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This paper is dedicated to Professor Maciej Wiewiórowski

Thermodynamic contribution of nucleoside modifications to yeast tRNA^{Phe} anticodon stem loop analogs*

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The determination of the structural and functional contributions of natural modified nucleosides to tRNA has been limited by lack of an approach that can systematically incorporate the modified units. We have produced a number of oligonucleotide analogs, of the anticodon of yeast tRNA^{Phe} by, combining standard automated synthesis for the major nucleosides with specialty chemistries for the modified nucleosides. In this study, both naturally occurring and unnatural modified nucleotides were placed in native contexts. Each oligonucleotide was purified and the nucleoside composition determined to validate the chemistry. The RNAs were denatured and analyzed to determine the van't Hoff thermodynamic parameters. Here, we report the individual thermodynamic contributions for Cm, Gm, m^{1}G, m^{5}C, Ψ. In addition m^{5}m^{6}U, m^{1}Ψ, and m^{3}Ψ, were introduced to gain additional understanding of the physicochemical contribution of Ψ and m^{5}C at an atomic level. These oligonucleo-

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Abbreviations: ASL, anticodon stem loop; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; t_m, melting temperature; UV, ultraviolet; one-letter symbols for modified nucleosides are presented in Fig. 1.

tides demonstrate that modifications have measurable thermodynamic contributions and that loop modifications have global contributions.

Cytoplasmic tRNAs are characterized by a significant level of post-transcriptional modification. The biological functions and physicochemical contributions [1] of most of the 79 modified nucleosides in tRNAs [2] have yet to be determined. Assigning the structural and functional contributions is a complex problem. The significant amount of genomic information allocated for modifications indicates their retention is evolutionarily favorable [3]. This supports an opinion that most modifications exist for yet unknown functional reasons. Four classes of modification are envisioned; those that exist with no function; those that exist with only a biological function or structural function, and those that have both structural and biological functions. Identification of modifications that have measurable thermodynamic contributions is a useful means to separate structural from biological roles for post-transcriptional modifications.

In order to study the contribution of modified nucleosides, an approach and model system had to be developed. Technically our approach links five different disciplines. By combining chemical synthesis of novel phosphoramidites [4], automated synthesis with optimized coupling and deprotection protocols, chromatographic methods tailored for purification and characterization of the synthetic products [5], thermal denaturations and thermodynamic analysis [6], and biochemical assays [7–9], a systematic approach to study the function of modified nucleosides is possible. We have chosen the Anticodon Stem and Loop, ASL, of yeast $tRNA^{Phe}$ ($tRNA^{Phe}_{ASL}$) as the model system to begin this research. In general, ASLs are modified to a higher degree and with a larger variety of modifications than the T or D domains [1]. One of the ways in which $tRNA^{Phe}_{ASL}$ is a good model is that it has five modified nucleotides, of which, four occur at the most frequently modified anticodon residues in all tRNAs, positions: 32, 34, 37 and 39. This study of yeast^{Phe} ASLs focuses on the introduction of modifications and their impact on thermodynamic parameters. A noteworthy example for the use of thermodynamics to understand biological function was its application to GNRA tetraloops. While a significant number of nucleotide sequences conform to the tetraloop motif, in nature only a few are biologically relevant [10]. Only after determination that many loops with the GNRA motif had similar stability was it possible to conclude there must be a biological bias in the selection of those few functional GNRA loops in nature. Another application of thermodynamic parameters is in the prediction of the stability of structural features. While many rules have been forwarded to predict thermodynamic contributions of sequence in stem-loop structures, much work remains if the contribution of modified nucleotides is to be used for structure predictions of RNA.

MATERIALS AND METHODS

Synthesis of ASLs. Oligonucleotides were synthesized on a Perkin-Elmer Applied Biosystems Model 394 automated synthesizer using standard RNA phosphoramidite chemistry [11]. The four standard nucleoside phosphoramidites and two additional modified phosphoramidites, Cm and Gm were purchased from Glen Research (Sterling, VA, U.S.A.). The phosphoramidite for m^5C was obtained from ChemGene (Waltham, MA, U.S.A.). Phosphoramidites for Ψ, m^1G, m^5m^6U, $m^3\Psi$ and $m^1\Psi$ are not commercially available. They were prepared by methods previously described [4]. The chemical structures and "single letter" symbols for the modified nucleosides are presented in Fig. 1.

