On the feasibility of 3-(hydroxyamino)propane-1,2-diol as a base filling scaffold in oligonucleotides.

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During the past three decades modified nucleotides and oligonucleotides have emerged in e.g. therapeutics and diagnostics. Depending on the application, modifications are carried out at the sugar, phosphate or base moieties. These modifications can lead to improved enzymatic resistance, binding affinity, biocompatibility, biodistribution and pharmacokinetics.

Base filling can be considered as an alternative to enzymatic or automated oligonucleotide synthesis. Base filling involves post synthetic introduction of a nucleobase to an abasic site on the backbone. This approach can be considered an application of dynamic combinatorial chemistry as equilibrium conditions are to be used in the base filling approach (most, but not all, of the reported base filling reactions are dynamic).

The aim of this study was to synthesize an oligonucleotide containing a base filling scaffold having a minimal structure for the reversible functionalization of oligonucleotides. The novel building block features a modified sugar phosphate backbone consisting of an oxazolidine ring in place of sugar moiety and an unusual N-O-P phosphodiester linkage instead of a conventional C-O-P linkage. Different synthetic routes were carried out to synthesize the base filling scaffold.

First, a phosphoramidite building block featuring the unusual N-O-P linkage was synthesized successfully. But this phosphoramidite was found to be unreactive and thus unsuitable for oligonucleotide synthesis. Therefore, formation of the desired phosphodiester bond was attempted by an alternative strategy, namely coupling the modified monomer with a commercially available nucleoside phosphoramidite to form a dimer, which will be used to study the compatibility of the carbamate protection and the N-O-P linkage with conditions of automated oligonucleotide synthesis and, ultimately, base filling.

Keywords: modified nucleotides, base filling, phosphoramidite, H-phosphonate diester

Abbreviations and Acronyms

ACN	-	Acetonitrile
ASO	-	Antisense Oligonucleotide
BMT	-	5-Benzylmercapto-1H-tetrazole
CPG	-	Controlled Pore Glass
COSY	-	Correlation Spectroscopy
DCC	-	Dynamic Combinatorial Chemistry
DCL	-	Dynamic Combinatorial Library
DCM	-	Dichloromethane
DIPEA	-	N,N-diisopropylethylamine
DMTr	-	Dimethoxy trityl
DNA	-	Deoxyribonucleic Acid
FANA	-	2-FluoroArabinose Nucleic Acid
GNA	-	Glycol nucleic acid
HNA	-	Hexitol Nucleic Acid
HMBC	-	Heteronuclear Multiple Bond Correlation
HSQC	-	Heteronuclear Single Quantum Correlation
LC-MS	-	Liquid Chromatography Mass Spectrometry
LNA	-	Locked Nucleic Acid
LPOS	-	Liquid Phase Oligonucleotide Synthesis
MOE	-	Methoxyethyl
NMR	-	Nuclear Magnetic Resonance
ON	-	Oligonucleotide
PCR	-	Polymerase Chain Reaction
PNA	-	Peptide Nucleic Acid
PS	-	Phosphorothioate
RNA	-	Ribonucleic acid
SPOS	-	Solid Phase Oligonucleotide Synthesis
TBDMS	-	*tert*-butyldimethysilyl
TdT	-	Terminal deoxynucleotidyl Transferase
TFA	-	Trifluoroacetic acid
TLC	-	Thin Layer Chromatography

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1 Introduction

Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are naturally occurring biomacromolecules that store, express and transmit genetic information in cells with sufficient stability and fidelity supporting the existence of the genome. These nucleic acids are also flexible which facilitates evolution[1]. Nucleic acids exhibit properties such as high water solubility, reversible denaturation and renaturation, predictable folding and programmable interactions[1]. These properties make nucleic acids useful in many applications.

During the last five decades, nucleic acids have been developed as potent therapeutics[2] in different forms. One form is antisense oligonucleotides (ASOs) which are short, single-stranded, synthetic oligonucleotides that can alter RNA and change protein expression through several mechanisms[3]. ASOs show positive effects in reducing oxidative stress which is a fundamental role in several conditions like cancers, Alzheimer's disease, cardiovascular and inflammation disorders and diabetes[4]. Another form is small interfering RNAs (SiRNAs) which are double-stranded RNAs with 20-25 nucleotides length involved in cell defence mechanisms and maintaining genome integrity through silencing of exogeneous nucleic acids and undesired RNA transcription[5]. siRNA drugs are designed to silence disease-causing genes by introducing synthetic siRNA molecules into the cells which can bind to messenger RNAs and interfere with the protein expression. These siRNAs are used to track genetic disorders, cancers, inflammatory diseases, viral infections and neurodegenerative diseases[6]. Aptamers are short, single-stranded DNA or RNA that can bind to specific target molecules. The ability of the single stranded oligonucleotides to fold into different shapes provides high affinity and specificity towards target molecules. Therefore, aptamers can be used as antibodies in therapeutics[7]. In addition, micro-RNA (miRNA), which are small non-coding RNA molecules that play a pivotal role in many biological processes,[8] are also used in many therapeutic applications such as spanning innate immunity[9], autoimmune disease[10] , depression[11], anxiety[12], different types of cancers[13,14], cardiovascular disease[15] and diabetes[16].

Nucleic acids can also be used for diagnostic purposes. DNA, messenger RNA and non-coding RNA are developed for bio sensing and imaging[17,18]. With the development of the

polymerase chain reaction (PCR) method, millions of copies of DNA and RNA target molecules are obtained through repeated enzymatic amplification with a high specificity and selectivity, making it the gold standard of nucleic acid detection[19]. Nucleic acids are developed to detect different targets such as pH[20,21], small molecules[22], RNAs[23,24], proteins[17] and cells[25]. Aptamers can identify changes in the mentioned targets and thus work as sensors.

In addition, nucleic acids are used in synthetic biology[26], material sciences[27] and also, they are used as important building blocks in DNA encoded libraries[28], storage of digital information[29], computing[30], as catalysts[31,32] and binders[33].

With the increasing demand for nucleic acids in different fields, modified nucleic acids are introduced to obtain the full potential as naturally occurring nucleic acids show poor chemical and biological stability and narrow chemical diversity[1].

1.1 Modified oligonucleotides

Nucleotides are the building blocks of nucleic acids which consist of three units. These units are a phosphate (1-3 phosphoryl groups), a pentose sugar and a nitrogenous base. The sugar molecule and the phosphate groups together make the backbone and the nitrogenous base forms the base pairs, stacking one nucleotide with another. Figure 1 shows the main three units of nucleotide.

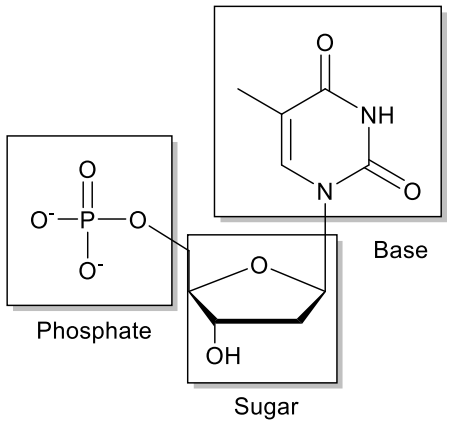


Figure 1. Chemical structure of a nucleotide, consisting of the sugar group, nitrogenous base group and phosphate group.

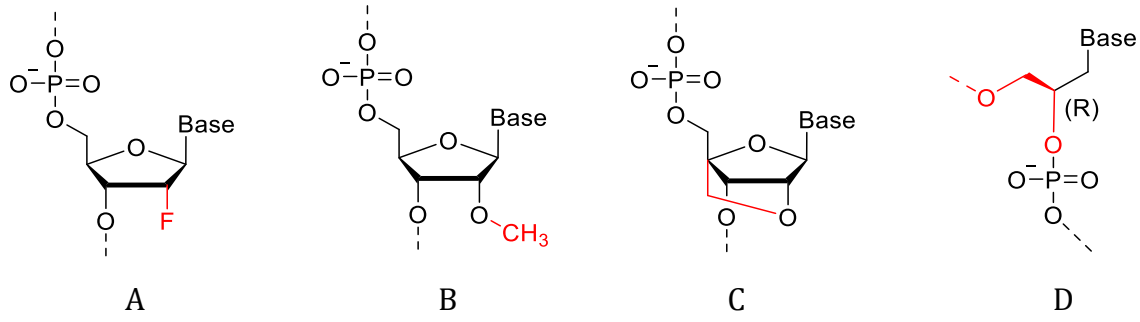
Modified nucleotides are obtained by performing modifications at the sugar group, phosphate groups or the nitrogenous base[1,18,34]. Modified oligonucleotides may show

improved sequence specificity, stability, hybridization affinity and various functional properties[18].

1.1.1 Sugar modifications

The sugar in natural nucleotides is deoxyribose in DNA and ribose in RNA. Sugar modifications can be performed by introducing substituents to the 2′position or by changing the nature of the sugar ring to trioses, tetroses, hexoses or heptoses or by introducing heteroatoms to the pentose sugar ring[35]. These modifications change conformation of the sugar pucker leading to enzyme resistance and, potentially, enhanced Watson-Crick hydrogen bonding and base stacking interactions[36].

Some of the examples for sugar modifications are 2′F RNA (Figure 2A) which is done by introducing fluorine to the 2′position of RNA. This modification has shown increased rigidity and stability compared to unmodified RNA due to the high electronegativity of the F atom compared to the OH group[36]. Another modification is methylation of the 2′O leading to 2′OMeRNA (Figure 2B). The methoxy group induces a strong gauche effect and stabilizes the 3′endo conformation[36]. In locked nucleic acids (LNA) (Figure 2C), a connection is made between 2′oxygen atom and the C4 carbon through a methylene bridge which locks the ribose in the 3′endo conformation. Oligonucleotides having LNA modification show a strong thermal stability with their target RNA molecules[37]. Glycol nucleic acid (GNA) (Figure 2D) has a sugar modification with an acyclic glycol-phosphate backbone which lacks one bond compared to conventional nucleic acids. This is more flexible and poses with two diasteriomers, namely (R)-GNA and (S)-GNA. These diasteriomers can form antiparallal self-pairs that show higher thermal stability than unmodified nucleic acids[35].



A B C D

Figure 2. Sugar modifications. A) 2′F RNA, B) 2′OMe RNA, C) LNA, D) GNA

1.1.2 Base modifications

Adenine, guanine, cytosine, thymine and uracil are the nitrogenous bases found in DNA and RNA. Alterations to these existing bases can expand the genetic alphabet[38], improve the stability of the double helices[39], enhance catalytic activity[40], enable incorporation of metal binding sites to nucleotides[41] and alter folding and functions of nucleic acids[42] . Incorporation of the naturally occurring nucleobase pseudouridine to a mRNA supresses RNA mediated immune activation in vivo and in vitro and leads to enhanced translational capacity of RNA[43]. Base modifications are mainly used in mRNA vaccines. For instance, the vaccine used against the covid-19 contained N1-methylpseudouridine instead of uridine[44]. Figure 3 presents some examples of base modifications.

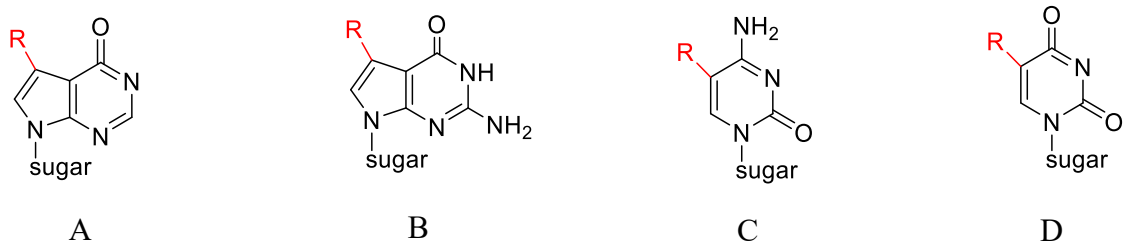


Figure 3. Base modifications**.** A) C7-modifided deazaadenine, B) C7-modified deazaguanine, C) C5-modified cytosine, D) C5-modified uracil.

1.1.3 Backbone modifications

Backbone modification may either be limited to the phosphodiester linkage or also replace the sugar moiety (PNA or morpholino oligos). Backbone modifications are used to improve membrane permeability[45], resistance to nucleases[46] and binding, for example to proteins in the plasma as well as in the cell[47]. One method of backbone modification is to substitute a non-bridging oxygen atom with an atom like sulphur or selenium. Phosphorothioates (PS) are backbone modified nucleotides where one non-bridging oxygen is substituted with a sulphur atom[45]. Boranophosphates are another type of backbone modification, carried out by replacing a non-bridging oxygen atom with a BH_3 moity. Due to the special nature of the borane group, this boranophosphates show unique properties such as change in polarity and nucleophilicity[48]. Peptide Nucleic Acid (PNA) is an example of a sugar linking backbone modification, where the deoxyribose phosphate backbone is replaced by a pseudo-peptide polymer to which the nitrogenous bases are

linked[49]. This backbone has a neutral nature and shows an outstanding binding affinity and specificity towards complementary DNA and RNA. In addition, it is resistant towards both nucleases and peptidases. PNA has been used in many applications such as antigen and anticancer drugs[50], for detection of genomic mutations, labelling chromosomes[51], as molecular probes[50] and modulating polymerase chain reactions (PCR)[52]. Triazole modified nucleic acids are obtained via click chemistry approach. This modification leads to several unique properties such as resistance towards hydrolysis, antibacterial, antiviral and antitumor properties[53]. The structures of PS (A), borano-phosphate (B), PNA (C) and 1,2,3-triazole (D) modified nucleic acids are presented in Figure 4.

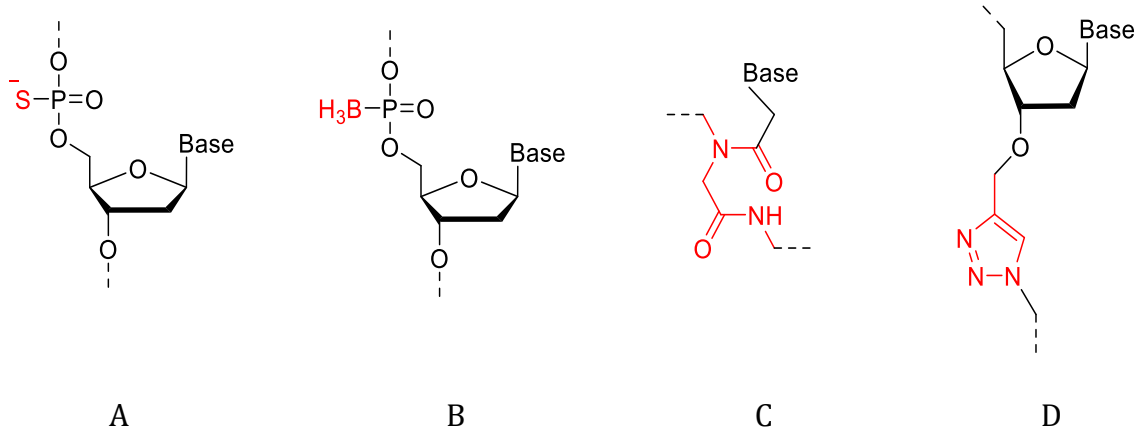


Figure 4. Backbone modifications. A) PS, B) Borano-phosphate, C) PNA, D) triazole modified nucleic acid.

1.2 Oligonucleotide synthesis

In general, oligonucleotide synthesis is done by linking DNA or RNA nucleotides (monomer units) together in a specified order forming the inter-nucleotide phosphodiester bond. The phosphodiester bond is illustrated in Figure 5.

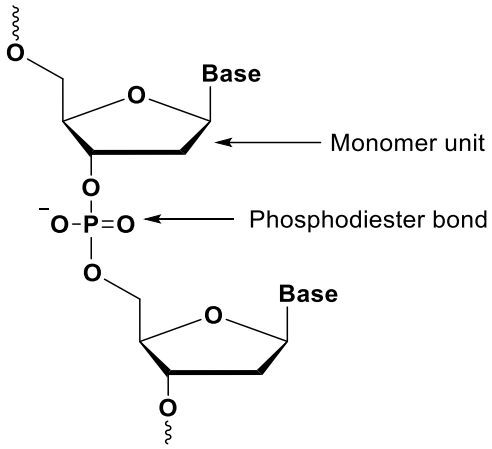


Figure 5. Two nucleoside monomer units connected with a phosphodiester bond.

Once the modified nucleotides are synthesized chemically, they are introduced to an oligonucleotide chain mainly by two methods, namely enzymatic or chemical synthesis. Enzymatic synthesis is used when the oligonucleotide synthesis is done using enzymes and chemical or automated synthesis is used when the oligonucleotide synthesis is done chemically.

1.2.1 Enzymatic synthesis.

In enzymatic synthesis, polymerase enzymes are used to link the nucleotides to the growing oligonucleotide chain. This method mimics the natural process of DNA and RNA synthesis in living cells. Enzymatic synthesis can be template dependent or template independent depending on the enzymes used. Different enzymes are used to polymerize nucleotides to DNA or RNA. For example, terminal deoxynucleotidyl transferase (TdT) enzyme adds deoxyribonucleotides to the 3′ terminus of the DNA chain[54,55]. T4 RNA ligase enzyme is used to join ribonucleotides between 5′ terminal of the phosphate group and 3′ hydroxyl group[56,57]. Polynucleotide phosphorylase enzyme is used to catalyze the synthesis of highly polymerized RNA like polynucleotides[58].

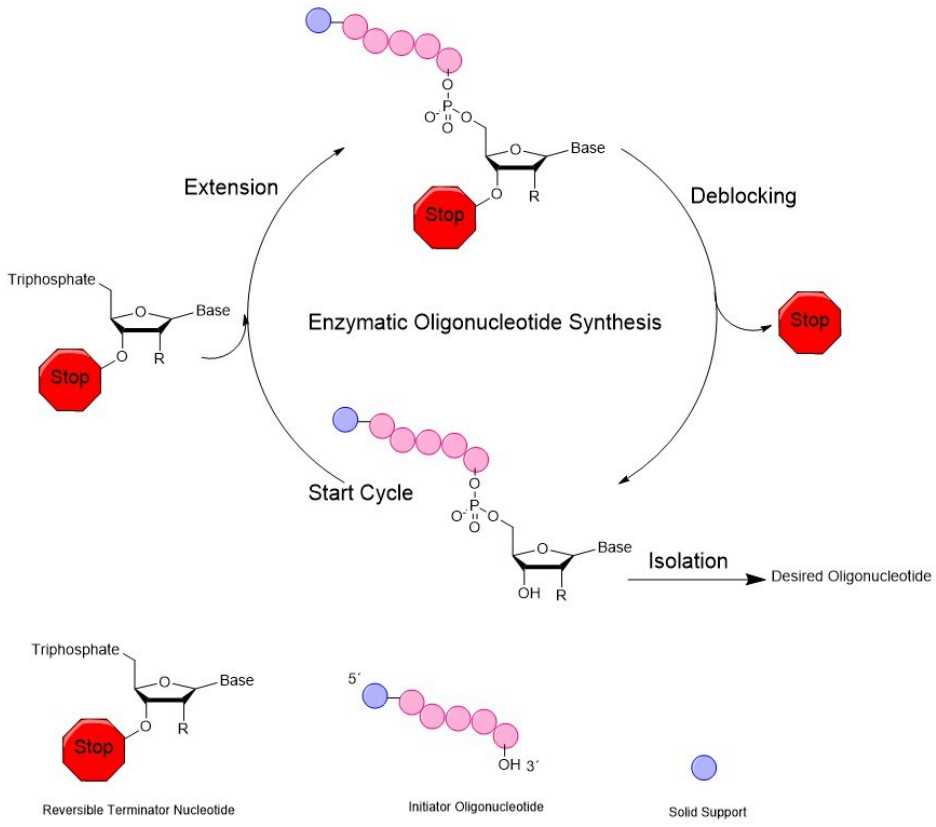
Advantages of enzyme synthesis over chemical synthesis are the ability to synthesize oligonucleotides using naturally occurring enzymes, producing less toxic waste, and the ability to synthesize longer oligonucleotide chains with higher coupling efficiencies[59].

There are also some limitations of this method. Most of the chemically modified nucleotides cannot be incorporated to an oligonucleotide chain using this method as the

enzymes cannot tolerate the modifications. Some diastereomeric analogues of nucleotides are also not recognized by some enzymes and cannot be used in enzymatic synthesis[60].

Controlled enzymatic synthesis is a method combining enzymatic oligonucleotide synthesis with elements of solid phase synthesis. In this method either the 3′- position of the sugar moiety or the nitrogenous base of the nucleotide is blocked and it is incorporated mainly by the template independent polymerases on to a primer which is bound to a polymer support[32].

In controlled enzymatic synthesis, nucleoside triphosphates which are blocked at the 3′ position of the sugar or at the base are introduced to a single stranded primers with the incorporation of polymerase enzymes. Once the nucleoside is bound to the oligonucleotide chain the blocking group is removed and it is ready for the next cycle. The steps of enzymatic synthesis are illustrated in Scheme 1.



