

# Chemical Synthesis of Modified RNA

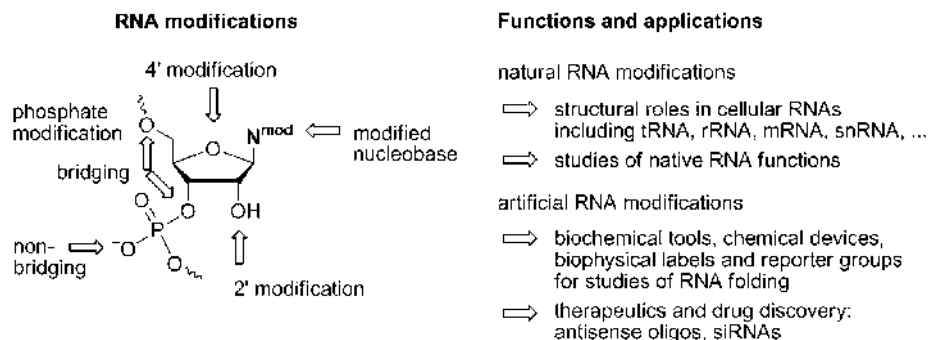
Claudia Höbartner and Falk Wachowius

## 1.1 Introduction

The synthesis of chemically modified RNA oligonucleotides is of paramount importance in many fields of nucleic acids research, ranging from studies of native RNA structure and function to applications in diverse areas not limited to chemistry, such as molecular biology, cell biology and medicine (Figure 1.1).

In Nature, more than 100 structurally distinguishable modified nucleotides have been identified in almost all classes of cellular RNAs [1]. The types of natural RNA modifications include simple nucleobase methylations and uridine isomerization, in addition to hypermodified nucleotides resulting from multistep biosynthetic transformations in complex RNA maturation processes [2]. A large number of structural and functional roles have been described for post-transcriptional RNA modifications, ranging from blocking or reinforcing single base pairs to increasing thermal stability and reducing conformational dynamics [3]. Changes in the physicochemical properties of RNA structures arising from nucleobase and ribose methylations have been attributed to enhanced base stacking due to increased hydrophobicity and polarizability and to changes in the hydration spheres of major and minor grooves [4]. Most insights into the roles of natural minor nucleotides come from extensive studies of highly modified tRNAs. However, the effects of individual nucleotide modifications in many other classes of RNAs are far from being fully understood. The availability of synthetic RNAs carrying natural modifications is therefore a prerequisite for studying the structural and functional contributions of individual RNA modifications. Advances in chemical synthesis of modified nucleotides will certainly lead to a more detailed understanding of natural phenomena.

In addition, artificial RNA modifications that introduce functional groups not found in Nature have proven to be useful tools for biochemical and biophysical investigations of RNA structure and function. Examples of reporter probes include fluorescent dyes to measure inter-helical distances or to report local and global changes during RNA folding [5–7], nitroxide spin probes for analysis of RNA structure and dynamics by EPR spectroscopy [8–10], disulfide crosslinks to restrict RNA helical elements for the investigation of structural models [11], selenium modifications to assist in solving the crystallographic phase problem [12–14] and



**Figure 1.1** Diagram illustrating sites for chemical modifications of ribonucleotides and versatile functions and applications of synthetic RNA

photolabile modifications for temporal control of RNA structure and activity [15]. These are only a few examples of the large diversity of approaches reported to date.

A separate motivation for the development of novel RNA analogs and methods for their chemical synthesis is their potential use as oligonucleotide-based therapeutics. Many modified oligonucleotides have been evaluated as antisense agents against different targets, but so far only a single antisense medium has been approved for clinical use [16]. The discovery of RNA interference (RNAi) as a natural gene-silencing pathway has energized the field of oligonucleotide-based technologies. The findings that synthetic small interfering RNAs (siRNAs) can be used as *in vitro* research tools and have the potential for the modulation of various diseases have increased the demand for modified and unmodified synthetic RNA oligonucleotides. Various modifications to the ribose, phosphate and nucleobase moieties of RNA have been investigated for their efficiency to decrease the susceptibility to nuclease degradation, reduce the risk of activating the innate immune response, decrease off-target effects and improve cellular uptake and pharmacological availability. Today, no single perfect modification pattern meets the significant biomedical challenges of delivery and biodistribution. Potential drug discovery is therefore a respectable motivation for the implementation of new RNA modifications. Recent reviews document the utility of various chemical modifications for use in siRNA technology [17–19].

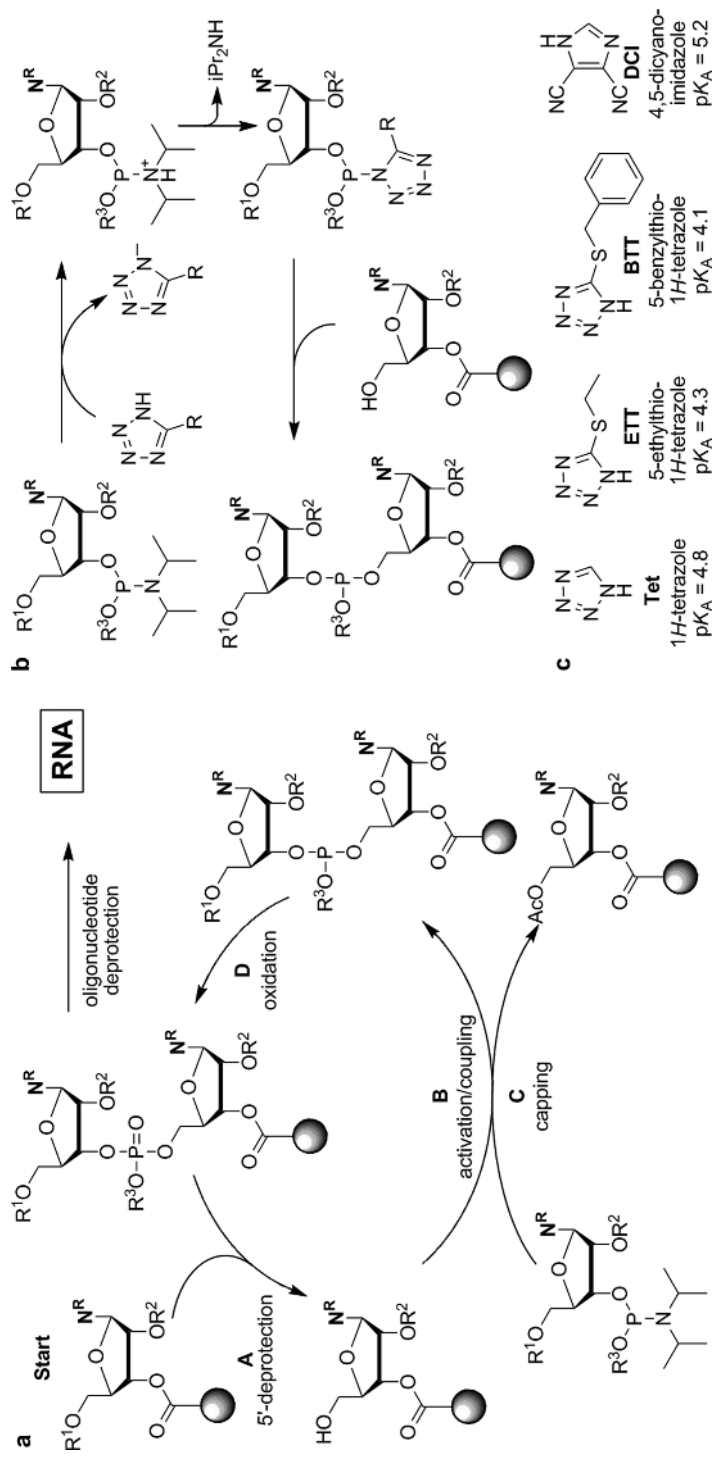
In this chapter we focus on RNA solid-phase synthesis using phosphoramidite chemistry and describe recent developments in protecting group strategies for RNA synthesis. We discuss general ways to introduce modifications into RNA oligonucleotides and we review selected examples of RNA modifications to illustrate various synthetic routes and give a flavor of the diversity of applications of modified RNAs.

## 1.2 The chemical synthesis of modified RNA

### 1.2.1 Solid-phase RNA synthesis using phosphoramidite building blocks

#### 1.2.1.1 The general chemistry of oligoribonucleotide synthesis

The currently most widely used strategy for the chemical synthesis of RNA oligonucleotides applies repeated coupling of ribonucleoside phosphoramidite building blocks on a solid support (Scheme 1.1a). Similarly to solid-phase DNA synthesis, the automated preparation of RNA oligonucleotides entails a four-step reaction cycle [20]. Chain elongation is initiated in step A by 5'-deprotection of an *N*,2',5'-*O*-protected ribonucleoside

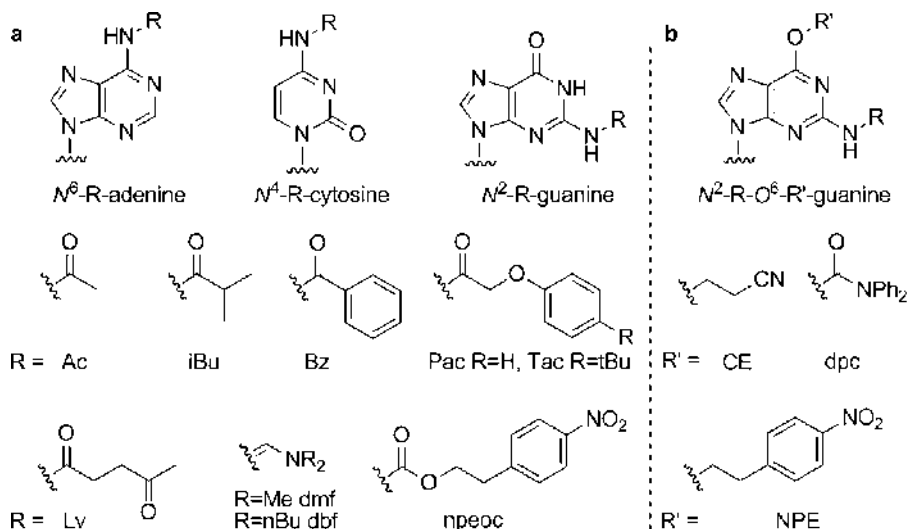


**Scheme 1.1** (a) RNA solid-phase synthesis cycle.  $N^R$ , protected nucleobase; R usually a base-labile protecting group (see Figure 1.2);  $R^1$ , transient 5'-protecting group (usually acid- or fluoride-labile);  $R^2$ , permanent 2'-protecting group, see text and Figures 1.4 and 1.5 for details;  $R^3$ , permanent phosphate protecting group, usually cyanoethyl or methyl. (b) Mechanism of phosphoramidite activation and coupling. (c). Commonly used activators

that is attached to a solid support through a bifunctional linker. The solid support is typically composed of controlled-pore glass with a high surface density of alkylamino groups (LCAA-CPG) or of amino-functionalized polystyrene resins. The bifunctional linker is usually an aliphatic dicarboxylic acid (succinic, adipic or pimelic acid), which is connected to the surface of the support via amide bond formation and comprises an ester linkage to the 3'-hydroxyl group of the ribonucleosides. In step B of the synthesis cycle, a suitably protected phosphoramidite building block is activated with a weak acid (such as an appropriately substituted 1*H*-tetrazole or imidazole derivative) and coupled to the 5'-hydroxyl group of the support-bound ribonucleoside, which results in the formation of a dinucleoside phosphite triester in a two-step reaction. This coupling reaction, as shown in Scheme 1.1b, proceeds via protonation of the diisopropylamino group of the phosphoramidite, followed by displacement of diisopropylamine by the conjugate base of the activator to form the active species, e.g., a tetrazolide intermediate. Subsequent nucleophilic substitution of the tetrazolide by the 5'-hydroxyl of the growing oligonucleotide forms the new phosphite triester linkage. Thus, a better proton donor and/or a better nucleophile to generate the reactive intermediate will increase the rate of the coupling reaction: for example, 5-ethylthio-1*H*-tetrazole (ETT) and 5-benzylthio-1*H*-tetrazole (BTT) improve the rate of the reaction compared with 1*H*-tetrazole due to their stronger acidity; 4,5-dicyanoimidazole (DCI) improves the reaction rate, presumably because it is a better nucleophile (Scheme 1.1c). The coupling step is followed by the capping step (step C in Scheme 1.1a) which involves 5'-*O*-acetylation of unreacted 5'-termini to prevent the subsequent extension to less than full-length oligonucleotide chains. The capping reagents also accomplish the cleavage of byproducts from nucleobase phosphorylation that may have formed during the coupling reaction. The solid-phase bound dinucleoside phosphite triester is then converted into a more stable phosphate triester (= phosphotriester) by oxidation with iodine or *tert*-butyl hydroperoxide (step D). Removal of the 5'-protecting group initiates the next chain extension cycle. The four-step synthesis cycle is repeated until chain assembly of the desired oligonucleotide length is completed. The full-length oligoribonucleotide is then released from the solid support and the nucleobase and phosphate protecting groups are removed, usually under ammonolytic conditions. Finally, 2'-deprotection affords the plain oligoribonucleotide product which is analyzed and purified for further utilization.

The key to successful solid-phase RNA synthesis is the choice of a suitable combination of orthogonal transient ( $R^1$ ) and permanent ( $R$ ,  $R^2$ ,  $R^3$ ) protecting groups for the reactive functional groups in ribonucleoside phosphoramidites. The levels of orthogonality that need to be considered are as follows: deprotection of 5'-hydroxyl groups, deprotection of phosphate backbone, deprotection of exocyclic amino groups on nucleobases, release from the solid support and deprotection of 2'-hydroxyl groups. Usually, nucleobase deprotection, phosphate deprotection and cleavage from the solid support are combined in a single step, which leaves at least three levels of orthogonality that must be met by any successful approach for RNA solid-phase synthesis. Occasionally, separate deprotection of the phosphate group is required. In general, base-labile protecting groups of the acyl, amidine or carbamoyl type are used for the exocyclic amino groups of nucleobases (Figure 1.2). Ammonolytic conditions are commonly applied for nucleobase deprotection and release of the oligonucleotide from the solid support. Simultaneously, the phosphotriester is converted into a phosphodiester (a few important exceptions are discussed below). It is imperative that the 2'-protecting groups are stable under these conditions, because generation of a free 2'-OH in the presence of a basic reaction medium results in strand cleavage and is also known to promote phosphate migration, which leads to the undesired formation of unnatural 2'-5' internucleotide linkages.

A plethora of protecting groups and solid-phase supports have been developed for the chemical synthesis of DNA and RNA oligonucleotides and a comprehensive review is beyond the scope of this chapter. Here, we describe the most commonly used and commercially available phosphoramidite building block families as a reference point and summarize recent developments in the field.



