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Invited review

Hybridization properties of base-modified oligonucleotides within the double and triple helix motif

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Abstract – In this review we describe the influence of modified nucleobases in order to understand the changes they introduce in double and triple helix forming oligomers with the aim to study their potential therapeutic use in the antisense/antigene/ribozyme field. The hybridization properties of the studied modified oligomers depend on the structure of the modified nucleoside, the number of modifications, the selected sequence and the location of the modified nucleoside along the sequence. Unfortunately, most available information is based on melting point determination, eventually CD experiments and only in a few cases on X-ray and NMR studies which makes it difficult to put forward general rules for predicting modified nucleic acid structures. On the other hand, several of the described base modifications allow us to select new oligonucleotides with improved properties in the different application fields. © Elsevier, Paris

modified nucleobase / oligomers / stability / hybridization properties / base pairing

1. Introduction

The chemistry and structure of modified nucleosides are studied intensively in order to understand the functional changes that they introduce into the nucleic acids in which they occur. In fact, there are many reasons why modified nucleosides are incorporated into oligonucleotides. Obvious reasons are the study of the influence of modified nucleosides on the shape and stability of DNA and RNA duplexes with the aim to study their potential therapeutic use in the antisense/antigene/ribozyme field. Increasing binding strength and improving binding kinetics are the main goals of this research. Other modifications were introduced to study the mechanism of action of biological active nucleoside analogues, to search after a good candidate for incorporation at ambiguous positions, to covalently modify DNA, to study the substrate specificity of polymerases, to broaden the genetic code, to use modified nucleosides for analytical purposes, to study protein-nucleic acid interactions, to study the function of naturally occurring non-canonical nucleosides in tRNA and so on.

The replacement of a natural nucleoside by a chemically modified congener within a certain sequence may give rise to fundamental changes in the duplex structure, dependent on which intra- and intermolecular interactions are influenced. These modifications may have an impact on stacking interaction, H-bonding, solvatation by influencing donor/acceptor properties of nucleobases, pK_a, tautomeric equilibria, cis/trans conformational changes of nucleosides, steric effects, electrostatic effects, hydrophobicity, planarity, van der Waals interactions, counterion organization. As a result of this, base pairing may alter (e.g. wobbling of base pairs), hydration spines in the grooves may be influenced, the helix form (A, B, Z, ...) may be altered, bases may be expelled from the double helix, the conformation of the DNA may be changed (groove width, curvature and bendability). The observed alterations are sequence-dependent (nearest-neighbor interactions) and dependent on the site of incorporation. Indeed structural consequences of replacing a nucleoside within a DNA or RNA helix are transmitted over a

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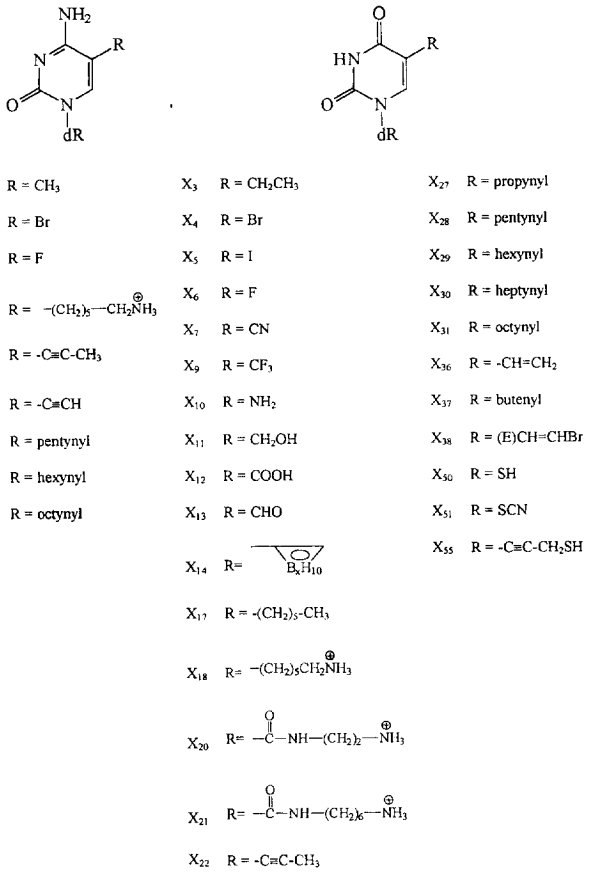


Figure 1.

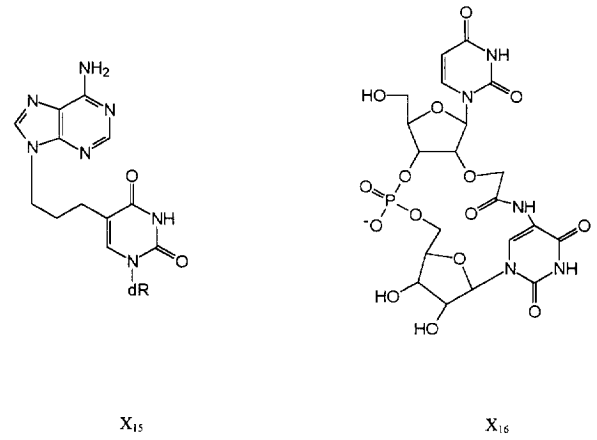


Figure 2.

significant distance and DNA conformation may be changed beyond the site of substitution. Drastic alterations of duplex structure can give rise to multi-state helix-coil transitions. There are simply no general rules which could predict the changes in duplex structure upon modification of individual nucleotides and only NMR or X-ray analysis may give a clear insight into these structural changes. However, this information is not available for most modifications. In most cases, only the change in thermal stability of the nucleic acids duplex upon incorporation of a modified nucleoside is reported. Giving T_m's of duplex structures is generally not very informative when it is not combined with some structural information from which CD measurements are the most simple, the most easily obtainable but also the least detailed, and with some thermodynamic parameters. Molecular dynamics studies may, to a certain extent, be usefull to propose a model for the altered duplex or triplex. A disadvantage of X-ray and NMR studies is that these structural analyses are done under different circumstances than found in nature, so they are not always convergent with the real situation.

This article reviews the present knowledge of stability/structural alterations of double and triple stranded nucleic acids when modified nucleosides are incorporated. Unfortunately, most available information is based on assumptions (after taking T_m's and eventually CD spectra) but not on detailed structural analysis. Much work still has to be done before we will have a clear insight in the effect of small molecular modifications on the micro- and macroconformational changes of biooligomers such as DNA and RNA.

2. Pyrimidine modifications

2.1. C-5 substitutions

The 5-position of pyrimidine nucleosides is one of the most favoured substitution sites because the chemistry for introducing substituents at that position is very well developed and moreover 5-substituted pyrimidines often demonstrate interesting biological properties. This 5-position is exposed to solvents in the major groove and substitution at this position is expected neither to interfere with base-pairing nor to influence the general structure of the double helix [1]. Small substituents in this 5-position may be expected to influence beneficially with neighboring bases.

Replacement of 2′-deoxyuridine and 2′-deoxycytidine by thymidine and 5-methyl-2′-deoxycytidine $\mathbf{X_1}$ into DNA has long been known to enhance duplex stability [2–5]. Replacement of the hydrogen atom by a methyl

Table I.

R	$(dU^R - dA)_{10}$	$(dC^R - dG)_6$
H	–	80 °C
CH_3	47 °C	92 °C
C_2H_5	–	81 °C
$CH{=}CH_2$	44 °C	–
C_3H7	35 °C	–
i-propyl	15 °C	–
1-butenyl	47 °C	–
butyl	34 °C	81 °C
pentyl	32 °C	79 °C
hexyl	< 5 °C	76 °C
octyl	< 5 °C	42 °C

group in the 5-position causes increased hyrophobic attractive interactions between the 5-methylpyrimidine residue and neighboring bases. The 5-methyl substituent may, however, influence the duplex structure to a greater extent than originally expected. The methylation of cytosine may not only stabilize the duplex structure against thermal perturbations [6, 7] but may reduce the cooperatively of the duplex melting process [6] as was observed for the self complementary sequence ATACGCGTAT ($T_m = 45$ °C) and $ATAX_1GX1GTAT$ ($T_m = 48$ °C). The presence of the methyl group in the major groove may affect the conformation of the oligonucleotide in solution [8] dependent on sequence and conditions. In some cases stabilization of the A form of DNA is reported, in other cases A to B or B to Z transitions are promoted [2, 6]. The presence of a 5-methylgroup (uracil versus thymine exchange) likewise gives enhancement in duplex stability of terminal wobble and Watson–Crick pairs (G:U, A:U, 2,6-diaminopurine:U versus T base pairs) [9].

Increasing the bulkiness of the substituent at the 5-position reduces duplex stability [GGAGAUCTCC ($T_m = 40$ °C), GGAGATCTCC (T_m: 42 °C), $GGAGAX_3CTCC$ ($T_m = 38$ °C)] [10]. The bulky hydrophobic substituent hampers efficient solvation of the duplex [10–13]. This decrease in duplex stability with increasing bulkiness of the substituent is more pronounced in a $(dU\text{-}dA)_{10}$ series than in a $(dC\text{-}dG)_6$ series [11]. Unsaturation of the 5-substituent has a beneficial effect on thermal stability (compare 5-butenyl versus 5-butyl) [11] (see *table I*).

Incorporation of 5-bromouracil (X_4) [14, 15] and 5-bromocytosine (X_2) bases [4, 7, 16], likewise stabilizes a duplex in comparison with the unmodified bases, which is attributed to the greater stacking potential of the modified base. The bromine substituent influences the

Table II.

Sequence	T_m of DNA (°C)	T_m of RNA (°C)
GCGAAUUCGC	44.2	53.0
$GCGAAX_6UCGC$	42.9	53.3
$GCGAAUX_6CGC$	44.3	54.0

electronic distribution within the pyrimidine ring which affects its interaction with neighboring bases [16]. In fact, the effect of a 5-methyl (T and X_1), a 5-bromo (X_2 and X_4), a 5-iodo (X_5) [4, 17, 18] and a 5-fluoro (X_6) substituent on duplex stability is very similar and these halogens slightly increases melting temperatures of the purine-pyrimidine base pairs, while pyrimidine-pyrimidine base pairs are not effected [4, 19, 20]. This is opposite to the influence of a cyano group (X_7). In contrast, a cyano group reduces the hydrophobicity and has a destabilization effect on duplex stability [10].

5-Bromouracil also enhances the photosensibility with respect to RNA or DNA-protein photo-cross linking [21, 22], UV-induced single- and double-stranded DNA cleavage [23] and creation of alkali-labile sites [21]. Using 5-iodouracil, cross linking yields of nucleoprotein complexes are three to five times higher than those achieved with 5-bromouracil [24, 25]. Likewise, 5-iodocytosine is an excellent chromophore for nucleoprotein photocross linking [25].

The situation with the 5-fluoro substituted derivatives is relatively complex. The incorporation of 5-fluorouracil nucleosides in DNA and RNA is extensively studied [26–32]. Replacement of uracil by 5-fluorouracil at a single site in A-form RNA, very slightly stabilizes the duplex. The observation that the fluorouracil:adenine base pair is somewhat more stable than the uracil:adenine base pair, suggest that the C-4 carbonyl group of fluorouracil is involved in H-bonding interactions. Substitution of uracil by 5-fluorouracil at a G-U wobble base pair slightly destabilizes the duplex, caused by a reduced stacking for the $G\text{-}X_6$ base pair relative to the G-U base pair [32]. Incorporation of 5-fluoro-2′-deoxyuridine into B-form DNA has a slight destabilization or no effect on duplex stability dependent on the studied sequence [27, 28] (see *table II*). The structure of 5-fluorouracil containing oligomers is very sensitive to pH changes [30].

Likewise, 5-fluoro-2′-deoxycytidine (X_8) does not significantly perturb either the structure or the stability of duplex DNA [33]. 5-Fluoro-2′-deoxycytidine was incorporated in DNA, mainly to elucidate cellular functions governed by DNA-methyltransferase [34–36]. This enzyme catalyses the transfer of a methyl group to the C-5 position of cytosine.

Many explanations have been given to try to explain the influence of 5-fluorinated nucleosides on duplex stability. Fluorine, because of its electron withdrawing inductive effect, lowers the pK_a value of the pyrimidine base rendering the imino hydrogen more acidic. The influence of the fluorine atom on the strength of the $(X_6)N^3 - H\cdots N^1$ (adenine) hydrogen bond is dependent on the sequence of the oligonucleotide as the pK_a of 5-fluorouracil nucleoside in an oligonucleotide is dependent on its environment [28, 31]. The pK_a of the $N^3 - H$ ionization of 5-fluorouracil range from 7.5 to 8.2. At physiological pH, a significant fraction of the incorporated fluorouracil residues in DNA will be ionized [31]. The N-3 hydrogen bond becomes less stable in a 5-fluorouracil:adenine base pair than in a thymine:adenine base pair [31]. It was observed that poly(U) and poly(FU) have similar conformation at low pH, but increasing pH induces conformational changes in poly(FU) [30], mediated by electrostatic interactions between the ionized phosphate groups and the negatively charged base moiety with raising pH. Secondly the fluorine atom may influence stacking interactions within dsRNA and dsDNA helix in a more or less favorable way. As a consequence of decreased stacking affinity in DNA for neighboring bases, the rate of base pairs opening at the site of incorporation of 5-fluoro-2′-deoxycytidine may be increased. Despite the fact that 5-fluorouracil may have several influences on the local structure of an oligomer, its incorporation generally has a negligible effect on the stability of dsRNA and dsDNA duplexes when compared with natural sequences. Likewise, 5-trifluoromethyl-2′-deoxyuridine (X_9) was incorporated in DNA and its influence on DNA replication was investigated [37]. The trifluoromethyl group has a more negative electrostatic potential and a larger van der Waals radius than a methyl group. 5-Trifluoromethyluracil forms Watson–Crick base pairs with adenine [38] and, except for hyperchromicity, the thermal melting profiles and CD spectra of the modified oligonucleotides is similar to that of the parent natural sequences [38].

A 5-amino group (X_{10}) slightly destabilizes [39, 40] or slightly stabilizes [41] duplexes probably dependent on both the neighbouring bases and the length of the oligonucleotides. The 5-acetamido group shows similar behaviour. In fact, the modifications of the base occurs at position 5, which is not directly involved in base-pairing. Moreover, the electron-donating properties of the amino group and acetamido has little influence in the base-pairing properties. A 5-amino group may be used for post-synthetic conjugation of DNA. Such modification, like dansylation, however, further destabilizes the duplex. 5-Hydroxymethyl-2′-deoxyuridine (X_{11}) [42, 43] and 5-carboxylate-2′-deoxyuridine (X_{12}) [44], likewise, were incorporated into DNA but information about their effect on duplex stability is lacking. 5-Formyl-2′-deoxyuridine (X_{13}) is a modified nucleoside isolated from γ-irradiated calf thymus DNA. Like the other 5-substituents, the formyl group of X_{13} could influence the stability of double stranded DNA either by its electron withdrawing effect (rendering the N^3-imino group more acidic) either by its influence on stacking and hydration [45–47]. 5-Formyluracil is recognized by the adenine base (DNA polymerase incorporate 2′-deoxyadenosine opposite to 5-formyl-2′-deoxyuridine) although with a reduced rate as natural nucleosides and incorporation of X_{13} destabilizes, for instance, the duplex d(CGCGAATTCGCG) [T_m = 67.5 °C; d(CGCGAAX_{13}TCGCG) T_m = 63.4 °C] [48]. The stability of the X_{13} – A base pair is dependent on the sequence of the duplex. Incorporation of X_{13} in the middle position of the sequence [GGAGATCTCC (T_m = 31 °C), GGAGAX_{13}CTCC (T_m = 40 °C] greatly stabilizes the self-complementary duplex.

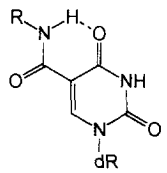
When non-radioactive boron-10 nuclei is irradiated with low-energy neutrons, an α-particle and lithium-7 nuclei are released, producing 100 million times more energy than initially used [49]. To further increase the selectivity of the boron neutron capture therapy for malignant tumors (or as probes for diagnosis), 5-(o-carboran-1-yl)-2′-deoxyuridine (X_{14}) was incorporated into oligonucleotides [49]. This bulky boron cage, however, sterically clash with the 5'-adjacent base which explains the decrease in duplex stability when incorporating 5-(o-carboran-1-yl)-2′-deoxyuridine in the centre or at the 3′-end of an oligonucleotide. The 5′-terminal modified oligonucleotides display a termal stability similar to the natural oligomer [49].

The C-5 position of pyrimidine nucleosides was used for tethering extrahelical bases which may stabilize nucleic acids complexes through hydrogen bonding to the opposite strand base and/or by intercalations [50]. The stability of the oligonucleotide containing one 5-(γ-adenylpropyl)-2′-deoxyuridine residue (X_{15}) is the same as that of the parent thymine:adenine containing duplex. A dinucleotide cyclized via an amide linker between the C-5 position of the second nucleoside and the 2′-hydroxyl group of the first nucleoside (X_{16}) was synthesized as a conformationally constrained model of a ^{t}RNA U-turn structure (the anticodon loop in ^{t}RNA has a sharply bent structure named U-turn) [51].

In a strategy to obtain dsDNA with a reduced net charge, zwitter ionic nucleotides were used to build up an oligonucleotide [52, 53]. This was realized using 5-ω-aminohexyl substituted pyrimidines (molecular model-

Table III.

Sequence	T_m of duplex (°C)
CTTTCTCTCCCT	39.4
$CX_{17}TX_{17}CX_{17}CX_{17}CCCT$	24.2
$CX_{18}TX_{18}CX_{18}CX_{18}CCCT$	39.1
$CTTTX_{19}TX_{19}TX_{19}CX_{19}T$	45.4
$CX_{18}X_{18}X_{18}X_{19}X_{18}X_{19}X_{18}X_{19}X_{19}X_{19}X_{18}$	41.5
$TX_1TX_1TX_1TX_1TX_1TX_1TX_1TX_1T$	49
$TX_1TX_1TX_1TX_1X_{20}X_1TX_1TX_1TX_1T$	53
$TX_1TX_1TX_1TX_1X_{21}X_1TX_1TX_1TX_1T$	53



X_{20} and X_{21}

Figure 3.

ling predicted that a propylether should be the minimum spacer required to make phosphate-ammonium ion interaction possible [52]). The hexyl substituent itself (X_{17}) gives severe duplex destabilization when positioned into the major groove due to disruption of groove-hydration and van der Waals strains [53].

Introduction of a positive ammonium ion (X_{18}) leads to ammonium-phosphate (or ammonium-base) interactions in the major groove and, hence, to charge neutralization. This neutralization (or binding interactions with functional groups) process is more effective than charge neutralization by free counterions. The negative influence of the hexyltether is balanced by the introduction of the amino group and, as a result, the oligonucleotide duplex becomes even somewhat more stable than the natural duplex. The duplex formed between a natural oligonucleotide and a complementary zwitter ion oligonucleotide exhibits little change in stability dependent on the ionic strength of the solution. The loss in counterions upon denaturation for this duplex is lower when compared to a natural duplex. The difference in charge density of the single- and double-stranded state is less pronounced than with natural DNA [53] (see *table III*).

These results were confirmed using another spacer arm like in $X_{20} - X_{21}$ [54–56]. As well end-modifications as centrally incorporated aminoalkyl substituents effectively stabilize duplex formation with both the complementary DNA and RNA strands, even when a bulky functional group such as folic acid, palmitiric acid as cholesterol is attached to the terminus of the amino-linkers [57]. The stability, however, is more pronounced than with the previous spacer, this may be due to the fact that the aminoalkyl spacer is bond at the C-5 position of the pyrimidine base using an amido group. This carbamide group has an electron withdrawing nature and may H-bond with the C-4 carbonyl group of the pyrimidine base, fixing the aminoalkyl group in one direction (*figure 3*) [54]. The pK_a of the modified base (pK_a 8.1) is 1.8 units lower than that of thymidine (pK_a 9.9). This increase in acidity may contribute to complex formation [54].

The situation, however, may be more complicated as initially believed. When the phosphate charges of DNA is asymmetric neutralized by incorporation of 5-aminohexyl substituted pyrimidine nucleosides, DNA bending may be induced [58].

The C-5 position of the uracil base is often used for connecting reporter groups [59] such as fluorescent groups, biotin, complexing agents, intercalators, groups for covalently cross linking of oligonucleotide strands, spin labels. Spin labelled oligonucleotides are used to study structural and dynamic properties of nucleic acids by electron paramagnetic resonance spectroscopy (EPR). This site-specific labelling technique of nucleic acids becomes more and more important, especially with fluorescent labels that gradually will replace radioisotopic labelling. Labelling of uracil at the 5-position will more generally produce duplexes with normal melting curve behaviour than cytosine labelling at the 4-position [60]. Hybrids are most stable when the modification is located near and off the end of the hybridizing sequence. Internally and relatively closed spaced modifications give less stable oligo-DNA hybrids [60, 61]. The duplex stability is significantly increased when potential intercalating agents are bind at the 5-position of the pyrimidine base using different spacers [54, 62].

The stability of DNA-RNA hybrids is increased by substituting 5-propynyl-2′-deoxyuridine (X_{22}) and 5-propynyl-2′-deoxycytidine (X_{23}) for the nucleosides thymidine and 2′-deoxycytidine into DNA [73]. At one side the entropy of binding is increased by the presence of the hydrophobic and bulky 5-propynyl substituent, but on the other hand stacking interactions may be increased [74] [$(TC)_5(T)_5$ DNA with RNA complement: $T_m = 65.5$ °C; $(TC)_3(X_{22}C)_2(X_{22}T)_2X_{22}$: $T_m = 74$ °C; $(TX_{23})_5(T)_5$: $T_m = 73$ °C]. A similar effect is seen when the 5-substituent of 2′-deoxycytidine is further elongated

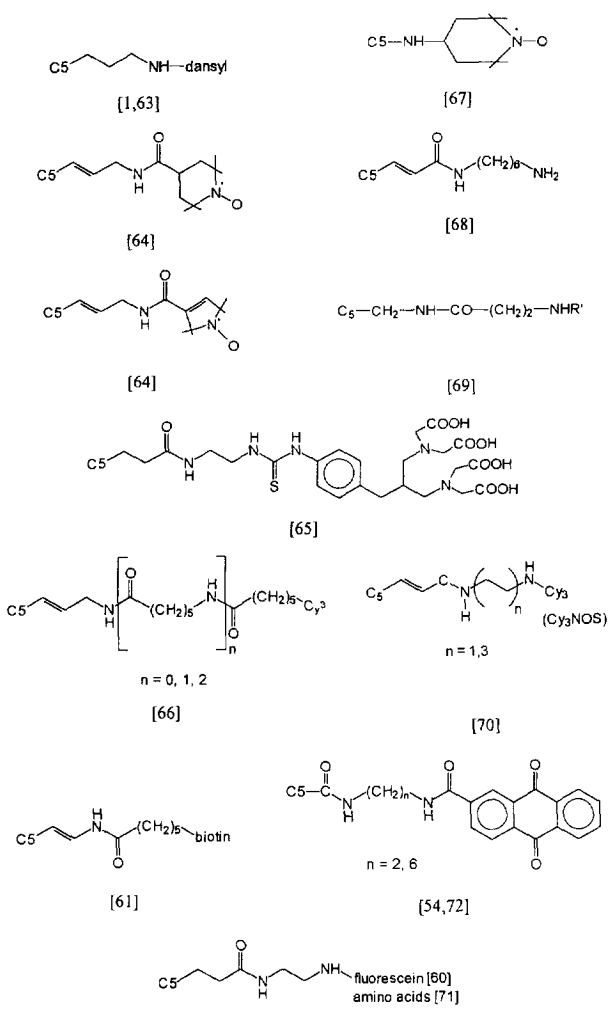


Figure 4. Examples of C-5 connected reported groups which have been incorporated in oligonucleotide.

till six carbon atoms [$(CG)_6$: T_m = 79.8 °C; $(X_{24}G)_6$: T_m = 95.2 °C; $(X_{25}G)_6$: T_m = 95.6 °C; $(X_{26}G)_6$: 95.9 °C; $(X_{27}G)_6$: 90.0 °C] [11]. The increase of stabilization, upon substitution of deoxycytidine in the 5-position with alkynes of growing chain length, is more uniform than on substitution of the deoxyuridines in the $(U\text{-}A)_n$ sequence. With deoxyuridine the stabilization effect decrease with increasing chain length and drops largely after hexynyl [$(TA)_{10}$: T_m = 47.4 °C; $(X_{22}A)_{10}$: T_m = 58.6 °C; $(X_{28}A)_{10}$: T_m = 54.4 °C; $(X_{29}A)_{10}$: T_m = 52.0 °C; $(X_{30}A)_{10}$: T_m = 44.0 °C; $(X_{31}A)_{10}$: T_m = 18.3 °C].

The 5-(1-pentynyl)-2′-deoxyuridine nucleoside was also used in a random nucleic acid library for the discovery of new aptamers [75]. Similar effects were

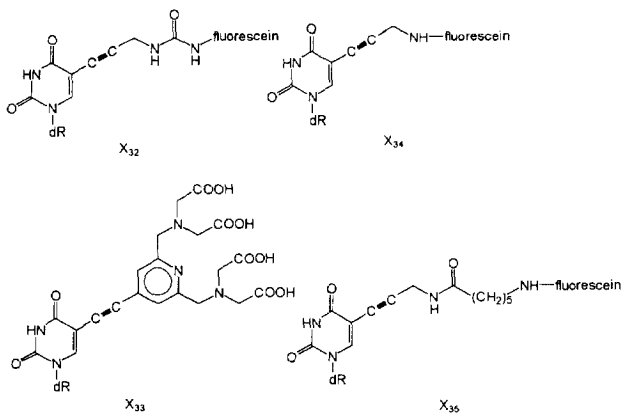


Figure 5.

observed with another sequence $[(TC)_5T_5]$ and RNA as complement [76]. When the five last thymidines are replaced by 5-propynyl-2′-deoxyuridine (X_{22}) the T_m increases from 62.5 °C to 70.5 °C. With 5-butynyluracil (T_m = 69.5 °C), 5-isopentynyluracil (T_m = 66.5 °C) and 5-^{t}butylethynyluracil (T_m = 66.5 °C) the stabilization effect is less pronounced. The influence of a 5-thiopropynyl substituent is marginal [CTTCTXTTTCTTC: T_m = 45 °C when X = thymidine and 45.6 °C when X = 5-thiopropynyl-2′-deoxyuridine] [77].

Due to the beneficial effect of the 5-positioned triple bond on duplex stability and the easy synthetic accessibility, this functional group was selected as an ideal spacer arm for binding reporter groups [78] (*figure 5*).

The T_m by incorporation of X_{32} into an oligonucleotide decreases by 2.5 °C [78]. The chelating function, present in X_{33}, form strongly fluorescent complexes with europium ions [79] and may be useful for the analysis of specific gene sequences in an oligonucleotide ligation assay. Fluorophores connected at the C-5 position with a short linker arm (X_{34}) hybridize considerably less efficiently than the corresponding probes carrying a longer linker (X_{35}) [80]. The presence of especially triple bonds increase stacking interactions and largely compensate for destabilizing effects seen with longer 5-alkyl substituents [11].

5-Vinyl-2′-deoxyuridine (X_{36}) and 5-(1-butenyl)-2′-deoxyuridine (X_{37}) destabilize the alternating $(U\text{-}A)_{10}$ duplex [11]. Short DNA fragments can accommodate one bulky bromovinyl residue (X_{38}) without changing its secondary structure [81]. This is also confirmed for a $(A\text{-}T)_6$ sequence. Complete replacement of the methyl groups of thymidine by bromovinyl, however, decreases duplex stability [82].

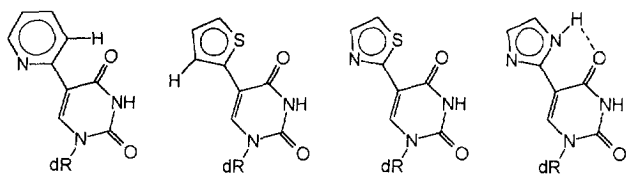
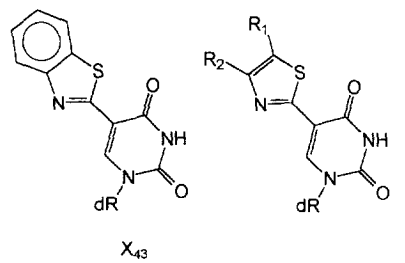


Figure 6.

Increased base stacking interactions of adjacent base pairs in a DNA-RNA duplex is also expected by introducing selected C-5 heteroaryl substituents that are coplanar with the pyrimidine ring [83]. The effect of a pyridinyl [$(TX_1)_5(X_{39})_5$: $T_m = 64$ °C] and thienyl [$(TX_1)_5(X_{40})_5$: $T_m = 64.5$ °C] substituent is similar to that of thymidine [$(TX_1)_5T_5$: $T_m = 62.5$ °C] while the effect of a thiazolyl substituent [$(TX_1)_5(X_{41})_5$: $T_m = 71$ °C] is similar to that of the stabilizing 5-propynyl group ($T_m = 70.5$ °C), the imidazolyl group being of intermediate stability [$(TX_1)_5(X_{42})_5$: $T_m = 66$ °C].

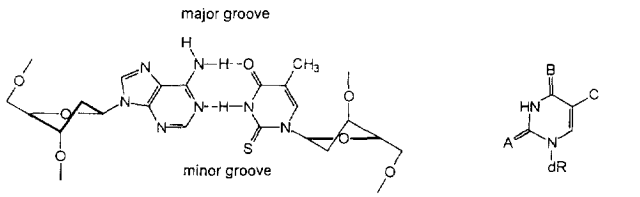
The different effect of the heteroaromatic substituents on the stability is explained based on steric interactions and hydrogen bonding leading either to deviation from coplanarity (X_{39}, X_{40}) either to the formation of weaker hydrogen bonds with complementary adenine base (X_{42}) [83].

