce in the solution phase with the short 3,6-dinitrocarbazole-acpcPNA sequence TXT because Raney-Ni was an insoluble solid that might not be suitable for solid-phase reduction. By this condition, a reduction of the 3,6-dinitrocarbazole moiety in the PNA oligomer was completed within 2 hours. The same procedure was applied for the reduction of longer 3,6-dinitrocarbazole-acpcPNA **P1** [$T_4\underline{X}T_4$ (X = DNC)]. Results from mass spectrum showed that *m/z* signal of the 3,6-dinitrocarbazole-acpcPNA (calcd mass=3310.4) decreased with concomitant increase of the 3,6-diaminocarbazole-acpcPNA (calcd mass=3250.4) as shown in Figure 28.

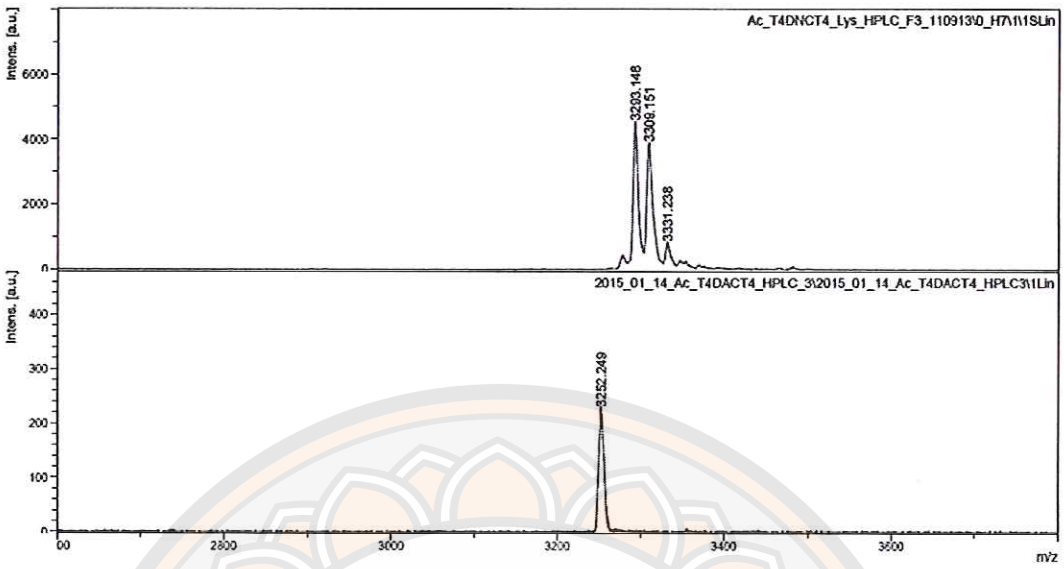


Figure 28 Mass spectra of PNA P1 sequence (T_4XT_4) (upper) X= 3,6-dinitrocarbazole and DAC-acpcPNA (lower) X= 3,6-diaminocarbazole

Thermal denaturation and fluorescence studies of 3,6-diaminocarbazole-acpcPNA

3,6-Diaminocarbazole-acpcPNA T_4XT_4 (X = 3,6-diaminocarbazole) was investigated its DNA hybridization properties with dA_9. The hybrid of 3,6-diaminocarbazole-acpcPNA·dA_9 provided its T_m value of 36°C which was even lower than that of the corresponding 3,6-dinitrocarbazole-acpcPNA·dA_9 hybrid (55.4°C) as shown in Table 9. This means that 3,6-diaminocarbazole-acpcPNA formed more unstable hybrid with DNA. The destabilization of 3,6-dinitrocarbazole-acpcPNA and 3,6-diaminocarbazole-acpcPNA hybrids comparative to unmodified acpcPNA system is reasonable from the consequence of steric hindrance of the carbazole moiety, which could interfere with the stacking arrangement in the PNA·DNA duplex. Although both 3,6-diaminocarbazole and 3,6-dinitrocarbazole should possess similar steric effects, but the difference in electronic nature of the substituent (nitro in 3,6-dinitrocarbazole and amino in 3,6-diaminocarbazole) could play some important roles. The electrostatic interaction of carbazole derivatives is in the order of 3,6-dinitrocarbazole more than carbazole itself. [39] It is assumed that the dipole moment of 3,6-dinitrocarbazole should be rather than carbazole and 3,6-diaminocarbazole respectively. Consequently, the 3,6-dinitrocarbazole could more stably stack with electron-rich nucleobase than

3,6-diaminocarbazole which lead to large destabilization of DAC-acpcPNA and DNA hybridization.

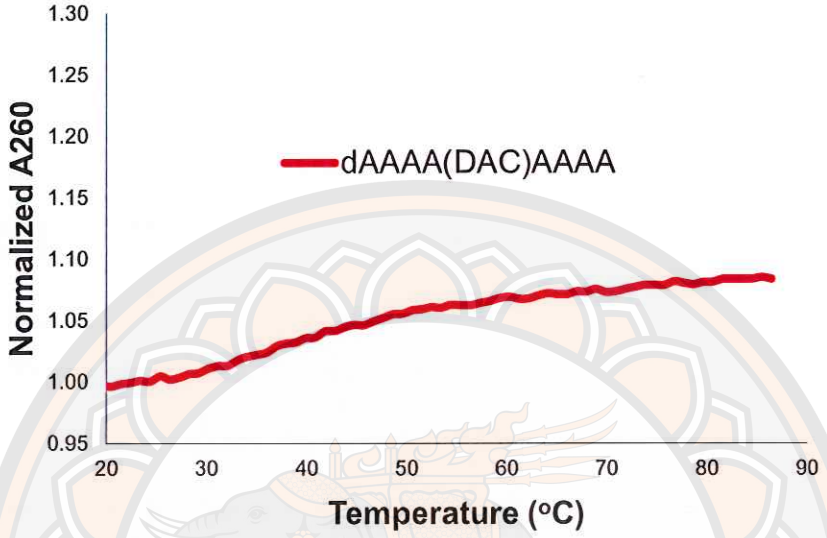


Figure 29 T_m **Thermal denaturation curves for PNA-DAC:DNA double**

Table 9 T_m **data of the DNC-acpcPNA·(P1) and (P4) DNA hybrids**

PNA	DNA(Y)	T_m	ΔT_m
N-TTTTXTTTT-C X=DAC	A	36.0	-
N-TTTTXTTTT-C X=DNC	A	55.4	17.7
	T	45.5	
	C	47.5	
	G	37.7	

Preliminary fluorescence experiments of the 3,6-diaminocarbazole-acpcPNA was shown in Figure 30. It was found that there is virtually no fluorescence change in the absence and presence of DNA. This result is in sharp contrast to the observation of the strong fluorescence increase in acpcPNA with 3,6-diaminocarbazole appended to

the backbone which has been reported before. [44] Further experimental and theoretical studies are required in order to explain this discrepancy.

