Peptide nucleic acid (PNA). A DNA mimic with a pseudopeptide backbone

CHEMICAL SOCIETY REVIEWS

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PNA (peptide nucleic acid) is a DNA mimic with a pseudopeptide backbone composed of *N*-(2-aminoethyl)glycine units with the nucleobases attached to the glycine nitrogen *via* carbonyl methylene linkers. PNA was first described in 1991 and has since then attracted broad attention within the fields of bioorganic chemistry, medicinal chemistry, physical chemistry and molecular biology due to its chemical and physical properties, in particular with regard to efficient and sequence specific binding to both single stranded RNA and DNA as well as to double stranded DNA. The present review discusses the structural features that provide the DNA mimicking properties of PNA and gives an overview of structural backbone modifications of PNA.

1 Introduction

Self-recognition by nucleic acids is one of the fundamental processes of life and also one of the most straightforward principles of molecular recognition in complex systems. Only four basal recognition units exist, the nucleobases adenine (A), cytosine (C), guanine (G) and thymine (T) [uracil (U) in RNA], that recognize each other two by two forming A–T and G–C base pairs by simple hydrogen bonding between complementary hydrogen bonding acceptor and donor sites of the nucleobases (Fig. 1). Thus, it is not surprising that this simple four building block system has been a paradigm and inspiration for chemists attempting to and succeeding in making self-organizing systems (*e.g.* ref. 1). Also, a large number of close as well as more distant analogues of DNA itself by modifications of the sugar phosphodiester backbone have also been made mainly with the aim of developing efficient antisense drugs

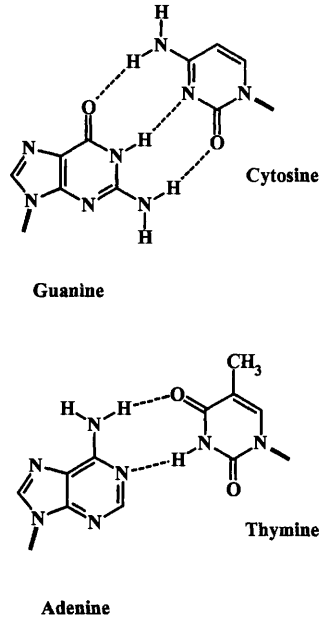

Fig. 1 Nucleobase pair recognition by Watson–Crick hydrogen bonding

(Fig. 2).[2] Peptide nucleic acid (PNA) represents a much more dramatic deviation from the natural DNA structure since the entire phosphodiester backbone has been replaced by a pseudopeptide backbone (Fig. 3). Thus, from a chemical point of view PNA is a hybrid between an oligonucleotide (the nucleobases) and a 'protein' (the backbone) and consequently shows properties from both 'worlds'.

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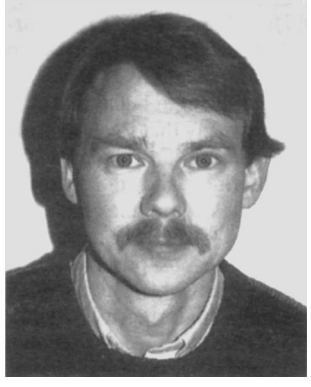

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Gerald Haaima hails from the 'Pride of the South', Otago, New Zealand. He obtained his BSc with first class honours in 1985 and followed this up with a PhD (1988) under the supervision of Rex Weavers. After a year with Gary Molander (Boulder, USA) and two years with Lew N. Mander (ANU, Australia) he relocated to Denmark and started work with the late Ole Buchardt and Peter Nielsen on Peptide Nucleic Acids. His research interests include nucleic acid recognition and the development of novel molecular recognition systems.

