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The influence of locked nucleic acid residues on the thermodynamic properties of 2′-O-methyl RNA/RNA heteroduplexes

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ABSTRACT

The influence of locked nucleic acid (LNA) residues on the thermodynamic properties of 2′-O-methyl RNA/RNA heteroduplexes is reported. Optical melting studies indicate that LNA incorporated into an otherwise 2′-O-methyl RNA oligonucleotide usually, but not always, enhances the stabilities of complementary duplexes formed with RNA. Several trends are apparent, including: (i) a 3′ terminal U LNA and 5′ terminal LNAs are less stabilizing than interior and other 3′ terminal LNAs; (ii) most of the stability enhancement is achieved when LNA nucleotides are separated by at least one 2′-O-methyl nucleotide; and (iii) the effects of LNA substitutions are approximately additive when the LNA nucleotides are separated by at least one 2′-O-methyl nucleotide. An equation is proposed to approximate the stabilities of complementary duplexes formed with RNA when at least one 2′-O-methyl nucleotide separates LNA nucleotides. The sequence dependence of 2′-O-methyl RNA/RNA duplexes appears to be similar to that of RNA/RNA duplexes, and preliminary nearest-neighbor free energy increments at 37°C are presented for 2′-O-methyl RNA/RNA duplexes. Internal mismatches with LNA nucleotides significantly destabilize duplexes with RNA.

INTRODUCTION

Understanding the thermodynamics of nucleic acid duplexes is important for many reasons. For example, such knowledge facilitates design of ribozymes (1), antisense and RNAi oligonucleotides (2–9), diagnostic probes including those employed on microarrays (10–23) and structures useful for nanotechnology (24–27). Many modified residues have been developed for such applications. Examples include propynylated bases (28–30), peptide nucleic acids (5,31–33), N3′–P5′ phosphoramidates (34–38) and 2′-O-alkyl RNA (39–43). A modification that is particularly stabilizing in DNA and RNA duplexes (44–51) is a methyl bridge between the 2′ oxygen and 4′ carbon of ribose to form a 'locked nucleic acid' or LNA as shown in Figure 1. McTigue *et al*. (48) have shown that the enhanced stability due to a single LNA residue in a DNA duplex can be predicted from a nearest-neighbor model.

Hybridization of oligonucleotides to RNA is important for applications, such as antisense therapeutics (4,8,21,46,52–54), diagnostics (32,33,42,55), profiling gene expression with microarrays (18–20,56), identifying bands by Northern blots of gels (57,58) and probing RNA structure (1,3,15,59–61). Oligonucleotides with 2′-O-alkyl modifications can be particularly useful for these applications because they are easily synthesized (39,43), chemically stable and bind relatively tightly to RNA (39–42). However, for many applications,

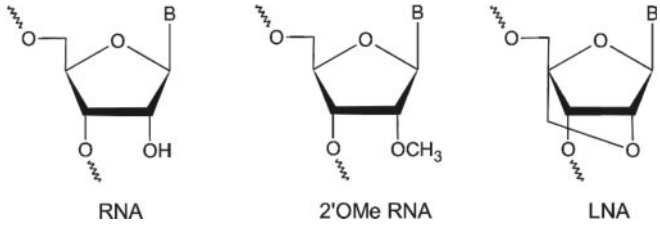

Figure 1. Covalent structures of sugars used.

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it is desirable to modulate the binding affinity. For example, sequence independent duplex stabilities would benefit applications that involve multiplex detection, such as microarrays. Here, we show that introduction of LNA into 2′-O-methyl RNA oligonucleotides can increase stabilities of 2′-O-methyl RNA/RNA hybrid duplexes and that the enhancements in stability can usually be predicted with a simple model.

MATERIALS AND METHODS

General methods

High-performance liquid chromatography (HPLC) was performed on a Hewlett Packard series 1100 HPLC with a reverse-phase Supelco RP-18 column (4.6 × 250 mm). Mass spectra were obtained on an LC MS Hewlett Packard series 1100 MSD with API-ES detector or on an AMD 604/402. Thin-layer chromatography (TLC) was carried out on Merck 60 F_{254} TLC plates with the mixture 1-propanol/aqueous ammonia/water = 55:35:10 (v/v/v).

Synthesis and purification of oligonucleotides

Oligoribonucleotides were synthesized on an Applied Biosystems DNA/RNA synthesizer, using β-cyanoethyl phosphoramidite chemistry (62). For synthesis of standard RNA oligonucleotides, the commercially available phosphoramidites with 2′-O-tertbutyldimethylsilyl groups were used (Glen Research). For synthesis of 2′-O-methyl RNA oligonucleotides, the 3′-O-phosphoramidites of 2′-O-methylnucleotides were used (Glen Research and Proligo). The 3′-O-phosphoramidites of LNA nucleotides were synthesized according to the published procedures with some minor modifications (44,47,63). The details of deprotection and purification of oligoribonucleotides were described previously (64).

UV melting

Oligonucleotides were melted in buffer containing 100 mM NaCl, 20 mM sodium cacodylate, 0.5 mM Na_2EDTA, pH 7.0. The relatively low NaCl concentration kept melting temperatures in the reasonable range even when there were multiple LNA substitutions. Oligonucleotide single-strand concentrations were calculated from absorbencies above 80°C and single-strand extinction coefficients were approximated by a nearest-neighbor model (65,66). It was assumed that 2′-O-methyl RNA and RNA strands with identical sequences have identical extinction coefficients. Absorbancy versus temperature melting curves were measured at 260 nm with a heating rate of 1°C/min from 0 to 90°C on a Beckman DU 640 spectrophotometer with a water cooled thermoprogrammer. Melting curves were analyzed and thermodynamic parameters were calculated from a two-state model with the program MeltWin 3.5 (67). For almost all sequences, the ΔH° derived from T_m^{-1} versus ln (C_T/4) plots is within 15% of that derived from averaging the fits to individual melting curves, as expected if the two-state model is reasonable.

Parameter fitting

Free energy parameters for predicting stabilities of 2′-O-methyl RNA/RNA and 2′-O-methyl RNA–LNA/RNA duplexes with the Individual Nearest-Neighbor Hydrogen Bonding (INN-HB) model (64) were obtained by multiple linear regression with the program Analyse-it v.1.71 (Analyse-It Software, Ltd, Leeds, England; www.analyse-it.com) which expands Microsoft Excel. Analyse-It was also used to obtain parameters for enhancement of stabilities of 2′-O-methyl RNA/RNA duplexes by substitution of LNA nucleotides internally and/or at the 3′ end when the LNAs are separated by at least one 2′-O-methyl nucleotide. Results from T_m^{-1} versus ln (C_T/4) plots were used as the data for the calculations.

RESULTS

Figures 2 and 3 show typical data from optical melting curves, and Table 1 lists the thermodynamic parameters for the helix to coil transition with either no or one LNA nucleotide in the primarily 2′-O-methyl strand of a hybrid with a Watson–Crick complementary RNA strand.

Single LNA substitutions at the 5′ end of heptamer duplexes have little effect on stability

The effects of single LNA substitutions at the 5′ end of the 2′-O-methyl strand were studied in duplexes of the form, 5′$\underline{N}C^MU^MA^MC^MC^MA^M$/3′r($\underline{Q}$GAUGGU), where superscript M denotes a 2′-O-methyl sugar, N is A, C, G, or U with a 2′-O-methyl or LNA sugar, r denotes ribose sugars, and Q is the Watson–Crick complement to N. As summarized in Table 1, 5′ terminal LNA substitutions make duplex stability more favorable by 0.3–0.6 kcal/mol at 37°C with an average enhancement of 0.45 kcal/mol. Thus, 5′ terminal LNA substitutions increase the binding constant for duplex formation by ~2-fold at 37°C.

The effects of single LNA substitutions at the 3′ ends of heptamer duplexes is idiosyncratic

The effects of single LNA substitutions at the 3′ end of the 2′-O-methyl strand was studied in duplexes of the form, 5′$A^MC^MU^MA^MC^MC^M\underline{N}$/3′r(UGAUGG$\underline{Q}$) and in 5′$A^MC^M$-$U^MA^MC^MG^M\underline{U}^{M/L}$/3′r(UGAUGC$\underline{A}$) (Table 1). If N is A, C

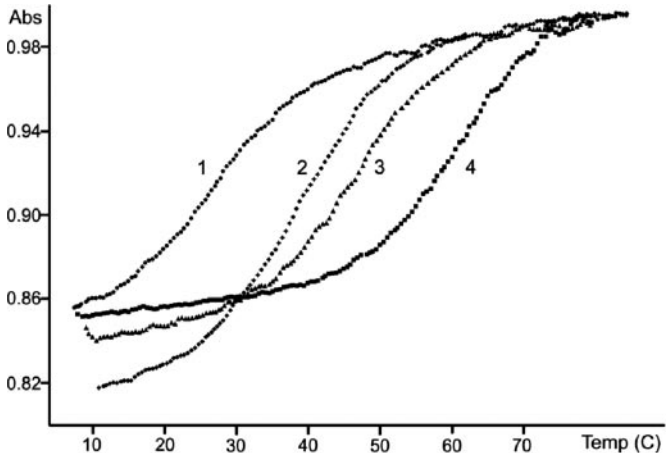

Figure 2. Representative UV melting curves 5′$A^MC^MU^M\underline{A}^LC^MC^MA^M$/5′UGG$\underline{C}$AGU (**1**, 52 μM), 5′$A^MC^MU^MA^MC^MC^MA^M$/5′UGGUAGU (**2**, 46 μM), 5′$A^MC^MU^M\underline{A}^LC^MC^MA^M$/5′UGGUAGU (**3**, 51 μM) and 5′$A^M\underline{C}^LU^M\underline{A}^IC^M\underline{C}^LA^M$/5′UGGUAGU (**4**, 51 μM). Note that sequence 1 has an A^LC mismatch in the middle.

