Chemical Approach for the Study of the 'Kissing Complex' of *Moloney murine leukaemia* Virus

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The replication of *Moloney murine leukaemia* virus relies on the formation of a stable homodimeric 'kissing complex' of a GACG tetraloop interacting through only two C·G base pairs flanked of 5′-adjacent unpaired adenosines A9. Previous NMR investigations of a model stem loop **1** has not permitted to reveal the origin of this interaction. Therefore, with the aim of deeper comprehension of the phenomena, the model sequence **10** was prepared where position 9 has been substituted for a nucleoside offering a wider π-stacking. In this context, the wyosine phosphoramidite building block **2** was prepared and incorporated by adapting the conditions of the automated synthesis and developing original templated enzymatic ligation. However, no 'kissing interaction' has been observed for this model sequence **10** due to steric hindrance as confirmed by computational simulation. Consequently, several other model sequences, **18**, **23**–**26**, containing modified nucleosides were prepared. Finally, the importance of the cross-loop H-bond between G8 and G11 nucleobases was revealed by preparing a 18mer RNA hairpin **27**, where the guanosine G8 has been substituted for inosine. The latter, which does not possess a C^3 amino function compared to guanosine, is unable to form any 'kissing complex' demonstrating the importance of this secondary interaction in the formation of the complex.

1. Introduction. – During the NMR investigation of a model stem loop **1** from retroviral RNA of *Moloney murine leukaemia* virus, *Kim* and *Tinoco Jr.* have observed that a highly conserved GACG tetraloop formed a stable homodimeric 'kissing complex' through the formation of only two C·G base pairs [1] (*Fig. 1*). By mutagenic

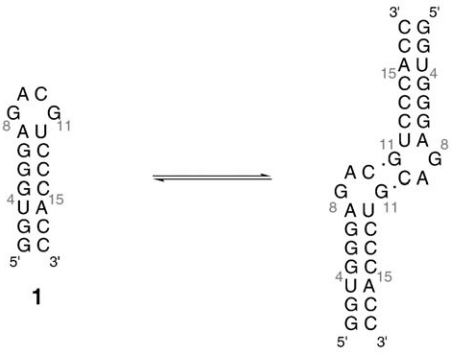

Fig. 1. *Model sequence designed by* Kim *and* Tinoco Jr. [1] *for revealing the existence of the 'kissing complex' interaction and used in the present work*

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approach, it has been demonstrated that the unpaired adenosines 5′-adjacent to the G · C base pair play an essential role for complex formation. This configuration may figure out the codon/anticodon interaction, where adjacent hypermodified purines stabilize the mRNA/tRNA interaction. The isolation of key interactions contributing significantly to the stabilization of the 'kissing complex' may help to evaluate the importance of 3′-end adjacent purines of the tRNA during the translation process. However, the parameters generating the 'unexpected stability' ($T_m = 48°$) of the 'kissing complex' remain difficult to isolate.

The complex has been spectroscopically investigated showing changes in the secondary structure and the tertiary folding of RNA sequence during the dimerization process. Deeper investigations, highlighting the essential interactions, require the preparation of modified RNA sequences. Whereas the mutagenic approach is widely used for long RNA sequences, it appears less practical for short RNA sequences, typically 20mers long [2]. For this latter application, the chemical preparation of synthetic oligonucleotides is a particularly fast and efficient technique especially because it allows the incorporation of non-canonical nucleosides into the sequence. However, previously in the context of preparation of fully modified tRNAs, several 2′-*O*-TOM nucleobase modified phosphoramidites were synthesized [3]. The variety of their structures and the singularity of their electronic properties offer the opportunity to use them as tools for revealing the importance of the adenosines 5′-adjacent to the G · C base pair in the stabilization of the kissing complex.

During their investigations, *Kim* and *Tinoco Jr.* have observed that these nucleobases exerted a strong electrostatic interaction around the base pairs, which can probably be related to π-stacking interaction, particularly favorable in the AC · G′ edifice. Consequently, we may suppose that the incorporation of an adjacent nucleoside offering a potential higher surface area [4] should produce a more stable 'kissing complex'. For this aim, the A9 of the 18mer RNA sequence was substituted for wyosine, which offers a wider π-stacking by its tricyclic structure (*Fig. 2*). This natural nucleoside is one of a wide variety of structurally related nucleosides which are presented in *Fig. 2*. The importance of this type of nucleoside in reading frame maintenance of the Phe^{UUU} codon reading has been poorly investigated. Most of these nucleosides have been synthesized (imG [5], mimG [5], yW [6], OHyW [7], imG-14 [8], imG2 [5]), but, with the exception of 4-desmethylwyosine [9], never transformed into phosphoramidite building blocks or incorporated into RNA sequences[2]).

Here, we report the optimized synthesis of 2′-*O*-[(triisopropylsilyl)oxy]methyl (2′-*O*-TOM) protected wyosine and its incorporation into the self-associating RNA sequence from *Moloney murine leukaemia* virus. Due to its lability towards acid ($t_{1/2} =$ 95 s at pH 1 and 25°) [10], wyosine itself is not fully compatible with the automated assembly of RNA sequences[3]) and can only be incorporated as the last nucleotide at the 5′-end. Therefore, its presence at an interior position within a RNA sequence requires the enzymatic ligation of the 5′-wyosine ending sequence with another strand (*Scheme 1*).

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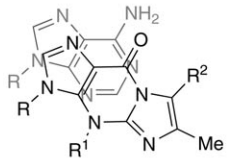
[2]) No synthesis of the corresponding phosphoramidite building block has been reported.

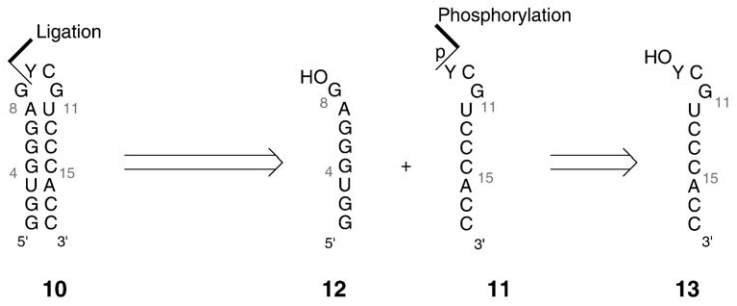
[3]) Within each cycle of the assembly, a detritylation reaction under acidic conditions (*e.g.*, 3% $CHCl_2COOH$ in $ClCH_2CH_2Cl$ [11]) is carried out.



Symbol	Common name	
imG	Wyosine	R^1 = Me, R^2 = H
mimG	Methylwyosine	R^1 = Me, R^2 = Me
yW	Wybutosine	R^1 = $-CH_2CH_2CH(NHCO_2Me)CO_2Me$, R^2 = Me
OHyW	Hydroxywybutosine	R^1 = $-CH_2CH(OH)CH(NHCO_2Me)CO_2Me$, R^2 = Me
o_2yW	Peroxywybutosine	R^1 = $-CH_2CH(OOH)CH(NHCO_2Me)CO_2Me$, R^2 = Me
OHyW*	Undermodified hydroxywybutosine	R^1 = $-CH_2CH(OH)CH(NH_2)CO_2Me$, R^2 = Me
imG-14	4-Desmethylwyosine	R^1 = H, R^2 = Me
imG2	Isowyosine	R^1 = H, R^2 = Me

Fig. 2. *Structure, names, and symbols of wyosine derivatives* (R = ribose moiety or backbone) *according to http://medlib.med.utah.edu/RNAmods*

