# Small Interfering RNAs and Their Chemical Synthesis

**Ronald Micura***

Only few years ago, it was discovered that RNA plays a fundamental role in post-transcriptional regulation of gene expression. When double-stranded RNA (dsRNA) of around 300 to 1000 base pairs (bp) in length was injected into the nematode *Caenorhabditis elegans*, the specific silencing of genes highly homologous in sequence to the delivered dsRNA was induced.[1] This phenomenon, termed RNA interference (RNAi), paved the way for a straightforward determination of gene function, once the sequence of the gene was known.[2]

### *Mechanism of RNA Interference*

Elucidation of the mechanism of RNAi is subject to intensive research. Transfection with dsRNA results in the degradation of the sequence-homologous mRNA, even in only substoichiometric amounts. The initial step is the processing of the dsRNA into fragments of 21–23 nucleotide (nt) strands. These short interfering RNA molecules (siRNA) are the mediators of mRNA degradation, as was recently established by T. Tuschl and co-workers, who demonstrated that chemically synthesized RNA duplexes with the fragment pattern mentioned above are capable of guiding mRNA cleavage.[3]

These siRNA duplexes readily associate to form target-cleaving RNA protein complexes (target = sense or antisense), which have not been well characterized to date. These complexes are referred to as small interfering ribonucleoprotein particles (siRNPs; Figure 1). The model of Tuschl and co-workers discriminates between two classes of siRNPs; namely, one that cleaves only the antisense and another that cleaves only the sense strand. Depending on the orientation of the duplex within an siRNP, only one of the two siRNA strands is capable of target-RNA recognition (the siRNAs at the proteins which are represented by black rounded rectangles in Figure 1). This model corresponds to earlier findings that certain chemical modifications, such as 2′-aminouridine or 2′-deoxythymidine, are well tolerated in the sense strand, but not in the mRNA cleavage-guiding antisense strand.[4]

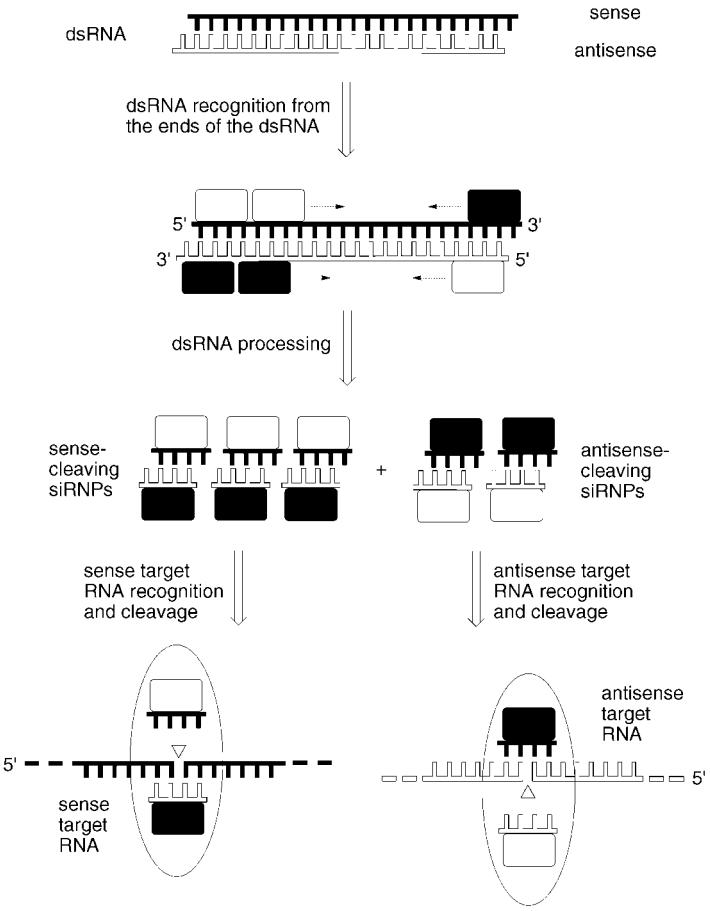



Figure 1. Model for RNA interference (RNAi) proposed by T. Tuschl and co-workers. Small interfering RNAs (siRNA) are the key molecules for targeted RNA cleavage (Figure adapted from reference[3]).

