This review summarizes the results of structural studies carried out with analogs of G-quadruplexes built from natural nucleotides. Several dozens of base-, sugar-, and phosphate derivatives of the biological building blocks have been incorporated into more than 50 potentially quadruplex forming DNA and RNA oligonucleotides and the stability and folding topology of the resultant intramolecular, bimolecular and tetramolecular architectures characterized. The TG4T, TG5T, the 15 nucleotide-long thrombin binding aptamer, and the human telomere repeat AG3(TTAG3)3 sequences were modified in most cases, and four guanine analogs can be noted as being particularly useful in structural studies. These are the fluorescent 2-aminopurine, the 8-bromo-, and 8-methylguanines, and the hypoxanthine. The latter three analogs stabilize a given fold in a mixture of structures making possible accurate structural determinations by circular dichroism and nuclear magnetic resonance measurements.

**Keywords:** quadruplex; modified nucleotides; thermodynamics; stability; folding topology

1. **Introduction**

Quadruplex nucleic acids are probably the most studied noncanonical DNA structures. The essential folding characteristics, structural features, stabilities (Neidle & Balasubramanian, 2006), methods of investigation (Baumann, 2010), ligand-binding properties, and potential therapeutic applications (Neidle, 2012; Neidle & Balasubramanian, 2006) of DNA and RNA quadruplexes have well been reviewed. A typical G-quadruplex consists of at least two guanine (G)-tetrads connected by loops of one or more nucleotides. The quadruplex fold is held together by stacking of the tetrads and by cation coordination via the O6 carbonyl oxygen atoms of G bases. The planar G-tetrads are formed through Hoogsteen-type circular double hydrogen bonds, and the cations are located either in the central cavity of the G-tetrad or in the spaces between the tetrads. Variation in the orientations of the sugar–phosphate backbones of the quadruplexes makes it possible for even a single sequence to adopt numerous folding topologies. A few major topologies are shown in Figure 1.

Chemical modification of natural building blocks has long been a method of studying the structure of nucleic acids. Beginning from the early 1990s this approach has also been used with quadruplexes. This review provides a comprehensive summary of the folding topologies and structural stabilities of quadruplexes incorporating base, sugar, and phosphate analogs of natural constituents, as compiled by the end of 2012.

Among the base substitutions, the tetrad-forming G has been the focus of modifications for its crucial role in the formation and maintenance of the quadruplex structure. Base analogs incorporation into the loops of quadruplexes has also been reported, and natural non-G bases and their derivatives have also been inserted into both the tetrads and loops.

The sequences listed in this review are all 5′-to-3′, and the nucleotide nomenclature, if available and applicable, is used for abbreviation of the modified nucleotides. Except for the paragraph on RNA quadruplexes, all sequences listed are DNA; therefore, the letter d (abbreviating 2′-deoxy) is omitted in the sequences printed in capital A, C, G, T and U letters. RNA sequences are written in lower case letters, such as a, c, g, and u. From the literature usage of the terms for quadruplexes, the G-quadruplex, quadruplex, and tetraplex the quadruplex, and from the terms for tetrads, the G-tetrad, tetrad, and quartet, the tetrad is favored in this review.
2. Guanine (G) analogs

2.1. 8-substituted guanines

2.1.1. 8-Bromo guanines (brG)

Among the G analogs, the 8-substituted G's have been most frequently incorporated into potentially quadruplex-forming DNA sequences. Insertion of bulky atoms or groups into position 8 of G will change its energetically preferred N-glycosidic conformation from anti to syn (Birnbaum, Lassota, & Shugar, 1984; Dias, Battiste, & Williamson, 1994; Dumas & Luedtke, 2010; He et al., 1998; Sugiyama et al., 1996; Uesugi & Ikehara, 1977; Xu, Ikeda, & Sugiyama, 2003; Xu & Sugiyama, 2006). The difference between the two forms has been estimated to be ~1 kcal/mol (Dumas & Luedtke, 2010; He et al., 1998; Xu et al., 2003). Consequently, some destabilizing effect can be anticipated if these 8-substituted analogs replace an anti-dG of the quadruplex. Dias et al. (1994) described for the first time in 1994 the incorporation of brG into a quadruplex forming synthetic oligodeoxynucleotide to probe the effect of glycosidic conformation on quadruplex stability. The sequence was the 20 nucleotide-long (20mer) (T3G2)4 that folded into a monomolecular antiparallel chair-type quadruplex (Figure 1, #1), similar to the quadruplex structure of the thrombin binding aptamer (TBA) sequence. The authors hypothesized that replacing a syn-dG with brG would stabilize the quadruplex, whereas replacing an anti-dG with brG would destabilize the quadruplex. Their data showed that substitution of four of the eight dG positions increased the thermostability of the parent quadruplex by 5–6 °C (Tm of parent was 45 °C in 100 mM K+), while the other four decreased it by 3–6 °C. Those substitutions that increased the Tm and ΔG25 were the first G-s of each Guanine-Guanine (GG) dimer step, and those that decreased the stability were the second G-s of the GG. Nuclear magnetic resonance (NMR) spectroscopy established that the first G-s were in the syn orientation in the parent quadruplex, whereas the second G-s were in the anti conformation. The range of free energy differences (ΔΔG25) between the parent and the eight brG-modified quadruplex was, +1 or −1 kcal/mol, correlating well with the supposed energy difference between the syn and anti forms of brG, 1–2 kcal/mol.

Figure 1. Schematic folding topologies of G-quadruplexes. Gray squares represent the G-tetrads, the lines the sugar-phosphate backbones, and the curved parts the loops. Number 1, two-tetrad antiparallel chair-type quadruplex of the TBA sequence with three edge (or lateral)-type loop; 2, three-tetrad chair-type antiparallel; 3, bimolecular antiparallel with diagonal loops; 4, antiparallel basket-type with two-edge and one diagonal loops; 5, type-1 hybrid with three parallel and one antiparallel sugar-phosphate backbones (hence the abbreviation 3+1) forming two edge and one reverse-chain propeller-type loops; 6, type-2 hybrid (3+1); 7, monomolecular parallel quadruplex with three propeller-type loops; 8, tetramolecular parallel quadruplex.
The TGGGT sequences form tetramolecular parallel quadruplexes, [TGGGT]₄ in K⁺ solution (Figure 1, #8) with all natural nucleotides and their tetrads in anti-glycosidic conformations. Esposito et al. (2004) described the circular dichroism (CD) spectrum of the tetramolecular quadruplex formed by TGGGT that contained a Br₈G in position 2 as showing the characteristics of an antiparallel quadruplex with a large positive peak near 295 nm and a negative one near 260 nm (Figure 2). On the other hand, Br₈G in the other two G-positions showed characteristics of a parallel quadruplex, similarly to that of the wild type [TGGGT]₄ (Figure 3), with a large positive peak near 263 nm and a small negative one at 242 nm. Xu, Noguchi, and Sugiyama (2006) described in 2006 the use of Br₈G to stabilize the quadruplex fold of the 22mer human telomere sequence (htel-22) AGGG(TTAGGG)₃ in K⁺ solution for CD studies. When the Br₈G was incorporated in place of an originally syn-dG position of the 3-tetrad monomolecular quadruplex, it proved to be a powerful tool in overcoming the difficulty of structure determination arising from the conformational heterogeneity of the folds in solution. As a result, this analog has often been used for stabilizing particular quadruplex conformations for structural studies with CD, UV, NMR techniques (Lim et al., 2009; Mashimo, Sannohe, Yagi, & Sugiyama, 2008; Matsugami, Xu, Noguchi, Sugiyama, & Katahira, 2007; Petraccone et al., 2007; Phan, Kuryavyi, Luu, & Patel, 2007; Sannohe et al., 2008; Xu, Sato, Sannohe, Shinohara, & Sugiyama, 2008; Xu, Suzuki, Lonnberg, & Komiyama, 2009). Among the many applications, we list a few specific ones below.

The specific use of Br₈G includes Sugiyama group’s work (Mashimo et al., 2008) that used the htel-22 sequence with multiple Br₈G nucleotides incorporated to elucidate the folding pathway of the hybrid-1 and hybrid-2 quadruplexes. Such sequence was the A₈GGGT-TABr₈GTTGTTABr₈Gbr₈GGGG. They have also incorporated (Xu et al., 2008) Br₈G into putative syn-dG positions of the following two sequences (a 16mer and a trimer), which formed an interstrand heterodimeric G-quadruplex with a hybrid scaffold in 0.1 M KCl, as deduced from the CD spectra:

\[
\text{br}^8\text{GGGGTTA}br^8\text{Gbr}^8\text{GGGGTTA}br^8\text{Gbr}^8\text{GGG} + \text{Tbr}^8\text{G}
\]

The \( T_m \) value of the quadruplex increased with increasing number of Br₈G in the sequences, from \( \sim 55^\circ \) C up to \( \sim 75^\circ \) C. Xu et al. (2009) used the same Br₈G-containing 16mer oligonucleotide to form another bimolecular hybrid (3+1) quadruplex in which the syn dG-s were replaced by Br₈G-s:

\[
\text{br}^8\text{GGGGTTA}br^8\text{Gbr}^8\text{GGGGTTA}br^8\text{Gbr}^8\text{GGG} + \text{br}^8\text{GGG}
\]

and an EDTphosphoric acid + Ce(IV) ‘scissor’ was attached.
to the 5'-end of the trimer sequence to carry out sequence-specific cleavage reaction (Xu et al., 2009). Phan et al. (2007) applied the br8G modification for NMR studies with the following quadruplex-forming htel sequences:

\[
\begin{align*}
\text{TA(htel-21)}: & \quad \text{TAGGG TTAGGG TTAGGG TT} \quad - \text{br}^8\text{G in position 16} \\
\text{TAGG TTAGGG TT} & \quad \text{br}^8\text{G TTAGGG TT} \\
\text{TA(htel-21)}TT: & \quad \text{TAGG TTAGGG TTAGGG TT} \quad - \text{br}^8\text{G in position 15} \\
\end{align*}
\]

The addition of different flanking nucleotides to the basic htel-21 sequence induces different folds: TA(htel-21) folds into the hybrid-1 form, whereas TA(htel-21)TT adopts the hybrid-2 form. Incorporation of br8G into wisely chosen sequence positions, position G16 in the middle tetrad for the former sequence, and G15 in one of the terminal tetrad of the latter sequence greatly stabilized their respective topologies. Both dG positions were originally syn, and thus, br8G contributed to the stability of each fold through its preferred syn glycosidic linkage, significantly improving the quality of the NMR spectra. In another study, Lim et al. (2009) determined that br8G increased the thermostability (\(T_m\)) of the following quadruplexes by 4–7°C, which were likewise analyzed by NMR.

\[
\begin{align*}
\text{htel-21)}T, \text{two-tetrad K}^+\text{-baskets:} & \quad \text{GGG TTA GGG TATA GGG TTA GGG T} \\
& \quad \text{GGG TTA br}^8\text{GG TTA GGG TTA GGG T} \\
& \quad \text{GGG TTA br}^8\text{GG TTA br}^8\text{GG TTA GGG T} \quad (\text{br}^8\text{G, 6-thioguanine}) \\
\text{TA(htel-21):} & \quad \text{TAGG TTAGGG TTA Gbr}^8\text{GG TTA GGG T} \\
& \quad \text{TAGG TTAGGG TTA br}^8\text{GG TTA GGG T} \quad \text{Hybrid-1} \\
& \quad \text{TAGG TTAGGG TTA br}^8\text{GG TTA GGG T} \quad \text{Hybrid-2} \\
\end{align*}
\]

With both the natural and the substituted forms of the 22mer (htel-21)T, the authors determined a new type of fold in K+ solution, a basket-type intramolecular quadruplex with only two-tetrad, containing two edge loops and one diagonal loop.

### 2.1.2. 8-Methylguanine (m8G)

\[
\text{syn 8-methyl-2'-deoxyguanosine}
\]

Similarly to br8G, m8G is also a quadruplex stabilizing modification. Xu and Sugiyama (2006) incorporated m8G into a 18mer, the CGGG GGG TTTT GGG CGGC sequence of the G-rich termini of retinoblastoma (Rb) gene DNA. (The Rb gene codes for a phosphoprotein that acts as a tumor suppressor, and the formation of the G-quadruplex in this sequence inhibits the replication of the gene.) The 18mer sequence formed an antiparallel quadruplex with two G-tetrads. The effect of m8G was studied by CD and fluorescent spectroscopy techniques. The CD-based thermal stability values were measured in 0.15 M KCl (Table 1).

<table>
<thead>
<tr>
<th>Glycosidic linkage of the dG replaced</th>
<th>(T_m) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGG GGG TTTT GGG CGGC</td>
<td>51</td>
</tr>
<tr>
<td>(\text{CG}^8\text{G}) CGGG GGG TTTT GGG CGGC</td>
<td>55</td>
</tr>
<tr>
<td>(\text{CG}^8\text{G}) CGGG GGG TTTT GGG CGGC</td>
<td>45</td>
</tr>
<tr>
<td>CGGG GGG TTTT (\text{CG}^8\text{G}) GGG CGGC</td>
<td>56</td>
</tr>
<tr>
<td>CGGG GGG TTTT (\text{m}^8\text{G}) GGG CGGC</td>
<td>54</td>
</tr>
<tr>
<td>CGGG GGG TTTT GGG (\text{m}^8\text{G}) CGGC</td>
<td>54</td>
</tr>
</tbody>
</table>

The quadruplex was stabilized by 3–5°C if the m8G was incorporated into positions where the original, unmodified dG nucleoside was in syn conformation, and the quadruplex was destabilized, by 6°C, if m8G replaced the originally anti guanosine. The quadruplex paid an energy penalty for accommodating the syn m8G, probably by converting it into an anti conformation. CD spectra of the m8G-containing structures showed antiparallel quadruplexes.

Virgilio, Esposito, Randazzo, Mayol, and Galeone (2005) determined that in the originally all anti nucleotide-containing four-molecular parallel quadruplex [TGGGT]4, the quadruplex fold depended on the position in which the m8G was incorporated: The m8G-tetrad in position 2 was all syn, and the CD spectrum was consistent with an antiparallel arrangement in K+, that is, to a spectrum with a large positive band near 290 nm, and a negative one near 260 nm. The m8G-tetrad in position 3 was all anti, and the CD showed parallel spectrum, that is, a spectrum with a large positive peak at 260 nm and a small negative peak around 240 nm. m8G in position 4 hindered the formation of a quadruplex. Another, recent systematic study of the same group also dealt with the positional effect of m8G on the formation of parallel and antiparallel tetramolecular quadruplexes of the TG3T and TG4T sequences (Virgilio et al., 2012).
2.1.3. 8-oxoguanine (o^8G)

7,8-Dihydro-8-oxoguanine or 8-oxoguanine, o^8G is one of the main DNA oxidation products of the reactive oxygen species (ROS) in vivo (Gannet & Sura, 1993). As G triplets act as sinks for oxidative damages, the o^8G predominantly forms in G-rich DNA sequences (Oikawa, Tada-Oikawa, & Kawanishi, 2001). Jean-Louis Mergny’s group (Gros et al., 2007) published an extensive study on the effect of twelve types of modified G bases and non-G bases on the kinetics of formation and on the thermal stability of modified tetramolecular parallel stranded quadruplexes composed of TG4T and TG5T sequences, the [TG4T]4 and [TG5T]4. In the tetramolecular quadruplexes each strand contains the modified base, and thus, modified-base tetrads are formed. Incorporation of o^8G into any G-position of TG4T and TG5T allowed the formation of tetramolecular parallel quadruplexes as determined by CD. Most modifications studied, such as 6-thioguanine (s^6G), 7-deazaguanine (c^7G), N6-methylguanine (m^6G), and hypoxanthine (I) decreased the thermal stability of quadruplexes formed from either of the two sequences; however, the o^8G, like br^8G, proved to be a stabilizing modification, especially, when incorporated at the 5' position of the G4 or G5 sequence. Stability was characterized by the T_{1/2} value, instead of the T_m, indicating non-equilibrium melting curves in most cases. T_{1/2} refers to the heat-induced dissociation of preformed quadruplexes (Table 2).

The preferred glycosidic conformation of o^8G is syn (Usug & Ikehara, 1977), and it was suggested by the authors that the o^8G nucleotide adopted this conformation when substituted at the 5' positions of the tetramolecular quadruplexes, at least in the nucleation part of the association process. The stabilization effect was explained by solid stacking interaction toward the next G-quartet in the 3' direction, although the 8-substitution changed the Hoogsteen-type circular H-bonding pattern. In the diketo form the N7 becomes a hydrogen donor, instead of an acceptor, and the O8 becomes an acceptor. As a consequence, the N7 does not take part in the circular hydrogen-bonding structure. Instead of two hydrogen bonds, a single circular H-bonding pattern remains in the o^8G-tetrad.