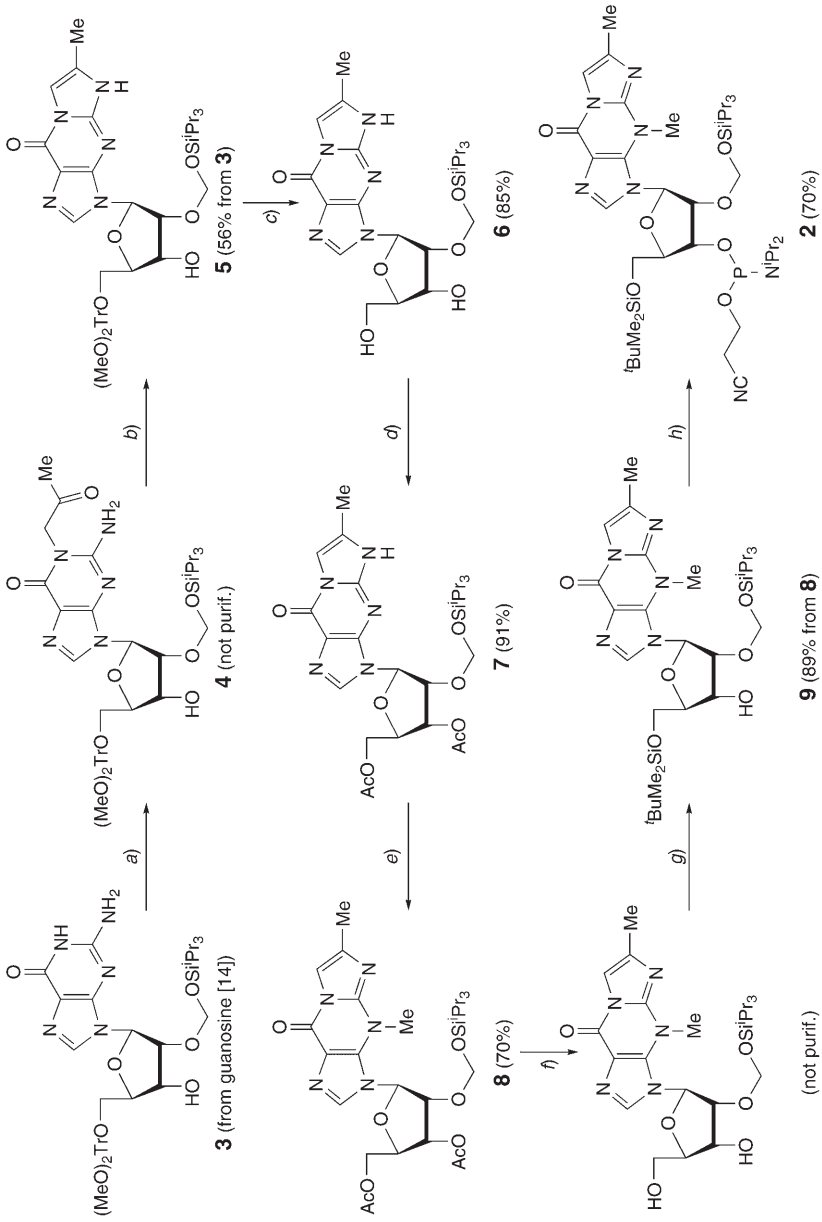
Scheme 1. *Retrosynthetic Approach for the Preparation of the Wyosine* (Y, imG) *Containing Sequence* **10** *and Relying on the Enzymatic Phosphorylation and Ligation of Chemically Prepared Oligonucleotides* (**12** and **13**)



2. Results and Discussion. – 2.1. *Synthesis of Phosphoramidite Building Block* **2**. The first attempt to introduce the 2′-*O*-TOM group under established conditions [11] into the unprotected nucleotide wyosine [12][13] resulted in depurination and the formation of several other unidentified products. Therefore, we decided to prepare the phosphoramidites **2** from the 2′-*O*-TOM and 5′-*O*-dimethoxytrityl ($(MeO)_2Tr$) protected guanosine **3** [14] (*Scheme 2*).

Under the conditions reported for the formation of 4-desmethylwyosine from guanosine (NaH/bromoacetone, DMF, $-15°$ [8]), partial decomposition of **3** was observed. By employing milder conditions (K_2CO_3 instead of NaH, and addition of KI) this problem was avoided, but now an efficient N(4)-alkylation of the product **4** was

Scheme 2



a) K_2CO_3, DMF, 20°; KI, then bromoacetone, −15°. *b*) Molecular sieve, CH_2Cl_2, 20°. *c*) $CHCl_2COOH$, CH_2Cl_2, 20°. *d*) 1. Ac_2O, pyridine, 20°; 2. pyridine, MeOH, H_2O, 20°. *e*) CH_2I_2, Et_2Zn, Et_2O, 4°. *f*) 1. NH_3, MeOH, 20°. *g*) TBDMS-Cl, imidazole, DMF, CH_2Cl_2, 4°. *h*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^iPr_2NEt$, CH_2Cl_2, 20°.

observed[4]). Finally, **5** was prepared in two steps. N(1)-alkylation of **3** with K_2CO_3/bromoacetone/KI in DMF at $-15°$, followed by work-up and extraction gave crude **4**, which then was transformed into the protected 4-desmethylwyosine **5** (56% yield) by dehydration and cyclization with 4-Å molecular sieves in CH_2Cl_2 at 20°. Removal of the $(MeO)_2Tr$ group with $CHCl_2COOH$ in CH_2Cl_2 gave **6** (85% yield). Treatment of this intermediate with Ac_2O in pyridine, followed by selective N(4)-deacetylation with MeOH/H_2O/pyridine 1:1:1 resulted in the formation of the 3′,5′-di-*O*-acetylated derivative **7** (91% yield). Efficient formation of the protected wyosine **8** (70% yield) was achieved by N(5)-methylation with CH_2I_2/Et_2Zn in Et_2O at 4°, according to [5]. The 5′-*O*-(*tert*-butyl)dimethylsilyl (5′-*O*-TBDMS) derivative **9** (89% yield) was obtained after deacetylation of **8** with 10M NH_3 in MeOH[5]), concentration of the reaction mixture and selective silylation with TBDMS-Cl/imidazole in DMF/CH_2Cl_2. The corresponding fully protected wyosine phosphoramidite building block **2** (70% yield) was obtained with 2-cyanoethyl diisopropylphosphoramidochloridite/$^i Pr_2NEt$ in CH_2Cl_2 (*Scheme 2*).

2.2. *Incorporation of Phosphoramidite* **2** *into RNA Sequences.* In preliminary studies with unprotected wyosine, its compatibility with RNA assembly and deprotection conditions was investigated. In addition to the instability of wyosine towards acid [10] and towards $MeNH_2$[5]), the nucleoside was found to be efficiently iodinated under the standard oxidation conditions (I_2/H_2O/pyridine in THF [11])[6]). Oxidation with ^{t}BuOOH in MeCN according to [14], and a milder deprotection which involved treatment of the solid support with $^i Pr_2NH$ in MeCN, followed by NH_3 in MeOH, however, were fully compatible with wyosine. Finally, employing these mild oxidation conditions, the RNA sequence **13** (10mer) containing a 5′-terminal wyosine was assembled from the phosphoramidite **2** and the 2′-*O*-TOM protected phosphoramidites of uridine, N^6-acetyladenosine, N^4-acetylcytidine [11] and guanosine (without base protecting group[7])) [14]. After deprotection with NH_3 in MeOH and $Bu_4NF \cdot 3\ H_2O$ in THF, the resulting product sequence was isolated in pure form by conventional anion exchange (AE) chromatography according to [11] (*Fig. 3*,*a*). In *Fig. 3*,*b*, an ESI mass spectrum of the purified product **13** is shown. The other RNA and DNA sequences were prepared under conventional conditions (see *Exper. Part*), and the 5′-terminal wyosine containing sequence was enzymatically ligated to longer target sequences (*Schemes 3* and *4*, *Fig. 4*).

2.3. *Preparation of a 18mer Wyosine-Containing RNA Hairpin* **10** *and Dimerization Studies.* The 18mer RNA sequence **10**, which contains a wyosine at position 9, was prepared by ligation of the wyosine-containing 10mer fragment **11** and the unmodified 8mer RNA fragment **12**. In a first attempt, the 5′-OH group of the chemically prepared RNA sequence **13** (*Scheme 3*) was phosphorylated with ATP in the presence of the

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4) H–N(4) in desmethylwyosine has a pK_a value of 3.24 [13].

5) The attempted deacetylation of **8** with $MeNH_2$ in EtOH resulted in ring-opening, presumably by attack on C(9) [10].

6) Iodination is known to occur in a selective and efficient manner at position 7 and has been applied for the preparation of wybutosine [7].

7) Complete deprotection of the usually employed 2′-*O*-TOM protected N^2-acetylguanosine phosphoramidite requires stronger conditions.

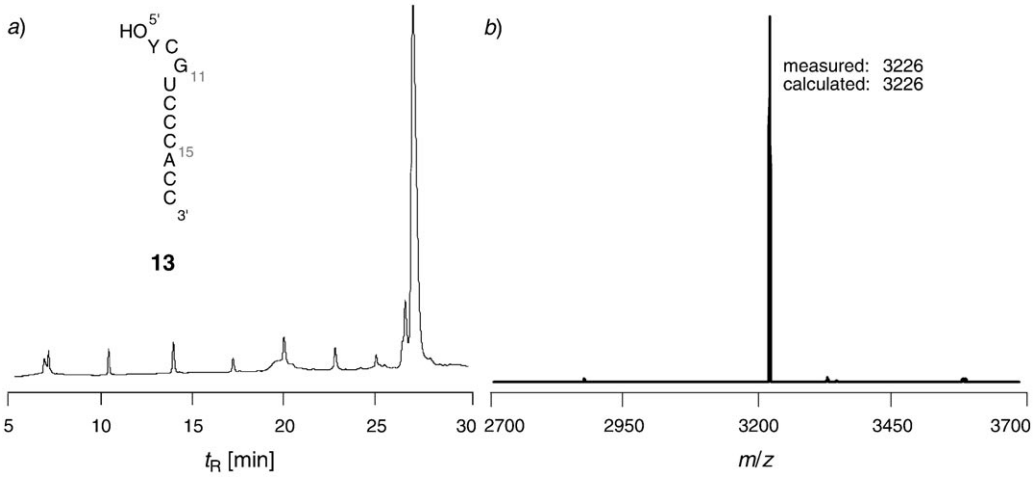
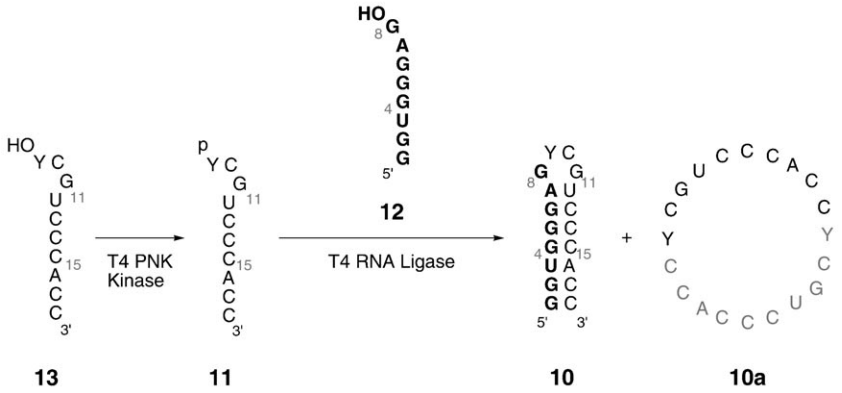


