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Studies on
A Novel Strategy for Reversible Control of DNA Recognition
by Peptide Ribonucleic Acids (PRNAs)

2004

HIROFUMI SATO
Studies on
A Novel Strategy for Reversible Control of DNA Recognition
by Peptide Ribonucleic Acids (PRNAs)

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HIROFUMI SATO

Department of Molecular Chemistry
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Preface

The work of this thesis has been carried out under the guidance of Professor Yoshihisa Inoue and Professor Takehiko Wada at the Department of Molecular Chemistry, Graduate School of Engineering, Osaka University.

The objective of this thesis is to develop novel and general strategies for nucleic acid recognition control. The author hopes that the results and conclusions presented in this thesis contribute to further development of nucleic acid recognition chemistry.

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List of Publications

1. Recognition control of the nucleic acid model through conformational switching of nucleobase induced by borate ester formation of cis-2',3'-diol
   Wada, T.; Minamimoto, N.; Satoh, H.; Inoue, Y.

2. Synthesis and conformation control of peptide ribonucleic acid containing 5'-amino-5'-deoxyribopurinenucleosides
   Sato, H.; Minamimoto, N.; Wada, T.; Inoue, Y.

3. Conformation and recognition control of peptide ribonucleic acid containing pyrimidine/purine mixed sequence
   Sato, H.; Wada, T.; Inoue, Y.

4. DNA recognition control of γ-PRNA and mismatched base effects upon complex stability
   Sato, H.; Hashimoto, Y.; Kikkawa, M.; Wada, T.; Inoue, Y.

5. A novel strategy for reversible control of conformation and DNA/RNA recognition of peptide ribonucleic acid (PRNA) by external factors
   Wada, T.; Sato, H.; Inoue, Y.

6. Solid-phase synthesis of peptide ribonucleic acids (PRNA)
   Sato, H.; Hashimoto, Y.; Wada, T.; Inoue, Y.
7. Synthesis and Conformation Control of Peptide Ribonucleic Acid (PRNA) Containing 5'-amino-5'-deoxyribopyrimidine and 5'-amino-5'-deoxyribopurine-nucleosides
Sato, H.; Wada, T.; Inoue, Y.

8. The Effect of Chirality of γ-Glutamic Acid Backbone upon Structure and Recognition of Peptide Ribonucleic Acids (PRNA)
Sato, H.; Hashimoto, Y.; Wada, T. and Inoue, Y.
in preparation.

9. Reversible Recognition Control of Peptide Ribonucleic Acid (PRNA) by change of pH
Sato, H.; Hashimoto, Y.; Wada, T. and Inoue, Y.
in preparation.
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Introduction

DNA and RNA are one of the most important and significant functional biopolymers of all living organisms, as the fundamental genetic materials. The genetic information is encoded as a linear array of triplet codons of DNA and this simple four bits of information (the four nucleobases), DNA encodes all the information necessary for development from sperm and egg to living things. Then, the regulation and control of gene expression or the process of replication, repair, and recombination of these genetic information would be achieved by interaction of specific proteins with regions of defined nucleobase sequences of DNA and/or RNA.1

The completion of the human genome project2 has driven our research interest and effort to the creation of new methodology of artificial regulation and/or control of genetic information, such as gene therapeutic drugs using the antisense and antigene strategies. Thus, a number of nucleic acid derivatives and analogues have been proposed for the use in gene therapy, for which both the improvement of enzyme tolerance and the hybridization affinity enhancement are required for not only the antisense molecules but also resulting antisense-mRNA hybrids.3 Studies on specific recognition and binding with synthetic (modified) nucleic acid, which was same and similar structures of natural nucleic acid.3 Nevertheless, furanose-phosphodiester backbone shows poor tolerance to nucleases and complex stabilities of these synthetic (modified) nucleic acid with target DNA/RNA was similar to natural nucleic acid hybrids.4 Thus, a number of modified nucleic acids, nucleic acid analogues, and nucleic acid model compounds, which mimicked the nucleic acid recognition moieties, have been proposed, designed, and synthesized, as antisense and/or antigene molecules.4,6 The modified nucleotides, in which the phosphodiester backbone is replaced by phosphorothioate,7 methyl phosphate,8 or phosphoroamidate,9 reveal a greatly improved tolerance to nucleases. Still, some drawbacks of these modified nucleotides have been their decreased stabilities of hybrids with the target DNA/RNA, compared with those of natural nucleic acid hybrids.10 Moreover, these modified phosphodiester backbones consist of chiral phosphorous center to produce complicated stereoisomer mixtures. Even though it is difficult in general to control the stereogenic center of the phosphorous in the preparation of these model compounds,7 its stereochemistry is known to significantly influence the hybridization efficiency.
Peptide nucleic acid (PNA), originally proposed by Nielsen,\textsuperscript{11,12} is one of the most successful nucleic acid analogues, which employ an oligo[N-(2-aminoethyl)glycine] backbone instead of the ribose-phosphate backbone in natural nucleic acid. However, these hitherto reported nucleic acid derivatives and analogues, including PNA, have an inherent limitation or drawback, lacking of a direct means to actively control the function of these antisense compounds by external stimuli. If one can find a methodology for controlling the recognition ability of nucleic acid model, the nucleic acid model will achieve much wider application through the active control of the function of nucleic acid.

It is well documented that the recognition/binding abilities of nucleic acid and nucleic acid analogues are critically affected by their conformational variations,\textsuperscript{13} especially thorough the syn-anti orientation switching of nucleobase unit. Certainly the nucleobase's anti orientation is essential for efficiently forming base pair, though syn-oriented nucleobase is obviously unfavorable for forming intermolecular hydrogen bonding with the complementary nucleobase. Thus, if one can switch the nucleobase orientation reversibly by external factor and/or stimuli, not only the external control of recognition behavior but also reversible regulation and control of the genetic information will be readily materialized and applied. Unfortunately few external agent or factor, which affects the syn-anti orientation and therefore the recognition behavior of nucleic acids and analogues has been found or proposed to date, although the unusual syn orientation is known to be induced by several internal factors, such as increased steric hindrance introducing large substituent into nucleobase and altered sugar puckering. Indeed, bulky substituents introduced at 6-position of the pyrimidine base or at 2-position of the purine base induce the syn orientation in the nucleobase, while ketalization of the cis-2',3'-diol of uridine enhances the syn/anti ratio of the resulting 2',3'-cUMP or 2'3'-O-isopropyridene uridine through the imposed 2'3'-planar-O$_4$-exo-furanose structure.\textsuperscript{14}

Recently, Minamimoto, Wada, and Inoue have proposed that one way complexation/decomplexation control of DNA with peptide ribonucleic acid (PRNA) was achieved thorough the anti-syn orientation switching of 5'-amino-5'-deoxy-pyrimidinenucleosides synergistically by changing of puckering of ribose through borate formation of 2',3'-diol and intra-molecular hydrogen bonding formation between 5'-amide proton and 2-carbonyl oxygen by adding borax as external factor.\textsuperscript{17} Such an
active control of DNA recognition by external factor has not been achieved and hence the PRNA approach is certainly useful as a unique method to realize the "on-demand" gene therapeutic drug in the antisense strategy and may find various biochemical and pharmaceutical applications as a powerful and versatile tool. However, the range of available PRNA is quite limited in variety at present, only simple 8-mers of homo-uracil sequence and of alternating uracil-cytosine sequence having been synthesized by fragment condensation method in solution. To further expand the scope and also to demonstrate the general validity of the PRNA strategy, it is absolutely necessary to establish simpler-and-versatile synthetic routes not only to the PRNA monomers of all four purine and pyrimidine nucleobases but also to 10- to 20-meric PRNA oligomers of purine-pyrimidine mixed sequences. Furthermore, reversible recognition and complexation/decomplexation control of DNA/RNA with PRNA by external factor and/or external stimuli should be required.

On the other hand, it is also well-known that boric acids and their derivatives form cyclic esters with a variety of cis-1,2-diols, including sugars and ribonucleosides, and this esterification process is reversible in aqueous solutions at moderate pH. From these results, reversible recognition control of DNA with PRNA will be achievable by pH in the presence of borax.

Thus, the present study focuses on the synthesis and reversible control of complexation/decomplexation process of DNA/RNA by external factors of a new type of the nucleic acid model named "peptide ribonucleic acid (PRNA)."

In chapter 1, the exploitation of synthesis methods for a series of four PRNA monomers, which contain uracil, adenine, cytosine, and hypoxanthine, and solid phase synthesis of PRNA oligomers with desired purine-pyrimidine mixed sequences will be demonstrated.

In chapter 2, synthesis and conformational control of PRNA containing 5'-amino-5'-deoxyribopyrimidine and 5'-amino-5'-deoxyribopurinenucleosides will be discussed.

In chapter 3, the effects of chirality of γ-glutamic acid backbone upon the structure and recognition behavior of PRNA will be discussed.

In chapter 4, on-off DNA recognition control of PRNA by adding borax as an external factor will be described. The sequence selectivities and effects of mismatch nucleobases upon stability of PRNA-DNA complex are investigated.
In chapter 5, reversible recognition and complex formation control of PRNA by pH in the presence of borax will be discussed.

References


94, 2561-2572.


CHAPTER 1

Solid-phase synthesis of peptide ribonucleic acids (PRNA)

Introduction

The completion of the human genome sequencing project has driven our research interest and endeavor to the creation of gene therapeutic drugs using the antisense and antigene strategies. Thus, a number of nucleic acid derivatives and analogues have been proposed for the use in gene therapy, for which both the resistance to nuclease, and the enhanced hybridization affinity are required for the oligomers as well as the resulting hybrids. Peptide nucleic acid (PNA), originally proposed by Nielsen, is one of the most successful nucleic acid analogues, which employ an oligo[N-(2-aminoethyl)glycine] backbone instead of the ribose-phosphate backbone in natural nucleic acid. However, these hitherto reported nucleic acid derivatives and analogues, including PNA, have an inherent limitation or drawback, lacking an ability to actively control the function of these nucleic acids by external stimuli.

We have recently proposed a new category of peptide ribonucleic acid (PRNA), in which the 5'-amino-5'-deoxypyrimidine ribonucleoside moiety is appended to an oligo(γ-L-glutamic acid) backbone through the 5'-amino group (Chart 1). Possessing improved solubility in water, longer ribose tether, and matched helical pitch, γ-PRNA 8-mers with an isopoly(L-glutamic acid) backbone form a stable complex with complementary DNAs. Furthermore, the recognition and complexation behavior of γ-PRNA 8-mers with target DNAs can be controlled by borax added as an external factor.

![Chart 1](image-url)
through the 2',3'-borate formation. Such an active control of DNA recognition by external factor has not been achieved and hence the PRNA approach is certainly useful as a unique method to realize the "on-demand" gene therapeutic drug in the antisense strategy and may find various biochemical and pharmaceutical applications as a powerful and versatile tool. However, the range of available PRNA is quite limited in variety at present, only simple 8-mers of homo-uracil sequence and of alternating uracil-cytosine sequence having been synthesized by fragment condensation method in solution. To further expand the scope and also to demonstrate the general validity of the PRNA strategy, it is absolutely necessary to establish simpler-and-versatile synthetic routes not only to the PRNA monomers of all four purine and pyrimidine nucleobases and to 10- to 20-meric PRNA oligomers of purine-pyrimidine mixed sequences.

In an effort to develop a simpler and more effective route to oligo-PRNAs, we found that the solid-phase peptide synthesis technique is applicable to the PRNA oligomer synthesis. In this strategy, fluorenemethyloxycarbonyl (Fmoc), which is readily removable under a mild basic condition, was employed as the protective group for the N-terminus of PRNA monomer, while benzoyl (Bz) group for the exocyclic amino residues of nucleobases. In this paper, we report the syntheses of four Fmoc-protected peptide ribonucleic acid monomers with uracil, N-Bz-cytosine, hypoxanthine, and N-Bz-adenosine nucleobases, which were subsequently used in the solid-phase syntheses of a couple of PRNA 12-mers with desired purine-pyrimidine mixed sequences.

\[
\text{Scheme 1. Proposed recognition control model of PRNA with complementary DNA.}
\]
Results and Discussion

Most of the existing antisense molecules are designed to simply inhibit the genetic information transfer, while little effort has been devoted to the active control of the DNA/RNA recognition processes. In our previous studies, we have proposed a new methodology and effective tools for controlling DNA/RNA recognition by external agent. This strategy employs a novel nucleic acid analogue, i.e. γ-peptide ribonucleic acid (γ-PRNA), as the recognition moiety with a built-in switch triggered by an external agent. The borate ester formation of ribose’s cis-2’,3’-diol and the synchronized hydrogen-bonding interaction between the ribose’s 5’-amide proton and the 2-carbonyl oxygen of nucleobase act as the external and internal switching devices (Scheme 1). Furthermore, we have demonstrated for the first time that stable complexes of γ-PRNA with complementary oligonucleotides are readily dissociated by adding borax. Thus, PRNA is certainly one of the most promising antisense molecules of the next generation and can be used as a powerful and versatile tool for switching biological functions.

In this study, we applied the solid-phase peptide synthesis technique to the construction of the peptide backbone of PRNA oligomer. In this strategy, we employed the Fmoc/benzotriazol-1-yloxytris(dimethylamino)phosphoniumhexafluoro-phosphate) (Bop), rather than tert-butyloxycarbonyl (Boc)/Bop, pair for the protection of N-termini, since the glycosyl bond of nucleoside derivatives is acid-sensitive and the unprotected cis-2’,3’-hydroxyl groups of PRNA is less stable under acidic condition. The Fmoc protection allows us to use the newly developed resin with a long ethylene glycol spacer, which is know to give high coupling yields particularly in the syntheses of bulky long peptides, whereas the Boc protection requires the use of conventional, but somewhat unstable, oxime resin as solid support, which is however somewhat unstable and less reliable in some cases. We first prepared the full set of Fmoc-protected PRNA monomers, which are the essential building blocks for the PRNA synthesis of desired sequence.

