Synthesis of Peptidoglycan Peptides for DNA Aptamer Selection

by

Wenxuan Zhang

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

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Examining Committee:

Martin Tanner

Supervisor

Harry Brumer

Supervisory Committee Member

Stephen Withers

Supervisory Committee Member

Abstract

Gram-positive bacteria possess a thick layer of peptidoglycan outside the cell membrane that is rigidified through crosslinks between neighboring peptide chains on the polysaccharide structure.

Vancomycin, a glycopeptide antibiotic, is effective at inhibiting the growth of Gram-positive bacteria by preventing the formation of crosslinks. The molecular basis of vancomycin's action is tight binding to a cell wall peptide precursor that terminates in D-Ala-D-Ala. In vancomycin resistant bacteria, the D-Ala-D-Ala linkage is replaced by D-Ala-D-Lac and the loss of a hydrogen bond to the amide NH accounts for a 1000-fold loss in potency.

In this research, we hope to generate DNA aptamers that will serve as an alternative to vancomycin and bind tightly to the peptidoglycan peptides. For DNA aptamer selection, two target compounds are synthesized. One molecule (**Target 2**) mimics the cell wall peptide precursor of vancomycin sensitive bacteria that terminates in L-Lys-D-Ala-D-Ala. Another molecule (**Target 3**) mimics the cell wall peptide precursor of vancomycin-resistant bacteria that terminates in L-Lys-D-Ala-D-Lac. This molecule will also be used to find catalytic unnatural DNA sequences (bearing primary amine groups) that can trans-amidate and displace the terminal lactate moiety. This will serve to form an agent that inactivates peptidoglycan biosynthesis of vancomycin resistant bacteria by forming a covalent linkage.

Lay Summary

The key goal in this research was to synthesize two target molecules for the selection of DNA aptamers and DNAzymes. The two targets will be mimicking vancomycin-sensitive and vancomycin-resistant bacterial peptidoglycan peptides respectively. Selected DNA aptamers and DNAzymes can potentially serve as alternatives to antibiotics in the treatment of Gram-positive bacterial infections.

Preface

This dissertation is original, unpublished, independent work by the author, Wenxuan Zhang.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

aq.	aqueous solution
Bn	benzyl
Boc	*tert*-Butyloxycarbonyl
Br	bromide
Cbz	benzyl
COMU	(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate
D-Ala	D-alanine
DCC	N, N'-dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	N-Ethyldiisopropylamine
D-Lac	D-lactate/ D-lactic acid
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
Fmoc	fluorenylmethyloxycarbonyl
GlcNAc	N-acetyl glucosamine
HBTU	N, N, N', N'-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole hydrate
L-Lys	L-lysine

LC-MS	liquid chromatography–mass spectrometry
M	molar
m/z	mass to charge ratio (mass spectrometry)
Me	methyl
Methanol-d4	deuterated methanol
MeOH	methanol
MurNAc	N-acetyl muramic acid
MHz	megahertz
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
OAMS	open-access mass spectrometer
Pd	palladium
PG	peptidoglycan
ppm	parts per million
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
rt	room temperature
TEA	triethylamine
TFA	trifluoro acetic acid

Acknowledgements

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My sincere thanks also go to all my lab colleagues who provided me with great academic support for my research and a wonderful working environment. Without they precious support it would not be possible to conduct this research.

Dedication

To my parents

And all my friends

Without whom none of my success would be possible

Chapter 1: Introduction

1.1 Bacterial cell walls

1.1.1 Cell wall structures of diverse types of bacteria

Bacteria can be categorized into two types: Gram-positive and Gram-negative, according to difference in their cell wall composition. The cell wall of Gram-positive bacteria is composed of a thick layer of peptidoglycan outside the plasma membrane; while the cell wall of Gram-negative bacteria is composed of an outer membrane and a thinner layer of peptidoglycan (Figure 1.1).

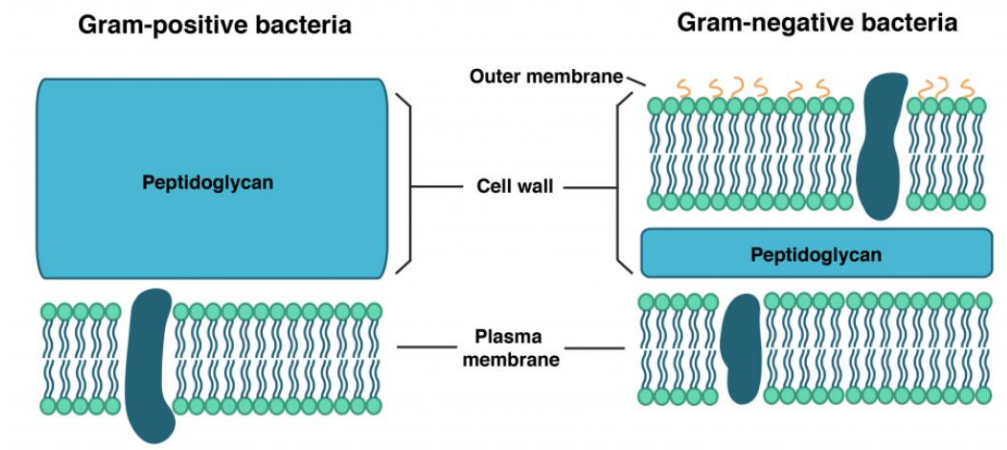

Figure 1.1. Schematic cell wall composition of Gram-positive and Gram-negative bacteria.

1.1.2 Peptidoglycan structure and its formation in Gram-positive bacteria

Peptidoglycan is a porous cross-linked polymer composed of three elements: glycan backbone, tetra-peptide side chains linked to N-acetyl muramic acid (MurNAc) and peptide cross linkage (Figure 1.2). The glycan backbone is made from repeating units of N-acetyl

muramic acid (MurNAc) and N-acetyl glycosamine (GlcNAc) linked by β-glycosidic bonds. The tetra-peptide side chain is linked to the MurNAc residue in each repeating unit and is crosslinked with its neighboring tetra-peptide side chain to guarantee the strength of bacterial cell wall.

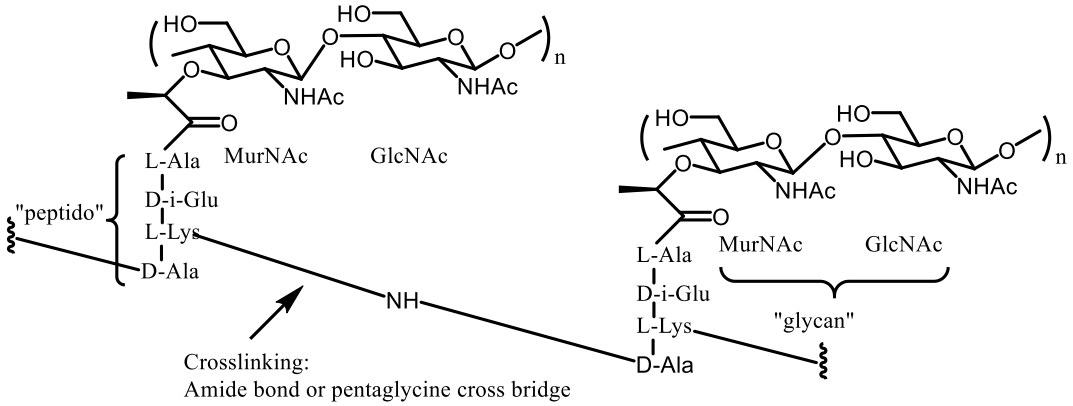

Figure 1.2. Peptidoglycan structure.

The tetra-peptide cross-linkage is formed in a late stage of the cell wall synthesis process from the MurNAc-GlcNAc polysaccharide bearing pentapeptide side chains (Figure 1.3). For Gram-positive bacteria, the pentapeptide side chain has an amino acid sequence L-Ala-D-*iso*-Glu-L-Lys-D-Ala-D-Ala. In some strains of bacteria, Lys forms a direct amide bond with D-Ala on the neighboring strand; in others the Lys residue is further modified by a pentaglycine peptide. In the crosslinking process, transpeptidase links one tetrapeptide chain to an adjacent precursor either directly via a Lys-D-Ala amide bond or via a pentaglycine cross bridge[1].

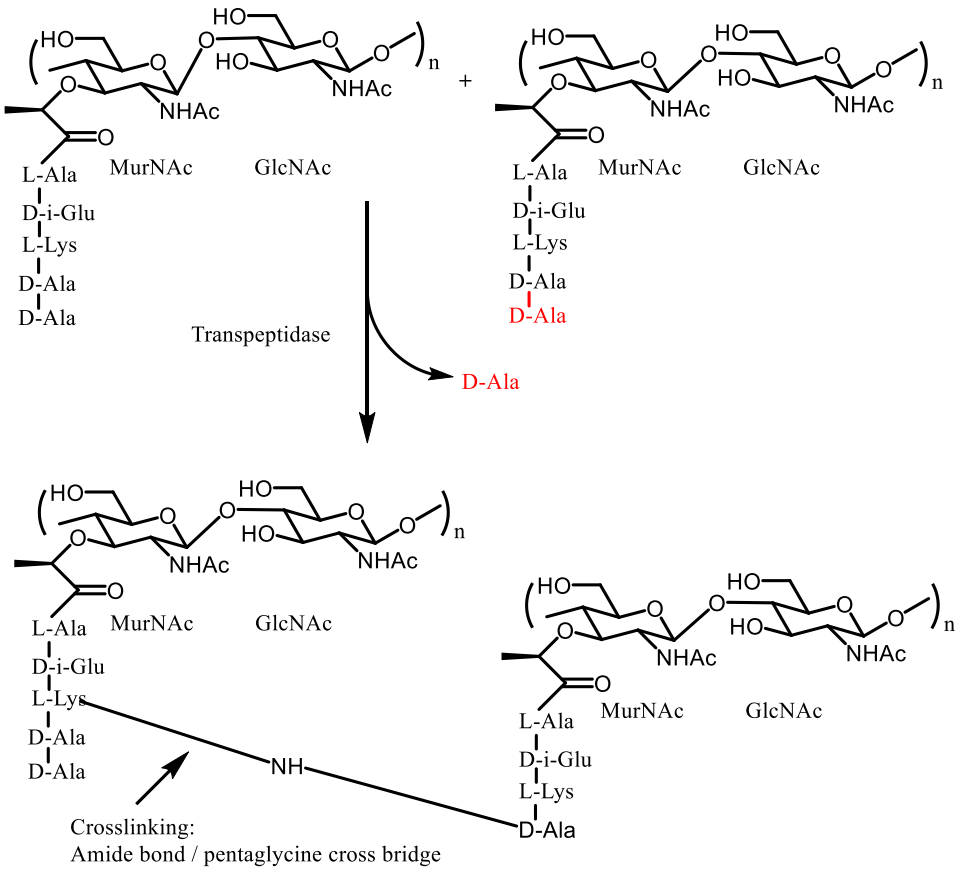

Figure 1.3. Schematic crosslink formation in peptidoglycan of Gram-positive bacteria.

1.2 Antibiotic treatment of Gram-positive bacterial infections: vancomycin

1.2.1 Working mechanism of vancomycin

Vancomycin is an important glycopeptide antibiotic drug for treating infections caused by Gram-positive bacteria. It was first isolated from a soil sample collected by the American pharmaceutical company Eli Lily in the mid-1950s [2]. The now-accepted chemical structure of vancomycin was first determined by Harris and Harris[3] (Figure 1.4). The working mechanism of vancomycin was found to involve its tight binding to the D-Ala-D-Ala moiety on the uncrosslinked cell wall precursor via five hydrogen bonds with a binding

constant of 1.6×10^{-5} M^{-1}[4]. This interaction prevents the cross-link formation between adjacent precursors, which will lead to incomplete cell wall formation and bacterial death.

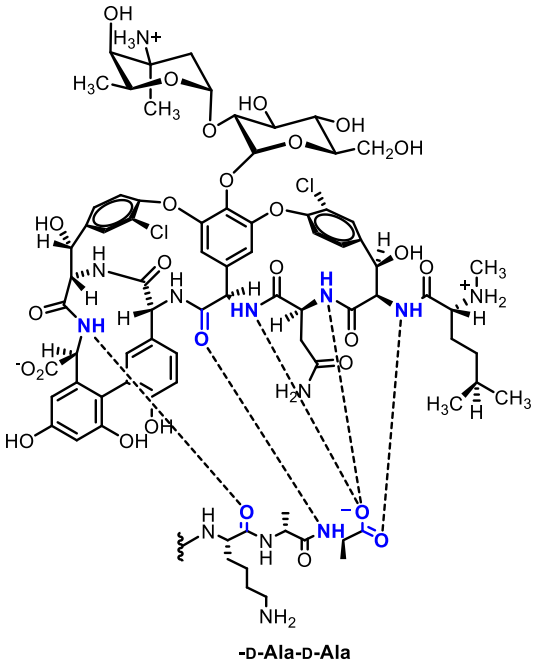

Figure 1.4. Structure of vancomycin and its interaction with the D-Ala-D-Ala moiety. Hydrogen binding is indicated with dotted lines. Atoms participating in interaction between two molecules are colored blue.

1.2.2 Vancomycin resistant bacteria

Vancomycin has been considered the last line of bacterial defense in the clinic for the treatment of Gram-positive bacteria[5]. The emergence of vancomycin resistant bacteria has caused a serious health problem for human beings. These bacteria are able to survive in the presence of vancomycin by structurally changing the pentapeptide side chain of the cell wall precursors. Walsh and co-workers have found that vancomycin resistance arises from

substitution of D-Ala-D-Ala with D-Ala-D-Lac[6] (Figure 1.5). The substitution of a nitrogen atom with an oxygen atom eliminates one out of the five significant hydrogen bonding interactions. Furthermore, the lone pairs on the two oxygen atoms introduce repulsion that further destabilizes the binding interaction between vancomycin and the cell wall precursor. These two factors account for a 1000-fold loss of efficiency for the drug in vancomycin resistant bacteria.

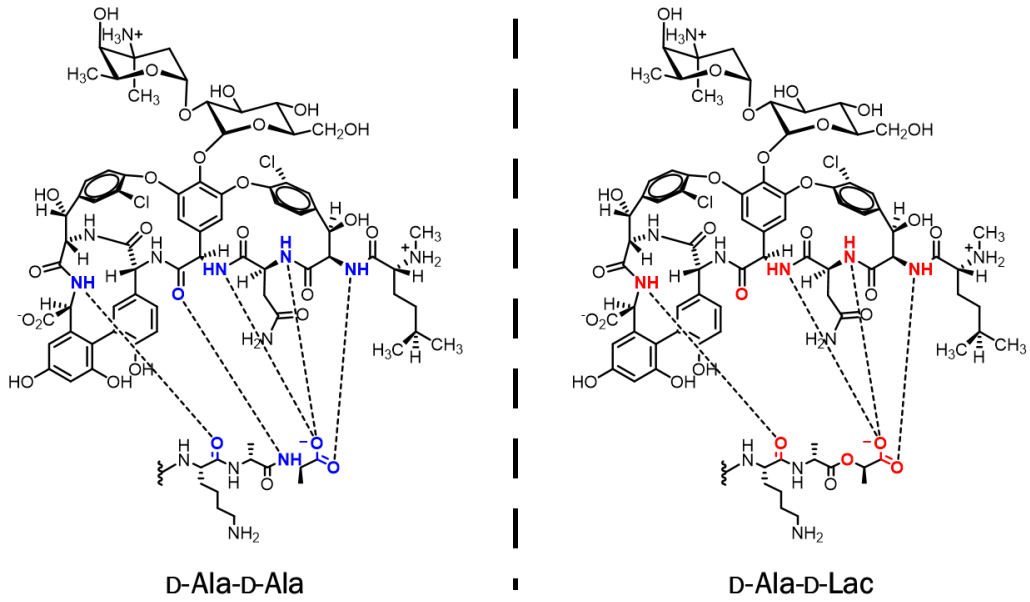

Figure 1.5. Interactions between vancomycin and the D-Ala-D-Ala and D-Ala-D-Lac moieties. Hydrogen bonding is indicated with dotted lines. Atoms participating in the interaction with D-Ala-D-Ala are colored blue and atoms participating in the interaction with D-Ala-D-Lac are colored red.

1.2.3 Peripherally modified vancomycin with retained activity and their problems

As vancomycin is derived from a peptide containing seven amino acid residues and possesses a very complicated crosslinked structure, it is very hard to engineer in new contacts to the modified peptidoglycan substrate[7][8]. However, the vancosamine nitrogen of the disaccharide moiety is easily modifiable and it was found that glycopeptides containing hydrophobic substituents at this position should increase activity against vancomycin resistant strains[9][10] (Figure 1.6). It is proposed that these carbohydrate-modified vancomycin compounds are effective due to their direct interaction with proteins functionalizing in the final steps of peptidoglycan biosynthesis, and there is no significant increase of binding potency to the D-Ala-D-Ala dipeptide moiety[11].

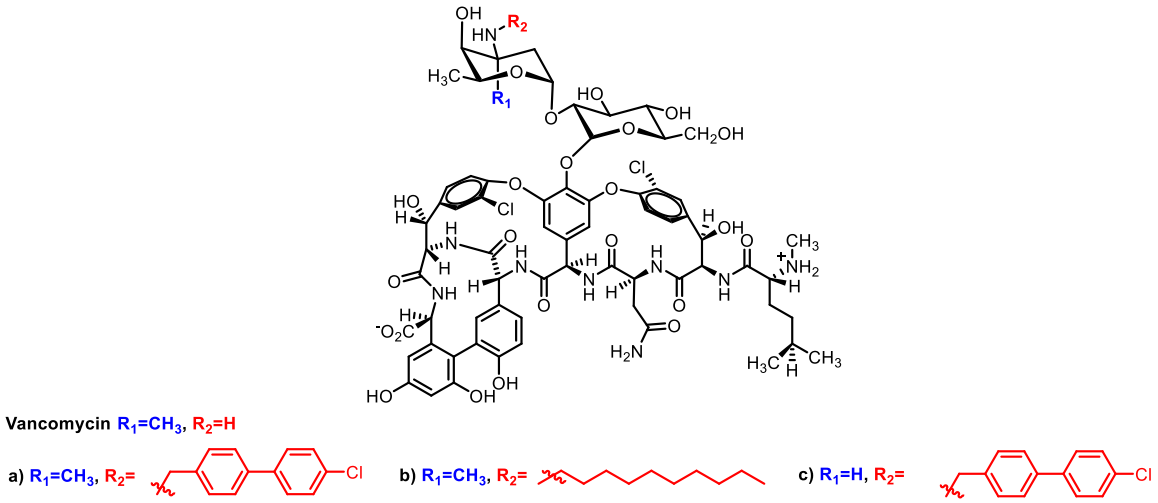

Figure 1.6. Structure of vancomycin and modified vancomycin compounds **a** to **c**.[Adapted from reference 9].

In further studies, it was found that the more independent but synergistic changes that are introduced, the better the potency of these modified compounds will be. Boger and co-workers developed several vancomycin analogs with three modifications: vancosamine nitrogen modified by (4-chlorobiphenyl)methyl (CBP), C- terminal modification and binding pocket modification[12]. These molecules were found to possess three independent and synergistic mechanisms in their antibiotic activity and showed comparable antibiotic potency to vancomycin when tested against resistant strains. The most potent vancomycin analogs with modifications are as shown in Figure 1.7 with ascending reactivity.

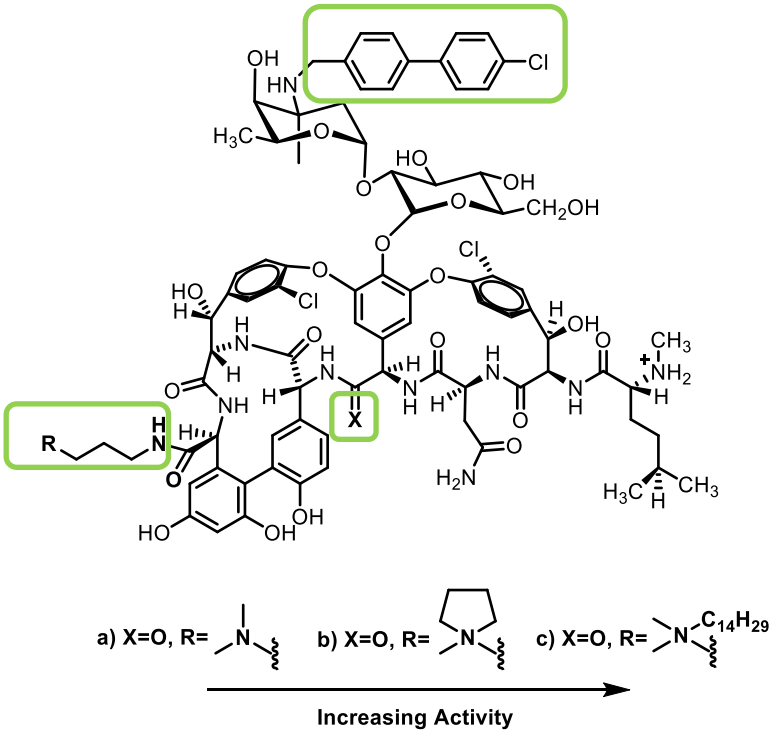

Figure 1.7. Combined CBP, C-terminal peripheral modifications and binding pocket modification to vancomycin. [Adapted from reference [12]].

Although vancomycin analogs with peripheral modifications show comparable antibiotic activity against resistant strains, synthesis of such compounds is costly. Finding replacements for vancomycin is therefore an attractive alternative.

1.3 DNA aptamers

1.3.1 DNA aptamers with natural and modified nucleobases

DNA aptamers are generally defined as single strand DNA oligonucleotides with a distinct folded three-dimensional pocket that can bind specific biomolecular targets with high specificity and affinity (Figure 1.8). Aptamers play a promising role in biological science and have been extensively applied as analytical tools, therapeutic agents and as vehicles for targeted drug delivery[13].

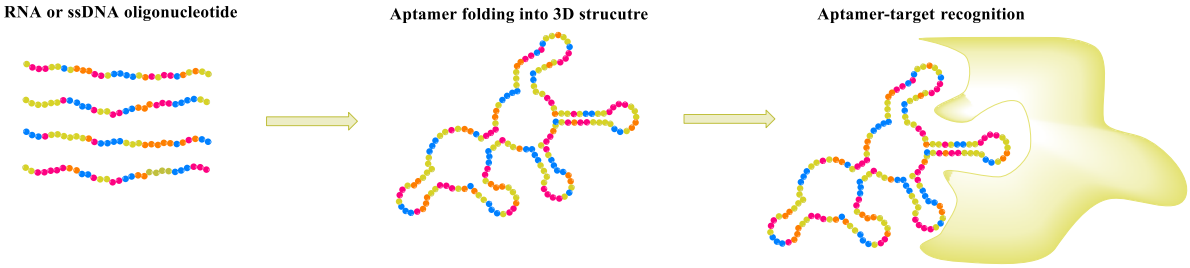

Figure 1.8. Schematic representation of aptamer-target interaction.[Adapted from reference [14]].

The nucleobases presenting in DNA aptamers can be either natural ones or artificially modified ones. As an alternative to antibodies, aptamers have an advantage due to the chemical nature of nucleic acids that allows for easy amplification, synthesis and modification of aptamers. At this stage, aptamers formed by purely natural nucleobases

have been applied to fulfill diverse functions including sensing, purification, and acting as diagnostic or therapeutic agents[15,16,17,18,19,20]. Ongoing efforts have focused on engineering aptamer molecules to enhance their bioavailability to act as regulatory switches and to perform multiple functions[21].

1.3.2 A common method for the rapid selection of highly specific DNA aptamers: SELEX selection

SELEX, Systematic Evolution of Ligands by Exponential Enrichment, is a common DNA aptamer selection procedure that was developed independently by Ellington's and Tuerk's group more than two decades ago[22]. In SELEX, a large pool of up to 10^{16} DNA sequences are used for the selection of aptamers that recognize their target through a three-dimensional binding pocket with high affinity and specificity. The targets can vary from small molecules to proteins, to cells [23,24,25].

The process begins with the synthesis of a very large library of oligonucleotides consisting of randomly generated sequences that are flanked by short constant regions for the sake of primer extension during PCR. In the case of DNA aptamers, a chemically synthesized DNA library is incubated with target molecules. Potential aptamers will bind to the target molecules and non-binding oligonucleotides will be washed away. With more stringent elution conditions, the bound oligonucleotide may be released. These DNA sequences are amplified by PCR and additional rounds of selection are performed. The process will be done for several rounds to select for only the tightest binding DNA aptamers (Figure 1.9)[14].

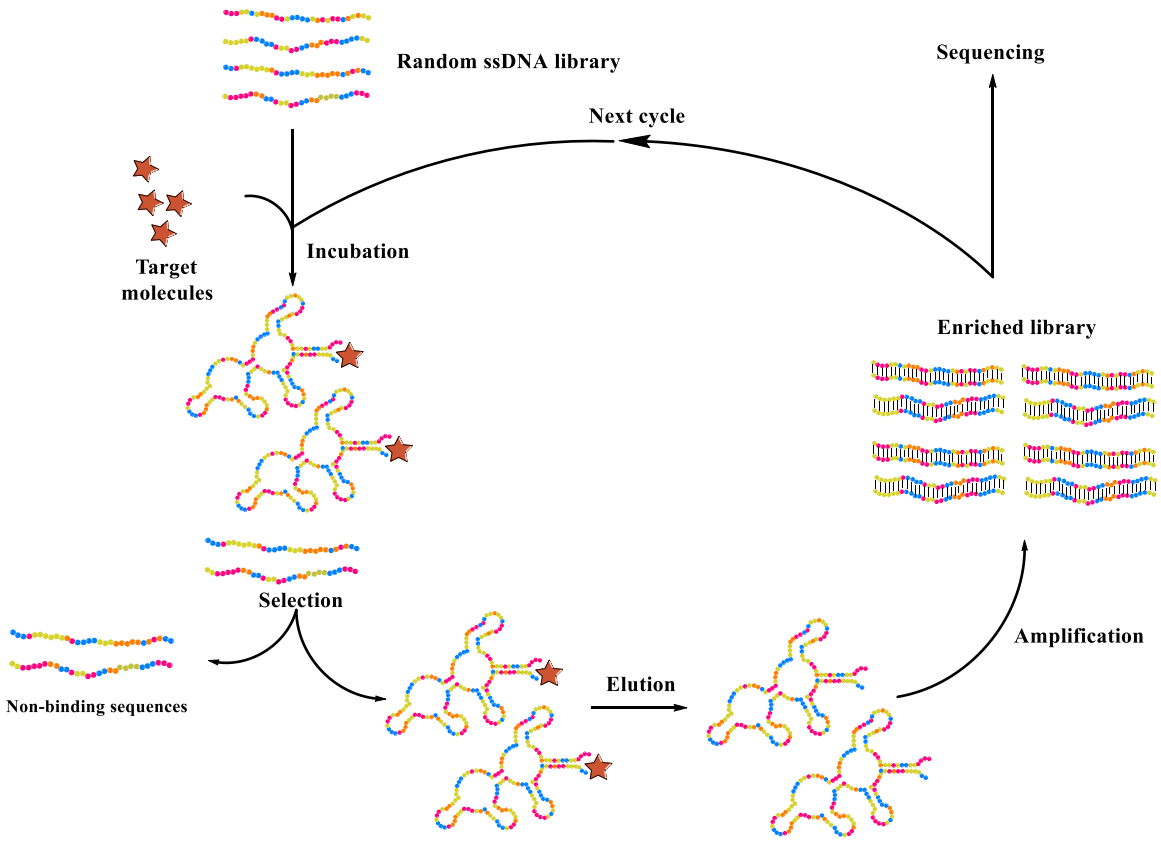

Figure 1.9. Schematic representation of SELEX selection of DNA aptamers. The random ssDNA library is first incubated with targets. Those aptamers binding with target will be split from unbound sequence, collected and amplified by PCR. After several rounds of selection, sequencing step is performed followed by evaluation of target affinity of the enriched aptamers. [Adapted from reference [26]].

1.3.3 DNA aptamers as oligonucleotide-based binding agents

DNA aptamers can be utilized to target a wide range of molecules varying from small molecules to proteins. Three-dimensional structural studies on several aptamer-target adducts have revealed that the binding specificity exhibit by DNA aptamers are due to the molecular interactions with their target molecules[27]. An AMP-binding DNA aptamer with

a dissociation constant of 6 μM shows stacking and hydrogen-bonding interactions with the aromatic ring on adenosine[28]. In the study of two arginine-binding DNA aptamers with dissociation constants of 125 μM, it was found that positively charged guanidinium group interacts exclusively with the nucleobases instead of the phosphodiester backbone[29]. And the binding affinity results from molecular shape complementary between the guanidinium groups and the cytosine groups[30].