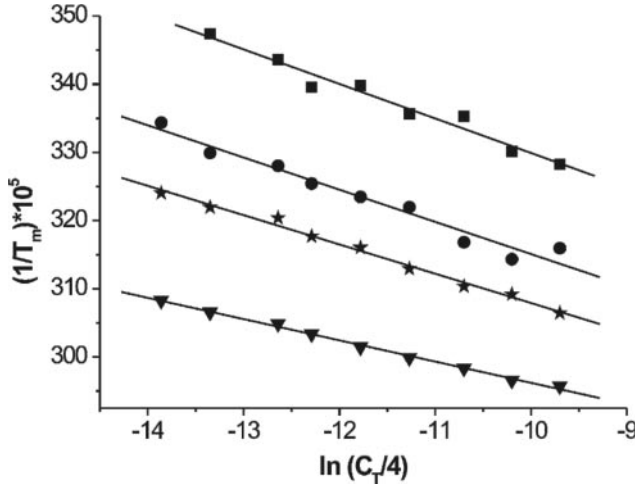

Figure 3. Reciprocal melting temperature versus ln (C_T/4) for 5′A^MC^M-$U^M\underline{A^L}C^MC^MA^M$/5′UGG$\underline{C}$AGU (solid squares), 5′$A^MC^MU^MA^MC^MC^MA^M$/5′UGGUAGU (solid circles), 5′$A^MC^MU^M\underline{A^L}C^MC^MA^M$/5′UGGUAGU (solid stars) and 5′$A^M\underline{C^L}U^M\underline{A^L}C^M\underline{C^L}A^M$/5′UGGUAGU (solid triangles) in 100 mM NaCl, 20 mM sodium cacodylate, 0.5 mM Na_2EDTA, pH 7.0. The thermodynamics of duplex formation were obtained by fitting the data points to the equation, $T_m^{-1} = (R/\Delta H^\circ) \ln (C_T/4) + \Delta S^\circ/\Delta H^\circ$. Here, T_m^{-1} is the inverse melting temperature in kelvin, R is the gas constant, 1.987 cal K^{-1} mol^{-1}, C_T is the total oligonucleotide strand concentration, and both strands have the same concentration.

or G, then LNA substitutions have similar effects. On average, an LNA substitution makes duplex stability more favorable by 1.2 kcal/mol at 37°C. In the two sequences with a 3′ terminal LNA U on the 2′-O-methyl strand, duplex stability is, however, affected little, averaging a destabilization of 0.08 kcal/mol at 37°C. In both cases, the terminal U is preceded by a GC pair, but both orientations of the GC pair give similar destabilization upon LNA substitution at the 3′ terminal U.

Single LNA substitutions in the interior of $A^MC^MU^MA^MC^MC^MA^M$ enhance the stability of the duplex formed with its complementary RNA by ~1.4 kcal/mol

The effect of interior position on the free energy increment for a single LNA substitution for a 2′-O-methyl RNA was studied for the duplex 5′$A^MC^MU^MA^MC^MC^MA^M$/3′r(UGAUGGU). As summarized in Table 1, a single interior LNA substitution makes duplex stability more favorable by 1.2–1.7 kcal/mol at 37°C, with an average of 1.4 kcal/mol. This corresponds to roughly a 10-fold increase in binding constant. Thus, interior and 3′ terminal LNA substitutions usually improve binding more than 5′ terminal LNA substitutions.

The effects of single LNA substitutions as a function of sequence context in the middle of heptamer duplexes

The effects from single LNA substitutions in the middle of heptamer duplexes were studied as a function of the LNA base and the 3′ adjacent 2′-O-methyl nucleotide in duplexes of the form, 5′$A^MC^MU^M\underline{NV}^MC^MA^M$/3′r(UGA$\underline{QW}$GU), where V^M is 2′-O-methyl A, C, G or U, and W is the ribose Watson–Crick complement to V. The effects on the ΔG°_{37} for duplex formation of substituting LNA for 2′-O-methyl at position N are summarized in Table 1. For 13 of 16 sequences, the LNA substitution makes duplex stability more favorable by 1.0–1.5 kcal/mol at 37°C, with an average enhancement of 1.3 kcal/mol. The enhancement for the other three sequences averages 2.1 kcal/mol at 37°C.

The dependence on the 5′ nearest-neighbor nucleotide of effects from substituting U^L for U^M was studied in duplexes of the form, 5′$A^MC^M\underline{V^MU^{L/M}}C^MC^MA^M$/3′r(UG$\underline{WA}$GGU) (Table 1). For three of the four sequences, the range for enhancement of duplex stability is 0.8–1.3 kcal/mol and the average is 1.0 kcal/mol. The exception is for the nearest neighbor 5′G^MU^L/3′r(CA), where LNA substitution destabilizes the duplex by 0.2 kcal/mol.

The results for single LNA substitutions in other contexts provide additional insight into the dependence of free energy increments on the 5′ nearest neighbor 2′-O-methyl nucleotide. In particular, the Watson–Crick complementary duplexes with 5′$A^M\underline{C^LU^M}A^MC^MC^MA^M$ and 5′$A^MC^MU^M\underline{C^LU^M}C^MA^M$ strands have a 5′C^LU^M/3′r(GA) nearest neighbor that is preceded by A^M and U^M, respectively. The LNA substitution for 2′-O-methyl in these cases results in duplex formation being more stable by 1.29 and 1.48 kcal/mol, respectively, at 37°C. Similarly, the duplexes with 5′$A^M\underline{G^LU^M}A^MC^MC^MA^M$ and 5′$A^MC^MU^M\underline{G^LU^M}C^MA^M$ each have a 5′G^LU^M/3′r(CA) nearest neighbor that is preceded by A^M and U^M, respectively. In both cases, the LNA substitution enhances duplex stability by 1.14 kcal/mol at 37°C. Thus, for seven duplexes, the enhanced stability from an LNA substitution is relatively independent of the nearest-neighbor nucleotide 5′ to the LNA. The one exception is for the nearest neighbor 5′G^MU^L/3′r(CA). Interestingly, this nearest-neighbor combination is also destabilized by LNA substitution at a 3′ terminal U (Table 1). Evidently, an LNA substitution in the middle of a 2′-O-methyl strand usually affects heteroduplex stability with an RNA strand by about the same amount as an LNA substitution at a 3′ terminus.

The effects of LNA substitutions are approximately additive when LNA nucleotides are spaced by at least one 2′-O-methyl nucleotide

Table 2 contains thermodynamic parameters measured for duplexes having more than one LNA substitution and Table 3 compares the stabilities at 37°C with those predicted from four simple models. The first model, labeled 'additivity', predicts the ΔG°_{37} for duplex formation in the 5′ACUACCA/3′UGAUGGU series by adding the free energy increments measured for single LNA substitutions in the same context to the ΔG°_{37} for duplex formation in the absence of LNA nucleotides. The second model predicts the ΔG°_{37} (kcal/mol) for duplex formation with the following equation as deduced from fitting the data in Tables 1 and 2 excluding sequences with adjacent LNAs.

$$\Delta G^\circ_{37}(\text{chimera/RNA}) = \Delta G^\circ_{37}(\text{2′-O-MeRNA/RNA}) - 0.45n_{5'\text{tL}} - 1.10n_{\text{iAL/UL}} - 1.60n_{\text{iGL/CL}} - 1.20n_{3'\text{tAL/CL/GL}} + 0.08n_{3'\text{tUL}} \quad \mathbf{1}$$

Here, ΔG°_{37} (2′-O-MeRNA/RNA) is the free energy change at 37°C for duplex formation in the absence of any LNA nucleotides, $n_{5'\text{tL}}$ is the number of 5′ terminal LNAs, $n_{\text{iAL/UL}}$ and $n_{\text{iGL/CL}}$ are the number of internal LNAs in AU and GC pairs, respectively, $n_{3'\text{tU}}$ and $n_{3'\text{tAL/CL/GL}}$ are the number of

Table 1. Thermodynamic parameters of duplex formation between RNA and 2′-O-methyl oligoribonucleotides with and without a single LNA substitution[a]