Naming of ASLs. All oligonucleotides used in this study are analogs of the Anticodon

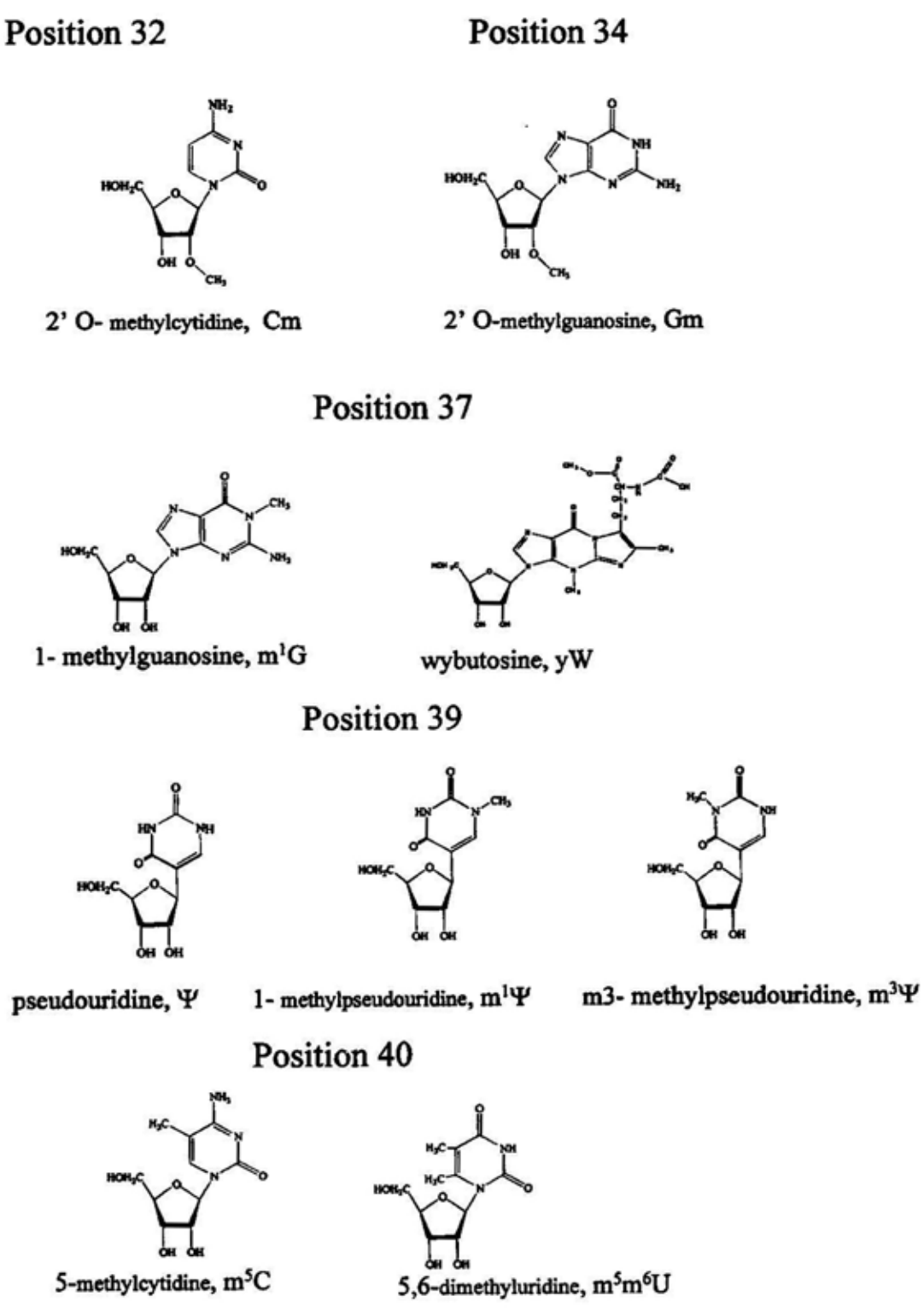

Figure 1. Chemical structures of modified nucleosides.