As the substituents on the 4- and 5-carbon atoms of the thiazole ring do not interfere with steric interactions with the H-6 or O_4 of the uracil ring, they were further substituted with methyl groups and a fused benzene ring [76]. Using RNA as complement, the benzothiazole substituent (X_{43}) gives increased stabilization as compared to a 5-methyl group [$(TC)_5(X_{43})_5$: $T_m = 68.5$ °C] but a decreased T_m with respect to the thiazole substituted oligonucleotides. This is in contrast with the results observed for fused tricyclic substituents [84].



	R_1	R_2
X_{44}	CH_3	H
X_{45}	H	CH_3
X_{46}	CH_3	CH_3

Figure 7.



	A	B	C
X_{47}	O	S	CH_3
X_{48}	S	O	CH_3
X_{49}	S	O	H

Figure 8.

The 5-methylsubstituent of the 5-methylthiazol ring of X_{44} may increase hydrophobic interactions with the thiazole ring of the adjacent base pair and further increase the affinity of the oligonucleotide for its RNA complement [$(TC)_5(X_{44})_5$: $T_m = 73.5$ °C]. This is not the case for the 4-methyl substituted compound X_{45} where the methyl group causes unfavorable steric interactions with H-2′ and H-3′ of the sugar of the adjacent base pair [$(TC)_5(X_{45})_5$: $T_m = 69.5$ °C]. The dimethyl substituted thiazole ring (X_{46}) has an intermediate effect ($T_m = 71.0$ °C) and the stability of the oligonucleotides containing the dimethylthiazole substituted nucleosides is the same as that of the thiazole containing oligonucleotide [76].

2.2. Sulfur-containing substituted pyrimidines

Several 2-thiopyrimidine and 4-thiopyrimidine nucleosides occur in ^{t}RNA molecules. s^2U substitution in a duplex may result in a preference for the 3′-endo sugar pucker, a better stacked structure of the duplex due to the more polarizable thio group and an increased acidity of the N-3 imino proton (stronger H-bond). The destabilization upon s^4U substitution is due to the lack of steric factors favoring the 3′-endo pucker and a weaker Watson–Crick base pair due to the replacement by a weaker hydrogen bond accepting sulfur despite the fact that the s^4U imino hydrogen is more acidic compared to s^2U and U (expected to form a stronger hydrogen bond). The acidity effect is counterbalanced by the weak hydrogen bond accepting property of the sulfur at C4 involved in Watson–Crick base pairing [85]. NMR studies and UV melting experiments coupled with thermodynamic analysis established that 2-thiouridine (s^2-U) containing RNA forms a very stable duplex structure [$Gs^2UUUC/G_mA_mA_mA_mC_m$: $T_m = 30$ °C] whereas substitution with s^4U [$Gs^4UUUC/G_mA_mA_mA_mC_m$:

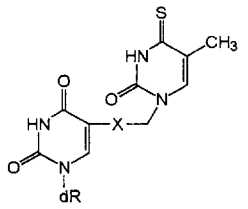
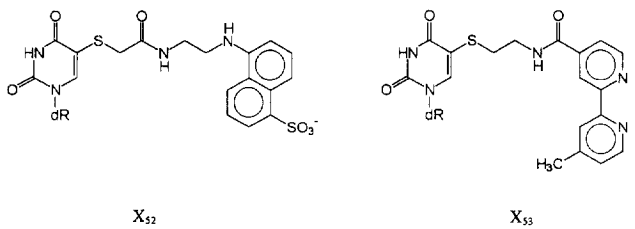


Figure 9.

T_m = 14.5 °C] leads to destabilization of the RNA duplex compared to the unmodified control duplex [GUUUC/$G_mA_mA_mA_mC_m$: T_m = 19.0 °C]. Sulfur is considered as a weaker hydrogen bond acceptor than oxygen, it is bulkier than oxygen and more polarizable, potentially increase stacking interactions when appropriate situated. Extensive studies on the incorporation of 4-thiothymine/4-thiouracil [85–96], N^3-methyl-4-thiothymine [97] and 2-thiothymine/2-thiouracil [8–87, 96, 98–100] in oligonucleotides are described. The main difference is that 2-thio analogues may be used for interaction studies with ligands in the minor groove of DNA (because of its altered hydrogen bonding characteristics) while 4-thiocarbonyl group is normally involved in the base pairing system in the major groove.

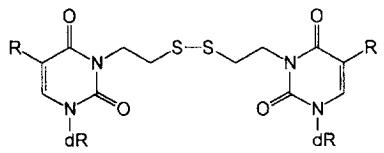
In biochemical studies, the thiocarbonyl group is used for photoaffinity labelling of nucleic acids binding proteins (via triplet state energy transfer to aromatic amino acid residues) [94, 96], and as a site-specific handle for the attachment of functionalized tethers. This functionalization may be carried out postsynthetically [101]. The 4-thiothymine base was also connected at the C-5 position of deoxyuridine and 2′-deoxy-4-thiouridine via a linker as a nucleic acid photoaffinity probe to investigate the photochemical behaviour of such oligonucleotide constructs towards their DNA and RNA complementary targets (*figure 9*) [102, 103].

Incorporation of 4-thiothymidine (X_{47}) does not alter duplex stability considerably [GACGATATCGTC: T_m = 53 °C; GACGAX_{53}ATCGTC: T_m = 52 °C] [86, 87].



X_{52} X_{53}

Figure 10.



X_{54}

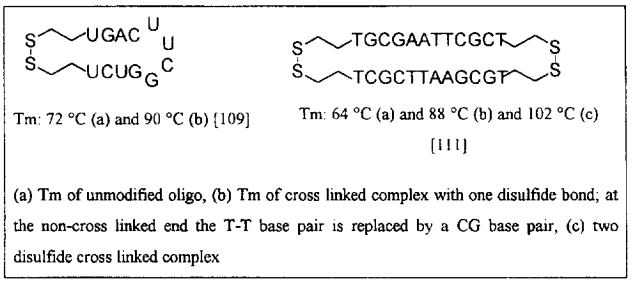


Figure 11.

The CD spectra of the 4-thiothymidine incorporated oligonucleotides are similar of those of B-DNA. The results obtained by incorporation of 2-thiothymidine (X_{48}) or 2′-deoxy-2-thiouridine (X_{49}) are different. Incorporation of 2-thiothymidine in the above mentioned sequence [GACGAX_{48}ATCGTC: T_m = 56 °C] gives stabilization of the duplex. After evaluation of the CD spectra, it was initially stated that these oligonucleotides do not show a typical B-DNA like helix structure but that the spectra are more resembling A-DNA (or distorted B-type DNA) [86, 87].

The occurrence of broad and complex melting curves and the increased T_m in palindromic sequences by incorporation of 2-thiothymidine, was also confirmed in another sequence [GGCGGAATTCCGCC: T_m = 64 °C; GGCGGAAX_{48}TCCGCC: T_m = 69 °C; GGCGGAA$X_{48}X_{48}$CCGCC: T_m = 71 °C] [96]. In non-palindromic sequences, where the T_m curves are sharp and more symmetrical, the stability of the duplex was slightly lower than that of the control oligo-

(a) Tm of unmodified duplex: 55 °C.

Figure 12.

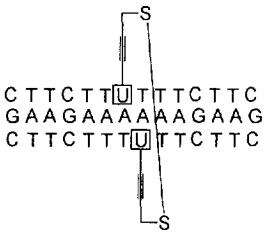


Figure 13.

nucleotide [GGCGGAATTCGCGG: T_m = 70 °C; GGCGGAAX$_{48}$TCGCGG: T_m = 66 °C; GGCGGAAX$_{48}$X$_{48}$CGCGG: T_m = 68 °C]. This holds also for 2′-deoxy-2-thiouridine (X$_{49}$), except for the disubstituted oligomer [GGCGGAAX$_{49}$X$_{49}$CGCGG: T_m = 77 °C] where possibly exist increased stacking interactions of the thiocarbonyl group [96]. The CD curves of the non-palindromic sequences are similar of the control spectra and the small difference is explained here by a widening of the minor groove to accommodate for the sterically bulkier sulfur atom [96].

The enhanced stability of the palindromic sequences may be due to interstrand interactions [96] while the more complex melting curves may be due to a multi-state helix-coil transition. The more complex CD spectra of the palindromic sequence (although a B-type DNA duplex is expected) is explained as the result of the presence of the thiopyrimidine chromophore and its nearest neighbour interactions [96].

While stable base pairs are formed between 2-thiothymine and adenine, no such base pairing occurs between 2-thiothymine and 2-aminoadenine. This is due to a steric clash between the 2-thio group of the pyrimidine base and the 2-amino group of the purine base, resulting in a tilt of the bases relative to each other. The 2-aminoadenine base forms very stable base pairs with thymine itself. This system has been used to create the so called selectively binding complementary oligonucleotides (SBC) [100]. These SBC invade the ends of homologous duplexes and form stable three-arm junctions [100] [GTAAGAGAATTATGCAGTGC: natural duplex T_m = 55 °C; replacing A by 2-aminoadenine and T by 2-thiothymidine in 1 strand: T_m = 63 °C and 65 °C; replacing A by 2-aminoadenine and T by 2-thiothymidine in both strands: T_m = 26 °C].

Other thio-containing substituents were mainly introduced at the C-5 position of the pyrimidine base. 5-Mercaptodeoxyuridine (X$_{50}$) or its 5-thiocyanato precursor (C$_{51}$) were used for the site specific incorporation of a reactive thiol group in DNA [104, 105]. A variety of molecular tags (photo-cross linkers, fluorescent groups, spin-label moieties) can be connected to the mercapto group by post-synthetic modifications exemplified by X$_{52}$ and X$_{53}$. The first one (X$_{52}$) gives fluorescence emission. The bipyridine structure (X$_{53}$) extrudes in the major groove and forms stable complexes with transition metals such as Zn^{2+} and Cu^{2+}, which are known to bind to phosphodiester bonds and activate them towards nucleophilic attach [106].

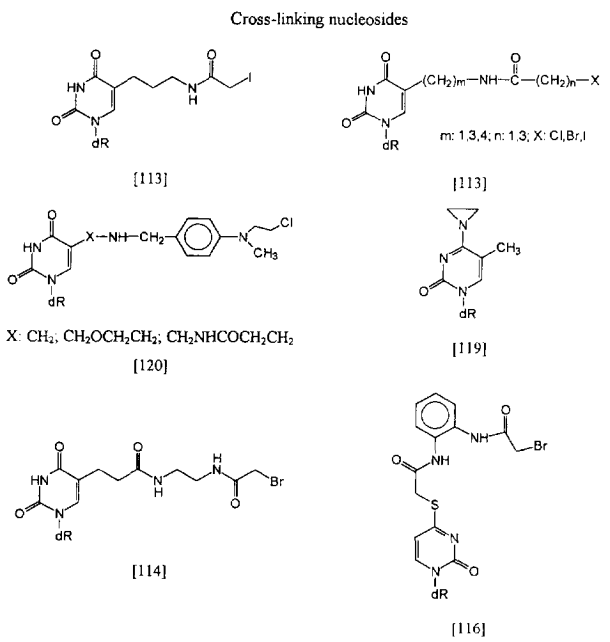
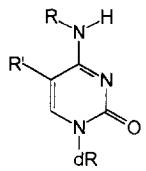


Figure 14.

2.3. *Cross-linking pyrimidines*

The most classical way of conformational stabilization of bioorganic molecules is by using a disulfide cross-link (X$_{54}$). This technique was used for the stabilization of hairpin structures (intramolecular) and nucleic acids duplexes (intermolecular) [107–112]. It may be used for stabilizing DNA conformations for NMR studies or to study torsional stress in DNA, like induced by the action of proteins [112]. The cross link increase the conformational homogenicity of the system and increase the thermal stability of hairpins and duplexes [110]. The introduction of a single cross-link changes the molecularity of the duplex from bimolecular to monomolecular and a large increase in thermal and thermodynamical stability is observed [110]. The second disulfide cross-link further increases the thermal stability due to an entropy decrease [110].



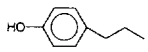
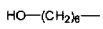
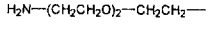
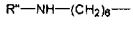
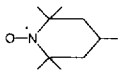
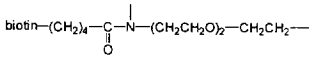
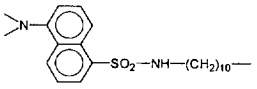
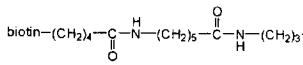
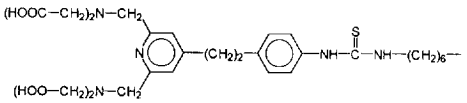
R	R'	Ref.
	H	[129]
	H	[123]
	H	[122]
 R*: biotinyl, dinitrophenyl, dansyl, pyrenyl	H	[121,125]
	H	[128]
	H	[124]
	H	[126]
	H	[127]
	H	[130,136]
-$(CH_2)_{10}$-NH-biotin	CH_3	[131]
-CH_2-pyrenyl	CH_3	[137]

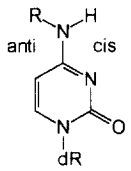
Figure 15. Examples of N^4-substituted cytosine base.

Cross-linking of two nucleic acids strands in the centre of the duplex and its influence on dsDNA structure was, likewise, studied [112]. By simple attaching the tether itself to DNA (without inducing cross-links), duplex destabilization was observed. The stabilization observed by introducing the disulfide bridge is dependent on the length of the cross link.

The 3-carbon link gives a relaxed structure. With the 2-carbon link the torsional stress is increased. The link is oriented in a syn-orientation with respect to the N^3 of the cytosine base, resulting in a steric clash between the α-methylene group of the tether and the carbonyl group of the complementary guanine base [112]. Instead of unwinding, the torsional stress is relieved by disrupting the Watson–Crick base pair system, forcing the guanine to slide into the minor groove.

The disulfide bond is also used for covalent cross-linking between two non-complementary DNA strands [77]. Using 5-thiopropynyl-2′-deoxyuridine (X_{55}) under aerobic conditions the two pyrimidines of a pyr-pur-pyr system were cross-linked. The melting temperature of the triplex which is normally 16 °C at pH 7 and 49 °C at pH 5, is increased to 52 °C and 66 °C respectively by introduction of one cross link. The thermal stability of the complex does not change substantially by introducing two (53 °C, 67 °C) or three (51 °C, 64 °C) linkages. The T_m of the natural duplex itself is 45 °C and of the duplex containing one thiopropynyl dUrd nucleotide: 46 °C.

The effectiveness of antisense oligonucleotides can also be improved by using other cross-linking reactive groups. The chemical groups used for irreversible inactivation of the complement target nucleic acids are



GCAAG(X)TTGC [133,134]			GACUU(X)GUA [138a]		
	R	Tm (°C)	X_n	R	Tm (°C)
cytosine	H	51.3	cytosine	H	50.5
X_{56}	CH_3	45.5	X_{56}	CH_3	48.0
X_{57}	$(CH_2)_2OH$	41.0	X_{57}	$(CH_2)_2OH$	48.7
X_{58}	$(CH_2)_2NH_2$	44.4	X_{58}	$(CH_2)_2NH_3^+$	50.5
X_{59}	$(CH_2)_4NH_2$	45.3	X_{59}	$(CH_2)_4NH_3^+$	54.3
X_{60}	CH_2COOH	36.7	X_{62}	CH_2-Ph	42.3
X_{61}	$(CH_2)_2$-SS-$(CH_2)_2NH_2$	40.9			

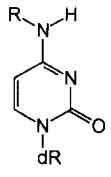
Figure 16.

haloacetyl groups [113–116], N-chloroethyl group [117] and N^4, N^4-ethanocytosine [118, 119]. An o-phenylenediamine tether to append an α-bromoacetamido function was used to covalent cross-links within duplex DNA through dG-N7 adduct formation [116]. The correct geometry of the acylamidoalkyl group is critical for activity [115]. Generally, however, the cross linking formation is too slow to use this system for in vitro inhibition of mRNA [119].

2.4. O^2-, N^4- and O^4-substitutions

One of the hydrogen atoms of the 4-amino group of the cytosine base is involved in W–C base pairing with the 6-carbonyl group of guanine. The other hydrogen atom protudes into the centre space of the major groove and, sterically, there should be no impedement for substituting an alkyl group for this hydrogen atom. Electronically, however, substituting an hydrogen atom indirectly involved in base pairing, may perturb the hybridization process. Because of the steric argument, the N^4-position of the cytosine base and of the 5-methylcytosine base has been considered as a potential substitution site for labelling oligonucleotides using different tethers [121–131] and for the electrochemically directed synthesis of solid support bearing oligonucleotides [132]. Although the thermal stability of duplexes obtained after incorporation of these modified nucleosides may sometimes be unaltered, the hybridization kinetics of the oligonucleotides may be affected with some of the tethers [130]. Their hybridization stabilities also depend upon the location along the oligodeoxynucleotide sequence [131].

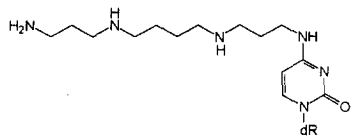
[138b]



$CTTC(T)_6(X)$			$CTTC(T)_6(X)(T)_4$		
	R	Tm (°C)		R	Tm (°C)
cytosine	H	40°	cytosine	H	47°
X_{59}	$(CH_2)_4NH_2$	37°	X_{59}	$(CH_2)_4NH_2$	45°
			X_{63}	$(CH_2)_3CH_3$	44°
			X_{64}	$(CH_2)_3COOH$	43°

Figure 17.

Generally, however, N^4-alkyl-2′-deoxycytidine residues do weakly stabilize duplex DNA when incorporated in oligonucleotides. This is demonstrated with the palindromic sequence GCAAG(X)TTGC [3, 133, 134] and GGA(X)CCGGGTCC [135]. It is explained by the need of the substituent to adopt the thermodynamically less stable anti-rotamer about the C4 – N^4 bond, to be able to form W–C base pairs with guanine. In the free nucleoside, the cis rotamer is favoured. It should also be



N^4-spermine substituent = X_{65}

$TT(X)(T)_6C(T)_6(X')T$	
substituent	Tm (°C)
X = X' = cytosine	51°
X = cytosine X' = X_{65}	46°
X = X' = X_{65}	42°

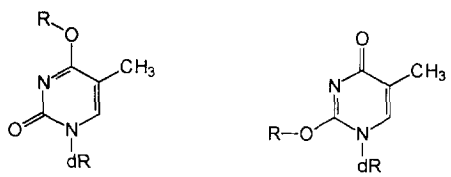
Figure 18.

remembered that N^4-methylcytosine, similarly to 5-methylcytosine, may favour B to Z transitions [135].

When the N^4-substituent becomes more bulkier (for example by introducing a spin label directly to N^4), the reduction in destabilization of the duplex is more pronounced [128].

The drastic decrease in thermal stability when acetate groups (X_{60}) are introduced may be the result of charge repulsion between neighboring carboxylate groups of the tether [133].

The slight destabilization effect of N^4-alkyl-2′-deoxycytidines was confirmed for non-palindromic sequences [60, 138a] as well at the 3′-end as in the middle of a duplex. The relative nonpolar tethers of methylamine, benzylamine perturbed stability more than the polar tether of ethanolamine. The identity of the functional group (NH_2, COOH) attached at the end of a



	R		R
X_{66}	CH_3	X_{71}	CH_3
X_{67}	C_2H_5	X_{72}	C_2H_5
X_{68}	C_3H_7		
X_{69}	C_4H_9		
X_{70}	CH_2-C_6H_5		

Figure 19.

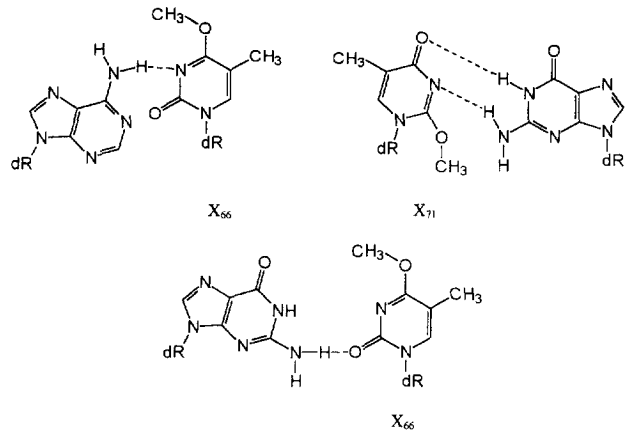


Figure 20.

(butyl) spacer is of low influence on duplex stability (X_{59}, X_{63}, X_{64}). This is somewhat unexpected. At neutral pH, the aminoalkyl substituent is protonated (X_{58}, X_{59}) and duplex stabilization might be expected by counteracting the electrostatic repulsion between the negatively charged oligonucleotides [6, 138a].

However, as the duplex is destabilized (instead of stabilized), one could argue that the protonated aminoalkyl group is involved in stabilization of the single strand oligonucleotide by internal electrostatic effects, while the influence on duplex stabilization is negligible. The introduction of polyamine substituents such as trieethylenetriamine and spermine gives, likewise, duplexes with a lower T_m than the unmodified control duplexes [139, 140].

The aminoalkyl substituents are indeed sterically undemanding. Termination into a protonated amino group assures its water solubility when the amino group is substituted with a reporter group (pyreensulfenyl, fluorescein, biotin-LC, pyreenbutyrate) [60]. The all-or-none T_m behaviour is changed to a different (at least two-phase hybridization) mechanism. This was studied for the sequence GCA(X)TCAG where X is a substituted N^4-aminopropyl-2′-deoxycytidine.

O-Alkylated pyrimidines are incorporated as models to study the effect of carcinogenic alkylating agents [141–148]. These agents are able to alkylate the thymine residues at its O-4 position (O-2 position) and eventually induce A:T to G:C transitions. Hydrogen bonding to O^4-MeT is very weak.

NMR studies demonstrated that the O-methyl group of O^4-methyl thymine (X_{66}) adopts a syn orientation with respect to the N3 of the base and is, thus, directed towards the purine base in the pu-py base pair [149] (*figure 20*).

G C A A T G G A X C C T C T A
X G T T A C C T Y G G A G A T

Y \ X	T	C	X_{67}	X_{70}
A	59°	49°	45°	33° and 48°
G	54°	62°	49°	38° and 50°

Figure 21.

C A G G A A T X C G C
G T C C T T A Y G C G

Y \ X	T	X_{71}	X_{66}
A	59°	39°	39°
G	51°	53°	47°

Figure 22.

This syn orientation of the OCH_3 group rules out both the wobble pairing for the G:O^4MeT lesion site and the postulated structure in which the iminoproton of G is hydrogen bonded to N^3 of O^4MeT [150]. Only one short hydrogen bond between the 2-amino group of G and the 2-carbonyl group of O^4MeT may be formed [150]. Only wobble pairing is possible for the A:O^4MeT base pair [149] (*figure 20*).

The base pair of O^4-alkyl T (X_{66}, X_{67}, X_{69}, X_{70}) with guanine is somewhat more stable than with adenine [151, 152]. The presence of X_{66} in an oligonucleotide opposing adenine or guanine destabilizes the helix. The melting curve of the oligonucleotide containing X_{66} base paired to guanine is biphasic which may be due to preferential melting of the base pairs around the modification site [153]. A biphasic melting profile is also reported for X_{66}:A containing duplexes and for X_{70}:A and G base pairs. The authors attributed this phenomenon as due to existence of two distinct types of duplexes having the alkyl group in syn and anti [151].

O^2-alkyl thymine preferentially pairs with guanine rather than with adenine. The stability of the duplex containing X_{71}:G wobble pairs is greater than those having a X_{66}:G base pair [154]. The weakness of the X_{71}:A interaction is explained by the observation that the presence of the O^2-methyl group prevents a close approach between the two bases and that only 1 hydrogen bond can be formed because of the lack of a proton on N^3 of X_{71} [154]. Despite of this, both dA and dT are incorporated opposite to X_{71} [155, 156].

Base modified pyrimidine nucleosides were synthesized to approach the problem of the degeneracy of the genetic code, generally at the codon third position [157]. To reduce the use of a complex mixture of oligonucleotides, a base may be used which has reduced hydrogen bonding specificity. The best known example is deoxyinosine. However, the stability of deoxyinosine containing duplexes is of wide variety depending on sequence and on the base to which it is paired [158]. As the pairing region of the natural nucleobases are of the d(onor), a(cceptor); a,d,a; a,d,d or d,a,a type, a base which should pair equally well with the four nucleobases should have the potential of appearing in a tautomeric equilibrium (with a tautomeric constant close to unity) or should have limited H-bonding characteristics. Stacking interactions will undoubtly play an important role during duplex stabilization using such modified bases.

The tautomeric constant (K_T) of N^4-methoxy-2′-deoxycytidine (X_{73}) is near unity [(10-30 in favour of the imino form) and duplexes containing X_{73}:A and X_{73}:G base pairs are of comparable stability but less stable than the corresponding duplexes containing normal base pairs [157]. The initial literature data [15] were somewhat confusing as it was first mentioned that the X_{73}:A base pair is more stable than the T:A pair, attributed to the increasing stacking properties due to the presence of the exocyclic N^4-methoxy group.

The tautomeric equilibrium of N^4-amino-2′-deoxycytidine (X_{74}) is shifted into the direction of the amino form [159]. N^4-amino-2′-deoxycytidine is a good substitute of cytidine (and less well for thymidine) and N^4-amino-2′-deoxycytidine preferentially directs the incorporation of deoxyguanosine monophosphate into DNA [159]. The order of base pair stability is C – G > X_{74} – G > X_{74} – A [160]. The compound, however, is a potent mutagen and

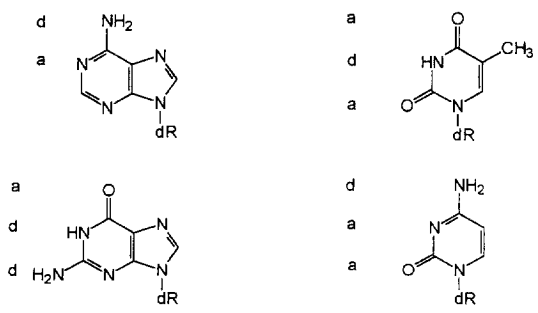


Figure 23.

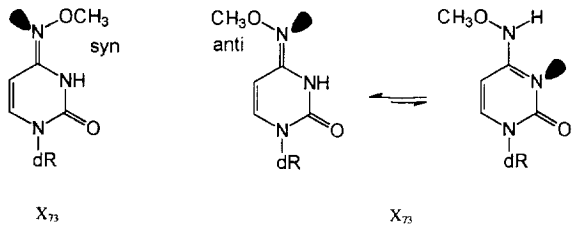


Figure 24.

incorporation of X_{74} into DNA can induce G to A as well as A to G transition [161].

When substituting the amino group with an alkyl substituent, steric hindrance prevents hybridization. N^4-dimethylaminocytosine is not a good substitute for either thymine or cytosine [162].

The preferred conformation of the methoxygroup in the nucleoside X_{73} itself is syn. This conformation, however, is not favored for a W–C base pair hydrogen bonding. In the duplex, the methoxygroup takes up the anti-conformation on base pairing.

Introducing a methylgroup in the 5-position of uracil or cytosine, generally increases considerably duplex stability due to stacking interaction. In this series, however, remarkable destabilization of the duplex together with broad melting transitions is observed when the 5-position of N^4-methoxy-2′-deoxycytidine is methylated (giving N^4-methoxy-5-methyl-2′-deoxycytidine (X_{75}) and X_{75}:A and X_{75}:G base pairs). For efficient base pairing the N-methoxy group must be anti-oriented with respect to N^3 of the pyrimidine base. The 5-methyl group sterically prevents the formation of this pairing conformation and the system is constrained in the unwanted syn-form of the N-methoxy group [15, 163]. When more than one N^4-methoxy-2′-deoxycytidine is incorporated (in an attempt to use probes for colony hybridization), a further reduction of duplex stability was observed. This was, likewise, attributed to the preferred syn conformer of X_{73} [157].

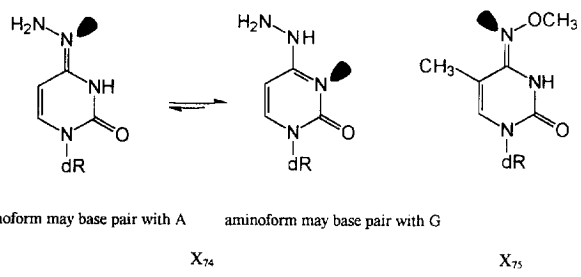


Figure 25.

A C T T G G C C X C C A T T T T G
T G A A C C G G Y G G T A A A A C

Y \ X	T	C	X_{73}	X_{75}	X_{76}
A	72°	64°	66°	39°	72°
G	70°	75°	66°	37°	73°

Figure 26.

In order to hold the N-O bond in the required anti-conformation with respect to the hydrogen bonding region, 6H,8H-dihydropyrimido[4,5-C][1,2]-oxazin-7-one nucleoside (X_{76}) was incorporated in an oligonucleotide. This modified pyrimidine base forms base pairs with A and G of comparable stabilities [157, 164, 165]. The duplexes are significantly more stable than with X_{73} and of similar stability as duplexes containing natural G:C and A:T pairs. By substituting two, three or four natural Pu-Py base pairs with Pu-X_{76} base pairs, thermal stability of the duplex, however, decreases considerably [157, 165]. All X_{76} containing duplexes show sharp melting transitions [157].

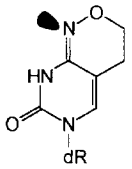
As the imino form is favoured over the amino tautomer for the X_{76} nucleoside, the equal stability of the A:X_{76} and G:X_{76} base pair is difficult to understand. This was further investigated in shorter self-complementary duplexes [166]. The preferential binding of X_{76} with A is easily observed in these duplexes. In the more stable 17-mer duplexes, used in the previous studies, this difference is less well marked (see *table IV*).

The base pairing system is different with adenine and guanine. An A:X_{76} base pair is a normal Watson–Crick system. Contrarily, G:X_{76} and G:X_{73} base pairs undergo slow exchanges between Watson–Crick and Wobble forms of base pairs.

The increased stability of the Wobble form of the G:X_{73} base pair (in the crystalline state, alone the Wobble structure is observed) may be explained by the preference

Table IV.