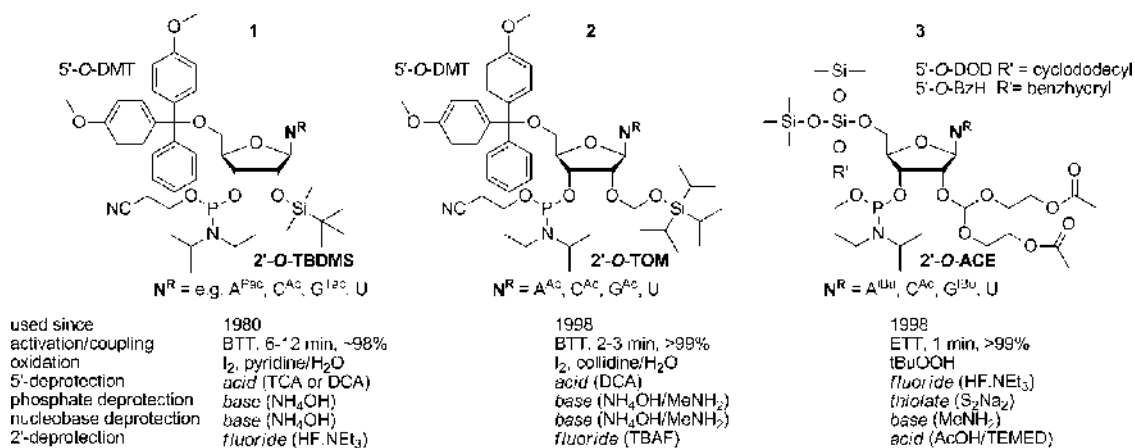
**Figure 1.2** (a) Natural nucleobases adenine (A), cytosine (C) and guanine (G) that need protecting groups at their exocyclic amino groups during solid-phase synthesis. Selected examples of base-labile nucleobase protecting groups (R): Ac = acetyl, iBu = isobutyl, Bz = benzoyl, Pac = phenoxyacetyl, Tac = 4-(tert-butylphenoxy)acetyl, Lv = levulinyl, dmf = dimethylaminomethylene (same as dimethylformamidine), dbf = dibutylaminomethylene, npeoc = (4-nitrophenyl)ethoxycarbonyl. (b) For special applications,  $O^6$ -protection of guanine is required. Examples of R': CE = 2-cyanoethyl, dpc = diphenylcarbamoyl, NPE = (4-nitrophenyl)ethyl

### 1.2.1.2 Commonly used protecting group strategies for ribonucleoside phosphoramidites

#### The 5'-O-dimethoxytrityl-2'-O-silyl strategy

The traditional approach for orthogonal protection of 5'- and 2'-hydroxyl groups of ribonucleoside phosphoramidites is based on 5'-O-dimethoxytrityl (DMT)-protected 3'- $\beta$ -cyanoethyl diisopropylphosphoramidites, and therefore capitalizes on the highly successful DNA synthesis strategy which routinely uses 5'-O-DMT protected phosphoramidites. The 5'-O-DMT group is rapidly removed by anhydrous acid and produces an orange-colored DMT carbocation that permits facile determination of sequential coupling yields by colorimetric detection. Various types of 2'-protecting groups have been described in combination with the 5'-O-DMT group, but the two most common representatives belong to the fluoride-labile class of silyl protecting groups. The 2'-O-tert-butyldimethylsilyl (TBDMS) and 2'-O-triisopropylsilyloxymethyl (TOM) protecting groups are described below. Conditions for phosphoramidite coupling and RNA deprotection are summarized in Figure 1.3.

**The tert-butyldimethylsilyl (TBDMS) protecting group** Since the 1980s, the tert-butyldimethylsilyl (TBDMS) group has been the most commonly used 2'-alkylsilyl protecting group for RNA solid-phase synthesis [21]. A wide variety of 5'-O-DMT-2'-O-TBDMS phosphoramidites of general formula **1** are commercially available. However, the performance of these building blocks in solid-phase RNA synthesis has not reached the level of deoxyribonucleoside phosphoramidites in DNA synthesis. The sluggish coupling kinetics (10–15 min coupling time) and relatively low coupling efficiencies (typically ~98% average coupling yield) have been attributed to steric interference of the TBDMS group with the coupling reaction [22]. For comparison, coupling times for deoxyribonucleoside phosphoramidites usually range from 0.5 to 2 min and coupling efficiencies exceed 99%. Traditionally, 1H-tetrazole was used as activator for 2'-O-TBDMS-protected RNA



**Figure 1.3** Ribonucleoside phosphoramidite building blocks for commercialized RNA synthesis methods using either the 5'-O-DMT-2'-O-silyl (1 and 2) or the 5'-O-silyl-2'-O-ACE chemistry (3). TCA = trichloroacetic acid; DCA = dichloroacetic acid; TBAF = tetrabutylammonium fluoride; S<sub>2</sub>Na<sub>2</sub> = disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate; TEMED = tetramethylethylenediamine

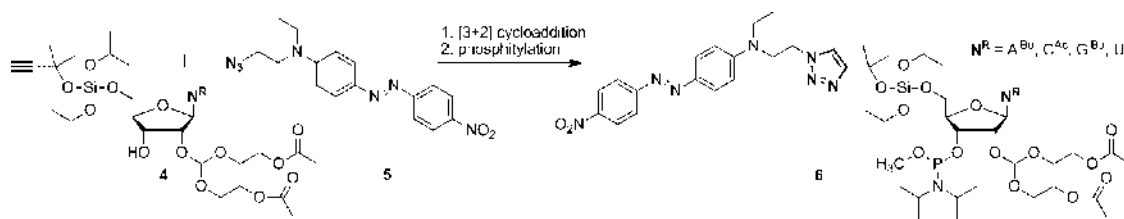
phosphoramidites, but today more powerful activators such as ETT and BTT are applied and coupling times can be reduced to 3–6 min to achieve coupling efficiencies of up to 99% [22]. The most critical issue associated with 2'-O-TBDMS protected ribonucleosides is the high potential of the alkylsilyl ether to migrate from the 2'- to the 3'-position during the phosphitylation step of phosphoramidite synthesis. Contamination of the 2'-O-TBDMS-protected 3'-phosphoramidite building blocks with the isomeric 3'-O-TBDMS-protected 2'-phosphoramidites will result in the undesired presence of unnatural 2'-5' phosphodiester linkages in the final RNA product. Since the 2'-O-TBDMS group is not indefinitely stable under harsh ammonia deprotection conditions, migration and eventually chain cleavage might also occur during basic deprotection. The use of nucleobase protecting groups cleavable under mild conditions, such as phenoxyacetyl (Pac) and 4-(*tert*-butylphenoxy)acetyl (Tbc), is therefore recommended.

**The triisopropylsilyloxymethyl (TOM) protecting group** Within the last decade, considerable advances in conventional 2'-silyl protection have been reported. Probably the most significant improvement in this context was the development of the 2'-O-triisopropylsilyloxymethyl (TOM) protecting group, reported by Wu and Pitsch in 1998 [23a]. The reduced steric demand of the TOM group compared with TBDMS during internucleotide bond formation allows for high coupling yields (>99%) of phosphoramidite building block 2 in short coupling times (2–3 min). In contrast to the TBDMS group, the acetal moiety of TOM does not undergo 2' to 3' isomerization during phosphitylation and therefore the risk of contamination with isomeric 2'-5' phosphodiester linkages in the final oligoribonucleotide is eliminated. The 2'-O-TOM group is entirely compatible with the 5'-O-DMT and the  $\beta$ -cyanoethyl groups. The exocyclic amino groups of the standard nucleobases are protected with acetyl groups that can be deprotected with methylamine in aqueous ethanol at room temperature within a few hours. The removal of the 2'-O-TOM protecting group is achieved with tetrabutylammonium fluoride (TBAF) in THF for 5–14 h at 25–30 °C. Data for RNA synthesis in high quality and high yield have been reported for oligonucleotides of up to 84 nucleotides (nt) in length [23b]. The 2'-O-TOM protection strategy is also compatible with a wide variety of modified nucleosides and has been used extensively for the preparation of modified RNA [24,25].

**The 5'-O-silyl-2'-O-bis(2-acetoxyethoxy)methyl orthoester (ACE) strategy**

In addition to the TOM chemistry, which has been commercialized and is currently widely used in many research laboratories, a second strategy was developed in the late 1990s that has advanced to a highly powerful and commercially offered RNA synthesis method. This second strategy, the 2'-O-bis(2-acetoxyethoxy)methyl orthoester (ACE) RNA synthesis method, was described in 1998 by Caruthers and co-workers and is based on a complete redesign of earlier protecting group strategies [26]. The new approach was designed under the notion that mildly acidic conditions would be most desirable for the final deprotection of RNA 2'-hydroxyl groups. For this purpose, the mildly acid-labile ACE protecting group was developed. The resulting loss of orthogonality with 5'-O-DMT protection, which is also sensitive to acidic conditions, necessitated the development of a novel class of 5'-protecting groups. Since the 2'-protecting group in ACE chemistry is no longer silyl based, the 5'-position can now be protected with a fluoride-labile silyl group. Two examples of extensively used substituted silyl ethers are the bis(trimethylsiloxy)cyclododecyloxysilyl ether (DOD) and the benzhydryloxybis(trimethylsiloxy)silyl ether (BzH), which are rapidly removed by fluoride ions under neutral conditions. This innovative 2'-O-ACE setup preserves only a few aspects of the traditional DNA synthesis strategy and requires alterations to commonly applied procedures and changes to standard instrumentation. The 5'-O-silyl-2'-O-ACE building blocks **3** (Figure 1.3) are prepared as methyl diisopropylphosphoramidites (i.e.  $R^3 = CH_3$ ) because the cyanoethyl protecting group is incompatible with fluoride treatment during repeating coupling cycles. An additional deprotection step is therefore required to cleave the methyl phosphate prior to release of the oligonucleotide from the solid support and nucleobase deprotection under alkaline conditions. During the basic deprotection step, the ACE group is converted to a 2'-O-bis(2-hydroxyethoxy)methyl orthoester, which is 10 times more acid labile than the acetylated form of the orthoester. The ACE RNA synthesis methodology yields a water-soluble oligonucleotide intermediate that can be rapidly 2'-deprotected (within 10 min) using mild aqueous acid immediately before use. Other advantages are the rapid coupling kinetics and high average stepwise coupling yields (>99% in less than 1 min). ACE chemistry permits RNA synthesis in excess of 70 nt in length, is easily scalable and applicable to high-throughput RNA production.

A feature that has been lacking in ACE chemistry since its development is the possibility of convenient tracking of repetitive coupling efficiencies in each cycle in analogy with the photometric detection of the dimethoxytrityl cation. This drawback has recently been overcome by the development of ACE phosphoramidite building blocks **6** that have a visible chromophore appended to the 5'-silyl protecting group via a 1,2,3-triazole linkage (Scheme 1.2) [27]. The chromophore is installed by the highly specific copper(I)-catalyzed [3 + 2] bipolar cycloaddition reaction between an alkyne on the silyl group of nucleosides **4** and an azide moiety on the chromophore **5**. The chromophore is a Disperse Red (DR) (DR = 2-{*N*-ethyl-*N*-[4-(4-nitrophenyldiazenyl)phenyl]}aminoethanol) derivative, which has similar absorption properties to the DMT cation ( $\lambda_{\max} = 498$  nm for DMT<sup>+</sup> and 470 nm for DR).



**Scheme 1.2** Synthesis of 5'-DRSil-2'-O-ACE ribonucleoside 3'-phosphoramidites **6** via 1,3-dipolar cycloaddition reaction and phosphorylation

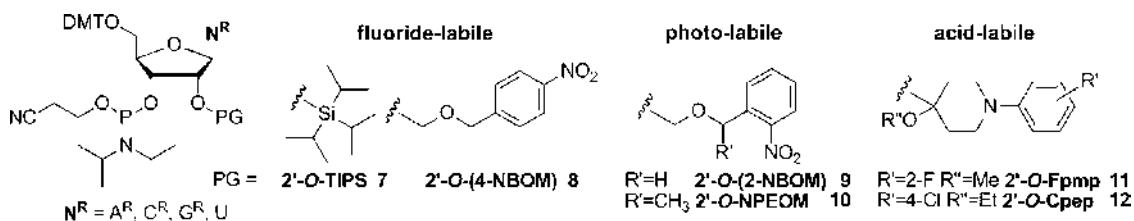
### 1.2.1.3 Recent developments in the area of 2'-protecting groups

Within the last few years, the increasing demand for synthetic oligonucleotides for RNAi applications has stimulated renewed research activities for the improvement of RNA synthesis technology beyond the capabilities of the current methods based on TBDMS, TOM and ACE chemistry. Almost all recent developments in this field involve variations of the 2'-protection strategies while maintaining orthogonality to the 5'-O-DMT group and compatibility with the cyanoethyl phosphate protecting group. Many of the novel 2'-protecting groups exploit a flexible formacetal moiety to take advantage of the minimized steric crowding in the vicinity of the 3'-phosphoramidite functionality, which allows for high coupling yields in short coupling times. According to the required deprotection conditions, 2'-protecting groups can traditionally be classified into the following types (Figure 1.4): fluoride-labile protecting groups [other examples apart from TBDMS and TOM are triisopropylsilyl (TIPS) (**7**) and 4-nitrobenzyloxymethyl (4-NBOM) (**8**)], photolabile nitrobenzyl protecting groups such as 2-nitrobenzyloxymethyl (2-NBOM) (**9**) and 1-(2-nitrophenyl)ethoxymethyl (NPEOM) (**10**) and acid-labile acetal/ketal protecting groups such as 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fmpmp) (**11**) and 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep) (**12**).

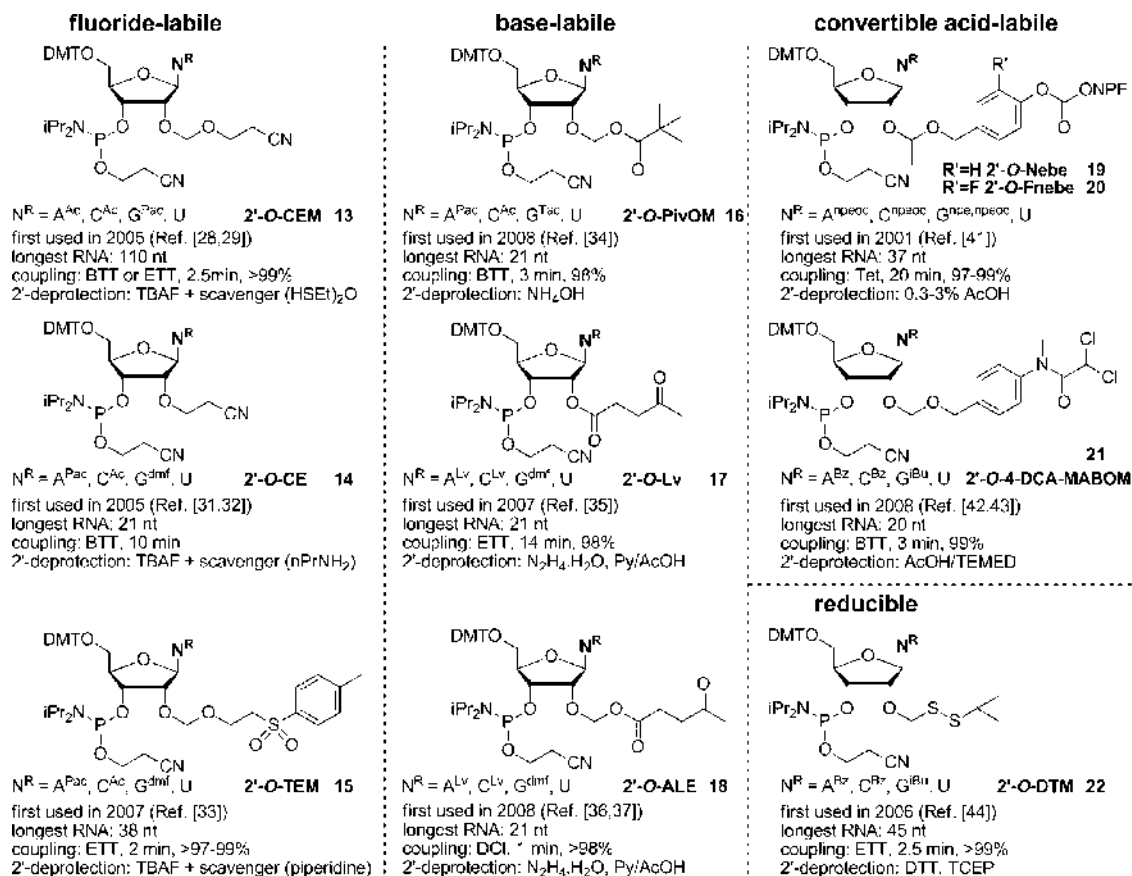
This classification is not absolutely strict and several exceptions are known. For example, some earlier reported protecting groups that are labile to UV irradiation are also cleaved by fluoride ions (e.g. 2-NBOM). More recently, base-labile and reducible protecting groups have also been found useful for RNA synthesis (Figure 1.5). A protection strategy that has been gaining increasing interest is the use of convertible or 'protected', protecting groups, in which fragmentation occurs under one set of conditions (usually base treatment) and leaves a modified protecting group that is then sensitive to a new set of conditions (usually more acid labile). This concept also applies to the design of the ACE group. Below we describe the most recent developments of fluoride-labile, base-labile, acid-labile and reducible 2'-protecting groups.

