A chemical synthesis of LNA-2,6-diaminopurine riboside, and the influence of 2′-*O*-methyl-2,6-diaminopurine and LNA-2,6-diaminopurine ribosides on the thermodynamic properties of 2′-*O*-methyl RNA/RNA heteroduplexes

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ABSTRACT

Modified nucleotides are useful tools to study the structures, biological functions and chemical and thermodynamic stabilities of nucleic acids. Derivatives of 2,6-diaminopurine riboside (D) are one type of modified nucleotide. The presence of an additional amino group at position 2 relative to adenine results in formation of a third hydrogen bond when interacting with uridine. New method for chemical synthesis of protected 3′-*O*-phosphoramidite of LNA-2,6-diaminopurine riboside is described. The derivatives of 2′-*O*-methyl-2,6-diaminopurine and LNA-2,6-diaminopurine ribosides were used to prepare complete 2′-*O*-methyl RNA and LNA-2′-*O*-methyl RNA chimeric oligonucleotides to pair with RNA oligonucleotides. Thermodynamic stabilities of these duplexes demonstrated that replacement of a single internal 2′-*O*-methyladenosine with 2′-*O*-methyl-2,6-diaminopurine riboside (D^M) or LNA-2,6-diaminopurine riboside (D^L) increases the thermodynamic stability ($\Delta\Delta G^\circ_{37}$) on average by 0.9 and 2.3 kcal/mol, respectively. Moreover, the results fit a nearest neighbor model for predicting duplex stability at 37°C. D-A and D-G but not D-C mismatches formed by D^M or D^L generally destabilize 2′-*O*-methyl RNA/RNA and LNA-2′-*O*-methyl RNA/RNA duplexes relative to the same type of mismatches formed by 2′-*O*-methyladenosine and LNA-adenosine, respectively. The enhanced thermodynamic stability of fully complementary duplexes and decreased thermodynamic stability of some mismatched duplexes are useful for many RNA studies, including those involving microarrays.

INTRODUCTION

Modified nucleotides are useful tools to study the structures, biological functions and chemical and thermodynamic stabilities of nucleic acids (1–7). Recently, microarray methods were introduced to study the structure of nucleic acids (8–11). In native RNA, a majority of nucleotides form canonical pairs and single-stranded regions are typically short, roughly 5–7 nucleotides long. Detection of these single-stranded regions by RNA binding to probes on microarrays requires that the hybrid formed be thermodynamically sufficiently stable to capture the RNA. The thermodynamic stability of nucleic acid duplexes is strongly dependent on sequence, however. For example, duplexes of RNA heptamers composed of only A-U or G-C base pairs can differ in stability ($\Delta\Delta G^\circ_{37}$) by up to 15 kcal/mol, which is over 10 orders of magnitude in K_d (12). This complicates interpretation of microarray data. Incorporation of modified nucleotides in microarray probes can increase the thermodynamic stability of hybrid duplexes and make the thermodynamic stability relatively independent of sequence. Consequently the single-stranded character of potential binding sites in target RNA becomes the dominant factor determining binding, thus simplifying interpretation to deduce target RNA secondary structure.

There are many ways to adjust the stabilities of nucleic acid duplexes (1,13–22). Initial microarray experiments to

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deduce RNA secondary structure (11) used 2′-*O*-methyl RNA probes because 2′-*O*-methyl RNA/RNA duplexes are more thermodynamically stable than DNA/RNA duplexes (14,17,23) and 2′-*O*-methyl RNA probes are also chemically stable. The thermodynamic stability of 2′-*O*-methyl RNA/RNA duplexes can be enhanced by incorporation of LNA nucleotides (16), much as LNA stabilizes DNA/DNA (15,18,19) and DNA/RNA (15,18) hybrids. Here, we show that 2,6-diaminopurine substitution for A in 2′-*O*-methyl RNA or LNA nucleotides can further increase thermodynamic stabilities of hybrids with RNA and thereby reduce the sequence dependence of hybrid formation.

The 2,6-diaminopurine riboside (D) is an analog of adenosine containing an additional amino group at position 2 of the purine ring. The 2-amino group allows formation of a third hydrogen bond with uridine in the complementary strand. Previous studies have shown that 2,6-diaminopurine can increase the thermodynamic stability of RNA and DNA duplexes (20–22). The data presented here demonstrate that substitution of D for A increases thermodynamic stability ($\Delta\Delta G^{\circ}_{37}$) of fully complementary 2′-*O*-methyl RNA/RNA duplexes by 0.4–1.2 and 1.0–2.7 kcal/mol at 37°C, respectively, for each 2′-*O*-methyl-2,6-diaminopurine riboside (D^M) or LNA-2,6-diaminopurine riboside (D^L) present in the duplex. The results for fully complementary 2′-*O*-methyl RNA/RNA duplexes fit a nearest neighbor model for predicting stability and the effects of D and LNA substitutions are additive. Measurements of duplexes with mismatches indicate that internal D-A, D-C and D-G pairs are very destabilizing relative to D-U, thus providing specificity.

MATERIALS AND METHODS

General methods

Mass spectra of nucleosides and oligonucleotides were obtained on an LC MS Hewlett Packard series 1100 MSD with API-ES detector or an MALDI-TOF MS, model Autoflex (Bruker). Thin-layer chromatography (TLC) purification of the oligonucleotides was carried out on Merck 60 F_{254} TLC plates with the mixture 1-propanol/aqueous ammonia/water = 55:35:10 (v/v/v). TLC analysis of reaction progress was performed on the same type of silica gel plates with various mixtures of dichloromethane and methanol (98:2 v/v, 95:5 v/v, 9:1 v/v and 8:2 v/v).

Chemical synthesis of phosphoramidite of 2′-*O*-methyl-2,6-diaminopurine riboside

The synthesis of protected 2′-*O*-methyl-2,6-diaminopurine riboside derivative was performed according to general procedures of the synthesis of 2′-*O*-methylnucleosides with some modifications (24). 2,6-Diaminopurine riboside was treated with 1,3-dichlorotetraisopropyldisiloxane (25) and then 5′,3′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2,6-diaminopurine riboside was methylated with iodomethane in the presence of sodium hydride (24). The 2′-*O*-methylated derivative was treated with isobutyryl chloride followed by triethylammonium fluoride (25,26). Treatment of the last derivative with dimethoxytrityl chloride followed by 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite gave 5′-*O*-dimethoxytrityl-2′-*O*-methyl-*N*2,*N*6-diisobutyryl-2,6-diaminopurine riboside-3′-*O*-phosphoramidite in overall yield *ca*. 35%.