Oligonucleotides (5′ to 3′)	RNA (5′ to 3′)	Average of curve fits $-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)	T_m^{-1} versus log (C_T/4) plots $-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)[c]	ΔT_m[b] (°C)
ACUACCA	UGGUAGU	58.5 ± 14.4	166.8 ± 45.7	6.80 ± 0.15	38.5	56.4 ± 6.6 (53.8)	160.0 ± 21.4 (146.6)	6.74 ± 0.23 (8.27)	38.2 (50.0)		
$A^MC^MU^MA^MC^MC^MA^M$	UGGUAGU	51.5 ± 7.7	143.0 ± 24.3	7.18 ± 0.21	41.0	44.4 ± 1.3 (53.8)	120.2 ± 4.0 (146.6)	7.13 ± 0.01 (8.27)	41.3 (50.0)		
$\underline{A^L}C^MU^MA^MC^MC^MA^M$	UGGUAGU	54.1 ± 4.9	149.3 ± 15.8	7.80 ± 0.10	44.4	52.1 ± 2.0	143.2 ± 6.5	7.72 ± 0.03	44.2	−0.59	2.9
$C^MC^MU^MA^MC^MC^MA^M$	UGGUAGG	65.5 ± 7.1	181.2 ± 21.8	9.27 ± 0.36	50.5	60.5 ± 3.5 (59.5)	165.9 ± 11.0 (160.3)	9.07 ± 0.13 (9.74)	50.6 (57.2)		
$\underline{C^L}C^MU^MA^MC^MC^MA^M$	UGGUAGG	66.5 ± 3.6	183.1 ± 10.8	9.74 ± 0.27	52.7	60.9 ± 5.6	165.9 ± 17.2	9.47 ± 0.28	52.7	−0.40	2.1
$G^MC^MU^MA^MC^MC^MA^M$	UGGUAGC	62.3 ± 6.9	171.5 ± 21.2	9.10 ± 0.36	50.4	54.3 ± 2.2 (61.0)	146.7 ± 6.9 (164.6)	8.78 ± 0.07 (9.90)	50.4 (57.6)		
$\underline{G^L}C^MU^MA^MC^MC^MA^M$	UGGUAGC	60.5 ± 4.6	164.6 ± 13.7	9.45 ± 0.34	52.7	52.7 ± 1.7	140.4 ± 5.3	9.11 ± 0.06	53.0	−0.33	2.6
$U^MC^MU^MA^MC^MC^MA^M$	UGGUAGA	50.7 ± 5.9	140.0 ± 18.4	7.25 ± 0.19	41.5	45.2 ± 1.6 (54.8)	122.5 ± 5.3 (149.6)	7.18 ± 0.03 (8.38)	41.5 (50.4)		
$\underline{U^L}C^MU^MA^MC^MC^MA^M$	UGGUAGA	55.7 ± 3.3	154.4 ± 10.0	7.84 ± 0.19	44.4	47.2 ± 3.0	127.5 ± 9.6	7.64 ± 0.08	44.5	−0.46	3.0
$A^MC^MU^MA^MC^MC^M\underline{A^L}$	UGGUAGU	60.7 ± 5.5	168.1 ± 17.3	8.54 ± 0.15	47.6	55.2 ± 2.6	150.9 ± 8.1	8.43 ± 0.06	48.0	−1.30	6.7
$A^MC^MU^MA^MC^MC^MC^M$	GGGUAGU	62.9 ± 4.0	176.1 ± 12.6	8.30 ± 0.09	46.0	61.2 ± 2.3 (60.4)	170.4 ± 7.2 (163.0)	8.29 ± 0.03 (9.87)	46.2 (57.6)		
$A^MC^MU^MA^MC^MC^M\underline{C^L}$	GGGUAGU	71.3 ± 4.9	200.4 ± 15.2	9.17 ± 0.23	48.9	76.6 ± 7.0	216.8 ± 21.9	9.34 ± 0.27	48.8	−1.05	2.6
$A^MC^MU^MA^MC^MC^MG^M$	CGGUAGU	56.8 ± 2.8	157.1 ± 8.9	8.11 ± 0.11	45.9	53.0 ± 2.5 (57.7)	145.3 ± 7.9 (157.0)	8.04 ± 0.05 (8.97)	46.1 (53.2)		
$A^MC^MU^MA^MC^MC^M\underline{G^L}$	CGGUAGU	61.4 ± 3.9	167.6 ± 11.8	9.44 ± 0.19	52.4	58.3 ± 1.3	158.0 ± 3.9	9.29 ± 0.04	52.4	−1.25	6.3
$A^MC^MU^MA^MC^MC^MU^M$	AGGUAGU	59.6 ± 5.7	168.4 ± 18.9	7.41 ± 0.28	41.2	62.2 ± 7.3 (53.8)	176.8 ± 23.6 (146.9)	7.37 ± 0.26 (8.24)	41.2 (49.8)		
$A^MC^MU^MA^MC^MC^M\underline{U^L}$	AGGUAGU	59.2 ± 5.2	167.7 ± 17.2	7.19 ± 0.17	40.5	55.6 ± 2.6	155.8 ± 8.5	7.24 ± 0.04	41.0	+0.13	−0.2
$A^MC^MU^MA^MC^MG^MU^M$	ACGUAGU	56.0 ± 5.3	157.7 ± 16.7	7.08 ± 0.16	40.1	50.9 ± 2.1 (52.0)	141.3 ± 6.9 (143.4)	7.05 ± 0.04 (7.50)	40.2 (45.6)		
$A^MC^MU^MA^MC^MG^M\underline{U^L}$	ACGUAGU	55.9 ± 3.5	157.6 ± 11.0	7.00 ± 0.17	39.6	51.1 ± 3.9	142.0 ± 12.8	7.03 ± 0.09	40.1	+0.02	−0.1
$A^M\underline{C^L}U^MA^MC^MC^MA^M$	UGGUAGU	58.3 ± 5.4	160.3 ± 16.9	8.57 ± 0.22	48.2	53.1 ± 2.9	143.9 ± 9.3	8.42 ± 0.09	48.4	−1.29	7.1
$A^MC^M\underline{U^L}A^MC^MC^MA^M$	UGGUAGU	54.4 ± 4.3	147.4 ± 13.7	8.62 ± 0.11	49.4	57.4 ± 2.7	157.1 ± 8.6	8.66 ± 0.08	48.9	−1.53	7.6
$A^MC^MU^M\underline{A^L}C^MC^MA^M$	UGGUAGU	58.9 ± 3.5	162.4 ± 11.2	8.53 ± 0.19	47.9	50.2 ± 1.8	134.8 ± 5.6	8.35 ± 0.05	48.7	−1.22	7.4
$A^MC^MU^MA^M\underline{C^L}C^MA^M$	UGGUAGU	58.8 ± 2.9	161.5 ± 8.9	8.75 ± 0,19	49.2	61.1 ± 3.8	168.8 ± 12.0	8.80 ± 0.13	49.0	−1.67	7.7
$A^MC^MU^MA^MC^M\underline{C^L}A^M$	UGGUAGU	58.6 ± 4.5	160.3 ± 13.9	8.89 ± 0.24	50.0	57.6 ± 3.7	157.3 ± 11.7	8.82 ± 0.13	49.9	−1.42	6.8
$A^MC^MU^MA^MA^MC^MA^M$	UGUUAGU	50.1 ± 4.8	144.7 ± 15.9	5.23 ± 0.18	29.2	48.9 ± 3.7 (47.2)	140.9 ± 12.5 (133.0)	5.23 ± 0.17 (5.94)	29.0 (35.9)		
$A^MC^MU^M\underline{A^L}A^MC^MA^M$	UGUUAGU	48.7 ± 5.2	135.6 ± 16.3	6.64 ± 0.15	37.7	42.2 ± 1.4	114.7 ± 4.7	6.65 ± 0.03	37.9	−1.42	8.9
$A^MC^MU^MA^MG^MC^MA^M$	UGCUAGU	75.8 ± 25.1	217.1 ± 81.4	8.47 ± 0.35	45.2	75.2 ± 6.3 (54.3)	216.0 ± 20.1 (148.5)	8.22 ± 0.15 (8.27)	44.1 (49.9)		
$A^MC^MU^M\underline{A^L}G^MC^MA^M$	UGCUAGU	58.4 ± 3.0	158.3 ± 9.3	9.30 ± 0.13	52.4	55.8 ± 1.3	150.4 ± 4.0	9.18 ± 0.05	52.5	−0.96	8.4
$A^MC^MU^MA^MU^MC^MA^M$	UGAUAGU	53.6 ± 3.5	154.1 ± 11.3	5.76 ± 0.07	32.6	52.1 ± 2.9 (50.8)	149.2 ± 9.7 (143.7)	5.82 ± 0.10 (6.22)	32.8 (37.7)		
$A^MC^MU^M\underline{A^L}U^MC^MA^M$	UGAUAGU	50.3 ± 3.4	139.3 ± 10.7	7.03 ± 0.14	40.1	45.5 ± 1.6	124.0 ± 5.2	7.02 ± 0.03	40.4	−1.20	7.6
$A^MC^MU^MC^MA^MC^MA^M$	UGUGAGU	64.7 ± 11.7	185.0 ± 37.6	7.33 ± 0.20	40.9	63.6 ± 6.1 (55.6)	181.7 ± 20.0 (152.9)	7.22 ± 0.17 (8.14)	40.4 (48.8)		
$A^MC^MU^M\underline{C^L}A^MC^MA^M$	UGUGAGU	58.0 ± 4.6	159.0 ± 14.7	8.70 ± 0.08	49.0	56.3 ± 2.3	153.8 ± 7.4	8.65 ± 0.04	49.1	−1.43	8.7
$A^MC^MU^MC^MC^MC^MA^M$	UGGGAGU	61.5 ± 6.8	166.8 ± 20.6	9.78 ± 0.42	54.3	55.9 ± 1.8 (60.5)	149.5 ± 5.7 (161.8)	9.51 ± 0.07 (10.31)	54.5 (60.2)		
$A^MC^MU^M\underline{C^L}C^MC^MA^M$	UGGGAGU	69.2 ± 2.7	184.9 ± 8.3	11.88 ± 0.18	63.0	66.4 ± 4.7	176.4 ± 14.1	11.68 ± 0.33	63.1	−2.17	8.6

