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The ups and downs of nucleic acid duplex stability: structure–stability studies on chemically-modified DNA:RNA duplexes

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ABSTRACT

In an effort to discover novel oligonucleotide modifications for antisense therapeutics, we have prepared oligodeoxyribonucleotides containing more than 200 different modifications and measured their affinities for complementary RNA. These include modifications to the heterocyclic bases, the deoxy-ribose sugar and the phosphodiester linkage. From these results, we have been able to determine structure–activity relationships that correlate hybridization affinity with changes in oligonucleotide structure. Data for oligonucleotides containing modified pyrimidine nucleotides are presented. In general, modifications that resulted in the most stable duplexes contained a heteroatom at the 2′-position of the sugar. Other sugar modifications usually led to diminished hybrid stability. Most backbone modifications that led to improved hybridization restricted backbone mobility and resulted in an A-type sugar pucker for the residue 5′ to the modified internucleotide linkage. Among the heterocycles, C-5-substituted pyrimidines stood out as substantially increasing duplex stability.

INTRODUCTION

The high affinity and specificity of Watson–Crick hybridization has made oligonucleotides attractive agents for diagnostic and therapeutic applications. Although unmodified DNA oligonucleotides have been reported to demonstrate antisense activity in cell assays, much research has been devoted to the discovery of modified oligonucleotides as antisense therapeutics (1). The primary goal of these modifications has been to improve biostability and cellular uptake of the oligonucleotides and to optimize tissue and cell distribution for a particular molecular target. It is important, however, that modified oligonucleotides maintain the hybridization characteristics of unmodified DNA. The mechanism of action of antisense oligonucleotides requires specific hybridization of the oligonucleotide at its complementary site on the mRNA. The importance of hybridization is demonstrated by the correlation of antisense activity observed in cell assays (2–5) and *in vivo* (6) with hybridization affinity and T_M. Described below is a strategy using six test sequences for evaluation of hybridization properties of chemically-modified oligonucleotides to RNA complement. Over 200 modifications were tested as part of our antisense drug discovery effort. The behavior of these modifications in this screening system will be discussed.

METHODS

Strategy for evaluation of oligonucleotide modifications

To maximize the number of oligonucleotide modifications that can be prepared and evaluated for utility in antisense applications, we adopted a two-phase strategy. In the first phase, only the modified T nucleoside was prepared as a 5′-*O*-DMT-protected phosphoramidite and a series of oligonucleotides containing only modified thymidines was prepared. Alternatively, if the modification was in the phosphodiester backbone, a T-T dimer containing the modified backbone between two thymidine residues was prepared. Block coupling of these modified dimers resulted in oligonucleotides with backbone modifications between consecutive T residues. Hybridization and nuclease resistance properties for this series of oligonucleotides with modifications only at the T residues or T-T linkages were evaluated *in vitro*. Only if the hybridization affinity, hybridization specificity and nuclease resistance of these modified oligonucleotides met some minimum requirements, modified amidites were prepared for the other nucleobases and the modification was incorporated into antisense oligonucleotides for testing in a cellular assay. This strategy has proved effective. Usually, synthesis of the modified T nucleoside phosphoramidite required fewer steps than the corresponding A, C or G amidites so the initial evaluation could be made rapidly. In general, hybridization properties of oligonucleotides which contain modifications only on a single nucleobase have been predictive of properties for uniformly modified oligonucleotides or ‘gapmers’ which contain a stretch of DNA flanked by regions of modification (3,7,8). More important, modifications that bind weakly to complementary RNA in this series have not demonstrated good antisense activity (K. H. Altmann, B. Monia and N. Dean, unpublished results). Thus preliminary evaluation of hybridization using only modified thymidines has been predictive of the value of a modification for antisense applications.

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Table 1. Sequences containing modified thymidine used for hybridization studies

sequence number	modified sequence[1]	# of modifications	
		T nucleosides	T-T dimers
seq1	tCCAGGtGtCCCGCAtC	4	NA
seq2	CTCGTACCtTTCCGGTCC or CTCGTACCt-tTCCGGTCC	1	1
seq3	CTCGTACttttCCGGTCC or CTCGTACt-tt-tCCGGTCC	4	2
seq4	GCGttttttttttGCG or GCGt-tt-tt-tt-tt-tGCG	10	5
seq5	TTTTtCTCTCTCTCT or TTTt-tCTCTCTCTCT	1	1
seq6[2]	tttttctctctctcT or t-tt-tt-ct-ct-ct-ct-cT	14	7

[1]t represents a modified thymidine nucleoside. t-t represents a TT dimer in which the phosphodiester linkage between the thymidines has been modified.
[2]This sequence contains modified thymidine and deoxycytidine residues.

Table 2. Effect of 5-methyl pyrimidine substitution on T_M

T dC dU 5-methyl dC

mod #		ΔT_M per mod (parent is DNA)					
		seq1	***seq2***	***seq3***	***seq4***	***seq5***	***seq6***
	PARENT FOR ALL MODIFICATIONS						
	T(dC)	0.0 (62.3)[1]	0.0 (63.3)[1]	0.0 (61.8)[1]	0.0 (50.2)[1]	0.0 (52.7)[1]	0.0 (52.7)[1]
	HETEROCYCLE MODIFICATIONS						
(1)	U(dC)	-0.6	-3.2	-0.2	-0.4	+0.1	-0.3
(2)	U(^{me}dC)						+0.0
(3)	T(^{me}dC)						+1.1

[1]Values in parentheses are the T_M (°C) for the unmodified DNA with its RNA complement.

Hybridization was evaluated using absorbance versus temperature profiles. The technique required only 2–4 OD units of modified oligonucleotide and reproducible results were easily obtained. Although T_M can be precisely measured, it is not a thermodynamic parameter and does not directly measure hybridization affinity. $\Delta G°_{37}$ is the appropriate parameter for evaluating hybridization affinity. It represents the free energy difference between duplex and single strands at 37°C. Unfortunately, for oligonucleotides longer than about 10 residues and ionic strengths less than ~0.5 M, coil to helix transitions tend to be non-two state (9–12) and this was observed for many of our transitions. $\Delta G°_{37}$ obtained from the melting curves was sensitive to analysis method, particularly to how baselines were drawn (13–15). T_M, on the other hand, was much less sensitive to analysis method. Consideration of the thermodynamic equations demonstrates that for changes in T_M <25°C and changes in $\Delta H°$ <25%, ΔT_M correlates quite well with $\Delta\Delta G°_{37}$. This correlation has also been observed experimentally (7,8). Thus T_M and ΔT_M were used to evaluate the effect of chemical modification on duplex stability. [Note: throughout this manuscript, we use the phrases 'duplex stability' and 'hybridization affinity' to refer to $\Delta G°_{37}$, the free energy difference between duplex and single strands at 37°C.]

The six sequences used for the initial hybridization studies are listed in Table 1. They included sequences with single modifications interspersed between unmodified residues (***seq1***, ***seq2*** and ***seq5***), sequences with short or long continuous stretches of modified residues (***seq3*** and ***seq4***, respectively) and one sequence (***seq6***) that was fully modified, except for the 3′ terminal nucleoside. Our primary interest was in the utilization of these modifications for antisense applications so we focused on hybridization of the modified oligonucleotides to complementary RNA.

To test the effect of our modifications in a uniformly modified oligonucleotide, the modified C amidite was synthesized and s***eq6*** was prepared. We opted to prepare the C amidite rather than to test homo-T oligomers for hybridization. When mixed with oligo-A, oligo-T can form many complexes including triple-stranded structures and high molecular weight aggregates with staggered duplexes (16). Due to the symmetry of the sequence, both parallel and antiparallel hybridization is possible. These complex structures can be difficult to characterize due to slow hybridization and coexistence of multiple species. Results with such complex structures can also be misleading. For example, triple-stranded complexes formed by PNA T_{10} and dA_{10} led to the conclusion that T_M values for short PNA–DNA duplexes were 50°C higher than their DNA–DNA counterparts (17,18). However, later work with mixed sequences demonstrated that, at physiological ionic strength, the ΔT_M value for a PNA–DNA 10mer duplex was only 17°C (19).

T_M measurements

Absorbance versus temperature curves were measured as described previously (7). Each sample contained 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, 4 µM modified oligonucleotide and 4 µM complementary, length matched RNA. ΔT_M per modification was calculated by subtracting T_M of the unmodified DNA–RNA parent duplex and dividing by the number of modified residues in the sequence. Average ΔT_M per substitution was calculated by summing the ΔT_M values for all oligonucleotides containing that modification and dividing by the total number of substitutions. Averages calculated by this method weigh each oligonucleotide by the number of substitutions it contains.