### Synthesis and purification of oligonucleotides

Oligonucleotides were synthesized on an Applied Biosystems DNA/RNA synthesizer, using β-cyanoethyl phosphoramidite chemistry (27). Commercially available A, C, G and U phosphoramidites with 2′-*O*-tertbutyldimethylsilyl or 2′-*O*-methyl groups were used for synthesis of RNA and 2′-*O*-methyl RNA, respectively (Glen Research, Azco, Proligo). The 3′-*O*-phosphoramidites of LNA nucleotides were synthesized according to published procedures (15,28,29) with some minor modifications. The details of deprotection and purification of oligoribonucleotides were described previously (12).

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 sodium chloride concentration kept melting temperatures in the reasonable range even when there were multiple substitutions and also allowed comparison with previous experiments (14,16). Oligonucleotide single-strand concentrations were calculated from absorbance above 80°C and single-strand extinction coefficients were approximated by a nearest-neighbor model with D approximated as A (30,31). Absorbance vs 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 thermoprogrammer. Melting curves were analyzed and thermodynamic parameters were calculated from a two-state model with the program MeltWin 3.5 (32). For most 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

Thermodynamic parameters for predicting stabilities of 2′-*O*-methyl RNA/RNA with the Individual Nearest Neighbor Hydrogen Bonding (INN-HB) model (12) 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 enhanced stability parameters for LNA-2′-*O*-methyl RNA/RNA duplexes when the LNAs are separated by at least one 2′-*O*-methyl nucleotide. Results from T_M^{-1} vs $\ln(C_T/4)$ plots were used as the data for the calculations.
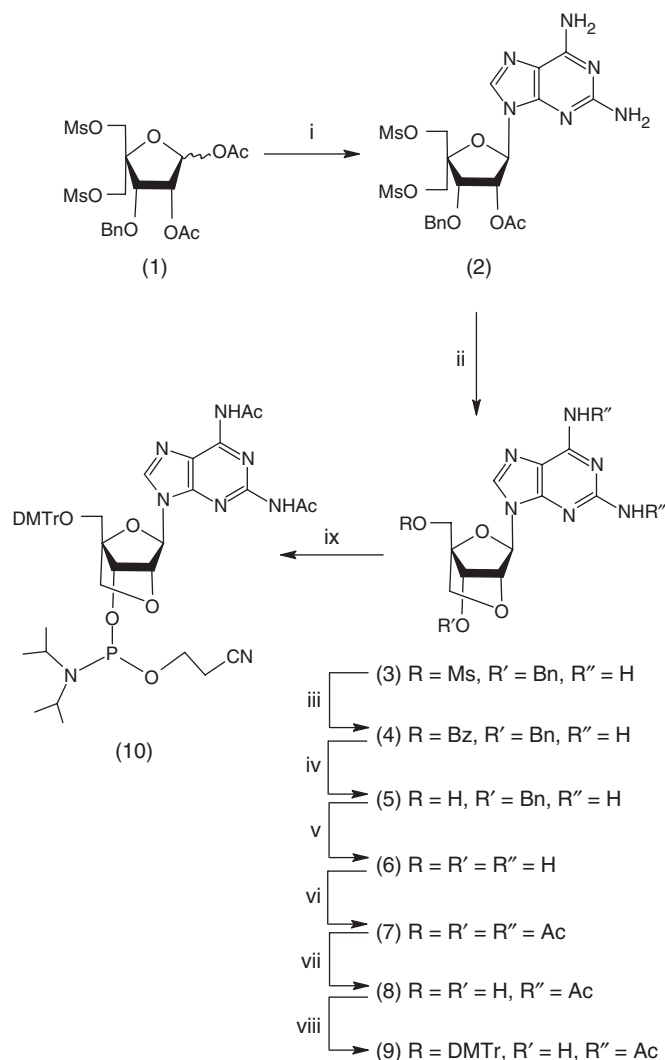
Figure 1. Synthesis of LNA-2,6-diaminopurine phosphoramidite. Reagents and conditions: (i) 2,6-diaminopurine, HMDS, TMSOTf, dichloroethane; (ii) LiOH·H$_2$O, THF, H$_2$O; (iii) BzOLi, DMF; (iv) conc. NH$_4$OH, Py; (v) Pd/C, HCOONH$_4$, MeOH; (vi) AcCl, Py; (vii) KOH, Py, H$_2$O, EtOH; (viii) DMTrCl, Py; (ix) 4,5-DCI, NC(CH$_2$)$_2$OP[N(iPr)$_2$]$_2$, CH$_3$CN.

RESULTS

Chemical synthesis of protected LNA-2,6-diaminopurine riboside derivative

The derivative of LNA-2,6-diaminopurine was synthesized with an approach similar to that described for synthesis of natural LNA nucleosides (15,28,29) (Figure 1). The derivative of pentafuranose (**1**) (33) was condensed with trimethylsilylated 2,6-diaminopurine in 1,2-dichloroethane in the presence of trimethylsilyl trifluoromethanesulfonate as catalyst (34). Treatment of derivative (**2**) with lithium hydroxide resulted in the 5′-*O*-methanesulfonyl derivative (**3**), which was converted with lithium benzoate into the 5′-*O*-benzoyl derivative (**4**). The application of lithium benzoate instead of sodium benzoate very significantly improved solubility of the benzoate salt in *N*,*N*-dimethylformamide. Treatment of 5′-*O*-benzoyl derivative (**4**) with aqueous ammonia