Eight different modified nucleosides were incorporated into the oligonucleotides used in this study. The structures of the modifications are shown. The structures are organized by the positions where they have been placed. In addition to the full name, the "one letter" symbol is provided below the structure. The structure of wybutosine is also shown because it is the native position 37 modification.

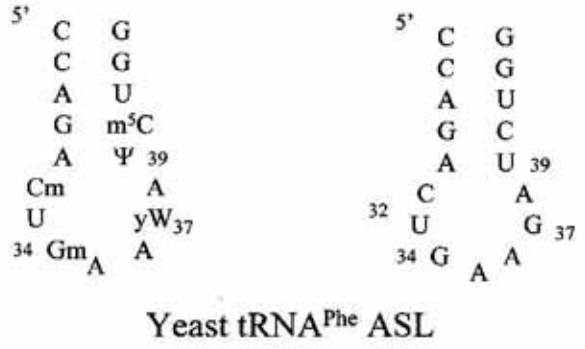

Figure 2. Oligonucleotides sequences.

All of the oligonucleotides used in this study are analogs of the Anticodon Stem Loop, ASL, of yeast $tRNA^{Phe}$. The sequence of the seventeen nucleotide ASLs including the five natural post-transcriptional modifications as well as the unmodified control are shown. The modified residues are Cm_{32}, Gm_{34}, yW_{37}, Ψ_{39} and m^5C_{40} with the positions numbered according to the standardized tRNA notation.

Stem-Loop, ASL, of yeast $tRNA^{Phe}$. The native and primary sequences are found in Fig. 2. To simplify discussion of the ASL analogs they are named in the following manner. The amino acid isoaccepting type and domain are stated first, for example the unmodified ASL of phenylalanine would be named $tRNA^{Phe}_{ASL}$. When modifications are present the "one letter" symbol [12] for the modification is followed by the sequence position, based on standardized tRNA numbering. An example is the ASL containing pseudouridine at tRNA position 39, $tRNA^{Phe}_{ASL\Psi 39}$.

Purification and chemical characterization of oligonucleotides. All ASLs were purified by anion exchange HPLC. Separations were made with a Rainin gradient controlled Rabbit HP system. A Machery-Nagel (Duren, Germany) 250/10 Nucleogen DEAE 60-7 column was employed for the separation. Figure 3 is a representative chromatograph of an HPLC separation. The purity of each oligonucleotide is confirmed by polyacrylamide gel electrophoresis, PAGE, as shown in Fig. 3 (inset). The PAGE was denaturing, 7 M urea, with 20% acrylamide. Successful incorporation of the modified nucleosides was verified by a quantitative nucleoside composition analysis [5]. Nucleotides are hydrolyzed with P1 nuclease and bacterial alkaline phosphatase. The constituent nucleosides were separated by reverse phase HPLC. Separa-

