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TOWARDS THE DISCOVERY OF OLIGONUCLEOTIDE CROSS-LINKING AGENTS

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TOWARDS THE DISCOVERY OF OLIGONUCLEOTIDE CROSS-LINKING AGENTS

By

Bhaskar Halami

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Chemistry

MICHIGAN TECHNOLOGICAL UNIVERSITY

2019

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This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Chemistry.

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Preface

More than 95% of the research included in this dissertation ‘Towards the Discovery of Oligonucleotide Cross-linking Agents’ was conducted by Bhaskar Halami under the guidance of Dr. Shiyue Fang.

The major portion of the research was previously published in *ChemistrySelect* **2018,** *3* (31), 8857-8862 under the title ‘Incorporation of Sensitive Ester and Chloropurine Groups into Oligodeoxynucleotide through Solid-Phase Synthesis’. The permission has been obtained for the re-use of this published work.

Research group members Shahien Shahsavari, Dhananjani Eriyagama, Zack Nelson, and Lucas Prehoda had synthesized some of the starting materials and linker included in Chapter 2.

Acknowledgements

This was an interesting journey to my Ph.D. degree. Lots of ups and downs while pursuing a dream of the life. This dissertation would not have been possible without a guidance, help, and support of many who encouraged me 24/7 till I finally made it. The first person in this long list of facilitators without any doubt is my Ph.D. advisor Dr. Shiyue Fang. His guidance, perseverance, expertise, and sincerity were the constant motivators throughout my studies. I take this opportunity to extend my sincere gratitude to Dr. Shiyue Fang.

The dissertation committee previously comprised of Dr. Rudy Luck, Dr. S. Komar Kawatra, and Xiaohu Xia while new committee members were Dr. Christo Christov, Dr. Lanrong Bi and Dr. Smitha Rao always supportive through their valuable feedback. The Graduate Programs Committee (GPC) of the chemistry department kept me on the track and helped me maintain balance between coursework, research, and other key requirements to succeed in Ph.D. program. The former chairs Dr. Sarah Green and Dr. Cary Chabalowski as well as the current chair Dr. Jahn Jaszczak have been truly troubleshooters. I acknowledge my Ph.D. committee, GPC, and all chairs for their help and their belief in me.

My special thanks to Dr. Marina Tanasova, Dr. Lynn Mazzoleni and their research groups for the help they extended during the entire period of my Ph.D. program. My teaching supervisors; Andrew Galerneau, Dr. Laleh Vahdat, Lorri Reilly and

Aparna Pandey were instrumental in improving my teaching skills. They made me feel confident as a teacher through their inputs time to time. My sincere thanks to them for all the efforts they put to make me a good teacher. Chemistry staff; Denise Laux, Charlene Page, Kimberly McMullan, Celine Grace, Jerry Lutz, and Dean Seppala were helpful all the times through their support for administrative and maintenance related issues. It was impossible to keep pace of the work without their help. I must thank them for all the kindness they have shown to make the work easy.

I never felt shortage of funds only because of the generous support from; the Ministry of Tribal Affairs, Government of India through the National Overseas Scholarship for first four years of the study. The remaining period of my study was supported by the chemistry department, NIH, NSF, David and Valeria Pruett Research Fellowship, Robert and Kathleen Lane Research Fellowship and Portage Health Foundation Graduate Student Assistantship. Also, ACS Upper Peninsula Local Section and Graduate Student Government, MTU provided me a travel grant to attend national meetings. I am thankful to every funding agency for supporting me throughout this journey.

I thank my lab mates; Dr. Durga Pokharel, Dr. Jinsen Chen, Dr. Ashok Khanal, Dr. Shawn Shahsavari, Mrs. Dhananjani (DJ), Zack Nelson, Lucas Prehoda, Criss, Komal, Liam and Brett for their help, support and nice time in the lab. Friends at MTU; Giri Vegesna, Wafa Mazi, Rashmi Adhikari, Vagho, Jianheng Bi, Jingtuo, Sudhir Khodwekar, Abdul Azeez, Niranjan, Hemant and Apurva were equally

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My mom and dad, wife and daughter were the sufferers during these six years but they faced every hardship with smile without letting me fall behind in my studies. I cannot even imagine to move an inch on my journey to the Ph.D. degree without their firm support. I am fortunate to have such caring parents and lovely family. I owe every bit of happiness they sacrificed for my education. Just thanking them is not enough therefore, I would like to promise them a better life ahead!

Abstract

Oligonucleotide cross-linking agents are expected to become a new class of antisense drugs. They function by first hybridizing with a complementary mRNA and then form a covalent bond to permanently link the mRNA to the oligonucleotide agent. Because of covalent cross-linking, this new class of antisense drugs are expected to have much higher potency than existing ones.

Some oligonucleotide cross-linking agents have appeared in the literature, but they have various drawbacks, which include slow cross-linking rate and reversible cross-linking. To address these problems, we designed a series of new cross-linking oligonucleotides with a range of different reactivities. These oligonucleotides were expected to react with complementary mRNA with higher rates and selectivity. To synthesize this new class of cross-linking agents, we used the Dmoc oligonucleotide synthesis technology recently developed in our laboratory. Using this new technology, oligonucleotide deprotection and cleavage were achieved under mild conditions, under which the cross-linking functions in our agents were kept intact. In contrast, if known oligonucleotide synthesis technologies had been used, the cross-linking functions would not have survived. After successful synthesis of the new class of cross-linking oligonucleotides, they were subjected to hybridization with complementary oligonucleotides under various conditions. The progress of the hybridizations was monitored and analyzed with RP-HPLC and MALDI-TOF. At the current stage of the project, the

properties of the cross-linking oligonucleotides were found to be less than ideal. However, successful synthesis of potential cross-liking oligonucleotides, development of key procedures and protocols for cross-linking experiments and development of analytical techniques to detect cross-linked products have paved the foundation for us to design and screen a series of similar oligonucleotides. Because as long as we can place the cross-linking function at proximity to a reactive site of complementary oligonucleotide, the two oligonucleotides will be forced to react, we believe that screening a series of oligonucleotides will provide desired cross-linking agents.

1 Introduction

1.1 Oligonucleotides

Oligonucleotides are short nucleic acids. There is no strict rule found about the length of oligonucleotides. The term oligonucleotide has been used for a chain of nucleotides as small as 2 nucleotides to couple hundred nucleotides long. The general structures of DNA and RNA strands are shown in Figure 1.1; DNA **11** composed of four nucleobases (adenine, cytosine, guanine and thymine), the phosphate backbone and a sugar moiety. RNA **12** on the other hand is slightly different with an extra hydroxyl group at 2' carbon on a sugar moiety and uracil replaces thymine.

Oligodeoxynucleotides (ODNs) or DNAs are important members of oligonucleotide family. Two types of nucleobases; purines (adenine and guanine) and pyrimidines (cytosine and thymine) are present in a certain order to make DNAs functional. DNAs are primary genetic materials present mainly in the nucleus of the cell. DNAs can interact efficiently with complementary DNAs as well as complementary RNAs to form a double helix.[1] They also play significant role in cellular metabolism by producing mRNAs through transcription which is followed by the synthesis of a protein. Proteins control metabolic processes of the cell through various enzymes. Formation of protein is very vital for all physiological processes occurring in the body of an organism.

RNAs on the other hand are like a carrier which carry different codes from genes

to other parts of the cell mainly to produce proteins and regulate cellular metabolism. They can be found both inside the nucleus and in the cytoplasm.

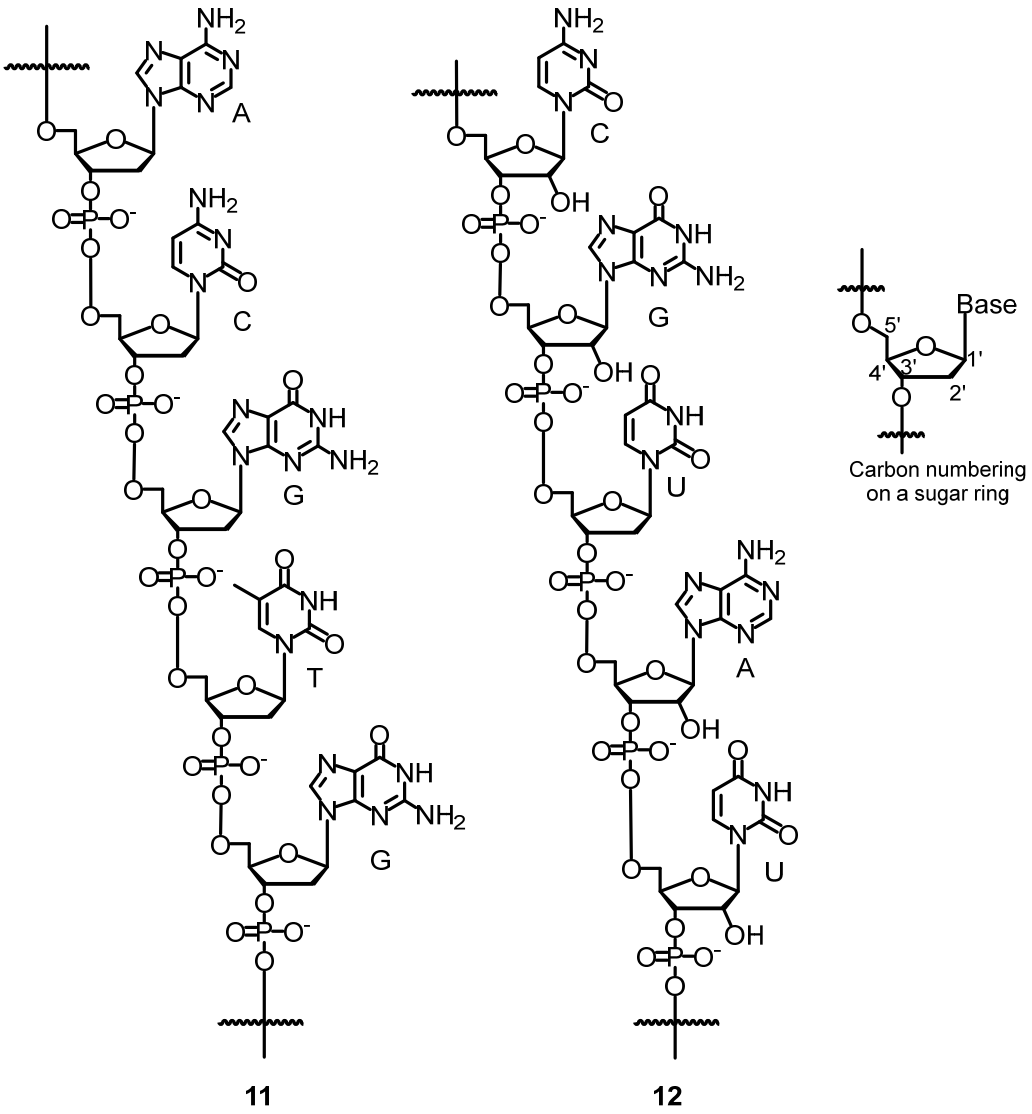
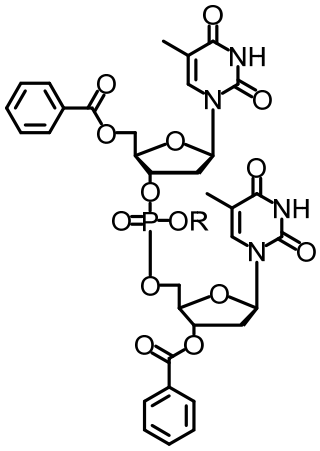


Figure 1.1 Structure of oligonucleotides

1.1.1 A brief history

The oligonucleotide synthesis was first reported in 1955. Michelson and Todd

successfully synthesized a dinucleotide, dithymidinyl **13** (Figure 1.2).[2] The molecule **13** they synthesized was protected by benzoyl protection from both 5' and 3' ends.



13

Figure 1.2 Dithymidinyl

Har Gobind Khorana and co-workers reported the synthesis of series of oligonucleotides in late 1960s and throughout 1970s using phosphodiester coupling approach.[3-5] They reported the synthesis of an active 72 mer tRNA in 1971.[3] During the same period Letsinger introduced solid support; first for the peptide synthesis and then for an oligonucleotide synthesis.[6] He also improved Khorana's phosphodiester approach by introducing phosphotriester method.[7] Beaucage and Caruthers introduced phosphoramidite chemistry by replacing chlorine by an amine as a leaving group from phosphotriester.[8] This simple change later became a reason for the construction of first automated oligonucleotide synthesizer, ABI in 1980s. Manual oligonucleotide synthesis needs special synthetic skills and is cumbersome. With the advancements in automated

oligonucleotide synthesis, manual synthesis became virtually extinct.

1.1.2 The current scenario

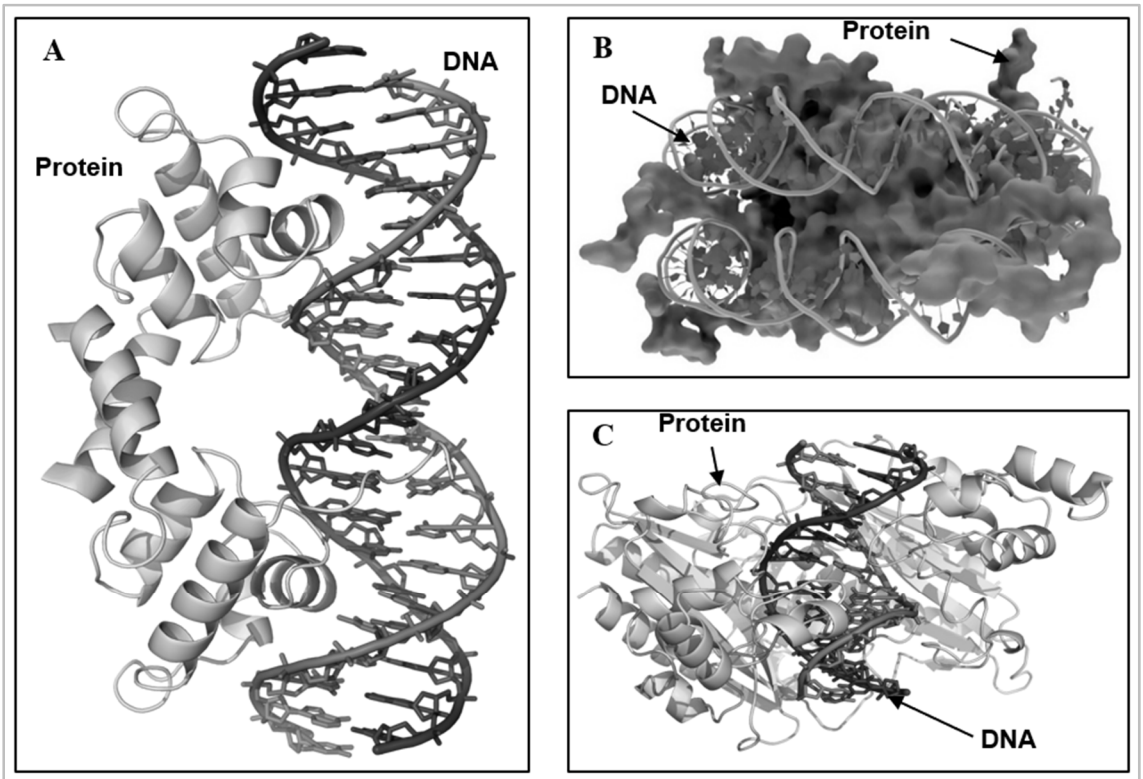
Today, we can synthesize oligonucleotides from few nucleotides long to few hundred nucleotides long. An automation of the synthesis process and solid phase synthesis by phosphoramidite approach have made it possible to synthesize millions of different oligonucleotides simultaneously from milligram to kilogram scales.[9-15] The ability to chemically assemble oligonucleotides on a solid support efficiently has made a broad impact in last few decades on many research areas including molecular biology,[16, 17] chemical biology,[17-19] synthetic biology,[20, 21] data storage,[22, 23] nanotechnology[24, 25] and medicine.[17, 19, 26] The current research in the field is visibly focused on the applications of oligonucleotides.

1.2 The need of modified oligonucleotides

Natural DNAs have been used successfully for gene synthesis,[27] understanding structure and functions of biomolecules,[28, 29] transgenic crop development[30, 31] and many more. But they need to be modified for some of the important purposes. To mention few; areas of epigenetics,[32, 33] chemical ligation,[34, 35] oligonucleotide conjugates,[36-38] DNA damage repair,[39-41] mutagenesis,[42, 43] peptide fusion[44] and antisense drug development[32, 45] need modified oligonucleotides. Out of several useful functions of modified oligonucleotides DNA-protein interactions, sensitive nucleic acids and cross-linking agents are of special interest to us. It is

because they involve binding of biomolecules together which is the basis requirement for the development of an oligonucleotide cross-linking agent. These interactions are discussed in following paragraphs.

1.2.1 DNA-protein interactions



A: The lambda repressor helix-turn-helix transcription factor bound to its DNA target
B: Interaction of DNA with histones
C: The restriction enzyme EcoRV-DNA complex

Figure 1.3 DNA-protein interactions

It is well known that several proteins interact with DNAs.[28, 46, 47] Out of many such interactions, three different interactions **A**, **B** and **C** are shown in a Figure 1.3.[48, 49] The interaction **A** shows the binding of a lambda repressor helix-turn-helix factor to its DNA target. Lambda repressor is a gene regulatory protein that binds to target

DNA by hydrogen bond formation and regulates a gene synthesis.[48, 50] The interaction **B** depicts the association of histones with DNA. This interaction checks the supply of transcription factor and makes gene expression slow.[29, 49] While third interaction **C** shows how restriction enzyme EcoRV binds to DNA. This binding eventually destroys the DNA.[51]

The DNA-protein interaction mainly manages the length of a genetic material by controlling gene expression.[52] Because binding of protein to DNA regulates the structure of chromatin (a genetic part of DNA), transcription and DNA repair processes.[29, 53, 54] These interactions are very important in gene therapies to understand and treat genetic disorders like cancers. The intervention of such interactions is possible by modified DNAs to regulate the expression of a disease-causing gene.[55-57]

1.2.2 Sensitive nucleic acids

Natural nucleic acids were considered only to have nucleobases adenine, guanine, cytosine and thymine in DNAs and adenine, guanine, cytosine and uracil in RNAs in their standard forms (Figure 1.1). Recent discoveries show that these nucleobases undergo different modifications which result into several sensitive nucleic acids containing modified nucleobases like N^4-acetylcytosine **14**, 5-methoxycarbonylmethyl-2-thiouridine **15** and wybutosine **16** (Figure 1.4).[58] The sensitive nucleic acids possess a second layer of information that is mostly noncoding in nature. These nucleic acids bind to the target proteins and RNAs and

play significant roles in cellular metabolism.[58] So far over 100 such modifications are reported for RNAs but they are present in the trace amounts. There is a need for the synthesis of sensitive nucleic acid with such modifications to target cellular metabolic process that causes health problems.

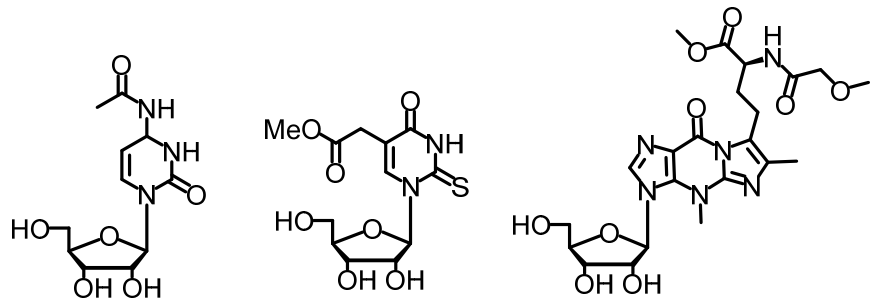


Figure 1.4 Sensitive nucleic acid bases

1.2.3 Cross-linking agents

Cross-linking oligonucleotide agents are usually synthetic and are recent entries to the field of nucleic acids. The modifications are designed to achieve cross-linking between two oligonucleotide strands.[59] Cross-linking can occur between DNA-DNA, DNA-RNA and RNA-RNA strands. Cross-linking agents are used successfully along with dyes for the detection of cancers and several other genetic disorders.[60] The cross-linking by modified oligonucleotides has an ability to alter or stop the replication,[61] transcription[62, 63] and translation[63, 64] processes by the formation of cross-linked product with DNA or RNA. Oligonucleotides as cross-linking agents have been widely studied by researchers for the development of

antisense drugs.[32, 65, 66] Antisense drugs can treat several genetic disorders selectively by stopping the synthesis of a disease-causing protein.

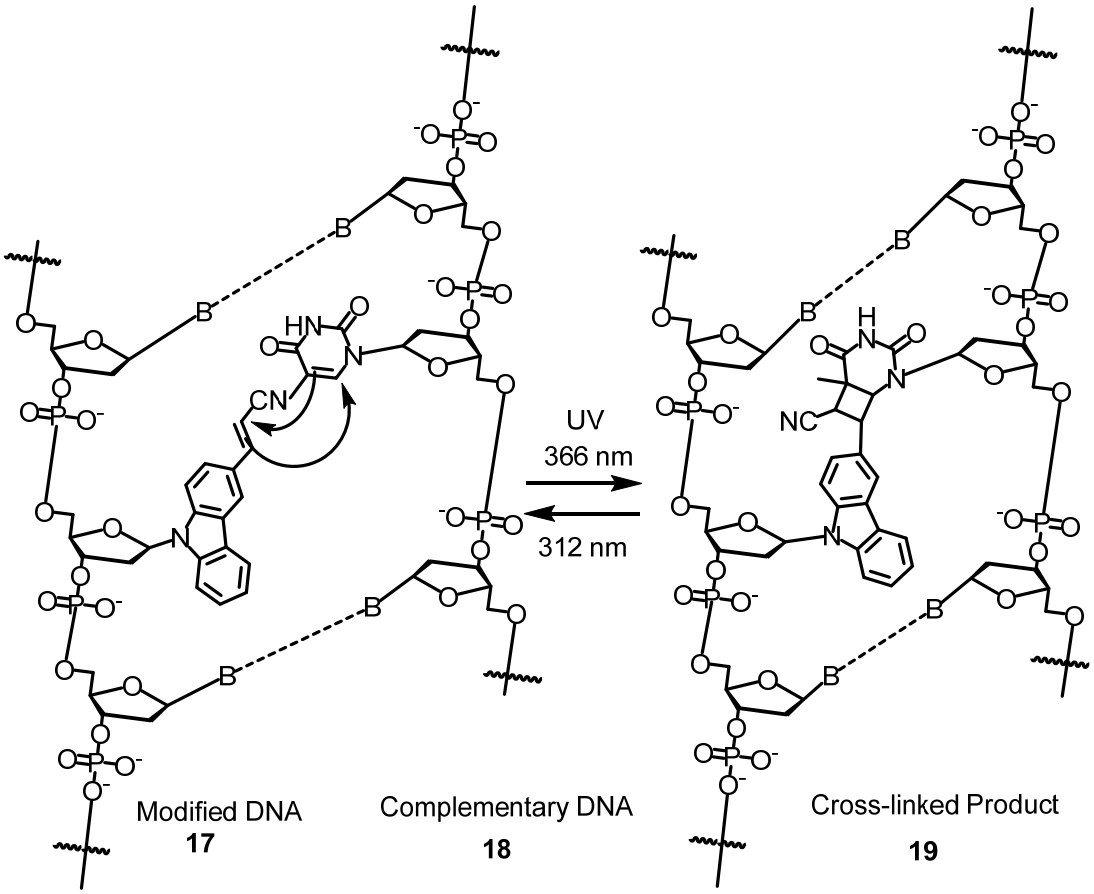
The binding affinity of biomolecules (DNAs, RNAs and proteins) is the useful tool for bringing two molecules together and holding them together through a permanent linkage. Several oligodeoxynucleotide (ODN) cross-linking agents are already reported with some serious limitations. The limitations of current ODN cross-linking agents provide an opportunity for the development of an 'ideal' ODN cross-linking agent for antisense drug development.

1.3 ODN cross-linking agent for an antisense drug development

1.3.1 Known cross-linking agents and their limitations

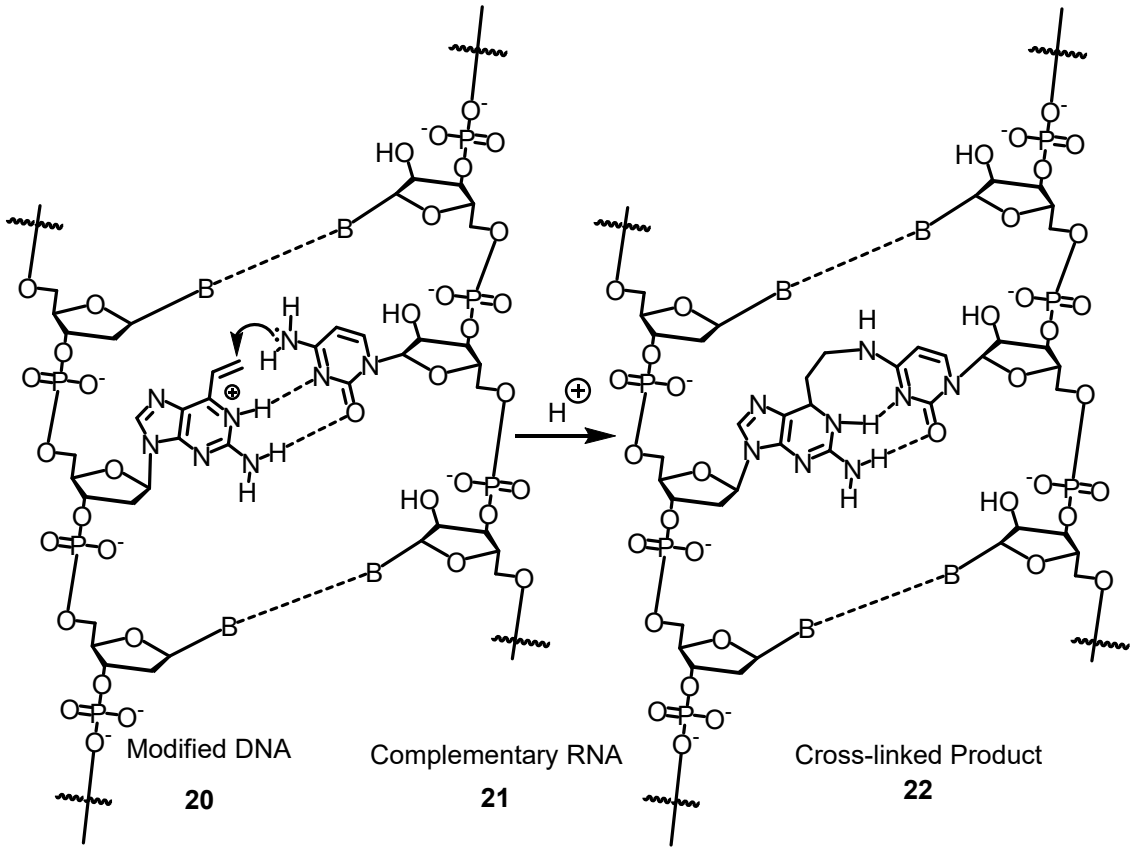
Several attempts have been made by synthetic chemists for the synthesis of an oligodeoxynucleotide cross-linking agent to facilitate antisense drug development. But almost all of them have a limited success.[67-70]

In one such attempt (Scheme 1.1) oligodeoxynucleotide **17** containing 3-cyanovinyl-carbazolenucleoside (CVCbN) found to be cross-linked with the complementary DNA strand **18** on irradiation of UV light at 366 nm to produce a cross-linked product **19**.[71] The change is temporary as cross-linking can be reversed by small increase in the energy to 312 nm to give back **17** and **18**. The use of UV light itself can cause the non-selective irreversible damage to the cell as well as can produce unwanted gene mutations.



Scheme 1.1 CVCbN oligonucleotide cross-linking agent

Another interesting cross-linking oligodeoxynucleotide agent was reported by Sasaki and co-workers in 2005 (Scheme 1.2).[59] They developed oligonucleotide **20** containing 6-vinylpurines as a cross-linking agent and got relatively stable cross-linked product **21** under acidic conditions. The slower action and selectivity are two major limiting factors to use vinylpurines for the development of an antisense drug agents. To overcome these limitations several derivatizations were carried out over vinylpurines with slightly better cross-linking properties.[70, 72, 73] Though some improvements have been made over the period, they are not suitable for the development of an antisense drug.



Scheme 1.2 Vinylpurine oligonucleotide cross-linking agent

1.3.2 An ideal oligodeoxynucleotide cross-linking agent

Antisense drug binds to the mRNA and stops its supply for protein synthesis. Because no more mRNA is available no protein synthesis is possible. Different mRNAs carry different information or codes specific to a protein. So different proteins are synthesized by different codes and need their own mRNAs. The synthesis of a protein responsible for diseases like cancer can be selectively stopped by cross-linking of a responsible mRNA to the modified oligonucleotide

cross-linking agent. To make this possible cross-linking agent should possess following properties.

1.3.2.1 Irreversible binding to mRNA

Covalent cross-linking perhaps is the only way to form a permanent bond between two interacting entities; a modified oligodeoxynucleotide and mRNA strand. But even covalent bonds are reversible in nature.[71] Modifications should be made to achieve irreversible covalent bond. This specificity and some other difficulties discussed later in the following paragraphs make structural modifications of nucleobases difficult and leaves synthetic chemist with very few choices. Therefore, attempts are being made in the direction of putting electrophilic group on the modified nucleobase so that nucleophilic nitrogen on the natural nucleobases can react with it to form an irreversible covalent bond.[59, 71] Examples of oligonucleotide cross-linking agents discussed are designed by considering this point of view. Results are not very exciting but electrophile-nucleophile chemistry provides the best platform for the design of oligonucleotide cross-linking agents by offering plenty of choices. So, we decided to stick to the electrophile-nucleophile chemistry for designing a cross-linking agent that can form a permanent bonding between modified oligodeoxynucleotide and mRNA.

1.3.2.2 Fast binding to targeted mRNA

Oligodeoxynucleotide cross-linking agents reported so far are slower in actions or

they are somewhat faster but have reversible nature.[59, 70, 74] Antisense drug needs to be quicker in action and slow reactivity of cross-linking agent is bound to affect the pace of the drug action.

1.3.2.3 High selectivity

Sequence selectivity is very important to target a mRNA fragment specific to a disease-causing mRNA strand. This problem is mostly addressed by designing a longer non-coding oligodeoxynucleotide strand complementary to the target mRNA fragment.

1.4 Our design of cross-linking agents for antisense applications

Keeping ideal assumptions about the oligonucleotide cross-linking agent in mind modified nucleobases **1**-**5** (Figure 1.5) have been designed. The design is based on nucleophile-electrophile chemistry which works better when an electrophile has a leaving group. Previous attempts failed to provide a suitable leaving group for a nucleophilic attack to produce a permanent covalent bond.[59, 70-73] Absence of a leaving group is one of the main drawbacks of cross-linking agents in the past.

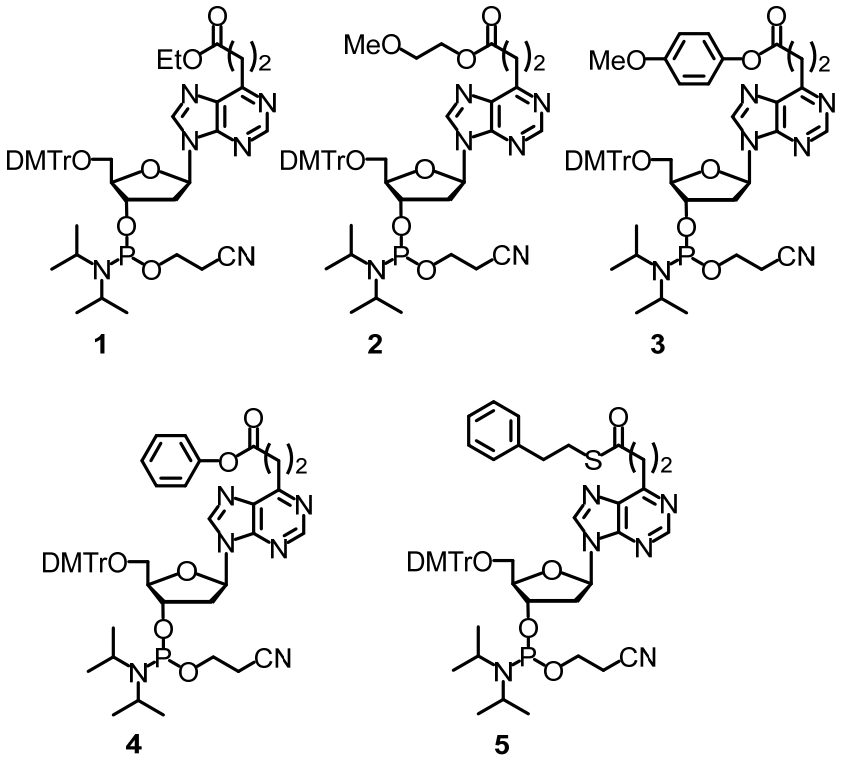


Figure 1.5 Modified nucleobases

Modified Nucleobases **1** through **5** contain ethyl ester, methoxyethyl ester, methoxyphenyl ester, phenyl ester and a thioester group respectively. Modified nucleobase **1** is the least reactive while nucleobase **4** is the most reactive. The reactivity modified nucleobase **5** is difficult to compare in absence of suitable data. But it is more reactive than nucleobases **1** and **2** as it has an electron withdrawing phenyl group attached to sulfur through an ethylene bridge. These modified nucleobases offer a great pool of esters with varying reactivities to facilitate cross-linking.

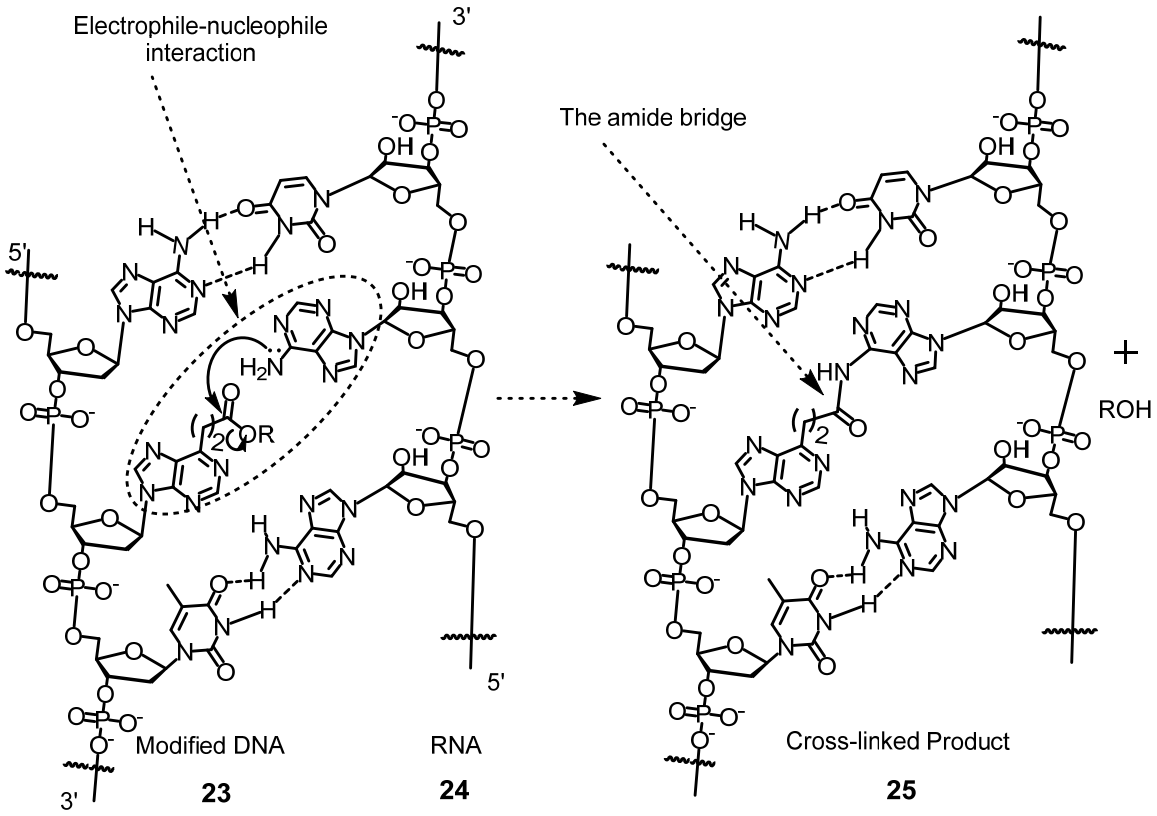
The proposed modified nucleobases have all features necessary for the design of a cross-linking agent which can be useful for the development of an antisense

drug. The modified nucleobase with an ester group are designed to incorporate them at any position into oligodeoxynucleotide chain. The major part of an oligodeoxynucleotide chain can be designed with natural nucleobases to form a double helix with complementary DNA or mRNA fragment. The position of modified nucleobase in a oligonucleotide strand was defined to have adenine or guanine as a base complementary to it on the targeted DNA or mRNA strand. As both adenine and guanine have exo-amine groups, the cross-linking can happen by the attack of an amine nitrogen (a nucleophile) on a carbonyl carbon (an electrophile) of an ester group leaving alcohol as a byproduct (Scheme 1.3).

1.4.1 The mechanism

The proposed design (Scheme 1.3) introduces an ester group on a modified oligonucleotide **23** to facilitate electrophile-nucleophile interaction with complementary RNA **24**. Modified DNA is complementary to the targeted RNA so that they can form double helix selectively. After they form a double helix nucleophilic nitrogen of adenine on RNA, present complementary to the ester nucleobase would attack the carbonyl carbon making a cross-linked product **24** with an amide bond. The byproduct alcohol will form as shown in the Scheme 1.3. Amide bond is known to be very stable and once it forms reaction cannot go backwards. Formation of an amide linkage will result into permanent bridge between RNA and modified DNA.

Many leaving groups like halides, water, tosylates etc. were considered before finalizing an alcohol as a leaving group. The reason for choosing an alcohol as a leaving group lies in the flexibility of esters. It is possible to modulate an ester group over the wide range of stability and selectivity using available synthetic chemistry methods.



Scheme 1.3 Proposed cross-linking mechanism

1.4.2 The challenge associated with their synthesis

The success of ODN synthesis is mainly limited to unmodified ODNs. For modified ODNs, we still face many challenges.[75-77] In one particular case, due to the use

of acyl protecting groups and linkers during solid phase synthesis and their need of strong nucleophiles for deprotection and cleavage, many nucleophile-sensitive groups could not be incorporated into this highly important class of materials. Examples of the sensitive groups include many common ones in organic chemistry such as esters, alkyl halides and epoxides. The designs of modified nucleobases **1** through **5** are very sensitive to these nucleophilic conditions.

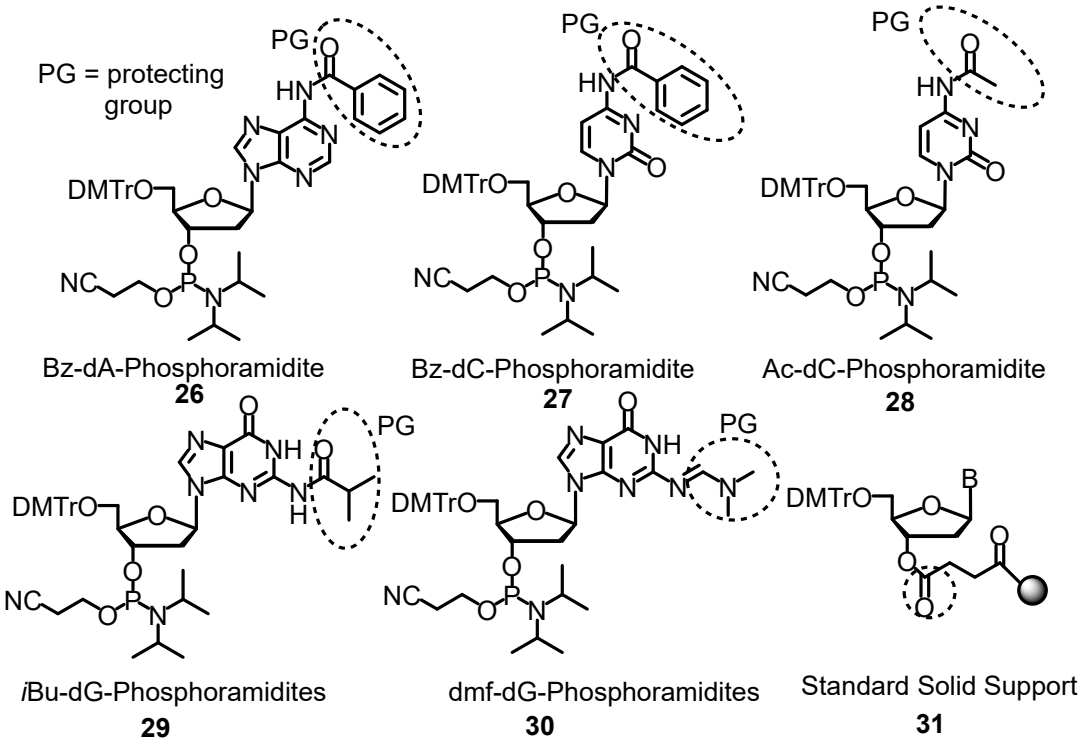


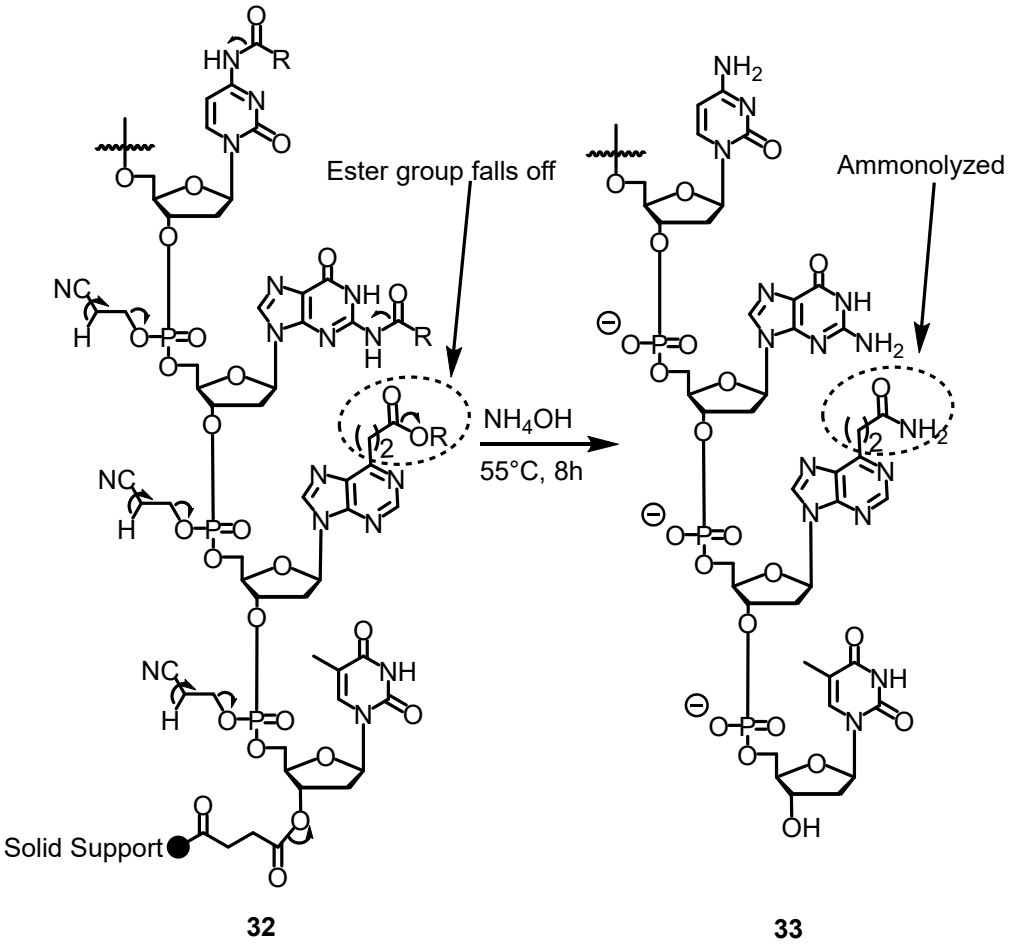
Figure 1.6 Phosphoramidites for standard DNA synthesis

The current standard DNA synthesis requires dA, dC, dG and dT phosphoramidites and a linker (Figure 1.6). As shown in Figure 1.6, exo-amine group of commercially available dA phosphoramidite is protected by benzoyl group **26**, dC phosphoramidite by benzoyl **27** or acetyl **28,** dG phosphoramidite by

isobutyryl **29** or dimethylaminoformamidine **30** and linker contains a strong ester group **31**. All protecting groups and a linker are designed to sustain DNA synthesis conditions which vary from slightly basic to moderately acidic. These protecting groups can be removed only by the strong bases like ammonia or methylamine. Therefore, the post DNA work-up needs the use of a strong base for deprotection and cleavage.

The use of strong base for post DNA synthesis work-up was the cause of concern as proposed nucleobases **1**-**5** containing variety of ester groups are not compatible with it. The nucleophilicity of bases used in standard DNA synthesis for work-up is very strong for esters to survive through the process (Scheme 1.4). The treatment of oligonucleotide **32** containing sensitive ester nucleobases **1-5** can easily fall off by the nucleophilic action of an ammonia to give ammonolyzed product **33**. This amide has no use in the designing of cross-linking oligonucleotides.

Therefore, to install these sensitive ester groups on oligonucleotide, needed a different DNA synthesis technology. Fortunately, the incorporation of sensitive groups into oligonucleotides has been made possible by 'Dmoc' oligonucleotide synthesis technology developed and introduced by professor Shiyue Fang.[76, 78] Though the stability of targeted esters using Dmoc technology was not known, the use of mildest possible post DNA synthesis work-up conditions and success in incorporation of some electrophiles into ODN,[76] made it the best choice of the time.



Scheme 1.4 Action of ammonia on the ester groups

1.4.3 The Dmoc technology of oligonucleotide synthesis

1.4.3.1 The synthesis

Unlike standard DNA synthesis, Dmoc technology uses phosphoramidites containing nucleobases protected by Dmoc protecting group. The abbreviation Dmoc stands for 1,3-dithian-2-yl-methoxycarbonyl. This abbreviated form has been chosen for naming the technology. All nucleobases (dA, dC and dG) requiring

exo-amine protection were protected by Dmoc and the linker was also developed using Dmoc protection on a CPG solid support (Figure 1.7). The oligodeoxynucleotide synthesis by Dmoc technology uses standard oligonucleotide synthesis cycle. Along with commercially available dT phosphoramidite, the reagents for detritylation, coupling and oxidation remain same. It requires suitable capping agent to avoid problems related to cap exchange.

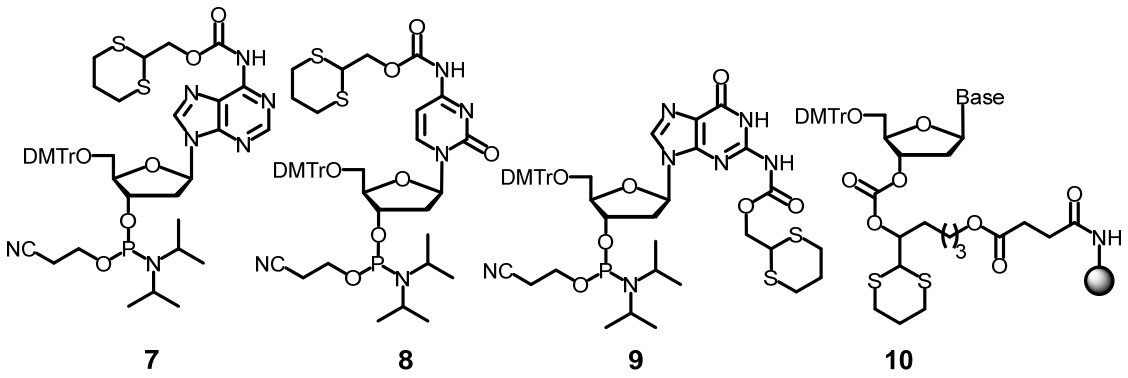


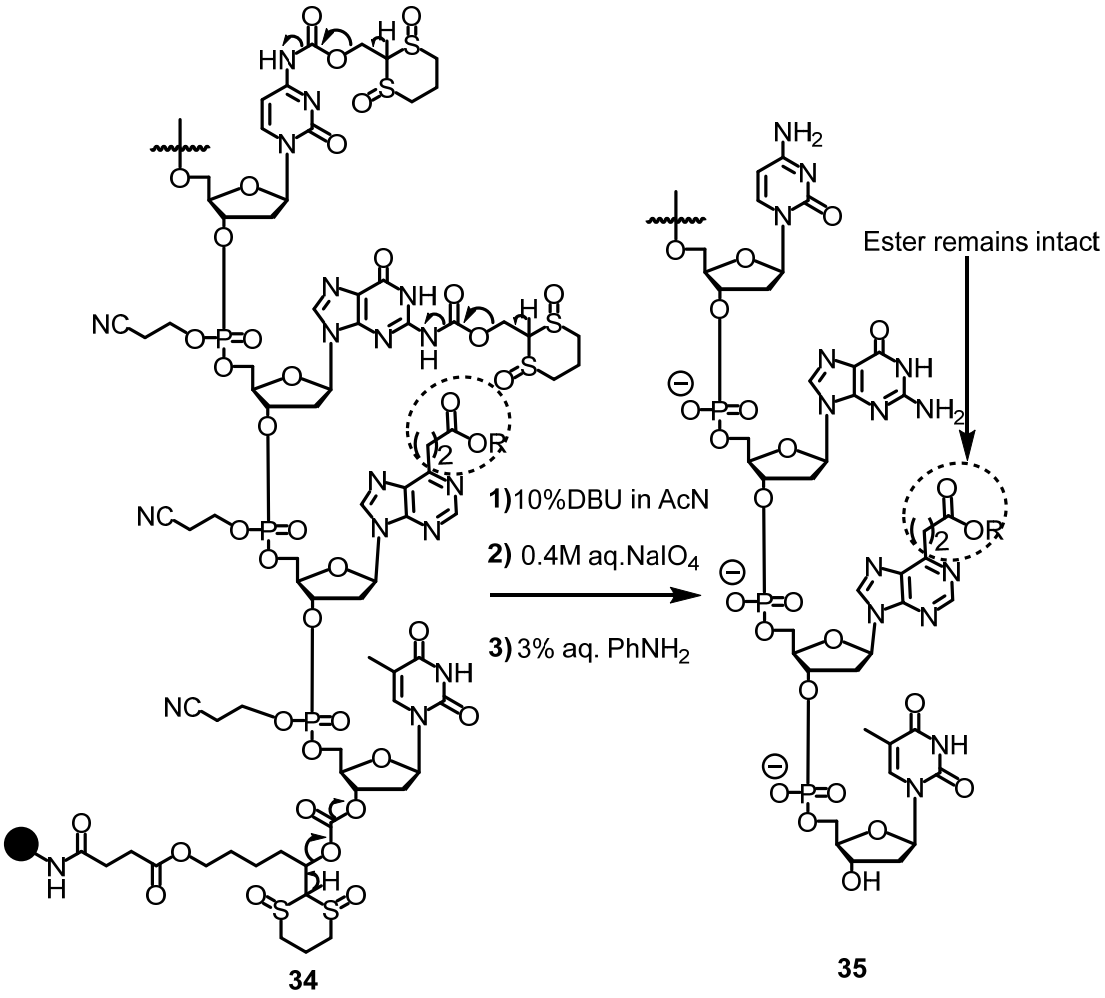
Figure 1.7 Dmoc protected phosphoramidites and linker

The major difference between standard DNA synthesis and synthesis by using Dmoc technology is post synthesis work-up. Standard technology uses strong bases like ammonia while Dmoc technology made work-up possible under nearly neutral conditions.

1.4.3.2 Post synthesis work-up using Dmoc technology

Dmoc technology carries post synthesis work-up (Scheme 1.5) in three key steps starting with the treatment of an oligodeoxynucleotide on CPG with 10% DBU in

acetonitrile



Scheme 1.5 Post synthesis work-up by Dmoc technology

. DBU being a base effects β-elimination to remove cyanoethyl group attached to the phosphorus backbone. In next step oxidation of all sulfurs present on Dmoc protections is carried out by using 0.4 M aqueous sodium periodate. Oxidation of sulfurs in Dmoc protecting group has been carried out to increase the acidic nature of tertiary proton adjacent to the sulfurs. Increased acidity of this proton then used to remove the protection by using 3% aqueous aniline suspension which effects

the cleavage and deprotection through β-elimination shown in Scheme 1.5 to give oligonucleotide. All steps involved in post synthesis work-up using Dmoc technology are conducted under nearly neutral conditions and therefore sensitive ester group on **34** survives in the final oligonucleotide product **35**.

1.5 References

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2 Synthesis of modified nucleobases

2.1 Introduction

Oligonucleotide cross-linking agents have the potential to be a next generation antisense drug. Reported ross-linking agents known to have several limitations to use them as antisense drugs.[1, 2] We have designed oligonucleotide cross-linking agents which can potentially overcome those limitations and serve the purpose. To synthesize oligonucleotides with cross-linking features we used Dmoc oligonucleotide synthesis technology. To accomplish the goal, we synthesized modified nucleobase phosphoramidites **1**-**4** with ester group and **5** with a thioester group. Also, we synthesized Dmoc protected standard phosphoramidites **7**-**9** and a linker **10** (Figure 2.1). This chapter is focused on the synthesis and characterization of these compounds.

2.2 Results and discussion

The synthesis started with the synthesis of compound **36** according to reported procedure.[3, 4] Cross-coupling of **36** with the commercially available 2-(ethoxy-carbonyl)ethylzinc bromide **37** using a palladium catalyst gave **38** containing one of the desired esters.[5] The other ester-containing monomers **2-5** were synthesized from **38**.[6] Hydrolysis of **38** under basic conditions gave the nucleoside carboxylic acid **39**. Hydrolysis conditions were defined through trial and errors to ensure the stability of glyosidic bond. Coupling **39** with the

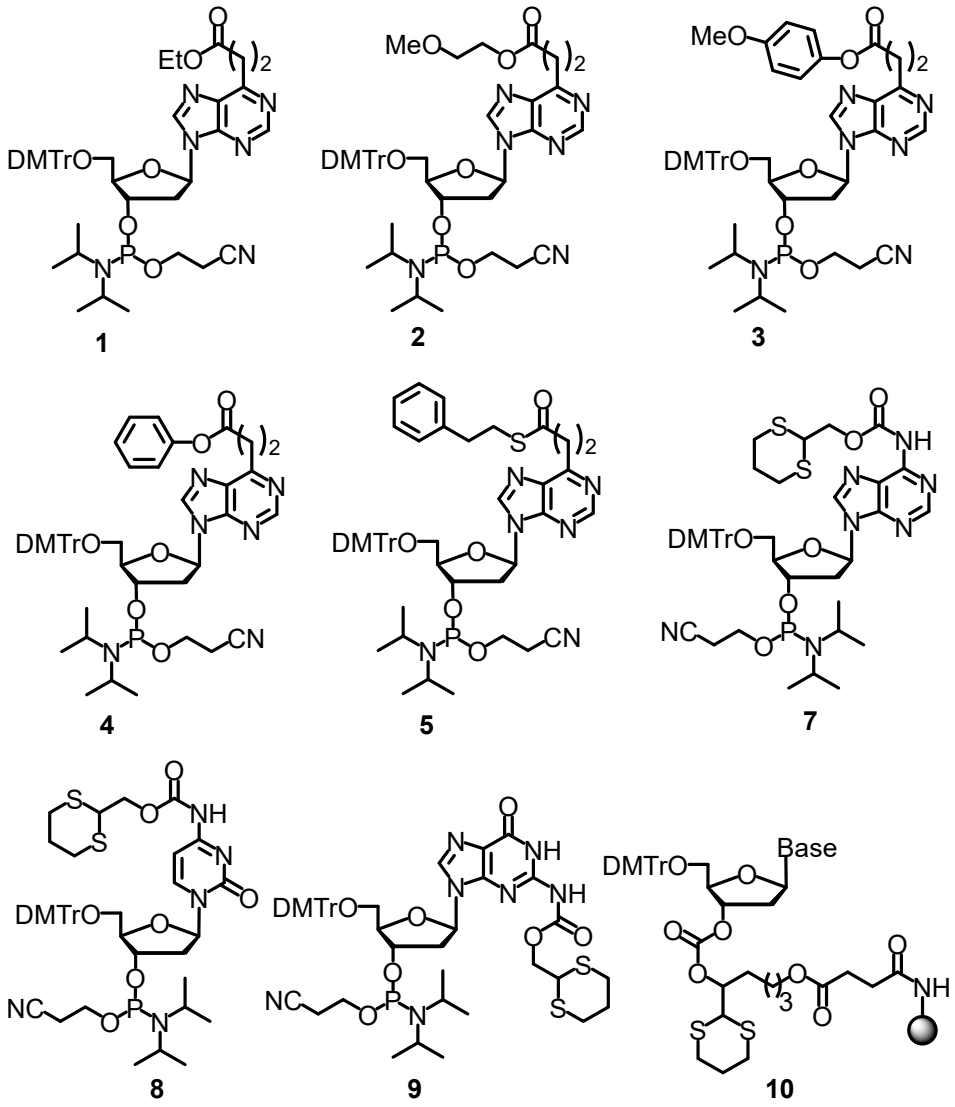
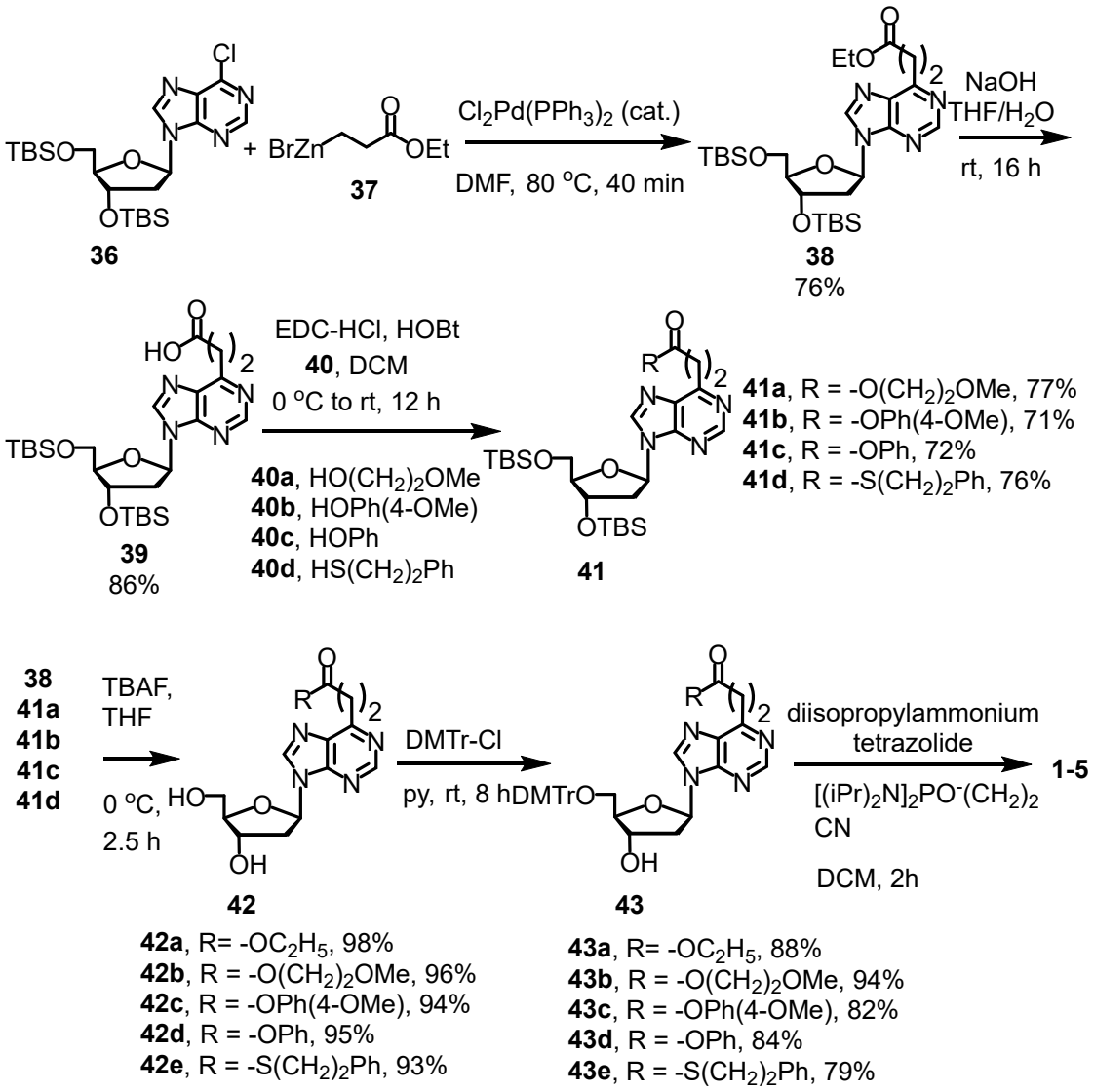


Figure 2.1 Modified and Dmoc nucleobase phosphoramidites, and linker

alcohols **40a-40c** and the thiol **40d** gave the esters **41a-41c** and the thioester **41d**, respectively. Removing the silyl protecting groups of **38 and 41a-41d** using TBAF gave **42a-42e**. Removal of silyl protecting group from **41a-41d** by TBAF at room temperature was destroying all the starting materials and resulting into very complex reaction mixture without the traces of products. Other deprotecting



Scheme 2.1 Synthesis of modified nucleobases **1-5**

agents like triethylamine hydrofluoride, pyridine hydrofluoride were tried but either they were slow or yielding the similar results. Finally, carrying deprotection strictly under 0 ºC and performing quick purification using neutralized silica gel worked. The yields obtained by this way were close to 100% in all cases. Later, compound **38** was found to was found to be synthesized with better results under these

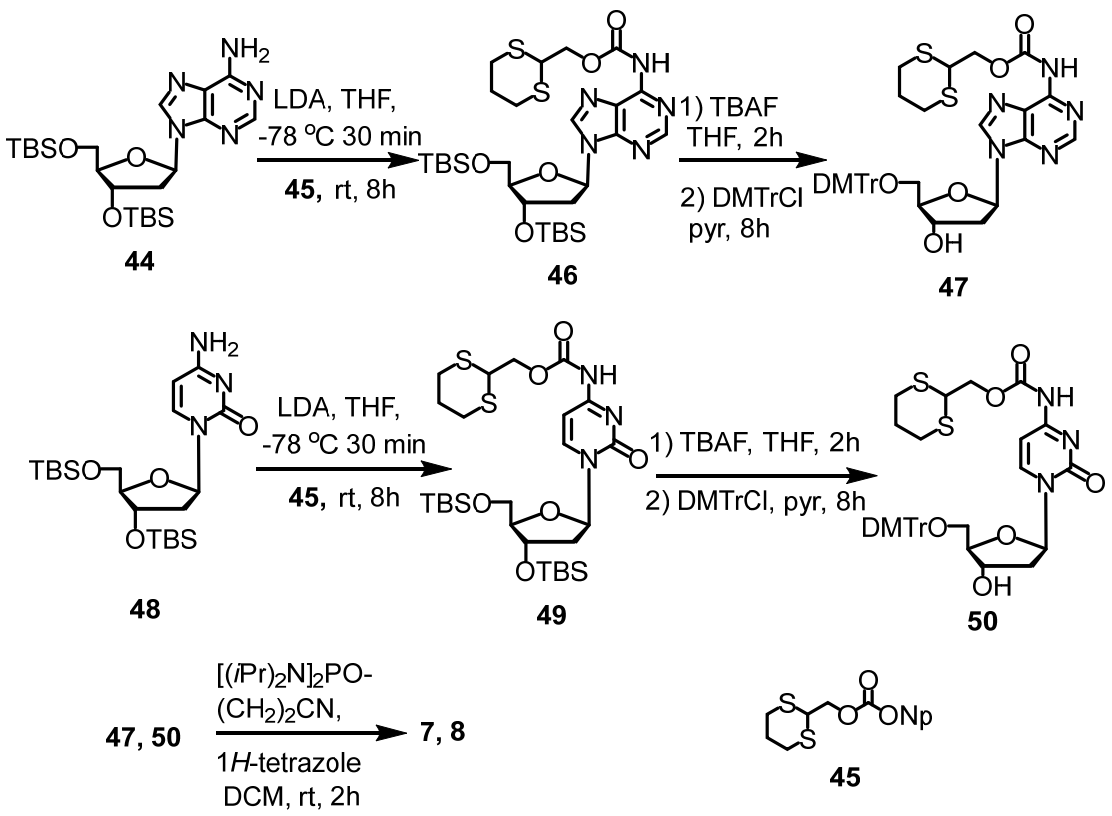
conditions. Selective tritylation of the primary hydroxyl group of **42a-42e** using DMTr-Cl gave **43a-43e**. This was again found to be working differently for different monomers.

For the substrates **42a** and **42b** tritylation was smooth but other substrates needed special precautions to perform column chromatography for their purification. Ensuring neutralization of silica gel and quick purifications solved the problems to get desired products in other cases. Converting **43a-43e** to the ester-containing phosphoramidite monomers **1-5** was achieved using the phosphitylation reagent 2-cyanoethyl *N*,*N*,*N′*,*N′*-tetraisopropylphosphordiamidite and an activator diisopropylammonium tetrazolide.[32]

Dmoc protected phosphoramidites **7**-**9** and linker **10** (Figure 2.1) were synthesized using procedures developed previously by our group with slight modifications for dG-Dmoc phosphoramidite **9**.[7]

Synthesis of Dmoc-dA-phosphoramidite **7** was accomplished in four steps (Scheme 2.2); compound **44** was protected by Dmoc successfully by treating it first with LDA using THF as a solvent below -78 ºC for 30 minutes followed by addition of **45** and stirring the reaction overnight at room temperature to give **47**. Compound **45** was then treated with TBAF for 2 hours in THF to deprotect TBS groups and selective tritylation of deprotected product was carried using DMTr-Cl, pyridine as a solvent and stirring the reaction mixture at room temperature for 8 hours to get **47**. The phosphitylation of **47** using DCM as a solvent, 2-cyanoethyl N,N,N',N'-

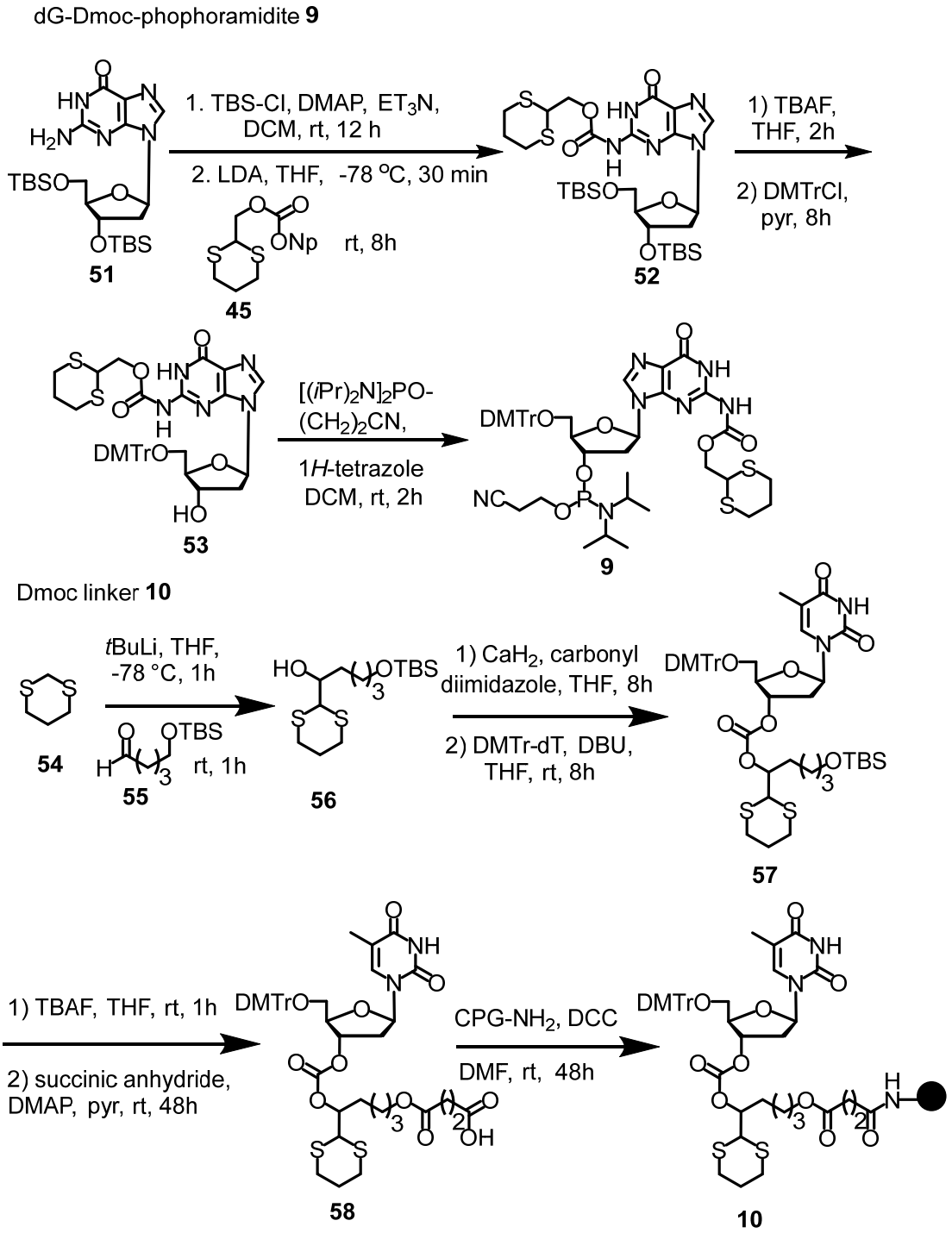
tetraisopropylphosphordiamidite and 1H-tetrazole was conducted to obtain **7**.



Scheme 2.2 Synthesis of dA and dC-Dmoc-phosphoramidite

Synthesis of Dmoc-dC-phosphoramidite **8** (Scheme 2.2) was completed in four steps using identical conditions used for the synthesis of **7**.

For the synthesis of Dmoc-dG-phosphoramidite **9** (Scheme 2.3) route was slightly modified for the first step compared to compounds **7** and **8**. Before Dmoc protection step carbonyl oxygen of **51** was temporarily protected to facilitate selective protection of an exo-amine group. The rest of the steps were same to the ones used for the synthesis of compounds **7** and **8**.



Scheme 2.3 Synthesis of dG-Dmoc-phosphoramidite and Dmoc linker

Synthesis of a linker **10** needed six steps (Scheme 2.3). To make **56,** 1,3-dithiane

54 was first deprotonated by using *t*-butyl lithium in THF under -78 ºC for 1 hour followed by addition of **55** and subsequent stirring by another hour. Compound **56** then converted **57** in two steps; 1) **56** was treated with calcium hydride and carbonyl diimidazole in THF for 8 hours to get an intermediate product. 2) The intermediate was then treated with DBU, DMTr-dT in THF for 8 hours to get compound **57**. TBS deprotection of **57** using TBAF followed by coupling with succinic anhydride gave compound **58**. Compound **58** was then attached to CPG through DCC coupling over hours to get **10** as a final linker.

2.3 Experimental section

2.3.1 General experimental

All reactions were performed in oven-dried glassware under argon using standard Schlenk techniques. Reagents and solvents available from commercial sources were used as received unless otherwise noted. THF and DCM were dried using an Innovative Technology Pure-Solv™ system. Pyridine was distilled over CaH_2 under nitrogen. Thin layer chromatography (TLC) was performed using Sigma-Aldrich TLC plates, silica gel 60F-254 over glass support, 250 μm thickness. Flash column chromatography was performed using SiliCycle silica gel, particle size 40-63 μm. ^{1}H and ^{13}C NMR spectra were measured on a Varian UNITY INOVA spectrometer at 400 and 100 MHz, respectively; chemical shifts (δ) were reported in reference to solvent peaks (residue $CHCl_3$ at δ 7.26 ppm for ^{1}H and $CDCl_3$ at δ 77.00 ppm for ^{13}C). HRMS was obtained on a Thermo HR-Orbitrap Elite Mass

Spectrometer.

2.3.2 Synthesis of modified monomers 1-5

Synthesis of modified monomers **1**-**5** was accomplished according to the Scheme 2.1 as follows;

Compound 38: Compound **36** (5.00 g, 10.02 mmol, 1.0 equiv.), synthesized according to reported procedure, [29-30] was dissolved in anhydrous DMF (20 mL) in a two-necked round-bottomed flask under argon. Commercially available 3-ethoxy-3-oxopropylzinc bromide **37** (0.5 M in THF, 30.08 mL, 15.04 mmol, 1.5 equiv.) was added via a syringe, and bis(triphenylphosphine)palladium(II) dichloride [$(Ph_3P)_2PdCl_2$ (140 mg, 0.20 mmol, 0.02 equiv.] was added under positive argon pressure. The reaction mixture was stirred at 80 ºC for 30 min. After cooling to r.t., EtOAc (150 mL) and sat. $NaHCO_3$ (50 mL) was added sequentially. The mixture was partitioned. The organic layer was washed with sat. NaCl (50 mL x 4), dried over anhydrous $NaSO_4$, filtered and evaporated to dryness under reduced pressure. Purification with flash chromatography (SiO_2, hexanes/EtOAc 6:1 to 5:2) gave compound **38** as a colorless oil (4.32 g, 76%): TLC R_f = 0.58 (hexanes/EtOAc 3:2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.33 (s, 1H), 6.53 (t, J = 6.4 Hz, 1H), 4.65 (m, 1H), 4.16 (q, J = 7.1 Hz, 2H), 4.04 (q, J = 3.3 Hz, 1H), 3.89 (dd, J = 4.2, 11.2 Hz, 1H), 3.79 (dd, J = 3.2, 11.2 Hz, 1H), 3.56 (t, J = 7.3 Hz, 2H), 2.99 (t, J = 7.0 Hz, 2H), 2.72 (m, 1H), 2.47 (m, 1H), 1.24 (t, J = 7.1 Hz, 2H), 0.92 (s, 9H), 0.90 (s, 9H), 0.11 (s, 6H), 0.08 (s, 3H), 0.07 (s, 3H) ppm; ^{13}C NMR

(100 MHz, $CDCl_3$) δ 172.8, 160.6, 152.3, 150.3, 142.5, 133.3, 88.2, 84.6, 72.2, 63.0, 41.3, 31.7, 28.0, 26.1, 25.9, 18.6, 18.2, 14.3, -4.4, -4.5, -5.1, -5.2 ppm; HRMS (ESI): calcd for $C_{27}H_{48}N_4O_5Si_2H$ $[M+H]^+$ 565.32415 found 565.32319, and $C_{27}H_{48}N_4O_5Si_2Na$ $[M+Na]^+$ 587.30609 found 587.30453.

Compound 39: Compound **38** (3.5 g, 6.20 mmol, 1.0 equiv.) and finely grounded NaOH (2.05 g, 1.15 mmol, 8.25 equiv.) in THF and H_2O (v/v 20:1) were stirred at r.t. for 16 h. The reaction mixture was cooled on an ice bath and quenched with citric acid (4.0 g). EtOAc (100 mL) was added and the mixture was partitioned. The organic phase was washed with sat. NaCl (50 mL x 3), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. Purification with flash chromatography (SiO_2, DCM/AcOH/MeOH 100:1:0 to 100:1:2) gave **39** as a colorless oil (2.85 g, 86%): TLC R_f = 0.36 (DCM/AcOH/MeOH 10:0.1:0.4); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (s, 1H), 8.43 (s, 1H), 6.51 (t, J = 6.4 Hz, 1H), 4.63 (m, 1H), 4.03 (q, J = 3.2 Hz, 1H), 3.88 (dd, J = 4.1, 11.2 Hz, 1H), 3.77 (dd, J = 3.1, 11.2 Hz, 1H), 3.58 (t, J = 7.1 Hz, 2H), 2.98 (t, J = 6.8 Hz, 2H), 2.68 (m, 1H), 2.48 (m, 1H), 0.89 (s, 9H), 0.87 (s, 9H), 0.08 (s, 6H), 0.06 (s, 3H), 0.05 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.3, 160.3, 151.8, 150.3, 143.2, 132.6, 88.3, 84.8, 72.1, 62.9, 41.5, 32.4, 27.5, 26.2, 26.0, 18.7, 18.3, -4.2, -4.4, -5.0, -5.1 ppm; HRMS (ESI): calcd for $C_{25}H_{44}N_4O_5Si_2H$ $[M+H]^+$ 537.29285 found 537.29200, and $C_{25}H_{44}N_4O_5Si_2Na$ $[M+Na]^+$ 559.27479 found 559.27328.