1.3.4 DNAzymes as oligonucleotide-based catalysts

DNA-based catalysts, also known as DNAzymes, have been developed due to the folding properties that enable them to adopt a discrete three-dimensional shape. DNAzymes with desired activities are selected by altering the stringency of selection in SELEX, including the length of random domains, buffer composition, metal cofactors, temperature, and ionic strength.

Applications of in vitro selections have led to the identification of numerous DNAzymes capable of catalyzing the scission of phosphodiester linkages. The majority of cleavage reactions catalyzed by DNAzymes happen at phosphodiester bonds within RNA (Figure 1.10a) and DNA oligonucleotides (Figure 1.10b)[31,32,33]. The reactivity of these DNAzymes always require the presence of one or more divalent metal cofactors such as Mg^{2+}, Pb^{2+} and Ca^{2+}. The presence of metal ions, depending on the reaction type, may serve to stabilize the negative charges of oligonuclear backbones, reduce repulsion between negative charged substrate species and the DNAzymes, activate hydrolysis reactions, and

stabilize reaction intermediates. For example, in the case of RNA phosphodiester cleavage, M^{2+} ions stabilize non-bridging negatively charged oxygen atoms (Figure 1.11a) and activate the 2'-hydroxyl group through coordination (Figure 1.11b)[34]. The negatively charged backbone oxygen of the 2',3'-cyclic phosphate intermediate is also stabilized by metal ions (Figure 1.11c).

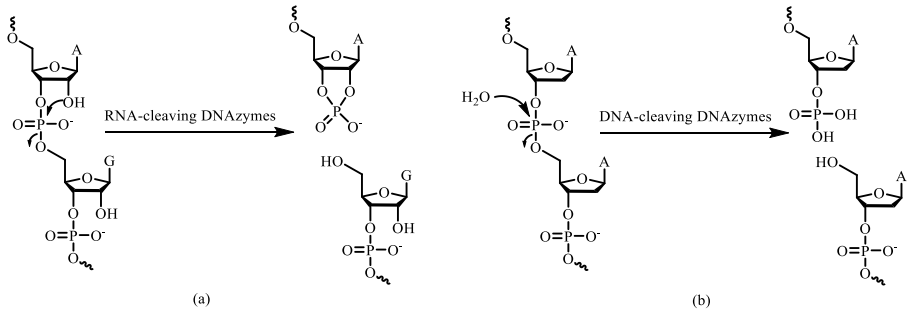

Figure 1.10. Scheme of phosphodiester hydrolysis reactions catalyzed by DNAzymes: (a) cleavage of ribophosphodiester linkages; (b) cleavage of deoxyribophosphodiester linkages [Adapted from references [31], [32] and [33]].

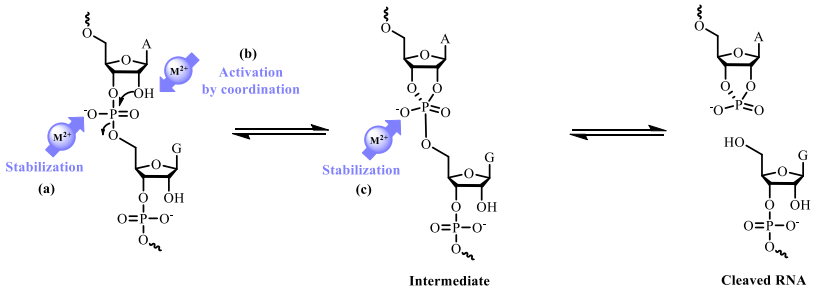

Figure 1.11. RNA cleavage reaction and putative sites of divalent metal ion interactions: (a) backbone non-bridging negative oxygen stabilization; (b) activation of 2'-hydroxyl group through coordination; (c) stabilization of negatively charged non-bridging oxygen atom.[Adapted from reference [34]].

The two most representative Mg^{2+}-dependent DNA metalloenzymes that cleave a broad variety of all-RNA substrates, coined Dz8-17(Figure 1.12a) and Dz10-23(Figure 1.12b), were isolated by Santoro and Joyce[35]. These DNAzymes work at high concentrations of Mg^{2+} (~100 mM) with efficiency approaching kinetic perfection (~10^9 $min^{-1}M^{-1}$). Dz10-23 shows an ability to cleave all purine-pyrimidine junctions with high tolerance; while Dz8-17 mostly prefers a G-A dinucleotide junction but can cleave all 16 dinucleotide junctions albeit with k_{obs} spanning over five orders of magnitude.

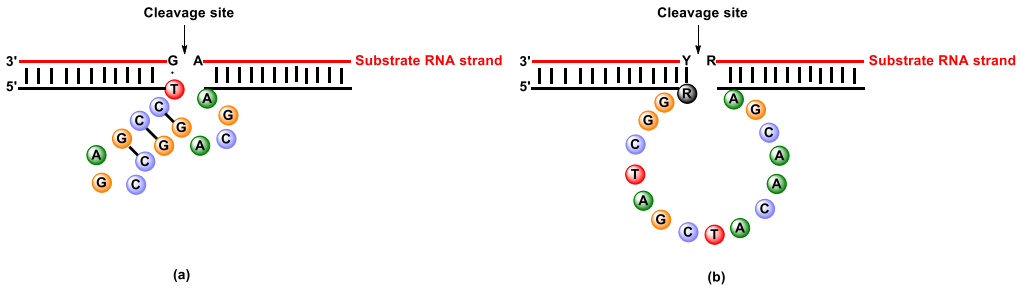

Figure 1.12. Schematic depiction of putative secondary structure of all-RNA-cleaving DNAzymes: (a) Dz8-17; (b) Dz10-23 (Y=U or C, R= A or G) [Adapted from reference [36]].

10MD5 is the most active DNAzyme that cleaves phosphodiester bonds at the ATG^T site (Figure 1.13)[31]. This enzyme shows high efficiency in cleaving all-DNA substrates in the presence of both Mn^{2+} and Zn^{2+} ions with a rate constant of k_{obs}= 0.045 min^{-1}. The limitation of this DNAzyme is that it possesses poor substrate tolerance (cleavage only happens at ATGT position) and it is very sensitive to even minute pH changes.

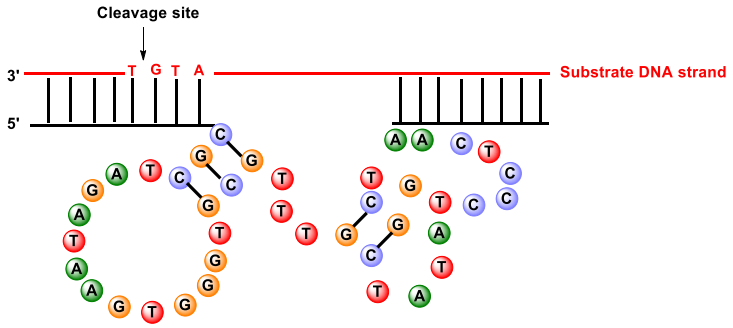

Figure 1.13. Sequence and hypothesized 2D structure of all-DNA-cleaving DNAzyme 10MD5. [Adapted from reference [31]].

Though the DNA-catalyzed cleavage of other bonds is rare, DNA-catalyzed hydrolysis of ester (maximum rate of k_{obs}= 0.05 min^{-1}) and anilides bonds (maximum rate of $k_{obs}= 3.5 \times 10^{-3}$ min^{-1}) have been reported recently[33]. This discovery promises a bright future for DNAzyme catalyzed cleavage applications to a wider variety of bio-organic molecules. Dz 14WM9 (Figure 1.14a), a DNAzyme selected by Silverman's group, is capable of hydrolyzing a monophosphoester bond attached to the side chain of tyrosine or serine on a polypeptide chain[37]. The hydrolysis reaction happens in the presence of Zn^{2+} at $k_{obs}= 5.2 \times 10^{-3} min^{-1}$. Another DNAzyme, named DhaDz1(Figure 1.14b), has been selected for elimination of a phosphate group on a serine residue of a peptide chain through C-O bond cleavage[38]. This DNAzyme's catalytic activity is dependent on three metal cofactors: Zn^{2+}, Mn^{2+} and Mg^{2+} and the cleavage reaction happens with a rate constant of $k_{obs}= 4.7 \times 10^{-3} min^{-1}$.

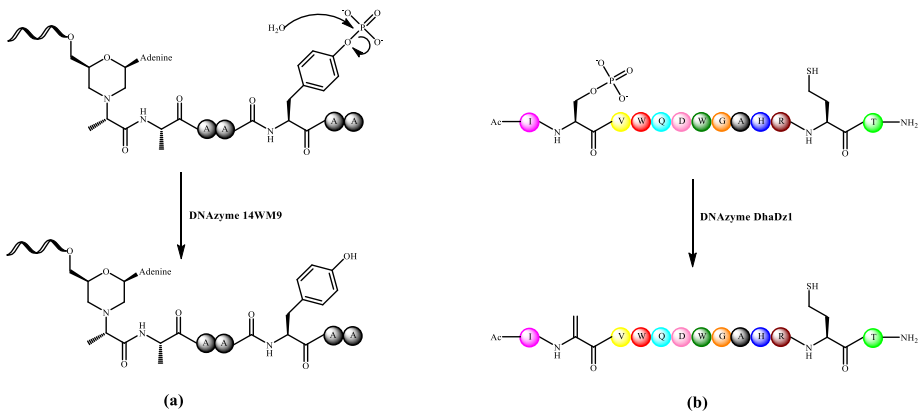

Figure 1.14. Reactions catalyzed by P-O and C-O bond cleavage DNAzymes: (a) DNAzyme 14WM9; (b) DNAzyme DhaDz1.[Adapted from references [37] and [38]].

1.3.5 DNA aptamers and DNAzymes containing modified nucleobases

Disadvantages of using natural nucleic acids for DNA aptamers selection include the poor tolerance to degradation by DNases, sensitivity to acidic conditions and limited diversity of nucleic acids libraries. To overcome such drawbacks exhibit by natural nucleic acids, modified nucleic acids are introduced to the existing nucleic acid library.

For DNA aptamers, modification on nucleobases can improve an aptamers' binding affinity and selectivity to target molecules. Modifications like introduction of positively charged species to hydrophobic nucleobases region enhances electrostatic interactions between nucleobases and DNA phosphate backbone, and these interactions will facilitate the formation of binding pockets on functional DNA aptamers. Besides, positively charged species can serve as alternatives to metal cofactors required in stabilizing aptamers' structure, and thus enable the discovery of metal-independent DNA aptamers. Nucleobase

modifications with flat aromatic rings introduce extra stacking interactions between aptamers and target molecules. In addition, modifications can also provide new hydrogen-bonding motifs as well as modified shape complementary to facilitate binding of aptamers against targets.

The Sawai group has developed a modified DNA aptamer containing modified deoxyuridine triphosphate named $dT^{HM}TP$ that can enantioselectively bind to R-thalidomide (Figure 1.15)[39]. The ammonium functional groups improve the stability against nucleases and improve the binding affinity to thalidomide by fixing the aptamer to a structure in favor of binding through electrostatic interactions with negatively charged phosphate backbones.

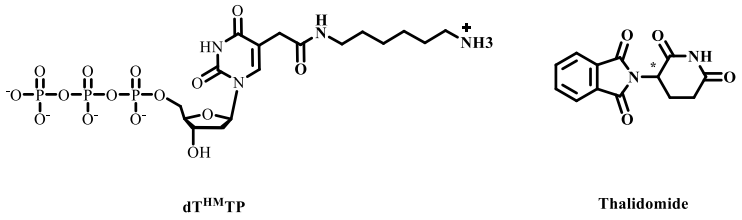

Figure 1.15. 5-modified dUTP molecule and thalidomide structures.

The Vaught group has developed other deoxyuridine triphosphate (dUTP) derivatives (Figure 1.16) that can be incorporated by DNA polymerase D. Vent (exo-) in primer extension reactions, and the polymerization reaction will give full length products with similar or even better yields than with thymine triphosphate (TTP) [40]. The amide bond

moiety mimics a protein backbone structure while providing new H-bonding motifs to facilitate the binding of aptamers to proteins. In addition, aromatic (Figure 1.16a, c-f) and aliphatic (Figure 1.16b) groups increase the hydrophobic content of DNA strands to mimic the properties of the binding domains of antibodies. It was reported that aptamers made from molecule **5d** showed the tightest binding to protein TNFRSF9 with a K_d of 5 nM, while aptamers made from TTP showed much weaker binding with a Kd value of more than 100 nM.

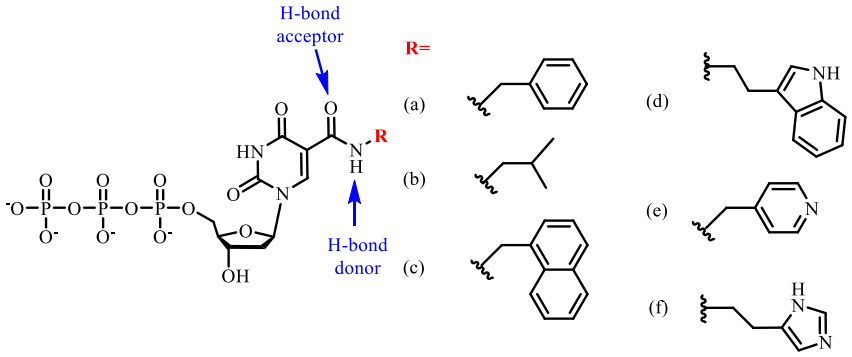

Figure 1.16. 5-modified dUTP molecules with new hydrogen bonding motifs [Adapted from reference [40]].

DNAzymes have also been prepared using modified nucleobases. The bases possess structures that assemble the side chains found in amino acids. The protein functionality introduced to DNA bases can greatly expand the catalytic possibilities of DNAzymes. Such modified bases can act as acids, bases, nucleophiles, as well as provide positive charges to recognize or stabilize negative charges through electrostatic interactions and reduce the need for divalent metal ions for activity.

A DNAzyme with three-protein functional groups, named Dz10-66c, was identified by the Perrin group to undergo a metal independent self-cleavage reaction with high activity (k_{cat} = 0.2 min^{-1}, K_M= 382 nM) (Figure 1.17)[41]. The positive charges serve to stabilize negative charges on phosphate backbone as well as act as substitutes for metal cofactors required in DNAzyme reactivity. The imidazole functionality is thought to act as a base to help deprotonate the C-2' hydroxyl group. The phosphodiester bond cleaving ability of DNAzymes containing modified DNA bases suggests many other reactions could be catalyzed in a similar manner.

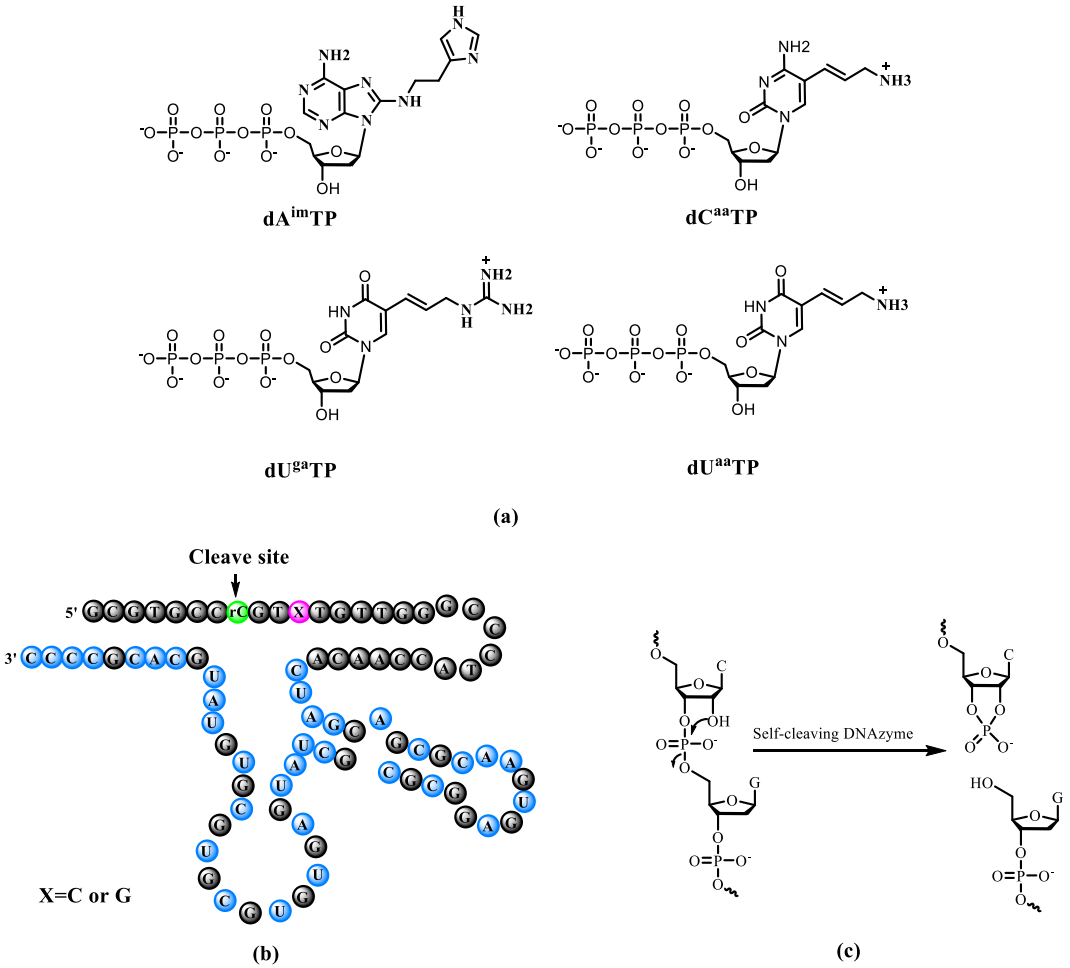

Figure 1.17. (a) Chemical structures of modified nucleic acids dA^{im}TP, dC^{aa}TP, dU^{ga}TP and dU^{aa}TP; (b) putative 2D structure of self-cleaving DNAzyme Dz10-66c, where A, G and C colored in red indicate 5-position modified nucleic acids mentioned in (a).[Adapted from reference [41]].

1.4 Project outlook

As DNA aptamers promise so many advantages including no immunogenicity, efficient penetration, less batch variation, easy modification, cost-effectiveness and short production time[42][43], they could serve as alternatives to vancomycin in the treatment of Gram-positive

bacterial diseases. DNA aptamers to peptidoglycan could also be used as sensors to detect bacterial infections.

The goal of this research is to synthesize one target compound for DNA aptamer selection (Figure 1.18a). This compound mimics the cell wall precursor of vancomycin-sensitive bacteria by containing a L-Lys-D-Ala-D-Ala tripeptide moiety. This target will be subjected to SELEX with DNA libraries containing either natural or unnatural nucleobases. The resulting DNA aptamers should bind tightly to the terminus of the peptidoglycan pentapeptides and prevent crosslinking. L-Lys is important in the design as the positive charge on its free amino group increases the potential for interactions with the negatively charged DNA backbone and hence could lead to better binding affinity.

A second target compound designed for the selection of catalytic DNAzymes is proposed (Figure 1.18b). This compound mimics the cell wall precursor of vancomycin resistant bacteria by containing a L-Lys-D-Ala-D-Lac moiety. Catalytic DNAzymes, which will contain artificial nucleobases with free amino moieties, will be selected to covalently attach to the target compound. It is anticipated that like could occur by a "transamidation" where an amino group on the catalytic DNA could attack the ester bond, displace the lactate moiety, and form an amide linkage with the D-Ala group. This could permanently inactivate the pentapeptide of vancomycin-resistant bacteria.

More molecular design and synthesis details will be discussed in Chapter 2 and chapter 3. SELEX experiment will be conducted by our collaborator Sombeb Paul from Prof. David M. Perrin's lab.

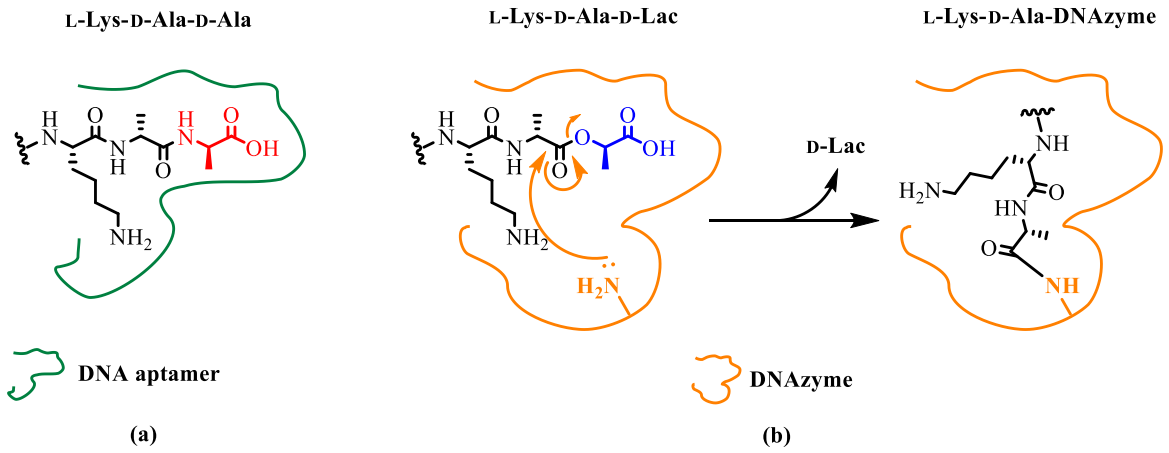

Figure 1.18. Scheme of (a) DNA aptamers bind vancomycin-sensitive bacteria cell wall precursor; (b) catalytic DNAzymes bind vancomycin-resistant bacteria cell wall precursor and the product after catalytic reaction

Chapter 2: First Generation of DNA Aptamer Selection Targets

2.1 Design of a molecule mimicking the vancomycin sensitive bacterial cell wall precursor

As introduced in Chapter 1, vancomycin sensitive bacteria are known to produce a pentapeptide side chain, in which the last three amino acids are L-Lys-D-Ala-D-Ala. The Tanner lab therefore decided to prepare a target for aptamer selection that bears this sequence and a means for immobilization on a solid support.

A biotin molecule was selected to help immobilize the target and facilitate the DNA-SELEX selection. The strong and specific non-covalent interaction between biotin and streptavidin allows for efficient separation of molecules containing biotin from those that do not (Figure 2.1).

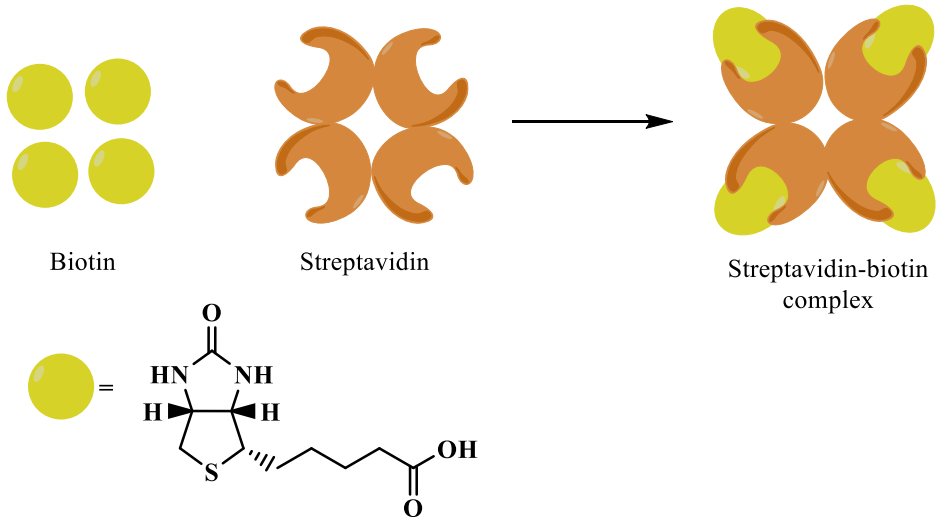

Figure 2.1. Scheme of the interaction between biotin and streptavidin.

Compound **YL-1** was designed to contain both biotin and the L-Lys-D-Ala-D-Ala tripeptide attached through a water-soluble linker (Figure 2.2). The linker is necessary to keep the tripeptide distant from the streptavidin protein and accessible to the oligonucleotides.

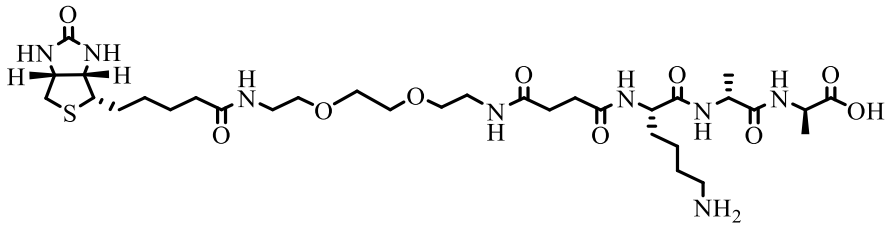

Figure 2.2. Chemical structure of compound **YL-1**.

Prior to my arrival in the group, Dr. Yanjie Liu had prepared compound **YL-1**, which was being used in the selection of DNA aptamers that bind to the termini of PG pentapeptides. The SELEX of aptamers was conducted by our collaborator Somdeb Paul from the laboratory of Prof. David M. Perrin.

2.2 Design and synthesis of a molecule mimicking the vancomycin resistant bacterial cell wall precursor

2.2.1 Design of Target 1

As introduced in the Chapter 1, vancomycin resistant bacteria are known to incorporate a D-Ala-D-Lac depsipeptide at the terminus of its pentapeptide side chain. Based on the design of compound **YL-1**, Target 1 was designed in which, the L-Lys-D-Ala-D-Ala tripeptide was substituted by a L-Lys-D-Ala-D-Lac moiety (Figure 2.3). This could be used

in either the selection of D-Ala-D-Lac specific aptamers or in the selection of catalytic DNAzymes that could covalently attach to the D-Ala group. The biotin and hydrophilic linker of compound **YL-1** were kept in **Target 1** for reasons discussed in section 2.1.

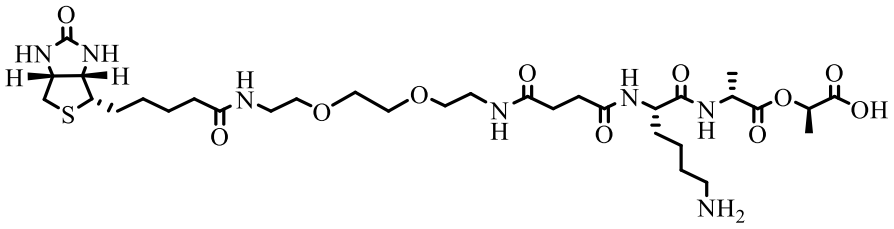

Figure 2.3. Chemical structure of **Target 1**.

2.2.2 Synthesis of Target 1

Target 1 can be broken down into four different parts and the synthetic route follows the logic as shown in Figure 2.4.

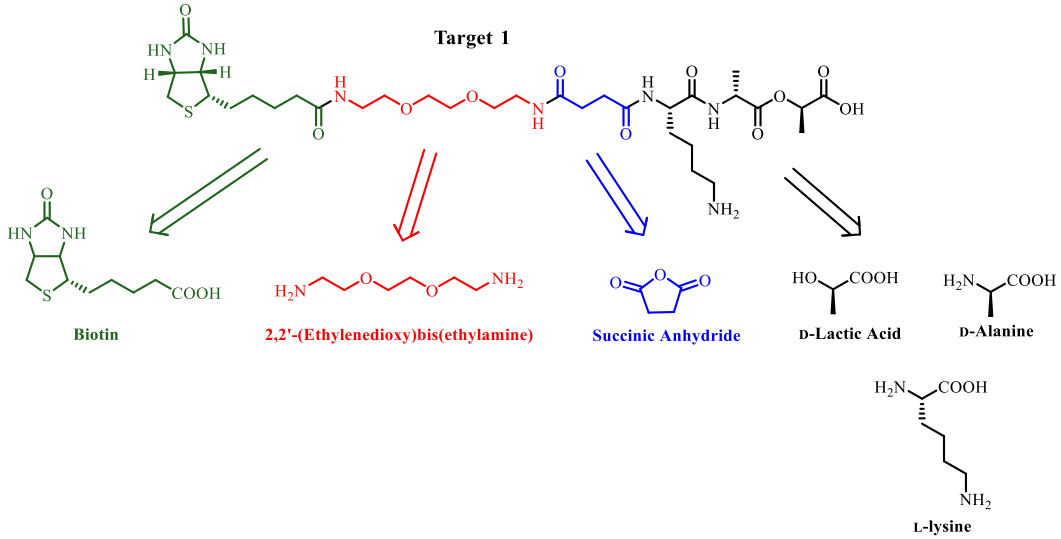

Figure 2.4. Synthetic logic behind the preparation of **Target 1**.

