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Review Article

DNA Analogues: From Supramolecular Principles to Biological Properties

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Abstract—Mainly driven by the needs of antisense research, a large number of oligonucleotide analogues have been prepared and evaluated over the last 15 years. Besides minor structural modifications of the building blocks of DNA and RNA itself, a considerable effort has been devoted to the de novo design of nucleoside analogues with improved binding properties. A paticularly successful concept turned out to be that of conformational restriction. This review focuses on recent advances in this area and tries to summarize scope and limitations of this design principle. © 2002 Elsevier Science Ltd. All rights reserved.

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Introduction

The discovery of the oligonucleotide directed inhibition of gene expression on the level of translation by Zamecnik and Stephenson[1,2] more than two decades ago set the basis of what is called the antisense approach in human therapy. Conceptually, the approach is based on an oligoribo- or deoxyribonucleotide (Fig. 1) or an analogue thereof, that is complementary (antisense) in sequence to a mRNA of interest. Watson–Crick duplex formation with the target thus inhibits its translation into a protein (Fig. 2). Inhibition has been shown to occur by a number of different mechanisms, depending on which section of a mature mRNA, or a non-spliced pre-mRNA is chosen as the target. The two most commonly known inhibition mechanisms rely on direct translational arrest effected by the antisense oligonucleotide, or on the recruitment of ubiquitous cellular enzymes as, for example RNaseH or RNaseL, that rapidly degrade the mRNA at the site of complexation. Clearly, the advantage of this concept over traditional small molecule protein targeting in drug design, lies in the fact that ideally only minimal structural knowledge about the target RNA, namely its base sequence, is required. Its specificity to one unique target sequence can be triggered by the number of nucleotide residues in the chain, ideally leading to a statistically unique base sequence within the complete sequence space of the genome. The universal nature of antisense technology not only makes it a key instrument for tomorrows therapies, but also a powerful tool in molecular biology for the manipulation of gene expression and the determination of protein function.

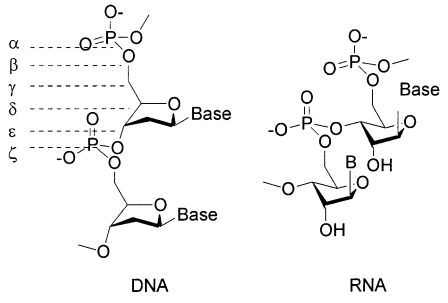

Figure 1. The chemical structures of DNA and RNA represented in their intrinsically preferred conformation.

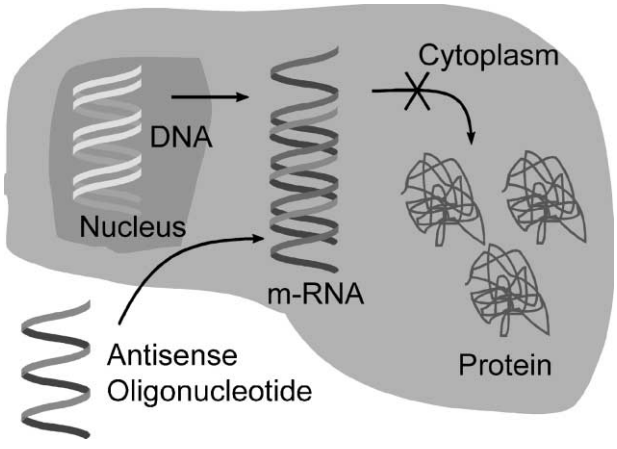

Figure 2. Schematic representation of the antisense priciple.

Key features to be met by antisense oligonucleotides are:

- high affinity and high specificity of an oligonucleotide to its mRNA target,
- rapid crossing of cellular and nuclear membranes (bioavailability),
- increased stability of oligonucleotide analogues in a cellular environment (biostability),
- minimization of toxicity, immunogenicity and non-antisense activities arising from unspecific binding to elements of the cellular matrix,
- feasibility of mass production at a realistic price.

A major effort over the last decade towards the search of oligonucleotide analogues meeting the mentioned criteria by various industrial and academic institutions has pushed forward the limits in related synthetic methodology and chemical development, as well as in the understanding of the physiological action of oligonucleotide analogues in vivo. A drug acting against cytomegalovirus (CMV) induced retinitis (Vitravene^{TM}), based on a phosphorothioate oligonucleotide was recently admitted to market and represents a first witness of the feasibility of the therapeutic antisense concept. Today, about one dozen of new antisense oligonucleotides are in various stages of clinical trials. These are still first generation antisense oligonucleotides, typically consisting of RNaseH activating phosphorothioate DNA, or second generation antisense oligonucleotides,[3] consisting of 2′*O*-modified ribooligonucleotides, or 'gapmers' composed of 2′*O*-modified RNA nucleotides at either end of the chain, and a core of unmodified or phosphorothioate DNA, responsible for RNaseH activation.

In order to increase RNA affinity, oligonucleotides with modifications in the phosphodiester backbone unit, in the sugar unit and, to a limited extent, in the base units have been prepared. From the estimated more than 1000 modifications reported to date (the ISIS/Novartis group itself has studied more than 200 modifications by 1997[4]), only a very small fraction shows a sequence-independent increase in affinity to DNA or RNA targets. This fact led to considerable frustration in the field and to consolidated research activities, especially on the industrial level. It fomidably highlights also, how poor current state of the art is in predicting the effect of structural changes in a monomeric nucleotide unit on its properties in a oligomeric context. This is the point where the medicinal chemistry aspect of antisense research meets basic questions in supramolecular chemistry. An analogy to the more complicated protein folding problem seems appropriate at this point, as it shares the same (mainly unsolved) questions on how the monomeric aminoacids as constituents of a peptide chain determine its secondary and tertiary structure.

One way to learn about the intrinsic structural and functional properties of DNA and RNA, and thus

about the interrelation between monomeric structure and oligomeric properties, consists in the preparation, analysis and comparison with each other, of well defined structural alternatives. This procedure stood at the outset of Eschenmoser's work on hexopyranosyl–, pentopyranosyl–, and tetrofuranosyl–NA in the context of defining a chemical etiology of nucleic acid structure.[5,6] Although these studies were completely driven by the question why nature selected the particular structures DNA and RNA as the molecules of life, the results of these investigations have dramatically influenced the design of new carbohydrate modified DNA analogues for antisense purposes. More specifically, the concept of conformational restriction of nucleosides as a measure for preorganizing oligonucleotide single strands for duplex formation[7,8] is a direct consequence of the studies on homo–DNA and resulted in novel DNA analogues, as tricyclo–DNA,[9] HNA,[10] or LNA[11,12] with the most promising RNA binding efficiencies known today.

This review focuses on a selection of carbohydrate modified, phosphodiester-based DNA analogues for which enough data is available to describe their differential binding properties to DNA and RNA in a depth that allows the establishment of more general structure/activity relationships.† Special emphasis is given to some DNA analogues originating from the author's laboratory. Neither the biological and clinical advances of antisense technology in general, nor aspects of base- or phosphate-modified DNA analogues, or analogues arising from the modification of the 2′-position of the natural nucleosides are covered here. For previous state of the art reviews on antisense technology,[13,14] aspects of delivery,[15] the medicinal chemistry of antisense oligonucleotides,[3,4] antisense mechanisms,[16,17] heterocyclic base-modifications,[18] conformational restriction,[19–21] and important modifications as 2′-carbohydrate-modified DNA,[22] N3′–P5′ phosphoramidate DNA,[23] morpholino–DNA[24] and locked nucleic acid (LNA),[11,12] the reader is referred to the indicated references.