Variable effects of o^8G were observed with quadruplexes of an htel sequence. Szalai, Singer, and Thorp (2002) incorporated o^8G into the following 25mer oligodeoxynucleotide, the A(htel-21)TGT, to construct site-specific probes and to study the effect of o^8G on the activity of the telomerase enzyme:

<table>
<thead>
<tr>
<th>AGGG TTAGGG TTAGGG TTAGGG TGT</th>
<th>Unmodified 25mer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGGG TTAGG GGG TTAGGG TTAGGG TGT</td>
<td>Pos. 8 = syn G position</td>
</tr>
<tr>
<td>AGGG TTAGG GGG TTAGGG TTAGGG TGT</td>
<td>Pos. 9 = anti</td>
</tr>
<tr>
<td>AGGG TTAGG TTag GGG GGG TTAGGG TTAGGG TGT</td>
<td>Pos. 14 = anti</td>
</tr>
<tr>
<td>AGGG TTAGG TTag GGG GGG TTAGGG TTAGGG TGT</td>
<td>Pos. 15 = syn</td>
</tr>
</tbody>
</table>

The authors found that if o^8G was in the 5'-position of a GGG triplet in the 25mer quadruplex (positions 8 & 14), the quadruplex fold was antiparallel (Figure 1, #4), and the telomerase activity significantly decreased. If o^8G was built into the middle position of a GGG triplet (positions 9 and 15) multiple structures were found and the activity of the telomerase enzyme did not decrease as compared to the control reaction with the unmodified quadruplex.

8-oxoguanine has also been substituted for each G of the three-tetrad quadruplex of the htel-21 sequence, G3(TTAG3)3 by Vorlícková, Tomasko, Sagi, Bednarova, and Sagi (2012). Results of the structural study revealed that although o^8G did not hinder the formation of monomolecular quadruplexes either in Na+ or K+ solutions, the quadruplexes were destabilized by o^8G by enthalpically-driven effects. The extent of this effect depended on the position of substitution and on the cation used according to UV absorption-based unfolding thermodynamics. Groups of 4 sequences below show the stability of the “upper”, the middle and the “bottom” tetrads, as shown in Figure 1, #4. The unmodified Na+-stabilized antiparallel quadruplex of thel-21 rearranges in K+ solution to a K+-stabilized monomolecular structure, whose CD spectrum is characterized (Renciuk et al., 2009) by a strong shoulder around 270 nm and the disappearing negative peak at 260 nm, while the 290 nm large positive peak of the Na+-antiparallel quadruplex remains also in K+ solution (Figure 2). The conformational rearrangement observed with the unmodified G3(TTAG3)3 on the Na+ to K+ exchange was however hindered in the qudruple-Roligodeoxynucleotide.
plexes that contained $\text{o}^8\text{G}$ in the two terminal tetrads. The middle tetrad-substituted quadruplexes were the most destabilized by $\text{o}^8\text{G}$ (Table 3), and probably as a consequence, $\text{o}^8\text{G}$ in the middle tetrad made the quadruplex flexible to folding changes (Vorlícková et al., 2012).

Not only htel-21 but also the A(htel-21), or htel-22, goes through the CD spectral changes on the Na$^+$ to K$^+$ exchange (Chang, Chien, Lin, Kang, & Chang, 2007). According to M. Vorlickova (Renciuk et al., 2009) the K$^+$-stabilized htel-21 and A(htel-21) quadruplexes are also basket-type monomolecular antiparallel quadruplexes, but the stacking interactions have changed due to the accommodation of the larger K$^+$ ions between two adjacent tetrads instead of the in-plane coordinating Na$^+$ ions. The explanation was based primarily on kinetic experiments. Other authors have however defined this K$^+$-stabilized monomolecular quadruplex as one of two hybrid (3 + 1) folds (Ambrus et al., 2006; Luu, Phan, Kuryavyi, Lacroix, & Patel, 2006; Phan, Luu, & Patel, 2006) or the antiparallel chair form (Matsugami et al., 2006) (Figure 1, #2) based mainly on NMR results. The discrepancy may arise from the very different strand concentrations used in the CD experiments (micromolars) and the NMR studies (lower millimolars) as quadruplex folding depends on the concentration of the quadruplex (Renciuk et al., 2009). Determination of a quadruplex fold by CD and NMR techniques using strand concentrations of orders of magnitude apart may lead to inconsistent conclusions.

The damaging effect of $\text{o}^8\text{G}$ on the stability of the monomolecular htel-21 quadruplex is surprising in light of the stabilizing effects observed with the tetramolecular quadruplexes (Gros et al., 2007). $\text{o}^8\text{G}$ caused mainly minor destabilization of duplex oligonucleotides, where the effect depended on duplex length: With duplexes longer than 15 nucleotides, small stabilization effects were even observed (Vorlicková et al., 2012, references therein). The htel-21 quadruplex was also destabilized by other tetrad modifications, such as adenine (A) (Sagi, Renciuk, Tomasko, & Vorlickova, 2010; Tomáško, Vorlíková, & Sagi, 2009) and G-abasic sites (AP sites) (Skoláková, Bednarova, Vorlickova, & Sagi, 2010). It is interesting to note that although $\text{o}^8\text{G}$, A, and the AP site are very different chemical structures the three modifications exerted similar positional effects on the stability and folding properties of the quadruplex of the htel-21 oligodeoxynucleotide.

### 2.1.4. 8-aminoguanine ($n^8\text{G}$)

![8-aminoguanine](image)

The 15mer TBA GGTTGGTGTGGTTGG oligodeoxynucleotide forms a monomolecular two-tetrad chair-type antiparallel quadruplex in both Na$^+$ and K$^+$ solutions (Figure 1, #1). Upon incorporation of $n^8\text{G}$ into position 2, an anti-dG position, the quadruplex became destabilized by 7 °C ($\Delta T_m$) in K$^+$ solution. Destabilization was enthalpic in origin with $\Delta\Delta H^\circ$ of 5.5 kcal/mol as determined by van’t Hoff analysis of CD melting curves by de la Osa et al. (2006). The negative effect on quadruplex stability contrasted with the effect of $n^8\text{G}$ on DNA triplexes and parallel duplexes, where $n^8\text{G}$ proved to be a stabilizing analog (references in de la Osa et al., 2006). The CD spectrum of the quadruplex of G$n^8\text{G}$TTGGTGTGGTTGG only slightly differed from the spectrum of the parent two-tetrad, antiparallel, chair-type TBA quadruplex.

### Table 3. Stability of 8-oxoguanine-containing htel-21 quadruplexes. (see Vorlickova et al., 2012)

<table>
<thead>
<tr>
<th>#</th>
<th>GGG TTAGGG TTAGGG TTAGGG</th>
<th>$T_m$ (°C)</th>
<th>$\Delta G_{37}$ (kcal/mol)</th>
<th>0.169 M Na$^+$</th>
<th>0.169 M K$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>GGG TTAGGG TTAGGG TTAGGG</td>
<td>67.7</td>
<td>4.7</td>
<td>75.4</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>GGG TTAGGG TTAGGG TTAGGG</td>
<td>60.2</td>
<td>3.0</td>
<td>66.1</td>
<td>4.6</td>
</tr>
<tr>
<td>7</td>
<td>GGG TTAGGG TTAGGG TTAGGG</td>
<td>52.1</td>
<td>2.0</td>
<td>61.2</td>
<td>3.7</td>
</tr>
<tr>
<td>15</td>
<td>GGG TTAGGG TTAGGG TTAGGG</td>
<td>50.4</td>
<td>1.7</td>
<td>61.2</td>
<td>3.4</td>
</tr>
<tr>
<td>19</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>53.5</td>
<td>2.0</td>
<td>60.6</td>
<td>3.3</td>
</tr>
<tr>
<td>2</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>48.2</td>
<td>1.4</td>
<td>48.0</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>41.9</td>
<td>0.6</td>
<td>44.9</td>
<td>nd</td>
</tr>
<tr>
<td>14</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>44.4</td>
<td>nd</td>
<td>47.0</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>43.6</td>
<td>nd</td>
<td>47.1</td>
<td>nd</td>
</tr>
<tr>
<td>1</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>64.0</td>
<td>3.8</td>
<td>63.2</td>
<td>4.1</td>
</tr>
<tr>
<td>9</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>64.1</td>
<td>3.8</td>
<td>64.2</td>
<td>4.0</td>
</tr>
<tr>
<td>13</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>65.3</td>
<td>4.0</td>
<td>64.4</td>
<td>4.2</td>
</tr>
<tr>
<td>21,T</td>
<td>GGG TTAGGG TTAGGG TTAGGG TTAGGG</td>
<td>60.8</td>
<td>3.4</td>
<td>64.9</td>
<td>4.4</td>
</tr>
</tbody>
</table>
2.1.5. 8-(2-pyridyl)guanine (Py8G)

Py8G is a highly fluorescent compound that has been used by Dumas and Luedtke (Dumas & Luedtke, 2010) to study the folding of quadruplexes and the energy transfer reactions within a quadruplex. They have incorporated Py8G into two positions of the 24mer Telo(wt) {TT(htel-21)A} and into a single position of the 22mer cKit DNA sequences. Py8G affected both the stability and the folding properties of the quadruplexes in a sequence-dependent manner, as determined in 110 mM Na+ or K+ or in 40% PEG 200 with or without salts. The following sequences were studied:

Telo(wt)  TT GGG TTA GGG TTA GGG
TeloG9   TT GGG TTA Py8GG TTA GGG
TeloG23  TT GGG TTA GGG TTA GGG
CKit (wt) AGGG AGGG CGCT
CKitG10 AGGG AGGG CGPy8GCT

While Py8G in position 9 of the 24mer (TeloG9) increased the Tm of the wild-type quadruplex by 7 and 10 °C in Na+ and K+ solutions, respectively, Py8G in position 23 (TeloG23) reduced the thermal stability by 2 and 9 °C, respectively. Apparently, the syn or anti orientation of the Py8G nucleoside is not related to these effects, and both positions were in one of terminal tetrads of the three-tetrad quadruplex. The effects of this substitution on folding also differed by position. Py8G in TeloG9 in K+ did not change the CD spectrum of the wild-type 24mer that was described as a hybrid (3+1) fold with a large positive band near 290 nm, a strong shoulder around 270 nm, and a negative peak near 240 nm. The use of Py8G in position 23 (TeloG23), however, induced a conversion into a typical antiparallel spectrum in K+ with a characteristic large positive peak near 295 nm, another small at 240 nm, and a medium negative band at 260 nm. 40% PEG (in 10 mM Li+-buffer), to simulate the macromolecular crowding conditions of cells, decreased the Tm of both TeloG9 and TeloG23, but addition of 110 mM Na+ to the PEG solution of TeloG9 increased the Tm by 18 °C. This solution stabilized the antiparallel folding of TeloG9. CD spectra of the 22mers, both the unmodified cKit and cKitG10 showed parallel quadruplex structures with large positive peaks at 260 nm and small negatives at 245 nm. Py8G in position 10 destabilized the parent quadruplex by 4 °C in K+, which was explained by the non-preferred glycosidic torsion of Py8G. PEG. This 8-substitution of G did not disrupt the Hoogsteen base pairing of the G-tetrads and did not influence the steric arrangement of stacking of tetrads. Thus, the stabilizing effect of Py8G in position 9 of a terminal tetrad can be compared with the effects of m8G and br8G. The reduction in quadruplex stability by TeloG23 in the other terminal tetrad remained unexplained (Dumas & Luedtke, 2010).

2.1.6. 8-(2-phenylethyl)-dG and 8-[2-(pyrid-4-yl)-ethenyl]-dG

In addition to Py8G, Dumas and Luedtke (2011) also incorporated 8-(2-phenylethyl)-dG and 8-[2-(pyrid-4-yl)-ethenyl]-dG into positions 9 and 23 of the 24mer TT (htel21)A DNA, the TT[G3(TTAG3)3]A. Interestingly, deoxynucleosides of these two 8-ethenyl group-containing derivatives of G adopted anti glycosidic torsion angles in solutions, like the dG does, contrary to the nucleoside of Py8G. Due to the in-plane conjugated ethenyl tethers the bulky aryl or heteroaryl groups are detached from the core of G to a distance that does not sterically hinder the anti orientation of the nucleosides. 8-Vinyl-dA has also been described to adopt an anti conformation (Gaied et al., 2005).

The quadruplex of the 24mer TT[G3(TTAG3)3]A has been described to adopt the hybrid (3+1) fold in K+ solutions, and the antiparallel topology in Na+ (Figure 1), based on NMR measurements (Dumas & Luedtke, 2011). The position 9-substituted 24mer quadruplex structures were stabilized by Py8G and also by the two vinyl group-containing derivatives, by the ΔTm of 7–8 °C in Na+ and by 5–9 °C in K+. Py8G proved to be the most stabilizing analog in this series. (The duplex of TT[G3(TTAG3)3]A with the complementary 24mer was destabilized by all three analogs by 1–5 °C.) The G derivatives incorporated into position 23 of the 24mer sequence destabilized the quadruplex structure by 1–6 °C in Na+, and by 6–8 °C in K+. (The duplex was slightly stabilized by the derivatives...
in position 23, by 0.2–0.9 °C, probably as the position 23 is at the chain end that might not have disrupted the duplex structure as much as a mid-chain substitution could.) In position 9 none of the G-analogs changed the CD spectrum of the wild-type 24mer in K⁺ that was thought to be a 3+1-fold, characterized by a large positive peak close to 290 nm, a strong shoulder at 270 nm, and a shallow negative minimum near 240 nm. However, the CD spectra of the 23-substituted 24mer quadruplexes showed definite transitions toward the antiparallel region of quadruplex folds in K⁺, that is the positive maximum of the wild-type quadruplex around 290 nm moved toward 295 nm, the shoulder of 270 nm diminished and became a negative peak at 260 nm, the 240 nm negative band became a small positive band at 240 nm. In Na⁺ solution the antiparallel character of the wild-type 24mer quadruplex did not change on substitution (Dumas & Luedtke, 2011).

2.1.7. 8-fluorenlyvinyl-dG (Fv₈G)

Ogasawara and Maeda (2009) developed a light-controlled reversible formation - disruption of a quadruplex structure based on the cis-trans isomerization of a photochromic nucleobase, Fv₈G, incorporated into the 15mer TBA sequence:

\[
\begin{align*}
\text{GG TT G} & \text{Fv₈G TGT Fv₈G TT GG} \\
\text{Fv₈G TT GG TGT GG TT Fv₈G} 
\end{align*}
\]

The wild-type TBA sequence forms a chair-type two-tetrad antiparallel quadruplex scaffold (Figure 1, #1) in K⁺ solution characterized by positive peaks near 295 and 250 nm, and a negative peak near 265 nm in its CD spectrum. The spectrum only slightly changed upon incorporation of Fv₈G into the anti-dG positions of either sequence shown if the 8-fluorenlyvinyl group of Fv₈G was in the trans form. In this case, thermally stabilized quadruplexes were formed. The \( T_m \)-s of both quadruplexes increased by about 10 °C, up to \( \sim 60 \) °C. On irradiation at 410 nm the \( \text{trans-Fv₈G} \) changed to the \( \text{cis-form} \), and the steric structure of the substituent disrupted the Hoogsteen-type base pairing of the tetrads, resulting in quadruplex unfolding as shown by both the CD spectra and the no-hyperchromicity thermal profiles recorded from 20 to 85 °C. Irradiation at 310 nm reversed the unfolding.

2.2. Non-8-substituted guanines

2.2.1. 6-Thioguanine (s₆G)

\[
\begin{align*}
\text{H}_2\text{N} & \text{N} \\
\text{NH} & \text{N} \\
\text{NH} & \text{N} \\
\text{O} & \text{NH}_2 \\
\text{N} & \text{N} \\
\text{O} & \text{NH}_2 \\
\text{N} & \text{N} \\
\text{O} & \text{NH}_2 \\
\end{align*}
\]

The s₆G incorporated into any G-position of TG₄T or TG₅T sequences did not change the CD characteristics of the parallel tetramolecular quadruplexes of \([\text{TG₄T}]₄\) and \([\text{TG₅T}]₄\) in K⁺, however, it decreased the thermostability \( (T_{1/2}) \) of \([\text{TG₅T}]₄\) in any position in Na⁺ (Gros et al., 2007). Similarly, the CD spectrum of the two-tetrad forming (htel-21)TT analog \( \text{G}_4(\text{TTs₆GG})(\text{TTAG}_4)_2\text{T} \) in K⁺ was not influenced by the presence of s₆G in position 7 (Lim et al., 2009). However, with the TBA analog \( \text{Gs₆GTGTTTGGTTGTTGG} \) the single s₆G in position 2 inhibited the formation of TBA’s chair-type quadruplex, and similarly, inhibited the formation of the two-tetrad basket-type quadruplex by the \( \text{Gs₆GTUT-UUGUTUTGGUUTTTGG} \) oligonucleotide, the latter containing also \( \text{U} \) bases (Marathias, Sawicki, & Bolton, 1999). Destabilization of various DNA secondary structures by s₆G was later confirmed by theoretical calculations: the destabilizing effect of this analog was greater for quadruplexes than it was for triplexes and duplexes (Spackova, Cubero, Sponer, & Orozco, 2004).
2.2.2. $\text{O}^6$-Methylguanine ($m^6G$)

The major natural base lesion $m^6G$ (Demple, Jacobsson, Olsson, Robins, & Lindahl, 1982) has been shown to be pro-mutagenic and pro-carcinogenic by causing G:C to A:T transition (Koike, Maki, Takeya, Hayakawa, & Sekiguchi, 1990). Incorporated into short quadruplex-forming sequences the $m^6G$ destabilized the tetramolecular quadruplex of $[\text{TG}_4\text{T}]_4$ in a position-dependent manner in Na$^+$ solution, by forming $m^6G$-tetrads. The presence of these tetrads either in $[\text{TG}_4\text{T}]_4$ or $[\text{TG}_5\text{T}]_4$ did not change the CD marks of the parallel tetramolecular quadruplexes exhibited by the unmodified parallel quadruplexes (Gros et al., 2007). $m^6G$ was incorporated by Mekmaysy et al. (2008) into each of the three G positions of the G-trimer at the 5′-end of the htel-22 AG$_3$(TTAGGG)$_3$. In this way, a single $m^6G$ was placed into each of the three G-tetrads. The authors found that the thermal stabilities of the substituted structures were reduced relative to the wild-type quadruplex (Table 4).

