

**Synthesis and Biological Investigation of New
Compounds Aiming at Immunomodulators:
Development of Novel Peptide Nucleic Acids and
New Topoisomerases Inhibitors**

**(新規イミュノモジュレーターの開発：新規ペプチド核酸なら
びにトポイソメラーゼ阻害剤の合成および生物活性研究)**

Mohamed Abdel-Aziz Mohamed Osman

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究)

Thesis presented by

Mohamed Abdel-Aziz Mohamed Osman

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医療薬科学専攻 医薬品化学研究室

Bioorganic Medicinal Chemistry Department

Graduate School of Pharmaceutical Sciences

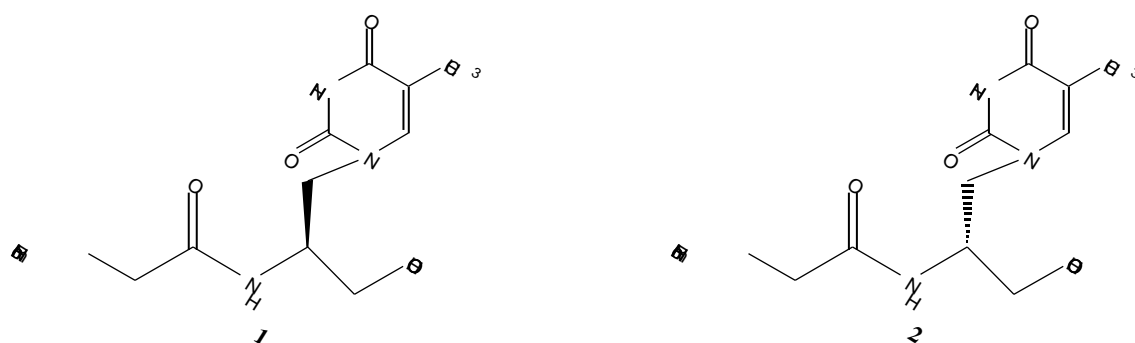
Kumamoto University

Kumamoto- Japan

To my parents, my
wife and my dear
children Hagar, Omar
and Sarah

Abstract

The flow of genetic information starting from DNA passing through RNA, finally leads to the formation of protein which have important and inevitable roles in metabolism and anabolism. Damaged DNA and or RNA developed either due to oxidation, virus, cancer, mutation, and infection lead to severe undesired side reactions (diseases). Several methods were developed affecting on protein synthesis all over its stages of synthesis, from these candidates the antisense methodology which have been observed to bind to DNA and RNA and inhibit their functions. Another candidate is the use of the strategy of enzyme inhibitors which have been attracted much attention in medicinal chemistry. In this thesis we tried to use both mechanisms, the antisense strategy through preparing novel PNAs, and the strategy of enzyme inhibitors through developing of new topoisomerase inhibitors.



In order to assess the hybridization properties of these monomers to complementary DNA and RNA, thymine isogaPNA monomers **1** (T_R) and **2** (T_S) were designed and synthesized. The two monomers were individually introduced into *aeg*PNA sequences in different positions to form different thymine dodecamers. The results illustrated in Table 1, show good binding properties to both DNA and RNA.

Table 1. T_m values of some complexes formed between various PNA oligomers and its complementary DNA and RNA.

PNA sequences	T_m (°C)		PNA sequences	T_m (°C)	
	Complex with dA ₁₂	Complex with rA ₁₂		Complex with dA ₁₂	Complex with rA ₁₂
H-T ₁₂ -Lys-NH ₂	65	73	H-T ₁₁ -T _S -Lys-NH ₂	61	67
H-T ₁₁ -T _R -Lys-NH ₂	65	67	H-T _S -T ₁₁ -Lys-NH ₂	64	68
H-T _R -T ₁₁ -Lys-NH ₂	65	67	H-T ₉ -T _S T _S T _S -Lys-NH ₂	56	63
H-T ₉ -T _R T _R T _R -Lys-NH ₂	60	67	H-T _S T _S T _S -T ₉ -Lys-NH ₂	58	64

Various acetylgalloylbenzamides **3a-f**, galloylbenzamides **4a-f**, acetylgalloylamino-

benzothiazoles **5a-b**, galloylaminobenzothiazoles **6a-b**, acetylgalloylaminobenzothiazole **7** and galloyl- aminothiazole **8** derivatives were prepared. Most of these compounds showed high inhibitory activity against topoisomerase I and topoisomerase II (Table 2).

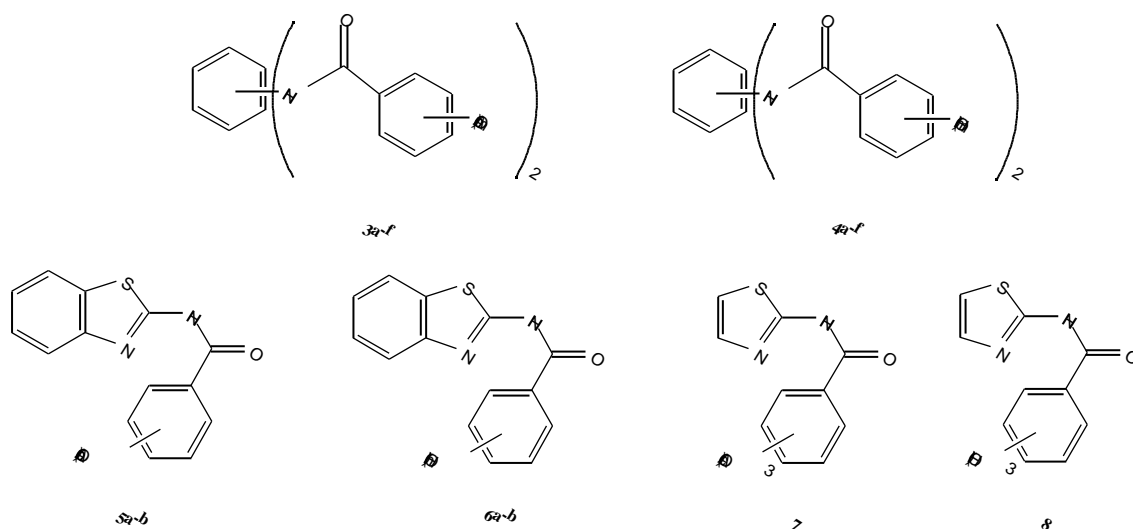


Table 2. Topoisomerases I and II inhibitory activities of compounds **3a-f**, **4a-f**, **5a-b**, **6a-b**, **7** and **8**.

Comp. #	# of acetyl	position	topo I inhibition (IC ₅₀ , μM)	Comp. #	# of hydroxy	topo I inhibition (IC ₅₀ , μM) (topo II)
3a (o)	3	3,4,5-	4.7	4a (o)	3	0.9 (0.09)
3b (o)	2	3,4-	88.0	4b (o)	2	6.4
3c (m)	3	3,4,5-	11.2	4c (m)	3	1.6 (0.08)
3d (m)	2	3,4-	43.2	4d(m)	2	10.0
3e (p)	3	3,4,5-	8.6	4e (p)	3	1.4 (0.08)
3f (p)	2	3,4-	> 100.0	4f (p)	2	2.6 (0.14)
5a	3	3,4,5	7.7	6a	3	8.2
5b	2	3,4-	> 100.0	6b	2	16.9
7	3		> 100.0	8	3	34.4

Preface

This thesis is the outcome of my four years and half research as a graduate student at the Bioorganic Medicinal Chemistry Department, Graduate School of Pharmaceutical Sciences, Kumamoto University, Japan. It is based on the following publications:

- 1) Synthesis and Hybridization Property of Novel 2',5'-isoDNA Mimic Chiral Peptide Nucleic Acids.
Mohamed Abdel-Aziz, Tetsuo Yamasaki and Masami Otsuka, *Bioorg. Med. Chem. Lett.*, **2003**, *13*, 1041.
- 2) New Strategy to Antiviral Agents from Peptide Nucleic Acid Derivatives.
Tetsuo Yamasaki, Mohamed Abdel-Aziz, Naotoshi Kiyota, Tokumi Maruyama and Masami Otsuka, *Heterocycles*, **2003**, *60*, 1561.
- 3) Inhibitory Activities against Topoisomerase I & II by Polyhydroxybenzoyl Amide Derivatives and Their Structure-Activity Relationship.
Mohamed Abdel-Aziz, Kazuya Matsuda, Masami Otsuka, Masaru Uyeda, Tadashi Okawara and Keitarou Suzuki, *Bioorg. Med. Chem. Lett.*, **2004**, *14*, 1669.
- 4) Synthesis of New Peptide Nucleic Acid Monomer with GlycylGlycine Backbone.
Tetsuo Yamasaki, Mohamed Abdel-Aziz, Takuji Iwashita, Tokumi Maruyama and Masami Otsuka, in preparation.

Abbreviations

PNA	Peptide nucleic acid.
isogaPNA	Isoglycylalanine peptide nucleic acid.
aegPNA	Aminoethylglycine peptide nucleic acid.
APNA	Aromatic peptide nucleic acid.
isoggPNA	Isoglycylglycine peptide nucleic acid.
Boc	<i>tert</i> -Butoxycarbonyl.
Bn	Benzyl.
Bz	Benzoyl.
DEAD	Diethyl azodicarboxylate.
DMF.....	Dimethylformamide.
DMSO	Dimethylsulfoxide.
Fmoc	9-Fluorenylmethoxycarbonyl.
Ac	Acetyl.
TEA	Triethylamine.
TFA	Trifluoroacetic acid.
THF	Tetrahydrofuran.
DIEA	<i>N</i> -Ethyl-diisopropylamine.
Gly.	Glycine.
Ala.	Alanine.
HOBt.....	1-Hydroxybenzotriazole.
TBTU	2-(1 <i>H</i> -Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.
DCHA	Dicyclohexylamine.
HATU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.
NMP	<i>N</i> -Methylpyrrolidone.
<i>T</i> _m	Melting temperature.
TFMSA	Trifluoromethanesulfonic acid.
DMS	Dimethylsulfide.
HIV	Human immunodeficiency virus.
HSV	Herpes simplex virus.
HTLV-III	Human T-lymphotropic virus type III.
RT	Reverse transcriptase.
<i>R</i> _t	Retention time.
AGBA	Acetyl-galloylbenzamide.
GBA	Galloylbenzamide.
AGABT	Acetyl-galloyl-2-aminobenzothiazole.
GABT	Galloyl-2-aminobenzothiazole.
AGAT	Acetyl-galloyl-2-aminothiazole.
GAT	Galloyl-2-aminothiazole.
<i>o</i> -GPD	<i>o</i> -galloylphenylenediamine.
SDS.....	Sodium dodecylsulfate.

MEM	Minimum essential medium.
<i>K_m</i>	Michaelis constant.
EDTA	Ethylene diamine tetraacetic acid.
CD	Circular dichroism.

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I. General Introduction

The information that enables a cell to live and to divide, giving rise to two identical daughter cells, is coded on genes. The chemical material of a gene is deoxyribonucleic acid (DNA) (in some viruses it is RNA). The genetic information for the synthesis of a particular protein is contained in a gene, a specific stretch of nucleotide sequence in DNA. DNA must be duplicated in a process called replication before a cell divides. The replication of DNA allows each daughter cell to contain a full complement of chromosomes (Fig. 1). DNA replication occurs at polymerization rates of about 500 nucleotides per second in bacteria and about 50 nucleotides per second in mammals. Several enzymes can affect on the process of replication including DNA polymerase I, DNA polymerase III and some helicases enzymes such as DNA ligase. For the genetic information to be expressed, a DNA sequence must first be copied into a strand of RNA.

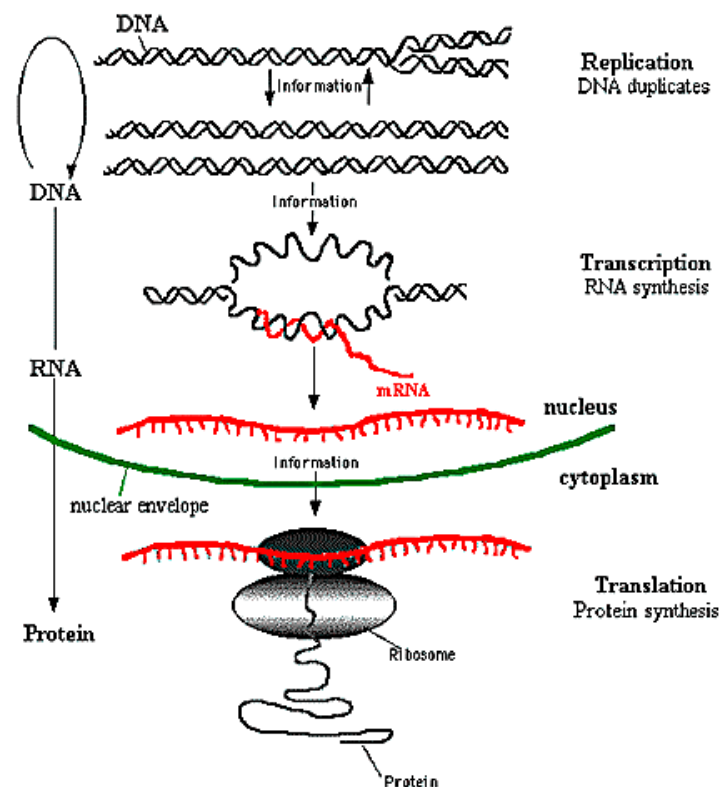


Fig. 1. The central dogma of molecular biology.

In this process called transcription in which the antisense (nontemplate) strand used as a

template for the synthesis of a messenger RNA (mRNA) strand that is complementary to the antisense strand but identical to the information in the sense (template) strand. Each gene gives rise to many identical mRNA molecules. The mRNA is then transported from the nucleus to the cytoplasm, where it is translated into the amino acid sequence of a protein in a process called translation. This unidirectional flow of genetic information transfer is sometimes called central dogma of molecular genetics⁽¹⁻³⁾ and this lead to the formation of protein which have important and inevitable roles in metabolism and anabolism. Gene expression can be regulated at many of the steps in the pathway from DNA to RNA to protein⁽⁴⁾ as shown in Fig. 2.

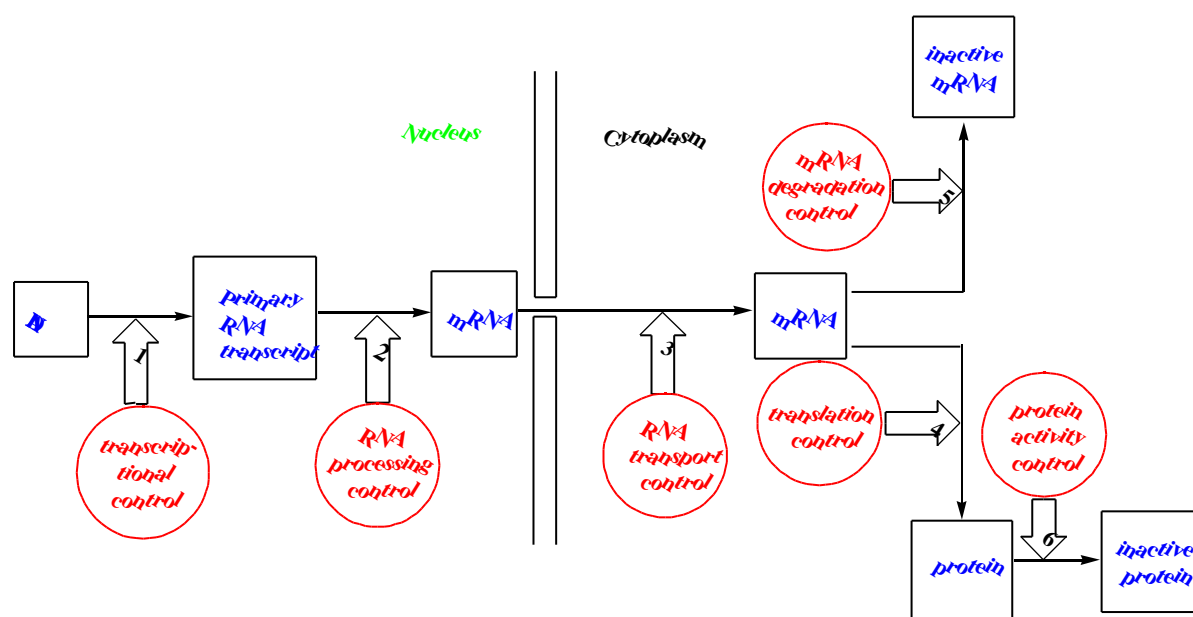


Fig. 2. Control of gene expression.

Damaged DNA and / or RNA caused by either oxidation, virus, cancer, mutation, and infection have received much attention of medicinal chemistry in order to repair the undesired side reactions (diseases) caused by these damage agents. The RNA component of ribosomes (rRNA) is essential for protein synthesis⁽⁵⁾ and therefore is an attractive target for antimicrobial drugs. Indeed, many natural antibiotics disrupt protein synthesis and most of these appear to act by binding rRNA.⁽⁶⁾ Several methods were developed affecting on protein synthesis all over its stages of synthesis including; antisense strategy (e.g. PNAs), intercalator (e.g. actinomycin D), alkylating agents of nucleobase (e.g. nitrosoureas) and inhibition of enzymes (e.g. topoisomerase I and II inhibitors), etc....especially in the antisense methodology, there is a potent ability for various genetic therapy because of the direct inhibition of abnormal nucleic acid sequences by antisense molecules with the complementary sequences. Peptide nucleic

acid (PNA) which is one of the artificial antisense molecules, is a DNA mimic that has shown considerable promise as a lead compound for developing gene therapeutic drugs. PNA is characterized by its ability to block DNA and RNA polymerases and ribosome progression when bound to DNA or RNA templates⁽⁷⁻⁹⁾ as well as its high potency to inhibit the activity of telomerase by binding to its RNA component.⁽¹⁰⁾ Antisense has been observed to bind to DNA and RNA and inhibit their functions. Some of them have been showed to inhibit cell growth of cancer and to give effect on cancer mouse and rat *in vivo*. A few of them has been applied clinically to cancer patients under phase II. When artificial antisense agents show no effect on such diseases, the next candidates come to the strategy of enzyme inhibitors. Inhibition of enzyme functions have been received much attention so far and many types of such drugs have been developed and explored to lead to various kinds of inhibitors which are commercially available. On the other hand, topoisomerases I and II are essential nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands. In addition to their normal cellular functions, both topoisomerase I and II enzymes have recently emerged as important cellular targets for chemical intervention in the development of antitumor drugs. Examples of these topoisomerase inhibitors are camptothecin, etoposide and doxorubicin. This year the human genome project has been sequenced and become more clear.⁽¹¹⁾ From these background we started our research which was focused on targeting at DNA, RNA and protein, and inhibit their functions. One is to inhibit the expression of DNA and RNA by artificial antisense, through developing of new PNA antisense. Another way is inhibition of enzymatic function by binding small molecules to them, through the preparation of galloyl derivatives as new topoisomerase inhibitors. By these two ways we can develop potent drugs which can inhibit the replication of DNA, transcription of DNA into RNA, translation of RNA and certain enzymes.

II. Development of Novel PNAs

2.1. Introduction

The antisense and antigene strategy for treatment of diseases at the level of gene expression has attracted wide attention in medicinal chemistry. On transcription, every gene gives rise to a relatively large number of copies of messenger ribonucleic acid (mRNA), which is translated into a large number of protein molecules. This is why inhibition of gene expression ought to be more efficient than inhibition of the resulting protein product. There are already a number of drugs on the market whose activity is based on direct interaction with deoxyribonucleic acid (DNA). Many of these compounds, which are mainly used for chemotherapy, intercalate or bind specifically only to DNA. On the other hand, it is possible to achieve sequence-specific recognition of nucleic acids using synthetic oligonucleotides that bind specifically by hydrogen bonding to complementary nucleic acids. These compounds are called antisense oligonucleotides based on their binding to the target sequence (sense strand). Zamecnik and Stephenson were the first to propose in 1978, the use of synthetic antisense oligonucleotides for therapeutic purposes.⁽¹²⁾ With a 13-mer oligonucleotide that was complementary to the RNA of Rous sarcoma virus they were able to inhibit the growth of this virus in cell culture. The specific inhibition is based on the specific Watson-Crick base pairing (Fig. 3) between the heterocyclic bases of the antisense oligonucleotide and of the viral nucleic acid. The process of binding of the oligonucleotides to a complementary nucleic acid is called hybridization.

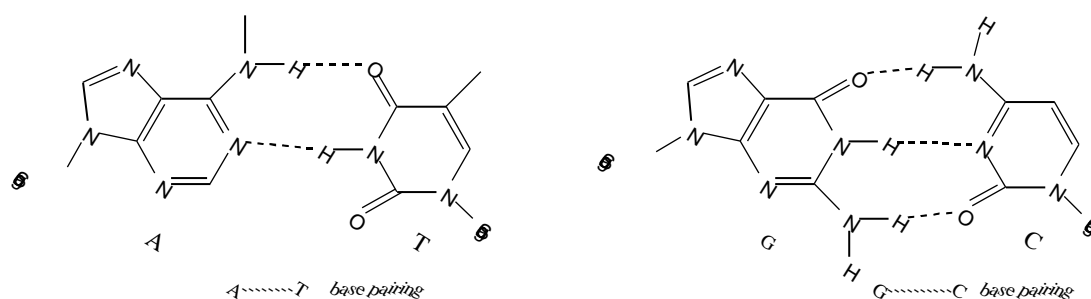


Fig. 3. Watson-Crick base pairing.

Various cellular processes can be inhibited depending on where the oligonucleotide hybridizes on single stranded regions of the DNA or mRNA. A simple model describes the inhibition of protein biosynthesis by an antisense oligonucleotide being bound to the mRNA (Fig. 4).

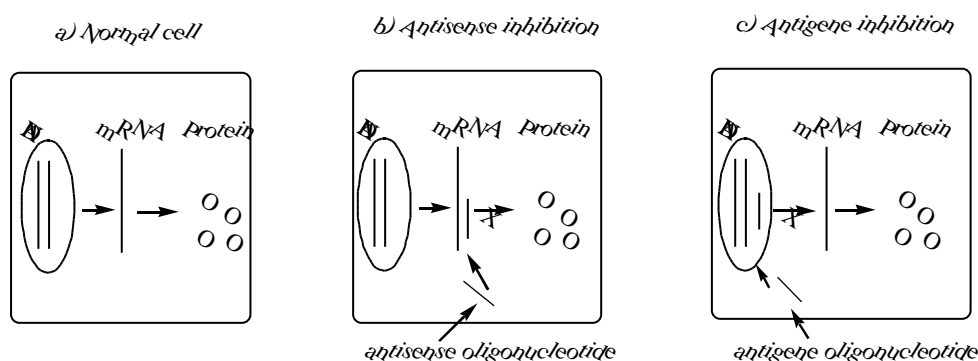


Fig. 4. Schematic illustration of (a) Normal gene expression. DNA is transcribed into mRNA followed by translation to give multiple copies of the protein gene product; (b) Antisense inhibition. An antisense oligonucleotide binds specifically to mRNA via Watson-Crick hydrogen bonding whereby it inhibits translation of mRNA into protein; (c) Antisense inhibition. Transcription is inhibited by the binding of an antisense oligonucleotide to DNA.

Several requirements needed for the antisense oligonucleotide to be able to inhibit translation including; (1) stability of the oligonucleotide toward extra- and intra- cellular enzymes, (2) specific interaction between the oligonucleotide and its target sequences, (3) stability of the complex formed between the oligonucleotides and its complementary target sequences under physiological conditions and (4) the ability to penetrate through the cell membrane.

Once it has reached the cytoplasm, it must bind specifically and with sufficient affinity to the target mRNA to inhibit its translation into the corresponding protein. Natural oligonucleotides have been shown to exhibit both antisense and antisense properties in vitro.⁽¹³⁻¹⁵⁾ However, both DNA and RNA are rapidly degraded by nucleases in vivo and in attempts to overcome this serious obstacle an impressive number of oligonucleotide analogues have been synthesized during the last two decades including; backbone modification⁽¹⁶⁻²⁸⁾, base modification⁽²⁹⁻³⁴⁾ and sugar modification.⁽³⁵⁻⁴⁰⁾ Chemical modifications generally improve the biological stability of oligonucleotides to nucleases and improve the passage through membranes. On the other hand, these chemical modifications have an adverse effects on the specificity and binding affinity.

Three practical problems block the application of a modified oligonucleotide as a therapeutic agent:-

- 1) Poor uptake into cells.
- 2) Digestion by cell nucleases.
- 3) Short life time of the duplex formed.

The recent invention of peptide nucleic acids, PNAs⁽⁴¹⁾, as strong and specific DNA and RNA binding agents has attracted much attention because of the ease and simplicity of the scale-up methods for peptide chemistry, in comparison with the synthetic procedures used for other modified sugar-phosphate backbones. The N-

aminoethylglycine backbone of the polyamide *aegPNA* (Fig. 5), carrying nucleobases through an acetyl linker, was designed from molecular modeling studies as the result of atom-by-atom replacement of the sugar-phosphate DNA backbone by a polyamide backbone.⁽⁴²⁾ PNAs act as excellent structural mimics of DNA/RNA and exhibit strong sequence specific binding with complementary oligonucleotide sequences following Watson-Crick base pairing rules, and thus fulfill the primary condition for their biological application. The relatively high binding affinity of PNAs towards the natural oligonucleotides is attributed to the lack of the electrostatic repulsion between the uncharged PNA backbone and the negatively charged sugar-phosphate backbone of the DNA and RNA⁽⁴²⁾ and the rigidity of the peptide backbone of PNA which arises from the SP^2 hybridization as well as the presence of tertiary amide linkage.

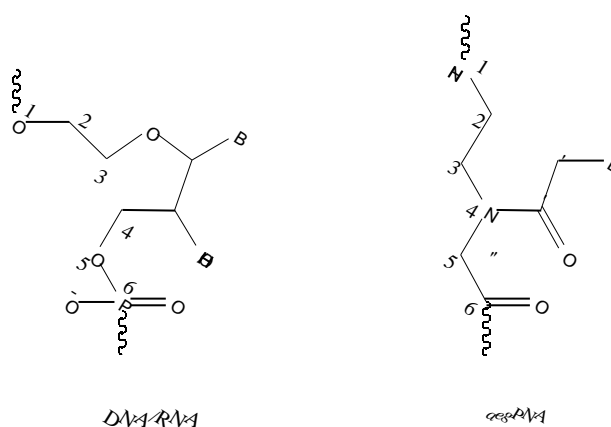


Fig. 5. Sugar-phosphate DNA/RNA and aminoethylglycyl PNA backbones.

The main drawbacks of *aegPNA* are; the relatively poor water solubility⁽⁴³⁾, poor cellular uptake of PNAs⁽⁴⁴⁾, and that the mixed purine/pyrimidine PNA oligomers bind to both parallel and antiparallel DNA target sequences with almost equal ability^(45,46), but with the antiparallel binding mode slightly preferred over the parallel mode (the C-terminus of the PNA is conventionally taken to be analogous to the 3' end of DNA/RNA). PNA oligomers bind strongly with highest sequence discrimination to complementary oligomers of DNA, RNA or PNA itself, and in general the hybrid thermal stability (T_m) for identical sequences in the following order; PNA-PNA > PNA-RNA > PNA-DNA (> RNA-DNA) > DNA-DNA.

2.1.1. Strategies for chemical modification of *aegPNA*

Various attempts for the modifications/substitutions in the PNA backbone, in the aminoethyl, glycyl or the acetyl segment carrying the nucleobase have been made, results in chiral PNA and is aimed to achieve selective binding with target DNA/RNA.⁽⁴⁷⁾ PNAs are suffixed with negatively charged DNA or positively charged polypeptide sequences at either the C- or the N-terminus. This modification results in PNA-DNA⁽⁴⁴⁾ or PNA-peptide chimeras⁽⁴⁸⁾ that have favorable aqueous solubility, cellular uptake and DNA binding/recognition properties. The PNA sequences have also

been appended with polyamines⁽⁴⁹⁾ to increase the aqueous solubility and improve binding to the target DNA. Some of these modified PNAs show improved water solubility, cellular uptake and/or directional binding selectivity relative to pure *aeg*PNAs, but in most cases exhibit a reduced binding towards the target sequences.

2.1.2. Construction of bridged PNA structures

The reduced entropy loss upon complex formation, can be achieved if the conformational freedom in *aeg*PNA is constrained by bridging the aminoethyl or glyceryl acetyl linker arms (Fig. 6) to give rise to cyclic analogues with pre-organized structures without affecting the nucleobase recognition ability through hydrogen bonding. Additionally, the introduction of chemical bridges into *aeg*PNA to provide cyclic structures may help in controlling the rotameric populations by fixing the nucleobase orientation and also in directional selective binding by virtue of the chirality in the backbone. Such a structural pre-organization approach using additional conformational constraint has been extremely successful in the case of DNA analogues. Prominent examples are conformationally locked nucleic acids⁽⁵⁰⁾ or conformationally frozen hexitol⁽⁵¹⁾ and altritol nucleic acids⁽⁵²⁾ (Fig. 7), which have pre-organized 3'-endo sugar conformations as prevalent in highly stable DNA-RNA duplexes.

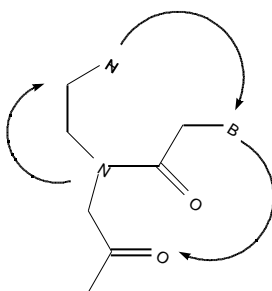


Fig. 6. Possible positions for introduction of methylene or ethylene bridges.

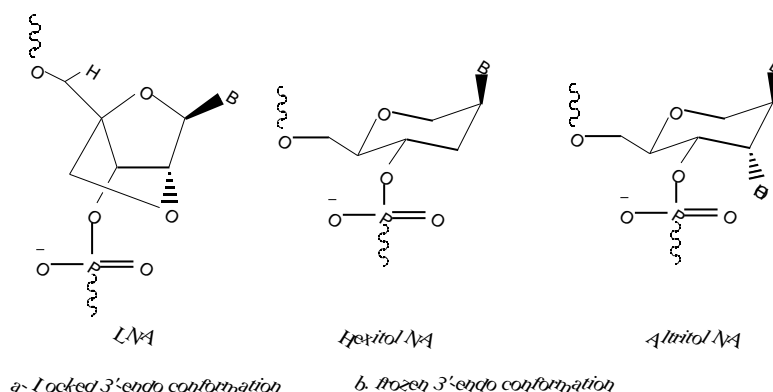


Fig. 7. a) Locked 3'-endo conformation in LNA; b) Frozen 3'-endo conformation in hexitol and altritol nucleic acids.

2.1.3. PNA with five-membered nitrogen heterocycles

Many researchers have exploited *trans*-4-hydroxy-L-proline for the synthesis of wide variety of chiral, constrained and structurally pre-organized PNAs (Fig. 8). Depending

on the construction strategy and the presence or absence of tertiary amine group in the monomers, the modifications afford either positively charged or uncharged cyclic PNA analogues.

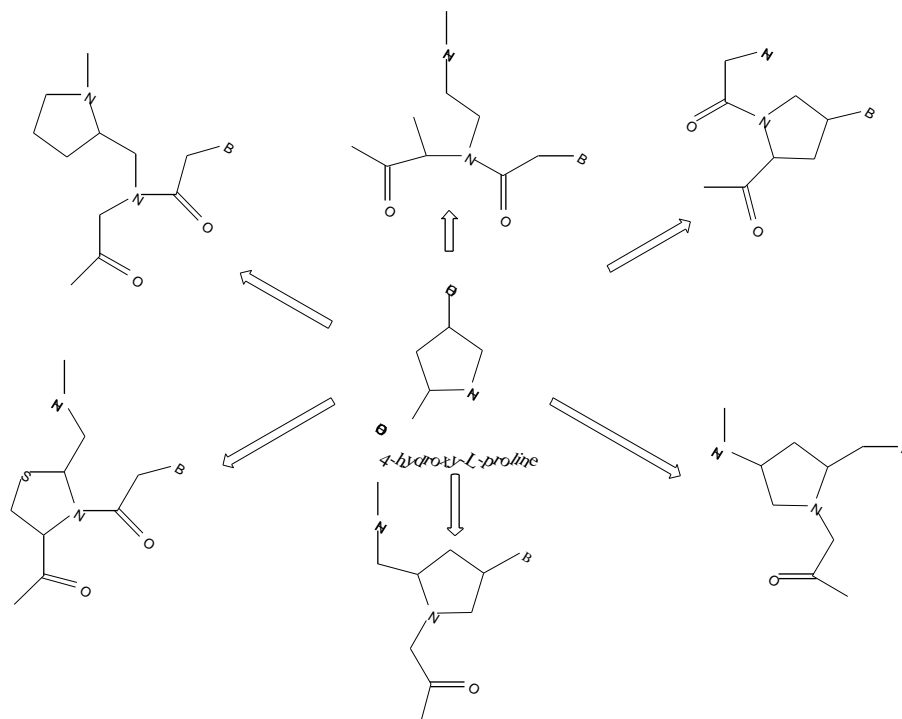


Fig. 8. PNA analogues with five-membered pyrrolidine rings.

2.1.3.1. Aminopropyl PNA

The introduction of a methylene bridge between the α -carbon atom of the aminoethyl segment and the γ -carbon atom of the glycine segment of the *aeg*PNA resulted in 4-aminopropyl PNA, with the introduction of two chiral centers (Fig. 9).⁽⁵³⁾ The incorporation of single chiral D-trans- and L-trans-prolyl PNA monomeric units in the PNA oligomers at the N-terminus resulted in discrimination in parallel/antiparallel binding orientation preferences towards the target DNA sequences. A backbone combining *aeg*PNA alternating with L-trans-4-aminopropyl PNA was later shown to bind to the target sequences with higher affinity than the pure *aeg*PNA oligomers.⁽⁵⁴⁾

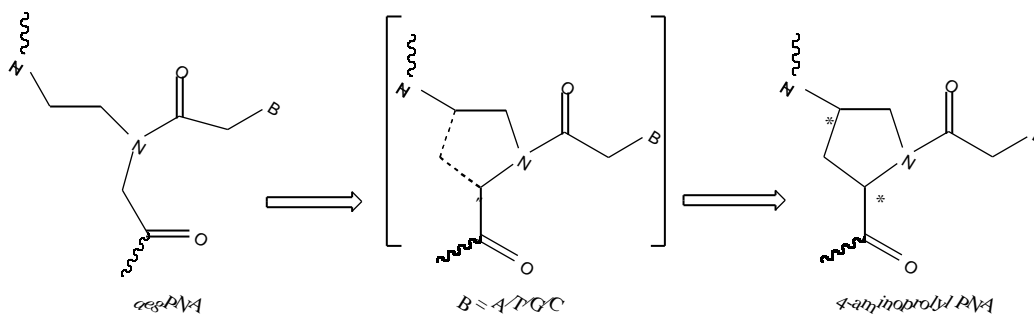


Fig. 9. Prolyl PNA with α - γ -methylene bridge.

2.1.3.2. Gly-pro-peptide PNA

4-Hydroxyproline was used by Lowe et al. for the synthesis of a novel chiral prolylglycyl PNA. The methylene bridge was inserted between the γ -carbon atom of the glycine unit and the α -carbon atom of the nucleobase linker of *aegPNA* (Fig. 10).⁽⁵⁵⁻⁵⁷⁾ The tertiary amide bond in the backbone between proline and glycine units replaced the aminoethylglycyl backbone. The backbone thus comprised alternate glycine-proline units, the nucleobase was directly attached to the proline ring by alkylation through the 4-hydroxy function. The oligomer with such a backbone did not bind to the target sequences and this may be due to the rigidity of the system which may lead to disfavored recognition for the target sequences. The sequences with *aegPNA* alternating with the proline-glycine PNA unit showed reduced binding to the target sequences, unlike the 4-aminoproline PNA.⁽⁵⁴⁾

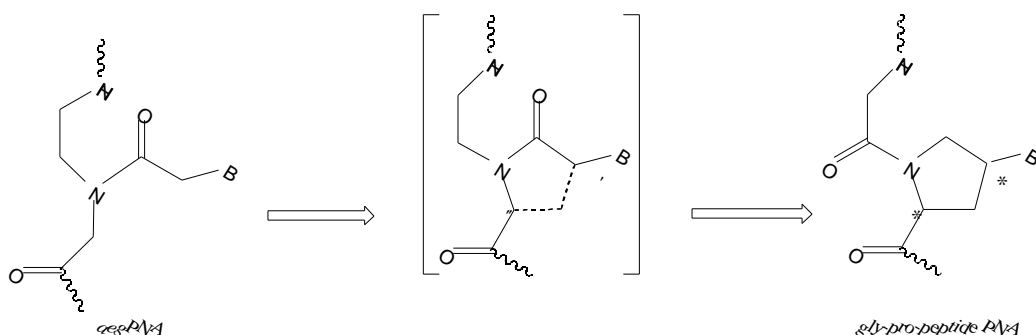


Fig. 10. Prolylglycyl PNA with a γ - α -methylene bridge.

2.1.3.3. Aminoethylprolyl PNA, *aepPNA*

In this type of PNA the γ -carbon atom of the glycine unit and the α -carbon atom of the nucleobase linker were joined through a methylene bridge (Fig. 11).⁽⁵⁸⁾ The flexibility in the aminoethyl segment of *aegPNA* was retained, unlike that in the proline-glycine PNA. The nucleobase attachment to the pyrrolidine ring was fixed by virtue of the chirality of C-4, thus removing the possibility of any rotameric populations. The oligomers comprising 4-(S)-2-(S/R) *aepPNA* thymine units showed very favorable binding properties towards the target sequences without compromising the specificity.

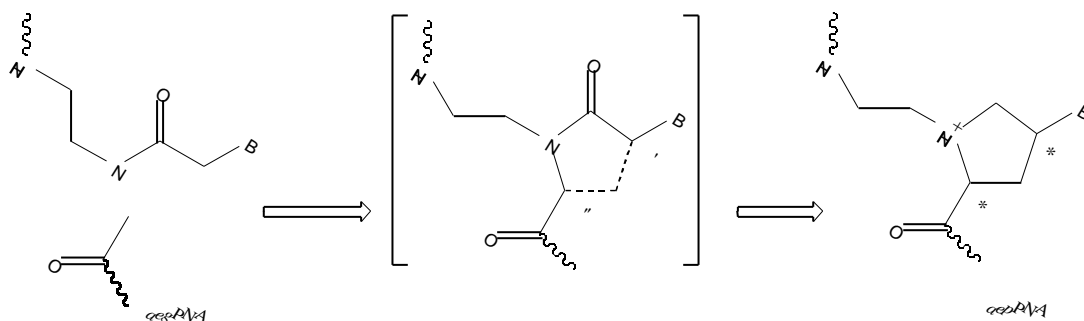


Fig. 11. Aminoethylprolyl PNA with a γ - α -methylene bridge.

2.1.3.4. Pyrrolidinone PNA

Another conformationally restricted cyclic PNA analogue was derived from a pyrrolidinone ring system.⁽⁵⁹⁾ A methylene bridge was inserted between the β -carbon atom of the aminoethyl segment and the α' -carbon atom of the acetyl linker to the nucleobase of *aeg*PNA (Fig. 12). The carbonyl group of the nucleobase linker was retained and was forced to point towards the carboxy terminus of the backbone. The bridge prevented rotation around the C-N bond of the acetyl segment connecting the nucleobase residue to the backbone, and pre-organized PNA in a rotameric conformation prevailing in complexes of PNA with nucleic acids.⁽⁶⁰⁾ The oligomers incorporating the (3*S*,5*R*) isomer carrying adenine as a base were shown to have highest affinity towards RNA in comparison with DNA.