Table 1. *Continued*

Oligonucleotides (5′ to 3′)	RNA (5′ to 3′)	Average of curve fits				T_m^{-1} versus log (C_T/4) plots					
		$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)[c]	ΔT_m[b] (°C)
$A^MC^MU^MC^MG^MC^MA^M$	UGCGAGU	64.2 ± 20.4	176.7 ± 64.2	9.36 ± 0.56	51.3	56.0 ± 2.5 (59.2)	152.5 ± 7.9 (160.1)	9.04 ± 0.7 (9.57)	51.5 (56.3)		
$A^MC^MU^MC^{L}G^MC^MA^M$	UGCGAGU	71.4 ± 2.5	193.1 ± 7.4	11.53 ± 0.20	60.4	65.0 ± 5.9	173.8 ± 17.9	11.13 ± 0.37	60.6	−2.09	9.1
$A^MC^MU^MC^MU^MC^MA^M$	UGAGAGU	50.8 ± 7.5	138.9 ± 24.1	7.72 ± 0.13	44.5	53.0 ± 1.7 (56.6)	146.1 ± 5.6 (156.1)	7.66 ± 0.02 (8.22)	43.8 (49.0)		
$A^MC^MU^MC^{L}U^MC^MA^M$	UGAGAGU	64.1 ± 3.3	167.4 ± 10.4	9.23 ± 0.01	51.3	58.1 ± 1.7	158.0 ± 5.3	9.14 ± 0.04	51.6	−1.48	7.8
$A^MC^MU^MG^MA^MC^MA^M$	UGUCAGU	54.2 ± 5.9	150.7 ± 18.5	7.46 ± 0.17	42.4	48.2 ± 0.7 (55.6)	131.8 ± 2.3 (152.9)	7.36 ± 0.00 (8.14)	42.4 (48.8)		
$A^MC^MU^MG^{L}A^MC^MA^M$	UGUCAGU	57.8 ± 5.3	157.0 ± 16.2	9.08 ± 0.27	51.3	52.6 ± 1.5	141.0 ± 4.8	8.86 ± 0.05	51.4	−1.50	9.0
$A^MC^MU^MG^MC^MC^MA^M$	UGGCAGU	59.1 ± 5.0	160.3 ± 15.2	9.41 ± 0.26	52.9	58.7 ± 2.3 (60.0)	159.2 ± 7.1 (160.4)	9.34 ± 0.09 (10.23)	52.6 (59.9)		
$A^MC^MU^MG^{L}C^MC^MA^M$	UGGCAGU	60.6 ± 2.2	161.1 ± 6.7	10.66 ± 0.20	59.6	55.5 ± 1.9	145.6 ± 5.9	10.35 ± 0.11	59.9	−1.01	7.3
$A^MC^MU^MG^MG^MC^MA^M$	UGCCAGU	60.5 ± 4.6	163.3 ± 14.0	9.86 ± 0.27	55.0	59.6 ± 1.5 (60.0)	160.7 ± 4.5 (160.4)	9.76 ± 0.06 (10.23)	54.8 (59.9)		
$A^MC^MU^MG^{L}G^MC^MA^M$	UGCCAGU	62.8 ± 2.3	164.5 ± 6.7	11.76 ± 0.25	65.2	63.7 ± 2.6	167.4 ± 7.7	11.82 ± 0.19	65.1	−2.06	10.3
$A^MC^MU^MG^MU^MC^MA^M$	UGACAGU	57.5 ± 6.3	159.5 ± 19.6	7.98 ± 0.25	45.0	52.5 ± 2.6 (55.6)	144.1 ± 8.2 (152.9)	7.83 ± 0.06 (8.14)	44.9 (48.8)		
$A^MC^MU^MG^{L}U^MC^MA^M$	UGACAGU	56.0 ± 6.1	150.9 ± 18.5	9.21 ± 0.34	52.6	51.3 ± 2.5	136.4 ± 7.9	8.97 ± 0.10	52.5	−1.14	7.6
$A^MC^MU^MU^MA^MC^MA^M$	UGUAAGU	50.4 ± 5.1	144.6 ± 16.5	5.58 ± 0.10	31.3	46.7 ± 2.1 (47.2)	132.2 ± 7.1 (133.0)	5.65 ± 0.08 (5.94)	31.3 (35.9)		
$A^MC^MU^MU^{L}A^MC^MA^M$	UGUAAGU	50.3 ± 6.2	139.8 ± 19.6	6.94 ± 0.13	39.5	46.8 ± 1.5	128.8 ± 4.7	6.88 ± 0.02	39.3	−1.23	8.0
$A^MC^MU^MU^MC^MC^MA^M$	UGGAAGU	50.2 ± 3.9	138.0 ± 12.2	7.42 ± 0.12	42.6	47.6 ± 1.3 (53.9)	129.6 ± 4.2 (148.1)	7.36 ± 0.01 (7.98)	42.5 (48.2)		
$A^MC^MU^MU^{L}C^MC^MA^M$	UGGAAGU	56.0 ± 3.2	152.2 ± 10.2	8.83 ± 0.14	50.3	50.5 ± 1.1	134.7 ± 3.6	8.68 ± 0.03	50.8	−1.32	8.3
$A^MC^MU^MU^MG^MC^MA^M$	UGCAAGU	61.2 ± 3.3	172.4 ± 10.0	7.71 ± 0.15	43.1	52.7 ± 0.7 (53.4)	145.3 ± 2.3 (146.7)	7.59 ± 0.01 (7.90)	43.4 (47.8)		
$A^MC^MU^MU^{L}G^MC^MA^M$	UGCAAGU	61.0 ± 2.3	167.7 ± 7.4	9.00 ± 0.12	50.1	59.5 ± 1.6	163.1 ± 5.0	8.96 ± 0.05	50.2	−1.37	6.8
$A^MC^MU^MU^MU^MC^MA^M$	UGAAAGU	46.1 ± 4.5	130.5 ± 14.8	5.59 ± 0.12	30.8	39.6 ± 1.0 (47.4)	109.2 ± 3.2 (134.5)	5.76 ± 0.04 (5.65)	31.1 (34.1)		
$A^MC^MU^MU^{L}U^MC^MA^M$	UGAAAGU	46.1 ± 5.3	126.5 ± 16.9	6.88 ± 0.16	39.4	39.9 ± 1.1	106.5 ± 3.4	6.85 ± 0.02	39.5	−1.09	8.4
$A^MC^MA^MU^MC^MC^MA^M$	UGGAUGU	47.9 ± 6.1	132.1 ± 19.7	6.92 ± 0.07	39.5	43.5 ± 0.8 (56.4)	118.2 ± 2.7 (155.6)	6.86 ± 0.01 (8.18)	39.4 (48.8)		
$A^MC^MA^MU^{L}C^MC^MA^M$	UGGAUGU	50.4 ± 5.1	136.9 ± 16.4	7.96 ± 0.12	46.0	46.4 ± 2.1	124.2 ± 6.7	7.85 ± 0.05	46.1	−0.99	6.7
$A^MC^MC^MU^MC^MC^MA^M$	UGGAGGU	59.7 ± 6.2	162.0 ± 19.4	9.49 ± 0.25	53.2	50.9 ± 2.3 (60.5)	134.6 ± 7.0 (161.8)	9.20 ± 0.09 (10.31)	54.1 (60.2)		
$A^MC^MC^MU^{L}C^MC^MA^M$	UGGAGGU	58.9 ± 5.1	156.5 ± 16.3	10.45 ± 0.08	59.1	50.2 ± 3.2	129.7 ± 9.9	10.02 ± 0.18	60.2	−0.82	6.1
$A^MC^MG^MU^MC^MC^MA^M$	UGGACGU	56.9 ± 5.4	154.4 ± 16.8	9.02 ± 0.23	51.2	55.8 ± 2.3 (58.7)	151.1 ± 7.2 (158.3)	8.96 ± 0.08 (9.57)	51.1 (56.5)		
$A^MC^MG^MU^{L}C^MC^MA^M$	UGGACGU	55.4 ± 5.1	149.5 ± 15.6	9.05 ± 0.29	51.8	50.4 ± 0.8	134.2 ± 2.6	8.78 ± 0.04	51.5	+0.18	0.4
$A^MG^MU^MA^MC^MC^MA^M$	UGGUACU	51.4 ± 8.1	141.6 ± 25.5	7.44 ± 0.20	42.6	44.5 ± 0.7 (53.8)	119.9 ± 2.3 (146.6)	7.33 ± 0.01 (8.27)	42.7 (50.0)		
$A^{M}G^{L}U^MA^MC^MC^MA^M$	UGGUACU	52.6 ± 6.5	141.9 ± 20.2	8.55 ± 0.25	49.4	51.7 ± 1.8	139.3 ± 5.7	8.47 ± 0.05	49.1	−1.14	6.4
$U^MC^MG^MG^MC^MA^M$	UGCCGA	65.6 ± 18.0	185.2 ± 56.9	8.18 ± 0.46	45.0	65.5 ± 5.7 (50.7)	185.1 ± 18.2 (136.2)	8.06 ± 0.16 (8.51)	44.4 (52.4)		
$U^MC^MG^MG^MC^{L}A^M$	UGCCGA	60.9 ± 5.3	164.2 ± 15.9	10.00 ± 0.39	55.7	57.5 ± 7.2	153.8 ± 22.0	9.80 ± 0.43	55.7	−1.74	11.3

[a]Solutions are 100 mM NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7. Values in parentheses are thermodynamics predicted for RNA/RNA duplexes in 1 M NaCl (64).
[b]Calculated for 10^{-4} M oligomer concentration. The ΔT_m is the difference in T_m due to the LNA substitution.
[c]The $\Delta\Delta G^\circ_{37}$ is the difference in ΔG°_{37} due to the LNA substitution.

