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Synthesis of modified D-allohexofuranosyl-uracil nucleoside analogs

Bio-organic research group
Department of Chemistry
University of Turku

MDP in Chemistry of Drug Development
Masters' thesis

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Abstract

The study of nucleosides, nucleotides, and their polymers is essential due to their critical roles in cellular processes such as DNA replication, RNA transcription, protein synthesis, signaling, and energy transfer. These molecules serve as the building blocks of life, making them fundamental to genetics, molecular biology, pharmacology, and other relevant scientific fields. Beyond their natural functions, chemically modified nucleosides, nucleotides, and their oligomers have emerged as powerful tools in medicine, biotechnology, and research. These advancements include the development of antiviral and anticancer therapies using modified nucleoside analogs, as well as therapies employing oligonucleotide-based treatments targeting pre-mRNA and mRNA. Furthermore, these modifications have enhanced diagnostic technologies and research tools. The ability to modify and efficiently synthesize these modified analogs and their oligomers opens new possibilities for therapeutic applications, offering improved stability, specificity, and efficacy.

This work builds on an extensive body of literature exploring the roles of nucleotides, nucleosides, and their modified analogs. Initially, the review covers the physiological significance of natural nucleos(t)ides, emphasizing their central role in genetic information transfer and cellular metabolism. Then focus shifts toward chemically modified nucleos(t)ides, which have become increasingly important in antiviral, anticancer, gene therapies, and biotechnological tools. Various synthetic strategies for altering sugar, nucleobase, and phosphate moieties are critically reviewed, with a particular emphasis on methods that enable precise structural alterations. Special attention is also given to the utility of D-allofuranose, an atypical sugar that served as a scaffold for modified nucleoside analogs developed in the practical part of this work. These insights underscore the potential of developing novel therapeutic agents with enhanced properties and directly inform the synthetic approaches. This review guided the selection of synthetic routes, protecting group strategies, and targeted modifications that were further practically explored in this study.

The experimental part of this research focused on synthesizing D-allofuranosyl-uracil analogs, with a special focus on modifying the 6'-hydroxyl group. The study explored the effectiveness of two different synthetic routes for the initial sugar configuration preparation, the separation of the α/β-anomer forms of the resulting uracil nucleosides, and the introduction of an azide group at the 6'OH-position. Despite encountering challenges, such as the unsuccessful addition of a triphosphate group, the research demonstrated the feasibility of synthesizing a modified nucleoside key intermediate. Further work is needed to optimize the phosphorylation process and fully evaluate the biological properties of consequently derived nucleotides' antiviral properties.

Keywords: D-allohexofuranose, uracil, modified nucleosides, synthesis, antiviral applications

Preface

This master's thesis was written between autumn 2023 and summer 2024. It was conducted at the University of Turku, in the Department of Chemistry, under the supervision of Professor Pasi Virta.

I sincerely thank my supervisor, Pasi Virta, for allowing me to work on such an exciting topic. His support, positive outlook, and insightful discussions have been immensely helpful throughout this project. I am also grateful for his numerous ideas that have contributed significantly to this research.

I would also like to thank the PhD and MSc students of the bio-organic group for their assistance and valuable advice. It has been a privilege to work alongside such a talented team.

Finally, I extend my heartfelt thanks to my family – my husband, our three children, and my parents and grandparents. Their patience, warmth, and unwavering support have been a source of motivation and strength, especially during challenging times.

Table of Content

List of abbreviations

Abbreviation	Name/Description
5-FU	5-fluorouracil
A	Adenine
ACN	Acetonitrile
ALL	Acute lymphoblastic leukemia
Ara-C	Cytarabine
ASOs	Antisense oligonucleotides
ATP	Adenosine triphosphate
AZT	Zidovudine
BrdU	5'-bromo-2'-deoxyuridine
C	Cytosine
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COSY	Correlation spectroscopy
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats associated protein 9
CuAAC	Cu(I) – catalyzed azide-alkyne cycloaddition
D4T	Stavudine
DAST	Diethylaminosulfur trifluoride
DCC	Dicyclohexylcarbodiimide
dCK	Deoxycytidine kinase
ddI	Didanosine
DEAD	Diethyl azodicarboxylate
DIAD	Diisopropyl azodicarboxylate
DIBAL-H	Diisobutylaluminium hydride
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
FAD	Flavine adenine dinucleotide
GTP	Guanosine triphosphate
G	Guanine
HDR	Homology-directed repair
HIV	Human immunodefficiency virus
HMDS	Hexamethyldisilazane
HONPht	N-hydroxyphthalimide
HSQC	Heteronuclear single quantum coherence
LC-MS	Liquid chromatography – Mass spectrometry
LNA	Locked nucleic acids
miRNA	Micro RNA
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NBS	N-bromosuccinimide
NDP	Nucleoside diphosphate
NGS	Next generation sequencing
NFSI	N-fluorobenzenesulfonimide
NMP	Nucleoside monophosphate
NMR	Nuclear magnetic resonance

NTP	Nucleoside triphosphate
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PNA	Peptide nucleic acid
Pre-mRNA	Pre-messenger RNA
PRR	Pattern recognition receptor
Py	Pyridine
qPCR	Quantitative PCR
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
sgRNA	Single guide RNA
siRNA	Small interfering RNA
T	Thymine
TBAP	Tetrabutylammonium pyrophosphate
TBDMS	tert-Butyldimethylsilyl
TEA	Triethylamine
THF	Tetrahydrofuran
TIPS	Triisopropylsilyl
TK	Thymidine kinase
TLC	Thin layer chromatography
TLR	Toll-like receptor
TMSCl	Trimethylsilyl
TMSOTf	Trimethylsilyl triflate
tRNA	Transfer RNA
p-TSA	p-Toluene sulfonic acid
UBP	Unnatural Base Pair
U	Uracil

1. Introduction

Natural nucleosides and nucleotides are the molecular pillars of life. They form the backbone of DNA and RNA and play essential roles in cellular and viral processes such as genetic information storage, replication, transcription, and translation.[1,2] Beyond their genetic functions, nucleotides also serve as key players in energy transfer, signal transduction, and enzymatic reactions, making them integral to a wide range of biological activities[3]. Their fundamental role explains their abundance in nature and underscores the importance of their research across various scientific fields, from genetics to pharmacology.

While natural nucleosides, nucleotides, and their polymers are foundational to life, their synthetically modified analogs have become indispensable tools in medicine, research, and biotechnology.[4–7] These chemically altered analogs are designed to have enhanced stability, binding affinity, biological activity, or other optimized critical properties achieved through various strategic synthetically introduced modifications.[8] These modifications can occur at all structural levels (base, sugar, phosphate groups), resulting in molecules with improved characteristics or entirely new functionalities that address specific needs. For example, in medicinal applications, certain modifications can make modified analogs, used as therapeutic agents, more resistant to enzymatic degradation, extending their half-life in the body and increasing their therapeutic efficacy.[6,9] They can also improve selectivity and potency against targets, such as viral or cancerous cells, making treatments more effective.[10] In research and biotechnology, specific chemical modifications can enhance the sensitivity, specificity, and efficiency of techniques like DNA amplification, sequencing, and detection. These enhancements optimize existing tools and enable the development of novel methods that facilitate advancements in scientific research and clinical diagnostics. [7,11]

The effectiveness of modified analogs is closely tied to developing efficient and precise synthetic methods. Efficient synthesis is essential to ensure sufficient yield and purity for practical use in clinical therapies or laboratory research. Depending on the complexity of molecules, the synthesis may require sophisticated strategies that would allow the introduction of the desired modifications with high accuracy while minimizing unwanted by-products. Efficient synthetic routes are particularly crucial in the pharmaceutical industry, where the scalability of production and the consistency of the product are important factors in the success of therapeutic agents. Furthermore, the economic viability of producing these compounds on a large scale is often directly linked to the efficiency of the synthetic process.[12,13]

Among the various strategies employed to diversify the chemical space of modified nucleos(t)ide analogs, the utilization of rare sugars and azidation appears particularly interesting.[14,15] D-allose, for example, is an uncommon aldohexose sugar [16] that combines potentially valuable and unique structural features. In its furanose form, D-allofuranosyl nucleoside has an additional hydroxymethyl group at the C5'-position, providing an extra site for chemical modifications. Coupled with its stereochemistry at C2' and C3' positions, which aligns with natural ribonucleoside, D-allofuranosyl nucleoside presents a compelling framework for further exploration. This combination enhances the likelihood of success in biological environments, as the structural similarity to ribonucleoside facilitates compatibility, while the extra hydroxymethyl group opens up new opportunities for functionalization.

Introducing an azide group at the C6'-position is particularly advantageous due to the azide's versatility in organic chemistry. It can serve as a precursor for further modifications or facilitate

the ligation process in oligonucleotide synthesis via the Staudinger reaction [15,17–20] or Cu(I) – catalyzed azide-alkyne cycloaddition (CuAAC)[21,22]. While many studies are related to azidonucleosides[15,20,23], D-allofuranose-based nucleos(t)ides remain underexplored, with only a few studies investigating their synthesis and biological effects. [24,25]

This study seeks to address the underexplored potential of D-allofuranose by developing efficient synthetic routes for C6' azide-modified D-allofuranose uracil nucleoside analogs and, if successful, subjecting them to biological evaluation. The study is divided into two main parts: a comprehensive literature review and an experimental investigation. The review will focus on the roles and applications of modified nucleos(t)ides, examining their synthesis and utility. The experimental part of the study will concentrate on developing a reliable synthetic pathway for C6' azidated D-allofuranose-based nucleos(t)ides to evaluate their antiviral properties.

2. Natural nucleos(t)ides: Building blocks of life

Natural nucleotides are the fundamental building blocks of DNA and RNA, essential macromolecules that store and transmit genetic information in all known life forms. DNA, or deoxyribonucleic acid, is a double-stranded helical molecule that serves as the genetic blueprint of an organism, encoding instructions necessary for the development, functioning, growth, and reproduction of all living things. As Figure 1 shows, each strand of DNA is composed of a backbone made of deoxyribose sugar, phosphate groups, and nucleobase – a nitrogen heterocycle: adenine (A), thymine (T), cytosine (C), and guanine (G) extending from the backbone. [1]

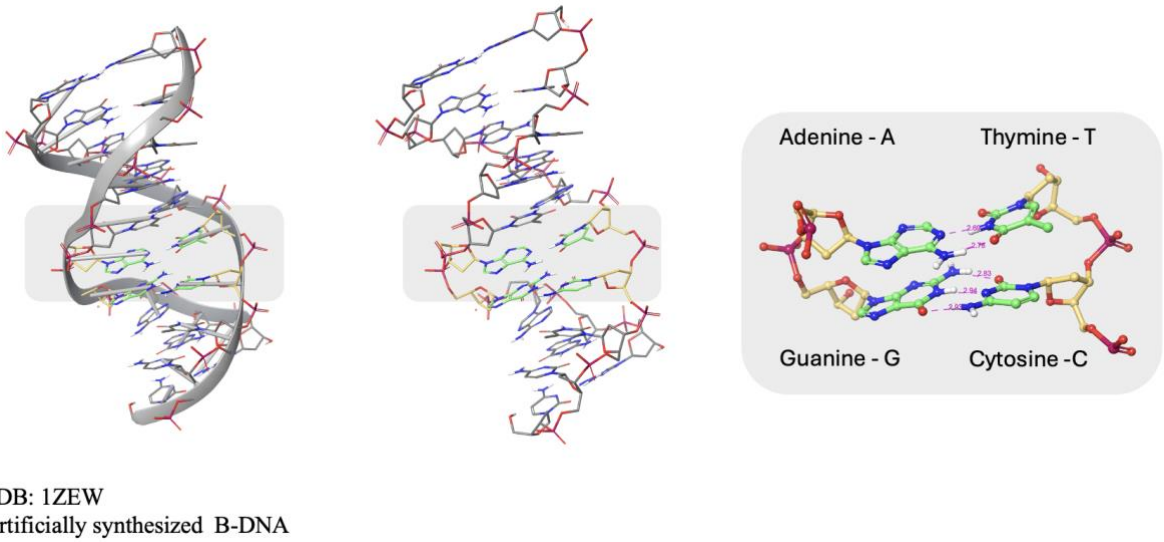


Figure 1: Structure of DNA - Figure illustrating the double-helix structure of DNA. 3D visualization of structures is created using Maestro, Schrodinger Release 2024-3 (LLC, New York, NY 2024)

The specific pairing between these bases—adenine (A) with thymine (T) and cytosine (C) with guanine (G)—is known as complementary base pairing. This pairing is crucial for the replication and transcription processes, where the genetic code from DNA is transferred to RNA to direct protein synthesis. DNA's primary role is to store and transmit genetic information during cell division and reproduction.[2,3]

Watson and Crick's 1953 discovery of DNA's double-helical structure marked a pivotal moment in the history of molecular biology. This discovery explained how genetic information is stored and replicated. It also laid the foundation for modern genetics and biotechnology, enabling the development of techniques such as genetic engineering, DNA sequencing, gene therapy, oligonucleotide drugs targeting to RNA (antisense oligonucleotides, splice switching oligonucleotides, siRNAs) and RNA-vaccines.

In contrast to DNA, RNA, or ribonucleic acid, typically exists as a single strand and plays several versatile roles in the cell. It is involved in protein synthesis, where different forms of RNA, such as messenger (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), work together to translate the genetic code into functional proteins. RNA also plays critical roles in gene regulation, where small RNAs, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), modulate gene expression by targeting pre-mRNA or degrading mRNA to inhibit translation. Structurally, RNA differs from DNA in that it contains ribose sugar and uracil (U) instead of thymine (T), which allows RNA to perform different functions within the cell.[2,3,26]

As mentioned, the monomers of DNA and RNA nucleotides comprise three main structural components: a nucleobase, a five-member sugar, and phosphate groups attached to 5'-carbon. While the nucleobases and their complementarity are key aspects of genetic processes, the presence of phosphate groups is crucial for cellular metabolism and energy transfer. The presence or absence of phosphate groups also distinguishes nucleotides from nucleosides structurally. Nucleosides consist solely of the nucleobase and sugar, lacking the phosphate groups, and nucleotides consist of all three components varying only in the number of phosphate groups attached at the C5' - position (*Figure 2*).

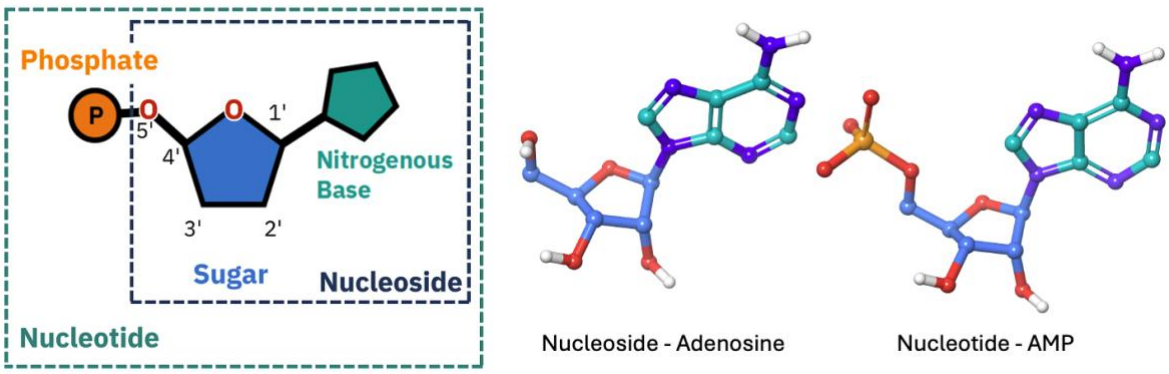


Figure 2: Structure of nucleotides & nucleosides. 3D visualization of structures is created using Maestro, Schrodinger Release 2024-3 (LLC, New York, NY 2024)

Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) are essential energy carriers in the cell. ATP, often called the "molecular currency" of intracellular energy transfer, is used in various cellular processes, including muscle contraction, nerve impulse propagation, and chemical synthesis. GTP, on the other hand, is involved in protein synthesis and signal transduction pathways, particularly those involving G-proteins, which are key players in transmitting signals from the cell surface to the cytosol. Other important examples include cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which function as secondary messengers helping to amplify signals from hormones or other stimuli, playing an important role in cell growth, differentiation, and metabolism. Nucleotides

also involved in synthesis of coenzymes such as adenine dinucleotide (NAD) and flavine adenine nucleotide (FAD). These coenzymes are critical for redox reactions in cellular respiration and other metabolic pathways, participating in hydride transfer.[2,3]

Understanding the structure and natural roles of nucleotides provides significant insights into potential applications of their modified analogs across various fields. These analogs play an important role in advancing medicine, synthetic biology, and research facilitating the development of effective drugs and molecular tools.

3. Chemically modified nucleos(t)ides: Expanding natural potential

Nucleos(t)ide modifications expand their utility beyond natural roles by introducing new properties and functionalities. These changes enable the design of molecules with enhanced traits tailored for specific scientific and therapeutic purposes. Synthetic analogs play a key role in advancing molecular interactions, developing innovative treatments, and creating biotechnological tools. The following sections will explore their applications across medicine, research, and biotechnology, emphasizing their significant impact and importance.

3.1 Modified nucleot(s)ides in medicine

3.1.1 Antiviral applications

In medicine, modified nucleos(t)ide analogs are pivotal in developing antiviral and anticancer therapies. For instance, they have been instrumental in treating human immunodeficiency virus (HIV), hepatitis B, and hepatitis C, significantly improving patient outcomes.[27,28] Nucleotide analogs, such as zidovudine (AZT) and acyclovir, due to their high similarity with natural nucleotides, can incorporate into viral DNA or RNA and terminate chain elongation, effectively inhibiting viral replication. [8] Figure 3 illustrates the structure of acyclovir co-crystallized with the herpes simplex virus DNA polymerase enzyme (PDB ID: 3KD5), highlighting acyclovir's binding interactions and mode of action. In detail, this figure demonstrates how acyclovir binds to the active site of the viral polymerase, inhibiting its function and preventing viral DNA synthesis due to the missing 3'OH group.[29]

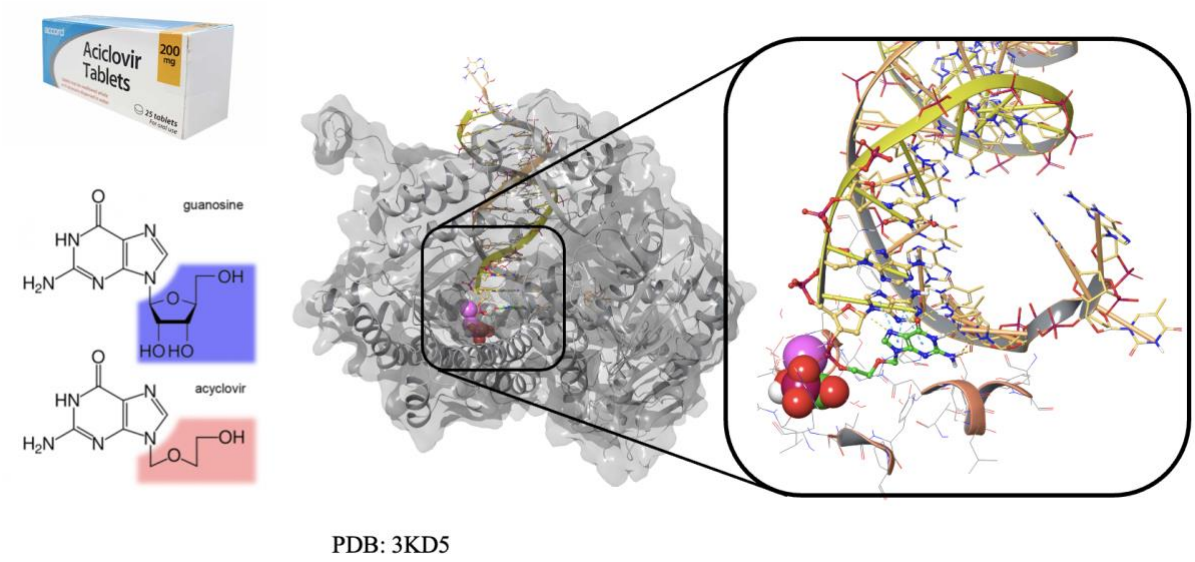


Figure 3. Structure of acyclovir and co-crystallized complex with the herpes simplex virus DNA polymerase enzyme (PDB: 3KD5). 3D visualization of structures is created using Maestro, Schrodinger Release 2024-3 (LLC, New York, NY 2024)

3.1.2 Anticancer applications

The ability of modified nucleosides to interfere with DNA synthesis and repair mechanisms is also highly relevant in cancer research and anticancer drug development.[8] Figure 4 provides a few examples of modified nucleosides widely marketed and used as chemotherapy agents.

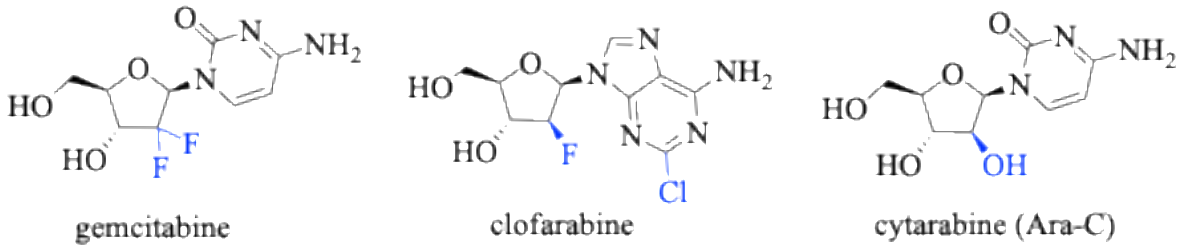


Figure 4. Modified nucleosides used in cancer treatment. 1 – Gemcitabine, 2- Clofarabine, 3 – Ara-C.

Gemcitabine

Gemcitabine (*Figure 4*) is characterized by the gem-difluoro substitution at the 2' position of the sugar ring. It is a potent anticancer agent in treating pancreatic, lung, breast, and ovarian cancers. Introducing two fluorine atoms enhances molecules' resistance to deamination by cytidine deaminase and increases their lipophilicity, allowing for improved cellular uptake. Once inside the cell, gemcitabine is phosphorylated to its active triphosphate form, which gets incorporated into DNA during replication. This results in chain termination after adding one more nucleotide–masked chain termination. This chain of events halts DNA synthesis and leads to apoptosis of rapidly dividing cells.[8,30]

Clofarabine
Clofarabine (*Figure 4*) is another modified nucleoside that combines a 2' -fluoro substitution with a chlorine atom on the purine base. It is used in pediatric acute lymphoblastic leukemia (ALL) and certain types of relapsed or refractory hematologic malignancies. Clofarabin's modifications play crucial roles in its mechanism of action. The 2'-fluoro group, similar to gemcitabine, helps improve stability against enzymatic degradation, increasing compounds' half-life and, thus, improving efficacy. The chlorine atom on the base enhances clofarabine's binding activity to DNA polymerase and ribonucleotide reductase, key enzymes required for DNA synthesis and repair. Once inside the cell, clofarabine is phosphorylated to its active form; as a nucleotide analog, it competes with natural analogs for incorporation into the DNA, leading to chain termination and inhibition of DNA synthesis. Additionally, clofarabine depletes deoxynucleotide pools by inhibiting ribonucleotide reductase, further enhancing its cytotoxic effects on cancer cells.[31]

Cytarabine (Ara-C)
Ara-C (*Figure 4*) is one of the earliest developed modified nucleoside analogs used as an anticancer agent, particularly effective in treating acute myeloid leukemia (AML) and non-Hodgkin's lymphoma. Structurally, it differs from deoxycytidine by having an arabinose sugar instead of ribose or deoxyribose, which makes it less stable and more prone to incorporation errors. Ara-C is also phosphorylated in the cell into its active form, Ara-CTP, which is consequently incorporated into the DNA of cancer cells. However, due to the altered sugar structure, the incorporation of Ara-C leads to faulty DNA elongation and chain termination, preventing further replication. As compared to the other two analogs, gemcitabine and clofarabine, having F at 2' position, the half-life of Ara-C is short due to rapid deamination to its inactive form by cytidine deaminase, which limits its use. [8,30]

The modifications introduced in these nucleoside analogs significantly enhance their therapeutic utility and make them highly effective in targeting rapidly dividing cells, though their effects are more pronounced in certain cancer types. Strategic chemical alterations optimize their pharmacokinetic profiles and increase their potency as chemotherapy agents.

3.1.3 Therapy applications of oligonucleotides

Oligonucleotide-based therapies have emerged as a promising drug modality, leveraging modified nucleotides to enhance therapeutic effectiveness. By modulating gene expression at the molecular level, they provide targeted treatment options for complex diseases. These treatments involve short, synthetic strands of nucleotides designed to specifically bind to complementary RNA – sequences, impacting pre-mRNA and mRNA processing. Antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) are notable examples, each with unique mechanisms. Splice-switching oligonucleotides (SSOs) bind pre-mRNA to alter splicing, gapmer ASOs degrade target RNA via RNase H-mediated cleavage, while siRNAs facilitate mRNA degradation via the RNA-induced silencing complex (RISC), effectively silencing specific genes. These oligonucleotides, incorporating chemically modified nucleotides, offer novel approaches for treating genetic disorders, cancer, and viral infections by directly interfering with gene expression pathways, providing a high degree of specificity with minimized side effects.[32]

Similarly to modified nucleos(t)ide analogs used as antiviral or anticancer agents, the above-mentioned oligonucleotides employed are also chemically altered to improve their stability and effectiveness. ASOs are often modified with the phosphorothioate backbones (replacing the

non-bridging oxygen in the phosphate group with sulfur) to improve stability against enzymatic degradation.. Modifications in the sugar moiety, like 2'-*O*-methyl or 2'-*O*-methoxyethyl, are also commonly used to enhance affinity and reduce immune response.[34] Just taking one example, Inotersen (Tegsedi®), a drug used for hereditary transthyretin-mediated amyloidosis treatment[33] utilizes these modifications. Modified siRNAs typically include 2'-fluoro or 2'-*O*-methyl groups to resist nuclease degradation and avoid immune detection. Patisran (Onpattro®) and Inclisiran (Leqvio®) are examples of siRNA drugs employing these modifications to improve safety and duration of action for conditions like hereditary transthyretin amyloidosis and hypercholesterolemia. Locked nucleic acids (LNAs) are modified forms where the ribose sugar is "locked" by a methylene bridge, which enhances thermal stability and binding affinity to target RNA. Peptide nucleic acids (PNAs) replace the sugar-phosphate backbone with a peptide-like structure, resisting enzymatic degradation and making them powerful tools for diagnostic and therapeutic uses. These modifications are in experimental stages for various indications.