Gerald Haaima

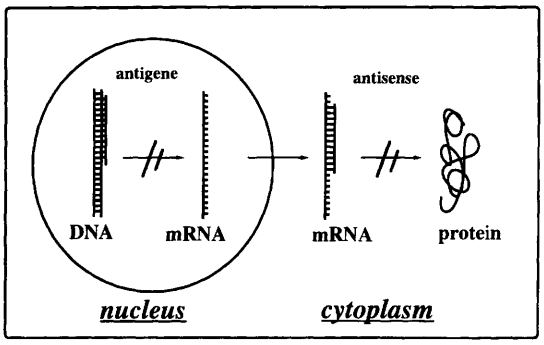


Fig. 2 Principle in 'antisense' and 'antigene' strategies. In the antisense strategy, an oligonucleotide (analogue) binds to the mRNA by Watson–Crick hybridization and thereby inhibits the translation to the protein product. This can occur either by inducing RNAse H degradation of the mRNA or by physical blocking of the ribosomes. An antigene agent binds to the gene itself, the double stranded DNA either by triplex formation (oligonucleotides) or duplex invasion (PNA) and thereby inhibits the transcription of the gene to mRNA.

PNA was originally designed as a reagent to sequence specifically target double stranded DNA as a mimic of triplex forming oligonucleotides which bind as a third strand in the major groove of a DNA double helix *via* T·A–T and C^+·G–C Hoogsteen base pairing (Fig. 4).[3] However, the PNA backbone turned out to be a much better substitute for the normal sugar phosphate backbone than anticipated,[4] and therefore much effort has been devoted to exploring the general DNA mimicking properties of PNA as well as its potential as an antisense and antigene drug, including being a sequence specific ligand for binding to double stranded DNA.

The chemistry,[5] physical chemistry[6] as well as molecular biology/drug aspects of PNA[7] have been presented quite extensively in the recent reviews. The present paper will focus on the 'structure–activity' relationships of peptide-like backbones in terms of DNA mimics.

Briefly, PNA is composed of a backbone built up from aminoethylglycine units (a reduced dipeptide backbone) in which the nucleobase is attached to the central amine *via* an acetyl linker. This particular arrangement of atoms resembles the '6 + 3' number of bonds arrangement found for DNA. A simple geometry dissection of the DNA backbone reveals that six bonds separate each nucleobase unit, while the distance between the backbone and the nucleobase is three bonds. There have been a number of other approaches to the synthesis of PNA monomers[8–10] since the first reports on PNA,[11–13] all of which essentially disconnect the molecule about the central amide bond presenting a synthesis of suitably protected nucleobase acetic acid and a protected backbone. The chemistry for these is well established in the literature and does not present new synthetic problems. More *de novo* approaches have been reported in which the monomer is built up from simple units during the solid phase oligomerization. This approach removes the need for monomer synthesis but its utility in producing high quality product has not been demonstrated.[10] As with peptide synthesis, PNA monomers come in two major varieties, Boc and Fmoc each of which present their own possibilities and limitations. This has been reviewed recently,[5] and by way of example a set of Boc-monomers are shown in Fig. 5.

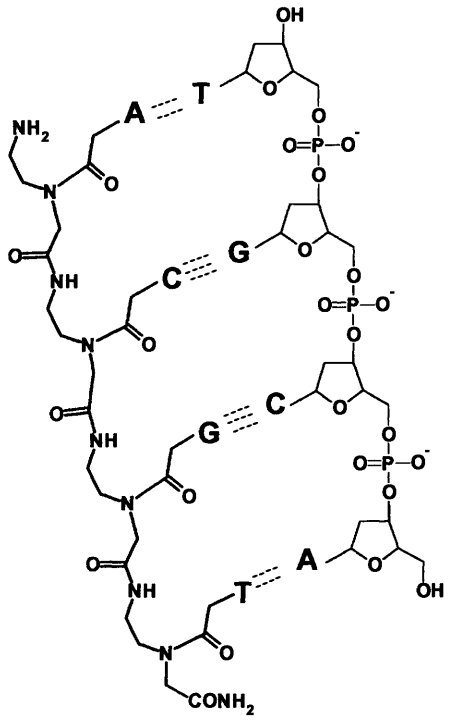


Fig. 3 Chemical structures of DNA and PNA. A, C, G and T designate the nucleobases adenine, cytosine, guanine or thymine.