Table 2. Thermodynamic parameters of duplex formation between RNA and 2′-O-methyl oligoribonucleotides with and without multiple LNA substitutions[a]

Oligonucleotides (5′ to 3′)	RNA (5′ to 3′)	Average of curve fits				T_m^{-1} versus log (C_T/4) plots			
		$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)
A^{L}C^M**U^L**A^M**C^L**C^M**A^L**	UGGUAGU	60.7 ± 7.0	157.5 ± 21.0	11.85 ± 0.53	66.8	59.5 ± 4.9	154.0 ± 14.7	11.68 ± 0.40	66.4
A^M**C^L**U^M**A^L**C^M**C^L**A^M**	UGGUAGU	66.3 ± 3.9	176.5 ± 11.7	11.55 ± 0.31	62.4	65.0 ± 2.5	172.7 ± 7.4	11.45 ± 0.16	62.4
A^M**C^L**U^{M}A^M**C^L**C^{M}A^M**	UGGUAGU	65.5 ± 7.6	179.3 ± 23.2	9.89 ± 0.35	53.8	77.5 ± 2.7	216.5 ± 8.6	10.36 ± 0.10	53.0
A^{L}C^{M}U^{M}A^M**C^L**C^{M}A^M**	UGGUAGU	60.7 ± 7.8	165.2 ± 23.7	9.42 ± 0.54	52.5	53.0 ± 1.6	141.7 ± 4.9	9.06 ± 0.06	52.5
A^{M}C^M**U^L**A^{M}C^M**C^L**A^M**	UGGUAGU	55.4 ± 1.6	145.5 ± 5.0	10.24 ± 0.09	59.3	53.1 ± 1.3	138.7 ± 3.9	10.12 ± 0.07	59.5
A^{L}C^{M}U^M**A^L**C^{M}C^M**A^L**	UGGUAGU	56.7 ± 7.2	150.0 ± 21.8	10.12 ± 0.45	58.0	53.6 ± 2.5	141.1 ± 7.7	9.87 ± 0.13	57.6
A^{L}C^{L}U^{L}A^{L}C^{L}C^{L}A^L	UGGUAGU	57.5 ± 3.2	146.8 ± 9.3	11.98 ± 0.35	69.5	51.0 ± 1.8	127.7 ± 5.4	11.40 ± 0.16	69.8
G^{M}C^{M}U^{M}A^{M}C^{M}U^{M}G^M	CAGUAGC	63.9 ± 2.6	178.6 ± 8.2	8.49 ± 0.09	46.8	66.2 ± 2.6 (61.8)	186.0 ± 8.2 (169.6)	8.51 ± 0.06 (9.17)	46.6 (53.2)
G^M**C^L**U^M**A^L**C^M**U^L**G^M**	CAGUAGC	81.3 ± 4.0	221.8 ± 12.2	12.52 ± 0.27	61.6	74.6 ± 4.9	201.6 ± 14.7	12.03 ± 0.34	61.7
G^M**C^L**U^{M}A^{M}C^M**U^L**G^M**	CAGUAGC	67.3 ± 7.8	182.7 ± 23.8	10.59 ± 0.42	56.9	64.6 ± 5.5	174.7 ± 16.9	10.44 ± 0.28	57.0
G^{L}C^{L}U^{L}A^{L}C^{L}U^{L}G^L	CAGUAGC	66.3 ± 5.7	171.7 ± 16.5	13.10 ± 0.58	71.1	58.6 ± 2.5	149.2 ± 7.3	12.37 ± 0.22	71.3
G^{M}C^{M}A^{M}U^{M}G^{M}G^M	CCAUGC	60.6 ± 1.1	172.4 ± 3.4	7.13 ± 0.16	40.1	51.9 ± 5.1 (54.9)	144.1 ± 16.6 (151.6)	7.19 ± 0.14 (7.91)	41.0 (47.5)
G^M**C^L**A^M**U^L**G^{M}G^M**	CCAUGC	57.1 ± 2.8	151.4 ± 8.7	10.13 ± 0.18	57.8	61.1 ± 5.4	163.7 ± 16.5	10.30 ± 0.31	57.4
G^{M}G^{M}C^{M}A^{M}U^{M}G^M	CAUGCC	60.7 ± 9.7	172.3 ± 31.5	7.29 ± 0.18	40.9	65.2 ± 4.1 (54.9)	187.1 ± 13.1 (151.6)	7.19 ± 0.07 (7.91)	40.2 (47.5)
G^{M}G^M**C^L**A^M**U^L**G^M**	CAUGCC	66.5 ± 4.9	180.9 ± 14.6	10.39 ± 0.33	56.1	68.8 ± 4.0	187.8 ± 12.4	10.49 ± 0.20	55.9
C^{M}G^{M}G^{M}C^{M}A^{M}U^M	AUGCCG	45.6 ± 2.6	124.4 ± 8.3	6.97 ± 0.08	40.0	45.3 ± 1.9 (51.4)	123.5 ± 6.0 (140.8)	6.98 ± 0.02 (7.71)	40.1 (47.0)
C^M**G^L**G^M**C^L**A^{M}U^M**	AUGCCG	47.6 ± 6.1	121.0 ± 18.3	10.10 ± 0.41	62.1	50.6 ± 5.5	130.2 ± 16.8	10.22 ± 0.35	61.4
U^{M}U^{M}C^{M}G^{M}G^{M}C^M	GCCGAA	59.3 ± 6.6	166.1 ± 20.6	7.83 ± 0.23	43.9	53.5 ± 8.0 (50.8)	147.3 ± 25.3 (138.8)	7.76 ± 0.33 (7.78)	44.3 (47.6)
U^{L}U^M**C^L**G^{M}G^{M}C^M**	GCCGAA	58.8 ± 8.4	155.6 ± 25.2	10.51 ± 0.59	59.5	55.7 ± 1.6	146.5 ± 4.9	10.26 ± 0.07	59.2
G^{M}U^{M}U^{M}C^{M}G^{M}G^M	CCGAAC	55.2 ± 4.9	155.5 ± 16.3	6.96 ± 0.17	39.4	54.6 ± 3.8 (51.1)	153.8 ± 12.5 (142.0)	6.95 ± 0.10 (7.05)	39.4 (42.9)
G^M**U^L**U^M**C^L**G^M**G^L**	CCGAAC	65.3 ± 6.1	170.6 ± 18.0	12.38 ± 0.50	67.5	61.7 ± 2.3	160.2 ± 6.7	12.00 ± 0.19	67.4
C^{M}G^{M}G^{M}C^{M}A^M	UGCCG	47.1 ± 8.8	131.6 ± 29.0	6.25 ± 0.19	35.1	46.0 ± 3.0 (42.0)	128.4 ± 9.8 (114.2)	6.19 ± 0.09 (6.61)	34.7 (40.8)
C^M**G^L**G^M**C^L**A^M**	UGCCG	46.5 ± 5.3	118.8 ± 16.0	9.61 ± 0.36	59.0	43.1 ± 5.3	108.8 ± 16.0	9.36 ± 0.42	58.8
U^{M}C^{M}G^{M}G^{M}C^M	GCCGA	50.9 ± 4.6	143.5 ± 14.9	6.39 ± 0.14	36.1	51.5 ± 7.9 (44.0)	145.4 ± 26.0 (119.9)	6.38 ± 0.35 (6.85)	36.1 (42.2)
U^M**C^L**G^M**G^L**C^M**	GCCGA	53.1 ± 5.3	139.4 ± 15.6	9.92 ± 0.47	58.1	49.5 ± 5.1	128.5 ± 15.2	9.64 ± 0.36	57.8

[a]Solutions are 100 mM NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7. Values in parentheses are thermodynamics predicted for RNA/RNA duplexes in 1 M NaCl (64).
[b]Calculated for 10^{-4} M oligomer concentration.