Modified oligonucleotides target previously "undruggable" genetic mutations and diseases. By using specific chemical alterations, these drugs improve safety profiles and extend half-lives, allowing lower dosages and reducing administration frequency. They are especially valuable for diseases with limited treatment options, like genetic disorders, certain cancers, and even viral infections.[32,35]

3.1.4 Vaccines

In more recent developments, the success of mRNA-based vaccines, particularly those developed for COVID-19 by Pfizer-BioNTech and Moderna, highlights another powerful application of modified nucleotides in medicine. These vaccines use chemically modified nucleotides in the mRNA strands to improve stability, reduce immunogenicity, and enhance protein translation efficiency within host cells. [36–38]

There are several existing strategies used to achieve the desired effects, and the pseudouridine substitution is one of them. Unmodified mRNA can trigger an immune response when detected by the body's innate immune system, particularly through pattern recognition receptors (PRRs) like Toll-like receptors (TLR) that recognize foreign RNA. This immune activation can cause unwanted inflammation and degrade the mRNA before it has a chance to produce the desired antigen. The substitution of uridine with pseudouridine prevents this recognition. [39] Pseudouridine is a naturally occurring isomer of uridine that alters the structure of the RNA without changing the genetic information it encodes. This structural change allows the mRNA to evade the immune system, reducing innate immune responses and inflammation while simultaneously enhancing its ability to be translated into protein inside the cells. Figure 5 shows structural differences between a standard nucleoside – uridine and modified analogs, pseudouridine and 1-methylpseudouridine.[36,40,41]

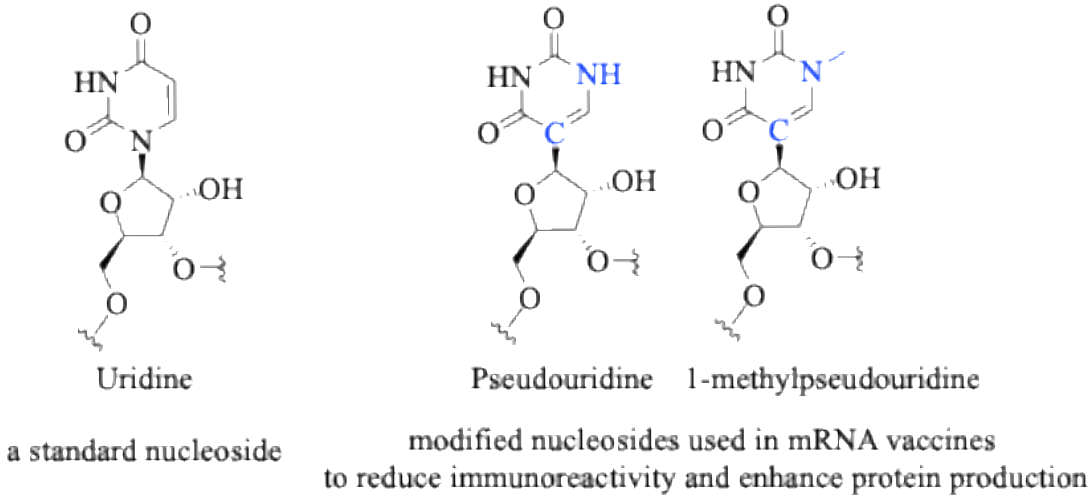


Figure 5. Pseudouridine substitution. Standard uridine (left), pseudouridine (middle), 1-methylpseudouridine (right).

The importance of this and other advancements was further emphasized when the 2023 Nobel Prize in Physiology and Medicine was awarded to Katalin Karikó and Drew Weissman for their pioneering research on mRNA technology, which laid the groundwork for mRNA vaccines. [36–38]

3.2 Modified nucleot(s)ides in biotechnology

In research and biotechnology, modified nucleotides, nucleosides, and oligonucleotides also play significant roles in various methods and applications. One key application is the polymerase chain reaction (PCR), a technique to amplify specific DNA sequences. Modified nucleotides, such as those with fluorescent labels, enable real-time PCR (qPCR), which allows for the quantification of DNA by measuring fluorescence during the amplification process. This modification enhances the accuracy and efficiency of DNA amplification by providing a way to monitor the reaction as it progresses.[42,43] In addition to fluorescent labels, modifications such as biotinylation are used to detect and quantify nucleic acids in various assays. Biotinylated nucleotides can be incorporated into DNA or RNA during synthesis and subsequently detected using streptavidin-conjugated enzymes or fluorophores, facilitating highly sensitive detection methods.[44,45]

Advanced techniques like next-generation sequencing (NGS) heavily rely on modified nucleotides. For example, reversible terminator nucleotides are used in Illumina sequencing platforms. These nucleotides have a reversible blocking group at the 3'-end, allowing for the sequential addition of nucleotides and enabling massively parallel sequencing.[46] This modification improves the accuracy and efficiency of sequencing by allowing precise control over nucleotide incorporation and detection. CRISPR-Cas9 - genome editing method also benefits from modified nucleotides and oligonucleotides. Single-guide RNAs (sgRNAs) with chemically modified nucleotides can enhance the stability and binding affinity of the guide RNA to the Cas9 enzyme, improving the efficiency and specificity of genome editing.[47] Also, modified donor DNA templates can be used in homology-directed repair (HDR) to increase the likelihood of successful gene insertion or correction.[48]

3.3 Modified nucleot(s)ides in synthetic biology

In synthetic biology, modified nucleotides are used to engineer novel genetic circuits and synthetic organisms with tailored functionalities. For instance, unnatural base pairs (UBPs) can be incorporated into DNA, expanding the genetic alphabet and allowing for the creation of proteins with novel amino acids and functions. [49] These innovations have potential applications in biofuel production, environmental remediation, and industrial biotechnology. [50]

4. D-allofuranosyl nucleos(t)ides: Exploration and progress in modified nucleoside research

D-allohexafuranose-based nucleosides represent a novel and underexplored class of modified nucleos(t)ides compared to the well-studied ribose or deoxyribose-based analogs. D-allose, a sugar component of D-allohexafuranosyl nucleosides, is an epimer of D-glucose and features a distinctive combination of structural features. These include stereochemistry at the C2' and C3' positions matching deoxyribose and ribose in their furanose forms, along with an additional CH_2OH group at the C5' position, which serves as an extra site for chemical modification (*Figure 6*). This combination offers potential advantages for functionalization, making D-allofuranose-based nucleos(t)ides promising targets for study and potential candidates for therapeutic applications.[14,16,51]

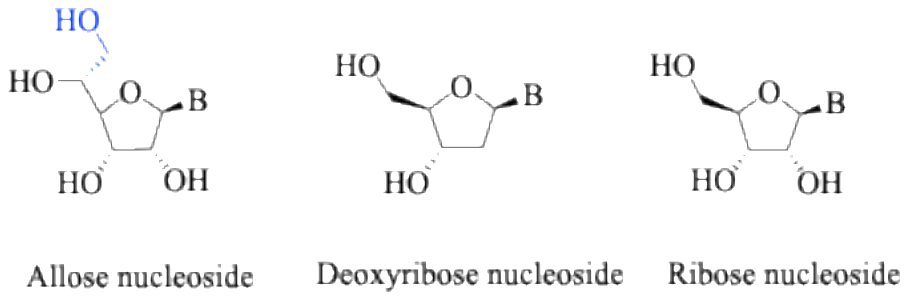


Figure 6. Comparison of D-allose-based nucleoside with deoxy- and ribose sugar-based analogs.

4.1 D-allose

The limited research on D-allofuranose-based nucleosides might be largely attributed to the natural scarcity of D-allose. D-allose is a rare aldohexose sugar, also known as "fetus sugar", because it was found in the umbilical cord blood and sera of pregnant women.[16] Although there is no significant evidence of D-allose naturally occurring in animals, small amounts of free D-allose have been found in certain medicinal plants like *Halodule pinifolia*, *Tamarindus indica,* and *Crataeva nurvala.*[52–54] Its levels are very low in nature, and its physiological role remains largely unknown.

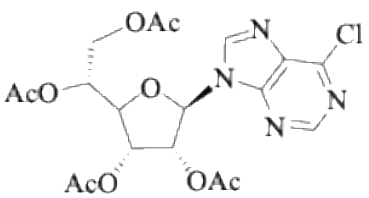
Despite its rarity, D-allose has been researched for its promising biological and pharmacological effects. Several studies demonstrated that D-allose, as such, exhibits anticancer properties, primarily through inhibiting cancer cell growth by altering energy metabolism and affecting glycolysis. By downregulating glucose transporters, D-allose reduces glucose availability to cancer cells, effectively starving them of the energy required for

proliferation. Studies have shown tumor suppression in liver and colon cancer models treated with D-allose.[55–59] Some attempts were undertaken to develop D-allose-based anticancer agents [60]. D-allose has also been shown to enhance the efficacy of existing anticancer treatments, such as 5-fluorouracil (5-FU) and cisplatin.[61,62] In combination therapies, D-allose can increase the sensitivity of cancer cells to these chemotherapeutic agents, thereby improving their effectiveness. In addition to anticancer potential, D-allose demonstrates protective effects in ischemic conditions, notably against reperfusion injury, a common issue in organ transplantation. Studies have highlighted D-allose's potential to reduce liver damage during hepatic ischemia and improve the overall success rate of organ transplants by mitigating oxidative damage and promoting cellular recovery.[63]

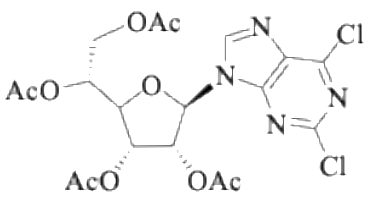
Due to its low natural abundance, D-allose is usually produced from more common sugars through enzymatic or chemical methods. One of the most widely used methods for its production involves the epimerization of D-glucose. This process changes the stereochemistry at the third carbon C3' of D-glucose to convert it to D-allose. The conversion is typically catalyzed by specific enzymes, such as L-rhamnose isomerase. The chemical synthesis of D-allose is complex, requiring multiple steps and harsh reagents, which can reduce overall yields and make large-scale production less feasible.[64,65]

4.2 D-allofuranosyl nucleosides

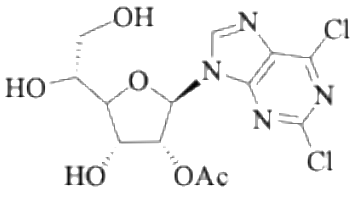
While D-allose has shown promising biological activities, research on D-allofuranose-based nucleosides remains limited. Only a few studies report the synthesis and initial biological evaluation of D-allofuranose derivatives. Besada et al. (2010) report the synthesis and cytostatic activity evaluation of D-allofuranose-based purine nucleosides. They utilized 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose as the starting material, a protected form of D-allofuranose that allows for selective modifications during the synthesis. The research focused on producing several derivatives using different purines and evaluating their cytostatic activity against various human cancer cell lines, including MCF-7 (breast cancer), HeLa-229 (cervical cancer), and HL-60 (leukemia). Among synthesized compounds, 9-(2,3:5,6-tetra-*O*-acetyl-β-D-allofuranosyl)-2,6-dichloropurine (compound 9, *Figure 7*) showed the most significant cytostatic activity, particularly against the HL-60 cell line, with efficacy similar to that of cisplatin, a well-known chemotherapy drug. Another compound, 9-(2,3:5,6-tetra-*O*-acetyl-β-D-allofuranosyl)-6-chloropurine (compound 7, *Figure 7*), displayed selective cytostatic activity against HL-60 cells, while 2,6-dichloropurine derivatives generally showed better activity across different cell lines. The results suggested that the acetylated nucleoside derivatives had increased lipophilicity, which enhanced their ability to penetrate cancer cells and exert cytotoxic effects. [66]



Compound 7



Compound 9



Compound 10

Figure 7. Cytostatically active β-D-allofuranosyl purine nucleosides derivatives. Compound 7 IC_{50} = 79.3 (±5.1) μM in HL-60 cells and > 100μM in MCF-7 and HeLa-229 cell lines. Compound 9 IC_{50} = 16.1 (±0.9) μM, 22.4 (±0.8) μM, and 24.2 (±1.1) μM in HL-60, MCF-7, and HeLa-229 cell lines, respectively. Compound 10 IC_{50} = 45.0 (±0.7) μM, 36.3 (±1.0) μM, and 36.3 (±1.2) μM in HL-60, MCF-7, and HeLa-229 cell lines, respectively.

Other studies have explored 6'-deoxy-β-D-allofuranosyl nucleoside analogs. In these cases, D-allofuranose served as a starting material or intermediate to achieve high yields of ribofuranose formation. The presence of the additional CH_2OH group at C5' was important in facilitating the prevalence of the furanose form, though this group was subsequently removed in later steps to form ribofuranosyl analogs.[67,68]

5. Synthetic methodologies for modified nucleos(t)ides: Approaches and challenges

Given the significant and broad applications of modified nucleos(t)ides in therapeutic, diagnostic, and research fields, their efficient synthesis is essential for advancing scientific understanding and practical use. Each stage in the synthesis process directly impacts the functionality and the utility of resulting analogs.

Typically, the synthesis process for modified nucleos(t)ide analogs involves several key steps: preparation of the desired sugar component, attachment of the nucleobase, introduction of specific modifications, and phosphorylation. The sequence and methods for each step depend on the chosen synthetic strategy, with every stage supported by thoughtful use of protecting groups' chemistry and purification techniques to ensure precision and high-quality results.

In the following sections, each of these steps will be explored in detail. The discussion will outline the rationale behind various synthetic strategies described in the literature while primarily focusing on the specific methods employed in this work. This discussion will highlight the advanced techniques and methodologies used in the field, providing a deeper understanding of the synthetic approaches explored in this study.

5.1 The synthesis of sugar components. D-allohexofuranose.

The synthesis of modified nucleosides often begins with the preparation of the desired sugar component, which is a critical step for ensuring the correct stereochemistry and functionality of the final nucleoside analog. [14] In this context, the sugar backbone refers to the modified carbohydrate component of the nucleoside. In this particular study, we worked with D-allose, trying to achieve the efficient preparation of its peracetylated hexofuranose and especially β-hexofuranose form over others.

One of the potential challenges associated with carbohydrate component preparation, in general, is achieving the desired sugar form, which is either a furanose or pyranose, a five- or six-membered ring, depending on the final application of the modified analog. Both forms exist in equilibrium, influenced by several factors, including the stereochemistry of the sugar, the nature of substituents, reaction conditions, and the presence of catalysts or enzymes. Furanose forms are typically less stable than pyranose forms due to ring strain in the five-membered furanose ring compared to the six-membered pyranose ring. [69] This inherent instability makes it more challenging to selectively synthesize or isolate the furanose form, as the reaction

conditions that favor furanose formation must be carefully controlled to prevent reversion to the more stable pyranose form. [70] Figure 8 illustrates the structural conformers of D-allose.

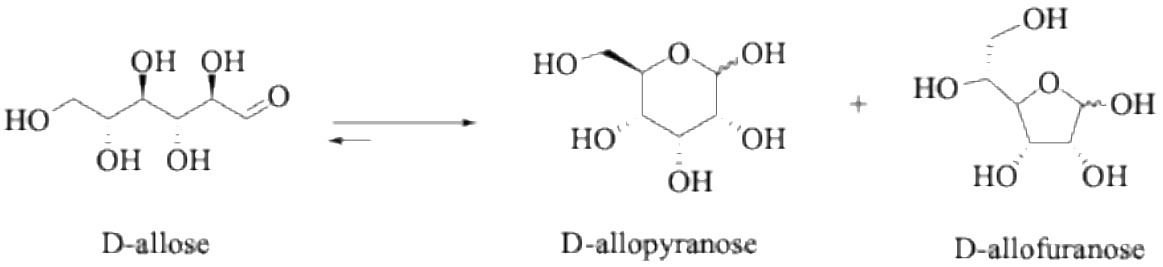


Figure 8. Furanose, pyranose, and linear forms of D-allose.

Several synthetic strategies to overcome the challenge of favoring the furanose form over the pyranose form have been previously reported in the literature for different sugars. Some notable approaches include selective protecting groups that favor and lock the furanose form during synthesis [71–73], manipulating the reaction conditions to favor furanose formation, and employing enzymatic catalysis [70,74].

In this work, we theoretically and practically explored two approaches. The first approach utilized boric acid to stabilize the reaction intermediate in its furanose form before forming the peracetylated compound. This method, based on the work of Furneaux et al. (2000), is particularly effective because boric acid forms complexes with the diols in the sugar, stabilizing the furanose over the pyranose form during acetylation. Removing boric acid from the mixture by co-evaporation in the form of trimethyl borate allows for the acetylation to proceed efficiently. In the context of D-glucose, this method resulted in a high yield of furanose forms, with more than 93% of the product being furanose and a ratio of β- to α-furanose of 1:1.8 (*Figure 9*). When applied to D-allose, this method also proved effective, although the specific outcomes were slightly different. The method yielded approximately 66% of the furanose forms (with a mixture of α- and β-furanose isomers) and significantly reduced the formation of pyranose isomers. Specifically, the β-furanose form of D-allose was produced in a yield of approximately 33%, which is noteworthy compared to traditional methods that do not employ boric acid stabilization.[73] This one-pot method appeared attractive due to the reduced number of synthetic and work-up steps. However, the main drawbacks of this method could be associated with the anomeric selectivity of the sugar's hydroxyl groups, impacting the yield of furanose formation, as well as concerns related to the effectiveness of subsequent purification steps for the different conformers/anomers. Additionally, the formation of a mixture of pyranose and furanose products may potentially complicate further processing.

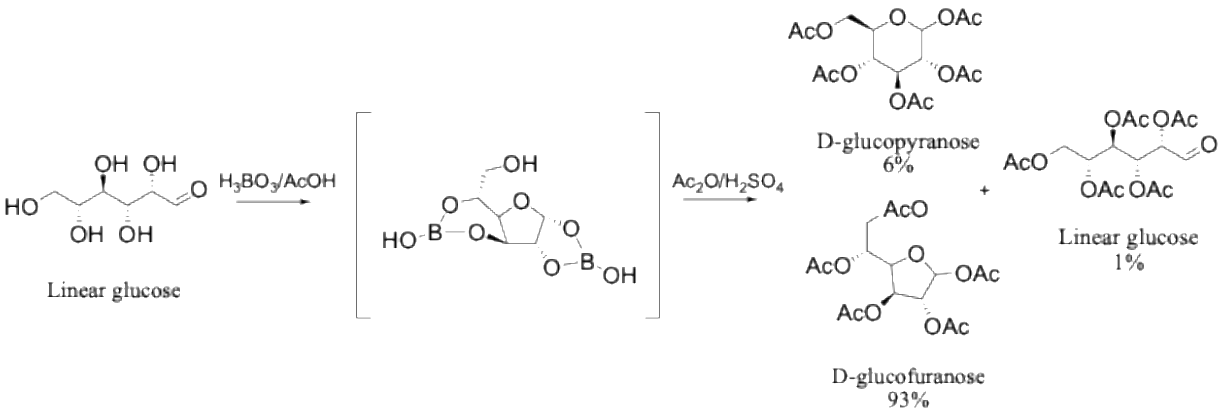


Figure 9. 'One-pot' synthesis of penta-*O*-acetyl-*β*-D-glucofuranose.[73]

The second approach considered in this work involved initiating the synthesis with a different starting material—commercially available 1,2:5,6-di-*O*-isopropylidene-D-allofuranose (**1**). This compound represents a protected furanose conformer, and according to the literature, the one-pot acid-catalyzed deacetalization - peracetylation of this starting material leads to the exclusive formation of the peracetylated D-furanose form [71] with α/β anomers in a 1:3 ratio [72]. (*Figure 10*)

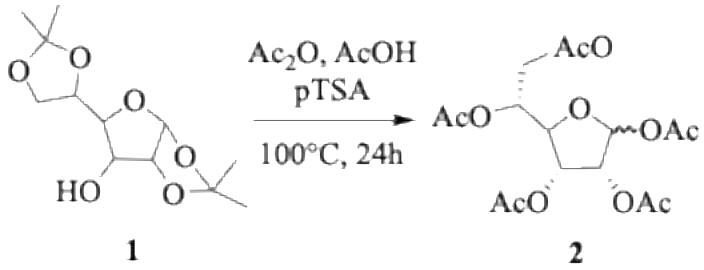


Figure 10. One-pot acid-catalyzed deacetalization - peracetylation of 1,2:5,6-di-*O*-isopropylidene-D-allofuranose resulted in peracetylated D-allofuranose.[71,72]

This approach was considered attractive due to the ease of preparing the desired hexafuranose form and the formation of a single product rather than a mixture of isomers. However, the separation of the α/β anomers would require further exploration in the base addition step or further.

5.2 N-glycosylation and anomeric control.

Adding the nucleobase is a more straightforward process compared to the complexity of preparing the sugar component. Once the desired sugar conformer is obtained, the effective introduction of the nucleobase at the C1' position can be achieved through several well-established methods. In the current work, we focused on the incorporation of uracil. A few synthetic approaches, proven successful in similar contexts, are outlined below

One of the most common methods for nucleobase addition is the Vorbrüggen glycosylation, a widely used approach due to its efficiency and versatility.[75] This method involves the activation of the sugar with a silylating agent, such as trimethylsilyl chloride (TMSCl) or hexamethyldisilazane (HMDS), followed by the addition of the nucleobase in the presence of a Lewis acid catalyst like trimethylsilyl triflate (TMSOTf). The reaction proceeds under mild

conditions and typically provides high yields of the desired nucleoside.[66,76] The Vorbrüggen method is particularly advantageous when working with uracil, as it facilitates the formation of the glycosidic bond at the C1' position with good regioselectivity and minimal side reactions. The Vorbrüggen reaction also follows the rules of trans-glycosidation and prefers the formation of β-anomer, which was the desired anomer in that work.[76]

During the glycosylation process, the formation of anomers is a common occurrence. Anomers are stereoisomers that differ at the anomeric carbon, which is the carbon at the site of the glycosidic bond (C1'). In the case of furanoses, both α- and β-anomers can form, but their ratio depends on the reaction conditions, the specific glycosylation method used, starting materials, and the usage of protecting groups.[76,77] The Vorbrüggen glycosylation method often favors the formation of β-anomer, which was the desired stereoisomer of this study. The β-anomer is typically more stable due to steric and electronic factors, and it also resembles the conformation of natural nucleos(t)ides, which makes it more attractive for possible biological applications.[78] The desired anomer formation is important to consider at early synthetic stages since the efficient separation of sugar or nucleos(t)ides anomers may be challenging using trivial chromatographic techniques.

Another method worth mentioning is the direct glycosylation of protected sugars with a pre-activated nucleobase. This approach can be efficient, especially when the sugar is already in its desired conformer, but it often requires more rigorous conditions and may lead to lower yields or the formation of unwanted side products.[79–81] Additionally, enzymatic glycosylation has been explored as a more selective but less commonly used method, relying on glycosyltransferases to catalyze the N-glycosidic bond formation under biocompatible conditions.[82]

In this work, the Vorbrüggen method was chosen for the addition of uracil to the D-allohexafuranose conformer. This decision was based on several factors. Firstly, the reaction conditions of the Vorbrüggen glycosylation are well-suited to preserving the integrity of the sugar conformer, which is crucial after the careful synthesis and stabilization of sugar in its furanose form. Secondly, the high efficiency and yield of the Vorbrüggen method make it an ideal choice for synthesizing nucleosides on a scale suitable for further modifications or applications. Finally, the method's compatibility with uracil, a relatively reactive nucleobase for the N-glycosidation, ensures that the glycosylation results in high purity and yields of the desired nucleoside and a high prevalence of its β -anomer form.

5.3 Phosphorylation

Phosphorylation, the attachment of phosphate groups to nucleosides to form nucleotides, is another critical step in synthesizing nucleotide analogs. This modification is essential for nucleotides' biological functionality, as it allows them to participate in enzymatic reactions, cellular energy transfer, and nucleic acid synthesis. Several approaches exist for the phosphorylation of nucleosides, ranging from direct phosphorylation to solid-phase chemical synthesis. The choice of method depends on the desired phosphate form (mono-, di-, or triphosphate), the specific nucleoside, and its reactivity.