General procedure for converting 39 to 41a-41d: Compound **39** (600 mg, 1.12 mmol, 1.0 equiv.) in anhydrous DCM (50 mL) was cooled to 0 ºC. 1-(3-

Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC-HCl, 257 mg, 1.34 mmol, 1.2 equiv.) was added. After stirring at 0 ºC for 20 min, 1-hydroxybenzotriazole hydrate (HOBt, 38 mg, 0.28 mmol, 0.25 equiv.) was added, and stirring was continued for 20 min at 0 ºC. Compound **40a** (133 µL, 1.68 mmol, 1.5 equiv.), **40b** (209 mg, 1.68 mmol, 1.5 equiv.), **40c** (158 mg, 1.68 mmol, 1.5 equiv.) or **40d** (226 µL, 1.68 mmol, 1.5 equiv.) was then added. After stirring at r.t. for 12 h, the mixture was transferred to a separatory funnel and washed with sat. NaCl (15 mL x 3). The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The products were purified with flash chromatography to give **41a**-**41d**, respectively.

Compound 41a: Flash chromatography (SiO_2, hexanes/EtOAc 5:1 to 3:1) gave **41a** as a thick oil (510 mg, 77%): TLC R_f = 0.51 (hexanes/EtOAc 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.32 (s, 1H), 6.51 (t, J = 6.5 Hz, 1H), 4.63 (m, 1H), 4.23 (m, 2H), 4.03 (q, J = 3.2 Hz, 1H), 3.87 (dd, J = 4.2, 11.2 Hz, 1H), 3.77 (dd, J = 3.2, 11.2 Hz, 1H), 3.57-3.51 (m, 4H), 3.34 (s, 3H), 3.04 (t, J = 7.5 Hz, 2H), 2.70 (m, 1H), 2.46 (m, 1H), 0.90 (s, 9H), 0.89 (s, 9H), 0.09 (s, 6H), 0.07 (s, 3H), 0.06 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 160.3, 152.1, 150.1, 142.4, 133.1, 88.1, 84.5, 72.1, 70.5, 63.7, 62.9, 59.0, 41.3, 31.5, 27.9, 26.1, 25.9, 25.8, 18.6, 18.2, -4.4, -4.5, -5.1, -5.2 ppm; HRMS (ESI): calcd for $C_{28}H_{50}N_4O_6Si_2H$ $[M+H]^+$ 595.33472 found 595.33371, and $C_{28}H_{50}N_4O_6Si_2Na$ $[M+Na]^+$ 617.31666 found 617.61455.

Compound 41b: Flash chromatography (SiO_2, hexanes/EtOAc 5:1 to 3:1) gave

41b as a thick oil (508 mg, 71%): TLC R_f = 0.55 (hexanes/EtOAc 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (s, 1H), 8.35 (s, 1H), 7.00 (m, 2H), 6.87 (m, 2H), 6.53 (t, J = 6.4 Hz, 1H), 4.65 (m, 1H), 4.04 (q, J = 3.3 Hz, 1H), 3.89 (dd, J = 4.2, 7.0 Hz, 1H), 3.78-3.75 (m, 4H), 3.67 (t, J = 7.2 Hz, 2H), 3.23 (m, 2H), 2.72 (m, 1H), 2.48 (m, 1H), 0.91 (s, 9H), 0.90 (s, 9H), 0.11 (s, 6H), 0.08 (s, 3H), 0.07 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 160.0, 157.1, 152.2, 150.2, 144.3, 142.5, 133.2, 122.3, 114.4, 88.1, 84.6, 72.1, 62.9, 55.7, 41.4, 31.7, 28.0, 26.2, 26.0, 18.6, 18.2, -4.3, -4.4, -5.0, -5.1 ppm; HRMS (ESI): calcd for $C_{32}H_{50}N_4O_6Si_2H$ $[M+H]^+$ 643.33471 found 643.33390, and $C_{32}H_{50}N_4O_6Si_2Na$ $[M+Na]^+$ 665.31666 found 665.31467.

Compound 41c: Flash chromatography (SiO_2, hexanes/EtOAc 5:1 to 3:1) gave **41c** as a thick oil (495 mg, 72%): TLC R_f = 0.63 (hexanes/EtOAc 2:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87(s, 1H), 8.34 (s, 1H), 7.35 (t, J = 7.6 Hz, 2H), 7.19 (t, J = 7.1 Hz, 1H), 7.07 (d, J = 8.0 Hz, 2H), 6.52 (t, J = 6.5 Hz, 1H), 4.63 (m, 1H), 4.03 (m, 1H), 3.88 (dd, J = 3.9, 11.2 Hz, 1H), 3.78 (dd, J = 2.6, 11.1 Hz, 1H), 3.67 (t, J = 7.1 Hz, 2H), 3.24 (t, J = 7.2 Hz, 2H), 2.70 (m, 1H), 2.47 (m, 1H), 0.90 (s, 9H), 0.89 (s, 9H), 0.09 (s, 6H), 0.07 (s, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 160.1, 152.3, 150.9, 150.4, 142.7, 133.3, 129.5, 125.8, 121.7, 88.2, 84.6, 72.1, 62.9, 41.4, 31.7, 27.9, 26.1, 25.9, 18.6, 18.2, -4.4, -4.5, -5.1, -5.2 ppm; HRMS (ESI): calcd for $C_{31}H_{48}N_4O_5Si_2H$ $[M+H]^+$ 613.32415 found 613.32328, and $C_{31}H_{48}N_4O_5Si_2Na$ $[M+Na]^+$ 635.30609 found 635.30441.

Compound 41d: Flash chromatography (SiO_2, hexanes/EtOAc 5:1 to 3:1) gave

41d as a thick oil (560 mg, 74%): TLC R_f = 0.81 (hexanes/EtOAc 3:2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82(s, 1H), 8.32 (s, 1H), 7.27-7.16 (m, 5H), 6.50 (t, J = 6.5 Hz, 1H), 4.63 (m, 1H), 4.02 (q, J = 3.3 Hz, 1H), 3.87 (dd, J = 4.2, 11.2 Hz, 1H), 3.77 (dd, J = 3.2, 11.2 Hz, 1H), 3.57 (t, J = 7.3 Hz, 2H), 3.25 (t, J = 7.8 Hz, 2H), 3.11 (t, J = 7.2 Hz, 2H), 2.84 (t, J = 8.2 Hz, 2H), 2.70 (m, 1H), 2.46 (m, 1H), 0.90 (s, 9H), 0.88 (s, 9H), 0.09 (s, 6H), 0.06 (s, 3H), 0.05 (s, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.7, 159.9, 152.1, 150.2, 142.4, 139.9, 133.0, 128.5, 128.4, 126.4, 88.0, 84.4, 72.1, 62.9, 41.3, 41.1, 36.0, 30.4, 28.2, 26.1, 25.9, 18.6, 18.1, -4.4, -4.5, -5.1, -5.2 ppm; HRMS (ESI): calcd for $C_{33}H_{52}N_4O_4SSi_2H$ $[M+H]^+$ 657.33260 found 657.33026.

General procedure for converting 38 and 41a-41d to 42a-42e: Compound **38** (700 mg, 1.24 mmol, 1.0 equiv.), **41a** (510 mg, 0.86 mmol, 1.0 equiv.), **42b** (495 mg, 0.77 mmol, 1.0 equiv.), **42c** (490 mg, 0.80 mmol, 1.0 equiv.) or **42d** (550 mg, 0.82 mmol, 1.0 equiv.) in anhydrous THF (20 mL) was cooled on an ice bath. A solution of tetrabutylammonium fluoride (TBAF) in THF (1.0 M, 2.5 equiv.) was added via a syringe dropwise. After stirring at 0 °C for 2.5 h, volatiles were removed under reduced pressure. The products were purified with flash chromatography to give **42a**-**42e**, respectively.

Compound 42a: Flash chromatography (SiO_2, DCM/MeOH 100:2 to 100:7) gave **12a** as a colorless oil (410 mg, 98%): TLC R_f = 0.35 (DCM/MeOH 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 8.10 (s, 1H), 6.41 (q, J = 5.5 Hz, 1H), 5.82 (d, 10.4 Hz, 1H), 4.82 (d, 5.0 Hz, 1H), 4.23 (s, 1H), 4.15 (q, J = 7.2 Hz, 2H), 3.99 (d,

13.7 Hz, 1H), 3.84 (t, *J* = 11.1 Hz, 1H), 3.57 (m, 2H), 3.14 (m, 1H), 3.01 (m, 2H), 2.37 (m, 1H), 1.24 (t, *J* = 7.1 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 172.9, 162.3, 151.8, 149.7, 143.9, 134.8, 90.0, 88.1, 73.9, 63.9, 61.0, 41.2, 31.8, 28.4, 14.7 ppm; HRMS (ESI): calcd for $C_{15}H_{20}N_4O_5H$ $[M+H]^+$ 337.15119 found 337.15058, and $C_{15}H_{20}N_4O_5Na$ $[M+Na]^+$ 359.13314 found 359.13194.

Compound 42b: Flash chromatography (SiO_2, DCM/MeOH 100:2 to 100:7) gave **42b** as a colorless oil (310, 99%): TLC R_f = 0.47 (DCM/MeOH 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.81 (s, 1H), 8.14 (s, 1H), 6.42 (q, *J* = 3.6 Hz, 1H), 4.79 (d, *J* = 3.8 Hz, 1H), 4.23-4.21 (m, 3H), 3.97 (d, *J* = 12.7 Hz, 1H), 3.81-3.79 (m, 1H), 3.57-3.52 (m, 4H), 3.35 (s, 3H), 3.06-3.02 (m, 3H), 2.37 (q, *J* = 5.7 Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 172.3, 161.4, 151.1, 149.1, 143.3, 134.1, 89.2, 87.3, 72.9, 70.2, 63.5, 63.1, 58.7, 40.6, 30.9, 27.6 ppm; HRMS (ESI): calcd for $C_{16}H_{22}N_4O_6H$ $[M+H]^+$ 367.16176 found 367.16106, and $C_{16}H_{22}N_4O_6Na$ $[M+Na]^+$ 389.14370 found 389.14235.

Compound 42c: Flash chromatography (SiO_2, EtOAc/acetone 10:1 to 10:5) gave **42c** as colorless oil (300 mg, 94%): TLC R_f = 0.37 (EtOAc/acetone 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.87 (s, 1H), 8.23 (s, 1H), 6.99 (d, *J* = 9.2 Hz, 2H), 6.87 (d, *J* = 9.2 Hz, 2H), 6.41-6.33 (m, 1H), 4.81 (d, *J* = 5.0 Hz, 1H), 4.41 (q, *J* = 3.7 Hz, 1H), 3.99 (d, *J* = 12.0 Hz, 1H), 3.77 (s, 3H), 3.67-3.64 (m, 2H), 3.25-3.22 (m, 2H), 3.12-3.02 (m, 1H), 2.63 (d, *J* = 15.2 Hz, 1H), 2.37 (dd, *J* = 6.2, 13.7 Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 171.6, 161.5, 157.2, 151.5, 151.4, 144.5, 144.2, 143.6, 122.3, 114.4, 89.9, 89.6, 63.5, 63.5, 55.7, 40.9, 31.4, 28.0 ppm; HRMS (ESI): calcd

for $C_{20}H_{22}N_4O_6H$ $[M+H]^+$ 415.16176 found 415.16118, $C_{20}H_{22}N_4O_6Na$ $[M+Na]^+$ 437.14370 found 437.14251.

Compound 42d: Flash chromatography (SiO_2, EtOAc/acetone 10:1 to 10:5) gave **42d** as colorless oil (292 mg, 95%): TLC R_f = 0.40 (EtOAc/acetone 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.12 (s, 1H), 7.36 (t, J = 7.5 Hz, 2H), 7.21 (t, J = 7.4 Hz, 1H), 7.08-7.05 (m, 2H), 6.42 (q, J = 3.7 Hz, 1H), 4.80 (d, 4.9 Hz, 1H), 3.99 (d, 13.1 Hz, 1H), 3.83 (t, 11.4 Hz, 2H), 3.69 (m, 2H), 3.56 (t, 7.4 Hz, 1H), 3.29-3.24 (m, 2H), 3.11-2.97 (m, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 161.4, 151.5, 151.4, 150.7, 143.7, 134.4, 129.4, 125.8, 121.5, 89.6, 87.7, 73.4, 63.5, 40.9, 31.4, 27.9 ppm; HRMS (ESI): calcd for $C_{19}H_{20}N_4O_5H$ $[M+H]^+$ 385.15119 found 385.15057, and $C_{19}H_{20}N_4O_5Na$ $[M+Na]^+$ 407.13314 found 407.13234.

Compound 42e: Flash chromatography (SiO_2, EtOAc/MeOH 100:1 to 100:5) gave **42e** as a colorless oil (335 mg, 92%): TLC R_f = 0.51 (EtOAc/MeOH 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.15 (s, 1H), 7.29-7.25 (m, 2H), 7.21-7.17 (m, 3H), 6.43 (q, J = 3.5 Hz, 1H), 4.81 (d, J = 5.0 Hz, 1H), 4.23 (s, 1H), 3.99 (dd, J = 1.6, 12.8 Hz, 1H), 3.58 (t, J = 7.3 Hz, 2H), 3.27 (t, J = 7.4 Hz, 2H), 3.13-3.02 (m, 4H), 2.85 (t, J = 8.1 Hz, 2H), 2.39 (q, J = 5.4 Hz, 1H) ppm; ^{13}C (100 MHz, $CDCl_3$) δ 197.9, 161.3, 151.5, 149.4, 143.7, 139.8, 134.3, 128.5, 128.4, 126.5, 89.5, 87.6, 73.2, 63.4, 40.9, 35.9, 30.5, 28.2 ppm; HRMS (ESI): calcd for $C_{21}H_{24}N_4O_4SH$ $[M+H]^+$ 429.15965 found 429.15810.

General procedure for converting 42a-42e to 43a-43e: To compound **42a** (400

mg, 1.19 mmol, 1.0 equiv.), **42b** (310 mg, 0.85 mmol, 1.0 equiv.), **42c** (300 mg, 0.72 mmol, 1.0 equiv.), **42d** (290 mg, 0.75 mmol, 1.0 equiv.), or **42e** (330 mg, 0.74 mmol, 1.0 equiv.) in freshly distilled pyridine (15 mL) was added 4,4-dimethoxytrityl chloride (DMTr-Cl, 1.1 equiv.) with rigorous stirring at r.t. under positive argon pressure. The reaction was allowed to proceed at r.t. for 8 h. Volatiles were removed under reduced pressure. The products were purified with flash chromatography to give **43a**-**43e**, respectively.

Compound 43a: Flash chromatography (SiO_2, DCM/MeOH/Et_3N 100:0:4 to 100:3:4) gave **43a** as a white foam (668 mg, 88%): TLC R_f = 0.65 (DCM/MeOH 100:6); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (s, 1H), 8.15 (s, 1H), 7.38 – 7.36 (m, 2H), 7.27 – 7.16 (m, 7H), 6.79 (d, J = 8.8 Hz, 4H), 6.48 (t, J = 6.5 Hz, 1H), 4.68 (s, 1H), 4.15 – 4.08 (m, 3H), 3.76 (s, 6H), 3.52 (t, J = 7.4 Hz, 2H), 3.43-3.35 (m, 2H), 2.97 (t, J = 7.4 Hz, 2H), 2.88-2.81 (m, 1H), 2.55 – 2.49 (m, 1H), 1.21 (t, J = 7.1 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.5, 160.4, 158.4, 152.0, 149.9, 144.3, 142.2, 135.4, 135.4, 133.0, 129.8, 127.9, 127.7, 126.8, 113.0, 86.4, 85.9, 84.2, 72.4, 63.6, 60.3, 55.0, 39.9, 31.3, 27.7, 14.0 ppm; HRMS (ESI): calcd for $C_{36}H_{38}N_4O_7H$ $[M+H]^+$ 639.28187 found 639.28000.

Compound 43b: Flash chromatography (SiO_2, (DCM/MeOH/Et_3N 100:0:4 to 100:3:4) gave **43b** as a white foam (530 mg, 94%): TLC R_f = 0.62 (DCM/MeOH 100:6); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (s, 1H), 8.14 (s, 1H), 7.41 – 7.35 (m, 2H), 7.30 – 7.16 (m, 7H), 6.79 (d, J = 8.7 Hz, 4H), 6.48 (t, J = 6.5 Hz, 1H), 4.69 – 4.66 (m, 1H), 4.22 – 4.19 (m, 2H), 4.14 – 4.11 (m, 1H), 3.76 (s, 6H), 3.58 – 3.49

(m, 4H), 3.43 – 3.37 (m, 2H), 3.33 (s, 3H), 3.03 (t, *J* = 7.4 Hz, 2H), 2.88-2.81 (m, 1H), 2.56-2.49 (m, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 172.7, 160.4, 158.5, 152.2, 150.1, 144.4, 142.3, 135.6, 135.6, 133.2, 130.0, 128.1, 127.9, 127.0, 113.2, 86.7, 86.1, 84.4, 72.7, 70.5, 63.9, 63.7, 59.1, 55.3, 40.2, 31.5, 27.9 ppm; HRMS (ESI): calcd for $C_{37}H_{40}N_4O_8H$ $[M+H]^+$ 669.29244 found 669.29233, and $C_{37}H_{40}N_4O_8Na$ $[M+Na]^+$ 691.27438 found 691.27271.

Compound 43c: Flash chromatography (SiO_2, EtOAc/acetone/Et_3N 100:10:4 to 100:50:6) gave **43c** as a white foam (425 mg, 82%): TLC R_f = 0.68 (EtOAc/acetone 5:3); ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.80 (s, 1H), 8.16 (s, 1H), 7.39 – 7.35 (m, 2H), 7.31 – 7.15 (m, 7H), 6.99 – 6.94 (m, 2H), 6.85 – 6.74 (m, 6H), 6.49 (t, *J* = 6.5 Hz, 1H), 4.70 (m, 1H), 4.15 (q, *J* = 4.9 Hz, 1H), 3.80 – 3.70 (m, 9H), 3.64 (t, *J* = 7.3 Hz, 2H), 3.40 (t, *J* = 4.7 Hz, 2H), 3.21 (t, *J* = 7.3 Hz, 2H), 2.88 – 2.82 (m, 1H), 2.56-2.50 (m, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 171.6, 160.1, 158.5, 157.1, 152.2, 150.1, 144.4, 144.2, 142.4, 135.6, 135.6, 133.2, 130.0, 128.1, 127.9, 127.0, 122.3, 114.4, 113.2, 86.7, 86.2, 84.5, 72.7, 63.8, 55.7, 55.3, 40.3, 31.6, 27.9 ppm; HRMS (ESI): calcd for $C_{41}H_{40}N_4O_8H$ $[M+H]^+$ 717.29244 found 717.29166, and $C_{41}H_{40}N_4O_8Na$ $[M+Na]^+$ 739.27438 found 739.27257.

Compound 43d: Flash chromatography (SiO_2, EtOAc/acetone/Et_3N 100:1:4 to 100:5:4) gave **43d** as a white foam (435 mg, 84%): TLC R_f = 0.71 (EtOAc/acetone 10:6); ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.80 (s, 1H), 8.17 (s, 1H), 7.39 – 7.15 (m, 14H), 7.06 (m, 2H), 6.79 (d, *J* = 8.9 Hz, 4H), 6.49 (t, *J* = 6.5 Hz, 1H), 4.70 – 4.66 (m, 1H), 4.16 (q, *J* = 4.6 Hz, 1H), 3.75 (s, 6H), 3.65 (t, *J* = 7.2 Hz, 2H), 3.49 (q, *J* = 7.0 Hz,

1H), 3.40 (t, *J* = 4.8 Hz, 2H), 3.24 (t, *J* = 7.4 Hz, 2H), 2.88 (dd, *J* = 6.6, 13.3 Hz, 1H), 2.56 (m, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 170.9, 159.7, 158.2, 151.8, 150.4, 149.8, 144.1, 142.1, 135.3, 135.3, 132.9, 129.7, 129.0, 127.7, 127.6, 126.6, 125.4, 121.2, 112.9, 86.4, 85.9, 84.2, 72.3, 63.5, 55.0, 40.0, 31.3, 27.6 ppm; HRMS (ESI): calcd for $C_{40}H_{38}N_4O_7H$ $[M+H]^+$ 686.28188 found 687.28009.

Compound 43e: Flash chromatography (SiO_2, DCM/MeOH/Et_3N 100:0:4 to 100:3:4) gave **43e** as a white foam (435 mg, 79%): TLC R_f = 0.77 (DCM/MeOH 20:1); ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.76 (s, 1H), 8.15 (s, 1H), 7.38 (d, *J* = 7.1 Hz, 2H), 7.28 – 7.17 (m, 13H), 6.79 (d, *J* = 8.8 Hz, 4H), 6.49 (t, *J* = 6.4 Hz, 1H), 4.71 – 4.67 (m, 1H), 4.15 (q, *J* = 5.2, Hz, 1H), 3.76 (s, 6H), 3.55 (t, *J* = 7.4 Hz, 2H), 3.41 – 3.36 (m, 2H), 3.25 (t, *J* = 7.4 Hz, 2H), 3.11 (t, *J* = 7.1 Hz, 2H), 2.90 – 2.80 (m, 3H), 2.57 – 2.42 (m, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 197.8, 160.1, 158.6, 152.2, 150.2, 144.4, 142.4, 135.6, 130.0, 128.6, 128.5, 128.1, 127.9, 127.0, 126.5, 113.2, 86.7, 86.1, 84.5, 72.8, 63.8, 55.4, 41.2, 40.2, 36.0, 30.5, 29.9, 28.3 ppm; HRMS (ESI): calcd for $C_{42}H_{42}N_4O_6SH$ $[M+H]^+$ 731.29033 found 731.28609, and $C_{42}H_{42}N_4O_6SNa$ $[M+Na]^+$ 753.27227 found 753.26971.

General procedure for converting 43a-43e to 1-5: To a solution of **43a** (600 mg, 0.94 mmol, 1.0 equiv.), **43b** (500 mg, 0.75 mmol, 1.0 equiv.), **43c** (400 mg, 0.56 mmol, 1.0 equiv.), **43d** (400 mg, 0.58 mmol, 1.0 equiv.), or **43e** (400 mg, 0.54 mmol, 1.0 equiv.) in anhydrous DCM (50 mL) was added diisopropylammonium tetrazolide (1.5 equiv.) under positive argon pressure and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (1.5 equiv.) via a syringe at r.t. with rigorous

stirring. The reaction was allowed to proceed at r.t. for 2 h. Volatiles were removed under reduced pressure. The products were purified with flash chromatography to give **1-5**, respectively.

Compound 1: Flash chromatography (SiO_2, hexanes/EtOAc/Et_3N 50:10:2 to 5:30:3) gave **1** as a white foam (695 mg, 88%): two diastereomers; TLC R_f = 0.57, 0.53 (hexanes/EtOAc 4:3); ^1H NMR (400 MHz, $CDCl_3$) δ 8.77 (s, 1H), 8.19 and 8.18 (s, 1H), 7.39 – 7.35 (m, 2H), 7.28 – 7.15 (m, 7H), 6.78 – 6.74 (m, 4H), 6.49 – 6.45 (m, 1H), 4.80 – 4.72 (m, 1H), 4.13 (q, J = 7.1 Hz, 2H), 3.86 – 3.80 (m, 1H), 3.76 – 3.75 (m, 6H), 3.67 – 3.57 (m, 4H), 3.52 (t, J = 7.5 Hz, 2H), 3.40-3.28 (m, 3H), 2.98 – 2.92 (m, 2H), 2.62 (t, J = 6.3 Hz, 1H), 2.47 (t, J = 6.4 Hz, 1H), 1.22 – 1.09 (m, 15H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 160.5, 158.5, 152.2, 144.5, 142.4, 135.6, 135,6, 133.2, 130.1, 130.0, 130.0, 128.1, 128.1, 127.8, 126.9, 126.9, 113.1, 86.5, 84.6, 84.6, 86.5,84.6, 84.6, 63.6, 63.4, 60.6, 55.3, 55.3, 43.5, 43.3, 39.5, 31.7, 28.0, 24.8, 24.7, 24.7, 24.7, 14.4 ppm; ^{31}P NMR (162 MHz, $CDCl_3$) δ 150.2, 150.0 ppm; HRMS (ESI): calcd for $C_{45}H_{55}N_6O_8PH$ $[M+H]^+$ 839.38972 found 839.38853, and $C_{45}H_{55}N_6O_8PNa$ $[M+Na]^+$ 861.37162 found 861.36923.

Compound 2: Flash chromatography (SiO_2, hexanes/EtOAc/Et_3N 50:10:2 to 50:40:4) gave **2** as a white foam (mg 510 mg, 80%): two diastereomers; TLC R_f = 0.45, 0.43 (hexanes/EtOAc 4:3); ^1H NMR (400 MHz, $CDCl_3$) δ 8.76 (s, 1H), 8.17 (s, 1H), 7.37 – 7.34 (m, 2H), 7.29 – 7.14 (m, 7H), 6.77-6.74 (m, 4H), 6.49 (t, J = 7.3 Hz, 1H), 4.71-4.76 (m, 1H), 4.31 – 4.27 (m, 1H), 4.23 – 4.19 (m, 2H), 3.88-3.78 (m, 2H), 3.75 (s, 6H), 3.57 – 3.49 (m, 5H), 3.36 – 3.30 (m, 4H), 3.02 (t, J = 7.4 Hz,

2H), 2.93 – 2.87 (m, 1H), 2.75-2.71 (m, 1H), 2.61 (t, *J* = 6.3 Hz, 2H), 2.46 (t, *J* = 6.4 Hz, 1H), 1.28 – 1.22 (m, 3H), 1.18 – 1.14 (m, 6H), 1.11-1.09 (m, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 172.6, 160.4, 158.5, 152.1, 150.2, 144.5, 142.4, 135.6, 135.6, 133.2, 130.0, 128.1, 128.1, 127.8, 126.9, 117.5, 113.1, 86.5, 85.9, 84.6, 70.5, 63.7, 63.6, 59.1, 58.5, 58.3, 55.3, 43.5, 43.3, 39.5, 31.5, 27.9, 24.8, 24.7, 24.6, 23.09, 20.58 ppm; ^{31}P NMR (162 MHz, $CDCl_3$) *δ* 150.2, 150.1 ppm; HRMS (ESI): calcd for $C_{46}H_{57}N_6O_9PH$ $[M+H]^+$ 869.40029 found 869.40006, and $C_{46}H_{57}N_6O_9PNa$ $[M+Na]^+$ 891.382223 found 891.37988.

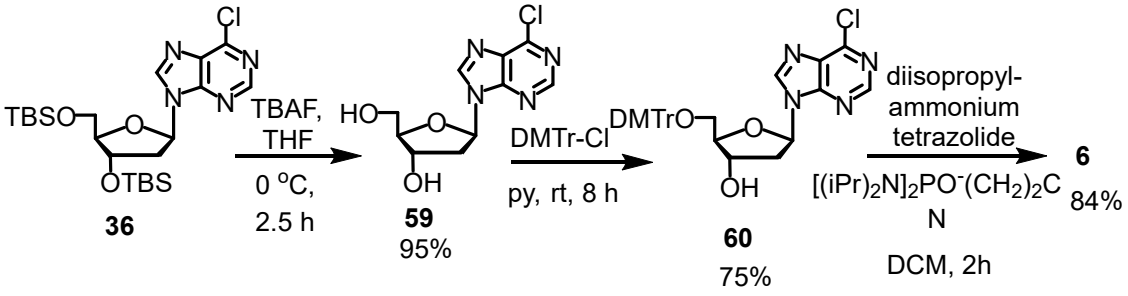
Compound 3: Flash chromatography (SiO_2, hexanes/EtOAc/Et_3N 50:10:2 to 50:40:4) gave **3** as a white foam (415 mg, 81%): two diastereomers; TLC R_f = 0.46, 0.44 (hexanes/EtOAc 4:3); ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.80 (s, 1H), 8.21 and 8.19 (s, 1H), 7.38 – 7.36 (m, 2H), 7.28 – 7.16 (m, 7H), 6.98 (d, *J* = 9.0 Hz, 2H), 6.84 – 6.74 (m, 6H), 6.51 – 6.46 (m, 1H), 4.76 (s, 1H), 4.29 (s, 1H), 4.21 – 4.07 (m, 2H), 3.84-3.79 (m, 1H), 3.76 (s, 6H), 3.66 – 3.54 (m, 4H), 3.40 – 3.29 (m, 2H), 3.21 (t, *J* = 7.2 Hz, 2H), 2.95 – 2.88 (m, 1H), 2.77 – 2.56 (m, 4H), 2.47 (t, 6.36 Hz, 1H), 1.28 – 1.24 (m, 3H), 1.21 – 1.14 (m, 6H), 1.12 (d, *J* = 6.7 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 171.6, 160.0, 158.5, 157.1, 152.2, 144.5, 144.3, 142.5, 135.7, 133.2, 130.0, 128.1, 127.8, 126.9, 122.3, 114.4, 113.2, 86.6, 84.7, 55.7, 55.3, 43.5, 43.5, 43.4, 39.5, 31.6, 28.0, 24.8, 24.8, 24.7, 24.7, 23.2, 23.2, 23.1, 23.1 ppm; ^{31}P NMR (162 MHz, $CDCl_3$) *δ* 150.2, 150.0 ppm; HRMS (ESI): calcd for $C_{50}H_{57}N_6O_9PH$ $[M+H]^+$ 917.40029 found 917.40014 and $C_{50}H_{57}N_6O_9PNa$ $[M+Na]^+$ 939.38223 found 939.37995.

Compound 4: Flash chromatography (SiO_2, hexanes/EtOAc/Et_3N 50:10:2 to 50:40:4) gave **4** as a white foam (420 mg, 81%): two diastereomers; TLC R_f = 0.50, 0.47 (hexanes/EtOAc 4:3); ^1H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H), 8.22 and 8.20 (s, 1H), 7.38 – 7.35 (m, 2H), 7.33 – 7.12 (m, 10H), 7.06 (d, J = 8.0 Hz, 2H), 6.77 (dd, J = 4.9, 8.5 Hz, 4H), 6.50 (m, 1H), 4.78 (m, 1H), 4.31 (m, 1H), 4.14 – 4.04 (m, 1H), 3.83 (m, 1H), 3.74 (s, 3H), 7.35 (s, 3H), 3.65 (t, J = 6.6 Hz, 2H), 3.57 – 3.29 (m, 4H), 3.24 (t, J = 7.2 Hz, 2H), 2.99 – 2.89 (m, 1H), 2.71-2.67 (m, Hz, 1H), 2.59 (t, J = 6.3 Hz, 1H), 2.46 (t, J = 6.3 Hz, 1H), 1.25 (d, J = 6.5 Hz, 4H), 1.18 (s, 6H), 1.11 (d, J = 6.8 Hz, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 160.0, 158.5, 152.2, 150.8, 150.2, 144.5, 142.5, 135.7, 133.2, 130.0, 129.3, 128.2, 128.1, 127.8, 126.9, 125.7, 121.6, 117.4, 113.2, 86.6, 86.0, 84.7, 73.6, 63.6, 63.4, 58.6, 58.4, 55.4, 55.3, 43.5, 43.4, 39.5, 31.6, 27.9, 24.70, 23.1, 20.4 ppm; ^{31}P NMR (162 MHz, $CDCl_3$) δ 150.1, 150.0 ppm; HRMS (ESI): calcd for $C_{49}H_{55}N_6O_8PH$ $[M+H]^+$ 887.38972 found 887.38934, and $C_{49}H_{55}N_6O_8PNa$ $[M+Na]^+$ 909.337167 found 909.36998.

Compound 5: Flash chromatography (SiO_2, hexanes/EtOAc/Et_3N 50:0:2 to 50:20:3) gave **2.5e** as a white foam (400 mg, 79%): two diastereomers; TLC R_f = 0.63, 0.58 (hexanes/EtOAc 2:1); ^1H NMR (400 MHz, $CDCl_3$) δ 8.77 and 8.76 (s, 1H), 8.20 and 8.18 (s, 1H), 7.38 – 7.35 (m, 2H), 7.32 – 7.09 (m, 12H), 6.78-7.74 (m, 4H), 6.48 – 6.43 (m, 1H), 4.32- 4.27 (m, 1H), 4.16 – 4.09 (m, 1H), 3.86 – 3.80 (m, 1H), 3.76 (s, 3H), 3.75 (s, 3H), 3.60 – 3.57 (m, 1H), 3.58 (t, J = 7.2 Hz, 2H), 3.48 – 3.40 (m, 2H), 3.39 – 3.30 (m, 2H), 3.24 (t, J = 7.3 Hz, 2H), 3.11 (t, J = 7.2

Hz, 2H), 2.95-2.88 (m, 1H), 2.85 (t, *J* = 8.2 Hz, 2H), 2.74 (t, *J* = 6.2 Hz, 1H), 2.61 (t, *J* = 7.9 Hz, 1H), 2.46 (t, *J* = 6.4 Hz, 1H), 1.24-1.20 (m, 4H), 1.19-1.17 (m, 6H), 1.15-1.10 (m, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.8, 160.0, 158.5, 152.2, 150.2, 144.5, 142.5, 140.0, 135.7, 133.2, 130.1, 130.0, 130.0, 128.6, 128.5, 128.2, 128.1, 127.8, 126.9, 126.4, 113.2, 86.6, 84.7, 63.6, 63.4, 55.4, 55.3, 58.6, 58.4, 45.8, 45.7, 43.5, 43.4, 41.2, 39.5, 36.1, 30.5, 29.9, 28.3, 24.8, 24.7, 23.3, 22.4, 20.7, 20.5 ppm; ^{31}P NMR (162 MHz, $CDCl_3$) δ 150.1, 150.0 ppm; HRMS (ESI): calcd for $C_{51}H_{59}N_6O_7PSH$ $[M+H]^+$ 931.39818 found 931.39691.

2.4 Synthesis of chloropurine monomer 6



Scheme 2.4 Synthesis of chloropurine monomer **6**

While we were studying the cross-linking properties of oligodeoxynucleotides we came across the applications of chloropurines. Chloropurines shown to have cytotoxic,[8-12] antiviral,[13-15] antitumor[8, 10, 11, 16] and antioxidant[9, 17] properties. Chemical synthesis of chloropurines is quite possible[10, 17, 18] but their incorporation into oligonucleotide is complicated by ammonia work-up just like our sensitive esters. Their applications compel us to synthesize chloropurine monomer **6**.

Another reason to extend our synthesis is to check the applicability of Dmoc technology in making of oligonucleotides having chloropurines in them. We had already synthesized a compound **36** to make monomers **1**-**5**. Taking advantage of availability of the starting material we gave the way to our curiosity and synthesized it as shown in the Scheme 2.4.

Compound 59: In an ice-cold solution of **36** (600 mg, 1.2 mmol, 1.0 equiv) in 20 mL anhydrous THF under argon, slowly added 1.0 M tetrabutylammonium fluoride solution in THF (3.0 mL, 3.0 mmol, 2.5 equiv). The reaction mixture was stirred at 0 ºC for 2.5 h. Solvent was evaporated in vacuo, residue was purified by flash chromatography (SiO_2, DCM/MeOH 100/2 to 100/7) to get compound **59** as a light-yellow oil (310 mg, 95%). TLC R_f = 0.44 (DCM/ MeOH 10/1); ^{1}H NMR (400 MHz, DMSO) δ 8.88 (s, 1H), 8.64 (s, 1H), 6.50 (t, J = 6.6 Hz, 1H), 4.5 (p, J = 2.84, 2.92, 5.76 Hz, 1H), 3.97 (q, J = 3.3 Hz, 1H), 3.70 (dd, J = 3.6, 11.8 Hz, 1H), 3.62 (dd, J = 3.5, 11.9 Hz, 1H), 2.68 (m, 1H), 2.42 (m, 1H) ppm; ^{13}C (100 MHz, DMSO) δ 150.7, 150.7, 149.2, 144.7, 131.2, 87.9, 84.3, 70.3, 70.2, 61.2, 61.1, 40.2 ppm; HRMS (negative ACPI): Calcd For $C_{10}H_{11}ClN_4O_3$ + e [M+e] 270.05197 found 270.05329 and For $C_{10}H_{11}ClN_4O_5$ +e [M+O_2+e] 302.04179 found 302.04273.

Compound 60: Compound **59** (300 mg, 1.11 mmol, 1.0 equiv) was dissolved in 15 mL freshly distilled anhydrous pyridine under argon and 4,4-dimethoxytrityl chloride (414 mg, 1.22 mmol, 1.1 equiv) was added with rigorous stirring at room temperature. Reaction mixture was stirred at r.t. for 8 h, solvent was removed by evaporation. The residue was purified by flash chromatography (SiO_2,

DCM/MeOH/Et_3N 10/0/4% to 10/3/4%) to get **60** as a yellow foam (478 mg, 75%). TLC R_f = 0.71 (DCM/MeOH 10/0.6); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (s, 1H), 8.25 (s, 1H), 7.37 – 7.35 (m, 2H), 7.27 – 7.17 (m, 7H), 6.79 – 6.76 (m, 4H), 6.48 (t, J = 6.5 Hz, 1H), 4.71 – 4.69 (m, 1H), 4.18 (q, J = 4.6 Hz, 1H), 3.77 (s, 6H), 3.49 – 3.34 (m, 3H), 2.89 – 2.82 (m, 1H), 2.60 (m, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.6, 151.9, 151.1, 144.3, 143.7, 135.5, 135.5, 130.0, 130.0, 128.0, 128.0, 127.1, 113.3, 86.8, 86.4, 85.0, 72.7, 63.7, 55.4, 40.5 ppm; HRMS (ESI): Calcd For $C_{31}H_{29}ClN_4O_5Na^+$ [M+Na^+] 595.17241 found 595.17133.

Compound 6: In a reaction flask at r.t. **60** (450 mg, 0.79 mmol, 1.0 equiv) was dissolved in 50 mL anhydrous DCM. To this solution added diisopropylammonium tetrazolide (208 mg, 1.19 mmol, 1.5 equiv) followed by the addition of 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (378 µL, 1.19 mmol, 1.5 equiv) via syringe with rigorous stirring. Reaction mixture was stirred at r.t. for 2 h. Solvent was evaporated and the residue was purified by flash chromatography (SiO_2, Hexanes/EtOAc/Et3N 5/1/4% to 5/4/4%) to get **6** as a white foam (510 mg, 84%), TLC for two diastereomers R_f = 0.55 and 0.51; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.32 (two diastereomeric s, 1H), 7.39-7.36 (m, 2H), 7.29 – 7.19 (m, 7H), 6.80 – 6.76 (m, 4H), 6.50-6.47 (m, 1H), 4.78 – 4.77 (m, 1H), 4.35– 4.33 (m, 1H), 3.89 – 3.84 (m, 1H), 3.79 – 3.78 (m, 6H), 3.66 – 3.58 (m, 2H), 3.34 (dd, J = 10.2, 4.6 Hz, 2H), 2.96 – 2.88 (m, 2H), 2.69-2.65 (m, 1H), 2.64 (t, J = 6.3 Hz, 1H), 2.49 (t, J = 6.4 Hz, 1H), 1.21-1.18 (m, 9H), 1.16 – 1.13 (m, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.5, 151.8, 144.4, 143.8, 135.6, 135.5, 130.1, 130.0, 128.1,

127.9, 127.0, 113.2, 86.7, 86.4, 85.2, 63.3, 58.6, 55.3, 43.5, 43.4, 39.8, 24.8, 20.5 ppm; ^{31}P NMR (162 MHz, $CDCl_3$) δ 150.2, 150.1 ppm; HRMS (ESI): Calcd For $C_{40}H_{46}ClN_6O_6PNa^+$ [M+Na$^+$] 795.28027 found 795.27901 and For $C_{40}H_{46}ClN_6O_6PH^+Na^+$ [M+ H$^+$Na$^+$] 796.28809 found 796.28147.

2.5 References

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3 Oligodeoxynucleotide Synthesis

3.1 Introduction

As discussed in Chapter 2, nucleobases **1**-**5**, Dmoc phosphoramidites **7**-**9** and Dmoc linker **10** were synthesized and characterized**.** With these materials we were ready for the synthesis of oligonucleotides using Dmoc technology. The synthesis was accomplished over automated oligonucleotide synthesizer MerMade-6. No strong base was used for post synthesis work-up. Instead, post-synthesis work-up was conducted under nearly neutral conditions. Total of 16 modified oligonucleotides were synthesized. Also, five standard ODNs were made in order to use them as a natural complementary strand to the modified ODNs. This Chapter discusses the synthesis and work-up of ODNs in detail.

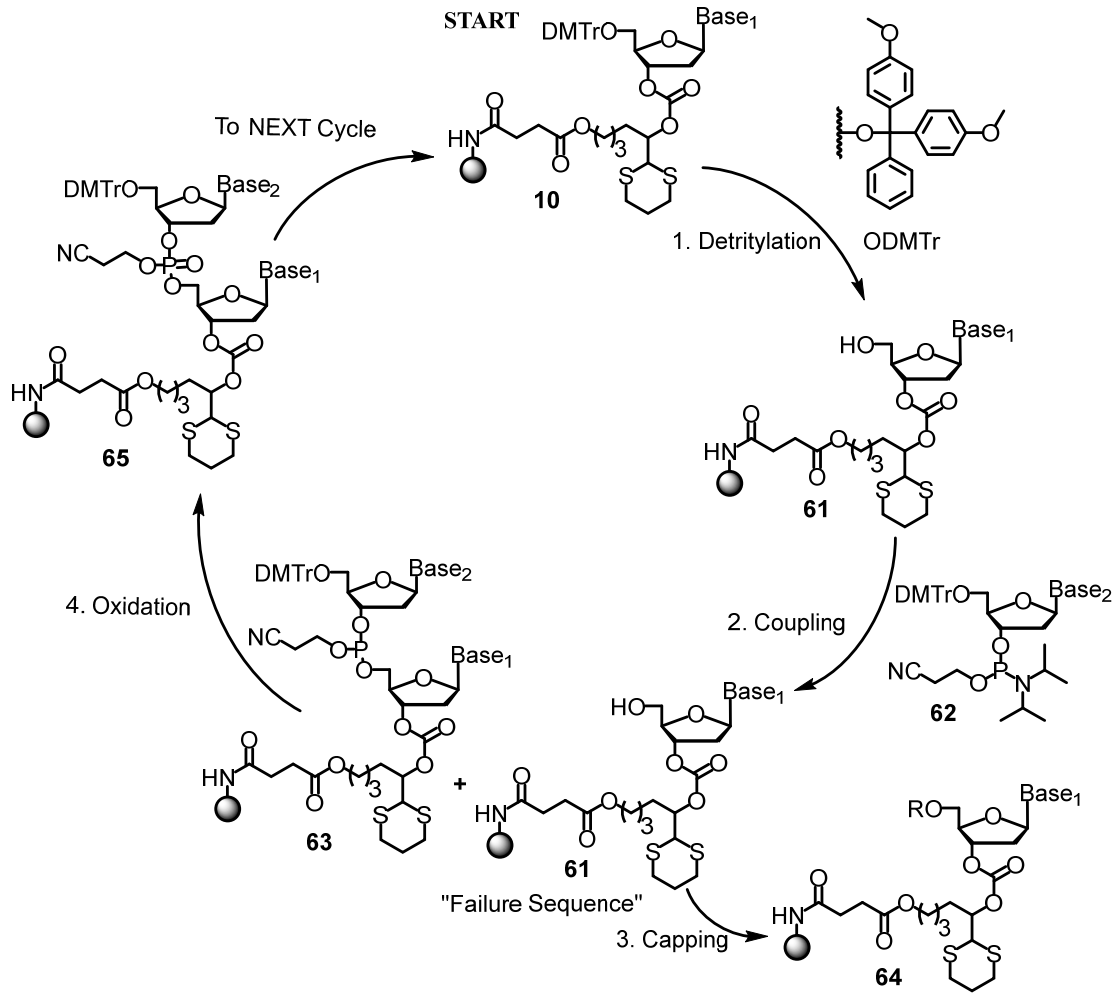
3.2 Results and discussion

Oligodeoxynucleotide synthesis was carried out using solid phase chemistry.[1] In general the ODN synthesis was carried out as follows (Scheme 3.1);[2] A linker **10** was a standard support with a nucleobase DMTr-dT already installed on it. The ODN synthetic cycle started with detritylation of **10**. The DMTr was removed by using acidic conditions to give a product **61** with unprotected alcohol at the 5' end.

The next step was coupling of **61** with a specific nucleobase **62**. The nucleobase **62** was different for every cycle and was decided based on the ODN sequence under synthesis. The first coupling gave a dimer **63**, while subsequent couplings

resulted into trimer, tetramer etc. and finally the 20 mer ODN. The efficiency of coupling was assumed to be less than 100% as real-time analysis at this stage is unreasonable. Less than 100% coupling efficiency means there was some unreacted **61** in the synthesis column. This unreacted **61** generates the failure sequence. The failure sequence needed to be terminated to avoid the formation of complex ODN mixture at the end of the ODN synthesis. The capping of failure sequence was carried out during every synthesis cycle immediately after coupling step to give a process inert **64**. In the last step trivalent phosphorus on the growing chain was converted to pentavalent phosphorus. For that oxidation was carried out using iodine to give **65**. With the oxidation the first ODN synthesis cycle completed and an attachment of a nucleobase was achieved. The cycle was repeated to give an ODN of desired length. In our case all ODNs were 20 nucleobase long and all were starting with thymine at the 3' end as our solid support has dT already installed on it.

Total of 16 modified ODN strands were synthesized using one modified monomer in each of them. All sequences were 20 nucleotides long and selected from DNA-polymerase template. Synthesis was arranged according to their sensitivity; starting from a less sensitive to end with the most sensitive. The most stable modified monomer **1** was used first to make ODNs, followed by **2** and continued to monomer **5** in the order.



Scheme 3.1 Oligonucleotide synthesis cycle by Dmoc technology

Two 20 mer deoxyoligonucleotide strands using modified monomer **1** were synthesized. **Strand-1** was synthesized with modified monomer **1** at 10th position from 5' end while it was at the 1st position **Strand-2** from the 5' end (Figure 3.1). This monomer was most stable of all synthesized monomers for the purpose. and the ODN synthesis was expected to be less troublesome. The trityl graph was excellent for every cycle and was an indication of a good ODN synthesis. Though trityl graph may be misleading sometime but it gives accurate information for the

synthesis of shorter oligonucleotides like the ones we targeted. So, for time being we decided to rely on it for the success of an ODN synthesis. Only two ODN strands were synthesized using monomer **1**.

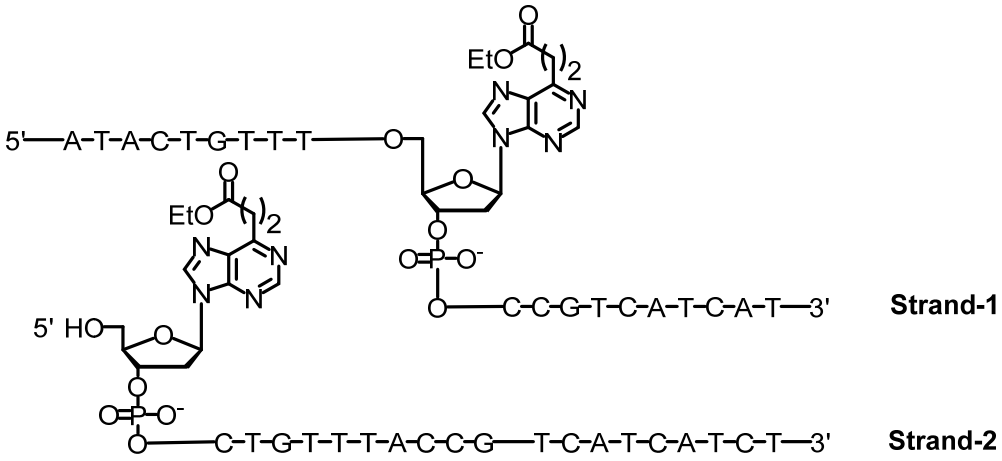


Figure 3.1 Sequences of the ODN strands with monomer **1**

ODN synthesis by using modified monomer **2** was carried out next. Four ODN strands were synthesized with one modified monomer **2** in each of them. In **Strand-3** it was placed at 9th position from 5' end while placed at 1st, 6th, and 19th position for **Strand 4**, **5**, and **6**, respectively (Figure 3.2). More ODN strands for this monomer were synthesized considering its sensitivity and likely work-up related problems. Another reason for making more strands with higher sensitivity monomers was their reactivity towards the nucleophiles. The chances of cross-linking of ODN strands with monomers **2**-**5** were greater as they were having better leaving group than the monomer **1**. This made us plan more ODN strands using monomers **2**-**5**.

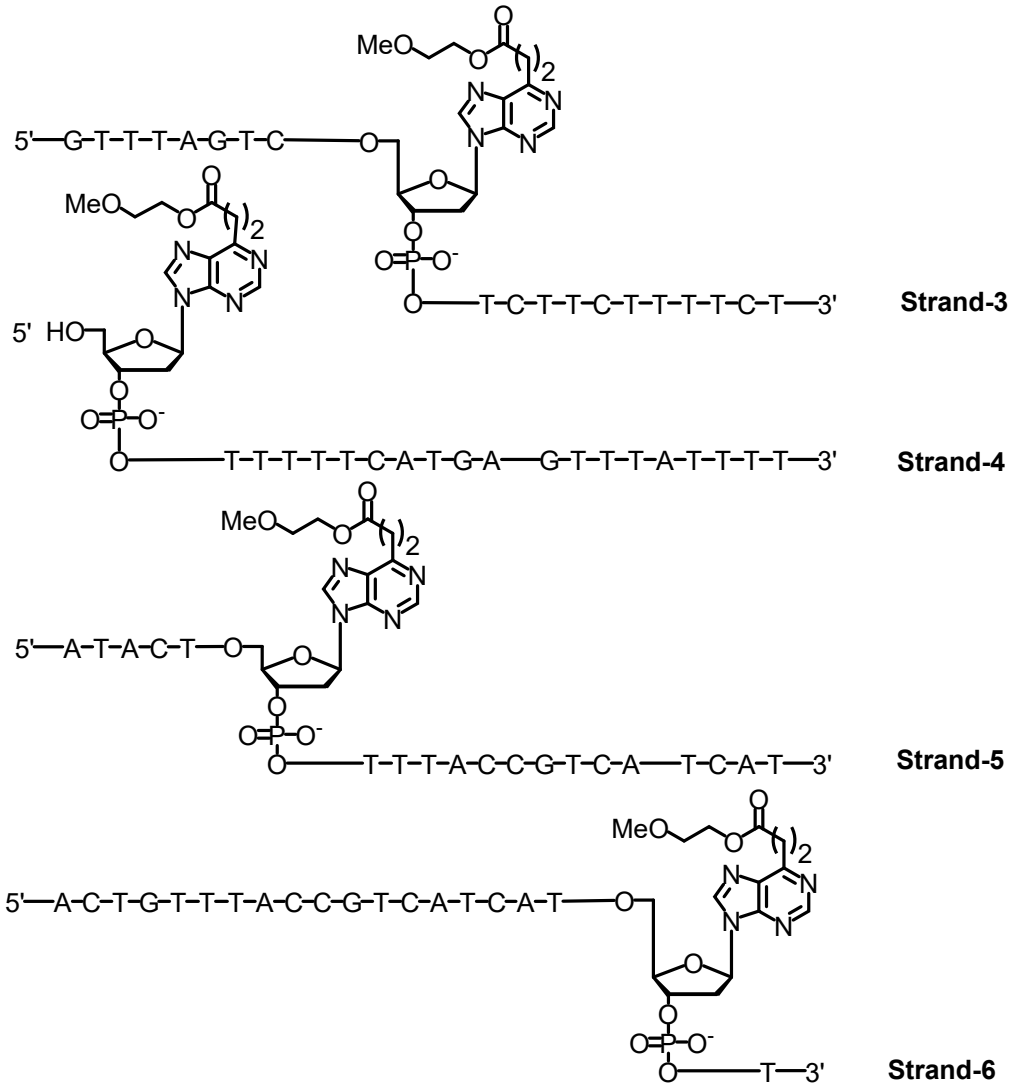


Figure 3.2 Sequences of the ODN strands with monomer **2**

Monomer **3** with methoxyphenyl ester was expected to be more sensitive to the acidic conditions employed during the ODN synthesis. four ODN strands (Figure 3.3) using this monomer were synthesized. All preparations were double checked carefully before the synthesis. The **Strand-7 8**, **9**, and **10** were synthesized with **3** at 8th, 1st, 9th and 1st positions, respectively from 5' end (Figure 3.3). The trityl graph was normal for all ODNs and for all cycles in each one of them. As it was one of

the very sensitive monomers, it was difficult to say anything about the success of ODN synthesis.

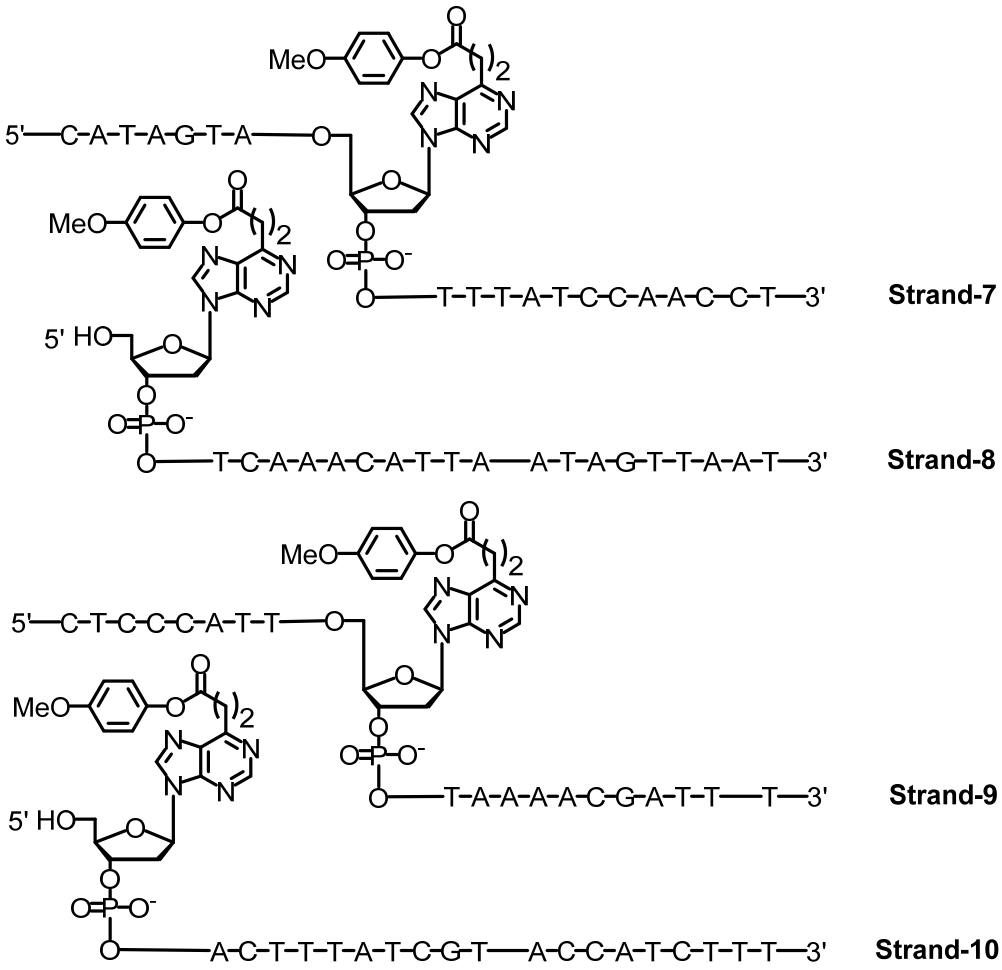


Figure 3.3 Sequences of the ODN strands with monomer **3**

We then moved on to the most sensitive monomers in the series we synthesized, monomer **4**. Position of the modified monomer kept closer to 5' end to minimize its exposure to the acidic conditions. The results for ODN syntheses were normal. We synthesized three ODN strands using this monomer; **Strands 11**, **12** and **13** with monomer **4** at 1st, 5th and 1st positions (Figure 3.4), respectively from the 5' end.

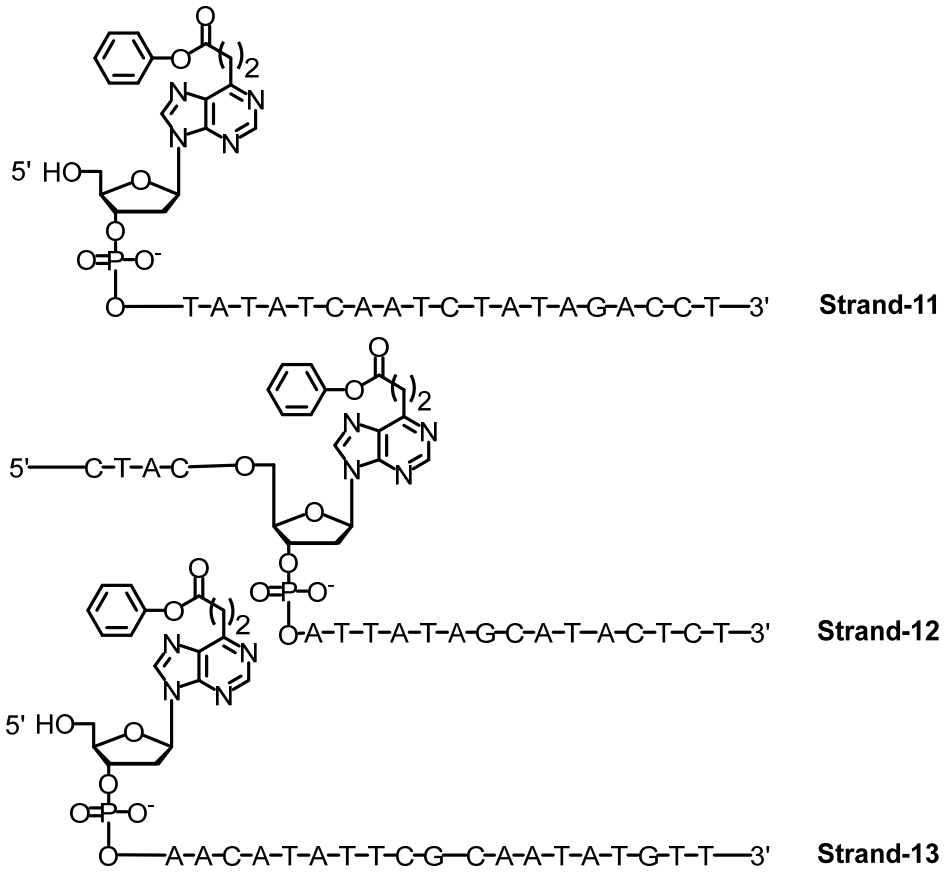


Figure 3.4 Sequences of the ODN strands with monomer **4**

All ODN syntheses using monomers **1**-**4** were excellent, at least on trityl monitor. So, we moved on to the monomer **5**. The sensitivity of monomer **5** was expected to be like monomer **3** and **4**. The ODN **Strands 14**, **15**, and **16** were synthesized with monomer **5** at 1st, 12th, and 10th positions (Figure 3.5), respectively form 5' end.

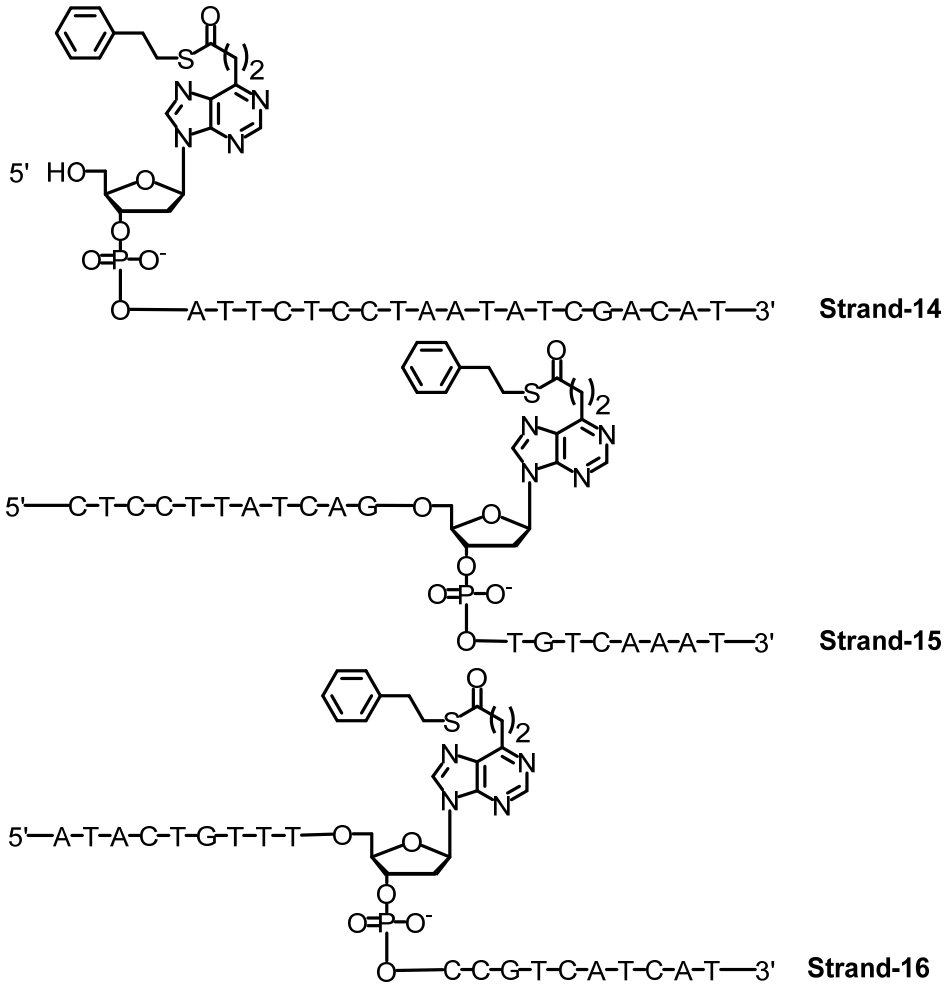
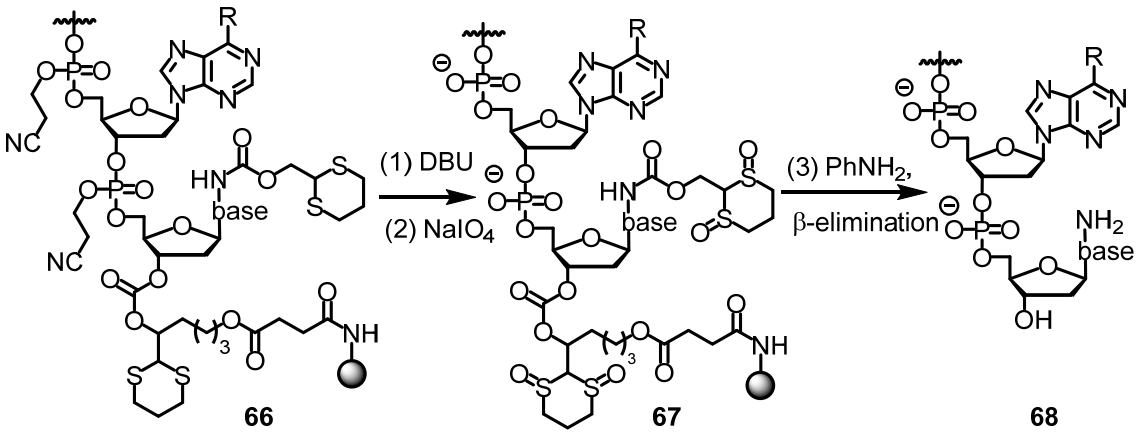


Figure 3.5 Sequences of the ODN strands with monomer **5**

The synthesis of ODNs with modified monomers at different positions and synthesis of multiple strands using each monomer was necessary in order tackle the challenges we were about to face during ODN work-up and purification. The ODN synthesis of even a very sensitive monomer such as the one with phenyl ester was excellent on trityl monitor but we were little suspicious about its survival during the work-up. So, we kept multiple options ready to explore all possible ways in the event of any such problems during work-up and purification.



R = -$(CH_2)_2COOC_2H_5$, -$(CH_2)_2COO(CH_2)_2OMe$, -$(CH_2)_2COOPh$,
-$(CH_2)_2COOPh(4\text{-}OMe)$, -$(CH_2)_2COS(CH_2)_2Ph$

Scheme 3.2 Post synthesis work-up of ODNs

After their synthesis the ODN strands were worked-up to get them cleaved from CPG and to remove Dmoc protections (Scheme 3.2). ODN strands with sensitive ester groups on **66** were treated with DBU to remove cyanoethyl group followed by oxidation of sulfurs on Dmoc protections using sodium periodate to give **67**. In the next step β-elimination by aniline gave final ODNs **68** in their crude forms. These crude ODNs were purified by reverse phase high performance liquid chromatography (RP-HPLC) to get pure ODN strands. Purification by RP-HPLC followed by MALDI-Tof mass determination have shown very good results for ODNs containing modified nucleobases **1** (Figures 3.6, 3.7 and 3.8 shown for ODN **Strand-1**), nucleobase **2** (Figures 3.9, 3.10 and 3.11 shown for ODN **Strand-5**) and nucleobase **5** (Figure 3.12, 3.13 and 3.14 shown for ODN **Strand-14**).

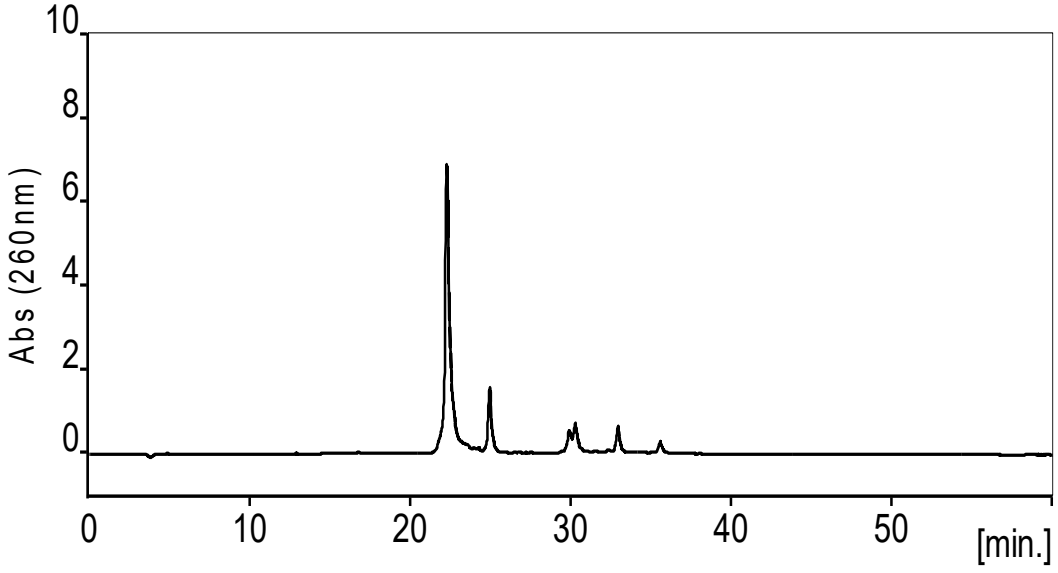


Figure 3.6 HPLC profile for ODN **Strand-1** (crude)

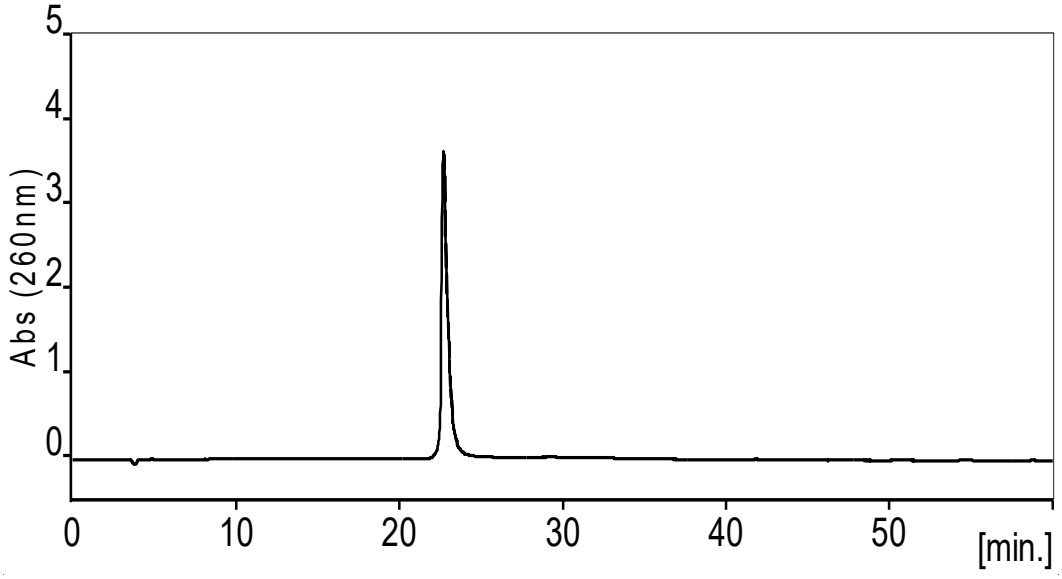


Figure 3.7 HPLC profile for ODN **Strand-1** (pure)

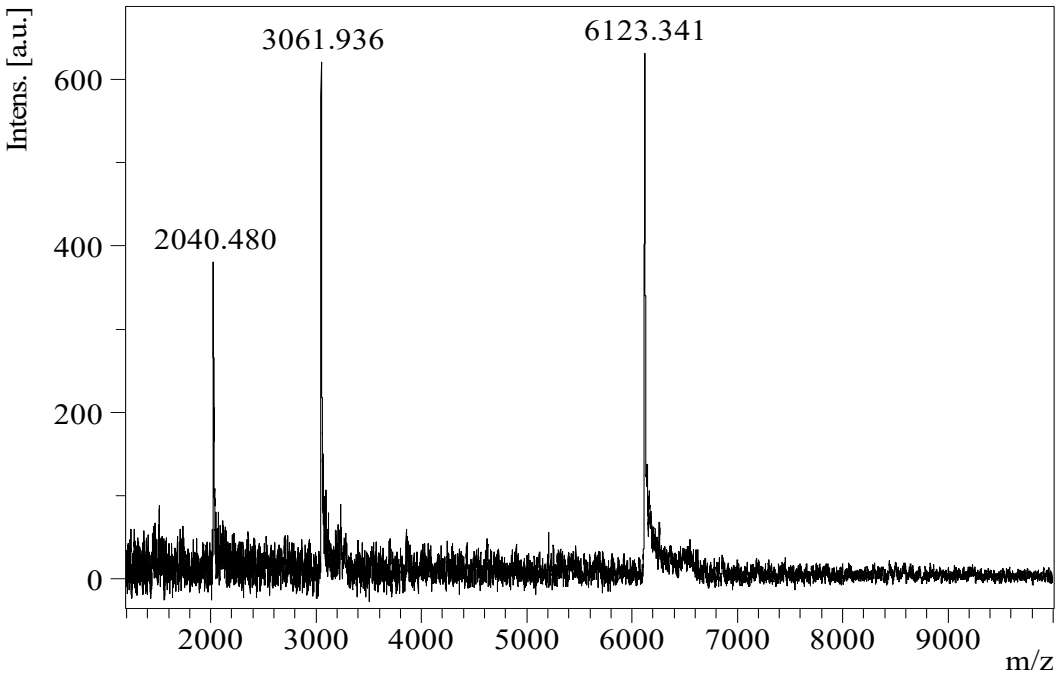


Figure 3.8 MALDI-Tof spectrum ODN **Strand-1**

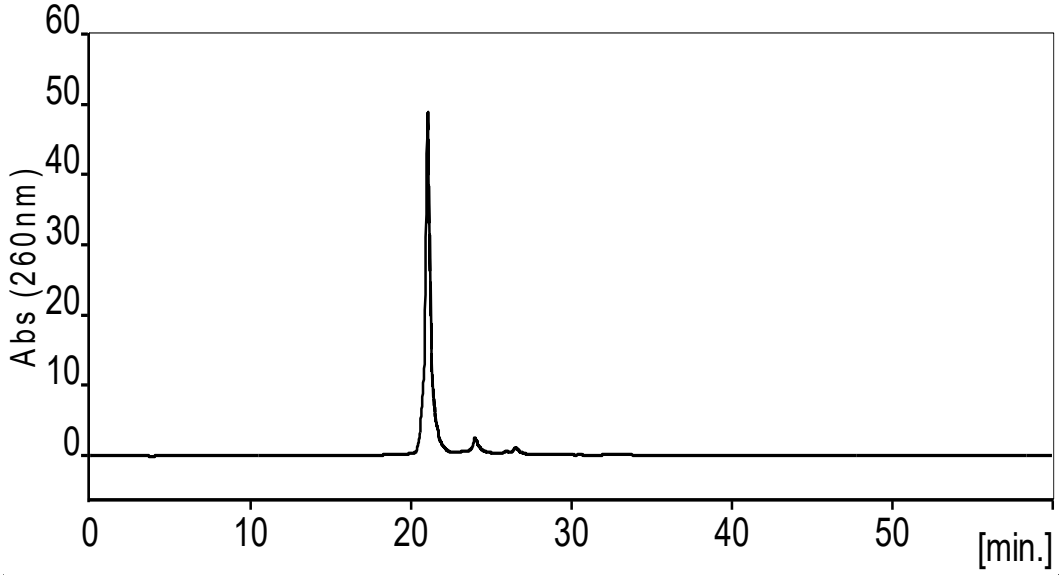


Figure 3.9 HPLC profile for ODN **Strand-5** (crude)

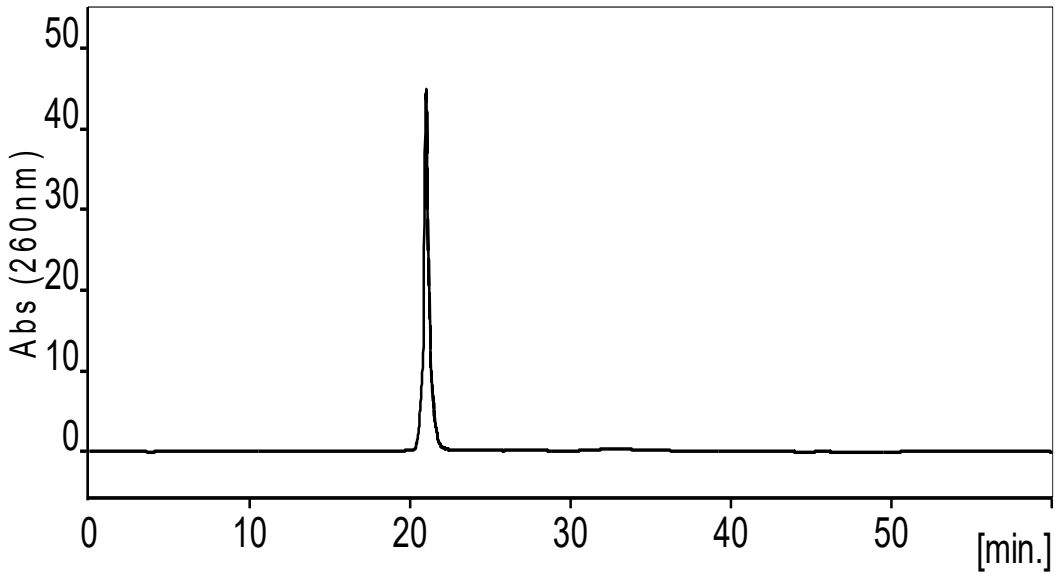


Figure 3.10 HPLC profile for ODN **Strand-5** (pure)

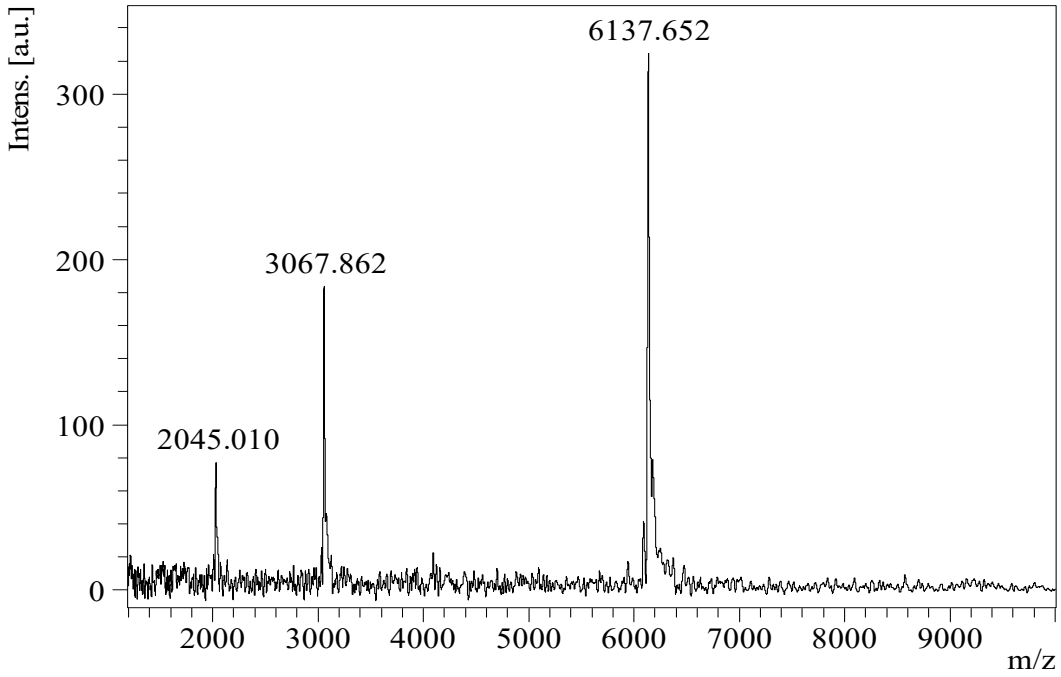


Figure 3.11 MALDI-Tof spectrum ODN **Strand-5**

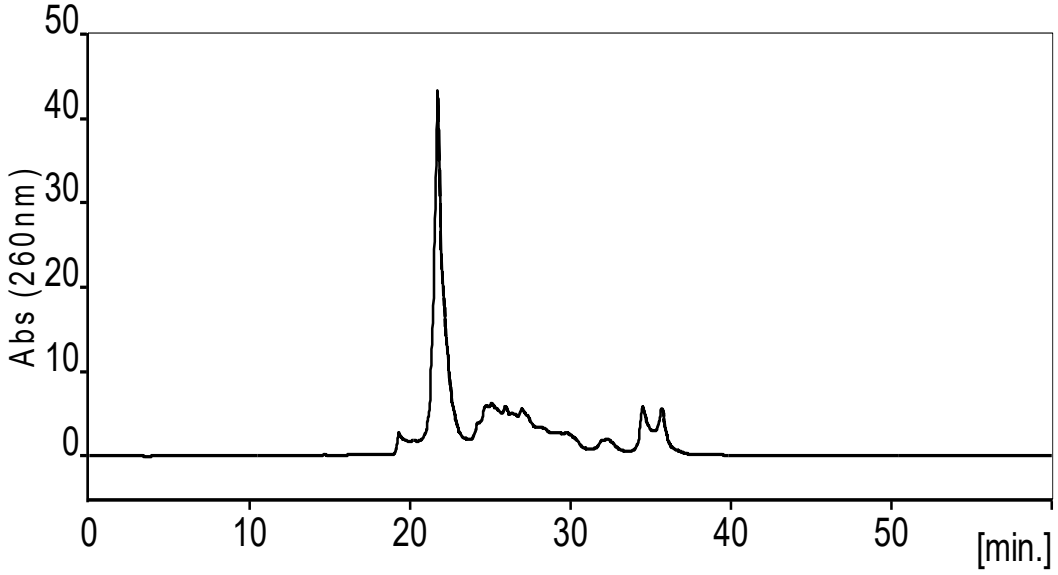


Figure 3.12 HPLC profile for ODN **Strand-14** (crude)

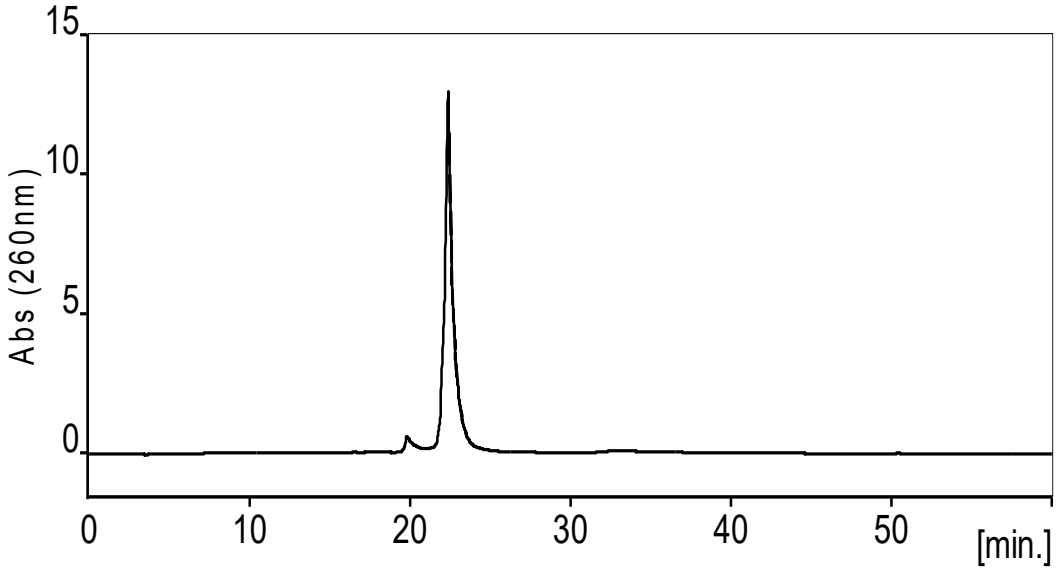


Figure 3.13 HPLC profile for ODN **Strand-14** (pure)

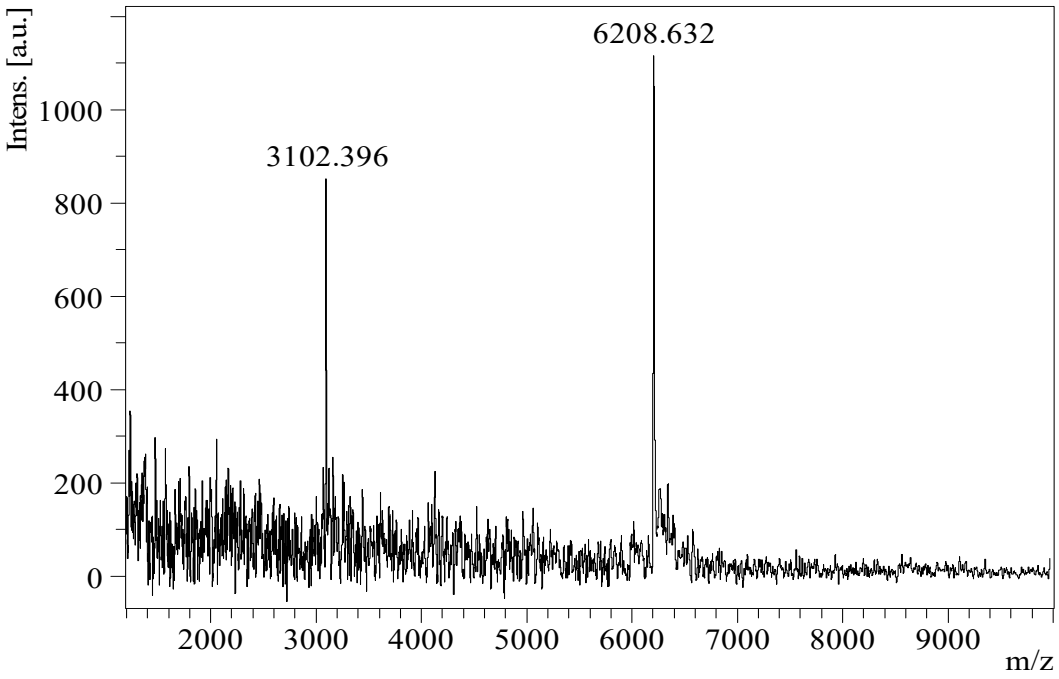
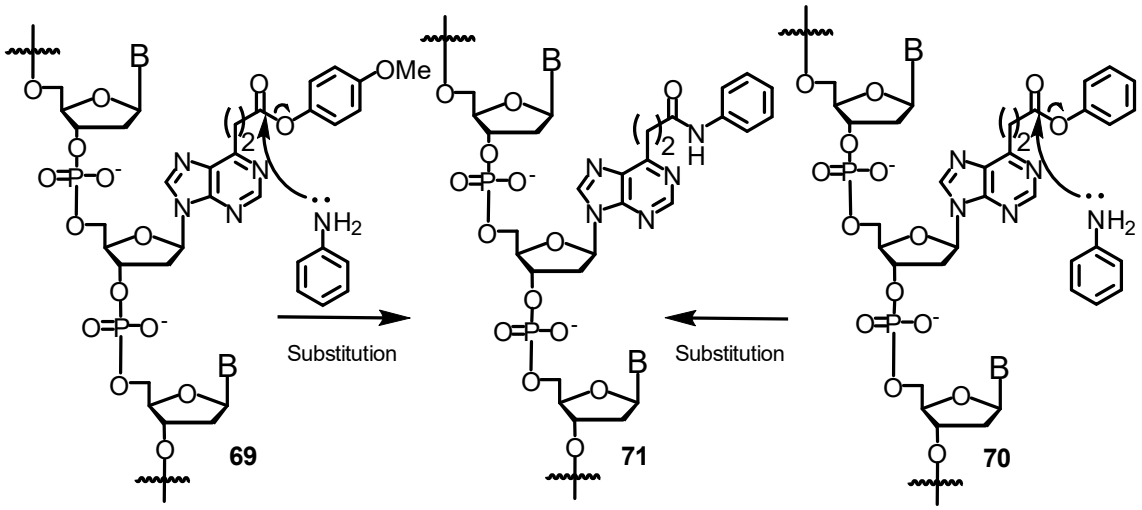


Figure 3.14 MALDI-Tof spectrum ODN **Strand-14**

But there was a problem with the work-up of ODNs with modified monomers **4** and **5**. We used aniline for β-elimination during the work-up of ODNs according to the Dmoc technology protocol. But, when ODNs with methoxyphenyl ester (a group on monomer **4**) and phenyl ester (a group on monomer **5**) were worked-up with this protocol, aniline was found to be replacing methoxyphenyl and phenyl groups buy nucleophilic substitution reaction.

