Structural Preorganization of Peptide Nucleic Acids: Chiral Cationic Analogues with Five- or Six-Membered Ring Structures

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The advent of aminoethylglycyl peptide nucleic acids, *aeg*PNAs, as strong and specific DNA/RNA binding agents has triggered much research activity directed towards the development of PNA-based antisense/antigene therapeutics. These efforts have mainly been directed towards further refinement of *aeg*PNA properties such as water solubility, cellular uptake and discrimination between parallel and antiparallel binding modes. Introduction of chirality and also of positive/negative charges in the PNA backbone has met with some success in this direction. The conformational freedom in the nucleobase linker arm and in the backbone aminoethyl and glycyl segments in the *aeg*PNA backbone were found to be causes of unfavourable entropic loss during complex formation. Suitable clamping in the *aeg*PNA backbone may reduce entropic loss and help produce a conformation appropriate for maximum enthalpic benefits from nucleobase recognition. Introduction of constraint by means of five- or six-membered ring structures in the *aeg*PNA and their contributions to maintaining the balance between rigidity and flexibility in the backbone have shown interesting effects on the overall stability of PNA-DNA/RNA complexes. This review presents an account of the literature in this direction. The significant promise of our approach, which makes use of the naturally occurring *trans*-4-hydroxy-L-proline to arrive at different chirally pure cyclic PNA analogues, is presented in this review, together with the DNA binding properties of the compounds.

1. Introduction

Replacement of the phosphodiester backbone linkages in nucleic acids DNA/RNA (Figure 1) has been extensively studied in the last decade in order to improve the viability of DNA-based antisense/antigene therapeutics.[1−4] The recent invention of peptide nucleic acids, PNAs,[5] as strong and specific DNA binding agents has attracted much attention because of the ease and simplicity of the scale-up methods for peptide chemistry, in comparison with the synthetic procedures used for other modified sugar-phosphate backbones. The *N*-aminoethylglycyl backbone of the polyamide, pseudo-peptide nucleic acids, *aeg*PNA (Figure 1), carrying nucleobases through an acetyl linker, was designed from molecular modelling studies as a result of atom by atom replacement of the sugar-phosphate DNA backbone by a polyamide backbone.[6] PNAs act as excellent struc-

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Vaijayanti A. Kumar was born in 1958 in Wai, a small town in Maharashtra State, India. She received her M. Sc. degree in Organic Chemistry in 1978 from University of Pune. She then completed her doctoral research at the National Chemical Laboratory under the supervision of the late Dr. V. N. Gogte in the area of phase-transfer catalysis and asymmetric synthesis using chiral auxiliaries. The University of Pune awarded her a Ph.D. in 1984. She continued her research career at NCL as a research scientist working particularly in developing synthetic routes to drugs and drug intermediates. She was introduced to the fascinating field of nucleic acids chemistry in 1988 and since then has been devoted to learning about synthetic chemistry providing modified nucleosides, nucleotides and oligonucleotides for biological applications. Her research interests are polyamine conjugated and fluorescent-labelled nucleic acids and peptide nucleic acids. During the past five years she has devoted her time to the conceptual development and synthesis of conformationally locked, stereodefined PNAs from the versatile naturally occurring amino acid 4-hydroxyproline.

MICROREVIEWS: This feature introduces the readers to the authors' research through a concise overview of the selected topic. Reference to important work from others in the field is included.

tural mimics of DNA/RNA and exhibit strong sequence-specific binding with complementary oligonucleotide sequences following Watson−Crick base pairing rules, and thus fulfil the primary condition for their biological application. The internucleobase distances in PNA are conserved, such that they complement the internucleobase distances in DNA for effective recognition of the base sequences. A unique property of PNAs is their ability to displace one strand of a DNA double helix to form strand invasion complexes, which is a favourable attribute for their application as antisense/antigene agents. This strand invasion process is inefficient or absent in DNA or in any other DNA analogues studied so far. The strand invasion by PNA (Figure 2) is dictated by the formation of triple helical structures through Watson−Crick and Hoogsteen binding modes and is overwhelmingly confined to the polypyrimidine PNA oligomers. The relatively high binding affinity of PNAs towards the natural oligonucleotides is attributed to the lack of electrostatic repulsion between the uncharged PNA backbone and the negatively charged sugar-phosphate backbone of DNA and RNA.[6] PNA being acyclic, the single-stranded form is conformationally flexible in its aminoethyl and glycyl segments, as well as in the acetyl linker arm carrying the nucleobase. Consequently, PNA-DNA or PNA-RNA complex formation is accompanied by conformational freezing of PNA such that the complex attains a structure for optimum hydrogen bonding and base stacking interactions to achieve maximum enthalpic advantage. Thus, the PNA-DNA or PNA-RNA duplex formation results in decreased entropy.[7,8] Any further increase in the conformational freedom in *aeg*PNA through extended structures with additional methylene groups in the aminoethyl, glycyl or nucleobase linker segments[9] or through the replacement of the tertiary amide linker to the nucleobase by an ethylene linker[10] has shown substantial reduction of the stability of the resulting complexes with target DNA/RNA.

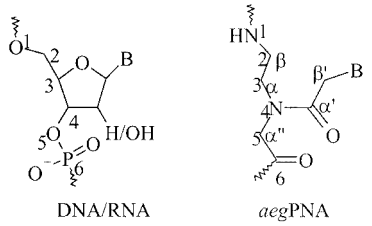

Figure 1. Sugar-phosphate DNA/RNA and aminoethylglycyl PNA backbones

The positive attributes of *aeg*PNA are accompanied by some disadvantages causing restrictions in the biological applications of these otherwise extremely promising DNA mimics. The main drawbacks are the relatively poor water solubility[11] and poor cellular uptake of PNAs,[12] both the result of their uncharged backbone as opposed to the negatively charged DNA backbone. Secondly, the mixed purine/pyrimidine PNA oligomers bind to both parallel (Figure 3a) and antiparallel (Figure 3b) DNA target sequences

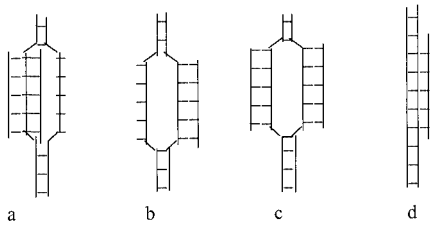

Figure 2. Strand invasion complexes of PNA-DANN: a) triplex invasion; b) duplex invasion; c) double duplex invasion; d) triplex formation.

