ABSTRACT

Synthesis of Modulators of TLR2, TLR4, and TLR7 Functionalized for Conjugation to Biomolecules

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A wide range of pathogen-associated molecules including acylated lipopeptides, lipopolysaccharides (LPS), and single-stranded viral RNAs initiate innate immune responses via binding to members of a protein family called Toll-like receptors (TLRs). Toll-like receptors recognize general molecular patterns associated with various pathogens. Recognition of pathogen-associated molecular patterns (PAMPs) by TLRs results in specific signaling pathways which initiate an immune defense against the pathogen. Herein is described the synthesis of small-molecule modulators that can activate or inhibit Toll-like receptors in order to modulate immune responses. In order to avoid systemic effects, our compounds are designed to allow their conjugation to biomolecules thus allowing targeted delivery.

A variety of compounds have been designed and characterized as agonists of TLR7, an endosomal receptor recognizing viral ligands. Among these compounds are imidazoquinolines and adenine derivatives. A significant part of the synthetic work presented herein is focused on the synthesis of adenine derivatives which can be developed

as vaccine adjuvants. In the first part of the work, a concise and efficient synthesis of adenine derivatives, several of which were functionalized for antibody conjugation, is presented. The second part of this project was directed towards the chemical modification of a previously studied TLR4 antagonist - TAK-242. The anti-inflammatory potential of this compound inspired us to explore its potential for the control of the blood-mediated inflammatory response which occurs subsequent to islet transplantation. We describe our efforts to synthesize TAK-242 and attach it to a β-eliminative bifunctional linker, which is expected to facilitate a slow release of active drug after transplantation. Finally, an analog of the TLR2 agonistic lipopeptide Pam3CSK4 was also synthesized for bioconjugation.

Synthesis of Modulators of TLR2, TLR4, and TLR7 Functionalized for Conjugation to Biomolecules

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF ABBREVIATIONS

Boc – tert-Butyloxycarbonyl

CD – Cluster of differentiation

CDI – 1,1'-Carbonyldiimidazole

COMU – (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate

COSY – Correlation spectroscopy

DBCO – Dibenzocyclooctyne

DCC – Dicyclohexylcarbodiimide

DCM – Dichloromethane

DC-SIGN – Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DEC-205 – Dendritic and epithelial cells, 205 kDa

DEPT-135 – Distortionless enhancement by polarization transfer - 135

DIC – *N*,*N*'-Diisopropylcarbodiimide

DIPEA – *N*,*N*-diisopropylethylamine

DMF – Dimethylformamide

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

EC_{50} – Half maximal effective concentration

EI – Electron impact

+ESI – Positive electrospray ionization

-ESI – Negative electrospray ionization

EtOAc – Ethyl acetate

F_{ab} – Fragment antigen binding

F_c – Fragment crystallizable

GC-MS – Gas Chromatography – Mass spectrometry

GPI – Glycophosphatidylinositol

HEK293 – Human embryonic kidney 293

HIV – Human immunodeficiency virus

HRMS – High resolution mass spectrometry

HSQC – Heteronuclear single quantum correlation

IFN – Interferon

IL – Interleukin

IV – Intravenous

LC-MS – Liquid Chromatography – Mass spectrometry

MALP-2 – Macrophage-activating lipopeptide-2

MAPK – mitogen-activated protein kinase

μW – microwave

MyD88 – myeloid differentiation primary response gene 88

NF-κB – Nuclear factor kappa B

NK cell – Natural killer cell

NMR –Nuclear magnetic resonance

PBMC – Peripheral blood mononuclear cell

pDC – Plasmacytoid dendritic cells

PEG – Polyetheylene glycol

Rf – Retention factor

RNA –Ribonucleic acid

RSV – Respiratory syncytial virus

RT – Retention time; room temperature

SAR – Structure-activity relationship

SEAP – Secreted embryonic alkaline phosphatase

SMCC – (Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate)

t-Bu – tert-Butyl

TEA – Triethylamine

THF – Tetrahydrofuran

TFA – Trifluoroacetic acid

TIR – Toll-interleukin-1 receptor

TIS – triisopropylsilane

TLC – thin-layer chromatography

TNF-α – Tumor necrosis factor alpha

TRAM – TRIF-related adaptor molecule

TRIF – TIR domain-containing adapter inducing interferon-β

ACKNOWLEDGMENTS

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To my family: my dad and mom, Ayo and Tosin, thank you for really making this possible and for your support along the way.

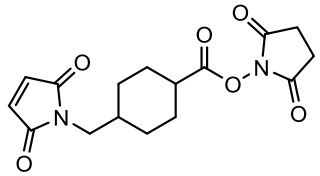
CHAPTER ONE

Introduction

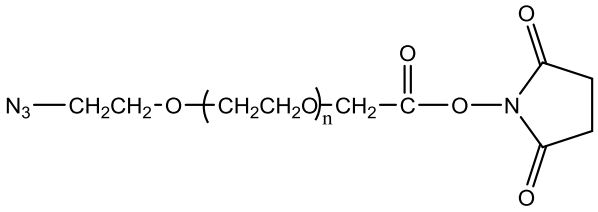
This dissertation reports studies on the synthesis of bioactive small molecules (specifically, Toll-like receptor modulators) that are functionalized for conjugation to biomolecules. The compounds are designed to be useful in vaccine development and in the transplant of islet cells.

Bioconjugation

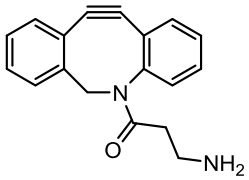
Bioconjugation is the chemical strategy of linking a biomolecule with other molecules to form a complex or a conjugate.[1] Often, bioconjugates are constructed from monoclonal antibodies (mAb) which are proteins that can exhibit high specificity and avidity for binding to a wide range of targets.[2] Bioconjugation is usually carried out using a synthetic linker which can possess different or the same functional groups at two ends.[3] Crosslinkers with two different reactive functional groups are known as hereobifunctional linkers, while those in which the reactive functional groups are the same are homobifunctional. Heterobifunctional crosslinkers possess the advantage that they can be used to couple two different functional targets (examples are shown in Figure 1). To produce a bioconjugate, one end of the linker is reacted with the biomolecule and the other end is reacted with a second molecule. Conjugation strategies are often described in terms of the nature of bonding with the biomolecule (more commonly), with the second molecule, or the nature of both.



succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)
Thiol and amine reactive



NHS-PEG-azide
Amine and nitrile or alkyne reactive



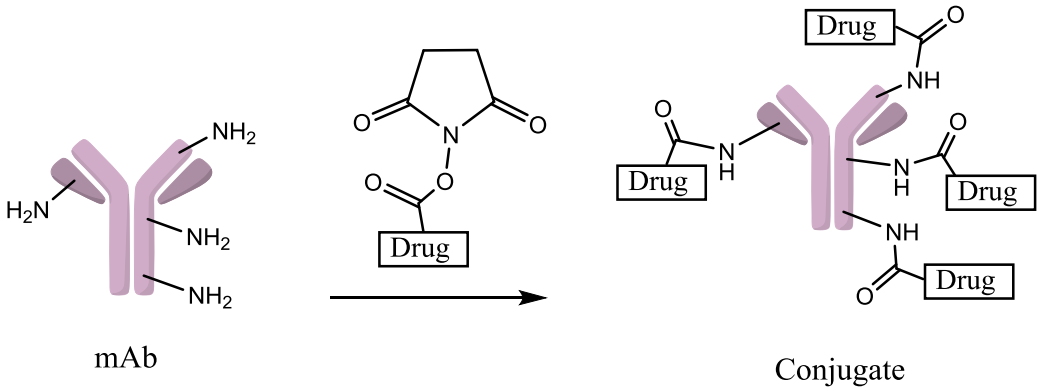
Dibenzocyclooctyne-amine (DBCO-amine)
Carbonyl or carboxylic acid and azide reactive

Figure 1. Heterobifunctional Linkers

NHS Ester and Maleimide Conjugations

Two of the classic bioconjugation methods that were used to make the first generation of antibody-drug conjugates involve acylation of lysine residues on an antibody with activated esters (e.g. *N*-hydroxysuccininimide (NHS) esters) and alkylation of cysteine thiols on an antibody with maleimides.[4] Figure 2 shows how lysine residues on an antibody would react with an NHS functionalized drug. A human IgG antibody typically contains about 100 lysine residues whose side chains terminate with primary amines. Mass spectrometry analysis has shown that potentially 40 of these residues can be modified (acylated).[2] Typically, conjugation reactions result in zero to eight drug molecules conjugated to an antibody. This implies that both unconjugated and overloaded antibodies can be produced in a heterogeneous fashion - over a million different antibody-drug conjugate species can be produced by this approach. This is referred to as a nonspecific conjugation. Problems associated with this nonspecific conjugation include

the competition of unconjugated antibodies with drug loaded ones for antigen binding, conjugation at the antigen binding region (Fab) of the antibody (reducing activity), conjugation at the Fc domain (which may affect antibody effector functions), antibody aggregation and increased toxicity and decreased stability (half-life) of conjugates.[2,5]



mAb Conjugate

Figure 2. Schematic representation of an NHS ester bioconjugation strategy

Cysteine conjugation relies on the reduction of the four interchain disulfide bonds on the antibody, which leads to eight thiol groups that can be available for conjugation (Figure 3). The free thiols are reacted with maleimides or other electrophiles on other molecules.[2] The formation of regioisomeric mixtures of conjugates still result in over a hundred different drug-loaded species. The cysteine-maleimide conjugation strategy therefore results in a smaller number of heterogeneous species than the lysine-NHS strategy. The two FDA approved antibody-drug conjugates Adcetris (lysine-NHS) and Kadcyla (cysteine-maleimide) were produced using the conjugation strategies described above.[4,6]

Site-Specific Conjugations

A number of site-specific reactions have been developed to achieve homogeneity and to limit drug-to-antibody ratio. The earlier mentioned cysteine-maleimide strategy can be used to produce a homogeneous antibody-drug conjugate in a site-specific reaction if all the interchain disulfide bonds are reduced and coupled with the drug molecules (Figure 3).[2,7] This approach was used by Doronina et al. to produce a conjugate of anti-CD30 antibody with monomethyl auristatin E.[8,9]

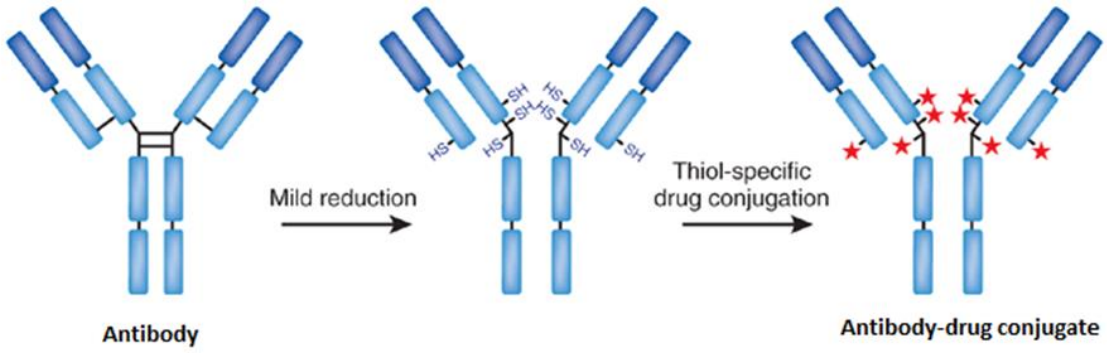


Figure 3. Site-specific conjugation using sulfhydryls after reduction of the antibody disulfide chains[7]

Other site specific conjugations that have been reported include selenocysteine-maleimide reaction,[10] oxime ligation and glycoconjugation,[11-13] microbial transglutaminase conjugation,[14] hydrazino-*iso*-Pictet-Spengler reaction,[15] and trapped-Knoevenagel ligation.[16] Another recently discovered but less utilized technique involves conjugating indole-based molecules to the conserved nucleotide binding site (NBS) at the Y/F42 residue (tyrosine or phenylalanine) of the variable light chain of an antibody, activated by UV energy (Figure 4).[5,18] A number of the site-specific conjugation techniques mentioned above require an initial chemical or enzymatic modification of the biomolecule.

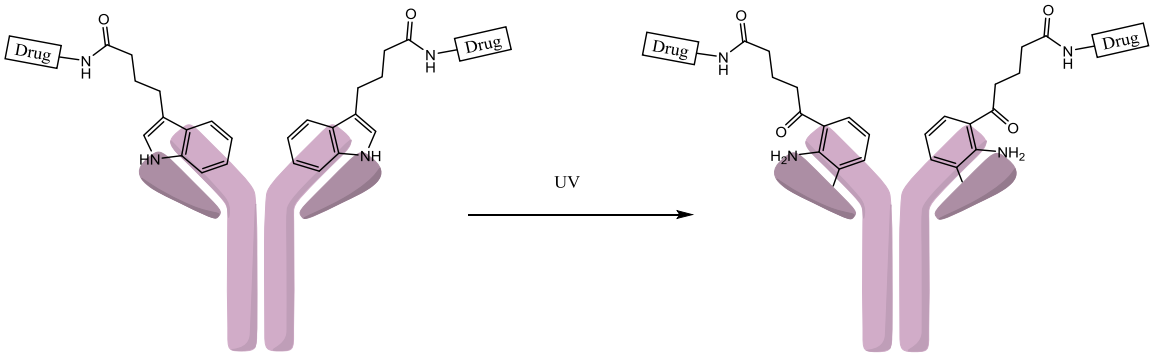


Figure 4. Photochemical conjugation of an indole-based Compound to an antibody at the conserved nucleotide binding site[5]

Click Chemistry Conjugation

A reaction qualifies to be described by the term "click chemistry" if it is high yielding, wide in scope, stereospecific, biorthogonal (functional groups of the reagents

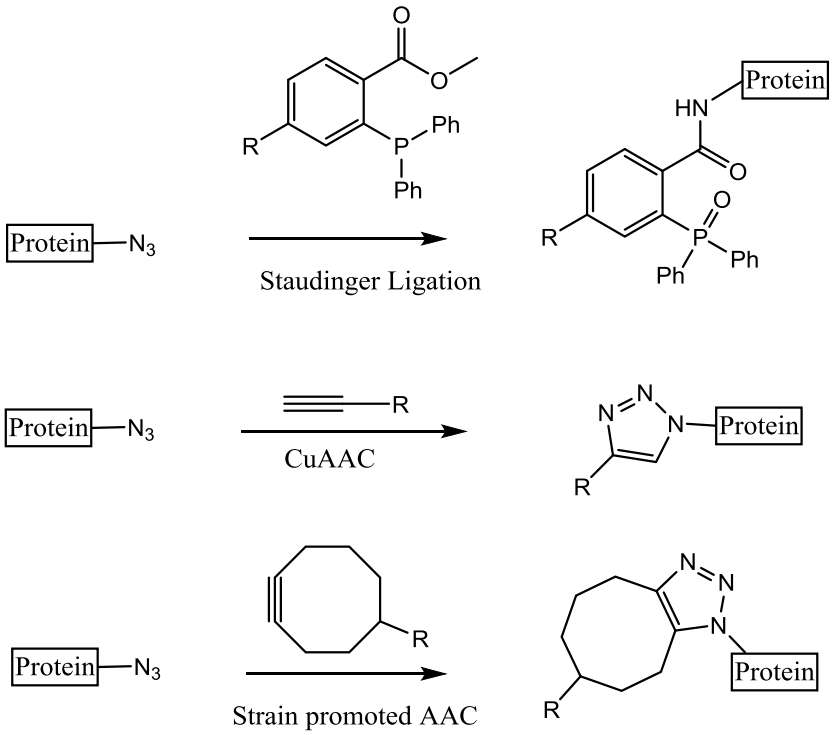


Figure 5. Examples of click chemistry reactions. R = Bioactive small molecule or drug.

are inert to those of biomolecules), involves non-toxic byproducts that can be removed without column chromatography, and can be carried out in benign solvents such as water.[19,20] The term click chemistry was introduced in 2001 by Sharpless et al. to describe reactions that satisfy these conditions.[19] By implication, these reactions can be used for bioconjugations and the products are stable under physiological conditions. Examples of click chemistry reactions that can be used to conjugate proteins (antibodies) and drugs are copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC), strain-promoted azide-alkyne cycloaddition, and azide-phosphine (Staudinger) ligations (Figure 5).[20]

Cleavable Linkers

The connector between the components of a bioconjugate is often called a linker. For example, an antibody drug conjugate may be considered to consist of an antibody, a drug and a synthetic linker (Figure 6). Often, the drug is attached to a suitably functionalized linker before it is conjugated to the antibody under mild (aqueous) conditions.[4] Accordingly, it becomes important for the linker to exhibit orthogonal reactivity, the ability to solubilize the drug (which is often hydrophobic), and some degree of stability during circulation (so the drug is not prematurely released in vivo).

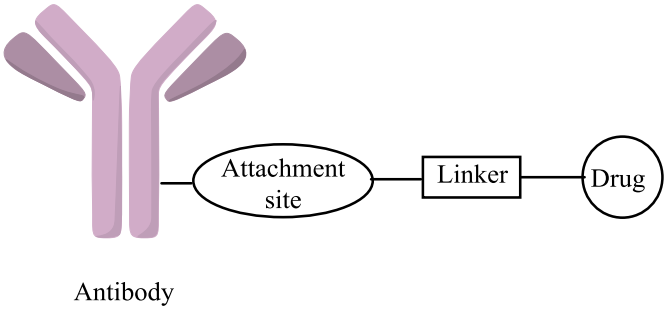


Figure 6. Components of a Conjugate

In certain cases, the need to minimize off-target effects of the conjugate, or other considerations including targeted drug release, has led to the use of conjugates which have cleavable linkers.[21] Cleavable linkers are often required to allow the release of a drug intracellularly. Conditions to be satisfied by cleavable linkers include cleavage under mild (physiological) conditions, and the ease of elimination of any by-products. One of the applications of cleavable linkers is in the development of pro-drugs. Prodrugs are designed to have little or no pharmacological activity while undergoing biotransformation (that is, until cleavage) to a therapeutically active metabolite. In other words, they are only active after a process such as enzymatic hydrolysis, specific to the diseased site in vivo, which facilitates the release of the drug. A number of cleavable linkers are known and have been used to make conjugates and several examples are shown in Figure 7. A disulfide group in a conjugate can be reduced by glutathione or by enzymes. Esters are cleaved hydrolytically

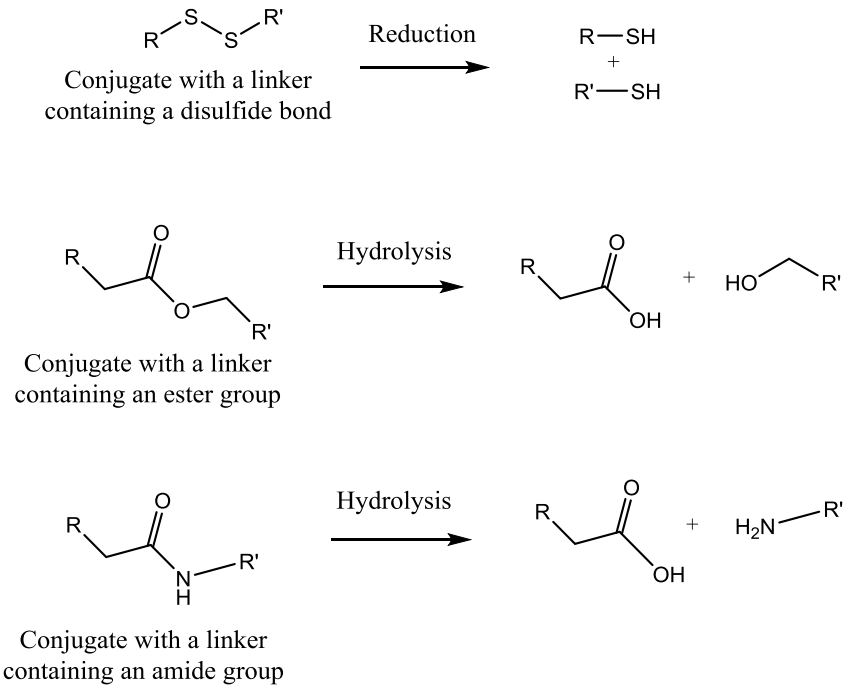
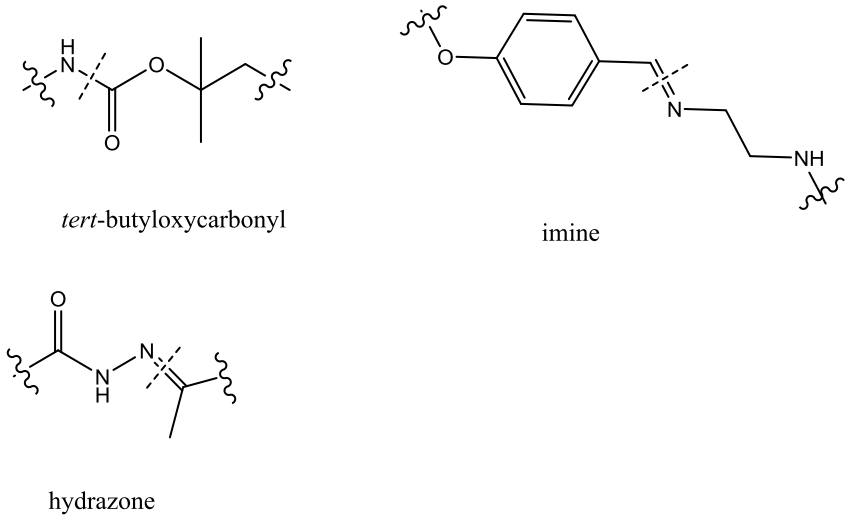


Figure 7. Different functionalities present in cleavable linkers and the cleaving reagents. R and R′ groups represent the molecules conjugated by the linker.

by esterases. Amide bonds can also be cleaved by proteases.[3] In addition, some linkers can be cleaved under acidic pH. Examples of these include linkers with the *tert*-butyloxycarbonyl (Boc), hydrazone, and imine functionalities (Figure 8).[21]



tert-butyloxycarbonyl

imine

hydrazone

Figure 8. Acid Labile Linkers; (Dashed line = Sites of cleavage).

β-Eliminative Cleavable Sulfone Linker

To improve pharmacokinetics, cleavable linkers which allow a controlled release of active drugs from the linker have been employed.[21] With the cleavable linker conjugation strategies so far described, the rate of drug release may not be well-defined. For example, variability in serum esterases often leads to unpredictable cleavage rates and, therefore, variable drug exposure.[22] A β-eliminative bifunctional linker that allows a tunable rate of the cleavage was described by Santi et al. (Figure 9).[22,23] In contrast to the previously described cleavable linkers, which are cleaved under acidic conditions or by enzymatic (or chemical) hydrolysis, this linker is cleaved under mildly basic and non-enzymatic conditions. The rate of release of drug is dependent on the acidity of the

indicated hydrogen in Figure 9. Basic hydrolysis is triggered by the removal this hydrogen, leading to β-elimination and release of the drug.

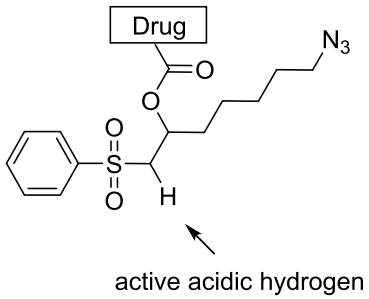
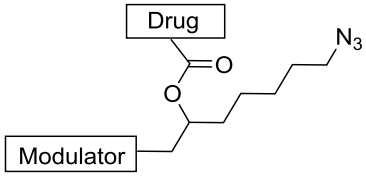


Figure 9. β-Eliminative linker with the phenyl sulfone modulator



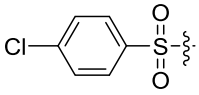
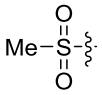
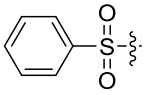
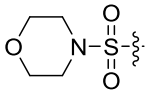
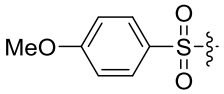
Modulators	Release half-lives in days	Modulators	Release half-lives in days
	1.5		19
	3		31
	6.7		100

Figure 10. β-Eliminative linker in vitro half-lives for conjugates with different modulators (pH 7.4 – 9.5, calculated to pH 7.4, 37ºC)[22]

Several other linkers have been synthesized with the phenyl sulfone group replaced with other groups (modulators).[22] This allows a variation of the rate of drug release in conjugates synthesized using different linkers (Figure 10). Physiological conditions have

been explored and in vitro cleavage rates correlated well with in vivo cleavage rates both in rats and mice.

Vaccine Development

Vaccines are substances that can mimic a disease-causing agent, introduced into the host to stimulate protective immunity against the disease.[24] Vaccination have successfully helped in the eradication of smallpox and the reduction of polio and measles. Traditionally, vaccines were produced from either live attenuated or heat-inactivated organisms. With the evolvement of pathogenic viral strains (that is, influenza, HIV, human papillomavirus etc.), efforts have been directed towards the development of vaccines that are based on recombinant proteins or subunit vaccines that can be prepared to stimulate an immune response against specific microbial antigens.[24,25] However, these proteins can be less immunogenic than traditional vaccines that are based on the whole organism. Therefore, co-administration with an adjuvant such as Toll-like receptor agonists is often required.[25]

Toll-Like Receptors as Part the Immune System

Immunity refers to the mechanisms used by the body as a protection against environmental agents that are foreign to the body. The English word arose from the Latin term *immunis*, meaning "exempt."[26] Immunity may be either innate or acquired. Innate immunity is conferred by a diverse array of cellular and subcellular components with which an individual is born. These components are always present and available at a short notice to protect the individual from challenges by foreign invaders. Elements of the innate immune system include pattern recognition molecules (innate receptors), such as Toll-like receptors, which can bind to various microorganisms.[27] Acquired immunity is present only

in vertebrates. Following contact with an antigen, antigen-presenting cells and lymphocytes (B and T cells) bearing antigen-specific receptors are activated, leading to their proliferation.[27,28] The program of events that follows leads to humoral or cell-mediated responses. These events take days to weeks to unfold. In this manner, the individual acquires a durable immunity to withstand and resist a subsequent attack by the same foreign agent.[27] Some properties highlighting key differences between innate and adaptive immune system are presented in Table 1.

Table 1. Features of the Innate and Adaptive Immune System[27]

Property	Innate	Adaptive
Characteristics	*Antigen nonspecific* *Rapid response* *No memory*	*Antigen specific* *Slow response (days)* *Memory*
Immune components	*Natural barriers (e.g. skin, mucous membranes), phagocytes and Natural Killer cells, soluble mediators (e.g. complement), pattern recognition molecules*	*Lymphocytes, antigen recognition molecules (B- and T-cell receptors), secreted molecules (e.g. antibody)*

Toll-Like Receptors

Microbes display highly conserved molecular patterns which are recognized by pattern recognition receptors such as Toll-like receptors (TLR) in the host; this recognition in turn helps the host in distinguishing self from non-self (foreign) molecules.[29] The Toll gene was originally identified for its role in dorsoventral patterning in the fruit fly *Drosophila melanogaster* embryos.[30] Later studies showed that Toll genes encode proteins that play a critical role in the fly's innate immune response to fungal infection. The human

Table 2. Known Toll-like Receptors, their Ligands and Locations[34-36]

Receptor	Ligands	Microorganisms recognized	Cell carrying receptor	Cellular location of receptor	Synthetic Ligands
TLR1:TLR2	Triacyl lipopeptides, lipoarabinomannan	Bacteria	DCs, monocytes, eosinophils, basophils, mast cells	Plasma membrane	Lipopeptides e.g. Pam_3CSK_4 (triacylated), zwitterionic polysaccharides (TLR2)
	GPI	Parasites e.g. trypanosomes (*T. cruzi*)			
TLR2:TLR6	Lipoteichoic acid, peptidoglycan, diacyl lipopeptides	Gram-positive bacteria	DCs, monocytes, eosinophils, basophils, mast cells	Plasma membrane	Lipopeptides e.g. MALP-2 (diacylated)
	Zymosan	Yeasts (fungi)			
TLR3	Double-stranded viral RNA	Viruses e.g. West Nile virus	NK cells	Endosomes	Poly (I:C)
TLR4:TLR4	Lipopolysaccharide, human RSV fusion protein, pertussis toxin, Taxol (from *Taxus brevifolia*)	Gram-negative bacteria	DCs, macrophages, eosinophils, mast cells	Plasma membrane	E6020, E5531, E5564
TLR5	Flagellin	Motile bacteria having a flagellum	Intestinal epithelium	Plasma membrane	
TLR7	Single-stranded viral RNA	Viruses e.g. HIV	pDCs, eosinophils, NK cells, B cells	Endosomes	Purines; nucleosides e.g. loxoribine (a guanosine analog); bropirimine (pyrimidinone); imidazoquinolin-es e.g. imiquimod, R848, CL097, gardiquimod
TLR8	Single-stranded viral RNA	Viruses e.g. influenza	NK cells	Endosomes	Imidazoquinolin-es
TLR9	Unmethylated CpG-rich DNA	Bacteria, viruses e.g. herpes virus	pDCs, eosinophils, basophils, B cells	Endosomes	
	Hemozoin	Malaria parasite			
TLR10, TLR 10:1 & TLR10:2			pDCs, eosinophils, basophils, B cells		
TLR11	*T. gondii* profilin, apicomplexan profilins	Uropathogenic bacteria			

homolog of the *Drosophila* Toll protein, now known as TLR4, was cloned in 1997. TLR4 was later shown to be involved in the recognition of lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria.[31,32] LPS is only one example of the many pathogen-associated molecular patterns (PAMPS) recognized by Toll-like receptors. Thirteen Toll-like receptors have been identified so far: TLR1 to 9 are conserved in both humans and mice, while TLR10 is only functional in humans, and TLR11 to 13 are found in mice, having been lost from the human genome.[33] Table 2 shows some of the TLRs, their natural ligands, location and agonists.[34-36]

TLR3, 7, 8, 9 are localized in intracellular organelles such as the endosomes, lysosomes, and the endoplasmic reticulum, while TLR1, 2, 4, 5, 6, 10 and 11 are localized on the plasma membrane (Figure 11). As such, the endosomal TLRs generally recognize

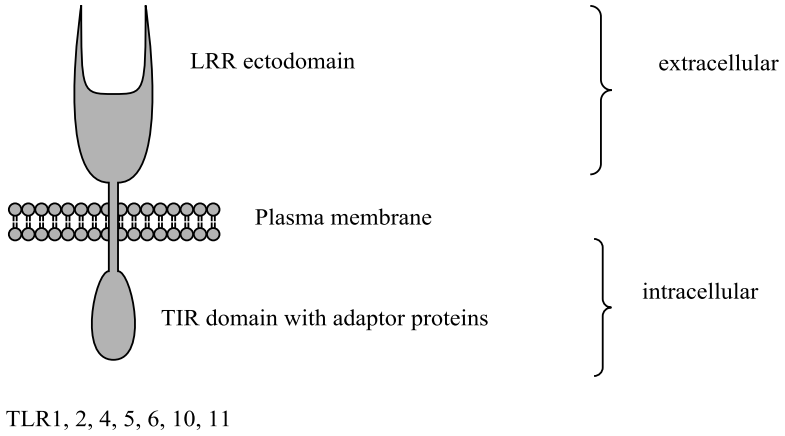


Figure 11. Plasma-membrane TLRs[37]

nucleic acid (viral mostly) components, while the plasma membrane TLRs recognize microbial (bacterial, fungal) components such as lipids and lipoproteins.[33,37] For example, TLR7 (an endosomal TLR) is expressed in plasmacytoid dendritic cells where it can stimulate response to RNA viruses.[33] Plasma membrane TLRs consist of extracellular and

intracellular domains. The extracellular (horse-shoe shaped) domain interacts with PAMPS while the intracellular (toll-interleukin-1 receptor, TIR) tail is the signaling region.[37] The extracellular domain (ectodomain) is characterized by the presence of a leucine-rich repeat (LRR) motif which is primarily used for mediating ligand recognition.

TLR7 Signaling and IFN Inducers

Activation of TLRs leads to activation and maturation of dendritic cells (DCs). TLR-mediated DC activation results in enhanced phagocytosis, upregulation of the major histocompatibility complex (MHC) and costimulatory molecules, secretion of cytokines (e.g. type I IFNs) and antigen presentation to lymphocytes.[33,34,38-43] TLR7 signaling follows a MyD88-dependent pathway. The TIR domain of TLR7 interacts with MyD88, a signal adaptor protein, which also has a TIR domain. Inflammatory responses are regulated by the activation of NF-κB (a nuclear transcription factor) and AP-1 (activator protein-1).[39,40] Cellular responses resulting from this TLR7 signaling include the production of type I IFNs (IFN-α) and proinflammatory cytokines (IL-6 IL-12, TNF-α).[38-40,42,43] It has been established that TLR7 recognizes single stranded viral RNA (derived from the human immunodeficiency type I virus (HIV-1), vesicular stomatitis virus (VSV), and influenza virus), certain siRNAs (small interfering RNA), and low molecular weight synthetic compounds.[42-44]

Imiquimod and Resiquimod (imidazoquinolines, Figure 12) are examples of small molecule TLR7 and TLR8 agonists that have been developed for treating skin and allergic disorders.[45,46] The clinical use of these compounds also results in some adverse effects including vomiting and fever.[47,48] Structures of other TLR7 and TLR8 agonists are presented in Figure 12.[41,45]

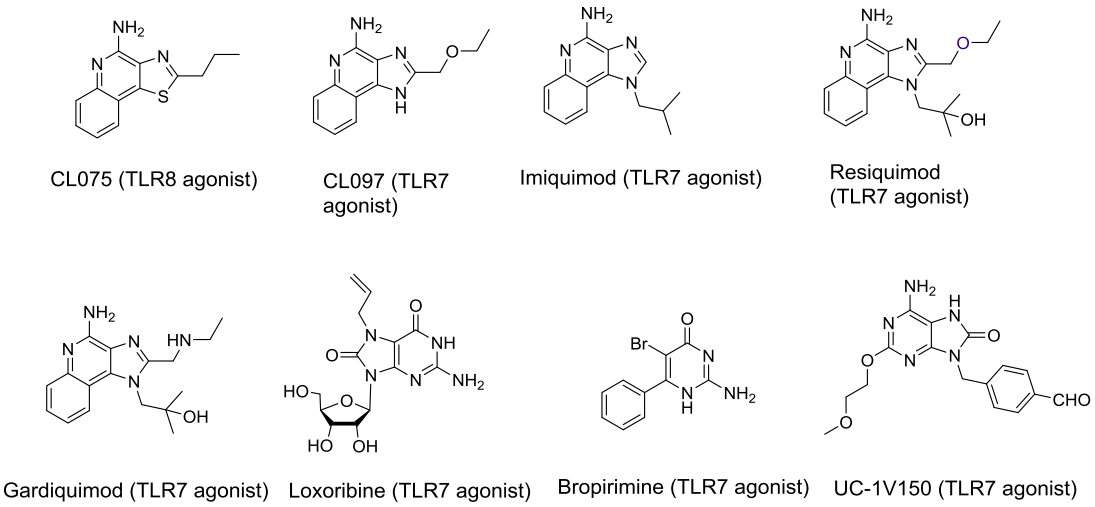


Figure 12. Structures of Known TLR7 and TLR8 Agonists

Previous SAR Studies on Adenine-Based TLR7 Agonists

Several adenine derivatives with structural modifications at the 8-, 9-, and 2-positions were screened by Hirota et al. for their IFN-inducing activities.[47,49] In vitro and in vivo studies in mice spleen cells and mice plasma samples resulted in the selection of 8-hydroxyl-9-benzyl derivatives (the 8-mercapto also showed moderate activity) when compared to the lead compound (R = H, Figure 13).

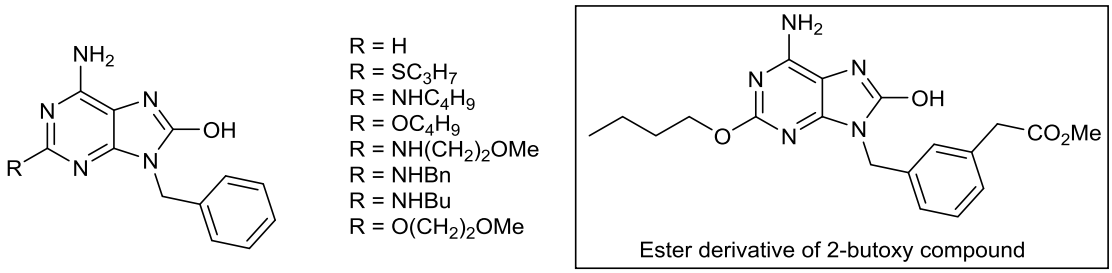


Figure 13. 2-Substituted, 6-Amino, 8-Hydroxyadenine Derivatives

For the 2-position, the authors found that the 2-propylthio, 2-butylamino, and 2-butoxyl derivatives (R = SC_3H_7, NHC_4H_9, OC_4H_9 respectively, Figure 13) had excellent

IFN-inducing activities. These compounds showed greater than 100-fold improvement of in vivo activity over the lead compound.

With the establishment of the importance of the amino group at the 6-position,[49] the 2-methoxyethylamino adenine derivative (R = $NH(CH_2)_2OMe$, Figure 13) was found to possess good IFN-inducing activity (30-fold greater potency in vivo than Imiquimod) and good oral bioavailability when compared with the equally potent 2-benzylamino (R = NHBn) and 2-butylamino (R = NHBu) derivatives.[50] For the 2-alkoxy and 2-mercapto analogs, the 2-propylthio-, 2-butoxy- and 2-methoxyethyloxy derivatives (R = SC_3H_7, OC_4H_9, $O(CH_2)_2OMe$ respectively) were satisfactory, showing about 100-fold greater potency in vivo than Imiquimod.[51] In addition, the 2-methoxyethyloxy compound (R = $O(CH_2)_2OMe$) showed preferable oral bioavailabilty.

A search for an IFN inducer with reduced cytokine induction and its associated side effects led to the synthesis of the ester derivative of the 2-butoxy compound as the best candidate (Figure 13).[46] The metabolism of this ester to the much less active carboxylic acid was expected to minimize side effects such as flu-like symptoms which results from systemic cytokine induction.

In their study of 9-substituted 2-butylaminoadenine derivatives, Isobe et al. found that the *para*-substituted 9-benzyl compounds were 10-fold more potent than the unsubstituted 9-benzyl and were preferable in activity to the *ortho*- and *meta*-substituted compounds.[52]

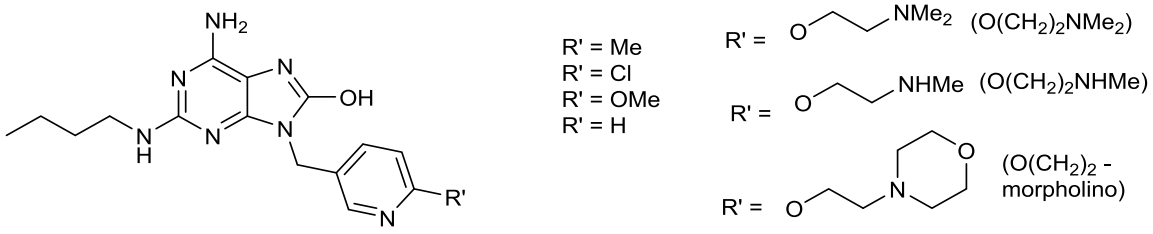


Figure 14. 9-Benzyl and 9-(3-Pyridylmethyl) Adenine Derivatives

Notably, the 6-methyl-3-pyridylmethyl compound (*para*-substituted: R' = Me, Figure 14) had a clear dose-dependent strong IFN induction when orally administered into a monkey model and did not cause emetic activity in ferrets (whereas emesis was observed with the reference compound Resiquimod, Figure 12). Equally strong IFN-inducing were analogs with R' = Cl, R' = OMe, and R' = H (Figure 14). In 2013, the same authors demonstrated that the mechanism of action of these compounds is TLR7 agonism.[53] A reporter gene assay based on HEK293 cells which were transfected with human TLR7 along with the NF-κB SEAP reporter was used to evaluate the in vitro activities of the compounds. The EC_{50} of the 6-methyl-3-pyridylmethyl compound (R' = Me, Figure 14) was determined to be 32 nM. The 6-methoxy analog (R' = OMe, Figure 14) had a better EC_{50} of 28.1 nM. The authors modified their compounds further and identified a dimethylaminoethoxy compound (R' = $O(CH_2)_2NMe_2$) with an EC_{50} of 7.2 nM. Analogs with R' = $O(CH_2)_2NHMe$ and $O(CH_2)_2$–morpholino (Figure 14) had poorer profiles in terms of potency and solubility. Given this observed trend in their studies, the authors concluded that the extra basicity of the NMe_2 (compared to the morpholino) as well as the tertiary amine functionality of NMe_2, as opposed the secondary NHMe, were essential. The dimethylaminoethoxy compound (DSR-6434) has been used in combination with ionizing radiation in the treatment of solid tumor in mice.[54]

8-Aminoadenine compounds have also been studied for their IFN-inducing activity. 2-alkoxy-8-aminoadenines (Figure 15) were evaluated by Jin et al. for IFN-α induction in PBMC from healthy human donors. Notably, the 8-morpholinoethylamino derivative (Figure 15) was the most active (compared to the lead compound with R = $O(CH_2)_2OMe$, Figure 13) with an EC_{50} value in the submicromolar range.[55]

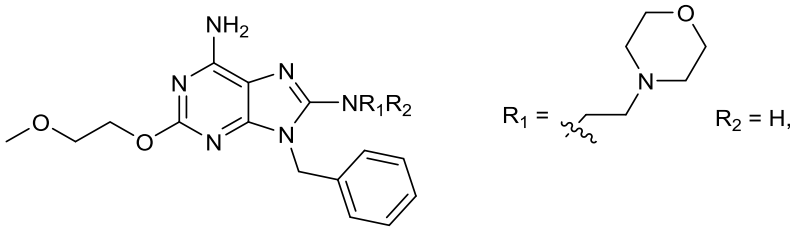


Figure 15. 8-Aminoadenine Compounds

In order to be able to add additional functionality (for conjugation chemistry) to TLR7 agonists, Filippov and his workers synthesized 9-benzyl-8-oxoadenines with an azide group introduced at the 2-position.[56] The azidoethyl- and azidopropyl-substituted compounds retained moderate TLR7 activity (Figure 16).

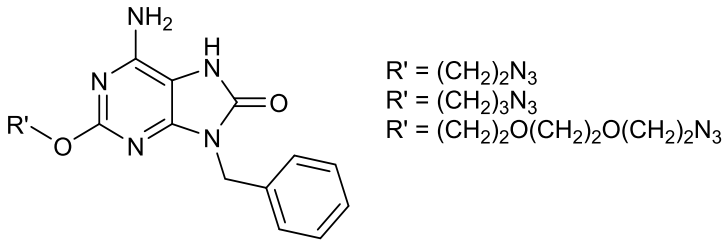


Figure 16. Azide-Bearing 2-Substituted Adenine Compounds

In order to enhance innate immune activation and improve the in vivo pharmacodynamics of their compounds, Carson and his co-workers synthesized phospholipid and phospholipid-polyethylene glycol (PEG) conjugates (A and B,

respectively, Figure 17).[57] Both were synthesized from a benzoic acid starting material. The phospholipid-TLR7 agonist conjugate (A) was estimated to be about 1000-fold more potent than Imiquimod as a cytokine inducer. The hybrid conjugate containing both phospholipids and PEG (B) was less potent, but possessed greater water solubility which

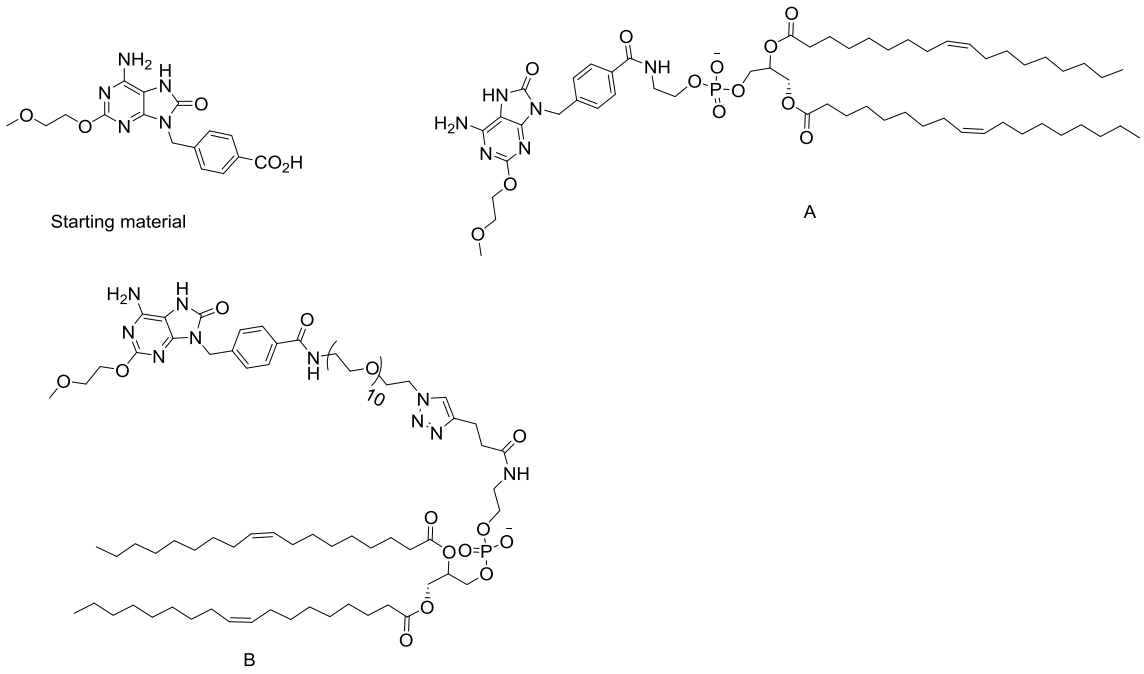


Figure 17. TLR7 Agonist Conjugates with Phospholipids

makes it useful for systemic administration. Recently, the phospholipid-TLR7 agonist conjugate (A) was used as an adjuvant in combination with a substituted pyrimido[5,4-b]indole TLR4 ligand to induce a balanced Th1- and Th2-type response protective against homologous and heterologous influenza viruses.[58]

Further supporting the general structural motif for IFN-induction by TLR7 agonists was the modeling study carried out by Musmuca et al. on a set of published IFN inducing compounds.[59] Using 3-dimensional quantitative structure-activity relationship (3-D QSAR) models, they identified important features including a steric filling region (near

adenine C-2), hydrophobic area (near N-9), an acceptor hydrogen bonding region (near C-6), and a polarized area - possibly with donor hydrogen bonding characteristics (near C-8) (Figure 18).

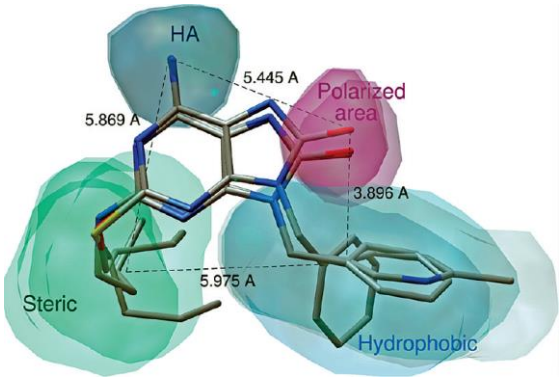


Figure 18. QSAR modeling depicting IFN induction regions on adenine TLR7 compounds[59]

TLR7-Antibody Conjugation

The clinical utilization of TLR7 agonists has been limited by side-effects resulting from the systemic induction and release of cytokines such as IL-6, IL-12, and type I IFN.[46] Conjugation of TLR7 agonists to an antibody that targets dendritic cells could potentially reduce side effects associated with systemic cytokine release.[60] It has also been shown that the covalent attachment of TLR ligands to antigenic proteins can result in vaccines with improved immunogenic activity.[61-63] Vaccines consisting of recombinant antibodies assembled with antigenic proteins that target dendritic cells have been produced.[64-66]

A DC-targeting conjugate was developed by Tacken et al. by encapsulating TLR3 and TLR7/8 agonists (poly I:C and R848) within nanoparticles coated with DC-targeting anti-DC-SIGN and anti-DEC-205 antibodies.[60,67] Their studies showed that the conjugation led to enhanced DC maturation and allowed the induction of cellular immune responses at lower adjuvant doses, thereby reducing toxicity.

The TLR7 agonist UC-1V150 (as an NHS ester; see Figure 12) has been recently conjugated with the antitumor antibody anti-hCD20 rituximab.[68] According to studies carried out on this conjugate, the antigen-binding activity and specificity of the conjugate was retained and the in vitro proinflammatory activity was increased over that of unconjugated UC-1V150.

Filippov and his co-workers conjugated an azide-bearing 2-alkoxy-8-hydroxyadenine (R' = $(CH_2)_2O(CH_2)_2O(CH_2)_2N_3$, Figure 16) TLR7 ligand with peptides from ovalbumin.[69] Using the Huisgen cycloaddition, they synthesized three conjugates with different peptides and discovered that the conjugates enhanced antigen presentation in vitro.

Wu et al. also conjugated UC-1V150 with mouse serum albumin (MSA) bearing

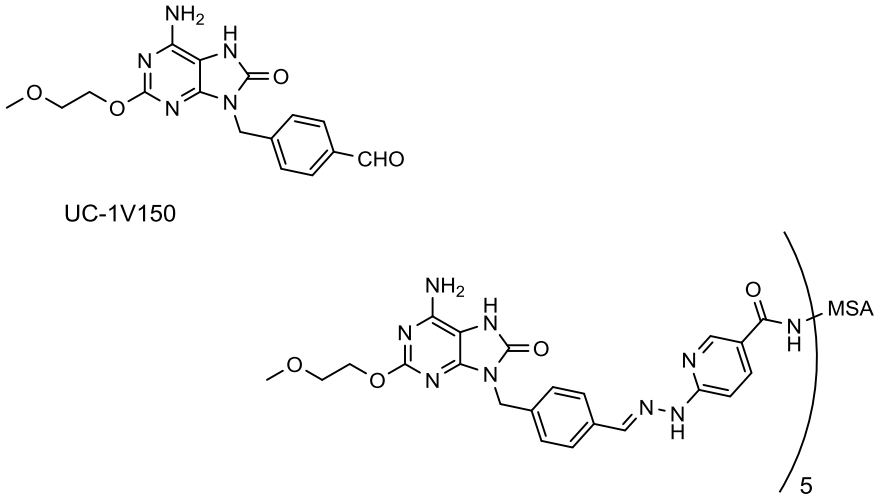


Figure 19. TLR7 agonist conjugates with mouse serum albumin (MSA)

a hydrazone linker (succinimidyl 4-hydrazinonicotinate acetone hydrazone) – Figure 19.[41] A standard UV absorption curve was used to determine the ratio of UC-1V150 to MSA in the conjugate to be approximately 5:1. In vitro studies in a murine model showed that the

conjugate produced higher levels of cytokines than the free drug. After intravenous injection, the conjugate improved the production of cytokines 10- to 100-fold over the free drug.

