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Review

Chemical methods for the modification of RNA

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Abstract:

RNA is often considered as being the vector for the transmission of genetic information from DNA to the protein synthesis machinery. However, besides translation RNA participates in a broad variety of fundamental biological roles such as gene expression and regulation, protein synthesis, and even catalysis of chemical reactions. This variety of function combined with intricate three-dimensional structures and the discovery of over 100 chemical modifications in natural RNAs require chemical methods for the modification of RNAs in order to investigate their mechanism, location, and exact biological roles. In addition, numerous RNA-based tools such as ribozymes, aptamers, or therapeutic oligonucleotides require the presence of additional chemical functionalities to strengthen the nucleosidic backbone against degradation or enhance the desired catalytic or binding properties. Herein, the two main methods for the chemical modification of RNA are presented: solid-phase synthesis using phosphoramidite precursors and the enzymatic polymerization of nucleoside triphosphates. The different synthetic and biochemical steps required for each method are carefully described and recent examples of practical applications based on these two methods are discussed.

Keywords: RNA solid-phase synthesis • phosphoramidites • sugar-base modifications • phosphorothioates • modified nucleotides • SELEX • RNA aptamers • Mutant T7 RNA polymerases

1. Introduction

RNA is a unique type of nucleic acid since it adopts a range of biological functions and is organized in various two and three dimensional structures. This prevalence calls for chemical methods for the covalent modification of RNA in order to investigate the function, structure, cellular localization, and intracellular transport of this biopolymer. The necessity for the development of reliable protocols for the chemical modification of RNA is further spurred by the discovery of over 100 different alterations of the nucleosidic scaffold of RNA in all kingdoms of life [1-4]. These modifications appear to be located on non-coding RNAs including tRNA, rRNA, and small nuclear RNA (snRNA) as well as on less abundant species such as mRNA but their exact number and often their function have not been fully elucidated. Hence, synthetic protocols that enable the synthesis of these modified building blocks and the corresponding oligonucleotides are in dire need.

Besides an interest for the elucidation of the function and mechanism of naturally occurring (modified) RNAs, potent practical applications based on RNA oligonucleotides heavily rely on the inclusion of chemical modifications. In this context, gene silencing molecules including antisense oligonucleotides, siRNAs, and miRNAs would be rapidly degraded by nucleases following injection into a patient and thus require chemical modifications to resist against the assault of these ubiquitous enzymes. Similarly, aptamers are single-stranded oligonucleotides that bind to targets with high specificity and selectivity and have been proposed as diagnostic, therapeutic, and drug delivery systems. Besides being prone to rapid renal filtration and efficient degradation by nucleases, aptamers often require the help of additional modifications to bind to more complex targets such as glycoproteins [5].

Here, we discuss the main methods available for the chemical modification of RNA: automated solid-phase synthesis with modified phosphoramidite building blocks and enzymatic synthesis with modified nucleoside triphosphates. We have not included other elegant methods including the use of catalytic nucleic acids [6-10] or cofactor analogues [11-14] and direct the interested reader to other recent review articles [15-19]. We also discuss some recent examples of application of these methods, particularly in the fields of aptamer discovery and the understanding of the function of post transcriptional modifications.

2. Chemical synthesis and modification of RNA

2.1 Solid-phase ribo-oligonucleotide synthesis

Solid phase oligonucleotide synthesis of DNA is an established and reliable method for the synthesis of specific-sequence short-strand, up to ~ 150-mer, DNA. As outlined in the introduction, many exciting therapeutic and diagnostic avenues are being explored using synthetic RNA, further highlighting the need for dependable and efficient chemical synthetic routes for specific-sequence RNA. While the synthesis of short DNA sequences using commercially available nucleosides is highly efficient, solid-phase RNA synthesis has proven more difficult to establish [20]. Complicated by the presence of the reactive 2'-hydroxyl sugar group, the chemical synthesis requires adoption of an additional protection step which initially hindered the use of this chemical synthetic route. Exciting developments in the last two decades are beginning to allow for the widespread, and efficient, synthesis of sequence specific ribo-oligonucleotides using commercially available substrates at nearly the same efficiency as DNA.

2.1.1 Overview of the method

The cyclical RNA solid phase synthesis protocol is outlined below (*Figure 1*). This step-wise process is the most widely used synthetic route and the process closely follows that of solid-phase DNA synthesis [20, 21]. This synthetic route allows for the sequence-specific incorporation of natural and modified nucleotides in a time and resource efficient manner. The synthesis uses a solid-support bound primary nucleoside in combination with nucleoside phosphoramidites as building blocks in a specifically designed oligonucleotide synthesizer. Following a step wise sequence of deprotection, activation, coupling, oxidation and capping, nucleotides can be sequentially added. Covalent attachment to a solid support via the primary nucleotide is required throughout the synthesis with cleavage performed after oligonucleotide synthesis is completed. The use of supports soluble in organic solvents, involving a removal of excess reagents by precipitation, has also been reported for RNA and represents an interesting alternative especially with regards to large scale synthesis [22]. Oligonucleotides are usually synthesized in the 3' to 5' direction and once synthesized, oligonucleotides are purified by HPLC purification.

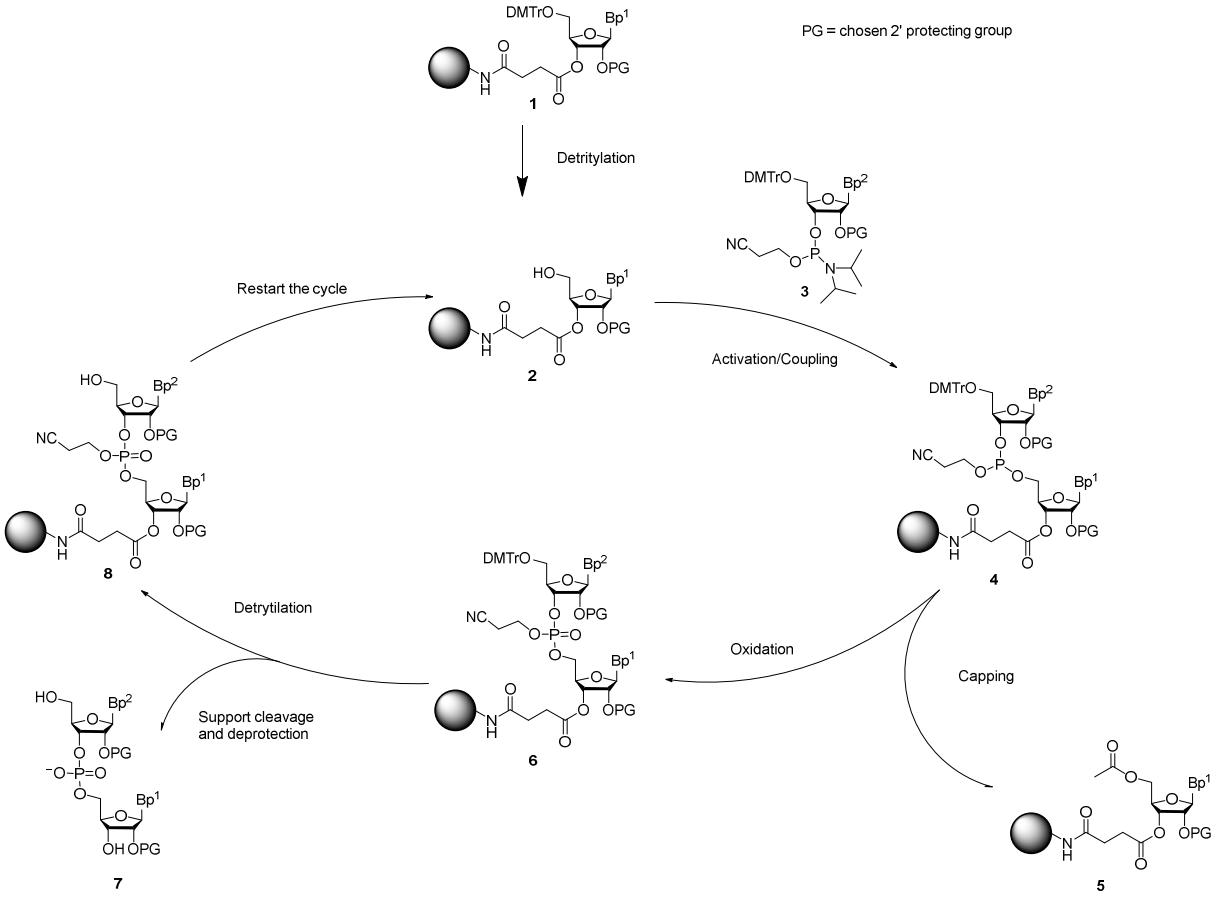


Figure 1. *A generalized scheme for the solid phase synthesis of RNA; DMTr - 4,4'-dimethoxytrityl; Bp – natural or modified, protected, nucleobase; PG- protecting group*

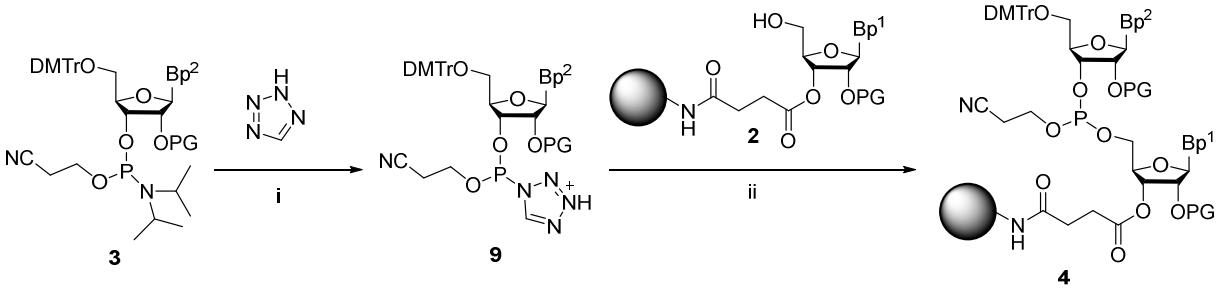
Synthesis commences with the initial ribonucleoside **1** in the sequence bound to solid support via the 3'-hydroxyl group. Typical protection at the 5'-hydroxyl position is by the acid labile 4,4'-dimethoxytrityl (DMTr). The 2'-hydroxyl position and exocyclic amino groups are also protected, as outlined below.

2.1.2 Detritylation step

The initial synthetic step is detritylation of the DMTr protected 5'-hydroxyl by acid. While a number of trityl derivatives have been proposed over the years, DMTr has gained most widespread popularity due to the efficiency of its use coupled with the bright orange nature of the cation produced during detritylation acting as a reliable indicator of successful detritylation [23]. Commonly used acids are trichloroacetic acid and dichloroacetic acid [20]. Following washing to remove the DMTr cation, the solid-bound nucleoside **2** bears the hydroxyl group required for the next step of the reaction.

2.1.3 Activation and coupling

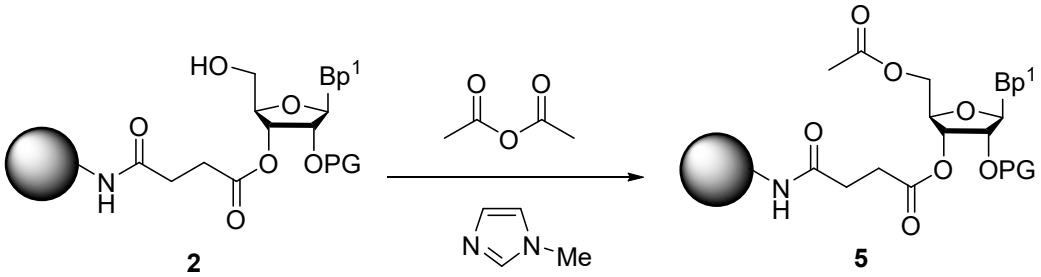
The incoming nucleoside **3** must first be activated to allow subsequent coupling. This activation typically takes place by addition of an acidic azole catalyst (*Scheme* 1) [23]. A number of activators can be selected for the activation step including 1*H*-tetrazole, 5-ethylthio-1*H*-tetrazole (ETT) and 5-benzylmercaptotetrazole (BMT) [24]. One of the most efficient activators for RNA synthesis is BMT which gives high coupling yields and clean crude products. Due to these interesting properties, the use of BMT has allowed the synthesis of very long RNA sequences (110-mer) [25]. In terms of mechanism, the protonated diisopropylamino group serves as a leaving group allowing nucleophilic substitution by the tetrazole. The intermediate tetrazole complex in turn serves as leaving group upon nucleophilic attack by the 5'-hydroxy group of the oligonucleotide. The resulting reaction leads to formation of a phosphite triester internucleotide bond **4** [20].



Scheme 1. *Activation and coupling of the incoming nucleobase phosphoramidite; i) activation of the phosphoramidite by acidic azole catalyst, here 1H-tetrazole; ii) Nucleophilic substitution by the oligonucleotide 5'hydroxyl moiety*

2.1.4 Capping, oxidation, and sequence completion

Coupling steps can typically achieve high yields though 100% yields are unlikely and, as a consequence, any unreacted solid bound nucleobases still sporting a 5' hydroxyl group **2** must be capped to prevent coupling in subsequent coupling steps (*Scheme 2*). Permanent blocking of the 5-hydroxyl group ensures protection of the desired genetic sequence from inclusion of 'shortmers' – oligonucleotide sequences missing specific nucleobases [20]. Adding DMAP to Cap B (which contains acetic anhydride and lutidine) yields the most effective capping which is beneficial when long oligonucleotides are to be synthesized but it can also lead to undesired dG side products [26].



Scheme 2. *Capping step of 5' termini during solid-phase synthesis*

The coupling step leads to formation of the P(III) phosphite triester **4** an unstable species which must be oxidized to the stable P(V) **6** which usually occurs by treatment with iodine or *tert*-butyl hydroperoxide [20, 21]. Upon completion of the oligonucleotide sequence release from the solid support, deprotection of the phosphate and nucleobase deprotection take place by treatment of concentrated ammonia [21]. The chosen 2'-protecting group will usually be removed as the final step.

Despite the advances, and an example of a 110-mer [25] sequence being synthesized, in practicality the length of RNA strands which can be reliably synthesized is around 60-mer.

2.1.5 Nucleobase phosphoramidite building blocks

A typical nucleobase phosphoramidite building block is shown below, **3** (*Figure 2*). Incorporation of a phosphoramidite moiety at the 3' position of the nucleoside allows for the cyclical addition of nucleosides in the oligonucleotide synthesis. This strategy is ubiquitous and well established. Phosphoramidites were first proposed in the early 1980's [27] and have since come to dominate oligonucleotide synthesis [28]. Phosphoramidites are generally highly reactive towards nucleophiles in the presence of even weak acids [29]. Previous to their introduction, chemical DNA synthesis had been conducted using H-phosphonate [30-33] , phosphodiester [34], phosphotriester [35, 36] and phosphite triester [37, 38] synthetic routes.

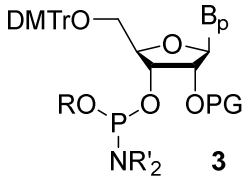
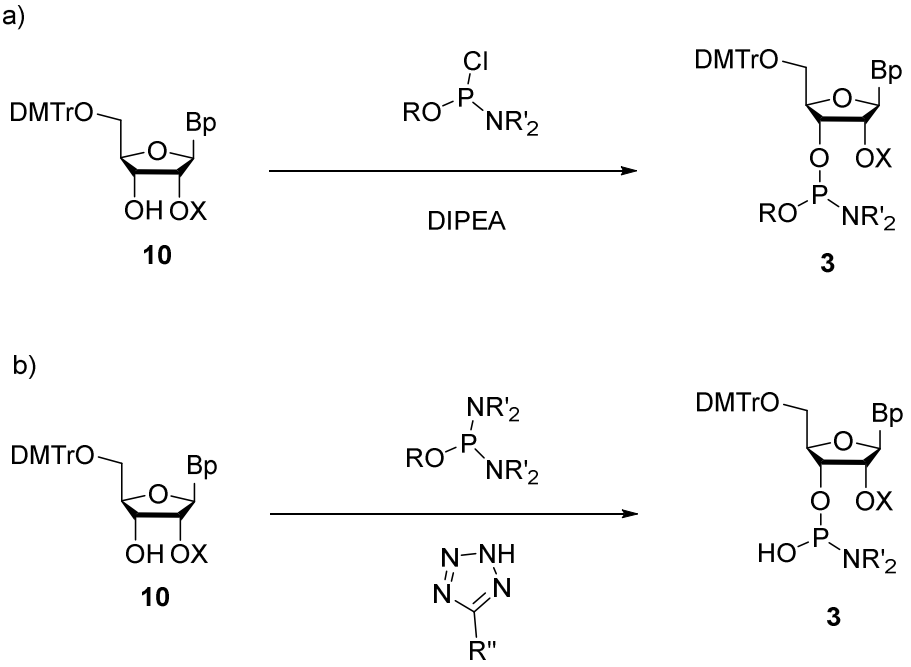


Figure 2. *A typical nucleobase phosphoramidite where Bp = protected base, PG = protecting group and DMTr = 4,4'-dimethoxytrityl*

The typical synthetic route to the phosphoramidite nucleotides is the reaction of the DMTr protected nucleoside with a phosphochloridite reagent [23] in the presence of the base DIPEA

or by bis-dialkylaminophosphines [39, 40] with a tetrazole based activator (*Scheme 3a*). In this context, 2-cyanoethyl *N*,*N*-diisopropylchlorophophoramidite (CEP-Cl) is the most frequently used phosphochloridite reagent. However, the use of phosphochloridites is complicated by their fast degradation in the presence of even small amounts of water.



***Scheme 3**. Phosphoramidite synthesis ; **a)** phosphochloridite, DIPEA, dry conditions (MeCN, DCM etc.) ; **b)** bis-dialkylaminophosphines , tetrazole activator, dry conditions (MeCN, DCM etc.)*

While widely used and relatively reliable, the use of phosphoramidites is not without problems. The relatively high reactivity of the moiety can lead to oxidation of the phosphoramidites in even mildly acidic conditions [23]. The oxidized product is incompatible with solid phase RNA synthesis rendering the oxide useless. Nonetheless phosphoramidite nucleosides remain the primary building blocks used in solid phase oligonucleotide synthesis.

Multiple phosphoramidite reagents have been developed over the years however the most widely used is 2-Cyanoethyl *N*,*N*,*N'*,*N'*-tetra*iso*propylphosphorodiamidite (where R = -CH_2CH_2CN and R' = -^{i}Pr in *Scheme 3b*).

This approach gained popularity due to the ease of deprotection by concentrated ammonia, an approach compatible with other commonly used deprotection methods [23].

2.1.6 Choice of the protecting groups

A number of reactive moieties present in nucleosides selected for oligonucleotide synthesis require protection prior to synthesis. As in DNA synthesis, the 5'-hydroxy group and the

exocyclic amino groups of the natural nucleobases require protection. While RNA synthesis differs significantly from DNA synthesis in the requirement for 2'-hydroxyl protection [20].