The succinic acid coupled depsipeptide **6** was ultimately synthesized using the synthetic route shown in Figure 2.5.

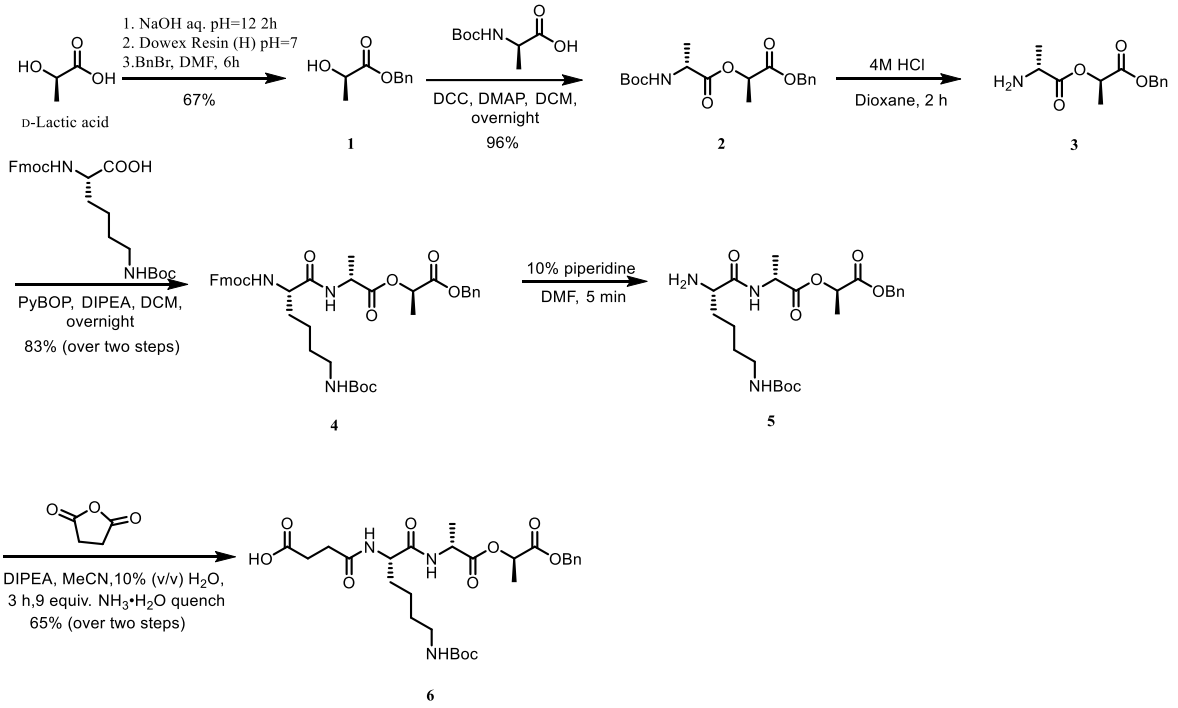

Figure 2.5. Synthetic procedures of succinic acid coupled compound **6**.

In our first attempt to synthesize the depsipeptide, D-lactic acid was directly converted to its benzyl ester, compound **1**, without the initial NaOH treatment shown in Figure 2.5, and then carried on in further steps. In these early reactions, an additional +72 peak was observed in mass spectra of all compounds **1**-**4** after column chromatography. Ultimately, we figured out that this +72-peak was also present in the starting material D-lactic acid,

indicating that the reagent contained dimerized impurities (Figure 2.6). NMR spectroscopy and MS spectrometry of the D-lactic acid reagent confirmed this assumption.

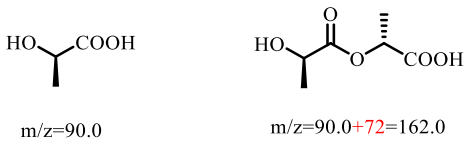

Figure 2.6. D-Lac and its dimerized impurity.

To rectify the situation, extra steps were added to the original procedure. An initial treatment of the **D**-Lac with NaOH aq. was used to hydrolyze the dimers at pH= 12. The pH was brought back to neutral by Dowex resin (H-form) to get rid of extra base without introducing cations. The material was then reacted with benzyl bromide to give ester **1** in an overall 67% yield.

The protected D-Lac(OBn) **1** was coupled to Boc-D-Ala-OH using DCC and DMAP to give compound **2**. At the beginning of this project, Fmoc-D-Ala-OH was used instead of Boc-D-Ala-OH. However, the solubility of ester Fmoc-D-Ala-D-Lac-(OBn) ester was very poor in a wide range of solvents, making the Fmoc deprotection reaction difficult. To solve this problem, the protecting group on D-Ala was changed from an Fmoc to a Boc group, and compound **2** was soluble during the deprotection step. It should be noted that the ^{1}H NMR spectrum of compound **2** showed only one isomer, indicating that racemization of D-lactic acid did not occur during the previous NaOH treatment.

Free amine **3** was obtained by Boc-deprotection of ester **2** and was coupled with Fmoc-L-Lys(Boc)-OH using PyBOP as coupling reagent to give compound **4**. Compound **4** was then deprotected with piperidine treatment to give compound **5**,

As compound **5** was directly carried on to the next step without column chromatography, no impurities were detected by mass spectroscopy. During the first several trials of the Fmoc-deprotection of compound **4**, a peak corresponding to a molecular weight of 384 was observed, in addition to the mass of the product at 479. The lower mass product corresponded to piperidine attacking the ester bond and forming a new amide bond (Figure 2.7).

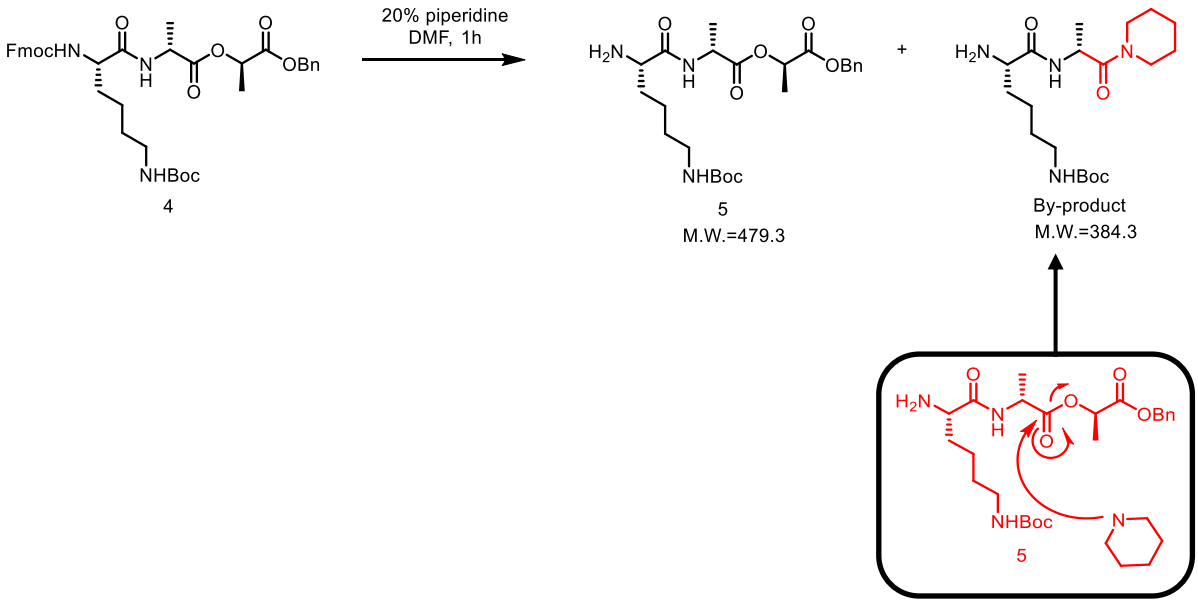

Figure 2.7. Deprotection reaction of Fmoc group on L-Lys gives both product and by-product. Mechanism of generation of by-product is indicated in red.

To minimize this side reaction, the reaction conditions were modified by shortening the reaction time and reducing the concentration of piperidine. Reactions were carried out for 5 min, 15 min and 30 min at 10% (v/v) and 20% (v/v) piperidine, respectively. The generation of by-product was monitored qualitatively by MS spectrometry. It was found that reaction with 10% (v/v) piperidine for 5 min gave the desired compound **5** without detectable side reaction. These conditions were optimal for the final condition for Fmoc-deprotection of compound **4**.

With compound **5** in hand, attempts were made to couple it to succinic anhydride in the presence of DIPEA and produce compound **6**. In early attempts, it was observed that the free amine on L-Lys would attack the ester bond between D-Ala and D-Lac to give an intramolecular cyclized by-product (Figure 2.8). To suppress this side reaction and accelerate intermolecular coupling between succinic anhydride and compound **5**, a large access (10 equiv.) of succinic anhydride was added in the reaction mixture.

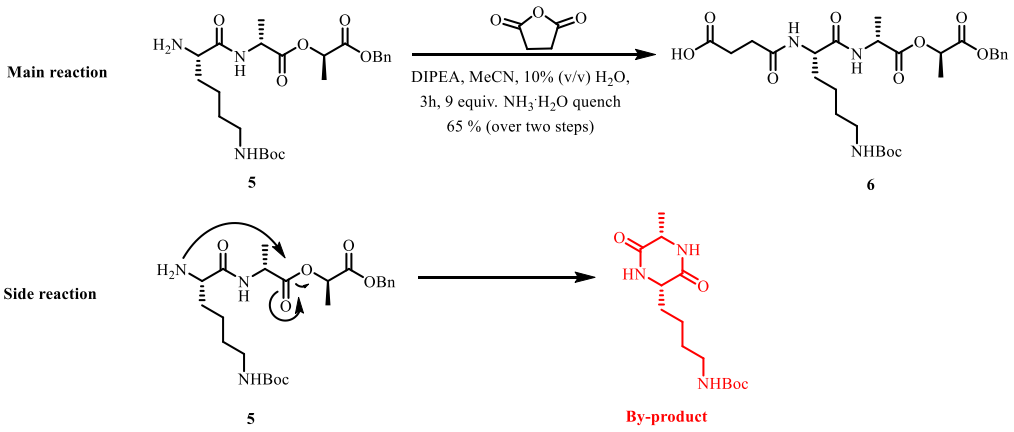

Figure 2.8. Main and side reactions in the synthesis of compound **6** from compound **5**.

Another problem with this reaction was the poor solubility of amine **5** in normal aprotic solvents that resulted in low yield (≤30%). 10% (v/v) water in MeCN were selected as a solvent system to facilitate dissolution of **5** and improve the yield.

As succinic anhydride was added in a large access, it would hydrolyze during column chromatography. The resulting succinic acid would elute together with the desired compound **6**. Therefore, ammonium hydroxide was used to quench the reaction and remove excess amount of succinic anhydride in reaction mixture prior to column chromatography. Ultimately, it was possible to obtain compound **6** in 65% yield over two steps from compound **4**.

The synthesis of the biotin-linker portion of **Target 2** is shown in Figure 2.9. Initially, 2,2'-(Ethylenedioxy) bis(ethylamine) was monoprotected with Boc anhydride to give the know compound **7**[44]. Biotin was then coupled with **7** using HBTU to generate the known compound **8**[45]. The Boc group of compound **8** was removed using 4M HCl to give free amine **9**.

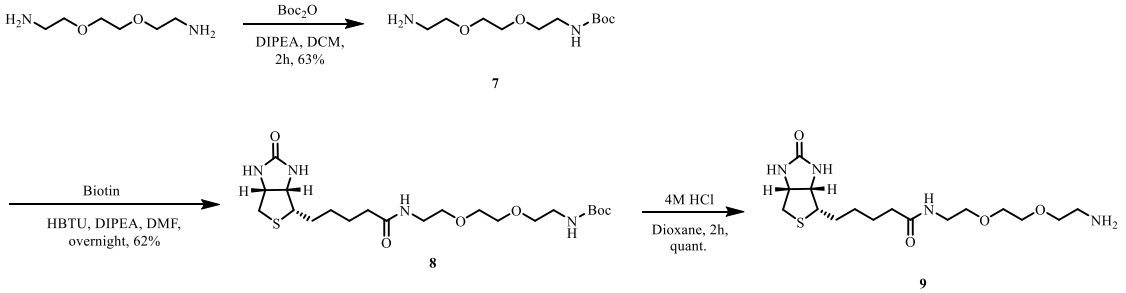

Figure 2.9. Synthetic procedures of coupling biotin with 1, 8-diamino-3,6-dioxaoctane.

The attachment of the biotin-linker with the succinic acid coupled L-Lys-D-Ala-D-Lac **6** was realized using HBTU to give fully protected **Target 1** as compound **10** (Figure 2.10). Compound **10** was present, as indicated by mass spectrometry, however, analysis of its ^{1}H NMR spetrum indicated that it was not a pure compound. Attempts were made to deprotect impure compound **10** via hydrogenolysis and acid treatment. Again, **Target 1** was evident by mass spectrometry, but the ^{1}H NMR spectrum indicated it was impure. At this point in the project, it became evident that the aptamers selected for binding to compound **YL-1** were in fact recognizing the streptavidin protein instead of the tripeptide. Therefore, the purification of compound **10** and **Target 1** were no longer pursued and new targets were designed.

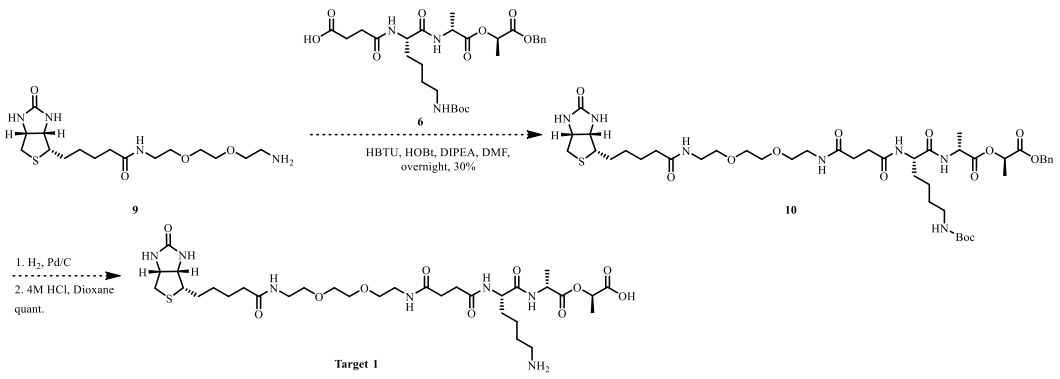

Figure 2.10. Coupling of biotin-linker with succinic acid-coupled depsipeptide to give **Target 1**.

2.3 Selection of DNA aptamers using compound YL-1

The selection of DNA aptamers was facilitated by the strong, non-covalent interaction between biotin and streptavidin. SELEX against compound **YL-1**(Figure 2.11) was carried

out by Somdeb Paul in the lab of Prof. David M. Perrin. During the selection, compound **YL-1** was immobilized onto streptavidin agarose after a negative selection step, followed by washing with the DNA aptamer pool. Aptamers that didn't bind were washed off and discarded, while those that bound were carried on for further rounds of amplification and selection.

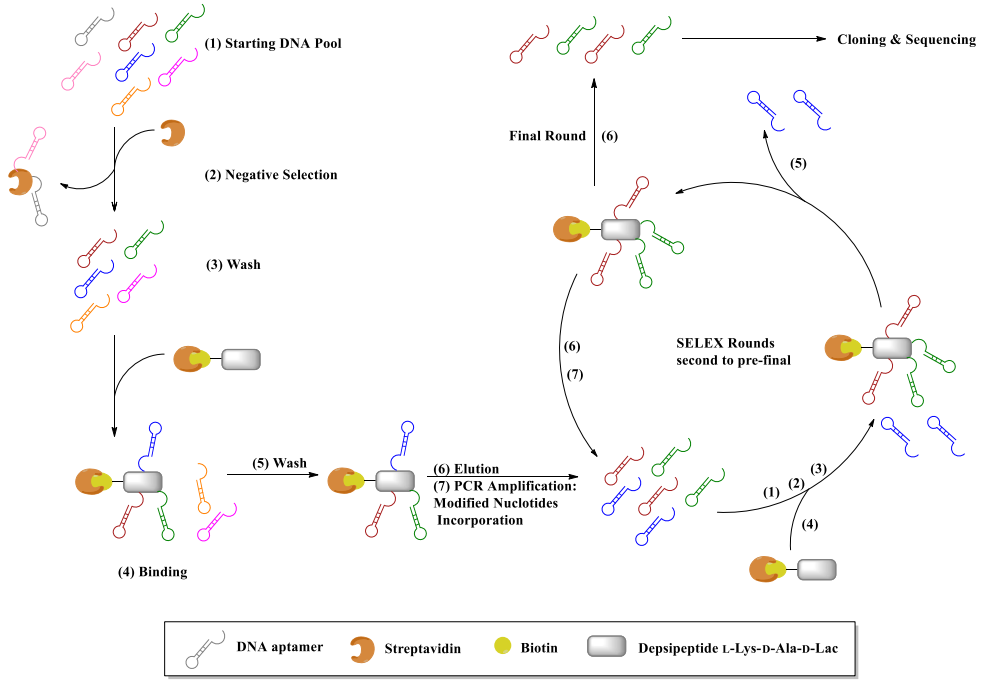

Figure 2.11. Scheme for the selection of DNA aptamers using target molecule **YL-1**.

However, despite the use of negative selection cycles against unmodified streptavidin agarose, DNA aptamers bond to streptavidin instead of target molecule without any specificity for compound **YL-1.** This result indicated that for the selection of DNA aptamers, a biotin-streptavidin based selection method should be abandoned. On the basis of the above

reasoning, **Target 1** was not subjected to SELEX. The design of second generation targets will be discussed in greater detail in Chapter 3.

2.4 Conclusion

Compound **YL-1,** and presumably **Target 1** as well, were not efficient targets for the selection of DNA aptamers due to the interference introduced by streptavidin. In future selections, a different method for immobilization was chosen.

2.5 Summary

Target 1 molecule was synthesized though not appropriately purified in this chapter. Together with compound **YL-1**, these two molecules did not serve as ideal targets for DNA aptamer selection. Molecular modifications are necessary in future research.

2.6 Future Direction

Possible solutions include the substitution of biotin with other moieties that enable immobilization and/or the introduction of a cleavable hydrophilic linker to serve as a secondary functional selection moiety.

2.7 Experimental

2.7.1 Material and general procedures

Chemicals were purchased from Sigma Aldrich, Alfa Aesar, Oakwood Products Inc., Toronto Research Chemicals, Oxchem and Chem-Impex. All chemicals were used directly

without further purification unless noted. TLC plates were purchased from EMD Chemical Inc. Flash silica chromatography was conducted using silica gel SiliaFlash F60 (Silicycle, 230-400 mesh). Ion exchange resin L13943 Dowex® 50WX2 50-100 (H) was purchased from Alfa Aesar. AG® 1-X8 Anion Exchange Resin was purchased from Bio-Rad Laboratories. DCM were distilled under Ar from CaH_2.

^{1}H NMR spectra and proton-decoupled ^{13}C NMR spectra were recorded by Bruker AV400sp spectrometer at a field strength of 400 MHz and 101 MHz, respectively. Mass spectra were obtained on a Waters LC-MS spectrometer (OAMS) and high-resolution mass spectrometer at the UBC Mass Spectrometry Facility.

2.7.2 Synthesis of Target 1

*Compound **1***

D-lactic acid (2.00 g, 22.2 mmol) was dissolved in water (10 mL) and a 0.01 M NaOH solution was added dropwise until pH reached 12. The reaction mixture was stirred at rt for 2 h and was then neutralized with Dowex resin (hydrogen form). Solvent was removed under reduced pressure to give D-lactic acid as colorless liquid which was used directly without further purification.

D-Lactic acid (2.00 g, 22.2 mmol) and benzyl bromide (4.90 g, 28.9 mmol) were dissolved in dry DMF (30 mL). The reaction mixture was stirred at rt for 15 h, quenched by brine, and extracted with ethyl acetate. The organic layer was then washed with water once and

brine once, and dried over $MgSO_4$. After filtration, the filtrate was concentrated under vacuum and further purified with flash column chromatography (silica, 4:1 petroleum ether: Ethyl acetate) to give compound **1** as colorless oil (3.28 g, 82 %). ^{1}H NMR (400 MHz, methanol-d4) δ 7.42 – 7.35 (m, 5H), 5.19 (dd, J = 2.3 Hz, 2H), 4.31 (q, J = 6.9 Hz, 1H), 1.39 (d, J = 6.9 Hz, 3H). MS (ESI) m/z= 203.2 $[M+Na]^+$.

*Compound **2***

Compound **1** (3.28 g, 18.2 mmol), Boc-D-Ala-OH (3.79 g, 20.0 mmol) and DMAP (2.45 g, 20.0 mmol) were dissolved in distilled DCM (90 mL) and stirred at 0 ˚C. A solution of DCC (4.15 g, 20.0 mmol) in distilled DCM (20 mL) was added dropwise over 10 min and it was stirred at rt for 15 h. The reaction mixture was filtered through celite and the celite was washed with 50 mL of ethyl acetate. The organic layer was washed by sat. $NaHCO_3$, 0.5 M HCl, sat. $NaHCO_3$ again, followed by brine. After drying over $MgSO_4$ and filtration, the filtrate was concentrated under reduced pressure and the resulting oil was purified using flash column chromatography (silica, 4:1 petroleum ether: ethyl acetate) to give compound **2** as a white powder (6.15 g, 96 %). ^{1}H NMR (400 MHz, methanol-d4) δ 7.38 – 7.35 (m, 5H), 5.19-5.13 (m, 3H), 4.18 (q, *J* = 7.3 Hz, 1H), 1.51 (d, *J* = 7.1 Hz, 3H), 1.45 (s, 9H), 1.34 (d, *J* = 7.3 Hz, 3H). MS (ESI) m/z= 374.2 $[M+Na]^+$.

*Compound **3***

To compound **2** (1.01 g, 2.9 mmol) was added 4 M HCl (25 mL) in dioxane and the reaction mixture was stirred at rt for 1 h. The volatiles were removed under reduced pressure.

Diethyl ether was added to the residue and then removed under reduced pressure. This procedure was repeated five times. Crude compound **3** was generated as a white solid and was used in subsequent reaction without further purification. MS (ESI) m/z= 252.2 $[M+H]^+$.

*Compound **4***

Crude compound **3** (719 mg) and Fmoc-Lys(Boc)-OH (1.22 g, 2.6 mmol) were dissolved in distilled DCM (100 mL). To the reaction mixture were added PyBOP (1.49 g, 2.9 mmol) and DIPEA (0.7 mL, 3.9 mmol). The mixture was stirred at rt for 15 h and followed by washing with 10% citric acid, sat. $NaHCO_3$ and brine. The organic layer was dried over MgSO4 and filtered. The filtrate was concentrated under vacuum and further purification was carried out by flash column chromatography (silica, 2:3 petroleum ether: ethyl acetate) to give compound **4** as a white solid (1.19 g, 60 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 7.82 (d, *J* = 7.5 Hz, 2H), 7.68 (t, *J* = 6.5 Hz, 2H), 7.41 (t, *J* = 7.7 Hz, 2H), 7.37 – 7.15 (m, 7H), 5.24 – 5.07 (m, 3H), 4.40 (d, *J* = 6.7 Hz, 2H), 4.24 (t, *J* = 6.8 Hz, 1H), 4.15 – 4.06 (m, 1H), 3.04 (t, *J* = 6.8 Hz, 2H), 1.80 – 1.60 (m, 6H), 1.50 (d, *J* = 7.0 Hz, 3H), 1.44 (s, 9H), 1.38 (d, *J* = 7.3 Hz, 3H). MS (ESI) m/z= 724.4 $[M+Na]^+$. HRMS (ESI): m/z calcd for $C_{39}H_{47}N_3O_9$ $[M+Na]^+$ 724.3210, found 724.3203.

*Compound **5***

Compound **4** (96 mg, 0.14 mmol) was dissolved in DMF (4.5 mL). After addition of piperidine (0.5 mL), the reaction mixture was stirred at rt for 5 min and then the volatiles

were removed under reduced pressure within another 5 min.The residue was washed with petroleum ether 5 times to give crude compound **5** as a white solid[46]. This material was carried on to the next step without further purification. MS (ESI) m/z= 480.2 $[M+H]^+$, 502.2 $[M+Na]^+$.

*Compound **6***

Crude compound **5** (376 mg) and succinic anhydride (663 mg, 6.6 mmol) was dissolved in acetonitrile (10 mL) and H_2O (200 µL). The reaction mixture was stirred at rt for 5 min before the addition of DIPEA (0.35 mL, 2.0 mmol), and then it was stirred for another 3 h, The reaction was quenched by ammonium water. The volatiles were removed under vacuum and the residue was purified by flash column chromatography (silica, 96.5:3.5 DCM: methanol) to give compound **6** as a yellowish gummy solid (288 mg, 63 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 7.44 – 7.27 (m, 5H), 5.19 (d, J = 1.3 Hz, 2H), 5.16 – 5.11 (m, 1H), 4.45 – 4.40 (m, 1H), 4.35 – 4.32 (m, 1H), 3.06 (t, J = 6.7 Hz, 2H), 2.67 – 2.61 (m, 2H), 2.55 – 2.49 (m, 2H), 1.91 – 1.55 (m, 6H), 1.51 (d, J = 7.1 Hz, 3H), 1.45 (s, 9H), 1.41 (d, J = 7.3 Hz, 3H). MS (ESI) m/z= 578.3 $[M-H]^-$.

*Compound **7***

2,2′-(Ethylenedioxy)bis(ethylamine) (1.50 g, 10.0 mmol) and DIPEA (1.8 mL, 10.0 mmol) were dissolved in distilled DCM (20 mL). A solution of di-*tert*-butyl dicarbonate (1.10 g, 5.0 mmol) in DCM (10 mL) was added to the reaction mixture over 40 min. The reaction mixture was stirred at rt for 2 h, and then the volatiles were removed under vacuum. The

residue was purified by flash column chromatography (silica, 91:4:5 DCM: methanol: NEt_3 to give compound **7** as a colorless oil (788 mg, 63 %). ^{1}H NMR (400 MHz, methanol-d4) δ 3.64 (s, 4H), 3.54 (dt, *J* = 10.0, 5.4 Hz, 4H), 3.24 (t, *J* = 5.7 Hz, 2H), 2.83 (t, *J* = 5.3 Hz, 2H), 1.46 (s, 9H). MS (ESI) m/z= 249.3 $[M+H]^+$.

*Compound **8***

Biotin (512 mg, 2.1 mmol, 1.5 equiv.), HBTU (689 mg, 1.8 mmol) and DIPEA (0.4 mL, 2.2 mmol) were dissolved in DMF (40 mL). The mixture was stirred for 10 min at rt, and then a solution of compound **7** (348 mg, 1.4 mmol, 1.0 equiv.) in DMF (10 mL) was added to the reaction mixture dropwise. The mixture was stirred at rt for 1 h, and the volatiles were removed by vacuum. The residue was purified by flash column chromatography (silica, 92:3:5 DCM: methanol: NEt_3) to give compound **8** as a yellowish foamy solid (414 mg, 62 %). ^{1}H NMR (400 MHz, Methanol-d4) δ 4.51 (dd, *J* = 7.6, 4.7 Hz, 1H), 4.32 (dd, *J* = 7.9, 4.5 Hz, 1H), 3.64 (s, 4H), 3.58 – 3.52 (m, 4H), 3.39 (t, *J* = 5.5 Hz, 2H), 3.24 (t, *J* = 5.8 Hz, 2H), 2.95 (dd, *J* = 12.7, 5.0 Hz, 1H), 2.72 (d, *J* = 12.7 Hz, 1H), 2.24 (t, *J* = 7.4 Hz, 2H), 1.83 – 1.55 (m, 8H), 1.46 (s, 9H). MS (ESI) m/z= 475.3 $[M+H]^+$.

Chapter 3: Second Generation of DNA Aptamer Selection Targets

3.1 Synthesis of a DNA aptamer target mimicking a vancomycin sensitive bacterial cell wall precursor

3.1.1 Design of Target 2

As introduced in Chapter 1, vancomycin sensitive strains of Gram-positive bacteria possess a D-Ala-D-Ala dipeptide at the end of the pentapeptide side chain in the cell wall precursor. This dipeptide is recognized in the interaction between vancomycin and the precursor molecules. For the selection of peptidoglycan-targeting DNA aptamers, the L-Lys-D-Ala-D-Ala tripeptide was chosen as the binding target. As the presence of streptavidin impacted the effectiveness of the SELEX selection mentioned in Chapter 2, the biotin moiety was substituted with thiol group. Thiols are widely used in SELEX selections since they allow for immobilization on an iodoacetyl-activated resin (Figure 3.1). As free thiols are subject to oxidation, a convenient way of synthesizing them is to prepare a dimeric disulfide and then cleave them to the thiol immediately before immobilization on the resin.

