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RNA Two-State Conformation Equilibria and the Effect of Nucleobase Methylation**

Claudia Höbartner, Marc-Olivier Ebert, Bernhard Jaun, and Ronald Micura*

The constitutional diversity of RNA is impressively reflected in more than 90 chemically modified nucleosides.[1, 2] It was reported recently that the modified nucleosides of a human mitochondrial $tRNA^{Lys}$ are required for the formation of the correct cloverleaf three-dimensional structure.[3] In contrast, the corresponding unmodified in vitro transcript did not fold into a cloverleaf but into an extended bulged hairpin. To the best of our knowledge, these studies provide the first and so far only experimental data that document the significant influence of nucleoside modifications on RNA folding. These results encouraged us to investigate systematically the effect of nucleobase methylation in short palindromic oligoribonucleotide duplexes. We found that the replacement of single nucleosides with the corresponding methylated counterparts, such as 1-methylguanosine (m^1G), N^2,N^2-dimethylguanosine ($m^2{}_2G$), N^6,N^6-dimethyladenosine ($m^6{}_2A$), or 3-methyluridine (m^3U), led to the formation of a hairpin rather than of a duplex structure.[4] These remarkable changes in the secondary structure motif form the basis of our investigations. Herein we report on single-stranded oligoribonucleotides that exist in monomolecular two-state conformation equilibria. By methylation of selected nucleobases, it is possible to shift these equilibria to a significant extent.

The methylation pattern of the studied sequences corresponds to the naturally occurring helix 45 loop located at the 3′-end of ribosomal RNA of the small subunit (SSU) (Figure 1).[5] The two successive $m^6{}_2A$ nucleosides within the four-membered loop are conserved almost universally in bacteria and eukaryotes. These modified adenosines are functionally significant; however, the exact reason for their methylation is not yet understood. We have chosen 5′-…$CCm^2GGm^6{}_2A$-$m^6{}_2AGG$…-3′ as a lead sequence for a comparison of the conformation of methylated versus nonmethylated sequence constructs (Table 1, Figure 2). Two questions are important in the sequence design. First, is a complementary sequence partition such as 5′-…CCUUCC…-3′ able to break up the nonmethylated stem-loop structure of 5′-…CC*GGAA*GG…-3′?

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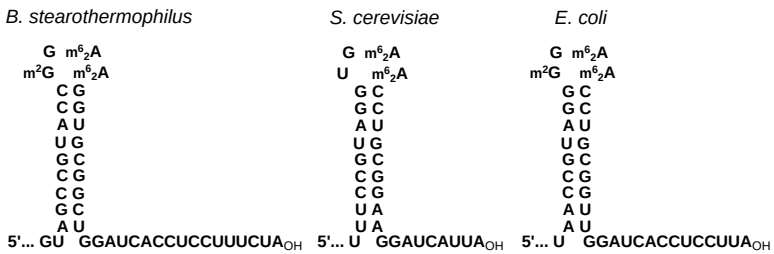

Figure 1. Selected helix 45 motifs located at the 3′-end of rRNA of the SSU. The successive dimethylated m^6_2A nucleotides within the loop are conserved in most organisms. The reason for the methylation is unclear. $m^2G = N^2$-methylguanosine.

Second, if so, does the methylation according to the helix 45 pattern 5′-…CCm^2GGm^6_2Am^6_2AGG…-3′ cause a reconstitution of the original stem-loop structure?

Figure 2 shows a set of sequences that is ideal to investigate these questions experimentally.[6] In principle, both the nonmethylated sequence **1** and the methylated sequence **2** are able to adopt two different conformations **1′/1″** and **2′/2″**, respectively (Figure 2a). These conformations are easily assigned by comparison with a pair of appropriate references (Figure 2b). The reference sequences **1a**/**1b** and **2a**/**2b** represent short sections of sequences **1** and **2** that are only able to adopt a single conformation. This defined conformation of **1a**, **1b** (and **2a**, **2b**) corresponds to one of the two possible conformations **1′** or **1″** of sequence **1** (and **2′** or **2″** of sequence **2**).