The largest destabilizing effect was observed when the $m^6G$ was incorporated into position 3, located in the middle tetrad of the three-tetrad monomolecular quadruplex. The stabilizing effect of K$^+$ versus Na$^+$ on quadruplex structure also diminished or even disappeared by the $m^6G$ substitutions. The large negative effect of substitution of G in the middle tetrad has also been observed with other natural base lesions, such as adenine (Sagi et al., 2009; Tomáško et al., 2009), 8-oxoguanine (Vorlicková et al., 2012), and guanine abasic sites (Školáková et al., 2010). The CD spectrum of the unmodified 22mer, indicating a monomolecular antiparallel quadruplex in Na$^+$ solution was only slightly modified by $m^6G$ substitutions. In K$^+$, the unmodified sequence was described by the authors as adopting a hybrid (3 + 1) structure, whereas the spectra of the terminal tetrads-modified quadruplexes, $m^6G$ in positions 2 and 4, were rather reminiscent of their Na$^+$-spectra. The spectrum of the middle tetrad-modified structure was qualitatively different from the others, showing two positive peaks near 260 and 295 nm. Based on a 5-ns molecular dynamics simulation in Na$^+$ the authors concluded that the O-methylated guanine did not participate in the tetrad formation and was instead flipped out of the stacked tetrads. Thus, the decreased thermal stability observed might well be the consequence of the missing cation coordination, the disrupted circular H-bonding scheme, and the reduced stacking interaction. In K$^+$ the central tetrad was completely distorted according to the simulation results, which may explain the anomalous CD spectrum as well as the greatly reduced thermal stability of AG$^m6G$ (TTAGGG)$_3$ (Mekmaysy et al., 2008).

2.2.3. Hypoxanthine (I for inosine)

In vivo deamination of adenine in DNA leads to hypoxanthine-2′-deoxyribose or 2′-deoxyinosine (Wang, 2008). The I base can be viewed as an analog of G with a missing 2-amino group. In this way, the Hoogsteen-bonded G-tetrad loses a hydrogen bond if I is formed in a quadruplex in vivo or is incorporated synthetically. I was first incorporated into a quadruplex in 1993 by Smith and Feigon (1993) who inserted it into the Oxystichia telomere repeat oligonucleotide G$_4$T$_4$G$_4$, that is, GGGG TTTT GGII. The authors studied the structure by $^1$H and $^{31}$P NMR spectroscopy. The sequences formed diagonally looped symmetrical bimolecular antiparallel quadruplexes with four G-tetrads and T4-loops in both Na$^+$ and K$^+$ solutions (Figure 1, #3). In another study, Smith, Schultze, and Feigon (1995) described the incorporation of both I and U into G$_4$(T$_4$G$_4$)$_3$ (Ox$^y$=3.5), G$_4$TTUGT$_4$G$_4$UUTTGGGII, and concluded from $^1$H and $^{31}$P NMR measurements that both the unmodified and the I- and U-modified sequences formed monomolecular quadruplexes with a diagonal T4 loop and two modified edge-wise loops. In another study, Gros et al. (2007) used the TG$_2$T and TG$_3$T sequences and found that the I-tetrad destabilized the tetramolecular quadruplex formed by these sequences. Destabilization by I was less extensive than by s$^7G$ or $m^6G$, and also, the I-tetrads did not change the parallel characteristics of the CD spectra of the parent [TG$_4$T]$_4$ and [TG$_5$T]$_4$ quadruplexes. With the fluorescent-labeled htel-21 (GGGTATA)$_3$GGG sequence, in which one of the guanines was substituted with I, Risitano and Fox (2005) showed that the relative stability of the I-modified quadruplex was different in Na$^+$ and K$^+$

![Diagram](https://via.placeholder.com/150)

**Table 4.** Effect of $\text{O}^6$-methylguanine on the stability of A (htel-21) quadruplexes. (see Mekmaysy et al., 2008)

<table>
<thead>
<tr>
<th></th>
<th>$T_m$ (°C)</th>
<th>Na$^+$</th>
<th>K$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGGG (TTAGGG)$_3$</td>
<td>62</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Am$^6$GGG (TTAGGG)$_3$</td>
<td>53</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Agm$^6$GG (TTAGGG)$_3$</td>
<td>37</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Ag0mGmG (TTAGGG)$_3$</td>
<td>48</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>
solutions, and that in Na\(^+\) the fold was a mixed parallel and antiparallel, while in K\(^+\) the fold was parallel.

In the quadruplex of the (TAGGG)_2TAIGG sequence a single I base did not change the CD signature of the antiparallel quadruplex (Hu, Lim, Bouaziz, & Phan, 2009), and similarly, the single I in GIGT(GGGT)_3 did not change the parallel CD spectrum of (GGGT)_4, but made the quadruplex structure more stable for NMR studies by significantly improving the spectral resolution (Do, Lim, Teo, Heddi, & Phan, 2011). Phan’s group (Do et al., 2011) has also incorporated I nucleotides into the GIGT (GGGT)_3 containing 5'-T0 and 3'-T0 nucleotides. They have also inserted I into T30117 and into an analog sequence of it (Mukundan, Do, & Phan, 2011). The T30177 oligonucleotide inhibits the integrase enzyme of HIV-1 in the nanomole range and it forms a dimeric quadruplex consisting of six G-tetrads by the 5'-to-5' stacking of two propeller-type parallel quadruplexes in K\(^+\) solution. The following sequences were studied:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T30177</td>
<td>GTGGT GG GTGGT GG GT</td>
</tr>
<tr>
<td>T30177-I1</td>
<td>GGGT GG GTGGT GG GT</td>
</tr>
<tr>
<td>T30177-TT</td>
<td>TT GTGGT GG GTGGT GG GT</td>
</tr>
<tr>
<td>T30177-I1-TT</td>
<td>TT GTGGT GG GTGGT GG GT</td>
</tr>
</tbody>
</table>

In the absence of the 2-amino group, a single I-base disrupts the symmetric, circular Hoogsteen-type double H-bonding of a G-tetrad. This makes possible the folding transition of unstable or equilibrium structures into another fold. An example for this is the study of Lim et al. (2010) that used the I for G substitution to change the conformational equilibrium between competing quadruplex folds in AG\(_2\)AG\(_2\)CTG\(_3\)AG\(_2\)C.

Zhang, Dai, Veliath, Jones, and Yang (2010) incorporated an I for a G in the A(1tel-21) and A(1tel-21)T sequences: AG\(_3\)TTAG\(_3\)TTAIGGTTAG\(_3\), and AG\(_3\)TTAG\(_3\)TTAIGGTTAG\(_3\)T. The NMR results showed that as a result of substitution of I in position 14, the structure formed contained two unusual triads, instead of tetrads, at both extremes of the now 2-tetrad quadruplex. The triad at the diagonal loop of the quadruplex was G22.I14:G10, held together by five H-bonds, and the triad at the other extreme was A19:G4.A7. Thermal stabilities in K\(^+\) of the I-containing quadruplexes were lower than those of the unmodified 22- or 23mers, and the I-22mer (\(T_m\) 54°C) was less stable than the I-23mer. CD spectra of the I-containing quadruplexes showed two positive maxima, at 290–295 and 253–256 in K\(^+\). These spectra were similar to those recorded in K\(^+\) with quadruplexes that contained A-for-G substitutions (Tomaško et al., 2009). The authors concluded that the two-tetrad conformations observed were probably just unstable transitional structures in the course of the interconversions of various conformations of quadruplex (Zhang et al., 2010).

2.2.4. Isoguanine (iG)

The guanine analog iG, in which the guanine’s 2-amino group and the 6-carbonyl group exchange positions was first incorporated into a synthetic DNA, poly(iG) by Shugar and coworkers in 1976 (Golas, Fikus, Kazimierczuk, & Shugar, 1976), iG-containing oligonucleotides were first prepared and characterized in the 1990s by Seela, Mertens, and Kazimierczuk (1992) and Switzer, Moroney, and Benner (1993). iG can pair with iC, in which the O2 and the NH\(_2\) of C4 of cytosine are exchanged, and these can form antiparallel double helices (Switzer et al., 1993; Tor & Dervan, 1993).

iG also pairs with C and they form parallel duplexes (Seela, Wei, & Kazimierczuk, 1995). Quadruplexes with iG base were first published by Frank Seela (Seela, Wei, & Melenewski, 1996): the sequence T\(_4\)(iG)\(_3\)T\(_4\) formed four-molecular parallel tetraplexes, [T\(_4\)(iG)\(_3\)T\(_4\)]\(_4\), with the assumed circular double hydrogen bonds of N6H-N2 and N1H-O2. The N7 is not involved in this base-pairing. This means that the iG-tetrads are not formed by Hoogsteen-pairing, as proved by the observation that the replacement of N7 of iG by CH did not hinder quadruplex formation (Seela, 1997). The [T\(_4\)(iG)\(_3\)T\(_4\)]\(_4\) was described as a more stable structure than the unmodified [T\(_4\)G\(_4\)T\(_4\)]\(_4\) in buffered 1M NaCl, 10 mM MgCl\(_2\), pH 7, based on CD spectroscopy. The unmodified tetraplex had a positive ellipticity around 270 nm and a negative one near 240 nm at 40°C, whereas the iG-containing tetraplex had a positive peak at 305 nm, a near zero peak at 275 nm, and a negative peak at 240 nm (Seela et al., 1996).

iG can form not only tetrads but also five-base pentads, giving rise to pentaplexes (Rosenzweig et al., 2009). Chaput and Switzer (Chaput & Switzer, 1999) synthesized [T\(_4\)(iG)\(_3\)T\(_4\)]\(_4\) and [T\(_8\)(iG)\(_4\)T\(_4\)]\(_4\) as well as [T\(_4\)(iG)\(_3\)T\(_4\)]\(_5\) and [T\(_8\)(iG)\(_4\)T\(_4\)]\(_5\). While the unmodified T\(_4\)G\(_4\)T and T\(_8\)G\(_4\)T formed tetraplexes in both K\(^+\) and Cs\(^+\) solutions (Venczel & Sen, 1993), the iG-containing T\(_4\)(iG)\(_3\)T and T\(_8\)(iG)\(_4\)T formed pentaplexes in K\(^+\) and, especially in Cs\(^+\) solu-
Gros et al. (2007) incorporated the nucleotide of \( c^2G \) into various G-positions of the TG3T and TG3T sequences, which sequences form tetramolecular parallel quadruplexes. The \( c^2G \)-tetrad-containing tetramolecular quadruplexes showed similar CD spectra in K\(^+\) or NH\(_4\)\(^+\) solution as the unmodified parallel tetramolecular quadruplexes did, although the Hoogsteen-type H-bonding could not be formed within the \( c^2G \)-tetrad as the acceptor N7 was missing. Instead of the N7, a CH group is present in \( c^2G \). Due to the steric requirements of the hydrogen atom of CH7 the \( c^2G \)-tetrad becomes sufficiently deformed, as compared to G-tetrad(s), to change the stacking(s) with neighboring G-tetrad(s). These effects resulted in large reduction in the thermal stability of the \([TG_5T_4]\) analogs, as compared to the \( T_m \) of the wild-types. The stability decrease was especially drastic in position 4 of G5 in Na\(^+\) solution.

### 2.2.5. 7-deazaguanine (\( c^7G \))

Not only the \( iG \)-nucleotides but also \( n^8c^7iG \) nucleotides can form tetrads and pentads, and thus, the sequences containing this analog of guanine can form quadruplex and pentaplex scaffolds. Seela and Kroschel (2001) observed by ion-exchange chromatography that the \( T_m(n^8c^7iG)_{12} \) oligodeoxynucleotide molecules self-assembled into pentaplexes in the presence of Cs\(^+\) cations, whereas quadruplexes were formed with Na\(^+\) or Rb\(^+\) ions.

### 2.2.6. 8-aza-7-deaza-isoguanine (\( n^8c^7iG \))

Esposito et al. (2010) have incorporated the AP-site mimic tetrahydrofuranyl residue in place of each G of the heptamer TG4T, and concluded that all modified sequences preserved the ability to form tetramolecular quadruplexes containing AP-tetrads. All these modified quadruplexes showed the characteristic CD spectrum of the parallel quadruplexes, just like the parent quadruplex did. Stability, however, changed in a sequence-dependent way in buffered 70 mM KCl (Table 5).

The effects of a guanine AP-site on the quadruplex structure have also been studied by Školáková et al. (2010) using the analogs of the htel-21 G5(TTAGGG)3 sequence. This sequence may better predict the structures existing in vivo than the tetramolecular model quadruplexes (Esposito et al., 2010) can do. The tetrahydrofuranyl AP-site was also used here to replace one-by-one each of the 12 G nucleotides of the G5(TTAGGG)3 that formed a 3-tetrad quadruplex in both Na\(^+\) and K\(^+\) (Table 6).

The number 0 stands for the wild-type htel-21; Oligonucleotides 3,7,15,19 contained the AP-site in the “top” terminal tetrad, 2,8,14,20 in the middle tetrad, and 1,9,13,21T in the “bottom” terminal tetrad that contained the diagonal loop in Na\(^+\). The 21T is a 22mer oligonucleotide. The structures formed by the sequences 8, 14, and 20 were mixed-fold quadruplexes, based on non-denaturing Polyacrylamide Gel Electrophoresis (PAGE); therefore, the \( T_m \)s refer to averages, and the free energies were not calculated.

None of the incorporated AP-sites hindered the formation of quadruplex structures in either salt solution, but each AP-site reduced the thermodynamic stability, as compared to that of the parent quadruplex in both Na\(^+\) and K\(^+\). Destabilizations depended on the sequence position of substitution, and were enthalpic in their origin. The loss of a single G base in any position did not change the Na\(^+\)-stabilized monomolecular antiparallel architecture of the parent quadruplex, based on CD spectra (Figure 2). In K\(^+\) solution however, the AP-site in
most quadruplex analogs hindered the conformational transition to the K⁺-stabilized fold that formed in the unmodified G₃(TTAGGG₃)₃ (Figure 2). Only two, middle-tetrad-modified quadruplexes showed CD spectral changes similar to that observed with G₃(TTAGGG₃). Spectra of all the other AP-quadruplexes remained rather the same in K⁺ as they were in Na⁺. These findings were very similar to those observed with the 6°G-modified quadruplexes of G₃(TTAGGG₃) (Vorliková et al., 2012).

Fujimoto, Nakano, Miyoshi, and Sugimoto (2011) also used the one-by-one substitution model to replace each G by a single AP site in the A(htel-21) AG₃(TTAGGG)₃. The abasic sites did not change the CD spectra of parallel architectures regardless of the position of the modified tetrad in [TG₄T]₄ or [TG₅T]₄ quadruplexes. The authors concluded that the stable docking platforms provided by the full G-quartets neighboring the modified tetrad were the structural bases of quadruplex formation (Gros et al., 2007).

### 3.2. A-tetrads

Gavathiotis and Searle (Gavathiotis & Searle, 2003) found that the A-tetrad in the tetramolecular quadruplex [TTAGGGT]₄ was relatively stable due to its anti glycosidic torsion angles and the H-bonding network of N6-H-to-N1. A-tetrads were also formed in the tetramolecular parallel quadruplexes of other truncated htel sequences, such as the AG₃T and TAG₃T in K⁺ solution, as determined in an NMR study by Patel, Koti, and Hosur (1999). Contrary to the A-tetrad in [TAG₃T]₄ quadruplex, the A-s in the A-tetrad of [AG₃T]₄ were in syn N-glycosidic conformation, whereas the G-s in the G-tetrads were all anti. The syn A-tetrad stacked well with the adjacent G-tetrad. An A-tetrad was also supposed to have formed by a modified htel-21 oligodeoxy-nucleotide, the AGGG TTAGGA TTAGG TAGGA in both Na⁺ and K⁺ solutions, according to the CD spectra, non-denaturing PAGE, and dimethyl sulphate (DMS) footprinting results. The A-tetrad formed in the terminal tetrad with the diagonal loop (in Na⁺) of the three-tetrad

<table>
<thead>
<tr>
<th>Table 6. Guanine abasic sites in a human telomer quadruplex. (see Školáková et al., 2010)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In 0.169 M Na⁺</strong></td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td><strong>Tₘ (°C)</strong></td>
</tr>
<tr>
<td>0 GGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>1 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>2 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>3 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>4 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>5 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>6 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>7 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>8 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>9 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>10 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>11 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>12 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>13 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>14 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>15 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>16 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>17 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>18 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>19 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>20 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>21 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>22 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>23 APGGTTAGGGTTAGGGT</td>
</tr>
<tr>
<td>24 APGGTTAGGGTTAGGGT</td>
</tr>
</tbody>
</table>

3. A, C, T, and U bases and their analogs in place of G in tetrads, pentads, hexads, heptads, and octads

### 3.1. A-, C-, T-, and U-tetrads in tetramolecular quadruplexes of TG₄T and TG₅T

In a basic structural study, Mergny’s group (Gros et al., 2007) found that incorporation of any non-G base, such as the A, C, T, and U was detrimental to the thermal stability (T₁/2) of the tetramolecular parallel quadruplexes formed by the TG₄T and TG₅T sequences, and the association rates also decreased. (T₁/2 is midpoint temperature of thermal unfolding of quadruplexes, and is used in cases if refolding occurs with hysteresis.) The destabilization effect depended on the type of the substituting base and the position of the G replaced. On the other hand, all A₄, C₄, T₄, and U₄ tetrad-containing tetramolecular quadruplexes displayed CD spectra characteristic of parallel architectures regardless of the position of the modified tetrad in [TG₄T]₄ or [TG₅T]₄ quadruplexes. The authors concluded that the stable docking platforms provided by the full G-quartets neighboring the modified tetrad were the structural bases of quadruplex formation (Gros et al., 2007).
3.3. The G-to-A substitutions

Contradictory results have been published on the role of an A base in a G-tetrad of a quadruplex: several studies have found that incorporation of a single A prevented the formation of a quadruplex, and a few other studies have shown that quadruplexes do form with an A base in a G-tetrad even if the quadruplexes are significantly destabilized. Pedroso, Duarte, Yanez, Baker, and Fletcher (2007) and Pedroso, Duarte, Yanez, Burkevitz, and Fletcher (2007) found the alteration of the TTAGGG partial sequence to TTAGAG in the 1st, 3rd, or 4th repeat of the 24mer (TTAGGG)₄ sequence (not a tetramolecular quadruplex) the quadruplex formed was intermolecular in Sr²⁺ solution, but an intramolecular structure was observed when the second repeat was modified by an A for G substitution. The CD spectra suggested that the structures, both the inter- and the intramolecular, were parallel-stranded quadruplexes. The same research group (Pedroso, Hayward, & Fletcher, 2009) also described that the (TTAGGG)₄ₙ sequence (Pedroso, Hayward, & Fletcher, 2009) also described that parallel-stranded quadruplexes. The same research group structures, both the inter- and the intramolecular, were for G substitution. The CD spectra suggested that the loops, and the connector Tₙ (n = 1–7) separated the two quadruplex units. The authors also studied the quadruplexes formed by adenine-mutated sequences, stating that the A nucleotides prevented the formation of a quadruplex. Table 8 shows the stability data.