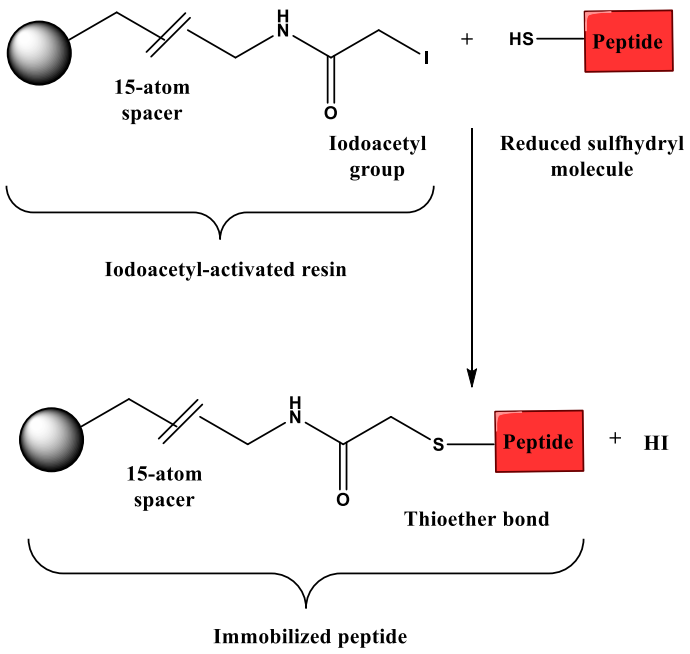

Figure 3.1. Peptide immobilization chemistry for iodoacetyl-activated resin.

Based on the concepts discussed above, molecule **Target 2** was designed to consist of two L-Lys-D-Ala-D-Ala tripeptides linked via a disulfide bond (Figure 3.2).

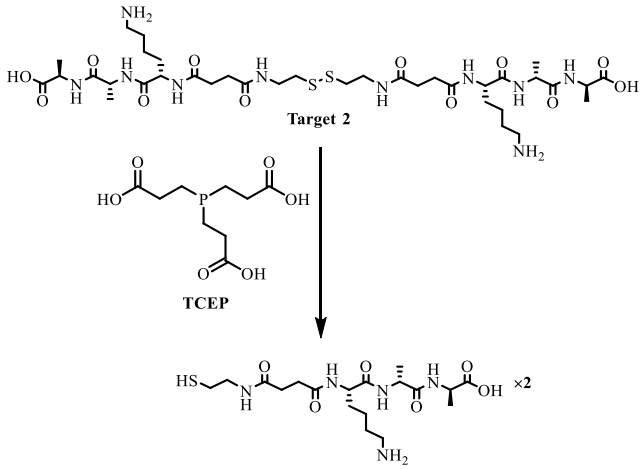

Figure 3.2. Chemical structure of **Target 2** and disulfide cleavage to give free thiol.

3.1.2 Synthesis of Target 2

Target 2 can be broken down into three different parts and the synthetic route follows the logic as shown in Figure 3.3.

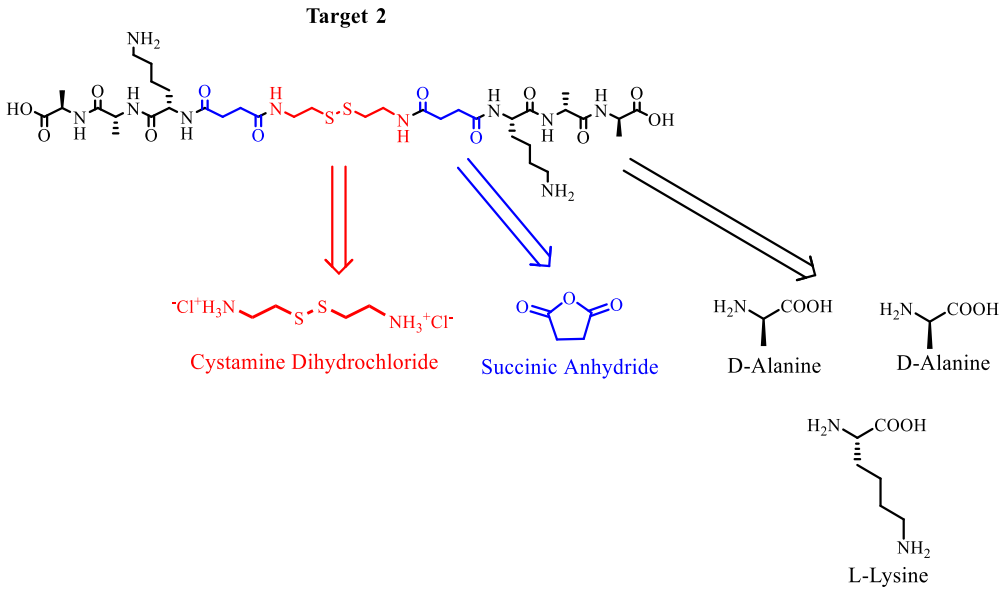

Figure 3.3. Synthetic logic behind the preparation of **Target 2.**

Target 2 was prepared via solution phase synthesis and the synthetic route for **Target 2** is shown in Figure 3.4.

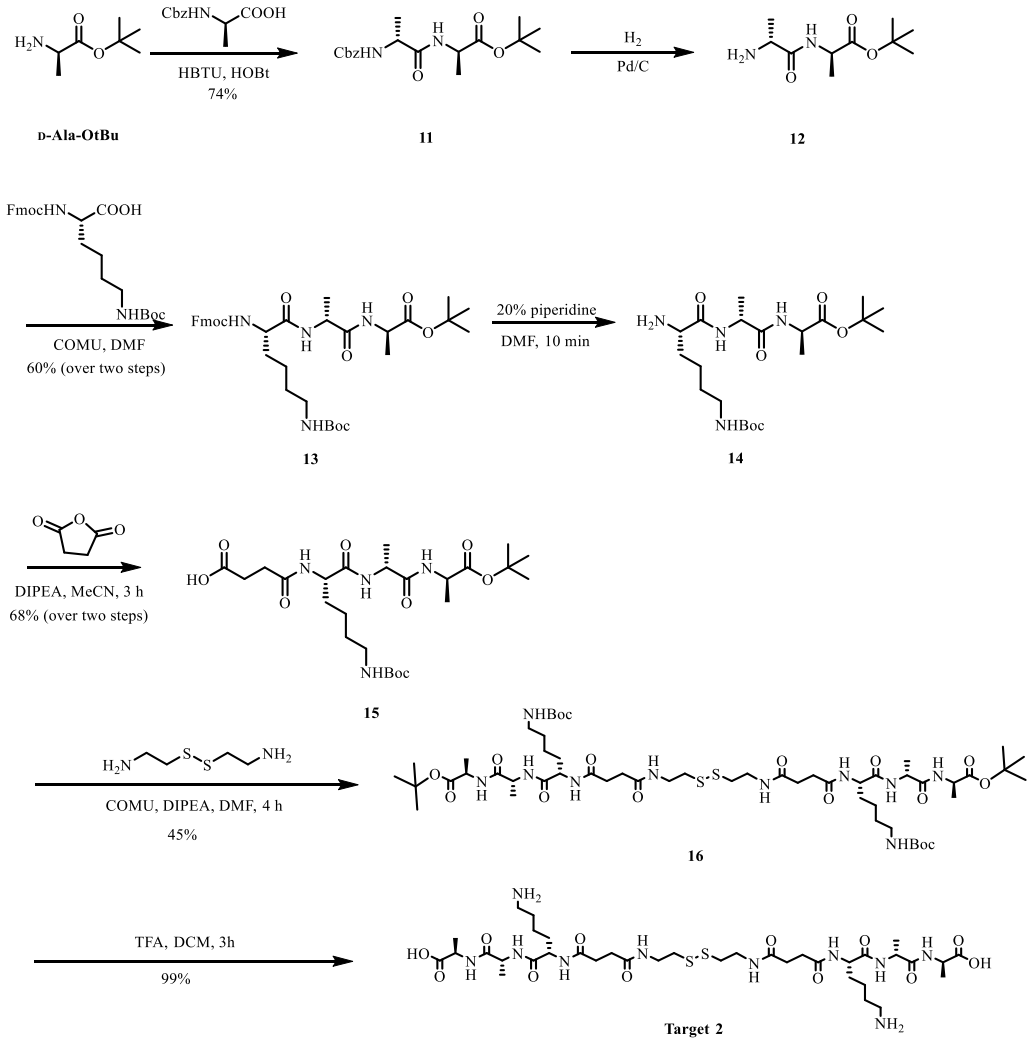

Figure 3.4. Synthetic procedures to prepare **Target 2**.

The starting material D-Ala-OtBu ester was coupled to N-Cbz-D-Ala-OH using the coupling reagent HBTU in the presence of HOBt to give the known compound **11**[47]. Free amine **12** was obtained via hydrogenolysis and was coupled with Fmoc-L-Lys(Boc)-OH using COMU as the coupling reagent to give compound **13**.

Initially, PyBOP was used as coupling reagent in this reaction as had been previously done with compound **3**. However, the purification of compound **13** from the reaction mixture was difficult, and there were always impurities in the product fractions after column chromatography as indicated by NMR spectroscopy. To improve the purity of the product, COMU, one of the newest and most efficient coupling reagents, was selected. Although there was a sacrifice in yield in the COMU-mediated coupling, the product was pure. Thus, COMU was chosen as the standard coupling reagent for this step.

Fmoc-deprotection was carried out on compound **13** to give free amine **14**. The amide bond formation between succinic anhydride and compound **14** was then facilitated by DIPEA in acetonitrile at room temperature, followed by quenching with ammonium hydroxide to give acid **15.** Compound **15** was then coupled to both ends of cystamine to give disulfide **16.**

As in the previous example, PyBOP was selected as the first coupling reagent to use in this reaction. However, impurities that couldn't be separated by column chromatography were generated during the reaction. From the experience of the synthesis of compound **13**, COMU was used as the second-choice for coupling and it worked well to give disulfide **16** in moderate yield and high purity.

Compound **16** was then subjected to global deprotection using TFA to generate the final product, **Target 2**.

3.1.3 SELEX Selection of DNA aptamers using Target 2

SELEX selection of DNA aptamers was carried out on oligonucleotides containing either purely natural nucleotides or a combination of natural and modified nucleotides. The modified nucleotides chosen were aminoallyl deoxycytidine triphosphate ($dC^{aa}TP$) and guanidinoallyl deoxyuridine triphosphate ($dU^{g}TP$) (Figure 3.5). These two oligonucleotides replaced the deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP) in the unmodified selections, respectively. Positively charged "lysine-like" and "arginine-like" side chains were chosen as they could help to recognize the C-terminal carboxylate of the tripeptide, help in folding of the oligonucleotide, and reduce the need for high divalent metal ion concentrations. Another reason why the "lysine-like" side chains were chosen is that they can serve as nucleophiles in the catalytic ester bond cleavage (section 3.2).

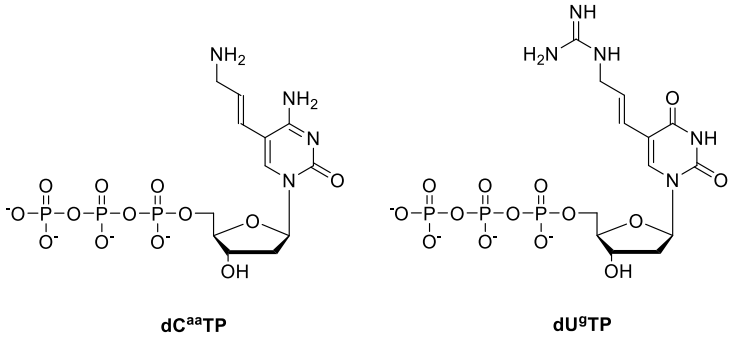

Figure 3.5. Chemical structures of modified nucleotides $dC^{aa}TP$ and $dU^{g}TP$.

The selections were done in the laboratory of Prof. David Perrin by Somdeb Paul. In the selection process (Figure 3.6), **Target 2** was first treated with TCEP to cleave the disulfide bonds and generate the tripeptide L-Lys-D-Ala-D-Ala linked to a free thiol group. The thiol

compound was immobilized onto an iodoacetyl based resin. The resin was then washed with a synthetic library of random oligonucleotides dissolved in a buffer containing divalent metal ions ([Mg^{2+}] = 5 mM or 2.5 mM). Aptamers that bound to **Target 2** via non-covalent interactions were retained on the resin while those that did not were discarded. The bound aptamers were washed off from the resin using deionized water (lacking metal ions that give the aptamers structure) and PCR amplified. Then the new aptamer pool was carried on for the next several rounds of selection to find out the best binding sequence. The aptamers are radioactively labeled by ^{32}P to quantify binding to the resin.

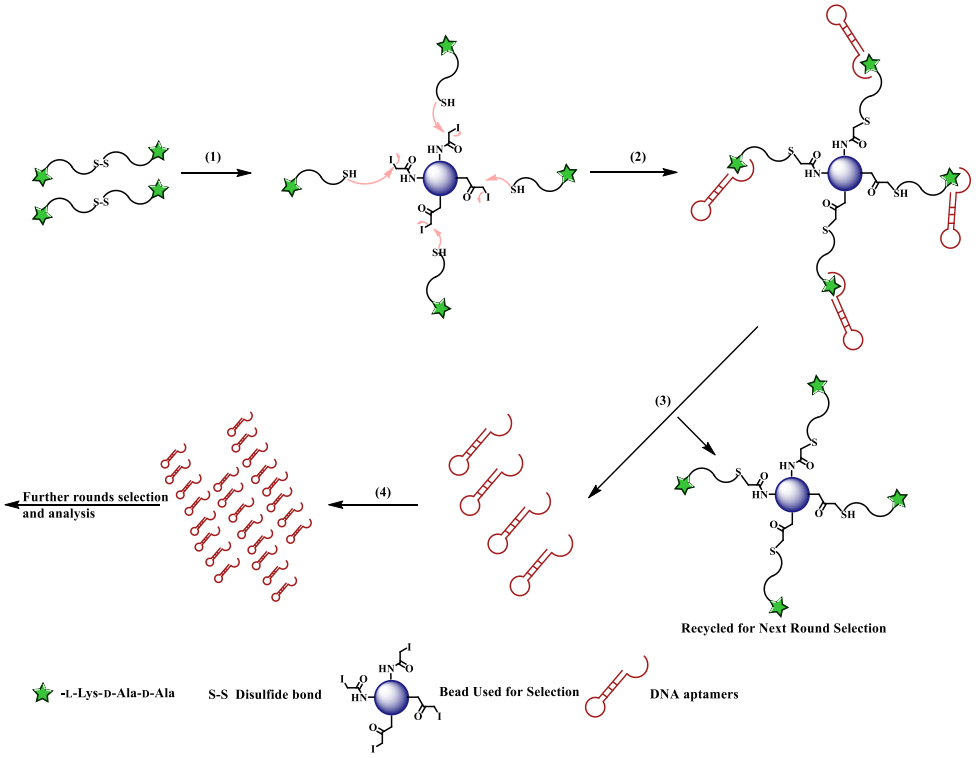

Figure 3.6. Selection procedures using **Target 2**. (1) TCEP cleavage of disulfide bond in **Target 2** and immobilization of peptide target onto iodoacetyl activated resin; (2) Binding of DNA aptamers with

target peptide; (3) Wash and release of binding-based DNA aptamers from resin, resin with peptide adduct will be recycled for next round selection; (4) Amplification of binding aptamers with PCR using artificial nucleotides and the pool of aptamers will be carried on for several more rounds of selection.

In the selection (Figure 3.7), several modified conditions were employed to gradually select the strongest aptamers with weak metal dependency and an ability to bind our tripeptide target at body temperature.

Two different resins were utilized in negative selections at different stages of the selection. In G1 to G3 no negative selection was conducted, while in G4 a negative selection was conducted utilizing resin **NS1** (Figure 3.8a). The **NS1** resin bears a cysteamine group instead of our tripeptide. It will serve to remove aptamers that bind only to the resin or non-specifically to primary amino groups, such as the lysine side chain in our target. From G5 to G12, both **NS1** and **NS2** resins were used in negative selections (Figure 3.8b). **NS2** served as a random amino acid mimic to exclude aptamers that bind non-specifically to amino acids. The selection results of G4 to G6 indicated that with more negative selections introduced, the stronger binding sequences were selected as the percentage eluted dropped significantly over increasing number of selection rounds. The percentage of modified aptamers binding to the target-resin adduct was always larger than that of the unmodified ones, indicating that modified aptamers possessed better affinity.

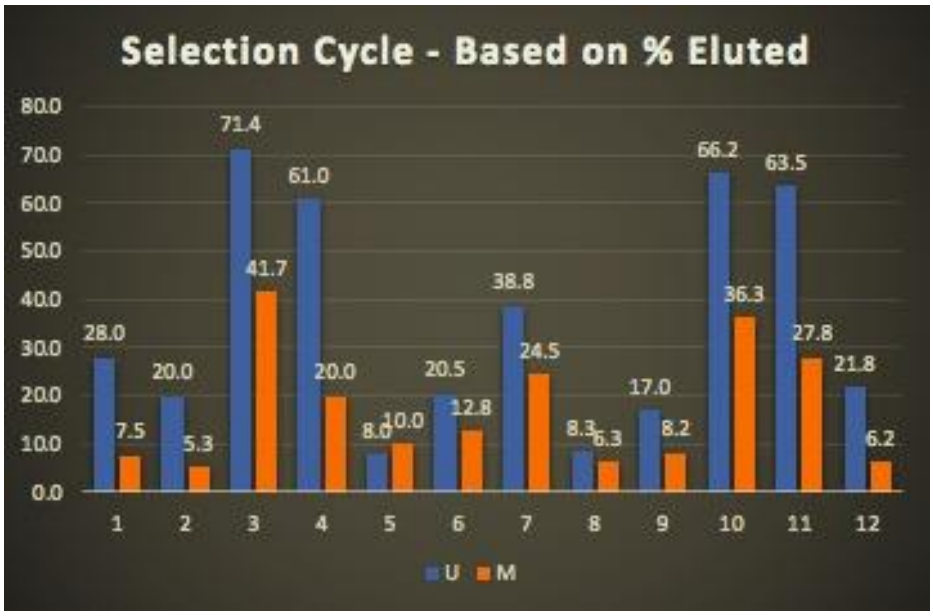

Figure 3.7. The percentage of sequences eluted in each generation (G1-12). U represents unmodified aptamers, M represents modified aptamers.

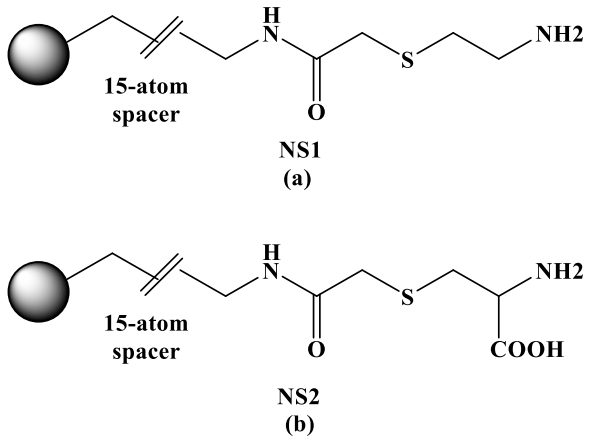

Figure 3.8 Structures of (a) **NS1** resin; (b) **NS2** resin used in negative selections.

A more stringent selection condition was applied from G7 to G12 by decreasing the concentration of divalent metal ions ($[Mg^{2+}]$ = 5 mM) by half ($[Mg^{2+}]$ = 2.5 mM)). As Mg^{2+}

plays an essential role in the folding of the structures of DNA aptamers, less metal cofactor-dependent aptamers would be selected under such conditions. The increase of percentage eluted from G6 to G7 indicated that a large number of aptamers selected in previous six rounds were highly metal dependent. The percentage drop in following two rounds G8, G9 proved that successful selection of less metal dependent aptamers was realized.

In the last three generations G10 to G12, aptamers binding at body temperature were further selected from the existing aptamer pool. In these rounds, the incubation temperature was increased from rt to 37 ˚C to mimic the environment of the human body. The increase in percentage eluted from G9 to G10 indicated that approximately half of the aptamers selected in previous rounds did not bind at 37 ˚C. At G12, the most specific aptamers with less metal dependency and activity at body temperature were selected.

Both unmodified and modified aptamers in G12 were collected and have been submitted for Next-generation sequencing. This data will be analyzed to determine if consensus sequences have "evolved" during the selection process. Aptamers collected from G1 were also submitted to ascertain that the synthesis of starting oligonucleotides library did not have any tendency to generate specific sequences.

Once specific sequences have been identified, tests will be done to analyze the binding efficiency of the aptamers towards our peptide. For example, we could use fluorescently

labeled peptides and size exclusion spin columns to measure the affinity of aptamers to the target.

3.1.4 Conclusion

An efficient synthesis of the L-Lys-D-Ala-D-Ala tripeptide linked to a thiol has been achieved. As the SELEX of DNA aptamers against **Target 2** is still in progress, it is hard to give a solid conclusion regarding the selection results at the present stage. However, the DNA aptamers with modified nucleobases has shown a higher fraction of retention on the resin when compared to the natural nucleotide pool. It is hopeful that DNA aptamers that can bind with the Gram-positive bacterial cell wall precursor will be identified soon.

3.2 Synthesis of catalytic DNAzyme target mimicking vancomycin-resistant bacterial cell wall precursor

3.2.1 Design of Target 3

As mentioned in Chapter 1, the mutation from D-Ala to D-Lac at the end of the pentapeptide side chain on the bacterial cell wall precursor results in a 1000-fold loss in binding potency between some Gram-positive bacteria and vancomycin, and thus leads to vancomycin resistance. In the design of the catalytic DNAzyme target molecule, **Target 3**, the last three residues L-Lys-D-Ala-D-Lac on the pentapeptide side chain of vancomycin resistant bacteria were retained. This could be used to generate either aptamers that simply bind, or DNAzymes that will covalently link to the target via amide bond formation.

As discussed in Section 2.2, the binding of aptamers to streptavidin can be problematic. However, a reversible or cleavable attachment is essential for the selection of DNAzymes that form covalent linkages, since active DNAzymes cannot be washed off from the resin once they form a covalent bond with the immobilized target molecule. A cleavable linkage is necessary to facilitate the removal of the aptamer-target adduct for further analysis. For this work, the cleavable linker cystamine with a disulfide bond was considered as the best choice. Oligonucleotides that covalently attach to the depsipeptide could be released by reducing conditions, however, those that bind to streptavidin would not.

To that end, **Target 3** was designed to contain three essential elements: L-Lys-D-Ala-D-Lac, disulfide linker and biotin (Figure 3.9).

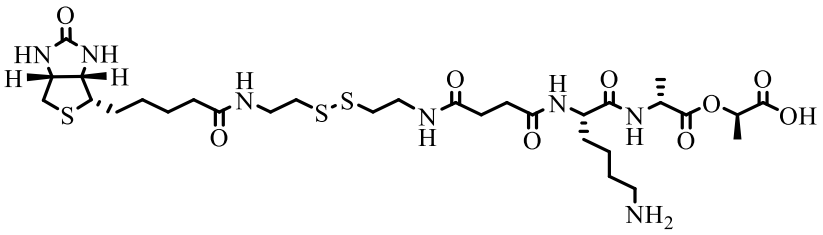

Figure 3.9. Chemical structure of **Target 3**.

3.2.2 Synthesis of Target 3

Target 3 can be broken down into four different parts and the synthetic route follows the logic as shown below in Figure 3.10.

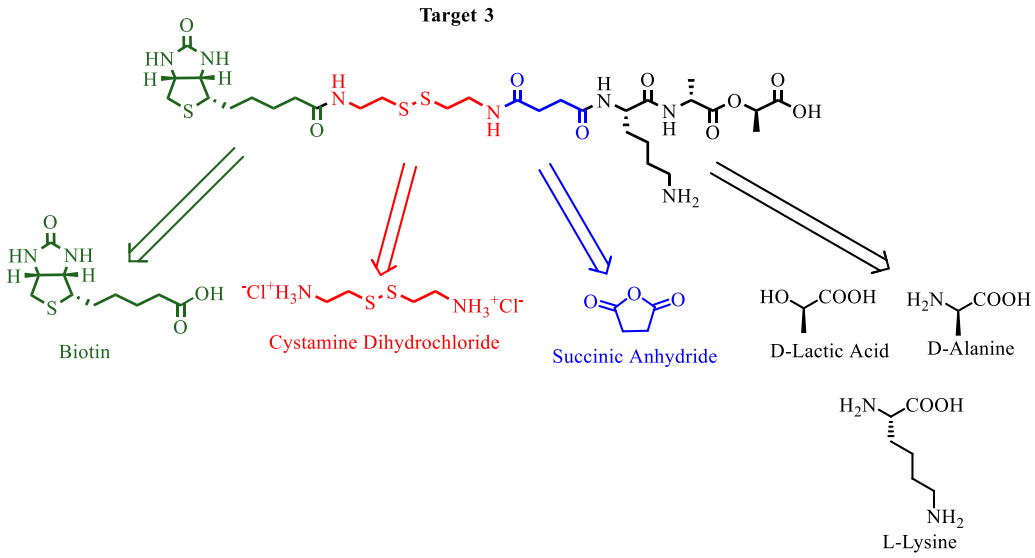

Figure 3.10. Synthetic logic behind the preparation of **Target 3.**

The synthesis of the succinic acid-coupled L-Lys(Boc)-D-Ala-D-Lac(OtBu) depsipeptide is shown in Figure 3.11.

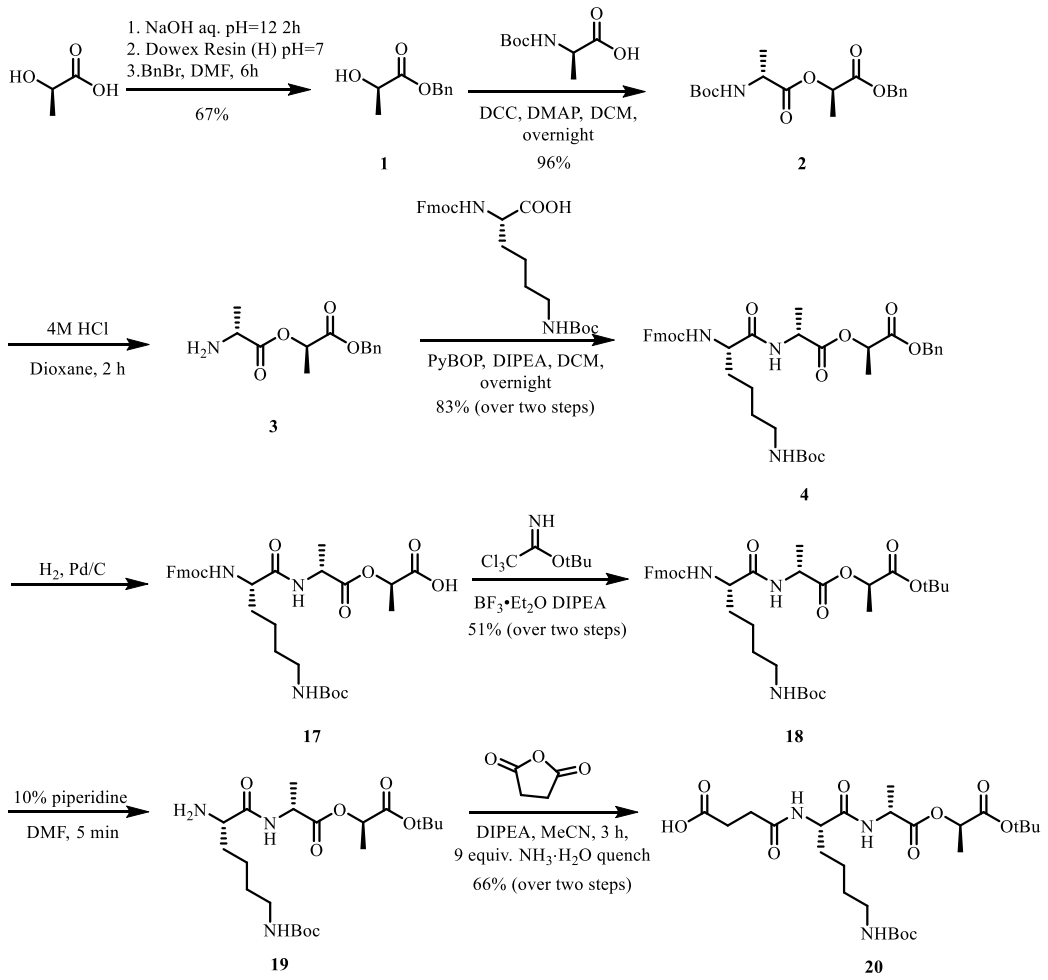

Figure 3.11. The synthesis of succinic acid-coupled L-Lys(Boc)-D-Ala-D-Lac(OtBu) depsipeptide.