We used ^{1}H NMR spectroscopy to determine the conformations of **1** and **2** (Figure 3). The NH signals in the ^{1}H NMR spectrum of **1** show characteristic slow H/D-exchange; the spectrum displays the NH resonances as measured for the individual reference sequences **1a** and **1b** in a 25:75 ratio.[7] This clearly shows that sequence **1** exists in a conformation equilibrium between **1′** and **1″** at 298 K.[8] In contrast, the methylated sequence **2** gives rise to the NH resonances of reference **2a** only, which indicates that sequence **2** exists exclusively in a single conformation (**2′**). It is thus clear that methylation forces the former formation of the unfavorable secondary structure.

The approach presented allows an estimation of the thermodynamic properties of base pairing of the competing substructures within sequences **1** and **2**. The thermodynamic parameters of references **1a**/**1b** and **2a**/**2b** were derived from UV melting profiles (Figure 4).[9] A comparison of the T_m values and the corresponding $\Delta G^{298\,K\text{-}UV}$ values of the reference substances can be used to predict qualitatively the position of the conformation equilibrium of **1** or **2**. However, we wish to stress that the references do not necessarily represent the precise thermodynamic behavior of the corre-

Table 1. Methylated and nonmethylated oligoribonucleotides that contain a helix 45 sequence motif of rRNA; melting temperatures T_m, thermodynamic data of double-helix formation derived from UV melting profiles, conformation equilibrium constants derived from ^{1}H NMR spectra, mass spectral data.[a-e]

No.	Base sequences	T_m[a]	Thermodynamic data of double-helix formation[a, b]			Conformation equilibrium constant[c,d]		Mass spectral data[e]	
			ΔH°	ΔS°	$\Delta G^\circ_{298\,K}$	$K^{298\,K\text{-}NMR}_{1'' \rightarrow 1'}$		$M_{calcd.}$	$M_{obs.}$
		[°C]	[kcal mol^{-1}]	[cal mol^{-1} K^{-1}]	[kcal mol^{-1}]			[amu]	
1	GACCGGAAGGUCCGCCUUCC	**78.0**				3.0	(25:75)	6356.8	6355.9
1a	GACCGGAAGGUCC	**77.7**	− 54.7	− 157.1	− **7.9**			4178.6	4178.1
1b	CGGAAGGUCCGCCUUCC	**73.3**	− 55.0	− 160.0	− **7.3**			5377.2	5376.3
2	GACCm^2GGm^6_2Am^6_2AGGUCCGCCUUCC	**72.7**	− 48.3	− 140.7	− **6.3**	< 0.02	(> 98: < 2)	6427.0	6426.4
2a	GACCm^2GGm^6_2Am^6_2AGGUCC	**72.7**	− 49.8	− 145.1	− **6.5**			4248.7	4247.9
2b	Cm^2GGm^6_2Am^6_2AGGUCCGCCUUCC	**54.0**	− 34.5	− 104.2	− **3.4**			5447.4	5445.0
3	GACCGGAAGGUCCGCCUUCCG	**81.2**				5.7	(15:85)	6702.0	6702.1
4	GACCm^2GGm^6_2Am^6_2AGGUCCGCCUUCCG	**72.4**	− 41.9	− 122.0	− **5.5**	< 0.02		6772.2	6771.8
5	GACCGGAAGGUCUUCCUUCC	**78.2**				1.2	(45:55)	6318.8	6318.3
5a	GACCGGAAGGUCU	**77.9**	− 56.4	− 161.4	− **8.3**			4179.6	4178.6
5b	CGGAAGGUCUUCCUUCC	**70.5**	− 48.0	− 140.8	− **6.0**			5339.2	5339.0
6	GACCm^2GGm^6_2Am^6_2AGGUCUUCCUUCC	**73.2**	− 49.3	− 142.5	− **6.8**	< 0.02		6388.9	6386.8
6a	GACCm^2GGm^6_2Am^6_2AGGUCU	**73.2**	− 48.8	− 141.1	− **6.7**			4249.7	4248.8
6b	Cm^2GGm^6_2Am^6_2AGGUCUUCCUUCC	**41.6**	− 34.7	− 108.4	− **2.4**			5409.3	5408.1
7	GACCGGAAGGUCE$_3$E$_3$CCUUCC	**70.8**				1.5	(40:60)	6130.7	6131.0
7a	GACCGGAAGGUC	**72.4**	− 51.3	− 149.3	− **7.1**			3874.1	3874.9
7b	CGGAAGGUCE$_3$E$_3$CCUUCC	**68.2**	− 62.4	− 183.3	− **7.7**			5151.1	5152.0
8	GACCm^2GGm^6_2Am^6_2AGGUCE$_3$E$_3$CCUUCC	**67.5**	− 50.9	− 149.2	− **6.4**	< 0.02		6200.9	6200.6
8a	GACCm^2GGm^6_2Am^6_2AGGUC	**64.9**	− 39.5	− 116.6	− **4.7**			3945.2	3945.5
8b	Cm^2GGm^6_2Am^6_2AGGUCE$_3$E$_3$CCUUCC	**40.6**	− 31.4	− 99.2	− **1.8**			5221.3	5222.5
9	GAGGGGAACCUCCGGGUUCC	**81.8**				19.0	(5:95)	6436.9	6436.5
9a	GAGGGGAACCUCC	**67.0**	− 43.9	− 129.6	− **5.3**			4178.6	4176.7
9b	GGGAACCUCCGGGUUCC	**79.9**	− 48.4	− 138.4	− **7.1**			5417.3	5418.2
10	GAGGm^{2}GGm$^6_2$Am$^6_2$ACCUCCGGGUUCC	**61.0**	− 40.0	− 120.0	− **4.3**	< 0.02		6507.0	6504.5
11	GAGGGGAACCUCCGGGUUC	**73.9**				4.0	(20:80)	6131.7	6130.4
11a	GAGGGGAACCUCC	**67.0**	− 43.9	− 129.6	− **5.3**			4178.6	4176.7
11b	GGAACCUCCGGGUUC	**71.7**	− 43.7	− 127.9	− **5.6**			4766.9	4766.1
12	GAGGm^{2}GGm$^6_2$Am$^6_2$ACCUCCGGGUUC	**60.7**	− 40.1	− 119.5	− **4.5**	< 0.02		6201.8	6199.0

