The Chemical Biology of Nucleic Acids

Edited by

GÜNTER MAYER
Strathclyde Institute for Pharmacy and Biological Sciences,
University of Strathclyde, Glasgow, UK

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Foreword

Chemists have played a key role not only in establishing the chemical structure of DNA and RNA but also in helping to understand their structure–function relationships. This fascination has continued over the years and, as the present book demonstrates, is unabated. The many chapters are great testimony to the fruitful interplay in nucleic acid chemistry and biology. As chemists have continued over the years to modify the structure and architecture of DNA and RNA for an even better insight into their biological functions, Nature has lately provided new challenges by disclosing some unanticipated novel structures and pathways. These new insights into Nature pose questions where chemistry helps to obtain a deeper understanding.

Even though the chemistry of nucleic acids has been described over the years in many textbooks and reviews, the chapters in this book describe the efforts and successes achieved in the last few years in combination with more recent developments. Hence this book comprises an appreciated update review of the chemical biology of nucleic acids as it stands today.

The book covers a wide scope, with chapters on specific topics written by experts in the particular area. Given the new horizons on the role of RNA which we have experienced over recent years, it is not surprising that several chapters describe RNA chemistry as an essential component for the deeper analysis of these phenomena. The chemical synthesis of modified RNA is, of course, fundamental and is rightly described at the beginning. RNA occurs in a surprisingly wide range of architectural motifs commensurate with the multitude of functions as a detailed account describes. The versatility of structural motifs is also apparent in the naturally occurring riboswitches. These are responsible for the control of translation or transcription by binding of small cellular metabolites. This area is still very much in flux and novel riboswitches are being discovered every year, supporting RNA as an important regulator for gene expression. MicroRNAs are a novel type of small RNAs whose mechanism of regulation of gene expression is still under investigation. So far, chemistry has not generally been applied to these, even though for medical applications this looks rather enticing. The functions of small non-coding RNAs in bacteria are attracting increasing attention and deserve the in-depth review presented. This should make chemists aware of a field which has been neglected by them so far.

RNA and DNA can actually share certain functions. This is seen in the catalytic properties which both can adopt in catalytic RNA (ribozymes) and DNAzymes, which by themselves occur in a variety of architectures. Aptamers can also consist of RNA or DNA to bind proteins and other molecules extremely specifically. The structures with these properties have been obtained by *in vitro* selection from random sequences where selective pressure plays an important role in sampling the sequence space. The examples provided demonstrate again the architectural and, with it, the functional versatility of RNA and DNA. As reviewed in several chapters, these in turn represent excellent opportunities for modulation by chemical derivatization.

Besides riboswitches, small molecules bind to RNA also in a different setting, such as to the ribosomal RNA, where aminoglycosides are the paradigm and where they exert antimicrobial effects. The fundamental

features playing a role in such interactions of small molecules with nucleic acids in general is an interesting subject.

Sugar- and base-modified nucleotides have been a subject of study for many years, yet novel aspects are still being explored. One example is the use of such modified nucleotides to study the selectivity of DNA polymerases. Another is the creation of new base pairs to expand the scope of the genetic alphabet by incorporation into DNA or RNA by replication and transcription. Of the sugar-modified nucleotides, the locked nucleic acids (LNAs) have found wide application because of their strong binding to RNA. This property makes them most suitable for interaction with mRNA and microRNAs for interference with gene expression. An entirely different analogue is the peptide nucleic acids (PNAs), where the phosphate backbone and the deoxyribose are replaced by amide linkages. Even though this is a drastic change in structure, the PNAs still hybridize favourably with DNA and RNA but are, of course, completely resistant to nucleases. They represent very powerful DNA mimics with interesting properties.

The potential of nucleic acids for therapy is a well-studied area where various strategies are being explored and being examined in clinical trials. There is high potential in this applied area and we look forward to a general breakthrough for the approval of drugs.