It is suggested that the relative orientation of the siRNA duplex within the siRNP is preserved from the preceding step of dsRNA processing.[3] The proteins involved, among them a nuclease of the RNase III family, assemble on the dsRNA in an asymmetric fashion and remain at least partly associated with the siRNA products. Thus, the molecular information of directionality is passed on, which results in distinct roles of the siRNA strands, only one being capable of guiding targeted RNA cleavage. The siRNA duplex is thought to be temporarily disrupted during target recognition and reformed after release of the cleaved target RNA. The position of RNA

[*] Dr. R. Micura
Leopold Franzens University
Institute of Organic Chemistry
Innrain 52a, 6020 Innsbruck (Austria)
Fax: (+43) 512-5072892
E-mail: ronald.micura@uibk.ac.at

  1433-7851/02/4113-2265 $ 20.00+.50/0 

cleavage relative to the guide siRNA is near the center of the region covered by the 21 or 22 nt siRNA.

Synthetic oligoribonucleotides were also valuable, as it was observed that 21 or 22 nt siRNA duplexes with double nucleotide 3′-overhangs were more efficient in degrading target RNA than similar blunt-ended duplexes.[3] This observation is consistent with the cleavage pattern of an RNase III-like nuclease activity during dsRNA processing.[5] It is of further note that dsRNAs of less than 38 bp are inefficient mediators of RNAi, as the reaction rate of siRNA formation is significantly reduced compared with longer dsRNA.[3]

### *siRNA in Mammalian Cells*

Although RNAi has become routine in many laboratories and has been studied for a wide range of organisms, its use in mammalian cells has long been considered problematic. However, with the understanding of siRNAs as key components in RNAi, the breakthrough for RNAi applications in mammalian cells has followed. The problem was that dsRNA in mammalian cells activates a nonspecific viral defense mechanism, the interferon response, which results in a global shutdown of protein synthesis and leads to cell death.[6] This pathway masks any sequence-specific effects that might arise from an RNAi pathway. However, the nonspecific pathway requires longer dsRNA, and does not occur with dsRNAs shorter than 30 bp. So, Tuschl and co-workers tested the ability of siRNA to target various luciferase transgenes in mammalian cell cultures (COS-7, NIH/3T3, HeLa, and 293 cells) and indeed observed reproducible, sequence-specific siRNA inhibition, which was easily assayed by luminescence spectroscopy.[7] In addition, siRNAs were also effective when targeting endogenous genes. Non-sequence-specific effects started to emerge for the transfection with 50 bp and longer RNAs, which suggested that both pathways (RNAi and interferon response) can operate simultaneously. However, gene expression in mammalian cells was not eliminated completely, as it was observed in the reference system of *Drosophila* cells.[7] That apart, the work above represents a landmark for siRNA as the upcoming gene-silencing methodology which, at this time, seems to be more promising than the alternatives, which include antisense and ribozyme-based strategies.

### *Two Major Improvements in the Chemical Synthesis of RNA*

Chemically synthesized RNA oligonucleotides are key components of RNAi technology. Although the widespread 5′-*O*-dimethoxytrityl(DMT)-2′-*O*-*tert*-butyldimethylsilyl(TBDMS) phoshoramidite chemistry enables their synthesis, this method has not reached the level of chemical DNA synthesis in terms of product quality and the accessible oligonucleotide length.[8, 9] In the need for more robust RNA routine-synthesis strategies, two novel methodologies have been developed and demonstrated to be successful.