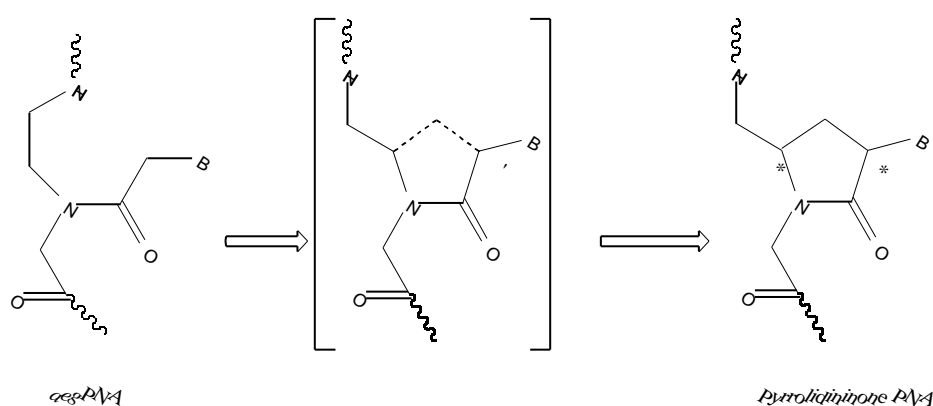


Fig. 12. Pyrrolidinone PNA with β - α' -methylene bridge.

2.1.3.5. Pyrrolidine PNA-II with β - α' -methylene bridge

Introduction of a β - α' -methylene bridge, resulting in another pyrrolidine based PNA modification (Fig. 13).⁽⁶¹⁾ This should probably ease the rigidity of the direct attachment of the nucleobase to the pyrrolidine ring, as in the case of the β - α' -methylene-bridged pyrrolidine PNA-I or *aep*PNA. Incorporation of (2*R*,4*S*) pyrrolidine PNA monomer in the center of the *aeg*PNA T₈ sequence, was able to bind to the target DNA better than pure *aeg*PNA, while incorporation of the other isomer (2*S*,4*S*) was found to make destabilization with DNA.

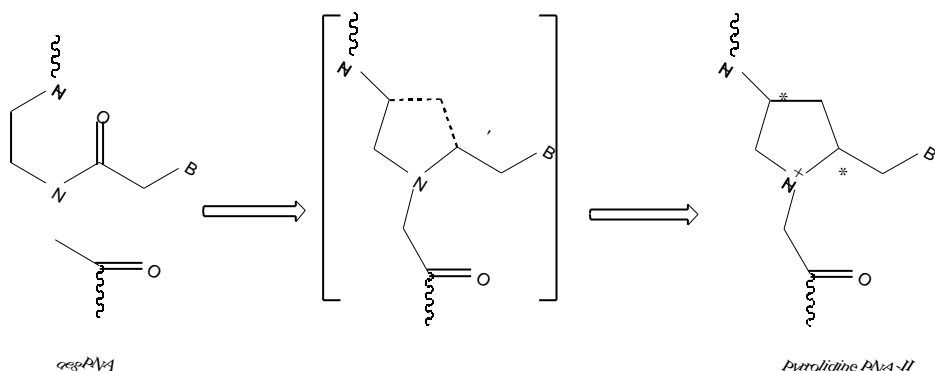


Fig. 13. Pyrrolidine PNA-II with β - α' -methylene bridge.

2.1.3.6. Aminomethylthiazolidine PNA, *amtPNA*

A thiomethylene bridge was inserted between the α -carbon atom in the aminoethyl segment and the β -carbon atom of the glycine segment of the *aegPNA*, providing aminomethylthiazolidine PNA, *amtPNA*.⁽⁶²⁾ The *amtPNA* thus constructed restricts movement in both the aminoethyl and the glycyI segments of the *aegPNA* (Fig. 14). The *syn* and *anti* isomers of the *amtPNA* were incorporated into *aegPNA* sequences in the central position, the triplex formed with this PNA with both DNA and RNA were significantly destabilized.

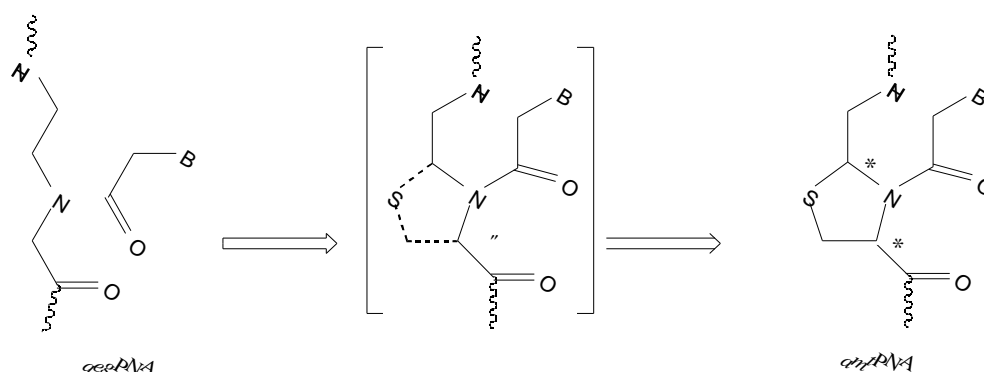


Fig. 14. Aminomethylthiazolidine PNA, *amtPNA*.

2.1.4. PNA with six-membered ring structures

2.1.4.1 Glucosamine nucleic acids, GNAs

The binding affinities and selectivities of the oligomers containing GNAs indicated selective recognition of RNA, the entropy changes were found to be smaller for GNA-DNA/RNA than for DNA-DNA/RNA⁽⁶³⁾ (Fig. 15).

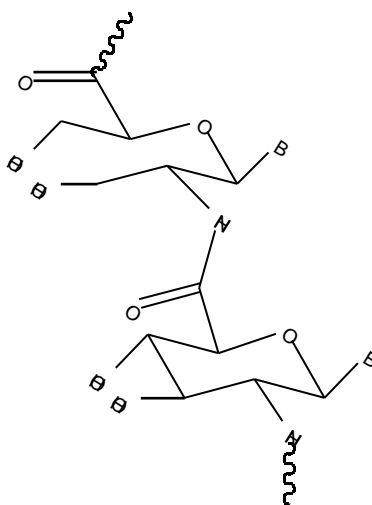


Fig. 15. A six-membered cyclic PNA derived from glucosamine.

2.1.4.2. Piperidinone PNA

Introduction of an ethylene bridge between the α - and γ -carbon atoms in the ethylenediamine and acetyl linkers resulting in a six-membered ring structure as in piperidinone PNA (Fig. 16).⁽⁶⁴⁾ Incorporation of these monomers containing adenine nucleic acid base into the *aeg*PNA resulted in a large decrease in the duplex stability.

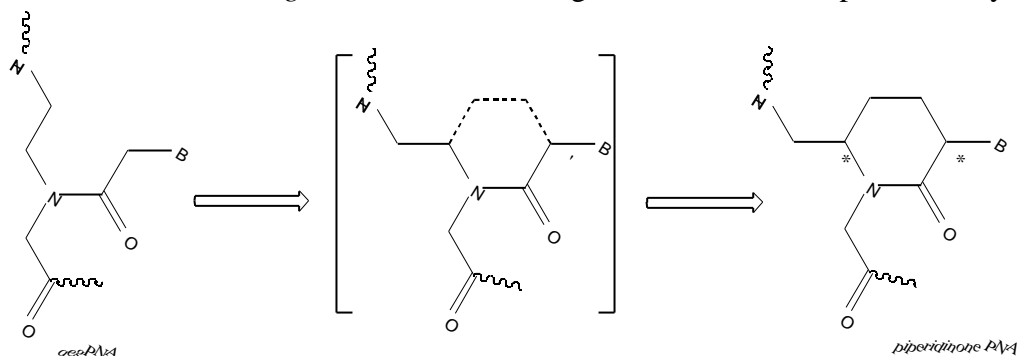


Fig. 16. Piperidinone PNA with α - γ -methylene bridge.

2.1.4.3. Morpholino PNA

The attractive morpholino antisense oligomers⁽⁶⁵⁾, with high sequence specificity, water solubility and low production cost, have sparked research into further modifications in morpholino-based DNA analogues. Set of morpholino analogues with phosphonate ester, amide or ester linkages between the morpholino nucleoside residues was synthesized (Fig. 17).⁽⁶⁶⁾ Preliminary evaluation indicated that the uridine homo- oligomer with either amide or ester linkage stabilized the complex with the DNA targets relative to the DNA-DNA complexes. The adenine homo-oligomers showed destabilizing effects when complexed with the target homo-thymidine DNA sequences. The amide-linked morpholino PNAs were better accommodated in the complexes than the ester- or the phosphonate-linked oligomers.

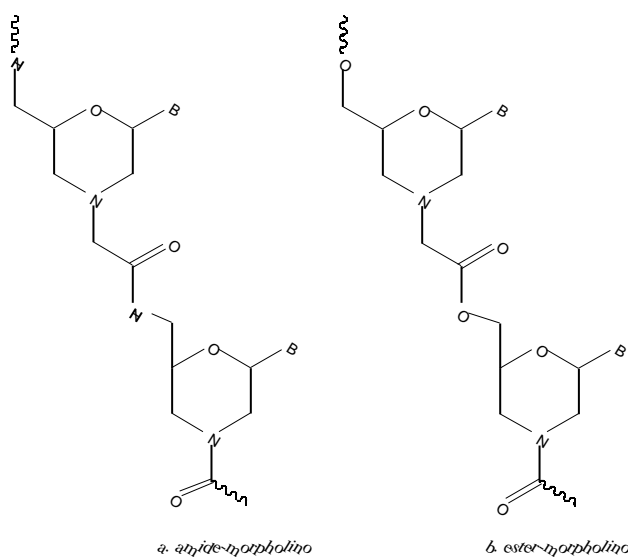


Fig. 17. PNA with morpholino amide and ester backbones.

2.1.5. APNA and PNA-APNA chimera

An aromatic PNA analogue in which the backbone *o*-aminophenylbutanoic acid derivative carries the nucleobase through an ether linkage has been synthesized (Fig. 18).⁽⁶⁷⁾ These analogues were designed in order to investigate possible stacking interactions and their stabilizing effects on duplex-triplex structures.⁽⁶⁸⁾

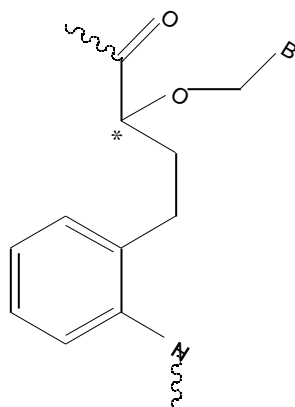


Fig. 18. Aromatic peptide nucleic acid, APNA-I.

The aromatic PNA was subjected to further modification by replacing it with another aromatic *N*-(2-aminobenzyl)glycine backbone (Fig. 19).⁽⁶⁹⁾ The optimum distance of six bonds between the nucleobases along the backbone was extended to seven bonds in this modification. The direct incorporation of aromatic rings in the backbone renders at least three bonds in the backbone coplanar. Furthermore, the internucleobase distance was altered by additional methyl substitution in the glycyl segment, affording *N*-(2-aminobenzyl)-(*R*-or *S*)-alanine or by replacing it with α -alanine, as in *N*-(2-aminobenzyl)- α -alanine backbones. The incorporation of these modified units in *aeg*PNA produced PNA-APNA chimera. The modified oligomers exhibited decreased binding affinities relative to the pure PNA.

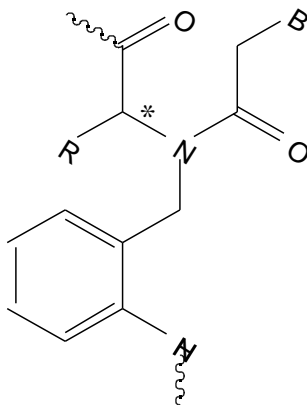


Fig. 19. APNA-II-PNA chimera.

2.2. Scope of Investigation

A large variety of structural modifications of oligonucleotides have been investigated as an exciting new concept for drug design to modulate the expression of genetic information by antisense and antigene oligonucleotide. Aminoethylglycine peptide nucleic acids (*aeg*PNAs) developed by Nielsen and co-workers are oligonucleotide analogues in which the sugar phosphate backbone is replaced by a peptide chain.^(41,43,45,70a-c) PNA is a very potent DNA mimic capable of hybridization to complementary DNA, RNA or PNA itself. Therefore many types of nucleotides with peptide backbone have been synthesized in order to improve the limited solubility and to investigate the structural flexibility and requirements for nucleic acid recognition (e.g., introduction of a certain side chain^(60a-c) and double bond⁽⁷²⁾ to peptide backbones, conversion of carbonyl group to sulfonyl group⁽⁷³⁾, and construction of ethereal and /or cyclic structural peptide backbones).^(58,74a-c) However, there is no report on the peptide nucleic acids corresponding to the 'non-genetic' 2',5'-linked oligonucleotide (2',5'-isoDNA)⁽⁷⁵⁾, which is one of the important candidates to search the effective antisense oligonucleotides due to the intrinsic selective RNA-binding activity of 2',5'-isoDNA (although the hybridization affinity of 2',5'-DNA or -RNA/ 3',5'-RNA is somewhat inferior to that of the 3',5'-DNA/ 3',5'-RNA duplex, despite 2',5'-DNA or -RNA shows binding selectivity for 3',5'-RNA over 3',5'-ssDNA) (Fig. 20). The favorable RNA- and DNA- binding properties of PNA and the selective binding affinity of 2',5'-isoDNA to RNA have led us to design 2',5'-isoDNA mimic chiral PNAs (isoPNAs) in order to develop novel PNAs with higher affinity to complementary RNA sequences. An antisense strategy in which the antisense molecules binding strongly and more selectively to RNA seems to be one of the most effective gene targeting therapy. This is attributed to that the selective binding to a specific region in the RNA sequences can alter several characters in the structure of RNA such as loop and turn forms of the RNA.

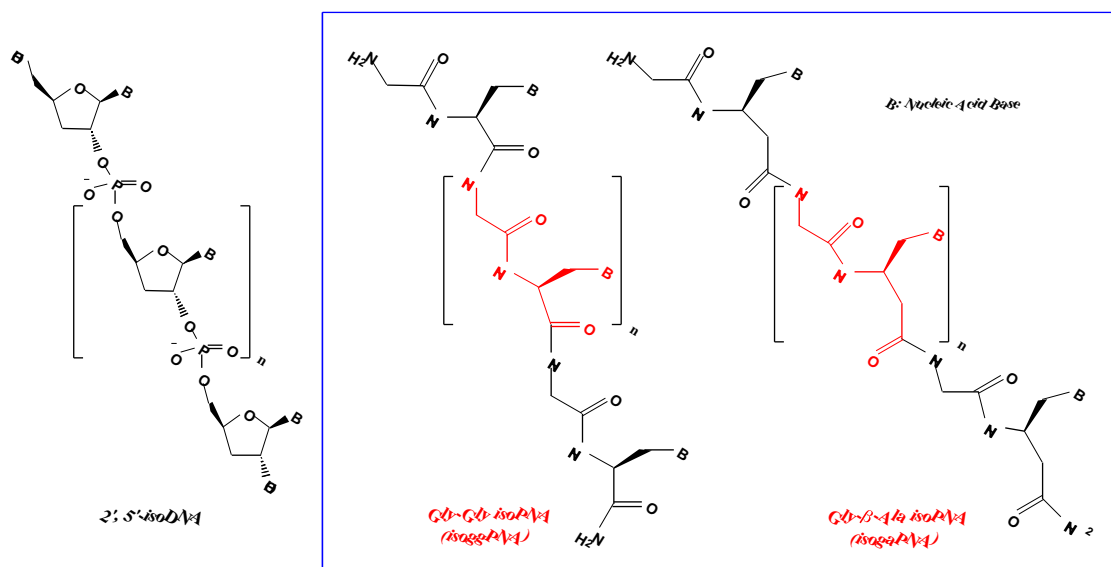


Fig. 20. Design of 2',5'-isoDNA mimic peptide nucleic acids.

The sugar phosphate backbone of 2', 5'-isoDNA consists of a repeating unit of seven atoms, configurationally and conformationally constrained by the 3'-deoxy-D-ribose ring. If these seven atoms could be replaced by an isostructural dipeptide unit that consists of repeating of six atoms glycyl-glycine isoPNA (isoggPNA) or seven atoms glycyl- -alanine isoPNA (isogaPNA) (Fig. 20), the new backbone would be amenable for preparation by automated peptide synthesizer and this may lead to structural analogues of isoDNA. In this context, we examined the synthesis and hybridization properties of PNAs containing novel isogaPNA monomers with glycyl- -alanine backbone derived from D and L-aspartic acid, together with the synthesis of isoggPNA monomer with glycyl-glycine backbone derived from L-serine (Fig. 21).

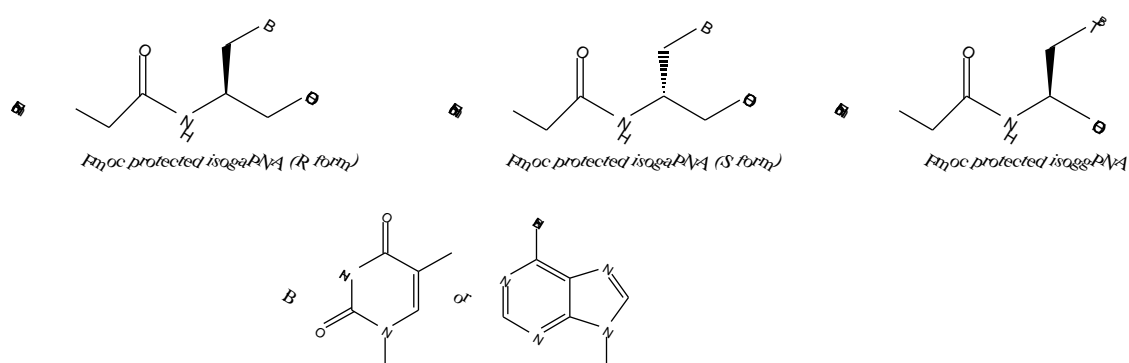


Fig. 21. Various isoPNA monomers.

We also studied on the strategy to antiviral agents from some derivatives of PNA monomers, which are potent isosters of natural nucleoside, in our effort of developing new PNAs (Fig. 22).

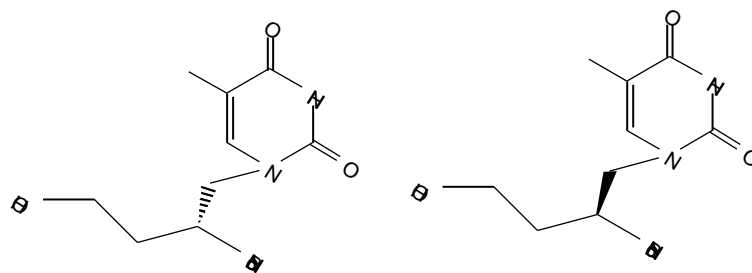


Fig. 22. Structures of some derivatives of isogaPNA tested as antiviral agents.

2.3. Results and Discussion

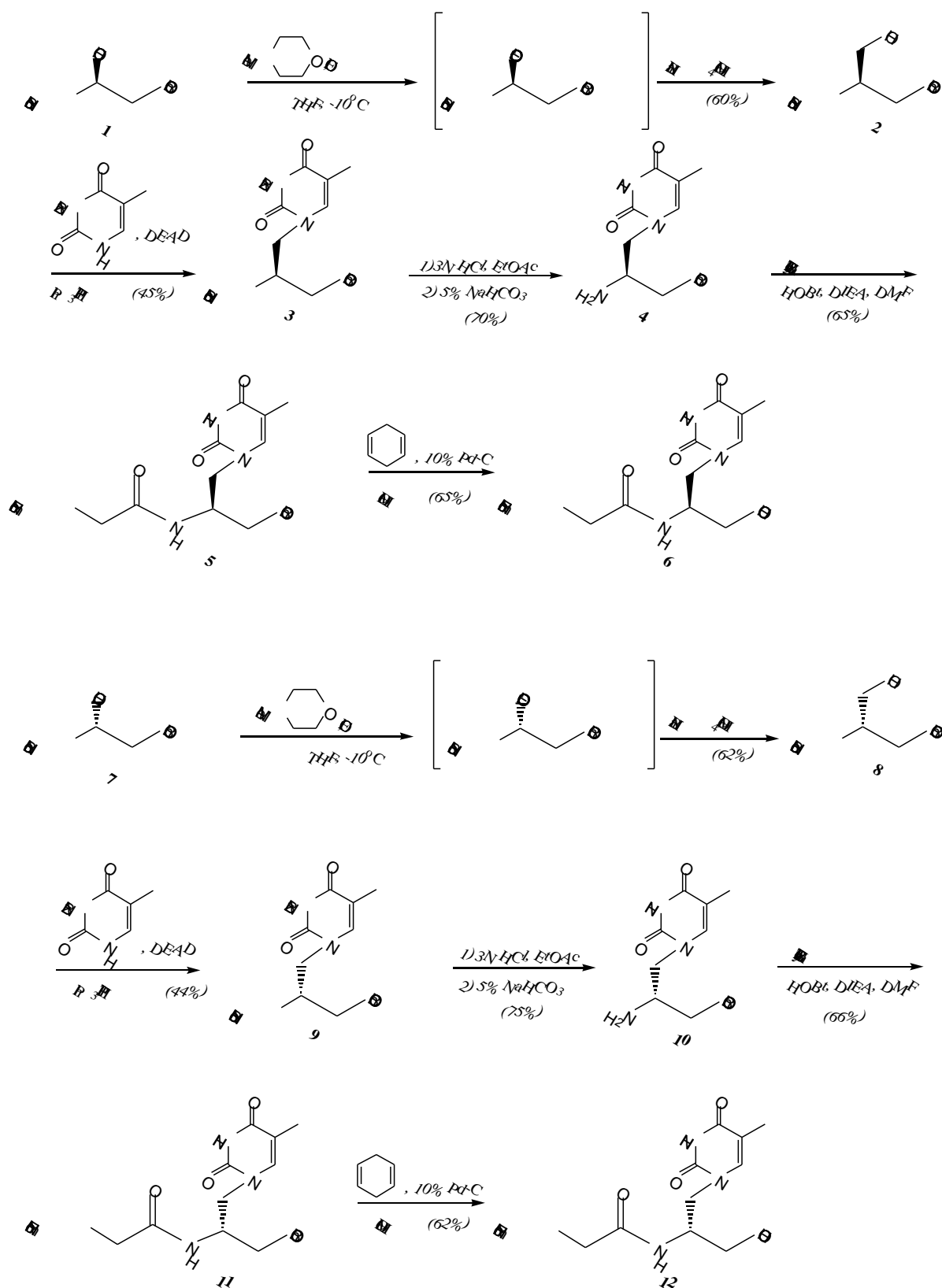
2.3.1. Synthesis of glycyl- β -alanine isoPNA (isogaPNA) monomers

The synthesis of glycyl- β -alanine isoPNA (isogaPNA) monomers proceeds through D- or L-aspartic acids protected with Boc and benzyl groups.

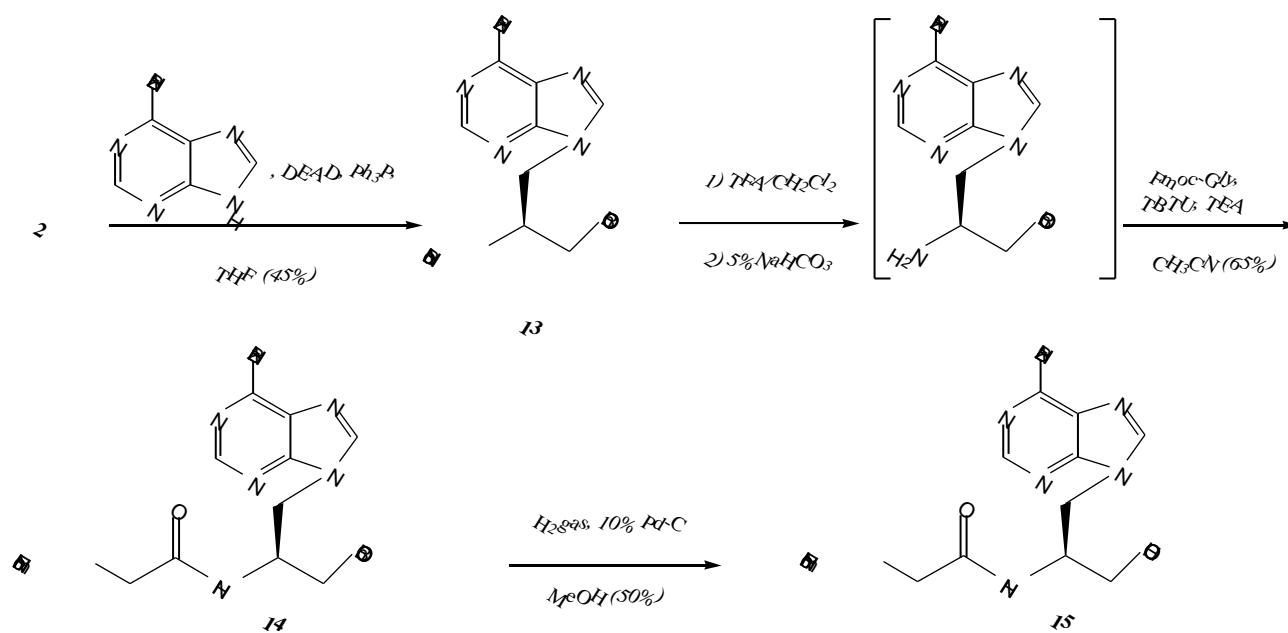
Thymine monomers were prepared as follows (Scheme 1). Compounds **1** and **7** could be reduced in a rapid and convenient method under mild conditions to its corresponding alcohols **2** and **8**.⁽⁷⁶⁾ Treatment of compounds **1** and **7** with equimolar amounts from *N*-methylmorpholine and ethylchloroformate in dry THF at -10°C for 5-10 minutes under argon gas gave the corresponding mixed anhydrides as intermediate. Treatment of these mixed anhydrides with sodium borohydride in MeOH at 0°C for 10 minutes followed by another 10 minutes at room temperature gave the corresponding alcohols **2** and **8** in 60% and 62% yields, respectively. Coupling between alcohols **2** and **8** with *N*³-benzoylthymine under Mitsunobu condition^(77,78) in the presence of *N,N*-diethyl azodicarboxylate and triphenylphosphine in dry THF under argon gas at 0°C for 1 hour followed by overnight stirring at room temperature afforded compounds **3** and **9** in 45% and 44% yields, respectively. Treatment of compounds **3** and **9** with 3N HCl in EtOAc at room temperature for 2 hours⁽⁷⁹⁾ afforded the HCl salt of compounds **4** and **10**, respectively. In this reaction removal of both Boc and benzoyl groups was observed, neutralization of the HCl salt with 5% NaHCO_3 gave compounds **4** and **10** in 70% and 75% yields, respectively. Coupling between compounds **4** and **10** with equimolar equivalent from Fmoc-glycine, in the presence of TBTU, HOBt and DIEA in DMF afforded compounds **5** and **11** in 65% and 66% yields, respectively. Finally deprotection of the benzyl group of compounds **5** and **11** was carried out using 1,4-cyclohexadiene and 10% Pd/C in MeOH successfully gave Fmoc-glycyl- β -alanine(thymine) PNA monomers (R and S form) **6** and **12** in 65% and 62% yields, respectively (Scheme 1).⁽⁸⁰⁾

Adenine monomer was also prepared in a similar method for the preparation of thymine monomer **6** (Scheme 2). Coupling of the alcohol **2** with *N*⁶-benzoyladenine under Mitsunobu condition in the presence of *N,N*-diethyl azodicarboxylate and triphenylphosphine in dry THF under argon gas at 0°C for 1 hour followed by overnight stirring at room temperature afforded compound **13** in 45% yield.⁽⁷⁷⁾ Removal of the Boc moiety from compound **13** was carried out by stirring with TFA/ CH_2Cl_2 (1:1) at 0°C for 1 hour followed by stirring at room temperature for 6 hours.⁽⁸¹⁾ Neutralization of the TFA salt with 5% NaHCO_3 gave a crude compound which was used directly for the next step without purification. Coupling between this crude compound and Fmoc-glycine in the presence of TBTU and TEA in CH_3CN afforded compound **14** in 65% yield.⁽⁸²⁾ Finally debenzylation of compound **14** was carried out using hydrogen gas and 10% Pd/C provided the Fmoc-glycyl- β -alanine(adenine^{Bz}) PNA monomer **15** in 50% yield (Scheme 2).⁽⁸³⁾

All of the prepared compounds were identified by their melting points, IR, ^1H -NMR, ^{13}C -NMR, mass spectrum, elemental analysis and angle of optical rotation.



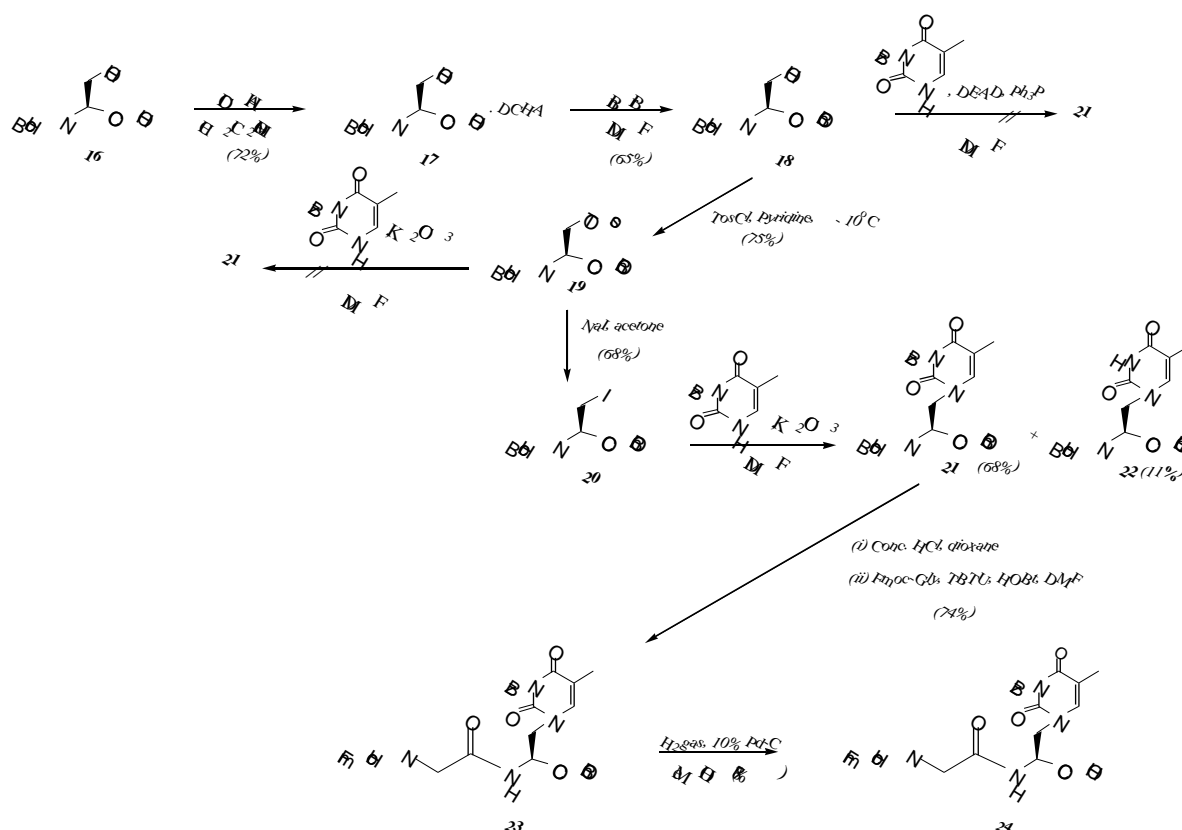
Scheme 1. Synthesis of thymine isogaPNA monomers (R and S).



Scheme 2. Synthesis of adenine^{Bz} isogaPNA monomer.

2.3.2. Synthesis of glycyl-glycine isoPNA (isoggPNA) monomer

The synthesis of thymine isoggPNA monomer **24** is the key step for the preparation of thymine isoggPNA monomer **24**. Attempts for the coupling of L-serine with N³-benzoylthymine⁽⁸⁴⁾ under the Mitsunobu conditions, previously applied to the coupling between hydroxymethylalanine and N³-benzoylthymine in the preparation of isogaPNA monomers, were proven to be unsuccessful (the starting material N³-benzoylthymine was recovered). The coupling using the tosylated compound **19**, prepared by the reaction of **18** with *p*-toluenesulfonyl chloride in pyridine⁽⁸⁵⁾ and N³-benzoylthymine under basic conditions but unfortunately, did not proceed well (tosylation of Boc-ser methyl ester underwent facile elimination at the tosylated position to give methyl acrylate compound). To this extent, compound **19** was converted to the corresponding iodide **20** using sodium iodide in dry acetone.⁽⁸⁵⁾ The iodide compound **20** was further reacted with N³-benzoylthymine under basic conditions, afforded compounds **21** and **22** in 68 % and 11 % yields successfully after flash silica gel chromatography and subsequent recrystallization. Removal of the Boc group of **21** using conc. HCl in dioxane⁽⁸⁶⁾ followed by coupling with Fmoc-glycine in the presence of TBTU gave compound **23** in 74% yield. Finally reductive cleavage of the benzyl ester of **23** was carried out using H₂ gas and 10% Pd-C gave thymine isoggPNA monomer **24** in 87% yield (Scheme 3).⁽⁸³⁾



Scheme 3. Synthesis of serine PNA monomer.

2.3.3. Solid-phase synthesis of thymine isogaPNA oligomers

In order to assess the hybridization properties of thymine isogaPNA monomers **6** and **12** to complementary DNA and RNA, the two monomers were individually introduced into *aeg*PNA sequences in different positions to form various thymine dodecamers, in addition to another two thymine dodecamers that consist only from complete isogaPNA monomers **6** and **12** (Table 1). The preparation of these oligomers was carried out using automated synthesis on an expedite 8909 (Applied Biosystems, Foster city, CA, USA) (Fig. 23) which can offer a routine method for producing PNAs on relatively small (2 μ M) scale and standard Fmoc chemistry on Fmoc-XAL PEG-PS resin. L-Lysine was introduced at the C-terminus of the PNAs to reduce their self aggregation and to ensure adequate water solubility.⁽⁴¹⁾ The monomers were coupled by activation with HATU in DMF/NMP (1/1). After the final coupling, Fmoc-group was removed by 20% piperidine in DMF and cleavage of the PNAs from the resin was performed using TFA/*m*-cresol (4/1).

Table 1. Various oligomers of *aeg*PNA and isogaPNA thymine monomers.

PNA sequences	
25	H-T₁₂-Lys-NH₂
26	DNA T₁₂
27	H-T₁₁-T_R-Lys-NH₂
28	H-T_R-T₁₁-Lys-NH₂
29	H-T₉-T_RT_RT_R-Lys-NH₂
30	H-T_RT_RT_R-T₉-Lys-NH₂
31	H-T₆-T_R-T₅-Lys-NH₂
32	H-T_{RI2}-Lys-NH₂
33	H-T₁₁-T_S-Lys-NH₂
34	H-T_S-T₁₁-Lys-NH₂
35	H-T₉T_ST_ST_S-Lys-NH₂
36	H-T_ST_ST_S-T₉-Lys-NH₂
37	H-T₆T_S-T₅-Lys-NH₂
38	H-T_{SI2}-Lys-NH₂

T = thymine *aeg*PNA monomer; T_R = thymine isogaPNA monomer (**6**); T_S = thymine isogaPNA monomer (**15**).

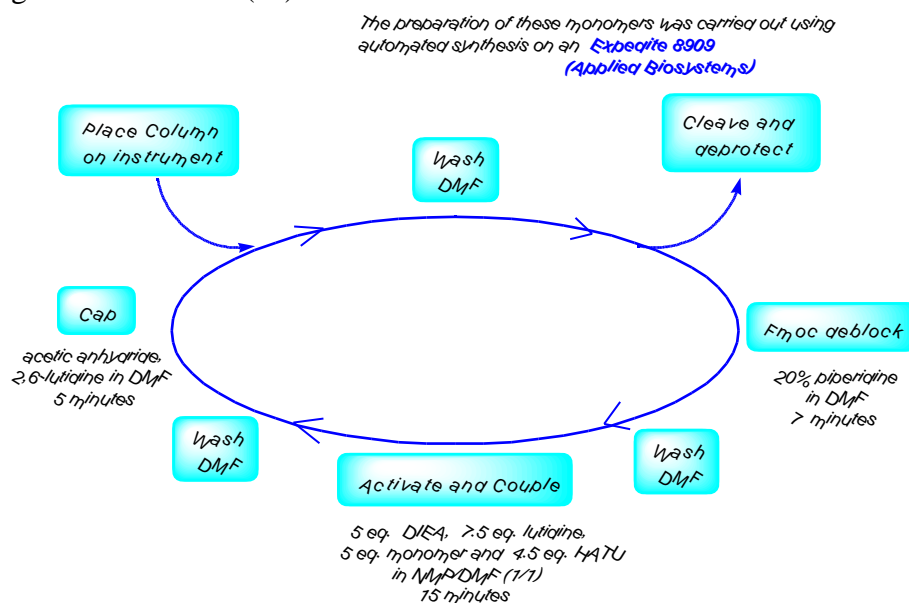


Fig. 23. PNA synthesis cycle.

The crude products were purified by C18-reverse-phase HPLC using gradients of 0.1% TFA in acetonitrile. The purity of the oligomers was rechecked by HPLC and characterized by Mass spectral analysis (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) (MALDI-TOF)⁽⁸⁷⁾, which gave a signal for the expected molecular weight (3338.90 (observed) versus 3338.34 (calculated)) (Fig. 24).

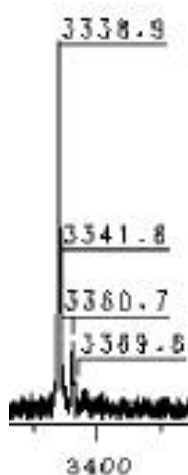


Fig. 24. MALDI-TOF mass analysis of PNA oligomers.

2.3.4. Hybridization property of thymine isogaPNA oligomers

The hybridization properties of PNA oligomers **27-38** with its complementary DNA and RNA were investigated by UV thermal melting experiments (T_m) at 260 nm and the

T_m results of these oligomers was compared with those formed with an unmodified thymine *aeg*PNA **25** (Table 2. entry 1) and with those which formed with DNA T_{12} **26** (Table 2. entry 2). These results show that; *aeg*PNA T_{12} -Lys-NH₂ control strand **25** gave T_m values of 65 and 73°C when hybridized to complementary dA₁₂ and rA₁₂, respectively (Table 2, entry 1). Natural DNA T_{12} **26** gave T_m values of 32 and 27°C

when hybridized to dA₁₂ and rA₁₂, respectively (Table 2, entry 2). PNA dodecamer **27**, **28** with a single isogaPNA (*T_R*) unit in either the C- or the N-terminus of the PNA dodecamer gave the same *T_m* values of 65 and 67°C when hybridized to complementary dA₁₂ and rA₁₂, respectively (Table 2, entries 3 and 4). PNA dodecamer **29** which contains three units of isogaPNA (*T_R*) towards C-terminus of the PNA dodecamer gave *T_m* values of 60 and 67°C when hybridized to dA₁₂ and rA₁₂, respectively (Table 2, entry 5). While the PNA dodecamer **30** which contains three units of isogaPNA (*T_R*) towards the N-terminus of the PNA dodecamer gave *T_m* values of 59 and 62°C when hybridized to dA₁₂ and rA₁₂, respectively (Table 2, entry 6). Incorporation of a single isogaPNA (*T_R*) unit in the middle of the PNA dodecamer, PNA dodecamer **31** gave *T_m* values 53 and 56°C when hybridized to complementary dA₁₂ and rA₁₂, respectively (Table 2, entry 7). Unfortunately no thermal melting transition was observed with the PNA dodecamer which consists mainly from 12 units of isogaPNA (*T_R*) **32** when hybridized either with the complementary dA₁₂ and rA₁₂, respectively (Table 2, entry 8). The above data of the PNA dodecamers **27-32** imply decreased *T_m* values of 1.5°C, 2°C and 12°C for fragments **29**, **30** and **31**, respectively, calculated from the unmodified *aeg*PNA dodecamer **25** complex with DNA (see Table 2 entries 5, 6, 7 vs. 1), and decreased *T_m* values of 6°C, 6°C, 2°C, 3.5°C and 17°C for fragments **27**, **28**, **29**, **30** and **31**, respectively, calculated from the unmodified *aeg*PNA dodecamer **25** complex with RNA (see Table 2 entries 3, 4, 5, 6, 7 vs. 1). However, the incorporation of one and three isogaPNA (*T_R*) into the dodecamers **27-31** resulted in significant close *T_m* values (Δ*T_m* 2-3°C) observed between the PNA dodecamers **27-31** DNA complexes and the corresponding PNA dodecamers **27-31** RNA complexes, contrary to the characters that *aeg*PNA and isoDNA hybridize with RNA preferentially.

