RNA Structural Rearrangements

Triggering of RNA Secondary Structures by a Functionalized Nucleobase**

*Claudia Höbartner, Harald Mittendorfer, Kathrin Breuker, and Ronald Micura**

The ability of nucleic acids to undergo conformational changes is a prerequisite for their immense functional repertoire. The definition of nucleic acid conformational changes is rather broad and ranges from local structural transformations, such as base flipping, to complex reorganizations of the secondary and tertiary structure.[1] The most prominent examples of the latter are metabolite-binding riboswitches, which have been recognized as a general means for gene regulation in many organisms.[2] Other examples concern ligand-responsive aptamer–ribozyme constructs with the ribozyme activity directed by allosteric effects upon binding of the ligand to a receptor domain.[3] The modular nature of these molecular sensors makes them highly flexible with respect to the rational design of diagnostic tools, such as the recently introduced target-assisted signal-amplifying ribozymes for the detection of single nucleotide polymorphism (SNP) or of microRNAs.[4,5]

A crucial feature of the above examples is that an effector molecule, either a small ligand or another oligonucleotide, induces the nucleic acid conformational change. As for natural riboswitches, the conformational changes in the designed molecules also occur at the secondary-structure level and lead, for example, to the formation of defined, mutually dependent stem–loop motifs (terminator–antiterminator) that are responsible for an on/off signal for transcription or translation.[6,7]

With the present work we introduce a novel concept for the manipulation of nucleic acid secondary structures. The idea relies on chemically functionalized nucleotides that play the role of an effector and that have potential as a previously unrecognized tool for designable secondary-structure modulation.

The underlying basic concept is depicted in Figure 1. A natural nucleoside is modified by a functional group at the Watson–Crick base-pairing site. When incorporated into an oligoribonucleotide this functionalized nucleoside is responsible for the selection of a distinct, defined secondary structure due to its deficiency of base pairing. However,

[*] Dipl.-Ing. C. Höbartner, Mag. H. Mittendorfer, Dr. K. Breuker, Prof. Dr. R. Micura
Leopold Franzens Universität
Institut für Organische Chemie
Innrain 52a, 6020 Innsbruck (Austria)
Fax: (+43) 512-507-2892
E-mail: ronald.micura@uibk.ac.at

[**] Financial support from the Austrian Science Fund (Grants P15042 to R.M. and P15767 to K.B.) is gratefully acknowledged.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200460068

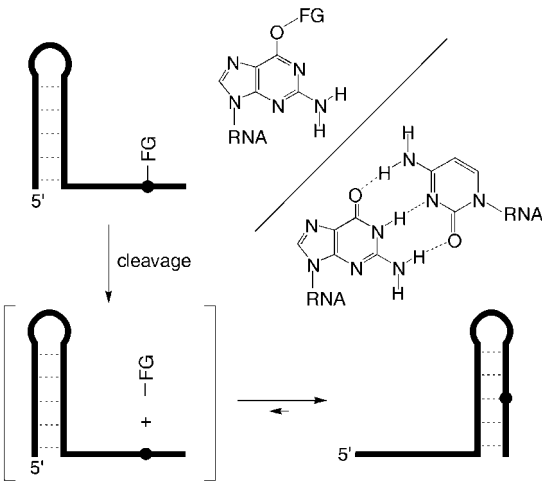
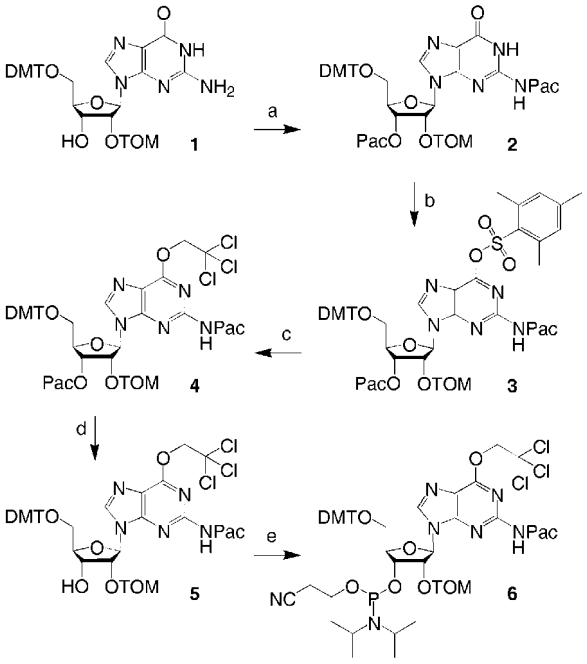


Figure 1. Novel concept for the modulation of nucleic acid secondary structures. A nucleobase, for example, guanine, is chemically functionalized at the Watson–Crick base-pairing site. Upon release of the functional group (FG) the base-pairing properties of the nucleobase are restored; the fold then becomes metastable and reorganizes into a thermodynamically favorable one.