Table 3. Comparison of measured and predicted stabilities

Sequence	$-\Delta G^\circ_{37}$ (kcal/mol) Measured	Additivity	ΔG°_{37} (measured) − ΔG°_{37} (predicted) (kcal/mol) Equation 1	Equations 1 + 2 + 3	Equations 1 + 4
5′**A**^{L}C^{M}**U**^{L}A^{M}**C**^{L}C^{M}**A**^{L}/3′UGAUGGU	11.68	0.54	−0.20	0.22	−0.05
5′A^{M}**C**^{L}U^{M}**A**^{L}C^{M}**C**^{L}A^{M}/3′UGAUGGU	11.45	−0.39	−0.03	0.40	0.13
5′A^{M}**C**^{L}U^{M}A^{M}**C**^{L}C^{M}A^{M}/3′UGAUGGU	10.36	−0.27	−0.04	0.39	0.12
5′**A**^{L}C^{M}U^{M}A^{M}**C**^{L}C^{M}A^{M}/3′UGAUGGU	9.06	0.33	0.12	−0.54	0.27
5′A^{M}C^{M}**U**^{L}A^{M}C^{M}**C**^{L}A^{M}/3′UGAUGGU	10.12	−0.04	−0.30	0.13	−0.14
5′**A**^{L}C^{M}U^{M}**A**^{L}C^{M}C^{M}**A**^{L}/3′UGAUGGU	9.87	0.37	0.01	0.43	0.16
5′G^{M}**C**^{L}U^{M}**A**^{L}C^{M}**U**^{L}G^{M}/3′CGAUGAC	12.03	–	0.27	0.22	0.48
5′G^{M}**C**^{L}U^{M}A^{M}C^{M}**U**^{L}G^{M}/3′CGAUGAC	10.44	–	0.76	0.71	0.97
5′G^{M}**C**^{L}A^{M}**U**^{L}G^{M}G^{M}/3′CGUACC	10.30	–	−0.42	−0.29	−0.56
5′G^{M}G^{M}**C**^{L}A^{M}**U**^{L}G^{M}/3′CCGUAC	10.49	–	−0.61	−0.48	−0.75
5′C^{M}**G**^{L}G^{M}**C**^{L}A^{M}U^{M}/3′GCCGUA	10.22	–	−0.48	0.09	0.26
5′**U**^{L}U^{M}**C**^{L}G^{M}G^{M}C^{M}/3′AAGCCG	10.26	–	−0.45	−1.03	−0.66
5′G^{M}**U**^{L}U^{M}**C**^{L}G^{M}**G**^{L}/3′CAAGCC	12.00	–	−1.15	−1.65	−1.68
5′C^{M}**G**^{L}G^{M}**C**^{L}A^{M}/3′GCCGU	9.36	–	0.02	−0.03	0.39
5′U^{M}**C**^{L}G^{M}**G**^{L}C^{M}/3′AGCCG	9.64	–	−0.07	−0.07	0.13

3′ terminal LNAs that are U or not U, respectively. Both methods that use experimental data for ΔG°_{37} (2′-O-MeRNA/RNA) provide reasonable predictions that are within 1 kcal/mol of the measured value (Table 3). Two other methods that use nearest-neighbor models to approximate ΔG°_{37} (2′-O-MeRNA/RNA) provide somewhat less accurate, but still reasonable predictions as described below. The duplex with the worst prediction, 5′G^{M}U^{L}U^{M}C^{L}G^{M}G^{L}/3′CAAGCC has a 5′G^{M}U^{L}/3′CA nearest neighbor, consistent with this motif being unusually unstable by ∼1.2 kcal/mol. Thus, it is likely that the ΔG°_{37} of Equation 1 should be made less favorable by 1.2 kcal/mol for every internal 5′G^{M}U^{L}/3′CA nearest neighbor in a duplex. Evidently, the effects of multiple LNA substitutions are approximately additive when the LNAs are spaced by at least 1 nt.

The data may also be fit to a nearest-neighbor model containing 30 of the LNA enhancement parameters associated with duplexes of RNA strands bound to 2′-O-methyl RNA/LNA chimeras. These parameters are listed in Supplementary Material. The number of occurrences for each nearest neighbor is limited, however, so the values are only roughly determined.

Predictions for RNA/RNA duplexes at 1 M NaCl can be used to approximate stabilities of 2′-O-methyl RNA/RNA duplexes at 0.1 M NaCl

The stabilities of RNA/RNA duplexes at 37°C and 1 M NaCl are predicted well by an Independent Nearest-Neighbor Hydrogen Bonding (INN-HB) model (64). In this model, the stability of an RNA/RNA duplex is approximated by:

$$\Delta G^\circ_{37}(\mathrm{RNA/RNA}) = \Delta G^\circ_{\mathrm{init}} + \sum n_j \Delta G^\circ_j(\mathrm{NN}) + m_{\text{term-AU}} \Delta G^\circ_{\text{term-AU}} + \Delta G_{\mathrm{sym}} \quad \mathbf{2}$$

Here, $\Delta G^\circ_{\mathrm{init}}$ is the free energy change for initiating a helix; each $\Delta G^\circ_j(\mathrm{NN})$ is the free energy increment of the jth type nearest neighbor (see Table 4) with n_j occurrences in the sequence; $m_{\text{term-AU}}$ is the number of terminal AU pairs; $\Delta G^\circ_{\text{term}-\text{AU}}$ is the free energy increment per terminal AU pair; ΔG_{sym} is 0.43 kcal/mol at 37°C for self-complementary duplexes and 0 for non-self-complementary duplexes.

Table 4. Preliminary nearest-neighbor free energy parameters (kcal/mol at 37°C) for 2′-O-methyl RNA/RNA duplexes in 0.1 M NaCl compared with parameters for RNA/RNA duplexes in 1 M NaCl

2′-O-MeRNA/RNA parameters	ΔG°_{37} (kcal/mol)	RNA/RNA parameters[a]	ΔG°_{37} (kcal/mol)[a]
5′A^{M}A^{M}3′	(−0.79 ± 0.44)[b]	5′AA3′	−0.93 ± 0.03
3′U U 5′		3′UU5′	
5′U^{M}U^{M}3′	−0.99 ± 0.20		
3′A A 5′			
5′A^{M}U^{M}3′	−0.73 ± 0.26	5′AU3′	−1.10 ± 0.08
3′U A 5′		3′UA5′	
5′U^{M}A^{M}3′	−1.28 ± 0.29	5′UA3′	−1.33 ± 0.09
3′A U 5′		3′AU5′	
5′C^{M}U^{M}3′	−2.18 ± 0.26	5′CU3′	−2.08 ± 0.06
3′G A 5′		3′GA5′	
5′A^{M}G^{M}3′	(−1.53 ± 0.41)[b]		
3′U C 5′			
5′C^{M}A^{M}3′	−1.96 ± 0.26	5′CA3′	−2.11 ± 0.07
3′G U 5′		3′GU5′	
5′U^{M}G^{M}3′	−1.58 ± 0.36		
3′A C 5′			
5′G^{M}U^{M}3′	−2.65 ± 0.35	5′GU3′	−2.24 ± 0.06
3′C A 5′		3′CA5′	
5′A^{M}C^{M}3′	−1.60 ± 0.23		
3′U G 5′			
5′G^{M}A^{M}3′	(−2.62 ± 0.52)[b]	5′GA3′	−2.35 ± 0.06
3′C U 5′		3′CU5′	
5′U^{M}C^{M}3′	−1.97 ± 0.24		
3′A G 5′			
5′C^{M}G^{M}3′	−2.11 ± 0.39	5′CG3′	−2.36 ± 0.09
3′G C 5′		3′GC5′	
5′G^{M}G^{M}3′	−2.88 ± 0.27	5′GG3′	−3.26 ± 0.07
3′C C 5′		3′CC5′	
5′C^{M}C^{M}3′	−2.85 ± 0.19		
3′G G 5′			
5′G^{M}C^{M}3′	−3.49 ± 0.36	5′GC3′	−3.42 ± 0.08
3′C G 5′		3′CG5′	
Initiation	3.59 ± 0.95		4.09 ± 0.22
Per terminal AU	0.29 ± 0.15		0.45 ± 0.04
Symmetry correction ('self-complementary')	0		0.43
Symmetry correction (non-self-complementary)	0		0

[a]Xia *et al*. (64).
[b]There are only one or two occurrences of these nearest-neighbor sequences in the database.

Similar sequence dependent parameters may also be applicable to 2′-O-methyl RNA/RNA heteroduplexes because they are expected to have A-form conformations similar to those of RNA/RNA homoduplexes (68). This was tested by comparing the predicted stabilities of RNA/RNA duplexes in 1 M NaCl at 37°C with those measured for 2′-O-methyl RNA/RNA duplexes in 0.1 M NaCl at 37°C. The predicted thermodynamics are listed in parentheses in Tables 1 and 2. On average at 37°C, the RNA/RNA duplexes in 1 M NaCl are 0.12 ± 0.01 kcal/mol of phosphate pairs more stable than the 2′-O-methyl RNA/RNA duplexes in 0.1 M NaCl. Presumably, much of this difference is due to a sequence independent effect of salt concentration, which would primarily affect the ΔS° for duplex formation (22,69). Thus, a reasonable approximation for the first term on the right hand side of Equation 1 is:

$$\Delta G^\circ_{37}(2'\text{-O-MeRNA/RNA}, 0.1\text{ M NaCl}) \approx \Delta G^\circ_{37}(\text{RNA/RNA}, 1\text{ M NaCl}) + 0.12(\#\text{phosphate pairs}) - \Delta G_{\text{sym}}(\text{RNA/RNA}) \quad \mathbf{3}$$

Note that ΔG_{sym} from the RNA/RNA calculation is subtracted because a 2′-O-methyl RNA/RNA duplex cannot be self-complementary because the backbones differ. For the duplexes studied here, the number of phosphate pairs is one less than the number of base pairs.