The first four steps in the synthesis of **Target 3** followed the same procedures as mentioned in the synthesis of **Target 1**. After cleavage of the ester dimer impurities, the starting material D-Lac was esterified with benzyl bromide to give compound **1**. This was coupled to Boc-protected D-Ala to give ester **2**, followed by removal of the Boc group to give amine **3**. Then amine **3** was coupled with Fmoc-L-Lys(Boc)-OH to give compound **4**. At this point, the benzyl protecting group was changed to a *tert*-butyl group by hydrogenolysis to

give acid **17**, followed by treatment with *tert*-butyl 2,2,2-trichloroacetimidate to give compound **18**. The reason for this protecting group change is described in the following paragraph.

In our initial attempt to synthesize **Target 3**, the benzyl protecting group was retained until the second last deprotection step before global deprotection involving hydrogenolysis of compound **26** (Figure 3.12).

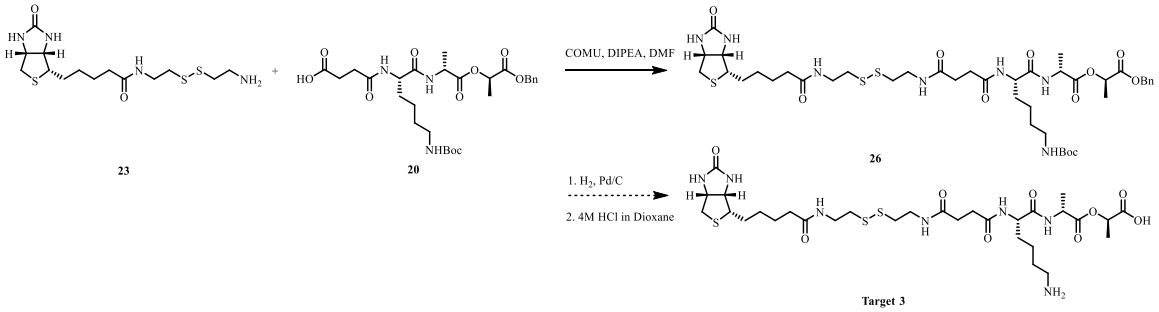

Figure 3.12. Original synthetic route of **Target 3**.

However, it was found that the hydrogenolysis step was unsuccessful regardless of the reaction conditions used (Table 3.1). In each case, only starting material was observed by mass spectroscopy. Different solvents were used as it was known that hydrogen gas possesses different solubility and reactivity in variable solvents[48,49], and different hydrogen sources were tested to potentially increase the activity and concentration of active hydrogen gas in the solvent[50]. Basic hydrolysis conditions were not attempted due to stability considerations of the ester bond present in the L-Lys-D-Ala-D-Lac depsipeptide.

Hydrogen Source	***Solvent***
H_2	MeOH
	EtOH
	EtOAc
	MeOH: EtOH: EtOAc= 1:1:1
10 equiv. Et_3OSi 4.4% HCOOH	MeOH

Table 3.1. Reaction conditions for hydrogenolysis in attempt to deprotect the benzyl ester group of compound **26**.

It was proposed that small amounts of the disulfide bond underwent a palladium metal-catalyzed cleavage to give free thiol, which inactivated the catalyst from functioning in benzyl ester deprotection. This assumption was supported by the study of Rooney's group in 2007 [51]. To avoid the interference of disulfide cleavage in the benzyl ester deprotection step, the protecting group on the D-Lac moiety was switched to a *tert*-butyl ester. Another advantage of using the *tert*-butyl ester was it can be removed along with the Boc protecting group on the L-Lys moiety via global deprotection with TFA.

At first, instead of changing the benzyl ester protecting group on molecule **4** in the middle of the synthesis, an alternative synthetic pathway to protect the free acid group on D-lactic acid as a *tert*-butyl ester was proposed, as shown in Figure 3.13. A common method to produce D-Lac(OtBu) utilizes distillation which is difficult on small scales[52]. Instead, the

method utilized by Wu's group was chosen as the purification method involving flash column chromatography[53]. However, for unknown reasons, the second step of this procedure was not successful in several trials.

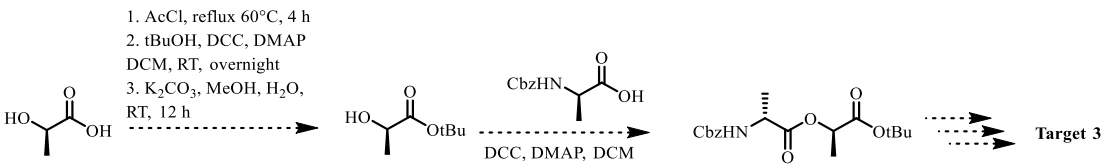

Figure 3.13. Proposed synthetic route for **Target 3** starting with the protection of D-lactic acid as a *tert*-butyl ester.

To solve this problem, we decided to keep the benzyl ester as a carboxylic acid protecting group in the beginning of the synthesis and then change it to a *tert*-butyl group at the stage of compound **4** (Figure 3.14). Although the yield of esterification was not ideal, the product showed high purity.

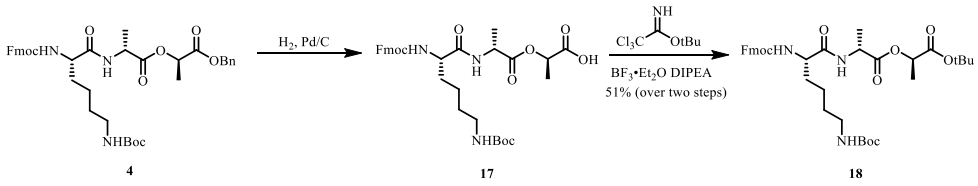

Figure 3.14. Synthetic route for switching benzyl ester group to *tert*-butyl ester group in **Target 3**.

The deprotection of the benzyl ester group in compound **4** also contributed to a low overall yield of compound **18** after two steps. Hydrogenolysis was conducted with the assistance of a palladium catalyst. During the first several trials, compound **4** was reacted with 10%

(w/w) of Pd/C following common hydrogenolysis procedures. However, deprotection of the Fmoc group on the L-Lys moiety occurred as a side reaction. To minimize this side reaction, the weight percentage of the palladium catalyst and reaction time was modified. Ultimately, the reaction was carried out using 10% Pd/C for 10 min to give optimal results. Other deprotection methods such as hydrolysis under basic conditions were not tested due to concerns around the cleavage of the D-Ala-D-Lac ester bond.

The succinic acid moiety was then coupled to molecule **18** to give acid **20** by Fmoc-deprotection giving free amine **19**, followed by treatment with excess amount of succinic anhydride under basic conditions (Figure 3.11).

For the synthesis of the other half of **Target 3** that contains the biotin and cystamine groups, cystamine dihydrochloride salt was first basified using a 1M NaOH solution to give free cystamine. This was then monoprotected with a Boc group using di-*tert*-butyl dicarbonate under basic conditions to give the known amine **21** (Figure 3.15)[54]. Compound **21** was coupled to biotin using TBTU to give the known compound **22**, which was then deprotected with 4 M HCl to generate free amine **23**.

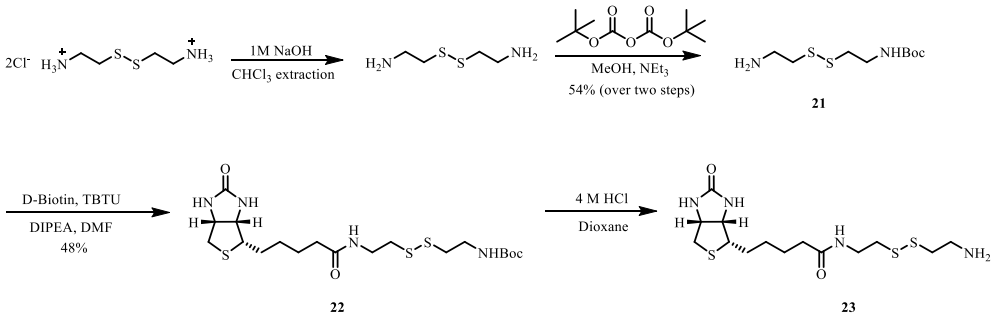

Figure 3.15. Synthetic procedures for the coupling of biotin with cystamine.

The attachment of biotin to L-Lys-D-Ala-D-Lac was realized by first activating the carboxylic group on compound **20** as a NHS ester to give compound **24** (Figure 3.16). The activated ester was carried on directly to the next step without any purification. Compound **24** was linked with compound **23** to give molecule **25**.

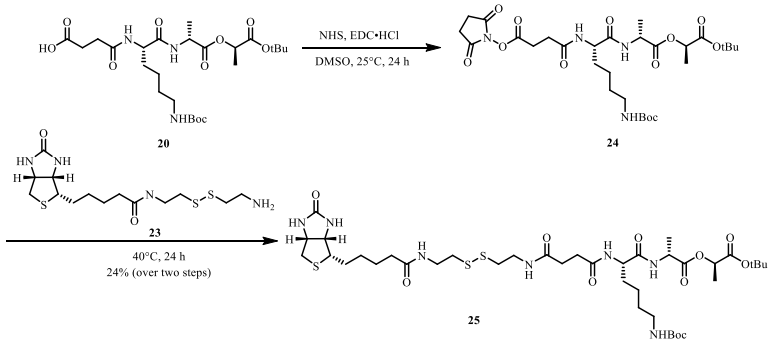

Figure 3.16. Coupling of compound **23** with tripeptide **20** to give compound **25**.

Initial attempts at the coupling of molecule **20** and **23** when using PyBOP, COMU or HBTU as the coupling reagent gave either no, or very low, yields of the desired product. The low yield of the above coupling reaction might be due to the low reactivity of the

carboxylic acid of compound **20**. Therefore, it was first activated by N-hydroxysuccinimide (NHS) using a procedure provided by Li's group [55], followed by coupling to **23** to give a better yield of compound **25**. Compound **25** then underwent global deprotection in the presence of 50% (v/v) of TFA in DCM to generate **Target 3** (Figure 3.17).

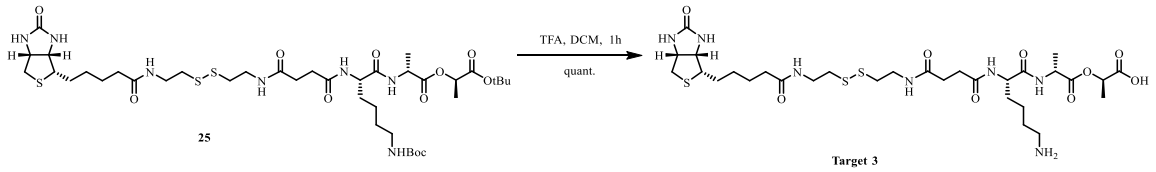

Figure 3.17. Global deprotection of compound **25** to give **Target 3**.

3.2.3 Selection of catalytic DNAzyme using Target 3

SELEX of DNAzymes against **Target 3** has not been started yet. In the selection (Figure 3.18), **Target 3** molecules will first be immobilized on a streptavidin-based resin. The resin will be washed with a synthetic library of oligonucleotides containing the modified bases $dC^{aa}TP$ and $dU^{g}TP$.(the corresponding triphosphates are shown in Figure 3.5). Catalytic aptamers that cleave the ester bond and form a covalent bond with the target will be collected while unbound ones will be discarded. The DNAzyme-target adduct will be released from the resin through a TCEP assisted disulfide bond cleavage. They will be amplified by PCR and carried on for the next several rounds of selection to identify the most active catalysts.

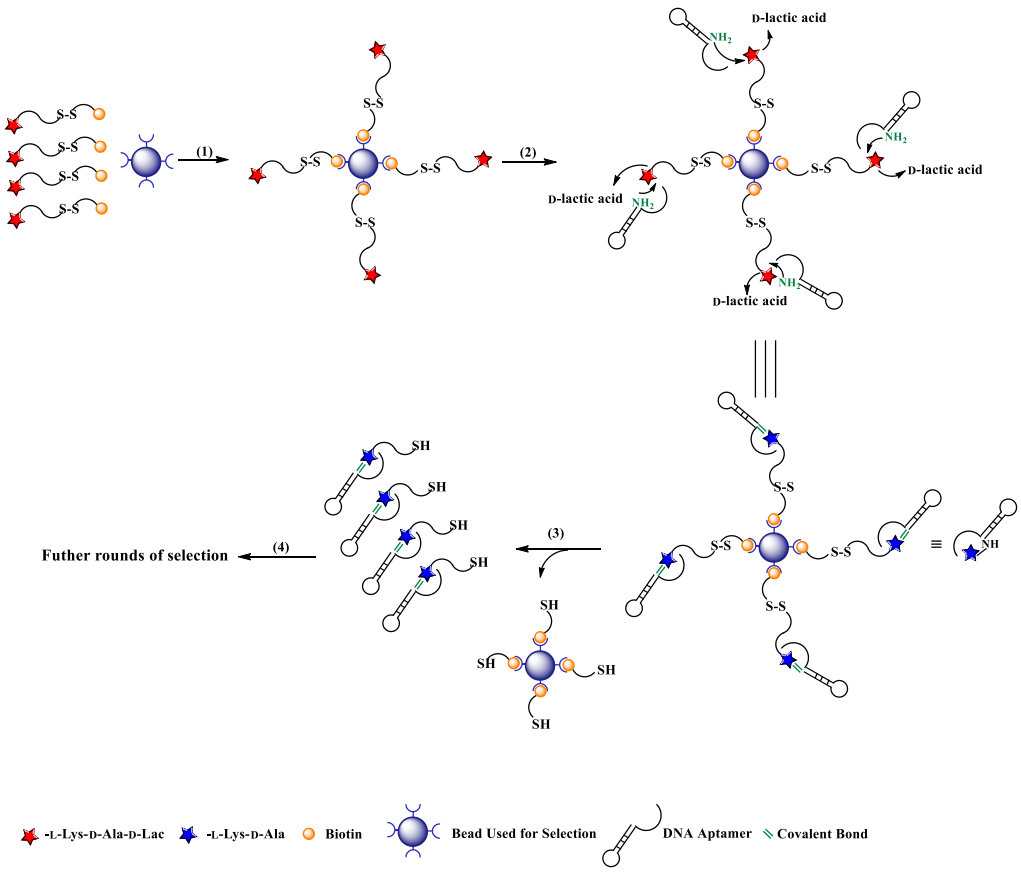

Figure 3.18. Selection procedures of DNAzymes using **Target 3**. (1) Immobilization of biotin-peptide **Target 3** onto streptavidin-activated resin; (2) Ester bond cleavage catalyzed by catalytic DNAzymes and covalent binding with target depsipeptide; (3) TCEP assisted disulfide cleavage and collection of DNAzyme-target adduct; (4) Amplification of binding aptamers with PCR and further rounds of selection.

3.2.4 Conclusion

A target compound suitable for the selection of catalytic DNAzymes against vancomycin-resistant peptidoglycan has been synthesized. At this point, SELEX selection of catalytic DNAzymes is ongoing. If one or several catalytic DNAzymes can be selected with the

desired activity, they could be used as either sensors for, or antibiotics against vancomycin-resistant strains of bacteria.

3.3 Summary

Molecules mimicking the vancomycin-sensitive and resistant bacterial cell wall precursor **Target 2** and **Target 3** were successfully synthesized in this section. Selections of corresponding DNA aptamers are still underway.

3.4 Future Directions

To demonstrate the interaction between a DNA aptamer and the peptide target might require the visualization of the DNA aptamers separated via gel electrophoresis. The synthesis of a tripeptide or depsipeptide bearing a fluorescent label could be useful in this regard. If only weakly binding aptamers or DNAzymes of low catalytic activity are isolated, it may be necessary to synthesize targets containing the full pentapeptide side chain of peptidoglycan precursor and repeat the selections.

3.5 Experimental

3.5.1 Material and general procedures

Chemicals were purchased from Sigma Aldrich, Alfa Aesar, Oakwood Products Inc., Toronto Research Chemicals, Oxchem and Chem-Impex. All chemicals were used directly without further purification unless noted. TLC plates were purchased from EMD Chemical Inc. Flash silica chromatography was conducted using silica gel SiliaFlash F60 (Silicycle,

230-400 mesh). Ion exchange resin L13943 Dowex® 50WX2 50-100 (H) was purchased from Alfa Aesar. AG® 1-X8 Anion Exchange Resin was purchased from Bio-Rad Laboratories. DCM were distilled under Ar from CaH_2.

^{1}H NMR spectra and proton-decoupled ^{13}C NMR spectra were recorded using a Bruker AV400sp spectrometer at a field strength of 400 MHz and 101 MHz, respectively. Mass spectra were obtained on a Waters LC-MS spectrometer (OAMS) and high-resolution mass spectrometer at the UBC Mass Spectrometry Facility.

3.5.2 Synthesis of Target 2

*Compound **11***

Cbz-D-Ala-OH (3.68 g, 16.5 mmol), HBTU (6.90 g, 18.2 mmol), HOBt (2.46 g, 18.2 mmol) and DIPEA（3.0 mL, 17.0 mmol) were dissolved in DMF (30 mL). The mixture was stirred at 0 °C, and then a solution of D-Ala(OtBu)·HCl (3.00 g, 16.5 mmol) and DIPEA (3.0 mL, 17.0 mmol) in DMF (10 mL) was added to the reaction mixture dropwise. Then the mixture was stirred at rt for 15 h. The solution was diluted with 100 mL of H_2O and extracted with ethyl acetate (3×100 mL). The organic layer was washed with brine 3 times and dried over $MgSO_4$. The volatiles were removed under vacuum and the residue was purified by flash column chromatography (silica, 7:3 petroleum ether: ethyl acetate) to give compound **11** as a white solid (4.23 g, 74 %). The spectral properties of compound **11** were identical to those reported in the literature.

*Compound **12***

Compound **11** (1.04 g, 2.9 mmol) was dissolved in methanol (10 mL) and Pd/C (0.1 g) was added. The reaction mixture was stirred at rt under hydrogen gas for 2.5 h. Then the mixture was filtered with celite and filtrate was concentrated under reduced pressure to give crude compound **12** as a white solid and was used in subsequent reactions without further purification. MS (ESI) m/z= 217.3 $[M+H]^+$.

*Compound **13***

Crude compound **12** (318 mg) and Fmoc-L-Lys(Boc)-OH (689 g, 1.5 mmol) were dissolved in DMF (30 mL). To the reaction mixture, COMU (630 mg, 1.5 mmol) and DIPEA (0.5 mL, 2.9 mmol) were added. The mixture was stirred at 0 °C for 1 h and then at rt for 3 h. The mixture was diluted with ethyl acetate (200 mL), and then washed with 10% citric acid, sat. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under vacuum and purification was carried out by flash column chromatography (silica, 2:8 petroleum ether: ethyl acetate) to give compound **13** as a white solid (571.3 mg, 58 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 7.82 (d, J = 7.5 Hz, 2H), 7.69 (t, J = 6.7 Hz, 2H), 7.41 (d, J = 8.1 Hz, 2H), 7.37 – 7.29 (m, 2H), 4.48 – 4.34 (m, 3H), 4.31 – 4.22 (m, 2H), 4.03 (t, J = 7.2 Hz, 1H), 3.06 (t, J = 6.8 Hz, 2H), 1.91 – 1.51 (m, 6H), 1.51 – 1.06 (m, 24H). ^{13}C NMR (101 MHz, methanol-d_4) δ 171.96, 171.96, 170.37, 155.79, 142.53, 142.52, 142.37, 142.36, 139.82, 139.82, 125.99, 125.37, 123.44, 118.13, 79.70, 77.08, 65.26, 54.03, 47.47, 47.14, 47.05, 38.22, 29.51, 29.51, 27.78, 26.00,

25.37, 21.33, 15.25, 14.47. HRMS (ESI): m/z calcd for $C_{36}H_{50}N_4O_8$ $[M+Na]^+$ 689.3526, found 689.3519.

*Compound **14***

Compound **13** (303 mg, 0.45 mmol) was dissolved in DMF (2.7 mL). After addition of piperidine (0.3 mL), the reaction mixture was stirred at rt for 5 min, and then the volatile was removed by under reduced pressure within 5 min. The residue was washed with petroleum ether 5 times to give crude compound **14** as a white solid. This was used in subsequent reactions without further purification. MS (ESI) m/z= 445.2 $[M+H]^+$, 467.2 $[M+Na]^+$.

*Compound **15***

Crude compound **14** (381 mg) and succinic anhydride (155 mg, 1.7 mmol) was dissolved in acetonitrile (5 mL). The reaction mixture was stirred at rt for 3 h. The volatiles were removed under vacuum and the residue was purified by flash column chromatography (silica,92:8 of DCM: methanol) to give compound **15** as a yellowish foamy solid (316 mg, 68 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 4.39 (q, J = 7.0 Hz, 1H), 4.30 – 4.18 (m, 2H), 3.06 (t, J = 6.3 Hz, 2H), 2.68 – 2.60 (m, 2H), 2.57 – 2.50 (m, 2H), 2.02 – 1.58 (m, 6H), 1.58 – 1.35 (m, 24H). ^{13}C NMR (101 MHz, methanol-d4) δ 174.98, 173.58, 173.22, 172.88, 171.83, 157.15, 81.11, 78.45, 60.13, 53.81, 48.87, 48.71, 39.66, 30.74, 29.92, 29.18, 28.74, 28.55, 27.40, 26.83, 22.76, 19.47, 16.57, 15.94, 13.08. HRMS (ESI): m/z calcd for $C_{25}H_{44}N_4O_9$ $[M+Na]^+$ 567.3006, found 567.3007.

*Compound **16***

Cystamine dihydrochloride (66 mg, 0.29 mmol) was dissolved in H_2O (3 mL). 1 M NaOH solution (1 mL) was added and the aqueous layer was extracted with 5×20 mL of chloroform. The combined organic layers were dried over $MgSO_4$ and filtered. The filtrate was concentrated under vacuum to give crude cystamine as a white solid which was used without further purification.

Compound **15** (350 mg, 0.64 mmol) and crude cystamine (44 mg) were dissolved in DMF (30 mL). COMU (274 mg, 0.64 mmol) and DIPEA (0.2 mL, 1.2 mmol) were added to the reaction mixture. The mixture was stirred at 0 °C for 1 h and then rt for 3 h. The mixture was diluted with ethyl acetate (200 mL) and was washed by 10% citric acid (2 times), sat. $NaHCO_3$ (2 times) and brine. The organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under vacuum and purification was carried out by flash column chromatography (silica, 9:1 DCM: methanol) to give compound **16** as a white solid (173 mg, 45 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 4.39 (q, J = 7.2 Hz, 2H), 4.30 – 4.21 (m, 4H), 3.49 (t, J = 6.8 Hz, 4H), 3.06 (t, J = 6.7 Hz, 4H), 2.82 (t, J = 6.8 Hz, 4H), 2.60 – 2.48 (m, 8H), 2.07 – 1.52 (m, 12H), 1.51 – 1.08 (m, 48H). ^{13}C NMR (101 MHz, methanol-d4) δ 173.75, 173.26, 173.21, 172.88, 171.91, 81.15, 53.74, 48.88, 48.78, 48.44, 48.32, 48.23, 39.68, 38.26, 37.06, 30.77, 30.59, 30.52, 27.41, 26.85, 22.80, 16.67, 15.99. HRMS (ESI): m/z calcd for $C_{54}H_{96}N_{10}O_{16}S_2$ $[M+H]^+$ 1205.6525, found 1205.6530.

Target 2

Compound **16** (78 mg, 0.065 mmol) was dissolved in DCM (1 mL) and TFA (1 mL) was added to the solution. The reaction mixture was stirred at rt for 2 h. The volatile was removed under vacuum. The residue was washed with diethyl ether 5 times to give final product **Target 2** as a white solid (71.5 mg with trifluoroacetate as conjugate base, 99 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 4.43 – 4.36 (m, 4H), 4.35 – 4.30 (m, 2H), 3.49 (t, *J* = 6.8 Hz, 4H), 2.96 (t, *J* = 7.2 Hz, 4H), 2.82 (t, *J* = 6.8 Hz, 4H), 2.59 – 2.50 (m, 8H), 1.99 – 1.53 (m, 12H), 1.52 – 1.39 (m, 12H). ^{13}C NMR (101 MHz, methanol-d4) δ 174.36, 173.94, 173.34, 173.24, 172.68, 53.23, 49.00, 39.09, 38.27, 37.08, 30.53, 30.48, 30.29, 26.66, 22.28, 16.60, 16.18. HRMS (ESI): m/z calcd for $C_{36}H_{64}N_{10}O_{12}S_2$ $[M+Na]^+$ 893.4225, found 893.4232.

3.5.3 Synthesis of Target 3

The synthesis of compounds **1**-**4** refer to Chapter 2.

*Compound **17***

Compound **4** (1.00 g, 1.4 mmol) was dissolved in methanol (20 mL) and Pd/C (0.10 g) was added to the solution. The reaction mixture was stirred at rt under an atmosphere of hydrogen gas for 10 min. Then the mixture was filtered through celite and the filtrate was concentrated under vacuum to give crude compound **17** as a white solid. Compound 17 was used in subsequent reactions without further purification.

*Compound **18***

Crude compound **17** (887 mg) was dissolved in DCM (10 mL) and added to a solution of *tert*-butyl 2,2,2-trichloroacetimidate (0.52 mL, 2.90 mmol) in cyclohexane (20 mL). To the reaction mixture, boron trifluoride diethyl etherate (36.4 µl, 0.29 mmol) was added. The reaction mixture was then stirred at rt for 15 h, and $NaHCO_3$ (122 mg, 1.45 mmol) was added to quench the reaction. The mixture was concentrated under vacuum and purification was carried out by flash column chromatography (silica, 2:3 petroleum ether: ethyl acetate) to give compound **18** as a white solid (336 mg, 51 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 7.82 (d, *J* = 7.5 Hz, 2H), 7.69 (t, *J* = 6.5 Hz, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.36 – 7.31 (m, 2H), 4.95 – 4.89 (m, 1H), 4.50 – 4.37 (m, 3H), 4.25 (t, *J* = 6.8 Hz, 1H), 4.19 – 4.07 (m, 1H), 3.13 – 2.98 (m, 2H), 2.05 – 1.53 (m, 6H), 1.52 – 0.91 (m, 24H). ^{13}C NMR (101 MHz, methanol-d4) δ 173.30, 171.94, 169.88, 157.15, 156.95, 143.94, 143.77, 141.21, 141.19, 128.17, 127.86, 127.37, 126.77, 124.80, 124.76, 119.51, 81.83, 78.46, 69.71, 66.48, 54.88, 39.63, 31.68, 27.39, 26.96, 26.75, 22.64, 16.10, 15.66. HRMS (ESI): m/z calcd for $C_{36}H_{49}N_3O_9$ $[M+Na]^+$ 690.3366, found 690.3365.

*Compound **19***

Compound **18** (101 mg, 0.15 mmol) was dissolved in DMF (1.9 mL). After addition of piperidine (0.1 mL), the reaction mixture was stirred at rt for 5 min and then the volatiles were removed under vacuum within 5 min. The residue was washed with petroleum ether 5 times to give crude compound **19** as a white solid. Compound **19** was used in subsequent reactions without further purification.

*Compound **20***

Crude compound **19** (119 mg) and succinic anhydride (267 mg, 2.7 mmol) was dissolved in acetonitrile (5 mL). The reaction mixture was stirred at rt for 3 h and was quenched by ammonium water. The volatiles were removed under vacuum and the residue was purified by flash column chromatography (silica, 92:8 DCM: methanol) followed by C18 reversed phase silica column chromatography (Sep-Pak C18 20 cc Vac Cartridge, 2:3 H_2O: methanol) to give compound **20** as a white foamy solid (94 mg, 65 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 4.92 (q, J = 7.1 Hz, 1H), 4.45 (q, J = 7.3 Hz, 1H), 4.39 – 4.30 (m, 1H), 3.06 (t, J = 6.7 Hz, 2H), 2.73 – 2.43 (m, 4H), 2.05 – 1.53 (m, 6H), 1.52 – 0.91 (m, 24H). ^{13}C NMR (101 MHz, methanol-d4) δ 175.20, 173.58, 172.88, 172.03, 169.90, 157.13, 81.83, 78.44, 69.68, 53.17, 39.66, 31.91, 31.20, 30.19, 29.14, 29.01, 27.39, 26.76, 22.66, 15.93, 15.68. HRMS (ESI): m/z calcd for $C_{25}H_{43}N_3O_{10}$ $[M+Na]^+$ 568.2846, found 568.2848.