Scheme 1. Nucleotide addition cycle of enzymatic oligonucleotide synthesis[61]

1.2.2 Chemical synthesis

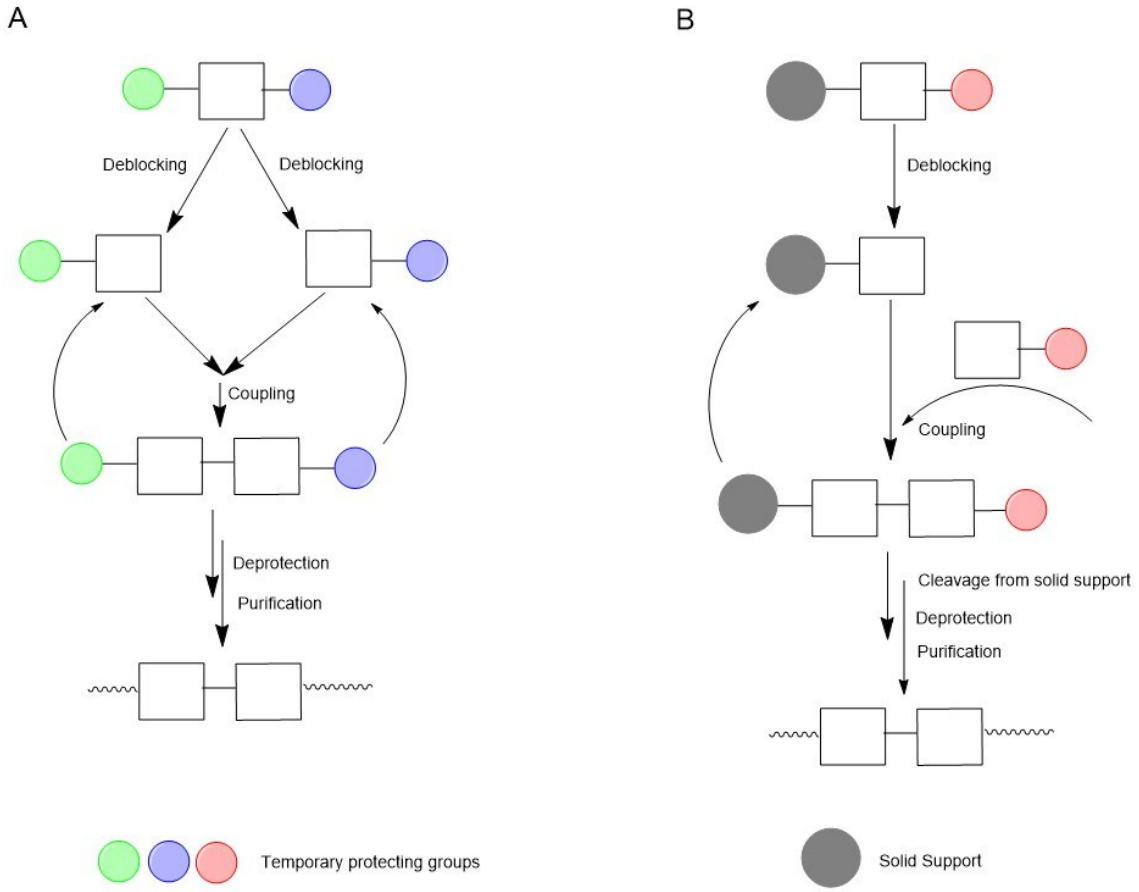
In chemical oligonucleotide synthesis, a single stranded DNA or RNA is synthesised by adding one nucleotide at a time in 3′ to 5′ direction. There are two strategies used in chemical synthesis, namely solid phase oligonucleotide synthesis (SPOS) and liquid phase oligonucleotide synthesis (LPOS).

Solid phase synthesis is done by using a solid support (usually a resin bead) to attach the nucleotides from the 3′-end. Once the oligonucleotide chain is formed, the linker is cleaved and the solid support is removed by simply washing with solvents. This method is one of the breakthrough findings in nucleic acids chemistry, which allows automated assembly of oligonucleotides with the desired sequence[62]. This method reduces laborious purification leading to a high coupling efficiency[63], affording oligonucleotides with a high purity. More importantly, this method facilitates the introduction of modified nucleotides to the growing oligonucleotide chain. However, solid phase synthesis is limited to < 10 kg batches of oligonucleotide production due to the reduction of product purity when increasing the column size[64]. This method is very expensive due to use of expensive resins and solvents for the synthesis. Huge amounts of solvents are used for each synthesis cycle and thus the waste production is very high, resulting in a large carbon footprint[62].

In liquid phase synthesis, instead of an insoluble solid support, a soluble polymer support is present where the reaction takes place. This polymer support is soluble in organic solvents making a homogeneous environment for the oligonucleotide synthesis[65]. Once the oligonucleotide is synthesised, it is freed from the unreacted reagents and soluble by-products by crystallization or membrane filtration[66–68]. Liquid phase synthesis can be performed easily using a conventional batch reactor. This reduces the cost of installing an automated synthesizer, the amount of solvents used in the synthesis and is thus an environmentally friendlier method. However, customised soluble supports are required for different oligonucleotide synthesis depending on the length, loading and the chemistry of the desired oligonucleotide sequence[65].

In both solid phase and liquid phase oligonucleotide synthesis, the linkage between the nucleotides is formally achieved by esterification of phosphoric acid with 3′OH of one nucleoside and with 5′OH of the other nucleoside. For this, the phosphate group should be activated and functional groups in the nucleotide which do not participate in the

reaction should be permanently or temporary protected[69]. Both of these methods consist of four common steps, deblocking, coupling, deprotection and purification. A comparison of solid phase and liquid phase oligonucleotide synthesis is illustrated in Scheme 2.



Scheme 2. Chemical oligonucleotide synthesis. A) liquid phase synthesis. B) solid phase synthesis[69].

Usually, the primary amino groups of the nucleobases are protected with acyl groups and the 5´OH of group is protected with a 4,4-dimethoxytrityl (DMTr) group. The coupling rection takes place at P atom at III or V oxidation state[70]. There are different approaches used in synthesizing oligonucleotides. They are phosphotriester approach[71], phosphoramidite approach[72] and H-phosphonate approach[73]. Phosphotriester approach is mainly used in liquid phase synthesis while phosphoramidite and H-phosphonate approaches are used in both liquid phase and solid phase synthesis. These different

approaches have some common steps. The common steps are deprotection, coupling and capping.

In deprotection step, the protecting group at 5′OH is removed, making it available for the next coupling reaction. For example, a weak acid is used to remove the DMTr protection. In the coupling step, the reactive 5′OH reacts with the activated monomer unit. In the capping step, uncoupled oligonucleotides with free 5′OH groups are masked by acetylation. This is done to minimize the formation of deletion products. In addition to these three steps, a fourth step, oxidation, is commonly performed in both phosphoramidite and H-phosphonate approaches. This occurs after each capping step in phosphoramidite approach and once after the assembly of the whole chain in the H phosphonate approach[74].

1.2.2.1 Phosphotriester approach for chemical oligonucleotide synthesis

Phosphotriester approach was introduced by Robert Letsinger in 1965[75]. In this method, oligonucleotides are synthesized by directly condensing 3′-O protected nucleoside 5′-phosphates or oligonucleotides having 5′-phosphomonoester group with nucleosides or oligonucleotides having a free 3′ terminal. The coupling forms a phophotriester linkage and after the chain assembly, protection groups are removed to obtain the natural phosphodiester bond. This coupling reaction takes place at P(V), therefore, the oxidation step is avoided which simplifies the coupling cycle[75]. In addition, another advancement of this method was the introduction of DMTr protection at the 5′OH. This method was successful for small scale oligonucleotide production[75,76].

This phosphotriester approach is carried out mainly in liquid medium[77], but methods have been investigated to perform using soluble solid supports and solid phase synthesis[78–80]. There are many advantages of this method. Firstly, the nucleotide building blocks and the phosphotriester intermediates are very stable and hence easy to handle[81]. Secondly, large nucleotides, monomer or dimer units can be used for the synthesis. Thirdly, there is no need to use large excess amount of nucleotide building blocks and lastly, coupling reactions are not affected by the presence of a small amount of water[81]. Limitations of this method are lower yield obtained when compared to phosphoramidite method and that the coupling reaction is very slow[81]. In addition, this method cannot be used to synthesize longer oligonucleotide chains[76] .

1.2.2.2 Phosphoramidite approach

Phosphoramidite approach was introduced in 1980s, which led to the development of automated solid phase synthesis[82]. This approach is known as the gold standard of oligonucleotide manufacturing[64] and it has been used in the oligonucleotide manufacturing industry for more than three decades. The 5′ OH of the nucleoside is protected with a dimethoxytrityl (DMTr) group, and the 3′ position is functionalized with the phosphoramidite moity which consists of a 2-cyanoethyl group and a diisopropylamino group attached to phosphorous (III) centre[83]. In RNA synthesis the 2′ position is also protected with a protecting group such as *tert*-butyldimethysilyl (TBDMS)[84]or tetrahydropyranyl (Thp)[85]. Figure 6 shows the structure of a general phosphoramidite building block.

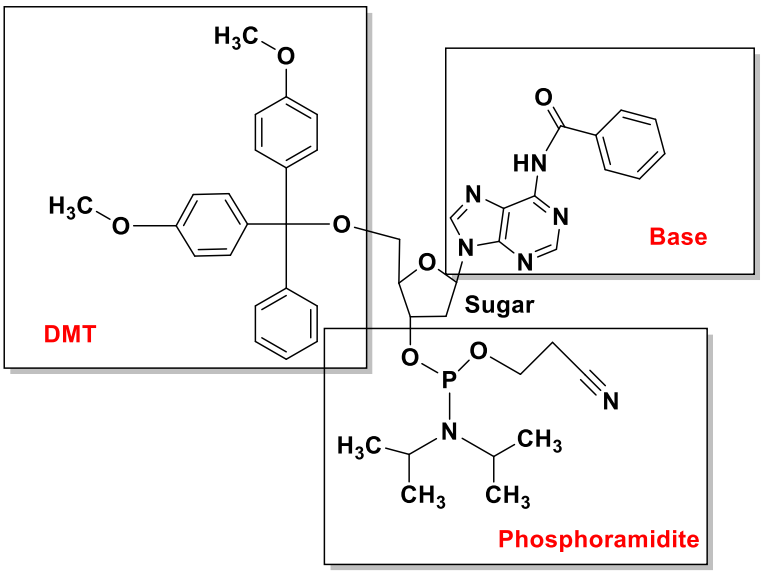
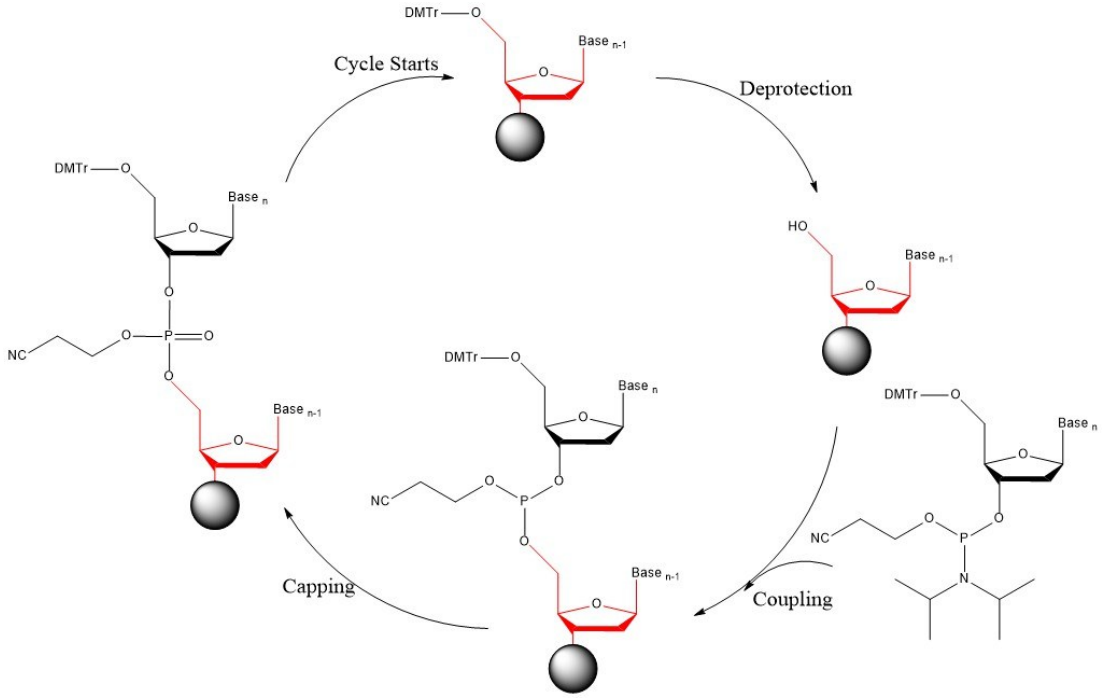


Figure 6. A phosphoramidite building block.

This method involves a four-step chain elongation cycle that successively adds nucleotides to the growing oligonucleotide chain which is bound to a solid support. Scheme 3 presents the four steps in phosphoramidite-based synthesis of oligonucleotides.



Scheme 3. Cyclic process of phosphoramidite-based synthesis of oligonucleotides.

The four steps include 1) DMTr deprotection, 2) coupling, 3) capping and 4) oxidation. After synthesizing the sequence, the oligonucleotide chain is released from the solid support and the protecting groups are removed[86].

In the first step, the dimethoxytrityl protection is removed by adding trifluoroacetic acid (TFA) which activates the 5′ position, allowing chain elongation with the next phosphoramidite monomer. In the second step, the next nucleotide is introduced as a DMTr protected phosphoramidite to the previous nucleotide and coupled with the 5′ OH group, forming a phosphite triester. In the third step, any unreacted 5′ OH groups are capped by acylation to prevent the elongation of sequences with deletion errors. The last step is oxidation, which is done by using iodine solution to convert the formed phosphite triester to a phosphate triester leading to a cyanoethyl protected phosphate backbone. This process is repeated. Once the desired sequence is synthesized, the oligonucleotide chain is released from the solid support and the protecting groups are removed by treating with ammonia solution[86].

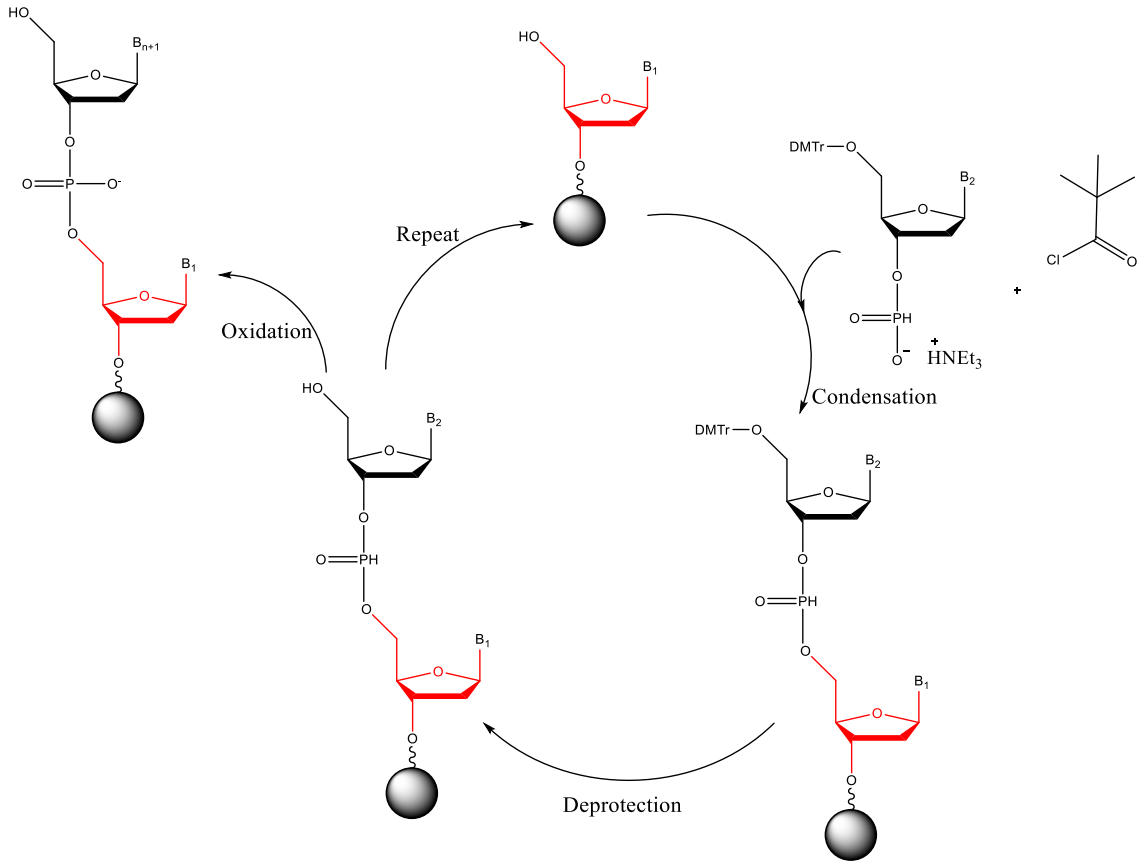
1.2.2.3 H-phosphonate approach

This is an alternative method to phosphoramidite approach where the 3′ OH of the nucleotide is functionalized with an H-phosphonate moiety. This method was first introduced by Sir Alexander Todd and later that method was developed to form H phosphonates which were then used as synthons for solid and liquid phase ON synthesis[87,88].

Scheme 4 illustrates the general steps in ON synthesis using H- phosphonate approach. The first step is the removal of DMTr protection using acid treatment. Second step, condensation, is carried out by introducing a properly 2′ protected ribonucleoside or deoxyribonucleotide H phosphonate monomer and activating it with pivaloyl or adamantoyl chloride. After activation, the H-phosphonate building block undergoes a nucleophilic attack at the phosphorous centre by the 5′OH of a nucleoside bound to a polymer support, forming an H-phosphonate diester[86]. Then oxidation is done to convert the H phosphonate to a phosphodiester using aqueous iodine solution. In contrast to phosphoramidite strategy, oxidation is not a part of the coupling cycle. It is done after the whole oligonucleotide chain is assembled. Finally, ammonia treatment is done to remove the protecting groups and release the ON chain from the polymer support.

In comparison to phosphite triester method, the linkage of H-phosphonate diester is stable under the acidic conditions.

This method is particularly suitable in RNA synthesis and acid labile ON analogues as there can be several problems such as double activation and phosphorous acylation due to the 2′-protecting group[89]. The protection at the 2′ OH position gives rise to steric hindrance and coupling of ribonucleotides on a solid support is less sensitive than coupling with the phosphoramidite method. RNA molecules with 50-60 molecules length can be synthesized in high yields with this method[86].



Scheme 4. Cyclic process of H-phosphonate based synthesis of oligonucleotides.

Modified nucleotides or backbone topologies can be incorporated into an oligonucleotide chain through the three approaches outlined above. Monomeric nucleobases can be added to an abasic site of an oligonucleotide through a process called base filling. Additionally, Dynamic Combinatorial Chemistry (DCC) has enabled screening and incorporation of natural or modified nucleobases into oligonucleotides with modified backbone topologies, facilitating the synthesis of novel oligonucleotide analogues.

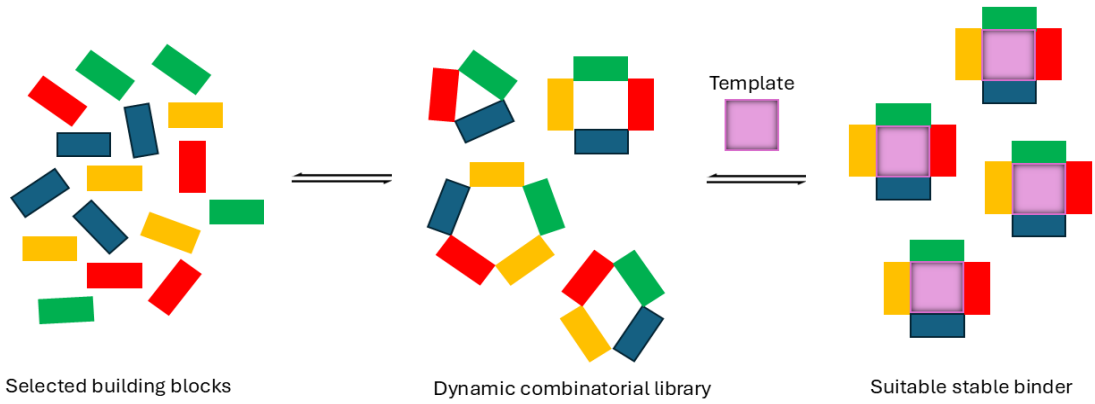
1.3 Dynamic combinatorial chemistry (DCC)

Over the past few decades, different methodologies have been introduced to screen efficient lead compounds for biological targets. Dynamic combinatorial chemistry is an approach in chemical synthesis which is based on reversible reactions to generate libraries of molecules in equilibrium. Unlike traditional combinatorial chemistry, the DCC

approach works under thermodynamic control. Therefore, the library in dynamic combinatorial chemistry is known as a dynamic combinatorial library (DCL)[90].

The unique nature of DCC is that the composition of the library depends on the thermodynamic stability of each member of the library under the conditions used in the experiment. This means that any alteration in experimental conditions can lead to corresponding changes in the library's composition. A DCL will respond to external influences such as pH, temperature or pressure, light and electric field[91]

The purpose of this approach is to allow the selected building blocks (a library) to adapt to an external "template," such as a target molecule. All building blocks contain functional groups that can form reversible bonds to one or more components, allowing the formation of molecules with different affinities for the template. When one or more molecule in this system forms a stable complex with the template, the equilibrium shifts towards the direction of formation of complex, according to the Le Chatelier principle. According to this principle, the compounds which binds strongly or form stable complex with the target will enrich while the week binders will be eliminated[91–93]. Therefore, DCC enables the screening of large numbers of compounds without synthesizing each compound separately.



Scheme 5. Schematic diagram of DCC.

Scheme 5 represents DCC where a selected set of initial building blocks are allowed to react reversibly to form a DCL. When a suitable complex is formed to bind with the biological template, the equilibrium moves towards the formation of that stable complex. Later such stable complexes are isolated by first freezing or controlled termination of the equilibrium reaction. This freezing can be achieved by adjusting the pH, by removing the

catalyst, adjusting the temperature etc. This is done to prevent the formed stable complexes from dissociation during the separation process. After freezing the equilibrium, the stable complexes are separated using primary separation techniques such as HPLC, Size Exclusion Chromatography (SEC) (for protein templated DCC), electrophoresis (for large biomolecules such as proteins and nucleic acids)[94].

In contrast to the traditional combinatorial chemistry, DCC facilitates the access to complex topologies that are challenging to achieve with traditional methods. However, there are some drawbacks in DCC compared to traditional methods. The number of suitable reactions for DCC is limited due to the requirement of reversibility, while traditional methods can utilise many irreversible reactions. Another critical limitation of DCC is the requirement for solubility of all the library members which can lead to kinetic traps if some materials precipitate[95].