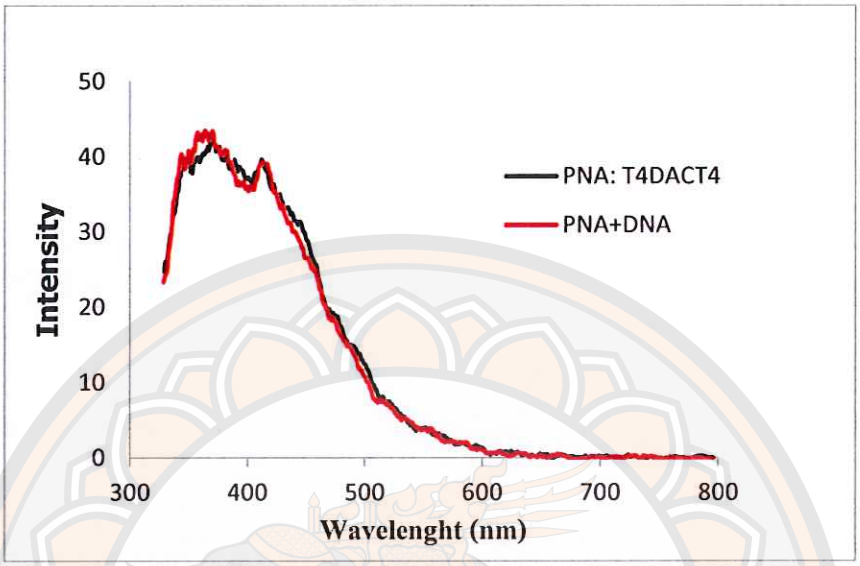


Figure 30 Fluorescence spectra of PNA (P4) and its hybrids with DNA (condition 1μM, PNA 1.2 μM DNA in 10 mM sodium phosphate buffer pH = 7, λ_{excit} = 315 nm)

In summary, the synthesis of mix-sequence acpcPNA **P2** carrying 3,6-dinitrocarbazole as a base replacement was successful. Studies in thermal denaturation revealed that the 3,6-dinitrocarbazole moiety in acpcPNA can behave as a universal base by providing the small ΔT_m of 2.0°C, which is in sharp contrast to the homothymine acpcPNA **P1** (ΔT_m of 17.7°C). In addition, 3,6-diaminocarbazole-acpcPNA was prepared from a direct reduction of 3,6-dinitrocarbazole-acpcPNA using a mild and effective condition from Raney-Ni in aqueous solution. This procedure should be generally useful for post-synthetic modification of PNA bearing various functional groups. The attempt to use 3,6-diaminocarbazole as a acpcPNA fluorescence probe in this case was unsuccessful in this stage.

CHAPTER V

CONCLUSION

In this research, 3,6-DNC acpcPNA and 3,6-DAC acpcPNA were synthesized and evaluated DNA binding properties. 3,6-dinitrocarbazole was designed and prepared to act as a universal base in original acpcPNA. The sequence TTTTXTTTT (X = DNC) was firstly investigated its binding ability with four DNA sequences (A_4YA_4); Y = A, T, C or G). The studies of UV-melting temperature (T_m) values of PNA·DNA hybrids revealed that our PNA shows large discrimination between the four natural nucleobases (ΔT_m ~1 7 . 7 °C). This means that DNC cannot act as a universal base in acpcPNA with homothymine sequence. Surprisingly, mix-sequence acpcPNA carrying 3,6-dinitro carbazole (DNC) was further synthesized in sequence GTAGCXGCACT (X = DNC) and thermal denaturation studies revealed that the DNC in acpcPNA can act as a universal base as shown by the small ΔT_m of only 2.0 °C, which is in sharp contrast to the homothymine sequence from previous study. The results indicated that DNC in mix-sequence acpcPNA with neighboring G·C pairs can potentially act as a universal base. The specific interactions of DNC-PNA with DNA during the binding was observed from the difference between CD spectra of PNA·DNA hybrid and summed spectra of the two components, leading to the observed conformational change.

Moreover, a mild and effective condition to reduce the nitro groups in DNC-acpcPNA to DAC-acpcPNA using Raney-Ni in aqueous solution was developed. Such condition should be generally useful for post-synthetic modification of PNA bearing various functional groups. Unfortunately, the study of DAC-acpcPNA by fluorescence technique revealed that this PNA cannot change its fluorescence in the presence of DNA.