TLR2 Agonists

TLR2 belongs to the class of cell surface TLRs. Among TLRs, TLR2 recognizes the broadest range of microbial derived components.[70] Studies of mice deficient in TLR2 have helped to demonstrate that this TLR has a distinct function in terms of PAMP recognition and immune responses.[71] TLR2-agonizing components include lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, tGPI-mucin from *Trypanosoma cruzi*, soluble factor from *Neisseria meningitides*, phenol-soluble modulin from *Staphylococcus epidermis*, and the hemagglutinin protein from measles virus.[33,34,43] TLR2 interacts with other host cell receptors such as TLR1, TLR6, CD14, CD36 and Dectin-1.[72] With TLR1 and TLR6, TLR2 forms heterodimers TLR1/2 and TLR2/6. With CD14 (a high affinity LPS binding receptor), TLR2 forms an LPS receptor complex.[73] CD36 acts together with TLR2/6 to mediate the sensing of some TLR2 agonists.[74] These interactions provide a way by which TLR2 can distinguish different ligands. For examples, triacyl lipopeptides (Pam3CSK4) and lipoarabinomannan can be recognized by TLR1/2, while diacyl lipopeptides (Pam2CSK4, MALP-2), zymosan and lipoteichoic acid are recognized by TLR2/6.[70,74] Interaction of TLR2 with Dectin-1, a lectin family receptor for the fungal cell wall component β-glucan, also demonstrates the ability of TLR2 to recognize fungal-derived components.[75,76] Additionally, TLR2 reportedly recognizes LPS preparations from non-enterobacteria such as *Leptospira interrogans*,[77] *Porphyromonas*

gingivalis and *Helicobacter pylori*.[78] These LPS structurally differ from the typical LPS of Gram-negative bacteria recognized by TLR4. It has also been reported that TLR2 signaling is stimulated in response to a number of viruses including Epstein-Barr virus,[79] measles virus,[80] Varicella-Zoster virus,[81] hepatitis C virus,[82,83] human and murine cytomegalovirus,[84,85] herpes simplex virus,[86-89] vaccinia virus,[90,91] lymphocytic choriomeningitis virus[92] and respiratory syncytial virus.[93]

As with most TLRs, TLR2 signaling involves the MyD88-dependent pathway, resulting in cellular responses such as activation of NF-κB and MAPKs and the production of proinflammatory cytokines.[94] TLR2 activation by viral ligands, such as vaccinia, triggers the production of type I IFNs by inflammatory monocytes.[95]

TLR2-ligand complexes are among the five TLR-ligand complexes to have been characterized structurally to date.[35] Figure 20 shows the structures of the TLR2/1 and TLR2/6 ectodomains binding lipopeptides Pam3CSK4 and Pam2CSK4 respectively.[96,97] The structures of the lipopeptides Pam3CSK4 and Pam2CSK4 are shown in Figure 21.[96,97] In both the TLR2/6-ligand and TLR1/2-ligand complexes, the dimeric arrangement consists of an 'm' shaped heterodimer, with the two N-termini extending in opposite directions and the C-termini converging in the middle region (Figure 20A, 20C). In the TLR2/1-ligand complex, two of the three lipid chains (the ester-bound ones) of Pam3CSK4 insert into the hydrophobic pocket of TLR2, while the third chain (the amide-bound) inserts into the narrow hydrophobic channel of TLR1 (Figure 20B).[96] The amide- bound lipid

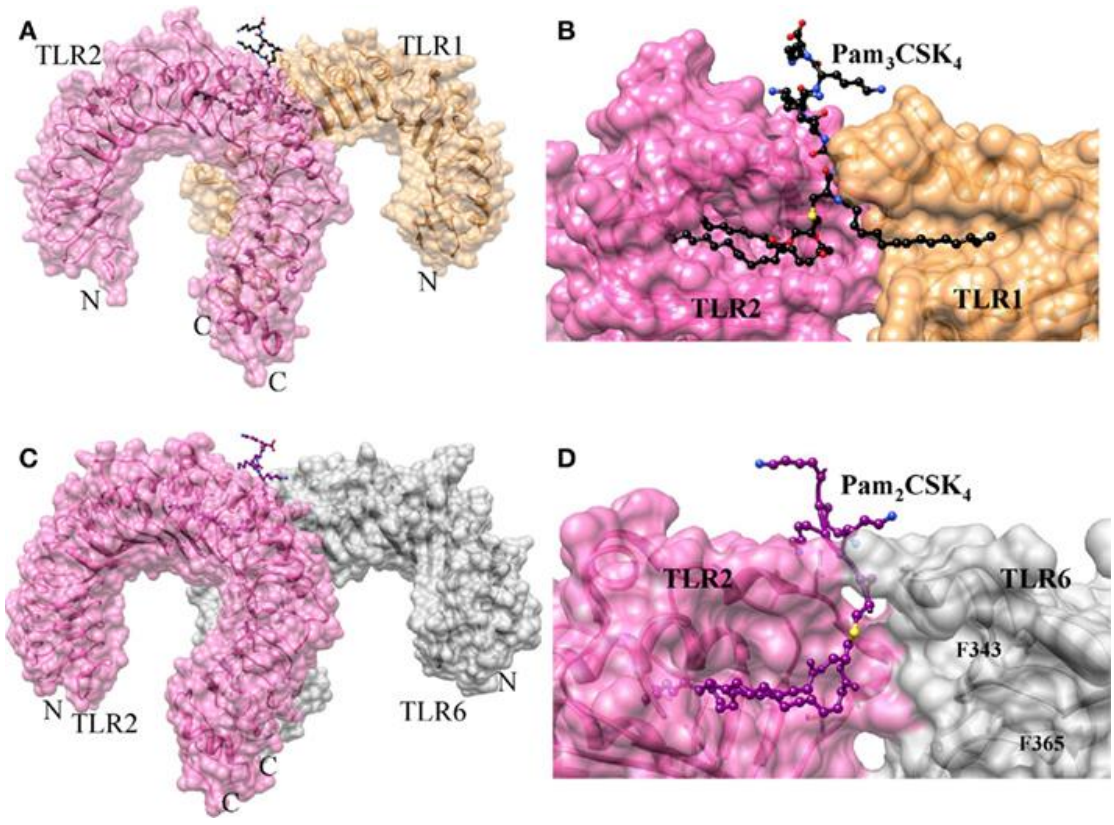


Figure 20. Crystal structures of TLR2:1 and TLR2:6 heterodimers showing their binding to lipopeptides[35]

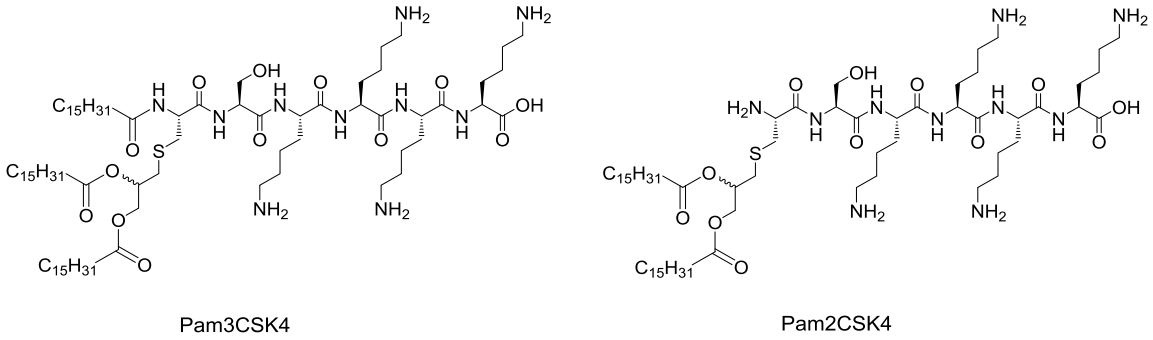


Figure 21. Structures of Pam3CSK4 and Pam2CSK4

chain plays an important role in bridging the two TLRs in the TLR2/1-triacylated lipopeptide. On the other hand, the hydrophobic channel is absent from TLR6, as the side chains of two phenylalanine residues (F343 and F365) block the lipid binding pocket,

leading to a pocket that is less than half the length of TLR1.[97] Therefore, the TLR2/6 heterodimer selectively binds to the diacylated lipopeptide using just the ester-bound lipid chains inserted into TLR2's hydrophobic pocket (Figure 20D). Dimerization in the TLR2/6-diacylated lipopeptide is strengthened by protein- protein interactions between the two TLRs (which is greater in the TLR2/6-ligand than in the TLR2/1-ligand) and also by hydrogen bonding. A hydrophobic interaction between the sulfur atom (on the cysteine) of the ligand and the TLRs also contributes to dimerization in the complexes.[97]

SAR studies using HEK293 cells transfected with human TLR2 have revealed the essential contribution of the ester-bound fatty acids for TLR2 signaling induction.[98] The importance of the fatty acid chain length was also demonstrated. While the incorporation of short chain fatty acids (such as acetic acid and hexanoic acid) resulted in little or no TLR2-dependent response, longer chains (octanoyl to palmitoyl) led to an increased response. The length and nature of the peptide are also important for recognition. Lipopeptide analogs Pam3CSSNA, Pam3CSK4, Pam3CAG, and Pam3CS were investigated by Reitermann et al.[99] Out of these, Pam3CSK4 was found to be a potent immune adjuvant in combination with dinitrophenylated bovine serum albumin, presumably due to its hydrophilic lysine residues.

TLR4 Modulators

TLR4 is the first discovered member of the TLR family. It is also a cell surface receptor, similar to TLR2. Lipopolysaccharide (LPS) is an integral component of the outer membrane of Gram-negative bacteria and is also a natural ligand and agonist for TLR4. Apart from LPS, other exogeneous ligands recognized by TLR4 include heat shock protein (HSP60) from *Chlamydia pneumoniae*, fusion protein from respiratory syncytial virus,

envelope proteins from respiratory syncytial virus and murine mammary tumor virus, GPI from *Trypanosoma cruzi*, and Taxol (a diterpene also known as paclitaxel).[35,43] Curcumin, cinnamaldehyde, caffeic acid phenethyl ester, isoliquiritigenin, L-sulforaphane, and morphine have also been identified as TLR4 ligands from natural sources.[100] Endogenous ligands known as danger associated molecular patterns (DAMPs) have also been shown to activate TLR4. Endogenous DAMP ligands for TLR4 include heat shock proteins (HSP60 and HSP70), β-defensin 2, the extra domain A of fibronectin, hyaluronic acid, heparan sulfate and fibrinogen.[34,35] These endogenous factors, usually released in the host as a consequence of injury or inflammation, typically require very high concentrations to activate TLR4.[34] LPS (or endotoxin), on the other hand, is a very potent activator of TLR4. Activation of TLR4 by small amounts of LPS often complicated studies of endogenous ligand preparations. LPS is composed of a hydrophilic polysaccharide portion and a hydrophobic lipid A portion. The biologically active (toxic) part of LPS is the membrane-anchoring moiety, lipid A.[100] Lipid A is composed of a glucosamine disaccharide core with phosphate esters and fatty acid chains. Activation of TLR4 by LPS is a complex process initiated by LPS binding protein (LPB), a soluble plasma protein. LPB catalyzes the extraction and transfer of individual LPS molecules from aggregated LPS to CD14 (a GPI-linked, LRR-containing protein that binds LPB), and from CD14 to myeloid differentiation protein 2 (MD-2).[33] Engagement of TLR4 and dimerization leads to the formation of the $(LPS.MD\text{-}2.TLR4)_2$ complex. The intracellular signal that results from this dimerization follows either the MyD88-dependent pathway or the MyD88-independent (TRIF- or TRAM-dependent) pathway.[34,100] TLR4 activation and signaling that results from LPS engagement can lead to a fatal septic syndrome if the inflammatory response is amplified

or uncontrolled.[101] The resulting inflammatory response includes the production of mediators such as cytokines or nitric oxides. Therefore, the release of these mediators can be used as a basis for evaluating antisepsis agents.[102] Sepsis (a systemic inflammatory response syndrome induced by bacterial infection) is one of the chief causes of death in the intensive care units today.[103] Therefore, the inhibition of TLR4 stimulation with TLR4 antagonists is an area of active research.

A synthetic small molecule with a structure totally different from that of Lipid A, known as TAK-242 (or resatorvid), has been developed as a TLR4 antagonist and a potential antisepsis agent.[102] TAK-242 is the (*R*)-enantiomer of ethyl 6-(*N*-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate (Figure 22). According to Takashima et al., TAK-242 inhibits TLR4 by binding directly (presumably as a Michael acceptor) to Cys747 in the intracellular domain of TLR4. It exhibited potent therapeutic effects in an *E. coli*-induced mouse sepsis model.[104] Recent work by Hua et al. has also shown that TAK-242 is able to cross blood-brain barrier, blocking TLR4 signaling, mediating the expression of inflammatory cytokines, and protecting the brain from acute damage induced by cerebral ischemia/reperfusion.[105]

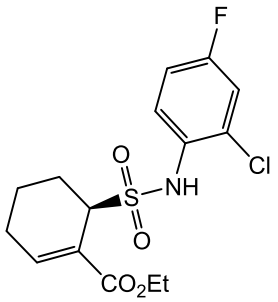


Figure 22. Structure of TAK-242

Islet Transplants

Islet cell transplantation is a viable option for treating people with type I diabetes. Islets constitute only about 2% of the pancreatic volume and help to produce insulin.[106] In people with type I diabetes, their islet cells have been destroyed. One approach to treating type I diabetes is to transfer islet from a healthy donor to the patient. A significant advantage of this procedure is that it involves simple IV transfer of islets into the portal vein, and is thus much less invasive than a whole organ transplant.[107] Challenges associated with this procedure, however, include the difficulty of obtaining sufficient islets from a single donor and the death of islets, especially after transplantation, by a variety of mechanisms including especially the instant blood mediated inflammatory response (IBMIR). IBMIR occurs instantaneously when allogenic or xenogenic islets come into contact with blood and results in a significant loss of transplanted islets.[108]

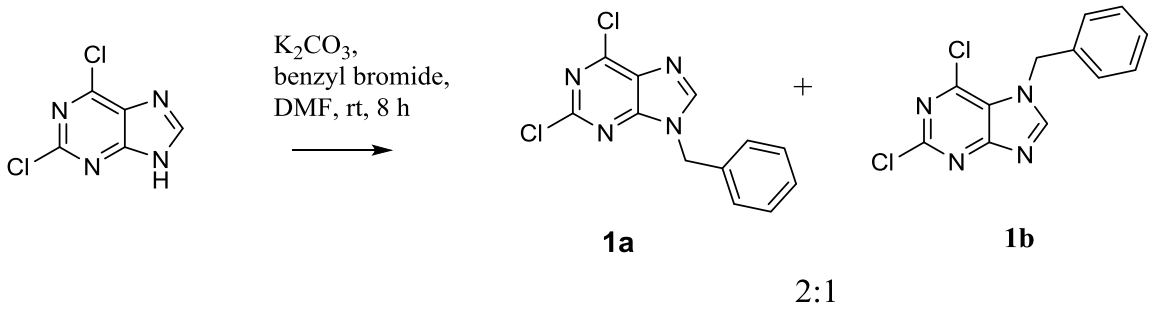
Apart from IBMIR, poor islet engraftment or graft loss has also been attributed to poor quality islets and hypoxia. IBMIR is characterized by features such as blood coagulation and complement activation.[108,109] In concert with these, a variety of approaches have been used to prevent graft loss or reduce IBMIR, including administration of low molecular weight immunosuppresants, anticoagulants, thrombin-inhibitors or anti-inflammatory drugs, as well as islet surface modifications with PEG and protein conjugates.[109-110]

CHAPTER TWO

Materials and Method

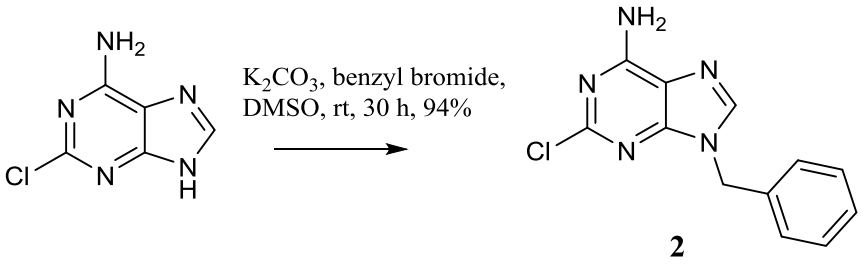
General Section

Solvents (ethyl acetate, ethyl ether, hexane and methanol) were obtained from the Baylor Sciences Building stockroom and distilled before use. All other reagents and solvents were purchased from Acros Organics, Alfa Aesar, Sigma-Aldrich, VWR, Pierce, Bio-synthesis, Bachem, and Click Chemisty Tools. LC/MS grade methanol from Fisher Scientific was used for running mass spectrometry experiments. Reactions were monitored by thin layer chromatography (TLC) using silica XG TLC plates w/UV254 from Sorbent Technologies and the final products purified by flash chromatography using silica gel 60 (230-400 mesh). A Varian 500 MHz NMR and an AVANCE III HD Bruker 600 MHz NMR spectrometer were used for obtaining ^{1}H and ^{13}C NMR. Chemical shifts (δ) are reported in ppm and coupling constants (J) are expressed in Hz. High resolution mass spectra (HRMS) were obtained using the electrospray ionization (ESI) technique on a Thermo Scientific LTQ Orbitrap Discovery mass spectrometer in the Baylor University Mass Spectrometry Core Facility. GC-MS was carried out using Thermo Scientific DSQ II GC/MS with FOCUS GC. Microwave-assisted synthesis was performed using the CEM Discover microwave system in sealed reaction vessels.



Scheme 1. Synthesis of **1**

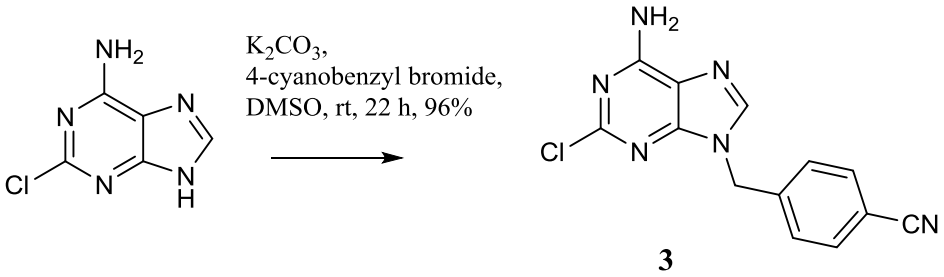
*9- and 7- Benzyl-2,6-dichloropurine (**1a, 1b**).* To a mixture of 0.7 g (3.7 mmol) of 2,6-dichloropurine and 1.5 g (10.9 mmol) of potassium carbonate was added 13.5 ml (174 mmol) of DMF. After stirring for 8 minutes, 0.62 ml (3.7 mmol) of benzyl bromide was added and stirring was continued for 8 hours at ambient temperature. The mixture was partitioned between DCM (30 ml) and water (100 ml). The aqueous phase was further extracted with DCM (2 x 20 ml). The combined organic phase was dried with magnesium sulfate and dried by rotary evaporation and high vacuum, leaving a yellow liquid. The isomers were separated using silica gel column chromatography (50% ethyl acetate/hexanes), giving 36 % of the less polar N-9 and 20% of the more polar N-7 product (which showed more downfield proton NMR chemical shifts). N-7 Product: ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.05 (s, 1H), 7.38 - 7.28 (m, 3H), 7.21 (d, J = 7.0 Hz, 2H), 5.74 (s, 2H). N-9 Product: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.85 (s, 1H), 7.39 - 7.27 (m, 5H), 5.50 (s, 2H).



2

Scheme 2. Synthesis of **2**

*9-Benzyl-2-chloroadenine (**2**).* To 2-chloroadenine (1.3 g, 7.7 mmol) and potassium carbonate (3 g, 21.7 mmol) was added DMSO (26 ml). After stirring for 2 minutes, benzyl bromide (1.25 ml, 10.5 mmol) was added. Stirring was continued for 30 hours at room temperature. The reaction mixture was poured into 140 ml of cold water and kept cold (4°C) overnight. The precipitate was filtered giving the product in 94% yield. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.25 (s, 1H), 7.77 (s, 2H), 7.38 - 7.26 (m, 5H), 5.33 (s, 2H).

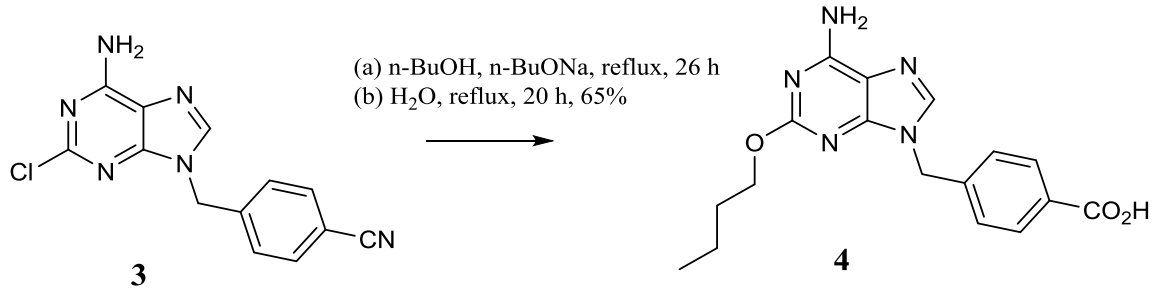


3

Scheme 3. Synthesis of **3**

*2-Chloro-9-(4-cyanobenzyl)adenine (**3**).* To 2-chloroadenine (2.00 g, 11.8 mmol) and potassium carbonate (5.07 g, 36.7 mmol) in a reaction flask was added DMSO (44 ml). After stirring the mixture for 5 minutes, 4-cyanobenzyl bromide (3.20 g, 16.3 mmol) was added and stirring was continued for 22 hours at room temperature. After allowing the reaction mixture to settle in the reaction flask, the mixture was poured into ethyl acetate (250 ml) and water (180 ml) in a glass-capped round-bottom flask. The contents of the

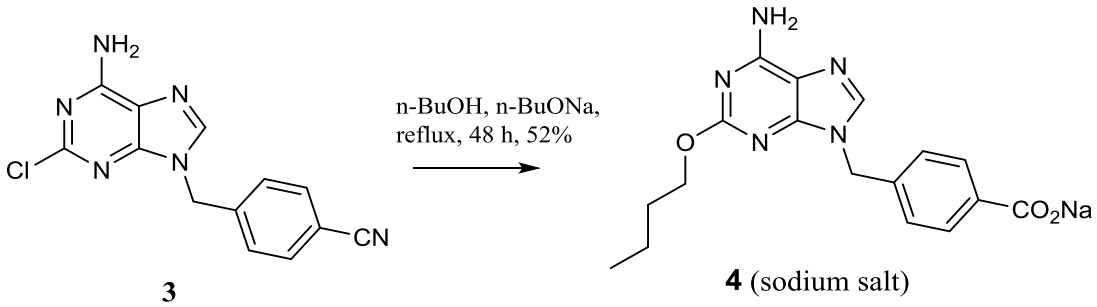
round-bottom flask were mixed thoroughly (sealed). The mixture was concentrated in vacuo. The concentrated mixture was placed in ice to cool and the precipitate obtained was filtered and rinsed with cold water. Drying under vacuum was continued overnight to give the product in 96% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.27 (s, 1H), 7.85-7.79 (m, 4H), 7.42 (d, J = 8.4 Hz, 2H), 5.45 (s, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.8, 153.2, 150.5, 142.2, 141.5, 132.7, 128.1, 118.6, 117.8, 110.6, 45.9; HRMS (+ESI) calcd for $C_{13}H_{10}ClN_6$ (MH^+) 285.0650, found 285.0640 (Δ 3.5 ppm); (-ESI) calcd for $C_{13}H_8ClN_6$ ($M\text{-}H^+$) 283.0504, found 283.0503 (Δ 0.3 ppm).



Scheme 4. Synthesis of **4**

*4-((6-Amino-2-butoxy-9H-purin-9-yl)methyl)benzoic acid (**4**).* Sodium metal (0.16 g, 7.0 mmol) was allowed to react completely with dry *n*-butanol (18.5 ml) to give sodium butoxide. Dry *n*-Butanol (54.0 ml) was added to the nitrile **3** (0.71 g, 2.5 mmol) in a round-bottom flask and the mixture was stirred for a minute. The sodium butoxide solution was added to **3** and *n*-butanol in the flask and the resulting mixture was refluxed with stirring for 26 hours. The reflux was paused to cool the mixture. Deionized water (6.0 ml, 333.1 mmol) was added, and the reflux was continued for additional 20 hours. The reaction mixture was extracted with 110 ml of water. The organic layer was further extracted with 80 ml of water, allowing 12 hours for the layers to separate (the

middle emulsion was separated, allowed to settle and extracted with additional water). The combined aqueous layers were acidified to pH 3 using 4M hydrochloric acid and cooled overnight. The precipitate obtained was filtered and dried to give the carboxylic acid in 65% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.16 (s, 1H), 7.91 (d, J = 8.2 Hz, 2H), 7.40 (d, J = 8.2 Hz, 2H), 5.37 (s, 2H), 4.23 (t, J = 6.7 Hz, 2H), 1.67-1.60 (m, 2H), 1.42-1.34 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 167.0, 159.2, 154.6, 150.5, 141.4, 140.4, 130.3, 129.7, 127.8, 113.9, 67.0, 46.2, 30.4, 18.7, 13.7; HRMS (+ESI) calcd for $C_{17}H_{20}N_5O_3$ (MH^+) 342.1561, found 342.1568 (Δ 2.0 ppm); (-ESI) calcd for $C_{17}H_{18}N_5O_3$ ($M-H^+$) 340.1415, found 340.1412 (Δ 0.9 ppm).

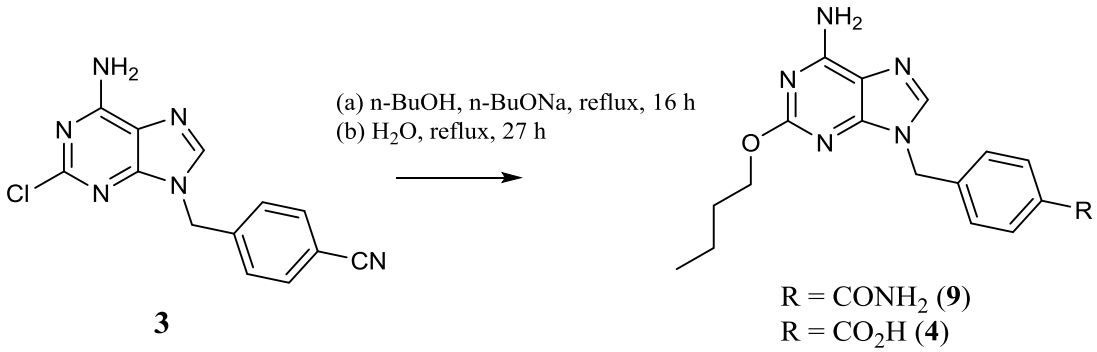


3 **4** (sodium salt)

Scheme 5. Synthesis of sodium salt of **4**

*Sodium 4-((6-amino-2-butoxy-9H-purin-9-yl)methyl)benzoate (**4**).* 0.3 g (13 mmol) of sodium metal was allowed to react completely with 45 ml of n-butanol. 130 ml of n-butanol was added to 1.7 g (6 mmol) of **3** in a round bottom flask and the mixture was stirred for a minute. The prepared sodium butoxide solution was added to **3** in butanol in the flask. The mixture was refluxed with stirring for 48 hours. The reaction mixture was cooled to room temperature and kept overnight at 5^{o}C. Vacuum filtration of the precipitate was carried out using minimal cold ethyl acetate to rinse the solid. The product was used without further purification (52%). ^{1}H NMR (500MHz, DMSO-d_6) δ 8.02 (s, 1H), 7.81 (d,

J = 8.0 Hz, 2H), 7.21 (d, J = 8.0 Hz, 2H), 7.17 (s, 2H), 5.24 (s, 2H), 4.21 (t, J = 6.6 Hz, 2H), 1.68-1.62 (m, 2H), 1.44-1.36 (m, 2H), 0.92 (t, J = 7.4 Hz, 3H); ^{1}H NMR (500 MHz, D_2O) δ 7.94 (s, 1H), 7.81 (d, J = 8.1 Hz, 2H), 7.31 (d, J = 8.1 Hz, 2H), 5.28 (s, 2H), 4.21 (t, J = 6.6 Hz, 2H), 1.64 – 1.57 (m, 2H), 1.37 – 1.29 (m, 2H), 0.86 (t, J = 7.4 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.4, 161.5, 156.7, 151.2, 140.1, 139.3, 137.2, 129.2, 126.4, 115.0, 65.8, 45.9, 30.6, 18.8, 13.7.



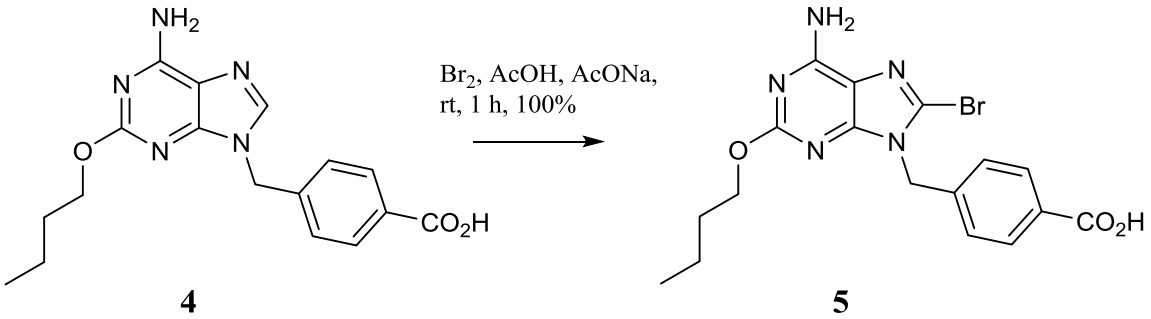
3

Scheme 6. Synthesis of amide product and **4**

*4-((6-Amino-2-butoxy-9H-purin-9-yl)methyl)benzamide and 4-((6-amino-2-butoxy-9H-purin-9-yl)methyl)benzoic acid (mixture of amide and acid: **9, 4**).* Sodium metal (0.76 g, 33 mmol) was allowed to react completely with dry n-butanol (88 ml). n-Butanol (256 ml) was added to the nitrile **3** (3.38 g, 11.9 mmol) in a 1000 ml round bottom flask and the mixture was stirred for a minute. The sodium butoxide solution was added to the **3**/butanol mixture and the resultant mixture was refluxed with stirring for 16 hours. The reflux was paused to cool the mixture, 29 ml of deionized water was added, and the reflux was continued for 27 hours. The reflux apparatus was replaced with a short-path distillation apparatus. 516 ml of deionized water was added to the reaction mixture. Distillation was carried out to remove 560 ml of the azeotrope. The reaction mixture was cooled and

acidified to pH 3 using 4M HCl. The mixture was kept at 4°C overnight, filtered using cold water to rinse, and dried to give a mixture of the amide and carboxylic acid **4** in 57% and 43% yields respectively.

Proton NMR of the amide: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.05 (s, 1H), 7.93 (s, 1H), 7.83 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 3H), 7.20 (s, 2H), 5.31 (s, 2H), 4.19 (t, J = 6.6 Hz, 2H), 1.66 – 1.59 (m, 2H), 1.42 – 1.36 (m, 2H), 0.91 (t, J = 7.4 Hz, 3H). HRMS (+ESI) calcd for $C_{17}H_{21}N_6O_2$ (MH^+) 341.1721, found 341.1716 (Δ 1.5 ppm).

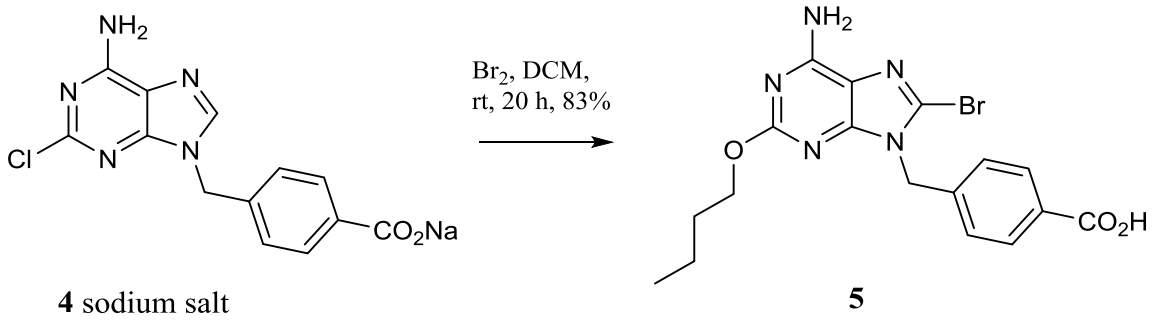


4 **5**

Scheme 7. Synthesis of **5**

*4-((6-Amino-8-bromo-2-butoxy-9H-purin-9-yl)methyl)benzoic acid (**5**).* **4** (1.3 g, 3.8 mmol) was dissolved in acetic acid (50 ml). Bromine (0.97 ml, 18.9 mmol) and sodium acetate (1.54 g, 18.8 mmol) were added and the resulting mixture was stirred for 1 hour at room temperature (the product formed as a precipitate). The reaction mixture was filtered and washed with cold water and 10% sodium thiosulfate solution (removing the red-brown color) to give the desired product. More product was recovered from the filtrate by allowing the filtrate to settle overnight. The precipitate formed in the filtrate was then filtered washing with cold water. The dried combined yellow powder was obtained in 100% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.90 (d, J = 8.2 Hz, 2H), 7.44 (brs, 2H), 7.29 (d, J = 8.2 Hz, 2H), 5.32 (s, 2H), 4.19 (t, J = 6.6 Hz, 2H), 1.67 – 1.59

(m, 2H), 1.41 – 1.33 (m, 2H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 167.1, 161.6, 155.8, 152.5, 140.5, 129.7, 127.1, 123.7, 115.3, 66.1, 46.2, 30.6, 18.8, 13.7; HRMS (+ESI) calcd for $C_{17}H_{19}BrN_5O_3$ (MH^+) 420.0666, found 420.0652 (Δ 3.3 ppm); (-ESI) calcd for $C_{17}H_{18}N_5O_3$ ($M\text{-}H^+$) 418.0520, found 418.0526 (Δ 1.4 ppm).

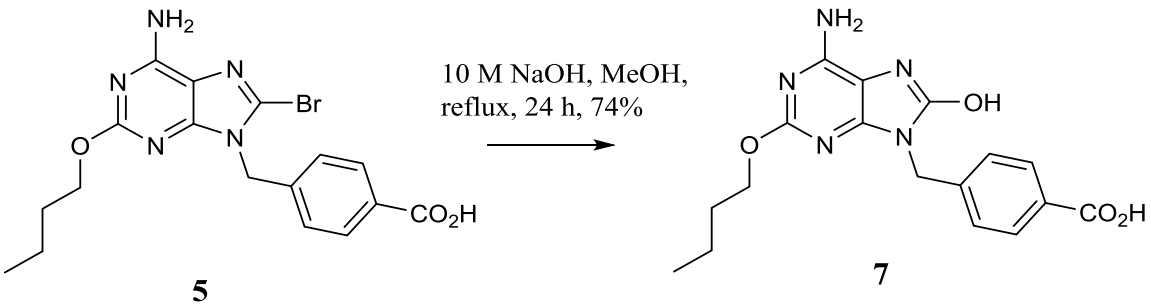


4 sodium salt **5**

Scheme 8. Synthesis of **5** from the sodium salt of **4**

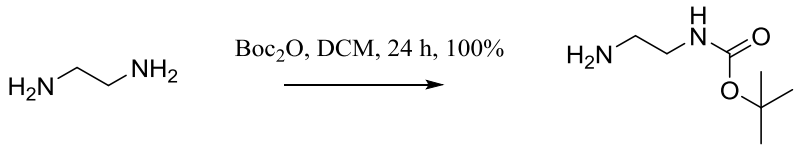
*4-((6-Amino-8-bromo-2-butoxy-9H-purin-9-yl)methyl)benzoic acid (**5**).* To 1.3 g (4 mmol) of powdered **4** (sodium salt) was added 80 ml (1248 mmol) of dichloromethane with stirring and 10 ml of bromine (195 mmol). The mixture was stirred at room temperature for 20 hours. The mixture was poured into a 1000 ml Erlenmeyer flask along with 115 ml of dichloromethane and 175 ml of 10% sodium thiosulfate solution (poured in slowly) using the sodium thiosulfate and spatula to remove and transfer all the solid that remained in the bottom of the round bottom flask into the Erlenmeyer flask. The flask was covered with aluminum foil and the mixture was stirred vigorously for 3 hours. The slurry obtained was filtered slowly and washed with 45 ml of saturated sodium bicarbonate and with cold water. The fully dried product was obtained with a yield of 83%. ^{1}H NMR (500MHz, DMSO-d_6) δ 7.92 (d, J = 8.4 Hz, 2H), 7.32 (d, J = 8.4 Hz, 2H), 5.34 (s, 2H), 4.23 (t, J = 6.6 Hz, 2H), 1.67-1.61 (m, 2H), 1.42-1.34 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C

NMR (126 MHz, DMSO-d_6) δ 166.9, 158.2, 152.9, 151.7, 140.3, 130.4, 129.8, 127.4, 125.4, 114.9, 67.6, 46.6, 30.2, 18.6, 13.7.



Scheme 9. Synthesis of **7**

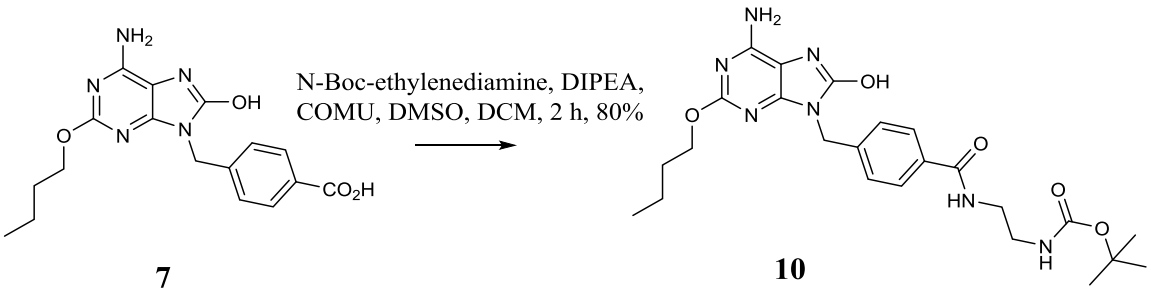
4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl) methyl benzoic acid (7). To compound **5** (1.50 g, 3.6 mmol) was added methanol (27 ml) in a round-bottom flask and the mixture was stirred for a minute. Sodium hydroxide (28 ml, 10 M, 280 mmol) was added to the mixture. The mixture was then refluxed for 24 hours while stirring. After the reflux, the solution was cooled to room temperature and poured to a 250 ml round bottom flask. 80 ml of methanol was added and the mixture was acidified using 60 ml of 6 M hydrochloric acid. The mixture was concentrated under reduced pressure. After cooling the concentrated mixture in ice, the white precipitate obtained was filtered, washing with cold water and dried to give **7** in 74% yield. ^{1}H NMR (500MHz, DMSO-d_6) δ 10.13 (s, 1H), 7.89 (d, J = 8.3 Hz, 2H), 7.38 (d, J = 8.3 Hz, 2H), 4.93 (s, 2H), 4.14 (t, J = 6.6 Hz, 2H), 1.64-1.57 (m, 2H), 1.39-1.31 (m, 2H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 167.0, 159.5, 152.1, 149.0, 147.2, 142.0, 129.9, 129.6, 127.4, 98.3, 66.2, 42.2, 30.5, 18.7, 13.7; HRMS (+ESI) calcd for $C_{17}H_{20}N_5O_4$ ($M+H^+$) 358.1510, found 358.1513 (Δ 0.8 ppm); (-ESI) calcd for $C_{17}H_{18}N_5O_4$ ($M-H^+$) 356.1364, found 356.1365 (Δ 0.3 ppm).



6

Scheme 10. Synthesis of *N*-Boc-ethylenediamine

*N-Boc-ethylenediamine (**6**).* A solution of di-tert-butyl dicarbonate (8.2 g, 38 mmol) in dichloromethane (200 ml) was added dropwise (using a 250 ml drip funnel) to a vigorously stirred solution of ethylenediamine (13.49 g, 15 ml, 224 mmol) in dichloromethane (200 ml) over a period of 8 hours at room temperature. The reaction mixture was stirred for a further 24 hours before the mixture was evaporated in vacuo to leave a crude oily residue. The residue was diluted with 120 ml dichloromethane and washed with 100 ml of 2 M sodium carbonate. The aqueous layer was extracted with 3 x 100 ml of dichloromethane. The combined organic layer was dried with magnesium sulfate and evaporated to give the product as a yellow oil in 100% yield (without further purification). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.13 (s, 1H), 3.13 (q, J = 5.9 Hz, 2H), 2.76 (t, J = 5.9 Hz, 2H), 1.99 (s, 2H), 1.39 (s, 9H); MS (EI) *m/z* (relative intensity) 103 $[M - tBu]^+$ (2), 57 (100), 43 (79), 30 (82).

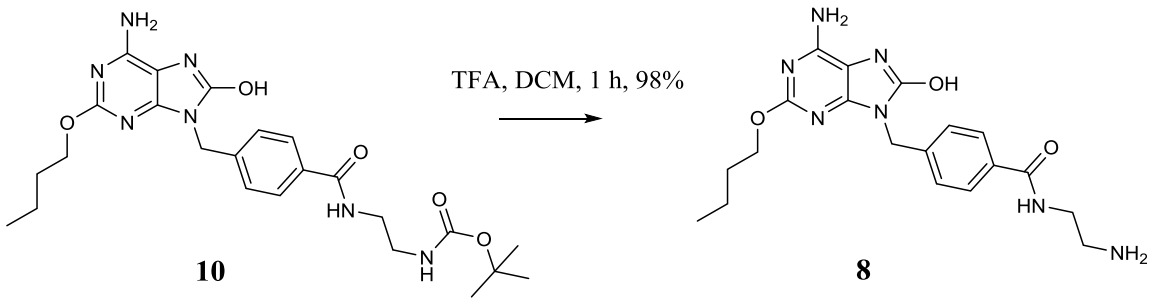


7 **10**

Scheme 11. Synthesis of **10**

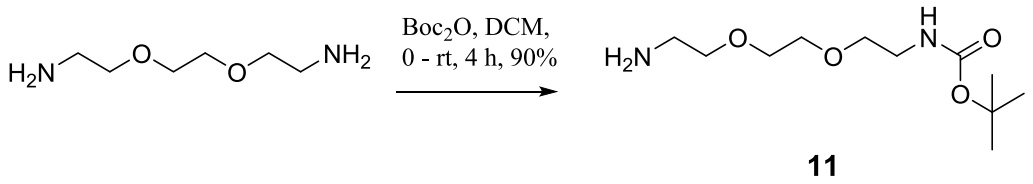
*Tert-Butyl-(2-(4-((6-amino-2-butoxy-8-hydroxy-9H-purin-9- yl)methyl)benzamido)-ethyl)carbamate (**10**).* Compound 7 (0.50 g, 1.4 mmol) was dissolved in DMSO (20.0 ml). *N*-Boc-ethylenediamine (0.70 g, 4.4 mmol) and *N*,*N*-diisopropylethylamine (1.2 ml, 6.9 mmol) were mixed and dissolved with dichloromethane (0.7 ml) in a vial and added with a syringe to the stirring solution of **7** in DMSO. After placing the reaction flask in ice, COMU (1.55 g, 3.6 mmol) was added. The flask was allowed to warm to room temperature and stirring was continued for 2 hours. The reaction mixture was poured into ethyl acetate (130 ml) and cooled (4ºC) for an hour. The mixture was then poured into a 500 ml glass-capped round-bottom flask. 1N sodium bicarbonate (90 ml) was added and the contents were mixed and allowed to settle. After concentrating the mixture in vacuo, the flask was cooled at 0ºC for one hour. The solid obtained was filtered under vacuum, washing with ice-cold water and dried to give the product in 80% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.07 (s, 1H), 8.47 – 8.38 (m, 1H), 7.77 (d, J = 7.9 Hz, 2H), 7.34 (d, J = 7.9 Hz, 2H), 6.94 – 6.85 (m, 1H), 6.50 (s, 2H), 4.90 (s, 2H), 4.12 (t, J = 6.5 Hz, 2H), 3.26 (d, J = 5.9 Hz, 2H), 3.08 (d, J = 5.9 Hz, 2H), 1.65 – 1.57 (m, 2H), 1.41 – 1.32 (m, 11H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.1, 160.1, 155.7, 152.3, 149.1, 147.8, 140.2, 133.7,

127.4, 127.2, 98.3, 77.7, 65.9, 42.1, 39.5, 39.5, 30.6, 28.2, 18.8, 13.7; HRMS (-ESI) calcd for $C_{24}H_{32}N_7O_5$ ($M\text{-}H^+$) 498.2470, found 498.2470 (Δ 0.0 ppm).



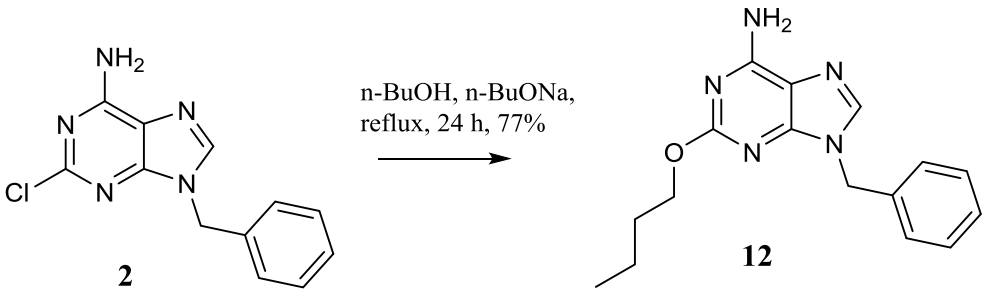
Scheme 12. Synthesis of **8**

*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(2-aminoethyl)-benzamide (**8**).* To **10** (0.3 g, 0.6 mmol) was added trifluoroacetic acid (0.92 ml, 12.0 mmol) and dichloromethane (5 ml) in a round-bottom flask. After stirring at room temperature for 1 hour the reaction mixture was evaporated until a viscous oil was left. Methanol was added to dissolve the oil and the mixture was re-evaporated. Diethyl ether was added to precipitate the product (by mixing the contents of the round-bottom flask thoroughly with ether and cooling for an hour at 4°C). The product was filtered, washing with cold ether and oven-dried to obtain the TFA salt in 98% yield. ^{1}H NMR (500MHz, DMSO-d_6) δ 10.01 (s, 1H), 8.55 (t, J = 5.4 Hz, 1H), 7.81 (d, J = 8.1 Hz, 2H), 7.74 (s, 3H), 7.37 (d, J = 8.1 Hz, 2H), 6.47 (s, 2H), 4.91 (s, 2H), 4.13 (t, J = 6.6 Hz, 2H), 3.51-3.45 (m, 2H), 3.01-2.94 (m, 2H), 1.65-1.58 (m, 2H), 1.40-1.32 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.6, 160.0, 152.2, 149.1, 147.8, 140.5, 133.1, 127.5, 127.2, 98.3, 65.8, 42.1, 38.7, 37.1, 30.6, 18.7, 13.7; HRMS (+ESI) calcd for $C_{19}H_{26}N_7O_3$ (MH^+) 400.2092, found 400.2098 (Δ 1.5 ppm).



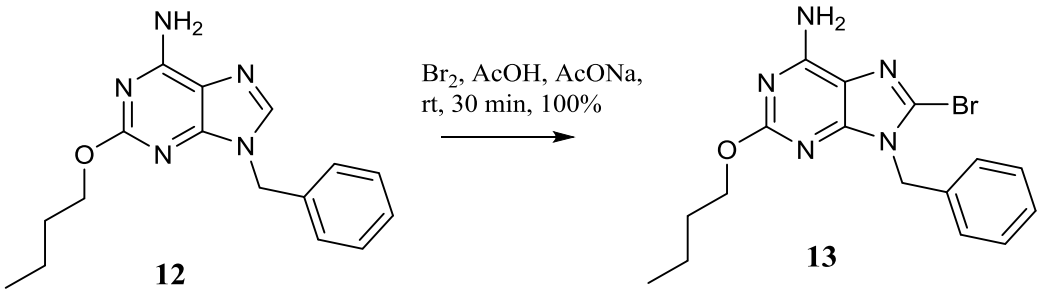
Scheme 13. Synthesis of *N*-Boc- 2,2′-(ethylenedioxy)diethylamine

N-Boc-2,2′-(ethylenedioxy)diethylamine ***(11)****.* A solution of di-tert-butyl dicarbonate (1.198 g, 5.5 mmol) in dichloromethane (80 ml) was added dropwise (using a 250 ml drip funnel) to a vigorously stirred solution of 2,2′-(Ethylenedioxy)bis(ethylamine) (8.120 g, 8 ml, 54.8 mmol) in dichloromethane (81 ml) at 0°C. The reaction mixture was then stirred for 4 hours at room temperature before the mixture was evaporated in vacuo to leave a crude oily mixture. The mixture was transferred to a separatory funnel with the addition of water (80 ml) and extracted with dichloromethane (3 x 80 ml). After evaporation of the combined organic phase, the residue was diluted with 50 ml of DCM and further washed with water (50 ml). The organic phase was dried with magnesium sulfate and evaporated to give the product in 90% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.18 (s, 1H), 3.60 (s, 4H), 3.52 (dt, *J* = 10.1, 5.1 Hz, 4H), 3.30 (d, *J* = 4.7 Hz, 2H), 2.87 (t, *J* = 5.0 Hz, 2H), 1.42 (s, 9H).



Scheme 14. Synthesis of **12**

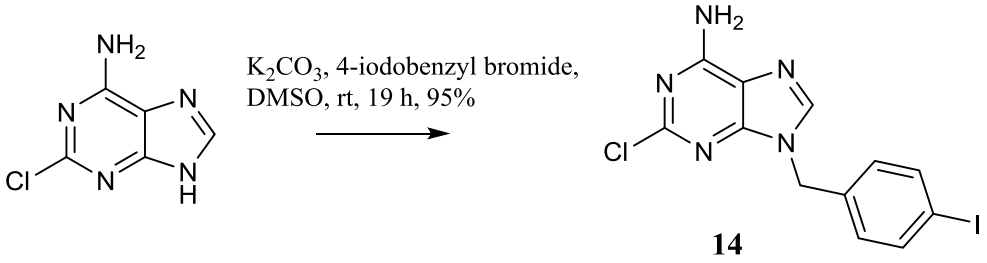
*9-Benzyl-2-butoxyadenine (**12**).* Sodium metal (0.41 g, 17.8 mmol) was allowed to react completely with dry *n*-butanol (62 ml) to give sodium butoxide. Dry *n*-butanol (178 ml) was added to **2 (**2.10 g, 8.2 mmol) in a round bottom flask and the mixture was stirred to dissolve the solid. The sodium butoxide solution was added to the *n*-butanol solution of **2** and the combined solution refluxed with stirring for 24 hours. The mixture was concentrated under reduced pressure and cooled (4ºC) overnight. The precipitate obtained was filtered and washed with water to give the product in 77% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.03 (s, 1H), 7.36 – 7.25 (m, 5H), 7.20 (s, 2H), 5.25 (s, 2H), 4.20 (t, J = 6.6 Hz, 2H), 1.68 – 1.61 (m, 2H), 1.44 – 1.35 (m, 2H), 0.91 (t, J = 7.4 Hz, 3H).



Scheme 15. Synthesis of **13**

*9-Benzyl-8-bromo-2-butoxyadenine (**13**).* **12** (1.99 g, 6.7 mmol) was dissolved in acetic acid (88 ml). Bromine (1.67 ml, 32.6 mmol) and sodium acetate (2.66 g, 32.4 mmol) were added and the resulting mixture was stirred for 30 minutes at room temperature. The reaction mixture was poured into 90 ml of 10% sodium thiosulfate in a round bottom flask (500 ml flask) and placed on ice for 10 minutes. The flask was fitted with a septum cap and vented and 100 ml of saturated sodium bicarbonate was added dropwise, swirling the flask on ice. The precipitate obtained was filtered, washed with cold water, and dried to give the bromide product (crude). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.44 (brs, 2H),

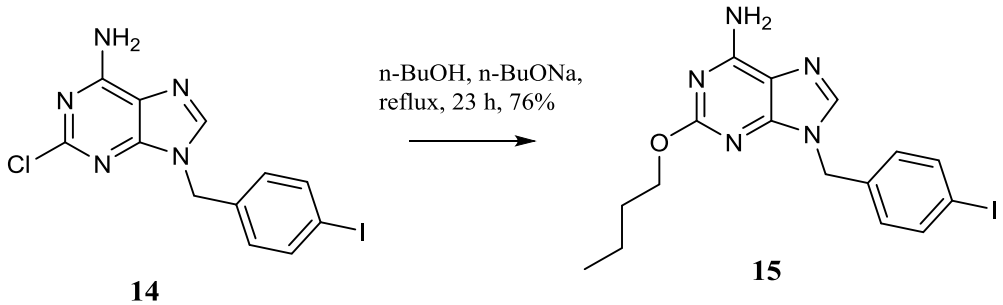
7.36 – 7.28 (m, 3H), 7.25 – 7.23 (m, 2H), 5.25 (s, 2H), 4.20 (t, *J* = 6.6 Hz, 2H), 1.66 – 1.62 (m, 2H), 1.42 – 1.35 (m, 2H), 0.90 (t, *J* = 7.4 Hz, 3H).



14

Scheme 16. Synthesis of **14**

*2-Chloro-9-(4-iodobenzyl)adenine (**14**).* To 2-chloroadenine (0.80 g, 4.7 mmol) and potassium carbonate (2.10 g, 15.2 mmol) was added DMSO (18 ml). After stirring for 2 minutes, 4-iodobenzyl bromide (1.9 g, 6.4 mmol) was added. Stirring was continued for 19 hours at room temperature. The reaction mixture was poured into 80 ml of methanol and 13 ml of water and cooled (4°C) overnight. The precipitate was filtered to give the product in 95% yield. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.25 (s, 1H), 7.81 (s, 2H), 7.72 (d, *J* = 8.0 Hz, 2H), 7.07 (d, *J* = 8.0 Hz, 2H), 5.29 (s, 2H).