The cationic porphyrin TmPyP4 is a known DNA G-quadruplex stabilizer (Qin, Rezler, Gokhale, Sun, & Hurley, 2007) although it has been observed to destabilize a bimolecular DNA quadruplex (Weisman-Shomer et al., 2003). Recently, Morris, Wingate, Silwal, Leeper, and Basu (2012) described another example for quadruplex destabilization by TmPyP4. The ligand was noted to unfold the extremely stable all purine RNA G-quadruplex, called M3Q, in a concentration-dependent manner: 50% unfolding of 4 μM of RNA quadruplex by 11 μM of TmPyP4 in 100 mM KCl. The M3Q RNA sequence is in the 5'-untranslated region MT3-MMP (matrix metalloproteinase) mRNA, and forms a three-tetrad monomolecular parallel quadruplex with a Tₘ value of 72°C in 1 mM KCl. The 20-nucleotide long RNA sequence, ga ggg a gag a ggg a, its DNA version, and a mutated RNA sequence containing both adenosine and uridine mutations, the ga gua a gua a gua a were studied by the authors. The TmPyP4 also destabilized the DNA version of the M3Q quadruplex (Tₘ = 71°C in 100 mM KCl), but the unfolding would have required higher concentrations of the ligand than was needed to unfold the RNA quadruplex. In a eukaryotic cell culture, TmPyP4 enhanced the translational activity of a reporter gene construct that contained M3Q by unfolding the RNA quadruplex, whereas the incorporated adenine and uridine-mutated RNA sequence did not affect the translation inhibition. The authors found earlier (Morris & Basu, 2009) that this mutated RNA sequence did not form quadruplex. A recent study by Beckett, Burns, Broxson, and Tomaletti (2012) also showed the negative effect of the G-to-A substitut-
occurring at the nuclease hypersensitive element III of the human c-myc promoter, did not prevent but instead modulated the formation of quadruplex structures. This five G-repeat sequence could fold into two types of hybrid (3 + 1) structures, myc-2345 and myc-1245 quadruplexes. The analogs in position G12 hindered the formation of the transcription regulator myc-2345 quadruplex but promoted the formation of the myc-1245 quadruplex. Either base lesion accommodated into a large loop containing the 3rd G-run. An additional G-to-A substitution in position 3 also influenced the folding: the myc-1245 was unable to form, instead the myc-2345 quadruplex formed although it contained the base lesion.

Contrary to the above mixed results, Tomaško et al. (2009) demonstrated that a single A substitution for any G base of the 21mer G3(TTAGGG)3 sequence did not hinder the formation of intramolecular antiparallel three-tetrad quadruplexes in either Na+ or K+ solution. In Na+, the A-for-G exchange in the two terminal tetrad did not change the CD characteristics of the unmodified htel-21 quadruplex; however, the substitution in the middle quartet led to 2-maximum CD spectra, one at 290–295 nm, the other at 245–250 nm, and these spectra resulted from a mixture of quadruplex architectures based on non-denaturating PAGE runs. The unmodified quadruplex underwent a conformational rearrangement when Na+ ions were replaced by K+, but none of the A-substituted quadruplexes did so. The A-for-G substitution in each G-position decreased the thermal stability of the parent quadruplex in both Na+ and K+, and the effect depended on the sequence position and the tetrad of substitution. Reduction in Tm ranged from 1.5 to 29 °C in Na+, and from 10 to 28 °C in K+.

The middle tetrad of the three-tetrad quadruplex was the most sensitive to the base exchange in both salts; the average destabilizations were 24.6 and 25.3 °C, respectively, in Na+ and K+ solutions (Tomaško et al., 2009). Results of another study from the same group (Sagi et al., 2010) showed that even double-A substitutions in the sequence positions corresponding to the diagonal-looped terminal tetrad (as defined in Na+) of the 21mer G3(TTAGGG)3 hindered the formation of monomolecular antiparallel quadruplexes in Na+ or K+ solutions. Double-A substitutions gave rise to G:A-G:A and G:G:A tetrad. Table 9 displays the sequences studied and the thermodynamic stability data determined.

The full A-tetrad (A4) in K+, however, was close to half-unfolded at 37 °C (Sagi et al., 2010). Another study (Derecka et al., 2010) also reported that a single G-to-A polymorphism did not affect the formation of stable parallel quadruplexes in a 22mer sequence, a G-rich insert in the 5′-exon of the bovine estrogen receptor α gene. Triple mutations of GGG to AAA, however, destabilized the quadruplex, giving rise to enhanced translational efficiency. The transcribed RNA also formed stable parallel quadruplex.

### 3.4. Comparison of the effect of natural base lesions on the structure and stability of the quadruplex of an htel-21 sequence

The adenine nucleotide (A) can be incorporated in vivo into telomeric DNA through spontaneous deamination of 5-methylcytosine and cytosine to thymine and uracil, respectively, and then by the G-to-A transition during replication (references in Tomaško et al., 2009)). Another lesion, the abasic (AP) site is the most common lesion of the genomic DNA formed by spontaneous base loss, mainly by depurination, and as an intermediate in the enzymatic base excision repair process of various base lesions (references in Školáková et al., 2010). ROS damage the DNA in vivo, and hydroxyl radical is thought to be the major causative agent. The hydroxyl radical can react with the sugars and the bases of nucleic acids. 8-oxo-7,8-dihydroguanine, or 8-oxoguanine (8OG) and its further oxidized derivatives are the most frequent products. Recent studies estimated the levels of 8OG to be

<table>
<thead>
<tr>
<th>G-to-A substitutions</th>
<th>In 0.169 M Na+</th>
<th>In 0.169 M K+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tm (°C)</td>
<td>∆G37 (kcal/mol)</td>
</tr>
<tr>
<td>0 GGG TTAGGG TTAGGG TTAGGA</td>
<td>67.7</td>
<td>−4.43</td>
</tr>
<tr>
<td>1–21 AGG TTAGGG TTAGGG TTAGGA</td>
<td>63.4</td>
<td>−3.69</td>
</tr>
<tr>
<td>9–13 AGG TTAGGA TTAGGG TTAGGG</td>
<td>62.6</td>
<td>−4.02</td>
</tr>
<tr>
<td>1–13 AAGG TTAGGG TTAGGG TTAGGA</td>
<td>60.0</td>
<td>−3.16</td>
</tr>
<tr>
<td>9–21 GGG TTAGGA TTAGGG TTAGGA</td>
<td>53.4</td>
<td>−2.12</td>
</tr>
<tr>
<td>An: A4: A4G TTAGGA TTAAAG TTAGGA</td>
<td>53.5</td>
<td>−2.18</td>
</tr>
</tbody>
</table>

Note: Symmetry of substitutions: o, for opposite; n, neighbors; A₄, A-tetrad by 1–9–13–21 substitutions; the bold numbers are for the syn-dG positions in Na+ solution.

### References

between 0.3 and 4 lesions in 10⁶ bases (references in Vorlicková et al., 2012). The A, o8G, and the AP-site are very different nucleotide derivatives from structural point of view. The A lacks oxygen atoms, thus cation coordination becomes less effective in any G1:A tetrad than it can be in the G4-tetrad, and the A inevitably leads to disruption of the circular H-bonding of the G-tetrad. The AP-site, which lacks the heterocyclic base, was thought to have a dramatic effect on the physical properties of a quadruplex. o8G has the closest resemblance to guanine, and thus a much smaller effect on structure would be predicted relative to the AP-site. Despite the great differences in the steric structures of these three derivatives, very similar effects on the properties of the three-tetrad quadruplex of htel-21 G₃(TTAGGG)₃ (Figure 4) were observed.

3.4.1. Effects on the folding properties
The presence of A₃, AP-site or o8G in the terminal (top or bottom) tetrad hindered the rearrangement of the modified quadruplexes to the K⁺-stabilized fold that was observed to happen with the unmodified htel-21 quadruplex. The middle tetrad-substituted sequences formed bimolecular structures in addition to monomolecular scaffolds in Na⁺ and/or K⁺ solutions.

3.4.2. Effects on stability
The middle tetrad was the most sensitive part with respect to stability, with the largest destabilization effects being observed with these modified quadruplexes, −12 to −31 °C in ΔTm. Unexpectedly, it was not the missing G base (the AP-site) that caused the largest negative change (Školáková et al., 2010). The G analog m6G can also be used in this comparison: among the single m6G nucleotides incorporated into each tetrad of the three-tetrad 22mer A(htel-21) quadruplex (Mekmaysy et al., 2008) the middle-tetrad-modified quadruplex was destabilized by the largest extent. The replacement of G in the bottom diagonal loop-connected tetrad caused the smallest destabilizations in Na⁺ but this effect almost disappeared in K⁺ solution. Substitution of G by A₃, AP-site or o8G in position 3 of htel-21 caused the smallest negative effects among the substituted other G-s of the top tetrad, especially in Na⁺.

These three natural base lesions, the o8G, the guanine abasic (AP) site and the A were studied together in one sequence recently Beckett et al. (2012). The 27mer nuclease hypersensitive element III₁ of human c-myc proto-oncogene has the following sequence,

\[
\text{TGGGG A GGG TGGGGA GGG T GGGG AAGG.}
\]

that can form two types of parallel G-quadruplexes depending on which G-triplet is involved: one with the 1245 G-triplets and the other with the 2345 triplets in K⁺ solution. The 1245 quadruplex contains a 6-nucleotide loop composed of TGGGGA. The authors found that a single substitution of the nucleotide G12 (in bold) by o8G or an AP-site prevented the formations of the 2345 quadruplex, but not the formation of the 1245 quadruplex that accommodated the lesion in the long loop. However, if in addition to the these lesions a single A was also incorporated into the position G3 (bold), which was supposed to hinder the formation of the 1245 quadruplex, the 2345 quadruplex did form, thus incorporating the o8G or the AP-site into the G-tetrad. (This study is also discussed under the heading Loop substitutions.)

3.5. T-tetrads
Using NMR techniques David Lilley’s group (Aboul-ela, Murchie, Norman, & Lilley, 1994), determined in 1994 the solution structure of the hexamer oligodeoxynucleotide TG₄T in 100 mM Na⁺ and found that it formed a parallel-stranded quadruplex with hydrogen-bonded guanine tetrads, [TG₄T]₄. The quadruplex structure was stabilized in K⁺, relative to Na⁺, without any significant change in the quadruplex conformation. The authors did not observe the formation of T-tetrads. The research group later determined also the crystal structure of [TG₄T]₄ at 0.95 Å resolution (Phillips, Dauter, Murchie, Lilley, & Luisi, 1997). The four strands formed a right-handed helix stabilized by hydrogen-bonded tetrads of co-planar guanine bases. The sodium ions were found between and within tetrad planes and were coordinated with the guanine O6 groups. Hydrogen-bonding water patterns were observed within the quadruplex’s helical grooves and clustered about the phosphate groups. The thymine bases did not contribute to the four-stranded fold, but stacked to stabilize the crystal lattice. Subirana’s group has later also determined the crystal structure.
of the quadruplex formed by TG₄T molecules (Cáceres, Wright, Gouyette, Parkinson, & Subirana, 2004). The parallel tetramolecular quadruplex had a very uniform structure independently of the ions present, whereas the terminal thymines could adopt a variety of structures: stacked T-triad at the 3′-ends of the quadruplexes, T-tetrad between two guanine tetrads, and completely disordered thymines which did not show any clear location in the crystal. The tetrads were stabilized by either Na⁺ or Ti⁺ ions. Formation of T-tetrad in solution have also been observed, for example, with symmetrical pairing of the T-s via O4-H3 H-bonds in the center and the 5′-end of the quadruplex of TGGTGGC sequences (Borbone et al., 2005; Oliviero et al., 2006; Patel & Hosur, 1999).

3.6. G-to-T mutations

The htel-24 sequence T₂G₃(TTAG₃)₂A, or TT(h tel-21)A, adopted an intramolecular antiparallel fold in Na⁺, and a hybrid (3 + 1) form in K⁺ solution (Dumas & Luédtké, 2011). Substitutions of G9 and G23 by T did not change the wild-type’s antiparallel fold in Na⁺ according to CD spectra, but in K⁺ the T substitutions of both G-positions rearranged the hybrid-characteristic of the CD spectrum to the antiparallel:

HT-G9  dTT GGG TTA GGG TTA GGGA
HT-G23  dTT GGG TTA GGG TTA GGGA

The positions of G replaced by T were in one of the two terminal tetrads of the three-tetrad quadruplex. Both substitutions caused drastic reduction of thermal stabilities in both salts. In Na⁺ solutions the ΔTm values were −14 and −8.4 °C, respectively, for the HT-G9 and the HT-G23 quadruplexes, whereas the duplexes formed with the complementary 24-mers were destabilized only by 8.2 and 1.5 °C, respectively. In K⁺, the destabilizations were even more drastic; the ΔTm was −23 °C for both quadruplexes (Dumas & Luédtké, 2011). It was observed with various base analogs of G that the quadruplex was significantly more destabilized than the duplex, for example o₈G (Vorlicková et al., 2012).

3.7. Mixed-base tetrads, pentads, hexads, heptads, octads

The cytosine (C)-containing dodecanucleotide GGGCT₄GGGC in Na⁺ formed a quadruplex through head-to-tail dimerization of two symmetry-related stem-hairpin loops with the adjacent strands antiparallel to each other. The connecting T₄ loops were lateral-type, resulting in a quadruplex structure containing two internal G-G:G-G tetrads flanked by G:C:G:G tetrads (Kettani et al., 1998). The quadruplex formed by the GCGGTGGC sequence in Na⁺ solution was a dimer of dimers containing two parallel stranded G:G:G:G tetrads flanked by an A:A mismatch and by four-stranded G:G:G:G tetrad (Webba da Silva, 2003). The CD spectrum of the htel-22 analog AG₃(C-TAG₃)₂ in K⁺ was similar to its spectrum in Na⁺ solution, but NMR determined a novel chair-type antiparallel fold with two G-tetrad sandwiched between a G:C base pair and a G:C:G:C tetrat (Lim et al., 2009).

A G:C:G:C tetrad and a non-G hybrid tetrad of A:T:A:T were formed by the cyclic octamer sequences <TGCTCGT> and <CATTCATT> (Escaja, Pedroso, Rico, & Gonzalez, 2000) and also by the GAGCAGGT sequence in 1 M NaCl. In this solution the GAGCAGGT formed a head-to-head dimeric quadruplex (Zhang et al., 2001).

Each of the two short linear sequences TGCTCGT and TCGTTCGT, and one cyclic oligonucleotide <CGCTCGGT> self associated and formed symmetric dimer quadruplexes containing another unusual tetrad, the C:G:G:C (Viladoms et al., 2009). The quadruplex of the CGGTGGC sequence contained G(C):G(C):G(C):G(C):G(C):G(C)<octads through 5′-5′ stacking of two tetramolecular quadruplex subunits, as revealed by CD and NMR studies. The C bases in parentheses were external to the G-tetrad (Borbone et al., 2011).

The quadruplex formed by GCGGTGGAT contained a T:A:A:T tetrad and a T:(G:G:G:G):T hexad Webba da Silva, 2005. A quadruplex with an all four-base tetrad, G:C:A:T was described as being the solution structure of the cyclic oligonucleotide <CGCTCAATT> (Escaja et al., 2003).