Direct phosphorylation

This method involves using phosphorylating agents to directly attach one or more phosphate groups to the nucleoside to form monophosphates or phosphate esters. Common reagents

include phosphorus oxychloride ($POCl_3$) and tributylphosphine (PBu_3), which react with nucleosides to form phosphate esters. For triphosphates, more complex phosphorylation reagents, such as cyclic triphosphates or ATP analogs, may be used.[83] One of the most common direct phosphorylation methods is the Khorana method, where nucleosides are treated with phosphorylating agents such as $POCl_3$ or tetra-n-butylammonium phosphate in the presence of dicyclohexylcarbodiimide (DCC). This reaction typically occurs in an organic solvent such as pyridine or tetrahydrofuran (THF) and is followed by purification steps to isolate the desired nucleotide.[84,85]

Chemical synthesis of triphosphates

The synthesis of nucleotide triphosphates can be challenging due to the instability of triphosphate bonds. One common strategy involves using imidazole or carbodiimide-based reagents to activate phosphate groups, followed by coupling with the nucleoside. These reactions are typically conducted in aqueous or polar aprotic solvents like dimethylformamide (DMF) to stabilize the intermediates. Another method is the Yoshikawa phosphorylation process, where the nucleosides is first reacted with $POCl_3$, followed by the addition of inorganic phosphate. This reaction allows for the selective introduction of a triphosphate group under mild conditions.[84,86,87] A method similar to Yoshikawa's has been chosen for the practical exploration in this work.

Enzymatic phosphorylation

Enzymatic phosphorylation is a biocompatible method that involves the use of kinases, enzymes that transfer phosphate groups from donor molecules like ATP to nucleosides. This method is commonly used in biochemistry and biotechnology applications where high selectivity is required. Kinases such as nucleoside monophosphate kinases or nucleosides diphosphate kinases are used to convert nucleoside monophosphates to di- or triphosphates.[88,89]

Metabolic incorporation & Intracellular phosphorylation

Many nucleoside analogs, especially those used in antiviral and anticancer therapies, rely on intracellular phosphorylation for their activation. The prodrug approach, where a nucleoside is administered in an unphosphorylated form and then phosphorylated by cellular enzymes, is commonly employed to improve bioavailability, reduce toxicity, and allow the drug to reach its target cells efficiently. Intracellular phosphorylation occurs in three sequential stages: mono-, di-, and triphosphorylation, each facilitated by different enzymes. The first step is monophosphorylation, where the nucleoside is converted to its monophosphate (NMP) form. This reaction is carried out by specific nucleoside kinases.

For example, thymidine kinase (TK) is responsible for the monophosphorylation of pyrimidine analogs like thymidine, while deoxycytidine kinase (dCK) is responsible for phosphorylating cytidine and other purine analogs. The nucleoside monophosphate is further converted to its diphosphate form (NDP) by nucleoside monophosphate kinases and finally reaches its activated triphosphate form (NTP) by nucleoside diphosphate kinase conversion.[88,90,91] This phosphorylation cascade is essential for the activity of many therapeutic nucleoside analogs. For the previously mentioned acyclovir, the phosphorylation is carried out specifically by viral kinases, allowing the drug to be activated predominantly in infected cells, thus reducing toxicity in normal cells. Similarly, gemcitabine and clofarabine, both anticancer agents, rely on intracellular phosphorylation to form their active NTP form, which inhibits DNA replication in rapidly dividing cells.[91]

5.4 The introduction of different modifications

Modifications can be introduced at various stages of nucleos(t)ide synthesis, allowing for strategic alterations to various structural parts of the nucleoside, including the sugar moiety, the nucleobase, or the phosphate groups in the case of nucleotides. The successful introduction of such modifications requires a well-planned protecting group strategy to ensure the reactivity of desired functional groups while protecting others. Protecting groups are essential because they allow selective reactions to occur at specific sites of the nucleoside, preventing undesired reactions at other reactive positions. For instance, hydroxyl groups in the sugar moiety can interfere with reactions intended for the nucleobase or a phosphate group, so they must be temporarily masked by protecting groups like tert-butyldimethylsilyl (TBDMS) or acetyl groups during intermediate stages of synthesis. [92]

From the perspective of the practical work in this study, azidation was the most relevant modification. The introduction of an azide group at the C6'-position in D-allofuranose nucleoside was particularly interesting due to its versatile applications in click chemistry and broader functional potential. The extra C5' CH_2OH group in D-allofuranose makes this sugar especially suited for this functionalization, distinguishing it from other sugars used in nucleosides. While C6' azidation was the primary focus of the practical work, the literature review includes an overview of other relevant modifications equally significant in the broader context of nucleoside chemistry.

5.4.1 Modifications to the Sugar

The sugar moiety is a common site for modifications. Modifications to the sugar substructure can significantly impact chemical properties, structural conformation, biological activity, and stability of designed modified nucleos(t)ides analogs.[14] Figure 11 illustrates the key positions within the nucleoside structure available for introducing modifications using D-allofuranose as an example, alongside examples of molecules showcasing different types of modifications, as discussed in the following sections.

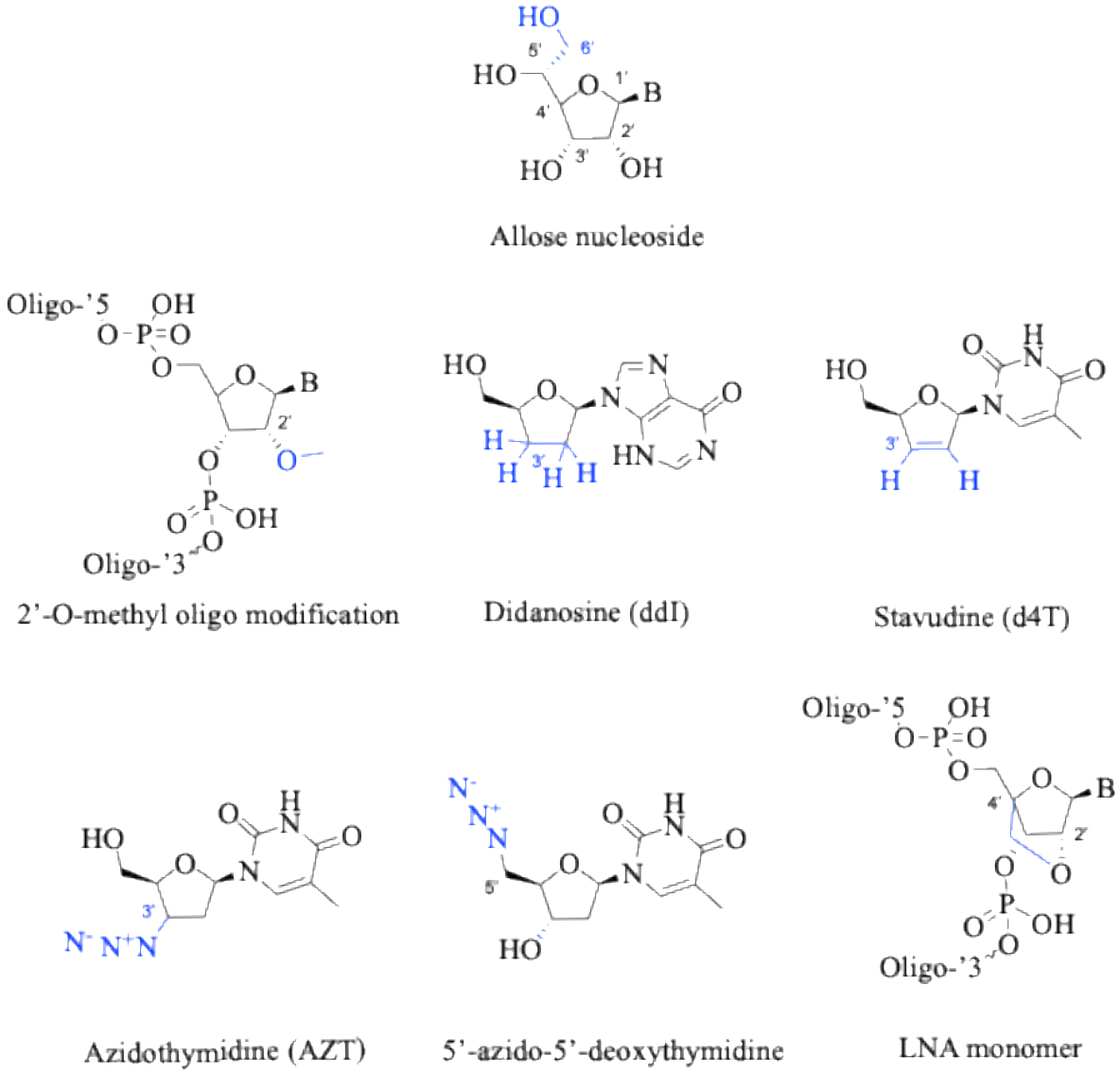


Figure 11. Allose nucleoside modification positions at the sugar core are enumerated 1' - 6'. Examples showcasing different modifications with the modifications highlighted in blue color.

Azide's introduction at the C6'/OH position

Azides are particularly useful in carbohydrate chemistry due to their role as valuable precursors of amines and their widespread applications in click chemistry [15,93]. In this study, we sought to develop efficient methods for introducing the azide group at the C6' position of D-allofuranose-uracil nucleoside. The C6' site was selected because it offers an additional functional handle absent in natural nucleosides. This additional group allows for greater flexibility in derivatization strategies, making it especially suitable for applications such as molecular labeling, bioconjugation, and drug design.

Introducing the azide into sugar typically involves substituting a hydroxyl group with a better-leaving group, followed by azidation. The azidation of the 6'OH group requires the protection of other present hydroxyl groups and is usually achieved through a nucleophilic substitution, where the hydroxyl group is first converted into a mesylate[94], or tosylate, which is then displaced by an azide ion (N_3^-). Activating agents such as methane sulfonyl chloride (MsCl),

tosyl chloride (TsCl) may be used. The activation is carried out in the presence of a base such as triethylamine (TEA) or pyridine (Py). The source of azide ions is commonly sodium azide NaN_3, and the reaction is often carried out in DMF – dimethylformamide. Heating might be required to ensure the complete substitution. The resulting azide-modified sugar can then be used as such, further modified or linked ato an alkyne via click chemistry, and form stable 1,2,3-triazole linkages.[15,22]

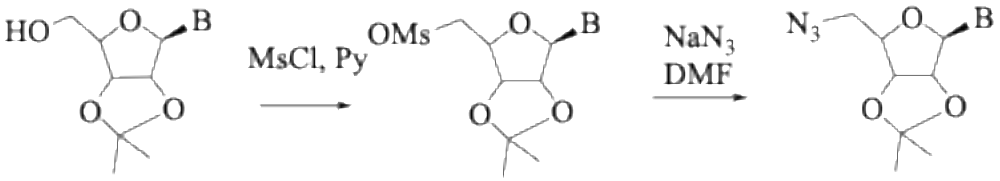


Figure 12. Azidation protocol.[94]

Alternative azidation reactions include one-step azidation of halogenated nucleoside such as commercially available 5'-chloro adenosine or the one-pot azidation with PPh_3 and CBr_4 halogenating agents, resulting in excellent yields.[18] (*Figure 13*).Yet another method for introducing an azide group involves the Mitsunobu reaction, which can be particularly useful when direct nucleophilic substitution is not feasible or yields poor results. In the Mitsunobu reaction, a primary or secondary alcohol is transformed into a nucleophile by reacting it with triphenylphosphine (PPh_3) and a diethyl azodicarboxylate (DEAD) reagent. This allows the alcohol to undergo inversion and react with hydrazoic acid (HN_3) or sodium azide.[95]

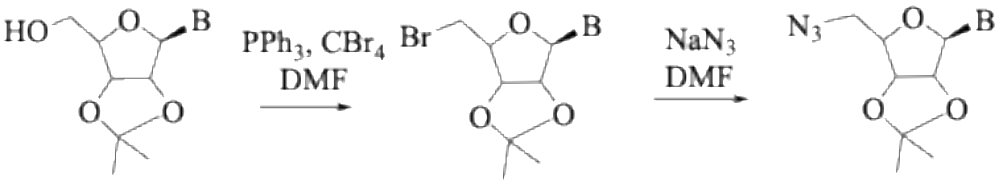


Figure 13. Azidation protocol.[18]

One example of a modified nucleoside with azide functionality used as a therapeutic agent is azidothymidine (AZT, *Figure 11*), an antiretroviral drug used to treat HIV in pediatric and adult patients. [96] In AZT, the azide group replaces the 3'OH group, preventing the elongation of the DNA chain during reverse transcription and thus inhibiting viral replication. Another example, 5'-azido-5'-deoxythymidine, has been used to synthesize clickable DNA probes, enabling the attachment of various reporter groups via click chemistry.[15]

Other modifications

2'-Fluoro and 2'-O-Methyl modifications

Modifications at the C2' position, such as introducing a fluorine atom or a methoxy group, are widely employed to enhance stability and improve therapeutic efficacy in anticancer agents and RNA-based therapies. Previously discussed anticancer agents such as gemcitabine and clofarabine (*Figure 11*) rely on fluorine substitutions at the C2' position to improve resistance to enzymatic degradation, thereby increasing their stability and half-life in biological systems.[9] The synthesis of both compounds is similar and involves an early-stage fluorination of a precursor molecule, typically using reagents like diethylaminosulfur trifluoride (DAST) or

Deoxo-Fluor. These fluorinating agents selectively replace hydrogen atoms at the C2' position with fluorine, requiring controlled reaction conditions to avoid side reactions and ensure high yield.
The protocol described for synthesizing gemcitabine and clofarabine by Yana Cen and Anthony A. Sauve (2010) involves another efficient method that relies on 2-deoxyribonolactone as a starting material. In both cases, lactone is first protected by triisopropylsilyl (TIPS) groups. For gemcitabine, the fluorination step is carried out using N-fluorobenzenesulfonimide (NFSI) and LiHMDS at low temperatures (-78ºC), producing a 2 -deoxy-2,2-difluororibonolactone. Following this, the compound undergoes reduction with DIBAL-H to give a lactol intermediate, which is then mesylated and coupled with bis(trimethylsilyl)cytosine. After TMAF deprotection, the final gemcitabine is obtained with an improved yield of 17%.[97] Similarly the synthesis of clofarabine begins with fluorination of lactone with NFSI, followed by reduction with DIBAL-H. The mesylated sugar is then coupled with 2,6-dichloropurine to form the nucleoside. The nucleoside undergoes further aminolysis to replace one of the chlorines with an amino group. Finally, the desired b-anomer is obtained in an overall yield of 38%, which is higher in this method. This selective fluorination is crucial for the drug's ability to terminate DNA chains during replication. It ensures the necessary stability to evade rapid degradation by enzymes like adenosine deaminase, prolonging their activity in the bloodstream.

2'-O-methyl modifications are widely used to enhance the stability of RNA molecules, particularly in siRNA and antisense oligonucleotide therapies. [98–100]The synthesis of these modified nucleosides involves methylating the hydroxyl group at the C2' position of ribose. Methyl iodide (MeI) or dimethylsulfate (DMS) might be used as methylating agents. Prior to methylation, protecting groups such as TBDMS are often used to block other reactive hydroxyl groups, ensuring the methyl group is introduced selectively at the C2' position.[101] 2'-O-methylated oligoribonucleotide gains resistance to nucleases, improving the stability and efficacy of RNA-based therapies in biological systems.[98]

O2' – C4' Methylene bridge

Locked Nucleic Acids, or LNAs, are a prominent example of sugar modifications in oligonucleotide design. LNAs enhance the stability of nucleotides by introducing a methylene bridge that locks the ribose in the C3'-endo conformation (*Figure 11*). This structural rigidity enhances the thermal stability of the resulting nucleic acid duplexes, making LNAs highly effective in antisense oligonucleotide (ASO) and siRNA therapies. LNAs show an increased affinity for complementary RNA sequences, a critical advantage in improving hybridization efficiency and specificity in gene silencing applications.[102]

The synthesis of LNA-modified nucleosides is complex and requires multiple synthetic steps. Typically, it starts with protecting functional groups to prevent unwanted side reactions. The methylene bridge might be introduced using bridging agents such as methylene iodide in the presence of a base.[103]

3' Modifications and their synthesis

Beyond the C2' position of nucleoside analogs, modifications at C3' are also critical for enhancing and optimizing analog properties. For example, 3'-deoxy modifications eliminate the hydroxyl group at the C3' position, which can halt the DNA synthesis by preventing the elongation of the nucleotide chain, thus acting as chain terminators. The synthesis of 3'

modifications typically involves selective protection of other hydroxyl groups and the subsequent removal of the C3' hydroxyl group through chemical or enzymatic methods. Chemical methods include reduction with triphenylphosphine (PPh3) in the presence of diethyl azodicarboxylate (DEAD), commonly referred to as the Mitsunobu reaction.[104] This 3' modification is essential in halting DNA or RNA elongation, making them invaluable in the treatment of viral infections. In addition to previously discussed acyclovir (*Figure 2*), didanosine (ddI) and stavudine (d4T) (*Figure 11*) exploit the same mechanism of action and are used in HIV treatment.[4,10,28,105]

5.4.2 Modifications to the Nucleobase

Modifying the nucleobase can alter the base-pairing properties, influence gene regulation, and improve the efficacy and functionality of nucleosides and nucleotides in various applications. The synthetic strategies employed depend on the specific nature of the base – whether it's a purine or pyrimidine – and its available reactive sites. The timing of these modifications within the overall synthetic route also impacts their efficiency and practicality. While the current work does not focus on base modifications, some relevant examples and brief discussions of their applications and synthetic strategies are presented for context.

Introducing halogen atoms into nucleobases, such as bromine and chlorine, can enhance their binding affinity and specificity. Halogenation is commonly used in nucleotide analogs designed for molecular imaging and structural studies. For example, 5'-bromo-2'-deoxyuridine (BrdU) is a well-known thymidine analog where bromine substitutes for a hydrogen atom at the 5-position. BrdU is widely used in molecular biology as a tool for labeling proliferating cells during DNA synthesis, allowing to track cell division. Its synthesis involves brominating 2'-deoxyuridine using reagents like bromine or N-bromosuccinimide (NBS). The reaction typically occurs in an aqueous or alcohol solvent at low temperatures to achieve high selectivity and avoid overbromination. The resulting BrdU is then used in research and clinical applications, especially in cancer studies, where cell proliferation is a key metric. [106,107]. Halogenated bases are also used in X-ray and NMR studies to investigate the structures of nucleic acids, as the halogen enhances the contrast in imaging techniques [108]

The chlorine atom at the 2-position of the purine base in the previously discussed clofarabine (*Figure 4*) also offers several key advantages. Some to mention include increased lipophilicity, stability, and binding affinity. In the case of clofarabine, the base is chlorinated before the glycosylation step. Common reagents for chlorination include phosphorus oxychloride ($POCl_3$) or thionyl chloride ($SOCl_2$) in the presence of a suitable base, such as triethylamine (TEA). These reagents facilitate the selective substitution of a hydrogen atom at the 2-position with a chlorine atom. The reaction typically proceeds under heated conditions to ensure a high degree of substitution.[109] After the successful chlorination, the modified base is attached to the desired sugar moiety via Vorbrüggen or other relevant glycosylation methods. [97]

5.4.3 Modifications to the Phosphate Group

The phosphate group can also be modified to alter the properties of nucleotides, affecting their stability, pharmacokinetics, and cellular uptake. The following section briefly discusses a few examples (*Figure 14*).

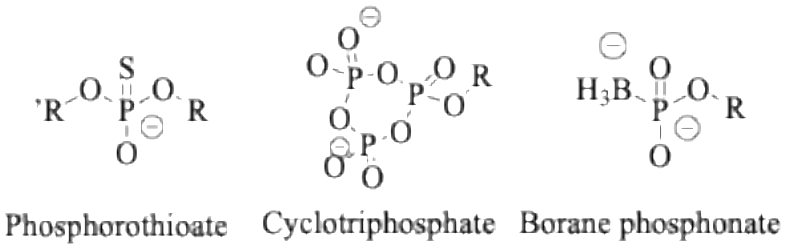


Figure 14. Phosphate group modifications. R-nucleoside.

Phosphorothioate Modifications

Substituting one of the non-bridging oxygen atoms in the phosphate group with sulfur forms phosphorothioate linkages. (*Figure 14*) These modifications increase the stability of nucleic acids against nucleases and are used in antisense oligonucleotides (ASOs) and small interfering RNA (siRNA) therapeutics [110,111]. The synthesis of phosphorothioate-modified nucleotides involves using sulfurizing agents, such as phosphorus pentasulfide or sulfur transfer reagents, during solid-phase oligonucleotide synthesis. The process is relatively simple. [112]

Cyclophosphate modifications

Cyclophosphate-modified nucleotides contain cyclic phosphate groups (*Figure 14*), enhancing their metabolic stability and enabling controlled release in prodrug strategies. These modifications are particularly relevant in delivering nucleoside-based drugs. Cyclophosphate nucleotides are synthesized using cyclization agents like imidazole or carbodiimides to convert the terminal phosphate into a cyclic form. This cyclic structure resists hydrolysis until activated within the cell, where the nucleotide is released to exert its therapeutic effect.

Borane Phosphonate Modifications

Replacing one of the oxygen atoms in the phosphate group with a borane group results in borane phosphonate nucleotides (*Figure 14*),. These modifications enhance chemical stability and reduce the negative charge of nucleotides, improving their cellular uptake. The synthesis of borane phosphonate nucleotides typically involves reacting nucleotide precursors with borane complexes to selectively replace the oxygen atom with borane (BH_3). The process is considered moderately challenging due to the need to carefully control the reaction environment.[113]

6. Research focus and Aim of the study

The primary aim of this study was to gain a comprehensive understanding of the existing methodologies, strategies, and advancements in the field of modified nucleoside synthesis and to develop and optimize an efficient and reliable synthetic route for the preparation of novel C6'- modified D-allohexafuranosyl-uracil nucleoside or nucleotide analogs. The literature review helped to identify key strategies and potential challenges and served as a guide in designing and optimizing the synthetic routes explored in this study. The search also uncovered studies detailing biological applications and therapeutic potential of modified nucleos(t)ides, providing insight into potential antiviral or anticancer uses of synthesized compounds.

6.1 Synthesis plan and goals

The synthetic plan involves several stages:

1) **Preparation of the peracetylated D-allofuranose.**
   Two different approaches are tested for preparing D-allofuranose, with the focus on analyzing their effectiveness in producing the desired furanose form.

   Approach 1: The first method utilizes boric acid stabilization to favor the furanose form during acetylation. This approach was chosen based on its potential to stabilize the sugar conformation, which was expected to yield a higher proportion of furanose over pyranose isomers. The method's simplicity was also one of the main criteria.[73]

   Approach 2: The second approach started with commercially available di-*O*-isopropylidene-D-allofuranose, which is already in the furanose form, followed by one-pot acid-catalyzed deactelization-peracetylation. This method was evaluated for its potential to simplify the synthesis process by reducing the need for further isomer separation.[71,72]

2) **Glycosylation.**
   The next step involved coupling the prepared D-allofuranose sugar with uracil via the Vorbrüggen glycosylation method.[75,114] This method was selected for its effectiveness in promoting the formation of β anomers, often preferred for biological applications.

3) **Separation of α/β anomers**
   After the glycosylation step, the resulting mixture contained both α and β anomers of the nucleoside. The separation of anomers was reported in the literature and involved introducing the isopropylidene-protecting groups.[66,72] These groups help to stabilize the sugar conformation and improve the separation of anomers using column chromatography.

4) **Functionalization and modifications.**
   The goal was to explore various modifications at C6' position (*Figure 15*) on the nucleoside to increase its versatility for future applications. The only modification successfully tested and implemented was the azidation of the 6'OH group.

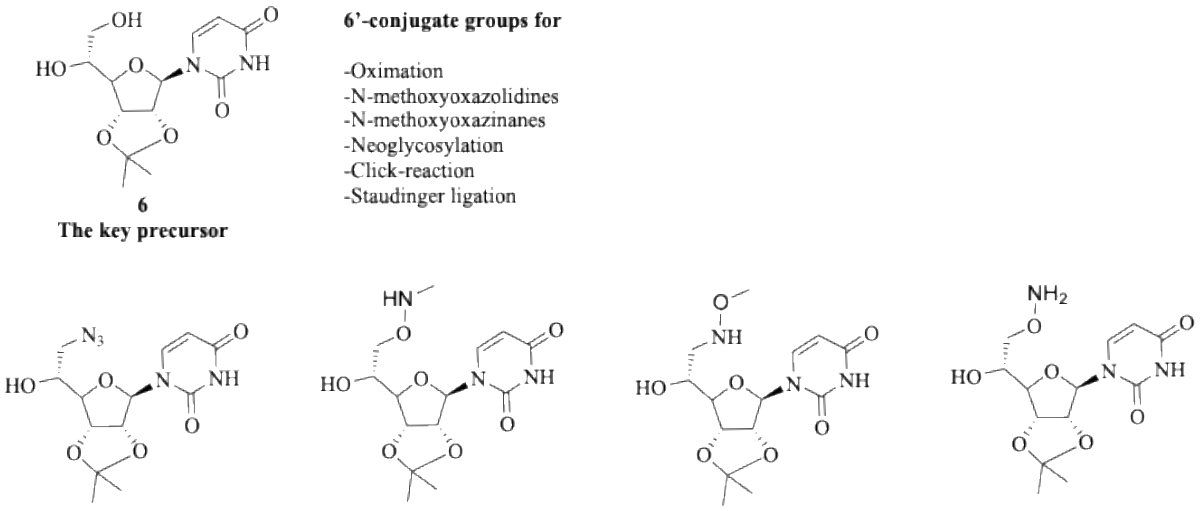


Figure 15. Planned C6'/6'OH modifications

The chosen azidation process involved activating 6'-OH using a tosylation step, followed by nucleophilic substitution with sodium azide. Other methods, such as Mitsunobu[95] and direct azidation[18], were also tested.

5) **Assessment of yield and purity.**
Each step of the synthetic process was evaluated for yield, stereoselectivity, and purity, which helped refine the synthetic pathway.