Table 2. UV thermal melting data (*T_m* values) of complexes between PNAs **25-38** and complementary DNA and RNA.

Entry	Compound #	Complex with dA ₁₂	Complex with rA ₁₂	<i>T_m</i> (°C) ^a	
				<i>T_m</i> (RNA vs. DNA) (°C)	<i>T_m/mod.</i> (°C)
1	25	65	73	8.0	
2	26	32	27	-	
3	27	65	67	2.0	-, (-6.0) ^f
4	28	65	67	2.0	-, (-6.0) ^f
5	29	60	67	7.0	(-1.5) ^e , (-2.0) ^f

6	30	59 (50) ^b	62	3.0	(-2.0) ^e , (-3.5) ^f
7	31	53 (44) ^c	56	3.0	(-12.0) ^e , (-17.0) ^f
8	32	nd ^d	nd	-	-
9	33	61	67	6.0	(-4.0) ^e , (-6.0) ^f
10	34	64	68	4.0	(-1.0) ^e , (-5.0) ^f
11	35	56	63	7.0	(-3.0) ^e , (-3.5) ^f
12	36	58 (56) ^b	64	6.0	(-2.5) ^e , (-3.0) ^f
13	37	46 (39) ^c	53	7.0	(-9.0) ^e , (-20.0) ^f
14	38	nd	nd	-	-

^a The *T_m* was measured at a ratio of PNA/DNA or RNA = 1:1, concentration of PNA strand = 2.5 μ M, 10 mM phosphate buffer, pH 7.4, heating rate 0.5°C/min. All of these oligomers can dissolve easily in this buffer.

^b The values with triple mismatching DNA A₉T₃.

^c The values with single mismatching DNA A₅TA₆.

^d nd, Not detected.

^e Compared to the *T_m* of PNA:DNA triplex for one monomer unit modification.

^f Compared to the *T_m* of PNA:RNA triplex for one monomer unit modification.

PNA dodecamer **33** with a single isogaPNA (*T_S*) unit at the C-terminus of the PNA dodecamer gave *T_m* values of 61 and 67°C when hybridized to complementary dA₁₂ and rA₁₂, respectively (Table 2, entry 9). While PNA dodecamer **34** with a single isogaPNA (*T_S*) unit at the N-terminus of the PNA dodecamer gave *T_m* values of 64 and 68°C when hybridized to complementary dA₁₂ and rA₁₂, respectively (Table 2, entry 10). PNA dodecamer **35** with three units of isogaPNA (*T_S*) at the C-terminus of the PNA dodecamer gave *T_m* values of 56 and 63°C when hybridized to dA₁₂ and rA₁₂, respectively (Table 2, entry 11). While the PNA dodecamer **36** which contains three units of isogaPNA (*T_S*) at the N-terminus of the PNA dodecamer gave *T_m* values of 58 and 64°C when hybridized to dA₁₂ and rA₁₂, respectively (Table 2, entry 12). Incorporation of a single isogaPNA (*T_S*) unit in the middle of the PNA dodecamer, PNA dodecamer **37** gave *T_m* values 46 and 53°C when hybridized to complementary dA₁₂ and rA₁₂, respectively (Table 2, entry 13). Unfortunately no thermal melting transition was observed with the PNA dodecamer which consists mainly from 12 units of isogaPNA (*T_S*) **38** when hybridized either with the complementary dA₁₂ and rA₁₂, respectively (Table 2, entry 14). The above data of the PNA dodecamers **33-38** imply decreased *T_m* values of 4°C, 1°C, 3°C, 2.5 °C and 9°C for fragments **33**, **34**, **35**, **36** and **37**, respectively, calculated from the unmodified *aeg*PNA dodecamer **25** complex with DNA (see Table 2 enteries 9, 10, 11, 12, 13 vs. 1), and decreased *T_m* values of 6°C, 5°C, 3.5°C, 3°C and 20°C for fragments **33**, **34**, **35**, **36** and **37**, respectively, calculated from the unmodified *aeg*PNA dodecamer **25** complex with RNA (see Table 2 enteries 3, 9, 10, 11, 12,13 vs. 1). Comparing to the corresponding *T_m* values measured between PNA **27-31** incorporated (*T_R*) and DNA dA₁₂, the complexes between **32-37** incorporated of isogaPNA (*T_S*) and dA₁₂ showed decreased *T_m* values (*T_m* 1-7°C). Introduction of mismatch base T to dA₁₂ at the position to hybridize with isogaPNA (*T_R*) and (*T_S*) resulted in decrease of the *T_m* (*T_m* 9°C for **30** and **31**, *T_m* 2°C and 7°C for **36** and **37**,

respectively). These results show that the conformation of backbone of (T_R) is more effective sterically than that of (T_S).

Furthermore, in order to determine the binding stoichiometry of these oligomers to DNA and RNA, we used the (Job-plot) titration method^(88a,b) for one of these oligomers (fragment **31**) with DNA. The total concentration which was used is 3.0 μ M, heating was carried out at 95°C for 10 minutes, then cooling to room temperature and keep standing for 8 hours and measure the absorbance of different concentrations of **31** and dA₁₂ at 260 nm. The binding stoichiometry was found to be 2:1 (**31** vs dA₁₂) indicating a PNA₂-DNA triplex structure (Fig. 25).

The decreased T_m values compared to unmodified *aeg*PNA oligomer indicate that the glycyl- -alanine backbone of isogaPNA may not be optimal length to hybridize with DNA and RNA.

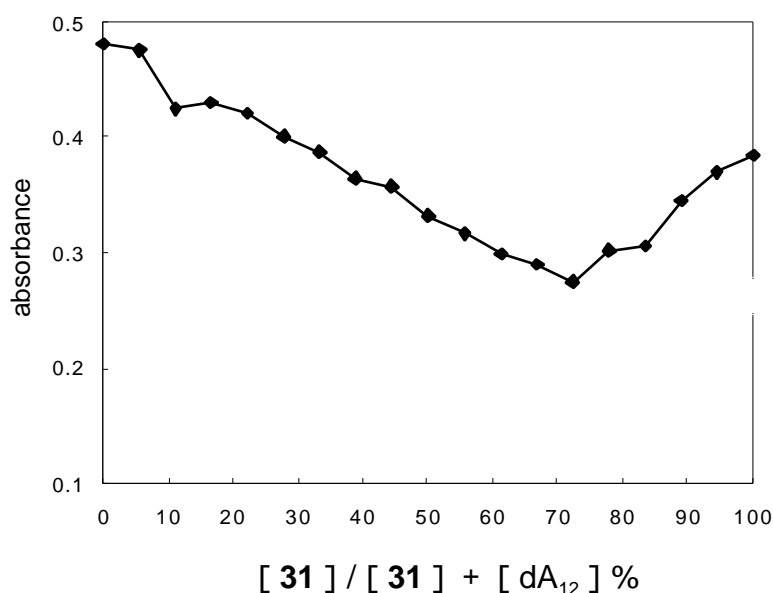


Fig. 25. Job plots of absorbance at 25°C for compound **31** and dA₁₂ at 260 nm. The following extinction coefficients were used: $T = 8.6$ mL/mmol.cm for PNA, $A = 15.3$ mL/mmol.cm, [**31**] and [dA₁₂] = 3.0 μ M, 10 mM phosphate buffer, pH 7.4.

2.3.5. Solid-phase synthesis of adenine isogaPNA oligomers

By the same way to assess the hybridization properties of adenine isogaPNA monomer **15** to its complementary DNA and RNA, adenine PNA monomer **15** was individually introduced into *aeg*PNA sequences in different positions to form various adenine octamers, in addition to another adenine octamer consists mainly from complete adenine PNA monomers **15**. The preparation of these oligomers was carried out using automated synthesis by the same method as the preparation of thymine isogaPNA oligomers. The crude products were characterized by Mass spectral analysis (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) (MALDI-TOF)⁽⁸⁷⁾, which gave a signal for the expected molecular weight (2347.2 (observed) vs. 2346.03 (calculated)).

Table 3. Various oligomers of *aeg*PNA and isogaPNA adenine monomers.

PNA sequences	
39	H-A₇-A_x-Lys-NH₂
40	H-A_x-A₇-Lys-NH₂
41	H-A₄-A_x-A₃-Lys-NH₂
42	H-A_{x8}-Lys-NH₂

A = adenine *aeg*PNA monomer, A_x adenine isogaPNA monomer (**15**).

After the preparation of these adenine oligomers the crude products were identified by MALDI-TOF mass spectral analysis, which gave a signal for the expected molecular weight (2347.2 (observed) vs. 2346.03 (calculated)) for all of these adenine oligomers. Unfortunately, despite several attempts to purify these oligomers using C18-reverse-phase HPLC under various conditions of mobile phases, we could not obtain pure oligomer to check its hybridization properties with complementary DNA and RNA.

2.3.6. Synthesis and evaluation of isogaPNA derivatives tested as anti-HIV and anti-HSV

Many antivirals are focused on the inhibition of viral polymerases and reverse transcriptases, which are the key enzymes in the replicative cycle of any virus for chemotherapy.^(89a,b) Most available drugs such as acyclovir and AZT (Fig. 26) are nucleoside analogues, and extensive research towards derivatisation on the sugar moiety of natural nucleoside to enhance antiviral activity has been carried out in numerous laboratories.^(90a-d) On the other hand, aminoethylglycine peptide nucleic acids (*aeg*PNAs)⁽⁴¹⁾, reported in 1991 by Nielsen, is a nucleic acid analogue in which the sugar-phosphate backbone is replaced by peptide linkage. Many kinds of nucleotides have been synthesized in order to improve the binding specificity to DNA and RNA, solubility and uptake into cells.^(91a-e) However there is no report on the derivatization of

peptide nucleic acid monomers, which are potent isosters of natural nucleoside, in order to explore new antivirals. In this context, we examined the approach to antiviral agents from some derivatives **44** and **48** with hydroxyl group in backbone, which is an important group for the phosphorylation by cellular kinases in the activation mechanism of antiviral nucleoside analogues, of isogaPNA⁽⁹²⁾ developed in our laboratory.

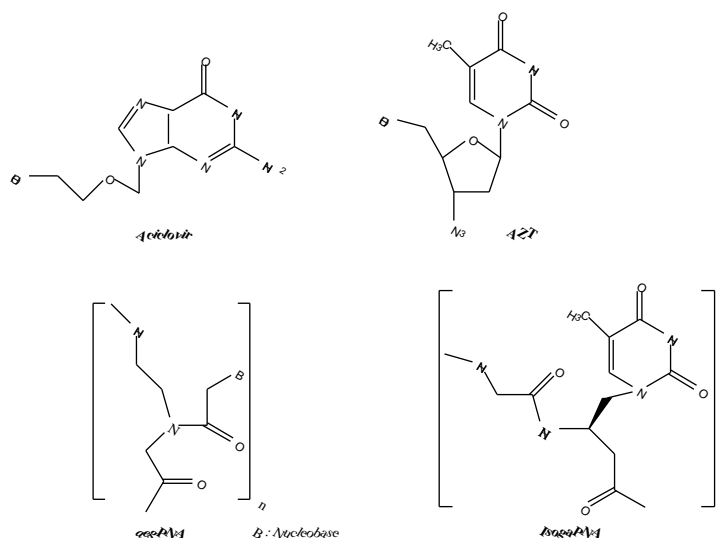
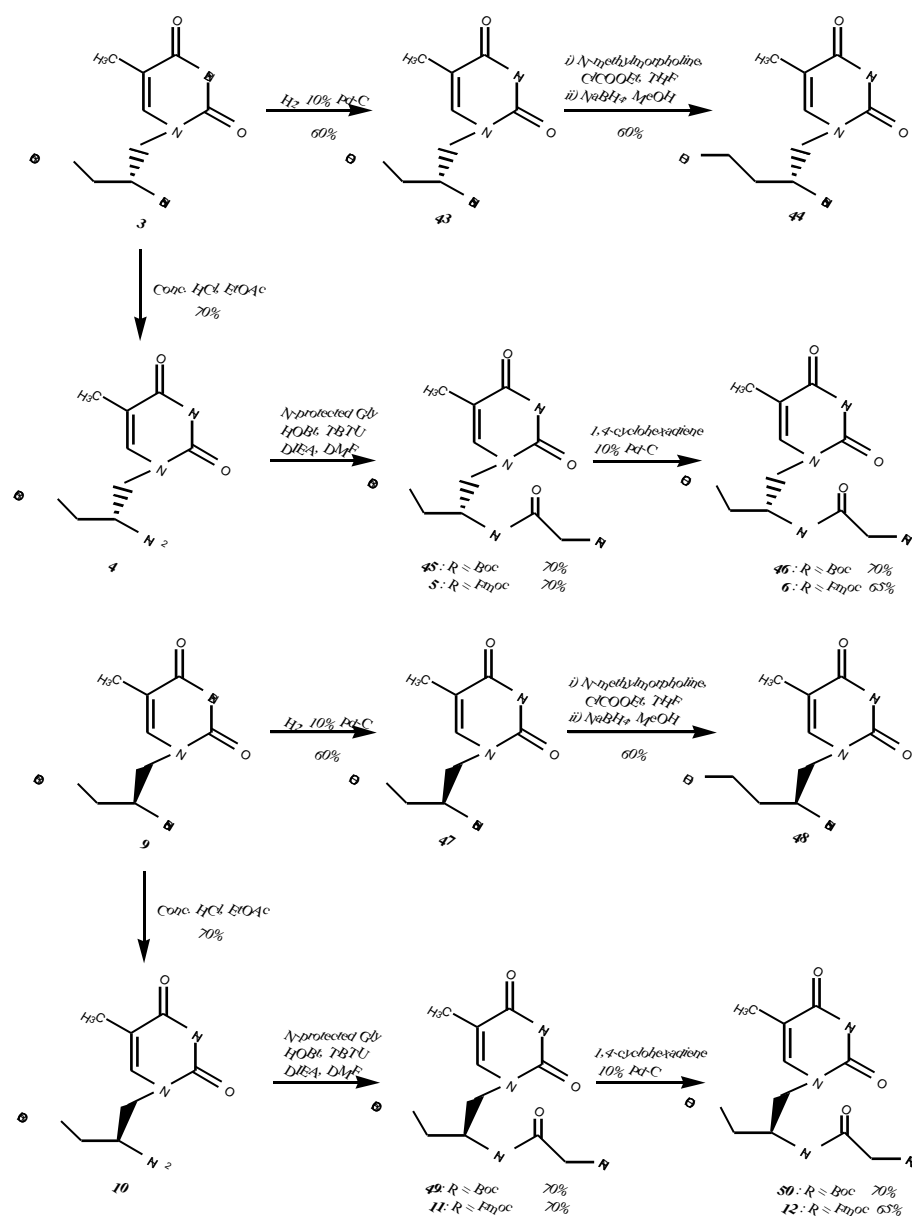


Fig. 26. Comparison between isogaPNA, aegPNA, aciclovir and AZT.

2.3.6.1. Synthesis of isogaPNA derivatives tested as anti-HIV and anti-HSV

The synthesis of 3-hydroxy-1-(thymine-1-ylmethyl)propylamine **44**, **48** proceeds through benzyl ester compounds **3**, **9**⁽⁹²⁾ which are intermediates of isogaPNA monomers (Scheme 1). Debenzoylation of compounds **3**, **9** was carried out by the use of H₂ gas in the presence of 10% Pd-C affording the corresponding acids **43**, **47** in 60% yield for each. Reduction of the carboxylic acid compounds **43**, **47** was carried out using ethyl chloroformate and sodium borohydride provided the objective compounds **44**, **48** in 60% yield for both.⁽⁷⁶⁾ Also the Boc-protected isogaPNA monomers **46**, **50** were prepared and used with the previously prepared Fmoc-protected isogaPNA monomers **6**, **12** for the comparison with hydroxyl compounds **44**, **48**. The Boc-protected isogaPNA monomers **46**, **50** were prepared from compounds **4**, **10** which are intermediates of isogaPNA monomers (Scheme 1). Coupling between compounds **4**, **10** with equimolar equivalent of Boc-glycine was carried out in the presence of TBTU, HOBt and DIEA in DMF gave compounds **45**, **49** in yield of 70% for each. Deprotection of the benzyl group was carried out by the use of 1, 4-cyclohexadiene and 10% Pd-C afforded **46**, **50** in yield of 70% for each.



Scheme 4. Synthesis of various isogaPNA derivatives.

2.3.6.2. Evaluation of isogaPNA derivatives as anti-HIV and anti-HSV

Anti-HIV-1 and anti-HSV-1 activities of hydroxyl compounds **44**, **48** isogaPNA monomers **6**, **12**, **46** and **50** and the intermediate **4**, **5**, **10**, **11**, **43**, **47** were examined by the use of Lenti RT activity kit of CAViDiTECH and plaque reduction assay with HSV-1 and VERO cells⁽⁹³⁾, respectively. The Lenti RT activity kit is optimized for the quantification of HIV RT activity in cell culture supernatants. The principle of the test depend on Step 1: Poly-rA bound to the microtiter plate wells serves as template for the reverse transcriptase enzyme. Oligo-dT serves as primer. Bromo-deoxyuridine-triphosphate is the substrate used by the enzyme to build the new DNA strand. Step 2: A

specific monoclonal anti-bromo-deoxyuridine-triphosphate antibody, conjugated to alkaline phosphatase detects this strand of DNA. The alkaline phosphatase acts on the chromogenic substrate para-nitrophenyl phosphate, and the colour change is measured photometrically. The assay has been optimized for Lenti type virus reverse transcriptase that depend on Mg^{2+} . Unfortunately, the antiviral activities against HIV-1 and HSV-1 were not observed in these assays.⁽⁹⁵⁻⁹⁷⁾ However, interestingly compound **12** (50 μ g/ml) derived from L-aspartic acid only showed potent cytotoxicity on VERO cells that was not shown by compound **6** derived from D-aspartic acid. The chirality of the molecules is one of the most important factors to show pharmacological activities. Therefore, it seems to be a merit to develop new antiviral drugs that the construction of chiral backbone of PNA monomers is relatively easy because of derivatization from chiral amino acids.

III. Development of New Inhibitors of Topoisomerases

3.1. Introduction

Developments in cancer treatment, although definite, have so far not resulted in a large increase in survival, especially in solid tumors.⁽⁹⁸⁾ Drug resistance to cancer chemotherapy is one of the causes of difficulty in the treatment of several cancer types. Among the anticancer drugs, the DNA topoisomerase (topo) inhibitors represent an important group of agents. Topoisomerases are essential nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, and the enzymes are involved in producing the necessary topological and conformational changes in DNA which are critical to many cellular processes such as replication, recombination and transcription.^(99a-c) There are two types of topoisomerases, topoisomerase I⁽¹⁰⁰⁾ and topoisomerase II⁽¹⁰¹⁾ (topo I and topo II) which have been isolated from mammalian cells. Topoisomerase I, which is ATP independent and introduces transient breaks (or nick) on a single-strand of duplex DNA, catalyzes the passage of another DNA strand through the nick. The level of topoisomerase I remains essentially constant through the cell cycle. The mechanism of action of topoisomerase I is illustrated in Fig. 27. On the other hand topoisomerase II which is ATP dependent and introduces double strand breaks of the DNA, and catalyzes the passage of another DNA double strands through this break. Topoisomerase II plays an important role in chromosome assembly, condensation and segregation of chromosomes in anaphase as well as in the completion of transcription.⁽¹⁰²⁻¹⁰⁶⁾ The level of topoisomerase II reaches to its peak in S-phase and then decline rapidly. The mechanism of action of topoisomerase II is illustrated in Fig. 28.

3.1.1. Mechanism of action of topoisomerase I

The mechanism of action of topoisomerase I enzyme is illustrated in Fig. 27.

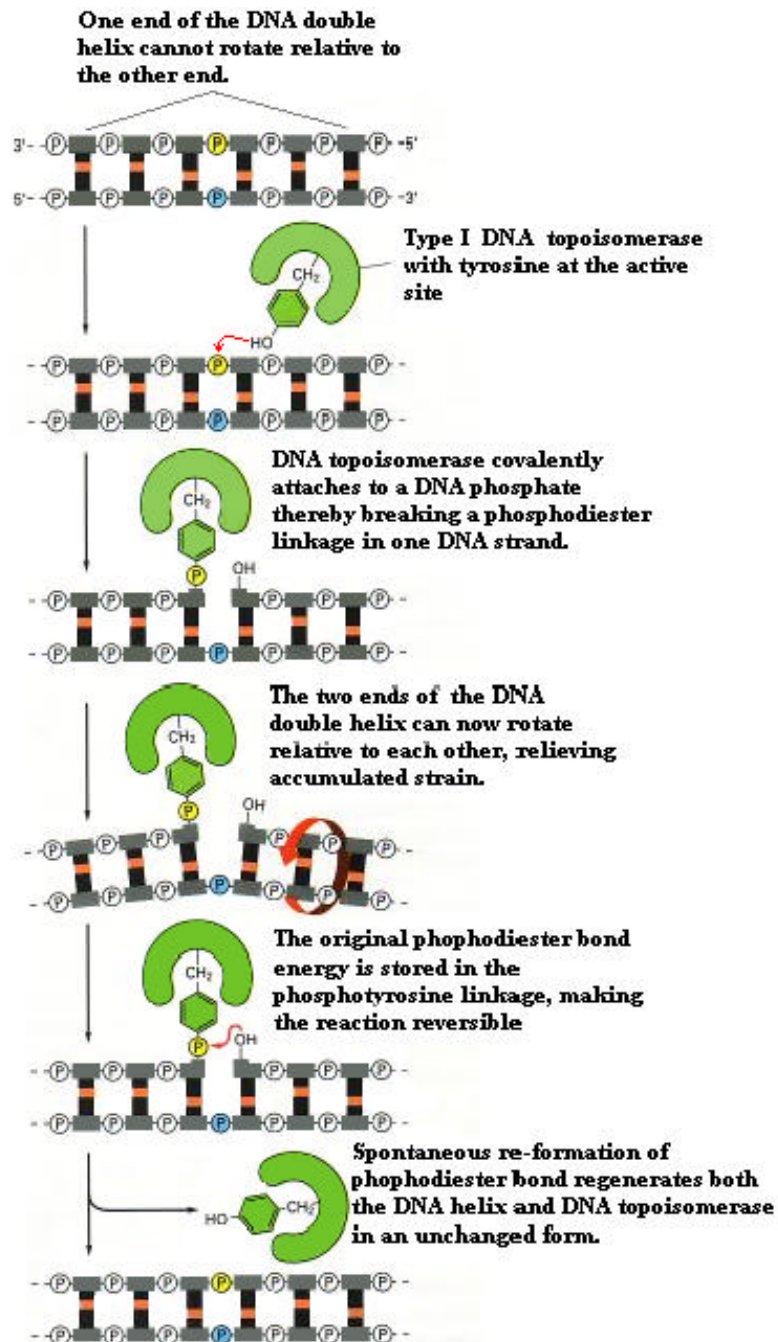


Fig. 27. The reversible nicking reaction catalyzed by DNA topoisomerase I enzyme.

3.1.2. Mechanism of action of topoisomerase II

The mechanism of action of topoisomerase I enzyme is illustrated in Fig. 28.

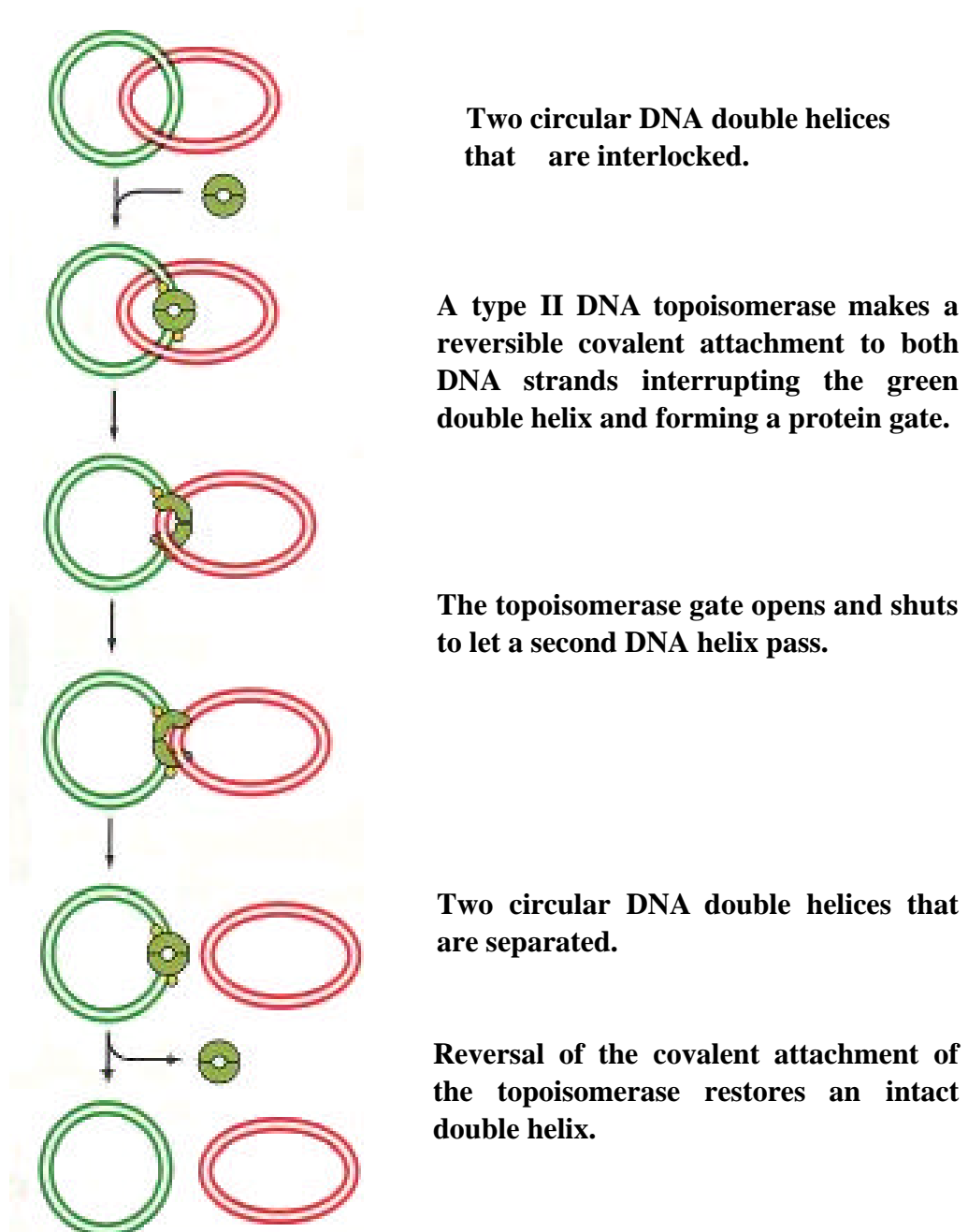


Fig. 28. Mechanism of action of topoisomerase II.

In addition to their normal cellular functions, both topoisomerase I and II enzymes have recently emerged as important cellular targets for chemical intervention in the development of antitumor drugs⁽¹⁰⁷⁾ and some drugs preventing the actions of the enzymes are used clinically in the treatment of a variety of cancers. Many topoisomerase inhibitors have been reported and these inhibitors are classified into three types; the cleavable complex-forming types with and without DNA intercalation, and the cleavable complex-nonforming type. The inhibitors of the first and second types inhibit the DNA rejoining reaction of topoisomerase by holding together the tight cleavable complex consisting of the enzyme and broken DNA.

3.1.3. Topoisomerase inhibitors that stabilize the cleavable complex and intercalate into DNA

The first group of drugs intercalate into DNA, representative agents of this group are doxorubicin⁽¹⁰⁸⁾, amsacrine⁽¹⁰⁹⁾ and ellipticines⁽¹¹⁰⁾ as topoisomerase II inhibitors and saintopin⁽¹¹¹⁾ as topoisomerase I and II inhibitor (Fig. 29).

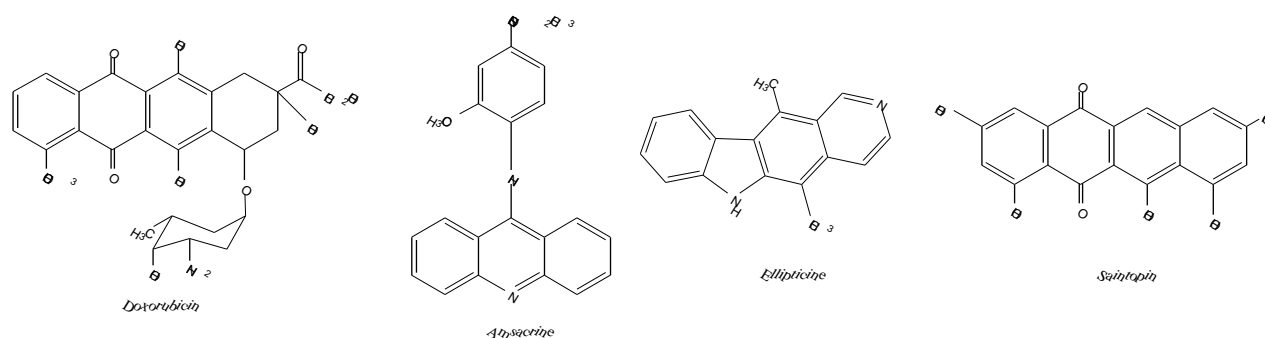


Fig. 29. Examples of cleavable complex-forming types with DNA intercalation topoisomerase inhibitors.

3.1.4. Topoisomerase inhibitors that stabilize the cleavable complex without intercalation into DNA

The second group of drugs stabilize the cleavable complex without intercalating into DNA. This group includes camptothecin⁽¹¹²⁾ and its synthetic derivatives such as CPT-11⁽¹¹³⁾ and topotecans⁽¹¹⁴⁾ as topoisomerase I inhibitor and epipodophyllotoxin (etoposide)⁽¹¹⁵⁾ as topoisomerase II inhibitor (Fig. 30). These antitumor drugs, referred to as topoisomerase inhibitors, trap the enzyme in an intermediate complex with DNA, termed cleavable complex which prevents the final rejoining step of the reaction and result in increased DNA strands breaks. The inhibitors of the two types promote the

accumulation of damaged DNA in the cells show strong cytotoxicity and therefore arrest cell cycle progression.^(116,117)

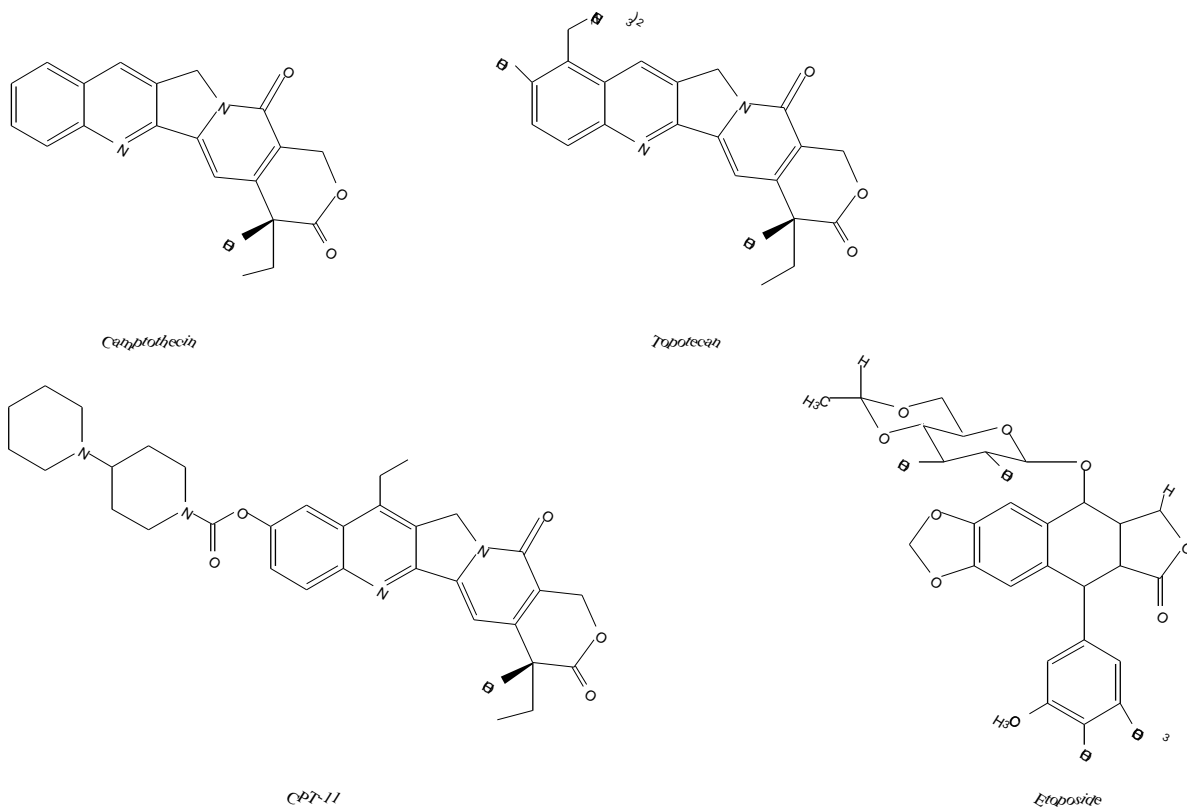


Fig. 30. Examples of cleavable complex-forming types without DNA intercalation topoisomerase inhibitors.

3.1.5. Cleavable complex-nonforming type topoisomerase inhibitors

The third group are cleavable complex-nonforming type topoisomerases inhibitors, they inhibit the DNA breaking and rejoining reactions of topoisomerases by a direct action on the enzyme molecule without forming the cleavable complex. Inhibitors of this type do not cause DNA damage. There are several examples of this group including synthetic compounds and natural compounds. Representative agents of the synthetic one include;

bisdioxopiperazine family⁽¹¹⁸⁾ (topoisomerase II inhibitor), 15-deoxy-_-12,14-prostaglandin J₂⁽¹¹⁹⁾ (topoisomerase II inhibitor), epigallocatechin-gallate⁽¹²⁰⁾ (topoisomerase I and II inhibitor), and petroselinic acid⁽¹²¹⁾ (topoisomerase I and II inhibitor) (Fig. 31).

3.1.5.1. Synthetic cleavable complex-nonforming type topoisomerase inhibitors

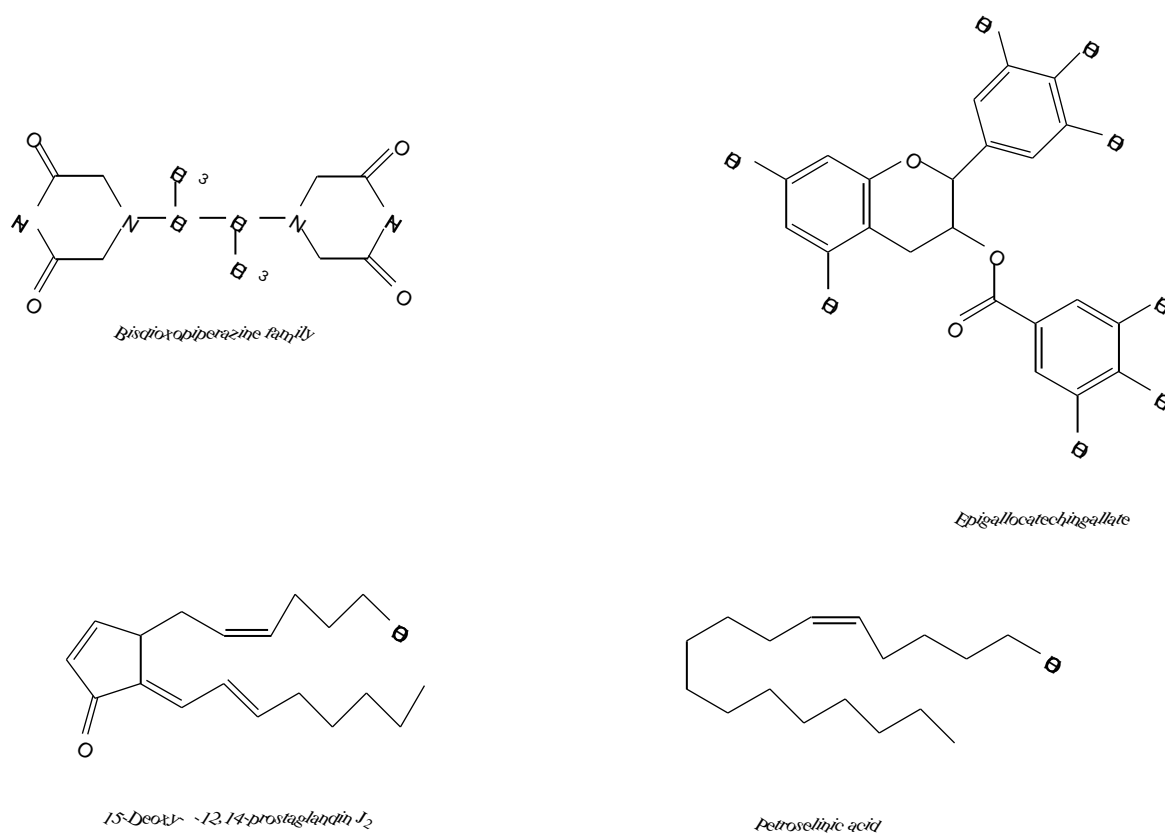


Fig. 31. Examples of synthetic cleavable complex-nonforming type topoisomerase inhibitors.

Representative agents of the natural one include; natural products from actinomycetes (low molecular compound) such as Isoaurostatin⁽¹²²⁾ (topoisomerase I inhibitor), Topostatin⁽¹²³⁻¹²⁵⁾ (topoisomerase I and II inhibitor), and 2070-DTI⁽¹²⁶⁾ (topoisomerase I and II inhibitor) and natural products from actinomycetes (high molecular compound) such as 2280-DTI (topoisomerase I inhibitor), 2890-DTI⁽¹²⁷⁾ (topoisomerase I and II inhibitor) and Macrostatin⁽¹²⁸⁾ (topoisomerase I and II inhibitor) (Fig. 32)

3.1.5.2. Natural cleavable complex-nonforming type topoisomerase inhibitors

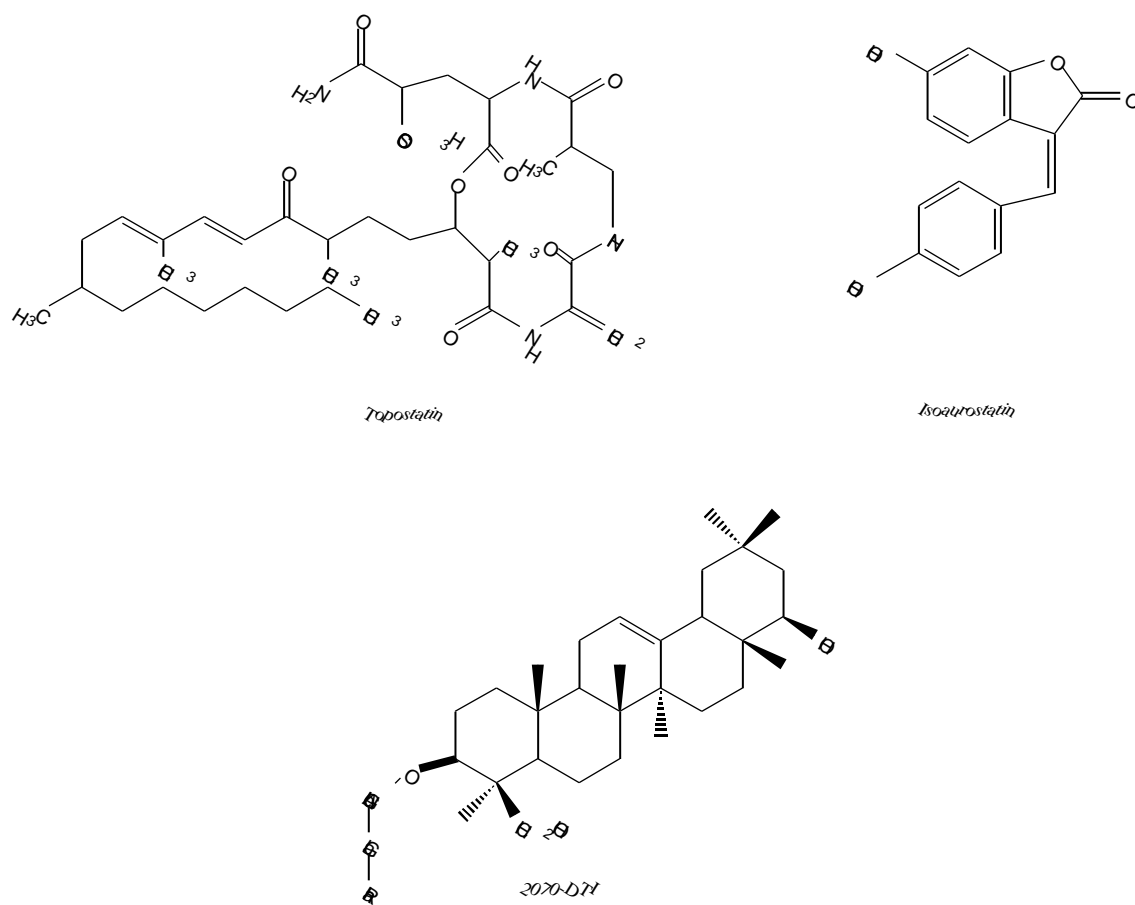


Fig. 32. Examples of natural cleavable complex-nonforming type topoisomerase inhibitors.