#### Fluoride-labile protecting groups

**The 2-cyanoethoxymethyl (CEM) and 2-cyanoethyl (CE) protecting groups** A recently introduced fluoride-labile, acetal-type 2'-hydroxyl protecting group is the 2'-O-2-cyanoethoxymethyl (CEM) group. Initially reported by Ohgi and co-workers in 2005 [28], this group showed satisfactory performance for the synthesis of up to 110 nt long RNAs [29,30]. Activation of the 2'-O-CEM protected phosphoramidites **13** was accomplished by BTT or ETT within a coupling period of 2.5 min, resulting in >99% coupling efficiency. The CEM group is modestly sensitive to the basic conditions used for nucleobase and phosphate deprotection and it is preferably removed by treatment with TBAF in THF or DMSO. The presence of bis(2-mercaptoethyl) ether or nitromethane as an acrylonitrile scavenger is essential to prevent the formation of nucleobase adducts during fluoride-mediated CEM deprotection.



**Figure 1.4** Examples of conventional 2'-protecting groups classified according to required deprotection conditions



**Figure 1.5** Overview of 5'-O-DMT-protected phosphoramidites with various recently developed 2'-O-protecting groups, classified according to 2'-deprotection conditions

The analogous 2'-O-cyanoethyl (CE) group without the formacetal moiety was also used as 2'-protecting group in RNA oligonucleotide synthesis [31,32]. The corresponding phosphoramidites **14** were activated with BTT over a coupling time of 10 min. Complete deprotection of the 2'-cyanoethyl group was accomplished with 1 M TBAF in THF in the presence of 5% *n*-propylamine as acrylonitrile scavenger. Interestingly, the cyanoethyl group proved to be stable upon exposure to triethylamine trihydrofluoride. This property could be used to produce partially 2'-O-cyanoethylated RNA oligonucleotides by using a combination of 2'-O-CE and 2'-O-TBDMS phosphoramidites in solid-phase synthesis. It was proposed that partially cyanoethylated oligoribonucleotides, which were shown to form stable duplexes with RNA and DNA oligonucleotides, could be suitable candidates for RNAi experiments [32].

**The 2-(4-tolylsulfonyl)ethoxymethyl (TEM) protecting group** Another fluoride-sensitive 2'-acetal-type protecting group is the 2'-O-2-(4-tolylsulfonyl)ethoxymethyl (TEM) group that was reported by Chattopadhyaya and co-workers in 2007 [33]. The TEM group was designed to be more stable towards ammonia deprotection conditions compared with the CEM group, which reduces the risk of chain cleavage during nucleobase and phosphate deprotection. The 2'-O-TEM-protected phosphoramidites **15** were

activated with ETT and allowed to couple for 2 min. The stepwise coupling yields ranged from 97 to 99%. RNA oligonucleotides of up to 38 nt in length have been produced. The deprotection proceeds under similar conditions as used for CEM deprotection. However, it was observed that during fluoride-assisted removal of TEM, the released 4-tolyl vinyl sulfone modified the exocyclic amino groups on nucleobases via Michael-type addition. Adduct formation was largely suppressed by using piperidine or morpholine as scavenger [33].

### ***Base-labile protecting groups***

*The pivaloyloxymethyl (PivOM) protecting group* The goal of reducing the number of steps for RNA deprotection after solid-phase synthesis for a rapid and efficient preparation of RNA relies on the design of base-labile protecting groups for the 2'-hydroxyl group. However, this is a major challenge due to the inherent instability of fully deprotected RNA under alkaline conditions. First success towards this goal has recently been reported by using the base-labile 2'-*O*-pivaloyloxymethyl (PivOM) protecting group for ribonucleoside phosphoramidites **16**. Debart and co-workers demonstrated the synthesis of up to 21 nt long RNAs with this base-labile acetal ester protecting group [34]. The average coupling efficiency upon activation with BTT was > 99% within a 3 min coupling time. The PivOM group was designed for a fast, two-step, all-base deprotection scheme, which consists of the selective cleavage of the phosphate cyanoethyl protecting groups by a non-nucleophilic base (DBU or piperidine), followed by ammonia treatment for the release of the oligomer from the solid support, with simultaneous deprotection of nucleobases and cleavage of the 2'-*O*-PivOM groups. It has been proposed that the formacetal intermediate on the 2'-position, which is generated upon ammonolysis of the acetal ester, is stable enough to ensure that the RNA does not degrade in aqueous ammonia. Upon evaporation of the ammonia deprotection mixture, the decrease in the pH results in fragmentation of the hemiacetal to liberate the unmodified RNA. The activity of an siRNA that was prepared by the new PivOM strategy was shown to have gene silencing activity comparable to a commercial sample of the same siRNA sequence [34].

*The levulinyl (Lv) and levulinyl acetal ester (ALE) protecting groups* Despite the difficulties associated with using base-sensitive 2'-protecting groups, 2'-*O*-levulinyl ribonucleoside phosphoramidites **17** were recently reported for use in RNA solid-phase synthesis on a fluoride-labile hydroquinone-*O,O'*-diacetic acid (Q-linker) CPG support [35]. The nucleobases also carried levulinyl protecting groups on the exocyclic amino groups for adenine and cytosine and the dimethylformamidine group for guanosine. These phosphoramidites were activated with ETT and coupled for 14 min to reach an average coupling yield of 98.5%, which is comparable to the standard 2'-*O*-TBDMS phosphoramidites. Release and deprotection of 21 nt long RNA oligonucleotides proceeded in three steps: first, the cyanoethyl groups were removed by triethylamine in acetonitrile, then hydrazinolysis cleaved the nucleobase and 2'-*O*-Lv groups, and finally, the completely deprotected RNA was released from the support by 1 M TBAF in THF. The Lv group is orthogonal to the TBDMS group and can therefore be used to prepare partially 2'-protected RNA oligonucleotides that might be useful for RNAi applications. One major obstacle is the difficult synthesis of pure 2'-*O*-Lv 3'-phosphoramidites because of the tendency of the levulinyl group to undergo facile 2' to-3' isomerization.

To overcome the issue of protecting group migration and to improve the coupling efficiency, the analogous 2'-*O*-levulinyl acetal ester (ALE) protecting group was developed, which can also be released with buffered hydrazine solutions [36,37]. The ribonucleoside phosphoramidites **18** were coupled with DCI as activator and yielded a coupling efficiency of >98% in a coupling time as short as 1 min. One additional motivation for the development of the ALE protecting group was the goal of allowing deprotection of RNA oligonucleotides that remain attached to a solid support such as a glass or chip surface [37].

### ***Acid-labile protecting groups***

A class of acid-labile acetal protecting groups that are compatible with the 5'-O-DMT group has also been reinvestigated recently. Early examples were the 2'-O-Fmp (11) and 2'-O-Cmp (12) protecting groups that showed great promise in the 1990s [38,39]. The Fmp-protected phosphoramidites 10 have been commercially available for some time, but there have been few reports of successful RNA synthesis using this method [40]. A next generation of acid-labile acetal protecting groups was proposed by Matysiak and Pfeleiderer in 2001 [41]. The protected benzylacetal derivatives 2'-O-Nebe (19) and 2'-O-Fnebe (20) are also compatible with the 5'-O-DMT group provided that the 4-nitrophenyloxycarbonyl group is present on the acetal moiety. Upon DBU treatment for nucleobase and phosphate deprotection, the 2'-O-acetal is converted into a much more acid-sensitive derivative that can subsequently be released under mildly acidic conditions. Nebe-protected phosphoramidites 19 have been applied for the synthesis of up to 37 nt long RNAs, but the coupling kinetics of these building blocks were very slow (a 20 min coupling time was required for optimal performance) [41].

In 2008, Beaucage and co-workers reported the 2'-O-4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxymethyl (4-DCA-MABOM) group for 2'-hydroxyl protection of ribonucleoside phosphoramidites 21, which is designed according to a similar strategy for convertible acetal protecting groups [42]. It was found that a 4-aminobenzyloxymethyl (4-ABOM) group resulting from reduction of a 4-NBOM derivative was sensitive to 0.1 M acetic acid at 90 °C [43]. Deprotection presumably proceeded through formation of an iminoquinone methide intermediate and elimination of formaldehyde. It was shown that the electronic and structural parameters of the benzyl acetal critically influence the acid sensitivity and that the presence of a single electron-donating methyl group on the aminobenzyl moiety is most favorable. Accordingly, phosphoramidites 21 with 2'-O-4-DCA-MABOM were prepared in which the methylaminobenzyl group was protected by dichloroacetylation. Activation of 21 was achieved with BTT for 3 min and an average stepwise coupling efficiency of 99% was reported for the synthesis of a 20 nt RNA oligonucleotide [42]. Release of the oligonucleotide from the support and removal of nucleobase and phosphate protecting groups were achieved by incubation with ammonia for 10–16 h at 55 °C. Under these conditions, the dichloroacetyl group of 4-DCA-MABOM was also cleaved. The residual 2'-O-4-MABOM group was removed in a TEMED-buffered acetic acid solution at pH 3.8 within 30 min at 90 °C. This final deprotection step is essentially identical with the release of the orthoester moiety in ACE chemistry. So far, the 4-DCA-MABOM-protected phosphoramidites have only been used for the synthesis of a model 20-mer oligoribonucleotide. It remains to be demonstrated if the combination of the advantageous features from 5'-O-DMT and acid-labile 2'-O-acetals will become a powerful alternative to the currently most widely used RNA synthesis strategies.

### ***The reducible 2-tert-butylthiomethyl (DTM) protecting group***

Ribonucleoside phosphoramidites 22 protected with the 2'-O-*tert*-butylthiomethyl (DTM) protecting group were developed by Kwiatkowski and co-workers in 2006 [44]. Upon activation of phosphoramidites 22 with ETT, coupling efficiencies of up to 99.8% were achieved within a coupling time of 2.5 min. The longest RNA synthesized with the 2'-O-DTM protecting group consisted of 45 nt. Release of the oligonucleotide and deprotection of nucleobase and phosphate groups proceeded under standard ammonolytic conditions. Cleavage of the DTM group was achieved with 1,4-dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) in a buffered pH 7.6 solution at 55 °C. The moderate stability of 2'-O-DTM-protected phosphoramidites in solution at room temperature (sufficiently stable within 12–24 h) currently seems to be a limitation of this otherwise attractive method.

Most of these newer approaches have not yet been evaluated extensively in the RNA research community and their suitability for widespread applications has yet to be demonstrated. The 2'-protecting groups that are orthogonal to the traditional TBDMS and TOM silyl protecting groups hold special promise to become useful for the preparation of partially protected RNAs that might have important features for siRNA delivery as they increase hydrophobicity and might facilitate cell uptake. In this context, it has been proposed that RNAs

modified with novel biolabile 2'-protecting groups should enable RNAi experiments in which the finally unprotected RNAs would be liberated only after administration inside the cells [45].

### 1.2.2 Synthetic strategies for RNA modification

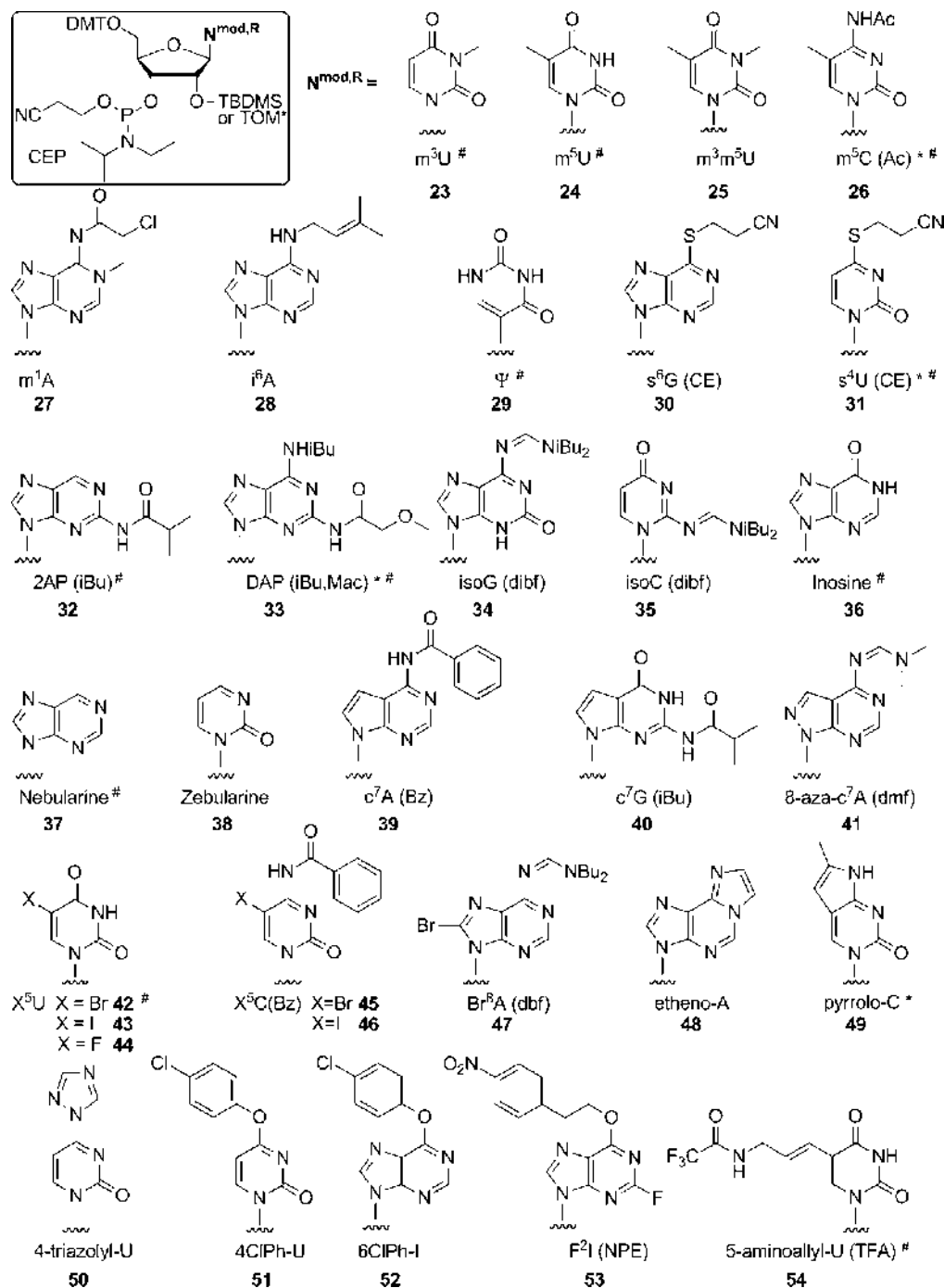
The site-specific incorporation of nucleoside modifications by RNA solid-phase synthesis generally follows one of two common strategies. The most widespread and versatile strategy is the direct incorporation of nucleotide analogs by replacing standard phosphoramidites with modified derivatives during solid-phase synthesis (see Section 1.2.2.1). Alternatively, the standard oxidation and/or capping solutions can be substituted by specialized reagents, which results in the synthesis of backbone-modified RNA (e.g. phosphorothioate or phosphoroselenoate RNA). The second common strategy for nucleoside functionalization involves post-synthetic modification, for which various strategies are feasible (see Section 1.2.2.2). Other important strategies for the synthesis of modified RNA involve combinations of chemical and enzymatic methods (see Section 1.2.2.3).