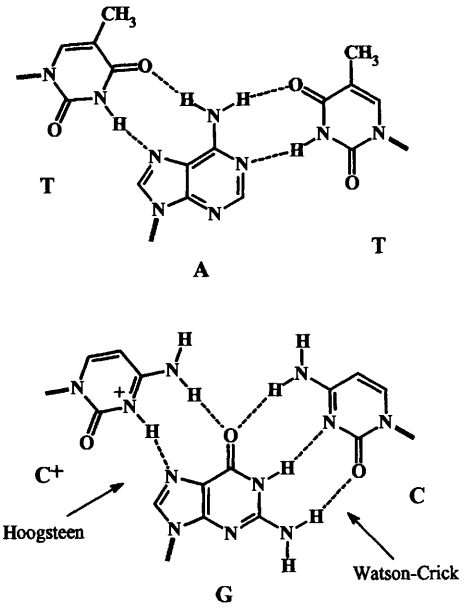


Fig. 4 T·A–T and C^+·G–C triplets involving Hoogsteen and Watson–Crick base pairings. Note, that N^3 of cytosine needs to be protonated in order to donate a hydrogen bond to the N^7 of guanine.

2 PNA hybridization

PNA oligomers bind strongly and with high sequence discrimination to complementary oligomers of DNA, RNA or another PNA, and in general the hybrid thermal stabilities (T_m) for identical sequences follow the order: PNA–PNA > PNA–RNA > PNA–DNA (> RNA–DNA > DNA–DNA).[4,14] Furthermore, the stabilities of PNA hybrids are, in contrast to hybrids between two anionic oligomers like DNA–DNA or RNA–DNA, fairly independent of ionic strength because of the neutral PNA backbone.[15] It is also noteworthy that PNA hybrids can be formed both in the antiparallel (amino-terminal of PNA facing the 3′-end of the oligonucleotide) and the parallel configuration even though the antiparallel complexes have the higher stability (in general a ΔT_m of 1–2 °C per base pair between antiparallel and parallel complexes are observed).[4]

Homopyrimidine PNAs distinguish themselves by forming PNA–NA–PNA triplexes of extremely high thermal stability.[8,16] Again, the charge neutral backbone of PNA can account for at least part of the triplex stabilization, but an X-ray crystal structure of a PNA_2–DNA triplex shows specific interactions (hydrogen bonding) between each amide N–H of the backbone

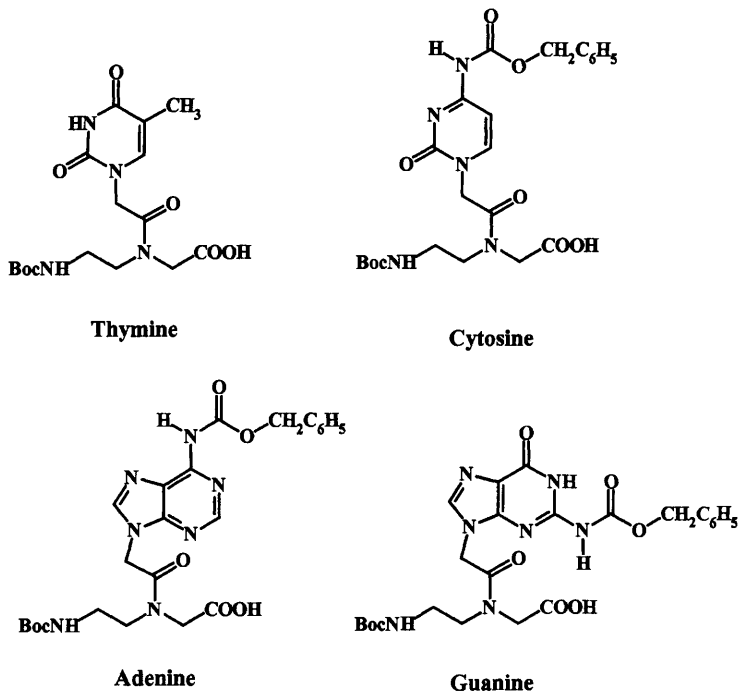


Fig. 5 PNA monomers used for oligomerization using the Boc (*tert*-butoxycarbonyl) strategy

of the Hoogsteen PNA strand and a phosphate oxygen of the DNA backbone[17] thereby also contributing to the stability.