Preparation of 5’-amino-5’-deoxynucleosides

In this study, adenosine, cytidine, uridine, and inosine were used as nucleic acid-recognizing moieties of PRNA. Inosine, lacking the 2-amino group, is a substitute for the guanosine nucleoside of natural nucleic acids and is also a complementary nucleobase of cytosine. 5’-Azido-5’-deoxynucleosides were prepared by reacting the
corresponding nucleosides with carbon tetrabromide and triphenylphosphine in the presence of lithium azide. The exocyclic amino group of adenosine and cytidine was protected by a benzoyl group, which can survive the catalytic hydrogenation to deprotect the benzyl group and also the trifluoroacetic acid (TFA) treatment to deprotect the Boc group, yet be removed by the treatment with 28% aqueous ammonia. The nucleosides with and without protected amino group were converted to 5'-azido-5'-deoxy-nucleosides in moderate to high yields (56-90%). 5'-Azido-5'-deoxyuridine (5'-N<sub>3</sub>-Urd) and -inosine (5'-N<sub>3</sub>-Ino) were purified by column chromatography on silica gel, while exocyclic amino-protected 5'-azido-5'-deoxycytidine (5'-N<sub>3</sub>-Cyd(N<sub>4</sub>-Bz)) and -adenosine (5'-N<sub>3</sub>-Ado(N<sub>6</sub>-Bz)) by recrystallization from ethanol. The azido residue of 5'-azido-5'-deoxynucleoside was converted to an amino group by catalytic hydrogenation. These 5'-azido-5'-deoxynucleosides were readily hydrogenated in quantitative yields under a mild condition. The 5'-amino-5'-deoxynucleosides thus obtained were purified by reprecipitation from methanol by adding ether (Scheme 2).
Preparation of N-Boc-protected γ-PRNA monomers

PRNAs, in which 5'-amino-5'-deoxyribonucleoside moiety is appended to each amino acid residue of the oligo(γ-L-glutamic acid) backbone, are designed not only to enhance the hybridization affinity as well as the stability of antisense molecule and its hybrid in cytosol, but also to reversibly control the recognition behavior by external factors. In PRNA monomers, 5'-amino-5'-deoxyribonucleoside unit is tethered to the α-carboxyl of α-amino/γ-carboxyl-protected L-glutamic acid through the 5'-amino group, thus reserving the ribose's cis-2',3'-diol for the cyclic borate formation and the 5'-amide proton for the hydrogen-bonding interaction with the pyrimidine's 2-carbonyl. Since the treatment with diisopropylethylamine (DIEA) is unavoidable in the synthesis of PRNA monomer, Boc-protected L-glutamic acid was chosen as the starting compound (as the Fmoc protective group is unstable even under mild basic condition). In all PRNA monomer syntheses, N-Boc-L-glutamic acid α-benzyl ester (Boc-Glu-OBzl) was condensed with 5'-amino-5'-deoxynucleosides in the presence of Bop reagent and 1-hydroxybenzotriazole (HOBt) as condensation reagents. The protected PRNA monomers showed solubilities in methanol varying from highly soluble Boc-isoGln(5'U)-OBzl (3a) and Boc-isoGln(5'A(N6-Bz))-OBzl (3d) to less soluble Boc-isoGln(5'I)-OBzl (3b), and then to slightly soluble Boc-isoGln(5'C(N4-Bz))-OBzl (3c). In particular, 3c could not be subjected to open column chromatography. Hence, the PRNA monomers containing uracil (3a), hypoxanthine (3b), and adenine (3d) were purified by column chromatography on silica gel, while the cytosine-containing PRNA monomer (3c) was purified by reprecipitation from ether with methanol (Scheme 3).

\[ \text{Scheme 3. Preparation of PRNA Monomers (3a-d)} \]
Preparation of N-Fmoc-protected γ-PRNA monomers

Fmoc, which is readily removable under a mild basic condition, is one of the most frequently employed α-amino-protecting groups in solid-phase peptide synthesis. An alternative conventional solid-phase peptide synthesis employs Boc as the protective group for the N-terminus and trifluoroacetic acid (TFA) as the deprotecting reagent. However, this method often causes depurination of purinenucleoside derivatives upon treatment with TFA. We therefore used the Fmoc method in the solid-phase synthesis of PRNAs.

Fmoc-protected PRNA monomers (6a-d) were prepared from the corresponding Boc-PRNA-Bzl monomers 3a-d. The Boc group of 3a-d was removed by the treatment with TFA at room temperature to give the TFA salts of PRNA-Bzl monomers (4a-d). Then, these PRNA-Bzl monomers, possessing a free amino group at the α-position, were reacted with N-(9-fluorenylmethylloxycarbonyloxy) succinimide (Fmoc-OSu) to give Fmoc-protected PRNA-Bzl monomers (5a-d). Fmoc-isoGln(5'U)-OBzl 5a was purified by reprecipitation from a methanol solution by adding diethyl ether, while Fmoc-isoGln(5'I)-OBzl (5b) and Fmoc-isoGln(5'A(N²-Bz))-OBzl (5d) were purified by short column chromatography. The Bzl protection of Fmoc-PRNA-Bzl monomers was quantitatively removed by catalytic hydrogenation over palladium/charcoal to afford the corresponding Fmoc-PRNA monomers, carrying a free carboxylic acid at the γ-position for solid-phase peptide synthesis. Since the Fmoc-isoGln(5'C(N²-Bz))-OBzl (4c) showed a worse solubility in DMF (although the fully deprotected 5'C(N²-Bz)-PRNA monomer was sufficiently soluble in DMF), then we employed a different approach to Fmoc-isoGln(5'C(N²-Bz))-OH (6c). In this strategy, the Bzl group of 4c was first removed by catalytic hydrogenation, and then the Fmoc group was introduced by reaction with Fmoc-OSu to give 6c. Now, a full set of the four Fmoc-protected PRNA monomers with a free carboxyl group (Fmoc-isoGln(5'N)-OH (6a-d)) were prepared in moderate overall yields (Scheme 4). The obtained Fmoc-PRNA monomers were soluble and stable enough to be handled in N-methylpyrrolidone (NMP), a representative solvent employed in solid-phase peptide synthesis.
Solid-phase synthesis of PRNA oligomers

First of all, the compatibility of Fmoc/Bz-protected PRNA monomers with the standard solid-phase peptide synthesis protocol (Chart 2) was checked. It was thus examined whether or not the Bz-protected exocyclic amino residue of nucleobase in PRNA monomers can survive the piperidine treatment used in the Fmoc deprotection. HPLC and UV spectral analyses indicated that both PRNA monomers, containing N'-benzoylcytosine and N6-benzoyladenine, are resistant to the piperidine treatment at ambient temperature, and that the Bz protection is efficiently removed in quantitative yield upon treatment with 28% aqueous ammonia. It was further demonstrated that the conventional solid-phase synthesis protocol does not damage the ribose’s unprotected cis-2',3'-diol of PRNA monomers appended to the polymer support. Thus, practically no side reactions, such as esterification, were found to occur in the oligomeric PRNAs recovered from the solid support. These results clearly indicate that oligomeric PRNAs can be prepared from the Fmoc/Bz-protected PRNA monomers using the standard solid-phase peptide synthesis protocol.
In this study to prepare a series of long-tethered oligomeric PRNAs, we employed a newly developed solid support with a long oxyethylene spacer, which was shown to give high coupling yields particularly in the synthesis of bulky or long peptides. Fmoc-PRNA monomer (3 eq), Bop reagent (3 eq), HOBt (3 eq), and DIEA (6 eq) were added to an NMP slurry of resin (1 eq), and the peptide backbone was elongated step by step (Chart 2). The Fmoc protective group was removed by the treatment with 20-30% piperidine in NMP. In each coupling step, the backbone elongation was confirmed by the conventional Kaiser test and the UV detection of Fmoc-piperidine adduct. If the result of Kaiser test was positive, the coupling reaction was repeated 2-3 times until the Kaiser test became negative and the Fmoc-piperidine adduct was formed quantitatively. All of the four PRNA monomers displayed excellent coupling efficiency and the quantitative coupling was achieved even in the synthesis of 12-mers, as demonstrated below. In the final step of the solid-phase synthesis, the Bz protection at the exocyclic amino group of adenosine and cytosine was removed by the treatment with 28% aqueous ammonia. After being thoroughly washed with NMP and chloroform, the target PRNA oligomer synthesized on the solid support was cleaved and isolated from the resin by TFA containing 2.5% water and 2.5% triisopropylsilane as scavengers. The crude PRNA oligomers in TFA were reprecipitated by adding cold ether to give TFA salts of PRNA oligomers as white powder. The PRNA oligomers thus obtained were purified by reversed phase preparative HPLC. Excellent yields of up to 98.5%/step were obtained and no byproduct was detected, while lower coupling yields around 80% were reported for the solution-state fragment condensation synthesis.

![Chart 2. Solid-phase peptide synthesis using fmoc protective group](image-url)
A couple of PRNA 12-mers, which contain either uracil and hypoxanthine or uracil, adenine, and cytosine, were synthesized indeed. Thus, PRNA 12-mer \( \text{NH}_2\text{-CCU-UAC-UAU-CUC-Lys-OH} \) (7) was prepared by the Fmoc solid-phase synthesis on the newly developed solid support with an amino residue content of 0.21 mmol/g. In the first step, the solid support (100 mg) was suspended in an NMP solution of Fmoc-Lys(Boc)-OH (29.5 mg, 0.63 mmol), Bop reagent (28.0 mg, 0.63 mmol, 1 eq), and HOBT (12.2 mg, 0.63 mmol, 1 eq), to which was added DIEA (23.1 µL, 1.26 mmol, 2 eq), and then the resulting solution was gently stirred at room temperature. After 15 min of stirring, the resin was washed 5 times with NMP, and if the Kaiser test was negative Fmoc was removed by treating the Lys(Boc)-appended resin with 30% piperidine in NMP for 15 min. After the deprotection, the resin was washed 5 times with NMP. The same procedure was repeated until the PRNA 12-mer of desired sequence was obtained by using the appropriate Fmoc-PRNA monomers, i.e. Fmoc-isoGln(5'U)-OH (37.5 mg, 0.63 mmol), Fmoc-isoGln(5'A(N6-Bz)-OH (45.5 mg, 0.63 mmol), and Fmoc-isoGln(5'C(N`-Bz)-OH (44.0 mg, 0.63 mmol). After the 12-mer was reached, the resin was washed with water and the benzoyl protection was removed by the treatment with 28% aqueous ammonia, and finally the resin was thoroughly washed with NMP and chloroform. After drying under a reduced pressure, the total resin weight was measured and the crude yield of PRNA 12-mer was determined as 83.4% (98.5%/step). A consistent yield of 82% was obtained from the UV spectrometric determination of ammonium benzoate solution obtained in the deprotection process of the solid-phase PRNA synthesis. The deprotected PRNA 12-mer was cleaved from the resin by TFA treatment and purified by reversed phase preparative HPLC eluted with aqueous acetonitrile. HPLC fractions, containing the PRNA 12-mer, were collected and the combined fraction was freeze-dried to give the target PRNA 12-mer, \( \text{NH}_2\text{-III-CCL-CCC-Lys-OH} \) (7), as white powder. The isolated PRNA 12-mer was analyzed by analytical HPLC on an RP18 column to show a single peak (>99% pure); see Figure 1. MALDI-TOF mass spectrometric analysis (negative mode) also indicated a >98% purity of PRNA 12-mer (Found \( m/z \) 4436.51 (M); Calcd 4436.65).

Similarly, the self-complementary PRNA 12-mer, \( \text{NH}_2\text{-III-CCI-CII-CCC-Lys-OH} \) (8), was obtained in good yield (88.0%, 98.4%/step). The purity was checked with \(^1\text{H} \text{NMR} \) and MALDI-TOF MS (positive mode; Found \( m/z \) 4535.15 (M+1); Calcd 4533.68)
Conclusion

We established the synthetic routes to a series of PRNA monomers, carrying adenine, cytosine, hypoxanthine, and uracil nucleobases as recognition sites. This enabled us to fully expand the range of available PRNA monomers. We further demonstrated that the newly synthesized Fmoc-protected PRNA monomers are compatible with the standard solid-phase peptide synthesis protocol, and oligomeric PRNAs with purine-pyrimidine mixed sequences can be prepared. Indeed, representative PRNA 12-mers of mixed sequences were synthesized in high yields by the Fmoc solid-phase peptide synthesis, and were characterized by HPLC and MALDI-TOF mass spectrometric analyses. It is noted that the solid-phase synthesis is much more convenient, efficient, and reliable than the conventional fragment condensation method in solution reported previously. The present synthetic strategy should lead us to a wide variety of PRNA oligomers with desired sequences, which function as reversibly controllable antisense molecules upon complexation with the complementary DNAs. Studies along this line are currently in progress.

Experimental Section

Standard abbreviations for amino acids and protecting groups are as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature.

All starting materials, reagents, and solvents were commercially available and
used without further purification. Nucleosides were purchased from Seikagaku Co. Ltd. (Tokyo, Japan). Boc-L-amino acids, Fmoc-L-amino acids, and 1-hydroxybenzotriazole (HOBT) were purchased from Peptide Institute, Inc. (Osaka, Japan). Other chemicals of guaranteed grade were purchased Tokyo Kasei Kogyo Co. (Tokyo, Japan) or Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Column chromatography was performed on silica gel (70-230 mesh) from Kanto Kagaku. Gel filtration was performed on Sephadex G-25 from Pharmacia. Nucleic acid analogues were purified on an ODS column by elution with 10% acetonitrile in water at a flow rate of 3 mL/min.