RESULTS

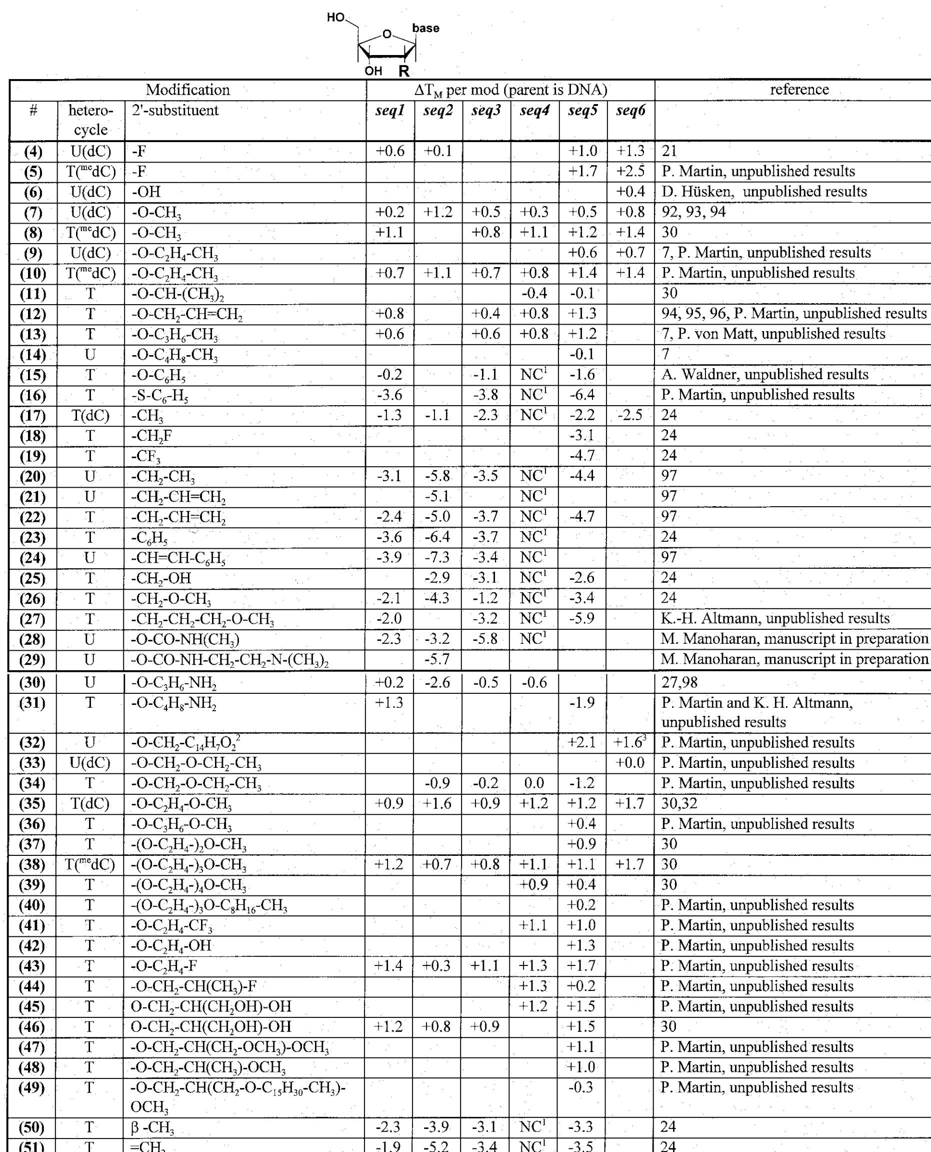
T_M values for the unmodified controls

T_M values for the six unmodified DNA sequences versus their RNA complements are listed in Table 2. In addition, ΔT_M values are listed for the same sequences containing dU **(1)** (bold numbers in parentheses refer to modification numbers from the tables and figures) and for ***seq6*** with 5-methyl dC **(2–3)**. Each substitution of dU for T resulted in an average change of about –0.5°C in T_M and substitution of 5-methyl dC for dC resulted in an average increase of about +0.5°C per substitution (20). For all modifications discussed below, ΔT_M values were calculated relative to the unmodified DNA parent (containing T and dC) even though some of the sugar and backbone modifications were prepared on dU rather than T or on 5-methyl C rather than dC. For each modification, the nucleobase is listed. If dU was used, a destabilization of approximately –0.5°C per substitution would be expected in addition to any stabilization or destabilization caused by the modified sugar or backbone.

Sugar modifications

Effect of 2′-sugar substitution. Table 3 lists ΔT_M values for oligonucleotides containing substitutions at the 2′ position of deoxyribose. It is clear from Table 3 that the exact value of ΔT_M per substitution depended on sequence. Thus, effects of modifications on duplex stability can be compared quantitatively only if the same sequences were used for all modifications. Trends,

Table 3. Effect of 2′ sugar substitution of T_M



Modification			ΔT_M per mod (parent is DNA)						reference
#	hetero-cycle	2'-substituent	*seq1*	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(4)	U(dC)	$-F$	+0.6	+0.1			+1.0	+1.3	21
(5)	T(^{me}dC)	$-F$					+1.7	+2.5	P. Martin, unpublished results
(6)	U(dC)	$-OH$						+0.4	D. Hüsken, unpublished results
(7)	U(dC)	$-O-CH_3$	+0.2	+1.2	+0.5	+0.3	+0.5	+0.8	92, 93, 94
(8)	T(^{me}dC)	$-O-CH_3$	+1.1		+0.8	+1.1	+1.2	+1.4	30
(9)	U(dC)	$-O-C_2H_4-CH_3$					+0.6	+0.7	7, P. Martin, unpublished results
(10)	T(^{me}dC)	$-O-C_2H_4-CH_3$	+0.7	+1.1	+0.7	+0.8	+1.4	+1.4	P. Martin, unpublished results
(11)	T	$-O-CH-(CH_3)_2$				-0.4	-0.1		30
(12)	T	$-O-CH_2-CH=CH_2$	+0.8		+0.4	+0.8	+1.3		94, 95, 96, P. Martin, unpublished results
(13)	T	$-O-C_3H_6-CH_3$	+0.6		+0.6	+0.8	+1.2		7, P. von Matt, unpublished results
(14)	U	$-O-C_4H_8-CH_3$					-0.1		7
(15)	T	$-O-C_6H_5$	-0.2		-1.1	NC[1]	-1.6		A. Waldner, unpublished results
(16)	T	$-S-C_6-H_5$	-3.6		-3.8	NC[1]	-6.4		P. Martin, unpublished results
(17)	T(dC)	$-CH_3$	-1.3	-1.1	-2.3	NC[1]	-2.2	-2.5	24
(18)	T	$-CH_2F$					-3.1		24
(19)	T	$-CF_3$					-4.7		24
(20)	U	$-CH_2-CH_3$	-3.1	-5.8	-3.5	NC[1]	-4.4		97
(21)	U	$-CH_2-CH=CH_2$		-5.1		NC[1]			97
(22)	T	$-CH_2-CH=CH_2$	-2.4	-5.0	-3.7	NC[1]	-4.7		97
(23)	T	$-C_6H_5$	-3.6	-6.4	-3.7	NC[1]			24
(24)	U	$-CH=CH-C_6H_5$	-3.9	-7.3	-3.4	NC[1]			97
(25)	T	$-CH_2-OH$		-2.9	-3.1	NC[1]	-2.6		24
(26)	T	$-CH_2-O-CH_3$	-2.1	-4.3	-1.2	NC[1]	-3.4		24
(27)	T	$-CH_2-CH_2-CH_2-O-CH_3$	-2.0		-3.2	NC[1]	-5.9		K.-H. Altmann, unpublished results
(28)	U	$-O-CO-NH(CH_3)$	-2.3	-3.2	-5.8	NC[1]			M. Manoharan, manuscript in preparation
(29)	U	$-O-CO-NH-CH_2-CH_2-N-(CH_3)_2$		-5.7					M. Manoharan, manuscript in preparation
(30)	U	$-O-C_3H_6-NH_2$	+0.2	-2.6	-0.5	-0.6			27,98
(31)	T	$-O-C_4H_8-NH_2$	+1.3				-1.9		P. Martin and K. H. Altmann, unpublished results
(32)	U	$-O-CH_2-C_{14}H_7O_2$[2]					+2.1	+1.6[3]	P. Martin, unpublished results
(33)	U(dC)	$-O-CH_2-O-CH_2-CH_3$						+0.0	P. Martin, unpublished results
(34)	T	$-O-CH_2-O-CH_2-CH_3$		-0.9	-0.2	0.0	-1.2		P. Martin, unpublished results
(35)	T(dC)	$-O-C_2H_4-O-CH_3$	+0.9	+1.6	+0.9	+1.2	+1.2	+1.7	30,32
(36)	T	$-O-C_3H_6-O-CH_3$					+0.4		P. Martin, unpublished results
(37)	T	$-(O-C_2H_4-)_2O-CH_3$					+0.9		30
(38)	T(^{me}dC)	$-(O-C_2H_4-)_3O-CH_3$	+1.2	+0.7	+0.8	+1.1	+1.1	+1.7	30
(39)	T	$-(O-C_2H_4-)_4O-CH_3$				+0.9	+0.4		30
(40)	T	$-(O-C_2H_4-)_3O-C_8H_{16}-CH_3$					+0.2		P. Martin, unpublished results
(41)	T	$-O-C_2H_4-CF_3$				+1.1	+1.0		P. Martin, unpublished results
(42)	T	$-O-C_2H_4-OH$					+1.3		P. Martin, unpublished results
(43)	T	$-O-C_2H_4-F$	+1.4	+0.3	+1.1	+1.3	+1.7		P. Martin, unpublished results
(44)	T	$-O-CH_2-CH(CH_3)-F$				+1.3	+0.2		P. Martin, unpublished results
(45)	T	$O-CH_2-CH(CH_2OH)-OH$				+1.2	+1.5		P. Martin, unpublished results
(46)	T	$O-CH_2-CH(CH_2OH)-OH$	+1.2	+0.8	+0.9		+1.5		30
(47)	T	$-O-CH_2-CH(CH_2-OCH_3)-OCH_3$					+1.1		P. Martin, unpublished results
(48)	T	$-O-CH_2-CH(CH_3)-OCH_3$					+1.0		P. Martin, unpublished results
(49)	T	$-O-CH_2-CH(CH_2-O-C_{15}H_{30}-CH_3)-OCH_3$					-0.3		P. Martin, unpublished results
(50)	T	β $-CH_3$	-2.3	-3.9	-3.1	NC[1]	-3.3		24
(51)	T	$=CH_2$	-1.9	-5.2	-3.4	NC[1]	-3.5		24

[1]NC, non-cooperative transition.
[2]The structure of this anthraquinone derivative is given in Figure 1C.
[3]This oligonucleotide contained substitutions at positions 4 and 13 only.

however, were consistent across all sequences studied. Modifications that stabilized the duplex did so for all sequences; modifications that destabilized the duplex reduced T_M for all sequences.

Figure 1 plots the average ΔT_M per substitution for the substitutions in Table 3. Among the 2′ substitutions reported here, a 2′-fluoro substituent **(4–5)** was the most stabilizing. In general, 2′-*O*-alkyl substitution **(7–14)** also stabilized the duplex, with smaller substituents resulting in greater duplex stability than larger ones. A clear correlation between substituent size and duplex stability has been reported previously for a large series of 2′-*O*-alkyl substitutions (7) and is confirmed by the data in Figure 1A. The improved hybridization of 2′-F and 2′-*O*-R-substituted oligonucleotides to complementary RNA has been attributed to the tendency of these electronegative substituents to shift the conformational equilibrium in the sugar moiety toward the northern (C3′-*endo*) conformation consistent with the A-form geometry of RNA duplexes (7,21–23). Destabilization by larger 2′-*O*-alkyl substitutions, on the other hand, may be caused by steric interference of the larger alkyl chains with other parts of the duplex or disruption of water structure in the minor groove (7).

In contrast to the increase in duplex stability observed with electronegative substituents at the 2′ position, 2′-sulfur linked **(16)** or 2′-carbon linked **(17–27)** modifications were very destabilizing (Fig. 1B). Destabilization due to 2′-α alkyl substitution was explained by the tendency of these substituents to shift the conformational equilibrium of the sugar toward the C2′ *endo* pucker and away from the C3′ *endo* pucker found in RNA duplexes (24). Destabilization by 2′-*S*-phenyl **(16)**, 2′-*S*-methyl (25) and 2′-amino (26) substitution likely has a similar explanation.