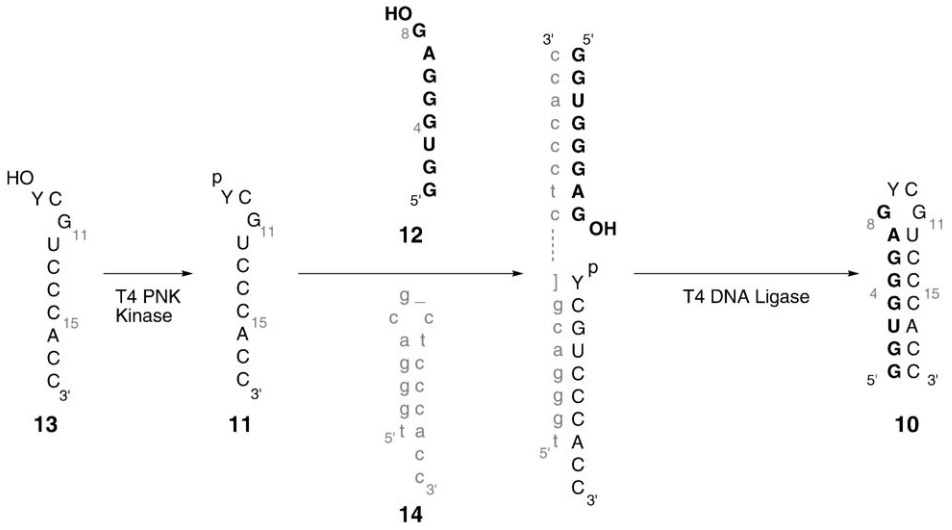
Fig. 3. a) *Ion-exchange HPLC trace* (detection 260 nm) *of crude* **13***, and* b) *deconvulated ESI-MS* (neg. mode) *analysis of the purified wyosine-containing sequence* **13**

Scheme 3. *Description of the Preparation of Sequence* **10** *Relying on Direct Enzymatic T4 RNA Ligation*



enzyme T4 PNK kinase. After thermal inactivation of the kinase, the phosphorylated product sequence **11** was treated with various amounts of the complementary 8mer RNA sequence **12** and ATP, in the presence of the enzyme T4 RNA ligase [15][16]. HPLC Analyses revealed the formation of a new product, but according to ESI-MS analyses, it was not the desired hairpin sequence **10**, but the cyclic homodimeric sequence **10a** (*Scheme 3*). This product is probably the result of autocomplexation of the guanosine-rich RNA sequence **12**, which prevents the required duplex formation with the sequence **11**. Therefore, the template-mediated ligation of **12** and **11** with ATP

Scheme 4. *Description of the Preparation of Sequence* **10** *Relying on Modified Template Assisted T4 DNA Ligation*



in the presence of the enzyme T4-DNA ligase[8]) (*Scheme 4*) was investigated. For this reaction, a 16mer DNA template **14** was designed and prepared, which is fully complementary to the 8mer RNA sequence **11**, and which can form seven base pairs with the 10mer RNA sequence **10**. Since the tricyclic wyosine cannot form any *Watson–Crick* interactions, an apurinic site derived from propane-1,3-diol **15** was introduced at the opposing site of the template (*Scheme 4* and *Fig. 5*). Incubation of the two substrate sequences **11** and **12** (20 μM each) and the template **14** (30 μM) with ATP (0.5 mM) and T4 DNA ligase (8 *Weiss* units) in an aqueous buffer (40 mM *Tris*·HCl, 2 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) at 37° resulted in clean and efficient (90% conversion) formation of the product sequence **10** after 26 h (*Fig. 4*)[9]). According to these conditions, a large-scale ligation was carried out, and 2.05 mg (18% yield) of the RNA sequence **10** was isolated in pure form according to HPLC and ESI-MS (*Fig. 4*). The wyosine containing 18mer RNA hairpin **10** was then analyzed by NMR imino proton spectroscopy to detect the eventual formation of the corresponding 'kissing' complex (*Fig. 6*).

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[8]) It is well-known that this DNA-processing enzyme catalyzes also the ligation of RNA sequences in the presence of a DNA template [17]. It has been discovered that 2′-OMe-RNA templates or DNA/2′-OMe-RNA hybride-templates are often superior and meanwhile, this reaction has been optimized [18].

[9]) The first experiments were carried out according to the conventional one-pot protocol, by first phosphorylating **13** in the presence of T4 PNK kinase (→**11**, followed by thermal inactivation (30 min at 60°), cooling to 4°, and addition of the other ligation components. However, under these conditions, the kinase was not completely inactivated and a part of the product **13** was phosphorylated. In order to completely avoid this side reaction, it was decided to isolate the intermediate **11** by HPLC prior to carry out the ligation reaction.

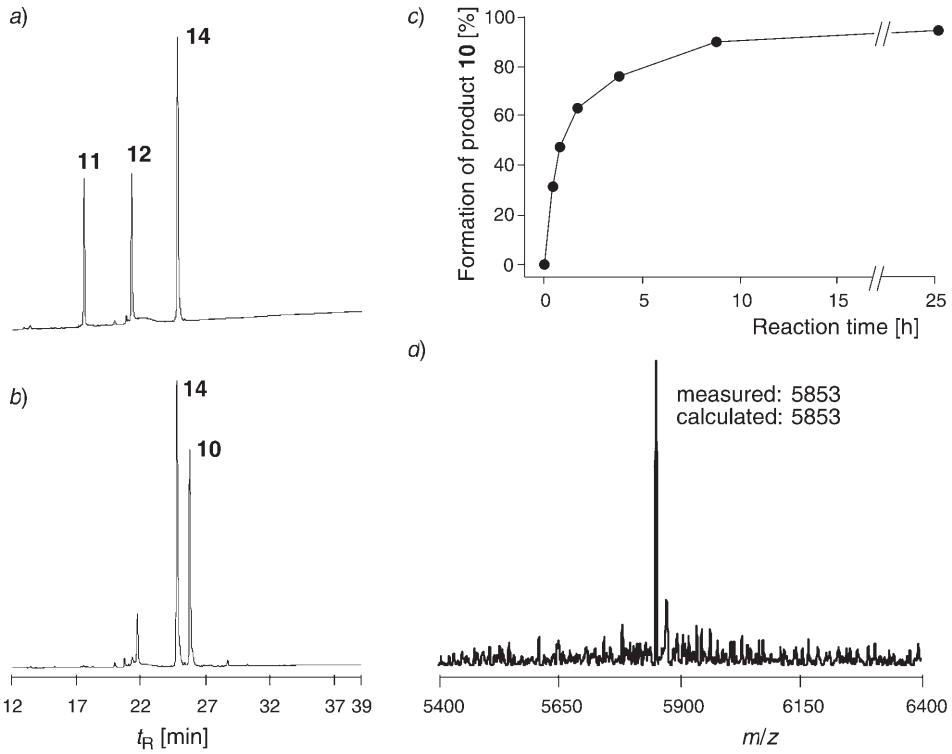

Fig. 4. *Ion-exchange HPLC trace* (detection 260 nm) *of the template* **14** *assisted ligation reaction* (T4 DNA ligase) *of the wyosine-containing sequence* **13** *with the complementary strand* **12** a) *after 0 min and* b) *after 24 hours.* c) *Plot against time of formation of product* **10** *and* d) *deconvulated ESI-MS* (neg. mode) *analysis of the purified corresponding sequence.*

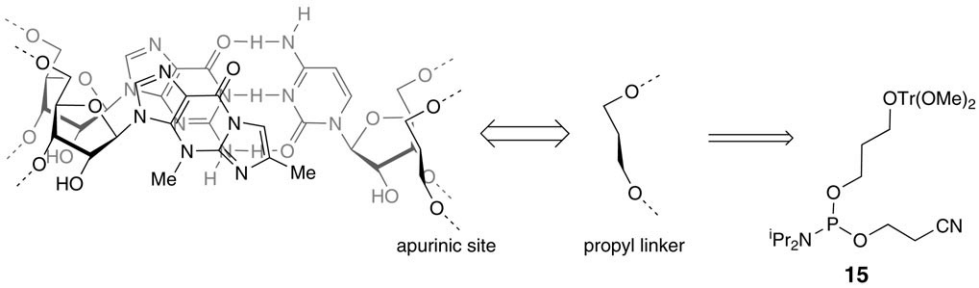

Fig. 5. *Design and structure of the 'apurinic site phosphoramidite'* (propane-1,3-diol derivative) *building block* **15**