IR spectra were recorded on a JASCO FT/IR-230 spectrometer. UV spectra were recorded on a JASCO V-550 UV/vis spectrophotometer equipped with a temperature controller. $^1$H NMR spectra were obtained on a JEOL GSX-270 at 270 MHz or a Varian INOVA-600 at 600 MHz. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane ($\delta_H = 0.0$ ppm) as internal standard. Mass spectral measurements were performed on a JEOL AX-500 instrument by fast-atom bombardment (FAB) ionization with nitrobenzyl alcohol (NBA) as a matrix or on a Voyager RP from PerSeptive Biosystems with $\alpha$-cyano-4-hydroxycinnamic acid (CHCA) or picolinic acid as a matrix (MALDI-TOF).

\[
N^2\text{-Fluorenylmethyloxycarbonyl-}\text{N}^2\text{-}(5'\text{-deoxy-5'\text{-uridy})\text{-L-isoglutamine}} \quad (\text{Fmoc-isoGln}(5'U)-\text{OBzl}) \quad (6a)
\]

$N^2\text{-}(5'\text{-Deoxy-5'\text{-uridy})\text{-L-isoglutamine benzyl ester trifluoroacetic acid salt (TFA\text{-isoGln}(5'U)-OBzl)}$ was synthesized as described previously.\textsuperscript{4} To a solution of TFA\text{-isoGln}(5'U)-OBzl (2.20 g, 3.81 mmol) and 9-fluorenylmethyl $N$-succinimidyl carbonate (Fmoc-OSu) (1.54 g, 4.57 mmol) in DMF (100 mL), diisopropylethylamine (1.19 g, 7.62 mmol) was added at 0 °C. The mixture was stirred for 10 min at room temperature. The solvent was removed under a reduced pressure and methanol was added to the residue to precipitate Fmoc-isoGln(5'U)-OBzl (5a) as a white precipitate, which was filtered, washed with methanol, and dried under a reduced pressure to give the product (2.16 g, 82.8%). A catalytic amount of 10% Pd/C (ca. 0.1 g) was added to a solution of Fmoc-isoGln(5'U)-OBzl (2.16 g, 3.15 mmol) in methanol-DMF mixture (1:1 v/v). The mixture was stirred for 2 h under a hydrogen atmosphere, and then the catalyst was filtered. The filtrate was concentrated by rotary evaporation under a reduced pressure, to which a small amount of methanol and then ether were added to
give the title compound as white powder (2.08 g, 3.12 mmol).

**Fmoc-isoGln(5'U)-OBzl (5a):**

δ (KBr disc) (270 MHz; DMSO-d$_6$) 3.69-2.00 (2H, m, β-CH$_2$), 2.37, (2H, t, γ-CH$_2$), 3.20-3.44 (2H, m, 5'-H), 3.75-3.89 (2H, m, fluorene-CH$_2$-O), 3.97-4.08 (2H, m, 3', 4'), 4.15-4.30 (3H, m, α, 2’, fluorene methine), 5.07 (2H, s, OCH$_2$-Ph), 5.17-5.30 (1H, bs, 3'-OH), 5.38-5.50 (1H, bs, 2'-OH), 5.61 (1H, d, 5-H), 5.73 (1H, d, 1'-H), 7.25-7.88 (15H, m, fluorene (8H), α-NHCO (1H), benzyl (5H), 6-H (1H)), 8.13 (1H, t, 5'-NH), 11.34 (1H, s, 3-NH); Anal. Found: C, 63.18; H, 5.39 N, 8.10. Calcd for C$_{36}$H$_{36}$N$_4$O$_{10}$: C, 63.15; H, 5.30; N, 8.18; FAB MS Found m/z 685 (M+1), Calc 684.24.

**Fmoc-isoGln(5'U)-OH (6a):**

δ (KBr disc) (270 MHz; DMSO-d$_6$) 1.67-2.00 (2H, m, β-CH$_2$), 2.29, (2H, t, γ-CH$_2$), 3.20-3.48 (2H, m, 5'-H), 3.73-3.89 (2H, m, fluorene-CH$_2$-O), 3.96-4.09 (2H, m, 3', 4'), 4.16-4.32 (3H, m, α, 2’, methine of fluorene), 5.23-5.51 (2H, bs, 2'-OH, 3'-OH), 5.63 (1H, d, 5-H), 5.73 (1H, d, 1'-H), 7.25-7.76 (10H, m, fluorene (8H), α-NHCO (1H), 6-H (1H)), 8.10 (1H, t, 5'-NH), 11.35 (1H, s, 3-NH); HR-FAB MS Found m/z 595.2015 (M+1, error [ppm/mmu, -4.2 / -2.5]), Calc 595.2040 (M+H).

**N$^a$-Fluorenlymethyloxycarbonyl-$N^\xi$-(5'-deoxy-5'-cytidyl($N^4$-benzoyl))-L-isoglutamine (Fmoc-isoGln(5'C($N^4$Bz))-OH) (6c)**

TFA-isoGln(5'C($N^4$Bz))-OBzl was synthesized as described previously. A solution of NH$_2$-isoGln(5'C($N^4$Bz))-OBzl (3.20 g, 4.71 mmol) in DMF-methanol (1:1 v/v) (500 mL) was vigorously stirred under a hydrogen atmosphere with 10% Pd/C (ca. 0.3 g) for 6 h. The mixture was filtered and evaporated under a reduced pressure. The residue was precipitated from ether to give 2.00 g of TFA·NH$_2$-isoGln(C($N^4$-Bz))-OH as a white amorphous solid. To a solution of TFA·NH$_2$-isoGln(C($N^4$-Bz))-OH (2.00 g) and 9-fluorenlymethyl $N$-succinimidyl carbonate (Fmoc-OSu) (1.36 g, 4.03 mmol) in DMF (100 mL), diisopropylethylamine (526, 4.20 mmol) was added at 0°C. The mixture was continuously stirred for 10 min at room temperature. The solvent was evaporated under a reduced pressure and methanol was added to the residue to precipitate Fmoc-isoGln(C($N^4$-Bz))-OH as white powder. The powder was filtered and washed with methanol and dried under a reduced pressure to give the title compound.
(1.40 g, 61%): $\nu_{\text{max}}$(KBr disc)/cm$^{-1}$ 3300, 3060, 2930, 1700, 1660, 1560, 1490, 1390, 1320, 1260, 1150, and 1090; $\delta_{\text{H}}$ (270 MHz; DMSO-$d_6$) 1.67-2.00 (2H, m, $\beta$-CH$_2$), 2.26, (2H, t, $\gamma$-CH$_2$), 3.40-3.55 (2H, m, 5'-H), 3.79-4.13 (4H, m, fluorene-CH$_2$-O, 3', 4'), 4.16-4.31 (3H, m, $\alpha$, 2', methine of fluorene), 5.19 (1H, d, 3'-OH), 5.52 (1H, d, 2'-OH), 5.79 (1H, d, 1'-H), 7.25-8.22 (17H, m, fluorene (8H), $\alpha$-NHCO (1H), 6-H (1H), 5-H (1H), $\sigma$, m, p-H (5H), 5'-NH (1H)), 11.27 (1H, s, 4-NHCO); HR-FAB MS Found m/z 698.2469 (M+1, error [ppm/mmu, +1.0 / +0.7]), Calcd 698.2462 (M+H).

5'-Azido-5-deoxy-inosine (5'-N$_3$-Ino)

Inosine (890 mg, 3.0 mmol), triphenylphosphine (2.20 g, 8.4 mmol), and lithium azide (1.02 g, 21.0 mmol) were suspended in dry DMF and carbon tetrabromide (2.78 g, 8.14 mmol) was added to the suspension. After stirring for 3h at room temperature, the solvent was evaporated to concentrate. The residue was purified by column chromatography on silica gel with chloroform-methanol (5:1 v/v) to give the title compound (775 mg, 88.09%): $\delta_{\text{H}}$ (270 MHz; DMSO-$d_6$) 3.51-3.72 (2H, m, 5'-H), 3.94-4.27, (2H, m, 3'-H, 4'-H), 4.63 (1H, q, 2'-H), 5.4 (1H, d, 3'-OH), 5.63 (1H, d, 2'-OH), 5.9 (1H, d, 1'-H), 8.08 (1H, s, 2-H), 8.33 (1H, s, 8-H); Anal. Found: C, 40.95; H, 3.68; N, 33.32. Calcd for C$_{10}$H$_7$N$_7$O$_4$: C, 40.94; H, 3.78; N, 33.44; FAB MS Found m/z 294 (M+1), Calcd 293.09.

5'-Amino-5'-deoxy-inosine (5'-NH$_2$-Ino) (2b)

10% Pd/C (ca. 0.1 g) was added to a solution of 5'-N$_3$-Ino (239 mg, 1.0 mmol) in methanol-DMF mixed solvent (1:1 v/v) (50 mL). After being stirred for 2 h under a hydrogen atmosphere (1 atm), the mixture was filtered and the filtrate was evaporated to concentrate and a small amount of methanol was added to the solution. Further, ether was added to give the title compound as white powder (245 mg, 92%): $\delta_{\text{H}}$ (270 MHz; DMSO-$d_6$) 2.71-2.91 (2H, m, 5'-H), 3.45 (2H, bs, 5'-NH$_2$), 3.88, (1H, q, 4'-H), 4.13 (1H, q, 3'-H), 4.57 (1H, t, 2'-H), 5.2-5.4 (2H, bs, 3'-OH, 2'-OH), 5.84 (1H, d, 1'-H), 8.06 (1H, s, 2-H), 8.36 (1H, s, 8-H); Anal. Found: C, 44.67; H, 5.11; N, 25.91. Calcd for C$_{10}$H$_7$N$_5$O$_4$: C, 44.94; H, 4.90; N, 26.21); FAB MS Found m/z 268 (M+1), Calcd 267.10.
**N'-tert-Butyloxycarbonyl-N5-(5'-deoxy-5'-inosyl)-L-isoglutamine (Boc-isoGln(5'I)-OBzl) (3b)**

To a solution of N-tert-butyloxycarbonyl-L-glutamic acid γ-benzyl ester (Boc-L-Glu(OBzl)) (1.32 g, 3.90 mmol), HOBt (526 mg, 3.9 mmol), and BOP reagent (1.15 g, 3.9 mmol) in DMF (50 mL), diisopropylethylamine (613 mg, 3.9 mmol) was added. After 30 s, 5'-NH2-Ino (1.04 g, 3.9 mmol) was added and stirring was continued for 1 h at room temperature. The solvent was removed under a reduced pressure and the residue was purified by column chromatography on silica gel with chloroform-methanol (5:1 v/v) to give the title compound as white powder (1.56 g, 68.0%): νmax(KBr disc)/cm⁻¹ 3420, 1680, 1520, 1460, 1390, 1250, and 1170; δH (270 MHz; DMSO-d6) 1.33 (9H, s, t-Bu-H), 1.63-2.00 (2H, m, P-CH₂), 2.36 (2H, γ-CH₂), 3.24-3.43 (2H, m, 5'-H), 3.76-4.06 (3H, m, 2'-H, 3'-H, 4'-H), 4.53 (1H, q, α-CH₂), 5.06 (2H, s, PhCH₂), 5.31 (1H, d, 3'-OH), 5.47 (1H, d, 2'-OH), 5.83 (1H, d, 1'-H), 6.98 (1H, d, Boc-NH), 7.27-7.40 (5H, m, Ar-H), 8.00-8.12 (2H, m, 2'-H, 5'-NH) 8.30 (1H, s, 8-H); Found: C, 55.01; H, 6.12; N, 14.01. Calcd for C₂₇H₃₄N₆O₉: C, 55.28; H, 5.84; N, 14.33; FAB MS Found m/z 587 (M+1), Calcd 586.2387.

**N5-(5'-Deoxy-5'-inosyl)-L-isoglutamine Benzyl Ester Trifluoroacetic Acid Salt (TFA-isoGln(5'I)-OBzl) (4b)**

Boc-isoGln(5'I)-OBzl (1.01 g, 1.72 mmol) was dissolved in TFA (10 mL) and the solution was kept at 0 °C for 30 min. Ether was added to the reaction mixture to give the title compound as an amorphous solid (1.03 g, 99%).

**N5-Fluorenlymethoxycarbonyl-N5-(5'-deoxy-5'-inosyl)-L-isoglutamine (Fmoc-isoGln(5'I)-OH) (6b)**

To a solution of TFA-isoGln(5'I)-OBzl (910 mg, 1.51 mmol) and 9-fluorenlymethyloxycarbonyl N-succinimidyl carbonate (Fmoc-OSu) (611 mg, 1.81 mmol) in DMF (50 mL), diisopropylethylamine (475 mg, 3.02 mmol) was added at 0°C. The reaction mixture was continuously stirred for 10 min at room temperature. The solvent was removed under a reduced pressure and the residue was purified by column chromatography on silica gel with chloroform-methanol (7:1 v/v) to give Fmoc-isoGln(5'I)-OBzl as white powder. The powder was filtered and washed with methanol and dried under a reduced pressure to give Fmoc-isoGln(5'I)-OBzl (943.8 mg, 87.7%).
10% Pd/C (ca. 0.1 g) was added to a solution of Fmoc-isoGln(5'T)-OBzl (943.8 mg, 1.32 mmol) in methanol-DMF (1:1 v/v) (250 mL). After the reaction mixture was stirred for 2 h under a hydrogen atmosphere (1 atm), the catalyst was filtered and the filtrate was evaporated to concentrate and a small amount of methanol was added to the solution. Ether was further added and the precipitate formed was filtered to give 880 mg of the title compound (1.32 mmol, 87.4%): \( \nu_{\text{max}}(\text{KBr disc})/\text{cm}^{-1} \) 3300, 3060, 2930, 1700, 1660, 1320, 1260, 1150, and 1090; \( \delta_{\text{H}} \) (270 MHz; DMSO-\(d_6\)) 1.63-1.97 (2H, m, \( \beta-\text{CH}_2 \)), 2.23 (2H, \( \gamma-\text{CH}_2 \)), 3.33-3.46 (2H, m, 5'-H), 3.91-4.07 (3H, m, 4'-H, fluorene-\( \text{CH}_2\)-O), 4.16-4.30 (3H, m, 2'-H, 3'-H, methine of fluorene), 4.53 (1H, t, \( \alpha-\text{CH} \)), 5.83 (1H, d, 1'-H), 7.26-7.93 (9H, m, fluorene (8H), \( \alpha-\text{NH} \)), 8.11 (1H, s, 2-H), 8.19 (1H, t, 5'-NH), 8.33 (1H, s, 8-H); HR-FAB MS Found \( m/z \) 619.2151 (M+1, error [ppm/mm], -0.2 / -0.1), Calcd 619.2153 (M+H).