1.3.1 Applications of DCC

There are different types of reversible reactions, namely reactions involving covalent bonds and reactions involving non-covalent bonds. Reversible reactions such as transesterification[96], transamidation[97], aldol exchange[90], acetal exchange[98] and Michael reactions[99] are mostly reported as the reactions involving covalent bonds. Meanwhile, metal-ligand coordination[100] and hydrogen bonding[101] were mainly reported under non-covalent reactions. Thus, DCLs can be generated with almost any type of reversible reactions if the final products can be isolated properly and if the equilibrium of the reaction can be controlled by controlling the thermodynamic factors such as pH[90,91].

DCC is a crucial strategy for identifying lead compounds with pharmacological significance. Proteins and nucleic acids are widely used as templates in DCC. Proteins are the most common biological templates in DCC because their binding sites naturally accommodate small molecules which are used in potential drug leads. Therefore, protein-directed DCC has been widely reported in past research, while the applications on nucleic acid-directed DCC are comparatively limited[91].

In protein directed DCC, the small molecules or fragments interconvert through reversible reactions such as disulfide exchange and imine formation. When protein is introduced, only the library members that bind well to its active site or allosteric pocket

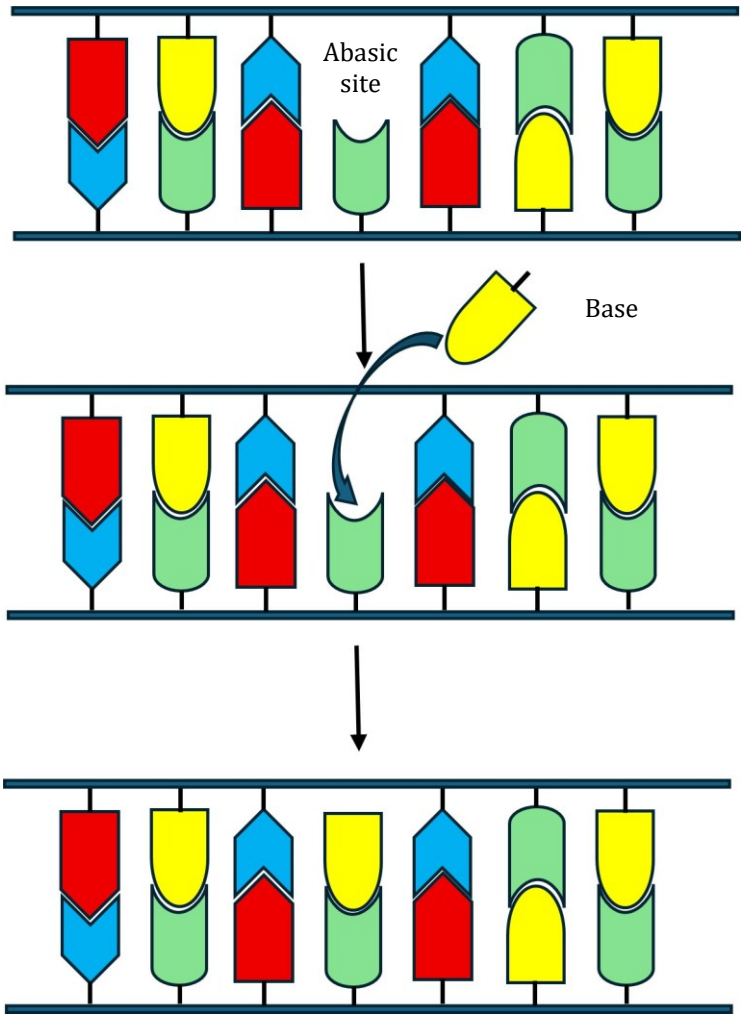
are stabilized. This shifts the equilibrium toward those protein–ligand complexes, effectively amplifying the best binders[102].

Even though nucleic acid templates are underexplored, DNA and RNA have also been successfully targeted with DCC in many studies. Out of them, early studies were carried out with reversible salicylaldimine‑metal complexation interactions where metal ions (Zn^{2+}, Cu^{2+}) were probed to bind with A-T oligonucleotides[103]. In this approach, a DCL composed of rapidly interconverting metal complexes was generated by incubating a series of salicylaldimines with zinc chloride ($ZnCl_2$) in aqueous solution. Zinc(II) was selected as the coordinating metal because of its well-defined tetrahedral coordination geometry with salicylaldimines and its biocompatibility with DNA.

Another application of DCC with nucleic acid templates is finding suitable binders for the G-quadruplex target which is a guanine rich region in genomic DNA. Small molecules having similar size, shape or interactions were used as a DCL to screen the best ligands [104].

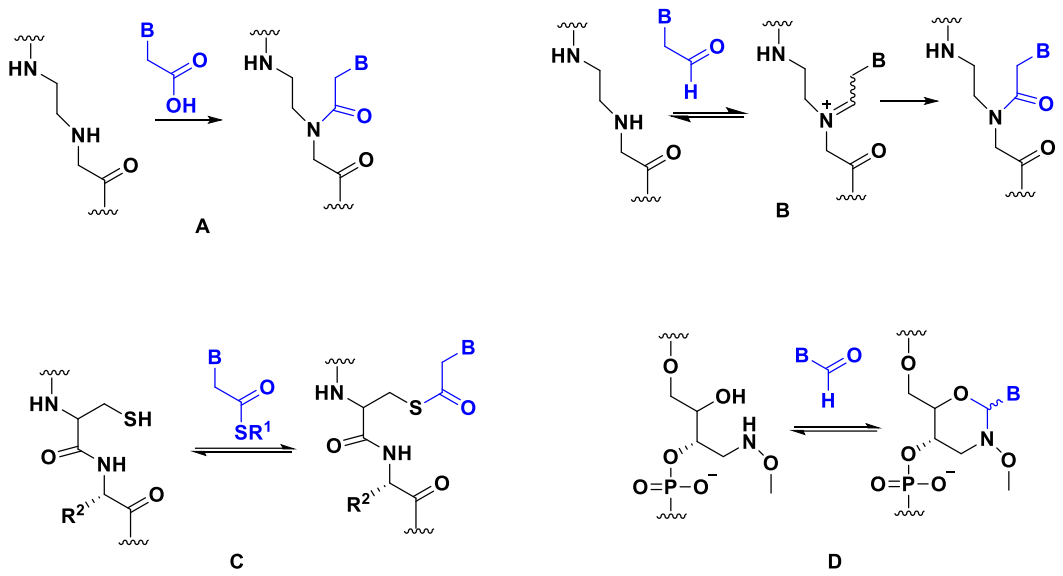
1.3.2 Base Filling

"Base filling" is the introduction of nucleobases or their analogues to abasic sites on the backbone of nucleic acids[105]. Base filling can be a reversible process which allows the use of dynamic combinatorial chemistry. Different backbone modifications such as PNA, N-methoxy-1,3-oxazinane nucleic acid (MOANA)[106] and D-threoninol[107] have been explored for base filling. The main advantage of base filling is that it can be used to test the introduction of different aromatic and aliphatic aldehydes including analogues of canonical bases, modified or natural backbone structures into an oligonucleotide scaffold[106]. Base filling can also be considered as an alternative way to sequential coupling of natural or modified nucleotides. Scheme 6 presents the process of base filling.



Scheme 6. Schematic diagram of base filling.

Natural nucleotides link the base to the sugar phosphate backbone through a N-glycosidic bond. This bond can be formed through N-glycosylation, but this coupling is difficult to carry out in aqueous medium. Therefore, different coupling mechanisms were introduced for the coupling of base moiety to oligonucleotide scaffolds. Some of the successful coupling reactions are peptide coupling to a PNA backbone, reductive amination with a PNA backbone, transthioesterification with a cysteine-containing peptide backbone and oxazinane formation with a (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol backbone[105]. Different coupling reactions for base filling are outlined in scheme 7.



Scheme 7. Different coupling reactions for base filling. A) peptide coupling to a PNA backbone, B) Reductive amination with a PNA backbone, C) transthioesterification with a cysteine containing peptide backbone, (D) oxazinane formation with a (2R,3S)-4-(methoxyamino)butane-1,2,3,-triol backbone[105].

Apart from peptide coupling, reactions outlined in scheme 7 are coupling reactions that are at an equilibrium under certain conditions. For example, a study carried out with the incorporation of (2R,3S)-4-(methoxyamino)butane-1,2,3-triol unit to oligonucleotides as post synthetic derivatization with different base moities, forming a N-methoxyoxazinane ring which is reversible at pH 5 and irreversible at pH 7[106,108]. Reversible base filling reactions have gained attention in the field as they allow the use of DCC and the reversibility shows a higher fidelity as the equilibrium can shift towards the matched Watson-Crick base pair[105].

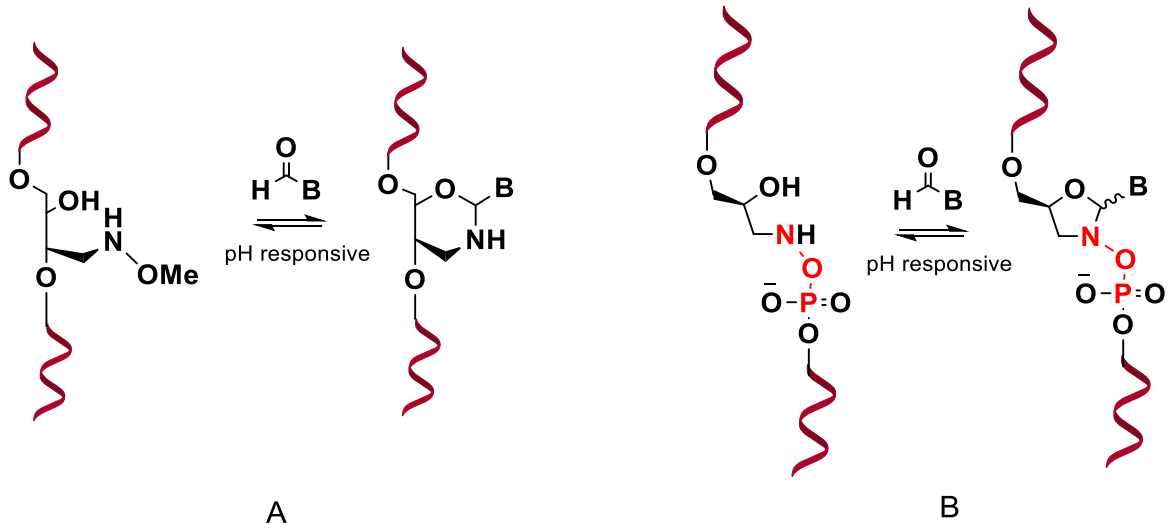
1.4 Significance of the study

The objective of this research was to synthesize a base filling scaffold having a minimal structure, which would be geometrically more compatible with a DNA double helix than previously reported ones.

A previous study has been carried out on nucleoside analogues featuring a 1,3- oxazinane ring in place of the sugar which was used for the base filling approach. Here, the

phosphitylation was done forming a C-O-P linkage similar to that of a conventional nucleoside analogue[109].

In contrast to the above mentioned study, this research aimed at synthesizing a novel building block featuring an 1,3-ozazolidine ring in place of sugar and an unusual N-O-P linkage upon phosphitylation. Scheme 8 presents the comparison between the previously reported base filling scaffold named N-methoxy-1,3-oxazinane nucleic acid and the newly synthesized base filling scaffold in this study.



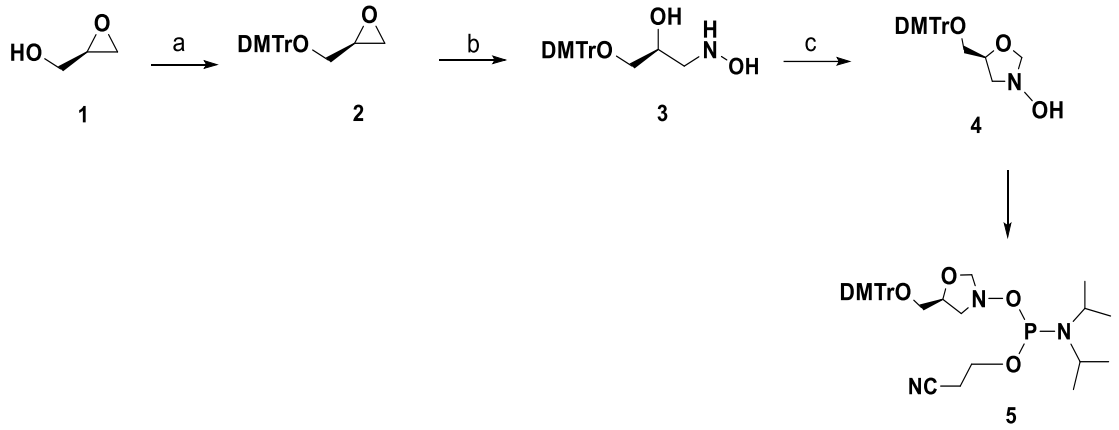
Scheme 8. Reversible attachment of the base to A) previously reported modified scaffold [109], B) novel building block featuring 3-(hydroxyamino)propane-1,2-diol scaffold with the unusual N-O-P linkage.

After the synthesis of the modified building block, it will be incorporated to an oligonucleotide chain. This oligonucleotide chain can be used in dynamic combinatorics. In other words, different natural or modified bases can be introduced to this oligonucleotide which will open up new possibilities. The aim of this research was to investigate the properties of the modified nucleotide with the N-O-P linkage and study the compatibility of the base filling scaffold to DNA double helix, rather than to improve the coupling yield of the oligonucleotide synthesis.

2 Results and discussion.

2.1 Synthesis of a methylene-protected phosphoramidite building block

The route for the synthesis of the methylene-protected phosphoramidite building block **5** is presented in scheme 9.



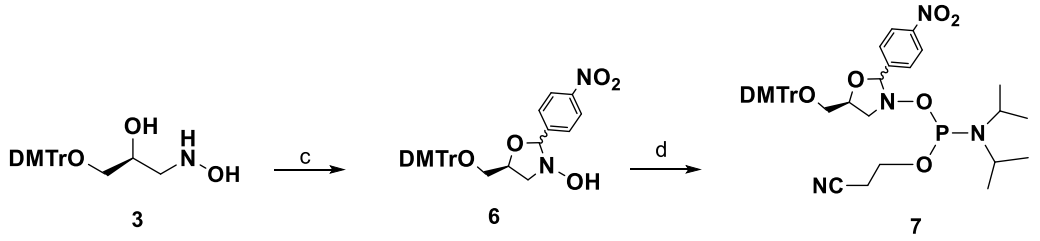
Scheme 9. Synthetic route for building block **5**. Reagents and conditions: (a) DMTrCl, CH_2Cl_2, DIPEA, room temperature, overnight; (b) hydroxylamine, DIPEA, methanol, dropwise addition of compound **2**, 40 °C, overnight; (c) formaldehyde, room temperature, overnight.

Commercially available glycidol was used as the starting material and the OH group was protected with the DMTr by a previously reported protocol[110], affording compound **2**. Then the epoxide ring was opened using hydroxylamine as the nucleophile and N,N-diisopropylethylamine as a non- nucleophilic base. First the reaction was carried out at room temperature, but it was found out that the reaction had not completed. Therefore, this reaction mixture was stirred at 40 °C overnight to obtain compound **3**. To a separate round bottom flask hydroxylamine hydrochloride, methanol and N,N-diisopropylethylamine were added and stirred well until all the hydroxylamine hydrochloride was dissolved, after which a solution of compound **2** in dichloromethane was added dropwise while stirring over 1 hour. Product formation was confirmed with LC-MS. The crude product **3** was treated with formaldehyde overnight to obtain compound **4**. The formation of compound **4** was tested with TLC, LC-MS and NMR. TLC, LC-MS and NMR profiles were all very complicated due to the presence of many compounds. The reason for this could be the high reactivity of compound **3** towards aldehydes present in the atmosphere and as contaminants in solvents, such as

formaldehyde and acetaldehyde [106]. After several purification rounds, the obtained yield of compound **4** was insufficient to proceed with the phosphitylation step. Therefore compound **5** could not be synthesized.

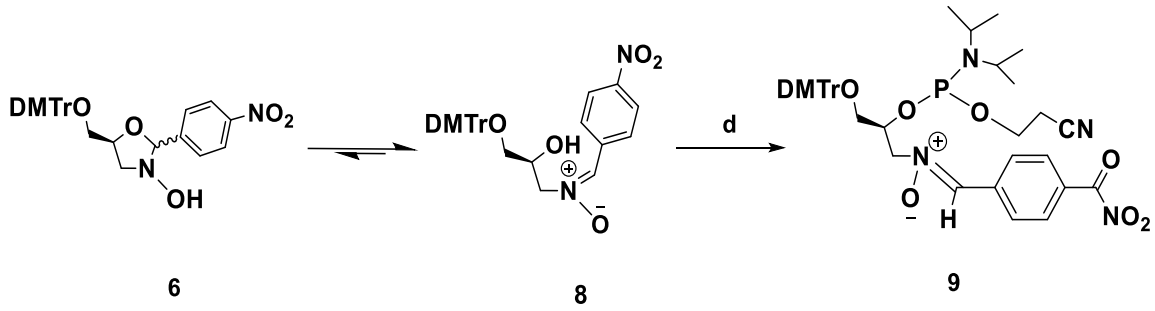
2.2 Synthesis of a p-nitrobenzylidene-protected phosphoramidite building block

The route for the synthesis of p-nitrobenzylidene-protected phosphoramidite building block **7** is presented in scheme 10.



Scheme 10. Synthetic route for building block **7**. Reagents and conditions: (c) 4-nitrobenzaldehyde, methanol, 2 d, room temperature; (d) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, dry triethylamine, N_2 atmosphere, room temperature, 40 min.

Compound **3** was synthesized according to the method described in chapter 2.1. Since compound **3** is very reactive, it was not isolated or characterised but instead treated immediately with 4-nitrobenzaldehyde to obtain compound **6**. Dry methanol was used as the solvent and the reaction mixture was stirred for two days to complete the reaction. After many purifications rounds, compound **6** was obtained but the purity was still less than 80%. Then compound **6** was phosphitylated by conventional methods and the product mixture analyzed by NMR spectroscopy. According to the spectra, compound **9** was obtained instead of the expected compound **7**. The disappearance of the 5.71 ppm signal in the ^{1}H NMR spectrum indicated opening of the oxazolidine ring and the ^{31}P signal at 150.09 ppm indicated that phosphitylation had taken place at a C-O-H position as this value is similar to those of conventional phosphoramidite building blocks. Taking ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, COSY and HMBC spectra into consideration, the following mechanism (Scheme 11) can be proposed.

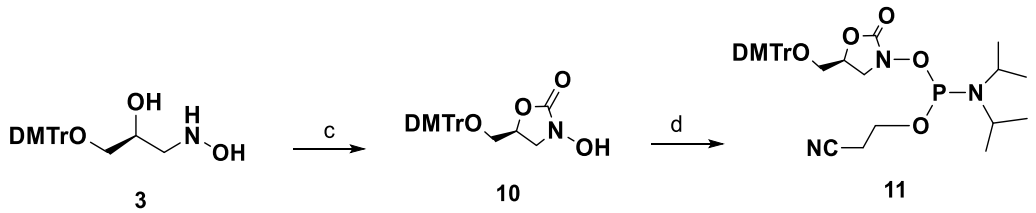


Scheme 11. Phosphitylation of compound **6**. Reagents and conditions: d) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, dry triethylamine, N_2 atmosphere, room temperature, 40 min.

Therefore, this synthetic route failed to deliver the desired building block.

2.3 Synthesis of a carbamate-protected phosphoramidite bulding block

The route for the synthesis of the carbamate-protected phosphoramidite building block **11** is presented in Scheme 12.



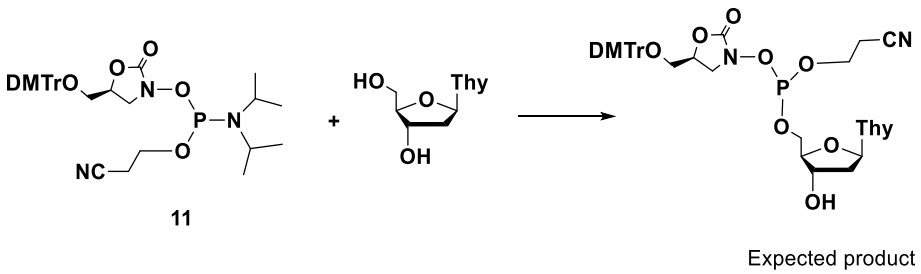
Scheme 12. Synthetic route for building block **11**. Reagents and conditions: (c) dry 1,4-dioxane, dry pyridine, dry *N*,*N*-diisopropylethylamine, 4-nitrophenylchloroformate, room temperature, overnight; (d) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, dry triethylamine, N_2 atmosphere, room temperature, 1 h.

Compound **3** was dissolved in a mixture of dry 1,4-dioxane, N,N-diisopropylethylamine and dry pyridine. Pyridine was used as a solvent to maintain the basic medium. Once compound **3** is completely dissolved in the above reagent mixture, 4-nitrophenylchloroformate was added to form the carbamate protection to compound **3**. After many purifications rounds compound **10** was obtained. The phosphoramidite building block **11** was synthesized by phosphitylation of compound **10**. This compound was dissolved in dry toluene and divided it into two portions. One portion was tested with oligonucleotide synthesizer, and the other portion with solution-phase coupling to a nucleoside.

2.4 Coupling of compound 11

The first portion was added to a solid support. Since the amount of compound **11** was too small to be used in the automated oligonucleotide synthesizer, the activation, capping and oxidation steps were done manually. Compound **11** was incorporated into the middle of a 7 mer oligonucleotide using automated oligonucleotide synthesizer. The sequence was TTTXTTT, where T is the thymidine phosphoramidite and X is the phosphoramidite building block **11**. According to trityl response, coupling of compound **11** failed.