When ODN **69** and ODN **70** containing methoxyphenyl and phenyl groups, respectively were treated with aniline. The substitution was occurring instead of β-elimination to give substituted product **71** (Scheme 3.3). The substitution was verified by purification of **Strand-7** (a methoxyphenyl strand) and **Strand-12** (a phenyl strand) using RP-HPLC and by MALDI-Tof analysis (Figures 3.15, 3.16,

3.17 and 3.18). The substituted and non-substituted products were observed almost in 1:1 ratio.



Scheme 3.3 Substitution by an aniline

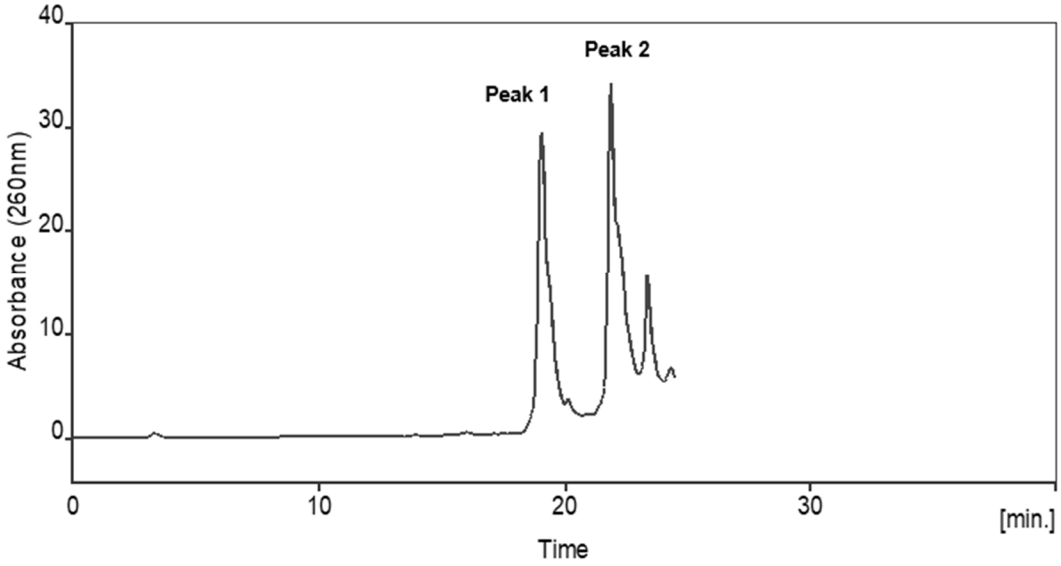


Figure 3.15 HPLC profile of ODN **Strand-7** after aniline work-up

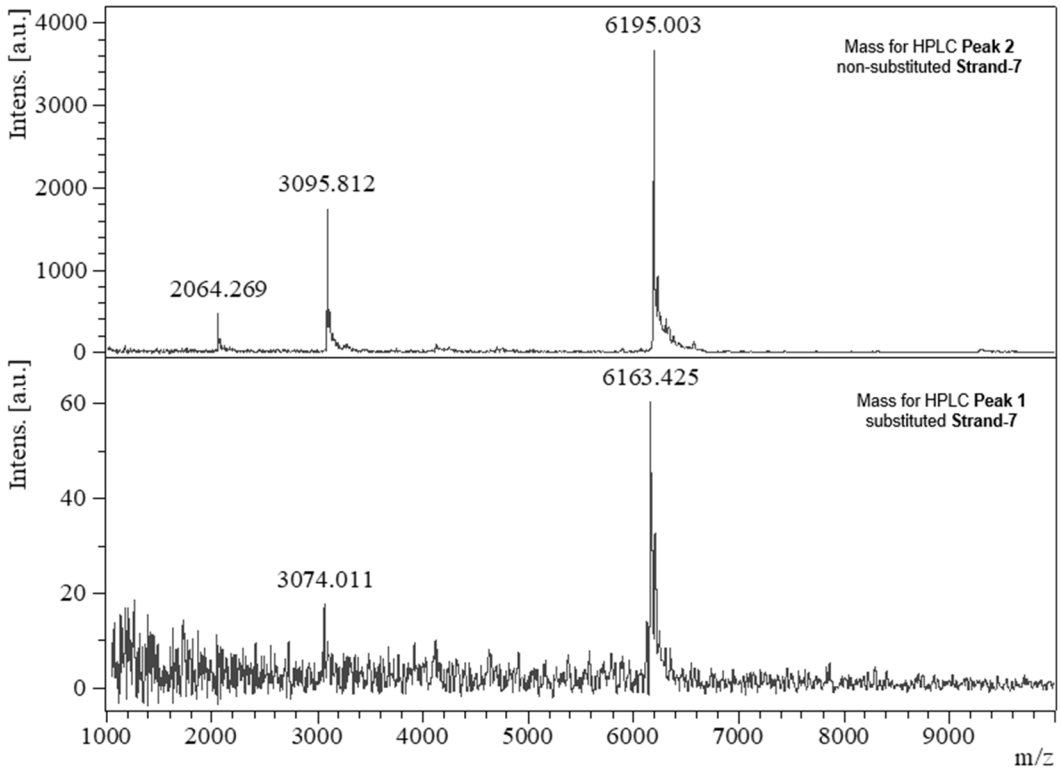


Figure 3.16 MALDI-Tof spectra of ODN **Strand-7** HPLC fractions after aniline work-up

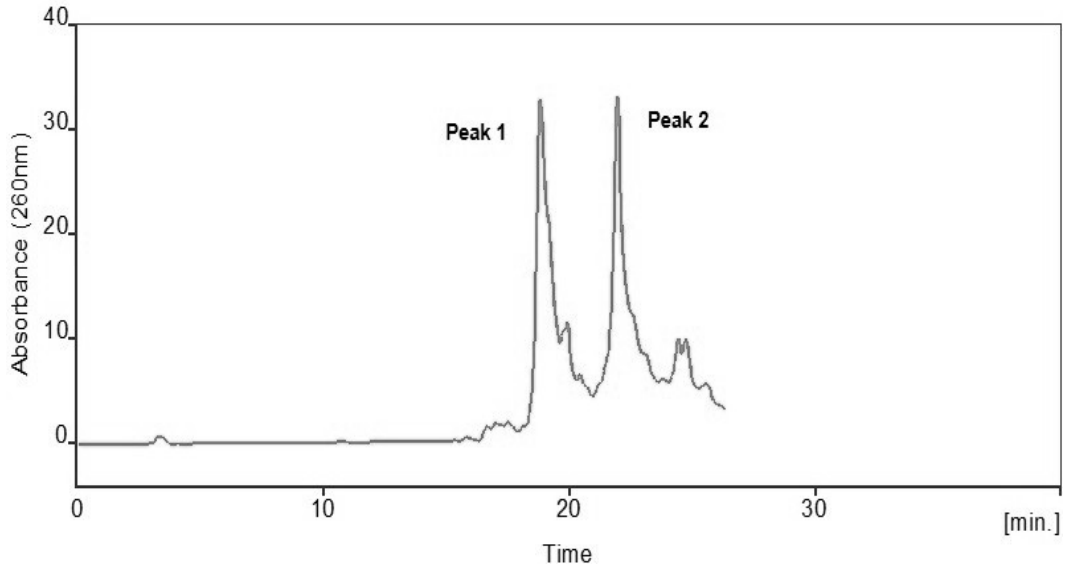


Figure 3.17 HPLC profile of ODN **Strand-12** after aniline work-up

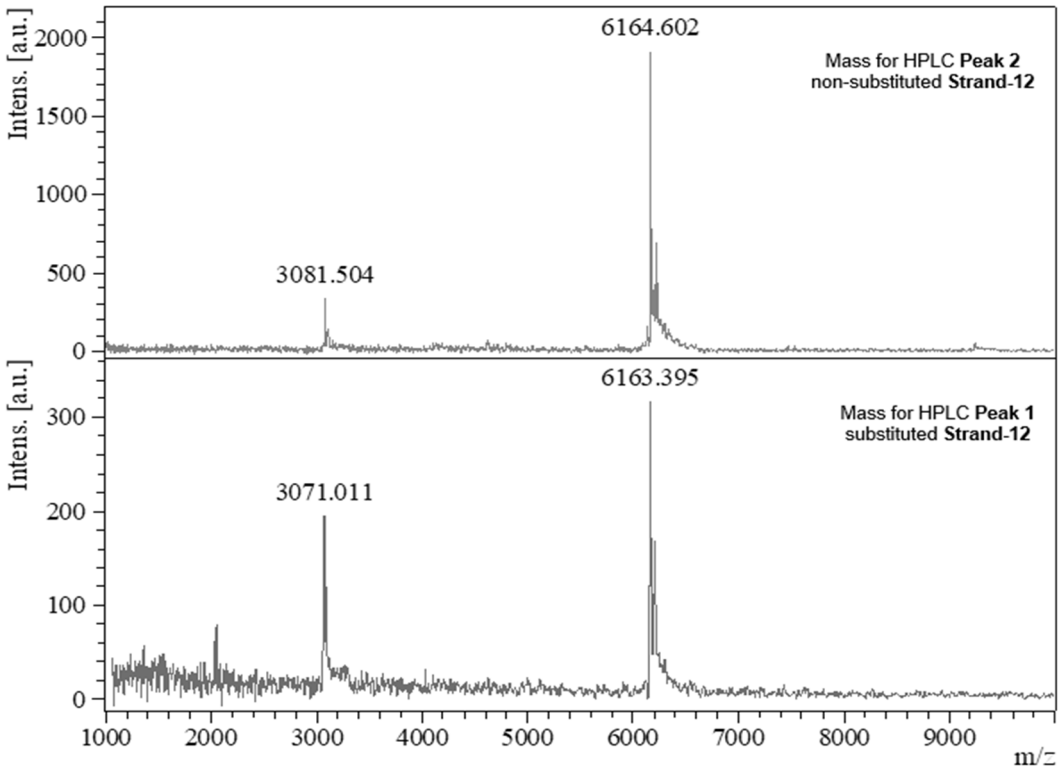
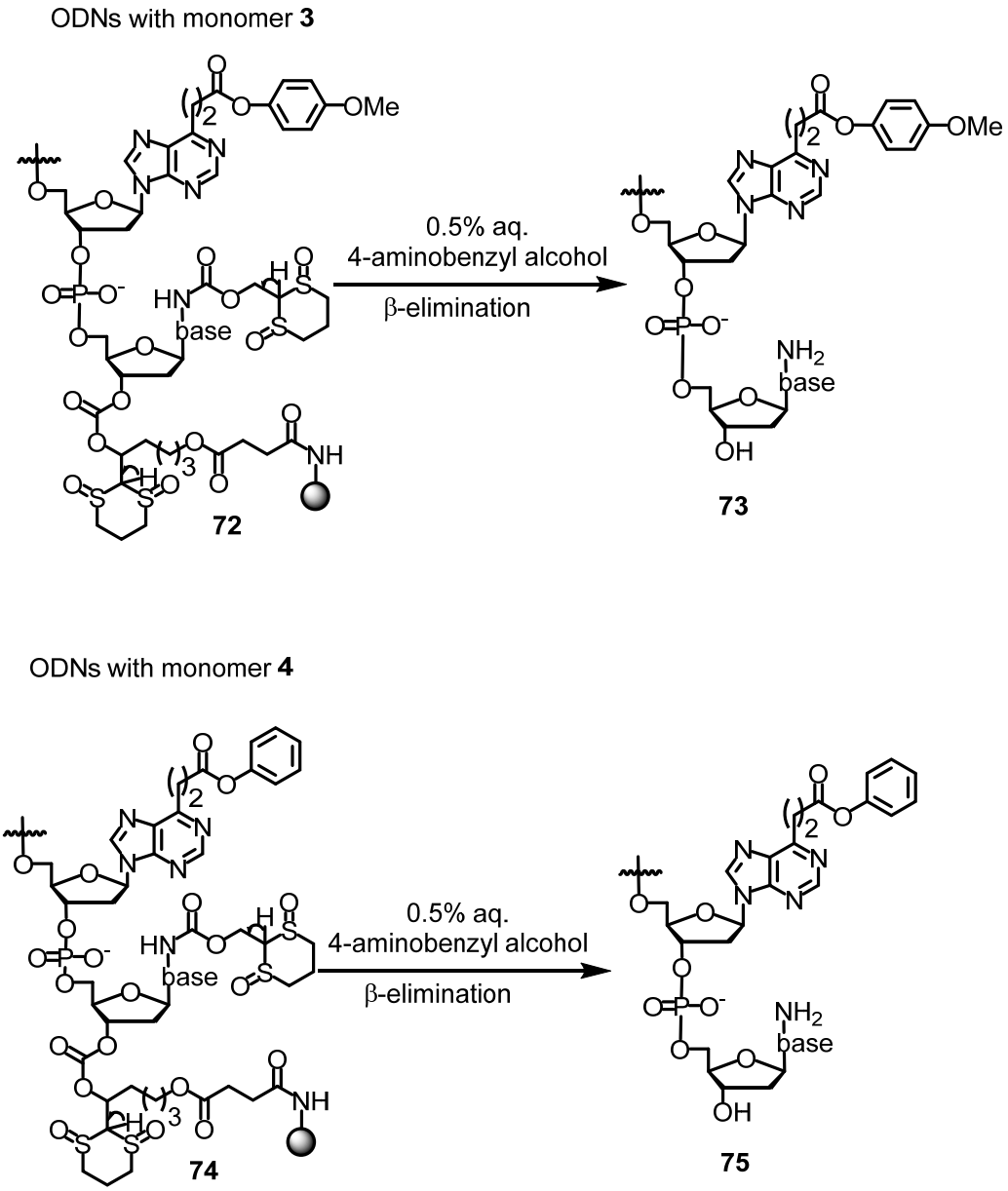


Figure 3.18 MALDI-Tof spectra of ODN **Strand-12** HPLC fractions after aniline work-up

The substitution instead of β-elimination was a big problem on the way to the development of potential oligonucleotide cross-linking agents using modified monomers **4** and **5**. To address this problem, we replaced 3% aniline suspension in water with 0.5% 4-aminobenzyl alcohol and carried the deprotection and cleavage (Scheme 3.4) under otherwise identical conditions. The lower nucleophilicity of 4-aminobenzyl alcohol was expected to minimize the substitution of sensitive methoxyphenyl and phenyl esters. Indeed, the results were much better. The analytical data for **Strand-7** (Figure 3.19, 3.20 and 3.21) and for **Strand-12** (Figure 3.22, 3.23 and 3.24). When the interval was long, more hydrolysis products were observed, but the yields of target ODNs were high

without any substituted ODNs.



Scheme 3.4 β-Elimination using 4-aminobenzyl alcohol

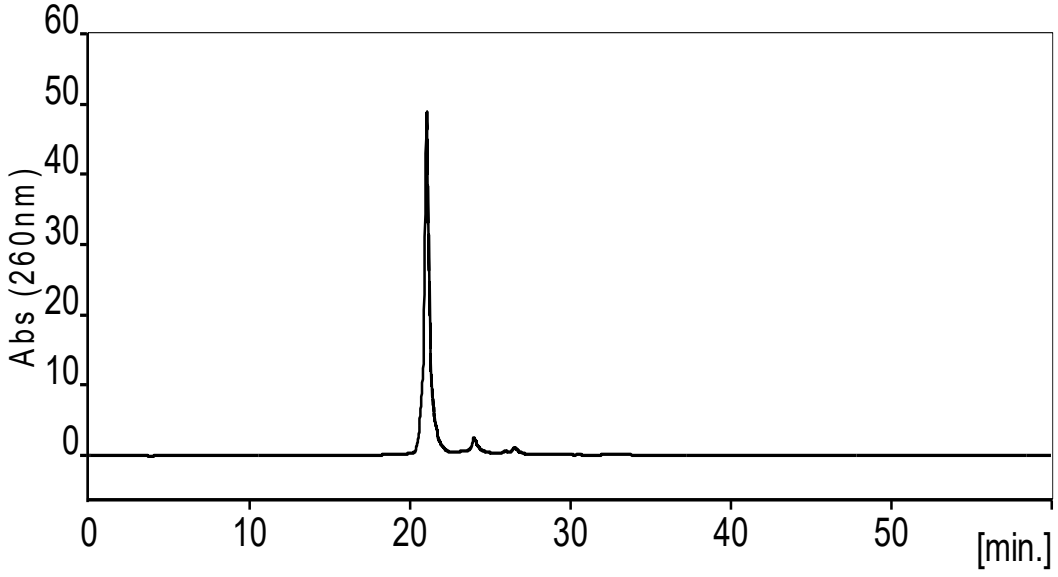


Figure 3.19 HPLC profile of ODN **Strand-7** (crude) after 4-aminobnzyl alcohol work-up

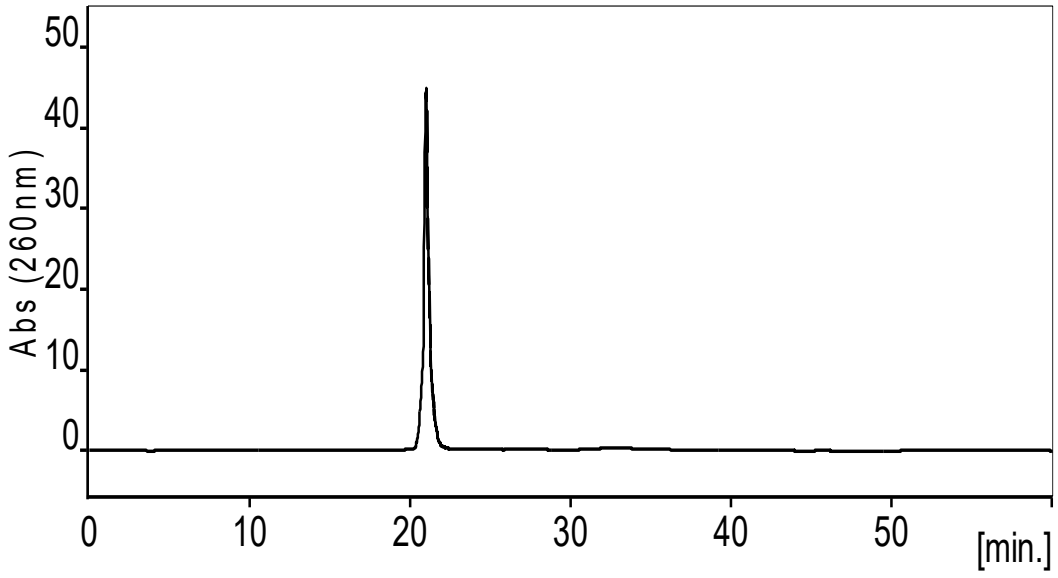


Figure 3.20 HPLC profile of ODN **Strand-7** (pure) after 4-aminobnzyl alcohol work-up

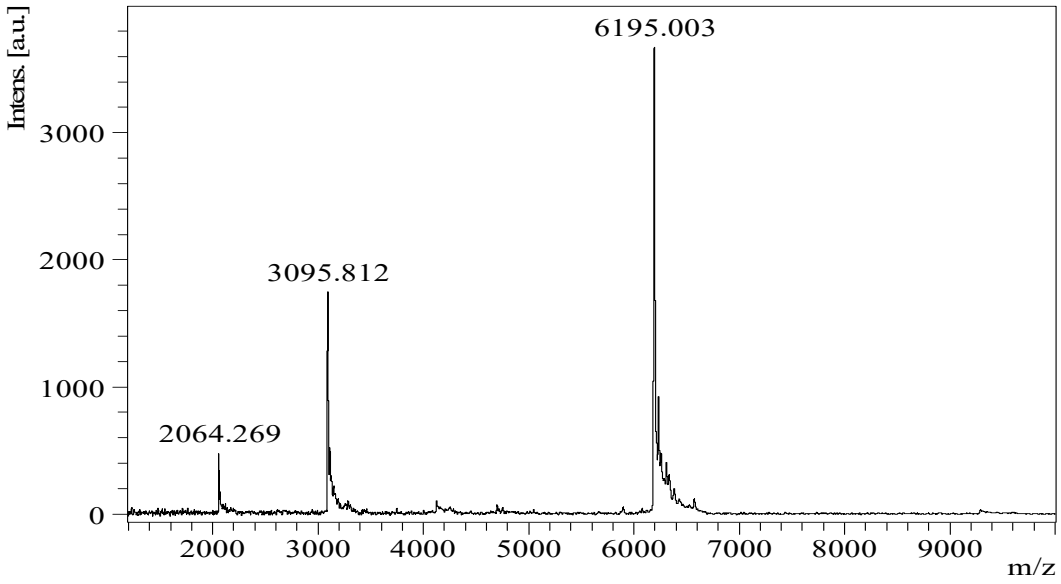


Figure 3.21 MALDI-Tof spectrum of ODN **Strand-7**

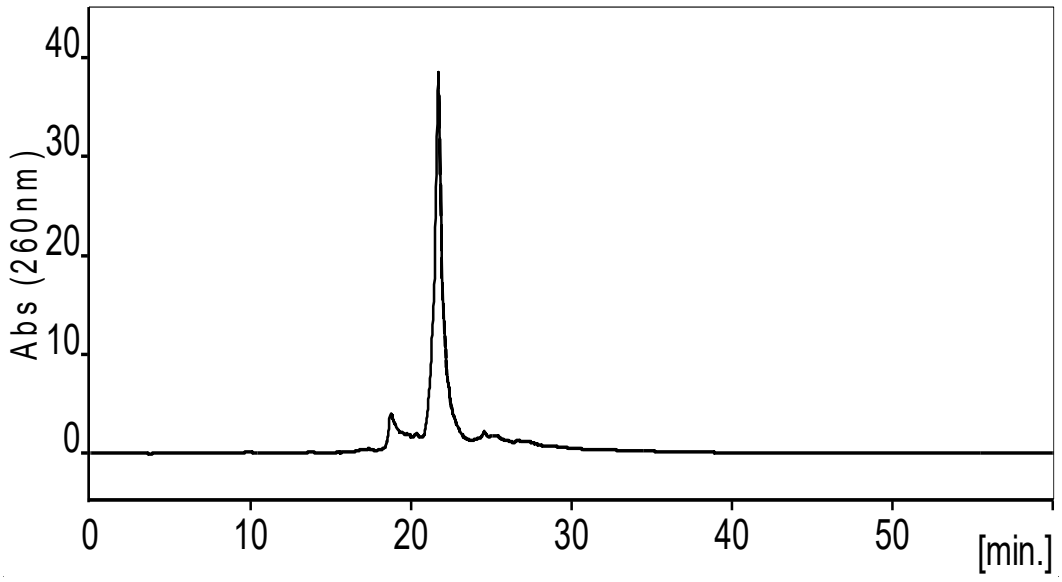


Figure 3.22 HPLC profile of ODN **Strand-12** (crude) after 4-aminobnzyl alcohol work-up

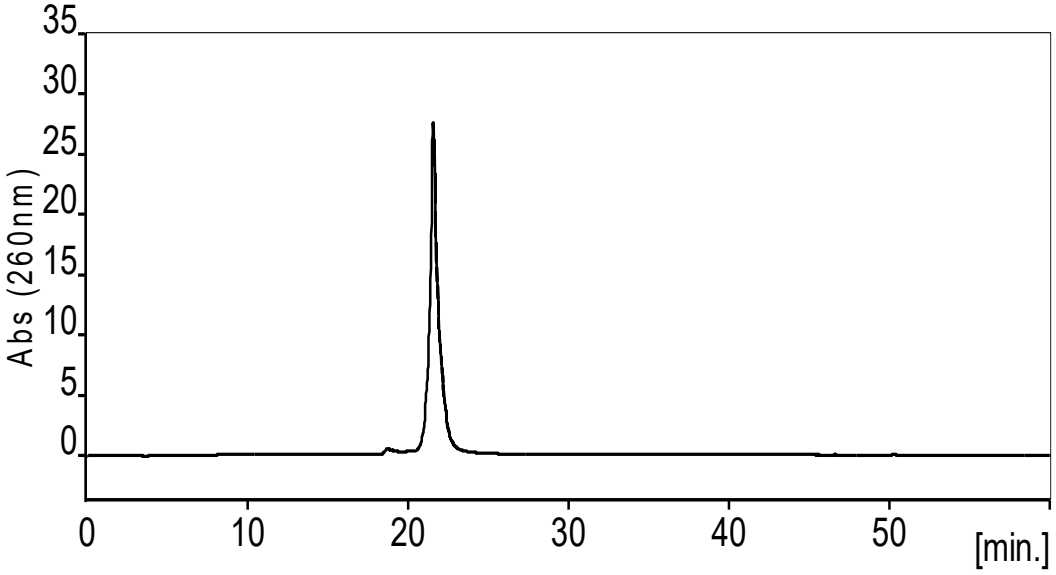


Figure 3.23 HPLC profile of ODN **Strand-12** (pure) after 4-aminobnzyl alcohol work-up

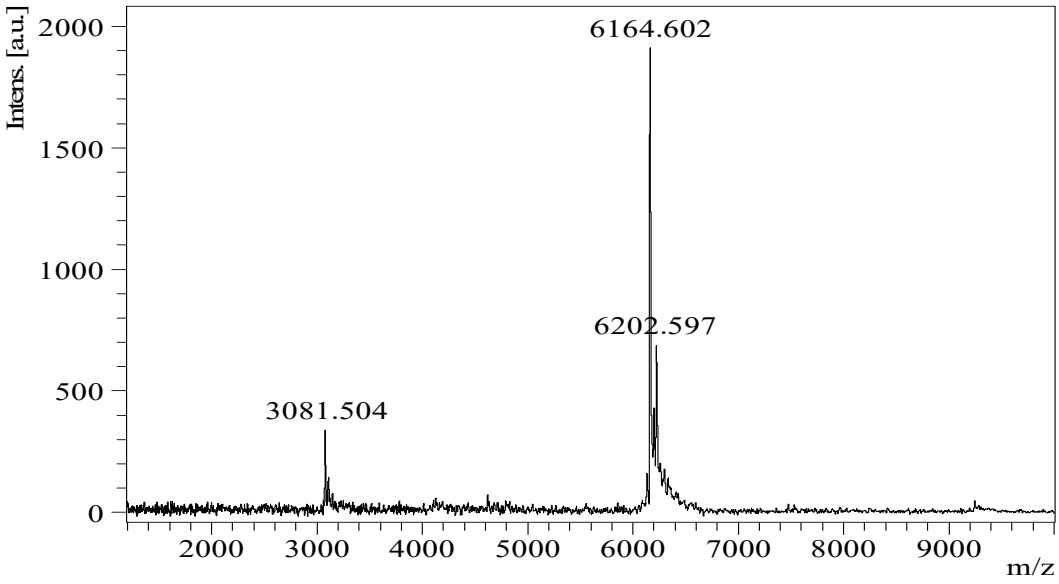


Figure 3.24 MALDI-Tof spectrum of ODN **Strand-12**

For work-up of ODNs with modified monomers **1**, **2** and **5** an aniline was used as a scavenger while for ODNs with modified monomers **3** and **4** the scavenger 4-

aminobenzyl alcohol was used. All ODNs were then purified by RP-HPLC followed by a characterization using MALDI-Tof. The yields for ODN strands were calculated using UV-Visible absorbance at 260 nm.

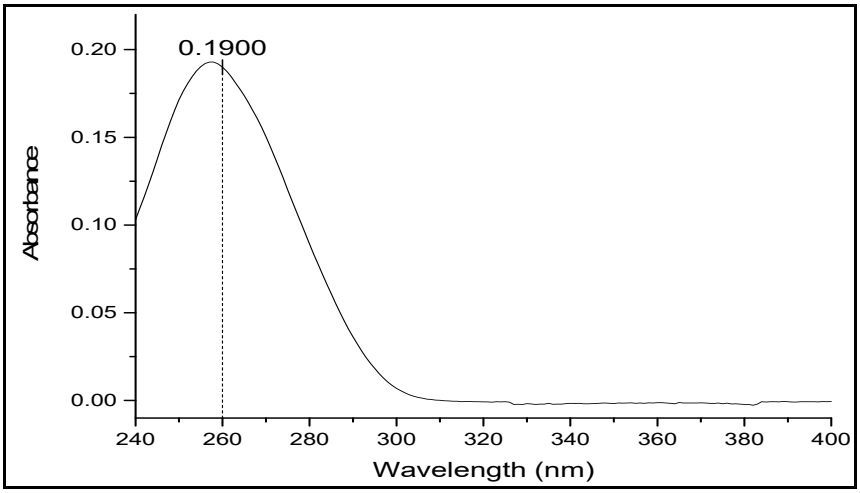


Figure 3.25 UV-Visible scan spectrum for **Strand-1**

For optical density measurement UV-Visible scan spectrum was obtained for each modified ODN like the one shown in Figure 3.25 for ODN **Strand-1**. The absorbance at 260 nm was taken as a maximum absorbance.

The sample calculations for **Strand-1** are shown as follows;

Molar mass of the ODN **Strand-1** was obtained using an online tool developed by Northwestern university[3] as 6125 da (or g/mol). Synthesis of ODNs calculated for 20 mg of solid support **10** to be 0.52 μmol = 0.52 $\times 10^{-6}$ mol. Molar Extinction Coefficient (ε) = 190300.00 liter.mol^{-1}.cm^{-1} calculated by considering modified monomer equivalent to adenine. Length of the cell was (*l*) = 1 cm. Absorbance (A) for ODN **Strand-1** at 260 nm = 0.1900 (Figure 3.25). Dilution factor = 10 (1/10th of

product worked-up) × 10 (1/10th of worked-up product was purified) × 2.5 (the purified product was diluted to 2.5 mL DI water) = 250

ODN Strands	(ε)	Expected Yield	A at 260 nm	OD	c	Yield	% Yield
2	183500.00	3.1725	0.1905	47.63	10.38×10^{-7}	1.5832	49.90
3	176000.00	3.1944	0.2859	71.48	16.24×10^{-7}	2.4941	78.08
4	188600.00	3.2271	0.2108	52.70	11.18×10^{-7}	1.7346	53.75
5	193600.00	3.1923	0.3257	81.43	16.82×10^{-7}	2.5814	80.86
6	190300.00	3.2006	0.2405	60.13	12.64×10^{-7}	1.9450	60.77
7	203900.00	3.2188	0.2160	54.00	10.59×10^{-7}	1.6388	50.91
8	194300.00	3.2063	0.3225	80.63	16.60×10^{-7}	2.5589	79.81
9	203000.00	3.2396	0.1833	45.83	9.03×10^{-7}	1.4064	43.41
10	195100.00	3.2219	0.1476	36.90	7.57×10^{-7}	1.1716	36.39
11	212300.00	3.2594	0.1357	33.93	6.39×10^{-7}	1.0013	30.72
12	192900.00	3.2219	0.1443	36.08	7.48×10^{-7}	1.1587	35.96
13	186300.00	3.2126	0.1469	36.73	7.89×10^{-7}	1.2179	37.91
14	195300.00	3.1652	0.2169	54.23	11.11×10^{-7}	1.6907	53.41
15	199200.00	3.1517	0.2258	56.45	11.34×10^{-7}	1.7176	54.50
16	206100.00	3.1850	0.1970	49.25	9.56×10^{-7}	1.4639	45.96

Table 3.1 Yields for modified ODN strands

Optical density (0.1900 × 250) = 47.5

Expected yield (6125 ×0.52 ×10^{-6}) = 3.1850 mg

Concentration (c = A/ ε.*l*) = 9.98×10^{-7} M

The number of moles in 1.0 ml sample solution = 9.98×10^{-10} mol

Yield (6125 g/mol × 9.98×10^{-10} mol × 250) = 1.5282 mg

Percent Yield (1.5282/3.1850 × 100) = 48.98%

The OD and yield calculations were performed for all remaining modified ODN strands using same method described above (Table 3.1). The highest yield was

found for ODN **Strand-5** at 80.86% and lowest yield was reported for ODN **Strand-8** at 30.72%.

For cross-linking studies, we needed complementary unmodified ODNs which were synthesized by using standard phosphoramidites and standard ODN synthesis cycle as well as work-up. We synthesized five unmodified 20 mer ODN strands complementary to modified ODN strands **1**, **3**, **7**, **12** and **15**. Modified ODN strands were selected based on the position of modified monomer. In all selected modified strands, the position of respective monomer was somewhere in the middle of the ODN. The middle position of the modified monomer in an ODN was expected to be better for the formation of double helical configuration with complementary ODN strand. Both ends with natural nucleobases could interact with complementary ODN strand to facilitate double helix configuration holding modified monomer tightly to one place.

3.3 Experimental section

3.3.1 General Experimental

ODNs were synthesized on MerMade 6 solid phase oligo synthesizer. Solvents and reagents were bought from various commercial sources and used as they were. For ODN synthesis 0.1 M solutions of all phosphoramidite (including modified) were prepared in anhydrous acetonitrile. For ODN Synthesis volumes of phosphoramidite solutions were calculated according to the amount to be delivered

to avoid wastage. Vacuum calibration of MerMade 6 was performed before each new batch of the synthesis using Dmoc linker **10** and standard phosphoramidite solutions to ensure exact delivery of reagents and for maximum exposure of linker **10** to the reagents. Standard Cap-A was replaced by phenoxyacetic anhydride for the synthesis of modified ODNs. RP-HPLC was performed on a JASCO LC-2000Plus System: pump, PU-2089Plus Quaternary Gradient; detector UV-2075Plus. For RP-HPLC a C-18 reverse phase analytical column (5µm diameter, 100 Å, 250 × 3.20 mm) was used. RP-HPLC mobile phase: Solvent A: 0.1 M triethylammonium acetate, 5% aqueous acetonitrile. Solvent B: 90% aqueous acetonitrile. RP-HPLC profiles were generated by detection of absorbance of ODN at 260 nm using the linear gradient solvent system: solvent B (0%-45%) in Solvent A over 60 min followed by Solvent B (45%-100%) in Solvent A over 20 min at a flow rate of 1.0 mL/min. Products were analyzed by Bruker Microflex LRF MALDI-Tof to confirm the synthesis of desired ODN. UV-Visible scan spectrum was recorded for each purified ODN using Perkin Elmer Lamda 35 UV-Visible spectrophotometer.

3.3.2 Synthesis of oligodeoxynucleotides

An ODN synthesis was carried out using standard ODN synthesis cycle (Scheme 3.1) The slight changes were incorporated to use Dmoc technology efficiently. Other than the Dmoc protected phosphoramidites **7**-**9** and Dmoc linker **10**, commercially available dT-phosphoramidite was used. Dmoc technology requires

same reagents so all other reagents were obtained from commercial sources and used as they were. 20 mg of Dmoc linker **10** with a loading of 26 μmol/gm was taken in the synthesis column and column was packed with suitable frits from both ends. The synthesis of ODN was expected to be 0.52 μmol. The ODN synthesis cycle involved sequential detritylation, coupling, capping and oxidation. Each step was programmed correctly to ensure the sequence of events throughout the ODN synthesis cycle.

3.3.2.1 Detritylation

The ODN synthesis cycle started with the detritylation of a base already present on a solid support **10.** Detritylation was carried out using 3% difluoro acetic acid in dichloromethane. 180 μL of detritylating agent was delivered to the ODN synthesis column and vacuum pulse for 30 ms was applied immediately to pull the reagent down to the bottom. Wait time for the step was adjusted to 45 s before another vacuum pulse. Wait time after second vacuum pulse was same i.e. 45 s. At the end of 90 second reagent present in the column was drained off and fresh detritylating agent was delivered one more time and all previous steps were repeated to ensure complete detritylation. After the completion of detritylation product was washed thoroughly using anhydrous acetonitrile (180 μL× .3 times). After washing the cycle was allowed to proceeded to the coupling step.

3.3.2.2 Coupling

During this step 90 µL solution of a desired phosphoramidite (0.1 M in acetonitrile) was delivered to the column with 90 µL of an activator 5-ethythio-1H-tetrazole (0.25 M in acetonitrile). A vacuum pulse of 35 ms was applied. After 60 s of wait time another vacuum pulse was applied. After second wait time for 60 s, the reagent was drained off and fresh phosphoramidite solution was delivered. To maximize the yield**,** coupling was carried out three times by repeating the steps above. At the end solid support was washed thoroughly using anhydrous acetonitrile (180 µL× .3 times). Capping was the next step to complete.

3.3.2.3 Capping

Capping was performed by delivering 90 µL of Cap-A (THF: phenoxyacetic acid: 2,6-lutidine 80:10:10) and 90 µL of Cap-B (16% v/v solution of 1-methyl-immidazole in THF). Two vacuum pulses for 30 s each and two wait times of 45 s each were applied. Capping was carried out only once. Washing by anhydrous acetonitrile (180 µL× .3 times) was carried out at the end before moving to next step.

3.3.2.4 Oxidation

180 µL of oxidizing solution (0.02 M iodine in v/v 1/1/1 THF/pyridine/H_2O) was delivered to the column. Two vacuum pulses for 30 s each and two wait times of 45 s each were applied. Oxidation was carried out only once. At the end solid

support was washed thoroughly using anhydrous acetonitrile (180 µL× .3 times).

3.3.2.5 Next cycle

After the first synthesis cycle dimer was a desired ODN strand and ready for the next round of synthetic cycle. The synthesis cycle was repeated another 18 times by delivering appropriate phosphoramidite solution during each cycle to get 20 mer ODNs. Each 20 mer ODN was synthesized using one modified nucleobase from modified nucleobases **1**-**5**.

3.3.3 Post synthesis ODN work-up

Unmodified complementary ODN strands were worked-up by treating CPG after synthesis with ammonium hydroxide solution at 55 °C for 8 hours. They were purified by RP-HPLC using preparative column to get enough amount to conduct cross-linking experiments. Modified ODNs were worked-up by Dmoc protocol.

3.3.3.1 Work-up using Dmoc technology protocol

Following procedure for the ODN work-up was adopted[2] to keep sensitive esters intact.

Taken 1/10th (2.2 mg) of ODN on CPG after ODN synthesis in 2.0 mL Eppendorf tube. DBU (10% in an anhydrous acetonitrile) 1.0 mL was added to it, tube was capped, sealed, vortexed gently, and rotated for 15 minutes. The tube with sample was centrifuged at 2.5 × 10^3 rpm for 30 seconds and DBU solution was removed

carefully by micropipette. To wash residual DBU from ODN sample, 200 µL acetonitrile was added in to the tube, tube was vortexed gently, centrifuged at 2.5 × 10^3 for 30 seconds and supernatant was removed carefully. The washing was repeated twice to ensure no more DBU was left in the ODN sample tube. To oxidize sulfur protecting groups on DNA as well as sulfurs on the linker, 1.0 mL, 0.4M $NaIO_4$ solution was added to the tube. Tube was capped, sealed, vortexed gently, and rotated for 3 h. $NaIO_4$ solution in the sample tube was changed with the fresh 1.0 mL, 0.4M $NaIO_4$ solution every 60-65 minutes. The tube with ODN and $NaIO_4$ solution from last step was centrifuged at 2.5 × 10^3 rpm for 30 seconds and $NaIO_4$ solution was removed carefully by micropipette. Washing was carried out by following the same procedure used to wash DBU except DI water (200 µL × 3) was used for washing instead of acetonitrile. Cleavage and deprotection of DNA was carried out in an aniline suspension/solution (3 % in water). 1.0 mL aniline solution was added into the sample tube, tube was capped, sealed, vortexed gently, and rotated for 1 hour. The tube with sample was centrifuged at 5.0 × 10^3 rpm for 3 minutes and supernatant was removed carefully and collected in a different 2.0 mL Eppendorf tube. DI water was added to the ODN sample tube, vortexed gently and centrifuged at 5.0 × 10^3 rpm for 3 minutes. Supernatants were combined in the Eppendorf tube. Obtained about 1.4 mL of the DNA solution. Solvent (mostly water and very little aniline) was evaporated over high vacuum and volume was reduced to approximately 50 µL. To this residue 1-Butanol (500 µL) was added, the tube was capped, vortexed strongly for 1 minute, and the solution was centrifuged at 14.5 × 10^3 rpm for 15 minutes. Supernatant was removed by micropipette

completely to get invisible or barely visible precipitate with crude DNA. The precipitate was dissolved in 200 µL DI water, vortexed and centrifuged at 5.0×10^3 rpm for 2 minutes. 20 µL crude ODN sample was injected to HPLC by sample syringe and purified it to get pure ODN. Rest of the 180 µL of crude sample was purified by using a preparative column by RP-HPLC to obtain enough amount of ODN for cross-linking experiments.

3.3.3.2 Modification of Dmoc ODN work-up protocol

An aniline was replaced by 0.5% 4-amonobenzyl alcohol solution for ODNs containing monomers **3** and **4** while rest of the procedure from Dmoc work-up protocol kept same. Except phenyl esters, all other monomers were cleaved and deprotected by using an aniline suspension/solution (3 % in water) with the procedure described earlier. After each successful work-up ODNs were purified by RP-HPLC followed by MALDI-Tof analysis and optical density measurements by UV-Visible spectroscopy.

3.3.4 MALDI-Tof mass analysis

3.3.4.1 Matrix preparation

3-HPA (3- hydroxy picolinic acid) was selected as a matrix for ODNs and Bruker Daltonics protocol[4] for matrix preparation was used with appropriate adjustments as follows;

Prepared 1 mL 0.1% solution of TFA (trifluoroacetic acid) in water. 500 µL of this 0.1% TFA solution was taken in an Eppendorf tube and to it added 500 µL acetonitrile. To it added 10 mg of diammonium hydrogen citrate and dissolved it completely by vortexing. This solution is named as TA50 by Bruker Daltonics. 500 µL of TA50 was taken in another Eppendorf tube and approximately 5 mg of 3-HPA was added to it. The mixture was vortexed strongly for about 1 minute to dissolve all 3-HPA. Another batch of about same amount of 3-HPA was added to previously obtained solution and vortexed to dissolve it. The addition of 3-HPA and vortexing was continued till some of 3-HPA was found insoluble. The mixture was sonicated for 5 minutes to dissolve maximum of 3-HPA. This saturated mixture was centrifuged at 5.0×10^3 rpm for 3 minutes. Supernatant was removed and clear solution was used as a matrix solution.

3.3.4.2 Sample preparation

All ODN samples modified as well as unmodified were purified and then the pure ODNs were repurified again by RP-HPLC to ensure maximum purity. Pure ODNs were collected manually in Eppendorf tubes. 5 µL of the repurified sample from about 2 mL of total volume collected during repurification was taken separately for MALDI-Tof mass analysis. This 5 µL of pure ODN sample was dried over Centrivac and then dissolved in 50 µL of DI water. This solution served as an ODN sample for MALDI-Tof mass analysis.

0.5 µL of matrix was applied on a ground steel Maldi Target and let it dry for 30 minutes. On this dried matrix 0.5 µL of ODN sample was applied and allowed to dry it for another 30 minutes. Drying was extended up to 2 hours whenever necessary.

3.3.4.3 Preparation of calibration standards

Laboratory synthesized standard DNAs, purified and characterized accurately were used as standards for calibration of MALDI-Tof. ODN selected for calibration was always within the mass range ± 100 da of the sample ODN. The standards were prepared using the same procedure used for sample preparation.

3.3.4.4 Data acquisition

The MALDI data were acquired in negative mode. First the mass for a standard calibrant was recorded and the equipment was calibrated to this exact mass. Calibration was checked by acquiring data for the standard calibrant at least twice after calibration. After calibration was found correct the data for sample ODNs were recorded. The recorded mass for all modified ODNs and unmodified complementary ODNs was found within acceptable range (± 0.01%).

3.3.5 UV-Visible measurements

For optical density measurement and yield calculations UV-Visible scan spectrum was obtained for each modified ODN.

The ODN sample was purified and then repurified by RP-HPLC as described earlier. This repurified ODN sample was then dried over Centrivac. The completely dried ODN sample was the dissolved in 2.5 mL of DI water. The UV-Visible absorbance scan spectrum was recorded over 400 nm – 200 nm range. The absorbance at 260 nm was taken as a maximum absorbance.

3.4 Synthesis of ODNs using monomer 6

As discussed earlier in last chapter another modified monomer **6** was synthesized to check the feasibility of Dmoc technology. Using same modified ODN synthesis cycle used for making ODN **Strands 1-16**, synthesis of two ODN **Strands 17** and **18** (Figure 3.26) using modified monomer **6** was accomplished. The ODN work-up with the use of aniline as a scavenger worked great. Purification of ODNs was carried out using RP-HPLC by following the same procedure to get pure ODNs (Figures 3.27 and 3.28). MALDI-Tof mass analysis confirmed the successful incorporation of modified monomer **6** into ODN (Figure 3.29).

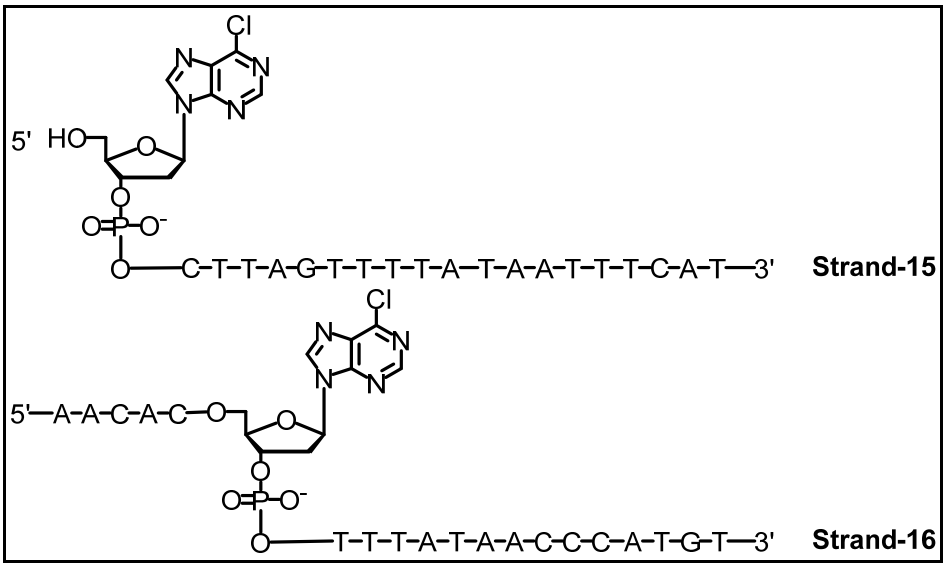


Figure 3.26 Sequences of the ODN strands with monomer **6**

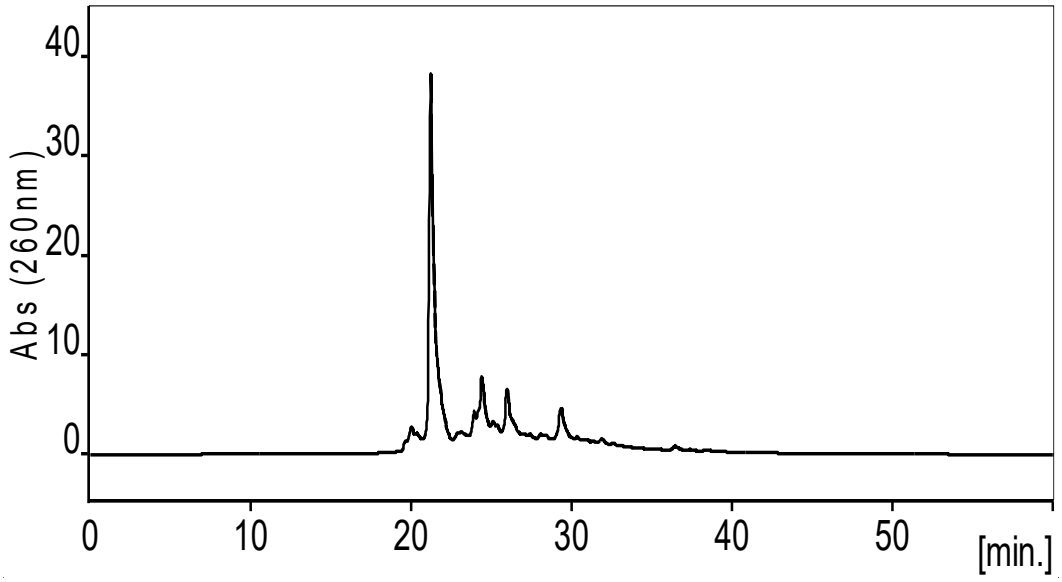


Figure 3.27 HPLC profile of ODN **Strand-17** (crude)

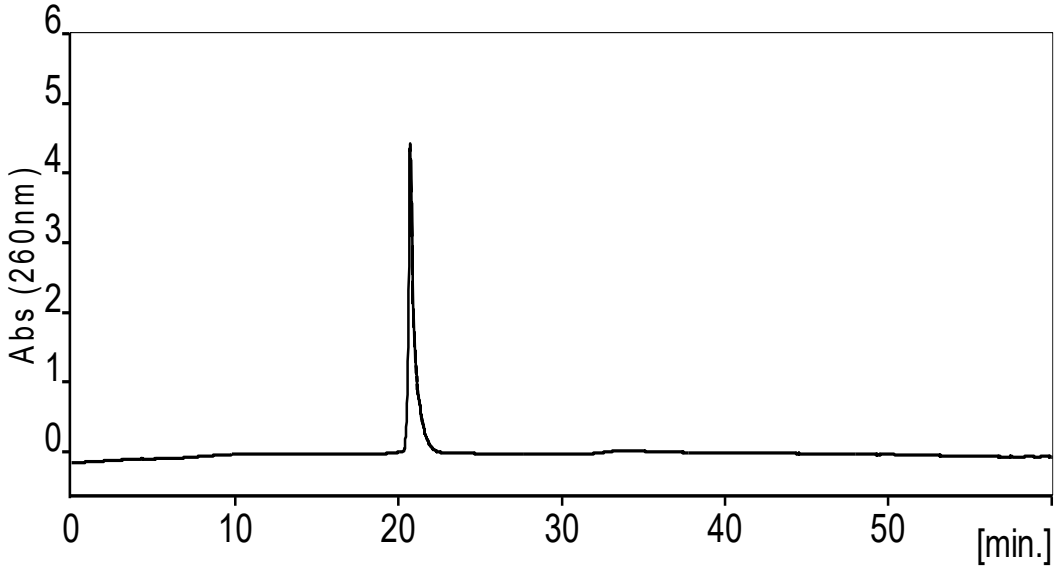


Figure 3.28 HPLC profile of ODN **Strand-17** (pure)

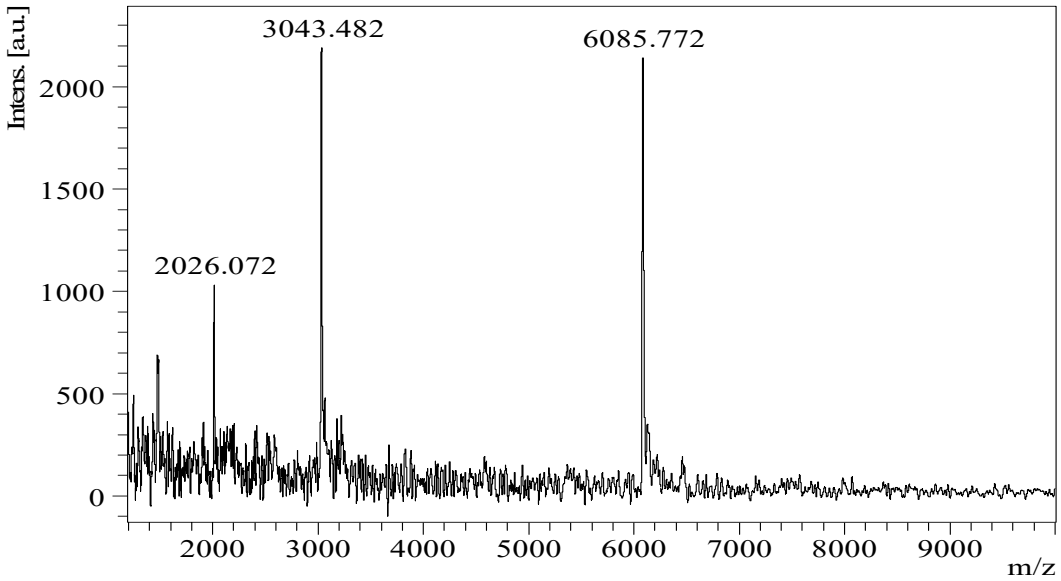


Figure 3.29 MALDI-Tof spectrum ODN **Strand-17**

3.5 References

[1] R. L. Letsinger, V. Mahadevan, *J. Am. Chem. Soc.* **1966**, *88*, 5319-+.

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[3] N. University, http://biotools.nubic.northwestern.edu/OligoCalc.html.

[4] *Buker Daltonics Instructions*, https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/InstructionForUse/8702557_IFU_Bruker_Guide_MALDI_Sample_Preparation_Revision_E.pdf.

4 Cross-linking Experiments

4.1 Introduction

The synthesis of modified and unmodified oligonucleotide has been discussed in the previous chapter. The series of cross-linking experiments have been performed by mixing a modified ODN strand and its complementary ODN strand in MES buffer. These hybrid mixtures were monitored over a length of time by HPLC and mass of different fractions collected from HPLC were analyzed by MALDI-Tof. The cross-linking was found to be less effective. However, the study established the foundation for future research through the development of detailed protocols for cross-linking experiments.

4.2 Design of cross-linking studies

Cross-linking experiments needed to be designed carefully to observe exact changes occurred during the hybridization. The goals of these experiments were set as follows;

4.2.1 The goals

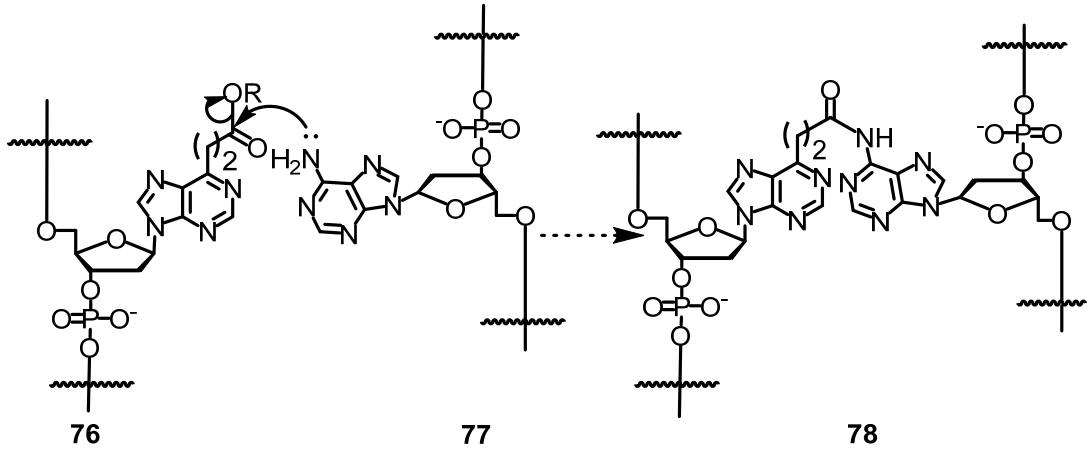
The synthesis of potential ODN cross-linking agents (modified ODN **Strands 1-16**) was already accomplished. The formation of covalent cross-linking between modified ODN strand and an unmodified complementary ODN strand was the basis for the development of antisense drug. Therefore, the goals of cross-linking

experiments were set to a) to hybridize modified ODN strand with an unmodified complementary strand, and b) to detect the potential covalent cross-linking between them in real-time. Experiments were carefully designed accomplish these to two goals.

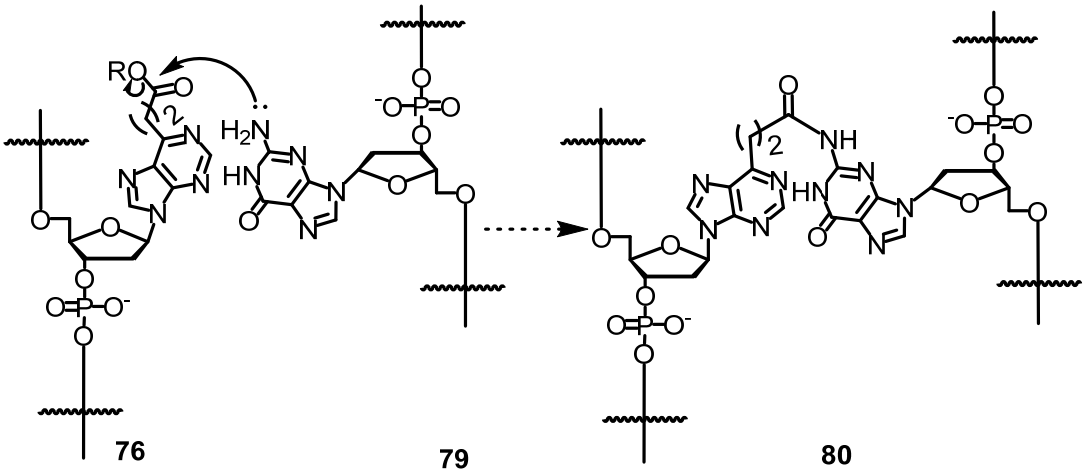
4.2.2 Experimental design

4.2.2.1 Design of complementary ODN strands

Senario 1: modified monomer on 76 reacts with adenine on 77



Senario 2: modified monomer on 76 reacts with guanine on 79



Scheme 4.1 Design of expected cross-linking

Unmodified complementary ODN strands were designed by considering their potential interactions with corresponding modified ODNs. Nucleobases with exo-amine groups can react with electrophiles better than the ones without it.[1-7] Therefore, complementary ODNs were designed to have either adenine or guanine against the modified nucleobase on modified ODN. The potential interactions during hybridization are shown in the Scheme 4.1. It is expected that modified ODN **76** with ester group could interact with an adenine complementary to it on an ODN **77** to give a cross-linked product **78**. Similarly, when guanine on an unmodified ODN **79** is complementary to the ester group on an ODN **76**, the cross-linked product **80** could be the result. Based on these assumptions complementary ODN strands for modified ODN **Strand 1**, **3**, **7**, **12** and **15** were designed.

4.2.2.2 Hybridization conditions

Several hybridization conditions for ODN cross-linking experiments were studied carefully.[7-18] Based on these studies we had finalized the following conditions for cross-linking experiments.

The MES buffer (0.1M NaCl, 50 mM MES, pH 5.00) was used as a buffer for hybridization experiments. While there are several other buffers mentioned in the literature, this is one of the best buffers for fast Interstrand ODN cross-linking.[4, 6, 16] We decided to move with it keeping all other options open for the future studies. The sample amount for OD studies was 2.5 mL and concentration of modified ODNs was already available. Based on this concentration quantities of modified

ODNs were calculated. We decided to use excess amount of an unmodified complementary ODN in each case. The reason for using excess amount of complementary ODN was to ensure 100% cross-linking of modified ODN strand with it.

Physiological temperature i.e. 37 °C was the obvious choice for hybridization experiments.

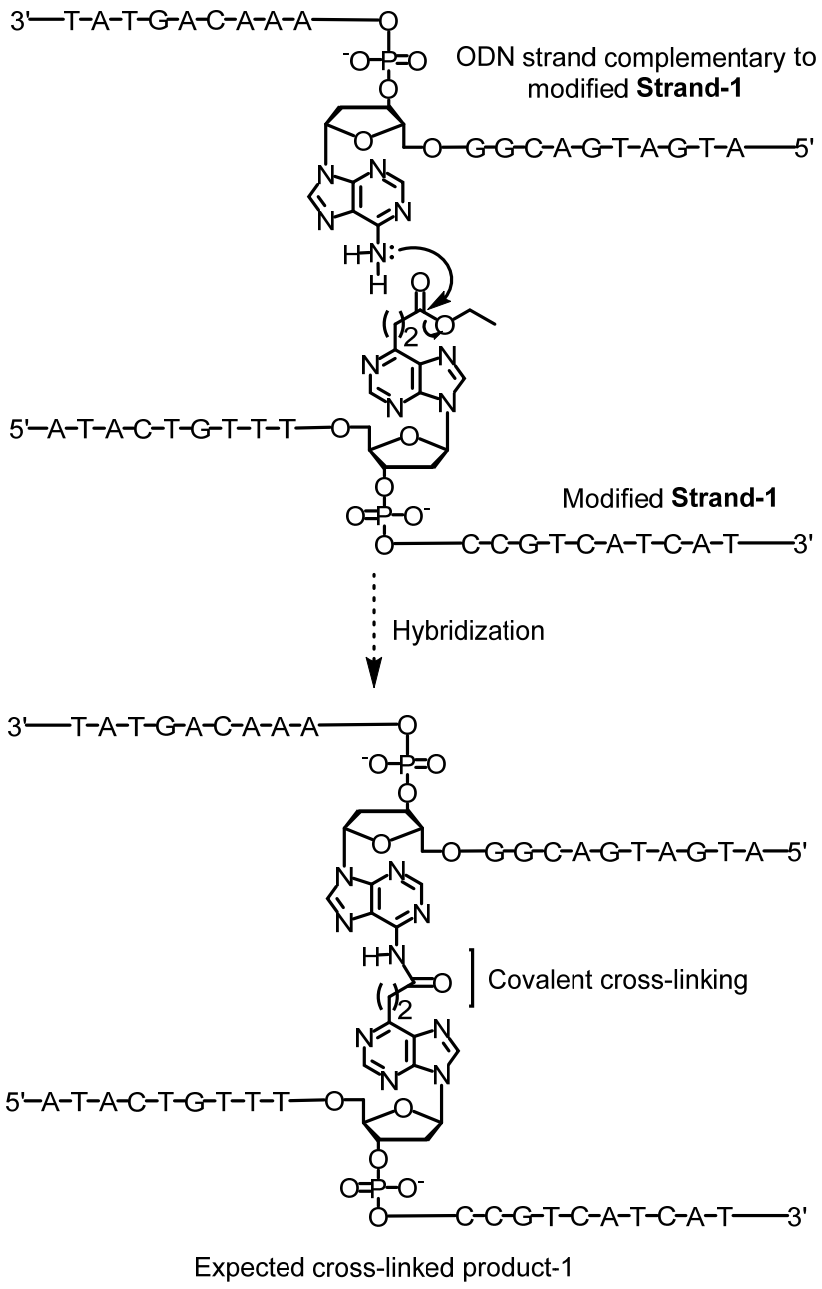
4.2.2.3 Real-time analysis of hybrid mixtures

The RP-HPLC and MALDI-Tof mass analysis were the best analytical techniques to analyze hybrid mixtures. Only a little portion of hybrid mixture needed for RP-HPLC and even a trace amount of this portion was enough for MALDI-Tof analysis. It was possible to study this very little sample over several weeks with these two techniques.

4.3 Results and discussion

In all five hybridizations were studied under the same conditions mentioned in experimental design. One modified ODN strand for each of monomer **1**-**5** were selected. All hybridizations were monitored by RP-HPLC and mass spectra of separated fractions were acquired using MALDI-Tof.

4.3.1 Hybrid Mixture-1



Scheme 4.2 Expected cross-linking for Hybrid Mixture-1

The modified ODN **Strand-1** was put for hybridization with its complementary ODN strand (Hybrid Mixture-1). Cross-linked product-1 was expected to be the result of

this interaction (Scheme 4.2). For this hybridization adenine was incorporated on the complementary strand facing against a modified nucleobase.

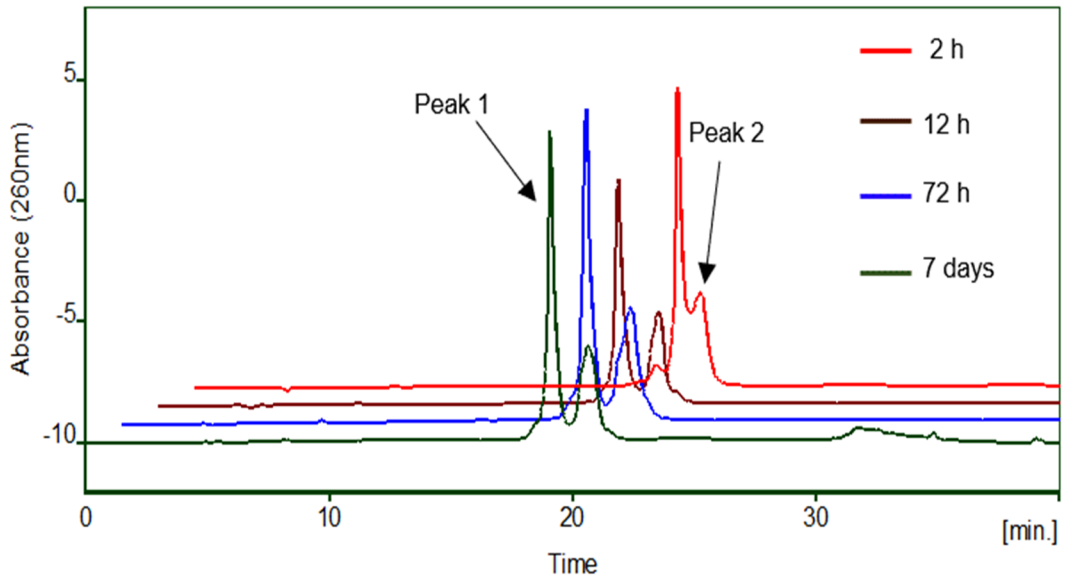


Figure 4.1 HPLC profiles for Hybrid Mixture-1

The Hybrid Mixture-1 was analyzed by RP-HPLC over the time range of 2h, 12h, 72h and finally 7 days (Figure 4.1). All RP-HPLC profiles were almost identical with two peaks in each one of them. MALDI-Tof mass analysis for two RP-HPLC separated peaks was performed. Mass corresponding to RP-HPLC Peak-1 confirmed the mass for unmodified complementary ODN strand while a mass for Peak-2 was found to be for the modified **Strand-1** (Figure 4.2). Results found indicated that the covalent cross-linking using this modified monomer was not achieved, at least for the hybridization conditions used. It was also evident from the studies that the modified ODN was stable under the conditions for study without any traces of possible hydrolysis product. This stability was perhaps another

reason for no cross-linking in this case. Then we moved on to more sensitive ODN containing modified monomer **2**.