[a] 2 μM, 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0. [b] ΔH° and ΔS° were derived from α/T-plots by curve fitting; see ref.[9]. [c] 0.2 mM; 25 mM sodium arsenate buffer, pH 7.4, 298 K, H_2O/D_2O 9:1. [d] Conformer ratio (%). [e] MALDI-TOF or ESI mass spectrometry was used.

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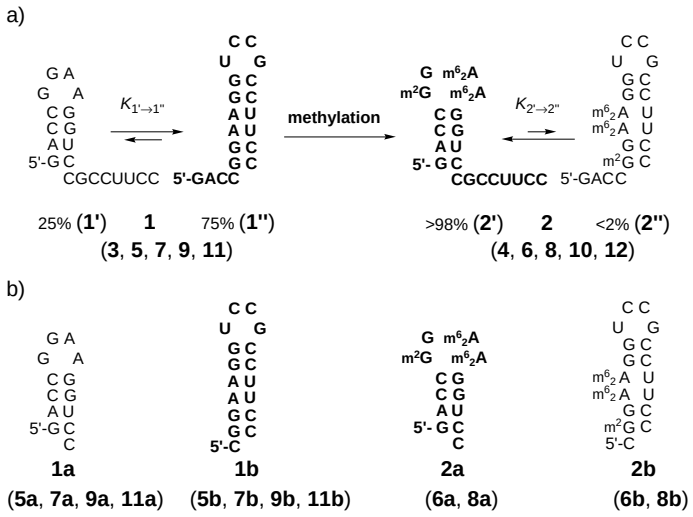

Figure 2. a) General approach for the investigation of monomolecular RNA two-state conformation equilibria and the equilibrium shifts induced by nucleobase methylation. This example illustrates sequence constructs that comprise a helix 45 motif (compare Figure 1). Both **1** and **2** are, in principle, able to adopt two different conformations (**1′**/**1″** and **2′**/**2″**). b) With the corresponding set of references (**1a**/**1b** and **2a**/**2b**), the conformation was easily determined by means of ^{1}H NMR spectroscopy (compare Figure 3). At 298 K, the nonmethylated sequence **1** exists in a two-state conformation equilibrium (**1′**/**1″** 25:75). The position of the equilibrium is sensitive to sequence variations (compare equilibrium constants for **3**, **5**, **7**, **9**, and **11** in Table 1). Upon methylation, the equilibrium is shifted in the direction of a single conformer (>98 % **2′**), which corresponds to the former unfavorable conformer.

