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SYNTHESIS OF SHORT OLIGONUCLEOTIDES ON A SOLUBLE SUPPORT BY THE PHOSPHORAMIDITE METHOD

Alejandro Gimenez Molina

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ABSTRACT

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GIMENEZ MOLINA, ALEJANDRO: Synthesis of Short Oligonucleotides on a Soluble Support by the Phosphoramidite Method

Doctoral thesis, 110 p.
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In the last decades, the chemical synthesis of short oligonucleotides has become an important aspect of study due to the discovery of new functions for nucleic acids such as antisense oligonucleotides (ASOs), aptamers, DNAzymes, microRNA (miRNA) and small interfering RNA (siRNA). The applications in modern therapies and fundamental medicine on the treatment of different cancer diseases, viral infections and genetic disorders has established the necessity to develop scalable methods for their cheaper and easier industrial manufacture. While small scale solid-phase oligonucleotide synthesis is the method of choice in the field, various challenges still remain associated with the production of short DNA and RNA-oligomers in very large quantities. On the other hand, solution phase synthesis of oligonucleotides offers a more predictable scaling-up of the synthesis and is amenable to standard industrial manufacture techniques.

In the present thesis, various protocols for the synthesis of short DNA and RNA oligomers have been studied on a peracetylated and methylated β-cyclodextrin, and also on a pentaerythritol-derived support. On using the peracetylated and methylated β-cyclodextrin soluble supports, the coupling cycle was simplified by replacement of the typical 5′-*O*-(4,4′-dimethoxytrityl) protecting group with an acid-labile acetal-protected 5′-*O*-(1-methoxy-1-methylethyl) group, which upon acid-catalyzed methanolysis released easily removable volatile products. For this reason monomeric building blocks 5′-*O*-(1-methoxy-1-methylethyl) 3′-(2-cyano-ethyl-*N*,*N*-diisopropylphosphoramidite) were synthesized. Alternatively, on using the precipitative pentaerythritol support, novel 2′-*O*-(2-cyanoethyl)-5′-*O*-(1-methoxy-1-methylethyl) protected phosphoramidite building blocks for RNA synthesis have been prepared and their applicability by the synthesis of a pentamer was demonstrated. Similarly, a method for the preparation of short RNAs from commercially available 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethyl-silyl)ribonucleoside 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) building blocks has been developed.

Key words: Oligonucleotides, Nucleosides, DNA, RNA, Soluble Support, Phosphoramidite method, large scale synthesis

PREFACE

This thesis is based on the experimental work carried out in the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku during the years 2010-2014. The financial support from the European Comission's Marie Curie 7th Framework Programme and the University of Turku Graduate School are gratefully acknowledged.

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This thesis is dedicated to all of you!

Turku, August 2015

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. Molina, A. G.; Kungurtsev, V.; Virta, P.; Lönnberg, H. Acetylated and Methylated β-Cyclodextrins as Viable Soluble Supports for the Synthesis of Short 2'-Oligodeoxyribonucleotides in Solution. *Molecules* **2012**, *17*, 12102-12120.

II. Kungurtsev, V.; Laakkonen, J.; Molina, A. G.; Virta, P. Solution-Phase Synthesis of Short Oligo-2′-deoxyribonucleotides by Using Clustered Nucleosides as a Soluble Support. *Eur. J. Org. Chem*. **2013**, 6687–6693.

III. Molina, A. G.; Jabgunde, A. M.; Virta, P.; Lönnberg, H., Solution Phase Synthesis of Short Oligoribonucleotides on a Precipitative Tetrapodal Support. *Beilstein J. Org. Chem*. **2014**, *10*, 2279-2285.

IV. Molina, A. G.; Jabgunde, A. M.; Virta, P.; Lönnberg, H., Assembly of Short Oligoribonucleotides from Commercially Available Building Blocks on a Tetrapodal Soluble Support. *Curr. Org. Synth*. **2015**, *12*, 202-207.

ABBREVIATIONS

Ac	acetyl
ACE	bis(2-acetoxyethoxy)methyl
Ade	adenine
ASOs	antisense oligonucleotides
β-CD	β-cyclodextrin
BTT	5-benzylthio-1*H*-tetrazole
Bz	benzoyl
CDs	cyclodextrins
CE	2-cyanoethyl
CEE	2-cyanoethoxyethyl
CEM	2-cyanoethoxymethyl
CPG	controlled pore glass
Cyt	cytosine
DCA	dichloroacetic acid
DCC	*N,N′*-dicyclohexylcarbodiimide
DCE	1,2-dichloroethane
DCI	4,5-dicyanoimidazole
DCM	dichloromethane
DIC	*N,N′*-diisopropylcarbodiimide
DIEA	*N,N*-diisopropylethylamine
DMAc	*N,N*-dimethylacetamide
DMAP	4-dimethylaminopyridine
dmf	dimethylaminomethylene
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMTr	4,4′-dimethoxytrityl
DMTrCl	4,4′-dimethoxytrityl chloride
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
DTM	*tert*-butyldithiomethyl
DTT	dithiothreitol
EA	ethyl acetate
ESI-MS	electrospray ionization mass spectrometry
Et	ethyl
ETT	5-ethylthio-1*H*-tetrazole
FDA	Food and Drug Administration
Gua	guanine
HELP	High Efficiency Liquid Phase
HPLC	high-performance liquid chromatography
HPX	hexakis(*p*-hydroxyphenyl)xylene
HRMS	high resolution mass spectrometry
ibu	isobutyryl

IEHPLC	ion exchange high-performance liquid chromatography
Im	imidazole
iPr	isopropyl
Kg	kilogram
LPS	liquid phase synthesis
Me	methyl
MeOH	methanol
miRNA	microRNA
MMTr	4-methoxytrityl
MMTrCl	4-methoxytrityl chloride
MS	mass spectrometry
Mthp	4-methoxytetrahydropyran-4-yl
NBC	*S*-nitrobenzylcysteine
NBOM	[(2-nitrobenzyl)oxy]methyl
NMI	*N*-methylimidazole
NMR	nuclear magnetic resonance
ONs	oligonucleotides
OSN	organic solvent nanofiltration
PBI	polybenzimidazole
PEG	polyethylene glycol
PGs	protecting groups
PS	polystyrene
Py	pyridine
RISC	ribonucleic acid induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RPHPLC	reversed-phase high-performance liquid chromatography
SELEX	systematic evolution of ligands by exponential enrichment
siRNA	small interfering ribonucleic acid
SPS	solid-phase synthesis
SSOs	splice switching oligonucleotides
TBAF	tetrabutylammonium fluoride
TBAI	tetrabutylammonium iodide
TBDMS	*tert*-butyldimethylsilyl
t-Bu	*tert*-butyl
TC	1,1-dioxo-1λ^6-thiomorpholine-4-carbothioate
TEA	triethylamine
TEAA	triethylammonium acetate
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thy	thymine
TIPDS	1,1,3,3,-tetraisopropyl-1,3-disiloxane
TLC	thin layer chromatography
TMS	tetramethylsilane
TOM	triisopropylsilyloxymethyl

TPM	tetrakis(*p*-hydroxyphenyl)methane
Tr	trityl
TsCl	tosyl chloride
Ura	uracil
USD	United States Dollar
UV	ultraviolet

1. INTRODUCTION

While the central role of nucleic acids in storage, transmission and dissemination of genetic information has been known since the middle of the twentieth century, the interest in nucleic acids as drugs and drug targets dates back only to the begining of 1980s. Zamecnik & Stephenson[1] (1978) found that Rous Sarcoma virus replication may be inhibited by a specific oligonucleotide. This pioneering work brought up the question: could oligonucleotides also be used as drugs to combat against tumors, viral and hereditary diseases? Contrary to the "classical" drug treatment concept, the underlying idea of these antisense drugs (**Figure 1**) is that arresting of mRNA selectively prevents the transfer of information from DNA to ribosomes and, hence, stops the protein synthesis. In theory, any mRNA may be entirely selectively arrested by a complementary 17-mer oligonucleotide[2].

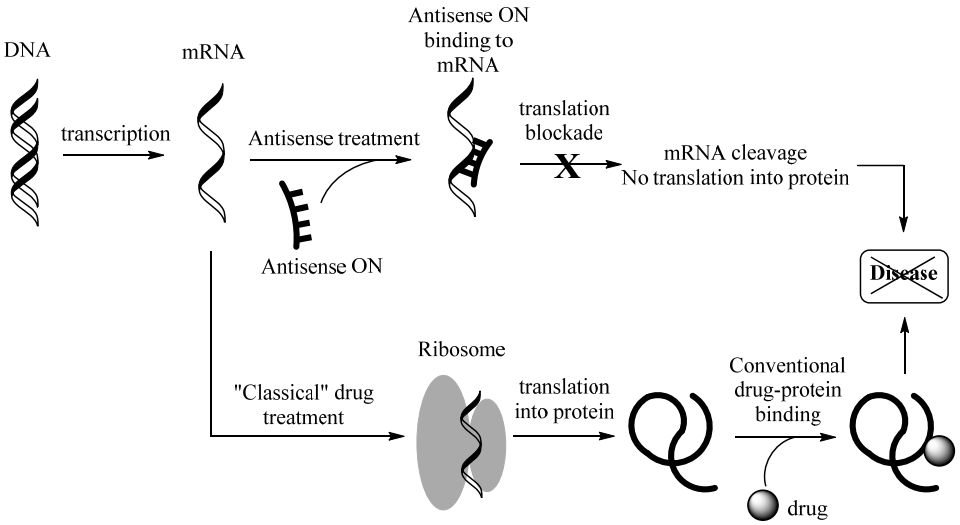


Figure 1. The underlying principle of selective inhibition of protein synthesis by antisense oligonucleotides (ASOs) and the "classical" drug treatment.

More recently splice switching oligos (SSOs) have been used for targeting of various disease-related genes[3]. The concept of SSOs is simple: chemically modified oligonucleotides block the access of the spliceosome to a splice site in the targeted pre-mRNA, producing the redirection of the process to another pathway (**Figure 2**). The modulation of the pre-mRNA via the SSOs has important therapeutic values, since it may be used to correct defective pre-mRNA and restore the translation when a reading frame is re-established by exon skipping and also to encode novel proteins with beneficial properties[4]. Splice switching oligos interfere with mRNA splicing, but do not arrest mRNA.

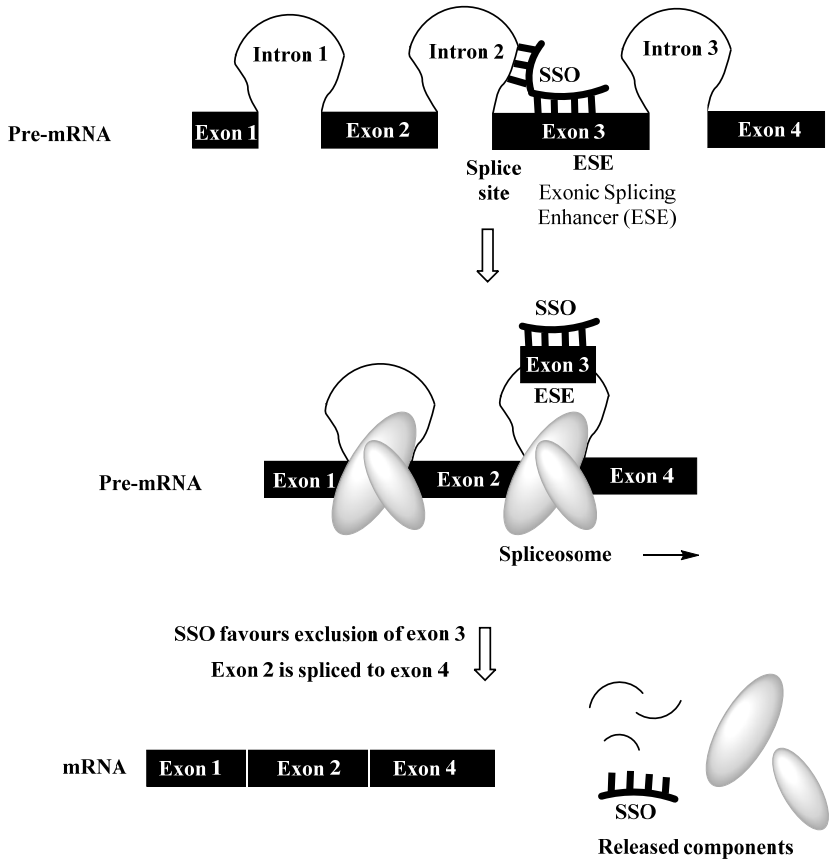


Figure 2. Exon skipping, an example of the action of splice-switching oligo-nucleotides (SSOs). The exclusion of a particular exon is carried out in the presence of complementary SSOs.

In late 1990s, the phenomenon of RNA interference (RNAi) had an important impact in the field of cell biology when Fire *et al*[5]. discovered that double stranded RNA (dsRNA) could efficienty inhibit gene expression. Afterward, the interest of oligonucleotide-based therapeutic technologies gradually shifted to RNA-oligonucleotides. Small interfering RNAs (siRNA)[6] that form the basis of RNAi-therapy, are 21 nucleotides long dsRNAs with 2 nucleotide long overhangs. siRNAs can modulate the gene expression to eliminate a gene-related disease[7] by first loading the antisense strand into the RISC (RNA-induced silencing complex) protein complex and inducing the enzymatic degradation upon binding to the target mRNA (**Figure 3**).

Another mode of RNAi-based therapy uses endogenous microRNA (miRNA)[8] as targets of antisense oligonucleotides (called now antagomirs). miRNAs are processed by the same enzyme system (RISC complex) as siRNAs and they, hence, play a role in regulation of gene expression.

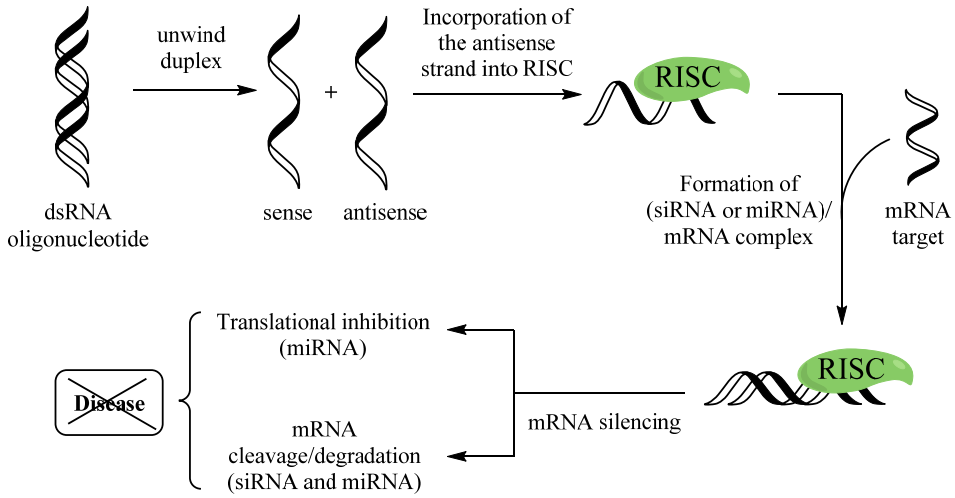


Figure 3. Model involving RNAi (siRNA and miRNA) principle.

Besides the therapies discussed above, Tuerk and Gold[9] in 1990s developed a technique called SELEX (Systematic Evolution of Ligands by EXponential Enrichment) for the isolation of synthetic oligonucleotides known as aptamers. Contrary to the previous therapeutic approaches in which the target was a mRNA molecule, aptamers are short single stranded oligonucleotides that form 3D structures with the capacity of binding to a ligand target by non-covalent bonds. In other words, they are able to form stable complexes with non-nucleic acid targets (**Figure 4**). High affinity and specificity together with low toxicity and immunogeneicity are the main advantages of these therapeutic agents. A special subtype of aptamers are spiegelmers[10-11] that offers good resistance to nucleases and, hence, excellent stability from degradation. They are composed of unnatural L-nucleosides and the recognition of the targets takes place as with the other aptamers.

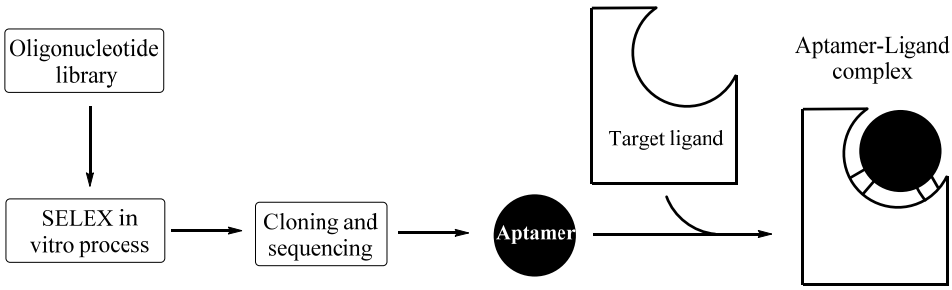


Figure 4. General schematic diagram of selection and production of an RNA aptamer as a binding agent.

Over the last two decades, the importance of nucleic acids as drug targets and oligonucleotides as drugs has considerably expanded due to the promises that they have shown on treatment of different cancer diseases, viral infections and genetic disorders[12]. As of year 2015, two antisense oligonucleotides named Vitravene[13] and Kynamro[14] (21-mer and 20-mer oligos, respectively) have been approved by the FDA for clinical use and several antisense drug candidates are in phase III clinical trials[15]. Similarly, one aptamer named Macugen[16] (28-mer oligonucleotide) has received approval by the FDA for the treatment of macular degenerations. In addition, more than hundred oligonucleotide drug candidates are currently undergoing clinical trials of various phases[17] and it is expected that some of them will soon be approved for pharmaceutical commercialization. As a proof of concept, one SSO is in phase III clinical trial and two are in phase II[15]. Also several siRNA drug candidates are in phase II clinical trials[18] and one antagomir is in phase II[15].