In summary, this collection of reviews is testimony to the fruitful role that chemists can play in helping to understand structure–function relationships in nucleic acids and their mechanisms of action. Recent years have provided big surprises, particularly in the field of RNA, which indicates that the field is wide open with lots of opportunities and challenges.

Fritz Eckstein
Göttingen
2010

Preface

In early 2007, Paul Deards from Wiley approached me and asked whether I would like to edit a book on the topic of light-responsive nucleic acids. Upon this impulse we started a fruitful discussion on that topic. An initial survey of books that cover nucleic acids in general, and on light-responsive nucleic acids in particular, resulted in the finding that almost no comprehensive compendium starting with the synthesis of nucleic acids and their derivatives and also covering their biological applications was available at that time. Therefore, we decided to withdraw the initial idea of putting together a book on light-responsive nucleic acids only, and instead we set out to edit a more general book on the topic of *The Chemical Biology of Nucleic Acids*. The result of this effort finally led to what is compiled within the present book.

Fortunately, many outstanding scientists within their respective scientific fields agreed to contribute to the book. Without their efforts this compilation would not have come to life. It is amazing to learn which central role RNA, DNA and derivatives thereof play in Nature, apart from the sole storage and transmission of genetic information, and it is even more remarkable to gain knowledge on the versatile application of artificial, synthetic nucleic acids to investigate biological phenomena. Being a part within this scientific area is exciting and I am curious to learn what comes next. I am very proud that Fritz Eckstein could be won over to write the Foreword to this book. He definitely is the right and most competent person to do so – thank you very much. Of course, the book cannot cover every aspect of the complex and diverse field of nucleic acids. I apologize if we have missed any subjects that others might consider invaluable. Owing to the inevitable space constraints, we definitely had to make compromises that owing to their inherent nature will not satisfy everyone's opinions completely. Finally, I would like to thank all the authors again for their willingness to make *The Chemical Biology of Nucleic Acids* an outstanding contribution – it will certainly become an important reference in the field. I am also grateful to Paul Deards who initiated the process leading to this book, and Richard Davis, Gemma Valler and Rebecca 'Becki' Ralf at Wiley for their helpful support and encouragement during the making of this book.

Günter Mayer
Glasgow, 2009

List of Contributors

Sergio Aguirre, School of Biomedical Engineering, McMaster University, Hamilton, Ontario, Canada.

Tyler D. Ames, Department of Molecular Cellular and Developmental Biology, Howard Hughes Medical Institute, Yale University, 266 Whitney Avenue, New Haven, CT 06520, USA.

Stefan Bauer, Institut für Immunologie, Philipps-Universität Marburg, BMFZ, Hans-Meerweinstrasse 2, 35043 Marburg, Germany.

Sabine Brantl, AG Bakteriengenetik, Friedrich-Schiller-Universität Jena, Philosophenweg 12, 07743 Jena, Germany.

Ronald R. Breaker, Department of Molecular Cellular and Developmental Biology and Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University, 266 Whitney Avenue, New Haven, CT 06520, USA.

Valérie Fritsch, Institut de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Université de Strasbourg 15 rue R. Descartes, 67084 Strasbourg Cedex, France.

Alexander Heckel, Goethe University Frankfurt, Cluster of Excellence Macromolecular Complexes, Max-von-Laue-Strasse 9, 60438 Frankfurt am Main, Germany.

Ichiro Hirao, Nucleic Acid Synthetic Biology Research Team, Systems and Structural Biology Center, Yokohama Institute, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, 230-0045, Japan.

Claudia Höbartner, Max Planck Institute for Biophysical Chemistry, Research Group Nucleic Acid Chemistry, Am Fassberg 11, 37077 Göttingen, Germany.

Britta Hoehn, Department of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, CA 91010, USA.

Andres Jäschke, University of Heidelberg, Institute of Pharmacy and Molecular Biotechnology, Department of Chemistry, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany.

Albert Jeltsch, School of Engineering and Science, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany.