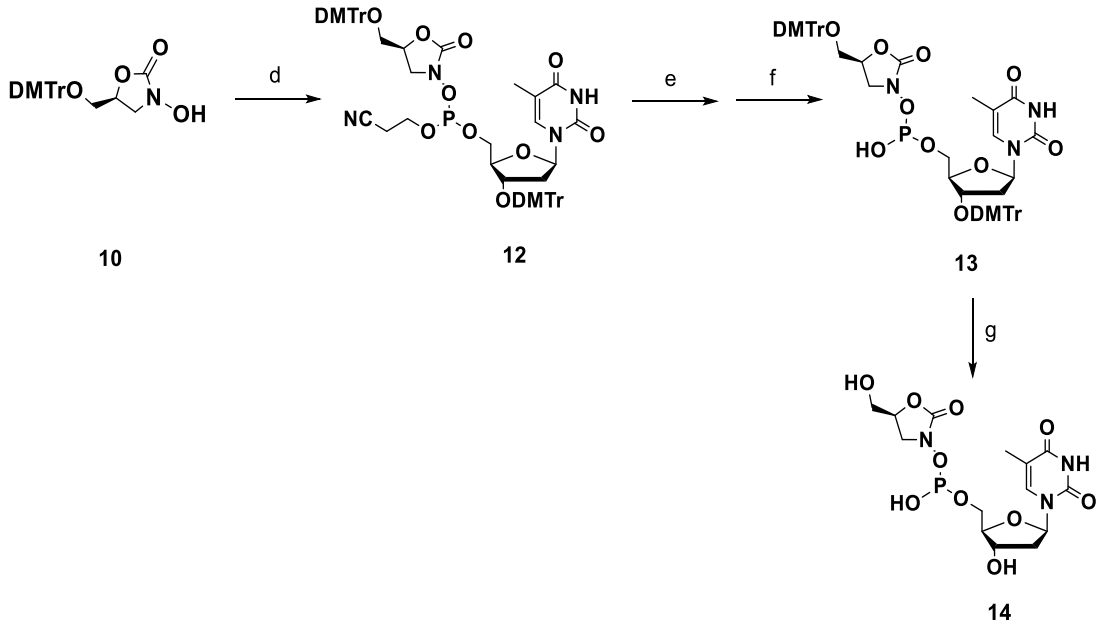
The second portion was treated with 3'-O-TBDMS-thymidine and the activator was added. The reaction was monitored by ^{31}P NMR but no change in the spectrum was observed. In other words, the ^{31}P NMR chemical shift value of 3.07 ppm obtained for compound **11** remained unchained even after coupling. This confirmed the unreactivity of compound **11** towards coupling. Scheme 13 illustrates the intended reaction.



Scheme 13. Coupling reaction of compound **11** with 3′-*O*-TBDMS-thymidine. Reagents and conditions: CD_3CN, Hyacinth BMT activator (0.3M 5-Benzylmercaptotetrazole in anhydrous acetonitrile).

2.5 Coupling of the carbamate-protected hydroxylamine 10 with a nucleoside building block

The route for the synthesis of the phosphite diester **14** is presented in scheme 14.



10 12 13 14

Scheme 14. Synthesis of compound **14**. Reagents and conditions: (d) dT-5-CE phosphoramidite, ACN, Hyacinth BMT activator (0.3M 5-Benzylmercaptotetrazole in anhydrous acetonitrile) , N_2 atmosphere, room temperature; (e) oxidizer reagent (0.05M I_2 in water and pyridine); (f) 25% aqueous NH_3, room temperature, overnight; (g) 3% trifluoracetic acid in dichloromethane.

Compound **10** was synthesized according to the methods described above. Since the phosphoramidite building block **11** did not couple to the oligonucleotide chain, an alternative method was tested by coupling compound **10** with 3′-***O***-DMTr-thymidine to obtain a dimer with the same N-O-P linkage. Successful dimer formation indicates the ability of this building block to be incorporated to an oligonucleotide chain or used as a model compound for base filling on its own.

dT-5'-CE phosphoramidite was dissolved in deuterated acetonitrile and it was treated with compound **10**. This reaction was done under inert atmosphere to avoid oxidation and hydrolysis. Then the activator solution (0.3M Hyacinth BMT activator) was added to the phosphoramidite building block to protonate which facilitates nucleophilic attack of the N-OH in compound **10**, forming an unstable phosphite triester linkage[111]. Coupling of compound **10** with the dT-5'-CE phosphoramidite was successful leading to compound **12**.

Phosphite triester **12** was then treated with 0.05 M I_2 in a mixture of water and pyridine to oxidize it to the phosphate triester **15** (Figure 7). This mixture was added until the

brown colour remained, at which point the reaction solution was analyzed with ^{31}P NMR. The chemical shift value of ^{31}P NMR obtained was -6.19 ppm which was different from a conventional phosphoramidite building block which is usually around 150 ppm. Deviation of the ^{31}P NMR value of compound **12** could be due to the formation of the new N-O-P linkage. The product formation was next tested with UPLC-MS.

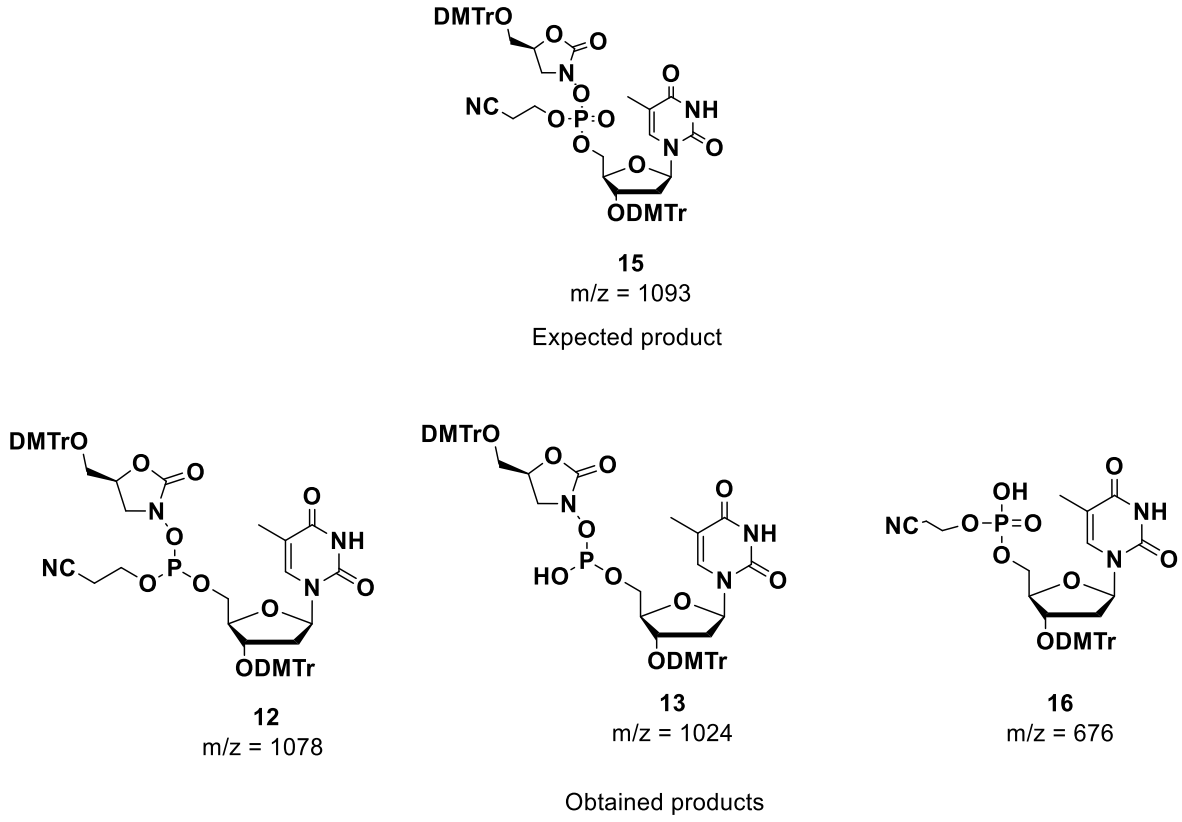


Figure 7. The expected product (Compound **15**) and obtained products (compound **12**,**13** and **16**) after oxidation.

Instead of the expected *m/z* (1093), values of 1078, 1024 and 676 were detected, consistent with compounds **12**, **13** and **16**.

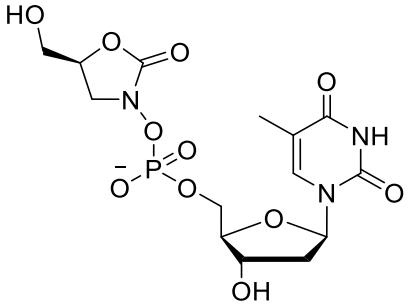
In other words, compound **12** appeared unusually resistant to oxidation under the typical conditions. Compound **13** would be formed by cleavage of the cyanoethyl protecting group. Cyanoethyl group is a base labile group which is normally cleaved by ammonia treatment. But during oxidation, either the cyanoethyl protection had been removed to some extent or compound **13** could be formed during negative ionization in MS.

Compound **16** was obtained by oxidation of thymidine cyanoethyl phosphite, a hydrolysis product of the dT-5′-CE phosphoramidite which used in excess. This indicates that compound **12** is much more difficult to oxidize than normal phosphite triesters or diesters. Stronger oxidizing conditions such as peroxides can be tested to oxidize compound **12**.

NH_3 treatment was done after the oxidation step to remove the remaining cyanoethyl protection. Compound **12** was dissolved in a mixture of methanol, acetonitrile and water. Then NH_3 solution (25% NH_3) was added, and the reaction mixture was stirred at room temperature for overnight. Usually, this NH_3 treatment is a fast reaction which is complete within few minutes. But compound **12** showed less reactivity towards the NH_3 treatment. A small portion of this reaction mixture was withdrawn and treated with more oxidizer solution to find out whether compound **13** would be oxidized more easily than compound **12**, but there was no change in the oxidation state of phosphorous centre.

Cyanoethyl protection was surprisingly difficult to remove even with a prolonged reaction time.

Finally, the acid labile DMTr protection was removed by treating with 3% trifluoroacetic acid (TFA) in dichloromethane. Compound **14** was obtained as a precipitate, and it was separated by centrifugation. The supernatant was neutralized using Et_3N and another precipitate was obtained. Both precipitates were analysed with HPLC-MS.



17
m/z = 436

Figure 8. Expected product after TFA treatment.

According to LC-MS data compounds having m/z values $[M–H]^-$ of 420, 374 and 321 were obtained. Figure 9 illustrates the compounds corresponding to those masses.

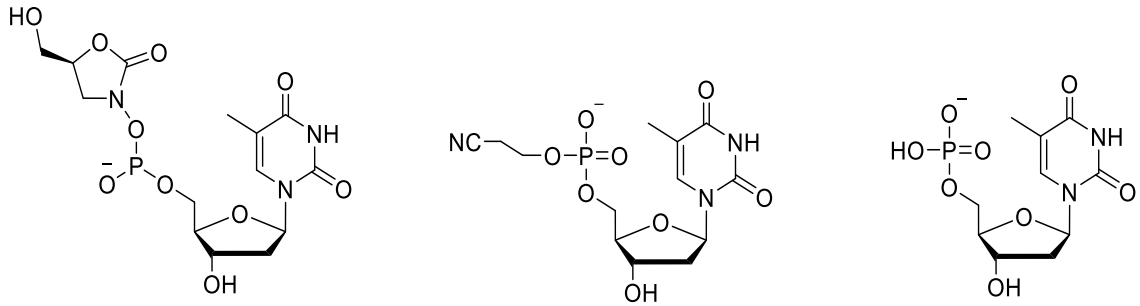
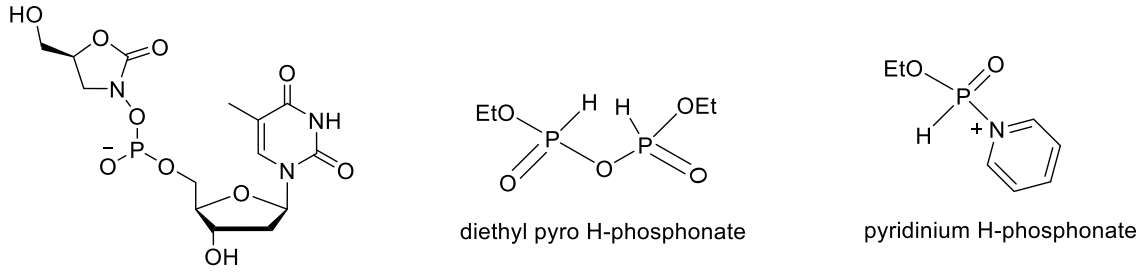


Figure 9. Compounds obtained after TFA removal.

Compound **14** was the expected product, most likely an H-phosphonate diester. Compound **14** was purified by reverse phase HPLC (RP-HPLC) and characterized by NMR spectroscopy.

According to ^{1}H NMR, all the expected peaks were present except the one corresponding to the P-O-H proton. Therefore, the sample in the NMR tube was freeze dried overnight and dissolved in DMSO-d_6 and ^{1}H NMR spectrum was measured again. However, signals of the exchangeable protons were still absent. But the molecular weight was a perfect match with compound **14**.

The ^{31}P NMR chemical shift of compound **14** was -6.19 ppm. However, phosphite diesters and triesters have different chemical shift values. In general, the chemical shift values range from -15 to + 30 ppm. Studies show that some H-phosphonates have a chemical shift value similar to that of compound **14**. For example, diethyl pyro H-phosphonate showed a ^{31}P NMR chemical shift at -3 ppm and pyridinium H-phosphonate at -2 ppm [112]. Finally, this compound was found to be surprisingly stable towards oxidation and hydrolysis. Efforts to find appropriate reagents and reaction conditions for oxidation of compound **14** are ongoing.



14

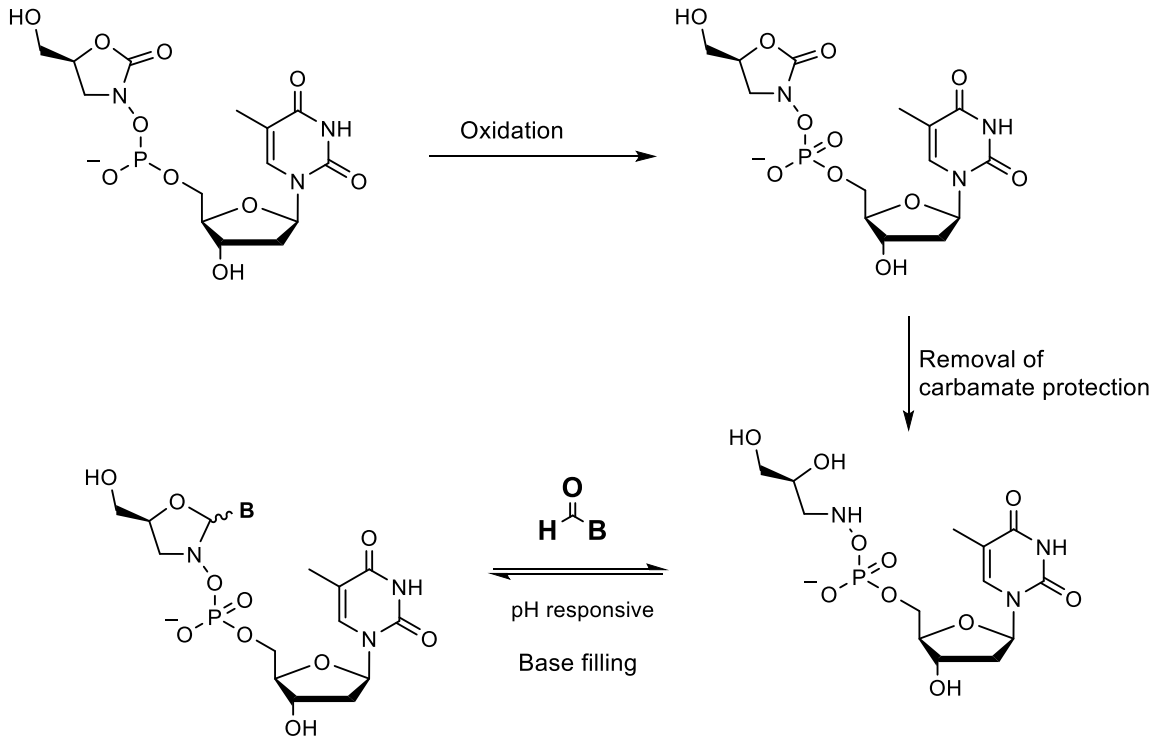
diethyl pyro H-phosphonate

pyridinium H-phosphonate

Figure 10. Comparison of the compound **14** with diethyl pyro H-phosphonate and pyridinium H-Phosphonate.

2.6 Future work

First, compound **14** needs be oxidised to make it more stable and then the carbamate protection should be removed to obtain the desired model compound. To introduce a nitrogenous base, base filling approach is to be used. The following scheme 15 represents further steps to be developed to obtain the monomeric building block for automated oligonucleotide synthesis.



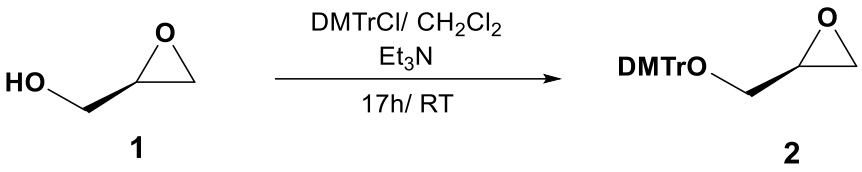
Scheme 15. Steps to be carried out to synthesize the final product.

3 Experimental Methods

3.1 General methods

Commercially available chemicals and solvents were used for the synthesis. Et_3N was dried over CaH_2 and solvents over activated 4 Å molecular sieves. NMR spectra were recorded on a Bruker Biospin 500 MHz NMR spectrometer and mass spectra on an Agilent 6120 LC-MS spectrometer. Reaction progress was monitored by Thin Layer Chromatography (TLC). Purification was done mainly using silica gel column chromatography. RP-HPLC was used to purify the H-phosphonate diester **14**.

3.1.1 (S)-(4,4´-dimethoxytrityl)glycidol (2)



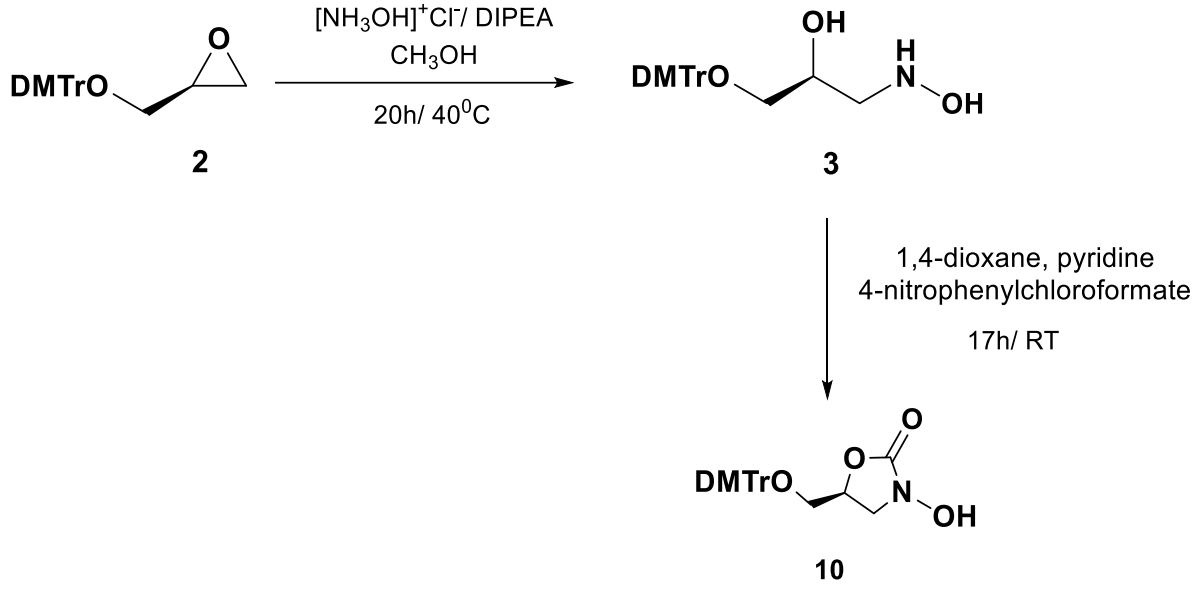
1 2

To a solution containing (R)-(+) glycidol (**1**, 1 mL, 15.1 mmol) and dry Et_3N (5.4 mL, 40.7 mmol) in CH_2Cl_2 (34 mL), dimethoxytritylchloride (6.4584 g, 19.0 mmol) was added and the mixture was stirred overnight at room temperature. Next day the reaction mixture was diluted with CH_2Cl_2 (100 mL), and extracted with 5% $NaHCO_3$ (3×100 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvents were evaporated to dryness. The residue was purified using silica gel column chromatography (CH_2Cl_2: EtOAc: Et_3N =84:15:1, v/v) yielding 2.8791 g (50.65%) of compound **2** as a colourless oil.

^{1}H-NMR (500 MHz, $CDCl_3$) : δ 7.48 (d, J = 7.4 Hz, 2H, aromatic H_7), 7.37 (d, J = 8.9 Hz, 4H, aromatic H_4), 7.31 (t, J = 7.6 Hz, 2H, aromatic H_8), 7.24 (d, J = 7.2 Hz, 1H, aromatic H_9), 6.86 (d, J = 8.9 Hz, 4H, aromatic H_5), 3.82 (s, 6H, H_6), 3.34 (dd, J = 2.6, 10.3 Hz, 1H, H_3), 3.15 (m, 2H, H_2), 2.80 (t, J = 4.6 Hz, 1H, H_1), 2.65 (dd, J = 2.6, 5.1 Hz, 1H, H_1).

^{1}H NMR spectrum of compound **2** is included in appendix 1 and COSY of compound **2** is included in appendix 2.