Oligonucleotides Derived from Hexoses and Pentoses

The hexopyranosyl–NA family

Within the family of the β-D-configured hexopyranosyl oligonucleotide analogues the four members depicted in Figure 3 have been investigated in detail by Eschenmoser et al. as an experimental entry towards understanding the evolutionary processes that lead to the selection of RNA and DNA as today's genetic material.[5,6]

Homo–DNA was the first to be synthesized[25] and its pairing behavior to be analyzed.[26] Homo–DNA, representing a deoxygenated model system within the hexopyranosly–NA family, shows much stronger Watson–Crick base-pairing than natural DNA. Besides this, a pronounced propensity for adenine and guanine self-pairing in the reversed Hoogsteen mode (for a structural representation of the relevant modes of association of bases see Fig. 9) is observed.[26,27] The base-pairing of homo–DNA is orthogonal to that of the natural system. There is no formation of duplexes with DNA and RNA observable. The high thermodynamic stability of homo–DNA duplexes was determined to be entropical in nature and was ascribed to the higher rigidity of the pyranose ring as compared to the furanose rings, resulting in a higher degree of preorganization of the single strands towards duplex formation. Structural analysis of a Watson–Crick homo–DNA duplex by NMR[28] showed it to adopt an almost linear (non-helical) structure with nearly ideal staggered conformations around the six torsion angles α–ζ‡ of the repeating phosphodiester backbone unit.

The fully hydroxylated counterparts of the 4′→6′ linked pyranosyl–NAs namely the gluco–, allo– and altropyranosyl–NAs, in contrast, show moderate to nonexistant Watson–Crick base pairing. Adenine–uracil pairing is virtually nonexistant, and guanine–cytosine pairing is much weaker than in RNA. Weak purine–purine self-pairing in the reverse-Hoogsteen mode occurs in the allo- and altropyranosyl series, but not in the glucopyranosyl series. Moreover, a strong sequence dependence with regard to pairing mode is observed. The failure of efficient duplex formation was interpreted as the consequence of intrastrand steric hindrance in the pairing conformation of the bulkier hydroxylated systems. Models based on the NMR structure of homo–DNA indicated that the insertion of an equatorially oriented hydroxy group in the 2′-position of the pyranose unit results in a steric clash between this hydroxy group and the neighboring nucleobase.

The 2′→4′ linked pentopyranosyl family

In continuation on the search for potential functional alternatives to RNA, the base-pairing properties of a series of 2′→4′ linked pentopyranosyl nucleic acids were studied by the Eschenmoser group (Fig. 4). The best investigated system in this family is the pyranosyl–RNA (pRNA) analogue of natural RNA. As predicted by conformational analysis[29] pRNA is a Watson–Crick base-pairing system that forms duplexes with a quasi-linear structure.[30] In contrast to the hexopyranosyl series, no other pairing mode as, for example the self-pairing of guanine-rich sequences in the Hoogsteen or reverse-Hoogsteen mode, was observed. Duplex formation occurs strictly in the antiparallel mode and exceeds that of natural RNA by ca. 2–4 °C in T_m per base-pair.§

†The intriguing chemical and biological properties of the charge neutral polyamide nucleic acid analogue PNA are not subject of this review. For a comprehensive discussion on the properties of PNA, the reader is referred to the following reviews.[109–111]

‡The six torsional angles describing the backbone geometry of the repetitive unit in DNA and RNA are outlined in Figure 1. The same nomeclature is used, in analogy, for describing the structural properties of DNA analogues. For a detailed discussion on terms and conventions describing nucleic acid structure, see ref 106.

§The melting temperature (T_m) is defined as the temperature at which two complementary oligonucleotides occur to 50% as single strands and as duplex. Typically these data are extracted from UV–melting curves as the maximum of the first derivative. Although T_m data do not reflect necessarily thermodynamic stability, they are a reliable and widely used measure for thermal stability of duplexes.

A NMR structure of a self-complementary octamer pRNA duplex shows it to adopt a ladder structure with a weak left-handed twist and a large inclination of the backbone relative to the base-pair axes. As a consequence, base stacking is overwhelmingly interstrand as opposed to intrastrand[31] and correlates well with observed sequence dependent differences in thermal stability of pRNA.[32] It has been shown that pRNA, as also natural RNA, is capable of secondary structure formation as, for example hairpin loops.[33] Efficient Watson–Crick duplex formation is not only restricted to the pRNA system but extends to all pentopyranosyl–NAs depicted in Figure 4.[34] In terms of thermal stability the arabinopyranosyl isomer ranks highest with increases in T_m per base-pair relative to natural RNA as high as 7 °C. All members of the family can efficiently crosspair between each other as was demonstrated in the case of four different base sequences 8–12 nucleotides in length.[35] This demonstrates a remarkable capacity of their diastereomeric backbones to adopt a common type of pairing conformation. It occurs though that systems with similar attachment of the phosphodiester bridges (ribo and xylo, lyxo and arabino) crosspair more efficiently. However, none of the members of the 2′→4′-linked pentopyranosyl–NAs show crosspairing with RNA.

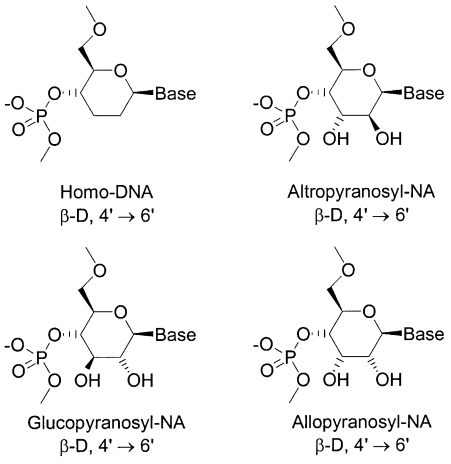

Figure 3. Members of the 4′→6′ linked hexopyranosyl–NA family.

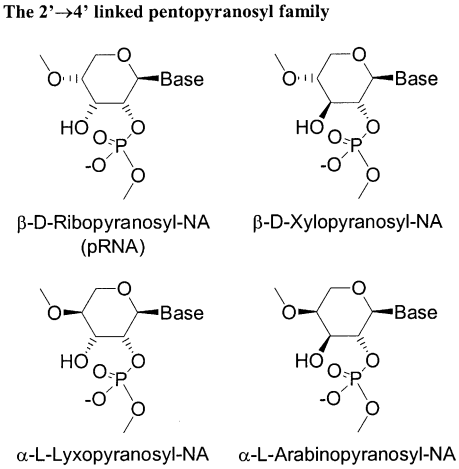

Figure 4. Chemical structures of 2′→4′ linked pentopyranosyl–NAs.