The high stability of PNA_2–DNA triplexes also helps explain why hompyrimidine PNAs when targeted to double stranded DNA prefer not to form traditional PNA–DNA_2 triplexes, but instead invade the DNA double helix forming an internal PNA_2–DNA triplex (having conventional Hoogsteen and Watson–Crick nucleobase interactions) in a strand displacement complex[18,19] (Fig. 6). This novel binding mode has opened a new avenue for the attempts to develop sequence specific dsDNA binding ligands, *e.g.* as gene therapeutic agents (antigene strategy)[20,21] or as biomolecular tools in genome analyses.[22]

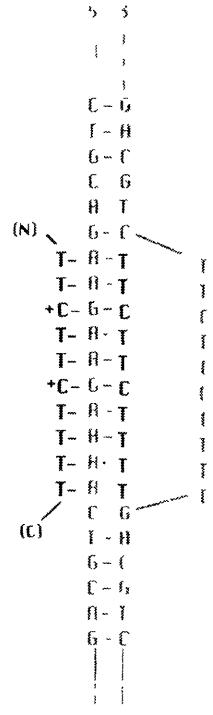


Fig. 6 Schematic representation of a PNA–triplex strand displacement complex involving a PNA–DNA–PNA triplex *via* Watson–Crick and Hoogsteen hydrogen bonding (PNA is shown in heavier type than DNA)

3 Biological aspects of PNA

PNA has many of the properties of a promising antisense or antigene drug, such as stable and highly sequence specific binding to the complementary mRNA or dsDNA gene target, high biological and chemical stability.[23] The easy synthetic accessibility and not least synthetic flexibility of PNA should also allow further optimization of the structure, especially with regard to bioavailability and pharmacokinetic properties. Thus it is not surprising that the drug aspects together with the utility as a molecular biology tool of PNA technology is being actively pursued, and the results so far are very encouraging.[7]

4 PNA structure

Four high resolution structures of PNA complexes are available at present. Two structures, a PNA–RNA[24] and a PNA–DNA[25] duplex, were determined by NMR methods, while a PNA_2–DNA triplex[17] and a PNA–PNA duplex[26] were solved by X-ray crystallography. It is quite clear from these structures that the PNA oligomer is to some extent able to structurally adapt to the oligonucleotide complement, but it is equally clear that the PNA has a preferred structure of its own termed the 'P-form'.[17,26] This is, of course, most obvious from the structure of the pure PNA duplex which is a very wide (28 Å diameter) helix with an accordingly large base pair helical displacement and a very large pitch (18 bp) [Fig. 7(*a*)]. A canonical B-form helix which is typical for DNA duplexes has a diameter of *ca.* 20 Å and a pitch of ten base pairs per turn. The base pairs are perpendicular to the helix axis and stack through the centre of the helix. A canonical A-form helix, typical of RNA duplexes, also has a diameter of *ca.* 20 Å but a pitch of 11 base pairs per turn, and the base pairs are tilted *ca.* 20° relative to the helix axis. Furthermore, the base pairs are displaced away from the helix leaving a central 'tunnel' in the helix, analogous to that seen in the P-form. It is also noteworthy that the base pairs in the P-form are practically perpendicular to the helix axis (B-like) with only minor variations in slide, tilt and propeller twist angles between individual base pairs, and with an interbase-pair stacking overlap that is remarkably close to that found in canonical A-form RNA helices (Fig. 7). It is likewise notable that the