#### 1.2.2.1 Solid-phase synthesis of modified RNA via phosphoramidite chemistry

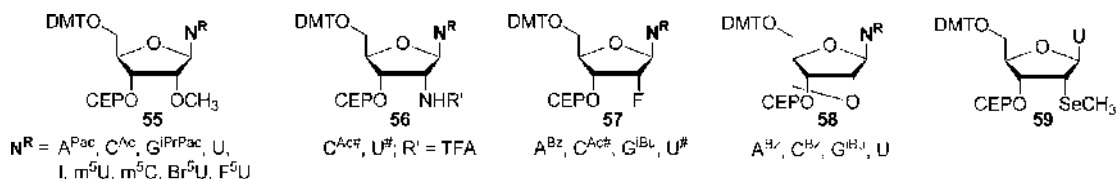
##### *Nucleobase and ribose modification via nucleoside analog phosphoramidites*

*Modified phosphoramidites for 5'-O-DMT-2'-O-silyl chemistry* (i) *Commercially available phosphoramidites* The foremost requirement for successful incorporation of nucleoside analogs via solid-phase phosphoramidite chemistry is that the desired modification is chemically compatible with all reactions and reagents used in the synthesis cycle and also remains unchanged under the deprotection conditions. Various modified phosphoramidites compatible with TBDMS and TOM chemistries are commercially available from different sources (e.g. GlenResearch, ChemGenes, Berry Associates). Figures 1.6 and 1.7 show most of the currently obtainable nucleobase- and ribose-modified RNA building blocks according to the 5'-O-DMT-2'-O-silyl protection scheme; the nucleoside analogs are shown with appropriate protecting groups. The following classes of nucleobase modifications are represented:

- Nucleosides containing natural RNA modifications, such as alkylated nucleobases [ $m^3U$  (**23**),  $m^5U$  (**24**),  $m^3m^5U$  (**25**),  $m^5C$  (**26**),  $m^1A$  (**27**),  $i^6A$  (**28**)], pseudouridine [ $\Psi$  (**29**)] and thio-substituted nucleobases [ $s^6G$  (**30**),  $s^4U$  (**31**)], are mainly used to mimic native systems closely and to study biochemical and biophysical properties of modified RNAs. Thio-substituted nucleosides are also used for photo-crosslinking studies or for derivatization via disulfide or thioether bonds.
- Nucleosides with altered patterns of exocyclic functional groups as compared with their natural counterparts [2AP (**32**), DAP (**33**), isoG (**34**), isoC (**35**)] and other derivatives entirely missing certain exocyclic functional groups [inosine (**36**), nebularine (**37**), zebularine (**38**)] are used to study the specific roles of nucleobase amino and carbonyl groups in RNA folding and catalysis.
- Nucleoside analogs with altered patterns of ring nitrogen atoms [ $c^7A$  (**39**),  $c^7G$  (**40**), 8-aza- $c^7A$  (**41**)] are primarily used for structural and mechanistic studies of RNAs.
- Halogenated nucleosides, such as  $Br^5U$  (**42**),  $Br^5C$  (**45**) and  $Br^8A$  (**47**), can be used for heavy atom isomorphous replacement studies in X-ray crystallography. 5-Halopyrimidine nucleosides are photoreactive and have also been used for RNA-protein crosslinking studies. The iodinated nucleosides  $I^5U$  (**43**) and  $I^5C$  (**46**) are useful for further derivatization by Pd-catalyzed cross-coupling reactions (see Section 1.2.2.2.2), whereas  $F^5U$  (**44**) is mainly used as a structural probe for studies on enzymes.
- Fluorescent nucleoside analogs such as etheno-A (**48**), pyrrolo-C (**49**) and 2AP (**32**) allow for monitoring of conformational changes during RNA folding.
- Convertible nucleosides, such as 4-triazolyluridine (**50**),  $O^4$ -(4-chlorophenyl)-U (**51**),  $O^6$ -(4-chlorophenyl)-I (**52**) and  $F^2I$  (**53**) and amino-tethered nucleosides such as 5-aminoallyl-U (**54**) are used to prefunctionalize RNA oligonucleotides for post-synthetic derivatization (see Section 1.2.2.2.1).



**Figure 1.6** Collection of commercially available modified phosphoramidites compatible with the 5'-O-DMT-2'-O-silyl protection scheme. The modified nucleobases are shown in their protected forms. The asterisk (\*) indicates commercial availability as 2'-O-TOM-protected phosphoramidite; # denotes availability via custom synthesis service using 2'-O-ACE chemistry. Mac = methoxyacetyl, dibf = diisobutylaminomethylene, TFA = trifluoroacetyl, ClPh = chlorophenyl



**Figure 1.7** Selection of commercially available 2'-ribose-modified phosphoramidites compatible with the 5'-O-DMT-2'-silyl protection scheme. The symbol # denotes availability via custom synthesis service using ACE chemistry. *iPrPac* = 4-isopropylphenoxyacetyl

The most important classes of commercially available 2'-modified RNA phosphoramidites:

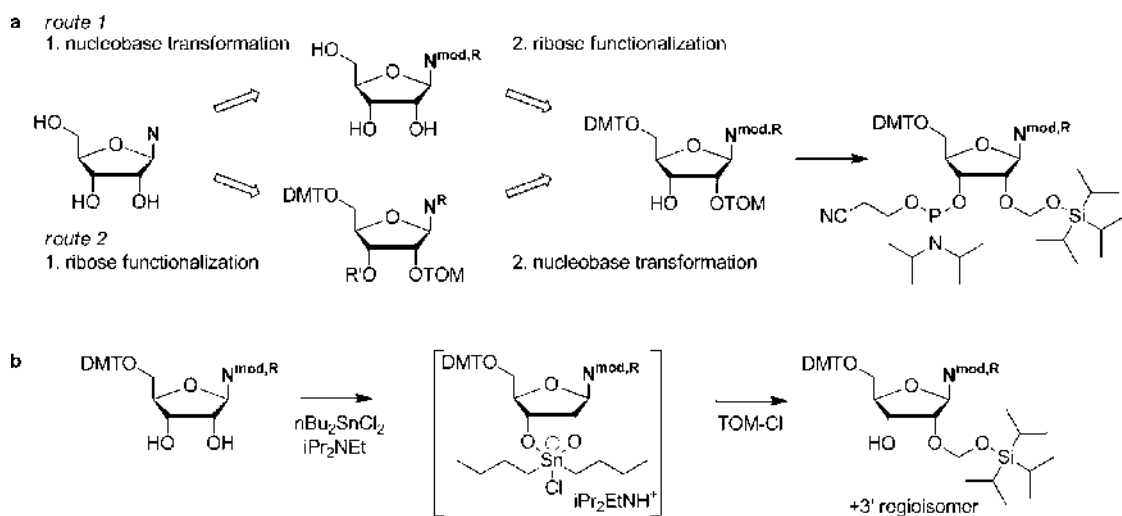
- 2'-O-Methyl modified nucleoside phosphoramidites **55**. 2'-OMe groups are common natural ribose modifications found in all classes of RNA. In addition to the four standard nucleosides A, C, G and U, various nucleobase-modified 2'-OMe-derivatized ribonucleoside analogs are available as phosphoramidite building blocks.
- 2'-Amino-2'-deoxy-modified nucleoside phosphoramidites **56**, of which only uridine and cytidine monomers are currently commercially available, but the analogous adenosine and guanosine phosphoramidites have been reported [46,47]. 2'-Amino-modified RNA was used in studies of the ribosomal peptidyl transferase reaction mechanism [48] and for thermodynamic analysis of ribozyme mechanisms [49]. The 2'-amino functionality can easily be derivatized with any biophysical label that can be supplied as activated carboxylic acid, isocyanate or isothiocyanate (see Section 1.2.2.2.4).
- 2'-Deoxy-2'-fluoro-modified nucleoside phosphoramidites **57**. 2'-Fluoro-modified oligonucleotides have been used as nuclease-resistant RNA analogs for antisense and RNAi applications [17], and as probes for  $^{19}\text{F}$  NMR in the determination of RNA conformational equilibria and ligand binding [50,51].
- LNA nucleosides **58**, in which the 2'-oxygen and the 4'-carbon atoms are linked with a methylene unit to lock the ribose in the C3'-endo conformation. This artificial ribose modification has been designed for improved base pairing behavior to complementary RNA targets as compared with unmodified DNA or RNA strands and it confers nuclease stability to the oligonucleotide [52].
- 2'-Methylseleno RNA phosphoramidites **59**, of which currently only 2'-SeMe-U is purchasable, but synthetic strategies for the nucleosides C, A and G are known [53,54].

In addition to the nucleobase- and ribose-modified nucleoside analogs depicted in Figures 1.6 and 1.7, several non-nucleoside phosphoramidites are commercially available and can be used to incorporate inter-nucleotide spacers (mono-, tri- or hexaethylene glycol units) or abasic site analogs into oligoribonucleotides. Moreover, phosphoramidites of fluorophores (e.g. fluorescein and its derivatives, tetramethylrhodamine, cyanine dyes), quenchers, biotin, acridine, psoralen and cholesterol are available for conjugation to the 5'- or 3'-termini of oligonucleotides. Most of these modifications are available as DMT-protected analogs, but several are also offered for combination with ACE chemistry.

(ii) *Chemical synthesis of modified nucleoside phosphoramidites* Although the number of commercially obtainable nucleoside analogs is constantly increasing, many modifications desired by researchers for specific experiments are not easily accessible and it remains the task of chemists to develop efficient synthetic routes for the preparation of suitable phosphoramidite building blocks.

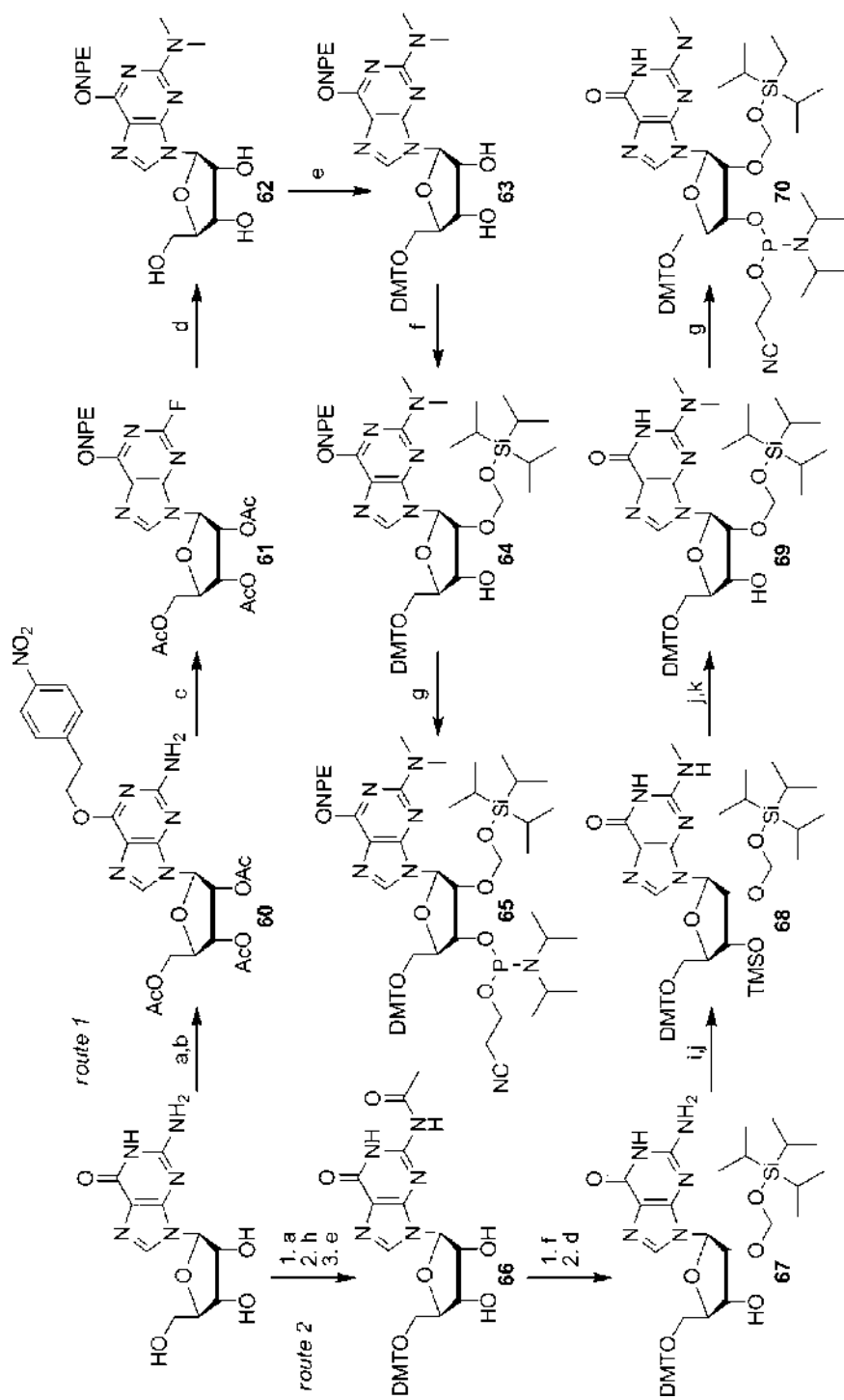
The synthesis of modified phosphoramidites usually involves installation of the desired nucleobase or ribose modification on a partially protected nucleoside, followed by protecting group manipulations that

finally result in functionalization of the modified nucleoside according to an orthogonal protecting group scheme for RNA solid-phase synthesis. Two general strategies for the synthesis of nucleobase-modified 2'-*O*-TOM-protected RNA phosphoramidites are depicted in Scheme 1.3a. These two routes differ in the order of nucleobase modification and introduction of the 2'-*O*-TOM group. Route 1 entails the transformation of the nucleobase early in the synthetic scheme, with preparation of the free (i.e. ribose-unprotected) nucleoside analog as intermediate. The ribose is then functionalized in at least three more steps. Alkylation of the vicinal 2',3'-diol, which is achieved via activation as dibutylstannylidene diacetal (Scheme 1.3b), yields a mixture of 2'-*O*- and 3'-*O*-alkylated isomers that need to be separated chromatographically (similar considerations apply to direct silylation with TBDMS-Cl or similar reagents, which also gives a mixture of 2'-*O*- and 3'-*O*-silylated isomers). In route 2, the 2'-*O*-TOM group is introduced before the nucleobase transformation is performed. This strategy generates only the desired 2'-*O*-alkylated isomers of precious modified compounds and is required for modifications that are not stable under the conditions for installation of the TOM protecting group. The majority of reported syntheses followed one of these two general routes. In principle, alternative synthetic approaches are conceivable and the exact sequence of synthesis steps is largely depending on the chemical stability of the targeted nucleobase modification.