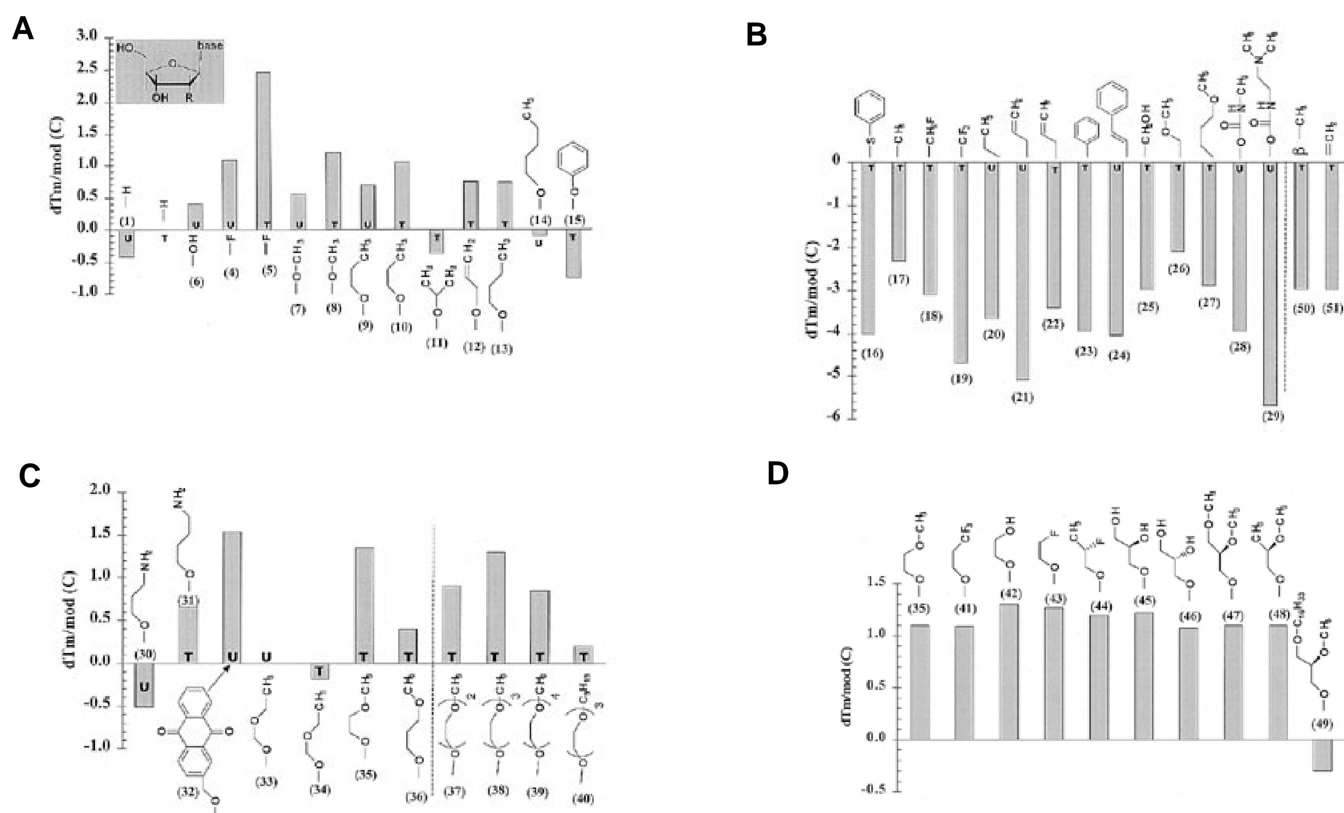


Figure 1. Average ΔT_M (°C) per substitution for 2′-substituted oligonucleotides. (**A**) 2′-fluoro-, 2′-*O*-alkyl-, 2′-*O*-allyl- and 2′-*O*-phenyl-substituted oligonucleotides; (**B**) oligonucleotides with 2′-sulphur or 2′-carbon linked substitutions or 2′-carbamate linkages; (**C**) 2′-*O* substitutions with heteroatoms in the side chain; (**D**) substitutions with the structure 2′-O-CH_2-CH_2-X where X = O, F or CF_3. For Figures 1–7, bold numbers in parentheses refer to the modification numbers in Tables 3–15.

In contrast, 2′-β-methyl substitution **(50)** drives the sugar equilibrium toward C3′ *endo* but the 2′-β-methyl substituent causes steric conflict in an A-form duplex (24). Also shown in Figure 1B are two 2′-carbamate substitutions **(28–29)**. These substitutions were very destabilizing. Apparently the rigid carbamate causes steric interference in the minor groove (M. Manoharan, manuscript in preparation).

Figure 1C reports the effect of additional heteroatoms in 2′-*O*-R substituents. 2′-*O*-amino-alkyl substitution **(30–31)** results in a zwitterionic oligonucleotide and, on average, had little effect on duplex stability. Similar results have been reported for 2′-*O*-amino-propyl substitution in other sequences (27). The 2′-*O*-anthraquinolylmethyl U modification **(32)** was very stabilizing suggesting the anthraquinone may intercalate into the hybrid duplex. Similar stabilization has been observed for oligonucleotides with intercalators attached to the 3′ end (28,29). When a second oxygen was incorporated into the 2′-*O*-alkyl side chain, the 2′-*O*-methoxy-ethyl modification **(35)** stood out as uniquely stabilizing. This stabilization is apparently associated with the ethylene glycol motif; 2′ substituents with as many as four ethylene glycol units **(37–39)** still stabilized the duplex. Even a nonyl group was well tolerated at the end of the ethylene glycol chain **(40)**. This contrasts with a destabilization of 2–3°C per substitution reported for 2′-*O*-nonyl substitution (7). The observation that 2′-O-$(CH_2)_n$-O-CH_3 substitution stabilized the duplex for $n = 2$ **(35)** but had little effect on duplex stability for $n = 1$ **(34)** or $n = 3$ **(36)** led to the hypothesis that, due to the *gauche* effect, the second oxygen of the 2′-ethylene glycol results in a conformation of the side chain consistent with duplex formation (30–32). Results in Figure 1D provide further support for this hypothesis. Substituents with the structure 2′-O-CH_2-CHR-X where X = OH, F, CF_3 or OCH_3 and R = H, CH_3, CH_2OH or CH_2OCH_3 **(41–48)** all resulted in substantial stabilization of the duplex. This suggests an electronegative group at X and any group at R results in duplex stabilization. The only exception was substitution with a very long hydrocarbon on the second carbon (R = $OC_{16}H_{33}$) **(49)** which was destabilizing.

Table 4. Effect of 3′-substituted thymidines on T_M

Modification			ΔT_M per mod (parent is DNA)						
#	R_1	R_2	*seq1*	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	ref.
(52)	-CH_3	-H	-0.1	-1.3			-1.5		24
(53)	-CH_3	-O-CH_3	-1.4	-4.8			-1.3		24

Effect of 3′-β substitution. Table 4 reports ΔT_M values for oligonucleotides modified at the 3′ position. 3′-β methyl substitution **(52)** resulted in reduced duplex stability. Additional 2′-α-*O*-methyl substitution **(53)** decreased duplex stability even further. Destabilization by these substitutions has been attributed to a strong preference of the 3′-β methyl nucleoside for the 2′ *endo* conformation which is incompatible with an A-form duplex and to unfavorable steric interactions in the modified duplex (24).

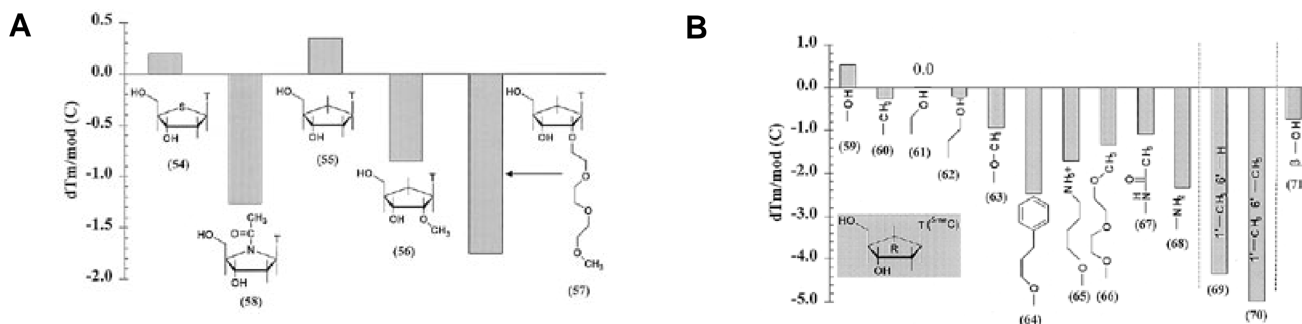


Figure 2. Average ΔT_M (°C) per substitution for oligonucleotides containing replacements for the furanose oxygen O4′. (**A**) Replacement of the ring oxygen with S, CH_2 or $NCOCH_3$ and (**B**) substitution at the 6′ carbon of carbocyclic nucleosides.

Table 5. Effect of 4′ oxygen substitution on T_M

Modification			ΔT_M per mod (parent is DNA)						
#	**-X-**	**-R**	***seq1***	***seq2***	***seq3***	***seq4***	***seq5***	***seq6***	ref.
(54)	-S-	-H				+0.2			34
(55)	$-CH_2-$	-H		+0.3			+0.4		99
(56)	$-CH_2-$	$-O-CH_3$		-1.9	-0.7	-1.0	+1.1		37
(57)	$-CH_2-$	$-(O-C_2H_4-)_3-O-CH_3$	-1.1	-2.9	-2.6	NC[1]	+0.2		37
(58)	$-N(COCH_3)-$	-H	-1.2	-0.6	-1.5				36

[1]NC, non-cooperative transition.

Effect of 4′-oxygen replacement. Table 5 lists ΔT_M values for oligonucleotides in which the ring oxygen of the furanose has been replaced with sulfur **(54)**, carbon **(55)** or nitrogen **(58)**. Averaged values are plotted in Figure 2A. Replacement of the oxygen with S **(54)** or CH_2 **(55)** had little effect on T_M consistent with the DNA-like conformation adopted by these nucleosides (33–35). 2′-*O*-methyl **(56)** or 2′-*O*-methoxy-diethoxy-ethyl **(57)** substitution on the carbocycle destabilized the duplex. This contrasts with the stabilizing effect these 2′ substituents had on a ribonucleoside and emphasizes the importance of the *gauche* effect between the ring oxygen and the 2′ oxygen in duplex stability.

In contrast to the slightly stabilizing effect of S or CH_2, replacement of the ring oxygen with an *N*-acetyl moiety **(58)** destabilized the duplex. It has been suggested that this destabilization is due to distortion in duplex structure caused by the acetyl group or the tertiary amide bond (36). ΔT_M values for oligonucleotides containing carbocyclic nucleosides modified at the 6′ position (in the carbocyclic nucleoside, the CH_2 which replaces the ring oxygen is designated 6′) **(59–71)** are reported in Table 6 with average values plotted in Figure 2B. 6′-α substitution with a methyl **(60)**, hydroxymethyl **(61)**, hydroxyethyl **(62)** or a hydroxy **(59)** group was well tolerated while 6′-α-OR **(63–66)**, 6′-α-amino **(68)** or 6′-α-acetylamino **(67)** substitution was destabilizing. It has been suggested that the stability of hybrid duplexes containing 6′-α-OH substitution is due to favorable solvation of the hydroxyl modified duplexes and their potential for H-bonding with adjacent residues (37). Model building suggested that 6′-α substituents can be accommodated in an A-form duplex so destabilization by the 6′-α-OR and other substituents may be due to unfavorable solvation effects.

Oligonucleotides containing 1′-β methyl-substituted carbocyclic nucleosides **(69–70)** hybridized very poorly (Table 6 and Fig. 2B). This destabilization might be due to a tendency of the 1′-α methyl carbocyclic nucleoside to adopt a 1′ *exo* conformation which is inconsistent with an A-form duplex structure (38). In contrast to 6′-α-hydroxy substituents, 6′-β-OH groups **(71)** led to duplex destabilization. This might be related to unfavorable effects on base conformation such as a preference of the base for a *syn* rather than the usual *anti* orientation.