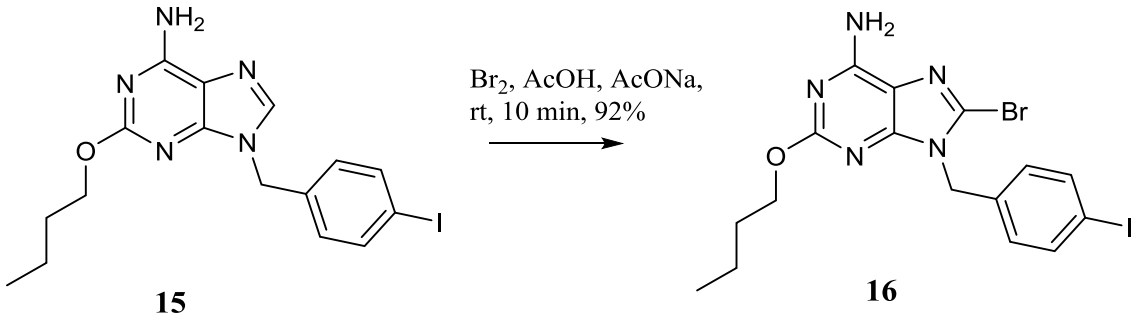


14 **15**

Scheme 17. Synthesis of **15**

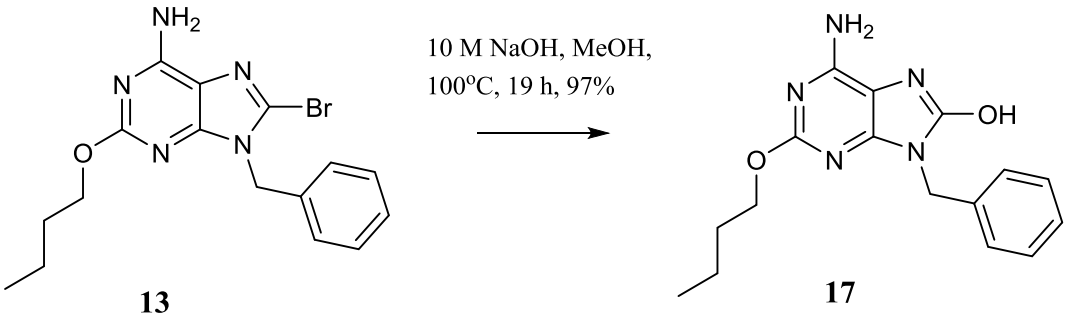
*2-Butoxy-9-(4-iodobenzyl)adenine (**15**).* Sodium metal (0.41 g, 17.8 mmol) was allowed to react completely with dry *n*-butanol (62 ml) under anhydrous conditions to give

sodium butoxide. Dry *n*-butanol (178 ml) was added to **14 (**2.10 g, 8.2 mmol) in an oven-dried round bottom flask. The sodium butoxide solution was added to **14** and *n*-butanol in the flask and the mixture was refluxed with stirring for 23 hours. The mixture was concentrated in vacuo and cooled (4°C) overnight. The solid obtained was filtered and washed with hexanes and cold water to give the product in 76% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.02 (s, 1H), 7.70 (d, J = 8.3 Hz, 2H), 7.19 (s, 2H), 7.11 (d, J = 8.3 Hz, 2H), 5.21 (s, 2H), 4.19 (t, J = 6.6 Hz, 2H), 1.67 – 1.59 (m, 2H), 1.43 – 1.34 (m, 2H), 0.91 (t, J = 7.4 Hz, 3H).



Scheme 18. Synthesis of **16**

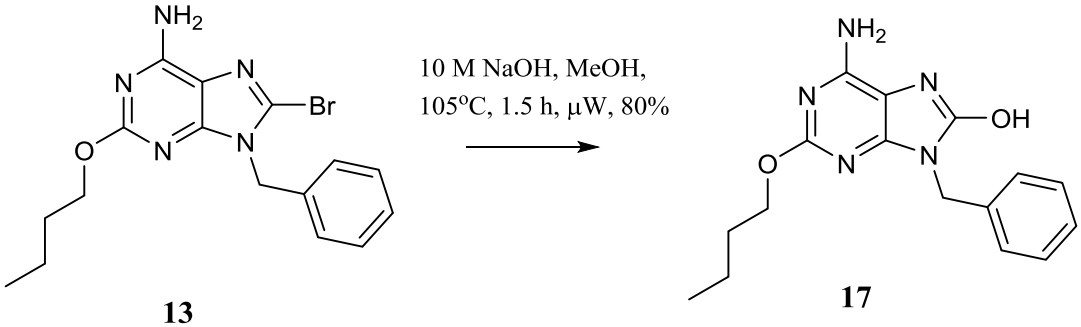
*8-Bromo-2-butoxy-9-(4-iodobenzyl)adenine (**16**).* **15** (2.10 g, 5.0 mmol) was dissolved in acetic acid (66 ml). Bromine (1.24 ml, 24.2 mmol) and sodium acetate (1.97 g, 24.0 mmol) were added and the resulting mixture was stirred for 10 minutes at room temperature (upon which the product formed as a precipitate). The reaction mixture was filtered and washed with 10% sodium thiosulfate solution (removing the red-brown color) and cold water and dried to give the desired product in 92% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.72 (d, J = 8.1 Hz, 2H), 7.04 (d, J = 8.1 Hz, 2H), 5.22 (s, 2H), 4.21 (t, J = 6.6 Hz, 2H), 1.67 – 1.61 (m, 2H), 1.41 – 1.35 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H).



13 **17**

Scheme 19. Synthesis of **17**

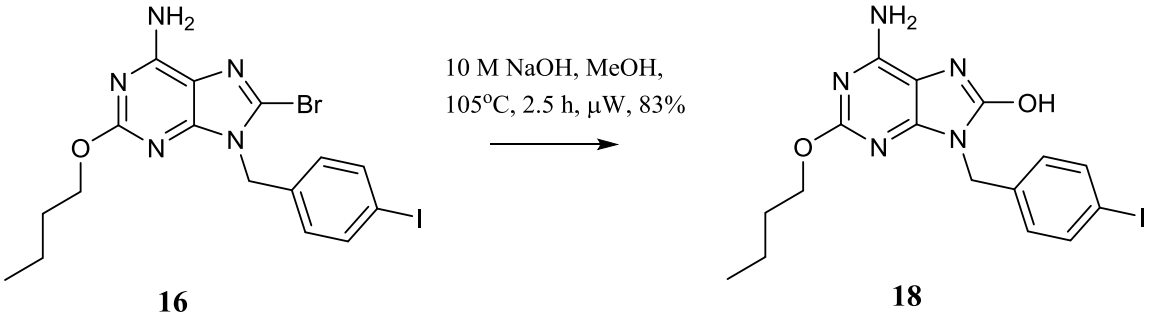
*9-Benzyl-2-butoxy-8-hydroxyadenine (**17**).* To the bromide **13** (2.44 g, 6.5 mmol) in a round-bottom flask was added sodium hydroxide (49 ml, 10 M, 490 mmol) and methanol (130 ml). The mixture was refluxed with stirring for 19 hours. After the reflux, the solution was cooled to room temperature and poured to a 1000 ml round bottom flask. 160 ml of methanol was added and the mixture was acidified using 85 ml of 6 M hydrochloric acid. The mixture was concentrated under reduced pressure. After cooling the concentrated mixture in ice, the precipitate obtained was filtered, washing with cold water and dried to give **17** in 97% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.94 (s, 1H), 7.34 – 7.24 (m, 5H), 6.43 (s, 2H), 4.85 (s, 2H), 4.14 (t, J = 6.6 Hz, 2H), 1.65 – 1.58 (m, 2H), 1.41 – 1.32 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H).



13 **17**

Scheme 20. Microwave Synthesis of **17**

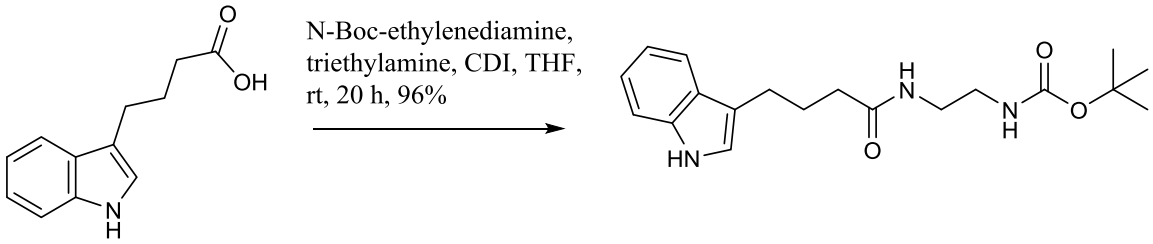
*9-Benzyl-2-butoxy-8-hydroxyadenine (**17**).* To **13** (0.32 g, 0.85 mmol) in a CEM Discover Microwave reaction vessel was added sodium hydroxide (6.4 ml, 10 M, 64 mmol) and methanol (17.0 ml). The mixture was heated with stirring at 105°C (42 psi) for 1.5 hours. The solution was cooled to room temperature and poured to a 250 ml round bottom flask. 20 ml of methanol was added and the mixture was acidified using 11 ml of 6 M hydrochloric acid. The mixture was concentrated under reduced pressure. After cooling the concentrated mixture in ice, the precipitate obtained was filtered, washing with cold water and dried to give **17** in 80% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.94 (s, 1H), 7.34 – 7.24 (m, 5H), 6.43 (s, 2H), 4.85 (s, 2H), 4.14 (t, J = 6.6 Hz, 2H), 1.65 – 1.58 (m, 2H), 1.41 – 1.32 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H).



Scheme 21. Synthesis of **18**

*2-Butoxy-8-hydroxy-9-(4-iodobenzyl)adenine (**18**).* To **16** (0.33 g, 0.66 mmol) in a CEM Discover Microwave reaction vessel was added sodium hydroxide (5.8 ml, 10 M, 58 mmol) and methanol (5.6 ml). The mixture was heated with stirring at 105°C (42 psi) for 2.5 hours. The solution was cooled to room temperature and poured to a 250 ml round bottom flask. 20 ml of methanol was added and the mixture was acidified using 11 ml of 6 M hydrochloric acid. The mixture was concentrated under reduced pressure. After cooling the concentrated mixture in ice, the precipitate obtained was filtered, washing with cold

water and dried to give **18** in 83% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.95 (s, 1H), 7.68 (d, J = 8.3 Hz, 2H), 7.09 (d, J = 8.3 Hz, 2H), 6.44 (s, 2H), 4.80 (s, 2H), 4.12 (t, J = 6.6 Hz, 2H), 1.64 – 1.57 (m, 2H), 1.41 – 1.32 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H).

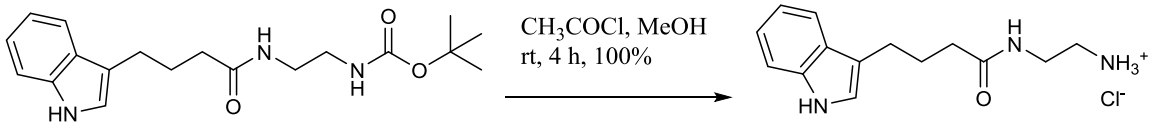


19

Scheme 22. Synthesis of **19**

*Tert-Butyl (2-(4-(1H-indol-3-yl)butanamido)ethyl)carbamate (**19**).* Indole-3-butyric acid (0.3 g, 1.48 mmol) and 1,1'-carbonyldiimidazole (0.26 g, 1.6 mmol) were dissolved in THF (5 ml) and stirred under nitrogen for 20 minutes. Triethylamine (0.5 ml, 3.6 mmol) and N-Boc-ethylenediamine (0.24 g, 1.48 mmol) were added and the mixture was stirred at room temperature for 20 hours. The mixture was evaporated, leaving a yellow liquid in the bottom of flask. 40 ml of water was added and the mixture was extracted with dichloromethane (3 x 50 ml). After washing the combined organic phase with 1 M sodium hydroxide (3 x 30 ml) and drying with magnesium sulfate the solvent was removed under reduced pressure to give the product in 96% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.99 (s, 1H), 7.59 (dd, J = 7.8, 1.0 Hz, 1H), 7.35 (d, J = 8.1 Hz, 1H), 7.18 (t, J = 7.0 Hz, 1H), 7.10 (t, J = 7.0 Hz, 1H), 7.00 – 6.99 (m, 1H), 6.01 (s, 1H), 4.87 (s, 1H), 3.33 – 3.31 (m, 2H), 3.24 – 3.23 (m, 2H), 2.81 (td, J = 7.4, 0.9 Hz, 2H), 2.24 (t, J = 7.5 Hz, 2H), 2.06 (q, J = 7.5 Hz, 2H), 1.42 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.0, 157.0, 136.5, 127.6, 122.0,

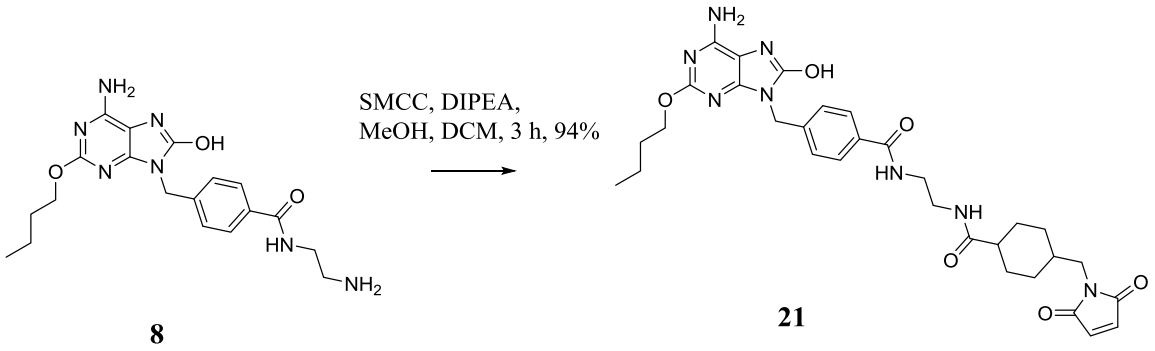
121.7, 119.3, 119.0, 115.6, 111.3, 79.8, 40.6, 40.5, 36.2, 28.5, 26.1, 24.6; HRMS (+ESI) calcd for $C_{19}H_{28}N_3O_3$ (MH^+) 346.2125, found 346.2119 (Δ 1.7 ppm).



19 **20**

Scheme 23. Synthesis of **20**

*N-(2-Aminoethyl)-4-(1H-indol-3-yl)butanamide. (**20**).* Acetyl chloride (0.23 ml, 3.2 mmol) was added to methanol (0.73 ml, 18.0 mmol) at 0ºC. The solution was stirred for 15 minutes at 0ºC. A solution of **19** (0.08 g, 0.2 mmol) in methanol (4 ml) was then added to the prepared hydrochloric acid solution (from acetyl chloride and methanol) at 0ºC. The resulting mixture was stirred for an additional 4 hours at room temperature. Methanol was then slowly evaporated under vacuum, without heating. Compound **20** was obtained as its hydrochloride salt in a yield of 100%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.90 – 10.71 (m, 1H), 8.11 (t, J = 5.6 Hz, 1H), 8.00 (s, 3H), 7.49 (d, J = 7.9 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.11 - 7.10 (m, 1H), 7.05 (ddd, J = 8.1, 6.9, 1.2 Hz, 1H), 6.96 (td, J = 7.4, 6.9, 1.0 Hz, 1H), 3.28 (q, J = 6.2 Hz, 2H), 2.83 (q, J = 6.1 Hz, 2H), 2.66 (t, J = 7.5 Hz, 2H), 2.16 (t, J = 7.6 Hz, 2H), 1.87 (p, J = 7.6 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.9, 136.3, 127.2, 122.3, 120.8, 118.3, 118.1, 114.1, 111.4, 38.6, 36.4, 35.2, 25.9, 24.4; HRMS (+ESI) calcd for $C_{14}H_{20}N_3O$ (MH^+) 246.1601, found 246.1609 (Δ 3.2 ppm).

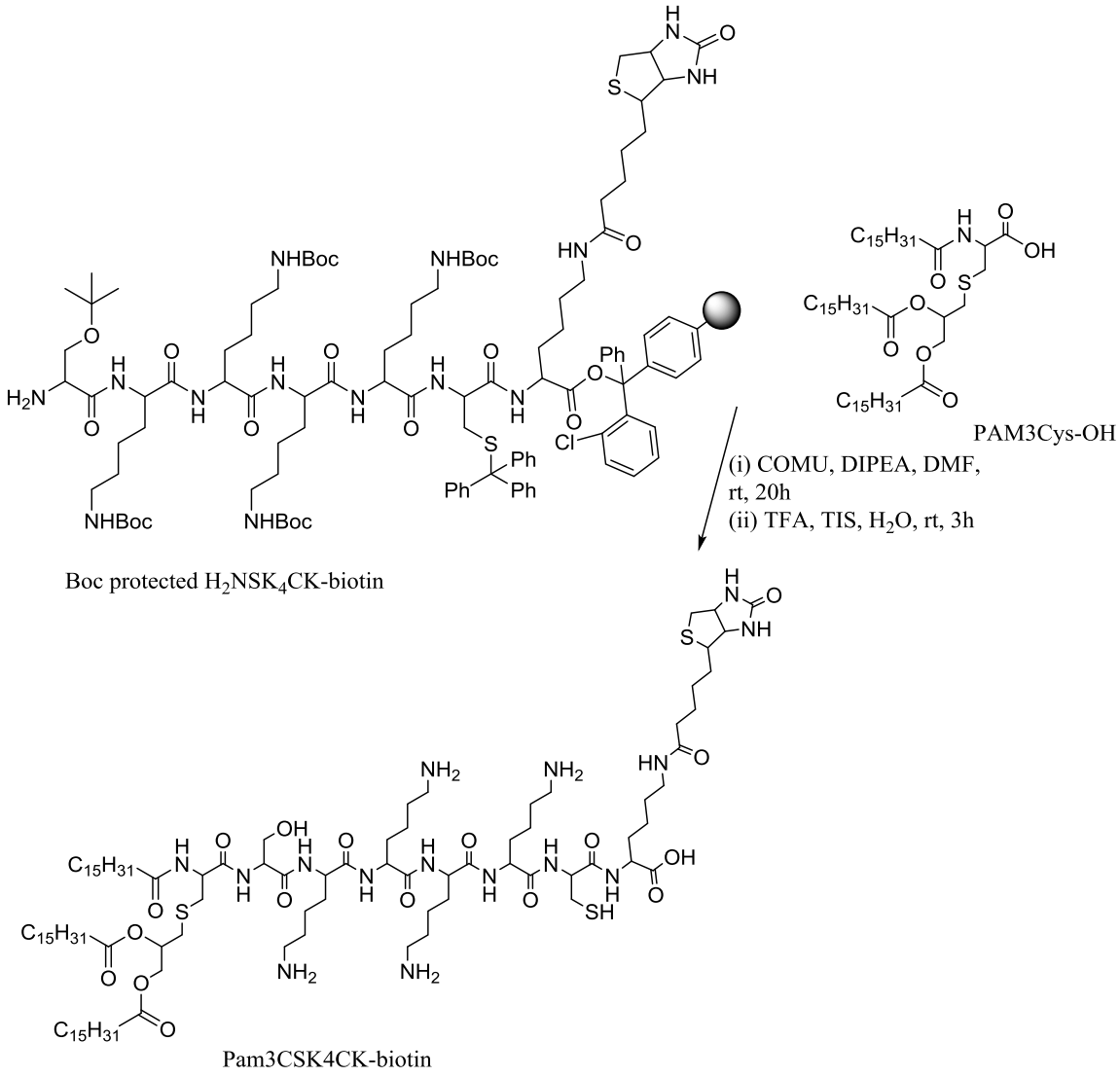


8 **21**

Scheme 24. Synthesis of **21**

*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(2-(4-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)cyclohexanecarboxamido)ethyl)benzamide(**21**).*

Compound **8** (23 mg, 0.045 mmol) and SMCC (15 mg, 0.045 mmol) were dissolved in dichloromethane (1 ml) and methanol (4 ml). *N*,*N*-diisopropylethylamine (10 μL, 0.057 mmol) was added and the mixture was stirred at room temperature for 3 hours. After evaporating the sample, the residue obtained was purified by flash chromatography (5 to 20% methanol/CH_2Cl_2) to give **21** in 94% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.03 (s, 1H), 8.41 (t, J = 5.7 Hz, 1H), 7.79 (t, J= 5.8 Hz, 1H), 7.76 (d, J = 8.3 Hz, 2H), 7.34 (d, J = 8.3 Hz, 2H), 7.00 (s, 2H), 6.48 (s, 2H), 4.90 (s, 2H), 4.13 (t, J = 6.6 Hz, 2H), 3.28-3.15 (m, 6H), 2.03-1.95 (m, 1H), 1.73-1.67 (m, 2H), 1.65-1.57 (m, 4H), 1.54-1.48 (m, 1H), 1.41-1.25 (m, 4H), 0.92-0.84 (m, 5H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.2, 171.2, 166.0, 160.1, 152.2, 149.1, 147.9, 140.2, 134.3, 133.6, 127.4, 127.1, 98.3, 65.8, 43.8, 43.0, 42.1, 38.1, 36.1, 30.6, 29.4, 28.5, 25.2, 18.7, 13.7; HRMS (–ESI) calcd for $C_{31}H_{37}N_8O_6$ ($M\text{-}H^+$) 617.2842, found 617.2849 (Δ 1.1 ppm).



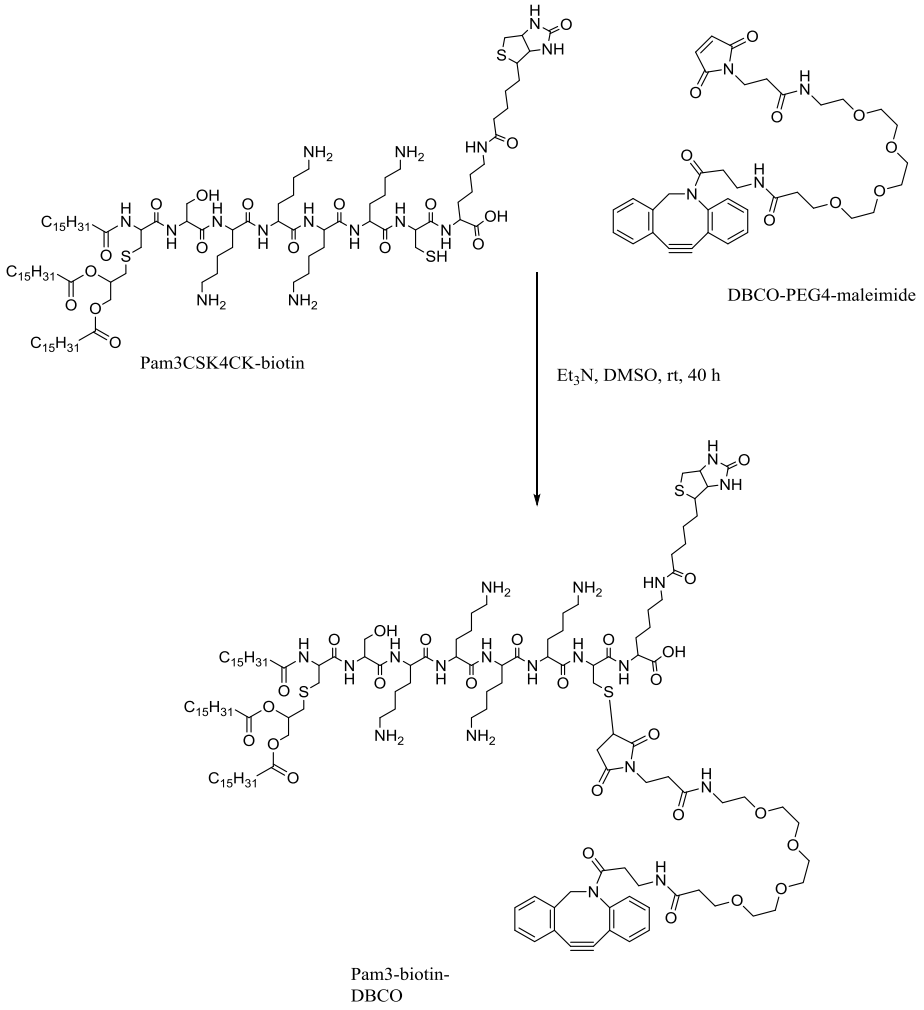
Scheme 25. Synthesis of **22**

Peptide Coupling between H_2NSK_4CK-biotin resin and PAM3Cys-OH. PAM3Cys-OH (60 mg, 0.07 mmol) was dissolved with 0.6 ml dichloromethane (by sonication) in a clean reaction vial. N,N-diisopropylethylamine (0.02 ml, 0.11 mmol), COMU (28 mg, 0.07 mmol) and DMF (0.2 ml) were added. After further sonication of the reaction mixture, the reaction vial was allowed to stand for 20 minutes. H_2NSK_4CK-biotin resin (14.1 mg) was added and reaction was allowed for 20 hours with occasional

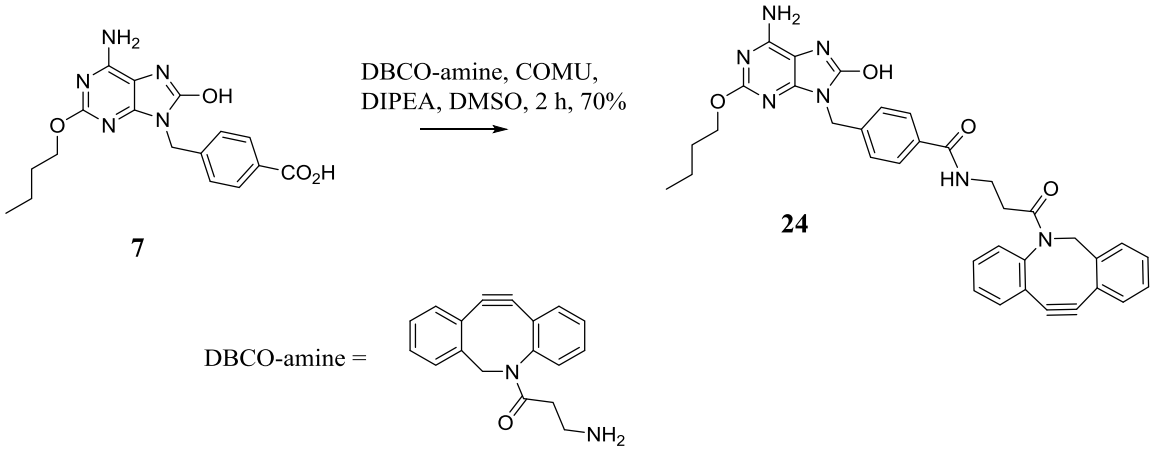
swirling. The resin was filtered (using DMF to rinse on a fritted glass funnel) and transferred to another vial. The cleaving cocktail (561 µL TFA, 31 µL water, 18 µL triisopropylsilane) was added to the resin in this vial. After swirling the vial occasionally for 3 hours, the resin was filtered using a glass-wool-fitted Pasteur pipette and the filtrate was evaporated to give 7.3 mg of product (Pam3CSK4CK-biotin). HRMS (+ESI) calcd for $C_{100}H_{190}N_{15}O_{17}S_3$ ($M+3H^+/3$) 656.45, found 656.79; LC-MS RT: 6.22 min, 99.9%.

*Synthesis of Pam3-biotin-DBCO (**23**).* DBCO-PEG4-maleimide (2 mg, 3 µmol) was dissolved in 0.4 ml DMSO. Synthesized Pam3CSK4CK-biotin (7.3 mg, 3 µmol) and triethylamine (7.3 µL, 52 µmol) were added. The mixture was stirred for 40 hours at room temperature and the resulting solution was analyzed by LC-MS. HRMS (+ESI) calcd for $C_{136}H_{232}N_{19}O_{26}S_3$ ($M+3H^+/3$) 881.22, found 881.22; LC-MS RT: 15.30 min, 86.9%.

*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl) methyl benzoic acid (**7**) with Dibenzocyclooctyne-amine linker (product **24**).* DBCO-amine (50 mg, 0.18 mmol) and *N,N*-diisopropylethylamine (42 µL, 0.24 mmol) were added to a solution of **7** (43 mg, 0.12 mmol) in DMSO (1.4 ml). After placing the reaction flask in ice, COMU (77 mg, 0.18 mmol) was added. The flask was allowed to warm to room temperature while stirring and the reaction was continued for 2 hours. The reaction mixture was poured into ethyl acetate (12 ml) and refrigerated (4°C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (7.8 ml) was added and the

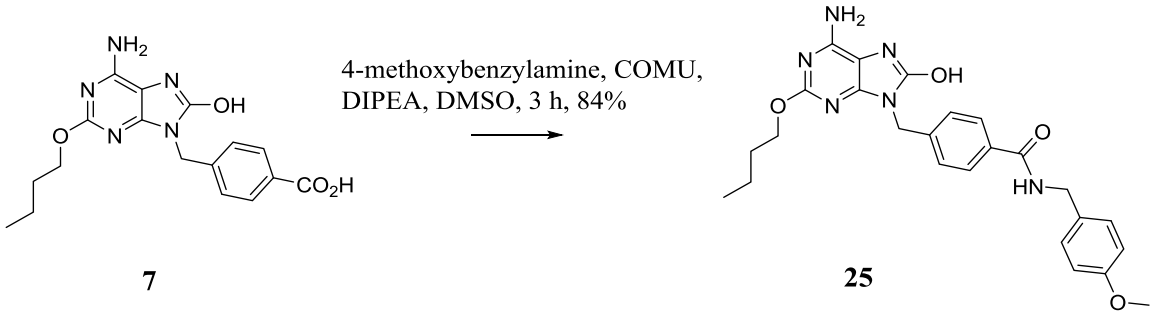


Scheme 26. Synthesis of **23**



Scheme 27. Synthesis of **24**

contents were mixed and allowed to settle. After concentrating the mixture in vacuo, the flask was cooled at 0°C for one hour. The solid obtained was filtered under vacuum and purified by flash chromatography (7% methanol/CH_2Cl_2) to give compound **24** in 70% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.98 (s, 1H), 8.20 (t, J = 5.6 Hz, 1H), 7.68 – 7.52 (m, 4H), 7.50 – 7.46 (m, 1H), 7.46 – 7.36 (m, 3H), 7.34 – 7.25 (m, 3H), 7.20 (dd, J = 7.5, 1.1 Hz, 1H), 6.47 (s, 2H), 5.05 (d, J = 14.1 Hz, 1H), 4.87 (s, 2H), 4.11 (t, J = 6.6 Hz, 2H), 3.62 (d, J = 14.0 Hz, 1H), 3.33 – 3.29 (m, 1H), 3.10 (dt, J = 13.7, 6.0 Hz, 1H), 2.56 – 2.52 (m, 1H), 2.00 – 1.94 (m, 1H), 1.64 – 1.56 (m, 2H), 1.35 (dq, J = 14.8, 7.4 Hz, 2H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.2, 165.7, 160.1, 152.2, 151.4, 149.2, 148.3, 147.8, 140.1, 133.5, 132.4, 129.5, 128.9, 128.2, 128.0, 127.7, 127.3, 127.1, 126.8, 125.2, 122.5, 121.4, 114.33, 108.1, 98.3, 65.9, 54.8, 48.0, 42.1, 35.8, 34.2, 30.6, 18.8, 13.7; HRMS (+ESI) calcd for $C_{35}H_{34}N_7O_4$ (MH^+) 616.2667, found 616.2676 (Δ 1.5 ppm); (-ESI) calcd for $C_{35}H_{32}N_7O_4$ ($M\text{-}H^+$) 614.2521, found 614.2534 (Δ 2.1 ppm).

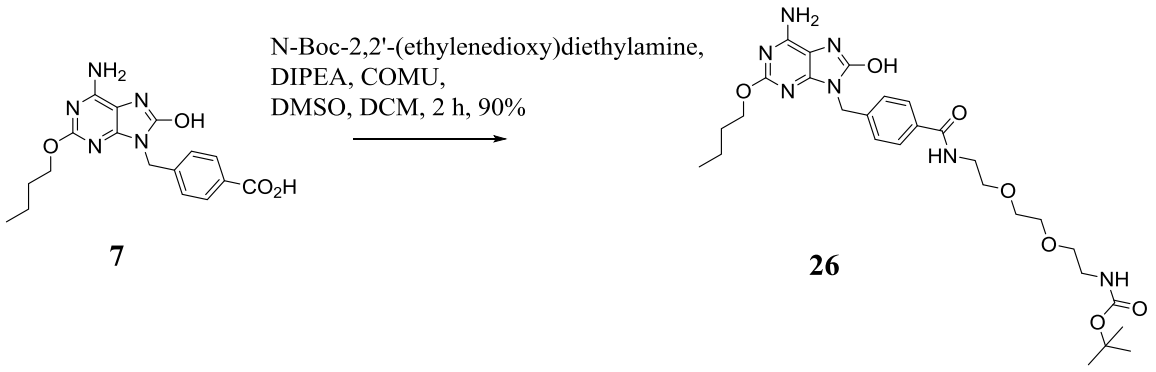


7 **25**

Scheme 28. Synthesis of **25**

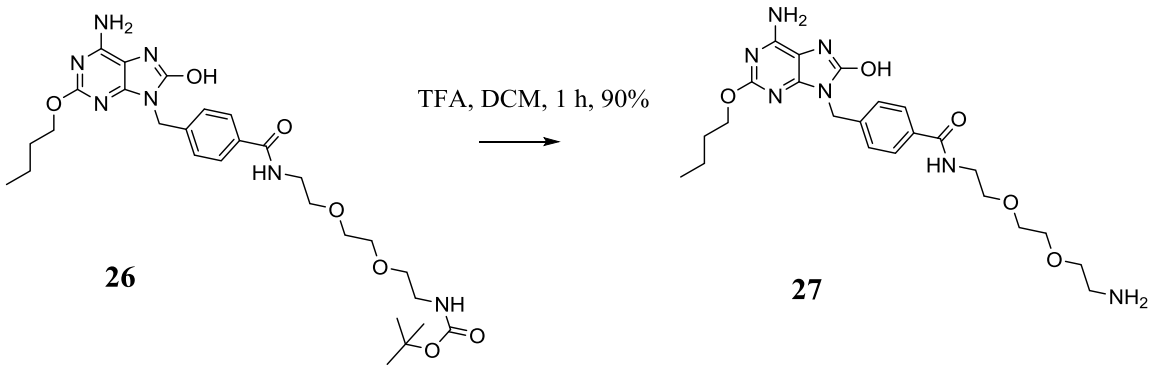
*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(4-methoxybenzyl)-benzamide (**25**).* 4-Methoxybenzylamine (16.5 µL, 0.13 mmol) and *N,N*-diisopropyl-

ethylamine (29.2 µL, 0.17 mmol) were added to a solution of **7** (30 mg, 0.08 mmol) in DMSO (1.0 ml). After placing the reaction flask in ice, COMU (54 mg, 0.13 mmol) was added. The flask was allowed to warm to room temperature while stirring and the reaction was continued for 3 hours. The reaction mixture was poured into ethyl acetate (8 ml) and refrigerated (4°C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (5.5 ml) was added and the contents were mixed and allowed to settle. After concentrating the mixture in vacuo and cooling, the solid obtained was filtered under vacuum, washing with ice-cold water, to give the product in 84% yield. ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 8.93 (t, *J* = 5.9 Hz, 1H), 7.81 (d, *J* = 8.2 Hz, 2H), 7.33 (d, *J* = 8.2 Hz, 2H), 7.21 (d, *J* = 8.6 Hz, 2H), 6.86 (d, *J* = 8.6 Hz, 2H), 4.90 (s, 2H), 4.38 (d, *J* = 5.9 Hz, 2H), 4.10 (t, *J* = 6.6 Hz, 2H), 3.71 (s, 3H), 1.63 – 1.57 (m, 2H), 1.39 – 1.33 (m, 2H), 0.89 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-*d*$_6$) δ 165.8, 158.1, 149.4, 149.4, 148.4, 140.9, 133.4, 131.6, 128.5, 127.4, 127.1, 126.9, 113.7, 109.6, 65.6, 55.0, 42.0, 39.5, 30.7, 18.8, 13.8; HRMS (+ESI) calcd for $C_{25}H_{29}N_6O_4$ (MH^+) 477.2245, found 477.2245 (Δ 0.0 ppm).



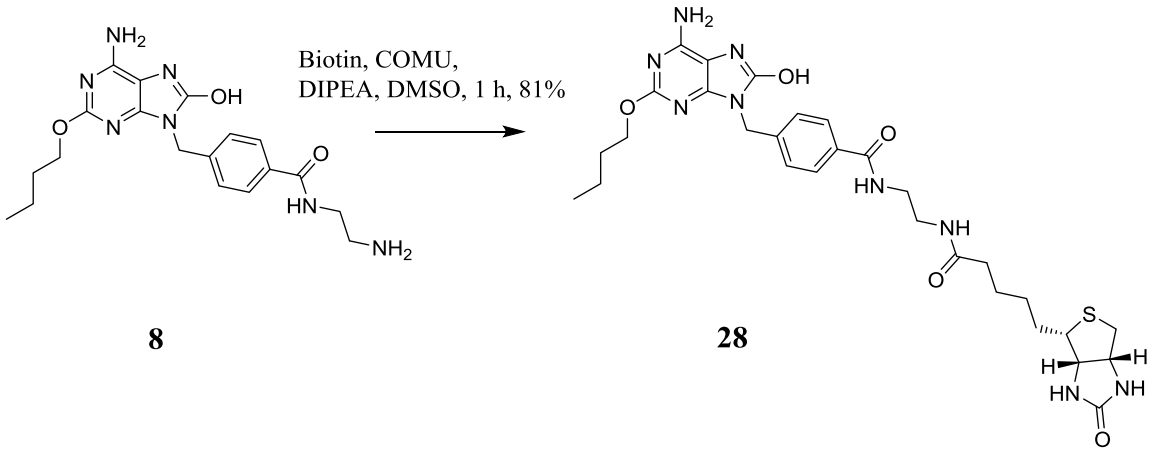
Scheme 29. Synthesis of **26**

*Tert-butyl (2-(2-(2-(4-((6-amino-2-butoxy-8-hydroxy- 9H-purin-9-yl) methyl) benzamido) ethoxy)ethoxy)ethyl)carbamate (**26**).* Compound **7** (0.23 g, 0.6 mmol) was dissolved in DMSO (9.2 ml). *N*-Boc-2,2'-(ethylenedioxy)diethylamine (0.49 g, 2.0 mmol) and *N*,*N*-diisopropylethylamine (0.56 ml, 3.2 mmol) were mixed and dissolved with dichloromethane (0.3 ml) in a vial and added with a syringe to the stirring solution of **7** in DMSO. After placing the reaction flask in ice, COMU (0.7 g, 1.6 mmol) was added. The flask was allowed to warm to room temperature and stirring was continued for 2 hours. The reaction mixture was poured into ethyl acetate (60 ml) and cooled (4°C) for an hour. The mixture was then poured into a 250 ml glass-capped round-bottom flask. 1N sodium bicarbonate (40 ml) was added and the contents were mixed and allowed to settle. After concentrating the mixture in vacuo, the flask was cooled at 0°C for one hour. The solid obtained was filtered under vacuum, washing with ice-cold water and oven-dried to give the product in 90% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.99 (s, 1H), 8.49 (t, J = 5.6 Hz, 1H), 7.78 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 6.77 (t, J = 5.5 Hz, 1H), 6.48 (s, 2H), 4.90 (s, 2H), 4.12 (t, J = 6.6 Hz, 2H), 3.52 – 3.47 (m, 6H), 3.41 – 3.35 (m, 4H), 3.04 (dd, J = 11.9, 6.0 Hz, 2H), 1.63 – 1.58 (m, 2H), 1.39 – 1.33 (m, 11H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.0, 160.1, 155.6, 152.2, 149.1, 147.8, 140.2, 133.6, 127.4, 127.2, 98.3, 77.6, 69.6, 69.5, 69.2, 68.9, 65.9, 42.1, 39.5, 30.6, 28.2, 18.8, 13.7; HRMS (-ESI) calcd for $C_{28}H_{40}N_7O_7$ ($M-H^+$) 586.2995, found 586.2983 (Δ 2.0 ppm).



Scheme 30. Synthesis of **27**

*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(2-(2-(2-aminoethoxy)-ethoxy)ethyl)benzamide (**27**).* To **26** (0.179 g, 0.3 mmol) were added trifluoroacetic acid (0.47 ml, 6.1 mmol) and dichloromethane (2.50 ml). After stirring at room temperature for 1 hour, the reaction mixture was evaporated until a viscous oil was left. Methanol was added to dissolve the oil and the mixture was re-evaporated. After cooling the residue, the product was precipitated by mixing the residue thoroughly with diethyl ether with sonication (dissolving residual dark brown oil with drops of methanol). After cooling for an hour at 4°C, the product was filtered and oven-dried to obtain the TFA salt in 90% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.04 (s, 1H), 8.50 (t, J = 5.6 Hz, 1H), 7.78 (d, J = 8.2 Hz, 2H), 7.74 (s, 2H), 7.34 (d, J = 8.2 Hz, 2H), 6.50 (s, 2H), 4.90 (s, 2H), 4.12 (t, J = 6.6 Hz, 2H), 3.56 (s, 6H), 3.52 (t, J = 6.1 Hz, 2H), 3.41 (dd, J = 11.8, 5.9 Hz, 2H), 2.95 (d, J = 4.7 Hz, 2H), 1.63 – 1.58 (m, 2H), 1.39 – 1.33 (m, 2H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.0, 160.1, 152.3, 149.2, 147.8, 140.3, 133.5, 127.4, 127.2, 98.3, 69.7, 69.5, 68.9, 66.7, 65.9, 42.1, 39.5, 38.6, 30.6, 18.8, 13.7; HRMS (+ESI) calcd for $C_{23}H_{34}N_7O_5$ (MH^+) 488.2616, found 488.2616 (Δ 0.0 ppm); (-ESI) calcd for $C_{23}H_{32}N_7O_5$ ($M\text{-}H^+$) 486.2470, found 486.2466 (Δ 0.8 ppm).

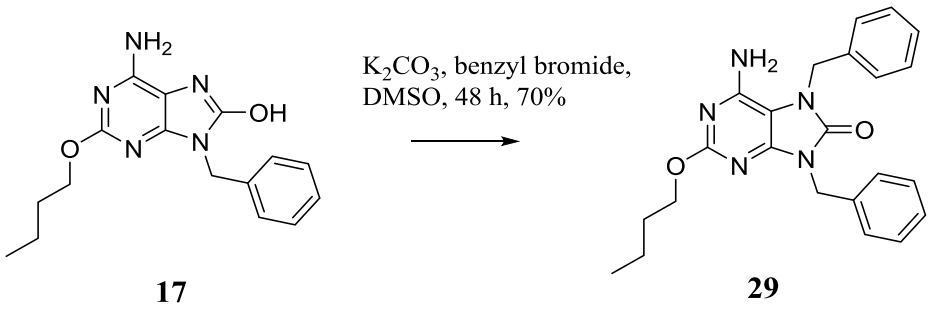


8 **28**

Scheme 31. Synthesis of **28**

*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethyl)benzamide (**28**).* Biotin (49.8 mg, 0.20 mmol) and N,N-diisopropylethylamine (48 µL, 0.28 mmol) were added to a solution of **8** (70.0 mg, 0.14 mmol) in DMSO (2.8 ml). After placing the reaction flask in ice, COMU (87.4 mg, 0.20 mmol) was added. The flask was allowed to warm to room temperature while stirring and the reaction was continued for 1 hour. The reaction mixture was poured into ethyl acetate (15 ml) and refrigerated (4°C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (8.7 ml) was added and the contents were mixed and allowed to settle. Partial evaporation was carried out to remove ethyl acetate. The flask was cooled at 0°C for one hour. The solid obtained was filtered under vacuum, washing with ice-cold water and dried to give the product in 81% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 (s, 1H), 7.90 (s, 1H), 7.77 (d, J = 8.0 Hz, 2H), 7.34 (d, J = 8.1 Hz, 2H), 6.57 (s, 2H), 6.42 (s, 1H), 6.35 (s, 1H), 4.90 (s, 2H), 4.31 – 4.26 (m, 1H), 4.15 – 4.07 (m, 3H), 3.28 (d, J = 5.8 Hz, 2H), 3.19 (d, J = 5.7 Hz, 2H), 3.07 – 3.01 (m, 1H), 2.89 – 2.76 (m, 2H), 2.05 (t, J = 7.4 Hz, 2H), 1.60 (dd, J = 14.2, 7.0 Hz, 3H),

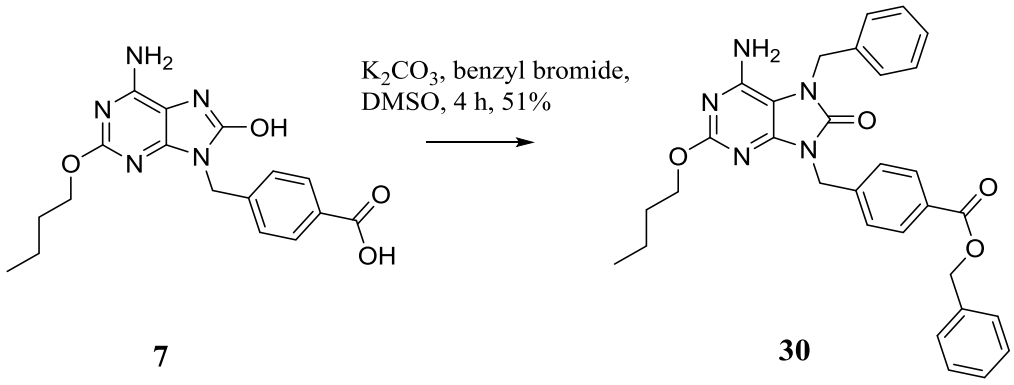
1.53 – 1.45 (m, 2H), 1.36 (dd, *J* = 14.8, 7.4 Hz, 2H), 1.31 – 1.22 (m, 2H), 0.89 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-*d*$_6$) δ 172.4, 166.0, 162.7, 160.0, 152.7, 149.2, 148.0, 140.3, 133.6, 127.4, 127.2, 65.8, 61.0, 59.2, 55.4, 42.1, 40.4, 38.2, 35.3, 30.6, 28.2, 28.0, 25.2, 18.8, 13.8; HRMS (+ESI) calcd for $C_{29}H_{39}N_9NaO_5S$ ($M+Na^+$) 648.2687, found 648.2684 (Δ 0.5 ppm).



17 **29**

Scheme 32. Synthesis of **29**

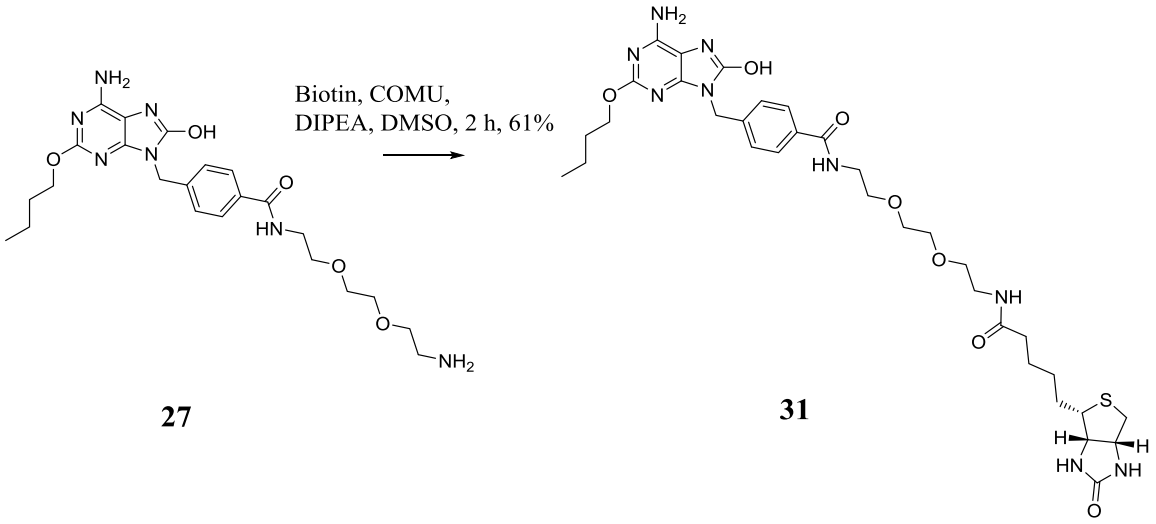
*6-Amino-7,9-dibenzyl-2-butoxy-7,9-dihydro-8H-purin-8-one (**29**).* To **17** (0.12 g, 0.38 mmol), dissolved in DMSO (7 ml), was added potassium carbonate (0.20 g, 1.45 mmol) and benzyl bromide (0.1 ml, 0.84 mmol). The mixture was stirred for 48 hours at room temperature, poured into 22 ml of water and cooled (4°C) for 4 hours. The precipitate obtained was filtered and boiled in diethyl ether (mixing thoroughly). After cooling, the product was filtered (70% yield). ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 7.35 – 7.16 (m, 10H), 6.55 (s, 2H), 5.21 (s, 2H), 4.95 (s, 2H), 4.13 (t, *J* = 6.6 Hz, 2H), 1.64 – 1.58 (m, 2H), 1.39 – 1.32 (m, 2H), 0.89 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-*d*$_6$) δ 160.0, 153.0, 149.4, 148.3, 137.9, 137.0, 128.6, 128.5, 127.5, 127.4, 126.7, 99.3, 65.9, 44.2, 42.9, 30.6, 18.7, 13.7; HRMS (+ESI) calcd for $C_{23}H_{26}N_5O_2$ (MH^+) 404.2081, found 404.2080 (Δ 0.2 ppm).



7 **30**

Scheme 33. Synthesis of **30**

*Benzyl 4-((6-amino-7-benzyl-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl) methyl) benzoate (**30**).* To **7** (0.40 g, 1.1 mmol), dissolved in DMSO (23 ml), was added potassium carbonate (0.93 g, 6.7 mmol) and benzyl bromide (0.25 ml, 2.1 mmol). The mixture was stirred for 4 hours at room temperature, diluted with water (102 ml), acidified with 4M HCl (19 ml) and filtered, rinsing with cold water. The solid obtained was boiled in diethyl ether (mixing thoroughly). After cooling, the product was filtered (51% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.96 (d, J = 8.0 Hz, 2H), 7.47 – 7.16 (m, 12H), 6.57 (s, 2H), 5.34 (s, 2H), 5.21 (s, 2H), 5.03 (s, 2H), 4.11 (t, J = 6.5 Hz, 2H), 1.61 – 1.54 (m, 2H), 1.36 – 1.30 (m, 2H), 0.86 (t, J = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.3, 160.0, 152.9, 149.3, 148.4, 142.5, 137.8, 136.1, 129.6, 128.7, 128.6, 128.5, 128.1, 127.9, 127.6, 127.4, 126.7, 99.3, 66.1, 65.9, 44.3, 42.7, 30.5, 18.7, 13.7; HRMS (+ESI) calcd for $C_{31}H_{32}N_5O_4$ (MH^+) 538.2449, found 538.2449 (Δ 0.0 ppm).