Pentopyranosyl– and threofuranosyl–NAs with vicinal linkage

The latest systems investigated by the Eschenmoser group are depicted in Figure 5. While all of the members of the hexo- and pentopyranosyl families discussed so far show six covalent bonds per repetitive backbone unit, as the natural nucleic acids, the three oligonucleotide analogues depicted in Figure 4 only have five such bonds. This requires that the phosphodiester groups are arranged adjacent to each other on the sugar units and are aligned in a way as to structurally compensate for the missing bond. While the 3′→4′ ribopyranosyl-series, having the vicinal phosphodiester groups arranged in an 3′-axial 4′-equatorial position, does not produce a competent oligonucleotidic pairing system, the 3′→4′-lyxopyranosyl oligonucleotides do self-pair in the form of Watson–Crick duplexes, as well as triplexes in the case of homoadenine/homothymidine strands.[36] The thermal stabilities of these duplexes are typically weaker and more sequence dependent than that of the 2′→4′ series. Importantly this system is capable of crosspairing with natural DNA and RNA. In a mixed base context, T_m values for hybrid duplexes are only moderately inferior to those of the pure DNA or RNA duplexes of the same sequence. These findings lead subsequently to the synthesis and evaluation of TNA (Fig. 5), in which the diaxiality of the phosphodiester substituents is maintained, while the ring structure has contracted from pyranose to furanose. TNA is an extraordinary oligonucleotidic system, in that it does not only self-pair into antiparallel duplexes of the Watson–Crick type with stabilities similar to RNA, but in that it also efficiently crosspairs with DNA and RNA.[37] Duplexes of TNA with RNA are of the same order of stability as RNA duplexes, with the exception of homobasic sequences. In the latter cases, there is a strong bias for oligopurine strands carrying the TNA backbone to be distincly more stable than pyrimidine strands with a TNA backbone. In contrast to the members of the pentopyranosyl series, TNA is, as expected, chemically much more stable towards hydrolytic cleavage due to the missing internal nucleophile in form of an adjacent hydroxy function. The intriguing base-pairing properties of TNA

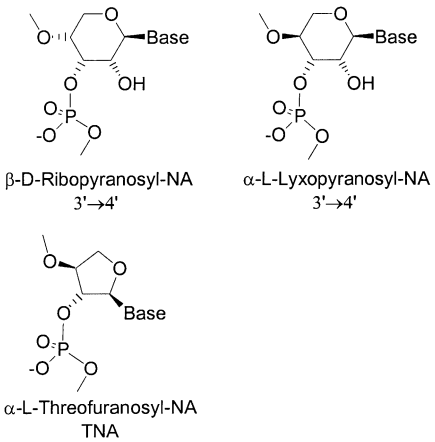

Figure 5. Chemical structures of 3′→4′-linked pentopyranosyl–NAs and of a tetrofuranosyl–NA.

with RNA not only opens new dimensions to its eventual role as an intermediate in the molecular evolution of life, but makes it also a relevant system to look at more closely in the antisense branch of medicinal chemistry.

Conformationally Restricted Oligonucleotide Analogues

The members of the family of conformationally restricted oligonucleotides that were designed to target single stranded DNA and RNA with the background of antisense applications has kept rapidly growing since its introduction in 1993.[7,8] The field has already been reviewed in the past.[19–21] The following section therefore focuses on selected families of structures with extraordinary base-pairing properties, rather than on a full account of the whole field.

The hexitol nucleic acid family

Driven by the needs of antisense technology the Herdewijn group has developed a series of hexitol nucleic acid analogues (Fig. 6). The most prominent representative of this class of conformationally constrained oligonucleotide analogues is the 1,5-anhydrohexitol-NA (HNA).[10,38] HNA can be regarded as a close congener to homo–DNA with the only difference, that the base has moved from the 1′ to the 2′ position. This seemingly minor change has a large effect on the base-pairing properties of this system. Not only does HNA form duplexes in its own series, but it also crosspairs with natural DNA and RNA.

Typically, HNA/DNA duplexes show increased thermal stability (ΔT_m/mod = +1.3 °C)[‖] with DNA as complement and by (ΔT_m/mod = +3 °C) with RNA as complement. HNA sequences clearly discriminate between matched and mismatched sequences. A recent NMR structural analysis of a HNA–RNA hybrid duplex clearly shows it to form a helical structure with a conformation that belongs to the A family.[39] The pyranose units occur in a chair conformation with the nucleobases in an axial position to minimize 1,3-diaxial strain. The two remaining groups are in equatorial positions. The axial orientation of the base is necessary to achieve efficient base stacking in the double helix. The minor groove dimension (P–P distance) was found to be similar to that of a pure RNA duplex which might explain the fact that HNA/RNA duplexes are resistant to RNase H. The biological activity of HNA was evaluated in an in vitro translation arrest system targeting the oncogene H-ras mRNA and in a cellular system targeting intracellular adhesion molecule-1 (ICAM-1) expression. HNA selectively inhibited H-ras mRNA translation [IC(50) of 50 nM] when targeted at the translation initiation region. Similarly, HNA inhibited intracellular adhesion molecule-1 (ICAM-1) expression in keratinocytes when targeting at codon sequences. However, a gap-mer approach was needed to inhibit mRNA translation. In this test system, HNA is less active but more selective than phosphorothioates. For HNA to be efficiently internalized into cells the cationic lipid lipofectin had to be used concomittantly.[40]

The two hyroxylated versions of HNA, namely ANA and MNA (Fig. 6) have also been prepared and their base-pairing properties with DNA and RNA studied. Complexes between ANA and RNA or DNA are reported to be more stable than between HNA and natural oligonucleotides. Interestingly with the introduction of the axially oriented hydroxy group, affinity for DNA increases more than for RNA compared to HNA.[41] A fully modified short sequence (hexamer) displays higher ΔT_m/mod (ca. +7 °C) compared to a longer sequence (dodecamer) with ΔT_m/mod of ca. +3.5 °C with RNA as a complement (interestingly, the effect of chain length on thermal stability of modified duplexes has never been looked at in detail for any conformationally constrained system). The diastereoisomer MNA displays considerably less affinity to RNA as compared to ANA or HNA. Molecular modelling suggested the reason for the reduced RNA affinity to lie in intrastrand interresidue H-bond formation between the equatorial 3′*O*-hydroxy function and the 6′*O* of the phosphodiester unit of the following nucleotide unit, tying the MNA single strand into a non-pairing conformation. A comparison to the hexopyranosyl-NAs discussed earlier (Fig. 3) is striking, as also in this case the presence or absence, or the configuration of a hydroxy group at position 2′ makes a big difference in pairing efficiency, although for a completely different (steric) reason.

The carba analogue of HNA namely the cyclohexanyl–NA (CNA) is another example demonstrating how seemingly minor changes can have a profound impact on the association behavior. CNA which was prepared in

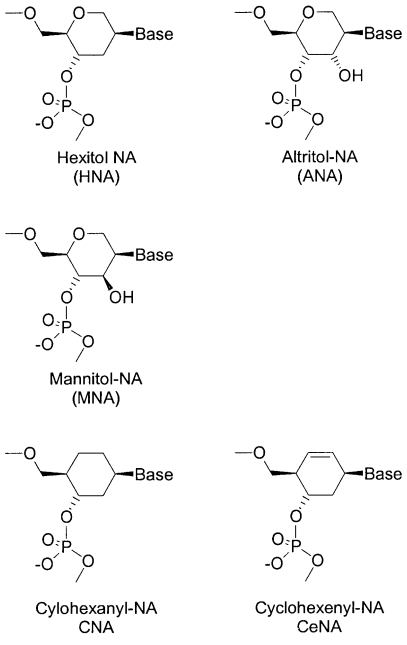

Figure 6. The family of the hexitol–NAs.

[‖] ΔT_m/mod data, as used throughout this text, refer to an (averaged) increase or decrease in duplex T_m originating from the replacement of one deoxyribonucleotide unit by a modified nucleotide unit within a DNA strand.

both enantiomeric (D/L) forms does form duplexes of the Watson–Crick type with itself under the premise that the backbones on both strands are homochiral.[42] D-CNA hybridizes preferentially with complementary RNA (as compared to DNA), however with reduced affinity compared to HNA or ANA. An explanation for the reduced affinity to RNA and DNA could be that in CNA the bases have a higher propensity to be equatorially aligned, due to higher 1,3-diaxial strain of a C–H bond relative to a (oxygen) lone-pair in the chair conformation with the axial base. Such a conformation is not compatible with duplex formation with RNA or DNA.