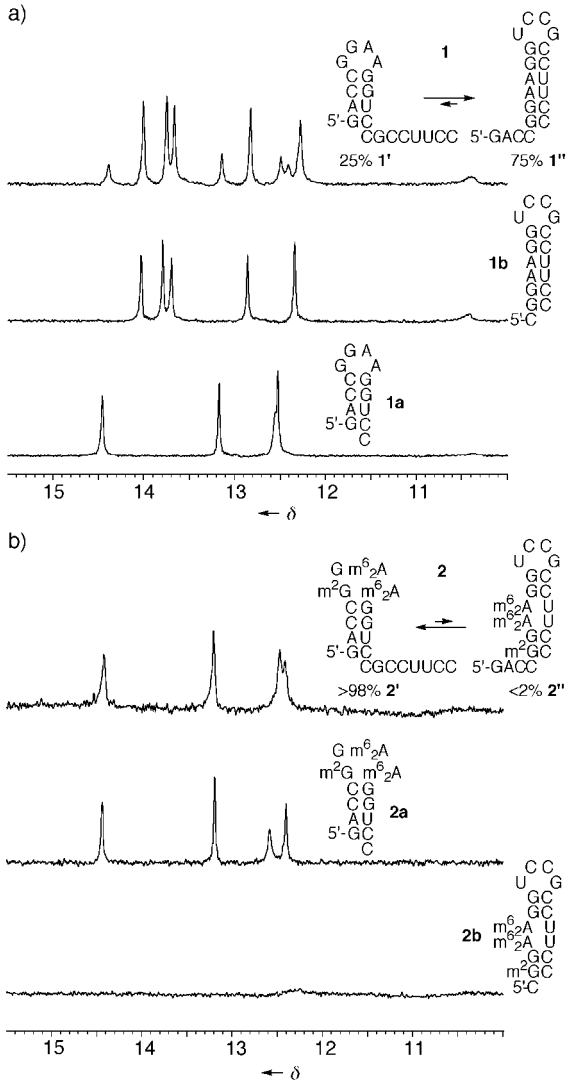

Figure 3. Determination of RNA conformation equilibria by means of ^{1}H NMR spectroscopy. a) The ^{1}H NMR spectrum of **1** shows signals according to the NH-resonances of both references **1a** and **1b** and ascertains a 25:75 equilibrium of **1′** and **1″** (for sample conditions see ref. [7]). b) In contrast, the ^{1}H NMR spectrum of **2** reflects the NH resonances corresponding to reference **2a**. Reference **2b** does not show any NH resonances at 298 K—evidence for the conformation depicted was obtained from the UV melting profile (compare Figure 4).

sponding substructures within **1** or **2**, and moreover, that the determination of ΔG derived from UV melting curves is subject to a 2 to 5 % error. The error value for references **1a**/**1b** is $\pm 0.2-0.4$ kcal mol^{-1} and therefore within the order of the equilibrium to be estimated. The energy difference $\Delta\Delta G^{298\,K\text{-}UV}_{\mathbf{1a},\mathbf{1b}}$ of $+0.6$ kcal mol^{-1} derived for the individual references favors **1a** over **1b** as the more stable hairpin, and allows an estimate of $K^{298\,K\text{-}UV}_{\mathbf{1}'\rightarrow\mathbf{1}''}$ (ca. 0.37). However, the exact position of the equilibrium can only be determined from the ^{1}H NMR spectrum and in case of sequence **1**, is clearly in favor of conformation **1″** ($K^{298\,K\text{-}NMR}_{\mathbf{1}'\rightarrow\mathbf{1}''} = 3.0$).[10] A $\Delta\Delta G^{298\,K\text{-}UV}_{\mathbf{2a},\mathbf{2b}}$ value of $+3.1$ kcal mol^{-1} is derived from the UV data of methylated references **2a** and **2b** and suggests a $K^{298\,K\text{-}UV}_{\mathbf{2}'\rightarrow\mathbf{2}''}$ of 0.0053. These values further corroborate that **2′** is the dominant conformation of sequence **2**, as determined from the ^{1}H NMR spectrum ($K^{298\,K\text{-}NMR}_{\mathbf{2}'\rightarrow\mathbf{2}''} < 0.02$; Figure 3b). We point out that the methylations destabilize **1′** relative to **2′** and, much more prominently, **1″** relative to **2″**. At a molecular level, this can be explained by the disruption of the GA sheared base pair for **1′** relative to **2′**, and the disruption of two Watson–Crick base pairs for **1″** relative to **2″**.