2.1.7 Nucleobase protecting groups

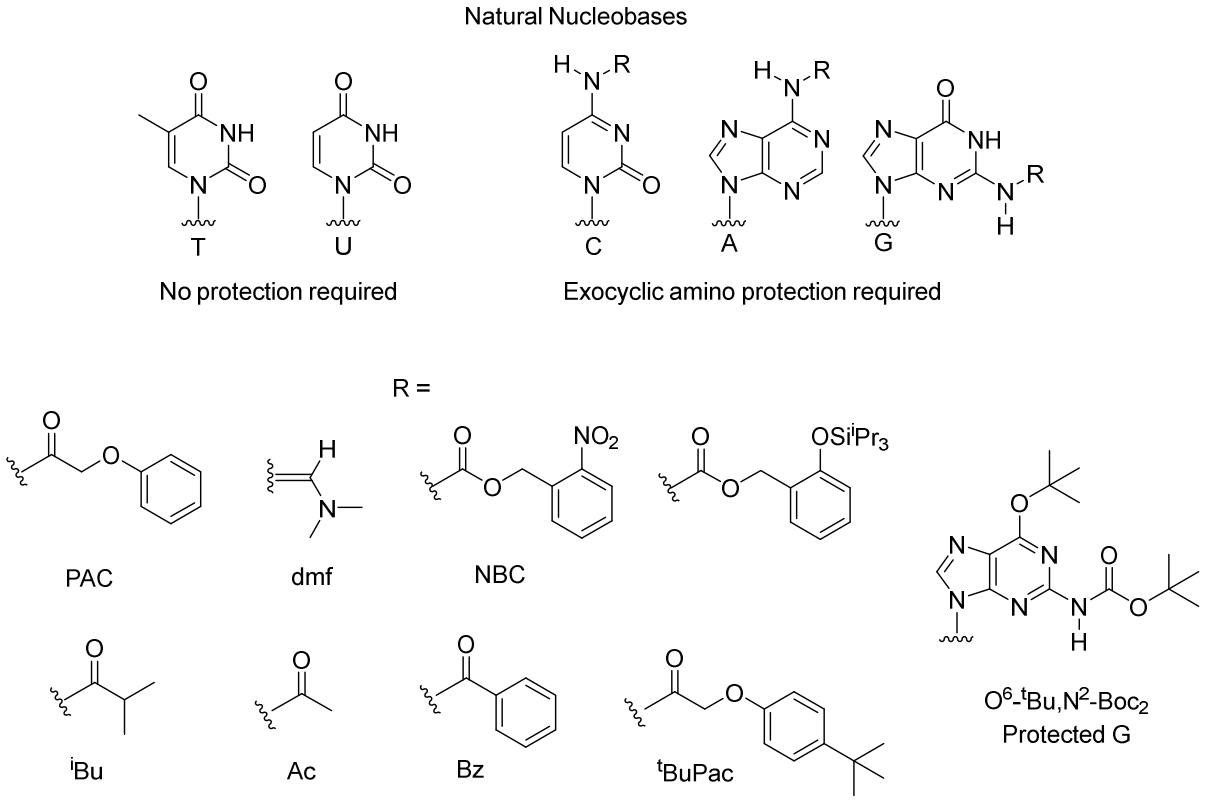


Figure 3. *Examples of common exocyclic amino protecting groups [23]*

The natural RNA bases adenine, cytosine and guanine require additional protection of their exocyclic amino groups (*Figure 3*) [21, 23]. Protecting groups are added prior to 5'-hydroxyl tritylation in order to prevent tritylation of the exocyclic amino group and to subsequently prevent the nucleophilic amino group from reacting with the phosphoramidite during oligonucleotide synthesis. This can be achieved in a number of ways with the most common approach, analogous to the use in DNA synthesis, being the use of acyl derivatives including acetyl, benzoyl and isobutyryl [20]. Some care needs to be taken to ensure compatibility with 2'-protecting groups as selective cleavage of the exocyclic amino groups is required. As such ^{t}BuPac can be selected in place of acetyl, benzoyl and isobutyryl as rapid cleavage in ammonia may preserve the 2'-protecting groups such as TBDMS [41]. Protection of guanosine nucleosides can be more challenging especially when modifications such as 2'-*O*-aminoalkyl units [42-44], 2'-$SeCH_3$ [45], or 2'-N_3 [46] are present on the scaffold. Hence, alternative protecting group patterns such as O^6-*tert*-butyl, N^2(bis-[*tert*-butyloxycarbonyl]) (O^6-^{t}Bu,N^2-Boc_2)

have been proposed for the guanosine nucleobase [47]. Also, modified nucleobases such as the epigenetic modification 5-Hydroxymethylcytosine (hm^5C) require alternative protection patterns to avoid potential side-reactions. In the case of hm^5C, acetyl groups were installed on both the nucleobase and the hydroxyl moiety in order to avoid an S_N2 reaction by $MeNH_2$ which is used during the deprotection of the RNA oligonucleotides following solid-phase synthesis [48].

2.1.8 Protection of the 2'-position

The most important distinguishing factor between DNA and RNA synthesis is the requirement for protection of the 2'-hydroxyl group. In fact, it has been said that selection of 2'-protecting groups during RNA synthesis is 'the single most critical decision that has to be made in undertaking the chemical synthesis of RNA sequences' [49]. A naked hydroxyl group at the 2'-position is primed to wreak havoc on the synthetic process (*Figure 4*). In both basic and acidic conditions phosphate migration can occur leading to cleavage of the oligonucleotide chain [50].

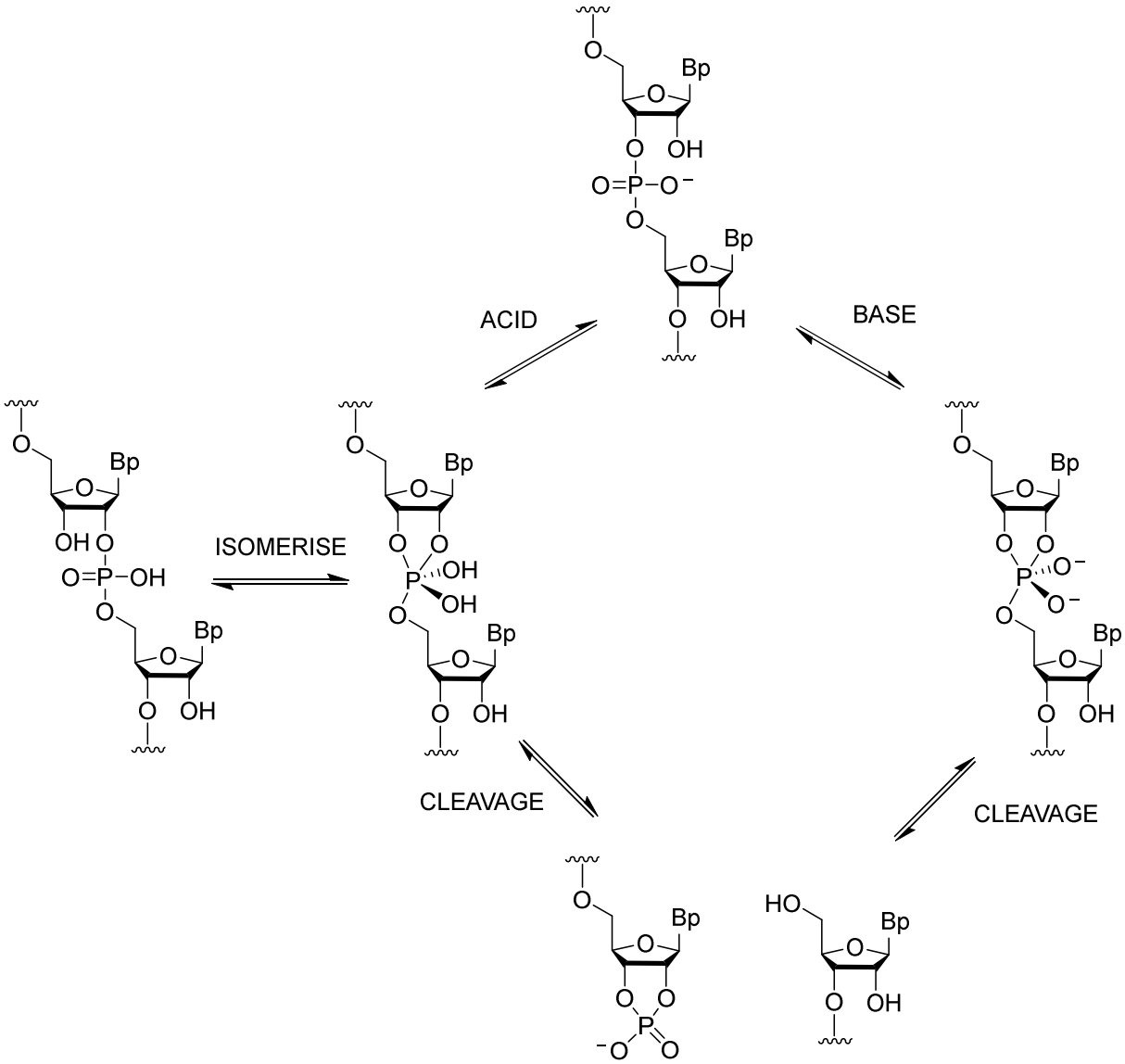


Figure 4. *Overview of phosphate migration and internucleotide chain cleavage possible with unprotected 2'-hydroxyl group. Recreated with permission from [50]*

A wide range of strategies have been employed for protection of the 2'-hydroxyl position with an in depth review cited covering the major findings [49]. Here we present a general overview of the main 2'-protecting groups proposed in the literature (*Figure 5*).

Protection of the 2'-hydroxyl moiety has proven difficult due to the wide range of chemical constraints placed on its selection. The primary requirement of the protecting group is that it must be sustained throughout the synthesis of the oligonucleotide, not being susceptible to deprotection during detritylation, exocyclic amino deprotection and release from the solid support. The design must allow for facile and efficient deprotection in one step, post oligonucleotide synthesis, while also being easily incorporated pre solid-phase synthesis. The

protecting group should not undergo migration to the 3’-hydroxyl position and must not hinder solid phase activation and coupling steps.

Perhaps the most widely employed class of 2’-protecting groups have been the fluoride-labile 2’-protection groups with *tert*-butyldimethylsilyl (TBDMS) being the classic example [51, 52] (*Figure 5*). While TBDMS proved chemically suitable with reliable incorporation pre-oligo synthesis, and maintenance throughout oligonucleotide synthesis, issues relating to rate of coupling were initially encountered. While coupling rates for DNA synthesis were typically below 100 seconds, coupling rates with TBDMS were around 600 seconds or more. This impediment was attributed to the bulky nature of the protecting groups, with increasing bulkiness of protecting groups associated with increased reduction in reaction rates [20]. Although rates of coupling were dramatically increased due to selection of the more potent activators ETT and BMT, many strategies have been explored including other fluoride-labile, base-labile [59, 60], acid-labile [61] and photo-labile [62, 63] groups. A table summarizing some of the reaction rates achieved with various protecting groups is given below (*Table 1*).

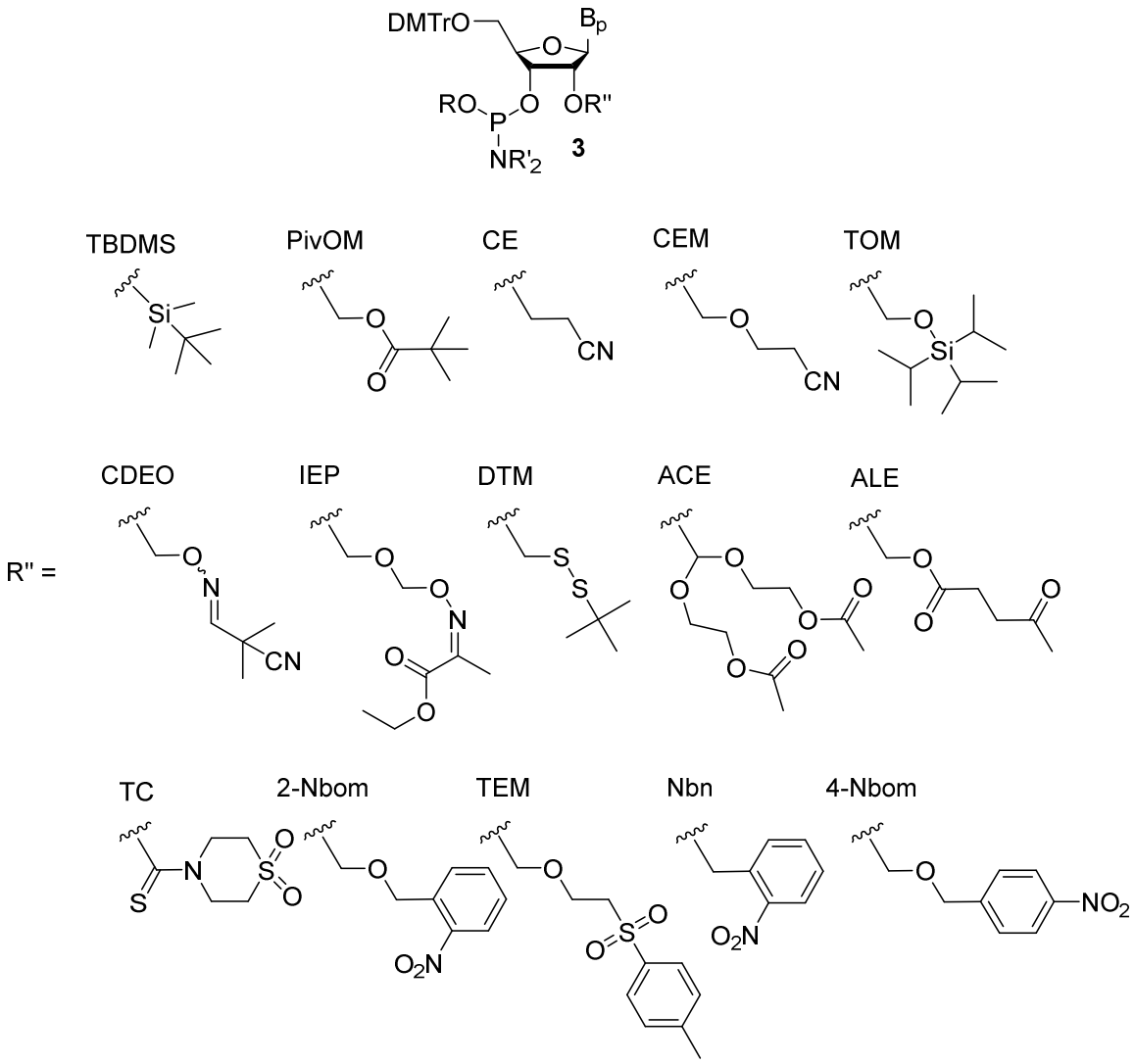
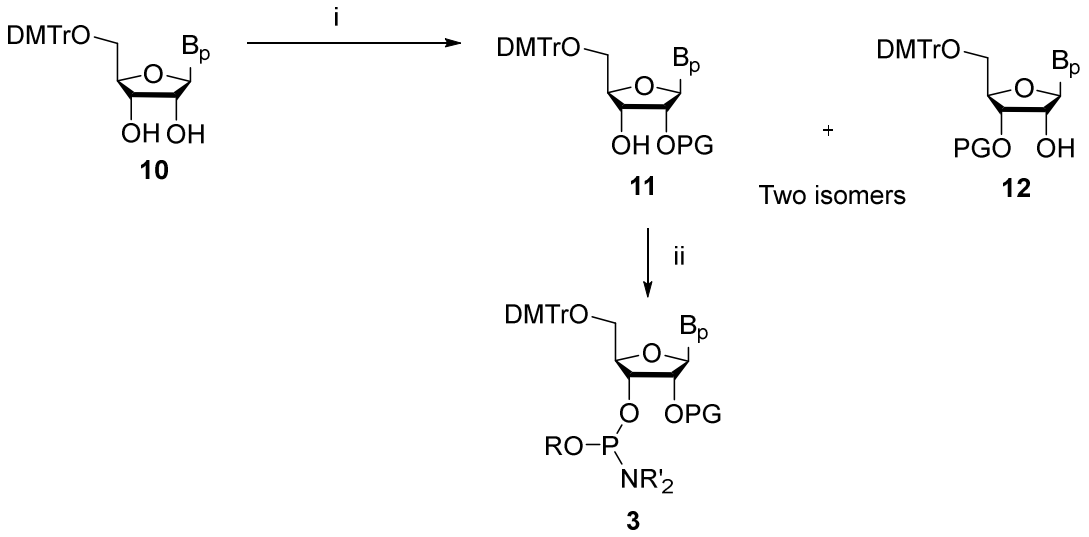


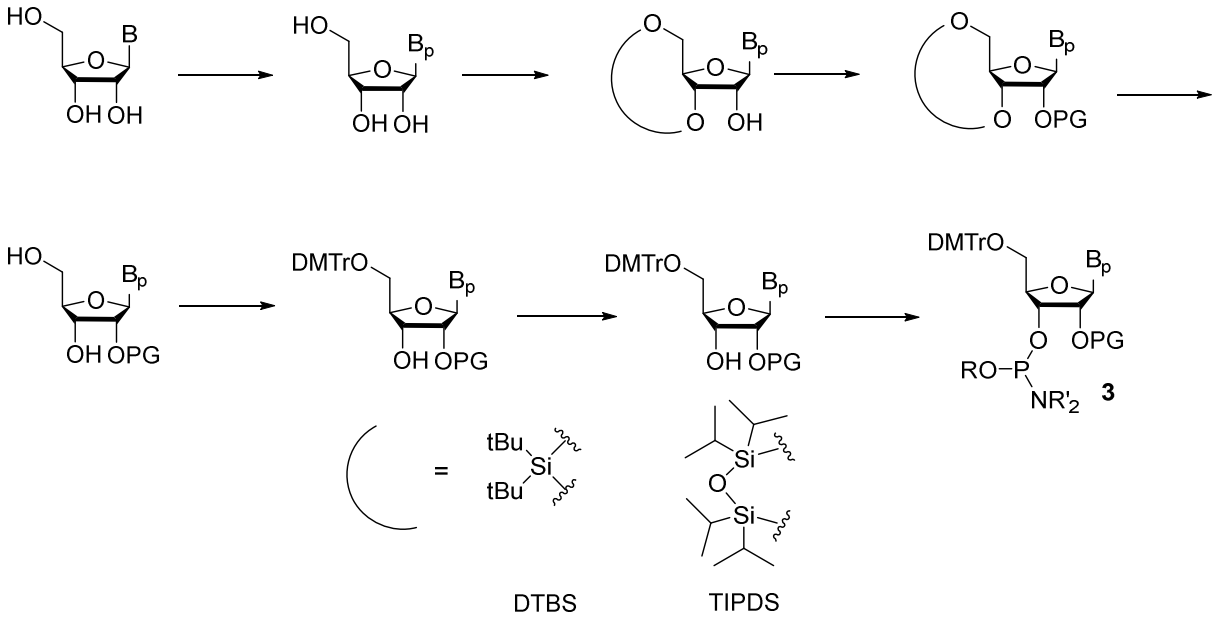
Figure 5. *A selection of 2'-hydroxyl protecting groups proposed in the literature. A summary of chemical properties of the groups is presented in Table 1*

While selective protection of the 5'-hydroxyl position and the exocyclic amino groups can be fairly trivially achieved, selective protection of the 2'-hydroxyl position is complicated by the presence of the neighboring 3'-hydroxy group [20]. Intended protection at the 2'-hydroxyl position can lead to non-regiospecific 2'- and 3'- protection, **11** and **12** (*Scheme 4*). This greatly reduces reaction yields and creates issues for large scale production.



Scheme 4. *Overview of non-regiospecific 2'-protection route and subsequent phosphoramidization; i) typical protection step producing mix of 2'- and 3'-protection; ii) Phosphitylation of the 2'-protected isomer*

Strategies to overcome this and increase yields of 2'-protection include the use of the 'Markiewicz method' [50, 64] (*Scheme 5*). In this approach, bidentate protection of the 3'- and 5'-hydroxyl groups allows for regiospecific protection of the 2'-hydroxyl. While tetra-isopropylsisiloxane (TIPDS) was originally proposed and is still widely used, the incompatibility of TIPDS with fluoride-labile 2'-protecting groups led Beigelman to propose the use of di-tert-butylsilylene (DTBS) where deprotection of the bidentate protecting group is compatible with the 2'-protecting group TBDMS [65]. The use of these synthetic routes leads to dramatic increases in yields and are widely employed.