#### *The 2′-O-TOM Method*

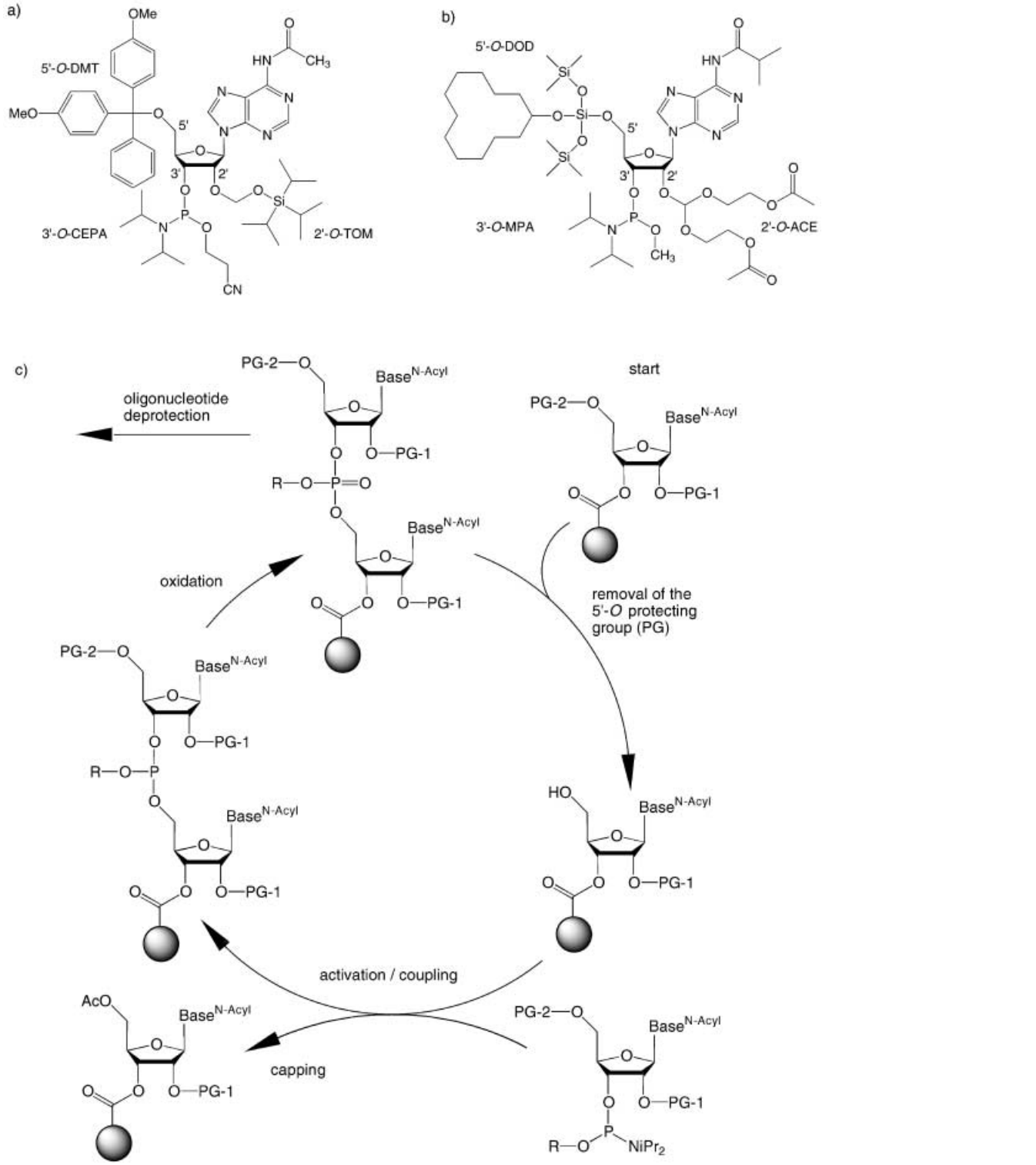
The first improvement was achieved by S. Pitsch and co-workers and maintains the principle of 5′-*O*-DMT-protection and 3′-*O*-(2-cyanoethyl)diisopropylphosphoramidite coupling (Scheme 1 a,c, Table 1). The key feature is the 2′-*O*-triisopropylsilyloxymethyl (TOM) protecting group of the nucleotide building blocks which was first introduced in 1998, but was recently published with the synthetic details.[10] The TOM protecting group guarantees an average coupling yield of 99.4 % under DNA coupling conditions and the usage of benzylthiotetrazole as activator. This superior coupling behavior, compared to the 2′-*O*-TBDMS building blocks (typical coupling yield: $\leq$98 %), can be attributed to the minimal steric demand of the TOM protecting group and allows the synthesis of up to 80mers. Furthermore, the combination of this coupling with the simple *N*-acetyl protection at the exocyclic amino groups of the nucleobases enables a reliable and complete two-step deprotection, first with $MeNH_2$ in $EtOH/H_2O$, followed by $Bu_4NF$ in THF, without concomitant degradation of the RNA products. The HPLC chromatograms of these RNA species are comparable to those obtained for the corresponding DNA sequence homologues. The TOM method is offered commercially as an oligoribonucleotide production service, and the nucleotide building blocks are also available commercially, which has contributed to the fast propagation of the method.[11] This availability resulted, for example, in the development of a solid-phase approach for the preparation of small circular RNA species, and in the usage of a variety of TOM-protected modified nucleoside building blocks.[12] A further strength of the TOM method is that it can be easily combined with the existing large pool of nucleoside labelling and marker building blocks.