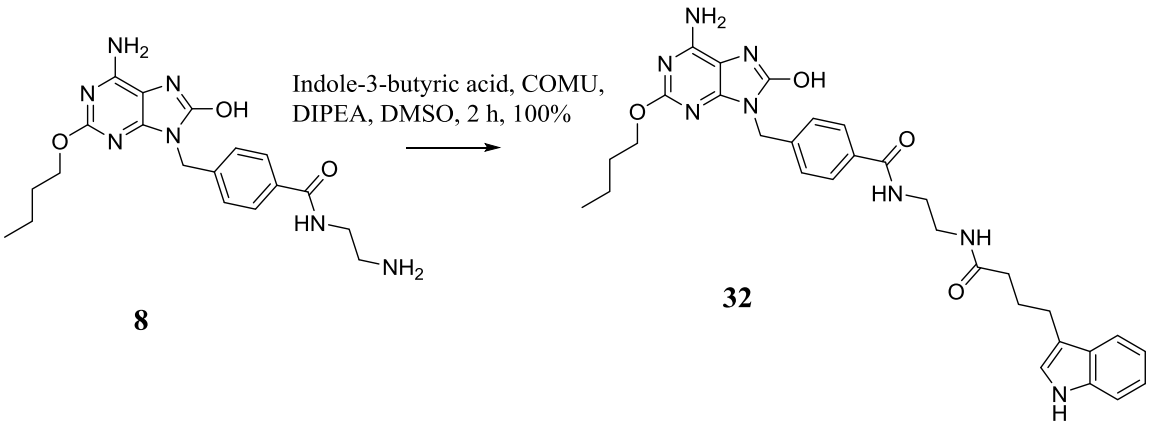


27 **31**

Scheme 34. Synthesis of **31**

*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(2-(2-(2-(5-((3aS,4S, 6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)-ethoxy) ethyl)benzamide (**31**).* Biotin (53.6 mg, 0.22 mmol) and *N,N*-diisopropylethylamine (50 µL, 0.29 mmol) were added to a solution of **27** (67.0 mg, 0.11 mmol) in DMSO (2.3 ml). After placing the reaction flask in ice, COMU (80.0 mg, 0.19 mmol) was added. The flask was allowed to warm to room temperature while stirring and the reaction was continued for 2 hours. The reaction mixture was poured into ethyl acetate (10 ml) and refrigerated (4°C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (7 ml) was added and the contents were mixed and allowed to settle. Partial evaporation was carried out to remove ethyl acetate. The flask was cooled at 0°C for one hour. The solid obtained was filtered under vacuum, washing with ice-cold water and dried to give the product in 61% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.14 (s, 1H), 8.51 (t, J = 5.5 Hz, 1H), 7.85 (t, J = 5.5 Hz, 1H), 7.79 (d, J = 8.1 Hz, 2H), 7.34 (d, J = 8.1 Hz, 2H), 6.56 (s, 2H), 6.44 (s, 1H), 6.38 (s, 1H), 4.90 (s, 2H), 4.31 – 4.27 (m, 1H), 4.12 (t, J = 6.5 Hz,

3H), 3.53 – 3.48 (m, 6H), 3.40 – 3.37 (m, 4H), 3.19 – 3.14 (m, 2H), 3.10 – 3.05 (m, 1H), 2.80 (dd, *J* = 12.4, 5.0 Hz, 1H), 2.56 (d, *J* = 12.4 Hz, 1H), 2.05 (t, *J* = 7.4 Hz, 2H), 1.64 – 1.56 (m, 3H), 1.52 – 1.43 (m, 3H), 1.35 (dd, *J* = 14.9, 7.4 Hz, 2H), 1.31 – 1.23 (m, 2H), 0.89 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.1, 166.0, 162.7, 159.7, 149.3, 148.2, 133.4, 127.4, 127.1, 69.6, 69.2, 68.9, 65.7, 61.0, 59.2, 55.4, 42.1, 40.4, 38.4, 35.1, 30.7, 28.2, 28.0, 25.3, 18.8, 13.8; HRMS (+ESI) calcd for $C_{33}H_{48}N_9O_7S$ (MH^+) 714.3392, found 714.3401 (Δ 1.3 ppm).

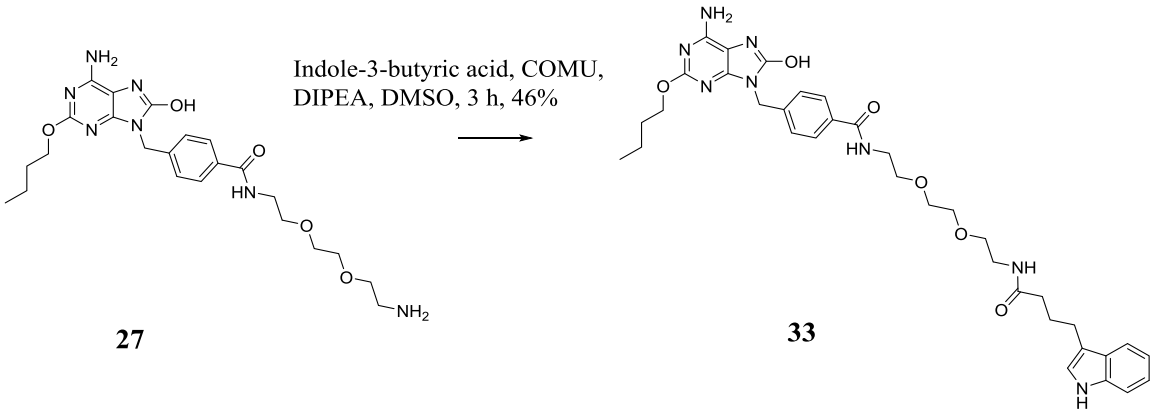


8 **32**

Scheme 35. Synthesis of **32**

*N-(2-(4-(1H-indol-3-yl)butanamido)ethyl)-4-((6-amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)benzamide (**32**).* Indole-3-butyric acid (0.12 g, 0.59 mmol) and *N,N*-diisopropylethylamine (0.16 ml, 0.92 mmol) were added to a solution of **8** (0.20 g, 0.39 mmol) in DMSO (8 ml). After placing the reaction flask in ice, COMU (0.25 g, 0.58 mmol) was added. The flask was allowed to warm to room temperature while stirring and the reaction was continued for 2 hours. The reaction mixture was poured into ethyl acetate (36 ml) and refrigerated (4°C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (25 ml) was added and the contents were

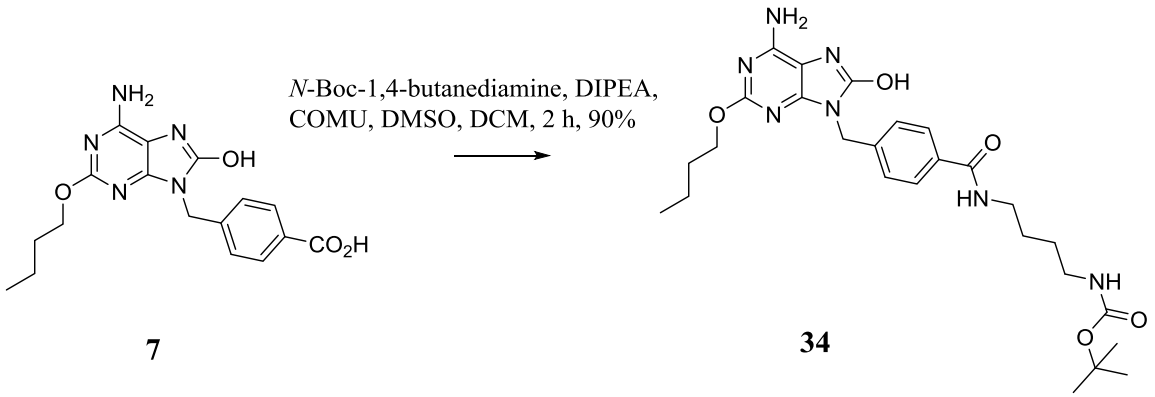
mixed and allowed to settle. Partial evaporation was carried out to remove ethyl acetate. The flask was cooled at 0°C for one hour. The solid obtained was filtered under vacuum, washing with ice-cold water and dried to give the product in 100% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.75 (s, 1H), 10.02 (s, 1H), 8.46 (t, J = 5.5 Hz, 1H), 7.93 (t, J = 5.7 Hz, 1H), 7.76 (d, J = 8.2 Hz, 2H), 7.47 (d, J = 7.8 Hz, 1H), 7.32 (dd, J = 11.8, 8.2 Hz, 3H), 7.07 (s, 1H), 7.03 (t, J = 7.4 Hz, 1H), 6.93 (t, J = 7.4 Hz, 1H), 6.49 (s, 2H), 4.89 (s, 2H), 4.12 (t, J = 6.6 Hz, 2H), 3.28 (dd, J = 12.2, 6.2 Hz, 2H), 3.23 – 3.16 (m, 2H), 2.64 (t, J = 7.4 Hz, 2H), 2.12 (t, J = 7.5 Hz, 2H), 1.85 (dt, J = 14.7, 7.4 Hz, 2H), 1.63 – 1.57 (m, 2H), 1.35 (td, J = 14.9, 7.5 Hz, 2H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.5, 166.0, 160.1, 152.3, 149.1, 147.8, 140.2, 136.3, 133.6, 127.4, 127.2, 122.2, 120.8, 118.3, 118.1, 114.1, 111.3, 98.3, 65.9, 42.1, 38.2, 35.3, 30.6, 26.1, 24.3, 18.8, 13.7; HRMS (+ESI) calcd for $C_{31}H_{37}N_8O_4$ (MH^+) 585.2932, found 585.2932 (Δ 0.0 ppm); (-ESI) calcd for $C_{31}H_{35}N_8O_4$ ($M\text{-}H^+$) 583.2787, found 583.2783 (Δ 0.7 ppm).



Scheme 36. Synthesis of **33**

*N-(2-(2-(2-(4-(1H-indol-3-yl)butanamido)ethoxy)ethoxy)ethyl)-4-((6-amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)benzamide (**33**).* Indole-3-butyric acid (33.0 mg,

0.16 mmol) and *N*,*N*-diisopropylethylamine (44.3 μL, 0.25 mmol) were added to a solution of **27** (64.9 mg, 0.11 mmol) in DMSO (2.2 ml). After placing the reaction flask in ice, COMU (69 mg, 0.16 mmol) was added. The flask was allowed to warm to room temperature while stirring and the reaction was continued for 3 hours. The reaction mixture was poured into ethyl acetate (10 ml) and refrigerated (4°C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (7 ml) was added and the contents were mixed and allowed to settle. Partial evaporation was carried out to remove ethyl acetate. The flask was cooled at 0°C for one hour. The solid obtained was filtered under vacuum, washing with ice-cold water and dried to give the product in 46% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.75 (s, 1H), 10.03 (s, 1H), 8.49 (t, J = 5.3 Hz, 1H), 7.87 – 7.84 (m, 1H), 7.78 (d, J = 8.0 Hz, 2H), 7.48 (d, J = 7.8 Hz, 1H), 7.32 (dd, J = 14.8, 8.0 Hz, 3H), 7.08 (s, 1H), 7.04 (t, 1H), 6.94 (t, J = 7.4 Hz, 1H), 6.49 (s, 2H), 4.89 (s, 2H), 4.12 (t, J = 6.5 Hz, 2H), 3.53 – 3.46 (m, 5H), 3.39 – 3.36 (m, 3H), 3.34 – 3.31 (m, 2H), 3.17 (dd, J = 11.6, 5.7 Hz, 2H), 2.64 (t, J = 7.5 Hz, 2H), 2.12 (t, J = 7.4 Hz, 2H), 1.83 (dt, J = 15.1, 7.4 Hz, 2H), 1.63 – 1.57 (m, 2H), 1.35 (dt, J = 14.8, 7.3 Hz, 2H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.2, 166.0, 160.1, 152.2, 149.1, 147.8, 140.2, 136.3, 133.5, 127.4, 127.2, 122.2, 120.8, 118.3, 118.1, 114.1, 111.3, 98.3, 69.6, 69.5, 69.2, 68.9, 65.9, 42.1, 38.5, 35.2, 30.6, 26.1, 24.3, 18.8, 13.7; HRMS (+ESI) calcd for $C_{35}H_{44}N_8NaO_6$ ($M+Na^+$) 695.3276, found 695.3275 (Δ 0.1 ppm).

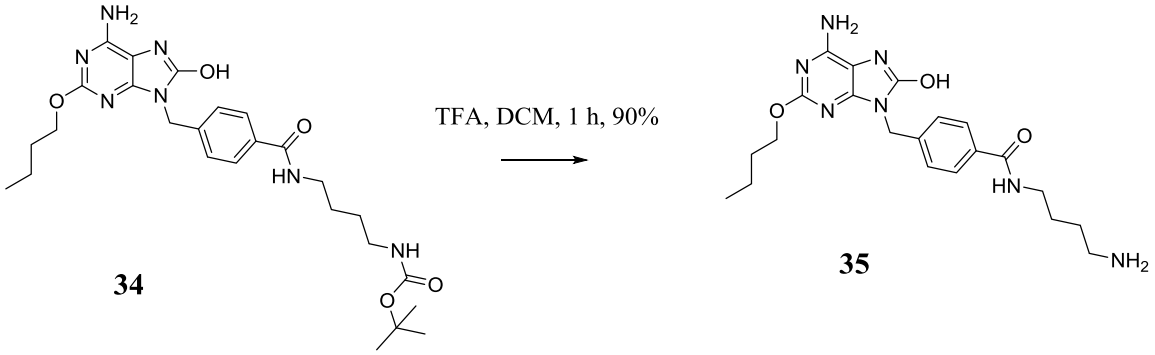


7 **34**

Scheme 37. Synthesis of **34**

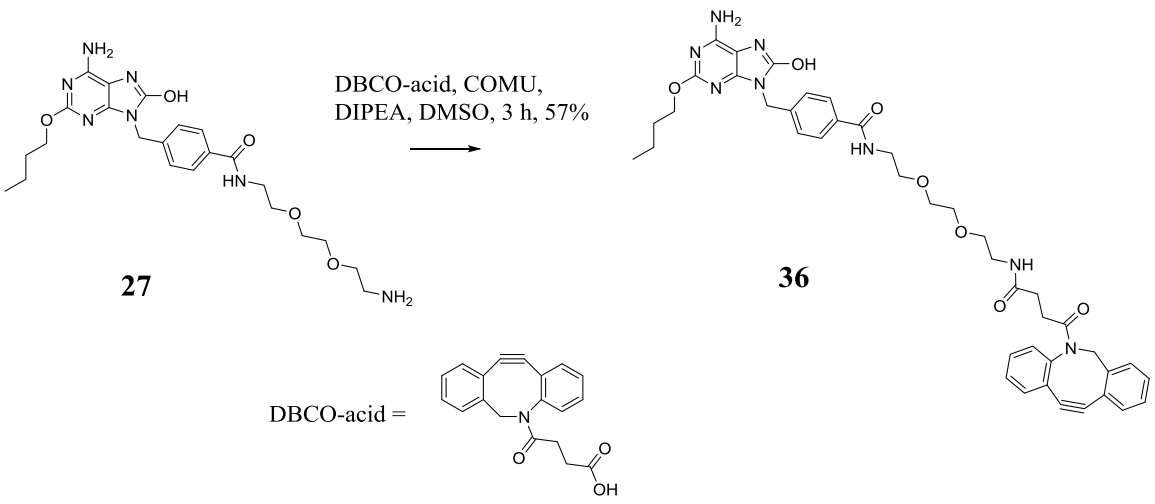
*Tert-Butyl (4-(4-((6-amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl) benzamido) butyl)carbamate (**34**).* Compound **7** (0.113 g, 0.3 mmol) was dissolved in DMSO (4.60 ml). *N*-Boc-1,4-butanediamine (0.189 g, 1.0 mmol) and *N,N*-diisopropylethylamine (0.27 ml, 1.6 mmol) were mixed and dissolved with dichloromethane (0.6 ml) in a vial and added with a syringe to the stirring solution of **7** in DMSO. After placing the reaction flask in ice, COMU (0.353 g, 0.8 mmol) was added. The flask was allowed to warm to room temperature and stirring was continued for 2 hours. The reaction mixture was poured into ethyl acetate (30 ml) and refrigerated (4^{o}C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (20 ml) was added and the contents were mixed and allowed to settle. After concentrating the mixture in vacuo and cooling, the solid obtained was filtered under vacuum, washing with ice-cold water, to give the product in 90% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.99 (s, 1H), 8.43 – 8.37 (m, 1H), 7.76 (d, J = 7.9 Hz, 2H), 7.33 (d, J = 7.9 Hz, 2H), 6.82 – 6.77 (m, 1H), 6.48 (s, 2H), 4.89 (s, 2H), 4.12 (t, J = 6.4 Hz, 2H), 3.24 – 3.18 (m, 2H), 2.93 – 2.88 (m, 2H), 1.63 – 1.58 (m, 2H), 1.48 – 1.43 (m, 2H), 1.38 – 1.33 (m, 13H), 0.89 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.8, 160.1, 155.6, 152.2, 149.1, 147.8, 140.1, 133.8, 127.4, 127.2, 98.3, 77.3, 65.9,

42.1, 39.5, 39.5, 30.6, 28.3, 27.1, 26.6, 18.8, 13.7; HRMS (+ESI) calcd for $C_{26}H_{38}N_7O_5$ (MH^+) 528.2929, found 528.2928 (Δ 0.2 ppm).



Scheme 38. Synthesis of **35**

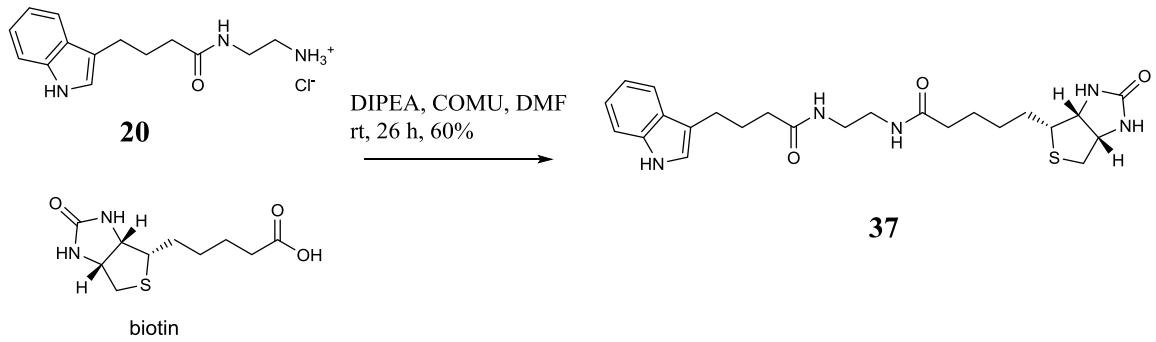
*4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(4-aminobutyl)benzamide (**35**).* To **34** (0.088 g, 0.17 mmol) was added dichloromethane (1.40 ml) and trifluroacetic acid (0.25 ml, 3.26 mmol) in a round-bottom flask. After stirring at room temperature for 1 hour the reaction mixture was evaporated until a viscous oil was left. Methanol was added to dissolve the oil and the mixture was re-evaporated. After cooling the residue, the product was precipitated by mixing the residue thoroughly with diethyl ether with sonication. After cooling further, the product was filtered and dried to obtain the TFA salt in 90% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.12 (s, 1H), 8.49 (t, J = 5.2 Hz, 1H), 7.78 (d, J = 8.0 Hz, 2H), 7.68 (s, 3H), 7.34 (d, J = 8.0 Hz, 2H), 6.54 (s, 2H), 4.90 (s, 2H), 4.12 (t, J = 6.5 Hz, 2H), 3.29 – 3.23 (m, 2H), 2.85 – 2.75 (m, 2H), 1.64 – 1.58 (m, 2H), 1.54 (s, 4H), 1.36 (dq, J = 14.6, 7.3 Hz, 2H), 0.89 (t, J = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.9, 159.8, 152.3, 149.1, 147.6, 140.2, 133.7, 127.4, 127.2, 98.3, 66.0, 42.2, 38.7, 30.6, 26.2, 24.6, 24.1, 18.8, 13.7; HRMS (+ESI) calcd for $C_{21}H_{30}N_7O_3$ (MH^+) 428.2405, found 428.2408 (Δ 0.7 ppm).



Scheme 39. Synthesis of **36**

4-((6-Amino-2-butoxy-8-hydroxy-9H-purin-9-yl)methyl)-N-(2-(2-(2-aminoethoxy)-ethoxy)ethyl)benzamide with Dibenzocyclooctyne-acid linker. DBCO-acid (33 mg, 0.108 mmol) and *N,N*-diisopropylethylamine (29.4 µL, 0.169 mmol) were added to a solution of **9** (45.0 mg, 0.075 mmol) in DMSO (1.6 ml). After placing the reaction flask in ice, COMU (46.3 mg, 0.108 mmol) was added. The flask was allowed to warm to room temperature while stirring and the reaction was continued for 3 hours. The reaction mixture was poured into ethyl acetate (7 ml) and cooled (4°C) for an hour. The mixture was then poured into a 100 ml glass-capped round-bottom flask. 1N sodium bicarbonate (5 ml) was added and the contents were mixed and allowed to settle. After concentrating the mixture in vacuo, the flask was cooled at 0°C for one hour. The solid obtained was filtered under vacuum, washing with ice-cold water and dried to give the product in 57% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.00 (s, 1H), 8.48 (t, J = 5.7 Hz, 1H), 7.78 (d, J = 7.9 Hz, 3H), 7.70 – 7.31 (m, 9H), 7.28 (d, J = 5.9 Hz, 1H), 6.48 (s, 2H), 5.01 (d, J = 14.1 Hz, 1H), 4.89 (s, 2H), 4.11 (t, J = 6.6 Hz, 2H), 3.60 (d, J = 14.0 Hz, 1H), 3.50 – 3.36 (m, 8H), 3.30 – 3.26 (m, 2H), 3.12 – 3.02 (m, 2H), 2.60 – 2.54 (m, 1H), 2.22 (dt, J = 15.4, 7.7 Hz, 1H), 2.03 – 1.94 (m, 1H), 1.80 –

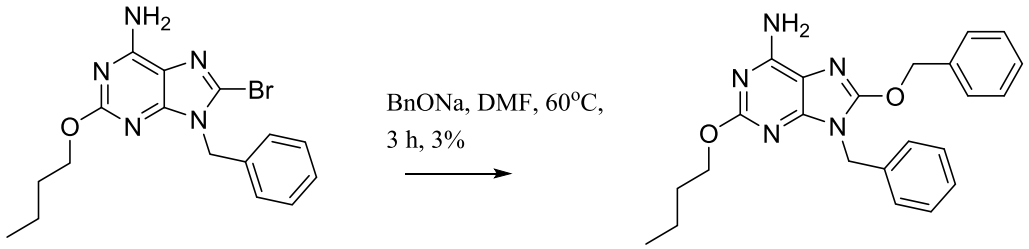
1.70 (m, 1H), 1.60 (p, J = 6.8 Hz, 2H), 1.35 (q, J = 7.4 Hz, 2H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.1, 171.0, 166.0, 160.1, 152.2, 151.6, 149.1, 148.4, 147.8, 140.2, 133.5, 132.4, 129.6, 128.9, 128.1, 128.0, 127.7, 127.4, 127.2, 126.8, 125.1, 122.5, 121.4, 114.2, 108.2, 98.3, 69.5, 69.5, 69.0, 68.9, 65.9, 54.9, 42.1, 38.5, 30.6, 30.3, 29.7, 18.8, 13.7; HRMS (+ESI) calcd for $C_{42}H_{46}N_8NaO_7$ (MH^+) 797.3382, found 797.3379 (Δ 0.4 ppm).



Scheme 40. Synthesis of **37**

*N-(2-(4-(1H-indol-3-yl)butanamido)ethyl)-5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**37**).* To a mixture of biotin (0.057 g, 0.23 mmol), DIPEA (0.16 ml, 0.92 mmol) and DMF (1.5 ml) in a reaction flask was added **20** (0.06 g, 0.21 mmol) with DMF (1 ml). The reaction flask was placed on ice and stirred for 5 minutes. COMU (0.1 g, 0.23 mmol) and DMF (0.4 ml) were added and the reaction flask was allowed to warm to room temperature and stirred for 26 hours. The DMF was removed in vacuo by a Centrivap concentrator. The dark pink solid obtained was mixed with ethyl acetate and filtered and then purified by flash column chromatography (10% MeOH/DCM and 30% MeOH/DCM; TLC: 10% MeOH/DCM). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.78 (d, J = 2.4 Hz, 1H), 7.86 – 7.85 (m, 2H), 7.49 (d, J = 7.8 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H),

7.10 (d, *J* = 2.2 Hz, 1H), 7.06 - 7.03 (m, 1H), 6.97 – 6.94 (m, 1H), 6.45 (s, 1H), 6.38 (s, 1H), 4.27 (dd, *J* = 7.7, 5.0 Hz, 1H), 4.11 – 4.07 (m, 1H), 3.08 - 3.04 (m, 5H), 2.79 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.65 (t, *J* = 7.5 Hz, 2H), 2.58 – 2.53 (m, 1H), 2.12 (t, *J* = 7.5 Hz, 2H), 2.04 (t, *J* = 7.8 Hz, 2H), 1.85 (p, *J* = 7.5 Hz, 2H), 1.63 – 1.55 (m, 1H), 1.54 – 1.40 (m, 3H), 1.35 – 1.20 (m, 2H); ^{13}C NMR (151 MHz, DMSO) δ 172.3, 172.2, 162.8, 136.3, 127.2, 122.3, 120.8, 118.4, 118.1, 114.1, 111.4, 61.0, 59.2, 55.4, 39.9, 38.4, 35.3, 35.3, 28.2, 28.1, 26.1, 25.3, 24.4; HRMS (+ESI) calcd for $C_{24}H_{33}N_5NaO_3S$ ($M+Na^+$) 494.2196, found 494.2197 (Δ 0.2 ppm).



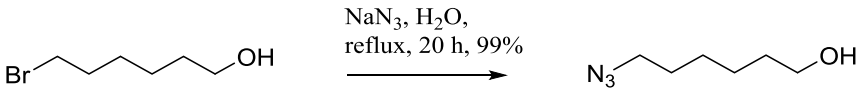
13 **38**

Scheme 41. Synthesis of **38**

*9-Benzyl-8-(benzyloxy)-2-butoxy-9H-purin-6-amine (**38**).* Sodium hydride (0.019 g, 0.79 mmol) was allowed to react completely with anhydrous benzyl alcohol (1.7 ml, 16.43 mmol) in a septum-fitted vial. The benzyloxy solution was transferred to compound **13 (**0.0798 g, 0.21 mmol) in DMF (0.8 ml) in a round bottom flask under nitrogen and the mixture was heated with stirring at 55-60°C for 3 hours. The reaction was then quenched with acetic acid (25 μL) and evaporated by a Centrivap concentrator. The residue was combined with ethyl acetate (30 ml) and washed with water (3 x 30 ml) and brine (20 ml). The organic phase was dried with magnesium sulfate, concentrated and purified by flash chromatography (3% methanol/CH_2Cl_2 and 60% EtOAc/hexanes) to give the product in

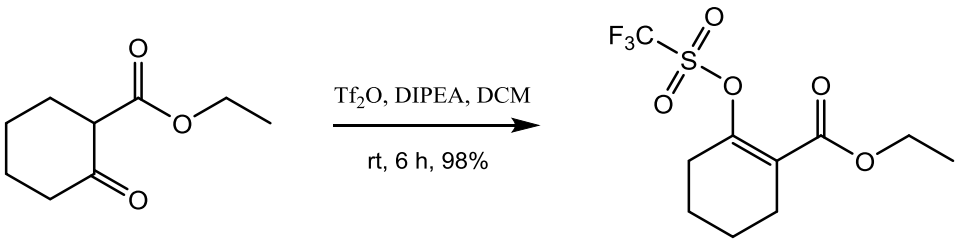
3% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.41 – 7.20 (m, 10H), 6.83 (s, 2H), 5.48 (s, 2H), 5.06 (s, 2H), 4.18 (t, J = 6.6 Hz, 2H), 1.67 – 1.61 (m, 2H), 1.42 – 1.37 (m, J = 7.5 Hz, 2H), 0.91 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.3, 154.6, 152.7, 150.9, 136.7, 135.8, 128.6, 128.4, 128.3, 127.8, 127.6, 127.4, 110.0, 70.6, 65.7, 43.9, 30.7, 18.8, 13.8; HRMS (+ESI) calcd for $C_{23}H_{26}N_5O_2$ (MH^+) 404.2081, found 404.2082 (Δ 0.2 ppm).

Data for **46**: ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.18 (s, 1H), 7.38 (d, J = 7.2 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.26 (t, J = 7.3 Hz, 1H), 6.86 (s, 3H), 5.26 (s, 2H), 4.18 (t, J = 6.6 Hz, 2H), 1.66 – 1.59 (m, 2H), 1.40 – 1.34 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.0, 161.3, 150.9, 148.7, 136.3, 128.4, 127.9, 127.5, 102.7, 66.2, 45.1, 30.5, 18.7, 13.7; HRMS (+ESI) found 330.1386.



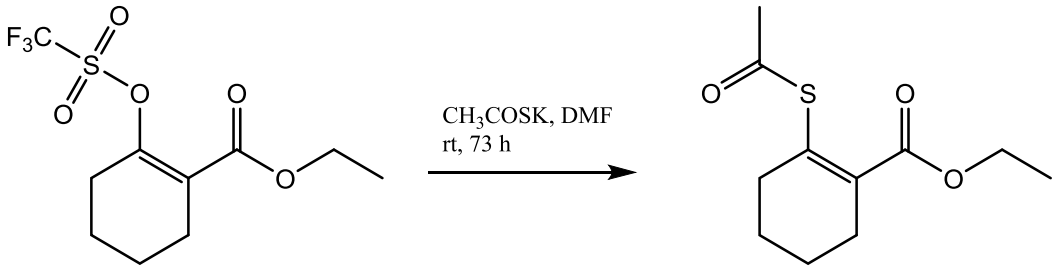
Scheme 42. Synthesis of 6-Azidohexan-1-ol

6-Azidohexan-1-ol. A mixture of 6-bromohexanol (1.20 g, 6.63 mmol) and sodium azide (0.86 g, 13.26 mmol) in 9 ml water was refluxed for 20 h and cooled to room temperature. The mixture was diluted with water (50 ml), extracted with DCM (3 x 50 ml). The organic layer was dried with magnesium sulfate and concentrated to give the product as colorless oil in 99% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 4.34 (t, J = 5.1 Hz, 1H), 3.41 – 3.36 (m, 2H), 3.31 (t, J = 6.9 Hz, 2H), 1.53 (p, J = 6.9 Hz, 2H), 1.41 (p, J = 6.6 Hz, 2H), 1.32 – 1.30 (m, 4H).



Scheme 43. Synthesis of Ethyl 2-(((trifluoromethyl)sulfonyl)oxy)cyclohex-1-ene-1-carboxylate

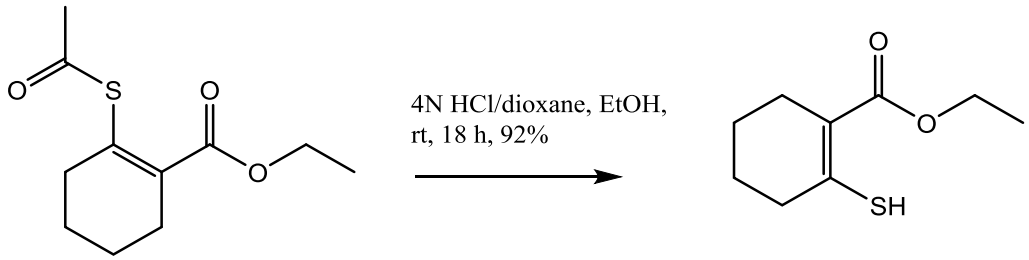
Ethyl 2-(((trifluoromethyl)sulfonyl)oxy)cyclohex-1-ene-1-carboxylate. To ethyl 2-oxocyclohexane-1-carboxylate (2 g, 11.8 mmol), dissolved in DCM (66 ml), was added *N,N*-diisopropylethylamine (2.2 ml, 12.6 mmol). After cooling the mixture to 0ºC, trifluoroacetic anhydride (3.5 ml, 20.8 mmol) was added and stirring was continued at room temperature for 6 hours. The reaction mixture was diluted with additional 55 ml of DCM and poured into 175 ml of saturated sodium bicarbonate in a separatory funnel. Extraction was carried out. The organic layer was washed with 130 ml of water, dried with magnesium sulfate and concentrated under reduced pressure to give the product in 98% yield. This was used without further purification.



Scheme 44. Synthesis of Ethyl 2-(acetylthio)cyclohex-1-ene-1-carboxylate

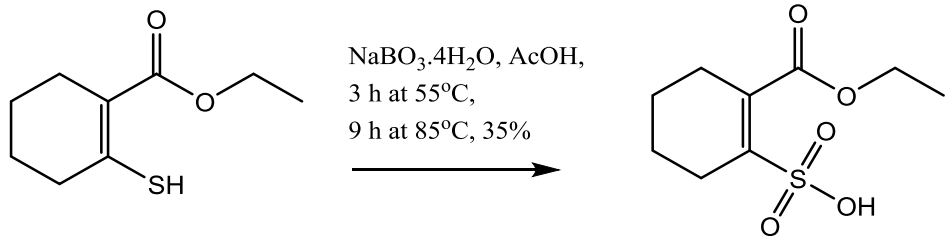
Ethyl 2-(acetylthio)cyclohex-1-ene-1-carboxylate. Ethyl 2-(((trifluoromethyl) sulfonyl)oxy)cyclohex-1-ene-1-carboxylate (2.3 g, 7.6 mmol) was dissolved in anhydrous DMF (28 ml). Potassium thioacetate (1.3 g, 11.4 mmol) was added and the mixture was

stirred at room temperature for 73 hours. The reaction mixture was poured into 100 ml of cold water and extracted with 3 x 90 ml of ethyl acetate. The combined organic phase was washed with 4 x 100 ml of water, dried with magnesium sulfate and concentrated in vacuo to give the product as a brown oil (1.8 g), which was used without further purification.



Scheme 45. Synthesis of Ethyl 2-mercaptocyclohex-1-ene-1-carboxylate

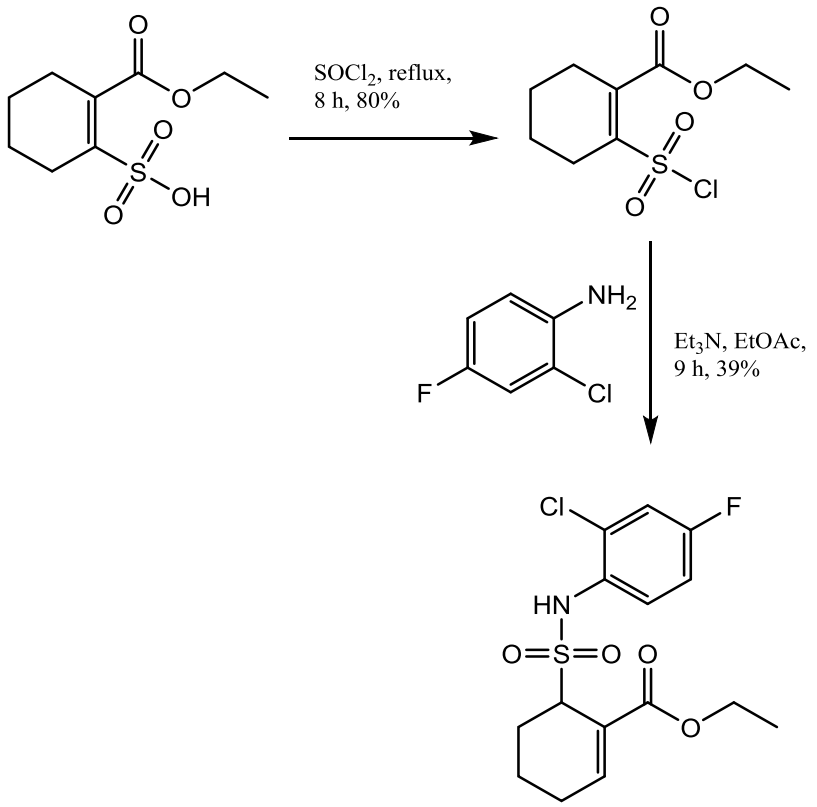
Ethyl 2-mercaptocyclohex-1-ene-1-carboxylate. Ethyl 2-(acetylthio)cyclohex-1-ene-1-carboxylate (2.6 g, 11.4 mmol) was dissolved in anhydrous ethanol (80 ml). 4N HCl/dioxane solution (27 ml, 108 mmol) was added via a septum and the mixture was stirred at room temperature for 18 hours. The reaction mixture was concentrated under reduced pressure and the residue was eluted through a plug of silica gel (14 x 3 cm^2) using 1:1 ethyl acetate/hexanes (800 ml). The eluate was concentrated under reduced pressure to give the thiol product as a brown oil (92% yield), which was used without further purification.



Scheme 46. Synthesis of 2-(Ethoxycarbonyl)cyclohex-1-ene-1-sulfonic acid

2-(Ethoxycarbonyl)cyclohex-1-ene-1-sulfonic acid. To ethyl 2-mercaptocyclohex-1-ene-1-carboxylate (0.49 g, 2.6 mmol) was added sodium perborate tetrahydrate (1.3 g, 8.4 mmol) and acetic acid (10 ml). The mixture was stirred for 3 hours at 55°C and for 9 hours at 85°C. After cooling the mixture, the suspension obtained was evaporated completely under reduced pressure to a light yellow gel. Acetonitrile (15 ml) was added and the mixture was stirred for 2 hours and filtered. The filtrate was evaporated completely to a yellow oil. Acetonitrile (10 ml) was added and the mixture was stirred for 2 hours (or until well mixed) and filtered. The filtrate was evaporated completely. The solid obtained was subjected to column chromatography using a gradient of 5%, 10% and 20% methanol in DCM. UV-active fractions giving an Rf of 0.2 with 10% MeOH/DCM were selected and concentrated to give the product in 35% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.98 (q, J = 7.1 Hz, 2H), 2.24 – 2.20 (m, 2H), 2.09 – 2.08 (m, 2H), 1.55 – 1.49 (m, 4H), 1.16 (t, J = 7.1 Hz, 3H).

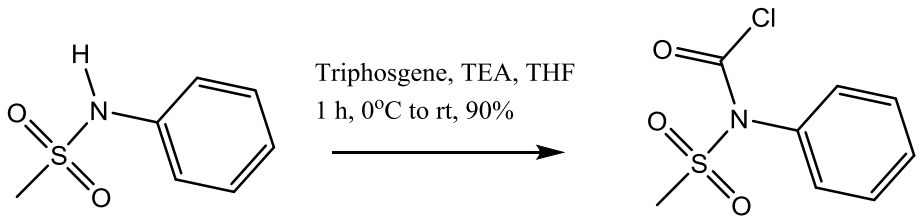
Ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate. 2-(Ethoxycarbonyl)cyclohex-1-ene-1-sulfonic acid (0.26 g, 1.1 mmol), dissolved in thionyl chloride (2.2 ml, 30.2 mmol), was heated under reflux for 8 hours and concentrated in vacuo. The evaporated residue was mixed with DCM (20 ml) and washed with water (25 ml) saturated sodium bicarbonate (25 ml) and brine (20 ml). The DCM solution was dried with magnesium sulfate and concentrated under reduced pressure to give the sulfonyl chloride intermediate in 80% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.30 (q, J = 7.2 Hz, 2H), 2.59 (m, 4H), 1.79 (m, 4H), 1.34 (t, J = 7.2 Hz, 3H).



Scheme 47. Synthesis of Ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate

A solution of ethyl 2-(chlorosulfonyl)cyclohex-1-ene-1-carboxylate (0.209 g, 0.83 mmol), obtained above, in ethyl acetate (1.1 ml) was added dropwise to an ice-cooled solution of 2-chloro-4-fluoroaniline (0.130 g, 0.89 mmol), triethylamine (0.23 ml, 1.65 mmol) and ethyl acetate (1.1 ml) under nitrogen. After having been stirred for 9 hours under nitrogen, the reaction mixture was diluted with 5 ml of ethyl acetate and washed successively with water, 0.5 M HCl, water, and brine (12 ml of each) and the ethyl acetate solution was dried with magnesium sulfate and concentrated by rotary evaporation. The residue was purified by flash column chromatography (15% - 20% EtOAc/hexanes) to yield the product in 39% yield as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.74 (s, 1H), 7.61 – 7.47 (m, 2H), 7.30 – 7.23 (m, 1H), 7.09 (t, J = 3.9 Hz, 1H), 4.31 – 4.28 (m, 1H),

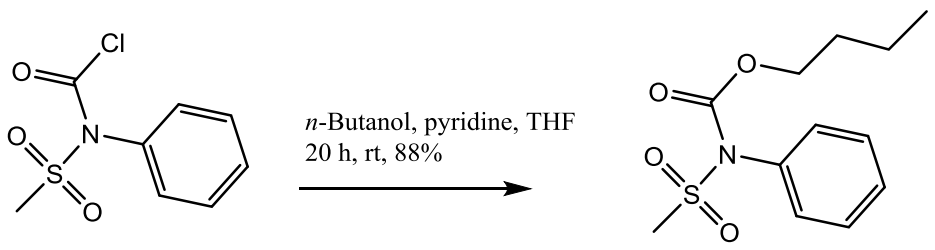
4.04 – 3.89 (m, 2H), 2.47 – 2.06 (m, 4H), 1.78 – 1.59 (m, 2H), 1.04 (t, J = 7.1 Hz, 3H); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.70 (dd, J = 9.1, 5.3 Hz, 1H), 7.31 (t, J = 3.9 Hz, 1H), 7.16 (dd, J = 7.9, 2.9 Hz, 1H), 7.04 – 6.97 (m, 2H), 4.50 – 4.46 (m, 1H), 4.22 – 4.10 (m, 2H), 2.53 – 2.44 (m, 2H), 2.30 – 2.22 (m, 1H), 2.20 – 2.12 (m, 1H), 1.79 – 1.71 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H).



39

Scheme 48. Synthesis of **39**

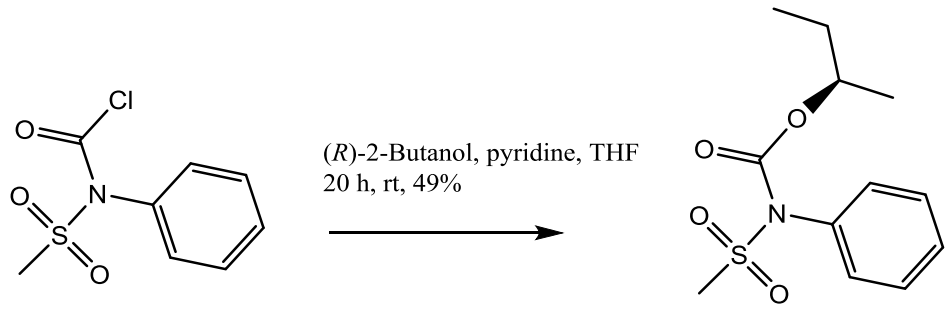
*(Methylsulfonyl)(phenyl)carbamic chloride (**39**).* To a solution of *N*-phenylmethanesulfonamide (0.2 g, 1.17 mmol) and triethylamine (0.23 ml, 1.65 mmol) in tetrahydrofuran (2 ml) was added, portion-wise and under ice-cooling, triphosgene (0.485 g, 1.63 mmol). The mixture was stirred for 30 minutes under ice-cooling and for 1 hour at room temperature. The reaction mixture was diluted with ethyl acetate and washed with water and brine. The organic phase was dried over magnesium sulfate, concentrated in vacuo and then taken up in hexanes. The precipitated product was filtered to give the carbamic chloride as an off-white solid in a yield of 90%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.55 – 7.48 (m, 3H), 7.34 – 7.31 (m, 2H), 3.50 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 149.2, 135.4, 130.8, 130.0, 129.9, 41.6; HRMS (+ESI) calcd for $C_8H_8ClNNaO_3S$ ($M+Na^+$) 255.9806, found 255.9807 (Δ 0.4 ppm).



39 **40**

Scheme 49. Synthesis of **40**

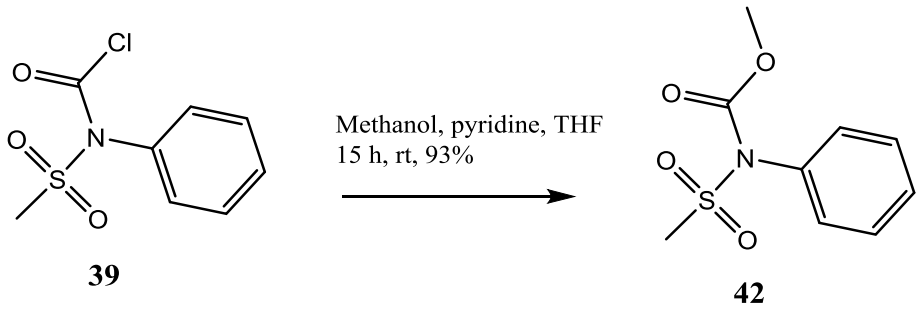
*Butyl (methylsulfonyl)(phenyl)carbamate (**40**).* To a stirred solution of *n*-Butanol (0.6 ml, 6.56 mmol) and (methylsulfonyl)(phenyl)carbamic chloride (0.33 g, 1.41 mmol) in anhydrous tetrahydrofuran (12 ml) was added pyridine (0.46 ml, 5.69 mmol) slowly. The mixture was stirred at room temperature for 20 hours (TLC eluent: 1:2 EtOAc: hexanes). The mixture was partitioned between ethyl acetate (40 ml) and water (40 ml). The phases were separated and the aqueous phase was extracted with ethyl acetate (30 ml x 2). The combined organic phase extracts were washed with water (30 ml), 1 M HCl (30 ml) and brine (30 ml) and dried over magnesium sulfate. The solvent was removed to give the product in 88% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.43 – 7.41 (m, 3H), 7.26 – 7.23 (m, 2H), 4.19 (t, J = 6.6 Hz, 2H), 3.45 (s, 3H), 1.58 – 1.52 (m, 2H), 1.27 – 1.21 (m, 2H), 0.84 (t, J = 7.4 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 153.0, 135.2, 129.4, 129.4, 129.3, 67.7, 41.6, 30.4, 18.8, 13.5; HRMS (+ESI) calcd for $C_{12}H_{17}NNaO_4S$ ($M+Na^+$) 294.0770, found 294.0771 (Δ 0.3 ppm).



39 **41**

Scheme 50. Synthesis of **41**

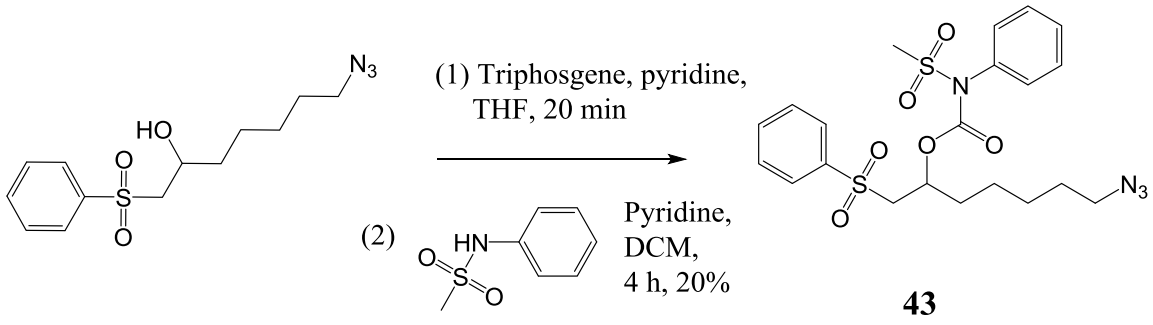
*(R)-sec-Butyl (methylsulfonyl)(phenyl)carbamate (**41**).* To a stirred solution of (*R*)-2-Butanol (50 µL, 0.54 mmol) and (methylsulfonyl)(phenyl)carbamic chloride (27.5 mg, 0.12 mmol) in anhydrous tetrahydrofuran (1 ml) was added pyridine (38 µl, 0.47 mmol) slowly. The mixture was stirred at room temperature for 20 hours (TLC eluent: 1:2 EtOAc: hexanes). The mixture was partitioned between ethyl acetate (10 ml) and water (10 ml). The phases were separated and the aqueous phase was extracted with ethyl acetate (10 ml x 2). The combined organic phase extracts were washed with water (10 ml), 1 M HCl (10 ml) and brine (10 ml) and dried over magnesium sulfate. The solvent was removed and the crude product purified by flash column chromatography (15% - 25% EtOAc/hexanes; removing the starting material sulfonamide) to give the product in 49% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.45 – 7.40 (m, 3H), 7.26 – 7.23 (m, 2H), 4.88 (h, J = 6.2 Hz, 1H), 3.46 (s, 3H), 1.52 (p, J = 7.3 Hz, 2H), 1.22 (d, J = 6.2 Hz, 3H), 0.80 (t, J = 7.4 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 152.8, 135.4, 129.5, 129.4, 129.4, 77.2, 41.8, 28.7, 19.3, 9.4; ^{13}C NMR (126 MHz, DMSO-d_6) δ 151.9, 135.6, 129.7, 129.0, 129.0, 75.9, 41.5, 28.0, 19.0, 9.1; HRMS (+ESI) calcd for $C_{12}H_{17}NNaO_4S$ ($M+Na^+$) 294.0770, found 294.0771 (Δ 0.3 ppm).



39 **42**

Scheme 51. Synthesis of **42**

*Methyl (methylsulfonyl)(phenyl)carbamate (**42**).* To a stirred solution of methanol (50 µL, 1.23 mmol) and (methylsulfonyl)(phenyl)carbamic chloride (62.4 mg, 0.27 mmol) in anhydrous tetrahydrofuran (2.1 ml) was added pyridine (87 µl, 1.1 mmol) slowly. The mixture was stirred at room temperature for 15 hours (TLC eluent: 1:2 EtOAc: hexanes). The mixture was partitioned between ethyl acetate and water. The phases were separated and the aqueous phase was extracted with ethyl acetate (x2). The combined organic phase extracts were washed with water, 1 M HCl and brine and dried over magnesium sulfate. The solvent was removed to give the product in 93% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.46 – 7.43 (m, 3H), 7.27 – 7.25 (m, 2H), 3.79 (s, 3H), 3.47 (s, 3H); ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.47 – 7.42 (m, 3H), 7.38 – 7.36 (m, 2H), 3.71 (s, 3H), 3.57 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.6, 135.1, 129.7, 129.5, 129.5, 54.6, 41.7; ^{13}C NMR (126 MHz, DMSO-d_6) δ 152.7, 135.4, 129.7, 129.2, 54.2, 41.3; HRMS (+ESI) calcd for $C_9H_{11}NNaO_4S$ ($M+Na^+$) 252.0301, found 252.0301 (Δ 0.0 ppm).

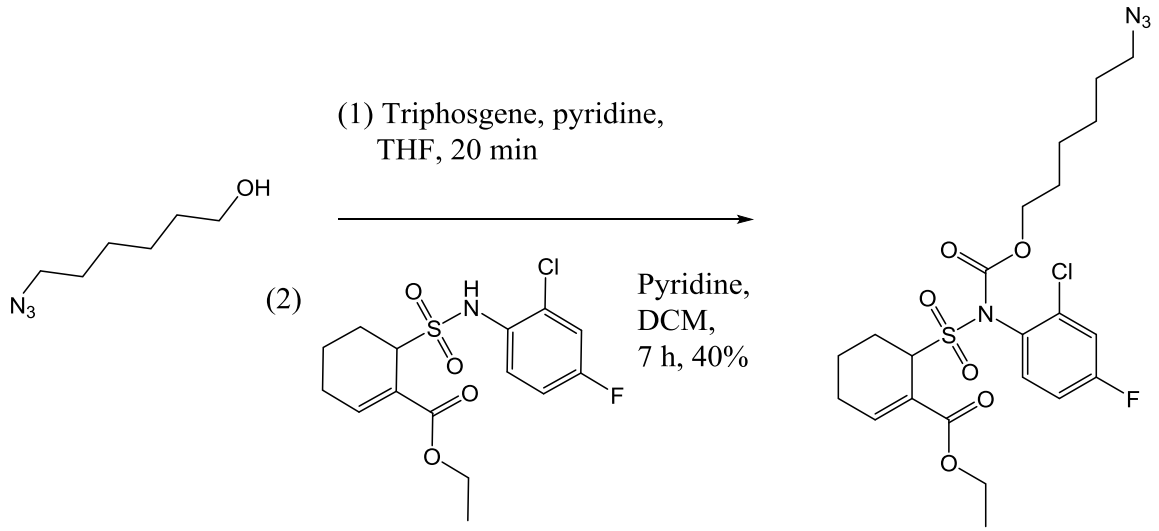


Scheme 52. Synthesis of **43**

*7-Azido-1-(phenylsulfonyl)heptan-2-yl (methylsulfonyl)(phenyl)carbamate (**43**).*

Pyridine (90 μL, 1.11 mmol) was added dropwise to a stirred solution of 7-azido-1-(phenylsulfonyl)heptan-2-ol (165 mg, 0.55 mmol) and triphosgene (277 mg, 0.93 mmol) in 9 ml of anhydrous tetrahydrofuran. The resulting suspension was stirred for 20 minutes and filtered and concentrated to give the crude chloroformate as an oil. To the solution of the crude chloroformate in dichloromethane (1 ml) was added N-phenylmethanesulfonamide (86 mg, 0.5 mmol) and pyridine (85 μL, 1.05 mmol). The solution was stirred for 4 hours at room temperature and then partitioned between dichloromethane (10 ml) and water (25 ml). The aqueous phase was extracted with dichloromethane (35 ml). The combined organic phases were washed with water (35 ml), dried over magnesium sulfate, concentrated and purified by flash chromatography using first 20 – 30% ethyl acetate/hexanes, then 100% dichloromethane to give the product in 20% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.96 – 7.94 (m, 2H), 7.72 (t, J = 7.5 Hz, 1H), 7.65 – 7.60 (m, 2H), 7.48 – 7.41 (m, 3H), 7.32 – 7.28 (m, 2H), 5.34 – 5.30 (m, 1H), 3.50 (s, 3H), 3.32 – 3.28 (m, 1H), 3.23 – 3.15 (m, 3H), 1.66 – 1.58 (m, 1H), 1.50 (p, J = 7.0 Hz, 3H), 1.32 – 1.18 (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 152.2, 138.9, 135.3, 134.4, 129.7,

129.7, 129.6, 129.5, 128.4, 71.0, 59.1, 51.2, 41.5, 34.1, 28.6, 26.1, 24.1; HRMS (+ESI) calcd for $C_{21}H_{26}N_4NaO_6S_2$ ($M+Na^+$) 517.1186, found 517.1190 (Δ 0.8 ppm).