The effects of LNA substitutions are likely not very dependent on salt concentration. Thus, it is probable that in 1 M NaCl or in the presence of Mg^{2+} (70) that ΔG°_{37} (2′-O-MeRNA/RNA) can be approximated by ΔG°_{37} (RNA/RNA, 1 M NaCl).

Table 3 compares measured values for duplexes with more than one LNA to predictions from combining Equation 1–3. The measured ΔG°_{37} values average −10.5 kcal/mol and the root-mean-square difference between measured and predicted ΔG°_{37} values is 0.6 kcal/mol with the largest difference being 1.7 kcal/mol. Again, the sequence with the largest difference contains a 5′G^MU^L/3′CA nearest neighbor so the prediction would be improved if Equation 1 was corrected for the apparent instability of this motif.

The results for 2′-O-methyl RNA/RNA duplexes provide preliminary nearest-neighbor free energy increments for predicting stabilities of such duplexes

The comparison of predicted RNA/RNA stabilities with those measured for 2′-O-methyl RNA/RNA duplexes suggests that the INN-HB model will also be applicable to 2′-O-methyl RNA/RNA duplexes (71). The results in Tables 1 and 2 were combined with those for several other duplexes (E. Kierzek, R. Kierzek, and D.H. Turner, unpublished data) to give preliminary INN-HB parameters for 2′-O-methyl RNA/RNA duplexes in 0.1 M NaCl (see Table 4). Three nearest neighbors are only represented once or twice in the database, and these parameters are in parentheses. The parameters for 2′-O-methyl RNA/RNA and RNA/RNA duplexes are similar, especially if the RNA/RNA Watson–Crick nearest-neighbor parameters are each made less favorable by 0.12 kcal/mol, which largely accounts for the difference in salt concentration as suggested above. Evidently, the first term on the right hand side of Equation 1 can also be approximated by:

$$\Delta G^\circ_{37}(2'\text{-O-MeRNA/RNA}) = \Delta G^\circ_{\text{init}} + \sum n_j \Delta G^\circ_j(\text{NN}) + m_{\text{term-AU}} \Delta G^\circ_{\text{term-AU}} \quad \mathbf{4}$$

Table 3 compares predictions from combining Equations 1 and 4 with measured values for duplexes with more than one LNA. The root-mean-square difference between measured and predicted ΔG°_{37} values is 0.6 kcal/mol with the largest difference being the 1.7 kcal/mol associated with the duplex containing a 5′G^MU^L/3′CA nearest neighbor. Undoubtedly, this model can be expanded and refined by more measurements, but it appears sufficient to aid sequence design for many applications.

Complete LNA substitution is no more stabilizing than substitution at every other nucleotide starting at the second nucleotide from the 5′ end

The effect of complete LNA substitution for a 2′-O-methyl RNA backbone was studied for the sequences 5′$A^LC^LU^LA^LC^LC^LA^L$/3′r(UGAUGGU) and 5′$G^LC^LU^LA^LC^LU^LG^L$/3′r(CGAUGAC). As summarized in Table 2, the stabilities of these duplexes at 37°C are within experimental error of those measured for 5′$A^MC^LU^MA^LC^MC^LA^M$/3′r(UGAUGGU) and 5′$G^MC^LU^MA^LC^MU^LG^M$/3′r(CGAUGAC), respectively. Evidently, the most effective use of LNA nucleotides is to space them every other nucleotide with the first LNA placed at the second nucleotide from the 5′ end.

Internal mismatches make duplex formation less favorable

Table 5 contains thermodynamic parameters measured for the formation of duplexes containing single mismatches and the difference in stabilities relative to completely Watson–Crick complementary duplexes (Tables 1 and 2). All internal mismatches make duplex formation less favorable by at least 2 kcal/mol at 37°C corresponding to at least a 25-fold less favorable equilibrium constant for duplex formation. In general, terminal mismatches destabilize much less than internal mismatches. In fact, when the 3′ terminal U^L of 5′$A^MC^MU^MA^MC^MC^MU^L$ makes a GU pair, the duplex is stabilized by 0.14 kcal/mol at 37°C relative to a terminal AU pair.

For four cases, the effect of a mismatch with an LNA nucleotide was compared with that for the equivalent 2′-O-methyl nucleotide. In each case, the mismatch penalty for the LNA was less than that for 2′-O-methyl RNA. However, for an A^M-G mismatch flanked by LNAs in the context 5′$A^LC^MU^L\underline{\mathbf{A^M}}C^LC^MA^L$/3′r(UGA**G**GGU), the LNAs enhanced the mismatch penalty by ∼1 kcal/mol relative to a completely 2′-O-methyl RNA strand. Thus, oligonucleotides containing LNA may discriminate best against mismatches flanked by LNAs.

DISCUSSION

Oligonucleotide hybridization to RNA has many applications, ranging from quantifying gene expression (18–20,56) to designing therapeutics (4,8,21,46,52–54). LNA nucleotides have characteristics useful for these purposes. For example, LNA usually stabilizes duplexes (4,44,48,51) and is more resistant than RNA and DNA to nuclease digestion (4,6,51). The results presented here provide insights that are useful for designing 2′-O-methyl RNA/LNA chimeric oligonucleotides for various purposes. Some trends may be general for RNA A-form helixes and thus may also be relevant to other chimeras with nucleotides that favor A-form conformations.

Table 5. Thermodynamic parameters of duplex formation between RNA and 2′-O-methyl oligoribonucleotides with and without LNA substitutions: effects of mismatches[a]

Mismatch	Oligonucleotides (5′ to 3′)	RNA (5′ to 3′)	Average of curve fits				T_m^{-1} versus log (C_T/4) plots					
			$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_m[b] (°C)	$\Delta\Delta G^\circ_{37}$[c] (kcal/mol)	ΔT_m[b] (°C)
A^L-C	$A^MC^MU^M\underline{A^L}C^MC^MA^M$	UGG**C**AGU	48.7 ± 4.5	140.9 ± 14.8	5.00 ± 0.17	27.5	44.7 ± 6.3	127.4 ± 20.9	5.15 ± 0.30	27.7	3.20	−21.0
A^L-C	$A^LC^MU^M\underline{A^L}C^MC^MA^L$	UGG**C**AGU	51.4 ± 8.6	144.8 ± 27.7	6.44 ± 0.18	36.4	47.8 ± 8.7	133.2 ± 28.1	6.47 ± 0.43	36.6	3.40	−21.0
A^L-G	$A^MC^MU^M\underline{A^L}G^MC^MA^M$	UGC**G**AGU	46.6 ± 20.5	127.5 ± 65.2	7.07 ± 0.32	40.6	40.2 ± 6.3	107.2 ± 20.6	6.93 ± 0.31	40.1	2.25	−12.4
A^M-G	$A^MC^MU^M\underline{A^M}G^MC^MU^M$	UGC**G**AGU	48.9 ± 4.9	144.6 ± 16.4	4.08 ± 0.27	22.2	45.3 ± 2.7	132.2 ± 9.2	4.32 ± 0.16	22.6	4.86	−29.9
A^M-G	$A^LC^MU^L\underline{A^M}C^LC^MA^L$	UGG**G**AGU	49.4 ± 3.8	135.4 ± 11.9	7.39 ± 0.16	42.5	40.8 ± 1.9	108.0 ± 6.2	7.29 ± 0.03	42.9	4.39	−23.5
A^M-G	$A^MC^MU^M\underline{A^M}C^MC^MA^M$	UGG**G**AGU	42.4 ± 4.3	122.9 ± 14.4	4.26 ± 0.25	21.2	49.3 ± 5.4	146.6 ± 18.5	3.81 ± 0.37	20.8	3.32	−20.5
C^L-A	$A^MC^MU^M\underline{C^L}C^MC^MA^M$	UGG**A**AGU	39.3 ± 6.2	110.0 ± 20.5	5.17 ± 0.22	26.6	42.5 ± 5.9	120.8 ± 19.7	5.01 ± 0.36	26.3	6.67	−36.8
C^L-A	$A^MC^MU^MA^MC^MC^M\underline{C^L}$	**A**GGUAGU	52.2 ± 6.3	147.2 ± 19.9	6.50 ± 0.36	36.8	44.5 ± 16.3	122.1 ± 52.6	6.55 ± 1.71	37.8	2.69	−11.0
C^L-U	$A^MC^MU^M\underline{C^L}G^MC^MA^M$	UGC**U**AGU	59.1 ± 8.9	177.2 ± 30.0	4.18 ± 0.37	25.1	62.2 ± 3.4	187.7 ± 11.5	4.00 ± 0.19	24.9	7.13	−35.7
C^M-U	$A^MC^MU^M\underline{C^M}G^MC^MA^M$	UGC**U**AGU	62.4 ± 5.7	188.7 ± 19.4	3.90 ± 0.29	24.4	69.2 ± 2.9	211.7 ± 9.8	3.55 ± 0.16	24.2	7.58	−36.4
C^L-U	$A^MC^MU^M\underline{C^L}U^MC^MA^M$	UGC**U**AGU	62.3 ± 10.6	187.0 ± 35.3	4.29 ± 0.46	26.2	62.9 ± 5.9	188.9 ± 19.7	4.32 ± 0.31	26.5	4.82	−25.1
G^L-A	$A^MC^MU^M\underline{G^L}C^MC^MA^M$	UGG**A**AGU	35.6 ± 7.6	98.7 ± 26.5	5.02 ± 0.63	24.4	37.5 ± 2.3	105.6 ± 7.9	4.73 ± 0.16	22.8	5.62	−37.1
G^L-A	$\underline{G^L}C^MU^MA^MC^MC^MA^M$	UGGUAG**A**	46.1 ± 5.1	127.3 ± 15.8	6.59 ± 0.16	37.4	43.8 ± 2.9	120.0 ± 9.5	6.59 ± 0.09	37.4	2.52	−15.6
G^L-U	$A^MC^MU^M\underline{G^L}C^MC^MA^M$	UGG**U**AGU	52.5 ± 4.3	145.8 ± 13.5	7.32 ± 0.12	41.7	49.7 ± 1.7	136.8 ± 5.4	7.27 ± 0.02	41.7	3.23	−18.4
G^M-U	$A^MC^MU^M\underline{G^M}C^MC^MA^M$	UGG**U**AGU	53.6 ± 8.2	152.4 ± 26.6	6.36 ± 0.09	36.0	53.5 ± 1.4	152.3 ± 4.5	6.28 ± 0.03	35.6	4.22	−24.5
G^L-U	$A^MC^MU^M\underline{G^L}U^MC^MA^M$	UGA**U**AGU	51.3 ± 3.6	146.4 ± 11.8	5.85 ± 0.10	32.9	47.7 ± 1.9	134.7 ± 6.3	5.93 ± 0.06	33.1	3.04	−19.4
G^L-U	$\underline{G^L}C^MU^MA^MC^MC^MA^M$	UGGUAG**U**	56.1 ± 6.4	156.8 ± 20.5	7.53 ± 0.26	42.6	48.0 ± 2.3	130.5 ± 7.3	7.48 ± 0.04	43.2	1.63	−9.8
U^L-C	$A^MC^MU^M\underline{U^L}A^MC^MA^M$	UGU**C**AGU	47.1 ± 10.4	141.0 ± 36.1	3.33 ± 0.77	17.2	53.0 ± 13.6	161.9 ± 46.4	2.84 ± 1.13	16.8	4.04	−22.5
U^L-C	$A^MC^MU^M\underline{U^L}U^MC^MA^M$	UGA**C**AGU	43.2 ± 11.6	125.9 ± 39.9	4.17 ± 0.88	20.9	43.2 ± 10.8	126.4 ± 37.0	4.01 ± 0.97	19.9	2.84	−19.6
U^L-G	$A^MC^MU^M\underline{U^L}G^MC^MA^M$	UGC**G**AGU	60.8 ± 11.0	174.2 ± 35.5	6.78 ± 0.19	38.3	55.9 ± 4.7	158.3 ± 15.5	6.76 ± 0.13	38.3	2.20	−11.9
U^M-G	$A^MC^MU^M\underline{U^M}G^MC^MA^M$	UGC**G**AGU	65.4 ± 7.9	195.8 ± 26.3	4.70 ± 0.21	28.5	61.6 ± 7.2	182.9 ± 24.1	4.84 ± 0.35	28.7	4.12	−21.5
U^L-G	$A^MC^MU^MA^MC^MC^M\underline{U^L}$	**G**GGUAGU	57.6 ± 11.2	161.3 ± 35.9	7.52 ± 0.29	42.4	58.4 ± 9.0	164.6 ± 29.0	7.38 ± 0.38	41.6	−0.14	0.6
U^L-U	$A^MC^MU^M\underline{U^L}G^MC^MA^M$	UGC**U**AGU	59.1 ± 3.8	175.7 ± 12.4	4.60 ± 0.14	27.2	71.5 ± 4.8	217.3 ± 16.1	4.07 ± 0.22	26.7	4.89	−23.5