3.2. Scope of Investigation

In the second part of the thesis we explain the preparation of several compounds with multi hydroxy groups on phenyl moiety aiming at inhibitory activities against topoisomerases. Topoisomerases (topos) are DNA-associated enzymes that control and modify DNA topology by causing transient single (topo I) or double (topo II) strand DNA breaks during processes such as transcription, translation and chromatid separation. Clinically potent anticancer agents, topo inhibitors like camptothecin, etoposide, and doxorubicin bind to the cleavable complex formed between topo and DNA, and keep it from going back the original DNA. This action is associated with severe side effects as well as other anticancer agents targeted at DNA. Now agents directly inhibiting topo are urgently being requested. Suzuki and co-workers reported isoaurostatin A isolated from *Thermomonospora alba* showed topo I inhibition ($IC_{50} = 307 \mu M$), and they proved that this compound directly inhibited topo I. Isoaurostatin A was modified to a lot of derivatives by chemical synthesis, including one that showed potent activity 100 times than the original compound ($IC_{50} = 3.0 \mu M$). In the same time, it was found that gallocatechin and epigallocatechin containing several hydroxy groups on phenyl moiety have strong inhibitory activities against topoisomerases (Fig. 33). From these results we tried to prepare compounds with polyhydroxy groups on phenyl moiety aiming to improve the inhibitory activities against topoisomerases and also we examine structure-activity relationship of the prepared compounds. Our objective compounds consist from *o*-, *m*-, *p*-phenylenediamines and 2-aminobenzothiazole coupled with either gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid or benzoic acid (Fig. 34). Thus the prepared compounds having hydroxy groups ranging from 6 to zero would enable us to study the structure-activity relationship to identify the essential parts of the structure which is responsible for the activity. Also the acetyl derivatives of those compounds were tested for the inhibitory activities against topoisomerases.

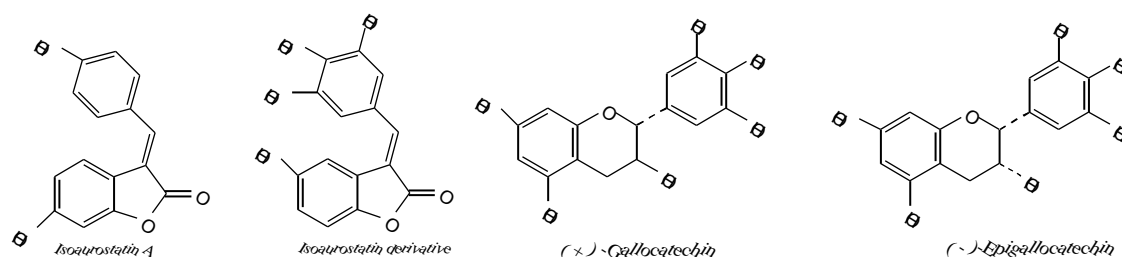


Fig. 33. Various examples of polyhydroxyphenyl compounds which have strong topoisomerases inhibitory activity.

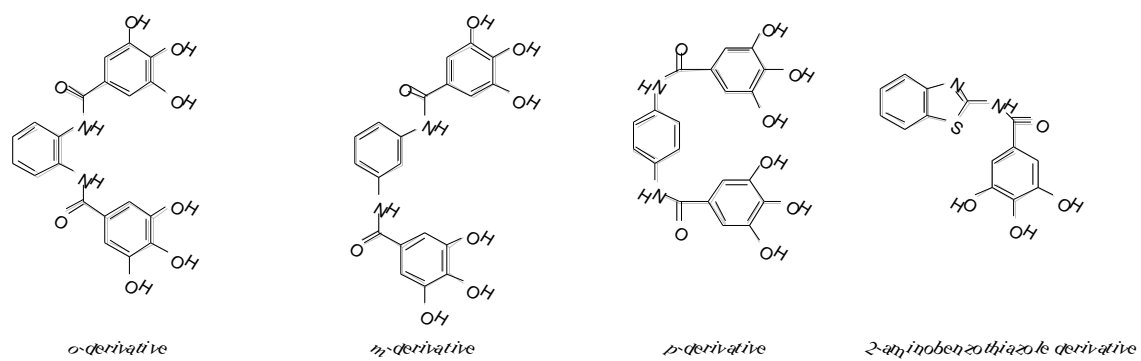
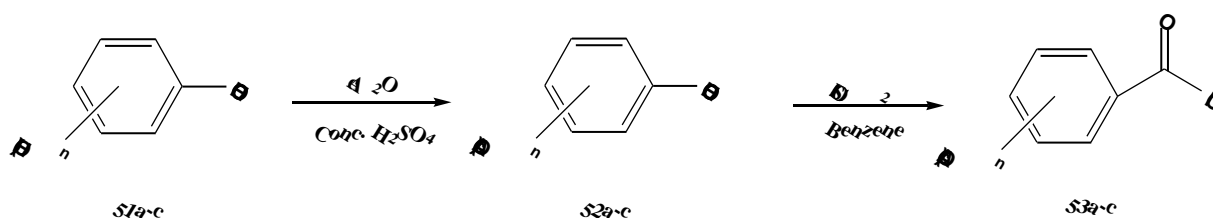


Fig. 34. Designed *o*-, *m*-, *p*-, and 2-aminobenzothiazole derivatives.

3.3. Results and Discussion

3.3.1. Synthesis of acetyl and acid chloride derivatives of gallic acid, 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid

The synthesis of acetyl derivatives **52a-c**⁽¹²⁹⁾ were carried out by acetylation of gallic acid, 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid **51a-c**, respectively using acetic anhydride in the presence of few drops of concentrated H₂SO₄ and heating the reaction mixture at 80°C for 10 minutes, the yields of these reactions were 77%, 77% and 81.6%, respectively. Treatment of the acetyl derivatives **52a-c** with thionyl chloride in benzene gave the corresponding acid chlorides **53a-c** in 82.1%, 82.6% and 79.6% yields, respectively. The results were shown in (Scheme 5) and (Table 4).



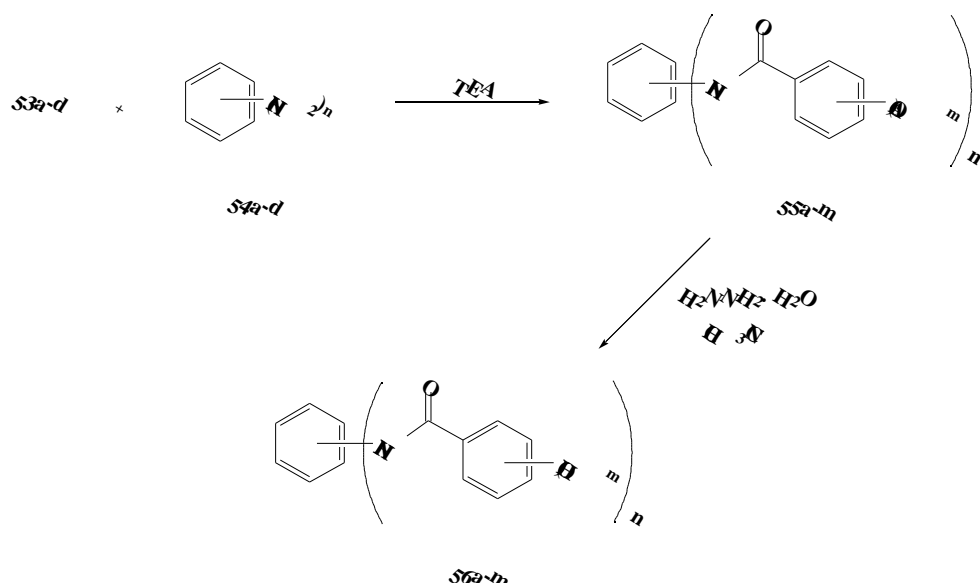
Scheme 5. Synthesis of various acetyl and acid chloride derivatives of gallic acid, 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid.

Table 4. Acetyl and acid chloride derivatives of gallic acid, 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid.

comp. #	n	position	comp. #	position	yield %	comp. #	position	yield %
51a	3	3, 4, 5-	52a	3, 4, 5-	77.0	53a	3, 4, 5-	82.1
52b	2	3, 4-	52b	3, 4-	77.0	53b	3, 4-	82.6
53c	1	4-	52c	4-	81.6	53c	4-	79.6

3.3.2. Synthesis of acetylgalloylbenzamide (AGBA) and galloylbenzamide (GBA) derivatives

The preparation of AGBA derivatives **55a-l** was carried out through coupling of various acid chlorides **53a-c** and benzoyl chloride **53d** with *o*-phenylenediamine **54a**, *m*-phenylenediamine **54b** and *p*-phenylenediamine **54c**, respectively in the presence of TEA either in CH₂Cl₂ in case of **53a-c** or in dry benzene and THF in case of **53d** (Scheme 6). In order to examine the structure-activity relationship compound **55m** was similarly prepared in 81% yield by coupling of acid chloride **53a** with aniline in the presence of TEA in CH₂Cl₂. Treatment of AGBA **55a-m**, respectively with hydrazine monohydrate in CH₃CN gave the corresponding GBA **56a-m** in good yields (Table 5).



Scheme 6. Synthesis of various derivatives of AGBA and GBA.

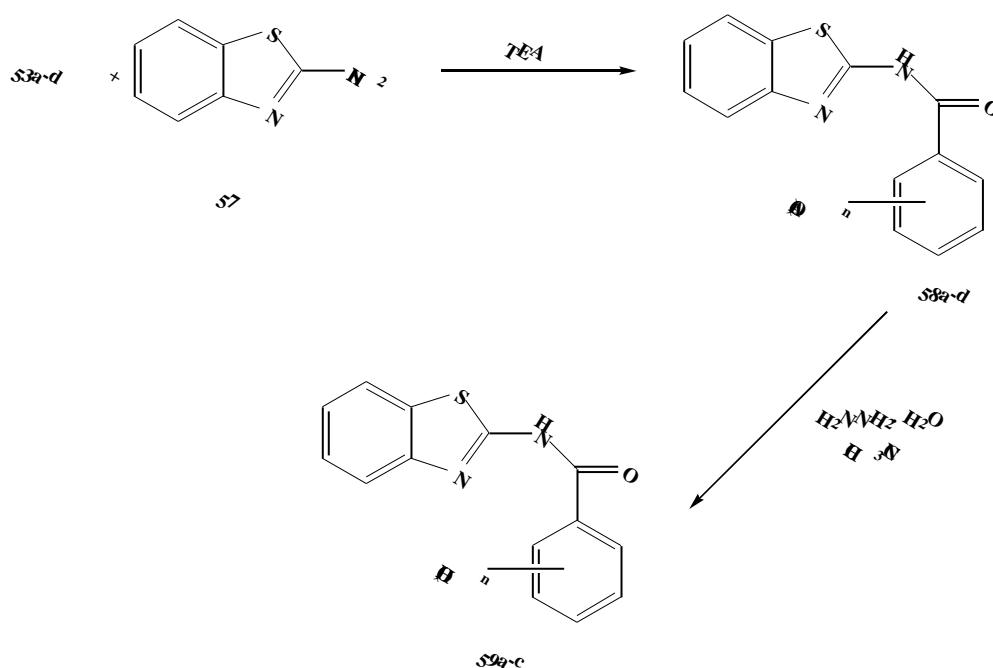
Table 5. Various derivatives of AGBA and GBA.

	n	comp. #	m	position	yield %	comp. #	position	yield %
54a	2	55a (<i>ortho</i> -)	3	3, 4, 5-	74.5	56a (<i>ortho</i> -)	3, 4, 5-	60.7
	2	55b (<i>ortho</i> -)	2	3, 4-	73.9	56b (<i>ortho</i> -) ⁽¹³⁰⁾	3, 4-	63.1
	2	55c (<i>ortho</i> -) ⁽¹³¹⁾	1	4-	70.6	56c (<i>ortho</i> -)	4-	57.5
	2	55d (<i>ortho</i> -) ⁽¹³²⁾	0		75.9			
54b	2	55e (<i>meta</i> -)	3	3, 4, 5-	75.3	56e (<i>meta</i> -)	3, 4, 5-	60.7
	2	55f (<i>meta</i> -)	2	3, 4-	75.7	56f (<i>meta</i> -) ⁽¹³⁰⁾	3, 4-	71.0
	2	55g (<i>meta</i> -) ⁽¹³¹⁾	1	4-	81.0	56g (<i>meta</i> -) ⁽¹³³⁾	4-	66.6
	2	55h (<i>meta</i> -) ⁽¹³⁴⁾	0		76.0			
54c	2	55i (<i>para</i> -)	3	3, 4, 5-	67.8	56i (<i>para</i> -) ⁽¹³⁵⁾	3, 4, 5-	56.0
	2	55j (<i>para</i> -)	2	3, 4-	77.5	56j (<i>para</i> -) ⁽¹³⁰⁾	3, 4-	68.4
	2	55k (<i>para</i> -) ⁽¹³¹⁾	1	4-	80.2	56k (<i>para</i> -) ⁽¹³⁶⁾	4-	66.0
	2	55l (<i>para</i> -) ⁽¹³⁷⁾	0		76.0			
54d	1	55m ⁽¹³⁸⁾	3	3, 4, 5-	81.0	56m ⁽¹³⁹⁾	3, 4, 5-	73.5

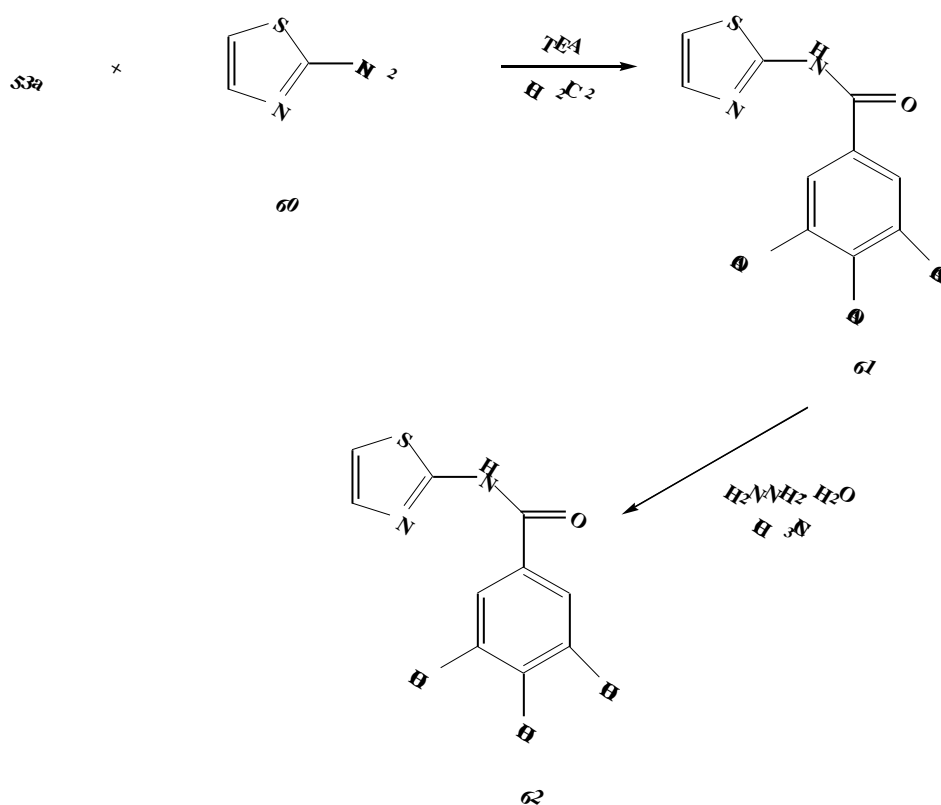
3.3.3. Synthesis of acetylgalloyl-2-aminobenzothiazole (AGABT), galloyl-2-amino-benzothiazole (GABT), acetylgalloyl-2-aminothiazole (AGAT) and galloyl-2-

aminothiazole (GAT) derivatives

Coupling of compounds **53a-d** with 2-aminobenzothiazole **57** (Scheme 7) was carried out in the presence of TEA either in CH_2Cl_2 in case of **53a-c** or in THF in case of **53d** affording the corresponding acetylgalloyl derivatives **58a-d** in 77.3%, 78.6%, 77.1% and 78.8% yields, respectively. In a similar way compound **61** was prepared in a good yield for the comparison of the structure-activity relationship (Scheme 8) by direct coupling of the acid chloride **53a** with 2-aminobenzothiazole **60** in the presence of TEA in CH_2Cl_2 . Complete deacetylation of compounds **58a-d** and **61** using hydrazine monohydrate in CH_3CN gave the corresponding galloyl derivatives **59a-c** and **62** in 66.2%, 59.4%, 66.7% and 67.4% yields, respectively (Scheme 7 and 8). The data of these compounds were shown in Table 6.



Scheme 7. Synthesis of various derivatives of AGABT and GABT.



Scheme 8. Synthesis of various derivatives of AGAT and GAT.

Table 6. Various derivatives of AGBAT, GBAT, AGAT and GAT.

comp. #	n	position	yield %	comp. #	position	yield %
58a	3	3, 4, 5-	77.30	59a	3, 4, 5-	66.20
58b	2	3, 4-	78.60	59b	3, 4-	59.40
58c ⁽¹⁴⁰⁾	1	4-	77.10	59c	4-	66.67
58d ⁽¹⁴¹⁾	0		78.77			
61	3	3, 4, 5-	74.10	62	3, 4, 5-	67.40

3.3.4. Topoisomerases inhibitory activities

Inhibition against relaxation activity of topoisomerases I and II was measured by detecting the conversion of supercoiled pBR322 DNA to its relaxed form.⁽¹⁰¹⁾

3.3.4.1. Topoisomerase I inhibitory activities of AGBA derivatives

Several derivatives of acetylalloylbenzamide (AGBA) (Fig. 35) were prepared by direct coupling of ortho-, meta- and para- phenylenediamine with either mono-, di-, and tri- acetylbenzoyl chloride, and the prepared compounds were evaluated for their inhibitory activities against topoisomerases I. Structure-activity relationship was examined for the prepared compounds in order to identify the essential parts responsible for the activity (Table 7).

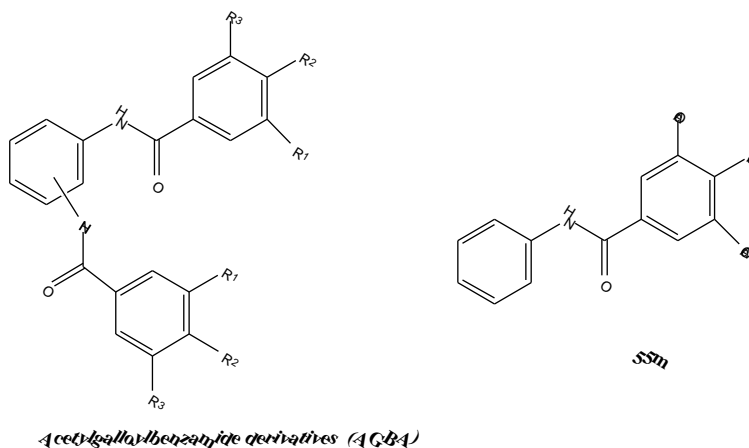


Fig. 35. Various AGBA derivatives.

Table 7. Topoisomerase I inhibitory activities of AGBA derivatives.

AGBA #	# of OAc	R ₁	R ₂	R ₃	Topo I inhibitory activity (IC ₅₀ , μM)
55a	6	OAc	OAc	OAc	4.7
55b	4	OAc	OAc	H	88.0
55c	2	H	OAc	H	> 100.0
55e	6	OAc	OAc	OAc	11.2
55f	4	OAc	OAc	H	43.2
55g	2	H	OAc	H	> 100.0
55i	6	OAc	OAc	OAc	8.6
55j	4	OAc	OAc	H	-
55k	2	H	OAc	H	> 100.0
55m	3	OAc	OAc	OAc	> 100.0

As shown in Table 7, compounds **55a**, **55b** and **55c** which consist from *o*-phenylenediamine coupled with either tri-, di- and mono- acetylbenzoyl chloride, exhibited inhibitory activity against topoisomerase I with IC₅₀ of 4.7, 88.0 and > 100

μM , respectively. These results show that according as the number of acetyl moiety in the AGBA derivatives increases, the inhibitory activity against topoisomerase I increased, i.e., IC_{50} of **55a** which contains six acetyl moieties was $4.7 \mu\text{M}$ while the IC_{50} of **55b** which contains four acetyl moieties was $88 \mu\text{M}$ (**55a** is 19 folds potent activity over **55b**), and the IC_{50} of **55c** which contains two acetyl moieties was $> 100 \mu\text{M}$. This indicated that the acetyl moiety is essential for activity in such AGBA derivatives. On the other hand, compounds **55e**, **55f** and **55g**, which were prepared from *m*-phenylenediamine and either tri-, di- and mono- acetylbenzoyl chloride, showed inhibitory activity against topoisomerase I with IC_{50} of 11.2, 43.2 and $> 100 \mu\text{M}$, respectively. These results suggest that by increasing the number of acetyl moiety in the AGBA derivatives, the inhibitory activity against topoisomerase I was enhanced, i.e., IC_{50} of **55e** which contains six acetyl moieties was $11.2 \mu\text{M}$ while the IC_{50} of **55f** which contains four acetyl moieties was $43.2 \mu\text{M}$ (**55e** is 4 folds potent activity over **55f**), and the IC_{50} of **55g** which contains two acetyl moieties was $> 100 \mu\text{M}$. This also support that the acetyl moiety is essential for activity of such AGBA derivatives. In contrast, compounds **55i**, **55k** which were prepared from *p*-phenylenediamine with tri- and mono- acetylbenzoyl chloride, respectively, showed inhibitory activity against topoisomerase I with IC_{50} of 8.6 and $> 100 \mu\text{M}$, respectively, while compound **55j** which was prepared by coupling of *p*-phenylenediamine with diacetylbenzoyl chloride did not show any inhibitory activity against topoisomerase I, this may be related to the incomplete solubility of this compound in the reaction mixture during the assay. These results indicated that by increasing the number of acetyl moiety in this type of AGBA derivatives, the inhibitory activity against topoisomerase I was enhanced, i.e., IC_{50} of **55i** which contains six acetyl moieties was $8.6 \mu\text{M}$ while the IC_{50} of **55k** which contains two acetyl moieties was $> 100 \mu\text{M}$. This also support that the acetyl moiety is essential for activity of such AGBA derivatives. It is also clear that if a comparison was made between the most potent compounds of these groups **55a**, **55e** and **55i** which contain six acetyl moieties, the inhibitory activity against topoisomerase I will be in the following order; **55a** more potent than **55i** which is more potent than **55e**. In order to check the effect of presence either one or two amino groups on phenylenediamine moiety on the inhibitory activity against topoisomerase I, compound **55m** was prepared from aniline and triacetylbenzoyl chloride and it showed inhibitory activity against topoisomerase I of $\text{IC}_{50} > 100 \mu\text{M}$. From these results of AGBA derivatives, it was apparent that the presence of two amino groups for coupling with mono-, di- and tri- acetylbenzoyl chloride is essential for the activity, and the presence of acetyl moiety is essential for activity (at least 4 acetyl moieties required), enhancing the inhibitory activity against topoisomerase I.

3.3.4.2. Topoisomerase I and topoisomerase II inhibitory activities of GBA derivatives

The GBA derivatives (Fig. 36) were prepared by deacetylation of their corresponding acetyl derivatives mentioned before using hydrazine monohydrate and evaluated of their inhibitory activities against topoisomerase I and II. Structure-activity relationship was examined for the prepared compounds in order to identify the essential parts responsible for the activity (Table 8).

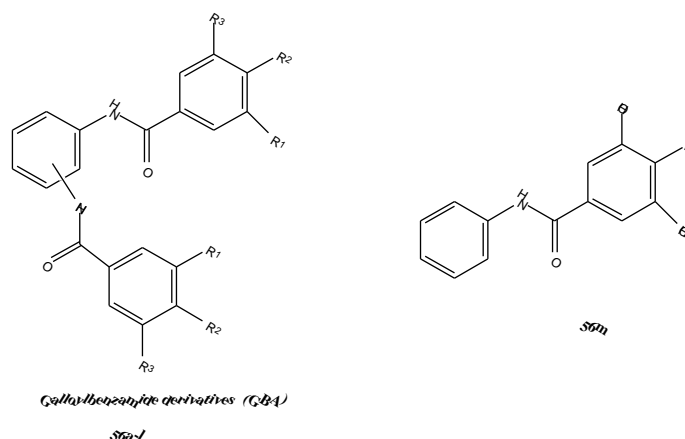


Fig. 36. Various GBA derivatives.

Table 8. Topoisomerase I and topoisomerase II Inhibitory activities of GBA derivatives.

GBA #	# of OH	R ₁	R ₂	R ₃	Topo I inhibitory activity (IC ₅₀ , μM)	Topo II inhibitory activity (IC ₅₀ , μM)
56a	6	OH	OH	OH	0.9	0.09
56b	4	OH	OH	H	6.4	nd
56c	2	H	OH	H	> 100.0	> 100.0
55d	0	H	H	H	> 100.0	> 100.0
56e	6	OH	OH	OH	1.6	0.08
56f	4	OH	OH	H	10.0	nd
56g	2	H	OH	H	> 100.0	> 100.0
55h	0	H	H	H	> 100.0	> 100.0
56i	6	OH	OH	OH	1.4	0.08
56j	4	OH	OH	H	2.6	0.14
56k	2	H	OH	H	> 100.0	nd
55l	0	H	H	H	> 100.0	> 100.0
56m	3	OH	OH	OH	> 100.0	nd

nd, not determined

As shown in Table 8, compounds **56a**, **56b**, **56c**, and **55d** which represent the ortho-forms of galloylbenzamide derivatives were prepared by deacetylation of their corresponding acetyl derivatives, except for **55d** which was prepared by direct coupling of *o*-phenylenediamine with benzoyl chloride; these compounds exhibited inhibitory activity against topoisomerase I with IC₅₀ of 0.9, 6.4, > 100 and > 100 μM, respectively. These results show that with increasing the number of hydroxy groups in ortho- form of

GBA derivatives, enhancement of the inhibitory activity against topoisomerase I was observed, i.e., IC_{50} of **56a** which contains six hydroxy groups was 0.9 μM while the IC_{50} of **56b** which contains four hydroxy groups was 6.4 μM (**56a** is 7 folds potent activity over **56b**) and both **56c** which contains two hydroxy groups and **55d** without any hydroxy group showed $IC_{50} > 100 \mu\text{M}$. This indicates that the presence of hydroxy moiety is essential for activity in such *o*-GBA derivatives. Compound **56a** exhibited inhibitory activity against topoisomerase II with IC_{50} of 0.09 μM , while compounds **56c** and **55d** exhibited inhibitory activity against topoisomerase II with IC_{50} of $> 100 \mu\text{M}$. The results indicated that compound **56a** has topoisomerase II inhibitory activity 10 times more than topoisomerase I inhibitory activity. On the other side, compounds **56e**, **56f**, **56g**, and **55h** which represent the meta- derivatives of galloylbenzamide were prepared by a similar method through deacetylation of the corresponding acetyl derivatives, except for **55h** which was prepared by coupling of *m*-phenylenediamine with benzoyl chloride; these derivatives exhibited inhibitory activity against topoisomerase I with IC_{50} of 1.6, 10.0, > 100 and $> 100 \mu\text{M}$, respectively. These results show that with increasing the number of hydroxy groups in the meta- form of GBA derivatives resulted in enhancement of the inhibitory activity against topoisomerases I i.e., IC_{50} of **56e** which contains six hydroxy groups was 1.6 μM while the IC_{50} of **56f** which contains four hydroxy groups was 10 μM (**56e** is 6 folds potent activity over **56f**) and both **56g** which contains two hydroxy groups and **55h** without any hydroxy group indicated $IC_{50} > 100 \mu\text{M}$. This means that the presence of hydroxy moiety is essential for activity in such *m*-GBA derivatives. Compound **56e** exhibited inhibitory activity against topoisomerase II with IC_{50} of 0.08 μM , while compounds **56g** and **55h** exhibited inhibitory activity against topoisomerase II with IC_{50} of $> 100 \mu\text{M}$. The results indicated that compound **56e** has topoisomerase II inhibitory activity 20 times more than topoisomerase I inhibitory activity. Compounds **56i**, **56j**, **56k**, and **55l** which represent the para- derivatives of such galloylbenzamide and prepared by the same way through deacetylation of their corresponding acetyl derivatives, except for **55l** which was prepared by direct reaction of *p*-phenylenediamine with benzoyl chloride, showed inhibitory activity against topoisomerase I with IC_{50} of 1.4, 2.6, > 100 and $> 100 \mu\text{M}$, respectively. These results show that with increasing the number of hydroxy groups in these para- GBA derivatives, enhancement of the topo I inhibitory activity was observed, i.e., IC_{50} of **56i** which contains six hydroxy groups was 1.4 μM while the IC_{50} of **56j** which contains four hydroxy groups was 2.6 μM (**56i** is 1.8 folds potent activity over than **56j**) and the IC_{50} of both **56k** which contains two hydroxy groups and **55l** without any hydroxy group was $> 100 \mu\text{M}$. This proves that the presence of hydroxy moiety in such *p*-GBA derivatives is essential for the activity. Compound **56i** exhibited inhibitory activity against topoisomerase II with IC_{50} of 0.08 μM , while compound **56j** indicated inhibitory activity against topoisomerase II with IC_{50} of 0.14 μM (compound **56i** is about 1.7 folds potent activity over than **56j**). On the other side, compound **55l** exhibited inhibitory activity against topoisomerase II with IC_{50} of $> 100 \mu\text{M}$. The results indicated that compound **56i** has topoisomerase II inhibitory activity 17.5 times more than topoisomerase I inhibitory activity. If we compare the most potent compounds **56a**,

56e and **56i** which contain six hydroxy groups, the topoisomerase I inhibitory activity will be in the following order; **56a** more potent than **56i** which is slightly potent than **56e**, the topoisomerase II inhibitory activity will be in the following order; **56e** has the same potency of **56i** and slightly potent than **56a**. Also these results indicated that at any case the topoisomerase I inhibitory activity of the deacetylated forms (GBA) are more potent than that of the corresponding acetyl derivatives (AGBA); **56a** is 5.2 folds potent over **55a** while **56e** is 7 folds potent over **55e** and **56i** is 6 folds potent over **55i**. In order to check the effect of presence either one or two amine groups on the phenylenediamine on the topoisomerase I inhibitory activity in such GBA derivatives, compound **56m** was prepared by deacetylation of its corresponding acetyl derivative **55m**. The results showed that **56m** exhibited inhibitory activity against topoisomerase I with $IC_{50} > 100 \mu M$. From these results of GBA derivatives, it was apparent that the presence of two amino groups is essential for activity, by increasing the number of hydroxyl groups which is essential for activity (at least 4 hydroxy groups required), the inhibitory activity against topoisomerase I increased and the activity of the hydroxyl derivatives of these compounds became more potent compared with the corresponding acetyl derivatives.

3.3.4.3. Topoisomerase I inhibitory activities of AGABT derivatives

AGABT derivatives (Fig. 37) were prepared by direct coupling of 2-aminobenzo-thiazole with either tri-, di- and mono- acetylbenzoyl chloride. The prepared compounds were evaluated for its inhibitory activities against topoisomerases I, structure- activity relationship was examined for the prepared compounds in order to identify the essential parts responsible for the activity (Table 9).

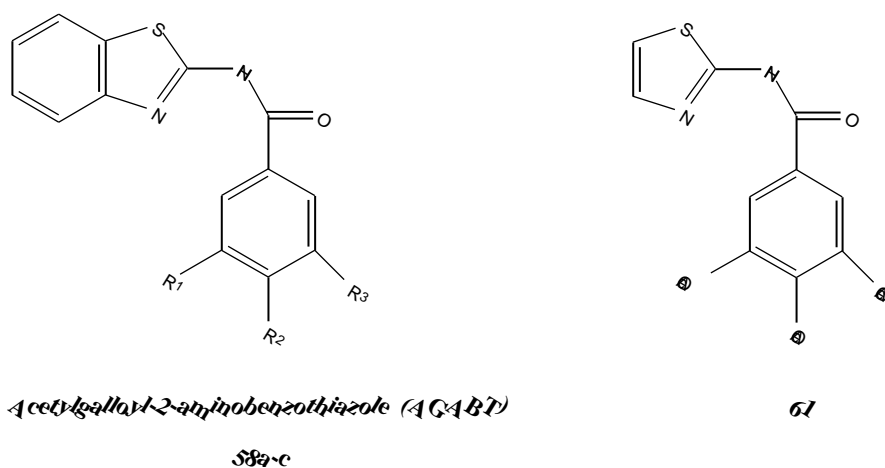


Fig. 37. Various AGABT derivatives.

Table 9. Topoisomerase I Inhibitory activities of AGABT derivatives.

AGABT #	# of OAc	R ₁	R ₂	R ₃	Topo I inhibitory activity (IC ₅₀ , μM)
58a	3	OAc	OAc	OAc	7.7
58b	2	OAc	OAc	H	> 100.0
58c	1	H	OAc	H	> 100.0
61	3	OAc	OAc	OAc	> 100.0

As shown in Table 9, compound **58a** which contains three acetyl moieties indicated strong inhibitory activity against topoisomerase I with IC₅₀ of 7.7 μM, while other derivatives **58b** (which contains two acetyl moieties) and **58c** (which contains one acetyl moiety) showed inhibitory activity against topoisomerase I with IC₅₀ > 100.0 μM. In order to check the effect of presence or absence of benzene ring on topoisomerase I inhibitory activity of such derivatives, compound **61** was prepared by coupling of 2-aminothiazole with triacetylbenzoyl chloride. Compound **61** showed inhibitory activity against topoisomerase I with IC₅₀ > 100.0 μM. From these results of AGABT derivatives

it was apparent that, the presence of benzene ring is essential for inhibitory activity against topoisomerase I and at least three acetyl moieties are required for the inhibitory activity against topoisomerase I.

3.3.4.4. Topoisomerase I inhibitory activities of GABT derivatives

GABT derivatives (Fig. 38) were prepared by the same method through deacetylation of its corresponding acetyl derivatives using hydrazine monohydrate except for **58d** which was prepared from 2-aminobenzothiazole with benzoyl chloride. The prepared compounds were subjected for evaluation of its inhibitory activities against topoisomerase I. Structure activity relationship was examined for the prepared compounds in order to identify the essential parts responsible for the activity (Table 10).

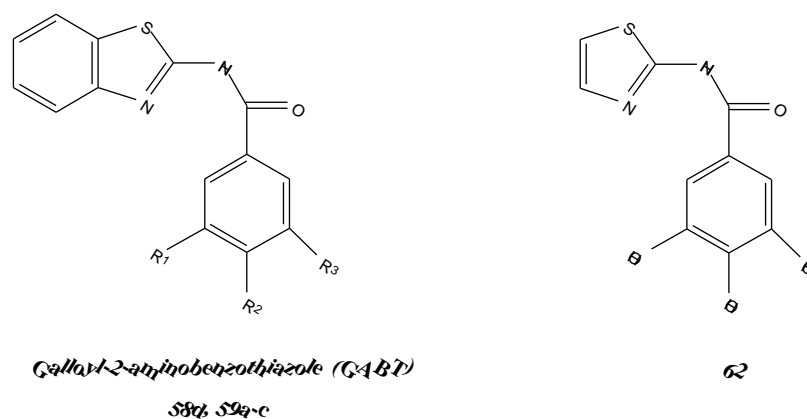


Fig. 38. Various GABT derivatives.

Table 10. Topo I inhibitory activities of GABT derivatives.

AGABT #	# of OH	R ₁	R ₂	R ₃	Topo I inhibitory activity (IC ₅₀ , μ M)
59a	3	OH	OH	OH	8.2
59b	2	OH	OH	H	16.9
59c	1	H	OH	H	> 100.0
58d	0	H	H	H	> 100.0
62	3	OH	OH	OH	34.4

As shown in Table 10, compounds **59a**, **59b**, **59c**, and **58d** showed inhibitory activity against topoisomerase I with IC₅₀ of 8.2, 16.9, > 100 and > 100 μ M, respectively. These results show that with increasing the number of hydroxy groups in these derivatives, enhancement of the inhibitory activity against topoisomerase I was observed, i.e., IC₅₀ of

59a which contains three hydroxy groups was 8.2 μM while the IC_{50} of **59b** which contains two hydroxy groups was 16.9 μM (**59a** is 2 folds potent activity over **59b**) and the IC_{50} of both **59c** which contains one hydroxy group and **58d** without any hydroxy group was $> 100 \mu\text{M}$. This indicates that the presence of hydroxy moiety is essential for the activity in such GABT derivatives. In order to identify the effect of presence or absence of benzene ring of 2-aminobenzothiazole on the inhibitory activity of topoisomerase I, compound **62** was prepared by the same method of deacetylation from its corresponding acetyl derivatives. The IC_{50} of **62** was 34.4 μM . From these results of GABT it was apparent that the presence of hydroxy groups is essential for the activity (at least two hydroxy groups required for the activity) as the enhancement of the inhibitory activity against topoisomerase I was observed upon increasing the number of hydroxy groups. The presence of benzene ring of 2-aminobenzothiazole is also essential for the activity because compound **59a** which includes benzene ring is about 4.2 folds potent than **62** which did not contain benzene ring.

3.3.5. Inhibitory property

The inhibitory properties of *o*-GPD **56a** were examined using these methods;

3.3.5.1. Inhibition of topoisomerase I and II by *o*-GPD

As shown in Fig. 39, in the presence of increasing *o*-GPD, topoisomerase I and II activities were inhibited in concentration dependence, and 0.9 μM and 0.09 μM of *o*-GPD gave 50% inhibition (IC_{50}) against topoisomerase I and II activities, respectively. The type of inhibition exhibited by *o*-GPD was determined by Lineweaver-Burk plots⁽¹⁴²⁾ of substrate concentrations against the rate of relaxation of supercoiled pBR322 DNA by topoisomerase I and II in the presence and absence of *o*-GPD. As shown in Fig. 40, the relaxation activities of pBR322 DNA by topoisomerase I and II were inhibited in a noncompetitive manner by *o*-GPD, and the K_i values were 0.4 μM and 74.1 nM, respectively. The Michaelis constants (K_m values) of relaxation by topoisomerase I and II were 3.7 nM and 10.5 nM, respectively. In view of inhibitory potency (K_i / K_m) against DNA relaxation by topoisomerase I and II, *o*-GPD was 15-fold potent inhibitor against topoisomerase II than topoisomerase I. From these results, *o*-GPD was found to be highly potent inhibitor for topoisomerase I and II and it was considered to bind to a different site from the binding site of the substrate DNA on the enzyme molecule.