The most recently introduced member of the HNA family, is the cyclohexenyl–NA (CeNA) (Fig. 6).[43] CeNA corresponds to a dehydrogenated analogue of CNA. Complementary recognition of RNA by the CeNA modification is more efficient as pure DNA (average ΔT_m/mod = +1.2 °C) and about half as efficient as the HNA modification (average ΔT_m/mod +2.7 °C) within the sequence context evaluated. A NMR investigation of a self-complementary duplex containing one CeNA unit suggests that the cyclohexenyl ring can more easily conformationally adapt when incorporated into DNA, compared to a CNA unit. A striking feature of CeNA is that it activates RNase H within a CeNA/RNA duplex. It is thus the only member of the whole family which displays this activity. All the results obtained so far are based on oligonucleotides containing CeNA units with the adenine base only. Validation of the observed effects with partly and fully modified CeNAs containing all four nucleobases still needs to come.

The LNA family

β-D-ribo-LNA (LNA). The first member of the family of locked nucleic acids (Fig. 7), the β-D-ribo-LNA, was introduced in 1998 by the Imanishi[44] and the Wengel[44,45] group.

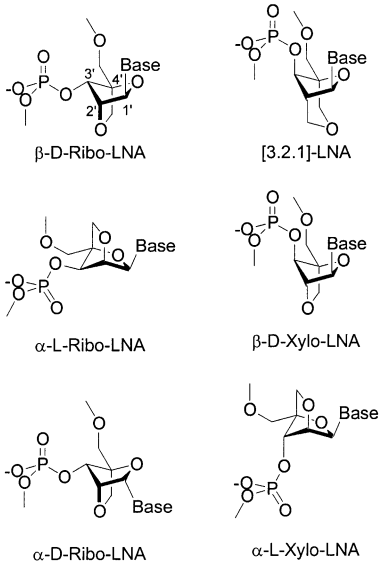

Figure 7. The locked nucleic acid (LNA) family.

Structurally, this modification corresponds to a ribonucleoside in which the 2′-oxygen and the 4′-carbon atoms are linked via a methylene unit. This modification locks the ribofuranose subunit into the 3′-endo conformation which is responsible for the A conformation of a nucleic acid double helix. β-D-ribo-LNA is the system which forms the strongest duplexes with complementary RNA known to date. This has been demonstrated in a series of recent original papers[44–48] and reviews,[11,21] and is certainly the reason why LNA–oligonucleotides are already commercially available (Proligo, Boulder, CO, USA).

Single and multiple LNA substitutions within a mixed-sequence DNA oligonucleotide stabilize duplex formation with a complementary RNA typically by ΔT_m/mod = 4–8 °C. depending on the sequence and the number of LNA units used. Amazingly, pure LNA duplexes reach thermal stabilities that exceed 90 °C already at the level of nonamers.[46] This is certainly the record for any phosphodiester-based oligonucleotide analogue reported so far. Single substitutions within a DNA oligonucleotide stabilize complementary duplex formation with DNA or RNA slightly more on a per modification basis than multiple substitutions suggesting that an energetically favorable cooperative change of conformation extends to the nearest neighbours of the LNA unit in the chain.[48] A single crystal X-ray investigation on a self-complementary DNA decamer duplex containing one LNA–thymine (T^L) residue in the center of the sequence (resulting in two consecutive T^L–A and A–T^L base-pairs, resp.) shows it to adopt an A-form structure.[49] No significant changes concerning the backbone torsion angles α–ζ and the torsion angle around the glycosidic bond χ is observed for the LNA units, compared to a natural nucleotide unit in a A-form duplex, demonstrating that the bicyclic sugar moieties fit seamlessly into an A-form double helix.

These results are consistent with those obtained from NMR solution studies of DNA and RNA duplexes with either single or multiple LNA residues in one of the strands.[50–53] Regardless of the type of complement (RNA or DNA) the LNA containing DNA-strand shows the LNA units locked in the 3′-endo conformation (as expected) while the remaining deoxynucleotides are in a dynamic interchange between 3′-endo and 2′-endo conformation. The LNA units exert a cooperative effect on the strand conformation. In the case of a decamer duplex with RNA, 3 LNA units suffice to drive the strand conformation completely to the A form, underlining the power of a LNA unit in reorganizing the conformational states of its nearest neighbor nucleotides. According to these structural analyses, the exceptional thermodynamic stability seen with LNA-modified duplexes (both in the DNA and RNA context) are interpreted as a result of the conformational preorganization of modified single strands for the duplex state (entropic contribution) as well as of the improved stacking both in single- and double-stranded states (enthalpic contribution). To date, a structural analysis of a duplex with a completely modified LNA strand, either with RNA or with itself, is still missing.

There is one caveat to be added concerning the binding properties of fully modified LNA. It has been noted by Wengel and coworkers that a 15-mer all-LNA mixed-base sequence oligonucleotide shows considerable self-structuring with high thermal stability. This could be one limitation in the applications of fully modified LNA to target RNA and might explain results by the Imanishi group on the use of LNA for triple-helix formation, in which strong enhancements of binding affinity to a homopurine/homopyrimidine duplex target in the parallel binding motif is observed for partially modified homopyrimidine third strands, but where fully modified third strands fail to bind to their target at all.[54]

Due to the fact that only a few LNA residues are needed in order to obtain high RNA affinity the 'gapmer' strategy is a very promising way for antisense applications. It has been shown, that LNA/DNA copolymers are not degraded readily in blood serum and cell extracts, but were capable of activating RNase H.[55] Even fully modified LNA was reported to activate RNase H in complex with RNA, however with greatly reduced efficiency. In contrast to phosphorothioate oligonucleotides, LNA showed no toxic reactions in rat brain, and LNA/DNA copolymers were shown to exhibit potent antisense activity in vivo.[55]

As already shortly mentioned, DNA homopyrimidine oligonucleotides containing LNA units, designed to bind in the parallel triple helical binding motif to duplex DNA were investigated by the Imanishi group. They were able to demonstrate that single substitutions of thymine- or 5-methylcytosine–LNA units could increase thermal stability of a triplex by 7–13 °C relative to a third strand with a deoxyribose unit in the requisite position.[56] In systems with multiple substitutions, a limited additivity of the stabilizing effect with a certain positional dependence of the LNA units in the chain was found.[54,57] A thermodynamic analysis by isothermal titration calorimetry (ITC) revealed a 10–18-fold increase in binding constant at near-neutral pH, attributable uniquely to the sugar replacement, for third strand 15-mers containing 5 or 7 modifications. Furthermore a kinetic evaluation revealed the increase in association constant to be entirely due to a decrease in the rate of dissociation.[58] As already mentioned, a fully modified LNA 15-mer does not bind anymore to its target, possibly due to self-structure formation.