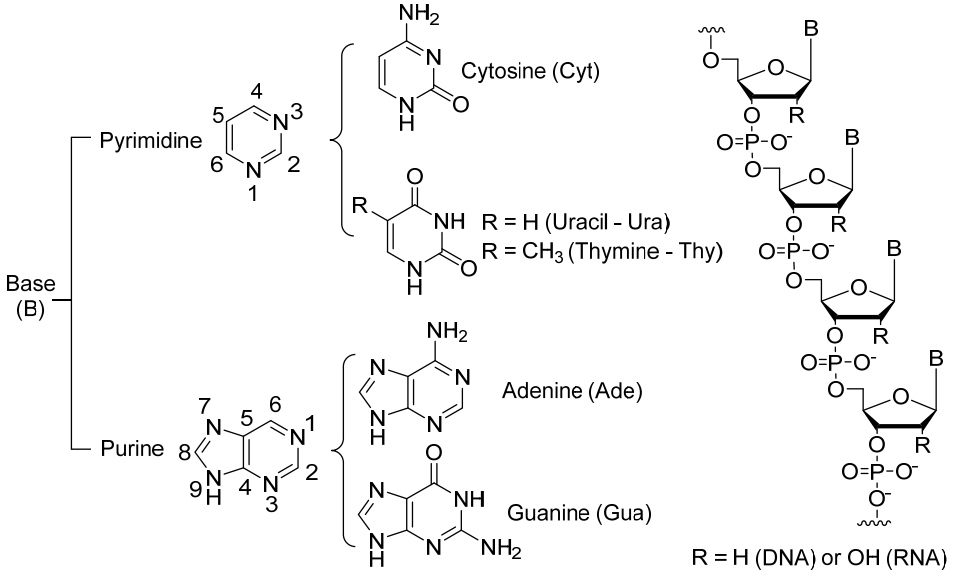
Apart from these potential forms of chemotherapy, another aspect to be considered is the use of oligonucleotides as raw materials for the construction of nano scale objects and devices. By using oligonucleotides, different nanostructured materials have been assembled for medical and computational applications[19]. Nanomechanical devices based on structural transitions induced by sequence dependent interactions have already been realized at a proof of concept level. Although the field has rapidly expanded in the last decade, the study of oligonucleotide-based nanotechnology still is at the early stage and significant progress will soon provide novel materials with different applications.

The synthesis of large amounts of oligonucleotide-based drugs is fundamental for their development as potential therapeutic agents and as starting material for various nano-scale constructs. The increased demand of larger quantities of oligonucleotides for these purposes has urged the need for novel large scale production platforms of oligonucleotides[20].

The current mainstream platform for the manufacture of ONs is the well-known solid phase synthesis. It is the method of choice for the small scale assembly of oligonucleotides and for more than 10 years has been routinely practiced on a higher scale[21-22]. Although the actual manufacture of oligonucleotides on an automated synthesizer is fast and efficient, the synthesis scale still is limited. The synthesis platforms available do not allow production in multikilogram quantities per batch[23]. The development of new methodologies and strategies for scaling-up the process is a crucial task nowadays for their successful large scale commercialization[24].

1.1 DNA and RNA structures

Both DNA and RNA are composed of three main constituents: a nitrogenous base, a pentose sugar and a phosphate group. The nitrogenous base is either a purine base [adenine (Ade) or guanine (Gua)] or a pyrimidine base [thymine (Thy), uracil (Ura) or cytosine (Cyt)] (**Scheme 1**). The N1 of pyrimidine base or N9 of purine base is bound to the C1 position of the pentose by a β-glycosyl linkage, and the resulting nucleosides form a linear polymer by 3',5'-phosphodiester bonds. There are only two differences in the structure of DNA and RNA oligonucleotides: 1) DNA cotains a 2-deoxyribose sugar whereas RNA is composed of a ribose sugar, and 2) RNA nucleosides have uracil bases instead of thymine bases (DNA).



Scheme 1. Purine and pyrimidine bases, and general structure of single-stranded DNA and RNA.

1.2 General remarks of oligonucleotide synthesis

The chemical synthesis of oligonucleotides usually takes place in the 3'→5' direction. It is carried out in a stepwise manner by linking monomeric building blocks in a predesign order to the growing chain by phosphodiester bonds. The procedures utilized nowadays for the synthesis of these polymers are based on the use of a solid support and the so-called phosphoramidite chemistry. This approach utilizes the P(III) oxidation state instead of the P(V) state during the coupling step, because of its higher reactivity[25]. Accordingly, short coupling time and high coupling efficiency have been achieved. In fact, as shown in **Figure 1**, a high stepwise coupling yield is needed in order to obtain an acceptable overall yield,

because of the exponential dependence of the overall yield on the number of coupling steps[26].

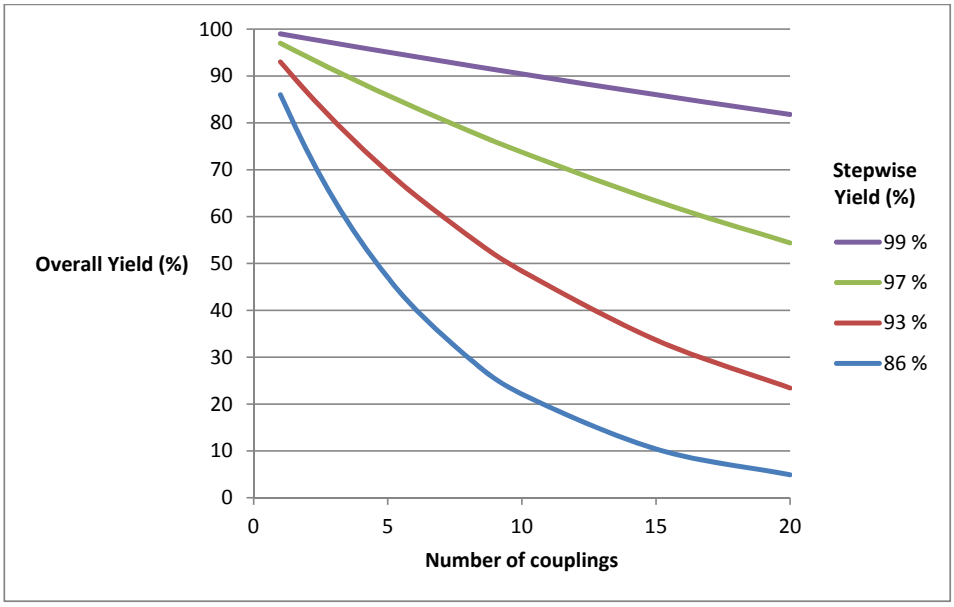
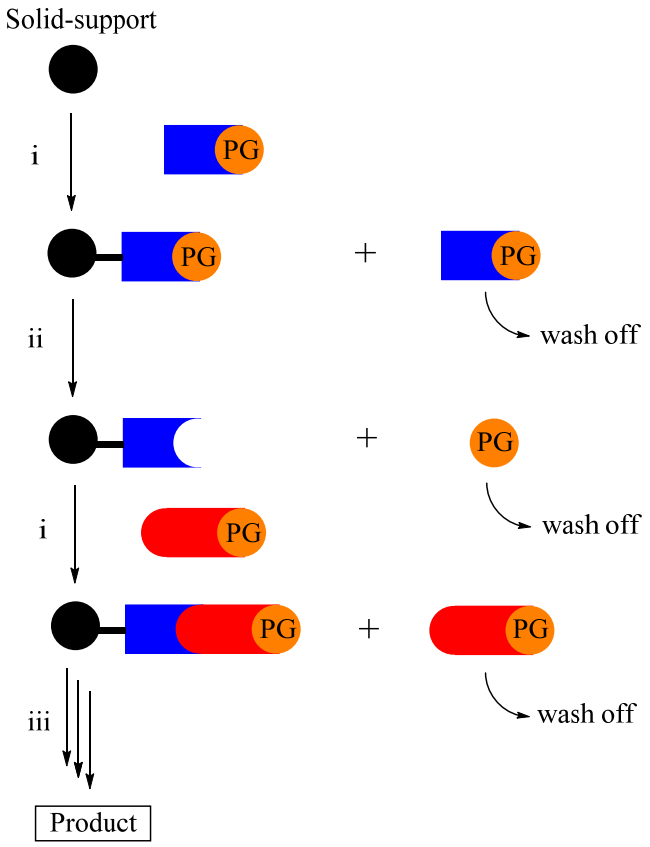


Figure 1. Overall yield vs number of steps coupling yield at different stepwise coupling yields[26].

1.3 Solid Phase Synthesis.

The general solid-phase synthesis (SPS) strategy is simple: the 3´-terminal nucleoside is bound to an insoluble polymer or silica support on which the chain assembly takes place. After each coupling, impurities and excess of reagents are washed away. The process is repeated as many times as the desired product is accomplished and finally the oligonucleotide is released from the solid support (**Scheme 2**).



Scheme 2. General oligonucleotide solid-phase synthesis strategy. PG stands for the commonly used acid labile 4,4′-dimethoxytrityl (DMTr) protecting group; (i) coupling, oxidation and capping; (ii) removal of the 5′-*O*-protecting group; (iii) cleavage of the fabricated oligonucleotide from the solid support.

There are many attributes regarding the dominance of SPS of oligonucleotides. The first and most important benefit is that the use of a solid support [generally Controlled Pore Glass (CPG), insoluble polymer or silica] allows the removal of excess reagents and impurities by simple washing after every step. In addition, a single automated synthesizer can do nearly any oligonucleotide by the standard phosphoramidite chemistry in a really fast and reproducible manner, eliminating any human error during the production.

The mechanization and automation of the solid-phase synthesizers is based on packing the solid-support into a small column and making the reagents to pass through it. This methodology allows the synthesis of oligonucleotides inside the column while the unreacted excess reagents and building blocks are washed out to the waste. Both DNA and RNA solid-phase synthesizers using phosphoramidite chemistry extend the chain one nucleotide at a time by a four step coupling cycle

including the 5'-terminus deprotection of the solid-supported chain, coupling of the next building block, capping of unreacted chains and oxidation of the phosphite diester linkage obtained to a phosphate triester (**Figure 2**).

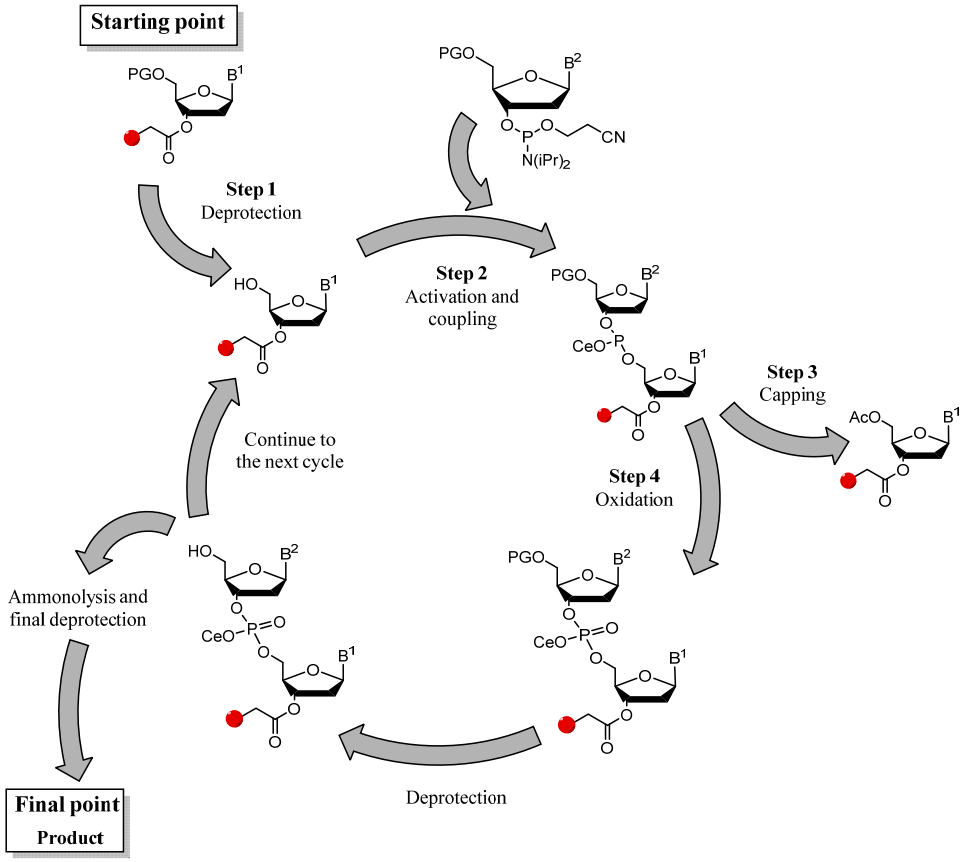
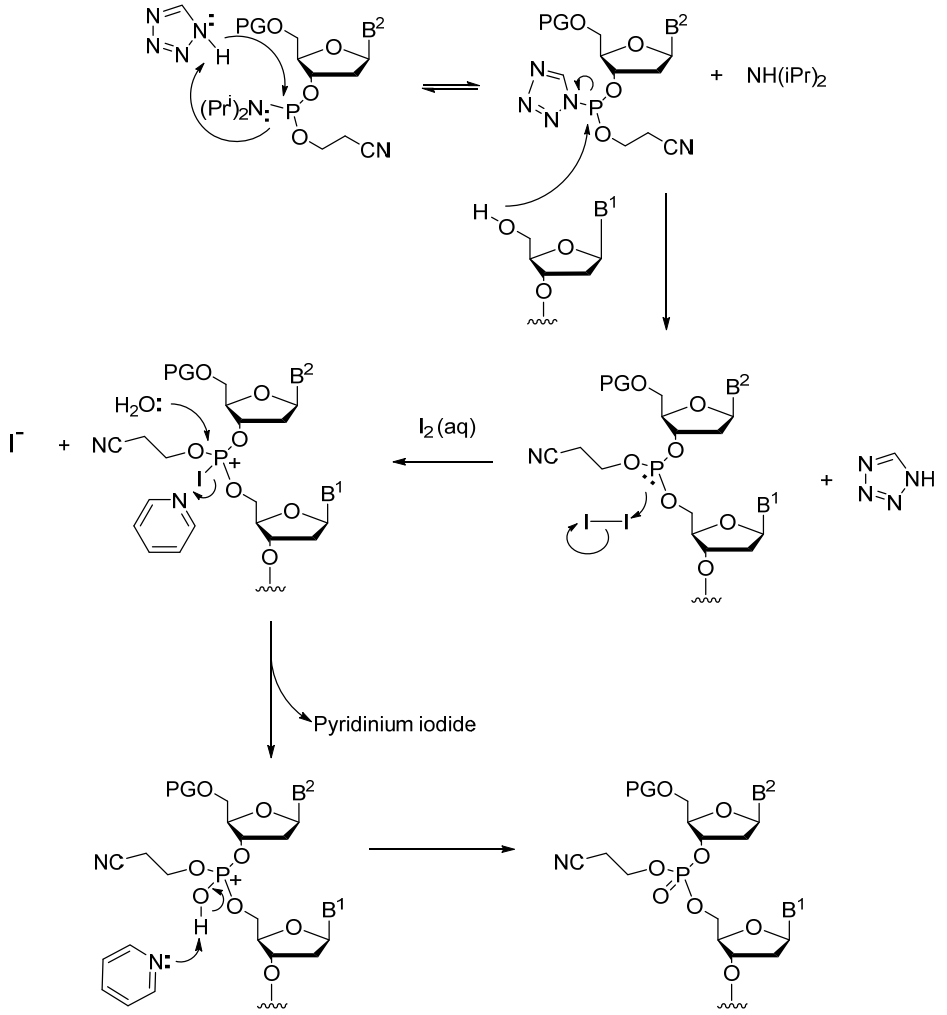


Figure 2. Oligonucleotide synthetic cycle. These 4 steps are repeated as many times as required for the synthesis of the desired oligonucleotide[25]. PG stands for protecting group and Ce stands for 2-cyanoethyl protection.

1.4 Phosphoramidite coupling activators

An activator is needed to enable the coupling, *i. e.* the nucleophilic displacement of the dialkylamino group of the nucleoside 3´-(*O*-alkyl-*N*,*N*-dialkyl-phosphoramidite) with the 5´-OH of the support anchored chain **(Scheme 3)**. The role of the activator is dual: to protonate the dialkylated amino group of the phosphoramidite and to act as a nucleophile that displaces it. In more detail, nucleophilic attack of the activator on phosphorus tends to elongate the P-NR_2 bond, which increases the electron density at the departing nitrogen, leading to its protonation and eventually departure of dialkylamine as a neutral species[25,27,28].

Finally, the phosphorus bound activator becomes, as a good leaving group, displaced by the 5'-hydroxyl group. The unstable phosphite triester is later oxidized to phosphate triester with aqueous iodine.



Scheme 3. Mechanism of activation, coupling and oxidation of the phosphor-amidite chemistry.

The first activator reported[29] for phosphoramidite coupling was 1-*H*-tetrazole, which still continues to be one of the most extensively used activators. Its acidic strength ($pK_a = 4.8$) indicates that it is a reasonably efficient proton donor, but leaves still 4,4´-dimethoxytrityl group (DMTr), the most extensively used acid labile protecting group for the 5´-OH, untouched. Slightly more acidic tetrazole-derived activators have more recently been developed by modifying the C5 position. **Figure 3** represents a few examples of common activators. All of them

are azoles, including modified tetrazoles, such as ETT ($pK_a = 4.28$) and BTT ($pK_a = 4.08$), and less acidic but more nucleophilic DCI ($pK_a = 5.2$)[30].

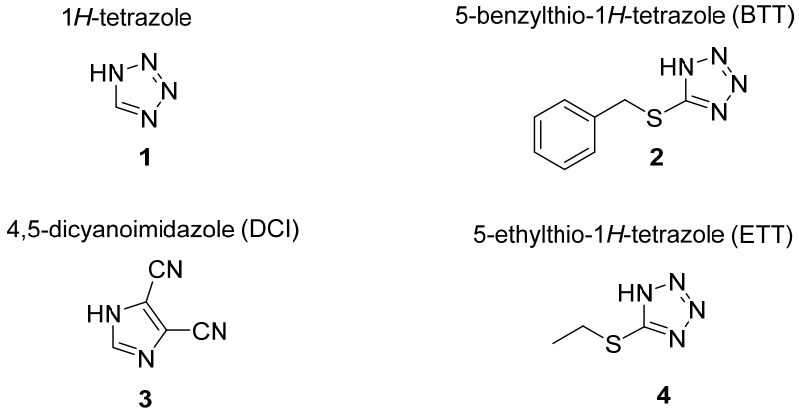


Figure 3. Examples of coupling activators commonly used in the phosphor-amidite chemistry.

1.5 Protecting groups

1.5.1 Base protecting groups

The utilization of appropriate protecting groups (PGs) in oligonucleotide synthesis is crucial. The standard oligonucleotide protecting groups still widely used were developed by Khorana and co-workers[31,32,33,34]. In spite of the rather harsh hydrolytic conditions needed for their deprotection, exocyclic amines of the base moieties are usually protected with an isobutyryl (for guanosine) and benzoyl or acetyl groups (for adenosine and cytidine)(**Figure 4**). The reason behind these choices is the stability of these groups under neutral and slightly acidic conditions. However, somewhat more base labile dimethylaminomethylene or much more labile phenoxyacetyl or 4-(*tert*-butyl)phenoxyacetyl groups may be used when milder conditions for the exocyclic deprotection step are for some reason needed. Thymidine and uridine are not usually protected. In general, once the synthesis of the intended oligonucleotide chain is completed, the final deprotection of N-acyl groups of DNA is usually performed by treatment with concentrated aqueous ammonia. For the deprotection of RNA, somewhat milder treatment with ethanolic ammonia is usually preferred or pressurized ammonia gas is used.