44

Scheme 53. Synthesis of **44**

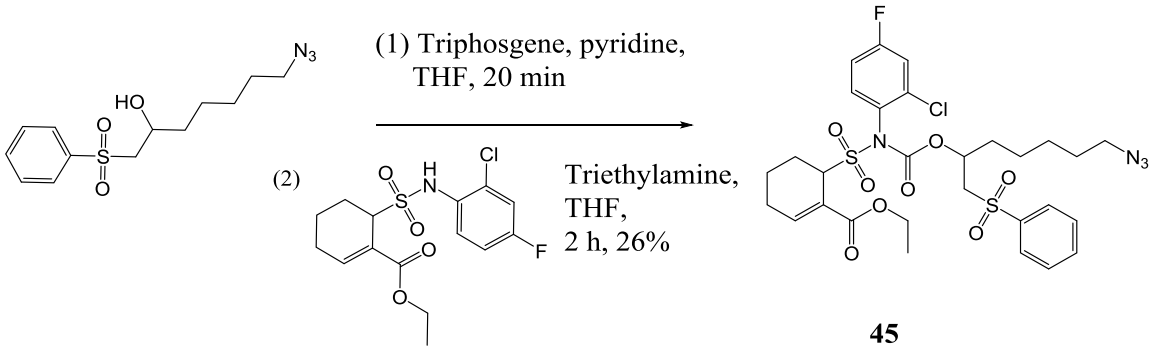
*Ethyl 6-(N-(((6-azidohexyl)oxy)carbonyl)-N-(2-chloro-4-fluorophenyl)sulfamoyl) cyclohex-1-ene-1-carboxylate (**44**).* Pyridine (62 µL, 0.77 mmol) was added dropwise to a stirred solution of 6-azido-1-hexanol (0.056 g, 0.39 mmol) and triphosgene (0.19 g, 0.64 mmol) in 1 ml of anhydrous tetrahydrofuran. The resulting suspension was stirred for 20 minutes and filtered and concentrated to give the crude chloroformate as an oil. To the solution of the crude chloroformate in dichloromethane (3 ml) was added ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate (0.128 g, 0.35 mmol) and pyridine (55 µL, 0.68 mmol). The solution was stirred for 7 hours at room temperature and then partitioned between dichloromethane (5 ml) and water (15 ml). The aqueous phase was extracted with dichloromethane (20 ml). The combined organic phases were washed

with water (20 ml), dried over magnesium sulfate, concentrated and subjected to by flash chromatography using ethyl acetate/hexanes (20% - 25%) to a provide **44**.

Major isomer (separated peaks): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38 (1H), 7.23 (1H), 7.19 (1H), 6.96 (1H), 5.41 (1H), 4.32 – 4.09 (4H), 3.21 (t, *J* = 6.9 Hz, 2H), 2.82 (1H), 2.47 (1H), 2.28 (1H), 2.04 (1H), 1.81 – 1.73 (2H), 1.62 (2H), 1.53 (2H), 1.31 (7H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.1, 162.3 (d, *J* = 253.1 Hz), 152.1, 148.6, 136.2 (d, *J* = 11.0 Hz), 132.4 (d, *J* = 9.4 Hz), 129.8 (d, *J* = 3.8 Hz), 124.0, 117.5 (d, *J* = 25.8 Hz), 114.7 (d, *J* = 22.3 Hz), 67.7, 61.3, 58.9, 51.2, 28.7, 28.3, 26.1, 25.2, 23.9, 16.8, 14.3.

Minor isomer (separated peaks): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60 (1H), 7.38 (1H), 7.23 (1H), 7.04 (1H), 5.03 (1H), 4.32 – 4.08 (4H), 3.21 (t, *J* = 6.9 Hz, 2H), 2.68 (1H), 2.47 (1H), 2.28 (1H), 2.19 (1H), 1.81 – 1.73 (2H), 1.62 (1H), 1.53 (2H), 1.31 (7H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.0, 162.4 (d, *J* = 253.2 Hz), 152.0, 147.5, 136.3 (d, *J* = 10.9 Hz), 132.1 (d, *J* = 9.6 Hz), 130.5 (d, *J* = 3.9 Hz), 123.8, 117.6 (d, *J* = 25.9 Hz), 114.9 (d, *J* = 22.3 Hz), 67.7, 61.1, 59.5, 51.2, 28.7, 28.2, 26.1, 25.0, 23.7, 16.3, 14.2.

HRMS (+ESI) calcd for $C_{22}H_{28}ClFN_4NaO_6S$ ($M+Na^+$) 553.1294, found 553.1295 (Δ 0.2 ppm).



Scheme 54. Synthesis of **45**

*Ethyl 6-(N-(((7-azido-1-(phenylsulfonyl)heptan-2-yl)oxy)carbonyl)-N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate (**45**).* Pyridine (27.3 µL, 0.34 mmol) was added dropwise to a stirred solution of 7-azido-1-(phenylsulfonyl)heptan-2-ol (45.5 mg, 0.15 mmol) and triphosgene (79 mg, 0.27 mmol) in 2.1 ml of anhydrous tetrahydrofuran. The resulting suspension was stirred for 20 minutes and filtered and concentrated to give the crude chloroformate as an oil. To the solution of the crude chloroformate in tetrahydrofuran (2.5 ml) was added ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate (55 mg, 0.15 mmol) and triethylamine (38.4 µL, 0.28 mmol). The solution was stirred for 2 hours at room temperature and diluted with ethyl acetate and washed with 1 M HCl, water, saturated sodium bicarbonate and brine (5 ml each). The organic phase was dried over magnesium sulfate, concentrated and subjected to flash chromatography using ethyl acetate/hexanes (25% - 30%) to provide **45**. ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.3, 166.1, 163.5, 163.5, 161.5, 161.5, 151.4, 151.3, 148.1, 146.8, 139.1, 139.0, 137.0, 136.9, 135.8, 135.7, 134.2, 134.2, 133.8, 133.0, 133.0, 132.2, 132.1, 130.6, 130.6, 129.5, 129.5, 129.5, 129.4, 128.4, 128.3, 127.5, 124.0, 123.6, 118.1, 117.9, 117.6, 117.4, 115.3, 115.1, 114.9, 114.8, 71.8, 71.8, 61.3, 61.2, 59.4, 58.7, 58.6, 58.5, 51.2, 44.6, 33.9, 33.6, 28.6, 26.2, 26.1, 25.2, 24.8, 24.0, 23.9, 23.8, 23.4, 16.8, 16.1, 14.4, 14.3; HRMS (+ESI) calcd for $C_{29}H_{34}ClFN_4NaO_8S_2$ ($M+Na^+$) 707.1383, found 707.1383 (Δ 0.0 ppm).

CHAPTER THREE

Results and Discussion

Synthesis of Adenine Derivatives

The goal of the work presented herein is to synthesize TLR agonists which bear functional groups that can serve to conjugate the compounds with biomolecules. With the established SAR of several adenine-based compounds, we designed and synthesized new adenine analogs in order to investigate their TLR agonistic activity and sought to achieve a desirable method for their bioconjugation. Our synthesis of TLR7 agonists and conjugates with substituents X, R, and OR' (Figure 23) began with the utilization of commercially available 2-chloroadenine (also called 6-amino-2-chloropurine).

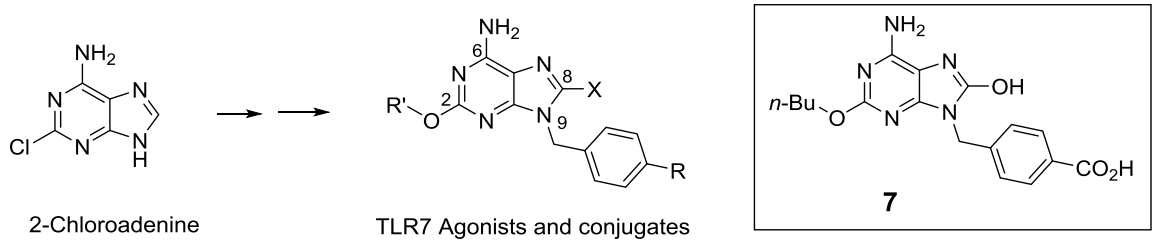


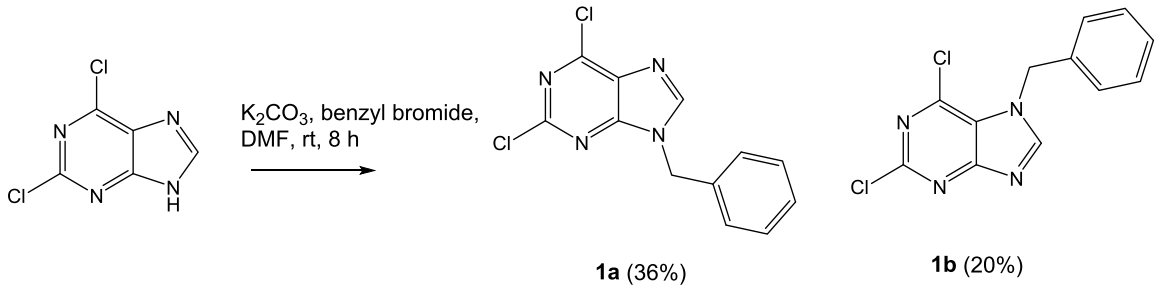
Figure 23. Synthesis of adenine analogs as TLR7 agonists and conjugates from 2-chloroadenine

The synthesis typically involved a benzylation at N-9, an alkoxy substitution at C-2, an electrophilic aromatic bromination at C-8, a nucleophilic aromatic hydroxyl substitution at C-8 and, finally, modifications of the *para*-substituted R group. A key intermediate in this series of transformations was the carboxylic acid **7**, which served as a useful synthon for the synthesis of a variety of amide derivatives. This compound allowed

modifications at the *para*-position that were used to introduce linkers bearing amine, free thiol, alkyne and indole functionalities for antibody bioconjugation.

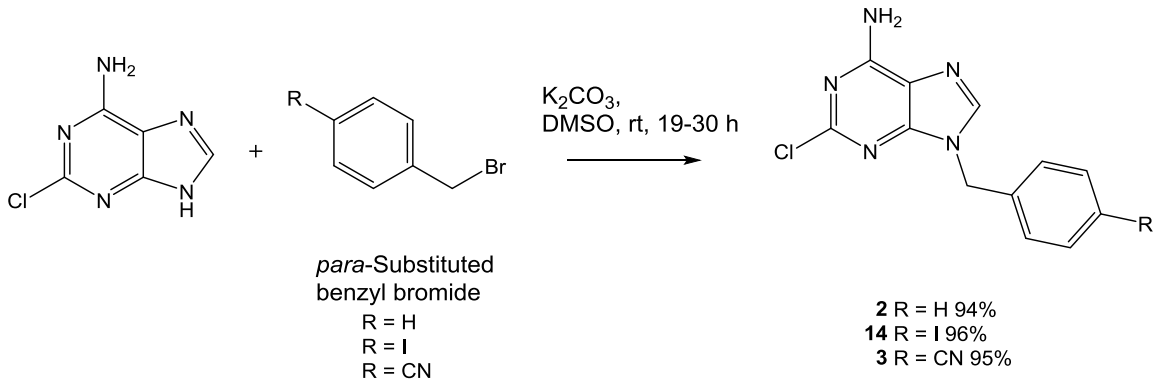
Alkylation at N-9

Benzylation of 2,6-dichloropurine afforded two constitutional isomers, the N-7 alkylated and the N-9 alkylated products, with the ratio of the less polar N-9 product (compound **1a**) to the N-7 product (compound **1b**) being ~2:1 (Scheme 55). As previously reported, amination of these constitutional isomers (or amination of 2,6-dichloropurine itself) provided the 6-amino compounds.[41,69]



Scheme 55. Benzylation of 2,6-dichloropurine to give two different constitutional isomers

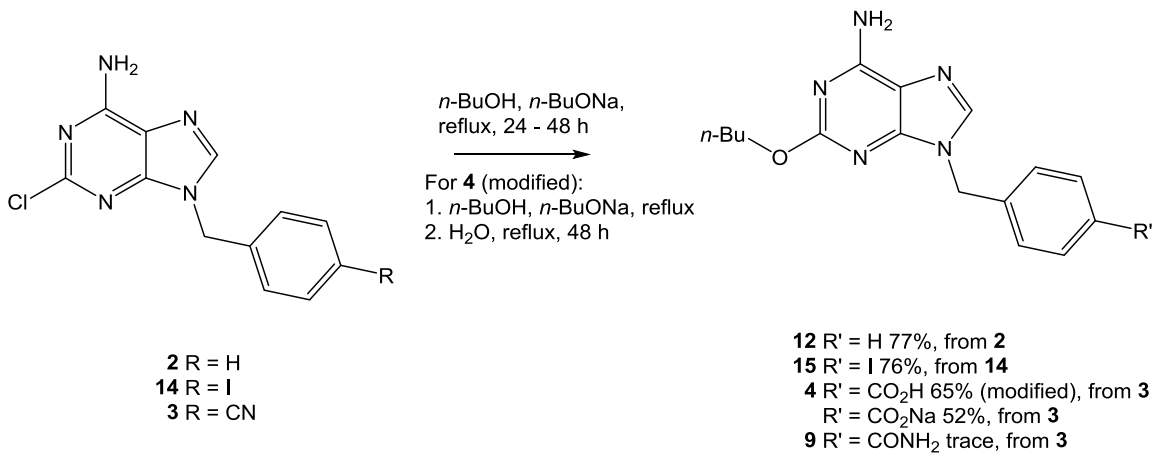
Alkylation of 2-chloroadenine (the 6-amino analog of 2,6-dichloropurine), however, produced only the N-9 alkylated product (Scheme 56).[46,50] Presumably, the regioselectivity observed in this alkylation is a result of steric or electronic (hydrogen bonding) effects from the 6-amino group. Benzyl bromide and *para*-substituted benzyl bromides (4-iodo- and 4-cyanobenzyl) were reacted with 2-chloroadenine in a nucleophilic substitution in DMSO as solvent (Scheme 56). Simple precipitation of the products from aqueous solutions gave the desired compounds **2** (unsubstituted), **3** (4-cyano substituted), **14** (4-iodo substituted) in high yields (94-96%).



Scheme 56. Alkylation of 2-Chloroadenine

Nucleophilic Aromatic Substitution at C-2

The next step in our synthesis was to introduce the butoxy group at carbon-2. In agreement with previous literature procedure, the method of refluxing a solution of sodium butoxide in butanol with the benzyl adenines provided the desired products.[46] The reactions were complete in 24 to 48 hours in yields from 65-77%. We found we could isolate some of the desired products by simple precipitation after concentrating the reaction mixture. This isolation worked well with the unsubstituted benzyl and 4-iodobenzyl compounds (**12** and **15**, Scheme 57). However, with the 4-cyanobenzyl compound, undesired reactions at the nitrile (alcoholysis and hydrolysis) led to the production of the sodium carboxylate (R' = CO_2Na, Scheme 57) as well as the intermediate amide (R' = $CONH_2$, **9**). We therefore modified the procedure to directly afford the carboxylic acid (R' = CO_2H) by first installing the butoxy group with refluxing butoxide solution, and then completely hydrolyzing the nitrile by introducing an excess water to the butoxide solution and continuing reflux. Protonation of the carboxylate gave a fair yield (65%) of the carboxylic acid **4** as a white powder.

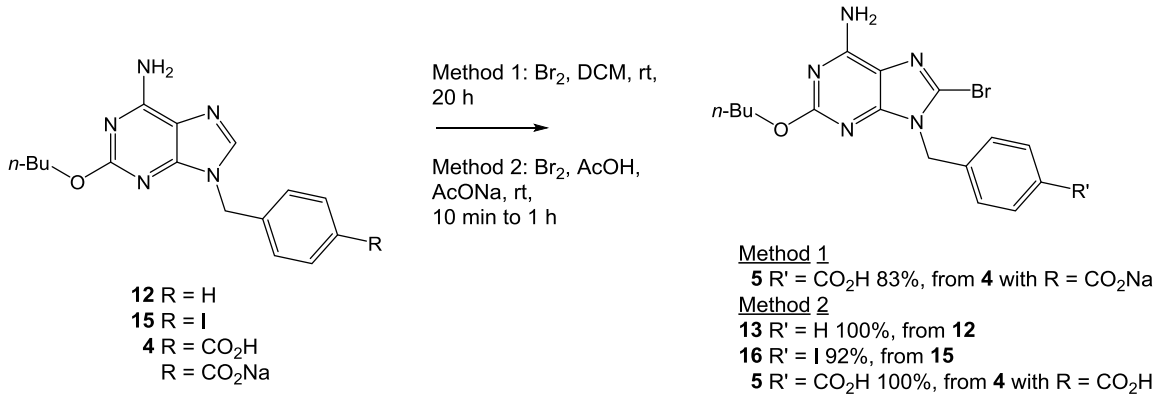


Scheme 57. Nucleophilic aromatic substitution of butoxy group for chlorine at C-2

Electrophilic Aromatic Substitution at C-8

Our initial work on bromination at the 8-position involved the use of dichloromethane as solvent and about 300 equivalents of bromine, which effected the complete bromination after approximately 20 h in yields between 75% to 85% yields.[41,111] The starting materials (compounds **12**, **15**, and the sodium salt of **4**, Scheme 58) were not well dissolved in dichloromethane. In our effort to optimize the reaction conditions, we tested several solvents for this transformation including 2,2,2-trifluoroethanol, 2-propanol, benzene, chloroform, ethanol, tetrahydrofuran, and acetic acid. Out of these solvents, acetic acid proved to be most desirable, in terms of solubility of reactants. It is noteworthy that with only acetic acid and bromine (3 to 11 equivalents), the reaction did not go to completion (as monitored by TLC: 7% MeOH/DCM) when allowed to continue for up to 3 days. We reasoned that the progress of the reaction was halted by the protonation of the substrate by hydrogen bromide produced as the reaction progressed, which, in turn, deactivated the ring towards further reaction. Therefore, we introduced sodium acetate (5 equivalents), which would serve to capture HBr. With acetic acid and sodium acetate, only

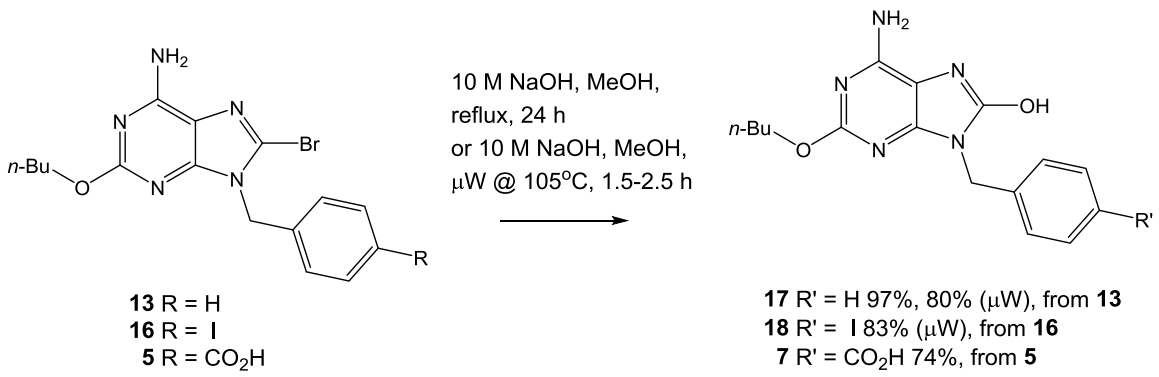
5 equivalents of bromine were sufficient to complete the bromination, the reaction times were reduced, and the yields were also improved (as shown in Scheme 58).[112,113]



Scheme 58. Bromination at C-8

Nucleophilic Aromatic Substitution at C-8

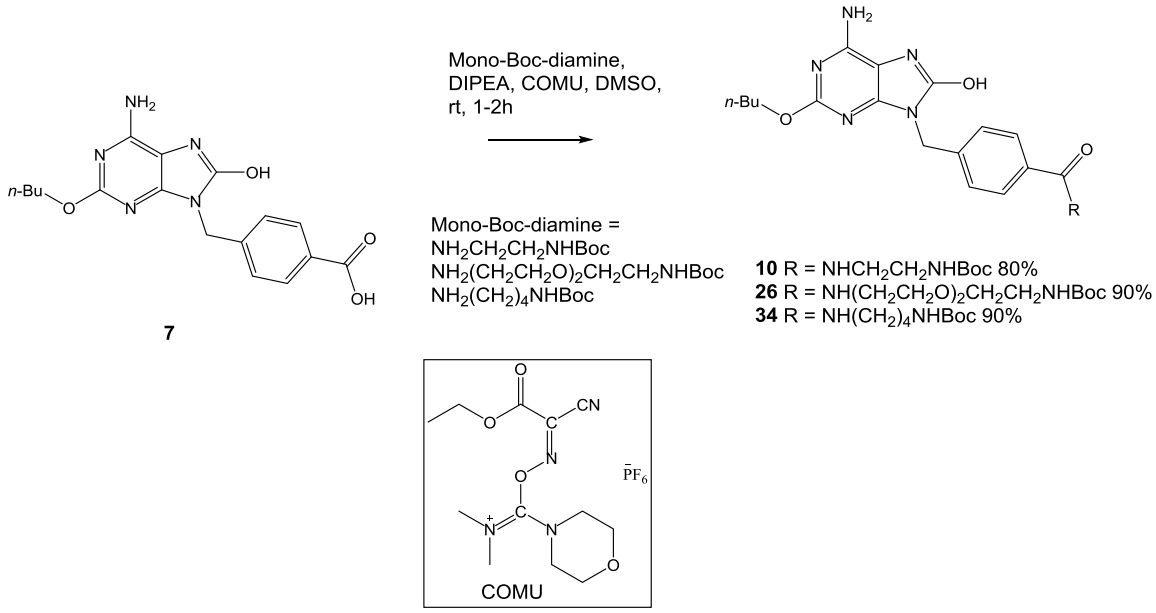
The C-8 hydroxyl derivative is commonly prepared from the C-8 bromo compound by a 2-step procedure which involves, first, the substitution of the bromine by a methoxy group and then a hydrolytic cleavage of the methoxy to give the 8-hydroxyl compound.[51,56] We employed a more efficient way of carrying out this transformation by directly installing the OH on the C-8. Literature examples of this direct hydroxylating procedure involve the use of either strongly acidic[50,53] or strongly basic solutions.[46,57] Our procedure involved a nucleophilic aromatic substitution using a strongly basic solution (10 M) of sodium hydroxide under refluxing conditions for 24 hours (Scheme 59). For compounds **17** and **18,** a significant reduction of reaction time was achieved via microwave heating. The 8-hydroxyadenine carboxylic acid product (**7**) is desirable in that it bears the carboxyl group which serves to conjugate biomolecules, with the 8-OH intact for IFN induction.



Scheme 59. Nucleophilic aromatic substitution of the OH at C-8

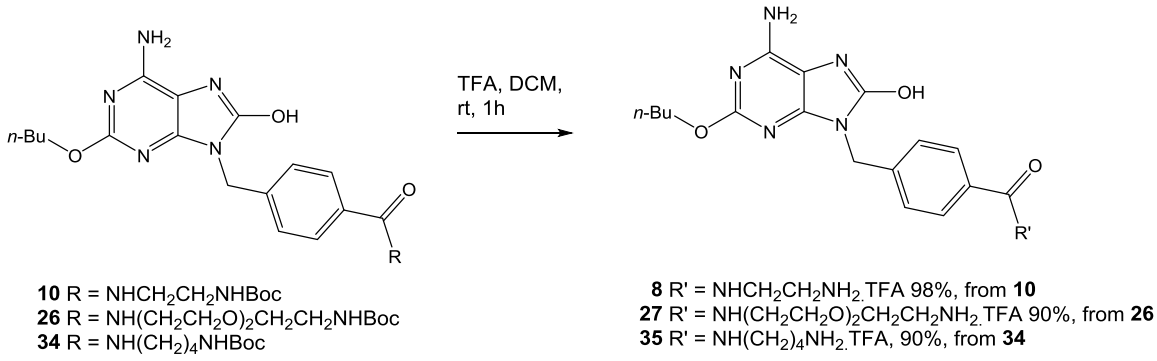
Synthesis of Amides

The next series of reactions involved the transformation of carboxylic acid **7** to Boc-protected amines. We proceeded to mono-protect ethylenediamine with the *tert*-butyloxycarbonyl (Boc) group using literature procedures[114-115] before reacting its remaining amino group with the carboxylic acid **7** using COMU[116] as the coupling agent (Scheme 60). The reaction proceeded smoothly, giving compound **10**. Using COMU, a uronium-based reagent, coupling reactions were carried out in DMSO with reaction times of 1 to 3 hours, and amide products were isolated by precipitation. In a similar manner, amides **26** and **34** were also prepared from compound **7** by using other diamines. Mono-Boc-protected 2,2′-(Ethylenedioxy)bis(ethylamine) and 1,4-butanediamine were synthesized using known literature procedure[117] and reacted with **7** to give compounds **26** and **34** respectively. The amides were obtained in 80-90% yields.



Scheme 60. Synthesis of Boc-protected Amines

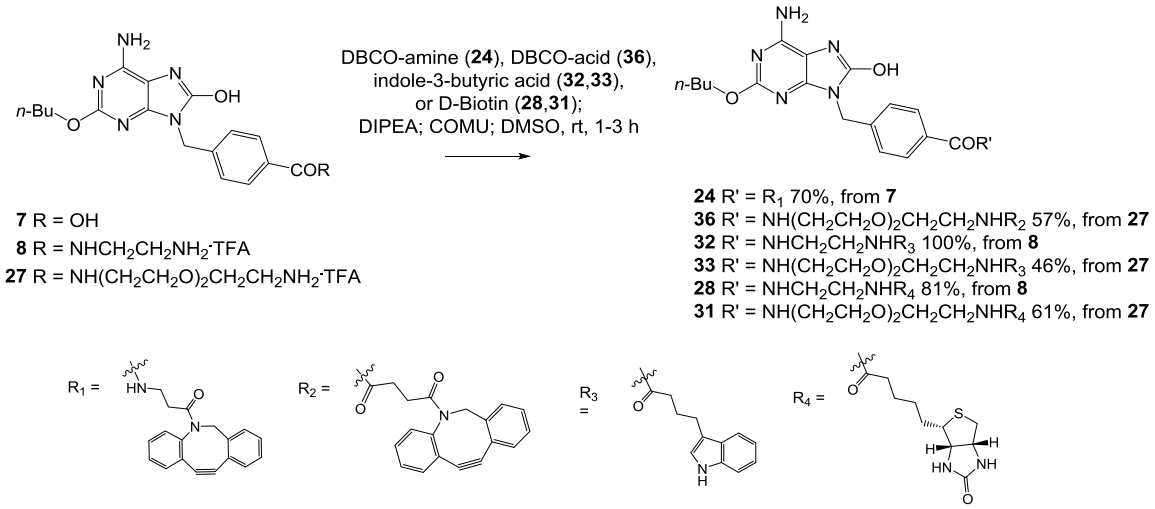
The Boc protecting group was readily removed on amides **10**, **26**, and **34** by treatment with trifluoroacetic acid (TFA) in DCM, giving amines **8**, **27** and **35**, respectively, as trifluoroacetic acid salts (Scheme 61).



Scheme 61. Removal of the Boc group to give free amine compounds

With the carboxyl and amine groups present on the TLR7 agonists, we were able to add functional groups that we planned to use for mechanistic studies and bioconjugations. The COMU coupling procedure was again employed to attach dibenzocyclooctyne (for

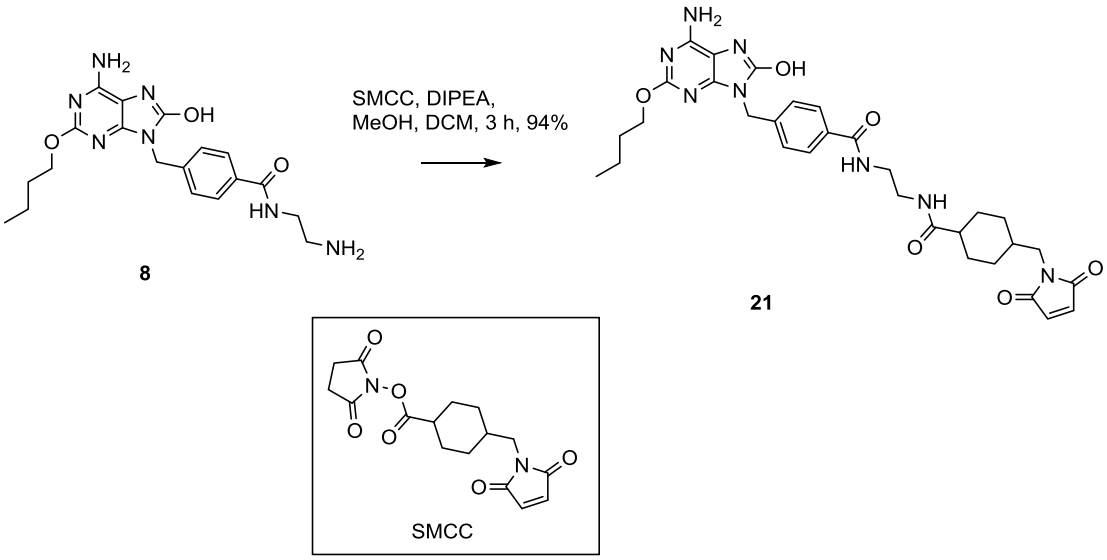
click chemistry with azido groups), indole-3-butyric acid and biotin, as shown in Scheme 62. The dibenzocyclooctyne derivatives were compound **24** (from the carboxylic acid **7**) and **36** (from amine **27**). The indole-3-butyric acid derivatives were compound **32** (from amine **8**) and compound **33** (from amine **27**). The biotinylated derivatives were compound **28** (from amine **8**) and compound **31** (from amine **27**). For conjugations with free thiols, maleimide **21** was also prepared from amine **8** using the NHS-maleimide crosslinker SMCC in 94% yield (Scheme 63).



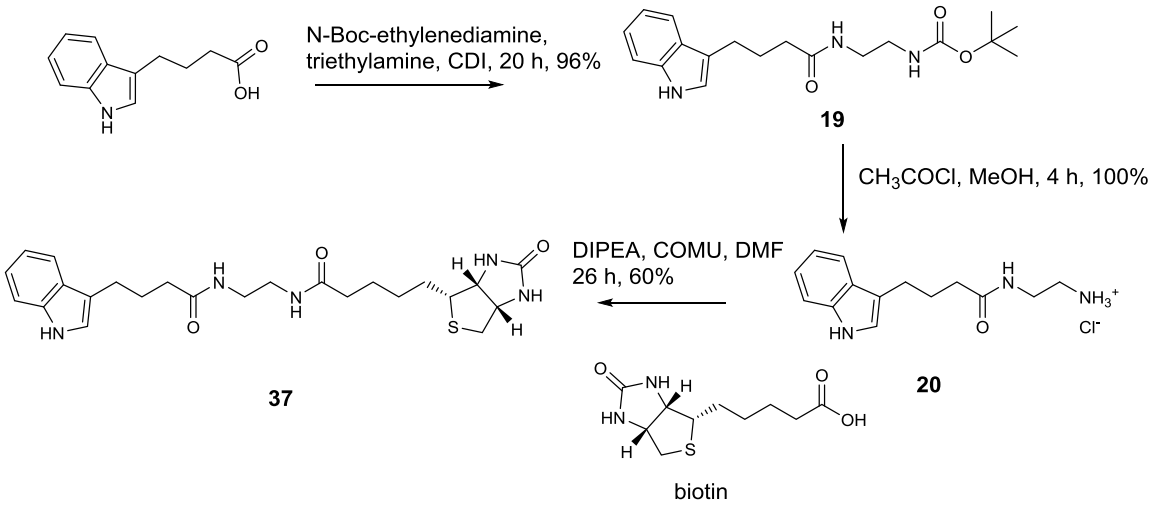
Scheme 62. Synthesis of compounds for antibody conjugation and bioassay

Additionally, **37**, a compound needed for mechanistic studies, was prepared with a slight modification in the coupling procedures. This compound contains indole-3-butyric acid and D-biotin connected through an ethylenediamine linker (Scheme 64). 1,1'-carbonyldiimidazole (CDI) was used to couple indole-3-butyric acid with *N*-Boc-ethylenediamine.[118,119] The cleavage of the Boc group was carried out efficiently by in-situ generated hydrogen chloride from methanol and acetyl chloride at 0°C.[120] The coupling of

the hydrochloride indole derivative (**20**) to biotin was then carried out using COMU as coupling agent.



Scheme 63. Synthesis of maleimide compound **21**



Scheme 64. Synthesis of Indole-3-butyric acid – Biotin

Other Adenine Derivatives for SAR Studies

Other 8-hydroxy adenine derivatives shown in Figure 24 were also synthesized for SAR studies.

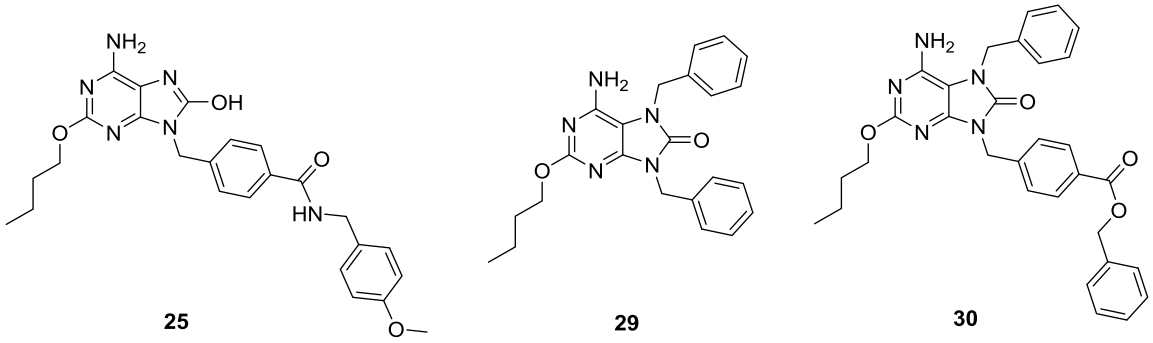
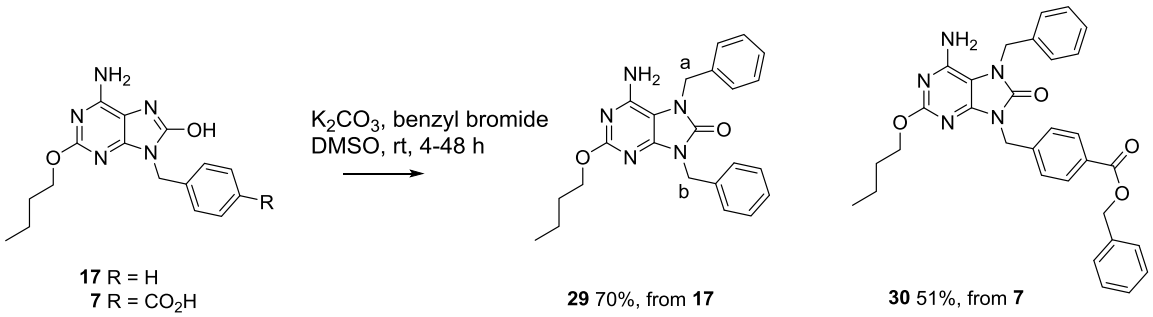


Figure 24. Other Adenine Derivatives

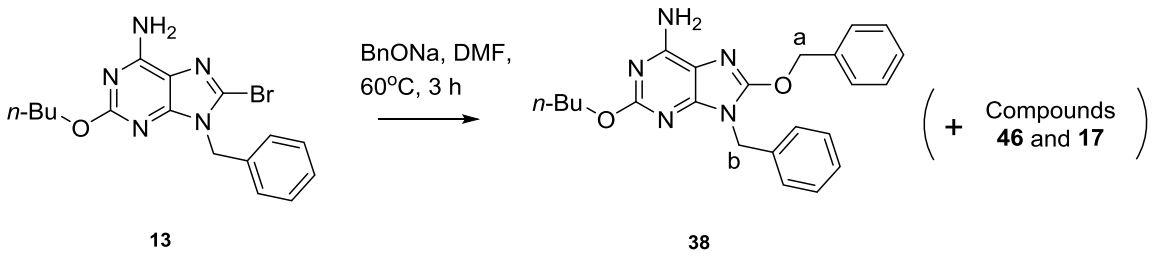
Compound **25** was synthesized using the COMU coupling procedure involving carboxylic acid **7** and 4-methoxybenzylamine. Compounds **29** and **30** were prepared by alkylating the 8-hydroxy compounds **17** and **7**, respectively, with benzyl bromide (Scheme 65).



Scheme 65. Synthesis of Alkylated Compounds **29** and **30**

As shown by the assigned structures of compounds **29** and **30**, the reaction proceeded by N-alkylation (rather than O-alkylation). In order to verify this, NAS of the

bromide **13** was carried out to give the *O*-benzyl product **38**, isomeric to compound **29** (Scheme 66).



13 **38**

Scheme 66. Synthesis of *O*-benzyl compound **38** by nucleophilic substitution

^{1}H and ^{13}C NMR data were used to confirm the assigned structures of **29** and **38**. The proton and carbon NMR chemical shifts for the two compounds were different; also the difference in the chemical shifts for the benzylic CH_2 protons (a and b) is smaller for compound **29** (0.26 ppm) than for compound **38** (0.42 ppm), as expected. Unfortunately, a low yield (3%) was recorded in the synthesis of **38**, which involved two column chromatographic runs to separate the product from starting material, the 8-hydroxy compound **17**, and an unexpected compound **46** whose NMR spectrum is presented in Figure 25. We also carried out other analyses in order to determine the structure of compound **46**. Identifiable features from the NMR (Figure 25) include the adenine 2-butoxy peaks (0.9 to 1.6 ppm and 4.18 ppm), the 9-benzyl (5.26 ppm and 7.24 to 7.39 ppm), and possibly the 6-amino (6.86 ppm). An additional singlet at 12 ppm was also present. After shaking the NMR sample with D_2O and obtaining the NMR again, the peaks at 6.86 ppm and 12 ppm disappeared – suggesting that these signals are from exchangeable protons (OH, NH). On analysis of the sample by +ESI-MS, a singly charged species at m/z 330.1386 was observed. Table 3 presents a comparison of the chemical shifts for the butoxy OCH_2 triplet and the benzylic CH_2 singlet and the observed masses (M+1) for this compound **46**, **17**, the

starting material **13**, and compounds **38** and **29**. The mass of **46** suggests that the product is monobenzylated rather than dibenzylated. From TLC information, the polarity order for the compounds (Rf values with 3% methanol/ DCM in parentheses) is **29** (0.61), **13** (0.51), **38**

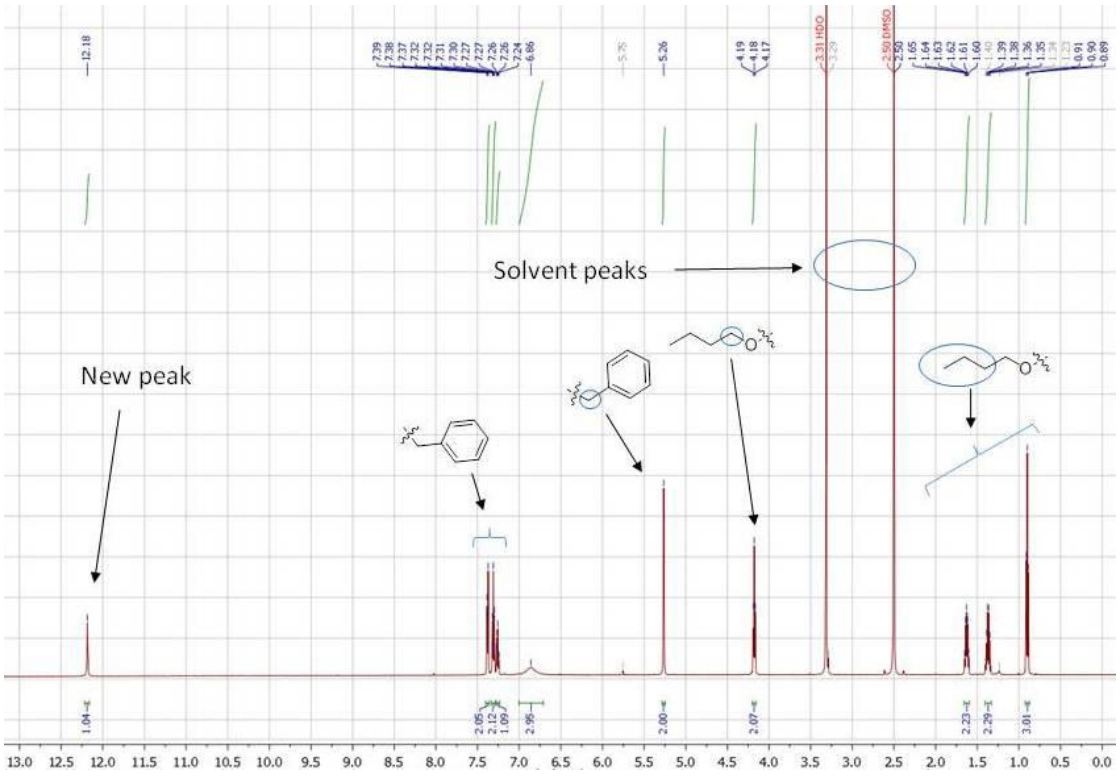


Figure 25. Proton NMR for compound **46**

Table 3. Butoxy OCH_2 and Benzylic CH_2 NMR peaks and HRMS data for compound **46** and compounds **13**, **17**, **38**, and **29**

Proton NMR/ MS	Compound **46**	Compound **13**	Compound **17**	Compound **38**	Compound **29**
Butoxy OCH_2 (ppm)	4.18	4.23	4.14	4.18	4.13
Benzylic CH_2 (ppm)	5.26	5.25	4.85	5.06, 5.48	4.95, 5.21
+ESI MS (m/z)	330.1386	376.0768	314.1613	404.2082	404.2080

(0.41), **46** (0.25) and **17** (0.12). We can infer from the information acquired that both benzylic and butoxy groups are present in this compound **46**, protic functional groups such as OH are present, and the polarity of the compound is close to that of alcohol **17**.

*Structure of Compound **46***

In agreement with the analytical data given above, we concluded that compound **46** is the thiol compound (Figure 26).[95,121]

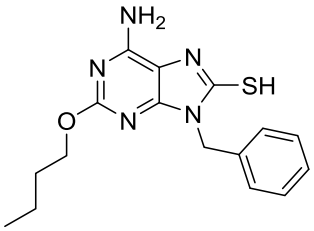


Figure 26. Compound **46**

The next challenge was to explain how compound **46** formed in the synthesis of compound **38** from the bromide **13** (Scheme 66). An accidental use of NaSH instead of NaH could explain this. The level of purity of the starting bromide could also yield an explanation. The isolation of the bromide involves the addition of sodium thiosulfate solution to an acidic (acetic acid) solution. It is known that thiosulfate ions can disproportionate to sulfur in acidic solutions: $SO_3^{2-} + H^+ \rightarrow HSO_3^- + S$.[122] Therefore, this could imply that the isolated crude product **13** contained an amount of sulfur, which could generate a reagent that can produce thiol **46** under the conditions used in the synthesis of **38**.[123] We purified the bromide starting material **13** by column chromatography using 2% MeOH/DCM. The isolated materials were a yellow powder - which had no signal in ^{1}H NMR - and the bromide **13** (purified). The Rf values (in 2% MeOH/DCM) of the yellow powder and **13** were 0.86 and 0.32 respectively.

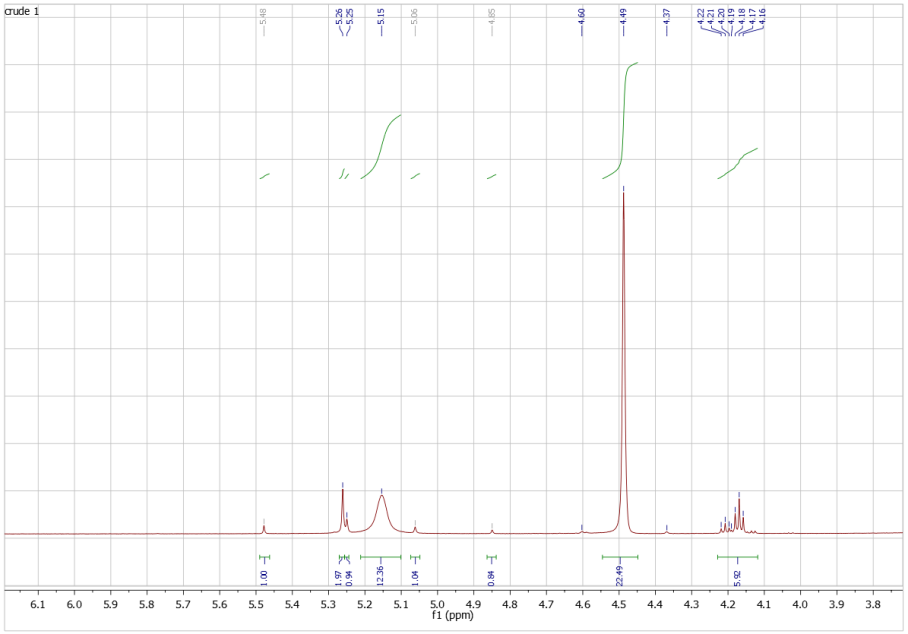


Figure 27. Crude NMR analysis of the benzyloxy nucleophilic substitution reaction on crude bromide **13**. Signal for the benzylic CH_2 of **46** was present at 5.26 ppm.

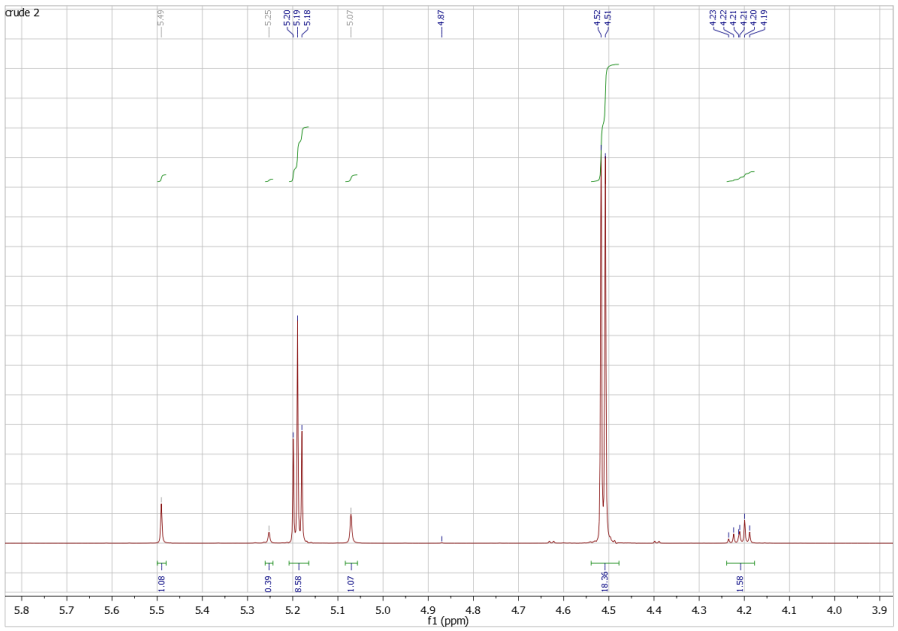


Figure 28. Crude NMR analysis of the benzyloxy nucleophilic substitution reaction on purified bromide **13**. (Signal for the benzylic CH_2 of **46** is expected at 5.26 ppm.)

A comparison of the crude NMR of the oily material (after extraction work up) obtained when crude **13** and purified **13** were subjected to the benzyloxy substitution reaction that gives **38** is given in Figures 27 and 28. It is noticeable that the contaminant product **46** appears to be absent when purified **13** was used (Figure 28). In addition, crude TLC and ESI-MS analyses of the reaction involving purified **13** did not indicate the presence of compound **46**. Lastly, GC-MS analysis of the yellow powder that was separated from the crude compound **13** is in good agreement with that of elemental sulfur (see Spectra 105). We can therefore conclude that the use of purified bromide starting material is preferable in the synthesis of the O-benzylated compound **38** from the bromide.

Reporter Cell Assay Analysis of TLR7 Conjugates

To confirm the TLR activity of new compounds a bioassay was performed using the HEK-Blue Reporter Cell assay. This assay utilizes cells with the TLR receptor of interest (TLR7, in our case) linked to a NF-κB reporter gene. Stimulation by the TLR compounds induces the production of an alkaline phosphatase enzyme which reacts with a precipitating substrate to produce a blue/purple colored product that can be measured by spectrophotometry. Compounds and conjugates were analyzed at the Baylor Institute of Immunology Research, Dallas, Texas. The graph in Figure 29 shows the signal intensity versus concentration for different compounds. R848 is Resiquimod, a standard reference TLR7 agonist. For each material being analyzed, TLR signaling was measured at a number of selected concentrations (5, 50, 500 and 1000 ng/ml).