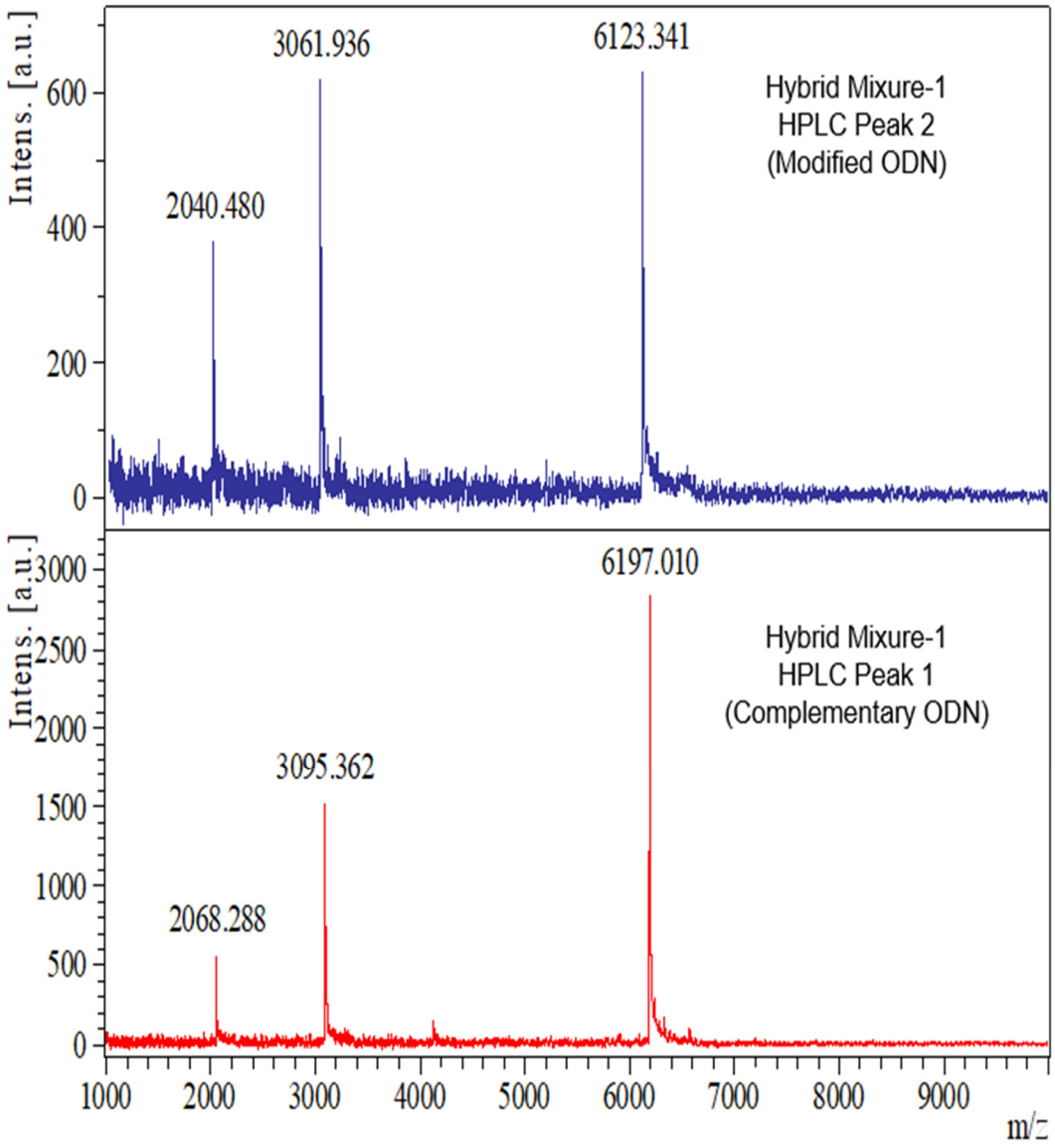


Figure 4.2 Maldi mass analysis of Hybrid Mixture-1

4.3.2 Hybrid Mixture-2

Hybrid Mixture-2 was prepared using modified ODN **Strand-3** and its

complementary strand. The covalent cross-linking product-2 (Scheme 4.3) was expected out of this interaction. The Hybrid Mixture-2 was analyzed by RP-HPLC over the same time range of 2h, 12h, 72h and finally 7 days (Figure 4.3). For this mixture we got only two peaks over the period. Through mass analysis RP-HPLC Peak-1 was found to be modified ODN **Strand-3** while Peak-2 was confirmed to be unmodified complementary ODN strand (Figure 4.4). The results we got for this hybridization were not satisfactory either. The reactivity might be the reason again just like the first hybridization.

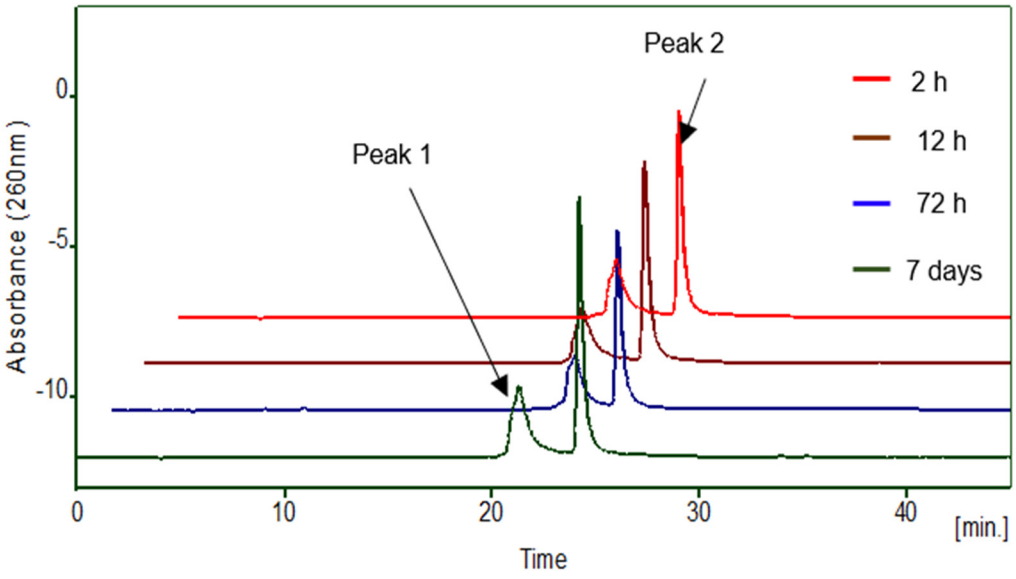
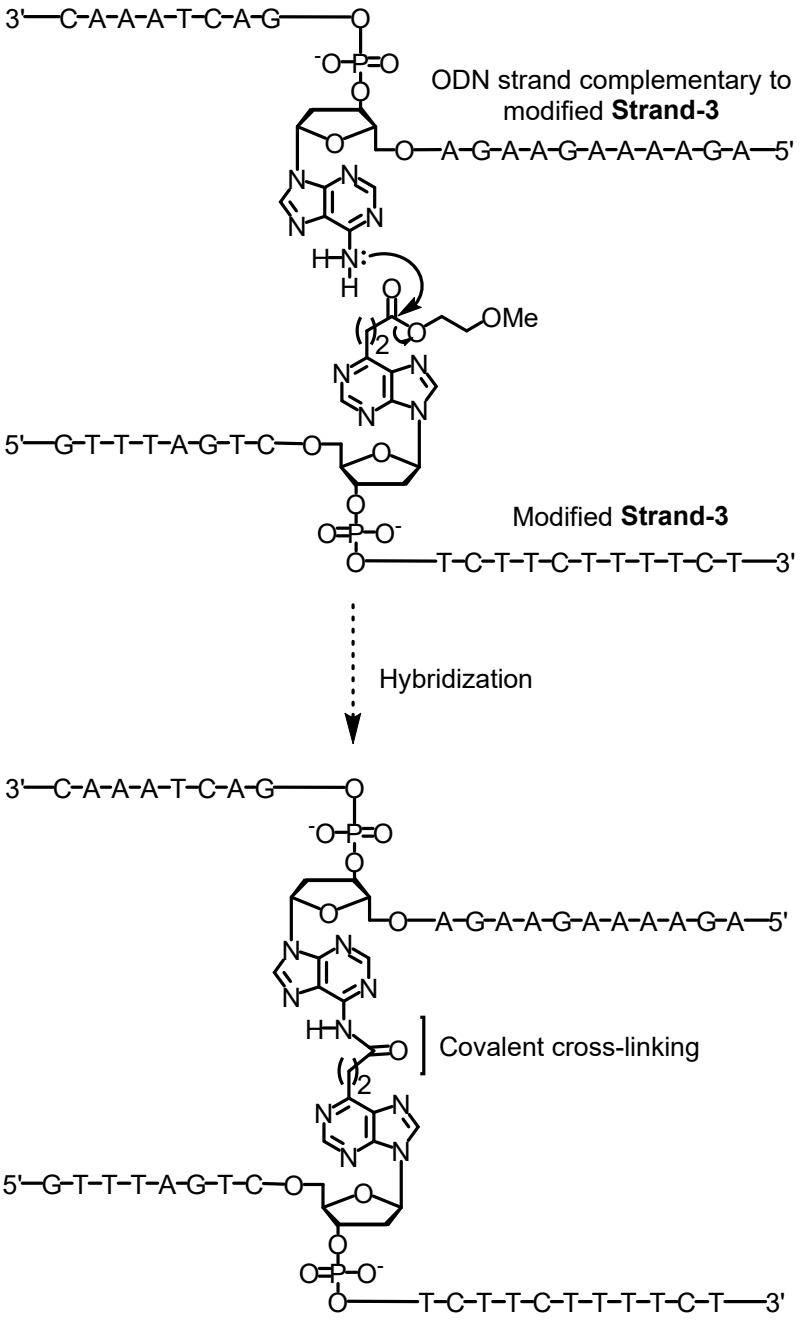


Figure 4.3 HPLC profiles for Hybrid Mixture-2



Expected cross-linked product-2

Scheme 4.3 Expected cross-linking for Hybrid Mixture-2

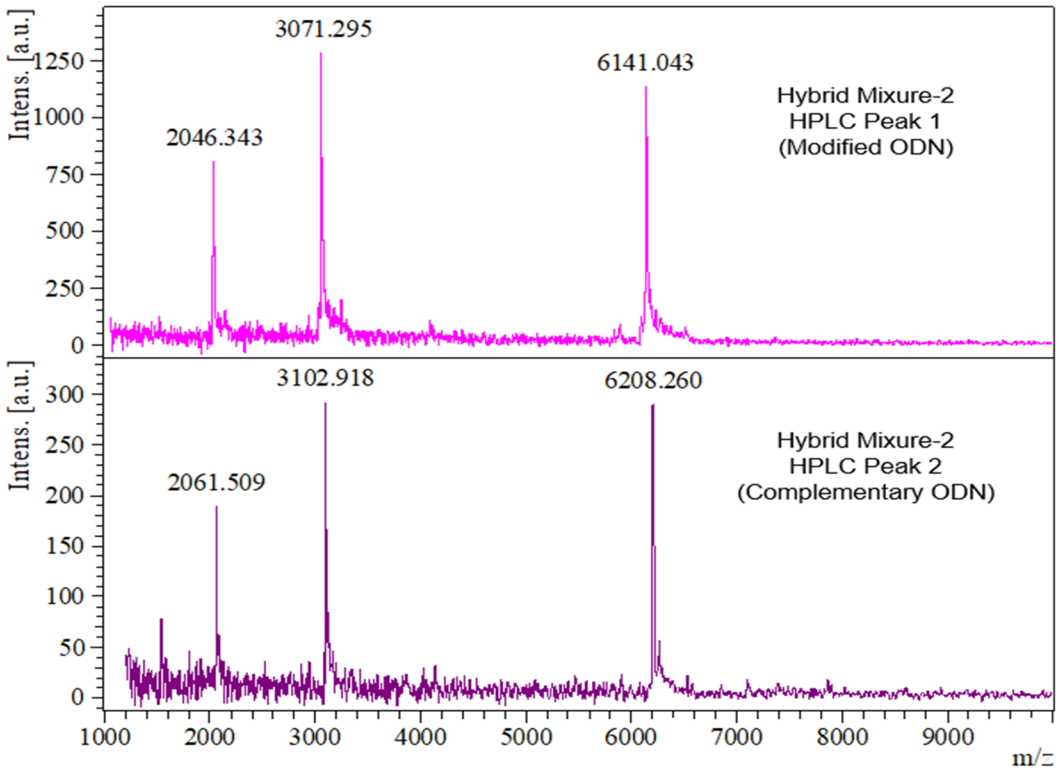
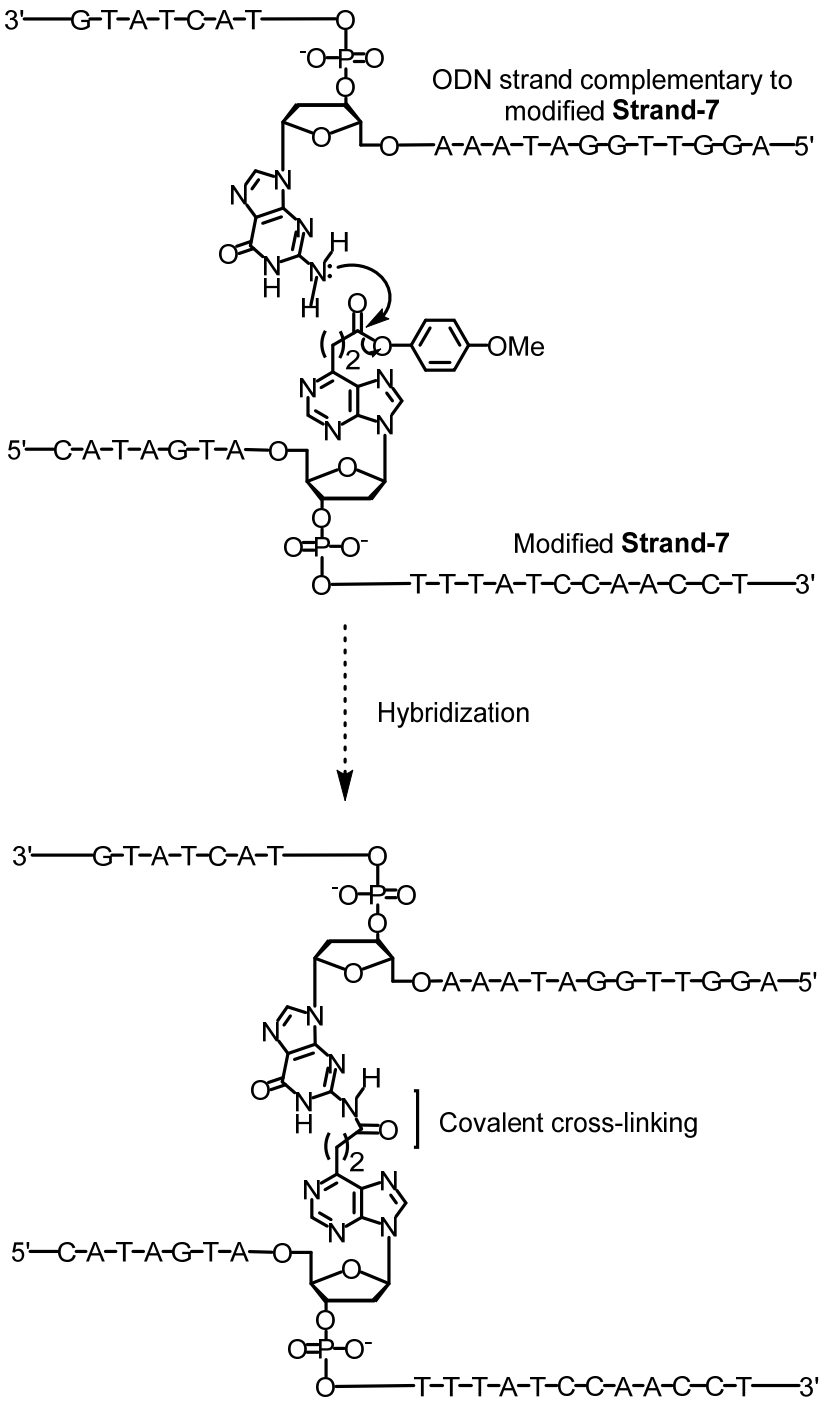


Figure 4.4 Maldi mass analysis of Hybrid Mixture-2

4.3.3 Hybrid Mixture-3

The Hybrid Mixture-3 was constituted modified ODN **Strand-7** and its complementary ODN strand. It was expected to give cross-linked product-3 (Scheme 4.4) Three RP-HPLC analyses were conducted; first immediately after mixing two ODN strands, second after 2h of mixing them and the final one after 24h (Figure 4.5). The RP-HPLC profile of a mixture immediately after mixing two strands together shown two peaks. The rightmost peak on RP-HPLC profiles of the mixture labeled as Peak-3 was found to be modified ODN **Strand-7** and the one labeled as Peak-2 was found to be a complementary ODN strand.



Expected cross-linked product-3

Scheme 4.4 Expected cross-linking for Hybrid Mixture-3

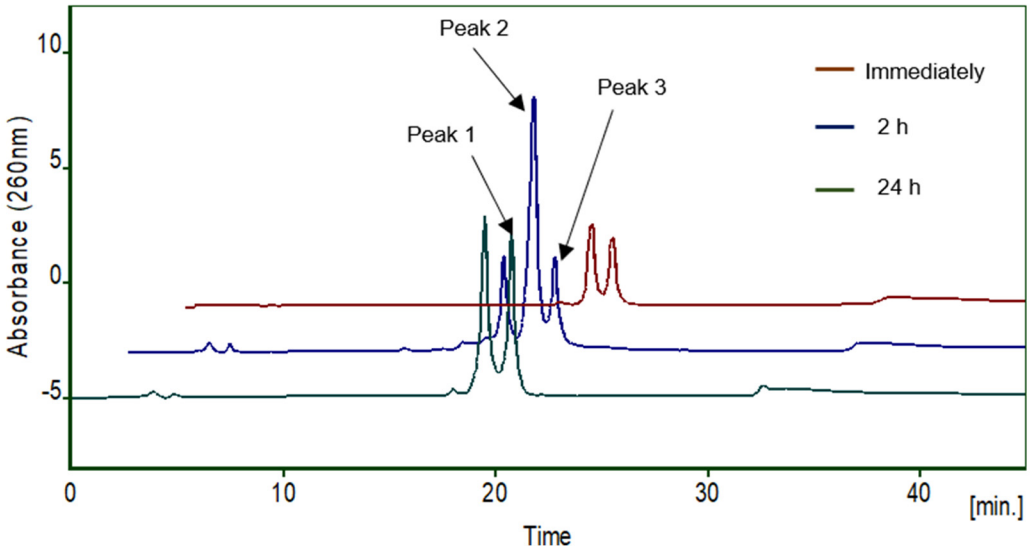


Figure 4.5 HPLC profiles for Hybrid Mixture-3

The RP-HPLC profile after 2 hours had shown 3 peaks and we were excited to see an additional peak. Unfortunately, the mass analysis of a new peak (Peak-1) was found to be a mass for the hydrolyzed product of the modified ODN **Strand-7** (Figure 4.5). Another RP-HPLC analysis was conducted after 24 hours. This time the HPLC profile showed only two peaks. The retention time for peak-1 was matching with the hydrolyzed product seen previously. Without relying too much on HPLC we did MALDI-Tof analysis (Figure 4.6) of the both fractions collected. Our guess was found correct that the modified ODN Strand-7 was completely hydrolyzed and mass for HPLC peak-1 was found corresponding to the hydrolyzed product only. The other peak was unmodified complementary ODN strand. This hybridization also found not working under these conditions. The next monomer i.e. monomer **4** was expected to be more sensitive than the monomer **3** and we were kind of sure about its hydrolysis as well.

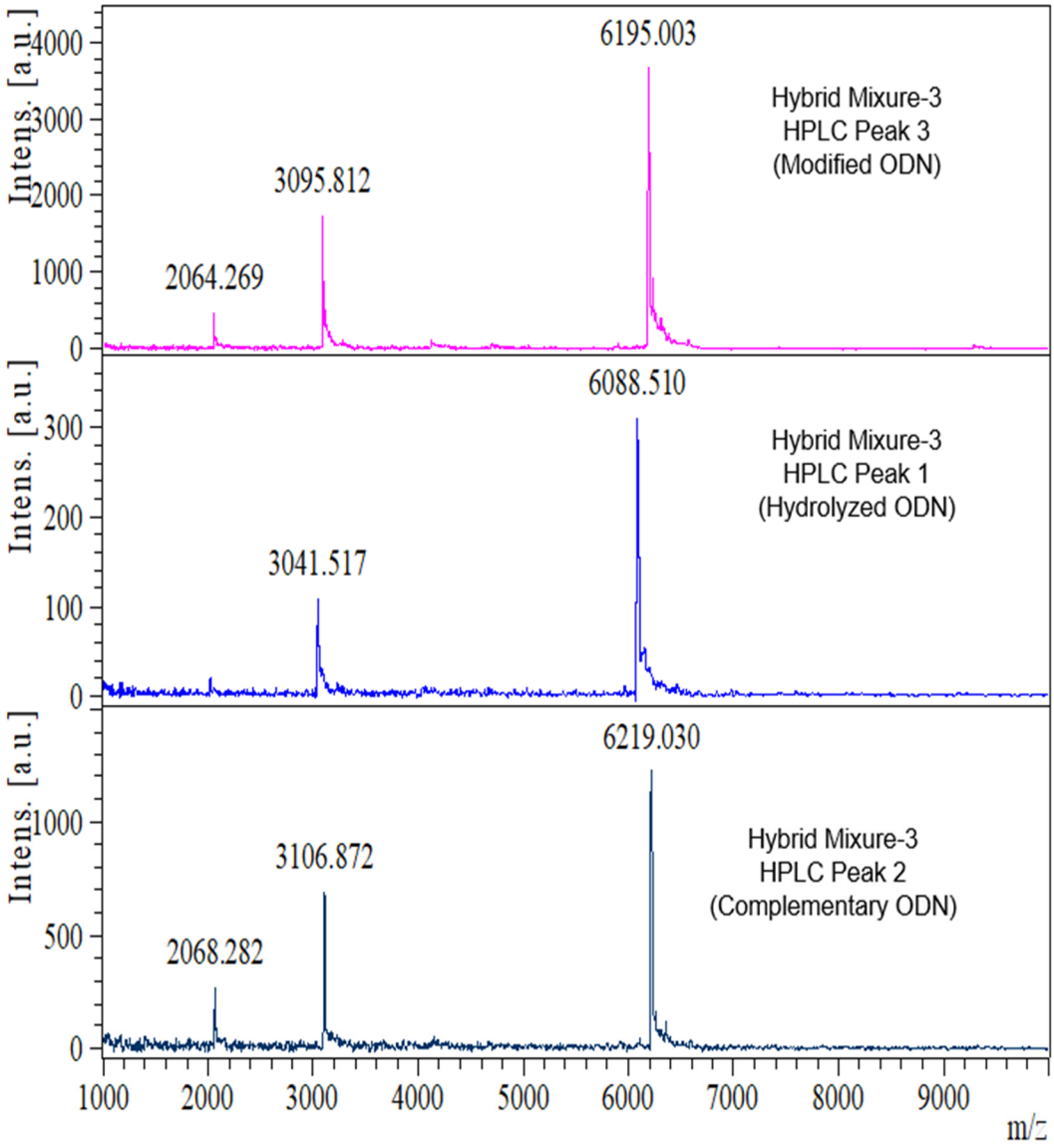
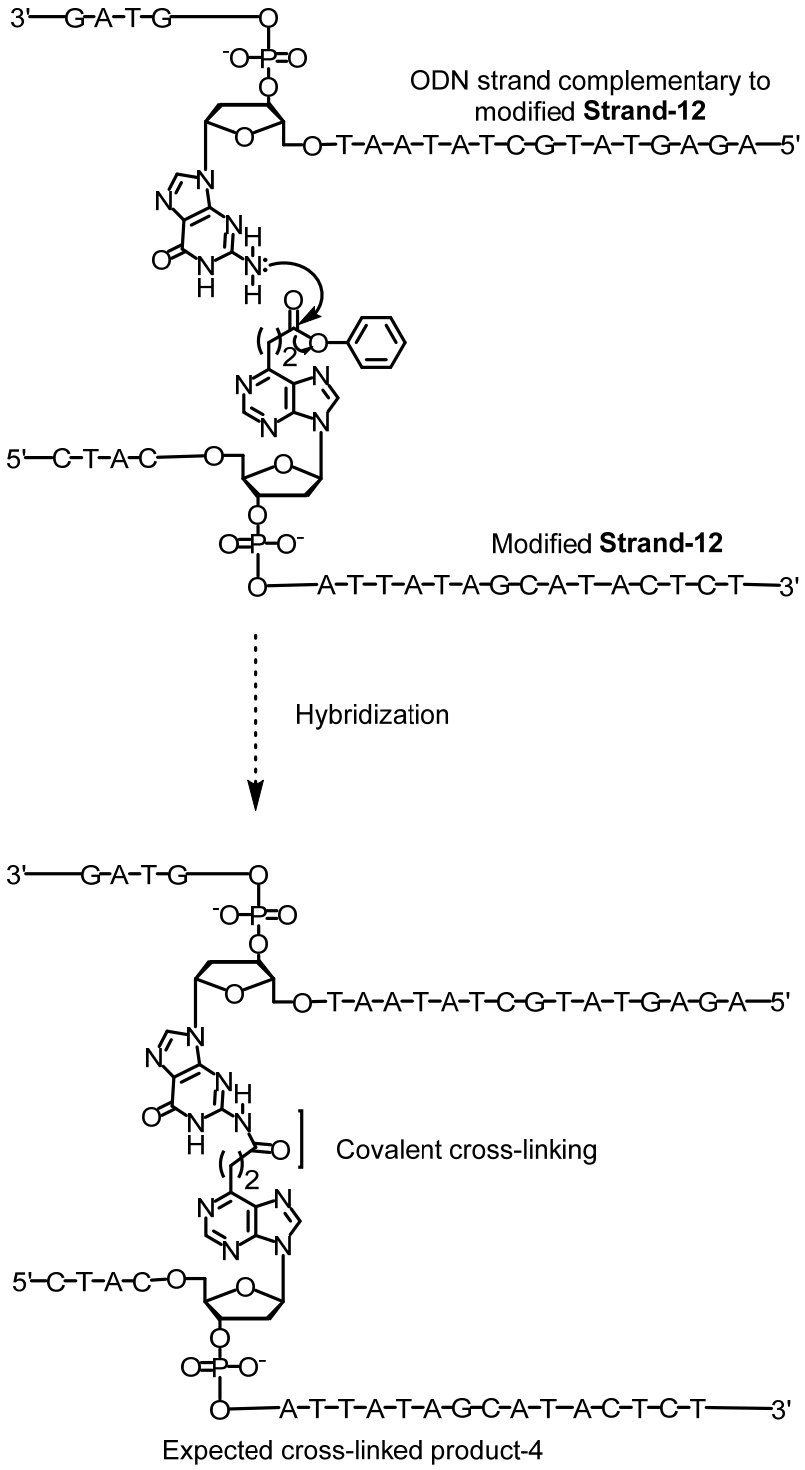


Figure 4.6 Maldi mass analysis of Hybrid Mixture-3

4.3.4 Hybrid Mixture-4

As expected results like Hybrid Mixture-3 were obtained for Hybrid Mixture-4. ODN **Strand-12** with its unmodified complementary strand were the components of this hybrid mixture (Scheme 4.5).



Scheme 4.5 Expected cross-linking for Hybrid Mixture-4

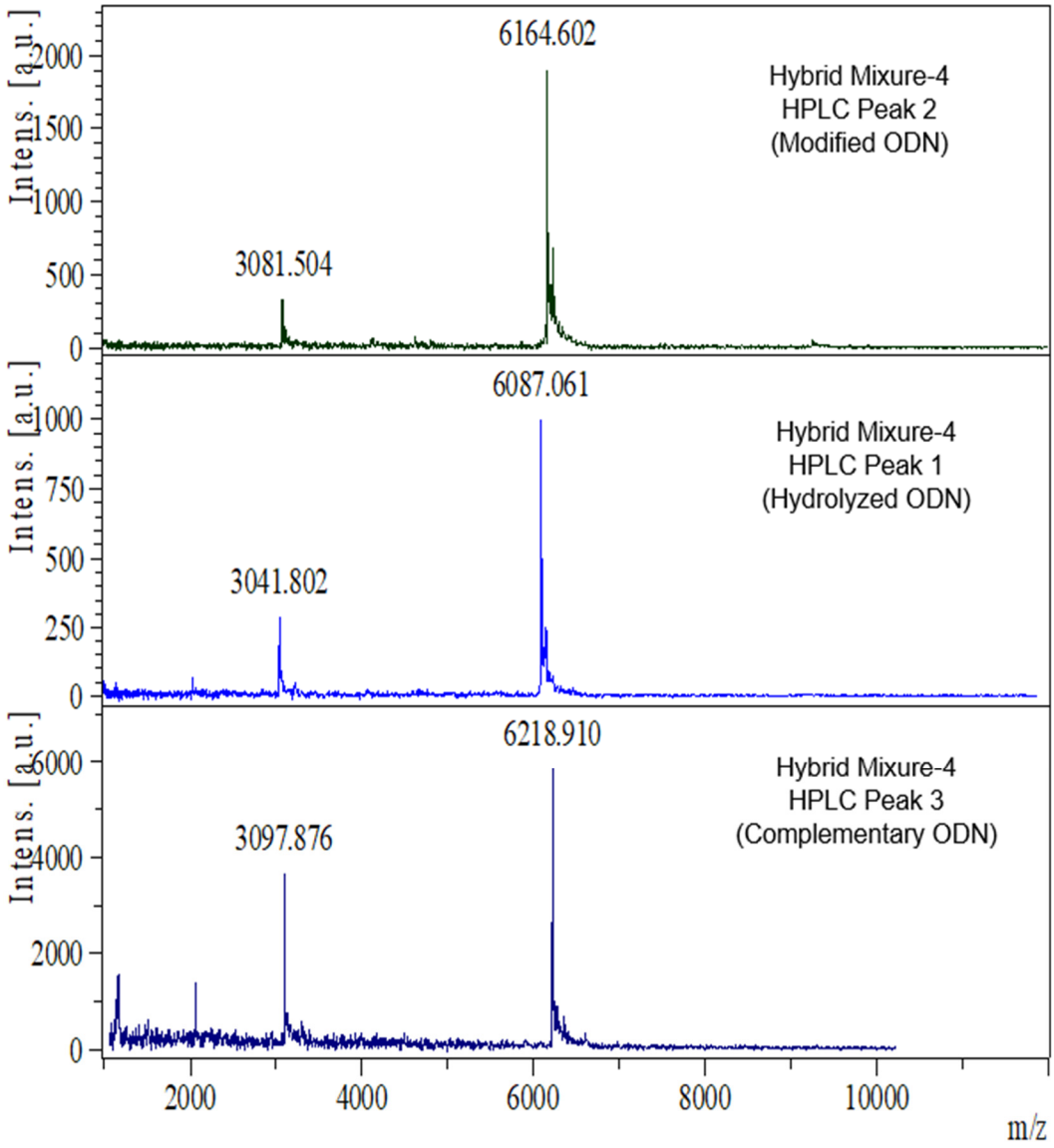


Figure 4.7 Maldi mass analysis of Hybrid Mixture-4

Two RP-HPLC analysis was conducted; one 2 hours after mixing two strands and other after 24h (Figure 4.8). The RP-HPLC profile of a mixture after 2 hours of mixing two strands showed three peaks. Peak-1 for hydrolyzed product, Peak-2 for modified ODN **Stand-12** and Peak-3 for unmodified ODN strand. After 24 hours modified ODN Strand-12 was completely hydrolyzed and only two peaks on

RP-HPLC profile could be seen (Figure 4.8). Mass for each one of them was confirmed by MALDI-Tof mass analysis (Figure 4.7).

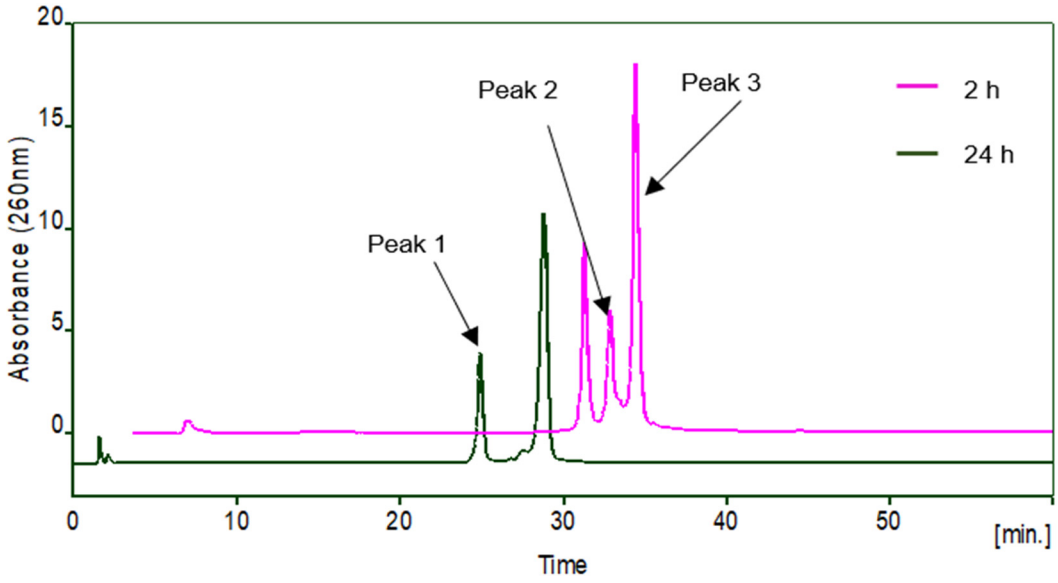
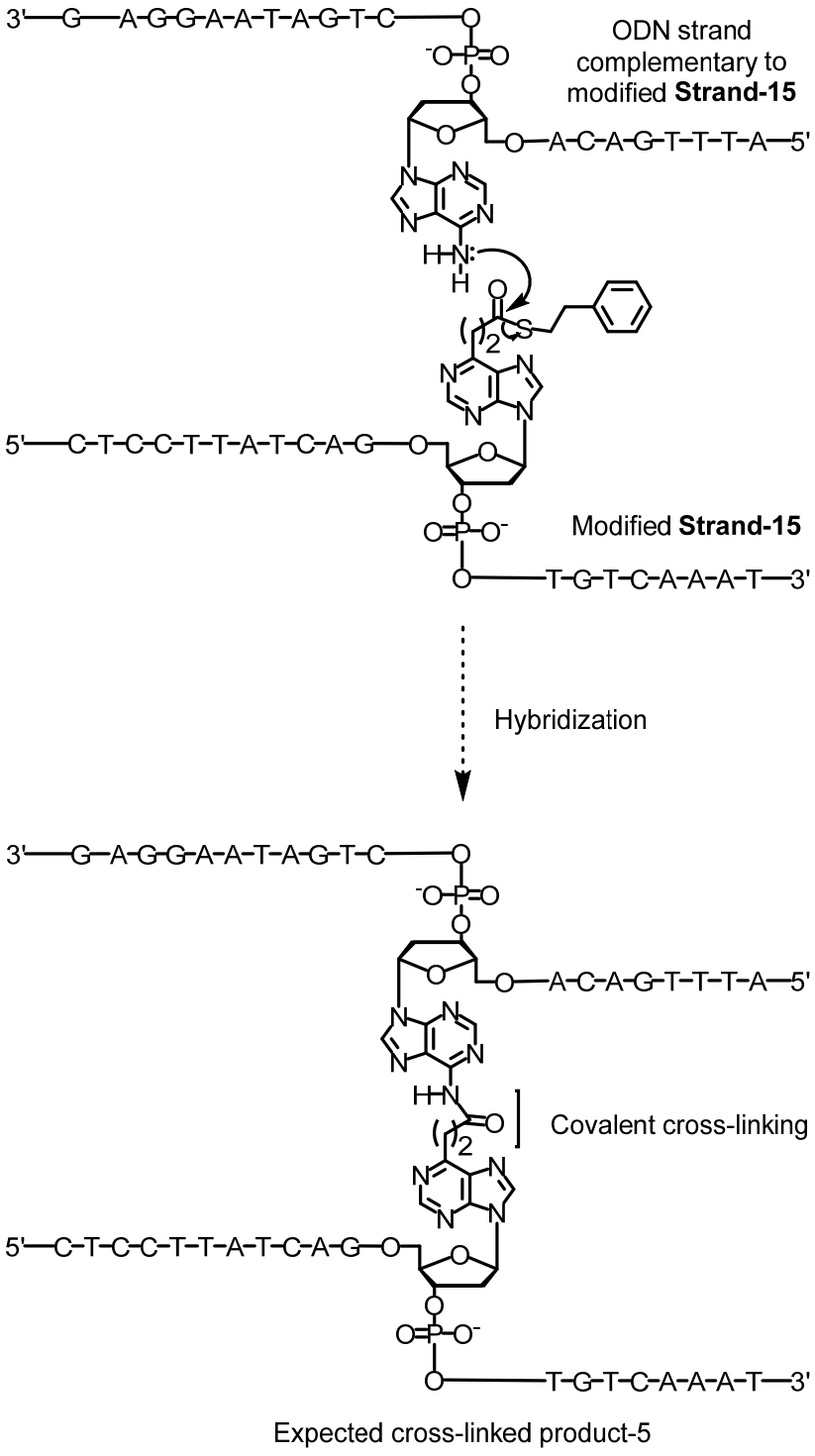


Figure 4.8 HPLC profiles for Hybrid Mixture-4

4.3.5 Hybrid Mixture-5

The Hybrid Mixture-5 was composed of modified ODN Strand-15 and the ODN strand complementary to it and was expected to give cross-linked product-5 (Scheme 4.6). The RP-HPLC analyses (Figure 4.9) for 2 hours showed two distinct peaks. Peak-1 was found to be the modified ODN **Strand-15** and Peak-2 was unmodified complementary ODN strand. After 48 hours RP-HPLC was conducted to see the changes but this time got very complex profile. The mass analysis (Figure 4.10) for all fractions collected confirmed presence of modified and unmodified strands in their original forms.



Scheme 4.6 Expected cross-linking for Hybrid Mixture-5

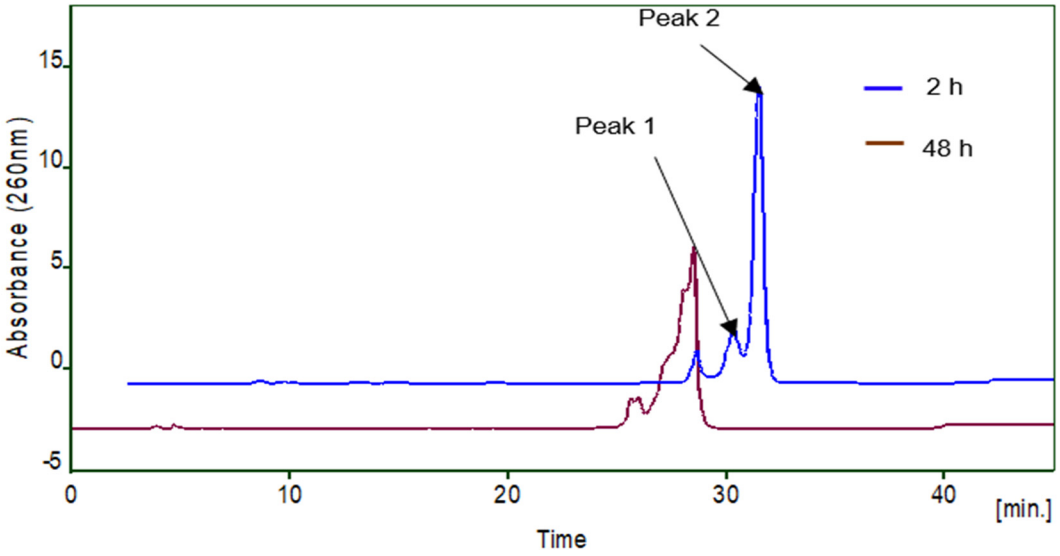


Figure 4.9 HPLC profiles for Hybrid Mixture-5

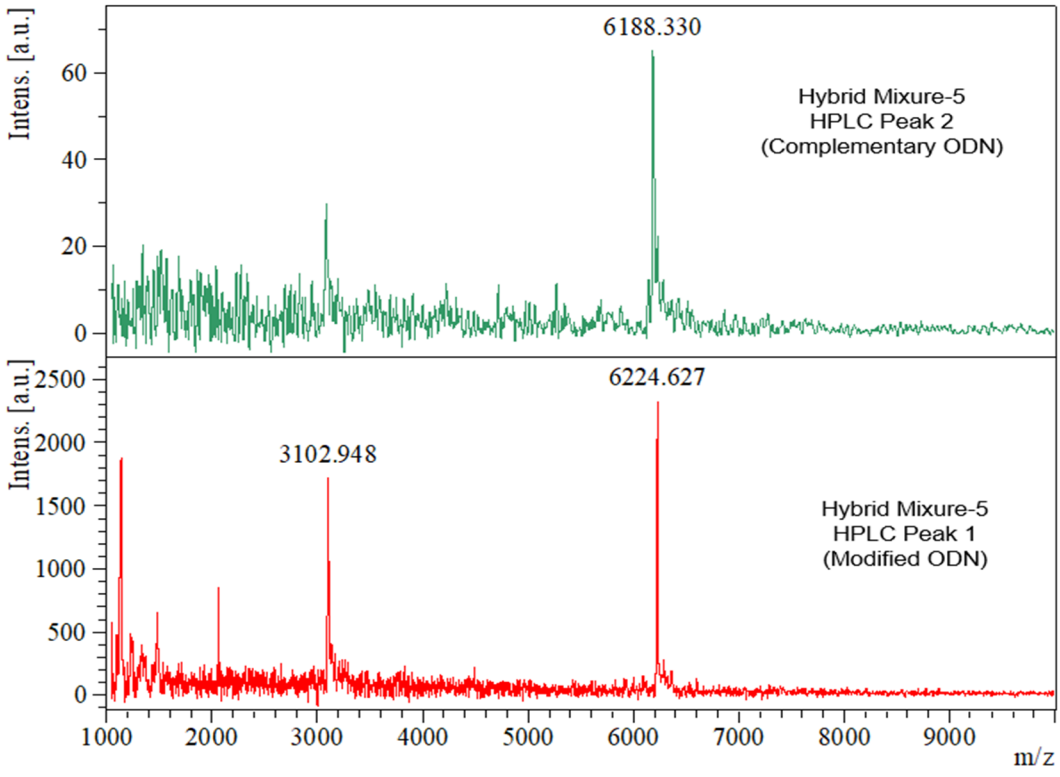


Figure 4.10 Maldi mass analysis of Hybrid Mixture-5

The experiments conducted for cross-linking studies were not satisfactory but the synthesis of ODNs with very sensitive groups was first of its kind of achievement. Therefore, we decided to publish a synthesis part of the study. Accordingly it was published in one of the reputed journal in the field of chemistry.[19]

4.4 Experimental section

4.4.1 General experimental

The RP HPLC was performed on a JASCO LC-2000Plus System: pump, PU-2089Plus Quaternary Gradient; detector UV-2075Plus. For RP-HPLC a C-18 reverse phase analytical column (5μm diameter, 100 Å, 250 × 3.20 mm) was used. RP-HPLC mobile phase: Solvent A: 0.1 M triethylammonium acetate, 5% aqueous acetonitrile. Solvent B: 90% aqueous acetonitrile. RP-HPLC profiles were generated by detection of absorbance of ODN at 260 nm using the linear gradient solvent system: solvent B (0%-45%) in Solvent A over 60 min followed by Solvent B (45%-100%) in Solvent A over 20 min at a flow rate of 1.0 mL/min. The RP-HPLC separated peaks were analyzed by Bruker Microflex LRF MALDI-Tof to confirm their mass.

4.4.2 Preparation of Hybrid Mixtures 1-5

MES buffer (0.1 M NaCl, pH=5.01, 50 mM) was prepared according the standard procedure.[4, 6] The solutions 50 μL of each of modified ODNs and unmodified complementary ODNs were prepared in the freshly prepared buffer in Eppendorf

tubes. Annealing of ODN solutions were carried out at 90 °C for 5 minutes. Annealing of ODN solutions for **Strand-7**, **12** and **15** was avoided to check the potential hydrolysis of very sensitive ester groups on them. Appropriate ODNs were mixed together to get **Hybrid Mixtures 1-5**.

4.4.3 RP-HPLC and MALDI-Tof analysis

The RP-HPLC was conducted using the conditions mentioned in general experimental. The peaks shown on RP-HPLC profiles were collected cautiously to avoid any mixing in 100 µL fractions. The collected RP-HPLC fractions were used as samples for MALDI-Tof analysis without further processing. Further processing might have destroyed the products or at least could hydrolyzed the sensitive ester groups. MALDI-Tof mass determination was conducted by following the same procedure mentioned in Chapter 3. Results obtained were compared with the theoretical values.

4.5 Conclusion and future work

The ODN cross-linking agents we designed and synthesized did not cross-link as we expected. Some of them remained unreacted over the period of several days while the ones with sensitive esters were completely hydrolyzed within 24 hours without cross-linking. However, we had successfully incorporated sensitive groups into oligonucleotide which are not possible to install by standard oligonucleotide synthesis. The procedures and protocols developed for the synthesis of monomers

and ODNs, cross-linking experiments, real-time monitoring of hybrid mixtures, analysis by HPLC and MALDI-Tof is the foundation for our future studies.

In the future we intend to design and synthesize more potential cross-linking agents and search will continue till we get the positive results. The search for an ideal oligonucleotide cross-linking agent has become easy because of this study. There is a need of several trial experiments to accomplish the desired cross-linking agent.

4.6 References

[1] P. P. Ghodke, M. E. Albertolle, K. M. Johnson, F. P. Guengerich, *Curr. Protoc. Nucleic Acid Chem.* **2019**, *76*, e74.

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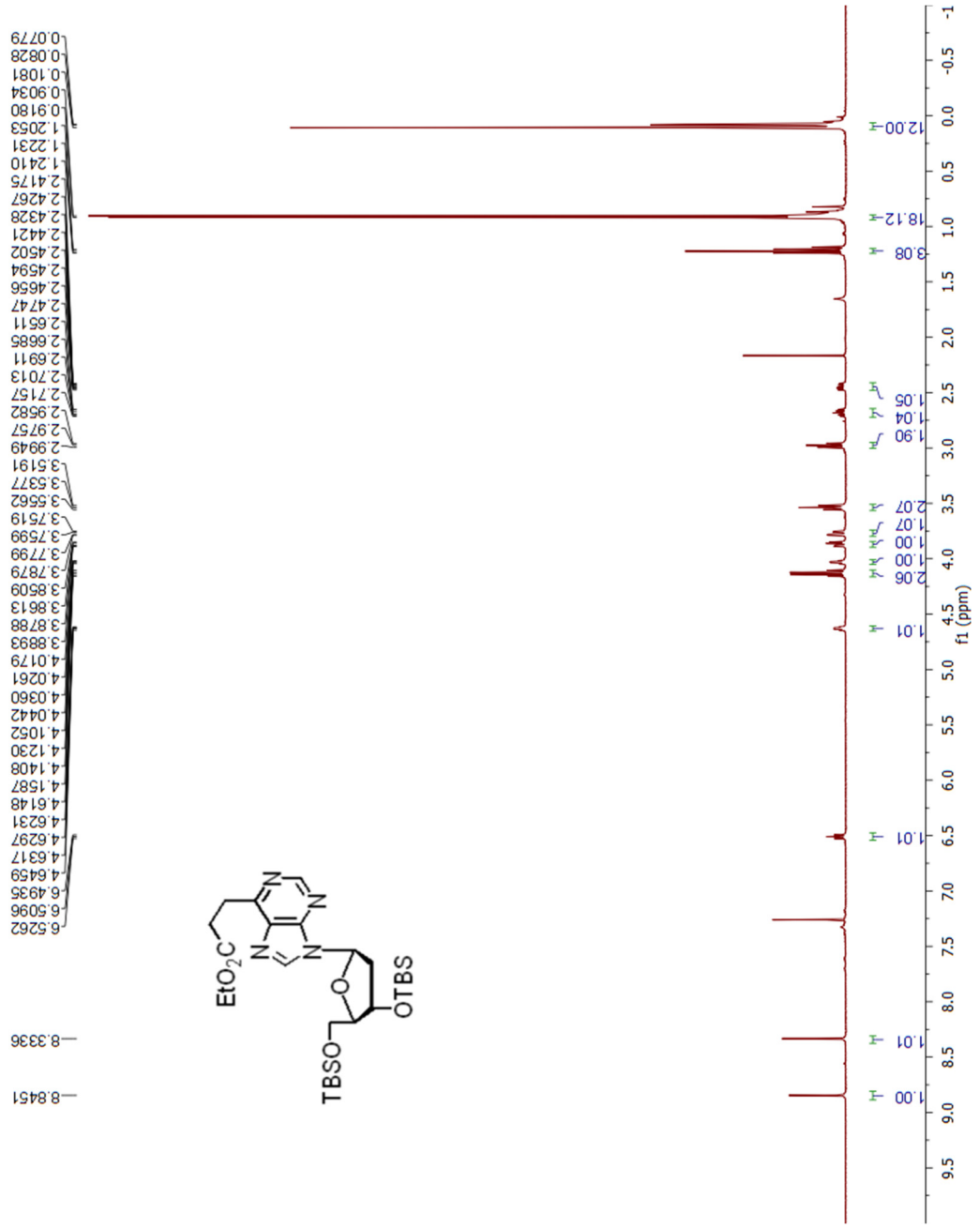
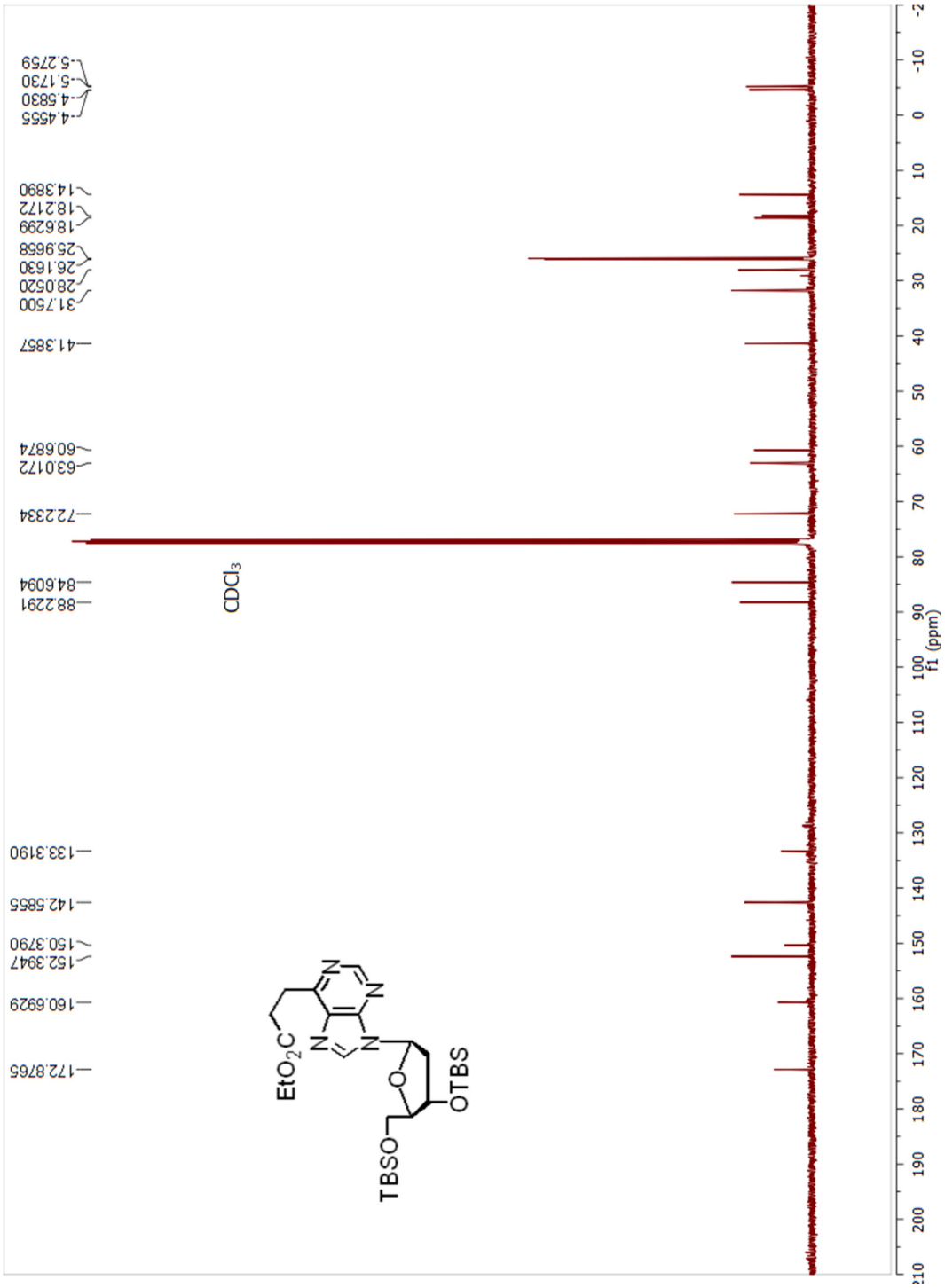
5 Appendices

A.1 NMR data for Chapter 2

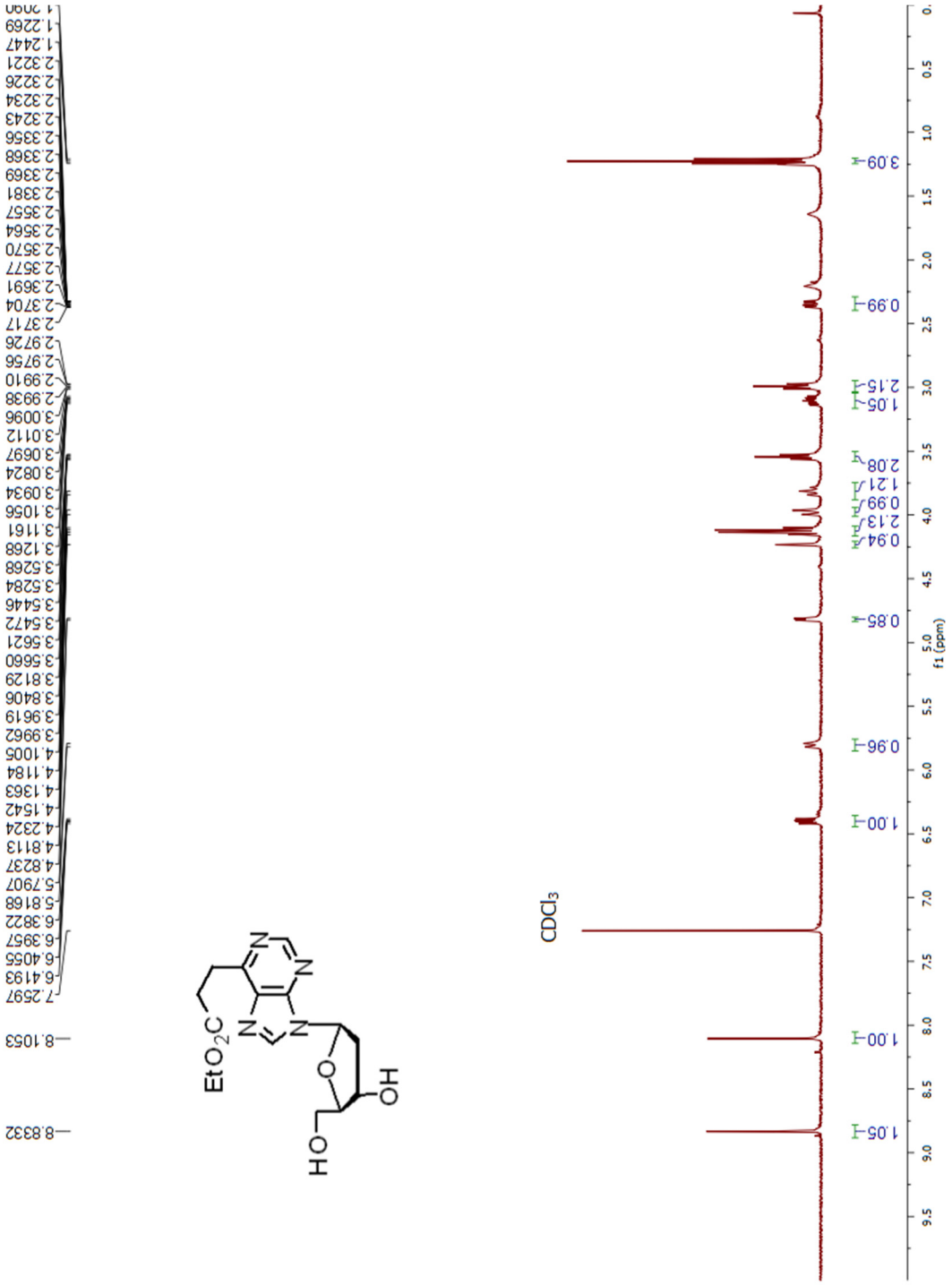
A.1.1 ^{1}H NMR of Compound **38** in $CDCl_3$, 400 MHz



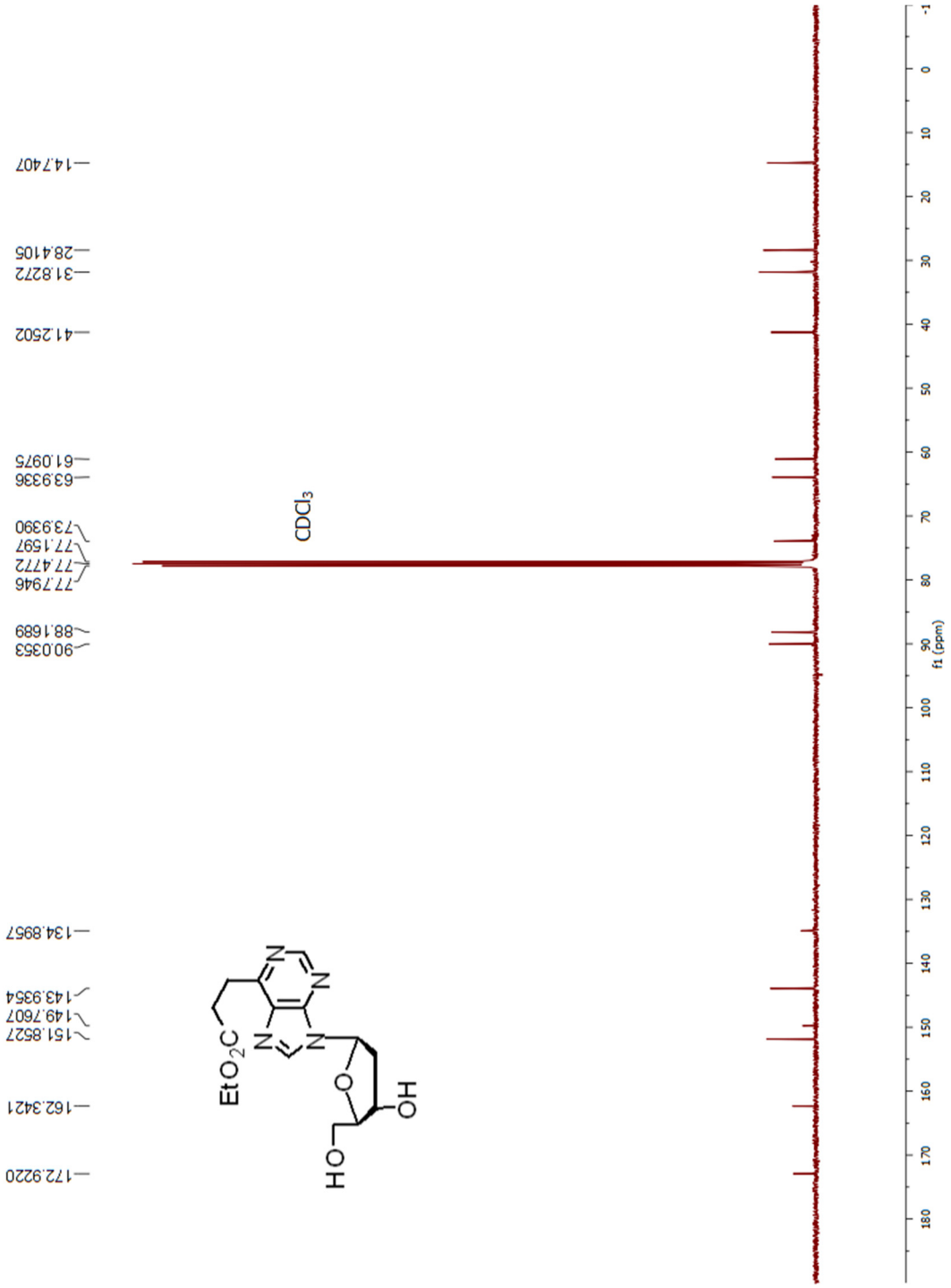
A.1.2 ^{13}C NMR of Compound **38** in $CDCl_3$, 100 MHz



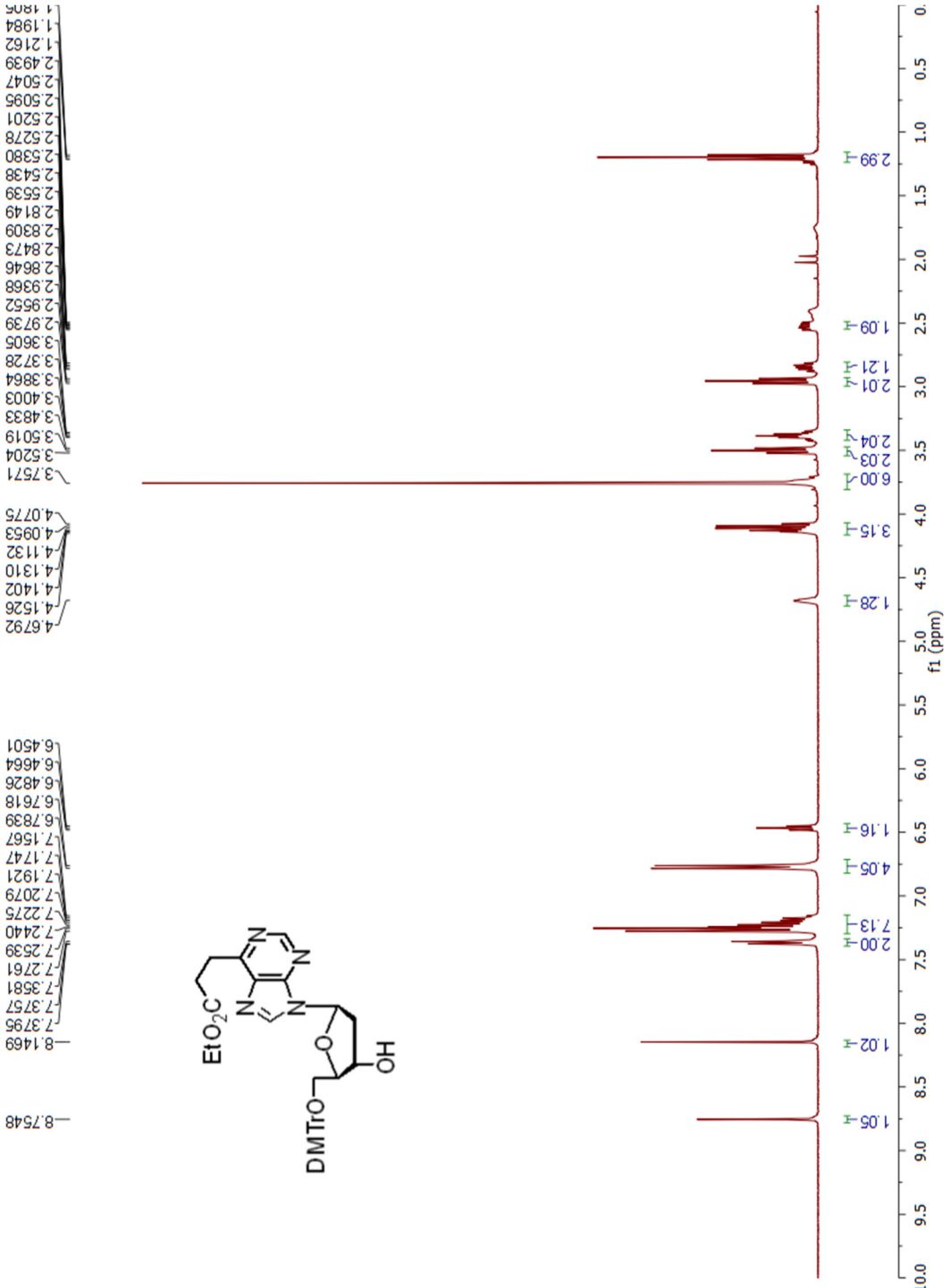
A.1.3 ^{1}H NMR of Compound **42a** in $CDCl_3$, 400 MHz



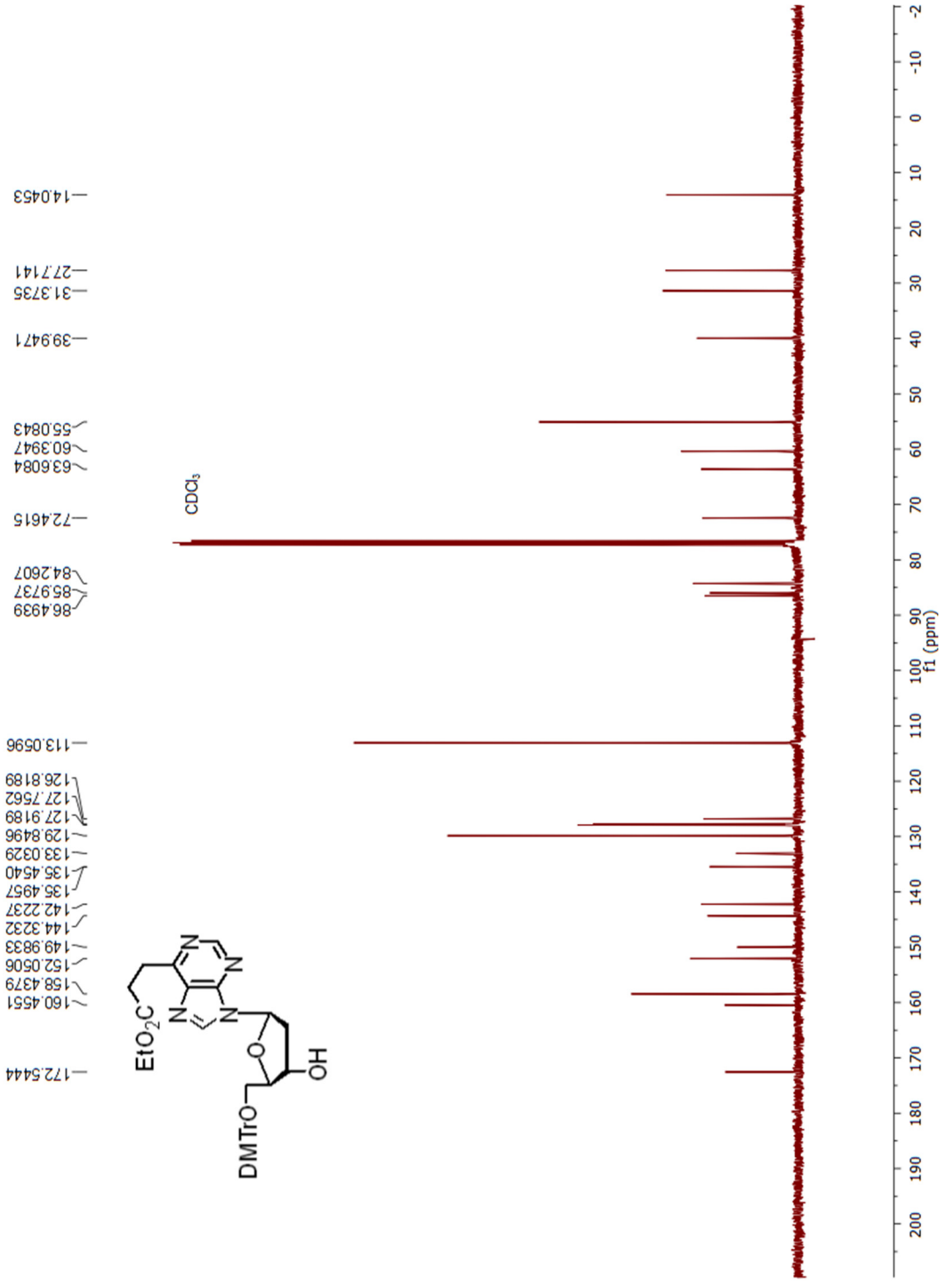
A.1.4 ^{13}C NMR of Compound **42a** in $CDCl_3$, 100 MHz



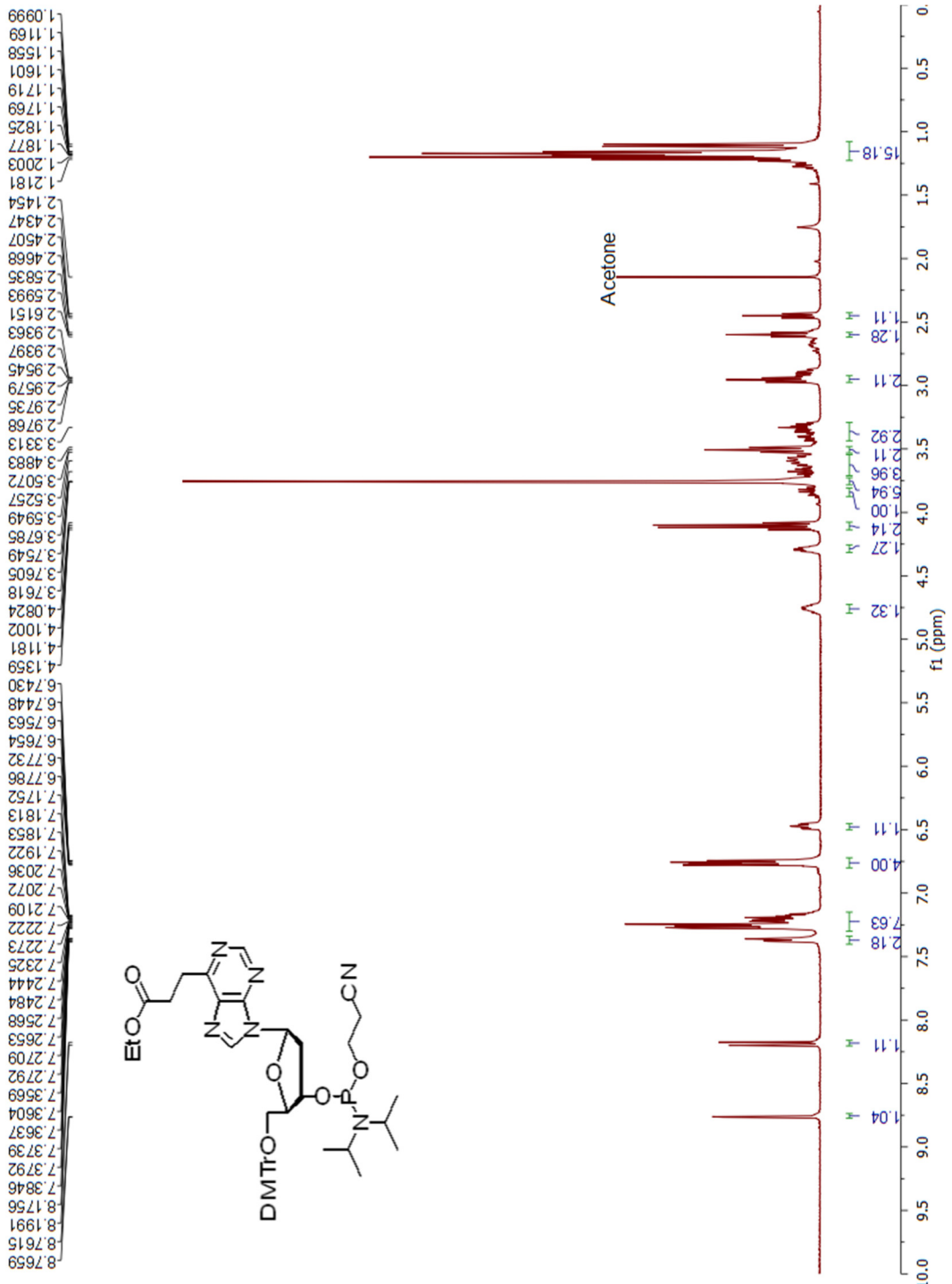
A.1.5 ^{1}H NMR of Compound **43a** in $CDCl_3$, 400 MHz



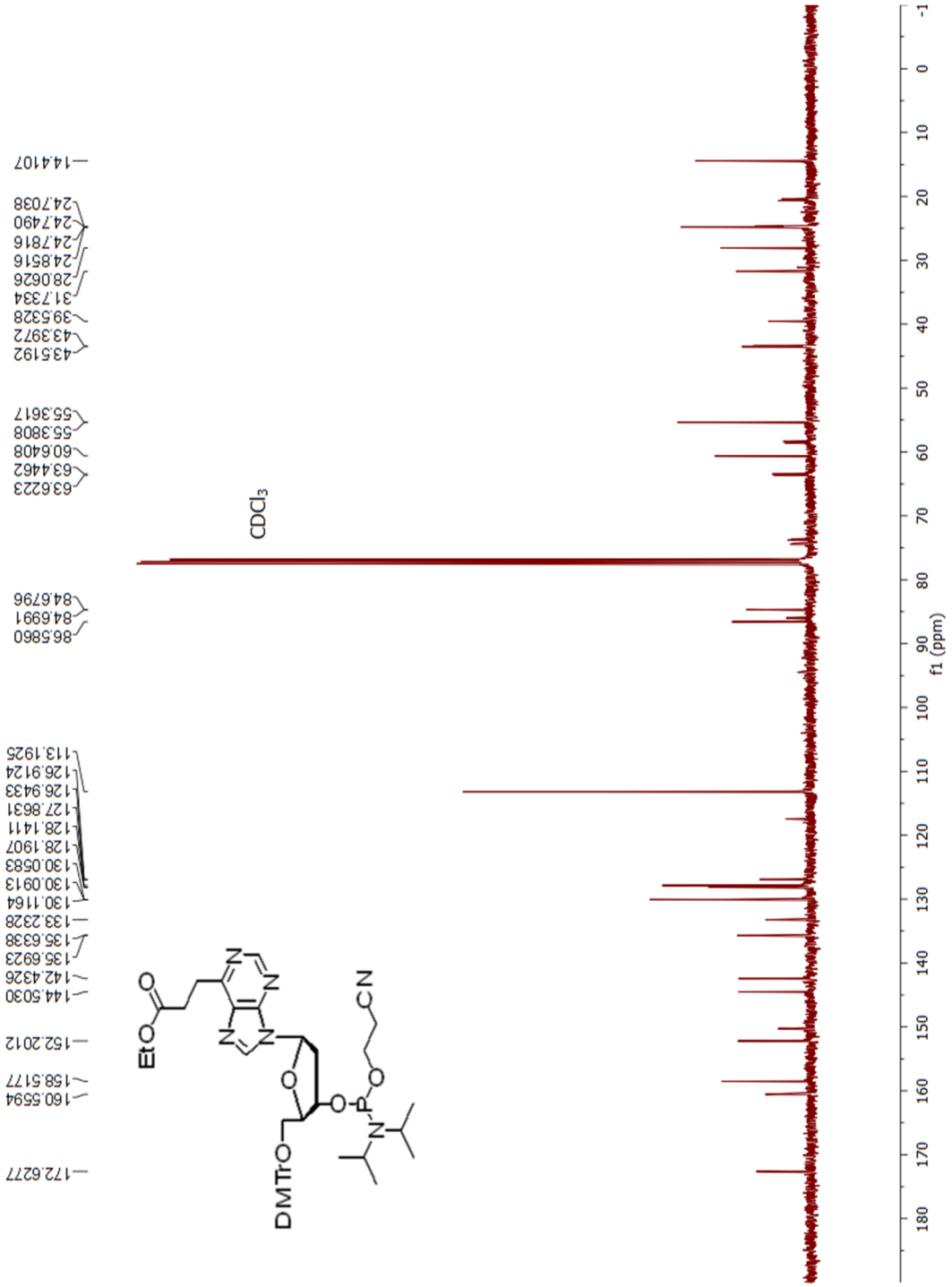
A.1.6 ^{13}C NMR of Compound **43a** in $CDCl_3$, 100 MHz