Scheme 1. Synthesis of O^6-(2,2,2-trichloroethyl)guanosine phosphoramidite **6**. a) Phenoxyacetyl chloride (5.5 equiv), pyridine/CH_2Cl_2, 0 °C, 2 h, 74%; b) mesitylenesulfonyl chloride (1.2 equiv), triethylamine (5.0 equiv), DMAP (0.1 equiv), CH_2Cl_2, RT, 1 h; c) 2,2,2-trichloroethanol (2.5 equiv), ethyldimethylamine (5.0 equiv), DBU (1.0 equiv), CH_2Cl_2, RT, 2 h, 58% over two steps; d) 0.05 M NaOH, THF/methanol/H_2O, 4 °C, 3 min, 97%; e) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.5 equiv), ethyldimethylamine (10 equiv), CH_2Cl_2, RT, 2 h, 90%. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DMAP = 4-dimethylaminopyridine, DMT = 4,4′-dimethoxytrityl, Pac = phenoxyacetyl, THF = tetrahydrofuran, TOM = triisopropylsilyloxymethyl.[9]

upon release of the functional moiety the capability for Watson–Crick base pairing is restored and the secondary structure becomes metastable and reorganizes into a thermodynamically favorable one.

To prove this concept, we considered O^6-(2,2,2-trichloroethyl)-modified guanosine (^{tce}G) to be appropriately functionalized. On the one hand, the O^6-tce group alters the donor–acceptor pattern of the guanosine Watson–Crick base-pairing site and is therefore expected to significantly lower the residue's base-pairing probability. On the other hand, release of this group should be easily achieved in aqueous solution by zinc, thereby restoring the guanosine as a potential base-pairing candidate that then represents the driving force for a secondary-structure rearrangement.

The synthesis of the O^6-tce-guanosine phosphoramidite **6** used in our studies is shown in Scheme 1. The 2′-*O*-TOM-guanosine derivative **1**[8] was treated with phenoxyacetyl chloride in pyridine to give compound **2**. Then, mesitylation of the O^6 atom of the lactam moiety and substitution of the corresponding mesitylenesulfonyl ester **3** with 2,2,2-trichloroethanol in the presence of DBU and ethyldimethylamine yielded **4**. Selective cleavage of the 3′-*O*-phenoxyacetyl group with aqueous NaOH in THF/methanol resulted in compound **5**, which was readily transformed into the O^6-tce-guanosine cyanoethyl phosphoramidite **6**.[9]