**Scheme 1.3** (a) General routes for the synthesis of nucleobase-modified 2'-*O*-TOM-protected phosphoramidites. (b) Introduction of 2'-*O*-TOM protecting group via cyclic 2',3'-dibutylstannylidene diacetal

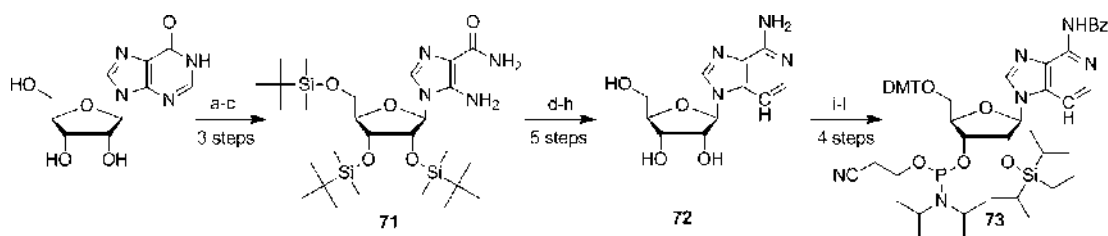
To illustrate the synthetic routes outlined in Scheme 1.3a, two reported syntheses of m<sup>2</sup><sub>2</sub>G building blocks are depicted in Scheme 1.4. The phosphoramidites **65** and **70** differ only in the presence or absence of the *O*<sup>6</sup>-NPE protecting group. In route 1 [24], the dimethylamino group is installed via nucleophilic aromatic substitution of the *O*<sup>6</sup>-NPE-protected 2-fluorinosine derivative **61**, which was prepared from guanosine in three steps. The resulting m<sup>2</sup><sub>2</sub>G nucleoside **62** was transformed into the phosphoramidite building block **65** by stepwise introduction of the 5'-*O*-DMT (**63**) and 2'-*O*-TOM protecting groups (**64**), followed by 3'-phosphitylation. In route 2 [25], the stepwise methylation of the 5'-*O*-DMT-2'-*O*-TOM-protected guanosine **67** was achieved by treatment with 1,3-benzodithiolium tetrafluoroborate and subsequent reduction of the intermediate with (Me<sub>3</sub>Si)<sub>3</sub>SiH-AIBN to give the monomethylated intermediate **68**. The second *N*<sup>2</sup>-methyl group on **69** was installed by repeating this reaction sequence. Again, 3'-phosphitylation as the



**Scheme 1.4** Two synthetic routes to 5'-O-DMT-2'-O-TOM-protected  $m^2G$  phosphoramidite building blocks that differ only in their nucleobase protecting groups. (a)  $Ac_2O$ , DMF-pyridine; (b) 4-nitrophenylethanol, diisopropyl azodicarboxylate,  $PPh_3$ , dioxane; (c)  $HBF_4$ ,  $NaNO_2$ , acetone- $H_2O$ ; (d)  $MeNH_2$ ,  $EtOH-H_2O$ ; (e)  $DMT-Cl$ , pyridine; (f) 1,  $Bu_2SnCl_2$ ,  $iPr_2NEt$ ; 2,  $TOM-Cl$ ,  $(CH_2Cl)_2$ ; (g)  $CEP-Cl$ ,  $Me_2NEt$ ,  $CH_2Cl_2$ ; (h)  $NaOH-MeOH-THF$ ; (i)  $Me_3SiCl$ , in pyridine; (j) 1, 1,3-benzodithiolium tetrafluoroborate; 2,  $(Me_3Si)_3SiH-AIBN$ , in benzene; (k)  $NH_3$ , in  $MeOH-THF$

last synthetic step yielded the phosphoramidite **70** for solid-phase RNA synthesis. Similarly, both routes have been implemented for the synthesis of a collection of nucleobase-methylated ribonucleoside phosphoramidites including m<sup>1</sup>G, m<sup>2</sup>G, m<sup>1</sup>I, m<sup>4</sup>C, m<sup>6</sup>A and m<sup>6</sup><sub>2</sub>A [24,25]. These methylated nucleosides have been used to study the influence of nucleobase methylations on equilibria of RNA secondary structures [55–57].

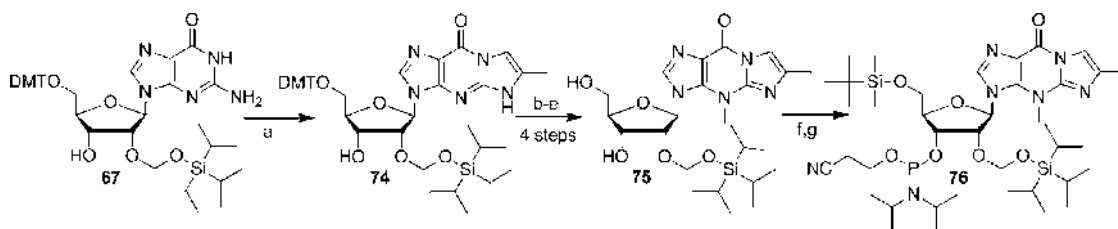
The general routes 1 and 2 in Scheme 1.3a are also applicable to slightly deviating hydroxyl group protection schemes. For example, the synthesis of 3-deazaadenosine (c<sup>3</sup>A) phosphoramidite **73** (Scheme 1.5) followed general route 1, although the fluoride-labile triisopropylsilyl (TIPS) protecting group was used instead of TOM or TBDMS (TIPS favorably gave higher 2'-regioselectivity in the silylation step). Construction of the modified nucleoside phosphoramidite **73** followed a 12-step synthetic route starting from inosine, via the crucial 5-amino-4-imidazolecarboxamide (AICA) ribonucleoside intermediate **71** [58]. The modified ribonucleoside 3-deazaadenosine **72** was obtained as free nucleoside intermediate and was further derivatized to the corresponding phosphoramidite **73** via benzylation, tritylation, silylation and phosphitylation. The c<sup>3</sup>A phosphoramidite **73** was incorporated into a 26 nt fragment of the 23S rRNA and was used for site-specific modification of the peptidyl transferase center in the large ribosomal subunit for mechanistic studies of ribosome-catalyzed peptide bond formation [58].



**Scheme 1.5** Synthesis of 3-deazaadenosine phosphoramidite **73**. (a) TBDMS-Cl, imidazole, DMF; (b) 2,4-dinitrochlorobenzene, K<sub>2</sub>CO<sub>3</sub>, DMF; (c) ethylenediamine; (d) p-toluenesulfonyl chloride, pyridine; (e) isoamyl nitrite, CH<sub>2</sub>I<sub>2</sub>; (f) ethynyltrimethylsilane, NEt<sub>3</sub>, (PhCN)<sub>2</sub>PdCl<sub>2</sub>, CH<sub>3</sub>CN; (g) NH<sub>3</sub>, MeOH; (h) Amberlite IRA 900 fluoride form, toluene; (i) 1, Me<sub>3</sub>SiCl, pyridine; 2, benzoyl chloride, pyridine; (j) DMT-Cl, pyridine; (k) TIPS-Cl, AgNO<sub>3</sub>, pyridine–THF; (l) CEP-Cl, Me<sub>2</sub>NEt, CH<sub>2</sub>Cl<sub>2</sub>

The compatibility of the nucleoside modification with the synthesis and deprotection conditions is of vital importance for successful use in solid-phase synthesis. In this regard, the hypermodified nucleoside analogs of the wyosine family, which are often found in the anticodon loop of tRNAs, are very challenging candidates for chemical synthesis. Wyosine is known to be particularly sensitive to acidic conditions and therefore it had not been successfully incorporated into synthetic RNA oligonucleotides until recently. With the exception of 4-demethylwyosine [59], no other syntheses of wyosine analog phosphoramidite building blocks have been reported [60]. In 2008, Porcher developed a synthetic method that allows for coupling of wyosine phosphoramidite **76** at the 5'-terminus of an RNA oligonucleotide [60]. A synthetic approach related to general route 2 of Scheme 1.3a was employed for the preparation of the wyosine phosphoramidite building block **76** (Scheme 1.6), because the wyosine nucleoside was not stable under conditions for introduction of the TOM group. The 5'-O-DMT-2'-O-TOM-protected guanosine **67** was transformed into the 4-demethylwyosine intermediate **74** via alkylation of N1 with bromoacetone in the presence of potassium iodide and subsequent cyclization in the presence of molecular sieves. Detritylation and ribose acetylation afforded a diacetylated intermediate that was methylated at N5 with CH<sub>2</sub>I<sub>2</sub>–Et<sub>2</sub>Zn. After deacetylation, the 5'-OH of the new wyosine building block **75** was protected with the TBDMS group

and subsequent phosphitylation produced the 2',5'-silyl-protected phosphoramidite **76**. Wyosine was incorporated at the 5'-end of a 10 nt RNA via solid-phase synthesis under optimized oxidation and deprotection conditions. The short modified RNA oligonucleotide was then enzymatically phosphorylated and used in a ligation reaction to generate 18 nt long RNA hairpins for studying the kissing-loop interaction of a fragment of the retroviral RNA of Moloney murine leukemia virus [60].



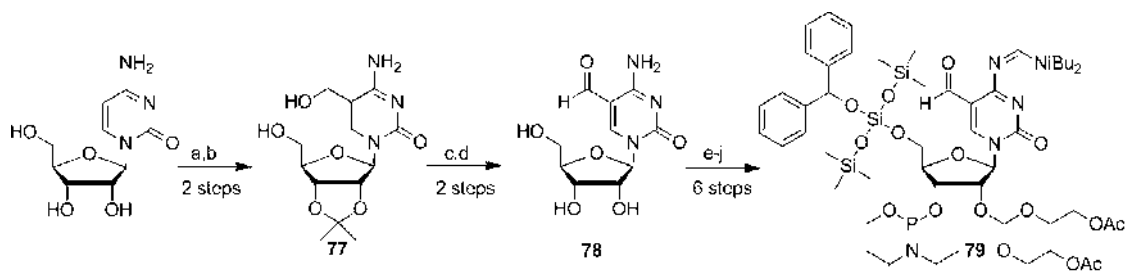
**Scheme 1.6** Synthesis of wyosine phosphoramidite **76**. (a) 1,  $K_2CO_3$ , DMF; KI, then bromoacetone; 2, molecular sieves,  $CH_2Cl_2$ ; (b) dichloroacetic acid,  $CH_2Cl_2$ ; (c) 1,  $Ac_2O$ , pyridine; 2, pyridine, MeOH,  $H_2O$ ; (d)  $CH_2I_2$ ,  $Et_2Zn$ ,  $Et_2O$ ; (e)  $NH_3$ , MeOH; (f) TBDMS-Cl, imidazole, DMF- $CH_2Cl_2$ ; (g) CEP-Cl,  $iPr_2NEt$ ,  $CH_2Cl_2$

Examples of ribose-modified nucleosides not commercially available include the 2'-O-2-aminoethyl- and 2'-O-2-mercaptoethyl-modified nucleosides that are appropriately protected to be compatible with the 5'-O-DMT-2'-O-silyl chemistry (also known as 2'-tethered amine and 2'-tethered thiol, respectively). Several synthetic routes have been described for the 2'-alkylation of various nucleosides [61–63]. Silverman and co-workers reported a complete collection of 2'-tethered thiol and 2'-tethered amine building blocks for each of the four common ribonucleotides A, C, G and U [64]. The most recent approach for the synthesis of 2'-O-aminoethyl nucleosides involves direct alkylation of the 2'-OH with phthalimidoethyl triflate [65]. RNA analogs with 2'-tethered amino substituents are useful substrates for post-synthetic labeling [66] (see Section 1.2.2.2, *Derivatization of artificial amino groups at ribose and nucleobase residues*) and have also been studied for RNAi applications [67]. A variety of other RNAs derivatized with lipophilic and zwitterionic 2'-alkylations, such as guanidinoethyl or aminopropyl modifications, have been analyzed for their properties as antisense or siRNA agents. The improved chemical stability and higher affinity for RNA targets and also the ability partially to neutralize the negatively charged phosphate backbone furnish the modified RNAs with better physicochemical and pharmacokinetic properties for medical applications [17,18,68].

In a different area of RNA research, a diversity of 2'-modified nucleotide analogs allowed the construction of an atomic mutation cycle to determine whether 2'-hydroxyl groups donate functionally important hydrogen bonds in the catalytic mechanisms of RNA enzymes and splicing machineries [49,69,70]. In a similar context, 2'-C- $\alpha$ -hydroxyalkyl-modified cytidine analogs have recently been used to probe RNA-solvent interactions in the catalytic core of the group II intron during the spliced exon reopening (SER) reaction [71,72]. Also, phosphorothiolate linkages in RNA oligonucleotides, in which a sulfur atom replaced the 3'- or 5'-bridging oxygen atom of the phosphodiester linkage, have yielded fundamental insights into RNA functions and revealed the participation of metal ions and hydrogen bonds in catalytic mechanisms [73–75]. A recent addition to the pool of highly functionalized probes to uncover complex interaction networks within ribozyme active sites is the combination of 3'-phosphorothiolate and 2'-ribose modifications. This was illustrated by the synthesis of a 2'-O-methyl-3'-thioguanosine phosphoramidite and its incorporation into an oligoribonucleotide that served as a substrate for investigations of group I intron catalysis [76].

**Modified phosphoramidites for 5'-O-silyl-2'-O-ACE chemistry** The commercial 2'-O-ACE RNA synthesis service by Dharmacon (part of Thermo Fisher Scientific) now also offers certain RNA modifications to be incorporated into custom oligoribonucleotides, but the great variety of accessible analogs known from 2'-O-silyl chemistry has not yet been achieved with the 2'-O-ACE method (the modifications currently offered are marked with # in Figures 1.6 and 1.7). There have also been some recent reports on the synthesis of new nucleobase- and ribose-modified ACE-phosphoramidites and their incorporation into RNA.

Lusic *et al.* synthesized a 5-formylcytidine ( $f^5C$ ) 5'-O-silyl-2'-O-ACE-protected building block (**79**, Scheme 1.7) that allowed the incorporation of  $f^5C$  into an RNA oligonucleotide for the first time [77]. The  $f^5C$  modification is found at the wobble position of human mitochondrial methionine-specific tRNA, but its function during decoding, chain initiation or chain elongation is still unknown. The recent synthesis of an  $f^5C$ -modified tRNA anticodon domain now allows the analysis of its thermodynamic properties and allows structural investigations of the modified anticodon stem-loop. The key step in the synthesis of phosphoramidite **79** was the installation of the hydroxymethylene unit into a protected cytidine nucleoside via a Baylis–Hillman-type reaction with formaldehyde. Selective oxidation of the allylic alcohol in **77** and acetone deprotection gave the free  $f^5C$  nucleoside **78**, which was appropriately functionalized to the 2'-O-ACE-protected phosphoramidite **79** [77].



**Scheme 1.7** Synthesis of 5-formylcytidine phosphoramidite **79**. (a) Dimethoxypropane, acetone,  $\text{HClO}_4$  (cat.); (b) paraformaldehyde, KOH; (c)  $\text{RuO}_2 \cdot \text{H}_2\text{O}$ , dioxane; (d) 1 M HCl; (e) 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane, pyridine–DMF; (f)  $i\text{Bu}_2\text{NCH(OMe)}_2$ , DMF; (g) tris(2-acetoxyethoxy)orthoformate, pyridinium *p*-toluenesulfonate, TBDMS-pentadione,  $\text{CH}_2\text{Cl}_2$ ; (h) HF–TEMED,  $\text{CH}_3\text{CN}$ ; (i)  $\text{BzH-Cl}$ ,  $i\text{Pr}_2\text{NH}$ ,  $\text{CH}_2\text{Cl}_2$ ; (j)  $(\text{MeO})\text{P}(i\text{Pr}_2\text{N})_2$ ,  $i\text{Pr}_2\text{NH}$ , ETT,  $\text{CH}_2\text{Cl}_2$

Micura and co-workers described the preparation of all four (A, C, G, U) 5'-silyl protected 2'-methylselenoribonucleoside phosphoramidites and successfully incorporated them into RNA oligonucleotides [54]. Expanding seleno-RNA synthesis to be compatible with ACE chemistry was an important contribution to making selenium-modified RNA generally accessible.

Other examples for the recent syntheses of modified ACE phosphoramidites include 2-iodoadenosine and 5-iodocytidine analogs; their application for post-synthetic RNA modification is discussed in Section 1.2.2.2, *Palladium-catalyzed cross-coupling of halogenated nucleobases* [78]. The increasing demand for modified RNAs and the growing popularity of ACE chemistry will likely result in a broader spectrum of ACE-containing modified RNA building blocks in the future.