6.2 Biological evaluation.

Although the focus of this thesis is on synthesis, modified nucleosides such as D-allofuranosyl-uracil analogs hold significant potential in several fields, such as antiviral and anticancer therapies and biotechnology. [51,66] Upon successful synthesis of the desired analog, the viral polymerase assay could be used to evaluate its antiviral properties. [4,22] Additionally, the azide-modified nucleosides could be applied in bio-orthogonal reactions for molecular labeling and click chemistry in biotechnology.[15,17,19,22]

The viral polymerase assay was initially planned to evaluate the antiviral properties of the designed compound. This method would assess whether the azide-modified nucleotide could interfere with viral RNA polymerization, one of the key mechanisms targeted by antiviral drugs. Several studies support our aim.[10,22,28,105]

Computational modeling of the designed modified nucleoside into the active site of T7 viral polymerase (PDB ID: 1S76) revealed possible compounds' orientation and binding interactions (*Figure 16*), which supported the hypothesis that the azide-modified nucleoside could effectively interact with the viral enzyme. These findings indicate that azide modifications in D-allofuranosyl-uracil analogs, while not yet explored, hold promise for both therapeutic and technological applications.

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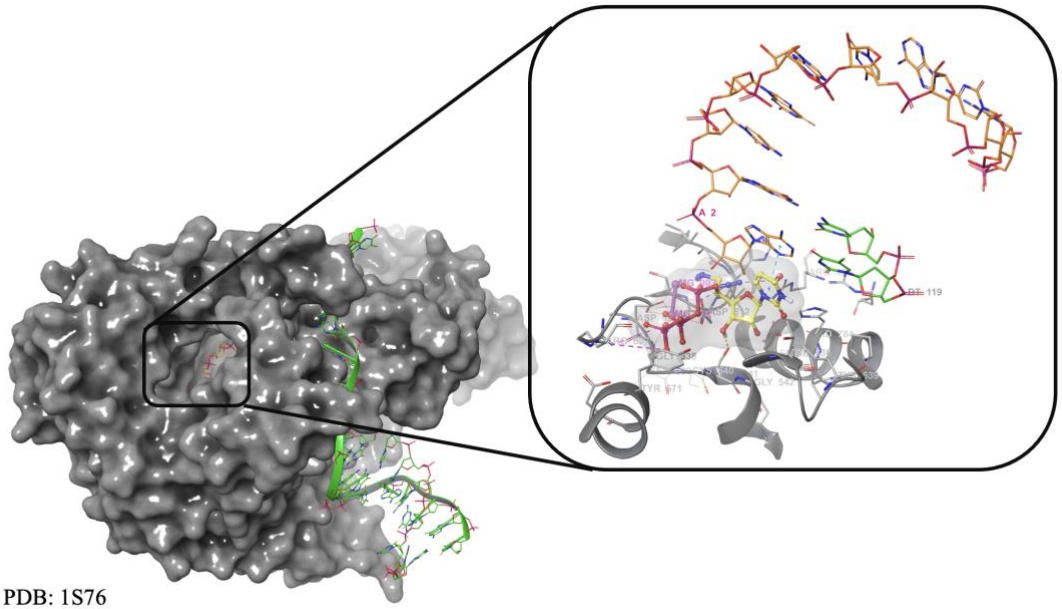


Figure 16. Designed 6'-azide D-allofuranosyl-uracil nucleotide analog aligned in place of natural substrate in the active site of T7 RNA Polymerase elongation complex. Protein (gray), growing RNA-chain (orange), template RNA (green), designed nucleoside analog's rotamers (yellow). Image is created using Maestro, Schrodinger Release 2024-3 (LLC, New York, NY 2024)

7. Materials and methods

7.1 General information

All reagents were obtained from commercial suppliers and used without further purification. The only exception was phosphoryl chloride, which was preliminary distilled before its further use in the phosphorylation reaction. All moisture-sensitive reactions were carried out in anhydrous conditions with an N_2 atmosphere using flamed and oven-dried glassware. Moisture-sensitive solid reagents were dried over P_2O_5 in a desiccator *in vacuo* preliminary to the usage. Solvents were dried over activated molecular sieves (3-4 Å) in flamed and oven-dried glassware.

NMR data were obtained on a Bruker AV-500B Spectrometer operating at 500.12 MHz for ^{1}H-NMR, the spectrometer was equipped with a Smartprobe, BB/1H and BCU I probe head cooling unit. Chemical shifts (δ values) were reported in parts per million (ppm) with two decimals. Reported ^{1}H NMR signals were referenced relative to residual solvent peak ($CDCl_3$ 7.26 ppm and 77.16 ppm, for DMSO-d_6 fixed at 2.50 ppm and 39.52 ppm) relative to tetramethylsilane (TMS). COSY and HSQC experiments were performed in order to confirm proton assignments. Coupling constants, J, are reported in Hertz. TopSpin version 4.0 was used for NMR data processing (Bruker Corporation. TopSpin, version 4.0; Bruker BioSpin GmbH: Billerica, MA, 2019.). High-resolution electrospray ionization mass spectra were recorded on a Waters ACQUITY RDa detector using an XBridge BEH C18 Column, 130 Å, 5 um, 4.6 x 30 mm (Waters) and using solvent A (H_2O/0.1% HCOOH) and solvent B (CH_3OH/0.1% HCOOH) with a flow rate of 0.8 mL/min. The gradient run consisted of 0-2.20 min, 2-100% B; 2.20 – 2.50 min, 100% B; 2.50 – 2.80 min, 100-2% B; 2.80 – 3.00 min, 2% B.

7.2 Chemicals and reagents

Reagent	CAS Number	Vendor
12:56-di-*O*-isopropylidene-D-allofuranose	2595-05-3	BIOSYNTH s.r.o
2,2-Dimethoxypropane	77-76-9	Sigma Aldrich
Acetic anhydride (Ac_2O)	108-24-7	J.T. Baker
Acetic acid (AcOH)	64-19-7	Lab-Honeywell.com
Acetone $(CH_3)_2CO$	67-64-1	Not provided
Acetonitrile (ACN)	75-05-8	Lab-Honeywell.com
Ammonia (NH_3 ca 4% in Methanol MeOH ca 2.0 mol/L)	7664-41-7	TCI
Ammonium sulfate ($(NH_4)_2SO_4$)	7783-20-2	Merck
Diisopropyl azodicarboxylate (DIAD)	2446-83-5	Sigma Aldrich
Dimethylformamide (DMF)	68-12-2	Fischer Scientific
Dimethylsulfoxide (DMSO)	2206-27-1	Sigma-Aldrich
Ethyl acetate (EtOAc)	141-78-6	Honeywell
Hexamethyldisilazane (HMDS)	999-97-3	Sigma Aldrich
Methanol (MeOH)	67-56-1	Fisher Scientific
Phosphoryl chloride ($POCl_3$)	10025-87-3	Not provided
Sodium azide (NaN_3)	26628-22-8	Aldrich Chemistry
N-hydroxyphthalimide (HONPht)	524-38-9	Not provided
p-Toluene sulfonic acid (p-TSA)	104-15-4	Merck
Pyridine (Py)	110-86-1	Fisher Scientific
Trimethylsilyl triflate (TMSOTf)	17875-18-2	Sigma-Aldrich
Triphenylphosphine (PPh_3)	603-35-0	Aldrich
Tetrabromomethane (CBr_4)	558-13-4	Aldrich
Mesyl Chloride (CH_3SO_2Cl)	124-63-0	Acros Organics
Sodium bicarbonate ($NaHCO_3$)	144-55-8	Not provided
Magnesium sulfate ($MgSO_4$)	7487-88-9	Merck
Tetrabutylammonium pyrophosphate (TBAP)	7411-21-8	Not provided

7.3 Instruments

Instrument	Model/Description
Rotavap/Vacuum concentrator	Laborota 4000
NMR	Brucker AV-500B Spectrometer
LC-MS	Waters ACQUITY RDa detector
TLC plates	TLC Silica gel 60 F254, Merck

7.4 Synthesis

7.4.1 Investigated Synthetic Routes: Successful route

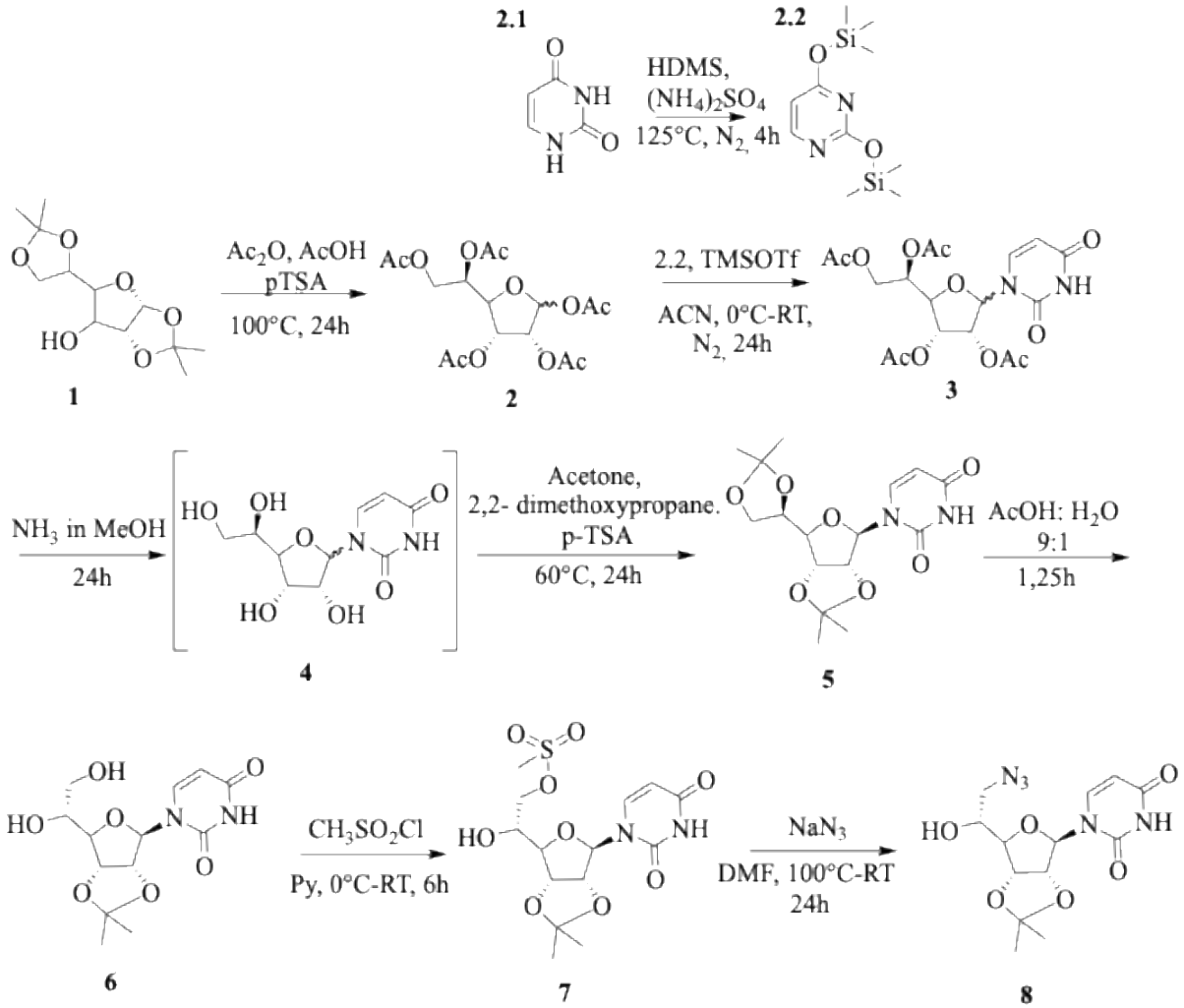


Figure 16. Successful synthetic route

Nucleoside synthesis

1,2,3,5,6-penta-*O*-Acetyl-(*α/β*)-D-allofuranose (2)[71,72]

To a solution of 1,2:5,6-di-*O*-isopropylidene-D-allofuranose (2.00 g, 7.68 mmol) in acetic acid (40 mL), acetic anhydride (Ac_2O, 7.26 mL, 76.84 mmol, 10 eq) and p-toluenesulfonic acid monohydrate (p-TSA 438.4 mg, 2.305 mmol, 0.3 eq) were added. This mixture was stirred at 100 °C for an overnight period. After cooling to room temperature, the mixture was concentrated under vacuum. The concentrated residue was then dissolved in ethyl acetate (EtOAc), followed by washing with saturated (1M) sodium bicarbonate ($NaHCO_3$) solution, drying with magnesium sulfate ($MgSO_4$), filtration, and further concentration by evaporation. The resulting dark oily substance was then subjected to column chromatography using a mixture of ethyl acetate and petroleum ether (1:1, *v/v* ratio), yielding the desired product **2** (2.21 g, 74% yield) as a mixture of α and β anomers in a 1:1 ratio.

Analytical data for α-anomer: Rf (Petroleum ether/EtOAc, 1/1, *v/v*) 0.28;
^{1}H NMR (500.12 MHz, $CDCl_3$, 25 °C) δ 6.38 (d, J = 4.55 Hz, 1H, H-1), 5.49 (dd, J = 2.74, 6.74 Hz, 1H, H-3), 5.16-5.21 (m, 2H, H-2, H-5), 4.37-4-40 (m, 2H, H-4, H6a), 4.10-4.14 (m, 1H, H-6b), 2.15, 2.13, 2.12, 2.08, 2.07 (5s, 15H, CH_3); ^{13}C NMR (125.75 MHz, $CDCl_3$, 25°C) δ 170.5, 169.9, 169.9, 169.7 169.3 (CO), 93.6 (C-1), 82.8 (C-4), 70.3, 70.0, 69.0 (C-2, C-3, C-5), 62.0 (C-6), 21.1, 21.0, 20.7, 20.7, 20.4 (CH_3). (SI *Figure 3, 4)*

Analytical data for β-anomer: Rf (Petroleum ether/EtOAc, 1/1, *v/v*) 0.51;
^{1}H NMR (500.12 MHz, $CDCl_3$, 25 °C) δ 6.16 (d, J = 0.86 Hz, 1H, H-1), 5.50 (dd, J = 5.06, 6.36 Hz, 1H, H-3), 5.32 (dd, J = 0.93, 4.97 Hz, 1H, H-2), 5.19 (m, 1H, H-5), 4.42 (dd, 3.60, 12.17 Hz, 1H, H-6a), 4.31 (t, J = 6.30 Hz, 1H, H-4), 4.05 (dd, J = 5.79, 12.16 Hz, 1H, H-6b), 2.12, 2.11, 2.09, 2.05, 2.05 (3s, 1d 15H, CH_3); ^{13}C NMR (125.75 MHz, $CDCl_3$, 25°C) δ 170.6, 169.8, 169.38, 169.36, 168.9 (CO), 98.3 (C-1), 80.05 (C-4), 74.4 (C-2), 71.00 (C-3, C-5), 62.1 (C-6), 21.0, 20.8, 20.7, 20.5, 20.4 (CH_3). (SI *Figures 1, 2*)

1-(2,3,5,6-tetra-*O*-Acetyl-*α/β*-D-allofuranosyl) uracil (2.2 and 3)

This reaction was carried out under the N_2-atmosphere. Dry uracil (3.69 g, 32.83 mmol, 5.8 eq) was mixed with $(NH_4)_2SO_4$ (325 mg, 2.46 mmol, 7.5 mol%), and the mixture was dissolved in excess of HMDS (50 mL). The reaction was stirred and refluxed for 1 day at 125 °C under the N_2 atmosphere. The excess of HMDS was evaporated *in vacuo*. Persilylated uracil was immediately used in the next step, where it was first dissolved in dry acetonitrile (40 mL). Dissolved uracil was further added to *in vacuo* dried **2** (2.21 g, 5.66 mmol) obtained in the previous step. After that, the mixture was kept stirring in an ice bath for 5-10 min. Next, the TMSOTf (7.68 mL, 42.5 mmol, 7.5 eq) was added dropwise into the reaction mixture. After 5-10 min of stirring, the reaction mixture was left stirring overnight at RT in a regular fume hood. The reaction was quenched by adding 50 ml of $CHCl_3$, followed by the extraction with saturated $NaHCO_3$ and further evaporation, which yielded compound **3** as a white, slightly brownish foam (crude mixture 2.52 g, 93.8%), displaying a mixture of α- and β-anomers with a ratio of 1/3. This time, the crude mixture was not purified by column chromatography since no meaningful amounts of side-products were formed; neither chromatography would allow for anomer separation at that stage. NMR - spectra presented for **3** were obtained while performing the same reaction during synthesis route exploration.

The Rf value of **3** in a CH_2Cl_2/EtOAc (1:1, *v/v*) solvent system was recorded as 0.24[72]
The Rf value of **3** in a EtOAc solvent system was recorded as 0.6

^{1}H NMR (500.12 MHz, $CDCl_3$, 25 °C) δ 8.97 (bs, H-1, NH a), 8.90 (bs, H-1, NH b), 7.33 (d, J = 8.18 Hz, H-1, H6 a), 7.20 (d, J = 8.16 Hz, H-1, H6 b), 6.02 (d, J = 2.30 Hz, H-1, H1’ a), 5.93 (d, J = 6.18 Hz, H-1, H1’ b), 5.76 – 5.80 (m, H-2, H5 a, H5 b), 5.52 (dd, J = 4.14, 6.03 Hz, H-1, H3’ b), 5.35 – 5.37 (m, H-2, H2’ a, H2’ b), 5.27 – 5.32 (m, H-3, H5’ b, H5’a, H3’ a), 4.44 – 4.49 (m, H-3, H6’a, H6’b, H4’ a), 4.24 (dd, J = 0.94, 9.46, H-1, H4’b), 4.10 – 4.13 (m, H-2, H6’’a, H6’’b), 2.03, 2.06, 2.08, 2.09, 2.12, 2.13, 2.17 (6s, CH_3); (SI *Figure 5*)

^{13}C NMR (125.75 MHz, $CDCl_3$, 25 °C) δ 170.4, 169.7, 169.5, 169.2, 168.8 (CO), 162.3 (C4 a/b), 149.9 (C2 a/b), 139.5, 139.2 (C6 b, C6 a), 103.5, 102.3 (C5 b, C5 a), 90.8 (C1’ a), 87.6 (C1’ b), 84.0 (C4’ a), 80.6 (C4’ b), 79.9 (C2’ a), 71.8 (C2’ b), 75.5 (C3’ a), 70.1 (C5’ b), 69.9 (C3’ b), 69.0 (C5’ a), 61.8 (C6’ a, C6’’a, C6’ b, C6’’ b), 20.7, 20.6, 20.5, 20.3 (CH_3).

(SI *Figure 6*)

Analytical data for 2.2
^{1}H NMR (500.12 MHz, $CDCl_3$, 25 °C) δ 8.28 (d, J = 5.58 Hz, H-1, H6 2.2), 6.47 (d, J = 5.58, H-1, H5 2.2), 0.33, 0.05 (2s, CH_3);
^{13}C NMR (125.75 MHz, $CDCl_3$, 25 °C) δ 169.9 (C4 2.2), 163.1 (C2 2.2), 160.8 (C6 2.2), 104.6 (C5 2.2). (SI *Figures 7, 8*)

α/β-D-allofuranosyl uridine (4)[72]

Compound **3** (1.23g, 2.77 mmol) was dissolved in a solution of ammonia (ca. 4% in Methanol, ca. 2.0 mol/l) (25 mL) and the mixture was stirred overnight at RT. Next, the solvent was evaporated *in vacuo* using a rotary evaporator resulting in the crude product as white solid powder (crude mixture 860 mg/ theoretical 100% yield of **4** = 760.5 mg) being used for the next step without further purification.

2,3;5,6-*O*-di-isopropylidene-β-D-allofuranosyl uridine (5)[72]

The crude product **4** obtained in a previous step (crude mixture 860 mg) was dissolved in acetone (17 mL). 2,2'-dimethoxypropane (1.8 mL, 5 eq assuming SM mass ≈ 800mg) and p-toluenesulfonic acid (p-TSA, 166 mg, 0.3eq assuming SM mass ≈ 800mg) were consequently added into the reaction mixture. The reaction mixture was left stirring overnight at 60°C. The reaction mixture was quenched by adding EtOAc. The organic phase was washed with saturated (1M) $NaHCO_3$, dried with Na_2SO_4, filtered, and concentrated by evaporation. The desired product **5** (780 mg) was obtained as a white foam after the column chromatography purification (CH_2Cl_2/EtOAc, 1:1, *v/v*)

The R*f* value in a CH_2Cl_2/EtOAc (1:1, *v/v*) solvent system was recorded as 0.32.

^{1}H NMR (500.12 MHz, DMSO d6, 25°C) δ 11.41 (bs, 1H, NH), 7.71 (d, J = 8.05 Hz, 1H, H-6), 5.81 (d, J = 2.26 Hz, 1H, H-1'), 5.65 (d, J = 8.00 Hz, 1H, H-5), 5.03 (dd, J = 2.28, 6.48 Hz, 1H, H-2'), 4.83 (dd, J = 3.78, 6.46 Hz, 1H, H-3'), 4.24 (dd, J = 5.35, 18.65 Hz, 1H, H-5'), 4.00 – 4.03 (m, 1H, H-6'), 3.95 (dd, J = 3.76, 7.08 Hz, 1H, H-4'), 3.72 (dd, J = 5.10, 8.67 Hz, 1H, H-6''), 1.49, 1.36, 1.30, 1.28 (4s, 12H, CH_3)
; ^{13}C NMR (125.75MHz, DMSO d6, 25°C) δ 163.2 (C-4), 150.3 (C-2) 142.7 (C-6), 113.2, 108.6 ($\underline{C}(CH_3)_2$), 101.9 (C-5), 92.0 (C-1'), 86.7 (C-4'), 83.5 (C-2'), 80.9 (C-3'), 74.7 (C-5'), 66.0 (C-6'), 27.0, 26.5, 25.1, 25.0 ($C(\underline{C}H_3)_2$). (SI *Figures 9, 10*)

The introduction of modification at 6'OH position

2,3-*O*-isopropylidene-β-D-allofuranosyl uridine (6)*

Compound **5** (780 mg, 2.2 mmol) was dissolved in a mixture of acetic acid and water (9:1, *v/v*, 5 mL) and heated to 60 °C for 1.5 hours. Afterward, the reaction mixture was diluted with toluene, concentrated under reduced pressure, and co-evaporated with toluene to obtain a white foam. This crude product was purified by precipitation in cold EtOAc. The resulted product **6** was obtained as a white powder (420 mg, 61%).

The R*f* value in the EtOAc solvent system was recorded as 0.16.

^{1}H NMR (500.12 MHz, DMSO-d_6): δ 11.37 (bs, 1H, NH), 7.78 (d, J = 8.07, 1H, H-6), 5.84 (d, J = 2.69 Hz, 1H, H-1'), 5.78 (dd, J = 5.59 Hz, 1H, OH5), 5.17 (d, J = 4.92, H1, OH5'), 4.86 (m, 2H, H-2', H-3'), 4.65 (t, 1H, OH6'), 4.03 (dd, J = 1.72, 7.91 Hz, 1H, H-4'), 3.65 (m, 1H, H-3'), 3.36 – 3.41 (m, 2H, H-6', H-6''), 1.47, 1.28 (2s, C(CH_3)$_2$);
^{13}C NMR (125.75 MHz, $CDCl_3$, 25°C) δ 163.1 (C-4), 150.4 (C-2), 141.9 (C-6), 112.9 (C(CH_3)$_2$), 101.9 (C-5), 90.7 (C-1'), 86.0 (C-4'), 83.4 (C-2'), 79.9 (C-3'), 71.4 (C5'), 62.6 (C6'), 27.1, 25.2 (C(CH_3)$_2$. (SI *Figures 11, 12*)

2,3-*O*-isopropylidene-6'-*O*-methanesulfonyl-*β*-D-allofuranosyl uridine (7)

The 6'OH mesylation of compound **6** was carried out under an N_2 atmosphere. Compound **6** (212 mg, 0.67 mmol) was dissolved in dry pyridine (11.5 mL), and the reaction mixture was cooled to 0°C. Next, mesyl chloride CH_3Cl (68 µL, 0.877 mmol, 1.3 eq) was added, and the reaction mixture was left stirring at 0 °C for 5 h. The reaction was quenched by the addition of water. The organic phase was extracted using EtOAc and dried over Na_2SO_4, then filtered and co-evaporated with DCM. The crude product was purified using column chromatography (50-100% EtOAc in DCM), resulting in the desired compound **7** (194.3 mg, 73.4 %).