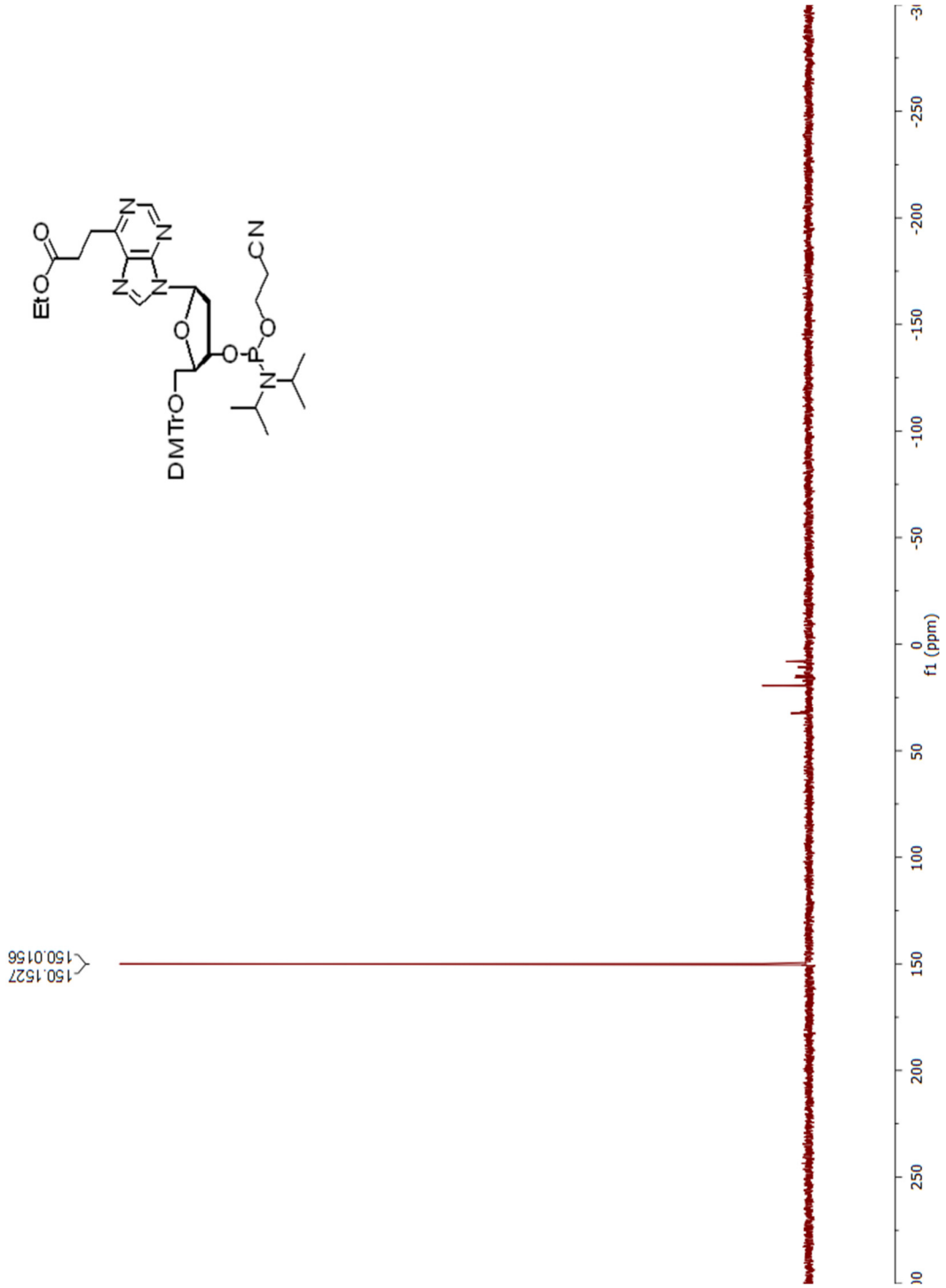
A.1.7 ^{1}H NMR of Compound **1** in $CDCl_3$, 400 MHz



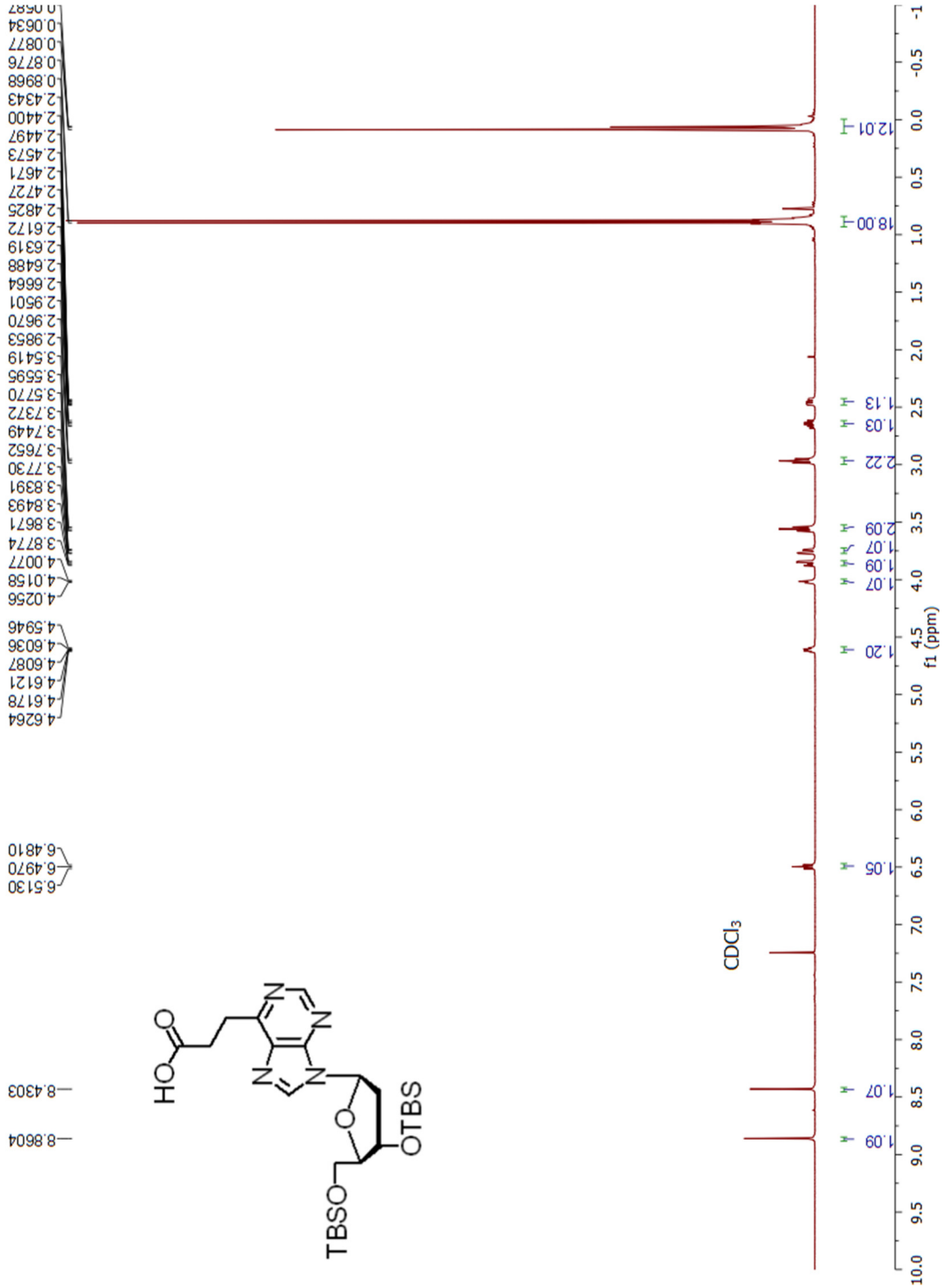
A.1.8 ^{13}C NMR of Compound **1** in $CDCl_3$, 100 MHz



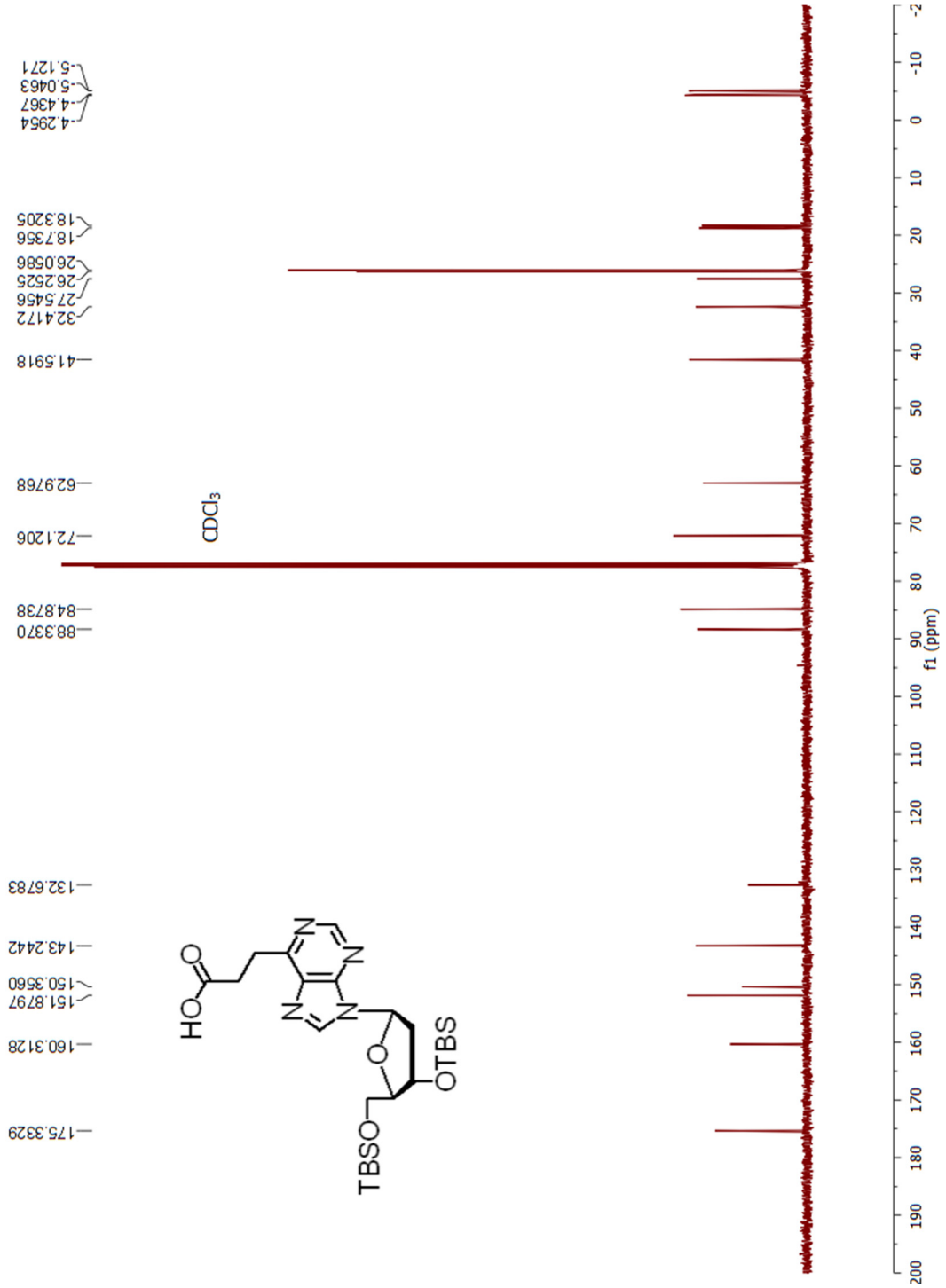
A.1.9 ^{31}P NMR of Compound **1** in $CDCl_3$, 162 MHz



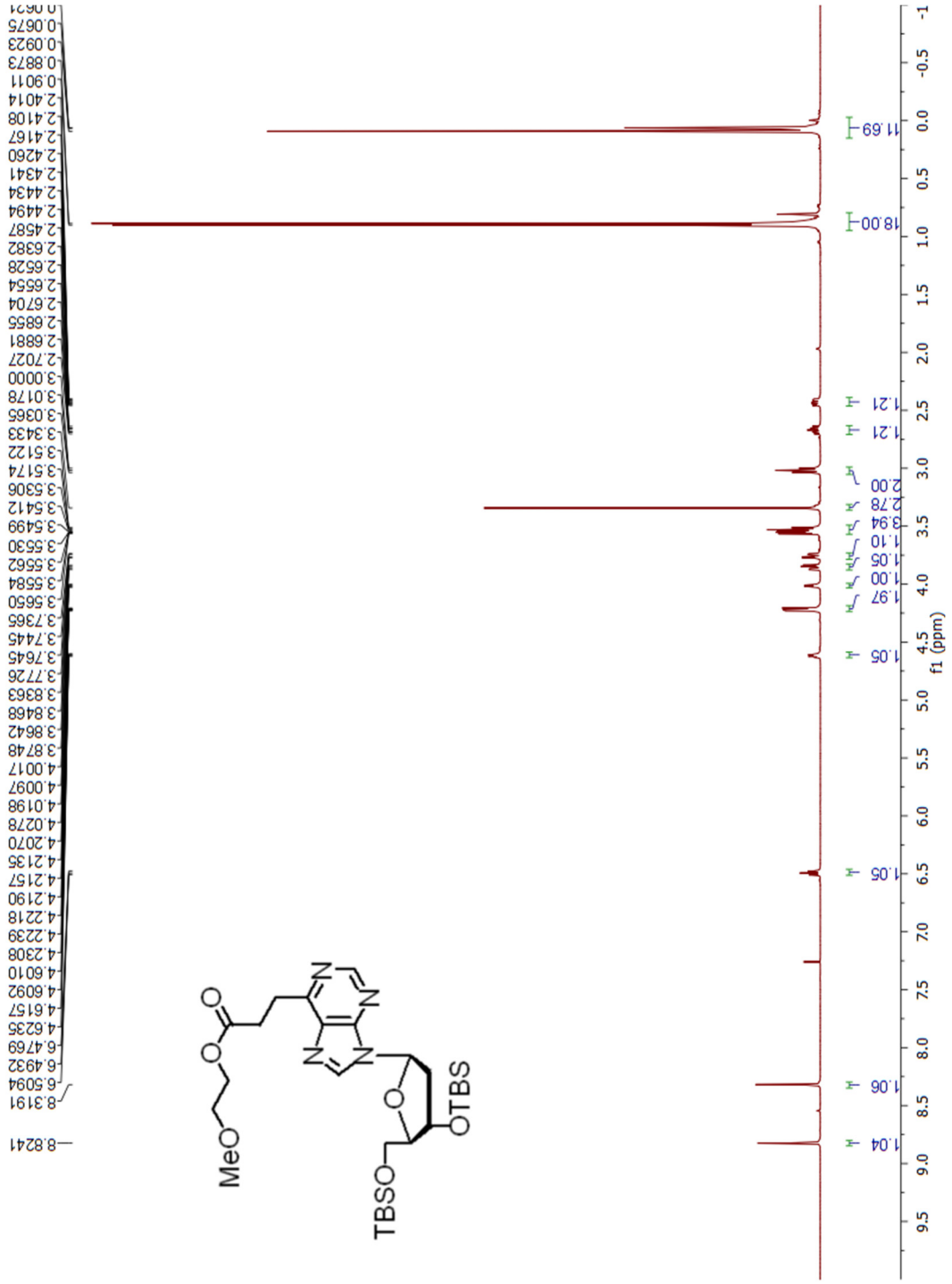
A.1.10 ^{1}H NMR of Compound **39** in $CDCl_3$, 400 MHz



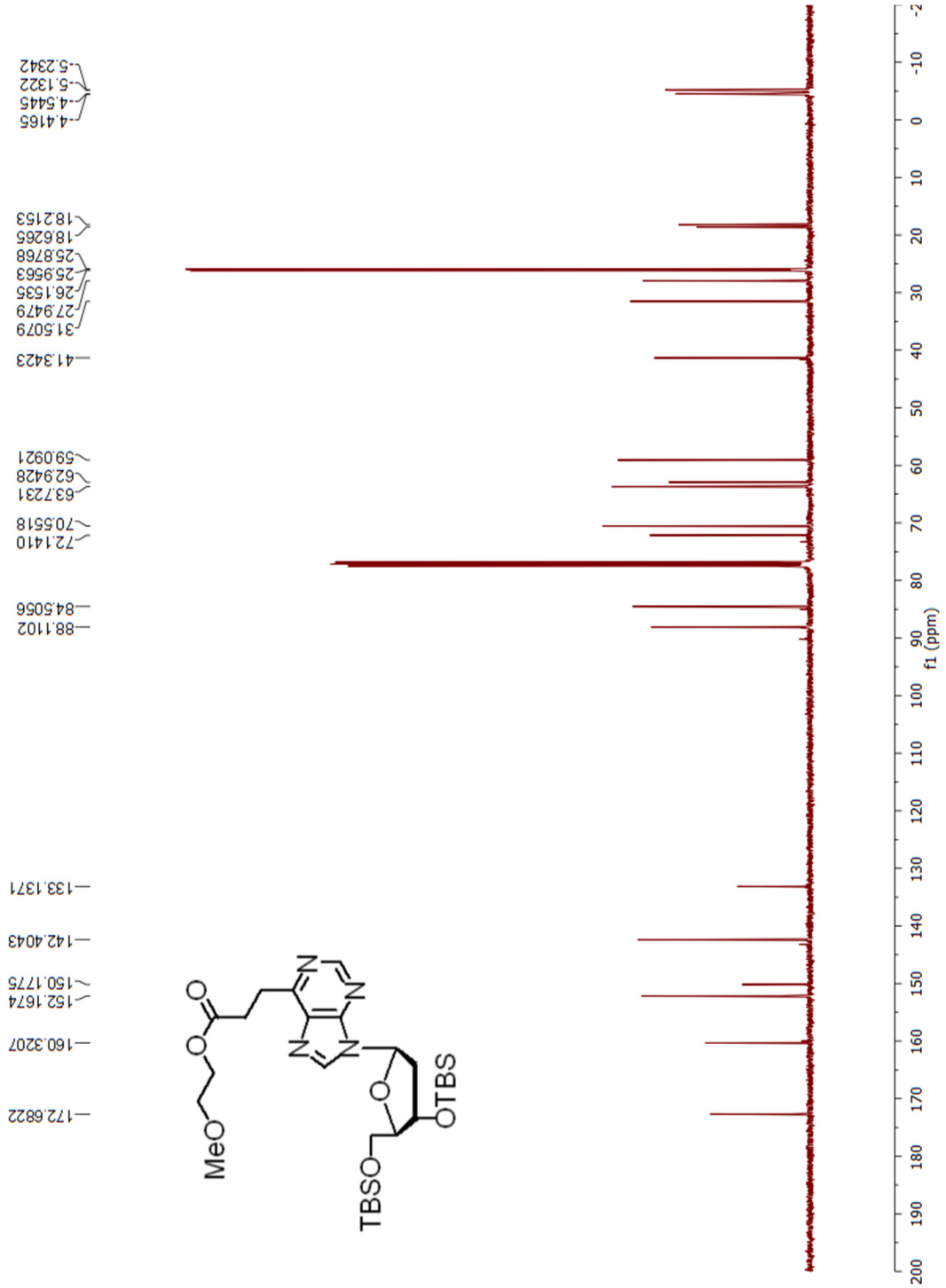
A.1.11 ^{13}C NMR of Compound **39** in $CDCl_3$, 100 MHz



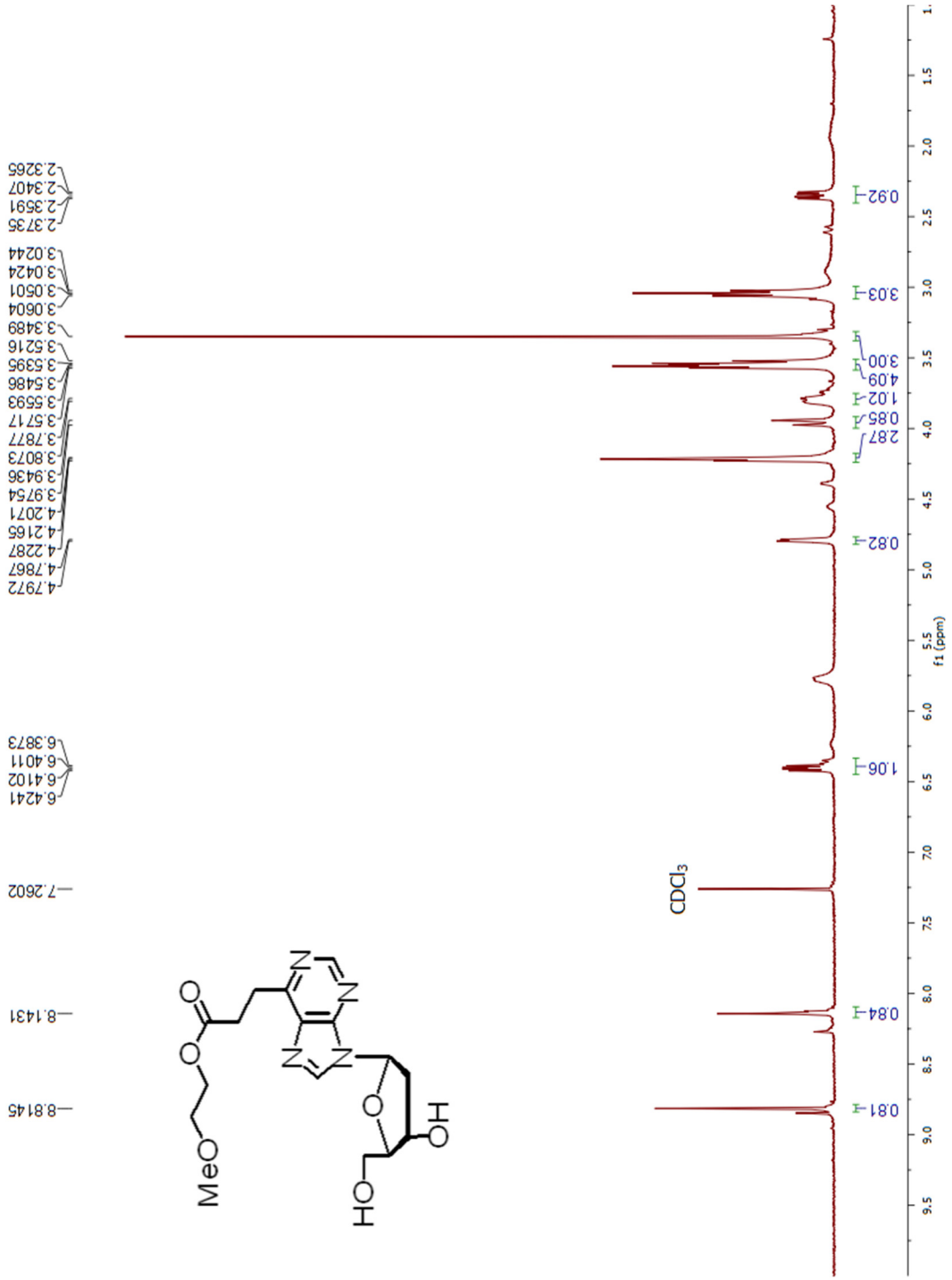
A.1.12 ^{1}H NMR of Compound **41a** in $CDCl_3$, 400 MHz



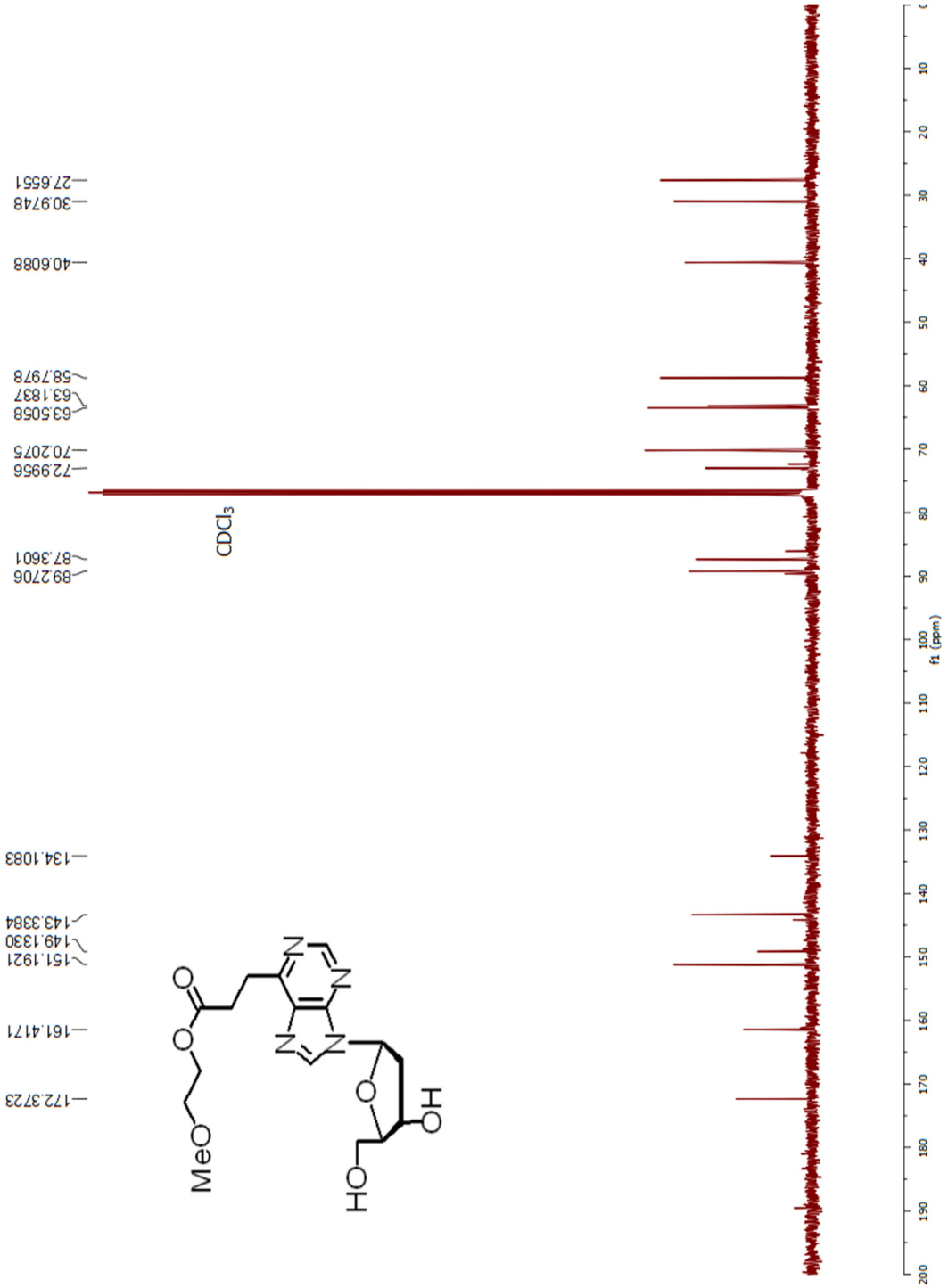
A.1.13 ^{13}C NMR of Compound **41a** in $CDCl_3$, 100 MHz



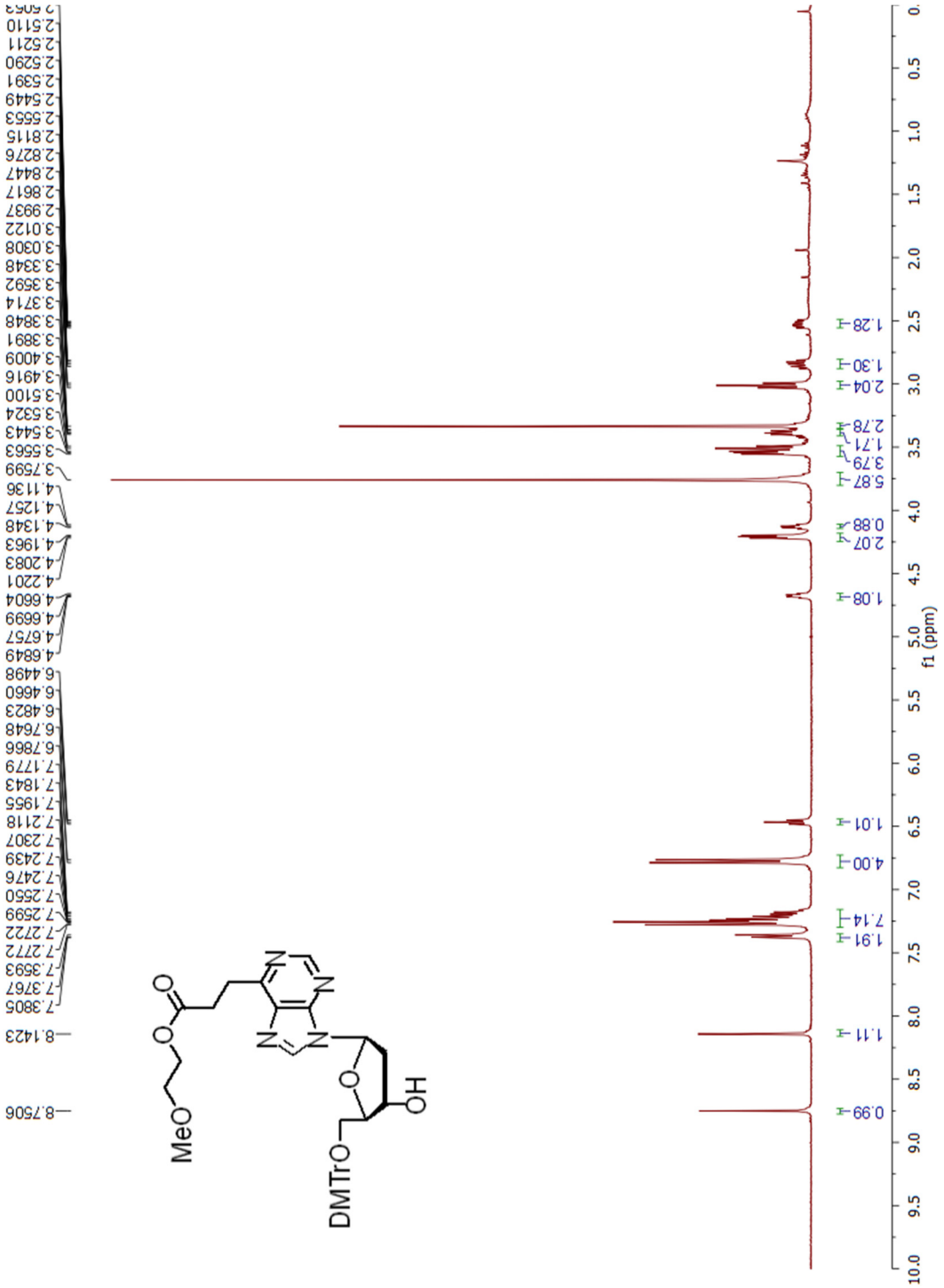
A.1.14 ^{1}H NMR of Compound **42b** in $CDCl_3$, 400 MHz



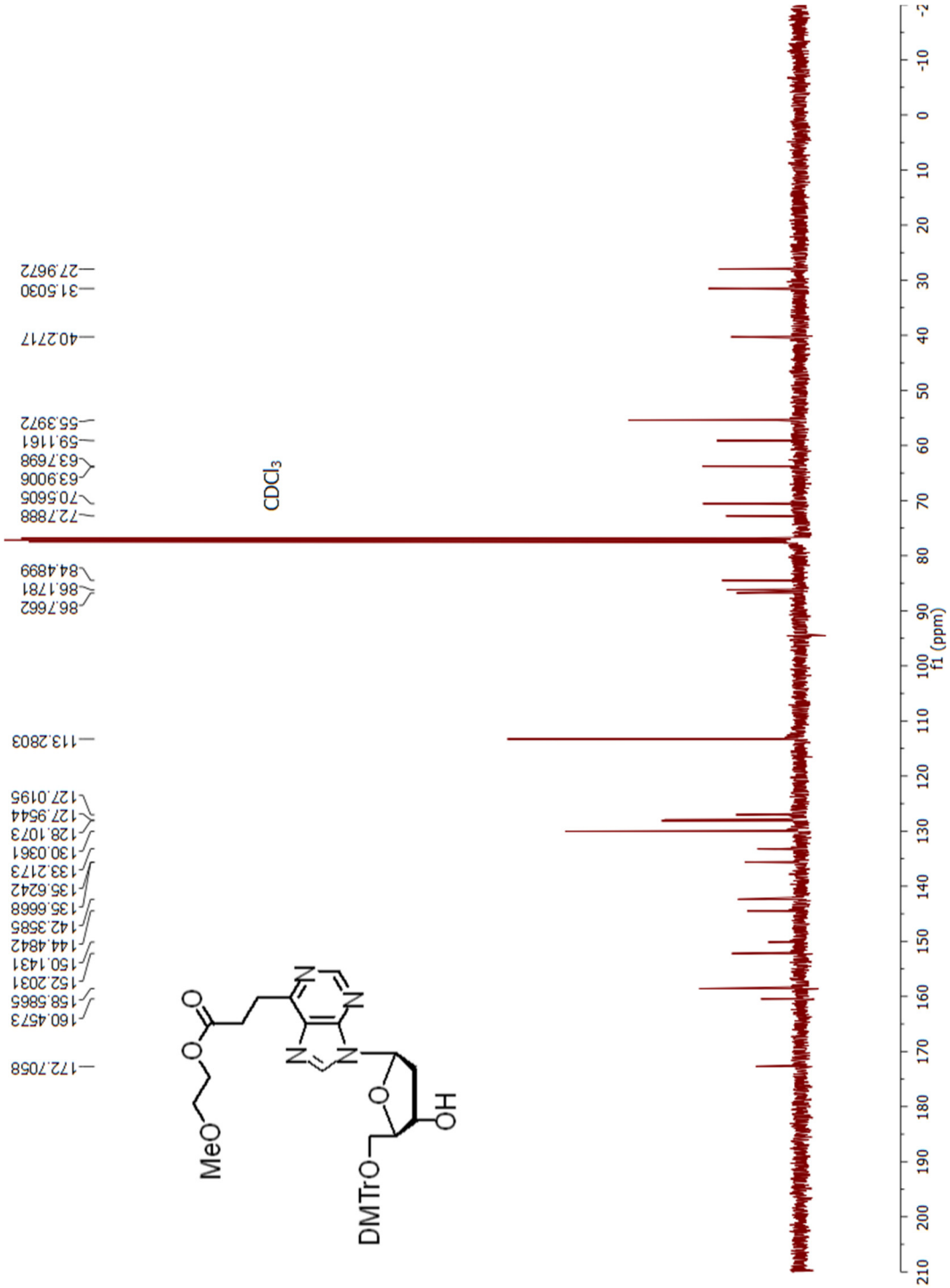
A.1.15 ^{13}C NMR of Compound **42b** in $CDCl_3$, 100 MHz



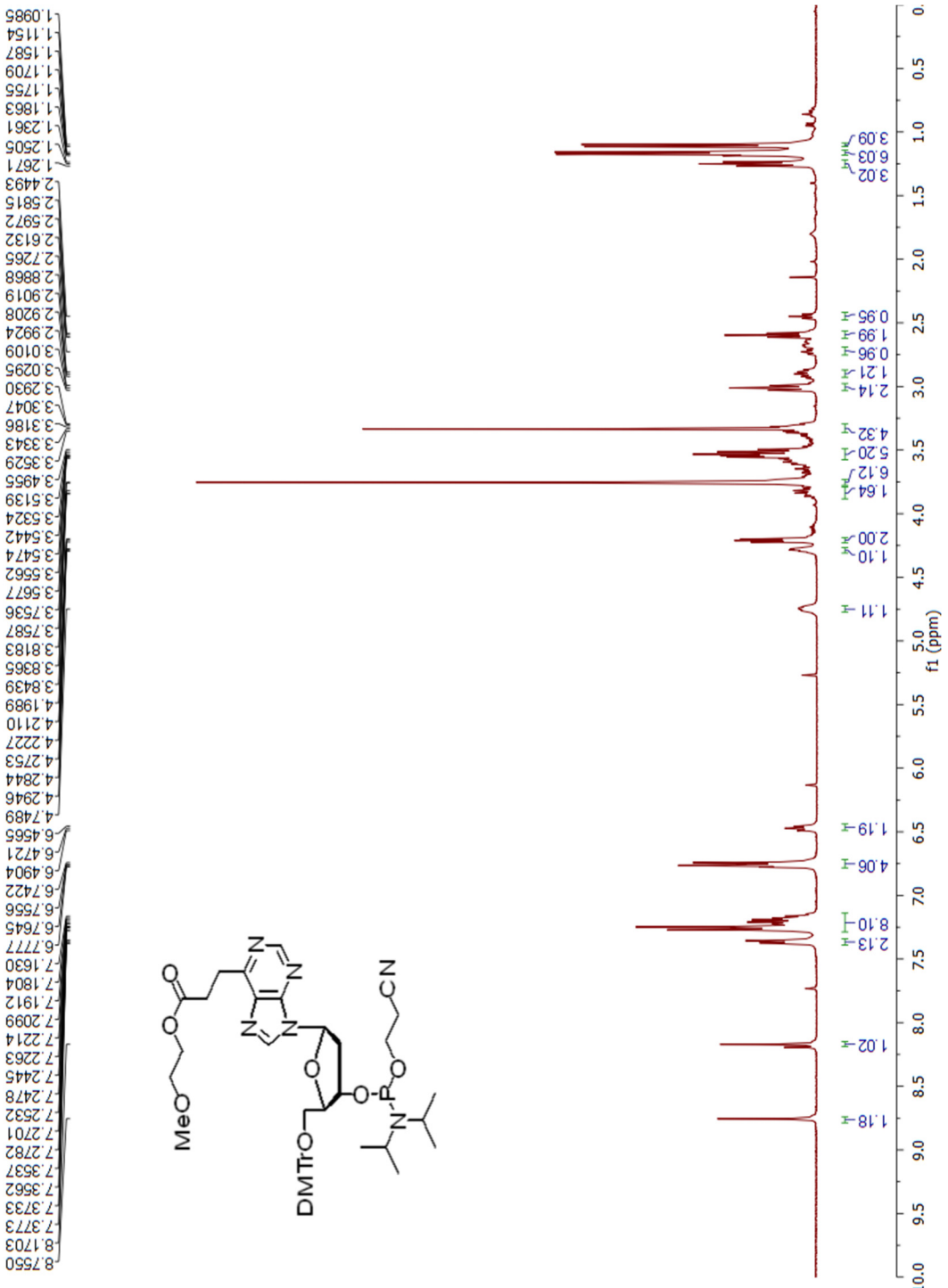
A.1.16 ^{1}H NMR of Compound **43b** in $CDCl_3$, 400 MHz



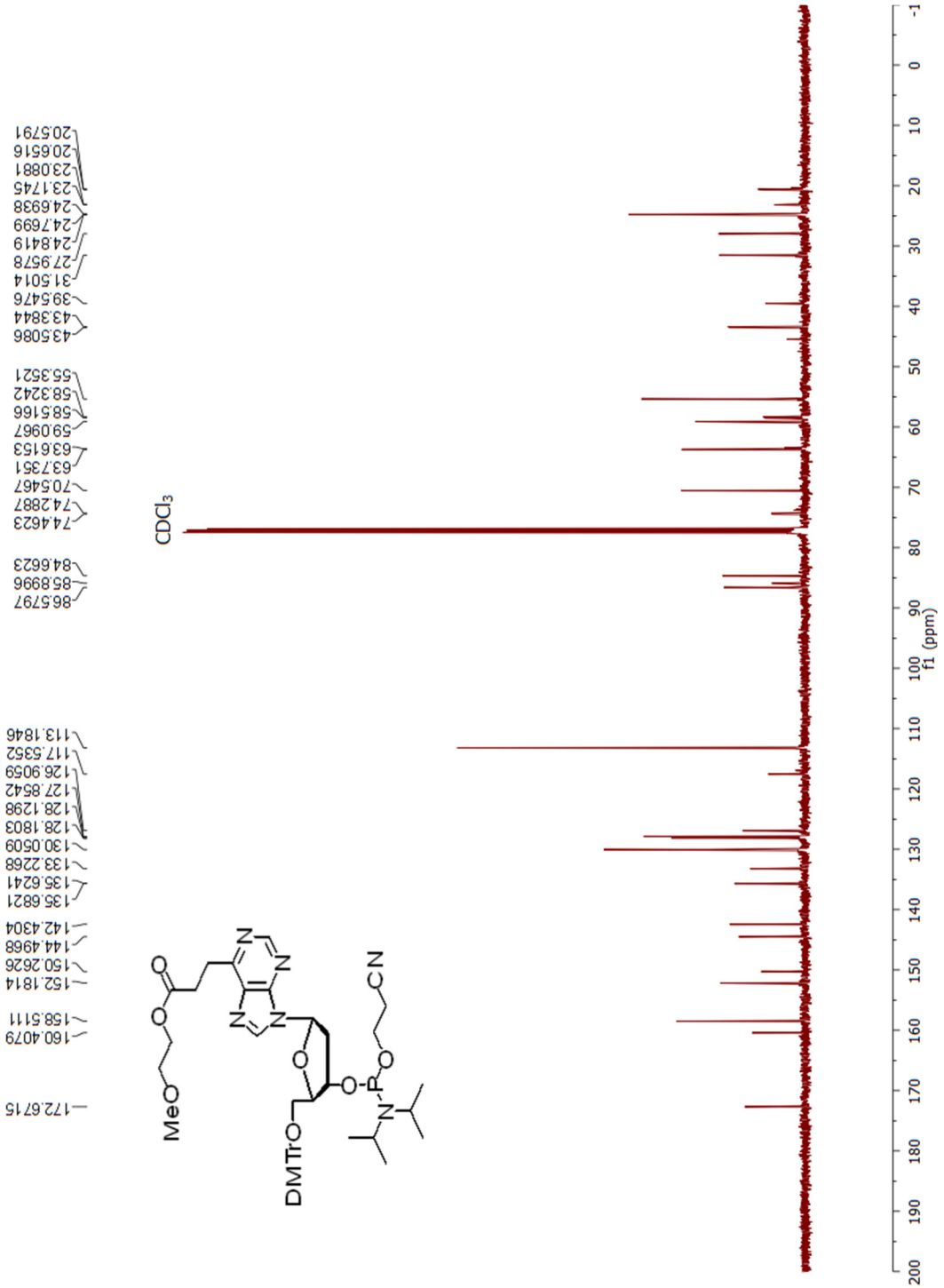
A.1.17 ^{13}C NMR of Compound **43b** in $CDCl_3$, 100 MHz



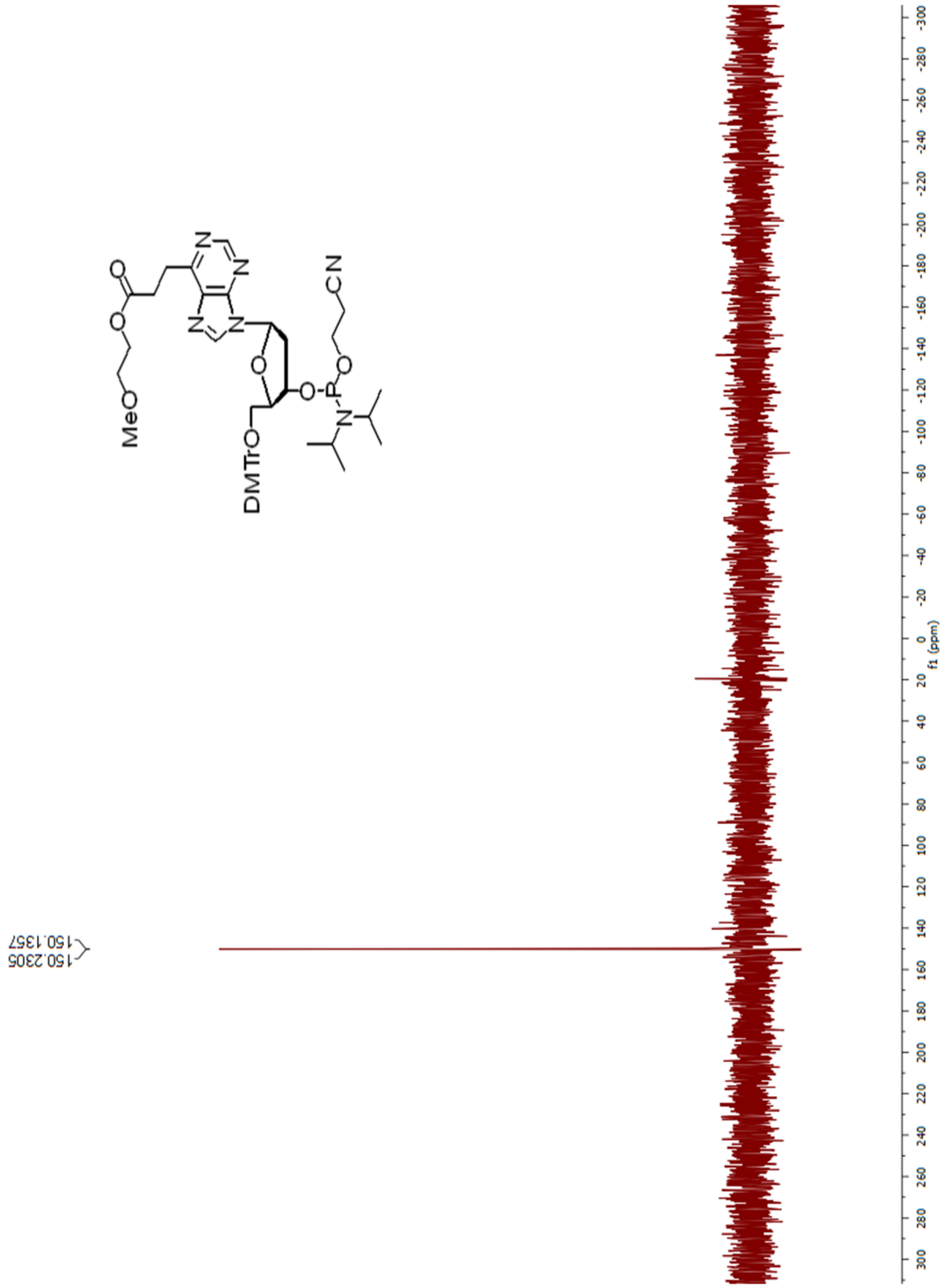
A.1.18 ^{1}H NMR of Compound **2** in $CDCl_3$, 400 MHz



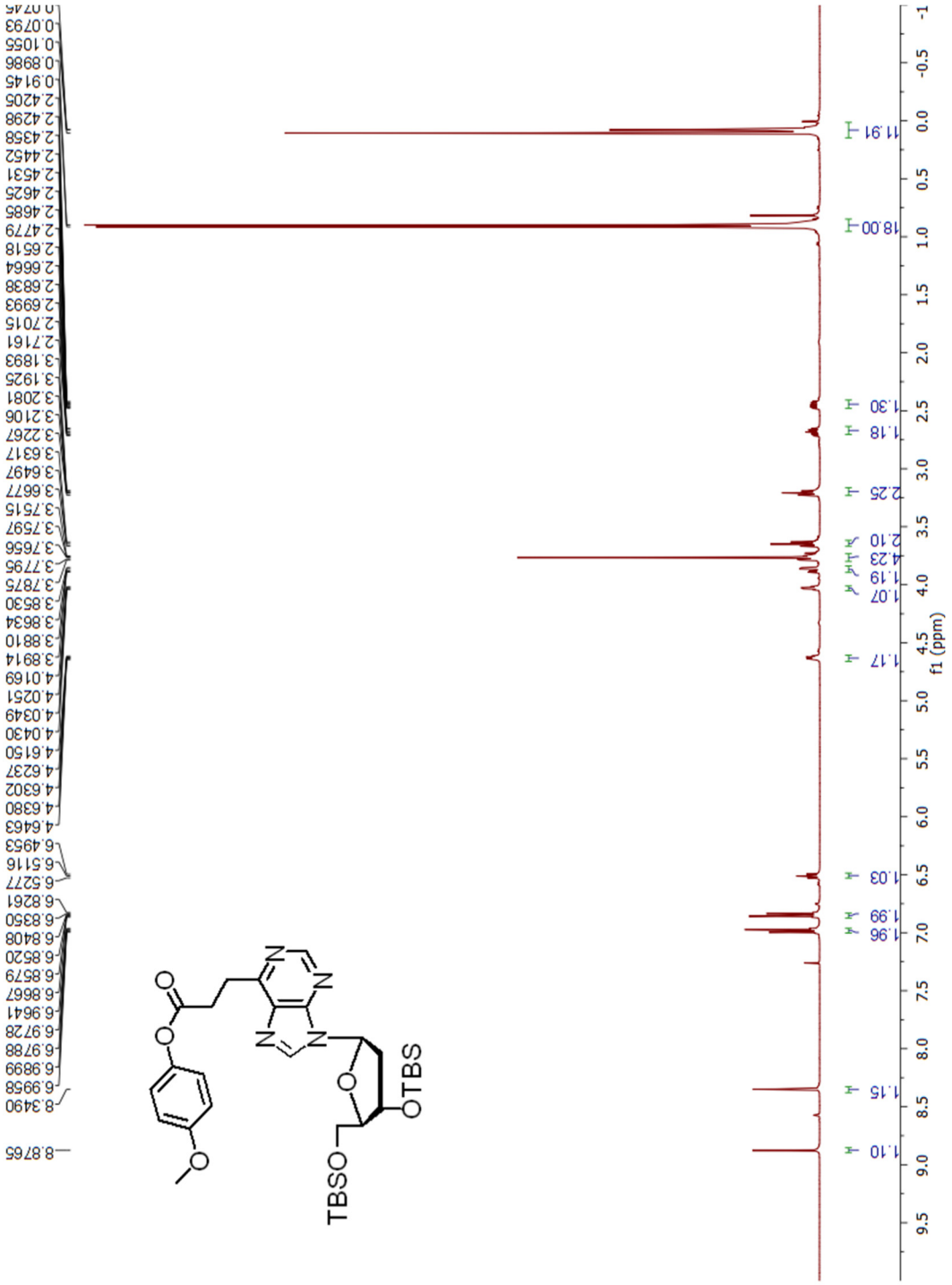
A.1.19 ^{13}C NMR of Compound **2** in $CDCl_3$, 100 MHz



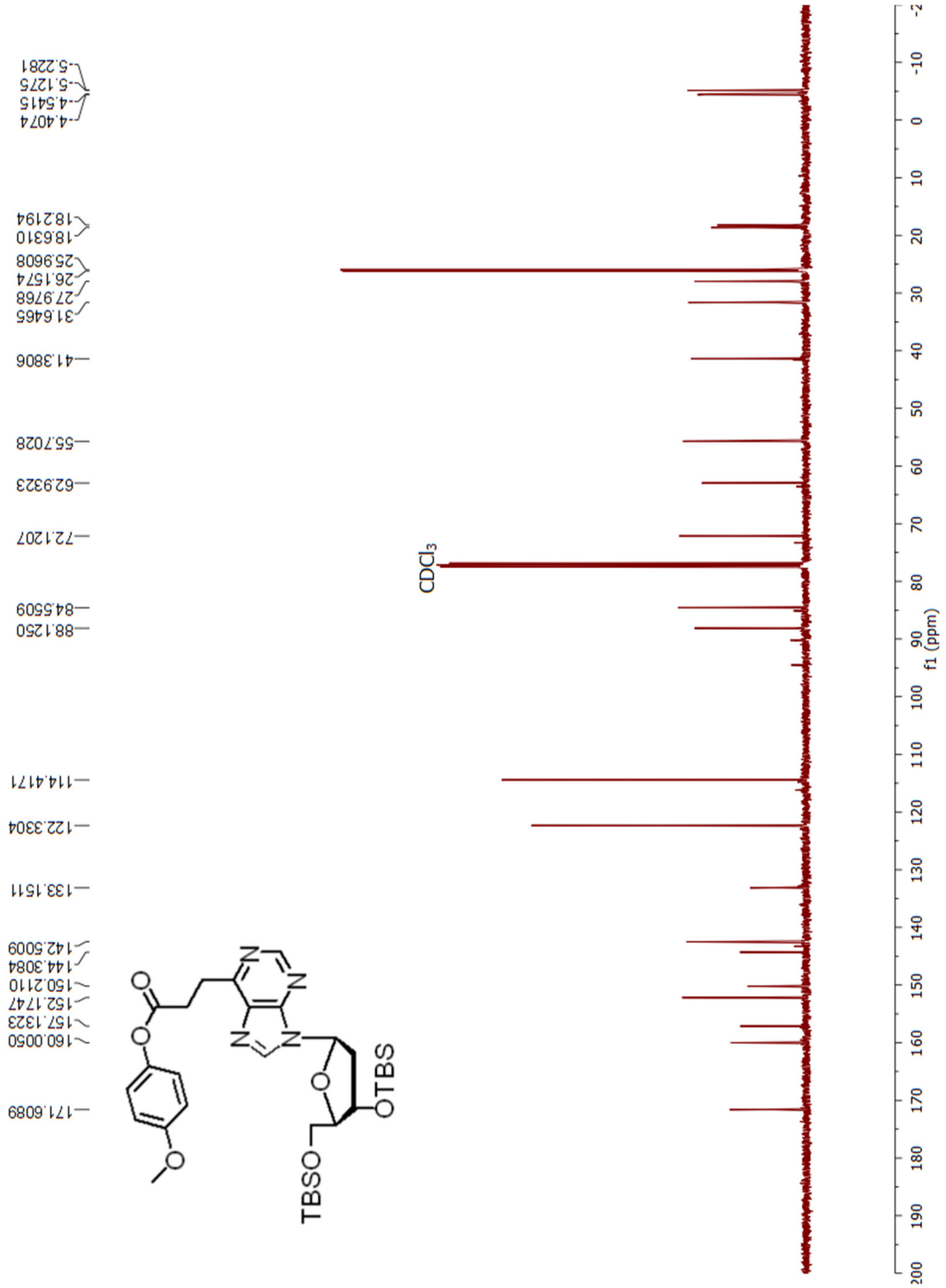
A.1.20 ^{31}P NMR of Compound **2** in $CDCl_3$, 162 MHz



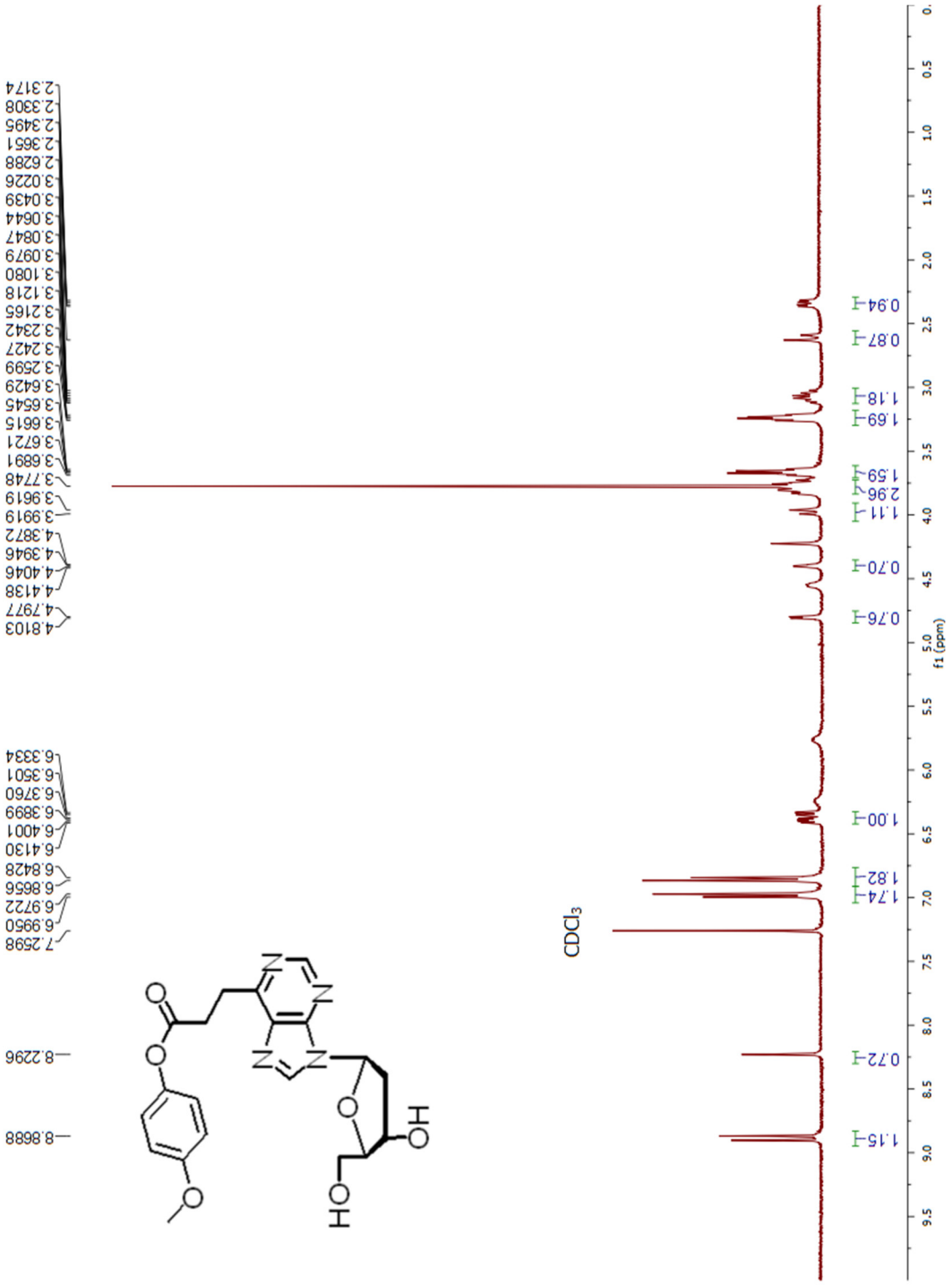
A.1.21 ^{1}H NMR of Compound **41b** in $CDCl_3$, 400 MHz



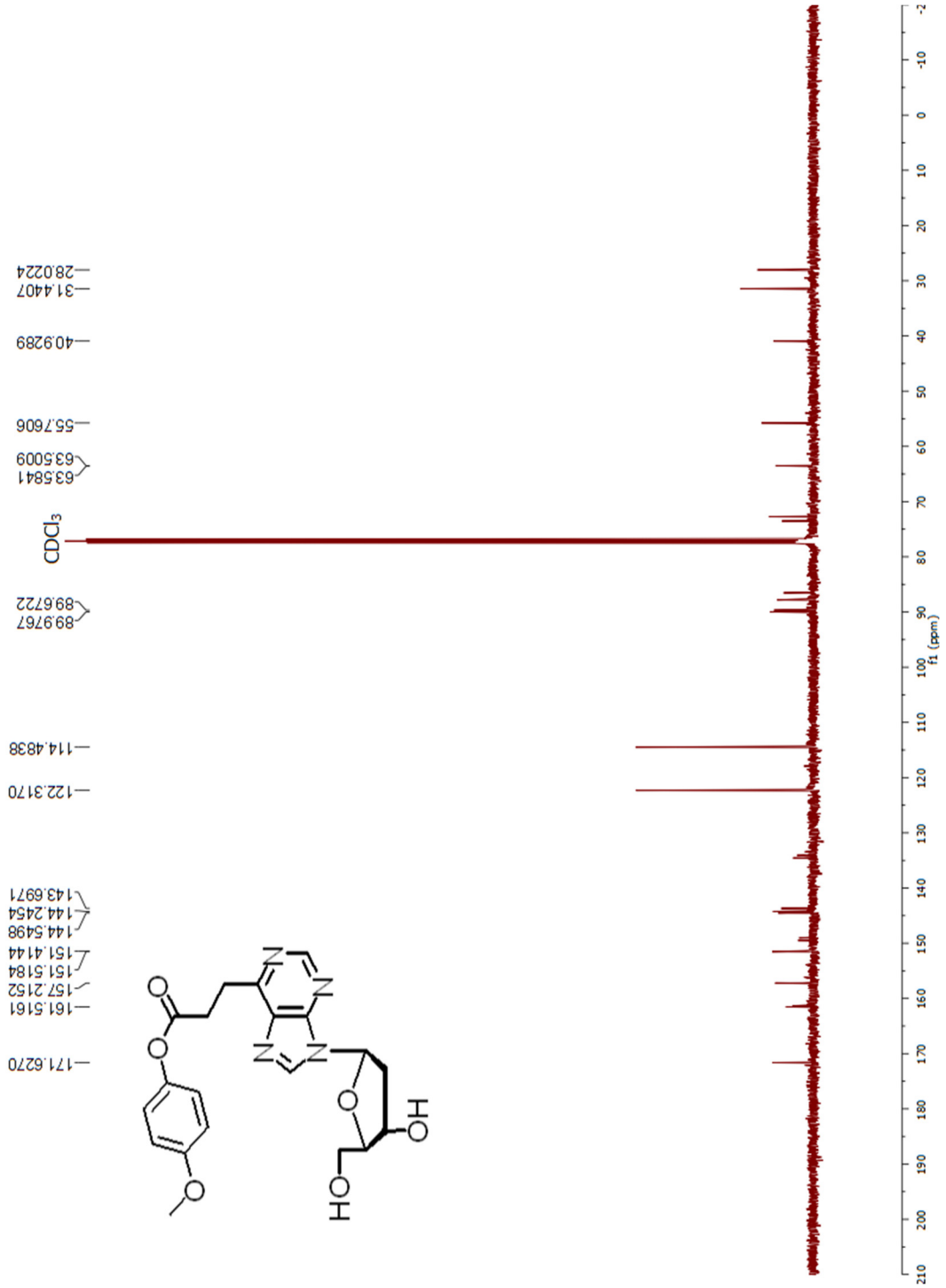
A.1.22 ^{13}C NMR of Compound **41b** in $CDCl_3$, 100 MHz



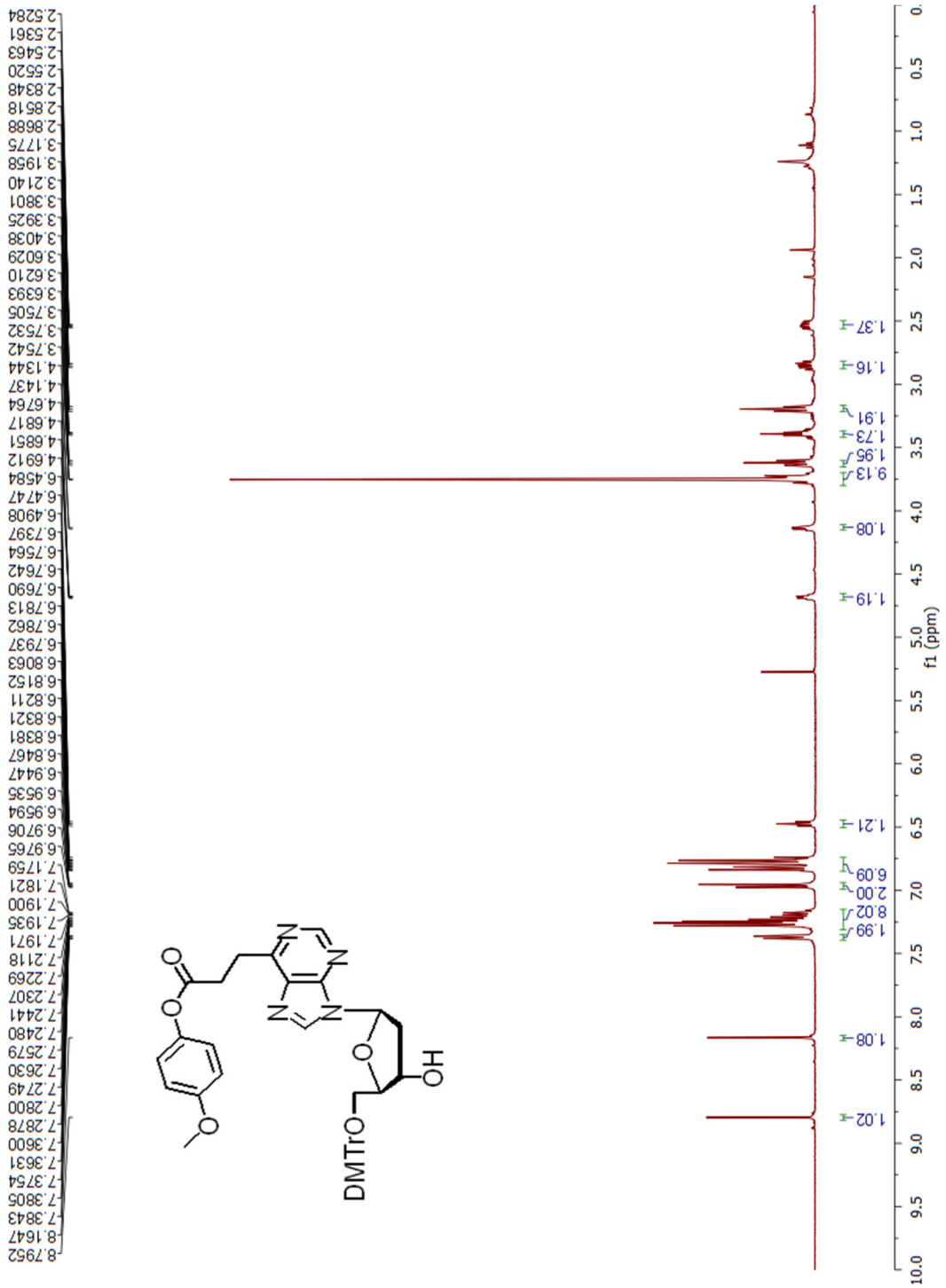
A.1.23 ^{1}H NMR of Compound **42c** in $CDCl_3$, 400 MHz



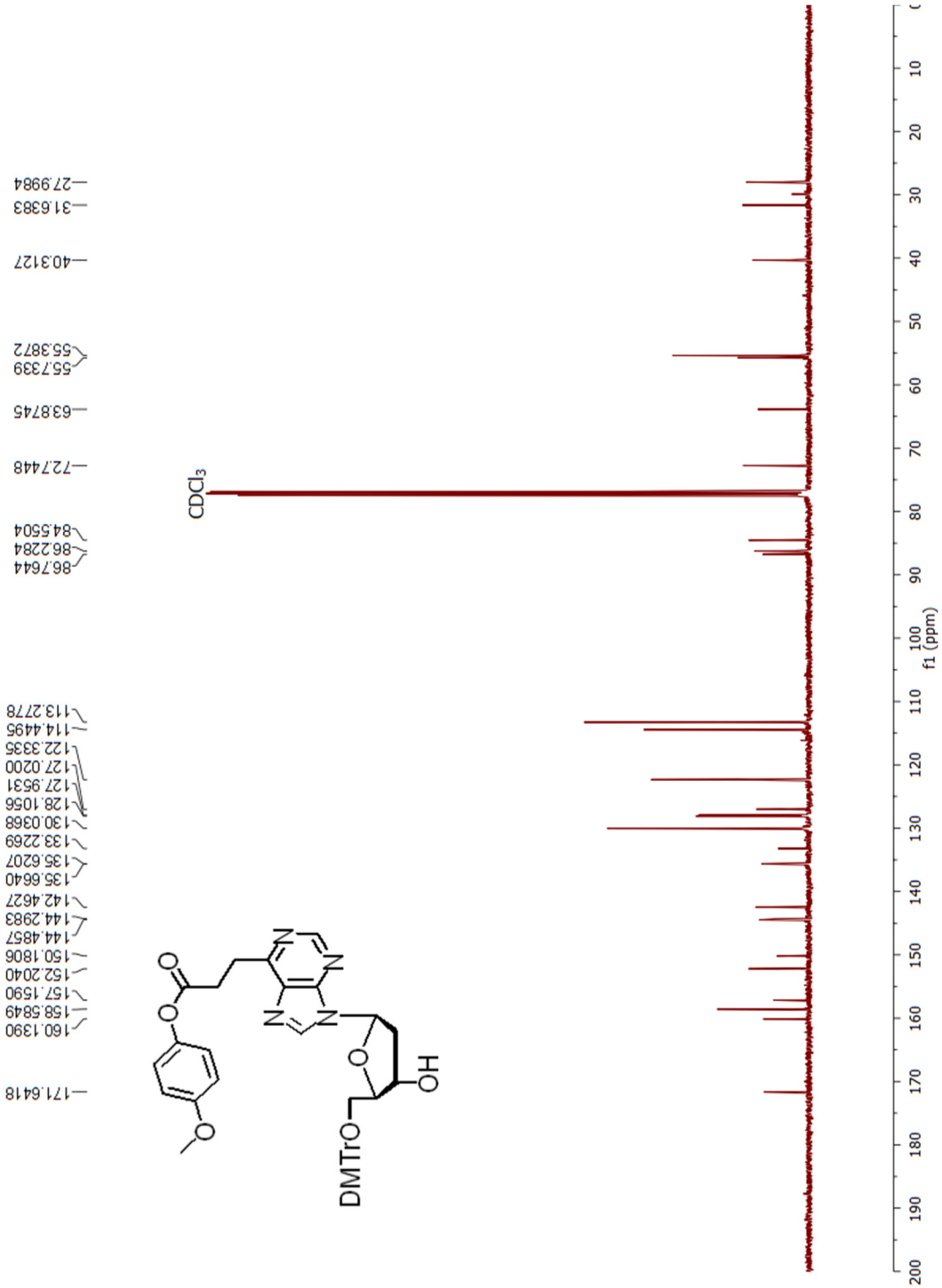
A.1.24 ^{13}C NMR of Compound **42c** in $CDCl_3$, 100 MHz



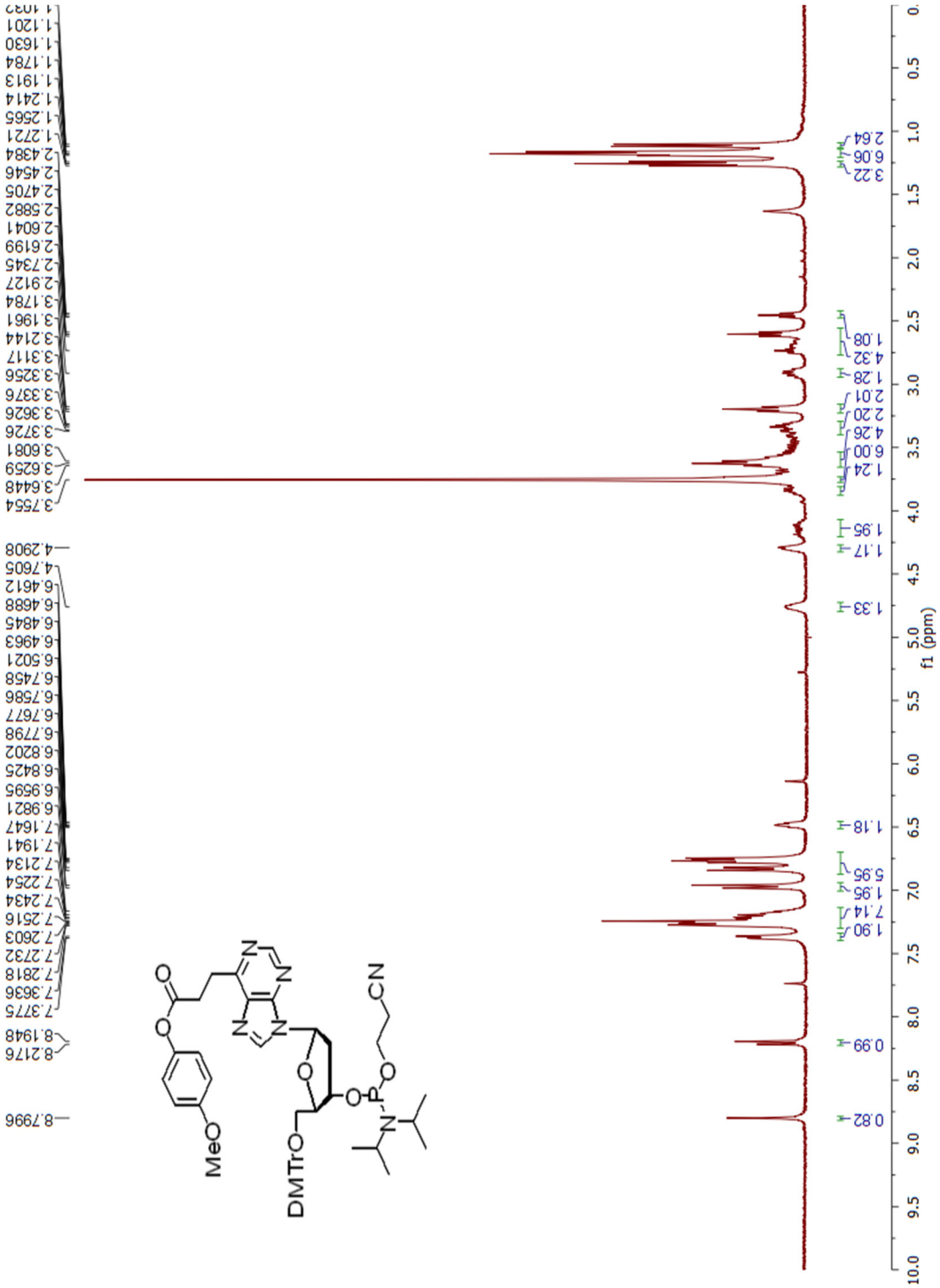
A.1.25 ^{1}H NMR of Compound **43c** in $CDCl_3$, 400 MHz



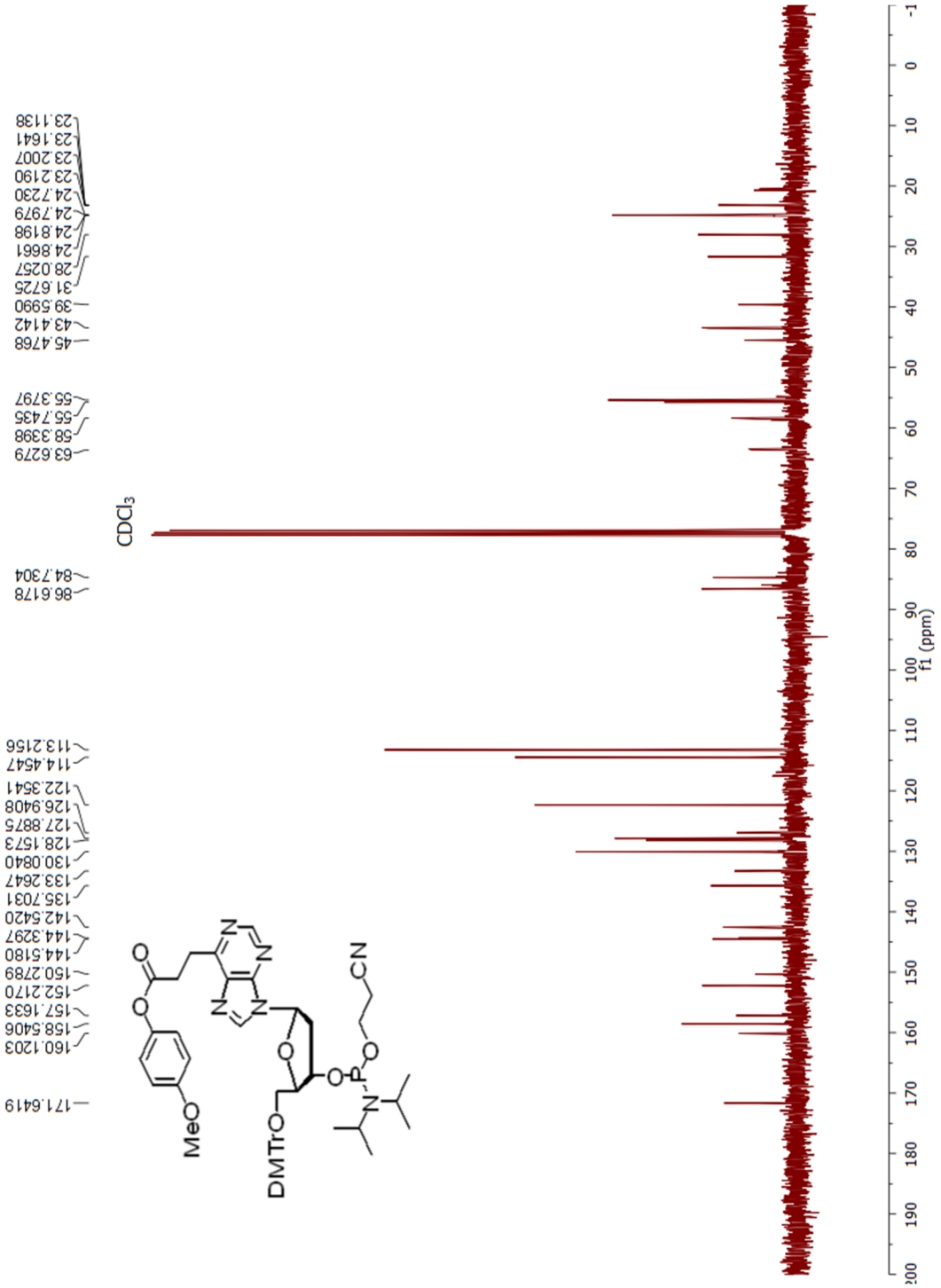
A.1.26 ^{13}C NMR of Compound **43c** in $CDCl_3$, 100 MHz