with almost equal ability,[7,13] but with the antiparallel binding mode slightly preferred over the parallel mode (the C-terminus of PNA is conventionally taken to be analogous to the 3′ end of DNA/RNA). This ambiguity in parallel/antiparallel DNA/RNA binding modes by PNA oligomers is probably a result of the orientation-independent base sequence recognition of the chiral DNA sequences with distinct 5′→3′ directionality by the achiral PNA. The attachment of the nucleobases to the achiral, acyclic, flexible *aeg*PNA backbone through an acetamide linker presumably allows the nucleobases to attain a spatial disposition such that both parallel and antiparallel binding in either Watson−Crick or Hoogsteen modes are almost equally efficient. This property is a significant deviation from that of the natural sugar-phosphate backbones of nucleic acids, where antiparallel duplex formation is the most common.[14] Three-dimensional structures of hybrids between PNA and DNA/RNA have been studied by X-ray crystallography[15] and NMR spectroscopy.[16,17] The conformations of oligonucleotides in such hybrids are found to be quite different from A- or B-DNA conformations. NMR studies of single-stranded PNA oligomers have also clearly shown these to be composed of complex mixtures of conformational isomers and *cis*/*trans* rotamers.[16,18] The PNA hybridization with the target DNA/RNA is therefore influenced by the slow rotameric equilibrium of the acetamide nucleobase linker to the backbone amide (Figure 4a).

2. Strategies for Chemical Modification of *aeg*PNA

Various attempts to address the above-mentioned shortcomings of PNAs have been made. Introduction of various modifications/substitutions in the PNA backbone, in the aminoethyl, glycyl or the acetyl segment carrying the nucleobase, results in chiral PNA and is aimed to achieve directional selective binding with target DNA/RNA.[19] PNAs are suffixed with negatively charged DNA or positively charged polypeptide sequences at either the C- or the N-terminus. The thus modified PNAs result in PNA-DNA[12] or PNA-peptide chimeras[20] that have favourable aqueous solubility, cellular uptake and DNA binding/recognition properties. The PNA sequences have also been appended with polyamines[21] to increase the aqueous solubility and improve binding to the target DNA. Attempts to control the rotameric populations have resulted in the construction of olefinic polyamide nucleic acids, OPAs, through the in-

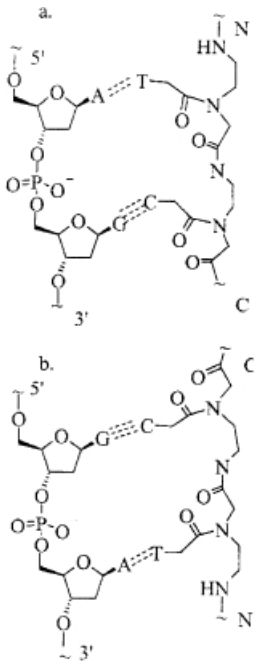

Figure 3. A schematic representation of DNA-PNA duplexes in a) parallel and b) antiparallel PNA strand orientation with respect to DNA

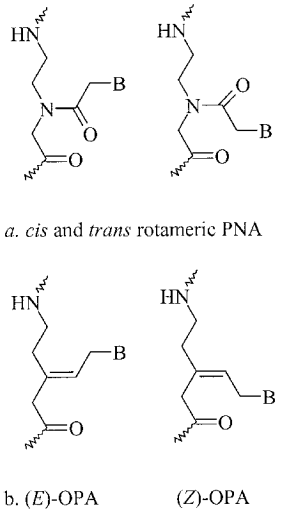

Figure 4. a) *cis* and *trans* PNA rotamers; b) (*E*)- and (*Z*)-olefinic peptide nucleic acids

troduction of a defined (*E*)- or (*Z*)-C−C double bond (Figure 4b) instead of the tertiary amide linker.[22] Some of these modified PNAs show improved water solubility, cellular uptake and/or directional selective binding relative to pure *aeg*PNAs, but in most cases exhibit a reduced binding affinity towards the target sequences. Commendable parallel/antiparallel sequence recognition has been achieved by the incorporation of aminoethyl-D-lysine PNA as a chiral box in the middle of the strand in mixed purine/pyrimidine sequences.[23]

3. Construction of Bridged PNA Structures

In the last few years, we have concentrated our efforts towards the objective of enriching populations of single-stranded PNA conformers that may be preferred forms in the duplex/triplex structures after complexation with the target. Any favourable structural preorganization of PNA may trigger the shift in the equilibrium towards the desired complex form because of the reduced entropy loss upon complex formation, provided that the enthalpic contributions suitably compensate. This may be achieved if the conformational freedom in *aeg*PNA is curtailed by bridging the aminoethyl or glycyl acetyl linker arms (Figure 5) to give rise to cyclic analogues with preorganized structures without affecting the nucleobase recognition ability through hydrogen bonding. Additionally, the introduction of chemical bridges into *aeg*PNA to provide cyclic structures may help in controlling the rotameric populations by fixing the nucleobase orientation and also in directional selective binding by virtue of the chirality in the backbone. Such a structural preorganization approach using additional conformational constraint has been extremely successful in the case of DNA analogues. Prominent examples are conformationally locked nucleic acids[24] or conformationally frozen hexitol[25] and altritol nucleic acids[26] (Figure 6), which have preorganized 3′-*endo* sugar conformations as prevalent in highly stable DNA-RNA duplexes. The structural preferences of PNA in PNA/DNA or PNA-RNA complexes are not yet as well deciphered as those of DNA-DNA/RNA complexes and may also be sequence-dependent. The research in our laboratory is focussed on preorganization of *aeg*PNA through the introduction of methylene bridges into the classical *aeg*PNA structure. Such modifications introduce chiral centres in the PNA monomeric units, with the possibility of further fine-tuning the structural features of PNA to mimic DNA. In this article, we present our own efforts and the contribution of other researchers giving rise to the creation of novel, chiral, structurally preorganized polyamide constructs with five- or six-membered cyclic structures and the consequences on their DNA/RNA recognition properties.