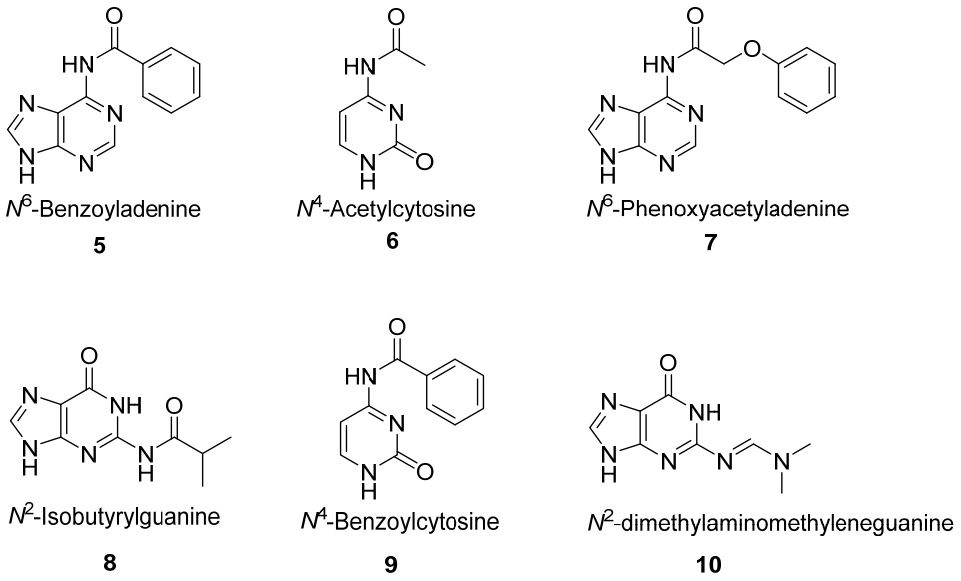


Figure 4. Standard exocyclic amino group protections.

1.5.2 DNA and RNA 5'-OH protection

The sugar moiety 5'-hydroxyl group is usually protected by the traditional acid-labile 4,4'-dimethoxytrityl (DMTr) protecting group. One of the attributes regarding its dominance is the stable carbocation formed upon the acidolytic removal. The characteristic orange color of the carbocation allows quantification of the stepwise yield of the synthesis.

1.5.3 RNA 2'-*O*-protection

Contrarily to the predominance of the 5′-*O*-DMTr protection, a wide selection of protecting groups has been investigated over the past years for the 2'-*O*-protection of ribonucleosides. As a general requirement, the 2'-hydroxyl protecting group should not retard the coupling reaction and, hence, bulky groups should be avoided. In addition, the protection should withstand the acidic conditions used for removal of 5′-*O*-DMTr group and the basic conditions required for removal of the phosphate protecting group, since 2′-OH attacks extremely rapidly on protected phosphate group[35,36]. The most popular among the 2′-*O*-protecting groups is the fluoride ion labile *tert*-butyldimethylsilyl (TBDMS) group.[37] The wide range of alternatives includes bis(2-acetoxyethoxy)methyl (ACE)[38], *tert*-butyldithiomethyl (DTM)[39], [(2-nitrobenzyl)oxy]methyl (NBOM)[40], 4-methoxytetrahydropyran-4-yl (Mthp)[41], triisopropylsilyloxymethyl (TOM)[42], 2-cyanoethoxymethyl (CEM)[43], 2-cyanoethyl (CE)[44] group and 1,1-dioxo-1λ^6-thiomorpholine-4-carbothioate (TC)[45] group (**Figure 5**).

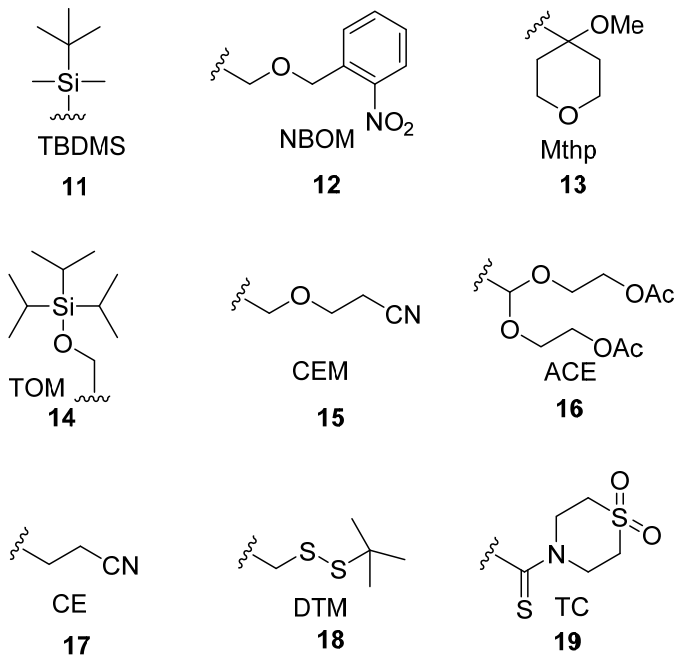


Figure 5. Examples of 2'-OH protecting groups and their structures.

1.5.4 Phosphate protection

In general, a 2-cyanoethyl group is utilized as the phosphate protection when using the phosphoramidite approach. Taking into consideration that P(III) compounds are more vulnerable to nucleophilic attack than P(V) compounds, an oxidation reaction is needed to convert the phosphite into phosphate triester immediately after each coupling. After completion of the chain assembly, final cleavage of this protecting group is achieved by rapid β-elimination upon treatment with concentrated aqueous ammonia. However, the acrylonitrile which is generated during the ammonolysis is able to alkylate the amino base moieties[46], and this phenomenon is more harmful when larger amounts of long oligonucleotides are required. To avoid this post-synthetic amino alkylation, alternative protecting groups, such as 2-benzamidoethyl or 2-[*N*-isopropyl-*N*-(4-methoxybenzoyl)-amino]ethyl groups, have been screened[47]. In addition to these, an *O*-methyl phosphate protection has been employed for the same purpose. In this case, the methyl group can be removed by treatment with soft nucleophiles, usually with 1 M disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate in DMF.[48]

1.6 Linkers

In general, the growing oligonucleotide chain is immibilized to the solid support via 3'-*O*-succinyl linker. Succinic anhydride reacts with the unprotected 3'-OH

group of the nucleoside in the presence of a nucleophilic catalyst (i.e. DMAP) to form an ester linkage and the exposed carboxy function is then coupled to a support bound amino group with the aid of activators. The ester linkage is cleaved during the ammonolysis carried out after completion of the chain assembly. Apart from the succinyl linker, a large variety of linker chemistries have been developed for making the synthesis of oligonucleotides more versatile[49,50]. For instance, more base-labile linkers such as oxalyl[51], malonic acid[52] or Q-linker[53] (hydroquinone-O,O'-diacetic acid) may be utilized (**Figure 6)**. The Q-linker, for example, can be rapidly cleaved by using milder reagents such as potassium carbonate, *t*-butylamine, 5% aqueous ammonia or fluoride ions, and still the linker is stable enough under the general oligonucleotide synthesis conditions. Solid-supported nucleosides anchored through the Q-linker are commercially available[54].

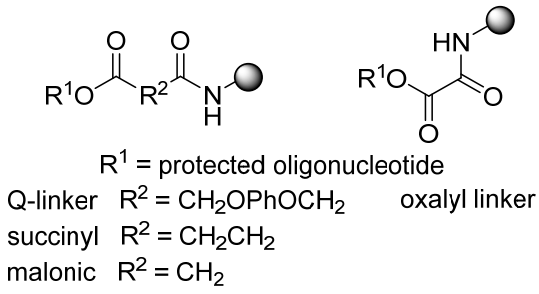


Figure 6. Examples of linkers commonly used in oligonucleotide synthesis.

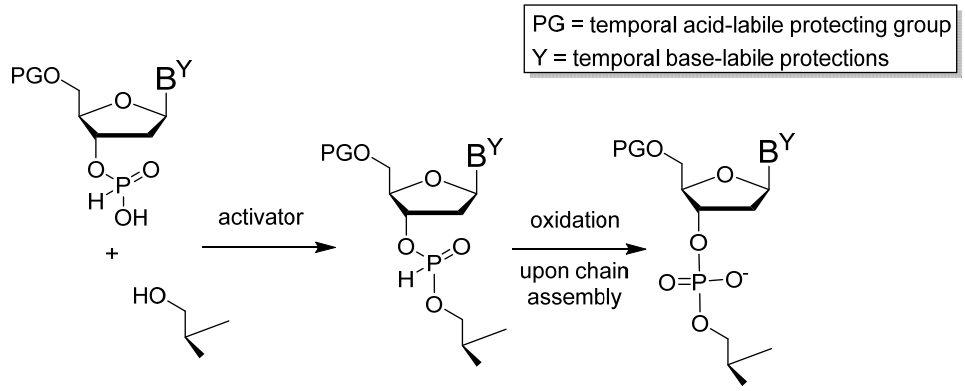
1.7 Alternative coupling methods

Besides the phosphoramidite chemistry, oligonucleotides may be obtained by the so-called phosphotriester[55] and H-phosphonate[56,57] chemistry (**Figure 7**). The advantage of phosphotriester chemistry is that the oxidation step is avoided, as the coupling gives directly the stable phosphate triester bond. In addition, the building blocks used in excess during the coupling step may be recovered, a fact of considerable importance when scaling up the process. However, the longer reaction times, the lower stepwise yield and, hence, overall efficiency, and the presence of coupling agents that may disturb the next coupling by capping the 5'-OH group are features that need to be taken into consideration.

On utilizing the H-phosphonate strategy, oxidation is not required after each coupling but only upon completion of the chain assembly. As in the phoshotriester chemistry, the excess of building blocks can be recovered on using the H-phosphonate approach. Nevertheless, coupling agents may as well disturb the next coupling by capping the 5'-OH group. Accordingly, careful optimization may be

needed to find the workable coupling conditions. The stepwise coupling yield is slightly lower than in the phosphoramidite chemistry.

H-Phosphonate strategy:



Phosphotriester strategy:

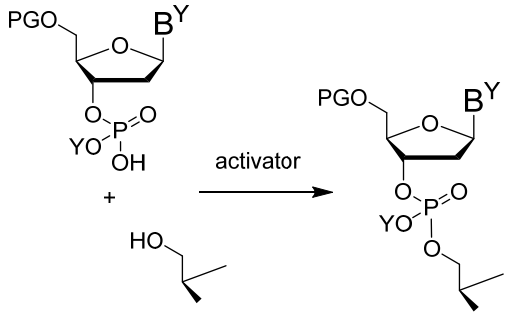


Figure 7. Alternative H-Phosphonate and phosphotriester coupling methods.

1.8 From small to large-Scale synthesis

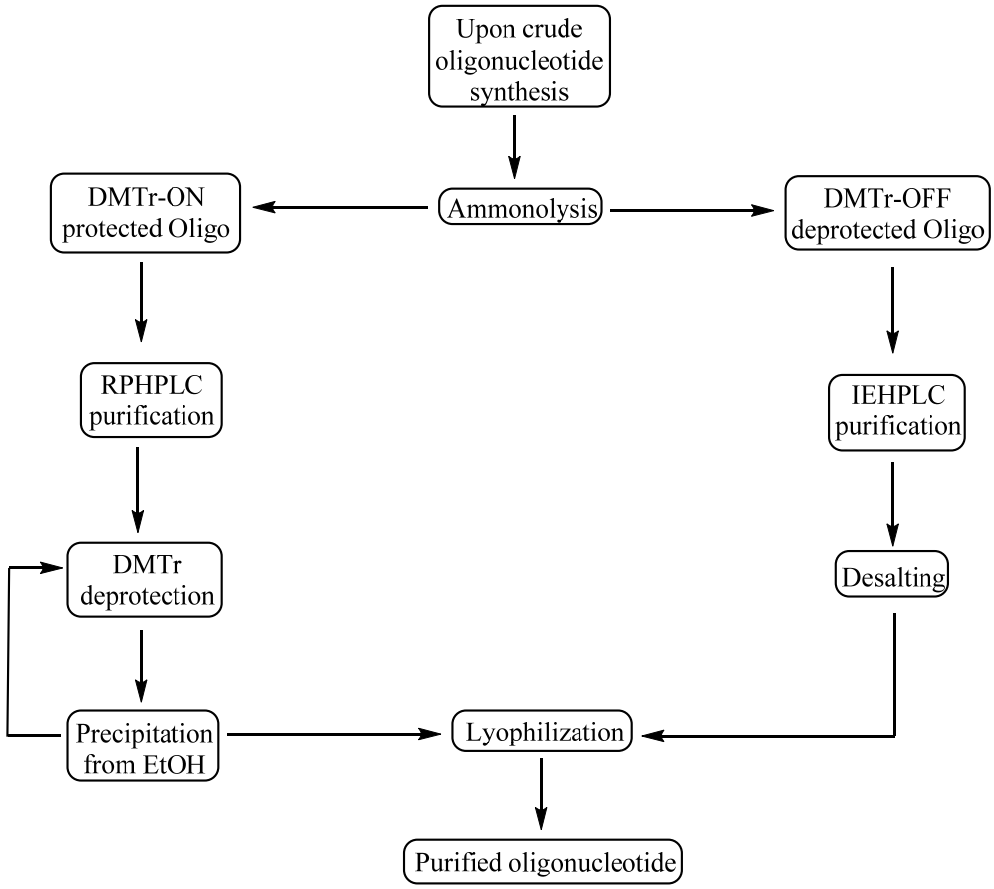
Undoubtedly solid-phase synthesis by the phosphoramidite chemistry is the method of choice for the small scale synthesis of oligonucleotides. On scaling the synthesis from milligram to kilogram quantities, various challenges still remain to be overcome. The considerable excess of monomeric building blocks and large volumes of solvents used in the solid-phase synthesis increase notably the cost of the production, as also does the low loading capacity and high cost of the solid-support. Although the solid-supported synthesis of oligodeoxyribonucleotides has already been performed in kilogram quantities, these factors nevertheless form an impediment for scaling up the process. The synthesis on an automated synthesizer is currently limited to 1 mole scale and the waste management cost is high[58]. In

addition, the use of explosive chemicals such as the tetrazole activator is not appropriate for safety reasons, but these safety risks have largely been eliminated by using non-explosive coupling activators for the same purpose[25]. In spite of development in technology and considerable reduction in consumption of phosphoramidite monomers, the scale and cost of the production still form a significant barrier for large-scale commercialization of oligonucleotides.

In spite of the challenges indicated above, the solid phase synthesis of oligonucleotides still is the method currently used for the large-scale synthesis of ONs. The method has remarkably changed over the last two decades: the efficiency of the process has been carefully optimized, and serious efforts have been done for decreasing the amounts of reagents and solvents, which also has led to substantial reduction of the process time. In addition, various highly-toxic reagents have been replaced while preserving a high purity and efficiency of the synthesis[59].

1.9 Large-Scale purification of oligonucleotides

In large-scale synthesis of oligonucleotides, purification upon cleavage from the support and removal of all temporal protecting groups is currently performed by two types of strategies. These methods are essentially conditioned by the final application of the oligomers, their overall length and the amount of truncated sequences produced during the synthesis. The method of choice also depends on the type of the oligonucleotide fabricated, as RNA, DNA and their modified analogs may require different type of purification. Among such techniques, chromatographic methodologies are at the vanguard of post-manufacture purification procedures for large scale synthesis of oligonucleotides. For instance, a standard purification strategy for the separation of oligonucleotides from undesired impurities is generally performed by either reversed phase HPLC or ion exchange HPLC, as shown in **Scheme 4.** The main difference between these two methods is essentially that reversed phase separation (RPHPLC) is based on the differences in matrix affinity whereas ion exchange separation (IEHPLC) depends on the length of the oligonucleotide or actually on the number of negative charges present in the molecule. In practice, RPHPLC allows an easy separation of the truncated sequences lacking the 5'-O-DMTr functional group from the corresponding 5'-O-DMTr protected oligomers, but it can also be used to remove many other impurities due to the high resolution of the technique. On the other hand, IEHPLC is the method of choice for the purification of RNA oligonucleotides upon detritylation, since the resolution and the capacity properties of the column provide excellent results with these types of compounds[58,60].



Scheme 4. Currently used solid-phase large scale oligonucleotide purification protocol. Model constructed on the basis of Sanghvi et al.[58,60]

1.10 General remarks of Solution Phase Synthesis

The development of solution phase synthesis as an alternative to the solid-supported oligonucleotide chemistry has received significant interest due to more predictable scaling-up of the synthesis. Potentially lower cost, owing to limited solvent and reagents consumption and the lower cost of the support, makes solution phase synthesis worthwhile to study in more detail. In particular, linear liquid phase synthesis of oligonucleotides on a soluble support offers an alternative to the solid-phase synthesis, as the purification of the growing oligonucleotide may well be facilitated by an appropriate soluble support. As long as the soluble support is relatively small, nearly quantitative coupling by using only a stoichiometric amount of the building block appears achievable. Furthermore, the possibility to characterize the elongated sequences by using MS or NMR methods at any time of the synthesis is an important feature to take into consideration, although not all the supports allow this. The major challenge for successful large scale synthesis in solution phase is the convenience of separation of the growing oligonucleotide chain from small molecular reagents after each coupling cycle. As discussed below, novel innovations in separation technology may, however, offer solutions to this problem.

As mentioned, on operating in solution, all byproducts and excess of reagents need to be removed after each coupling. Although chromatography is a good choice for small scale synthesis conditions, the technique is not convenient when scaling up the process. On the other hand, the use of modern membrane separation such as the cross-linked polybenzimidazole (PBI) facilitates the removal of small molecular reagents and byproducts by filtration and potentially offers a solution for this problem. Extraction is another possible alternative, but large solvent consumption may well limit the applicability of such approaches. In addition, extreme care needs to be exercised for prevention of hydrolysis of the phosphite triester moiety after coupling. Apart from these approaches, the development of precipitation techniques seems to be another realistic approach for the separation of growing oligonucleotides. The majority of the research on solution phase synthesis has been built around it. Precipitation may as well be applied from small to medium or even large quantities of oligonucleotides, affording in optimal cases nearly quantitative yields. Since a large number of soluble supports can utilize this methodology, it is a versatile tool with high potential for the purification of oligonucleotides.

1.11 Previous studies on solution phase synthesis of ONs by using soluble supports.

A considerable amount of research has been focused on developing a solution phase oligonucleotide synthesis strategy on using a soluble-supported platform. Among various soluble supports, a wide variety of polyethylene glycol (PEG) polymers has been at the forefront[61,62,63,64,65,66,67]. PEG allows the use of acetonitrile as the solvent for the coupling reaction and the isolation of the growing oligonucleotide from excess reagents and small molecular weight impurities can be carried out by simple precipitation from diethyl ether and filtration. Since this carrier avoids the heterogeneity as the reaction medium, the method can be favorably used to produce large quantities of ONs. As an example of this, Bonora and co-workers[62] used the standard phosphoramidite chemistry on a PEG soluble support (weight ranging between 5 and 12 kDa), and applied a new protocol called HELP Plus (High Efficiency Liquid Phase) to the large scale synthesis of ONs in a hundred of μmol scale. During the chain elongation cycle, 4 precipitations from Et_2O and filtration were required, one after each synthetic cycle step (coupling, capping, oxidation and detritylation) (**chart 1**). Analogously, the same protocol was utilized to produce phosphorothioate ONs in a mmol scale[63,64] and even chimeric ONs containing phosphorothioate linkages at selected positions.[65] In addition, a PEG support has been used for the synthesis of oligonucleotides by phosphotriester[66,67] and H-phosphonate[61] strategies.