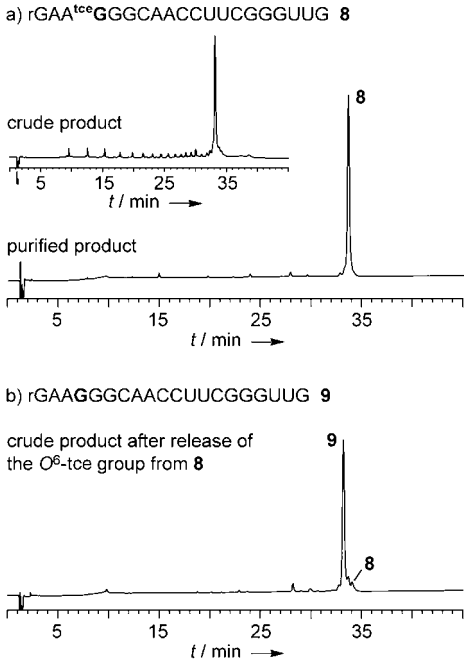


Figure 2. a) Representative example for the deprotection and purification of an O^6-tce-containing RNA sequence. HPLC trace of the product **8**, after treatment with aqueous NH_3/ethanol (1:1) at 55 °C for 5 h, treatment with 0.9 M TBAF in THF/H_2O (9:1) at 25 °C for 14 h, and purification by anion-exchange chromatography; MALDI-TOF MS: *m*/*z* calcd for $[M+H]^+$: 6594.3; found: 6597.5 ± 5. The inset shows the crude product before purification. b) Representative example for the release of the functional group, the O^6-tce group from **8**. HPLC trace of the crude product **9**, after treatment with zinc dust and 1 M NH_4OAc at pH 7 and 25 °C; $t_{1/2}$ = 5 min; MALDI-TOF MS: *m*/*z* calcd for $[M+H]^+$: 6462.9; found: 6466.3 ± 5. Anion-exchange HPLC: Dionex DNAPac column (4 × 250 mm), 80 °C, 1 mL min^{-1}, 0→60% B in 45 min (A: 25 mM tris(hydroxymethyl)aminomethane/HCl, 6 M urea, pH 8.0; B: same as A with addition of 0.5 M $NaClO_4$).

The incorporation of phosphoramidite **6** into oligoribonucleotides was achieved with coupling yields higher than 99% by using standard DNA/RNA protocols and 2′-*O*-triisopropylsilyloxymethyl-protected nucleoside phosphoramidites.[10–12] For capping, phenoxyacetic anhydride was used instead of acetic anhydride. The deprotection of the synthesized O^6-tce-guanosine-containing oligoribonucleotides was optimized thoroughly, as the 2,2,2-trichloroethyl moiety was unstable under standard RNA-deprotection conditions based on methylamine. We finally found that treatment with aqueous ammonia in ethanol (1/1) at 55 °C for 5–10 hours followed by treatment with 0.9 M tetrabutylammonium fluoride (TBAF) in THF/water (9/1) at 25 °C for 14 hours resulted in complete deprotection without strand degradation (Figure 2). A prerequisite for this procedure was the use of either N^2-methoxyacetylated or N^2-unprotected 2′-*O*-TOM-guanosine phosphoramidites instead of the commonly used N^2-acetylated counterparts. Otherwise, cleavage of the guanosine N^2-acetyl groups was incomplete under the conditions tolerated by the O^6-tce group.[13]

Release of the O^6-tce group from the oligoribonucleotides as suggested in our concept (Figure 1) was optimized experimentally and proceeded smoothly in aqueous ammonium acetate buffer at pH 7 in the presence of zinc dust. Clean and complete cleavage was achieved at room temperature and under slight agitation. The half lives of the O^6-tce groups depended on the length of the oligoribonucleotide and ranged from 0.5 minutes for a pentamer to 5 minutes for the 20-mer oligoribonucleotides (see the Supporting Information and Figure 2).[14]

The sequence set in Figure 3 documents the feasibility of our proposed concept for the modulation of nucleic acid secondary structures. The RNA 20-mer 5′-rGAAGG-(GCAA)CC(UUCG)GGUUG (**9**) is a rationally designed sequence that exists in a monomolecular equilibrium of two equally populated secondary structures.[15,16] However, upon replacement of a single guanosine by O^6-tce-guanosine in strand positions 4 or 17, respectively, only one of the two folds is selected. The functionalized 20-mer 5′-rGAA^{tce}GGG-CAACC(UUCG)GGUUG (**8**) exclusively adopts a UUCG hairpin conformation, whereas the 20-mer 5′-rGAAGG-(GCAA)CCUUCGG^{tce}GUUG (**7**) solely forms the alternative hairpin with a GCAA tetraloop. Cleavage of the O^6-tce group in **7** or **8** results in sequence **9** and restores the secondary-structure equilibrium.

The second sequence example was designed to satisfy the requirements for a complete transformation of the secondary structure and is represented by the RNA sequence of 5′-rUGUCUCCG(GACA)CGGAGAC (**12**). This oligonucleotide forms a highly stable GACA hairpin with a seven-base-pair stem. However, when a tandem of O^6-tce labels is introduced into this sequence, 5′-rUGUC(UCCG)GACAC ^{tce}G^{tce}GAGAC (**11**) selects the UCCG hairpin with a four-base-pair stem as the exclusive conformation. Upon release of the labels, the secondary structure switches to the thermodynamically favorable one, **12**. All secondary structures discussed above were ascertained by comparative imino proton NMR spectroscopy, a simple approach that we have introduced recently for the reliable probing of secondary structures of small RNAs (see Figure 3 and the Supporting Information).[16,17]

We have demonstrated here that chemical functionalization of the Watson–Crick base-pairing site is an efficient tool for inducing defined rearrangements of RNA secondary