### Backbone modification via alternative oxidation reagents

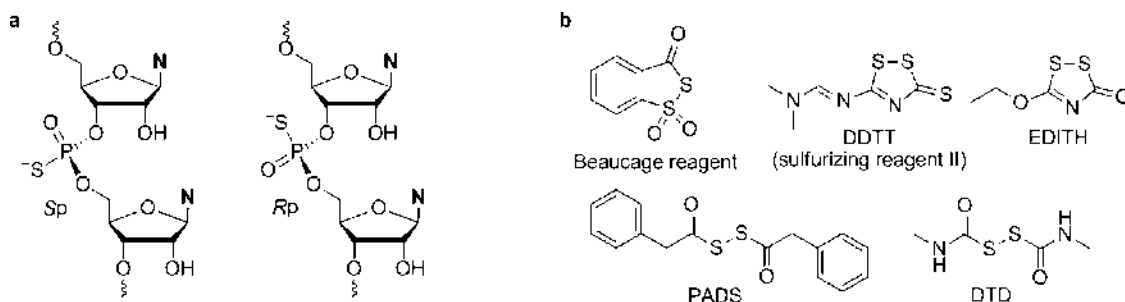
Solid-phase phosphoramidite chemistry can also be used to introduce backbone modifications into RNA oligonucleotides. The most important example is represented by the phosphorothioate group, in which a non-bridging oxygen of the phosphodiester internucleotide linkage is replaced by a sulfur atom. The single-atom

exchange can be classified as a conservative modification, because sulfur is situated in group VI of the periodic table right below oxygen. The van der Waals radius of sulfur is 0.3 Å larger than that of oxygen (1.5 vs 1.8 Å) and the P–S versus P–O bond length is increased by 0.5 Å (1.5 vs 2.0 Å). Phosphorothioates possess a number of chemical properties that clearly distinguish them from natural phosphodiester linkages, such as facile cleavage by iodine, their nucleophilicity and the preferred coordination of soft metal ions according to the HSAB (hard and soft acids and bases) principle. An important feature of the phosphorothioate modification is the chirality of the phosphorus center and the resulting existence of two diastereomers, denoted *Rp* and *Sp* (Figure 1.8a), which – in the case of short oligonucleotides – can be separated by HPLC. The availability of distinguishable isomers makes phosphorothioates ideally suited to probe enzymatic reaction mechanisms and offers the opportunity via metal ion rescue experiments (with soft metal ions such as  $\text{Mn}^{2+}$  or  $\text{Cd}^{2+}$ ) to determine whether a particular phosphate is involved in metal ion interactions.

Phosphorothioate-modified oligonucleotides are the best known representatives of the first generation of antisense oligonucleotides. These analogs have been intensively investigated due to their increased nuclease resistance and attractive pharmacokinetic properties [79]. Reported shortcomings include off-target effects and observed cellular toxicity [80,81].

Interestingly, the phosphorothioate modification was the first observed naturally occurring DNA backbone modification. In 2007, phosphorothioate linkages were discovered in bacterial DNA from *Streptomyces lividans* and related bacteria [82]. This novel aspect of natural nucleic acid modification will stimulate additional investigations to understand why Nature has chosen phosphorothioates and by which biosynthetic pathways they are installed. Synthetic phosphorothioate oligonucleotides will play a significant role in such experiments.

The key step in the solid-phase synthesis of phosphorothioate oligoribonucleotides by the 2'-*O*-silyl or 2'-*O*-ACE chemistry is the replacement of the standard (iodine or *tert*-butyl hydroperoxide) oxidation solution by a sulfurizing reagent. Elemental sulfur was one of the first reagents used by Burgers and Eckstein [83]. Since then, a number of alternative sulfurizing reagents have been described. Among others, the Beaucage reagent, 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Figure 1.8b), is one of the best known backbone-modifying reagents [84]. Despite its limited stability in solution and suboptimal kinetics for the sulfurization of RNA, it has been widely used for the synthesis of phosphorothioate oligonucleotides. Other examples include 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) and [3-(dimethylaminomethylidene)amino]-3*H*-1,2,4-dithiazole-5-thione (DDTT), which feature improved performance for RNA sulfurization and extended shelf-life but are rather expensive [85]. More recently, cheaper reagents such as phenylacetyl disulfide (PADS) [86] and dimethylthiuram disulfide (DTD) [87] have been used successfully in large-scale syntheses of RNA phosphorothioates.

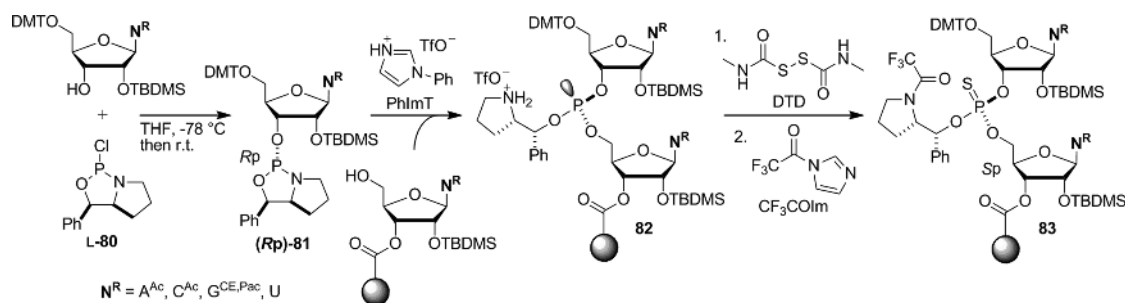


**Figure 1.8** (a) Phosphorothioate RNA diastereomers. (b) Selected sulfurizing reagents used for oligonucleotide phosphorothioate synthesis

The stereodefined chemical synthesis of phosphorothioate oligonucleotides has been a longstanding goal, because the stereorandom synthesis of oligonucleotides containing  $n$  phosphorothioate modifications provides a mixture of  $2^n$  diastereomers, each of which could interact differently with other biomolecules. At least two strategies have been described for the stereoselective synthesis of phosphorothioate DNA analogs. The method developed by Guga and Stec is based on 3'-O-(2-thio-1,3,2-oxathiophospholane) monomers that are condensed under strongly basic conditions [88]. This strategy is incompatible with standard coupling of phosphoramidite-based monomers, but stereodefined dinucleoside phosphoramidite building blocks with nitrobenzyl-protected sulfur atoms at the phosphorothioate junction were subsequently reported [89]. To construct any desired dinucleotide junction in either the *Sp* or *Rp* configuration, 32 different dinucleotide building blocks would be required, of which four have been reported so far [89]. Moreover, the necessary separation of the oxathiophospholane diastereomers is tedious and time consuming. Nevertheless, it seems possible that such dinucleoside building blocks could also be generated for the synthesis of stereodefined RNA phosphorothioates, but at present it is unclear whether such significant efforts would be worthwhile.

An alternative, phosphoramidite-based method has been investigated by the Agrawal [90,91] and Wada groups [92,93] for several years. The diastereoselective synthesis of proline-derived bicyclic 1,3,2-oxazaphospholidines as synthons for stereoselective synthesis of phosphorothioate oligonucleotides has been reported and conditions for activation and coupling have been carefully optimized [93]. The choice of activator reagent is critical because oxazaphospholidines are prone to epimerization in the presence of acidic activators. (*N*-Cyanomethyl)pyrrolidinium triflate (CMPT) was described as an optimal activator with low nucleophilicity; up to 12 nt long stereodefined phosphorothioate DNA oligonucleotides were obtained with excellent diastereoselectivities [93].

Wada's group has recently reported the successful stereodefined synthesis of phosphorothioate RNA oligonucleotides [94]. Their strategy is based on diastereomerically pure, 2'-*O*-TBDMS-protected, configurationally stable, bicyclic 1,3,2-oxazaphospholidine derivatives (*Rp*)- or (*Sp*)-**81** that were diastereoselectively prepared from 2-chloro-1,3,2-oxazaphospholidines L- or D-**80** (derived from L- and D-proline, respectively; Scheme 1.8). The best coupling performance of phosphoramidites **81** was observed with *N*-phenylimidazolium triflate (PhImT) as activating reagent; 99% coupling efficiency was achieved in a 15 min coupling time for formation of (*Sp*)-phosphorothioate linkages and 97% for (*Rp*)-phosphorothioate linkages. Sulfurization of the stereodefined phosphite triester **82** was performed with DTD. Acylation of the secondary amino group of the chiral auxiliary to afford the fully protected phosphorothioate **83** and capping



**Scheme 1.8** Synthesis of stereodefined phosphorothioate RNA **83**. Activation of 3'-O-oxazaphospholidine monomers **81** with *N*-phenylimidazolium triflate (PhImT) and coupling to the 5'-OH of a solid-phase attached (oligo)nucleotide gives phosphite intermediate **82**, which is followed by sulfurization with *N,N'*-dimethylthiuram disulfide (DTD) and capping with trifluoroacetylimidazole ( $CF_3COIm$ )

of unreacted 5'-OH groups was achieved with trifluoroacetylimidazole. After completion of chain assembly, the stereodefined phosphorothioate RNA was cleaved from the solid support and deprotected with  $\text{NH}_3\text{-EtOH}$  and the TBDMS groups were removed with TBAF. The largest all-*Rp* and all-*Sp* phosphorothioate RNA oligonucleotides reported so far are only 10 nt long. Nevertheless, this recent report of successful diastereoselective synthesis of RNA phosphorothioate linkages is encouraging for future developments in the field and for syntheses of longer stereodefined RNA oligonucleotides with mixed sequences.

It should be noted that stereodefined phosphorothioates can also be prepared by *in vitro* transcription. T7 RNA polymerase accepts the *Sp* diastereomers of nucleoside 5'-O- $\alpha$ -thiotriphosphates as substrates. The polymerization reaction leads to inversion of configuration at the  $\alpha$ -P and produces solely *Rp* phosphorothioates. However, enzymatic synthesis usually does not allow for site-specific incorporation of individual phosphorothioate modifications.

Other backbone modifications incorporated by solid-phase synthesis include phosphoroselenoates, for which similar principles apply as for phosphorothioates. In this case, the selenium modification is introduced by oxidation with  $\text{KSeCN}$ . Boranophosphates and methylphosphonates should also be mentioned as important backbone modifications. These derivatives have mainly been applied as antisense oligonucleotides [17], and will not be discussed in further detail in this chapter.

### 1.2.2.2 *Post-synthetic RNA modification strategies*

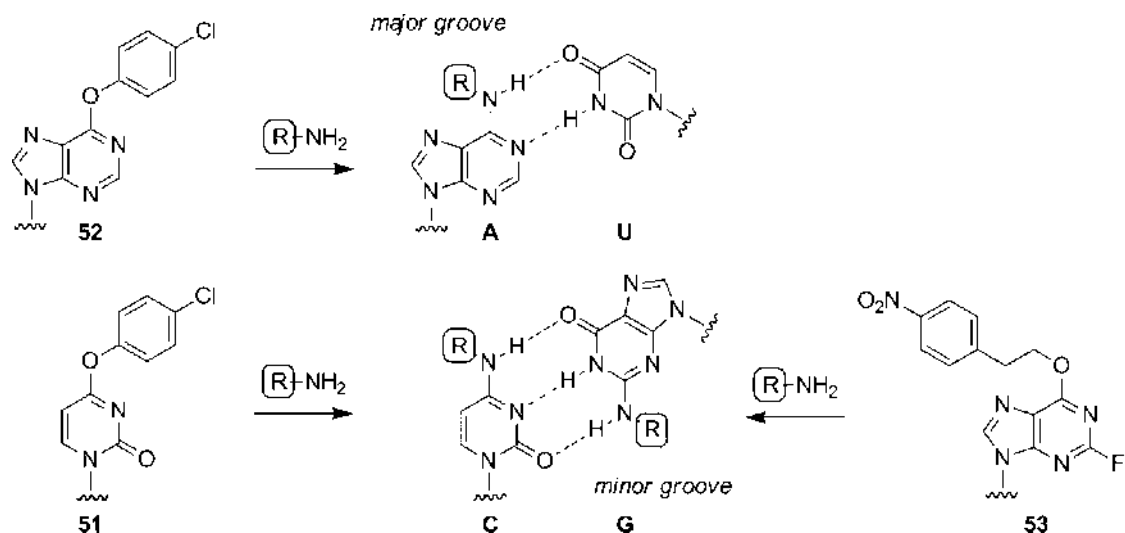
The post-synthetic modification of RNA oligonucleotides relies on the introduction of nucleoside analogs containing reactive functionalities by solid-phase synthesis and permits the site-specific attachment of a variety of reporter groups and chemical devices. Useful types of nucleoside derivatization reactions include nucleophilic aromatic substitution of appropriate leaving groups on nucleobases, palladium-catalyzed cross-coupling reactions, formation of thioether or disulfide bonds and functionalization of amino groups via formation of amide bonds or ureido groups.

#### *Nucleophilic aromatic substitution of convertible nucleosides*

A convertible nucleobase contains an appropriate leaving group that can be displaced by a nucleophile, which in turn becomes attached to that nucleobase. The most prominent examples of convertible ribonucleoside phosphoramidites, introduced by Verdine and co-workers in 1997 [95], are currently commercially available as 2'-*O*-TBDMS protected building blocks (see Figure 1.6, compounds **51–53**). Displacement of the 4-chlorophenyl leaving group with a nucleophilic primary alkylamine at uridine and inosine nucleosides leads to  $N^4$ -modified cytidine and  $N^6$ -modified adenosine analogs, respectively. Labeling of guanines at position N2 is achieved by fluoride displacement in *O*<sup>6</sup>-protected 2-fluoroinosine derivatives. These convertible nucleosides allow for attachment of novel functionalities or biophysical probes at either the major (A and C) or minor (G) groove of A-form RNA double helices while maintaining Watson–Crick base pairing capabilities of modified nucleosides (Scheme 1.9). The most prominent examples of modifications installed by this approach include disulfide crosslinking reagents, photo-crosslinking reagents and <sup>15</sup>N isotope labels. Correct incorporation of the modification needs to be verified by careful analysis of the isolated product by LC–ESI-MS or MALDI-MS. For new modifications, validation of incorporation by enzymatic digestion and base composition analysis by reversed-phase HPLC is recommended.

#### *Palladium-catalyzed cross-coupling of halogenated nucleobases*

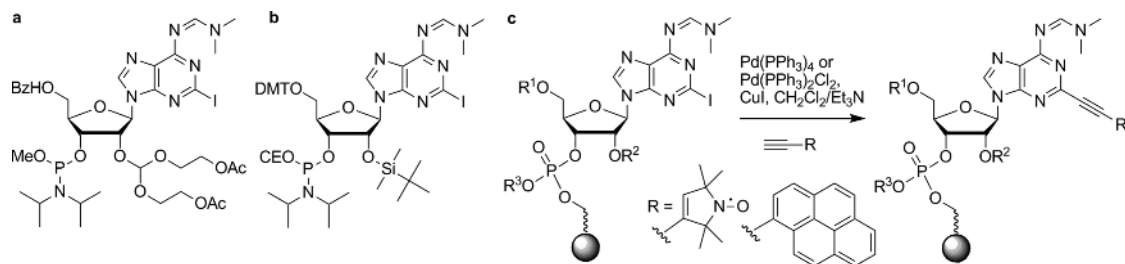
Functionalization of the nucleobase with a biophysical reporter group can also be achieved by a carbon–carbon bond-forming reaction. The prime example is the palladium-catalyzed Sonogashira cross-coupling reaction for the derivatization of halogenated nucleosides with terminal alkynes. This strategy has been employed extensively for the derivatization of DNA oligonucleotides with ethynylpyrene [96–98].