Scheme 5. *An overview of regiospecific 2'-protection protection of the nucleobase using the 'Markiewicz method' [50]*

2' – PG[a]	**5' – PG**[b]	PA[c]	2’ deprotection	AY[d] (CL[e])	CT[f]	LCL[g] (OY[h])	2’ –PY[i]	year	citation
privaloxyloxymethyl (**PivOM**)	DMTr	Standard[j]	Base (NH_4OH)	99.0 % (21-mer) - 99.7 % (3-mer)	180	21 (83.8 %)		2008	[66], [60]
2-(4-Tolysulfonyl)ethoxymethyl (**TEM**)	DMTr	standard	TBAF (fluoride)	97 % (14-mer) - 99.1 % (20-mer)	120	38 (40 %)	26 - 38 %	2007	[53]
2-cyanoethoxymethyl (**CEM**)	DMTr	standard	TBAF (fluoride)	> 99 % (55-mer)	150 (BMT or ETT)	55 (15 %)		2005	[54] [25] [67]
Cyanoethylated (**CE**)	DMTr	standard	Base (NH_4OH)	NQ	600 (1-h tetrazole and ETT)	17 (33 %)	82.2 – 96.3	2005	[59]
tert-butyldimethylsilyl (**TBDMS**)	MMT	R = OME, R’ = iPr	TBAF (fluoride)	98 % (42)	885 (tetrazole)	42	NQ	1974, 1987	[51] [52]
tris-iso-propylsilyloxymethyl (**TOM**)	DMTr	standard	TBAF (fluoride)	99.3 % (average over >50000)	90 (BMT)	82 (20 – 40 %)	40 – 60 %	2001	[55]
tert-butyldithiomethyl (**DTM**)	DMTr	standard	1,4-dithiothreitol or tris(2-carboxyethyl)phosphine (following NH_4OH treatment)	98.5 – 99.8 % (NQ)	150 (ETT)	45 (NQ)	34 – 57 %	2006	[68]

bis(2-acetoxyethyloxy)methyl (**ACE**)	silyl ethers	R = OME, R' = iPr	pH 3, 10 min., 55 °C	>90 % (NQ)	<90 s	12 (71 %)	65 %	1998	[61]
thionocarbamate (**TC**)	DMTr	standard	ethylenediamine	NQ	600 (ETT)	54 (NQ)	60 – 70 %	2011	[50]
(o-nitrobenzyloxymethyl) (**2-Nbom**)	DMTr	standard	Photolabile	>98 % (33-mer)	120 (NQ)	33 (NQ)	39 %	1992	[62]
p-nitrobenzyloxymethyl (**4-Nbom**)	DMTr	standard	TBAF	99 % (13-mer)	120	13 (28 %)	37 %	1996	[56]
acetal levulinyl ester (**ALE**)	DMTr	standard	Hydrazine hydrate	97 % (12-mer)	60 (DCI)	12 (NQ)	78 %	2008	[69]
2-cyano-2,2-dimethylethanimine-N-oxymethyl (**CDEO**)	DMTr	standard	TBAF	99 % (20-mer)	180 (BMT)	20 (NQ)	54 – 82 %	2013	[58]
iminooxymethyl ethyl proponate (**IEP**)	DMTr	standard	TBAF	NQ (20-mer)	NQ	20 (NQ)	78 %	2016	[57]

Table 1. *Summary of 2'-protecting groups proposed for use in solid phase RNA synthesis. Values are quoted from original publication, improved values may have been published in subsequent publications; [a] 2'-protecting group; [b] 5'-protecting group; [c] Phosphoramidite moiety; [d] average stepwise coupling yield (%); [e] Chain length ; [f] coupling time (s) ; [g] longest chain length achieved (mer); [h] overall yield (%); [i] 2' protection yield; [j] standard phosphoramidite:, R = CH_2CH_2CN, R' = iPr; NQ – not quoted*

2.2 Synthesis of chemically modified RNA

2.2.1 Synthesis of phosphorothioates

As outlined in the introduction, the lifetime of RNA in biological settings is severely reduced by the susceptibility of RNA to hydrolysis by nucleases. Therefore, phosphorothioates are important analogues of the phosphate backbone, where a non-bridging oxygen atom has been replaced by sulfur. Fortunately, the phophorothiate backbone can be efficiently, cheaply and easily achieved through modification of the oxidation step of oligonucleotide synthesis. Rather than oxidation, a sulfur transfer step is introduced using one of the commercially available sulfur transfer reagents including the Beaucage reagent [70] and DDTT [21] (*Figure 6*) . The resulting phosphorotioate linkage, post deprotection, is chiral and the sulfurization step results in a mixture of diastereoisomers (*S*p/*R*p ratio), *Figure 11*. Interestingly, the nature of the activator and the 2'-*O*-protecting groups have a direct influence on the ratio of diastereoisomers during solid-phase synthesis of phosphorothioate containing RNA sequences. For instance, with 2'-O-TBDMS protected amidites (see above) and BMT as activator the *R*p isomer is predominantly formed while with tetrazole and the same protecting group the *S*p isomer is mainly produced [71]. This observation can have strong implications since *S*p and *R*p containing oligonucleotides have different stabilities and enzyme recognition capacities.

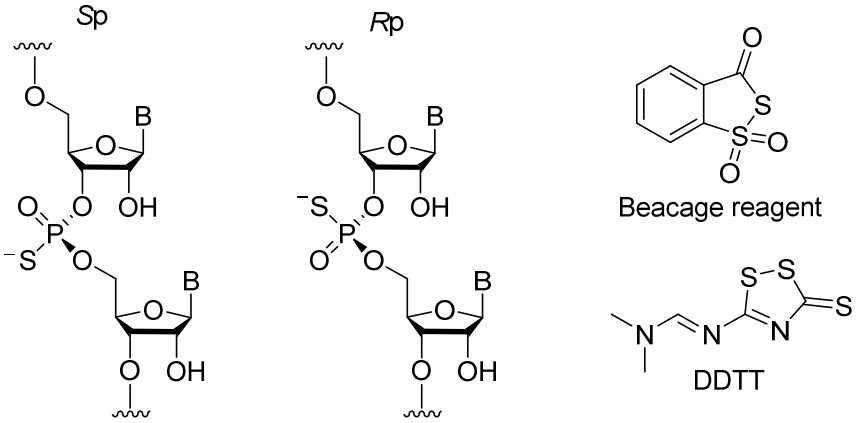
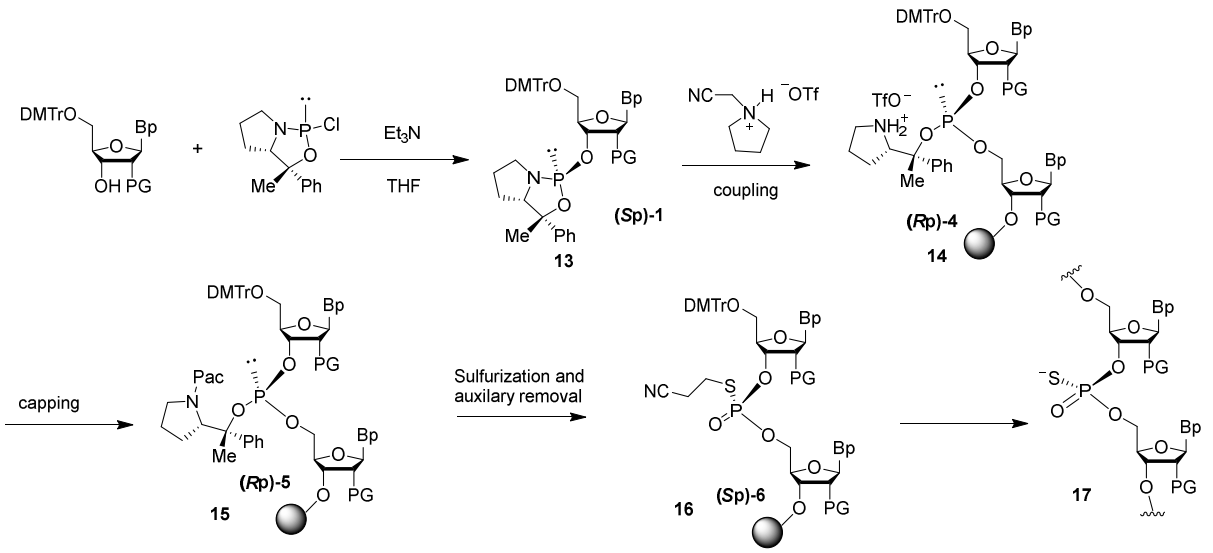


Figure 6. *Structures of the phosphorothioate backbone diasteroisomers and sulfurizing reagents*

Recently, a method based on a chiral auxiliary was developed to control the stereochemical outcome of phophorothiate RNA (and DNA) oligonucleotides [72]. The process involves introduction of a chiral phospho-group in place of the phosphoramidite moiety, **13**. In combination with specialized activators, sterically pure oligonucleotides can be achieved (*Scheme 6*). Alternatively, the issue of *S*p/*R*p ratios can be overcome by introducing achiral phosphorodithioate units where both non-bridging oxygens are replaced by sulfur [73].



Scheme 6. *Overview of the synthetic strategy employed in the solid phase synthesis of sterically pure phosphorothioate oligonucleotides; PG = -H(DNA); -OCH_3 (2'-OMe RNA); -$OCH_2CH_2OCH_3$ (2'-MOE RNA)*

2.2.2 Incorporation of sugar modifications

A wide range of sugar modifications have been proposed and employed in the synthesis of AONs, siRNAs, and anti-miRNAs [74-76]. Dozens of modifications involve incorporation of exotic groups at the 2'-hydroxyl group and are generally compatible with solid phase RNA synthesis, with the added benefit of acting as 2'-protecting groups.

Some sugar modifications involve more fundamental changes to the sugar structure [74]. Arabino Nucleic Acids (ANAs), where the 2'-hydroxyl group has been inverted, were introduced by Dahma [77] and shown to be compatible with solid phase RNA synthesis with acetyl protection at the 2' position using standard phosphoramidite chemistry (*Figure 7*). Recently, the ANA scaffold was used to install dyes on siRNAs [78]. Bridged nucleic acids (BNAs) are a class of nucleic acid employed extensively in ASOs due to their enhanced affinity to complimentary RNA and DNA [79]. The original BNA, locked nucleic acid (LNA) is compatible with solid phase synthesis using DMTr and standard phosphoramidite chemistry [80]. Many analogues of LNA have subsequently been proposed [74]. Other sugar modifications will be described in the section dedicated to the generation of aptamers (Section 3.4).

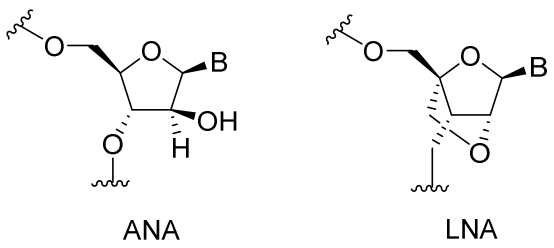


Figure 7. *General chemical structures of the sugar modified analogs ANA and LNA*

2.2.3 Incorporation of base modifications

The solid phase synthetic route to RNA is perfectly primed for the incorporation of chemically modified bases. Dozens of modified bases have been shown compatible with many being used for anti-sense oligonucleotides (AONs) [81], small interfering RNAs (siRNAs), micro-RNA-targeting oligonucleotides (anti-miRNAs) [75], and fluorescent labelling of RNAs [82, 83]. There is growing interest in the role RNA modification plays in the regulation of gene expression [3]. As such a need for synthetically modified RNA mimicking the natural modifications present in RNA is increasing.

2.2.4 Incorporation of methylated nucleobases

There have been six modified bases discovered at mRNA internal positions so far [84] (*Figure 8a*), while many other naturally occurring methylated bases have been discovered with a selection shown below (*Figure 8b*) *[85] .*

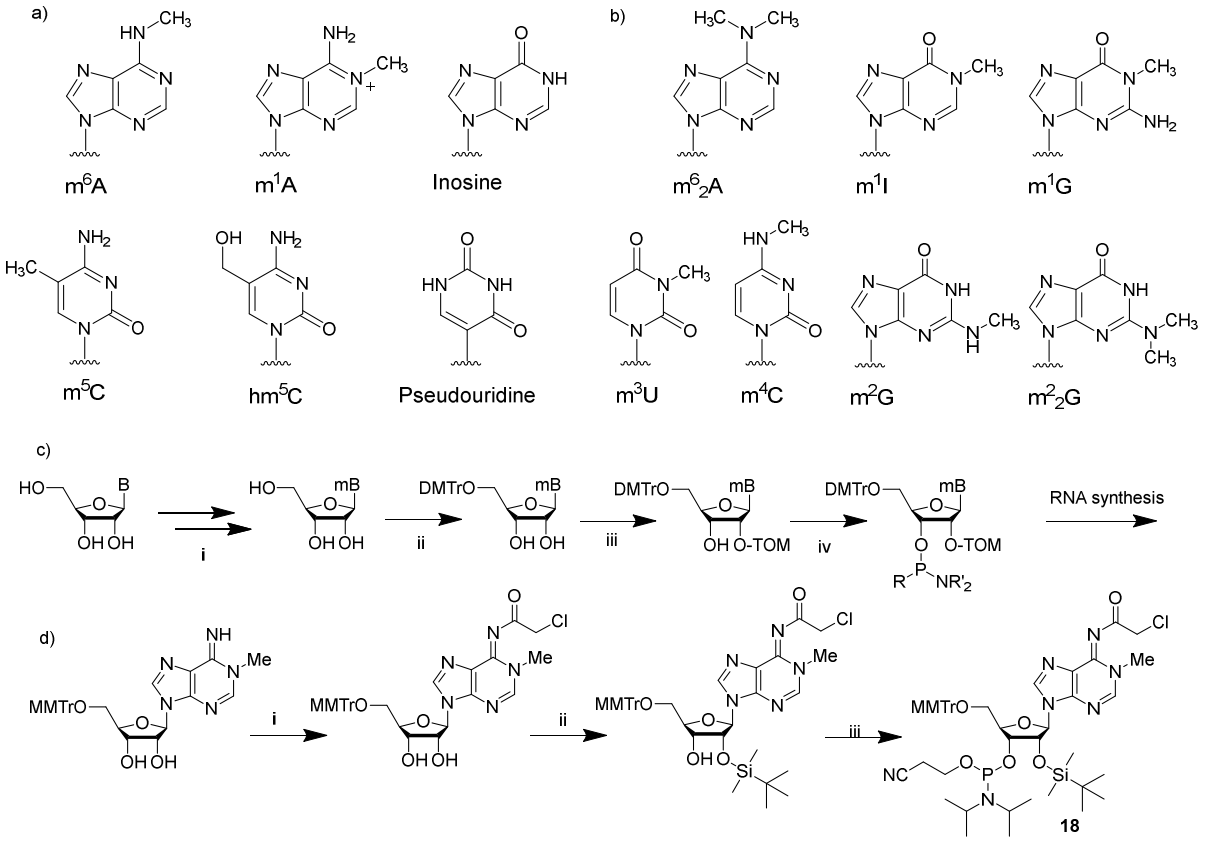


Figure 8. a) *Structures of the modified bases known to be present at central positions in mRNA [84];* ***b)*** *structures of the naturally occurring modified bases incorporated using RNA synthesis by Micura and coworkers;* ***c)*** *General synthetic route to the modified nucleobase amidites in [85]; B = A, C, G, U, I; mB =* m^6A, $m^6{}_2A$, m^4C, m^3U, m^1I, N^2-Ac-m^1G, O^6-NPE-m^2G, O^6-NPE-$m^2{}_2G$; ***d)*** *Synthetic route to the 1-Methyladenosine amidite building block used in [86]; i) MMTrCl in pyridine; ii) 1)* $(ClCH_2CO)_2O$ *in pyridine 2)* $tBuMe_2SiCl$*-imidazole*

Extensive work by Micura and coworkers led to a general synthetic route to methylated ribonucleoside amidites for use in solid phase RNA synthesis [85]. The general route was shown to be suitable for synthesis of the ribonucleosides 1-methylguanosine (m^1G), *N*^{2}-methylguanosine (m^2G), *N*^{2},*N*^{2}-methylguanosine ($m^2{}_2G$), 1-methyinosine (m^1I), 3-methyluridine (m^3U), *N*^{4}-methylcytidine (m^4C), *N*^{6}-methyladenosine (m^6A) and *N*^{6}, *N*^{6}-dimethyladenosine ($m^6{}_2A$), *Figure 13b*. The nucleobase is first modified and protected before trytilation by DMTr. 2'-hydroxyl protection is provided by 2'-*O*-TOM and the standard 3'-*O*-2-cyanoethyl-diisopropylphosphoramidite (CEP) reagent was used to yield the corresponding phosphoramidites (*Figure 8c*). The resulting phosphoramidites displayed coupling yields ranging from 97 to 99.5 % [87-90].

Herdewijn and coworkers successfully incorporated the chemically modified base 1-methyladenosine (m^1A) into RNA using solid phase synthesis [86]. 1-Methyladenosine was first prepared by methylation of adenosine by an established synthetic route [91]. Following addition of the 4-Methoxytrityl (MMTr) protecting group at the 5'-hydroxy position, the exocyclic amino group was protected by an alpha chloro acetyl group. TBDMS protection and the standard phosphoramidite were chosen for the 2'- and 3'- hydroxyl positions (*Figure 8d*). The modified nucleoside was then successfully incorporated once in a 17-mer sequence. The coupling time was set to 10 minutes with base-labile groups removed by 2 M NH_3 in MeOH and TBDMS removed by TBAF in THF.

More recently, Li and coworkers also successfully incorporated 1-Methyladenosine (m^1A) alongside 3-Methylcytosine (m^3C) [92]. Importantly they described formation of undesired byproducts with Dimroth rearrangement of the m^3C leading to formation of m^6A and deamination of m^3C leading to formation of m^3U. As m^1A and m^6A have identical molecular weights and m^3C and m^3U differ by only 1 dalton the formation of these byproducts could potentially be missed. A detailed characterization method to distinguish products is outlined with the paper serving as a warning to the potential pitfalls of relying on MS for characterization.

3. Enzymatic synthesis of RNA.

3.1 Overview of the method

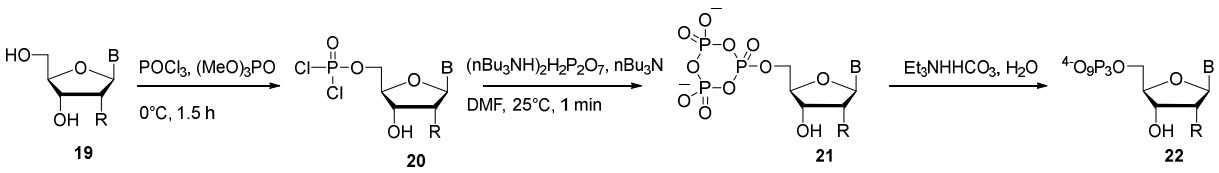
Automated solid phase synthesis grants access to larger quantities but is only suitable for synthesis of rather short RNA oligonucleotides (up to 60 nucleotides). However, for certain practical applications, long RNA sequences are required or sensitive chemical moieties need to be incorporated and both requirements cannot be met by solid support chemical synthesis. Alternatively, modified RNA oligonucleotides can be obtained by enzymatic synthesis. Particularly, modified nucleoside triphosphates (N*TPs) can be synthesized and used as substrates for RNA polymerases which incorporate these nucleotide analogs in the growing RNA chain during *in vitro* transcription [93]. Provided the polymerase accepts the modified substrate, the mild conditions allow for the incorporation of a broad array of functional groups with no apparent size limitation for the oligonucleotides [94-98]. Prior to the enzymatic synthesis of modified RNA oligonucleotides, several steps are necessary which will be described in this section.

3.2. Common synthetic pathways for modified nucleoside triphosphates.

Modified nucleoside triphosphates often need to be synthesized chemically or enzymatically. However, even though some biocatalytic methods have been developed for the preparation of nucleoside triphosphates equipped with unnatural bases or sugar units [99, 100], they do not provide a general route for the preparation of N*TPs. This is mainly due to the need of finding a suitable and generally applicable enzyme that would accept the modified nucleosides or nucleotides. Similarly, a generally applicable synthetic protocol has not yet been established due to substrate-dependent variations in both yields and ease of purification. Therefore, this part of this review article focuses on the description of the most common chemical ways for the crafting of N*TPs. The interested reader is directed to other excellent review articles for the description of other existing synthetic protocols [101-106].

3.2.1 Yoshikawa one-pot methodology

Yoshikawa et al. reported in 1967 one of the first methods for the synthesis of nucleoside triphosphates [107, 108], which was improved in 1981 by Ludwig (*Scheme 7*) [109]. In this 5'-regioselective one-pot reaction, the unprotected nucleoside **19** reacts with phosphorus oxychloride in trimethylphosphite to give the reactive phosphorodichlorate **20**. The latter can directly react with bis-tributylammonium pyrophosphate in presence of an amine base to generate a cyclic triphosphate **21**. The reaction is then quenched with triethylammonium bicarbonate buffer to give the corresponding NTP **22**.