**N\textsuperscript{2}-tert-Butyloxycarbonyl-N\textsuperscript{5}-(5'-deoxy-5'-adenyl(N\textsuperscript{6}-benzoyl))-L-isoglutamine Boc-isoGln(5'A(N\textsuperscript{6}-Bz))-OBzl (3d)**

To a solution of \( N\text{-tert-butyloxycarbonyl-L-glutamic acid-}\gamma\text{-benzyl ester (Boc-L-Glu(OBzl))} \) (1.40g, 4.04 mmol), HOBt (1.36g, 4.04 mmol), and BOP reagent (546 mg, 4.04 mmol) in DMF (100 mL), diisopropylethylamine (653 mg, 4.04 mmol) was added. After 30 s, 5'-NH\textsubscript{2-Ado(N\textsuperscript{6}-Bz)\textsuperscript{12}} (1.5 g, 4.04 mmol) was added and stirring was continued for 1 h at room temperature. The solvent was removed under a reduced pressure and the residue was purified by column chromatography on silica gel with chloroform-methanol (7:1 v/v) to give the title compound as white powder (2.11g, 75.8%): \( \nu_{\text{max}}(\text{KBr disc})/\text{cm}^{-1} \) 3320, 1680, 1540, 1450, 1380, 1250, and 1170; \( \delta_{\text{H}} \) (270 MHz; DMSO-\(d_6\)) 1.33 (9H, s, t-Bu-H), 1.66-2.00 (2H, m, \( \beta-\text{CH}_2 \)), 2.36 (2H, \( \gamma-\text{CH}_2 \)), 3.29-3.54 (2H, m, 5'-H), 3.92-4.13 (3H, m, 2'-H, 3'-H, 4'-H), 4.76 (1H, q, \( \alpha-\text{CH} \)), 5.06 (2H, s, Ph\text{CH}_3), 5.37 (1H, d, 3'-OH), 5.58 (1H, d, 2'-OH), 6.00 (1H, d, 1'-H), 6.98 (1H, d, Boc-NH), 7.27-7.40 (5H, m, Ar-H), 7.56 (2H, t, Ar-\( \text{m-H} \)), 7.66 (1H, t, Ar-\( \text{p-H} \)), 8.05 (2H, d, Ar-\( \text{m-H} \)), 8.13 (1H, t, 5'-NH), 8.71 (1H, s, 2-H) 8.80 (1H, s, 8-H), 11.25 (1H, s, 4-NH); Found: C, 56.66; H, 5.59; N, 14.22. Calcd for C\text{34}H\text{39}N\text{7}O\text{9}: C, 59.21; H, 5.70; N, 14.22; FAB MS Found \( m/z \) 690 (M+1), Calcd 689.28.
\(N^2-(5'\text{-deoxy}-5'\text{-inosyl})\)-L-isoglutamine Benzyl Ester Trifluoroacetic Acid Salt TFA-isoGln(\(5'A(N^6\text{-Bz})\))-OBzl (4d)

Boc-isoGln(\(5'A(N^6\text{-Bz})\))-OBzl (2.11 g, 3.03 mmol) was dissolved in TFA (10 mL) and the solution was kept at 0 °C for 30 min. Ether (500 mL) was added to the solution to give the title compound as an amorphous solid (1.28 g, 60%).

\(N^2\text{-Fluorenylmethyloxycarbonyl-}N^2-(5'\text{-deoxy}-5'\text{-adenyl(N}^6\text{-benzoyl)})\)-L-isoglutamine (Fmoc-isoGln(\(5'A(N^6\text{-Bz})\))-OH) (6d)

To a solution of TFA-isoGln(\(A(N^6\text{-Bz})\))-OBzl (3.60 g, 5.12 mmol) and 9-fluorenylmethyl \(N\)-succinimidyld carbonate (Fmoc-OSu) (2.07 g, 6.14 mmol) in DMF (100 mL), diisopropylethylamine (1.32 g, 10.5 mmol) was added at 0 °C. The mixture was continuously stirred for 10 min at room temperature. The solvent was removed under a reduced pressure and methanol was added to the residue to precipitate Fmoc-isoGln(A(N^6-Bz))-OBzl as white powder. This powder was filtered and washed with methanol and dried under a reduced pressure (2.20 g, 62.3%). 10% Pd/C (10%, ca. 1 g) was added to a solution of Fmoc-isoGln(\(A(N^6\text{-Bz})\))-OBzl (3.30 g, 4.78 mmol) in methanol-DMF mixed solvent (30:70 v/v, 800 mL). After the reaction mixture was stirred for 12 h under a hydrogen atmosphere (1 atm), the catalyst was filtered and the filtrate was evaporated to concentrate and the residue was purified by column chromatography on silica gel with chloroform-methanol (1:1 v/v). The fraction (Rf = 0.3 with 1:5 chloroform-methanol) was evaporated to concentrate and the title compound was deposited as powder (1.64 g, 49%): \(\nu_{\text{max}}\) (KBr disc) cm\(^{-1}\) 3300, 3060, 1690, 1550, 1440, 1390, 1290, and 1100; \(\delta_{\text{H}}\) (270 MHz; DMSO-\(d_6\)) 1.73-2.07 (2H, m, \(\beta\)-CH\(_2\)), 2.38 (2H, \(\gamma\)-CH\(_2\)), 3.35-3.64 (2H, m, 5'-H), 3.95-4.34 (6H, m, 4'-H, fluorene-CH\(_2\)-O, 2'-H, 3'-H, methine of fluorene), 4.77 (1H, q, \(\alpha\)-CH), 5.38 (1H, d, 3'-OH), 5.62 (1H, d, 2'-OH), 6.02 (1H, d, 1'-H), 7.22-8.05 (14H, m, fluorene (8H), \(\alpha\)-NH, Ar (5H)), 8.24 (1H, t, 5'-NH), 8.72 (1H, s, 8-H), 8.80 (1H, s, 2-H), 11.25 (1H, s, 6-NH); HR-FAB MS Found m/z 722.2584 (M+1, error [pmu/mmu, +1.5 / +1.1]), Calcd 722.2574 (M+H).

\(NH_2\text{-CCU-UAC-UAU-CUC-Lys-OH (7): }\nu_{\text{max}}\) (KBr disc) cm\(^{-1}\) 3400, 1660, 1550, 1480, 1400, 1230, 1150, and 1050; \(\delta_{\text{H}}\) (270 MHz; DMSO-\(d_6\)) 1.34 (4H, m, \(\gamma\)-CH\(_2\) (Lys) and \(\delta\)-CH\(_2\) (Lys)), 1.76-1.90 (24H, m, \(\beta\)-CH\(_2\) (Glu)), 2.14 (24H, m, \(\gamma\)-CH\(_2\) (Glu)), 2.99 (2H, m, \(\varepsilon\)-CH\(_2\) (Lys)), 3.33-3.46 (24H, m, 5'-H (U, C and A)), 3.91-4.07 (12H, m, 4'-H
(U, C and A)), 4.16-4.30 (24H, m, 2'-H, 3'-H (U, C and A)), 4.53 (13H, m, α-CH (U, C, A and Lys)), 5.17 (12H, m, 3'-OH (U, C and A)), 5.40-5.52 (12H, m, 2'-OH (U, C and A)), 5.64 (5H, m, 5-H (U)), 5.72-5.83 (12H, m, 1'-H (U, C and A)), 7.23 (2H, bs, ε-NH₂ (Lys)), 7.32 (5H, m, 5-H (C)), 7.64 (5H, m, 6-H (U)), 7.92-8.13 (14H, m, α-NH and NH₃), 8.11 (2H, s, 2-H(A)), 8.19-8.22 (17H, m, 5'-NH (U, C and A) and 6-H (C)), 8.33 (2H, s, 8-H(A)), 11.33 (12H, m, 3-NH (U), 4-NH (C), 6-NH (A)); MALDI-TOF MS (α-CHCA), Found m/z 4436.51 (M), Calcd 4436.65.

\[ \text{NH₂-III-CCI-II-CCC-Lys-OH (8): } \nu_{max} \text{ (KBr disc)/cm}^{-1} \text{ } 3420, 1660, 1560, 1490, 1390, 1260, 1170, \text{ and } 1050; \delta_{H} (270 \text{ MHz; DMSO-}d_{6}) 1.34 \text{ (4H, m, } \gamma\text{-CH}_{2} \text{ (Lys) and } \delta\text{-CH}_{2} \text{ (Lys)}), 1.75-1.92 \text{ (24H, m, } \beta\text{-CH}_{2} \text{ (Glu))}, 2.11 \text{ (24H, m, } \gamma\text{-CH}_{2} \text{ (Glu))}, 2.99 \text{ (2H, m, } \varepsilon\text{-CH}_{2} \text{ (Lys))}, 3.32-3.47 \text{ (24H, m, } 5\text{-H (C and I)}), 3.91-4.07 \text{ (12H, m, } 4\text{-H (C and I))}, 4.16-4.34 \text{ (24H, m, } 2\text{-H, 3'}\text{-H (C and I))}, 4.54 \text{ (13H, m, } \alpha\text{-CH (C, I and Lys))}, 5.17 \text{ (12H, m, } 3\text{-OH (C and I))}, 5.40-5.52 \text{ (12H, m, } 2\text{-OH (C and I))}, 5.72-5.83 \text{ (12H, m, } 1\text{-H (C and I))}, 7.23 \text{ (2H, bs, } \varepsilon\text{-NH}_{2} \text{ (Lys))}, 7.32 \text{ (5H, m, } 5\text{-H (C))}, 7.92-8.13 \text{ (14H, m, } \alpha\text{-NH and NH}_{3}), 8.19 \text{ (2H, s, } 2\text{-H(I))}, 8.20-8.25 \text{ (17H, m, } 5\text{-NH (C and I) and 6-H (C))}, 8.33 \text{ (2H, s, } 8\text{-H(I))}, 11.35 \text{ (6H, m, } 4\text{-NH (C))}; \text{ MALDI-TOF MS (} \alpha\text{-CHCA), Found m/z 4535.15 (M+1), Calcd 4533.68.}

\textbf{Reference}\n


CHAPTER 2

Synthesis and Conformation Control of Peptide Ribonucleic Acid (PRNA) Containing 5'-Amino-5'-deoxyribopyrimidine and 5'-Amino-5'-deoxyribopurinenucleosides

Introduction

The antisense and antigene molecules have recently attracted the interest not only as agents of genetic therapies but also as tools of molecular biology. So many nucleic acid analogs, such as nucleotide derivatives with modified phosphodiester backbone and peptide nucleic acids (PNA) have been designed and synthesized. However, an inherent crucial drawback of these model systems is the lack of a direct means to actively control the function of these nucleic acids.

It is well-documented that the recognition and binding ability of nucleic acids and their analogues are critically affected by their conformational variations, especially through the syn-anti orientational change of nucleic acid base moiety. Certainly, the nucleobase's anti orientation is the essential for performing efficient recognition, since the syn-oriented nucleobases are obviously unfavorable for forming the intermolecular hydrogen bonds with the complementary base. This means that, if the nucleobase orientation could be switched by some additive, one can readily materialize external control of the recognition behavior. Recently, we reported that the nucleobase orientation of 5'-amino-5'-deoxypyrimidine nucleosides are switched from anti to syn simply by using borate buffer in place of conventional phosphate buffer. Thus we have designed and synthesized a new category of nucleic acid analogues, i.e. peptide ribonucleic acid (PRNA), in which the 5'-amino-5'-deoxypyrimidinenucleoside moiety was tethered to mono and oligo α- and γ-L-glutamic acid (Scheme 2-1).
It has been demonstrated that PRNAs can easily change base orientation by borate as external factor through intramolecular hydrogen bonding between the 5'-amide proton and the 2-carbonyl oxygen accompanying borate ester formation at 2',3'-cis-diol of nucleoside unit. Moreover, the on-off switching of nucleic acid recognition has been realized for the first time using a borate-induced orientational switching of γ-PRNA. However, we have been demonstrated PRNA containing only pyrimidinenucleosides.