**Scheme 1.9** The convertible nucleoside approach allows installation of nucleobase modifications at the major or minor groove side in A, C and G nucleosides while maintaining Watson–Crick base pairing capability

RNA oligonucleotides have been derivatized by the Sonogashira reaction with pyrene fluorophores and with nitroxide spin labels [78,99]. The halogenated pyrimidine or purine nucleosides are incorporated via 2'-O-TBDMS- or 2'-O-ACE-protected phosphoramidites (see Figure 1.6 and Scheme 1.10) [78,99,100]. The palladium-catalyzed derivatization reaction is usually performed while the oligonucleotide is still fully protected and attached to the solid support (Scheme 1.10c). The solid-phase synthesis is interrupted after coupling of the halogenated nucleoside phosphoramidite and the column is removed from the synthesizer. The reagent mixture for the Sonogashira coupling containing the ethynyl reagent, the palladium catalyst [e.g.  $(\text{PPh}_3)_4\text{Pd}(0)$  or  $(\text{PPh}_3)_2\text{Pd}(\text{II})\text{Cl}_2$ ], CuI and triethylamine in dichloromethane is injected into the synthesis column. After coupling (usually 2–3 h), the solid support is washed and dried under vacuum, followed by continued chain elongation on the synthesizer to obtain the desired full-length oligonucleotide.

Other palladium-catalyzed cross-coupling reactions such as the Suzuki–Miyaura coupling of boronic acids and the Stille coupling of unsaturated stannanes have been used to derivatize solid-phase-bound



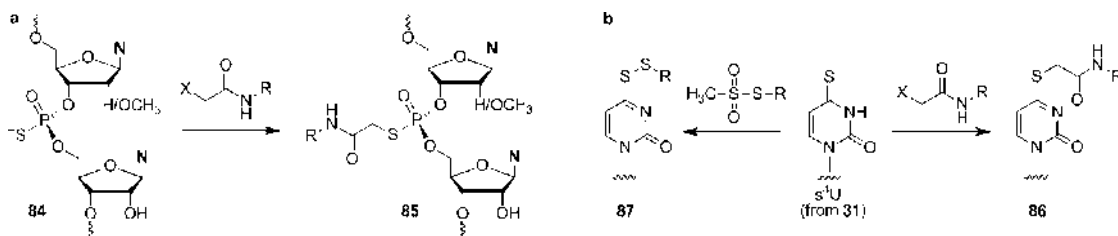
**Scheme 1.10** 2-Iodoadenosine phosphoramidite building blocks (a) for 2'-O-ACE chemistry and (b) for 2'-O-TBDMS chemistry. (c) Pd-catalyzed Sonogashira cross-coupling reaction of terminal alkynes to 2-iodoadenosine on the solid support. Prominent examples of successfully coupled alkynes include alkynylpyrene derivatives and nitroxide radical-containing spin labels

2'-deoxy-5-iodouridine nucleotides [101,102]. The major difficulties associated with these palladium-catalyzed techniques arise from the strict requirements for carefully controlled coupling conditions in dry and deoxygenated solutions under an argon atmosphere. The reaction temperatures and times (often 80–100 °C for up to 20 h) are not easily compatible with solid-phase RNA synthesis. Nevertheless, the recent success with Sonogashira coupling reactions in modifying RNA and the proof-of-concept work for Suzuki–Miyaura and Stille coupling to polystyrene-bound mononucleotides are promising examples for the development of new C–C bond-forming methods for on-column RNA derivatization.

### Derivatization of sulfur-containing RNA

Post-synthetic modification of RNA oligonucleotides can be achieved by derivatization of sulfurylated RNA residues that have been incorporated during solid-phase synthesis. In contrast to the convertible nucleoside approach and to Pd-catalyzed cross-coupling reactions, derivatization reactions of thio- and amino-modified RNA are performed in solution after complete deprotection of the oligonucleotide.

One example of post-synthetic RNA backbone modification applies the reaction of phosphorothioate residues **84** with  $\alpha$ -haloacetyl compounds (Scheme 1.11a) [103]. The nucleophilic displacement reaction leads to the formation of a phosphotriester species **85**, which is highly unstable in the presence of an adjacent 2'-hydroxyl group in standard RNA. Phosphotriesters are prone to hydrolysis in a reaction analogous to that used for phosphorothioate RNA sequencing [104] and nucleotide analog interference mapping (NAIM) [105]. It is therefore necessary to introduce a 2'-deoxyribonucleotide or a 2'-*O*-methyl derivative directly 5' to the phosphorothioate modification. Examples of phosphorothioate labeling include derivatization of RNA with photo-crosslinking reagents and with nitroxide spin labels [103,106,107]. One of the reported complications is non-specific modification of RNA with highly reactive  $\alpha$ -haloacetyl reagents, particularly at exocyclic amino groups of adenine and cytosine [103]. Increased selectivity for phosphorothioates is achieved by a low concentration of the derivatization reagent, low temperature and short reaction time.



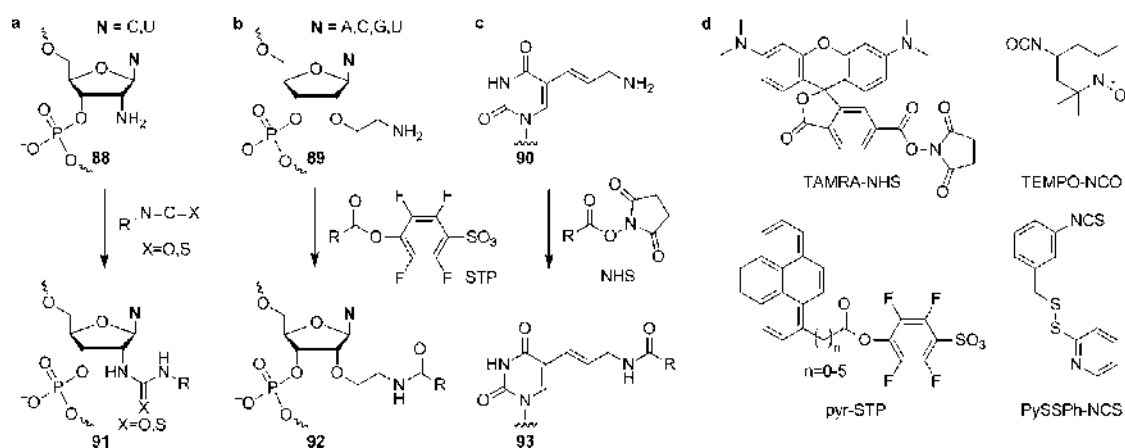
**Scheme 1.11** (a) Phosphorothioate modification with an  $\alpha$ -haloacetamide reagent leads to formation of a phosphotriester (**85**). (b) 4-Thiouridine modification with an  $\alpha$ -haloacetyl compound gives a lactim thioester linkages (**86**) and reaction with a methanethiosulfonate reagent gives a disulfide bond (**87**). X = Cl, Br, I; R = biophysical or chemical label or reporter group, such as a fluorophore, ion complexation reagent, photo-crosslinking reagent or spin label

Post-synthetic modification can also be applied to thio-modified nucleobases, in particular to 4-thiouridine, which is introduced by solid-phase synthesis with phosphoramidite **31** (see Figure 1.6). The reaction with  $\alpha$ -haloacetyl compounds leads to the formation of a lactim thioester linkage as in **86** (often called thioether for simplicity) [108]. A biophysical label or a chemical probe can alternatively be attached to the s<sup>4</sup>U nucleobase via a disulfide linkage (**87**, Scheme 1.11b). In this case, the label or probe is applied as a methanethiosulfonate reagent [109].

The formation of disulfide crosslinks between two site-specifically attached thiol moieties on nucleosides also represents a useful approach for studying the structure and function of nucleic acids. Sulfhydryl groups have been directly attached to the N3 position of pyrimidine bases and were used for intrahelical disulfide crosslinking in tRNA [110]. Disulfide crosslinks involving the ribose moiety have been installed at the 2'-position via ribonucleosides containing 2'-*O*-(2-thioethyl) substitutions. Inter- and intra-helical disulfide crosslinks in tRNA were introduced by air oxidation of site-specifically incorporated 2'-thioalkyl groups on pyrimidine nucleosides [110]. All four common RNA nucleotides A, C, G and U were more recently synthesized as 2'-thioethyl-modified ribonucleoside phosphoramidites [64]. 2'-Thioethyl-modified RNA was used for the reversible connection of 5'-thiolated DNA oligonucleotides on to RNA in a disulfide exchange reaction [111]. In this manner, DNA duplexes were attached to specific 2'-positions of the P4-P6 domain of the *Tetrahymena* group I intron RNA and were used as conformational constraints to control RNA folding. The reversibility of disulfide crosslinking by reduction with DTT (1,4-dithiothreitol) was utilized as one of several methods to modulate the structural effects of DNA constraints.

### Derivatization of artificial amino groups at ribose and nucleobase residues

Amino-modified nucleotides provide a very important class of functional groups for post-synthetic labeling of RNA. Similarly to labeling and crosslinking reactions of sulfur residues, the derivatization of amino groups is usually performed in solution after deprotection of the synthetic RNA. Three major types of amino-modified RNA nucleotides include 2'-amino-modified (**88**) and 2'-aminoethyl-modified (**89**) ribonucleotides and also 5-(aminoallyl)pyrimidine nucleotides (**90**) (Scheme 1.12), all of which are incorporated into RNA oligonucleotides via the corresponding 3'-phosphoramidite building blocks.



**Scheme 1.12** (a) Derivatization of 2'-amino-modified RNA **88** with isocyanate or isothiocyanate reagents to form urea or thiourea linkages as in **91**. (b) Reaction of 2'-aminoethyl-modified RNA **89** with STP esters. (c) Amide bond formation of 5-(aminoallyl)uridine-modified RNA **90** with NHS esters. (d) A small collection of common amino-modifying reagents, showing one example per class of NHS ester (TAMRA-NHS), STP ester (pyrene-STP esters with variable linker length), TEMPO-isocyanate for spin labeling and aromatic isothiocyanate for disulfide installation. NHS = N-hydroxysuccinimide, STP = 4-sulfonyltetrafluorophenyl, TAMRA = tetramethylrhodamine

Site-specifically incorporated 2'-amino groups can be selectively conjugated by reactions with aromatic isothiocyanates or aliphatic isocyanates (Scheme 1.12a) to produce the corresponding urea or thiourea linkages in **91**. Primary amino groups of 2'-aminoethyl ribonucleosides and 5-(aminoallyl)pyrimidines are

often conjugated via amide bond formation (**92**, **93**) with active esters (Scheme 1.12b,c) such as *N*-hydroxysuccinimide (NHS) esters and 4-sulfonyltetrafluorophenyl (STP) esters; the latter offer the advantage of higher solubility in buffered aqueous reaction media commonly used for RNA derivatization [112]. Although not explicitly shown in Scheme 1.12, it should be noted that 2'-amino-modified RNA can also react with active esters to form amide bonds and 2'-aminoethyl and 5-aminoallyl groups can be derivatized with isocyanates or isothiocyanates. Examples of different types of amino-labeling reagents are shown in Scheme 1.12d.

One notable advantage of post-synthetic RNA labeling strategies is that a relatively small number of phosphoramidites are needed to incorporate site-specifically suitable amino groups to which a wide variety of labels can easily be conjugated. Such a convergent approach facilitates the optimization of important parameters, such as the search for ideal tether lengths for the attachment of biophysical probes [66,113].

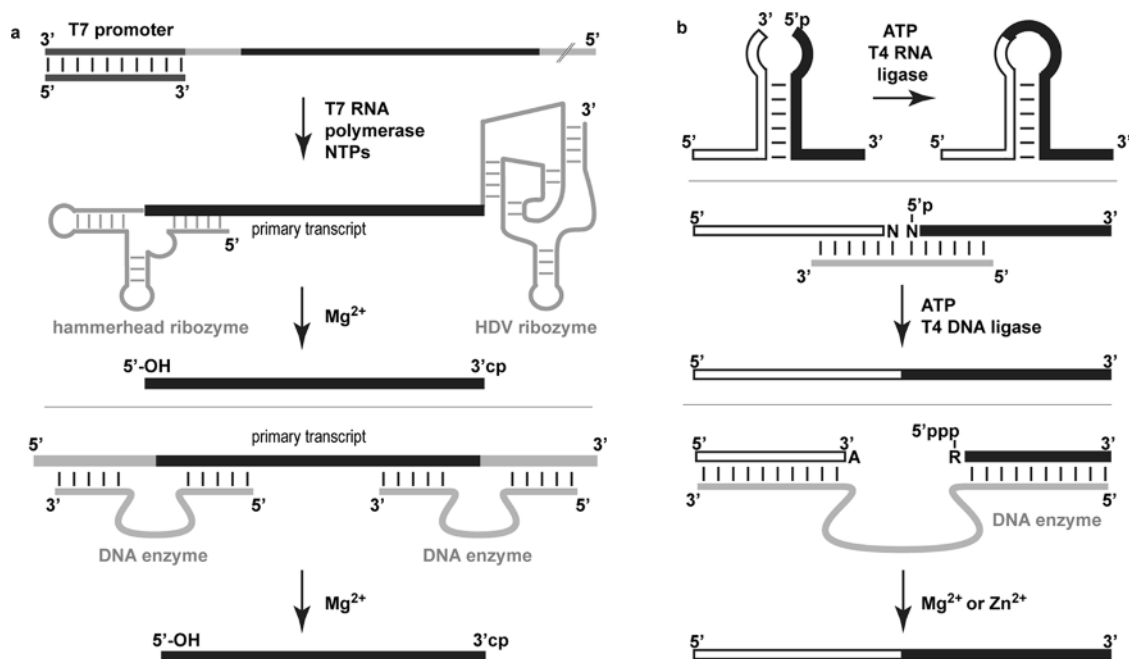
A different strategy for the derivatization of 2'-amino-modified RNA **88** involves reductive amination reactions with carbonyl groups. This approach has been used for the irreversible attachment of DNA constraints to RNA in the context of controlling RNA conformations [114]. The DNA oligonucleotide was modified on the 5'-terminus with a 1,2-diol which was converted into an aldehyde functional group upon oxidation with NaIO<sub>4</sub>. The reductive amination reaction with the 2'-amino RNA **88** proceeded in the presence of NaCNBH<sub>3</sub> and NiCl<sub>2</sub> to give an engineered RNA–DNA conjugate [115].

### 1.2.2.3 Combined chemical and enzymatic methods

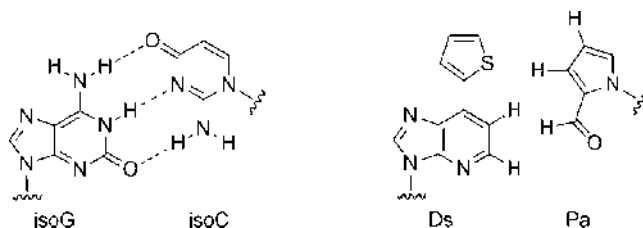
The preparation of modified RNAs up to a length of 50–60 nt can be reliably achieved by solid-phase synthesis. However, the combination of one or more protein- or nucleic acid-based enzymes is needed to incorporate chemically synthesized, modified oligonucleotide fragments into larger RNAs.

Either the required RNA segments can all be prepared by chemical synthesis, or larger fragments can be generated enzymatically by *in vitro* transcription. T7 RNA polymerase and related phage enzymes use synthetic DNA templates, double-stranded DNA fragments generated by polymerase chain reaction (PCR) or sections of linearized plasmids. Transcription is initiated from a 17 nt double-stranded T7 promoter region and continues to the 3'-end of the DNA template (Figure 1.9a). Several methods have been developed to circumvent problems of length heterogeneity that result from non-templated nucleotide addition at the 3'-end or, in rare cases typically involving multiple G nucleotides in a row, at the 5'-end. For example, the desired transcript can be engineered between a hammerhead ribozyme at the 5'-end and a hepatitis delta virus (HDV) ribozyme sequence at the 3'-end. [116] Upon transcription, the *cis*-acting ribozyme constructs will precisely excise the desired RNA by self-cleavage. It should be noted that hairpin and HDV ribozyme can also be used independently of each other, if processing of only the 5'- or 3'-end of the RNA transcript is required. Alternatively, deoxyribozymes (= DNA enzymes) can be used for the site-specific cleavage of RNA transcripts to prepare homogenous termini for further utilization in chemical or enzymatic reactions [117,118].