#### *The 2′-O-ACE Method*

The second novel method for the chemical synthesis of RNA was introduced by S. Scaringe and co-workers, and represents an impressive strategy which was first communicated in 1998, recently followed by the detailed procedures.[13] Based on the literature, mildly acidic aqueous conditions were considered the most desirable for the final 2′-*O* deprotection of the synthesized RNA. The loss of orthogonality in the combination with the 5′-*O*-DMT group was an obstacle to using a mildly acid-labile 2′-*O* protecting group. The new RNA synthesis strategy is therefore based on the fluoride-labile 5′-*O*-bis(trimethylsiloxy)cyclododecyloxysilyl ether (DOD), together with the 2′-*O*-bis(2-acetoxyethyloxy)methyl (ACE) orthoester (Scheme 1 b,c, Table 1). The 3′-OH group is derivatized as the methyl-*N*,*N*-diisopropylphosphoramidite, as the cyanoethyl group turned out to be unstable with fluoride reagents. The coupling yields are higher than 99 %, in less than 90 s and are therefore also superior to those observed for the TBDMS building blocks.

After the oligonucleotide assembly, the phosphate methyl protecting groups are removed with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate ($S_2Na_2$) in DMF (Scheme 2). Then basic conditions (40 % aqueous $MeNH_2$) cause oligonucleotide cleavage from the solid support, along with the removal of the acyl protecting groups on the exocyclic amino groups and, importantly, of the acetyl groups on the 2′-orthoesters. The resulting 2′-*O*-bis(2-hydroxyethyloxy)methyl orthoesters are ten times more acid labile than prior to the removal of the acetyl groups; very mild acidic

  1433-7851/02/4113-2266 $ 20.00+.50/0 




Scheme 1. a) Nucleoside building blocks used in the 2′-*O*-TOM method (other nucleobases: $N^4$-acetylcytosine, $N^2$-acetylguanine, uracil); b) nucleoside building block used in the 2′-*O*-ACE method (other nucleobases: $N^4$-acetylcytosine, $N^2$-isobutyrylguanine, uracil); c) General scheme for the automated synthesis of oligoribonucleotides. PG = protecting group.

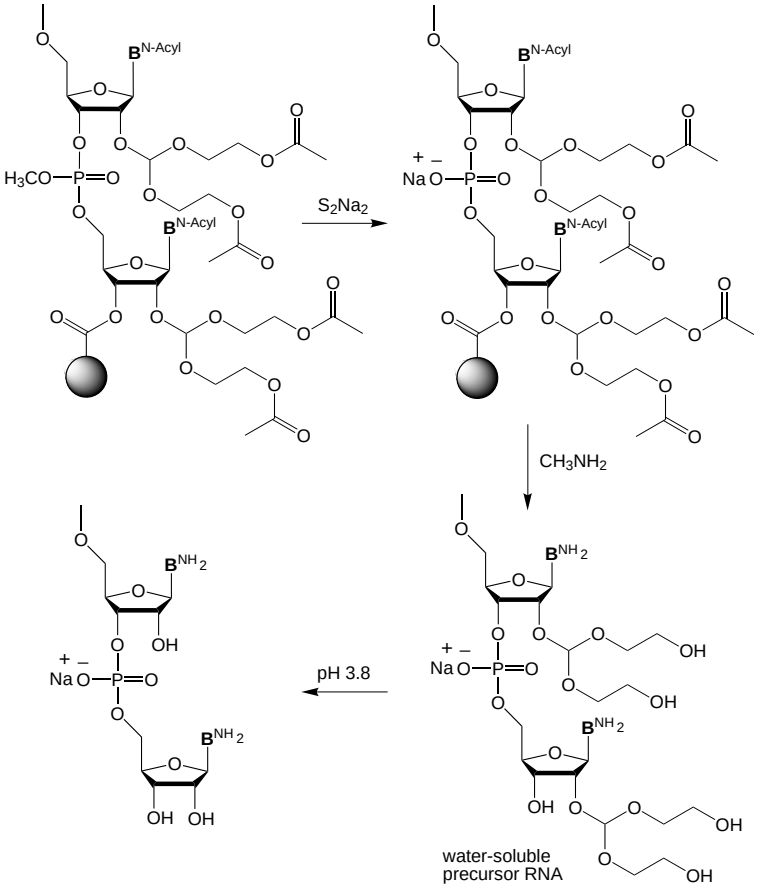
conditions (pH 3.8, 30 min, 60 °C) are therefore required for the final deprotection step. The HPLC chromatograms of the crude RNAs impress by showing hardly any by-products.