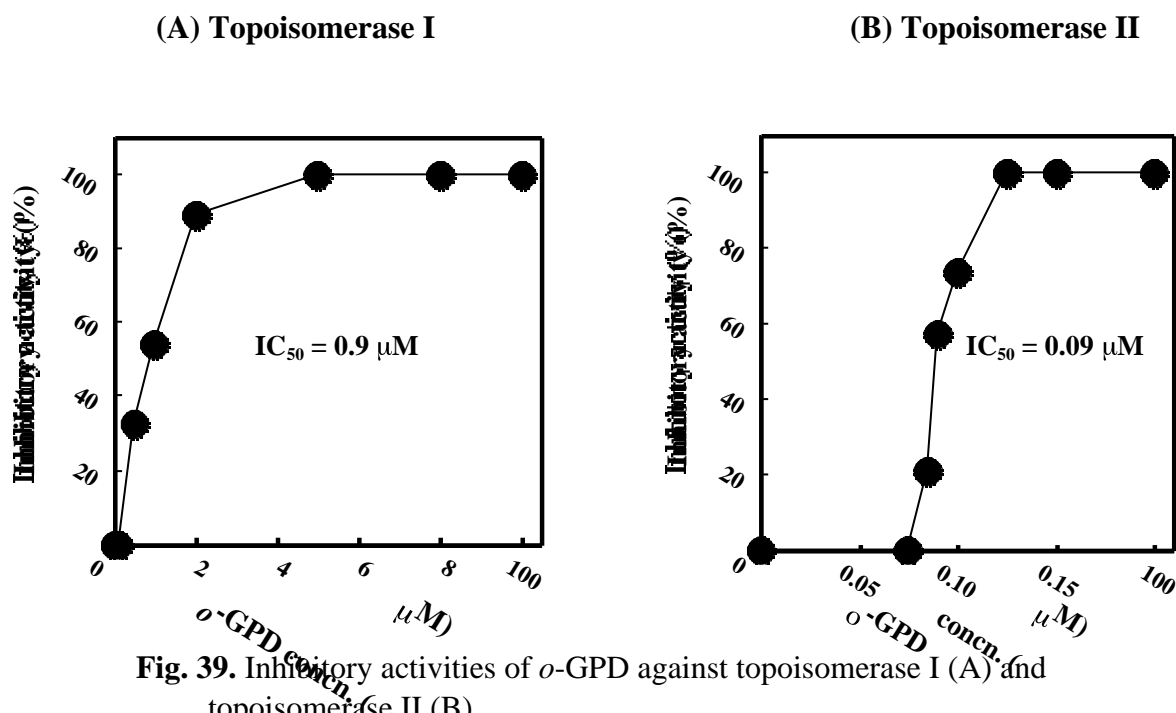
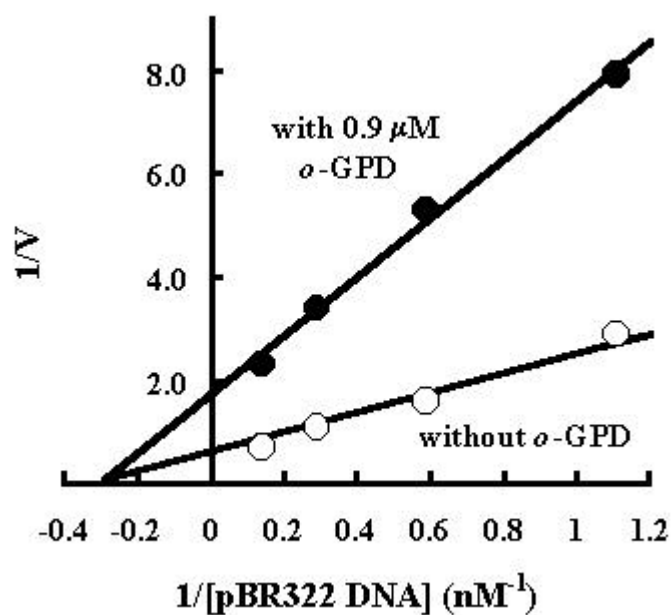


Fig. 39. Inhibitory activities of *o*-GPD against topoisomerase I (A) and topoisomerase II (B).

(A) Topoisomerase I



(B) Topoisomerase II

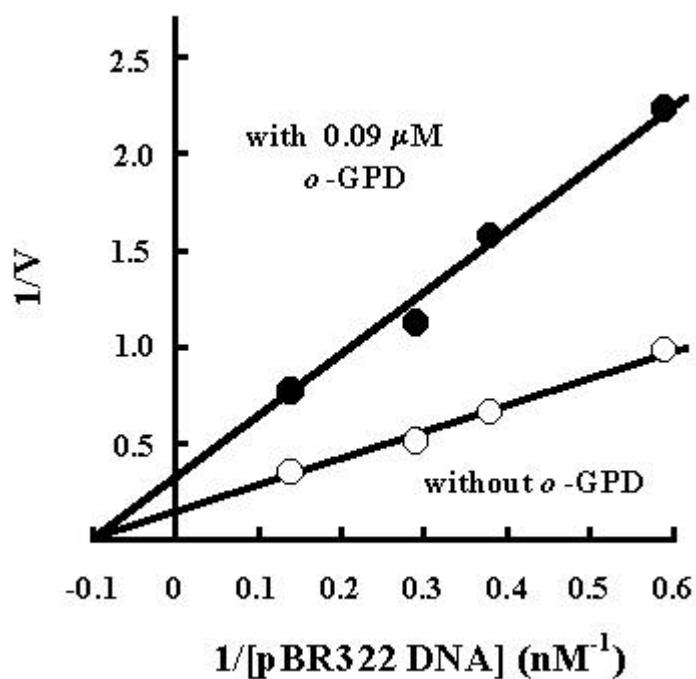


Fig. 40. Lineweaver-Burk plots of substrate (supercoiled pBR322 DNA) concentrations against rate of relaxation by (A) topoisomerase I (B) topoisomerase II with and without $o\text{-GPD}$.

3.3.5.2. Stabilization of topo-cleavable complex by $o\text{-GPD}$

Topoisomerase inhibitors of the cleavable complex-forming type, such as camptothecin

and etoposide, stabilize the cleavable complex (topo-DNA reaction intermediate) and inhibit the DNA rejoining reaction of topoisomerase; this inhibitory mechanism of the inhibitors induces nicked or linearized DNA in the cleavage assay.^(143, 144) To determine whether *o*-GPD is an inhibitor of the cleavable complex-forming type or not, cleavage assays were carried out. Camptothecin and etoposide were used as the controls for cleavable complex-forming inhibitors against topoisomerase I and II, respectively. As shown in Fig. 41, camptothecin induced nicked DNA with increasing concentrations. Unlike camptothecin, *o*-GPD could not induce nicked DNA even at 100 μ M. The results for the stabilization of topoisomerase II-cleavable complex are shown in Fig. 41. Etoposide induced the linearized DNA, but *o*-GPD failed to linearize DNA even at 1000 μ M. These results suggest that *o*-GPD did not inhibit topoisomerase I and II by stabilizing the cleavable complex and *o*-GPD is considered as an inhibitor of the cleavable-nonforming type. The inhibition mechanism of *o*-GPD against topoisomerase I and II differed from camptothecin and etoposide. *o*-GPD may directly act on topoisomerase I and II molecules in an earlier step than the formation of the topoisomerase-DNA complex and inhibit the DNA breaking and rejoining reactions by the enzymes.

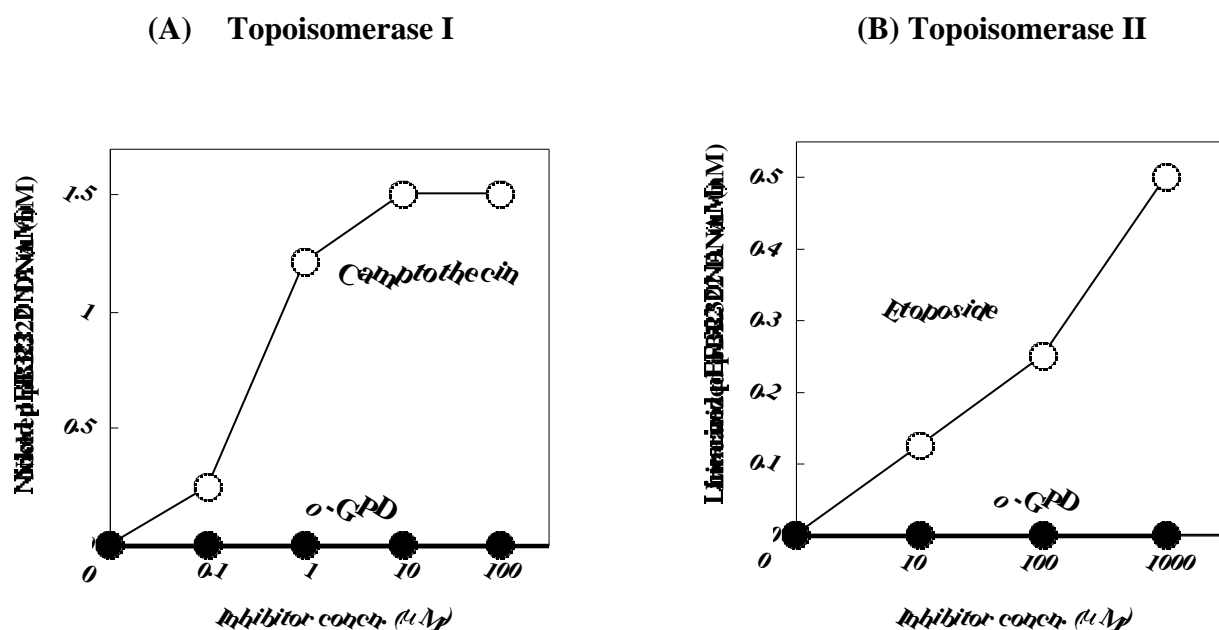


Fig. 41. Stabilization of topoisomerase I and II cleavable complexes by *o*-GPD, camptothecin and etoposide.

3.3.5.3. DNA interaction by *o*-GPD

Some topoisomerase inhibitors such as doxorubicin and amascarine are DNA intercalators. To determine whether *o*-GPD has the ability to intercalate into DNA strands, CD (circular dichroism) spectral change of DNA by addition of *o*-GPD was measured, since the spectrum is sensitive to the conformation changes induced in DNA by intercalators.^(145,146) Doxorubicin was used as control of intercalator at the same concentration. As shown in Fig. 42, the spectrum of DNA changed greatly with increasing the concentrations of doxorubicin. On the other hand, there is no spectral changes by *o*-GPD was noticed, therefore, it is clear that *o*-GPD has no ability to intercalate into DNA. Thus, *o*-GPD is a selective inhibitor against topoisomerase I and II, and it is different from inhibitors causing DNA damage such as cleavable complex-forming inhibitors and DNA intercalators.

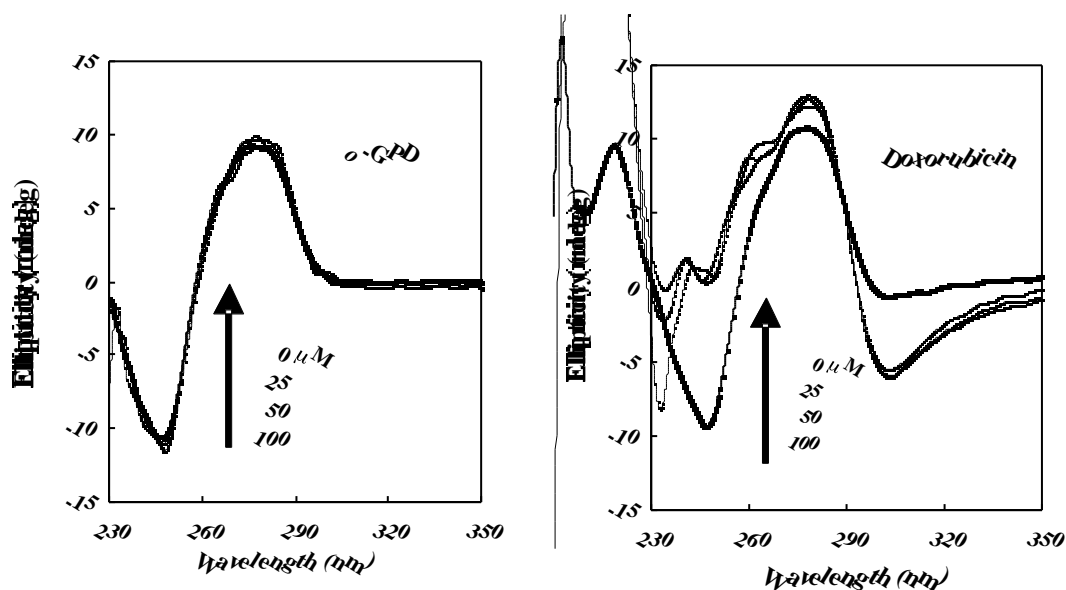


Fig. 42. CD spectral changes of DNA by addition of *o*-GPD and doxorubicin.

3.3.5.4. Effect of *o*-GPD on the growth and cell cycle of HeLa cells

The cell growth inhibition of *o*-GPD was determined in HeLa cell by Alamar blue assay.⁽¹⁴⁷⁾ As shown in Fig. 43, the values of cell growth inhibition (GI_{50}) of *o*-GPD, camptothecin and etoposide were 30, 0.6 and 40 μ M, respectively. Camptothecin and etoposide promote the accumulation of damaged DNA by stabilization of the cleavable complex in the cells, thereby arresting cell cycle progression. The cell cycle progression was analyzed with a flow cytometer. As shown in Fig. 44, HeLa cells were arrested at the S phase and G2/M phase when cultured with 0.1 μ M camptothecin and 2 μ M etoposide, respectively. On the other hand, *o*-GPD did not affect on the cell cycle even at an extremely high concentration (100 μ M). The results suggest that the cytotoxicity of *o*-GPD is clearly different from that of camptothecin and etoposide, and these results are in a good agreement with the results of the cleavage assay of *o*-GPD. The results suggest that *o*-GPD do not cause damage of DNA by cleavage complex formation in the cells.

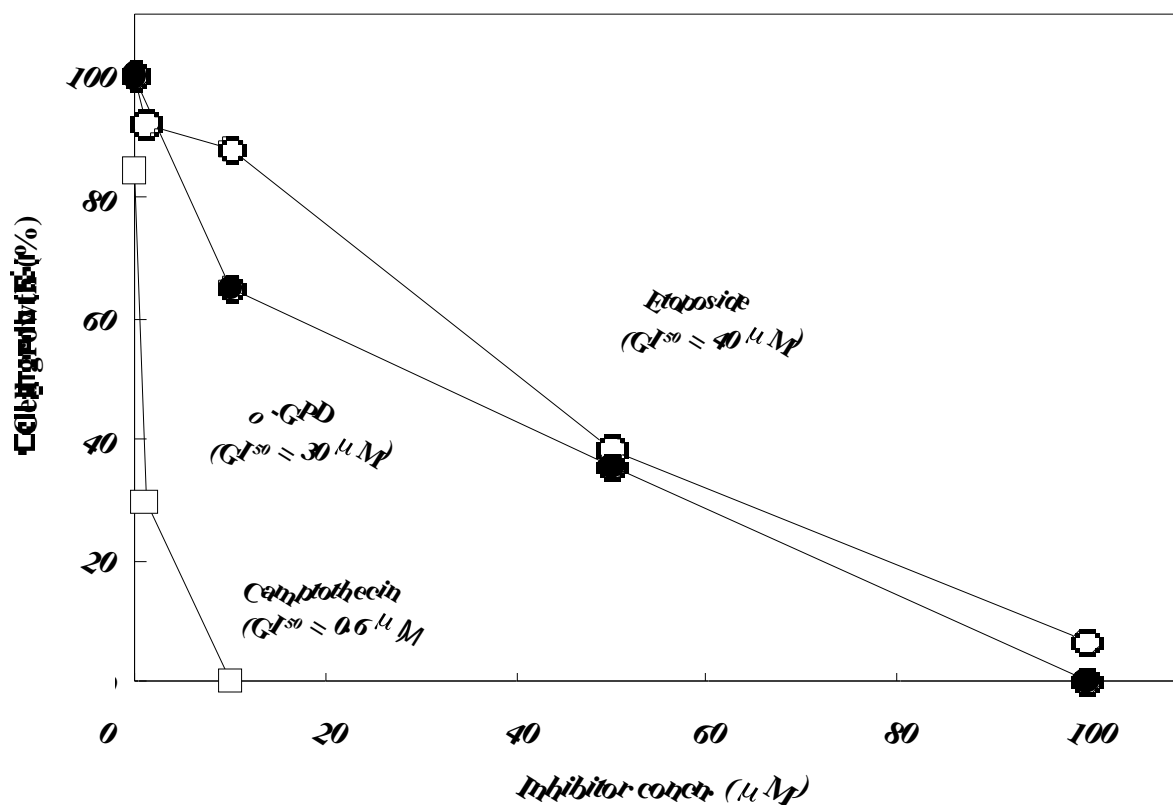


Fig. 43. Inhibitory effects of *o*-GPD, camptothecin and etoposide on cell growth.

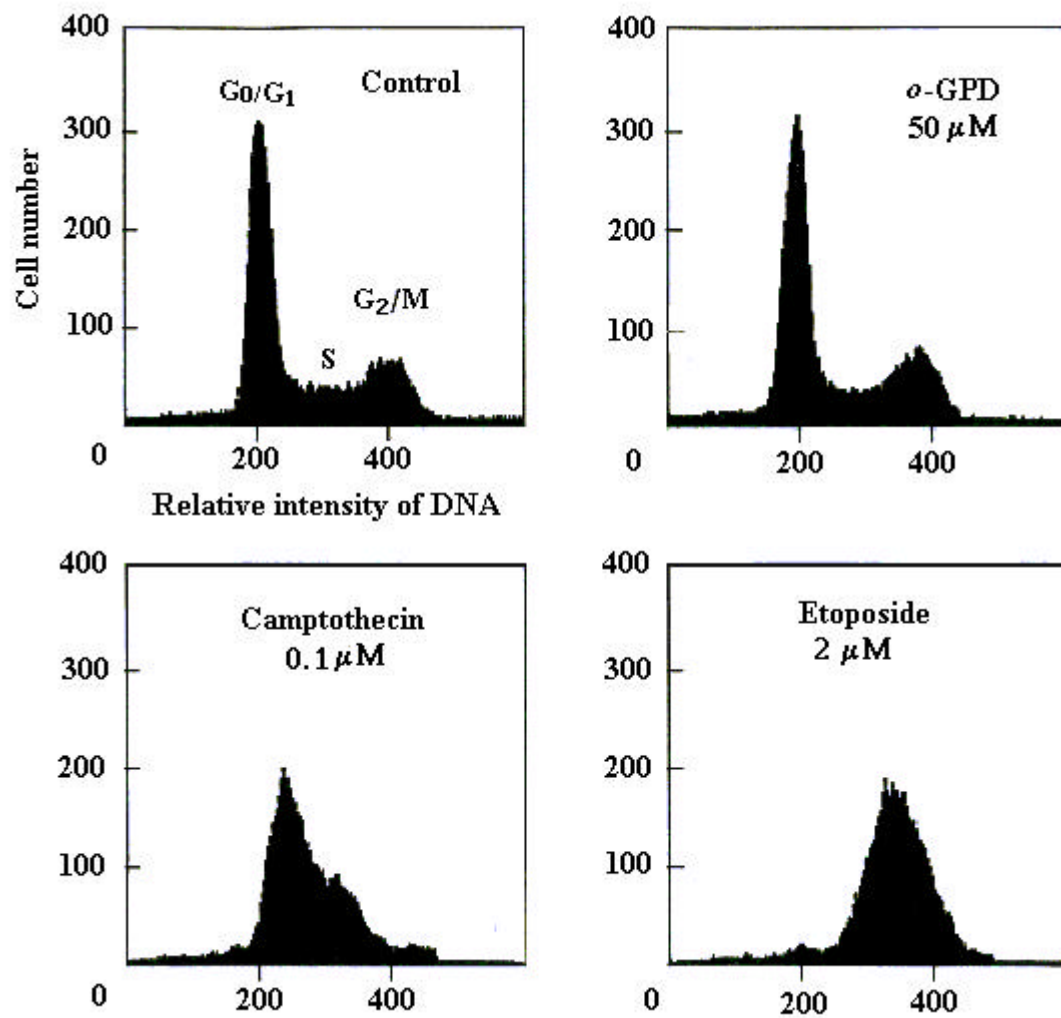


Fig. 44. Effects of *o*-GPD, camptothecin and etoposide on cell cycle of HeLa cells.

IV. General Conclusion

4.1. General conclusion of peptide nucleic acid chapter

This chapter was concentrated about peptide nucleic acids, which are very potent DNA mimic capable of hybridization to complementary DNA, RNA or PNA. Due to the favorable RNA- and DNA-binding properties of PNA and the selective binding of 2',5'-isoDNA to RNA this have led us to design a novel type of 2',5'-isoDNA mimic chiral peptide nucleic acids (isoPNAs). The novel PNA was glycyl- -alanine isoPNA (isogaPNA) in R- and S- forms. They were prepared in high degree of purity through several steps starting from D- and L- aspartic acids protected with Boc and benzyl groups. In order to assess the hybridization properties of these monomers to complementary DNA and RNA, the two monomers were individually introduced into *aeg*PNA sequences in different positions to form varied thymine dodecamers. The hybridization properties of these PNA oligomers with complementary DNA and RNA were investigated in UV-melting experiments (260 nm) and the results of these PNA oligomers were compared to the dodecamer prepared totally from unmodified *aeg*PNA. The results indicted that most of these PNA oligomers have good binding affinity to complementary DNA and RNA in the same time, and some of these oligomers show similar T_m values to the unmodified *aeg*PNA oligomer when hybridized to DNA. On the other hand, decreased T_m values lower than that of the unmodified *aeg*PNA oligomer was observed upon binding of these oligomers to its complementary RNA. Most of these oligomers did not show selectivity to either DNA or RNA. Unfortunately, no hybridization was found for the complexes of complete isogaPNA T_R or T_S with DNA or RNA, respectively. Introduction of mismatch base thymine to dA₁₂ at the position to hybridize with isogaPNA T_R and T_S resulted in decrease of the T_m . These results show the conformation of backbone of T_R is more effective sterically than that of T_S . Furthermore, the binding stoichiometry was found by UV titration (Job-Plot) to be 2:1, indicating a PNA₂-DNA triplex structure. The decreased T_m values compared to unmodified *aeg*PNA oligomer indicate that the length of the glycyl- -alanine backbone of isogaPNA may not be optimal to hybridize with DNA and RNA. This led us to think and design of new type of PNA, glycyl-glycine isoPNA (isoggPNA) monomer which is a lower homolog of the isogaPNA. This new isoggPNA was prepared starting from L-serine after several trials and obtained in a high degree of purity. The work now is in progress for the preparation of various oligomers containing isoggPNA monomer to check its hybridization properties to complementary DNA and RNA. Also another PNA monomer which is isogaPNA containing adenine as a nucleobase in stead of thymine was prepared in high degree of purity starting from D-aspartic acid by the same procedure as that for the preparation of isogaPNA thymine monomer. Several oligomers

consisting of 8 monomers (octomers) were prepared by introducing isogaPNA adenine monomer in different sequences of *aeg*PNA adenine monomers. The prepared oligomers were checked by MALDI-TOF mass spectrum analysis. Unfortunately, despite the several trials to purify these oligomers using HPLC under various conditions, we could not obtain pure oligomers to investigate the hybridization properties to complementary DNA and RNA. On the other side there is no report on the derivatization of PNA monomers, which are potent isosters of natural nucleosides, in order to develop new antivirals. Several PNA monomers with a backbone containing a hydroxyl group, which is important for the phosphorylation by cellular kinases in the activation mechanism of antiviral nucleoside, were prepared through reduction of their corresponding carboxylic acid derivatives. These monomers with hydroxyl group and other monomers with carboxylic group in addition to some intermediates were investigated for the anti-HIV and anti-HSV activities but unfortunately the antiviral activities against HIV-1 and HSV-1 were not observed. However, interestingly the isogaPNA thymine monomer (S) derived from L-aspartic acid (50 µg/ml) only showed potent cytotoxicity on VERO cells whereas the other isogaPNA thymine monomer (R) derived from D-aspartic acid show no activity. This proves that the chirality of the molecules is one of the important factors to show pharmacological activities.

4.2. General conclusion for the topoisomerases inhibitors chapter

This chapter was focused on the topoisomerases inhibitors, topoisomerases are essential nuclear enzymes involved in producing the necessary topological and conformational changes in DNA which are critical to many cellular processes such as replication, recombination and transcription. In addition to their normal cellular functions, both topoisomerase I and II enzymes have emerged as important targets for chemical intervention in the development of antitumor drugs. From this idea we begin to study several polyhydroxyphenyl compounds prepared by the coupling of various acetylated acid chlorides with *o*-, *m*-, *p*- phenylenediamine and 2-aminobenzothiazole. The formed acetylated coupled compounds were completely deacetylated using hydrazine monohydrate, and the obtained polyhydroxy derivatives in addition to the acetylated ones were evaluated for their topoisomerases inhibitory activities. Structure-activity relationship was examined in order to determine the essential parts for the activity of these compounds. The results show that most of these compounds show strong inhibitory activity against topoisomerase I and II, the polyhydroxyl form of the *o*-phenylenediamine (*o*-GPD), indicating strong topoisomerase I inhibitory activity with $IC_{50} = 0.9 \mu M$ and very strong topoisomerase II inhibitory activity with $IC_{50} = 0.09 \mu M$.

On the other hand, the polyhydroxyl form of the *m*-phenylenediamine indicating strong topoisomerase I inhibitory activity with $IC_{50} = 1.6 \mu M$ and very strong topoisomerase II inhibitory activity with $IC_{50} = 0.08 \mu M$, while the polyhydroxyl form of the *p*-phenylenediamine indicating strong topoisomerase I inhibitory activity with $IC_{50} = 1.4 \mu M$ and very strong topoisomerase II inhibitory activity with $IC_{50} = 0.08 \mu M$. Other derivatives show good inhibitory activity against topoisomerase I and II. A study on the inhibitory properties of *o*-GPD was carried out and the results indicated that, *o*-GPD is highly potent inhibitor for topoisomerase I and II and it was consider to bind to a different site from the binding site of the substrate DNA on the enzyme molecule. Cleavage assays were carried out also on *o*-GPD to determine whether this compound is an inhibitor of the cleavable complex-forming type or not. The results show that *o*-GPD is considered as an inhibitor of the cleavable-nonforming type and the inhibition mechanism of *o*-GPD against topoisomerase I and II differed from camptothecin and etoposide, *o*-GPD may act directly on topoisomerase I and II molecules in an earlier step than the formation of the topoisomerase-DNA complex and inhibit the DNA breaking and rejoining reactions by the enzymes. CD spectral change of DNA by addition of *o*-GPD was also measured, and there is no spectral changes noticed by *o*-GPD, indicating that *o*-GPD has no ability to intercalate into DNA. Also the cell growth inhibition of *o*-GPD was determined in HeLa cell by Alamar blue assay, and indicated that the GI_{50} of *o*-GPD was $30 \mu M$. *o*-GPD did not affect on the cell cycle even at an extremely high concentration ($100 \mu M$), the results suggest that the cytotoxicity of *o*-GPD is clearly different from that of camptothecin and etoposide.

v. Experimental

5.1. Development of novel peptide nucleic acids

Materials

All reagents which used for the preparation of PNA monomers and their intermediates were purchased from Wako, Aldrich, Nacalai tesque, Tokyo Kasei and Novabiochem and used without purification. Solvents were HPLC grade from Wako, Aldrich and Sigma. Dry solvents were obtained by use of appropriate molecular sieves, except for tetrahydrofuran was distilled from sodium/benzophenone. The water content in dry solvents did not exceed 50 ppm as determined by Karl-Fischer titration. Reagents for PNA synthesis were obtained from Applied Biosystems and other commercial sources.

General methods

TLC was performed on analytical Merck 9385 silica glass plates with F_{254} indicator. TLCs were viewed either under 254 nm UV or by staining with phosphomolybdic acid in ethanol or iodine crystals where appropriate. Column chromatography was performed as flash chromatography on Merck 9385 silica gel 60 (0.040-0.063 mm). Reactions were carried out under Argon gas. Accurate masses were obtained on Micromass LCT mass spectrometer which was recorded in the positive ion mode with leucine enkephalin as an internal lock mass standard. Elemental analysis was performed at the microanalytical laboratory, Institute of Resource Development and Analysis, Kumamoto University. Melting points were determined on electrothermal melting point apparatus and were uncorrected. IR data were obtained on Jasco Fourier Transform-410 Infrared spectrometer as KBr discs for the crystalline products and on NaCl cell for the oily products. NMR spectra were obtained on a 300 MHz AL spectrometer. δ -Values are in ppm relative to DMSO- d_6 (2.50 for proton and 39.5 for carbon) or $CDCl_3$ (7.29 for proton and 76.9 for carbon). MALDI-TOF mass spectrometry measurements of PNA oligomers were performed in a (Kratos) MALDI mass spectrometer using α -cyanocinnamic acid as the matrix. The angle of optical rotation of different isogaPNA monomers and its intermediates was measured on Jasco DIP-1000 KUY digital polarimeter. Reversed-phase HPLC chromatography was performed on a Shodex HPLC system equipped with Wakosil-II 5C18-100, 7.5 mm x 300mm (W). For the purification of different isogaPNA oligomers the following conditions were used. Buffer A: 0.1% (v/v) trifluoroacetic acid in water (pH 2.0), buffer B: 0.1% (v/v) trifluoroacetic acid in acetonitrile. Flow rate: 1 or 0.7 ml/min. Condition A) a 65 minutes linear gradient from 90 to 0% and B) from 10 to 100%, all runs were performed at 50°C. Fractions containing the PNA were lyophilized. Pure isogaPNAs oligomers were stored frozen at -

30°C) solutions in deionized water. Complementary DNA was purchased from Greiner-Japan, RNA was purchased from Takara, Tokyo, Japan. DNA and RNA sequences were purified by both cation exchange and reversed-phase HPLC, yielding chromatographically pure materials. Synthesis of isogaPNA oligomers was carried out using Expedite 8909 Synthesizer, all the reagents used for automated PNA synthesis including *aeg*PNA monomers (thymine and adenine), HATU, PNA base solution (0.2 M DIEA, 0.3M 2,6-lutidine), PNA deblocking solution (20% piperidine in DMF), PNA capping solution (5% acetic anhydride, 6% 2,6-lutidine in DMF), PNA diluent (N-methylpyrrolidone, NMP), Wash A (DMF), and Wash B (99.8% anhydrous DMF) were purchased from Applied Biosystems, columns for solid phase synthesis containing Fmoc-XAL PEG resin (GEN063053, Applied Biosystems) should be stored in a desiccant box at –20°C and allowed to warm to room temperature prior to use. Powders such as monomers, activators and lysine should be unpacked on arrival and placed in a sealed container containing Drierite desiccant at –20°C and to ensure proper synthesis, dry the powdered reagents *in vacuo* overnight prior to use. A PNA:DNA annealing buffer and PNA:DNA melting temperature buffer was 0.1 M Na₂HPO₄, pH 7.4. Thermal melting experiments including UV absorption spectra and melting curves (absorbance versus temperature) were performed on 3-cm path-length cells using Jasco V-530 UV-VIS spectrophotometer with Jasco ETC-505T temperature controller with a programmed temperature increase of 0.5°C/min. Lenti RT activity kit for examination of anti-HIV-1 was from CAViDiTECH, MEM (minimum essential medium) used for the anti-HIV-1 and anti-HSV-1 assay was purchased from Gibco.

5.1.1. Synthesis of isogaPNA thymine monomer (R)

3-*tert*-Butoxycarbonylamino-4-hydroxybutyric acid benzyl ester (R) (2)⁽⁷⁶⁾

To a stirred solution of **1** (1.94 g; 6.00 mmol) in distilled THF (30 ml) at –10°C, under Argon atmosphere, N-methylmorpholine (0.61 g; 6.00 mmol) was added in a dropwise manner followed by ethyl chloroformate (0.65 g; 6.00 mmol), after 10 minutes sodium borohydride (0.68 g; 18.00 mmol) was added in one portion, methyl alcohol (30 ml) was then added in a dropwise manner to the mixture over a period of 10 minutes at 0°C. The solution is stirred for additional 10 minutes, and then neutralized with 1N HCl or 1N H₂SO₄ (12 ml). The organic solvents were evaporated under reduced pressure and the product was extracted with ethyl acetate (3 x 20 ml). The combined organic phase was washed consecutively with 1N HCl or 1N H₂SO₄ (40 ml), H₂O (50 ml), 5% NaHCO₃ (50 ml), H₂O (2 x 50 ml), dried over MgSO₄ or Na₂SO₄, and the solvent was

evaporated under reduced pressure. The crude product was purified with silica gel column chromatography using ethyl acetate: n-hexane (1:4) and then with ethyl acetate: n-hexane (1:1) to give **2** as white crystals in yield of 1.12g (60.0%): mp 59.5-60°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3482 (OH), 3372 (NH), 1724, 1684 (C=O); $^1\text{H-NMR}$ (300 MHz, CDCl_3)

1.45 (9H, s, $\text{C}(\text{CH}_3)_3$), 2.69 (2H, d, $J = 6.30$ Hz, CH_2), 2.94 (1H, s, OH), 3.69 (2H, t, $J = 5.10$ Hz, CH_2), 3.96-4.08 (1H, m, CH), 5.14 (2H, s, CH_2), 5.30 (1H, d, $J = 12.00$ Hz, NH), 7.37 (5H, s, C_6H_5); $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) 28.26 (CH_3), 35.97 (CH_2), 49.36 (CH), 64.19 (CH_2), 66.52 (CH_2), 79.73 (C), 128.15 (CH), 128.24 (CH), 128.50 (CH), 135.52 (C), 155.74 (C), 171.55 (C), FAB-Mass: m/z 310 ($\text{M}+1$).

4-(3-Benzoyl-5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-3-tert-butoxy-carbonylaminobutyric acid benzyl ester (R) (3)^(77,78)

To a stirred suspension of **2** (0.31 g; 1.00 mmol), N^3 -Benzoylthymine (0.23 g; 1.00 mmol) and triphenylphosphine (0.29 g; 1.10 mmol) in dry THF (10 ml) was added diethyl azodicarboxylate (182 μl ; 1.10 mmol) in a dropwise manner at -15°C . The reaction mixture was stirred at room temperature for 24 hours, and then the clear solution was evaporated to dryness. The residue was purified by silica gel column chromatography using CH_2Cl_2 : acetone (50:1) as a mobile phase a foamy product was formed which was recrystallized from ethyl acetate: n-hexane to give **3** as white crystals in yield of 0.24 g (45.0%): mp 76-77°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3359 (NH), 1747, 1705, 1654 (C=O); $^1\text{H-NMR}$ (300 MHz, CDCl_3) 1.39 (9H, s, $\text{C}(\text{CH}_3)_3$), 1.86 (3H, s, CH_3), 2.60 (2H, d, $J = 5.40$ Hz, CH_2), 3.45-4.22 (2H, m, CH_2), 4.25-4.37 (1H, m, CH), 5.10 (2H, s, CH_2), 5.55 (1H, d, $J = 9.00$ Hz, NH), 7.08 (1H, s, CH-6 of T), 7.32 (5H, s, C_6H_5), 7.45 (2H, t, $J = 7.65$ Hz, PhC=O), 7.60 (1H, t, $J = 7.35$ Hz, PhC=O), 8.07 (2H, d, $J = 7.80$ Hz, PhC=O); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) 12.22 (CH_3), 28.34 (CH_3), 36.25 (CH_2), 46.24 (CH), 52.04 (CH_2), 66.83 (CH_2), 79.94 (C), 110.07 (C), 128.30 (CH), 128.46 (CH), 128.65 (CH), 129.05 (CH), 130.71 (CH), 131.81 (C), 134.86 (CH), 135.41 (C), 140.82 (CH), 150.20 (C), 155.42 (C), 163.22 (C), 169.19 (C), 170.88 (C); FAB-Mass: m/z 522 ($\text{M}+1$); (found C, 64.53; H, 5.74; N, 8.13. $\text{C}_{28}\text{H}_{31}\text{N}_3\text{O}_7$ requires C, 64.48; H, 5.99; N, 8.06).

3-Amino-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid benzyl ester (R) (4)⁽⁷⁹⁾

A solution of 3N HCl in ethyl acetate (3 ml) was added to compound **3** (0.10 g; 0.20 mmol). After stirring of the reaction mixture for 30-45 minutes at room temperature, the reaction was monitored by TLC and excess of the reagent was removed under reduced

pressure. The obtained oily hydrochloride salt was dried for 2 hours in *vacuo* over potassium hydroxide, neutralized with 5% sodium bicarbonate until pH (7-8) and then extracted with ethyl acetate several times. The ethyl acetate extracts were washed with saturated sodium chloride and then with distilled water, evaporation of the ethyl acetate extracts gave **4** as an oily compound in yield of 0.04 g (70.0%): ¹H-NMR (300 MHz, CDCl₃) 1.88 (3H, s, CH₃), 2.43 (1H, dd, *J* = 9.00 and 9.00 Hz, CHH), 2.62 (1H, dd, *J* = 3.90 and 3.90 Hz, CHH), 3.57-3.67 (2H, b, NH₂), 3.90-4.05 (2H, m, CH₂), 4.17 (1H, q, *J* = 7.20, CH), 5.12 (2H, s, CH₂), 6.98 (1H, s, CH-6 of T), 7.34 (5H, s, C₆H₅); ¹³C-NMR (125 MHz, CDCl₃) 12.10 (CH₃), 36.10 (CH₂), 45.47 (CH), 52.87 (CH₂), 66.46 (CH₂), 107.08 (C), 128.07 (CH), 128.14 (CH), 129.00 (CH), 135.98 (C), 136.11 (CH), 163.97 (C), 168.32 (C), 169.96 (C); FAB-Mass: *m/z* 318 (*M*+1), (found C, 60.48; H, 6.13; N, 13.18. C₁₆H₁₉N₃O₄ requires C, 60.56; H, 6.03; N, 13.24).

3-[2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)acetylamino]-4-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)butyric acid benzyl ester (R**) (**5**)**

To a stirred solution of **4** (1.00 g; 3.15 mmol), Fmoc-glycine (0.94 g; 3.15 mmol) and *N*-ethyldiisopropylamine (1.10 ml; 6.30 mmol) in DMF (20 ml) at 0°C under Argon gas, HOBt.H₂O (0.48 g, 3.15 mmol) was added followed by TBTU (1.01 g; 3.15 mmol). The mixture was stirred at 0°C for 1 hour and then at room temperature for additional 4 hours. After evaporation of DMF completely, the residue was dissolved in ethyl acetate (100 ml). The ethyl acetate was washed successively with 5% aqueous KHSO₄ (20 ml), water (20 ml), 5% NaHCO₃ (20 ml) and water (20 ml). The solution was dried over MgSO₄ and then the solvent was evaporated under reduced pressure to give a crude crystalline product, which was recrystallized from EtOAc: n-hexane to give **5** as a white crystalline product in yield of 1.32 g (70.0%): mp 128-129°C; [α]_D²⁵ – 26.77° (c 0.1, methanol); ^{max}/cm⁻¹ 3329 (NH) 1724, 1645 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 1.74 (3H, s, CH₃), 2.52-2.60 (2H, m, CH₂), 3.48 (2H, t, *J* = 5.70 Hz, CH₂), 3.90 (2H, d, *J* = 6.60 Hz, CH₂), 4.18-4.27 (3H, m, CH₂, CH), 4.46-4.57 (1H, m, CH), 5.01 (2H, s, CH₂), 7.24 (1H, s, CH-6 of T), 7.30 (2H, d, *J* = 7.50 Hz, Ar-H), 7.33 (5H, s, C₆H₅), 7.40 (2H, dd, *J* = 7.20 and 7.50 Hz, Ar-H), 7.70 (2H, d, *J* = 7.20 Hz, Ar-H), 7.87 (2H, d, *J* = 7.50 Hz, Ar-H), 10.83 (1H, b, NH); ¹³C-NMR (125 MHz, DMSO-*d*₆) 12.29 (CH₃), 36.85 (CH₂), 42.55 (CH₂), 43.58 (CH₂), 44.28 (CH), 44.51 (CH), 65.44 (CH₂), 66.22 (CH₂), 107.02 (C), 119.85 (CH), 121.20 (CH), 127.12 (CH), 127.74 (CH), 127.77 (CH), 128.22 (CH), 128.76 (CH), 136.24 (CH), 137.32 (C), 139.32 (C), 142.50 (C), 151.43 (C), 164.04 (C), 168.89 (C), 170.21 (C), 171.96 (C); FAB-Mass: *m/z* 597 (*m*+1); (found C, 65.93; H, 5.51; N, 9.54. C₃₃H₃₂N₄O₇ requires C, 66.43; H, 5.41; N, 9.39).