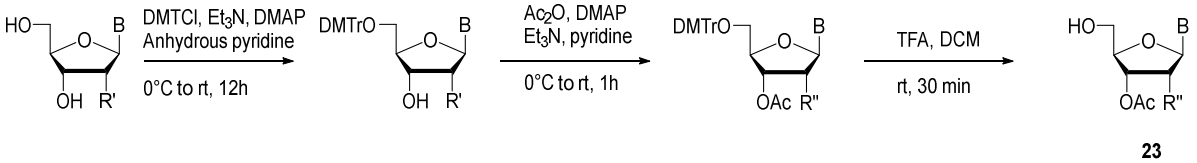
B = natural or modified nucleobase
R = OH or modification

Scheme 7. *Yoshikawa protocol for the synthesis of nucleoside triphosphates.*

Even if no protecting group is required, reports have shown the formation of side-products which could complicate the purification step of the nucleoside triphosphates [110, 111]. It should also be noted that these triphosphorylation reactions are highly water-sensitive and the use of hygroscopic alkylammonium salts could have deleterious consequences. Recent examples of N*TPs synthesized by this method include a fluorescent GTP analog [112] and various 5-substituted pyrimidine and 7-substituted 7-deazapurine nucleotides [93, 113]. A variant of the Yoshikawa protocol was recently reported by Korhonen et al. where tris(bis(triphenylphosphoranylidene)ammonium) (PPN) pyrophosphate was proposed as an alternative to the hygroscopic salts [114].
However, the use of the strong electrophilic phosphorus oxychloride might not be suitable with all modified nucleosides.

3.2.2 Ludwig-Eckstein “one-pot, three-steps” synthesis

Ludwig and Eckstein developed, in 1989, an alternative route for the preparation of NTPs [115]. As this pathway is non-regioselective, the 2’- and 3’-positions of NTPs need to be protected prior to the triphosphorylation step. One popular protecting strategy is presented in *Scheme 8*, where the 5’-position of the nucleotide is tritylated, followed by a 2’- and 3’-*O*-acetylation. The deprotection of the DMTr group leads to the suitably protected precursor **23**.

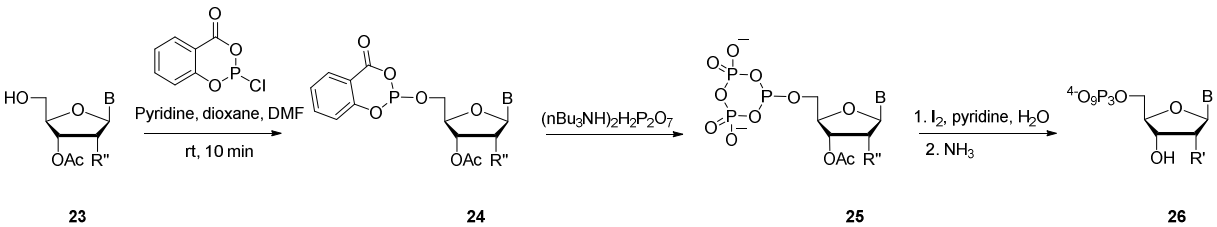


B = natural or modified nucleobase
R' = OH or modification
R'' = OAc or modification

Scheme 8. *Common protecting method used prior to Ludwig-Eckstein triphosphorylation.*

The free hydroxyl group of **23** can react with salicyl phosphorochlorite to give the activated 5’-phosphite intermediate **24** (*Scheme 9*). Tris(tetra-*n*-butylammonium) hydrogen pyrophosphate is then added to form the cyclic intermediate **25**. The latter is finally oxidized

by iodine and the acetyl groups are deprotected by the use of ammonia, to yield the NTP **26**. The reaction can easily be followed by ^{31}P NMR.



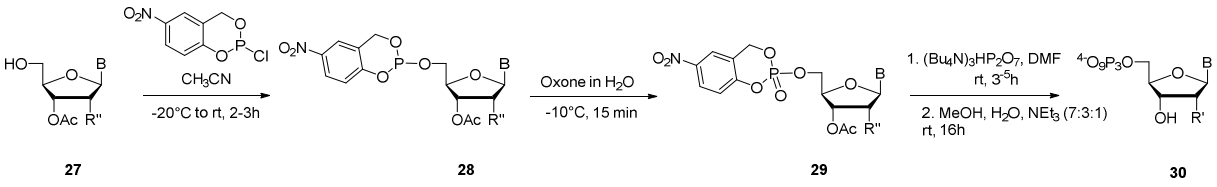
B = natural or modified nucleobase
R' = OH or modification
R" = OAc or modification

Scheme 9. *Ludwig-Eckstein triphosphorylation method.*

Despite the longer route of this protocol, the protection of the hydroxyl groups consequently reduces the number of undesirable by-products, easing the HPLC purification. The yields obtained by this method usually vary from poor to moderate because the reaction depends on the nature of the substrate.

3.2.3 Alternatives to the Ludwig-Eckstein strategy

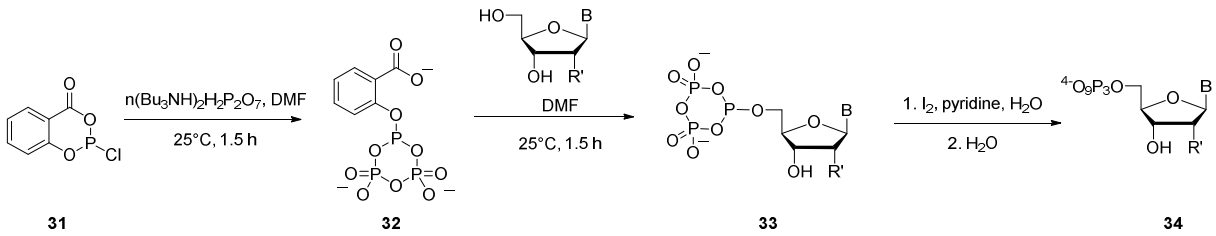
In 2009, Warnecke and Meier published a complementary method to the Ludwig-Eckstein strategy and demonstrated its efficiency for both natural and modified nucleosides (*Scheme 10*) [116]. It is based on the use of 5-nitro-*cyclo*Sal-phosphochloridite [117] which can react rapidly with the 5'-OH group of a nucleoside, to give intermediate **28**. The 5-nitro substitution was chosen in order to increase the electrophilicity of the phosphorus atom, due to the electron-withdrawing effect of the nitro group. **28** is then oxidized in the presence of oxone to form the *cyclo*Sal-triester **29**. After extraction, **29** is reacted with pyrophosphate and the acetyl group is deprotected. Triphosphate **30** is then purified by chromatography with increased yields compared to the Ludwig-Eckstein process (40-80%).



B = natural or modified nucleobase
R' = OH or modification
R" = OAc or modification

Scheme 10. *Synthesis of triphosphates via cycloSal activated nucleosides.*

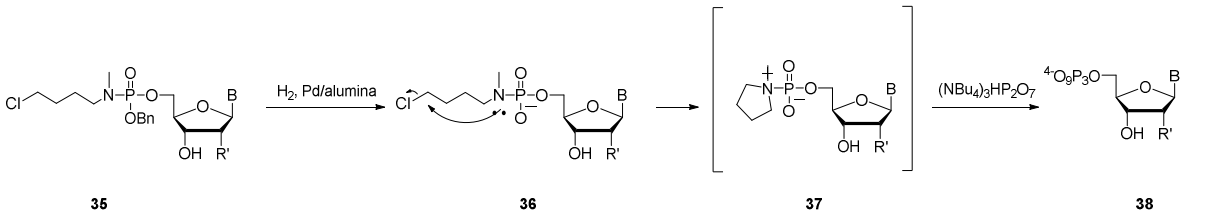
A few years later, Caton-Williams et al. reported an altered one-pot method based on the use of salicyl phosphorochlorite (*Scheme 11*) [118]. The latter is first reacted with a pyrophosphate to give **32**. The bulky phosphitylating reagent can then selectively react with an unprotected nucleoside to give compound **33**. After oxidation, hydrolysis and purification by RP-HPLC, nucleotide triphosphate **34** can be obtained [119].



Scheme 11. *One-pot triphosphorylation method developed by Caton-Williams et al. [118].*

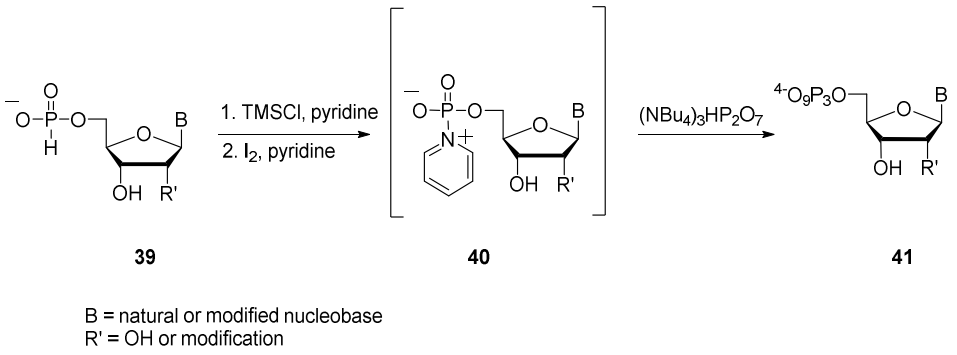
This synthesis can be easily monitored by ^{31}P NMR and does not need any protection of the nucleoside. However, the same group demonstrated that 3'-triphosphates constituted the major by-products with 5-10% formation [120]. Recent examples of N*TPs obtained by this method include photocaged nucleotides [121], arabinose [122] and threose-based analogues [123, 124], 2'-(R)-fluorinated derivatives of epigenetic bases [125], 2'-methylseleno-nucleotides [126], and 2'-*O*-carbamoyl uridine triphosphates [127].

An original method involving a reactive zwitterionic intermediate has been developed by Borch (*Scheme 12*) for the synthesis of both natural and modified triphosphates [128]. The non-protected nucleoside bearing an *O*-benzyl-phosphoramidate ester **35** is activated by catalytic hydrogenation. The removal of the protecting group causes a spontaneous rearrangement of molecule **36** which leads to a pyrrolidinium phosphoramidite zwitterion **37**. The latter reacts *in situ* with a nucleophile pyrophosphate, to give the corresponding triphosphate **38** with high yields (50-80%).



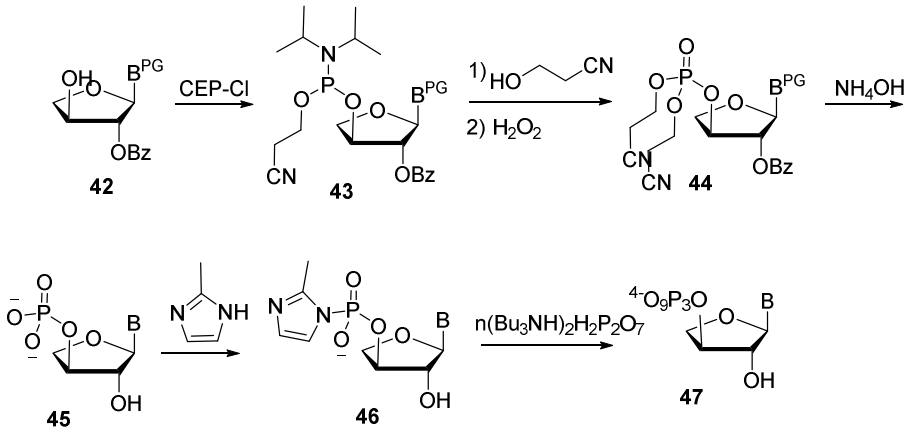
Scheme 12. *Borch alternative for the synthesis of nucleoside triphosphates.*

However, as preparation of **35** is long and tedious, Peterson et al. proposed to use more accessible *H*-phosphonate nucleosides **39** as starting materials (*Scheme 13*) [129]. Silylation and oxidation lead to a pyridinium phosphoramidate **40**, which can react easily with pyrophosphate. The usefulness of approach for the synthesis of N*TPs has been demonstrated since the triphosphates of ribavirin and 6-methylpurine were obtained by this variation of the Borch method.



Scheme 13. *Use of H-phosphonates in Borch synthesis.*

Lastly, an elegant synthetic approach was designed for the synthesis of threose nucleic acid (TNA) triphosphates **47** (*Scheme 14*). Unlike other RNA analogues, TNA contains vicinal 2',3' phosphodiester linkages and the synthesis of the corresponding triphosphates requires the phosphorylation of the 3'-OH moiety which is less reactive than a primary 5'-hydroxyl group of unmodified nucleotides [130]. In order to circumvent the low reactivity of the 3'-hydroxyl group, the Chaput laboratory has devised a number of synthetic strategies to obtain the corresponding triphosphates equipped with either canonical [130-132] or modified nucleobases [97, 133, 134]. A recent and elegant method is depicted in Scheme 8 which involves the building of the corresponding 3'-monophosphate TNA nucleosides **45** via 3'-*O*-phosphoramidite intermediates **43**. The resulting 3'-monophosphates **45** are then converted to the corresponding triphosphates **47** via the activated 3'-phosphoro-(2-methyl)-imidazolide intermediates **46**.



Scheme 14*. Synthetic pathway leading to TNA triphosphates [130].*

3.3. Modified NTPs and enzymatic synthesis.

Chemical modification of RNA oligonucleotides is an alluring strategy for numerous applications in biotechnology, biomedical research as well as the biophysical investigation of the structural and functional properties of RNA [135, 136]. Amongst all possible modifications, a commonly used pattern is the inclusion of functional groups at position 2' of the sugar since it induces an increase in nuclease resistance, chemical stability [137] and in some cases influences RNA structure, hydration and base selectivity [138-140]. However, due to the high substrate specificity of natural polymerases, the enzymatic incorporation of modified nucleotides into RNA still represents a challenge [141]. In this part, different mutant polymerases as well as ribozymes are discussed that have proven their acceptance for specific types of modification (summary in *Table 2*). Other strategies and examples can be found in the recent review by Holliger et al. [142].

3.3.1 RNA polymerases

Bacteriophage T7 RNA polymerase has been extensively studied because of its high efficiency of polymerization of the canonical nucleotides. Unlike DNA polymerases, the DNA-dependent T7 RNA polymerase does not require a primer for RNA synthesis but recognizes a specific promoter region [143, 144]. Transcription initiation occurs if guanosines are present in the +1 and/or +2 positions [145]. Wild type T7 RNA polymerase has been reported to accept a reasonable variety of chemically modified nucleotides [93] as substrates and hence can be used to synthesize RNAs with various functionalities including cross-linking agents [146, 147], amino acid-like side-chains [148-152], groups for post-transcriptional modification and labelling [16, 94, 95, 146, 153-158], fluorophores [96, 112, 159, 160], and 2'-*O*-carbamoyl- [127] and 2'-fluoro-substituents [161] (*Figure 9*).

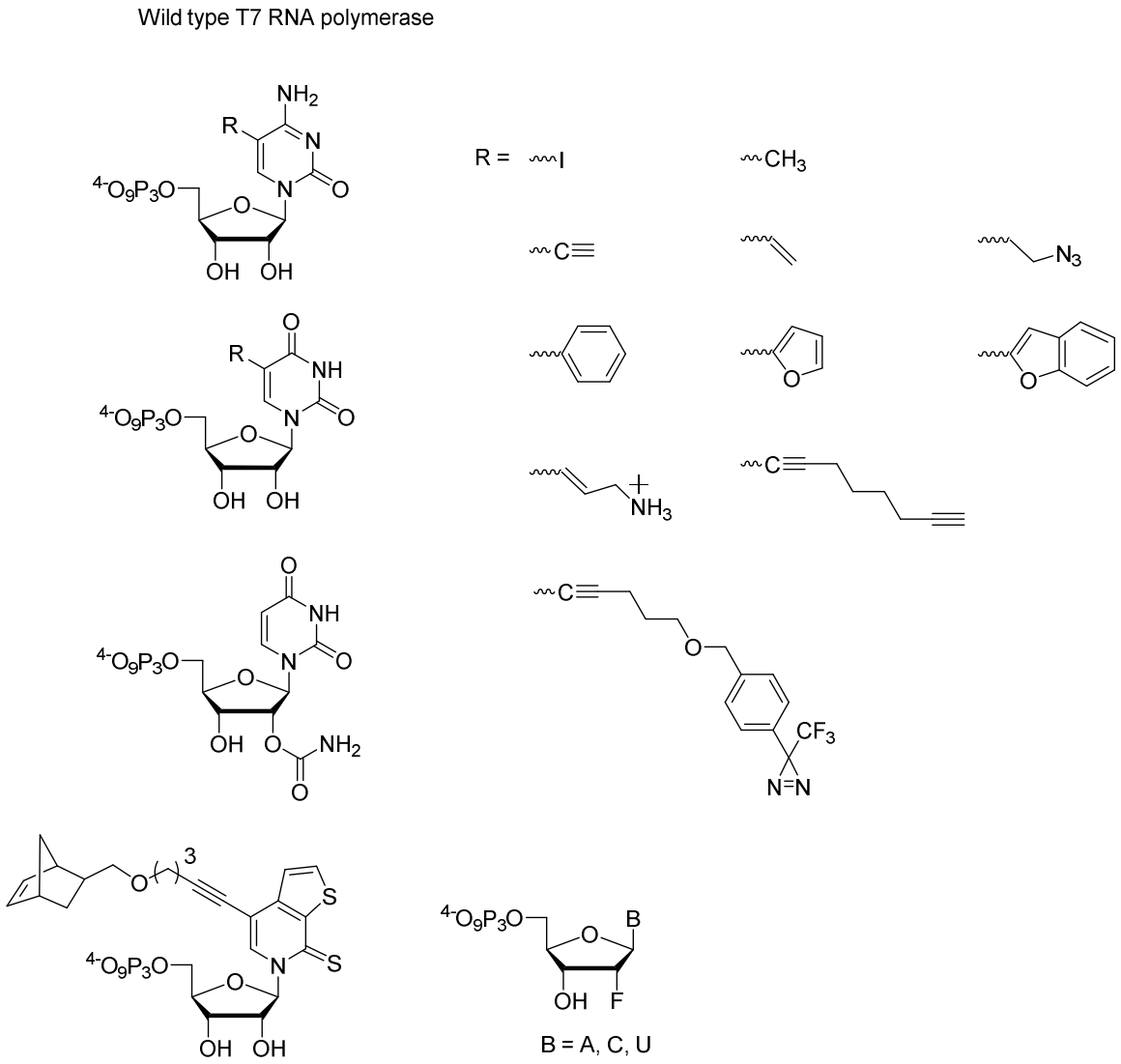


Figure 9. *Structures of recent modified triphosphates accepted as substrates by wild type T7 RNA polymerase.*

However, as some modifications are not accepted as substrates by the wild-type T7 RNA polymerase, enzyme engineering has been developed to create mutants that could efficiently incorporate these nucleotide analogues. Sousa and Padilla published in 1995 the T7 RNA Y639F mutant that readily accepts 2'-Fluoro pyrimidines as substrates [152, 162]. A few years later, a second mutation led to the identification of T7 RNA Y639F H784A which demonstrated the ability to accept bulky substituents at the 2'-position such as 2'-OMe or 2'-azido [163]. Even though these two engineered polymerases are among the most commonly used, libraries of the T7 RNA polymerase have been screened to find yet other mutants with increased substrate acceptance of N*TPs [164-166]. Despite the continuous effort in the engineering of the T7 polymerase, the incorporation of modified GTPs still remains challenging due to the need of guanosine in the +1 and/or +2 position of the transcript.

In 2012 a thermophilic RNA polymerase, homologous to bacteriophage T7, was reported [167]. The marine cyanophage Syn5 was found to be able to initiate polymerisation without the need of GTP. This polymerase also requires a lower temperature and lower concentrations of nucleotides than the parent T7 RNA polymerase. A few years later, Richardson et al. demonstrated that the Syn5-Y564F mutant could incorporate 2'-Fluoro nucleotides with higher efficiency compared to the T7-Y639F RNA polymerase and may be a suitable alternative for the synthesis of transcripts that are not recognised by the T7 polymerase [168].

3.3.2 DNA polymerases

DNA polymerases favour the incorporation of dNTPs by 10^3-10^6 fold compared to the corresponding NTPs [169]. The discrimination between ribo- and deoxyribonucleotides for DNA polymerases is made by a steric gate residue which prevents the incorporation of nucleotides with 2'-substitutions [170]. As a consequence, no wild type DNA polymerase is able to accept modified NTPs for RNA synthesis. Yet, a common mutation of Tyr12 into Ala12 leads to an increase of NTP tolerance [171] and several groups have used directed evolution to create DNA polymerases able to recognise natural and unnatural NTPs [142].