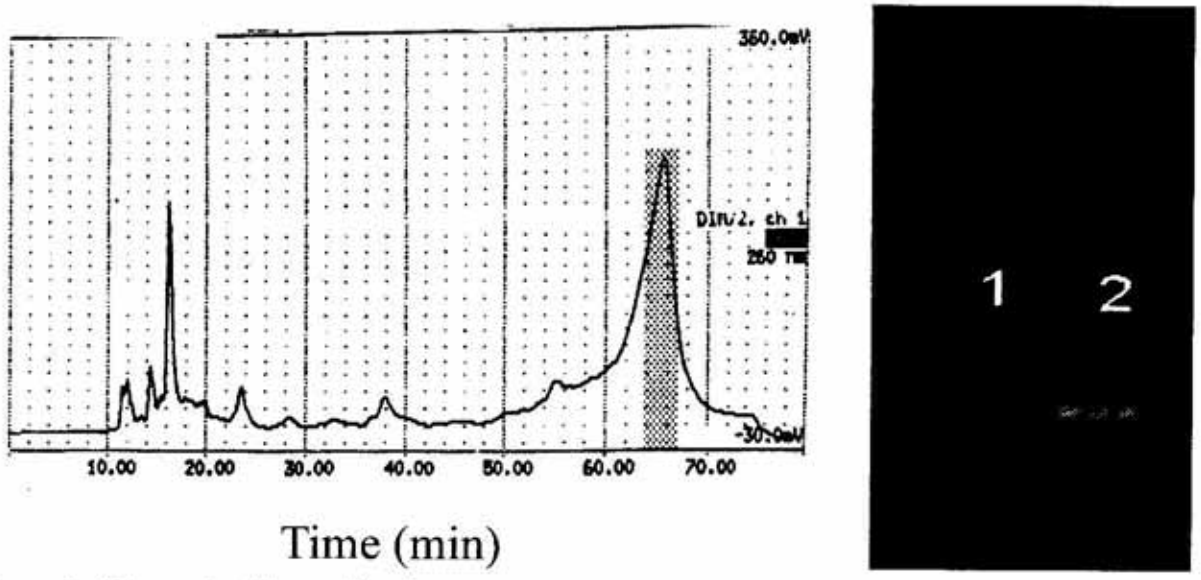

Figure 3. Oligonucleotide purification.

Purification of all ASLs was made by anion exchange HPLC. A chromatograph showing a representative separation of an ASL is shown. The fractions from the shaded region were pooled and desalted. Purity was confirmed with polyacrylamide gel electrophoresis, PAGE, see inset. Lane 1 is $tRNA^{Phe}_{ASL}$ $m^5m^6U_{40}$ and lane 2 is the unmodified ASL control.

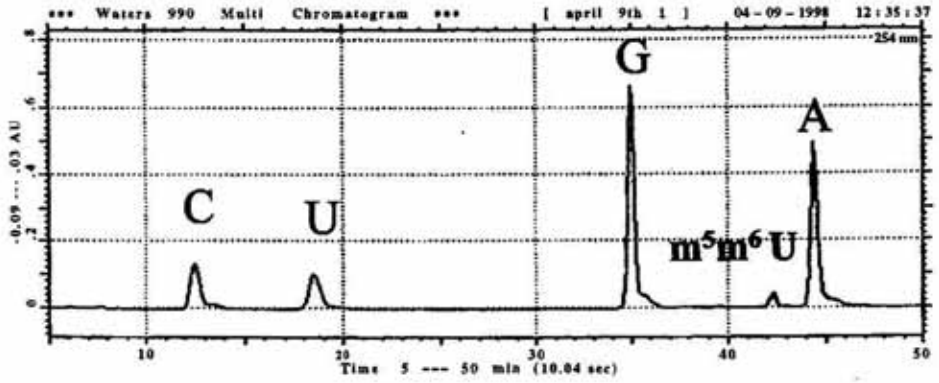

Figure 4. Compositional analysis confirms proper incorporation.

For ASLs that contained a modified nucleotide, a portion of the purified product was hydrolyzed. Reversed phase chromatography was used to resolve the major and modified nucleosides. Shown as an example of the method is the ASL containing m^5m^6U.

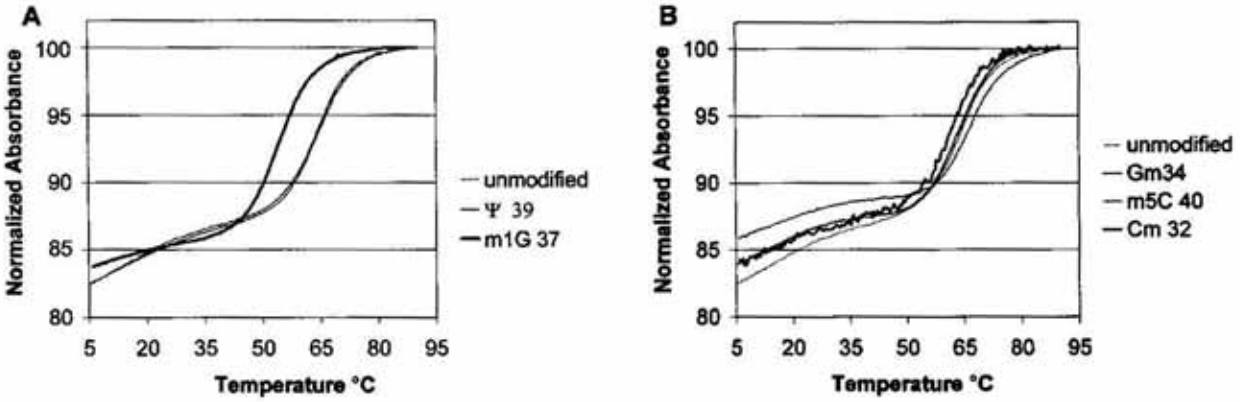

Figure 5. UV monitored thermal denaturations of oligonucleotides containing single native modifications.