resulted in formation of (**5**). Removal of the 3′-*O*-benzyl with ammonium formate in the presence of Pd/C (35) resulted in formation of LNA-2,6-diaminopurine riboside (**6**). Derivative (**6**) was treated with acetyl chloride to produce (**7**), which was converted into LNA-*N*2,*N*6-diacetyl-2,6-diaminopurine riboside (**8**), using classical Khorana's procedure (36), and later into the 5′-*O*-dimethoxytrityl derivative (**9**). The overall yield of synthesis up to this step was 18%. In reaction of LNA-5′-*O*-dimethoxytrityl-*N*2,*N*6-diacetyl-2,6-diaminopurine riboside (**9**) with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl-phosphordiamidite was converted into LNA-5′-*O*-dimethoxytrityl-N2,N6-diacetyl-2,6-diaminopurine riboside-3′-*O*-phosphoramidite (**10**) in 93% yield. It was possible to use acetyl instead of isobutyryl to protect the 2,6-amino groups of LNA-2,6-diaminopurine riboside because LNA-5′-*O*-dimethoxytrityl-*N*2,*N*6-diacetyl-2,6-diaminopurine riboside-3′-*O*-phosphoramidite (**10**) is soluble in acetonitrile. This is in contrast to 5′-*O*-dimethoxytrityl-2′-*O*-methyl-*N*2,*N*6-diacetyl-2,6-diamino-purine riboside-3′-*O*-phosphoramidite. The details concerning chemical synthesis of derivatives (**2–10**) are described in Supplementary Data.

The thermodynamic stability of 2′-O-methyl RNA/RNA duplexes containing 2′-O-methyl-2,6-diaminopurine riboside or LNA-2,6-diaminopurine riboside

The adenosines in duplexes of the form 5′A^{M}C^{M}W^{M}A^{M}X^{M}C^{M}A^M/r(3′UGZUYGU) were replaced singly or completely by 2′-*O*-methyl D (D^M) or LNA D (D^L), and the thermodynamics for duplex formation were measured (Table 1). Here WZ and XY are Watson–Crick base pairs. The results can be compared with previous measurements (14,16) for the unsubstituted duplexes and for the A^{M}s substituted by LNA A (Table 1, see also Supplementary Data for complete thermodynamic data). When only a 5′ or 3′ terminal A is substituted by D with the same type of sugar, the average enhancement in stability at 37°C is 0.37 kcal/mol. If the middle A is substituted by D with the same type of sugar, then the average enhancement is 0.94 kcal/mol. Comparisons of $\Delta\Delta G°_{37}$ values for the A^M to D^L replacements in Table 1 to the sum of corresponding A^M to D^M and A^M to A^L replacements, which in Table 1 are listed immediately below in square brackets, indicate that the effects of replacing A with D and 2′-*O*-methyl with LNA are essentially additive.

The results with D^{M}C^{M}U^{M}A^{M}C^{L}C^{M}A^M, D^{L}C^{M}U^{M}A^{M}C^{L}C^{M}A^M, D^{M}C^{M}U^{M}D^{M}C^{M}C^{M}D^M, and D^{L}C^{M}U^{M}D^{L}C^{M}C^{M}D^L suggest that the effects of multiple substitutions are also additive. For these sequences, the enhancement in heteroduplex stability relative to A^{M}C^{M}U^{M}A^{M}C^{M}C^{M}A^M differs from the sum of enhancements due to the individual replacements by only 0.48, 0.29, 0.27 and 0.07 kcal/mol, respectively.

The results in Table 1 can be combined with previous results (14,16) to obtain nearest neighbor parameters for 2′-*O*-methyl RNA/RNA duplexes (Table 2). The nearest neighbor parameters with D are preliminary due to the small number of occurrences

Table 1. Thermodynamic parameters of duplex formation to RNA 7-mers complementary to the sequence shown[a]