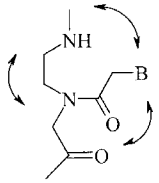

Figure 5. Possible positions for introduction of methylene or ethylene bridges

4. PNA with Five-Membered Nitrogen Heterocycles

The naturally occurring amino acid *trans*-4-hydroxy-L-proline, a five-membered nitrogen heterocycle with useful

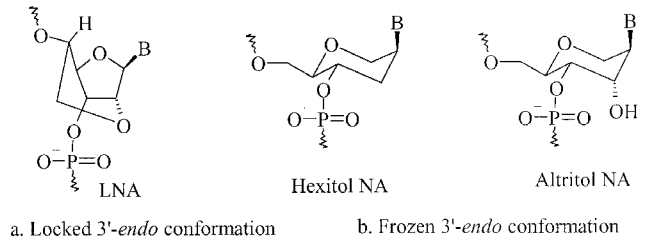

Figure 6. a) Locked 3′-*endo* conformation in LNA; b) frozen 3′-*endo* conformation in hexitol and altritol NA

substitutions and well known and easily manipulated stereochemistry,[27,28] is a versatile, commercially available starting material amenable for creating structural diversity to mimic the DNA/PNA structures. Many researchers have exploited *trans*-4-hydroxy-L-proline for the synthesis of a wide variety of chiral, constrained and structurally preorganized PNAs (Figure 7). Depending on the construction strategy and the presence or absence of the tertiary amine group in the monomers, the modifications afford either positively charged or uncharged cyclic PNA analogues.

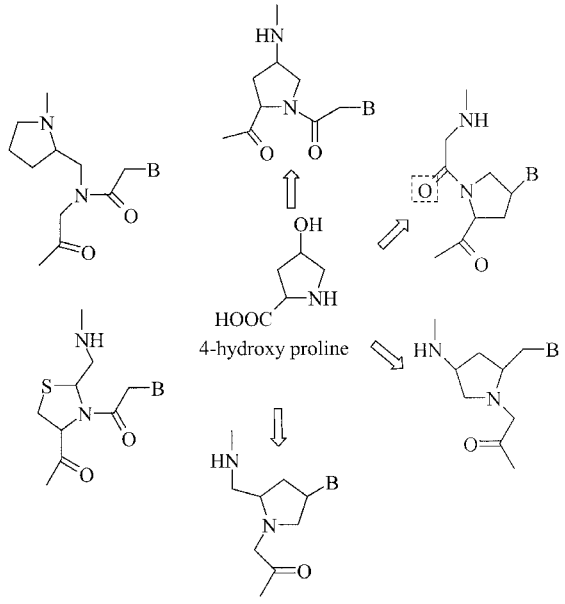

Figure 7. PNA analogues with five-membered pyrrolidine rings

4.1 Aminoprolyl PNA

The introduction of a methylene bridge between the β-carbon atom of the aminoethyl segment and the α′′-carbon atom of the glycine segment of the *aeg*PNA resulted in 4-aminoprolyl PNA, with the introduction of two chiral centres (Figure 8).[29] All four diastereoisomers arising due to the two chiral centres were synthesized from *trans*-4-hydroxy-L-proline and were incorporated into PNA oligomers. The incorporation of single chiral D-*trans*- and L-*trans*-prolyl PNA monomeric units in the PNA oligomers at the N-terminus resulted in very interesting CD profiles in the single strands and discrimination in parallel/antiparallel binding orientation preferences towards the target DNA sequences. Homochiral aminoprolyl PNAs based on thymine monomers did not bind to the target sequences.[30,31] Probably the bridged structure of the monomers imparted high rigidity to the oligomers, making them structurally incompatible with the DNA geometry for any effective nucleobase recognition interactions. A backbone combining *aeg*PNA alternating with L-*trans*-4-aminoprolyl PNA was later shown to bind to the target sequences with higher affinity than the pure *aeg*PNA oligomers.[32] Efforts directed towards releasing the structural strain in aminoprolyl PNA resulted in the synthesis of prolyl carbamate nucleic acids in which the backbone amide bond was replaced by a carbamate linkage, extended by two additional atoms (Figure 9a)[33] in comparison to the unmodified *aeg*PNA oligomers.

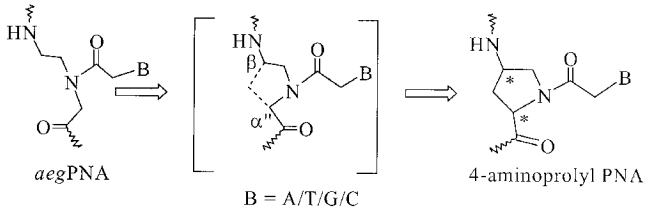

Figure 8. Prolyl PNA with α′′-β-methylene bridge

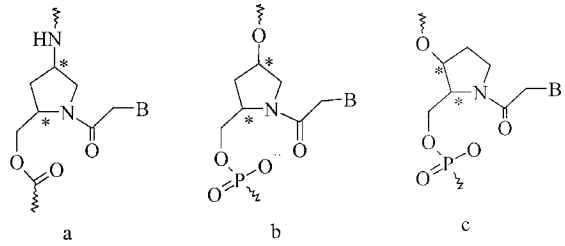

Figure 9. a) Prolyl carbamate NA; b) 4-hydroxyprolinol NA; c)3-hydroxyprolinol NA

Thymine or mixed pyrimidine oligomers of L-*cis*-prolyl carbamate nucleic acids were not attuned for binding with the target DNA. Another modification of prolyl PNA in which the internucleobase distance could be compatible to DNA was that formed by the replacement of the amide linkage by the phosphate linkage. No additional advantage was observed by the use either of 4-hydroxy-[34] or of 3-hydroxyprolinol[35] derivatives (Figure 9 b,c).

4.2 Gly-Pro-Peptide PNA

4-Hydroxyproline was used by Lowe et al. for the synthesis of a novel chiral prolylglycyl PNA. The methylene bridge was inserted between the α′′-carbon atom of the glycine unit and the β′-carbon atom of the nucleobase linker of *aeg*PNA (Figure 10).[36−38] The tertiary amide bond in the backbone between proline and glycine units replaced the aminoethylglycyl backbone. The backbone thus comprised alternate glycine-proline units that might be the cause of undesired rotameric populations. The nucleobase was directly attached to the proline ring by alkylation

through the 4-hydroxy function. The oligomers with such a backbone did not bind to the target sequences, the geometry imposed by the rigidity of the system probably being inappropriate for correct recognition. The sequences with *aeg*PNA alternating with the proline-glycine PNA unit showed reduced binding to the target sequences, unlike the 4-aminoproline PNA.[32]