Renata Z. Jurkowska, School of Engineering and Science, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany.

Harleen Kaur, Institute of Genomics and Integrative Biology, CSIR, Mall Road, Delhi 110 007, India.

Michiko Kimoto, Nucleic Acid Synthetic Biology Research Team, Systems and Structural Biology Center, Yokohama Institute, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, 230-0045, Japan.

Jeffrey C.F. Lam, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada.

Yingfu Li, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada.

Joe C. Liang, Department of Bioengineering, 473 Via Ortega, MC 4201, Stanford University, Stanford, CA 94305, USA.

Souvik Maiti, Institute of Genomics and Integrative Biology, CSIR, Mall Road, Delhi 110 007, India.

Andreas Marx, Department of Chemistry, Konstanz Research School Chemical Biology, Universität Konstanz, Universitätsstrasse 10, Box 726, 78457 Konstanz, Germany.

Günter Mayer, Strathclyde Institute for Pharmacy and Biological Sciences, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, UK.

Gunter Meister, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany.

Peter E. Nielsen, Department of Cellular and Molecular Medicine, Faculty of Health Sciences, The Panum Institute, University of Copenhagen, Blegdamsvej 3c, 2200 Copenhagen N, Denmark.

Michael Oberhuber, Institute of Organic Chemistry and Center for Molecular Biosciences (CMBI), University of Innsbruck, Innrain 52a, 6020 Innsbruck, Austria. Present address: Laimburg Research Centre for Agriculture and Forestry, Laimburg 6, Pfatten (Vadena), 39040 Auer (Ora), Italy.

John J. Rossi, Department of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, CA 91010, USA.

Christina D. Smolke, Department of Bioengineering, 473 Via Ortega, MC 4201, Stanford University, Stanford, CA 94305, USA.

Victor K. Tam, Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093-0358, USA.

Yitzhak Tor, Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093-0358, USA.

Peter J. Unrau, Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, V5A 1S6, Canada.

Falk Wachowius, Max Planck Institute for Biophysical Chemistry, Research Group Nucleic Acid Chemistry, Am Fassberg 11, 37077 Göttingen, Germany.

Qing Wang, Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, V5A 1S6, Canada.

Eric Westhof, Institut de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Université de Strasbourg 15 rue R. Descartes, 67084 Strasbourg Cedex, France.

Maung Nyan Win, Department of Bioengineering, 473 Via Ortega, MC 4201, Stanford University, Stanford, CA 94305, USA.

Bernhard Wulffen, University of Bonn, Life and Medical Sciences Bonn, c/o Kekulé Institut für Org. Chemie und Biochemie, Gerhard-Domagk-Strasse 1, 53121 Bonn, Germany.

Yun Xie, Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093-0358, USA.

1

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

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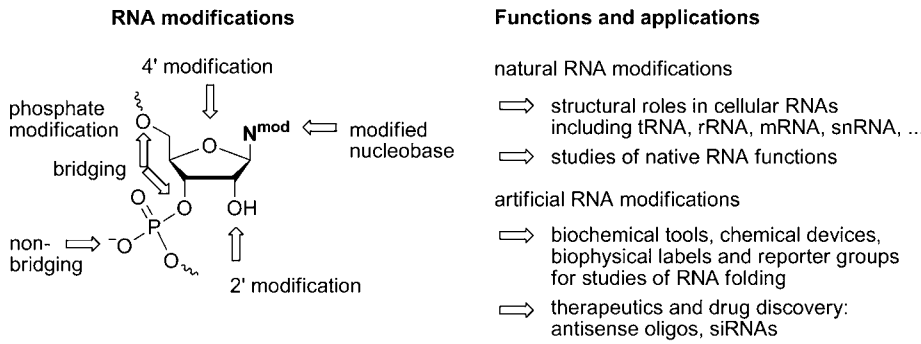


Figure 1.1 *Diagram illustrating sites for chemical modifications of ribonucleotides and versatile functions and applications of synthetic RNA*

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].