[a]Solutions are 100 mM NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7.
[b]Calculated for 10^{-4} M oligomer concentration. The ΔT_m is the difference in T_m between the mismatched and fully Watzon-Crick complementary duplexes.
[c]The $\Delta\Delta G^\circ_{37}$ is the diffence in ΔG°_{37} between the mismatched and fully ΔG°_{37} between the mismatched and fully Watson-Crick complementary duplexes.

The results suggest several principles for the design of 2′-O-methyl RNA/LNA chimeras for hybridization to RNA

The database in Tables 1 and 2 is too small to generate a complete model for the design of 2′-O-methyl RNA/LNA chimeras. Nevertheless, several trends are apparent: (i) LNA substitution at the 5′ end has little effect on duplex stability. (ii) Except for a 3′ terminal U^L, interior and 3′ terminal LNA substitutions have similar effects. (iii) Most of the stability enhancement is achieved when LNA nucleotides are separated by one 2′-O-methyl nucleotide. (iv) The effects of LNA substitutions are approximately additive when the LNA nucleotides are separated by at least one 2′-O-methyl nucleotide. Thus, in most cases, LNA nucleotides are used most effectively for duplex stabilization when separated by at least one 2′-O-methyl nucleotide and not placed on the 5′ end of the chimera. The additivity observed suggests that a nearest-neighbor model (64,72–75) will be able to predict well the stabilities of duplexes formed between RNA and 2′-O-methyl RNA/LNA chimeras. While the database is not sufficient to determine all the parameters for such a model, the simpler model of Equation 1 provides reasonable approximations for stabilities at 37°C, though a correction should probably be applied for duplexes containing a $5'G^MU^L/3'CA$ nearest neighbor. It also appears that approximations for the stabilities of 2′-O-methyl RNA/RNA duplexes can be provided by nearest-neighbor models for 2′-O-methyl RNA/RNA duplexes (Equation 4) or even for RNA/RNA duplexes (Equations 2 and 3).

The magnitude and sequence dependence of the stabilization due to LNAs are surprising. Ribose and therefore probably 2′-O-methyl ribose sugars in single strands are typically found in roughly equal fractions in C2′-endo and C3′-endo conformations. If the methylene bridge of an LNA only locks the sugar into the C3′-endo conformation, then the expected stabilization due to preorganization would be: $\Delta\Delta G^\circ = -RT \ln 2$, which is −0.4 kcal/mol at 37°C (310.15 K). The stabilization observed for a 5′ terminal LNA is roughly −0.4 kcal/mol, but the average stabilizations for internal LNAs and 3′ terminal A^L, C^L and G^L are more favorable at −1.3 and −1.2 kcal/mol, respectively. Moreover, if stabilization was only due to preorganization of an LNA sugar, then the effect would not saturate when alternate sugars are LNA. Evidently, the LNA substitution also affects the 5′ neighboring base pair in a way that enhances the stabilization beyond that expected from preorganization of a single sugar. Interestingly, NMR structures of DNA/LNA chimeras bound to RNA show that only the DNA sugar 3′ of the LNA is driven to a C3′-endo conformation for the sequence $d(5'CTGAT^LATGC)/3'GACUAUACG$, but all non-terminal DNA sugars are C3′-endo when all three Ts are LNAs (76).

Comparison with single LNA substitutions in DNA/DNA duplexes

McTigue *et al*. (48) measured the thermodynamic effects of single internal LNA substitutions in 100 DNA/DNA duplexes. The free energy increments at 37°C for LNA substitutions ranged from +0.83 to −1.90 kcal/mol with an average of −0.55 kcal/mol. This compares with a range from +0.18 to −2.17 kcal/mol and an average of −1.32 kcal/mol for the single internal LNA substitutions in Table 1. The comparision suggests that single LNA substitutions are on average more stabilizing to 2′-O-methyl RNA/RNA duplexes than to DNA/DNA duplexes. This may reflect the expectation that LNA substitutions do not have a large effect on the conformations of 2′-O-methyl RNA/RNA duplexes, but alter the conformations of DNA/DNA duplexes.

LNA substitutions should be useful for probing RNA with short 2′-O-methyl RNA oligonucleotides

RNA structure can be probed with short oligonucleotides on microarrays (3). To optimize such methods, it is necessary to have tight binding that is sequence independent and that discriminates against mismatches. It appears that LNA nucleotides can be used to achieve this. For example, free energy increments for 2′-O-methyl RNA/RNA nearest neighbors range from −0.7 to −3.5 kcal/mol, corresponding to $5'A^MU^M/3'UA$ and $5'G^MC^M/3'CG$, respectively (Table 4). The average increment of −1.3 kcal/mol of internal and 3′ terminal LNA can help compensate for such less favorable stability of AU relative to GC pairs. The stability enhancement from LNA can also allow the use of shorter oligonucleotides.

The potential disadvantage to LNA substitutions in 2′-O-methyl RNA oligonucleotides is that discrimination against mismatches containing an LNA may be less than with a complete 2′-O-methyl RNA backbone. This was clearly true for three of the four cases where such direct comparisons were made. Nevertheless, internal mismatches with LNA nucleotides are considerably destabilizing, averaging a penalty of 4.1 kcal/mol at 37°C (Table 5), which translates to almost a 1000-fold weaker binding due to a single mismatch. When LNAs flanked an A^M-G mismatch, the mismatch penalty at 37°C was 4.4 kcal/mol compared with 3.3 kcal/mol in the absence of LNAs. Such an effect may reflect enhanced rigidity due to LNA, which thereby prevents a mismatch from adopting a favorable conformation. Thus, it may be advantageous to use LNAs to flank nucleotides likely to give small mismatch penalties.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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