Table 6. Effect of 1′ and 6′ substituted carbocyclic nucleoside analogs on T_M

Modification			ΔT_M per mod (parent is DNA)						
#	**-R1**	**-R2**	***seq1***	***seq2***	***seq3***	***seq4***	***seq5***	***seq6***	reference
(59)	-OH	-H	-0.4		+0.2	+0.8	-0.8	+0.8	37
(60)	$-CH_3$	-H	-1.5	-0.8	-0.3	-0.2	-0.1	+0.1	100
(61)	$-CH_2-OH$	-H	-1.0			+0.2	-0.1	+0.2	100
(62)	$- C_2H_4-OH$	-H					-0.2		K.-H. Altmann, unpublished results
(63)	$-O-CH_3$	-H	-1.3	+0.2	-0.7	-0.9	-2.0		37
(64)	$-O-C_3H_6-C_6H_5$	-H	-2.8	-2.8	-1.7	NC	-4.0		37
(65)	$-O-C_4H_8-NH_2$	-H	-1.4	-1.8		-1.9	-1.1		37
(66)	$-(O-C_2H_4-)_2-O-CH_3$	-H					-1.8	-1.3[2]	37
(67)	$-NH-CO-CH_3$	-H					-1.1		K.-H. Altmann, unpublished results
(68)	$-NH_3$	-H	-3.0	-4.0	-1.6		-1.1		K.-H. Altmann, unpublished results
(69)	-H	$-CH_3$	-5.2	-5.9	-3.3	NC[1]	-3.7		38
(70)	$-CH_3$	$-CH_3$	-6.1	-7.6	-3.8	NC[1]	-2.8		K.-H. Altmann, unpublished results
(71)	β -OH	-H				-0.4	-4.3		K.-H. Altmann, unpublished results

[1]NC, non-cooperative transition.
[2]The heterocycles for this oligonucleotide were thymine and cytosine.

Effect of bicyclic sugars. In an attempt to pre-organize the antisense oligonucleotide into a structure compatible with A-type duplex formation, several bicyclic sugar modifications have been investigated. Structures for four of such conformationally constrained building blocks and averaged values for ΔT_M per substitution are shown in Figure 3 and exact T_M data for our sequences are listed in Table 7. Among these bicycles, only the 4′-6′-methano carbocyclic thymidine **(73)** stabilized the duplex. DNA:RNA duplex stabilization correlates with the tendency of this nucleoside to adopt a northern conformation (39,40). The 1′-6′-methano carbocyclic thymidine **(72)**, in contrast, favors the Southern conformation and resulted in a decrease in duplex stability (41). The other two bridged nucleosides **(74–75)** destabilized the duplex substantially. Destabilization by **(75)** may be due to the rigidity of this modification.

Table 7. Effect of bicyclic modification or cyclobutyl substitution on T_M

Modification #[1]	ΔT_M per mod (parent is DNA)						reference
	seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(72)	-1.8	-0.9	-1.1	-0.8	-1.8	-1.1	41
(73)					+0.8	+2.1[2]	39,40
(74)		-2.3					H. Moser and R. Mah, unpublished results
(75)	-4.6	-8.8	-3.9	NC[3]	-5.1		101
(76)	-3.3	-3.7	-3.2	NC[3]			G. Baschang and F. Gasparini, unpublished results
(77)	-3.3	-4.0	-3.2	NC[3]			G. Baschang and F. Gasparini, unpublished results

[1]Structures for these modifications are given in Figure 3.
[2]This oligonucleotide had a single modification in position 10.
[3]NC, non-cooperative transition.

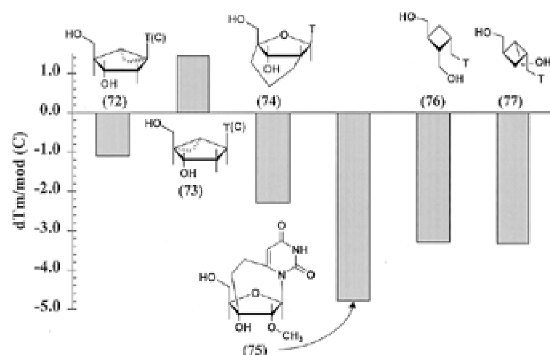


Figure 3. Average ΔT_M (°C) per substitution for oligonucleotides containing bicyclic sugar analogs.

Furanose replacement by four-membered rings. Two cyclobutyl nucleoside analogs **(76–77)** were incorporated into oligonucleotides. They greatly destabilized the duplex (Fig. 3).

Nucleobase modifications

Effect of substitution at the 5 or 6 position of thymine. ΔT_M values for oligonucleotides containing substitutions at the 5 and 6 positions of uracil are reported in Table 8 with average values plotted in Figure 4A. Removal of the 5-methyl group of T to generate dU **(1)** resulted in a slight decrease of duplex stability. Substitution of the 5-methyl group with a halogen **(78–80)** had little effect and substitution with a methoxy-ethoxy-methyl group **(83)** was destabilizing. Among the bases substituted at the 5 position, 5-propynyl dU **(81)** stood out as most stabilizing. This stabilization has been explained by increased stacking (42) and has also been observed for 5-methylthiazole-substituted dU (43) and tricyclic dC analogs (44).

A single positively charged amino-propyl group at the 5 position of U **(82)** had a slight positive effect on duplex stability at this ionic strength. Slight stabilization has also been reported for 5-amino-hexyl-substituted pyrimidines and has been attributed to shielding of the negative phosphate charges in unmodified hybrid duplexes (45). Interestingly, in another sequence, substitution of five thymidines with an analog containing a six-atom, amino-ethyl-3-acrylimido modifier at the 5 position of dU **(84)** (Glen Research, Sterling, VA) resulted in an increase in T_M of 1.2°C per substitution (M. Manoharan, unpublished results). Perhaps the acrylimido group contributes to stacking in a manner similar to the propyne substitution.

In contrast to the stabilizing or neutral effect of substituents at the 5 position, substitution at the 6 position **(85–87)** was very destabilizing. This destabilization is most likely related to the inability of these nucleosides to adopt the anti conformation due to the bulk of the substituent at position 6 (20).

Figure 4B summarizes the effect of combinations of 5 and 2′ substituents. In all cases the effects were roughly additive. Combination of two stabilizing modifications such as 2′ fluoro and 5-propynyl **(88)** resulted in a very stable hybrid. When stabilizing and destabilizing modifications were combined, for example, 2′-*O*-methoxy-ethyl with 5-methoxy substitution, the effect on duplex stability was essentially neutral.

Effect of other pyrimidine heterocycle modifications. Tables 9 and 10 report ΔT_M values for other pyrimidine modifications. Substitution of O4 or O2 of 2′-*O*-methyl U **(92–94)** resulted in extreme duplex destabilization (Fig. 4C). This is likely due to the

Table 8. Effect of 5 or 6 pyrimidine substitution on T_M

Modification				ΔT_M per mod (parent is DNA)						reference
#	-R1	-R2	-R3	*seq1*	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(1)	-H	-H	-H	-0.6	-3.2	-0.2	-0.4	+0.1	-0.3	20
(78)	-F	-H	-H	-0.2						20
(79)	-Br	-H	-H	+0.3						20
(80)	-I	-H	-H	-0.1						20
(81)	-C≡C-CH_3	-H	-H	+0.9	+2.6	+1.7	+2.1	+2.6		5,42,102, P. Martin, unpublished results
(82)	-$C_3H_6NH_3$	-H	-H		+0.7			-0.2		P. Martin, unpublished results
(83)	-CH_2-O-C_2H_4-O-CH_3	-H	-H	-1.0	-2.2	-1.6		-1.2		P. Martin, unpublished results
(85)	-H	-CH_3	-H	-3.9						20
(86)	-CH_3	-CH_3	-H	-3.3						20
(87)	5-6 propyl bridge[1]		-H	-2.7	-1.3					Y. S. Sanghvi, unpublished results
(88)	-C≡C-CH_3	-H	-F		+2.6	+3.1				O. Acevedo, unpublished results
(89)	-C≡C-CH_3	-H	-O-C_2H_4-O-CH_3		+2.1	+2.3		+3.6		103
(90)	-O-CH_3	-H	-O-C_2H_4-O-CH_3	-0.2			+0.3	+0.4		P. Martin, unpublished results
(91)	-C≡C-CH_3	-H	-CH_3		-2.2		-1.0	-0.6		C. Schmit, unpublished results

[1]See Figure 4A for structure.

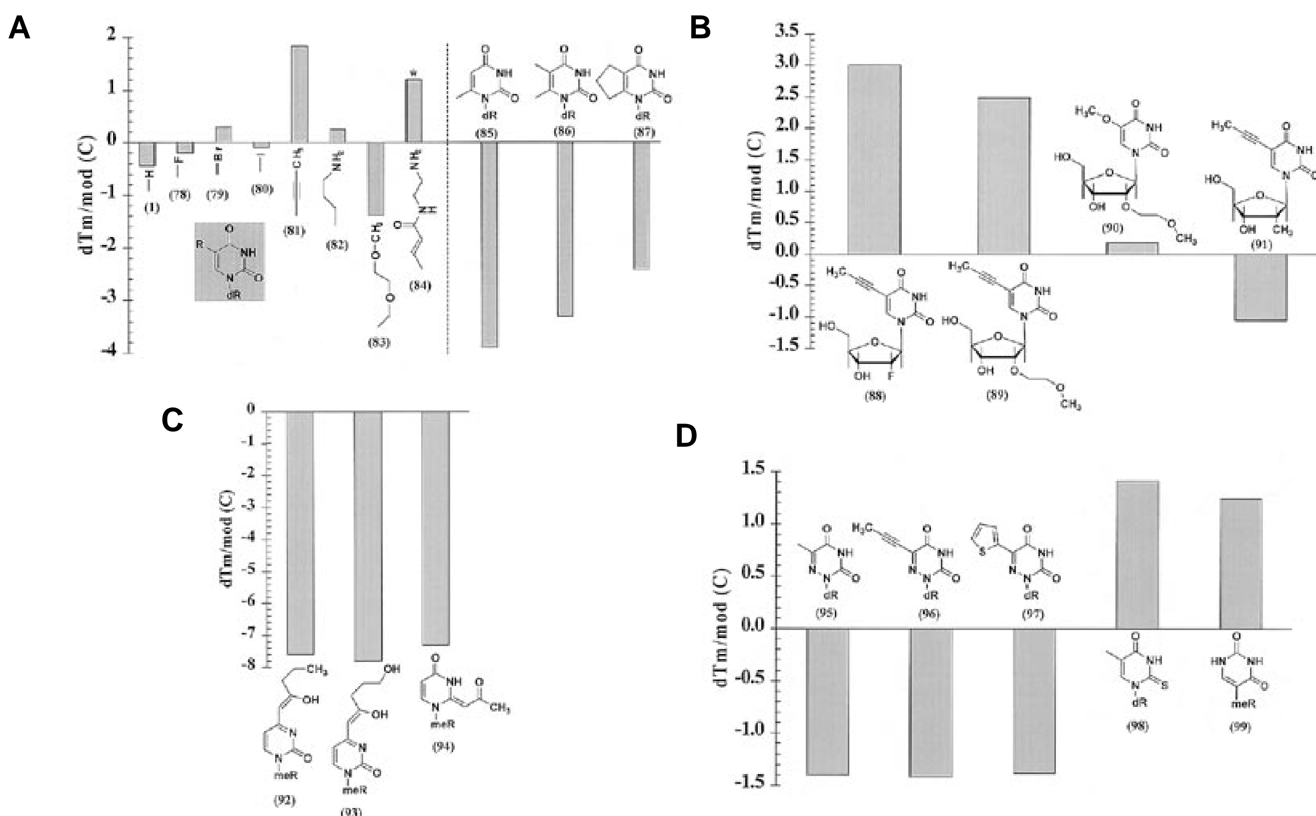


Figure 4. Average ΔT_M (°C) per substitution for oligonucleotides containing modified heterocycles. (**A**) 5- and 6-substituted thymidines; (**B**) 5 substitution combined with 2′ substitution; (**C**) substitution at O4 or O2 on 2′-*O*-methyl U; (**D**) 6 aza T analogs, 2 thio T and 2′-*O*-methyl pseudo uridine. The modification marked with an asterisk was evaluated in the sequence CtCGtACCttCCGtCC.