The observations described above are not limited to sequences **1** and **2**. To understand further the properties of two-state conformation equilibria and the impact of methylation on these equilibria, we investigated the related sequences **3** to **12** (compare Table 1 and Supporting Information). The oligonucleotides **3** and **4** differ from **1** and **2** by an additional guanosine at the 3′-end. Consequently, the equilibrium of the nonmethylated sequence **3** is further shifted towards the UCCG-hairpin conformation ($K^{298\,K\text{-}NMR}_{\mathbf{3}'\rightarrow\mathbf{3}''} = 5.7$). The equilibrium of the methylated sequence remains reversed; **4** exclusively adopts the m^{2}GGm$^6{}_2$Am$^6{}_2$A stem-loop structure, which corresponds to **2′** ($K^{298\,K\text{-}NMR}_{\mathbf{4}'\rightarrow\mathbf{4}''} < 0.02$).

The oligonucleotides **5** and **6** differ from **1** and **2** by a two-nucleoside exchange (C13G14 by U13U14). Therefore, the two-state conformation equilibrium of sequence **5** no longer reflects the competition between a GNRA and a UNCG hairpin, both of which are known to adopt extraordinarily stable secondary structures.[11] In **5**, the latter sequence segment is replaced by UCUU. This exchange causes a conformation equilibrium in which conformer **5″** is only slightly favored over **5′** ($K^{298\,K\text{-}NMR}_{\mathbf{5}'\rightarrow\mathbf{5}''} = 1.2$). Upon methylation, the position of the equilibrium is pushed entirely to the m^{2}GGm$^6{}_2$Am$^6{}_2$A hairpin **6′** ($K^{298\,K\text{-}NMR}_{\mathbf{6}'\rightarrow\mathbf{6}''} < 0.02$).

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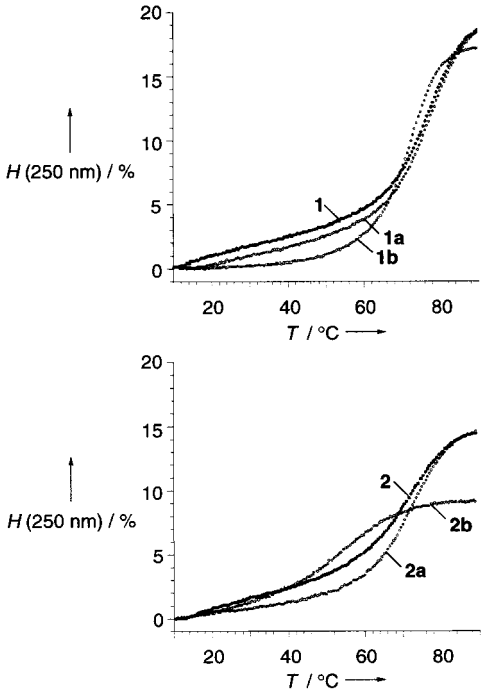

Figure 4. UV melting profiles: a) GACCGGAAGGUCCGCCUUCC **1** (T_m = 78.0 °C), references GACCGGAAGGUCC **1a** (T_m = 77.7 °C), CGGAAGGUCCGCCUUCC **1b** (T_m = 73.3 °C). b) GACCm^{2}GGm$^6{}_2$Am$^6{}_2$AGGUCCGCCUUCC **2** (T_m = 72.7 °C), references GACCm^{2}GGm$^6{}_2$Am$^6{}_2$AGGUCC **2a** (T_m = 72.7 °C), Cm^{2}GGm$^6{}_2$Am$^6{}_2$AGGUCCGCCUUCC **2b** (T_m = 54.0 °C); c = 2 μM, 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0.