CGAATXCG		CGGATXCG	
Base pair	T_m (°C)	Base pair	T_m (°C)
A.T	52	G.C	70
A.X_{76}	51	G.X_{76}	42
A.X_{73}	32	G.X_{73}	24



X_{76}

ACTTG GCCA CCA TTTT G	
TGAA Y_1CGG Y_2GG Y_3AAAA Y_4	
	Tm (°C)
$Y_1 = Y_4 = C$; $Y_2 = Y_3 = X_{73}$	69°
$Y_1 = Y_4 = C$, $Y_2 = Y_3 = X_{76}$	71°
$Y_2 = T$, $Y_4 = C$; $Y_1 = Y_3 = X_{73}$	57.5°
$Y_2 = T$, $Y_4 = C$; $Y_1 = Y_3 = X_{76}$	67°
$Y_4 = C$; $Y_1 = Y_2 = Y_3 = X_{73}$	52.5°
$Y_4 = C$; $Y_1 = Y_2 = Y_3 = X_{76}$	66°
$Y_1, Y_2, Y_3, Y_4 = X_{76}$	63°
unmodified duplex	75°

Figure 27.

of the N^4-methoxcytidine nucleoside to adopt a syn isomeric form in the imino tautomeric structure [166].

Likewise, the 2,7-dioxopyrido[5,6-d]pyrimidine nucleoside (X_{77}) is considered to form hydrogen bonds with guanine as well as with adenine [167]. When incorporated into a self-complementary oligonucleotide, it was demonstrated that the stability of the base pair between pyridopyrimidine and guanine is much stronger than those with adenine.

Moreover, X_{77}:G stabilizes the DNA duplex more than a natural G:C base pair. These results were explained by the formation of three W–C hydrogen bonds between the guanine base and pyridopyrimidine and increased stacking interactions [167]. For the formation of a Wobble base pair with A, the backbone of the oligonucleotide has to be shifted. Later it was suggested that the pyridopyrimidine nucleoside may exist in different tautomeric forms within duplex or triplex complexes [168]. The pairing system of X_{77} with A in a duplex DNA is still an open question.

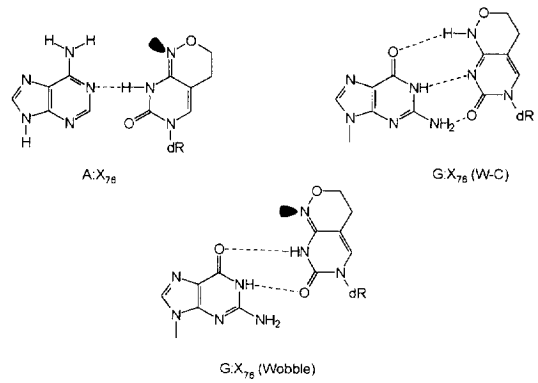
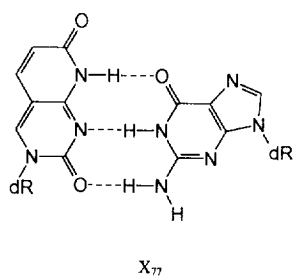


Figure 28.

2.5. Miscellaneous modified nucleosides

An increased stability of duplexes may, likewise, be obtained by enhancing the stacking interactions by way of extending the heteroaromatic system [169, 170]. Therefore, several tricyclic nucleosides (i.e. carbazole, phenoxazine, phenothiazine) were incorporated in oligonucleotides. The influence of the extended aromatic system (with a mixed RNA purine sequence as complement) is most pronounced when the modified nucleosides are clustered [TCTCCCTCTCTTTTT $T_m = 69$ °C; TCT$(X_{78})_3$TCTCTTTTT $T_m = 77$ °C; TCT$(X_{79})_3$TCTCTTTTT $T_m = 84$ °C; TCT$(X_{80})_3$TCTCTTTTT $T_m = 81$ °C].

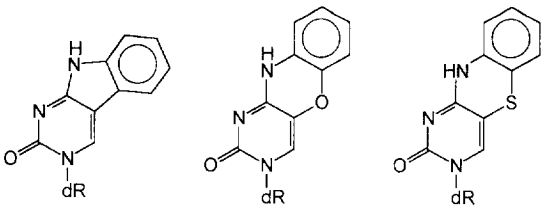
The increased stability of the phenoxazine and phenothiazine system is explained by the excellent π–π overlap between the second ring of the heterocycle and the third ring of the adjacent tricycle [170]. The nonpolarizable benzene ring, however, increases the hydrophobicity in the major groove and it might be more



X_{77}

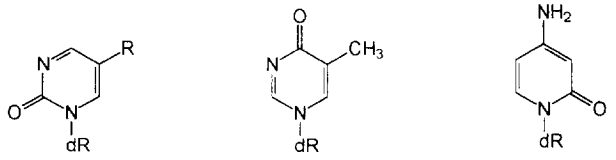
GGGAAXYTTCCC	
XY	Tm (°C)
C:G	50.5
T:A	47.9
X_{77}:A	39.4
X_{77}:G	58.4

Figure 29.



X_{78} X_{79} X_{80}

Figure 30.



X_{84} R = H
X_{85} R = CH_3

X_{86}
[178b]

X_{87}

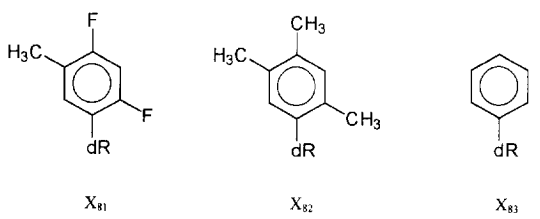
Figure 32.

advantageous to replace it by a heterocyclic ring. The pairing system is specific for the guanine base. Likewise, G-selectivity and increased duplex stability (with DNA as complement) is observed by incorporating the fluorescent 2,7-dioxopyrido(2,3-d(pyrimidine nucleoside (X_{77}) into a self-complementary DNA [GGGAACGTTCCC T_m = 50.5 °C; GGGAA(X_{77})GTTCCC T_m = 58.5 °C] [167].

It is generally accepted that stacking interactions of the aromatic π-systems of the nucleobases contribute more to the stability of nucleic acids structures than hydrogen bonding interactions between the base pairs. The generation of hydrogen bonds between base pairs implies desolvatation of the bases. These hydrogen bonds are important for the specificity of the interactions and for additional stabilization. The stacking phenomenon was studied using hydrophobic isosters of pyrimidines (and purines) [171].

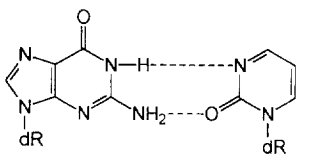
Due to the absence of obvious hydrogen bonding capabilities, these thymine isosters pair very poorly and are non-selective in pairing with natural bases. The self-pairing systems are more stable than the pairing with the natural bases. This may be due to the fact that the desolvatation of the natural bases is not compensated by the formation of new hydrogen bonds within the duplex structure [171]. The desolvation costs of X_{81} and X_{82} is neutral [171, 172]. Incorporation of the modified nucleosides in an internal position gives a decrease in duplex stability which is explained by imperfect steric mimicry and the formation of non-ideal duplex geometry [171] in addition to lacking hydrogen bonds: $[C(T)_4C(T)_3C(T)_2$ with DNA complement: T_m = 39.4 °C; $C(T)_4CX_{81}(T)_2C(T)_2$ with $G(A)_4GX_{81}(A)_2G(A)_2$ as complement: T_m = 28.6 °C; with a X_{82}:X_{82} base pair: T_m = 29.4 °C]. However, X_{81} serves as a very good template for DNA synthesis and the Klenow fragment (KF, eno-mutant) of Escherichia coli DNA polymerase I can efficiently replicate a base pair (A – X_{81}/X_{81} – A) that is inherently very unstable, and the replication occurs with very high fidelity despite a lack of inherent base-pairing selectivity suggesting that hydrogen bonds may be less important in the fidelity of replication and that nucleotide/template shape complementarity may play a more important role [173, 174].

Contrarily, the stability of the duplex is markedly increased when the modified base pairs are placed at the end of the duplexes. In this case the X_{81}:X_{81} pair is more stabilizing than an A:T base pair [TCGCGCGA duplex: T_m = 51 °C; X_{81}CGCGCGX_{81} duplex: T_m = 63 °C]. This stabilization by positioning the base pair at the end position is explained by the greater freedom of the nucleic acids to adopt non-β-like conformation at the end position and by the increased hydrophobicity. The hydrophobic pyrimidine mimics (X_{81}, X_{82}), likewise, increase



X_{81} X_{82} X_{83}

Figure 31.



	Tm (°C)
CTGAATTCAG	41 °C
CTGAATTX_{84}AG	21 °C
CTGAATX_{85}CAG	<15 °C

Figure 33.

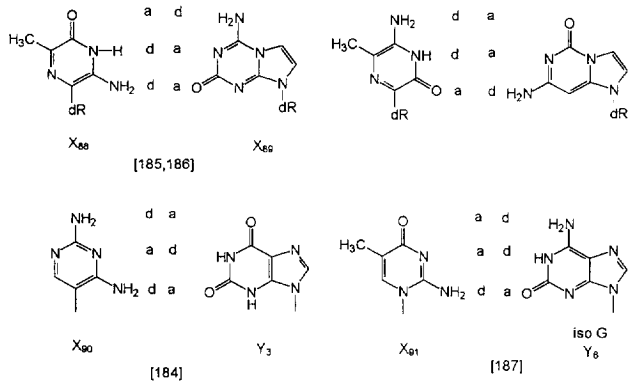


Figure 34.

loop stabilization [172, 175] due to better base stacking. In order to better analyze the system and separate stacking from pairing interactions, the non-natural nucleotides were placed in a dangling position at the end of the duplex [176]. From these data it is clear that the less polar X_{81} stacks more strongly than the thymine base, which is indeed due to increased hydrophobicity and not different polarizability, as difluorotoluene is equally as polarizable as thymine [176].

A method to study indirectly the details of protein-nucleic acid interactions is by replacing a group on the base capable of interacting with a protein with one that cannot form this interaction [177]. Some of these modified oligonucleotides are able to form stable covalent adducts with enzymes (for example: 2-pyrimidinone X_{84} and methylase) [177] and/or are hydrolytically unstable so that they can be used for the generation of abasic sites in oligonucleotides (the same 2-pyrimidinone may be used for this purpose) [178a].

Incorporation of 2-pyrimidinone (X_{84}) and its 5-methyl analogue (X_{85}) in DNA (in the position of a cytosine or thymine nucleoside, respectively) [178a–181] results in a large destabilization of the duplex. The 2-pyrimidinone and its 5-methyl analogue do not pair with adenine bases [57, 178a]. The melting curves of the CGCGAATTGCG sequence, after incorporation of X_{84} and X_{85} at the place of T show anomalous behaviour and multiple transitions seemed to be present [178a]. A mixture of hairpin and duplex structures may be present. The deletion of the carbonyl group at the C-4 position of the thymine(uracil) base changes the tautomeric character of the N-3 nitrogen, inhibiting formation of W–C hydrogen bonding with adenine. With some wobbling of the base pair, a single H-bond could be formed but this is, likewise, not the case as no duplex formation with a 9-mer is observed. With the guanine base, the formation of a Watson–Crick hydrogen

Table V.

	T_m (°C)
GACGATATCGTC	53
GACGAX_{85}ATCGTC	50
GACGATAX_{85}CGTC	50

bonding scheme is consistent with the observed thermal behaviour [178a].

Most surprising, in another self-complementary sequence incorporation of 5-methyl-2-pyrimidinone nucleosides does not cause appreciable helix destabilization, although it was mentioned that the modified base cannot form either of its normal Watson–Crick hydrogen bonds with a complementary A [86]. The CD spectra of the modified oligo only slightly distort away from the B-DNA structure (see *table V*).

Removing the N^3-atom of the cytosine base gives a 4-amino-2-(1H)-pyridinone nucleoside (X_{87}) which hydrogen bonds to 2′-deoxycytidine [31, 182, 183]. The greater basicity of the 2-carbonyl group of 3-deaza-cytidine stabilizes the imino-enol form of the compound which can then base pair with the cytosine base [182].

Several new pyrimidine and purine nucleosides were synthesized to study new base-pair systems based on hydrogen bonding complementarity, size complementarity, hydrophobicity and planarity of the bases [184]. When specific, these new systems may be used to expand the genetic alphabet. The 6-amino-3-methyl-pyrazin-2-(1H)-one nucleosides (X_{88}) base pairs with a 5-aza-7-deaza-isoguanine (X_{89}) nucleoside more efficiently than with A and G [185, 186]. A 5-(2,4-diamino-pyrimidine) nucleoside (X_{90}) pairs with a xanthine base (Y_3) [184]. The 5-methylisocytidine (X_{91}):isoguanine (Y_6) base pair is slightly stronger than the natural C:G base pair [187].

5-Methylisocytosine (X_{91}) and isoguanine (Y_6) form base pairs with guanine and cytosine, respectively, in a parallel strand orientation. The stability of the duplex is similar to a normal antiparallel duplex [188]. The unnatural pair formed by C/iso-G is observed to be isoenergetic with U/A Watson–Crick pairs [189]. Ab initio data suggest that iso-G may appear as an trans-imino oxo tautomer (*figure 35*). Isocytosine also forms base pairs with isoguanine itself in normal antiparallel duplexes [190], i.e. isoguanine prefers pairing with isocytosine over thymine. The unnatural iso-C/iso-G is found to be as stable as a C/G Watson–Crick pairs in DNA/RNA duplexes consistent with the formation of three-hydrogen bonds between iso-G and iso-C [189].

Isoguanosine can be site-specifically incorporated into RNA using deoxy-5-methylisocytidine containing DNA

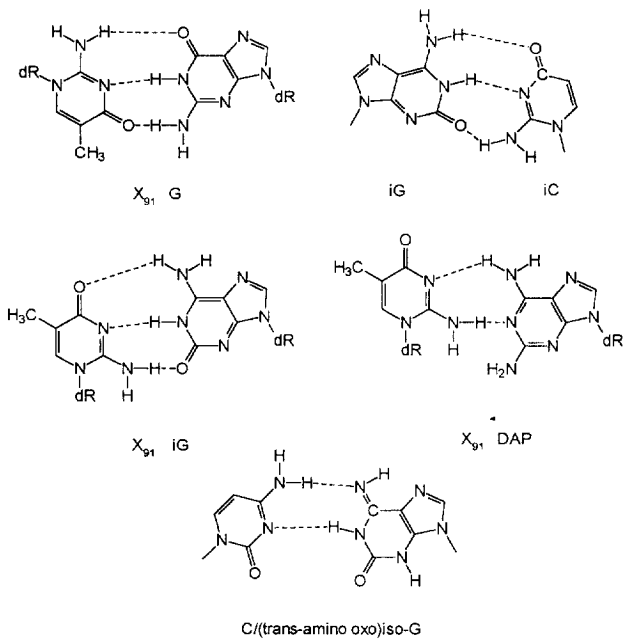


Figure 35.

templates [191] and T_7 RNA polymerase. This also holds for N^6-(6-aminohexyl)isoguanosine incorporation. The free reactive primary amino group may then be used for derivatization reactions. Isocytidine is misincorporated opposite T in a DNA template [191].

Also the 5-methylisocytosine:2,6-diaminopurine (DAP) Wobble base pair was investigated [9]. The DAP:X_{91} pair is conformationally similar to a G:U pair but with a reversed hydrogen bonding pattern and a different major and minor groove constitution. At the end of an RNA duplex, both base pair systems (DAP:X_{91} and G:U) bring about equivalent stability to the duplex. In fact, all Watson–Crick and Wobble base pairs that form two hydrogen bonds are approximately energetically equal at terminal position [9]. The presence of diverse non-hydrogen bonding groups with major or minor

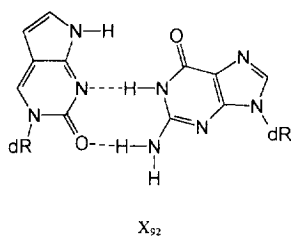


Figure 36.

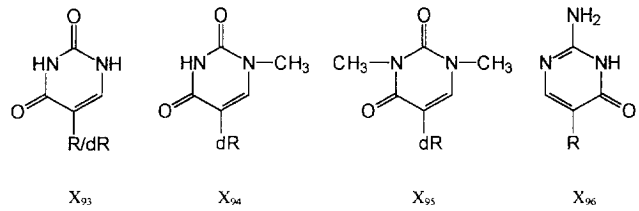


Figure 37.

groove at the terminal position of a duplex seems to have only a modest effect on stability [9].

Selective binding complementary oligonucleotides are obtained based on a inosine-cytosine and pyrrolo-[2,3-d]-pyrimidine-2(3H)-one (X_{92}):guanosine base pair system because inosine does not form a stable base pair with the pyrrolopyrimidinone [192]. The modified bases form only one hydrogen bond when paired to each other, but form at least two hydrogen bonds when paired to the natural partner. The third hydrogen bond between pyrrolopyrimidinone and guanine is very weak due to suboptimal orientation of the N-H group of the pyrrool ring. Self-complementary oligonucleotides uniformly substituted with inosine and pyrrolopyrimidinone nucleotides have single strand character and are able to invade the end of natural nucleic acid duplex [192]. The hybridization to the target generate more new base pairs than in the start situation. In this way the accessible dsDNA and RNA hairpin structures that may function as target for oligonucleotides, increases.

Several articles describe that the incorporation of pseudouridine (X_{93}) [193, 194] and alkylated-pseudouridine (deoxy)nucleosides [195, 196] are isosteric and isoelectronic to thymine analogues. Incorporation of 2′-O-methylpseudouridine in place of 2′-O-methyluridine increases the stability of the hybrid with an RNA target [(T_m: 1°C/substitution) [194]. Incorporation of 1-methyl (X_{94}) or 1,3-dimethylpseudodeoxyuridine (X_{95}) in an oligothymidylate results in duplex destabilization [196].

The tautomerization (N-1 or N-3 hydrogen) in pseudoisocytidine (X_{96}) has a negative impact on W–C base pairing and duplex stability decreases [197].

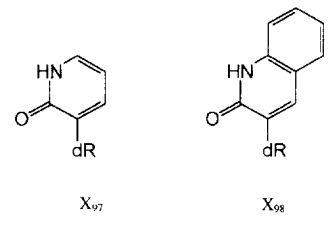


Figure 38.

C C G A T [X] T A G C C
G G C T A [Y] A T C G G

Y \ X	T	X_{97}	X_{98}
A	43°	ND	ND
G	ND	38°	35°
2-aminopurine	ND	42°	38°

Figure 39.

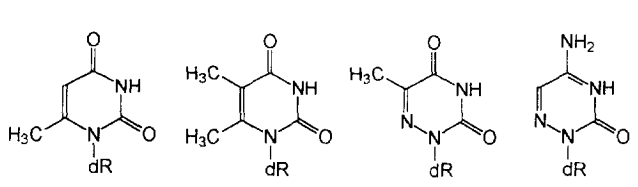
Fusion of a benzene ring across the 5 and 6 carbon atoms of 2-hydroxypyridine (X_{97}) gives a 2-hydroxyquinoline (X_{98}) nucleoside with the sugar moiety bond at the C-3 position [198]. These modified C-nucleosides preferentially pair with 2-aminopurine nucleosides, representing a novel base-pairing system.

The stability of the base pair is only slightly lower than that of the natural A-T base pair. Wobble base pairing with G is also observed.

The fused C-nucleoside system is also used to incorporate a nitroxide spin probe for studying DNA dynamics [198, 199].

A phenyl ring itself makes little or no contribution to base stacking and is as destabilizing as having no base present [200].

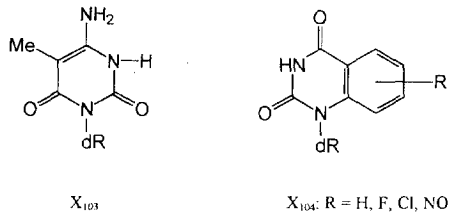
The 5- and 6-position of pyrimidines are located in the major groove and should, theoretically, not effect directly the hydrogen bonding pattern. However, indirect effects on Watson–Crick base pairing may be seen because the substituent may alter the syn/anti conformational equilibrium around the glycosidic bond (for example a 6 methyl



X_{99} X_{100} X_{101} X_{102}

	Tm (°C)
TCCAGGT G T C C GCATC	63 °C
TCCAGGX_{99} G X_{99} C C GCATC	54 °C
TCCAGGX_{100} G X_{100} C C GCATC	55 °C
TCCAGGX_{101} G X_{101} C C GCATC	58 °C
TCCAGGT G T X_{102} X_{102} GCATC	56 °C

Figure 40.



X_{103} X_{104}: R = H, F, Cl, NO

Figure 41.

or 5,6-dimethyl substituent stabilizes syn-conformation) or may change the electronic distribution within the heterocyclic ring (for example a 6 aza group as electronegative centre). Incorporation of 6-methyl-2′-deoxyuridine (X_{99}), 5,6-dimethyl-2′-deoxyuridine (X_{100}), 6-azathymidine (X_{101}), 6-aza-2′-deoxycytidine (X_{102}) invariably gives duplex destabilization [4, 7]. Likewise, the 6-ketoderivative of 5-methylcytosine and cytosine (X_{103}) gives duplex stabilization because the N^3-H of the pyrimidine base is oriented opposite to the N^1-H of guanine [201, 202].

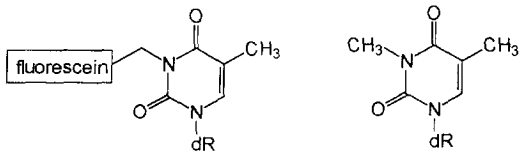
By introducting 2,4-quinazolinedione nucleoside dervatives (X_{104}), duplexes are clearly destabilized [203, 204] which is not the case for the substituted congeners.

Some examples of N^3-substituted thymines for end-modification of oligonucleotides are known [205] (X_{105}, X_{106}). The N^3-methyl group (X_{106}) lowers T_m and results in broad melting ranges [206].

Modification at the base pairing site may, however, increase duplex stability when the modified nucleoside is introduced at an unpaired site [207] which was demonstrated using pyrimido-pyrimidinone (X_{107}) and naphtimidazo-pyrimidinone nuleosides (X_{108}).

The stability is greatest when the extra base pair is present between two terminal base pairs. An additive on single insertion is observed when the two extra bases are present in the opposite strands.

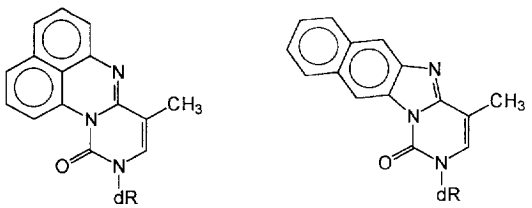
Several other modified pyrimidine and related nucleosides (X_{109}–X_{118}) were incorporated but information on



[205] [138]

X_{105} X_{106}

Figure 42.



X_{107} X_{108}

Figure 43.

their influence on duplex stability is scarce. They were introduced for several reasons, i.e. as model for site specific oxidation lesion [208] (X_{109}), as fluorescent probes [209, 210] (X_{110}), as component of a ^{t}RNA cloverleaf structure [211] (X_{111}, X_{112}), to study the correlation of changes induced by mutated nucleosides on the function and structure of nucleic acids (X_{113}) and so on. Several of them are represented in *figure 44*.

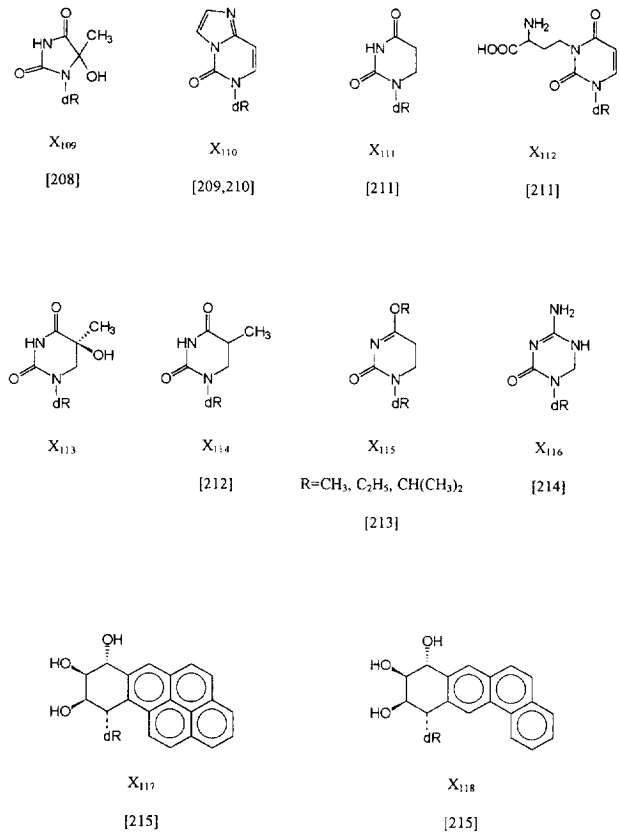
3. Purine modifications

3.1. C-2 and C-6 substitutions

Deoxyinosine has been used as so called 'universal nucleoside'. Indeed, the genomic sequence is ambiguous at positions where the genetic code is redundant. An ideal probe base would pair with equal stability to all bases. The stability of the base pairs, however, is sequence specific (large neighboring base effects due to stacking interactions and local structural variations) which limits the universality of parameters obtained in studies of small groups of oligonucleotides [158].

Oligonucleotides containing deoxyinosine (Y_1) residues at ambiguous positions (third position) of the amino acid codon are used as hybridization probes for cloning genes. Likewise, some ^{t}RNA molecules have inosine at the 5'-hydroxyl ends of their anticodon enabling pairing with A, C or U and also in the middle position of an anticodon to pair with A [216–218].

The most stable base pair is Y_1:C. However, it contributes less to stability of a duplex than a standard Watson–Crick A:T and G:C base pair. The deletion of the N^2-exocyclic amino group of guanine significantly destabilizes a Watson–Crick base pair (G:C versus Y_1:C) but has little effect on the stability of a terminal wobble pair [9] (see further). The Y_1:A base pair is more stable than the G:A base pair. The stability of the Y_1:G and Y_1:T



X_{109} [208] X_{110} [209,210] X_{111} [211] X_{112} [211]

X_{113} X_{114} [212] X_{115} R=CH_3, C_2H_5, $CH(CH_3)_2$ [213] X_{116} [214]

X_{117} [215] X_{118} [215]

Figure 44.

pairs may be higher or lower than the G:G and G:T pairs, dependent on sequence environment [158, 219].

The general order of stability is $Y_1C > Y_1A > Y_1T > Y_1G$ [158, 220, 221] although stability of Y_1T and Y_1G may be reversed [222a,b]. When Y_1 is incorporated at the 5'-end of an oligonucleotide, it pairs preferentially in the order seen with Y_1 in internal positions but with reduced discrimination [222a]. The stability of the duplexes are dependent on the counterions present (base pairs are less stable in 1 M NaCl than in 3.5 M tetramethylammonium chloride). The lowered stability in 1 M NaCl results in greater discrimination between paired and mispaired bases [222a]. Deoxyinosine incorporated at the 3'-end of the oligonucleotide shows lower discrimination [222a]. When two adjacent deoxyinosines are incorporated in the centre of an oligonucleotide with sequence $CCCY_1Y_1TTT$, the order of stability is:

$$CC > CA > AA > AC > GC > GA > CG > TA > > TC > CT \simeq AG > AT > GT > TT$$ [222a]

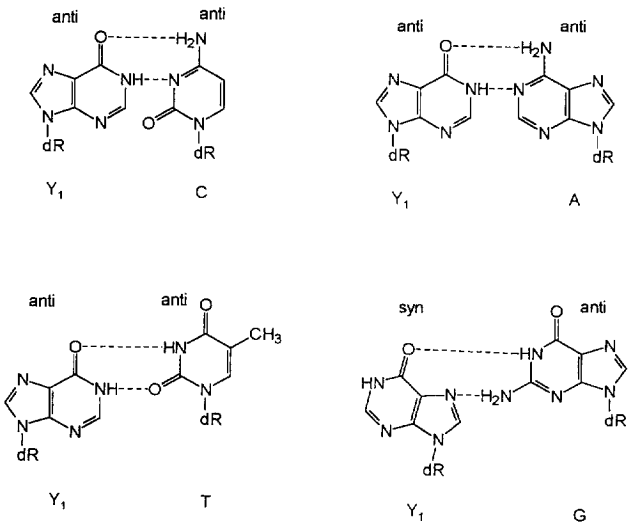


Figure 45.

In the Y_1:C base pair, both bases are *anti* oriented [223]. The Y_1:C pair shows Watson–Crick geometry similar to hydrogen bonding found in the G:C base pair [224]. The Y_1:C base pair resembles A:T in the minor groove and G:C in the major groove of DNA, in the arrangement of potential H-bond donors and acceptors available for interaction with proteins [224].

Hydrogen bonding between Y_1 and G is of the Hoogsteen type [Y_1(syn):G(anti)] and that of Y_1(anti):T(anti) is a wobble base pair [158, 219, 220, 222b]. The internal Y_1:U pair (when compared to G:U) is more destabilizing than a terminal pair and one of the reasons may be that the exocyclic 2-amino group of G may stabilize an internal wobble pair [9].

The Y_1:C base pair occurs in Z-DNA, the Y_1:T wobble pair in A-DNA (GGY_1GCTCC) and the Y_1(anti):A(syn) base pair in B-DNA ($CGCY_1AATTAGCG$) [225]. The CD pattern of the Y_1 (replacing G) containing duplex is very different due to a difference in the UV spectrum of

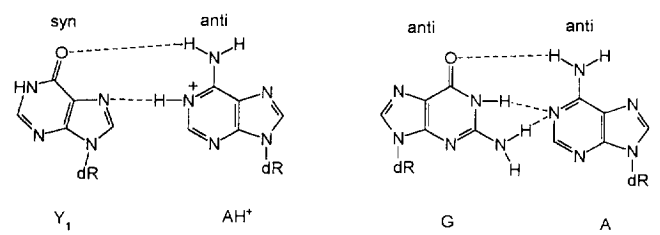


Figure 46.