3-[2-(9H-Fluoren-9-ylmethoxycarbonylamino)acetylamino]-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid (R) (6)⁽⁸⁰⁾

To a stirred solution of **5** (0.60 g; 1.00 mmol) in ethanol (10 ml) under Argon gas 10 % Pd/C (1.01 g) was added followed by 1,4-cyclohexadiene (1.64 g, 20.00 mmol). The mixture was stirred over night and then filtered through celite. After washing several times with hot ethanol, the combined organic solvent was evaporated and the residue was dried in vacuum then 5 % NaHCO₃ (10 ml) was added. The mixture was extracted with ethyl acetate for the removal of byproduct. The sodium salt was neutralized with 10 % KHSO₄ till pH (2-3) and then extracted with ethyl acetate (3 x 20 ml). The combined ethyl acetate extracts were washed consequently with saturated sodium chloride solution and distilled water, dried over MgSO₄ and evaporated under reduced pressure to give a crude product, which was recrystallized from ethanol to give **6** as white crystals in yield of 0.33 g (65.0%): mp 144°C; $[\alpha]_D^{25} - 30.56^\circ$ (c 0.1, methanol); $\nu_{\text{max}}/\text{cm}^{-1}$ 3311 (NH) 1714, 1638 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 1.75 (3H, s, CH₃), 2.35-2.46 (2H, m, CH₂), 3.30 (1H, br, NH), 3.41-3.58 (2H, m, CH₂), 3.84-3.98 (2H, m, CH₂), 4.18-4.30 (3H, m, CH₂, CH), 4.40-4.53 (1H, m, CH), 7.23 (1H, s, CH-6 of T), 7.32 (2H, dd, *J* = 7.20 and 7.50 Hz, Ar-H), 7.40 (2H, dd, *J* = 7.20 and 7.50 Hz, Ar-H), 7.71 (2H, d, *J* = 7.50, Ar-H), 7.87 (2H, d, *J* = 7.50 Hz, Ar-H), 10.81 (1H, br, NH), 12.14 (1H, br, COOH); ¹³C-NMR (75 MHz, DMSO-*d*₆) 12.48 (CH₃), 37.03 (CH₂), 42.72 (CH₂), 43.39 (CH₂), 44.55 (CH), 46.63 (CH), 65.74 (CH₂), 107.13 (C), 120.06 (CH), 125.27 (CH), 127.06 (CH), 127.60 (CH), 136.28 (CH), 140.69 (C), 143.85 (C), 151.54 (C), 156.30 (C), 164.14(C), 168.48 (C), 171.94 (C); FAB-Mass: *m/z* 507 (M+1); (found C, 61.21; H, 5.19; N, 10.94. C₂₆H₂₆N₄O₇ requires C, 61.65; H, 5.17; N, 11.06).

5.1.2. Synthesis of isogaPNA thymine monomer (S)

3-tert-Butoxycarbonylamino-4-hydroxybutyric acid benzyl ester (S) (8)⁽⁷⁶⁾

To a stirred solution of **7** (1.94 g; 6.00 mmol) in distilled THF (30 ml) at -10°C, under Argon atmosphere, N-methylmorpholine (0.61 g; 6.00 mmol) was added in a dropwise manner followed by ethyl chloroformate (0.65 g; 6.00 mmol), after 10 minutes sodium borohydride (0.68 g; 18.00 mmol) was added in one portion, methyl alcohol (30 ml) was then added in a dropwise manner to the mixture over a period of 10 minutes at 0°C. The solution is stirred for additional 10 minutes, and then neutralized with 1N HCl or 1N H₂SO₄ (12 ml). The organic solvents were evaporated under reduced pressure and the product was extracted with ethyl acetate (3 x 20 ml). The combined organic phase was washed consecutively with 1N HCl or 1N H₂SO₄ (40 ml), H₂O (50 ml), 5%

NaHCO₃ (50 ml), H₂O (2 x 50 ml), dried over MgSO₄ or Na₂SO₄, and the solvent was evaporated under reduced pressure. The crude product was purified with silica gel column chromatography using ethyl acetate: n-hexane (1:4) and then with ethyl acetate: n-hexane (1:1) to give **8** as white crystals in yield of 1.15g (62.0%): mp 62°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3482 (OH), 3372 (NH), 1724, 1685 (C=O). ¹H-NMR (300 MHz, CDCl₃) 1.42 (9H, s, C(CH₃)₃), 2.65 (2H, d, *J* = 6.30 Hz, CH₂), 3.09 (1H, br, NH), 3.63 (2H, d, *J* = 4.80 Hz, CH₂), 3.95-4.05 (1H, m, CH), 5.11 (2H, s, CH₂), 7.34 (5H, s, C₆H₅); ¹³C-NMR (125 MHz, CDCl₃) 28.28 (CH₃), 36.07 (CH₂), 49.49 (CH), 64.29 (CH₂), 66.52 (CH₂), 79.73 (C), 128.14 (CH), 128.24 (CH), 128.51 (CH), 135.62 (C), 155.73 (C), 171.49 (C); FAB-Mass: *m/z* 310 (M+1).

4-(3-Benzoyl-5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-3-tert-butoxy-carbonylaminobutyric acid benzyl ester (9)^(77,78)

To a stirred suspension of **8** (0.31 g; 1.00 mmol), N³-Benzoylthymine (0.23 g; 1.00 mmol) and triphenylphosphine (0.29 g; 1.10 mmol) in dry THF (10 ml) under Argon gas was added diethyl azodicarboxylate (182 μ l; 1.10 mmol) in a dropwise manner at -15°C. The reaction mixture was stirred at room temperature for 24 hours, and then the clear solution was evaporated to dryness. The residue was purified by silica gel column chromatography using CH₂Cl₂: acetone (50:1) as a mobile phase, gave a foamy product which was recrystallized from ethyl acetate: n-hexane to give **9** as white crystals in yield of 0.23 g (44.0%): mp 77°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3360 (NH) 1747, 1705, 1654 (C=O); ¹H-NMR (300 MHz, CDCl₃) 1.37 (9H, s, C(CH₃)₃), 2.02 (3H, s, CH₃), 2.57 (1H, dd, *J* = 4.80 and 5.10 Hz, CHH), 2.66 (1H, dd, *J* = 4.80 and 5.10 Hz, CHH) 3.81 (1H, d, *J* = 9.60 Hz, NH), 4.11-4.30 (2H, m, CH₂), 4.32-4.42 (1H, m, CH), 5.12 (2H, s, CH₂), 7.11 (1H, s, CH-6 of T), 7.33 (5H, s, C₆H₅), 7.47 (2H, t, *J* = 7.50 Hz, PhC=O), 7.88 (1H, d, *J* = 7.50 Hz, PhC=O), 8.11 (2H, d, *J* = 7.20 Hz, PhC=O); ¹³C-NMR (125 MHz, CDCl₃) 12.06 (CH₃), 28.19 (CH₃), 36.10 (CH₂), 46.07 (CH), 51.88 (CH₂), 66.67 (CH₂), 79.79 (C), 109.90 (C), 128.14 (CH), 128.31 (CH), 128.49 (CH), 128.91 (CH), 130.55 (CH), 131.64 (C), 134.73 (CH), 135.25 (C), 140.69 (CH), 150.04 (C), 155.27 (C), 163.08 (C), 169.04 (C), 170.71 (C), FAB-Mass: *m/z* 522.5 (M+1); (found C, 64.02; H, 5.95; N, 8.12. C₂₈H₃₁N₃O₇ requires C, 64.48; H, 5.99; N, 8.06); (found C, 64.38; H, 5.95; N, 8.03. C₂₈H₃₁N₃O₇ requires C, 64.48; H, 5.99; N, 8.06).

3-Amino-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid benzyl

ester (S) (10)⁽⁷⁹⁾

A solution of 3N HCl in ethyl acetate (3 ml) was added to compound **9** (0.10 g; 0.20 mmol). After stirring the reaction mixture was for 30-45 minutes at room temperature, the reaction was monitored by TLC and excess of the reagent was removed under reduced pressure. The obtained oily hydrochloride salt was dried for 2 hours in *vacuo* over potassium hydroxide, neutralized with 5% sodium bicarbonate until pH (7-8) and then extracted with ethyl acetate several times, the ethyl acetate extracts were washed with saturated sodium chloride and then with distilled water, evaporation of the ethyl acetate extracts gave **10** as an oily compound in yield of 0.04 g (75.0%): ¹H-NMR (300 MHz, CDCl₃)_ 1.89 (3H, s, CH₃), 2.43 (1H, dd, *J* = 8.98 and 8.98 Hz, CHH), 2.63 (1H, dd, *J* = 3.85 and 4.03, CHH) 3.63 (2H, b, NH₂), 3.90-4.10 (2H, m, CH₂), 4.15-4.22 (1H, m, CH), 5.1 (2H, s, CH₂), 6.98 (1H, s, CH-6 of T), 7.34 (5H, s, C₆H₅),); ¹³C-NMR (125 MHz, CDCl₃)_ 12.21 (CH₃), 36.11 (CH₂), 45.52 (CH), 52.82 (CH₂), 66.53 (CH₂), 107.10 (C), 128.03 (CH), 128.12 (CH), 129.10 (CH), 135.90 (C) 136.01 (CH), 164.11 (C), 168.38 (C), 169.98 (C); FAB-Mass: *m/z* 318 (*m*+1); (found C, 60.42; H, 6.03; N, 13.21. C₁₆H₁₉N₃O₄ requires C, 60.56; H, 6.03; N, 13.24).

3-[2-(9H-Fluoren-9-ylmethoxycarbonylamino)acetylamino]-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid benzyl ester (S) (11)

To a stirred solution of **10** (1.00 g; 3.15 mmol), Fmoc-glycine (0.94 g; 3.15 mmol) and *N*-ethyldiisopropylamine (1.10 ml; 6.30 mmol) in DMF (20 ml) at 0°C under Argon gas, HOBt.H₂O (0.48 g, 3.15 mmol) was added followed by TBTU (1.01 g; 3.15 mmol). The mixture was stirred at 0°C for 1 hour and then at room temperature for additional 4 hours. After evaporation of DMF completely, the residue was dissolved in ethyl acetate (100 ml), the ethyl acetate was washed successively with 5% aqueous KHSO₄ (20 ml), water (20 ml), 5% NaHCO₃ (20 ml) and water (20 ml). The solution was dried over MgSO₄ and then the solvent was evaporated under reduced pressure to give a crude product which was recrystallized from EtOAc: n-hexane give **11** as a white crystalline product in yield of 1.24 g (66.0%): mp 130-131°C; [α]_D²⁵ 26.47° (c 0.1, methanol); ^{max}/cm⁻¹ 3330 (NH); 1736, 1668 (C=O), ¹H-NMR (300 MHz, DMSO-*d*₆)_ 1.74 (3H, s, CH₃), 2.53 (2H, d, *J* = 6.9 Hz, CH₂), 3.48 (2H, t, *J* = 5.7 Hz, CH₂), 3.90 (2H, d, *J* = 6.9 Hz, CH₂), 4.19-4.26 (3H, m, CH₂, CH), 4.45-4.59 (1H, m, CH), 5.01 (2H, s, CH₂), 7.24 (1H, s, CH-6 of T), 7.32 (5H, s, C₆H₅), 7.28-7.44 (4H, m, Ar-H), 7.70 (2H, d, *J* = 7.2 Hz, Ar-H), 7.87 (2H, d, *J* = 7.5 Hz, Ar-H), 10.82 (1H, b, NH); ¹³C-NMR (75 MHz, DMSO-*d*₆)_ 12.45 (CH₃), 36.92 (CH₂), 42.65 (CH₂), 43.37 (CH₂), 44.53 (CH), 46.60 (CH), 65.57 (CH₂), 65.72 (CH₂), 107.11 (C), 120.06 (CH), 125.25 (CH), 127.05 (CH), 127.59

(CH), 127.87 (CH), 127.89 (CH), 128.33 (CH), 135.99 (C), 136.36 (CH), 140.68 (C), 143.83 (C), 151.51 (C), 156.30 (C), 164.12 (C), 168.55 (C), 170.27 (C); FAB-Mass: m/z 597 ($m+1$); (found C, 66.15; H, 5.54; N, 9.51. $C_{33}H_{32}N_4O_7$ requires C, 66.43; H, 5.41; N, 9.39).

3-[2-(9H-Fluoren-9-ylmethoxycarbonylamino)acetylamino]-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid (S) (12)⁽⁸⁰⁾

To a stirred solution of **11** (0.60 g; 1.00 mmol) in ethanol (10 ml) under Argon gas 10 % Pd/C (1.01 g) was added followed by 1,4-cyclohexadiene (1.64 g, 20.00mmol). The mixture was stirred over night and then filtered through celite. After washing several times with hot ethanol, the combined organic solvent was evaporated and the residue was dried in a vacuum then 5 % $NaHCO_3$ (10 ml) was added. The mixture was extracted with ethyl acetate for the removal of the byproduct. The sodium salt was neutralized with 10 % $KHSO_4$ till pH (2-3) and then extracted with ethyl acetate (3 x 20 ml). The combined ethyl acetate extracts were washed consequently with saturated sodium chloride solution (20 ml) and distilled water (2 x 20 ml), dried over $MgSO_4$ and evaporated under reduced pressure to give a crude product which was recrystallized from ethanol to give **12** as a white crystalline product in yield of 0.31 g (62.0%): mp $145^\circ C$; $[\alpha]_D^{25}$ 30.15° (c 0.1, methanol); ν_{max}/cm^{-1} 3310, 3295 (NH) 1715, 1635 (C=O); 1H -NMR (300 MHz, $DMSO-d_6$) δ 1.75 (3H, s, CH_3), 2.39-2.44 (2H, m, CH_2), 3.42-3.58 (2H, m, CH_2), 3.85-3.96 (2H, m, CH_2), 4.18-4.28 (3H, m, CH_2 , CH), 4.41-4.52 (1H, m, CH), 7.23 (1H, s, CH-6 of T), 7.31 (2H, t, $J = 7.35$ Hz, Ar-H), 7.40 (2H, t, $J = 7.20$ Hz, Ar-H), 7.71 (2H, d, $J = 7.80$ Hz, Ar-H), 7.87 (2H, d, $J = 7.80$ Hz, Ar-H), 10.81 (1H, br, NH), 12.14 (1H, br, COOH); ^{13}C -NMR (75 MHz, $DMSO-d_6$) δ 12.48 (CH_3), 37.03 (CH_2), 42.72 (CH_2), 43.39 (CH_2), 44.55 (CH), 46.63 (CH), 65.73 (CH_2), 107.13 (C), 120.06 (CH), 125.27 (CH), 127.06 (CH), 127.60 (CH), 136.28 (CH), 140.69 (C), 143.85 (C), 151.54 (C), 156.30 (C), 164.15 (C), 168.48 (C), 171.93 (C); FAB-Mass: m/z 507 ($M+1$); (found C, 61.29; H, 5.01; N, 11.28. $C_{26}H_{26}N_4O_7$ requires C, 61.65; H, 5.17; N, 11.06).

5.1.3. Synthesis of isogaPNA adenine monomer

4-(6-Benzoylaminopurin-9-yl)-3-*tert*-butoxycarbonylaminobutyric acid benzyl ester (13)⁽⁷⁷⁾

To a stirred suspension of **2** (5.57 g, 18.00 mmol), triphenylphosphine (5.19 g, 19.80 mmol) and N⁶-benzoyladenine (12.92 g, 54.00 mmol) in dry THF (150 ml) was added *N,N*-diethyl azodicarboxylate (2.83 ml, 18.00 mmol) in a dropwise manner at 0°C. The reaction mixture was stirred at 0°C for 2 hours followed by overnight stirring at room temperature under stream of N₂ gas. The insoluble products were filtered off and the filtrate was evaporated till dryness. The crude product was purified with silica gel column chromatography using dichloromethane: acetone (50:1) as a mobile phase to give **13** as white crystals in yield of 4.30 g (45.0%): mp 127-129°C, $\nu_{\text{max}}/\text{cm}^{-1}$ 3296 (NH) 1725, 1644 (C=O)), ¹H-NMR (300 MHz, DMSO-*d*₆) 1.22 (9H, s, C(CH₃)₃), 2.49-2.76 (2H, m, CH₂), 4.22-4.45 (3H, m, CH, CH₂), 5.07 (2H, s, CH₂), 7.01 (1H, d, *J* = 7.8 Hz, NH), 7.35 (5H, s, C₆H₅), 7.53 (2H, t, *J* = 7.35 Hz, PhC=O), 7.62 (1H, d, *J* = 7.2 Hz, PhC=O), 8.02 (2H, d, *J* = 7.2 Hz, PhC=O), 8.27 (1H, s, CH), 8.70 (1H, s, CH), 11.10 (1H, s, NH); ¹³C-NMR (75 MHz, DMSO-*d*₆) 27.88 (CH₃), 36.69 (CH₂), 46.41 (CH₂), 47.41 (CH), 65.68 (CH₂), 77.96 (C), 127.83 (CH), 127.95 (CH), 128.36 (CH), 128.41 (CH), 128.66 (CH), 128.80 (CH), 132.32 (CH), 133.56 (C), 135.99 (C), 144.84 (CH), 149.96 (C), 151.26 (C), 154.73 (C), 170.11 (C); FAB-Mass: *m/z* 531 (M+1); (found C, 63.05; H, 5.71; N, 15.36. C₂₆H₃₀N₆O₅ requires C, 63.38; H, 5.70; N, 15.84).

4-(6-Benzoylaminopurin-9-yl)-3-[2-(9H-fluoren-9-ylmethoxycarbonylamino)-acetylaminobutyl]butyric acid benzyl ester (14)^(81,82)

To a stirred solution of **13** (1.06 g, 2.00 mmol) in dichloromethane (8 ml) at 0°C and under stream of Argon gas was added trifluoroacetic acid (8 ml). The mixture was stirred at room temperature for 24 hours, once the TLC analysis indicated complete acidolysis of the Boc group, the solvents were completely evaporated. The residue was dried under reduced pressure then dissolved in water (20 ml). The aqueous solution was extracted with dichloromethane (3 x 20 ml) to remove byproduct. The aqueous phase was neutralized with 5% NaHCO₃ until pH 7 and then extracted with ethyl acetate. The combined ethyl acetate extracts were washed with saturated NaCl (30 ml) and H₂O (2 x 30 ml), dried over MgSO₄ and then evaporated till dryness to give crude white crystals which was used for the next step without purification. To a stirred solution of the crude product (1.12 g, 2.60 mmol), Fmoc-glycine (0.71 g, 2.40 mmol) and TEA (0.35 ml, 2.50 mmol) in acetonitrile (30 ml) at 0°C under argon gas, TBTU (0.83 g, 2.60 mmol) was

added. The mixture was stirred at 0°C for 1 hour then at room temperature for 24 hours. The reaction was quenched by the addition of saturated NaCl (30 ml), the resulting mixture was extracted with ethyl acetate (4 x 30 ml). The combined ethyl acetate extracts were washed consequently with 2N HCl (20 ml), H₂O (20 ml), 5% NaHCO₃ (20 ml) and H₂O (2 x 20 ml). The solution was dried over MgSO₄, and then the solvent was completely evaporated under reduced pressure to give a crude oily product, which was purified using silica gel column chromatography eluted with CH₂Cl₂: MeOH (10:1) as a mobile phase to give **14** as a white crystalline product in yield of 1.4 g (75.8 %): mp 133-135°C; $\nu_{\text{max}}/\text{cm}^{-1}$; ¹H-NMR (300 MHz, DMSO-*d*₆) 2.74 (2H, dd, *J* = 5.70 and 5.70 Hz, CH₂), 3.5 (2H, d, *J* = 5.86 Hz, CH₂), 4.22 (1H, d, *J* = 6.23 Hz, CH), 4.28 (2H, d, *J* = 6.23 Hz, CH₂), 4.32-4.48 (2H, m, CH₂), 4.52-4.64 (1H, m, CH), 5.07 (2H, s, CH₂), 7.29-7.43 (4H, m, Ar-H), 7.34 (5H, s, C₆H₅), 7.53 (2H, t, *J* = 6.70 Hz, Ar-H), 7.61 (1H, d, *J* = 7.33 Hz, Ar-H), 7.70 (2H, d, *J* = 7.51 Hz, Ar-H), 7.87 (2H, d, *J* = 7.51 Hz, Ar-H), 8.03 (2H, d, *J* = 7.51 Hz, Ar-H), 8.33 (1H, s, CH), 8.70 (1H, s, CH), 11.12 (1H, s, NH); ¹³C-NMR (75 MHz, DMSO-*d*₆) 36.23 (CH₂), 43.44 (CH₂), 45.76 (CH₂), 46.08 (CH), 46.59 (CH), 65.75 (CH₂, CH₂), 120.04 (CH), 125.07 (C), 125.21 (CH), 127.02 (CH), 127.57 (CH), 127.94 (CH), 128.36 (CH), 128.39 (CH), 132.31 (CH), 133.47 (C), 135.91 (C), 140.67 (C), 143.80 (C), 144.98 (CH), 149.99 (C), 151.37 (CH), 152.81 (C), 156.44 (C), 165.49 (C), 168.99 (C), 169.99 (C); FAB-Mass: *m/z* 710 (*m*+1); (found C, 67.11; H, 5.14; N, 13.49. C₄₀H₃₅N₇O₆ requires C, 67.69; H, 4.97; N, 13.81).

4-(6-Benzoylamino-purin-9-yl)-3-[2-(9H-fluoren-9-ylmethoxycarbonylamino)acetylaminobutyrac acid (15**)**⁽⁸³⁾

To a stirred solution of **14** (0.71 g, 1.00 mmol) in methanol (10 ml) under Argon gas 10% Pd-C (0.70 g) was added. The mixture was stirred under stream of hydrogen gas for 24 hours and then the mixture was filtered through celite. After washing several times with hot methanol, the combined methanol solution was completely evaporated in vacuum then 5 % NaHCO₃ (10 ml) was added. The mixture was extracted with ethyl acetate for the removal of byproduct. The sodium salt was neutralized with 10 % KHSO₄ till pH (2-3) and then extracted with ethyl acetate (3 x 20 ml). The combined ethyl acetate extracts were washed consequently with saturated NaCl (30 ml), distilled water (2 x 30 ml), dried over MgSO₄ and then completely evaporated under reduced pressure to give a crude product, which was recrystallized from ethanol to give **15** as a white crystalline product in yield of 0.30 g (50.0%): mp 213-215°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3309 (OH), 1697, 1646 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.42-2.62 (2H, m,

CH₂), 3.49 (2H, d, $J = 5.2$ Hz, CH₂), 4.13-4.56 (4H, m, CH₂, CH, CH), 7.10 (1H, s, NH), 7.31 (2H, d, $J = 7.50$ Hz, Ar-H), 7.39 (2H, t, $J = 7.20$ Hz, Ar-H), 7.54 (2H, d, $J = 7.2$ Hz, Ar-H), 7.61 (1H, d, $J = 8.1$ Hz, Ar-H), 7.70 (2H, d, $J = 7.2$ Hz, Ar-H), 7.87 (2H, d, $J = 7.2$ Hz, Ar-H), 8.03 (2H, d, $J = 7.8$ Hz, Ar-H), 8.32 (1H, s, CH), 8.71 (1H, s, CH), 11.13 (1H, s, NH); ¹³C-NMR (75 MHz, DMSO-*d*₆) 36.45 (CH₂), 43.42 (CH₂), 45.80 (CH₂), 46.13 (CH), 46.58 (CH), 65.73 (CH₂), 120.02 (CH), 125.05 (C), 125.21 (CH), 127.01 (CH), 127.54 (CH), 128.37 (CH), 132.28 (C), 133.52 (CH), 140.66 (C), 143.80 (C), 144.96 (CH), 149.94 (C), 151.24 (CH), 152.76 (C), 156.40 (C), 165.58 (C), 168.88 (C), 171.73 (C); FAB-Mass: m/z 620 ($m+1$); (found C, 63.87; H, 4.81; N, 15.61. C₃₃H₂₉N₇O₆ requires C, 63.97; H, 4.72; N, 15.82).

5.1.4. Synthesis of isoggPNA thymine monomer

2-*tert*-Butoxycarbonylamino-3-hydroxypropionic acid dicyclohexylamine salt (**17**)

To a stirred solution of **16** (24.62 g, 0.12 mol) in a mixture of dichloromethane-methanol (100:50 ml) was added dicyclohexylamine (24.30 ml, 0.12 mol) in a dropwise manner. The mixture was stirred at 0°C for 30 minutes followed by overnight stirring at room temperature. The solvents were completely evaporated under reduced pressure and the resulting oily product was recrystallized from ethyl acetate: n-hexane to give **17** as a white crystalline substance in yield of 34.00 g (73.3%).

2-*tert*-Butoxycarbonylamino-3-hydroxypropionic acid benzyl ester (**18**)

To a stirred solution of **17** (44.84 g, 0.12 mol) in DMF (200 ml) was added benzyl bromide (13.85 ml, 0.12 mol). The mixture was stirred at room temperature for 24 hours and then The DMF was completely evaporated under reduced pressure. Water (200 ml) and ethyl acetate (100 ml) were added, and the aqueous layer was extracted with ethyl acetate (4 x 30 ml). The combined ethyl acetate extracts were washed consequently with saturated NaCl (30 ml) and water (2 x 30 ml), dried over MgSO₄. The ethyl acetate extracts were completely evaporated to give a crude oily product which was recrystallized from ethyl acetate: n-hexane to give **18** as a white crystalline product in yield of 28.20 g (82.3%): mp 70-71°C; $\nu_{\max}/\text{cm}^{-1}$ 3420 (OH), 3365 (NH), 1760, 1670 (C=O); ¹H-NMR (300 MHz, CDCl₃) 1.44 (9H, s, C(CH₃)₃), 2.1 (1H, br, OH), 3.90 (1H, dd, $J = 11.30$ and 3.5 Hz, CHH), 3.98 (1H, dd, $J = 11.20$ and 4 Hz, CHH), 4.42 (1H, br, CH), 5.21 (2H, s, CH₂Ph), 5.4 (1H, br, NH), 7.35 (5H, s, C₆H₅); ¹³C-NMR (75 MHz,

CDCl₃) 28.08 (CH₃), 55.80 (CH), 66.60 (CH₂), 71.80 (CH₂), 79.42 (C), 127.81 (CH), 128.06 (CH), 128.35 (CH), 135.62 (C), 155.30 (C), 169.17 (C); FAB-Mass: M/z 296 (M+1).

2-tert-Butoxycarbonylamino-3-(toluene-4-sulfonyloxy)propionic acid benzyl ester (19)⁽⁸⁵⁾

To a stirred solution of **18** (6.91 g, 23.42 mmol) in dry pyridine (40 ml) was added tosyl chloride (4.91 g, 25.76 mmol). The resulting solution was stirred at -10°C under stream of N₂ gas for 2 hours followed by stirring at room temperature for 18 hours. The resulting mixture was poured into a beaker of ice-water (200 ml) with continued stirring, a white solid was formed. The resulting solid was filtered and washed with distilled water, dried in a vacuum desiccator over phosphorus pentoxide. The obtained crude product was recrystallized from ethanol to give **19** as a white crystalline solid in yield of 8.86 g (84.2%): mp 95-96°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3374 (NH), 1753, 1694 (C=O); ¹H-NMR (300 MHz, CDCl₃) 1.41 (9H, s, C(CH₃)₃), 2.42 (3H, s, CH₃), 4.27-4.33 (1H, m, CHH), 4.38-4.44 (1H, m, CHH), 4.51-4.57 (1H, m, CH), 5.08 (1H, d, *J* = 12.00 Hz, PhCHH), 5.17 (1H, d, *J* = 12.3 Hz, PhCHH), 5.34 (1H, d, *J* = 8.10 Hz, NH), 7.29-7.37 (2H, m, Ar-H), 7.34 (5H, s, C₆H₅), 7.71 (2H, d, *J* = 8.10 Hz, Ar-H); ¹³C-NMR (75 MHz, CDCl₃) 21.61 (CH₃), 28.15 (CH₃), 53.02 (CH), 67.79 (CH₂), 69.46 (CH₂), 80.43 (C), 127.92 (CH), 128.23 (CH), 128.52 (CH), 128.58 (CH), 129.88 (CH), 132.25 (C), 134.72 (C), 145.08 (C), 154.89 (C), 168.39 (C); FAB-Mass: M/z 450 (M+1).

2-tert-Butoxycarbonylamino-3-iodopropionic acid benzyl ester (20)⁽⁸⁵⁾

A solution of sodium iodide (3.90 g, 26.06 mmol) in dry acetone (20 ml) was added in a dropwise manner to a stirred solution of **19** (7.81 g, 17.37 mmol) in dry acetone (20 ml) under N₂ gas. The resulting yellow solution was stirred in the dark at ambient temperature for 24 hours and then filtered and concentrated under reduced pressure. The residue was dissolved in chloroform (100 ml), washed sequentially with distilled water (2 x 50 ml), sodium thiosulfate (50 ml, 1M), and distilled water (3 x 50 ml), and then dried over anhydrous sodium sulfate. Concentration under reduced pressure gave a light-sensitive colorless oil which solidified on standing. Recrystallization from a minimum amount of hot ethanol, followed by filtration and trituration with light petroleum ether to give the iodide **20** as a white crystalline solid in yield of 5.66 g (80.4%): mp 79-80°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3362 (NH), 1759, 1685 (C=O); ¹H-NMR (300 MHz, CDCl₃) 1.37 (9H, s, C(CH₃)₃), 3.29-3.38 (1H, m, CHH), 3.47-3.55 (1H, m, CHH),

4.22-4.31 (1H, m, CH), 5.13 (2H, s, CH₂), 7.35 (5H, s, C₆H₅), 7.45 (1H, d, *J* = 8.10 Hz, NH); ¹³C-NMR (75 MHz, CDCl₃)_ 4.42 (CH₂), 28.08 (CH₃), 55.55 (CH), 66.43 (CH₂), 78.67 (C), 127.78 (CH), 128.06 (CH), 128.35 (CH), 135.59 (C), 155.10 (C), 169.13 (C); FAB-Mass: M/z 406 (M+1).

3-(3-Benzoyl-5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-2-tert-butoxy-carbonylaminopropionic acid benzyl ester (21) and 2-tert-Butoxycarbonylamino-3-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)propionic acid benzyl ester (22)

To a stirred solution of iodide compound **20** (4.05 g, 10.00 mmol), N³-Benzoylthymine (4.60 g, 20.00 mmol) in DMF (30 ml) was added anhydrous K₂CO₃ (2.76 g, 20.00 mmol). The mixture was stirred at room temperature for 12 hours, the solvent was evaporated completely to dryness. The resulting residue was dissolved in ethyl acetate (200 ml), washed with water (200 ml), and then dried over MgSO₄. The ethyl acetate was completely evaporated under reduced pressure and the resulting crude products were purified by flash chromatography on silica gel using ethyl acetate: n-hexane (1:2) as a mobile phase to give **21** as a white crystalline substance in 3.45 g (68.0% yield) and **22** as a white crystalline substance in yield of 0.45g (11.2%). Analytical samples were further purified by recrystallization from ethyl acetate: n-hexane. **21**: mp 133-134°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3361 (NH) 1746, 1703, 1655 (C=O); ¹H-NMR (300 MHz, CDCl₃) 1.43 (9H, s, C(CH₃)₃), 1.90 (3H, s, CH₃), 3.70-3.85 (1H, m, CHH), 4.34-4.42 (1H, m, CHH), 4.59-4.70 (1H, m, CH), 5.07 (1H, d, *J* = 12.00 Hz, PhCHCH), 5.19 (1H, d, *J* = 12.00 Hz, PhCHCH), 5.47 (1H, d, *J* = 6.60 Hz, NH), 7.02 (1H, s, CH-6 of T), 7.33 (5H, s, C₆H₅), 7.48 (2H, dd, *J* = 7.80 and 7.80, PhC=O), 7.63 (1H, dd, *J* = 7.80 and 7.80 Hz, PhC=O), 8.04 (2H, d, *J* = 7.80 Hz, PhC=O); ¹³C-NMR (75 MHz, CDCl₃)_ 12.30 (CH₃), 28.21 (CH₃), 50.47 (CH₂), 52.26 (CH), 68.08 (CH₂), 80.68 (C), 110.41 (C), 128.53 (CH), 128.63 (CH), 129.01 (CH), 130.68 (CH), 131.59 (C), 134.68 (C), 134.89 (CH), 140.46 (CH), 150.06 (C), 155.22 (C), 163.04 (C), 168.86 (C), 169.39 (C); FAB-Mass: M/z 508 (M+1); (found: C, 63.98; H, 5.73; N, 8.30. C₂₇H₂₉N₃O₇ requires: C, 63.89; H, 5.76; N, 8.28).

22: mp 191-192°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3358 (NH) 1745, 1703, 1653 (C=O); ¹H-NMR (300 MHz, CDCl₃)_ 1.41(9H, s, C(CH₃)₃), 1.85 (3H, s, CH₃), 4.02 (1H, dd, *J* = 14.20 and 6.80 Hz, CHH), 4.20 (1H, dd, *J* = 14.20 and 5.60 Hz, CHH), 4.52 (1H, dd, *J* = 6.80 and 5.60 Hz,

CH), 5.16 (1H, d, $J = 12.00$ Hz, PhCHH), 5.22 (1H, d, $J = 12.00$ Hz, PhCHH), 5.46 (1H, br, NH), 6.92 (1H, s, CH-6 of T), 7.34 (5H, s, C₆H₅), 8.25 (1H, brs, NH); ¹³C-NMR (75 MHz, CDCl₃) 11.95 (CH₃), 27.98 (CH₃), 48.12 (CH₂), 51.70 (CH), 66.38 (CH₂), 78.72 (C), 108.03 (C), 127.89 (CH), 128.11 (CH), 128.39 (CH), 135.58 (C), 141.80 (CH), 150.87 (C), 155.26 (C), 164.20 (C), 169.76 (C); FAB-Mass: M/z 404 (M+1); (found: C, 59.65; H, 5.95; N, 10.23. C₂₇H₂₉N₃O₇ requires: C, 59.54; H, 6.25; N, 10.42).

3-(3-Benzoyl-5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-2-[2-(9H-fluoren-9-ylmethoxycarbonylamino)acetylamino]propionic acid benzyl ester (23)⁽⁸⁶⁾

To a stirred solution of **21** (0.51 g, 1.00 mmol) in 1,4-dioxane (10 ml) was added concentrated HCl (8 ml). The reaction was continued stirring until the TLC analysis indicated complete acidolysis of the Boc group. The solvents were completely evaporated under reduced pressure and the residue was neutralized with saturated NaHCO₃. The solution was extracted with CH₂Cl₂ (3 x 100 ml), the combined CH₂Cl₂ extracts washed with saturated NaCl (20 ml) and water (2 x 30 ml) then dried over MgSO₄. The organic solvent was completely evaporated till dryness to give a crude deprotected compound from Boc as an oily product. After dryness under reduced pressure for 24 hours, the oily compound was used in the next coupling without purification. *N*-Ethyl-diisopropylamine (0.35 ml, 2.00 mmol) was added to a stirred solution of the crude oily compound, Fmoc-glycine (0.30 g, 1.00 mmol), HOBT.H₂O (0.15g, 1.00 mmol), and TBTU (0.32 g, 1.00 mmol) in DMF (6 ml) under argon gas at room temperature. After stirring for 1.5 hour, the solvent was evaporated under reduced pressure. The resulting residue was dissolved in CH₂Cl₂ (100 ml) and the solution was washed consecutively with H₂O (80 ml), 4% NaHCO₃ (3 x 80 ml), 5% aqueous KHSO₄ (3 x 80 ml) and H₂O (2 x 80 ml), dried over MgSO₄. The organic solvent was evaporated completely under reduced pressure, and the resulting residue was purified by flash chromatography on silica gel with ethyl acetate: n-hexane (3:1) to give an oily compound. The resulting oily compound was recrystallized from ethyl alcohol-diethyl ether to give **23** as a white crystalline solid in yield of 0.51 g (74.0% from **21**): mp 194-195°C; [α]_D²⁵ 5.00° (c 0.1, methanol); ν_{max} /cm⁻¹ 3330 (NH) 1726, 1645 (C=O); ¹H-NMR (300 MHz, CDCl₃) 1.87 (3H, s, CH₃), 3.78-3.85 (2H, m, CH₂), 4.13-4.28 (2H, m, CH₂), 4.39 (2H, d, $J = 6.90$ Hz, CH₂), 4.82-4.91 (1H, m, CH), 5.08 (1H, d, $J = 12.00$ Hz, PhCHH), 5.17 (1H, d, $J = 12.00$ Hz, PhCHH), 5.30-5.38 (1H, m, CH), 7.02 (1H, d, $J = 5.20$ Hz, NH), 7.28-7.48 (7H, m, Ar-H), 7.33 (5H, s, C₆H₅), 7.52-7.62 (5H, m, Ar-H

and NH), 7.76 (2H, d, $J = 7.40$ Hz, Ar-H), 7.93 (2H, d, $J = 7.40$, Ar-H); ^{13}C -NMR (75 MHz, CDCl_3)_ 12.24 (CH_3), 44.54 (CH_2), 47.04 (CH), 49.51 (CH_2), 52.11 (CH), 67.33 (CH_2), 68.28 (CH_2), 111.37 (C), 120.00 (CH), 125.05 (CH), 127.08 (CH), 127.76 (CH), 128.63 (CH), 128.72 (CH), 128.81 (CH), 129.10 (CH), 130.60 (CH), 131.44 (C), 134.58 (C), 135.07 (CH), 140.21 (CH), 141.29 (C), 143.67 (C), 150.52 (C), 156.67 (C), 162.88 (C), 168.77 (C), 169.49 (C); FAB-Mass: M/z 687 ($M+1$); (found: C, 67.92; H, 4.95; N, 8.18. $\text{C}_{39}\text{H}_{34}\text{N}_4\text{O}_8$ requires: C, 68.21; H, 4.99; N, 8.16).

3-(3-Benzoyl-5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-2-[2-(9H-fluoren-9-ylmethoxycarbonylamino)acetylamino]propionic acid (24**)⁽⁸³⁾**

To a stirred solution of **23** (0.85 g, 1.20 mmol) in methyl alcohol (20 ml) was added 10 % Palladium on carbon (0.60 g), under Argon gas. After stirring for 0.5-1 hour under atmospheric pressure of hydrogen gas, the solvent was filtered through celite. After washing several times with hot methanol, the solvent was evaporated under reduced pressure. The resulting residue was purified by flash chromatography on silica gel (ethyl acetate: methyl alcohol gradient) to give **24** as a white crystalline solid in yield of 0.64 g (87.0%). Analytical sample was purified by recrystallization from dichloromethane: n-hexane: mp 183-184°C; $[\alpha]_D^{25}$ -9.44° (c 0.1, methanol); $\nu_{\text{max}}/\text{cm}^{-1}$ 3328 (NH) 1713, 1645 ($\text{C}=\text{O}$); ^1H -NMR (300 MHz, CDCl_3)_ 1.78 (3H, s, CH_3), 3.55-3.70 (2H, m, CH_2), 4.18-4.28 (4H, m, CH_2 , CH_2), 4.37-4.45 (2H, m, CH, CH), 7.27 (1H, s, CH-6 of T), 7.32 (1H, d, $J = 7.5$ Hz, Ar-H), 7.39 (2H, t, $J = 7.35$ Hz, Ar-H), 7.45-7.50 (1H, m, Ar-H), 7.53 (2H, d, $J = 7.80$ Hz, Ar-H), 7.62-7.73 (4H, m, Ar-H and NH), 7.80 (1H, d, $J = 7.50$ Hz, Ar-H), 7.87 (2H, d, $J = 7.50$ Hz, Ar-H), 8.04 (2H, d, $J = 7.50$ Hz, Ar-H); ^{13}C -NMR (75 MHz, $\text{DMSO}-d_6$)_ 11.74 (CH_3), 43.67 (CH_2), 46.60 (CH), 51.27 (CH_2), 52.28 (CH), 65.80 (CH_2), 107.63 (C), 120.09 (CH), 125.21 (CH), 127.09 (CH), 127.61 (CH), 129.24 (CH), 130.56 (CH), 131.32 (C), 135.13 (CH), 140.69 (C), 142.78 (CH), 143.80 (C), 149.45 (C), 156.47 (C), 163.03 (C), 168.94 (C), 170.01 (C), 171.12 (C); FAB-Mass: M/z 597 ($M+1$); (found: C, 64.44; H, 4.88; N, 9.12. $\text{C}_{32}\text{H}_{28}\text{N}_4\text{O}_8$ requires: C, 64.42; H, 4.73; N, 9.39).