Surprisingly, no 'kissing interaction' was observed even at lower temperature (15°). It has been immediately hypothesized that the extended π-system of wyosine was not efficient since this nucleoside was incorporated adjacent to a C · G base pair instead of

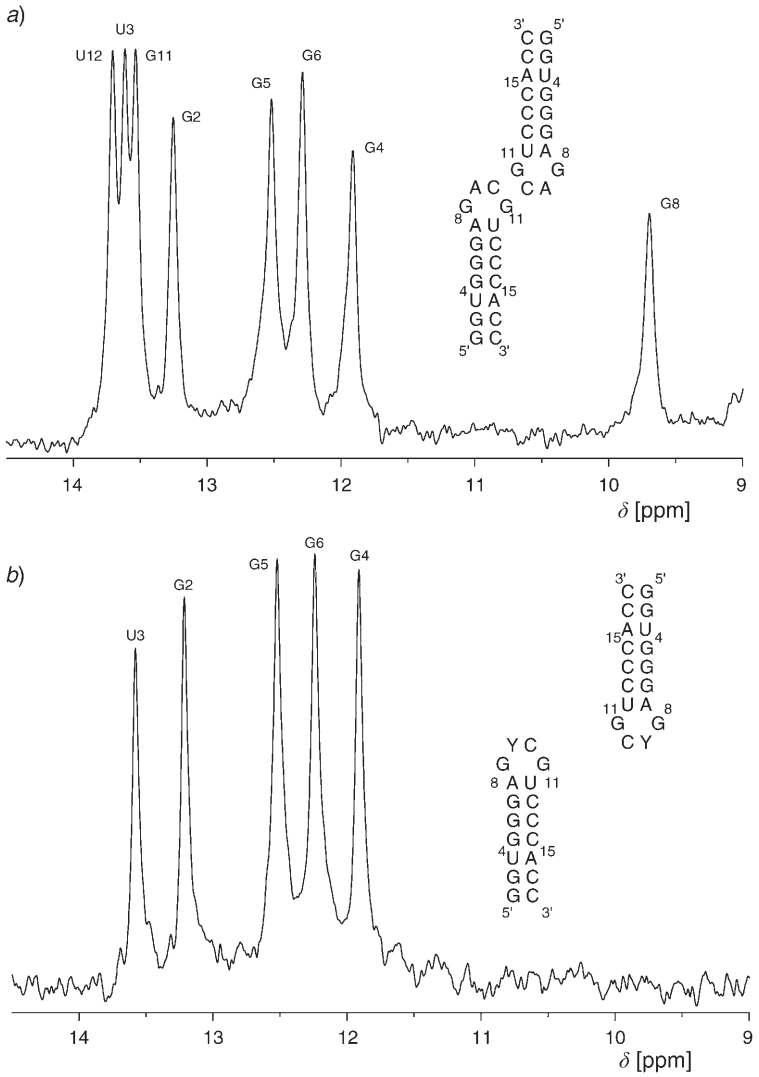

Fig. 6. *NMR Analysis of the imino H-atoms of* a) *the natural sequence* **1** *and* b) *the wyosine-containing sequence* **10** *permitting the direct detection of the 'kissing complex interaction'* (scale in ppm)

the naturally encountered A·U base pair. In order to evaluate the stacking energy provided by wyosine, the four analogous substrate strands **16**, **17**, **19**, **21** containing the canonical nucleosides at the 5′-position were prepared and submitted to the same ligation conditions. The course of these reactions was monitored by HPLC. The efficiency of these reactions varied to a great extent. After 24 h reaction time, the following conversion ratios were obtained as a function of the terminal nucleoside: wyosine (**11** → **10**, 90%) ≫ guanosine (**16** → **18**, 48%) > adenosine (**17** → **1**, 37%) ≫ uridine (**19** → **20**, 21%) ≫ cytidine (**21** → **22**, 2%). These values correlated very well

with the ΔG values of stacking energies for canonical base pairs according to the nearest neighbor model [19]. By linear extrapolation, the stacking energy for wyosine to $\Delta G_{stacking} = -2.5$ kcal mol^{-1} (*Fig. 7,b*)[10]) can be estimated. Therefore, the first expectation on the favorable stacking stabilization provided by wyosine was confirmed. Nevertheless, this result gave no information about the reason why the 18mer RNA hairpin **10** offered no formation of a 'kissing complex'. Further computational investigations revealed that the steric hindrance of the wyosine nucleobase itself prevented the connection of the two hairpins.

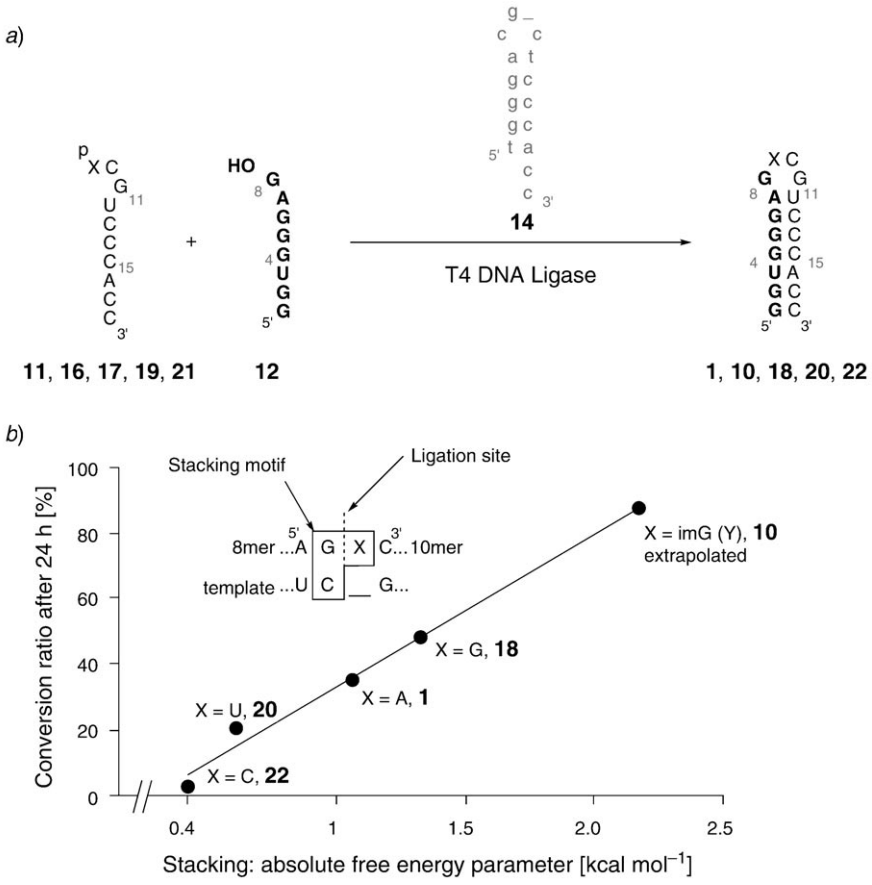

Fig. 7. a) *Description of the template* **12** *assisted ligation reaction of specially designed RNA sequence* (X = imG(Y), **11**; A, **17**; G, **16**; U, **19**; C, **21**). b) *Plot of the amount of the ligated products* (**1**, **10**, **18**, **20**, **22**) *relative to the sum of starting material 8mer* **12** *and 10mers of the respective* (**11**, **16**, **17**, **19**, **21**) *RNA sequences.*

[10]) In this extrapolation, the positive stacking energy of cytosine ($\Delta G = +0.4$ kcal mol^{-1}) was considered, but the intercept was not set to zero, assuming that a minimal energy maybe required for structuration of the double helix [20].

To pursue the question about the 'unexpected stability' of the 'kissing complex', several analogues of the 18mer RNA sequence have been prepared by substituting the A9 moiety for different modified nucleosides (*Table*). Therefore, nucleosides naturally found at position 37 of the tRNA were incorporated: Guanosine **18**, Methyl-1-guanosine (m^1G) **23**, and N^6-methyladenosine **24** (m^6A), but also some other nucleosides found within the tRNA for which we have already prepared the 2′-*O*-TOM phosphoramidites: inosine **25** and N^2,N^2-dimethylguanosine **26** ($m^2{}_2G$) [5]. Only the adenosine derivatives provided sufficient π-stacking stabilisation energy, correlating well the fact that, in tRNAs, adenosine derivatives are adjacent to C·G base pairs, whereas guanosine derivatives are found after G·C base pairs [21]. Thanks to the NMR data accumulated by *Kim* and *Tinoco Jr.* attention was paid to the cross-loop interactions, especially the one existing between the G8 and G11 nucleobases (*Fig. 8*). Although this H-bond ($NH_2 \cdots N^7$) may play a role in the structuration and the rigidification of the loop, it could also provide an electrophilic center. This delocalization of the lone pair of N^7 involved in the bonding with the adjacent H-atom could stabilize significantly the G·C base pair, as already described for the interaction of the guanine N^7 N-atom with metallic cations (up to 70%) [22]. As a last example, the 18mer RNA hairpin **27** was prepared, where the guanosine G8 was replaced by inosine. This nucleoside does not possess, in contrast to guanine, a C^3 amino function (*Fig. 8*). As expected, this modified sequence did not form any 'kissing complex', demonstrating the importance of this secondary H-bond in the formation of the complex. Unfortunately, this observation led to the conclusion that this sequence was not suitable for modelling of the codon/anticodon interaction.