Since lower consumption of phosphoramidite building blocks is employed during the oligonucleotide assembly in comparison to the solid-phase synthesis, the process is cost-saving. In addition, the methodology reported by Bonora's group[62] allows monitoring the progress of the reaction by NMR spectroscopy at any time during the synthesis. Despite the fact that the protocol has very convenient features for the large scale synthesis of ONs in solution, the use of 4 precipitations and recrystallizations in ethanol after each chain extension cycle makes the process time-consuming and, hence, any effort aimed at reducing the precipitation and recrystallization steps would be desirable. Since the feasibility of the method for the large-scale synthesis of oligonucleotides has been demonstrated for the synthesis of oligomers up to 20 nucleotides in length, introducing the above-mentioned improvements would provide a realistic approach for industrial applications.

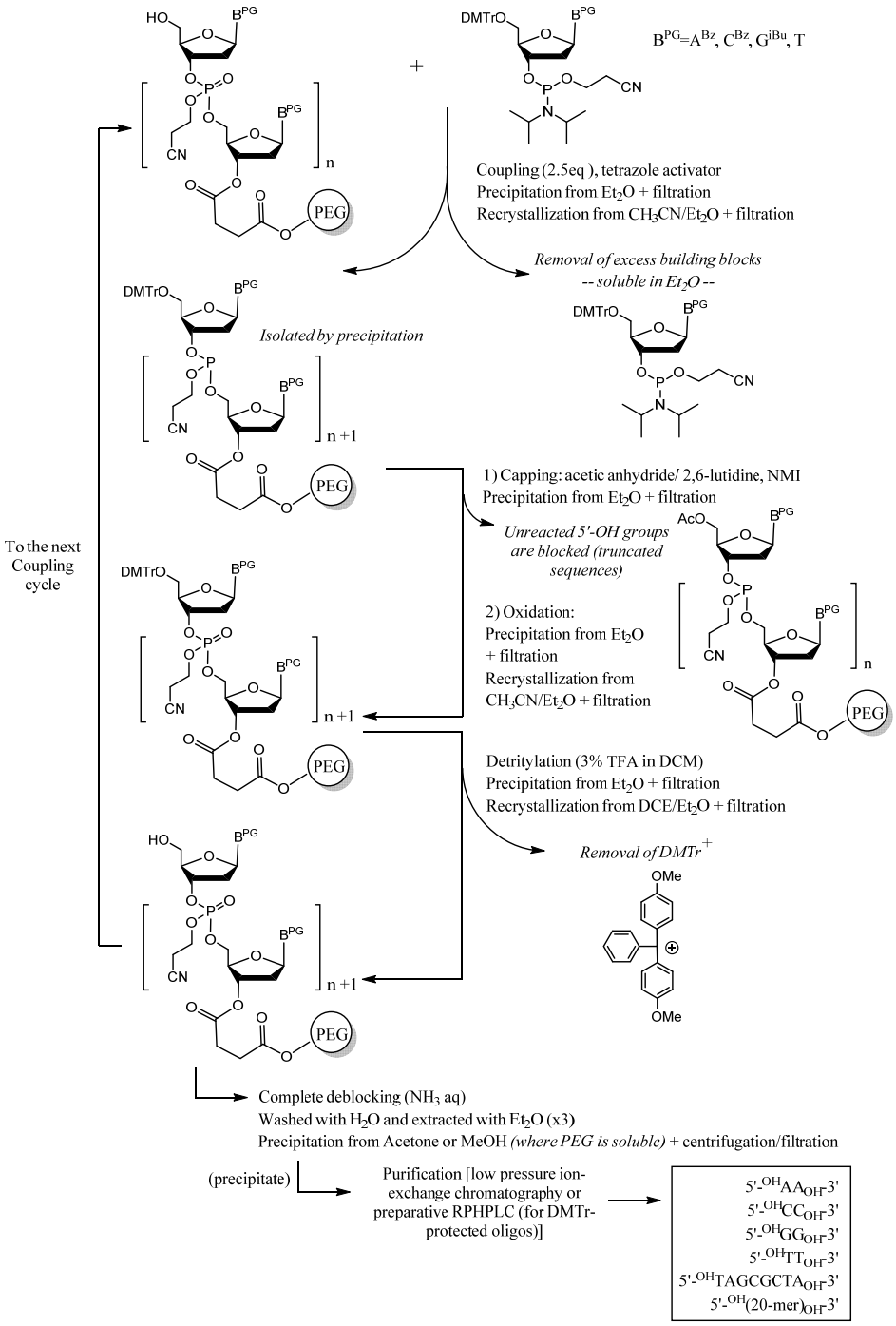


Chart 1: Strategy of Bonora's phosphoramidite approach[62] on using the HELP Plus protocol on a PEG soluble support.

More recently, a new approach for the solution phase synthesis of oligonucleotides, based on (adaman-1-yl)acetyl as a soluble support, was introduced by van der Marel's group[68]. The advantage of this support comes from feasibility of an extractive work-up procedure as a method for removal of small molecular impurities and excess reagents. Eight extractions were required in each synthetic cycle in order to remove completely the activator, DMTr byproduct, small molecular weight reagents and unreacted excess of monomeric building blocks: four times after the oxidation ($Na_2S_2O_3$, 10% $KHSO_4$, 10% $NaHCO_3$ and brine) and another four times after the detritylation step (heptane/Et_2O, EtOAc/H_2O, 10% $NaHCO_3$ and brine) (**chart 2**). The extractive work-up procedure allowed a quantitative separation of the growing oligonucleotide chain from the small excess reagents and impurities, providing pure product in high yield. In addition, the efficiency of the synthesis was demonstrated up to the 6-mer level. N^3-Pivaloyloxymethyl protected thymidine was used to increase the solubility of the growing oligonucleotide. Since this methodology is amenable to scale up, an industrial manufacturing platform could be developed. However, it would be highly desirable to reduce the number of extractions per chain extension cycle in the procedure, due to shorten the process time and decrease the solvent consumption.

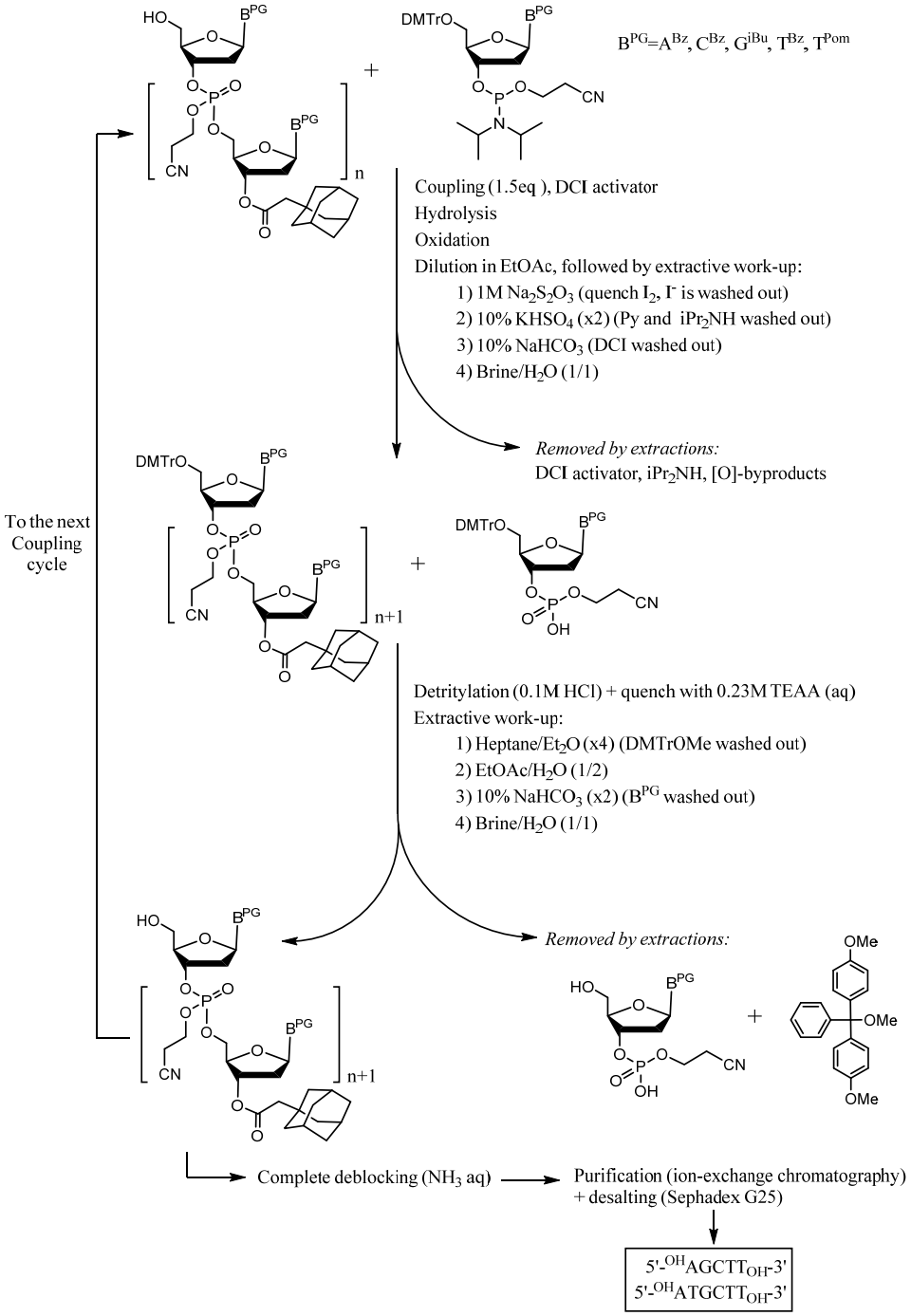


Chart 2: The strategy of van der Marel's group for the solution-phase synthesis of ONs by the phosphoramidite approach on using (adaman-1-yl)acetyl[68] as a soluble support.

An imidazolium ion tag, having tetrafluoroborate as a counterion, has been utilized as a soluble support for the synthesis of oligonucletodies by the group of Damha[69]. This ionic liquid supported synthesis allowed an easy purification of the growing oligonucleotide chain from excess reagents and impurities by applying a combination of precipitative and extractive techniques. Selective precipitation without temperature cycling was carried out four times per synthetic cycle: twice upon the coupling and prior to oxidation, and another twice upon the detritylation step. In both cases a mixture of EtOAc/Et_2O was successfully used for the purpose, allowing a rapid elimination of excess reagents and soluble by-products. In addition, convenient extractive work-up on using initially 5% *w/v* aqueous $NaHSO_3$ in DCM and later in water were carried out upon the oxidation step for the removal of resultant salts and uncoupled soluble supported nucleotides. Successful application of this support was demonstrated by the synthesis of oligomers in different length: four dinucleotides, a thymidine homo-trimer and a thymidine homo-tetramer (**chart 3**). Since the protocol allows scaling up due to the homogeneous reaction processes and the higher (30 fold) loading capacity of the ionic soluble support compared to the CPG solid polymers, the industrial manufacturing platform appears possible to achieve. Nevertheless, similarly to the adamantly-derived core, the reduction of the number of operations (particularly precipitations) would be desirable.

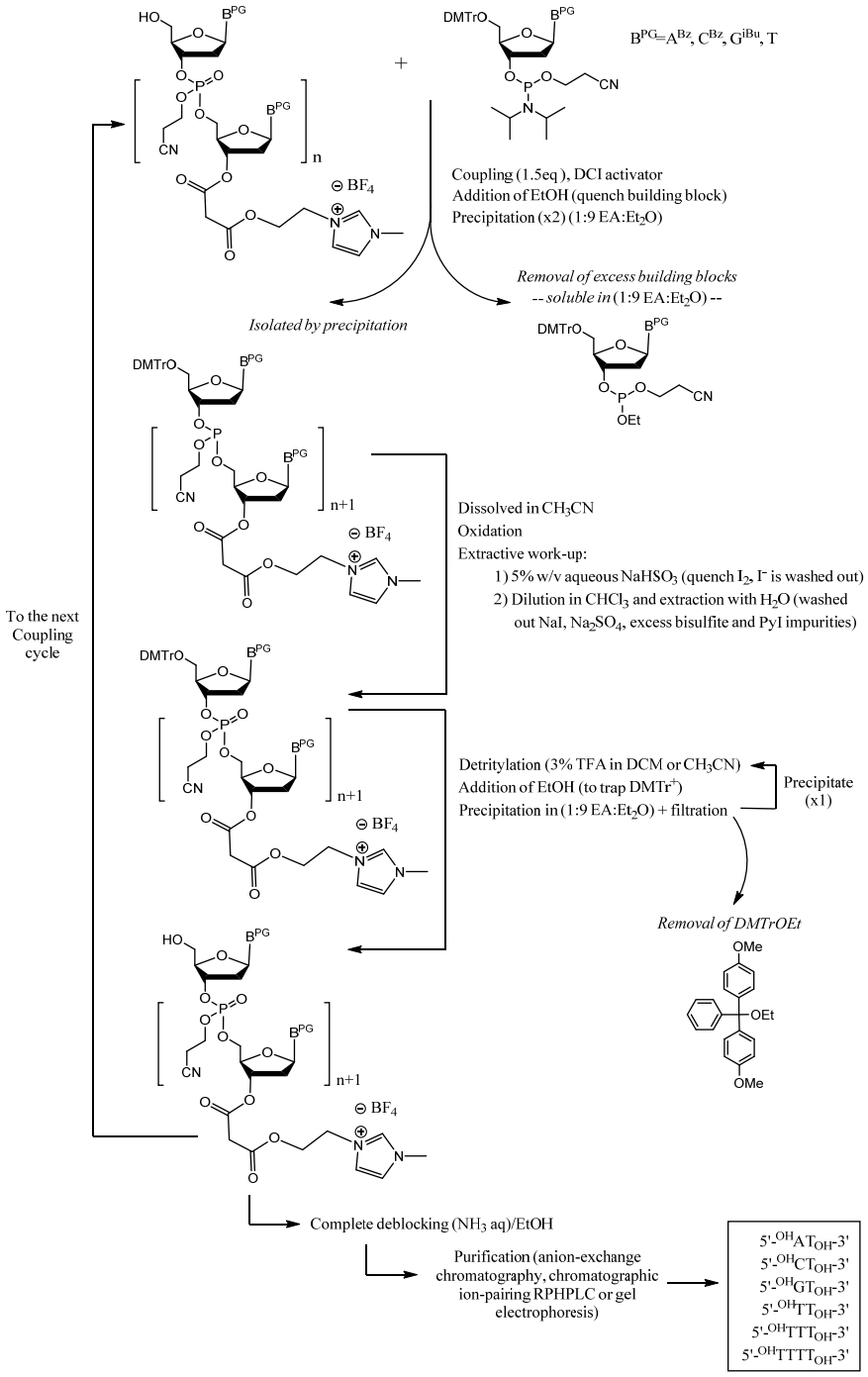


Chart 3: Strategy of Damha's group for the solution-phase synthesis of ONs by the phosphoramidite approach on using an imidazolium ion tag[69] as a soluble support.

More recently, attempts to employ an adamantine-based and two different phenolic cores named tetrakis(*p*-hydroxyphenyl)methane (TPM, four-arm core) and hexakis(p-hydroxyphenyl)xylene (HPX, six-arm core) were investigated by Richert and colleagues[70,71,72]. In these cases, only one coupling was carried out on using phosphoramidite dinucleotide building blocks for the synthesis of branched DNA hybrids with four arms to an adamantine-based support, and also to four or six arms to the phenolic cores. Despite the fact that these studies do not contain any oligonucleotide synthesis, the usefulness of these kinds of supports comes from the point of view of their precipitative properties. For instance, precipitation of TPM and HPX-derived cores from hexane removed easily excess of dimeric building blocks. However, since only one coupling and purification by precipitation were carried out during the process, no real synthetic method was developed and, hence, no oligonucleotides were really prepared.

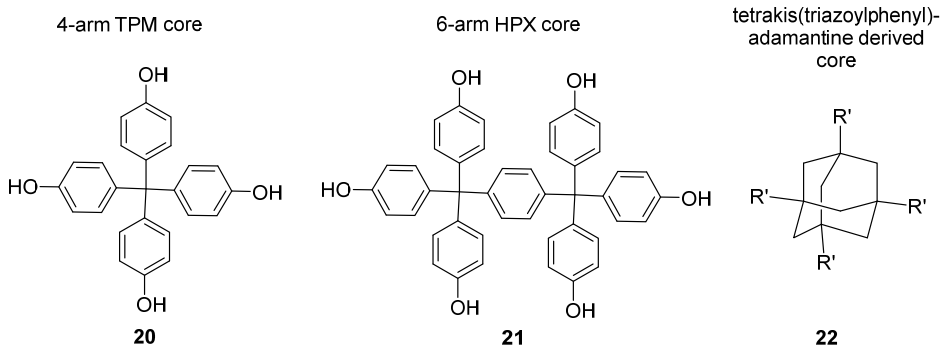


Figure 8. Representation of soluble supports employed by Richert's group[70,71,72].

2. AIMS OF THE THESIS

The research on which this thesis is based was initiated as part of the MEMTIDE project "Novel soluble supports and linkers for membrane enhanced synthesis of oligonucleotides, a new paradigm for large scale synthesis", under the FP7 Marie Curie Actions. The project consortium was composed of 6 partners: Imperial College (UK), University of Barcelona (Spain), University of Turku (Finland), Lonza (Switzerland), Janssen (Belgium) and Evonik-MET (UK). The main objective comprised the development of a scalable oligonucleotide and peptide synthesis platform on using the emergent Organic Solvent Nanofiltration (OSN) purification technique. The role of the University of Turku was, in collaboration with researchers at Imperial College of London, to develop a large scale method for preparation of oligonucleotides useful for the membrane platforms. Accordingly, the primary aims of the present theses were i) to develop such new soluble supports for solution phase synthesis of oligonucleotides that could be exploited in membrane-supported approaches, ii) to introduce alternative protecting group strategies compatible with the OSN methodology, and III) to design novel synthesis protocols compatible with the large scale synthesis and the membrane filtration technique. The secondary aim was to develop in parallel a related methodology that could be used in normally equipped research laboratories for the preparation of oligonucleotides in hundreds of milligrams scale, which is the amount often required for extensive physico-chemical and structural studies of oligonucleotides by techniques such as NMR spectroscopy, X-ray crystallography or calorimetry.