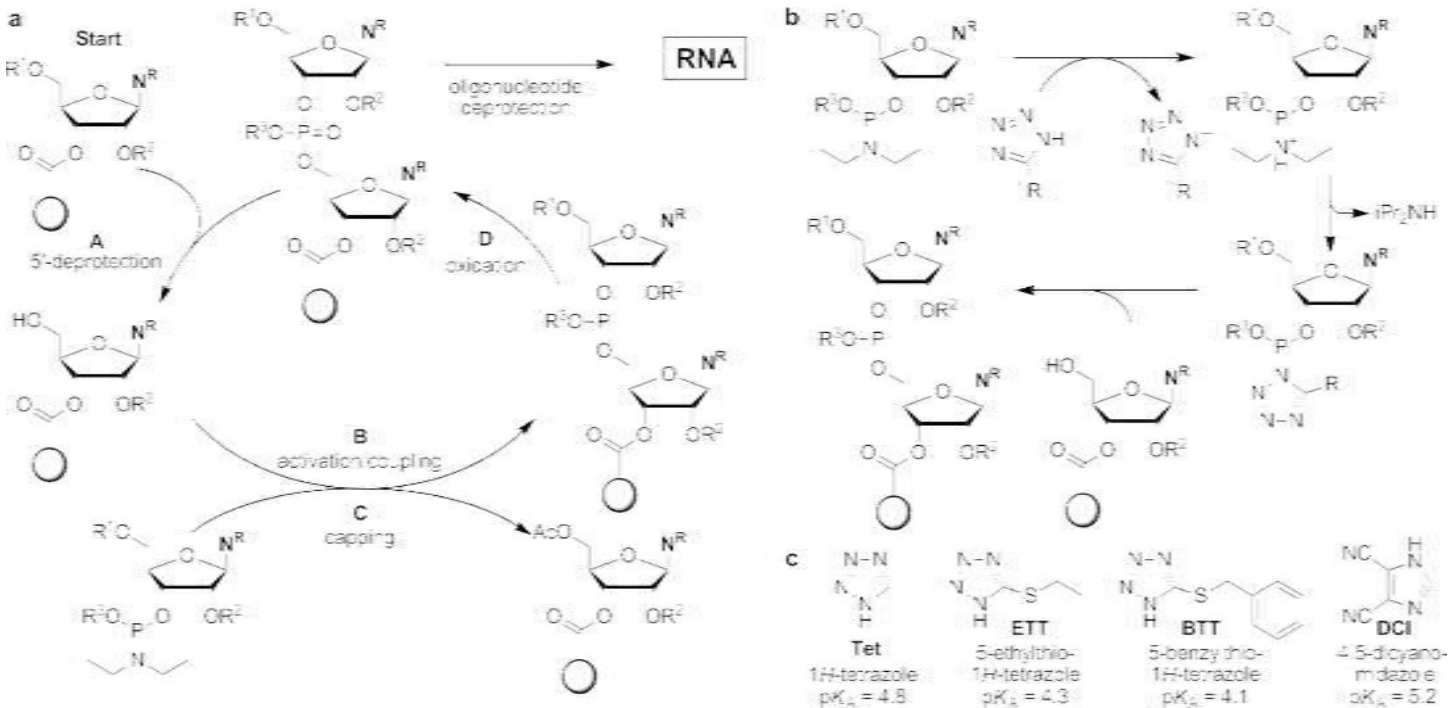
In this chapter we focus on RNA solid-phase synthesis using phosphoramidite chemistry and describe recent developments in protecting group strategies for RNA synthesis. We discuss general ways to introduce modifications into RNA oligonucleotides and we review selected examples of RNA modifications to 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.