Table. *RNA Sequences Employed for Investigation of the 'Kissing Complex Interaction'*

5′-GGUGGGAGXCGUCCCACC-3′				
X[a])	Compound	*m/z* calc.	*m/z* found[b])	Interaction[c])
A	**1**	5786	5786	Yes
imG (Y)	**10**	5854	5854	No
G	**18**	5802	5802	No
m^1G	**23**	5816	5816	No
m^6A	**24**	5800	5800	Yes
I	**25**	5787	5787	No
$m^2{}_2G$	**26**	5830	5830	No

[a]) Structure, name, and symbol of derivatives (R = ribose moiety or backbone) according to http://medlib.med.utah.edu/RNAmods. For clarity of the RNA sequence drawing, imG has been substituted for the letter Y. [b]) LC/ESI-MS spectrometry (neg. mode) analysis. Detailed procedure in *Exper. Part.* [c]) NMR Spectroscopy analysis. Detailed procedure in *Exper. Part.*

Conclusions. – The presented results prove the efficiency of the methodology, combining chemical synthesis and template assisted enzymatic ligation, in the preparation of modified oligonucleotides. This approach has allowed, for the first time, the incorporation of the wyosine nucleoside and evaluation of the stacking stabilization provided by its tricyclic moiety. Ultimately, all prepared modified RNA

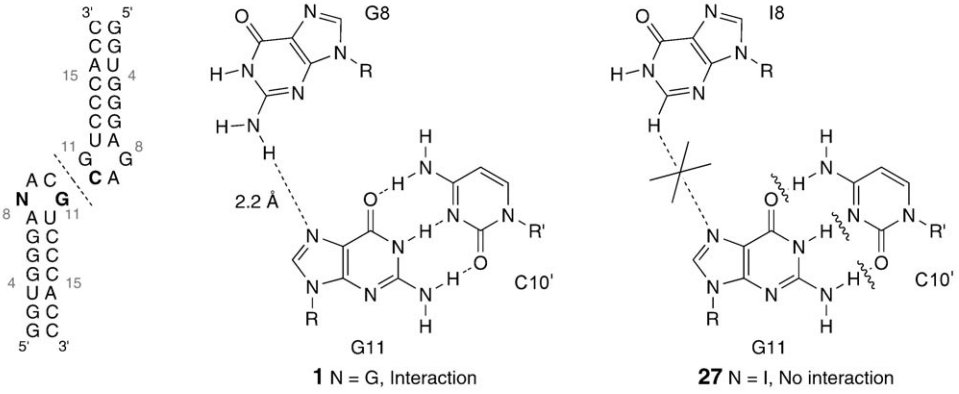

Fig. 8. *Design of modified sequence* **27** (Inosine at position 8 instead of G occurring in the natural sequence **1**) *preventing formation of the 'kissing complex interaction'*

sequences give new insight into the interactions contributing to the formation of the 'kissing complex'. In this context, these studies have demonstrated that secondary H-bonds ($NH_2 \cdots N^7$) could contribute significantly to the stabilization of inter-strand connections. Although these cross-strand bonds do not exist in the codon/anticodon interaction, it can be expected that secondary H-bonds may play an important role in the strength of mRNA/tRNA complex formation [23].

I am grateful to EPFL and to the *Swiss National Science Foundation* (Grant Nr. 2000-06890) for their generous support. I would like to thank Prof. *S. Pitsch*, my supervisor of thesis, for introducing me to the RNA world. I am grateful to Dr. *Cyrille Denarié* for his support in the ligation experiments, Prof. *Ursula Röthlisberger* and Dr. *Christian Gossens* for their support in the computational simulation of the wyosine-containing sequence. Dr. *Philipp Wenter* is also thanked for his continuous support in carrying out NMR experiments.

Experimental Part

1. *General.* Reagents and solvents (highest purity) from various suppliers, used without further purification; [(triisopropylsilyl)oxy]methyl chloride (= TOM-Cl) was prepared according to [5]. Workup implies distribution of the reaction mixture between CH_2Cl_2 and sat. aq. $NaHCO_3$ soln., drying of the org. layer ($MgSO_4$), and evaporation. TLC: precoated SiO_2 plates from *Merck*, stained by dipping into a soln. of anisaldehyde (10 ml), H_2SO_4 (10 ml), and AcOH (2 ml) in EtOH (180 ml) and subsequent heating with a heat-gun. CC (column chromatography): SiO_2 *60* (230–400 mesh) from *Fluka*. The eluent was systematically conditioned with few percent of Et_3N (*Fluka*). ESI-MS (positive mode): *SSQ 710* (*Finnigan*), measurements in MeCN/H_2O/AcOH 50:50:1. Py = pyridine, r.t. = room temperature (*ca.* 20°), DMAP = *N*,*N*-dimethylpyridin-4-amine, TMS-Cl = Me_3Si-Cl, DME = Dimethoxyethane.

The usual 2′-*O*-TOM protected RNA phosphoramidites according to [11] and DNA phosphoramidites from *Glen Research*; size exclusion cartridges: *NAP-10* columns from *Pharmacia*, elution according to the manufacturer's instruction; *Sepak* cartridges from *Waters*. Sterile H_2O from *B. Braun Medical AG*. Anion exchange-HPLC: *DNAPAC PA-100* (9.0 × 250 mm; *Dionex*), flow 2.5 ml/min; eluent *A*: 12 mM *Tris* · HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris* · HCl (pH 7.4), 0.5m $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°. ESI-MS (pos. mode): *SSQ 710* (*Finnigan*), measurements in MeCN/H_2O/AcOH 50:50:1. LC/ESI-MS (neg. mode): *Q-Tof-Ultima* (*Micromass*/*Waters*) coupled to *Cap-LC* (*Waters*), injection: 2 µl aq. sample (*c*(RNA) = 2.5 µM, *c*(EDTA) = 1 mM); chromatography on

Xterra RP-C18 column (*Waters*, 5 μM, 0.32 × 50 mM; flow 8 μl/min, *eluent A:* 25 mM aq. $Me_2NBu \cdot H_2CO_3$ (pH 8.4); *eluent B:* MeCN, elution at 60°, sheath flow 25 μl/min (MeCN)); gradient $A \rightarrow A/B$ 1:1 (15 min.); deconvolution by MaxEnt1 software. Ligation buffer for T4 DNA ligase (*Labforce*) mediated ligation 40 mM *Tris* · HCl (*Sigma*), 2 mM $MgCl_2$ (*Fluka*), 10 mM DTT (1,4-Dithio-DL-threitol) (*Fluka*), 0.5 mm ATP (*Fluka*). Same buffer employed for T4 polynucleotide kinase mediated phosphorylation (*NewEnglandBiolabs*). NMR-spectroscopy: *Bruker* 400 MHz (^{1}H: 400 MHz, ^{13}C: 100 MHz, ^{31}P: 162 MHz). Chemical shift δ in ppm, relative to external standards (^{1}H and ^{13}C: Me_4Si, ^{31}P: 85% aq. H_3PO_4); coupling constants *J* in Hz; multiplicities (^{13}C) according to DEPT-spectra. NMR Experiments were carried out on a *Bruker AV 600* MHz spectrometer equipped with a 5 mM *TX1-HCN* cryogenic probe with *z*-gradients. The RNA samples (amounts determined spectrophotometrically) were dissolved in 0.3 ml potassium arsenate buffer (25 mM, pH 7.0) in H_2O/D_2O 9:1. Restricted volume *Shigemi* tubes were used for all experiments. 1D ^{1}H-NMR Spectra were recorded by combining a water flip-back pulse and 3-9-19 WATERGATE for suppression of the water signal.