3. RESULTS

3.1 Synthesis of oligodeoxyribonucleotides on acetylated and methylated β –cyclodextrins.

3.1.1 Assembly of oligonucleotides from 5'-*O*-(4,4'-dimethoxytrityl)-protected building blocks on acetylated β–cyclodextrin.

The objective of this subproject was to describe a new protocol for oligonucleotide synthesis based on utilization of acetylated β-cyclodextrin (β-CD) as a soluble support. β-CD (**23**) is a cyclic oligosaccharide that consists of 7 (α-1,4)-linked α-D-glucopyranose units. The general appearance of the structure gives an impression of a truncated torus, with a lipophilic inner cavity and a hydrophilic outer part (**Figure 9**). Accordingly, secondary OH groups are positioned on the wider extremity, whereas the primary OH groups are located on the narrower bottom of the torus.

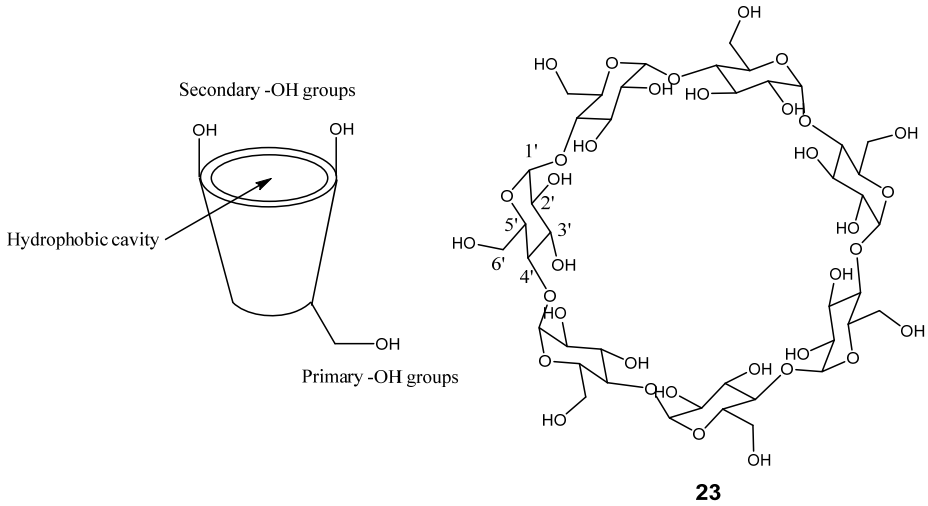


Figure 9. Structure of β-CDs.

The use of β-CD as a soluble support was expected to enable a workable large-scale synthesis of ONs. First of all, it is commercially available and can be found easily in marketed pharmaceutical products. In addition, compared to many other supports, β-CD is inexpensive (~5 USD/Kg)[73] and would, hence, have a very low contribution to the overall cost of the preparation of oligonucleotides. From the point of view of nanofiltration, the size of β-CD is convenient as it is still small enough to allow normal solution phase chemistry and characterization of the reaction products by MS or NMR methods at any stage of the synthesis.

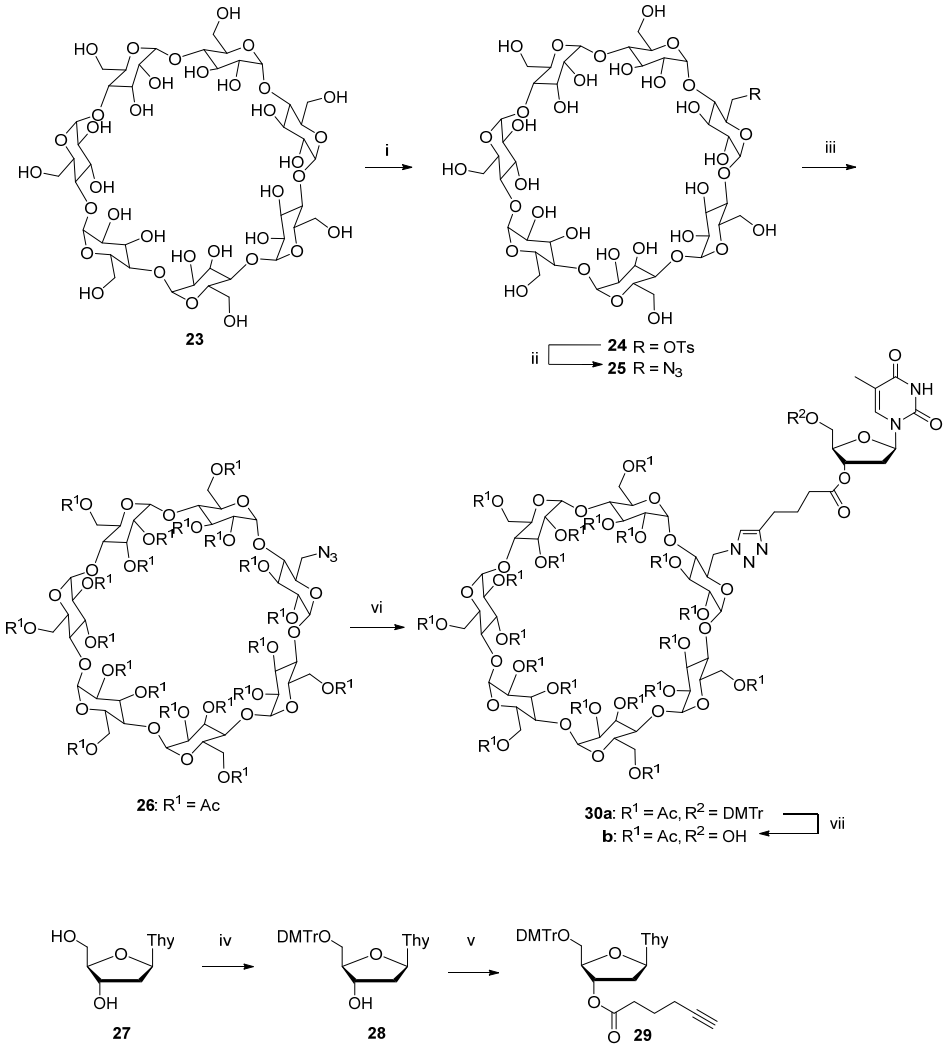
Furthermore, acetylated β-CDs are hydrophobic enough to allow a convenient purification of the growing oligonucleotide chain from excess reagents and impurities by flash column chromatography with small solvent consumption.

Apart from the advantages indicated above, a few challenges were encountered during this study. All the hydroxyl groups of β-CD have to be kept protected during the oligonucleotide synthesis to prevent the phosphoramidite building blocks to react with them and to increase the solubility into organic solvents.[74] The most obvious approaches for the hydroxyl function protection are acylation[75] or alkylation[76,77],which both expectedly improve the solubility in organic solvents, such as acetonitrile and dichloromethane often used in oligonucleotide synthesis.

Monofunctionalization of β-CD is another problem to overcome. The aim was to immobilize the 3´-terminal nucleoside to β-CD by the so-called click-reaction, *i. e.* by Cu(I) catalyzed 1,3-dipolar cycloaddition between and alkyne and azide.[78] That is why, monoazidation of β-CD was attempted. Conventional synthesis starts with tosylation of one primary hydroxyl functions by treatment with one equiv. of tosyl chloride to obtain 6-*O*-tosyl-β-CD (**24**).[79] In practice, derivatization of only one OH group is difficult to achieve, and the presence of a mixture of mono-, di-, and even tri-functionalized β-CD is usually observed. Although this problem does not disturb to the subsequent reactions, the monofunctionalized product is difficult to obtain in pure state before acetylation or methylation of the remaining hydroxyl groups, which disturbs characterization. Despite the fact that there are several easily accessible anchoring sites, it appeared useful to use only the monofuctionalized product for the chain assembly, since it was the simplest study model and also was less difficult due to steric hindrance. The tosyloxy group is subsequently displaced with azide ion, giving the monofunctionalized 6-azido-6-deoxy-β-CD (**25**)[80]. In our first study, the remaining free hydroxyl groups were acetylated with acetic anhydride in pyridine[75] to afford **26**.

Afterward the 3´-terminal nucleoside was then converted to a 3´-*O*-(hex-5-ynoyl) derivative. The 5´-hydroxy function of thymidine was first protected by treatment with DMTrCl in pyridine (to obtain **28**), and the 3´-hydroxy group was acylated with hex-5-ynoic anhydride prepared *in situ*. The 5´-*O*-DMTr-3´-*O*-(hex-5-ynoyl)thymidine (**29**) obtained was then subjected to the click reaction with the azido function of β-CD **26** to yield **30a**, as shown in **Scheme 6**. Among the great diversity of linkers that can be used for immobilization of the 3'-terminal nucleoside, the 3′-*O*-(hex-5-ynoyl) linker that bears a terminal alkyne was chosen, owing to the known efficiency of the Cu(I) promoted chemoselective conjugation reaction with the azide group.[81 ,82] The reaction generates a stable 1,2,3-triazole structure, which remains intact during the oligonucleotide synthesis, even under acidic and oxidizing conditions used in the synthesis, while the ester function

allows conventional release by ammonolysis. Support **30a** was finally detritylated by treatment with a solution of 3% dichloroacetic acid in DCM followed by DCM/HCO_3^- extractive work-up to obtain **30b.** The identity and homogeneity of support **30b** was verified by ESI-MS and RPHPLC.

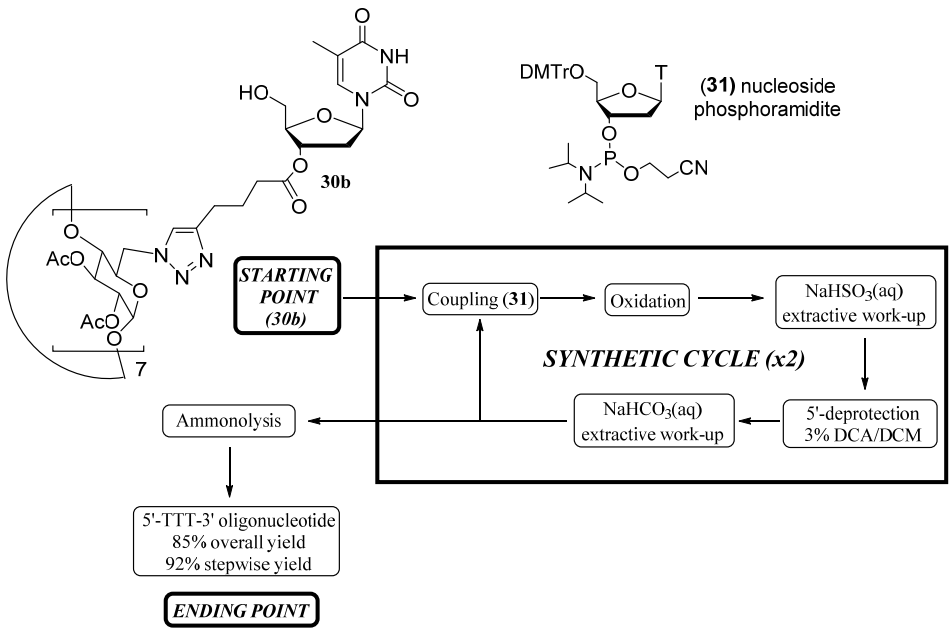


Scheme 6. Preparation of peracetylated CD support for oligonucleotide synthesis. *Reagents and conditions*: (i) TsCl, Py;(ii) NaN_3, KI, DMF;(iii) Ac_2O, Py;(iv) DMTrCl, Py (v) 1. 5-hexynoic acid, DCC, dioxane, 2. **28**, Py; (vi); $CuSO_4$, sodium ascorbate and **29**; (vii) 3% DCA/DCM.

To test the applicability of support **30b** to the synthesis of oligonucleotides, a 5'-TTT-3'-β-CD trimer was first assembled on using 1.5 eq of commercially available 5′-(4,4′-dimethoxytrityl)thymidine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (**31**) building block on the peracetylated β-CD soluble support. Tetrazole (1.5 eq), added as a 0.45 M solution in MeCN, was used

as an activator. Upon 1h stirring under nitrogen, an aqueous iodine oxidation solution (I_2:H_2O:THF:2,6-lutidine 0.43g: 4mL: 8mL: 2mL; 1.2 equiv.) was added and the reaction was left for 30min at r.t. After the phosphoramidite coupling step and subsequent aqueous iodine oxidation, the mixture was concentrated under reduced pressure and the excess of iodine was removed by extractive aqueous sodium bisulfite / DCM work-up. The product was retained quantitatively in the organic phase. The organic phase was dried over anhydrous sodium sulfate, filtrated and evaporated to dryness. The identity of the product before the detritylation step was confirmed by MS, which also verified the absence of starting material **30b**. The 5´-*O*-DMTr group was then removed by 1h treatment with 3% dichloroacetic acid in DCM. Afterwards, the mixture was washed with saturated aqueous sodium bicarbonate. The aqueous phase was back-extracted twice with DCM, the combined organic phases were dried over sodium sulfate, and the solvent removed under reduced pressure. The identity and purity of the product was confirmed by MS and RPHPLC analysis, respectively. Although the detritylated dimer still contained DMTrOH impurities, the product was subjected to the next coupling without further purification. This synthetic coupling cycle was repeated in a similar manner by using the same building block (**31**). Upon completion of the trimer assembly, the chain was cleaved from the support by aqueous ammonia treatment. ESI-MS confirmed the identity of the expected product, and the homogeneity of the trimer was assessed by RPHPLC analysis (Figure 2A, article I). According to the UV-spectrophotometric assay in comparison to the support **30b**, the overall yield of the trimer was 85%, corresponding to 92% stepwise yield.

Scheme 7 illustrates the general procedure utilized for the synthesis of the 5'-TTT-3' oligonucleotide synthesis by using commercially available 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) building blocks.



Scheme 7. General strategy for the assembly of oligodeoxynucleotides from 5'-*O*-DMTr protected thymidine building blocks on acetylated β-CD.

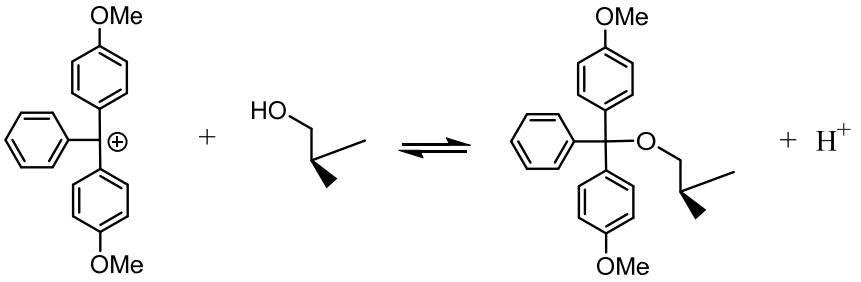
The low molecular weight of the CD support, allowed mass spectrometric analysis at any time of the synthesis and, hence, verification of the completeness of coupling. However, upon final ammonolytic treatment, the acetyl groups on the β-CD were also cleaved and the isolation of the oligonucleotide chain from the cyclodextrin support became difficult. In addition, hydrophobic DMTr alcohol and unreacted building blocks were accumulated in the reaction mixture. In spite of this, synthesis of a trimer was successful.

3.1.2 Assembly of oligonucleotides from 5'-*O*-(1-methoxy-1-methyl-ethyl)-protected building blocks on acetylated β–CD.

Since the shortcomings mentioned above did not permit assembly of longer sequences, the coupling cycle was simplified by replacing the 5'-O-DMTr protection with a 5'-*O*-(1-methoxy-1-methylethyl) protecting group and the synthesis of the 5'-TTT-3' trimer was repeated by using chromatographic purification for removal of impurities and the excess of reagents after each coupling cycle.

The main reason for the replacement of the 5′-*O*-DMTr protection with an acetal protection was that an equilibrium between tritylated and detritylated material was observed to be settled during the detritylation reaction (**Scheme 8**). To push the reaction to completion (to obtain detritylated product) different approaches can be

used, including: 1) increasing the acidity of the solution; 2) removing the DMTr cation from the mixture; 3) using a scavenger to trap the DMTr cation. The first alternative is not possible, since an increase of the acid concentration results in cleavage of purine bases.[83] In fact, this depurination is the major limitation of all current protocols applied to synthesis of oligodeoxyribonucleotides. The second alternative can be realized by simply making the solution more diluted by increasing the volume of the reaction mixture. However, this will in large scale synthesis lead to excessive solvent consumption. Finally, the third alternative, use of scavangers, is a method that has been rather extensively studied.[69,84,85,86,87,88] While undoubtedly feasible, even this approach increases reagent consumption and complicates separation steps. In addition to pyrrole[84,85,86], triethylsilane[87,88] and borane-amine complexes[88], even methanol[84,87] or ethanol[69,84] have been used as scavengers. Although the equilibrium is significantly shifted towards the formation of the detritylated material, the approach does not seem to eliminate completely this problem.[69]

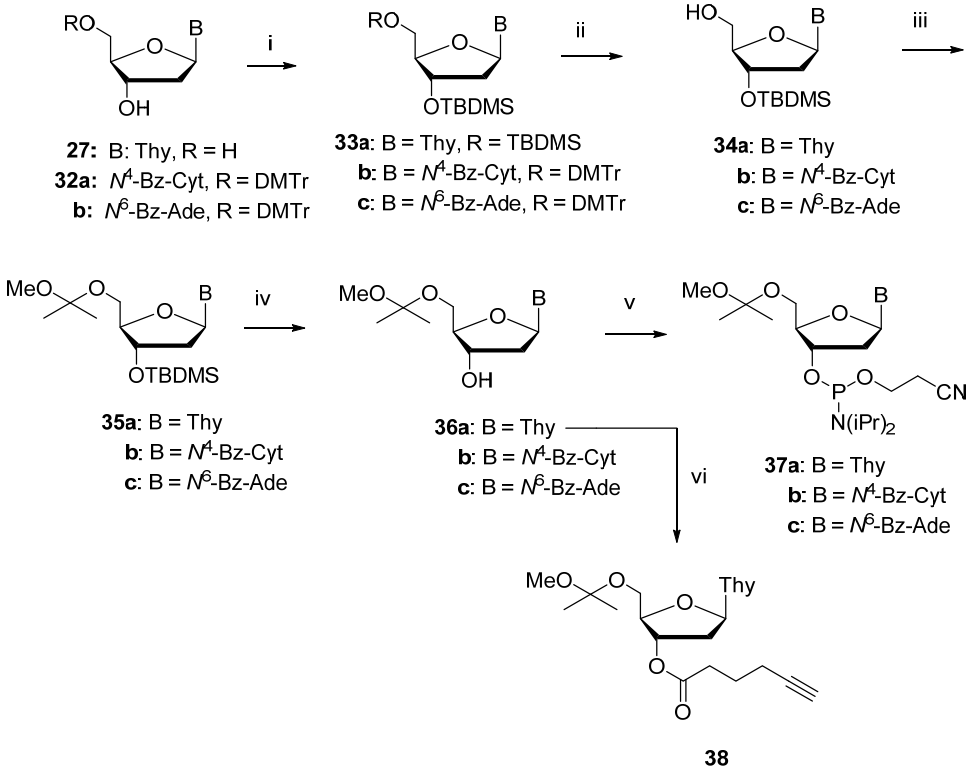


Scheme 8. Equilibrium reaction during the DMTr deprotection in ON synthesis.