Compounds **7**, **8**, **21**, **24**, **25**, **26**, **27**, **29**, **30**, **32**, **33**, **35**, **36** were analyzed. The results were in agreement with predictions of TLR7 agonistic activity by structure-activity relationship.[121] The parent carboxylic acid **7** and the amine linkers **8**, **27** and **35** all showed

good TLR7 activity. The indole-based compounds **32** and **33** also showed good activity. Both DBCO compounds **24** and **36** were also active - with a higher activity recorded by the PEG-2 compound **36**. This is in agreement with the significant activity of its PEG-2 amine precursor **27**. The maleimide compound **21** showed a reduced activity, while compounds **29** and **30**, lacking the C-8 OH group, were inactive. Notably, TLR7 active compounds that were inactive at or below 50 ng/ml are compounds **21**, **24**, **25** and **26**. A more extensive activity data for these compounds has recently been published.[121]

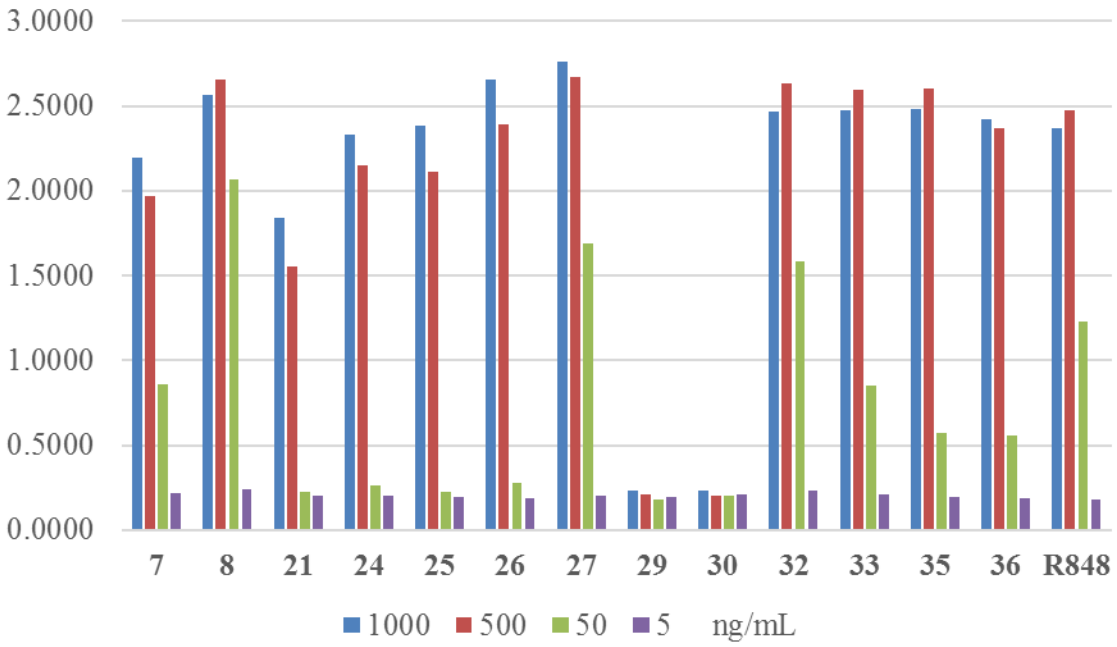


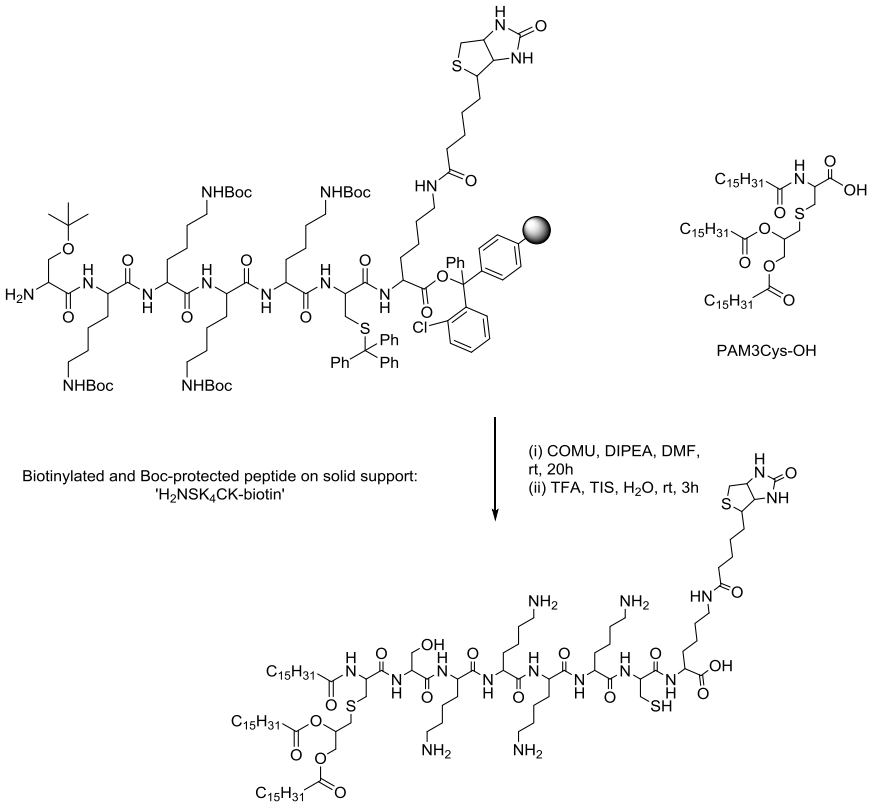
Figure 29. TLR7 Activation (AU) measured by HEK Blue Cell Reporter Assays

Solid Phase Synthesis and Peptide Conjugation

Studies have shown the importance of the fatty acid chain length, the ester-bound fatty acid linkage, and the length and nature of the peptide in TLR2 ligands such as Pam3CSK4 for TLR2 signaling induction.[98,99] We proceeded to synthesize a novel lipopeptide **22**, based on the Pam3CSK4 structure as a potential TLR1:TLR2 agonist

(Scheme 67). The presence of the additional cysteine moiety bearing the free sulfhydryl group enables us to explore bioconjugation using the thiol-maleimide chemistry. Additionally, the biotin molecule allows us to monitor antibody conjugation.

Peptide H_2NSK_4CK-biotin was purchased and used in its Boc protected form on solid support. The Boc protection ensures that only the desired amino group reacts with cysteine carboxylic acid on the reagent PAM3Cys-OH. This coupling reaction was carried out using COMU and DIPEA in DMF. The PAM3Cys-OH reagent was dissolved in DCM to initiate reaction.[124,125] After reaction, the product was cleaved and deprotected using a cleaving cocktail (30:2:1 TFA/water/triisopropylsilane by volume) to remove the Boc, t-Bu, trityl, and 2-chlorotrityl groups along with the polymer support. The mixture



22

Scheme 67. Synthesis of Functionalized Pam3CSK4 Analog **22**

was filtered to remove insoluble material and evaporated. In order to analyze the reactions by LC-MS, the peptide starting material H_2NSK_4CK-biotin was initially deprotected by the deprotecting protocol above and analyzed by LC-MS. For the deprotected H_2NSK_4CK-biotin with a product theoretical mass of m/z 1075.61, the triply charged and doubly charged species were observed at m/z 359.21 and m/z 538.31 respectively. In addition, the model TLR2 ligand Pam3CSK4 was also analyzed. With a product theoretical mass of m/z 1510.16, the triply charged and doubly charged species were also observed (m/z 504.06 and m/z 755.59 respectively).

The lipopeptide product (**22**) with a product theoretical mass of m/z 1967.35 showed the triply and doubly charged species at m/z 656.79 and m/z 984.68 respectively (Figure 30).

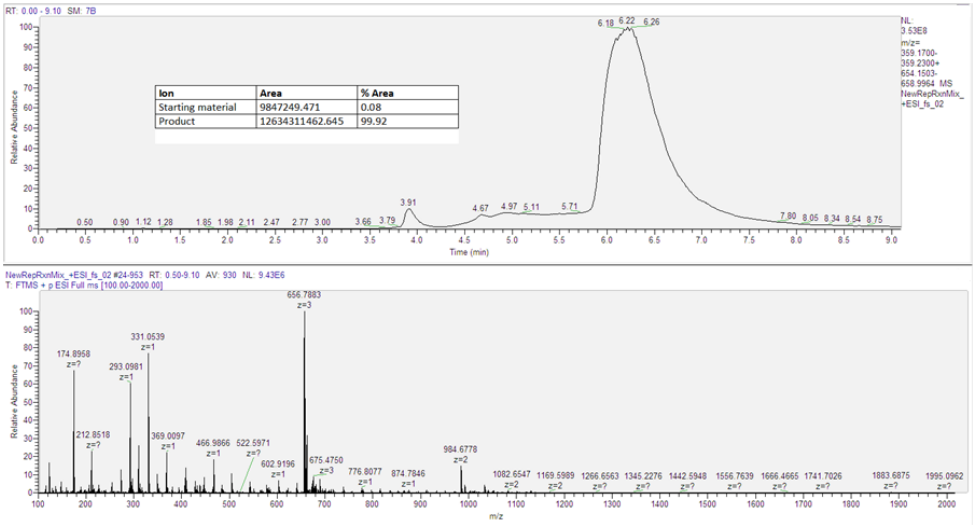
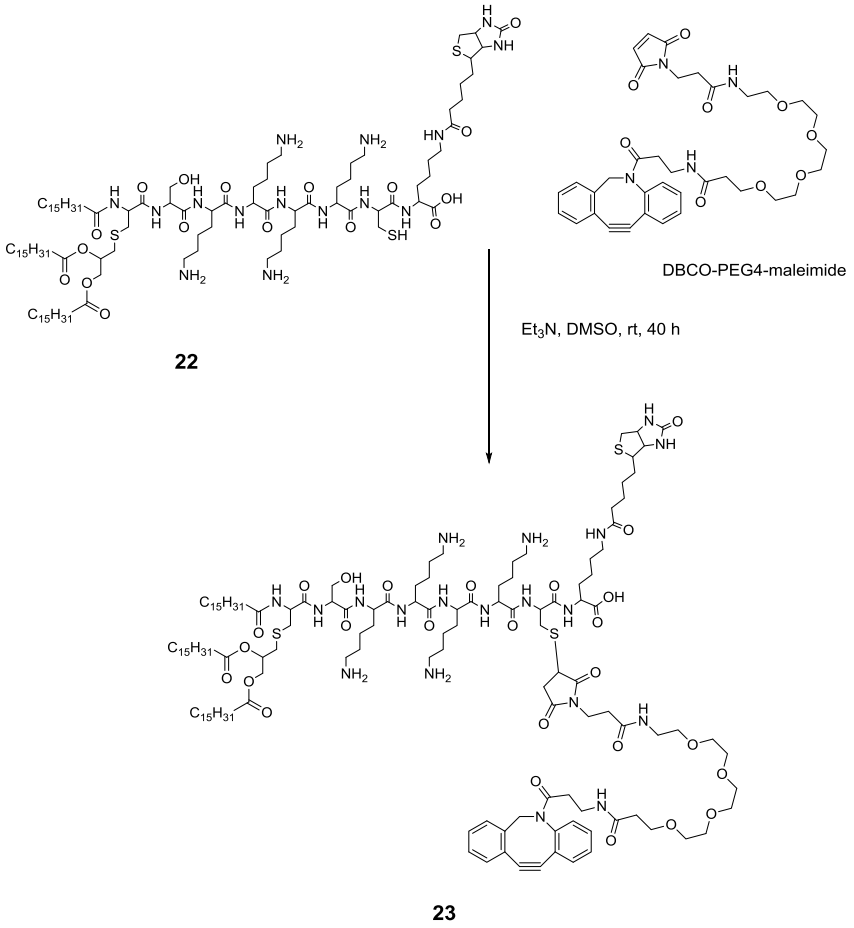


Figure 30. LC-MS spectra for Pam3CSK4CK-biotin peptide

We then carried out a thiol-maleimide reaction between compound **22** and DBCO-PEG4-maleimide using triethylamine in DMSO (Scheme 68). After 40 hours, the solution was analyzed directly by LC-MS without purification. The product (**23**) was observed at

m/z 881.55 (for the triply charged). The unreacted peptide was observed at m/z 656.45 (for the triply charged) and the unreacted crosslinker was observed at m/z 675.30 (for the singly charged).

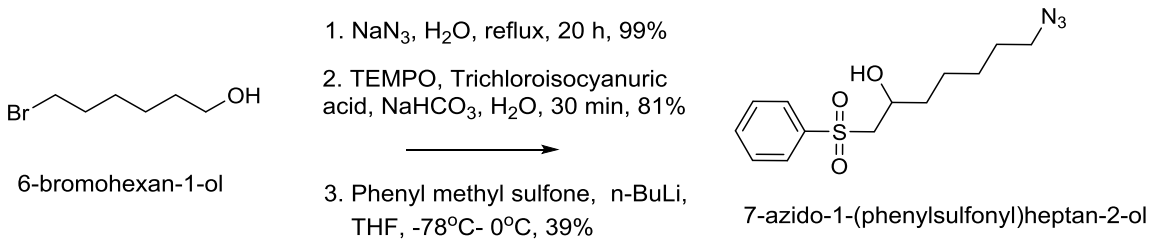


Scheme 68. Thiol-maleimide addition reaction between functionalized peptide **22** and DBCO-PEG4-maleimide linker

Bifunctional Releasable Linker Synthesis

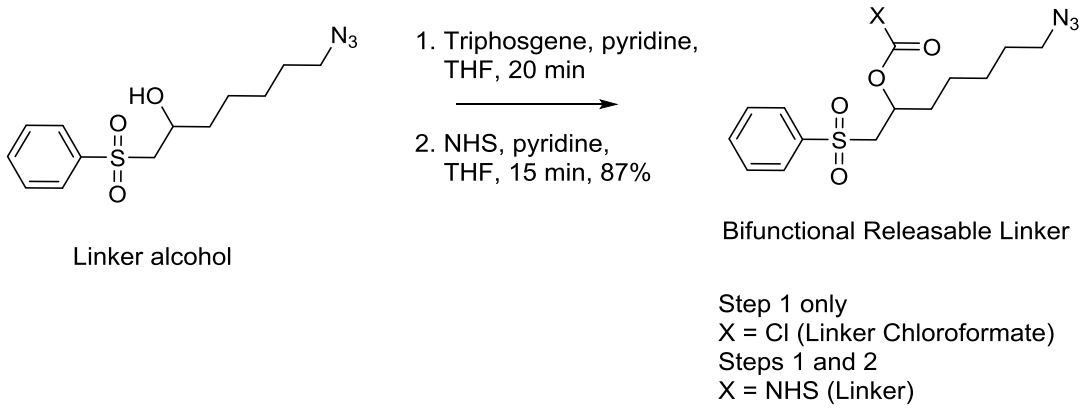
To be able to achieve the slow release of conjugated drugs in vivo, we synthesized a β-eliminative linker according to the protocol used by Santi et al.[22] 7-azido-1-(phenylsulfonyl)heptan-2-ol was synthesized in 3 steps from 6-bromohexanol (Scheme

69). These steps involve azide substitution for the bromine, primary alcohol oxidation (to aldehyde), and nucleophilic addition at the carbonyl by the carbanion of the sulfone.



Scheme 69. 7-Azido-1-(phenylsulfonyl)heptan-2-ol synthesis by a 3-step literature protocol

The linker was then synthesized from the linker alcohol (7-azido-1-(phenylsulfonyl)heptan-2-ol) via the chloroformate, according to Scheme 70.[22]

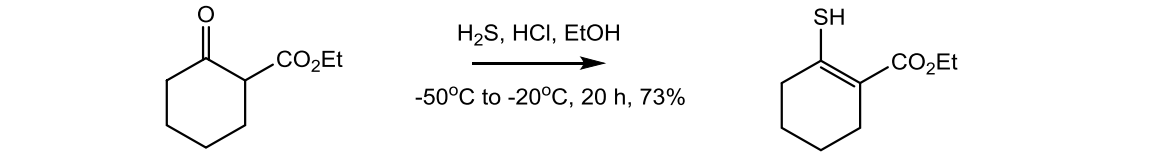


Scheme 70. Synthesis of the bifunctional releasable linker or its chloroformate

Sulfonamides Synthesis

TAK-242 Synthesis

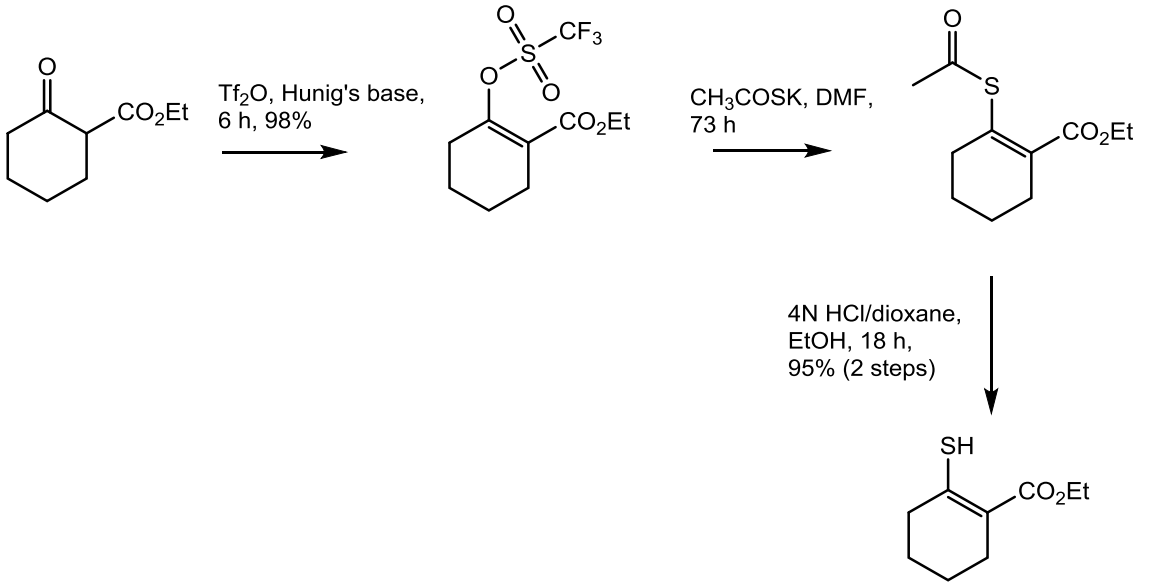
The literature approach to the synthesis of TAK-242 begins with the direct synthesis of the intermediate thiol from the starting ethyl 2-oxocyclohexane-1-carboxylate by using nucleophilic hydrogen sulfide gas under acidic conditions (Scheme 71).[102]



ethyl 2-oxocyclohexane-1-carboxylate ethyl 2-mercaptocyclohex-1-ene-1-carboxylate

Scheme 71. Direct synthesis of ethyl 2-mercaptocyclohex-1-ene-1-carboxylate from starting keto ester

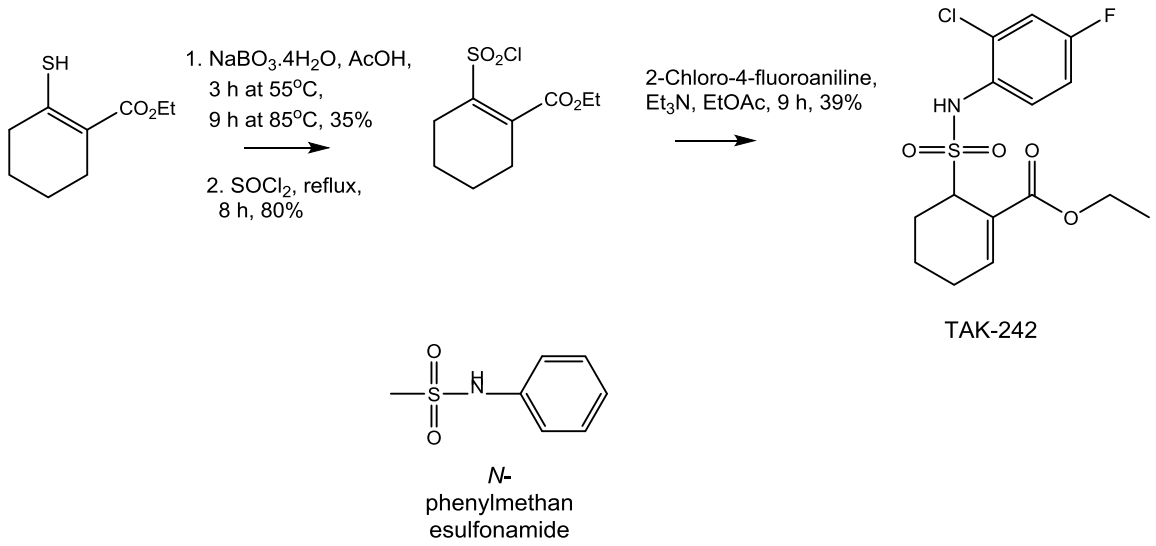
In order to avoid the use of H_2S, we initially explored the use of Lawesson's reagent to synthesize the thiol using different solvents (toluene, benzene or hexane) at an elevated temperature. Evaporation of solvent gave mixtures that contained <10% product to starting material. Accordingly, we adopted an alternative approach which involves the sulfonation of the starting ethyl 2-oxocyclohexane-1-carboxylate with triflic anhydride,



Scheme 72. Successful approach to the synthesis of ethyl 2-mercaptocyclohex-1-ene-1-carboxylate

followed by a nucleophilic substitution of the triflate with thioacetate to give the thioester (Scheme 72).[126] Hydrolysis of the thioester by 4N HCl under anhydrous conditions provided the desired thiol. To complete the synthesis of TAK-242, the thiol was oxidized

using sodium perborate in acetic acid and the sulfonic acid intermediate obtained was treated with thionyl chloride to give the sulfonyl chloride (Scheme 73). A difference between our procedure and the literature procedure[102] for the synthesis of the sulfonic acid was that we carried out column chromatography to remove inorganic impurities that could be present after product isolation. The final step of the synthesis involves nucleophilic substitution with 2-chloro-4-flouroaniline using triethylamine. This reaction proceeds with isomerization involving the alkene bond of the cyclohexene carboxylate. Results from the literature (Yamada et al.)[102] suggest that the base is a necessary part of the isomerization mechanism, as isomerization did not occur when DMF was used in the place of triethylamine/ethyl acetate in this reaction.

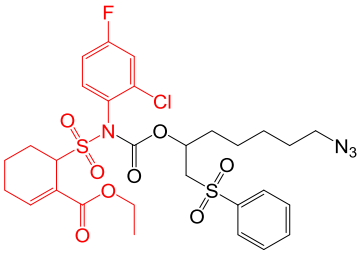


Scheme 73. Final stages in the synthesis of TAK-242

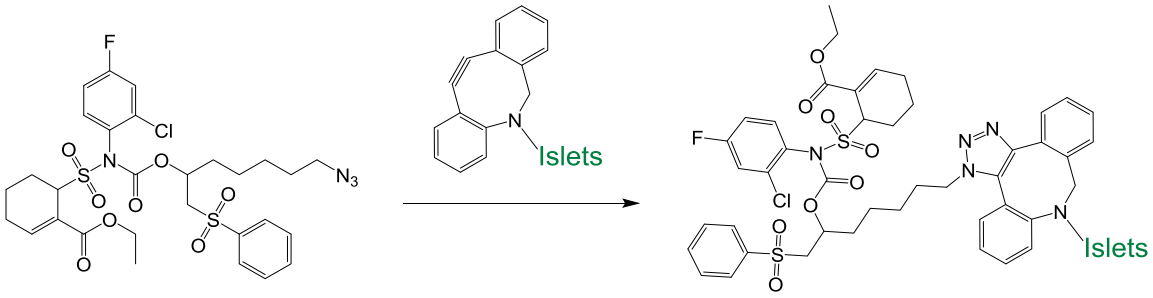
As a model compound in the place of TAK-242, *N*-phenylmethanesulfonamide was synthesized from aniline and mesyl chloride in the presence of triethylamine.

Carbamates Synthesis

The primary goal of our synthesis of carbamates is to attach the sulfonamide drug TAK-242 to the linker alcohol (7-azido-1-(phenylsulfonyl)heptan-2-ol) via the carbamate moiety, generating a molecule which slowly releases the active drug in vivo. Studies by Yamada et al. showed that alkylation at the sulfonamide nitrogen resulted in a significant loss of activity.[102] Therefore the synthesis of the carbamate **45** which bears a cleavable group attached to TAK-242 (in red, Scheme 74) will allow us to use the drug as a prodrug (See also Figures 9 & 10). The azide functionality on **45** allows the conjugation to islets via click chemistry.



45

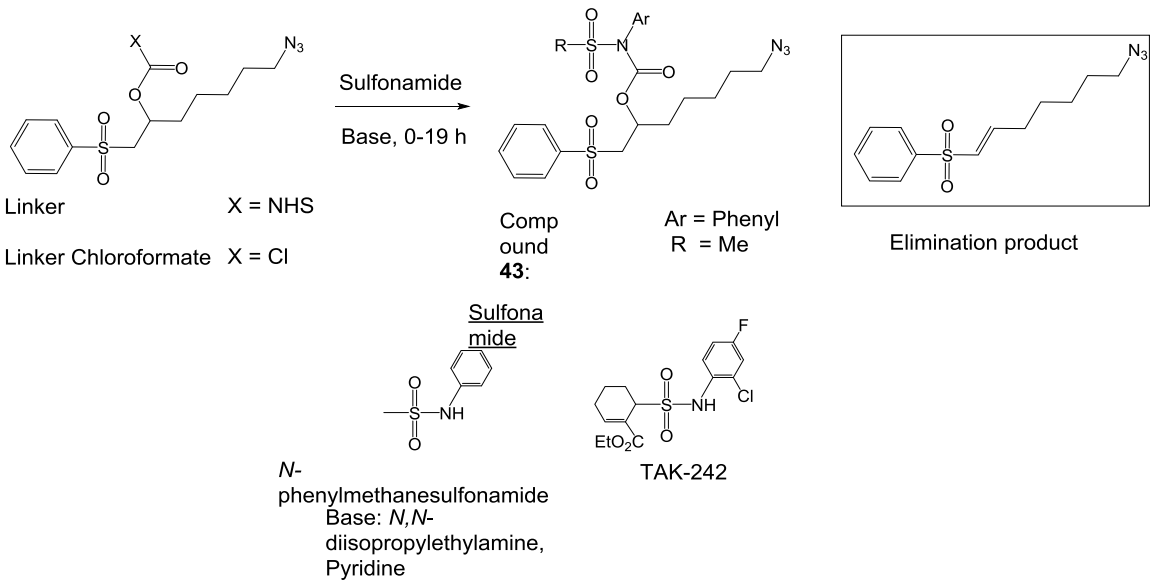


45

Scheme 74. Conjugation of anti-inflammatory drug TAK-242 to islets via a releasable linker

General Route: Substitution of X on Bifunctional Releasable Linker Using Sulfonamide

Our initial attempt was to directly react the linker (X = NHS, Scheme 75) with TAK-242 or *N*-phenylmethanesulfonamide. This approach failed, mainly due to the generation of the elimination product. For this reaction, we used pyridine (with TAK-242) or diisopropylethylamine (with *N*-phenylmethanesulfonamide) as the base. No reaction occurred when pyridine was used, while the elimination product resulted from the use of diiisopropylethylamine. The chloroformate (X = Cl, Scheme 75) is expected to be more reactive than the NHS.[127,128] (The chloroformate was obtained by reacting the alcohol with triphosgene in the presence of pyridine, Scheme 70.) Indeed, the substitution reaction

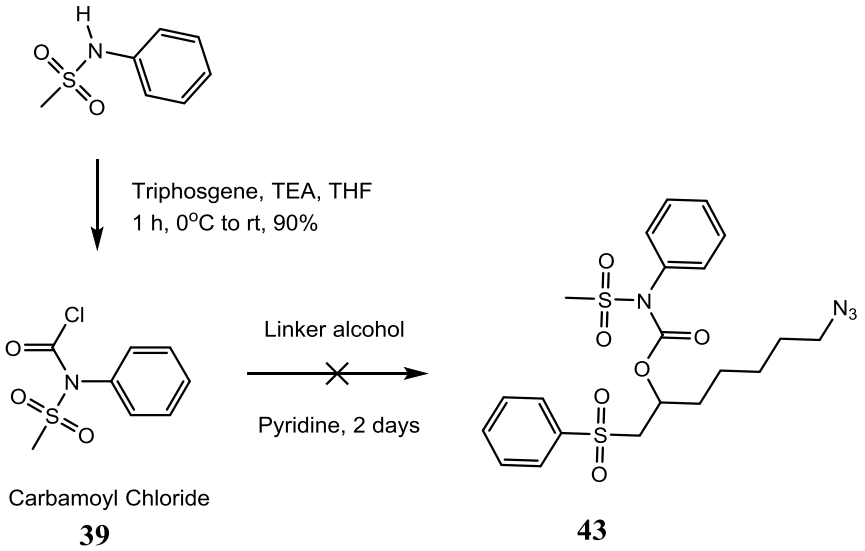


Scheme 75. Substitution of X at Bifunctional Releasable Linker

was successful - albeit with a poor yield - when the linker chloroformate was reacted with *N*-phenylmethanesulfonamide using pyridine as the base. This gave compound **43**. However, an attempt to reproduce this result in the reaction of TAK-242 (in the place of the *N*-phenylmethanesulfonamide) with the linker chloroformate failed.

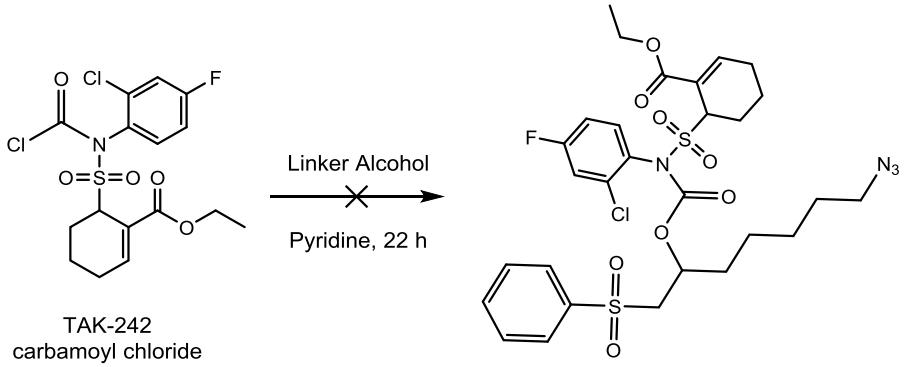
Use of Carbamoyl Chlorides; Substitution by Alcohols

We proceeded to use a different approach which is a reverse of the first approach in terms of the substrate that is first reacted triphosgene. In this case the sulfonamide is reacted with triphosgene to generate the carbamoyl chloride before treating the carbamoyl chloride with the alcohol as nucleophile (Scheme 76).[129] This approach first led to the synthesis of the surprisingly stable carbamoyl chloride **39** (from *N*-phenylmethanesulfonamide and triphosgene in the presence of triethylamine) as a white solid. The reaction of **39** with the linker alcohol was unsuccessful (no reaction).



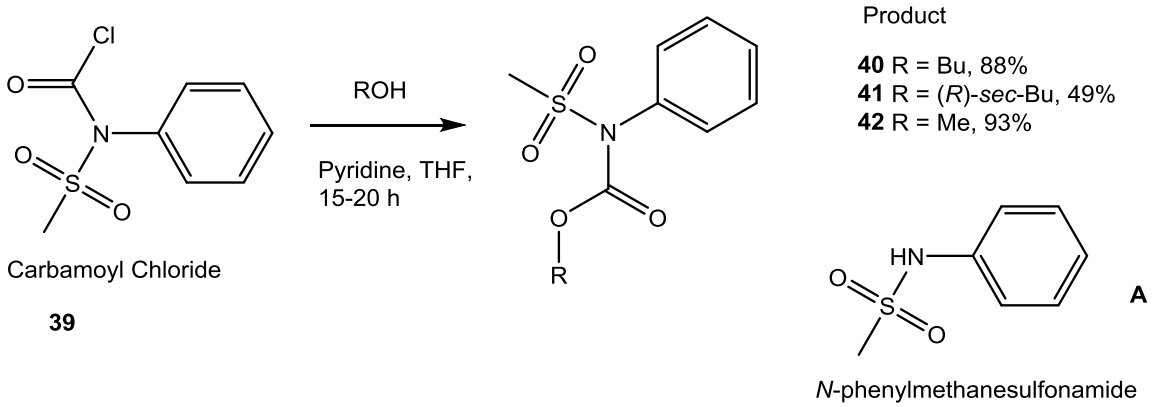
Scheme 76. Reaction of Compound **39** with Linker Alcohol

The synthesis of TAK-242 carbamoyl chloride itself (Scheme 77) was low yielding. Further reaction with the linker alcohol also failed to give the target carbamate at an appreciable yield.



Scheme 77. Reaction of TAK-242 Carbamoyl Chloride with Linker Alcohol

The reactions of the model carbamoyl chloride **39** with n-butanol, 2-butanol, and methanol as the alcohol were successful, giving **40**, **41**, and **42** (Scheme 78).[130] Before purification, analysis of the crude product by NMR indicated the presence of the sulfonamide **A** alongside the 3 products as shown in Table 4. The relative amounts of **A** present (%**A**) for the three different alcohols suggests that this reaction is particularly sensitive to sterics as the secondary alcohol 2-butanol is more hindered than the two primary alcohol. The reaction of the carbamoyl chloride with 2-butanol led to more of the hydrolysis side product (sulfonamide) than the desired product.



Scheme 78. Reactions of **39** with *n*-Butanol, Methanol and Secondary Butanol

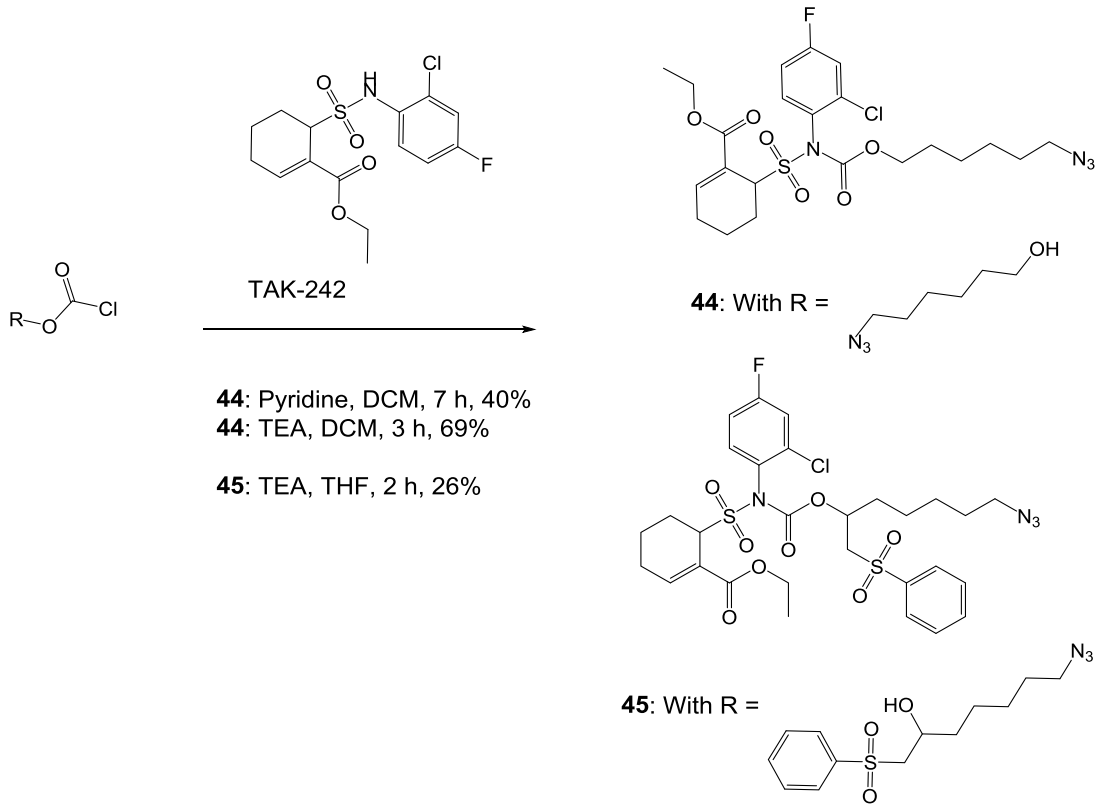
Table 4. Relative Reactivity of **39** with *n*-Butanol, Methanol and *Sec*-Butanol

ROH	R = *n*-Bu	R = *Sec*-Bu	R = Me
% of **A** present*	12%	52%	3%

*Determined by NMR spectrum**

Synthesis of Target Product

Reaction of 6-azidohexanol via its chloroformate with TAK-242 was successful to give a carbamate that lacks β-eliminative functionalities (acidic C-H, sulfone, or modulator), compound **44** (Scheme 79). To react the linker alcohol (7-azido-1-(phenylsulfonyl)heptan-2-ol) with TAK-242 successfully, a stronger and more hindered base triethylamine was needed, giving compound **45**.



Scheme 79. Synthetic Scheme for the Synthesis of Carbamates from TAK-242 and Chloroformates

An improvement in the reaction yield was also recorded when triethylamine was used in the synthesis of **44**. It is worthwhile to investigate the effect of the hindered base in this reaction by using other more hindered bases (Hunig's base or 2,6-lutidine). The use of a hindered base would imply a less tendency to produce β-elimination side products, thus resulting in a greater yield of desired product.

Identity and Characterization of the Carbamates

By examining the proton NMR of compound **44**, we noticed that this compound was formed as a mixture of compounds that were structurally similar. We carried out a series of characterization experiments including COSY (correlation spectroscopy) NMR, carbon-13 NMR, DEPT-135, multiplicity-edited HSQC to determine the nature of this compound. The results of these experiments are consistent with the existence of two isomers with one being the major and the other being the minor one. The possibility of rotational isomers is depicted in Figure 31. Compound **45**, on the other hand, is expected to exist as diastereomers arising from the two asymmetric centers present and some conformational isomerism is also possible.

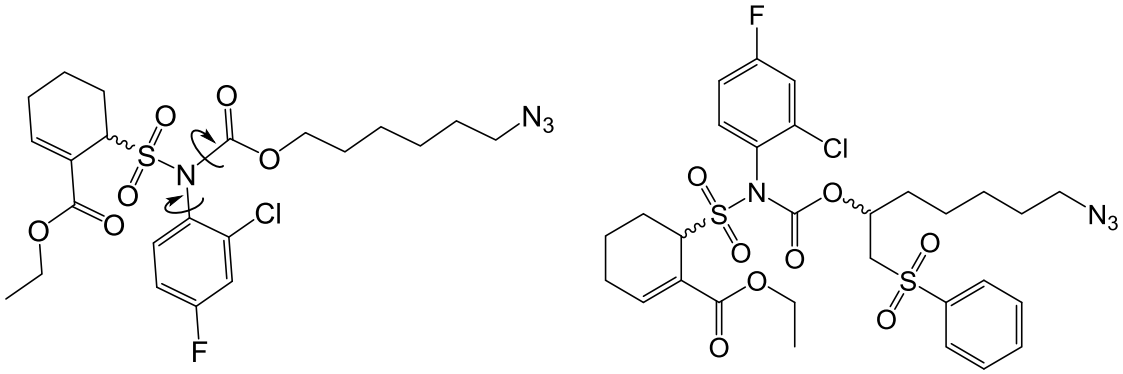


Figure 31. Possible isomerism in compounds **44** and **45**

A necessary experiment to determine if compound **44** is a mixture of rotational isomers was a variable temperature NMR. Proton NMR of **44** was obtained at temperatures 25°C, 35°C, 50°C, 75°C, and 90°C. Our tentative assignment for the signal of the proton

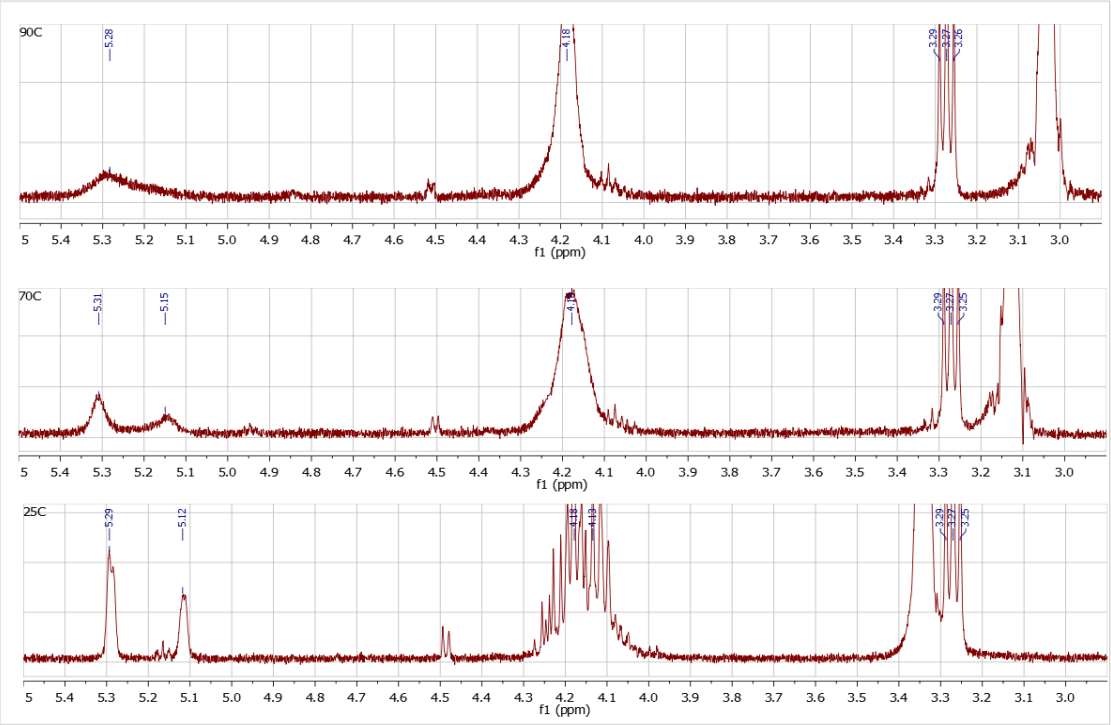


Figure 32. Variable Temperature NMR experiment for compound **44** (Temperatures: 25°C, 70°C, 90°C, NMR solvent: DMSO-d_6)

on the asymmetric carbon was at ~5 ppm. Beginning from 70°C to 90°C, two separate signals in this region began to coalesce and eventually became one - expected as a result of fast rotation and equilibration at higher temperatures (Figure 32).

The observed ratio of the major isomer to the minor isomer of compound **44**, according the proton NMR, is 1: ~0.37 (Figure 33) in $CDCl_3$ and 1: 0.65 in DMSO-d_6. The proton NMR assignments shown in Figure 33 were determined using ^{1}H NMR, COSY, ^{13}C NMR, and edited HSQC (^{1}H-^{13}C) spectra.

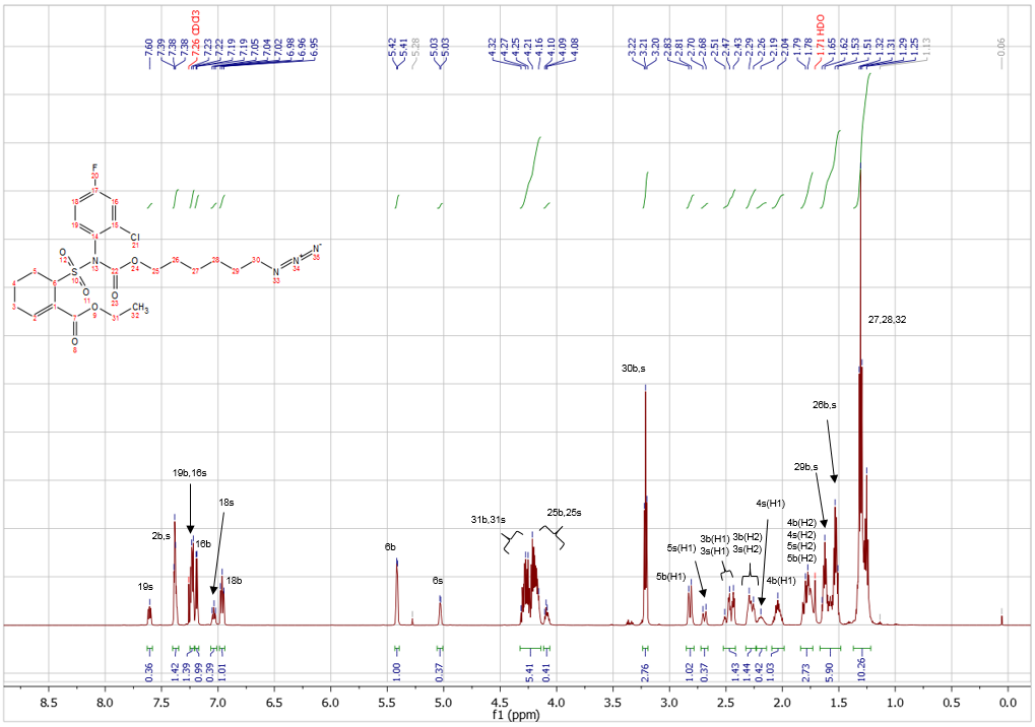


Figure 33. ^{1}H NMR Chemical Shift Assignments for Compound **44.** Key: b-bigger percent (major) isomer; s-smaller percent (minor) isomer; H1,H2- methylene protons on same carbon.

Figure 34 shows the general numbering of the protons and carbons of compound **44** for spectral assignment purposes. The assignment of peaks in the aromatic region of the proton NMR spectrum of **44** is presented in Table 5. This assignment was carried out according to measurements of coupling constants. The coupling constant values are consistent with standard values for H-H and H-F couplings.

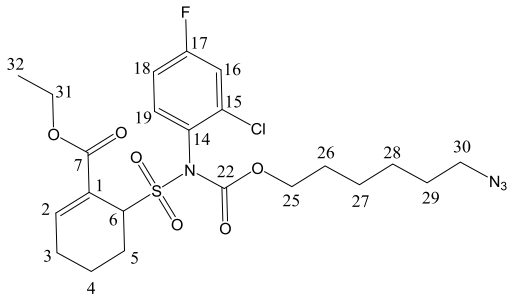


Figure 34. General structural numbering of **44** for spectral assignments

Table 5. ^{1}H NMR Coupling Constants (H-H and H-F couplings) and Assignments for the Aromatic Region for Compound **44**

Proton index number	Type of Coupling	J value (Hz)
19	HC-C(H)-CF	5.5
	HC-CH	8.8
16	HC-CF	8.0
	HC-C(F)-CH	2.8
18	HC-CH	8.6
	HC-C(F)-CH	2.7
	HC-CF	Not observed[131]

Table 6. ^{13}C NMR Chemical Shift Assignments for Compound **44**

Carbon index number	δ (ppm)	Line(s) for major isomer (ppm)	Line(s) for minor isomer (ppm)
32	14.18, 14.30	14.30	14.18
4	16.28, 16.80	16.80	16.28
5	23.70, 23.87	23.87	23.70
3	25.03 – 25.23	25.20	25.03
27,28	26.13		
26	28.22, 28.29	28.29	28.22
29	28.68		
30	51.24		
6	58.92, 59.49	58.92	59.49
31	61.10, 61.29	61.29	61.10
25	67.67, 67.73	67.73	67.67
18	114.64 – 114.96	114.64, 114.82	114.78, 114.96
16	117.35 – 117.74	117.35, 117.56	117.53, 117.74
1	123.77, 123.97	123.97	123.77
14	129.75 – 130.49	129.75, 129.78	130.46, 130.49
19	132.04 – 132.45	132.38, 132.45	132.04, 132.12
15	136.14 – 136.35	136.14, 136.23	136.27, 136.35
2	147.49, 148.61	148.61	147.49
22	152.00, 152.11	152.11	152.00
17	161.28 – 163.38	161.28, 163.29	161.37, 163.38
7	166.02, 166.08	166.08	166.02

The assignment of carbon-13 NMR peaks for compound **44**, determined using ^{1}H NMR, ^{13}C NMR, and edited HSQC (^{1}H-^{13}C) spectra, is shown in Table 6. Additionally, Table 7, shows the assignment of peaks in the aromatic region of the carbon NMR spectrum

according to measurements of coupling constants. The coupling constant values are consistent with standard values for C-F couplings.

Table 7. ^{13}C NMR Coupling Constants (C-F couplings) and Assignments for the Aromatic Region for Compound **44**

Carbon index number	Type of Coupling	J value (Hz)
17	1-bond coupling	253
14	4-bond coupling	3.8
15	3-bond coupling	11
19	3-bond coupling	9.4
18	2-bond coupling	22.3
16	2-bond coupling	25.8

For compound **45**, while the ratio of isomers present, according to proton NMR, is approximately 1:1 in DMSO-d_6, this ratio is close to 1: 0.7 in $CDCl_3$.

Concluding Remarks

In conclusion, several new adenine derivatives and linkers were synthesized with a handful of them showing good activity as TLR7 agonists (compounds **7**, **8**, **21**, **24-28**, **31-33**, **35**, **36**).[121] We have begun the preparation of protein conjugates from a number of these compounds. Interesting results were obtained in the benzyloxy substitution reaction on bromide **13** to give **38**. This reaction was of value in that it helped to elucidate the structures of dibenzyl products (**29**, **38**). Compound **29** was determined to be N-benzylated while **38** was the O-benzylated product.

We also synthesized the TLR4 inhibitor TAK-242 and attached this compound to 6-azidohexanol and the β-eliminative sulfone linker[22] via the chloroformate to give carbamates **44** and **45**, respectively. The compounds are expected to allow the mechanistic study of slow release of TAK-242 when used to modify pancreatic islets. The release of the active drug prevents the immediate blood-mediated inflammatory response (IBMIR) which is a leading cause of graft loss in islet transplantation.

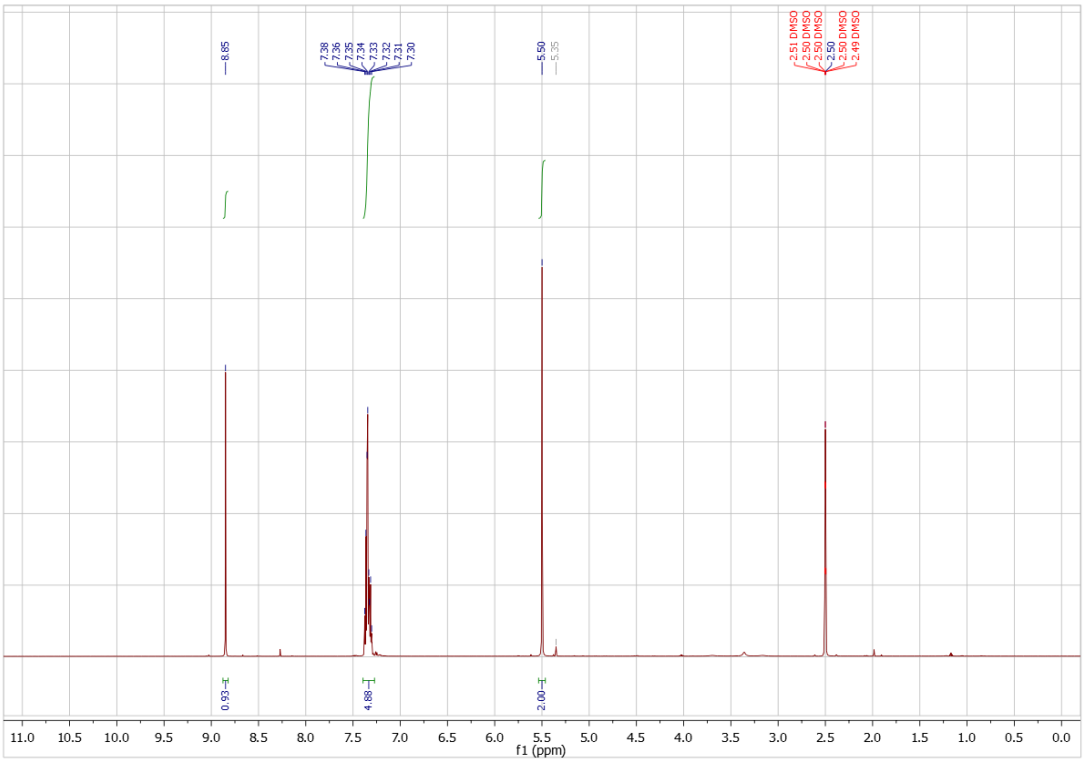
Finally, new lipopeptides **22** and **23** were synthesized as potential TLR2 agonists that are functionalized for bioconjugation.