5.1.5. Synthesis of some derivatives of isogaPNA thymine monomer (R)

3-*tert*-Butoxycarbonylamino-4-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)butyric acid (R) (43)⁽⁸³⁾

To a stirred solution of **3** (1.01 g, 2.00 mmol) in methyl alcohol (20 ml) under Argon gas was added 10 % Palladium on carbon (0.50 g). The mixture was stirred for 0.5-1 hour under atmospheric pressure of hydrogen gas, the solvent was filtered through celite. After washing several times with hot methanol, the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (dichloromethane-methanol 5:1 gradient) to give **43** as a white crystalline solid in yield of 0.39 g (60.0%). Analytical sample was purified by recrystallization from ethanol: n-hexane: mp 170-171°C; $[\alpha]_D^{25} - 26.56^\circ$ (c 0.1, methanol); $\nu_{\max}/\text{cm}^{-1}$ 3365 (OH), 1720, 1708 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 1.27 (9H, s, C(CH₃)₃), 1.74 (3H, s, CH₃), 2.35 (2H, d, $J = 6.60$ Hz, CH₂), 3.64-4.02 (2H, m, CH₂), 4.12-4.25 (1H, m, CH), 6.48 (1H, d, $J = 9.00$ Hz, NH), 7.22 (1H, s, CH-6 of T), 10.78 (1H, br, NH), 12.10 (1H, br, COOH); $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6) 12.32 (CH₃), 27.95 (CH₃), 37.00 (CH₂), 43.33 (CH₂), 45.52 (CH), 77.35 (C), 106.98 (C), 135.94 (CH), 151.38 (C), 154.83 (C), 163.93 (C), 172.02 (C); FAB-Mass: M/z 328 (M+1); (found: C, 51.41; H, 6.44; N, 12.96. C₁₄H₂₁N₃O₆ requires: C, 51.37; H, 6.47; N, 12.84).

[3-Hydroxy-1-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-ylmethyl)propyl] carbamic acid-*tert*-butyl ester (R) (44)⁽⁷⁶⁾

To a stirred solution of **43** (1.96 g; 6.00 mmol) in distilled THF (30 ml) at -10°C, under Argon atmosphere, N-methylmorpholine (0.61 g; 6.00 mmol) was added in a dropwise manner followed by ethyl chloroformate (0.65 g; 6.00 mmol). After 10 minutes stirring sodium borohydride (0.68 g; 18.00 mmol) was added in one portion, and then methyl alcohol (30 ml) was added in a dropwise manner to the reaction mixture over a period of 10 minutes at 0°C. The reaction mixture was stirred for additional 10 minutes at 0°C, and then neutralized with 1N HCl or 1N H₂SO₄ (12 ml). The organic solvents were evaporated under reduced pressure and the product was extracted with ethyl acetate (3 x 20 ml). The combined ethyl acetate was washed consecutively with 1N HCl or 1N H₂SO₄ (40 ml), H₂O (50 ml), 5% NaHCO₃ (50 ml) and H₂O (2 x 50 ml), dried over MgSO₄ or Na₂SO₄, and then the solvent was evaporated under reduced pressure. The crude product was purified with silica gel column chromatography using dichloromethane: methanol (15:1) then dichloromethane: methanol (5:1) as mobile phases to give an oily compound. The resulting oily product was recrystallized from ethyl acetate: n-hexane to give **44** as white crystals in yield of 1.30 g (69.0%): mp

180°C; $[\alpha]_D^{25} +8.47^\circ$ (c 0.1, methanol); $\nu_{\max}/\text{cm}^{-1}$ 3356 (OH), 1720, 1687, 1645 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 1.27 (9H, s, C(CH₃)₃), 1.49-1.51 (2H, m, CH₂), 1.74 (3H, s, CH₃), 3.59-3.62 (2H, m, CH₂), 3.89-3.92 (2H, m, CH₂), 4.35-4.37 (1H, m, CH), 6.39 (1H, d, $J = 7.80$ Hz, NH), 7.22 (1H, s, CH-6 of T), 10.76 (1H, br, OH); $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6) 12.32 (CH₃), 27.98 (CH₃), 34.96 (CH₂), 44.04 (CH₂), 45.72 (CH), 57.72 (CH₂), 77.12 (C), 106.96 (C), 135.85 (CH), 151.37 (C), 155.28 (C), 163.93 (C); FAB-Mass: m/z 314 (M+1); (found C, 53.59; H, 7.51; N, 13.44. C₁₄H₂₃N₃O₅ requires C, 53.66; H, 7.40; N, 13.41).

3-[(2-*tert*-Butoxycarbonylamino)acetylamino]-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid benzyl ester (R) (45)

To a stirred solution of **4** (1.00 g; 3.15 mmol), Boc-glycine (0.55 g; 3.15 mmol) and *N*-Ethyl-diisopropylamine (1.10 ml; 6.30 mmol) in DMF (20 ml) at 0°C under Argon gas was added HOBt.H₂O (0.48 g, 3.15 mmol) followed by TBTU (1.01 g; 3.15 mmol). The mixture was stirred at 0°C for 1 hour and then at room temperature for additional 4 hours. After complete evaporation of DMF under reduced pressure, the formed residue was dissolved in ethyl acetate (100 ml). The ethyl acetate was washed successively with 5% KHSO₄ (20 ml), water (20 ml), 5% NaHCO₃ (20 ml) and water (20 ml). The solution was dried over MgSO₄ and then the solvent was completely evaporated under reduced pressure to give a crude crystalline product which was recrystallized from ethyl acetate: n-hexane to give **45** as white crystals in yield of 1.00 g (70.0%): mp 103°C; $\nu_{\max}/\text{cm}^{-1}$ 3332 (NH), 1715, 1650 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 1.36 (9H, s, C(CH₃)₃), 1.75 (3H, s, CH₃), 2.54 (2H, d, $J = 6.00$ Hz, CH₂), 3.36-3.40 (2H, m, CH₂), 3.89 (2H, d, $J = 7.20$ Hz, CH₂), 4.45-4.55 (1H, m, CH), 5.01 (2H, s, CH₂), 6.70-6.73 (1H, m, NH), 7.24 (1H, s, CH-6 of T), 7.33 (5H, s, C₆H₅), 7.72 (1H, d, $J = 8.70$ Hz, NH), 10.82 (1H, s, NH); $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6) 12.27 (CH₃), 28.06 (CH₃), 36.80 (CH₂), 42.55 (CH₂), 43.14 (CH₂), 44.45 (CH), 65.44 (CH₂), 77.88 (C), 107.02 (C), 127.74 (CH), 127.77 (CH), 128.22 (CH), 135.93 (C), 136.21 (CH), 151.40 (C), 155.45 (C), 164.04 (C), 168.67 (C), 170.15 (C); FAB-Mass: m/z 475 (M+1).

3-[(2-*tert*-Butoxycarbonylamino)acetylamino]-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid (R) (46)⁽⁸⁰⁾

To a stirred solution of **45** (0.47 g, 1.00 mmol) in methyl alcohol (20 ml) under Argon

gas was added 10 % Palladium on carbon (0.20 g) followed by 1,4-cyclohexadiene (1.64 g, 20.00 mmol). The mixture was stirred for 24 hours and then filtered through celite. After washing several times with hot methanol, the combined solvents were evaporated under reduced pressure. The obtained residue was purified by silica gel column chromatography using dichloromethane: methanol 5:1 as a mobile phase to give an oily compound which was recrystallized from ethanol: ether gave **46** as a white crystalline substance in yield of 0.25 g (65.0%): mp 115-117°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3324 (OH), 1713, (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 1.36 (9H, s, C(CH₃)₃), 1.75 (3H, s, CH₃), 2.37 (2H, d, J = 5.4 Hz, CH₂), 3.35-3.40 (2H, m, CH₂), 3.82-3.95 (2H, m, CH₂), 4.35-4.50 (1H, m, CH), 6.70-6.80 (1H, m, NH), 7.23 (1H, s, CH-6 of T), 7.64 (1H, d, J = 8.40 Hz, NH), 10.81 (1H, br, NH), 12.15 (1H, br, COOH); $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6) 12.32 (CH₃), 28.07 (CH₃), 36.83 (CH₂), 42.60 (CH₂), 43.16 (CH₂), 44.40 (CH), 77.89 (C), 107.02 (C), 136.13 (CH), 151.42 (C), 155.45 (C), 164.04 (C), 168.60 (C), 171.77 (C); FAB-Mass: m/z 385 (M+1).

5.1.6. Synthesis of some derivatives of isogaPNA thymine monomer (S)

3-tert-Butoxycarbonylamino-4-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid (S) (**47**)⁽⁸³⁾

To a stirred solution of **9** (1.01 g, 2.00 mmol) in methyl alcohol (20 ml) under Argon gas was added 10 % Palladium on carbon (0.50 g). After stirring for 0.5-1 hour under atmospheric pressure of hydrogen gas, the solvent was filtered through celite. After washing several times with hot methanol, the combined methanol solution was evaporated under reduced pressure. The resulting residue was purified by silica gel column chromatography using dichloromethane: methanol 5:1 as a mobile phase to give **47** as a white crystalline solid in yield of 0.39 g (60.0%). Analytical sample was purified by recrystallization from ethanol: n-hexane: mp 170-171°C; $[\alpha]_D^{25}$ 27.23° (c 0.1, methanol); $\nu_{\text{max}}/\text{cm}^{-1}$ 3365 (OH), 1716, 1708 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 1.26 (9H, s, C(CH₃)₃), 1.73 (3H, s, CH₃), 2.36 (2H, d, J = 6.60 Hz, CH₂), 3.60-4.00 (2H, m, CH₂), 4.15-4.25 (1H, m, CH), 6.48 (1H, d, J = 9.00 Hz, NH), 7.22 (1H, s, CH-6 of T), 10.77 (1H, br, NH), 12.11 (1H, br, COOH); $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6) 12.35 (CH₃), 27.98 (CH₃), 37.00 (CH₂), 43.35 (CH₂), 45.56 (CH), 77.41 (C), 107.04 (C), 135.97 (CH), 151.43 (C), 154.87 (C), 163.97 (C), 172.01 (C); FAB-Mass: M/z 328 (M+1); (found: C, 51.09; H, 6.47; N, 12.93. C₁₄H₂₁N₃O₆ requires: C, 51.37; H, 6.47; N, 12.84).

[3-Hydroxy-1-(5-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-ylmethyl)propyl] carbamic acid-*tert*-butyl ester (S) (48)⁽⁷⁶⁾

To a stirred solution of **47** (1.96 g; 6.00 mmol) in distilled THF (30 ml) at -10°C , under Argon atmosphere, N-methyl morpholine (0.61 g; 6.00 mmol) was added in a dropwise manner followed by ethyl chloroformate (0.65 g; 6.00 mmol). After 10 minutes stirring sodium borohydride (0.68 g; 18.00 mmol) was added in one portion and then methyl alcohol (30 ml) was added in a dropwise manner to the reaction mixture over a period of 10 minutes at 0°C . The solution was stirred for additional 10 minutes at 0°C , and then neutralized with 1N HCl or 1N H_2SO_4 (12 ml). The organic solvents were evaporated under reduced pressure and the product was extracted with ethyl acetate (3 x 20 ml). The ethyl acetate extracts were washed consecutively with 1N HCl or 1N H_2SO_4 (40 ml), H_2O (50 ml), 5% NaHCO_3 (50 ml) and H_2O (2 x 50 ml), dried over MgSO_4 or Na_2SO_4 . The solvents were evaporated completely under reduced pressure to give the crude product which was purified with silica gel column chromatography using dichloromethane: methanol 15:1 then dichloromethane: methanol 5:1. the resulting oily product was recrystallized from ethyl acetate: n-hexane gave **48** as white crystals in yield of 1.41 g (69.0%): mp 179°C ; $[\alpha]_{\text{D}}^{25} -7.96$ (c 0.1, methanol); $\nu_{\text{max}}/\text{cm}^{-1}$ 3356 (OH), 1720, 1687, 1645 (C=O); $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) 1.27 (9H, s, $\text{C}(\text{CH}_3)_3$), 1.50-1.52 (2H, m, CH_2), 1.74 (3H, s, CH_3), 3.59-3.62 (2H, m, CH_2), 3.89-3.92 (2H, m, CH_2), 4.34-4.38 (1H, m, CH), 6.41 (1H, d, $J = 8.40$ Hz, NH), 7.23 (1H, s, CH-6 of T), 10.75 (1H, br, OH); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$) 12.32 (CH_3), 27.98 (CH_3), 34.96 (CH_2), 44.04 (CH_2), 45.72 (CH), 57.72 (CH_2), 77.12 (C), 106.96 (C), 135.85 (CH), 151.37(C), 155.28 (C), 163.93(C); FAB-Mass: m/z 314 (M+1); (found C, 53.50; H, 7.48; N, 13.29. $\text{C}_{14}\text{H}_{23}\text{N}_3\text{O}_5$ requires C, 53.66; H, 7.40; N, 13.41).

3-[(2-*tert*-Butoxycarbonylamino)acetyl]amino]-4-(5-methy-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)butyric acid benzyl ester (S) (49)

To a stirred solution of **10** (1.00 g; 3.15 mmol), Boc-glycine (0.55 g; 3.15 mmol) and N-Ethyldiisopropylamine (1.10 ml; 6.30 mmol) in DMF (20 ml) at 0°C under Argon gas was added HOBt. H_2O (0.48 g, 3.15 mmol) followed by TBTU (1.01 g; 3.15 mmol). The mixture was stirred at 0°C for 1 hour and then at room temperature for additional 4 hours. After complete evaporation of DMF under reduced pressure, the formed residue was dissolved in ethyl acetate (100 ml). The ethyl acetate was washed successively with 5% KHSO_4 (20 ml), water (20 ml), 5% NaHCO_3 (20 ml) and water (20 ml). The solution was dried over MgSO_4 and then the solvent was evaporated completely under reduced

pressure to give a crude crystalline product which was recrystallized from ethyl acetate: n-hexane to give **49** as white crystals in yield of 1.00 g (70.0%): mp 102-103°C; ν_{max} /cm⁻¹; ¹H-NMR (300 MHz, DMSO-*d*₆) 1.37 (9H, s, C(CH₃)₃), 1.74 (3H, s, CH₃), 2.54 (2H, d, *J* = 6.00 Hz, CH₂), 3.36-3.40 (2H, m, CH₂), 3.89 (2H, d, *J* = 7.20 Hz, CH₂), 4.46-4.56 (1H, m, CH), 5.01 (2H, s, CH₂), 6.70-6.73 (1H, m, NH), 7.24 (1H, s, CH-6 of T), 7.33 (5H, s, C₆H₅), 7.72 (1H, d, *J* = 8.70 Hz, NH), 10.81 (1H, s, NH); ¹³C-NMR (125 MHz, DMSO-*d*₆) 12.28 (CH₃), 28.04 (CH₃), 36.78 (CH₂), 42.53 (CH₂), 43.12 (CH₂), 44.45 (CH), 65.42 (CH₂), 77.87 (C), 107.02 (C), 127.73 (CH), 127.75 (CH), 128.22 (CH), 135.92 (C), 136.22 (CH), 151.42 (C), 155.47 (C), 163.98 (C), 168.65 (C), 170.13 (C); FAB-Mass: *m/z* 475 (M+1).

3-[(2-*tert*-Butoxycarbonylamino)acetylamino]-4-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)butyric acid (S) (50**)⁽⁸⁰⁾**

To a stirred solution of **49** (0.47 g, 1.00 mmol) in methyl alcohol (20 ml) under Argon gas was added 10 % Palladium on carbon (0.20 g) followed by 1,4-cyclohexadiene (1.64 g, 20.00 mmol). The mixture was stirred for 24 hours and then filtered through celite. After washing several times with hot methanol, the combined solvents were evaporated under reduced pressure. The obtained residue was purified by silica gel column chromatography using dichloromethane: methanol 5:1 as a mobile phase to give an oily product which was recrystallized from ethanol: ether to give **50** as a white crystalline substance in yield of 0.27 g (70.0%): mp 114-116°C; ν_{max} /cm⁻¹ 3323 (OH), 1713, 1655 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 1.36 (9H, s, C(CH₃)₃), 1.75 (3H, s, CH₃), 2.37 (2H, d, *J* = 6.00 Hz, CH₂), 3.32-3.41 (2H, m, CH₂), 3.85-3.91 (2H, m, CH₂), 4.35-4.48 (1H, m, CH), 6.70-6.80 (1H, m, NH), 7.22 (1H, s, CH-6 of T), 7.63 (1H, d, *J* = 8.70 Hz, NH), 10.80 (1H, d, *J* = 5.10 Hz, NH), 12.16 (1H, br, COOH); ¹³C-NMR (125 MHz, DMSO-*d*₆) 12.32 (CH₃), 28.07 (CH₃), 36.83 (CH₂), 42.60 (CH₂), 43.17 (CH₂), 44.42 (CH), 77.91 (C), 107.04 (C), 136.14 (CH), 151.43 (C), 155.46 (C), 164.05 (C), 168.61 (C), 171.77 (C); FAB-Mass: *m/z* 385 (M+1).

5.1.7. Synthesis of PNA oligomers

Several PNA oligomers were prepared using automated peptide synthesizer on an Expedite 8909 (Applied Biosystems, Foster City, CA) uses Fmoc PNA monomers and offers a routine method for producing PNAs on a relatively small (2 μ M) scale, five oligomers **27-31** were prepared each of which consists from 12 units by introducing thymine monomer **6** (T_R) (either one unit or three units) into *aeg*PNA thymine oligomer in different positions either toward the C-terminal, N-terminal or in the middle of the 12-mer oligomer. In addition to another oligomer **32** which consists mainly from 12 units of thymine monomer **6** (T_R). Another five oligomers **33-37** were prepared each of which consists from 12 units by introducing thymine monomer **12** (T_S) (either one unit or three units) into *aeg*PNA thymine oligomer in different positions either toward the C-terminal, N-terminal or in the middle of the 12-mer oligomer. In addition to another oligomer **38** which consists from 12 units of thymine monomer **12** (T_S). Also another four oligomers **39-41** were prepared each of which consists from 8 units by introducing adenine monomer **15** (A_x) (one unit) into *aeg*PNA adenine oligomer in different positions either toward the C-terminal, N-terminal or in the middle of the 8 mer oligomer. In addition to another oligomer **42** which consists from 8 units of adenine monomer **15** (A_x). Lysine was introduced at the C-terminus of the PNAs to reduce their self-aggregation and to ensure adequate solubility in UV-melting experiments.⁽⁴¹⁾ *aeg* PNA thymine and *aeg*PNA adenine monomers should be warmed to room temperature prior to solubilization to prevent water from condensing inside the container, *aegT* PNA and *aeg*PNA adenine monomers were dissolved in diluent (*N*-methylpyrrolidone (NMP)) (3.25 ml) and attached directly to the amber bottle in which monomer was shipped and allow the mixture to sit undisturbed for 10 minutes. In the same way monomers **6**, **12** and **15** dissolved in NMP and then introduced into the amber bottle in which monomer was shipped, PNA activator (HATU is dissolved in anhydrous DMF 13.5 ml, PNA base solution (0.2 M DIEA, 0.3M 2,6-lutidine in DMF), PNA deblocking solution (20% piperidine in DMF), PNA capping solution (5% acetic anhydride, 6% 2,6-lutidine in DMF) and wash B (99.8% anhydrous DMF), wash A (DMF). Columns for solid-phase synthesis containing Fmoc-XAL PEG resin should be stored in a desiccant box at -20°C and allowed to warm to room temperature prior to use. After finishing the preparation of these PNAs oligomers according to the protocol of Applied Biosystems for the preparation of PNA oligomers, the columns were removed and washed from both sides with DMF (3 ml) and then with CH_2Cl_2 (3 ml) and leave to dryness using vacuum pump. Cleavage from the resin was carried out using 20% (v/v) *m*-cresol in TFA (200 μ l) for 90 minutes. Centrifugation of the tubes containing the samples was carried out at 4000 rpm for 5 minutes and then allow filtration and keep the filtrate in ice bath for 5

minutes. Precipitation for the PNAs oligomers was carried out using anhydrous dry ether (800 μ l), keep cool in ice bath for 10 minutes and vortex for 1 minute. Centrifugation again at 4000 rpm for 5 minutes and then decantation of the solvents, the obtained precipitate was washed with anhydrous dry ether (3 x 300 μ l), and then drying in *vacuo*. In the case of Adenine oligomers **39-42** the removal of the benzoyl protected the amino group of adenine was carried out using (TFMSA/ TFA/DMS/*m*-cresol (1/10/6/2, v/v/v/v)⁽⁹⁴⁾, leaving for 3.5 hours). Analysis and purification of crude PNAs was carried out using reversed-phase HPLC, using C18 column and maintain the water jacket around column at 50°C to reduce aggregation and sharpen peaks, flow rate was ranged from 0.7 to 1 ml/min. using the mobile phase previously described, after identification of the objective PNAs using MALDI-TOF mass spectrometry using -cyanocinnamic acid as the matrix, which gave the expected molecular weight.

5.1.7.1. Synthesis of thymine PNA oligomers

H-T₁₁-T_R-Lys-NH₂ (27)

Oligomer **27** was prepared as described above, (*R_t* 25.6 minutes, flow rate was 0.9 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺, 3361.31 [M+Na]⁺ and 3377.32 [M+K]⁺.

H-T_R-T₁₁-Lys-NH₂ (28)

Oligomer **28** was prepared as described above, (*R_t* 27.1 minutes, flow rate was 0.8 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺, 3361.31 [M+Na]⁺ and 3377.32 [M+K]⁺.

H-T₉-T_R-T_R-T_R-Lys-NH₂ (29)

Oligomer **29** was prepared as described above, (*R_t* 29.6 minutes, flow rate was 0.8 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3340.3 [M+H]⁺, 3362.31 [M+Na]⁺ and 3378.32 [M+K]⁺.

H-T_R-T_R-T_R-T₉-Lys-NH₂ (30)

Oligomer **30** was prepared as described above, (*R_t* 29.4 minutes, flow rate was 0.8 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed

3339.3 [M+H]⁺, 3361.31 [M+Na]⁺ and 3377.32 [M+K]⁺.

H-T₆-T_R-T_S-Lys-NH₂ (31)

Oligomer **31** was prepared as described above, (*R*_t 28 minutes, flow rate was 1 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺ and 3361.31 [M+Na]⁺.

H-T_{RI2}-Lys-NH₂ (32)

Oligomer **32** was prepared as described above, (*R*_t 37 minutes, flow rate was 0.9 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺.

H-T₁₁-T_S-Lys-NH₂ (33)

oligomer **33** was prepared as described above, (*R*_t 25.3 minutes, flow rate was 0.9 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺, 3361.31 [M+Na]⁺.

H-T_S-T₁₁-Lys-NH₂ (34)

Oligomer **34** was prepared as described above, (*R*_t 27.6 minutes, flow rate was 0.8 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺, 3361.31 [M+Na]⁺ and 3377.32 [M+K]⁺.

H-T₉-T_S-T_S-T_S-Lys-NH₂ (35)

Oligomer **35** was prepared as described above, (*R*_t 29.6 minutes, flow rate was 0.8 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3340.3 [M+H]⁺, 3362.31 [M+Na]⁺.

H-T_S-T_S-T_S-T₉-Lys-NH₂ (36)

Oligomer **36** was prepared as described above, (*R*_t 29.1 minutes, flow rate was 0.8 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺, 3361.31 [M+Na]⁺ and 3377.32 [M+K]⁺.

H-T₆-T_S-T_S-Lys-NH₂ (37)

Oligomer **37** was prepared as described above, (*R*_t 27.4 minutes, flow rate was 1 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed

3340.3 [M+H]⁺ and 3362.31 [M+Na]⁺.

H-*T*₅₁₂-Lys-NH₂ (38)

Oligomer **38** was prepared as described above, (*R*_t 40.2 minutes, flow rate was 0.8 ml/min). MS (MALDI-TOF): calculated for C₁₃₈H₁₈₃N₅₁O₄₉ *m/z* = 3338.34, observed 3339.3 [M+H]⁺ and 3361.31 [M+Na]⁺.

After complete dryness of the collective samples using lyophilizer the samples were kept in the refrigerator until *T*_m measurements.

5.1.7.2. Synthesis of adenine PNA oligomers

H-A₇-A_x-Lys-NH₂ (39)

Oligomer **39** was prepared as described above, (*R*_t 14.5 minutes, flow rate was 1 ml/min). MS (MALDI-TOF): calculated for C₉₄H₁₁₉N₅₉O₁₇ *m/z* = 2346.03, observed 2347.2 [M+H]⁺ and 2369.1 [M+Na]⁺ in addition to many peaks of byproducts.

H-A_x-A₇-Lys-NH₂ (40)

Oligomer **40** was prepared as described above (*R*_t 14.3 minutes, flow rate was 1 ml/min). MS (MALDI-TOF): calculated for C₉₄H₁₁₉N₅₉O₁₇ *m/z* = 2346.03, observed 2347.2 [M+H]⁺ in addition to many peaks of byproducts.

H-A₄-A_x-A₃-Lys-NH₂ (41)

Oligomer **41** was prepared as described above, (*R*_t 15 minutes, flow rate was 1 ml/min). MS (MALDI-TOF): calculated for C₉₄H₁₁₉N₅₉O₁₇ *m/z* = 2346.03, observed 2347.2 [M+H]⁺ in addition to many peaks of byproducts.

H-A_{x8}-Lys-NH₂ (42)

Oligomer **42** was prepared as described above, (*R*_t 28 minutes, flow rate was 1 ml/min). MS (MALDI-TOF): calculated for C₉₄H₁₁₉N₅₉O₁₇ *m/z* = 2346.03, observed 2347.2

$[M+H]^+$ in addition to many peaks of byproducts.

5.1.8. Melting experiments

Melting experiments between different PNAs oligomers (**25-38**) with its complementary DNA or RNA were carried out using a 1:1 molar mixtures of PNA and the corresponding target DNA or RNA (amounts determined by the UV absorption at 260 nm), each at a concentration of 2.5 μ M in 10 mM phosphate buffer, pH 7.4, containing 100 mM sodium chloride, and 0.1 mM EDTA. Prior to the measurement of the melting profiles, the solutions were heated to 95°C for 5 minutes at a fast rate, then cooled to 0.5°C over 90 minutes and equilibrated at this temperature for 45 minutes subsequently, the absorbance A_{260} was recorded versus temperature for both dissociation and annealing at the rates 0.5°C/min. and 0.2°C, respectively. In (Job-plot) titration method for determination the binding stoichiometry between PNA and DNA, the total concentration used was 3 μ M (20 μ M DNA, 20 μ M PNA), the mixture of PNA and DNA allowed for heating at 95°C for 10 minutes and then cooled to room temperature and then allowed to stay for 8 hours in dark place and then the absorbance was measured at 260 nm.

5.1.9. Biological procedures for anti-HSV⁽⁹³⁾

Monolayer cultures of VERO cells were prepared in plastic petri dishes 6.0 cm in diameter at 37°C in a 5% CO₂ incubator in Eagle's minimum essential medium (MEM) with 10% calf serum and 0.03% L-glutamine. After the medium was discarded and washed with phosphate buffer saline once, 0.2 ml of the PH strain of HSV, type 1, at 5 x 10² plaque forming units/ml was inoculated over the VERO cells. One hour later the cell sheets were overlaid with a solution of the test compounds at various concentrations, prepared in MEM with 0.5% methyl cellulose and 1% calf serum. The cultures were incubated at 37°C in a 5% CO₂ incubator for 2 days and then the cells were stained with crystal violet and the plaques were counted. Petri dishes without an added compound served as controls, and the percentage inhibition of plaque development by test compounds was calculated. Adenine arabinoside (and acyclovir) served as a positive control for comparison.

5.1.10. HIV cytopathic effect inhibition assay⁽⁹⁵⁻⁹⁷⁾

MT2 cells or ATH8 cells (2 x 10⁵), which are sensitive to the cytopathic effect of

HTLV-III, exposed to a high multiplicity of infectious HTLV-III_B virus (2000 virus particles per cell) for 45 minutes, resuspended in 2 ml of 15% (v/v) fresh culture medium [the culture medium supplemented with 15 % heat inactivated fetal calf serum, 4mM L-glutamine, Penicillin (50 unit/ml), and streptomycin (50 µg/ml)], and cultured in the presence or absence of various concentrations of the tested compounds in culture tubes. The culture tubes were incubated in at 37°C in 5% CO₂-containing humidified air. Control cells were treated similarly but were not exposed to the virus. The assays were all performed in duplicate. On days 6, 8, and 10 the total viable cells were counted in a hemocytometer under the microscope by the trypan blue dye exclusion method.

5.2. Development of novel topoisomerases inhibitors

Materials

All reagents which used for the preparation of final compounds and their intermediates were purchased from Wako, Aldrich, Nacalai tesque, Tokyo Kasei and Novabiochem and used without purification. Solvents were HPLC grade from Wako, Aldrich and Sigma. Dry solvents were obtained by use of appropriate molecular sieves, except for tetrahydrofuran was distilled from sodium/benzophenone. The water content in dry solvents did not exceed 50 ppm as determined by Karl-Fischer titration.

General methods

TLC was performed on analytical Merck 9385 silica glass plates with F_{254} indicator. TLCs were viewed either under 254 nm UV or by staining with phosphomolybdic acid in ethanol or iodine crystals where appropriate. Column chromatography was performed as flash chromatography on Merck 9385 silica gel 60 (0.040-0.063 mm). Reactions were carried out under Argon gas. Accurate masses were obtained on Micromass LCT mass spectrometer which was recorded in the positive ion mode with leucine enkephalin as an internal lock mass standard. Elemental analysis was performed at the microanalytical laboratory, Institute of Resource Development and Analysis, Kumamoto University. Melting points were determined on electrothermal melting point apparatus and were uncorrected. IR data were obtained on Jasco fourier transform-410 infrared spectrometer as KBr discs. NMR spectra were obtained on a 300 MHz AL spectrometer. δ -Values are in ppm relative to DMSO- d_6 (2.50 for proton and 39.5 for carbon) or $CDCl_3$ (7.29 for proton and 76.9 for carbon).

5.2.1. Synthesis of acetyl and acid chlorides of gallic acid, 3,4-dihydroxy- benzoic acid and 4-hydroxybenzoic acid

3,4,5-Triacetoxybenzoic acid (52a)⁽¹²⁹⁾

To a stirred suspension of gallic acid **51a** (30.00 g; 0.17 mol) in acetic anhydride (120 ml) was added concentrated sulfuric acid (10 drops). The resulting solution was heated at 80°C for 10 minutes, then left to cool to room temperature. The solution was decomposed with caution by adding it to ice water (300 ml). After two hours leaving at room temperature the formed white precipitate was filtered off and dried to give **52a** as white crystals in yield of 40.00 g (77.0%): mp 163°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3546 (OH), 1789, 1696 (C=O); $^1\text{H-NMR}$ (300 MHz, CD_3OD) 2.17 (6H, s, $\text{CH}_3 \times 2$), 2.18 (3H, s, CH_3), 7.67 (2H, s, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, CD_3OD) 19.91 (CH_3), 20.32 (CH_3), 123.21 (CH), 130.04 (C), 139.95 (C), 144.82 (C), 167.32 (C), 168.21 (C), 169.41 (C); FAB-Mass: m/z 297 (M+1).

3,4-Diacetoxybenzoic acid (52b)⁽¹²⁹⁾

Compound **52b** was prepared as described above for compound **52a**, starting from 3,4-dihydroxybenzoic acid **51b** (26.20 g; 0.17 mol) to give **52b** as white crystals in yield of 31.20 g (77.0%): mp 155-156°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3540 (OH), 1774, 1686 (C=O); $^1\text{H-NMR}$ (300 MHz, CD_3OD) 2.28 (6H, s, $\text{CH}_3 \times 2$), 7.32 (1H, d, $J = 8.40$ Hz, Ar-H), 7.84 (1H, d, $J = 2.10$ Hz, Ar-H), 7.93 (1H, dd, $J = 8.40$ and 2.10 Hz, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, CD_3OD) 20.36 (CH_3), 20.41 (CH_3), 124.70 (CH), 126.08 (CH), 129.05 (CH), 130.56 (C), 143.61 (C), 147.47 (C), 168.13 (C), 169.43 (C), 169.75 (C); FAB-Mass: m/z 239 (M+1).

4-Acetoxybenzoic acid (52c)⁽¹²⁹⁾

Compound **52c** was prepared as described above for compound **52a**, starting from 4-hydroxybenzoic acid **51c** (23.48 g; 0.17 mol) to give **52c** as white crystals in yield of 25.00 g (81.6%): mp 195-197°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3512 (OH), 1755, 1682 (C=O); $^1\text{H-NMR}$ (300 MHz, CDCl_3) 2.33 (3H, s, CH_3), 7.21 (2H, d, $J = 8.40$ Hz, Ar-H), 8.15 (2H, d, $J = 8.40$ Hz, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, CDCl_3) 21.01 (CH_3), 121.65 (CH), 126.85 (C), 131.78 (CH), 155.08 (C), 168.68 (C), 171.12 (C); FAB-Mass: m/z 181 (M+1).

2,3-Diacetoxy-5-chlorocarbonylacetic acid phenyl ester (53a)⁽¹²⁹⁾

To a suspension of **52a** (7.00 g; 23.6 mmol) in dry benzene (50 ml) was added thionyl chloride (5 ml; 70.0 mmol) in a dropwise manner. The mixture was refluxed for 1.5

hours then leave to cool to room temperature, and then the solvent was completely evaporated using vacuum pump. A crude white precipitate formed which was washed several times with ether and dried using vacuum pump to give **53a** as a white precipitate in yield of 6.10 g (82.1%).

2-Acetoxy-5-chlorocarbonylacetic acid phenyl ester (53b)⁽¹²⁹⁾

Compound **53b** was prepared as described above for compound **53a**, starting from **52b** (5.62 g; 23.6 mmol) to give **53b** as an oily compound in yield of 5.00 g (82.6%).

4-Chlorocarbonylacetic acid phenyl ester (53c)⁽¹²⁹⁾

Compound **53c** was prepared as described above for compound **53a**, starting from **52c** (4.25 g; 23.6 mmol) to give **53c** as a white crystalline substance in yield of 3.73 g (79.6%): mp 169-170°C.

5.2.2. Synthesis of AGBA derivatives

2,3-Diacetoxy-5-[2-(3,4,5-triacetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55a)

To a stirred solution of *o*-phenylenediamine **54a** (0.11 g; 1.0 mmol) in CH₂Cl₂ (20 ml) was added **53a** (0.63 g; 2.0 mmol) followed by triethylamine (0.31 ml; 2.2 mmol). The mixture was stirred for 1 hour at room temperature, the reaction was quenched by the addition of saturated NaCl (20 ml) and EtOAc (20 ml). The aqueous layer was extracted with EtOAc (2 x 20 ml), the combined EtOAc extracts were washed subsequently with 1 N HCl (10 ml), H₂O (2 x 10 ml), NaHCO₃ (10 ml), H₂O (2 x 10 ml) and saturated NaCl (10 ml), and dried over MgSO₄. After complete evaporation of the organic solvents using vacuum pump an oily crude product was formed which was purified by silica gel column chromatography using CH₂Cl₂: acetone 10:1 as a mobile phase. The obtained oily product was then recrystallized from CH₂Cl₂: n-hexane to give **55a** as white crystals in yield of 0.99 g (74.5%): mp 114-115°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3423 (NH), 1780, 1659 (C=O); ¹H-NMR (300 MHz, CDCl₃) 2.26 (12H, s, CH₃ x 4), 2.27 (6H, s, CH₃ x 2), 6.99 (2H, q, *J* = 3.60 Hz, Ar-H), 7.36 (2H, q, *J* = 3.60 Hz, Ar-H), 7.70 (4H, s, Ar-H), 9.64 (2H, s, NH x 2); ¹³C-NMR (75 MHz, CDCl₃) 20.07 (CH₃), 20.45 (CH₃) 120.61 (CH), 125.61 (CH), 126.67 (CH), 130.08 (C), 132.06 (C), 137.83 (C), 143.49 (C), 164.14 (C), 166.54 (C), 167.79 (C); FAB-Mass: *m/z* 665 (M+1); (found C, 57.79; H, 4.22; N, 4.17. C₃₂H₂₈N₂O₁₄ requires C, 57.83; H, 4.25; N, 4.22).

2-Acetoxy-4-[2-(3,4-diacetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55b)

Compound **55b** was prepared as described above for compound **55a**, starting from **53b** (0.51 g; 2.0 mmol) to give a crude product which was purified by silica gel column chromatography using CH₂Cl₂: acetone 15:1 as a mobile phase. The oily product which was obtained was then recrystallized from EtOAc: n-hexane to give **55b** as white crystals in yield of 0.81 g (73.9%): mp 219-220°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3273 (NH), 1770, 1678, 1653 (C=O); ¹H-NMR (300 MHz, CDCl₃) 2.31 (6H, s, CH₃ x 2), 2.33 (6H, s, CH₃ x 2), 6.87 (2H, q, *J* = 3.60 Hz, Ar-H), 7.25-7.34 (4H, m, Ar-H), 7.83 (2H, d, *J* = 2.10 Hz, Ar-H), 7.86 (2H, s, Ar-H) 9.49 (2H, s, NH x 2); ¹³C-NMR (75 MHz, CDCl₃) 18.75 (CH₃), 18.85 (CH₃) 121.81 (CH), 121.90 (CH), 124.01 (CH), 124.09 (CH), 124.83 (CH), 130.40 (C), 132.09 (C), 142.29 (C), 145.32 (C), 164.81 (C), 167.64 (C), 167.89 (C); FAB-Mass: *m/z* 549 (M+1); (found C, 61.27; H, 4.38; N, 4.95. C₂₈H₂₄N₂O₁₀ requires C, 61.31; H, 4.41; N, 5.11).

4-[2-(4-Acetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55c)⁽¹³¹⁾

Compound **55c** was prepared as described above for compound **55a**, starting from **53c** (0.40 g; 2.0 mmol) to give a crude product which was purified by recrystallization from EtOH to give **55c** as white crystals in yield of 0.61 g (70.6%): mp 200°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3277 (NH), 1755, 1648 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.28 (6H, s, CH₃ x 2), 7.26-7.30 (6H, m, Ar-H), 7.66 (2H, q, *J* = 3.60 Hz, Ar-H), 7.98 (4H, d, *J* = 8.40 Hz, Ar-H), 10.04 (2H, s, NH x 2); ¹³C-NMR (75 MHz, DMSO-*d*₆) 20.85 (CH₃), 121.99 (CH), 125.56 (CH), 125.88 (CH), 129.08 (CH), 131.30 (C), 131.79 (C), 153.08 (C), 164.75 (C), 168.94 (C); FAB-Mass: *m/z* 433 (M+1).

***N*-[2-(Benzoylamino)phenyl]benzamide (55d)⁽¹³²⁾**

Compound **55d** was prepared as described above for compound **55a**, starting from benzoyl chloride **53d** (0.28 g; 2.0 mmol) the reaction was carried out in dry benzene and stirred for 5 hours to give a crude product which was purified by recrystallization from EtOH to give **55d** as a brownish white precipitate in yield of 0.48 g (75.9%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3272 (NH), 1655 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 7.30-7.35 (4H, m, Ar-H), 7.50-7.63 (6H, m, Ar-H), 7.93 (4H, d, *J* = 7.20, Ar-H), 10.05 (2H, s, NH x 2); ¹³C-NMR (75 MHz, DMSO-*d*₆) 125.34 (CH), 125.57 (CH), 127.24 (CH), 128.33

(CH), 131.15 (C), 131.58 (CH), 134.07 (C), 165.31 (C); FAB-Mass: m/z 317 (M+1).