Patel and coworkers (Zhang et al., 2001) recognized a stable A-(G-G-G-G)-pentad in the dimeric quadruplex formed by G₃AG₂T₃G₃AT in 100 mM NaCl. They also reported (Kettani et al., 2000) on the formation of a A-(G-G-G-G)-A hexad in the dimeric quadruplex of the GGAGGAG sequence in 150 mM Na⁺ (or K⁺) solution. The A bases in the pentad and hexad were in extra-tetrad positions of the G-tetrad.

3.8. Adenine and thymine analogs in tetrads

Patracco et al. (2005) and Esposito et al. (2005) replaced the adenines by 8-bromo adenine (br₈A), and 8-(1-propynyl)adenine (py₈A) in the TAGGGT and AGGGT sequences. According to the CD and NMR spectra the substituted sequences formed parallel-stranded tetramolecular quadruplexes, similarly to the unmodified sequences. Unfolding thermodynamics have
also been determined from the CD melting curves measured in buffered 1 M KCl (Table 10).

<table>
<thead>
<tr>
<th></th>
<th>( T_m ) (°C)</th>
<th>( \Delta G_{298} ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[TAGGGT](_4)</td>
<td>51</td>
<td>20.1</td>
</tr>
<tr>
<td>[Tbr(_8)AGGGT](_4)</td>
<td>60</td>
<td>21.5</td>
</tr>
<tr>
<td>[Tp(_8)AGGGT](_4)</td>
<td>53</td>
<td>21</td>
</tr>
<tr>
<td>[AGGGT](_4)</td>
<td>37</td>
<td>19.3</td>
</tr>
<tr>
<td>[br(_8)AGGGT](_4)</td>
<td>48</td>
<td>19.8</td>
</tr>
<tr>
<td>[py(_8)AGGGT](_4)</td>
<td>53</td>
<td>19.6</td>
</tr>
</tbody>
</table>

Both analogs of adenine stabilized the tetramolecular quadruplex. Stabilizations were clearly reflected by the \( T_m \) values, whereas free energy change values were close to each other in the case of the [AGGGT]\(_4\) analogs. The quadruplexes of the TAGGGT sequences did not contain T, A or 8-substituted-A tetrads. On the other hand, the quadruplex of the shorter unmodified sequence formed an A-tetrad at the 5'-end. There was no word about the effect of the 8-substituted adenines on this A-tetrad. The same research group also studied 8-methyladenine (m\(_8\)A) in m\(_8\)AGGGT and Tm\(_8\)AGGGT, which formed fourfold symmetric parallel G-quadruplexes just as the unmodified sequences did. The m\(_8\)A residues in the m\(_8\)A-tetrad were in the syn-orientation and a symmetrical arrangement of m\(_8\)A bases stacking onto the adjacent G-tetrad was observed (Randazzo et al., 2005; Virgilio, Esposito, Randazzo, Mayol, & Galeone, 2004). They also studied the quadruplexes formed by the TAGGGT and AGGGT sequences with 8-oxoadenine (o\(_8\)A) (Esposito, Randazzo, Virgilio, Cozzuto, & Mayol, 2005) and 8-hydroxyadenine (oh\(_8\)A) (Petracco et al., 2007) replacing the adenines. CD spectra showed that these modified sequences were also able to form tetramolecular parallel-stranded quadruplexes.

Hypoxanthine (I) was incorporated into the *Oxytricha* telomere repeat oligonucleotide G\(_4\)T\(_4\)G\(_4\) and studied by \(^1\)H and \(^31\)P NMR spectroscopy by Smith & Feigon (Smith & Feigon, 1993). The I replaced a G in a tetrad: GGGG TTTT GGGG. The oligonucleotide formed a diagonally looped, symmetrical bimolecular antiparallel quadruplex in both Na\(^+\) and K\(^+\) with four G-tetrads and T-loops at the opposite ends of the quadruplex. In another study, Smith et al. (1995) described the incorporation of both I and U into G\(_4\)(T\(_i\)G\(_i\))\(_3\) (Oxy-3.5), GGGG T\(_U\)U GGGG TTTT GGGG U\(_T\)TTT GGGG, where again, the I was a tetrad substitution. They concluded from \(^1\)H and \(^31\)P NMR measurements that both the unmodified and the I- and U-modified sequences formed a monomolecular quadruplex with a diagonal T4 loop.

Petracco et al. (2003) incorporated the thymine analog 5-hydroxymethyluracil (hm\(_5\)U) into the TGGG\(_T\)T sequence that formed [TGGGT]\(_4\) parallel quadruplexes. The three-dimensional structure of the hm\(_5\)U-containing quadruplex was very similar to those of other parallel stranded quadruplexes. The hm\(_5\)U bases were able to form, at least in the minimised structures a tetrad that contained extra H-bonds via the hydroxyl groups. In this new tetrad the hm\(_5\)U bases were slightly open toward the solvent with respect to the unmodified T-tetrad.

### 4. Loop substitutions

In the loop-forming sequences of synthetic oligodeoxyribonucleotides the thymine and adenine have been replaced by the natural bases U, C, A, I, and T, respectively, and also by base analogs, such as br\(_8\)U, io\(_8\)U, n\(_8\)P, o\(_8\)G, and AP sites.

#### 4.1. Substitutions of loop’s thymines

Smith and Feigon published first on the effect of uracil U on the structure of a DNA quadruplex formed by the oligonucleotide G\(_4\)U\(_T\)T\(_4\)G\(_4\), which they determined by NMR techniques. Both the parent G\(_4\)T\(_4\)G\(_4\) and the G\(_4\)U\(_T\)T\(_4\)G\(_4\) folded into a symmetrical bimolecular quadruplexes with four G-quartets and diagonal (U)T-loops at the opposite ends of the G-quartets in both Na\(^+\) and K\(^+\) solutions (Smith & Feigon, 1993). In another paper, Smith et al. (1995) described the incorporation of U into G\(_4\)(T\(_i\)G\(_i\))\(_3\) (Oxy-3.5), GGGG T\(_U\)U GGGG TTTT GGGG U\(_T\)TTT GGGG, where the I was a tetrad substitution. They
concluded from $^1$H and $^3$P NMR measurements that both the unmodified and the $U$- and $I$-modified sequences formed a monomolecular quadruplex with a diagonal T4 loops. On the other hand, Philip Bolton and coworkers have found that quadruplex formation was inhibited by a single $s^5G$ in position 2 of Gs$^5$GTUUGGUTTG-GGUUTTGG, a $U$-containing, extended loop-modified TBA oligodeoxynucleotide (Marathias et al., 1999).

Phan and Patel (Phan & Patel, 2003) published an NMR study on the structures of quadruplexes in which uracil and 5-bromouracil were incorporated into the TTA loop of the htel-12 oligonucleotide TAGGGTTAGGGT. This sequence could form both parallel and antiparallel dimeric quadruplexes with three G-tetrads in K$^+$. The TAGGG$U$ATTAGGGT formed predominantly a parallel quadruplex with double-chain reversal loops, with all guanines in the *anti* position. The other analog, TAGGG$br^5U$TATTAGGGT predominantly formed an antiparallel dimeric quadruplex with edgewise loops with six *syn* and six *anti* guanines. The folding was temperature-dependent.

For structural photochemistry, that is, for the formation of deoxyribolactones by 30-min UV irradiation at 302 nm of antiparallel and parallel quadruplexes, each T base of the three loops has been replaced one-by-one by 5-iodouracil (i$U$) in the htel-22 AG$_3$(TAG)$_3$ and the [G$_4$T$_4$G$_4$]$_2$ sequences by Xu and Sugiyama (Xu & Sugiyama, 2004). Results showed that the lactones formed only in the diagonal loop of antiparallel quadruplexes, either of the Na$^+$-form of the htel-22 or in the bimolecular antiparallel quadruplexes of both the [G$_4$TTi$5$UG$_4$]$_2$ and the [G$_4$TTi$5$UG$_4$]$_2$. In the htel quadruplex the lactone formed in the Na$^+$-AG$_3$TAAAGG-Ti$5$UAGGGTTAGGG sequence. No formation of lactones was observed in the K$^+$-stabilized fold of the htel-22, or in duplexes of the same sequences, hairpins or single stranded DNAs. The authors regarded this reaction as a specific probe for the antiparallel G-quadruplexes containing diagonal loops.

Sacca, Lacroix and Mergny (2005) incorporated multiple U bases in place of T-s in the loop-forming region of various sequences, such as the intramolecular quadruplex-forming 15mer TBA, the 18mer and 22mer htel sequences, a dimer forming 12mer and the tetramolecular parallel quadruplex forming hexamer. UV absorption based thermodynamics, and in some cases DSC-based thermodynamics of unfolding/folding was determined with 5 or 10 μM strand concentrations of the oligonucleotides in 10 mM sodium cacodylate buffer, pH 7, and 100 mM of either NaCl or KCl (Table 11).

Substitution of T-s by U-s in various sequences had different effects on thermostability. The effect depended on the quadruplex fold and also on the cations present in the solution. An important result of the experiments came from the determination of the transition enthalpy for quadruplex unfolding of the htel-22 by two methods, the model-dependent UV absorption-based and the model-independent calorimetric techniques. Both techniques provided similar results: $\pm$45.0 and $\pm$41.5 kcal/mol, respectively. This result provided proof, for the first time, that the melting of this 22mer quadruplex was a two-state process: only the folded and unfolded molecules were present in significant amounts during melting and refolding.

Similarly to the results obtained by Sacca et al. (2005) for the T-to-U substitution of the TBA quadruplex, Olsen and Marky (2009) also described that the removal of methyl groups of all six thymines in the loops of the TBA sequence G$_2$T$_2$G$_2$TGTG$_2$T$_2$G$_2$, result-

![Diagram](https://via.placeholder.com/150)

**Table 11.** Thermodynamics of the T-to-U exchanges in quadruplex loops. (see Sacca, Lacroix & Mergny, 2005)

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>$T_m$ in Na$^+$ (°C)</th>
<th>$T_m$ in K$^+$ (°C)</th>
<th>Δ$G_{m}$ in K$^+$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGTTGGTGTGGTTGG</td>
<td>20</td>
<td>48</td>
<td>$\pm$1.57</td>
</tr>
<tr>
<td>GGUUGGUGUGUGUGG</td>
<td>30</td>
<td>54</td>
<td>$\pm$2.52</td>
</tr>
<tr>
<td>AGGGTTAGGTAGGTAGG</td>
<td>40</td>
<td>42</td>
<td>$\pm$0.70</td>
</tr>
<tr>
<td>AGGUUAGGUAGGUAGG</td>
<td>40</td>
<td>56</td>
<td>$\pm$2.81</td>
</tr>
<tr>
<td>AGGGTTAGGTAGGGTTAGG</td>
<td>55</td>
<td>62; 64$^a$</td>
<td>$\pm$3.37$^a$</td>
</tr>
<tr>
<td>AGGUUAGGUAGGUAGG</td>
<td>54</td>
<td>62; 63$^a$</td>
<td>$\pm$3.13$^a$</td>
</tr>
<tr>
<td>[GGGGTTGGTTGG]$^2$</td>
<td>53</td>
<td>Non-reversible, multiphase profile</td>
<td></td>
</tr>
<tr>
<td>[GGGGUUUGGGG]$^2$</td>
<td>50</td>
<td>Non-reversible, multiphase profile</td>
<td></td>
</tr>
<tr>
<td>[TGGGGT]$^4$</td>
<td>55</td>
<td>$\geq$90</td>
<td></td>
</tr>
<tr>
<td>[UGGGGU]$^4$</td>
<td>65</td>
<td>$\geq$90</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Calorimetric determination.
ing in G2U2G2UGUGU2G2G2, increased the stability of the two-tetrad chair-type antiparallel quadruplex, as determined also by both spectroscopic (UV, CD) and calorimetric methods in 10 mM Cs-Hepes, pH 7.5 and 50 mM KCl (Table 12).

They explained the increased stability by the contribution of increased stacking (contrary to the effect observed with DNA duplexes), release of structural water and uptake of counterions. That is, the favorable formation of the U-containing quadruplex (more negative free energy) resulted from the compensation of the unfavorable entropy contribution by the favorable enthalpy, by the uptake of ions and the release of water molecules. The U-loops also contributed with favorable enthalpy through better stacking. The T-to-U substitutions did not change the CD spectrum of TBA quadruplex, only slight blue shifts of the three peaks were observable. As a continuation, Olsen, Lee, and Marky (2009) published again on the thermodynamic effect of base replacements in the loops of the 15mer TBA quadruplex. Both calorimetric (cal) and spectroscopic-derived data were collected, and data shown below refer to folding thermodynamics in buffered 100 mM KCl. The middle TGT loop’s G by was replaced by A, C, T, U, and nPP (2-aminopurine) nucleotides. The more negative free energy of folding was a result of the favorable enthalpy contribution of substitution, partially compensated for by the unfavorable entropy contribution, and the uptake of counterions and release of water molecules. It was interesting that while the exothermic heat of folding increased (more negative enthalpy and free energy) with all loop substitutions, the thermostability decreased in all but two cases. These two cases were the result of T-to-U substitutions of the double T-loops. Based on the CD spectra, all modified TBA sequences formed intramolecular, chair-type antiparallel quadruplexes. Their results showed the importance of the effect of the stacking of loop bases on the stability of a quadruplex (Table 13).

Keith Fox and coworkers (Rachwal, Brown, & Fox, 2007) found by fluorescence melting and CD studies that the replacement of T nucleotides by C or A nucleotides or AP-sites of the three single-base loops of the 17mer TG3TG3TG3TG3T did not change the ability of the oligonucleotides to fold into intramolecular parallel, double-chain reversal loop quadruplexes, as the wild-type sequence did. Their fold was characterized by a strong maximum near 260 nm and a small minimum at around 240 nm in the CD spectra, in both Na+ and K+. Stability was, however, affected by the substitutions. The thermostabilities are shown in Table 14.

While the A nucleotides significantly decreased the stability of the quadruplex, relative to that of the wild-type, C nucleotides only slightly decreased it in both salts. On the other hand, the no-base sugar derivative 1′,2′-dideoxyribose AP-site increased the stability. These results imply that both the sequence and the modification of the loops influence quadruplex stability, in addition to the loop length. However, the stacking of the loop bases may or may not be of primary importance, at least not with the double-chain reversal single-base loops.

With the similar hexadecamer sequence (G3T)4 Anh T. Phan’s group (Do et al., 2011) also found that the T-s were the single-nucleotide loops in the propeller-type parallel-stranded quadruplexes. These quadruplexes formed dimers stacked through their 5′-tetrads in K+ solution. Substitution of all four T-s by C nucleotides in

---

**Table 12.** Effect of the T-to-U exchange in loops on the stability of TBA quadruplex. (see Olsen & Marky, 2009)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>ΔHcal (kcal/mol)</th>
<th>ΔG290 (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2T2G2TGTG2TGTG2</td>
<td>48.1</td>
<td>-22.4</td>
<td>-2.3</td>
</tr>
<tr>
<td>G2U2G2UGUGU2G2G2</td>
<td>53.9</td>
<td>-39.0</td>
<td>-4.0</td>
</tr>
</tbody>
</table>

**Table 13.** Thermodynamic parameters for loop-modified TBA quadruplexes. (see Olsen, Lee, & Marky, 2009)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>ΔHcal (kcal/mol)</th>
<th>ΔG290 (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG TT GG TGT GG TT GG</td>
<td>52.4</td>
<td>-22.9</td>
<td>-2.3</td>
</tr>
<tr>
<td>GG TT GG TAT GG TT GG</td>
<td>45.5</td>
<td>-32.3</td>
<td>-2.6</td>
</tr>
<tr>
<td>GG TT GG TCT GG TT GG</td>
<td>43.3</td>
<td>-34.6</td>
<td>-2.5</td>
</tr>
<tr>
<td>GG TT GG TTT GG TT GG</td>
<td>47.0</td>
<td>-35.7</td>
<td>-3.0</td>
</tr>
<tr>
<td>GG TT GG TnPT GG TT GG</td>
<td>51.6</td>
<td>-36.6</td>
<td>-3.6</td>
</tr>
<tr>
<td>GG TT TT TTT TGT TGG TT TGG</td>
<td>43.2</td>
<td>-33.5</td>
<td>-2.5</td>
</tr>
</tbody>
</table>

**Table 14.** Substitution of loops of the quadruplex formed by TG1TG3TG3TG1T. (see Rachwal, Brown, & Fox, 2007)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>Stability</th>
</tr>
</thead>
</table>
| TG1TG3TG3TG1T | 68.3 | Unmodified with three T-
| TG1TG3AG3TG1T | 60.5 | Two T- & one A-
| TG1AG3TG1AG3T | 52.2 | One T & two A-
| TG1AG3TG1AG3T | 43.7 | Three A-
| TG1CG3CG3CG3T | 67.1 | Three C-
| TG1AP3AP3AP3T | 72.5 | Abasic sites |
| TG1TG3TG3TG1T | 85.6 | Unmodified with three T-
| TG1TG3AG3TG1T | 79.3 | Two T- & one A-
| TG1AG3TG1AG3T | 72.1 | One T & two A-
| TG1AG3TG1AG3T | 64.4 | Three A-
| TG1CG3CG3CG3T | 83.2 | Three C-
| TG1AP3AP3AP3T | 88.1 | Abasic sites |
(GGGC)$_4$ did not change the parallel-stranded dimeric structures, based on NMR and CD spectra.