LNA-2'OMe RNA (5'–3')	$-\Delta G^{\circ}_{37}$ (kcal/mol) [predicted][b]	$T_M{}^c$ (°C)	$\Delta\Delta G^{\circ}_{37}$ (kcal/mol)		
			$D^M\text{-}A^M$ or $(D^L\text{-}A^M)^d$	$D^L\text{-}A^{L,e}$	L-M[f]
A. Duplex formation with D^M-U or D^L-U at terminal positions.					
$\mathbf{D^M}C^M U^M A^M C^M C^M A^M$	7.65 ± 0.01 [7.82]	43.7	−0.52		
$\underline{\mathbf{D^L}}C^M U^M A^M C^M C^M A^M$	8.15 ± 0.03 [8.35]	47.8	−1.02 [−1.11]^g	−0.43	−0.50
$\mathbf{D^M}C^M U^M A^M \underline{\mathbf{C^L}}C^M A^M$	8.84 ± 0.07 [9.40]	51.0	−1.71		
$\underline{\mathbf{D^L}}C^M U^M A^M \underline{\mathbf{C^L}}C^M A^M$	9.53 ± 0.07 [9.93]	54.9	−2.40		−0.69
$A^M C^M U^M A^M C^M C^M \mathbf{D^M}$	7.52 ± 0.02 [7.69]	43.4	−0.39		
$A^M C^M U^M A^M C^M C^M \underline{\mathbf{D^L}}$	8.57 ± 0.04 [8.92]	49.7	−1.44 [−1.69]^g	−0.14	−1.05
B. Duplex formation with D^M-U or D^L-U at internal positions.					
$A^M C^M A^M \underline{\mathbf{A^L}}C^M C^M A^M$	6.98±0.01 [7.76]	40.4	−0.67		−0.67
$A^M C^M A^M \underline{\mathbf{D^M}}C^M C^M A^M$	7.12 ± 0.01 [7.10]	40.8	−0.81		
$A^M C^M A^M \underline{\mathbf{D^L}}C^M C^M A^M$	8.03 ± 0.02 [8.44]	47.4	−1.72 [−1.48]^g	−1.05	−0.91
$A^M C^M C^M \mathbf{D^M}C^M C^M A^M$	9.21 ± 0.12 [9.06]	52.4	−0.71		
$A^M C^M C^M \underline{\mathbf{D^L}}C^M C^M A^M$	11.22 ± 0.16 [10.40]	62.2	−2.72 [−1.89]^g	−1.54	−2.01
$A^M C^M G^M \underline{\mathbf{A^L}}C^M C^M A^M$	9.27 ± 0.04 [9.72]	53.9	−0.99		−0.99
$A^M C^M G^M \underline{\mathbf{D^M}}C^M C^M A^M$	9.21 ± 0.12 [9.03]	52.4	−0.93		
$A^M C^M G^M \underline{\mathbf{D^L}}C^M C^M A^M$	10.19 ± 0.12 [10.37]	59.3	−1.91 [−1.92]^g	−0.92	−0.98
$A^M C^M U^M \mathbf{D^M}C^M C^M A^M$	8.09 ± 0.04 [7.97]	47.2	−0.96		
$A^M C^M U^M \underline{\mathbf{D^L}}C^M C^M A^M$	9.45 ± 0.04 [9.31]	55.5	−2.32 [−2.18]^g	−1.10	−1.36
$A^M C^M A^M A^M A^M C^M A^M$	4.06 ± 0.31 [4.27]	21.6			
$A^M C^M A^M \underline{\mathbf{A^L}}A^M C^M A^M$	5.07 ± 0.07 [5.55]	27.3	−1.01		−1.01
$A^M C^M A^M \underline{\mathbf{D^M}}A^M C^M A^M$	5.29 ± 0.06 [5.32]	29.1	−1.23		
$A^M C^M A^M \underline{\mathbf{D^L}}A^M C^M A^M$	(6.41 ± 0.03) [6.66]	(36.1)	(−2.35) [−2.24]^g	(−1.34)	(−1.12)
$A^M C^M C^M A^M A^M C^M A^M$	6.69 ± 0.02 [6.48]	38.1			
$A^M C^M C^M \underline{\mathbf{A^L}}A^M C^M A^M$	7.90 ± 0.01 [7.76]	46.2	−1.21		−1.21
$A^M C^M C^M \underline{\mathbf{D^M}}A^M C^M A^M$	7.45 ± 0.01 [7.28]	43.2	−0.76		
$A^M C^M C^M \underline{\mathbf{D^L}}A^M C^M A^M$	8.73 ± 0.02 [8.62]	51.8	−2.04 [−1.97]^g	−0.83	−1.28
$A^M C^M G^M A^M A^M C^M A^M$	6.37 ± 0.01 [6.23]	35.9			
$A^M C^M G^M \underline{\mathbf{A^L}}A^M C^M A^M$	7.36 ± 0.07 [7.51]	42.8	−0.99		−0.99
$A^M C^M G^M \underline{\mathbf{D^M}}A^M C^M A^M$	7.07 ± 0.01 [7.25]	40.4	−0.70		
$A^M C^M G^M \underline{\mathbf{D^L}}A^M C^M A^M$	8.34 ± 0.03 [8.59]	48.4	−1.97 [−1.69]	−0.98	−1.27
$A^M C^M U^M \mathbf{D^M}A^M C^M A^M$	6.23 ± 0.21 [6.19]	35.2	−1.00		
$A^M C^M U^M \underline{\mathbf{D^L}}A^M C^M A^M$	7.77 ± 0.02 [7.53]	45.5	−2.54 [−2.54]^g	−1.12	−1.54
$A^M C^M U^M \mathbf{D^M}G^M C^M A^M$	8.59 ± 0.08 [8.59]	48.4	−1.00		
$A^M C^M U^M \underline{\mathbf{D^L}}G^M C^M A^M$	10.17 ± 0.11 [9.93]	59.0	−2.58 [−2.59]^g	−0.99	−1.58
$A^M C^M U^M \mathbf{D^M}U^M C^M A^M$	6.92 ± 0.06 [6.92]	39.4	−1.10		
$A^M C^M U^M \underline{\mathbf{D^L}}U^M C^M A^M$	8.20 ± 0.07 [8.26]	47.9	−2.38 [−2.30]^g	−1.18	−1.28
C. Duplex formation with three D^M-U or D^L-U base pairs.					
$\mathbf{D^M}C^M U^M \mathbf{D^M}C^M C^M \mathbf{D^M}$	8.73±0.03 [8.88]	50.3	−1.60		
$\underline{\mathbf{D^L}}C^M U^M \underline{\mathbf{D^L}}C^M C^M \underline{\mathbf{D^L}}$	11.84±0.15 [11.45]	68.5	−4.71	−3.11	−3.11

[a]Solutions are 100 mM NaCl, 20 mM sodium cacodylate and 0.5 mM Na$_2$EDTA, pH 7. Values are from $T_M{}^{-1}$ versus log ($C_T/4$) plots. Values in parentheses are from non-two-state melts.
[b]Values in square brackets are predicted on the basis of the INN-HB model (Table 2) and equation **1**.
[c]Calculated for 10^{-4} M total oligonucleotide strand concentration.
[d]The differences in ΔG°_{37} compared with completely 2'-*O*-methyl RNA strand without any D (14,16).
[e]The differences in ΔG°_{37} due to substitution D^L for A^L in the same 2'-*O*-methyl RNA strand (16).
[f]The differences in ΔG°_{37} due to substitution of LNA for 2'-*O*-methyl RNA,
[g]The sum of differences in ΔG°_{37} for A^M to D^M and A^M to A^L substitutions. The thermodynamic data of some reference 2'-*O*-methyl RNA/RNA duplexes were published earlier (14,16).

for them. The thermodynamic parameters for $5'A^M C^M U^M A^M G^M C^M A^M/3'r(UGAUCGU)$ were re-measured and the values in Table 1 and Supplementary Data were used for deriving the nearest neighbor parameters. The parameters for nearest neighbors without D are similar to those reported previously (14).