2, 3-Diacetoxy-5-[3-(3,4,5-triacetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55e)

Compound **55e** was prepared as described above for compound **55a**, starting from *m*-phenylenediamine **54b** (0.11 g; 1.0 mmol) to give a crude product which was purified by silica gel column chromatography using CH₂Cl₂: acetone 5:1 as a mobile phase. The resulting oily compound was recrystallized from CH₂Cl₂: n-hexane to give **55e** as white crystals in yield of 1.00 g (75.3%): mp 252-253°C; $\nu_{\max}/\text{cm}^{-1}$ 3382 (NH), 1779, 1675 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.32 (12H, s, CH₃ x 4), 2.33 (6H, s, CH₃ x 2), 7.34 (1H, t, *J* = 8.40 Hz, Ar-H), 7.48 (2H, d, *J* = 8.40 Hz, Ar-H) 7.83 (4H, s, Ar-H), 8.27 (1H, s, Ar-H) 10.40 (2H, s, NH x 2); ¹³C-NMR (75 MHz, DMSO-*d*₆) 19.7 (CH₃), 20.15 (CH₃) 112.92 (CH), 116.33 (CH), 120.39 (CH), 128.56 (CH), 132.75 (C), 137.17 (C), 138.87 (C), 143.00 (C), 162.97 (C), 166.75 (C), 167.80 (C); FAB-Mass: m/z 665 (M+1); (found C, 57.67; H, 4.08; N, 4.17. C₃₂H₂₈N₂O₁₄ requires C, 57.83; H, 4.25; N, 4.22).

2-Acetoxy-4-[3-(3,4-diacetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55f)

Compound **55f** was prepared as described above for compound **55e**, starting from **53b** (0.51 g; 2.0 mmol) to give a crude product which was purified by silica gel column chromatography using CH₂Cl₂: Acetone 15:1 as a mobile phase. The obtained oily compound was then recrystallized from EtOAc: n-hexane to give **55f** as white crystals in yield of 0.83g (75.7%): mp 211-212°C; $\nu_{\max}/\text{cm}^{-1}$ 3366 (NH), 1771, 1654 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.31 (6H, s, CH₃ x 2), 2.32 (6H, s, CH₃ x 2), 7.32 (1H, t, *J* = 8.00 Hz, Ar-H), 7.44 (2H, d, *J* = 8.70 Hz, Ar-H), 7.49 (2H, dd, *J* = 9.60 and 1.46 Hz, Ar-H) 7.87 (2H, s, Ar-H), 7.92 (2H, dd, *J* = 8.40 and 1.83 Hz, Ar-H), 8.30 (1H, s, Ar-H),

10.38 (2H, s, NH x 2); ^{13}C -NMR (75 MHz, DMSO- d_6) 20.30 (CH_3), 20.38 (CH_3), 112.87 (CH), 116.22 (CH), 123.21 (CH), 123.70 (CH), 126.22 (CH), 128.68 (CH), 133.39 (C), 139.15 (C), 141.83 (C), 144.59 (C), 163.90 (C), 168.03 (C), 168.23 (C); FAB-Mass: m/z 549 (M+1); (found C, 61.52; H, 4.42; N, 5.05. $\text{C}_{28}\text{H}_{24}\text{N}_2\text{O}_{10}$ requires C, 61.31; H, 4.41; N, 5.11).

4-[3-(4-Acetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55g)⁽¹³¹⁾

Compound **55g** was prepared as described above for compound **55e**, starting from **53c** (0.40 g; 2.0 mmol) to give a crude product which was purified by recrystallization from EtOH to give **55g** as a white crystalline in yield of 0.70 g (81.0%): mp 280-281°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3330 (NH), 1754, 1645 (C=O); ^1H -NMR (300 MHz, DMSO- d_6) 2.30 (6H, s, CH_3 x 2), 7.28 (4H, d, J = 8.70 Hz, Ar-H), 7.32-7.35 (1H, m, Ar-H), 7.49 (2H, d, J = 8.10 Hz, Ar-H) 8.00 (4H, d, J = 8.70 Hz, Ar-H), 8.30 (1H, s, Ar-H) 10.31 (2H, s, NH x 2); ^{13}C -NMR (75 MHz, DMSO- d_6) 20.86 (CH_3), 112.81 (CH), 116.04 (CH), 121.79 (CH), 128.59 (CH), 129.21 (CH), 132.47 (C), 139.30 (C), 152.88 (C), 164.79 (C), 168.99 (C); FAB-Mass: m/z 433 (M+1).

N-[(3-Benzoylamino)phenyl]benzamide (55h)⁽¹³⁴⁾

Compound **55h** was prepared as described above for compound **55e**, starting from **53d** (0.28 g; 2.0 mmol), the reaction was carried out in dry THF and stirred for 24 hours. The obtained crude product washed several times with ether and dried under reduced pressure, and purified by recrystallization from EtOH to give **55h** as a white precipitate in yield of 0.48 g (75.9%): mp 245°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3268 (NH), 1648 (C=O); ^1H -NMR (300 MHz, DMSO- d_6) 7.31 (1H, t, J = 8.10 Hz, Ar-H), 7.48-7.58 (8H, m, Ar-H), 7.96 (4H, d, J = 6.90 Hz, Ar-H), 8.33 (1H, s, Ar-H) 10.30 (2H, s, NH x 2); ^{13}C -NMR (75 MHz, DMSO- d_6) 112.95 (CH), 116.08 (CH), 127.67 (CH), 128.34 (CH), 128.55 (CH), 131.52 (CH), 134.94 (C), 139.34 (C), 165.54 (C); FAB-Mass: m/z 317 (M+1).

2,3-Diacetoxy-5-[4-(3,4,5-triacetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55i)

Compound **55i** was prepared as described above for compound **55a**, starting from *p*-phenylenediamine **54c** (0.11 g; 1.0 mmol) to give a crude product which was purified by

silica gel column chromatography using CH₂Cl₂: acetone 10:1 as a mobile phase. The obtained oily product was then recrystallized from CH₂Cl₂: n-hexane to give **55i** as white crystals in yield of 0.90 g (67.8%): mp 280-281°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3284 (NH), 1774, 1649 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.32 (12H, s, CH₃ x 4), 2.33 (6H, s, CH₃ x 2), 7.73 (4H, s, Ar-H), 7.82 (4H, s, Ar-H), 10.36 (2H, s, NH x 2); ¹³C-NMR (75 MHz, DMSO-*d*₆) 20.27 (CH₃), 20.72 (CH₃), 120.86 (CH), 121.27 (CH), 133.33 (C), 135.27 (C), 137.69 (C), 143.58 (C), 163.32 (C), 167.32 (C), 168.37 (C); FAB-Mass: m/z 665 (M+1); (found C, 57.75; H, 4.15; N, 4.26. C₃₂H₂₈N₂O₁₄ requires C, 57.83; H, 4.25; N, 4.22).

2-Acetoxy-4-[4-(3,4-diacetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55j)

Compound **55j** was prepared as described above for compound **55i**, starting from **53b** (0.51 g; 2.0 mmol) to give a crude product which was purified by silica gel column chromatography using CH₂Cl₂: acetone 10:1 as a mobile phase. The obtained oily product was then recrystallized from CH₂Cl₂: n-hexane to give **55j** as a white crystalline product in yield of 0.85g (77.5%): mp 288-290°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3347 (NH), 1770, 1649 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.31 (6H, s, CH₃ x 2), 2.32 (6H, s, CH₃ x 2), 7.44 (2H, d, *J* = 8.40 Hz, Ar-H), 7.74 (4H, s, Ar-H), 7.86 (2H, s, Ar-H), 7.92 (2H, d, *J* = 8.10 Hz, Ar-H), 10.32 (2H, s, NH x 2); ¹³C-NMR (75 MHz, DMSO-*d*₆) 20.29 (CH₃), 20.35 (CH₃), 120.60 (CH), 120.70 (CH), 123.13 (CH), 123.68 (CH), 126.08 (CH), 133.40 (C), 134.86 (C), 141.83 (C), 144.51 (C), 163.64 (C), 168.00 (C), 168.19 (C); FAB-Mass: m/z 549 (M+1); (found C, 61.37; H, 4.39; N, 5.11. C₂₈H₂₄N₂O₁₀ requires C, 61.31; H, 4.41; N, 5.11).

4-[4-(4-Acetoxybenzoylamino)phenylcarbamoyl]acetic acid phenyl ester (55k)⁽¹³¹⁾

Compound **55k** was prepared as described above for compound **55i**, starting from **53c** (0.40 g; 2.0 mmol) to give a crude product which was purified by silica gel column chromatography using CH₂Cl₂: acetone 10:1 as a mobile phase. The obtained oily product was then recrystallized from CH₂Cl₂: n-hexane to give **55k** as white crystals in yield of 0.69 g (80.2%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3315 (NH), 1756, 1644 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.30 (6H, s, CH₃ x 2), 7.28 (4H, d, *J* = 8.40 Hz, Ar-H), 7.74 (4H, s, Ar-H), 8.00 (4H, d, *J* = 8.40 Hz, Ar-H), 10.25 (2H, s, NH x 2); ¹³C-NMR

(75 MHz, DMSO- d_6) 20.85 (CH₃), 120.49 (CH), 120.59 (CH), 121.79 (CH), 129.11 (CH), 132.48 (C), 134.92 (C), 152.83 (C), 164.53 (C), 168.96 (C); FAB-Mass: m/z 433 (M+1).

***N*-[(4-Benzoylamino)phenyl]benzamide (**55l**)⁽¹³⁷⁾**

Compound **55l** was prepared as described above for compound **55i**, starting from **53d** (0.28 g; 2.0 mmol), the reaction was carried out in dry THF and stirred for 24 hours. The formed crude product was washed several times with ether and dried under pressure and purified by recrystallization from EtOH to give **55l** as a white precipitate in yield of 0.48 g (75.9%): mp >300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3332 (NH), 1649 (C=O); ¹H-NMR (300 MHz, DMSO- d_6) 7.49-7.58 (6H, m, Ar-H), 7.75 (4H, s, Ar-H), 7.94-7.96 (4H, m, Ar-H), 10.23 (2H, s, NH x 2); ¹³C-NMR (75 MHz, DMSO- d_6) 120.64 (CH), 127.57 (CH), 128.35 (CH), 131.46 (CH), 134.94 (C), 134.97 (C), 165.30 (C); FAB-Mass: m/z 317 (M+1).

2, 3-Diacetoxy-5-phenylcarbamoylacetic acid phenyl ester (55m**)⁽¹³⁸⁾**

Compound **55m** was prepared as described above for compound **55a**, starting from aniline **54d** (0.09 ml; 1.0 mmol), **53c** (0.40 g; 2.0 mmol) and triethylamine (0.15 ml; 1.1 mmol). And stirring for 2 hours to give a crude product which was purified by recrystallization from EtOH to give **55m** as white crystals in yield of 0.30 g (81.0%): mp 162-163°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3330 (NH), 1782, 1764, 1652 (C=O); ¹H-NMR (300 MHz, CD₃OD) 2.29 (6H, s, CH₃ x 2), 2.30 (3H, s, CH₃) 7.12-7.20 (1H, m, Ar-H), 7.32-7.40 (2H, m, Ar-H), 7.64-7.70 (2H, m, Ar-H), 7.74 (2H, s, Ar-H); ¹³C-NMR (75 MHz, CD₃OD) 19.96 (CH₃), 20.36 (CH₃), 108.28 (CH) 121.38 (CH), 122.32 (CH), 122.37 (CH), 125.84 (CH), 129.80 (CH), 134.39 (C), 139.57 (C), 145.04 (C), 146.74 (C), 166.03 (C), 168.34 (C), 169.52 (C); FAB-Mass: m/z 372 (M+1).

5.2.3. Synthesis of GBA derivatives

3,4,5-Trihydroxy-*N*-[2-(3,4,5-trihydroxybenzoylamino)phenyl]benzamide(56a)

To a stirred solution of **55a** (0.66 g; 1.0 mmol) in acetonitrile (20 ml) was added hydrazine monohydrate (0.29 ml; 6.0 mmol). The mixture was stirred for 30 minutes at room temperature, then 10% aqueous KHSO₄ was added until pH = 3. The total mixture was extracted with EtOAc (3 x 20 ml), the combined EtOAc extracts were washed with saturated NaCl solution (10 ml) and H₂O (2 x 10 ml) and then dried over MgSO₄. Complete evaporation of the EtOAc was carried out using vacuum pump to give a crude product which was recrystallized from water to give **56a** as yellowish white crystals in yield of 0.25 g (60.7%): mp 261°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3479 (OH), 3400 (NH), 1591 (C=O); ¹H-NMR (300 MHz, CD₃OD) δ 6.87 (4H, s, Ar-H), 7.18 (2H, q, *J* = 3.60 Hz, Ar-H), 7.47 (2H, q, *J* = 3.60 Hz, Ar-H); ¹³C-NMR (75 MHz, CD₃OD) δ 108.34 (CH), 125.78 (C), 126.87 (CH), 127.18 (CH), 132.85 (C), 138.85 (C), 146.84 (C), 169.04 (C); FAB-Mass: *m/z* 413 (M+1); (found C, 58.01; H, 4.11; N, 6.66. C₂₀H₁₆N₂O₈ requires C, 58.25; H, 3.91; N, 6.79).

3,4-Dihydroxy-*N*-[2-(3,4-dihydroxybenzoylamino)phenyl]benzamide (56b)⁽¹³⁰⁾

Compound **56b** was prepared as described above for compound **56a**, starting from **55b** (0.55 g; 1.0 mmol) and hydrazine monohydrate (0.19 ml; 4.0 mmol). A crude product was formed which was purified by recrystallization from water to give **56b** as white crystals in yield of 0.24 g (63.1%): mp 269-270°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3295 (OH), 3218 (NH), 1635 (C=O); ¹H-NMR (300 MHz, CD₃OD) δ 6.73 (2H, d, *J* = 8.40 Hz, Ar-H), 7.16-7.24 (4H, m, Ar-H), 7.31 (2H, d, *J* = 2.40 Hz, Ar-H), 7.48 (2H, q, *J* = 3.30 Hz, Ar-H); ¹³C-NMR (75 MHz, CD₃OD) δ 116.06 (CH), 116.12 (CH), 121.04 (CH), 126.63 (C), 126.88 (CH), 127.21 (CH), 132.85 (C), 146.54 (C), 150.82 (C), 168.68 (C); FAB-Mass: *m/z* 381 (M+1).

4-Hydroxy-*N*-[2-(4-hydroxybenzoylamino)phenyl]benzamide (56c)

Compound **56c** was prepared as described above for compound **56a**, starting from **55c** (0.43 g; 1.0 mmol) and hydrazine monohydrate (0.1 ml; 2.0 mmol). A crude product was formed which was recrystallized from methanol to give **56c** as white crystals in yield of 0.20 g (57.5%) mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3342 (OH), 3233 (NH), 1646 (C=O); ¹H-

NMR (300 MHz, DMSO- d_6) 6.84 (4H, d, J = 8.70 Hz, Ar-H), 7.24 (2H, q, J = 3.45 Hz, Ar-H), 7.60 (2H, q, J = 3.60 Hz, Ar-H), 7.81 (4H, d, J = 8.40 Hz, Ar-H), 9.87 (2H, s, OH x 2), 10.14 (2H, s, NH x 2); ^{13}C -NMR (75 MHz, DMSO- d_6) 114.45 (CH), 124.47 (CH), 124.83 (CH), 125.18 (C), 128.74 (CH), 131.05 (C), 160.38 (C), 164.61 (C); FAB-Mass: m/z 349 (M+1); (found C, 68.68; H, 4.59; N, 7.89. $\text{C}_{20}\text{H}_{16}\text{N}_2\text{O}_4$ requires C, 68.96; H, 4.63; N, 8.04).

3,4,5-Trihydroxy-*N*-[3-(3,4,5-trihydroxybenzoylamino)phenyl]benzamide (56e)

Compound **56e** was prepared as described above for compound **56a**, starting from **55e** (0.66 g; 1.0 mmol) and hydrazine monohydrate (0.29 ml, 6.0 mmol). A crude product was formed which was recrystallized from EtOH: n-hexane to give **56e** as white crystals in yield of 0.25 g (60.7%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3377 (OH), 3325 (NH), 1656 (C=O); ^1H -NMR (300 MHz, CD_3OD) 6.87 (4H, s, Ar-H), 7.20 (1H, q, J = 7.20 Hz, Ar-H), 7.29-7.33 (2H, m, Ar-H), 7.83 (1H, t, J = 1.95 Hz, Ar-H); ^{13}C -NMR (75 MHz, CD_3OD) 108.20 (CH), 115.37 (CH), 118.33 (CH), 126.62 (C), 129.89 (CH), 138.44 (C), 140.39 (C), 146.72 (C), 169.15 (C); FAB-Mass: m/z 413 (M+1); (found C, 57.93; H, 3.99; N, 6.50. $\text{C}_{20}\text{H}_{16}\text{N}_2\text{O}_8$ requires C, 58.25; H, 3.91; N, 6.79).

3,4-Dihydroxy-*N*-[3-(3,4-dihydroxybenzoylamino)phenyl]benzamide (56f)⁽¹³⁰⁾

Compound **56f** was prepared as described above for compound **56a**, starting from **55f** (0.55 g; 1.0 mmol) and hydrazine monohydrate (0.19 ml; 4.0 mmol). A crude product was formed which was purified by recrystallization from EtOH: n-hexane to give **56f** as a white crystalline product in yield of 0.27 g (71.0%): mp 280-281°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3490 (OH), 3348 (NH), 1646 (C=O); ^1H -NMR (300 MHz, DMSO- d_6) 6.81 (2H, d, J = 8.10 Hz, Ar-H), 7.23 (1H, t, J = 7.95 Hz, Ar-H), 7.33-7.42 (6H, m, Ar-H), 8.22 (1H, s, Ar-H), 9.41 (2H, s, OH x 2), 9.93 (2H, s, NH x 2); ^{13}C -NMR (75 MHz, DMSO- d_6) 112.94 (CH), 114.87 (CH), 115.49 (CH), 115.67 (CH), 119.66 (CH), 125.98 (C), 128.29 (CH), 139.57 (C), 144.92 (C), 148.82 (C), 165.21 (C); FAB-Mass: m/z 381 (M+1).

4-Hydroxy-*N*-[3-(4-hydroxybenzoylamino)phenyl]benzamide (56g)⁽¹³³⁾

Compound **56g** was prepared as described above for compound **56a**, starting from **55g** (0.43 g; 1.0 mmol) and hydrazine monohydrate (0.10 ml; 2 mmol). A crude product was formed which was purified by recrystallization from EtOH: n-hexane to give **56g** as white crystals in yield of 0.23 g (66.0%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3410 (OH), 1643 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 6.86 (4H, d, J = 8.40 Hz, Ar-H), 7.26 (1H, t, J = 8.10 Hz, Ar-H), 7.44 (2H, d, J = 9.90 Hz, Ar-H), 7.87 (4H, d, J = 8.70 Hz, Ar-H), 8.25 (1H, s, Ar-H), 10.00 (2H, s, OH x 2), 10.10 (2H, s, NH x 2); $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) 112.95 (CH), 114.90 (CH), 115.76 (CH), 125.46 (C), 128.39 (CH), 129.76 (CH), 139.57 (C), 160.53 (C), 165.09 (C); FAB-Mass: m/z 349(M+1).

3,4,5-Trihydroxy-*N*-[4-(3,4,5-trihydroxybenzoylamino)phenyl]benzamide (56i)⁽¹³⁵⁾

Compound **56i** was prepared as described above for compound **56a**, starting from **55i** (0.66 g; 1.0 mmol) and hydrazine monohydrate (0.29 ml, 6.0 mmol). A crude product was formed which was recrystallized from EtOH: n-hexane to give **56i** as white crystals in yield of 0.23 g (56.0%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3380 (OH), 3204 (NH), 1649 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 6.94 (4H, s, Ar-H), 7.65 (4H, s, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) 106.90 (CH), 119.95 (CH), 120.05 (CH), 124.86 (C), 134.50 (C), 136.14 (C), 144.99 (C), 164.76 (C); FAB-Mass: m/z 413 (M+1).

3,4-Dihydroxy-*N*-[4-(3,4-dihydroxybenzoylamino)phenyl]benzamide (56j)⁽¹³⁰⁾

Compound **56j** was prepared as described above for compound **56a**, starting from **55j** (0.55 g; 1.0 mmol) and hydrazine monohydrate (0.19 ml; 4.0 mmol). A crude product was formed which was purified by recrystallization from EtOH: n-hexane to give **56j** as a white crystalline product in yield of 0.26 g (68.4%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3450 (OH), 3311 (NH), 1642 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 6.81 (2H, d, J = 8.40 Hz, Ar-H), 7.32 (2H, d, J = 2.25 Hz, Ar-H), 7.37 (2H, s, Ar-H), 7.66 (4H, s, Ar-H), 9.25 (2H, s, OH x 2), 9.87 (2H, s, NH x 2); $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) 114.87 (CH), 115.38 (CH), 119.52 (CH), 120.43 (CH), 125.99 (C), 134.93 (C), 144.92 (C), 148.73 (C), 164.99 (C); FAB-Mass: m/z 381 (M+1).

4-Hydroxy-*N*-[4-(4-hydroxybenzoylamino)phenyl]benzamide (56k)⁽¹³⁶⁾

Compound **56k** was prepared as described above for compound **56a**, starting from **55k** (0.43 g; 1.0 mmol) and hydrazine monohydrate (0.10 ml; 2 mmol). A crude product was formed which was purified by silica gel column chromatography using CH_2Cl_2 : MeOH

10:1. The obtained oily product was recrystallized from EtOAc: n-hexane to give **56k** as a white crystalline substance in yield of 0.23 g (66.0%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3422 (OH), 3324 (NH), 1647 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 6.85 (4H, d, J = 8.70 Hz, Ar-H), 7.68 (4H, s, Ar-H), 7.84 (4H, d, J = 8.70 Hz, Ar-H), 9.93 (2H, s, OH x 2), 10.07 (2H, s, NH x 2); $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) 114.88 (CH), 120.51 (CH), 125.46 (C), 129.61 (CH), 134.94 (C), 160.44 (C), 164.85 (C); FAB-Mass: m/z 349 (M+1).

3,4,5-Trihydroxy-*N*-phenylbenzamide (56m)⁽¹³⁹⁾

Compound **56m** was prepared as described above for compound **56a**, starting from **55m** (0.37 g; 1.0 mmol) and (0.14 ml; 3.0 mmol). A crude product was formed which was purified by recrystallization from water to give **56m** as white crystals in yield of 0.18 g (73.5%): mp 211-212°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3356 (OH), 3276 (NH) 1652 (C=O); $^1\text{H-NMR}$ (300 MHz, CD₃OD) 6.96 (2H, s, Ar-H) 7.08-7.12 (1H, m, Ar-H), 7.28-7.34 (2H, m, Ar-H), 7.61 (2H, d, J = 7.50 Hz, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, CD₃OD) 108.28 (CH), 122.34 (CH), 125.32 (CH), 126.79 (C), 129.69 (CH), 138.40 (C), 139.98 (C), 146.73 (C), 169.10 (C); FAB-Mass: m/z 246 (M+1).

5.2.4. Synthesis of AGABT and AGAT derivatives

2,3-Diacetoxy-5-(benzothiazol-2-ylcarbamoyl)acetic acid phenyl ester (58a)

Compound **58a** was prepared as described above for compound **55a**, starting from 2-aminobenzothiazole **7** (0.15 g; 1.0 mmol) to give a crude product which was purified by silica gel column chromatography using CH₂Cl₂: acetone 25:1 as a mobile phase. The obtained oily product was recrystallized from EtOAc: n-hexane to give **58a** as white crystals in yield of 0.33 g (77.3%): mp 132-134°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3286 (NH), 1779, 1754, 1677 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 2.33 (6H, s, CH₃ x 2), 2.34 (3H, s, CH₃), 7.31-7.36 (1H, m, Ar-H), 7.44-7.49 (1H, m, Ar-H), 7.74-7.79 (1H, m, Ar-H), 7.99-8.05 (1H, m, Ar-H), 8.03 (2H, s, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) 19.86 (CH₃), 20.31 (CH₃), 121.31 (CH), 121.91 (CH), 123.84 (CH), 126.36 (CH), 130.16 (C), 138.21 (C), 143.28 (C), 166.94 (C), 167.98 (C); FAB-Mass: m/z 429 (M+1); (found C, 56.07; H, 3.81; N, 6.52. C₂₀H₁₆N₂O₇S requires C, 56.07; H, 3.76; N, 6.54).

2-Acetoxy-4-(benzothiazol-2-ylcarbamoyl)acetic acid phenyl ester (58b)

Compound **58b** was prepared as described above for compound **58a**, starting from **53b** (0.25 g; 1.0 mmol) to give a crude product which was purified by silica gel column chromatography using CH₂Cl₂: acetone 15:1 as a mobile phase. The obtained oily product was recrystallized from EtOAc: n-hexane to give **58b** as a white crystalline product in yield of 0.29g (78.6%): mp 182-183°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3175 (NH), 1775, 1675 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.31 (3H, s, CH₃), 2.34 (3H, s, CH₃), 7.18-7.52 (3H, m, Ar-H), 7.73 (1H, d, *J* = 8.1 Hz, Ar-H), 7.99-8.12 (3H, m, Ar-H), 12.98 (1H, s, NH); ¹³C-NMR (75 MHz, DMSO-*d*₆) 20.24 (CH₃), 20.32 (CH₃), 114.13 (CH), 121.75 (CH), 123.71 (CH), 123.92 (CH), 123.98 (CH), 126.20 (CH), 126.82 (CH), 141.77 (C), 142.02 (C), 145.33 (C), 145.55 (C), 167.84 (C), 168.04 (C), 168.14 (C); FAB-Mass: *m/z* 371 (M+1); (found C, 58.32; H, 3.81; N, 7.18. C₁₈H₁₄N₂O₅S requires C, 58.37; H, 3.81; N, 7.56).

4-(Benzothiazol-2-ylcarbamoyl)acetic acid phenyl ester (**58c**)⁽¹⁴⁰⁾

Compound **58c** was prepared as described above for compound **58a**, starting from **53c** (0.20 g; 1.0 mmol) to give a crude product which was purified by recrystallization from EtOH to give **58c** as white crystals in yield of 0.24g (77.1%): mp 212-213°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3311 (NH), 1745, 1674 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 2.30 (3H, s, CH₃), 7.06-7.48 (4H, m, Ar-H), 7.76-7.78 (1H, d, *J* = 7.89 Hz, Ar-H), 7.99-8.02 (1H, d, *J* = 7.89 Hz, Ar-H), 8.06-8.09 (1H, d, *J* = 8.80 Hz, Ar-H), 8.16-8.19 (1H, m, Ar-H), 12.91 (1H, s, NH); ¹³C-NMR (75 MHz, DMSO-*d*₆) 20.74 (CH₃), 121.58 (CH), 121.85 (CH), 121.93 (CH), 122.70 (CH), 123.55 (CH), 126.03 (CH), 129.85 (CH), 130.69 (CH), 132.22 (C), 132.67 (C), 153.79 (C), 155.42 (C), 168.66 (C); FAB-Mass: *m/z* 313 (M+1).

N-Benzothiazol-2-yl-benzamide (**58d**)⁽¹⁴¹⁾

Compound **58d** was prepared as described above for compound **58a**, starting from **53d** (0.14 g; 1.0 mmol), the reaction was carried out in dry THF and was stirred for 6 hours to give a crude product which was washed several times with ether and dried under pressure to give **58d** as a white precipitate in yield of 0.20 g (78.8%): mp 130°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3156 (NH), 1673 (C=O); ¹H-NMR (300 MHz, DMSO-*d*₆) 7.28 (1H, t, *J* = 7.80 Hz, Ar-H), 7.44-7.63 (5H, m, Ar-H), 7.77 (1H, t, *J* = 7.05 Hz, Ar-H), 7.96-8.01 (2H, m, Ar-H); FAB-Mass: *m/z* 255 (M+1).

2,3-Diacetoxy-5-(thiazol-2-ylcarbamoyl)acetic acid phenyl ester (61)

Compound **61** was prepared as described above for compound **58a**, starting from 2-aminothiazole **60** (0.10 g; 1.0mmol) to give a crude product which was purified by recrystallization from EtOH to give **61** as white crystals in yield of 0.28 g (74.1%): mp 205-206°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3340 (NH), 1774, 1676 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 2.32 (6H, s, CH₃ x 2), 2.33 (3H, s, CH₃), 7.28 (1H, d, J = 3.90 Hz, Ar-H), 7.556 (1H, d, J = 3.90 Hz, Ar-H), 7.98 (2H, s, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) 19.77 (CH₃), 20.21 (CH₃), 113.94 (CH), 121.08 (CH), 130.50 (C), 137.93 (C), 143.23 (C), 166.93 (C), 167.97 (C); FAB-Mass: m/z 379 (M+1); (found C, 50.78; H, 3.71; N, 7.28. C₁₆H₁₄N₂O₇S requires C, 50.79; H, 3.73; N, 7.40).

5.2.5. Synthesis of GABT and GAT derivatives

***N*-Benzothiazol-2-yl-3,4,5-trihydroxybenzamide (59a)**

Compound **59a** was prepared as described above for compound **56a**, starting from **58a** (0.43 g; 1.0 mmol) and hydrazine monohydrate (0.14 ml; 3.0 mmol). A crude product was formed which was purified by recrystallization from EtOH: n-hexane to give **59a** as white crystals in yield of 0.2 g (66.2%): mp > 300°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3505 (OH), 3368 (NH), 1673 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 7.07 (2H, s, Ar-H), 7.24 (1H, t, J = 7.60 Hz, Ar-H), 7.37 (1H, t, J = 7.65 Hz, Ar-H), 7.68 (1H, d, J = 8.10 Hz, Ar-H), 7.91 (1H, d, J = 7.80 Hz, Ar-H); $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) 108.00 (CH), 120.26 (CH), 121.61 (CH), 123.45 (CH), 126.07 (CH), 131.54 (C), 138.39 (C), 145.64 (C); FAB-Mass: m/z 303 (M+1); (found C, 55.37; H, 3.32; N, 9.17. C₁₄H₁₀N₂O₄S requires C, 55.62; H, 3.33; N, 9.27).

***N*-Benzothiazol-2-yl-3,4-dihydroxybenzamide (59b)**

Compound **59b** was prepared as described above for compound **56a**, starting from **58b** (0.37 g; 1.0 mmol) and hydrazine monohydrate (0.10 ml; 2.0 mmol). A crude product was formed which was purified by recrystallization from EtOH to give **59b** as a white crystalline substance in yield of 0.17 g (59.4%): mp 266-267°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3303 (OH), 3175 (NH), 1648 (C=O); $^1\text{H-NMR}$ (300 MHz, DMSO- d_6) 6.84 (1H, d, J = 8.10 Hz, Ar-H), 7.27-7.32 (1H, m, Ar-H), 7.40-7.46 (1H, m, Ar-H), 7.53 (1H, d, J = 2.40 Hz, Ar-H), 7.58 (1H, d, J = 2.25 Hz, Ar-H), 7.74 (1H, d, J = 8.10 Hz, Ar-H), 7.97 (1H, d, J =

7.20 Hz, Ar-H); ^{13}C -NMR (75 MHz, DMSO- d_6) 114.90 (CH), 115.72 (CH), 120.51 (CH), 121.30 (CH), 122.55 (C), 123.16 (CH), 125.75 (CH), 130.54 (C), 144.96 (C), 150.04 (C); FAB-Mass: m/z 287 (M+1).

***N*-Benzothiazol-2-yl-4-hydroxybenzamide (59c)**

Compound **59c** was prepared as described above for compound **56a**, starting from **58c** (0.31 g; 1.0 mmol) and hydrazine monohydrate (0.05 ml; 1.0 mmol). A crude product was formed which was purified by silica gel column chromatography using CH_2Cl_2 : acetone 5:1. The obtained oily product was then recrystallized from EtOAc: n-hexane to give **59c** as a white crystalline substance in yield of 0.18 g (66.7%): mp 258°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3352 (OH), 3146 (NH), 1675 (C=O); ^1H -NMR (300 MHz, DMSO- d_6) 6.83 (2H, d, J = 8.7 Hz, Ar-H), 7.2-7.28 (1H, m, Ar-H), 7.36-7.41 (1H, m, Ar-H), 7.70 (1H, d, J = 7.80 Hz, Ar-H), 7.93 (1H, d, J = 7.20 Hz, Ar-H), 7.99 (2H, d, J = 8.70 Hz, Ar-H), 10.35 (2H, s, OH x 2), 12.57 (2H, s, NH x 2); ^{13}C -NMR (75 MHz, DMSO- d_6) 115.03 (CH), 121.31 (CH), 122.15 (C), 123.20 (CH), 125.77 (CH), 130.34 (CH), 161.49 (C); FAB-Mass: m/z 271 (M+1); (found C, 62.07; H, 3.76; N, 10.30. $\text{C}_{14}\text{H}_{10}\text{N}_2\text{O}_2\text{S}$ requires C, 62.21; H, 3.73; N, 10.36).

3,4,5-Trihydroxy-*N*-thiazol-2-yl-benzamide (62)

Compound **62** was prepared as described above for compound **56a**, starting from **61** (0.38 g; 1.1 mmol) and hydrazine monohydrate (0.14 ml; 3.0 mmol). A crude product was formed which was purified by recrystallization from EtOH: n-hexane to give **62** as a white crystalline substance in yield of 0.17 g (67.4%): mp 282-283°C; $\nu_{\text{max}}/\text{cm}^{-1}$ 3339 (OH), 3226 (NH), 1654 (C=O); ^1H -NMR (300 MHz, DMSO- d_6) 7.06 (2H, s, Ar-H), 7.19 (1H, d, J = 3.60 Hz, Ar-H), 7.49 (1H, d, J = 3.60 Hz, Ar-H), 9.20 (3H, s, OH x 3), 12.16 (3H, s, NH x 3); ^{13}C -NMR (75 MHz, DMSO- d_6) 107.72 (CH), 113.20 (CH), 122.03 (C), 137.79 (C), 145.64 (C), 164.92 (C); FAB-Mass: m/z 253 (M+1); (found C, 47.40; H, 3.23; N, 10.75. $\text{C}_{10}\text{H}_8\text{N}_2\text{O}_4\text{S}$ requires C, 47.61; H, 3.20; N, 11.11).

5.2.6. Biological evaluation of topoisomerases inhibitors

Materials

Topoisomerase I (EC 5.99.1.2) from calf thymus gland and topoisomerase II (EC 5.99.1.3) from human placenta were purchased from TopoGEN. Propidium iodide, RNase A and calf thymus DNA were purchased from Sigma. Proteinase K, pBR322 DNA and HeLa cell were purchased from Boehringer Mannheim GmbH, MBI fermentas and from ATCC, respectively. MEM (minimum essential medium), Alamar Blue reagent were purchased from Gibco and Biosource, respectively. Camptothecin, etoposide and doxorubicin hydrochloride were obtained from Aldrich, Calbiochem and Sigma, respectively.

5.2.6.1. DNA relaxation and cleavage assays of topoisomerase I

Relaxation activity of topoisomerase I was determined by detecting the conversion of supercoiled pBR322 DNA to its relaxed form.⁽¹⁴⁸⁾ Topoisomerase I reaction was performed in 20 μ l of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.6 μ g bovine serum albumin, tested compound solution in 10% DMSO/MeOH, 1 unit topoisomerase I (20 units for DNA cleavage assay) and 0.15 μ g supercoiled pBR322 DNA. Enzyme reaction proceeded for 40 minutes at 37°C and terminated by adding 5 μ l loading buffer consisting of 200 mM Tris (pH 7.5), 200 mM boric acid, 5 mM EDTA, 50% glycerine and 10 % bromophenol blue. Fifteen μ l of the mixture was subjected to 1.0% agarose gel electrophoresis at 50V for 60 minutes in 100 mM Tris-borate buffer (pH 8.5) containing 2.5 mM EDTA. The agarose gel was stained with ethidium bromide and washed thoroughly with deionized water, and the remaining supercoiled pBR322 DNA on the gel was measured by a densitometer (Atto Co., AE-6900M). One unit of inhibitory activity (IC_{50}) was defined as the amount of inhibitor inhibited 50% of the relaxation of supercoiled pBR322 DNA by 1 unit of topoisomerase I under the above assay conditions. For DNA cleavage reaction⁽¹⁴³⁾, the reaction mixture was terminated by the addition of 5 μ l of a solution containing 5% SDS and 12.5 mg proteinase K and incubated for additional 30 minutes at 37°C. Loading buffer was added and the mixture was run into 1.0% agarose gel containing 0.1% SDS and ethidium bromide (0.5 μ g/ml) at 50V for 2 hours. After agarose gel electrophoresis, the nicked pBR322 DNA on the gel was measured by a densitometer. The increase of nicked pBR322 DNA (topoisomerase I-mediated DNA cleavage) was estimated as the stabilization of cleavable complex by an inhibitor.

5.2.6.2. DNA relaxation and cleavage assays of topoisomerase II

DNA topoisomerase II reaction was performed⁽¹⁰¹⁾ in 20 µl of reaction buffer supplemented with 50 mM Tris-HCl buffer (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.06 µg bovine serum albumin, 0.15 µg supercoiled pBR322 DNA, tested compound solution in 10% DMSO/MeOH, 1 unit topoisomerase II. Incubation for 60 minutes at 37°C, followed by the addition of loading buffer (4 µl). The mixture was electrophoresed in 1% agarose gel in 100 mM Tris-borate buffer (pH 8.5) containing 2.5 mM EDTA and reaction products were quantitated by densitometric analysis. One unit of inhibition was defined as the amount that inhibits 50% of the relaxation of supercoiled pBR322 DNA by 1 unit of topoisomerase II under the above assay conditions. For DNA cleavage activity⁽¹⁴⁴⁾, the same procedure for topoisomerase I but 10 units topoisomerase II were used. After agarose gel electrophoresis, the increase of linearized pBR322 DNA (topoisomerase II-mediated DNA cleavage) was estimated as the stabilizing of cleavable complex by an inhibitor.

5.2.6.3. Cell cycle and cell growth analysis

HeLa cells were grown at 37°C in a humidified atmosphere containing 5% CO₂ in MEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 unit/ ml penicillin and 100 µg/ ml streptomycin. For cell cycle analysis by flow cytometry, HeLa cells (1 x 10⁶ cells) in exponential growth were treated with inhibitor at several concentrations for 24 hr. After treatment, the cells were washed with phosphate buffer saline (PBS; 8.4 mM Na₂HPO₄·12H₂O, 1.5 mM KH₂PO₄, 136.9 mM NaCl, 2.7 mM KCl, pH 7.2) and fixed in cold 70% MeOH and stored at -20°C. The cell pellet was incubated with PBS containing 0.1% RNase A for 40 minutes at 37°C and then 100 µl of propidium iodide at 500 µg/ ml was added. The cells were analyzed with a flow cytometer (Becton Dickinson FACS Calibur) using the ModFit LT which is software for determining the percentage of cells in G0/G1, S and G2/M phases.⁽¹²⁶⁾

HeLa cells (1 x 10⁴ cells/well) were grown for 21 hours in 12-well plate on the same conditions as the cell cycle analysis. Aliquots of 100 µl of Alamar Blue reagent were added to each well containing 1 ml of medium. After incubation for 3 hours at 37°C, the absorbance was read on the plate reader (System Instrument Co., Immuno Mini NJ-2300) at 560 and 620 nm. Cell growth was calculated from the subtracting value of the dual wavelength (OD₅₆₀ to OD₆₂₀).

5.2.6.4. DNA Intercalation

CD (circular dichroism) spectral change of DNA was obtained by subtracting of the CD of inhibitor from the CD of DNA with inhibitor. CD spectra was recorded in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 1 mM MgCl₂ and salmon sperm DNA (25 mg/ml) by a spectropolarimeter (JASCO J-720).

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