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APPENDIX

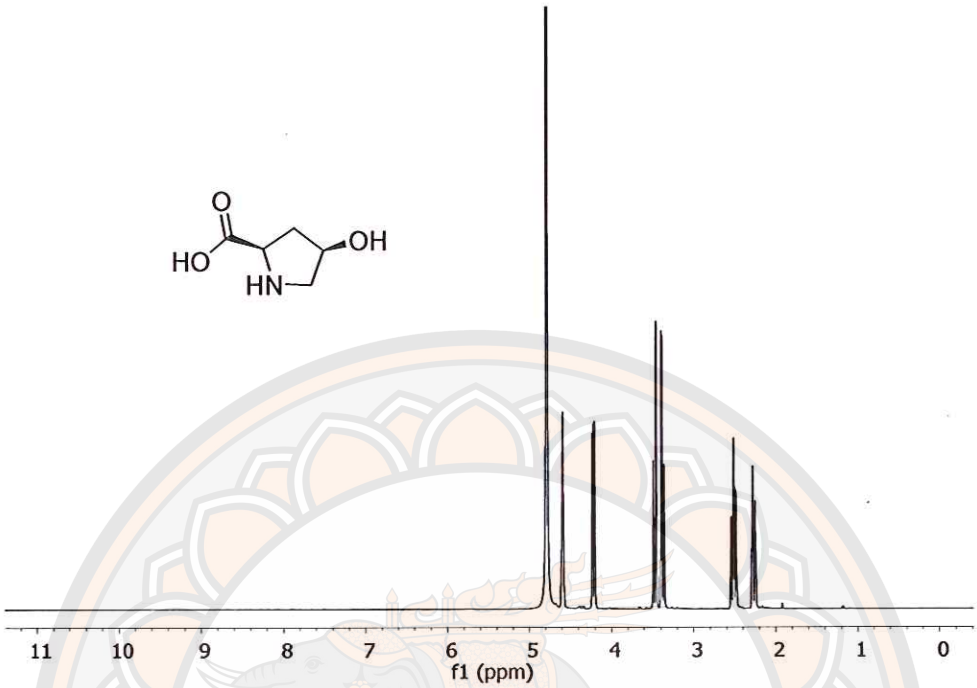


Figure 31 ^{1}H NMR spectrum of *cis*-4-hydroxy-D-proline

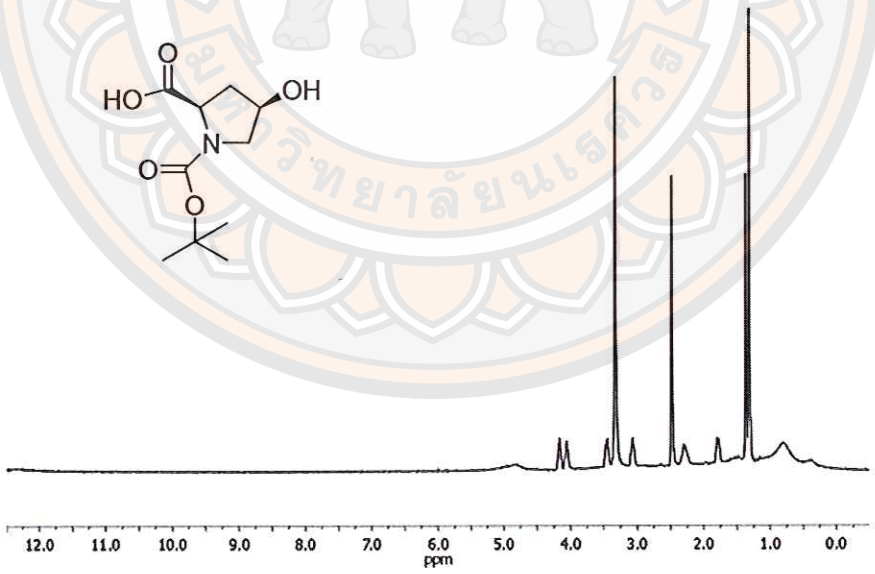


Figure 32 ^{1}H NMR spectrum of *cis*-*N*-(*tert*-butoxycarbonyl)-4-hydroxy-D-proline

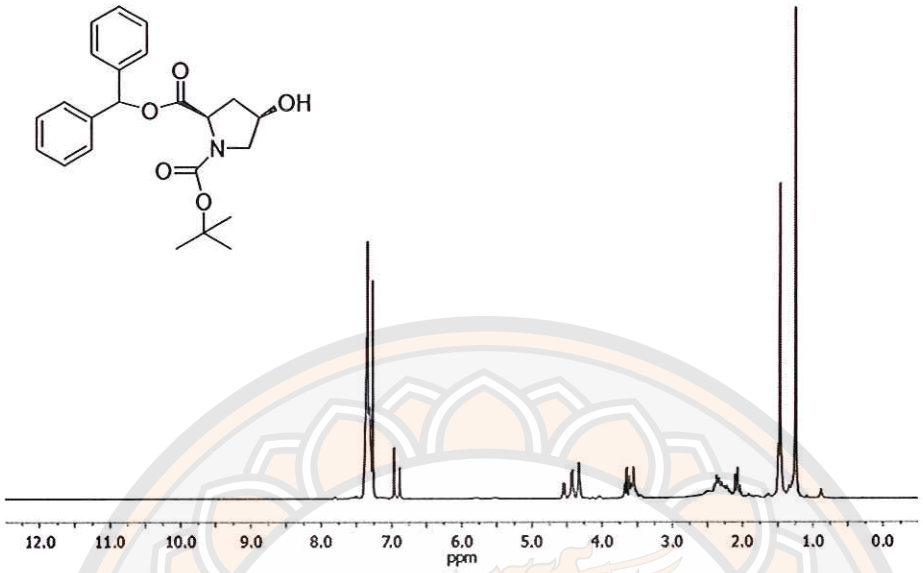


Figure 33 ^{1}**H NMR spectrum of *N*-tert-Butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester**

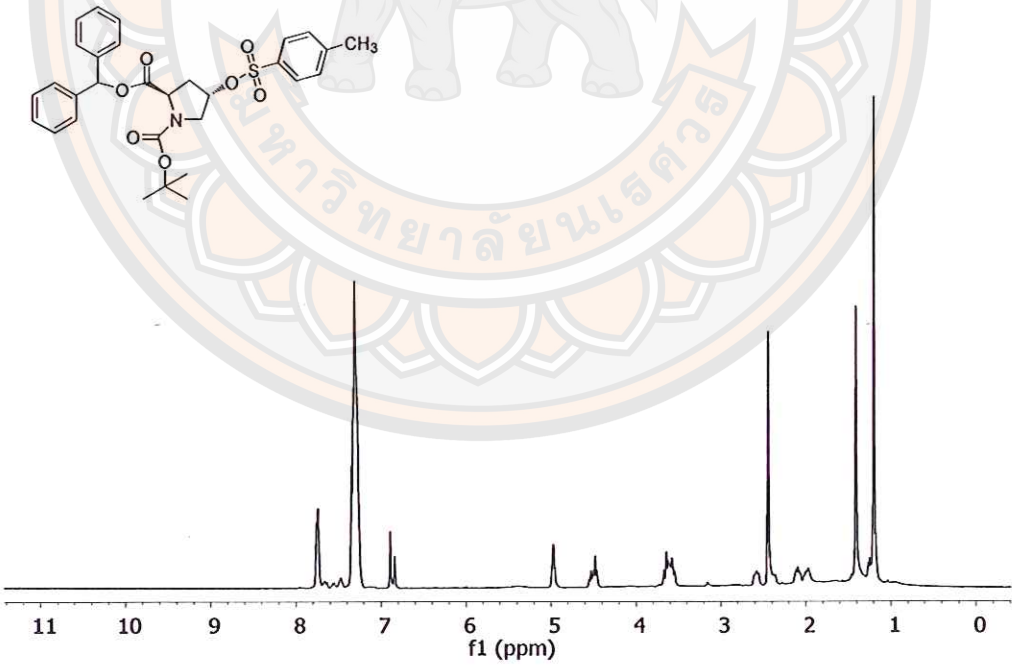


Figure 34 ^{1}**H NMR spectrum of *N*-tert-Butoxycarbonyl-trans-4-tosyl-D-proline diphenylmethyl ester**