For these reasons, a new approach involving the replacement of the conventional DMTr by a 5'-*O*-(1-methoxy-1-methylethyl) group was studied. Upon acidic treatment, this acetal protection generates an easily removal volatile product dimethyl acetal of acetone. In addition, dichloroacetic acid was replaced by volatile HCl. After the deprotection step, HCl can be removed easily from the reaction mixture by evaporation under reduced pressure. Since 5'-*O*-(1-methoxy-1-methylethyl)-protected building blocks are not commercially available, they were prepared as described below.

The synthesis was initiated by protection of the 3'-OH group of nucleosides **32a-c** with a *tert*-butyldimethylsilyl (TBDMS) group (**Scheme 9**). On using thymidine (**27**) as a starting material, both 3'- and 5'-OH groups were first silylated, and the 5′-*O*-protection was selectively removed with aqueous TFA[89] in THF to obtain **34a**. 3′-*O*-Silylated N^4-benzoyl-2′-deoxycytidine (**34b**) and N^6-benzoyl-2′-deoxyadenosine (**34c**) were, in turn, prepared by silylation of the commercially available base protected 5′-*O*-DMTr-nucleosides (**32a,b**) to obtain **33b**,**c**, followed

by acidolytic detritylation. All three nucleosides were then subjected to acid-catalyzed acetalization with 2-methoxypropene to obtain **35a-c**. Cleavage of the 3'-*O*-TBDMS group with tetrabutylammonium fluoride (TBAF) in THF then resulted in 3′-unprotected nucleosides **36a-c**, which were phoshitylated to the desired acetal protected phosphoramidite building blocks (**37a-c**). In addition, the 3′-*O*-(hex-5-ynoyl) linker was attached to the 3'-OH- group of **36a** to obtain **38.**

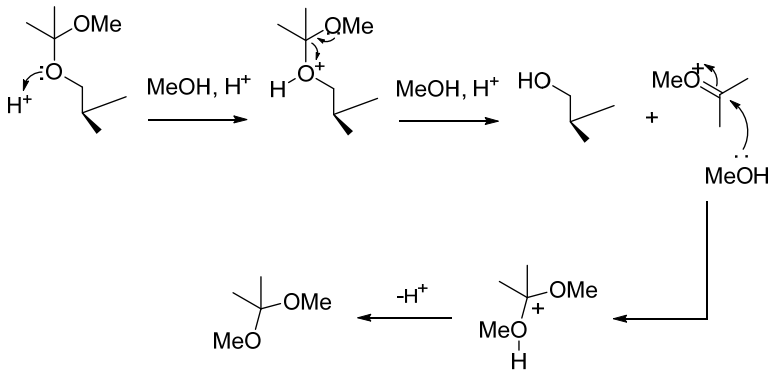


Scheme 9. General strategy for the synthesis of acetal protected nucleosidic building blocks. *Reagents and conditions:* (i) TBDMSCl, Im, DMF; (ii) TFA, aq. THF for **33a**; 3% DCA/MeOH/DCM for **33b-c**; (iii) 2-methoxypropene, TsOH, dioxane; (iv) TBAF, THF; (v) 1-chloro-1-(2-cyanoethoxy)-*N*,*N*-diisopropylphosphinamine, Et_3N, DCM; (vi) 1. 5-hexynoic acid, DCC, dioxane, 2. **36a**, Py;

For comparative purpose, assembly of a 5'-TTT-3' trimer was performed on the peracetylated β-CD on using 5'-*O*-(1-methoxy-1-methylethyl)thymidine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (**37a**) building block. The coupling was carried out using 1.5 eq of **37a** and 4,5-dicyanoimidazole (1.5 eq, 0.25 M solution in dry MeCN) as an activator. After 1h, the phosphite triester was oxidized to the phosphate triester by treatment for 30 min with the standard aqueous iodine solution (0.2M I_2 in 2,6-lutidine/H_2O/THF 2:4:8). The reaction mixture was then concentrated under reduced pressure and sodium bisulfite/DCM extractive work-up was used to remove the excess of iodine. The organic phase was dried over

sodium sulfate, filtrated and evaporated to dryness. The expected product was identified by ESI-MS analysis. Traces of lutidine were still observed in the mixture even after evaporation under reduced pressure. 5'-*O*-Deprotection was then performed by adjusting the pH to 4 with HCl (using a 0.1 M stock solution in a 2:1 (*v/v*) mixture of dioxane and MeOH) and allowing the reaction to proceed for 30 min. Both HCl and the released dimethyl acetal of acetone were then removed by simple evaporation under reduced pressure.

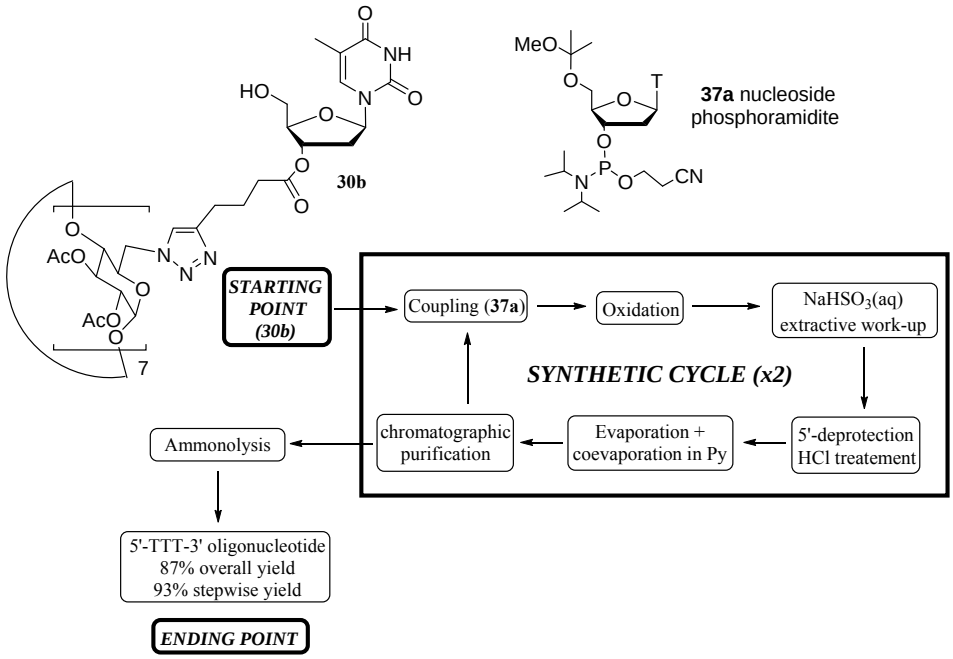
The cleavage of the 5'-*O*-(1-methoxy-1-methylethyl) group was fast enough to almost entirely avoid depurination (Figure 3, article I). Coevaporation with dry pyridine removed any traces of acid still present in the solution. As regards the mechanism of the acid-catalyzed removal of the 5'-O-(1-methoxy-1-methylethyl) group in MeOH, the rapid initial protonation of the 5'-O-function is followed by departure of 2-methoxypropane oxocarbenium ion that is converted to 2,2-dimethoxypropane by nucleophilic attack of MeOH and subsequent deprotonation (**Scheme 10**).



Scheme 10. Mechanism for the deprotection of the 5'-*O*-(1-methoxy-1-methyl-ethyl) protecting group.

After the 5´-deprotoection, a flash chromatographic purification was carried out to remove the excess of unreacted building block, and, hence, avoiding the accumulation of impurities after repeated couplings. This chromatographic purification gave the desired dimer free of any contaminants. The coupling cycle was then repeated in a similar manner and upon completion of the trimer, the oligonucleotide was released from the β-CD soluble support by ammonolysis. ESI-MS confirmed the identity of the expected trimer, and RPHPLC analysis (Figure 2B, article I) indicated high purity of the product. Compared to the assembly by using 5'-*O*-DMTr protected building blocks, this study showed slightly better yield in the synthesis of 5'-TTT-3' in solution. Accordingly, an overall yield of 87% (according to UV-analysis in comparison to the support **30b**), corresponding to

93% stepwise yield was obtained. **Scheme 11** represents the general procedure carried out to obtain 5'-TTT-3' on using **37a** as the building block.



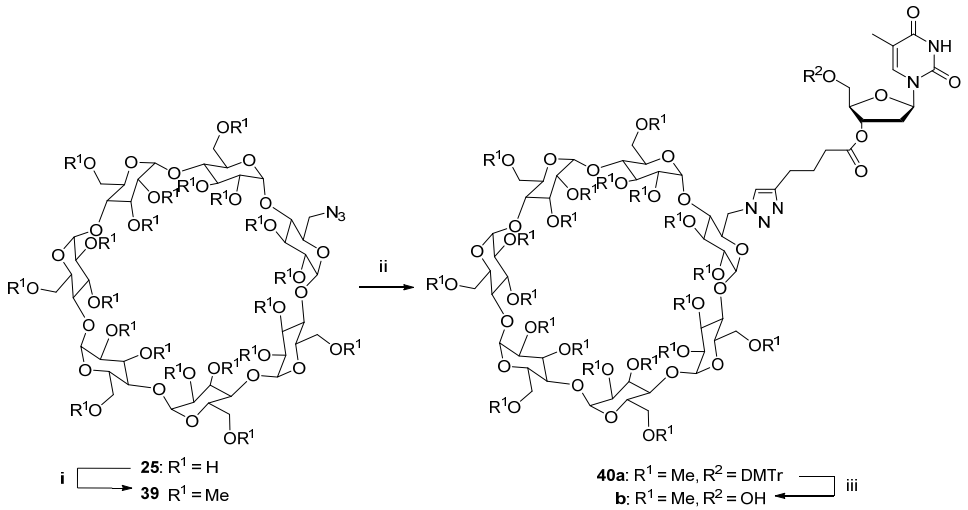
Scheme 11. General strategy for the assembly of oligodeoxynucleotides on using 5'-*O*-(1-methoxy-1-methylethyl) protected thymidine building blocks on a peracetylated β-CD soluble support.

3.1.3 Assembly of oligonucleotides from 5'-*O*-(1-methoxy-1-methyl-ethyl)-protected building blocks on methylated β–CD.

Since peracetylated β-CD does not withstand the final ammonolytic treatment, it appeared attractive to replace it with a more stable methylated β-CD, which upon ammonolytic treatment may be removed by extractive work-up procedures without the need of RPHPLC purification. In addition, a longer oligonucleotide was assembled by using a hetero-sequence composed of 5'-O-(1-methoxy-1-methylethyl) protected building blocks (**37a-c**).

Methylation was carried out with a mixture of sodium hydroxide and methyl iodide in DMSO[76,77] to afford **39**. As with the acetylated β-CD, the azido function of **39** was subjected to Cu^+ promoted 1,3-dipolar cycloaddition[81,82] to attach the 3′-terminal nucleoside bearing a 3′-*O*-(hex-5-ynoyl) linker (**29**) to the CD support. Finally, detritylation of support **40a** was performed by treatment with HCl in a 2:1 mixture of dioxane and MeOH followed by removal of volatiles under reduced pressure to afford **40b** (**Scheme 12**). Subsequent coevaporation with pyridine and MeCN eliminated any acid still present in the mixture. Finally, chromatographic

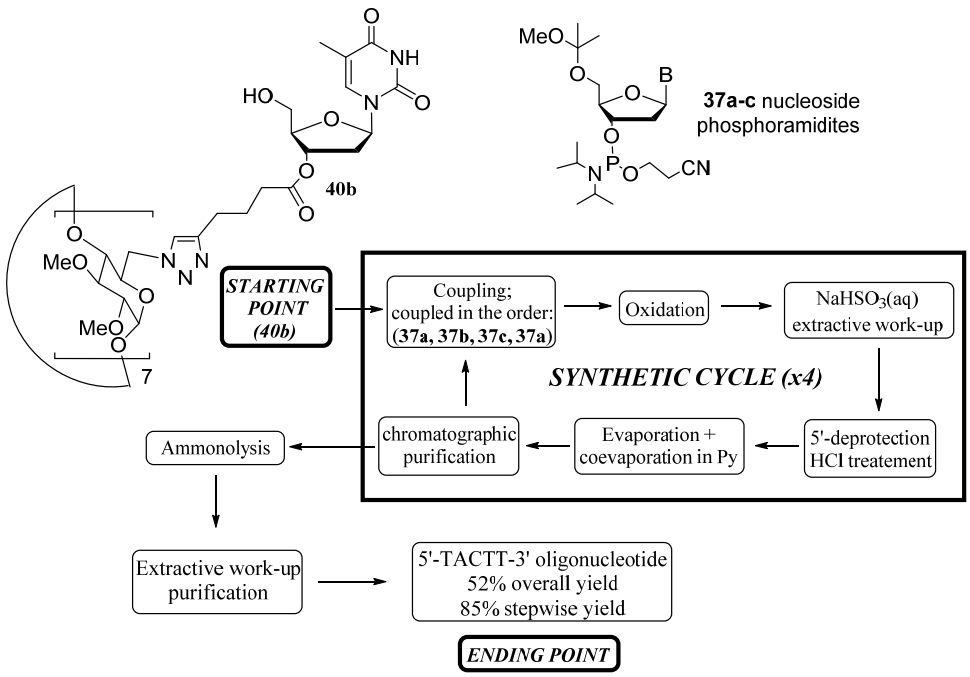
purification was carried out in order to remove trityl impurities, such as DMTrOMe and DMTrOH. The identity and homogeneity of support **40b** was confirmed by ESI-MS and RPHPLC, respectively. (Figure 1B, article I).



Scheme 12. Preparation of permethylated β-CD support for oligonucleotide synthesis. *Reagents and conditions*: (i) NaOH, MeI, DMSO;(ii) $CuSO_4$, sodium ascorbate and **29**; (iii) HCl in (dioxane:MeOH) (2/1).

As regards oligonucleotide synthesis on the methylated β-CD-T-5'-OH, **40b** was firstly subjected to 6 h coupling by using **37a (**1.5 eq) as the building block and 4,5-dicyanoimidazole (1.5 eq, 0.25 M solution of 4,5-dicyanoimidazole in anhydrous MeCN) as the activator. Upon completion of the coupling, oxidation of the phosphite triester to phosphate triester was carried out as described above. The mixture was dried over sodium sulfate, filtrated and the solvent removed by evaporation under reduced pressure. The expected product was confirmed by ESI-MS analysis. Traces of lutidine were still observed in the mixture. The 5'-*O*- acetal protecting group was removed by adjusting the pH to 4 (pH paper indicator) with HCl (0.1 M solution in a 2:1 mixture of dioxane and MeOH). Flash column chromatography in DCM/MeOH removed the byproducts formed from the unreacted building block. Upon completion of the synthetic coupling cycle from monomer to dimer, the protocol was repeated three times in a similar manner, but using in a sequential order **37b**, **37c** and again **37a** as building blocks to obtain pentamer 5'-TdAdCTT-3'. The identity and homogeneity of the products were confirmed upon each coupling cycle by ESI-MS and RPHPLC. Finally the oligonucleotide was released from the permethylated β-CD support by ammonolysis, followed by extractive work-up in DCM/H_2O. Since the permethylated β-CD could be quantitatively removed by extraction, isolation of the pentamer was considerably simplified compared to the situation on using the

acetylated support. ESI-MS confirmed the structure of the expected pentamer, and the homogeneity of the product was determined by RPHPLC analysis (Figure 4, article I). The overall yield of the pentamer synthesis was 52% (according to gravimetric measures in comparison to the support **40b**), corresponding to 85% stepwise yield. **Scheme 13** represents the general procedure used for the synthesis of 5'-TdAdCTT-3' on using **37a-c** as the building blocks.



Scheme 13. General strategy for the assembly of oligodeoxynucleotides on using 5'-O-(1-methoxy-1-methylethyl) protected building blocks on methylated β-CD.

Although the protocol described in **Scheme 13** turned out quite successful, it still suffered from some experimental inconveniences. In spite of extractive sodium bisulfite work-up after the oxidation step, traces of 2,6-lutidine were present in the mixture and had to be neutralized with HCl upon adjusting the pH to 4 for the removal of the 5′-*O*-protecting group. Accordingly, a time consuming titrimetric procedure had to be used to adjust the pH. Attention needed to be paid for this operation as too low pH led to increased amount of depurination. Another experimental inconvenience is the chromatographic separation of the growing oligonucleotide chain from excess of reactants, other reagents and impurities. Despite the fact that chromatography is a good choice for small scale synthesis, the technique is not convenient when scaling up the process. From the practical point of view, an alternative platform based on a precipitative support was explored.

3.2 Synthesis of oligodeoxyribonucleotides on a pentaerythritol-derived soluble support.

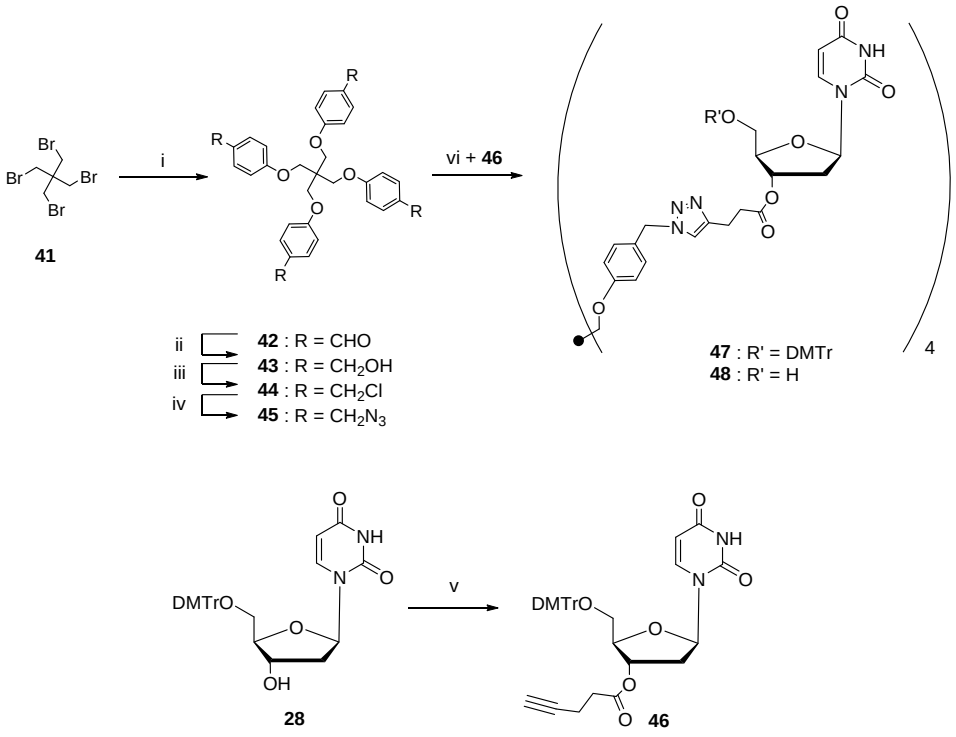
The underlying idea of this study was to describe a novel protocol for the synthesis of ONs by the phosphoramidite approach utilizing a tetrapodal pentaerythritol core as a soluble support. The interest on this symmetrical pentaerythritol-derived structure aroused from the necessity of searching a soluble support compatible with the membrane filtration purification technique. Although that was the original idea and the results of membrane filtration studies were encouraging, we found that precipitation offered an excellent alternative for the purification of the growing support-bound ON chain. In addition, the protocol employed commercially available building blocks, which undoubtedly would benefit to research groups that sporadically require DNA synthesis. Furthermore, since the branching molecule can be used to carry four ON arms, the atom economy of the synthesis is significantly increased compared to cyclodextrin supports. For that reason, the use of the pentaerythritol core as a soluble support was expected to permit large-scale synthesis of ONs.