The R*f* value in the EtOAc solvent system was recorded as 0.27

^{1}H NMR (500.12 MHz, DMSO-d_6): δ 11.41 (bs, 1H, NH), 7.75 (d, J = 8.05 Hz, 1H , H-6), 5.80 (d, J = 2.37 Hz, 1H, H-1'), 5.78 (d, J = 5.55 Hz, 1H, OH5'), 5.64 (dd, J = 8.10, 2.10 Hz, 1H, H-5'), 4.98 (m, 2H, H-2', H-3'), 4.21 (m, 1H, H-6'), 4.09 (m, 1H, H-6''), 3.90 – 3.96 (m, 2H, H-4', H-5'), 2.95 (s, 3H, SO_3CH_3), 1.49, 1.30 (2s, 6H, C(CH_3)$_2$);
; ^{13}C NMR (125.75MHz, $CDCl_3$, 25°C) δ 163.2 (C-4), 150.3 (C-2), 143.0 (C-6), 113.0 6 (C(CH_3)$_2$), 101.8 (C-5), 92.5 (C-1'), 85.8 (C-4'), 83.2 (C-2'), 81.2 (C-2'), 71.5 (C-6', 6''), 68.3 (C-5'), 36.6 (SO_3CH_3), 26.9, 25.1 (C(CH_3)$_2$). (SI *Figures 13, 14*)

2',3'-*O*-isopropylidene-6'-azido-6'-deoxy-*β*-D-allofuranosyl uridine (8)

Compound **7** (185.5 mg, 0.47 mmol) was dissolved in dry DMF (10 mL), and the NaN_3 (153.7 mg, 2.36 mmol, 5 eq) was added to the reaction mixture. The reaction mixture was heated at 100 °C for 2 h, then after it was left stirring at RT over the weekend. The reaction was quenched by adding water; the organic phase was thoroughly extracted with diethyl ether, washed with 1M saturated $NaHCO_3$, dried with Na_2SO_4, filtered, and evaporated. The desired compound was purified using gradient column chromatography (EtOAc/petroleum ether 50% - 100%), resulting in compound **8** as a colorless oil (80 mg, 50 %)

The R*f* value in the EtOAc solvent system was recorded as 0.61

^{1}H NMR (500.12 MHz, DMSO-d_6): δ 10.17 (bs, 1H, NH), 7.34 (d, J = 8.06 Hz, 1H, H-6), 5.73 (d, J = 8.01 Hz, 1H, H-5), 5.55 (d, J = 2.01 Hz, 1H, H-1'), 5.00 – 5.06 (m, 2H, H-2', H-3'), 4.02 (m, 2H, H-4', H-5'), 3.36 (m, 2H, H-6', H-6''), 1.52, 1.32 (2s, 6H, (C(CH_3)$_2$);
^{13}C NMR (125.75MHz, $CDCl_3$, 25°C) δ 163.8 (C-4), 150.8 (C-2), 143.4 (C-6), 114.6 (C(CH_3)$_2$), 102.9 (C-5), 95.8 (C1'), 87.9 (C-4'), 84.1 (C-3'), 80.3 (C-2'), 70.3 (C-5'), 53.5 (C-6', 6''), 27.1, 25.2 (C(CH_3)$_2$). (SI *Figures 15, 16*)

7.4.2 Investigated Synthetic Routes: Unsuccessful Attempts and Alternative Strategies

Synthesis of peracetylated D-allofuranose from D-allose

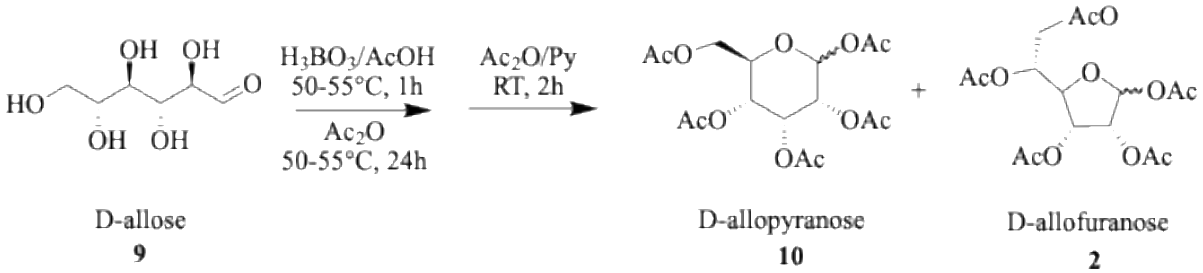


Figure 17. Synthesis of peracetylated D-allofuranose from linear D-allose using one-pot approach[73].

D-(+)-allose (1.0 g, 5.7 mmol), boric acid (H_3BO_3, 0.77 g, 12.5 mol), and acetic acid (20 mL) were mixed and stirred at 50-55°C for 1 h until all sugar had dissolved. Next, the reaction mixture was cooled to RT, allowing 20 mL of acetic anhydride (Ac2O) to be added. The reaction mixture was heated to 50-55°C and stirred overnight. The reaction was quenched by adding MeOH (4 mL) and partially (50% of volume) concentrated by evaporation *in vacuo.* The latter step was repeated twice by adding 2 and then 4 mL of MeOH. Ac_2O (20 mL) and dry pyridine (20 mL) were added to the reaction mixture, which was stirred at RT for 2 h. Next, the reaction mixture was concentrated by evaporation *in vacuo*. The reaction was quenched by adding ice, and the organic phase was extracted with $CHCl_3$. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated, resulting in a yellowish oily mixture of pyranose and furanose products. The mixture was further subjected to column purification (EtOAc/Petroleum benzine, 30%), but no sufficient separation of isomers was achieved. Further steps, such as the Vorbrüggen reaction and further deacetylation for isolated mixtures of compounds, were successful, but no desired separation of α/β anomers was achieved, which is why this path was discontinued.

The introduction of modifications at 6'OH position – Alternative approach 1

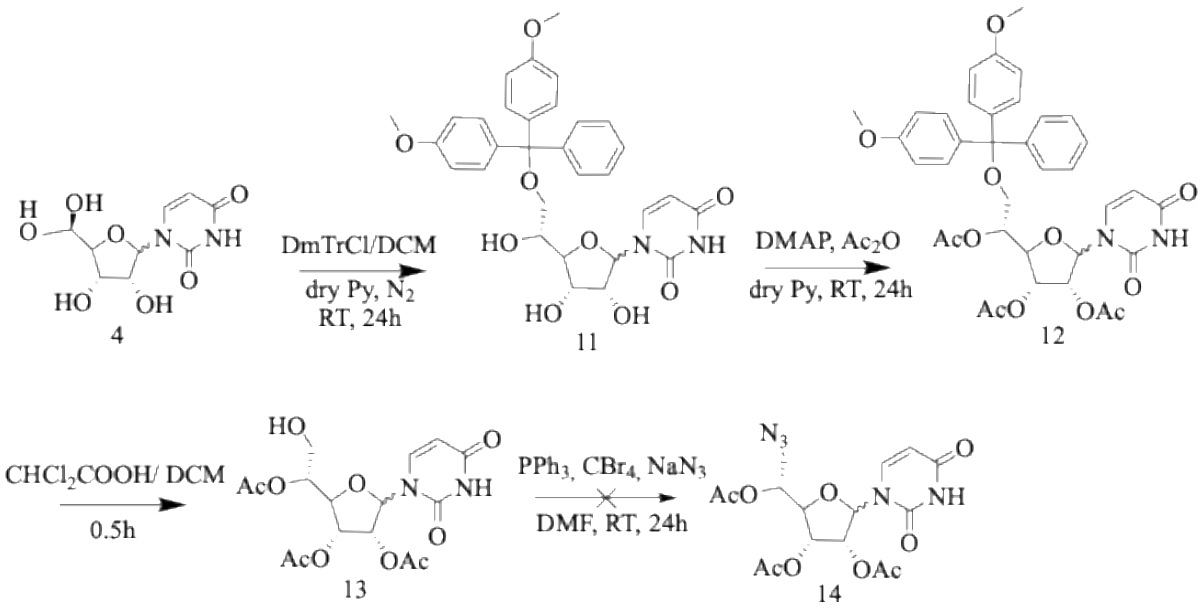


Figure 18. Unsuccessful attempts to introduce C6'/6'OH azide modification via tritylation of 6'OH.

Tritylation of 6'OH

6'-*O*-(4,4'-dimethoxytrityl)-*α/β*-D-allofuranosyl uridine (11)

Compound **4** (142.5 mg, 0.52 mmol) was dissolved in dry pyridine (3 mL). The reaction was continued under the N_2 atmosphere. The dimethoxytrityl chloride (DMTrCl, 186 mg, 0.55 mmol, 1.05 eq) was dissolved in DCM and transferred to the reaction mixture, which was left stirring overnight at RT. The reaction was stopped upon the addition of DCM, and the organic phase was washed with saturated (1M) sodium bicarbonate, dried over Na_2SO_4, and concentrated by evaporation *in vacuo*. The desired product was purified using column chromatography (MeOH/DCM 5% + 0.5% TEA), resulting in desired compound **11** as a mixture of α and β anomers (200 mg, 66.8%)

The R*f* value in the MeOH/DCM (1/9, *v/v*) solvent system was recorded as 0.45 – 0.56 (curved run line)

Fractions containing mainly β-anomer:
^{1}H NMR (500.12 MHz, $CDCl_3$) δ 11.84 (bs, 1H, NH), 7.67 (d, J = 8.12 Hz, 1H, H-6), 7.19 – 7.42 (m, 9H, Aromatics), 6.81 – 6.83 (m, 4H, Aromatics), 5.75 (d, J = 3.15 Hz, 1H, H-1'), 5.68 (d, J = 8.09 Hz, H-5, 1H), 4.31(m, 2H, H-2', H-4'), 4.05 (m, 2H, H-3', H-5'), 3.19 – 3.23 (m, 1H, H-6'), 3.31 – 3.35 (m, 1H, H-6''), 1.39 (m, C$\underline{H}_3$s). (SI *Figure 17*)

Peracetylation of tritylated compound

2',3',5'-tri-*O*-acetyl-6'-*O*-(4,4'-dimethoxytrityl)-*α/β*-D-allofuranosyl uridine (12)

Compound **11** (97 mg, mmol) was dissolved in dry pyridine (2.5 mL). DMAP (13 mg 0.106 mmol) and Ac_2O were added to the reaction mixture (96 µL, 2eq/OH). The reaction was left stirring overnight at RT. The reaction was quenched by addition of MeOH (1 mL). The organic phase was extracted with EtOAc, washed with saturated (1M) $NaHCO_3$, dried with Na_2SO_4,

filtered, and concentrated by evaporation/co-evaporation with DCM + toluene *in vacuo,* resulting in a dark brown syrup-like mixture containing compound **12** as a mixture of α and β anomers (138 mg/ expected yield 118mg)

Unassigned NMR of product mixture (α and β furanose + possibly small amounts of pyranose) SI Figures 18 and 19.

Deprotection of dimethoxytrityl – protecting group

2',3',5'-tri-*O*-acetyl-*α/β*-D-allofuranosyl uridine (13)

Compound **12** (90 mg, 0.168 mmol) was dissolved in a mixture of 3% dichloroacetic acid in DCM (100 mL). The reaction was stirred at RT for 0.5 h, and it was quenched by addition of MeOH, and following extraction of the organic phase with DCM, its repeated washing with saturated (1M) $NaHCO_3$, drying over Na_2SO_4, filtering and concentration by evaporation *in vacuo*. The reaction mixture was purified by column chromatography (MeOH/DCM, 5-10%), resulting in **13** (α/β) (17mg, 33%)

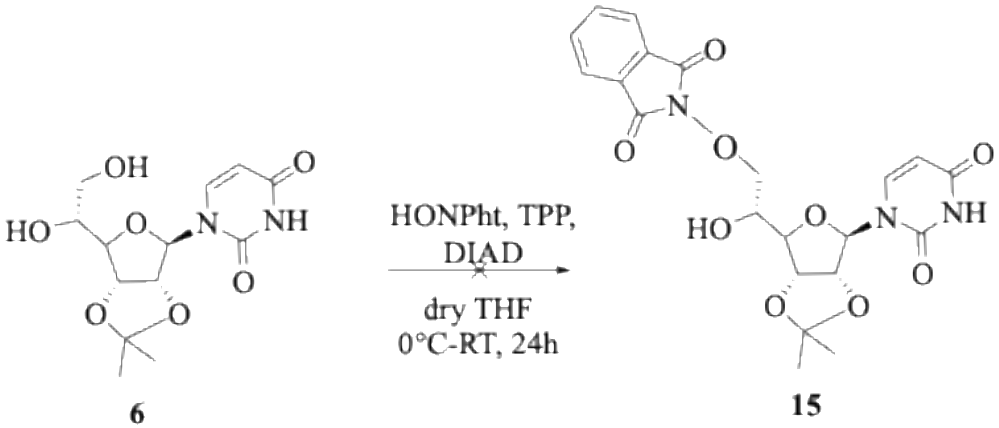
Unassigned NMR of product mixture (α and β furanose + possibly small amounts of pyranose) SI Figures 20 and 21.

Azidation

2',3',5'-tri-*O*-acetyl-6'-azido-6'-deoxy-*α/β*-D-allofuranosyl uridine (14)

Compound **13** (17mg, 0.043 mmol) was dissolved in DMF (0.5 mL). Triphenylphosphine (PPh_3, 14 mg, 0.0509 mmol, 1.2 eq), sodium azide (NaN_3, 8.3 mg, 0.127 mmol, 3 eq), tetrabromomethane (CBr_4, 17 mg, 0.509 mmol, 1.2 eq) were added to the reaction and it was left stirring overnight at RT. No product formation was observed.

The introduction of modifications at 6'OH position – Alternative approach 2



6 15

Figure 19. Unsuccessful attempts to introduce an oxime and other consequent modifications at the C6'/6'OH site via the Mitsunobu reaction

Mitsunobu to 6'OH

2',3'-*O*-isopropylidene-6'-*O*-phthalimido-6'-deoxy-*β*-D-allofuranosyl uridine (15)

Compound **6** (117 mg, 0.372 mmol) was dissolved in dry THF (4 mL). PPh_3 (99%, 98.6 mg, 0.376 mmol, 1eq) and N-hydroxy phthalimide (97%, 62.6 mg, 0.384 mmol, 1eq) were added to the reaction mixture, which was cooled to 0 °C. After that, DIAD (95%, 78 µL, 0.392 mmol, 1eq) was added slowly to the reaction mixture and it was left stirring in RT overnight. Partial new product formation was observed based on TLC; the addition of reagents did not improve the results, and the formed product could not be analyzed due to purification challenges. The reaction was repeated two times.

Triphosphorylation of an azide product

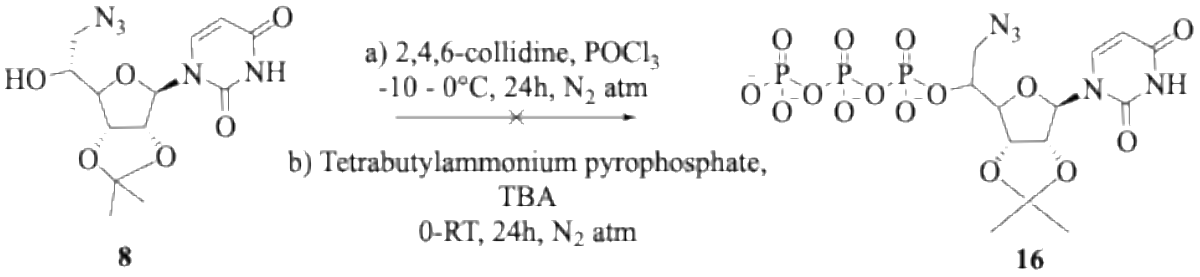


Figure 20. Unsuccessful mono- and triphosphorylation attempts

All the steps were carried out under N_2 atmosphere.

- Compound **8** (37 mg, 0.109 mmol) was dissolved in dry triethylphosphate (850 uL). Next 2,4,6-trimethylpyridine (14.5 µL, 0.109 mmol, 1eq) was added to the reaction mixture, which was further cooled to -10 - -20 °C in a salt bath. Freshly distilled phosphoryl chloride (15.3 µL, 0.166 mmol) was added to the reaction mixture and the reaction was left stirring at +4 °C overnight.
- Dry tetrabutylammonium pyrophosphate (197 mg, 0.218 mmol, 2eq) in ACN (2 mL) and dry tributylamine (52 µL, 0.218 mmol, 2eq) were added to the reaction mixture at 0°C, and the reaction was left stirring overnight 0 °C - RT.
- The reaction was quenched by adding TEAA buffer (50 mM, 4 mL) and $CHCl_3$ (4 mL). The aqueous phase was extracted and washed with $CHCl_3$. Next NaI (49 mg, 0.327 mmol, 3eq) was added to the aqueous phase, which after it was diluted with acetone. Aq.phase was vortexed for 2 min and moved to the "cold" room (-20 °C) for 0.5 h and consequently centrifugated at 3650 rpm for 15 min. The supernatant was decanted and evaporated *in vacuo*. The transparent pellet was moved to the "cold" room.
- According to HPLC analysis (50 mM TEEA buffered H_2O/ACN gradient: 0-5 min 0% ACN/100% H_2O; 5-20 min 0 – 50% ACN/ 100 – 50% H_2O; 20 – 30 min 50-100% ACN/ 50 – 0% H_2O; 30-35 min 100-0% ACN/ 0-100% H_2O; 35-45 min 0% ACN/100% H_2O. Detection wavelength = 260 nm) The main peak observed on the chromatogram was the starting material (Rt 22.27 min, data not shown).

8. Results

8.1 Synthesis of peracetylated D-allofuranose

The synthesis of peracetylated furanoses faces challenges, including isomeric complexity, polyfunctionality, epimerization, and difficulty separating α- and β-anomers. We initiated this

study by exploring the efficiency of synthetic routes for the preparation of peracetylated D-allofuranose, examining two different approaches and using two distinct starting materials: a D-allose sugar in the first approach and a commercially available protected derivative, 1,2:5,6-di-*O*-isopropylidene-D-allofuranose (**1**), in the second.

The peracetylation of D-allose sugar using the previously reported one-pot method[73] involved heating D-allose with boric acid (H_3BO_3) in acetic acid (AcOH), and then adding acetic anhydride (AcO_2) to facilitate acetylation. Boric acid was removed as trimethyl borate using methanol, followed by final acetylation with acetic anhydride and pyridine. This method was intended to simplify the process by reducing purification steps, increasing efficiency, and enhancing the yield and purity of the desired furanose isomers. Following this protocol, we achieved yields of approximately 45-50% of the desired product (**2**) analyzed by NMR from the product mixture. However, in this route, the significant presence of pyranose hindered the sufficient separation of the furanose and its anomers. The prevalence of α -anomer of the peracetylated allofuranose was also undesired for our purposes. Thus, we concluded that while the method has potential for other sugar types, especially for glucose, its application in the current route would be insufficient and necessitate the development of efficient separation techniques.

The second, also previously reported approach[71,72], involved the peracetylation of commercially available 1,2:5,6-di-*O*-isopropylidene-D-allofuranose, which resulted in nearly traceless amounts of pyranose and proved to be more efficient. This method involved using acetic anhydride and p-toluenesulfonic acid in acetic acid, with the reaction carried out at 100 °C overnight. This resulted in a significantly higher yield of 74%, producing a balanced mixture of α/β anomers in a 1:1 ratio (**2**), making it the method of choice for this work. The higher yield and better control over isomer formation highlight the effectiveness of using a protected derivative as the starting material, providing a more efficient and pure synthesis of peracetylated D-allofuranose.

8.2 Introduction of nucleobase

The optimized Vorbrüggen reaction used for introducing uracil at the 1'-OH position resulted in high yields of compound **3** as a mixture of α/β anomers in a 1:3 ratio.[72,76,114] However, we anticipated a higher prevalence of the β-anomer and thus had to consider additional steps to achieve better purity of the desired β-anomer (**5**).

8.3 Separation of α/β -nucleosides

We employed several derivatization strategies along the synthetic route to efficiently purify the β-anomer of the desired nucleoside in a gravimetry column. The most effective strategy involved the use of isopropylidene protective groups.[72] By providing additional steric hindrance or selective full protection of β-anomer mostly, this strategy resulted in more efficient anomers separation. This approach resulted in higher purity and yield of the desired β-anomer (**5**), making it the preferred method for our synthesis. Based on NMR results, only partial protection occurred for alpha anomer (not shown).

Other attempted strategies included optimizing column chromatography conditions to separate the mixture of nucleoside anomers as such and/or using other protecting groups such as dimethoxytrityl (DMTr) at 6'OH position (**11**, **12**) to induce more pronounced conformational differences between anomers. However, these strategies resulted in lower purity, reduced

stability, and smaller yields of the desired product than the method employing isopropylidene groups. Selective protecting and deprotecting of 6'OH with DMTr, for introducing the desired 6'OH modifications, is worth repeating with pure β-anomer, not a mixture of conformers as described. The attempts to separate intermediate structures anomers before the Vorbrüggen reaction step proved useless.

8.4 Introduction of modifications to 6'OH modification

The introduction of the desired modifications at the 5'-OH position of modified pentafuranosyl nucleosides is considered to be synthetically relatively easy. The main strategies for azidation at the 5'-OH position include previously described direct azidation methods on commercially available halogenated nucleosides, one-pot Appel reaction followed by azidation[23,115], mesylation followed by azidation as well as Mitsunobu, which are straightforward reactions and provide high yields and selectivity. However, introducing the desired azide or other modification at the 6'-OH position of the **5** - nucleoside derivative from allohexose proved complicated. Attempts using DMTr - protection followed by simultaneous deprotection and azidation did not yield the desired product (**14**), nor did the Mitsunobu reaction with N-hydroksiphthalimide as a nucleophile (*Figure 13, 14*). The inefficiency of DMTr protection and further incorporation of an azide could also be attributed to the isomers mix used as a starting material, for clarity, this method could be tested in the future with the pure β - anomer precursor. The reasons for the failed Mitsunobu reaction are not very clear since N-hydroksiphthalimide is considered an effective nucleophile, but according to the TLC - analysis, the reaction did not proceed at all during the first attempt and proceeded only partly in the second attempt with newly dried solvent. However, further purification steps were not sufficient enough to get a reasonable yield. Considerations regarding protecting the nitrogen N3 of nucleobase were brought up in the discussions but not verified or tested.

The strategy that proved effective included the mesylation of the 6'-OH group with methane sulphonyl chloride (CH_3SO_2Cl), isolation of the mesylated compound **7**, and subsequent azidation with an excess of sodium azide (NaN_3) in DMF. This method allowed for the successful conversion of 6'OH into an azide group. Previously, we also attempted the isolation of compound 6 without isolating the mesylated intermediate, which resulted in the presence of undesired side products as observed on NMR, even after the column purification (data not shown).

Despite the successful conversion of the hydroxyl group, the yields of an azide product were less than anticipated (50%). The low yield was primarily associated with the products' solubility in the aqueous phase during the extraction, suggesting that further improvement of the extraction strategy could potentially enhance the observed yield. Another suggestion for potential yield improvement may include the usage of other methods, such as introducing desired modification via the epoxide intermediate.

8.5 Triphosphorylation

The final step of the synthetic work was introducing the triphosphate group at the 5'-OH position of compound **8** (*Figure 15*), thus developing the modified nucleotide suitable for further biological evaluation in a viral polymerase assay. Unfortunately, this step was unsuccessful. According to HPLC-analysis (SI), the resulting product mixture mainly included the starting material. Some potential reasons explaining the results include the 5'-OH being a secondary alcohol, which is known to be less reactive than the primary OH

usually employed for triphosphorylation in pentoses and insufficient measures to avoid moisture contact during the reaction. Although, the formation of anhydrous derivatives was not observed, possibly the protection of uracil 3 NH group could be still tested in future settings. Other undiscussed reasons are also possible. These factors likely contributed to the low reactivity and incomplete conversion of the starting material to the desired triphosphate product. Further investigations are needed to verify and/or develop other sufficient approaches to overcome observed challenges.

9. Conclusion

This study explored the synthesis of 6'OH-modified D-allofuranosyl-uracil nucleoside analogs, highlighting opportunities for using rare sugars scaffolds in modified nucleos(t)ide synthesis and applications. [14] The azidation of the C6'-position was a central focus due to the versatility of the azide functionality and its prominent roles as a precursor of amines and utility in click chemistry.[15,20]

Initially, two synthetic pathways were explored for preparing the peracetylated allofuranose. The second approach utilizing a commercially available partly protected derivative of D-allofuranose was identified as a more efficient route delivering high yields of pure D-allofuranose. [71,72] The Vorbrüggen glycosylation method [76,78,114], commonly employed for coupling nucleobases, resulted in high yields of D-allofuranosyl-uracil nucleoside with β -anomer selectivity. However, subsequent derivatization and anomer separation were needed to purify the desired β-anomer for further modification. Anomer separation challenges were addressed by introducing protective groups, a method previously reported in other studies.[72]

After several unsuccessful attempts, azide functionality was finally introduced at the 6'-position by azidation of an initially mesylated intermediate. Main challenges, however, were encountered at the phosphorylation stage, revealing that the tested method was insufficient for the secondary (5'OH) alcohol of the D-allofuranosyl scaffold. This limitation underscores the need for further research to optimize phosphorylation techniques, which are essential for enabling these analogs' full biological evaluation and therapeutic potential.

The inability to complete phosphorylation limited biological assays, particularly initially planned tests involving viral RNA polymerase inhibition. Computational modeling hinted at the potential for these compounds to bind within the T7 RNA polymerase complex, highlighting their potential in future antiviral studies once the synthetic challenges are overcome.