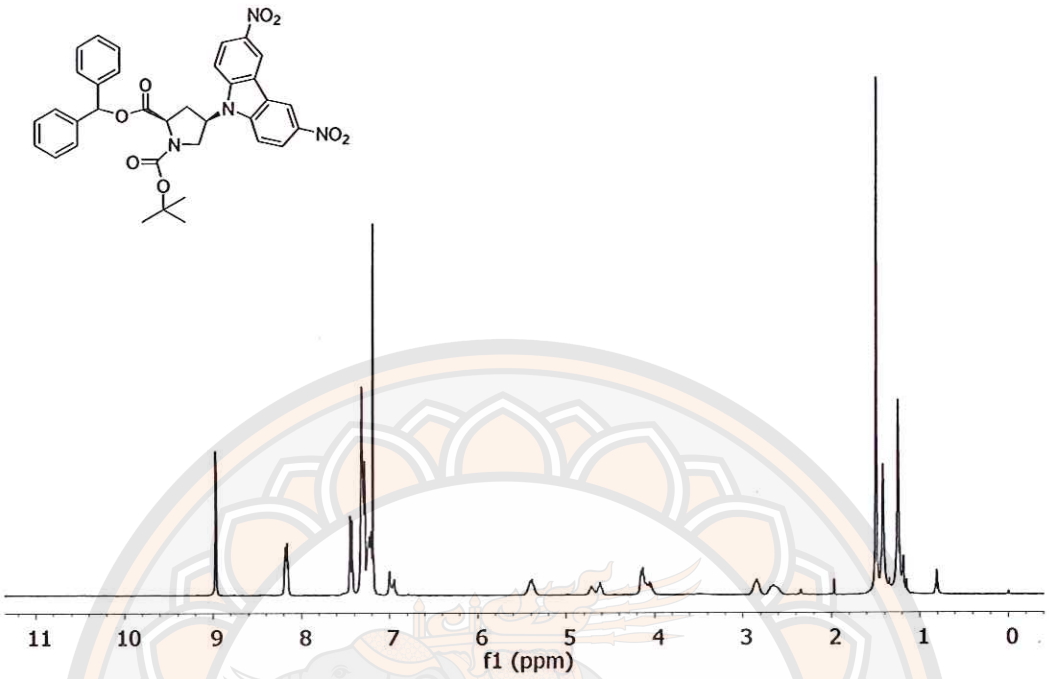


Figure 35 ^{1}**H NMR spectrum of *N-tert*-Butoxycarbonyl-*cis*-3,6-dinitro carbazole-D-proline diphenylmethyl ester**

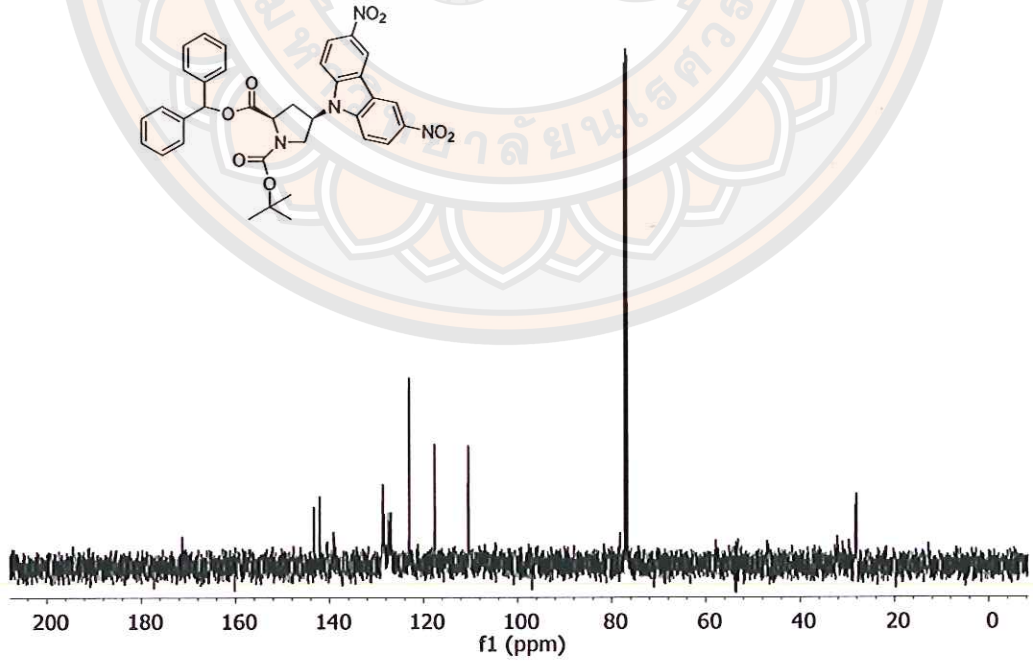


Figure 36 ^{13}**C NMR spectrum of *N-tert*-Butoxycarbonyl-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethyl ester**

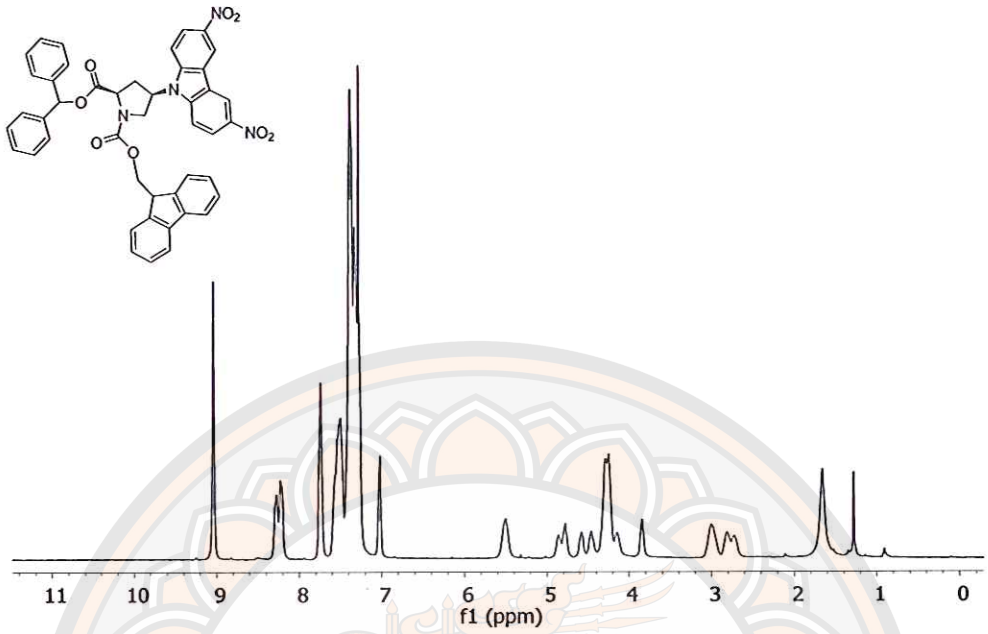


Figure 37 ^{1}**H NMR spectrum of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-prolinediphenylmethyl ester**