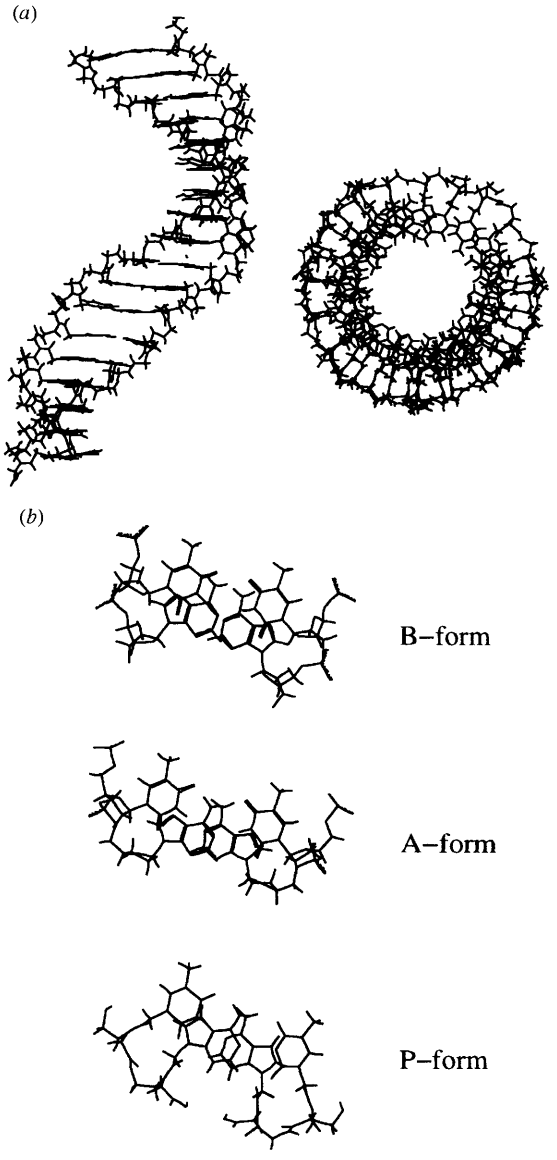


Fig. 7 (*a*) Structure of a PNA duplex from X-ray crystallography. The structure was determined from a self-complementary hexamer[26] but the full turn (18 bp) of the helix has been modelled from these data. Only the right-handed form is shown, but the unit cell contains both a right-handed and a left-handed form. (*b*) Base pair overlaps in A-, B- and P-form helices. Two consecutive A–T base pairs are shown as viewed from the end of the helix for a canonical B-form, a canonical A-form or for the crystal structure of the PNA–PNA duplex P-form.

backbone structure found in the PNA duplex[26] is almost identical to that seen in both the Watson–Crick and the Hoogsteen strand of the PNA_2–DNA triplex[17] and that the basic features, such as carbonyl orientations are also in common with the two PNA–oligonucleotide duplexes.[6,24,25]

One conclusion to be drawn from the above described structural data is that despite the ability of PNA to efficiently bind and recognize DNA or RNA, the conformation adopted by PNA in these hybrid complexes is not optimal, because the P-form helix preferred by PNA is distinct in terms of important parameters such as helical width and pitch from both the B-form preferred by DNA and the A-form of RNA. Thus, one should be able to obtain a better peptide nucleic acid DNA mimic if one could construct a backbone that in its lowest energy state would adopt a B-form (or A-form) helix. However, the compactness and simplicity of the PNA structure pose severe restrictions as to which modification can be implemented and still result in chemically reasonable structures. Some of these possibilities are discussed below.

5 PNA backbone modifications

Since the first reports on PNA, a large number of PNA backbone derivatives have been described and investigated (Tables 1 and 2) in order to explore the 'structural space' in which this type of PNA mimic operates, as well as in an effort to obtain a molecular understanding of the chemical and structural parameters that determine the DNA mimicking properties of a (peptide) nucleic acid analogue. Thereby we should hopefully also gain a better understanding of the DNA (and RNA) molecules themselves.