Lin, Zhang, Zeng, Luo, and Wang (2010) studied the effect of UVA irradiation of a br$_5$U-containing quadruplex. The irradiation gave rise to G(8-5)U intrastrand cross-links, where C8 of G of a G-tetrad was covalently linked to the C5 of the 3’-neighboring U in the loop. Cross-link production depended on the fold of the quadruplex, and thus, this method could be used to determine the conformation of quadruplexes. The experiments were carried out with the htel-22 AG$_3$(TTAGG)$_3$ in 100 mM Na$^+$ and with the htel-26 A$_3$(G$_3$TTA)$_3$G$_3$AA in 100 mM K$^+$. The 22mer formed a basket-type antiparallel quadruplex, whereas the 26mer formed a hybrid (3 + 1) type quadruplex. Incorporation of br$_5$U into any of the three loops and the formation of cross-links did not change the basket or the hybrid folds, based on CD spectra. In the presence of K$^+$ ions the yield for the cross-links was very similar to the case when br$_5$U was incorporated into any of the three loops of the 26mer. However, in the presence of Na$^+$ ions, the yield for the cross-links formed in the diagonal loop of the 22mer was 2–3-fold higher than those of formed in the two lateral loops. The reason might be that the first T (5’), thus also the isosteric br$_5$U in the same position of the diagonal loop stacks better to neighboring (5’) G in the Na$^+$-basket structure than the corresponding T and thus br$_5$U do in the two edge loops. This theory was reinforced by the authors (Lin et al., 2010) using the Oxytrichia sequence G$_4$(T$_4$G$_4$)$_3$ that also forms a basket-type quadruplex in Na$^+$ (Wang & Patel, 1995).

4.2. Substitutions of the adenine

Philip Bolton’s group (Rujan, Melaney, & Bolton, 2005) replaced the base A by T in the GGGTTAGGG oligodeoxynucleotide. This sequence folded into a bimolecular antiparallel quadruplex in Na$^+$, and converted into a bimolecular parallel quadruplex in K$^+$. The A-to-T substitutions were thus in the two diagonal loops. Based on the CD spectra, the substitutions did not change the ion-sensitivity of the [GGGTTTGGG]$_2$ quadruplex, as compared to that of the wild-type, although there were some differences in the ratio of intensities at 260 and 290 nm.

For an NMR study Lim et al. (2009) exchanged the A by C in one of three loops of the htel-22: G$_3$(TTAGG)$_3$TTCCGGGT. The effect of substitution was not detailed.

Wei, Parkinson, Reszka, and Neidle (2012) published on the 1.62 Å crystal structure of the intramolecular quadruplex of AG$_3$AG$_3$CGCGbr$_5$UG$_3$AGGAG$_3$, a modified deoxyoligonucleotide from the promoter region of the c-kit gene in which the A nucleotide was replaced by br$_5$U. The br$_5$U was incorporated to aid in crystallographic phasing. This was the first reported crystal structure of a promoter quadruplex and the first observation of localized magnesium ions in a quadruplex structure.

The fluorescent 2-aminopurine has been widely used in structure analysis of quadruplexes. For example, n$_2$P has been substituted for all four adenines of the htel-22 sequence for conformational studies by Li, Correia, Wang, Trent, and Chaires (2005):

\[
\begin{align*}
\text{n}_2\text{PGGG TTAGGG TTAGGG TTAGGG} \\
\text{AGGG TTn}_2\text{PGGG TTAGGG TTAGGG} \\
\text{AGGG TTAGGG TTn}_2\text{PGGG TTAGGG} \\
\text{AGGG TTAGGG TTAGGG TTn}_2\text{PGGG}
\end{align*}
\]

The authors pointed out that the folding topology of K$^+$-form of htel-22 was different in crystals and in solution. The n$_2$P was also incorporated in place of A in the three TTA loops of the 3-tetrad htel-22 AG$_3$(TTAGG)$_3$ and thermal stabilities were also determined by Kimura, Kawai, Fujitsuka, and Majima (2007) (Table 15).

The substitution reduced the thermal stability of the unmodified quadruplex only by a small extent in 50 mM sodium phosphate (pH 7), 100 mM NaCl, and there was also only a slight difference in the destabilizations caused by the substitutions of the different positions of A. On the other hand, significant changes were observed in fluorescence intensity of n$_2$P depending on whether it was present in the quadruplex or in the duplex formed by the same sequence and the complementary strand. Thus, n$_2$P can be used to monitor the duplex to quadruplex conformational transition, in addition to the CD method. These probes could also distinguish between the basket-type and propeller-type G-quadruplexes. (Kimura et al. (2007) also demonstrated the detection of formation of a complex between the quadruplex and TMPyP4, a telomerase inhibitor molecule, by using a n$_2$P-modified telomeric DNA. Gray, Petraccone, Trent, and Chaires (2010) serially incorporated n$_2$P in place of each A of the loops of the htel-22 AGGG(TTAGG)$_3$ sequence, as did Li et al. (2005) before, to use the fluorescence base as a sensitive probe of the kinetics of quadruplex formation in Na$^+$ and K$^+$ solutions. They found that the folding of the 22mer in Na$^+$ into the basket-type quadruplex

<table>
<thead>
<tr>
<th>Table 15. Effect of n$_2$P on thermostability of htel-22 quadruplex. (see Kimura, Kawai, Fujitsuka, &amp; Majima, 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_m$ (°C)</td>
</tr>
<tr>
<td>AGGG TTAGGG TTAGGG TTAGGG</td>
</tr>
<tr>
<td>AGGG TTn$_2$P(7) GGG TTAGGG TTAGGG</td>
</tr>
<tr>
<td>AGGG TTAGGG TTn$_2$P(13) GGG TTAGGG</td>
</tr>
<tr>
<td>AGGG TTAGGG TTAGGG TTn$_2$P(19) GGG</td>
</tr>
</tbody>
</table>
was a cooperative two-state process, whereas the K\textsuperscript{+}-dependent folding was biphasic, referring to the presence of more than two structures at low, 1–30 mM K\textsuperscript{+}. Their result suggested that the elevated K\textsuperscript{+} concentrations would increase the rigidity of the loops, thus stabilize a given (single) conformation. The substituted htel-22 sequences were also used by Buscaglia, Jameson, and Chaires (2012) to monitor the cation-driven folding by time-resolved fluorescence studies, applying phasor diagrams. Gray, Buscaglia, and Chaires (2012) also used these four n\textsuperscript{2}P-modified quadruplexes to analyze the temperature-induced mechanism of melting and concluded that there are two stable melting intermediates, in addition to the folded quadruplex and the unfolded structure in 25 mM K\textsuperscript{+} solution in the course of melting of the wild-type htel-22, and one stable intermediate in the case of the n\textsuperscript{2}P-modified htel-22. They have also determined the thermodynamic parameters of the intermediate structures, one of which was a triplex.

### 4.3. Substitution of the loops’ guanines

Phan, Modi, and Patel (2004) determined by NMR the 3D structure of two sequence analogs of the 27mer nuclease-hypersensitive element III\textsubscript{q} of the human c-myc promoter, called Pu27, as being intramolecular propeller-type parallel stranded quadruplexes at 0.5–2 mM strand concentrations in K\textsuperscript{+} solution. Thermostability of the structures was also determined by NMR (Table 16).

The underlined G triplets formed the three G-tetrads in the two analogs. Substitution of the four G-s in the myc-1245 by the T-cluster destabilized the structure as shown by the $T_m$ values in 9 mM K\textsuperscript{+}. This was probably the result of the extended loop structure formed by the 6mer TTTTTA sequence in place of the single-base loops.

Using the same 27mer sequence Beckett et al. (2012) replaced the loop guanines by the natural base lesions o\textsuperscript{8}G, and the guanine abasic (AP) site, and a tetrad-G by A. The authors studied the effect of these three lesions on the duplex-to-quadruplex transitions using DMS footprinting and RNA polymerase arrest assays. The 1245 quadruplex contains a 6-nucleotide loop composed of TGGGGA. By studying eleven modified duplexes the authors found that a single substitution of the G12 (in bold) by o\textsuperscript{8}G or an AP-site prevented the formation of the 2345 quadruplex, but did not hinder the formation of the 1245 quadruplex that accommodated the lesion in the long loop. Interestingly, when in addition to the former lesions the authors also incorporated a single A into G3 (bold), which was supposed to hinder the formation of the 1245 quadruplex, the 2345 quadruplex did form, incorporating the o\textsuperscript{8}G or the AP-site into the G-tetrad. The 2345 structure is biologically relevant in the transcription regulation of the c-myc gene. Destabilization of this quadruplex resulted in a 3-fold increase in transcription activity.

Dai, Chen, Jones, Hurley, and Yang (2006) determined by NMR the structure of the G-rich strand of the promoter region of the BCL2 gene. Strand concentrations were 0.2–2 mM, in 20 mM K-phosphate (pH 7), 40 mM KCl. A 23mer segment of the 39mer promoter sequence was selected and also modified by G-to-T mutations for the study:

| GGG CGC GGG AGGAAGG GGG C GGG |
| GGG CGC GGG AGGAATT GGG C GGG |
| mutated at pos. 15,16 |
| GGG CGC GGG AGGAATG GGT C GGG |
| mutated at pos. 15,19 |
| GGG CGC GGG AGGAAGG GTT C GGG |
| mutated at pos. 18,19 |

A mixed parallel/antiparallel-stranded, hybrid G-quadruplex was described that contained three G-tetrads connected via two lateral and one side loop. The first CGC loop formed a lateral loop, and the third single nucleotide C loop formed the double-chain-reversal side loop. The second lateral loop consisted of the sequence AGGAAGG, and its structure depended on mutations. Other mutations by C, T, U, A, and I nucleotides in the loop sequences have also been incorporated and the results showed that those in C4 and A10 (underlined) destabilized the G-quadruplex.

The fluorescent purine derivative n\textsuperscript{2}P was inserted by Xu and Sugiyama into one of the two edge loops of the two-tetrad forming sequence CGG\textsuperscript{n2P}TG\textsubscript{2}G\textsubscript{2}CG\textsubscript{2}C in which the G4 and G5 formed originally the loop. Analysis revealed that an increase in fluorescence in the

---

**Table 16. Thermostability of the myc quadruplexes.** (see Phan, Modi, & Patel, 2004)

<table>
<thead>
<tr>
<th>TGGGG A GGG TGGGGG A GGG T GGGG AAGG.</th>
<th>$T_m$ (°C) in 9 mM K\textsuperscript{+}</th>
<th>In 90 mM K\textsuperscript{+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The 22mer myc-2345

TG A GGG T GGGG A GGG T GGGG AA

The 24mer myc-1245

T GGGG A GGG T TTTT A GGG T GGGG A

| 63 | >80 |
| 47 | 75 |
Table 17. Effect of G-to-A substitution on the stability of a 23mer quadruplex. (see Chen et al., 2012)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAGGGGGGCGGCAGGGGCAAGGA</td>
<td>88.2</td>
</tr>
<tr>
<td>AAGGGAAGGGCGGGCGAGGGCAAGGA</td>
<td>74.4</td>
</tr>
<tr>
<td>AAGGGAAGGGCGGGGAGGGCAAGGA</td>
<td>69.7</td>
</tr>
<tr>
<td>AAGGGGAAGGCAGGGGCGAACAGGA</td>
<td>63.8</td>
</tr>
<tr>
<td>AAGGGAAGGGCGGGGCAAGGA</td>
<td>62.7</td>
</tr>
</tbody>
</table>

G-quadruplex relative to duplex could be attributed to the unstacked n3P within the loop (Xu & Sugiyama, 2006).

Chen et al. (2012) have found that the quadruplex formed by the G-rich 23mer nuclease-hypersensitive element of the promoter of the human PDGFR-β adopted a novel type of parallel-stranded intramolecular architecture with a broken G-strand and three single-nucleotide double-chain reversal loops. The G-to-A substitution in the loops destabilized the quadruplex but did not hinder the formation of parallel folds in 25 mM potassium phosphate, 70 mM KCl, pH 7 (the underlined nucleotides formed the loops, Table 17).

5. Modified sugar units, RNA quadruplexes
5.1. Ribonucleosides and 2′-O-methyl-ribonucleosides

Using two-dimensional NMR and molecular dynamics Cheong and Moore (1992) determined for the first time in 1992 the formation of an RNA quadruplex built from the hexamer ug4u, containing uridine and guanosine ribonucleosides. The structure was a tetramolecular parallel quadruplex fold with Hoogsteen-pairs connected g-tetrads, and at least one u-tetrad at one end held together by N3-H…O4 base pairs. The quadruplex proved to be an extremely stable structure in 50 mM KCl. Deng, Xiong, and Sundaralingam (2001) analyzed the same quadruplex, [ug4u]4 by X-ray method in the presence of Na+ ions only, that is, there was a selective metal ion bonding within the quadruplex. The gu octads formed in the groove.

Mergny’s group (Sacca et al., 2005) also studied the RNA quadruplexes. The full ribo, and the 2′-O-methyl-ribo (OMe) modifications destabilized the monomolecular and bimolecular quadruplexes (at least in Na+), whereas these modifications stabilized the tetramolecular parallel quadruplex in 100 mM NaCl or KCl solutions. They studied the following structures:

- 15-mer TBA sequence forms a two-tetrad intramolecular chair-type antiparallel quadruplex;
- htel-18 AGGG(TTAGG)3 forms two-tetrad antiparallel basket-type quadruplex in Na+;
- htel-22 AGGG(TTAGG)3 forms the three-tetrad antiparallel basket quadruplex in Na+;
- 12mer GGGTTTTGGGG forms a bimolecular antiparallel quadruplex; and
- the 6mer TGGGGT forms tetramolecular parallel quadruplex (Table 18).

Tang and Shafer (2006) incorporated ribonucleosides into the DNA sequence of TBA to study the influence of the ribo analogs on the quadruplex fold by CD, UV, PAGE and NMR techniques: The ribonucleotides could

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tm (°C) in Na⁺</th>
<th>Tm (°C) in K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>15mer TBA</td>
<td>G2T2G2TGGTG2T2G2</td>
<td>~20 48</td>
</tr>
<tr>
<td>15mer ribo</td>
<td>gguugg ugu gguugg</td>
<td>non non rev</td>
</tr>
<tr>
<td>15mer OMe</td>
<td>gguugg ugu gguugg</td>
<td>~10 32</td>
</tr>
<tr>
<td>18mer ribo</td>
<td>AGG(TTAGG)₃</td>
<td>~25 42</td>
</tr>
<tr>
<td>18mer OMe</td>
<td>agg uuagg uuagg uuagg</td>
<td>~14 22</td>
</tr>
<tr>
<td>22mer ribo</td>
<td>AGGG(TTAGG)₃</td>
<td>55 42</td>
</tr>
<tr>
<td>22mer OMe</td>
<td>agg uuagg uuagg uuagg</td>
<td>44 66</td>
</tr>
<tr>
<td>22mer OMe</td>
<td>agg uuagg uuagg uuagg</td>
<td>~41 62</td>
</tr>
<tr>
<td>12mer OMe</td>
<td>[G₄T₄G₄]₂</td>
<td>53 non rev</td>
</tr>
<tr>
<td>12mer OMe</td>
<td>[gggguuuuuuuuu]₂</td>
<td>40 non rev</td>
</tr>
<tr>
<td>12mer OMe</td>
<td>[gggguuuuuuuuu]₂</td>
<td>~41 62</td>
</tr>
<tr>
<td>6mer TBA</td>
<td>[TG,T]₄</td>
<td>~55 &gt;90</td>
</tr>
<tr>
<td>6mer OMe</td>
<td>[uggggu]₄</td>
<td>&gt;80 &gt;90</td>
</tr>
</tbody>
</table>

*Non rev. is for non reversible melting.
change the DNA quadruplexes’ antiparallel fold to parallel, the unimolecular to bimolecular, and the deoxyguanosines could reverse this changes in the dimeric parallel RNA quadruplexes. The changes were well illustrated by the systematically selected sequences studied in buffered 25 mM KCl (Table 19).

The underlined G-s are syn dG-s. This was the key structural feature directing the structural changes. The quadruplex of TBA is monomolecular chair-type antiparallel structure that contains syn and anti dG-s. The energetically preferred N-glycosidic conformation of the ribonucleosides is the anti, originating from the C3′-endo (North) sugar pucker. Therefore, replacing the syn dG-s in the TBA quadruplex by (ribo) g-s led to all-anti nucleosides in the tetrads. This caused thermal destabilization and, based on CD spectra, conversion to parallel quadruplexes that were probably bimolecular. On the other hand, replacing the anti dG-s by g-s did not change the glycosidic torsions, the unimolecular fold did not change, and the therm stability increased, probably as a result of the contribution of 2′-hydroxyl group to the stabilizing water structure around the quadruplex molecule. The full ribo TBA quadruplex is parallel and is less stable than the deoxy. Incorporating dG-s into the originally syn positions restored the antiparallel quadruplex folding and elevated the therm stability, whereas incorporation of dG-s into the anti positions left the ribo- quadruplex in the parallel folding but with a strong decrease in stability.

The human telomere RNA contains repeats of the 6mer uuagg. Xu, Kaminaga, and Komiyama (2008) studied the formation of RNA quadruplexes with the 12mer uaggguuagg and the 24mer [uaggg]4 by CD, NMR and MALDI-TOFMS methods. Both formed parallel quadruplexes based on the positive CD signal at 265 nm and the negative one at 240 nm in buffered 100 mM NaCl solution. CD-based thermostabilities were 41 and 51 °C, respectively, for the 12mer and 24mer quadruplexes. Thermal refolding of the 12mer showed hysteresis, referring to a bimolecular parallel quadruplex, whereas the melting and annealing curves of the 24mer were superimposabe. The authors prepared another 12mer, the uuaggguuagg, and its CD spectrum also showed a parallel quadruplex (no Tm data were given).