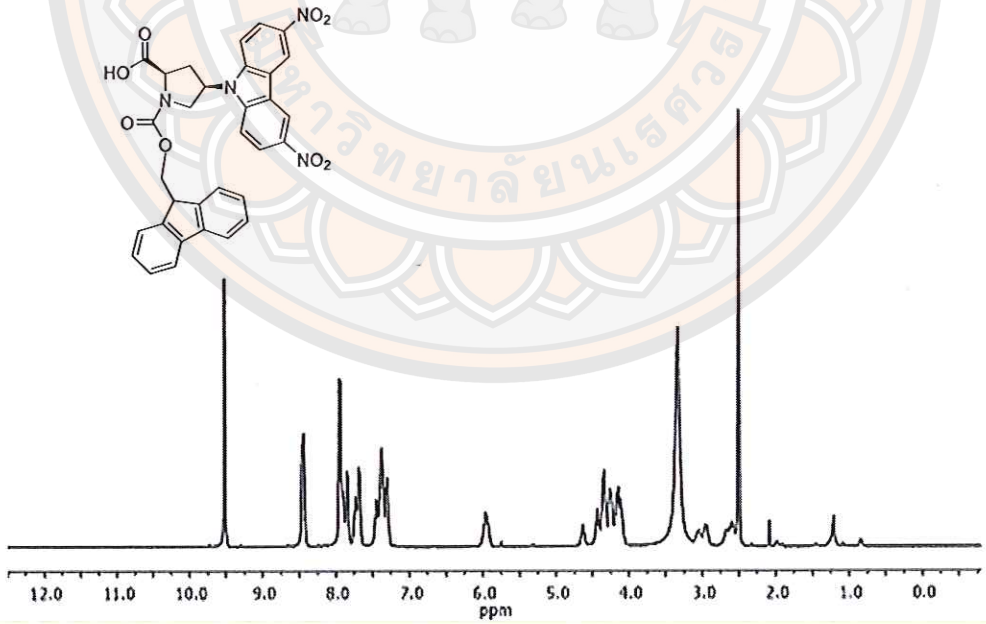


Figure 38 ^{1}**H NMR spectrum of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline carboxylic acid**

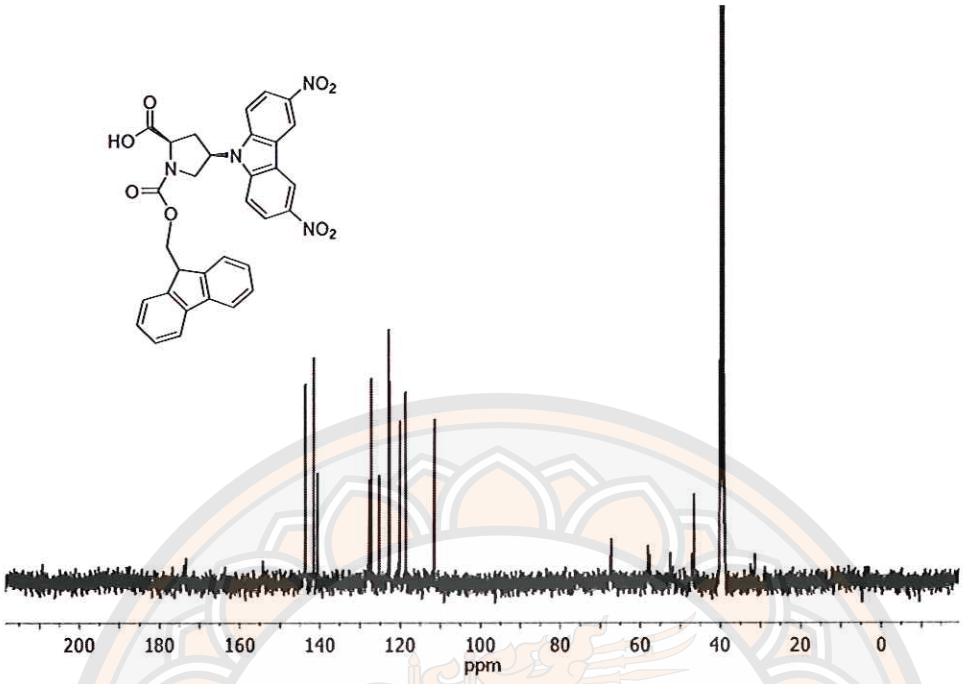


Figure 39 ^{13}**C NMR spectrum of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline carboxylic acid**

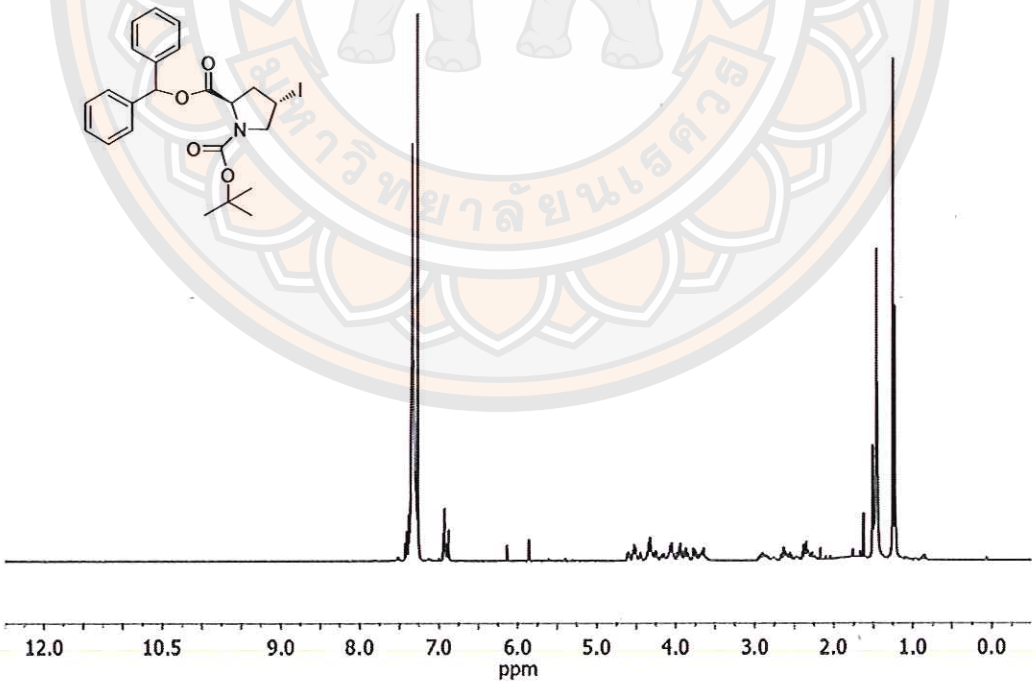


Figure 40 ^{1}**H NMR spectrum of (2R,4S)-1-((9H-fluoren-9-yl)methyl) 2-benzhydryl 4-iodopyrrolidine-1,2-dicarboxylate**