Unequivocally, the access to the 2′-*O*-bis(2-hydroxyethyloxy)methyl oligoribonucleotide is a major strength of the new approach as this precursor RNA is water soluble, can be analyzed by HPLC, and purified if necessary. Of further significance is that the 2′-*O*-bis(2-hydroxyethyloxy)methyl protection of the precursor oligoribonucleotide appears to interrupt secondary structures. This was demonstrated by the synthesis of a 23mer homopolymer of guanosine.[13d]

  1433-7851/02/4113-2267 $ 20.00+.50/0 

Table 1. Conditions for a complete coupling cycle in the 2′-*O*-TOM method and in the 2′-*O*-ACE method, and for the subsequent deprotection of the synthesized oligoribonucleotide.

| | 2′-*O*-TOM method (1.5 μmole scale) | 2′-*O*-ACE method (0.2 μmole scale) |
|---|---|---|
| Removal of the 5′-*O*-PG | 4% dichloroacetic acid in 1,2-dichloroethane, 90 s | 1.1 M HF/2.9 M triethylamine in dimethylformamide, 35 s |
| Activation/Coupling | 0.25 M benzylthiotetrazole (65 equiv)/0.1 M cyanoethyl-phosphoramidite (6 equiv) in $CH_3CN$, 90 s | 0.5 M ethylthiotetrazole (75 equiv)/0.1 M methylphosphor-amidite (15 equiv) in $CH_3CN$, 90 s |
| Capping | $Ac_2O$/2,6-lutidine/THF (1/1/8; v/v) and *N*-methylimidazole/THF (16/84; v/v), 60 s | $Ac_2O/CH_3CN$ (1/9; v/v) and *N*-methylimidazole/$CH_3CN$ (1/9; v/v), 30 s |
| Oxidation | $I_2/H_2O$/pyridine/THF (3/2/20/75; w/w), 45 s | 1 M *tert*-butylhydroperoxide in toluene, 45 s |
| Oligonucleotide deprotection | 1) 10 M $MeNH_2$ in EtOH/$H_2O$; 1 – 24 h, 25 – 33 °C<br>2) 1 M $Bu_4NF \cdot 3H_2O$ in THF; 1 – 50 h, 25 °C<br>3) 1 M Tris · HCl, $H_2O$, pH 7.4 | 1) 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate ($S_2Na_2$) in DMF; 15 min<br>2) 40% $MeNH_2$ in $H_2O$; 1 h, 55 °C<br>3) 100 mM tetramethylethylendiamine/acetic acid, pH 3.8; 30 min, 60 °C |




Scheme 2. Deprotection of an oligoribonucleotide synthesized by the 2′-*O*-ACE method proceeds via a water-soluble precursor RNA which cannot form strong secondary structures and is therefore easy to analyze by HPLC.

The 2′-*O*-ACE method can be used on commercial automated DNA synthesizers after some technical adjustments. This chemistry has been commercialized as an oligoribonucleotide production service, however, the corresponding nucleotide building blocks are not yet commercially available. First reports on the incorporation of nucleoside modifications also exist and, just recently, the combined chemical and enzymatic synthesis of tRNAs for high-throughput crystallization was published.[14]

Both methodologies, the 2′-*O*-TOM method and the 2′-*O*-ACE routes are major improvements in the synthesis of RNA oligonucleotides and offer unprecedented product quality. With respect to the incorporation of modifications, the TOM chemistry appears to have a slight advantage at the moment, as it benefits from the large existing "modifier" pool developed for use with TBDMS. It will be interesting to see if, in the long term, the 2′-*O*-ACE method is able to take the place of the 5′-*O*-DMT method, which is now strengthened by the TOM protecting group.


[1] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, C. C. Mello, *Nature* **1998**, *391*, 806 – 811.

[2] For a Highlight on RNA interference see: a) U. Schepers, T. Kolter, *Angew. Chem.* **2001**, *113*, 2503 – 2505; *Angew. Chem. Int. Ed.* **2001**, *40*, 2437 – 2439; for reviews see, for example: b) T. Tuschl, *ChemBioChem* **2001**, *2*, 239 – 245; c) R. Barnstead, *Curr. Opin. Chem. Biol.* **2001**, *5*, 63 – 66.