The oligonucleotides **7** and **8** differ from **1** and **2** again by a two-nucleoside exchange within the original UCCG sequence segment; C13G14 is replaced by non-nucleotide linker units, namely bis(triethylene glycol) phosphate (E_3E_3).[12] Interestingly, this modification does not cause a significant destabilization of **1″**. The constants of the conformation equilibria of **7** and **8** are comparable to those of **1** and **2**.

The oligonucleotides **9**/**10** and **11**/**12** have different loop-closing base pairs than the sequences discussed above (C/G converted into G/C; compare also Figure 1). The sequence pair of **9**/**10** represents a conformation switch induced by methylation ($K^{298\,K\text{-NMR}}_{\mathbf{9}'\rightarrow\mathbf{9}''} = 19$ and $K^{298\,K\text{-NMR}}_{\mathbf{10}'\rightarrow\mathbf{10}''} < 0.02$).

The ability of RNA molecules to adopt two (or more) stable conformations is generally discussed in terms of RNA tertiary structure and is often related to the concept of molecular switches.[13, 14] The latter implies that the conformation of a single molecule is forced into an alternative one by an extrinsic stimulator, for example, the addition of Mg^{2+},[14a] the addition of a complementary oligonucleotide,[14b] or a variation in the pH.[14c] At the secondary structure level and in the absence of extrinsic factors, monomolecular conformation equilibria have been postulated,[15] but to the best of our knowledge, the coexistence of conformers in equilibrium has not yet been verified experimentally for shorter RNA sequences. Our approach now allows the thorough investigation of monomolecular equilibria of competing stem-loop structures within single-stranded RNA molecules: that is, first, the identification of the base sequence prerequisites and the allowed sequence variety; second, the determination of the pairing properties of the competing substructures; and third, the extraction of the thermodynamics for the conformation equilibria. Furthermore, we consider the investigation of such equilibria fundamental for an understanding of the early stages of the RNA-folding processes. Equilibria of this kind may reflect the status of branching into different folding pathways.[16]

Beyond that, with the sequence design that includes the helix 45 motif, we have addressed equilibrium shifts ("switches") induced by methylation of the nucleobases. In particular, tetra-, penta-, and hexanucleotide sections that are complementary to the helix 45 loop region are found in the nucleotide sequences of rRNA.[17] These complementary sections may well constitute folding traps that are avoided upon methylation. This might be a reason for the highly conserved methylation pattern of helix 45 in the SSU of rRNA.[18]

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[7] ^{1}H NMR spectra were acquired on a Bruker Avance 500 MHz DRX by using excitation sculpting to suppress the water signal: a) T.-L. Hwang, A. J. Shaka, *J. Magn. Reson. A* **1995**, *112*, 275–279; or alternatively, on a Varian Unity 500, by applying a selective excitation-refocusing sequence that employs selective pulses shaped according to the G4 profile (excitation; b) L. Emsley, G. Bodenhausen, *Chem. Phys. Lett.* **1990**, *165*, 469–476; 2.62 ms, rf amplitude = 1.74 kHz) or the REBURP profile (refocusing; c) H. Geen, R. Freeman, *J. Magn. Reson.* **1991**, *93*, 93–141; 1.4 ms, rf amplitude = 4.47 kHz). Both shaped pulses were centered at 13 ppm. NMR sample preparation: oligonucleotide (0.2–0.3 mM), sodium arsenate buffer (25 mM; pH 7.4, 298 K, H_2O/D_2O 9:1). All samples were heated at 90 °C for 1 min, then rapidly cooled to room temperature and equilibrated for 1 h before measurements. The samples were stored at 4 °C and remeasured after a week, at which point some of the reference sequences had formed duplexes to a minor extent.
[8] The ^{1}H NMR spectra of sequences **7** and **9** were additionally recorded at temperatures of 305 K and 288 K: no significant change in the position of the equilibrium was observed. At 274 K the NH signals are slightly broadened.
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[10] The fact that **1a** is more stable than **1b** whereas conformer **1′** is 3-fold less populated than **1″** was unexpected. A possible explanation is that

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the 3′-overhang for state **1′** is fully complementary to its loop, whereas the 5′-overhang for **1″** is complementary to only two of the four bases, thus possibly providing a facile route to **1″** and a faster rate constant in the forward direction.