One of the attractive advantages of this support is that pentaerythritol tetrabromide used as a starting material is commercially available (755 USD/Kg). Although more expensive than β-CD, it still has a low contribution to the overall cost of the preparation of oligonucleotides. The other advantages of the pentaerythritol-derived soluble support includes: 1) easy preparation in the laboratory; 2) chemical and mechanical stablity (withstands the reaction conditions used during the oligonucleotide synthesis); 3) good solubility properties (not limited by the solvent); 4) several easily accessible anchoring sites. In addition, from the point of view of nanofiltration, the size of support is convenient as it is still small enough to permit normal solution phase chemistry and characterization of the reaction products by MS or NMR methods at any stage of the synthesis. Furthermore, one significant attribute of this support is that purification of the growing oligonucleotide chain can be achieved by quantitative precipitation from MeOH.

Preparation of the support started from commercially available pentaerythritol tetrabromide (**41**), which upon treatment with 4-hydroxybenzaldehyde, KOH and TBAI in DMF at 120ºC afforded (**42**). The aldehyde was then reduced to alcohol with $NaBH_4$ in MeOH, to obtain (**43**). The hydroxyl function was displaced by chlorination with $SOCl_2$ in dioxane to afford (**44**), and the chloride was subsequently displaced with azide ion (**45,** 55% overall yield from **41**).

5´-*O*-DMTr-thymidine (**28**) was converted to a 3´-*O*-(pent-4-ynoyl) derivative by acylation with pent-4-ynoic anhydride prepared *in situ*. The 5´-*O*-DMTr-3´-*O*-(pent-4-ynoyl)thymidine (**46**) obtained was then subjected to Cu(I) promoted 1,3-

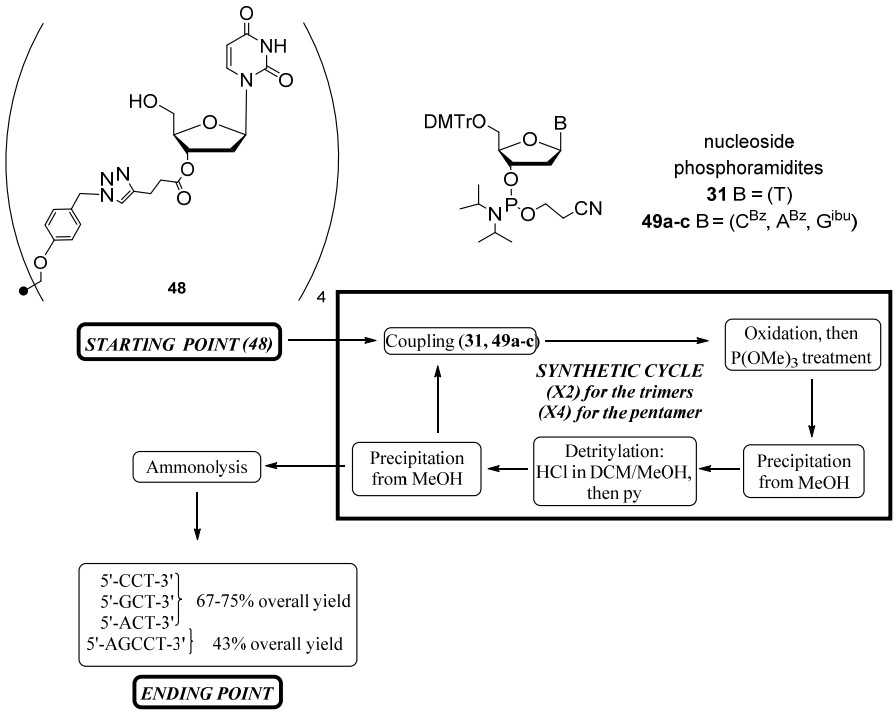
dipolar cycloaddition to attach the alkyne linker (**46**) to support **45**. After completion of conjugation by this so-called click reaction, clustered support **47** was detritylated with a solution of 10 mM HCl in MeOH/DCM (1:1 *v/v*). Addition of pyridine, followed by evaporation under reduced pressure and chromatographic purification, afforded the nucleoside cluster, tetrakis{[4-({4-[3-(thymidin-3'-*O*-yl)-3-oxoprop-1-yl]-1-*H*-1,2,3-triazol-1-yl}methyl)phenoxy]-methyl}methane (**48**), as shown in **scheme 14**. The identity and homogeneity of support **48** was verified by ESI-MS and NMR spectroscopy (Figure S3, supporting information, article II).



Scheme 14. General strategy for the immobilization of thymidine on a pentaerythritol-derived soluble support. *Reagents and conditions*: (i) 4-hydroxybenzaldehyde, KOH, TBAI, DMF, 120ºC; (ii) $NaBH_4$, MeOH, rt; (iii) $SOCl_2$, dioxane, 70ºC; (iv) NaN_3, DMF; (v) pent-4-ynoic anhydride, DMAP, pyridine; (vi) aq $CuSO_4$, sodium ascorbate, dioxane, 40ºC; (vii) HCl (13 mmol/L) in MeOH/DCM (1:1 *v/v*), rt.

To test the applicability of the clustered nucleoside support **48** to the synthesis of oligonucleotides, three trimer heterosequences, 5'-dCdCT-3', 5'-dGdCT-3' and 5'-dAdCT-3', and one pentamer containing all the bases, 5'-dAdGdCdCT-3', were assembled by using 6 equiv. (1.5 eq per free OH group on cluster **48**) of commercially available 5′-(4,4′-dimethoxytrityl)-3′-(2-cyanoethyl-*N*,*N*-

diisopropylphosphoramidite) building blocks (**31, 49a-c**). 4,5-Dicyanoimidazole (6 eq), added as 0.25 M solution in MeCN, was used as an activator. Upon 2h stirring under nitrogen, the standard aqueous iodine oxidation solution (I_2:H_2O:THF:2,6-lutidine 0.43g: 4mL: 8mL: 2mL; approximately 1.3 eq per mol of building block **31, 49a-c**) was added until the dark color remained and the reaction was left for 5 min at r.t. The excess of iodine was removed by the addition of 1 M solution of trimethyl phosphite in DMF (approximately 0.3 eq of trimethyl phosphite per mol of building block **31, 49a-c**). Completion of the oxidation reaction was confirmed by RPHPLC analysis (Figure 1, plots i-ii, article II). The mixture was then subjected to precipitatation from cold MeOH (267 mL/g). While the support precipitated quantitatively, small molecular weight reagents and impurities remained in solution. The product was then subjected to detritylation by using 13 mmol/L HCl in MeOH/DCM (1:1 *v/v*) and the progress of the reaction was monitored by TLC (approximately 15min). Upon detritylation, the acid was neutralized by addition of pyridine, the reaction mixture was concentrated to oil and quantitative precipitation of the supported oligonucleotide from cold MeOH was carried out for removal of undesired excess reagents and impurities. Afterwards, the white precipitate was isolated, dried under vacuum and the identity and homogeneity of the product was confirmed by ESI-MS and RPHPLC, respectively. Upon generation of unprotected 5'-OH group, the coupling cycle was repeated in a similar manner. The three trimers, 5'-dCdCT-3', 5'-dGdCT-3', 5'-dAdCT-3', and pentamer 5'-AGCCT-3', were cleaved from the pentaerythritol support by treatment with concentrated aqueous ammonia. **Scheme 15** represents the general protocol for the oligonucleotide synthesis on using the clustered pentaerythritol support (**48**) and commercially available building blocks (**31** and **49a-c)**. Finally, ESI-MS confirmed the identity of the compounds (Table 1, article II) and RPHPLC analysis revealed that nearly homogeneous products were obtained (Figure 1, plots viii-xi, article II). Only small traces of debenzoylation and depurination of adenosine were observed (Figure 1, plots iv-vii, article II). The overall yield of the pentamer synthesis was 43% (according to UV spectroscopic analysis in comparison to the support **48**), whereas the trimers were obtained in 67-75% yield (according to UV absorbance from **48**).



Scheme 15. General strategy for the assembly of oligodeoxynucleotides by using 5'-*O*-DMTr-protected building blocks on a pentaerythritol-derived soluble support.

As with the acetylated and methylated β-CDs supports, the pentaerythritol support also allowed a precise mass spectrometric analysis at any stage of the synthesis. From the practical point of view, the main advantage of this support is the convenient precipitation technique applied to the isolation of the growing support-bound oligonucleotide chains. In fact, excess reagents and impurities are soluble in MeOH, while the supported nucleotides precipitate in quantitative yields from this solvent. Accordingly, it appeared attractive to use the pentaerythritol support also for the assembly of short oligoribonucleotides (RNA) in solution.

3.3 Synthesis of oligoribonucleotides on a pentaerythritol soluble support.

3.3.1 Assembly of oligoribonucleotides from 2´-*O*-(2-cyanoethyl)- 5'-*O*-(1-methoxy-1-methylethyl)-protected building blocks.

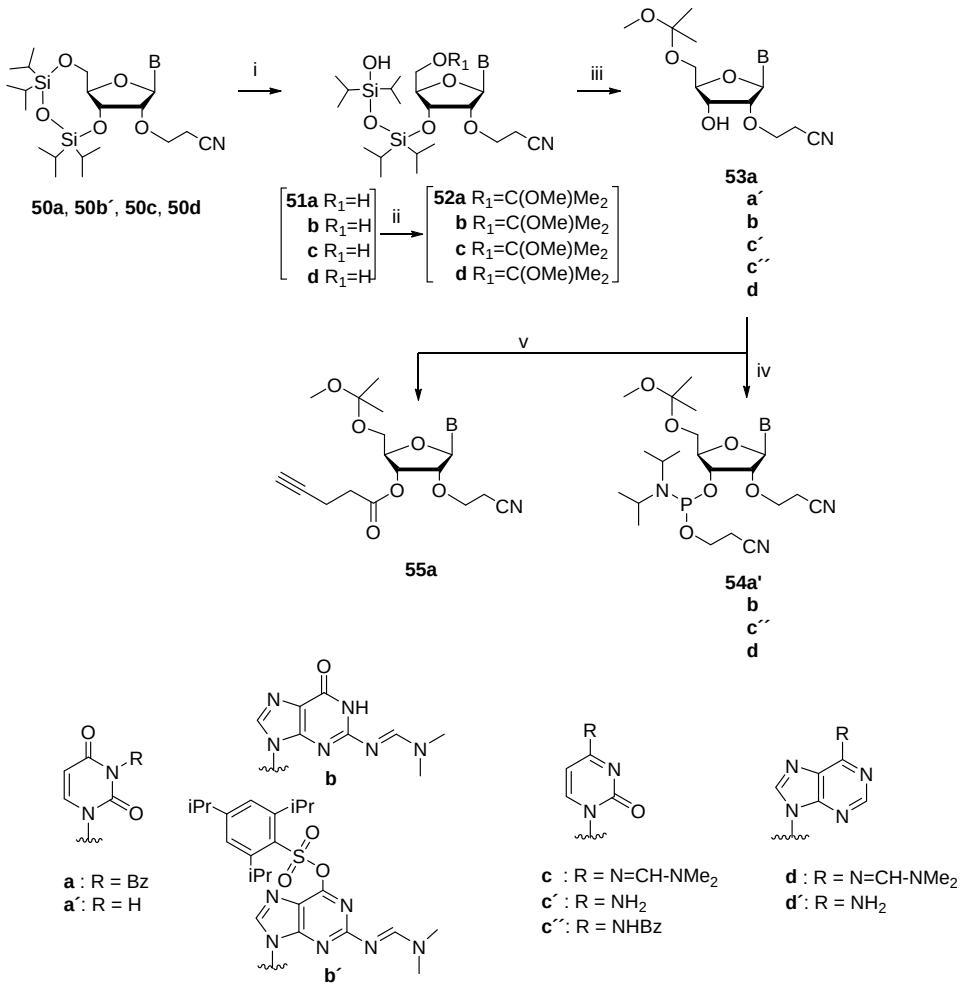
This study was aimed at providing a basis for the synthesis of short oligoribonucleotides on using the pentaerythritol-derived soluble support. During this study, a few relevant challenges were encountered. The first one was the difficulty to achieve quantitative Cu(I) promoted 1,3-dipolar cycloaddition by

using a mixture of aqueous $CuSO_4$, and sodium ascorbate in 1,4-dioxane as a solvent, since the reaction didn't proceed completely due to the precipitation of the pentaerythritol-derived soluble support in the presence of a small volume of water. To overcome this problem, catalytic amount of CuI and sodium ascorbate in dimethylacetamide (DMAc) was used followed by degassing in order to remove the oxygen during the reaction. These changes together with control of the water content in the solution resulted in formation of the expected product in nearly quantitative yield.

Preliminary studies showed that large hydrophobic 2'-O-protecting groups, such as TBDMS, prevented precipitation of the support-bound oligonucleotide from MeOH. Apart from 2'-*O*-TBDMS, 2'-*O*-methyl, 2'-*O*-TOM, 2'-*O*-PivOM and 2'-*O*-TC protections, no other phosphoramidite building blocks are commercially availaible and, hence, the monomeric blocks had to be prepared. To ensure precipitation, relatively small 2-cyanoethyl and 1-methoxy-1-methylethyl groups were used for the 2´-*O*- and 5´-*O* protection, respectively.

The starting materials of the synthesis of these building blocks were base moiety protected 2'-*O*-(2-cyanoethyl)-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) ribonucleosides (**50a**,**b'**,**c**,**d**), which were prepared as described in the literature[90]. Selective hydrolysis of the 5'-*O*-Si bond with aqueous TFA in THF was carried out to obtain **51a-d**, and the unprotected 5'-OH group was subjected to acetalization by treatment with 2-methoxypropene in THF in the presence of a catalytic amount of *p*-toluenesulfonic acid, to afford **52a-d**. Desilylation of the 3'-*O*-TIPDS was a problem to overcome. The removal was first attempted with TEA·3HF, but partial cleavage of the 5'-*O*-(1-methoxy-1-methylethyl) group turned out to be an impediment. TBAF was next employed for desilylation, but unfortunately partial cleavage of the 2'-*O*-(2-cyanoethyl) group was found to be a limitation. Finally, treatment with NH_4F in MeOH resulted in the desired desilylation leaving both 2'-*O*- and 5'-*O* protections intact (**53a-d**). Unfortunately, in some cases partial cleavage of base moiety protection took place. For instance, adenosine (**53d'**) lost the dimethylaminomethylene protection entirely and cytidine (**53c'**) partially. As the base moieties have to be kept protected during the oligonucleotide synthesis (except uracil), **53d´** and **53c´** were reprotected. In the case of adenosine, the dimethylaminomethylene protection was reintroduced (**53d**), whereas cytidine was *N*-benzoylated (**53c''**). In addition, upon NH_4F treatment, the *N*-benzoylated uridine derivative (**52a**) yielded a mixture of benzoylated (**53a**) and debenzoylated (**53a'**) products. Finally, phosphitylation of the free 3'-OH group was carried out by treatment with 1-chloro-1-(2-cyanoethoxy)-*N,N*-diisopropylphosphinamine to afford the phosphoramidite building blocks (**54a',b,c'',d**). Additionally, for immobilization of the 3'-terminal

nucleoside to the pentaerythritol-derived tetraazido support (**45**), the 3´-OH group of nucleoside **53a** was–esterified by acylation with pent-4-ynoic anhydride prepared *in situ*, affording **55a**. The synthesis of the monomeric building blocks is outlined in **Scheme 16**.

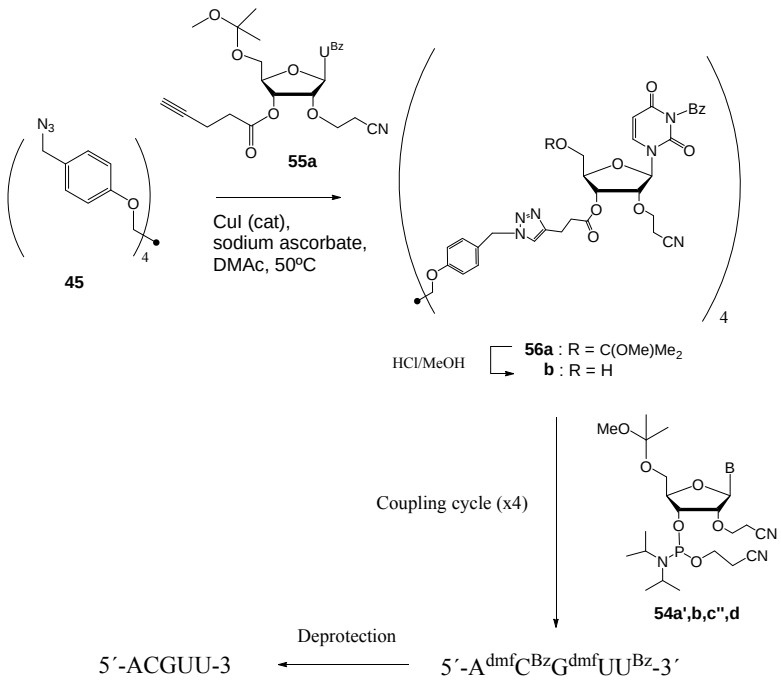


Scheme 16. Synthesis of nucleosidic building blocks. *Reagents and conditions*: (i) TFA, aq THF, 0ºC; (ii) 2-methoxypropene, TsOH, THF; (iii) NH_4F, MeOH; (iv) 1-chloro-1-(2-cyanoethoxy)-*N,N*-diisopropylphosphinamine, DIPEA, DCM; (v) 1. 4-pentynoic acid, DCC, dioxane, 2. Py, DMAP (cat).

Immobilization of the alkyne tether of the 3´-terminal nucleoside (**55a**) to the tetraazido support (**45**) was carried out by Cu(I) catalyzed 1,3-dipolar cycloaddition on using a catalytic amount of CuI and sodium ascorbate in dimethylacetamide (DMAc) followed by degassing[91], to obtain **56a**. The 1-methoxy-1-methylethyl protection was then removed with HCl (2.5 mM) in a

mixture of 2:1 (*v/v*) of dioxane and MeOH. After 1h reaction, the solvent was removed by evaporation under reduced pressure. Addition of pyridine neutralized any traces of acid sill present in the mixture and precipitation from Et_2O gave instantly **56b**. The white precipitate was isolated and the identity and homogeneity of **56b** was confirmed by ESI-MS and RPHPLC analysis.