The thermodynamic stability of 2'-*O*-methyl RNA/RNA duplexes containing mismatches formed by D^M and D^L nucleotides

Some single mismatches in RNA/RNA duplexes are particularly stable thermodynamically due to hydrogen

Table 2. Thermodynamic parameters for INN-HB nearest neighbor model applied to 2′-*O*-methyl RNA/RNA heteroduplexes in 0.1 M NaCl, pH 7[a]

Parameters	ΔG°_{37}(kcal/mol)	ΔH°_{37}(kcal/mol)	ΔS° [b](eu)	Number of occurrences
m(5′-AA)/r(3′-UU)	-0.60 ± 0.11	-9.16 ± 2.24	-27.6 ± 7.2	18
m(5′-AU)/r(3′-UA)	-0.83 ± 0.12	-6.39 ± 2.44	-17.9 ± 7.8	17
m(5′-UU)/r(3′-AA)	-0.94 ± 0.10	-5.67 ± 2.09	-15.3 ± 6.7	19
m(5′-AD)/r(3′-UU)	-0.97 ± 0.30	-7.45 ± 6.07	-20.9 ± 19.4	2
m(5′-UA)/r(3′-AU)	-1.13 ± 0.14	-5.26 ± 2.83	-13.3 ± 9.0	27
m(5′-DA)/r(3′-UU)	-1.28 ± 0.27	-11.25 ± 5.46	-32.1 ± 17.4	4
m(5′-UD)/r(3′-AU)	-1.55 ± 0.31	-3.05 ± 6.19	-4.8 ± 19.8	5
m(5′-AC)/r(3′-UG)	-1.59 ± 0.15	-6.14 ± 3.02	-14.7 ± 9.7	85
m(5′-DU)/r(3′-UA)	-1.72 ± 0.41	-10.73 ± 8.22	-29.1 ± 26.3	1
m(5′-AG)/4(3′-UC)	-1.75 ± 0.14	-12.46 ± 2.79	-34.5 ± 8.9	17
m(5′-DC)/r(3′-UG)	-1.84 ± 0.25	-8.95 ± 4.98	-22.9 ± 15.9	7
m(5′-CA)/r(3′-GU)	-1.87 ± 0.14	-4.82 ± 2.90	-9.5 ± 9.3	74
m(5′-UC)/r(3′-AG)	-1.88 ± 0.15	-9.61 ± 3.04	-24.9 ± 9.7	21
m(5′-UG)/r(3′-AC)	-1.94 ± 0.15	-12.68 ± 3.06	-34.6 ± 9.8	19
m(5′-CD)/r(3′-GU)	-1.99 ± 0.25	-4.03 ± 5.03	-6.6 ± 16.1	4
m(5′-GA)/r(3′-CU)	-2.08 ± 0.16	-5.37 ± 3.31	-10.6 ± 10.6	15
m(5′-CU)/r(3′-GA)	-2.16 ± 0.14	-9.69 ± 2.92	-24.3 ± 9.3	41
m(5′-GU)/r(3′-CA)	-2.18 ± 0.15	-7.15 ± 2.94	-16.0 ± 9.4	16
m(5′-DG)/r(3′-UC)	-2.29 ± 0.42	-17.84 ± 8.27	-50.1 ± 26.4	1
m(5′-CG)/r(3′-GC)	-2.35 ± 015	-9.79 ± 2.97	-24.0 ± 9.5	25
m(5′-GD)/r(3′-CU)	-2.42 ± 0.32	-7.98 ± 6.52	-17.9 ± 20.8	2
m(5′-CC)/r(3′-GG)	-2.81 ± 0.09	-9.80 ± 1.82	-22.5 ± 5.8	45
m(5′-GG)/r(3′-CC)	-2.85 ± 0.16	-10.29 ± 3.21	-24.0 ± 10.3	19
m(5′-GC)/r(3′-CG)	-2.98 ± 0.17	-10.01 ± 3.40	-22.7 ± 10.9	24
Initiation	3.31 ± 0.51	-12.57 ± 10.36	-51.2 ± 33.1	84
Per Terminal AU	0.27 ± 0.07	3.01 ± 1.49	8.8 ± 4.8	120

[a]Digits beyond experimental error are provided to allow better predictions of melting temperature. See (12) for INN-HB model.
[b]Calculated from $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/310.15$.

bonding (37). Interpretation of microarray and other data must consider potential hybridization involving mismatches. Because it is important to determine the specificity of base pairing to modified nucleotides, mismatches with D^M, D^L, A^M and A^L were studied. Most mismatches were placed at an internal position within duplexes because that is statistically the most likely occurrence. Some terminal mismatches were also measured, however. The results from optical melting experiments are listed in Table 3 (see also Supplementary Data for complete thermodynamic data) and the differences between free energies of duplex formation with A-U or D-U and mismatch pairing for single internal mismatches at 37°C are summarized in Table 4.

Many of the duplexes had melting temperatures <20°C, which makes measurements difficult. This is one reason that some of the transitions appear non-two-state as indicated by more than a 15% difference between ΔH° values derived from fitting the shapes of the melting curves or from T_M^{-1} vs ln $(C_T/4)$ plots. Slightly, non-two-state melts were also found for four duplexes with melting temperature >31°C. For these sequences, there was at most a 19% difference between the derived ΔH° values. The ΔG°_{37} values for these sequences are still reliable because errors in ΔH° and ΔS° compensate making ΔG°_{37} values near the T_M reliable (38). Values from non-two-state melts are listed in parentheses in Tables 3 and 4.

Mismatches at 5′- or 3′-terminal positions are typically less destabilizing than internal mismatches (16). To evaluate this effect, D^M-G and D^L-G mismatches were placed at 5′- or 3′-terminal positions of 2′-*O*-methyl RNA/ RNA duplexes (Table 3C). For $5'XC^MU^MA^MC^MC^MA^M/$ $3'$rGGAUGGU duplexes, where X is D^M or D^L, the destabilization ($\Delta\Delta G^\circ_{37}$) is 0.78 and 0.98 kcal/mol for D^M-G and D^L-G, respectively. This is similar to the destabilizations of 0.37 and 0.58 kcal/mol when X is A^M or A^L, respectively. For $5'A^MC^MU^MA^MC^MC^MX/$ $3'$rUGAUGGG duplexes, where X is D^M or D^L, the destabilization is 0.67 and 0.56 kcal/mol for D^M-G and D^L-G, respectively. This is similar to the destabilization of 0.32 and 0.48 kcal/mol when X is A^M or A^L, respectively. While the differences between destabilizing by terminal A-G and D-G mismatches are within experimental error, the D-G mismatches are all more destabilizing than A-G suggesting that this is a real, albeit small, effect. As expected, the destabilization effect ($\Delta\Delta G^\circ_{37}$) is reduced compared with the same mismatches at an internal position as listed in Table 4.