In this paper, we would like to extend this borates induced orientational switching of PRNA containing not only pyrimidinenucleosides by also purinenucleosides and pyrimidine-purine mixed sequences (Chart 2-1).
Materials and Methods

Standard abbreviations for amino acids and protecting groups are as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature. Other abbreviations include: HOBt, 1-hydroxybenzotriazole; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; isoGln(5'U), \(N^5\)-(5'-deoxy-5'-uridy)-L-isoglutamine residue; isoGln(5'I), \(N^5\)-(5'-deoxy-5'-inosyl)-L-isoglutamine residue; ClZ, 2-chlorobenzyloxycarbonyl group. All starting materials, reagents, and solvents were commercially available and used without further purification. Uridine and inosine was purchased from Seikagaku Co. (Tokyo, Japan). Boc-L-amino acids and 1-hydroxybenzotriazole (HOBt) were purchased from Peptide Institute, Inc. (Osaka, Japan). Oligonucleotides were purchased from Takara Co. (Kyoto, Japan). Other chemicals of guaranteed grade were purchased Tokyo Kasei Kogyo Co. (Tokyo, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Column chromatography was performed over 70-230 mesh silica gel from Merck. Gel filtration was performed over Sephadex G-25 from Pharmacia. Nucleic acid model was purified on a pre-packed Hiber column RT by elution with 10% acetonitrile in water at a flow rate of 3 mL/min, using a recycling preparative HPLC instrument LC-908 from Japan Analytical Industries. IR spectra were recorded on a JASCO FT/IR-230 spectrometer. \(^1\)H NMR spectra were obtained on a JEOL GSX-270 at 270 MHz or a Varian INOVA-600 at 600 MHz. Chemical shifts are reported as \(\delta\) value in parts per million (ppm) relative to tetramethylsilane \((\delta_H = 0.0 \text{ ppm})\) as internal standard. Mass spectral measurements were performed on a JEOL AX-500 instrument by fast-atom bombardment (FAB) ionization with nitrobenzyl alcohol (NBA) as a matrix and a Voyager RP from PerSeptive Biosystems with \(\alpha\)-cyano-4-hydroxycinnamic acid (\(\alpha\)-CHCA) or picolinic acid as a matrix (MALDI-TOF). For conformation/orientation studies and nucleic acids recognition studies, following aqueous buffers were used: phosphate buffer, 1/30 M \(\text{KH}_2\text{PO}_4\)-1/30 M \(\text{Na}_2\text{HPO}_4\) (pH 7.2); borate buffer, 1/10 M \(\text{KH}_2\text{PO}_4\)-1/20 M \(\text{Na}_2\text{B}_4\text{O}_7\) (pH 7.2). CD spectra were recorded on a JASCO J-720W spectrophotometer. Thermal dissociation experiments were performed on a JASCO V-550 UV/Vis spectrophotometer equipped with a temperature controller. Absorbance data at 260 nm was collected at 10 s intervals upon heating a solution of nucleic acid model \((6 \times 10^{-5} \text{ M})\) and oligonucleotide \((6 \times 10^{-5} \text{ M})\) at a rate of 0.5 °C/min to give the melting
Equilibrium constants \( (K_y) \) were determined for the borate ester formation of \( \gamma \)-PRNAs from the quantitative analysis of the CD spectral changes induced by adding borax (3.7—84 mM) to a borate-free phosphate buffer solution of 5'-aminonucleoside or \( \gamma \)-PRNA (0.7—1 \( \times \) 10\(^{-4} \) M). Non-linear least squares analysis of the differential molar ellipticity data \( (\Delta \theta_{\text{I}_{\text{max}}}) \) led to an excellent fit to the 1:1 stoichiometric curve, affording the equilibrium constants for the esterification with boric acid or borax.

\[
N^{\alpha}-t\text{-Butoxycarbonyl}-N^5-(5'\text{-deoxy-5'\text{-uridylyl}})-L\text{-isoglutamine} \quad (\text{Boc-isoGln}(5'U)\text{-OH}) \quad (9).
\]

Palladium on activated carbon (10%; ca. 0.2 g) was added to a solution of 3a (1.80 g, 3.20 mmol) in methanol (50 mL). After 2 h of continued stirring under hydrogen atmosphere (1 atm.), the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give compound 9 as a powder (1.44 g, 95%).

\[
\begin{align*}
\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1} & \quad 3320, 1680, 1530, 1460, 1390, 1250, \text{ and } 1170; \\
\nu_{\text{NIR}} & \quad \text{CHN,NH} = 1.37 \ (9 \ H, \ s, \ t-Bu-H), 1.69-1.82 \ (2 \ H, \ m, \ \beta-\text{CH}_2), 2.19 \ (2 \ H, \ t, \ J_{\gamma,\beta} = 7.3, \ \gamma-\text{CH}_2), 3.29-3.39 \ (2 \ H, \ m, \ 5'-\text{H}), 3.83 \ (2 \ H, \ m, \ 3'-\text{H} \text{ and } 4'-\text{H}), 3.92 \ (1 \ H, \ q, \ J_{\text{CH},\text{NH}} = J_{\text{CH},\beta} = 5.9, \text{ Boc-NHCH}) , 4.02 \ (1 \ H, \ t, \ J_{2',1'} = J_{2',3'} = 4.9, \ 2'-\text{H}), 5.63 \ (1 \ H, \ d, \ J_{5,6} = 8.3, \ 5'-\text{H}), 5.73 \ (1 \ H, \ d, \ J_{1',2'} = 5.9, \ 1'-\text{H}), 6.92 \ (1 \ H, \ d, \ J_{\text{NH},\text{CH}} = 8.3, \text{ Boc-NH}), 7.65 \ (1 \ H, \ d, \ J_{6,5} = 7.8, \ 6'-\text{H}), 7.98 \ (1 \ H, \ t, \ J_{\text{NH},5'} = 5.4, \ 5'-\text{NH}), 11.35 \ (1 \ H, \ s, \ 3'-\text{NH}); \\
\text{MALDI-TOF HRMS} (\alpha\text{-CHCA}), m/z \text{ found } 495.17 \ (M + Na), \text{ calculated } 495.170.
\end{align*}
\]

\[
N^{\alpha}-t\text{-Butoxycarbonyl}-N^5-(5'\text{-deoxy-5'\text{-inosyl}})-L\text{-isoglutamine} \quad (\text{Boc-isoGln}(5'I)\text{-OH}) \quad (10).
\]

Palladium on activated carbon (10%; ca. 0.2 g) was added to a solution of 3b (1.80 g, 3.20 mmol) in methanol (50 mL). After 2 h of continued stirring under hydrogen atmosphere (1 atm.), the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give compound 10 as a powder (1.44 g, 95%).

\[
\begin{align*}
\text{Boc-isoGln}(5'U)-isoGln(5'I)-OH \quad (11)
\end{align*}
\]

To a solution of Boc-isoGln(5'U)-OH (9) (945 mg, 2.0 mmol), HOBt (270 mg, 2.0 mmol), and BOP reagent (890 mg, 2.0 mmol) in DMF (20 mL), diisopropylethylamine (0.74 ml, 4.0 mmol) was added. After 30 s, TFA-isoGln(5'I)-
OBzl (12) (1.32 g, 2.2 mmol) was added and stirring was continued for 2 h at room temperature. The solvent was removed under a reduced pressure and the residue was purified by column chromatography on silica gel with ethyl acetate-methanol (9:1 v/v) to give the title compound as white powder. The powder was filtered and washed with ether and dried under a reduced pressure to give the purified compound (1.86 g, 97.2%).

10% Pd/C (ca. 0.1 g) was added to a solution of Boc-isoGln(5’U)-isoGln(5’I)-OBzl (13) (1.80 g, 1.91 mmol) in methanol-DMF (1:1 v/v) (250 mL). After the reaction mixture was stirred for 6 h under a hydrogen atmosphere (1 atm), the catalyst was filtered and the filtrate was evaporated to concentrate and a small amount of methanol was added to the solution. Ether was further added and the precipitate formed was filtered to give 1.53 g of the title compound (11) (1.80 mmol, 94.2%): υ̃ (KBr disc)/cm⁻¹: 3420, 1660, 1560, 1490, 1390, 1250, and 1170; δH (270 MHz; DMSO-d6): 1.36 (9H, s, t-Bu-H); 2.06-2.23 (4H, m, γ-CH₃ (U and I)); 3.18-3.51 (4H, m, 5’-H (U and I)); 3.73-4.16 (6H, m, 2’-H, 3’-H, 4’-H (U and I)); 4.25 (1H, q, α-CH₂ (U)); 4.55 (1H, q, α-CH₂ (I)); 5.18 (2H, d, 3’-OH (U)); 5.30 (2H, d, 3’-OH (I)); 5.40 (2H, d, 2’-OH (U)); 5.52 (2H, d, 2’-OH (I)); 5.64 (2H, d, 5’-H (U)); 5.73 (2H, d, 1’-H (U)); 5.83 (2H, d, 1’-H (I)); 6.89 (1H, d, Boc-NH (U)); 7.65 (1H, d, NH (U)); 7.90-8.06 (2H, m, 5’-NH (U), peptide); 8.12 (1H, s, 2-H (I)); 8.22 (1H, t, 5’-NH (I)); 8.32 (1H, s, 8-H (I)); 11.3 (1H, s, 3-NH (U)). Found: C, 47.88; H, 5.65; N, 16.31. Calcd for C₃₄H₄₆N₁₀O₁₆: C, 48.00; H, 5.45; N, 16.46; FAB MS Found m/z 851 (M+1), Calcd 850.31.

Boc-isoGln(5’U)-isoGln(5’I)-isoGln(5’U)-OH (12)

To a solution of Boc-isoGln(5’U)-isoGln(5’I)-OH (11) (80 mg, 94 μmol), HOBT (12.7 mg, 94 μmol), and BOP reagent (41.6 mg, 94 μmol) in DMF (10 mL), diisopropylethylamine (30 mg, 188 μmol) was added. After 30 s, TFA-isoGln(5’U)-OBzl (7) (54.2 g, 94 μmol) was added and stirring was continued for 2 h at room temperature. The solvent was removed under a reduced pressure and 1 ml of methanol and 30 ml of ether was added to give the title compound as white powder. The powder was filtered and washed with ether and dried under a reduced pressure to give the purified compound (98 mg, 88.0%). 10% Pd/C (ca. 50 mg) was added to a solution of Boc-isoGln(5’U)-isoGln(5’I)-isoGln(5’U)-OBzl (14) (98 mg, 75.7 μmol) in methanol-DMF (1:1 v/v) (20 mL). After the reaction mixture was stirred for 24 h under a hydrogen atmosphere (1 atm), the catalyst was filtered and the filtrate was evaporated to
concentrate and a small amount of methanol was added to the solution. Ether was further added and the precipitate formed was filtered to give 42.3 mg of the title compound 12 (35.1 μmol, 46.4%): ν<sub>max</sub>(KBr disc)/cm<sup>-1</sup> 3420, 1660, 1560, 1490, 1390, 1260, 1170 and 1050; δ<sub>h</sub> (270 MHz; DMSO-d<sub>6</sub>) 1.36 (9H, s, t-Bu-H), 1.61-1.98 (6H, m, β-CH<sub>2</sub> (U and I)), 2.05-2.22 (4H, m, γ-CH<sub>2</sub> (U and I)), 2.33 (1H, t, γ-CH<sub>2</sub> (U)), 3.16-3.51 (6H, m, 5'-H (U and I)), 3.72-4.09 (9H, m, 2'-H, 3'-H, 4'-H (U and I)), 4.15-4.30 (2H, q, α-CH<sub>2</sub> (U)), 4.57 (1H, q, α-CH<sub>3</sub> (I)), 5.14-5.21 (4H, m, 3'-OH (U)), 5.29 (2H, d, 3'-OH (I)), 5.37-5.43 (4H, m, 2'-OH (U)), 5.52 (2H, d, 2'-OH (I)), 5.59-5.68 (4H, d, 5'-H (U)), 5.73 (4H, d, 1'-H (U)), 5.83 (2H, d, 1'-H (I)), 6.90 (2H, d, Boc-NH (U)), 7.64 (2H, d, 6'-H (U)), 7.90-8.04 (3H, m, 5'-NH (U), peptide), 8.08-8.25 (3H, m, 2'-H (I), 5'-NH (I), 5'-NH (U)), 8.31 (1H, s, 8-H (I)), 11.4 (2H, s, 3-NH (U)); Found: C, 47.85; H, 5.62; N, 16.11. Calcd for C<sub>45</sub>H<sub>64</sub>N<sub>14</sub>O<sub>23</sub>: C, 48.00; H, 5.45; N, 16.46; FAB MS Found m/z 1205 (M+1), Calcd 1204.43.

Results and Discussion

In the recognition process involving DNA/RNA, the base orientation of nucleoside attached to the glycosyl group plays a major role, where an anti orientation is essential for base recognition. Hence, if the syn-anti orientation of nucleoside analogs can be switched by an external factor, this will be a convenient and powerful tool for control of nucleic acid recognition.

Pyrimidine nucleosides are known to favor an anti nucleobase orientation in solution phase. One plausible strategy to induce the unfavorable syn orientation is to utilize the cooperative effects of a hydrogen bonding interaction between 2-carbonyl and 5'-hydroxyl groups and a bridging substitution of the cis-2',3'-dihol. The use of the 5'-hydroxyl proton, which is inevitably lost in conventional nucleotides and nucleic acid model compounds, is unrealistic as a candidate for the possible hydrogen bonding interaction with 2-carbonyl. We therefore substituted the 5'-hydroxyl group of pyrimidine ribonucleosides, such as uridine and cytidine, with an amino group, and elucidated the orientational change and its effect in the resultant 5'-amino-5'-deoxyuridine in the presence/absence of borate, which was added as an external switching agent. It is well known that borates forms cyclic esters with a variety of cis-1,2-diols, including sugars and ribonucleosides, and this esterification process is
reversible in aqueous solutions at moderate pH. It was demonstrated that the nucleobase orientation of 5'-amino-5'-deoxypyrimidineribonucleosides were dramatically switched from anti to syn by using borate buffer instead of conventional phosphate buffer. This efficient anti to syn orientational switching is induced by the cooperation of the 2',3'-cyclic borate esterification and the hydrogen bonding interaction between the 5'-amino and 2-carbonyl groups of pyrimidine base. In this paper, with the purpose of elucidate the effect of borates for orientation and/or conformation of 5'-amino-5'-deoxypurineribonucleosides and PRNA containing purine bases and purine - pyrimidine mixed bases were estimated by the CD spectroscopic study and NMR NOE experiments both in a phosphate buffer (borate free, pH 7.2) and borate buffer (pH 7.2).

For this purpose, we synthesized the PRNA monomer 9 and 10 by reacting 5'-amino-5'-deoxyuridine and 5'-amino-5'-deoxyinosine with N- and γ-C-protected L-glutamic acid, using benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate (BOP reagent), as shown in Scheme 2-2 and Scheme 2-3. Then, a series of oligomeric PRNAs 11 and 12, containing pyrimidine - purine mixed sequence were prepared by progressive elongation reactions composed of a repeated selective deprotection-condensation cycle, as shown in Scheme 2. In the first step, the benzyl group of the protected PRNA monomer (3a and 3b) was removed by catalytic hydrogenation to give 9 and 10 with a free carboxyl terminus, while the Boc group of 3a and 3b was removed by treatment with TFA, affording 4a and 4b with a free amino terminus. The subsequent condensation of 9 and/or 10 and 4a and/or 4b using the BOP reagent yielded the dimeric and trimeric PRNAs (13). Each coupling process proceeded in 70-98% yield. The benzyl group of 13 was subsequently removed to give the N-protected dimer and trimer (10 and 12).
Scheme 2-2. Reagents and conditions: a, Boc-Glu(OBzl), BOP reagent, HOBt, (i-Pr)2NEt, DMF, rt, 1 h; b, H2, Pd/C(10%), MeOH, rt, 2 h; c, TFA, 0°C, 30 min.