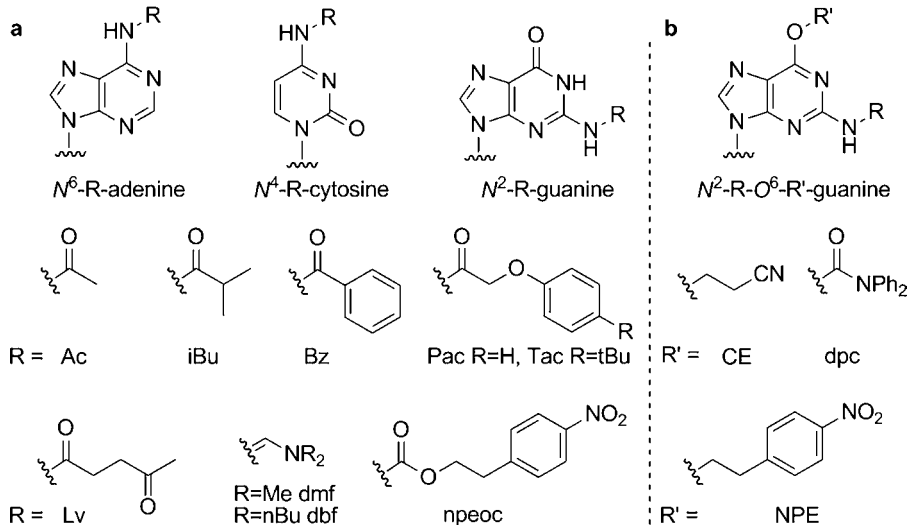


Figure 1.2 *(a) Natural nucleobases adenine (A), cytosine (C) and guanine (G) that need protecting groups at their exocyclic amino groups during solid-phase synthesis. Selected examples of base-labile nucleobase protecting groups (R): Ac = acetyl, iBu = isobutyl, Bz = benzoyl, Pac = phenoxyacetyl, Tac = 4-(*tert*-butylphenoxy)acetyl, Lv = levulinyl, dmf = dimethylaminomethylene (same as dimethylformamidine), dbf = dibutylaminomethylene, npeoc = (4-nitrophenyl)ethyloxycarbonyl. (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′-β-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, 1*H*-tetrazole was used as activator for 2′-*O*-TBDMS-protected RNA

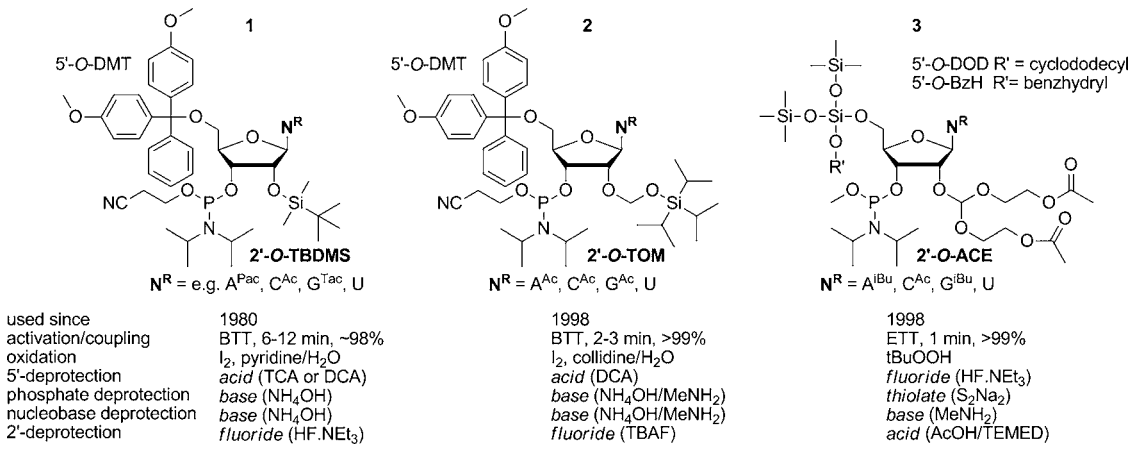


Figure 1.3 *Ribonucleoside phosphoramidite building blocks for commercialized RNA synthesis methods using either the 5′-O-DMT-2′-O-silyl (**1** and **2**) or the 5′-O-silyl-2′-O-ACE chemistry (**3**). TCA = trichloroacetic acid; DCA = dichloroacetic acid; TBAF = tetrabutylammonium fluoride; S_2Na_2 = disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate; TEMED = tetramethylethylenediamine*