Table 9. Effect of substitution of O4 or O2 of 2′-*O*-methyl uridine on T_M

mod #	Modification	ΔT_M per mod (parent is DNA)						ref.
		seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(92)	**R1 =** $-C(OH)=CH-C_4H_9$		-7.6					46
(93)	**R1 =** $-C(OH)=CH-C_3H_6-OH$		-7.8					46
(94)	**R2 =** $-CH_3-CO-CH_3$	-6.6	-10.2					46

Table 10. Effect of modified pyrimidines on T_M

mod #	Modification[1]	ΔT_M per mod (parent is DNA)						reference
		seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(95)	6 aza T	-1.4						20
(96)	6 aza, 5 propynyl dU		-2.3	-1.2				104
(97)	6 aza, 5 thienyl dU		-3.2	-1.9	-1.0			104
(98)	2 thio T	+0.9	+0.5		+1.7			E. Swayze, unpublished results
(99)	2'-O-methyl-pseudo U	+1.6	+0.3	+1.0	+1.3			50

[1]Structures of these modified nucleosides are shown in Figure 4D.

fact that these modifications remove hydrogen bonding sites in the heterocycle (46).

Substitution of T with 6-aza T **(95)** was also destabilizing (Fig. 4D). We speculate this destabilization is due to decreased H-bonding because the reduced pK_a for 6-aza T, compared to T, shifts the nucleoside toward the enol tautomer (47–49). In contrast to the results in Figure 4B, addition of a 5-propynyl group to 6-aza T **(96)** did not improve RNA binding affinity.

Figure 4D also plots data for 2-thio T **(98)** and 2′-*O*-methyl pseudo U **(99)**. 2-thio-T resulted in an average increase of T_M of +1.4°C per substitution (Fig. 4D). This may be due to a tendency of the 2-thio nucleoside to adopt a C3′ *endo* sugar conformation (E. Swayze, unpublished results). This modification also improved binding to DNA targets suggesting improved stacking also contributes to duplex stability. The stabilizing effect of 2′-*O*-methyl pseudo U **(99)** was greater than that of 2′-*O*-methyl U **(6)** suggesting that the modified heterocycle itself also contributes to enhanced duplex stability (50).

Effect of purine heterocycle modifications. Although the series of oligonucleotides used in this study contained modifications only on pyrimidine residues, it is important to note that modifications of the purine heterocycle have also been described which result in improved hybrid stability. Among the most stabilizing purine modifications are the 7-halo-7-deaza purines (51,52) and the 7-propyne-7-deaza purines (53). The likely cause of increased duplex stability for these modifications is increased stacking of the modified purine rings.

Another modification that stabilizes the duplex is 2-amino-adenosine (2,6-diamino-purine). The amino group allows an additional H-bond to form with U and results in an increase in T_M of ~1°C per substitution (54, E.Lesnik, unpublished results).

Backbone modifications

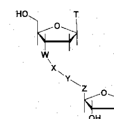
The effect of non-phosphorus containing backbone modifications. The unmodified phosphodiester backbone contains five bonds and four atoms (-O-PO_2-O-CH_2-) between the five-membered rings of adjacent residues. Several modifications were tested in which these four atoms were replaced with a non-phosphorous containing backbone (Table 11). Replacement of the phosphate backbone with four CH_2 groups **(100)** severely destabilized the duplex (Fig. 5A). Flexible glycol and ether linkages **(101–103)** were also very destabilizing. When C=C double bonds **(105–107)** or C≡C triple bonds **(108–111)** were incorporated into the backbone, destabilization was less pronounced but in no case did oligonucleotides with an all carbon backbone hybridize to complementary RNA with the same affinity as unmodified DNA (Fig. 5A). In contrast to these all carbon backbones, the thioformacetal backbone (-S-CH_2-O-CH_2-) increased T_M 0.8°C per substitution (55). This stabilization was attributed to the compatibility of the backbone with the conformation of the DNA:RNA duplex due to a shift of the sugar conformation toward C3′ *endo* because of the reduced electronegativity of sulfur compared to oxygen (54).

Figure 5B plots average ΔT_M values for oligonucleotides containing unsubstituted urea **(112)**, carbamate **(118** and **123)** and amide **(125, 128–129, 139–142)** linkages. Three-atom **(143–144)** and five-atom **(145)** amide linkages were destabilizing. Urea, carbamate and five of the four-atom amide backbones were also destabilizing. Only two amides did not destabilize the duplex, both of which had the amide moiety located in the middle position. They have been termed amide 3 **(129)** and amide 4 **(139)**. Modeling studies of the structures in Figure 5B suggest that the backbone conformers for these two amide modifications most closely approach backbone conformations in a hybrid duplex (56,57). Thus, the stability of these modifications is likely due to a tendency of the backbone in the single strand to preorganize in conformations favorable for duplex formation (58). Apparently the less flexible urea and carbamate backbones and the destabilizing amide backbones prefer backbone conformations unfavorable for duplex formation. The beneficial effect of a rigid bond in the middle position (as in amides 3 and 4) was also observed for a *trans* C=C double bond **(105)** which was the least destabilizing of the all carbon backbones (Fig. 5B). A single oligonucleotide uniformly modified with amide 3 was also investigated **(130)**. Its T_M was slightly lower than that of the unmodified DNA control suggesting that the flexibility of intervening phosphates is required to obtain improved hybridization compared to natural DNA.

To explore the effect of conformational rigidity in the backbone on duplex stability further, four analogs of amides 3 and 4 were tested with an additional bond between the 3′ methylene group and C2′ of the deoxyribose **(162–165)**. Structures of these analogs are given in Figure 5C. ΔT_M values are listed in Table 12. As is seen in Figure 5D, all of these constrained structures were much more destabilizing than the parent amides.

Data for more four-atom, non-phosphorous backbones are summarized in Figure 5E. Among these amine, hydroxylamine and hydrazino backbones, only two were stabilizing. These were the methylene(methylimino) or MMI **(148)** and the dimethylhydrazino (MDH) **(157)**. Stabilization by the MMI backbone has been attributed to the fact that the 3′ methylene group of the MMI linkage induces a C3′ *endo* sugar conformation in the sugar 5′ to the linkage (59).

Table 11. Effect of non-phosphorous backbones on T_M



mod #	Backbone (-W-X-Y-Z-)	ΔT_M per mod (parent is DNA)						reference
		seq1	***seq2***	***seq3***	***seq4***	***seq5***	***seq6***	
(100)	$-CH_2-CH_2-CH_2-CH_2-$		-4.2	-3.6	-5.9	-3.1		57,105,106
(101)	$-CH_2-CH_2-CH_2-O-$				NC[1]			107
(102)	$-O-CH_2-CH_2-O-$				-3.3			107
(103)	$-S-CH_2-CH_2-O-$				NC[1]			K. Teng, unpublished results
(104)	$-CH_2-CO-CH_2-CH_2-$				-2.8	-2.1		57,106
(105)	$-CH_2-CH=CH-CH_2-$ *(trans)*				-0.6	-1.0		108
(106)	$-CH_2-CH=CH-CH_2-$ *(cis)*				-1.3	-1.5		108
(107)	$-CH=CH-CH_2-CH_2-$				-2.2	-3.3		P. von Matt, unpublished results
(108)	$-O-CH_2-C\equiv C-$				-3.1	-1.8		109
(109)	$S-CH_2-C\equiv C-$					-4.6		109
(110)	-CH2-CH(OCH3)-C≡C-*(R)*				-2.3	-3.1		109
(111)	-CH2-CH(OCH3)-C≡C-*(S)*				-0.5	-3.6		109
(112)	$-NH-CO-NH-CH_2-$		-4.6	-3.9	NC[1]	-3.0		110
(113)	$-NH-CO-NCH_3-CH_2-$		-2.9	-3.4	-6.4	-3.7		110
(114)	$-NH-CO-NC_2H_5-CH_2-$		-3.9	-4.2		-3.2		110
(115)	$-NCH_3-CO-NH-CH_2-$		-7.7	-5.2		-2.7		110
(116)	$-NH-CO-NC_3H_7-CH_2-$		-3.3			-2.9		110
(117)	$-NCH_3-CO-NCH_3-CH_2-$					-5.2		110
(118)	$-O-CO-NH-CH_2-$		-3.4	-3.2	-4.8	-2.9		111
(119)	$-O-CO-NCH_3-CH_2-$		-3.2	-2.5	-4.0	-2.0		111
(120)	$-O-CO-NC_2H_5-CH_2-$		-2.5					111
(121)	$-O-CO-NC_3H_7-CH_2-$		-1.2	-2.3	-3.4	-1.2		111
(122)	$-S-CO-NCH_3-CH_2-$				-6.7	-4.0		112
(123)	$-NH-CO-O-CH_2-$		-7.0	-5.0	NC[1]	-3.8		111
(124)	$-NCH_3-CO-O-CH_2-$		-5.6	-4.8	NC[1]	-3.9		111
(125)	$-NH-CO-CH_2-CH_2-$		-2.2	-2.8	-3.5	-2.7		113
(126)	$-NCH_3-CO-CH_2-CH_2-$		-3.7	-2.9	-3.8	-2.4		113
(127)	$-NC_3H_7-CO-CH_2-CH_2-$		-2.5	-3.1	-5.0	-3.9		113
(128)	$-CH_2-CH_2-NH-CO-$		-1.3	-1.8	-2.8	-0.5		114
(129)	$-CH_2-CO-NH-CH_2-$		+0.9	+0.5	-0.1	+0.4	+0.6[2]	56,115
(130)	$-CH_2-CO-NH-CH_2-$ *uniform*[5]						-0.4[3]	K.-H. Altmann, unpublished results
(131)	$-CH_2-CO-NCH_3-CH_2-$	+1.0	-0.2			-1.1		56
(132)	$-CH_2-CO-NC_3H_7-CH_2-$	+0.9	-0.2	-0.2		-0.4		56
(133)	$-CH_2-CO-N(C_2H_4OC_2H_4OC_2H_5)-CH_2-$	-0.0	-0.3	-0.3		-0.7		116
(134)	$-CH_2-CO-N(C_6H_5)-CH_2-$					-0.7		116
(135)	$-CH_2-CO-N(C_6H_4-C_8H_{17})-CH_2-$			NC		-3.5		116
(136)	$-CH_2-CO-N(C_6H_4-O-C_2H_4-O-C_2H_4-O-CH_3)-CH_2-$			-1.3		-2.0		116
(137)	$-CH_2-CO-N(C_3H_6-N(CH_3)_2)-CH_2-$			-1.0				P. von Matt, unpublished results
(138)	$-CH_2-CO-NH-CH_2-$ *(with 5 propyne)*[4]				+0.8[4]	+0.4[4]		A. De Mesmaeker, unpublished results
(139)	$-CH_2-NH-CO-CH_2-$		-0.5	-0.3	+0.4	-0.8		117
(140)	$-CO-NH-CH_2-CH_2-$		-3.8	-3.3		-3.4		118
(141)	$-O-CH_2-CO-NH-$ (carbo sugar on bottom)				NC[1]	-4.3		K. H. Altmann, unpublished results
(142)	$-CH_2-CH_2-CO-NH-$ (carbo sugar on both)				-2.8			K. H. Altmann, unpublished results
(143)	$-CH_2-NH-CO-$					-1.8		119
(144)	$-CO-NH-CH_2-$					-5.0		119
(145)	$-CH_2-CH_2-CO-NH-CH_2-$				-2.6	-1.1		119
(146)	$-CH=N-O-CH_2-$		-3.1					120
(147)	$-CH_2-NH-O-CH_2-$		-0.5	-1.0	-1.2			120
(148)	$-CH_2-NCH_3-O-CH_2-$		+1.5	-0.2	+0.1		+1.5[2]	121
(149)	$-CH_2-N(-C_2H_5)-O-CH_2-$				-0.5			122
(150)	$-CH_2-N(-C_3H_7)-O-CH_2-$				-0.7			122
(151)	$-CH_2-N(-CH_2-CH(CH_3)_2)-O-CH_2-$				-1.8			122
(152)	$-CH_2-N(-C_2H_4-O-CH_3)-O-CH_2-$				-0.5			122
(153)	$-CH_2-N(-C_2H_4-NH_2)-O-CH_2-$				-0.8			122
(154)	$-CH_2-N(-C_2H_4-N-(CH_3)_2)-O-CH_2-$				-1.0			122
(155)	$-CH_2-N(CH_2-C_6H_5)-O-CH_2-$		+0.0	-1.2	-2.2			122
(156)	$-CH_2-N(-CH_2-C_3H_5)-O-CH_2-$				-0.2			122
(157)	$-CH_2-NCH_3-NCH_3-CH_2-$		+1.6	-0.1	+0.2			123
(158)	$-CH_2-O-N(CH_3)-CH_2-$			-1.3	-2.0			124
(159)	$-CH_2-N(CH_3)-CH_2-CH_2-$		-2.5	-2.1	-2.6			125
(160)	$-CH_2-CH_2-N(CH_3)-CH_2-$		-3.8	-3.3	-4.4			125
(161)	$-O-N(CH_3)-CH_2-CH_2-$		-1.2	-0.8	-0.9			126