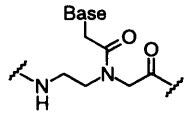
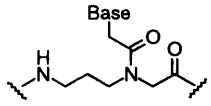
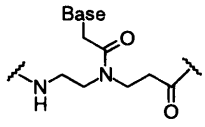
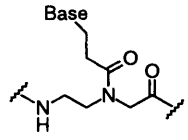
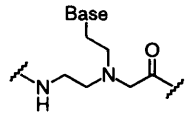
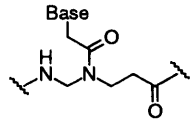
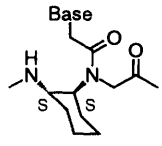
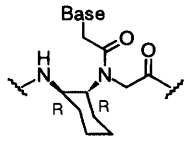
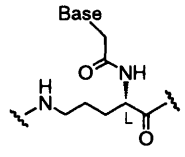
The results so far have shown (Tables 1 and 2) that only certain alterations of—or deviations from—the original aminoethyl-glycine backbone are 'allowed' without severe penalties in DNA/RNA–PNA hybrid stability. As an overall conclusion at this stage one cannot touch the basic structure of the PNA backbone (**1**), *e.g.* by extending either of the 'linkers' [ethyl → propyl (**2**), glycine → β-alanine (**3**) or acetyl → propionyl (**4**)],[27] reducing the nucleobase linker amide (**5**)[28] or even reverse the amide linkage within the backbone (**6**).[29] However, much freedom seems to be in placing (functional) substituents on the backbone as exemplified by exchanging the non-functional glycine for other natural amino acids[30] (**10–18**, Table 2), although the type of substituent and also the stereochemistry at the now created chiral centre have different effects on the PNA hybridization properties. Even cyclic substituents, as exemplified by the 'cyclohexyl substitution' at the amino ethyl linker (**8**, **9**) is possible provided the 'right' stereoisomer is chosen (Lagriffoule, Nielsen *et al.*, in prep.).

Naturally, the PNA analogues described to date have far from exhausted the imagination of chemists and more will undoubtedly be investigated, now that 'pure peptide chemistry' has been introduced successfully in the 'oligonucleotide analogue' field. More bold chemists may even do away with both sugar–phosphate and peptide backbones and come up with totally novel constructions.

6 Why is PNA a good DNA mimic?

One might ask: what features of the original PNA structure are responsible and required for its DNA mimicking properties and also what improvements might be possible? However, prior to engaging in this discussion, it is informative to make some thermodynamic considerations. Hybridization of complementary oligomers whether these being DNA, RNA or RNA is characterized by a large enthalpy gain and a significant entropy loss.[4,15] The decrease in entropy upon hybrid formation, naturally, is due to the formation of a highly ordered and fairly rigid duplex structure from two rather flexible and much less ordered single strands. (It should be kept in mind that an entropy gain which cannot compensate for the above loss is also accompanying hybrid formation due to release of ordered water molecules around the hydrophobic nucleobases). Therefore constraining the single stranded PNA (or other oligomer) in a conformation identical to or close to that found in the hybrid should greatly reduce the entropy loss and therefore increase the free energy upon binding. Thus restricting backbone flexibility, *e.g.* by introducing cyclic structures is an obvious strategy in the quest for oligomers of improved hybridization potency. This principle has been met with some success using bicyclic DNA analogues for triplex formation,[31] and the idea was also the rationale for making the cyclohexyl derivatives **7** and **8** of PNA (Lagriffoule, Nielsen *et al.*) (Table 1). Disappointingly, neither the (*SS*)- nor the (*RR*)-isomer conferred improved hybridization although the (*SS*)-isomer had no serious adverse effect. Very interestingly, however, a thermodynamic analysis revealed that when compared to a normal PNA, a 10-mer PNA containing three (*SS*)-cyclohexyl units only showed an entropy loss ($-\Delta S$) of 280 J mol^{-1} K^{-1} (*versus* 375 J mol^{-1} K^{-1} for the normal PNA), whereas the enthalpy gain ($-\Delta H$) was 127 kJ mol^{-1} (*versus* 153 kJ mol^{-1}). Therefore, one may conclude that the