2. *Preparation of the Wyosine Phosphoramidite Building Block.* N^4*-Desmethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-{[(triisopropylsilyl)oxy]methyl}wyosine* (= *3-[5-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2-*O*-({[tris(1-methylethyl)silyl]oxy}methyl)-β-*D*-ribofuranosyl]-3,5-dihydro-6-methyl-9*H*-imidazo[1,2-a]purin-9-one*; **5**). A soln. of **3** (1.00 g, 1.3 mmol) prepared according to [14] in DMF (12 ml) was treated with K_2CO_3 (197 mg, 1.4 mmol) and stirred at r.t. for 1 h. This suspension was cooled to −15° and successively treated with KI (107 mg, 0.6 mmol) and bromoacetone (178 mg, 1.3 mmol), and stirred for 8 h. The mixture was poured on a cold 10% soln. of $AlCl_3$ (150 ml) and AcOEt (150 ml). The aq. layer was extracted twice with each 150 ml AcOEt, and the combined org. layers were dried over $MgSO_4$. After evaporation of the solvent, the yellow oily residue **4** was redissolved in DMF (10 ml) and stirred overnight on K_2CO_3 (197 mg, 1.4 mmol) or 4-Å molecular sieve. Workup and CC (SiO_2 (30 g); CH_2Cl_2/acetone 0:1 → 2:8) gave **5** (588 mg, 56%) as green oil and **3** (199 mg, 20%). R_f (CH_2Cl_2/MeOH 9:1) 0.53. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.04–1.11 (*m*, ^{i}Pr$_3$Si); 2.26 (*s*, Me−C(6)); 3.15 (br. *s*, HO−C(3′)); 3.35 (*dd*, *J* = 3.4, 10.3, H−C(5′)); 3.51 (*dd*, *J* = 3.2, 10.3, H′−C(5′)); 3.77, 3.79 (2*s*, 2 MeO); 4.29 (*dd*, *J* = 2.6, 3.2, H−C(4′)); 4.64 (*dd*, *J* = 2.2, 4.4, H−C(3′)); 4.95 (*d*, *J* = 4.7, 1 H, OCH_2O); 5.08 (*t*, *J* = 5.7, H−C(2′)); 5.13 (*d*, *J* = 4.7, 1 H, OCH_2O); 6.02 (*d*, *J* = 6.2, H−C(1′)); 6.80–6.82 (*m*, 4 arom. H); 7.26–7.49 (*m*, 9 arom. H, H−C(7)); 7.81 (br. *s*, H−C(2)), 10.11 (br. *s*, H−N(5)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.3 (*q*, *Me*−C(6)); 12.2 (*d*, Me_2*C*H); 18.2 (*q*, *Me*$_2$CH); 55.7 (*q*, 2 MeO); 64.0 (*t*, C(5′)); 71.7 (*d*, C(4′)); 81.7 (*d*, C(2′)); 84.4 (*d*, C(3′)); 86.7 (*s*, arom. C); 86.9 (*d*, C(1′)); 91.4 (*t*, OCH_2O); 104.6 (*d*, C(7)); 113.4 (*d*, arom. C); 117.7 (*s*, C(9a)); 127.3, 128.3, 128.8, 130.7 (4*d*, arom. C); 136.1 (*s*, arom. C); 137.9 (*d*, C(2)); 145.1 (*s*, C(6)); 145.9 (*s*, C(4a)); 150.2 (*s*, C(3a)); 152.4 (*s*, C(9)); 158.9 (*s*, arom. C). ESI-MS: 810.30 (100, $[M + H]^+$).

N^4*-Desmethyl-2′-*O*-{[(triisopropylsilyl)oxy]methyl}wyosine* (= *3,5-Dihydro-6-methyl-3-[2-*O*-({[tris(1-methylethyl)silyl]oxy}methyl)-β-*D*-ribofuranosyl]-9*H*-imidazo[1,2-a]purin-9-one*; **6**). A soln. of **5** (823 mg, 1.0 mmol) in CH_2Cl_2 (10 ml) was treated with $CHCl_2COOH$ (0.4 ml, 6.0 mmol) and stirred at r.t. After 15 min, the mixture was quenched with MeOH (2.6 ml) on a 10% cold soln. of NH_4HCO_3 (50 ml) and CH_2Cl_2 (50 ml). The aq. layer was extracted twice with each 150 ml of AcOEt, and the combined org. layers were washed with brine. CC (SiO_2 (15 g); hexane/AcOEt 1:1 → 0:1) afforded **6** (431 mg, 85%). Light green foam. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.01–1.07 (*m*, ^{i}Pr$_3$Si); 2.30 (br. *s*, Me−C(6)); 3.05–3.60 (br. *s*, HO−C(3′), HO−C(5′)[11]); 3.83 (*d*, *J* = 11.7, H−C(5′)); 4.01 (*d*, *J* = 12.5, H′−C(5′)); 4.29 (br. *s*, H−C(4′)); 4.57 (*d*, *J* = 4.2, 1 H, OCH_2O); 4.88–4.95 (*m*, H−C(2′), OCH_2O); 5.08 (*d*, *J* = 4.6, H−C(3′)); 5.89 (*d*, *J* = 7.3, H−C(1′)); 7.28 (*s*, H−C(7)); 7.76 (br. *s*, H−C(2)); 11.50 (br. *s*, H−N(5)[11]). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.3 (*q*, *Me*−C(6)); 12.2 (*d*, Me_2*C*H); 18.1 (*q*, *Me*$_2$CH); 63.7 (*t*, C(5′)); 72.2 (*d*, C(3′)); 81.2 (*d*, C(2′)); 87.2 (*d*, C(4′)); 89.8 (*d*, C(1′)); 90.9 (*t*, OCH_2O); 104.5 (*d*, C(7)); 118.1 (*s*, C(9a)); 126.5 (*s*, C(6)); 139.4 (*d*, C(2)); 145.4 (*s*, C(4a)); 148.6 (*s*, C(3a)); 152.1 (*s*, C(9)). ESI-MS: 508.32 (100, $[M + H]^+$)[11]).

*3′,5′-Di-*O*-acetyl-*N^4*-desmethyl-2′-*O*-{[(triisopropylsilyl)oxy]methyl}wyosine* (= *3-[3,5-Di-*O*-acetyl-2-*O*-({[tris(1-methylethyl)silyl]oxy}methyl)-β-*D*-ribofuranosyl]-3,5-dihydro-6-methyl-9*H*-imidazo[1,2-a]-purin-9-one*; **7**). A soln. of **6** (360 mg, 0.7 mmol) in pyridine (5 ml) was treated with Ac_2O (0.4 ml,

[11]) Confirmed by H_2O exchange with D_2O.

6.0 mmol) and stirred at r.t. for 2 h. After workup, the solvent was removed under reduced pressure and the mixture was stirred for 2 h in pyridine/H_2O/MeOH 1:1:1 (6 ml). Workup, evaporation, and coevaporation with benzene (2 ml) gave **7** (382 mg, 91%). Yellow foam. No further purification was necessary. R_f (CH_2Cl_2/MeOH 9:1) 0.42. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.90–1.10 (*m*, ^{i}Pr$_3$Si); 2.09, 2.20 (2*s*, 2 MeCO); 2.38 (*s*, *Me*–C(6)); 4.45–4.47 (*m*, CH_2(5′)); 4.50–4.60 (*m*, H–C(4′)); 4.84 (*d*, *J* = 4.6, 1 H, OCH_2O); 4.89 (*d*, *J* = 4.7, 1 H, OCH_2O); 5.23 (*t*, *J* = 5.6, H–C(2′)); 5.53 (*dd*, *J* = 3.3, 5.0, H–C(3′)); 6.01 (*d*, *J* = 6.3, H–C(1′)); 7.38 (*d*, *J* = 5.5, H–C(7)); 7.74 (br. *s*, H–C(2)); 9.77 (br. *s*, H–N(5)). ^{13}C-NMR (100 MHz, $CDCl_3$): 11.5 (*q*, *Me*–C(6)); 12.1 (*d*, Me$_2$*C*H); 18.0 (*q*, *Me*$_2$CH); 21.2 (*q*, *Me*CO); 21.3 (*q*, *Me*CO); 64.4 (*t*, C(5′)); 72.2 (*d*, C(3′)); 76.0 (*d*, C(2′)); 80.7 (*d*, C(4′)); 88.4 (*d*, C(1′)); 89.9 (*t*, OCH_2O); 104.7 (*d*, C(7)); 118.0 (*s*, C(9a)); 125.6 (*s*, C(6)); 137.9 (*d*, C(2)); 146.1 (*s*, C(4a)); 149.9 (*s*, C(3a)); 152.3 (*s*, C(9)); 170.6, 171.6 (2*s*, Me*C*O). ESI-MS: 592.80 (100, $[M + H]^+$).