3.1.2 (S)-1-(4,4´-dimethoxytrityloxy)-3-(hydroxyoxazolidine-2-one) (10)



Hydroxylamine hydrochloride (2.237 g, 0.0321 mol) was dissolved in methanol (50 mL). To this, *N*,*N*-diisopropylethylamine (12.0 mL, 0.0643 mol) was added and the resulting mixture was stirred well until the hydroxylamine hydrochloride was completely dissolved. In a separate flask, a portion of compound **2** (1.211 g, 0.00321 mol) was dissolved in 4 mL of CH_2Cl_2. This solution was added dropwise over 1 h to the above mixture while stirring. This reaction was stirred at 40 ºC overnight. Next day, the reaction mixture was evaporated to dryness, and the crude product mixture was dissolved in CH_2Cl_2 (50 mL) and washed with 5% $NaHCO_3$ (3×50 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness, affording a mixture containing compound **3**. Compound **3** was dissolved in a mixture of dry 1,4-dioxane (20 mL) and dry pyridine (10 mL). Then *N*,*N*-diisopropylethylamine (12.0 mL, 0.0688 mol) was added and stirred well until compound **3** was completely dissolved. To this mixture, 4-nitrophenylchloroformate (0.832 g, 4.131 mmol) was added and the reaction mixture was stirred overnight at room temperature. Next day the solvent was partially evaporated and the remaining solution diluted with CH_2Cl_2 (50 mL) and washed with 5% $NaHCO_3$ (3×50 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The residue was purified using silica gel column chromatography three times eluting with three different solvent systems ((CH_2Cl_2: Methanol: Et_3N = 98:1:1, v/v), (CH_2Cl_2: Methanol: Et_3N = 96:3:1, v/v), (EtOAc: hexane: Et_3N = 70:30:1, v/v), respectively). After these three

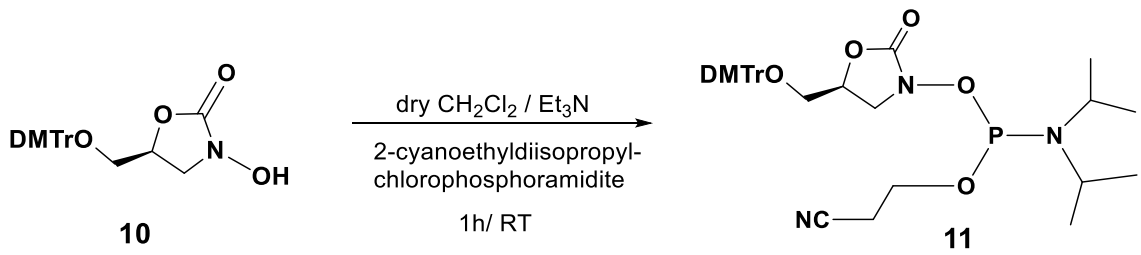
purification rounds, the product was further dried by coveporation from dry toluene (2.5 mL). Compound **10** was obtained (0.0492 g, 91.8 μmol).

^{1}H-NMR (500 MHz, $CDCl_3$) : δ 7.45 (d, *J* = 7.2 Hz, 2H, aromatic H_{13}), 7.33 (d, *J* = 9.0 Hz, 4H, aromatic H_8), 7.30 (t, *J* = 5.1 Hz, 2H, aromatic H_{14}), 7.23 (t, *J* = 7.3 Hz, 1H, aromatic H_{15}), 6.83 (q, *J* = 5.0 Hz, 4H, aromatic H_9), 4.60 (m, 1H,H_4), 3.79 (s, 6H,H_{11}), 3.76 (t, *J* = 7.9 Hz, 1H,H_5), 3.64 (t, *J* = 8.0 Hz, 1H, H_5), 3.40 (dd, *J* = 4.1, 10.7 Hz, 1H,H_3), 3.28 (dd, *J* = 4.8, 10.7 Hz, 1H, H_3).

^{13}C-NMR (126 MHz, $CDCl_3$) : δ 160.79 (C_1), 158.53 (aromatic C_{10}), 144.53 (aromatic C_{12}), 135.50 (aromatic C_7), 129.85 (aromatic C_{14}), 127.82 (aromatic C_8), 126.98 (aromatic C_{15}), 113.59 (aromatic C_9), 86.27 (C_6), 72.26 (C_4), 63.23 (C_5), 55.33 (C_{11}), 50.13 (C_3).

^{1}H NMR, ^{13}C NMR, COSY and HSQC spectra of compound **10** are included in appendix 3, 4, 5 and 6 respectively.

3.1.3 (S)-5-[(4,4´-Dimethoxytrityloxy)methyl]-2-oxooxazolidin-3-yl (2-cyanoethyl) diisopropylphosphoramidite (11)



Compound **10** (0.1772 g, 0.407 mmol) was dissolved in dry toluene (10 mL) and evaporated to dryness. This was repeated three times. To the dry compound **10**, dry CH_2Cl_2 (4 mL) was added, and the mixture was stirred until compound **10** had completely dissolved. Then dry Et_3N (283.5 μL, 2.03 mmol) was added. Finally, 2-cyanoethyldiisopropylchlorophosphoramidite (109 μL, 4.88 mmol) was added and the resulting mixture was stirred for 1 h at room temperature under inert (N_2) atmosphere.

After 1 h, the reaction mixture was taken from the N_2 chamber, diluted with CH_2Cl_2 (30 mL), and washed with 5% $NaHCO_3$ (3× 30 mL). The organic layer was collected, dried

over anhydrous Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel column chromatography (ethyl acetate: hexane: Et_3N = 69:30:1, v/v), affording compound **11** (44.4 mg) (Yield percentage 17.17%).

^{1}H-NMR (500 MHz, $CDCl_3$) : δ 7.49 (d, *J* = 8.7 Hz, 2H, aromatic H), 7.41 (m, 4H, aromatic H), 7.31 (m, 2H, aromatic H), 7.24 (m, *J* = 2.6 Hz, 1H, aromatic H), 6.86 (m, 4H, aromatic H), 4.68 (m, 1H, H_7), 3.96 (t, *J* = 9.2 Hz, 1H, H_3), 3.81 (s, 6H, H_{15}), 3.67 (q, *J* = 5.5 Hz, 1H, H_9), 3.55 (m, 2H, H_8), 3.32 (qd, *J* = 5.0, 61.5 Hz, 2H, H_2), 2.83 (m, 2H, H_4), 1.24 (dd, *J* = 6.8, 58.2 Hz, 12H, H_1).

^{13}C-NMR (126 MHz, $CDCl_3$) : δ 158.65 (C14), 144.28 (C6), 135.70 (C in benzene ring), 130.02 (C in benzene ring), 127.94 (C in benzene ring), 126.89 (C in benzene ring), 116.81 (C5), 113.33 (C in bezene ring), 110.09 (s, 1C), 86.45 (C10), 69.07 (C7), 65.59 (C9), 60.72 (C3), 57.25 (C15), 55.26 (C2), 46.59 (C8), 22.48 (C1), 19.70 (C4).

^{31}P NMR (202 MHz, $CDCl_3$) : δ 3.07.

^{1}H NMR, ^{13}C NMR, ^{31}P NMR, COSY, HSQC and HMBC spectra of compound **11** are included in apendix 7, 8, 9, 10, 11 and 12 respectively.

3.1.4 Oligonucleotide synthesis

Compound **11** (44.4 mg,0.0699 mmol) was dissolved in dry toluene (10 mL) and divided into two portions (5 mL).

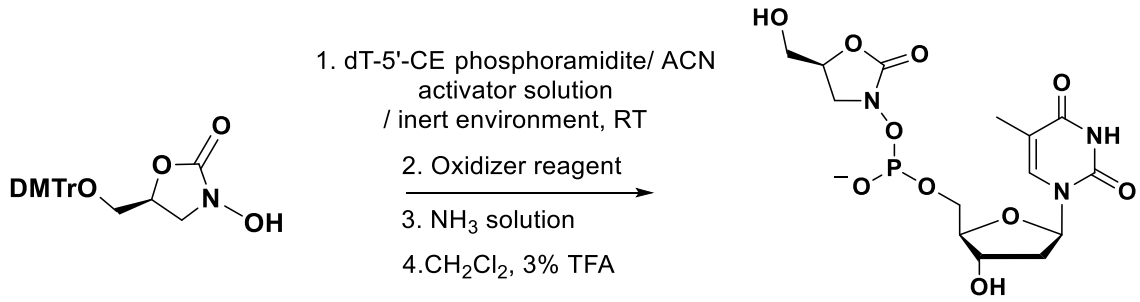
To the 1st portion, Ac-dC-CPG solid support (30 mg) and acetonitrile (160 µL) were added. Then the activator solution, Hyacinth BMT (0.3M 5-benzylmercaptotetrazole in anhydrous acetonitrile) (160 µL) was added. The mixture was shaken occasionally over 10 min and the solid support was recovered by filtration. Capping was done by mixing capping solution B1 (40% acetic anhydride in acetonitrile) (1 mL) and capping solution B2 (60% 2,6-Lutidine, 40% acetonitrile) (1 mL) together and mixing it with capping solution A (20% 1-methylimidazole in acetonitrile) (2 mL). This capping solution was passed through the activated compound **11** three times. After the capping step, compound

11 was treated with the oxidizer solution (0.05M I_2 in water and pyridine) (2 mL). Finally, excess solvents were removed by rinsing with dry acetonitrile using a vacuum pump.

Then compound **11** was coupled to a oligonucleotide chain TTTXTTT using the automated oligonucleotide synthesizer where T is thymidine phosphoramidite and X is Compound **11**. First three T´s coupled using the oligonucleotide synthesizer and then coupled with the compound **11** and then coupled with another three T´s to synthesize the desired oligonucleotide chain.

The 2nd portion of compound **11** in dry toluene (0.0124 mmol) was treated with 3'O-TBDMS- thymidine (4.4 mg, 0.0124 mmol). The mixture was dissolved in dry toluene and evaporated to dryness three times. The residue was then dissolved in deuterated acetonitrile and the ^{31}P NMR spectrum was measured. No change in ^{31}P NMR was observed. Therefore, activator solution (100 µL) was added to the reaction mixture and ^{31}P NMR spectrum was measured again immediately after the addition of the activator solution, after 2 hours, 1 day and 3 days. No change in the ^{31}P NMR spectrum was observed.

3.2 (S)-5-[(4,4´-Dimethoxytrityloxy)methyl]-2-oxooxazolidin-3-yl thymidine-5´-yl phosphite (14)



10 **14**

Compound **10** (32 mg, 0.074 mmol) was dried by coevaporation from dry toluene three times. To dry compound **10**, commercially available dT-5'-CE phosphoramidite building block (82.3 mg, 0.11 mmol) dissolved in deuterated ACN (750 µL) was added. This reaction was carried out in an inert atmosphere (in a N_2 chamber). Once the solid reactants had completely dissolved, activator solution (735.3 µL, 0.22 mmol) was added and the

resulting mixture stirred for a few minutes at room temperature. Then this solution was taken out from the N_2 chamber and treated with the oxidizer solution (0.05 M I_2 in pyridine and water 90:10 (V/V))(1mL) until the brown color remained. After a few minutes this reaction mixture was diluted with CH_2Cl_2 (20 mL) and washed with 0.2 M $Na_2S_2O_3$ solution ($3\times 20\,mL$). The organic layer was collected, dried over anhydrous Na_2SO_4 and evaporated to dryness.

The residue was then dissolved in aqueous NH_3 solution (200 µL). Since the compound did not dissolve completely, ACN (1 mL) was added. This reaction mixture was stirred overnight at room temperature.

A small portion (100 µL) of the reaction mixture was withdrawn and treated with the oxidizer solution (100 µL) and kept overnight at room temperature.

Next day, both samples were evaporated to dryness and analyzed by LCMS. Neither sample had oxidized. Therefore, they were combined and dissolved in CH_2Cl_2 (2 mL). This sample was treated with 3% TFA in dichloromethane solution and shaken. Upon shaking a precipitate was formed. This precipitate was separated from the supernatant by centrifugation. The supernatant was neutralized with Et_3N. Another precipitate was observed after neutralization with Et_3N. This precipitate was also separated using centrifugation.

Both the precipitates were dissolved in water (300 µL) and purified using RP-HPLC chromatography. The RP-HPLC separation was carried out using a Hypersil ODS C18 column (250×4.6 mm, 5 µm) and a 260 nm detection wavelength. In RP-HPLC method a binary solvent system was used with acetonitrile and ammonium formate buffer solution with a flow rate of 3 mL/min. The method started with 2% can, increased to 30% within 15 minutes, dropped down to 2% at 20 minutes and remained at 2% until 30 minutes. All the eluted fractions were collected and analyzed by LCMS and NMR spectrometry.

Compound **14** was obtained.

^{1}H-NMR (500 MHz, D_2O) : 7.65 (d, J = 1.2 Hz, 1H, H_3), 6.26 (m, 1H, H_{12}), 4.70 (m, 1H, H_6), 4.47 (m, 2H, H_9), 4.06 (m, 1H, H_8), 3.87 (t, J = 9.4 Hz, 1H, H_{10}), 3.87 (t, J = 9.4 Hz, 1H, H_{10}), 3.75 (m, 2H, H_{14}), 3.58 (m, 2H, H_{11}), 2.29 (m, 2H, H_7), 1.84 (s, 3H, H_5).

^{13}C-NMR (126 MHz, D_2O) : 166.18 (C_1), 158.96 (C_2), 151.96 (C_{13}), 137.05 (C_3), 111.77 (C_4), 85.12 (C_{12}), 75.64 (C_6), 71.01 (C_8), 61.87 (C_{10}), 46.74 (C_{11}), 38.84 (C_7), 11.29 (C_5).

^{31}P NMR (202 MHz, D_2O) : δ -6.19 ppm.

^{1}H NMR,^{13}C NMR, ^{31}P NMR, COSY, HSQC and ^{31}P HMBC spectra of compound **14** are included in apendix 13, 14, 15, 16, 17 and 18 respectively.

4 Conclusion

Different synthetic routes were explored to synthesize a 3-(hydroxyamino)propane-1,2-diol building block which is to be used as a base filling scaffold in oligonucleotides. Phosphoramidite approach was first used to synthesize the building block and the synthesis was successful, but the product was unreactive in oligonucleotide synthesis.
Therefore, H-phosphonate approach was used to link the synthesized building block to 3′-*O*-TBDMS-thymidine nucleoside, synthesizing a H phosphonate diester containing an unusual N-O-P linkage. This synthesis was successful, but the product was found to be unreactive towards oxidation and hydrolysis.
In order to succeed the synthesis of the building block, oxidation of the H-phosphonate monomer is very important. Reagents and reactions conditions should be investigated to oxidize the H-phosphonate monomer. Alternatively, phosphotriester approach of oligonucleotide synthesis could be used where the oxidation step is avoided altogether. Then the carbamate protection could be removed to afford the desired base-filling scaffold.

5 References

(1) Duffy, K.; Arangundy-Franklin, S.; Holliger, P. Modified Nucleic Acids: Replication, Evolution, and next-Generation Therapeutics. *BMC Biol*. **2020**, 18 (1), 112.

(2) Khvorova, A.; Watts, J. K. The Chemical Evolution of Oligonucleotide Therapies of Clinical Utility. *Nat Biotechnol.* **2017**, *35* (3), 238–248.

(3) Rinaldi, C.; Wood, M. J. A. Antisense Oligonucleotides: The next Frontier for Treatment of Neurological Disorders. *Nat Rev Neuro*. **2018**, 14 (1), 9–21.

(4) Collotta, D.; Bertocchi, I.; Chiapello, E.; Collino, M. Antisense Oligonucleotides: A Novel Frontier in Pharmacological Strategy. *Front Pharmacol*. **2023**, *14*, 1304342.

(5) Laganà, A.; Veneziano, D.; Russo, F.; Pulvirenti, A.; Giugno, R.; Croce, C. M. aria; Ferro, A. Computational Design of Artificial RNA Molecules for Gene Regulation. *Methods Mol Biol.* **2015**, *1269*, 393–412.

(6) Ebenezer, O.; Oyebamiji, A. K.; Olanlokun, J. O.; Tuszynski, J. A.; Wong, G. K. S. Recent Update on SiRNA Therapeutics. *IJMS.* **2025**, 26 (8), 3456.

(7) Adachi, T.; Nakamura, Y. Aptamers: A Review of Their Chemical Properties and Modifications for Therapeutic Application. *Molecules*. **2019**, *24* (23), 4229.

(8) Seyhan, A. A. Trials and Tribulations of MicroRNA Therapeutics. *IJMS.* **2024**, *25* (3), 1469.

(9) Gareev, I.; de Jesus Encarnacion Ramirez, M.; Goncharov, E.; Ivliev, D.; Shumadalova, A.; Ilyasova, T.; Wang, C. MiRNAs and LncRNAs in the Regulation of Innate Immune Signaling. *Non-coding RNA Res.* **2023**, 8 (4), 534–541.

(10) Pauley, K. M.; Cha, S.; Chan, E. K. L. MicroRNA in Autoimmunity and Autoimmune Diseases. *J. Autoimmun.* **2009**, *32* (3), 189–194.

(11) Li, Y. J.; Xu, M.; Gao, Z. H.; Wang, Y. Q.; Yue, Z.; Zhang, Y. X.; Li, X. X.; Zhang, C.; Xie, S. Y.; Wang, P. Y. Alterations of Serum Levels of BDNF-Related MiRNAs in Patients with Depression. *PLoS One.* **2013**, *8* (5).

(12) Scott, K. A.; Hoban, A. E.; Clarke, G.; Moloney, G. M.; Dinan, T. G.; Cryan, J. F. Thinking Small: Towards MicroRNA-Based Therapeutics for Anxiety Disorders. *Expert Opin Investig Drugs.* **2015**, *24* (4), 529–542.

(13) Peng, Y.; Croce, C. M. The Role of MicroRNAs in Human Cancer. *Sig. Transduct Target Ther.* **2016**, *1* (1), 15004.

(14) Hu, W. Z.; Tan, C. L.; He, Y. J.; Zhang, G. Q.; Xu, Y.; Tang, J. H. Functional MiRNAs in Breast Cancer Drug Resistance. *Onco Targets Ther*. **2018**, 11, 1529–1541.

(15) Nunez Lopez, Y. O.; Coen, P. M.; Goodpaster, B. H.; Seyhan, A. A. Gastric Bypass Surgery with Exercise Alters Plasma MicroRNAs That Predict Improvements in Cardiometabolic Risk. *Int J Obes.* **2017**, *41* (7), 1121–1130.

(16) Seyhan, A. A.; Nunez Lopez, Y. O.; Xie, H.; Yi, F.; Mathews, C.; Pasarica, M.; Pratley, R. E. Pancreas-Enriched MiRNAs Are Altered in the Circulation of Subjects with Diabetes: A Pilot Cross-Sectional Study. *Sci Rep.* **2016**, *6* (1), 31479.

(17) Heyduk, E.; Heyduk, T. Nucleic Acid-Based Fluorescence Sensors for Detecting Proteins. *Anal. Chem.* **2005**, *77* (4), 1147–1156.

(18) Wang, F.; Li, P.; Chu, H. C.; Lo, P. K. Nucleic Acids and Their Analogues for Biomedical Applications. *Biosensors.* **2022**, *12* (2), 93.

(19) Shin, S.; Kim, J.; Song, E.; Han, S.; Hohng, S. Analytical Techniques for Nucleic Acid and Protein Detection with Single-Molecule Sensitivity. *Exp Mol Med.* **2025**, *57* (5), 938–949.

(20) Liu, L.; Xiong, M.; Rong, Q.; Zhang, M.; Zhang, X. B. Nucleic Acid Sensors in Vivo: Challenges and Opportunities. *VIEW.* **2023**, *4* (3), 20220064.

(21) Nesterova, I. V; Nesterov, E. E. Rational Design of Highly Responsive PH Sensors Based on DNA I-Motif. *J. Am. Chem. Soc.* **2014**, *136* (25), 8843–8846.

(22) Abedi, R.; Raoof, J. B.; Bagheri Hashkavayi, A.; Jalayeri Darbandi, Z.; Abedi, P.; Barati Darband, G. Innovations in Aptamer-Based Biosensors for Detection of Pathogenic Bacteria: Recent Advances and Perspective. *Talanta.* **2025**, 295, 128330.

(23) Koksaldi, I. C.; Park, D.; Atilla, A.; Kang, H.; Kim, J.; Seker, U. O. S. RNA-Based Sensor Systems for Affordable Diagnostics in the Age of Pandemics. *ACS Synth Biol.* **2024**, 13 (4), 1026–1037.

(24) Choe, C. A.; Andreasson, J. O. L.; Melaine, F.; Kladwang, W.; Wu, M. J.; Portela, F.; Wellington-Oguri, R.; Nicol, J. J.; Wayment-Steele, H. K.; Gotrik, M.; Khatri, P.; Greenleaf, W. J.; Das, R. Compact RNA Sensors for Increasingly Complex Functions of Multiple Inputs. *Nat Chem.* **2025**, 17 (12), 1839–1852.

(25) Wu, L.; Wang, Y.; Xu, X.; Liu, Y.; Lin, B.; Zhang, M.; Zhang, J.; Wan, S.; Yang, C.; Tan, W. Aptamer-Based Detection of Circulating Targets for Precision Medicine. *Chem Rev.* **2021**, 121 (19), 12035–12105.

(26) Blackstock, C.; Walters-Freke, C.; Richards, N.; Williamson, A. Nucleic Acid Joining Enzymes: Biological Functions and Synthetic Applications beyond DNA. *Biochem J.* **2025***,* 482 (2), 39–56.

(27) Bonifazi, D.; Carloni, L. E.; Corvaglia, V.; Delforge, A. Peptide Nucleic Acids in Materials Science. *Artif DNA PNA XNA.* **2012**, 3 (3), 112–122.

(28) Fitzgerald, P. R.; Paegel, B. M. DNA-Encoded Chemistry: Drug Discovery from a Few Good Reactions. *Chem. Rev.* **2021**, 121 (12), 7155–7177.

(29) Lee, H.; Wiegand, D. J.; Griswold, K.; Punthambaker, S.; Chun, H.; Kohman, R. E.; Church, G. M. Photon-Directed Multiplexed Enzymatic DNA Synthesis for Molecular Digital Data Storage. *Nat Commun.* **2020**, 11 (1), 5246.

(30) Kieffer, C.; Genot, A. J.; Rondelez, Y.; Gines, G. Molecular Computation for Molecular Classification. *Adv Biol.* **2023**, 7 (3), 2200203.