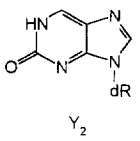


Figure 47.

hypoxanthine and guanine and not to essential differences in the DNA structure itself [226]. Removal of the minor groove amino functionality within guanine (replacing by inosine), affects DNA structure more in a d(TCG) sequence than in a d(TCC) sequence [227]. Changes in free energy upon substitution of inosine for guanine, is sequence dependent. The purine 2-amino group is a crucial requirement for binding of actinomycin D and echinomycin in the minor groove [228].

The conformational variability of the A:Y_1 base pair was studied more thoroughly and compared with the A:G base pair. For the Y_1:A system, both *anti–anti* as *anti–syn* pairing is reported [158, 219, 220, 223, 229, 230]. The base pairing is different depending on pH and sequence of the oligonucleotide, as is the case for the G:A base pair [219].

The AH^+(anti):Y_1(syn) base pair predominates below pH 6.5 and an A(anti):Y_1(anti) mispair is the major species present between pH 6.5 and 8.0 [225]. The pH-dependent stability profiles of $CGCAAATTY_1GCG$ and CGCAAATTGGCG indicate that at pH 6.5 there is almost a 1:1 ratio of the protonated *anti–syn* and non-protonated *anti–anti* base pair in solution. X-Ray analysis demonstrate preferentially crystallization of *anti–syn* [225].

The AH^+(anti):Y_1(syn) base pair is less stable than its AH^+(anti):G(syn) analogue. Contrary, the A(anti):

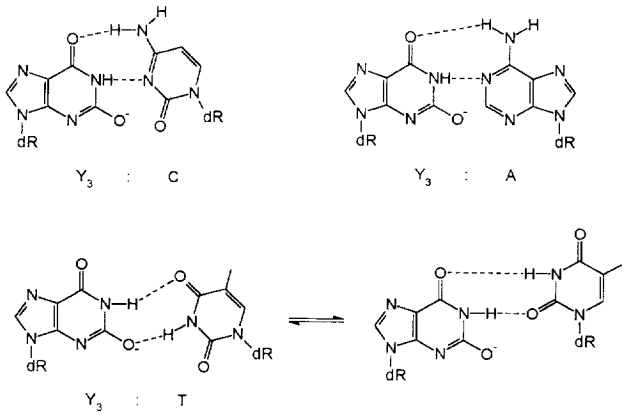


Figure 48.

Y_1(anti) base pair is more stable than the A(anti):G(anti) analogue. This last phenomenon was explained in two ways: (1) the formation of a reverse three-center interbase hydrogen bond between G(anti) and A(anti) would not completely alleviate the destabilization effect of the presence of an unfulfilled (2-NH_2) hydrogen bond donor; (2) the N^2-amino group in the minor groove interferes with the formation of the hydration spine. The AH^+(anti):G(syn) base pair is more stable than the AH^+(anti):Y_1(syn) couple because the 2-NH_2 group of guanine is no longer situated in the minor groove but is linked via a network of water mediated hydrogen bonds to a neighboring phosphate oxygen atom [225]. The hydration spine in the minor groove of the $(AAATTY_1)_2$ core region of the studied duplex is not perturbated by the presence of syn purine bases [225].

It may also not be forgotten that the presence of Y_1 may influence DNA bending (see further). When deoxyadenosine is replaced by Y_1, the d(AAY_1AA).d(TTCTT) tracts maintain bending while multimers containing d(AAGAA).d(TTCTT) tracts do not show bending. This is due to the fact that the hypoxanthine base does not contain a 2-amino group and the minor groove hydration can occur at hypoxanthine sites [231]. The 2-amino group of G interferes with formation of minor groove hydration. In the floor of the major groove, a three-center hydrogen bond patron involving an N-6 amino group of adenine and two O-4 carbonyl groups of the adjacent thymines in the opposite strand, are formed influencing the narrowness of the minor groove [231]. This bifurcated hydrogen bond can not be formed at the hypoxanthine and guanine site (no amino group at the 6-position is present) and this also influences the differences of the degrees of bending [231].

Deoxyisoinosine (Y_2) gives large destabilization (loop out) of a polyT sequence. The destabilization is less when the modified base is located at the ends of the oligonucleotide (breathing of terminal bases) [232].

The xanthine base (Y_3) may be present in DNA as a result of spontaneous deamination of guanine residues and modification by nitrous acid [233]. As the pK_a of the xanthine nucleoside is about 5.7, the anion form is expected to occur in DNA under physiological conditions. The xanthine base pairs have stabilities at pH 5.5 that are similar to those of the hypoxanthine base pairs at pH 7.5. The xanthine base pairs are less stable than hypoxanthine base pairs at neutral pH [217]. At physiological pH, Y_3:T is more stable than Y_3:G, while Y_3:A and Y_3:C containing duplexes have low melting temperature ($Y_3T > Y_3G > Y_3A > Y_3C$). This may be explained by the presence of an unsolvated negative charge on the xanthine in the Y_3:A and Y_3:C mismatch, and by the

G T C T C A T G T A ☐ T A A C A T C G
C A G A G T A C A T ☐ A T T G T A G C

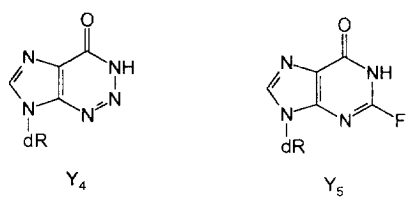
base pair	Tm (°C) at pH 5.5	Tm (°C) at pH 7.5	base pair	Tm (°C) at pH 7.5
A-Y_3	55.5	51.9	A-Y_1	55.8
C-Y_3	52.8	51.8	C-Y_1	57.7
G-Y_3	53.9	54.7	G-Y_1	53.7
T-Y_3	54.9	55.5	T-Y_1	53.1

Figure 49.

formation of H-bonds between the enolate oxygen of the xanthine base and H-1 of guanine or H-3 of thymine [217]. At neutral pH, it is postulated that Y_3:C is a Watson–Crick base pair and Y_3:T is a wobble pair. Adenine can form a base pair with Y_3 in a similar way to Y_3:C pair, although distance between the anomeric carbon atom is longer than a Watson–Crick pair [233]. At pH 5.5 (when compared with data at pH 7.5), the Y_3:A and Y_3:C mismatch are stabilized and the Y_3:T and Y_3:G base pairs are somewhat destabilized.

In spite of the very low stability of the Y_3:C base pair, dCMP is incorporated at the site opposite to Y_3 by Drosophila DNA polymerase (in vitro [233]. The order of incorporation preference is T > C > > A and G. The somewhat lower preference of dCMP over dTMP (although the former is a W–C system and the latter a wobble pair) may be due to destabilization of the Y_3:C pair originated from the presence of the unsolvated negative charge of Y_3 and repulsion between this ionized oxygen and the negatively polarized carbonyl group of dC [233].

Substitution of the keto group at position 2 of xanthine (Y_3) by a nitrogen atom gives 2-azahypoxanthine (Y_4). This nucleoside analogue has the same a-d-a configuration but a higher pK_a value (pK_a of 2-azainosine: 6.8) than xanthine (pK_a 5.5). Both at pH 6.0 and 8.0, the 2-azahypoxanthine (Y_4) base pairs (with T, C, A and G) are less stable than the hypoxanthine congeners [234] and little variation among different bases is observed.



Y_4 Y_5

Figure 50.

T T A C A Y_5 C T G G A
A A T G T X G A C C T

base pair	Tm (°C) at pH 8.0	base pair	Tm (°C) at pH 4.9
Y_5-A	22.9 (G-A 30.4)	Y_5-A	34.4 (G-A 30.6)
Y_5-C	23.3 (G-C 45.3)	Y_5-C	24.6 (G-C 45.8)
Y_5-G	16.3 (G-G 34.1)	Y_5-G	21.7 (G-G 32.5)
Y_5-T	15.1 (G-T 30.8)	Y_5-T	17.1 (G-T 31.3)

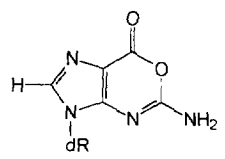
Figure 51.

At pH 8, 2-fluorohypoxanthine (Y_5) does not form strong base pairs with natural bases due to the presence of a negative charge, formed in the same manner as with xanthine (Y_3). The pK_a of 2-fluorohypoxanthine is 4.9. At pH 4.9 the duplexes are more stable but the stability is still low when compared to G containing base pairs [152].

Incorporation of 2′-deoxyisoguanosine (Y_6) instead of 2′-deoxyadenosine does not lead to large destabilization of $(dA - dT)_6$ ($T_m = 30$ °C) $[(Y_6 - dT)_6$ ($T_m = 27$ °C)] [235]. When alternating isoguanosine and adenosines are present in a $(A\text{-}U)_6$ duplex, giving $(AUY_6U)_3$, duplex becomes less stable (19 °C instead of 34 °C). The 2-oxo group in Y_6 sterically interferes with the 2-oxo group of uracil base [236]. Antiparallel as well as parallel strand orientation is possible, although the former one is more likely [236].

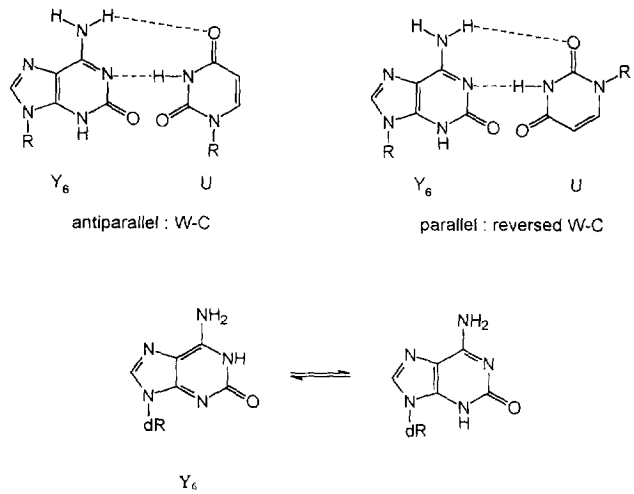
2′-Deoxyisoguanosine forms a reversed Watson–Crick base pair with 2′-deoxycytidine [237] resulting in DNA duplexes with parallel chain orientation [238, 239]. The aggregate $d(T_4 - iG_4 - T_4)$ containing four consecutive 2′-deoxyisoguanosine is shown to be a tetramer similar to that of $d(T_4 - G_4 - T_4)$ [239].

Nitrous acid or nitric oxide converts 2′-deoxyguanosine into 2′-deoxyoxanosine Y_7 which could not form a stable base pair with a specific natural base. Low T_m values are observed for Y_7-containing duplexes [240].



Y_7

Figure 52.



Y_6

Figure 53.

The formation of 1H- and 3H-tautomers of Y_6 may lead to mispairing in duplex DNA [235]. Base pairing between isoguanine (Y_6) and guanine was found by Eschenmoser and coworkers in pRNA [241, 242]. The presence of ^{Me}isoC (X_{91}) in a DNA template directs T7 RNA polymerase incorporation of isoGTP. The misincorporation of Y_6 opposite T is eliminated in the presence of ATP [191]. Isoguanine and ^{Me}isoC (X_{91}) form parallel stranded oligonucleotides with DNA and RNA oligomers with comparable stability as normal antiparallel duplexes [188]. Semiempirical PM3 calculations in the gas phase suggested that both iC-G and Y_6-C base pairs are more stable than a reverse Watson–Crick A-T base pair and that the base pair should possess a stability comparable to that of a Watson–Crick G-C base pair [188].

2-Aminopurine (Y_8) is a non-biomolecule despite its functional potential. It has a lower change of assembling itself prebiologically [243]. 2-Aminopurine (Y_8) preferentially forms base pairs with thymine [16, 244, 245]. The order of base-pair stability with 2-aminopurine is T > A > C > G. Duplexes of 2-aminopurine polynucleotides with polydT of polyU are less stable than natural duplexes [246, 247].

The stability of the duplexes, however, is dependent on the identity of the nearest neighbor bases [248]. As an example the stability of the Y_8:C base pair is increased when Y_8 is stacked between nearest neighbor C's instead of G's. The melting profiles of short duplexes containing Y_8 is relatively broad and the observed T_m is different at 330 nm than at 275 nm, indicating that melting around the modified base pair occurs prior to melting of the rest of the polymer [14, 248].

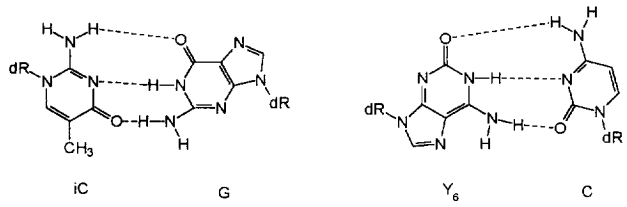


Figure 54.

In the Y_8:T base pair, the 2-amino group of Y_8 hydrogen bonds with the 2-oxo group of T in the minor groove [245]. For the Y_8:C pairing different suggestions have been made: as (i) a standard Watson–Crick base pair [249] stabilized by one hydrogen bond, (ii) a charged base pair with Watson–Crick geometry stabilized by two hydrogen bonds [250], (iii) an imino tautomer of either AP or C paired with the amino tautomer of the other base to form a Watson–Crick geometry [251a,b] and (iv) a wobble base pair [252]. An NMR study has shown that the 2-aminopurine:C mispair has a wobble geometry (in neutral and high pH). The structure and stability of this base mispair is dependent upon the local base sequence [253].

2-Aminopurine may generate genetic mutations in procaryotic systems [254]. The possibility that Y_8 could pair with either T or C might give an explanation for its mutagenic properties [245] (see *table VI*).

The 2-aminopurine:A wobble structure was studied in the sequence mentioned in the table [255]. Both bases are anti-oriented. The adenine is stacked in the helix, but the helix twist between the adenine and neighboring cytosine in the 3′-direction is unusually small. As a result the aminopurine in the opposite strand is somewhat pushed out of the helix. The two adjacent G-C base pairs are not significantly destabilized by the presence of the purine-purine wobble pair.

Table VI.

CGGXGGC
GCCYCCG

X–Y	T_m (°C)
G–C	40.2
A–C	11.5
A–T	34.6
Y_8–C	24.9
Y_8–T	31.4
Y_8–A	28.0
Y_8–G	20.4

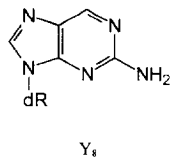


Figure 55.

Substitution of 2′-deoxyadenosine by 2-amino-2′-deoxyadenosine (Y_9) stabilizes hybrids formed with both DNA and RNA [5, 16, 256] due to stronger base pairing without disturbing the global or local conformation of the duplex [257]. On one side, the extra amino group disturbes the hydration spine in the minor groove which contributes to the stabilization of the DNA structure [256] and 2-aminoadenine may facilitate B → Z (or even B → A) transition in DNA structure [256, 258, 259]. On the other hand, the 2-amino group creates an additional hydrogen bond which contributes to the increased hybridization strength and selectivity when incorporated in an oligonucleotide [246, 259] (see *table VII*).

The width of the minor groove of DNA helix may approximately be correlated with AT/GC content. A:T tracts appear to be associated with a narrowed groove, G:C tracts often have a widened minor groove [260]. The 2-amino group of the guanine base, which protrudes out from the stack of base pairs, is the major chemical difference in the minor groove between A:T and G:C base pairs and may play an important role in local conformational changes. From the standpoint of a view into the minor groove the G → Y_1 and A → Y_9 replacement may convert a G:C base pair into an A – T base pair and vice versa [260]. Replacement of A by Y_9 may then widen the minor groove and replacement of G by Y_1 may narrow the minor groove [260].

The diaminopurine-thymine base pair, with three hydrogen bonds in a W–C arrangement, is more stable than the A-T base pair [14, 261, 262] in dC_3Y_9G3:dC_3TG_3, as stable as A:T in $dCT_3Y_9T_3G$:dCA_3TA_3G and less stable than A:T in $dCA_3Y_9A_3G$:dC_7TG [263], and less stable than the C-G pair [259, 261, 262]. The Y_9:U pair is more stable than A:U but less stable than G-C. This lower stability as compared to GC might be attributed to a

Table VII.

	T_m (°C) [256]
CTGAAACCGGCGAAG	60
$CTGY_9Y_9Y_9CCGGCGY_9Y_9G$	64

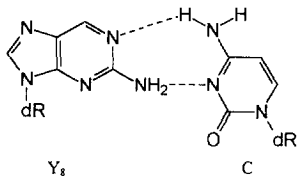


Figure 56.

difference in base stacking pattern and, possibly, in the geometry of the hydrogen bonds [25, 264]. The 2′-deoxyxanthosine-Y_9 and 2′-deoxy-7-deazaxanthosine-Y_9 base pairs also fit the Watson–Crick geometry [265], but are joined by a non-standard hydrogen-bonding pattern.

The increase in thermal stability of duplexes by introducing Y_9 is larger in the ribo-series than in the deoxy-series [266, 267] and the effect is different with homo-oligomers and with mixed-base oligomers. Magnesium ions enhance the RNA preference of Y_9 containing oligonucleotides. Oligoadenylates bind poorly to RNA because dA disfavors the A-form helix (favoured by RNA duplexes) and dA tracts destabilize RNA duplexes. Substitution of dA with Y_9 counter this destabilization, affecting most DNA-RNA hybrids [262]. The dA $\rightarrow$ Y_9 substitution enhances binding to RNA in homooligomers. However, with homooligomers conversion of dA to Y_9 does not improve binding to DNA. With mixed-base oligomers stabilization of the duplex is showed. Indeed, DNA with Y_9:T pairs are more thermostable than those with A:T pairs except when the stabilizing effect conferred by the spine of hydration to A:T tracts, is reduced by the inclusion of a Y_9:T pair [263, 268].

The Y_9:^{Me}iC base pair is as stable as the G:U base pair and more stable than the Y_9:C pair [9]. These base pairs are proposed to adopt a wobble configuration [9]. The Y_9:^{Me}iC and A:^{Me}iC pairs are energetically equivalent (as are Y_9:C and A:C).

3.2. *C-8 substitutions*

Deoxyguanosine residues in DNA are hydroxylated at the 8-position by various agents that produce oxygen

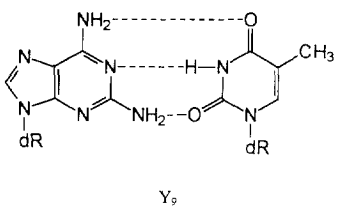


Figure 57.

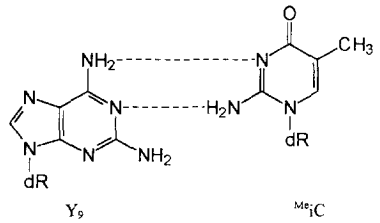


Figure 58.

radicals [269]. The presence of the 8-hydroxyguanine base (Y_{10}) in DNA has been linked to several diseases, e.g. rheumatoid arthritis, systemic lupus erythomatosus, cancer [270, 271]. Likewise, adenine residues in DNA may be oxidized under the action of ionizing radiation at the C-8 position to give 7,8-dihydro-8-oxo-adenine (Y_{12}) [272]. The 8-hydroxyguanine base (or 7,8-dihydro-8-oxoguanine) is mainly present in the 6,8-diketo conformation and the *syn* conformation is favoured over the more usual *anti* conformation (because of steric hindrance between C-8 substituent and sugar moiety) [273–276]. Generally, a bulky substituent at the 8-position of purine nucleosides favor adoption of *syn* conformation of the N-glycoside bond in solution. The addition of the oxygen function at position 8 also changes the electrostatic potential of the molecule giving it a more negative character.

Within a DNA duplex, 8-hydroxyguanine and 8-methoxyguanine (opposite cytosine) take an unfavourable *anti* glycosidic conformation to form a stable base pair. This may partially explain the lower duplex stability of Y_{10}.C and Y_{11}.C base pairs related to G:C [277]. 8-Hydroxyguanine is promutagenic which means that during replication the polymerase may insert dA instead of dC [270]. dCMP and dAMP are incorporated selectively opposite Y_7 and dTMP is incorporated opposite 8-hydroxyadenine (Y_{12}) [278, 279]. Y_{12} forms base pairs with T in the *anti*-keto form.

The phosphorylated Y_{10} itself is misincorporated opposite to template adenine [280]. The lower stability of duplexes containing Y_{11}:C with respect to that of Y_{10}:C can be explained by the presence of the 8-methoxy substituent which is more bulkier than the hydroxy group [277]. 8-Hydroxyguanine base pairs to C with W–C type hydrogen bonding in an *anti* conformation and with reduced thermal stability of the duplex. The Y_{10}:C base pair (although less stable than the natural G:C pair) is more stable than the base pair formed between Y_7 and the other bases [281]. The Y_{10}:A base pair is more stable than the natural G:A pair [281]. Oligonucleotide duplexes with Y_{10}:T and Y_{10}:G pairs are thermodynamically somewhat

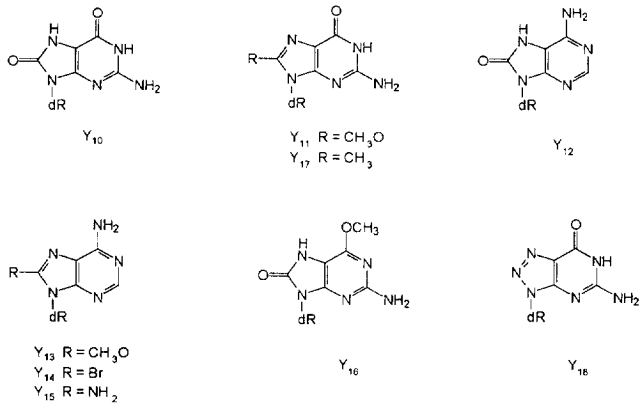


Figure 59.

more stable than the G:T and G:G pairs when incorporated at the end of the duplex, but not when incorporated in a central position. Conformational conversions of modified bases can occur more easily near the end than in the middle of oligonucleotide, which may facilitate hydrogen binding of unusual base pairs [281]. This is a general observation for 8-substituted nucleosides (the duplex structure becomes less stable when $Y_{10} - Y_{13}$ are incorporated in the centre of the recognition sequence than when the 8-substituted nucleoside is incorporated at the 5′-side [282, 283].

The 8-hydroxyguanine repair enzymes in human cells are effective at removing Y_{10} from Y_{10}:C, Y_{10}:T and Y_{10}:G base pairs but not at removing Y_{10}:A base pairs (most probably due to structural similarity to A-T base pairs, both having a carbonyl oxygen positioning in the minor groove of DNA) [270]. The Y_{10}:A base pair was studied by X-ray diffraction [270, 271, 284]. The A(anti):Y_{10}(syn) base Hoogsteen type pair has a similar structure as the A(anti):G(syn) base pair [270, 271, 274] but is thermodynamically more stable at physiological pH because the adenine base has to be protonated in the latter case (duplex becomes more stable at low pH) [275].

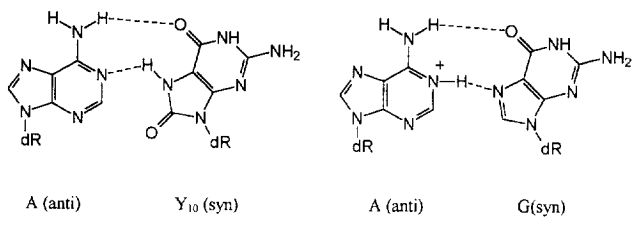


Figure 60.

G A G C T X G T G G C
C T C G A Y C A C C G

base pair X-Y	Tm (°C) at pH 7.0	base pair X-Y	Tm (°C) at pH 7.0
A-T	53	A-G	46
Y_{13}-T	47	Y_{13}-G	44
Y_{14}-T	48	Y_{14}-G	43

Figure 61.

NMR studies revealed that the Y_{10}.A base pair incorporated in the 12-mer duplex d(CCACTAY_{10}TCACC).d(GGTGAATAGTGG) give rise to two DNA conformations, due to possible keto-enol tautomers of Y_{10} which are in slow exchange with each other. In the major component, the Y_{10}(syn).A(anti) alignment at the lesion site places the 8-oxo group in the minor groove and the base pair can be stabilized by two hydrogen bonds from NH7 and O6 of Y_{10} to N1 and NH_2-6 of dA. The Y_{10}:A base pair stacks well between its two adjacent Watson–Crick base pairs and forms a stable pair in the interior of the helix [271].

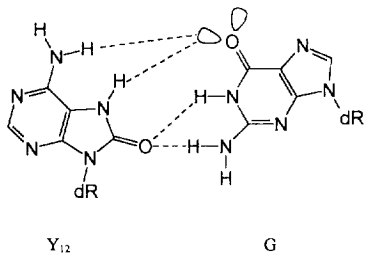
The guanine bases in DNA are not oxidized randomly to 8G but G residues located 5′ to a second G are the most easily oxidized [285a] and calculations have further shown that oxidation of 8G itself also becomes easier when it is located 5′ to a second G [285b].

In order to investigate the mutagenic effects and the repair of DNA lesions by γ-rays, 7,8-dihydro-8-oxodeoxyadenosine Y_{12} was introduced in DNA [286]. Crystallographic studies reveal that the G:Y_{12} base pair adopts the anti:syn wobble conformation with three-center hydrogen bonds centered on one lone pair each of O6(G) and O8(Y9) (reverse three-center hydrogen bonds) [279]. Likewise, G(anti):Y_{12}A(syn) is a non Watson Crick base pair where the presence of this reversed three-centered hydrogen-bonding system was observed. Alternatively hydrogen bond formation to a base in an adjacent base pair may stabilize the structure. These studies were carried out on the following sequence [279]:

G C C A C⊗A G C T C

Incorporation of 8-methoxyadenine (Y_{13}) at adenine sites, likewise, give duplexes with lowered T_ms. The Y_{13}:T and Y_{13}:G base pairs cause similar destabilization.

The Y_{13} base in the Y_{13} – G base pair can easily be accommodated in the *syn* conformation of DNA and steric clashes of the 8-methoxy group with the 2-amino group of guanine base seems to be not problematic [287].



Y_{12} G

Figure 62.

The effect of the Y_{13} substitution is sequence dependent. In sequences where the adenine moiety in the A-G mismatch prefer the *anti* conformation, Y_{13} substitution destabilizes duplexes. In A-G mismatches containing *syn* conformation of A, Y_{13} substitution results in duplex stabilization [287]. The $Y_{13} - Y_1$ base pair is less stable than the $Y_{13} - G$ base pair, indicating that the 2-amino group of G may contribute to duplex stability.

Likewise, substitution of bromine for hydrogen at the 8-position of adenine (giving 8-bromoadenine (Y_{14}) as photoactive probe) does not lead to major distortion of duplex DNA structure [288]. A similar destabilization as found for Y_{13} was observed [287].

8-Amino-2'-deoxyadenosine (Y_{15}) forms a reverse-Hoogsteen base pair with thymine [289]. 8-Oxo-7,8-dihydro-6-O-methyl-2'-deoxyguanosine (Y_{16}) was incorporated in oligonucleotides for studying the interactions with MutY enzyme (DNA repair enzyme which removes misincorporated adenine residues) [290, 291]. Introduction of a methyl group in the 8-position of guanine (giving 8-methylguanine or Y_{17}) incorporated into DNA sequences stabilizes the Z conformation ($CGCY_{17}CG$ with all guanines in the syn conformation). The Z-conformation is further stabilized by increasing the number of Y_{17} incorporated bases [292]. 8-Aza-2'-deoxyguanosine Y_{18} gives duplex stabilization in a $d(C-G)_3$ sequence (replacing G by Y_{18}), which may be the result of better proton-donor properties of NH(3) as well as the 2-NH_2 group [293].

3.3. Deaza analogues

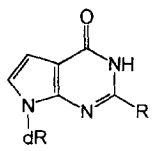
Removal of the 7-nitrogen atom of guanine, giving 7-deaza-2'-deoxyguanosine (Y_{19}), reduces duplex stability of a poly GC sequence [294] and of the palindromic GGAATTCC sequence [295]. This is explained by an helix distortion due to an altered overlap of the π-electron system of the modified base pair and by an increase in the pK_a of the amido group (pK_a of $Y_{19} = 10.3$; pK_a of dG = 9.3). Increase in pK_a results in less efficient hydrogen donor capacities of the $N^3 - H$ function and destabilization of the Watson–Crick base pair [294]. 7-Deazaguanosine gives disaggregation of G4-structures [296]. Incorporation of a methyl group in position 7, giving 7-deaza-7-methyl-2'-deoxyguanosine Y_{21} stabilizes the B-DNA duplex of a poly GC sequence compared to 7-deazaguanosine Y_{19}, coming close to the values of the parent purine oligonucleotide [297]. Homooligonucleotides of Y_{21} show sigmoidal melting indicating a highly ordered single-stranded structure.

Removal of the 2-amino group of Y_{19} gives 7-deaza-2'-deoxyinosine (Y_{20}). The altered dipole moment of the 7-deaza-hypoxanthine base (influencing stacking interactions) together with the reduction in hydrogen bonding capabilities and the reduced hydration of the hydrophobic base results in a further decrease of the T_m values [295]. The Y_{20}:C base pair is even less stable than the Y_1:C pair. The allopurinol base (Y_{22}), in contrast, destabilizes duplexes less strongly than Y_1 [298, 299] and may be a candidate for incorporation at ambiguous positions.

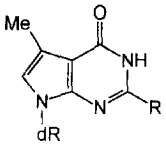
The 8-aza-7-deaza-adenine analogue (Y_{23}), when incorporated in a palindromic sequence (CTGGATCCAG) is not destabilizing when the deoxyribose moiety is bond at the N-9 position (Y_{23}) ($T_m = 47$ °C versus 48 °C) but is strongly destabilizing when the sugar moiety is connected at N-8 (Y_{24}) ($T_m = 35$ °C) [300]. Surprisingly, in an alternating $d(X - T)_n$ sequence, both modified bases are

G(anti):A(anti)		G(syn):A(anti)		G(anti):A(syn)	
GAGCT X GTGGC CTCGA Y CACCG		C G C X A A T T G G C G G C G G T T A A X C G C		CGC X AGCT Y GCG GCG Y TCGA X CGC	
XY	Tm (°C)	X	Tm (°C)	X-Y	Tm (°C)
G-A	47	G-A	38	A-G	42
G-Y_{13}	41	G-Y_{13}	<28	Y_{13}-G	54

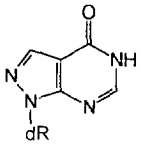
Figure 63.