The applicability of the branched support **56b** to the assembly of 5'-ACGUU-3' pentamer was carried out by using 6 equiv. (1.5 eq per free OH group in cluster **56b**) of 5'-*O*-(1-methoxy-1-methylethyl)-2'-(2-cyanoethyl) 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) building blocks (**54a',b,c'',d**). 4,5-dicyanoimidazole (6 eq), added as 0.25 M solution in MeCN, was used as an activator. Upon 12 h stirring under nitrogen, the standard aqueous iodine oxidation solution (I_2:H_2O:THF:2,6-lutidine 0.22g: 2mL: 4mL: 1mL) was added until the dark color remained and the reaction was left for 20min at r.t. The excess of iodine was removed by the addition of 1M $P(OMe)_3$ solution in DMF until the dark color disappeared. Then the reaction mixture was concentrated to oil, and quantitative precipitation of the growing oligonucleotide chain from cold MeOH removed small molecular weight reagents and impurities, as shown by RPHPLC studies (Figure 1A and 1B, article III). The white precipitate was isolated by filtration over celite (acid-washed). Acid-catalyzed methanolysis was then carried out by using 0.015 M HCl in MeOH/DCM (2:5 *v/v*), and the acid was neutralized by addition of pyridine and the reaction mixture was concentrated to oil. Finally, precipitation of the oligonucleotide from cold MeOH and filtration over celite removed undesired surplus reagents and impurities. RPHPLC analysis indicated that the product was nearly homogenous. Only traces of debenzoylation and removal of cyanoethyl from the phosphate groups were observed (Figure 2A and 2B, article III). The product was dried under vacuum and ESI-MS confirmed the identity of the compound. The coupling cycle was repeated in a similar manner by using the phosphoramidite building blocks **54a',b,c'',d** for the synthesis of the oligomer in the sequential order 5'-$A^{dmf}C^{Bz}G^{dmf}UU^{Bz}$-3'. Upon completion of the pentamer, treatment with TEA followed by ammonolysis and treatment with 1M TBAF in THF released the chain from the soluble support and removed all protecting groups. The oligomer was then subjected to precipitation from cold EtOH with the aid of NaOAc buffer (pH = 5.2) for 1.5h at -20C. **Scheme 17** represents the general procedure of the oligonucleotide synthesis on using the clustered pentaerythritol support (**48**) and building blocks (**54a',b,c'',d)**.The identity of the compounds was verified by ESI-MS at different stages of the synthesis (Table 1, article III) and final RPHPLC analysis revealed that nearly homogeneous pentamer product had been obtained (Figure 3, article III) in high yield (54% overall yield, according to UV spectroscopic analysis compared to support **56b**).



Coupling cycle: 1. Nucleoside phosphoramidite (**54a',b,c",d**), 4,5-dicyanoimidazole, MeCN/DMF, N_2;
2. I_2, H_2O, THF, 2,6-lutidine, then $P(OMe)_3$ in DMF
3. Precipitation in MeOH
4. HCl in DCM/MeOH, then Py
5. Precipitation in MeOH

The nuceloside phosphoramidites coupled in the order: **54a'** (B = uracil), **54b** (B = N^2-dimethylaminomethyleneguanine), **54c"** (B = N^4-benzoylcytosine), **54d** (B = N^6-dimethylaminomethyleneadenine).

Deprotection: 1. Et_3N, 2. NH_3 (aq), 3. 1M TBAF in THF.

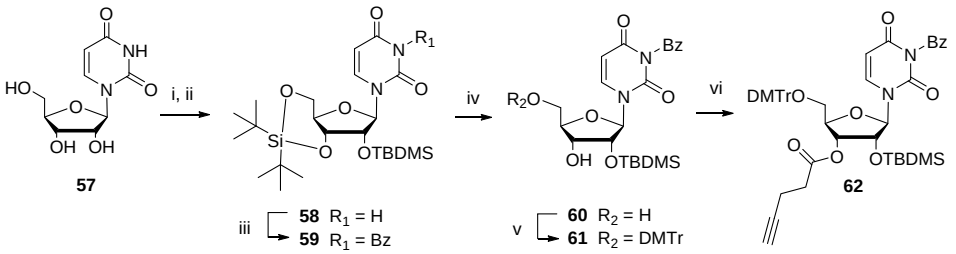
Scheme 17. General strategy for the assembly of oligoribonucleotides on using 5'-*O*-(1-methoxy-1-methylethyl)-2'-O-(2-cyanoethyl)-protected phosphoramidite building blocks on a pentaerythritol-derived soluble support.

Although the efficiency of the synthesis was demonstrated by the assembly of a pentameric ON in good yield and in hundreds of mg scale, one drawback of this protocol is the laborious preparation of the 5'-*O*-(1-methoxy-1-methylethyl)-protected phosphoramidite building blocks that are not commercially available. For the needs of researchers that only occasionally use RNA synthesis and are not used to synthesize phosphoramidite building blocks, an alternative platform was explored by using the pentaerythritol-derived support and commercially available 5′-*O*-(4,4′-dimethoxytriyl)-2′-*O*-(*tert*-butyldimethylsilyl)ribonucleo-side 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)s as building blocks.

3.3.2 Assembly of oligoribonucleotides from 5'-*O*-(4,4'-dimethoxy-trityl)-2´-*O*-(*tert*-butyldimethylsilyl)-protected building blocks.

The objective of this study was to develop a general protocol for the synthesis of short oligoribonucleotides in solution on using the branched pentaerythritol-derived soluble support and commercially available 5´-*O*-(4,4´-dimethoxytrityl)-2´-*O*-(*tert*-butyldimethylsilyl)-3´-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) building blocks (U, A^{Bz}, G^{ibu} and C^{Ac}). As stated previously, the use of more hydrophobic 2'-*O*-TBDMS groups do not allow precipitation of the growing oligonucleotide from MeOH and, hence, one precipitation from water after the oxidation step and one chromatographic purification upon the 5'-detritylation were needed in each synthetic cycle in order to remove contaminants from the reaction mixture.

The preparation of the 3'-terminal alkyne derivative of uridine was similar to that reported above except that 2´-*O*-(*tert*-butyldimethylsilyl) protection was used instead of 2´-*O*-(2-cyanoethyl). The synthesis started by selective silylation of the 5'-OH and 3'-OH groups of uridine (**57**) with di-*tert*-butylsilanediyl bis(trisfluoromethanesulfonate) in anhydrous DMF[92] (**Scheme 18**). The 2'-hydroxy group was then silylated with TBDMSCl in the presence of imidazole in DMF to afford **58**. The amino group of the base moiety was benzoylated by treatment with BzCl in the presence of Na_2CO_3, yielding **59**. To prevent *N*3 acylation during the introduction of the ω-alkynoyl linker to the 3´*O*, *N*3 was benzoylated before removal of the di-*tert*-butylsilanediyl group, which was accomplished by treatment with HF-Py in pyridine to afford (**60**). The 5´-*O*-DMTr protection was then introduced with DMTrCl in pyridine to obtain **61**. Finally the 3´-OH group of the nucleoside (**61**) was converted to a 3´-*O*-(pent-4-ynoyl) derivative by acylation with pent-4-ynoic anhydride prepared *in situ*, to afford **62**, as shown in **Scheme 18**.



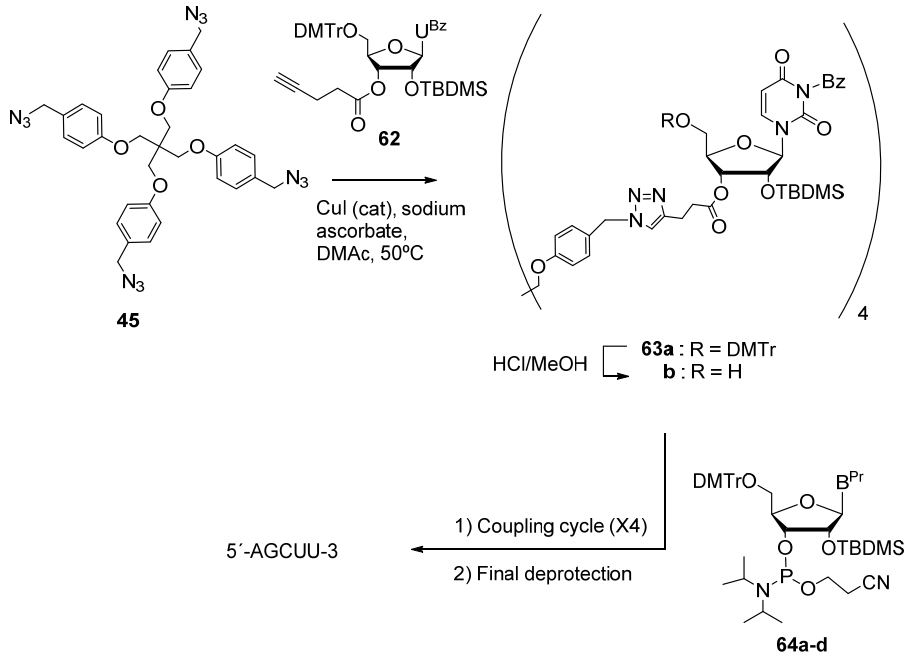
Scheme 18. Synthesis of fully protected uridine bearing a 3'-O-(pent-4-ynol) linker. *Reagents and conditions*: (i) *t*-$Bu_2Si(OTf)_2$, DMF; (ii) *t*-$BuMe_2SiCl$, Im, DMF; (iii) BzCl, Na_2CO_3; (iv) HF-Py, O °C, DCM; (v) DMTrCl, Py; (vi) 1. 4-pentynoic acid, DCC, dioxane, 2. Py, DMAP (cat).

The alkyne functionalized nucleoside (**62**) was conjugated to the tetrakis-*O*-(4-azidomethylphenyl)pentaerythritol support (**45**) by Cu(I) catalyzed 1,3-dipolar

cycloaddition using a catalytic amount of CuI and sodium ascorbate in dimethylacetamide (DMAc) followed by degassing[91], to obtain **63a**. The nucleoside bearing support (**63a**) was subjected to acid-catalyzed methanolysis with HCl (5 mM) in a mixture of DCM/MeOH 1:1 (*v/v*) and the DMTrOMe formed was removed by chromatographic purification to afford **63b**. The identity and homogeneity of the compound was confirmed by ESI-MS and RPHPLC (Figure 1, article IV), respectively.

A pentameric 5'-AGCUU-3' oligoribonucleotide was then assembled to demonstrate the applicability of the method (**Scheme 19**). Couplings were performed on using 1.5 equiv. of commercially available building blocks (**64a-d**) per each free 5'-hydroxyl group on the support and an equal amount of 4,5-dicyanoimidazole as the activator (added as 0.25 M solution in MeCN). After 12h reaction, the phosphite triester was oxidized to phosphate triester by 25 min treatment with an aqueous iodine oxidation, as described above. Excess of iodine was quenched with trimethylphosphite. Since precipitation from MeOH was not possible, owing to the higher hydrophobicity of the TBDMS group compared to the 2-cyanoethyl group, the system was readapted and quantitative precipitation from water was performed for partial removal of reagents, as shown in RPHPLC analysis (Figure 2A and 2B, article IV). Although the byproducts derived from unreacted building blocks were only partly removed by this method, this did not prevent continuation of the coupling cycle by the detritylation step. The white precipitate was filtered, isolated and subjected to detritylation by treatment with HCl (5 mM) in a mixture of DCM/MeOH 5:2 (*v/v*). Pyridine was added to the solution to neutralize the acid and the mixture was concentrated to oil. The support was then purified by column chromatography by using a 1-8% gradient of MeOH in DCM. The identity and homogeneity of the compound was confirmed by ESI-MS (Table 1, article IV) and RPHPLC (Figure 3, article IV), respectively. The coupling cycle was repeated in a similar manner by using phosphoramidite building blocks **64a-d** for the synthesis of the oligomer to obtain 5'-$A^{Bz}G^{iBu}C^{Ac}UU^{Bz}$-3'. During the synthesis, traces of debenzoylation and removal of the 2-cyanoethyl groups from the phosphate moiety occurred (Figure 1B, article IV). Upon completion of the pentamer, treatment with TEA followed by concentrated aqueous ammonia and 1:1 (*v/v*) mixture of dry DMSO and $(HF)_3Et_3N$ released the oligonucleotide from the support and cleaved all protecting groups. The oligomer was then subjected to precipitation from cold EtOH with NaOAc buffer (pH = 5.2) for 1.5h at -20C. **Scheme 19** represents the general procedure of the oligonucleotide synthesis on using the clustered pentaerythritol support (**63b**) and commercially available building blocks (**64a-d)**.The identity of the compounds was confirmed by ESI-MS at any stage of the synthesis (Table 1, article IV) and final RPHPLC analysis verified formation of the nearly

homogeneous pentamer (Figure 4, article IV) in high yield (46% overall yield according to UV spectroscopic analysis compared to support **63b**).



Coupling cycle: 1. Nucleoside phosphoramidite (**64a-d**), 4,5-dicyanoimidazole, MeCN/DMF, N_2;
2. I_2, H_2O, THF, 2,6-lutidine, then $P(OMe)_3$ in DMF
3. Precipitation in H2O
4. HCl in DCM/MeOH, then Py
5. Column chromatography

The nuceloside phosphoramidites coupled in the order: **64a** (B^{Pr} = uracil), **64b** (B^{Pr} = *N4*-acetylcytosine), **64c** (B^{Pr} = *N2*-isobutyrylguanine), **64d** (B^{Pr} = *N6*-benzoyladenine)

Final deprotection: 1. Et_3N, 2. NH_3 (aq), 3. $(HF)_3TEA$ in DMSO

Scheme 19. General strategy for the assembly of oligoribonucleotides on using 5'-*O*-(4,4´-dimethoxytrityl)-2´-*O*-(*tert*-butyldimethylsilyl)-protected phosphor-amidite building blocks on a pentaerythritol-derived soluble support.

Although the overall yield of the pentamer was slightly lower than the one obtained on using 5'-*O*-(1-methoxy-1-methylethyl)-2'-O-(2-cyanoethyl)-protected phosphoramidite building blocks (43% vs. 54%), the protocol is still workable in cases where researchers want to use only commercially available building blocks in a straightforward synthesis procedure.

4. DISCUSSION

The objective of the MEMTIDE project was to develop a scalable solution phase strategy for oligonucleotide synthesis that would offer an efficient and versatile platform for the emerging OSN technique. The general idea was to assemble the oligonucleotide attached to a soluble support and perform the separation of the growing oligonucleotide from excess reagents and impurities after each coupling by nanofiltration in organic solvent (OSN). Initial attempts using acetylated and methylated CDs and also the pentaerythritol soluble support in the laboratory of Prof A. Livingstone at Imperial College London have given engouraging results when the membrane filtration technique has been applied. However, significant optimization of chemistry and membranes has still to be done to increase the yield. While these approaches will hopefully lead to novel industrial technology, the approaches described in the present theses are believed to be useful in normal organic chemistry laboratories for preparation of oligonucleotides of limited length in the scale of hundreds of milligrams.

In the Results chapter, a description of various novel solution phase synthesis strategies has been presented. The syntheses of short oligonucleotides on acetylated and methylated β-CDs, and, in particular, on the pentaerythritol-derived soluble support have provided strong evidence that solution phase synthesis may well be applied in cases where large amounts of short ONs are needed without the need of any special infrastructure. For instance, we have demonstrated that hundreds of mg quantities may be easily produced in the laboratory. In addition, these approaches neatly have provided similar overall yields compared to those previously reported protocols on using different soluble supports, such as PEG (Polyethylene-glycol),[61,62,63,64,65,66,67] (adaman-1-yl)acetyl[68] or succinyl-tethered-1-ethyl-3-methylimidazolium tetrafluoroborate[69]. Furthermore, apart from extractive work-up, diafiltration and precipitation, we have also established chromatography as a tool for the purification of the growing oligonucleotide while using the acetylated and methylated β-CDs. Although the commonly used precipitation and extraction techniques essentially exhibit potential for the large-scale purification processes, none of them have yet been applied for the synthesis of ONs with commercial purposes. Since all these protocols commented above are operated entirely in solution phase, the high reaction rates and the large reaction volumes should not be a problem.

As regards oligonucleotide assembly on a soluble support, numerous protocols have been reported for the preparation of oligodeoxyribonucleotides, but none of these attempts have so far been applied to the synthesis of oligoribonucleotides.

For that reason, upon completion of the DNA studies, we decided to apply two different protocols on using the pentaerythritol-derived soluble support for the assembly of RNA. As mentioned in the Results chapter, in the first case the use of 5'-*O*-(1-methoxy-1-methylethyl)-2'-O-(2-cyanoethyl)-protected phosphoramidite building blocks gave good results on using the precipitation technique for the purification of the growing oligonucleotide. The second protocol utilized commercially available 5'-O-(4,4´-dimethoxytrityl)-2´-*O*-(*tert*-butyldimethylsilyl)-protected phosphoramidite building blocks and chromatographic purification on the same soluble support. In both cases, the simplicity and reproducibility of these protocols were suitable to ensure a nearly homogeneous pentameric product. Nevertheless, the methodology has not yet been applied for the synthesis of longer oligonucleotides, which could be affected due to some solubility problems of the soluble support upon each elongation of the chain assembly.

Although the current state-of-the-art platform is the well-known solid-phase oligonucleotide synthesis, it seems worthwhile to use a combination of both SPS and LPS to develop a scalable manufacture platform that permit the synthesis of oligonucleotide-based drugs in multi-Kg or ton scales for chemotherapeutical purposes in the pharmaceutical industry. Since the SPS uses a completely machine-automated procedure and avoids any human error during the synthesis, the platform could be combined with the LPS for a cost-effective scale-up manufacture process. Although the feasibility for a large scale process design is still difficult but not impossible, the optimization of the chemistry and new advances in technology could overcome this challenge in a near future.

5. EXPERIMENTAL

General synthesis and characterization of the compounds

The synthetic methods mentioned in this thesis are reported in the original publications (**I-IV**). The characterization of compounds was carried out by NMR (1D and 2D), HRMS and RP HPLC techniques. NMR spectra were recorded on a Bruker Avence 500 MHz or 400 MHz at 25 °C, in which chemical shifts are given in ppm and are referred to internal TMS (for ^{1}H and ^{13}C NMR) and to external orthophosphoric acid (for ^{31}P NMR). High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics MicrOTOF-Q spectrometer using ESI ionization. RP HPLC analyses were performed on an analytical Thermo ODS Hypersil C18 (250 × 4.6 mm, 5μm) column using UV detection at 260 nm. In addition, reactions were monitored by TLC (Merck, Silica gel 60 F254), using short wavelength UV for detection. Anhydrous solvents were prepared by storing them over baked 4Å molecular sieves.

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