Thermal denaturations of the unmodified control and the five single natural modified analogs were recorded using a Varian Cary 3 UV spectrophotometer. Panel A shows representative, normalized plots of the denaturations of ASLs m^1G_{37} (thick line), unmodified and Ψ_{39} (line farthest to the right). Panel B shows denaturations of ASLs Gm_{34} (moderate line), unmodified (thin line), m^5C_{40} (gray bold line) and Cm_{32} (bold line to left).

tions were accomplished with a Waters 600 system with a Supeleco (Bellfonte, PA, U.S.A.) Supelcosil LC 18-S 250 mm × 4.6 mm column. Separations were monitored over 250-400 nm with a Waters 990 photodiode array detector. Figure 4 is a representative chromatograph of the separation of the hydrolysate of $tRNA^{Phe}_{ASL}$ $m^5m^6U_{40}$ that confirms the incorporation of m^5m^6U.

Monitoring of thermal denaturations. Thermal denaturation of ASLs was monitored by UV absorbance at 260 nm over a temperature range 5-90°C using a Cary 3 Spectrophotometer. The denaturation of each oligonucleotide was monitored in triplicate at each of three concentrations, 0.5, 5 and 50 μM. Both 2 and 10 mm cuvettes were used in order to maintain proper optical response. Variation of t_m over the three concentrations was less than 1°C for all ASLs. The solution conditions were 10 mM sodium phosphate, pH 7.0, 100 mM NaCl. Samples were ther-

Table 1. Thermal denaturation results and van't Hoff thermodynamic parameters for the unmodified (control) and containing modification ASLs of yeast $tRNA^{Phe}$.

Results are the average of multiple runs and the standard deviation, S.D., is listed below.

ASL		t_m (°C)	ΔH (kcal/mol)	ΔS (cal/mol × K)	ΔG (kcal/mol)
Control	Avg:	63.8	-54.4	-161	-4.33
	S.D.:	0.45	2.4	7.6	0.1
Cm_{32}	Avg:	62.3	-58.1	-173.4	-4.38
	S.D.:	0.38	1.9	5.8	0.12
Gm_{34}	Avg:	62.4	-59.3	-177	-4.48
	S.D.:	0.53	3.1	9.2	0.2
m^1G_{37}	Avg:	54.4	-51.2	-156	-2.71
	S.D.:	0.4	2.1	6.4	0.09
Ψ_{39}	Avg:	65.1	-55.4	-166	-4.6
	S.D.:	0.43	2.5	7.4	0.18
m^5C_{40}	Avg:	66.1	-57.4	-169	-4.92
	S.D.:	0.24	1.2	3.6	0.11
$m^1\Psi_{39}$	Avg:	67.4	-57.8	-170	-5.16
	S.D.:	0.22	0.47	1.4	0.06
$m^3\Psi_{39}$	Avg:	50.6	-42.2	-131	-1.78
	S.D.:	0.41	2.1	6.7	0.04
$m^5m^6U_{40}$	Avg:	29.0	-24.4	-80.7	-0.62
	S.D.:	0.87	3.6	12	0.04

mally denatured and then renatured to determine reversibility of the transition with no hysteresis observed for any ASL. In all cases, a difference of less than 1°C in t_m was observed between denaturation and annealing. Concentrations were determined spectrophotometrically at 260 nm using an absorption coefficient of 1.6×10^{-6} M^{-1} cm^{-1}. Plots of thermal denaturation data are presented normalized, with the absorbance at 90°C equal to 100%.

Calculation of thermodynamic parameters. Thermodynamic parameters, ΔH, ΔS and ΔG, were calculated with a van't Hoff analysis of the data as described by Marky & Breslauer [6] using Origin software. The mono-phasic melting curves are consistent with a two-state transition assumption for the calculation. The values presented in Table 1 are the average of six thermal denaturations at three different sample concentrations. Calculation of the theoretical values of ΔH, ΔS and ΔG for the unmodified sequence, Table 2, were made from published values of sequence contributions, loop formation and the 3' residue of stem/loop junctions [13].