In 2002, Romesberg et al. developed mutants of the Stoffel fragments of *Taq* DNA polymerase I (SFR1, SFR2, SFR3) that were able to efficiently incorporate natural NTPs over dNTPs [172]. One of these mutant polymerases, *Taq*-SFM4-3, maintained the thermostability of the parent enzyme and could be used in an RNA equivalent of PCR called Polymerase Chain Transcription (PCT) to amplify mixed DNA and RNA sequences [173]. However, these engineered enzymes were found to be unable to synthesize modified RNA beyond 8 nucleotides. Consequently, the most potent of these mutant polymerases, SFM4-3, was further improved by an optimized phage display selection system. The resulting SFM19 enzyme readily accepts NTPs as well as 2'-OMe and 2'-Fluoro-NTPs as substrates and served in the synthesis of 60-mer long RNA oligonucleotides [174].

Holliger et al. reported on the isolation of the *Taq*-AA40 polymerase which can incorporate modified 2'-Fluoro nucleotides into short RNA polymers and to display reverse transcriptase activity [175]. However, the incorporation of 2'-amino or 2'-OMe nucleotides was found to be ineffective.

In 2012, a deep study of *Thermococcus gorgonarius* (Tgo DNA polymerase) has led to the discovery of two important mutations for the improvement of RNA synthesis (i.e. E664K and Y409G). The primer dependent polymerisation of long RNA strands (up to 1.7 kb) was reported with modified nucleic acids [176]. The Tgo mutant was also found to accept 2'-modifications such as 2'-Fluoro, 2'-OMe and 2'-azido nucleotides. The enzymatic synthesis of fully modified XNAs strands was also described for arabinose nucleic acids (ANA), 2'-Fluoro arabinose nucleic acids (FANA), cyclohexenyl nucleic acids (CeNA), locked nucleic acids (LNA) and 1,-anhydrohexitol nucleic acids (HNA) [177]. These impressive properties then

culminated in the selection of XNA-based aptamers [177] and catalysts [178].
More recently, the 352 amino acids constituting the DNA polymerase IV from *Sulfolobus solfataricus* were converted to their mirror image (D-amino acids) and the resulting thermostable D-DNA polymerase D-Dpo4-3C enabled the amplification of L-DNA oligonucleotides [179]. The authors envision the use of such polymerases to create modified and unmodified L-messenger and L-ribosomal RNAs for the production of mirror image life. As L-nucleic acids are neither recognised by the immune system nor by nucleases, mirror image polymerases could be useful for the production of L-RNA or DNA aptamers with a high plasma and serum stability, known as Spiegelmers [180]. A first step towards the synthesis of L-RNA oligonucleotides was taken by producing mutants of the D-Dpo4-3C polymerase: the Tyr12 side chain which is involved in the discrimination between dNTPs and NTPs was mutated to a serine and an alanine. Both mutants were capable of elongating natural D-DNA and D-RNA primers on an 81-nt long D-DNA template with both D-dNTPs and D-NTPs as well as synthesizing mirror image L-DNA oligonucleotides under PCR-like conditions [179].

Polymerase	Mutation	Type of accepted modification	Ref.
RNA polymerase T7	Y639F, H784A	2'-OMe, 2'-Fluoro, 2'-azido, base modifications	[162, 164]
RNA polymerase Syn5	Y564F	2'-Fluoro	[167, 168]
DNA polymerase *Taq* I	AA40, SFM4-3	2'-OMe, 2'-Fluoro, 2'-azido	[173, 181, 182]
DNA polymerase Tgo	E664K, Y409G, A485L	CeNA, ANA, HNA, FANA, LNA, 2'-azido, 2'-OMe, 2'-Fluoro	[176, 177]
DNA polymerase IV	D-Dpo4-3C Tyr12Ser, Tyr12Ala	L-NTPs	[179, 180]

Table 2. *Summary of different modifications accepted by polymerases for RNA synthesis.*

3.3.3 Ribozymes

Despite recent progress, engineering of polymerases still remains a rather complex and time-consuming procedure while chemically more evolved analogues are not readily incorporated by the T7 RNA polymerase. As a potential alternative to proteinaceous enzymes, various ribozymes have been evolved by *in vitro* selection to synthesize RNA oligonucleotides in a template-dependent manner [183-185]. Among the different existing ribozymes, the Z RNA polymerase ribozyme [185] displays a surprisingly high propensity at incorporating modified uridine nucleotides into RNA. Particularly, single and even multiple incorporation events could be observed with 2'-Fluoro-modified nucleotides, LNA-NTPs, and nucleotides displaying small modifications of the nucleobases suggesting that the Z polymerase ribozyme could be used for the introduction of chemically altered analogues into RNA despite reduced yields compared to the unmodified counterparts [186]. Besides the Z polymerase, the 24-3 polymerase ribozyme obtained by *in vitro* selection is amongst the most proficient RNA-based polymerases with primer extension rates reaching 1.2 nt/min on a broad variety of

templates [184]. Recently, the 24-3 polymerase was shown to be capable of 3'-end labelling of RNA (and DNA) primers with base- and sugar-modified NTPs [187]. These findings bode well for the application of this RNA polymerase ribozyme for the synthesis of longer modified RNA sequences.

As mentioned in the previous section, some DNA polymerases have been engineered to synthesize mirror image L-nucleic acids. As an alternative to these protein enzymes, Sczepanski and Joyce have evolved a cross-chiral RNA ligase that is capable of synthesizing L-RNA oligonucleotides [188]. A last promising strategy for the synthesis of RNA oligonucleotides is based on a recently isolated ribozyme that accepts 5'-triphosphorylated RNA trinucleotides (triplets) as substrates [189]. This ribozyme (coined $t5^{+1}$) copies RNA sequences by adding nucleotides three-by-three and is capable of synthesizing RNA in the presence of primers (and in the canonical 5'-3' as well as the reverse 3'-5' directions) and in the absence of primers. The possibility of incorporating modified nucleotides and extending the synthesis to longer sequences (> 50 nucleotides) has not been explored yet.

Even though RNA-based polymerases do not rival their proteinaceous counterparts in terms of rate of synthesis, fidelity of replication, and substrate tolerance, significant progress has been made in the field which suggests that these biopolymers will represent a serious alternative in the near future.

3.4. Aptamer selections

N*TPs have been used for a number of applications such as labelling of RNA [160] or chemical probing for structural investigations [16, 190, 191] but one important research topic involving these activated building blocks is the Darwinian evolution of aptamers [192]. Indeed, these functional nucleic acids have proven their utility in numerous applications such as therapeutics [193, 194], diagnostics [195, 196], drug delivery systems and nanotechnology [5, 197, 198]. Aptamers are short single-stranded DNA or RNA molecules that are able to bind a target with high affinity and specificity because of their unique three dimensional structures. They can recognise a variety of targets including small molecules, proteins or bigger structures such as cells, virus, bacteria or whole organisms [73, 199] with an affinity similar or superior to that of antibodies [200] (since the dissociation constants (K_D values) of bounded aptamers vary from µM to pM [201, 202]). Unlike antibodies, they can be easily synthetized and modified at larger scale and lower price, are able to penetrate rapidly into tissues and possess a low or a lack of immunogenicity [203].

3.4.1 RNA aptamer isolation by the SELEX process

Aptamers are produced by an *in vitro* selection process called SELEX (Systematic Evolution of Ligands with EXponential Enrichment) [204, 205]. A library of oligonucleotides (10^{13}-10^{15} molecules) is synthetized by primer extension reactions (PEX) or by PCR. All oligonucleotides constituting these library typically contain PCR primer binding regions flanking a central random region of 20-60 nucleotides. Choosing short randomized sequences ensures a complete sampling of the entire sequence space during the selection protocol. On the other hand, longer randomized sequences do not permit the exploration of the complete sequence space but increase the complexity and the number of possible three-dimensional structures [206-208]. For RNA selections, the pool of DNA oligonucleotides is first converted into RNA oligonucleotides by T7 RNA polymerase mediated transcription and incubated with the target molecule immobilized on solid support. After washing, only the species bound to the target will be retained. The latter are then eluted and the remaining RNA pool is reverse transcribed into complementary DNA (cDNA) which is then PCR amplified. Another round of the selection cycle is initiated by the transcription of the enriched library into RNA and the process is repeated several times (usually between 5 and 15 cycles) until no further enrichment occurs. The binders are finally cloned, sequenced and characterized.

3.4.2 Necessity for modified aptamers

The traditional SELEX process enables the identification of potent aptamers but also imparts certain limitations. For instance, selection protocols often involve buffers as media and hence the resulting aptamers will more easily recognise their targets in the buffer than under *in vivo* conditions [209]. To address this issue, new methodologies have been developed to increase the *in vivo* selectivity [210], in which selections are made directly on

live cells or animals [211]. In addition, RNA (and DNA) aptamers composed of natural nucleic acids are usually not suitable for *in vivo* applications because of two major shortcomings: a poor stability against nuclease degradation and insufficient pharmacokinetic properties. RNAs are indeed rapidly degraded *in vivo* through the cleavage of phosphodiester bonds by *exo*- and *endo*-nucleases, which are abundant in biological fluids [212]. The half-time of natural RNA aptamers in human blood was found to be only of a few minutes [213, 214]. In addition to enzymatic hydrolysis, unmodified RNA aptamers are prone to renal clearance. This could be explained by their small size (5 to 30 kDa) which is inferior to the renal filtration threshold of glomeruli (50 kDa), leading to their rapid excretion by the kidneys [215].
These concerns can mainly be addressed by introducing chemical modifications into the scaffolds of aptamers. In this context, a frequently used modification that blocks nuclease degradation is the substitution of the 2'-hydroxyl group of NTPs with 2'-amino, 2'-fluoro or 2'-O-methyl groups [216, 217]. The capping of the 5' and/or 3' position by a polyethylene glycol (PEG) moiety of high molecular weight is also commonly used to prevent renal clearance [218, 219]. A combination of modifications has been found to improve the residence half time of RNA aptamers up to a few days [220].

The first examples of modified RNA aptamers were created by post-SELEX optimisation. This method consists in isolating a natural RNA aptamer by the SELEX process and introducing chemical modifications at various locations using the solid-phase synthesis method described earlier. The effect of these chemical alterations is then assessed by structure activity relationship studies. For example, the properties of the only FDA-approved RNA aptamer drug called pegaptanib (Macugen®) have been drastically improved by such a post-SELEX modification protocol [221]. Pegaptanib is used for the treatment of neovascular age-related macular degeneration by binding to the human vascular endothelial growth factor $VEGF_{165}$ [222]. This aptamer was obtained by *in vitro* selection using a mixture of 2'-F pyrimidines N*TPs and unmodified purines NTPs. The latter were converted into the 2'-*O*-methyl nucleotides after the SELEX protocol. Moreover, a polyethylene glycol unit was added at the 5'-end and a deoxythymidine residue was appended at the 3'-end, in order to prevent nuclease degradation and reduce renal clearance [223]. Those post modifications did not affect the high binding affinity of the aptamer (K_D of pegaptanib against $VEGF_{165}$ is 200 pM) [222-224]. Other examples of post-SELEX aptamers can be found in the following recent reviews [225, 226].

3.4.3 Recent modified RNA aptamers selected by mod-SELEX strategy

The post-SELEX strategy encounters limitations because of the need of rather time-consuming and uncertain SAR studies to ensure that future modified aptamers will display similar K_D values as the unmodified aptamer selected through the SELEX process. Moreover, the post-SELEX process involves the addition of synthetic steps that might not be compatible with every functional group. For all these reasons, the direct incorporation of N*TPs into the

SELEX process has emerged as a promising strategy called mod-SELEX. In this approach, N*TPs serve for the construction of modified libraries which eventually will lead to the isolation of aptamers capable of recognizing their targets and displaying enhanced properties without having to resort to SAR studies.
In this part of the review, we will focus only on a few recent examples of modified RNA aptamers that have been generated through the mod-SELEX strategy.

3.4.5 Sugar modifications

As mentioned above, sugar modifications are often considered in order to improve the biostability of nucleic acids and of aptamers particularly. Hence, a large number of 2'-Fluoro modified aptamers has been reported for various applications. For instance, in 2014 Jayamaran et al. developed a library of oligonucleotides containing 2'-fluoro pyrimidines. A combination of positive and negative selection steps led to the isolation of an aptamer that specifically (K_D = 120 nM) recognises and inhibits the activation of GluNI/GluNA2 NMDA receptors without affecting AMPA and KA receptors [227]. Similarly, 2'-Fluoro pyrimidines were also used to create an aptamer against human prostatic acid phosphatase (K_D = 118 nM). Analysis of secondary structures revealed that two hairpin sequences and a 50 nucleotide sequence were required for binding [228]. Casella et al. generated two aptamers containing 2'-Fluoro nucleotides that can bind to sickle hemoglobin with moderate affinity (K_D = 1.68 and 3.56 μM) but with interesting polymerization-inhibitory properties [229]. Under hypoxic conditions, these aptamers were found to inhibit the polymerisation of sickle hemoglobin in lysates, making them potent candidates for therapeutic applications against sickle cell disease. Very recently, a 2'-fluoro pyrimidine containing aptamer against the methyltransferase of the dengue virus serotype 2 was created using the mutant Y639F T7 RNA polymerase. The isolated aptamer displays a high affinity (K_D = 28 nM) for its target and was capable of inhibiting the *N*-7 methylation activity of the protein [230].
Another common sugar modification is the replacement of the furanose ring oxygen by a sulfur atom at position 4' of the sugar. Matsuda et al. reported in 2005 the synthesis of 4'-thioUTP and 4'-thioCTP. Their incorporation into RNA by wild-type T7 RNA polymerase was demonstrated as well as the reverse transcription of RNA into DNA [231]. A 4'-thiomodified aptamer against α-thrombin was developed, which showed a greater stability and binding affinity than its corresponding unmodified RNA aptamer (K_D = 4.7 nM versus K_D = 85 nM, respectively). A few years later, the same group employed this methodology to create a fully modified 4'-thio aptamer against α-thrombin with improved nuclease resistance and dissociation constants in the low nanomolar range [232].
2'-deoxy-2'-fluoro-β-D-arabinose substitution (FANA) represents another type of important modification which was found to present an improved thermal stability, nuclease resistance, and binding affinity for complementary DNA or RNA strands [233-235]. Recently, a fully modified 2'-FANA aptamer against HIV-1 reverse transcriptase (HIV-1 RT) was identified by incorporation of the triphosphates into DNA using the TgoT DNA polymerase mutant D4K. The isolated aptamer showed a high affinity to its target with a K_D value of 4 pM [236].

FANA-based enzymes have also been reported by application of a similar selection strategy [178, 237].
Lastly, Kabza and Sczepanski reported in 2017 the creation of an L-RNA aptamer comporting a 5-aminoallyl-uridine cationic modification. As L-nucleic acids are unable to form Watson-Crick base pairs with natural D-sequences, this Spiegelmer aptamer was created against oncogenic precursor microRNA 19a (pre-miR-19a) [151]. The binding affinity was found to be high (K_D = 2.2 nM) and the L-aptamer was able to inhibit Dicer-mediated cleavage of pre-miR into mature pre-miR-19a (IC_{50} = 4 nM), showing its ability to modulate RNA function.

3.4.6 Base modifications

The base modification of NTPs is a convenient way to introduce additional functionalities and increase the chemical diversity of the selection library. The C5 position of pyrimidines and the C2 position of purines are known to be permissible sites for modifications that are accepted by polymerases. Surprisingly and despite the acceptance of numerous base-modified NTPs by wild-type T7 RNA polymerase (see section 3.3.1), no aptamers containing a base modification have been reported recently. A few years ago, Liu et al. published the *in vitro* selection of a photoresponsive RNA aptamer [238]. A modified adenosine triphosphate bearing an azobenzene moiety was first synthetized and shown to be accepted as a substrate by the T7 RNA polymerase. The corresponding RNA template was suitable for reverse transcription PCR. *In vitro* selection led to a modified aptamer that is able to bind hemin after visible light irradiation and to release its target following UV irradiation.

3.4.7 Phosphate backbone modifications

α-phosphorothioates are the most common modification of the phosphate backbone. They are synthetized chemically as a mixture of diastereoisomers. However, most polymerases only accept (*S*p) enantiomers to produce (*R*p) phosphorothiolate internucleotide linkages [239]. In order to circumvent this issue, Yang et al. considered the generation of achiral aptamers containing phosphorodithiolate linkages. The impact of this modification was explored by comparing the binding affinities of a modified and an unmodified aptamer [240]. An aptamer against $VEGF_{165}$ was generated and shown to exhibit a higher affinity compared to its natural counterpart (K_D = 1.0 pM vs K_D = 961.0 pM, respectively). The same trend was observed for a modified aptamer against thrombin (K_D = 1.8 pM vs K_D = 1871.0 pM, respectively). This discovery might allow the production of modified aptamers with improved binding affinity without the need of addressing the question of the chirality at the thiophosphate centers.
α-P-Borano modified triphosphates (α-B-GTP and α-B-UTP) were also introduced into the SELEX process with T7 RNA polymerase to generate aptamers against ATP that contain a boranophosphate linkage which is more resistant to nuclease degradation than the native phosphodiester linkage [241].

4. Conclusions

Chemical modification of RNA can be achieved by application of two main chemical methods: solid-support based chemical synthesis and enzymatic polymerization of nucleoside triphosphates. The phosphoramidite-based solid-phase method presents the advantage of being fully automatized and grants access to larger quantities of RNA oligonucleotides (up to the kilogram-scale [22]). This method is thus well-suited for the synthesis and development of therapeutic oligonucleotides including siRNAs, miRNAs, and antisense oligonucleotides. On the other hand, chemical synthesis is restricted to smaller sequences (< 100 nucleotides) and the rather harsh conditions employed during the repeated synthetic cycles do not permit the introduction of sensitive functional groups. As an alternative, modified RNA oligonucleotides can be produced by *in vitro* transcription using N*TPs in conjunction with natural or engineered RNA polymerases. This method is not limited in terms of oligonucleotide length and nature of the chemical modification. The polymerization of N*TPs is therefore well-suited for the generation of aptamers and ribozymes and potentially for the study of long non-coding RNAs. In addition, the discovery of natural nucleoside triphosphate transporters such as *Pt*NTT2 [242, 243] combined with the advent of efficient artificial transporters [244], provides a means for the introduction of N*TPs (and dN*TPs) directly into cells and bodes well for the development of methods of *in vivo* synthesis of modified RNAs. On the other hand, no universal and high yielding synthetic route has been reported for N*TPs as yet and the incorporation of the modified triphosphate heavily relies on the substrate acceptance by RNA polymerases. Recent progress in polymerase engineering and ribozyme evolution will certainly permit to further relax the latter requirement and expand the chemical diversity that can be obtained by enzymatic synthesis. Lastly, the combination of both chemical methods (or the inclusion of other emerging strategies) often helps in the synthesis of modified RNAs as exemplified by the first FDA approved commercialized oligonucleotide-based drug pegaptanib sodium (Macugen®) [203]. Chemical methods for the modification of RNA will certainly largely contribute to the refining our understanding of the biological properties of RNA and to the development of RNA-based tools and devices.