[11] a) M. E. Burkard, D. H. Turner, I. Tinoco, Jr. in *The RNA World*, *Second Edition* (Eds.: R. F. Gesteland, T. R. Cech, J. F. Atkins), CSHL Press, New York, **1999**, pp. 675 – 680; V. P. Antao, S. Y. Lai, I. Tinoco, Jr., *Nucleic Acids Res.* **1991**, *19*, 5901 – 5905; c) R. Kirchner, M. Vogtherr, S. Limmer, M. Sprinzl, *Antisense Nucleic Acid Drug Dev.* **1998**, *8*, 507 – 516.

[12] For a thermodynamic comparison of nucleotide loop replacements with non-nucleotide linkers, see: W.Pils, R. Micura, *Nucleic Acids Res.* **2000**, *28*, 1859 – 1863.

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[16] a) P. Brion, E. Westhof, *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 113 – 137; b) I. Tinoco, Jr., C. Bustamante, *J. Mol. Biol.* **1999**, *293*, 271 – 281.

[17] For an example of an rRNA databank, see: Y. Van de Peer, P. Rijk, J. Winkelmans, R. De Wachter, *Nucleic Acids Res.* **2000**, *28*, 175 – 176.

[18] It has been reported that mutations of the rRNA dimethylase ksgAp of *E. coli* block the $m^6{}_2Am^6{}_2A$ dimethylation of 16S rRNA; however, they do not interfere with rRNA processing. Consequently, the mutants synthesize the SSU of rRNA in which helix 45 is not modified. These allow the growth of the mutant strains at a reduced rate. The unmodified ribosomes are defective in several aspects of translation in vitro; see: a) P. H. van Knippenberg in *Structure, Function, and Genetics of Ribosomes* (Eds.: B. Hardesty, G. Kramer), Springer, New York, NY, **1986**, pp. 412 – 424; b) D. Nègre, C. Weizmann, J. Ofengand, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 4902 – 4906; c) D. L. J. Lafontaine, D. Tollervey in *Modification and Editing of RNA* (Eds.: H. Grosjean, R. Benne), ASM, Washington, DC, **1998**, pp. 281 – 288.

Iron-Catalyzed Cross-Coupling Reactions of Alkyl-Grignard Reagents with Aryl Chlorides, Tosylates, and Triflates**

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Classical cross-coupling processes such as the Kumada – Corriu, Negishi, Stille, or Suzuki reaction are of utmost importance for modern organic synthesis.[1] These transformations are usually catalyzed by palladium or nickel complexes, although no inherent restriction to these late transition metals exists. Aryl iodides and bromides are the best substrates; only recently have special ligands been designed that allow the scope of these methods to be extended to aryl chlorides.[2] Aryl triflates represent yet another class of suitable starting materials, whereas less expensive sulfonates have hardly been used so far because of their lack of activity in most cases.[3]

Herein we describe initial studies on the development of an alternative cross-coupling procedure which allows the attachment of alkyl groups to arenes in a very efficient way.[4] This method is distinguished by a number of notable advantages:

1) expensive nobel metal catalysts are replaced by cheap, stable, commercially available and toxicologically benign iron salts
2) aryl chlorides and triflates provide a priori better results than the corresponding bromides or iodides
3) aryl tosylates turned out to be suitable starting materials as well
4) the reaction is performed under "ligand-free" conditons
5) the reaction times are usually very short.

Although iron salts were proposed as catalysts for cross-coupling reactions by Kochi et al. as early as 1971,[5] they attracted little attention in the following decades.[6] Their scope remained essentially limited to reactions of Grignard reagents with *alkenyl* halides.[6, 7] Successful applications to other types of substrates, in particular to *aryl* halides, have not been reported. Moreover, the mechanism of the iron-catalyzed reactions remained rather obscure, whereas detailed insights into most of the prominent palladium-catalyzed processes have been gained over the years.[1] It has been proposed that Fe^0 or Fe^I species constitute the catalytically relevant intermediates,[5] although no secured information as to their structure or mode of action could be obtained. Alternatively, "super-ate" complexes of Fe^{II} have been suggested as the active species.[8]

Taking recent advancements in the field of "inorganic Grignard reagents" into consideration,[9] however, these hypotheses seem to be highly unlikely and called for a re-evaluation of iron-catalyzed cross-coupling chemistry. It is now well established that $FeCl_2$ reacts with *four* equivalents of

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