[3] S. M. Elbashir, W. Lendeckel, T. Tuschl, *Genes Dev.* **2001**, *15*, 188 – 200.

[4] S. Parrish, J. Fleenor, S. Xu, C. Mello, A. Fire, *Mol. Cell* **2000**, *6*, 1077 – 1087.

[5] E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* **2001**, *409*, 363 – 366.

[6] G. R. Stark, I. M. Kerr, B. R. Williams, R. H. Silverman, R. D. Schreiber, *Annu. Rev. Biochem.* **1998**, *67*, 227 – 264.

[7] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, *Nature*, **2001**, *411*, 494 – 498.

[8] G. M. Blackburn, M. Gait, *Nucleic Acids in Chemistry and Biology*, 2nd ed., Oxford University Press, Oxford, UK, **1996**.

[9] K. K. Ogilvie, K. L. Sadana, E. A. Thompson, M. A. Quilliam, J. B. Westmore, *Tetrahedron Lett.* **1974**, *15*, 2861 – 2863.

[10] a) X. Wu, S. Pitsch, *Nucleic Acids Res.* **1998**, *26*, 4315 – 4323; b) "Ribonucleoside-derivative and method for preparing the same": S. Pitsch, P. A. Weiss, L. Jenny, US Patent 5,986,084, **1999**; c) S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu, *Helv. Chim. Acta* **2001**, *84*, 3773 – 3795.

[11] a) M. Hünges, C. Rolz, R. Gschwind, R. Peteranderl, F. Berglechner, G. Richter, A. Bacher, H. Kessler, G. Gemmecker, *EMBO J* **1998**, *17*, 4092 – 4100; b) M. C. Nagan, P. Beuning, K. Musier-Forsyth, C. J. Cramer, *Nucleic Acids Res.* **2000**, *28*, 2527 – 2534; c) M. Brännvall, N. E. Mikkelsen, L. A. Kirsebom, *Nucleic Acids Res.* **2001**, *29*, 1426 – 1432.

[12] Small circular RNA species: a) R. Micura, *Chem. Eur. J.* **1999**, *5*, 2077 – 2082; b) R. Micura, W. Pils, K. Grubmayr, *Angew. Chem.* **2000**, *112*, 956 – 959; *Angew. Chem. Int. Ed.* **2000**, *39*, 922 – 926; modified


  1433-7851/02/4113-2268 $ 20.00+.50/0 

nucleosides: c) R. Micura, W. Pils, C. Höbartner, K. Grubmayr, M.-O. Ebert, B. Jaun, *Nucleic Acids Res.* **2001**, *29*, 3997 – 4005; d) V. Boudou, J. Langridge, A. Van Aerschot, C. Hendrix, A. Millar, P. Weiss, P. Herdewijn, *Helv. Chim. Acta* **2000**, *83*, 152 – 161; e) C. Höbartner, M.-O. Ebert, B. Jaun, R. Micura, *Angew. Chem.* **2002**, *114*, 619 – 623; *Angew. Chem. Int. Ed.* **2002**, *41*, 605 – 609.

[13] a) S. A. Scaringe, F. E. Wincott, M. H. Caruthers, *J. Am. Chem. Soc.* **1998**, *120*, 11 820 – 11 821; b) S. A. Scaringe, US Patent 6,111,086, **1998**; c) S. A. Scaringe, *Methods Enzymol.* **2000**, *317*, 3 – 18; d) S. A. Scaringe, *Methods* **2001**, *23*, 206 – 217.

[14] Nucleoside modifications: a) M. Meroueh, P. J. Grohar, J. Qiu, J. SantaLucia, Jr., S. A. Scaringe, C. S. Chow, *Nucleic Acids Res.* **2000**, *28*, 2075 – 2083; b) H. M.-P. Chui, M. Meroueh, S. A. Scaringe, C. S. Chow, *Bioorg. Med. Chem.* **2002**, 325 – 332; tRNAs: c) L. D. Sherlin, T. L. Bullock, T. A. Nissan, J. J. Perona, F. J. Lariviere, O. C. Uhlenbeck, S. A. Scaringe, *RNA* **2001**, *7*, 1671 – 1678.