List of abbreviations

BMT - 5-benzylmercaptotetrazole

CEP-Cl - 2-cyanoethyl *N*,*N*-diisopropylchlorophophoramidite

DCM – dichloromethane

DDTT - *N*,*N*-Dimethyl-*N*'-(3-thioxo-3*H*-1,2,4-dithiazol-5-yl)-formimidamide

DIPEA – *N*,*N*-diisopropylethylamine

DMTr - 4,4–Dimethoxytrityl

DNA - Deoxyribonucleic acid

DTBS - di-*tert*-butylsilylene

ETT - 5-ethylthio-1*H*-tetrazole

HPLC - High-performance liquid chromatography

iPr – isopropyl

MeCN – Acetonitrile

TBDMS - *tert*-butyldimethylsilyl

TIPDS - tetra-isopropylsisiloxane

ANA = arabinonucleic acid

CeNA = cyclohexenyl nucleic acid

FANA = 2'-fluoro-arabino nucleic acid

HNA = 1',5'-anhydrohexitol nucleic acid

LNA = locked nucleic acid

TNA = threose nucleic acid

NTP = nucleoside triphosphate

N*TP = modified nucleoside triphosphate

PEG = polyethylene glycol

PEX = primer extension reaction

PPN = tris(bis(triphenylphosphoranylidene)ammonium)

SELEX = Systematic Evolution of Ligands with Exponential Enrichment

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References

[1] J.H. Song, C.Q. Yi, Chemical Modifications to RNA: A New Layer of Gene Expression Regulation, ACS Chem. Biol. 12(2) (2017) 316-325.
[2] S. Nachtergaele, C. He, Chemical Modifications in the Life of an mRNA Transcript, Annu. Rev. Genet. 52(1) (2018) 349-372.
[3] I.A. Roundtree, M.E. Evans, T. Pan, C. He, Dynamic RNA modifications in gene expression regulation, Cell 169(7) (2017) 1187-1200.
[4] E.M. Harcourt, A.M. Kietrys, E.T. Kool, Chemical and structural effects of base modifications in messenger RNA, Nature 541(7637) (2017) 339-346.
[5] P. Rothlisberger, C. Gasse, M. Hollenstein, Nucleic Acid Aptamers: Emerging Applications in Medical Imaging, Nanotechnology, Neurosciences, and Drug Delivery, Int. J. Mol. Sci. 18(11) (2017) 39.
[6] M.V. Sednev, V. Mykhailiuk, P. Choudhury, J. Halang, K.E. Sloan, M.T. Bohnsack, C. Höbartner, N6-Methyladenosine-Sensitive RNA-Cleaving Deoxyribozymes, Angew. Chem. Int. Ed. 57(46) (2018) 15117-15121.
[7] T.J. Carrocci, L. Lohe, M.J. Ashton, C. Hobartner, A.A. Hoskins, Debranchase-resistant labeling of RNA using the 10DM24 deoxyribozyme and fluorescent modified nucleotides, Chem. Commun. 53(88) (2017) 11992-11995.
[8] L. Buttner, F. Javadi-Zarnaghi, C. Höbartner, Site-Specific Labeling of RNA at Internal Ribose Hydroxyl Groups: Terbium-Assisted Deoxyribozymes at Work, J. Am. Chem. Soc. 136(22) (2014) 8131-8137.
[9] B. Samanta, D.P. Horning, G.F. Joyce, 3 '-End labeling of nucleic acids by a polymerase ribozyme, Nucleic Acids Res. 46(17) (2018) 6.
[10] A.K. Sharma, J.J. Plant, A.E. Rangel, K.N. Meek, A.J. Anamisis, J. Hollien, J.M. Heemstra, Fluorescent RNA Labeling Using Self-Alkylating Ribozymes, ACS Chem. Biol. 9(8) (2014) 1680-1684.
[11] L. Anhauser, F. Muttach, A. Rentmeister, Reversible modification of DNA by methyltransferase-catalyzed transfer and light-triggered removal of photo-caging groups, Chem. Commun. 54(5) (2018) 449-451.
[12] M. Heimes, L. Kolmar, C. Brieke, Efficient cosubstrate enzyme pairs for sequence-specific methyltransferase-directed photolabile caging of DNA, Chem. Commun. 54(90) (2018) 12718-12721.
[13] K. Hartstock, B.S. Nilges, A. Ovcharenko, N.V. Cornelissen, N. Pullen, A.M. Lawrence-Dorner, S.A. Leidel, A. Rentmeister, Enzymatic or In Vivo Installation of Propargyl Groups in Combination with Click Chemistry for the Enrichment and Detection of Methyltransferase Target Sites in RNA, Angew. Chem. Int. Ed. 57(21) (2018) 6342-6346.
[14] C.M. Palumbo, P.A. Beal, Nucleoside analogs in the study of the epitranscriptome, Methods (2018) 10.1016/j.ymeth.2018.10.014.
[15] L. Anhauser, A. Rentmeistert, Enzyme-mediated tagging of RNA, Curr. Opin. Biotechnol. 48 (2017) 69-76.
[16] J.T. George, S.G. Srivatsan, Posttranscriptional chemical labeling of RNA by using bioorthogonal chemistry, Methods 120 (2017) 28-38.
[17] S.K. Silverman, Catalyic DNA: Scope, Applications, and Biochemistry of Deoxyribozymes, Trends Biochem.Sci. 41(7) (2016) 595-609.
[18] Y. Liu, R. Sousa, Y.X. Wang, Specific labeling: An effective tool to explore the RNA world, Bioessays 38(2) (2016) 192-200.

[19] D. Gillingham, D. Rasale, Direct and Selective Modification of RNA - An Open Challenge in Nucleic Acid Chemistry, Chimia 72(11) (2018) 777-781.
[20] A. Somoza, Protecting groups for RNA synthesis: an increasing need for selective preparative methods, Chem. Soc. Rev. 37(12) (2008) 2668-2675.
[21] C. Höbartner, F. Wachowius, Chemical synthesis of modified RNA, The chemical biology of nucleic acids (2010) 1-37.
[22] H. Lönnberg, Synthesis of oligonucleotides on a soluble support, Beilstein J. Org. Chem. 13 (2017) 1368-1387.
[23] X. Wei, N. Takahashi, H. Tamiaki, Y. Saga, Coupling activators for the oligonucleotide synthesis via phosphoramidite approach pp 3615e3637, Tetrahedron 69(18) (2013) 3609e3613.
[24] S. Ni, H. Yao, L. Wang, J. Lu, F. Jiang, A. Lu, G. Zhang, Chemical modifications of nucleic acid aptamers for therapeutic purposes, Int. J. Mol. Sci. 18(8) (2017) 1683.
[25] Y. Shiba, H. Masuda, N. Watanabe, T. Ego, K. Takagaki, K. Ishiyama, T. Ohgi, J. Yano, Chemical synthesis of a very long oligoribonucleotide with 2-cyanoethoxymethyl (CEM) as the 2′-O-protecting group: structural identification and biological activity of a synthetic 110mer precursor-microRNA candidate, Nucleic Acids Res. 35(10) (2007) 3287-3296.
[26] J.S. Eadie, D.S. Davidson, Guanine modification daring chemical DNA synthesis, Nucleic Acids Res. 15(20) (1987) 8333-8349.
[27] S. Beaucage, M.H. Caruthers, Deoxynucleoside phosphoramidites—a new class of key intermediates for deoxypolynucleotide synthesis, Tetrahedron Lett. 22(20) (1981) 1859-1862.
[28] S. Roy, M. Caruthers, Synthesis of DNA/RNA and Their Analogs via Phosphoramidite and H-Phosphonate Chemistries, Molecules 18(11) (2013) 14268-14284.
[29] S.L. Beaucage, R.P. Iyer, Advances in the synthesis of oligonucleotides by the phosphoramidite approach, Tetrahedron 48(12) (1992) 2223-2311.
[30] R. Hall, A. Todd, R. Webb, 644. Nucleotides. Part XLI. Mixed anhydrides as intermediates in the synthesis of dinucleoside phosphates, J. Chem. Soc. (1957) 3291-3296.
[31] A. Michelson, A.R. Todd, Nucleotides part XXXII. Synthesis of a dithymidine dinucleotide containing a 3′: 5′-internucleotidic linkage, J. Chem. Soc. (1955) 2632-2638.
[32] P.J. Garegg, I. Lindh, T. Regberg, J. Stawinski, R. Strömberg, C. Henrichson, Nucleoside H-phosphonates. III. Chemical synthesis of oligodeoxyribonucleotides by the hydrogenphosphonate approach, Tetrahedron Lett. 27(34) (1986) 4051-4054.
[33] B.C. Froehler, P.G. Ng, M.D. Matteucci, Synthesis of DNA via deoxynudeoside H-phosphonate Intermediates, Nucleic Acids Res. 14(13) (1986) 5399-5407.
[34] P.T. Gilham, H.G. Khorana, Studies on polynucleotides. I. A new and general method for the chemical synthesis of the C5 ”-C3 ”internucleotidic linkage. Syntheses of deoxyribo-dinucleotides1, J. Am. Chem. Soc. 80(23) (1958) 6212-6222.
[35] R.L. Letsinger, K.K. Ogilvie, Nucleotide chemistry. XIII. Synthesis of oligothymidylates via phosphotriester intermediates, J. Am. Chem. Soc. 91(12) (1969) 3350-3355.
[36] C.B. Reese, The chemical synthesis of oligo-and poly-nucleotides by the phosphotriester approach, Tetrahedron 34(21) (1978) 3143-3179.
[37] R. Letsinger, J. Finnan, G. Heavner, W. Lunsford, Nucleotide chemistry. XX. Phosphite coupling procedure for generating internucleotide links, J. Am. Chem. Soc. 97(11) (1975) 3278-3279.
[38] M.D. Matteucci, M.H. Caruthers, Synthesis of deoxyoligonucleotides on a polymer support, J. Am. Chem. Soc. 103(11) (1981) 3185-3191.
[39] A.D. Barone, J.Y. Tang, M.H. Caruthers, In situ activation of bis-dialkylaminophosphines - A new method for synthesizing deoxyoligonucleotides on polymer supports, Nucleic Acids Res. 12(10) (1984) 4051-4061.
[40] J. Nielsen, O. Dahl, Improves synthesis of (PRI2N)2POCH2CH2CN, Nucleic Acids Res. 15(8) (1987) 3626-3626.
[41] N. Sinha, P. Davis, N. Usman, J. Perez, R. Hodge, J. Kremsky, R. Casale, Labile exocyclic amine protection of nucleosides in DNA, RNA and oligonucleotide analog synthesis facililating N-deacylation, minimizing depurination and chain degradation, Biochimie 75(1-2) (1993) 13-23.

[42] K. Koizumi, Y. Maeda, T. Kano, H. Yoshida, T. Sakamoto, K. Yamagishi, Y. Ueno, Synthesis of 4 '-C-aminoalkyl-2 '-O-methyl modified RNA and their biological properties, Bioorg. Med. Chem. 26(12) (2018) 3521-3534.
[43] S. Milton, D. Honcharenko, C.S.J. Rocha, P.M.D. Moreno, C.I.E. Smith, R. Stromberg, Nuclease resistant oligonucleotides with cell penetrating properties, Chem. Commun. 51(19) (2015) 4044-4047.
[44] M. Teplova, S.T. Wallace, V. Tereshko, G. Minasov, A.M. Symons, P.D. Cook, M. Manoharan, M. Egli, Structural origins of the exonuclease resistance of a zwitterionic RNA, Proc. Natl. Acad. Sci. U. S. A. 96(25) (1999) 14240-14245.
[45] C. Höbartner, R. Rieder, C. Kreutz, B. Puffer, K. Lang, A. Polonskaia, A. Serganov, R. Micura, Syntheses of RNAs with up to 100 nucleotides containing site-specific 2'-methylseleno labels for use in X-ray crystallography, J. Am. Chem. Soc. 127(34) (2005) 12035-12045.
[46] K. Fauster, M. Hartl, T. Santner, M. Aigner, C. Kreutz, K. Bister, E. Ennifar, R. Micura, 2 '-Azido RNA, a Versatile Tool for Chemical Biology: Synthesis, X-ray Structure, siRNA Applications, Click Labeling, ACS Chem. Biol. 7(3) (2012) 581-589.
[47] L. Jud, R. Micura, An Unconventional Acid-Labile Nucleobase Protection Concept for Guanosine Phosphoramidites in RNA Solid-Phase Synthesis, Chem. Eur. J. 23(14) (2017) 3406-3413.
[48] C. Riml, A. Lusser, E. Ennifar, R. Micura, Synthesis, Thermodynamic Properties, and Crystal Structure of RNA Oligonucleotides Containing 5-Hydroxymethylcytosine, J. Org. Chem. 82(15) (2017) 7939-7945.
[49] S.L. Beaucage, C.B. Reese, Recent advances in the chemical synthesis of RNA, Curr. Protoc. Nucleic Acid Chem. 38(1) (2009) 2.16. 1-2.16. 31.
[50] D.J. Dellinger, Z. Timár, J. Myerson, A.B. Sierzchala, J. Turner, F. Ferreira, Z.n. Kupihár, G. Dellinger, K.W. Hill, J.A. Powell, Streamlined process for the chemical synthesis of RNA using 2'-O-thionocarbamate-protected nucleoside phosphoramidites in the solid phase, J. Am. Chem. Soc. 133(30) (2011) 11540-11556.
[51] K.K. Ogilvie, K.L. Sadana, E.A. Thompson, M.A. Quilliam, J.B. Westmore, The use of silyl groups in protecting the hydroxyl functions of ribonucleosides, Tetrahedron Lett. 15(33) (1974) 2861-2863.
[52] N. Usman, K. Ogilvie, M. Jiang, R. Cedergren, The automated chemical synthesis of long oligoribuncleotides using 2'-O-silylated ribonucleoside 3'-O-phosphoramidites on a controlled-pore glass support: synthesis of a 43-nucleotide sequence similar to the 3'-half molecule of an Escherichia coli formylmethionine tRNA, J. Am. Chem. Soc. 109(25) (1987) 7845-7854.
[53] C. Zhou, D. Honcharenko, J. Chattopadhyaya, 2-(4-Tolylsulfonyl) ethoxymethyl (TEM)—a new 2'-OH protecting group for solid-supported RNA synthesis, Org. Biomol. Chem. 5(2) (2007) 333-343.
[54] T. Ohgi, Y. Masutomi, K. Ishiyama, H. Kitagawa, Y. Shiba, J. Yano, A New RNA Synthetic Method with a 2 '-O-(2-Cyanoethoxymethyl) Protecting Group, Organic letters 7(16) (2005) 3477-3480.
[55] S. Pitsch, P.A. Weiss, L. Jenny, A. Stutz, X. Wu, Reliable chemical synthesis of oligoribonucleotides (RNA) with 2'-O-[(triisopropylsilyl) oxy] methyl (2'-O-tom)-protected phosphoramidites, Helvetica Chimica Acta 84(12) (2001) 3773-3795.
[56] G. Gough, T. Miller, N. Mantick, p-Nitrobenzyloxymethyl: a new fluoride-removable protecting group for ribonucleoside 2'-hydroxyls, Tetrahedron Lett. 37(7) (1996) 981-982.
[57] J. Cieślak, A. Grajkowski, C. Ausín, S.L. Beaucage, Protection of the 2'-Hydroxy Function of Ribonucleosides as an Iminooxymethyl Propanoate and Its 2'-O-Deprotection through an Intramolecular Decarboxylative Elimination Process, Eur. J. Org. Chem. 2016(35) (2016) 5817-5821.
[58] J. Cieślak, C. Ausín, A. Grajkowski, S.L. Beaucage, The 2-cyano-2, 2-dimethylethanimine-N-oxymethyl group for the 2'-hydroxyl protection of ribonucleosides in the solid-phase synthesis of RNA sequences, Chem. Eur. J. 19(14) (2013) 4623-4632.
[59] H. Saneyoshi, K. Seio, M. Sekine, A General Method for the Synthesis of 2 '-O-Cyanoethylated Oligoribonucleotides Having Promising Hybridization Affinity for DNA and RNA and Enhanced Nuclease Resistance, J. Org. Chem. 70(25) (2005) 10453-10460.
[60] T. Lavergne, J.R. Bertrand, J.J. Vasseur, F. Debart, A base-labile group for 2'-OH protection of ribonucleosides: A major challenge for RNA synthesis, Chem. Eur. J. 14(30) (2008) 9135-9138.

[61] S.A. Scaringe, F.E. Wincott, M.H. Caruthers, Novel RNA synthesis method using 5 ‘-O-Silyl-2 ‘-O-orthoester protecting groups, J. Am. Chem. Soc. 120(45) (1998) 11820-11821.
[62] M. Schwartz, R. Breaker, G. Asteriadis, G. Gough, Rapid synthesis of oligoribonucleotides using 2’-O-(o-nitrobenzyloxymethyl)-protected monomers, Bioorg. Med. Chem. Lett. 2(9) (1992) 1019-1024.
[63] A. Stutz, S. Pitsch, Automated RNA-synthesis with photocleavable sugar and nucleobase protecting groups, Synlett (1999) 930-934.
[64] W.T. Markiewicz, Tetraisopropyldisiloxane-1, 3-diyl, a group for simultaneous protection of 3’- and 5’-hydroxy functions of nucleosides, Chemischer Informationsdienst 10(23) (1979) no-no.
[65] V. Serebryany, L. Beigelman, An efficient preparation of protected ribonucleosides for phosphoramidite RNA synthesis, Tetrahedron Lett. 43(11) (2002) 1983-1985.
[66] A. Biscans, M. Bos, A.R. Martin, N. Ader, G. Sczakiel, J.J. Vasseur, C. Dupouy, F. Debart, Direct Synthesis of Partially Modified 2 '-O-Pivaloyloxymethyl RNAs by a Base-Labile Protecting Group Strategy and their Potential for Prodrug-Based Gene-Silencing Applications, ChemBioChem 15(18) (2014) 2674-2679.
[67] S. Nagata, T. Hamasaki, K. Uetake, H. Masuda, K. Takagaki, N. Oka, T. Wada, T. Ohgi, J. Yano, Synthesis and biological activity of artificial mRNA prepared with novel phosphorylating reagents, Nucleic Acids Res. 38(21) (2010) 7845-7857.
[68] A. Semenyuk, A. Földesi, T. Johansson, C. Estmer-Nilsson, P. Blomgren, M. Brännvall, L.A. Kirsebom, M. Kwiatkowski, Synthesis of RNA Using 2 ‘-O-DTM Protection, J. Am. Chem. Soc. 128(38) (2006) 12356-12357.
[69] J.G. Lackey, D. Mitra, M.M. Somoza, F. Cerrina, M.J. Damha, Acetal Levulinyl Ester (ALE) Groups for 2 '-Hydroxyl Protection of Ribonucleosides in the Synthesis of Oligoribonucleotides on Glass and Microarrays, J. Am. Chem. Soc. 131(24) (2009) 8496-8502.
[70] R.P. Iyer, W. Egan, J.B. Regan, S.L. Beaucage, 3H-1, 2-Benzodithiole-3-one 1, 1-dioxide as an improved sulfurizing reagent in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates, J. Am. Chem. Soc. 112(3) (1990) 1253-1254.
[71] H. Jahns, M. Roos, J. Imig, F. Baumann, Y.L. Wang, R. Gilmour, J. Hall, Stereochemical bias introduced during RNA synthesis modulates the activity of phosphorothioate siRNAs, Nat. Commun. 6 (2015) 9.
[72] N. Iwamoto, D.C.D. Butler, N. Svrzikapa, S. Mohapatra, I. Zlatev, D.W.Y. Sah, Meena, S.M. Standley, G.L. Lu, L.H. Apponi, M. Frank-Kamenetsky, J.J. Zhang, C. Vargeese, G.L. Verdine, Control of phosphorothioate stereochemistry substantially increases the efficacy of antisense oligonucleotides, Nat. Biotechnol. 35(9) (2017) 845-+.
[73] M. Mascini, I. Palchetti, S. Tombelli, Nucleic Acid and Peptide Aptamers: Fundamentals and Bioanalytical Aspects, Angew. Chem. Int. Ed. 51(6) (2012) 1316-1332.
[74] T.P. Prakash, An overview of sugar-modified oligonucleotides for antisense therapeutics, Chem. Biodiversity 8(9) (2011) 1616-1641.
[75] G.F. Deleavey, M.J. Damha, Designing chemically modified oligonucleotides for targeted gene silencing, Chem. Biol. 19(8) (2012) 937-954.
[76] P.R. Bohlander, M.L. Abb, F. Bestvater, H. Allgayer, H.A. Wagenknecht, Two wavelength-shifting molecular beacons for simultaneous and selective imaging of vesicular miRNA-21 and miRNA-31 in living cancer cells, Org. Biomol. Chem. 14(22) (2016) 5001-5006.
[77] P.A. Giannaris, M.J. Damha, Hybridization properties of oligoarabinonucleotides, Canadian journal of chemistry 72(3) (1994) 909-918.
[78] J. Steinmeyer, H.K. Walter, M.A. Bichelberger, V. Schneider, T. Kubar, F. Ronicke, B. Olshausen, K. Nienhaus, G.U. Nienhaus, U. Schepers, M. Elstner, H.A. Wagenknecht, "siRNA traffic lights": arabino-configured 2 '-anchors for fluorescent dyes are key for dual color readout in cell imaging, Org. Biomol. Chem. 16(20) (2018) 3726-3731.
[79] J.S. Jepsen, M.D. Sørensen, J. Wengel, Locked nucleic acid: a potent nucleic acid analog in therapeutics and biotechnology, Oligonucleotides 14(2) (2004) 130-146.