*Compound **23***

To compound **22** (50 mg, 0.10 mmol) was added 4 M HCl (2 mL) in dioxane and the reaction mixture is stirred at rt for 1 h. The volatiles were removed under vacuum and the residue was washed by diethyl ether 5 times. Crude compound **23** was generated as a white solid and was used in subsequent reactions without further purification.

*Compound **24***

Compound **20** (50 mg, 0.091 mmol), NHS (21 mg, 0.182 mmol) and EDC·HCl (35 mg, 0.182 mmol) were dissolved in DMSO (5 mL). The reaction mixture was stirred at 30 °C

for 24 h to give a solution containing compound **24**, which was used directly in subsequent reaction without further purification.

*Compound **25***

Crude compound **23** (34 mg) was dissolved in DMSO (5 mL), and then was added to the solution containing crude compound **24** followed by the addition of DIPEA (23.5 µl, 0.182 mmol) and EDC·HCl (35 mg, 0.182 mmol). The mixture was stirred at 40 °C for 24 h. The mixture was dissolved in 100 mL of ethyl acetate followed by washing with water (400 mL), a saturated $NaHCO_3$ solution, a 10% citric acid solution and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by flash column chromatography (silica, 12:88 methanol: DCM) to afford compound **25** as a white solid (20 mg, 24 %). ^{1}H NMR (400 MHz, methanol-d_4) δ 4.93 (q, J = 7.1 Hz, 1H), 4.56 – 4.49 (m, 1H), 4.45 (q, J = 7.3 Hz, 1H), 4.38 – 4.30 (m, 2H), 3.54 – 3.45 (m, 4H), 3.25 – 3.18 (m, 1H), 3.05 (t, J = 6.7 Hz, 2H), 2.95 (dd, J = 12.7, 4.9 Hz, 1H), 2.84 (m , 4H), 2.73 (d, J = 12.7 Hz, 1H), 2.62 – 2.46 (m, 4H), 2.24 (t, J = 7.3 Hz, 2H), 1.94 – 1.54 (m, 12H), 1.54 – 1.37 (m, 24H). HRMS (ESI): m/z calcd for $C_{39}H_{67}N_7O_{11}S_3$ $[M+Na]^+$ 928.3958, found 928.3950.

Target 3

Compound **25 (**20.2 mg, 0.022 mmol) was dissolved in DCM (2 mL), and TFA (2 mL) was added to the solution. The reaction mixture was stirred at rt for 1 h and then the volatile ws removed under vacuum to give a yellowish oil. The crude was purified by ion-exchange

column and the product comes in water fractions to give Target 3 as a white powder (18 mg in formate form, 100%). ^{1}H NMR (400 MHz, methanol-d_4) δ 4.98 – 4.94 (m, 1H), 4.54 – 4.49 (m, 1H), 4.49 – 4.42 (m, 1H), 4.42 – 4.36 (m, 1H), 4.36 – 4.29 (m, 1H), 3.58 – 3.44 (m, 4H), 3.27 – 3.18 (m, 1H), 2.95 (q, *J* = 7.3, 6.2 Hz, 3H), 2.89 – 2.79 (m, 4H), 2.73 (d, *J* = 12.9 Hz, 1H), 2.61 – 2.50 (m, 4H), 2.25 (t, *J* = 7.2 Hz, 2H), 1.82 – 1.58 (m, 12H), 1.58 – 1.46 (m, 6H). ^{13}C NMR (101 MHz, methanol-d4) δ 174.81, 173.78, 173.14, 172.56, 172.24, 168.91, 164.70, 72.22, 61.97, 60.23, 55.61, 39.67, 39.15, 38.28, 38.14, 37.19, 35.33, 30.74, 30.34, 28.34, 28.09, 27.25, 26.74, 25.44, 22.13, 22.01, 16.82, 16.77, 16.02, 15.97. HRMS (ESI): m/z calcd for $C_{30}H_{51}N_7O_9S_3$ $[M+H]^+$ 750.2989, found 750.2996.

Chapter 4. Conclusion

In our early target molecule **YL-1** designed to mimic the peptidoglycan precursor of vancomycin sensitive strains of Gram-positive bacteria, the tripeptide L-Lys-D-Ala-D-Ala was linked to biotin through a non-cleavable linker. In the SELEX selection using compound **YL-1**, DNA aptamers mainly bound to the streptavidin protein that was used for immobilizing the target**.** To rectify this situation, a thiol group was introduced to replace the biotin giving **Target 2. Target 2** contained a tripeptide L-Lys-D-Ala-D-Ala linked to a thiol group and was synthesized successfully with high purity and used in SELEX selection of DNA aptamers. SELEX results indicated a successful selection of DNA aptamers, which could potentially bind to peptidoglycan precursor of Gram-positive bacteria with high affinity and weak metal dependency at body temperature. Further analysis, such as binding affinity studies, will be conducted in the near future. If the aptamers selected have comparable binding constants to vancomycin, they could be used as possible candidates in the treatment of Gram-positive bacterial infections.

In our early **Target 1** that mimiced the peptidoglycan precursor of vancomycin resistant strains of Gram-positive bacteria, the depsipeptide L-Lys-D-Ala-D-Lac was linked to biotin through a non-cleavable linker. Based on experience from the selection using molecule **YL-1**, we realized that the streptavidin protein would interfere with the SELEX selection. Therefore, we decided to introduce a cleavable disulfide to our target, which resulted in **Target 3**. The introduction of a disulfide bond eliminated the interference of streptavidin during selection as only covalently-linked DNAzymes would be collected after cleavage

of the disulfide bond. Though the synthesis of the depsipeptide part of **Target 3** required careful consideration of the stability of the ester bond, **Target 3** was successfully synthesized with high purity. The SELEX selection of **Target 3** is still on-going. If possible DNA aptamers with comparable binding constants to vancomycin or DNAzymes with turnover numbers of greater than 1 s^{-1} could be selected, they could possibly be applied in the treatment of vancomycin resistant bacterial infections.

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Appendix

NMR Spectra of Selected Compound

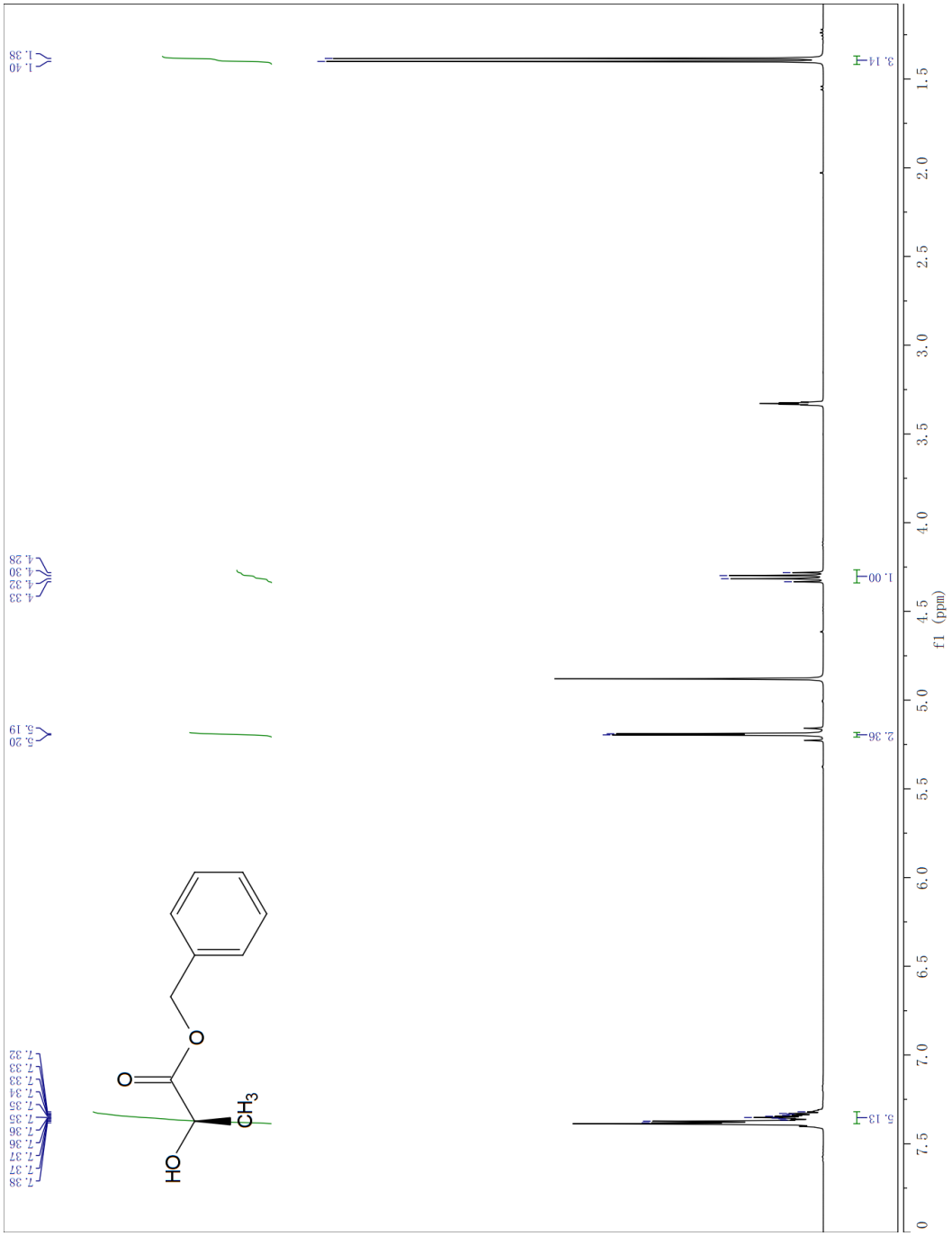

Figure A.1. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **1**.

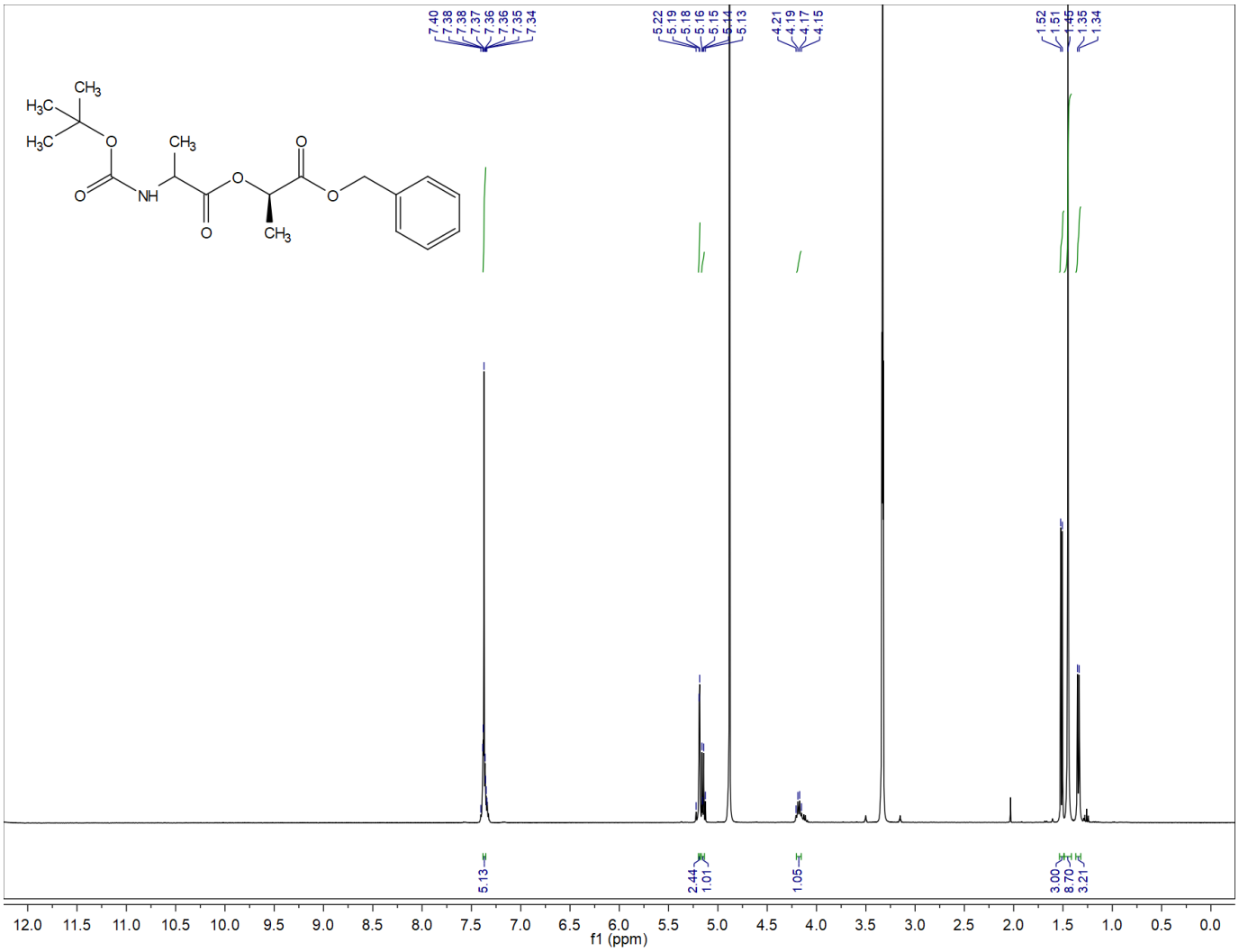

Figure A.2. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **2**.

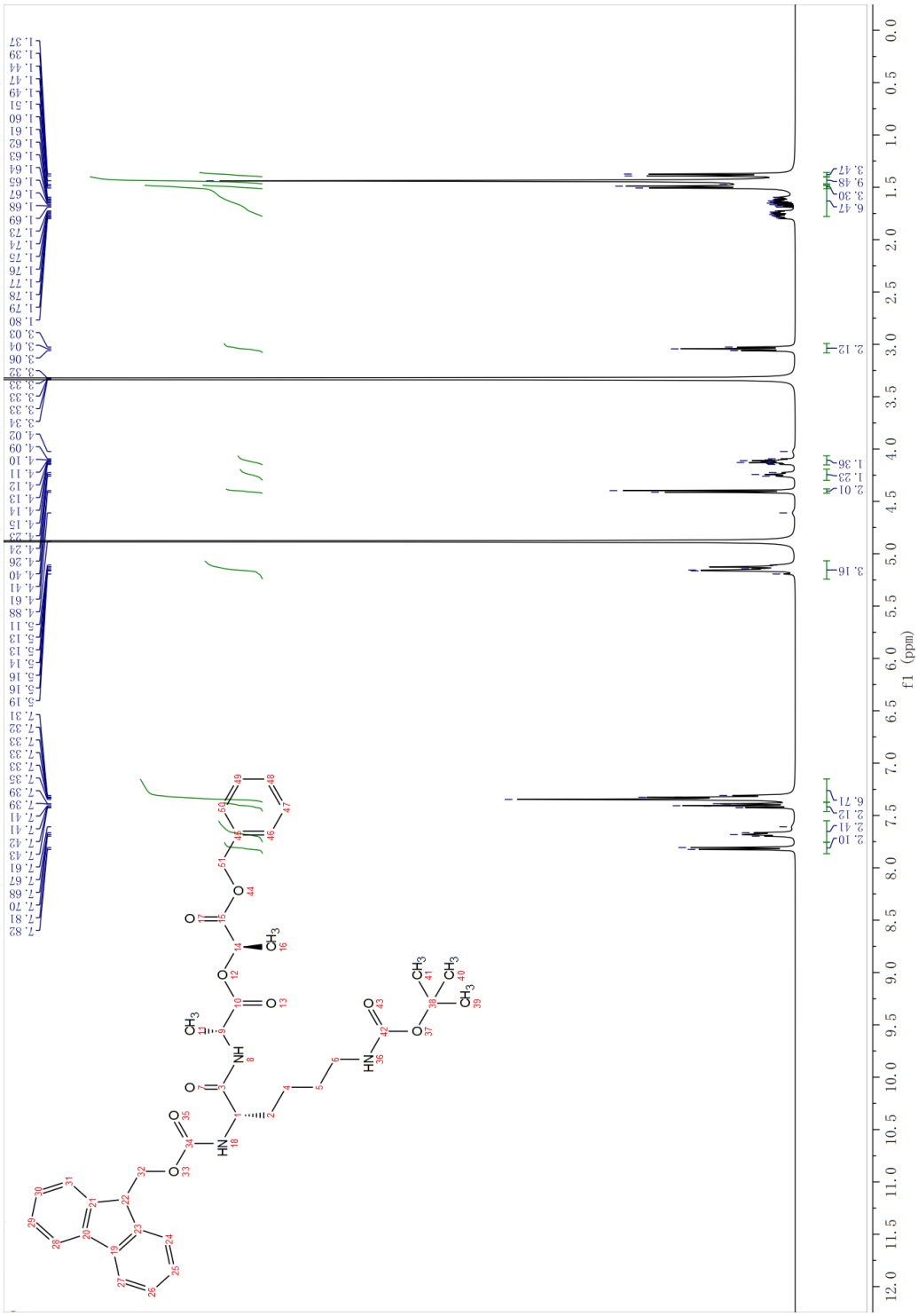

Figure A.3. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **4**.

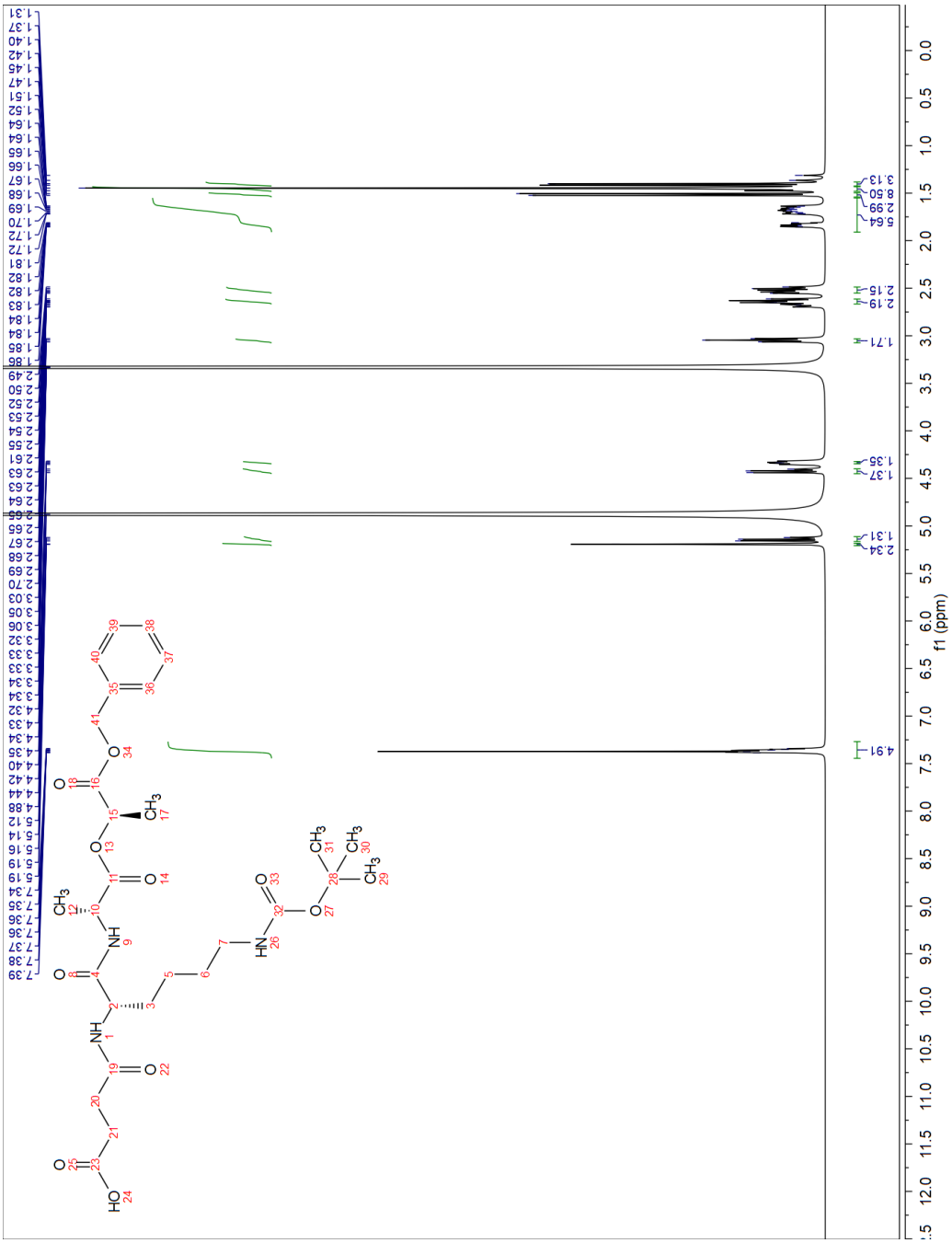

Figure A.4. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **6**.

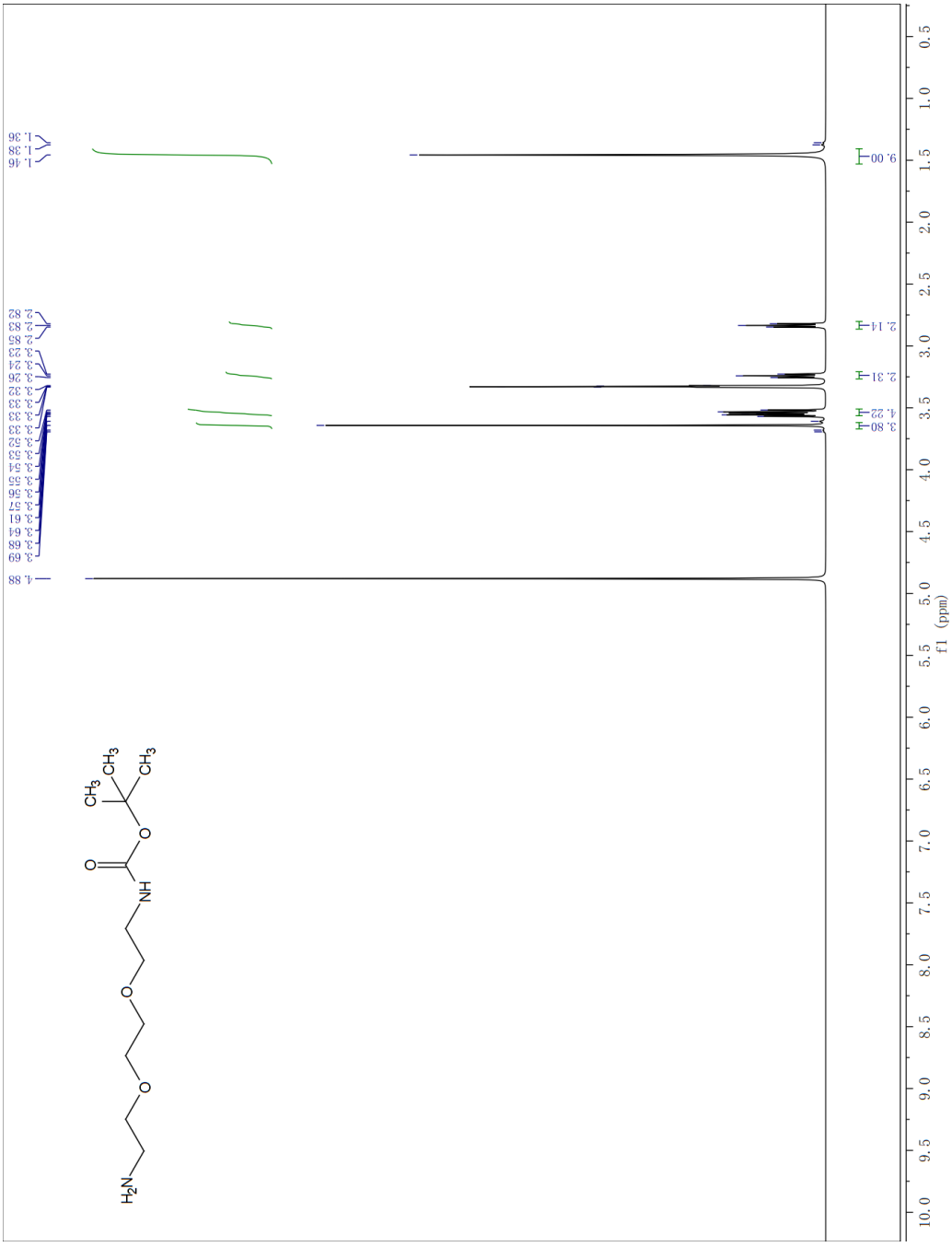

Figure A.5. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **7.**

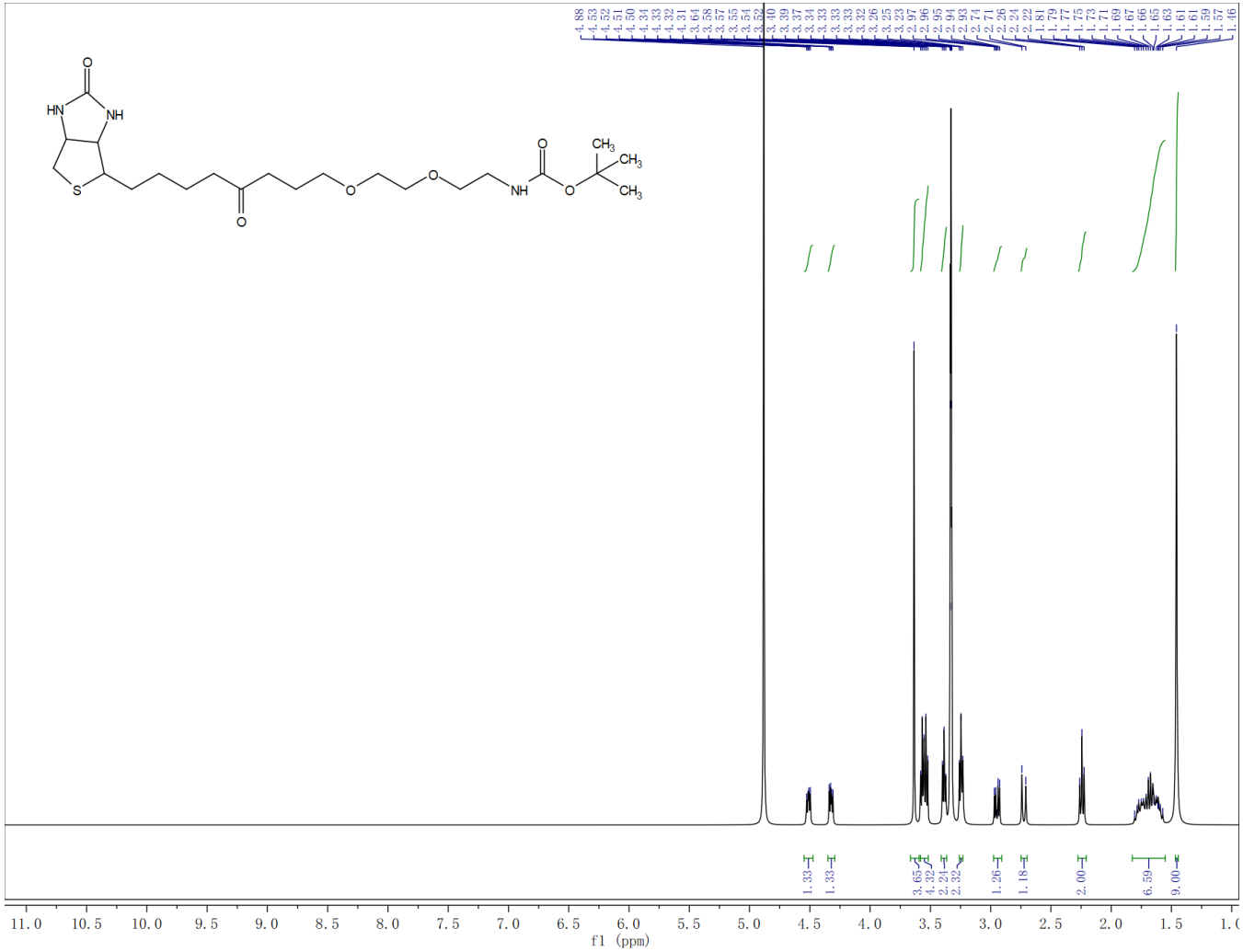

Figure A.6. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **8**.