DISCUSSION

There are many reasons to modify the thermodynamic stabilities of nucleic acid duplexes. The application of microarrays of short oligonucleotides to probe RNA secondary structure (11) is one case where it is particularly useful to have sequences that base pair strongly and isoenergetically to RNA targets. Strong pairing permits the use of short oligonucleotides so that self-folding of probe is largely avoided. Moreover, short oligonucleotides provide enhanced specificity of binding (10). Isoenergetic binding further simplifies interpretation of data because

Table 3. Effects of mismatches on thermodynamic parameters of helix formation[a]

LNA-2′OMe RNA (5′–3′)	RNA (5′–3′)	$-\Delta G^\circ_{37}$ (kcal/mol)	$T_M{}^b$(°C)	$\Delta\Delta G^\circ_{37}{}^c$ (kcal/mol)
A. Effects of D^M-A, D^L-A, A^M-A, and A^L-A mismatches at internal positions.				
$A^M C^M U^M \mathbf{D^M} C^M C^M A^M$	UG**GA**AGU	3.31 ± 1.60	18.4	4.78
$A^M C^M U^M \underline{\mathbf{D^L}} C^M C^M A^M$	UG**GA**AGU	5.09 ± 0.45	27.2	4.36
$A^M C^M U^M \mathbf{A^M} U^M C^M A^M$	UG**A**AAGU	(4.64 ± 1.18)	(9.8)	(1.18)
$A^M C^M U^M \mathbf{D^M} U^M C^M A^M$	UG**A**AAGU	4.25 ± 0.30	16.7	2.67
$A^M C^M U^M \underline{\mathbf{A^L}} U^M C^M A^M$	UG**A**AAGU	(3.95 ± 2.75)	(11.6)	(3.07)
$A^M C^M U^M \underline{\mathbf{D^L}} U^M C^M A^M$	UG**A**AAGU	2.99 ± 0.39	19.1	5.21
B. Effects of D^M-C, D^L-C, A^M-C, and A^L-C mismatches at internal positions.				
$A^M C^M U^M \mathbf{A^M} C^M C^M A^M$	UG**GC**AGU	(3.92 ± 0.27)	(20.2)	(3.21)
$A^M C^M U^M \mathbf{D^M} C^M C^M A^M$	UG**GC**AGU	(6.52 ± 0.02)	(36.9)	(1.57)
$A^M C^M U^M \underline{\mathbf{A^L}} C^M C^M A^M$	UG**GC**AGU	5.15 ± 0.30	27.7	3.20
$A^M C^M U^M \underline{\mathbf{D^L}} C^M C^M A^M$	UG**GC**AGU	6.46 ± 0.11	36.5	2.99
$A^M C^M U^M \mathbf{A^M} U^M C^M A^M$	UG**A**CAGU	(3.38 ± 0.66)	(14.8)	(3.64)
$A^M C^M U^M \mathbf{D^M} U^M C^M A^M$	UG**A**CAGU	4.67 ± 0.15	27.0	2.25
$A^M C^M U^M \underline{\mathbf{A^L}} U^M C^M A^M$	UG**A**CAGU	3.08 ± 0.14	15.9	3.94
$A^M C^M U^M \underline{\mathbf{D^L}} U^M C^M A^M$	UG**A**CAGU	5.17 ± 0.03	29.6	3.03
C. Effects of D^M-G, D^L-G, A^M-G, and A^L-G mismatches at internal and terminal positions.				
$A^M C^M U^M \mathbf{D^M} C^M C^M A^M$	UG**GG**AGU	3.95 ± 0.45	21.6	4.14
$A^M C^M U^M \underline{\mathbf{D^L}} C^M C^M A^M$	UG**GG**AGU	5.21 ± 0.14	27.8	4.24
$A^M C^M U^M \mathbf{A^M} U^M C^M A^M$	UG**A**GAGU	3.13 ± 0.54	12.1	2.69
$A^M C^M U^M \mathbf{D^M} U^M C^M A^M$	UG**A**GAGU	3.38 ± 0.03	18.2	3.54
$A^M C^M U^M \underline{\mathbf{A^L}} U^M C^M A^M$	UG**A**GAGU	2.81 ± 1.50	16.9	4.21
$A^M C^M U^M \underline{\mathbf{D^L}} U^M C^M A^M$	UG**A**GAGU	3.10 ± 0.29	19.7	5.10
$G^M A^M C^M \mathbf{A^M} A^M A^M C^M A^M G^M$	C**U**GUGUGUC	5.02 ± 0.05	28.9	3.28
$G^M A^M C^M A^M \mathbf{D^M} A^M C^M A^M G^M$	CUGU**GU**GUC	(5.61 ± 0.06)	(31.7)	(3.90)
$G^M A^M C^M A^M \underline{\mathbf{A^L}} A^M C^M A^M G^M$	CUGU**GU**GUC	5.51 ± 0.15	31.5	3.83
$G^M A^M C^M A^M \underline{\mathbf{D^L}} A^M C^M A^M G^M$	CUGU**GU**GUC	5.78 ± 0.30	33.2	5.14
$A^M C^M C^M \mathbf{A^M} A^M C^M A^M$	UG**U**GGGU	(1.32 ± 0.25)	(12.0)	(5.37)
$A^M C^M C^M \mathbf{D^M} A^M C^M A^M$	UG**U**GGGU	4.46 ± 0.55	15.1	2.99
$A^M C^M C^M \underline{\mathbf{A^L}} A^M C^M A^M$	UG**U**GGGU	(3.19 ± 0.36)	(17.0)	(4.71)
$A^M C^M C^M \underline{\mathbf{D^L}} A^M C^M A^M$	UG**U**GGGU	4.82 ± 0.02	25.0	3.91
$A^M C^M G^M \mathbf{A^M} A^M C^M A^M$	UG**U**GCGU	4.28 ± 0.52	21.2	2.09
$A^M C^M G^M \mathbf{D^M} A^M C^M A^M$	UG**U**GCGU	3.03 ± 0.78	17.2	4.04
$A^M C^M G^M \underline{\mathbf{A^L}} A^M C^M A^M$	UG**U**GCGU	(4.80 ± 0.59)	(21.0)	(2.56)
$A^M C^M G^M \underline{\mathbf{D^L}} A^M C^M A^M$	UG**U**GCGU	3.54 ± 0.50	19.3	4.80
$A^M C^M U^M \mathbf{A^M} A^M C^M A^M$	UG**U**GAGU	3.22 ± 0.53	11.8	2.01
$A^M C^M U^M \mathbf{D^M} A^M C^M A^M$	UG**U**GAGU	3.01 ± 0.63	16.1	3.22
$A^M C^M U^M \underline{\mathbf{A^L}} A^M C^M A^M$	UG**U**GAGU	3.36 ± 0.33	16.0	3.29
$A^M C^M U^M \underline{\mathbf{D^L}} A^M C^M A^M$	UG**U**GAGU	(2.03 ± 0.78)	(13.5)	(5.74)
$A^M C^M U^M \mathbf{A^M} C^M C^M A^M$	UGG**U**AGG	6.76 ± 0.02	38.6	0.37
$\mathbf{D^M} C^M U^M \mathbf{A^M} C^M C^M A^M$	UGG**U**AGG	6.87 ± 0.03	39.4	0.78
$\underline{\mathbf{A^L}} C^M U^M \mathbf{A^M} C^M C^M A^M$	UGG**U**AGG	(7.14 ± 0.02)	(41.3)	(0.58)
$\underline{\mathbf{D^L}} C^M U^M \mathbf{A^M} C^M C^M A^M$	UGG**U**AGG	7.17 ± 0.11	41.0	0.98
$A^M C^M U^M \mathbf{A^M} C^M C^M \mathbf{A^M}$	**G**GGUAGU	6.81 ± 0.02	39.1	0.32
$A^M C^M U^M \mathbf{A^M} C^M C^M \mathbf{D^M}$	**G**GGUAGU	6.85 ± 0.17	39.2	0.67
$A^M C^M U^M \mathbf{A^M} C^M C^M \underline{\mathbf{A^L}}$	**G**GGUAGU	(7.95 ± 0.01)	(46.5)	(0.48)
$A^M C^M U^M \mathbf{A^M} C^M C^M \underline{\mathbf{D^L}}$	**G**GGUAGU	8.01 ± 0.09	47.3	0.56
D. Effects of D^M-G, D^L-G, A^M-G, and A^L-G mismatches in the presence of D^M-U and D^L-U base pairs.				
$\mathbf{D^M} C^M U^M \mathbf{D^M} C^M C^M \mathbf{D^M}$	UG**GG**AGU	4.70 ± 0.08	25.7	4.03
$\underline{\mathbf{D^L}} C^M U^M \underline{\mathbf{D^L}} C^M C^M \underline{\mathbf{D^L}}$	UG**GG**AGU	7.25 ± 0.03	41.8	4.59
$\mathbf{D^M} C^M U^M A^M \underline{\mathbf{C^L}} C^M A^M$	UGG**U**AGG	8.14 ± 0.03	47.2	0.70
$\mathbf{D^M} C^M U^M A^M \underline{\mathbf{C^L}} C^M A^M$	UGG**U**AGG	8.54 ± 0.02	49.5	0.99