Scheme 2-3. Reagents and conditions: a, Li3, Ph3P, CBr4; b, H2, Pd/C (10%); c, Boc-Glu(BOzI), BOP reagent, HOBt, (i-Pr)2NEt, DMF, rt, 1 h; d, TFA, 0°C, 30 min; e, 1, BOP reagent, HOBt, (i-Pr)2NEt, DMF, rt, 2 h; f, H2, Pd/C (10%); g, 1, BOP reagent, HOBt, (i-Pr)2NEt, DMF, rt, 2 h; h, H2, Pd/C (10%).
From CD spectra of 5'-amino-5'-deoxyinosine (Figure 2-1a) and 5'-amino-5'-deoxyadenosine (Figure 2-1b) in borate buffer and phosphate buffer, no remarkable spectral change was observed. In these cases, although 5'-aminopurinenucleosides have 2',3'-cis-diol for borate ester formation, there is no hydrogen-accepting group for hydrogen bonding interaction between 5'-amino groups of furanose moiety. In contrast, interestingly, in cases of PRNA dimer and trimer containing pyrimidine-purine mixed sequence, dramatically CD spectral change was observed (Figure 2-2).

![CD spectra of 5'-amino-5'-deoxyinosine and 5'-amino-5'-deoxyadenosine](image)

**Figure 2-1.** The CD spectra (a) 5'-amino-5'-deoxyinosine in phosphate buffer (solid line) and in borate buffer (dashed line) and (b) 5'-amino-5'-deoxyadenosine in phosphate buffer (solid line) and in borate buffer (dashed line).

The CD spectra of γ-PRNA dimer (3) were measured in both borate and phosphate buffers (Figure 2-2a). In phosphate buffer, the [θ]_{max} value obtained (6600 deg cm² dmol⁻¹) was indicated anti orientation of nucleobase. In contrast, in borate buffer, the [θ]_{max} value of 11 was drastically reduced to 3250 deg cm² dmol⁻¹ and this [θ]_{max} value change indicated that efficient anti to syn orientational change was induced by borate. The CD spectra of γ-PRNA trimer (12), which is containing inosine residue between the two uridine units, were also measured in both borate and phosphate buffers.
(Figure 2-2b). In phosphate buffer, the \([\theta]_{\text{max}}\) value obtained (15600 deg cm\(^2\) dmol\(^{-1}\)) was indicated \textit{anti} orientation of nucleobase. In contrast, the \([\theta]_{\text{max}}\) value of 12 was drastically reduced to 3140 deg cm\(^2\) dmol\(^{-1}\) in borate buffer. Furthermore, this unique borate-driven conformational switching from \textit{anti} to \textit{syn} of the base orientation was confirmed by the NMR NOE measurements of 11 in both buffer solution. In the NOE spectra in phosphate buffer, where uracil H6 is presaturated, uracil H5 and/or furanose H1', H2', H3', and H5' gave notable NOE peaks. This NOE profile is entirely consistent with the \textit{anti}-orientation of 11. In borate buffer, 11 afforded a completely different NOE spectrum. The presaturation of uracil H6 gave NOE signals only with uracil H5 and furanose H1'. This unique NOE profile, in addition to the decreased CD intensity provides evidence in support of the predominant contribution of the \textit{syn} orientation for 11 in borate buffer, which is driven by the borate ester formation.

These results showed the \(\gamma\)-PRNA containing not only 5'-amino pyrimidinenucleoside but also 5'-aminopyrimidine - purinenucleosides could switch \textit{anti}-to-\textit{syn} base orientation of nucleosides induced by borates.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig22.png}
\caption{The CD spectra (a) 3 in phosphate buffer (solid line) and in borate buffer (dashed line) and (b) 4 in phosphate buffer (solid line) and in borate buffer (dashed line).}
\end{figure}
Conclusion

Current studies on antisense molecules are directed mostly toward the simple inhibition of genetic information transfer. Little effort has been devoted to the active control of the DNA/RNA recognition processes, possibly as a result of a lack of suitable fundamental strategy and the necessary practical tools. In this and preceding studies, we have proposed a new methodology and effective tools for controlling DNA/RNA recognition through the use of an external agent. This strategy employs novel nucleic acid analogues, i.e. γ-peptide ribonucleic acids (γ-PRNAs), as the recognition moiety which has a built-in switch triggered by an external factor. In the cases of not only homo-pyrimidine PRNAs but also pyrimidine - purine mixed sequenced PRNAs, the formation of a borate ester of the ribose’s cis-2’,3’-diol and the simultaneous hydrogen-bonding interaction between the 5’-amide proton and the 2-carbonyl oxygen act as the external and internal switching devices. The results obtained in these studies are encouraging, demonstrating that γ-PRNA form stable hybrid complexes with the complementary oligonucleotides, which are readily dissociated upon addition of borax or boric acid. Certainly, a definition of the scope and limitations of this strategy remain to be addressed, including the study of sequence selectivity and the search for other switching devices/agents. Moreover, the present concept of the external control of DNA/RNA recognition has the potential to be widely employed in the next generation of antisense molecules.

References

Res. Supple., 2: 159.


CHAPTER 3

The Effects of Chirality of γ-Glutamic Acid Backbone upon Structure and Recognition of Peptide Ribonucleic Acids (PRNA)

Introduction

Oligonucleotides that specifically recognize and bind to messenger RNA or double stranded genomic DNA offer unique properties for inhibiting protein syntheses system i.e. the antisense approach or for modulating gene expression via triple helix formation. For that purpose, a wide variety of nucleic acid analogues, which show sequence specific recognition/binding to mRNA and/or dsDNA, have been proposed, designed, and synthesized.1 The deoxyribose/ribose phosphate backbones of DNA/RNA have been modified in order to enhance the stabilities of not only antisense molecules but also antisense – mRNA hybrids in the presence of nucleases, proteases, and so on, including phosphodiester,2 mono,3 or dithiophosphate,4 methyl phosphonates,5 borano phosphates,6 phosphoramidate,7 as well as formacetal,8 carbamate,9 and siloxane.10 Even though it is difficult in general to control the stereogenic center of phosphorous in the preparation of these model compounds, its stereochemistry is known to significantly influence the hybridization efficiency.11 Nucleic acid analogues not to contain nonphosphodiester backbone but peptide backbone were strenuously investigated as resistance to enzyme such as nuclease. Peptide nucleic acid (PNA) was most successful nucleic acid analogue that consisted of 2-aminoethylglycine as a backbone and nucleobase as a recognition site.12 However, most of nucleic acid analogues, which have been reported, were primary-intended to inhibition of genetic information through the complexation with target RNA. We have been proposed the next generation antisense molecule, which named peptide ribonucleic acid (PRNA).13 Not only inhibition but also positive control of expression/inhibition will be required in novel nucleic acid analogues, thus the property of PRNA, which reversible nucleic acid recognition control thorough syn-anti orientational switching by ribose's hydroxyl group borate formation and changing pH system within physical condition was achieved, fitted the purpose i.e. active control of complexation with target gene.14
As it is expected that mRNAs form several higher-order structure, the PRNA complexation with RNA will eminently depend on higher-order structure of PRNA oligomer, which attributed to isoglutamic acid backbone and nucleoside side chain. Heretofore L-γ-glutamic acid was tethered to PRNA, thus studies on chirality of peptide backbone to impact on conformational variety of PRNA–oligonucleotide complex. On the other hand, for example, it has been reported that PNA, which linked achiral 2-aminoethylglycine backbone, were dominantly induced right-handed and/or left-handed coiled structure when D- and L-lysine were partially introduced in terminal end respectively, thus D-amino acid connected PNA, which induced right-handed coil as well as DNA duplex, formed more stable complex than opposite one. However, little differences were observed in melting temperature and selective direction of complex, which was inherent drawback to apply for antisense molecule in spite of forming higher-order structure.

In this chapter, PRNA containing D-isoglutamic acid as a backbone was prepared to investigate the conformational and DNA recognition differences between D- and L-PRNA. Considering the difference will directly lead us to an objective guide of modeling the antisense molecules and nucleic acid analogues.

Results and discussion

PRNA monomer tethered by D- and L-γ-glutamic acid

Nucleobase orientation of PRNA monomer tethered by L-γ-glutamic acid (L-PRNA monomer) was switched from anti to syn induced by borax. However, diastereomeric D-PRNA, which might have different conformation and affinity to right-handed coiled DNA, was not discussed. Thus D- and L-PRNA monomer (15 and 9 respectively) containing uracil base were prepared to investigate the conformation and base orientation switching ability. CD spectroscopy has been used to evaluate the syn-anti orientation of nucleobase.

Hence, the CD spectra of PRNA monomers 15 and 9 containing uridine unit were measured in phosphate and added borax buffer solutions at pH 7.2 in order to examine the effects of borax and backbone chirality. As can be seen from Figure 3-1, the [θ] value at 270 nm of 15 and 9 decreased similarly to indicate that the base orientation in monomer unit were efficiently switched from anti to syn in spite of backbone
chirality. From these results, the base orientations of ambidextrous D- and L-PRNA changed by adding small amount of borax indicating backbone chirality was not effected to controlling nucleobase orientation.

![Figure 3-1. CD spectral changes of D-PRNA monomer 15 (left) and L-PRNA monomer 9 (right) with increasing concentration of borax in phosphate buffer at pH 7.2.](image)

**Conformational comparison between D- and L-PRNA oligomer**

It is known that oligopeptide consisted of L-α-amino acid was right-handed coiled structure in contrast D-α-amino acid was left-handed, whereas γ-glutamic acid does not have determined structure. However in the case of γ-PRNA, two chiral unit existed both in the backbone glutamic acid and sugar moiety of nucleoside, thus D-PRNA and L-PRNA dodecamer (16 and 17 respectively) containing uracil base were diastereomeric relationship to result in quite different higher-order structure in oligomerization. The CD spectrum of an α-helical L-peptide has been well characterized and the right-handed spiral chirality of peptide reflected a negative CD peak around 208–222 nm. The CD spectra of 16 and 17, which contained homo-uridine sequence as DNA recognition site, were measured in both phosphate and borax added buffer solutions (Figure 3-2). Commonly, α-oligopeptide consisted by all D-amino acids gave positive CD value at 220 nm, in contrast to negative CD value for L-amino acids.
However, in the case of PRNA overlapped nucleoside absorption was observed around 220 nm,\textsuperscript{20} where the backbone was γ-glutamic acid not to determine coiled structure by CD shape. However, can be seen from Figure 3-2, CD shapes of 3 and 4, which were quite different from those of 1 and 2, indicated that PRNA oligomers were induced quite different structure each other. Compared D with L configuration especially around 220 nm in phosphate buffer, CD peak of 3 were negative to indicate that peptide bonding were right-handed coiled, in contrast that of 4 were positive. On the other hand, in phosphate buffer added borax, the $[\theta]_{\text{max}}$ value of these oligomers around 270 nm were both decreased to indicate that the orientation was changed from anti to syn.\textsuperscript{13} The orientation control by borax was efficiently achieved in spite of backbone chirality.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-2.png}
\caption{CD spectral changes of D-PRNA monomer 16 (left) and L-PRNA monomer 17 (right) with increasing concentration of borax in phosphate buffer at pH 7.2.}
\end{figure}

\section*{Nucleic Acid Recognition}
It was indicated that D-PRNA was induced right-handed coiled structure, which could form stable complex with right-handed DNA. It will be expected that PRNA, which had D-isoglutamic acid backbone, was predominant nucleic acid model to form stable complex with natural nucleic acid such as discussed in PNA.\textsuperscript{16} The hybridization ability of PRNA was evaluated from the melting temperature, $T_m$, which was determined from hypochromic changes observed in the UV spectrum upon mixing PRNA with the target oligonucleotide dodecamer (Table 3-1).\textsuperscript{21}
Compared PRNA with DNA, L-PRNA\textsuperscript{17} was formed 4 degrees more stable complex (Tm = 27.2 °C) with complementary DNA\textsuperscript{19} than DNA\textsuperscript{18}–DNA\textsuperscript{19} complex (Tm = 23.1 °C) in phosphate buffer. Interestingly, the d-PRNA\textsuperscript{16}–\textsuperscript{19} complex was more stable than L-PRNA\textsuperscript{17}–\textsuperscript{19} complex indicating that backbone chirality inducing right-handed coil was very important for forming stable complex. Moreover added borax to these systems, DNA-DNA complex enhanced the stability by salt effect, however, both PRNA-DNA complex dissociated by borate induced base orientation change from \textit{anti} to \textit{syn}. From these results, it was very important for the absolute configuration of backbone chirality to form stable complexes between nucleotide analogues consisted of peptide main chain and oligonucleotide.

**Table 3-1. Melting temperature of γ-PRNA and oligonucleotide complexes**

<table>
<thead>
<tr>
<th>PRNA or oligonucleotide complement</th>
<th>Tm°C</th>
<th>Borax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>20 mM</td>
</tr>
<tr>
<td>DNA \textsuperscript{19}</td>
<td>23.2</td>
<td>29.2</td>
</tr>
<tr>
<td>PRNA \textsuperscript{16} DNA \textsuperscript{18}</td>
<td>33.1</td>
<td>&lt; 3.5</td>
</tr>
<tr>
<td>PRNA \textsuperscript{17}</td>
<td>27.2</td>
<td>&lt; 3.5</td>
</tr>
</tbody>
</table>

[PRNA] = [DNA] = 5.0 x 10\textsuperscript{3} M in 1/30 M phosphate buffer at pH 7.2.

**Conclusion**

Present studies on antisense methodologies are directed mostly toward the
simple inhibition of genetic information transfer. PRNA were developing as the next generation antisense molecule that could control the genetic information transfer through switching complexation/decomplexation by external factors. Moreover two chiral moieties of peptide backbone and ribose in PRNA could induce different diastereomeric single strand, which d-PRNA is right-handed and l-PRNA is left-handed. The results obtained in chirality studies are encouraged, demonstrating that d-PRNA form the more stable complex with DNA. It was very important for modeling peptide derived nucleic acid model to select the chirality of backbone.