RESULTS AND DISCUSSION

Thermodynamic parameters of RNA derived from thermal denaturations have been widely used to gain a great deal of insight in structure and function understanding. A general feature of all the oligonucleotides in this study was a lack of change in t_m and denatura-

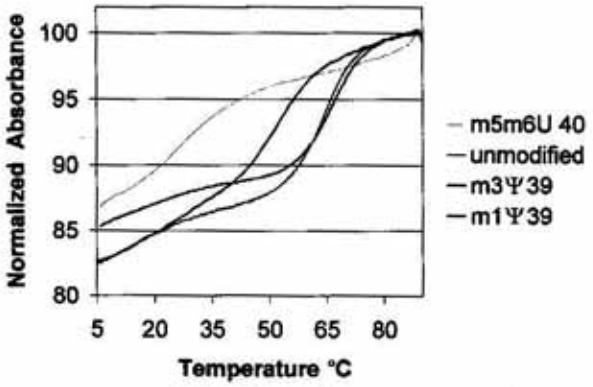

Figure 6. Thermal denaturation plots of ASL containing non-natural modified nucleotides.

Thermal denaturations of the unmodified control and three single non-natural modified ASLs were recorded using a Varian Cary 3 UV spectrophotometer. Denaturation of $m^5m^6U_{40}$ (thin line to left), unmodified (moderate line to right), $m^3\Psi_{39}$ (bold line to left) and $m^1\Psi_{39}$ (bold gray line) are shown.

tion profiles over a two magnitude, concentration range. This indicates that these ASLs are hairpins at the micromolar concentrations used for this study. For all the ASLs the denaturation appear mono-phasic which allows for analysis of the profile assuming a two-state model. The denaturations of the five ASL each containing a single natural, modification in the native context (Fig. 5) demonstrate that modifications can noticeably alter the denaturation profile. Depending on the modifications, t_m was either lowered or increased, as compared to the unmodified sequence.

Additional insight into the individual contribution of a nucleotide's modification is obtained by analysis of the van't Hoff parameters calculated from the UV monitored denaturation profiles, Table 1. For the single modification that significantly lowered t_m, m^1G_{37}, the most influenced van't Hoff parameter was ΔH. This suggests that the addition of m^1G disrupts some loop features present in the unmodified and other singly modified sequences. The size of the change in ΔG, > 1.5 kcal, is near the magnitude range for the loss of a base pair. The location of this modification, position 37, in the middle of the loop, is not one of those considered in theoretical analysis of hairpin stability. The high frequency of modification of residue 37, > 75% [12], in native tRNA indicates a functional advantage which appears to have a structural basis.

Two modifications, Cm and Gm, only slightly change t_m. There is some shifting of thermodynamic parameters, but no increase in stability over unmodified, based on similar

Table 2. The predicted thermodynamic stability of the unmodified yeast $tRNA^{Phe}_{ASL}$ calculated with sequence dependent contributions

		ΔH	ΔS	ΔG
		(kcal/mol)	(cal/mol × K)	(kcal/mol)
CG		-12.2	-29.7	-2.9
AU		-10.2	-26.2	-2.1
GC		-13.3	-35.5	-2.3
AU		-7.6	-19.2	-1.7
C/A[1]		-6.3	-17.7	-0.8
A/U		-5.7	-16.4	-0.7
Terminal	AU			0.4
Loop	Initiation			5
Calculated		-55.3	-144.7	-5.1
Observed		-54.4	-161	-4.3

[1]The identity of the terminal mismatch has a thermodynamic contribution.

ΔGs. This lack of contribution was a little unexpected because of the generally favorable contribution of 2'O methylation in stem/duplex contexts [14]. The enzymes responsible for these modifications have not been identified. Analysis of the tRNA database finds Cm_{32} and Gm_{34} occur only in phenylalanine tRNAs [12]. This observation, along with a lack of thermodynamic changes, favors a biological function or identity for these modifications in phenylalanine tRNAs.

For two of the modifications, Ψ and m^5C, their incorporation increased t_m compared to the unmodified sequence. The change of thermodynamic parameters when Ψ is present, reduced ΔH and ΔS, suggests that perhaps Ψ makes a structural contribution of increased 3' side base stacking rather than additional hydrogen bond formation. This idea has been forwarded for a lysine tRNA [14]. The structural feature appears to provide a selective advantage. Analysis of all known tRNA gene sequences [12] shows a significant bias that favors dT, 40%, at position 39. Analysis of mature tRNA sequences further supports the advantage that Ψ provides in that uridines at position 39 are almost completely modified to Ψ, 95%.