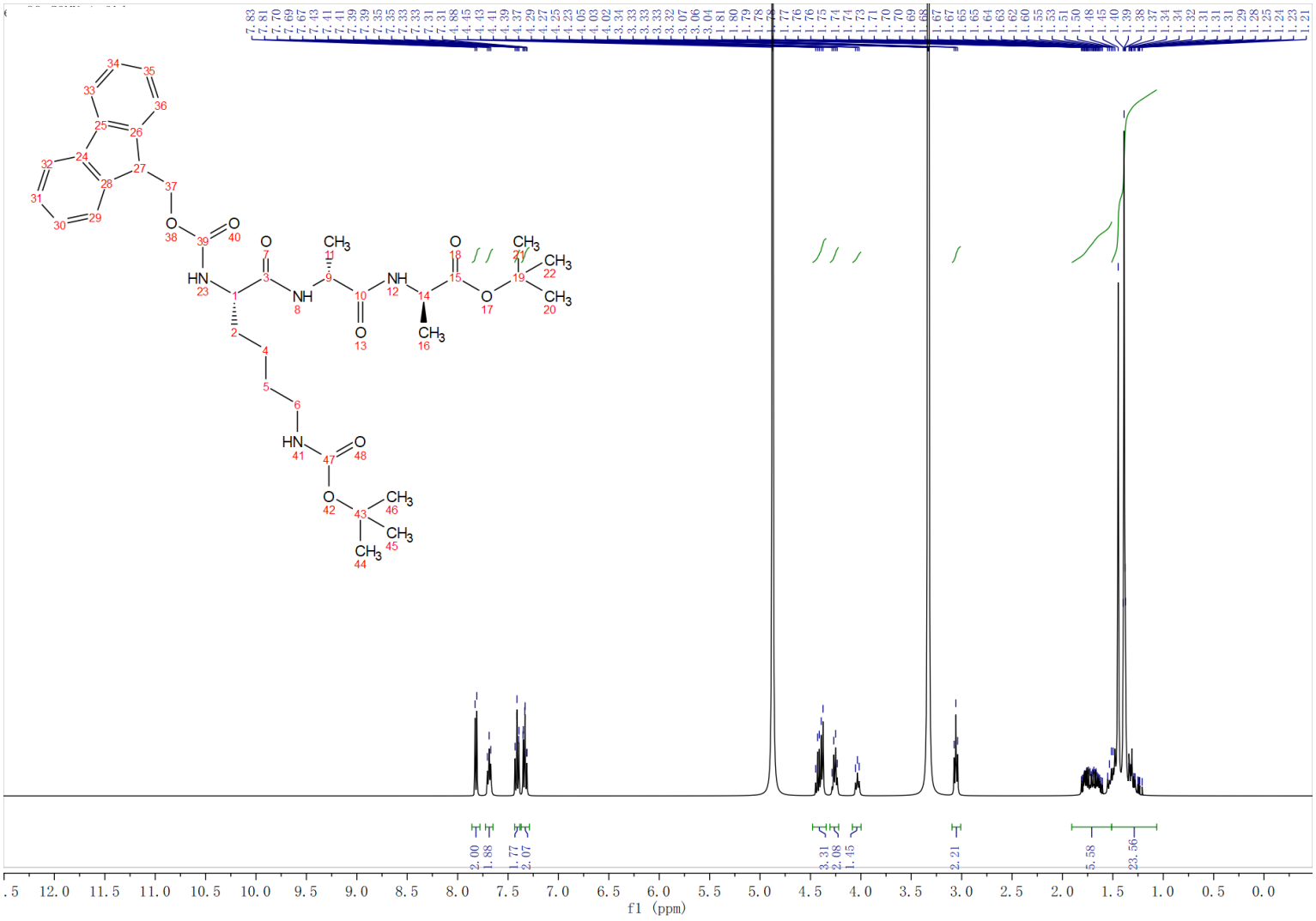

Figure A.7. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **13**.

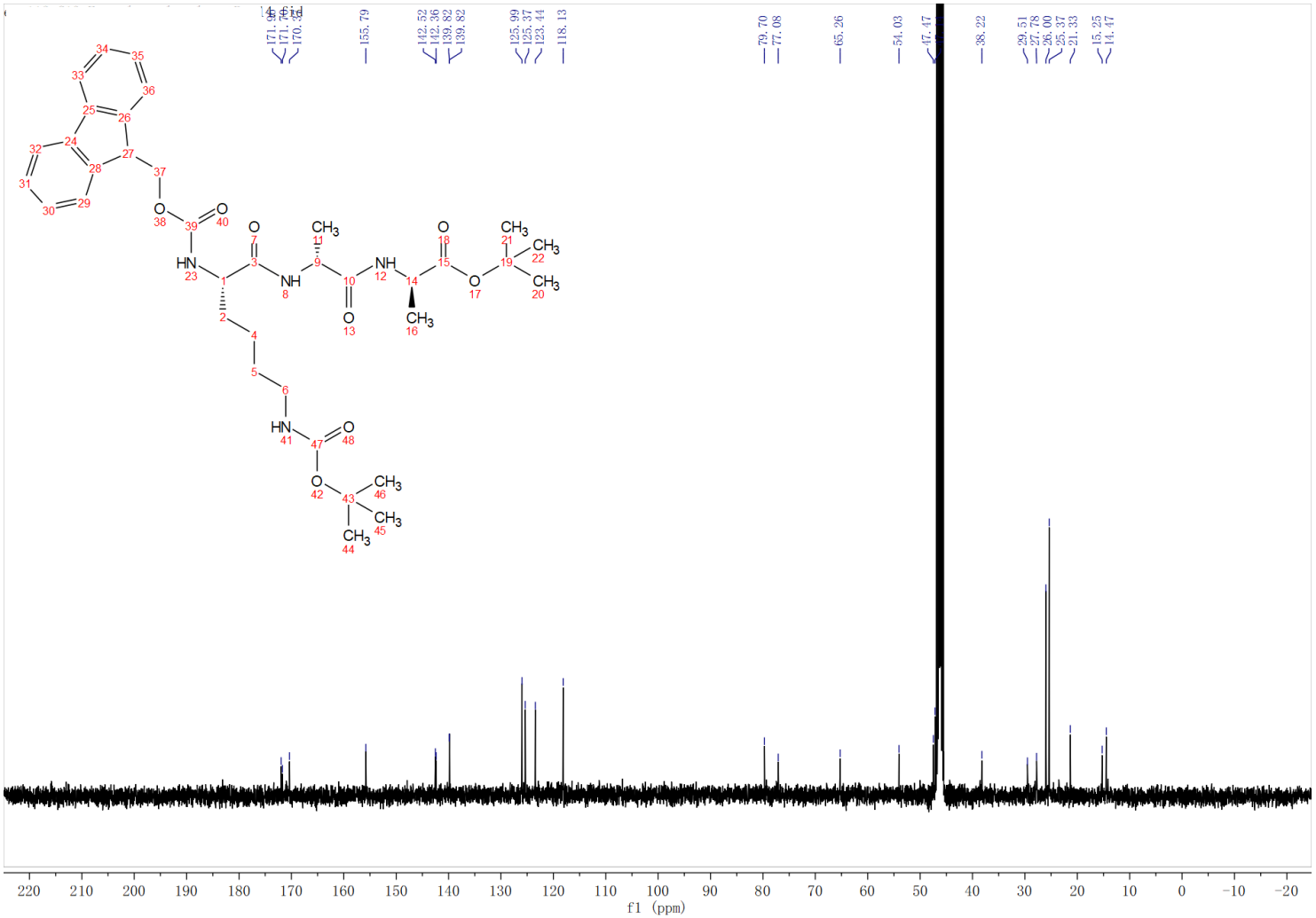

Figure A.8. ^{13}C NMR (101 MHz, methanol-d4) spectrum of compound **13**.

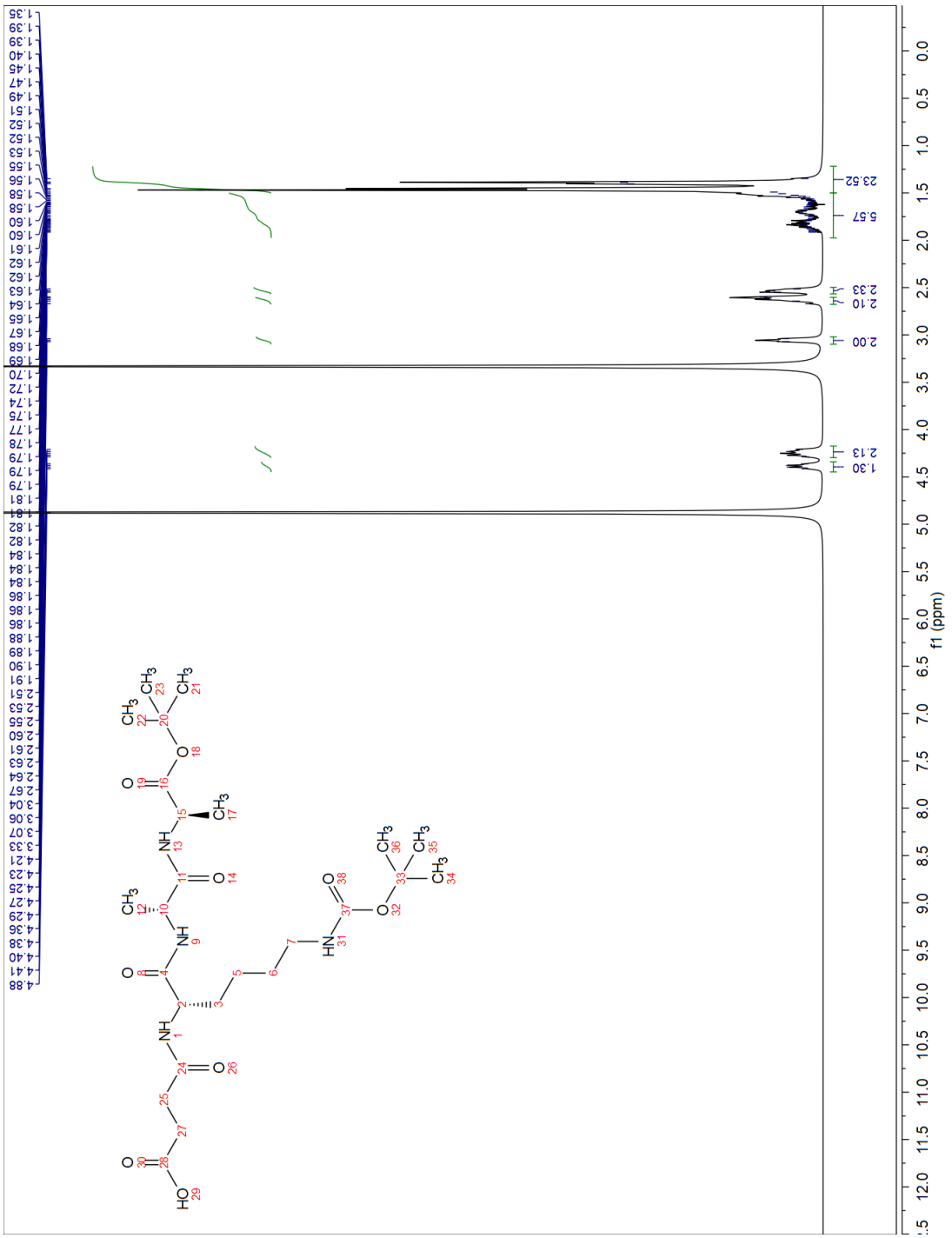

Figure A.9. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **15**.

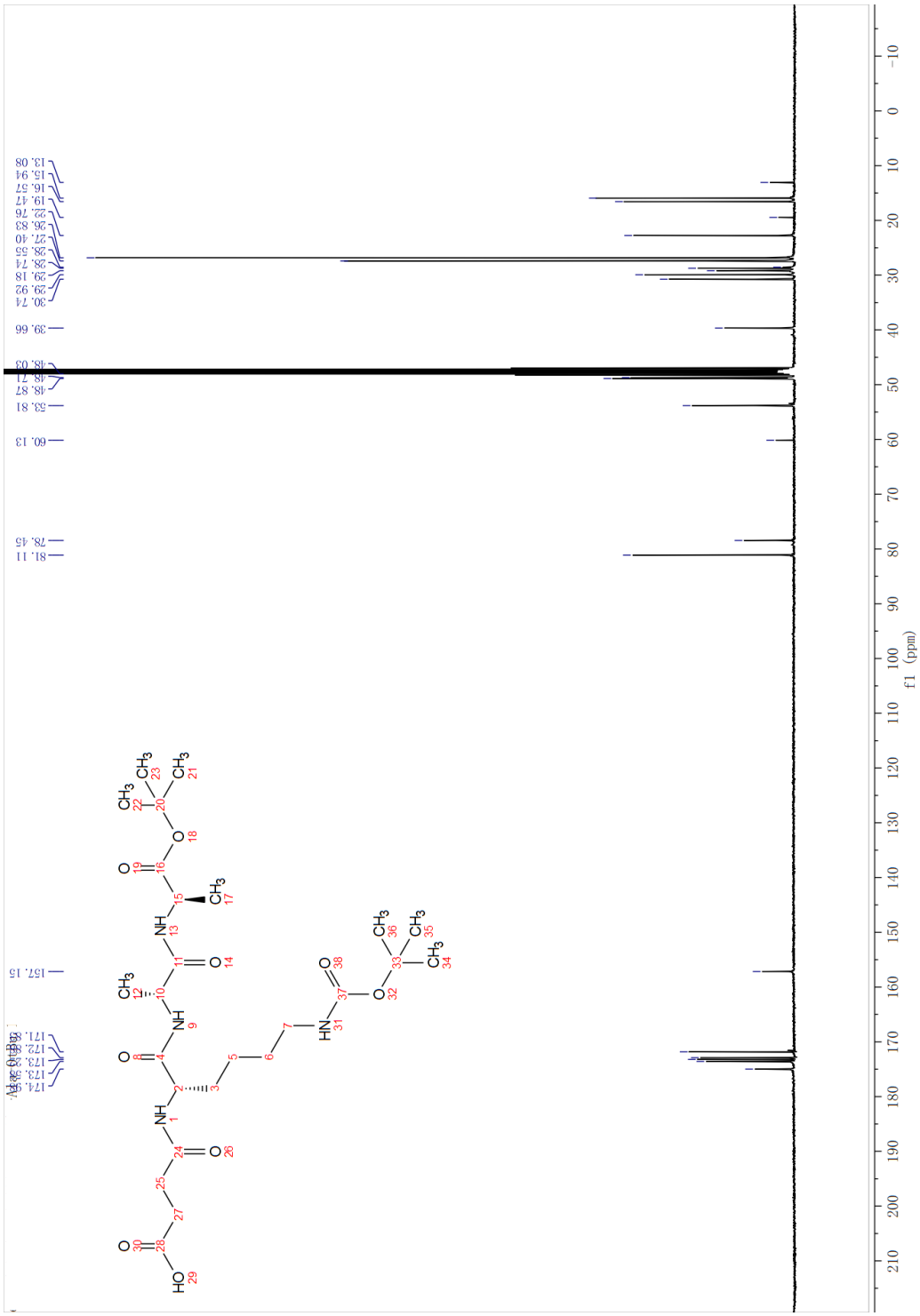

Figure A.10. ^{13}C NMR (101 MHz, methanol-d4) spectrum of compound **15.**

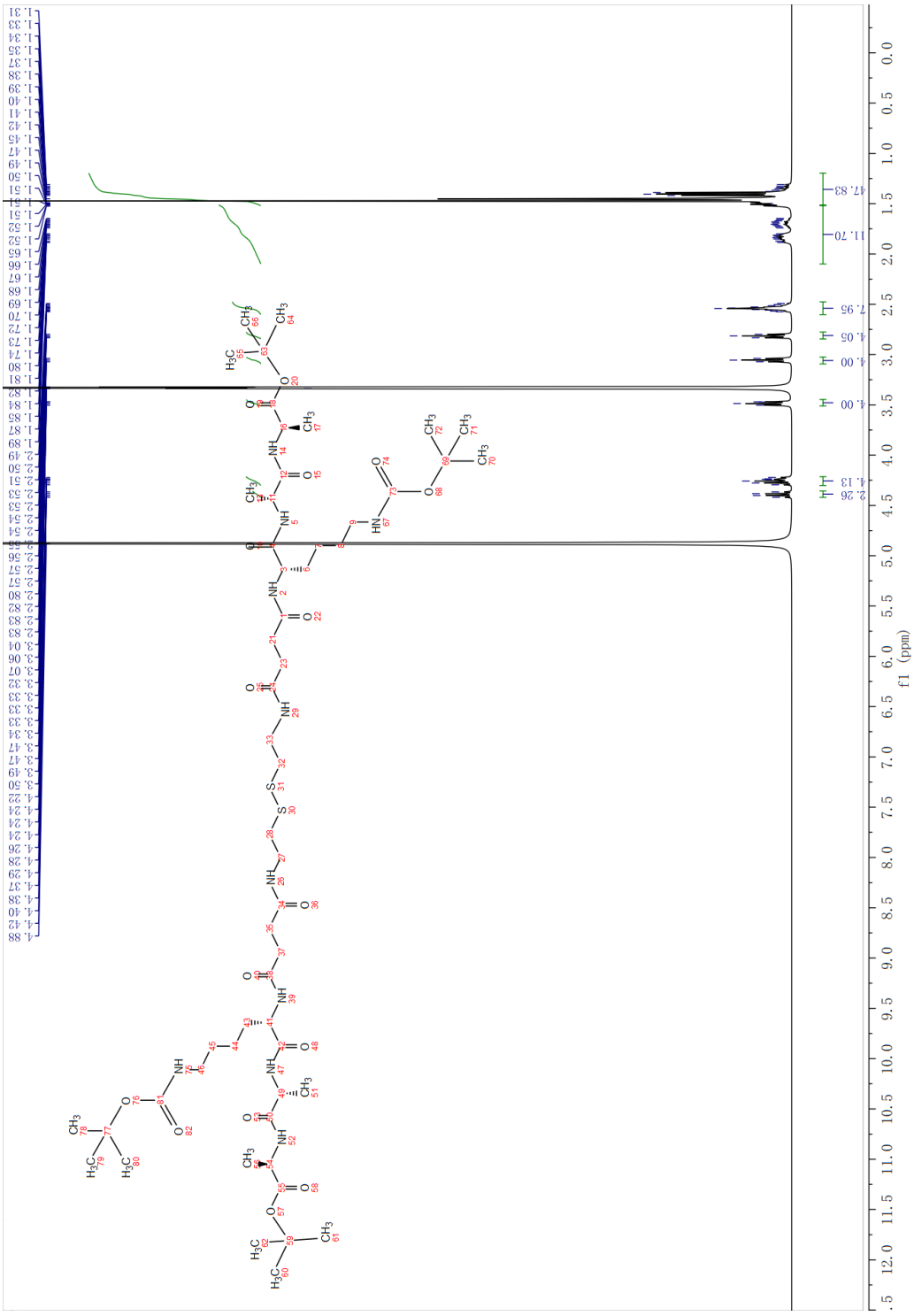

Figure A.11. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **16.**

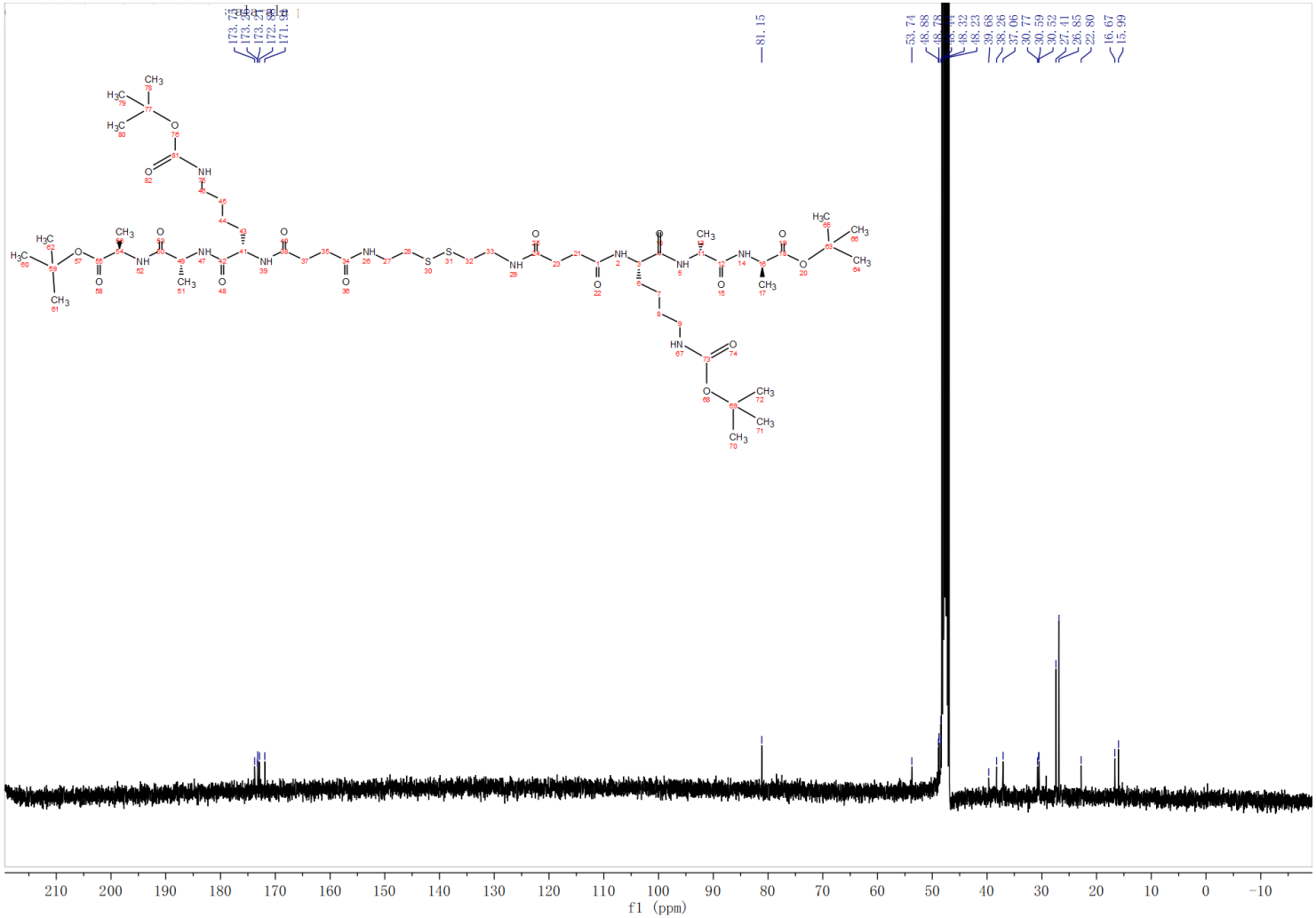

Figure A.12. ^{13}C NMR (101 MHz, methanol-d4) spectrum of compound **16.**

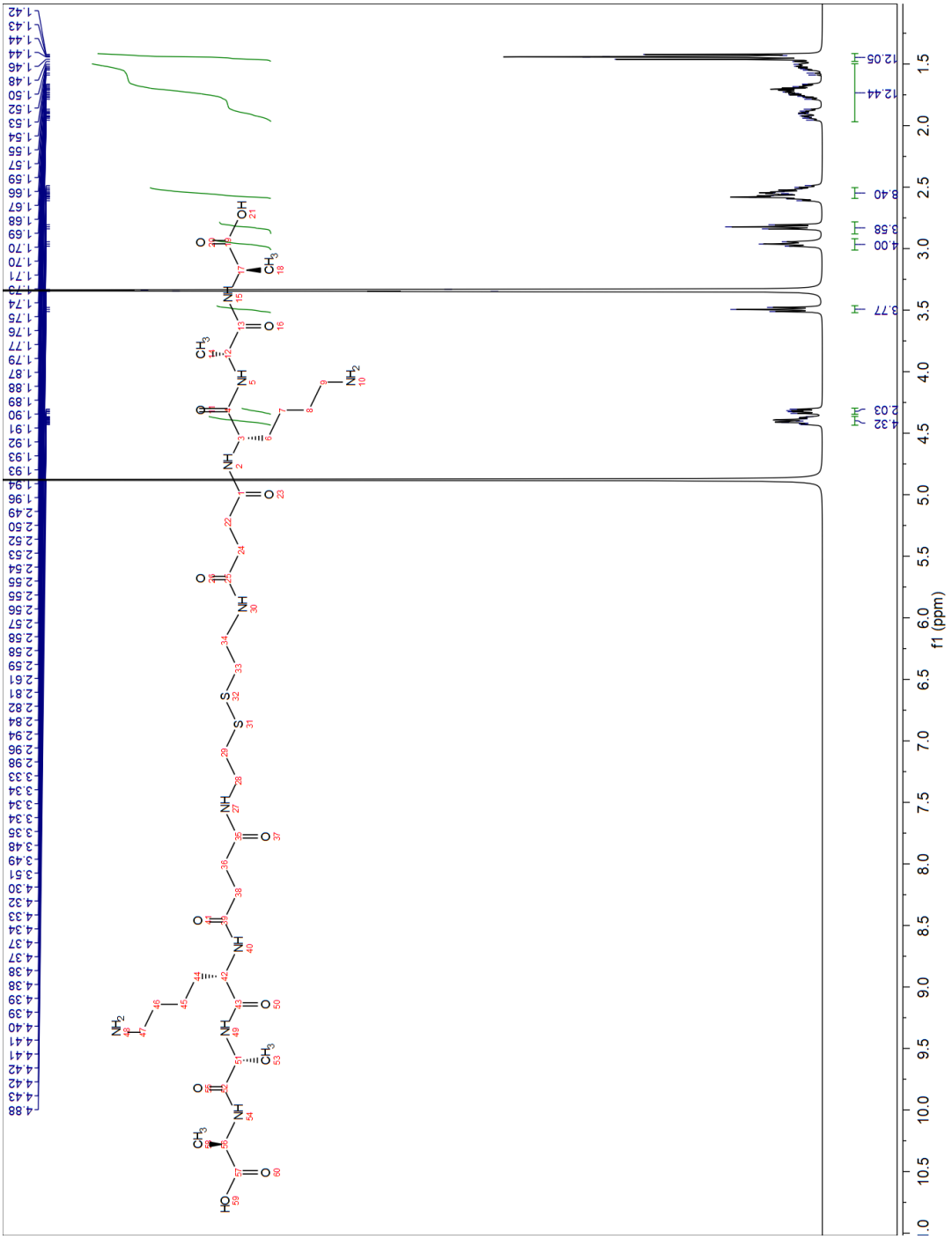

Figure A.13. ^{1}H NMR (400 MHz, methanol-d4) spectrum of **Target 2.**

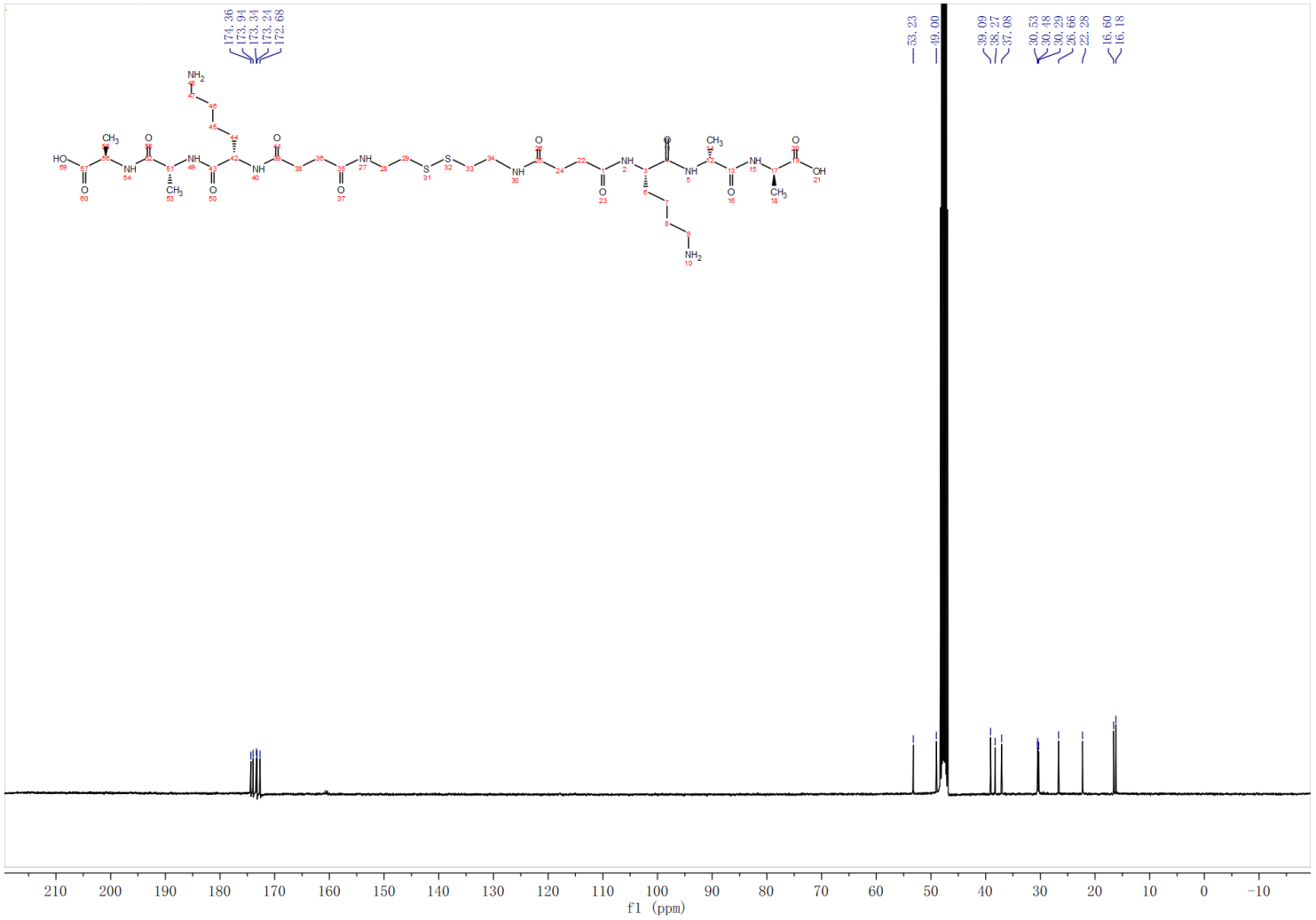

Figure A.14. ^{13}C NMR (101 MHz, methanol-d4) spectrum of **Target 2**.