APPENDIX

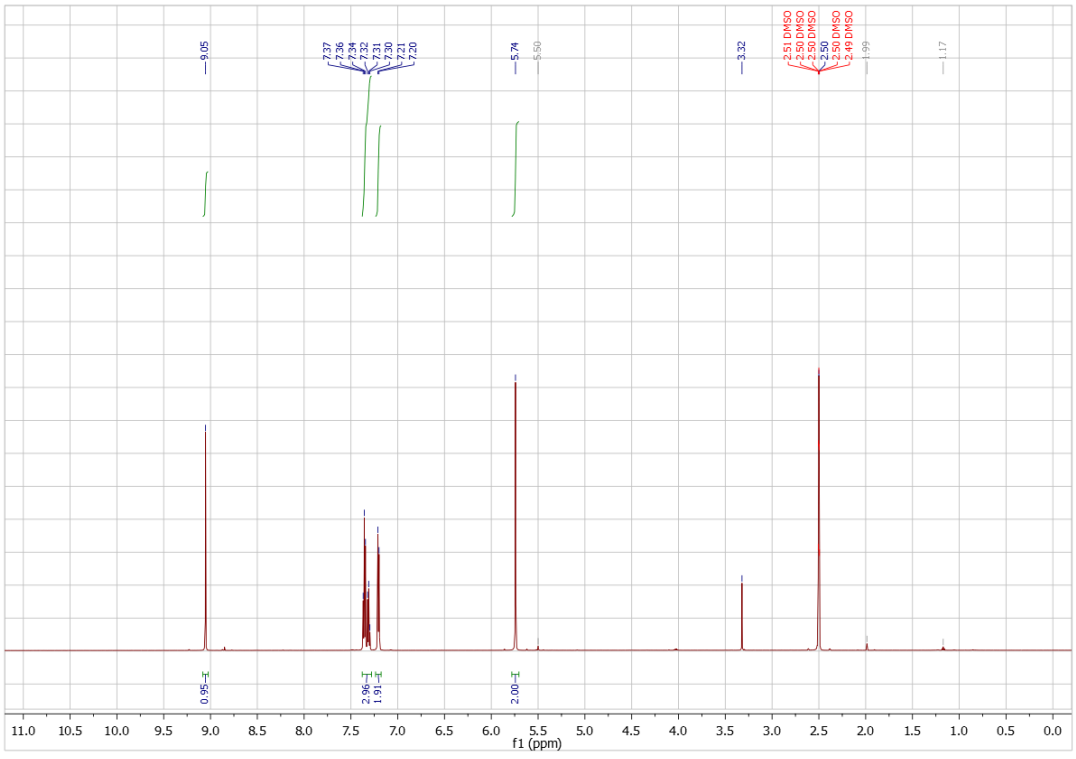
APPENDIX

Selected NMR Spectra, High Resolution Mass Spectra, and LC-MS Spectra of Compounds

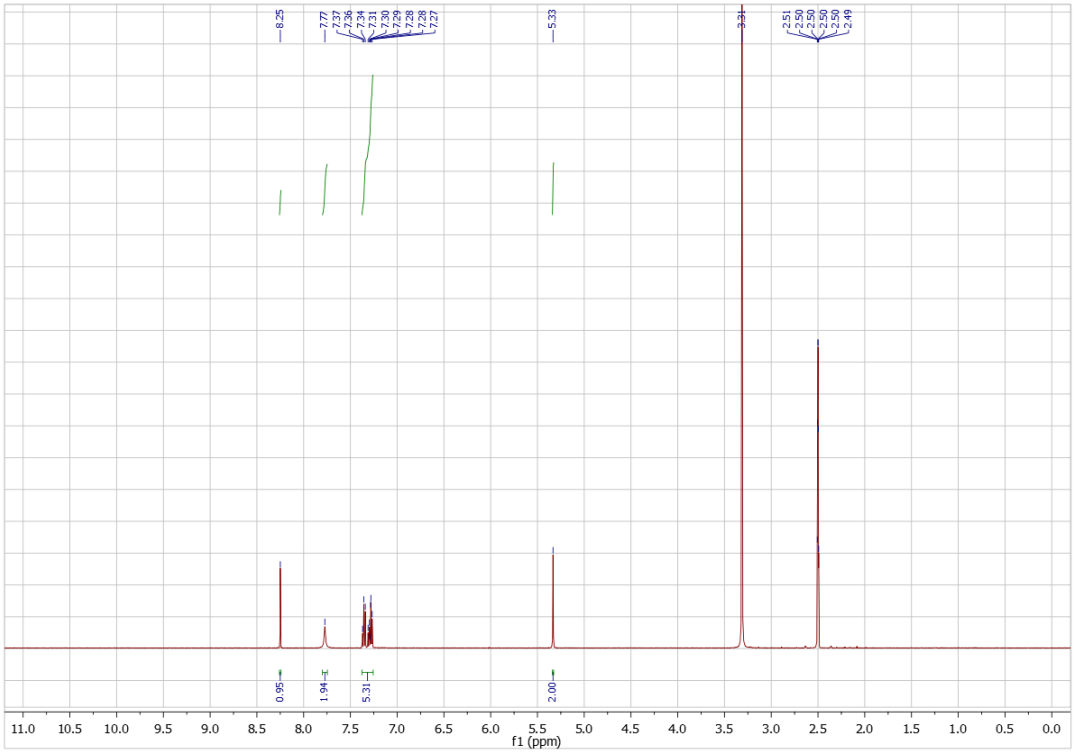
LIST OF SPECTRA



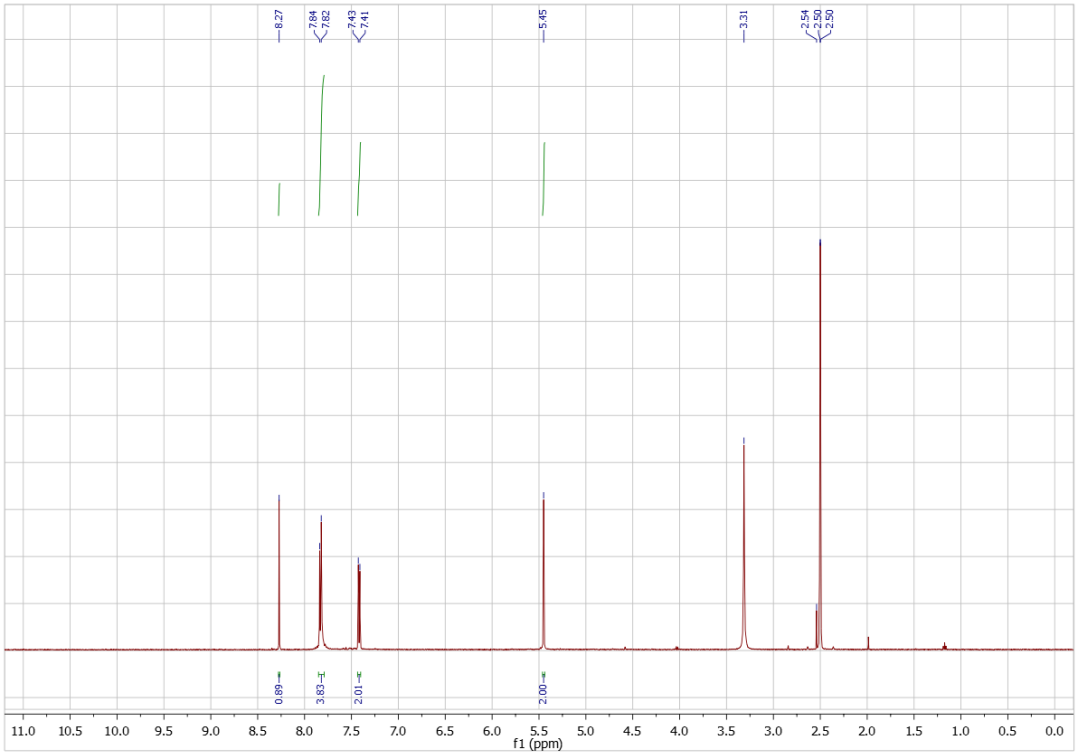
Spectra 1. ^{1}H NMR of **1a**



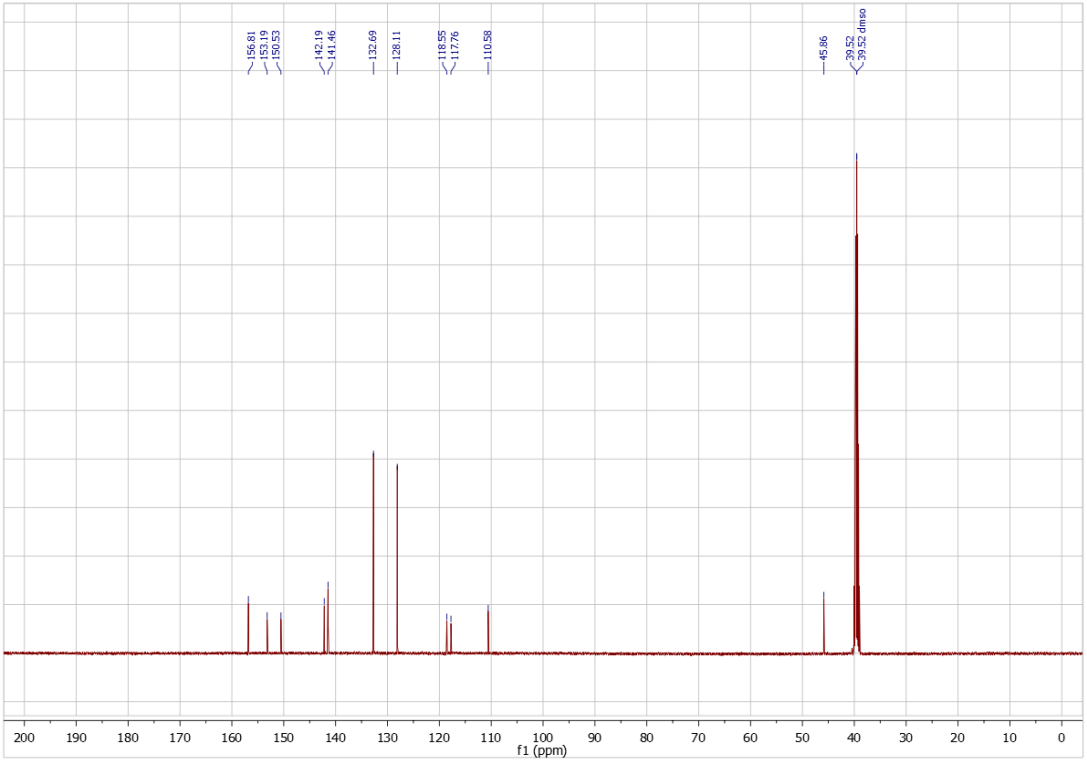
Spectra 2. ^{1}H NMR of **1b**



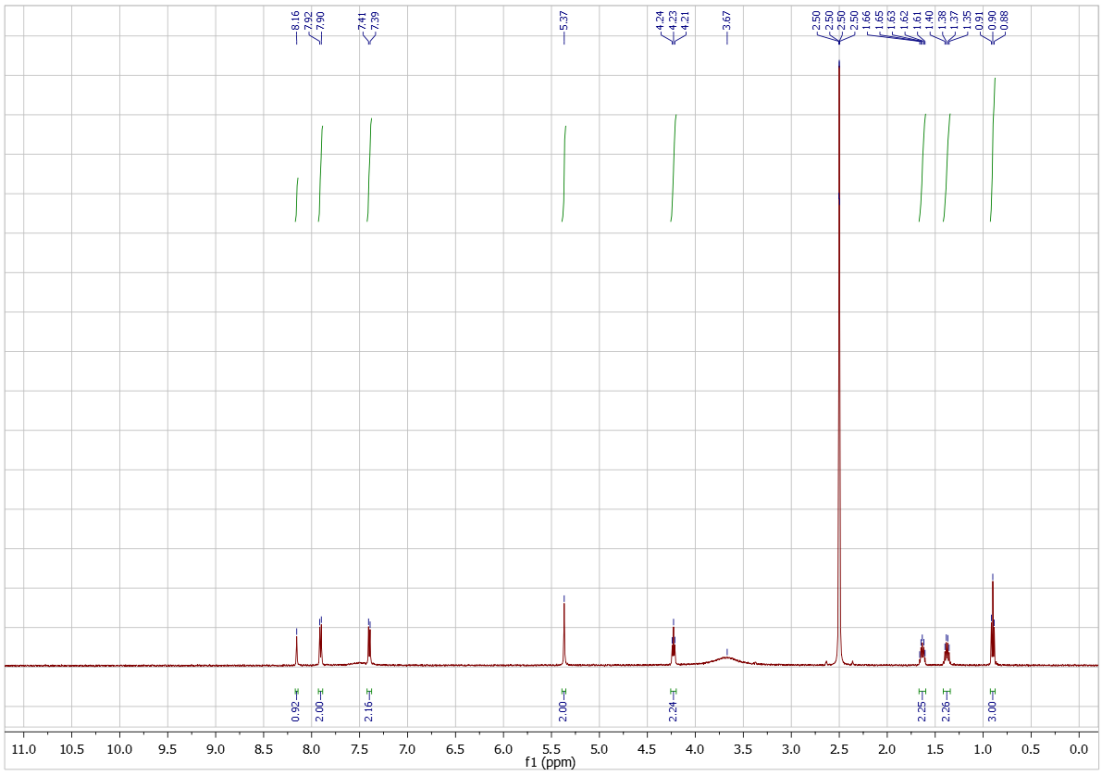
Spectra 3. ^{1}H NMR of **2**



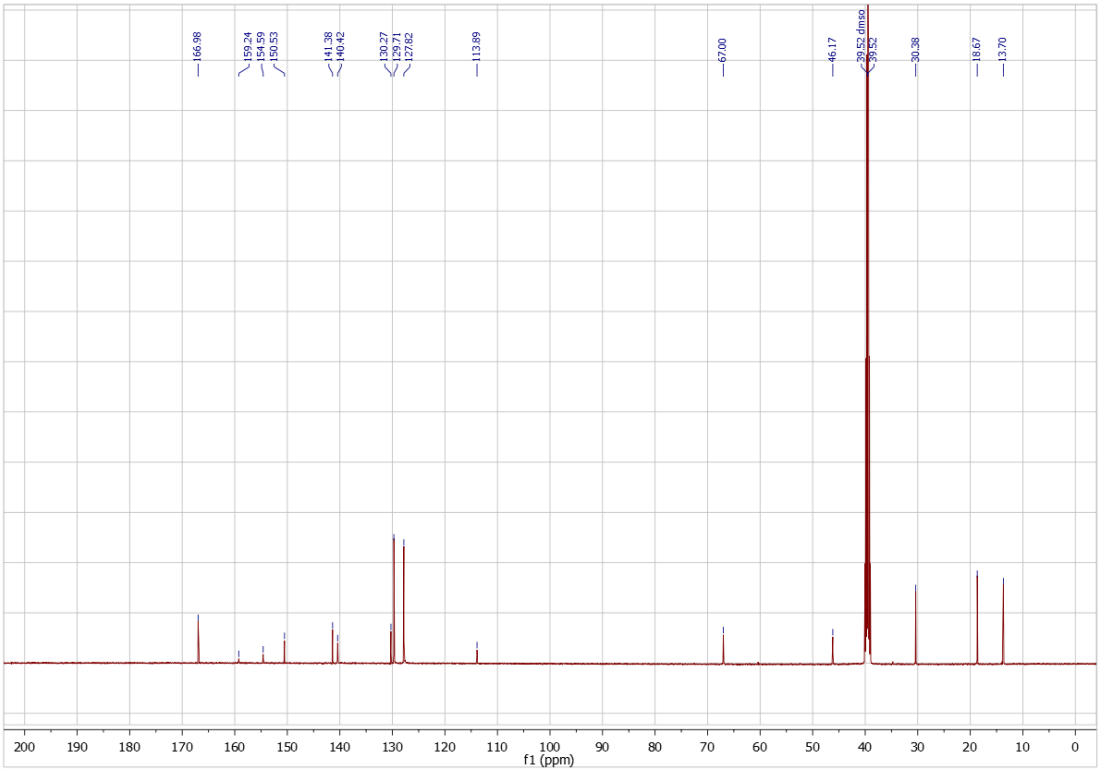
Spectra 4. ^{1}H NMR of **3**



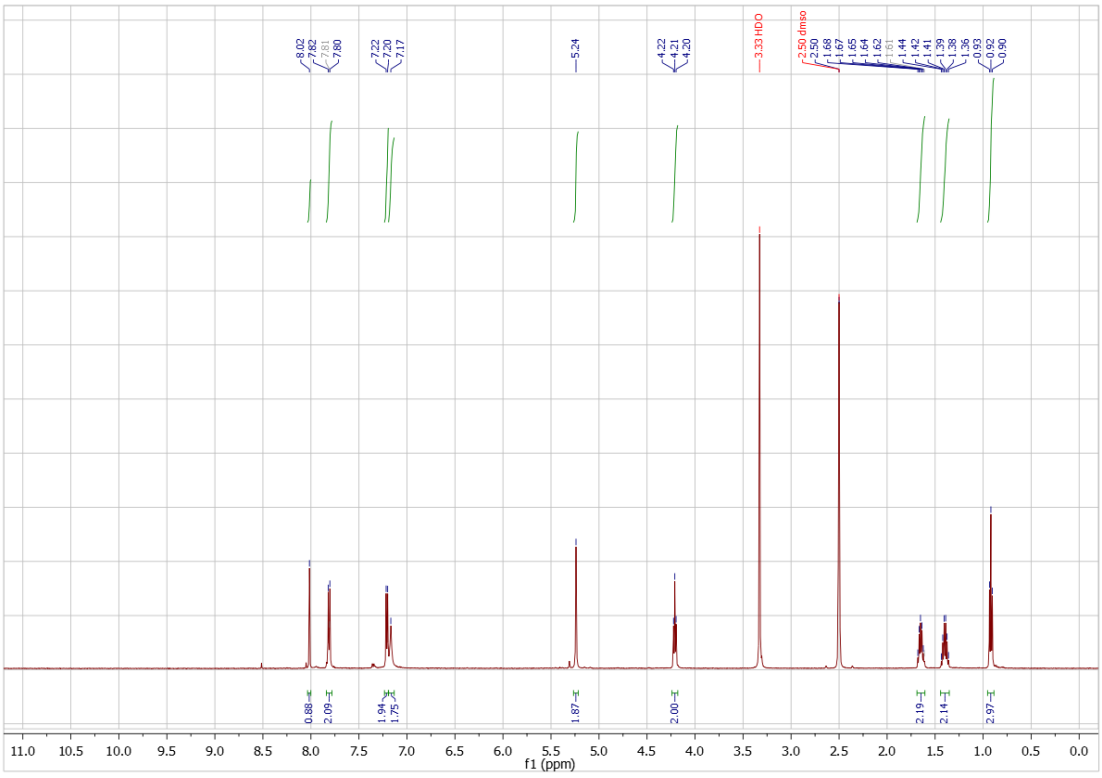
Spectra 5. ^{13}C NMR of **3**



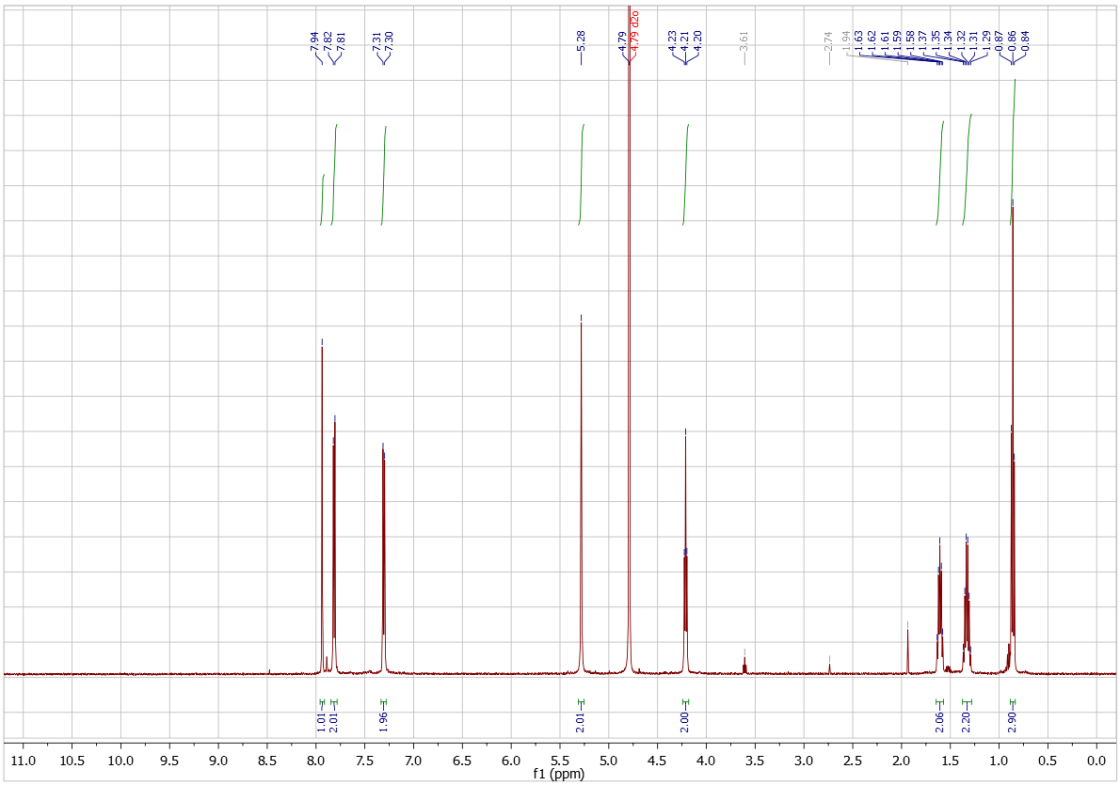
Spectra 6. ^{1}H NMR of **4**



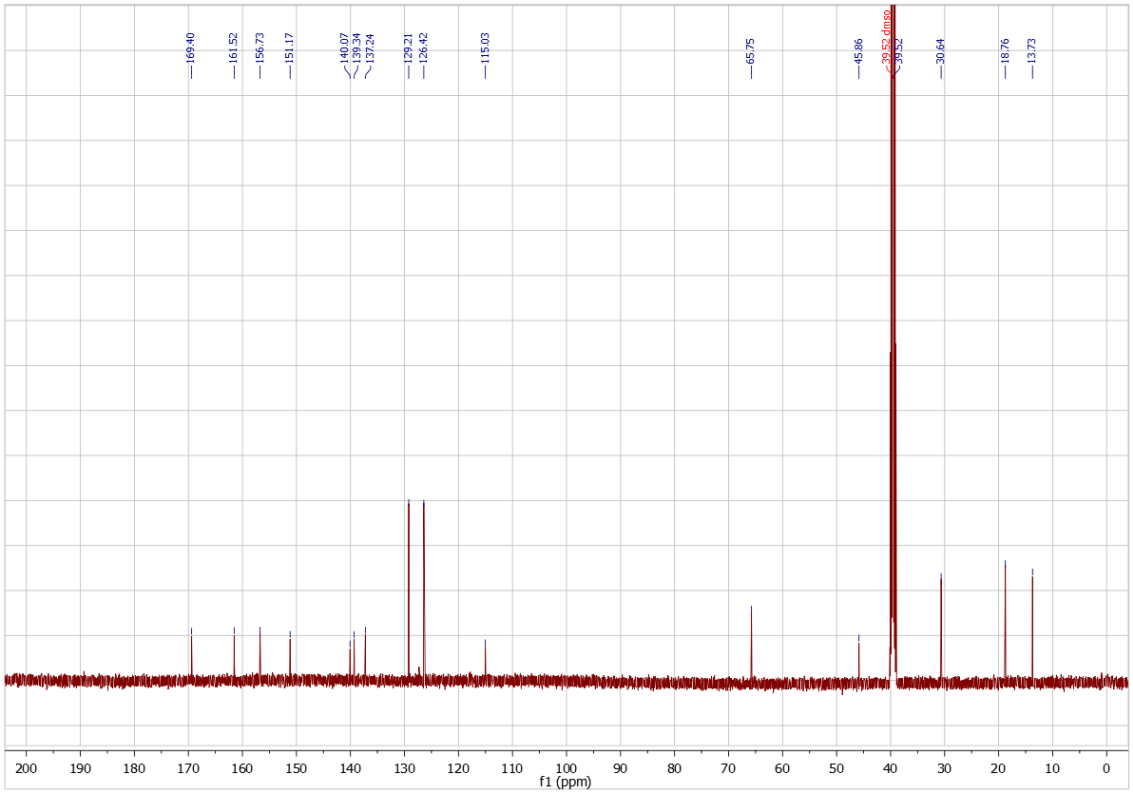
Spectra 7. ^{13}C NMR of **4**



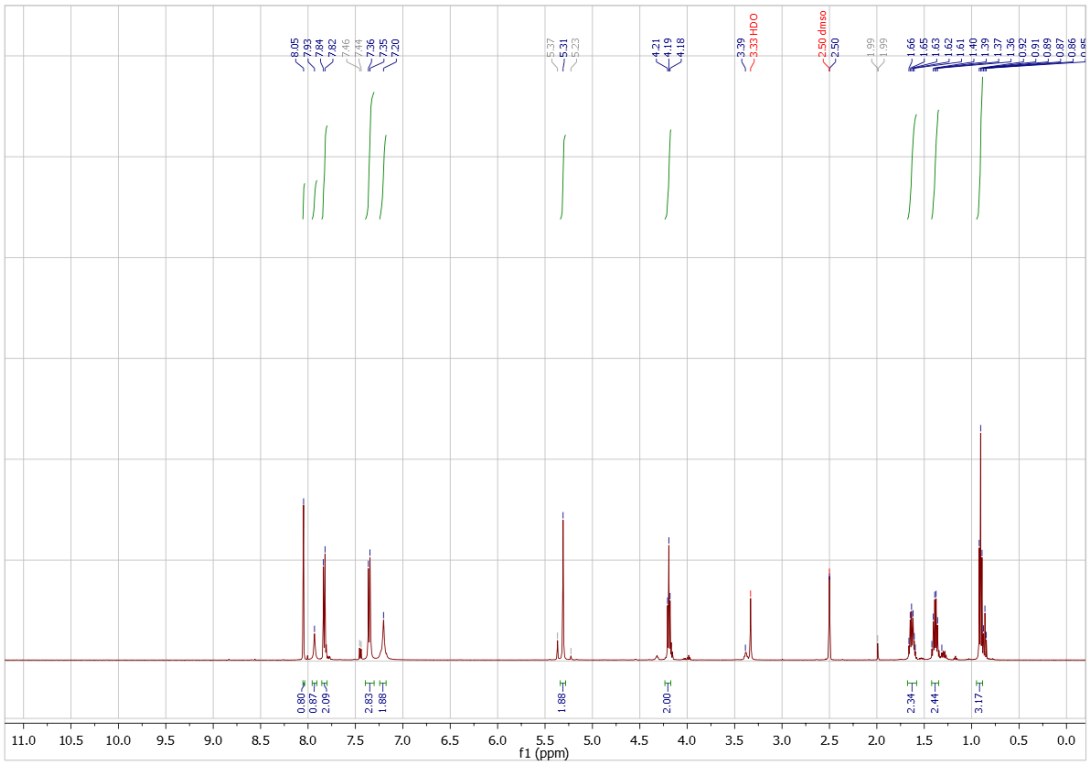
Spectra 8. ^{1}H NMR of Sodium Salt of **4**



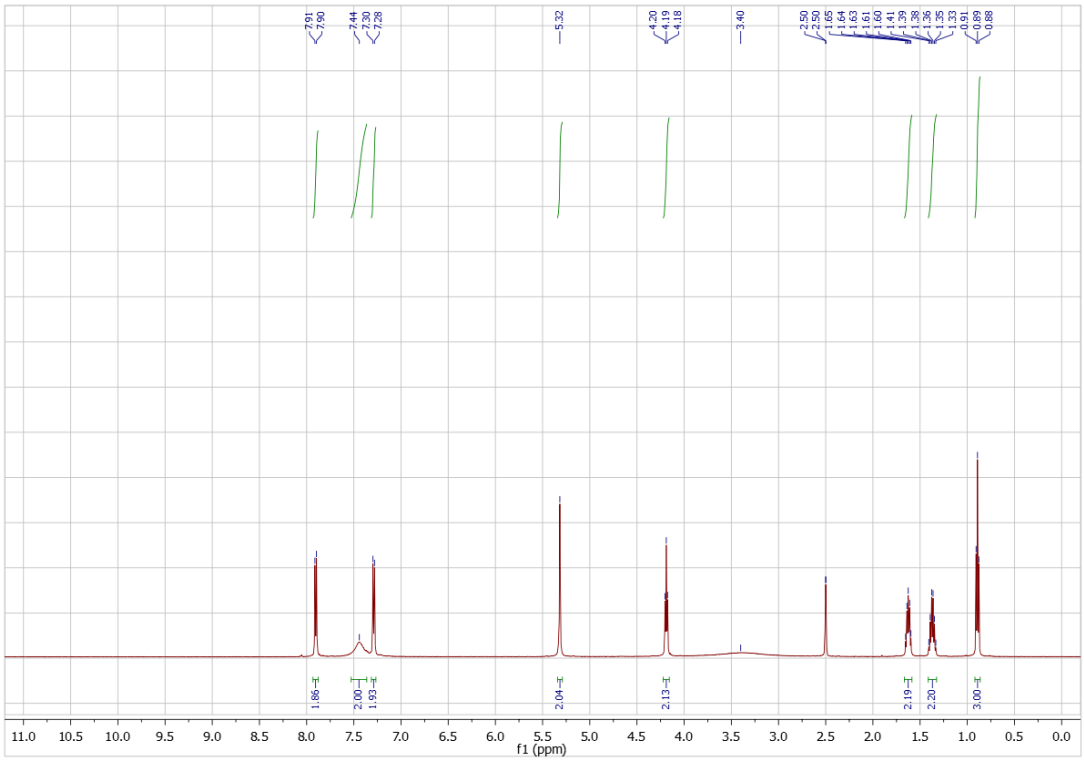
Spectra 9. ^{1}H NMR (D_2O) of Sodium Salt of **4**



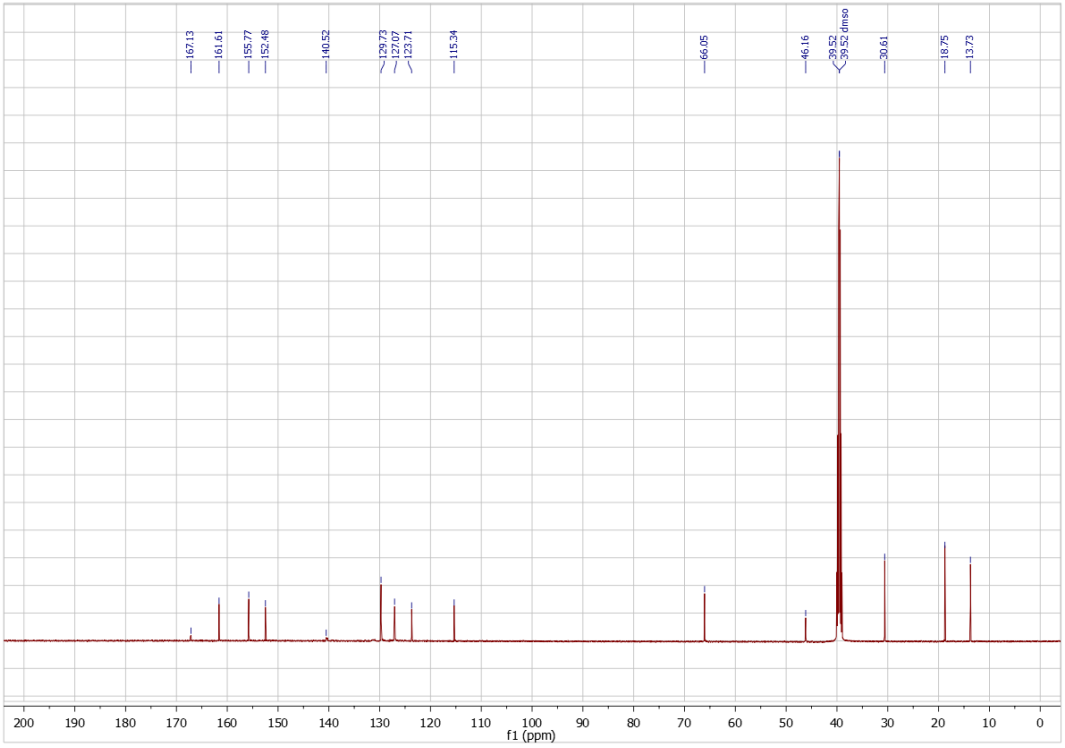
Spectra 10. ^{13}C NMR of Sodium Salt of **4**



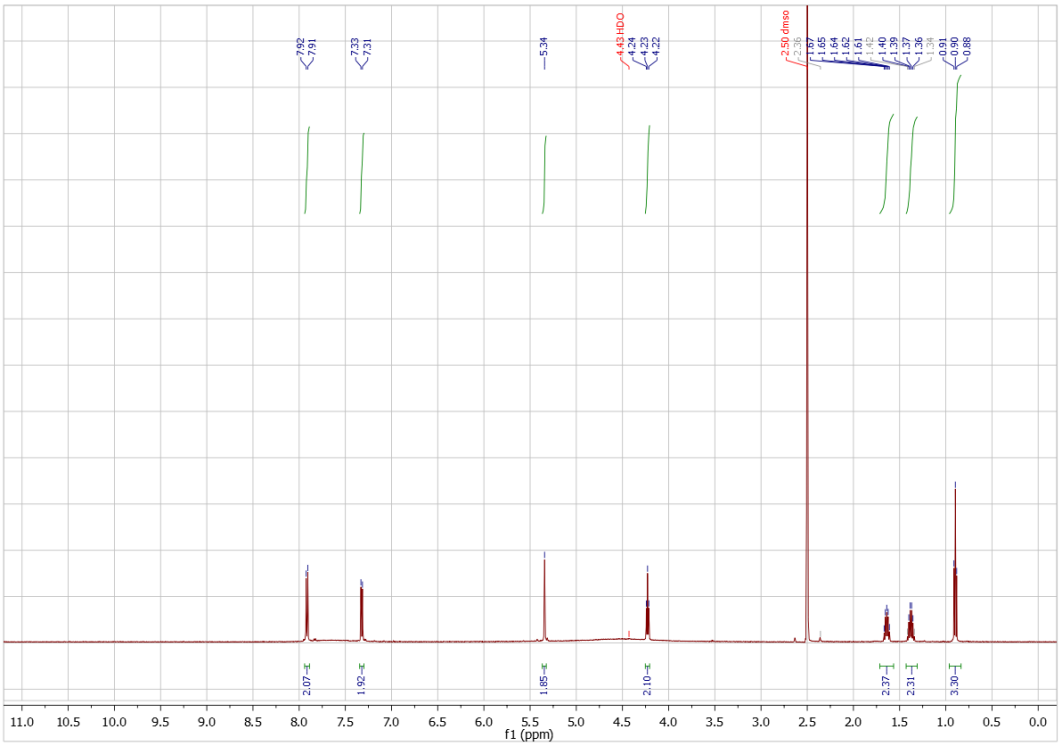
Spectra 11. ^{1}H NMR of **9**



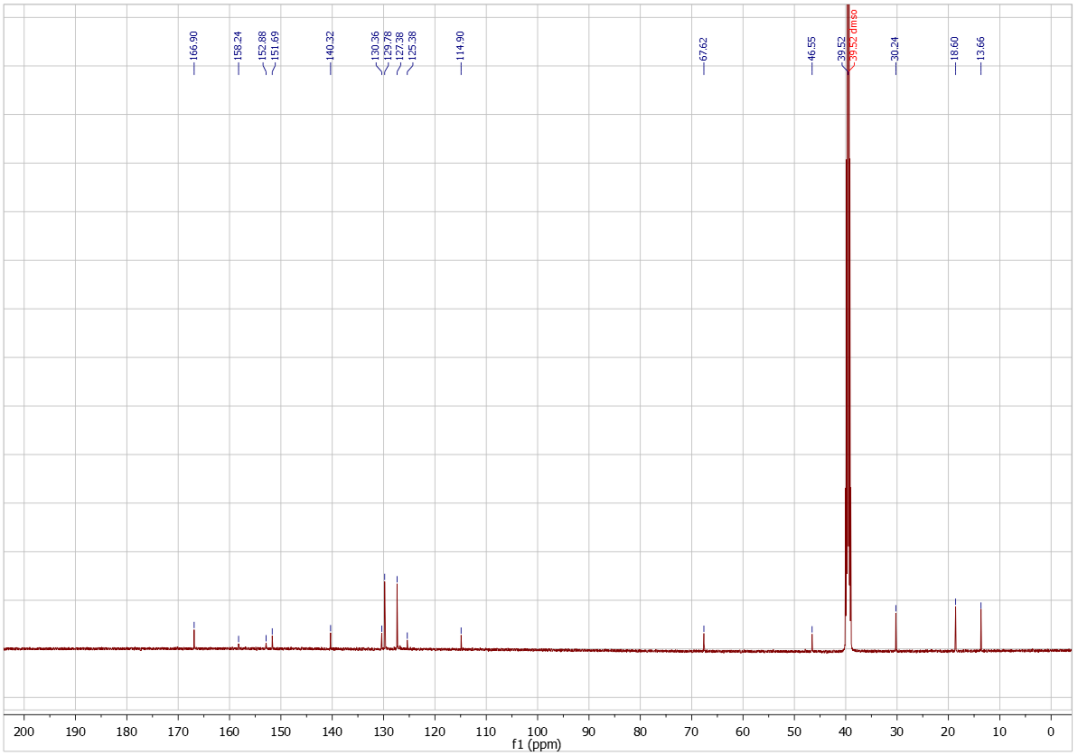
Spectra 12. ^{1}H NMR of **5**



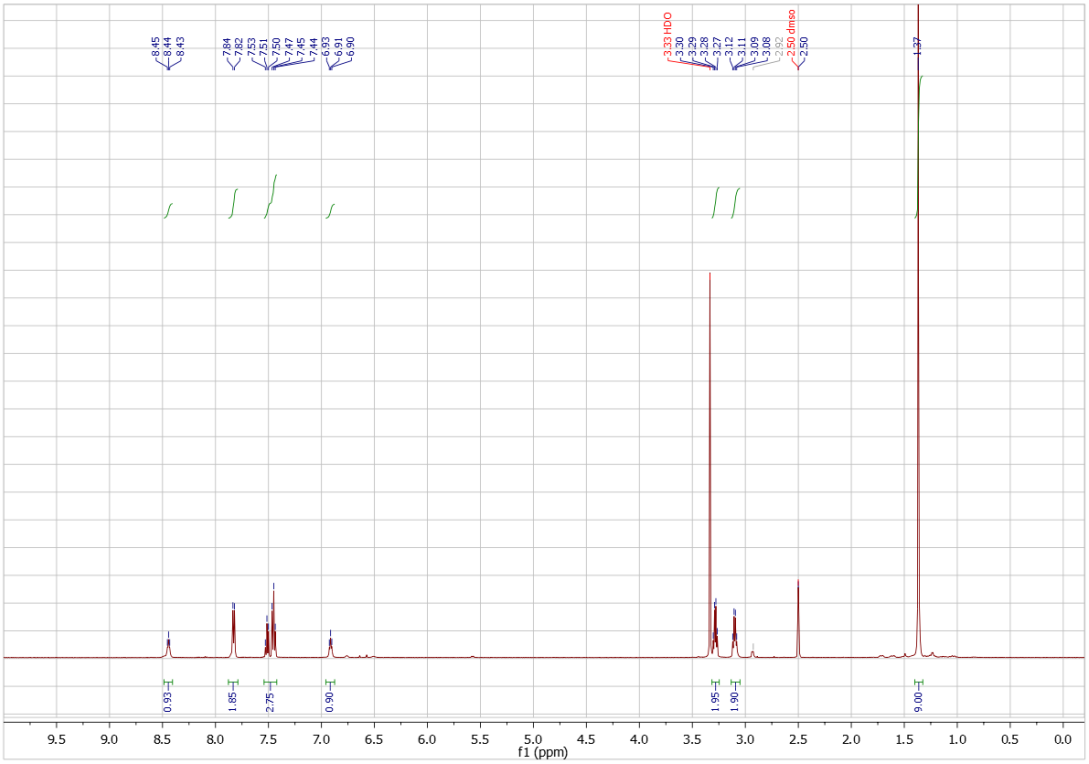
Spectra 13. ^{13}C NMR of **5**



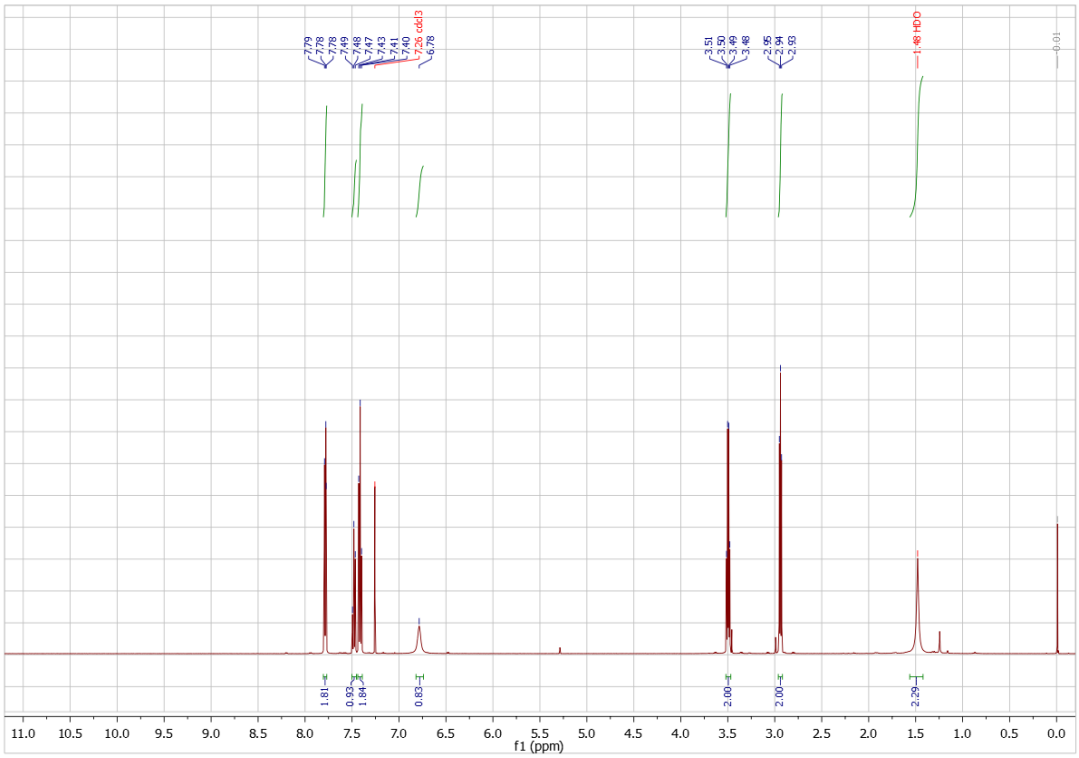
Spectra 14. ^{1}H NMR of **5** from **4** (Sodium Salt)



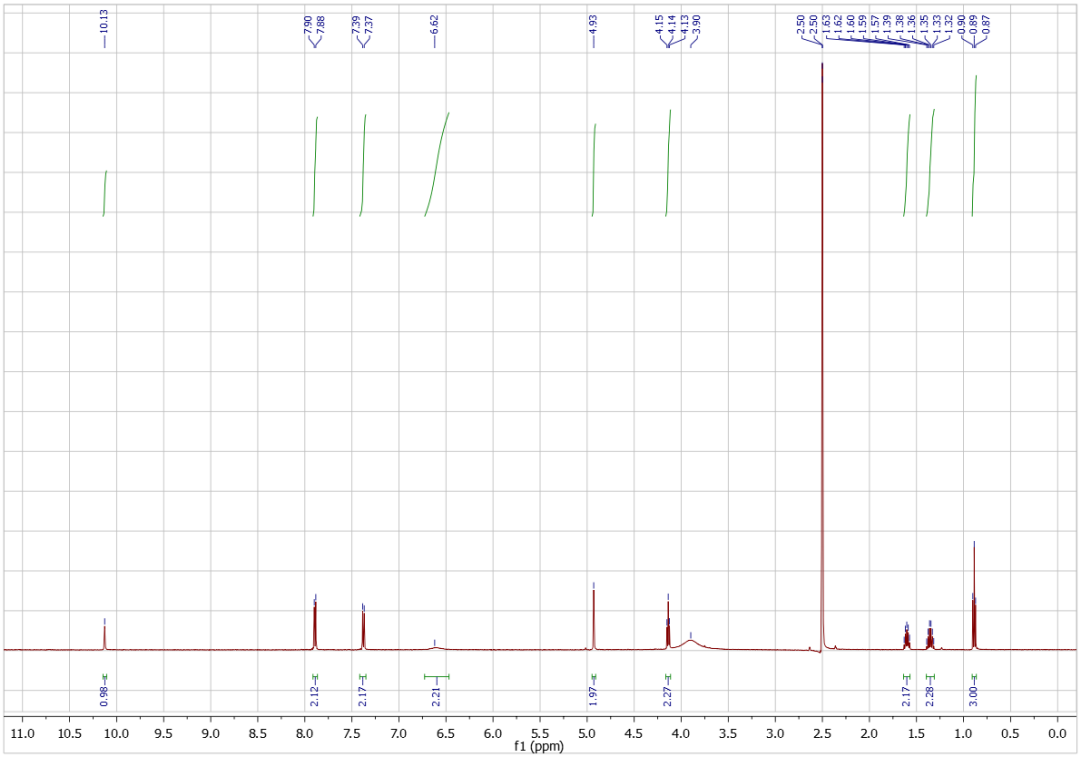
Spectra 15. ^{13}C NMR of **5** from **4** (Sodium Salt)