[80] A.A. Koshkin, S.K. Singh, P. Nielsen, V.K. Rajwanshi, R. Kumar, M. Meldgaard, C.E. Olsen, J. Wengel, LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition, Tetrahedron 54(14) (1998) 3607-3630.
[81] C. Rinaldi, M.J. Wood, Antisense oligonucleotides: the next frontier for treatment of neurological disorders, Nat. Rev. Neurol. 14(1) (2018) 9.
[82] A.F. Fuchtbauer, S. Preus, K. Borjesson, S.A. McPhee, D.M.J. Lilley, L.M. Wilhelmsson, Fluorescent RNA cytosine analogue - an internal probe for detailed structure and dynamics investigations, Sci Rep 7 (2017) 8.
[83] R.W. Sinkeldam, N.J. Greco, Y. Tor, Fluorescent Analogs of Biomolecular Building Blocks: Design, Properties, and Applications, Chem. Rev. 110(5) (2010) 2579-2619.
[84] E.M. Harcourt, A.M. Kietrys, E.T. Kool, Chemical and structural effects of base modifications in messenger RNA, Nature 541(7637) (2017) 339.
[85] C. Höbartner, C. Kreutz, E. Flecker, E. Ottenschläger, W. Pils, K. Grubmayr, R. Micura, The Synthesis of 2'-O-[(Triisopropylsilyl)oxy] methyl (TOM) Phosphoramidites of Methylated Ribonucleosides (m^1G, m^2G, $m^{22}G$, m^1I, m^3U, m^4C, m^6A, $m^{62}A$) for Use in Automated RNA Solid-Phase Synthesis, Monatshefte für Chemie/Chemical Monthly 134(6) (2003) 851-873.
[86] S.N. Mikhailov, J. Rozenski, E.V. Efimtseva, R. Busson, A. Van Aerschot, P. Herdewijn, Chemical incorporation of 1-methyladenosine into oligonucleotides, Nucleic Acids Res. 30(5) (2002) 1124-1131.
[87] R. Micura, W. Pils, C. Höbartner, K. Grubmayr, M.-O. Ebert, B. Jaun, Methylation of the nucleobases in RNA oligonucleotides mediates duplex–hairpin conversion, Nucleic Acids Res. 29(19) (2001) 3997-4005.
[88] C. Höbartner, M.O. Ebert, B. Jaun, R. Micura, RNA Two-State Conformation Equilibria and the Effect of Nucleobase Methylation, Angew. Chem. Int. Ed. 41(4) (2002) 605-609.
[89] R. Micura, W. Pils, K. Grubmayr, Bridged cyclic oligoribonucleotides as model compounds for codon–anticodon pairing, Angew. Chem. Int. Ed. 39(5) (2000) 922-925.
[90] R. Micura, W. Pils, K. Grubmayr, M.-O. Ebert, B. Jaun, Bridged cyclic oligoribonucleotides - towards models for codon-anticodon pairing, Nucleosides Nucleotides Nucleic Acids 20(4-7) (2001) 1287-1289.
[91] J.W. Jones, R.K. Robins, Purine nucleosides. III. Methylation studies of certain naturally occurring purine nucleosides, J. Am. Chem. Soc. 85(2) (1963) 193-201.
[92] Q. Tang, A. Cai, K. Bian, F. Chen, J.C. Delaney, S. Adusumalli, A.C. Bach, F. Akhlaghi, B.P. Cho, D. Li, Characterization of Byproducts from Chemical Syntheses of Oligonucleotides Containing 1-Methyladenine and 3-Methylcytosine, ACS omega 2(11) (2017) 8205-8212.
[93] N. Milisavljevic, P. Perlikova, R. Pohl, M. Hocek, Enzymatic synthesis of base-modified RNA by T7 RNA polymerase. A systematic study and comparison of 5-substituted pyrimidine and 7-substituted 7-deazapurine nucleoside triphosphates as substrates, Org. Biomol. Chem. 16(32) (2018) 5800-5807.
[94] A.A. Sawant, P.P. Mukherjee, R.K. Jangid, S. Galande, S.G. Srivatsan, A clickable UTP analog for the posttranscriptional chemical labeling and imaging of RNA, Org. Biomol. Chem. 14(24) (2016) 5832-5842.
[95] A.A. Sawant, A.A. Tanpure, P.P. Mukherjee, S. Athavale, A. Kelkar, S. Galande, S.G. Srivatsan, A versatile toolbox for posttranscriptional chemical labeling and imaging of RNA, Nucleic Acids Res. 44(2) (2016) 12.
[96] G. Stengel, M. Urban, B.W. Purse, R.D. Kuchta, Incorporation of the Fluorescent Ribonucleotide Analogue tCTP by T7 RNA Polymerase, Anal. Chem. 82(3) (2010) 1082-1089.
[97] H. Mei, C.H. Shi, R.M. Jimenez, Y.J. Wang, M. Kardouh, J.C. Chaput, Synthesis and polymerase activity of a fluorescent cytidine TNA triphosphate analogue, Nucleic Acids Res. 45(10) (2017) 5629-5638.
[98] M. Kimoto, A.J. Meyer, I. Hirao, A.D. Ellington, Genetic alphabet expansion transcription generating functional RNA molecules containing a five-letter alphabet including modified unnatural and natural base nucleotides by thermostable T7 RNA polymerase variants, Chem. Commun. 53(91) (2017) 12309-12312.

[99] S. Ericksson, Enzymatic synthesis of nucleoside triphosphates, in: M. Vaghefi (Ed.), Nucleoside Triphosphates and their Analogs., Boca Raton: CRC Press., 2005, pp. 23-37.
[100] L.E. Iglesias, E.S. Lewkowicz, R. Medici, P. Bianchi, A.M. Iribarren, Biocatalytic approaches applied to the synthesis of nucleoside prodrugs, Biotechnol. Adv. 33(5) (2015) 412-434.
[101] K. Burgess, D. Cook, Syntheses of Nucleoside Triphosphates, Chem. Rev. 100(6) (2000) 2047-2060.
[102] A.R. Kore, B. Srinivasan, Recent Advances in the Syntheses of Nucleoside Triphosphates, Curr. Org. Synth. 10(6) (2013) 903-934.
[103] M.A. Dellafiore, J.M. Montserrat, A.M. Iribarren, Modified Nucleoside Triphosphates for In-vitro Selection Techniques, Front. Chem. 4(18) (2016).
[104] M. Hollenstein, Nucleoside Triphosphates — Building Blocks for the Modification of Nucleic Acids, Molecules 17(11) (2012) 13569.
[105] M. Hocek, Synthesis of Base-Modified 2 '-Deoxyribonucleoside Triphosphates and Their Use in Enzymatic Synthesis of Modified DNA for Applications in Bioanalysis and Chemical Biology, J. Org. Chem. 79(21) (2014) 9914-9921.
[106] B. Roy, A. Depaix, C. Perigaud, S. Peyrottes, Recent Trends in Nucleotide Synthesis, Chem. Rev. 116(14) (2016) 7854-7897.
[107] M. Yoshikawa, T. Kato, T. Takenishi, A novel method for phosphorylation of nucleosides to 5'-nucleotides, Tetrahedron Lett. 8(50) (1967) 5065-5068.
[108] Y. Masaharu, K. Tetsuya, T. Tadao, Studies of Phosphorylation. III. Selective Phosphorylation of Unprotected Nucleosides, Bull. Chem. Soc. Jpn. 42(12) (1969) 3505-3508.
[109] J. Ludwig, A new route to nucleoside 5'-triphosphates, Acta Biochim. Biophys. Acad. Sci. Hung 16(3-4) (1981) 131-133.
[110] I. Gillerman, B. Fischer, An Improved One-Pot Synthesis of Nucleoside 5'-Triphosphate Analogues, Nucleosides Nucleotides Nucleic Acids 29(3) (2010) 245-256.
[111] V. Borsenberger, M. Kukwikila, S. Howorka, Synthesis and enzymatic incorporation of modified deoxyuridine triphosphates, Org. Biomol. Chem. 7(18) (2009) 3826-3835.
[112] L.S. McCoy, D. Shin, Y. Tor, Isomorphic Emissive GTP Surrogate Facilitates Initiation and Elongation of in Vitro Transcription Reactions, J. Am. Chem. Soc. 136(43) (2014) 15176-15184.
[113] P.A. Hopkins, L.S. McCoy, Y. Tor, Enzymatic incorporation and utilization of an emissive 6-azauridine, Org. Biomol. Chem. 15(3) (2017) 684-690.
[114] H.J. Korhonen, H.L. Bolt, L. Vicente-Gines, D.C. Perks, D.R.W. Hodgson, PPN Pyrophosphate: A New Reagent for the Preparation of Nucleoside Triphosphates, Phosphorus Sulfur Silicon Relat. Elem. 190(5-6) (2015) 758-762.
[115] J. Ludwig, F. Eckstein, Rapid and efficient synthesis of nucleoside 5'-0-(1-thiotriphosphates), 5'-triphosphates and 2',3'-cyclophosphorothioates using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one, J. Org. Chem. 54(3) (1989) 631-635.
[116] S. Warnecke, C. Meier, Synthesis of Nucleoside Di- and Triphosphates and Dinucleoside Polyphosphates with cycloSal-Nucleotides, J. Org. Chem. 74(8) (2009) 3024-3030.
[117] C. Meier, cycloSal phosphates as chemical Trojan horses for intracellular nucleotide and glycosylmonophosphate delivery - Chemistry meets biology, Eur. J. Org. Chem. (5) (2006) 1081-1102.
[118] J. Caton-Williams, L.N. Lin, M. Smith, Z. Huang, Convenient synthesis of nucleoside 5 '-triphosphates for RNA transcription, Chem. Commun. 47(28) (2011) 8142-8144.
[119] J. Caton-Williams, R. Hoxhaj, B. Fiaz, Z. Huang, Use of a Novel 5'-Regioselective Phosphitylating Reagent for One-Pot Synthesis of Nucleoside 5'-Triphosphates from Unprotected Nucleosides, Curr. Protoc. Nucleic Acid Chem. 0 1 (2013) Unit-1.30.
[120] J. Caton-Williams, M. Smith, N. Carrasco, Z. Huang, Protection-Free One-Pot Synthesis of 2 '-Deoxynucleoside 5 '-Triphosphates and DNA Polymerization, Org. Lett. 13(16) (2011) 4156-4159.
[121] K. Ohno, D. Sugiyama, L. Takeshita, T. Kanamori, Y. Masaki, M. Sekine, K. Seio, Synthesis of photocaged6-O-(2-nitrobenzyl) guanosine and 4-O-(2-nitrobenzyl)uridine triphosphates for photocontrol of the RNA transcription reaction, Bioorg. Med. Chem. 25(21) (2017) 6007-6015.

[122] M. Tsuda, K. Terada, M. Ooka, K. Kobayashi, H. Sasanuma, R. Fujisawa, T. Tsurimoto, J. Yamamoto, S. Iwai, K. Kadoda, R. Akagawa, S.Y.N. Huang, Y. Pommier, J.E. Sale, S. Takeda, K. Hirota, The dominant role of proofreading exonuclease activity of replicative polymerase epsilon in cellular tolerance to cytarabine (Ara-C), Oncotarget 8(20) (2017) 33457-33474.
[123] S.P. Sau, J.C. Chaput, A one-pot synthesis of alpha-L-threofuranosyl nucleoside triphosphates (tNTPs), Bioorg. Med. Chem. Lett. 26(14) (2016) 3271-3273.
[124] K.Y. Zou, A. Horhota, B. Yu, J.W. Szostak, L.W. McLaughlin, Synthesis of alpha-L-threofuranosyl nucleoside triphosphates (tNTPs), Org. Lett. 7(8) (2005) 1485-1487.
[125] A.S. Schroder, E. Parsa, K. Iwan, F.R. Traube, M. Wallner, S. Serdjukow, T. Carell, 2 '-(R)-Fluorinated mC, hmC, fC and caC triphosphates are substrates for DNA polymerases and TET-enzymes, Chem. Commun. 52(100) (2016) 14361-14364.
[126] T. Santner, V. Siegmund, A. Marx, R. Micura, The synthesis of 2 '-methylseleno adenosine and guanosine 5 '-triphosphates, Bioorg. Med. Chem. 20(7) (2012) 2416-2418.
[127] Y. Masaki, H. Ito, Y. Oda, K. Yamazaki, N. Tago, K. Ohno, N. Ishii, H. Tsunoda, T. Kanamori, A. Ohkubo, M. Sekine, K. Seio, Enzymatic synthesis and reverse transcription of RNAs incorporating 2 '-O-carbamoyl uridine triphosphate, Chem. Commun. 52(87) (2016) 12889-12892.
[128] W.D. Wu, C.L.F. Meyers, R.F. Borch, A novel method for the preparation of nucleoside triphosphates from activated nucleoside phosphoramidates, Org. Lett. 6(13) (2004) 2257-2260.
[129] Q. Sun, J.P. Edathil, R. Wu, E.D. Smidansky, C.E. Cameron, B.R. Peterson, One-pot synthesis of nucleoside 5 '-triphosphates from nucleoside 5 '-H-phosphonates, Org. Lett. 10(9) (2008) 1703-1706.
[130] S. Bala, J.Y. Liao, H. Mei, J.C. Chaput, Synthesis of alpha-L-Threofuranosyl Nucleoside 3 '-Monophosphates, 3 '-Phosphoro(2-Methyl)imidazolides, and 3 '-Triphosphates, J. Org. Chem. 82(11) (2017) 5910-5916.
[131] S. Bala, J.Y. Liao, L. Zhang, C.N. Tran, N. Chim, J.C. Chaput, Synthesis of 2 '-Deoxy-alpha-L-threofuranosyl Nucleoside Triphosphates, J. Org. Chem. 83(16) (2018) 8840-8850.
[132] S.P. Sau, J.C. Chaput, A Gram-Scale HPLC-Free Synthesis of TNA Triphosphates Using an Iterative Phosphorylation Strategy, Org. Lett. 19(16) (2017) 4379-4382.
[133] H. Mei, J.Y. Liao, R.M. Jimenez, Y.J. Wang, S. Bala, C. McCloskey, C. Switzer, J.C. Chaput, Synthesis and Evolution of a Threose Nucleic Acid Aptamer Bearing 7-Deaza-7-Substituted Guanosine Residues, J. Am. Chem. Soc. 140(17) (2018) 5706-5713.
[134] H. Mei, J.C. Chaput, Expanding the chemical diversity of TNA with tUTP derivatives that are substrates for a TNA polymerase, Chem. Commun. 54(10) (2018) 1237-1240.
[135] K. Phelps, A. Morris, P.A. Beal, Novel Modifications in RNA, ACS Chem. Biol. 7(1) (2012) 100-109.
[136] E. Paredes, M. Evans, S.R. Das, RNA labeling, conjugation and ligation, Methods 54(2) (2011) 251-259.
[137] A. De Mesmaeker, R. Haener, P. Martin, H.E. Moser, Antisense Oligonucleotides, Acc. Chem. Res. 28(9) (1995) 366-374.
[138] Y. Masaki, R. Miyasaka, A. Ohkubo, K. Seio, M. Sekine, Linear Relationship between Deformability and Thermal Stability of 2′-O-Modified RNA Hetero Duplexes, J. Phys. Chem. B. 114(7) (2010) 2517-2524.
[139] M. Egli, P.S. Pallan, Crystallographic Studies of Chemically Modified Nucleic Acids: A Backward Glance, Chem. Biodiversity 7(1) (2010) 60-89.
[140] K. Seio, R. Tawarada, T. Sasami, M. Serizawa, M. Ise, A. Ohkubo, M. Sekine, Synthesis and hybridization of 2′-O-methyl-RNAs incorporating 2′-O-carbamoyluridine and unique participation of the carbamoyl group in U–G base pair, Bioorg. Med. Chem. 17(20) (2009) 7275-7280.
[141] S.G. Wu, J.Y. Wang, X.M. Pu, L.C. Li, Q. Li, T7 RNA Polymerase Discriminates Correct and Incorrect Nucleoside Triphosphates by Free Energy, Biophys. J. 114(8) (2018) 1755-1761.
[142] G. Houlihan, S. Arangundy-Franklin, P. Holliger, Engineering and application of polymerases for synthetic genetics, Curr. Opin. Biotechnol. 48 (2017) 168-179.
[143] Y.W. Yin, T.A. Steitz, Structural basis for the transition from initiation to elongation transcription in T7 RNA polymerase, Science 298(5597) (2002) 1387-1395.

[144] I. Kuzmine, P.A. Gottlieb, C.T. Martin, Binding of the priming nucleotide in the initiation of transcription by T7 RNA polymerase, J. Biol. Chem. 278(5) (2003) 2819-2823.
[145] W.P. Kennedy, J.R. Momand, Y.W. Yin, Mechanism for de novo RNA synthesis and initiating nucleotide specificity by T7 RNA polymerase, J. Mol. Biol. 370(2) (2007) 256-268.
[146] M.B. Walunj, A.A. Tanpure, S.G. Srivatsan, Post-transcriptional labeling by using Suzuki-Miyaura cross-coupling generates functional RNA probes, Nucleic Acids Res. 46(11) (2018) 10.
[147] C.C. Smith, M. Hollenstein, C.J. Leumann, The synthesis and application of a diazirine-modified uridine analogue for investigating RNA-protein interactions, RSC Adv. 4(89) (2014) 48228-48235.
[148] T.M. Dewey, A.A. Mundt, G.J. Crouch, M.C. Zyzniewski, B.E. Eaton, New uridine dernivatives for systematic evolution of RNA ligands by exponential enrichment, J. Am. Chem. Soc. 117(32) (1995) 8474-8475.
[149] J.D. Vaught, T. Dewey, B.E. Eaton, T7 RNA polymerase transcription with 5-position modified UTP derivatives, J. Am. Chem. Soc. 126(36) (2004) 11231-11237.
[150] N.K. Vaish, R. Larralde, A.W. Fraley, J.W. Szostak, L.W. McLaughlin, A novel, modification-dependent ATP-binding aptamer selected from an RNA library incorporating a cationic functionality, Biochemistry 42(29) (2003) 8842-8851.
[151] A.M. Kabza, J.T. Sczepanski, An l-RNA Aptamer with Expanded Chemical Functionality that Inhibits MicroRNA Biogenesis, ChemBioChem 18(18) (2017) 1824-1827.
[152] T. Schoetzau, J. Langner, E. Moyroud, I. Roehl, S. Vonhoff, S. Klussmann, Aminomodified nucleobases: Functionalized nucleoside triphosphates applicable for SELEX, Bioconj. Chem. 14(5) (2003) 919-926.
[153] C.Y. Jao, A. Salic, Exploring RNA transcription and turnover in vivo by using click chemistry, Proc. Natl. Acad. Sci. U.S.A. 105(41) (2008) 15779-15784.
[154] P.N. Asare-Okai, E. Agustin, D. Fabris, M. Royzen, Site-specific fluorescence labelling of RNA using bio-orthogonal reaction of trans-cyclooctene and tetrazine, Chem. Commun. 50(58) (2014) 7844-7847.
[155] J.T. George, S.G. Srivatsan, Vinyluridine as a Versatile Chemoselective Handle for the Post-transcriptional Chemical Functionalization of RNA, Bioconj. Chem. 28(5) (2017) 1529-1536.
[156] T.J. Chen, F.E. Romesberg, Enzymatic Synthesis, Amplification, and Application of DNA with a Functionalized Backbone, Angew. Chem. Int. Ed. 56(45) (2017) 14046-14051.
[157] C. Domnick, F. Eggert, S. Kath-Schorr, Site-specific enzymatic introduction of a norbornene modified unnatural base into RNA and application in post-transcriptional labeling, Chem. Commun. 51(39) (2015) 8253-8256.
[158] J. Schoch, S. Ameta, A. Jaschke, Inverse electron-demand Diels-Alder reactions for the selective and efficient labeling of RNA, Chem. Commun. 47(46) (2011) 12536-12537.
[159] S.G. Srivatsan, Y. Tor, Fluorescent pyrimidine ribonucleotide: Synthesis, enzymatic incorporation, and utilization, J. Am. Chem. Soc. 129(7) (2007) 2044-2053.
[160] J.M. Holstein, A. Rentmeister, Current covalent modification methods for detecting RNA in fixed and living cells, Methods 98 (2016) 18-25.
[161] Z. Huang, W. Wen, A. Wu, L. Niu, Chemically Modified, alpha-Amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) Receptor RNA Aptamers Designed for in Vivo Use, ACS Chem. Neurosci. 8(11) (2017) 2437-2445.
[162] R. Sousa, R. Padilla, Mutant T7-RNA polymerase as a DNA polymerase, EMBO J. 14(18) (1995) 4609-4621.
[163] R. Padilla, R. Sousa, A Y639F/H784A T7 RNA polymerase double mutant displays superior properties for synthesizing RNAs with non-canonical NTPs, Nucleic Acids Res. 30(24) (2002) 4.
[164] J. Chelliserrykattil, A.D. Ellington, Evolution of a T7 RNA polymerase variant that transcribes 2 '-O-methyl RNA, Nat. Biotechnol. 22(9) (2004) 1155-1160.
[165] V. Siegmund, T. Santner, R. Micura, A. Marx, Screening mutant libraries of T7 RNA polymerase for candidates with increased acceptance of 2 '-modified nucleotides, Chem. Commun. 48(79) (2012) 9870-9872.