[1]NC, non-cooperative transition.
[2]This oligonucleotide contained modified TT and TC dimers.
[3]This oligonucleotide contained an amide backbone at all 14 positions with no intervening phosphates. The heterocycles were T and 5-methyl dC. ΔT_M is relative to a reference DNA oligo containing T and 5-methyl dC.
[4]In addition to the amide backbone, these oligonucleotides contained a 5-propyne substitution on the T 3′ to each backbone substitution.

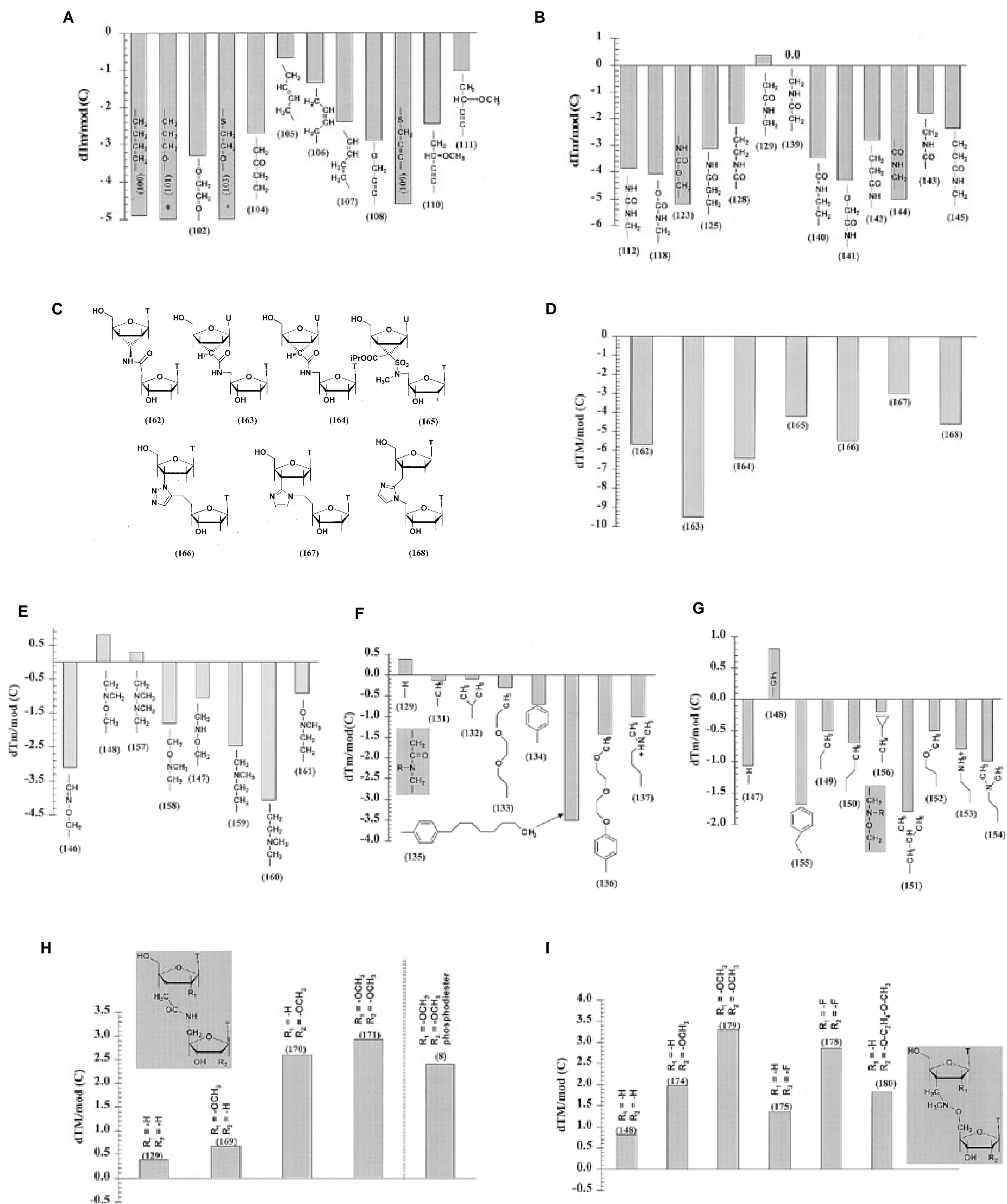


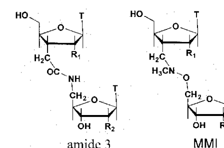
Figure 5. Average ΔT_M (°C) per substitution for oligonucleotides containing non-phosphorous backbone modifications. (**A**) Glycols, ethers and all carbon backbones; (**B**) urea, carbamate and amide backbones; (**C**) structures of cyclic backbone substitutions; (**D**) cyclic backbone substitutions; (**E**) amines, hydroxylamines and hydrazino backbones; (**F**) *N*-substituted amide 3 backbones; (**G**) *N*-substituted MMI backbones; (**H**) 2′-substituted amide 3 modifications; (**I**) 2′-substituted MMI modifications. Modifications marked with an asterisk resulted in a non-cooperative transition for the only sequence studied.

Table 12. Effect of cyclic backbone substitutions on T_M

mod #[1]	ΔT_M per mod (parent is DNA)						ref.
	seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(162)			-5.4	NC[2]			127
(163)				-4.2[3]			128
(164)				-9.5[3]			128
(165)				-6.4[4]			128
(166)				-6.1	-2.3		129
(167)				-3.7[5]	-1.6		129
(168)				-4.6[3]			129

[1]Structures of these backbone modifications are shown in Figure 5C.
[2]NC, non-cooperative transition.
[3]These oligonucleotides contained only three backbone modification, at positions 4–5, 8–9 and 12–13.
[4]This oligonucleotide contained only one backbone modification, at position 8–9.
[5]This oligonucleotide contained only two backbone modifications, at positions 6–7 and 12–13.

Table 13. Effect of 2′ substitution on T_M of amide 3 or MMI-modified oligonucleotides



mod #	Backbone	R1	R2	ΔT_M per mod (parent is DNA)						reference
				seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(169)	amide 3	$-O-CH_3$	-H				+0.8	+0.0		130
(170)	amide 3	-H	$-O-CH_3$				+2.0	+2.1	+3.1[1]	130
(171)	amide 3	$-O-CH_3$	$-O-CH_3$				+3.0	+2.6		130
(172)	amide 3	-OH	-H					-1.2		130
(173)	N-phenyl amide 3[2]	-H	$-O-CH_3$	+1.7[2]						A. Waldner, unpublished results
(174)	MMI	-H	$-OCH_3$		+0.9	+1.7	+2.3			131,132
(175)	MMI	-H	-F		-0.2	+1.0	+1.8			131,132
(176)	MMI	$-O-CH_3$	-F		+1.6	+2.5	+3.2			131,132
(177)	MMI	-F	$-O-CH_3$		+2.0	+3.0	+3.8			131,132
(178)	MMI	-F	-F		+1.5	+2.2	+3.4			131,132
(179)	MMI	$-O-CH_3$	$-O-CH_3$		+1.9	+2.8	+3.8			131,132
(180)	MMI	-H	$-O-C_2H_4-O-CH_3$		+1.0	+1.6	+2.1			Y. S. Sanghvi, unpublished results

[1]This oligonucleotide contained T and 5-methyl C heterocycles.
[2]In addition to the indicated 2′ substitutions, this oligonucleotide contained an *N*-phenyl substitution in the amide backbone.

In an attempt to identify analogs of amide 3 or MMI with improved hybridization properties, several *N*-substituted variants were investigated. Results for *N*-substituted amide 3 **(131–137)** and *N*-substituted MMI **(147–156)** are summarized in Figure 5F and G, respectively. Although small substituents on the nitrogen of amide 3 were tolerated, none improved hybridization compared to amide 3 itself **(129)** and large substituents were very destabilizing (Fig. 5F). For the methyleneimino backbone, only the *N*-methyl analog **(148)** was stabilizing (Fig. 5G). All other nitrogen substituents were destabilizing.