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Supporting material

NMR spectra

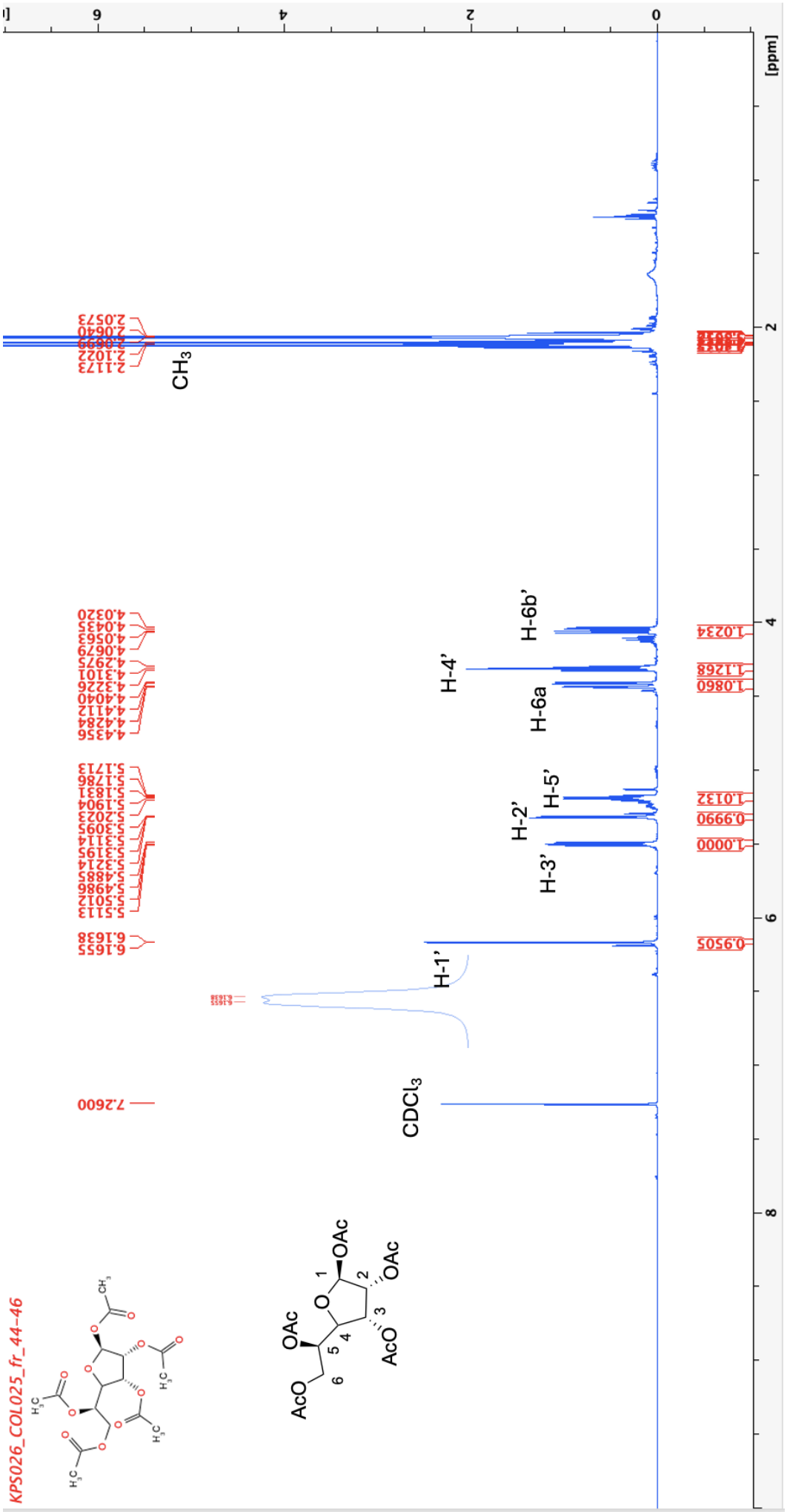

Figure 1. Compound 2 (β-anomer) 1H NMR

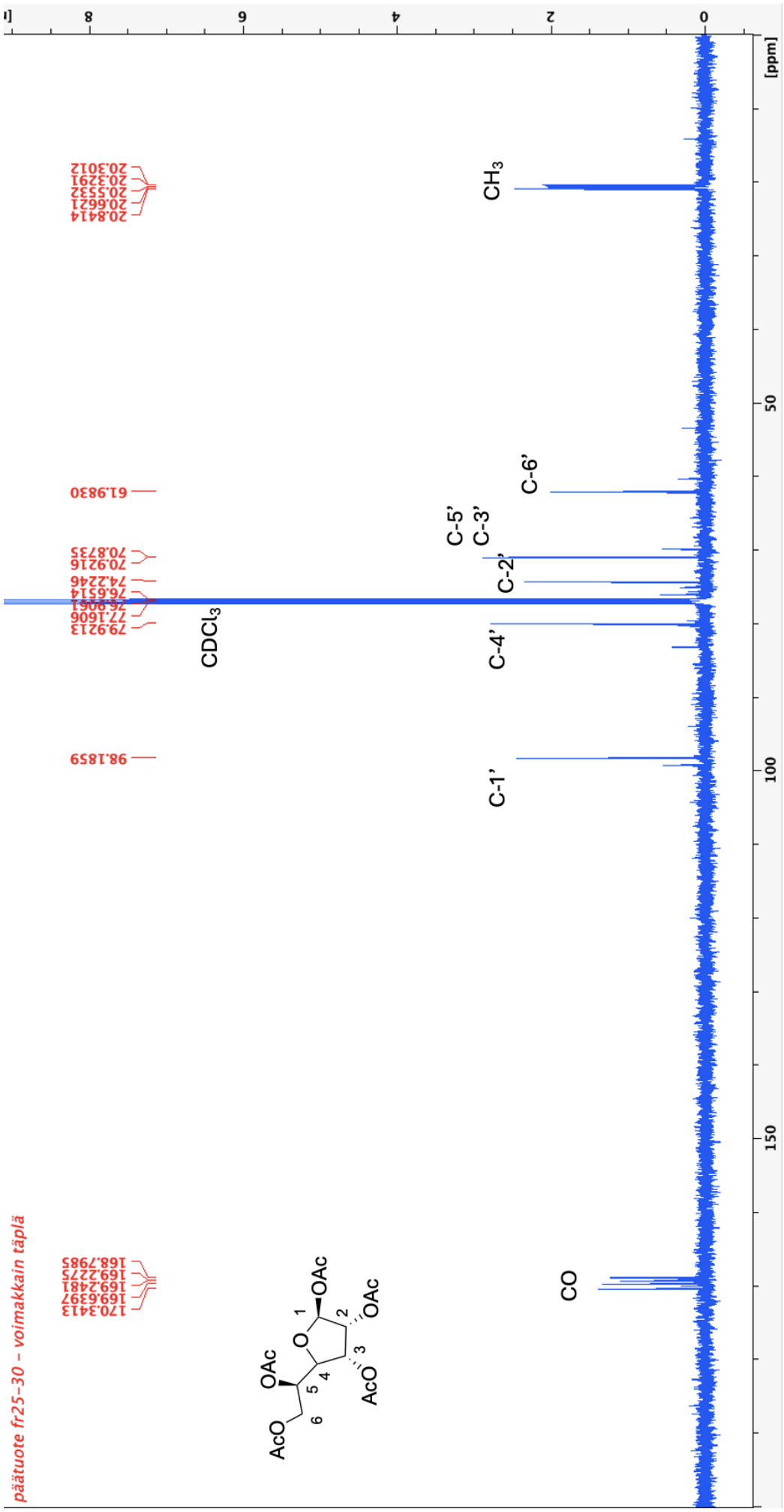

Figure 2. Compound 2 (β-anomer) ^{13}C NMR

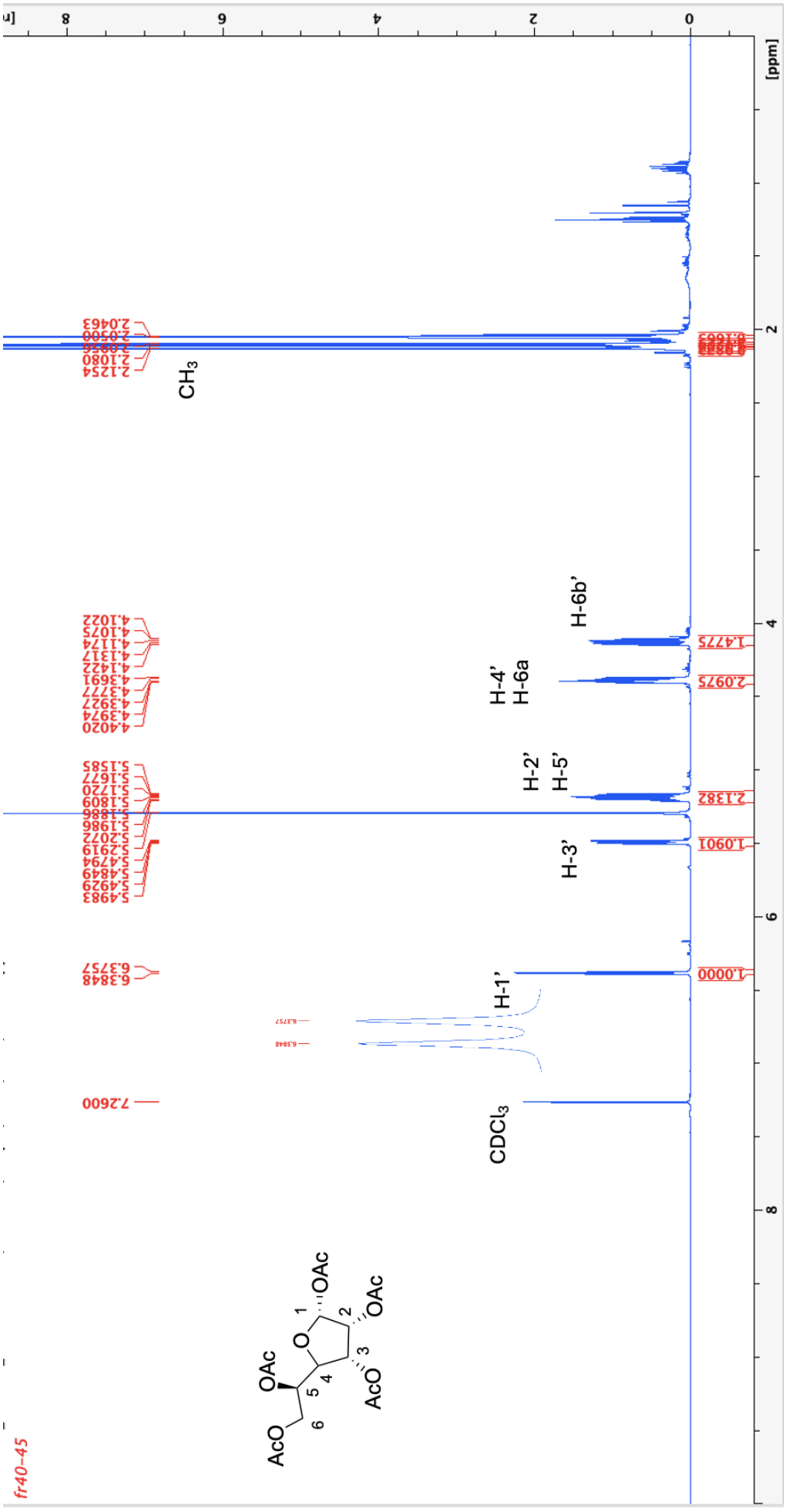

Figure 3. Compound 2 (α-anomer) ^{1}H NMR

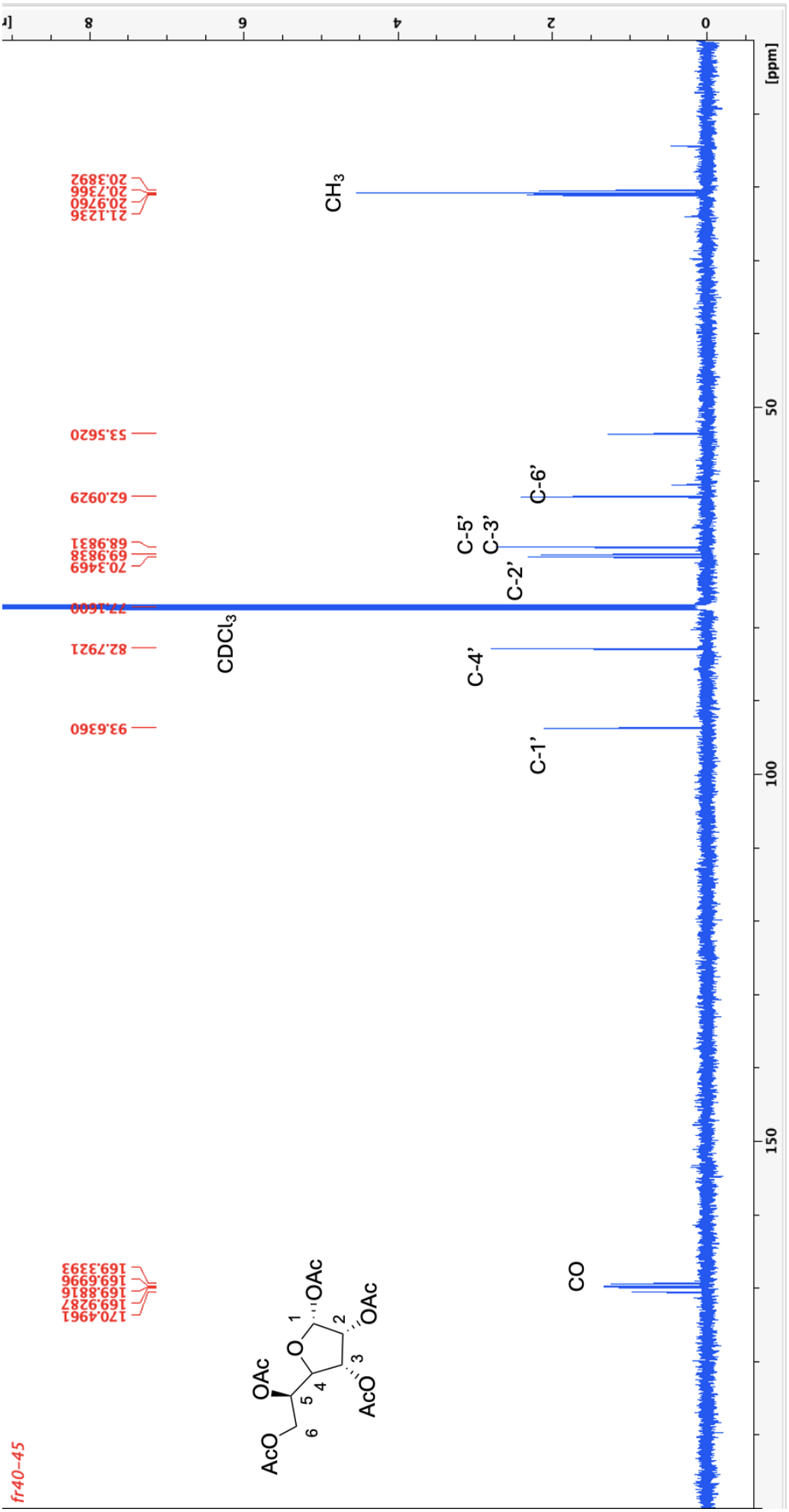

Figure 4. Compound 2 (α-anomer) ^{13}C NMR

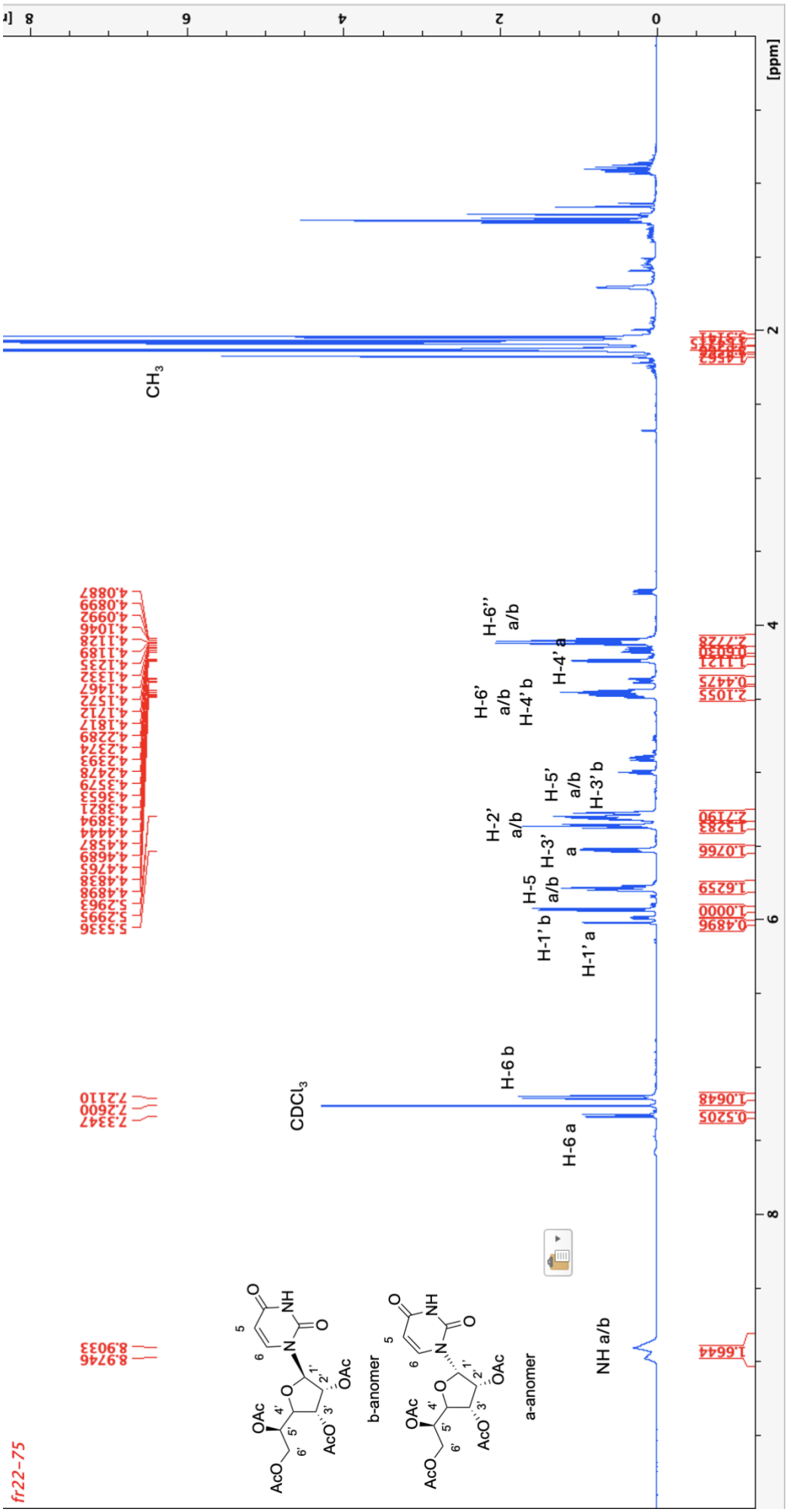

Figure 5. Compound 3 (α/β-anomer) ^{1}H NMR

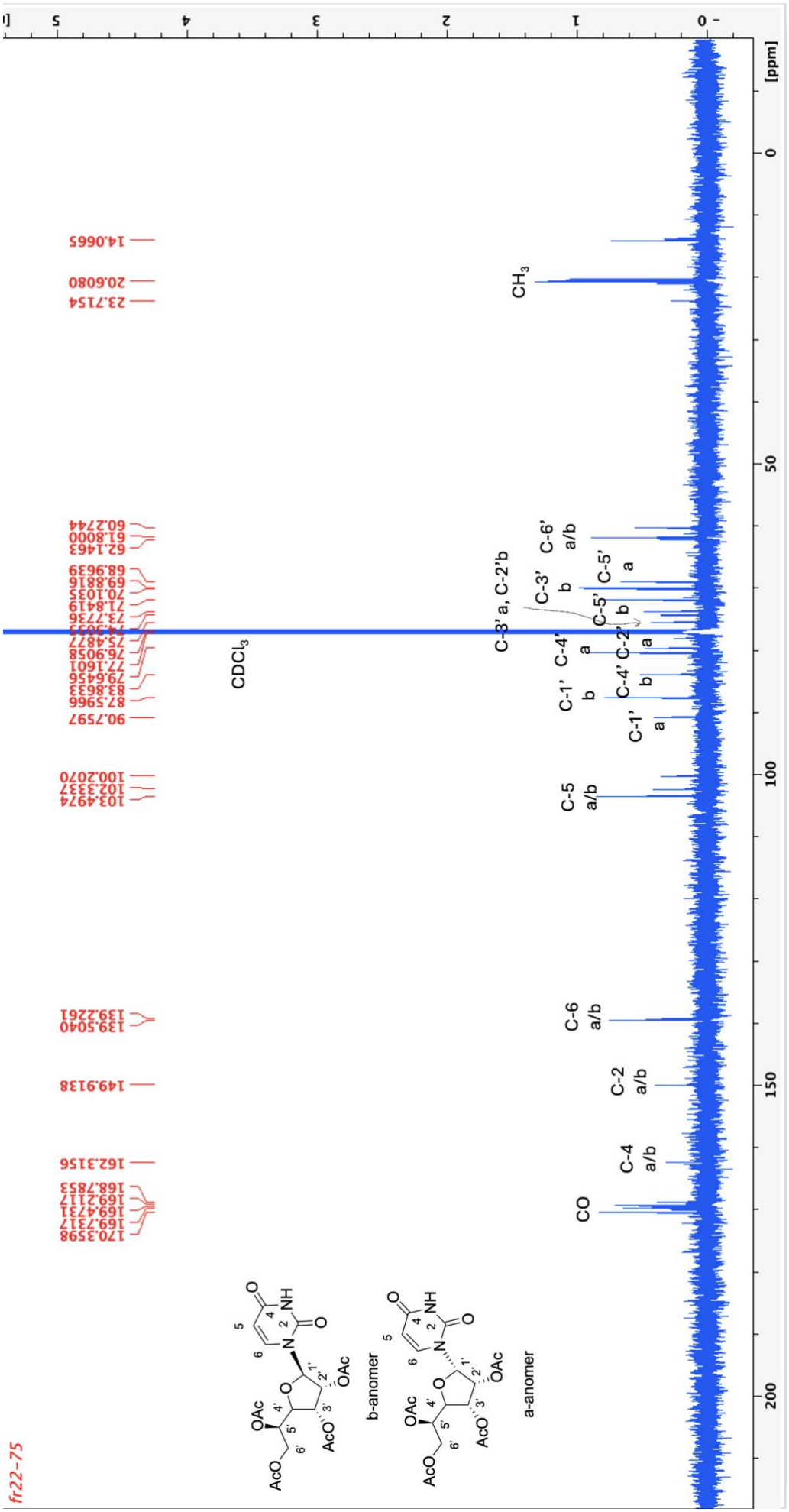

Figure 6. Compound 3 (α/β-anomers) ^{13}C NMR