[a]Solutions are 100 mM NaCl, 20 mM sodium cacodylate and 0.5 mM Na$_2$EDTA, pH 7. Values are form $T_M{}^{-1}$ vs log ($C_T/4$) plots. Values in parentheses are from non-two state melts.
[b]Calculated for 10^{-4} M oligonucleotide strand concentration.
[c]Difference compared with duplex formation when bold A or D is paired with U.

binding will be primarily dependent on target structure rather than probe sequence. The synthesis of oligonucleotides with 2,6-diaminopurine described here provides a way to improve recognition of U in RNA targets by enhancing both binding and specificity. Moreover, the thermodynamic results provide approximations that allow design of isoenergetic probes. The design is relatively straightforward because the effects of non-adjacent modifications are usually additive. Short modified oligonucleotides could also be applied as antisense oligonucleotides (ASO) (3,39,40). They could also be useful to modulate binding and biological activity related to single nucleotide polymorphism (SNP) (41,42) and microRNAs (43–47).

Table 4. Summary of destabilization (ΔΔG°) at 37°C due to internal mismatches[a]

5'U^{M}A$^{M/L}$A^M	2.01	5'U^{M}A$^{M/L}$U^M	2.69		
3'A G U	3.29	3'A G A	4.21		
5'U^{M}D$^{M/L}$A^M	3.22	5'U^{M}D$^{M/L}$U^M	3.54	5'U^{M}D$^{M/L}$C^M	4.14
3'A G U	(5.74)	3'A G A	5.10	3'A G G	4.24
5'A^{M}A$^{M/L}$A^M	3.28	5'U^{M}A$^{M/L}$U^M	(1.18)		
3'U G U	3.83	3'A A A	(3.07)		
5'A^{M}D$^{M/L}$A^M	(3.90)	5'U^{M}D$^{M/L}$U^M	2.67	5'U^{M}D$^{M/L}$C^M	4.78
3'U G U	5.14	3'A A A	5.21	3'A A G	4.36
5'G^{M}A$^{M/L}$A^M	2.09				
3'C G U	(2.56)				
5'G^{M}D$^{M/L}$A^M	4.04	5'U^{M}A$^{M/L}$U^M	(3.64)	5'U^{M}A$^{M/L}$C^M	(3.21)
3'C G U	4.80	3'A C A	3.94	3'A C G	3.20
5'C^{M}A$^{M/L}$A^M	(5.37)	5'U^{M}D$^{M/L}$U^M	2.25	5'U^{M}D$^{M/L}$C^M	(1.57)
3'G G U	(4.71)	3'A C A	3.03	3'A C G	2.99
5'C^{M}D$^{M/L}$A^M	2.99				
3'G G U	3.91				

[a]Top value is for A^M or D^M and bottom value is for A^L or D^L; values in parentheses are from non-two-state melts.