Figure 5H and I and Table 13 summarize the effects of adding 2′ substitutions to amide 3 **(129)** and MMI **(148)**, respectively. For both backbones, addition of a 2′-*O*-methyl group on the lower sugar (the sugar 3′ of the modified linkage) **(170, 174)** greatly stabilized the duplex and 2′-*O*-methyl substitution on both sugars of the modified backbone **(171, 179)** stabilized even more than 2′-*O*-methyl substitutions on a phosphate diester backbone **(8)**. Similar effects were observed for 2′-fluoro **(175–178)** and 2′-*O*-methoxy-ethyl **(180)** substitution. This stabilization was explained by the effect of the backbone and the 2′ substituents on the sugar pucker (60). For the bis-deoxy MMI modification **(148)** the conformational analysis indicated 68 and 31% northern conformation for the upper and lower sugar, respectively, compared to ~30% northern for sugars in unmodified DNA. Addition of a 2′-*O*-methyl group to the lower sugar of the MMI linked dimer units **(174)** shifted the conformational equilibrium to ~65% northern conformation for both sugars and resulted in a significant increase in T_M. Addition of a second 2′-*O*-methyl on the upper sugar **(179)** increased the fraction of C3′ *endo* conformation to 95 and 76% for the upper and lower sugar, respectively, and stabilized the duplex even further. Thus the high stability of modified DNA:RNA duplexes incorporating intrinsically favorable backbone modifications in combination with electronegative 2′ substituents appears to be strongly correlated with the conformational equilibria of the sugars.

Table 12 and Figure 5C and D report T_M data for backbones containing triazole **(166)** and imidazole **(167–168)** heterocycles. All of these cyclic backbones were destabilizing.

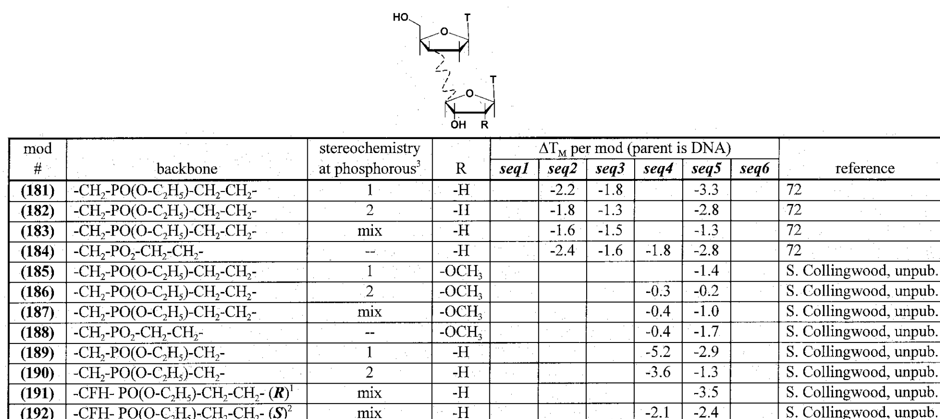
Modified backbones containing phosphorous. Among oligonucleotide modifications used for antisense applications, those that have been tested most extensively are phosphate-modified backbones. These include phosphorothioates (61), phosphoramidates (62–64) and methyl phosphonates (65) in which one of the non-bridging phosphate oxygens has been replaced by sulfur, -NHR or $-CH_3$, respectively. All of these modifications result in reduced hybrid stability. It has been suggested that this destabilization is caused by diastereoisomerism due to chirality at phosphorous, however, phosphorodithioates, which contain an achiral phosphorous atom, also destabilize the duplex (66–68).

In contrast, substitution of the bridging 3′-oxygen with NH (N3′→P5′ phosphoramidates) resulted in very stable duplexes with T_M increases of ~2°C per substitution (69). Even greater stabilization of 4°C per substitution was reported for 2′-fluoro, N3′→P5′ phosphoramidate oligonucleotides (70). These stabilizations, which are some of the largest reported to date have been attributed to the tendency of the sugar moieties to adopt a C3′ *endo* conformation when the 3′-O is replaced with 3′-NH (71).

ΔT_M data for oligonucleotides containing other types of phosphorous modifications are reported in Tables 14 and 15. Averaged data are plotted in Figure 6. Both isomers of an ethyl phosphinate moiety **(181–182)** were destabilizing, as was the free phosphinate **(184)** (Fig. 6A). Shorter, three-atom phosphinates **(189–190)** were also destabilizing. Although addition of a 2′-*O*-methyl group to the lower sugar of the four-atom ethyl phosphinate modified dimer units **(185–188)** improved hybridization, these modifications were still destabilizing. Because the phosphinate backbone modified oligonucleotides hybridized to DNA much more poorly than to RNA, it was suggested that the lack of an electronegative group at C3′ likely favors a northern sugar pucker (72). This is supported by the observation that replacement of the 3′CH_2 with a more electronegative CHF **(191–192)** reduced duplex stability even further.

Data for other phosphorous containing backbones are plotted in Figure 6B. Thio-phosphate **(193)**, which has been widely used for antisense applications, reduced T_M ~0.7°C per substitution. Averaged over several uniformly modified sequences, mixed diastereoisomers of thiophosphates reduced T_M by ~0.5°C per substitution (E. Lesnik, unpublished results). Phosphine oxide

Table 14. Effect of phosphinate substitutions on T_M



mod #	backbone	stereochemistry at phosphorous[3]	R	ΔT_M per mod (parent is DNA) seq1	seq2	seq3	seq4	seq5	seq6	reference
(181)	$-CH_2-PO(O-C_2H_5)-CH_2-CH_2-$	1	-H		-2.2	-1.8		-3.3		72
(182)	$-CH_2-PO(O-C_2H_5)-CH_2-CH_2-$	2	-H		-1.8	-1.3		-2.8		72
(183)	$-CH_2-PO(O-C_2H_5)-CH_2-CH_2-$	mix	-H		-1.6	-1.5		-1.3		72
(184)	$-CH_2-PO_2-CH_2-CH_2-$	--	-H		-2.4	-1.6	-1.8	-2.8		72
(185)	$-CH_2-PO(O-C_2H_5)-CH_2-CH_2-$	1	$-OCH_3$					-1.4		S. Collingwood, unpub.
(186)	$-CH_2-PO(O-C_2H_5)-CH_2-CH_2-$	2	$-OCH_3$				-0.3	-0.2		S. Collingwood, unpub.
(187)	$-CH_2-PO(O-C_2H_5)-CH_2-CH_2-$	mix	$-OCH_3$				-0.4	-1.0		S. Collingwood, unpub.
(188)	$-CH_2-PO_2-CH_2-CH_2-$	--	$-OCH_3$				-0.4	-1.7		S. Collingwood, unpub.
(189)	$-CH_2-PO(O-C_2H_5)-CH_2-$	1	-H				-5.2	-2.9		S. Collingwood, unpub.
(190)	$-CH_2-PO(O-C_2H_5)-CH_2-$	2	-H				-3.6	-1.3		S. Collingwood, unpub.
(191)	$-CFH-PO(O-C_2H_5)-CH_2-CH_2-$ (***R***)[1]	mix	-H					-3.5		S. Collingwood, unpub.
(192)	$-CFH-PO(O-C_2H_5)-CH_2-CH_2-$ (***S***)[2]	mix	-H				-2.1	-2.4		S. Collingwood, unpub.

[1]Stereochemistry at the site of fluoro substitution was *R*.
[2]Stereochemistry at the site of fluoro substitution was *S*.
[3]Isomers 1 and 2 represent the two diastereoisometrically pure isomers. Absolute stereochemistry, at phosphorous, of the isomers has not been determined.

Table 15. Effect of phosphate backbone modification on T_M

mod #	backbone	stereochemistry at phosphorous[1]	ΔT_M per mod (parent is DNA) seq1	seq2	seq3	seq4	seq5	seq6	reference
(193)	$-O-POS-O-CH_2-$	mix					-1.0	-0.7	D. Hüsken, unpublished results
(194)	$-CH_2-PO(CH_3)-CH_2-CH_2-$	mix				-3.9	-2.4		S. Collingwood, unpublished results
(195)	$-CH_2-PO(CH_3)-CH_2-CH_2-$	1					-3.5		S. Collingwood, unpublished results
(196)	$-CH_2-O-PO_2-O-CH_2-$	--		-1.8	-1.5	-1.8			133
(197)	$-O-CH_2-CH_2-O-PO_2-O-CH_2-$	--					-3.8		P. Martin, unpublished results

[1]Isomer 1 represents a diastereoisometrically pure isomer. Absolute stereochemistry, at phosphorus, has not been determined.

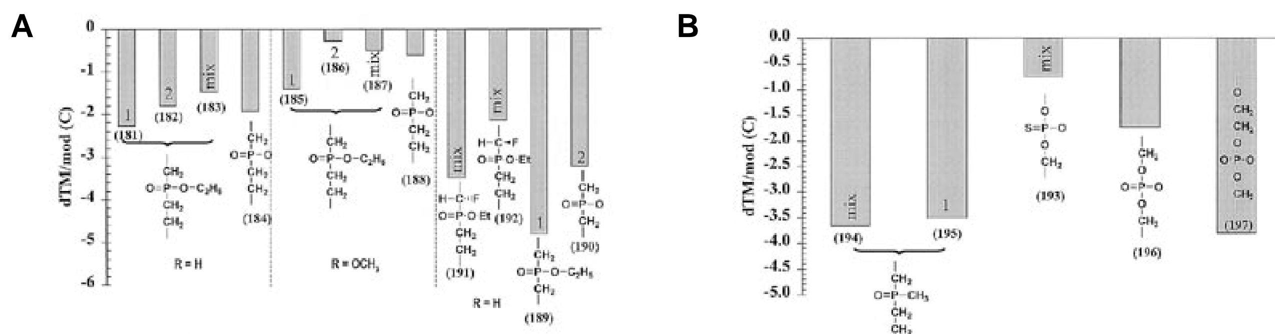


Figure 6. Average ΔT_M (°C) per substitution for oligonucleotides containing modified phosphate backbones. (**A**) Phosphinate analogs and (**B**) phosphate and phosphine oxide backbones.

modifications **(194–195)** and longer phosphate backbones **(196–197)** were very destabilizing.

Other neutral backbones. In addition to the modifications mentioned above, there are two interesting modifications that could not be studied in the partially modified sequences of Table 1 because the synthetic strategies used for these modifications could not easily be combined with DNA phosphoramidite chemistry. These modifications are the phosphoryl linked morpholino backbone **(199)** of Summerton and Weller (73–75) and the polyamide backbone called PNA **(198)** (17,19). Structures and ΔT_M values for these modifications are given in Figure 7. The increased hybrid stability observed for these modifications is likely due to their neutrality and probably reflects a tendency of the single strands to adopt conformations favorable for duplex formation.