Y_{19} R = NH_2
Y_{20} R = H



Y_{21}



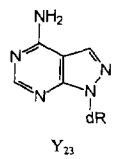
Y_{22}

sequence	Tm (°C)
GCGCGC	45° [294]
$Y_{19}CY_{19}CY_{19}C$	35° [294]
GCY_1CGC	27° [298]
$GCY_{20}CGC$	23° [298]
$GCY_{22}CGC$	30° [295]
GGAATTCC	38° [295]
$GY_{19}AATTCC$	34° [295]
$GY_{20}AATTCC$	22° [295]

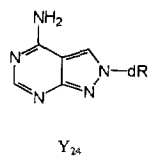
Figure 64.

strongly stabilizing. The stabilizing effect of the N-9 substituted 8-aza-7-deaza-adenine bases were explained by the authors as the result of the different π-electronic distribution of the base compared to adenine itself and stronger stacking interactions. The d(A – T) oligomer itself forms a special DNA type duplex with poor base overlap and weaker stacking interactions than random sequences [300]. The extraordinary stability of the N-8 substituted (Y_{24}) 8-aza-7-deaza-adenine containing oligomer may then be explained by the formation of a new, hitherto probably unknown, tertiary DNA structure. The CD spectrum points to a left-handed helix, as found for Z-DNA [301], with a reversed Watson–Crick base pair.

The guanine analogue Y_{25} is able to form Watson–Crick base pairs and gives duplex stabilization



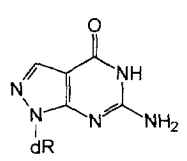
Y_{23}



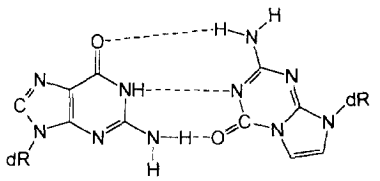
Y_{24}

	Tm
$d(A\text{-}T)_6$	32 °C
$d(Y_{23}\text{-}T)_6$	36 °C
$d(Y_{24}\text{-}T)_6$	48 °C

Figure 65.



Y_{25}



G

Y_{26}

Figure 66.

[302] when incorporated in the place of G in the sequence GGAATTCC.

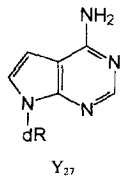
The 5 aza-7-deoxyguanine Y_{26} derivative forms a base pair with guanine resulting in stable oligonucleotide duplexes with parallel strand orientation [303].

Other examples of modified deaza-purines incorporated into oligonucleotides are deoxytubercidin (Y_{27}) [304, 305] and the 7-deaza analogue (Y_{29}) [306–308] of deoxynebularin (Y_{28}) [87, 309]. They have an altered proton acceptor site (Y_{27}, Y_{29}) or proton donor site (Y_{28}, Y_{29}) as well as an altered π-electron system.

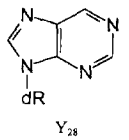
The dissociation temperature of 16 mer duplexes, evaluated during an investigation on the mismatch repair machinery, decreases in the order G – T > Y_1 – T > A – C > Y_{20} – T >> Y_{28} – C > Y_{27} – C ≈ Y_{29} – C [310].

Some of these mismatches do not form hydrogen bonds (Y_{28} – C; Y_{29} – C) and are mainly stabilized by stacking interactions. Some duplexes are stabilized by a single H-bond (A – C; Y_{28} – T; Y_{29} – T), some by two H-bonds (Y_1 – C; G – T; Y_1 – T) [87, 306, 310, 311].

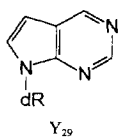
Two hydrogen bonds, but with reduced stability, may be formed between Y_{20} – C, Y_{20} – T and Y_{27} – T. The weaker base pair is presumably the result of a different electron delocalization in the deazapurine ring system (Y_{20} – C and Y_{20} – T) or due to steric hindrance (Y_{27} – T). In the latter case, the proton at position-7 of the purine analogue could force the exo-amino group out of the plane of the aromatic system and weakening the H-bond formation capacities. Some base pairing systems (G – T) implicate in addition major and minor groove modifications (in addition to the substitution of the



Y_{27}

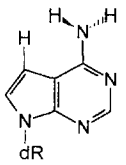


Y_{28}



Y_{29}

Figure 67.



G A T C C G T C X A C C T G C A
C T A G G C A G Y T G G A C G T

Y \ X	G	Y_1	Y_{20}	A	Y_{27}	Y_{28}	Y_{29}
C	49°	44°	42°	40°	35°	36°	35°
T	42°	41°	39°	47°	41°	41°	40°

Figure 68.

nitrogen atom by a CH function) due to displacement of the bases into the major or minor groove [310, 311].

7-Deaza-adenine (Y_{27}) gives duplex stabilization when replacing deoxyadenosine in an alternative $(A-T)_6$ sequence and strong duplex destabilization when replacing deoxyadenosine in an $(dA)_n - (dT)_n$ sequence [312–314]. This results from the different structures of the sequences. DNA sequences containing repeated $d(A)_n.d(T)_n$ tracts (H-DNA) adjacent to B-DNA demonstrate bending [218]. Due to the presence of a spine of hydration (water molecules hydrogen-bonded with thymine O-2 atoms and adenine N-3 atoms bridging adjacent bases belonging to different chains) in the minor groove of $d(A)_n.d(T)_n$, the minor groove is narrowed and base pairs fold into this groove resulting in the bending phenomenon [218]. The ordered water spine in the minor groove stabilizes the H-DNA form. When Y_{27} is replacing deoxyadenosine in $d(A)_n$ tracts, the bending decreases [218] due to destabilization of the H-form of the bending element by the 7-deaza-adenine base and an increasing imperfection of the sequence phasing relative to the helical screw axis of the oligomers [218]. The influence of Y_{27} on bending is dependent on the position of the adenine base which is replaced [231, 313].

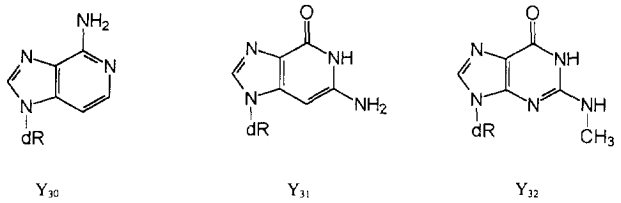
Alternating $poly(dA-T)_x$ is much more flexible and has an alternating B-DNA structure with a small helical twist of the d(A – T) steps and a larger one at the d(T – A) steps. This results in a polymorphic structure which can adopt more than one conformation depending on the environment. When high salt conditions are used $d(A-T)_6$ sequences are stabilized by substitution of dA by Y_{27}. At low salt concentrations destabilization of $d(A-T)_6$ is observed. The displacement of dA by Y_{27} in this sequence may result in a more stable uniform structure with almost equal stacking interactions along the oligonucleotide chain [313]. It is clear that the effect of incorporation of modified nucleotides is dependent on the studied sequence. Generally, replacement of deoxyadenosine by Y_{27} in oligodeoxynucleotides and replacement of adenosine by tubercidin in ribopolynucleotide duplexes result in a decrease of duplex stability [87, 218, 314]. Besides the altered stacking interactions and hydrophobization of the major groove, also the formation of bifurcated hydrogen bonds between the 6-amino group and adjacent O^4-thymine base may be affected by the displacement of dA by Y_{27}.

Generally, 3-deazapurines (Y_{30}, Y_{31}) destabilizes oligonucleotides more than 7 deazapurines [226, 315].

This phenomenon is sequence dependent and more pronounced in homopurine tracks [313]. The destabilization when introducing Y_{30} in a mixed sequence is minor, but the unmodified sequence produces a sharper melting curve indicating a more cooperative transition [87, 316]. The 3-deazapurines are still able to maintain Watson–Crick hydrogen bonding but it removes an essential hydrogen bond acceptor from the minor groove [316]. A difference in the delocalization of the π-electron system affecting base stacking interaction and hydration in the minor groove result in alteration of the overall conformation [231]. Another base modification facing into the minor groove is N^2-methylguanine (Y_{32}). The N^2-methylguanine containing oligonucleotides show higher T_m than the natural decamers [226].

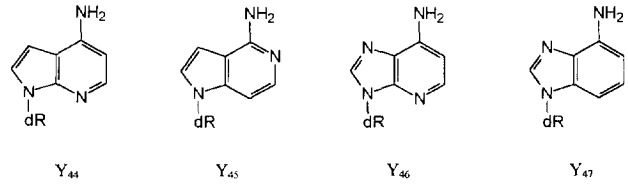
While unsubstituted 7-deaza-adenine gives duplex destabilization, introduction of a bromine (Y_{33}), chlorine (Y_{34}) or methyl (Y_{35}) substituent in the 7-position increases duplex stability compared to adenine [312]. This can be compared with the introduction of a methyl group or bromine atom in the 5-position of uracil where hydrophobic and/or stacking interactions are favoured. These substituents (5-substituent of pyrimidine and 7-substituent of purines) are located in the major groove and have steric freedom. The stabilization of the 7-substituent is due to favourable reaction enthalpy (H-bond and stacking interactions) [312].

The duplex stability can be tuned by an appropriate selection of a 7 substituent within the 7-deazaguanine-cytosine base pair. The structure of a B-DNA is such that a 7-substituent or a linker arm from the 7-position of a base paired Y_{19} to a reporter group should extend into the major groove with minimal distortion of the helix. 7-(1-Propynyl)-7-deaza-2′-deoxyguanosine (Y_{36}) and 7-(1-propynyl)-7-deaza-2′-deoxyadenosine (Y_{39}) likewise, increase duplex stability relative to the 7-unsubstituted deazapurines [317]. The 7-(hex-1-ynyl)-



Y_{30} Y_{31} Y_{32}

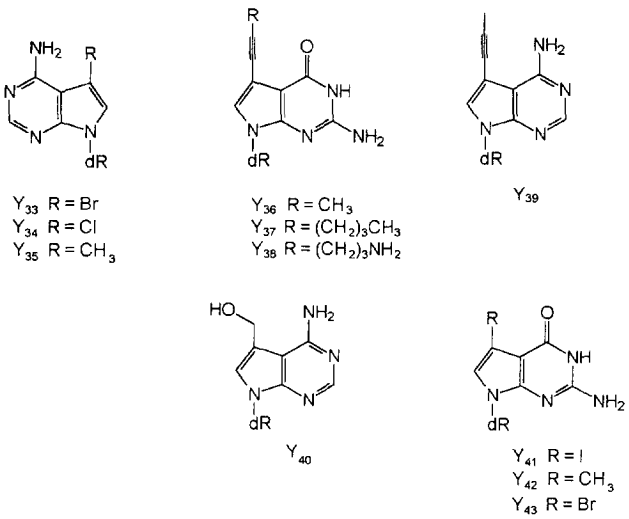
Figure 69.



Y_{44} Y_{45} Y_{46} Y_{47}

Figure 71.

substituted 7-deazaguanine containing oligomers $d(Y_{37}-C)_4$ and $d(C-Y_{37})_4$ exhibit similar T_m values as the G – C and C – G oligomers because the hexynyl chain fits exactly into the major groove without any protrusion. In order to examine the charge effects of 7 substituted 7-deazaguanines and not only steric effects on the duplex stability the oligonucleotide containing 7-(5-aminopent-1-ynyl)-7-deazaguanine Y_{38} $[d(Y_{38}.C)_4]$ was studied which gives no cooperative melting. In contrast, the duplex 5′-d(TAY$_{38}$.Y$_{38}$TCAATACT)-3′-d(ATCCY$_{38}$Y$_{38}$TTATY$_{38}$A)-5′ has the same stability as the native duplex [318]. 7-(Hydroxymethyl)-7-deaza-2′-deoxyadenosine (Y_{40}) was incorporated to mimic the role of structural waters in the major groove of DNA. Such structural H_2O molecules bridging specific hydrogen bond donors and acceptors in $d(A)_n$ tracts in DNA are postulated to contribute to the bending of DNA [319]. 7-Iodo-7-deaza-2′-deoxyguanosine (Y_{41}) and 7-bromo-7-deaza-2′-deoxyguanosine (Y_{43}) incorporated in a self-complementary $d(G-C)_n$ sequence in the place of G,



Y_{33} R = Br
Y_{34} R = Cl
Y_{35} R = CH_3

Y_{36} R = CH_3
Y_{37} R = $(CH_2)_3CH_3$
Y_{38} R = $(CH_2)_3NH_2$

Y_{39}

Y_{40}

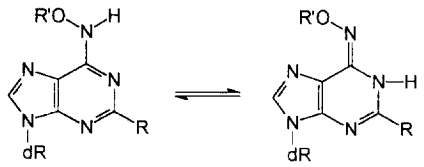
Y_{41} R = I
Y_{42} R = CH_3
Y_{43} R = Br

Figure 70.

stabilize the duplex enthalpically but lead to an entropic destabilization. The 7-substituents are located in the major groove and they do not interfere sterically with the sugar-phosphate backbone. However, the identity of the 7-substituent is of importance as a 7-CH_3 group (Y_{42}) destabilizes the same duplex [320]. Surprisingly, the heteroduplexes of $d(C_6)$ with $d[(Br^7C^7G)_5-G]$ (T_m = 23 °C) and $d[(I^7C^7H)_5-G]$ (T_m = 15 °C) are slightly less stable than $d[(C^7G)_5-G].d(C_6)$ (T_m = 27 °C) [321]. The stabilization of the iodine substituent has been declared by (a) hydrophobization of the major groove, (b) increased stacking interactions of modified base, (c) better proton donor properties of NH(1) and stronger H-bonding within base pair (pK_a of 7-deazaguanine = 10.3 and of 7-iodo derivative 10.0).

1,7-Dideaza-2′-deoxyadenosine (Y_{44}) [pK_a = 6.1] is less basic than 3,7-dideaza-2′-deoxyadenosine (Y_{45}) [pK_a = 8.6] but easier to protonate than 2-deoxyadenosine [pK_a = 3.8] and 7-deaza-2′-deoxyadenosine [pK_a = 5.3] [322]. It is not able to form Watson–Crick base pairs but may contribute to vertical base-stacking. 1,7-Dideaza-2′-deoxyadenosine (Y_{44}) destabilizes DNA duplexes. When incorporated in the middle of palindromic oligonucleotides, it stabilizes hairpin formation. Hairpin formation may be facilitated when decreasing the Watson–Crick base pairing interactions within the internal part of an oligonucleotide [322]. Homooligomers of 1-deaza-2′-deoxyadenosine (Y_{46}) [pK_a = 4.6] forms less stable duplexes with $d(T)_n$ than $d(A)_n.d(T)_n$ Watson–Crick duplexes [323]. Compound Y_{46}, most probably, forms Hoogsteen duplexes with thymine in a parallel chain polarity.

Contrary, oligonucleotide containing 1,3-dideaza-2′-deoxyadenine (Y_{47}) are not able to form Hoogsteen duplexes [324]. Indeed the 4-amino group of 1,3-dideaza-2′-deoxyadenosine is much more basic [pK_a of N^7 = 4.5; pK_a of NH_2 = 0.7] than that of 1-deaza-2′-deoxyadenosine and therefore a weak proton donor in (Hoogsteen and Watson–Crick) base pairing [324]. 4-Aminobenzimidazole (Y_{47}) interacts with regular nucleoside residue in a duplex structure mainly by



Y_{48} R = H, R' = CH_3

Y_{49} R = NH_2, R' = CH_3

Y_{50} R = H, R' = H

Figure 72.

vertical stacking. Alternatively the base may be oriented in a syn-position and one H-bond between N(7) of 1,3-dideazaadenine as acceptor and the H – N(3) of thymine as donor may be formed. Whatever the structure is, incorporation of 1,3-dideaza-adenine in an oligonucleotide leads to duplex destabilization [324].

3.4. N^6- and O^6-substitutions

Substitution by electronegative groups such as amino, hydroxyl and methoxy on the exocyclic amino group of cytosine increase the ratio of the imino tautomer [325] and this is similar for the N^6-substitution of the adenine base (Y_{48}). The equal stability of the N^6-methoxyadenine:cytosine and the N^6-methoxyadenine:thymine base pairs, suggest the existence of an imino-amino tautomeric equilibrium [326] where cytosine can pair with the imino tautomer. Both base pairs may occur with Watson–Crick geometry. The tautomerism is dependent on solvent polarity. The imino form is more stable than the amino form in polar solvents such as water and dimethylsulfoxide. The conformation of the methoxy group in solutions, syn or anti with respect to N-1 of the adenine ring, has not been clear yet. Likewise, N^6-methoxy-2,6-diaminopurine (Y_{49}) pairs with equal stability with T and C [165, 327, 328], although the overall stability of the oligomer is lower than the fully complementary ones. As mentioned before, the related 2,6-diaminopurine (Y_9) containing oligomers show different stabilities with C and T opposite Y_9. The Y_{48} containing duplexes are generally less stable than the Y_{50} series. The base pairs with Y_{48} and Y_{49} are less stable than the corresponding fully complementary duplexes, but more stable than those containing mismatch pairs. This lower stability may be partially attributed to syn→anti configurational changes of the methoxy group [328].

Methoxyadenine Y_{48} also forms fairly stable base pairs with A and G with similar stabilities to the Y_{48}:T and Y_{48}:C pairs. The T_m of the duplexes containing methoxyadenine pairs are within a relative small range (± 6 °C) [325]. The base pairing properties of Y_{48} in DNA strands with incoming dNTP substrates during replication of DNA are biologically important since formation of Y_{48} in DNA is expected to contribute to mutation.

dTMP and dCMP are only incorporated into DNA strands opposite to N^6-methoxyadenine by DNA polymerase because purine-purine type of base pairs are larger in size than pyrimidine-purine type of base pairs, the normal duplex conformation will be disordered more by the presence of Y_{48}:A and Y_{48}:G

A C T T G G C C [Y] C C A T T T T G
T G A A C C G G [X] G G T A A A A C

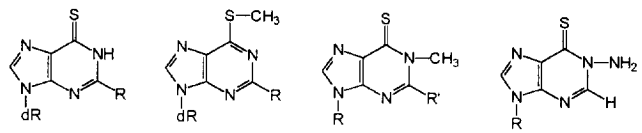
Y \ X	A	G	Y_{48}	Y_{49}
T	70°		65°	67°
C		75°	64°	66°

Figure 73.

C C T G G T A [Y] C A G G T C C
G G A C C A T [X] G T C C A G G

Y \ X	T	C	A	G
Y_{48}	50°	46°	52°	48°
Y_{50}	51°	42°	42°	50°
A	54°	44°	45°	53°
G	48°	59°	54°	52°

Figure 74.



Y_{51} R = NH_2

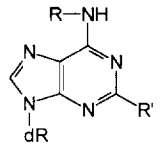
Y_{52} R = H

Y_{53} R = H or NH_2

Y_{54} R' = H or NH_2

Y_{55}

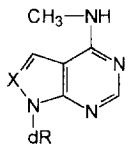
Figure 75.



Y_{56} R: CH_3; R' = H

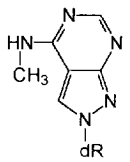
Y_{57} R = CH_3; R' = NH_2

Y_{58} R: $-CH_2CONH_2$



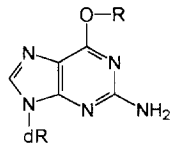
Y_{59} X = CH

Y_{60} X = N



Y_{61}

Figure 76.



Y_{62}	R = $-CH_3$	Y_{65}	$-(CH_2)_3CH_3$
Y_{63}	R = $-CH_2CH_3$	Y_{66}	$-CH(CH_3)_2$
Y_{64}	R = $-CH_2CH_2CH_3$	Y_{67}	$-(CH_2)_5CH_3$
		Y_{68}	$-(CH_2)_7CH_3$
		Y_{69}	$-CH_2Ph$

Figure 77.

pairs and this fact can explain why the formation of the Y_{48}:N base pairs by the DNA polymerase do not correlate directly with the thermal stabilities of the duplexes. Likewise Y_{50} is enzymatically incorporated in DNA strands opposite to thymine and cytosine. In case of Y_{50}, the base pair stability follows the order Y_{50}:T > Y_{50}:G > Y_{50}:A = Y_{50}:C [329].

The lower stability of the Y_{48}:T pair and the Y_{50}:T pair in comparison with the A-T pairs is probably due to steric hindrance between N^7 of Y_{48} or Y_{50} and the MeO- or HO-group [329]. The low stability of Y_{50}:C may be a reflection that Y_{50} prefers to be in the amino form. The Y_{48}:C base pair is more stable than A:C (iminoform is suitable for formation of Y_{48}:C pairs).

Incorporation of 6-thioguanine (Y_{51}) in DNA is believed to be the prime event in the mode of action of mercaptopurine antimetabolites. This last compound is used for the treatment of childhood and adult leukemias. The increase of the NH–S versus NH–O hydrogen bond length, the lower H-bond strength of the NH–S system and the alteration of the C^6 – S bond length may reflect in a distortion of nucleic acid helical structure [330]. Incorporation of 6-thioguanine leads to a decrease of duplex stability [277, 331–338]. The base pair Y_{51}:C is stronger than Y_{51}:T. The CD curve shows a transition at 340 nm due to the presence of the modified base.

The same is true for 6-mercaptopurine (Y_{52}) [334, 339]. It forms better base pairs with C than with T but the base pairs are less stable than the hypoxanthine analogues. This oxygen → sulphur replacement may be used to obtain probes for the study of protein-nucleic acid interactions, postsynthetic modifications and photo-cross linking [339].

The stability of the Y_{51}:C pair and of the Y_{52}:C pair is the same. The presence of the sulfur atom in thioguanine apparently does not allow the same close geometrical fit as found in the G:C base pair [334] which is essential for the formation of three hydrogen bonds. In the case of Y_{52}, the absence of the 2-NH_2 group makes the accommodation of the sulfur atom and the formation of two H-bonds easier to achieve [334]. Other thiopurine analogues which were incorporated into oligonucleotides are 6-methylthiopurine (Y_{53}) [291], 1-methyl-6-thioguanine and 1-methyl-6-thioinosine (Y_{54}) [340]. 1-Amino-6-thioinosine Y_{55} lacks the unique melting behavior characteristic and shows no cooperative transition in either the UV or CD spectra [341].

Base pairing with N^6-methyladenine (Y_{56}) and 2-amino-6-methylaminopurine (Y_{57}) [342] is only possible when the N-alkyl group occupy the anti-orientation with respect to the N-1 atom of the purine moiety. The methylation does remove the hydrogen atom that could otherwise be available for hydrogen bond formation in the major groove and it increases steric effects in the major groove [16]. N^6-methylation of 2′-deoxyadenosine gives a decrease in duplex stability [4, 14, 16, 301, 343, 344] as does incorporation of N^6-(carbamoylmethyl)-2'-deoxyadenosine (Y_{58}) [343].

Due to a restricted rotation about the bond linking C_6 with the exocyclic nitrogen two rotational isomers has been identified. The methylamino-group in both is coplanar with the purine ring but the Me-group is preferentially positioned cis to N_1 which interferes with correct Watson–Crick pairing. The forced anti orientation of the methyl group may lead to duplex destabilization [345]. Moreover, the CH_3NH-group is expected to be a weaker proton donor than an NH_2 group, thus destabilizing the H-bond to the carbonyl group of dT for electronic as well as for sterical reasons. Furthermore, N^6-methylation affects the electronic state of the nucleobase and thus stacking interactions [301].

Finally, the H_2O spine located within the major groove of an oligo duplex may be affected by the presence of the N-methyl group. Due to the anti location of the methyl

group with respect to N1, the H_2O accepting N(7) may be less accessible leading to destabilization of the duplex. Removal of the N-7 function, giving 7-deaza-N^6-methyl adenine (Y_{59}) further reduces duplex stability [301].

Introduction of a nitrogen substituent in the 8-position [giving 8-aza-7-deaza-N^6-methyladenine (Y_{60})] reverse somewhat the destabilizing effect of the N^6-methyl group. The nitrogen (N-8) in the major groove may again allow the formation of hydrates. Introduction of the N-8 isomer (Y_{61}) at one position, does not alter the structure of the whole duplex [301] which is in contrast with findings of single incorporation of the N-8 isomer of 8-aza-7-deaza-adenine [300].

The carcinogenicity and mutagenicity of N-nitroso compounds is believed to result primarily from alkylation of DNA on the O^6-position of guanine base and O^4 of thymine residues [153, 346]. When O^6-alkylguanines (alkyl = methyl, ethyl, propyl, butyl, isopropyl, hexyl, octyl, benzyl) ($Y_{62} - Y_{69}$) are incorporated opposite cytosine, duplex stability decreases. With O^6-isopropylguanine (Y_{66}) it has been demonstrated that the oligomers show significantly less hypochromicity [346]. Likewise, the O^6-alkylguanine:thymine base pairs give decreased hypochromicity and biphasic melting profiles [346]. The melting curves of sequences containing O^4-MeT:G base pairs and O^6-MeG:T base pairs are similar [153].

O^6-alkyl guanines create a region of localized instability in DNA with large destabilization of helix-coil transition [347, 348]. The stability of the base pair with O^6-methylguanine decreases in the order C > A > G > T. The decrease of T_m as a function of the alkyl group follows the order: wild type > Y_{69} > Y_{63} ≅ Y_{62}. For duplexes containing adjacent carcinogen-modified bases, more broader melting curves are observed, probably reflecting the presence of regions of greater localized instability [349]. It has been demonstrated that Y_{64} and Y_{65} induce G → A transitions [350] and that Y_{68} induces G → A transitions for 70% and G → T transversions for 30% [350], explaining their mutagenic activity.

An Y_{62}:T 12-mer duplex (CGT(C)GAATTCY_{62}CG) exhibits a transition midpoint that is 9 °C lower than that of the G:T 12-mer duplex [351, 352]. Several hypotheses have been formulated for the structure of the base pairs. NMR studies have shown that the Y_{62}:T pair is in a distorted Watson–Crick configuration (*figure 78a*) [353, 354]. However, the more Watson–Crick like structure illustrated in *figure 78b*, has been observed in the X-ray structure analysis [355] as well as with protected nucleosides in $CDCl_3$ [356]. The Y_{62}:C pair is much more stable than the Y_{62}:T pair [356]. Both wobble base pairing (*figures 78c* [357, 358] and *78d* [359]) as the formation of

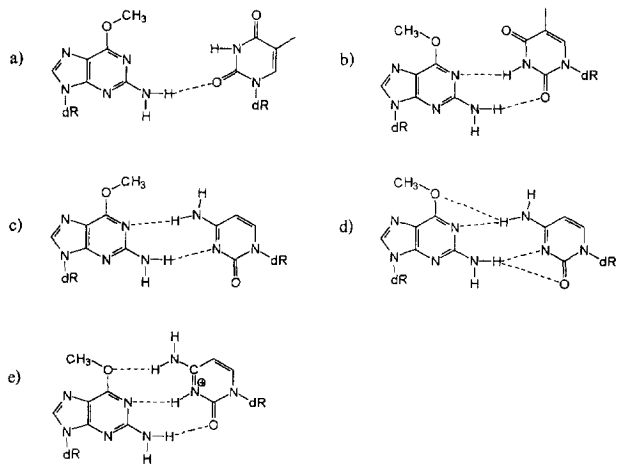


Figure 78.

a more Watson–Crick like protonated structure (*figure 78e*) [356] have been proposed for the Y_{62}:C [356, 360]. Spratt and Levy [361] investigated the structure of the hydrogen bonding complex for the base pairs Y_{62}:T and Y_{62}:C by using analogs of Y_{62} (S^6-methyl-6-thioguanine Y_{70}, O^6-methyl-1-deazaguanine Y_{71} and O^6-methylhypoxanthine Y_{72}) in which the Watson–Crick hydrogen bonding sites have been altered.

They have shown that incorporation of dTTP occurs via a Watson–Crick like structure (*figure 78b*) and the neutral Y_{62}:C pair occurs as the more distorted (*figure 78c* and *78d*) wobble structure.

In spite of the higher stability of Y_{62}:C over Y_{62}:T, it was demonstrated that DNA polymerases insert dTMP with preference for dCMP into sites opposite Y_{62} [233, 362]. Pairing of unprotonated C with Y_{62} in a wobble geometry and pairing of T with Y_{62} in a Watson–Crick base pair may explain this preference [233, 356]. It is then assumed that a wobble base pair or a non Watson–Crick pair which distorts DNA duplex, is not suitable for polymerisation [233]. When the Y_{62}:C mispair exists in a wobble pair conformation, it will be

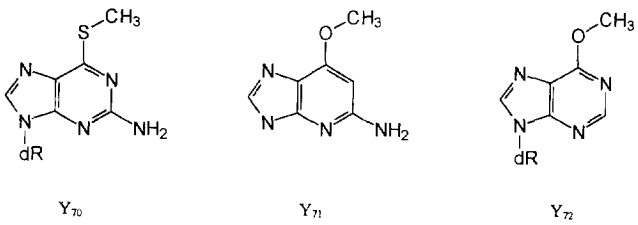
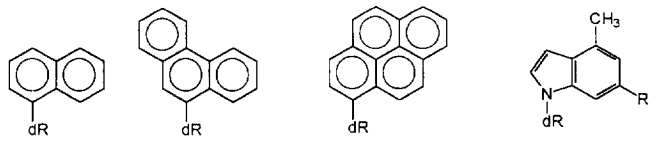


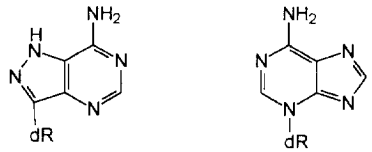
Figure 79.



Y_{73} Y_{74} Y_{75} Y_{76} R = CH_3

Y_{77} R = H

Figure 80.