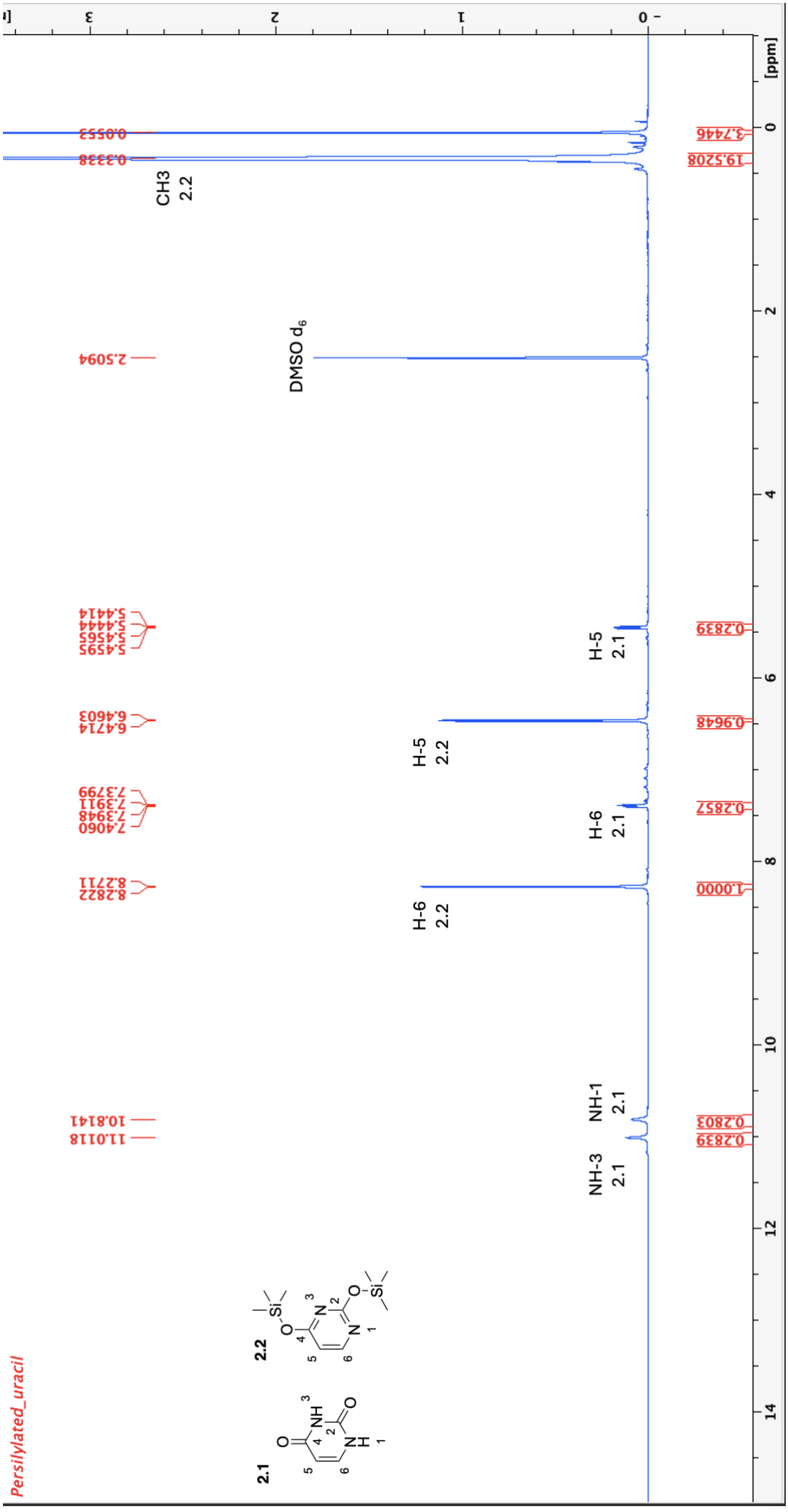

Figure 7. Compounds 2.1 & 2.2 ^{1}H NMR

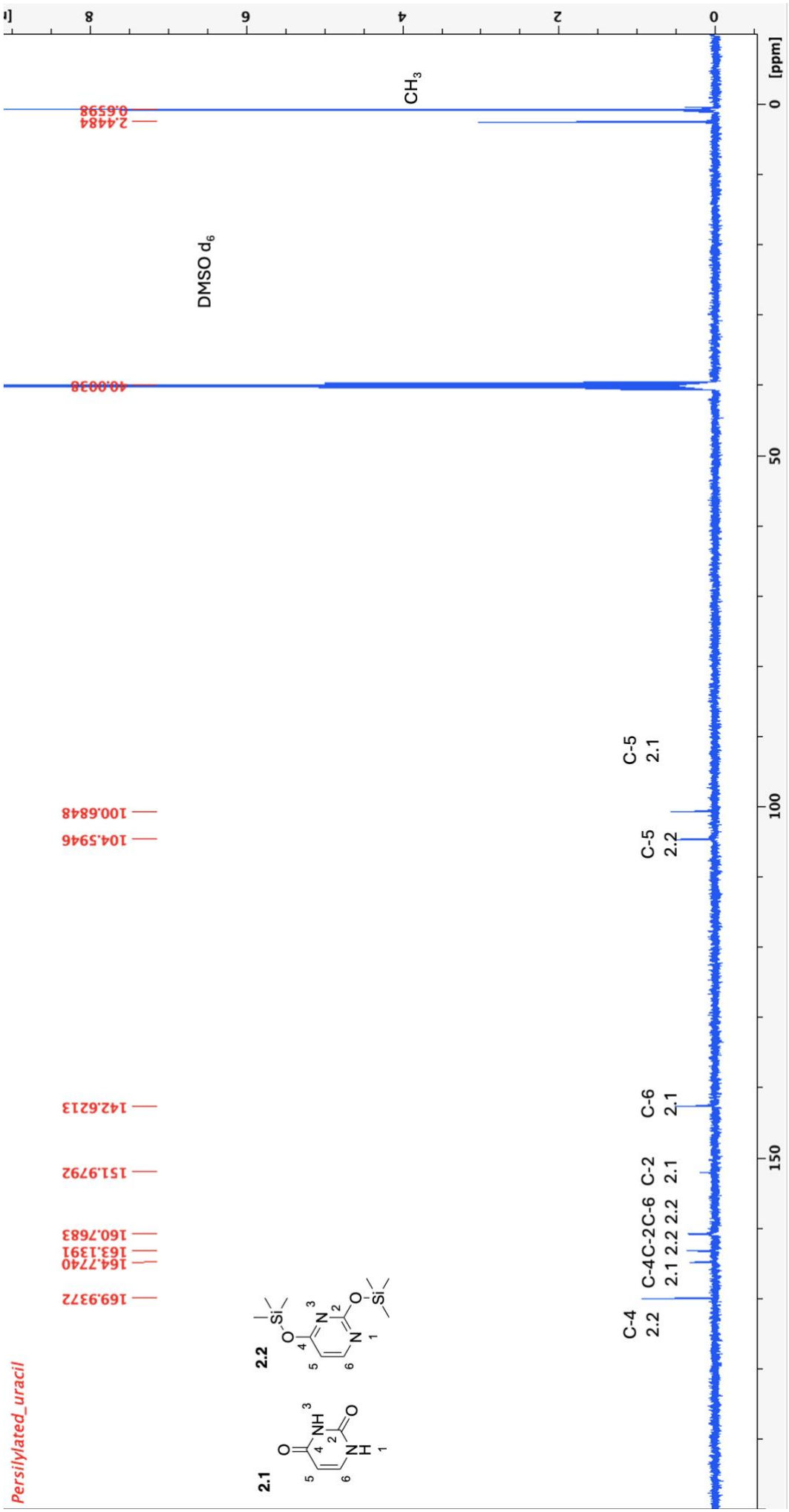

Figure 8. Compounds 2.1 & 2.2 ^{13}C NMR

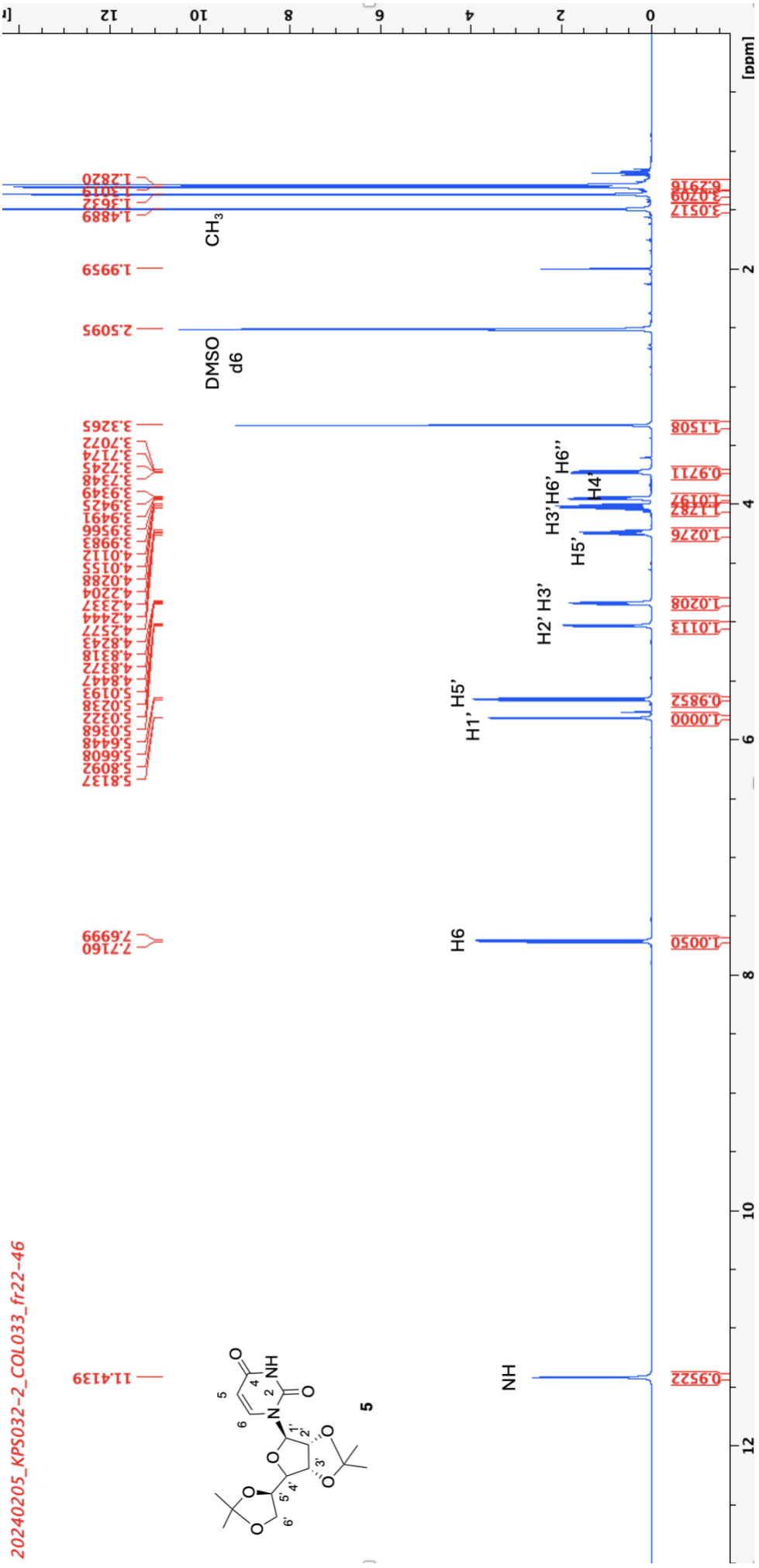

Figure 9. Compound 5 ^{1}H NMR

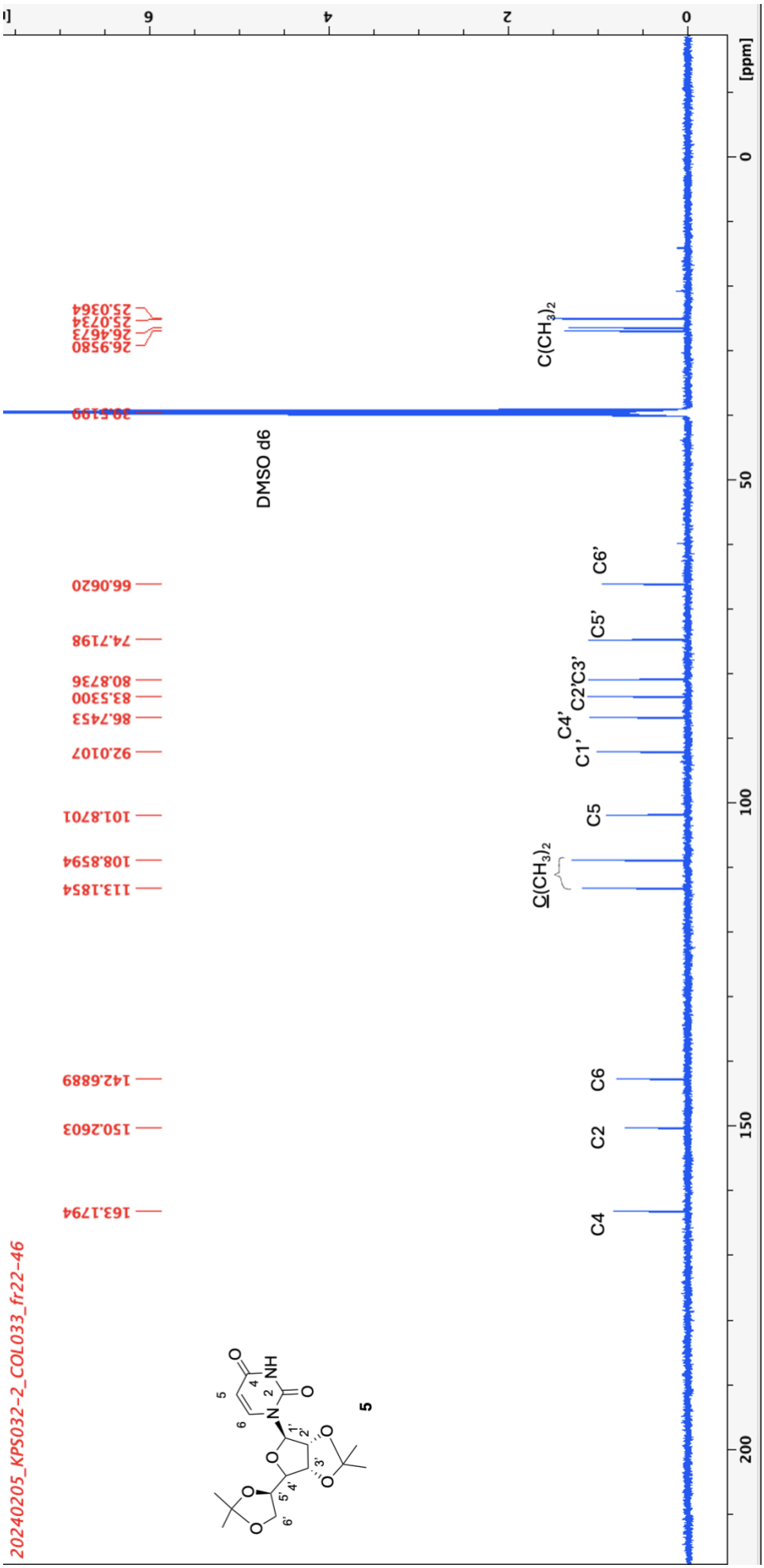

Figure 10. Compound 5 ^{13}C NMR

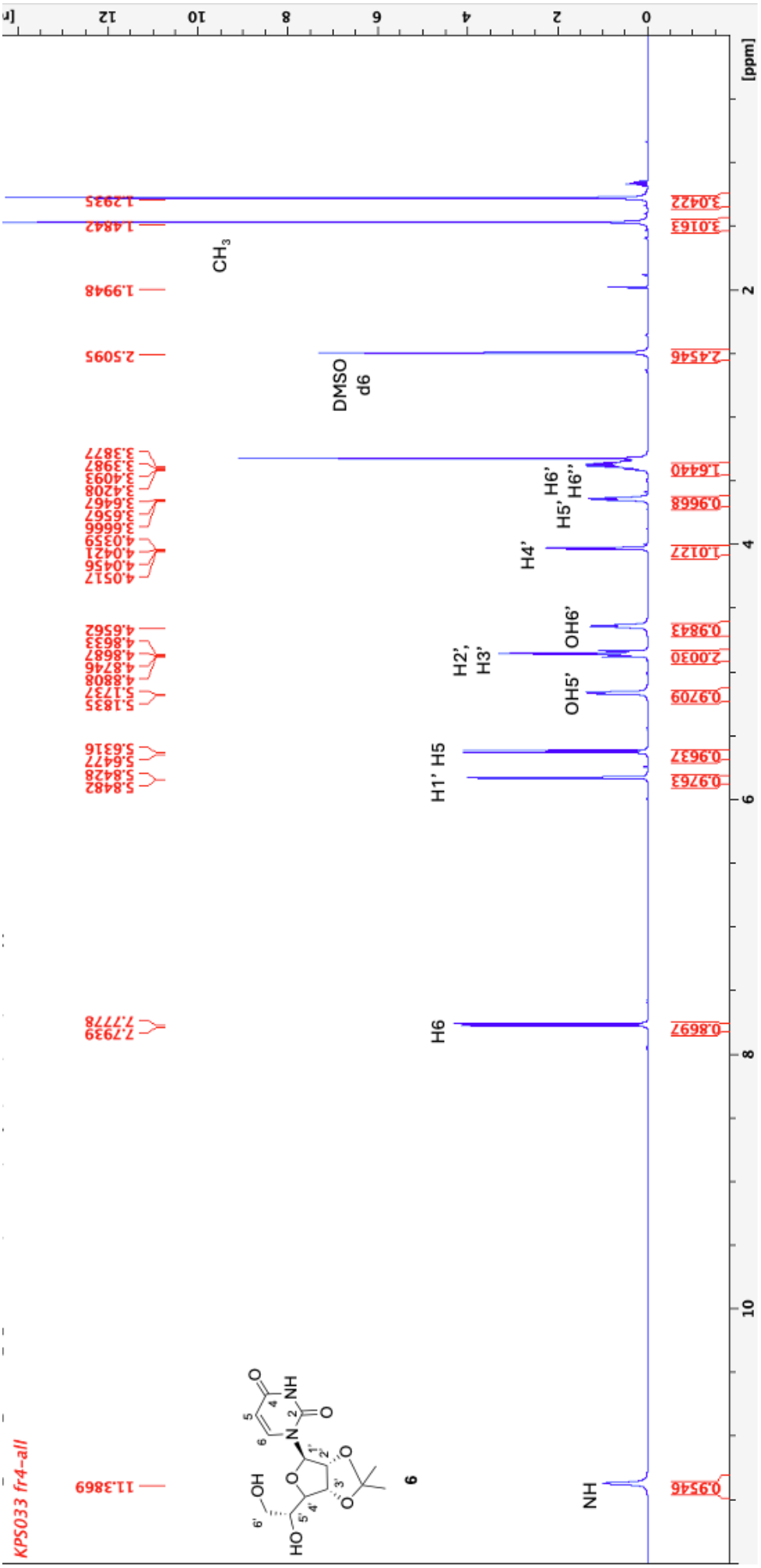

Figure 11. Compound 6 ^{1}H NMR

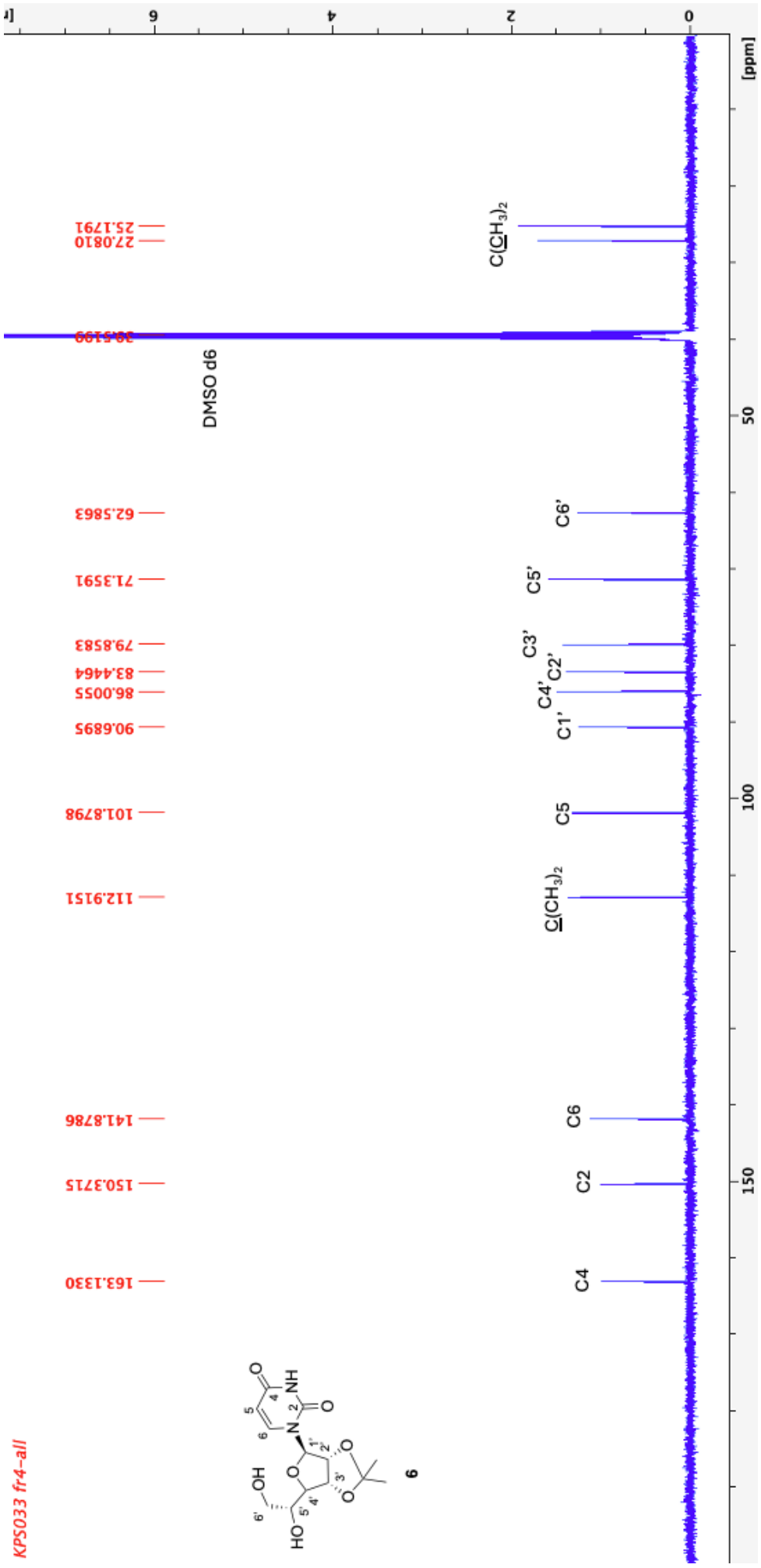

Figure 12. Compound 6 ^{13}C NMR

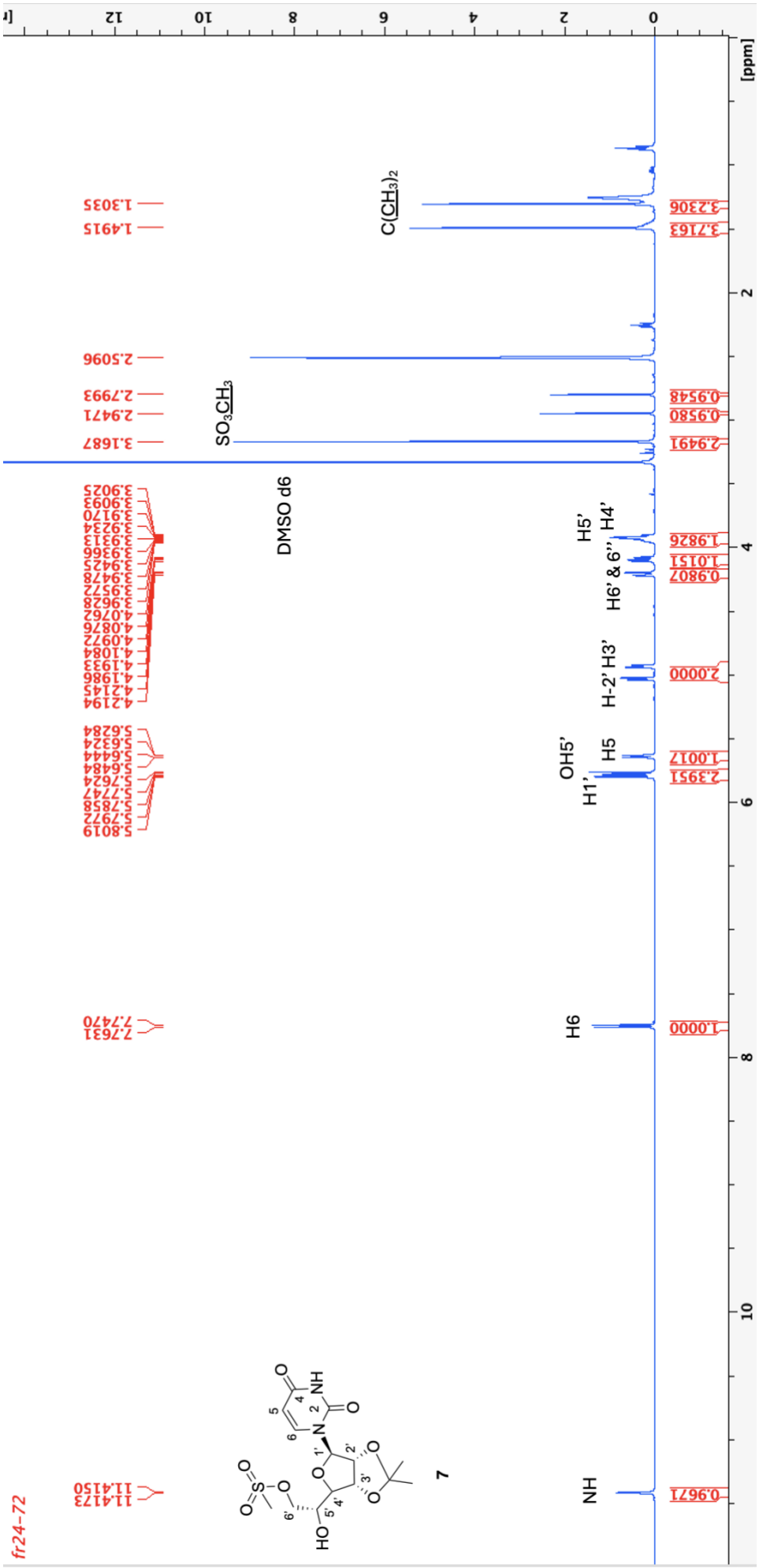

Figure 13. Compound 7 ^{1}H NMR

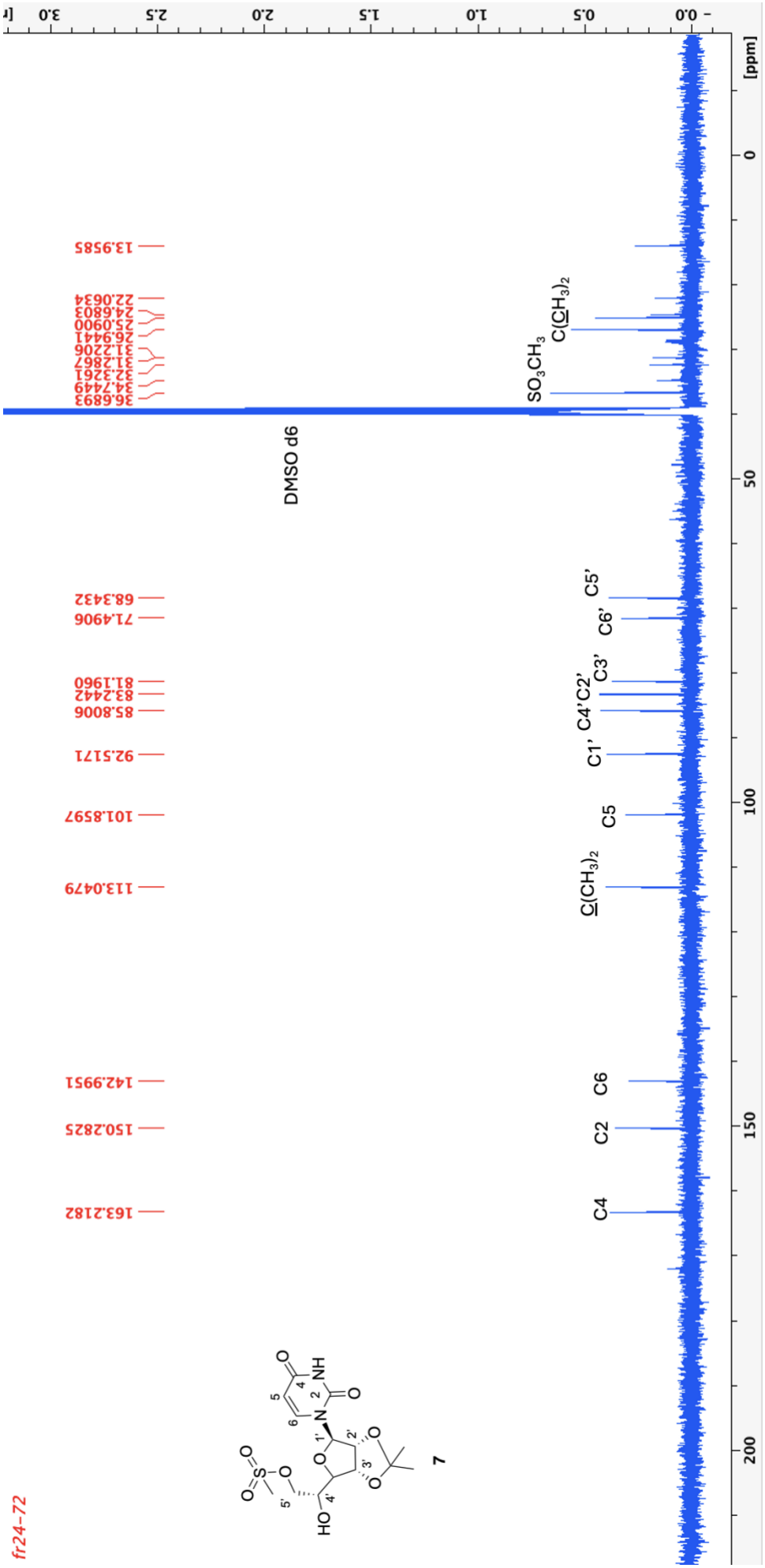