Synthesis of LNA-2,6-diaminopurine riboside was reported by Rosenbohm *et al.* (20) and Koshkin *et al.* (21). Both used 2-amino-6-chloropurine as precursor of 2,6-diaminopurine. Rosenbohm used a saturated solution of ammonia in methanol to convert derivative of 2-amino-6-chloropurine riboside into 2,6-diaminopurine riboside and this transformation was accompanied by formation of 6-*O*-methyl derivative. Efficient synthesis (65% yield) of 2,6-diaminopurine riboside required not only specific temperature but particularly control of the pressure during this reaction. Koshkin proposed to convert the derivative of 2-amino-6-chloropurine riboside into 2-amino-6-azidopurine riboside and then into 2,6-diaminopurine riboside derivative simultaneously with deprotection of 3'-*O*-benzyl. An advantage of Rosenbohm and Koshkin approaches is universal character of 2-amino-6-chloropurine riboside derivative which beside 2,6-diaminopurine riboside can be transformed into LNA-guanosine and LNA-2-aminopurine riboside. A disadvantage is the much higher price of 2-amino-6-chloropurine than 2,6-diaminopurine. Moreover, both authors propose to use benzoyl as amino protecting group and in consequence using 40% aqueous solution of methylamine at 60–65°C for 2–4 h for deprotection of oligonucleotides containing 2,6-diaminopurine riboside. The method described herein is based on standard and much cheaper substrate as well as many well established procedures and is therefore a simple and efficient method for synthesizing LNA-2,6-diaminopurine riboside. Moreover, the chemical synthesis and deprotection of many oligonucleotides carrying LNA-2,6-diaminopurine riboside demonstrate that acetyl is very suitable for protection of amino groups in 2,6-diaminopurine residue.

Facile synthesis and incorporation of 2'-*O*-methyl-2,6-diaminopurine riboside and LNA-2,6-diaminopurine riboside into oligonucleotides allowed measurements of the thermodynamics for formation of 2'-*O*-methyl RNA/RNA and LNA-2'-*O*-methyl RNA/RNA duplexes containing D^M and D^L. The results show that incorporation of 2,6-diaminopurine into oligonucleotides allows modulation of duplex stability over a wide range.

Replacement of adenosine by D^M and D^L always enhances the thermodynamic stability of fully complementary 2'-*O*-methyl RNA/RNA and LNA-2'-*O*-methyl RNA/RNA duplexes. The largest stabilization is observed at internal positions where enhancements range from 0.7 to 1.2 kcal/mol with an average of 0.9 kcal/mol and 1.7–2.7 kcal/mol with an average of 2.3 kcal/mol, respectively, for D^M and D^L substituting for A^M. The D^M stabilization is in the range expected for addition of a hydrogen bond in RNA (48). The D^L stabilization is the sum of the effects of an extra hydrogen bond and of the LNA. The enhancement (ΔΔG°$_{37}$) for D^M and D^L relative to A^M and A^L is less at 5'- and 3'-terminal positions where it averages 0.4 kcal/mol. This difference in stabilization at terminal and internal positions is likely due to the competition between stacking and hydrogen bonding at terminal base pairs (48) and to the particular sequences studied. Other sequences may show larger effects for 2,6-diaminopurine substitutions at terminal positions.

The stabilities of fully complementary 2'-*O*-methyl RNA/RNA and LNA-2'-*O*-methyl RNA/RNA duplexes at 37°C can be predicted reasonably well with simple models. The nearest neighbor parameters in Table 2 allow prediction of stabilities for 2'-*O*-methyl RNA/RNA duplexes using the INN-HB model (12) and the additional enhancement, ΔΔG°$_{37}$ (chimera/RNA), due to an LNA sugar can be predicted from:

$$\Delta\Delta G^0_{37}(\text{chimera/RNA}) = -0.53n_{5'tL} - 1.28n_{iAL/UL}$$
$$- 1.34n_{iDL} - 1.58n_{iGL/CL} - 1.23n_{3'tAL/CL/GL/DL} - 0.14n_{3'tUL}$$

1

Here $n_{5'tL}$ is the number of 5' terminal LNAs, $n_{iAL/UL}$, n_{iDL} and $n_{iGL/CL}$ are the number of internal LNAs in A-U, D-U and G-C pairs, respectively, $n_{3'tU}$ and $n_{3'tAL/CL/GL/DL}$ are the number of 3' terminal LNAs that are U or not U, respectively. The equation is similar to that suggested previously (14,16), but has been updated to include the new results in Table 1. The predicted values are listed in square brackets in Table 1.

Mismatches formed by D^M and D^L destabilize duplexes (Tables 3 and 4). At the central position of duplexes with seven pairs that melt in a two-state manner, the destabilization (ΔΔG°$_{37}$) ranges between 2.3 and 5.2 kcal/mol at 37°C when D was only present as a mismatch. This corresponds to K$_d$'s less favorable by 42 to 4600-fold at 37°C.

With the possible exception of the 5'CAA/3'GGU context, mismatches of D^M and D^L with G and A destabilize 2'-*O*-methyl RNA/RNA and LNA-2'-*O*-methyl RNA/RNA duplexes more than similar mismatches formed by A^M and A^L, respectively (Table 4). The trend of destabilization is reversed for D-C mismatches. The D-C mismatches might be stabilized by a hydrogen bond between the 2-amino group of D and the O2 of C.

Interestingly, the effect of a central D-G mismatch is enhanced when both terminal base pairs are D-U in the context 5′DC^{M}U^{M}DC^{M}C^{M}D/3′rUGAGGGU (Table 3). Here, the destabilization is 4.03 and 4.59 kcal/mol when each D is 2′-*O*-methyl or LNA, respectively, compared with 1.57 and 2.99 kcal/mol when the terminal nucleotides of the probe are 2′-*O*-methyl A. For mismatches at terminal positions, the destabilization ranges from 0.6 to 1.0 kcal/mol at 37°C. Mismatches with an LNA nucleotide are usually more destabilizing than those with a 2′-*O*-methyl nucleotide (Table 4).

The enhanced, variable and predictable duplex stability available from 2,6-diaminopurine substitutions with either 2′-*O*-methyl or LNA sugars makes them valuable for designing isoenergetic duplexes. The large destabilizations from internal mismatches means that oligonucleotides with 2,6-diaminopurine will be highly specific for their complementary sequence. Thus they should facilitate many applications of oligonucleotides, including microarray methods for probing RNA structure (11) and design of nanostructures (49–51).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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