NMR, MS and PAGE confirmed that the 12mer formed a dimeric parallel quadruplex with two double-chain reversal loops in Na+.

Recent studies have shown that mammalian telomeric DNA sequences are transcribed into RNA sequences that thus contain the telomeric repeats. Kimura, Xu, and Komiyama (2009) reported that the htel RNA uagggu formed tetramolecular parallel quadruplexes with a U-quartet at the 3′-end of the quadruplex in either Na+ or K+ solution. The same group, Komiyama and coworkers (Xu, Ishizuka, Kimura, & Komiyama, 2010) studied also the RNA quadruplexes folded from similar sequences, the uagggu, uuaggg, and those containing analogs of uridines, the zebularine (ribonucleoside of 2-pyrimidinone), and 4-thiouridine (s′u). The two unmodified sequences formed tetramolecular parallel quadruplexes in both Na+ and K+ solutions with positive bands at 265 nm and negative ones at 240 nm in their CD spectra. The CD-determined thermostabilities were surprisingly different in buffered 150 mM NaCl (Table 20).

NMR and MS techniques were also used to determine the structural features of the folds. The authors concluded that while an U-quartet was formed at the 3′-end of [uagggu]4 in addition to the three g-quartets, no U-tetrad was formed at the 5′-end due to the steric hindrance caused by the adenine nucleotides. In a similar way, the [uaggg]4 quadruplex contains only three g-tetrads. The stabilizing stacking effect of the extra 3′-U-tetrad in the former quadruplex was supposed to cause the drastic difference in stability. The circular, single H-bonded U-tetrad was held together via N3-H…O4 hydrogen bonds. The existence of the H-bonds and the formation of the stacked tetrad were further proved by the incorporation of zebularine, which does not have the O4 atom, and the s′u that cannot form H-bonds. The quadruplexes...
containing either of the two analogs at the 3'-end, instead of uracil, were strongly destabilized as compared to [uagggu]₄, as it is shown above with [uaggg]₄.

Matsugami et al. (2008) and Nishikawa, Murakami, Matsugami, Katahira, and Nishikawa (2009) described unique quadruplex structures formed by the gga triplet repeats that are present in RNA aptamers recognizing the bovine prion protein. One of the aptamers contains four repeats of the triplet, (gga)₄, and the authors determined the structure formed in physiological conditions (buffered 100 mM KCl) by NMR and CD. The 12mer (gga)₄ strands formed an intramolecular quadruplexes that consisted of one g:g:g:g tetrad and one g(a):g:g( a):g hexad plane. The adenines were in external positions to the guanine tetrad connected by (a)N6-H..N3(g) and (a)N7.–H–N2(g) H-bonds. Furthermore, the mono-meric quadruplexes formed thermostable dimeric ones through the stacking of the hexad planes. The dimeric structure of (gga)₄ is similar to that formed by (GGA)₄ but the deoxy triple repeat formed tetrads and heptads.

Martadinata and Phan (2009) reported on the folding properties of the htel-12 RNA quadruplex of uaggguuagggu determined by NMR in K⁺ solution. The sequence formed dimer propeller-type quadruplexes. Two such type of quadruplexes of the 10mer ggguuagggu stacked on each other by the 5'-terminal tetrads in 5'-to-5' orientation, and higher order (rod-like) G-quadruplex structures were observed with the quadruplexes formed by the 9mer gggguagggg, which meant terminal tetrad associations in 5'-to-5'-3'-to-3' orientations.

5.2. Arabinofuranose

Peng and Damha (2007) have studied the effect of 2'-deoxy-2'-flour-D-arabinofuranosylguanine (fl₂araG) is anti, so if it is incorporated in place of a syn dG of a G-tetrad, it would affect both the stability and folding. In fact, the authors observed the effects. While incorporating fl₂araG or fl₂araT in place of anti dG-s or T-s (T in the loop) either in antiparallel or parallel quadruplexes, the analogs stabilized the structure and maintained the fold of the wild-type quadruplex. With the TBA quadruplex the ΔTm was up to 3 °C per ara-nucleotide incorporated. For example, the Tm of the TBA quadruplex increased from 46.4 to 53.3 °C with GGGTTGTTGTTG, in which the underlined, bold G stands for fl₂araG and the glycosidic angles of the replaced dG-s were all anti. With GGGTTGTTGTTGGG, in which the dG-s replaced were syn, the Tm value only slightly decreased, to 45.4 °C, but the CD spectrum of the quadruplex changed from the antiparallel to parallel-type. The parallel fold was not specified. In case all nucleotides were substituted by ara-nucleotides the Tm was high, 54.1 °C, but the CD showed again parallel characteristics. If all T-s of the loops were substituted the Tm was even higher, 56.3 °C and the CD spectrum remained antiparallel. With the parallel tetramolecular quadruplex [T₂G₄T₂]₄, in which all nucleotides are anti, the substitutions with the anti-fl₂araG resulted in stabilization and no change in the folding topology. The phosphorothioate substitution alone led to elevated Tm, the 66 °C Tm of [T₂G₄T₂]₄ increased to 74 °C with P5-[T₂G₄T₂]₄, and increased further to 83 °C if all nucleotides were replaced by fl₂araG. The Tm of the four-tetrad bimolecular antiparallel [G₄T₄G₄] was 65 °C, and if all nucleotides were replaced by ara-nucleotides the Tm increased to 90 °C, but the antiparallel fold converted to a parallel fold. It was not specified whether the parallel structure was bimolecular or tetramolecular (Peng & Damha, 2007).

5.3. Locked ribonucleosides (LNA)

The locked nucleoside is a 2'-O-4'-C-methylene-linked ribonucleoside. The deoxyoligonucleotide (G₄T₄)₃G₄ of Oxytrichia trifalax (Oxy28) can form intramolecular, antiparallel crossover (diagonal)-basket quadruplex with four G-tetrads. Dominick and Jarstfer (2004) incorporated single locked nucleotides, G-LNA and T-LNA into certain positions of Oxy28, and found that a single LNA can change the folding topology of the quadruplex. The following G and T positions were substituted, one-by-one, by an LNA (the bold, underlined base is the LNA):

```
2 4 5 6 15 18
GGGTTTTGGGGTTTTGGGGTTTTGGGG
```

CD spectra and thermal stability of modified quadruplexes were determined in buffered 50 mM NaCl and KCl solutions. The Tm of the unmodified quadruplex (64 and 85 °C in Na⁺ and K⁺, respectively) was reduced by each single LNA, most extensively by the G-LNAs. G-LNA in position 2 caused the largest effects, −14 and −25 °C, respectively. This effect is similar in magnitude to that observed with other middle tetrad substitutions of
three-tetrad G-quadruplexes, such as A (Tomaško et al., 2009), AP-site (Školáková et al., 2010), 8-oxoG (Vorlicková et al., 2012), and m²G (Mekmaysy et al., 2008).

Substitution by G-LNA of the other non-terminal tetrad position in Oxy28, the G18, also reduced the \( T_m \) by 6 and 20 °C, respectively, in Na\(^+\) and K\(^+\). In Na\(^+\) solution the unmodified Oxy28 quadruplex displayed a typical antiparallel CD spectrum with a strong maximum at 295 nm and a strong minimum at 260 nm. In K\(^+\) the Oxy28 quadruplex showed again a strong maximum at 295 nm and a shallow minimum at 260 nm, still indicative of an antiparallel quadruplex. In Na\(^+\) all LNA-containing quadruplexes showed similar spectra as the wild-type Oxy28 quadruplex had. In K\(^+\), however, the presence and location of the LNA had various effects on the structure. The LNA did not affect the structure if it was inserted into positions 5 and 6 (T-s in the first loop), led to a mixtures of folds in positions 4 (tetrad G), 15 or 16 (T-s in the second loop), and caused formation of intramolecular parallel folds if incorporated into positions 2, 17 and 18 (tetrad G-s): a strong CD maximum at 260 nm and a minimum at 240 nm. LNA is constrained in the C3′-endo sugar pucker and favors the anti-N-glycosidic bonds (Dominick & Jarstfer, 2004).

Randazzo, Esposito, Ohlenschlager, Ramachandran, and Mayol (2004) have reported on the structural characterization of the LNA-modified parallel quadruplex of [TG3T]\(_4\) using NMR and CD techniques. The CD-measured thermostability (\( T_m \)) values in buffered 70 mM KCl are shown in Table 21.

Contrary to the thermal destabilization of the intramolecular quadruplex of (G4T4)3G4 (Oxy28) by LNA substitution (Dominick & Jarstfer, 2004), the tetramolecular parallel quadruplex structure was considerably stabilized, as much as by the ribo substitution was. The thermostabilities were T\(_{1/2}\) values (midpoint of the unfolding profiles), as there was significant hysteresis during the refolding of each quadruplex. It is noteworthy that the full LNA quadruplex showed the smallest hysteresis, probably as the consequence of the rigid, organized sugar part. The much higher stability of the LNA-quadruplex was explained by entropy gains (more ordered structure) due to the locked sugar pucker. NMR data showed that the helical parameters of the tetramolecular LNA-quadruplex were very similar to those of the unmodified deoxy-quadruplex, but all LNA nucleosides have 3′-endo (N-type) sugar pucker and are in anti conformation; therefore, the backbone structure of the duplex LNA resembles the A-type structure, in contrast to the B-type of the deoxyribose strands. The CD spectrum of the full LNA-quadruplex differed from the general parallel spectrum that in addition to the typical large 260 nm positive band there was also a smaller positive band, or it was rather a shoulder at 288 nm. The negative peak was at 239 nm (Randazzo et al., 2004).

LNA-modified tetramolecular quadruplexes were also studied by Nielsen, Arar, and Petersen (2006). They determined the NMR structure of the modified quadruplexes built by the Oxytricha nova sequence TG4T: The [TG(LG)G(LG)TG]\(_4\) and the [T(LG)4]T\(_4\) (LG stands for G-LNA). Both quadruplexes formed parallel-stranded tetramolecular, right-handed quadruplexes, similarly to the wild-type [TG4T]\(_4\) quadruplex. Only local structural alterations were observed, which were due to the C3′-endo sugar pucker of the LNA. The G-LNA can exist only in anti-N-glycosidic conformation. The syn cannot form owing to the steric clash between H3′ of the sugar and N3 of the base. Thus, the LNA nucleotides can preferentially be incorporated into parallel quadruplexes. The C3′-endo sugar pucker slightly unwinds the quadruplex causing significant widening of the grooves (from 2.5 to 7 Å) that increases the distance between the negatively charged phosphate groups. The decreased repulsion may lead to increased stability. Actually, the thermostability of the LNA-quadruplexes studied by Nielsen et al. (2006) increased relative to the unmodified [TG4T]\(_4\) quadruplex. The \( T_m \) of the latter was 82 °C, and with the two LNA-modified quadruplexes no hypochromic effect was observed up to 90 °C, the absorbance remained constant, that is, the \( T_m \) values must have been well above 90 °C. {The LNA nucleotides are known to increase the thermostability of the duplex and triplex DNA-s as well (Petersen & Wengel, 2003), and the B-type duplex is converted towards the A-type [RNA-like] geometry by the LNA substitutions (Petersen, Bondensgaard, Wengel, & Jacobsen, 2002).}

The G-LNA was also incorporated into the 3′-terminal G position of TBA oligodeoxynucleotide 5′-GGTTGGTGTGGTTG(LG)-3′ by Virno et al. (2007). The substitution did not change the antiparallel, two-tetrad intramolecular chair-type character of the unmodified TBA quadruplex as it was determined by CD and NMR techniques. Bonifacio, Church, and Jarstfer (2008) reported on the replacement by LNA-s of the G2, G5 (in the same G-tetrad), T4 (in the TT loop), T7 and G8 (TGT loop) of the 15-mer TBA oligodeoxynucleotide. In buffered 50 mM KCl the substitution at G2 by G-LNA reduced the \( T_m \) of the unmodified quadruplex to the largest extent, from 48.1 to 33.5 °C, although the dG in position 2 was anti, and G-LNA is in anti conformation. The TGT loop’s substitution at position T7 also caused

<table>
<thead>
<tr>
<th>Quadruplex</th>
<th>( T_m ) °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>[TG3T](_4)</td>
<td>46</td>
</tr>
<tr>
<td>[uggu](_4)</td>
<td>67</td>
</tr>
<tr>
<td>(<a href="LG">LT</a>(_4)(LT)](_4)</td>
<td>66</td>
</tr>
</tbody>
</table>
destabilization, by about 5 °C, whereas, G5-LNA (tetrad) and G8-LNA (central loop) increased the thermal stability of the unmodified quadruplex by 2.6 and 1.9 °C, respectively. With the latter two LNA- quadruplexes the thermal changes were apparently hyperchromic, while the fold-unfold thermal transitions reported so far for all other quadruplexes were hypochromic around 295 nm. No explanation was given for this unusual observation. The T4-LNA quadruplex was so unstable that \( T_m \) could not be determined. Thermal stability of the modified quadruplexes was more or less inversely related to their anti-thrombin activity: The G2-LNA that triggered the largest destabilization of the quadruplex produced about the same thrombin inhibitory activity as the wild-type TBA quadruplex did. The stabilized G5-, T7- and G8-substituted quadruplexes showed decreased activity in thrombin inhibition. The LNA substitutions studied here did not change the folding topology of the unmodified TBA quadruplex. CD spectra in K\(^+\) solution showed that each LNA-quadruplex formed an antiparallel quadruplex.

### 5.4. Unlocked ribonucleosides (UNA)

Pasternak, Hernandez, Rasmussen, Vester, and Wengel (2011) described the positional effect of an incorporated single unlocked-nucleoside (2′-3′-acyclic-rG or -rU; abbreviated as uG or uU) into the 15mer, 5′-biotinylated TBA sequence. (According to the authors’ unpublished results the 5′-biotin decreased the thermodynamic stability of the TBA quadruplex, and they assumed the effect was the same for all modified TBA quadruplexes.) The unlocked analog used is a flexible ribonucleoside derivative. Replacement of T by uU in certain loop positions, one in each of the three loops, proved stabilizing for the TBA quadruplex. These positions were the 3, 7, and 12.

The presence of uU in the other three T positions of loops destabilized the quadruplex. Uracil in the loops has been described by Sacca et al. (2005) to stabilize the TBA quadruplex, contrary to duplexes, by \(-0.16\ \text{kcal/mol}\) each, thus with the uU nucleosides this effect must have contributed to the stability values observed. CD spectra of quadruplexes with uU in positions 3, 7, and 12 were the same as that of the unmodified TBA, showing an intramolecular antiparallel chair-type quadruplex. uU in the other loop positions changed this spectra by significantly lowering the amplitudes and causing band shifts. Incorporation of uG in any tetrad position either destabilized the quadruplex or hindered the formation of it, as it was revealed by CD spectra and thermal profiles. Binding properties of the modified TBA quadruplexes to thrombin were also studied: the quadruplex with uU in position 7 proved to be the only one with more effective in blood clotting than the unmodified TBA quadruplex. Agarwal, Kumar, and Maiti (2011) have also studied the effect of unlocked nucleosides on quadruplex stability by incorporating uA, uT, or uC into the single-base diagonal loop or uG into the G-tetrad of the quadruplex formed by the \( \text{GGGT}_3\text{G}_3\text{A}_3\text{G}_4\text{T}_3\text{G}_5 \) sequence. The unmodified 23mer quadruplex is a 3-loop (two edge and one diagonal loop, according to their scheme), three-G-tetrad quadruplex of parallel folding topology, based on CD spectra. The underlined three G and one A or T or C nucleosides were replaced by a same-base uU-nucleoside. The UV absorption-based melting data showed that loop (A, T or C) modifications stabilized the quadruplex by 3–7 °C in the \( T_m \) and \(-0.9 \text{ to } -1.46 \text{ kcal/mol}. \) The purine uA had the greatest effect. Stabilization was explained by the flexibility of the unlocked nucleoside, which could ease the tension that might exist in a single-base diagonal loop. Contrary to the loop substitutions, replacement of G-s in the G-tetrads by uG-s led to significant destabilization of the quadruplex. The terminal tetrads were destabilized by about the same extent, by 5 °C (1.3 kcal/mol in the case of terminal tetrad with the diagonal loop), and uG substitution in the middle tetrad caused the largest effect, the \( \Delta T_m \) was \(-17.6 \text{ °C and } 4.17 \text{ kcal/mol}\) the free energy change. Flexibility of the sugar moiety did not prove to be a beneficial structural motif in the G-tetrads. Similarly, as found earlier by Pasternak et al. (2011), the CD spectra revealed that in some of these quadruplex analogs the unlocked nucleoside caused transition from the typical antiparallel to a hybrid-type fold in 100 mM K\(^+\) solution.

### 5.5. Other modifications of the sugar moiety

Risitano and Fox (2004) replaced the TTA trinucleotide loops by non-sugar alkyl-diol chains in F-GGG-X-GGG-X-GGG-X-GGG-Q, where X was a phosphate group connected to propanediol, octanediol, hexaethyleneglycol or (hexaethyleneglycol), and F is for the fluorophore and Q for the quencher. The oligonucleotides with these non-nucleoside linkers did form quadruplexes. Stability and kinetics of formation were greatly influenced by the linker. The quadruplexes with octyl, glycol and propyl were more stable than the wild-type, as characterized by thermodynamic data. This finding raises the question about the contribution of base stacking in loops to quadruplex stability. CD spectra of the unmodified quadruplex showed the typical characteristics of the monomolecular Na\(^+\)-basket and the K\(^+\)-stabilized non-parallel quadruplexes, and the spectra of the modified quadruplexes showed paral-
lel-types in both Na⁺ and K⁺ solutions with positive maxima at ~264 nm and negative minima at ~244 nm.