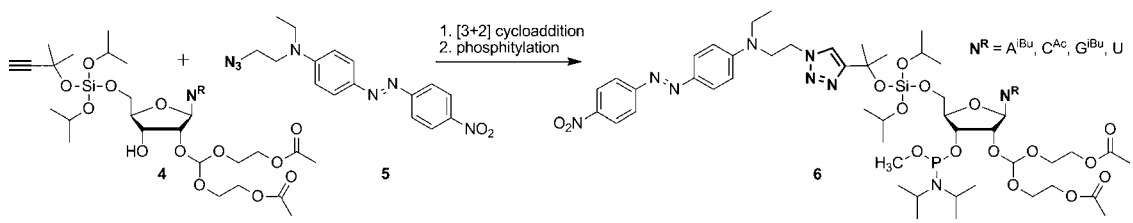
phosphoramidites, but today more powerful activators such as ETT and BTT are applied and coupling times can be reduced to 3–6 min to achieve coupling efficiencies of up to 99% [22]. The most critical issue associated with 2′-*O*-TBDMS protected ribonucleosides is the high potential of the alkylsilyl 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 (Tac), 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 β-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^+ 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 phosphitylation*

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 (Fpmp) (**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.

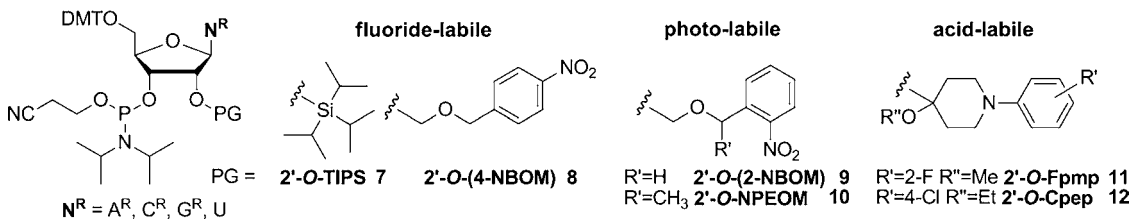


Figure 1.4 *Examples of conventional 2′-protecting groups classified according to required deprotection conditions*