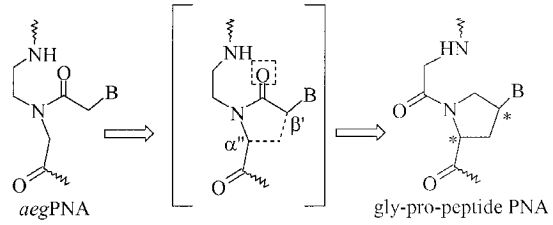

Figure 10. Prolylglycyl PNA with a α′′-β′-methylene bridge

4.3 Aminoethylprolyl PNA, *aep*PNA

We further envisaged a proline-based PNA in which a pyrrolidine ring would replace the tertiary amide linker to the nucleobase and thus might balance flexibility and rigidity in the PNA backbone. The α′′-carbon atom of the glycine unit and the β′-carbon atom of the nucleobase linker were joined through a methylene bridge (Figure 11).[39] The flexibility in the aminoethyl segment of *aeg*PNA was retained, unlike that in the proline-glycine PNA. The nucleobase attachment to the pyrrolidine ring was fixed by virtue of the chirality of C-4, thus removing the possibility of any rotameric populations. The monomers were synthesized in four steps starting from 4-(*R*)-hydroxy-2-(*R*/*S*)-proline. The tertiary amine function in the backbone was found to be at least partially protonated at physiological pH ($pK_a \approx 6.8$). Thus, in aminoethylprolyl PNA (*aep*PNA), all elements of the structural freedom of *aeg*PNA were conserved in addition to the restriction of the rotamers. The nucleobase with 4-(*S*) stereochemistry was expected to have a spatial disposition similar to that of the natural DNA. The oligomers comprising 4-(*S*)-2-(*S*/*R*) *aep*PNA thymine units showed very favourable binding properties towards the target sequences without compromising the specificity. The stereochemistry at the C-2 centre did not exert any significant effect on the binding ability of the homooligomeric sequences.

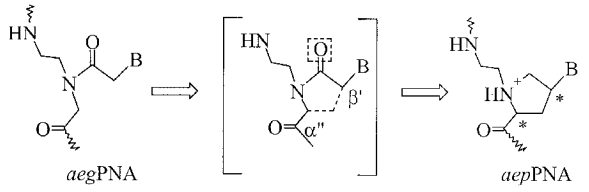

Figure 11. Aminoethylprolyl PNA with a α′′-β′-methylene bridge

The mixed pyrimidine hairpin sequences with cytosine and *N*-7 guanine *aep*PNA units exhibited directional discrimination in binding to parallel/antiparallel DNA sequences.[40] The *aep*PNA units carrying the individual nucleobases adenine, thymine, cytosine and guanine in a mixed purine/pyrimidine sequence exerted nucleobase-dependent binding efficacies and orientation selectivities towards target oligomers.[41] In another study, a thymine *aep*PNA homo-oligomer with (2*R*,4*R*) stereochemistry displayed significant stabilization of the complexes with target polyRNA.[42] The adenine homooligomer with (2*S*,4*S*) stereochemistry showed improved binding to the target DNA sequence, whereas the thymine homo-oligomers with same backbone geometry did not show any transition in UV melting experiments.[43] The results so far obtained with *aep*PNA are promising for further biological applications. The binding specificity of these *aep*PNA oligomers in the sequence context needs further investigation, and to this end synthesis of stereoregular mixed purine/pyrimidine *aep*PNA sequences and examination of their binding properties are currently underway in our laboratory.

4.4 Pyrrolidinone PNA

Another conformationally restricted cyclic PNA analogue was derived from a pyrrolidinone ring system.[44] A methylene bridge was inserted between the α-carbon atom of the aminoethyl segment and the β′-carbon atom of the acetyl linker to the nucleobase of *aeg*PNA (Figure 12). The carbonyl group of the nucleobase linker was retained and was forced to point towards the carboxy terminus of the backbone. The bridge prevented rotation around the C−N bond of the acetyl segment connecting the nucleobase residue to the backbone, and preorganized PNA in a rotameric conformation prevailing in complexes of PNA with nucleic acids as studied earlier.[22] The synthesis of all four diastereomers of adenin-9-yl *pyr*PNA was accomplished from D/L-pyroglutamic acid in an eighteen-step synthesis. The hybridization properties of PNA decamers containing this analogue with complementary DNA, RNA and PNA strands were investigated. The oligomers incorporating the (3*S*,5*R*) isomer were shown to have the highest affinity towards RNA in comparison with DNA. The fully modified decamer bound to rU_{10} with a small decrease in the binding efficiency relative to *aeg*PNA.

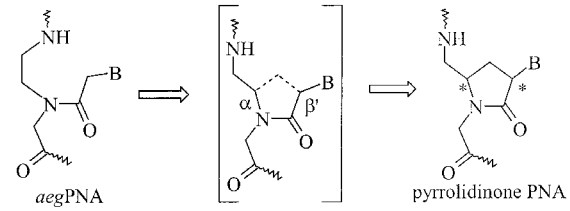

Figure 12. Pyrrolidinone PNA with α-β′-methylene bridge

4.5 Prolyl-(β-amino acid) Peptide PNA

The conformational strain in the alternating proline-glycine backbone was released by replacement of the α-amino acid residue by different β-amino acid spacers with appropriate rigidity (Figure 13).[45] β-Amino acids that gave favourable conformational freedom to the otherwise rigid backbone replaced the glycine unit in the prolyl-glycyl peptide backbone.[36] Novel pyrrolidinyl PNAs comprising al-

ternate units of nucleobase-modified D-proline and either L-aminopyrrolidine-2-carboxylic acid, D-aminopyrrolidine-2-carboxylic acid, (1*R*,2*S*)-2-aminocyclopentanecarboxylic acid or β-alanine were synthesized. Gel binding shift assay revealed that only the homothymine PNA oligomer with D-aminopyrrolidine-2-carboxylic acid spacer could bind with the DNA target. The cyclic nature and the stereochemistry of the β-amino acid in the backbone made important contributions to effective binding to the target oligomers. The constrained flexibility of the backbone geometry thus becomes an important feature of structural mimics of DNA.

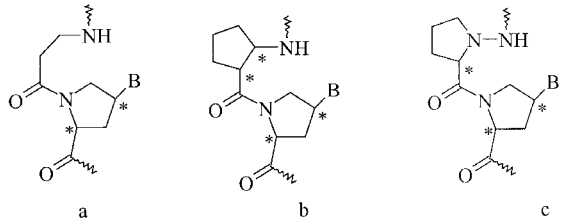

Figure 13. a) Prolyl-β-alanine PNA; b) prolyl-2-aminocyclopentanecarboxylic acid PNA; c) prolyl-D/L-aminopyrrolidinecarboxylic acid PNA

4.6 Pyrrolidine PNA and Pyrrolidine PNA-DNA Chimera with α-β′-Methylene Bridge

Three different research groups concurrently synthesized interesting structural isomers of another modified PNA that related the structural features of both PNA and DNA. The removal of the tertiary amide function of the pyrrolidone PNA resulted in the synthesis of (2*R*,4*S*)-pyrrolidine PNA (Figure 14).[46] A fully modified pyrrolidine PNA decamer, a homoadenylate sequence comprising this monomer formed very stable complexes with both DNA and RNA targets. The effect of protonation of the ring nitrogen atom was found to be insignificant, thereby indicating only a modest contribution from electrostatic interactions to the complex stability. Incorporation of a single pyrrolidine adenine unit in the centre position of *aeg*PNA mixed purine/pyrimidine sequence resulted in PNA-DNA/RNA duplex destabilization relative to the unmodified PNA-DNA/RNA. The destabilization was found to be greater in the case of the pyrrolidine analogue than in that of the pyrrolidone analogue. No synthesis of fully modified mixed sequences has yet reported, but again it will be quite interesting to determine their affinity towards DNA/RNA targets.