Y_{83} Y_{84}

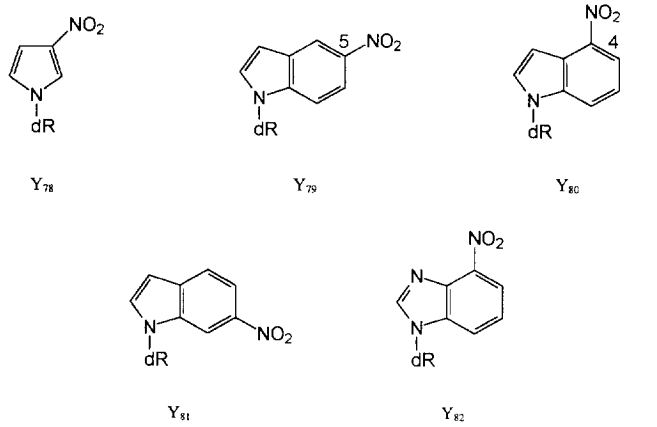
Figure 82.

removed by normal proofreading and repair enzymes but when it occasionally occurs as a protonated Watson–Crick pair the repair enzymes does not recognize this mispair and therefore the presence of m^6G in genomic DNA does not always give rise to a mutation [355].

Hairpin or cruciform structures are favoured when d(AT) rich sequences are flanked by d(GC) fragment. These (GC) induced changes can be altered if the number of H-bonds or the stacking interactions within alternating GC regions are reduced [363]. If the outher dG residues of the sequence C*G*CGAATTCGCC are replaced by Y_{62}, duplexes are formed with reduced stability. If the dG next to dA are replaced, increased hairpin formation is observed [363].

3.5. Miscellaneous modified nucleosides

Electrostatics, polarizability, hydrophobicity and surface area are factors influencing π-stacking interactions in aqueous solution [364]. Some of these factors were studied by introducing (poly)cyclic aromatic hydrocarbons in oligonucleotides [364] ($Y_{73} - Y_{77}$).



Y_{78} Y_{79} Y_{80}

Y_{81} Y_{82}

Figure 81.

Incorporation of Y_{76} as a 2-aminoadenine isoster, lacking hydrogen bonding capacity, in DNA give low and relative non-selective duplex binding, with slight preference for self-pairing and for pairing with other hydrophobic bases [171]. A $(Y_{76})_4$ loop is as stable than a T_4 loop and more stable than a A_4 loop [175]. The stacking capacity of the hydrophobic base was further studied by placing them in a 'dangling' position at the end of a base paired duplex [176]. The stacking order is as follows: $Y_{73} \sim Y_{74} \leq Y_{77} < Y_{75}$ [176]. The purine-sized compounds give increased stacking relative to the pyrimidine-sized cases. This might be explained by the increased surface area excluding from solvents, which allows stronger interactions with neighboring base pairs.

The interaction becomes more favorable with increasing size. Naphtalene interactions (Y_{73}) are stronger than adenine interactions. Phenanthrene (Y_{74}) is significantly more polarizable than naphtalene, but does not stack more strongly. The third ring of phenanthrene does not contact the adjacent bases. These results may be explained by solvophobic effects (rather than dipole-induced dipole effects). The amount of non polar surface area excluded from solvent by stacking is expected to be similar for naphtalene and phenanthrene in these structures. Pyrene models (Y_{75}) indicate that a large fraction of one face of this molecule can stack with the neighboring base pair.

A non-discriminatory base analogue should maximize intra- and/or interstrand stacking interactions, should not sterically disrupt DNA duplex formation and should demonstrate minimizing hydrogen-bonding interactions (which decreases selectivity) [365]. The electrostratic potential field of a π-aromatic system may be polarized (by which stacking is enhanced) by introduction of a nitro- or cyanogroup. Both groups are not strong hydrogen bonding acceptors. Therefore 1-(2′-deoxy-β-D-ribofuranosyl)-3-nitropyrrole (Y_{78}) was introduced into oligonucleotide and the ΔT_m with 4 bases was reported as fell within 3 °C range, although the modification greatly destabilizes DNA duplexes (ΔT_m 11–16 °C lower than

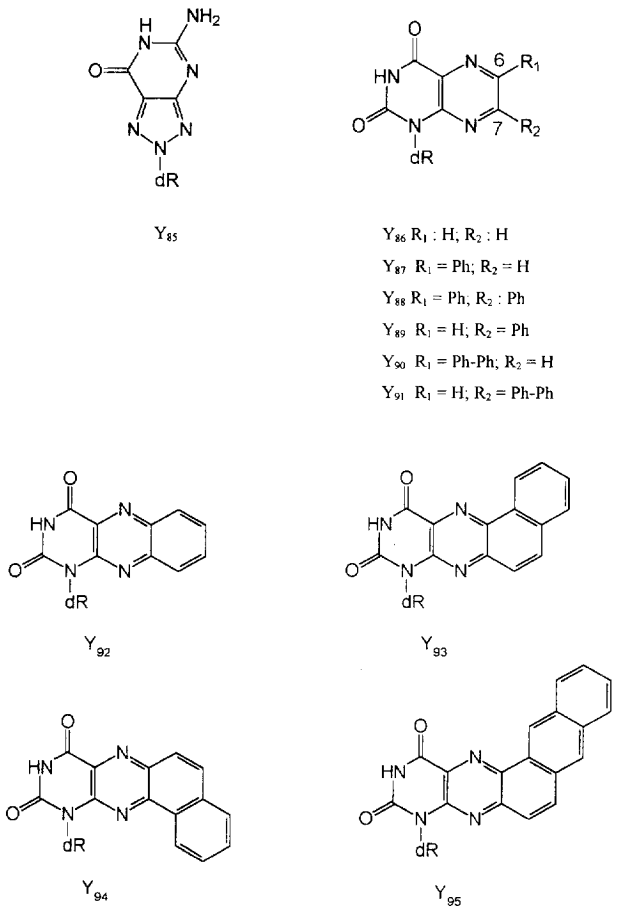


Figure 83.

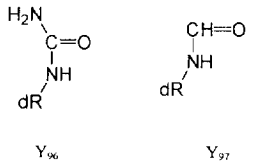


Figure 84.

natural AT and GC pairs). The 3-nitropyrrole base was selected first because of its structural and electronic resemblance to p-nitroaniline, derivatives of which are among the smallest known dsDNA intercalators [366]. The selection of a five membered heterocycle is based on the assumption that larger rings would not be easily accommodated opposite a purine without significant distortion of the duplex [366]. Although the author suggests that a five-membered ring heterocycle itself may be probably too small to provide adequate stacking interactions [366].

3-Nitropyrrole and 5-nitroindole nucleosides (Y_{79}) were tested as universal bases [367]. The nitrogen group increases the size of the base by the equivalent of an extra ring [367]. The 5-nitroindole nucleosides gave consistently higher duplex stability than the 3-nitropyrrole nucleoside together with a low discrimination towards each of the 4 natural bases [368]. Each individual residue is able to help stablize duplexes, however, both modifications are inferior to deoxyinosine as universal nucleoside. The hydrophobic bases are stacked together when consecutive substitutions are made [367].

The order of duplex stability decreases in the order $Y_{79} > Y_{80} > Y_{81} > Y_{78}$ [368], but all are of lower stability than an A:T pair. However, enhanced stability occurs when Y_{79} is present as an additional bulged base and as a pendant base in duplexes. The stabilisation is presumed to be due to enhanced stacking interactions for the nitroindole base. There is a strong evidence that when there are many consecutive 5-nitroindole residues in an oligomer there is a tendency for these bases to self-associate [369]. Likewise, the 4-nitro-1H-benzimidazole (Y_{82}) nucleosides do not form H-bonds with opposite bases but are stabilizing the duplex by stacking interactions and favorable entropic changes [370]. The destabilization effect is almost independent of the mismatch [370].

2′-Deoxyformycin A (Y_{83}) lowers duplex stability [371]. At pH 7.0 the thymine-formycin A duplex is more stable than G:A, C:A or A:A mismatches and base pairs formed between formycin A and the other natural nucleobases (G, C, A) [371]. 2′-Deoxy-3-isoadenosine (Y_{84}) forms Hoogsteen type base pairs with thymidine in the CGY_{84}TCG duplex [372, 373].

Some nucleosides with an unusual N-glycosidic bond linkage are able to form duplex structure with DNA: examples are N^7-(2′-deoxyribosyl)purines [300, 374] and N^8-(2′-deoxyribosyl)-8-azaguanine (Y_{85}) [293] although the latter with strongly reduced T_m.

Modification of the self-complementary sequence d(G-GTTCCATGCATGGAACC) with various lumazine nucleosides ($Y_{86} - Y_{91}$) at the 3′-end and 5′-end as well as by substitution of T units revealed interesting effects on the melting temperature of the various hybrids [375]. The parent lumazine nucleoside Y_{86} slightly decreases the small melting temperature depending on the number of substituents and their site in the chain whereas 6,7-diphenyllumazine (Y_{88}) did not alter the T_m much but raise T_m by 3 °C when two T substitutions were performed. Incorporation of two 6-phenyllumazine N-1 nucleosides (Y_{87}) in position 7 and 11 of the sequence

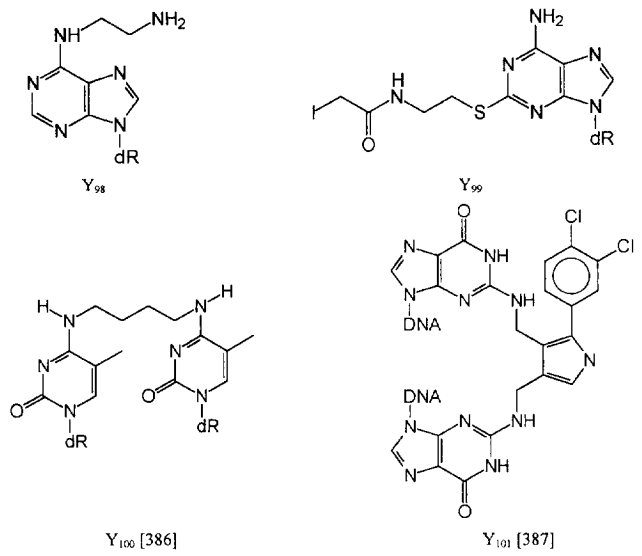


Figure 85.

CCAAGG7ACG11ACCTTGG raise the T_m by 5 °C. The 7-phenyllumazine nucleosides (Y_{89}) show only minor effects on T_m. Double substitution with 7-(4-biphenyl-lumazine (Y_{91}) gives a 10 °C increase in T_m while 6-(4-biphenyl)lumazine (Y_{90}) gives a decrease in thermal stability of the oligonucleotide. Incorporation of the various condensed areno[g] lumazine derivatives $Y_{92} - Y_{95}$ stabilizes the helical structure by improved stacking effects. Stabilization is strongly dependent on the site of the modified nucleobase in the chain.

Deoxyribosylurea (Y_{96}) [376–378] and deoxyribosylformamide (Y_{97}) [220, 379] are the major DNA modifications induced by the action of ionizing radiation [376], justifying studies on the mutagenic effect of these modifications and the repair of these lesions [380]. The lack of base-pairing and base-stacking by incorporation of 2'-deoxyribosylformamide leads to large duplex destabilization [379].

Deoxyribosylformamide incorporation or propanediol incorporation as abasic internucleotide linkers, reveal that the stability of the duplex containing abasic damage is rather dependent on the kind of nucleotide residue present on the opposite side of the abasic residue, than on the kind of abasic residue itself [381].

Deoxyribosylurea (Y_{96}) incorporated opposite thymine, exist in two isomers with respect to the orientation about the peptide bond [377]. The *trans* isomer is hydrogen bonded to T. In the *cis* isomer, deoxyribosylurea adopts an extrahelical position [377].

Several modified purines were incorporated into oligonucleotides to obtain inter- or intra-chain cross-linked oligomers ($Y_{98} - Y_{101}$). The NH_2-group of Y_{98} comes in close proximity of the 4-position of the pyrimidine residue in the complementary strand [382, 383]. After bisulphite addition across the 5,6-double bond of the pyrimidine ring, the 4-position may become activated for nucleophilic attack by the amino function. The 2-(N-iodoacetyl-aminoethyl)thioadenine (Y_{99}) was incorporated to target the N-3 of adenine in the minor groove [384, 385]. The authors claim that Y_{99} still forms Watson–Crick base pairing with T and Hoogsteen pairing with G.

Cross-linking confer a large degree of stabilization of DNA duplexes. Cross linking using a guanine modified disulphide bond is more efficient with a dithiobis(propane) link (Y_{104}) than with a dithiobis(ethane) link (Y_{103})

		Tm (°C)			Tm (°C)
	natural duplex	71 °C		natural duplex	70 °C
Y_{102}	G–SMe	73 °C	Y_{105}	A–SMe	45 °C
Y_{103}	G–S–S–G	86 °C	Y_{106}	A–S–S–A	89 °C
Y_{104}	G–S–S–G	90 °C	Y_{107}	A–S–S–A	86 °C

Figure 86.

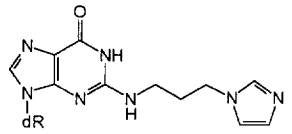
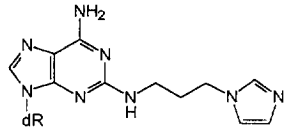
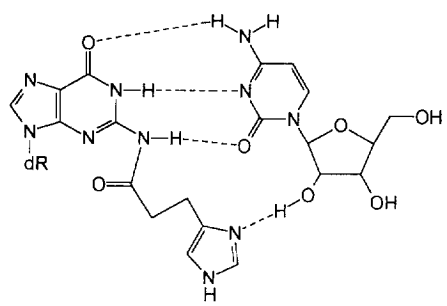
		ΔTm/md.	
	sequence GCCGAGGTCCATGTCGTACGC	DNA	RNA
Y_{108}		+2.0	+0.3
Y_{109}		+2.7	+0.6

Figure 87.

[388]. Molecular dynamics simulation demonstrate the accomodation of the disulfide cross-links in the major groove of DNA and they do not perturb significantly the DNA structure [389]. A shorter cross link induces more constraint on twisting [389]. The tether alone (Y_{102}) has a negligible effect on duplex stability [388].

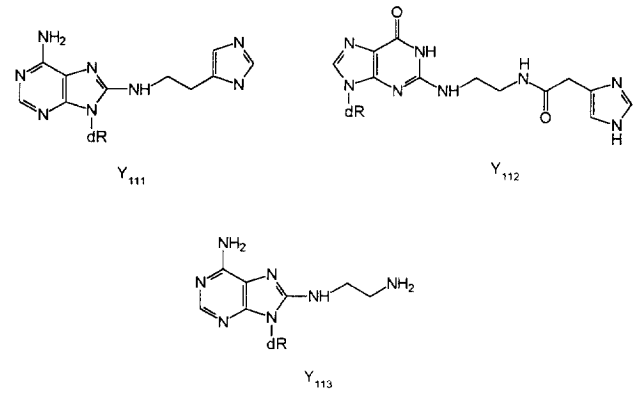
With N^6-derivatized adenine bases, the T_m of the C-2 cross-linked oligonucleotide (Y_{106}) is higher than of the C-3 cross-linked molecules (Y_{107}) [390]. The disulfphide cross link stabilizes DNA mainly at the base pairs immediately adjacent to the cross-link, but not over long distances [390].

Interstrand disulfide cross-link can significantly stabilize duplex DNA, which results from entropic destabilization of the denaturated state [391]. The cross-linked molecules are destabilized enthalpically relative to the unmodified oligos as a result from the requirement to



Y_{110}

Figure 88.



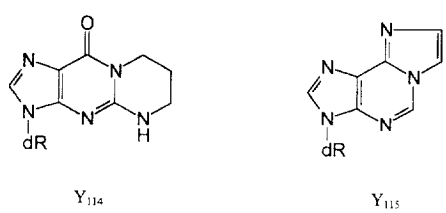
Y_{111} Y_{112} Y_{113}

Figure 89.

form W–C base pairs via the less stable anti-rotamer of the adenine nucleosides [391].

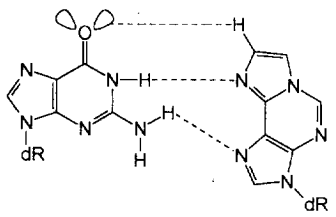
N^2-imidazolylpropylguanine (Y_{108}) and N^2-imidazolylpropyl-2-aminoadenosine (Y_{109}) are incorporated in DNA and RNA [392]. The 3-(1H-imidazol-1-yl)propyl group protrudes into the minor groove and stabilizes DNA-DNA duplexes more than DNA-RNA duplexes. In DNA-DNA duplexes the imidazole binds proximate to the phosphate backbone. In the case of a DNA-RNA duplex, the increased stabilization may be explained by the existence of other H-bond donor and acceptor interactions within the broad minor groove and not to specific binding to phosphates [392]. The N^2-modified 2-amino-adenine is as specific as dA, while N^2-modified guanine base is more specific (to complementary C) than the unmodified dG [392].

By incorporation of deoxyguanosine tethered through the exocyclic nitrogen via a 3-carbon chain to the 4 position of imidazole (Y_{110}), the stability of duplexes with ribonucleotide is increased and the stability of duplexes with oligodeoxynucleotide is decreased [393]. This effect is consistent with a model where the imidazole group forms a H-bond with a 2′-HO group of a ribonucleoside on a complementary strand. The mismatch



Y_{114} Y_{115}

Figure 90.



Y_{115}

Figure 91.

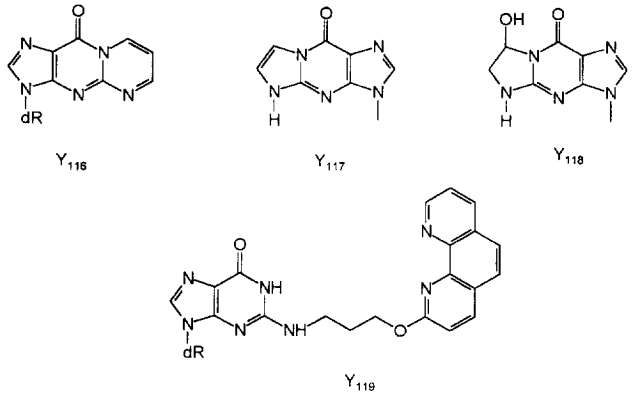
between the modified deoxyguanine and adenosine is more stable than a G:A mismatch [393].

In the case of deoxynucleotides, no such stabilization occurs. The disruption of the intramolecular hydrogen bond between N^1 – H of guanine base and the imidazole group, needed to form a Watson–Crick pair has, in this case, a significant energetic cost [393].

Conjugated imidazole and ethylene diamine chains are used as ribonuclease mimics ($Y_{111} - Y_{113}$) [394–396]. The self-hydrolysis of ribodimers is accelerated by the presence of imidazole attached to C-8 of adenine base Y_{111} [397, 398].

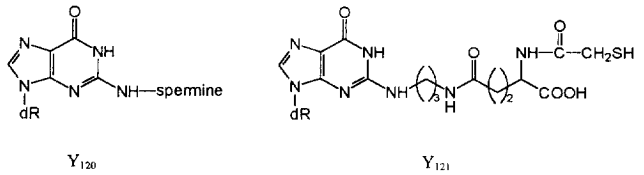
8-Amino-(2-aminoethyl)-2′-deoxyadenosine (Y_{113}) [394, 395, 399] incorporation drastically lowers ($\Delta T_m \simeq 15$ °C) the stability of the Dickerson dodecamer, perhaps due to repulsion of two positively charged ω-NH_3^+ group, situated spatialy close in this self complementary duplex DNA (CGCGY_{113}ATTCGCG) or due to a modified glycosidic torsion [399].

When guanine is exposed to acrolein, a common environmental pollutant and mutagenic, 1,N^2-propanedeoxyguanosine is formed (Y_{114}) which destabilizes duplexes insensitive whether the base opposite to the lesion is cytosine or adenine [400, 401].



Y_{116} Y_{117} Y_{118} Y_{119}

Figure 92.



Y_{120} Y_{121}

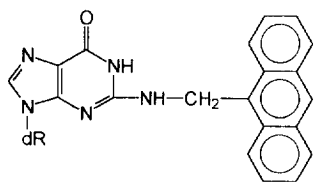
Figure 93.

Ethenodeoxyadenosine (Y_{115}) was incorporated for studying of fluorescence quenching as a result of base-base stacking interactions [402, 403] or as fluorescent probes [209].

The 1,N^6-ethenoadenosine:guanine (Y_{115}) base pair is more stable than the adenine(anti):G(syn) mispair [404] but less stable than the natural adenine:thymine pairing system. The pairing is proposed as a G(anti):Y_{115}(syn) system with 3-interbase hydrogen bonds (one weak interaction between O^6-carbonyl lone-pair orbitals and C–H of etheno bridge) and an altered sugar-phosphate backbone [404].

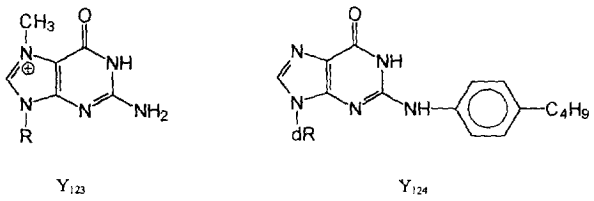
Malondialdehyde is, likewise, a major mutagenic and carcinogenic product generated during peroxidation of membrane lipids giving rise to the alkaline labile pyrimido(1,2-a(purin-10(3H)-one nucleoside (Y_{116}) which has been incorporated in oligonucleotides [405].

1,N^2-Ethenoguanine (Y_{117}) and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-a] purine (Y_{118}) are two modified bases formed in the reaction of DNA with 2-chlorooxirane, which have been found to have miscoding potential [406]. A phenanthroline ligand bound through N-2 of deoxyguanosine (Y_{119}) has been used to position a metal binding ligand within the minor groove of a duplex and sequence-specific cleaving of nucleic acids [407]. Spermine bind to DNA with low sequence preference ($K_a \simeq 10^4\ M^{-1}$). Covalent binding of spermine at the N^2 of guanine (Y_{120}) increase duplex stability



Y_{122}

Figure 94.



Y_{123} Y_{124}

Figure 95.

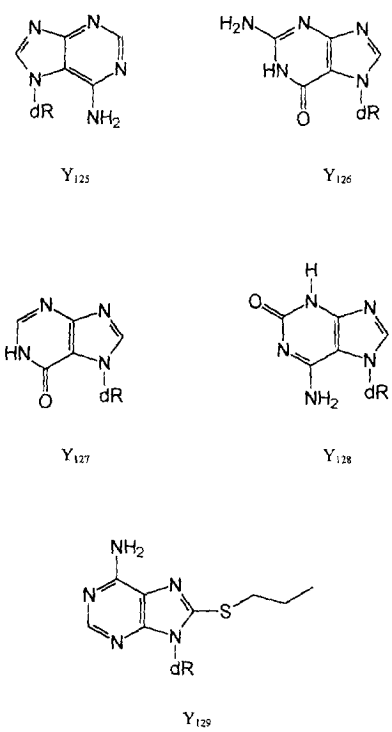
considerably (ΔT_m = 15 to 25 °C higher than natural duplexes) [408].

N-(2-Mercaptoacetyl)-glutamate modified guanine base (Y_{121}) was incorporated with the aim of decapitation of a complementary 5′-capped mRNA [409].

While attachment of one or two pyrene residues to a thymine base (via an 8-10 atom linker) results in stabilization of a double stranded octamer, the presence of an antracenylmethyl group at the N^2-position of the guanine base results in duplex destabilization. This polycyclic aromatic hydrocarbon (Y_{122}) is not intercalated but is nestled within the minor groove [410].

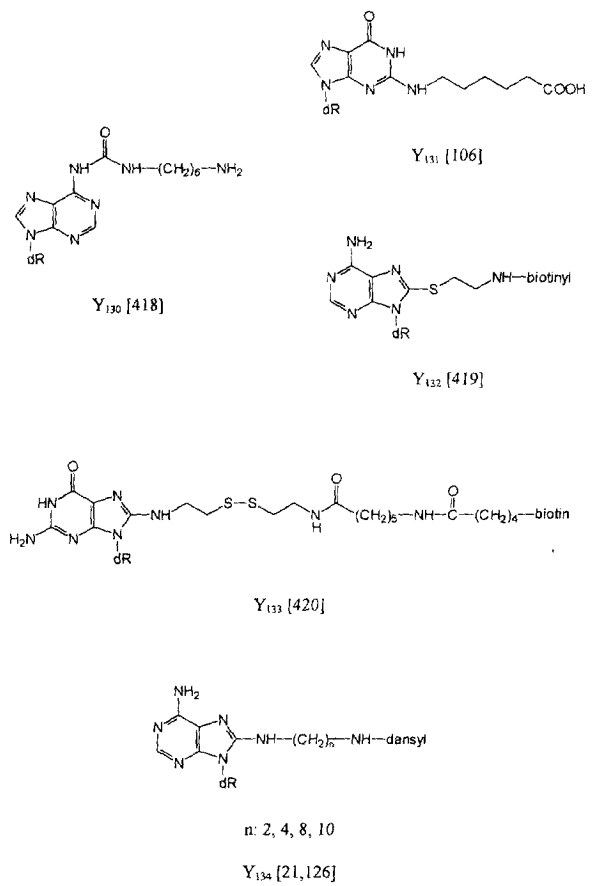
2-, 6- and 8-azido purine nucleosides are incorporated in DNA or RNA as photochemical cross-linking agents [411]. N^7-methylated guanosine Y_{123} are used for the synthesis of capped oligonucleotides [412].

Compound Y_{124} was incorporated because the nucleoside itself as triphosphate is a DNA polymerase (inhibitor [413]. N^7-(2-deoxy-β-D-erythropentofuranosyl)adenine (Y_{125}) was incorporated in DNA [374, 414]. The $(Y_{125})_{11}$A oligo forms a duplex with dT_{12} (T_m = 22 °C), probably via antiparallel chain polarity and reverse Watson–Crick base pairing [374]. The N^7-(2-deoxy-β-D-erythropentofuranosyl)guanine (Y_{126}), N^7-(2-deoxy-β-D-erythro-pentofuranosyl)hypoxanthine (Y_{127}) and N^7-(2-deoxy-β-D-erythro-pentofuranosyl)isoguanine (Y_{128}) likewise give duplex destabilization [415]. Compound Y_{126} can form a base pair with 2′-deoxyguanosine within an antiparallel duplex structure [416]. Oxidation of the sulfide Y_{129} to sulfone increases the rate of cleavage of glycosidic bond and this may be a route for the preparation of oligonucleotides containing an abasic site at a preselected position [417].



Y_{125} Y_{126} Y_{127} Y_{128} Y_{129}

Figure 96.



Y_{131} [106] Y_{130} [418] Y_{132} [419] Y_{133} [420]

n: 2, 4, 8, 10

Y_{134} [21,126]

Figure 97.

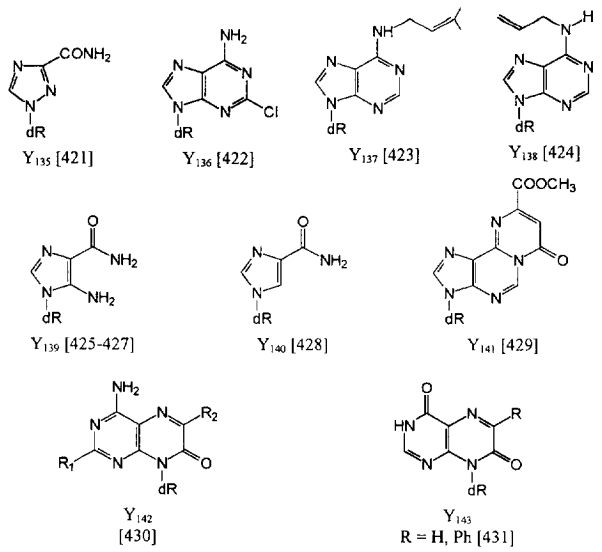


Figure 98.

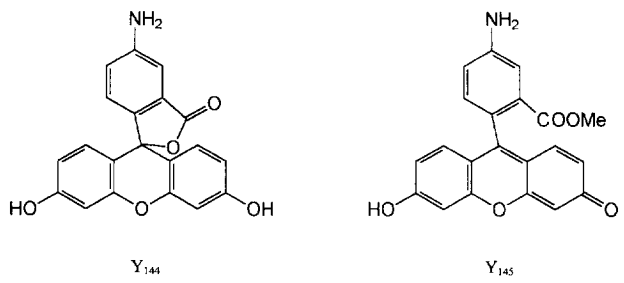


Figure 99.

Many purines have been modified in order to be able to connect reporter groups ($Y_{130} - Y_{134}$). A carboxylate group (Y_{131}) can serve as ligand to position a metal ion adjacent to phosphodiester groups, functioning as a general base for proton abstraction from 2′-OH.

Several modified purines with biological activity (Y_{135}, Y_{136}) or as constituent of ^{t}RNA (Y_{137}) or because of their potential to function as general base (Y_{139}, Y_{140}), or as self-cleaving DNA nucleoside (Y_{138}) were incorporated in oligonucleotides, in an effort to try to explain their function and/or activity. Compounds Y_{141}, Y_{142}, Y_{143} were incorporated because of their fluorescence properties.

5-Aminofluorescein Y_{144} and 5-aminofluorescein methylester Y_{145} have been coupled to the amino groups of the common nucleosides via a carbamoyl spacer in order to bring these fluorescent markers in close proximity to the nucleobases [432].

Finally, understanding structure and conformation of nucleic acid mutagen adducts is essential for the elucidation of events involved in chemical carcinogenesis. To this extent many adducts between environmental carcinogens and nucleosides were incorporated in oligos ($Y_{146} - Y_{156}$). However, the studies carried out with these modified oligomers are rather limited.

N-(deoxyguanosin-8-yl)-1-aminopyrene (Y_{156}) is proposed as a model for 1-nitropyrene adduct, which is the predominant nitropolycyclic hydrocarbon found in diesel exhaust and an established mutagen and tumorigen. Duplex of approximately equal thermal stability are formed with either T or dG opposite to the phenanthrene-diol epoxide-2′-deoxyadenosine adduct Y_{155}, whereas a less stable duplex is formed with dA in this position. The decrease in selectivity for the base in the complementary position may contribute to the mechanism of mutagenesis by the presence of the adduct.