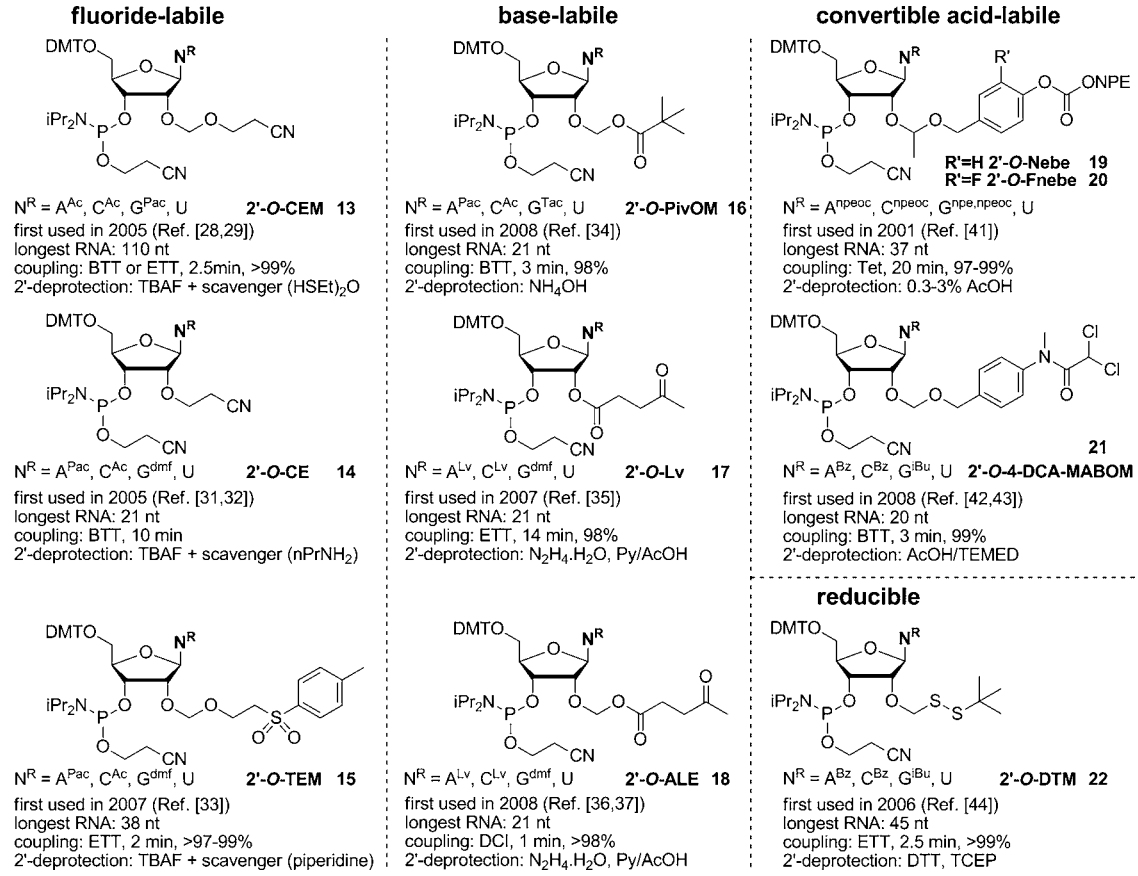


Figure 1.5 *Overview of 5′-O-DMT-protected phosphoramidites with various recently developed 2′-O-protecting groups, classified according to 2′-deprotection conditions*

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*-Fpmp (**11**) and 2′-*O*-Cpmp (**12**) protecting groups that showed great promise in the 1990s [38,39] The Fpmp-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 Pfleiderer 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-butyldithiomethyl (DTM) protecting group

Ribonucleoside phosphoramidites **22** protected with the 2′-*O*-*tert*-butyldithiomethyl (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 [Ψ (**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 photocrosslinking 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).