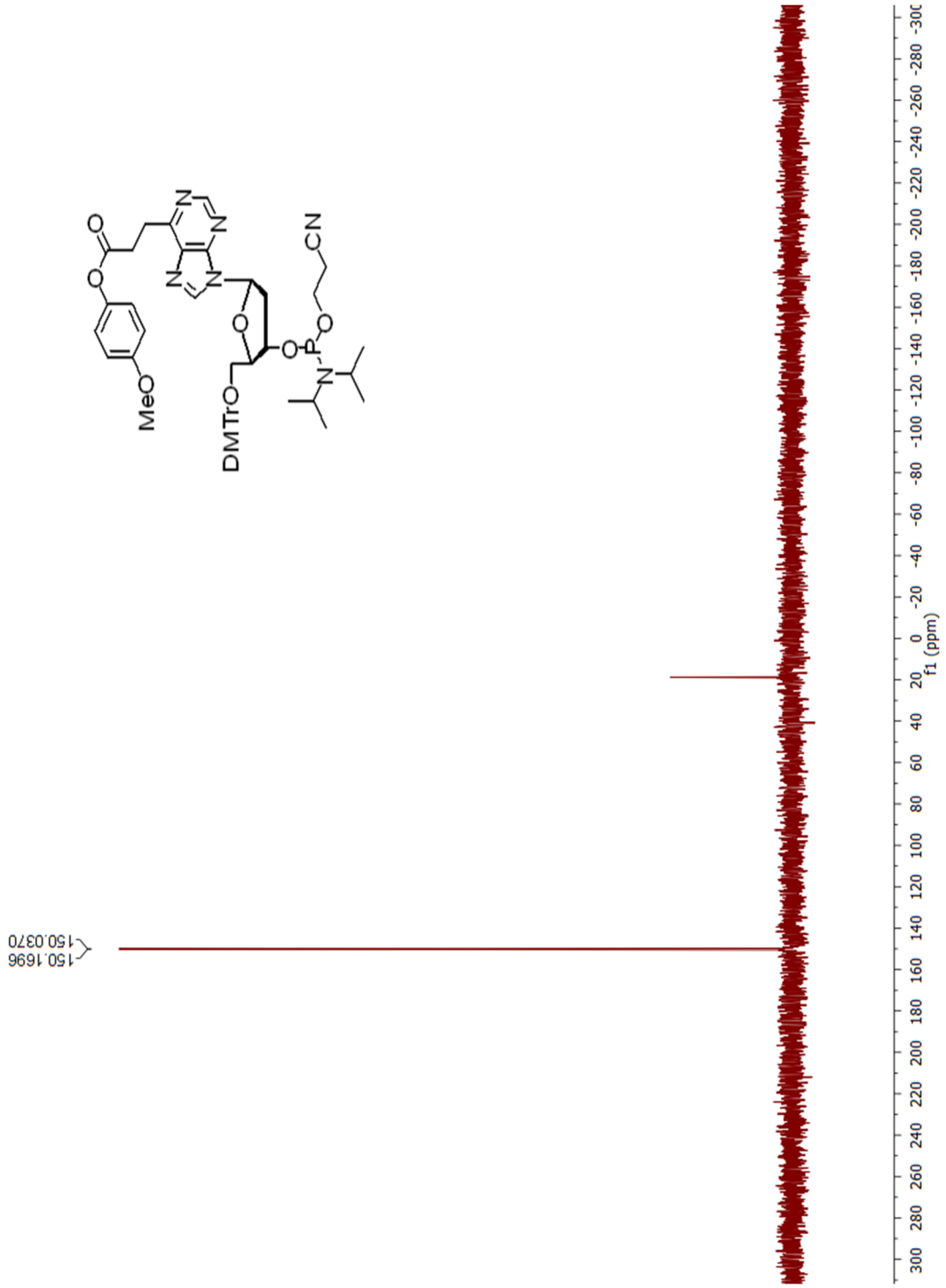
A.1.27 ^{1}H NMR of Compound **3** in $CDCl_3$, 400 MHz



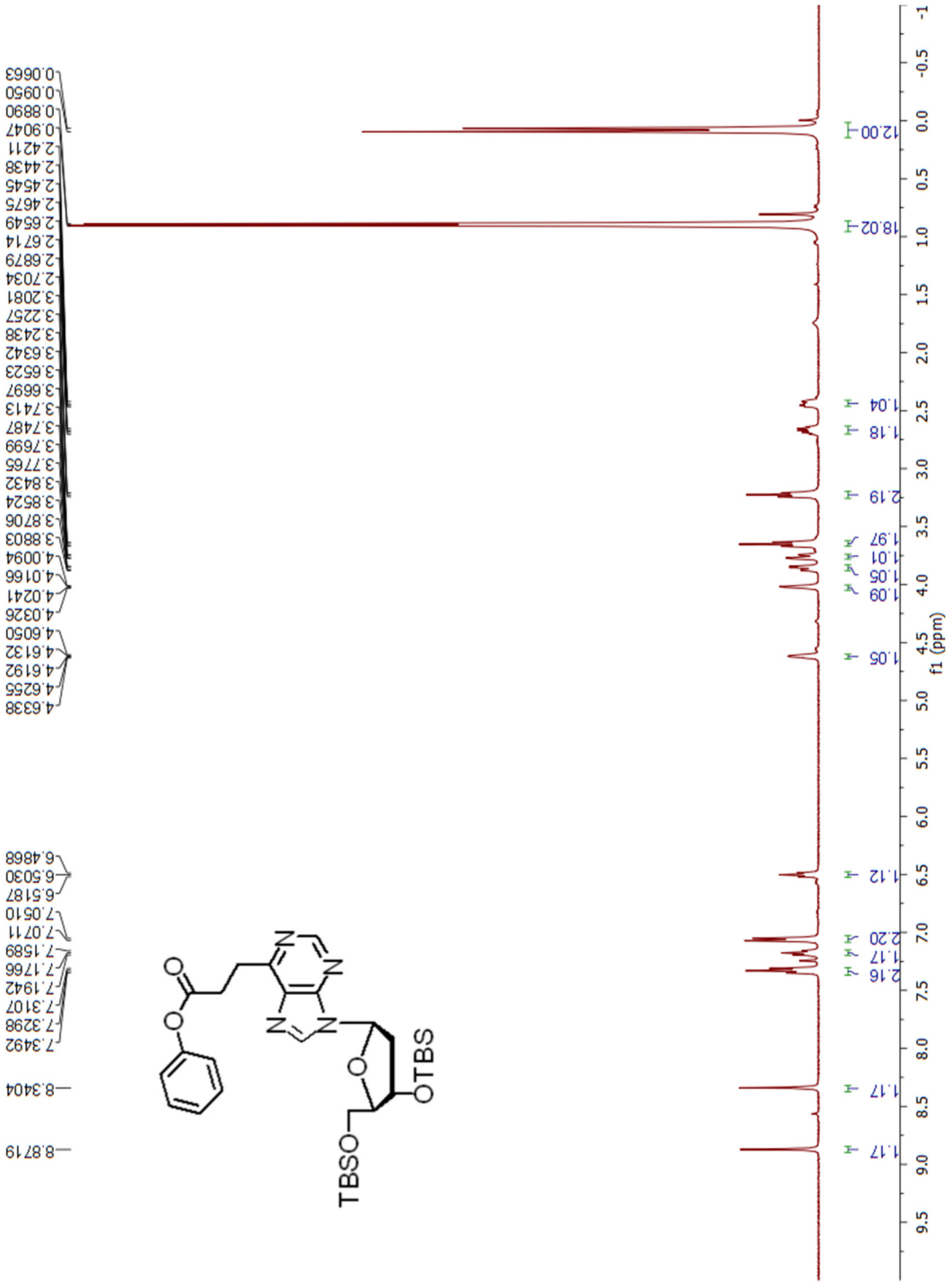
A.1.28 ^{13}C NMR of Compound **3** in $CDCl_3$, 100 MHz



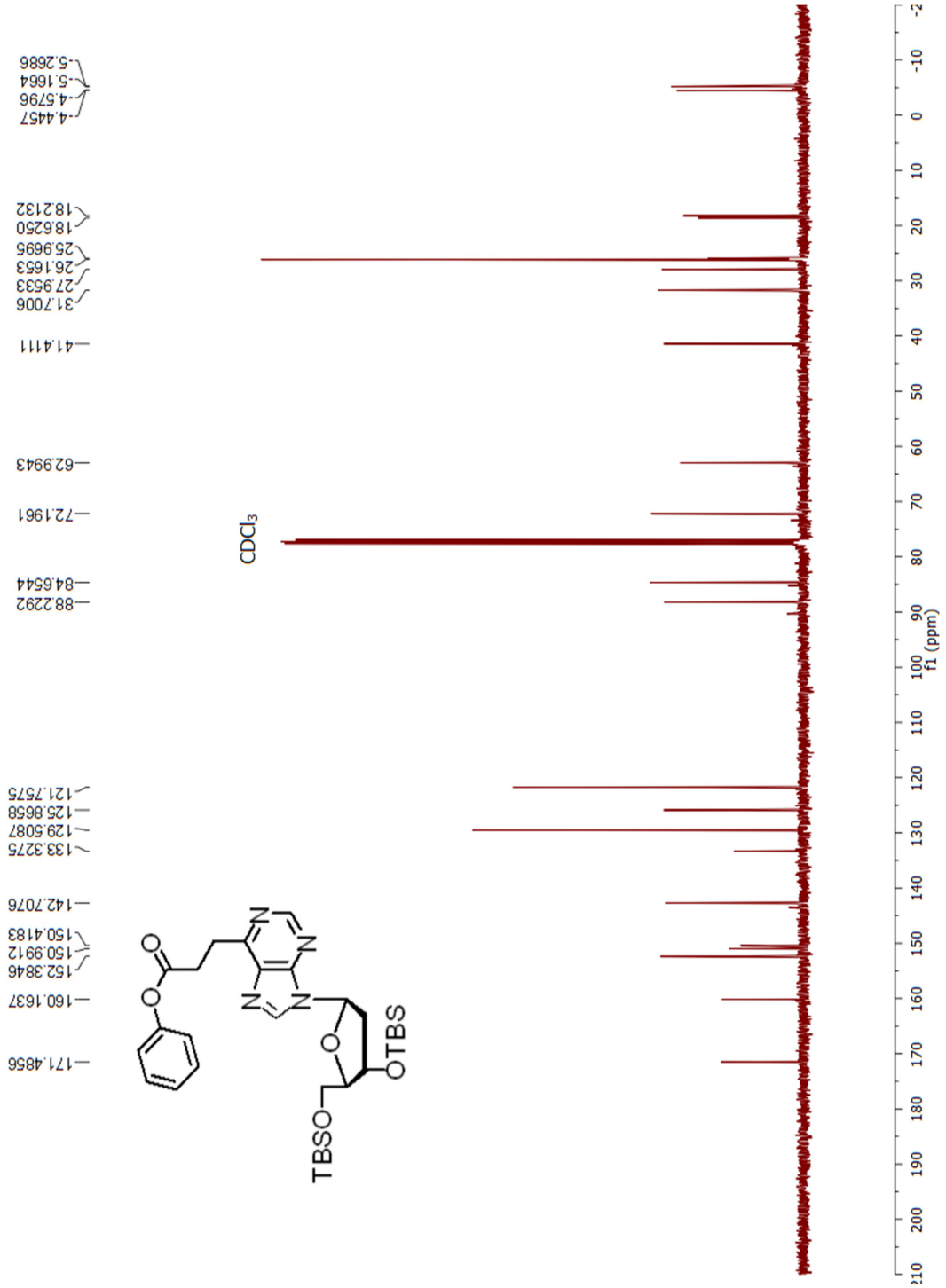
A.1.29 ^{31}P NMR of Compound **3** in $CDCl_3$, 162 MHz



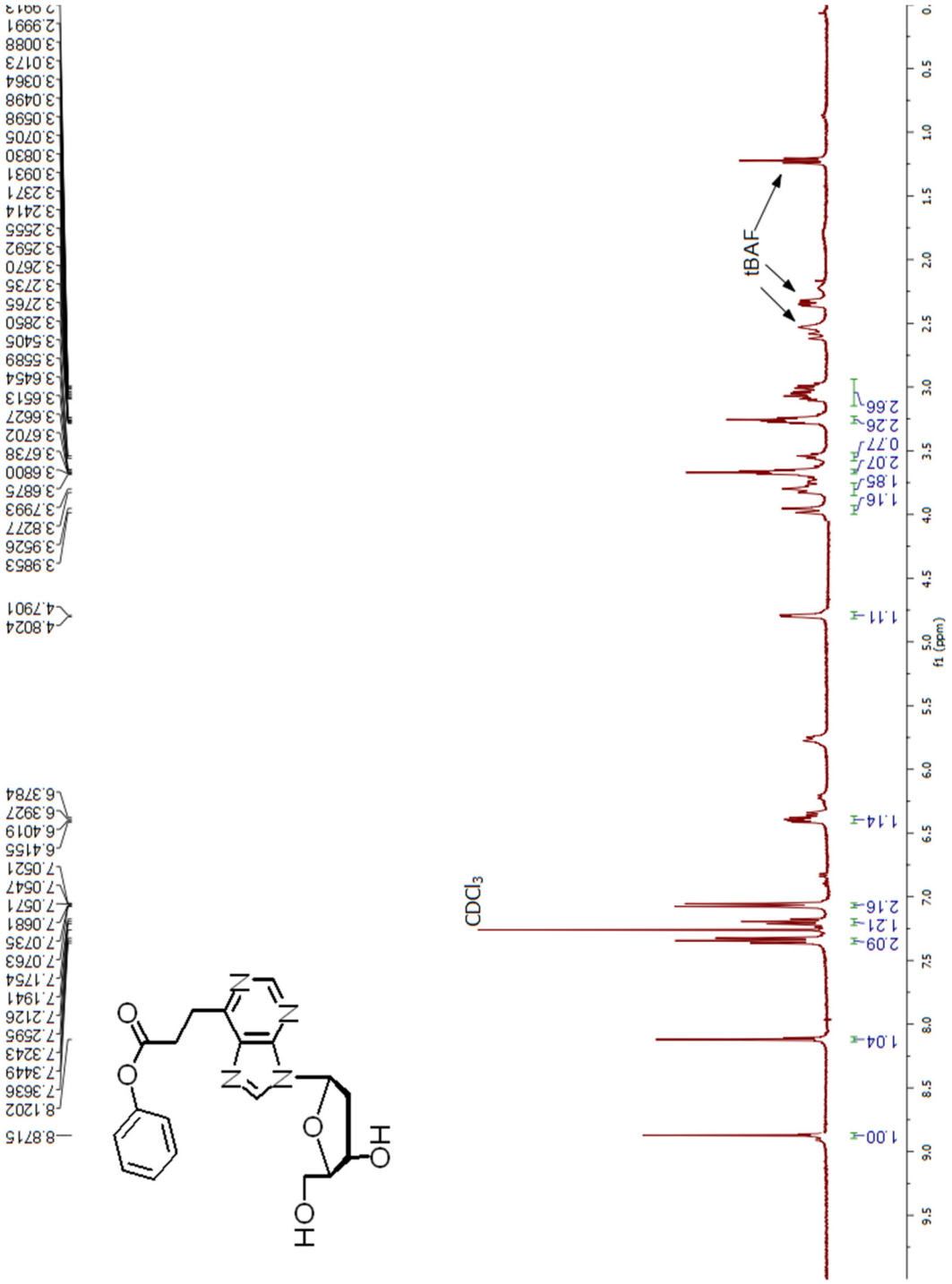
A.1.30 ^{1}H NMR of Compound **41c** in $CDCl_3$, 400 MHz



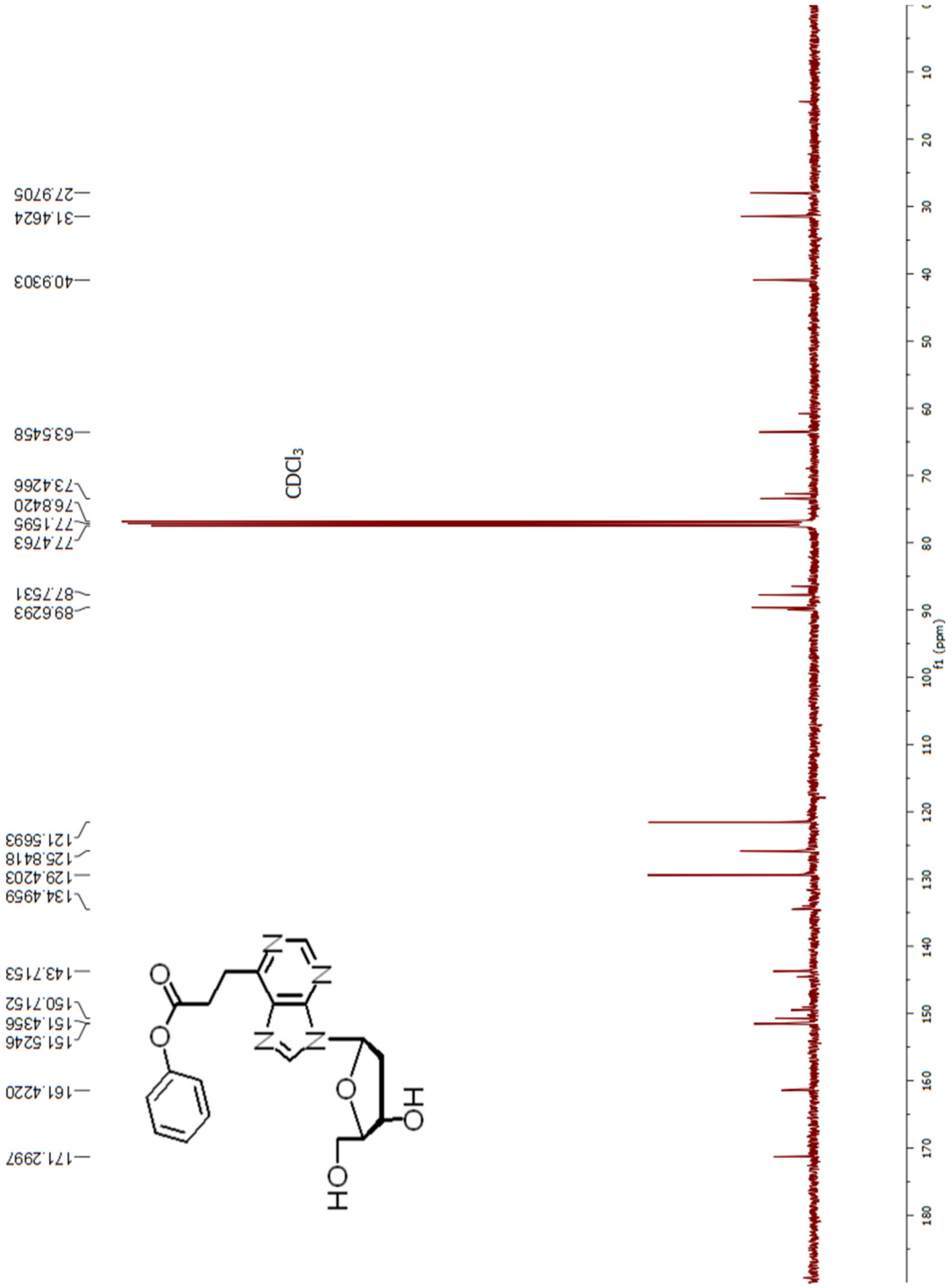
A.1.31 ^{13}C NMR of Compound **41c** in $CDCl_3$, 100 MHz



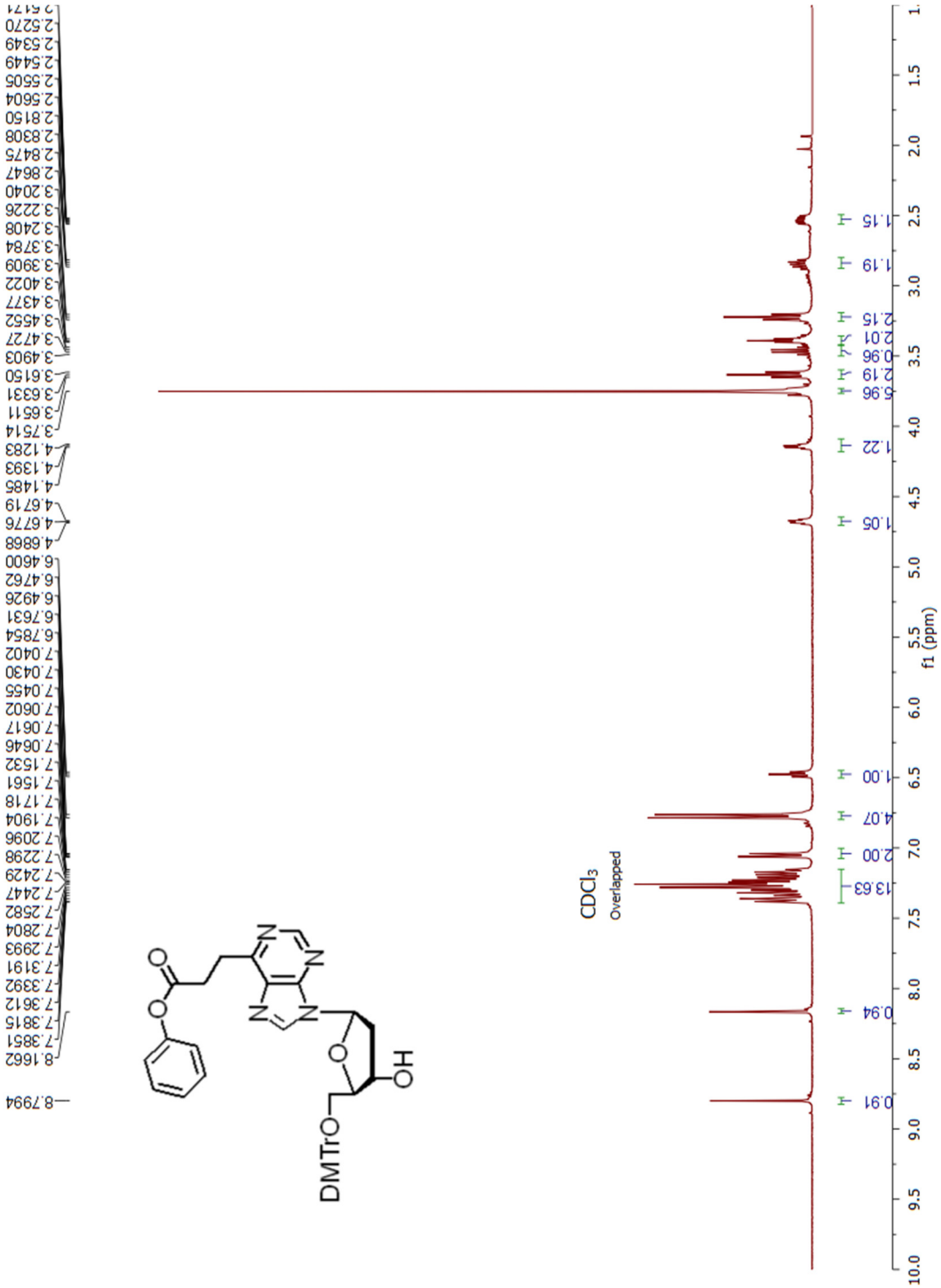
A.1.32 ^{1}H NMR of Compound **42d** in $CDCl_3$, 400 MHz



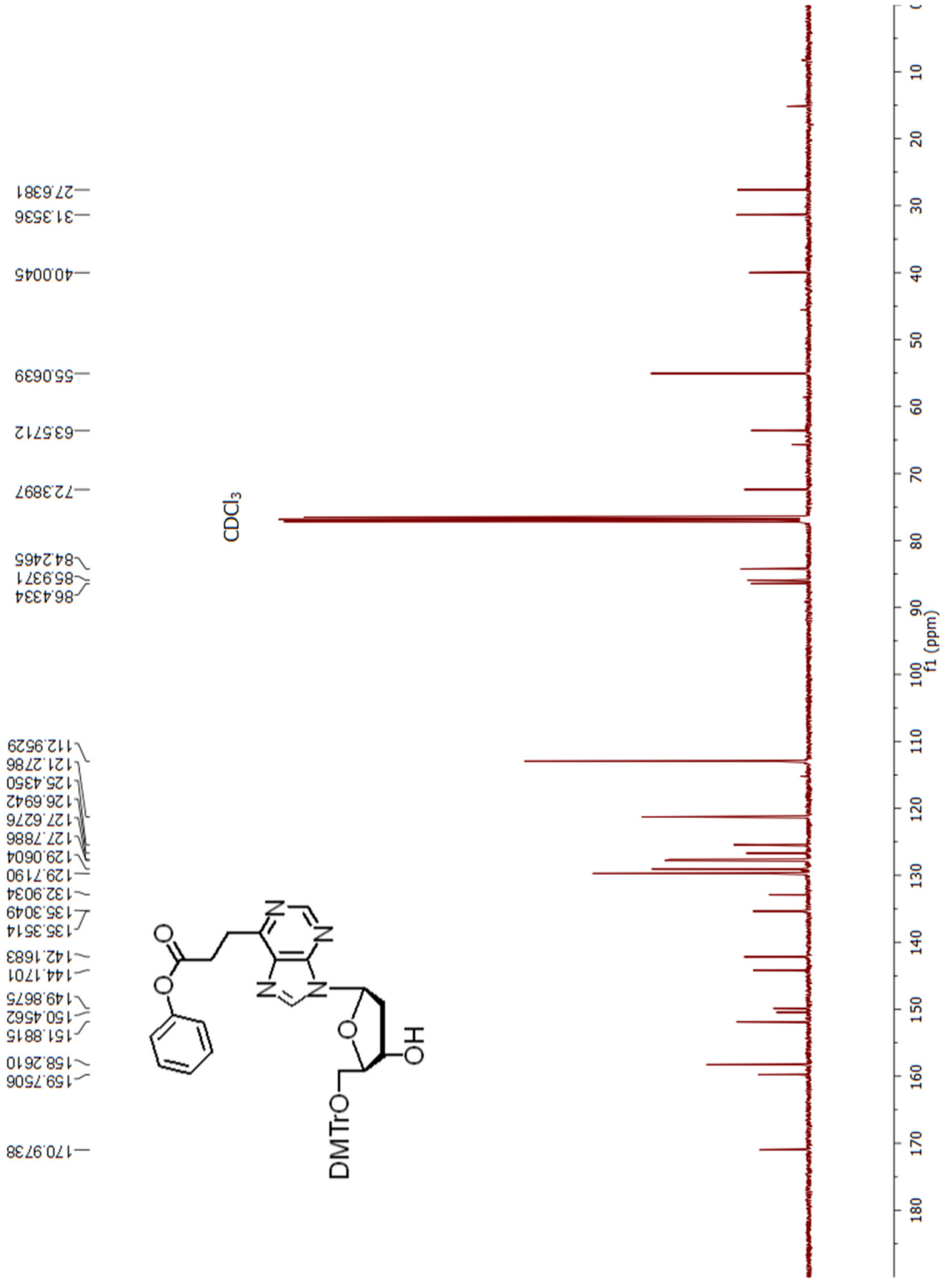
A.1.33 ^{13}C NMR of Compound **42d** in $CDCl_3$, 100 MHz



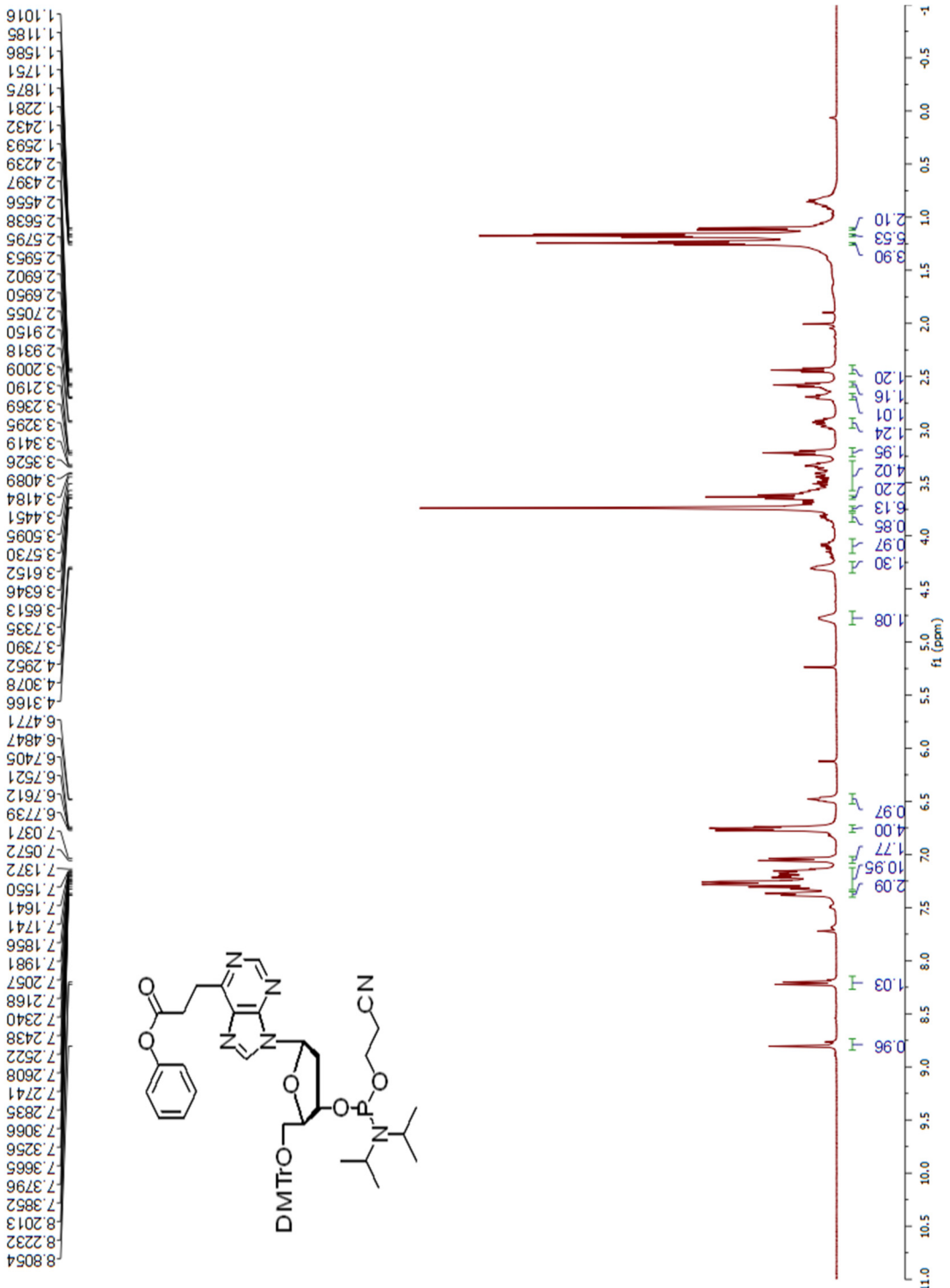
A.1.34 ^{1}H NMR of Compound **43d** in $CDCl_3$, 400 MHz



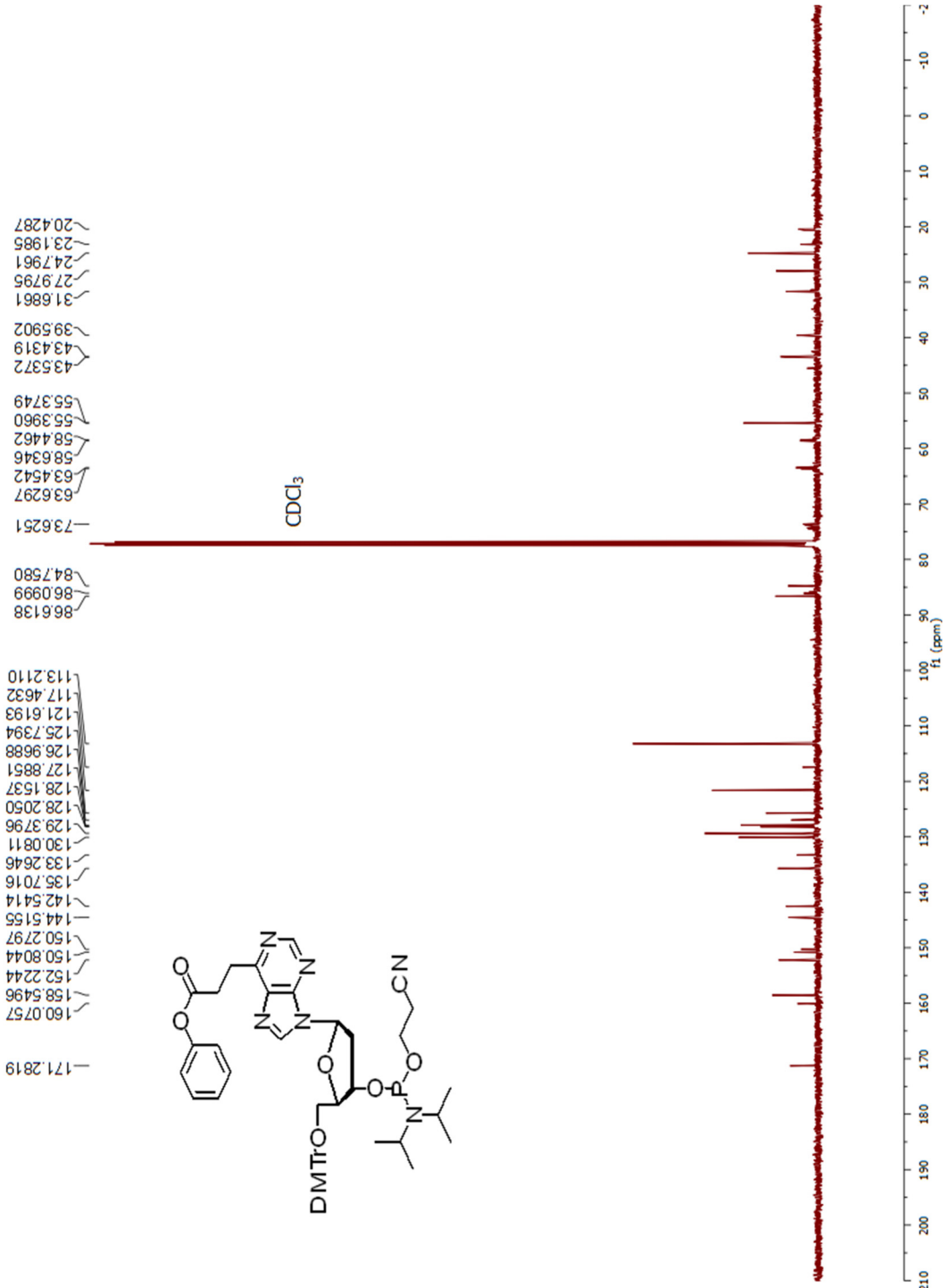
A.1.35 ^{13}C NMR of Compound **43d** in $CDCl_3$, 100 MHz



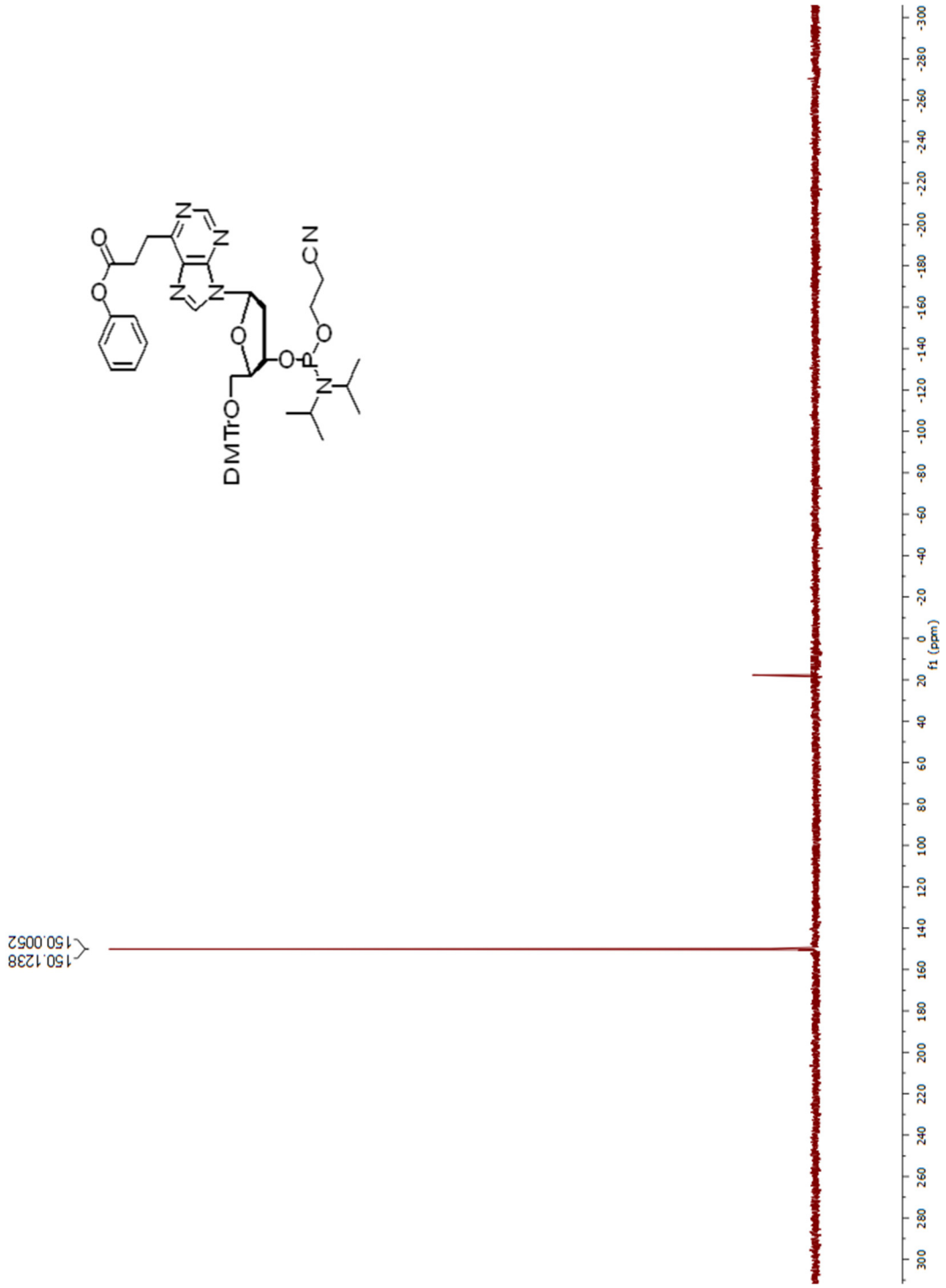
A.1.36 ^{1}H NMR of Compound **4** in $CDCl_3$, 400 MHz



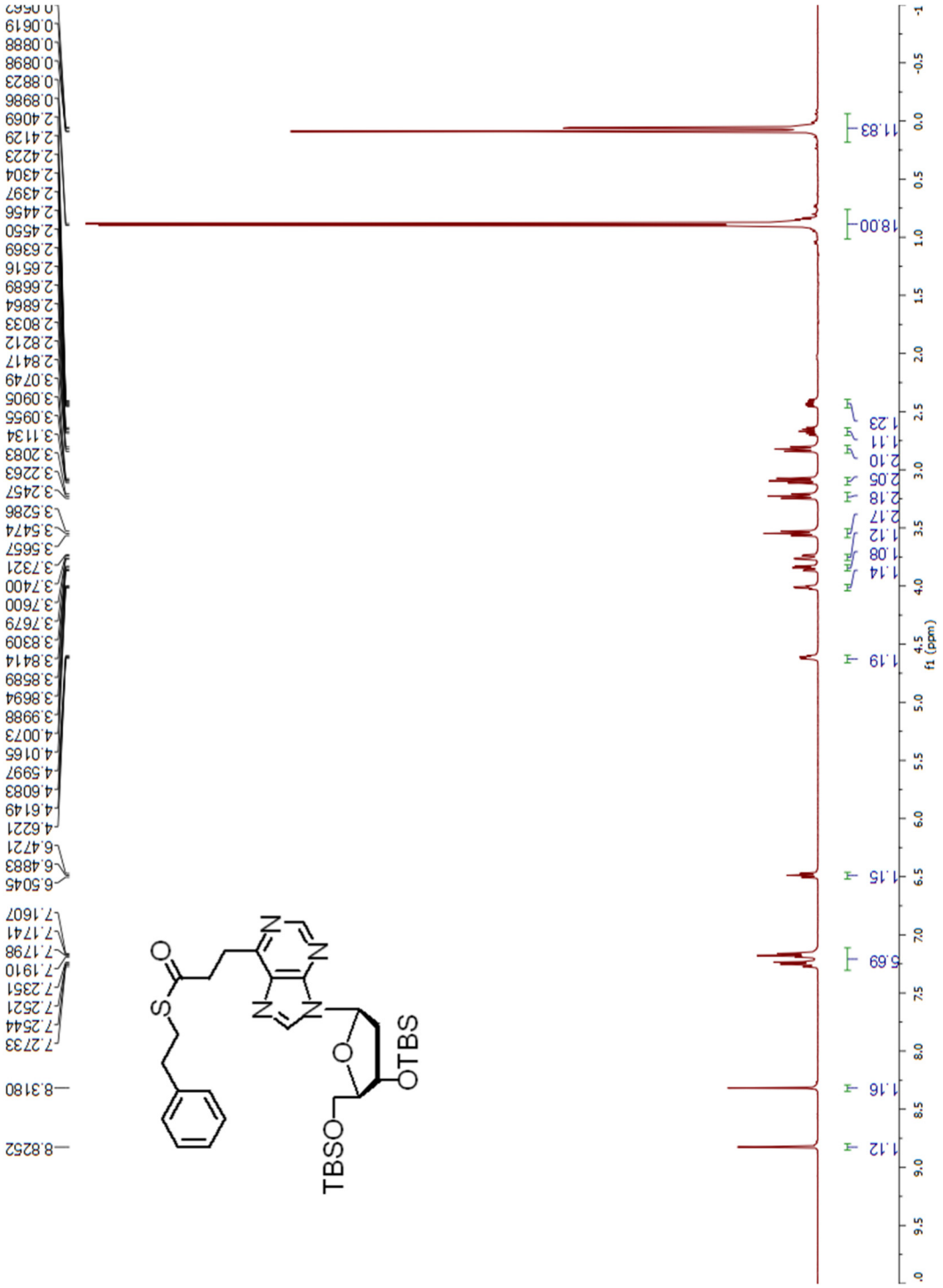
A.1.37 ^{13}C NMR of Compound **4** in $CDCl_3$, 100 MHz



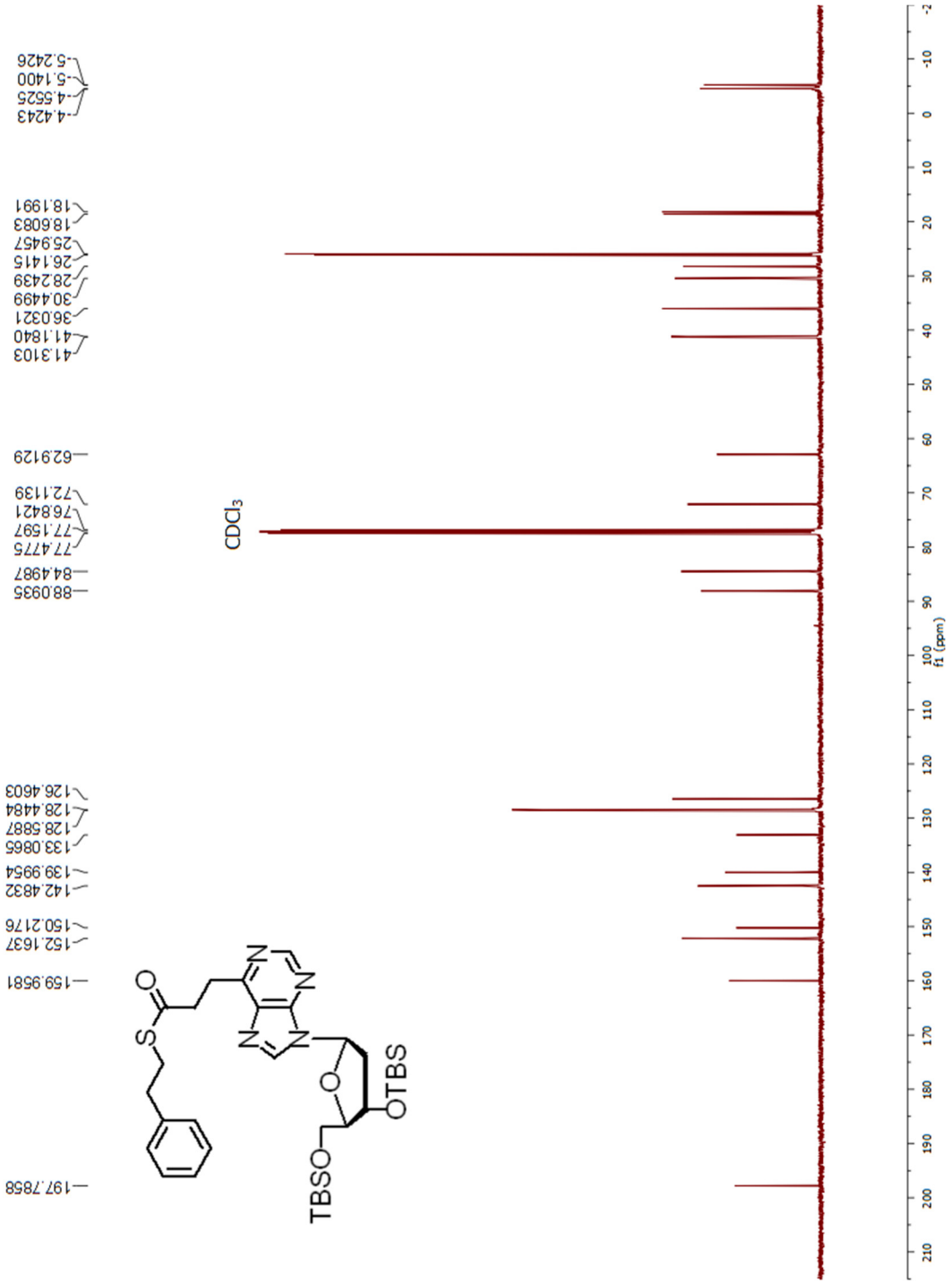
A.1.38 ^{31}P NMR of Compound **4** in $CDCl_3$, 162 MHz



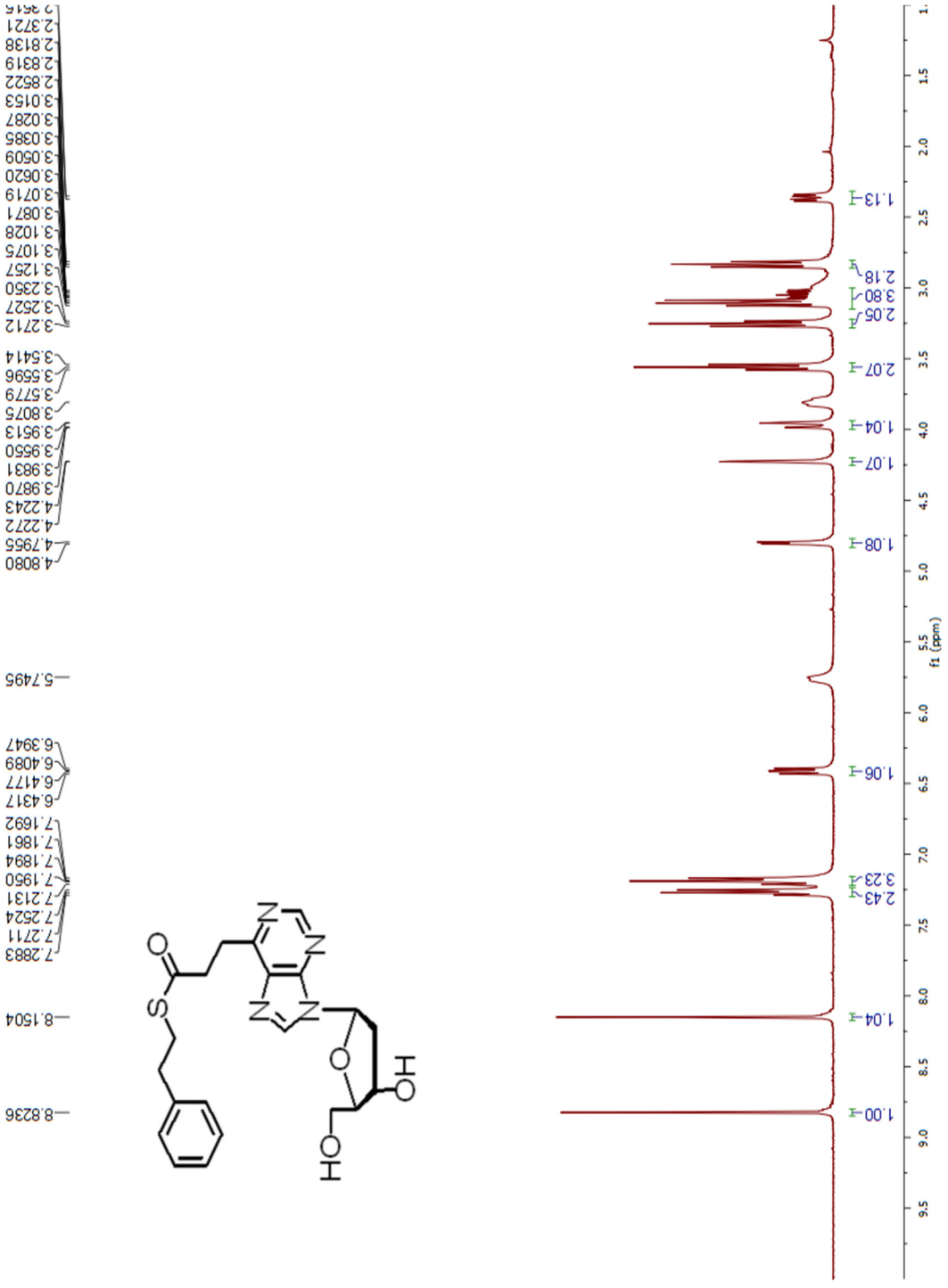
A.1.39 ^{1}H NMR of Compound **41d** in $CDCl_3$, 400 MHz



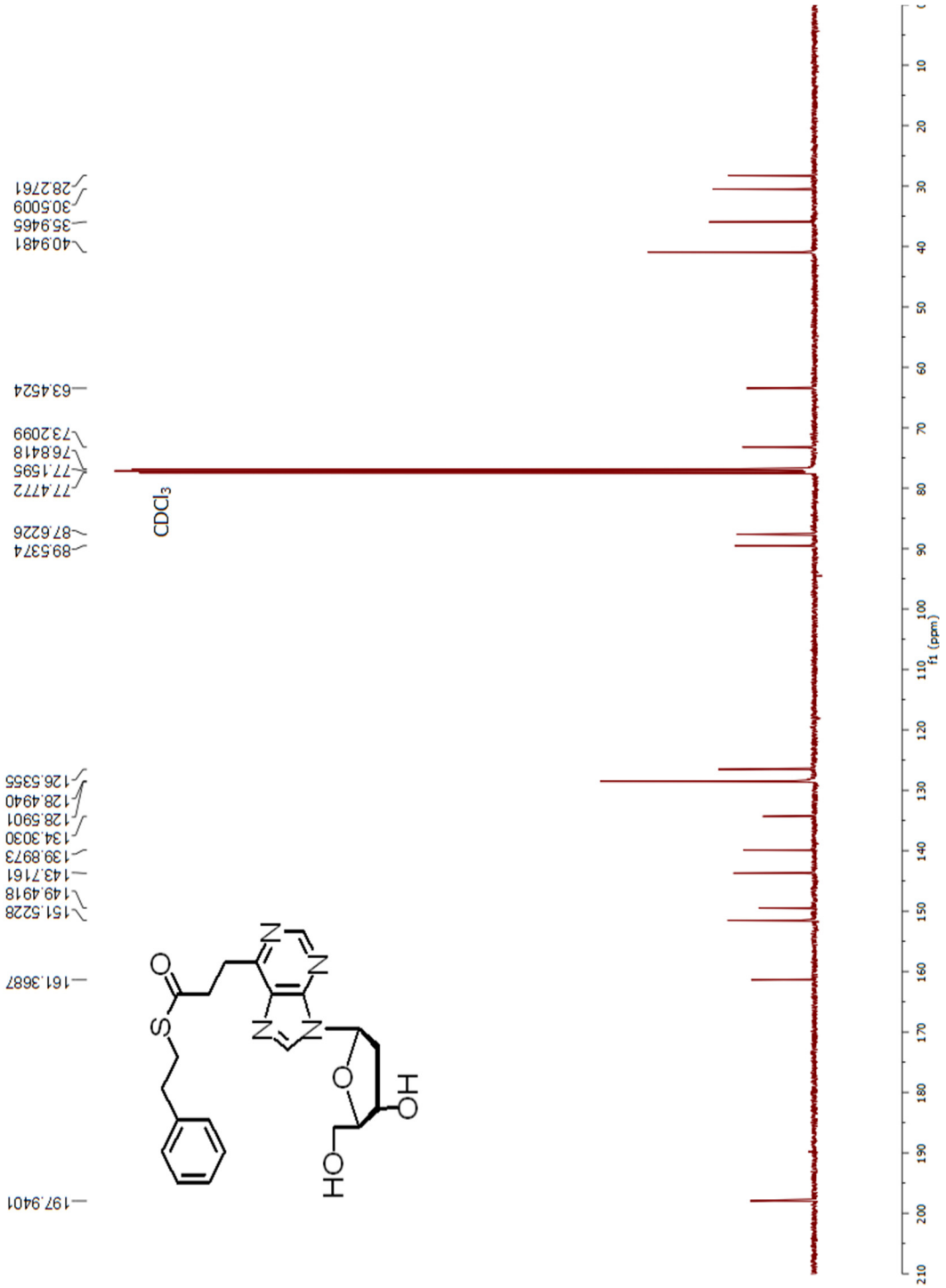
A.1.40 ^{13}C NMR of Compound **41d** in $CDCl_3$, 100 MHz



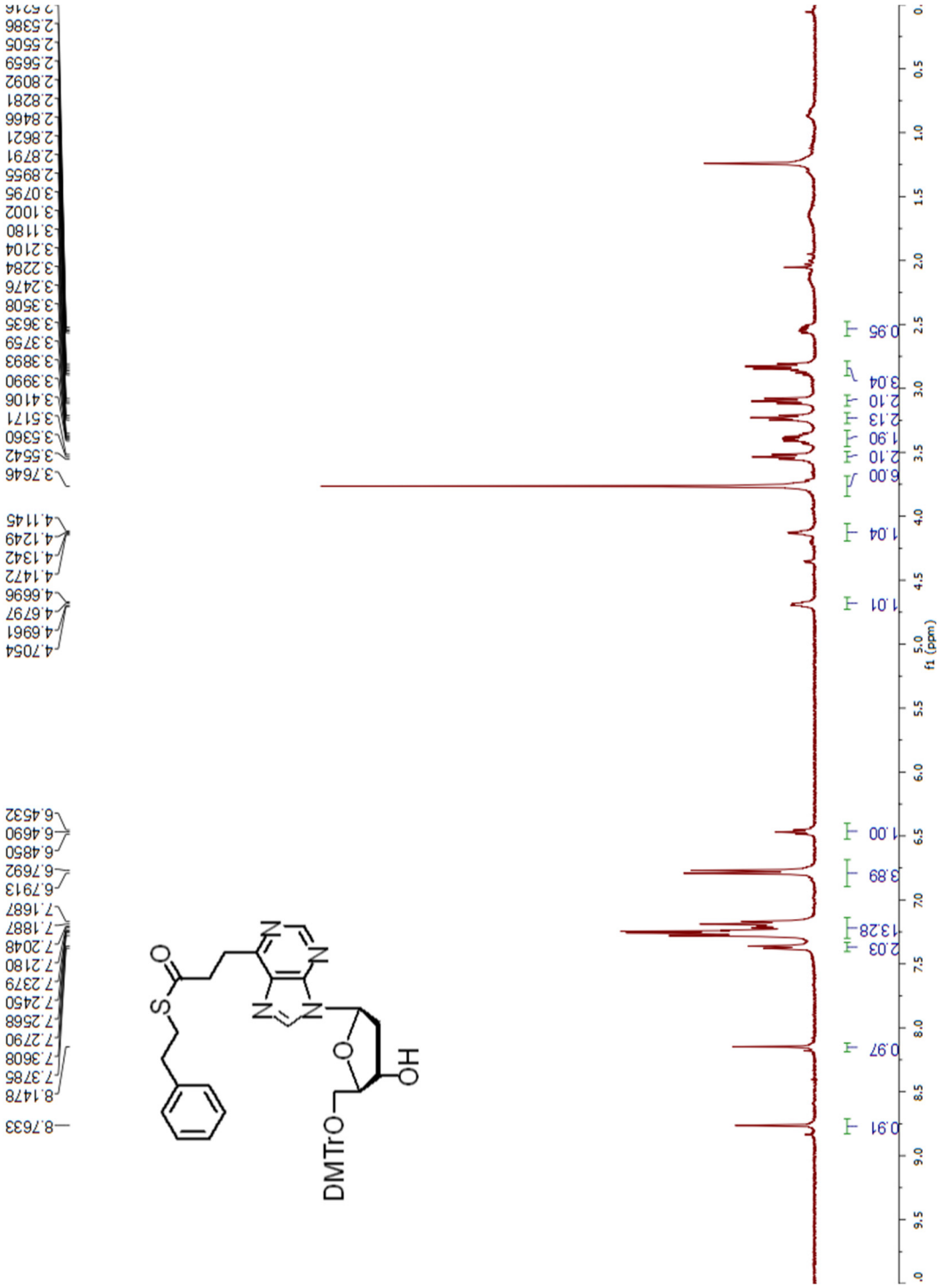
A.1.41 ^{1}H NMR of Compound **42e** in $CDCl_3$, 400 MHz



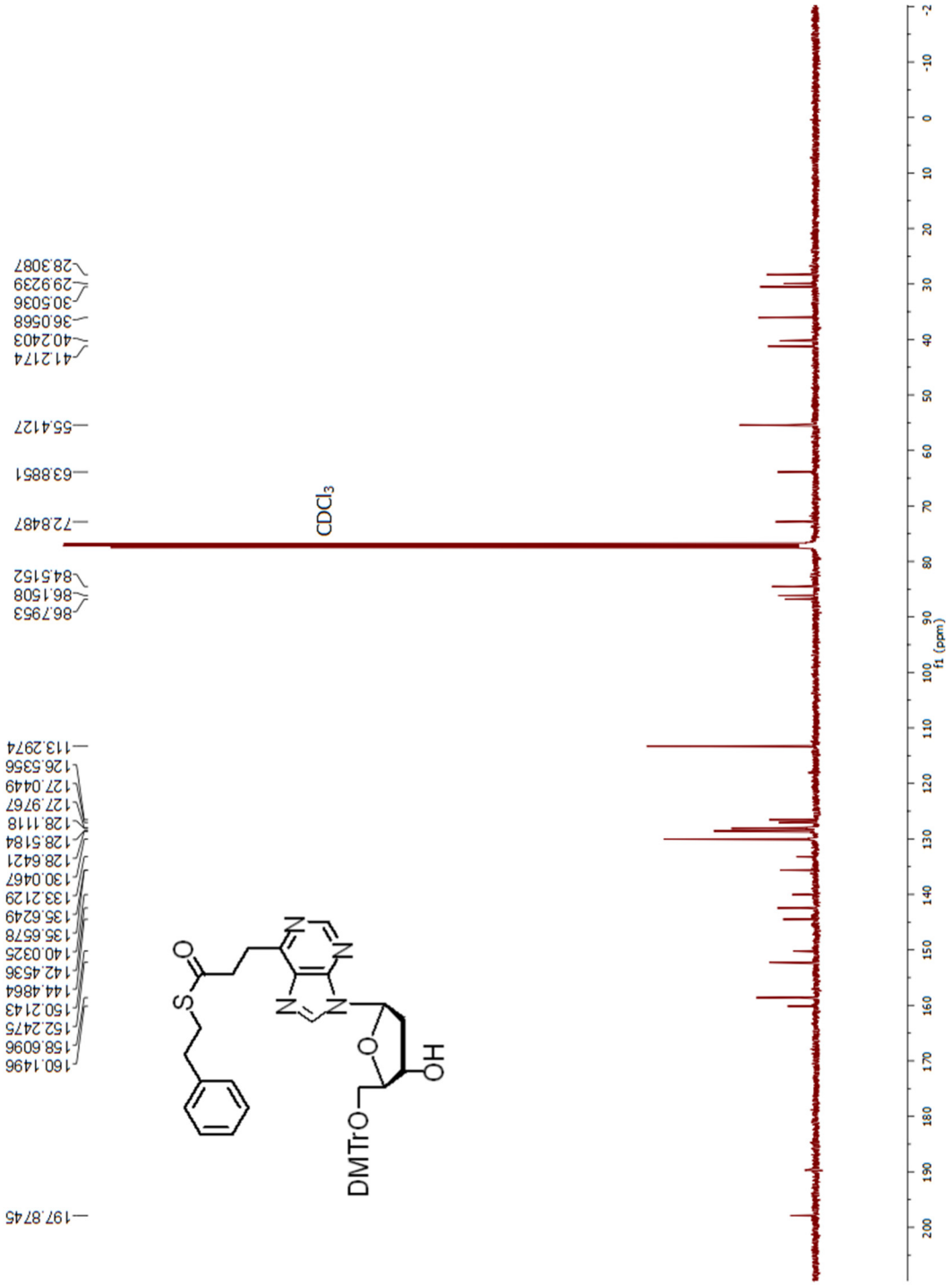
A.1.42 ^{13}C NMR of Compound **42e** in $CDCl_3$, 100 MHz



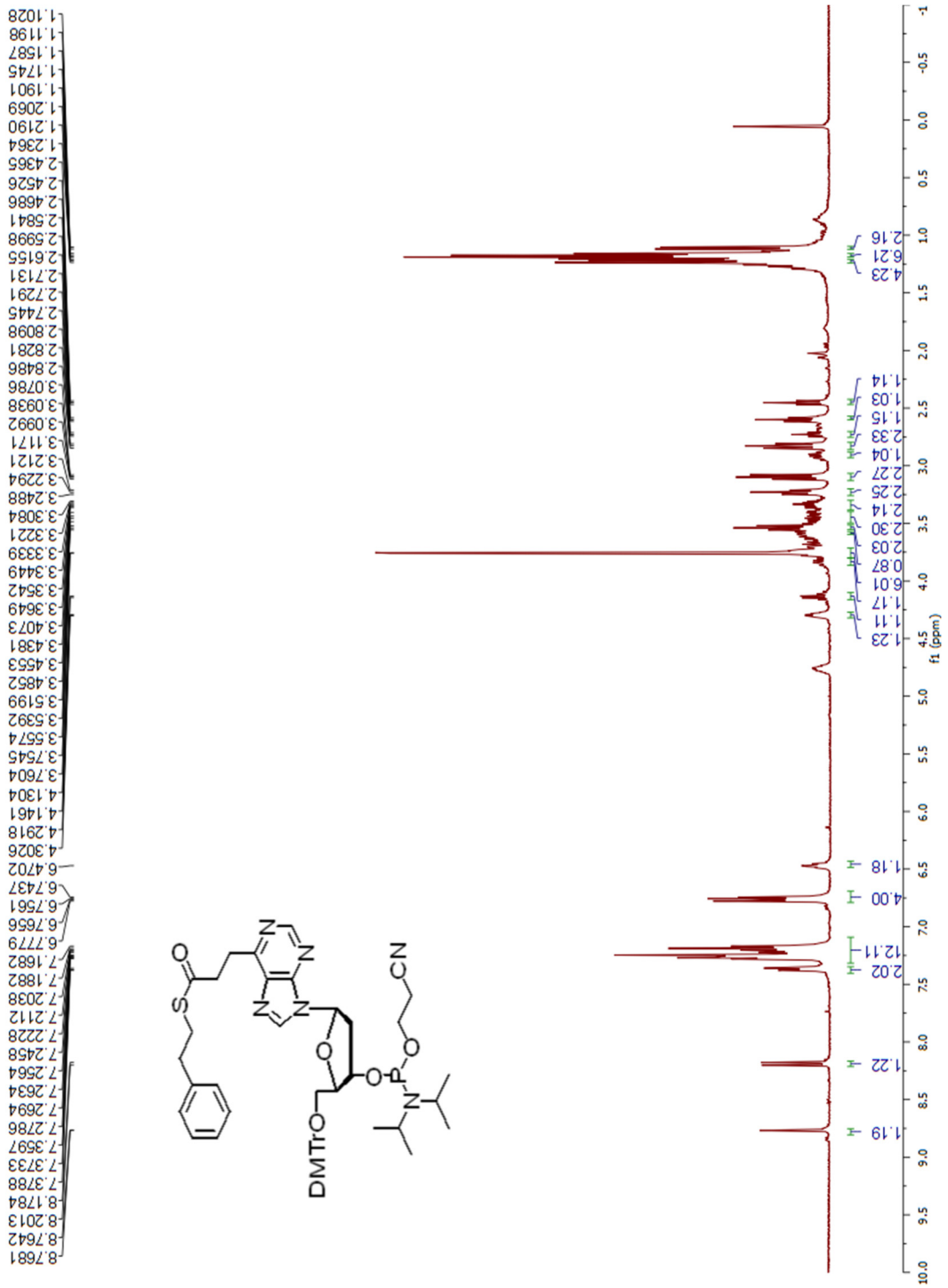
A.1.43 ^{1}H NMR of Compound **43e** in $CDCl_3$, 400 MHz



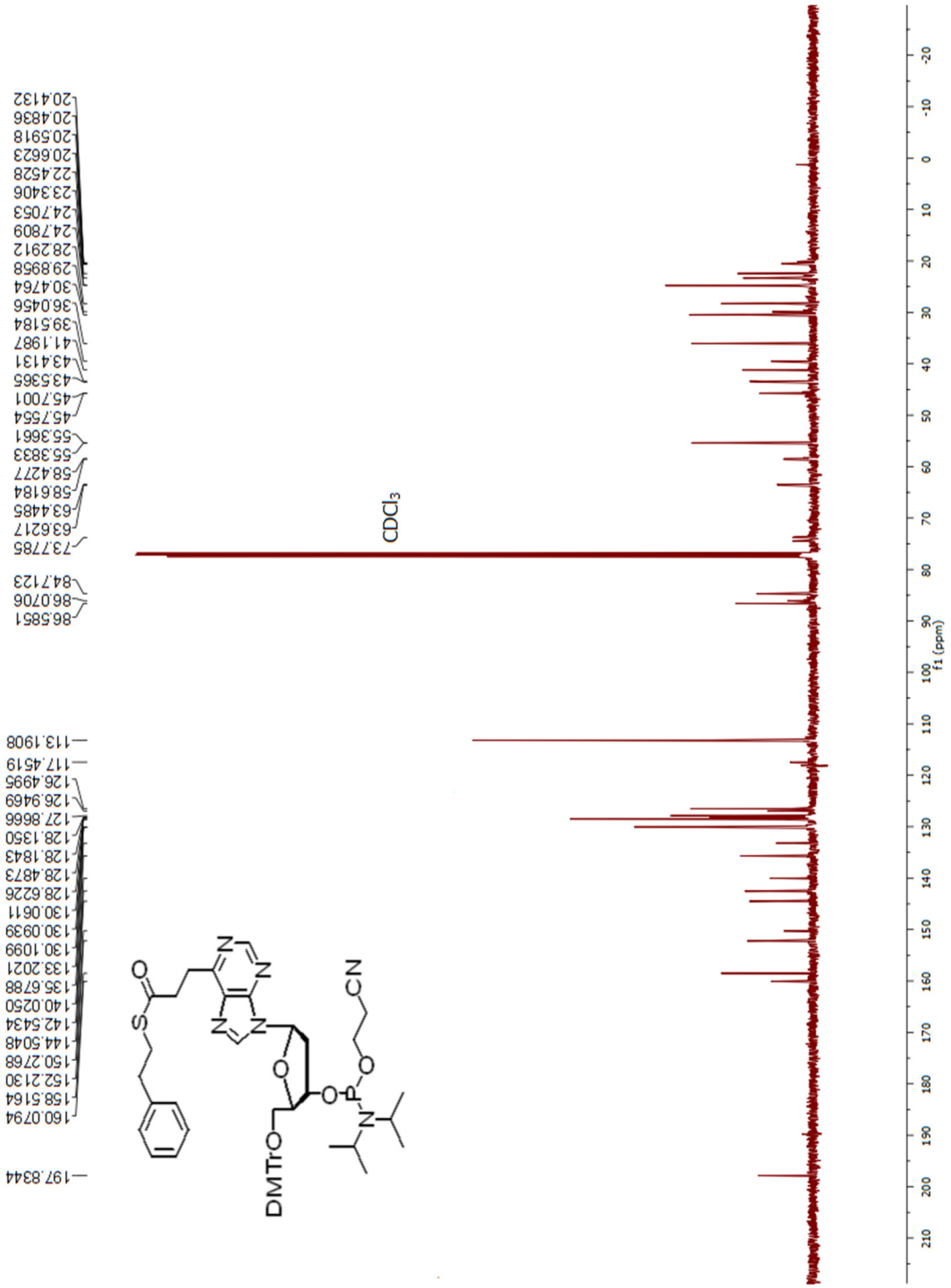
A.1.44 ^{13}C NMR of Compound **43e** in $CDCl_3$, 100 MHz



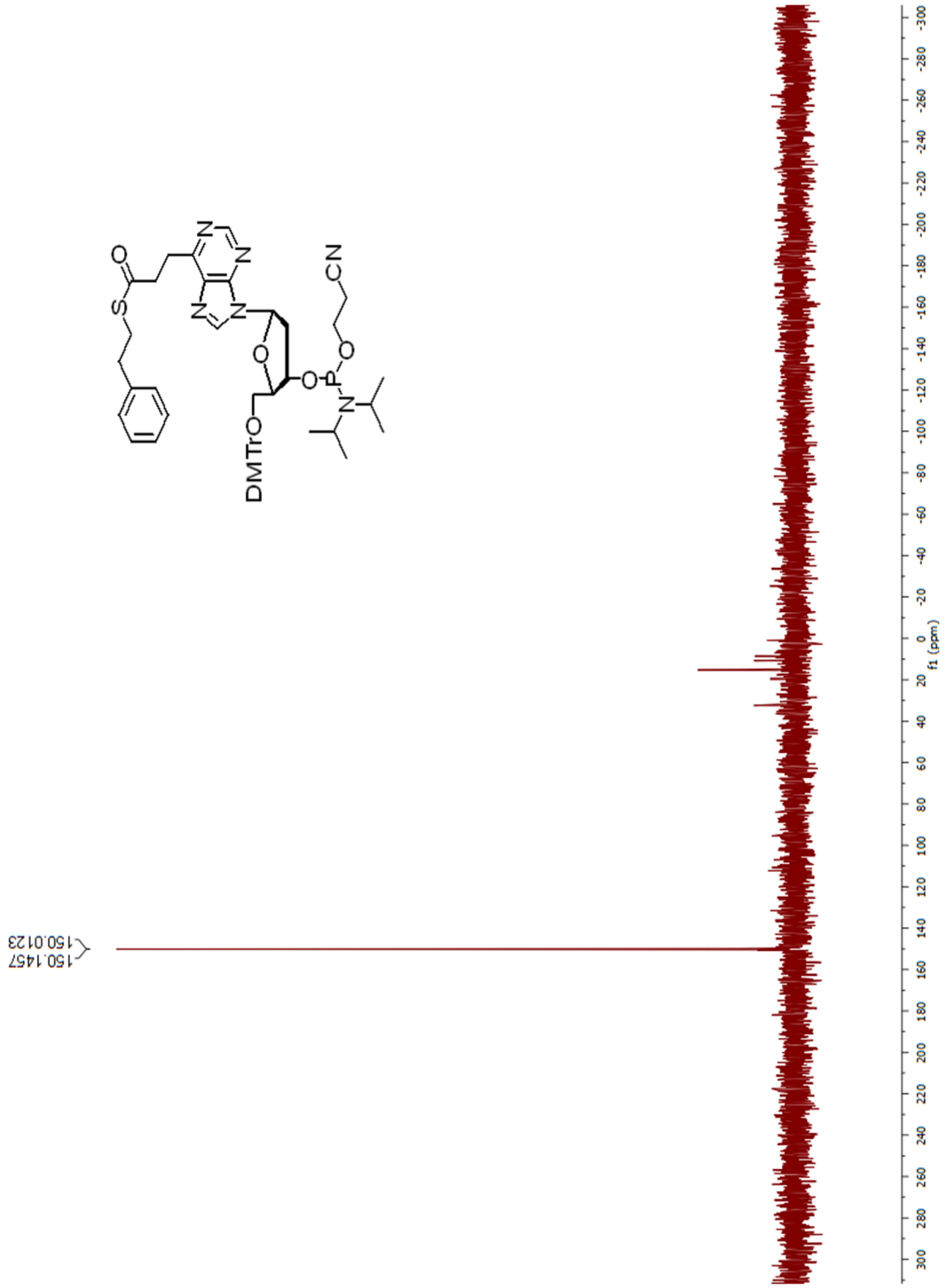
A.1.45 ^{1}H NMR of Compound **5** in $CDCl_3$, 400 MHz



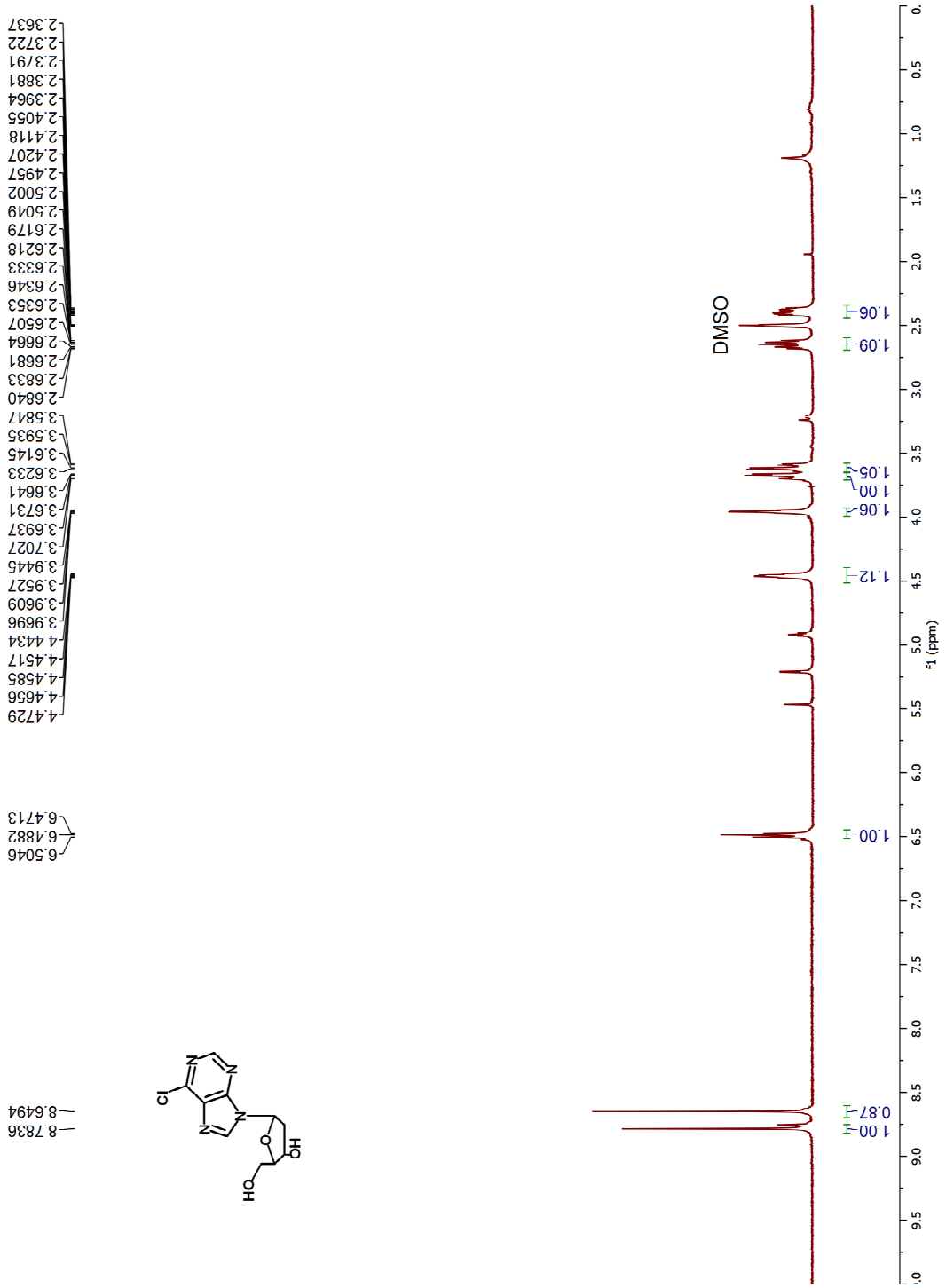
A.1.46 ^{13}C NMR of Compound **5** in $CDCl_3$, 100 MHz