(31) Kennebeck, M. M.; Kaminsky, C. K.; Massa, M. A.; Das, P. K.; Boyd, R. D.; Bishka, M.; Tricarico, J. T.; Silverman, S. K. DNAzyme-Catalyzed Site-Specific N-Acylation of DNA Oligonucleotide Nucleobases. *Angew Chem Int Ed.* **2024**, 63 (7). 202317565.

(32) Pichon, M.; Hollenstein, M. Controlled Enzymatic Synthesis of Oligonucleotides. *Commun Chem.* **2024**, *7* (1), 138.

(33) Zutterling, C.; Todeschini, A. L.; Fourmy, D.; Busso, D.; Veaute, X.; Ducongé, F.; Veitia, R. A. The Forkhead DNA-Binding Domain Binds Specific G2-Rich RNA Sequences. *Nucleic Acids Res.* **2023**, *51* (22), 12367–12380.

(34) Kulkarni, J. A.; Witzigmann, D.; Thomson, S. B.; Chen, S.; Leavitt, B. R.; Cullis, P. R.; van der Meel, R. The Current Landscape of Nucleic Acid Therapeutics. *Nat Nanotechnol.* **2021**, *16* (6), 630–643.

(35) Dhara, D.; Mulard, L. A.; Hollenstein, M. Natural, Modified and Conjugated Carbohydrates in Nucleic Acids. *Chem Soc Rev.* **2025**, *54* (6), 2948–2983.

(36) Das, G.; Harikrishna, S.; Gore, K. R. Influence of Sugar Modifications on the Nucleoside Conformation and Oligonucleotide Stability: A Critical Review. *Chem Rec.* **2022**, *22* (12), e202200174.

(37) Kauppinen, S.; Vester, B.; Wengel, J. Locked Nucleic Acid (LNA): High Affinity Targeting of RNA for Diagnostics and Therapeutics. *Drug Discov Today Technol.* **2005**, *2* (3), 287–290.

(38) Bag, S.; Banerjee, A.; Sinha, S. Expansion of Genetic Alphabets: Designer Nucleobases and Their Applications. *Synlett.* **2023**, a-2219-6680.

(39) Rausch, C.; Zhang, P.; Casas-Delucchi, C. S.; Daiß, J. L.; Engel, C.; Coster, G.; Hastert, F. D.; Weber, P.; Cristina Cardoso, M. Cytosine Base Modifications Regulate DNA Duplex Stability and Metabolism. *Nucleic Acids Res.* **2021**, *49* (22), 12870–12894.

(40) Hollenstein, M. Nucleic Acid Enzymes Based on Functionalized Nucleosides. *Curr Opin Chem Biol.* **2019**, *52*, 93–101.

(41) Jash, B.; Müller, J. Metal-Mediated Base Pairs: From Characterization to Application. *Chem Eur J.* **2017**, *23* (68), 17166–17178.

(42) Helm, M. Post-Transcriptional Nucleotide Modification and Alternative Folding of RNA. *Nucleic Acids Res* **2006**, *34* (2), 721–733.

(43) Karikó, K.; Muramatsu, H.; Welsh, F. A.; Ludwig, J.; Kato, H.; Akira, S.; Weissman, D. Incorporation of Pseudouridine into MRNA Yields Superior Nonimmunogenic Vector with Increased Translational Capacity and Biological Stability. *Mol Ther.* **2008**, *16* (11), 1833–1840.

(44) Bernard, M.-C.; Bazin, E.; Petiot, N.; Lemdani, K.; Commandeur, S.; Verdelet, C.; Margot, S.; Perkov, V.; Ripoll, M.; Garinot, M.; Ruiz, S.; Boudet, F.; Rokbi, B.; Haensler, J. The Impact of Nucleoside Base Modification in MRNA Vaccine Is Influenced by the Chemistry of Its Lipid Nanoparticle Delivery System. *Mol. Ther. Nucleic Acids.* **2023**, *32*, 794–806.

(45) Novikova, D.; Sagaidak, A.; Vorona, S.; Tribulovich, V. A Visual Compendium of Principal Modifications within the Nucleic Acid Sugar Phosphate Backbone. *Molecules* **2024**, *29* (13), 3025.

(46) Brown, D. A.; Kang, S. H.; Gryaznov, S. M.; De Dionisio, L.; Heidenreich, O.; Sullivan, S.; Xu, X.; Nerenberg, M. I. Effect of Phosphorothioate Modification of Oligodeoxynucleotides on Specific Protein Binding. *J Biol Chem.* **1994**, *269* (43), 26801–26805.

(47) Roberts, T. C.; Langer, R.; Wood, M. J. A. Advances in Oligonucleotide Drug Delivery. *Nat Rev Drug Discov.* **2020**, *19* (10), 673–694.

(48) Shaw, B. R.; Sergueev, D.; He, K.; Porter, K.; Summers, J.; Sergueeva, Z.; Rait, V. Boranophosphate Backbone: A Mimic of Phosphodiesters, Phosphorothioates, and Methyl Phosphonates. *ChemInform.* **2000**, *31* (25), chin.200025286.

(49) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. Sequence-Selective Recognition of DNA by Strand Displacement with a Thymine-Substituted Polyamide. *Science (1979).* **1991**, *254* (5037), 1497–1500.

(50) Nielsen, P. E.; Egholm, M. An Introduction to Peptide Nucleic Acid. *Curr Issues Mol Biol.* **1999**,1(1-2), 89-104

(51) Pellestor, F.; Paulasova, P. PRINS Combined with Peptide Nucleic Acid Labeling. *Methods Mol Biol* **2006**, 334, 15–22.

(52) Fiandaca, M. J.; Hyldig-Nielsen, J. J.; Gildea, B. D.; Coull, J. M. Self-Reporting PNA/DNA Primers for PCR Analysis. *Genome Res.* **2001**, *11* (4), 609–613.

(53) Baraniak, D.; Boryski, J. Triazole-Modified Nucleic Acids for the Application in Bioorganic and Medicinal Chemistry. *Biomedicines.* **2021**, *9* (6).

(54) Zhang, C.; Subthain, H.; Guo, F.; Fang, P.; Zheng, S.; Shen, M.; Jiang, X.; Gao, Z.; Meng, C.; Li, S.; Du, L. Terminal Deoxynucleotidyl Transferase: Properties and Applications. *Eng Microbiol.* **2025**, *5* (1), 100179.

(55) Gao, N.; Yu, A.; Yang, W.; Zhang, X.; Shen, Y.; Fu, X. Enzymatic de Novo Oligonucleotide Synthesis: Emerging Techniques and Advancements. *Biotechnol Adv.* **2025**, 108604.

(56) England, T. E.; Uhlenbeck, O. C. Enzymic Oligoribonucleotide Synthesis with T4 RNA Ligase. *Biochemistry.* **1978**, *17* (11), 2069–2076.

(57) Kikuchi, Y.; Hishinuma, F.; Sakaguchi, K. Addition of Mononucleotides to Oligoribonucleotide Acceptors with T4 RNA Ligase. *Proc Natl Acad Sci. U.S.A.* **1978**, *75* (3), 1270–1273.

(58) Grunberg-Manago, M.; Ortiz, P. J.; Ochoa, S. Enzymic Synthesis of Polynucleotides I. Polynucleotide Phosphorylase of Azotobacter Vinelandii. *Biochim. Biophys. Acta.* **1956**, *20*, 269–285.

(59) Prat, D.; Wells, A.; Hayler, J.; Sneddon, H.; McElroy, C. R.; Abou-Shehada, S.; Dunn, P. J. CHEM21 Selection Guide of Classical- and Less Classical-Solvents. *Green Chem.* **2016**, *18* (1), 288–296.

(60) Burgers, P. M. J.; Eckstein, F. A Study of the Mechanism of DNA Polymerase I from Escherichia Coli with Diastereomeric Phosphorothioate Analogs of Deoxyadenosine Triphosphate. *J Biol Chem.* **1979**, *254* (15), 6889–6893.

(61) Geronimo, I.; Vidossich, P.; Donati, E.; De Vivo, M. Computational Investigations of Polymerase Enzymes: Structure, Function, Inhibition, and Biotechnology. *WIREs Comput Mol Sci*. **2021**, 11 (6), e1534.

(62) Mohammed, A. A.; AlShaer, D.; Al Musaimi, O. Oligonucleotides: Evolution and Innovation. *Med Chem Res.* **2024***, 33* (12), 2204–2220.

(63) Lönnberg, H. Solid-Phase Synthesis of Oligonucleotide Conjugates Useful for Delivery and Targeting of Potential Nucleic Acid Therapeutics. *Bioconjugate Chem.* **2009**, *20* (6), 1065–1094.

(64) Obexer, R.; Nassir, M.; Moody, E. R.; Baran, P. S.; Lovelock, S. L. Modern Approaches to Therapeutic Oligonucleotide Manufacturing. *Science.* **2024**, 384 (6692), eadl4015.

(65) Molina, A. G.; Sanghvi, Y. S. Liquid-Phase Oligonucleotide Synthesis: Past, Present, and Future Predictions. *Curr. Protoc. Nucleic Acid Chem.* **2019**, *77* (1).

(66) Bonora, G. M.; Biancotto, G.; Maffini, M.; Scremin, C. L. Large Scale, Liquid Phase Synthesis of Oligonucleotides by the Phosphoramidite Approach. *Nucleic Acids Res.* **1993**, *21* (5), 1213–1217.

(67) Padiya, K. J.; Salunkhe, M. M. Liquid Phase Oligonucleotide Synthesis by Alkyl H-Phosphonate Approach. *Bioorg Med Chem.* **2000**, *8* (2), 337–342.

(68) Liang, Z.; Rosenqvist, P.; Pajuniemi, E.; Ora, M.; Heinonen, P.; Virta, P.; Oivanen, M. 5'-O-(2-Isopropoxyprop-2-Yl)-Protected Phosphoramidite Building Blocks in the Liquid Phase Oligonucleotide Synthesis. *European J. Org. Chem.* **2023**, *26* (36).

(69) Abramova, T. Frontiers and Approaches to Chemical Synthesis of Oligodeoxyribonucleotides. *Molecules.* **2013**, *18* (1), 1063–1075.

(70) Lönnberg, H. Synthesis of Oligonucleotides on a Soluble Support. *Beilstein J. Org. Chem.* **2017**, *13*, 1368–1387.

(71) Jones, S. S.; Rayner, B.; Reese, C. B.; Ubasawa, A.; Ubasawa, M. Synthesis of the 3'-Terminal Decaribonucleoside Nonaphosphate of Yeast Alanine Transfer Ribonucleic Acid. *Tetrahedron.* **1980**, *36* (20), 3075–3085.

(72) Dörper, T.; Winnacker, E.-L. Improvements in the Phosphoramidite Procedure for the Synthesis of Oligodeoxyribonucleotides. *Nucleic Acids Res.* **1983**, *11* (9), 2575–2584.

(73) Garegg, P. J.; Lindh, I.; Regberg, T.; Stawinski, J.; Strömberg, R.; Henrichson, C. Nucleoside H-Phosphonates. IV. Automated Solid Phase Synthesis of

Oligoribonucleotides by the Hydrogenphosphonate Approach. *Tetrahedron Lett.* **1986**, *27* (34), 4055–4058.

(74) Hughes, R. A.; Miklos, A. E.; Ellington, A. D. Gene Synthesis: Methods and Applications. *Methods Enzymol.* **2011**, *498*, 277–309.

(75) Letsinger, R. L.; Ogilvie, K. K. Nucleotide Chemistry. XIII. Synthesis of Oligothymidylates via Phosphotriester Intermediates. *J. Am. Chem. Soc.* **1969**, *91* (12), 3350–3355.

(76) Kungurtsev, V.; Virta, P.; Lönnberg, H. Synthesis of Short Oligodeoxyribonucleotides by Phosphotriester Chemistry on a Precipitative Tetrapodal Support. *European J. Org. Chem.* **2013**, No. 35, 7886–7890.

(77) Reese, C. B.; Quanlai Song. A New Approach to the Synthesis of Oligonucleotides and Their Phosphorothioate Analogues in Solution. *Bioorg. Med. Chem. Lett.* **1997**, *7* (21), 2787–2792.

(78) Efimov, V. A.; Reverdatto, S. V; Chakhmakhcheva, O. G. New Effective Method for the Synthesis of Oligonucleotides via Phosphotriester Intermediates. *Nucleic Acids Res.* **1982**, *10* (21), 6675–6694.

(79) Bonora, G. M.; Scremin, C. L.; Colonna, F. P.; Garbesi, A. HELP (High Efficiency Liquid Phase) New Oligonucleotide Synthesis on Soluble Polymeric Support. *Nucleic Acids Res.* **1990**, *18* (11), 3155–3159.

(80) Gait, M. J.; Singh, M.; Sheppard, R. C.; Edge, M. D.; Greene, A. R.; Heathcliffe, G. R.; Atkinson, T. C.; Newton, C. R.; Markham, A. F. Rapid Synthesis of Oligodeoxyribonucleotides IV. Improved Solid Phase Synthesis of Oligodeoxy-Ribonucleotides through Phosphotriester Intermediates. *Nucleic Acids Res.* **1980**, *8* (5), 1081–1096.

(81) Reese, C. B.; Pei-Zhuo, Z. Phosphotriester Approach to the Synthesis of Oligonucleotides: A Reappraisal. *J. Chem. Soc., Perkin Trans. 1* **1993**, No. 19, 2291.

(82) Beaucage, S. L.; Caruthers, M. H. Deoxynucleoside Phosphoramidites—A New Class of Key Intermediates for Deoxypolynucleotide Synthesis. *Tetrahedron Lett.* **1981**, *22* (20), 1859–1862.

(83) Sinha, N. D.; Biernat, J.; Köster, H. β-Cyanoethyl N,N-Dialkylamino/N-Morpholinomonochloro Phosphoamidites, New Phosphitylating Agents Facilitating Ease

of Deprotection and Work-up of Synthesized Oligonucleotides. *Tetrahedron Lett.* **1983**, *24* (52), 5843–5846.

(84) Ogilvie, K. K.; Theriault, N.; Sadana, K. L. Synthesis of Oligoribonucleotides. *J. Am. Chem. Soc.* **1977**, *99* (23), 7741–7743.

(85) Griffin, B. E.; Reese, C. B. Oligoribonucleotide Synthesis via 2′,5′-Protected Ribonucleoside Derivatives. *Tetrahedron Lett.* **1964**, *5* (40), 2925–2931.

(86) Roy, S.; Caruthers, M. Synthesis of DNA/RNA and Their Analogs via Phosphoramidite and H-Phosphonate Chemistries. *Molecules.* **2013**, *18* (11), 14268–14284.

(87) Froehler, B. C.; Ng, P. G.; Matteucci, M. D. Synthesis of DNA via Deoxynudeoside H-Phosphonate Intermediates. *Nucleic Acids Res.* **1986**, *14* (13), 5399–5407.

(88) Garegg, P. J.; Regberg, T.; Stawinski, J.; Strömberg, R. Nucleoside H-Phosphonates. V. The Mechanism of Hydrogen phosphonate Diester Formation Using Acyl Chlorides as Coupling Agents in Oligonucleotide Synthesis by the Hydrogen phosphonate Approach. *Nucleosides Nucleotides.* **1987**, *6* (3), 655–662.

(89) Kraszewski, A.; Stawinski, J. H-Phosphonates: Versatile Synthetic Precursors to Biologically Active Phosphorus Compounds. *Pure Appl Chem.* **2007**, *79* (12), 2217–2227.

(90) Corbett, P. T.; Leclaire, J.; Vial, L.; West, K. R.; Wietor, J. L.; Sanders, J. K. M.; Otto, S. Dynamic Combinatorial Chemistry. *Chem. Rev.* **2006**, *106* (9), 3652–3711.

(91) Aguanell, A.; Hennebelle, M.; Ortega, M. Á.; Pérez-Fernández, R. Dynamic Combinatorial Chemistry Directed by Proteins and Nucleic Acids: A Powerful Tool for Drug Discovery. *Chem. Soc. Rev.* **2025**, *54* (15), 7093–7113.

(92) Ramström, O.; Lehn, J. M. Drug Discovery by Dynamic Combinatorial Libraries. *Nat Rev Drug Discov.* **2002**, *1* (1), 26–36.

(93) Canal-Martín, A.; Pérez-Fernández, R. Protein-Directed Dynamic Combinatorial Chemistry: An Efficient Strategy in Drug Design. *ACS Omega.* **2020**, *5* (41), 26307–26315.

(94) Misuraca, M. C.; Moulin, E.; Ruff, Y.; Giuseppone, N. Experimental and Theoretical Methods for the Analyses of Dynamic Combinatorial Libraries. *New J. Chem.* **2014**, *38* (8), 3336–3349.

(95) Otto, S.; Furlan, R. L. E.; Sanders, J. K. M. Dynamic Combinatorial Chemistry. *Drug Discov Today.* **2002**, *7* (2), 117–125.

(96) Brady, P. A.; Bonar-Law, R. P.; Rowan, S. J.; Suckling, C. J.; Sanders, J. K. M. 'Living' Macrolactonisation: Thermodynamically Controlled Cyclisation and interconversion of Oligocholates. *Chem. Commun.* **1996**, No. 3, 319–320.

(97) Swann, P. A.; Casanova, R. A.; Desai, A.; Frauenhoff, M. M.; Urbancic, M.; Slomczynska, U.; Hopfinger, A. J.; Le Breton, G. C.; Venton, D. L. Nonspecific Protease-Catalyzed Hydrolysis/Synthesis of a Mixture of Peptides: Product Diversity and Ligand Amplification by a Molecular Trap. *Biopolymers.* **1996**, *40* (6), 617–625.

(98) Orrillo, A. G.; Escalante, A. M.; Furlan, R. L. E. Dithioacetal Exchange: A New Reversible Reaction for Dynamic Combinatorial Chemistry. *Chem Eur J.* **2016**, *22* (20), 6746–6749.

(99) Shi, B.; Greaney, M. F. Reversible Michael Addition of Thiols as a New Tool for Dynamic Combinatorial Chemistry. *Chem Commun.* **2005**, No. 7, 886–888.

(100) Huc, I.; Krische, M. J.; Funeriu, D. P.; Lehn, J. M. Dynamic Combinatorial Chemistry: Substrate H-Bonding Directed Assembly of Receptors Based on Bipyridine-Metal Complexes. *Eur. J. Inorg. Chem.* **1999**, No. 9, 1415–1420.

(101) Crego-Calama, M.; Reinhoudt, D. N.; Ten Cate, M. G. J. Templation in Noncovalent Synthesis of Hydrogen-Bonded Rosettes. *Top. Curr. Chem.* **2005**, *249*, 285–316.

(102) Hartman, A. M.; Gierse, R. M.; Hirsch, A. K. H. Protein-Templated Dynamic Combinatorial Chemistry: Brief Overview and Experimental Protocol. *Eur J Org Chem.* **2019**, *2019* (22), 3581–3590.

(103) Klekota, B.; Miller, B. L. Selection of DNA-Binding Compounds via Multistage Molecular Evolution. *Tetrahedron*. **1999,** 55 (39), 11687–11697.

(104) Bugaut, A.; Jantos, K.; Wietor, J. L.; Rodriguez, R.; Sanders, J. K. M.; Balasubramanian, S. Exploring the Differential Recognition of DNA G-Quadruplex Targets by Small Molecules Using Dynamic Combinatorial Chemistry. *Angew. Chem. Int. Ed.* **2008**, *47* (14), 2677–2680.

(105) Afari, M. N. K.; Lönnberg, T. Base-Filling in Double-Helical Nucleic Acids. *ChemistryOpen.* **2024**, *13* (9), e202400088.

(106) Afari, M. N. K.; Virta, P.; Lönnberg, T. N-Methoxy-1,3-Oxazinane Nucleic Acids (MOANAs) - a Configurationally Flexible Backbone Modification Allows Post-Synthetic Incorporation of Base Moieties. *Org. Biomol. Chem.* **2022**, *20* (17), 3480–3485.

(107) Kanlidere, Z.; Jochim, O.; Cal, M.; Diederichsen, U. DNA Functionalization by Dynamic Chemistry. *Beilstein J Org Chem* **2016**, *12*, 2136–2144.

(108) Aho, A.; Sulkanen, M.; Korhonen, H.; Virta, P. Conjugation of Oligonucleotides to Peptide Aldehydes via a pH-Responsive N-Methoxyoxazolidine Linker. *Org Lett.* **2020**, *22* (17), 6714–6718.

(109) Wallin, J.; Lönnberg, T. Improved Synthesis Strategy for N-Methoxy-1,3-Oxazinane Nucleic Acids (MOANAs). *Eur J Org Chem.* **2022**, *2022* (35), e202200538.

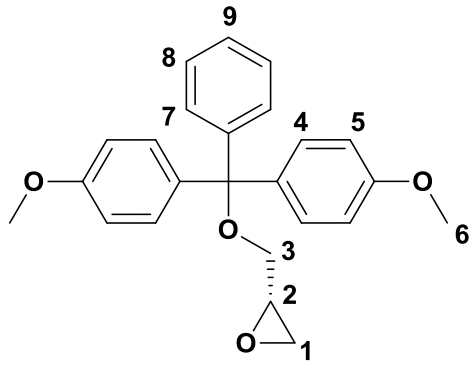
(110) Zhang, L.; Peritz, A. E.; Carroll, P. J.; Meggers, E. Synthesis of Glycol Nucleic Acids. *Synthesis (Stuttg).* **2006**, No. 4, 645–653.