The increase in t_m with the presence of m^5C is in a similar range to that of Ψ. It is likely that a structural change accounts for the thermodynamic change. For DNA in general and for an analog of yeast $^{Phe}_{ASL}$ specifically, it has been shown that m^5C adds a bend in the backbone near the modified residue [16]. The significance of the contribution to function is not clear. Additional biochemical analysis using m^5C modified ASLs may identify a functional role but, it has already been determined that m^5C is not essential for tRNA charging or ribosomal binding [17]. In known tRNA sequences m^5C_{40} is rare, with only five observations. Four of the five occurrences are phenylalanine tRNAs but that is only 10% of phenylalanine sequences in the database. The enzyme thought to be responsible for the m^5C_{40} is the same enzyme that modifies C at 49. The frequency of m^5C at 49 is quite different. Over 32% of known tRNA sequences have m^5C at position 48 or 49. Further, of those with the potential to be modified, i.e. C_{49}, over 80% are modified. A consensus substrate sequence for the m^5C methyl transferase has been proposed that requires a modified G three residues 5' of the modified C [18]. It is possible that m^5C at position 40 is not a functional modification but, a combined consequence of the position 37 modification and an enzyme specificity. While m^5C may induces a structural change when placed at position 40 it is likely its functional role is when placed at position 48 or 49.

A synthetic approach to production of modified analogs provides the opportunity to study the contribution of particular atoms of a nucleoside. Several analogs with non-natural modifications were produced to study, at the atom level, the contributions that selected modifications have. By the selective replacement of a hydrogen with a methyl group, the global contribution of a modification can be studied in detail. In some cases, the addition of a methyl only mildly alters the local residue conformation while in other cases it will significantly alter the conformation. The thermal denaturations of the non-natural modifications are shown in Fig. 6. The thermal denaturations show that one non-natural modification, $m^1\Psi$, increased t_m, one non-natural substitution, $m^3\Psi$, moderately lowered t_m and one substitution, m^5m^6U, significantly lowered t_m. The van't Hoff thermal dynamics parameters calculated from analysis of the denaturation plots are found in Table 1.

For the modification that increased t_m as compared to the unmodified, $m^1\Psi_{39}$, ΔH and ΔS are both affected. It is likely that the ΔH decrease was due to methylation, similar to that observed for Cm, Gm and m^5C (Table 1). The decreased ΔS is likely due to the same factors as observed with pseudouridine in $tRNA^{Phe}_{ASL}$ Ψ_{39}. The change in thermal parameters by the introduction of $m^3\Psi_{39}$, increasing ΔH 12 kcal and ΔS 30 cal/degree, al-

most equal the amount predicted by thermodynamic stability tables for the loss of the terminal base pair and change in the terminal loop closing mismatch (Table 2) [13]. This is consistent with what would be expected because the N3 proton is involved in the A_{31}-Ψ_{39} base-pair.

For the non-natural substitution that significantly lowered t_m, $m^5m^6U_{40}$, the destabilization was partially expected. It was known that methylation at the 6 position of uridine, at the nucleoside level, induces a *syn* conformation. It was not certain if this N-glycosidic bond conformation would be retained at the oligonucleotide level. If the *syn* conformation is retained the potential to form a $G_{30}U_{40}$ base pair would be lost. The calculated change for the disruption of the terminal base pair and change in stacking parameters is ΔH 33 kcal and ΔS of 89 cal/degree. The similarity observed of a change of 30 kcal for ΔH and 80 cal/degree ΔS supports the predicted structural changes.

In summary, it appears that Cm and Gm may have a biological function. In contrast, m^1G, Ψ and m^5C appear to have structural functions that may or may not have direct biological implications. The examples presented in this report were only possible by combining chemical synthesis, automated polymerization, chromatography and biophysical studies. They represent a novel, unified approach to understanding the biological and structural contributions of natural nucleoside modifications. The approach provides tools to quantitatively understand structure/function relationships. This approach when coupled with analysis of the tRNA modification database may provide a means to distinguish and understand the structural and functional roles of modifications.

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