Table 1 Effects on thermal stability (ΔT_m/°C) per modified unit for structurally modified PNA T-monomers when incorporated into the oligomer sequence H–GTA GA*T* CAC T–NH_2

Entry	Structure	Backbone/linker	ΔT_m DNA/ °C	ΔT_m RNA/ °C	Ref.
1		Ethylglycine	0	0	
2		Propylglycine	−8.0	−6.5	27
3		Ethyl-β-alanine	−10	−7.5	27
4		Propionyl linker	−20	−16	27
5		Ethyl linker	−22	−18	28
6		Retro-inverso	−6.5	nd	29
7		(*S,S*)-Cyclohexyl	−0.7	−0.5	Submitted
8		(*R,R*)-Cyclohexyl	−8	−7	Submitted
9		L-Ornithine	−14	−8	34, 35

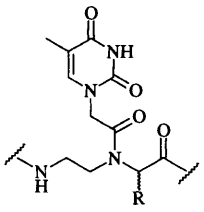
structural constraints most likely has had the desired effect of producing a more ordered single strand state, but, unfortunately, not in the optimal conformation(s) for DNA (or RNA) hybridization.

Although thermodynamic data are not yet available for other PNA derivatives, it is obvious that in addition to any adverse steric or structural constraints imposed by the changes of derivatives **1**–**5**, these will all result in more flexible molecules. This is especially illustrative for derivative **5**, in which all distances are retained, but one of the amide bonds has been reduced.

Therefore apart from having the proper 'intra-backbone' distances (6 + 3), we believe that the constrained flexibility imposed by the two amide functions in the PNA backbone is crucial. However, the poor DNA mimicking properties of the 'retro-inverso' PNA (**6**) which is a true isomer of the original aminoethylglycine PNA obeying both the '6 + 3-rule' as well as having the same number of constraining amide bonds, shows that more subtle factors such as dipole–dipole interactions and changes in hydration patterns that we do not fully understand also make significant contributions. On the other hand, using the 'PNA system'—due to the synthetic accessibility of a wide

variety of analogues—could help us further unravel the general principles for structure–activity relationships at the molecular level as well as improve our ability to translate chemical structures into three-dimensional structures.

Table 2 Effects on thermal stability per monomer (ΔT_m/°C) for the PNA sequence H–*G*T*A* G*A*T CAC *T*–NH_2[a] incorporating three chiral monomers as compared to an unmodified PNA[30]



Entry	R	Chirality	ΔT_m DNA/°C	ΔT_m RNA/°C
10	CH_3	L	−1.8	nd
11	CH_3	D	−0.7	nd
12	Bu[s]	L	−2.6	−3.0
13	CH_2OH	L	−1.0	−1.0
14	CH_2OH	D	−0.6	−1.0
15	CH_2CO_2H	L	−3.3	nd
16	$CH_2CH_2CO_2H$	D	−2.3	nd
17	$(CH_2)_4NH_2$	L	−1.0	−1.3
18	$(CH_2)_4NH_2$	D	+1.0	0

7 Outlook

The results obtained with PNA have bearing on many areas of chemistry and biology ranging from basal molecular recognition, self-assembly and chiral induction aspects[14] over molecular biology tools and gene therapeutic drugs to our understanding of the structure and function of Nature's genetic material, DNA, and its possible prebiotic predecessors and origin.[32] Even some novel materials have their origin in PNA.[33] Therefore, PNA should not be viewed only as a DNA mimic, but as a structural and self-recognizing system in its own right, and we foresee that the properties of PNA and related compounds will prove of increasing interest and utility in both the traditional 'oligonucleotide field' as well as in other areas of science—including ones which at this stage are not imagined.

8 Acknowledgements

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