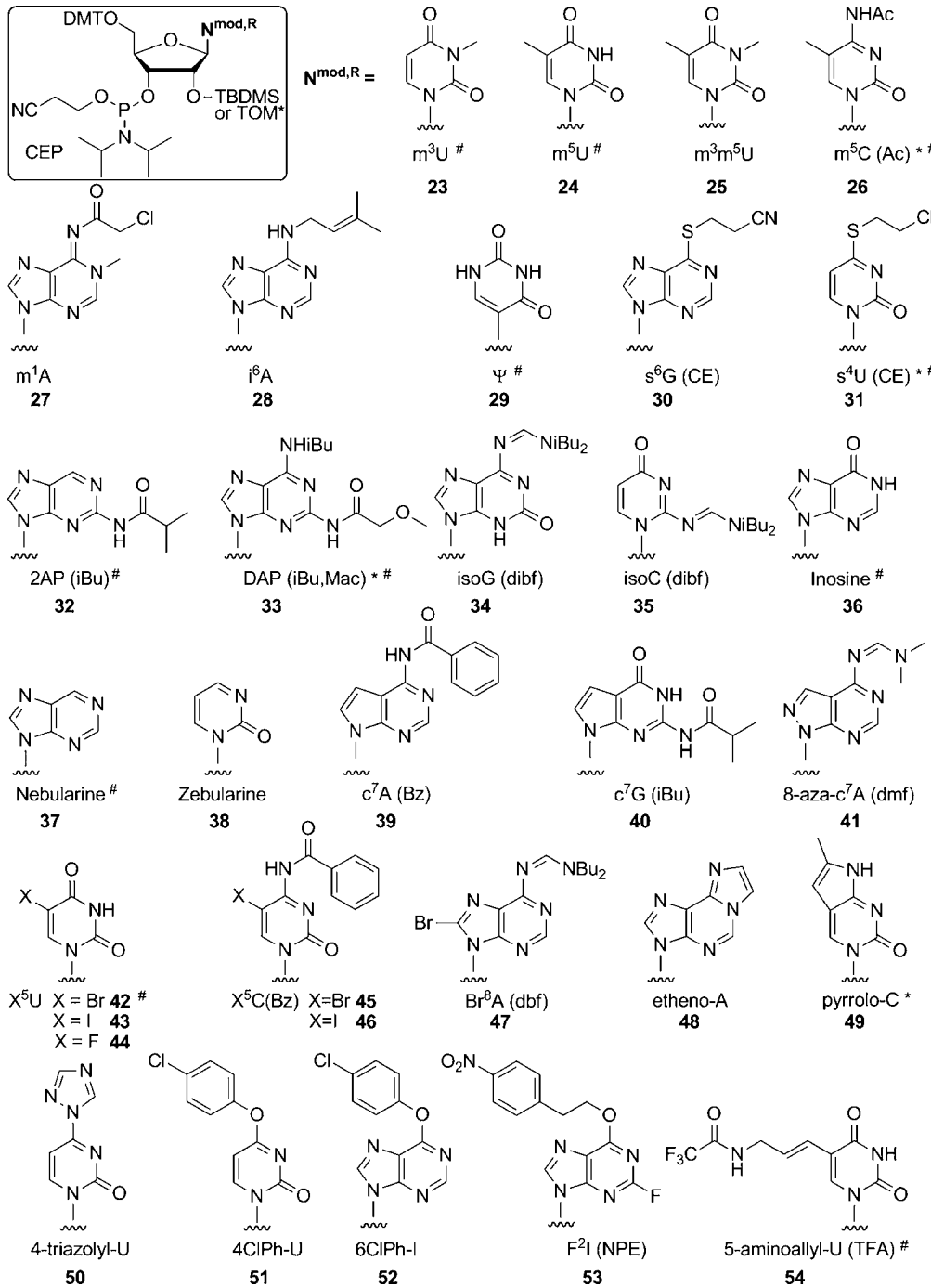


Figure 1.6 *Collection of commercially available modified phosphoramidites compatible with the 5′-O-DMT-2′-O-silyl protection scheme. The modified nucleobases are shown in their protected forms. The asterisk (*) indicates commercial availability as 2′-O-TOM-protected phosphoramidite; # denotes availability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

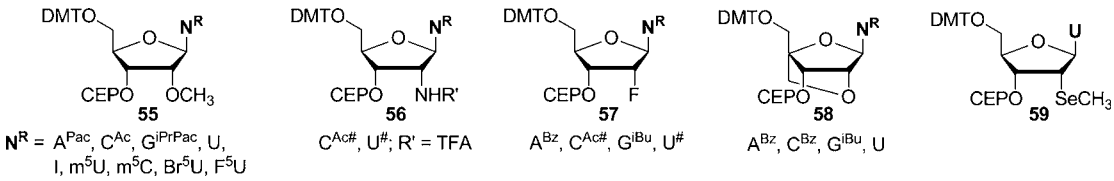


Figure 1.7 *Selection of commercially available 2′-ribose-modified phosphoramidites compatible with the 5′-O-DMT-2′-silyl protection scheme. The symbol # denotes availability via custom synthesis service using ACE chemistry. iPrPac = 4-isopropylphenoxyacetyl*

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}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 internucleotide 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