3′,5′-Di-O-*acetyl-2′*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (= *3-[3,5-Di*-O-*acetyl-2*-O-*({[tris(1-methylethyl)silyl]oxy}methyl)-β*-D-*ribofuranosyl]-3,4-dihydro-4,6-dimethyl-9*H-*imidazo[1,2*-a*]purin-9-one*; **8**). The regioselective methylation of the tricyclic moiety was performed according to [5]. A light-prevented soln. of CH_2I_2 (1.2 ml, 14.9 mmol) in dry Et_2O (9 ml) was treated with a 1M soln. of Et_2Zn in hexane (7.5 ml, 7.5 mmol) and stirred for 30 min at r.t. The clear soln. gave a cloudy white suspension upon addition of DME (0.8 ml, 7.5 mmol). After 30 min, the mixture was cooled to 4° and a soln. of starting material **7** (0.424 g, 0.71 mmol) in CH_2Cl_2 (1 ml) was quickly added. After 3 min of stirring, the mixture was poured on an ice cold 1M aq. soln. of ammonium carbonate (50 ml) and CH_2Cl_2 (50 ml). The aq. layer was extracted twice with each 150 ml of AcOEt, and the combined org. layers were washed with (100 ml) of a 0.1M soln. of thiosulfate. Drying over $MgSO_4$, evaporation, and CC (SiO_2 (15 g); CH_2Cl_2/acetone 99:1 → 95:5 (+1% Et_3N)) gave **8** (300 mg, 70%). Yellow foam. R_f (CH_2Cl_2/MeOH 9:1) 0.38. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.08 (*m*, ^{i}Pr$_3$Si); 2.10, 2.23 (2*s*, 2 MeCO); 2.35 (*s*, *Me*–C(6)); 4.20 (*s*, MeN(4)); 4.23 (*dd*, *J* = 2.6, 12.3, H–C(5′)); 4.32 (*dd*, *J* = 3.1, 12.1, H′–C(5′)); 4.51 (*dd*, *J* = 2.2, 4.2, H–C(4′)); 4.90 (*dd*, *J* = 4.6, 10.4, 1 H, OCH_2O); 4.93 (*dd*, *J* = 4.6, 10.4, 1 H, OCH_2O); 4.97 (*t*, *J* = 4.8, H–C(2′)); 5.42 (*dd*, *J* = 2.1, 4.9, H–C(3′)); 6.20 (*d*, *J* = 6.8, H–C(1′)); 7.47 (br. *s*, H–C(7)); 7.92 (br. *s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.2 (*d*, Me$_2$*C*H); 14.7 (*q*, *Me*–C(6)); 18.2 (*q*, *Me*$_2$CH); 21.1 (*q*, *Me*CO); 21.2 (*q*, *Me*CO); 34.3 (*q*, MeN(4)); 63.8 (*t*, C(5′)); 71.6 (*d*, C(3′)); 72.1 (*d*, C(2′)); 77.2 (*d*, C(4′)); 81.8 (*d*, C(1′)); 89.7 (*t*, OCH_2O); 107.1 (*d*, C(7)); 117.3 (*s*, C(9a)); 134.2 (*d*, C(2)); 138.4 (*s*, C(6)); 140.1 (*s*, C(3a)); 142.9 (*s*, C(4a)); 152.6 (*s*, C(9)); 170.4 (*s*, 2 Me*C*O). ESI-MS: 606.83 (100, $[M + H]^+$).

5′-O-tert-*Butyldimethylsilyl-2′*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine* (= *3-[5*-O-*{(1,1-Dimethylethyl)dimethylsilyl}-2*-O-*({[tris(1-methylethyl)silyl]oxy}methyl)-β*-D-*ribofuranosyl]-3,4-dihydro-4,6-dimethyl-9*H-*imidazo[1,2*-a*]purin-9-one*; **9**). A methanolic (0.5 ml) soln. of **8** (81 mg, 0.13 mmol) was treated with a sat. soln. of NH_3 in MeOH (2 ml) and stirred for 3 h at r.t. After evaporation to dryness, the residue was dissolved in CH_2Cl_2/DMF 2:1 (1.1 ml) and treated with imidazole (26 mg, 0.32 mmol). After 5 min, the mixture was cooled to 4° and TBDMS-Cl (26 mg, 0.15 mmol) was added, followed by stirring at 4° for 1 h. Workup and CC (SiO_2 (2 g); CH_2Cl_2/acetone 1:1 → 0:1) afforded **9** (85 mg, 98%). Light yellow oil. R_f (CH_2Cl_2/MeOH 1:9) 0.75. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.25, 0.26 (2*s*, ^{t}Bu*Me*$_2$Si); 1.00–1.29 (*m*, ^{i}Pr$_3$Si, ^{t}*Bu*Me$_2$Si); 2.47 (br. *s*, *Me*–C(6)); 2.60 (br. *s*, HO–C(3′)); 3.98 (*dd*, *J* = 1.6, 11.5, H–C(5′)); 4.06 (*dd*, *J* = 1.9, 11.5, H′–C(5′)); 4.29 (*s*, MeN(4)); 4.48 (br. *s*, H–C(4′)); 4.58–4.62 (*m*, H–C(2′), H–C(3′)); 5.00 (*d*, *J* = 5.1, 1 H, OCH_2O); 5.28 (*d*, *J* = 5.1, 1 H, OCH_2O); 6.39 (*d*, *J* = 6.3, H–C(1′)); 7.58 (br. *s*, H–C(7)); 8.17 (br. *s*, H–C(2)). ^{13}C-NMR (100 MHz, $CDCl_3$): −5.6, −5.5 (2*q*, *Me*$_2$Si); 11.9 (*d*, Me$_2$*C*H); 14.3 (*q*, *Me*–C(6)); 17.7 (*q*, *Me*$_2$CH); 18.4 (*s*, Me$_3$*C*); 26.0 (*q*, *Me*$_3$C); 34.3 (*q*, MeN(4)); 63.8 (*t*, C(5′)); 71.2 (*d*, C(3′)); 84.9 (*d*, C(2′)); 86.2 (*d*, C(4′)); 86.9 (*d*, C(1′)); 90.9 (*t*, OCH_2O); 106.6 (*d*, C(7)); 116.6 (*s*, C(9a)); 134.7 (*d*, C(2)); 137.9 (*s*, C(6)); 139.6 (*s*, C(3a)); 142.6 (*s*, C(4a)); 152.3 (*s*, C(9)). ESI-MS: 636.37 (100, $[M + H]^+$).

5′-O-tert-*Butyldimethylsilyl-2′*-O-*{[(triisopropylsilyl)oxy]methyl}wyosine 3′-(2-Cyanoethyl Diisopropylphosphoramidite* (= *3-[3*-O-*{[Bis(1-methylethyl)amino](2-cyanoethoxy)phosphino}-5*-O-*[(1,1-dimethylethyl)dimethylsilyl]-2*-O-*({[tris(1-methylethyl)silyl]oxy}methyl)-β*-D-*ribofuranosyl]-3,4-dihydro-4,6-dimethyl-9*H-*imidazo[1,2*-a*]purin-9-one*; **2**). A soln. of **9** (83 mg, 0.13 mmol) in CH_2Cl_2 (1.0 ml) was treated consecutively with ^{i}Pr$_2$NEt (0.06 ml, 0.32 mmol) and 2-cyanoethyl diisopropylphosphoramidochloridite (40 mg, 0.16 mmol). After stirring for 14 h at r.t., the mixture was subjected to CC (SiO_2 (3 g); hexane/AcOEt 4:1 → 1:4 (+3% Et_3N)): **2** (75 mg, 70%; 1:1 mixture of diastereoisomers). Colorless

foam. R_f (hexane/AcOEt 3:7) 0.50. ^{1}H-NMR (400 MHz, $CDCl_3$): 0.09–0.13 (*m*, ^{t}BuSi); 0.91–1.00 (*m*, ^{i}Pr$_3$Si); 1.17–1.25 (*m*, $(Me_2CH)_2N$); 2.34 (*s*, *Me*–C(6)); 2.66 (br. *d*, *J* = 5.1, CH_2CN); 3.65–3.99 (*m*, $(MeCH)_2N$, $CH_2(5')$, $POCH_2$, 6 H); 4.15, 4.18 (2*s*, MeN(4)); 4.34 (br. *s*, H–C(4′)); 4.53–4.61 (*m*, H–C(3′)); 4.75–4.81 (*m*, H–C(2′)); 4.96–5.03 (*m*, OCH_2O); 6.22–6.25 (*m*, H–C(1′)); 7.46 (br. *s*, H–C(7)); 8.04 (*s*, H–C(2)). ^{31}P-NMR (162 MHz, $CDCl_3$): 149.9, 151.6. MALDI-MS: 836.81 (100, $[M+H]^+$).

3. *Preparation of RNA Sequences. RNA Sequence r(GGUGGGAG)* (**12**). The assembly of the RNA sequence was carried out on a *Gene Assembler Plus* (*Pharmacia*), from 55 mg solid support (loading 32 μmol/g) and 2′-*O*-TOM-protected ribonucleoside phosphoramidites according to [11]. After the assembly, the solid support was treated with a mixture of 12M $MeNH_2$ in H_2O and 8M $MeNH_2$ in EtOH (1 ml) for 4 h at 35°. The supernatant was removed by centrifugation and evaporated to dryness; the residue was treated with a THF soln. (1 ml) of $Bu_4NF \cdot 3\ H_2O$ (1M) for 14 h at 20°, diluted with aq. *Tris*·HCl (1 ml, 1M, pH 7.4), and evaporated to a volume of 1 ml. The remaining soln. was applied on a *NAP-10* cartridge (*Pharmacia*) and eluted with H_2O. The first 1.5 ml of soln. was purified by ion-exchange HPLC: AE-HPLC (20–60% *B* in 30 min) flow 2.5 ml/min; eluent *A:* 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B:* 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; (detection at 260 nm, elution at 85°): t_R 11.0 min. The fraction containing pure product was diluted with 1M aq. $Et_3N \cdot H_2CO_3$ to a final 0.1M concentration and applied to a *Sepak* cartridge (conditioned by washing with MeCN (10 ml) and 0.1M aq. $Et_3N \cdot H_2CO_3$ (10 ml)). The cartridge was washed with 20 mM $Et_3N \cdot H_2CO_3$ (10 ml), and the RNA sequence was eluted with MeCN/H_2O 1:1 (4 ml). In order to completely remove the remaining $Et_3N \cdot H_2CO_3$, 0.5 ml H_2O were added to the residue, followed by lyophilization (this procedure was carried out twice). OD_{260} 46 (=0.48 μmol, 25%). MALDI-MS (neg mode): m/z = 2645 (calc. 2645).