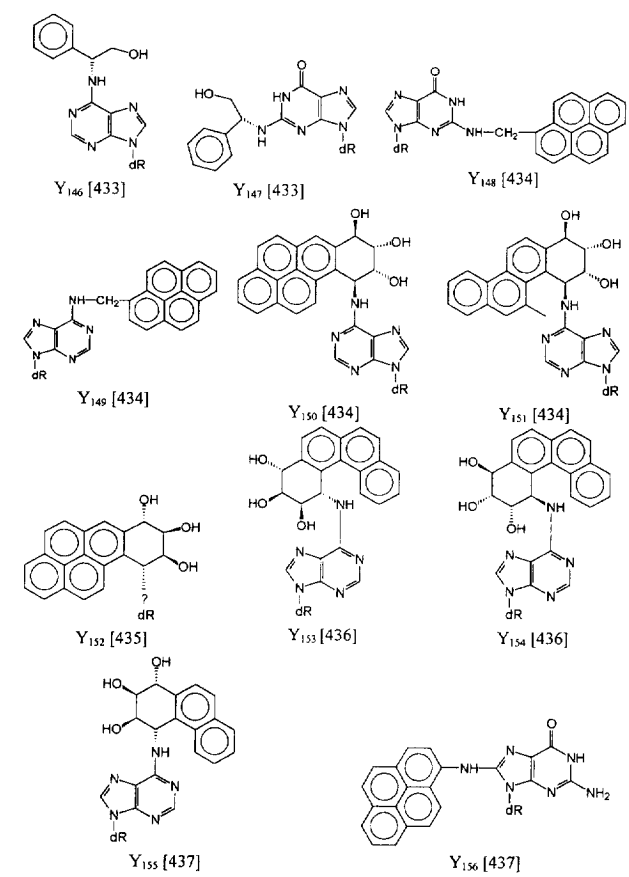


Figure 100.

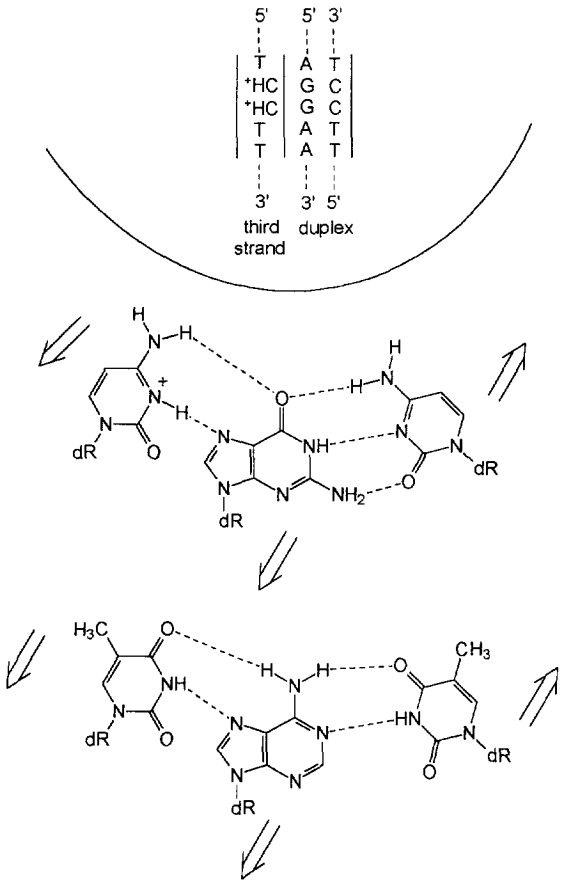


Figure 101.

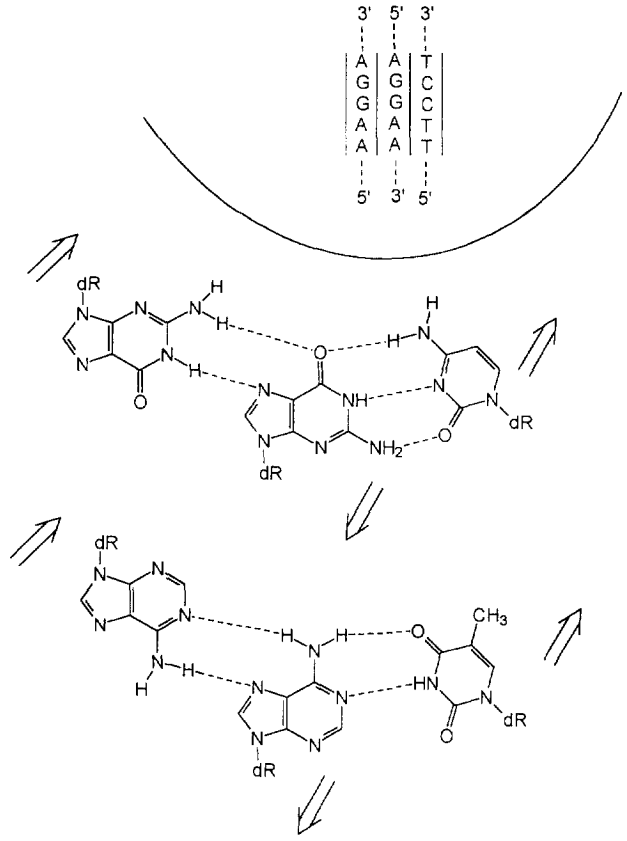


Figure 102.

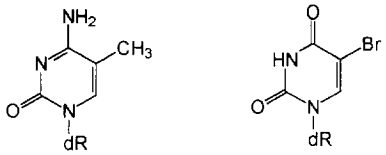
4. Triple helix formations

Three main triple helix motifs can be distinguished:

(a) Oligopyrimidines (thymine and protonated cytosine) recognize by Hoogsteen pairing oligopurine strands (adenine and guanine) of a DNA double helix. The oligopyrimidine triple helix forming strand is oriented parallel with respect to the oligopurine sequence of the DNA double helix. As the cytosine base in the third strand needs protonation to be able to bind to the guanine residue with 2 hydrogen bondings, this triple helix formation is strongly pH dependent. The complex dissociate with increasing pH.

(b) Oligopurines (adenine and guanine) recognize by reverse-Hoogsteen pairing oligopurine strands (adenine and guanine) of a DNA double helix. The oligopurine strand is oriented antiparallel with respect to the oligopurine sequence of the duplex. The triplex is stabilized by multivalent cations. As no bases have to be protonated, this triple helix formation is pH independent. Relative high G-content of one strand (60%) is necessary to achieve stable binding [439].

(c) A third possibility is to mix both previous systems. The adenine in the third strand in system 'b' may be replaced by thymine or, reversely, the protonated cytosine in the third strand of system 'a' may be replaced by guanine giving mixed purine-pyrimidine oligonucleotides as triple strand (TA*T* and CG*G*). These oligonucleotides recognize oligopurine strands (adenine and guanine) of a DNA double helix in a pH independent manner. The orientation of the third (G, T) strand may be as well parallel as antiparallel dependent on the sequence and on the number of 5′TpG3′ and 5′GpT3′ steps. This motif has the disadvantage that G-rich sequences may lead easily to



X_1 X_4

Figure 103.

self-association. The triple helix recognition motif may be extended through the recognition of T by G via one hydrogen bond (G.TA motif). The recognition selectivity is given by [440]:

$$T.AT > T.GC,\ T.CG,\ T.TA$$

$$^{Me}C.GC > {}^{Me}C.AT,\ {}^{Me}C.CG,\ {}^{Me}C.TA$$

$$G.TA > G.AT,\ G.CG,\ G.GC$$

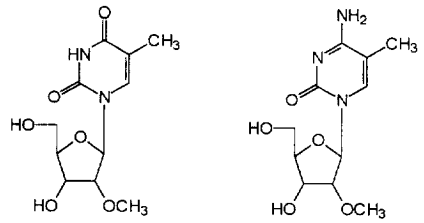
Triple helixes composed of a combination of recognition motifs (polypyrimidines and polypurines) can also be formed by alternate strand binding [441–443].

The main purpose to introduce modified nucleosides in a triple helix forming oligomer is to increase binding strength and binding kinetics and to develop a system which can be used to bind all four base pairs of DNA, mainly by using non-natural bases [440]. Indeed, the number of double-helical sequences that can be recognized has been further extended by the incorporation of nonnatural bases and by the design of 3-3′ and 5-5′ linked pyrimidine oligonucleotides for alternate strand triple helix formation. But a general method for recognition of mixed sequences containing all four base pairs of duplex DNA through triple-helix formation is still lacking. The modified oligomers should (i) not disrupt the backbone geometry, (ii) should have appropriately positioned hydrogen bond donors and acceptors for pairing in the edges of the AT/TA and GC/CG Watson–Crick base pairs and (iii) should cause optimal stacking within the helix.

X Y Y X X Y X X Y X Y
G A A G G A G G A G A
C T T C C T C C T C T (T_4)

X/Y	pH 5.5	pH 6.0	pH 6.5	pH 7.0
C/T	53°	20°	-	-
X_1/T	58°	38°	30°	-
C/X_4	55°	35°	28°	-
X_1/X_4	74°	45°	39°	20°

Figure 104.

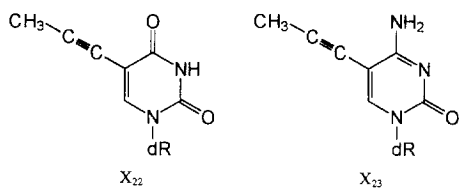


X_{119} X_{120}

sequence	Tm (triplex at pH 5.0)
UCCCUUCUC (ribo)	48.1 °C
$T^{Me}C^{Me}C^{Me}CTT^{Me}CT^{Me}C$ (deoxy)	43.4 °C
U*C*C*C*U*U*C*U*C* (2'-OMe)	54.9 °C
$X_{119}C*C*C*X_{119}X_{119}C*X_{119}C*$	55.3 °C
$U*X_{120}X_{120}X_{120}U*U*X_{120}U*X_{120}$	52.0 °C
$X_{119}X_{120}X_{120}X_{120}X_{119}X_{119}X_{120}X_{119}X_{120}$	49.3 °C

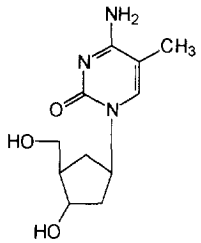
Figure 105.

In DNA triple helices, methylation of C-5 of uracil (thymine) or cytosine have similar stabilizing effects for both bases. However, methylation of the C-5 position of uracils in circular triplex-forming RNA oligonucleotides



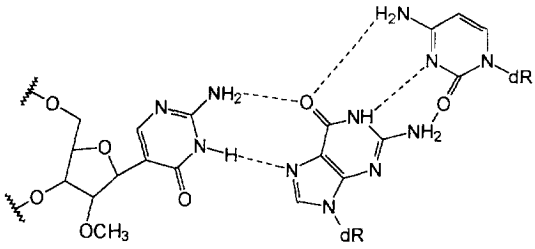
X_{22} X_{23}

Figure 106.



X_{121}

Figure 107.



X_{124}

Figure 109.

increases not only affinity but also sequence specificity (discrimination against mismatches) in binding an RNA strand but addiction of C-5 methyl groups to cytosines decreases affinity in binding RNA [446].

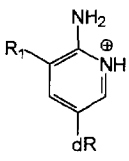
Binding of contiguous tracts of GC base pairs in a target duplex above pH 7.0, however, is inefficient with oligonucleotides containing 5-methyl-2'-deoxycytidine, which still have to be protonated [447].

The effect of triplex stabilization by introduction of a 5-methyl substituent in 2'dCyd may change upon sugar modifications. Like unmodified pyrimidine RNA (sequence UCCCUUCUC) the triplex of a 2'-O-CH_3 pyrimidine oligomer and dsDNA is more stable than the corresponding DNA triplex. Using 5-methyl-2'-O-methyluridine (X_{119}) instead of 2'-O-methyluridine in the same sequence (UCCCUUCUC), triple-helix becomes even more stable. In contrast 5-methyl-2'-O-methylcytidine (X_{120}) in place of 2'-O-methylcytidine destabilizes the triplex [444]. This effect has also been observed using 5-(1-propynyl)-2'-dUrd (enhances triplex stability) and 5-(1-propynyl)-2'-dCyd (opposite effect). These differences may be partially due to differences in pK_a of the cytosine base in the triplex structure [444].

The beneficial effect on binding entropy by introducing a 5-methyl substituent may be further increased by using the more bulky and hydrophobic 5-propyn substituent [74]. This substituent also allows increased stacking of the base which represents a more favourable enthalpic contribution to binding.

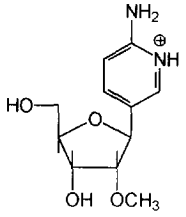
As mentioned before, 5-(1-propynyl)-2'-deoxyuridine (X_{22}) increases triplex stability (ΔT_m/substituent = + 2.4 °C) while 5-(1-propynyl)-2'-deoxycytidine (X_{23}) decreases triplex stability (ΔT_m/substituent = – 3.4 °C) (NMN 672). The pK_a of 5-(1-propynyl)-2'-deoxycytidine (pK_a = 3.30) is 1.05 units lower than that of 5-methyl-2'-deoxycytidine which has a drastic effect on the stability of oligonucleotide complexes.

5-(1-Propynyl)-2'-deoxyuridine also increases cooperative binding of two successive oligonucleotides to dsDNA [448]. A 44-fold enhancement in the associations constant of one oligonucleotide in the presence of a neighbor oligomer was observed (sequences: $TX_{22}{}^{Me}C^{Me}CX_{22}X_{22}X_{22}X_{22}$ and $X_{22}X_{22}X_{22}X_{22}{}^{Me}CX_{22}{}^{Me}CT$).



X_{122a} R_1 = H

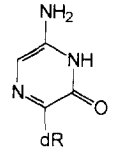
X_{122b} R_1 = Me



X_{123}

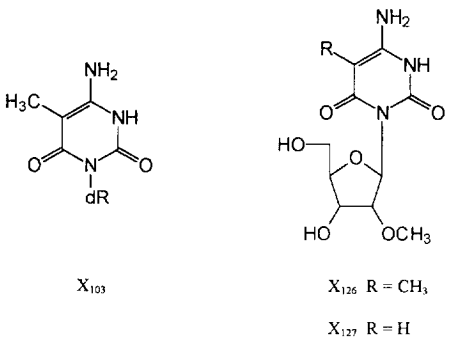
Z	TTTTTZTZTZTZTZT			
	pH 6.0	pH 7.0	pH 7.5	pH 8.0
X_{123a}	50.3	39.0	31.3	23.1
^{Me}C	53.3	31.5	20.1	12.2
C	42.5	20.3	9.8	< 4

Figure 108.



X_{125}

Figure 110.



X_{103}

X_{126} R = CH_3

X_{127} R = H

Figure 111.

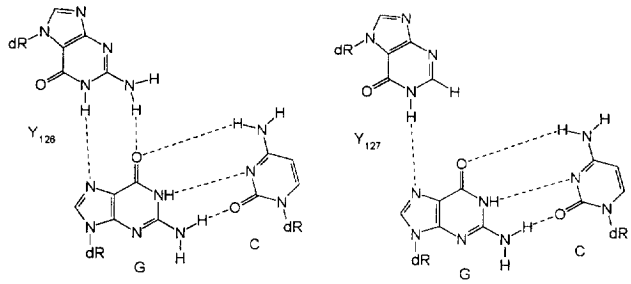


Figure 113.

The cooperative interaction is due to the structural transition between adjacent sites on DNA and the increased base stacking between the propyn-substituted 2′-deoxyuridines [448]. Stacking of the 5-propyn group of the base on the 3′-side of the junction into the 5′-base across the junction was observed (but not stacking of the propyn group at the 5′-site of the junction into the 3′-adjacent base) [448].

An alternative approach to increase the pK_a of the cytosine (mimic) and facilitate protonation at neutral pH is to use sugar modified ^{Me}C analogues. The pK_a of carbocyclic deoxycytidine (X_{121}) is 4.80 while the pK_a of deoxycytidine itself is 4.35. The replacement of 5-methyl-2′-deoxycytidine by its carbocyclic congener leads to approximately 100-fold increase in stability (ΔT_m = 3.9°/substituent) of the complex at pH 7.2 (sequence $T^{Me}CT^{Me}CT^{Me}CT^{Me}CT^{Me}CTTTTT$) [449].

The pK_a of a 2-aminopyridine-5-yl nucleoside (X_{122a}) is two units higher than natural cytidine. Above pH 7.0 oligonucleotides derived from T, X_{122a}, X_{122b} have considerable higher binding affinities for the target than oligonucleotides containing C and ^{Me}C [450, 451]. The pH dependence and sequence composition effects are much less pronounced for X_{122a} and X_{122b} containing oligonucleotides than for ^{Me}C containing ones [452]. It seems that the effect of the pK_a difference (0.4 pK_a units) between X_{122a} and X_{122b} becomes only operative at pH > 8.0.

The protonated 2-aminopyridine nucleoside may form two standard hydrogen bonds to the guanine base. Triplex forming oligonucleotides containing the α-anomeric 2-aminopyridine nucleoside have a slightly higher stability than those compared of the β-anomers, possible because the α-anomer has slightly increased basicity compared to the β-anomer [451].

Oligonucleotides containing 2-amino-2′-O-methylpyridine-5-yl X_{123} nucleosides show distinctly less stable triple helices [452].

A third possibility to raise the pK_a of the N3 position of the pyrimidine base is to use 6-amino-2′-O-methylcytosine (pK_a 6.8 or a ΔpK_a of 2.3 relative to 2′-deoxycytosine). However, the 6-amino group causes a perturbation of the conformation of the nucleoside to disfavor binding to the target duplex and the expectations were not fulfilled [453].

The requirements for base protonation (pH independent recognition of GC base pairs) can be eliminated by

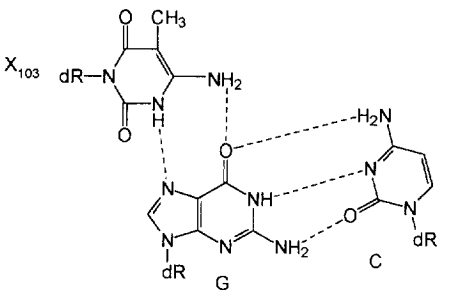


Figure 112.

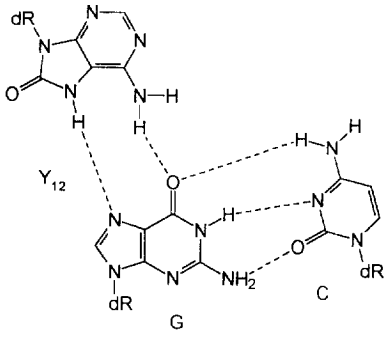
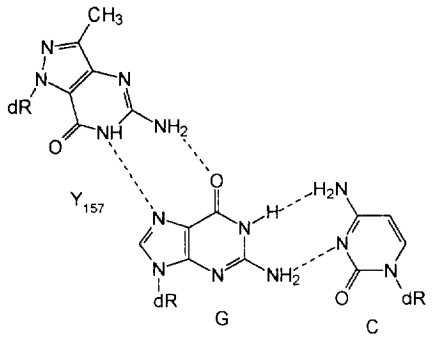
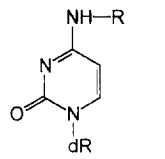


Figure 114.



sequence	binding affinity
$TTTT^{Me}CT^{Me}CT^{Me}CT^{Me}CT^{Me}CT$	$3.10^9\ M^{-1}$
$TTTTTY_{157}TY_{157}TY_{157}TY_{157}TY_{157}T$	$10^5\ M^{-1}$
$TTTT^{Me}CTTTT^{Me}C^{Me}C^{Me}C^{Me}C^{Me}C^{Me}CT$	$5\ 10^4\ M^{-1}$
$TTTT^{Me}CTTTTY_{157}Y_{157}Y_{157}Y_{157}Y_{157}Y_{157}T$	$4\ 10^9\ M^{-1}$

Figure 115.



X_{128} R : $-(CH_2)_2-COOH$

X_{59} R : $-(CH_2)_4-NH_2$

X_{129} R : $-(CH_2)_4-CH_3$

Figure 116.

using base analogues which possess suitable hydrogen-bond donating groups, capable of interacting with guanine of GC pairs [453].

2′-O-Methylpseudoisocytidine (X_{124}) may substitute for 2′-deoxycytidine for Hoogsteen type base pairing with guanine in neutral and basic conditions [197, 455].

The 2′-methoxy substituent stabilizes triplex formation. With the sequence 5′-$TTX_{124}TTX_{124}TT$-3′, two transitions were observed (T_m = 12 °C and 42 °C) between pH 7.0 and 8.7 while this was not the case with dCyd and 2′-O-methylcytidine [455]. 2′-O-Methylpseudoisocytidine forms, likewise, more stable triplexes than 5-methyl-2′-deoxycytidine [197]. Pseudoisocytosine is, in fact, tautomerically ambiguous and may occur as well as 'dda' as 'daa' system. This is not the case for the 2′-O-methyl pyrazine nucleoside (X_{125}) which occurs as 'dda' system and is able to form pH-independent triple helixes (pH range 6.3 to 8.0) [456].

The 6-ketoderivative of 5-methyl-2′-deoxycytidine (X_{103}) in the parallel stranded recognition motif, recognize GC base pairs in a largely pH independent manner (pH 6.5-8.5) [201]. It can be considered as an analog of an N^3 protonated cytosine derivative. At low pH, the cytosine and 5-methylcytosine base give more stable triplexes than X_{103}. At high pH (pH 8.0-8.5), the 4-amino-5-methyl-[1H,3H]-pyrimidin-2,6-dione base X_{103} is more stabilizing than C and 5-MeC. The modified base X_{103} is uncharged and charged H-bonding partners result in enhanced complex stability [201].

A similar study was undertaken using X_{126} and X_{127} opposite GC base pairs. Both nucleoside analogues results in pH independent (pH 6-8) triple helix formation [202]. The triplexes are less stable than the C.GC triplex at low pH (pH 6) but more stable at neutral and slightly basic pH (pH 7-8). The CH_3-group (X_{126}) has a slight destabilizing effect in the case of complementary GC base pairs and a stabilizing effect in the case of mismatch triplexes (AT, CG, TA) [202] (it should be mentioned that incorporation of 2′-O-CH_3 derivatives of 5-methyl-2′-deoxycytidine decreases triple helix stability [444]).

These results were explained by the authors as caused by weaker base stacking by the more electron-poor heterocycle of the 6-oxocytosine base. An effect, which is not compensated by more stable hydrogen bonding because of the electron-withdrawing oxo-group (this effect should increase the acidity of N^3 – H and 4-NH_2 group and stabilize H-bonding to guanine) [202].

The triple helix formation, however, is restricted to certain sequences. A DNA duplex containing a series of five contiguous GC base pairs can not be effectively targeted with either 5-methyl-2′-deoxycytidine or X_{103} [457]. In the first case, this is due to charge-charge

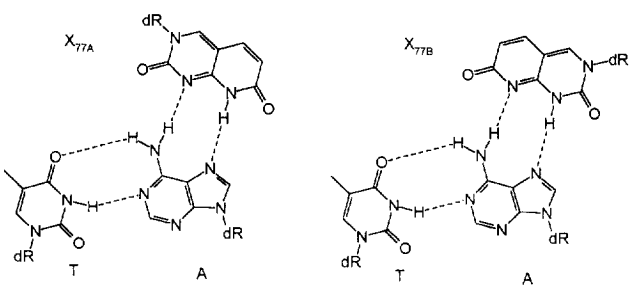
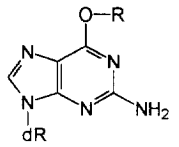


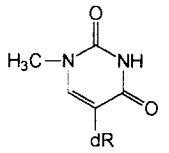
Figure 117.



Y_{158} R = -$(CH_2)_6$-O-psoralene

Y_{159} R = -$(CH_2)_5$NH-acridine

Figure 118.



X_{94}

Figure 120.

repulsion effects. In the latter case, it might be due to ineffective base stacking or undesirable steric effects of the carbonyl group at C-6 [457].

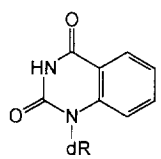
N^7-Glycosylated guanine base may be considered as a non-natural nucleoside with H-bonding functionalities similar to protonated cytosine [458, 459]. It does not fit in an antiparallel oriented motif [458], but Y_{126} binds to GC with affinity equal to the ^{Me}C.GC triplet in a parallel motif [Y_{126}.GC: 1.6 10^8; ^{Me}C.GC: 1.4 10^8; T.AT 2.1 10^8]. N^7-Glycosylated guanine binds to GC base pairs with both high affinity and sequence specificity.

Thus, by replacing G by N^7-glycosylated.G in the G.GC triplet, the third strand orientation is reversed and becomes parallel to the purine Watson–Crick strand [460]. The glycosylation of the guanine at N7 position permits it to adopt a conformation such that the Hoogsteen face of the base mimics the arrangement of hydrogen bond donors seen in protonated cytosine [461]. At neutral pH, the effect on incorporation of Y_{126} is sequence dependent. The sequence $(T)_5(Y_{126}T)_5T$, binds with three times lower affinity to its target than $(T)_5(^{Me}CT)_5T$. The sequence $(T)4^{Me}C(T)_4(Y_{126})_6T$ binds five times stronger than $(T)4^{Me}C(T)_4(^{Me}C)_6T$ [460]. This is explained by the lack of structural isomorphism of the Y_{126}.GC and T.AT triplets. The structural difference between AG and GA junctions and contiguous A or G sequences could explain the above observations [460]. This might broaden the accessible sequences for triple helix formation. At neutral pH, ^{Me}C might be used to bind isolated GC base pairs and Y_{126} to bind contiguous GC base pairs [460]. Contiguous ^{Me}C bases reduce triplex stability due to electrostatic repulsion between adjacent protonated ^{Me}C bases.

The nucleoside analog 7-(2′-deoxy-β-D-ribofuranosyl) hypoxanthine Y_{127} can recognize a G-C base pair with high selectivity and equal as slightly enhance efficiency compared to 5-methylcytidine at pH 7.0. The stabilizing interaction most likely relies on base-pairing of hypoxanthine to guanine via one H-bond [462].

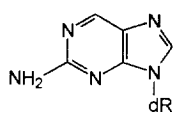
8-Oxo-2′-deoxyadenosine (Y_{12}) can be considered as an analogue of protonated 2′-deoxycytosine and oligonucleotides containing this modified base binds to double stranded DNA in a pH independent manner [454, 463]. 8-Oxo-2′-deoxyadenosine binds to GC base pairs with two hydrogen bonds and to the UA base pair with one hydrogen bond (between N^6-exocyclic amino group of 8-oxoadenine and the O^4 of uracil) [454]. The CD spectra of these triplexes are almost identical to those formed by the ^{Me}C.GC triplets [454]. It does not form triplexes with the AT and TA base pair [454]. Above pH 7.4, Y_{12} binds tighter to dsDNA compared to dCyd. The lower affinity of Y_{12} compared to dCyd at a pH below 7.4 is attributed to a less ideal geometry of the hydrogen bonding pattern. The stability of the triplex, however, is also dependent of the GC to AT base pair ratio and on the experimental conditions [463].

3-Methyl-5-amino-1H-pyrazolo[4,3-d]pyrimidin-7-one nucleoside (Y_{157}) mimics protonated cytosine. It binds to GC base pairs with no requirements for protonation [464, 465] and as selectively and strongly as 2'-deoxycytidine itself (over an extended pH range). The order of duplex



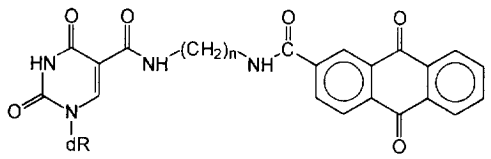
X_{104}

Figure 119.



Y_8

Figure 121.



sequence $Z(^{Me}CT)_8$

Z	Tm of triplex
T	36 °C
X_{130} (n = 2)	39 °C
X_{131} (n = 6)	41 °C

Figure 122.

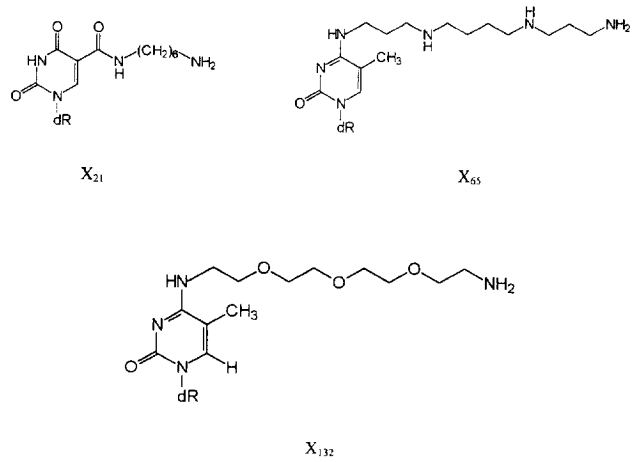


Figure 123.

recognition is Y_{157}.GC > Y_{157}.CG > Y_{157}.AT > Y_{157}.TA. The modified base Y_{157} enables the sequence-specific binding of dsDNA sites containing six contiguous GC base pairs at physiological pH [464]. This modified base, thus, also expands the sequence repertoire which may be targeted by triple helixes [465].

The difference in binding affinity of the modified oligomers for their dsDNA target, is attributed to the lack of structural isomorphism in the Y_{157}.GC and TAT triplets (while the T.AT and ^{Me}C.GC triplets are structurally isomorphous) and to repulsion between adjacent protonated ^{Me}C bases. The 5′-AG-3′ and 5′-GA-3′ junctions generate distortion in the backbone in triple helical complexes relative to contiguous A or G sequences [465].

By using the N^4-substituted C analogues (X_{59}, X_{128}, X_{129}), instead of 2′-deoxycytidine itself (GC recognition), the triplex stability decreases considerably. This is attributed to steric interactions between the side chain and major groove substituents and/or the different protonation degree at pH 7.0 [466].