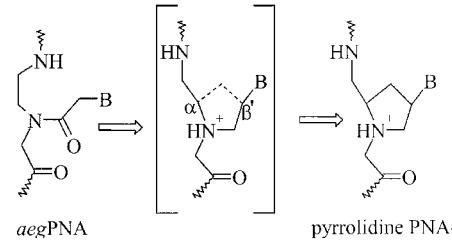

Figure 14. Pyrrolidine PNA-I with α-β′-methylene bridge

A different group has synthesized the pyrrolidine amide oligonucleotide mimic (2*R*,4*R*)-POM, a pyrrolidine PNA with different stereochemistry, derived by replacing the furanose ring of the native nucleic acids with a pyrrolidine ring, which may be protonated at physiological pH.[47] X-ray crystal structure determination and semiempirical quantum mechanical calculations on a protonated pyrrolidine model system stereochemically identical and stereoelectronically similar to the pyrrolidine monomer, found a close resemblance to the northern (N) conformation of uridine in the crystalline state. The solution-phase synthesis of a pentameric thymine homo-oligomer was carried out starting from *trans*-4-hydroxy-L-proline. It was shown to bind with very high affinity to complementary single-stranded RNA and DNA, whilst exhibiting kinetic binding selectivity for RNA over DNA.

At the same time, following the logic of exerting constrained flexibility in the *aeg*PNA through cyclic structures, we synthesized pyrrolidine PNA-I in our laboratory.[48] Insertion of a methylene bridge in *aeg*PNA, linking the α-carbon atom of the aminoethyl segment and the β′-carbon atom of the tertiary amide linker, afforded the envisaged pyrrolidine PNA (Figure 14). The tertiary acetamide linker to the nucleobase was absent, and as a result positive charge was introduced on the ring tertiary nitrogen atom. The monomer with (2*S*,4*S*) stereochemistry was synthesized by starting from the *trans*-4-hydroxy-L-proline. The incorporation of the modified monomer into homothymine oligomers and mixed pyrimidine oligomers resulted in a decreased binding efficiency of these oligomers with the target DNA/RNA sequences. The (2*R*,4*R*) isomer was synthesized and incorporated into a PNA:DNA dimer amenable to the synthesis of PNA:DNA chimeras (refer to section 5, Figure 18) by phosphoramidite chemistry. The chimeric PNA:DNA bound to the target DNA with decreased efficiency relative to the native DNA. Such a constrained PNA monomer may be useful in synthesis of PNA-5′-DNA or DNA-3′-PNA chimeras at the junction, as the cause of destabilization of chimeric complexes with target DNA/RNA has be proposed to be conformational freedom at the PNA-DNA junction.[49] More work is required to understand the utility of this constrained PNA analogue fully.

4.7 Pyrrolidine PNA-II with β-α′-Methylene Bridge

We further explored the possibility of the introduction of a β-α′-methylene bridge, resulting in another pyrrolidine-based PNA modification (Figure 15).[50] This should probably ease the rigidity of the direct attachment of the nucleobase to the pyrrolidine ring, as in the case of the α-β′-methylene-bridged pyrrolidine PNA-I or *aep*PNA. Two thymine monomeric units with either (2*S*,4*S*) or (2*R*,4*S*) stereochemistry were synthesized from 4-(*R*)-hydroxy-2-(*S*/*R*)-proline. It was observed that the (2*R*,4*S*)-pyrrolidine PNA monomer, when incorporated in the centre of the *aeg*PNA T_8 sequence, was able to bind to the target DNA better than pure *aeg*PNA. Incorporation of the other isomer, with (2*S*,4*S*) stereochemistry, actually destabilized the complex with DNA. To the best of our knowledge, this is

the first report where such stereochemical discrimination has been observed with a modified PNA. The tertiary amine function in this monomer, being protonated at physiological pH, may also affect the water solubility of the homo-oligomeric sequences. The results are preliminary and need to be verified in the context of other mixed sequences.

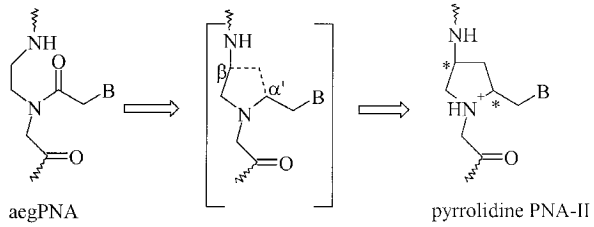

Figure 15. Pyrrolidine PNA-II with α′-β-methylene bridge

4.8 Pyrrolidine PNA-III with a N-β-Propylene Bridge

The introduction of a propylene bridge between the amino nitrogen atom and the β-carbon atom of the aminoethyl segment of the *aeg*PNA afforded yet another PNA modification with a five-membered pyrrolidine ring (Figure 16).[51] The synthesis of this modification involved both D- and L-prolinols, resulting in a chiral PNA monomer with a single asymmetric centre. The conformational constraint was in the aminoethyl segment of the *aeg*PNA. Incorporation of the (*R*/*S*)-thymine monomer in the *aeg*PNA afforded a constrained PNA, which bound to the target sequences with less efficiency than did the unmodified PNA. The PNA oligomer with a (2*R*)-pyrrolidine ring formed more stable complexes than did the PNA oligomer with a (2*S*)-pyrrolidine ring. Homochiral oligomer synthesis with all modified units with (*R*) stereochemistry may show interesting binding properties.