Saneyoshi et al. (2009) studied the effect on quadruplex fold of the conformationally rigid analogs of dG, the North- and South-bicyclo[3.1.0]hexane-dG by incorporating these analogs into position 14 and 15 of the two-tetrad chair-type antiparallel quadruplex of TBA. The unmodified dG in positions 14 and 15 were in syn and anti, respectively, N-glycosidic conformations, and all nucleotides had South (C2'-endo) sugar pucker. Four analogs of GTTTGGTGTGGTGG have been prepared:

1. GG TT GG TGT GG TT (S/sG)G pos. 14 is syn-dG in unmodified TBA
2. GG TT GG TGT GG TT (NaG)G
3. GG TT GG TGT GG TT G(S/sG) pos. 15 is anti-dG in unmodified TBA
4. GG TT GG TGT GG TT G(N/aG)

where S/s represent the South/syn, the N/a the North (C3'-endo) anti conformations of the conformationally constrained analogs of dG. The replacement of dG in position 14, which was S/syn, by S/sG did not change the stability and folding properties of TBA, as confirmed by NMR. However, the replacement by N/aG completely disrupted the quadruplex structure as a consequence of the opposite sugar pucker and glycosidic torsion angle. Substitution of dG in position 15, in which dG was S/anti, by S/sG caused a strong destabilization of TBA quadruplex, while substitution by N/aG produced less destabilization. The global folding pattern of the quadruplex was not affected in either case.

6. Phosphodiester analogs

In a systematic study, Sacca et al. (2005) elucidated the effect of the phosphorothioate (P-S) and methylphosphonate (P-Me) modifications of the internucleotide phosphodiester groups on the stability and folding features of quadruplexes built by various DNA sequences. In the phosphorothioate, a sulfur anion replaced the non-diester-bonded oxygen anion of the internucleotide phosphodiester group. The larger ion size was not well tolerated and thus the quadruplex of the 15mer intramolecular TBA in K⁺ was destabilized. Similarly to the TBA quadruplex, the stericity of the dimeric intermolecular quadruplex of the 12mer G₄T₄G₄ sequences and the tetramolecular quadruplex of the 6mer TG₄T was reduced in Na⁺, but the P-S substitution had no effect in Na⁺ and increased the stability in K⁺ of the quadruplexes of the intramolecular 18mer AGG(TTAGG), and also stabilized the intramolecular quadruplex of the htel-22 AGGG(TTAGGG)₃ in both Na⁺ and K⁺ solutions. In the methylphosphonate modification the non-diester-bonded oxygen anion of the phosphodiester group is replaced by a neutral methyl group, and thus the DNA becomes uncharged. In this way, the stabilizing waters spine along the backbone is disrupted. The loss of charge led to unfolded structures, and thus no thermal transition could be detected with the above sequences, except the htel-22 AGGG(TTAGGG)₃ that could form a quadruplex, but it was strongly destabilized in both salt solutions as compared to the stability of the unmodified htel-22 quadruplex (Sacca et al., 2005). The phosphorothioate substitutions were also studied by Zaitseva et al. (2010) with the TBA oligodeoxynucleotides. Physical properties of the 13 modified structures studied depended on whether the P-S substitution took place between the G-tetrads or in the loops. The basic characteristics of the CD spectra of the antiparallel intramolecular fold of TBA quadruplex were not changed by these modifications, only the peak size depended on the position of the substitution. Stability of the TBA quadruplex (Tₘ 52°C and ΔG₃₇ =−1.84 kcal/mol in buffered 0.1 M KCl) was reduced if the substitution was between G-nucleotides, but was not affected or even increased if the substitution was in the loops. Multiple substitutions in the G-planes, up to four, led to linear decrease and increase, respectively, of the Tₘ (−12°C at max) and ΔG₃₇ (1.3 kcal/mol max), and saturation-type of increase (4°C and −0.4 kcal/mol) in the Tₘ and ΔG₃₇, respectively, with the maximum number of substitution of ten.

Esposito, Oliviero, Pepe, Virgilio, and Galeone (2008) carried out an NMR study to determine the effect on quadruplex structure of the inversion of polarity of the TGGGT strand containing abasic sites (AP). Quadruplexes formed by the following sequences were studied:

1. 5’-AP-3’,3’,5’-GGGT-5’
2. 5’-T-3’,3’,5’-GGGT-5’
3. 5’-TG-3’,3’,5’-GGT-5’
4. 3’-AP-5’,5’,5’,synGGGT-3’
5. 3’-T5’,5’,5’,synGGGT-3’
6. 3’-TG-5’,5’,5’,synGGGT-3’

The wild type TGGGT chains form parallel-stranded tetramolecular quadruplexes with all anti-dG nucleosides, that is, containing anti-G tetrads. Each analog studied here also formed fourfold symmetric parallel quadruplexes with three G-tetrads, but the glycosidic angles of dG depended on polarity changes of the 3’,3’- or 5’,5’-type. The 3’,3’-quadruplexes of 1–3, and the 5’,5’-type 4 with the AP site at the 5’-junction had anti-dG tetrads. The other 5’,5’-tipped 5 and 6 sequences formed four-stranded quadruplexes with a syn-dG tetrad right at the 5’-junction inversion of polarity side. The presence or absence of base at the end of the 5’-5’ bond is important, as shown
by sequence 4. The results are in accord with these authors’ previous works on polarity changes in quadruplex-forming sequences (Esposito, Virgilio, Randazzo, Galeone, & Mayol, 2005; Galeone, Mayol, Virgilio, Virno, & Randazzo, 2008).

7. Incorporated nucleotide analogs listed according to the sequence of quadruplexes (Section, subsection, and references are indicated in parentheses.)

7.1. Nucleotide analog incorporated into intramolecular quadruplexes formed by

15mer
oligonucleotides:
- TBA, G2T2G2TGTG2T2G2
  nG (2.1.4, de la Osa et al., 2006),
  8-fluorenylmethyl-dG (2.1.7, Ogasawara & Maeda, 2009),
  sG (2.2.1, Marathias et al., 1999)
  U in loop (4.1, Olsen & Marky, 2009; Olsen et al., 2009; Sacca et al., 2005)
  A, C, T, nP for G in TGT loop (4.1, Olsen et al., 2009)
  faraG (5.2, Peng & Damha, 2007)
  faraT (5.2, Peng & Damha, 2007)
  LNA (5.3, Virno et al., 2007)
  oU, uU (5.4, Pasternak et al., 2011)
  North- and South-bicyclo[3.1.0]hexane-dG (5.5, Saneyoshi et al., 2009)
  P-S (6, Sacca et al., 2005)
- ribo TBA, g2u2g2ugug2u2g2
  O-methyl-ribo (5.1, Sacca et al., 2005)
- GGGXGGGXGGGXGGG, where X was in (5.5, Risitano & Fox, 2004):
  Propanediol
  Octanediol
  Hexaethylenglycol
  (hexaethylenglycol)2

16mer
- (G3T)4
  I (2.2.3, Do et al., 2011)
  C for T of loops (4.1, Do et al., 2011)

17mer
- T(G3T)4
  A, C, AP site for T of loops (4.1, Rachwal et al., 2007)
- T30177, TTGTG2T(GGGT)3
  I (2.2.3, Mukundan et al., 2011)

18mer
- CGT(G3T)4CG3C
  mG (2.1.2, Xu & Sugiyama, 2006)
  nP for G in loop (4.3, Xu & Sugiyama, 2006)
- htel-18, AG3TTAG3TTAG3TTAG3TTAG3TTAG2
  U in loop (4.1, Sacca et al., 2005)
  P-S (6, Sacca et al., 2005)
- htel-18 ribo, ag2u2ag2u2ag2u2ag2
  O-methyl-ribo (5, Sacca et al., 2005)

19mer
- T30177TT, TTGTG2T(GGGT)3
  I (2.2.3, Mukundan et al., 2011)

20mer
- (T3G2)4
  brG (2.1.1, Dias et al., 1994)
- (TAG3)4
  I (2.2.3, Hu et al., 2009)
- G2(TU)2G2(UT)2G2T2G2
  sG (2.2.1, Marathias et al., 1999)
  U in loop (4.1, Marathias et al., 1999)
- AG4AG4CTG3AG3C
  I (2.2.3, Lim et al., 2010)
- M3Q RNA, gag3ag3ag3ag3ag3a
  a for g (3.3, Morris et al., 2012)
  u for g (3.3, Morris et al., 2012)

21mer
- htel-21, G3(TTAG3)3
  oG (2.1.3, Vorlicková et al., 2012)
  I (2.2.3, Risitano & Fox, 2005)
  AP (2.3, Školáková et al., 2010)
  A (3.3 and 3.4, Sagi et al., 2010; Tomaško et al., 2009)
  A-tetrad (3.2 and 3.3, Sagi et al., 2010)

22mer
- A(htel-21), AG3(TTAG3)3
  brG (2.1.1, Mashimo et al., 2008; Xu et al., 2006)
  mcG (2.2.2, Mekmasy et al., 2008)
  I (2.2.3, Zhang et al., 2010)
  AP (2.3, Fujimoto et al., 2011)
  brU in loop (4.1, Lin et al., 2010)
  ioU in loop (4.1, Xu & Sugiyama, 2004)
  U in loop (4.1, Sacca et al., 2005)
  C for A in loop (4.2, Lim et al., 2009)
  nP for A in loops (4.2, Buscaglia et al., 2012; Gray et al., 2010, 2012; Kimura et al., 2007; Li et al., 2005)
  P-S (6, Sacca et al., 2005)
  P-Me (6, Sacca et al., 2005)
- AG3GCGC tetrad (3.7, Lim et al., 2009)
- htel-22 ribo, ag(uuag3)3
  O-methyl-ribo (5.1, Sacca et al., 2005)
- (htel-21)T, G3(TTAG3)3T
  brG (2.1.1, Lim et al., 2009)
- e-kit, AG3AG3CGCTG3AG2AG3
  PyG (2.1.5, Dumas & Luedtke, 2010)

23mer
- A(htel-21)T, AG3(TTAG3)3T
  I (2.2.3, Zhang et al., 2010)
- (htel-21)TT, G3(TTAG3)3TT
  sG (2.2.1, Lim et al., 2009)
- TA(htel-21), TAG3(TTAG3)3
  brG (2.1.1, Lim et al., 2009; Phan et al., 2007)
- BCL2 gene promoter, G3CGCG3AG2A2G5CG3
  T for G in loop (4.3, Dai et al., 2006)
- nucleosomehypersensitive element of the promoter of the human PGDFR-β, A2G-CGC-CGCAG3A
  A for G in loop (4.3, Chen et al., 2012)
- G3T2A(TC)TG3T3G3
  a for g (3.3, Morris et al., 2012)
  u for g (3.3, Morris et al., 2012)

24mer
- TT(htel-21)A, TTG3(TTAG3)3A
  8-(2-pyridyl)guanine (2.1.5, Dumas & Luedtke, 2010),
8-(2-phenylethenyl)-dG (2.1.6, Dumas & Luedtke, 2011),
8-[2-(pyrid-4-yl)-ethenyl]-dG (2.1.6, Dumas & Luedtke, 2011)
T for G (3.6, Dumas & Luedtke, 2011)
(TTAGGG)n, n = 4,7,8
A for G (3.3, Pedroso, Duarte, Yanez, Baker, et al., 2007; Pedroso, Duarte, Yanez, Burkevitz, et al., 2007)

25mer
- TA(htel-21)TT, TAG3(TTAG3)3TT
  br8G (2.1.1, Phan et al., 2007)
- A(htel-21)TGT, AG3(TTAG3)3TGT
  o8G (2.1.3, Szalai et al., 2002)

26mer
- htel-26 A3(G3TTA)3G3AA
  br5U in loop (4.1, Lin et al., 2010)

27mer
- Oxytricha telomere (Oxy-3.5), G4(T4G4)3
  I (2.2.3, Smith et al., 1995)
  U in loop (2.2.3, 3.8 and 4.1, Smith et al., 1995)

7.3. Tetramolecular and pentamolecular quadruplexes
by

5mer
- [TG,T]4
  - br8G (2.1.1, Esposito et al., 2004)
  - m8G (2.1.2, Virgilio et al., 2005, 2012)
  - hm8U for T (3.8, Petraccone et al., 2003)
  - T-LNA, G-LNA (5.3, Randazzo et al., 2004)
- [AGGGT]4
  - br8A (3.8, Esposito et al., 2005; Petraccone et al., 2005)
  - py8A (8-(1-propynyl)adenine) (3.8, Esposito et al., 2005; Petraccone et al., 2005)
  - m8A (3.8, Randazzo et al., 2005; Virgilio et al., 2004)
  - o8A (3.8, Esposito et al., 2005)
  - oh8A (3.8, Petraccone et al., 2007)
- ribo, [uagg]4
  - s4u (5.1, Xu et al., 2010)
  - zebularine (5.1, Xu et al., 2010)

6mer
- [TG,T]4
  - m8G (2.1.2, Virgilio et al., 2012)
  - o8G (2.1.3, Gros et al., 2007)
  - s6G (2.1.2, Gros et al., 2007)
  - m6G (2.2.2, Gros et al., 2007)
  - I (2.2.3, Gros et al., 2007)
  - c7G (2.2.5, Gros et al., 2007)
  - U (4.1 and 5.1, Sacca et al., 2005)
  - G-LNA (5.3, Nielsen et al., 2006)
  - P-S (6, Sacca et al., 2005)
  - A-, C-, U-tetrads (3.1, Gros et al., 2007)

7mer
- [TG,T]4
  - [G,C]-U-tetrads (3.1, Gros et al., 2007)
  - [TG,G]4
  - br8A (3.8, Esposito et al., 2005; Petraccone et al., 2005)
  - py8A (8-(1-propynyl)adenine) (3.8, Esposito et al., 2005; Petraccone et al., 2005)
  - m8A (3.8, Randazzo et al., 2005; Virgilio et al., 2004)
  - o8A (3.8, Esposito et al., 2005)
  - oh8A (3.8, Petraccone et al., 2007)
- ribo, [uagg]4
  - 2′-O-methyl-ribo (5.1, Sacca et al., 2005)
  - br5u (5.1, Deng et al., 2001)

8mer
- [TG,G]4
  - fl2’araG (5.2, Peng & Damha, 2007)
  - P-S (6, Peng & Damha, 2007)

9mer
- [T,G]4
  - iG (2.2.4, Chaput & Switzer, 1999)
10mer
- [T₄G₄T₄]₄
  iG (2.2.4, Seela & Kroschel, 2001)
  n°cIG (2.2.6, Seela & Kroschel, 2001)
- [T₄G₄T₄]₅
  iG (2.2.4, Seela & Kroschel, 2001)
  n°cIG (2.2.6, Seela & Kroschel, 2001)

12mer
- [T₄G₄T₄]₄
  iG (2.2.4, Chaput & Switzer, 1999; Seela et al., 1996)
  fl°araG, fl°araT (5.2, Peng & Damha, 2007)

13mer
- [T₅G₅T₅]₄
  iG (2.2.4, Chaput & Switzer, 1999)
- [T₅G₅T₅]₅
  iG (2.2.4, Chaput & Switzer, 1999)

8. Perspectives
The studies of the incorporated nucleotide analogs detailed in this review greatly contributed to the structural elucidation of the diverse folds adopted by DNA and RNA quadruplex structures. A few base analogs, such as the 2-aminopurine, the 8-bromo-, and 8-methylguanines, and hypoxanthine proved especially useful for these investigations. There remains much to be done with structural exploration of quadruplexes using non-natural constituents, in particular with the promoter region architectures. The application of nucleotide analogs to structural examination of other non-B conformations would also be advantageous. Additional areas of quadruplex research may also benefit from the application of modified components. Stabilizing nucleotide analogs may be of utility in determining the exact bindings sites of small molecular ligands and binding proteins on quadruplexes. Base lesions resulting from the in vivo spontaneous deamination and depurination of DNA constituents and from the reactions of ROS with the natural building blocks may damage the secondary structures of nucleic acids. Cancer cells are known to contain elevated level of ROS (Kuang, Balakrishnan, Gandhi, & Peng, 2011). Since the associated binding proteins (Shelterin) and the formation of T-loops at telomere ends hide the quadruplex-containing regions from the damage-sensing mechanisms and the DNA repair complexes (Choi1, Farrell, Lakamp, & Ouellette, 2011), these lesions may induce quadruplex unfolding or destabilization in the telomere, promoting in this way the telomerase-mediated immortalization of cancerous cells. Investigation of the structural properties of such damaged quadruplexes would be of significant importance in understanding the consequences of natural base lesions of this type in vivo.

Acknowledgements
The author is thankful to Dr. Michaela Vorlickova for the CD figures, and to Andras J. Sagi for his comments and corrections.

References


Ganet, P. M., & Sura, T. P. (1993). Base pairing of 8-oxoguanosine and 8-oxo-2'-deoxyguanosine with 2'-deoxyadenosine, 2'-deoxycytosine, 2'-deoxyguanosine, and thymidine. *Chemical Research in Toxicology, 6*, 690–700.