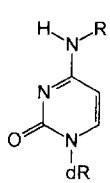
The 2,7-dioxopyrido[5,6-d]pyrimidine nucleoside (X_{77}) recognize with high selectivity AT base pairs within triple helical complexes [168, 467]. The X_{77}.AT triplet appears to be somewhat less stable than the T.AT triplet. This becomes more pronounced when a larger percentage of T residues are replaced by X_{77}. X_{77} May occur in two tautomeric forms and it may be possible that the duplex base pair may have an influence on the tautomeric equilibrium [168, 467]. This suggests that the predominant form of X_{77} in a parallel binding motif may be X_{77A} and in an antiparallel binding motif may be X_{77B}. The tautomer X_{77B} could only recognize TA in a parallel motif only after a backbone shift that permit wobble base pairing [168].

O^6-Modified 2′-deoxyguanosine (Y_{158}, Y_{159}) may possibly be used to recognize an AT base pair (Y_{158}/Y_{159}.AT) in a parallel motif or to recognize a TA base pair (Y_{158}/Y_{159}.TA) within a purine tract [468].

The replacement of T with 1-(2-deoxy-β-D-erythropentofuranosyl)quinazoline-2,4(3H)-dione (X_{104}) decreases the stability of triple helix formation in both the parallel and antiparallel motif [203, 439].

Introduction of one X_{104}, giving a X_{104}.AT triplet, in the PyPu.Py or PyPu.Pu motif, led to a ΔT_m of approximately − 1 °C to − 2 °C. Consecutive substitution of a second X_{104} (in the PyPu.Py motif) led to a more pronounced destabilization than when second X_{104} is introduced at a more remote site [203].

The third strand T residue in both parallel and antiparallel motifs are normally in 'anti' glycosidic conformation. This T has been replaced by N^1-methyl-2′-deoxypseudouridine (X_{94}). However, it was found to be not a good substitute for T [469]. In a parallel motif (for example $TX_{94}TTTX_{94}TTX_{94}TX_{94}TTT$), the presence of



X_{63} R = -$(CH_2)_3$-CH_3

X_{64} R = -$(CH_2)_3$-COOH

X_{133} R = -$(CH_2)_3$-NH_2

X_{134} R = -$(CH_2)_3$-NH-CO-CH_3

Figure 124.

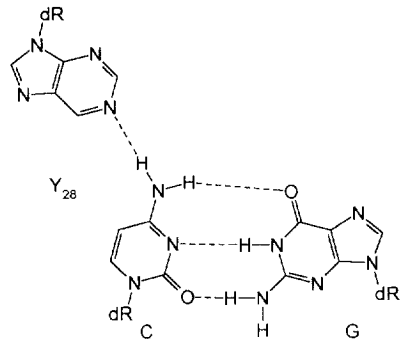


Figure 125.

X_{94} decreases triple helix stability. In an antiparallel motif ($GGGTTGGX_{94}TGGTTGG$) no improvement of stability in comparison with T was observed [469].

2-Aminopurine (Y_8).AT and G.GC triplets are considered as isomorphous (Pu.PuPy motif), just as T.AT and C^+.GC (Py.PuPy motif) [470].

The stability of triplexes may be slightly increased by substituting T for dUrd in which the 5-position is substituted with anthraquinone via a linker. As well the nature of the linker as the sequence itself is important for stable triplex formation [54].

Triple helix formation may also be stabilized by using aminoalkyl substituted dUrd (X_{21}) [54] and dCyd (X_{65}) [140, 471].

Spermine conjugated at the N^4 position of 5-methyl-2'-deoxycytidine show decreased triplex stability at low pH (pH 6.0) but increased stability at neutral pH (pH 7.3) [140, 471]. The stability order of the triplets are X_{65}.GC (X_{65}.AT > X_{65}.CG > X_{65}.TA. Using spermine modified oligomers, N^3-protonation of cytosine base is not required for triplex formation [471] and triplex formation is observed even in the absence of $MgCl_2$ (although Mg^{2+} still has a favourable effect on complex formation). The pH effect of spermine-oligonucleotides is opposite to that seen for normal triplexes containing 5-methyl-2'-deoxycytidine i.e. optimum triplex stability at physiological pH and low salt concentration and moderate destabilization at acidic pH [140, 471]. The triplex stability is lowered when increasing the degree of spermine substitution [140], most probably due to electrostatic repulsion between spermine chain or between spermine and protonated cytosine bases. The factors leading to triplex stabilization when using spermine conjugated nucleosides have been described extensively [471]. In summary they may be allocated to: (a) intra-/interstrand electrostatic phosphate neutralization; (b) less entropic changes

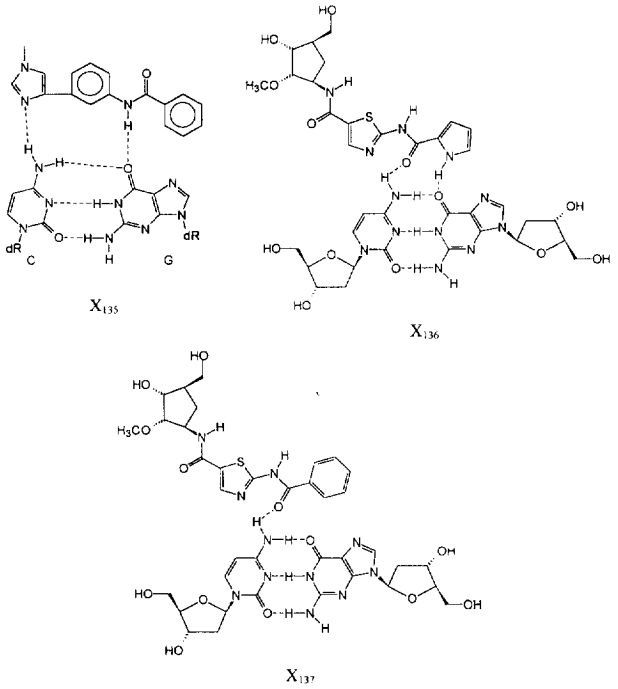


Figure 126.

from counter-ion effects during melting; (c) a more favoured association/dissociation equilibrium; (d) stabilization by additional hydrogen bonding interactions of spermine with complementary or adjacent bases; and not to an altered pK_a of the N-3 of the cytosine base.

Tetraethyleneoxyglycolamine conjugation at N^4 position of 5-methyl-2'-deoxycytidine (X_{132}) shows similar behaviour as spermine conjugated nucleosides, i.e. optimum triplex stability at physiological pH and destabilization at acidic pH [472]. However, X_{132} destabilizes the triplexes to a lesser extent as the spermine conjugated ODN because the presence of a single protonated amino function at terminus only slightly disfavours N-3 protonation due to electrostatic repulsion in contrast with spermine conjugated nucleosides where multiple protonation of the spermine side chain causes a much lower pK_a of N-3. The authors stated that tetraethyleneoxyglycolamine (X_{132}) conjugated ODN stabilizes the triplex through a fine tuning of the microenvironment by the polyether side chain in terms of hydrophobic desolvation [472].

Several approaches are based rather on shape-selective recognition than specific H-bonding interactions [473–475]. The N^4-(3-aminopropyl)-2'-deoxycytidine (X_{133}) and N^4-(3-acetamidopropyl)-2'-deoxycytidine (X_{134}) nucleoside allow either the 3-aminogroup hydro-

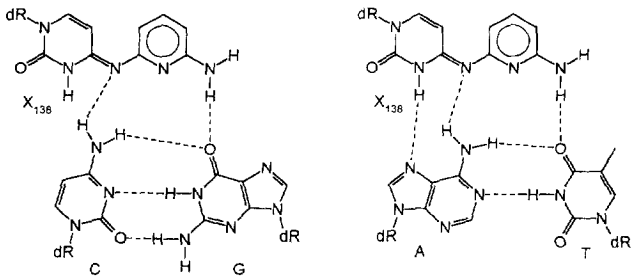


Figure 127.

gen or the amide hydrogen to form hydrogen bond to the O^6-carbonyl group of the guanine base. These modified nucleosides recognize selective CG base pairs, which is not the case with N^4-(butyl)-2′-deoxycytidine (X_{63}) and N^4-(carboxypropyl)-2′-deoxycytidine (X_{64}) [473].

At pH 7.4, the stability of the nebularine (Y_{28}) containing triplets decrease in the order Y_{28}.CG ~ Y_{28}.AT > > Y_{28}.GC ~ Y_{28}.TA. The stability of the Y_{28}.CG triplet is comparable in strength with the T.CG triplet and less stable than the A.AT, T.AT and G.GC combination [474].

At physiological pH, the triplet stability with 4-(3-benzamidophenyl)imidazole decreases in the order X_{135}.TA ~ X_{135}.CG > X_{135}.AT > X_{135}.GC. So, it has a preference for PyPu base pairs over PuPy motifs [474]. As stated by the authors, the incorporation of this modified nucleoside was based on following rationale: (i) a compound that should sterically match the edges of PyPu base pairs in the major groove; (ii) to position hydrogen bond donors and acceptors in such a way as to form hydrogen bonds in the major groove with CG or TA base pairs; (iii) to maintain a backbone geometry compatible with the Py.PuPy triple helix motif; iv) to allow energetically favorable stacking of bases in the third strand. The modified nucleoside may span both strands of the Watson–Crick CG base pair and form two hydrogen bonds.

A solution structure determined by NMR of a pyrimidine.purine.pyrimidine triplex containing X_{137} in the third strand showed that the observed selectivity for py-pu base pairs is due to selective intercalation of the nucleobase of X_{137} into the WC base pairs of the DNA duplex [476]. The nucleobases of X_{136} and X_{137} possess a system of aromatic rings fused by amide bonds comparable to the nucleobase X_{135} and exhibit the same selectivity pattern for py-pu over pu-py base pairs suggesting that these compounds share a common binding mode of sequence-specific intercalation [477].

The N-benzoylated nucleoside analog of X_{138} also showed a similar selectivity pattern as X_{135}, X_{136} and X_{137} but N^4-(6-aminopyridinyl)-2′-deoxycytidine X_{138} has a preference for AT and CG recognition (over TA and GC) [478]. The order of stability at pH 7 is X_{138}.CG > X_{138}.AT > X_{138}.TA > X_{138}.GC. It may be useful for CG interuption of polypurine tracts [479]. The removal of the 6-amino group eliminates triplex formation while removal of the pyridine nitrogen atom diminishes the stability of the duplex [479].

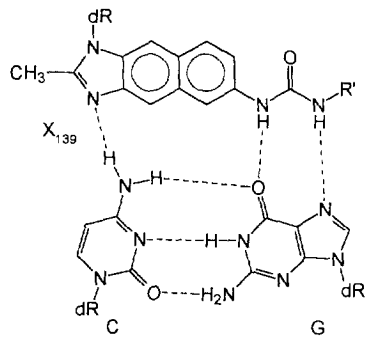


Figure 128.

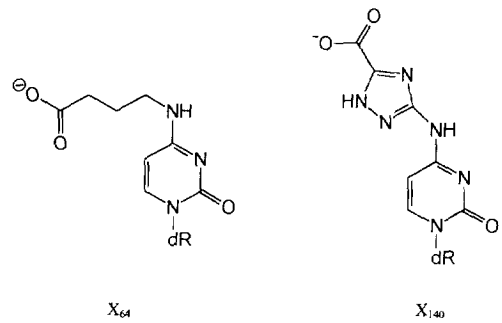
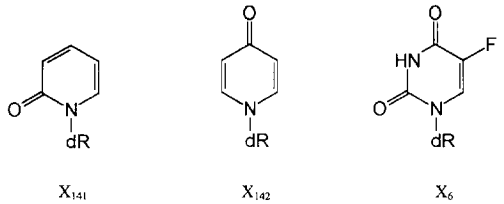


Figure 129.



X_{141} X_{142} X_6

Figure 130.

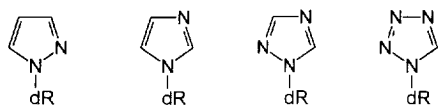
The higher stability of X_{138}.AT relative to that of T.AT (2H bonds) suggest the appearance of the unusual imino-tautomeric form of X_{138} in the base triads [478]. The pyridinyl group may contribute to triplex formation via hydrophobic interactions with neightbouring bases. The 6-amino-2-pyridinyl group spans the major groove of the target duplex and forms a hydrogen bond with the O^6 of guanine and, probably, an additional hydrogen bond between the N^4 of the imino-tautomer of X_{138} and the N^4-amino group of cytosine [479].

X_{139} binds a CG base pair in an organic solvent by spanning both bases [480].

The modified bases X_{64} and X_{140} were designed to recognize pyrimidine interruptions in an otherwise homopurine strand. It belongs to the strategies to employ modified nucleosides which can contact both bases of a py-pu interuption [481]. The NH may function as H-bond donor for interaction with the O^4-carbonyl group of T, while the carboxylate group may be the H-bond acceptor for the N^6-amino hydrogen of the adenine base in the TA base pair.

The 3-carboxypropyl nucleoside (X_{64}) recognizes TA base pairs and GC base pairs (but not UA, CG, AT). In MOPS buffer, triplex formation is only observed with GC and not with TA. The 5-carboxytriazole nucleoside (X_{140}) is less selective than X_{64} and forms triplexes with TA, UA and CG [481].

The difference between TA and UA recognition has been explained by the authors as due to the observation that the 5-methyl group (of T) may face the base slightly out and across the major groove to avoid steric clashes. This could result in a better positioning of the carboxylate



X_{143} X_{144} X_{145} X_{146}

Figure 131.

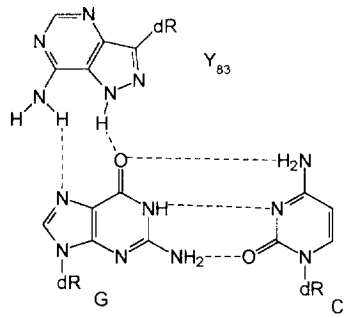


Figure 132.

group of X_{64} for hydrogen bonding with the N^6-amino group of adenine of the TA base pair [481].

'CG' inversions are sites where a cytosine base is present in an otherwise homopurine sequence. G and A do not accomodate at CG inversions because the large purine in the third strand may lead to steric clash with the cytosine in the duplex [482]. The steric problem is reduced by using pyrimidine nucleosides and the thymine base accomodate best. The cytosine base give no binding at all. As well the methyl group as the 4-carbonyl group of T contribute to the T.CG interactions, which is demonstrated by studying the binding of pyridin-2-one (X_{141}), pyridin-4-one (X_{142}) and dUrd [482].

The drawback of using thymine in the third strand is the loss of sequence specificity because the relative affinity of T for an AT and CG base pair is similar [482]. Several other 5-substituted pyrimidine analogues were tested for binding to CG inversions and to AT base pairs. The order of stability is 5-F > 5-CH_3 > 5-I > 5-Br > 5-H. Thus 5-FdUrd (X_6) enhances triplex formation as well at CG inversions as at AT base pairs.

Several azole nucleosides ($X_{143} - X_{146}$) have been investigated for antiparallel triplex formation at CG or TA

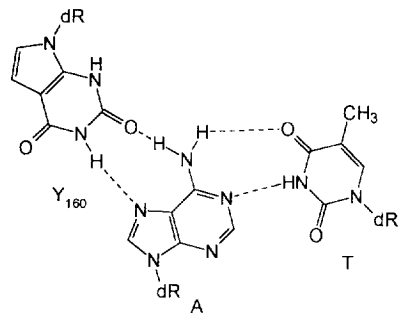
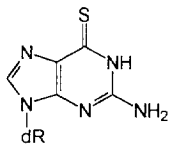


Figure 133.



Y_{51}

sequence a) GGGTGGGTGGTGGG

sequence b) $GY_{51}GTGY_{51}GTY_{51}GTGY_{51}G$

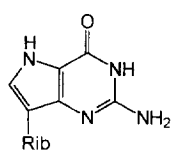
sequence c) $GGGY_{160}GGGY_{160}GGY_{160}GGG$

	affinity order of sequences [487]				
normal affinity	c	>	a	>	b
in presence of Na^+	b	≃	a	>	c
in presence of K^+	b	>>	c	≃	a

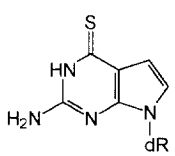
Figure 134.

interuptions. The azole nucleosides considerably enhance triplex formation, particularly at TA inversion sites [483]. They bind at least 10^2- to 10^3-fold more tightly to TA than natural nucleosides. The association constants, however, are still 10-30 times less than those of G.GC and T.AT [483]. The imidazole (X_{144}) and tetrazole (X_{146}) containing oligomers bind better to CG base pairs than triazole nucleosides (X_{145}). Pyrazole nucleosides (X_{143}) do not bind to CG base pairs [483]. The pyrazole nucleoside binds TA four times stronger than GC. Apart from binding to CG, the imidazole (X_{144}), triazole (X_{145}) and tetrazole nucleosides (X_{146}) also bind to GC and TA, but not to TA base pairs [483]. However, imidazole does not distinguish between TA and CG inversions and are not useful in triple helixes with closely spaced sites of pyrimidine/purine base pair inversions in the target duplex [486].

Another solution to the binding problem at the CG inversion site is to alter the position of the two hydrogen bond donors of guanine relative to the glycosylation site



Y_{161}



Y_{162}

Figure 135.

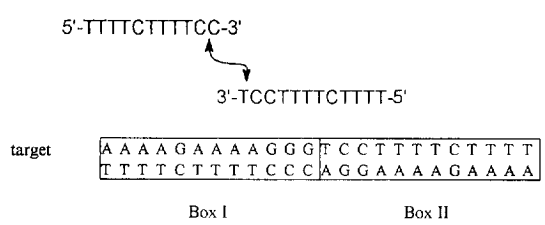
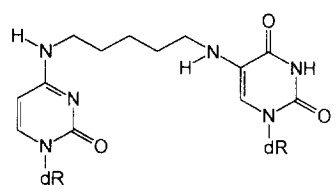


Figure 136.

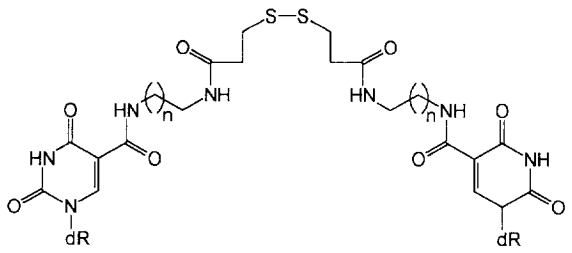
[484]. By using 2'-deoxyformycin A (Y_{83}) in an antiparallel triple helix, H-bonding may occur (with guanine base) at the 'other side' of the major groove. Introduction of 3 times compound Y_{83} in a 36 mer, results in a ten times increase in binding affinity as compared to its unmodified counterpart [484].

7-Deaza-2'-deoxyxanthosine (Y_{160}) can be considered as a purine analogue of the thymine base and, thus, potentially able to form Y_{160}:AT triplets, with the triple strand in an antiparallel orientation with respect to the polypurine strand of the target DNA [485]. The removal of the hydrogen bond acceptor at N-7 eliminates the ability of the oligomer to form G-quartets. Moreover, the replacement of the N-7 of xanthosine with carbon increases the pK_a to 7.3 (xanthine: $pK_a = 5.3$) which potentially allows triplet formation at physiological pH (xanthosine is negatively charged at neutral pH). Three other arguments were given by the authors [485] to demonstrate the logic of their research: (1) Y_{160} may decrease the propensity of the oligonucleotide to form secondary structure mediated by G quartets because the neighboring T's are known to enhance the stability of G quartets and other bases not; (2) oligomers containing only purines could have better binding characteristics due to the more regular geometry of the third strand; (3) as a homopurine strand places the third strand phosphodiester backbone in the middle of the major groove (and thus further away from the negative charges of the duplex than the T's in the GT oligos), a decrease in repulsive forces between the phosphodiester backbone in the third strand and phosphodiester backbone in the duplex may be expected.



X_{147}

Figure 137.



X_{148}

n = 1, 2, 3, 6

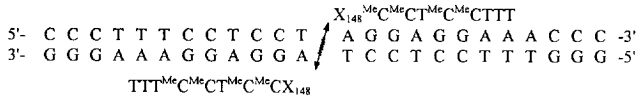
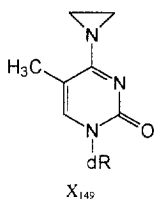


Figure 138.

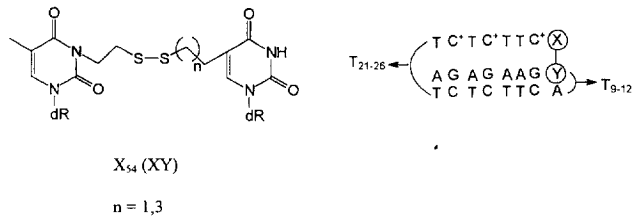
Substitution of 7-deaza-2′-deoxyxanthosine (Y_{160}) for T in a G/T oligomer indeed causes a greater than 100-fold increase in affinity [485]. When G is replaced by 7-deaza-2′-deoxyguanosine in a G/T or G/Y_{160} oligomer, a significantly lower affinity of the triple strand is observed [485], attributed to electronic effects.

Guanine-rich oligonucleotides form intramolecular tetrads which coordinate around a potassium ion. This makes G-rich sequences unavailable for intermolecular triplex formation [486]. Using modified nucleosides such as 7-deaza-2′-deoxyxanthosine (Y_{160}) and 6-thioguanine (Y_{51}), triplet formation should be facilitated due to resistance against guanine-quartet-mediated aggregation. In the absence of potassium, oligonucleotides containing Y_{160} (in place of T), T or 6-thioguanine (Y_{51}) have similar binding affinity for the duplex DNA [487]. Triplex formation with Y_{160} and T was abolished in the presence of potassium ions [487]. [Incorporation of Y_{160} into less guanine rich oligos, has been shown to stabilize triplexes in the presence of physiological K^+ concentrations [487].] Incorporation of 2′-deoxy-6-thioguanine (Y_{51}) into



X_{149}

Figure 139.



X_{54} (XY)

n = 1,3

Figure 140.

G-rich oligos inhibit G-tetrad formation and indeed facilitates triplex formation in the presence of potassium ions [486–488]. Triplexes involving oligonucleotides containing four 6-thioguanine bases substituted for guanine resist K^+ inhibition [487]. The overall binding affinity, however, is reduced as Y_{51}.GC triplets are weaker than G.GC triplets.

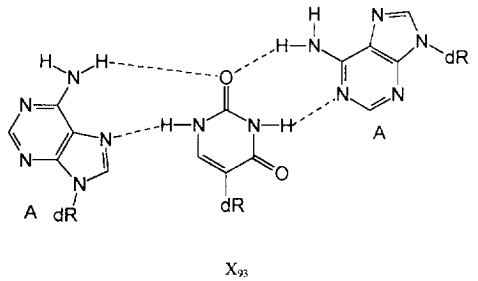
9-Deazaguanosine (Y_{161}), as substitute for G in preventing the formation of G-tetrads in G-rich oligonucleotides, reduces the strength of triple helix formation [489].

Neither 2′-deoxy-9-deazaguanosine (Y_{161}) nor 2′-deoxy-7-deaza-6-thioguanosine (Y_{162}) improve the binding affinity to form triple helixes when compared to the unmodified oligomers [490].

The 'switched triple helix' formation requires that the third strand oligonucleotides (of the triplex) are joined together via a linker between the two terminal bases with either a 3′-3′ or a 5′-5′ polarity [491].

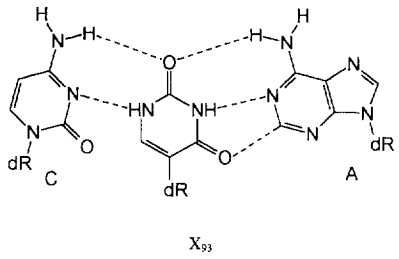
When using X_{147} as the junction, the switched-oligo forms a triple helix which exhibits a higher T_m than each of the separate oligonucleotides (ΔT_m = 15 °C, at pH 7.0). The thermal stability, however, of this triple helix is still lower (ΔT_m = 10 °C) than that of the full length 24 mer triple helix (in wich the pu-py sequence in Box II is reversed) [491].

The T_m's (± 30 °C) of 5′-5′-linked ODN's, using X_{148} as the junction, were significantly higher than those of



X_{93}

Figure 141.



X_{93}

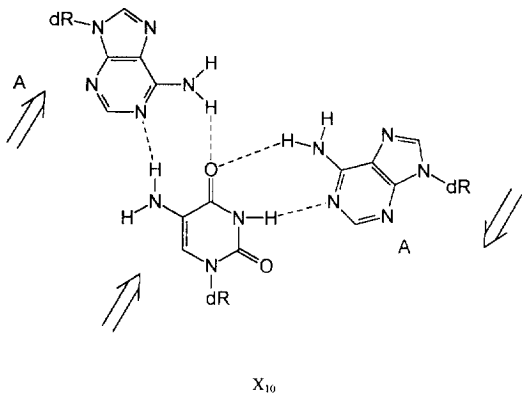
Figure 142.

unlinked ODN's (± 17 °C). The 5′-5′-linked ODN with the shortest linker group, formed the most stable triple helix [492].

The efficiency of the antigene approach may be increased by using modified bases which may cross-link with the target dsDNA. N^4,N^4-Ethano-5-methyl-2′-deoxycytidine (X_{149}) is an efficient cross-linking nucleosides, binding to the N^7 of a guanine base in the double-stranded DNA target [493].

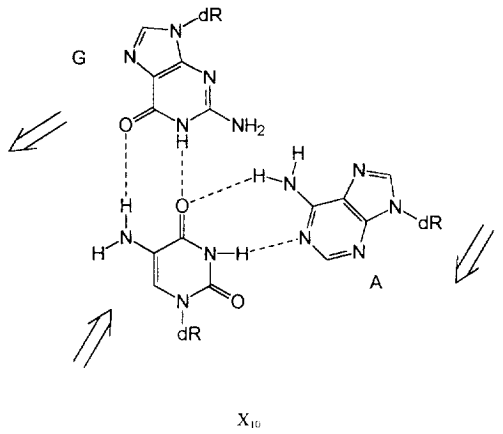
The stability of a triple helix structure was increased by introduction of a disulfide cross-link [494]. Therefore a nucleoside-dimer having a N^3-thioethylthymidine at the 3′-end of the Hoogsteen strand and a C^5-thioethyl-2′-deoxyuridine (X_{54}) of the opposite site in the Watson–Crick purine strand was introduced in the oligomer. This resulted in a monophasic thermal denaturation and a stabilization of the complex by ~ 40 °C [495]. CD and NMR measurements indicate that the cross-link does not significantly perturb the native helical geometry [496].

Several modified nucleosides were incorporated in the central strand of a DNA triplet (inverted motifs). This may be used to target single stranded DNA and RNA



X_{10}

Figure 143.



X_{10}

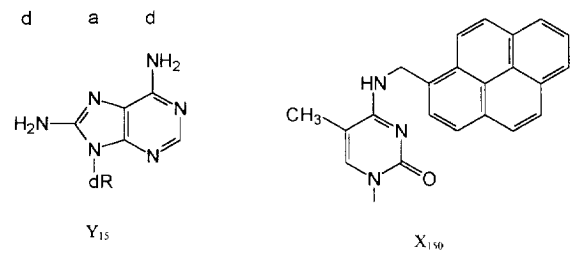
Figure 144.

sequences by the addition of two complementary oligonucleotides. Dual recognition of a modified (central) nucleoside may occur when both sites of the heterocycle are appropriately derivatized and 2′-deoxy-pseudouridine (X_{93}) is a prime candidate for this function. The A.X_{93}A motif gives more stable triplexes than the A.UA or T.AT triads [497]. Likewise the C.X_{93}A triplet is more stable than C.UA.

This means that a natural homopyrimidine third strand can bind to a mixed pyrimidine-purine duplex when the pyrimidine residues in the second strand contain an addition H-bonding site [498].

5-Amino-2′-deoxyuridine (X_{10}) may function as the central base in an A.X_{10}. A triad (parallel A containing Hoogsteen strand) and in an G.X_{10}.A triad (with an antiparallel oriented G) [499]. The 5-amino group may also contribute to stabilization by participation in the hydration network.

8-Amino-2′-deoxyadenosine (Y_{15}) triads of the T.Y_{15}.T type may be of the Hoogsteen–Watson–Crick or the reverse Hoogsteen–Watson–Crick type [12].



Y_{15} X_{150}

Figure 145.

The insertion of 2′-deoxy-5-methyl-N^4-(1-pyrenylmethyl)cytidine derivative X_{150} into a triplex forming oligonucleotide increases the stabilities of the corresponding triplexes [500].

5. Conclusions

Several ingenious strategies for the incorporation of modified nucleobases in oligonucleotides have been developed during the last decennia. However, incorporation of a new heterocyclic base often remains a synthetic challenge due to incompatibilities between reactivity and stability of the base and protection/deprotection strategies in oligonucleotide synthesis. As demonstrated in this review, several hundreds of modified bases have already been incorporated and this field of research may be considered as one of the most extensively studied in the nucleic acid field. The incorporation of modified bases and the study of their pairing behaviour has been carried out with the aim of increasing duplex stability, expanding the genetic alphabet, introduction of reporter groups, to study the correlations of changes induced by mutated nucleosides on the function and the structure of nucleic acids, to study protein-nucleic acids interactions, to study the origin of life, etc.

Modified nucleosides were also introduced in triple helix forming oligomers with the purpose to increase binding strength, binding kinetics and to develop a general method for recognition of mixed sequences containing all four base pairs of duplex DNA. The hybridization properties of the studied modified oligomers depend on the structure of the modified nucleoside, the number of modifications, the selected sequence and the location of the modified nucleoside along the sequence. Most available information is based on melting point determination, sometimes sustained by CD experiment and, only in a few cases on detailed structural analysis by X-ray and NMR spectrometry. This lack of detailed information prevents a profound analysis of the influence of modified bases on the stability of duplexes and triplexes and makes it difficult to put forward general rules for predicting the modified nucleic acids structure. Much work still has to be done. From this point of view it is a pity that most material is not available in sufficient amounts to carry out these structural analysis. On the other hand, the research on incorporation of modified nucleosides in oligonucleotides has given us new oligomeric constructs with improved properties (hybridization stability, enzymatic stability, base pairing selectivity) which open a new world for efficient control of transcription and translation processes.

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