An important development in DNA triplex chemistry is the use of the LNA sugar in combination with new bases in order to overcome the homopurine/homopyrimidine sequence restriction, which is obstructing a sequence-generalized antigene approach (for a recent review describing the current status in antigene research see ref 59). First steps into this direction untilizing LNA residues with oxazole[60] or a pyridone residue[61] as a base-replacment for targeting C–G interruption sites within a otherwise intact purin/pyrimidine sequence context by only one hydrogen bond are very promising. In this context it might be relevant to note, that current knowledge points to third strand *affinity* and not necessarily *selectivity* to be the major hurdle in the sequence generalization of the triple helix approach.[62]

Analogues of LNA. Due to the unprecedented binding properties of LNA, extensive structure/affinity studies were initiated. Minor geometric changes in the sugar unit of LNA, for example, are represented by LNA anlogues where the 2′-bridging oxygen was replaced by sulfur or nitrogen.[63] Replacement of the 2′-oxygen by an amino or methylamino function still preserves the high affinity of correspondingly modified DNA oligomers.[64] Extension of the bridge between the 2′- and 4′-position by one carbon ([3.2.1]-LNA, Fig. 7) was experimentally realized in the context of the nucleobase thymine,[65] and was shown to lead to oligonucleotides with intermediate affinities relative to pure DNA or RNA, and LNA.[66] ΔT_m/mod values to RNA as a complement range between +1.9 and +3.3 °C/mod. Interestingly, replacement of the O2′ by a methylene group in this bridge-enlarged LNA analogue has been shown by the Novartis group to lead to a T_m depression by −2.3 °C/mod, pointing towards a significant role of hydration to duplex stability at this position.[4]

An analysis of the base-pairing properties to complementary RNA of all eight possible stereoisomeric LNAs, arising from changes in configuration at the centers 1′, 2′, 3′ and 4′ was achieved by the Wengel group[67] in an indirect way. The four diastereomeric LNAs (β-D-ribo-LNA, α-L-ribo-LNA, β-D-xylo-LNA, and α-L-xylo-LNA, Figure 7), containing nine consecutive thymidine residues each, were synthesized and the remaining four stereoisomers which happen all to be enantiomers, were evaluated indirectly by crosspairing to the enantiomeric RNA (L-RNA). From these studies it became clear that three members out of the LNA family, namely β-D-ribo, the α-L-ribo-LNA and the β-D-xylo-LNA show strong binding to the respective D-RNA target in a sequence selective manner, while the α-L-xylo-LNA is unable to hybridize with RNA at all. Interestingly the former three members all bind also with slightly reduced affinity to L-RNA, but only the α-L-ribo-LNA in a sequence specific manner. For the β-D-ribo- and α-L-ribo-LNA the similar binding efficiencies to RNA were rationalized by the geometric similarities of the monomeric units, descernible from the close spacial arrangement of the C5′, O3′ and the base residues in a superposition of the two monomeric structures. The base-pairing properties of the β-D-xylo-, and the α-L-ribo-LNA were further investigated in the context of chimaeric DNA/LNA sequences. While monomodifications of DNA by one β-D-xylo-LNA unit is strongly destabilizing,[68,69] one or more subsitutions by α-L-ribo-LNA units is neutral to slightly stabilizing relative to DNA and strongly stabilizing by 4–5 °C relative to RNA as a complement[68–70] corroborating the structural rationalization given above. Meanwhile, the α-D-ribo-LNA has also been experimentally realized in the context of the nucleobase thymine and shown to destabilize duplex formation with complementary DNA and RNA, when part of a α-DNA strand and showing neutral behavior when fully modified, compared to α-DNA.[71] When introduced with polarity inversion (3′-3′ and 5′,5′ junctions at the modification site) either in β-DNA or α-DNA the destabilizing effect towards DNA or RNA single strands is even more pronounced.[72]

A synopsis of the properties of LNA at this point teaches us that conformational fixation of a ribose unit into a 3′-endo conformation goes along with dramatically increased thermal stabilities of corresponding duplexes. The effect is so remarkable that it rises the question whether all of the observed increase in affinity is due to structural preorganization or whether there are further factors fortuitously enhancing the recognition process. Moreover the data available show, that α-ribo-LNA is an almost perfect structural and functional analogue to the parent β-ribo-LNA. By analogy, this suggests that α-configured L-RNA, might have similar properties than the natural β-configured D-RNA. Whether this is the case remains open at this point.

The bicyclo–DNA family

Design principles and structure of the mononucleosides. Intrigued by the thermochemical properties of homo–DNA[26] we started out in the early nineties to search for nucleoside structures that allow for the transfer of the concept of conformational restriction into the world of the natural nucleic acids. In our design of bicyclo–DNA (Fig. 8) we intended to include both backbone bonds (cf. torsion angle γ and δ) that lie within the carbon framework of a natural nucleoside in a ring structure. We reasoned that the furanosyl backbone system provides the geometric basis for adopting helicity (in contrast to linearity),[6] and that the effect of restricting the C4′–C5′ bond, which has minimal conformational restriction in the natural nucleosides, could contribute even more to the preorganization of a single strand for duplex formation than the C3′–C4′ bond.

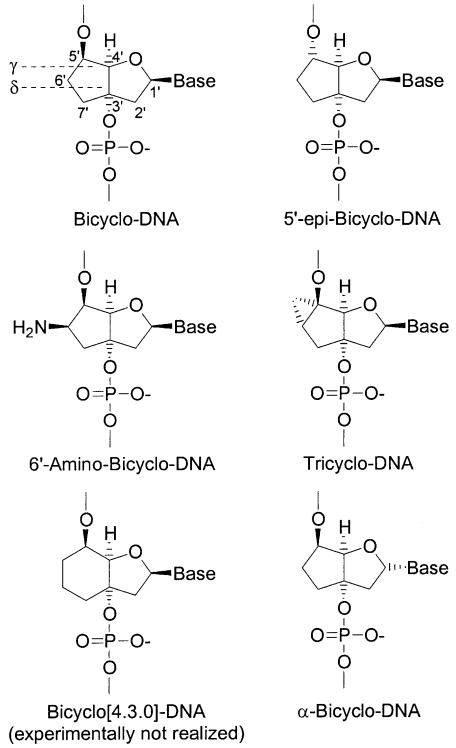

Figure 8. The bi- and tricyclo–DNA family.

Early molecular modeling of the nucleosides suggested that introduction of the carbocyclic ring into the [3.3.0] scaffold would drive the furanose ring into a 2′-endo/1′-exo conformation (*S*-type conformation) as observed in B-DNA.[7] This prediction was fully supported by structural analysis of the mononucleosides in the solid state and in solution by X-ray crystallography and variable temperature NMR, resp. The configuration at C5′ (*R*) was chosen as to match the geometry of torsion angle γ in DNA duplexes of the A- and B-type (synclinal, $+sc$) as close as possible. It was, however, clear from modeling and structural analysis of nucleoside monomers that the antiperiplanar (*ap*) arrangement of γ, orienting the hyroxy group in a pseudoequatorial position, is energetically preferred in bicyclo-nucleosides.[7] This was the price to be paid in order to include both γ and δ into a ring, and one reason why we did not experimentally realize the [4.3.0]-scaffold (Bicyclo[4.3.0]–DNA, Fig. 8) with a cyclohexyl ring, for which molecular modeling suggested an even larger energy difference between conformers having the C5′-OH function in equatorial versus axial arrangement.[7]

Homopurine/homopyrimidine sequences of bicyclo–DNA. The first sequences to be investigated were homodecamers of the bases adenine and thymine. These were prepared in high yield using the well established protocols of automated DNA synthesis from phosphoramidite building blocks.[8,73]