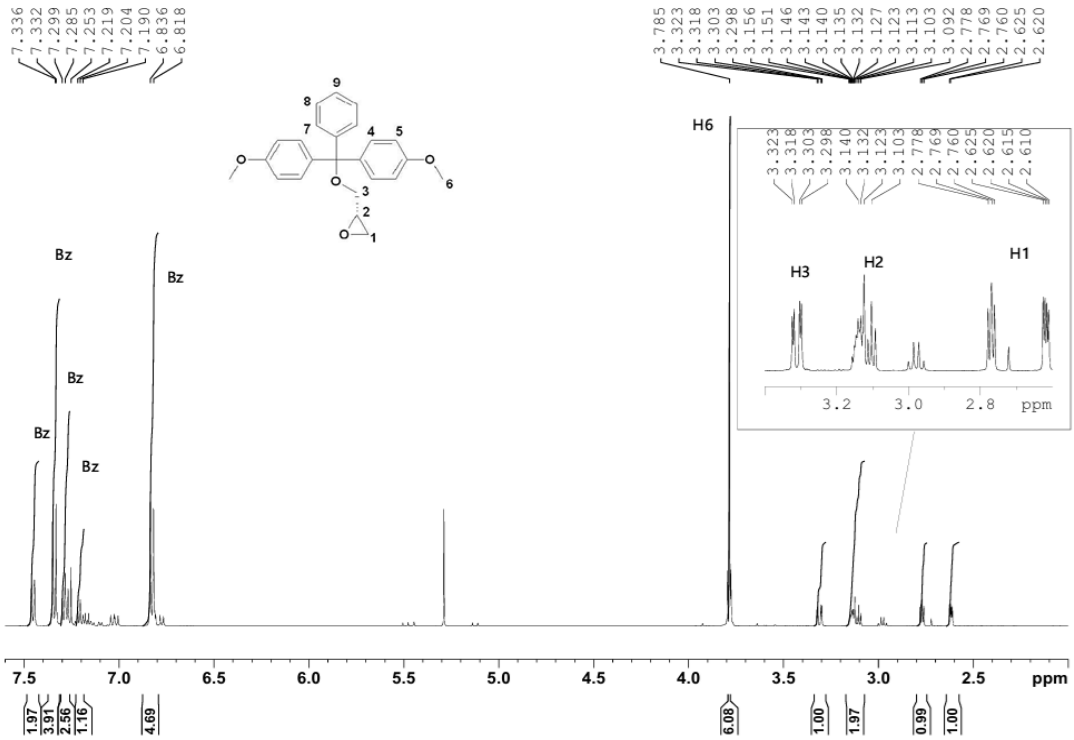
(111) Wei, X. Coupling Activators for the Oligonucleotide Synthesis via Phosphoramidite Approach. *Tetrahedron.* **2013**, *69* (18), 3615–3637.

(112) Sobkowski, M.; Kraszewski, A.; Stawinski, J. Recent Advances in H-Phosphonate Chemistry. Part 1. H-Phosphonate Esters: Synthesis and Basic Reactions. *Top. Curr. Chem.* **2014**, *361*, 137–178.

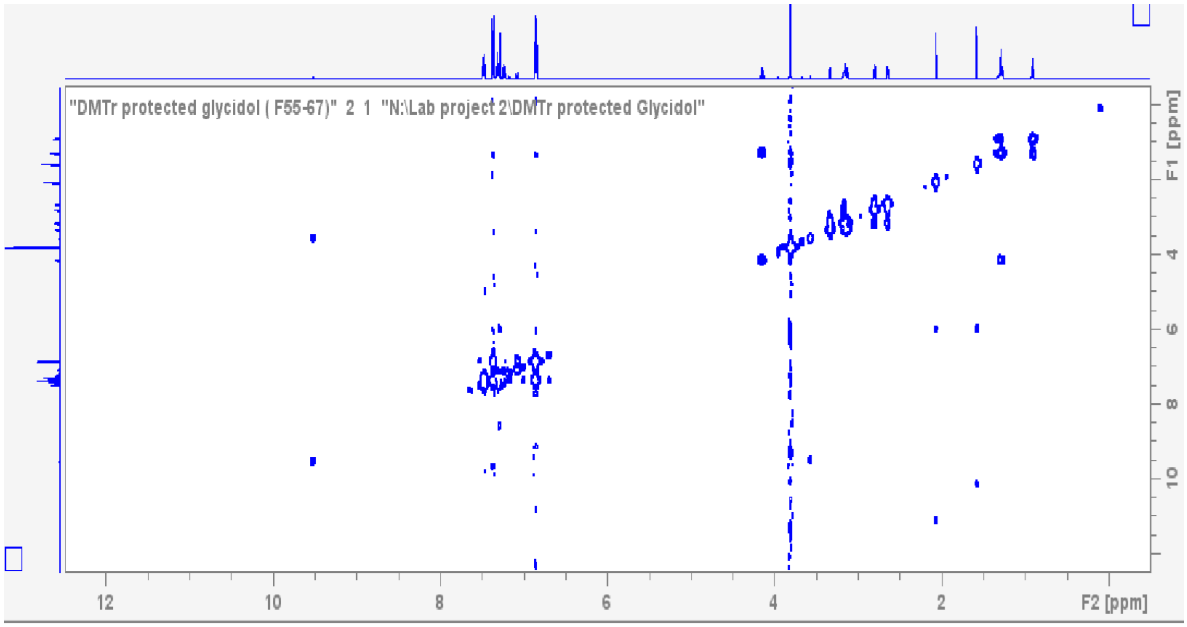
6 Appendix

Appendix 1. ^{1}H NMR spectrum of compound **2**





Appendix 2. COSY spectrum of compound **2**



Appendix 3. ^{1}H NMR spectrum of compound **10**

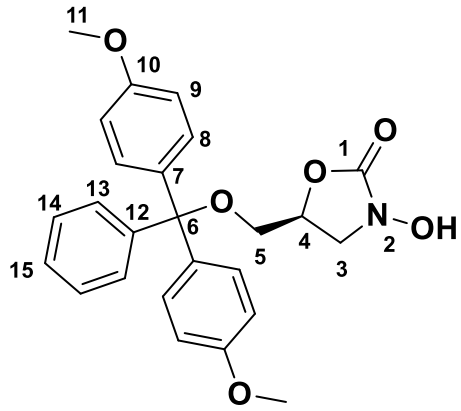
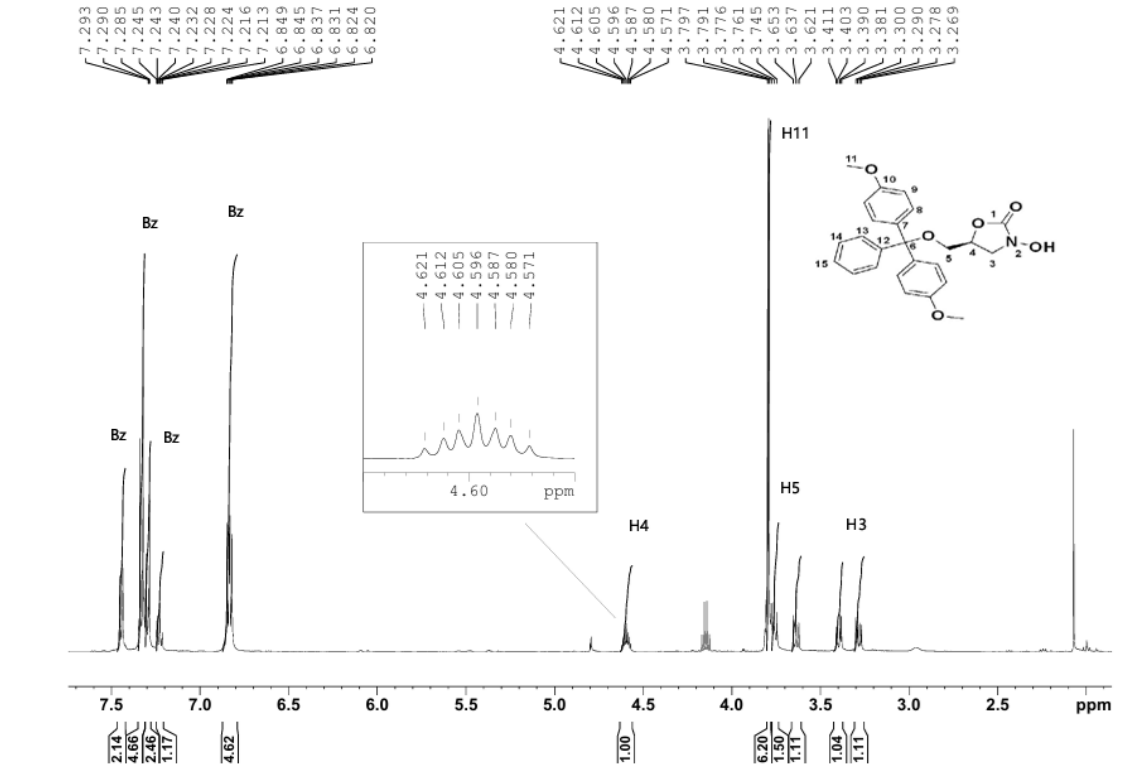
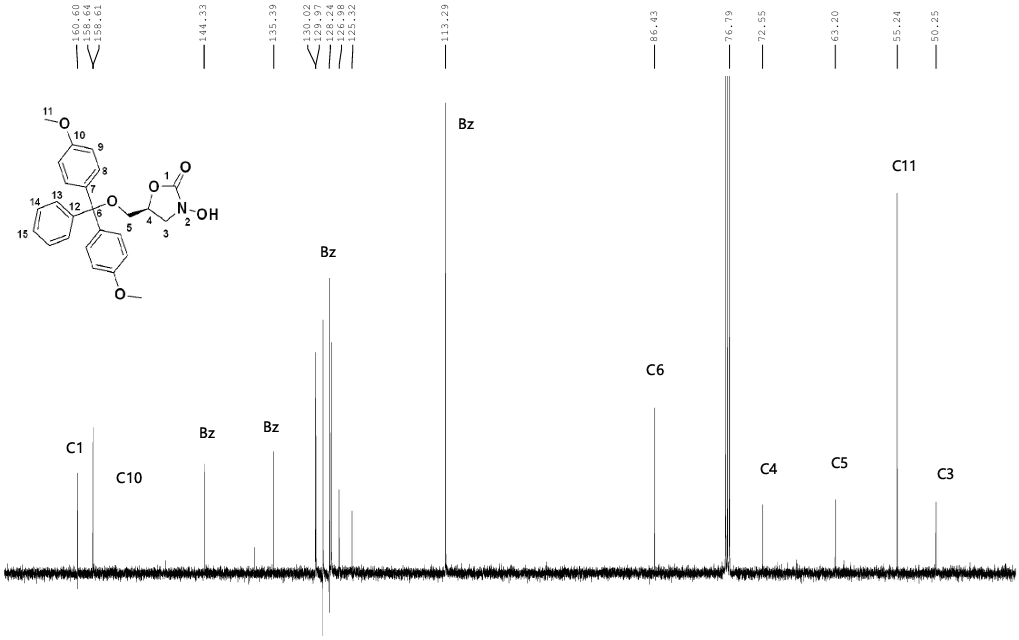


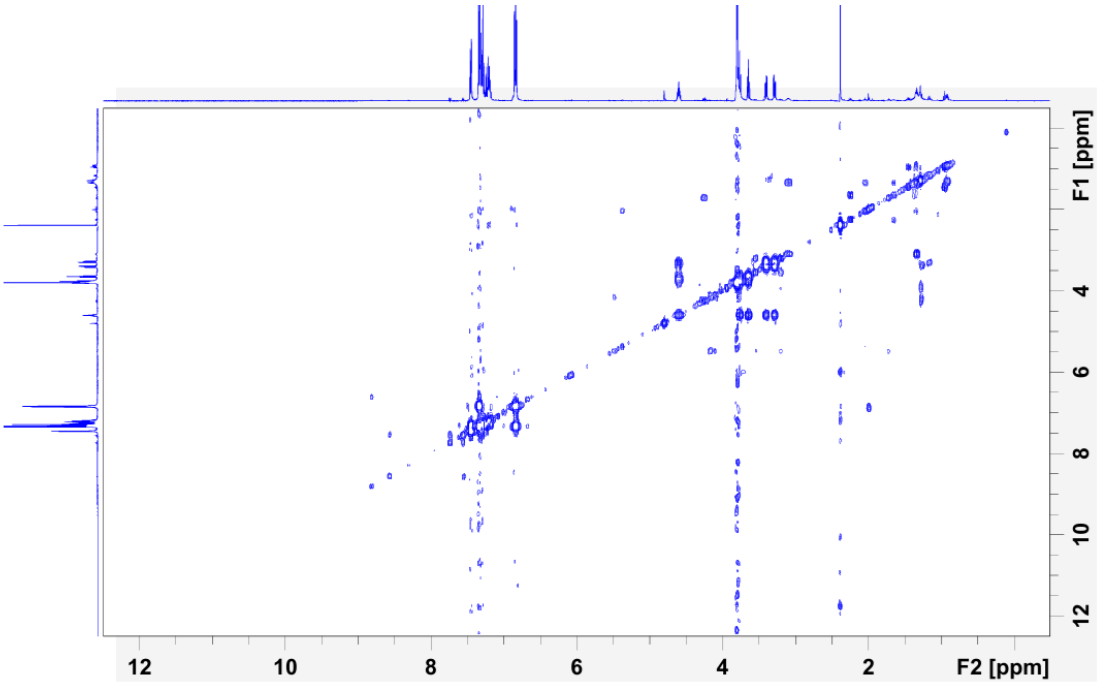
Figure 2 - Compound 10



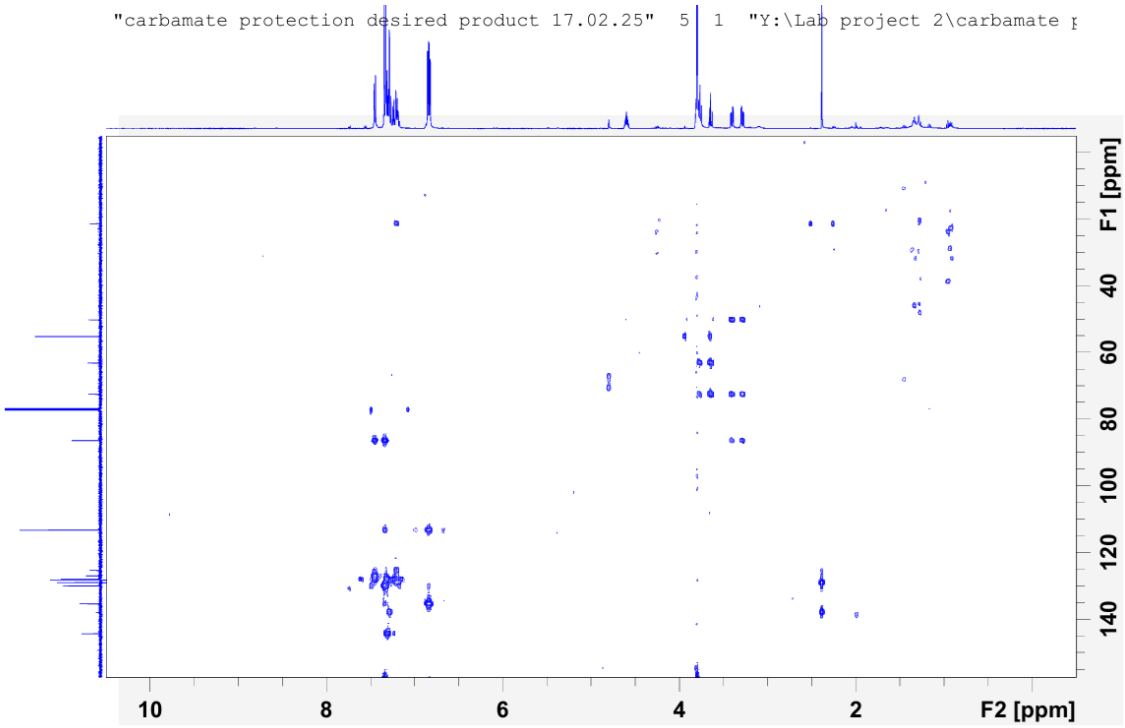
Appendix 4. ^{13}C NMR spectrum of compound **10**



Appendix 5. COSY spectrum of compound **10**



Appendix 6. HSQC spectrum of compound **10**



Appendix 7. ^{1}H NMR spectrum of compound **11**

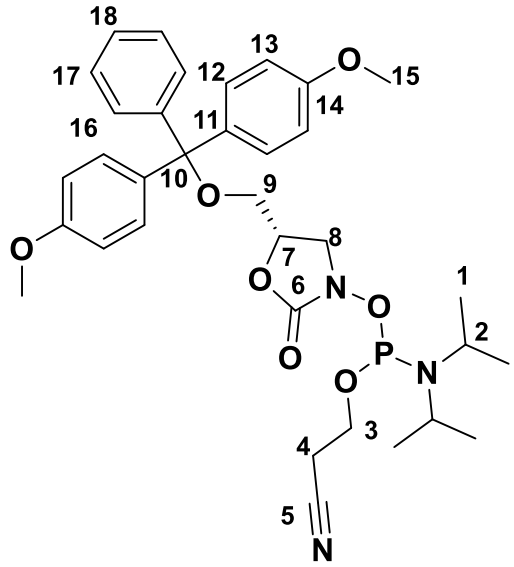
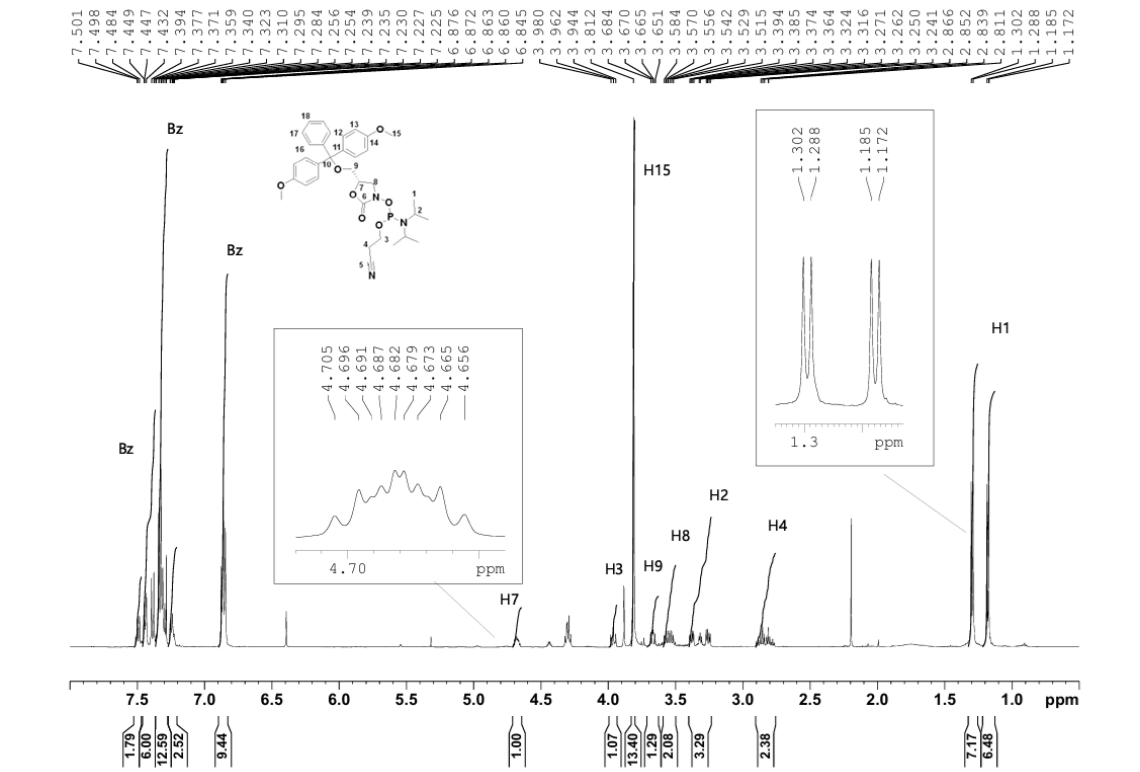
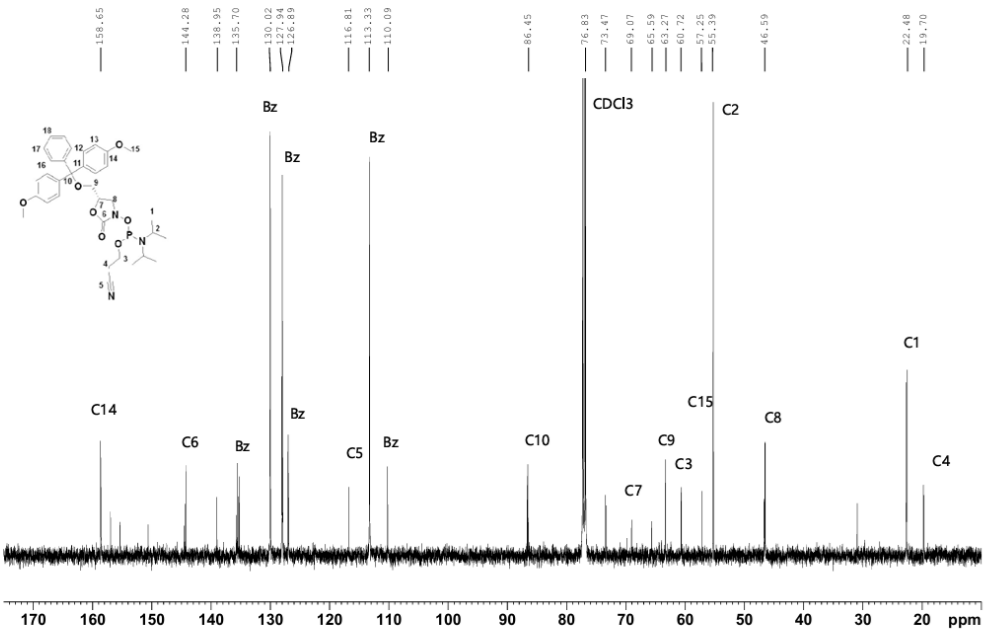