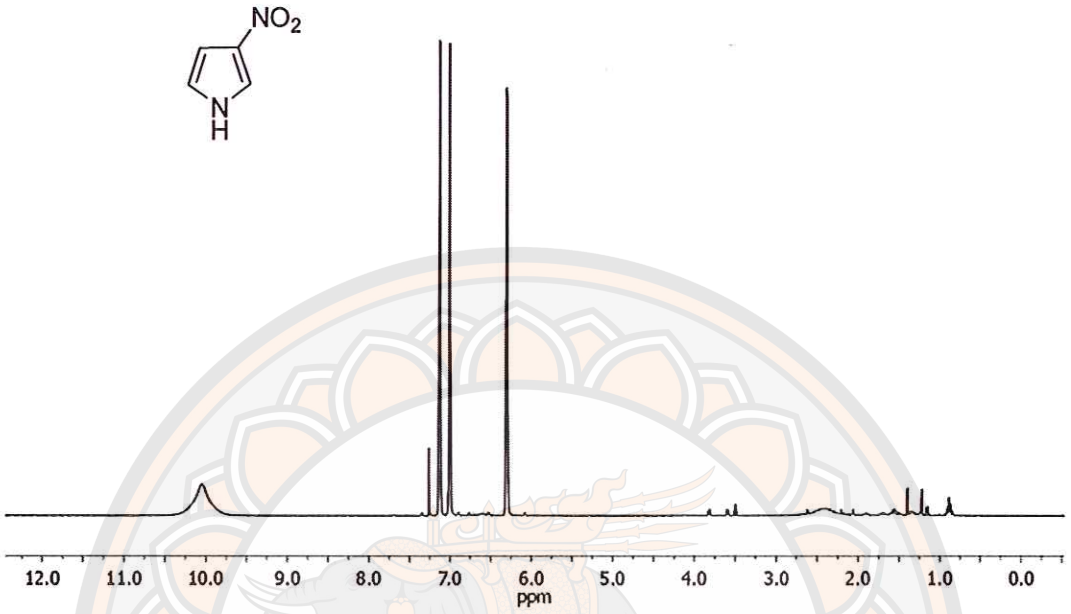


Figure 41 ^{1}H NMR spectrum of 3-nitro pyrrole

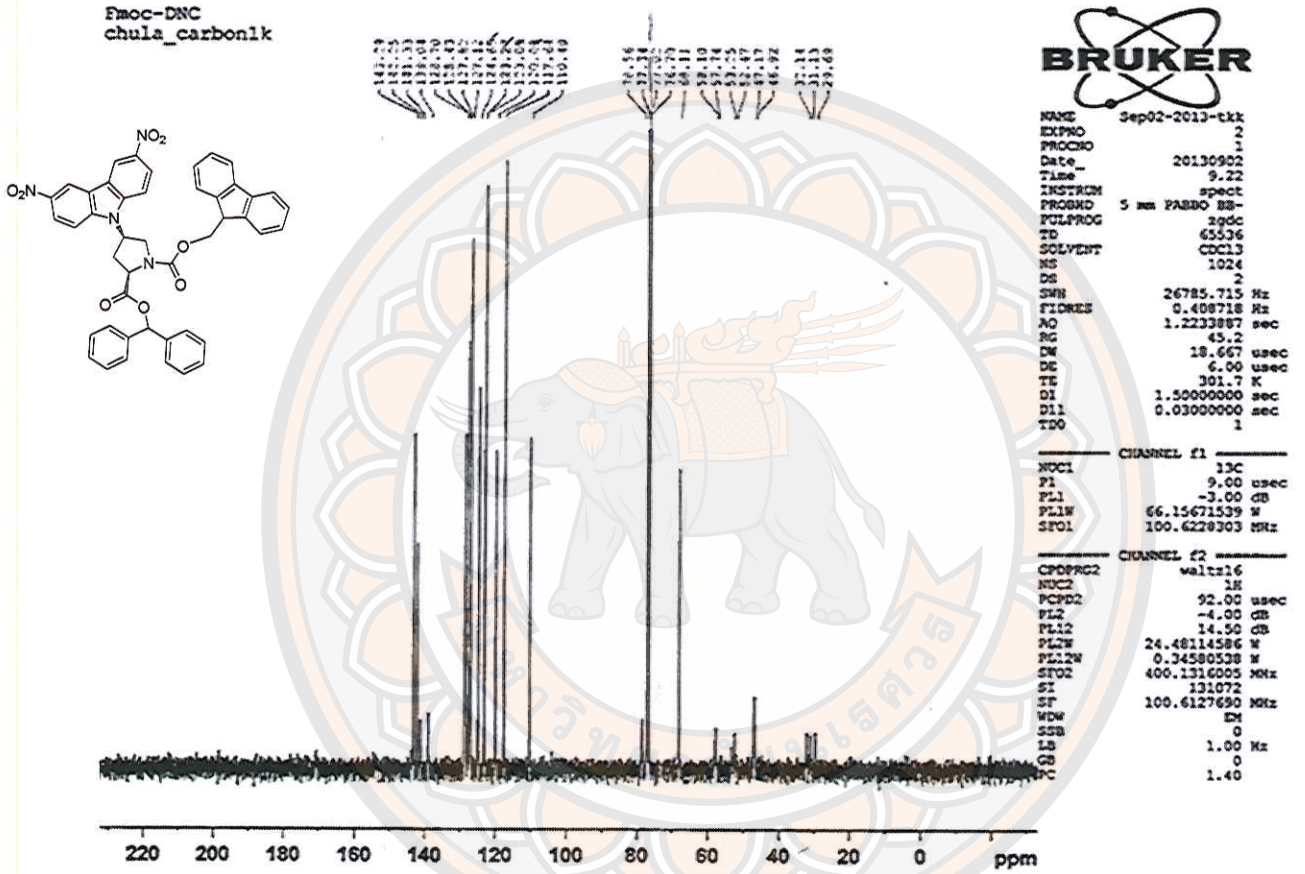


Figure 42 ^{13}C NMR spectrum of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline diphenylmethyl ester