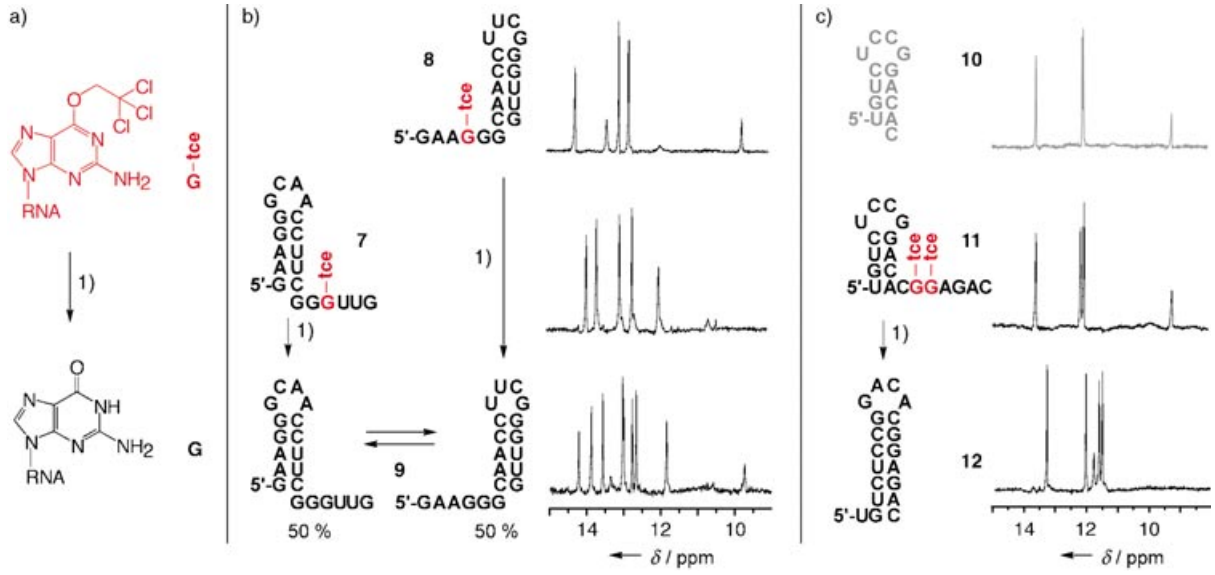


Figure 3. Secondary-structure modulation of short RNAs containing functionalized nucleosides. a) O^6-(2,2,2-trichloroethyl)guanosine (^{tce}G). b) Incorporation of ^{tce}G at positions 17 or 4 of the sequence rGAA(G^4)GGCAACCUUCGG(G^{17})UUG results in conformation selection of either the GCAA hairpin, **7**, or the UUCG hairpin, **8**. Release of the O^6-tce groups restores the conformational equilibrium of **9** (see also Figure 2). c) rUGUC(UCCG)GACAC^{tce}G^{tce}GAGAC (**11**) forms a stable hairpin with a UCCG tetraloop corresponding to reference sequence **10**. Upon release of the O^6-tce groups this RNA sequence converts into the thermodynamically favorable GACA hairpin, **12**. 1) Zn^0, 1 M NH_4OAc, pH 7, 25 °C. Structural probing of the RNA secondary structures by comparative imino proton NMR spectroscopy (500 MHz): 0.2–0.3 mM strand concentration, H_2O/D_2O (9:1), 25 mM sodium arsenate buffer, pH 7.4, 26 °C (see also the Supporting Information).

structures. The requirements for the functional labels are quite stringent. With respect to their incorporation into RNAs, they have to be orthogonal to the protecting groups used throughout the solid-phase synthesis. This is effectively achieved by use of the trichloroethyl group. In the late sixties, this group was introduced into oligonucleotide chemistry in the context of phosphate protection.[18] With respect to the applications of the trichloroethyl group for secondary-structure manipulation, we had to consider that the Zn^{2+} ions generated during the cleavage of the O^6-tce group may affect the secondary structure.[19–21] However, for the relatively small RNAs investigated so far we have no evidence for a structure-determining influence of the Zn^{2+} ions. In extending our approach towards larger RNAs, nucleobase functionalization by other chemical groups is conceivable; highly promising candidates are photolabile groups.

The control of conformational changes of nucleic acid structures is an important issue in RNA biotechnology. In particular, great efforts have been made in the development of allosterically triggered ribozymes for gene therapy and for nucleic acid diagnostics.[22–24] With the present concept for secondary-structure manipulation by functionalized nucleobases, a potential tool for applications in these fields has been created.