Although T7 RNA polymerase accepts certain modified NTPs as substrates (for example,  $\alpha$ -thiotriphosphates and even certain nucleobase-modified derivatives) [105], it is usually not possible to introduce modified nucleotides site-specifically using standard DNA templates, unless, in rare cases, the particular nucleotide in question is present only once in the entire transcript. Another exception is the extreme 5'-terminus at which modifications can be placed by using special dinucleotide constructs (XpG) as initiators. Moreover, several research groups have developed unnatural base pair systems to attempt direct site-specific modification by RNA polymerases. Since the first report of the unnatural base pair isoG–isoC [119], a variety of other innovative base pair analogs have been described. A recent example is the shape-complementary, hydrophobic base pair formed by 7-(2-thienyl)imidazo[4,5]pyridine (Ds) and pyrrole-2-carbaldehyde (Pa)



**Figure 1.9** Enzymatic methods for RNA manipulation. (a) In vitro transcription by T7 RNA polymerase from a synthetic DNA template with engineered 5'-hammerhead and 3'-HDV ribozyme sequences (gray) for production of desired RNA with homogenous termini (black); processing of primary RNA transcript can also be achieved with RNA-cleaving deoxyribozymes (bottom). (b) Enzymatic ligation of RNA fragments with T4 RNA ligase (top), T4 DNA ligase and splint (middle) or a deoxyribozyme (bottom)



**Figure 1.10** The first unnatural base pair isoG-isoC with non-natural hydrogen bonding pattern and the recently described Ds-Pa base pair formed by specific hydrophobic shape complementation without hydrogen bonding interactions

(Figure 1.10), which displays robust selectivity for the incorporation of these base substrates into RNA by transcription [120]. This approach is conceptually attractive, but it is still limited to highly specialized nucleoside analogs.

Another useful way for enzymatic incorporation of a modified nucleotide is the RNA ligase-catalyzed ligation of a modified nucleoside 3',5'-bisphosphate to the 3'-terminus of an RNA transcript [121]. For example, this method has been used to incorporate s<sup>4</sup>U and m<sup>1</sup>A into tRNAs for the purpose of further derivatization and RNA folding studies [122].

To generate longer RNAs than are routinely achievable by direct chemical synthesis, modified and non-modified RNA fragments can be covalently joined by enzymatic ligation. Most commonly, the protein enzymes T4 DNA ligase or T4 RNA ligase are used to activate the 5'-terminal phosphate of the donor RNA fragment by adenylation and join it to the 3'-hydroxyl group of the acceptor substrate [123,124]. T4 DNA ligase catalyzes the ligation of two RNA substrates that are precisely aligned in a fully base-paired RNA–DNA heteroduplex, whereas T4 RNA ligase is used to join two single-stranded RNAs in the absence of a splint oligonucleotide (Figure 1.9b). The T4 RNA ligase-catalyzed reaction is prone to generating side products, most notably circular RNAs, unless the 3'-end of the donor RNA is blocked, for example by 3'-phosphorylation. In a different setup, T4 RNA ligase can be used with a splint that leaves the RNA termini that will be joined unpaired; this could be useful if the ligation site is not in a preformed stem–loop.

Numerous examples have been reported for the successful combination of chemical synthesis of modified RNA with enzymatic ligation methods. A recent example is the efficient preparation of 2-aminopurine-labeled riboswitch domains for fluorescence spectroscopic RNA folding studies [125–127]. Folding of the RNA tertiary structure has also been monitored by fluorescence of covalently attached pyrene. Short 2'-amino- or 2'-tethered amino-modified RNAs were chemically derivatized with pyrene and enzymatically ligated to larger RNA transcripts to provide pyrene-labeled P4–P6 RNA [66,128]. Efficient enzymatic ligation was recently also described for small and highly structured fluorophore-labeled tRNA and ribozyme fragments [129]. A combination of the convertible nucleoside approach and thiol-specific RNA labeling together with enzymatic ligation was applied for engineering of pre-mRNA and snRNA constructs [130,131]. In these studies, a site-specifically attached hydroxyl radical probe (Fe-BABE) was used to investigate the architecture of early spliceosomal complexes.

A recent addition to the repertoire of available methods for covalent ligation of RNA fragments comes from the *in vitro* selection of deoxyribozymes. Silverman and co-workers reported practically useful DNA catalysts for the ligation of a 5'-triphosphate RNA donor substrate to the 3'-hydroxyl group of a second RNA fragment [132,133].

A different technique for post-synthetic or post-transcriptional RNA labeling is the application of RNA-ligating deoxyribozymes to attach fluorophore-labeled tagging RNAs to specific RNA 2'-hydroxyl groups via 2',5'-phosphodiester bond formation. This method has been termed DECAL (deoxyribozyme-catalyzed labeling of RNA) and has been applied for the preparation of fluorescein- and TAMRA-labeled P4–P6 RNA for studying RNA folding by fluorescence resonance energy transfer (FRET) [134].

### **1.3 Examples of modified RNA for the analysis and manipulation of RNA secondary and tertiary structures**

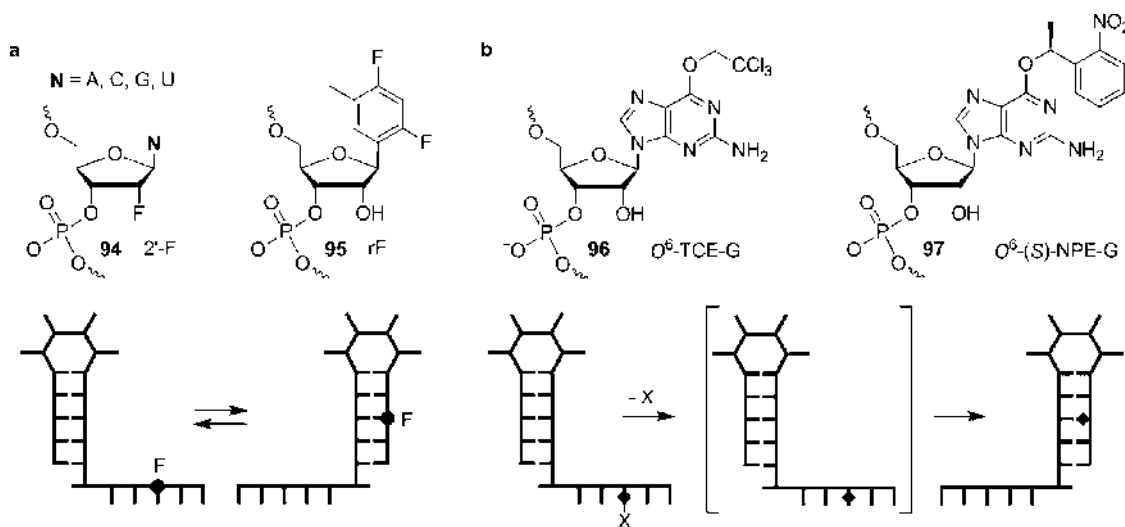
Interconvertible RNA secondary and tertiary structures play key roles in natural systems for the regulation of transcription and translation events. For instance, riboswitches are mRNA-based genetic control elements that rely heavily on the interplay of alternative RNA secondary structures. Defined single-stranded regions belong to mutually exclusive stem–loop motifs in the absence or presence of dedicated riboswitch ligands [135,136]. Such structural transitions between different folding states not only may occur in large RNAs, but also alternating conformations can exist in small RNAs of only 20–40 nt [137,138]. Several research groups have focused on specific properties of chemically modified RNA oligonucleotides to analyze secondary structure equilibria and to study RNA refolding processes. Recent examples include fluorine- or aminopurine-labeled RNAs to monitor RNA secondary structure populations by NMR or fluorescence spectroscopy and caged nucleotides to manipulate deliberately RNA conformations.

### 1.3.1 $^{19}\text{F}$ NMR spectroscopy of fluorine-modified RNA

Micura and co-workers incorporated fluorine atoms site-specifically into RNA oligonucleotides and capitalized on  $^{19}\text{F}$  NMR spectroscopy to analyze RNA secondary structure populations [50,139].  $^{19}\text{F}$  NMR spectroscopy offers high intrinsic sensitivity based on 100% natural abundance of the  $^{19}\text{F}$  nucleus. The 100-fold larger chemical shift dispersion of  $^{19}\text{F}$  compared with  $^1\text{H}$  results in strongly reduced signal overlap in  $^{19}\text{F}$  spectra compared with often severe resonance degeneracy observed in  $^1\text{H}$  NMR imino proton spectra. Applications of fluorine-labeled RNA and  $^{19}\text{F}$  NMR spectroscopy have recently been reviewed [140].

In one strategy, 2'-deoxy-2'-fluoro-modified nucleotides **94** were used as non-invasive spin labels for the analysis of RNA secondary structure equilibria. Thereby, the 2'-F-nucleotides were strategically positioned in the RNA to reside within a double helix in one RNA conformation, but to be part of a single-stranded region in an alternative conformational state (Figure 1.11a). The significantly different chemical environment experienced by the 2'-F atom in both conformations resulted in different chemical shifts for the  $^{19}\text{F}$  resonances, with slow exchange on the NMR time-scale. Integration of the  $^{19}\text{F}$  NMR signals allowed quantification of the equilibrium positions [50]. The combination of 2'-F-modified RNA and  $^{19}\text{F}$  NMR spectroscopy has also been applied in a proof-of-principle study for the identification of specific RNA–ligand binding events. Upon complexation of a small-molecule target, the RNA experienced local conformational alterations in the binding pocket that were monitored in form of chemical shift changes of specific 2'-F resonances [51].

In a different approach, the nonpolar uridine analog 2,4-difluorotoluylyl ribonucleoside (rF) was site-specifically incorporated into RNA (**95**) by solid-phase synthesis and was used for the analysis of RNA secondary structure populations and for the investigation of temperature-dependent conformational changes



**Figure 1.11** Analysis and manipulation of RNA conformations. (a) 2'-Deoxy-2'-fluoro nucleotides **94** and 2,4-difluorotoluylyl ribonucleoside **95** for characterization of interconvertible RNA secondary structures by  $^{19}\text{F}$  NMR spectroscopy. (b) O<sup>6</sup>-TCE-modified guanosine **96** and O<sup>6</sup>-(S)-NPE-caged guanosine **97** for the manipulation of RNA structures. Nucleobase functionalization with X (= NPE or TCE) specifically selects one conformation. Upon release of X by reductive elimination or photolysis, the base pairing properties of the nucleobase are restored, the RNA conformation becomes metastable and is reorganized into a thermodynamically more favorable folding state

by  $^{19}\text{F}$  NMR spectroscopy [139]. This strategy is highly valuable for the precise determination of RNA equilibrium positions at elevated temperatures because it is not dependent on the detection of intrinsically exchangeable NH-N nuclei (in  $^1\text{H}$  NMR spectroscopy, H–D exchange results in signal loss at elevated temperatures). The rF-labeling approach allows the measurement of RNA melting temperatures ( $T_m$ ) with good precision at high RNA concentrations that are generally not accessible by UV melting analysis. Moreover, the rF modification has recently been incorporated into siRNAs and was shown to confer improved nuclease resistance in serum. An internal rF:A base pair did not negatively affect gene silencing activity relative to native siRNAs in HeLa cells, despite slightly reduced affinity of the rF-modified RNA for the target strand [141–143]. In combination with  $^{19}\text{F}$  NMR spectroscopy, the rF modification could be applicable for conformational analysis of siRNAs and provide further insights for mechanistic investigations of RNAi.

### 1.3.2 Triggering of RNA structural transitions by functionalized nucleobases

Chemically functionalized nucleosides provide useful tools to control intentionally conformational changes of nucleic acid structures. Nucleobase modifications installed at the Watson–Crick base pairing site have been designed to prevent the formation of selected base pairs and specifically to destabilize predefined RNA secondary or tertiary structures. With respect to the site-specific incorporation into RNA, the functional labels must meet the stringent requirement of orthogonality to the protecting groups used throughout solid-phase synthesis. The first example of a functionalized RNA nucleotide described as an efficient tool for inducing defined rearrangements of RNA secondary structures is the  $O^6$ -trichloroethyl (TCE)-modified guanosine **96** [144]. The TCE group alters the hydrogen bonding donor–acceptor pattern of guanosine and therefore significantly reduces the residue’s base pairing ability. Upon release of the TCE functionality under reductive conditions (Zn–AcOH), the base pairing capability is restored and the then unmodified nucleobase allows secondary structure rearrangements of the metastable conformation to occur (Figure 1.11b). In this study, alternative secondary structures were monitored by comparative imino proton NMR spectroscopy [138]. In a similar approach, Pitsch and co-workers introduced the photolabile (*S*)-1-(2-nitrophenyl)ethyl (NPE)<sup>1</sup> group at the  $O^6$ -position of guanosine **97** to modulate the Watson–Crick base pairing capability of defined nucleotides in bistable RNAs [145]. The kinetics of RNA conformational switching upon release of the photolabile group was studied by monitoring characteristic imino proton resonances of specific RNA conformations by time-resolved NMR spectroscopy [146–148].

For a different application, Höbartner and Silverman synthesized all four caged (*S*)-NPE-modified RNA nucleotides and placed individual caging groups into the 160-nt P4–P6 domain of the *Tetrahymena* group I intron RNA [149]. The effects on RNA folding were studied by nondenaturing (native) PAGE, which reports on global folding of P4–P6 RNA. Caging groups at selected positions were shown to disrupt key tertiary contacts by alteration of hydrogen bonding interactions, by disruption of base-stacking interactions or by introduction of steric clashes. In general, chemical interference with specific tertiary contacts, such as disruption of a key tetraloop–receptor interaction, can be used to produce stable misfolded states of large RNAs that may be of functional significance. Upon release of the caging group, the refolding into the native state can be initiated. Phototriggered RNA folding in combination with time-resolved monitoring of conformational changes by spectroscopic methods is expected to yield further insights into the mechanisms of complex RNA refolding events.

<sup>1</sup> It should be noted that the same abbreviation NPE is used in the literature for two different isomers of nitrophenylethyl groups. Here, NPE denotes the photolabile 1-(2-nitrophenyl)ethyl group, whereas in Figures 1.2 and 1.6 and Scheme 1.4 NPE at the  $O^6$  of guanosine or inosine derivatives denotes the 2-(4-nitrophenyl)ethyl group that is cleaved by  $\beta$ -elimination during RNA deprotection.

## 1.4 Conclusion

In this chapter, we have summarized recent developments in protecting group strategies for RNA solid-phase synthesis and presented an overview of available methods for the preparation of chemically modified RNA. The diversity of chemically accessible RNA modifications is rapidly increasing, accompanied by an expanding number of applications in fundamental and applied research. Here, we could discuss only a small selection of studies demonstrating the power of oligoribonucleotide analogs for biochemical and biophysical applications.

Future research efforts will continue to yield more detailed insights into structural and mechanistic aspects of functionally important RNAs involved in various biochemical processes. The creativity and aptitude of chemists will ensure that tailor-made nucleoside modifications will be designed and synthesized to modulate RNA in various ways for multidisciplinary experiments.

## Acknowledgments

The authors are grateful for support from the Max Planck Society. C.H. thanks Professor Ronald Micura, Innsbruck, for stimulating discussions. Professor Scott K. Silverman, Urbana-Champaign, and Dr. Manfred Konrad, Göttingen, are gratefully acknowledged for critical reading of the original manuscript.

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