Experimental Section

D-, and l-PRNA monomers were prepared according to method reported previously. The PRNA 3 and 4 were prepared by solid-phase synthesis using N-terminus Fmoc protected PRNA monomers. PRNA oligomer was purified on a preparative ODS column (acetonitrile/H₂O = 9/91). For both conformation/orientation studies and nucleic acid recognition studies, following aqueous buffers were used: phosphate buffer, 1/30 M KH₂PO₄-1/30 M Na₂HPO₄ (pH 7.2). 1H NMR spectra were obtained on a JEOL GSX-270 at 270 MHz or a Varian INOVA-600 at 600 MHz. Mass spectral measurements were performed on a Voyager RP from PerSeptive Biosystems with α-CHCA or picolinic acid as a matrix (MALDI-TOF). CD spectra were recorded on a JASCO J-720W spectrophotometer equipped with temperature controller. Thermal dissociation experiments were performed on a JASCO V-560 UV/Vis spectrophotometer equipped with a temperature controller. Absorbance data at 260 nm was collected at 10 s intervals upon heating a solution of nucleic acid model (5.0 x 10⁻⁶ M) and DNA (5.0 x 10⁻⁶ M) at a rate of 0.5 °C/min to give the melting curve.

References

(5) (a) Miller, P. S.; Yano, J.; Yano, E.; Carroll, C.; Jayaraman, K.; Ts'o, P. O. P.


(14) Chapter 5 in this thesis


CHAPTER 4

DNA Recognition Control of γ-PRNA and Mismatched Base Effects upon Complex Stability

Introduction

Modified nucleotides and nucleic acid models that bind to single stranded RNA and/or double stranded DNA have received much attention in molecular biology and medical chemistry as they may be developed into antisense RNA and antigen agents for diagnostic and therapeutic purposes, and powerful tools for sequence specific modification and cleavage of DNA. Several models have been proposed, not only to improve the stability of the oligomers and/or hybrids in the presence of nuclease, but also to enhance the hybridization affinity. An inherent, crucial drawback of these model systems is the lack of direct means to actively control the function of these nucleic acids. We have been recently proposed that nucleic acid models named peptide ribonucleic acids (PRNAs), in which the entire phosphate sugar backbone has been replaced with a backbone consisting of iso-glutamic acid units to which 5’-amino-5’-deoxyribonucleosides are attached through α-amide or γ-amide bonding.2,3

The author here report conformation/orientation and recognition control of PRNA with DNA. Furthermore, the binding properties of the PRNA with mismatched DNA were discussed.

Experimental

PRNA monomers and oligomers were prepared according to method reported chapter 1. For both conformation/orientation studies and nucleic acids recognition studies, following aqueous buffers were used phosphate buffer, 0.033 M KH2PO4-0.033 M Na2HPO4; borate buffer, 0.1 M KH2PO4-0.05 M Na2B4O7. 1H NMR spectra were obtained on a JEOL GSX-270 at 270 MHz or a Varian INOVA-600 at 600 MHz. Mass spectral measurements were performed on a Voyager RP from PerSeptive Biosystems with α-CHCA as a matrix (MALDI-TOF). CD spectra were recorded on a JASCO J-720W spectrophotometer equipped with temperature controller. Thermal dissociation experiments were performed on a JASCO V-560 UV/Vis spectrophotometer equipped
with a temperature controller. Absorbance data at 260 nm was collected at 10 s intervals upon heating a solution of nucleic acid model (7 x 10^-6) and DNA (7 x 10^-6) at a rate of 1 °C/min to five the melting curve.

Results and discussion

CD spectral study of PRNA and DNA

CD spectroscopy has been used to evaluate the syn-anti orientation of nucleobases in DNA and nucleic acid models as discussed in the former chapters in this thesis. The UV and CD spectra of PRNA 12-mer (NH,-CCU-UAC-UAU-CUC-Lys-0H, 7), which synthesized in chapter 1, were measured in phosphate buffer in the absence and presence of 20 mM of borax (Figure 4-1, Figure 4-2). Little UV spectral change was observed between both conditions, however, CD spectra changed drastically. In the absence of borax in phosphate buffer, the $[\theta]_{max}$ value of PRNA7 obtained was nearly the same as the averaged value observed for 5'-amino-5'-deoxynucleosides, and this is compatible with the preferred anti orientation in the absence of borax. On the other hand, as can be seen from the CD spectra shown in figure 1, the $[\theta]_{max}$ value of PRNA7 was greatly reduced to 23000 deg cm$^2$ dmol$^{-1}$ in the presence of 20 mM of borax.

**Figure 4-1.** UV spectra of purine-pyrimidine mixed-sequence PRNA 7 in phosphate in the presence (dashed) and absence (solid) of 20 mM borax buffer at pH 7.2.

**Figure 4-2.** CD spectra of purine-pyrimidine mixed-sequence PRNA 7 in phosphate in the presence (dashed) and absence (solid) of 20 mM borax buffer at pH 7.2.
suggests that the borate added to the solution appreciably induces the syn orientation through cooperative effects of borate ester formation and an intramolecular hydrogen bonding interaction. The higher binding constant and much reduced CD intensity in the presence of borax observed for PRNA7 indicate that the borate formation and the hydrogen-bonding interaction synergistically enhance the anti-to-syn orientation switching of the nucleobase moiety in oligomeric PRNA.

**Stoichiometry and Structure of PRNA-DNA complex**

Generally, the decreased absorbance in UV spectrum around 260 nm, thus hypochromicity, is observed when nucleic acid forms complex with complementary strand. Furthermore, Job plot of the hypochromicity shows Stoichiometry of the bases in complex.

Mixing PRNA7 possessing the pyrimidine-purine mixed sequence and DNA20 (d(GAG-ATA-GTA-AGG)), which was complementary sequence to PRNA7, gave hypochromicity at 5 °C to indicate that PRNA7 could form a complex with complementary DNA20. Job plot was carried out to determine the complex stoichiometry was 1:1 complex with 26% reduction of the absorbance at 260 nm of UV spectra (Figure 4-3).

![Figure 4-3. Job plot of hypochromicity for PRNA1-DNA2 system.](image-url)
Complementary poly- and/or oligonucleotides can form double-stranded helical structures adenine hydrogen bonded to uracil or thymine, and guanine hydrogen-bonded to cytosine. Of course, these super asymmetric structures also have the base-base interactions that yield intense CD spectra. A-form like CD spectra of PRNA-DNA complex, where $\frac{\theta_{\text{max}}}{\theta_{\text{min}}}$ was almost 2/1,$^5$ was measured in phosphate buffer despite DNA-DNA complexes show typical B-form CD shapes, where $\frac{\theta_{\text{max}}}{\theta_{\text{min}}}$ was 1/1,$^5, 6$ to suggest that PRNA7 has RNA type 2'-exo-3'-endo ribose puckering conformation (Figure 4-4, Table 4-1).

![Figure 4-4. CD spectra of PRNA7-DNA20 (solid line) and DNA21-DNA20 (dotted line)](image)

**Table 4-1.** The $\frac{\theta_{\text{max}}}{\theta_{\text{min}}}$ ratio of DNA-DNA, RNA-DNA, and PRNA-DNA complexes

<table>
<thead>
<tr>
<th>PRNA or oligonucleotide</th>
<th>$\Delta[\theta]<em>{\text{max}}/\Delta[\theta]</em>{\text{min}}$</th>
<th>double stranded complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRNA7-DNA20</td>
<td>2.00</td>
<td>A type</td>
</tr>
<tr>
<td>DNA21-DNA20</td>
<td>0.92</td>
<td>B type</td>
</tr>
<tr>
<td>r(AC)$<em>{12}$-d(GT)$</em>{12}$</td>
<td>2.60$^5$</td>
<td>A type</td>
</tr>
<tr>
<td>d(AC)$<em>{12}$-r(GT)$</em>{12}$</td>
<td>2.30$^5$</td>
<td>A type</td>
</tr>
<tr>
<td>poly r(GC)-poly r(GC)</td>
<td>2.00$^6$</td>
<td>A type</td>
</tr>
<tr>
<td>d(AC)$<em>{12}$-d(GT)$</em>{12}$</td>
<td>0.91$^5$</td>
<td>B type</td>
</tr>
</tbody>
</table>

[PRNA] = [DNA] = 4.0 x 10$^{-6}$ in 1/30 phosphate buffer.
Control of Hybridization of γ-PRNA with Complementary Oligonucleotide

The hybridization ability of γ-PRNA7 with complementary DNA20 was evaluated from the melting temperature $T_m$, which is determined from the hypochromic changes in the UV spectrum upon mixing PRNA7 with the DNA20 of target sequence. The $T_m$ value, taken as a quantitative measure of the binding affinity, is known to be affected by the conformation of nucleic acid or its analogue as well as the ionic strength of the aqueous solution used.

It was noted that in the borax-free buffer the hybrid complex between antiparallel PRNA7-DNA20, which the direction between N-terminus of PRNA and 3'-terminus of DNA was complementary, gave a considerably higher $T_m$ of 39.2 °C than the antiparallel complementary double-stranded DNA complex (DNA21-DNA20) ($T_m = 33.9$ °C), indicating stronger interaction in the hybrid rather than the natural DNA pair. The higher stability of PRNA was caused by the PRNA’s structural property that no anionic moiety existed in peptide backbone to repel the anionic phosphodiester backbone of DNA. On the other hand, the hybrid complex between parallel directed PRNA7-DNA22, which N-terminus of PRNA7 complemented to 5'-terminus of DNA22, gave significantly 15 °C lower $T_m$ than that of antiparallel PRNA7-DNA20 complex. These results clearly indicated PRNA formed more stable complex with complementary DNA and PRNA-DNA complex preferred antiparallel direction.

In contrast, in the 20 mM borax-added buffer, the hybrid complex did not exhibit any melting behavior above 5 °C or hypochromic changes, while the complementary DNA21-DNA20 duplex gave even higher $T_m$ of 40.3 °C, probably due to the slight higher ionic strength. This contrasting behavior of the natural DNA-DNA and PRNA-DNA hybrid pairs in the presence and absence of borax is most likely
attributed synergetic anti-to-syn orientational switching of the pyrimidine and purine base in PRNA7, due to the cooperative borate ester formation of the cis-2',3'-diols and hydrogen bonding interaction between the 5'-amide proton and 2-carbonyl oxygen and also partly due to the electrostatic repulsion between the anion formation of borate formed at pH 7.2 as illustrated in Scheme 4-1.

Scheme 4-1. Complexation and decomplexation of PRNA by switching nucleobase orientation.

Sequence Selectivity of PRNA

DNA-DNA complex with mismatched base pair decreased the complex stability compared with full-matched complex. Especially, internal mismatch, which existed middle of sequence, drastically destabilized the complex. The mismatch base effect upon PRNA-DNA complex was also studied in phosphate buffer. In the case of natural DNA-DNA complex, $T_m$ of C-T mismatch near by 5'-terminus (d(GAT-ATA-GTA-AGG), DNA23) and the middle of sequence (d(GAG-ATA-TA-AGG-3', DNA24) were 11.5 °C and 14.5 °C lower than that of full-matched complex respectively. In contrast, in the case of mismatched PRNA-DNA complex, efficient decreased $T_m$s of PRNA7-DNA23 and PRNA7-DNA24 (17.3 °C and 23.8 °C respectively) were observed, especially for T-C mismatched base on the middle of the sequence. These results suggested that one mismatched especially the middle of sequence leaded no formation of nearer base-pairs because of rigidity derived from backbone amide bonding of PRNA.
From these results, PRNA showed the higher sensitivity for mismatched base and sequence selectivity, which were the essential properties of antisense molecule.

Conclusion
Genetic information of DNA was coded by a series of four bases, thus nucleic acid recognition by nucleic acid models containing purine-pyrimidine mixed sequence were very important. The recognition ability of purine-pyrimidine mixed sequence PRNA and its control by an external factor were investigated. Adding borax to the solution of PRNA-DNA complex as an external factor controlled the recognition of the purine-pyrimidine mixed PRNA in the first time, although homo-pyrimidine PRNA could control complexation/decomplexation behavior by orientation switching of pyrimidine base. Moreover nucleic acid models were improved the recognition selectivity as well as its recognition ability. PRNA formed a more stable complex with complementary DNA and binding of PRNA with complementary DNA was sequence specific.

References
CHAPTER 5

Reversible Recognition Control of Peptide Ribonucleic Acid (PRNA) by Change of pH

Introduction

Modified nucleotides and nucleic acid models that bind to mRNA and/or double stranded DNA have been received much attention in molecular biology and medical chemistry and many nucleic acid models have been reported not only to improve the stability of the oligomers and hybrids in the presence of nuclease but also to enhance the hybridization affinity. Antisense molecules bind to target in intracellular environment where is distinct from that of extracellular in terms of pH. Generally the inside of a neoplasm and cancer has low pH compared with a normal organization, and the cell under low pH accepts and has high warm temperature susceptibility. While many investigations on antisense and antigen and pH sensitive liposomes that delivered antisense oligonucleotides have so far been carried out, there were no antisense molecules that focused on pH susceptible nucleotide recognition control.

Peptide ribonucleic acid (PRNA) which can control a complex formation and decomplexation with DNA by borax addition as an external factor is a RNA mimic in which the phosphate backbone of RNA has been replaced by a γ-glutamic acid backbone. Homo-pyrimidine PRNA oligomers that synthesized by liquid elongation have been found to form stable hybrids with DNA and dissociate from DNA in the presence of borax. The driving forces of dissociation were the cooperating effects on borate ester formation of 2‘, 3‘ hydroxyl group of PRNA with undergoing furanose’s puckering changing, hydrogen bonding formation between 5‘-amide proton and 2-carbonyl oxygen located in nucleobase, and negative charge generated on boron. On the other hand, it is general that borate ester formation with 1, 2-cis-diol like saccharide was very stable at basic condition, whereas dissociate at acidic condition because of its lability. Thus combination between borate ester formation and pH system can completely and reversibly control the complex hybridization. In addition, while homo-pyrimidine PRNA oligomers had been found to switch the recognition to the complementary homo-purine DNA from on to off, for wider application complementary
nucleic acid recognition of purine-pyrimidine mixed sequence should be needed. We here show that, indeed, PRNA—DNA and PRNA—PRNA duplex is formed in purine-pyrimidine mixed sequence and self-complementary PRNA respectively. Furthermore, the self-complementary PRNA NH$_2$-III-CCI-II-CCC-Lys-OH (8) was controlled its recognition behavior using by borax and pH changing as external factors.