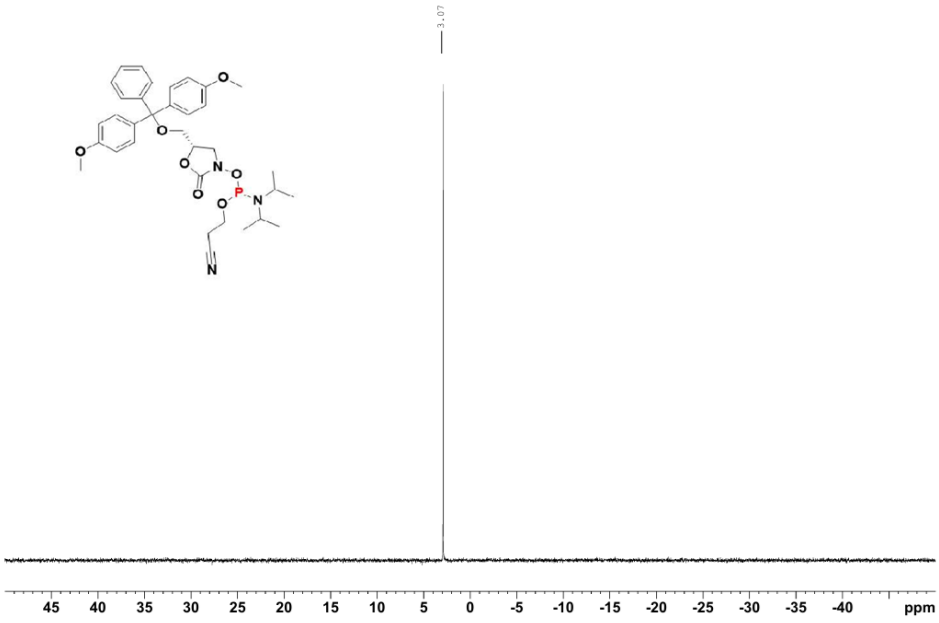
Figure 3 - Compound 11



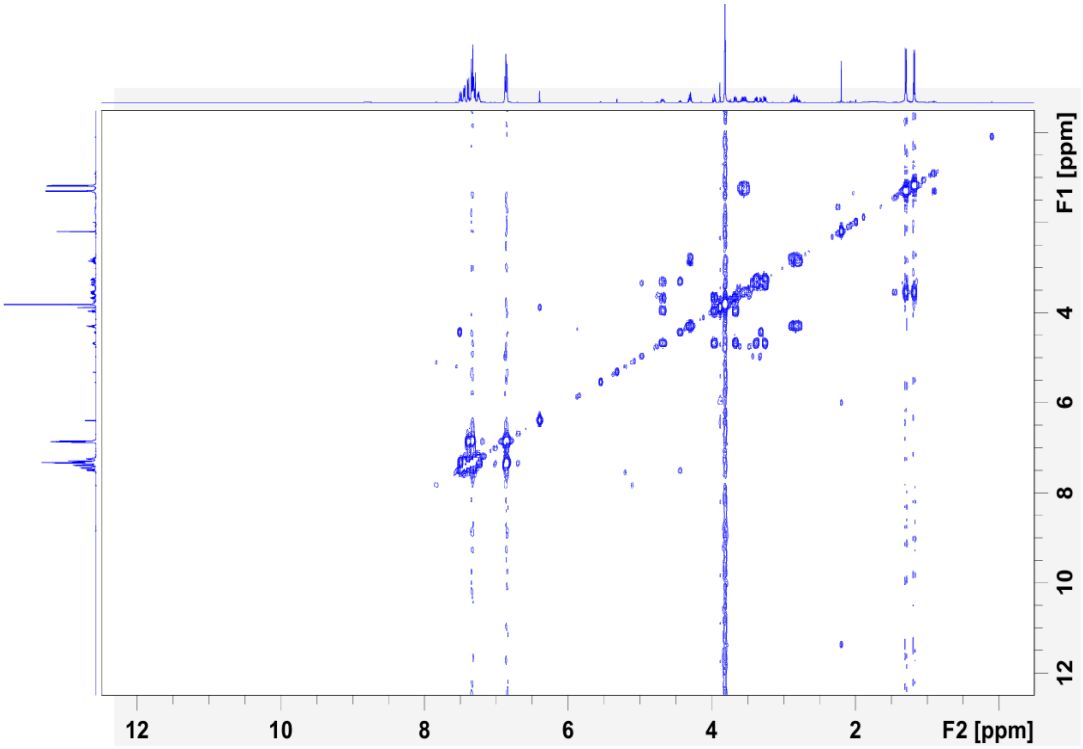
Appendix 8. ^{13}C NMR spectrum of compound **11**



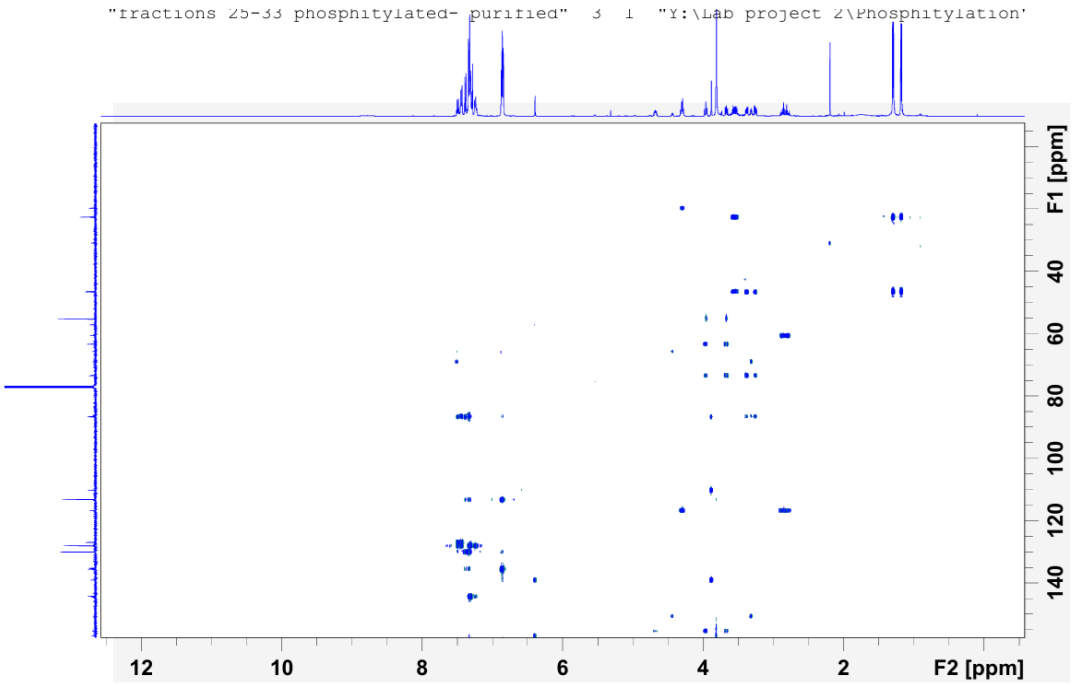
Appendix 9. ^{31}P NMR spectrum of compound **11**



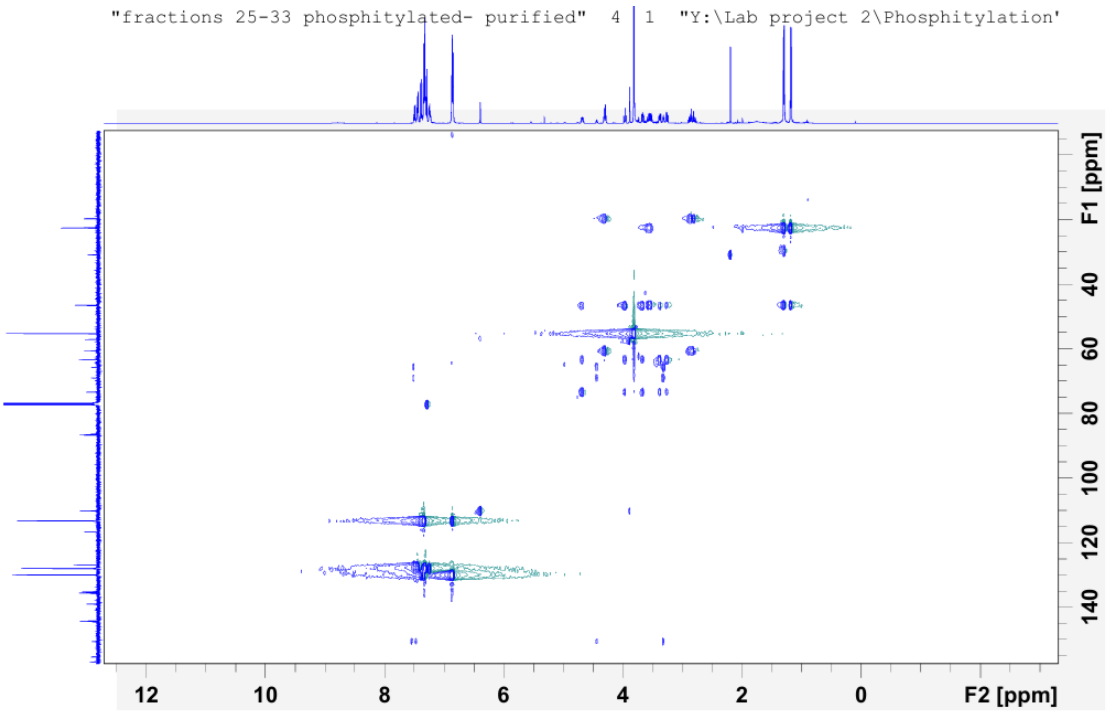
Appendix 10. COSY spectrum of compound **11**



Appendix 11. HMBC spectrum of compound **11**



Appendix 12. HSQC spectrum of compound **11**



Appendix 13. ^{1}H NMR of compound **14**

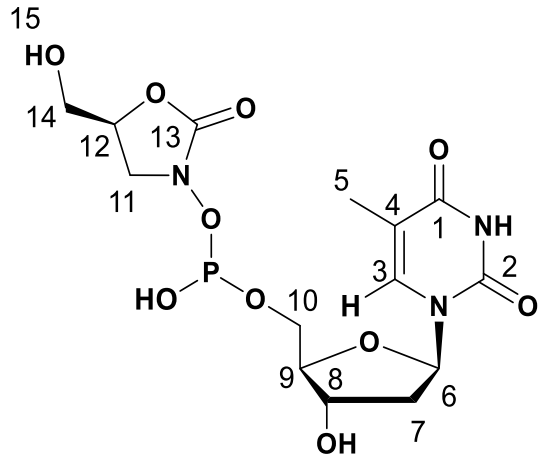
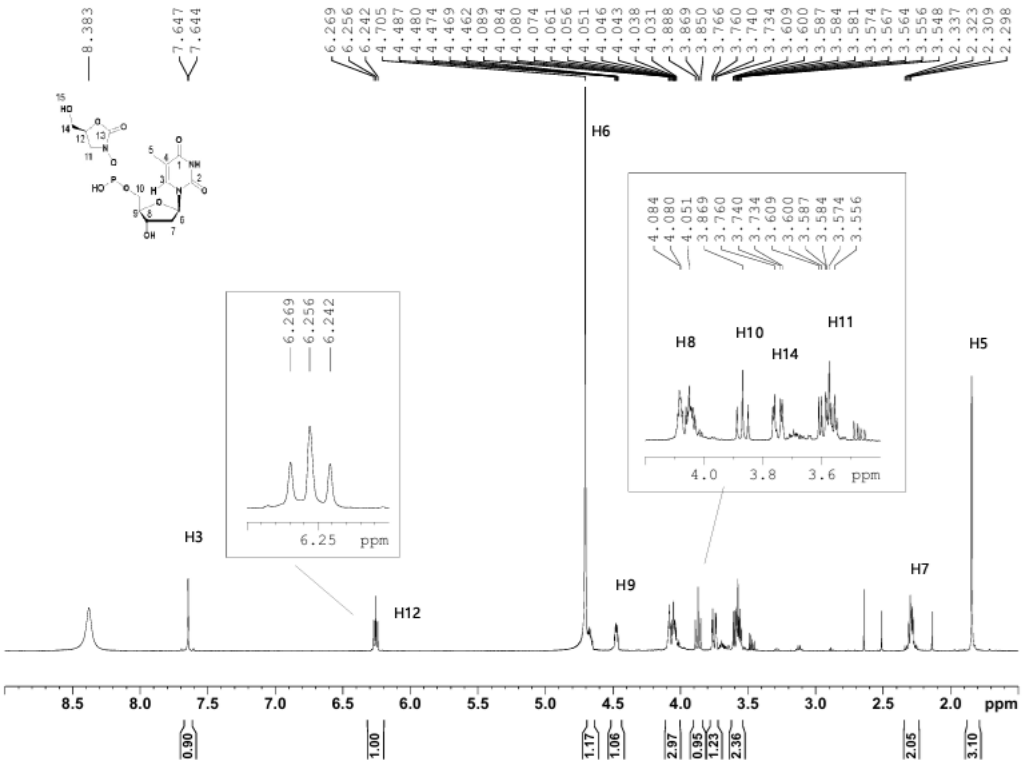
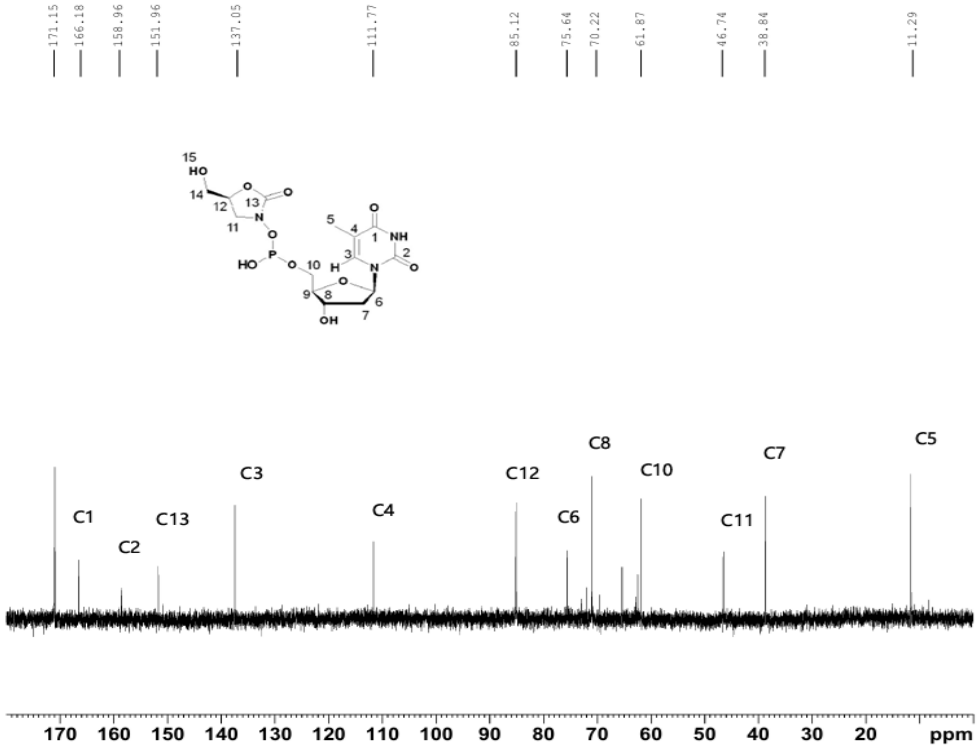


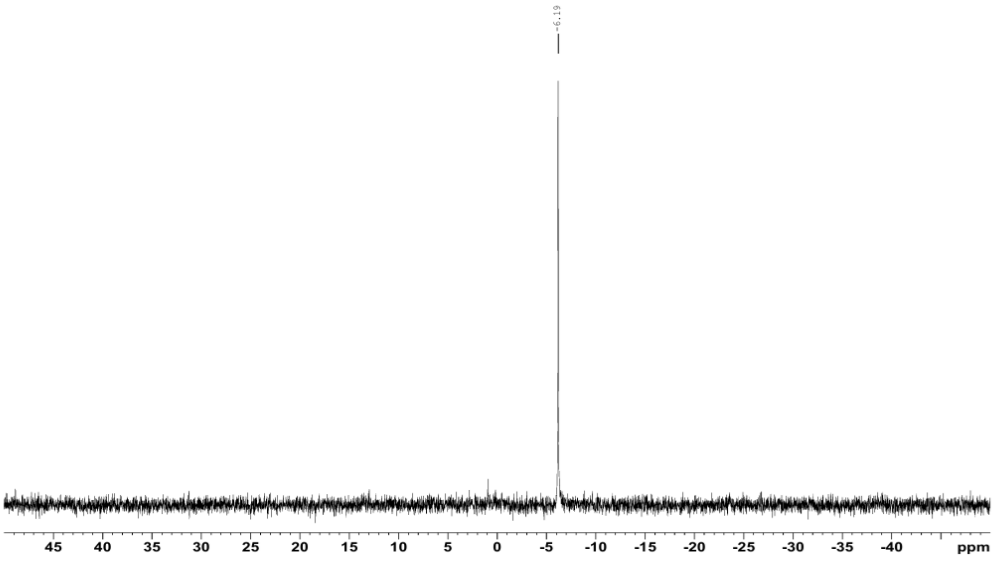
Figure 4 - Compound 14



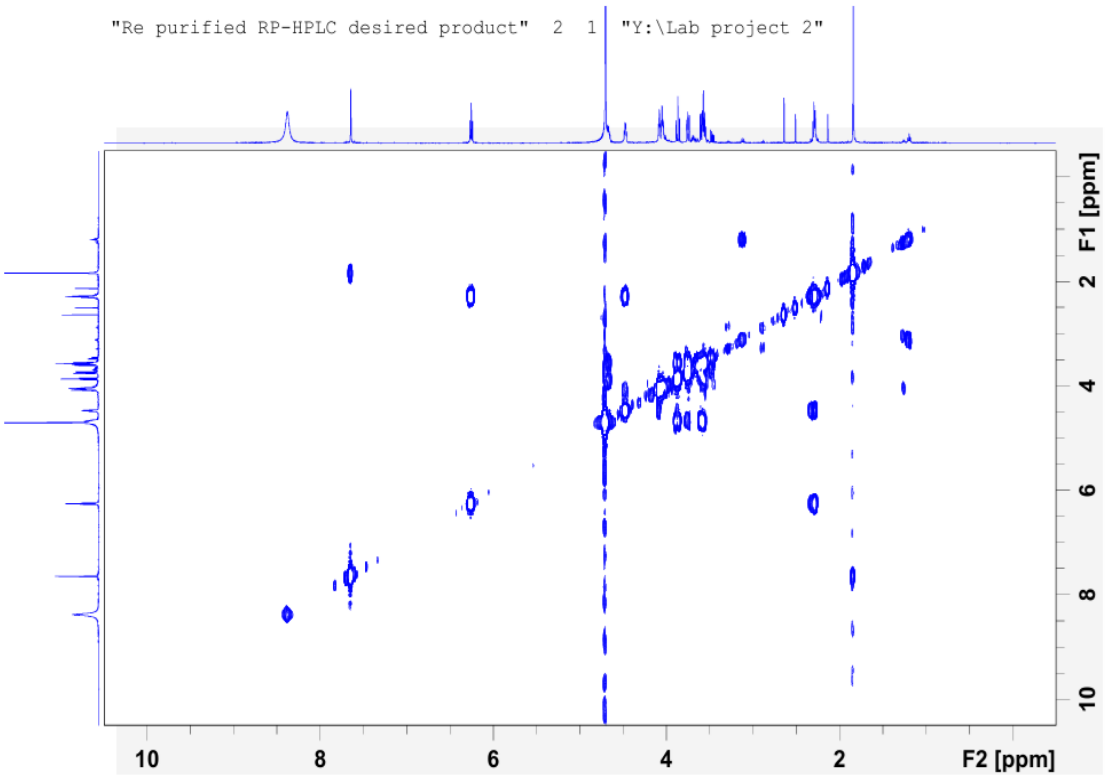
Appendix 14. ^{13}C NMR of compound **14**



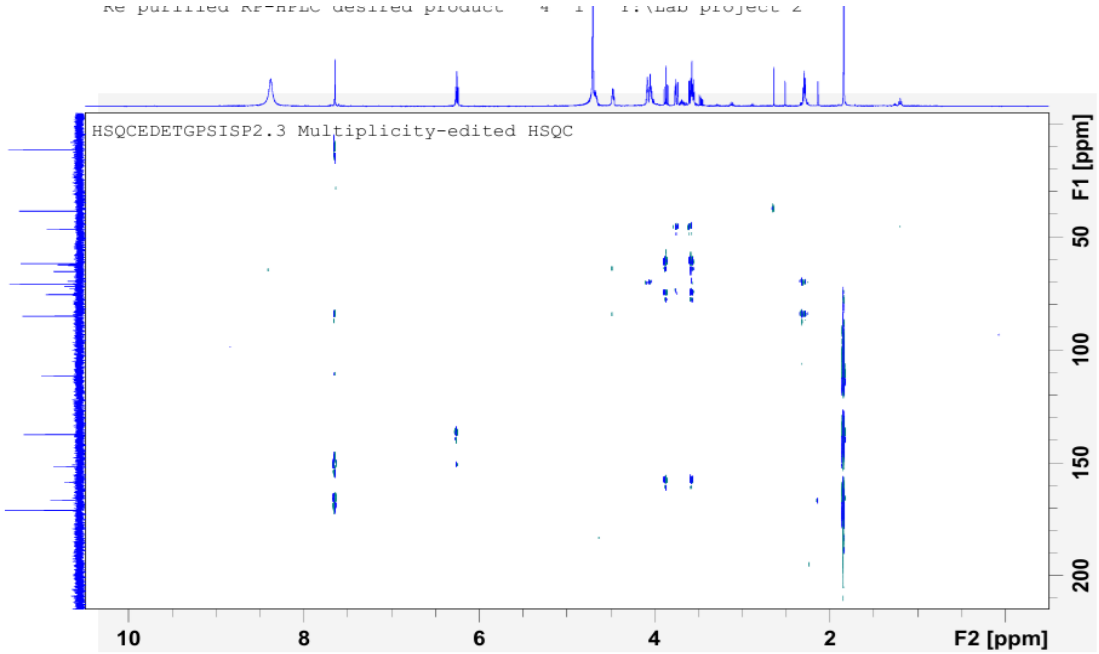
Appendix **15.** ^{31}P NMR of compound **14**



Appendix 16. COSY spectrum of compound **14**



Appendix 17. HSQC spectrum of compound **14**



Appendix 18. ^{31}P HSQC spectrum of compound **14**