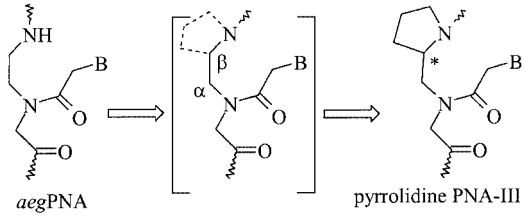

Figure 16. Pyrrolidine PNA-III with *N*-β-propylene bridge

4.9 Aminomethylthiazolidine PNA, *amt*PNA

Recently, a thiomethylene bridge was inserted between the α-carbon atom in the aminoethyl segment and the α′′-carbon atom of the glycine segment of the *aeg*PNA, providing aminomethylthiazolidine PNA, *amt*PNA.[52] The *amt*PNA thus constructed restricts movement in both the aminoethyl and the glycyl segments of the *aeg*PNA (Figure 17). The *syn* and *anti* isomers of the *amt*PNA were synthesized and incorporated into *aeg*PNA sequences in the central position by solid-phase synthesis. UV melting studies showed that the modified PNA-DNA and PNA-RNA triplexes were significantly destabilized. The stereochemical constraint in both the aminoethyl and the glycine segments, as in 4-aminoprolyl PNA with intact tertiary amide nucleobase linker arm, may confer to the system an unfavourable geometry for correct recognition.

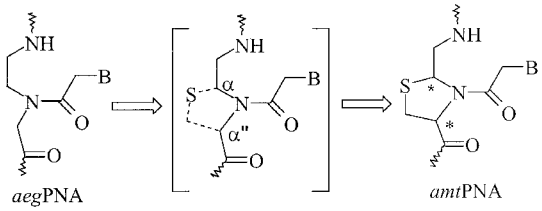

Figure 17. Aminomethylthiazolidine PNA, amtPNA

5. Deoxyribose- and Riboseamide Backbones

The replacement of the phosphodiester linkage in the DNA backbone by the amide unit was planned because of the several advantages over other modifications. The achiral amide moiety in the oligonucleotides (Figure 18) reduced the overall negative charges and could be accessed by simpler synthetic methods. The amide thymine dimers were constructed from appropriate starting materials by solution-phase peptide chemistry. These were then converted into dimer phosphoramidite blocks amenable for oligomer synthesis by employment of solid-phase phosphoramidite chemistry.[53] The oligonucleotides constructed from these modified dimer units with alternate phosphate-amide linkages showed interesting binding properties. The amide in which the amino function resided on the 5′-sugar carbon atom rendered the complexes with target RNA more stable than the unmodified complex. Alkyl substitution of the amino function had negligible effects on the stability when only one unit was present. More than two modified units in the oligonucleotides destabilized the complex to a small extent.[54] The complexes with DNA were all destabilized relative to those with RNA. The direction of the amide bond also significantly contributed to the stability of the complexes. Further modifications to this system, in which sugar conformations were restricted to 3′-*endo* forms, have also been reported. Additional 2′-methoxy groups in the dimer contributed to an increased stability of the complex.[55] An additional C5′-methyl group [(*S*) configuration] resulted in an increase in the stability of the complexes with RNA.[56] Use of five-atom linkers instead of four-atom linkers involving amide bonds was also found to provide favourable backbone geometry without affecting entropic contributions.[57] Recently, the solution-phase synthesis of a polyamide-ribose sugar backbone uracyl pentamer was reported.[58] It will be interesting to observe the binding preferences of such pure ribose-amide repeat backbones.

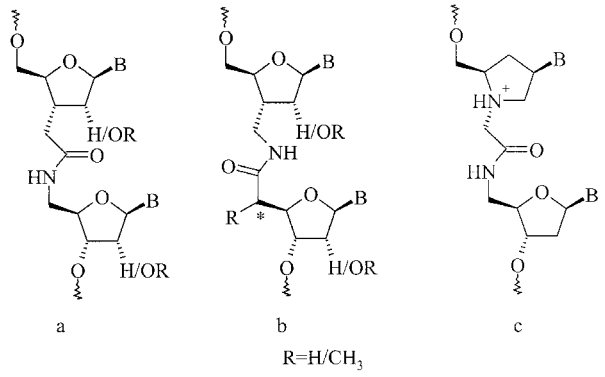

Figure 18. Amide-linked nucleoside dimer blocks: a) 5′-amino-3′-carboxylate linker; b) 5′-carboxylate-3′-amino linker; c) pyrrolidine-*N*-(acetic acid)-5′-amino sugar chimera

6. Peptide Ribonucleic Acids, PRNA

The synthesis of poly-L-glutamic acid in which ribonucleoside units are attached to the side chain as pendant groups through an amide linkage between the γ-carboxy function of the side chain and the 5′-amine of the 5′-amino-5′-deoxyribonucleoside afforded the α-peptide ribonucleic acid α-*PRNA* (Figure 19, a).[59] The strategy was actively to control the function of these oligomers through an external factor. The interconversion of the *syn*/*anti* nucleobase conformations of the ribonucleosides in *PRNA* with a free 2′,3′-diol system could be controlled by the formation of a borate ester.[60] After the formation of the borate ester with the *cis* diol system, the preferred nucleobase conformation was found to be to be *syn* (2-carbonyl oxygen atoms of the pyrimidine nucleobases towards the 5′-amino group), which is unfavourable for duplex formation, and thus complex formation can be controlled. Unfortunately, the efficiency of the external control was not very high because of the mismatched distance of the nucleobases and as a result the complexes were all destabilized.

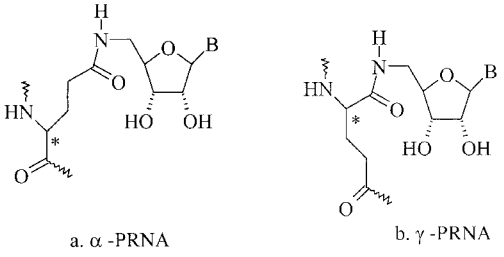

Figure 19. a) α-PRNA with polyglutamate backbone; b) γ-PRNA with polyisoglutamate backbone

The strategy was further improved by the synthesis of isopoly-L-glutamic acid in which ribonucleoside units were attached as pendant groups through an amide linkage between the α-carboxy function of the glutamic acid and the 5′-amine of the ribonucleoside. This gave rise to the γ-peptide ribonucleic acid γ-*PRNA* (Figure 19 b),[61] in which the nucleobases were in the correct positions for RNA/DNA recognition. The 1:1 complex of homothymine γ-*PRNA* with complementary DNA was considerably more stable than the unmodified PNA-DNA complex. The mixed base sequence was also able to exhibit high directional selectivity, the antiparallel complex being more stable than the parallel one. The presence of the ribose sugar could favourably improve the water solubility of γ-*PRNA*. As expected, in the presence of 20 mM borax, the complex with homothymine γ-*PRNA* was completely destabilized due to the conformational effects on the ribose sugar as a result of the formation of the borate ester. The concept of external control on DNA/RNA recognition through duplex formation is quite interesting and may have potential in the next generation of antisense molecules.