Adenine homodecamers with the bicyclo–DNA backbone showed equal to increased thermal and thermodynamic stability when complexed to DNA or RNA, resp. The opposite was the case for homo thymidine sequences with the bicyclo–DNA backbone. homoadenine sequences with the bicyclo–DNA backbone were able to form T–A–T triplexes with itself or DNA complements under corresponding stoichiometric ratios of strands. A thermochemical analysis via differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) revealed the higher thermodynamic stability to be entropic in origin, with the exception of the duplex involving the bicyclic thymidine and the natural deoxyadenosine strand. Moreover, the dependence of thermal stability on electrolyte concentration was enhanced for bicyclic relative to natural duplexes, and the CD spectra for duplexes containing bicyclo–adenine strands were distinctly different from that of the natural DNA duplex and that from A– or B–DNA duplexes in general. All these data suggested that bicyclo–DNA purine strands might prefer an association mode that is different from the Watson–Crick base-pairing mode (Fig. 9).[73]

Final proof for this came by introduction of a 1-deaza adenine base (Fig. 9) into the center of a bicyclic homoadenine decamer.[74] Such a modified duplex displays almost fully restored pairing behavior with regard to the non-modified bicyclo–DNA duplex, which ruled out Watson–Crick pairing to be operative in this system. In order to prove whether Hoogsteen or reversed Hoogsteen base-pairing occurs, an asymmetric purine nonamer containing adenine and guanine bases was

prepared and complexed to its parallel or antiparallel bicyclo–DNA complement. Indeed in the parallel arrangement, strong, pH dependent duplexation occurred due to the need of protonation of the cytosine base at N3, indicating Hoogsteen duplex formation. With the antiparallel pyrimidine strand, duplex formation was pH-dependent below pH 7.0 and pH independent above pH 7.0 indicative for reversed Hoogsteen duplex formation at low and Watson–Crick duplex formation at high pH.[74]

All these data taken together firmly allow the conclusion that within the homopurine sequence context bicyclo–DNA prefers the Hoogsteen and reverse-Hoogsteen over the Watson–Crick base-pairing mode. Structurally this change in association mode is solely triggered by the change of torsion angle γ from a $+sc$ to an *ap* arrangement. In this context it is worth noting that homopurine/homopyrimidine tracts in natural DNA tend also to change their preferred association mode from Watson–Crick to Hoogsteen in the case where triple-helix specific intercalators are inserted in between the base-pairs.[75] Although the structural adaption of the backbone conformation to admit for intercalation is not known in detail in this case, it would not be surprising if it is based on a change of torsion angle γ as in the case of bicyclo–DNA.

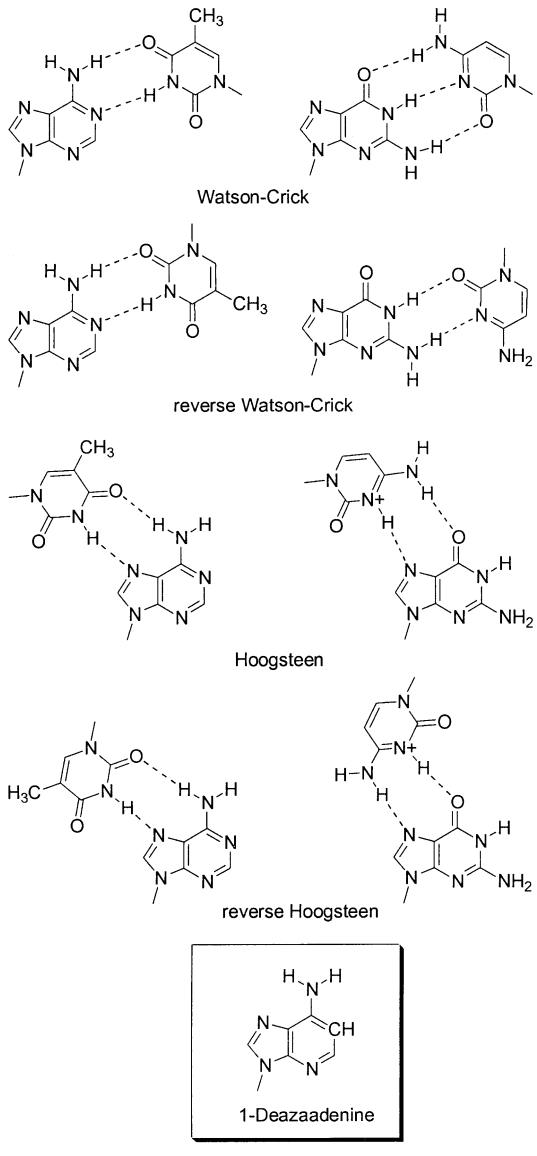

Figure 9. Structures and denomination of A–T (left) and G–C (right) base-pairs, relevant for the discussion, as well as of 1-deazaadenine.

Watson–Crick base-pairing behavior and enzymatic stability

DNA oligonucleotides with mixed-base sequences, for structural reasons, can not form Hoogsteen duplexes unless changes in the glycosidic bond from the anti- to the syn-conformation are admitted. We had shown previously in the case of the monomers by NMR–NOE spectroscopy that this is unlikely to occur in bicyclo–DNA. An investigation of a series of bicyclo–DNA sequences revealed that Watson–Crick duplex formation in its own series as well as with complementary DNA and RNA readily occurs.[76] The thermal stabilities of heteroduplexes with DNA and RNA are in the same order of that of DNA duplexes. Base pairing is sequence- and orientation-specific. The formation of left handed DNA (Z-DNA) in C-G-alternating sequences is not permitted by the bicyclo–DNA backbone. Bicyclo–DNA is more stable against 3′-exonucleases. A relative enhancement in stability of a factor of 10 against the enzyme snake venom phosphodiesterase, and a factor of 8 when incubated in fetal calf serum was measured.

5′-Epi-bicyclo–DNA: profiling torsion angle γ. In order to complete the structure/affinity analysis around the C4′–C5′-bond the bicyclonucleosides with inverted configuration at C5′ were prepared and incorporated into DNA.[77,78] This inversion brings about a shift of torsion angle γ to the $-sc$ range as determined by NMR on the mononucleosides containing the bases adenine and thymine. The strong decrease in affinity towards complementary DNA of partially modified oligonucleotides, and the failure of a fully modified homothymidine decamer to form a duplex with its DNA complement shows the incompetency of this local structural arrangement for right-handed, helical Watson–Crick duplex formation.

Taken all together, the general structure/activity profile for torsion angle γ, outlined in Figure 10, emerges in which the $+sc$ alignment in oligonucleotides leads to preferential Watson–Crick duplex formation, the *ap* alignment to preferential Hoogsteen or reverse Hoogsteen duplex formation, and the $-sc$ alignment is not compatible with a pairing conformation.

α-bicyclo–DNA. α-DNA strands, built from α-anomeric nucleosides, are known to form parallel stranded duplexes with natural (β)-DNA.[79–81] Duplex formation is base-selective and thermal stabilities are roughly equal to that of natural DNA duplexes. This prompted us to investigate the properties of α-bicyclo–DNA (Fig. 8). A conformational analysis of the α-bicyclodeoxynucleosides based on X-ray and ^{1}H NMR analysis revealed a rigid sugar structure with the furanose units in the 1′-exo/2′-endo conformation and the secondary

hydroxyl groups on the carbocyclic ring again in the pseudoequatorial orientation. Oligonucleotides consisting of α-bicyclothymidine and α-bicyclodeoxyadenosine were successfully synthesized from the corresponding nucleosides by phosphoramidite methodology on a DNA synthesizer. An evaluation of their pairing properties with complementary natural RNA and DNA by means of UV–melting curves showed that homodecamers of each base efficiently form complexes with complementary DNA and RNA.[82,83] The stability of these hybrids is comparable or slightly lower to those in the natural series. The strand orientation in α-bicyclo–DNA/β-DNA duplexes is parallel as was deduced from UV-melting curves of decamers with non-symmetric base sequences. Significant structural differences between α-bicyclo–DNA/β-DNA duplexes compared to α-DNA/β-DNA duplexes were observed by CD-spectroscopy, however not further investigated. α-bicyclo–DNA is ca. 100-fold more resistant to the enzyme snake venom phosphodiesterase with respect to β-DNA and about equally resistant as α-DNA itself.