Figure 14. Compound 7 ^{13}C NMR

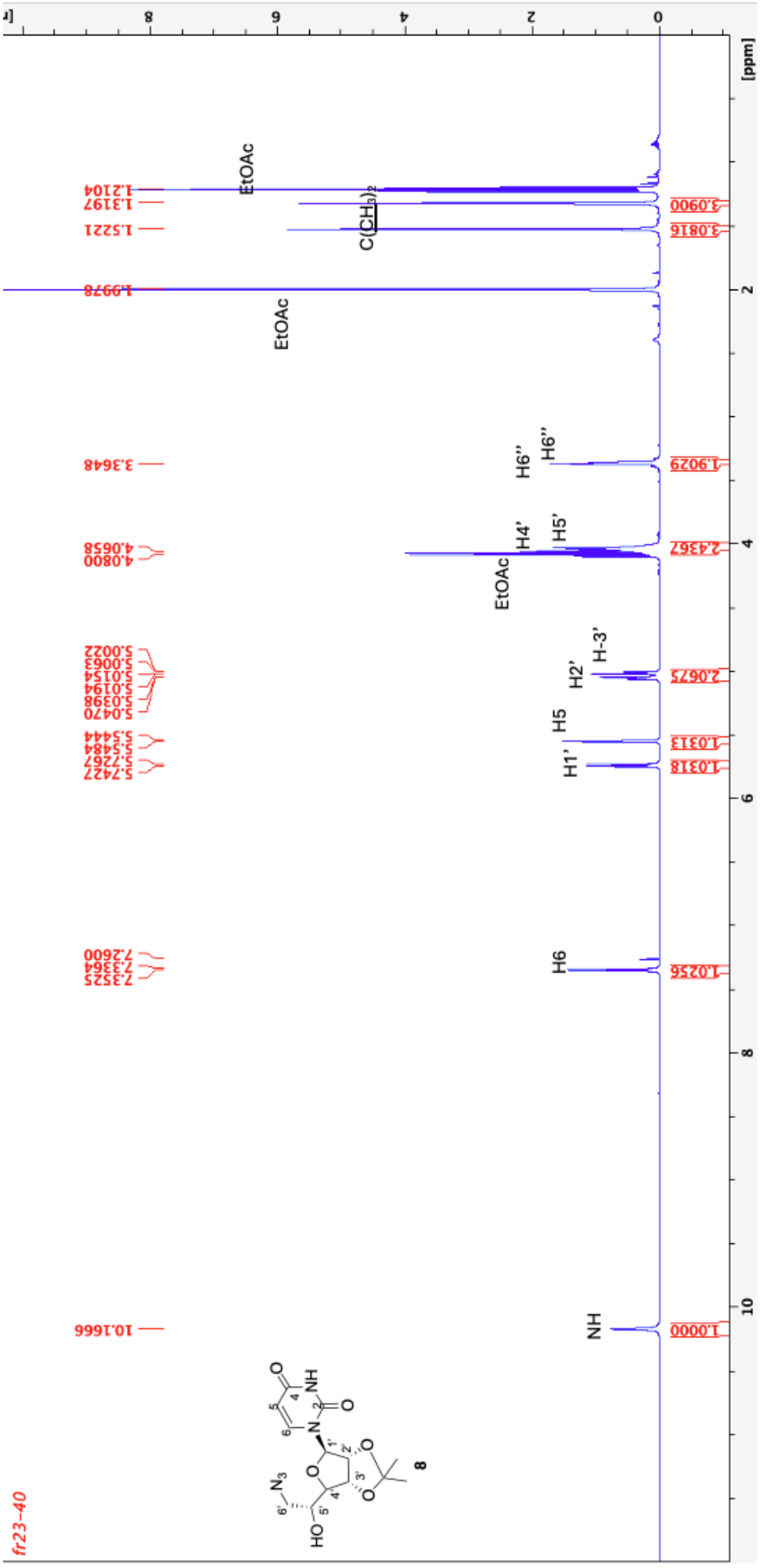

Figure 15. Compound 8 ^{1}H NMR

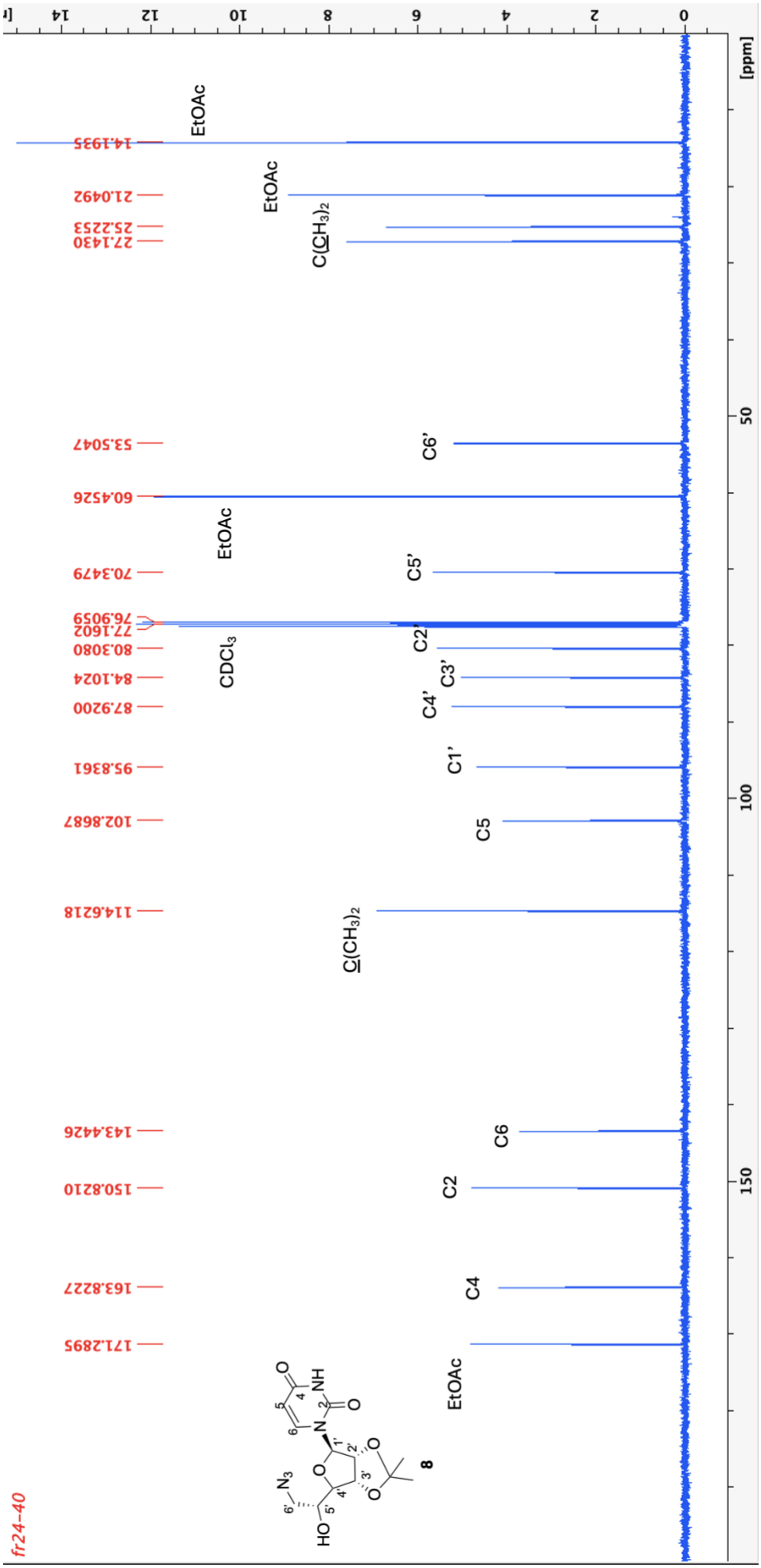

Figure 16. Compound 8 ^{13}C NMR

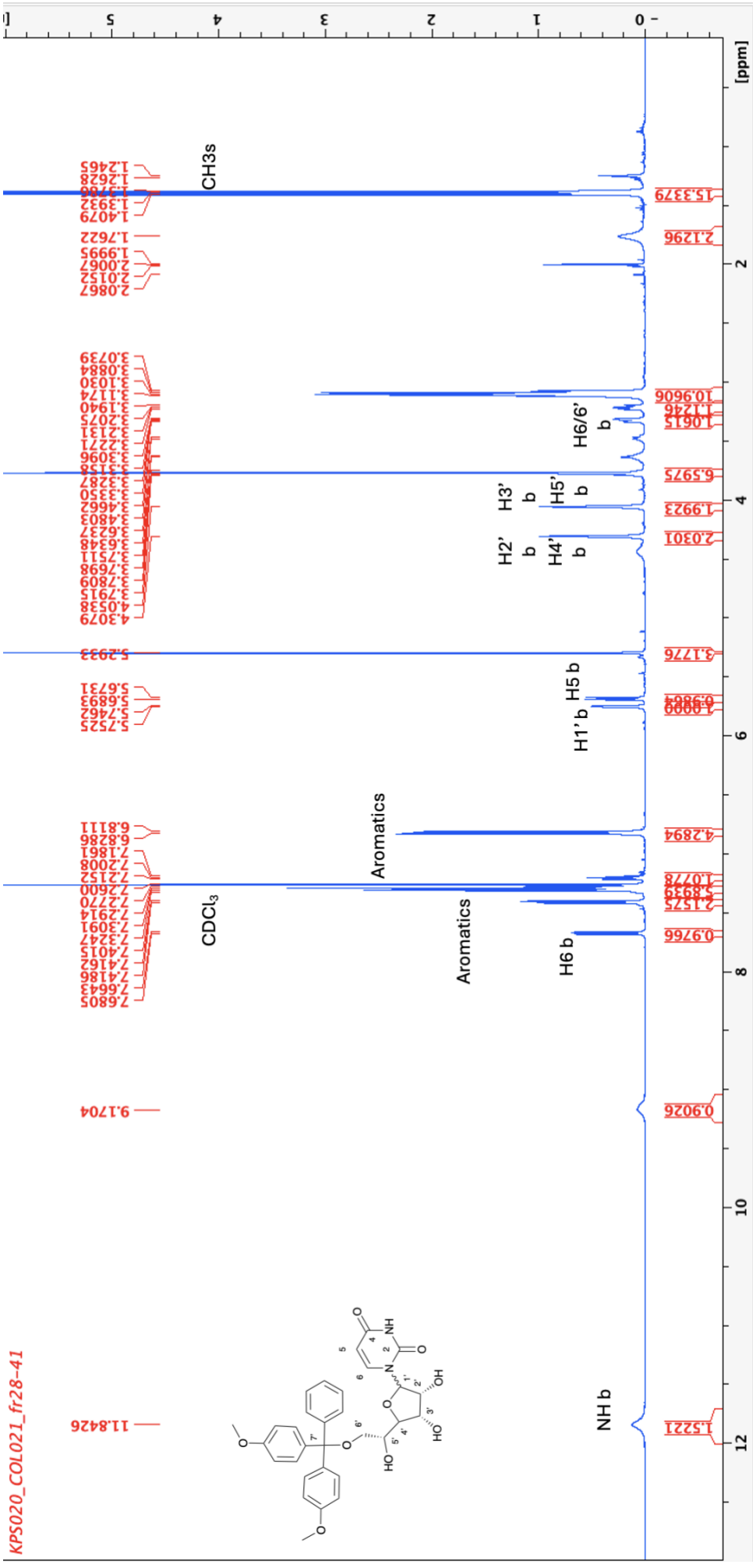

Figure 17. Compound 11 ^{1}H NMR

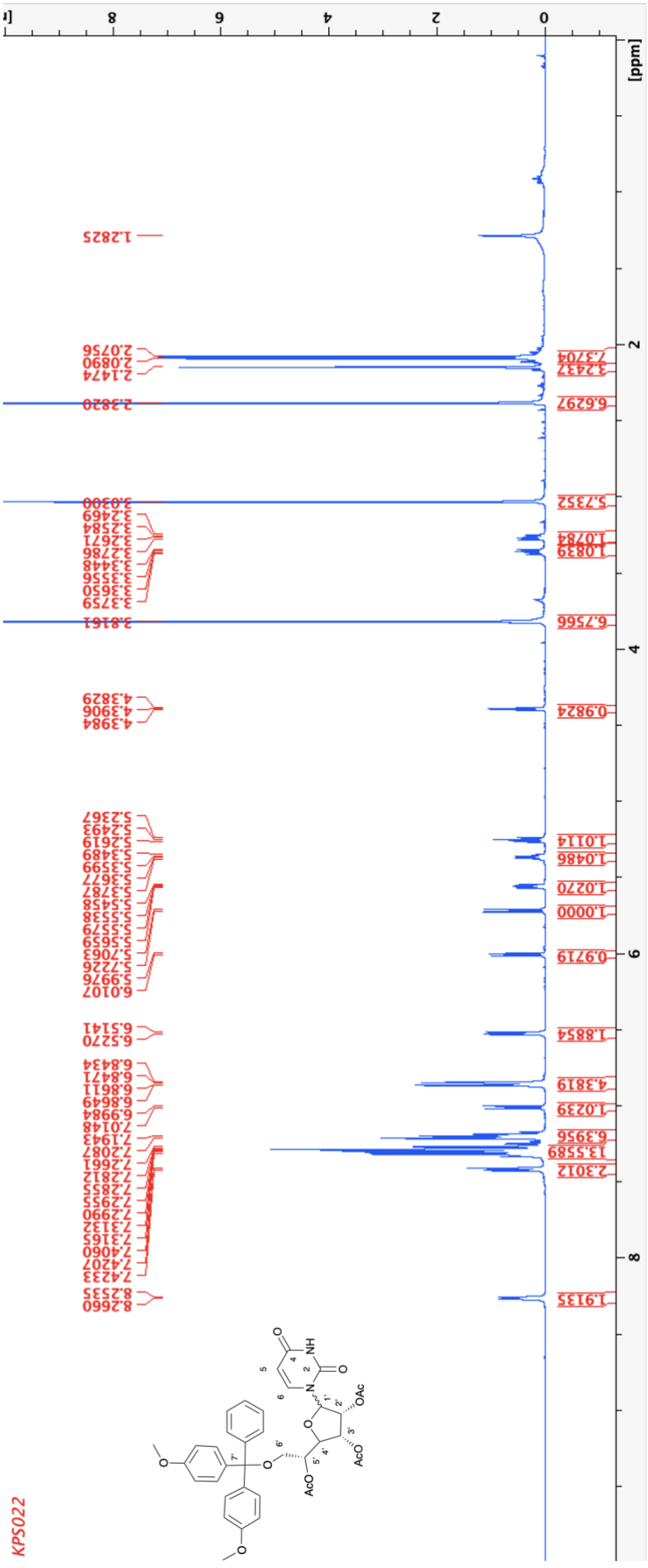

Figure 18. Compound 12 ^{1}H NMR

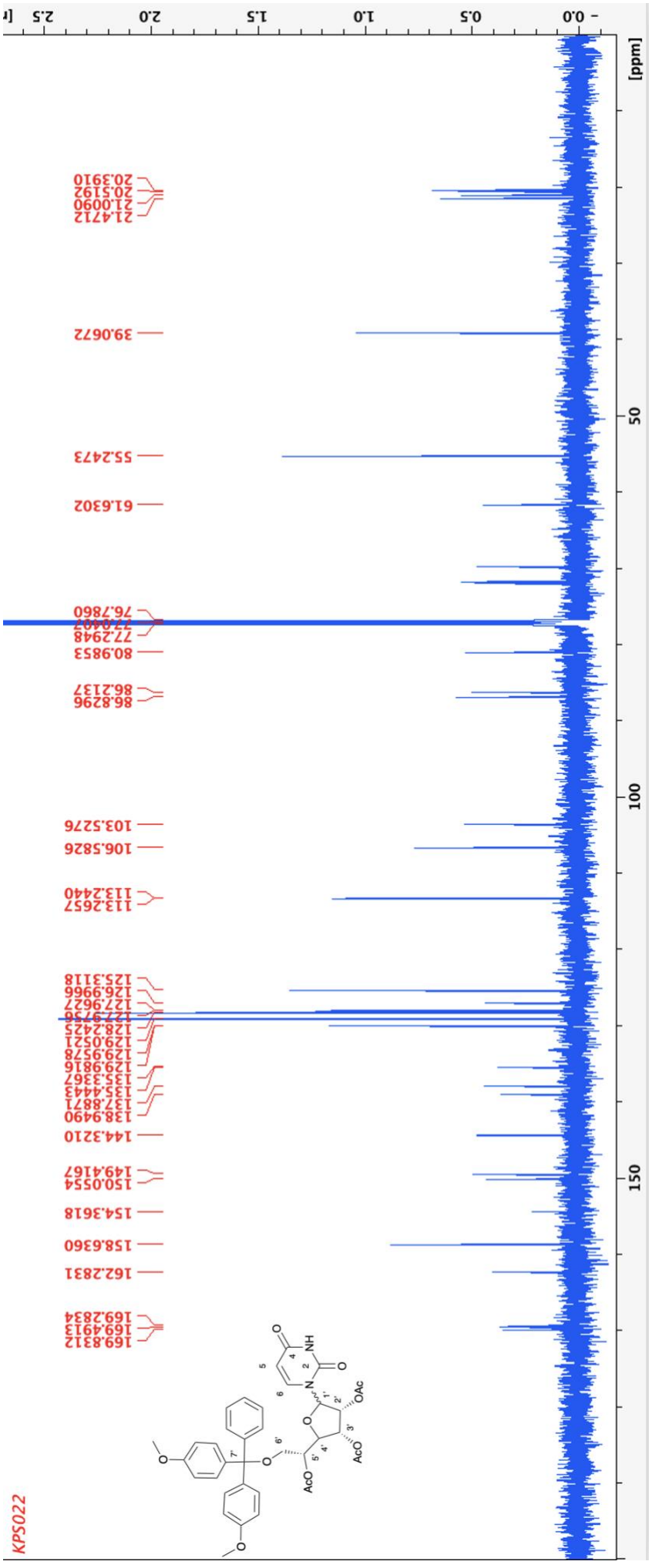

Figure 19. Compound 12 ^{13}C NMR

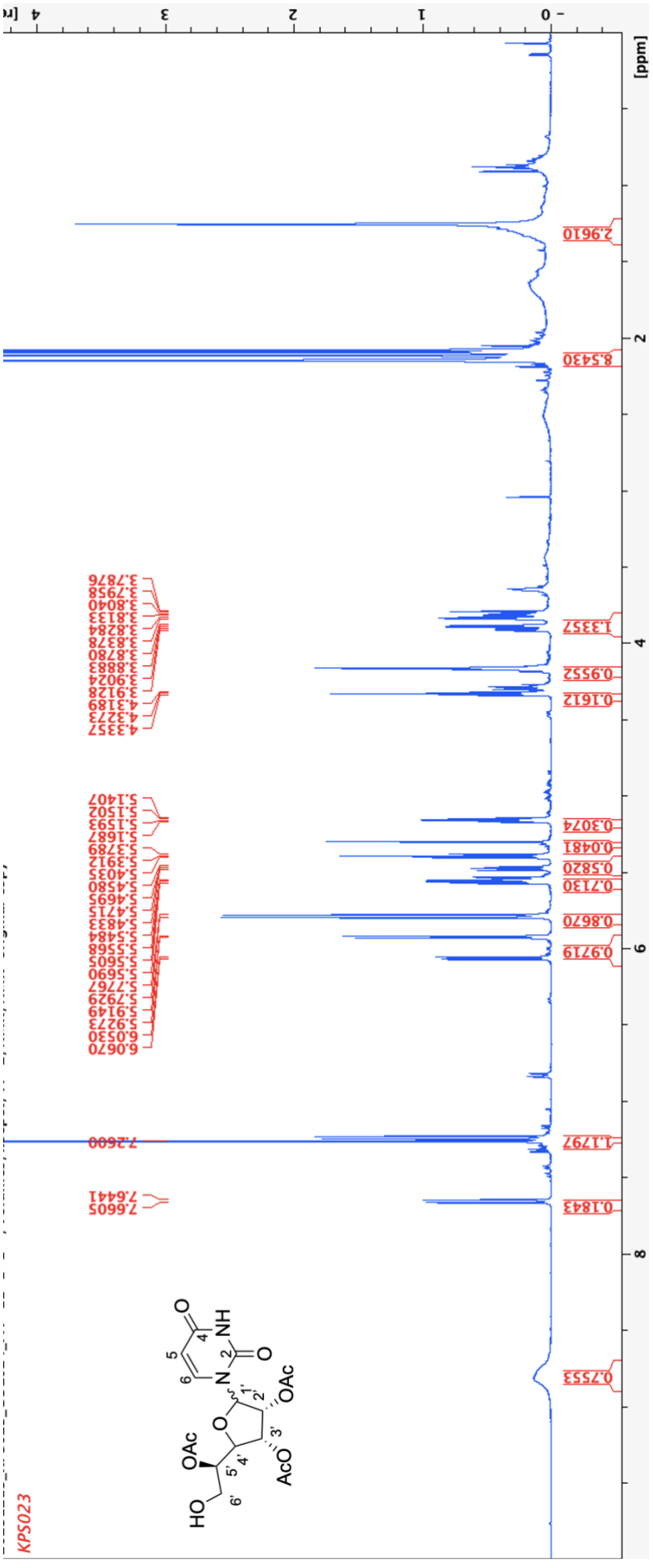

Figure 20. Compound 13 ^{1}H NMR

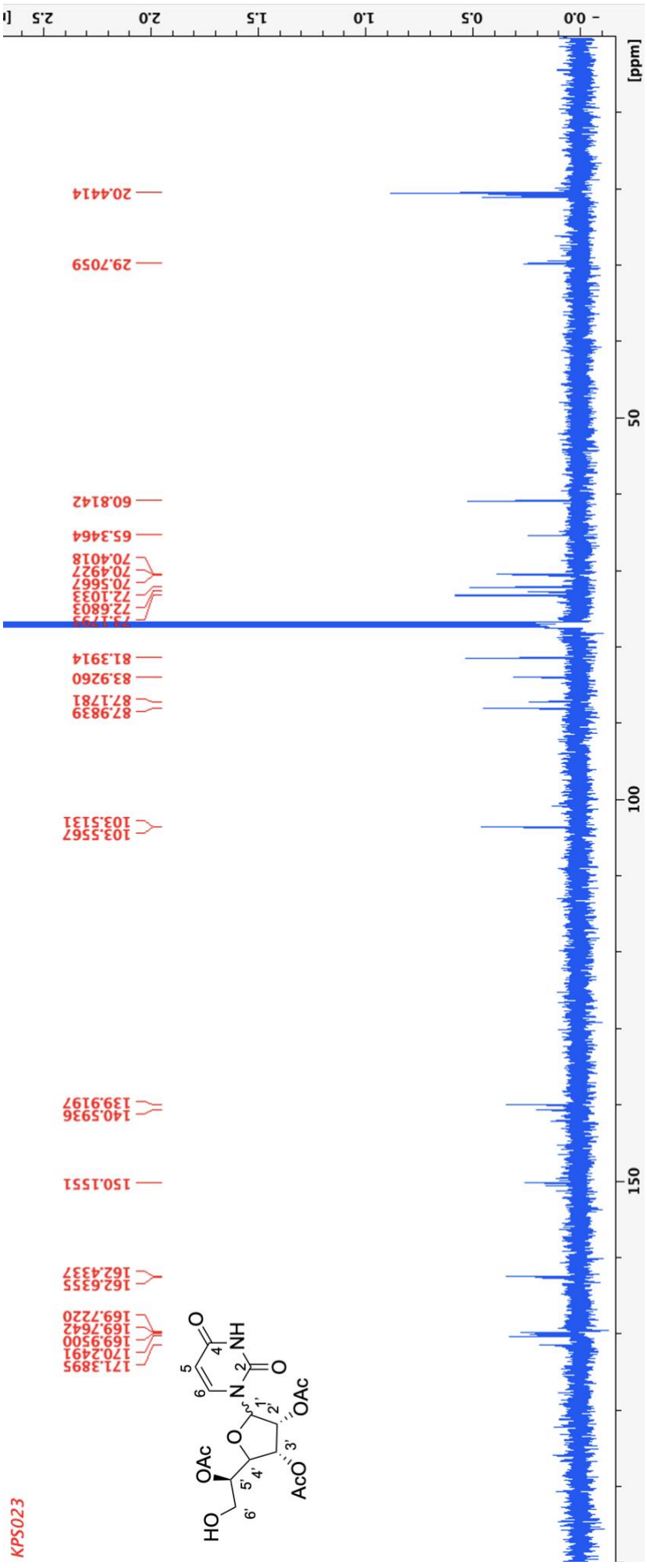

Figure 21. Compound 13 ^{13}C NMR