7. PNA with Six-Membered Ring Structures

Six-membered ring structures exhibit unique conformational preferences, and the binding abilities of hexose sugar phosphate containing oligonucleotides have been extensively studied by Eschenmoser and his group.[62] The ability of morpholino,[63] hexitol[25] and cyclohexene[64] oligonucleotides for DNA/RNA recognition are well established and are dictated by the conformational constraints of the six-membered ring structures. The use of such structures in PNA has not yet been extensively studied, but is briefly addressed in this article. Conformations in the six-membered ring structures are rigid, in contrast to the relatively flexible five-membered structures, and hence their influence on the stability of the resulting PNA-DNA/RNA complexes may be expected to make important contributions to the stabilities of the DNA/RNA complexes.

7.1 Glucosamine Nucleic Acids, GNAs

The six-membered glucosamine ring appeared to fulfil the requirements of optically pure and somewhat constrained conformational scaffolding for the attachment of nucleobases. The monomer units were synthesized from glucosamine by glycosylation of the nucleobases and oxidation of the primary hydroxy function to provide the appropriately protected amino acid building blocks for the synthesis of oligomers (Figure 20).[65] The homo-pyrimidine and mixed base sequences using GNA monomer were constructed. The binding affinities and selectivities of these oligomers to DNA and RNA targets indicated selective recognition of RNA consistent with the Watson−Crick binding model. These highly novel, water-soluble and stable oligonucleotide analogues mimic the binding properties of DNA and RNA.[66] The entropy changes were found to be smaller for GNA-DNA/RNA than for DNA-DNA/RNA, consistent with the idea that the GNA oligomer was preorganized for binding to the target sequences. These results show an interesting contrast to those reported for other six-membered DNA analogues, such as homo-DNA, pyranosyl-

RNA and hexose nucleoside analogues, which do not bind to DNA or RNA.[67]

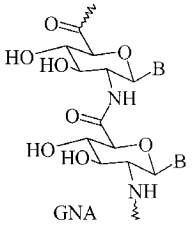

Figure 20. A six-membered cyclic PNA derived from glucosamine

7.2 Piperidinone PNA

Introduction of an ethylene bridge into *aep*PNA resulted in a six-membered ring structure as in piperidinone PNA (Figure 21).[68] The ethylene bridge was introduced between the α- and β′-carbon atoms in the ethylenediamine and acetyl linkers. Model building suggested that a six-membered homologue of the pyrrolidinone PNA could adjust better to a duplex structure. The synthesis involved a stereoselective oxidation step, as in the case of the pyrrolidone analogue. The two adenine diastereomeric monomers − (3*R*,6*R*) and (3*S*,6*R*) − differed in the stereochemistry of nucleobase attachment to the piperidinone ring. The incorporation of these monomers in the *aeg*PNA resulted in a large decrease in the duplex stability. The preorganization of the PNA induced by these cyclic piperidinone PNA seems to be inferior to the cyclic pyrrolidinone PNA in terms of producing a hybridization-competent conformation. The stereochemical effects in the backbone are not yet fully explored for these analogues and the implications due to the remaining two diastereomers have yet to be deciphered.

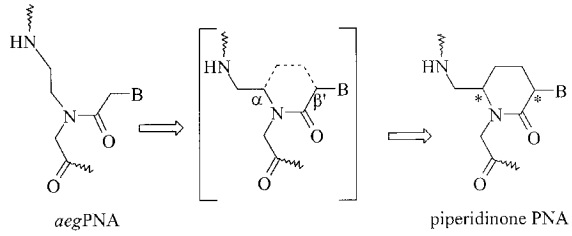

Figure 21. Piperidinone PNA with α-β′-methylene bridge

7.3 Cyclohexyl PNA

One of the earliest efforts to introduce conformational constraint in the *aeg*PNA structure resulted in the chiral cyclohexyl-derived backbone (Figure 22).[69] The aminoethyl segment of the *aeg*PNA was replaced with a 1,2-diaminocyclohexyl moiety, either in the (*S*,*S*) or the (*R*,*R*) configuration. The conformational freedom in the ethylene chain was thus locked in the six-membered cyclic structure. The oligomers with (*S*,*S*)-cyclohexyl residues were able to hybridize with DNA or RNA, with little effect on the thermal stability depending on their number and the sequence. From the thermal stabilities and molecular modelling based on the solution structure of a PNA-DNA duplex determined by NMR techniques, it was concluded that the right-handed hybrid duplex accommodated the (*S*,*S*) isomer more easily than the (*R*,*R*) isomer. Thermodynamic measurements also pointed to a decrease in entropy indicating a conformationally constrained structure. The favourable entropic contribution could not reflect on the stability of the complex that might be balanced by reduced enthalpic gain, indicating that the structure constrained by the cyclohexyl group was not well suited for hybridization with DNA. In contrast, incorporation of the (*R*,*R*) isomer resulted in a drastic decrease in the stability of PNA-DNA/RNA complexes. In PNA-PNA duplexes, however, the (*S*,*S*)- and the (*R*,*R*)-cyclohexyl residues exerted minor effects on the stability. The complexes formed with the two isomers were of the opposite handedness, as evidenced from CD spectroscopy.

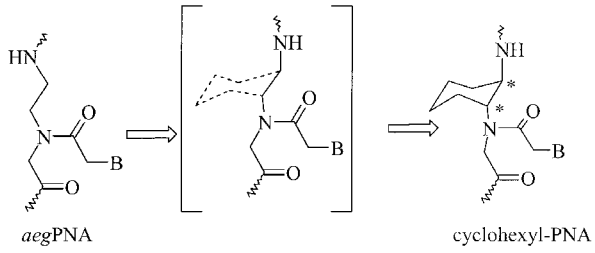

Figure 22. Cyclohexyl PNA

7.4 [(Aminoethyl)amino]cyclohexanoic Acid PNA

Rigidity was introduced into the *aeg*PNA by replacing the glycyl segment in the backbone by α-aminocyclohexanoic acid (Figure 23).[70] The synthesis was accomplished by a simple and efficient four-component Ugi reaction and provided many previously unknown and interesting PNA monomers. Incorporation of these monomers into oligomeric structures and their DNA/RNA binding properties have not yet been reported.