Modification of the carbocyclic ring in bicyclo–DNA: 6′amino-bicyclo–DNA

The bicyclo–DNA skeleton lends itself for the introduction of functional groups in the carbocyclic ring. This is of interest in order to confer chemically reactive or reporter groups into any desired position within an oligonucleotide duplex. Although both the C6′ and C7′ positions are structurally equally suited for modification, C6′ functionalization was easier from the synthetic point of view. In a case study we prepared and analyzed the properties of 6′-amino- and amido-bicyclo–DNA containing the base thymine.[84] Pairing properties with complementary DNA, as determined by UV–melting curves, of singly or alternatingly amino-modified oligonucleotides (that are partly zwitterionic in nature) showed duplex formation capabilities equal to DNA. Duplex destabilization occurs however, if the amino functions are replaced by the charge neutral acetamido group, most probably due to loss of the positive charge and steric interference of the acetamido substituent with the backbone P–O(5′) bond. In neither case was it possible to obtain fully modified oligonucleotides due to failure of efficient synthetic coupling of two consecutive

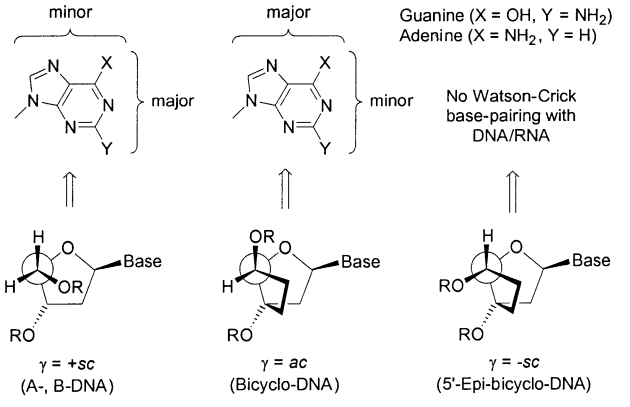

Figure 10. Structure–activity profile of purine oligonucleotides as a function of the relative orientation of torsion angle γ.

modified aminobicyclo–DNA building blocks. However, typical applications in molecular biology, taking advantage of this functional handle, do not need complete modification of oligonucleotides.

Tricyclo–DNA. The last member of the family discussed here, namly tricyclo–DNA, is a descendent of bicyclo–DNA (Fig. 8). Originally, the aim of the introduction of the cyclopropane ring in tricyclo–DNA was to further restrict conformational flexibility of the backbone and to correct at least in part for the altered geometry of backbone torsion angle γ. Taking advantage of intermediates along the synthetic pathway of bicyclo–DNA, tricyclo-nucleosides of the bases adenine and thymine became readily available,[85] were converted into oligonucleotides, and their complementary duplex formation investigated.[9,86] 5′-End phosphorylated tricyclo–DNA sequences are chemically stable in aqueous, pH-neutral media at temperatures from 0 to 90 °C. Tricyclo–DNA sequences resist enzymatic hydrolysis by the 3′-exonuclease snake venom phosphodiesterase. Homobasic adenine- and thymine-containing tricyclo–DNA octa- and nonamers are extraordinarily stable A–T base-pairing systems, not only in their own series but also with complementary DNA and RNA. Base mismatch formation is strongly destabilized. As in bicyclo–DNA, the tricyclo–DNA homoadenine sequences preferentially accept a complementary strand on the Hoogsteen face of the base. A thermodynamic analysis reveals entropic benefits in the case of hetero–backbone duplex formation (tricyclo–DNA/DNA duplexes) and both an enthalpic and entropic benefit for duplex formation in the pure tricyclo–DNA series compared to natural DNA. Homopyrimidine DNA sequences containing tricyclothymidine residues form triplexes with complementary double-stranded DNA. Triple-helix stability depends on the sequence composition and can be higher when compared to that of natural DNA. The use of one tricyclothymidine residue in the center of the self-complementary dodecamer duplex [d(CGCGAA**T**t**C**GCG), **t** = tricyclothymidine] strongly stabilizes its monomolecular hairpin loop structure relative to that of the corresponding pure DNA dodecamer ($\Delta T_m = +20$ °C), indicating (tetra)loop-stabilizing properties of this nucleoside analogue. Especially this latter fact is of interest in efforts to stabilize secondary structural elements of RNA or DNA, often found motifs in aptamers and ribozymes.¶

Meanwhile tricyclo–DNA oligonucleotides containing all four bases in a mixed sequence context have been made. Fully modified tricyclo–oligonucleotides efficiently form Watson–Crick duplexes with complementary DNA and RNA that show increased thermal stabilities by 1.2 and 2.4 °C/mod respectively. Tricyclo–DNA turns out to be completely resistant towards nucleases in heat deactivated fetal calf serum and does not elicit RNaseH activity in duplexes with

¶In vitro evolution strategies lead to new DNA or RNA sequences with highly specific ligand binding properties (aptamers) or with potential to catalyze a wide variety of chemical reactions (ribozymes). For recent reviews see refs 107 and 108, respectively.

RNA.[87] Especially the latter property does not particularly qualify tricyclo–DNA as an antisense probe against coding sequences of mature mRNA, but it is a prerequisite for antisense oligonucleotides targeted to non-coding regions as, for example intron sequences of pre-mRNA. We could recently show that aberrant pre-mRNA splicing in vivo in HeLa cells which stably express β-globin pre-mRNA carrying point mutations at the intron positions 654 and 705[88] can be efficiently restored when targeting the aberrant splice site by tricyclo–DNA.[87] A direct comparison with a 2′-OMe phosphorothioate oligoribonucleotide of the same sequence and length showed enhanced activity of tricyclo–DNA by a factor of up to 100. Thus tricyclo–DNA is definitely an interesting antisense candidate for further exploration.

The bicyclo[3.2.1]family

The interest in oligonucleotide analogues containing the nucleobases connected via a flexible linker arm to a rigid backbone structure, as in the bicyclo[3.2.1] family (Fig. 11) arose from fundamental questions on the role of the furanose ring (or any ring) as a connecting unit between backbone and base.

We were particularly interested in the question whether backbone restriction can compensate for loss of conformational preorganization of the base unit in terms of duplexation energy, and whether selectivity in strand orientation and base-mismatch discrimination would be compromized in such systems. At the outset of this project, there were two fully modified seco–DNA systems experimentally investigated in detail, namely glycerol–DNA[89,90] and 1′,2′-seco–DNA.[91] Both oligonucleotides were shown to have no capacity of duplex formation with complementary DNA and with itself — a fact that was ascribed to the high entropic costs of reorganization of single strand conformation upon duplex formation.

Bicyclo[3.2.1]–DNA. The bicyclo[3.2.1]DNA model turned out to be an interesting system. Having the torsion angles γ and δ (Fig. 11) fixed in a B-DNA conformation[92] complementary base-pairing with DNA and RNA and with itself occurs in oligomers 10–20 nucleotides in length. Complementary base-pairing with natural DNA is destabilized on the order of −3.0 to −0.6 °C/mod and typically increases (smaller ΔT_m) from shorter to longer and from pyrimidine-rich to purine-rich bicyclo[3.2.1]DNA sequences.[93,94] Only duplexes with antiparallel oriented strands are stable with DNA or with itself as complement. Base-mismatches were shown to contribute equally to duplex destabilization as in natural DNA. Given the major structural change with respect to DNA it is no surprise that bicyclo[3.2.1]–DNA is resistant to the action of the 3′-exonuclease snake venom phosphodiesterase.