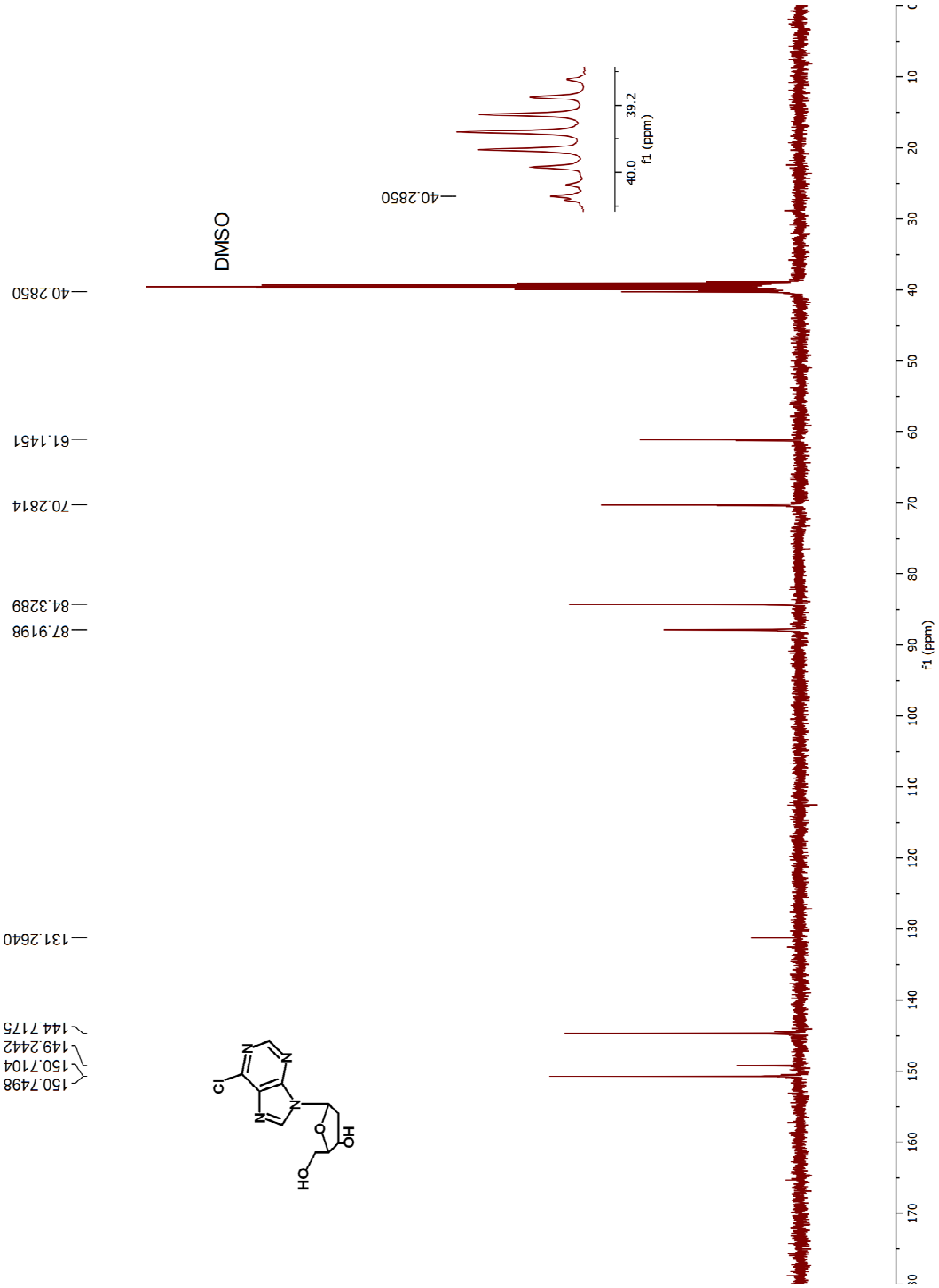
A.1.47 ^{31}P NMR of Compound **5** in $CDCl_3$, 162 MHz



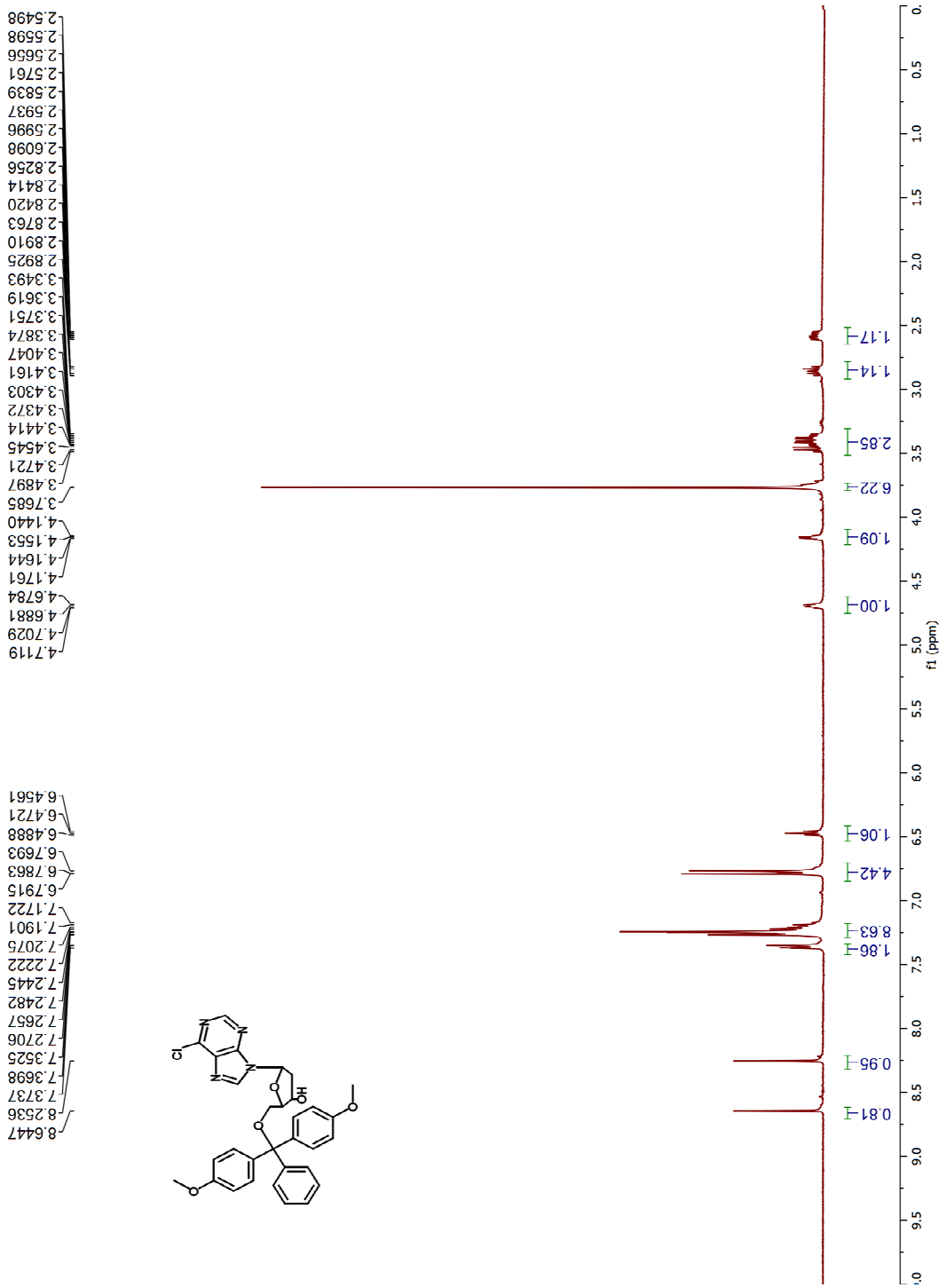
A.1.48 ^{1}H NMR of Compound **59** in DMSO, 400 MHz



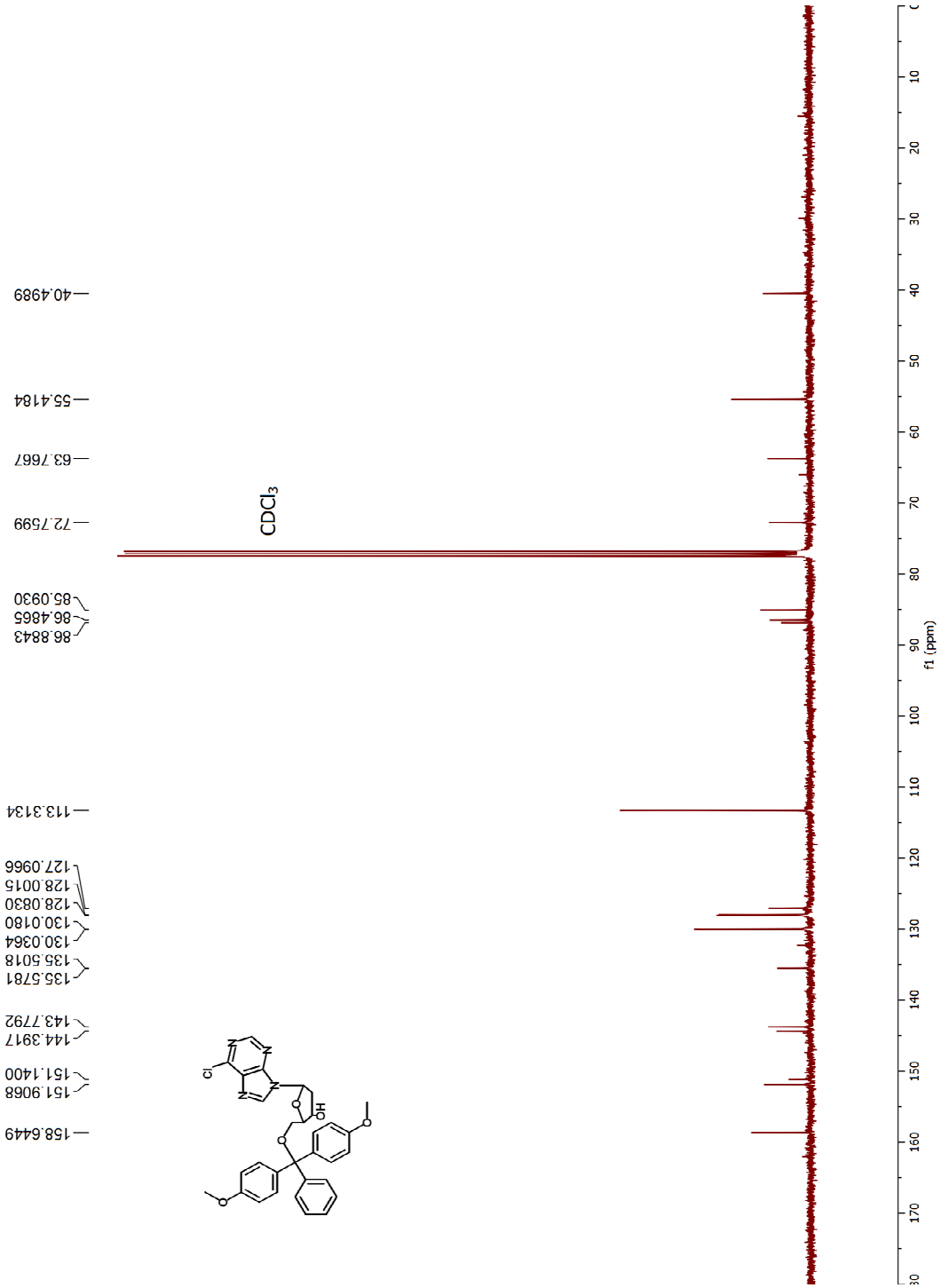
A.1.49 ^{13}C NMR of Compound **59** in DMSO, 100 MHz



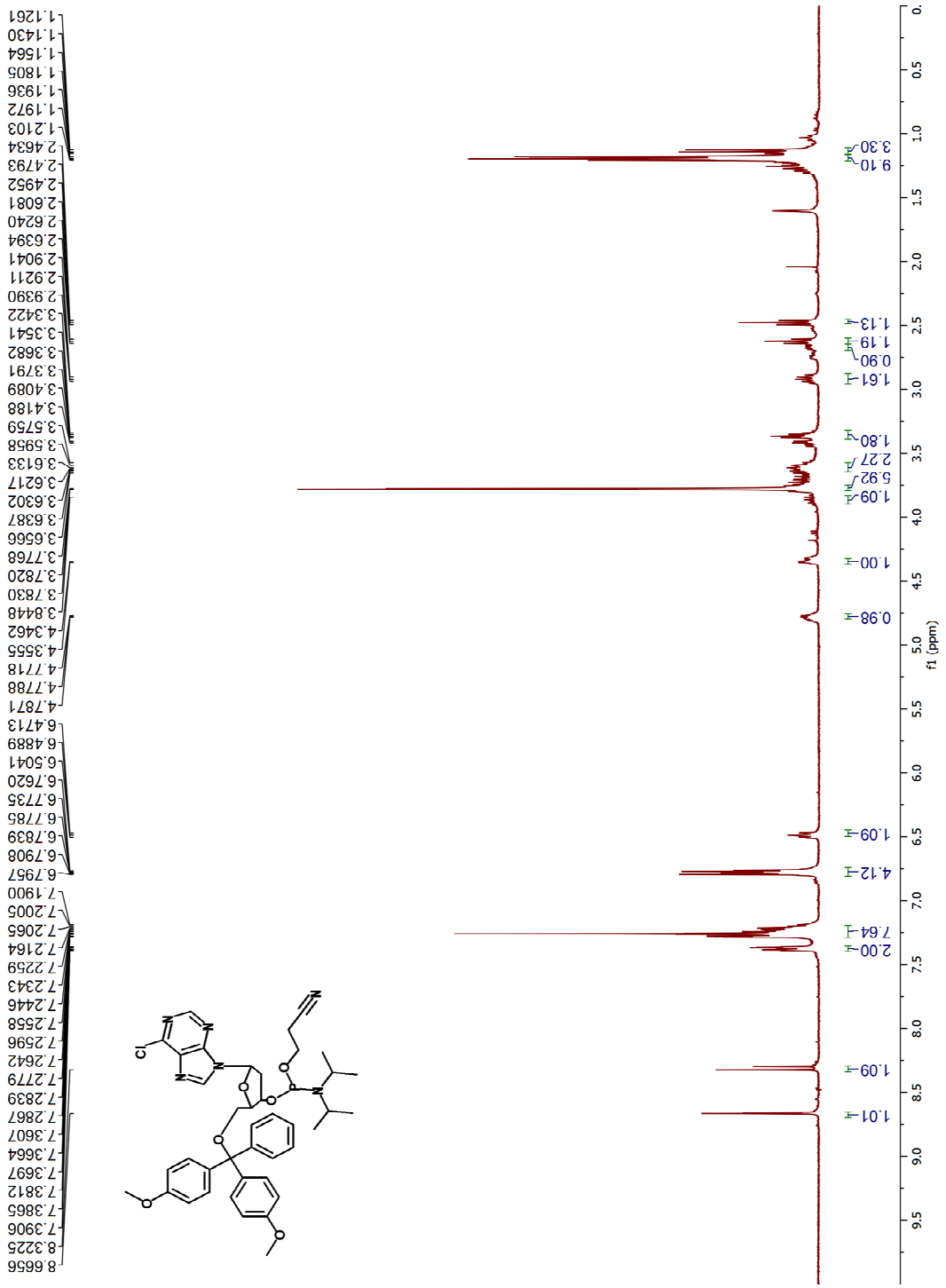
A.1.50 ^{1}H NMR of Compound **60** in $CDCl_3$, 400 MHz



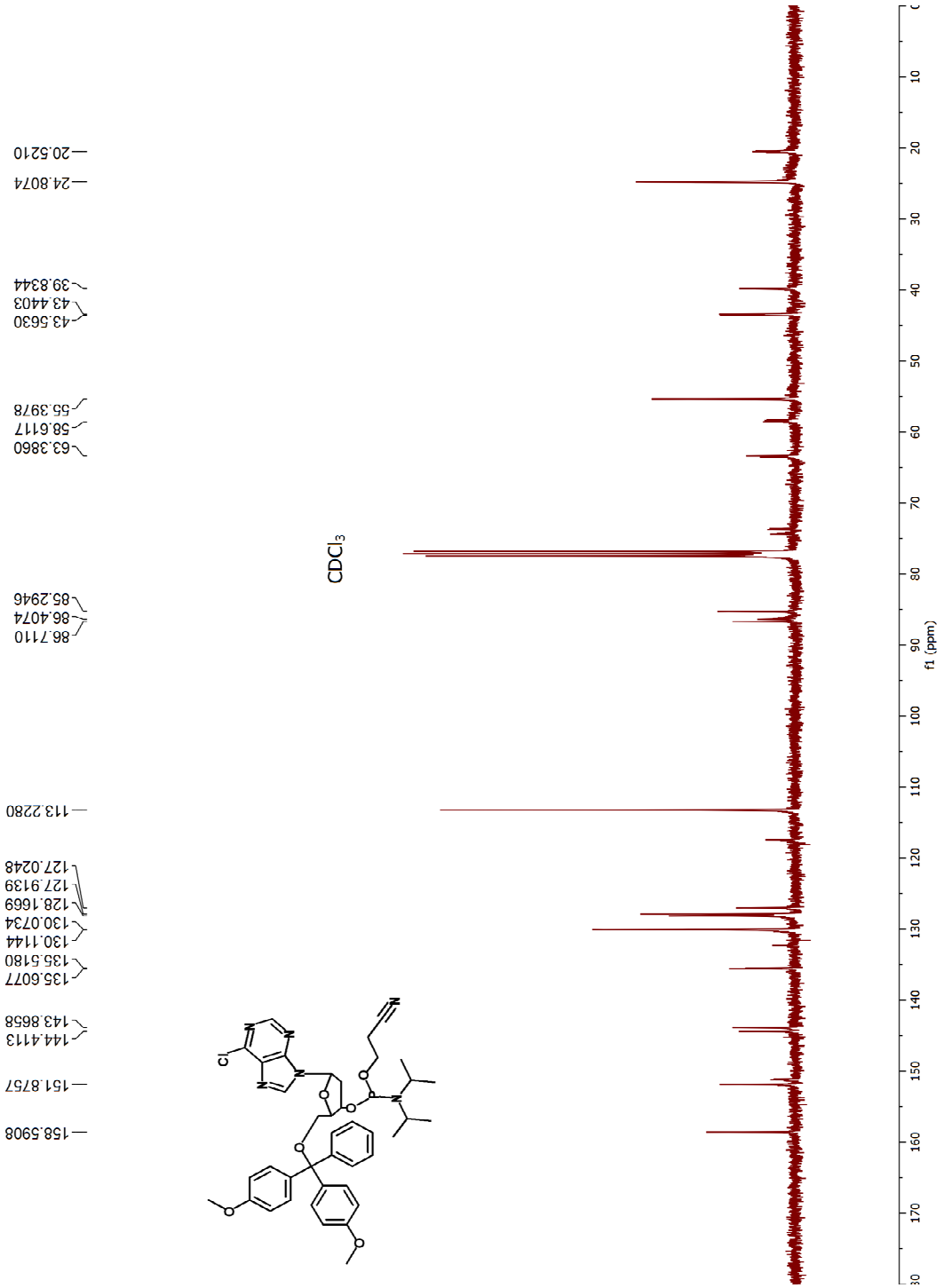
A.1.51 ^{13}C NMR of Compound **60** in $CDCl_3$, 100 MHz



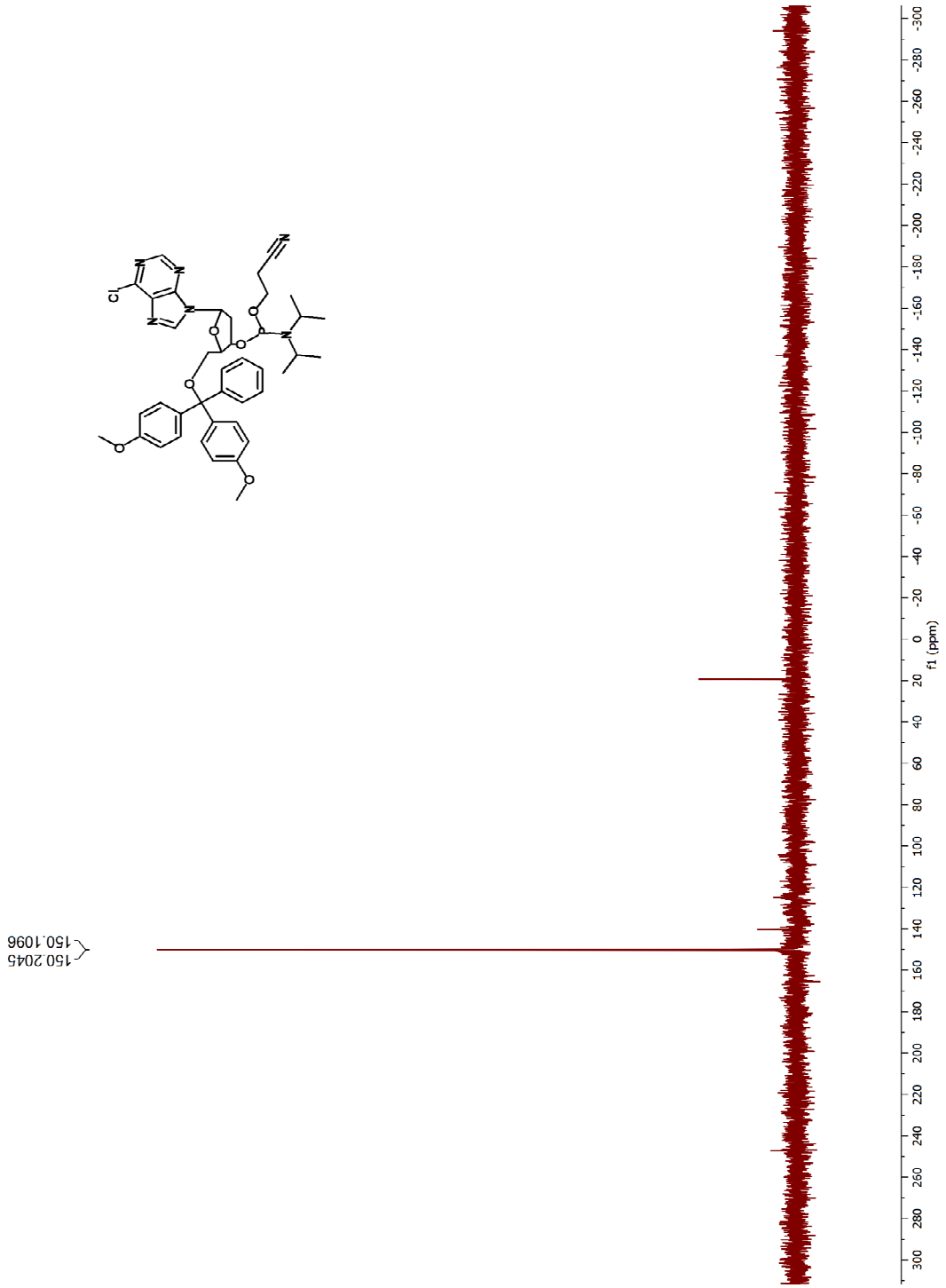
A.1.52 ^{1}H NMR of Compound **6** in $CDCl_3$, 400 MHz



A.1.53 ^{13}C NMR of Compound **6** in $CDCl_3$, 100 MHz

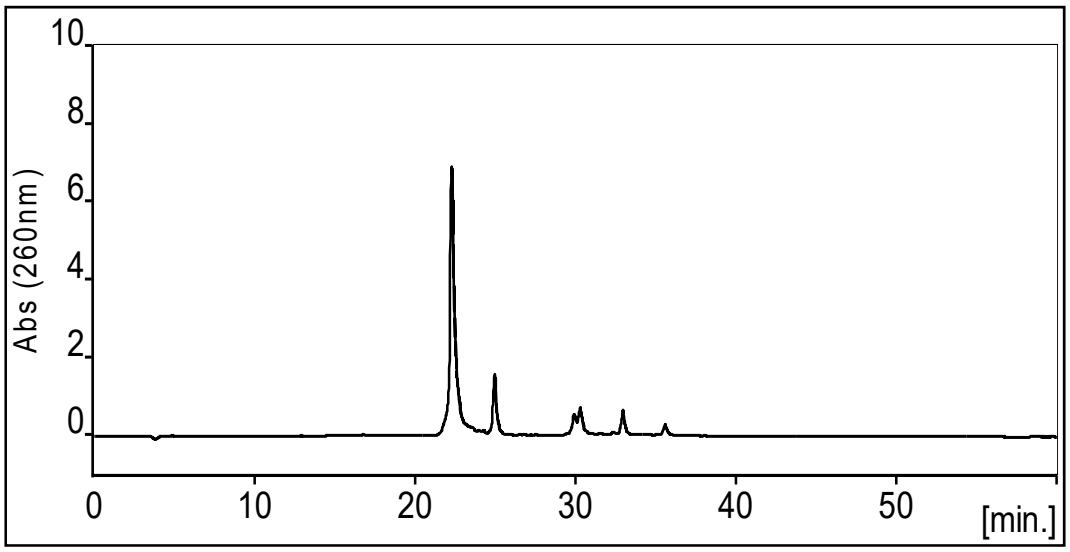


A.1.54 ^{31}P NMR of Compound **6** in $CDCl_3$, 162 MHz

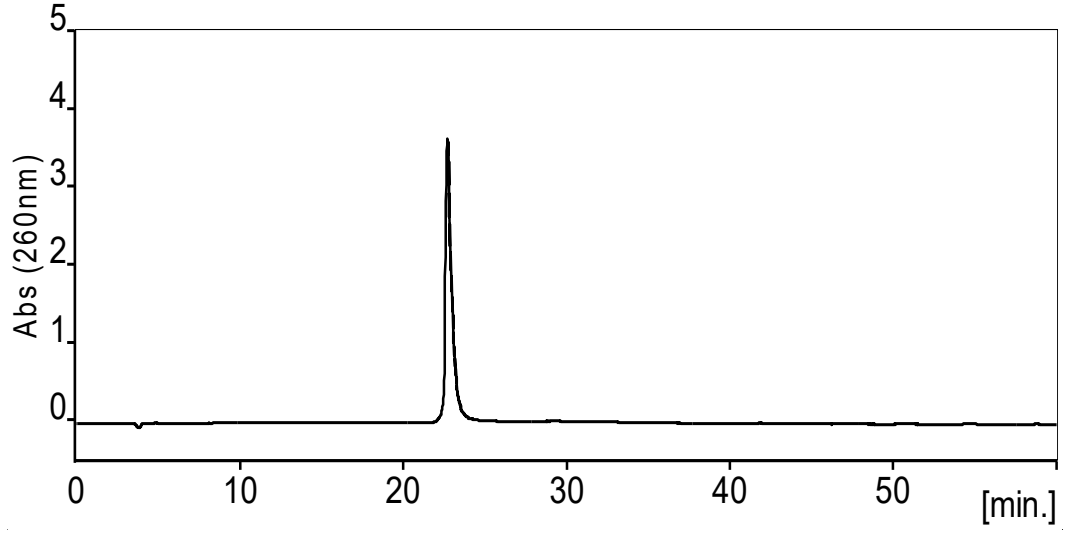


A.2 HPLC profiles of oligonudeoxynucleotides for chapter 3

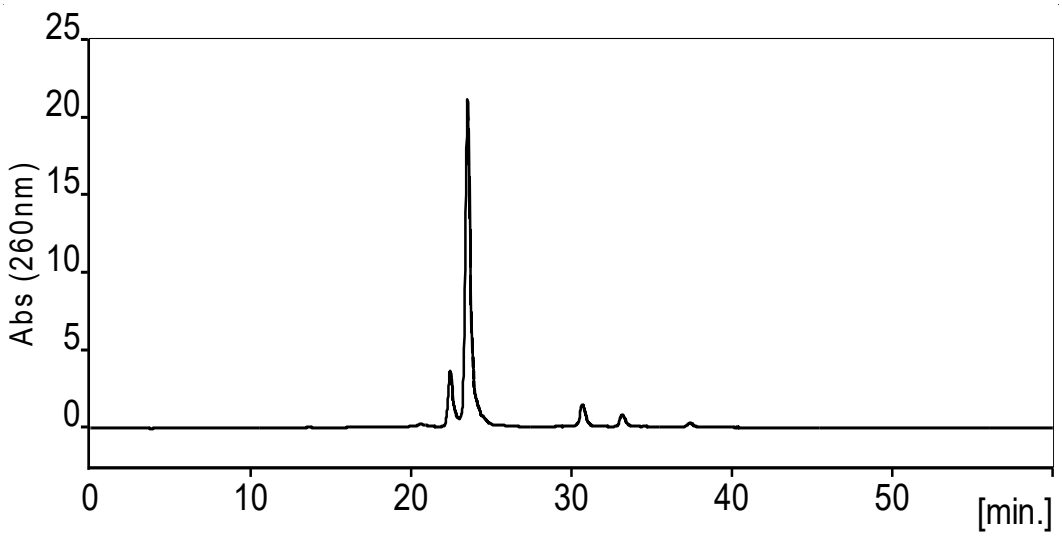
A.2.1 Crude oligodeoxynucleotide **Strand-1**



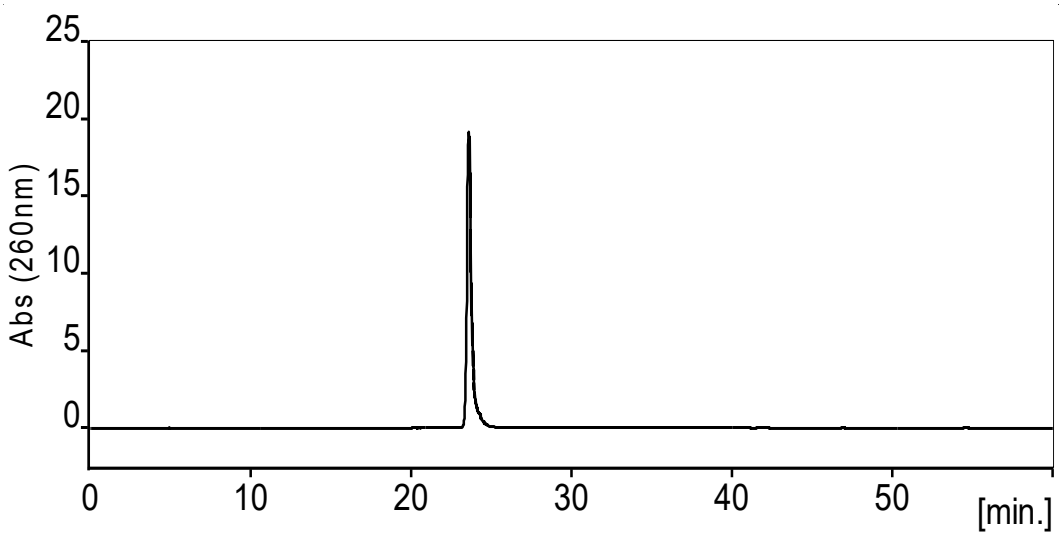
A.2.2 Pure oligodeoxynucleotide **Strand-1**



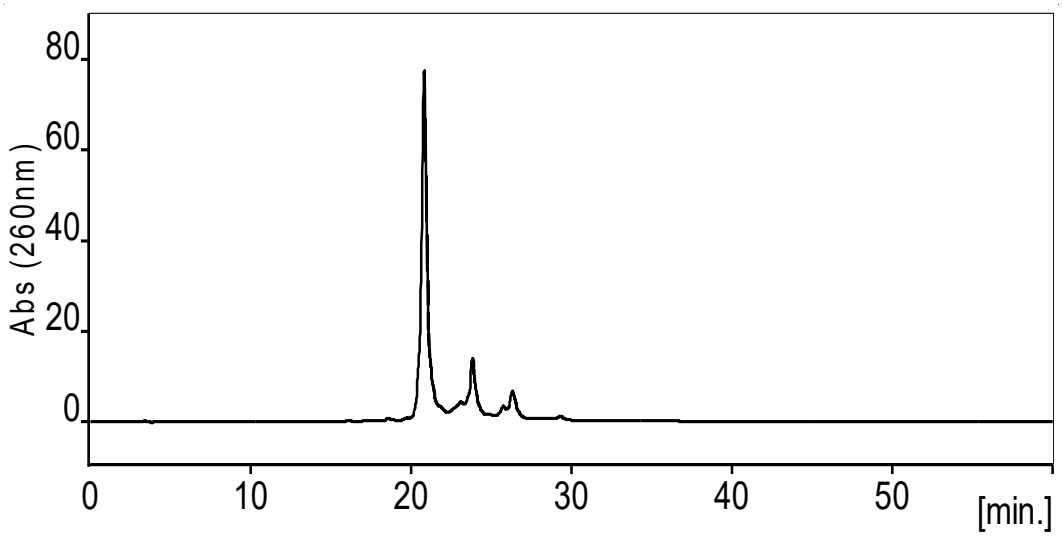
A.2.3 Crude oligodeoxynucleotide **Strand-2**



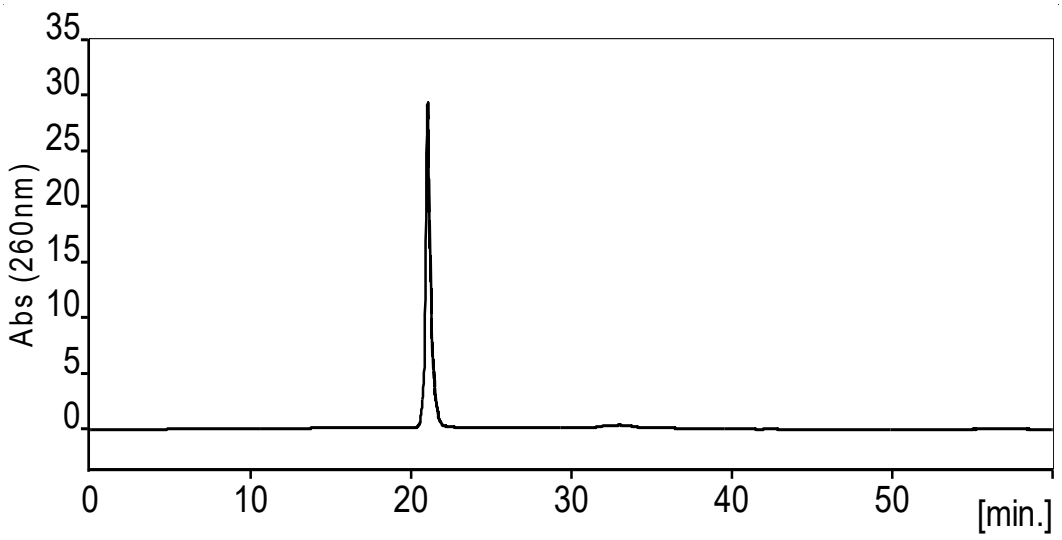
A.2.4 Pure oligodeoxynucleotide **Strand-2**



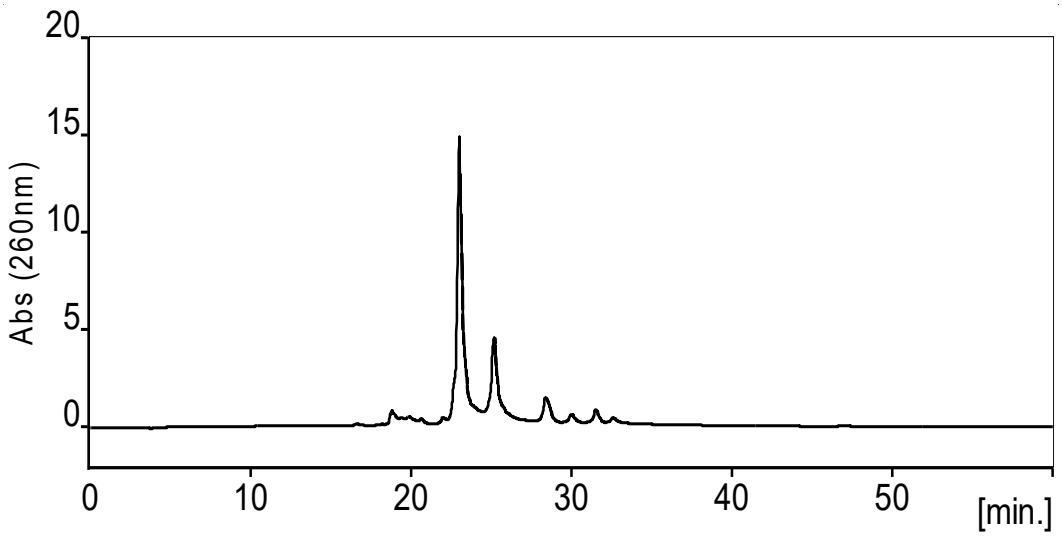
A.2.5 Crude oligodeoxynucleotide **Strand-3**



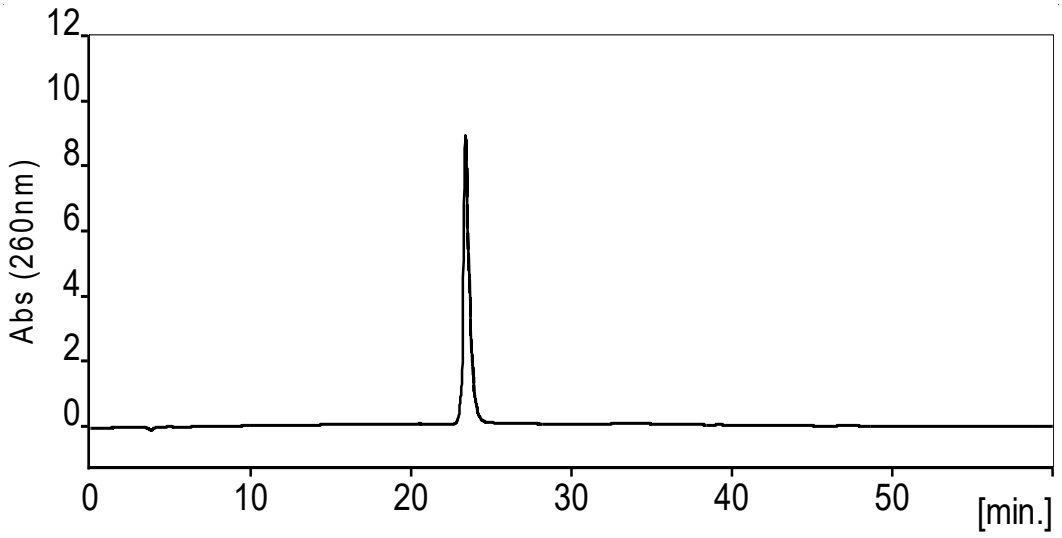
A.2.6 Pure oligodeoxynucleotide **Strand-3**



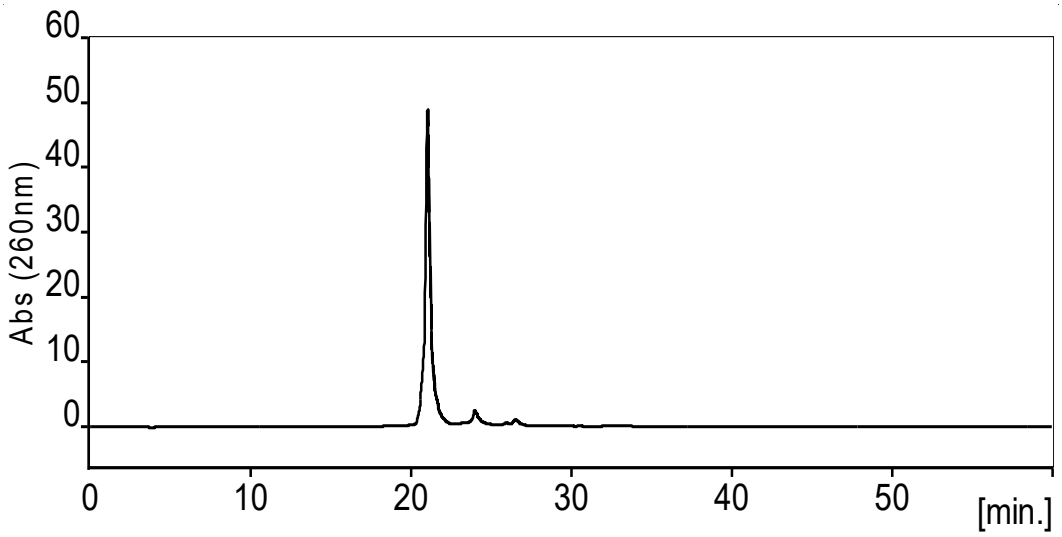
A.2.7 Crude oligodeoxynucleotide **Strand-4**



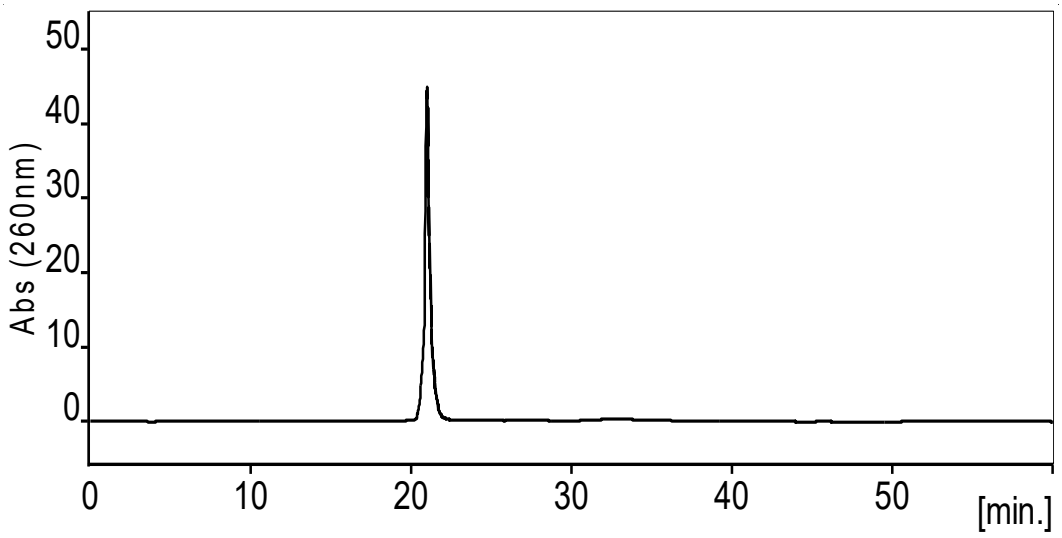
A.2.8 Pure oligodeoxynucleotide **Strand-4**



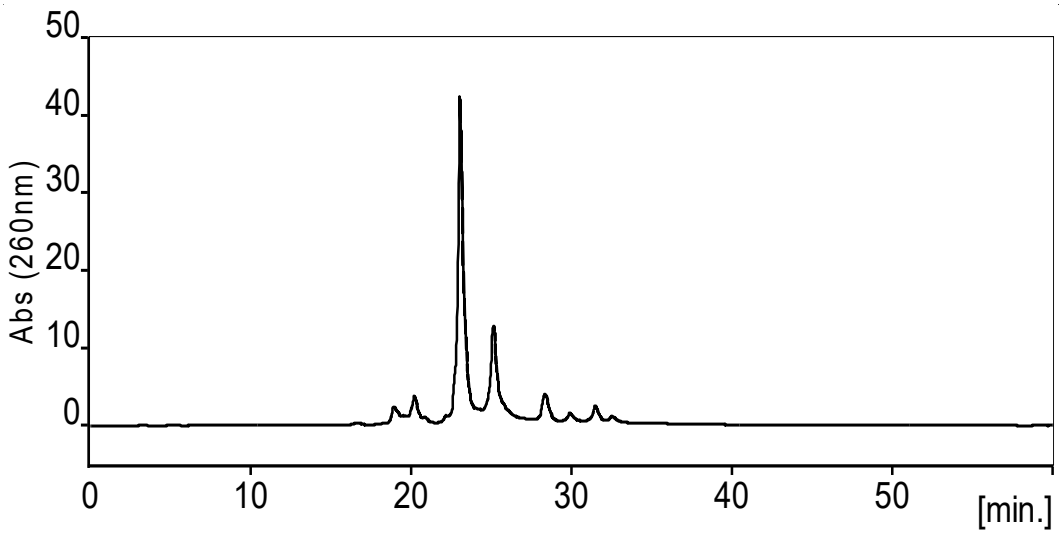
A.2.9 Crude oligodeoxynucleotide **Strand-5**



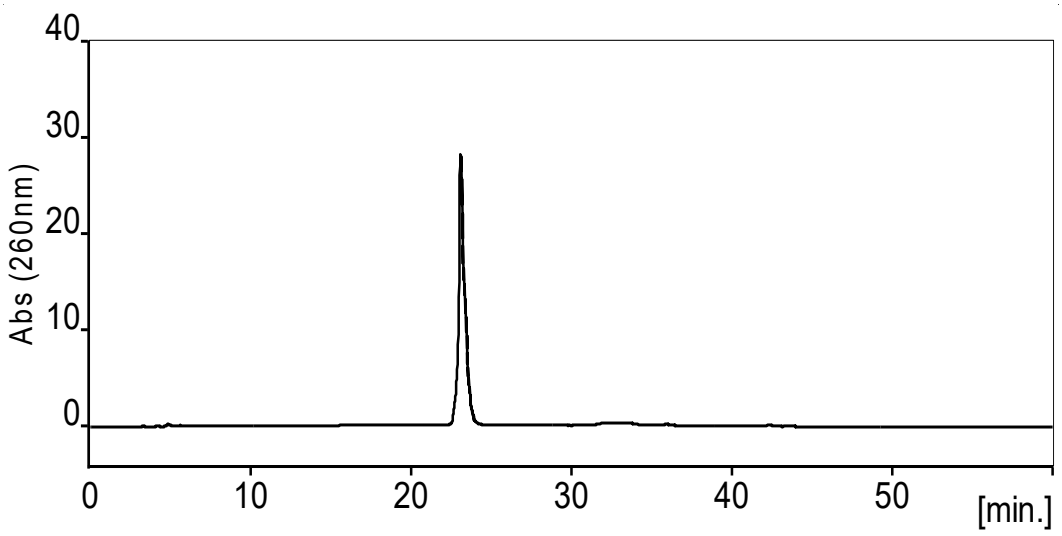
A.2.10 Pure oligodeoxynucleotide **Strand-5**



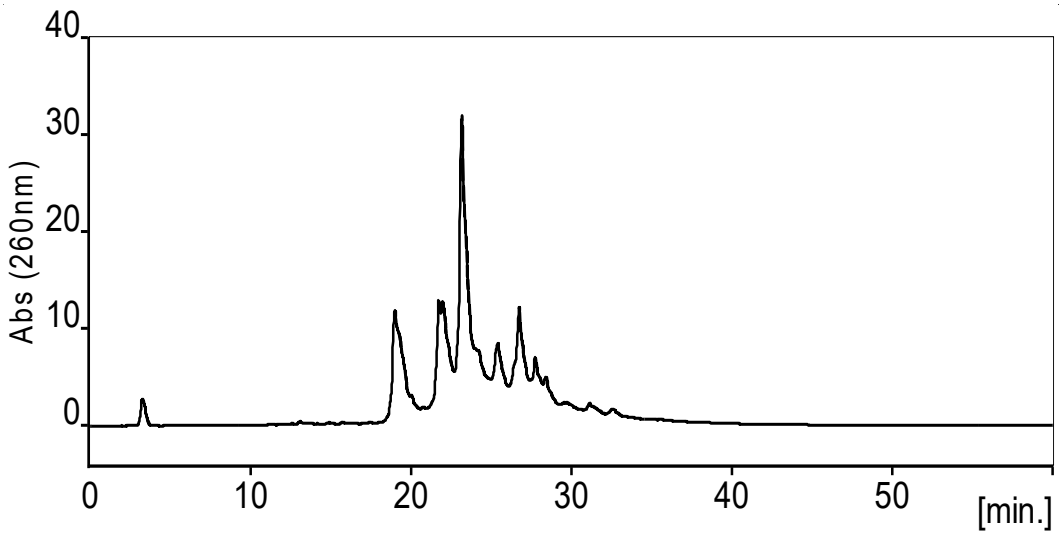
A.2.11 Crude oligodeoxynucleotide **Strand-6**



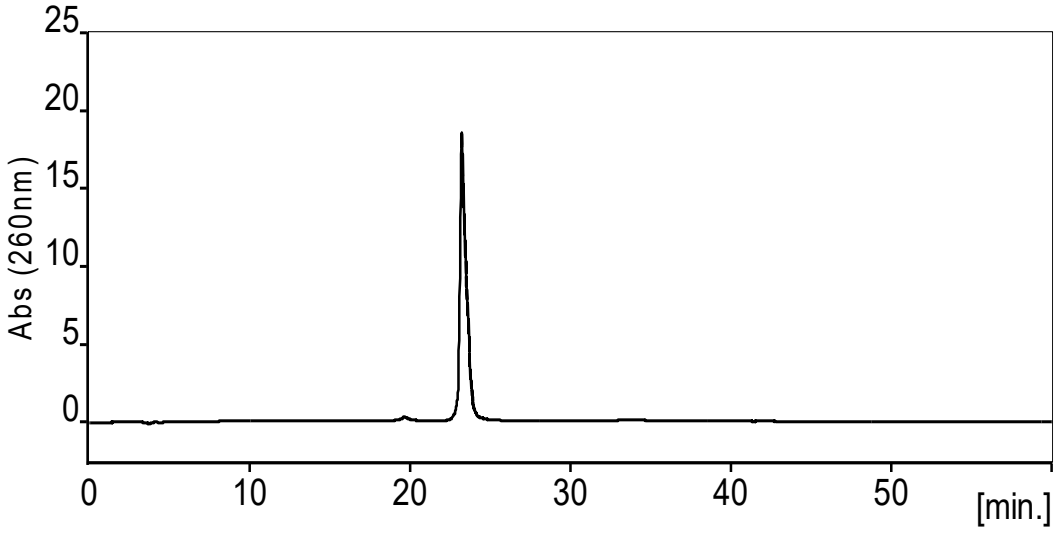
A.2.12 Pure oligodeoxynucleotide **Strand-6**



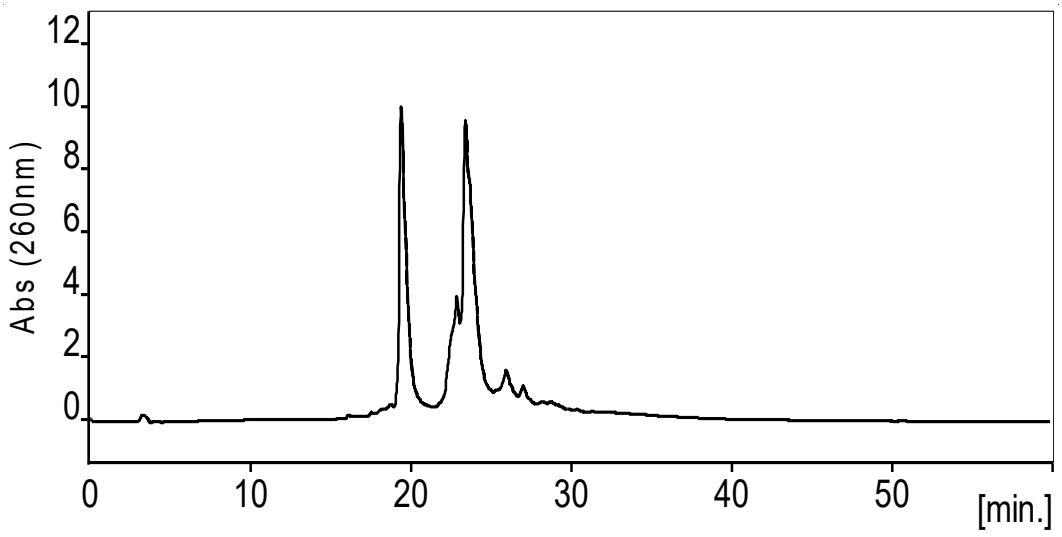
A.2.13 Crude oligodeoxynucleotide **Strand-7**



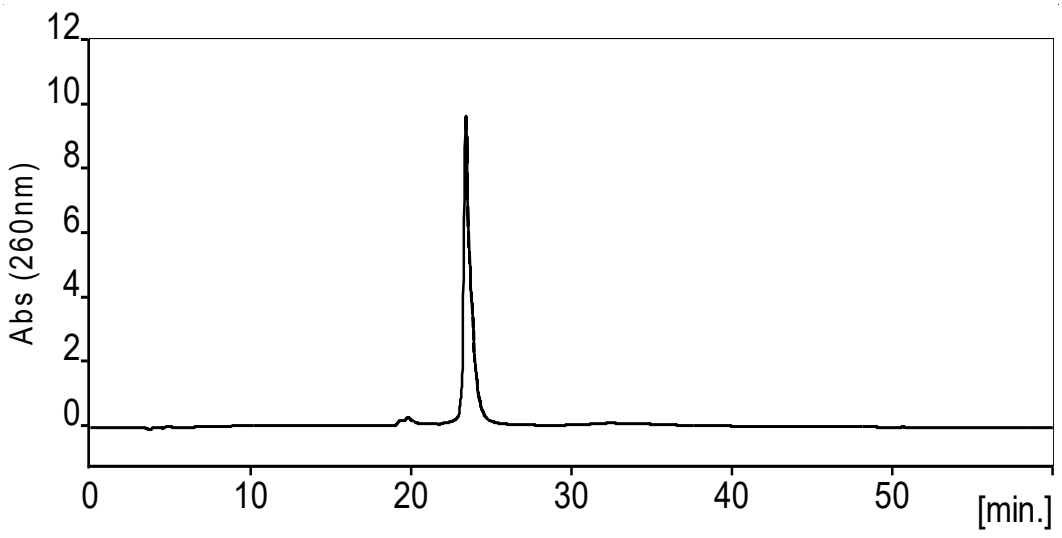
A.2.14 Pure oligodeoxynucleotide **Strand-7**



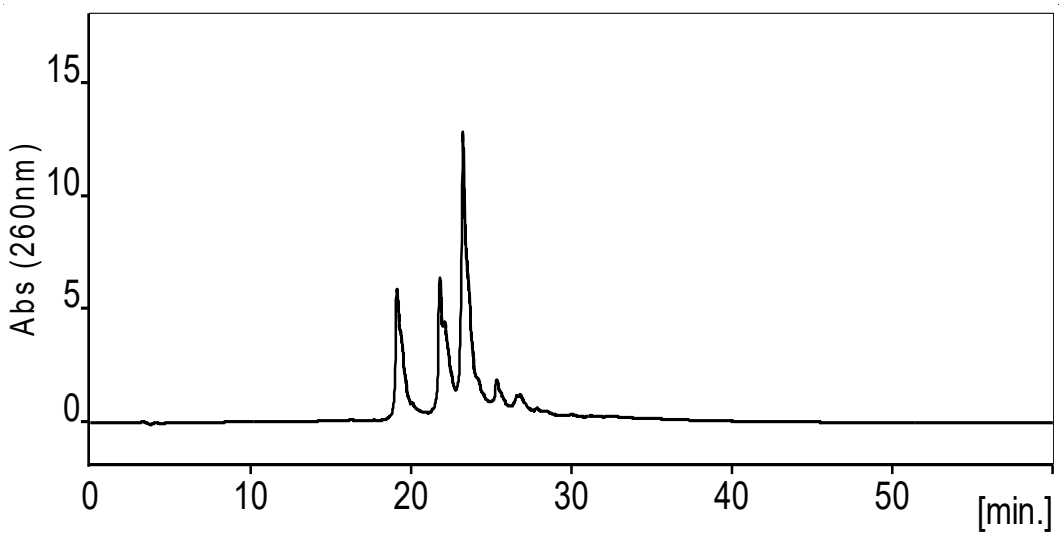
A.2.15 Cure oligodeoxynucleotide **Strand-8**



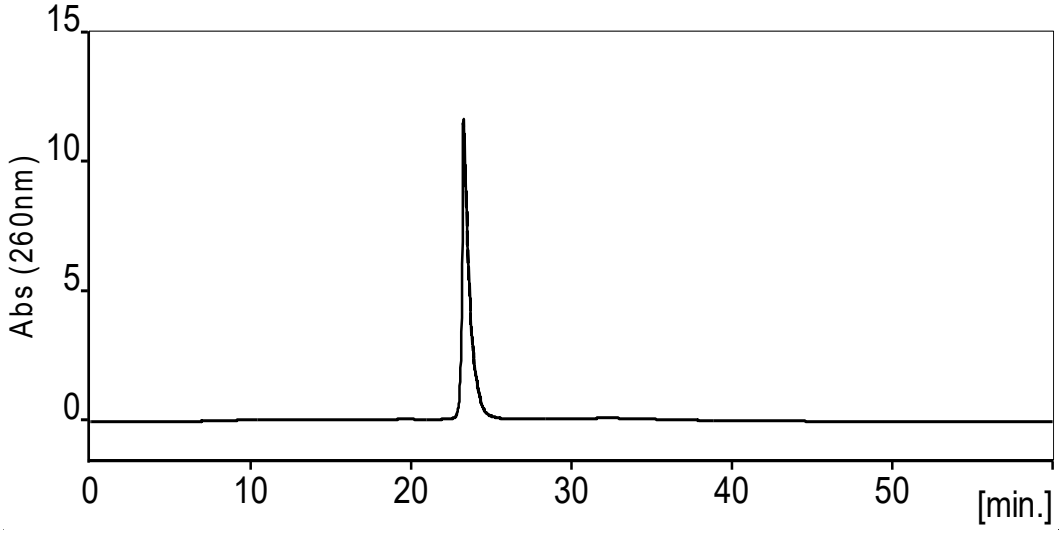
A.2.16 Pure oligodeoxynucleotide **Strand-8**



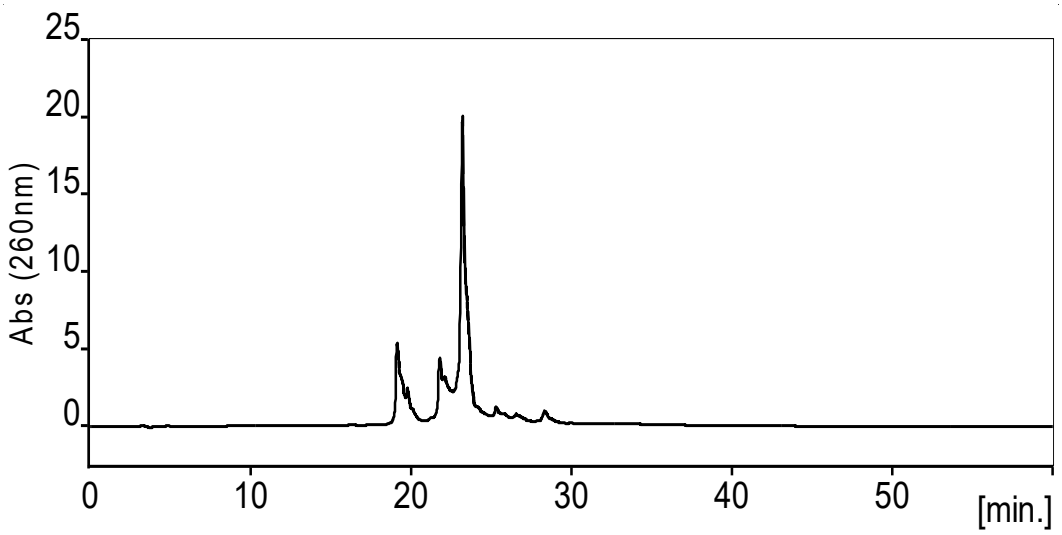
A.2.17 Crude oligodeoxynucleotide **Strand-9**



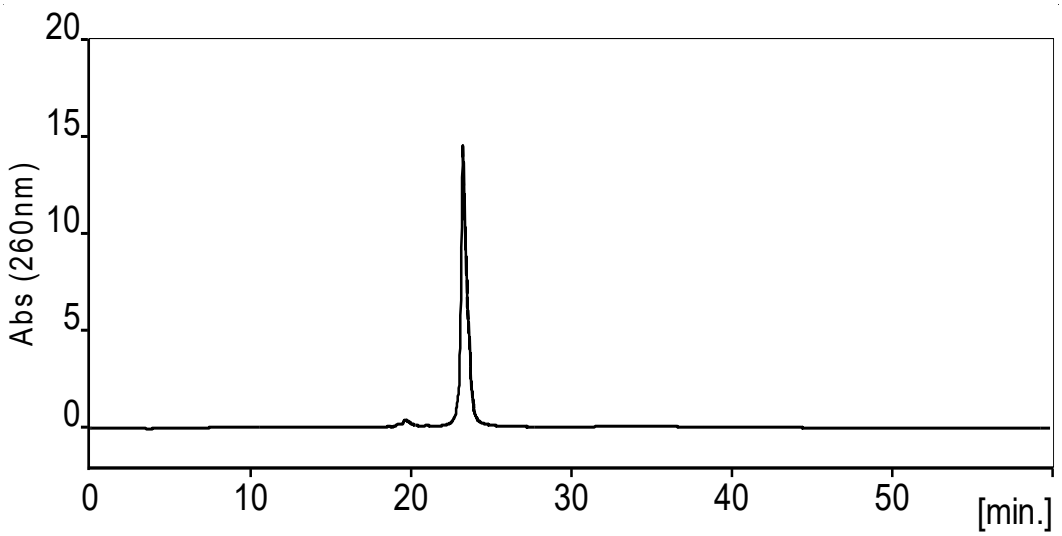
A.2.18 Pure oligodeoxynucleotide **Strand-9**



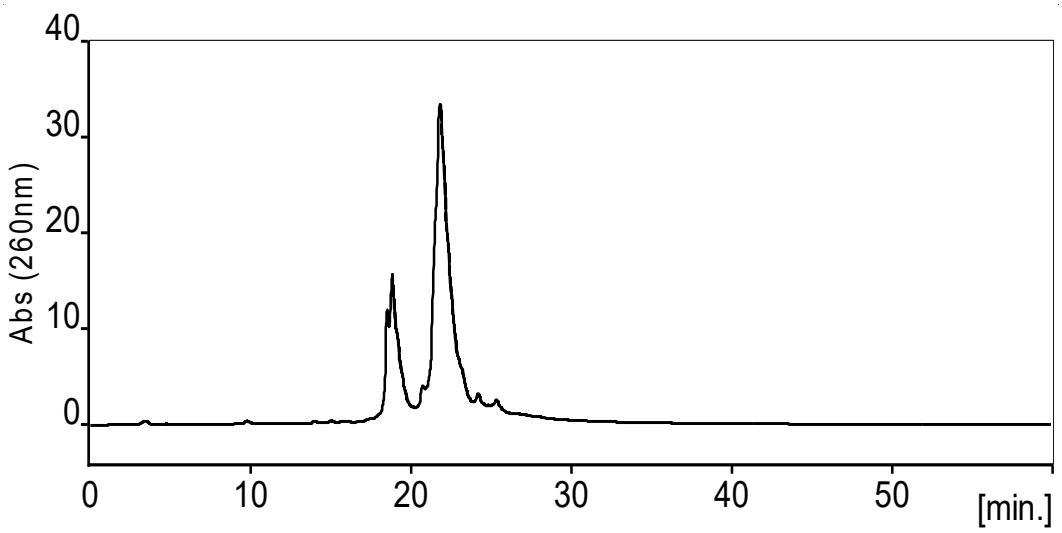
A.2.19 Crude oligodeoxynucleotide **Strand-10**



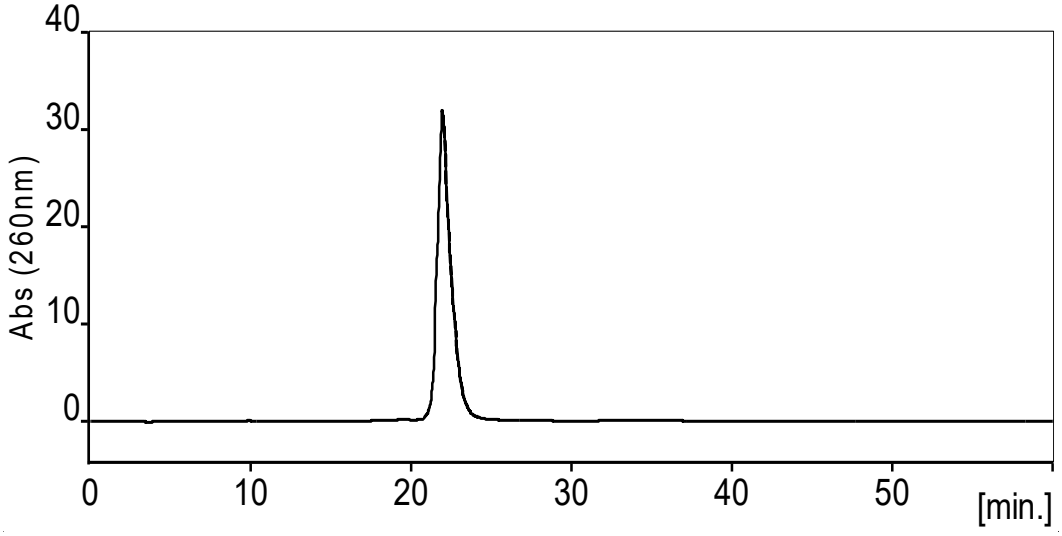
A.2.20 Pure oligodeoxynucleotide **Strand-10**



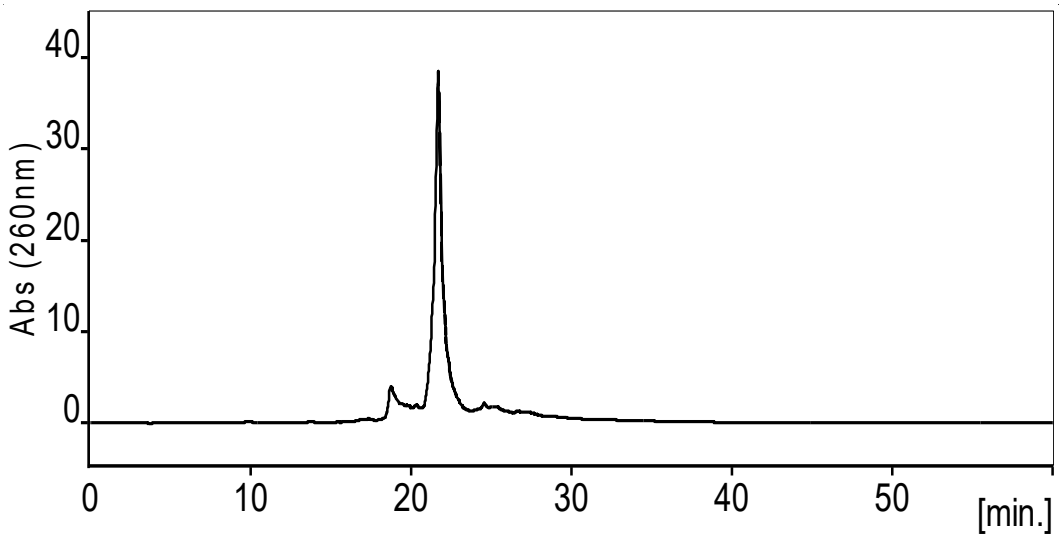
A.2.21 Crude oligodeoxynucleotide **Strand-11**



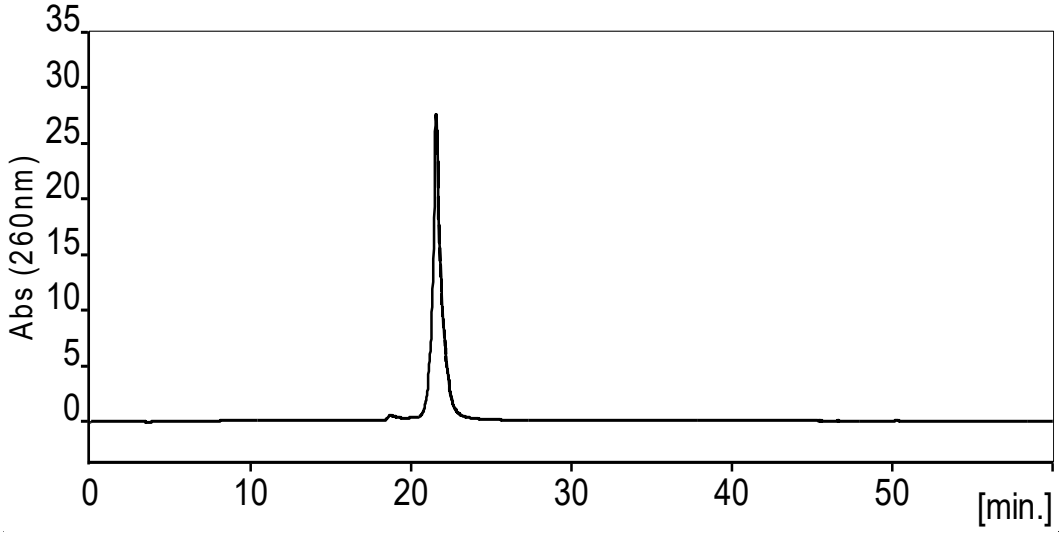
A.2.22 Pure oligodeoxynucleotide **Strand-11**



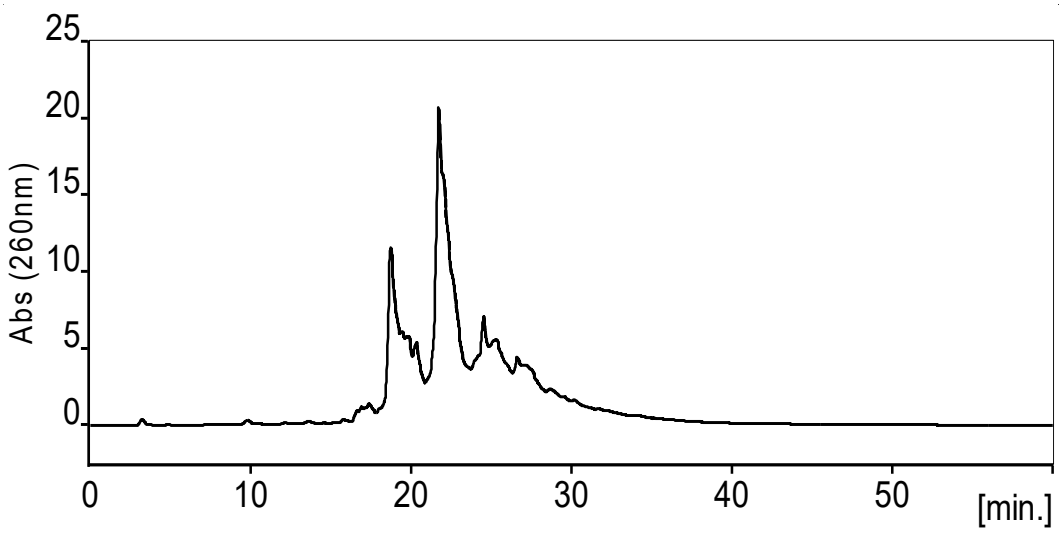
A.2.23 Crude oligodeoxynucleotide **Strand-12**



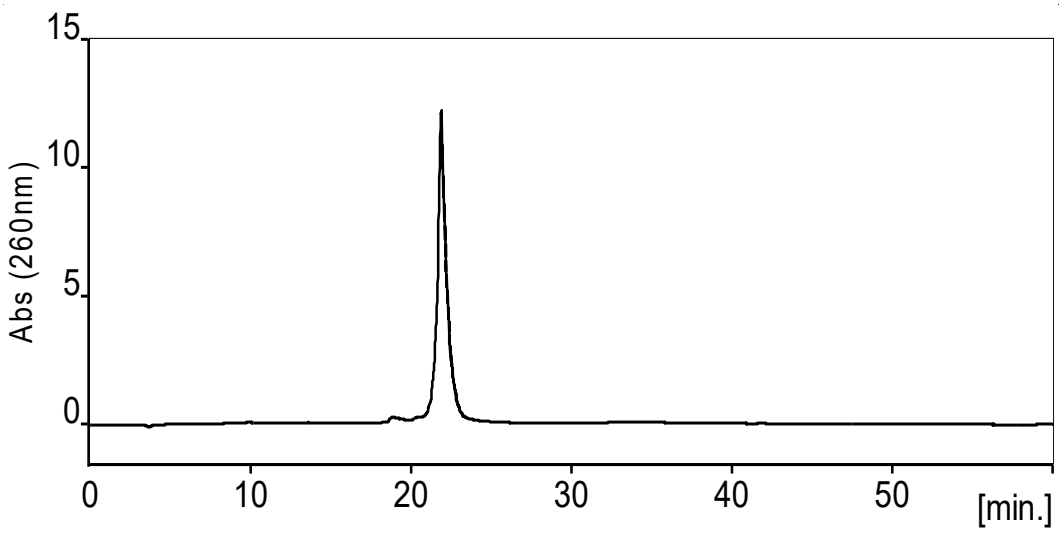
A.2.24 Pure oligodeoxynucleotide **Strand-12**



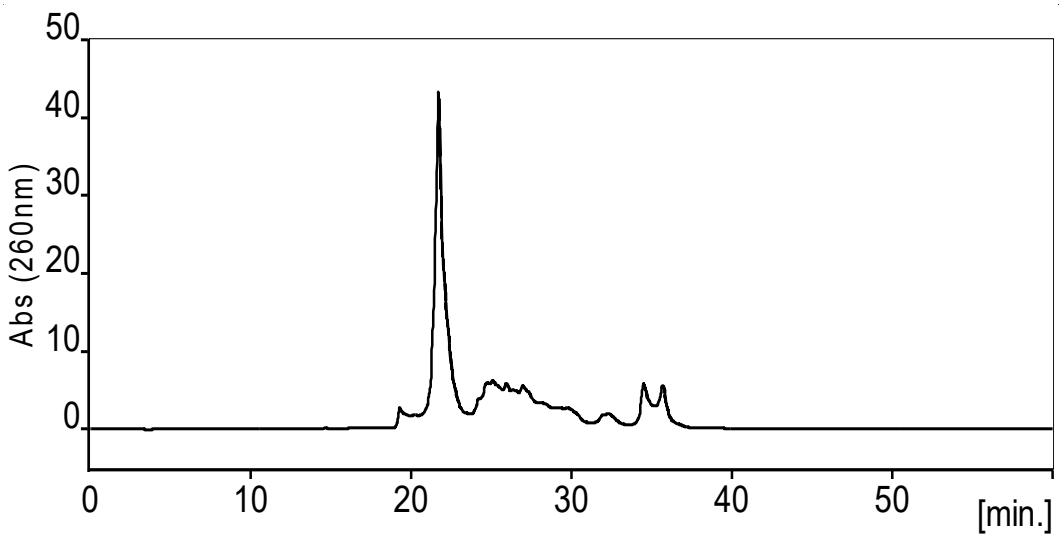
A.2.25 Crude oligodeoxynucleotide **Strand-13**