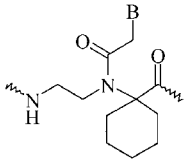

Figure 23. PNA based on aminoethylaminocyclohexanoic acid

7.5 Morpholino PNA

The attractive morpholino antisense oligomers,[63] with high sequence specificity, water solubility and low production cost, have sparked research into further modifications in morpholino-based DNA analogues. The set of morpholino analogues with phosphonate ester, amide or ester linkages between the morpholino nucleoside residues was synthesized (Figure 24).[71] Preliminary evaluation indicated that the uridine homo-oligomer with either amide or ester linkage stabilized the complex with the DNA targets relative to the DNA-DNA complexes. The adenine homo-oligomers showed destabilizing effects when complexed with the

target homo-thymidine DNA sequences. The amide-linked morpholino PNAs were better accommodated in the complexes than the ester- or the phosphonate-linked oligomers.

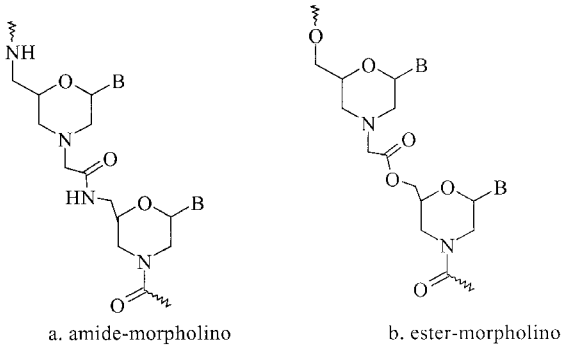

Figure 24. PNA with morpholino amide and ester backbones

7.6 Aminopipecolyl PNA, pipPNA

In our quest to produce a PNA analogue with favourable geometry for optimum binding to the target DNA sequences, we synthesized a PNA analogue with a six-membered ring. We arrived at this structure by introducing a methylene bridge between the γ-carbon atom and the α′′-carbon atom of the aminopropylglycyl PNA (Figure 25).[72] We envisaged that the increased conformational freedom and the internucleobase distance in the aminopropylglycyl PNA could be effectively curtailed by a bridged system in the monomeric unit. The *trans*-(2*S*,4*S*)-pipecolic acid thymine monomeric unit was synthesized and was incorporated into the homo-pyrimidine *aeg*PNA. The complexes with target DNA were destabilized and the additional methylene groups caused reduced water solubility in the modified oligomers. The synthesis of other stereoisomers is currently underway in our laboratory. It will be interesting to study the effects of these monomeric units on the properties of the modified PNA.

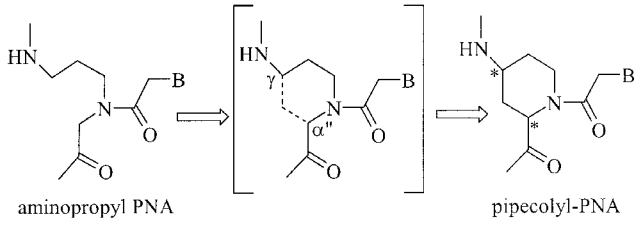

Figure 25. Pipecolyl PNA based on γ-α′′-methylene bridge in an aminopropylglycyl backbone

8. APNA and PNA-APNA Chimera

An aromatic PNA analogue in which the backbone *o*-aminophenylbutanoic acid derivative carries the nucleobase through an ether linkage has been synthesized (Figure 26).[73] The synthesis of a thyminyl derivative of (*S*)-2-hydroxy-4-(2-aminophenyl)butanoic acid was achieved in high enantiomeric purity and was shown to be a useful building block for the production of APNA oligomers. The nucleobase attachment was through a flexible ether linkage. As three of the bonds in the aniline moiety were coplanar, the spatial distance between the bases was expected to depend on the conformation. These analogues were designed in order to investigate possible π stacking interactions and their stabilizing effects on duplex-triplex structures.[74] The aromatic PNA was subjected to further modification by replacing it with another aromatic *N*-(2-aminobenzyl)glycine backbone (Figure 27).[75] An efficient synthetic procedure amenable for parallel or combinatorial preparation of APNA building blocks with high degree of structural diversity was developed. The optimum distance of six σ bonds between the nucleobases along the backbone was extended to seven σ bonds in this modification. The direct incorporation of aromatic rings in the backbone renders at least three bonds in the backbone coplanar. Furthermore, the internucleobase distance was altered by additional methyl substitution in the glycyl segment, affording *N*-(2-aminobenzyl)-(*R* or *S*)-alanine or by replacing it with β-alanine, as in *N*-(2-aminobenzyl)-β-alanine backbones. The incorporation of these modified units in *aeg*PNA produced PNA-APNA chimera. The modified oligomers exhibited decreased binding affinities relative to the pure PNA. An *N*-(2-aminobenzyl)glycine unit in the *aeg*PNA resulted in the smallest decrease in the thermal stability of the triplexes with DNA and RNA while maintaining the selectivity of base pairing recognition.

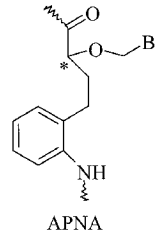

Figure 26. Aromatic peptide nucleic acid, APNA-I

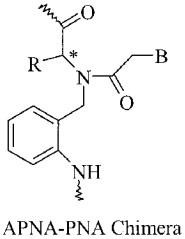

Figure 27. APNA-II-PNA chimera

9. Conclusions

Peptide/polyamide nucleic acids *aeg*PNAs, achiral, acyclic and uncharged DNA/RNA mimics, have recently invited the attention of chemists to improve their properties for useful therapeutic purposes. This review presents efforts initiated by us and others to design conformationally re-

stricted monomers in polyamide backbones with the aim of arriving at a structure that is prevalent in PNA-DNA/RNA complexes. In this way, maximum enthalpic and base-stacking interactions may be attained with a minimum loss of entropy during DNA/RNA recognition process. Introduction of five- and six-membered rings in acyclic *aeg*PNA opens up a large variety of structures with defined configurations and conformational preferences. As lead structures, these PNA analogues could be part of a chemical evolution process that might give rise to a synthetic nucleic acid analogue/mimic possessing optimum single-strand conformations for DNA/RNA recognition. From the literature presented here, it appears that the range of such conformations in PNA is quite narrow as, barring a few exceptions, a large number of the constrained structures destabilize the complexes with target DNA. Striking the balance between flexibility and rigidity of the ring structures in conjunction with correct nucleobase orientation seems to be the key factor responsible for sequence-independent specific DNA/RNA recognition process. Positively charged *aep*PNA and pyrrolidine PNAs, endowed with increased water solubility and affinity towards target sequences, could prove to be the lead structures for further development of PNAs with desired properties.

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