[166] M. Kimoto, A.J. Meyer, I. Hirao, A.D. Ellington, Genetic alphabet expansion transcription generating functional RNA molecules containing a five-letter alphabet including modified unnatural and natural base nucleotides by thermostable T7 RNA polymerase variants, Chem. Comm. 53(91) (2017) 12309-12312.
[167] B. Zhu, S. Tabor, D.A. Raytcheva, A. Hernandez, J.A. King, C.C. Richardson, The RNA Polymerase of Marine Cyanophage Syn5, J. Biol. Chem. 288(5) (2013) 3545-3552.
[168] B. Zhu, A. Hernandez, M. Tan, J. Wollenhaupt, S. Tabor, C.C. Richardson, Synthesis of 2 '-Fluoro RNA by Syn5 RNA polymerase, Nucleic Acids Res. 43(14) (2015) 11.
[169] P.H. Patel, L.A. Loeb, Multiple amino acid substitutions allow DNA polymerases to synthesize RNA, J. Biol. Chem. 275(51) (2000) 40266-40272.
[170] J.A. Brown, Z.C. Suo, Unlocking the Sugar "Steric Gate" of DNA Polymerases, Biochemistry 50(7) (2011) 1135-1142.
[171] S.M. Sherrer, D.C. Beyer, C.X. Xia, J.D. Fowler, Z.C. Suo, Kinetic Basis of Sugar Selection by a Y-Family DNA Polymerase from Sulfolobus solfataricus P2, Biochemistry 49(47) (2010) 10179-10186.
[172] G. Xia, L.J. Chen, T. Sera, M. Fa, P.G. Schultz, F.E. Romesberg, Directed evolution of novel polymerase activities: Mutation of a DNA polymerase into an efficient RNA polyrnerase, P.N.A.S. 99(10) (2002) 6597-6602.
[173] T.J. Chen, F.E. Romesberg, Polymerase Chain Transcription: Exponential Synthesis of RNA and Modified RNA, J. Am. Chem. Soc. 139(29) (2017) 9949-9954.
[174] T.J. Chen, N. Hongdilokkul, Z.X. Liu, R. Adhikary, S.S. Tsuen, F.E. Romesberg, Evolution of thermophilic DNA polymerases for the recognition and amplification of C2 '-modified DNA, Nat. Chem. 8(6) (2016) 557-563.
[175] J.L. Ong, D. Loakes, S. Jaroslawski, K. Too, P. Holliger, Directed evolution of DNA polymerase, RNA polymerase and reverse transcriptase activity in a single polypeptide, J. Mol. Biol. 361(3) (2006) 537-550.
[176] C. Cozens, V.B. Pinheiro, A. Vaisman, R. Woodgate, P. Holliger, A short adaptive path from DNA to RNA polymerases, P.N.A.S. 109(21) (2012) 8067-8072.
[177] V.B. Pinheiro, A.I. Taylor, C. Cozens, M. Abramov, M. Renders, S. Zhang, J.C. Chaput, J. Wengel, S.-Y. Peak-Chew, S.H. McLaughlin, P. Herdewijn, P. Holliger, Synthetic Genetic Polymers Capable of Heredity and Evolution, Science 336(6079) (2012) 341-344.
[178] A.I. Taylor, V.B. Pinheiro, M.J. Smola, A.S. Morgunov, S. Peak-Chew, C. Cozens, K.M. Weeks, P. Herdewijn, P. Holliger, Catalysts from synthetic genetic polymers, Nature 518(7539) (2015) 427-430.
[179] A. Pech, J. Achenbach, M. Jahnz, S. Schulzchen, F. Jarosch, F. Bordusa, S. Klussmann, A thermostable D-polymerase for mirror-image PCR, Nucleic Acids Res. 45(7) (2017) 3997-4005.
[180] D. Oberthur, J. Achenbach, A. Gabdulkhakov, K. Buchner, C. Maasch, S. Falke, D. Rehders, S. Klussmann, C. Betzel, Crystal structure of a mirror-image L-RNA aptamer (Spiegelmer) in complex with the natural L-protein target CCL2, Nat. Comm. 6 (2015) 11.
[181] M. Fa, A. Radeghieri, A.A. Henry, F.E. Romesberg, Expanding the substrate repertoire of a DNA polymerase by directed evolution, J. Am. Chem. Soc. 126(6) (2004) 1748-1754.
[182] H.J. Schultz, A.M. Gochi, H.E. Chia, A.L. Ogonowsky, S. Chiang, N. Filipovic, A.G. Weiden, E.E. Hadley, S.E. Gabriel, A.M. Leconte, Taq DNA Polymerase Mutants and 2'-Modified Sugar Recognition, Biochemistry 54(38) (2015) 5999-6008.
[183] W.K. Johnston, P.J. Unrau, M.S. Lawrence, M.E. Glasner, D.P. Bartel, RNA-catalyzed RNA polymerization: Accurate and general RNA-templated primer extension, Science 292(5520) (2001) 1319-1325.
[184] D.P. Horning, G.F. Joyce, Amplification of RNA by an RNA polymerase ribozyme, Proc. Natl. Acad. Sci. U.S.A. 113(35) (2016) 9786-9791.
[185] A. Wochner, J. Attwater, A. Coulson, P. Holliger, Ribozyme-Catalyzed Transcription of an Active Ribozyme, Science 332(6026) (2011) 209-212.
[186] J. Attwater, S. Tagami, M. Kimoto, K. Butler, E.T. Kool, J. Wengel, P. Herdewijn, I. Hirao, P. Holliger, Chemical fidelity of an RNA polymerase ribozyme, Chem. Sci. 4(7) (2013) 2804-2814.

[187] B. Samanta, D.P. Horning, G.F. Joyce, 3’-End labeling of nucleic acids by a polymerase ribozyme, Nucleic Acids Res. 46(17) (2018) e103-e103.
[188] J.T. Sczepanski, G.F. Joyce, A cross-chiral RNA polymerase ribozyme, Nature 515(7527) (2014) 440-442.
[189] J. Attwater, A. Raguram, A.S. Morgunov, E. Gianni, P. Holliger, Ribozyme-catalysed RNA synthesis using triplet building blocks, eLife 7 (2018).
[190] H.Y. Sun, S.B. Jiang, J. Caton-Williams, H.H. Liu, Z. Huang, 2-Selenouridine triphosphate synthesis and Se-RNA transcription, RNA 19(9) (2013) 1309-1314.
[191] V. Siegmund, T. Santner, R. Micura, A. Marx, Enzymatic synthesis of 2 '-methylseleno-modified RNA, Chem. Sci. 2(11) (2011) 2224-2231.
[192] K.T. Urak, S. Shore, W.M. Rockey, S.J. Chen, A.P. McCaffrey, P.H. Giangrande, In vitro RNA SELEX for the generation of chemically-optimized therapeutic RNA drugs, Methods 103 (2016) 167-174.
[193] A.D. Keefe, S. Pai, A. Ellington, Aptamers as therapeutics, Nat. Rev. Drug Discov. 9(7) (2010) 537-550.
[194] Y.H. Lao, K.K.L. Phua, K.W. Leong, Aptamer Nanomedicine for Cancer Therapeutics: Barriers and Potential for Translation, ACS Nano 9(3) (2015) 2235-2254.
[195] M. Vorobyeva, V. Timoshenko, P. Vorobjev, A. Venyaminova, Aptamers Against Immunologic Targets: Diagnostic and Therapeutic Prospects, Nucleic Acid Ther. 26(1) (2016) 52-65.
[196] M. Famulok, J.S. Hartig, G. Mayer, Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy, Chem. Rev. 107(9) (2007) 3715-3743.
[197] L. Yang, X.B. Zhang, M. Ye, J.H. Jiang, R.H. Yang, T. Fu, Y. Chen, K.M. Wang, C. Liu, W.H. Tan, Aptamer-conjugated nanomaterials and their applications, Adv. Drug Deliv. Rev. 63(14-15) (2011) 1361-1370.
[198] S. Kruspe, F. Mittelberger, K. Szameit, U. Hahn, Aptamers as Drug Delivery Vehicles, ChemMedChem 9(9) (2014) 1998-2011.
[199] A.S. Davydova, M.A. Vorobjeva, A.G. Venyaminova, Escort Aptamers: New Tools for the Targeted Delivery of Therapeutics into Cells, Acta Naturae 3(4) (2011) 12-29.
[200] M.R. Dunn, R.M. Jimenez, J.C. Chaput, Analysis of aptamer discovery and technology, Nat. Rev. Chem. 1(10) (2017) 16.
[201] E.N. Brody, L. Gold, Aptamers as therapeutic and diagnostic agents, Rev. Mol. Biotechnol. 74(1) (2000) 5-13.
[202] A.M. Gewirtz, Oligonucleotide therapeutics: Clothing the emperor, Curr. Opin. Mol. Tech. 1(3) (1999) 297-306.
[203] J.H. Zhou, J. Rossi, Aptamers as targeted therapeutics: current potential and challenges, Nat. Rev. Drug Discov. 16(3) (2017) 181-202.
[204] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment - RNA ligands to bacteriophage T4 DNA polymerase Science 249(4968) (1990) 505-510.
[205] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346(6287) (1990) 818-822.
[206] T.E. Velez, J. Singh, Y. Xiao, E.C. Allen, O.Y. Wong, M. Chandra, S.C. Kwon, S.K. Silverman, Systematic Evaluation of the Dependence of Deoxyribozyme Catalysis on Random Region Length, ACS Comb. Sci. 14(12) (2012) 680-687.
[207] R. Knight, M. Yarus, Analyzing partially randomized nucleic acid pools: straight dope on doping, Nucleic Acids Res. 31(6) (2003) 9.
[208] S.K. Silverman, Catalytic DNA (deoxyribozymes) for synthetic applications - current abilities and future prospects, Chem. Commun. (30) (2008) 3467-3485.
[209] S.M. Shamah, J.M. Healy, S.T. Cload, Complex target SELEX, Acc. Chem. Res. 41(1) (2008) 130-138.
[210] J. Mi, Y.M. Liu, Z.N. Rabbani, Z.G. Yang, J.H. Urban, B.A. Sullenger, B.M. Clary, In vivo selection of tumor-targeting RNA motifs, Nat. Chem. Biol. 6(1) (2010) 22-24.
[211] J. Barman, Targeting cancer cells using aptamers: cell-SELEX approach and recent advancements, RSC Adv. 5(16) (2015) 11724-11732.

[212] A.V. Lakhin, V.Z. Tarantul, L.V. Gening, Aptamers: Problems, Solutions and Prospects, Acta Naturae 5(4) (2013) 34-43.
[213] J.P. Shaw, K. Kent, J. Bird, J. Fishback, B. Froehler, Modified deoxyoligonucleotides stable to exonuclease degradation in serum, Nucleic Acids Res. 19(4) (1991) 747-750.
[214] N.C. Pagratis, C. Bell, Y.F. Chang, S. Jennings, T. Fitzwater, D. Jellinek, C. Dang, Potent 2'-amino-, and 2'-fluoro-2'-deoxyribonucleotide RNA inhibitors of keratinocyte growth factor, Nat. Biotech. 15(1) (1997) 68-73.
[215] C.P. Rusconi, J.D. Roberts, G.A. Pitoc, S.M. Nimjee, R.R. White, G. Quick, E. Scardino, W.P. Fay, B.A. Sullenger, Antidote-mediated control of an anticoagulant aptamer in vivo, Nat. Biotech. 22(11) (2004) 1423-1428.
[216] S. Shigdar, J. Macdonald, M. O'Connor, T. Wang, D.X. Xiang, H. Al Shamaileh, L. Qiao, M. Wei, S.F. Zhou, Y.M. Zhu, L.X. Kong, S. Bhattacharya, C.G. Li, W. Duan, Aptamers as Theranostic Agents: Modifications, Serum Stability and Functionalisation, Sensors 13(10) (2013) 13624-13637.
[217] C. Kratschmer, M. Levy, Effect of Chemical Modifications on Aptamer Stability in Serum, Nucleic Acid Ther. 27(6) (2017) 335-344.
[218] P.E. Burmeister, S.D. Lewis, R.F. Silva, J.R. Preiss, L.R. Horwitz, P.S. Pendergrast, T.G. McCauley, J.C. Kurz, D.M. Epstein, C. Wilson, A.D. Keefe, Direct in vitro selection of a 2 '-O-methyl aptamer to VEGF, Chem. Biol. 12(1) (2005) 25-33.
[219] R.M. Boomer, S.D. Lewis, J.M. Healy, M. Kurz, C. Wilson, T.G. McCauley, Conjugation to polyethylene glycol polymer promotes aptamer biodistribution to healthy and inflamed tissues, Oligonucleotides 15(3) (2005) 183-195.
[220] P. Sundaram, H. Kurniawan, M.E. Byrne, J. Wower, Therapeutic RNA aptamers in clinical trials, Eur. J. Pharm. Sci. 48(1) (2013) 259-271.
[221] C.A. Stein, D. Castanotto, FDA-Approved Oligonucleotide Therapies in 2017, Mol. Ther. 25(5) (2017) 1069-1075.
[222] E.W.M. Ng, D.T. Shima, P. Calias, E.T. Cunningham, D.R. Guyer, A.P. Adamis, Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease, Nat. Rev. Drug Discov. 5(2) (2006) 123-132.
[223] J. Ruckman, L.S. Green, J. Beeson, S. Waugh, W.L. Gillette, D.D. Henninger, L. Claesson-Welsh, N. Janjic, 2 '-fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF(165)) - Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain, J. Biol. Chem. 273(32) (1998) 20556-20567.
[224] J.H. Lee, M.D. Canny, A. De Erkenez, D. Krilleke, Y.S. Ng, D.T. Shima, A. Pardi, F. Jucker, A therapeutic aptamer inhibits angiogenesis by specifically targeting the heparin binding domain of VEGF(165), Proc. Natl. Acad. Sci. U.S.A. 102(52) (2005) 18902-18907.
[225] S.X. Gao, X. Zheng, B.H. Jiao, L.H. Wang, Post-SELEX optimization of aptamers, Anal. Bioanal. Chem. 408(17) (2016) 4567-4573.
[226] P. Röthlisberger, M. Hollenstein, Aptamer chemistry, Adv. Drug Deliv. Rev. 134 (2018) 3-21.
[227] G. Lee, D.M. MacLean, H. Ulrich, X. Zhao, J. Aronowski, V. Jayaraman, RNA Based Antagonist of NMDA Receptors, ACS Chem. Neurosci. 5(7) (2014) 559-567.
[228] H.Y. Kong, J. Byun, Screening and Characterization of a Novel RNA Aptamer That Specifically Binds to Human Prostatic Acid Phosphatase and Human Prostate Cancer Cells, Mol. Cells 38(2) (2015) 171-179.
[229] S.H. Purvis, J.R. Keefer, Y.M. Fortenberry, E.A. Barron-Casella, J.F. Casella, Identification of Aptamers That Bind to Sickle Hemoglobin and Inhibit Its Polymerization, Nucleic Acid Ther. 27(6) (2017) 354-364.
[230] J.I. Jung, S.R. Han, S.W. Lee, Development of RNA aptamer that inhibits methyltransferase activity of dengue virus, Biotechnol. Lett. 40(2) (2018) 315-324.
[231] Y. Kato, N. Minakawa, Y. Komatsu, H. Kamiya, N. Ogawa, H. Harashima, A. Matsuda, New NTP analogs: the synthesis of 4 '-thioUTP and 4 '-thioCTP and their utility for SELEX, Nucleic Acids Res. 33(9) (2005) 2942-2951.

[232] N. Minakawa, M. Sanji, Y. Kato, A. Matsuda, Investigations toward the selection of fully-modified 4 '-thioRNA aptamers: Optimization of in vitro transcription steps in the presence of 4 '-thioNTPs, Bioorg. Med. Chem. 16(21) (2008) 9450-9456.
[233] M.Y. Anzahaee, J.K. Watts, N.R. Alla, A.W. Nicholson, M.J. Damha, Energetically Important C−H···F−C Pseudohydrogen Bonding in Water: Evidence and Application to Rational Design of Oligonucleotides with High Binding Affinity, J. Am. Chem. Soc. 133(4) (2011) 728-731.
[234] J. Lietard, H. Abou Assi, I. Gomez-Pinto, C. Gonzalez, M.M. Somoza, M.J. Damha, Mapping the affinity landscape of Thrombin-binding aptamers on 2 ' F-ANA/DNA chimeric G-Quadruplex microarrays, Nucleic Acids Res. 45(4) (2017) 1619-1632.
[235] C.G. Peng, M.J. Damha, G-quadruplex induced stabilization by 2'-deoxy-2'-fluoro-d-arabinonucleic acids (2'F-ANA), Nucleic Acids Res. 35(15) (2007) 4977-4988.
[236] I.A. Ferreira-Bravo, C. Cozens, P. Holliger, J.J. DeStefano, Selection of 2 '-deoxy-2 '-fluoroarabinonucleotide (FANA) aptamers that bind HIV-1 reverse transcriptase with picomolar affinity, Nucleic Acids Res. 43(20) (2015) 9587-9599.
[237] Y.J. Wang, A.K. Ngor, A. Nikoomanzar, J.C. Chaput, Evolution of a General RNA-Cleaving FANA Enzyme, Nat. Comm. 9 (2018) 10.
[238] M.Z. Liu, H. Jinmei, H. Abe, Y. Ito, In vitro selection of a photoresponsive RNA aptamer to hemin, Bioorg. Med. Chem. Lett. 20(9) (2010) 2964-2967.
[239] F. Eckstein, Phosphorothioates, Essential Components of Therapeutic Oligonucleotides, Nucleic Acid Ther. 24(6) (2014) 374-387.
[240] N.D. Abeydeera, M. Egli, N. Cox, K. Mercier, J.N. Conde, P.S. Pallan, D.M. Mizurini, M. Sierant, F.E. Hibti, T. Hassell, T.Z. Wang, F.W. Liu, H.M. Liu, C. Martinez, A.K. Sood, T.P. Lybrand, C. Frydman, R.Q. Monteiro, R.H. Gomer, B. Nawrot, X.B. Yang, Evoking picomolar binding in RNA by a single phosphorodithioate linkage, Nucleic Acids Res. 44(17) (2016) 8052-8064.
[241] S.M. Lato, N.D.S. Ozerova, K.Z. He, Z. Sergueeva, B.R. Shaw, D.H. Burke, Boron-containing aptamers to ATP, Nucleic Acids Res. 30(6) (2002) 1401-1407.
[242] A.W. Feldman, E.C. Fischer, M.P. Ledbetter, J.Y. Liao, J.C. Chaput, F.E. Romesberg, A Tool for the Import of Natural and Unnatural Nucleoside Triphosphates into Bacteria, J. Am. Chem. Soc. 140(4) (2018) 1447-1454.
[243] D.A. Malyshev, K. Dhami, T. Lavergne, T.J. Chen, N. Dai, J.M. Foster, I.R. Correa, F.E. Romesberg, A semi-synthetic organism with an expanded genetic alphabet, Nature 509(7500) (2014) 385-388? .
[244] Z. Zawada, A. Tatar, P. Mocilac, M. Budesinsky, T. Kraus, Transport of Nucleoside Triphosphates into Cells by Artificial Molecular Transporters, Angew. Chem. Int. Ed. 57(31) (2018) 9891-9895.