# Hydrides and Iodides of Gold

**Margaret-Jane Crawford* and Thomas M. Klapötke***

The synthesis of gold hydrides in the solid state has long been desirable. Despite early investigations by Wiberg et al.,[1] to prepare $AuH_3$ utilizing a variety of reducing agents such as $LiAlH_4$, $AlH_3$, and $LiBH_4$, no direct evidence for the elusive $AuH_3$ could be obtained and only decomposition products, that is, Au and $H_2$ could be detected. Despite, or possibly because, of the lack of experimental evidence for gold hydrides in the solid state, a considerable number of theoretical studies have probed the structure of AuH and by using density functional methods.[2–6] Moreover, in the past few years, the chemistry of the gold halides and hydrides has received a great deal of attention, and utilizing a combination of computational and experimental techniques, the structures of many of the gold halides have been shown to agree with earlier structural predictions made by Schwerdtfeger et al.[5–7]

The similar electronegativities ($\chi_{AR}I = 2.2$; $\chi_{AR}H = 2.2$; $\chi_{AR}Au = 2.4$)[6b] of I and H, makes a comparison between gold hydride and iodide compounds relevant. Whereas AuH is a stable diatomic molecule that has been characterized in the gas phase, and although the analogous diatomic AuX species (X = F, Cl, Br) have been know in the gas phase for some time, it was only very recently that the gas-phase structure of AuI was determined by microwave spectroscopy.[8] However, whereas AuH was until very recently unknown in the solid state, AuI is a well-known and even commercially available (!) polymer, which is constructed of a zigzag chain with linear I-Au-I units (Figure 1).[9] The unusual chainlike structure found for AuI can be explained by relativistic effects (as opposed to correlation effects) which show an increased covalency in the Au–I interactions.[10] The structures of $AuI_3$ and $AuH_3$, however, were found to be a complex problem, not least because of the decreasing stability of the gold trihalides with increasing mass of the halide.[11–14] For $AuH_3$, the lowest-energy isomer was found not to be either the T-shaped or linear structure, but rather, a Y-shaped structure (singlet electronic state) which is better viewed as an adduct between AuH and $H_2$.

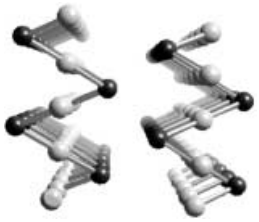


Figure 1. Solid state structure of gold(I) iodide.

Bayse recently reported detailed quantum-chemical DFT studies of the $AuH_3/Au_2H_6$ system, and suggested that in $AuH_3$ the AuH and $H_2$ units would be only loosely bound together [Eq. (1)].[3]

$$HAu(\eta^2\text{-}H_2) \xrightarrow{\Delta E_{diss}=\text{kJ mol}^{-1}} AuH + H_2 \qquad (1)$$

Moreover, with respect to the dimerization of $AuH_3$ to form $Au_2H_6$, the "classical" square-planar, $D_{2h}$ structure of $Au_2H_6$ was reported to be the only isomer located. The dimerization of both the Y- and T-shaped isomers of $AuH_3$, which formed $D_{2h}$ $Au_2H_6$, was found in both cases to be an exothermic process by $-84$ and $-305$ kJ mol$^{-1}$, respectively (Figure 2). The bonding in the lowest-energy Y-shaped isomer of $AuH_3$

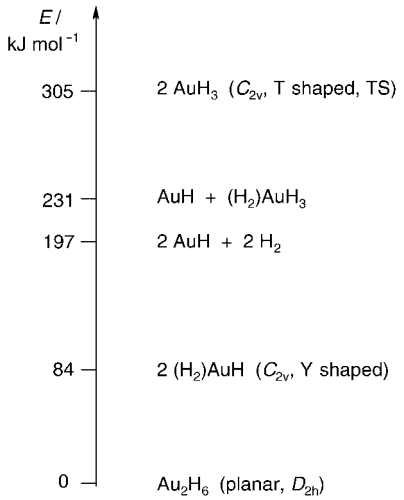



Figure 2. Relative energies of various gold hydride species (TS = transition state).

[*] Dr. M.-J. Crawford, Prof. Dr. T. M. Klapötke
Department of Chemistry
Ludwig-Maximilians University, Munich
Butenadtstrasse 5 – 13 (Haus D), 81377 Munich (Germany)
Fax: (+49) 89-2180-7492
E-mail: mjc@cup.uni-muenchen.de, tmk@cup.uni-meunchen.de

  1433-7851/02/4113-2269 $ 20.00+.50/0 