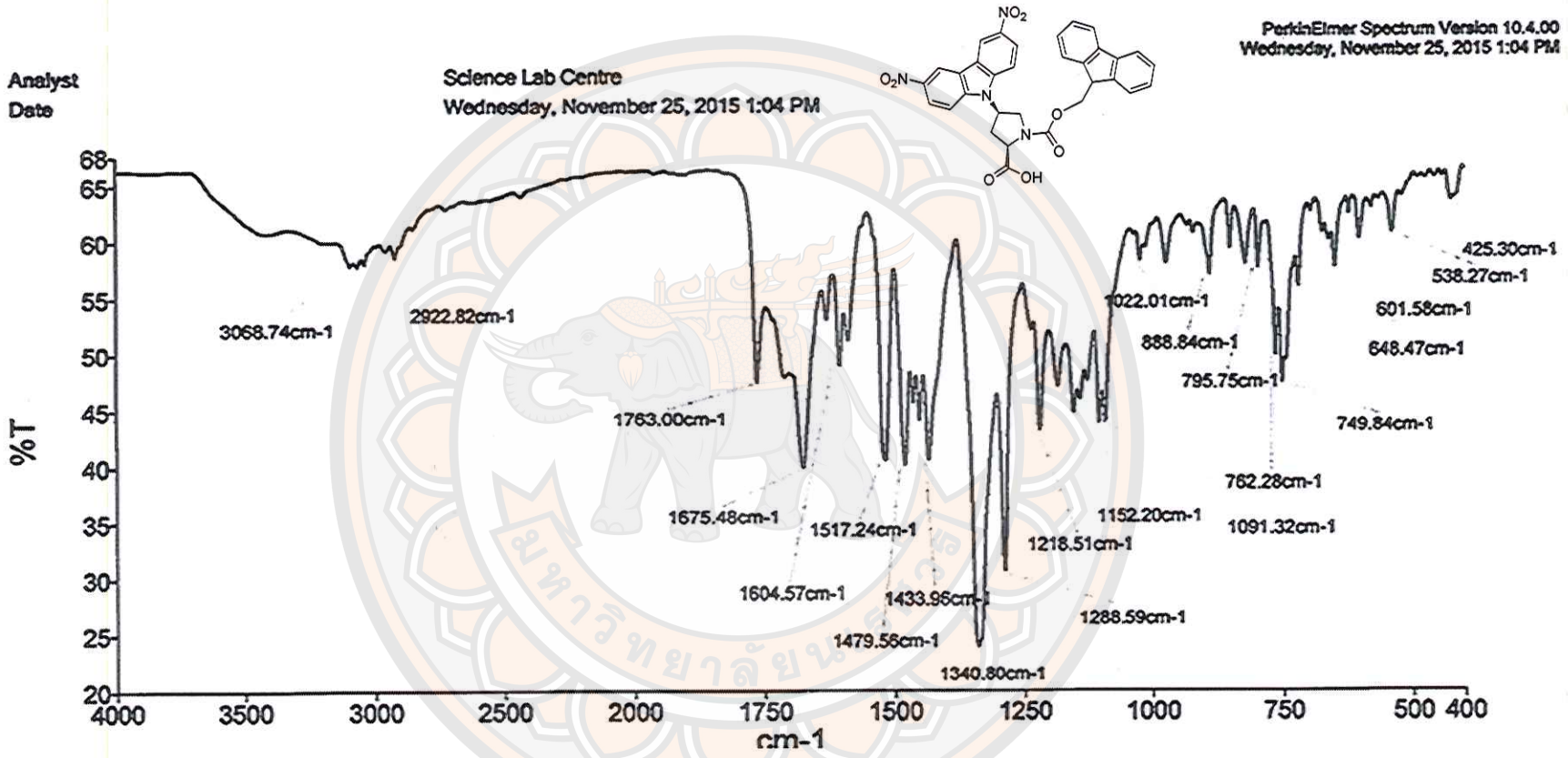


Figure 43 FT-IR spectrum of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6-dinitrocarbazole-D-proline carboxylic acid

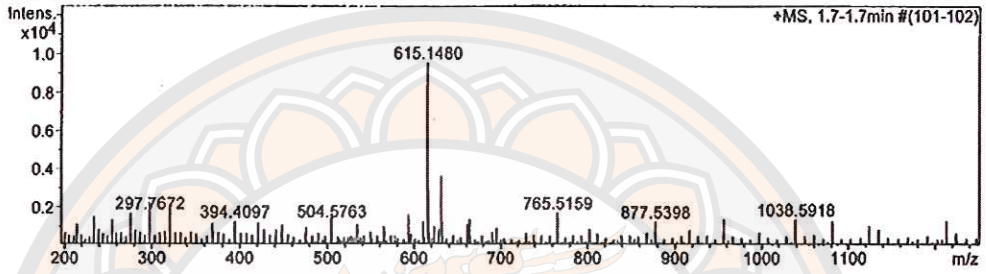
Mass Spectrum List Report

Analysis Info

Analysis Name	OSCU5810050033.d	Acquisition Date	10/5/2015 2:12:14 PM
Method	Tune_wide_POS_Natee20130403.m	Operator	Administrator
Sample Name	Fmoc-DNC Fmoc-DNC	Instrument	micrOTOF 72

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	92 V
Scan Range	n/a	Capillary Exit	180.0 V	Set Pulsar Pull	409 V
Scan Begin	50 m/z	Hexapole RF	600.0 V	Set Pulsar Push	391 V
Scan End	3000 m/z	Skimmer 1	70.0 V	Set Reflector	1300 V
		Hexapole 1	25.0 V	Set Flight Tube	9000 V
				Set Detector TOF	1980 V



#	m/z	I	I %	S/N	FWHM	Res.
1	56.1071	1934	20.2	30.2	0.0051	11030
2	58.6207	1512	15.8	23.6	0.0078	7471
3	77.3559	1415	14.8	22.1	0.0180	4300
4	89.1359	1574	16.5	24.5	0.0062	14465
5	101.9282	1982	20.7	31.0	0.0081	12581
6	145.1040	1377	14.4	21.5	0.0065	22236
7	145.1213	1446	15.1	22.6	0.0139	10441
8	196.0399	1921	20.1	32.3	0.0105	18732
9	254.4180	1342	14.0	24.7	0.0103	24706
10	275.7303	1686	17.6	32.2	0.0123	22398
11	297.6780	1658	17.4	32.8	0.0173	17227
12	297.7672	1694	17.7	33.6	0.0308	9656
13	593.1656	1611	16.9	24.1	0.0712	8330
14	615.1480	9555	100.0	146.5	0.0755	8149
15	616.1514	2873	30.1	43.7	0.0868	7096
16	631.1126	3619	37.9	55.6	0.0808	7814
17	663.4580	1386	14.5	21.2	0.0884	7501
18	765.5159	1707	17.9	27.6	0.0194	39402
19	1038.5918	1378	14.4	25.0	0.0343	30283
20	1258.6769	1532	16.0	28.7	0.0238	52914
21	1450.2131	1867	19.5	36.6	0.0270	53731
22	1603.0548	1358	14.2	27.4	0.0339	47308
23	1874.0197	1691	17.7	34.8	0.0300	62505
24	1874.2508	1631	17.1	33.6	0.0336	55844
25	2166.6545	1408	14.7	27.8	0.0372	58169
26	2351.8715	2543	26.6	49.6	0.0314	74970
27	2352.2257	3195	33.4	62.4	0.0326	72180
28	2814.7571	1335	14.0	27.3	0.0354	79560
29	2884.4803	1332	13.9	27.5	0.0713	40458
30	2972.6989	1323	13.8	27.4	0.0433	68621