Received: March 21, 2004 [Z460068]

Keywords: conformational switches · nucleobase modifications · oligonucleotides · RNA structures

[1] D. M. Crothers in *RNA* (Eds.: D. Söll, S. Nishimura, P. B. Moore), Pergamon Elsevier Science, Oxford, **2001**, pp. 61–70.
[2] W. C. Winkler, R. R. Breaker, *ChemBioChem* **2003**, *4*, 1024–1032.
[3] R. R. Breaker, *Curr. Opin. Biotechnol.* **2002**, *13*, 31–39.
[4] S. Sando, T. Sasaki, K. Kanatani, Y. Aoyama, *J. Am. Chem. Soc.* **2003**, *125*, 15720–15721.
[5] J. S. Hartig, I. Grüne, S. H. Najafi-Shoushtari, M. Famulok, *J. Am. Chem. Soc.* **2004**, *126*, 722–723.
[6] A. S. Mironov, I. Gusarov, R. Rafikov, L. E. Lopez, K. Shatalin, R. A. Kreneva, D. A. Perumov, E. Nudler, *Cell* **2002**, *111*, 747–757.
[7] M. Mandal, R. Breaker, *Nat. Struct. Biol.* **2004**, *11*, 29–35.
[8] A. Stutz, C. Höbartner, S. Pitsch, *Helv. Chim. Acta* **2000**, *83*, 2477–2503.
[9] For detailed experimental procedures, see the Supporting Information.
[10] R. Micura, *Angew. Chem.* **2002**, *114*, 2269–2373; *Angew. Chem. Int. Ed.* **2002**, *41*, 2265–2267.
[11] S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu, *Helv. Chim. Acta* **2001**, *84*, 3773–3795.
[12] C. Höbartner, C. Kreutz, E. Flecker, E. Ottenschläger, W. Pils, K. Grubmayr, R. Micura, *Monatsh. Chem.* **2003**, *134*, 851–873.
[13] Oligoribonucleotide synthesis and deprotection protocols were first elaborated for the sequence rCG^{tce}GUA by using N^2-acetyl-, N^2-methoxyacetyl-, or N^2-unprotected 2′-*O*-TOM-guanosine phosphoramidites in various combinations with N^2-acetyl-, N^2-methoxyacetyl-, or N^2-phenoxyacetyl-protected O^6-tce-2′-*O*-TOM-guanosine phosphoramidites. These experiments and the synthesis of the corresponding building blocks will be described elsewhere.
[14] Deprotection of the O^6-tce groups was first evaluated for the sequence rCG^{tce}GUA under conditions with various pH values, strand concentrations, and buffer compositions, as well as different metals (Zn, Cd). For details, see the Supporting Information.
[15] For other examples of short bistable RNAs, see: C. Höbartner, M.-O. Ebert, B. Jaun, R. Micura, *Angew. Chem.* **2002**, *114*, 619–623; *Angew. Chem. Int. Ed.* **2002**, *41*, 605–609.
[16] C. Höbartner, R. Micura, *J. Mol. Biol.* **2003**, *325*, 421–431.
[17] R. Micura, C. Höbartner, *ChemBioChem* **2003**, *4*, 984–990.
[18] F. Eckstein, I. Rizk, *Angew. Chem.* **1967**, *79*, 939; *Angew. Chem. Int. Ed. Engl.* **1967**, *6*, 949.
[19] E. Ennifar, P. Walter, P. Dumas, *Nucleic Acids Res.* **2003**, *31*, 2671–2682.
[20] E. B. Khomyakova, H. Gousset, J. Liquier, T. Huynh-Dinh, C. Gouyette, M. Takahashi, V. L. Florentiev, E. Taillandier, *Nucleic Acids Res.* **2000**, *28*, 3511–3516.
[21] M. Wu, I. Tinoco, Jr., *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11555–11560.
[22] M. Warashina, T. Kuwabara, H. Kawasaki, J. Ohkawa, K. Taira in *RNA* (Eds.: D. Söll, S. Nishimura, P. B. Moore), Pergamon Elsevier Science, Oxford, **2001**, pp. 277–308.
[23] B. A. Sullenger, E. Gilboa, *Nature* **2002**, *418*, 252–258.
[24] M. B. Long, J. P. Jones III, B. A. Sullenger, J. Byun, *J. Clin. Invest.* **2003**, *112*, 312–318.