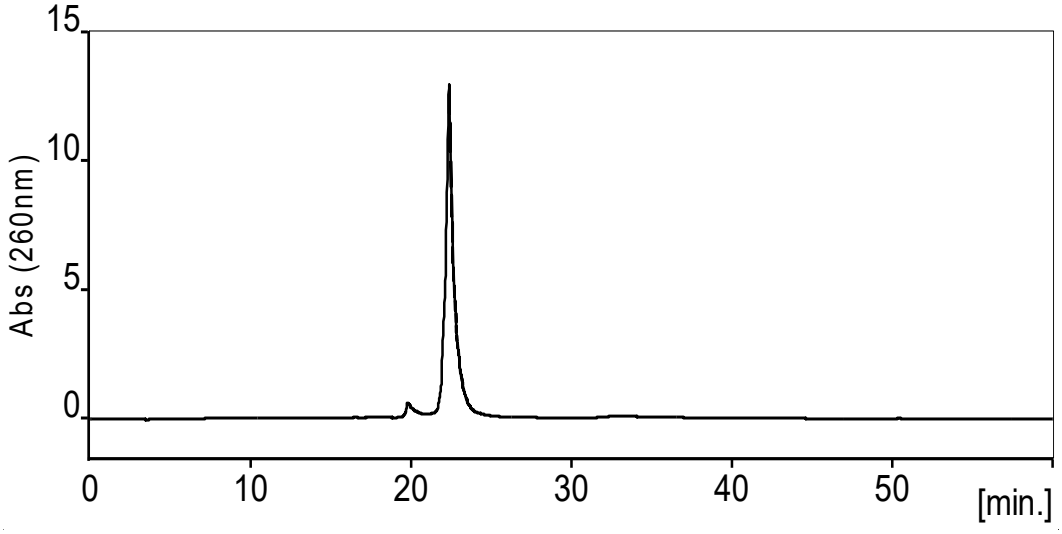
A.2.26 Pure oligodeoxynucleotide **Strand-13**



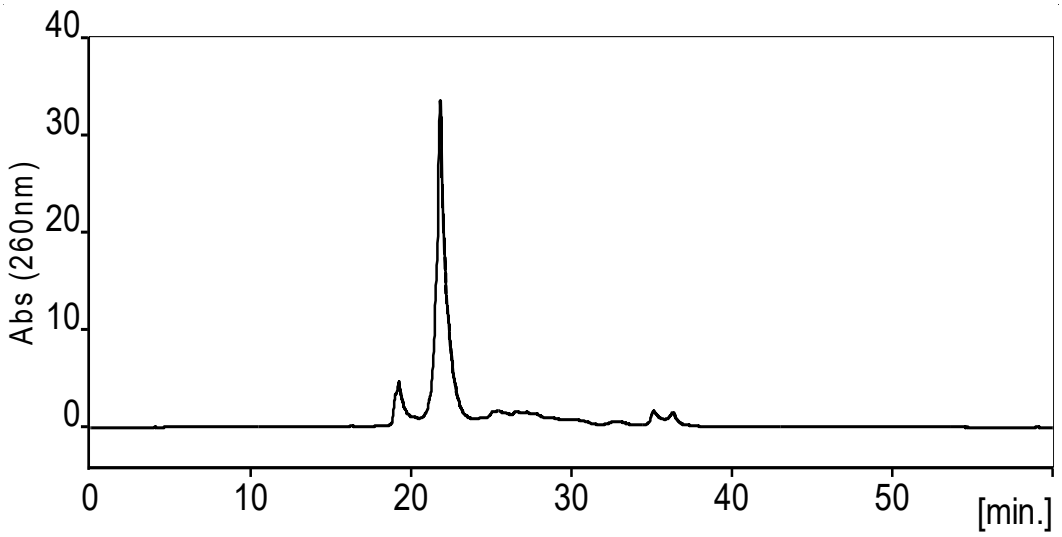
A.2.27 Crude oligodeoxynucleotide **Strand-14**



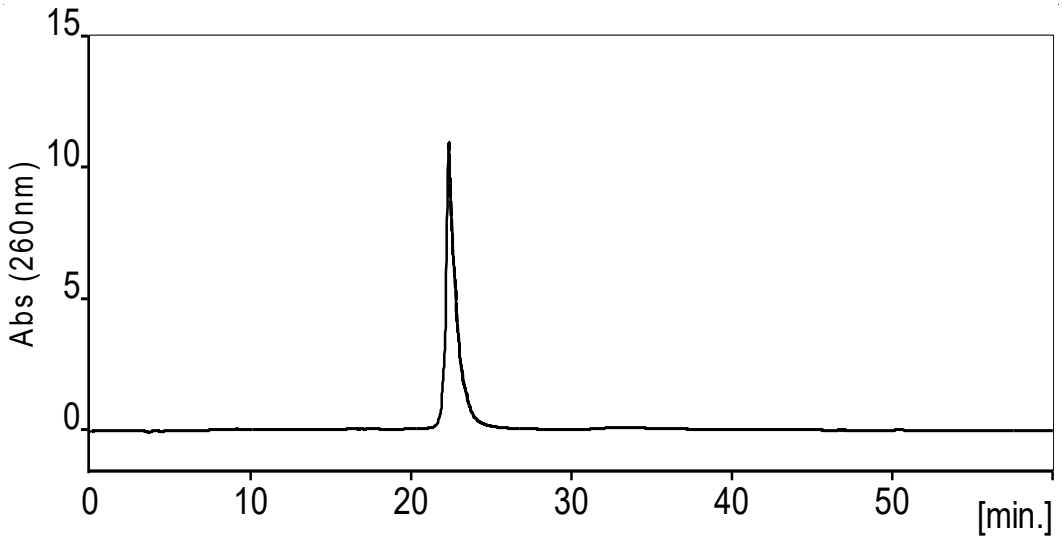
A.2.28 Pure oligodeoxynucleotide **Strand-14**



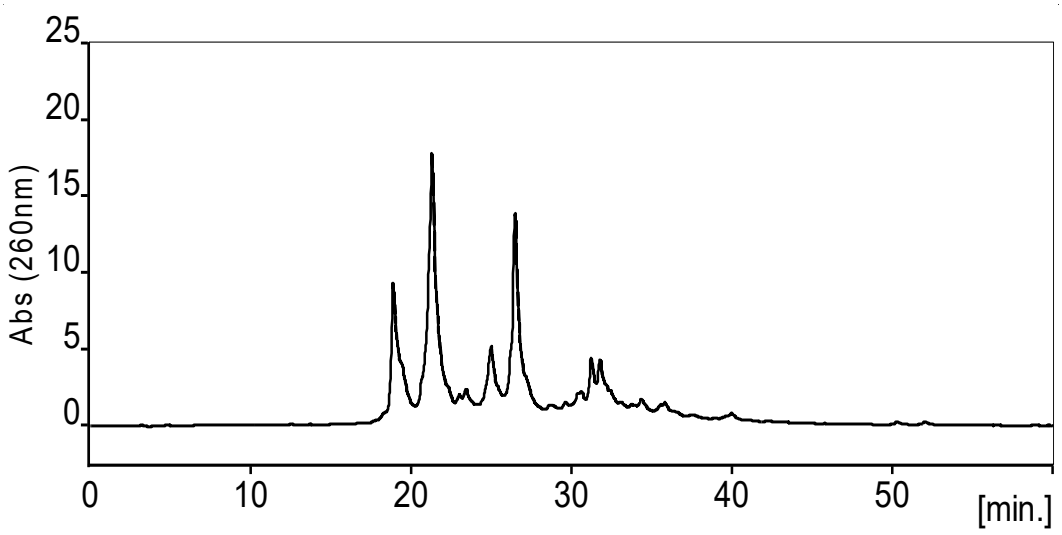
A.2.29 Crude oligodeoxynucleotide **Strand-15**



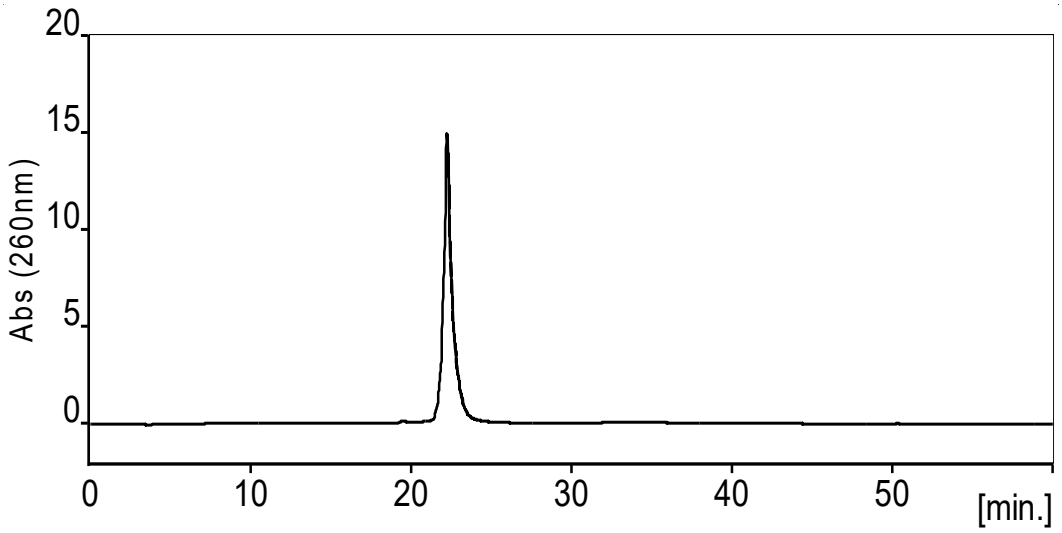
A.2.30 Pure oligodeoxynucleotide **Strand-15**



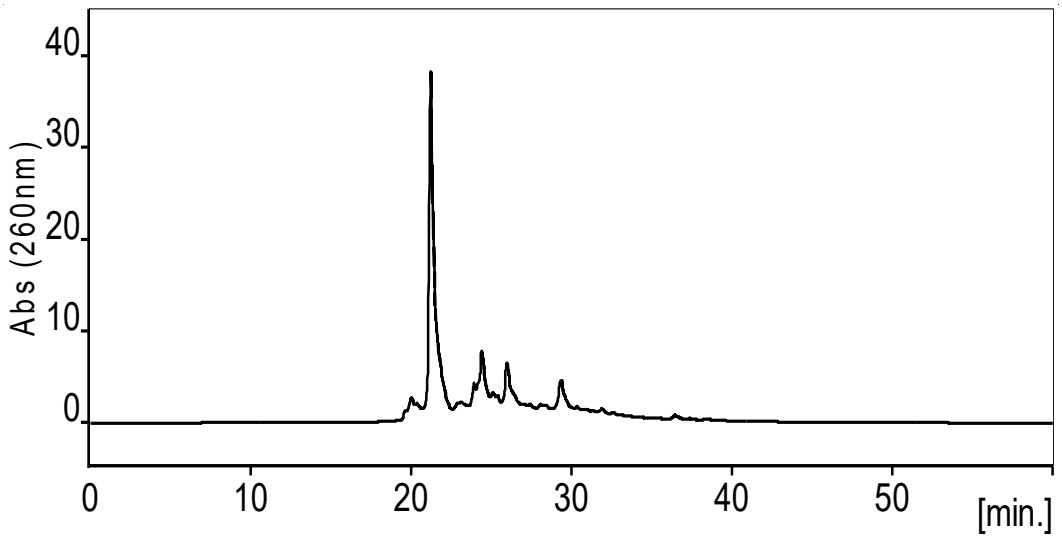
A.2.31 Crude oligodeoxynucleotide **Strand-16**



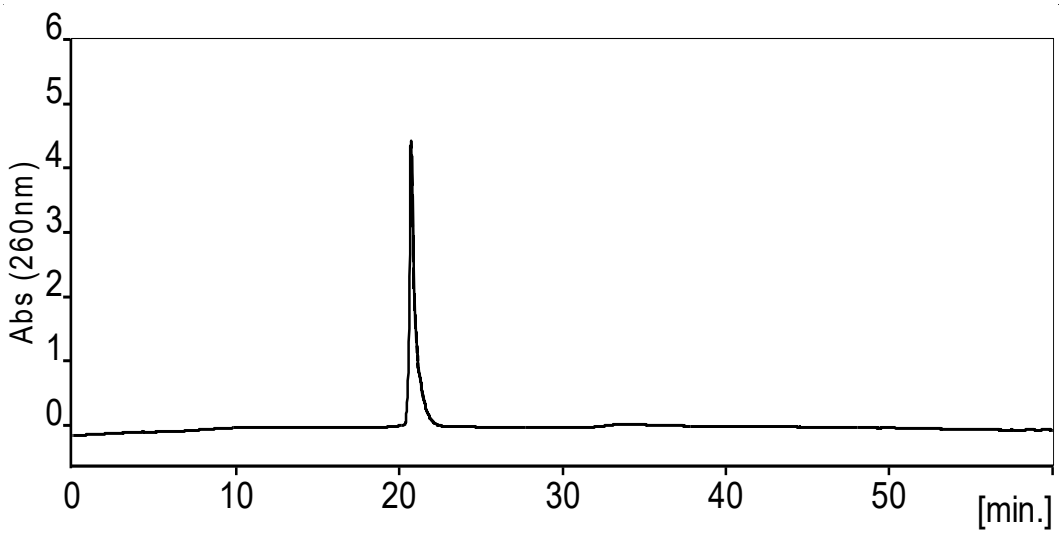
A.2.32 Pure oligodeoxynucleotide **Strand-16**



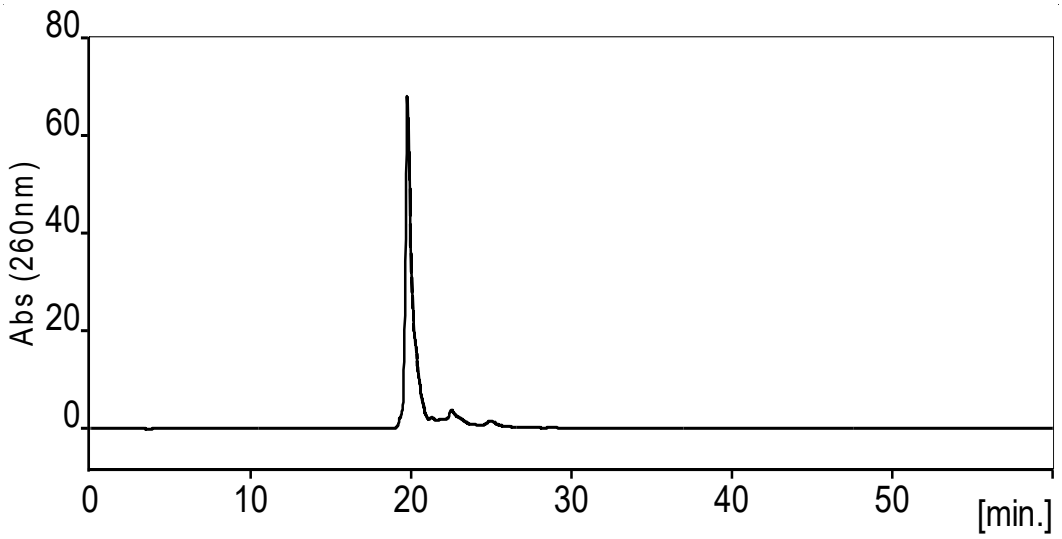
A.2.33 Crude oligodeoxynucleotide **Strand-17**



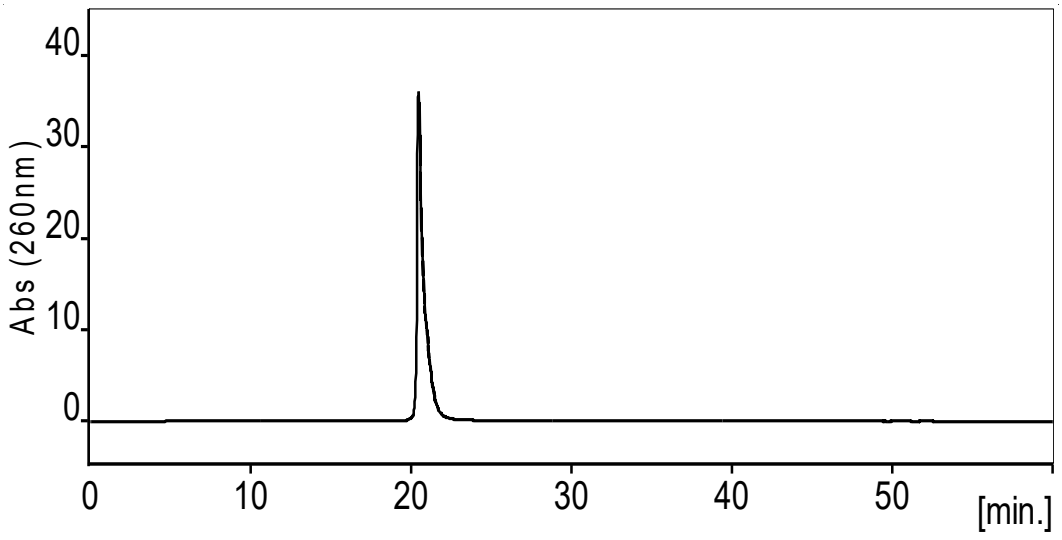
A.2.34 Pure oligodeoxynucleotide **Strand-17**



A.2.35 Crude oligodeoxynucleotide **Strand-18**

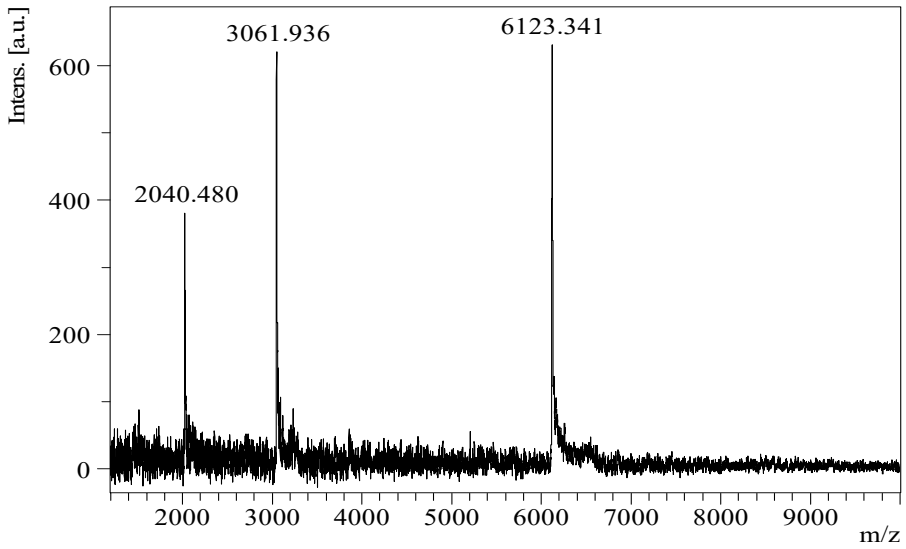


A.2.36 Pure oligodeoxynucleotide **Strand-18**

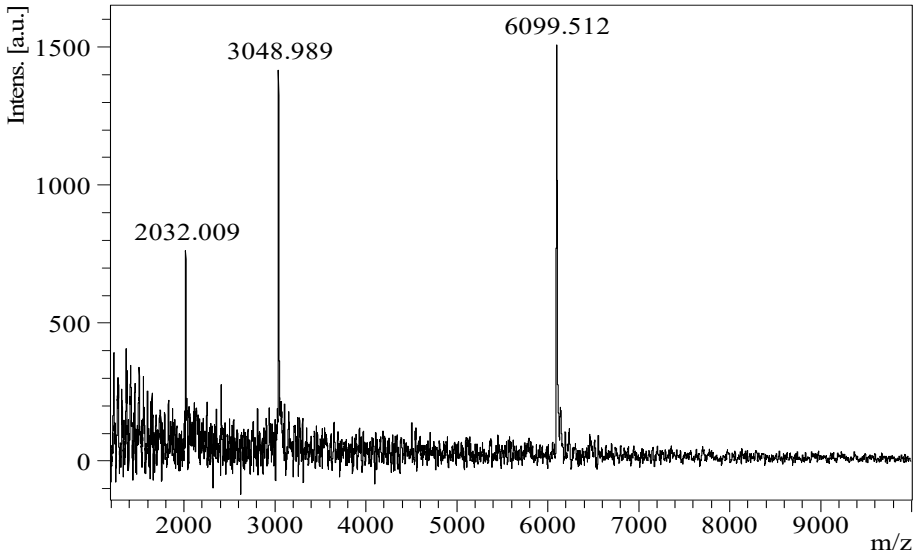


A.3 MALDI-Tof spectra of oligodeoxynucleotides chapter-3

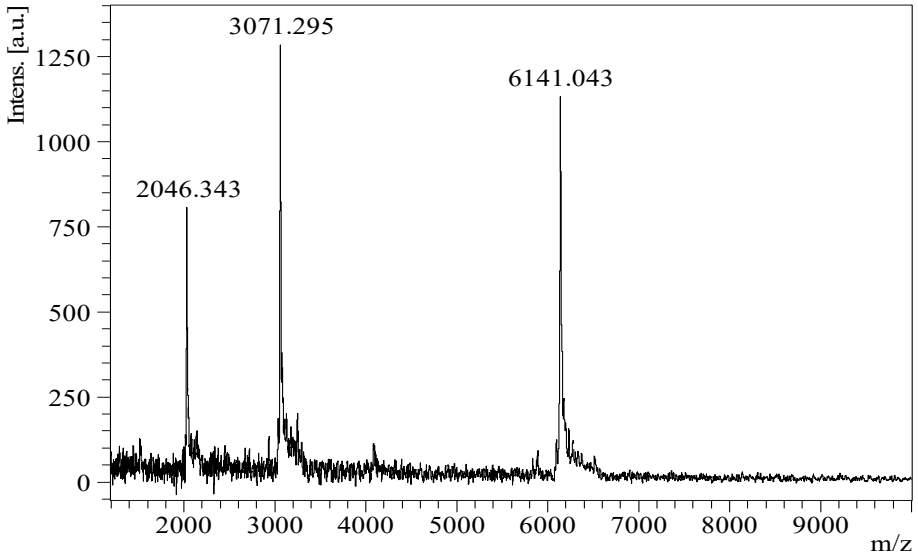
A.3.1 Mass spectrum for ODN **Strand-1**



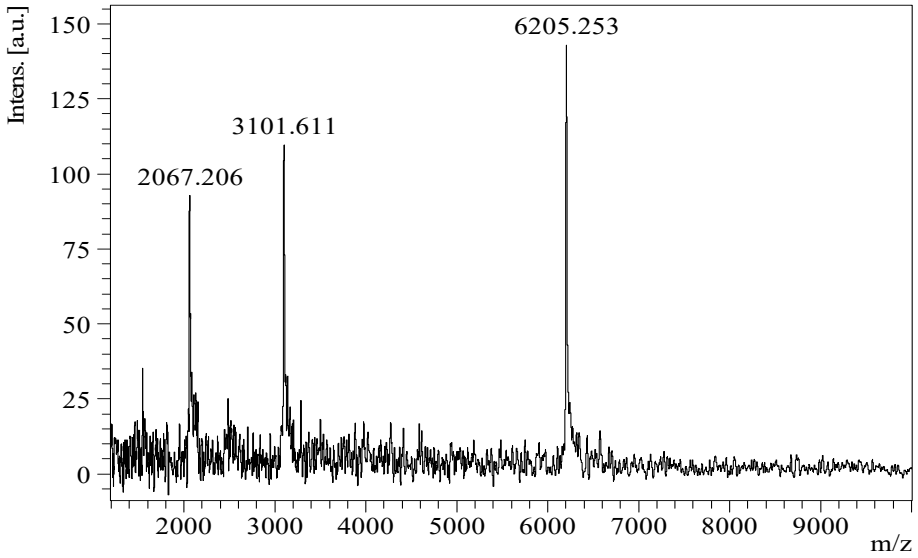
A.3.2 Mass spectrum for ODN **Strand-2**



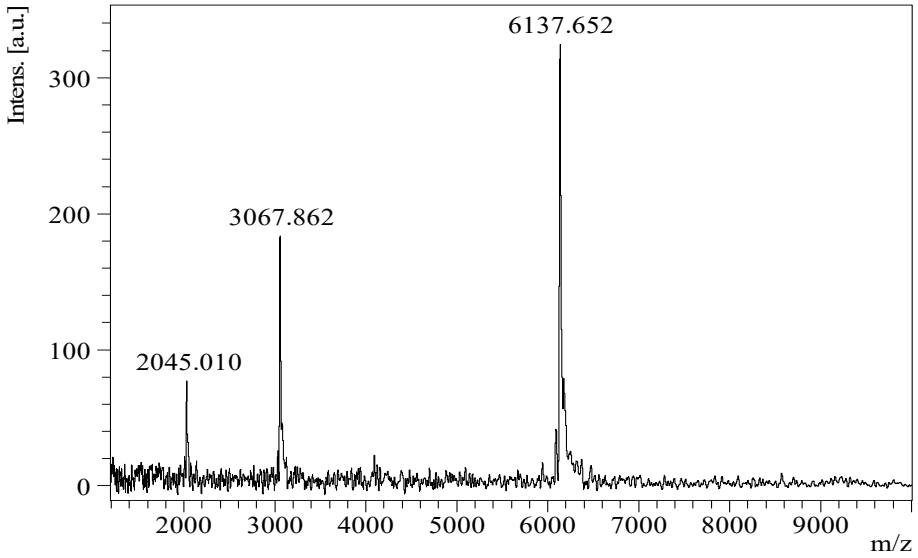
A.3.3 Mass spectrum for ODN **Strand-3**



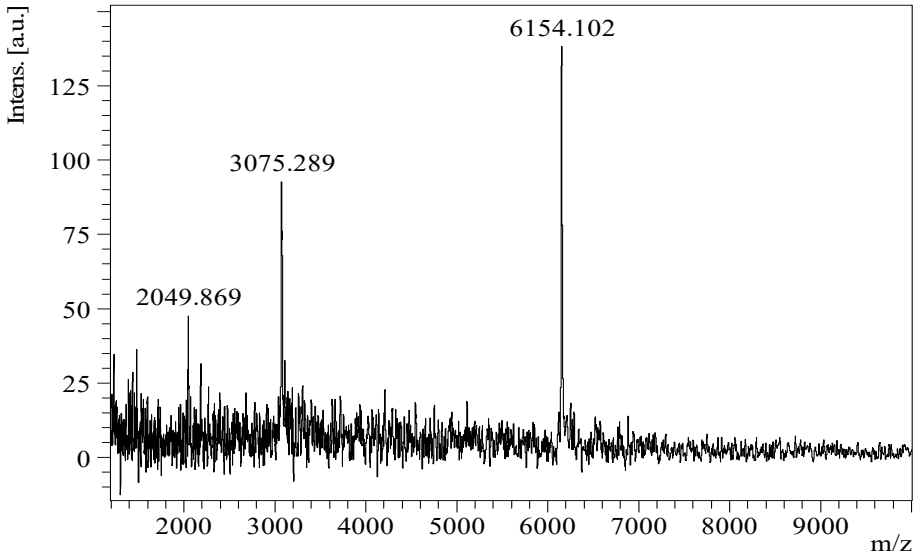
A.3.4 Mass spectrum for ODN **Strand-4**



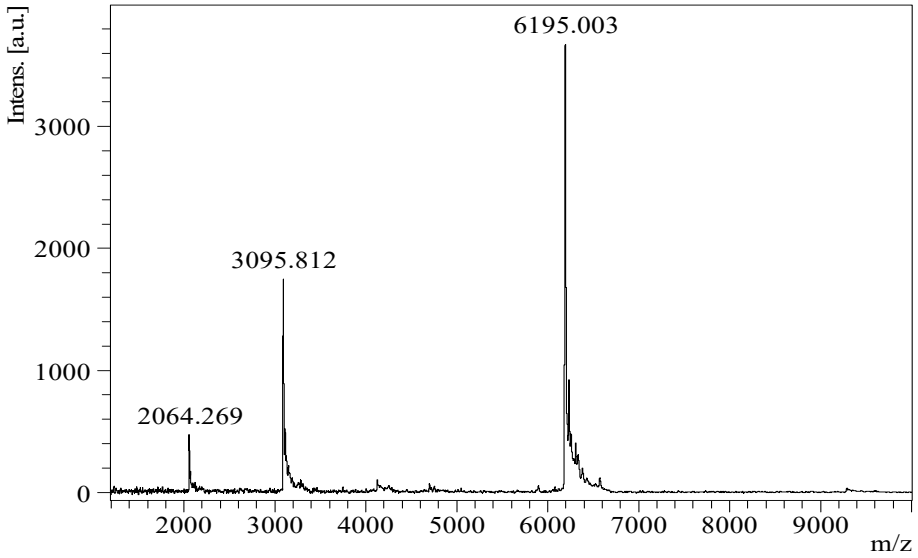
A.3.5 Mass spectrum for ODN **Strand-5**



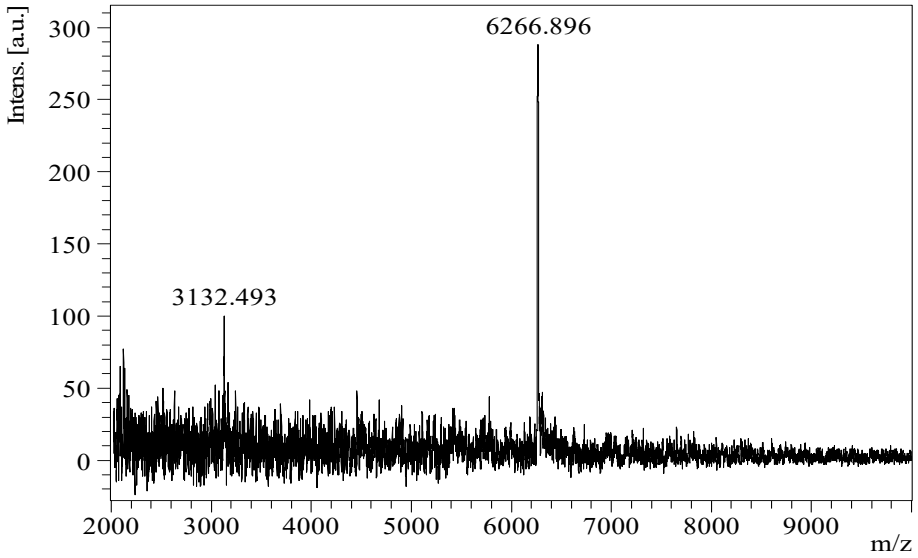
A.3.6 Mass spectrum for ODN **Strand-6**



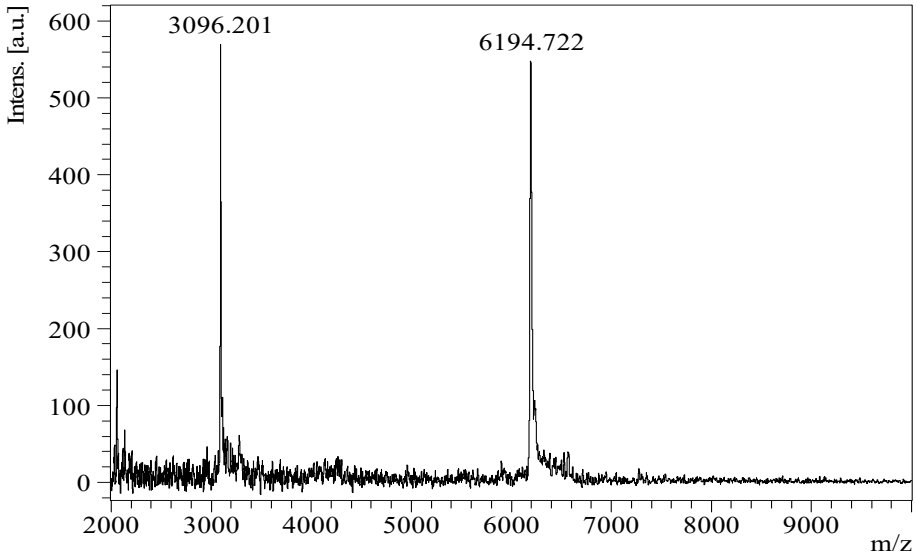
A.3.7 Mass spectrum for ODN **Strand-7**



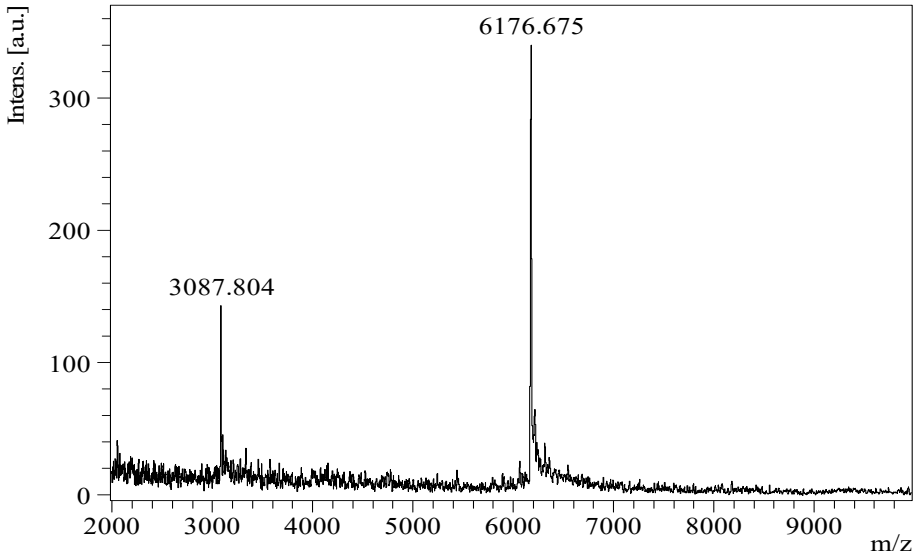
A.3.8 Mass spectrum for ODN **Strand-8**



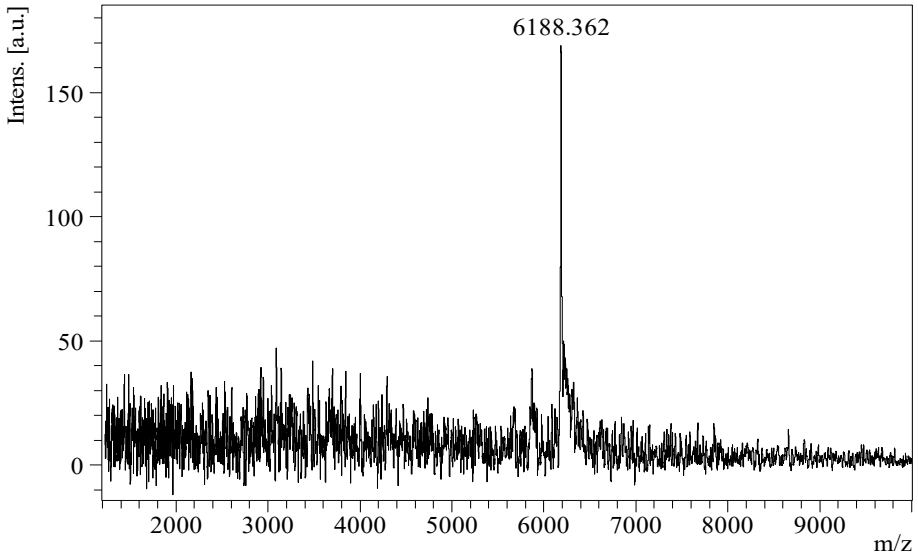
A.3.9 Mass spectrum for ODN **Strand-9**



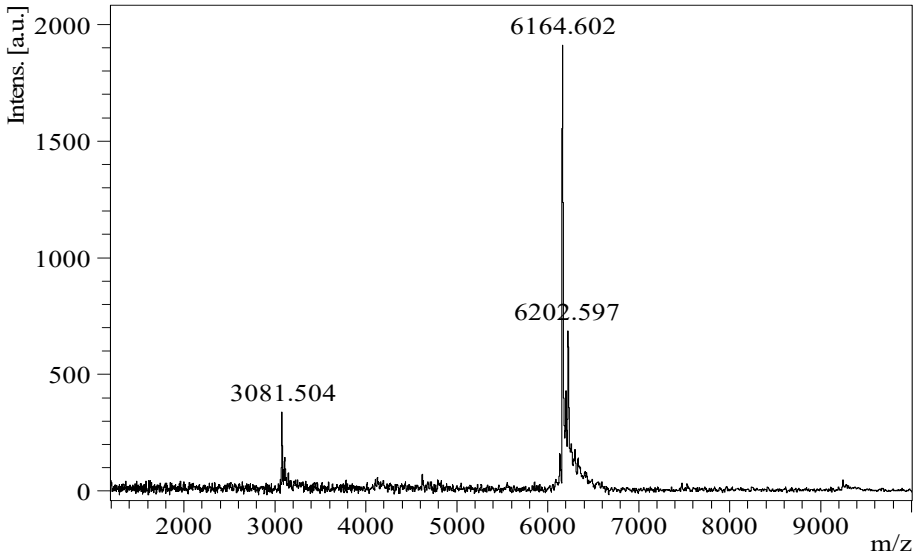
A.3.10 Mass spectrum for ODN Strand-10



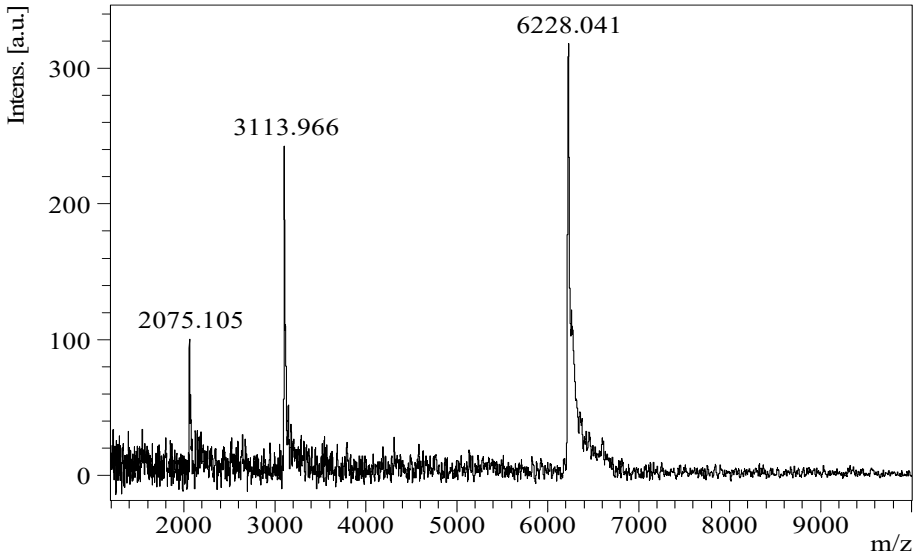
A.3.11 Mass spectrum for ODN **Strand-11**



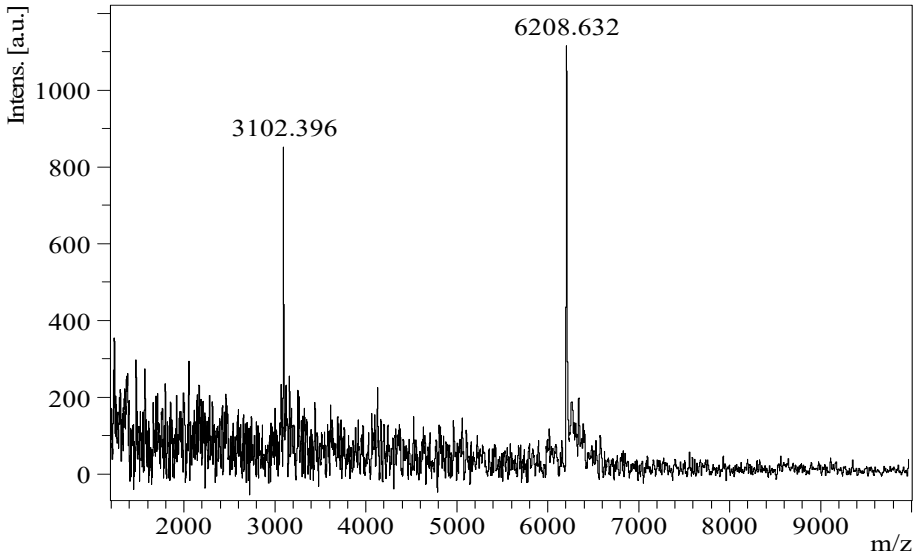
A.3.12 Mass spectrum for ODN **Strand-12**



A.3.13 Mass spectrum for ODN **Strand-13**



A.3.14 Mass spectrum for ODN **Strand-14**



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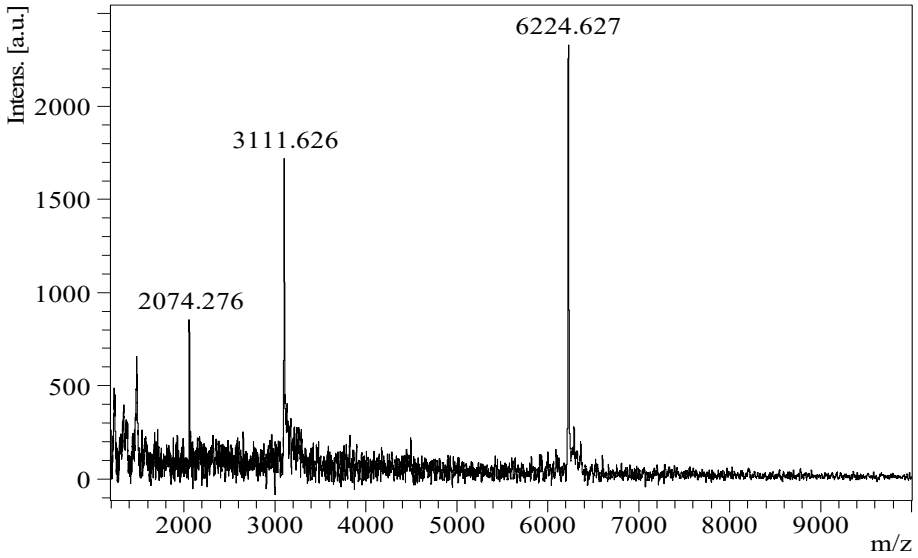

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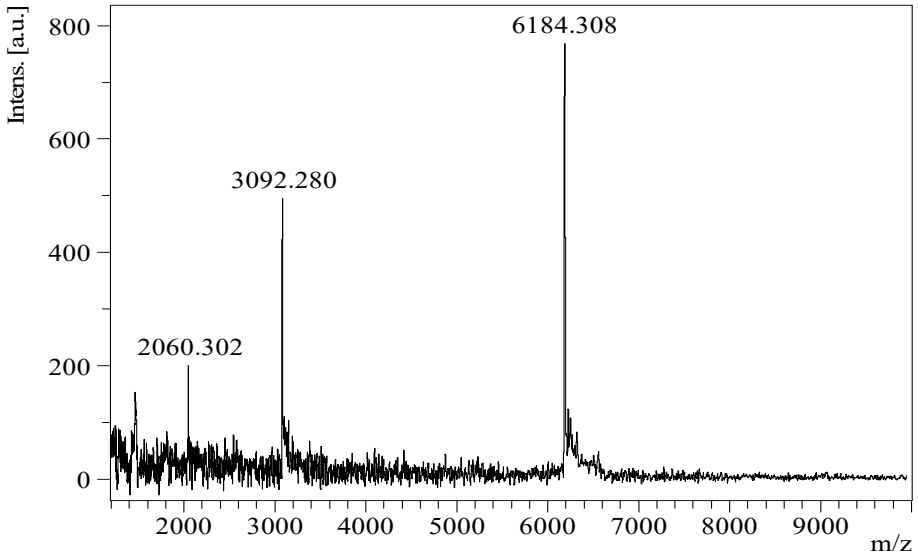
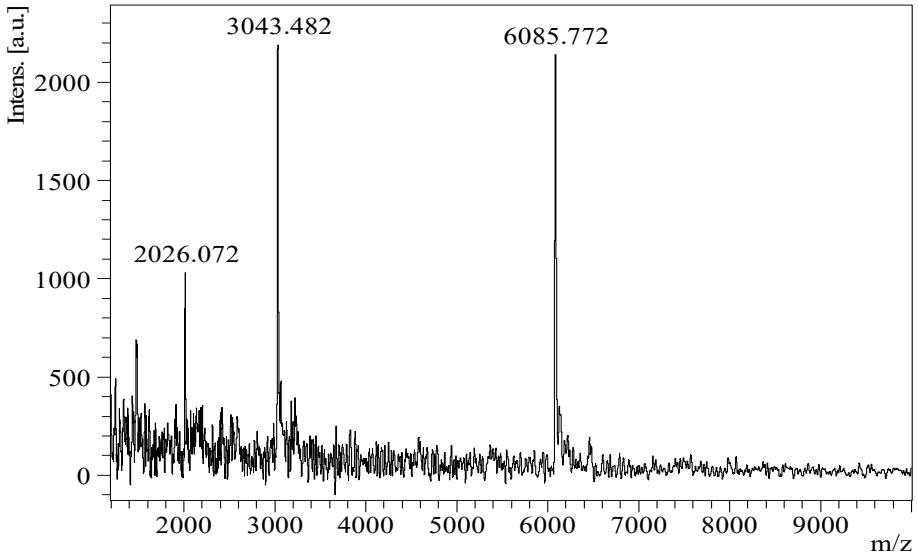
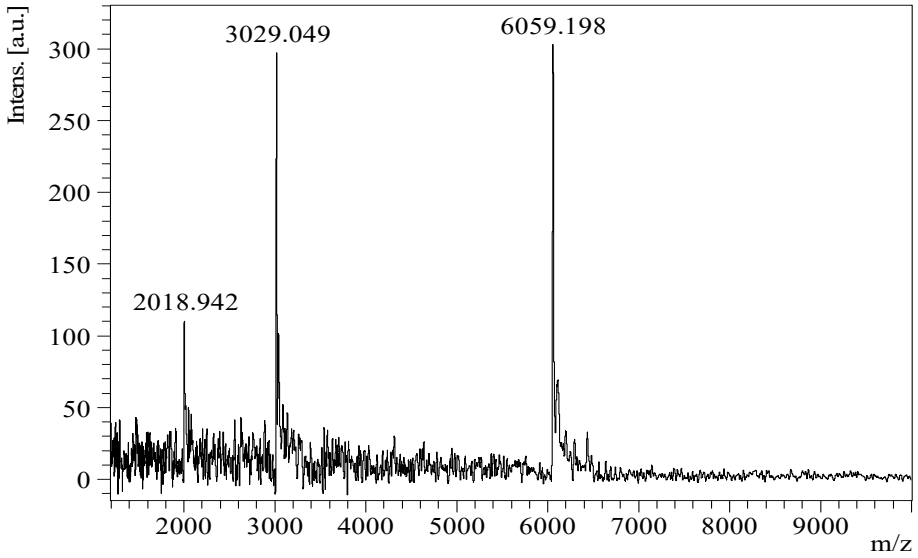


A.3.17 Mass spectrum for ODN **Strand-17**

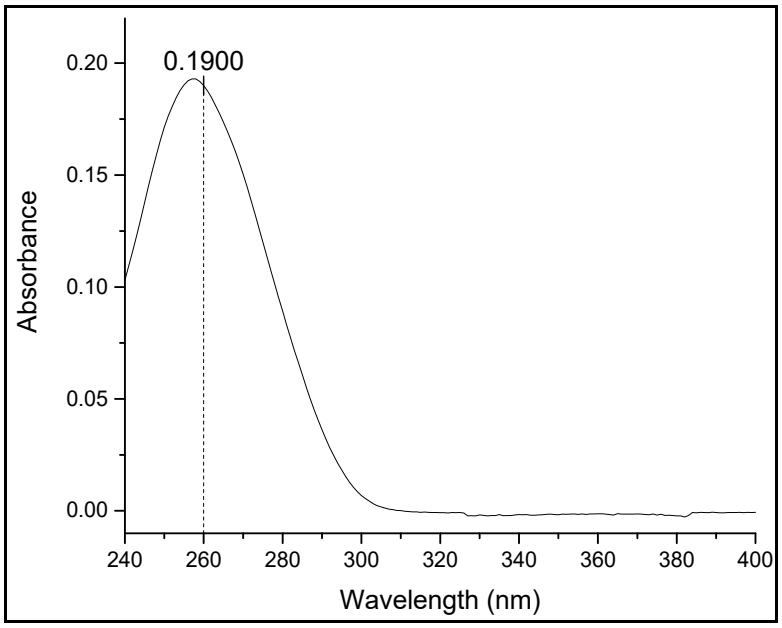


A.3.18 Mass spectrum for ODN **Strand-18**

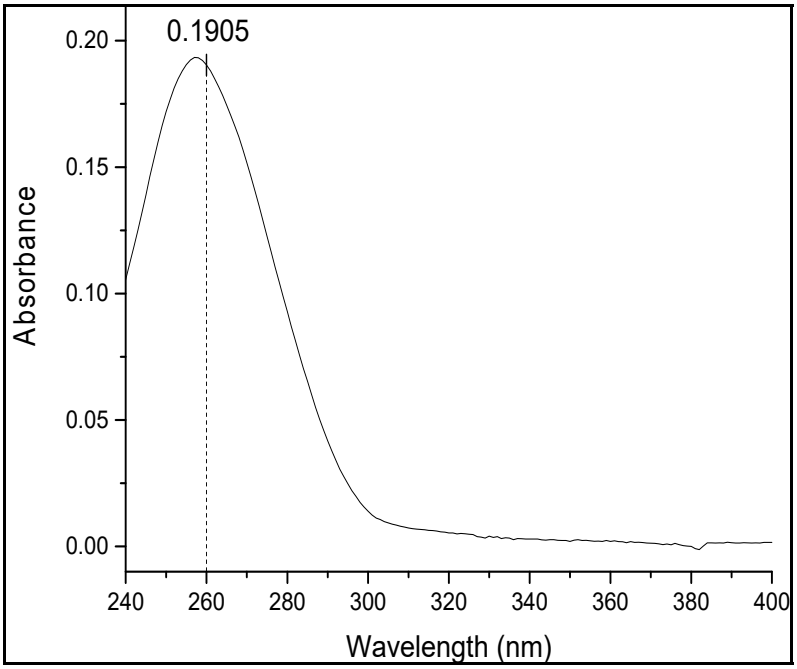


A.4 UV-Visible spectra of oligonucleotides chapter 3

A.4.1 UV-Visible spectrum for ODN **Strand-1**



A.4.2 UV-Visible spectrum for ODN **Strand-2**



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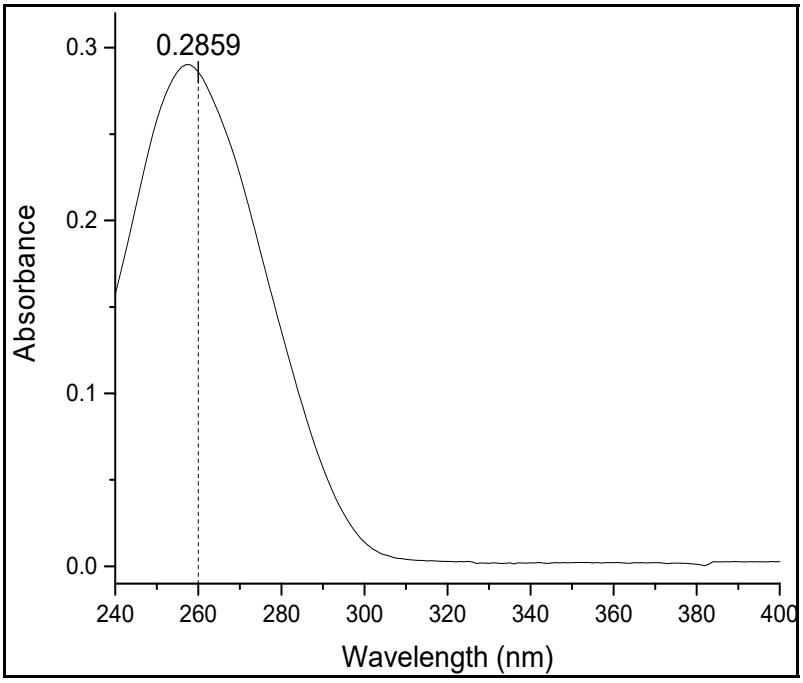
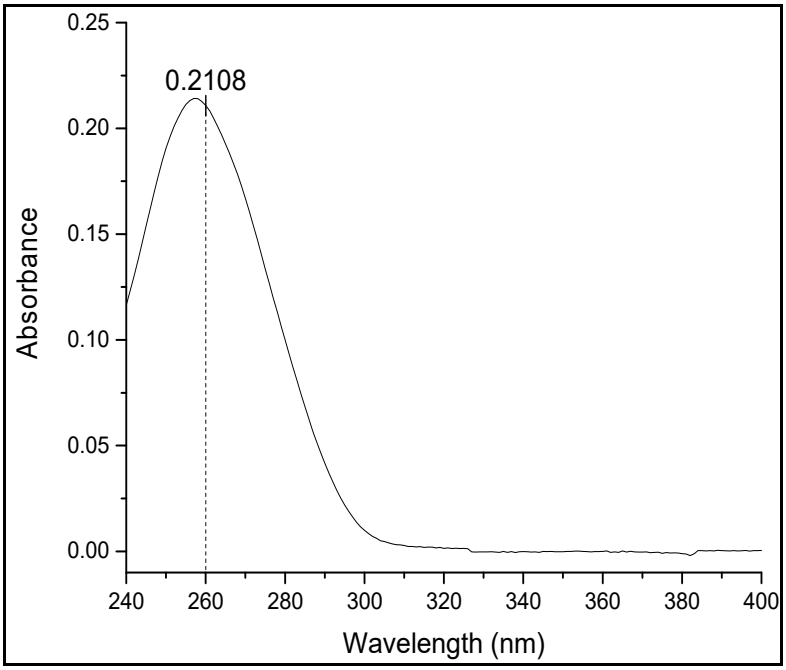
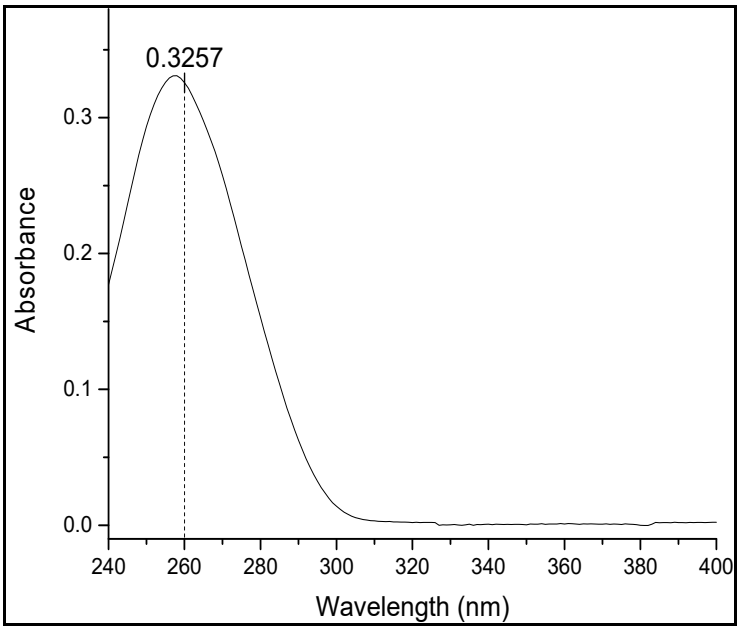


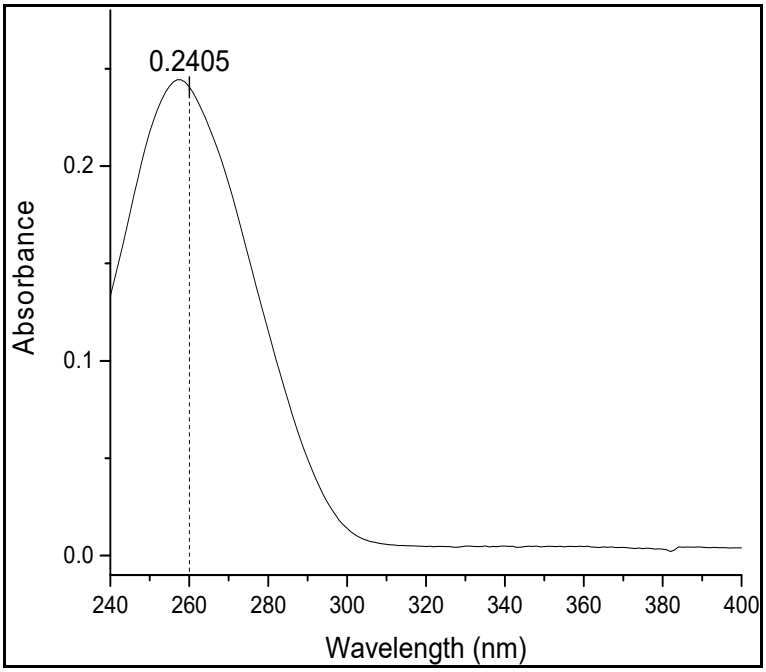
A.4.4 UV-Visible spectrum for ODN **Strand-4**



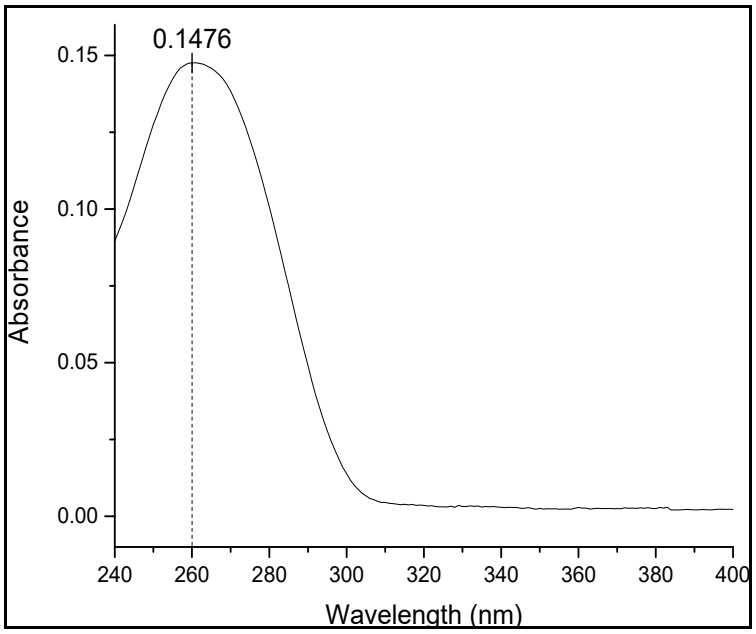
A.4.5 UV-Visible spectrum for ODN **Strand-5**



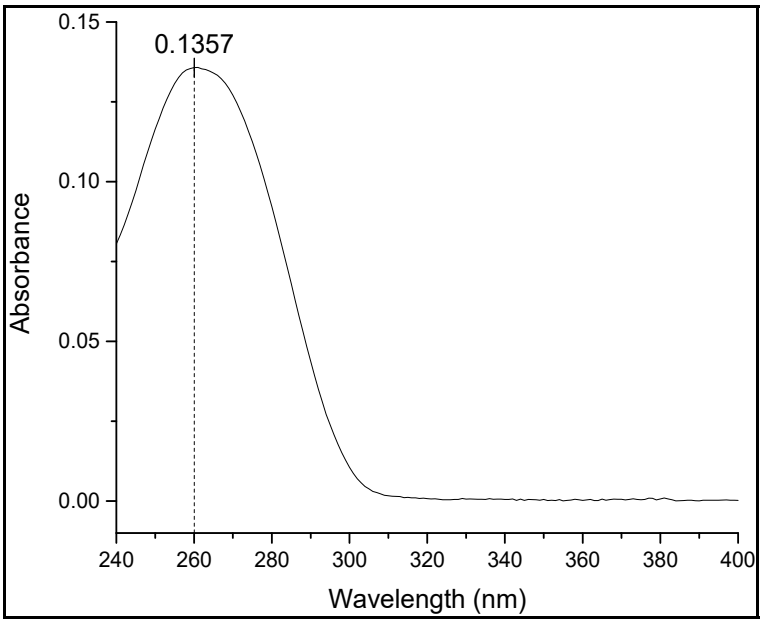
A.4.6 UV-Visible spectrum for ODN **Strand-6**



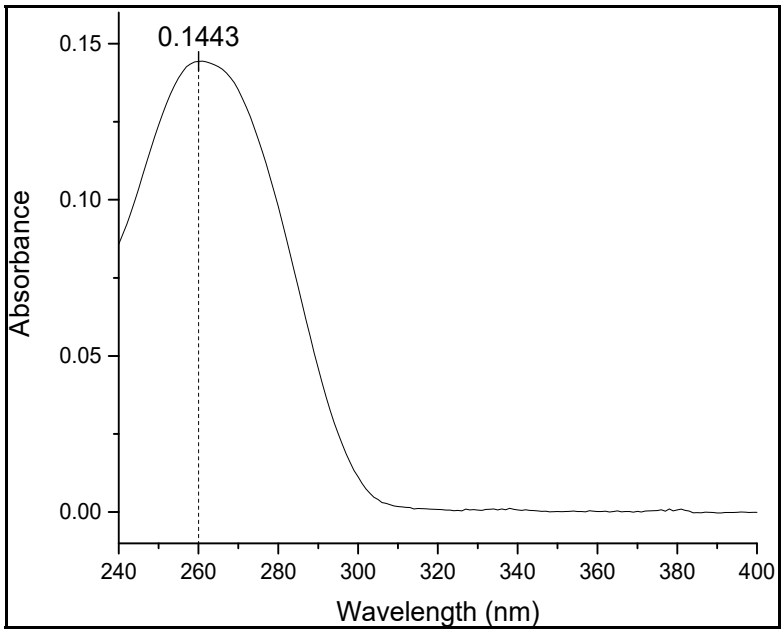
A.4.7 UV-Visible spectrum for ODN **Strand-7**



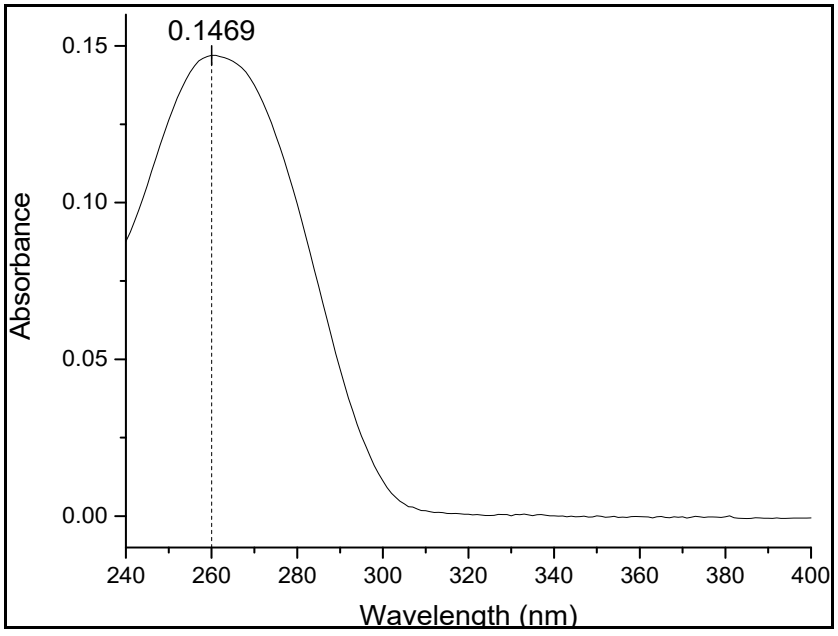
A.4.8 UV-Visible spectrum for ODN **Strand-8**



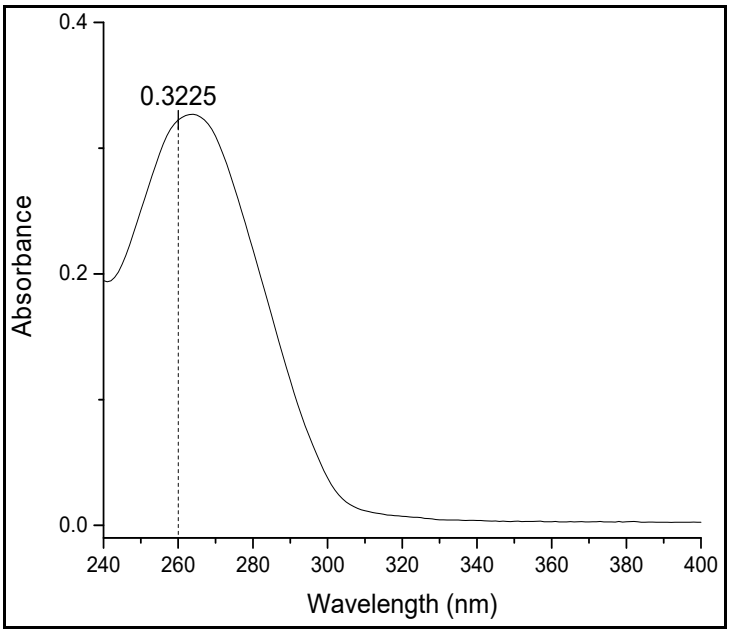
A.4.9 UV-Visible spectrum for ODN **Strand-9**



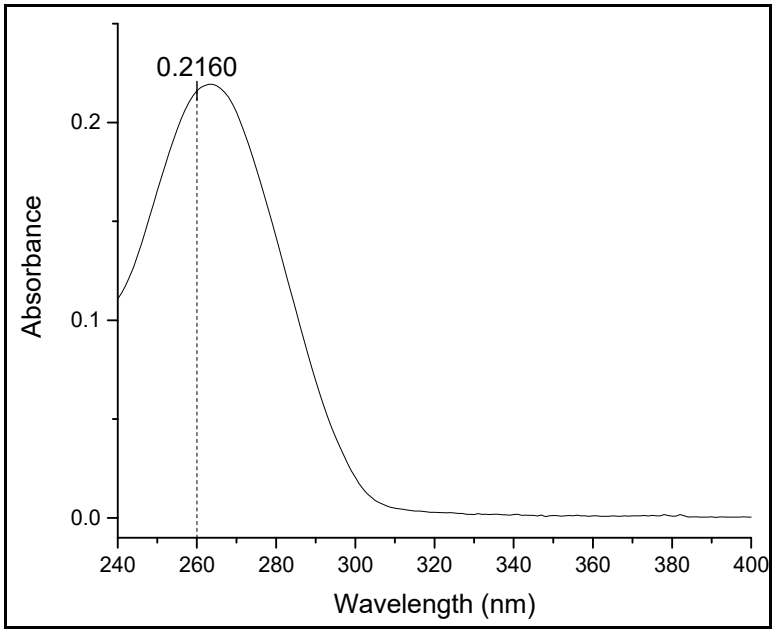
A.4.10 UV-Visible spectrum for ODN **Strand-10**



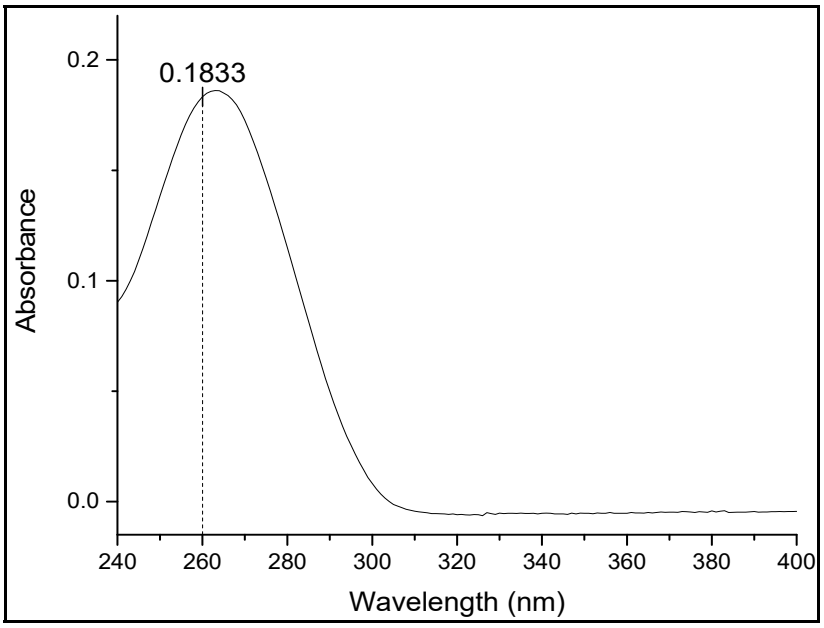
A.4.11 UV-Visible spectrum for ODN **Strand-11**



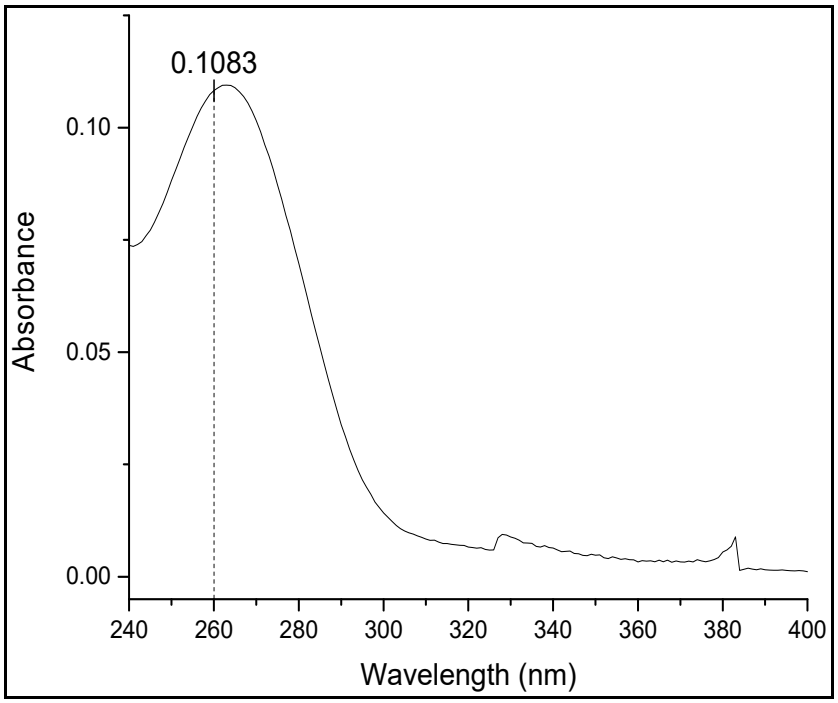
A.4.12 UV-Visible spectrum for ODN **Strand-12**



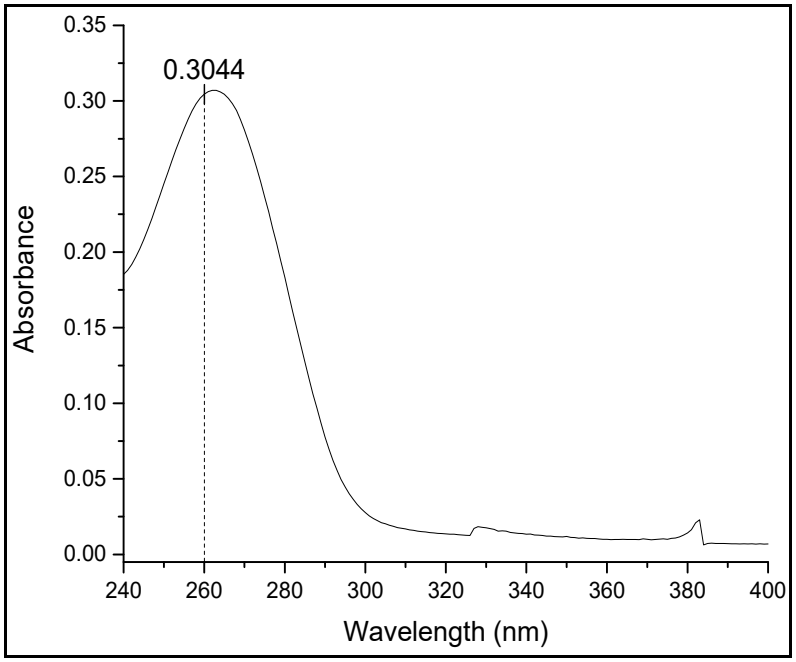
A.4.13 UV-Visible spectrum for ODN **Strand-13**



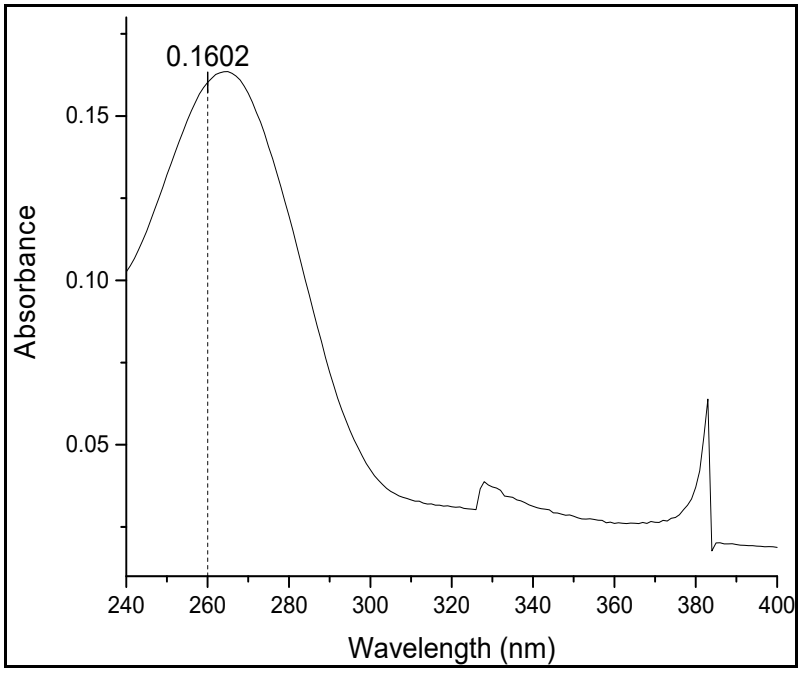
A.4.14 UV-Visible spectrum for ODN **Strand-14**



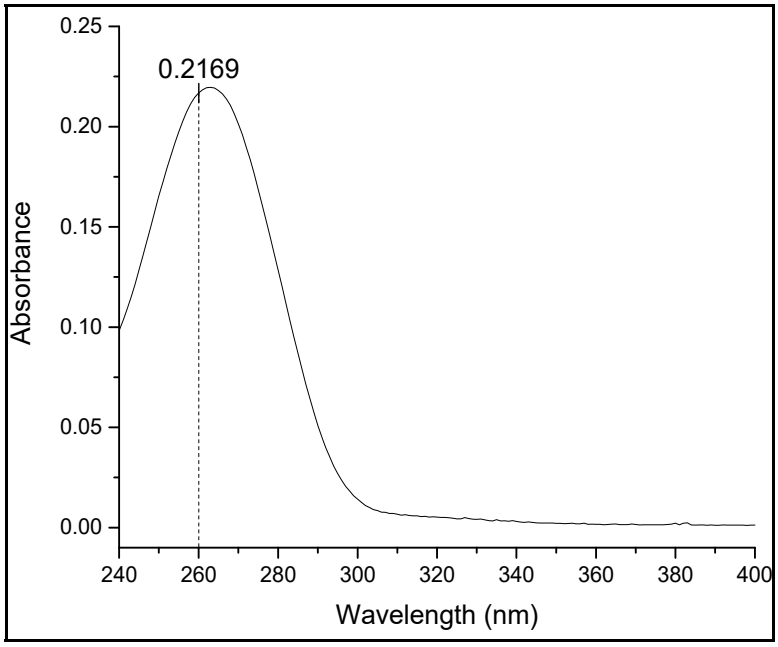
A.4.15 UV-Visible spectrum for ODN **Strand-15**



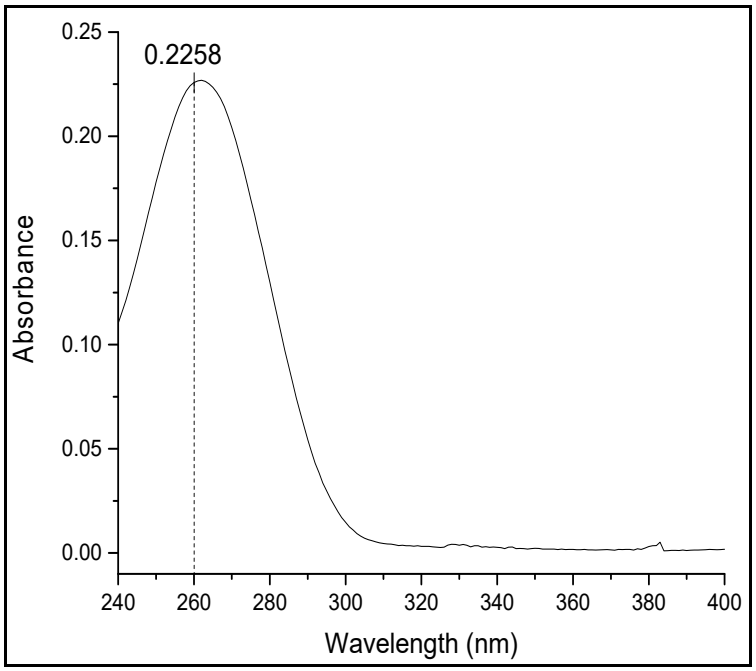
A.4.16 UV-Visible spectrum for ODN **Strand-16**



A.4.17 UV-Visible spectrum for ODN **Strand-17**



A.4.18 UV-Visible spectrum for ODN **Strand-18**



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