Figure 44 HRMS (ESI+) spectrum of (*N*-Fluoren-9-ylmethoxycarbonyl)-*cis*-3,6- dinitrocarbazole-D-proline carboxylic acid

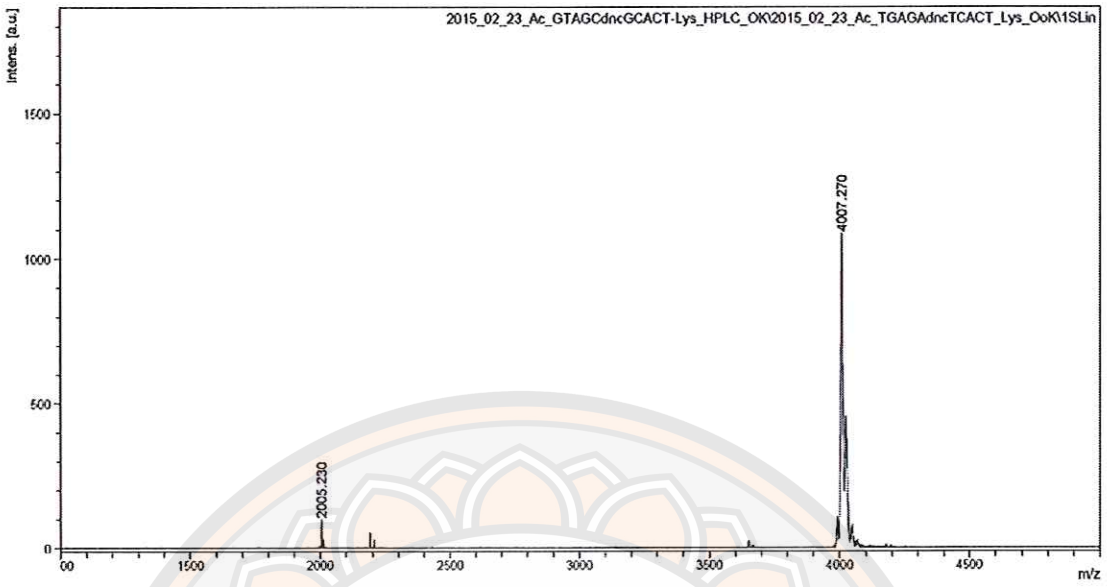


Figure 45 MALDI-TOF MS of purified PNA (P2)

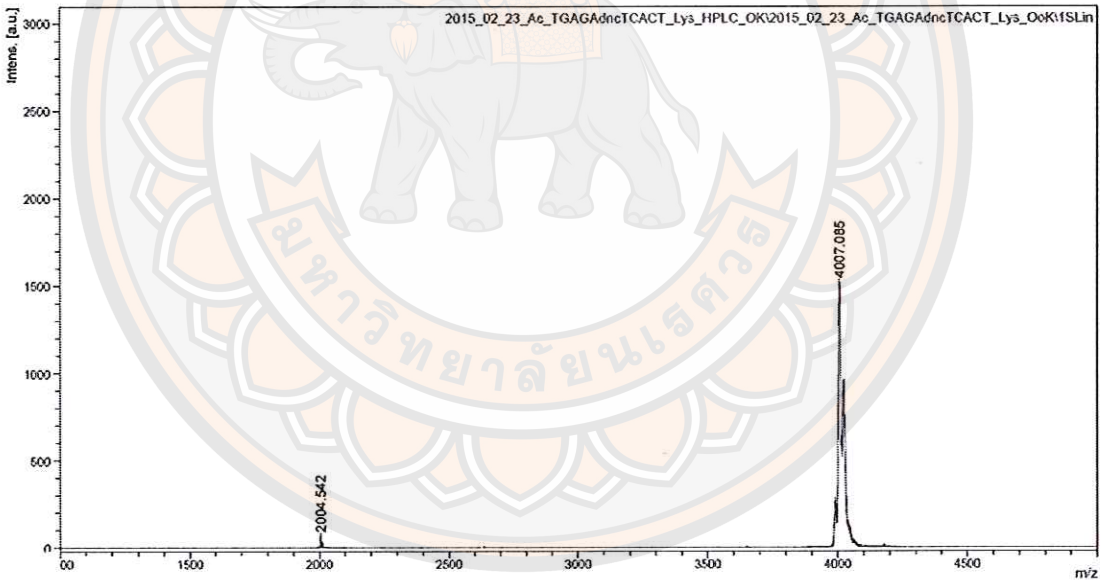


Figure 46 MALDI-TOF MS of purified PNA (P3)

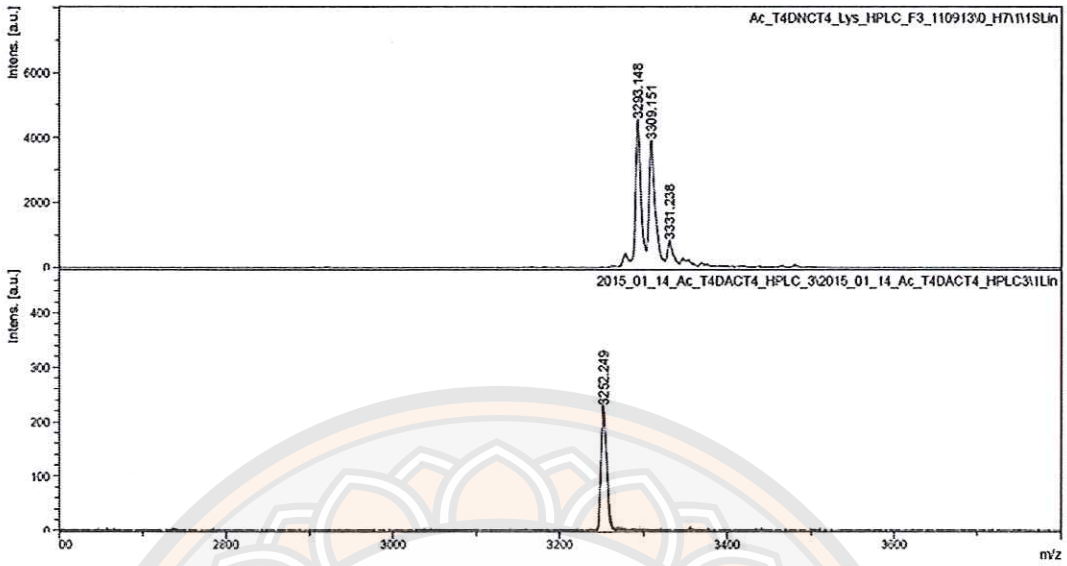


Figure 47 Mass spectra of PNA P1 sequence (T_4XT_4) (upper) X= 3,6-dinitrocarbazole and P2 (lower) X= 3,6-diaminocarbazole