Specificity of hybridization. For antisense applications, high specificity of Watson–Crick binding is as important as high affinity of hybridization. For evaluation of hybridization specificity, T_M was measured for ***seq2*** against RNA targets containing mismatched nucleotides (C, G or U) opposite the modified T. T_M with the matched target was compared to T_M with the mismatched

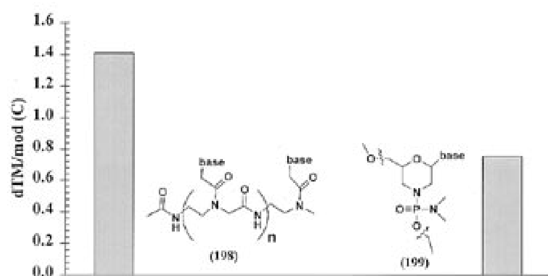


Figure 7. Structure and ΔT_M per substitution for the PNA **(198)** and morpholino **(199)** backbones used in this study. For PNA, ΔT_M per substitution was averaged over the sequences TGTACGTCACAACTA, GCACAGCC, TATTCCGTCATCGCTCCTCA, TTTAGGATTCGTGCTCATGG, GCCTTTCGCGACCCAACACT, CGCTCAAGTCCCATCGACCT, TAATGCGTACCATATGC, CGACTATGCAAGTAC, CGCTTGGCAGTCTC. For morpholino, ΔT_M was measured in a single sequence, UCUGAGUAGCAGAGGAGCUC.

targets. For all modifications that resulted in increased or only a slightly decreased duplex stability (not more than –1°C per substitution), specificity of the modified oligonucleotide was never worse than that of the unmodified DNA parent. The only modifications that showed poor specificity were those that resulted in sizable destabilization. These destabilizing modifications likely lead to distortions in duplex structure that cause disruption of base pairing at the site of modification and thus loss of Watson–Crick base pair specificity.

DISCUSSION

Analysis of the results presented above reveals four approaches that can be used to modify DNA for improved hybridization to RNA targets: (i) preorganize the sugars and phosphates of the DNA single strand into conformations favorable for hybrid formation, (ii) improve stacking by adding a polarizable group to the heterocycle, (iii) increase the number of H-bonds for an A–U pair and (iv) neutralize the backbone charge. Examples of each of these will be discussed below.

Modifications that shift the sugar conformation toward the northern pucker

Sugars in DNA:RNA hybrid duplexes frequently adopt a C3′ *endo* conformation. Thus modifications that shift the conformational equilibrium of the sugar moieties in the single strand toward this conformation should preorganize the antisense strand for binding to RNA. Several types of modifications reported above shifted the sugar toward a C3′ *endo* conformation. Substitution with an electronegative atom at the 2′ position [e.g. 2′-fluoro **(4–5)** (Fig. 1A) or 2′-OR **(6–15, 28–49)** (Fig. 1A, C and D)] resulted in a shift towards the northern conformation and, in general, increased T_M. Large 2′-*O*-alkyl substituents, however, were not well tolerated, presumably because of steric interference by the flexible alkyl chain with other parts of the duplex. However, large 2′-*O* substituents were tolerated if they contained the ethylene glycol motif **(35, 37–49)** (Fig. 1C and D). Apparently the *gauche* effect of the oxygen γ to the 2′ oxygen results in a configuration of the side chain favorable for duplex formation.

Shift of the sugar conformation towards a northern pucker and an increased T_M were also observed for modifications in which the 3′-oxygen was replaced with a non-electronegative group such as CH_2 in MMI **(148)** (Fig. 5E) or amide 3 **(129)** (Fig. 5B), with S in the thioformacetal backbone (-S-CH_2-O-CH_2-) **(55)**, or with NH in the N3′→P5′ phosphoramidate backbone (69). Thus an electronegative group at the 2′ position or a non-electronegative group at the 3′ position was effective in shifting the sugar conformation and improving T_M. Although it seems clear that the presence of a less electronegative group than oxygen at C-3′ represents an important feature for modifications that enhance duplex stability, this characteristic is by no means sufficient to enhance RNA binding affinity. This is amply illustrated by a whole range of backbone modifications incorporating a CH_2 group attached to C-3′ which did not lead to increased DNA:RNA duplex stability (Figs 5 and 6). Among these are Benner's sulfone modified oligonucleotides (-CH_2-CH_2-SO_2-CH_2-) which generate an A-type pucker but did not improve binding to RNA because the single sequence for which RNA binding has been reported formed a stable hairpin (76,77).

Another approach to shift the sugar conformation toward a northern pucker involves the introduction of conformational constraints using a 4′–6′ methylene bridge in the carbocyclic nucleoside **(73)** (Fig. 3). A change in the sugar conformational equilibrium toward a northern pucker can also be induced by certain base modifications without alterations in the 2′-deoxyribose. Thus, 2-thio T **(98)** in combination with an unmodified sugar–phosphate backbone still resulted in a shift of the sugar pucker towards a northern conformation and increased T_M (Fig. 4D).

The beneficial effect of preorganization of the sugar–phosphate backbone is also observed in the conformationally restricted 1′–5′ anhydrohexitol oligonucleotides which exhibit substantially improved hybridization compared to unmodified analogs (78–80). In this context, it should also be noted that the importance of conformational preorganization of the sugar–phosphate backbone is most impressively demonstrated by Eschenmoser's work on homo-DNA and related hexose-based nucleic acids (81–84). The stability of (2,3-dideoxy-D-glucopyranose-based) homo-DNA duplexes far exceeds that of natural DNA/DNA duplexes; however, due to their particular conformational properties, these analogs do not bind to natural nucleic acids and, in fact, would not be predicted to do so (81,82,84). On the other hand, incorporation of flexible, glycerol-based nucleoside analogs into oligodeoxyribonucleotides reduced binding affinity for complementary DNA (and presumably also RNA) dramatically (84–86) and neither did glycerol-based DNA analogs form stable self-duplexes (84,86). These findings may be rationalized by a reduction in appropriate conformational preorganization (increased entropy) similar to that observed for many flexible backbone modifications (see below).

Modifications that preorganize the backbone in conformations favorable for hybrid duplex formation

In addition to shifting the sugar to a conformation favorable for hybridization, modifications can also be made that preorganize the internucleotide backbone part of the modified DNA into conformations favorable for duplex formation. These modifications do not necessarily have to limit the single strand to a single conformation; they simply increase the population of single strands in conformations favorable for duplex formation and reduce the population in conformations incompatible with duplex formation. Modifications reported above that did this successfully were amide-3 **(129)** (Fig. 5B), amide-4 **(139)** (Fig. 5B), MMI **(148)** (Fig. 5E) and MDH **(157)** (Fig. 5E).

Preorganization of the backbone can also be detrimental for hybridization. Many modifications tested were less flexible than the normal phosphate backbone and likely resulted in preorganization of the antisense single strand but resulted in destabilization of the duplex. Examples include the three-atom **(143–144, 189–190)** and five-atom **(145, 196)** linkages in Figures 5B and 6. The conformations favored by these backbones likely were incompatible with duplex formation and resulted in a decrease in T_M. Clearly just the right amount of preorganization in just the right place was required for improved hybridization to occur.

Modifications that improve stacking by adding a polarizable group to the heterocycle

Favorable stacking of the heterocyclic bases contributes much of the favorable enthalpy of duplex formation for nucleic acid duplexes (14,87). This favorable stacking is due primarily to favorable interactions between dipoles and induced dipoles in adjacent residues. Thus modifications to the heterocycle that improve these interactions are likely to stabilize the duplex. Some examples include substitution at the 5 position of pyrimidine with propyne **(81)** (Fig. 4A), amino-ethyl-3-acrylimido **(84)** (Fig. 4A) or methylthiazole (43), tricyclic dC analogs (44) and 7-modified-7-deaza-purines (51–53).

Modifications that increase the number of H-bonds

H-bonds in RNA duplexes contribute ~1 kcal/mol of favorable free energy (88). This correlates well with the increase in T_M reported above for 2,6-diamino purine which can form three hydrogen bonds with U. Thus addition of a Watson–Crick H-bond can improve duplex stability.

Modifications that neutralize the negative phosphate charge

It has long been known that charge repulsion between phosphates on opposite strands provides a significant unfavorable contribution to the free energy of duplex formation at physiological ionic strengths (89,90). Thus removal of the negative charge on one strand is expected to increase duplex stability at physiological ionic strength. Several modifications described above reduced the net charge on the oligonucleotide and reduced the dependence of T_M on ionic strength (19,27, S. Freier, unpublished results). Only some of these resulted in an increase in T_M because often the favorable effect of the neutral charge was offset by an unfavorable effect such as preorganization into a structure incompatible with duplex formation or increased flexibility of the internucleotide linkage.

Some of the greatest increases in stability were observed for the PNA **(198)** and morpholino **(199)** modifications (Fig. 7) which are no longer negatively charged but whose backbone conformations are still compatible with duplex formation. A second approach to charge neutralization is to add a positive charge to the oligonucleotide. This was done most effectively at the 2′ position by addition of a 2′-*O*-amino-alkyl group **(30–31)** (Fig. 1C) and at the 5 position of T by addition of an amino alkyl **(82)** or an amino-ethyl-3-acrylimido group **(84)** (Fig. 4A).

Effect of combinations of stabilizing features

We have listed above four approaches for improving duplex stability and have presented examples for each approach. It is clear, however, that for most stabilizing modifications, more than one of these factors contributes to improved hybridization. For example, the stabilizing effect of MMI is a combination of the shift toward C3′ *endo* caused by the 3′ CH_2, restricted backbone flexibility and the neutral charge. Similarly, the stabilizing effect of 2-thio-T is likely a combination of the shift of the sugar pucker toward C3′ *endo* and improved stacking.

All four of the factors listed above also play a role in hybridization properties of destabilizing modifications. Frequently, in fact, one factor may contribute favorably but it is outweighed by another factor with a very unfavorable effect. For example, the ethyl phosphinates **(181–183)** (Fig. 6A) have a neutral backbone and the 3′-CH_2 helps to drive the sugar toward a C3′ *endo* conformation. In spite of these effects, however, the modifications were very destabilizing, probably because this backbone did not easily adopt conformations consistent with duplex formation. Of the modifications reported above, most were, in fact, very destabilizing. Usually, when a molecule was modified to favorably affect one of the factors listed above, the other factors were unfavorably affected for a net negative effect. Thus, net favorable effects were rare and the success rate was low.

The most stable duplexes reported above were formed with oligonucleotides that contained two different types of modification. These include 2′-*O*-methyl MMI backbones **(179)** (Fig. 5I), 2′-*O*-methyl amide 3 backbones **(171)** (Fig. 5H), 2′-*O*-methyl, 2-amino-adenosine (91), 2′-fluoro-5-propynyl dU **(88)** (Fig. 4B) and the 2′-fluoro, N3′→P5′ phosphoramidate oligonucleotides (70). The high T_Ms were achieved because each of the two modifications fulfilled one of the principles outlined above and no principle was violated. Thus careful combination of stabilizing modifications can produce even more stable duplexes.

It is important to note at this point that duplex stability will also be significantly affected by the difference in solvation energy between the single strands and the duplex. The importance of this parameter has been addressed in some detail in a recent review article by Elgi (23). Unfortunately, due to a lack of structural information, it is impossible to assess the relevance of solvation effects for the modified DNA:RNA duplexes discussed in this paper in any meaningful fashion. We do feel, however, that interactions with solvent may play an important role in distinguishing the effects of simple alkyl and ethylene glycol-based 2′-*O*-substitutent on RNA binding affinity (see Results: Sugar modifications).

In summary, we have tabulated above, T_M data for roughly 200 modifications that were incorporated into a single set of sequences. We also tried to include data for stabilizing modifications studied in other sequences. In spite of the large number of modifications tested, only relatively few structures that significantly stabilize DNA:RNA duplexes were identified. It appears that modified oligonucleotides with very high RNA binding affinity need to be constructed by the combination of two or more different types of modifications, each of which contributes favorably to one of the general factors outlined above.

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