RNA Sequence r(imG-CGUCCCACC) (**13**). The RNA sequence was prepared as previously described for the RNA sequence **12**, but employing the 2′-*O*-TOM wyosine phosphoramidite building block **2**. Furthermore, a 2′-*O*-TOM H_2N–C(2) unprotected guanosine phosphoramidite building block, prepared according to [14], was introduced by performing a double coupling cycle. For the wyosine building block, the oxidation step has been adapted. The final oxidation was achieved with a 1.1M soln. of ^{t}BuOOH in MeCN (flow 0.5 ml/min during 18 min). The solid support was washed with ^{i}Pr$_2$NH/MeCN 1:9 for 20 min (flow-rate 2.5 ml/min). Cleavage from the solid support and deprotection was carried out with 12M dry NH_3 in MeOH (1 ml) for 14 h at 20°. The supernatant was removed by centrifugation and evaporated to dryness; the residue was treated with a THF soln. (1 ml) of $Bu_4NF \cdot 3\ H_2O$ (1M) for 14 h at 20°, diluted with aq. *Tris*·HCl (1 ml, 1 M, pH 7.4), and concentrated to a volume of 1 ml. After desalting on a *NAP* cartridge, the crude product was purified by ion-exchange HPLC: AE-HPLC (0–40% *B* in 40 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°: t_R 30.0 min. OD_{260} 61 (=0.71 μmol, 37%). LC/ESI-MS: m/z = 3147 (calc. 3147).

RNA Sequence 5′-Monophosphate-r(imG-CGUCCCACC) (**11**). The oligonucleotide was dissolved to a final concentration of 20 μM for a volume of reaction of 60 μl. The phosphorylation reaction was performed according to the instructions from the supplier (*Fermentas*). The reaction was performed for 1.5 h at 37°. The crude mixture was purified by AE-HPLC. (0–40% *B* in 40 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°: t_R 30 min. OD_{260} 39 (=0.46 μmol, 64%). LC/ESI-MS: m/z = 3226 (calc. 3226).

Analytical HPLC for Kinetic Monitoring Control. AE-HPLC: (0–50% *B* in 30 min) flow 1.0 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°. *r(imG-CGUCCCACC):* t_R 20.3 min, *5′-monophosphate-r(imGCGUCCCACC):* t_R 21.8 min.

DNA Sequence d(GGUGGGACG-_-CUCCCA) (**14**). The assembly of the sequence was carried out under standard conditions [11]. 2′-Deoxyphosphoramidites from *Glen Research* and propylphosphoramidite linker prepared according to [24] were used. AE-HPLC: (0–60% *B* in 36 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°: t_R 25.0 min. OD_{260} 69 (=0.44 μmol, 23%). LC/ESI-MS: m/z = 4731 (calc. 4731).

4. *Ligation Experiments. RNA Sequence r(GGUGGGAG-imG-CGUCCCACC)* (**10**). The previously prepared 8mer **12**, 10mer **11**, and 16mer template **14** were mixed to a final concentration of 20 μM for the substrates and 30 μM for the template (1.5 equiv.). After the addition of *PEG 6000* (3 μl of 50% PEG stock soln.) and H_2O (q.s.p. 54 μl), the mixture was heated at 95° for 4 min and subsequently cooled down to 40° by steps of 0.1° per second and finally to 4° within 1.5 min. After the addition of ligation buffer (40mM *Tris*·HCl, 2 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) and 8 units of ribonuclease inhibitor (*Fermentas*), the reaction was initiated by adding T4 DNA ligase (8 *Weiss* units), and kept at 37°. For the ligation kinetics, aliquots were taken and diluted in 1 ml of H_2O and injected into an anal. AE-HPLC. AE-HPLC: (0–60% *B* in 36 min) flow 1.0 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°. *r(GGUGGGAG):* t_R 17.7 min (**12**). *5′-monophosphate-r(imG-CGUCCCACC):* t_R 21.4 min (**11**). *d(GGUGGGACG-_-CUCCCA):* t_R 24.9 min (**14**). *r(GGUGGGAG-imG-CGUCCCACC):* t_R 26.0 min (**10**). Yield: 90%. LC/ESI-MS: $m/z = 5854$ (calc. 5854).

Preparation of Unmodified RNA Sequences 5′-Monophosphate-r(X-CGUCCCACC) (**16**–**19**). The RNA sequences were prepared under conditions as previously described for **12** from the conventional 2′-*O*-TOM phosphoramidite building blocks and phosphorylating reagent [3-(4,4′-dimethoxytrityloxy)-2,2-dicarboxyethyl]propyl(2-cyanoethyl)(*N,N*-diisopropyl)phosphoramidite (*Glen Research*). The crude sequences were purified by AE-HPLC (0–50% *B* in 30 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°: t_R 21 min. LC/ESI-MS: m/z for X = A **16** (calc. 3159, found 3159), G **17** (calc. 3174, found 3174), U **19** (calc. 3135, found 3135), C **21** (calc. 3135, found 3135).

RNA Sequences r(GGUGGGAG-X-CGUCCCACC) (**10**). The previously prepared 8mer **12**, 10mers **16**, **17**, **19**, and **21**, and 16mer template **14** were mixed to a final concentration of 20 μM for the substrates and 30 μM for the template (1.5 equiv.) and ligated as previously described for sequence **10**. For the ligation kinetics, aliquots were taken and diluted in 1 ml of H_2O and injected into an anal. AE-HPLC. AE-HPLC: (0–60% *B* in 36 min) flow 1.0 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°. *r(GGUGGGAG)*: t_R 17.7 min (**12**). *5′-monophosphate-r(X-CGUCCCACC)*: t_R 21 min (**16**, **17**, **19**, and **21**). *d(GGUGGGACG-_-CUCCCA)*: t_R 24.9 min (**14**). *r(GGUGGGAG-X-CGUCCCACC)*: t_R 26 min (X = A **1** (37%); G **18** (48%); U **20** (21%); C **22** (2%)).

5. *NMR Experiments. Preparative Ligation of RNA Sequence r(GGUGGGAG-imG-CGUCCCACC)* (**10**). 160 Ligations of 60 μl batches each were performed, purified, and pooled as described above. The Et_3NH^+ form of the sequence **10** was transformed into its Na salt by dissolving in H_2O (2 × 1.5 ml), treatment with $NaHCO_3$ (10 equiv. per phosphate group), and evaporating to dryness. The residue was dissolved in H_2O (2 ml) and desalted on 2 *NAP*-columns according to the manufacturer's instructions: aq. soln. of RNA sequence. OD_{260} 6.12 (= 0.03 μmol). ^{1}H-NMR (400 MHz, D_2O, $c = 100$ μM).

Preparation of Modified RNA Sequences r(GGUGGGAG-X-CGUCCCACC) (**18**, **23**–**26**). The assembly of RNA sequences was carried out on a *Gene Assembler Plus* (*Pharmacia*), from 60 mg solid support (loading 32 μmol/g) and 2′-*O*-TOM-protected ribonucleoside phosphoramidites according to X = G **18** [11]. The modified nucleotides were introduced conventionally from the 2′-*O*-TOM-protected ribonucleoside phosphoramidites X = m^1G **23**, m^6A **24**, I **25**, m^2_2G **26**, prepared according to [3]. After the assembly, the solid supports were treated with a mixture of 12M $MeNH_2$ in H_2O/8M $MeNH_2$ in EtOH (1 ml) for 4h at 35°. Then, the same procedure as described for **12** was applied to the RNA sequences **18** and **23**–**26**. The crude sequences were purified by AE-HPLC: (0–60% *B* in 36 min) flow 2.5 ml/min; eluent *A*: 12 mM *Tris*·HCl (pH 7.4), 6M urea; eluent *B*: 12 mM *Tris*·HCl (pH 7.4), 0.5M $NaClO_4$, 6M urea; detection at 260 nm, elution at 85°: t_R 26 min. LC/ESI-MS: m/z for X = G **18** (calc. 5802, found 5802), m^1G **23** (calc. 5816, found 5816), m^6A **24** (calc. 5800, found 5800), I **25** (calc. 5787, found 5787), m^2_2G **26** (calc. 5830, found 5830). 0.1 μmol of each sequence was isolated and submitted to NMR experiments.

Preparation of the Modified RNA Sequence r(GGUGGGA-I-ACGUCCCACC) (**27**). The RNA sequence was prepared as previously described for RNA sequence **12**. LC-MS (ESI): $m/z = 5787$ (calc. 5787). 0.1 μmol of the sequence was isolated and submitted to NMR experiments.

Preparation of Samples for NMR Analysis of Sequences **1**, **10**, **18**, *and* **23**–**27**. The Et_3NH^+ form of the sequences was transformed into their Na salt by dissolving in H_2O (2 × 1.5 ml), treatment with $NaHCO_3$

(*Fluka*) (10 equiv. per phosphate group), and evaporating to dryness. The residue was dissolved in H_2O (2 ml) and desalted on 2 *NAP*-columns according to the manufacturer's instructions: aq. soln. of RNA sequence. ^{1}H-NMR (400 MHz, D_2O, $c = 100$ μM).

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Received March 3, 2008