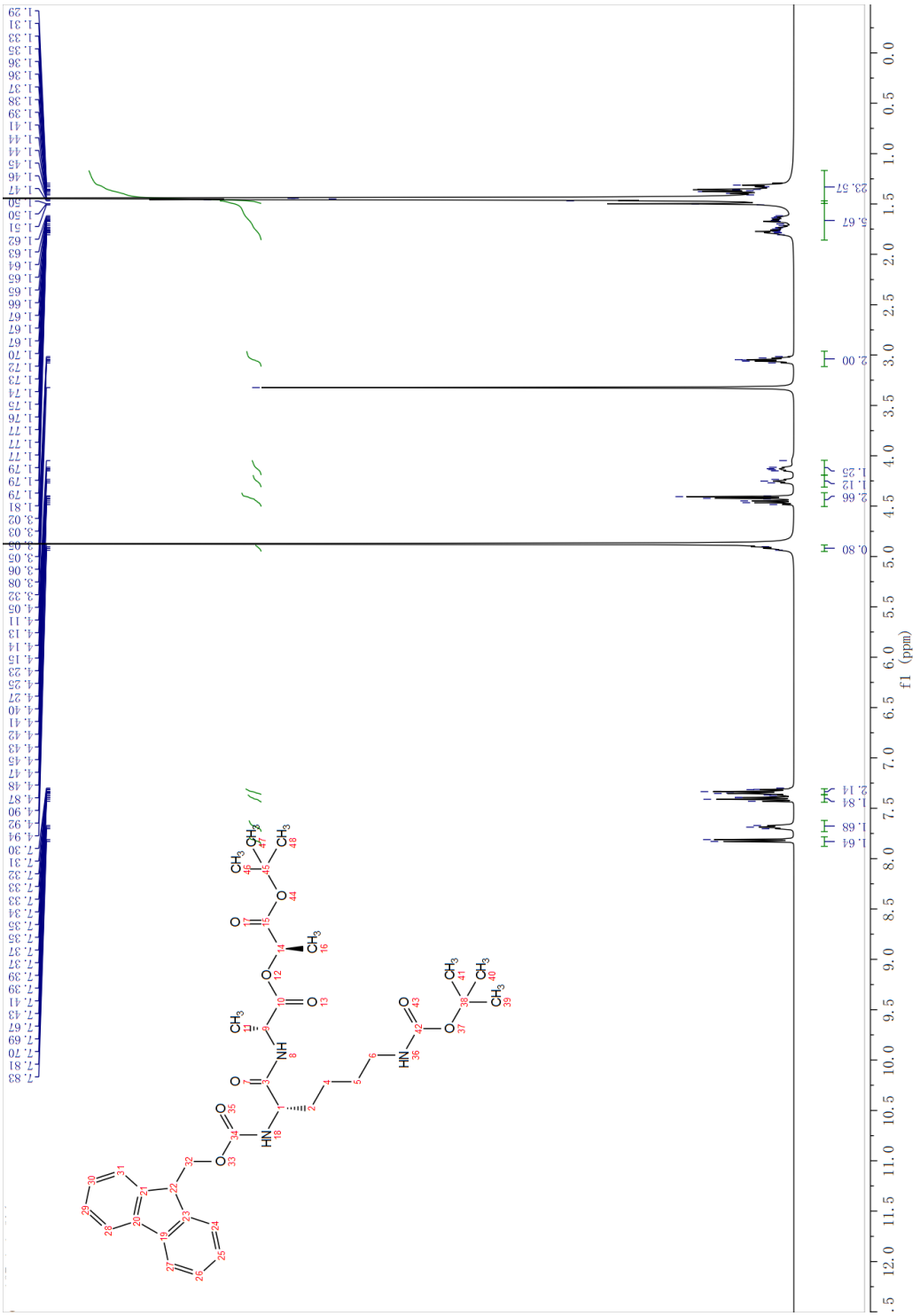

Figure A.15. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **18.**

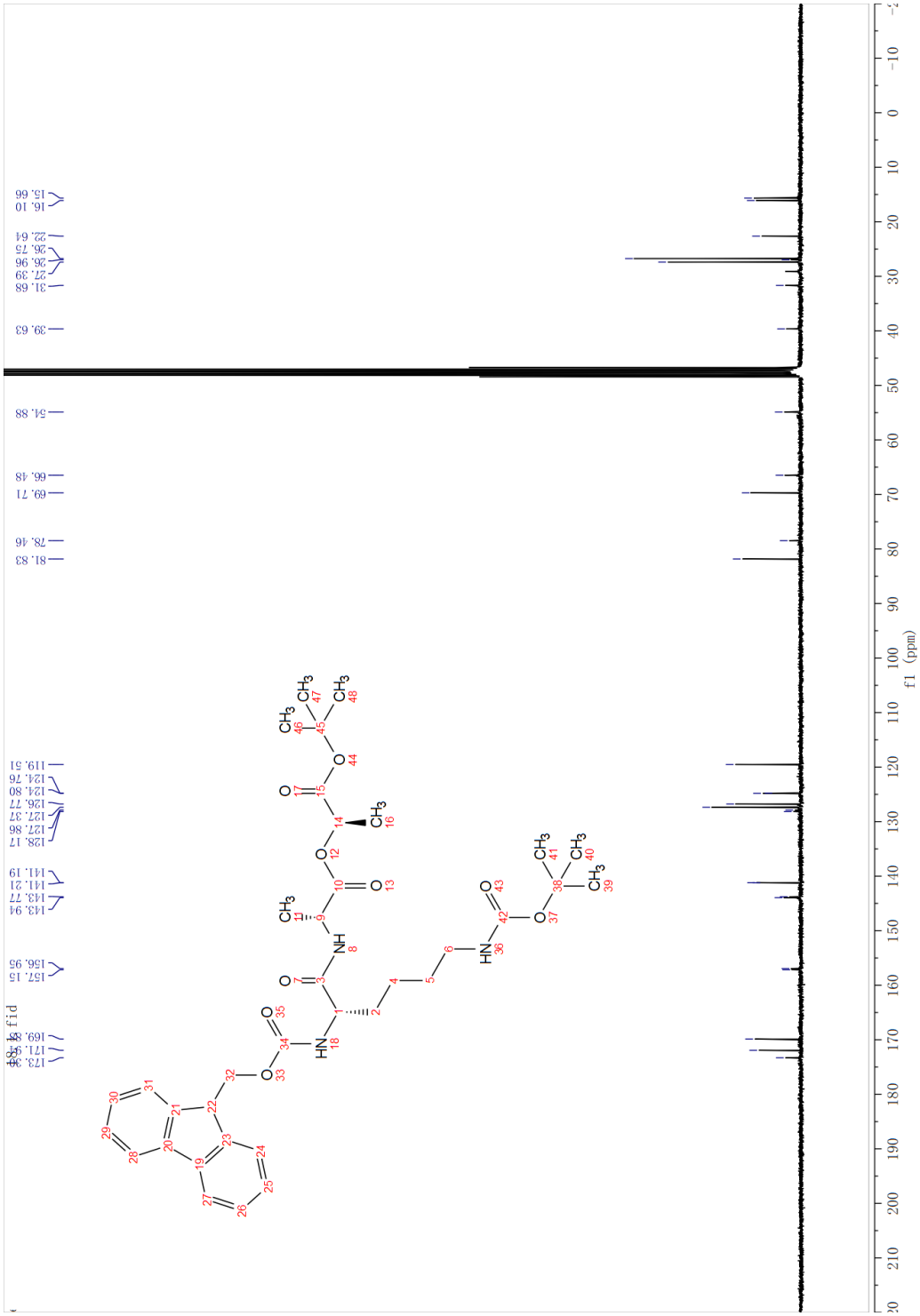

Figure A.16. ^{13}C NMR (101 MHz, methanol-d4) spectrum of compound **18.**

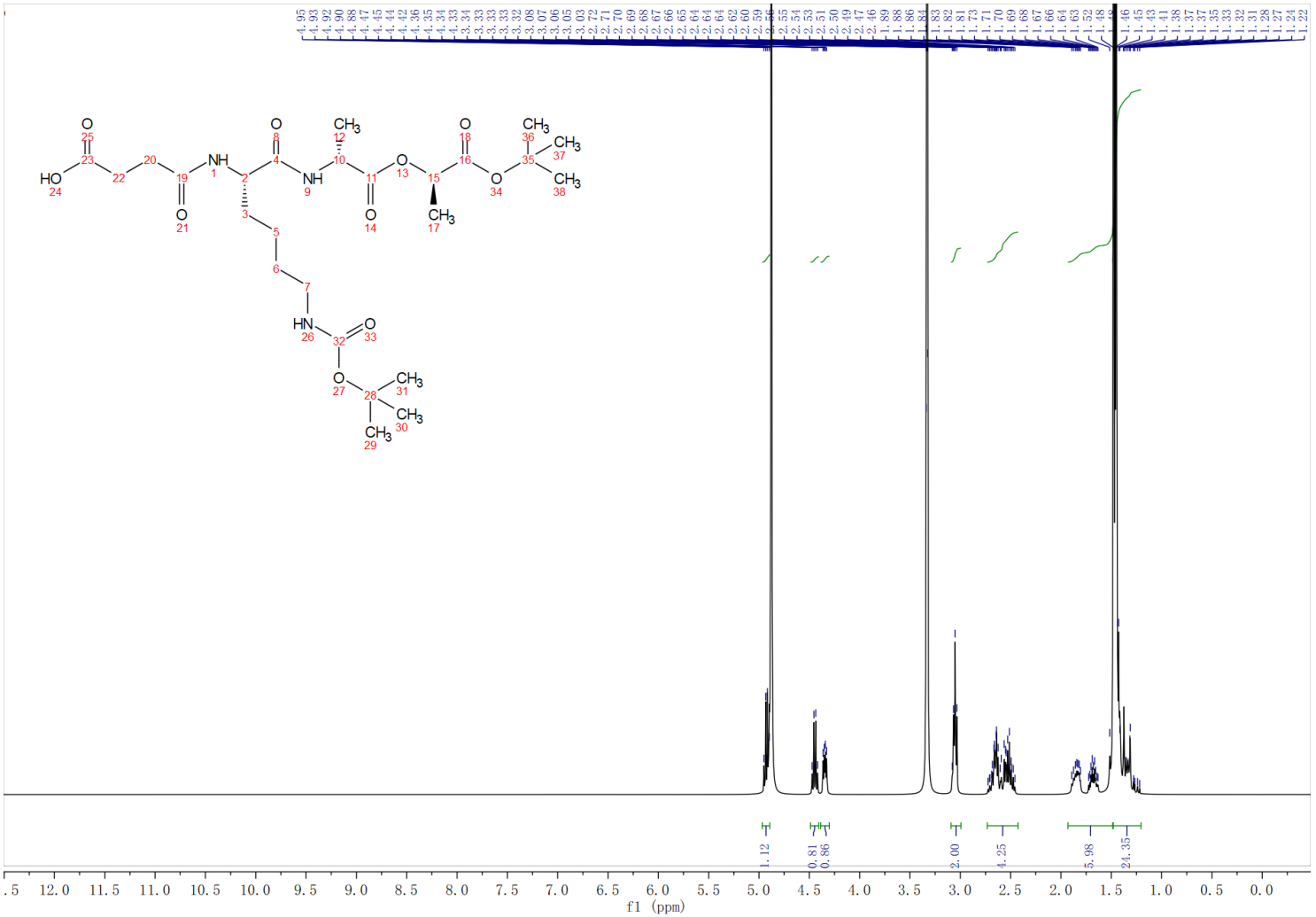

Figure A.17. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **20.**

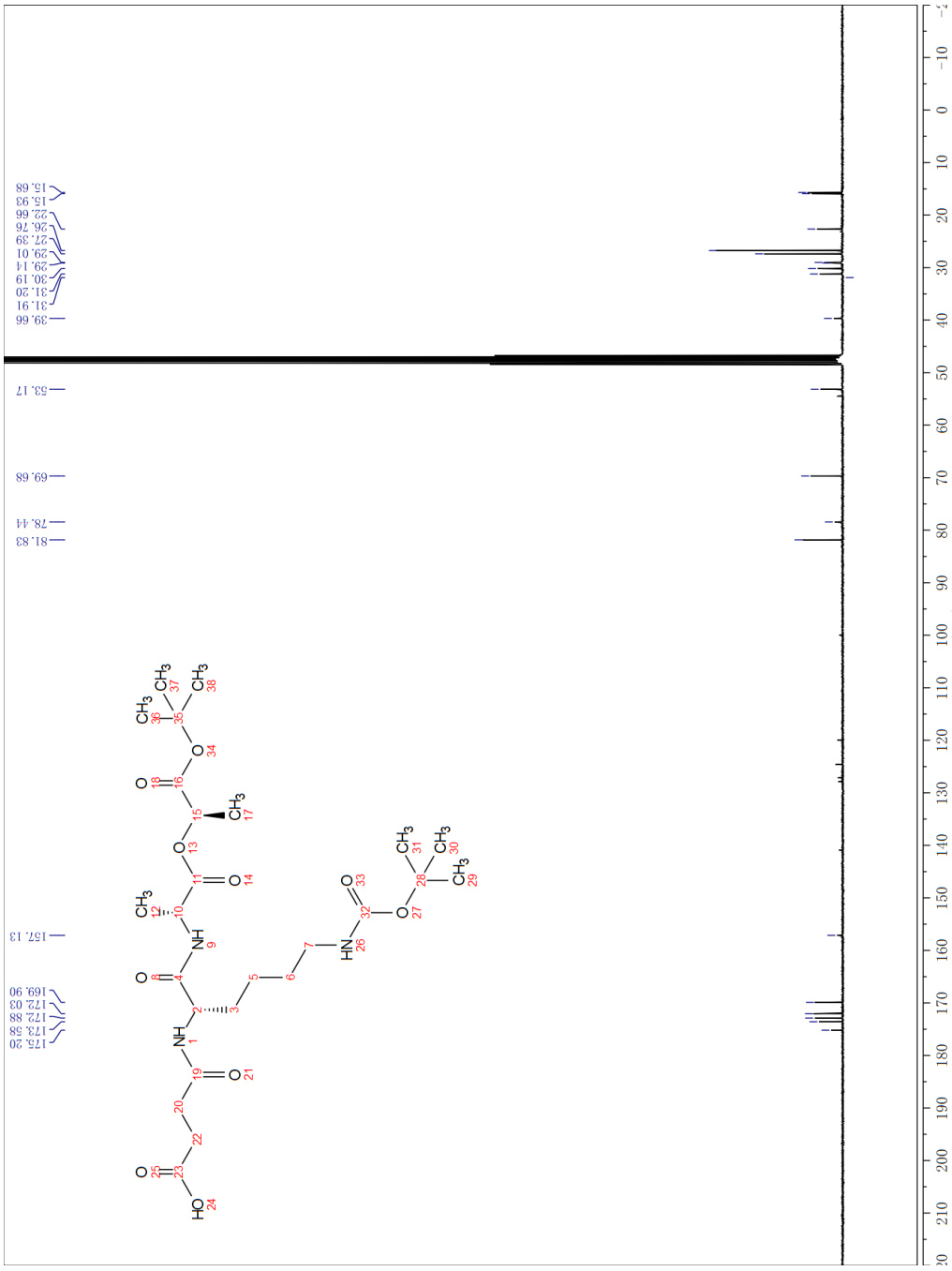

Figure A.18. ^{13}C NMR (101 MHz, methanol-d4) spectrum of compound **20**

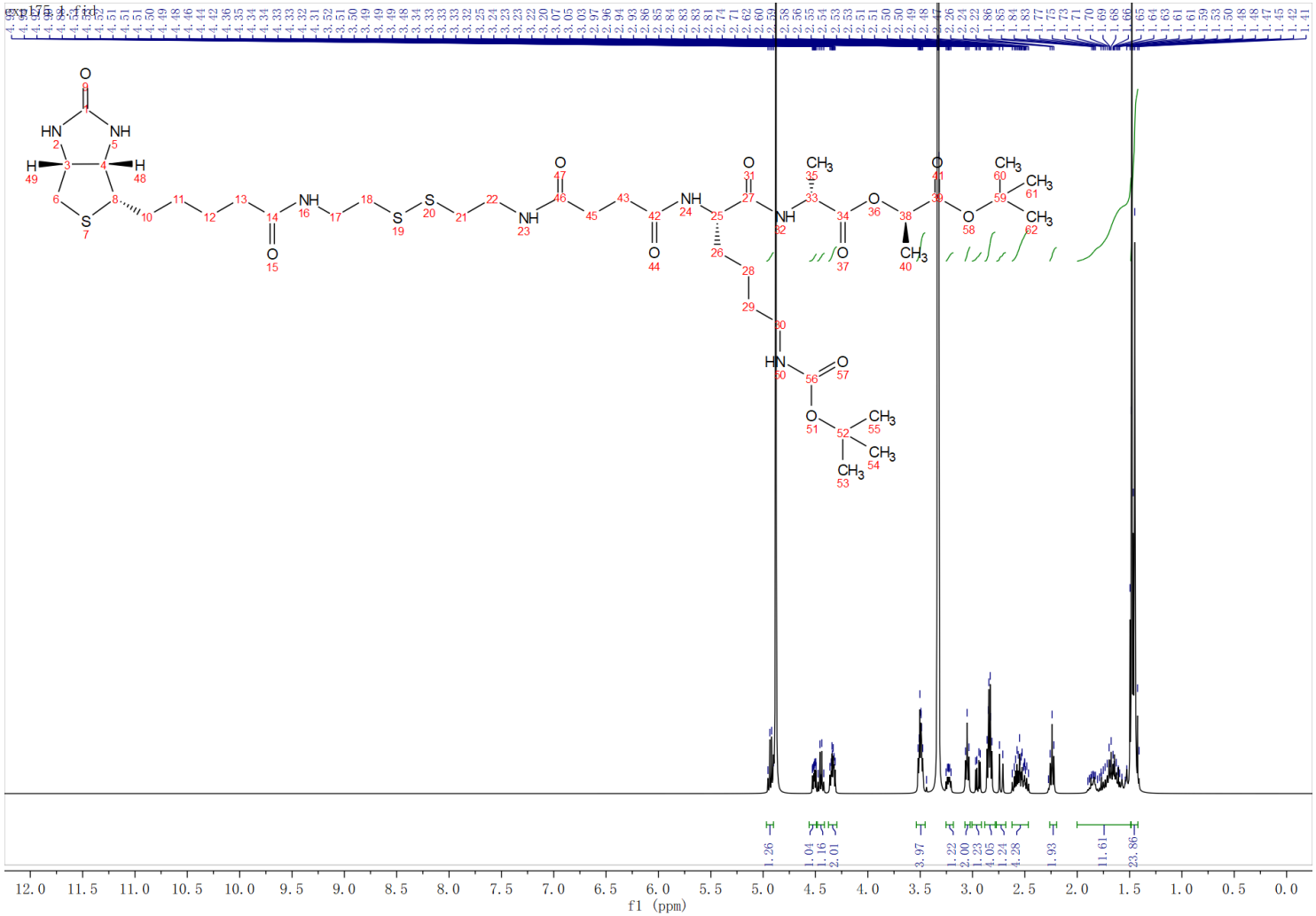

Figure A.19. ^{1}H NMR (400 MHz, methanol-d4) spectrum of compound **25.**

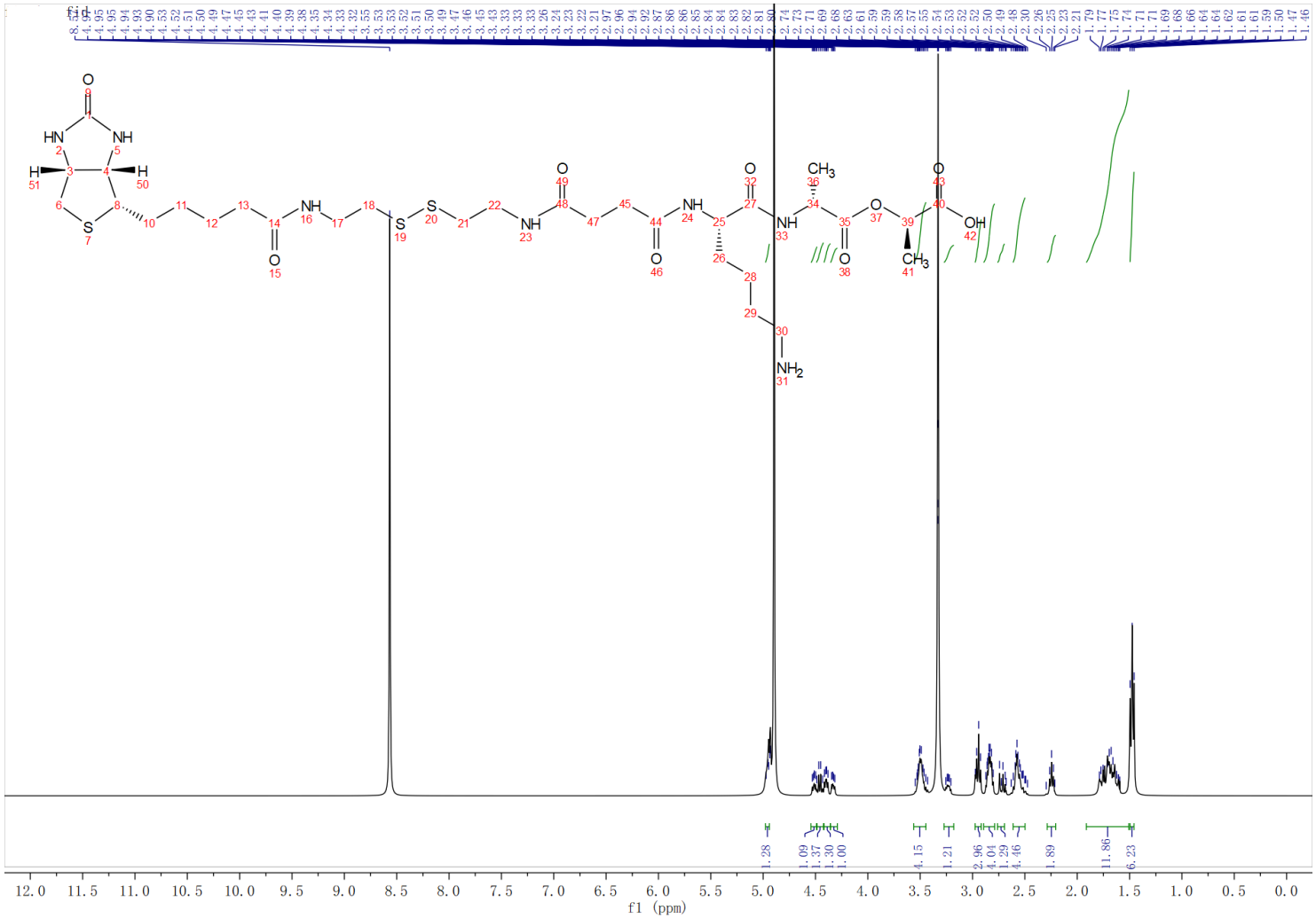

Figure A.20. ^{1}H NMR (400 MHz, methanol-d4) spectrum of **Target 3.**

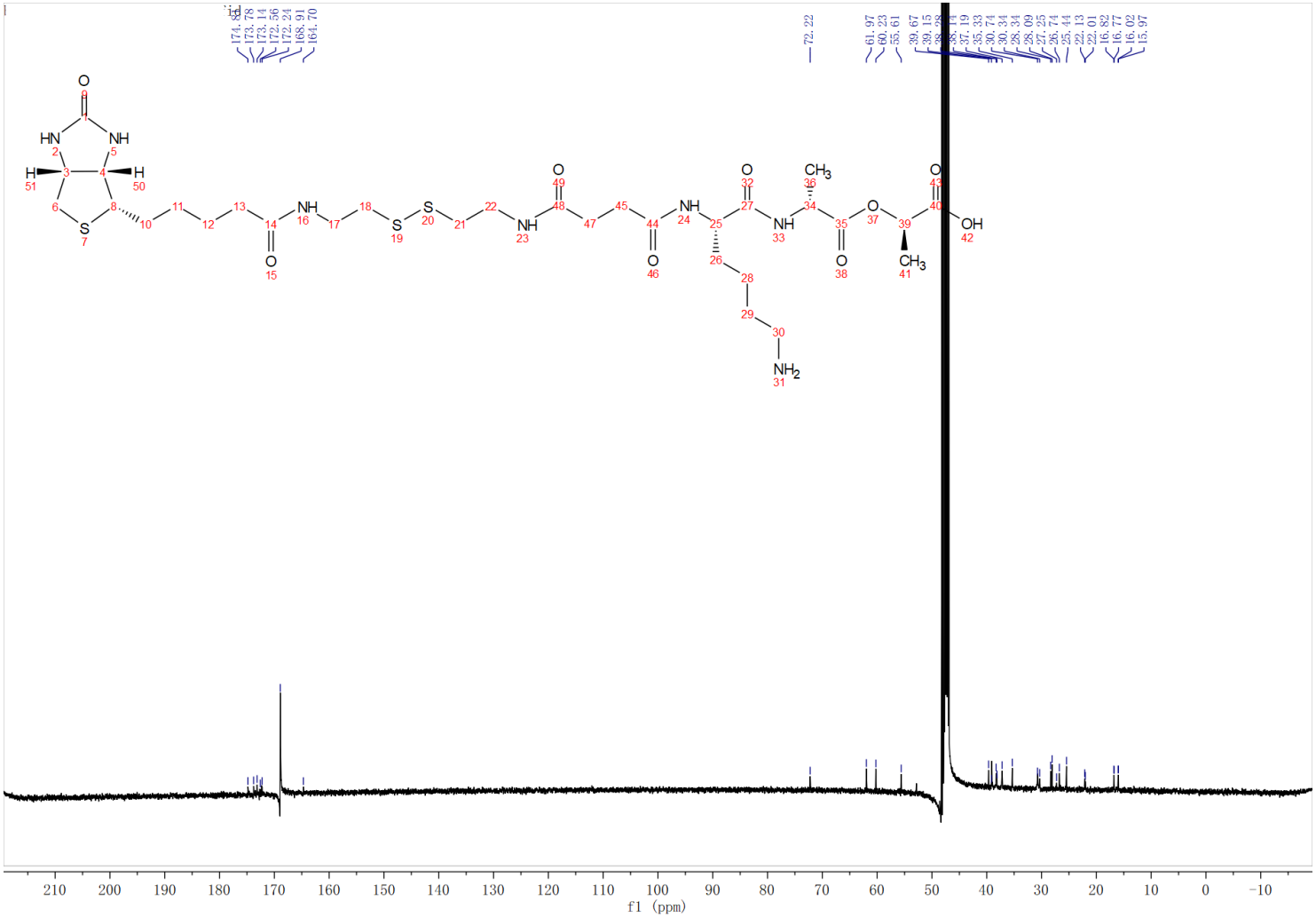

Figure A.21. ^{13}C NMR (101 MHz, methanol-d4) spectrum of **Target 3.**