Results and Discussions

Reversible nucleobase orientation switching by pH

It is well-known that borate ester formation with 1, 2- cis-diol like saccharide was very stable at basic condition, whereas dissociate at acidic condition because of its lability. The molar ellipticity of the major CD band around 270 nm ($[	heta]_{	ext{ext}}$) has been used to assess the glycosyl orientation as well as the puckering of the furanose ring. $[	heta]_{	ext{ext}}$ was efficiently decreased indicating that The Nucleobase orientation of Boc-isooGln(5'U)-OH (9) changed from anti to syn by adding a drop of borax at pH 8.8(Figure 5-1). In contrast, No $[	heta]_{	ext{ext}}$ change was observed at the acidic condition, pH 6.0, to indicate nucleobase anti orientation was kept by continuous borate formation of 2',3'-diol (Figure 5-1). Controlling borate formation by pH system, the reversible

![Figure 5-1. CD spectral change of PRNA monomer 9 by adding borax to phosphate buffer at pH 6.0 (left) and pH 8.8 (right)](image-url)
nucleobase orientation of pyrimidine nucleosides by pH under borax was investigated. The $[\theta]_{\text{ext}}$ value was decreased from 6400 to 3100 indicating that the nucleobase orientation changed to syn orientation by adding 5.5 mM borax in phosphate buffer, where nucleobase orientation was anti in borax free solution (pH 7.2). Adding HCl to pH 6.0 into borax system, $[\theta]_{\text{ext}}$ was quite recovered to 6200 to reveal that nucleobase orientation was reversibly changed from syn to anti by pH changing under borax existence. Furthermore, the reversible nucleobase orientation switching was observed in several times pH preparation in the range from pH 6.0 to pH 8.0 (Figure 5-2). This phenomena were clearly indicated that nucleobase orientation of PRNA was reversibly controlled from anti to syn completely to control complexation/decomplexation cooperatively by pH system and borax addition.

![Figure 5-2. pH dependence of $[\theta]_{\text{ext}}$ of 9 in the presence of borax.](image)
Recognition switching of self-complementary PRNA

The complexation behavior of γ-PRNA NH₂-III-CCI-II-CCC-Lys-OH (8), which possesses self-complementary sequence with cytidine and inosine unit, was studied in phosphate buffer, 20 mM borax into phosphate buffer and pH 6.0 prepared with 20 mM borax system. (Table 5-1) It has been reported γ-PRNA bound to antiparallel target sequence rather than parallel one.5, 6 Thus, Self-complementary sequence was expected to form a complex by itself. In the case of 8, 40 % of hypochromic effect was observed in phosphate buffer at 5°C. The hybridization ability of 8 was evaluated from the melting temperature, Tₘ, which was determined from hypochromic changes observed in the UV spectrum. In borax-free buffer, the hybrid complex of self-complementary PRNA 8 gave a Tₘ of 35°C indicating forming stable complex. Interestingly, adding 20 mM borax to this system resulted in a complete loss of Tₘ, indicating the dissociation of the complex through both ant-to-syn orientational switching of base and repulsion of anions by generated borate ester in single strands. Furthermore, preparing this system to pH 6.0 by use of aqueous hydrochloric acid, the hypochromicity was clearly and completely recovered to result in regenerating the stable complex. It was clearly indicating that switching behavior between complexation and decomplexation of hybrid formation was completely controlled cooperatively by both adding borax and the difference among only changing pH of one by external factors, which range was change in intracellular environment of neoplasm and cancer.3

Table 5-1. Melting temperature of self-complementary γ-PRNA complex.

<table>
<thead>
<tr>
<th>PRNA</th>
<th>Additive</th>
<th>Tₘ/°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRNA30</td>
<td>None (pH 7.2)</td>
<td>35.0</td>
</tr>
<tr>
<td></td>
<td>20 mM Borax</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>HCl (pH 6.0)</td>
<td>34.0</td>
</tr>
</tbody>
</table>

[PRNA30] = 1.0 x 10⁻⁵ M in phosphate buffer at pH 7.2

Circular dichroism (CD) spectroscopy has been used to evaluate the syn-anti orientation of nucleobase and hybrid formation of both natural and modified nucleotides.10 Hence the CD spectra of 8 was also measured in phosphate buffer and
adding 20 mM borax to examine the effects of borate ester formation to switch the base orientation and hybrid formation in the presence and absence of borax. As can be seen the CD spectra shown in Figure 5-2, the extreme maximum of molar ellipticity ([θ]_{ext}) around 270 nm was changed from 100000 at 60°C where it exist in single strand to 150000 deg cm² dmol⁻¹ at 5°C in phosphate buffer where it formed complex with 40% UV hypochromicity. The CD spectrum of PRNA—PRNA complex exhibited maximum θ value at 270 nm and minimum at 225 nm similar to RNA—RNA complex to indicate that PRNA—PRNA complex formation where the ribose’s puckering was 2’-exo-3’-endo might be similar to RNA—RNA complex, that is A-type double strand. In contrast, adding borax to the complex forming system resulted in significant decreasing of [θ]_{ext} indicating that nucleobase was changed from normal anti to syn orientation which is unsuit for complexation as reported in another sequence previously. Preparing pH down to 6.0 by use of hydrochloric acid, [θ]_{ext} was completely recovered to indicate that the base orientation was returned from syn to anti. At 25°C, [θ]_{ext} value in the presense of borax pH 6.0 was slightly lower than that at 60°C in phosphate buffer to indicate that PRNA was exist as single strand. Cooling down the system result in the complete

![Figure 5-2](image)

**Figure 5-2.** CD spectra of self-complementary PRNA (4) in phosphate buffer (pH 7.2) at 5°C (solid line), 60°C (dashed line), added 20 mM borax (dotted line), added 20 mM borax (pH 6.0) at 25°C (chain line) and added 20 mM borax (pH 6.0) at 5°C (broken line).
recovery of the [θ]_\text{eq} value to coincide with it of double strand. From these results, PRNA 8 was controlled recognition switching behavior with base orientation turning which was very sensitive to pH.

**Conclusion**

The author demonstrated that the reversible control between the complexation and decomplexation of purine and pyrimidine mixed sequence PRNA-DNA and PRNA—PRNA duplex with UV melting behavior and CD spectroscopy. Moreover, the recognition control was achieved in the range of physiological condition that is different one of pH around pH 7.2. Together with latter, PRNA can control DNA/RNA recognition for the purpose of active control of genetic recognition processes. The present concept of the external control of DNA/RNA recognition has the potential to be widely employed in the next generation of antisense molecules.

**Experimental section**

PRNA monomers and oligomer were prepared according to method reported previously. PRNA oligomer was prepared by solid-phase synthesis using N-terminus Fmoc protected PRNA monomers. For both conformation/orientation studies and nucleic acids recognition studies, following aqueous buffers were used. CD spectra were recorded on a JASCO J-720W spectrophotometer equipped with temperature controller. Thermal dissociation experiments were performed on a JASCO V-560 UV/Vis spectrophotometer equipped with a temperature controller. Absorbance data at 260 nm was collected at 10 s intervals upon heating a solution of nucleic acid model (7.51 \times 10^{-5} M) at a rate of 0.5 °C/min to give the melting curve.

**References**


Conclusion

The completion of the human genome-sequencing project has driven our research interest and effort to the creation of new methodology of artificial regulation and/or control of genetic information, such as gene therapeutic drugs using the antisense and antigene strategies. Nevertheless, the hitherto reported antisense and antigene molecules are directed mostly toward the simple inhibition of genetic information transfer. Little effort has been devoted to the active control of the DNA/RNA recognition processes, possibly as a result of a lack of suitable fundamental strategy and the necessary practical tools. Thus, an inherent, crucial drawback of these antisense/antigene systems is the lack of a direct means to actively control the function by external factors and/or external stimuli. If one can find a strategy for controlling the recognition ability of nucleic acid model, the dynamic and reversible regulation of genetic information could be materialized and then, the nucleic acid model will achieve much wider application through the active control of the function.

However, no such nucleic acid models have hitherto been proposed for the recognition control of nucleic acids. The subject of this work was dynamic and reversible control of nucleic acid recognition by an external factor, which cannot be realized by the conventional antisense molecule based on a structure of nucleic acids. In this work, focusing on the importance of the base orientation of nucleic acids in the nucleic acid recognition process and the stability of cyclic borate ester with the ribose’s cis-2′,3′-diol of PRNA upon pH in the reversible recognition process, the author designed novel peptide nucleic acids (PRNAs), which possess the reversible recognition control ability through the switching of the base orientation by an external factor.

In chapter 1, the author established the synthetic routes to a series of PRNA monomers, carrying adenine, cytosine, hypoxanthine, and uracil nucleobases as recognition moiety. This enabled us to fully expand the range of available PRNA monomers. The author further demonstrated that the newly synthesized Fmoc-protected PRNA monomers are compatible with the standard solid-phase peptide synthesis protocol, and oligomeric PRNAs with variety of purine-pyrimidine mixed sequences can be prepared. Indeed, representative PRNA 12-mers of mixed sequences were synthesized in high yields by the Fmoc solid-phase peptide synthesis, and were characterized by HPLC and MALDI-TOF mass spectrometric analyses. It is noted that
the solid-phase synthesis is much more convenient, efficient, and reliable than the conventional fragment condensation method in solution phase previously reported. The present synthetic strategy should lead us to a wide variety of PRNA oligomers with desired sequences, which function as reversibly controllable antisense molecules upon complexation with the complementary DNA/RNA.

In chapter 2, it was demonstrated that the *syn-anti* orientation of pyrimidine-purine mixed sequence PRNAs can readily be switched by adding borate as an external controlling factor. In the cases of not only homo-pyrimidine PRNAs but also PRNAs with pyrimidine-purine mixed sequence, the formation of a borate ester of the ribose’s cis-2’,3’-diol and the simultaneous hydrogen-bonding formation between the 5’-amide proton and the 2-carbonyl oxygen of pyrimidine bases act as the external and internal switching devices. Thus, the cooperative effects of the borate ester formation and the intramolecular hydrogen bonding formation effectively induce the *syn* orientation in PRNA oligomers not only homo-pyrimidine sequences but also pyrimidine—purine mixed sequences. It was shown that this rather simple, but effective, strategy using borate as an external factor to control base orientation could be applicable to the recognition control of DNA/RNA by nucleic acid model compounds.

In chapter 3, it was investigated that the effects of chirality of peptide backbone of PRNA upon the higher ordered structure of PRNA oligomers and upon DNA recognition behavior and stability of PRNA/DNA hybrids. The orientation of PRNA monomers and oligomers tethered to L- and D-γ-glutamic acid backbone could readily be switched from *anti* to *syn* by adding borax as an external controlling factor. The cooperative orientation switching and borate ester formation were enhanced by oligomerization of PRNA. Furthermore, D-, L-PRNAs were preferred right-handed and left-handed higher-ordered coiled structure, estimated by CD spectroscopic studies, respectively. The complex of right-handed coiled D-PRNA with complementary right-handed helical DNA was more stable than that of left-handed coiled L-PRNA with DNA.

In chapter 4, the *on-off* switching of DNA recognition behavior of PRNAs, which were consisting of pyrimidine-purine mixed sequence, was demonstrated. PRNAs with purine—pyrimidine mixed sequence formed stable complex with complementary DNA and stabilities of PRNA-DNA complexes were higher than those of natural DNA—DNA complexes. Additionally, it was revealed that the *on-off*
switching of the DNA recognition of PRNA could be materialized by borax as external factor. Furthermore, nucleic acid models were shown improved base-sequence specific recognition selectivity, as well as these complex stabilities.

In chapter 5, it was demonstrated that the complexation/decomplexation behavior control of self-complementary PRNA with purine - pyrimidine mixed sequence, discussed with UV and CD spectroscopic melting studies. Furthermore, the reversible complexation/decomplexation control was achieved in the range of physiological pH conditions between 7.2 and 6.5 in the presence of borax as external orientation controlling factor.

The present concept of the external reversible control of DNA/RNA recognition by pH in the presence of borates has the potential to be widely employed in the next generation of antisense molecules. Antisense method has been hitherto developed for the purpose of simple inhibitory limitation of genetic information transfer. However, current antisense strategy has an inherent drawback, lacking direct means to control the function of these antisense compounds actively by external factor or stimuli. The author proposed a new antisense methodology in this thesis that a novel antisense molecule i.e. peptide ribonucleic acid (PRNA) could control its nucleic acid recognition behavior reversibly within pH range 1.2, from pH 7.2 to pH 6.0, in the presence of borax as an external factor.

This methodology is the first revolutionary example, in which the reversible recognition control of nucleic acid has achieved within the cytoplasm pH range of living organisms. It is expected that these results will be promising strategy for next generation of antisense molecules, which would be combined the “on-demand” gene therapeutic function corresponding to cellular condition, such as cytoplasm pH.

In actual fact, the cytoplasm pH of cancer cell are decreased to pH range from ca. 6.0 to 6.5, as a result of over-expression of acidic saccharides, such as sialic acid, then this pH difference of cytoplasm might be applied for trigger of cancer cell specific PRNA based antisense molecules. Certainly, some issues to be solved exist for application of PRNAs as next generation of antisense molecule, such as antisense compound – RNA complex being substrate for RNase H, permeability through the cellular membrane, borax administration strategy into cell, and so on. Still, the present concept of reversible control of DNA/RNA recognition behavior with PRNA has
proposed a strategy of novel cancer specific antisense methodology.
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