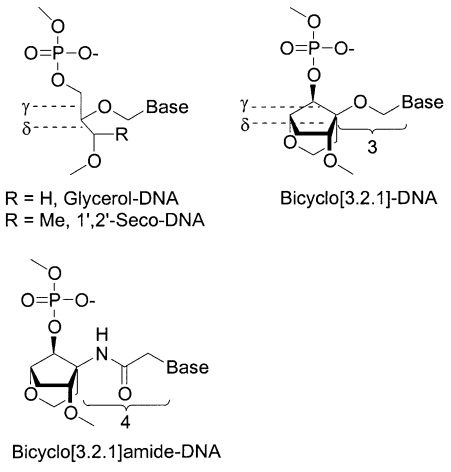

Figure 11. Structures of seco–DNA and the family of bicyclo[3.2.1]DNA.

Based on these results, we drew the conclusion that the structural feature of the furanose unit, as present in the natural nucleosides, is not a necessary prerequisite for obtaining a stable and selective base-pairing system, provided that the C–C bonds participating in the repetitive unit of the phosphodiester backbone are conformationally locked. Moreover, the information for the preference of antiparallel strand association in DNA must not necessarily be a consequence of the bases being attached to a specific (α or β) side of the furanose unit, but can also be encoded in the backbone itself. Furthermore, conformational flexibility in the base-pairing region does not lead to a loss of selectivity (match vs mismatch) in base-pair formation.

Bicyclo[3.2.1]amide–DNA. While the effect on base-pairing properties of shortening or enlarging the number of bonds of the repetitive backbone unit to 5 or 7 respectively, was recently discussed,[95] we were interested in the effect of lengthening the distance between the backbone and the bases by one bond. The bicyclo[3.2.1]amide–DNA (Fig. 11) is thus a good example for such a study. In this analogue the distance from the backbone to the bases, which is three bonds in DNA and RNA, is extended to four bonds in bicyclo[3.2.1]amide–DNA. Fully modified decamers of the base adenine were shown to base-pair with complementary DNA and RNA, with equal thermal stability, while decamers of the base thymine do not.[96] The structure of the duplexes were investigated by CD spectroscopy and were shown to be similar for the hybrid duplexes and the pure DNA or DNA/RNA duplexes, indicating the presence of a right handed Watson–Crick helix. An unexpected fact was that A–T pairing in the pure bicyclo[3.2.1]-series also readily occurs, but here a CD structural investigation pointed to the presence of a left handed Watson–Crick helix. As a consequence we investigated whether bicyclo[3.2.1]amide–DNA also forms duplexes with enantio-RNA. We could show indeed that such duplexes exist and that their thermal stabilities are only moderately lower compared to those with D-ribo complements. Current efforts focus on a rationalization of this unusual pairing behavior.[97] Bicyclo[3.2.1]amide–DNA is thus the first example of a chiral, non-chirospecific oligonucleotidic base-pairing system. From the three levels of selectivity, namely chiroselectivity, constitutional selectivity (parallel vs. antiparallel stranded duplexes) and base-pair formation selectivity, it has only lost the former.

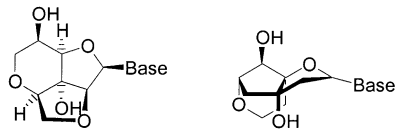

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Figure 12. Two examples of second generation conformationally constrained DNA analogues.

Conclusions and Outlook

Following the recent activities in oligonucleotide chemistry it becomes apparent that progress has been made in understanding the interrelation between nucleoside structure and oligonucleotide properties. While we start having a fairly good qualitative understanding on how geometric variations in the repeating backbone unit of DNA affects complex formation, we are still far away from being able to make quantitative predictions and control the non-conformational aspects as, for example hydration, coulombic and dipolar interactions, base stacking and so on in a rational way. Most of this lack of control comes from the difficulty of predicting and analyzing *single-strand* oligonucleotide structures.

Reflections on conformational restriction

The term 'preorganization' has been coined by the late Donald Cram in the context of supramolecular host–guest interactions.[98] Strictly speaking it means that the reduction of all possible conformational states of a guest molecule to those that fit with the geometric and stereochemical requirements of a host molecule leads to an entropic benefit of complex formation, and thus to enhanced complex stability. Conformational restriction as used throughout this text is synonymous for preorganization in that the conformational states of oligonucleotide single strands are reduced to those that match the geometry of this strand in duplexed form. Although this concept is very successful and has lead to nucleic acid analogues with promising properties for applications in therapy and diagnosis, a few basic questions still have to be addressed. The most important question is that of a possible limitation of the concept in general. Will continuous constraining of all rotational degrees of freedom of an oligonucleotide backbone be associated with a continuous increase in complex strength at physiological temperatures? For an ideal system, and concentrating on preorganization only, one has to expect a maximum in entropic complex stabilization along the coordinate of increasing preorganization of a single strand at the point where the *possible conformational states of the single strand is reduced to those appearing in its complexed form*, (maximum increase in $\Delta\Delta G^0$ between single strands and complex due to elimination of the population of non-preorganized conformers without reducing the number of possible conformational states of the complex). A decrease has to be expected, whenever the *number of conformational states of the complex* is reduced (complex destabilization due to overpreorganization of single strands).

Is the conformational locking of one out of six torsion angles of the backbone, as in LNA, just right, and that of two, as in tricyclo–DNA, already too much? Or is the restriction of a particular torsion angle, for example γ in a single strand inherently coupled to a restriction of conformational states of it in the duplexed form? In principal we do not know. One way to gain insight into such questions offers molecular dynamics simulations of oligonucleotide duplexes, in which the rotational freedom of individual bonds of the repetitive backbone unit in one strand are frozen out. Perhaps this leads to additional help in the design of new, more active, conformationally constrained DNA analogues and eventually define the limits of the preorganization concept.

Future trends

The search for new conformationally constrained oligonucleotide analogues will certainly go on and will eventually lead to shorter oligonucleotides that bind with equal or enhanced affinity and selectivity compared to those known today. This would decrease the molecular size and potentially reduce the cost of an antisense oligonucleotide; both factors that are of considerable importance for therapeutic applications. The structural complexity of mononucleosides that will be synthesized and incorporated into oligonucleotides will still increase. Two examples, one by Nielsen and coworkers,[99] and the other from our group (unpublished results) are given in Figure 12.

Such structures will be important in order to advance our basic knowledge on the interaction of biomolecular systems.

A large field that has not yet been tapped is the use of conformationally constrained nucleosides for the stabilization of secondary structural elements as, for example hairpin loops and bulges. Such structural elements are ubiquitous in ribozymes and aptamers,[100] and can be useful for enhancing their catalytic or binding efficiency. Moreover, triphosphates of sugar modified nucleoside analogues, in conjunction with natural or chemically engineered DNA polymerases, might provide new tools for molecular biology[101,102] or lead to new oligoncleotides for binding (aptamers) and catalysis (ribozymes) via in vitro evolution techniques. Increased activity in the use of oligonucleotide analogues is likely to be seen also in a non-biological context as, for example in nanotechnology or biocomputing.[103–105]

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