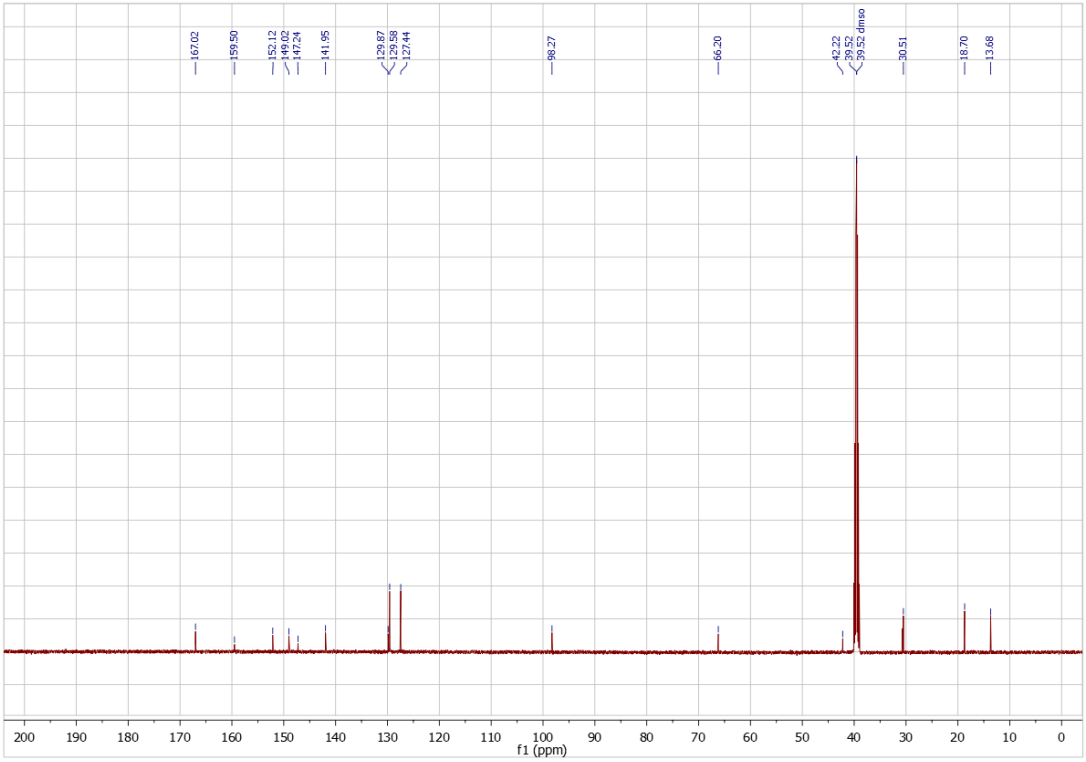
Spectra 16. ^{1}H NMR of **6**



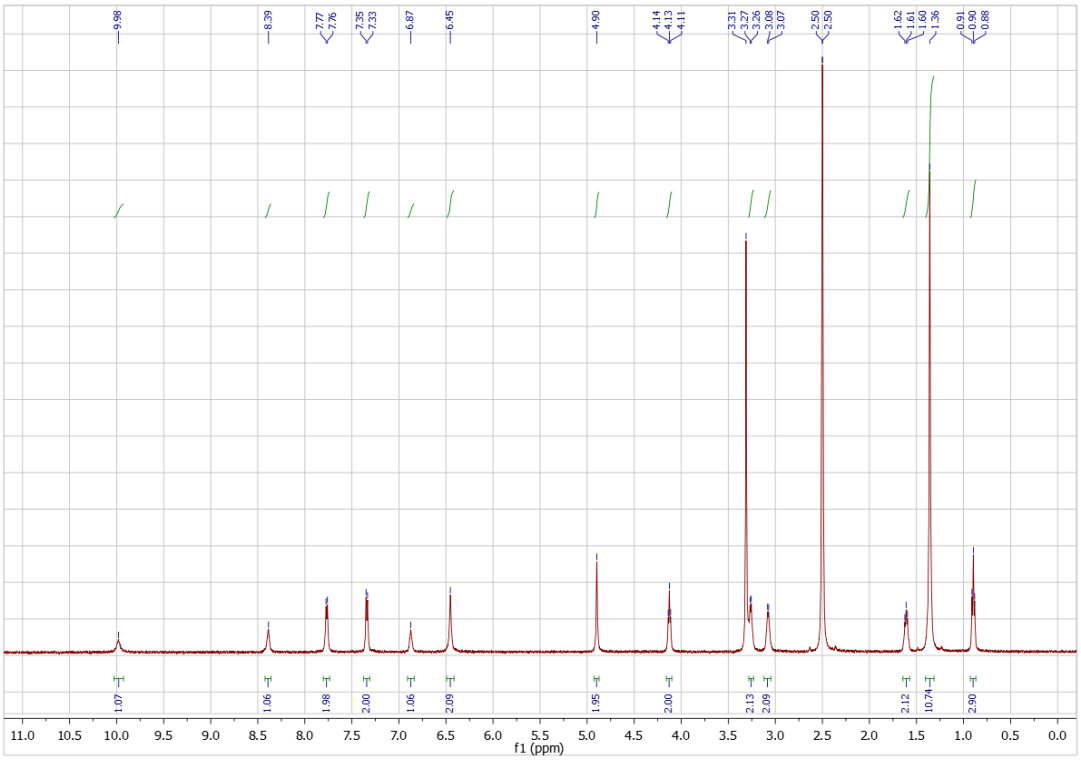
Spectra 17. ^{1}H NMR of **11**



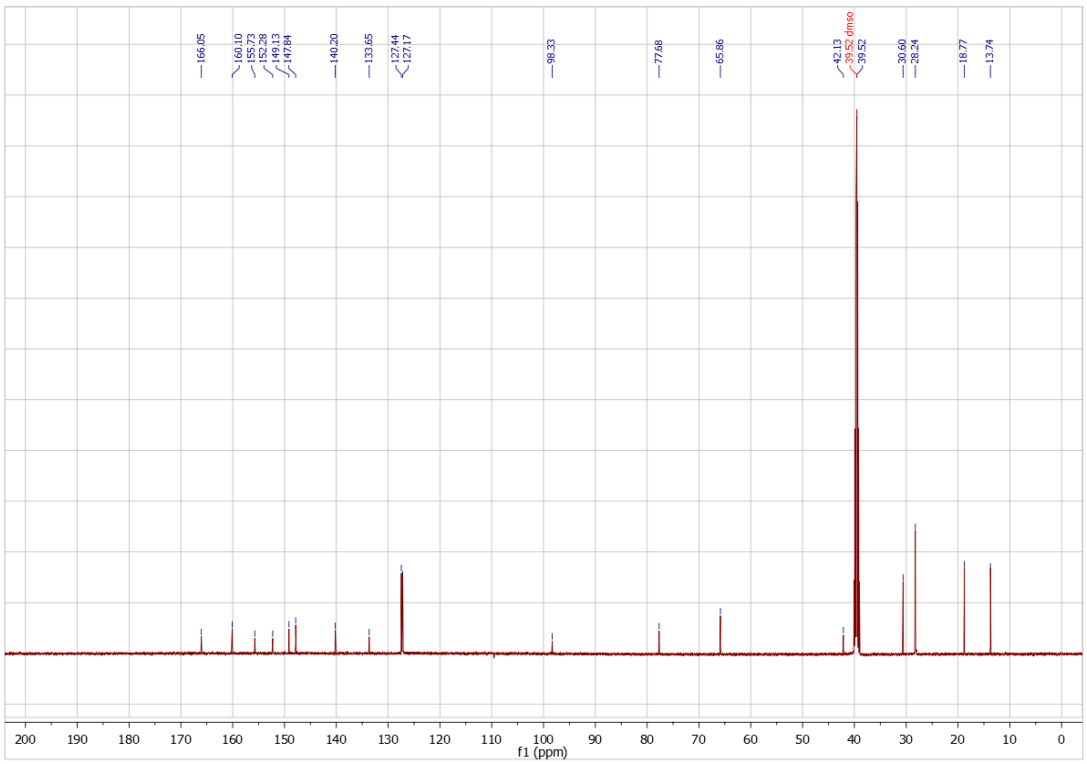
Spectra 18. ^{1}H NMR of **7**



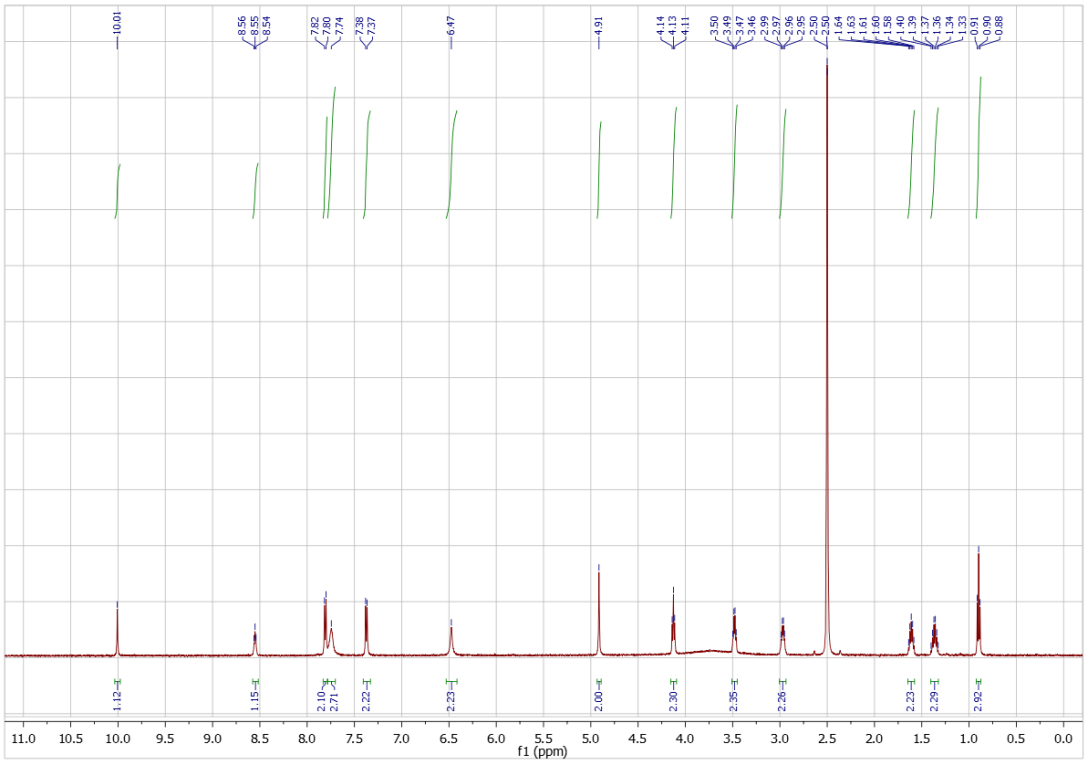
Spectra 19. ^{13}C NMR of **7**



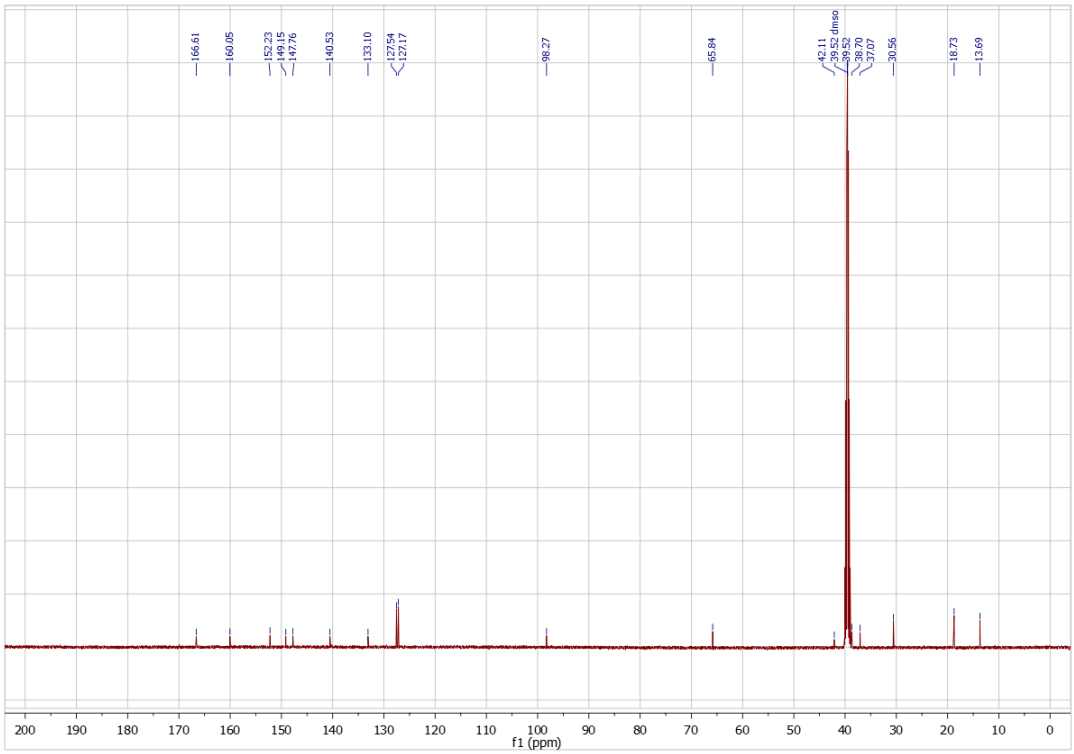
Spectra 20. ^{1}H NMR of **10**



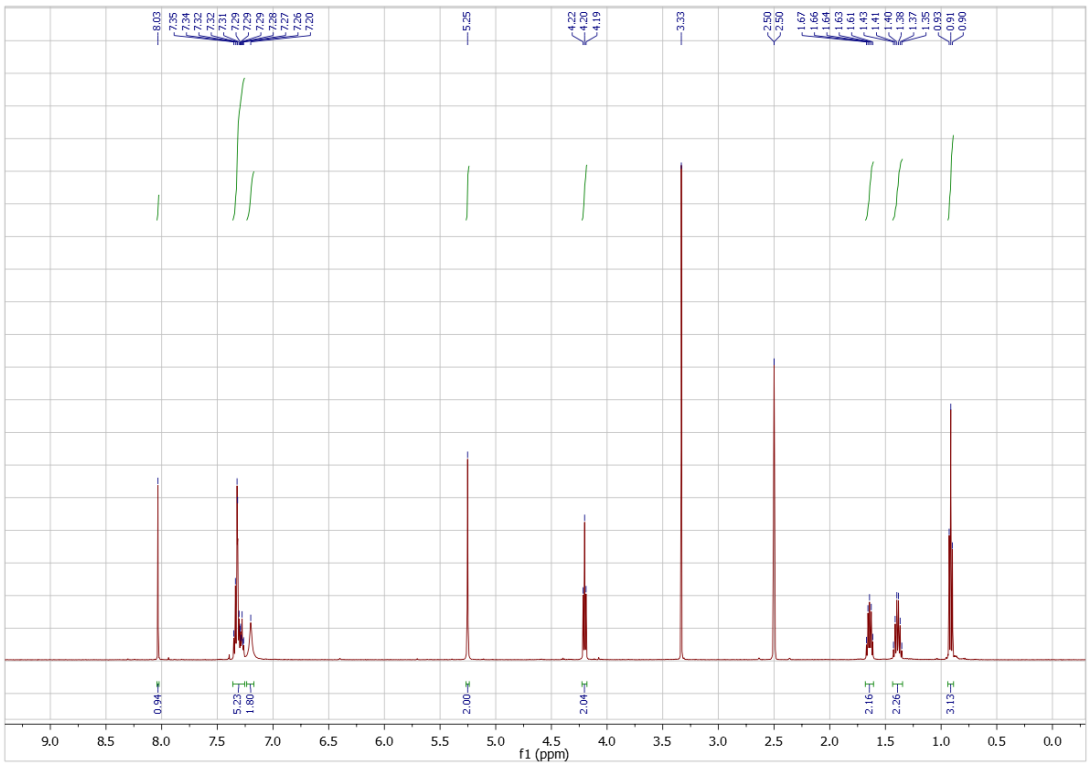
Spectra 21. ^{13}C NMR of **10**



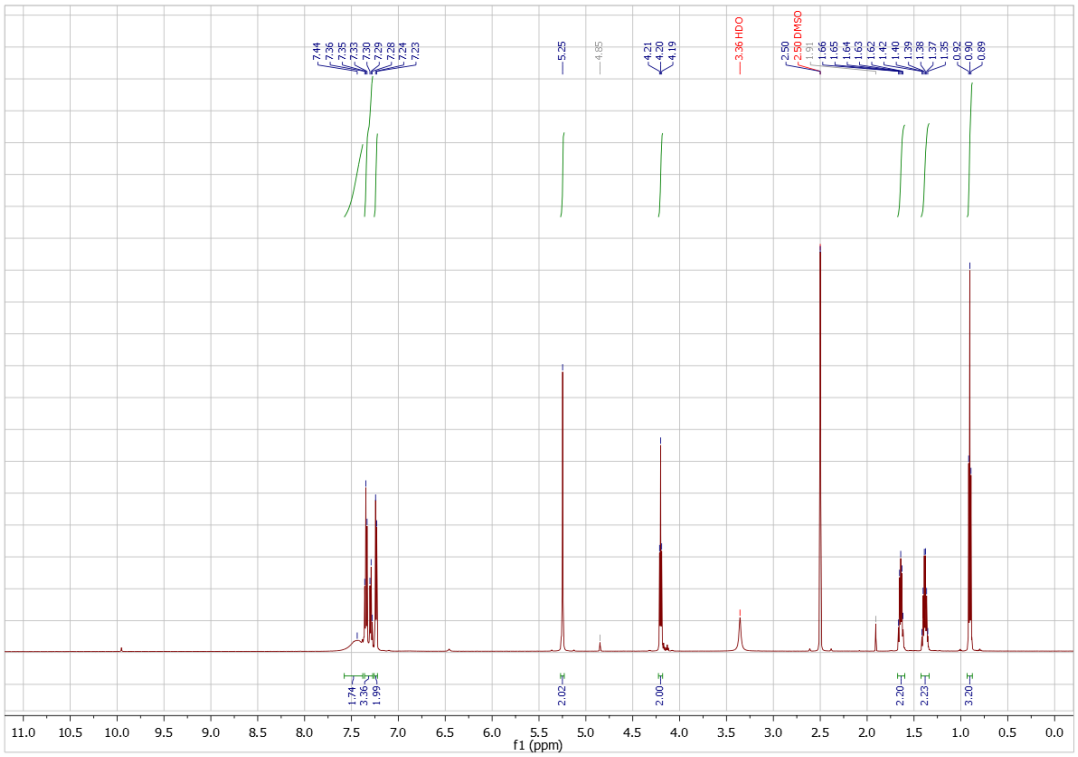
Spectra 22. ^{1}H NMR of **8**



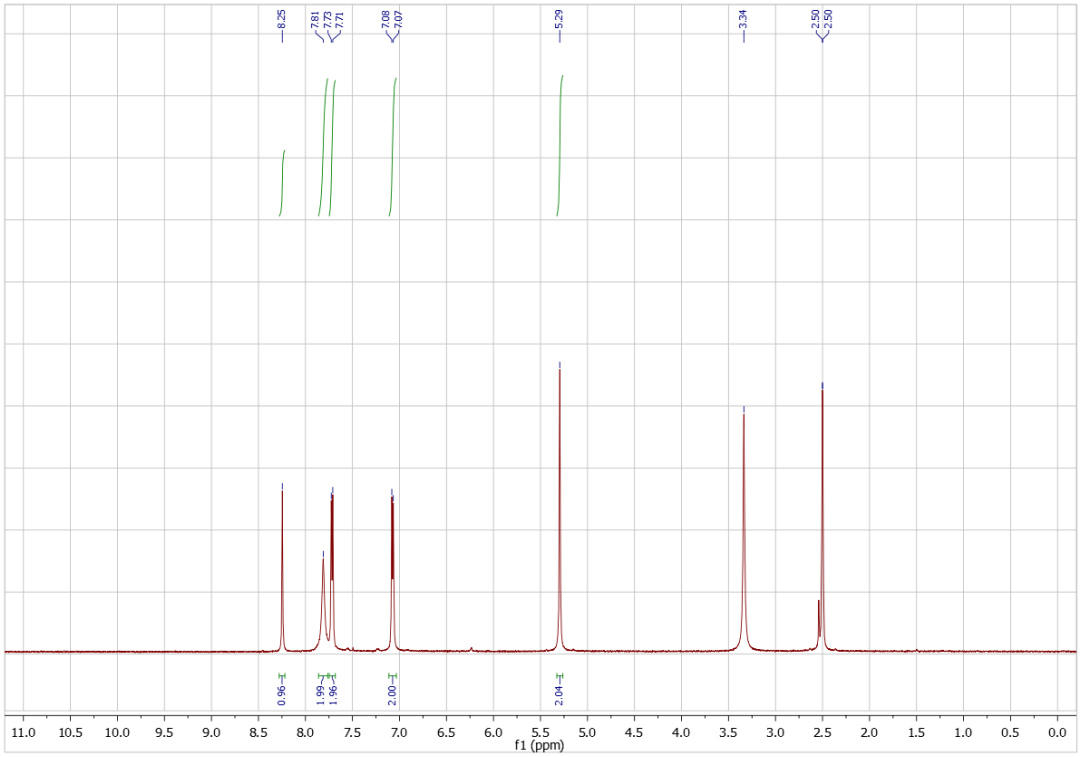
Spectra 23. ^{13}C NMR of **8**



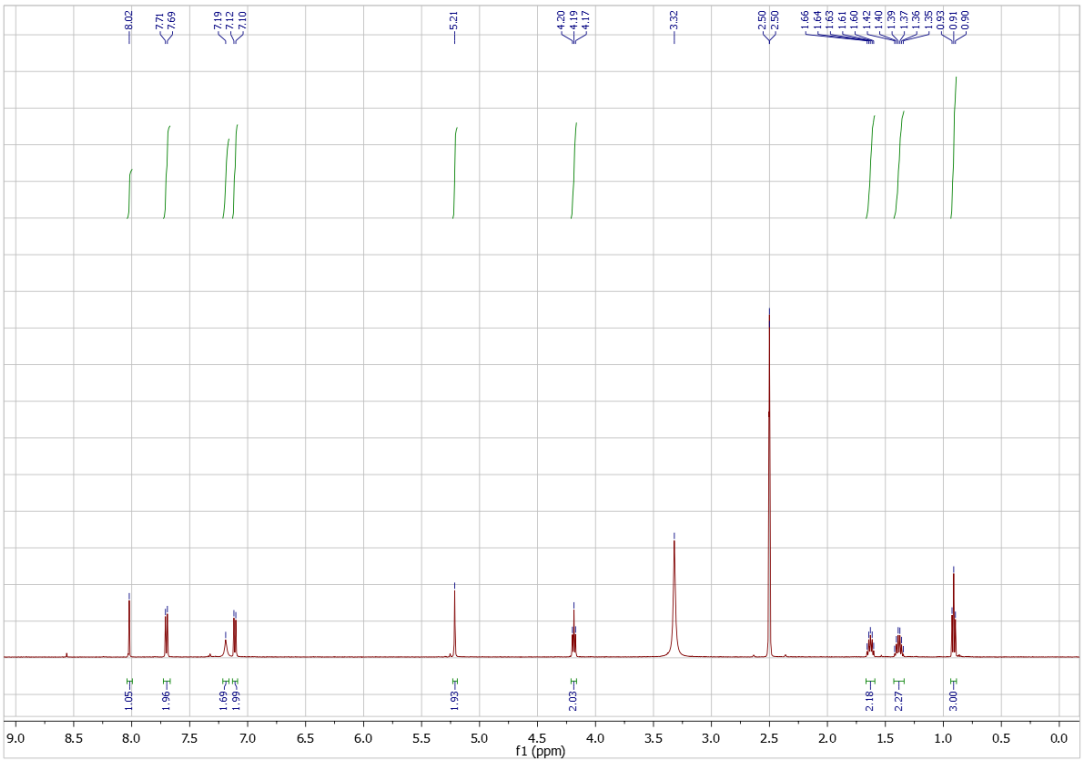
Spectra 24. ^{1}H NMR of **12**



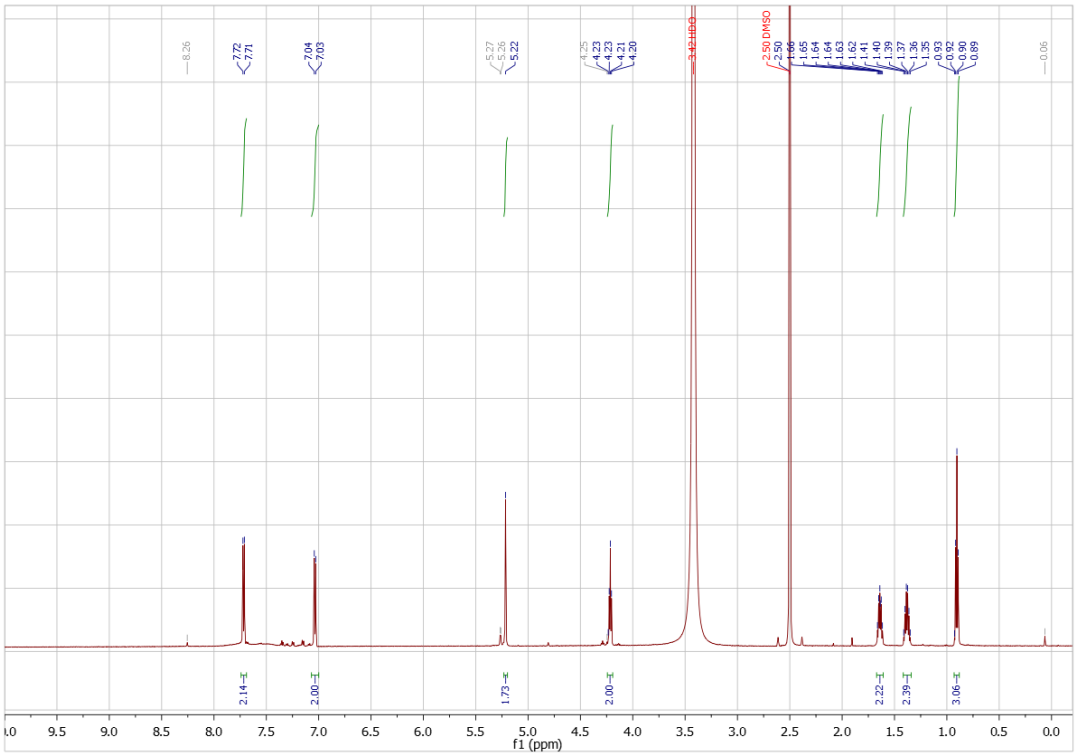
Spectra 25. ^{1}H NMR of **13**



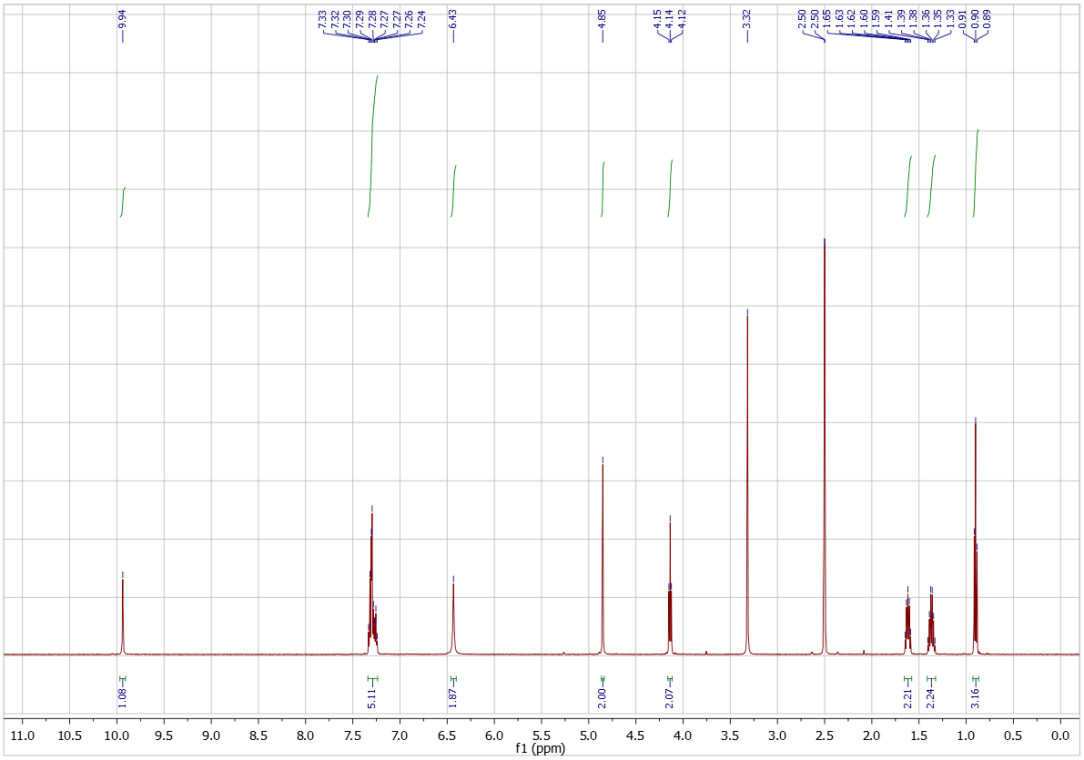
Spectra 26. ^{1}H NMR of **14**



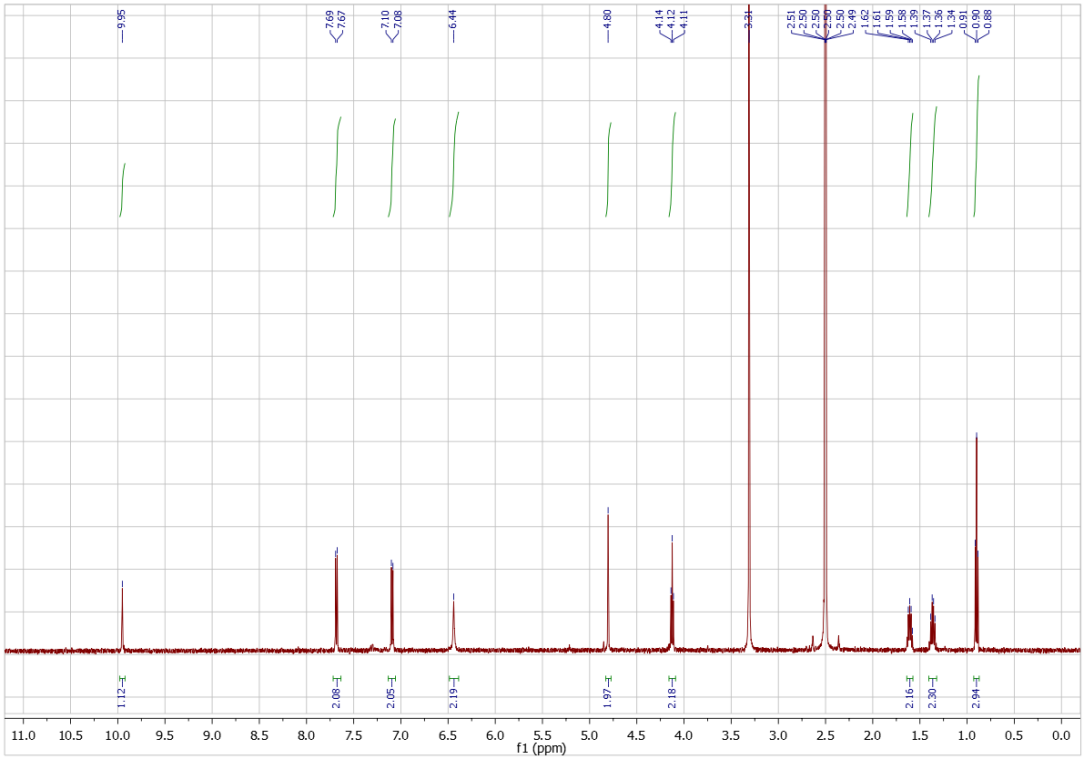
Spectra 27. ^{1}H NMR of **15**



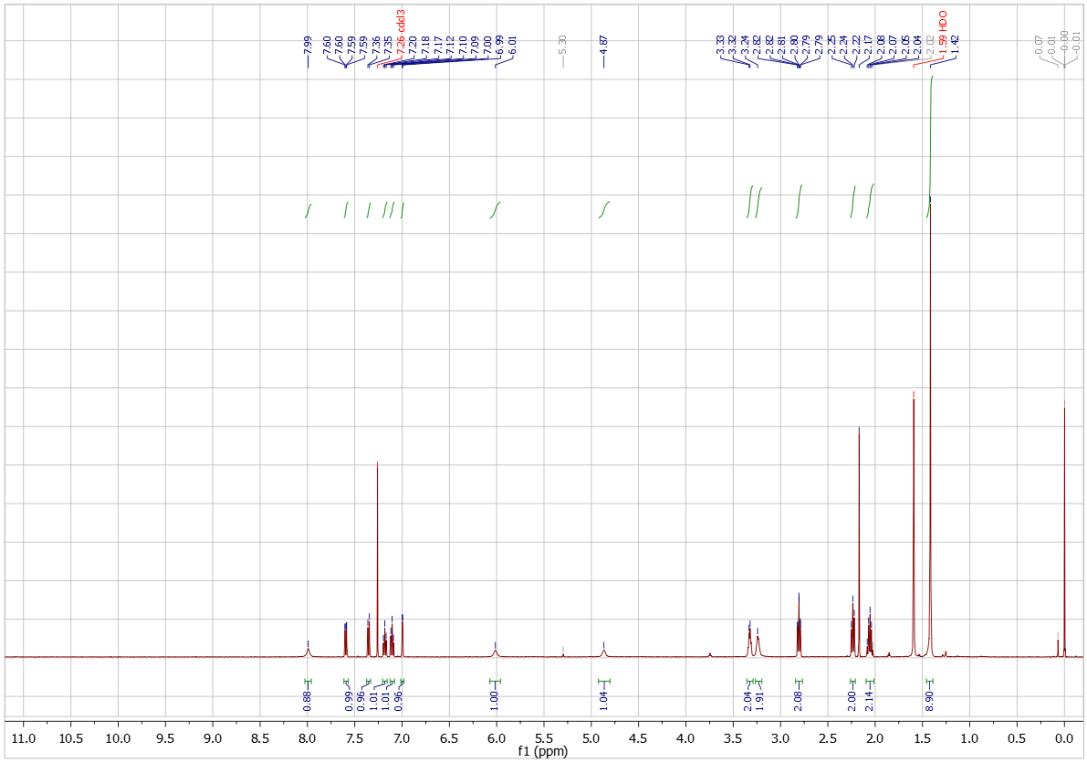
Spectra 28. ^{1}H NMR of **16**



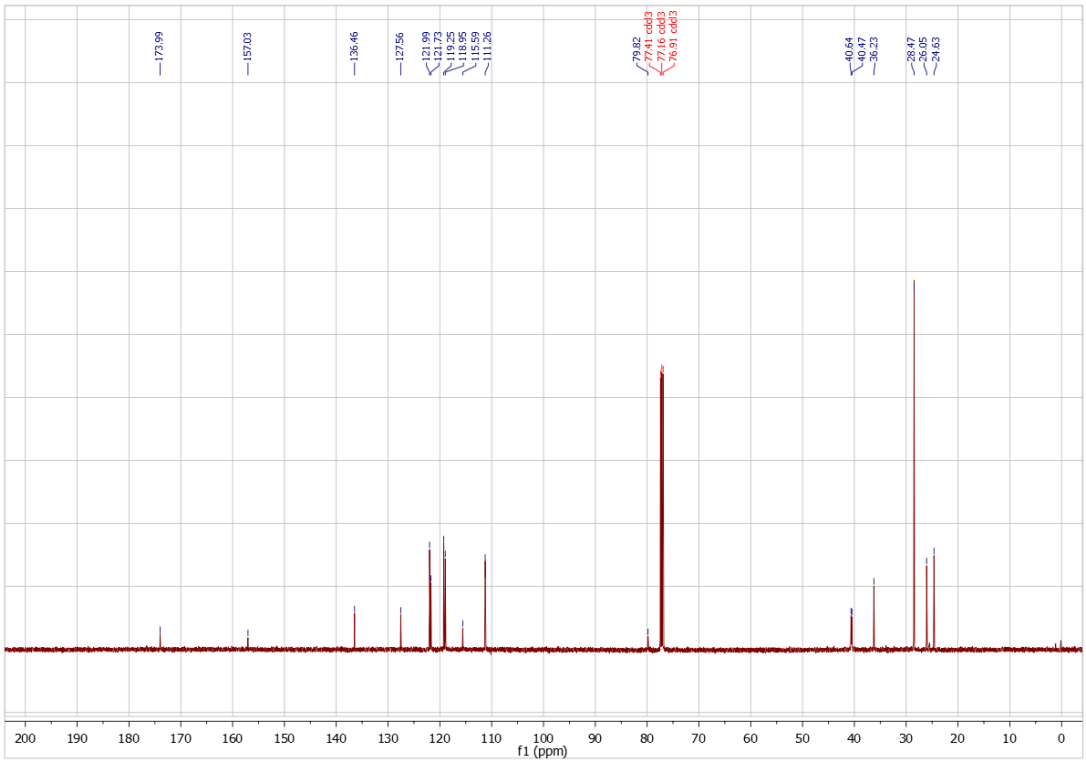
Spectra 29. ^{1}H NMR of **17**



Spectra 30. ^{1}H NMR of **18**

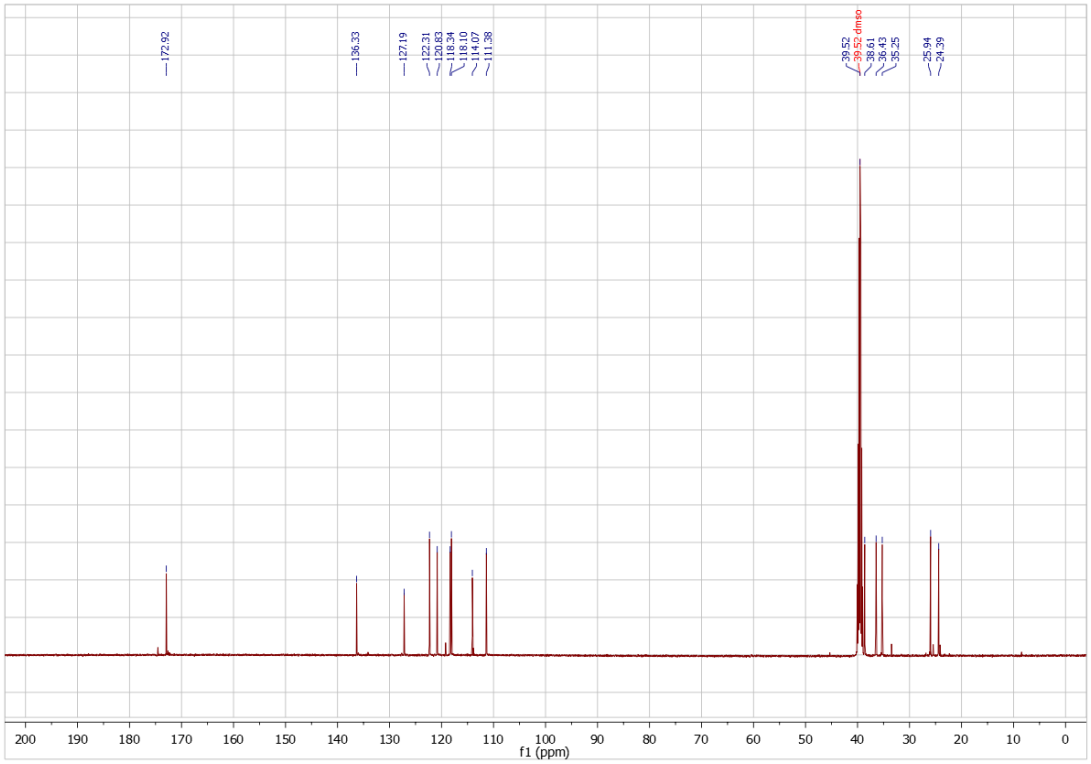


Spectra 31. ^{1}H NMR of **19**

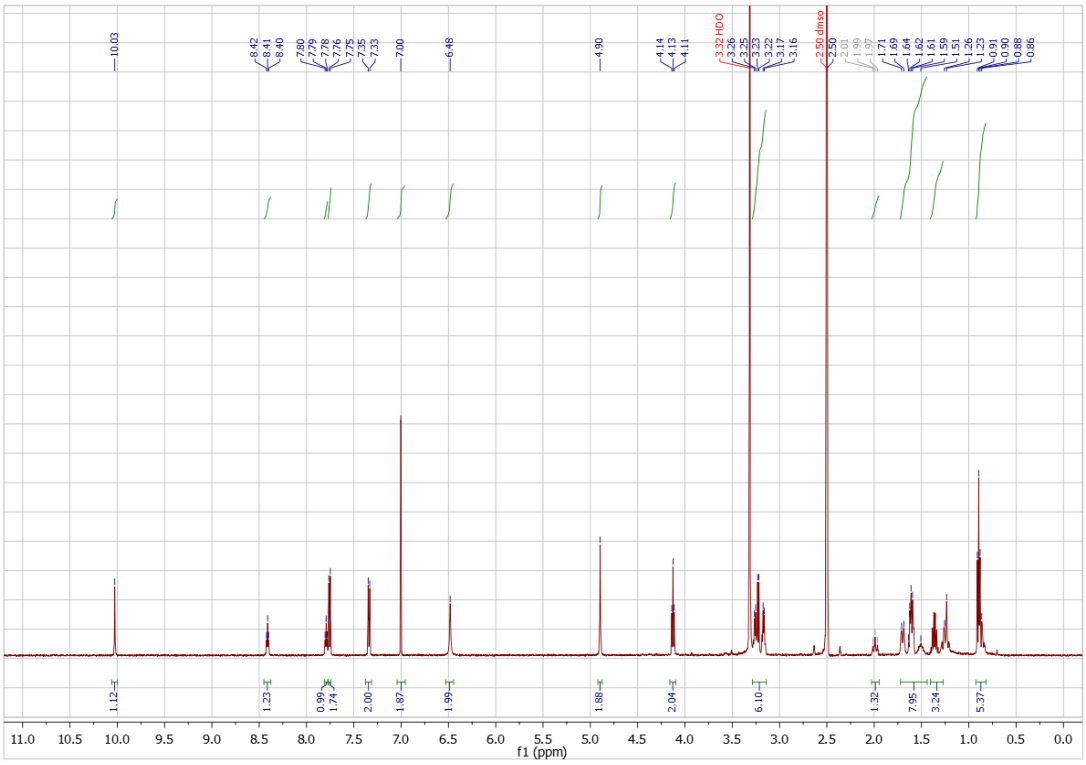


Spectra 32. ^{13}C NMR of **19**

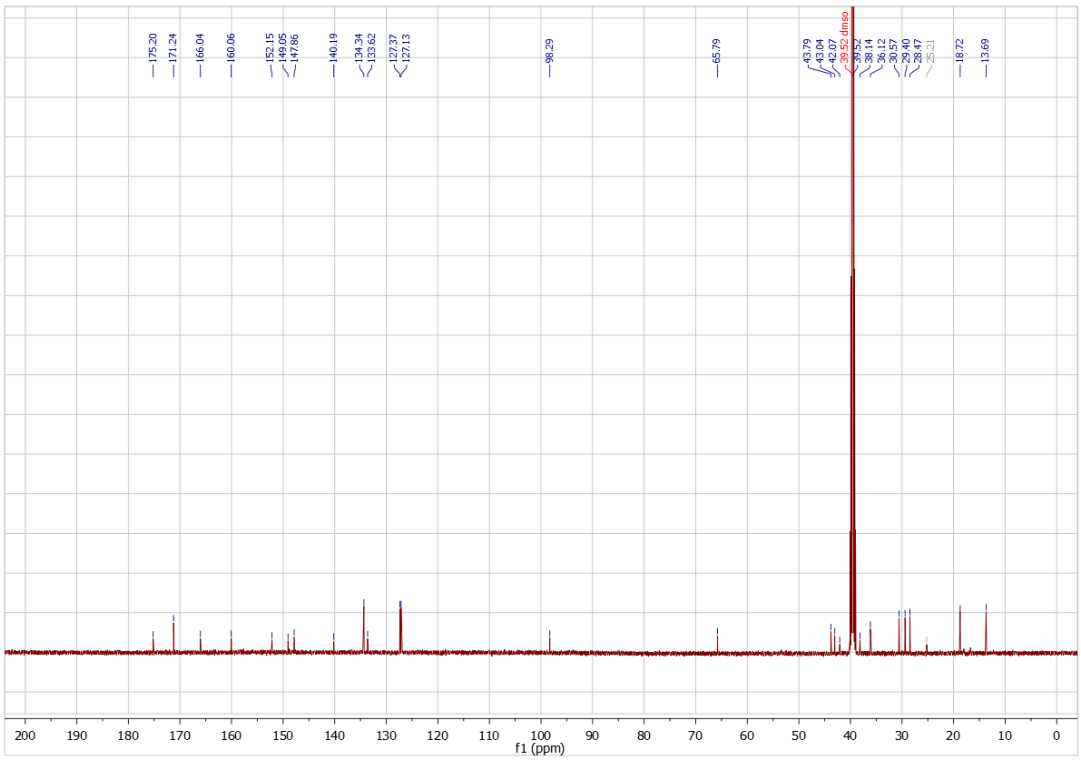
Spectra 33. ^{1}H NMR of **20**



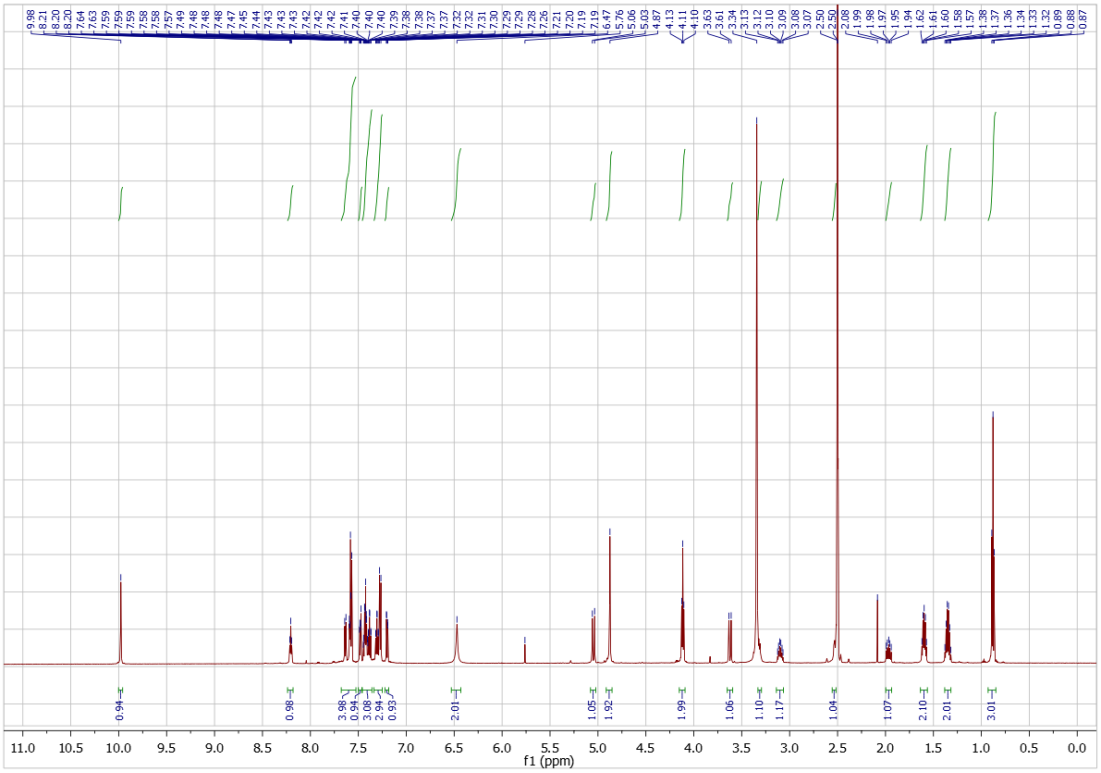
Spectra 34. ^{13}C NMR of **20**



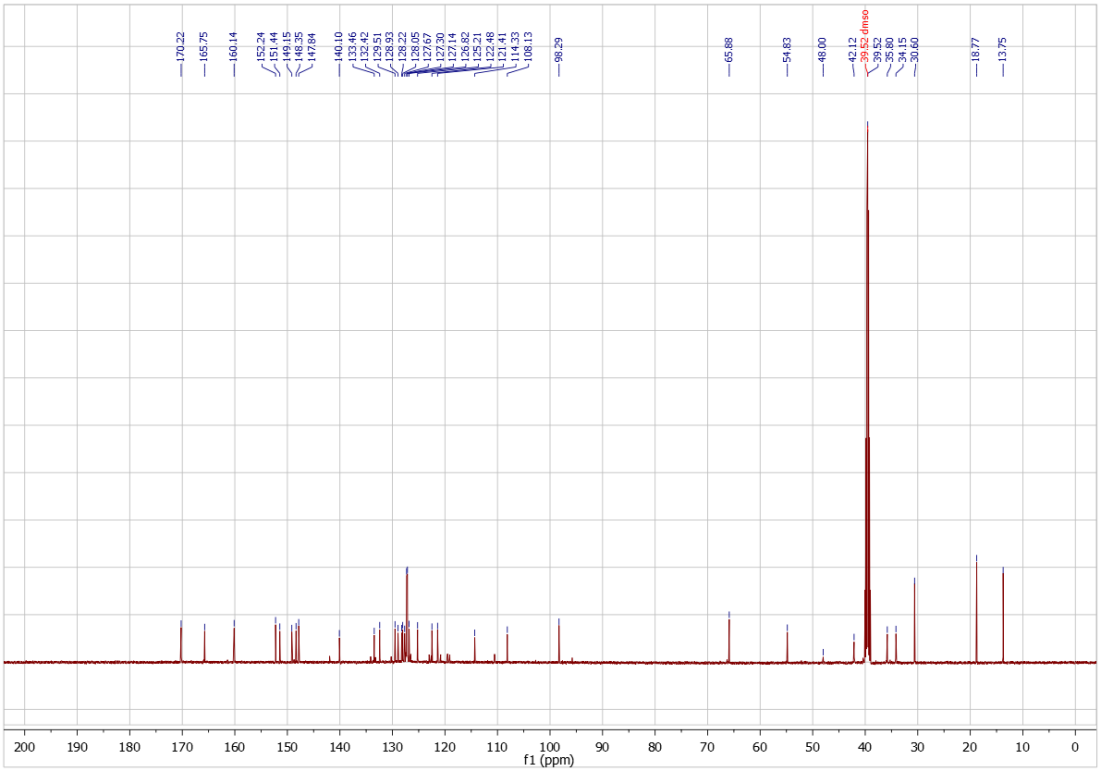
Spectra 35. ^{1}H NMR of **21**



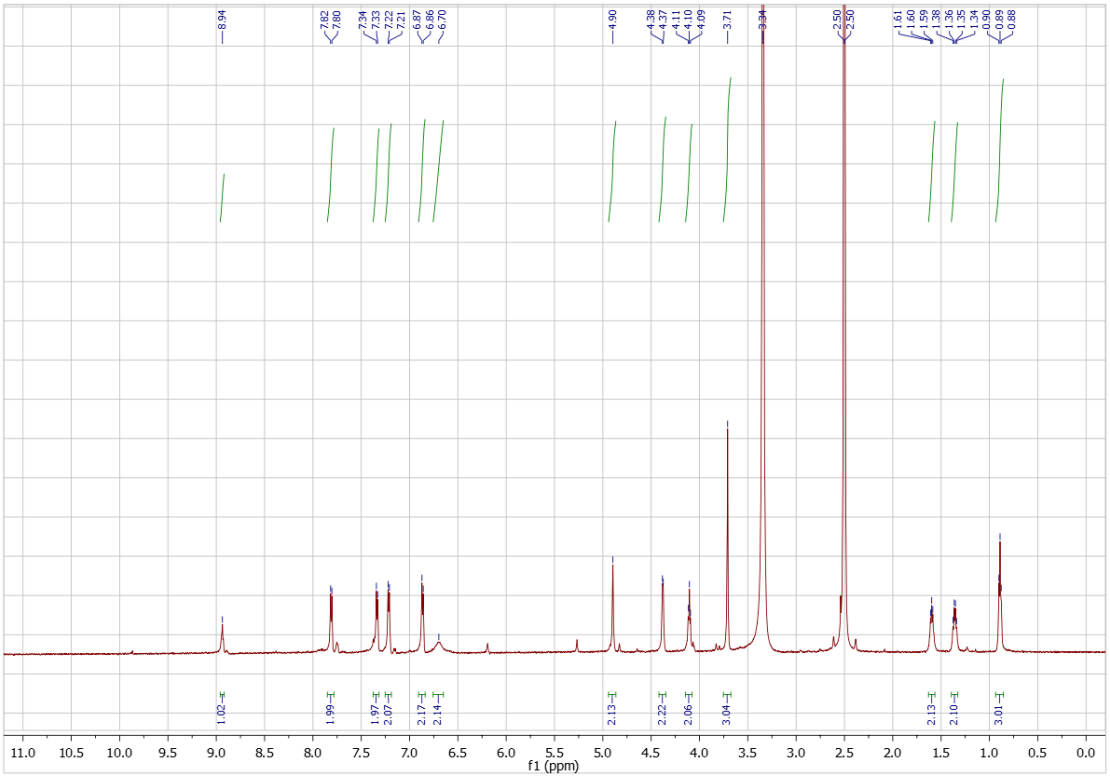
Spectra 36. ^{13}C NMR of **21**



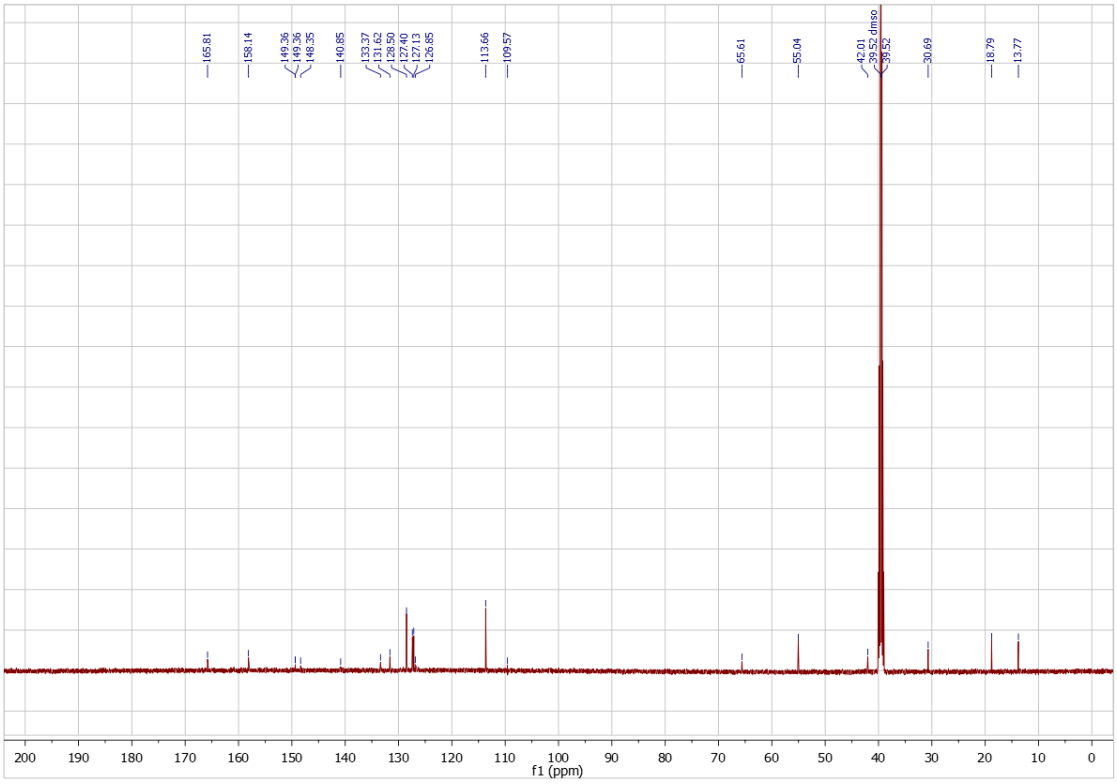
Spectra 37. ^{1}H NMR of **24**



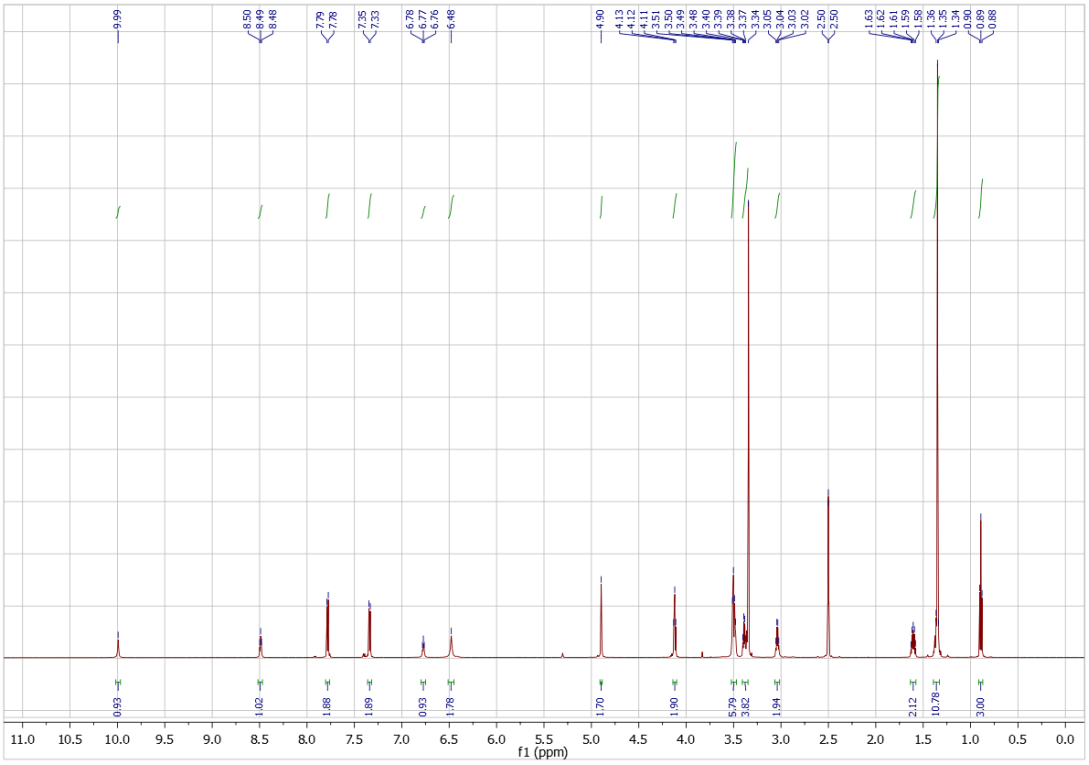
Spectra 38. ^{13}C NMR of **24**



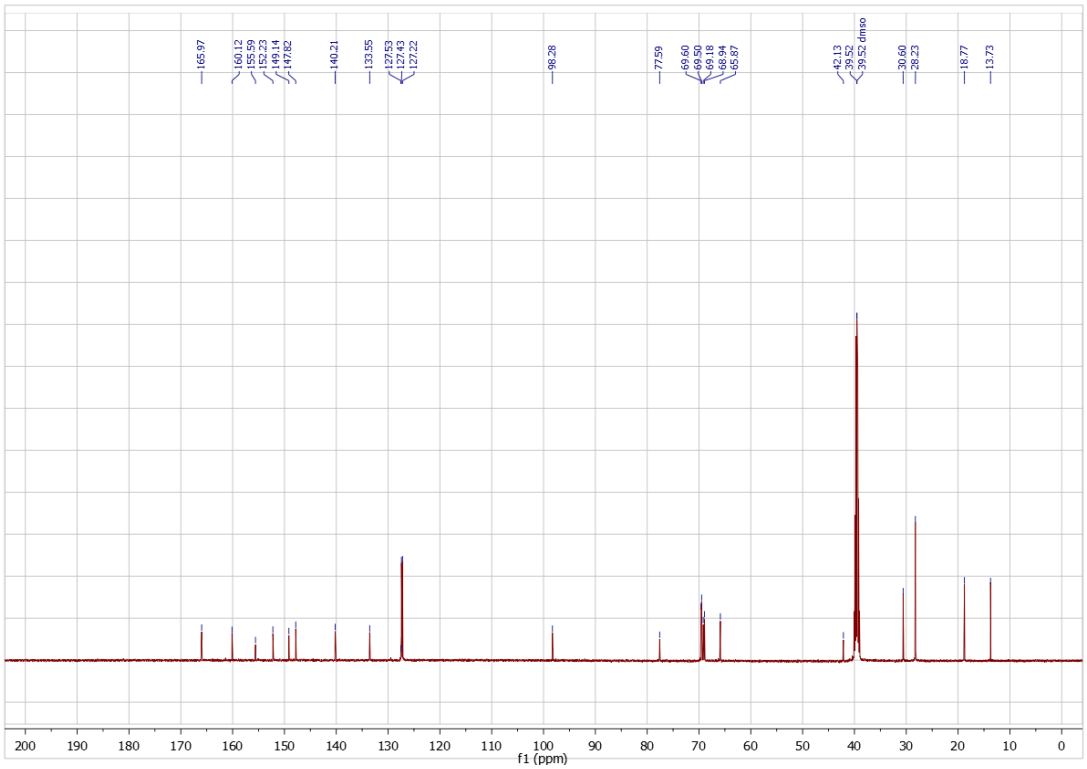
Spectra 39. ^{1}H NMR of **25**



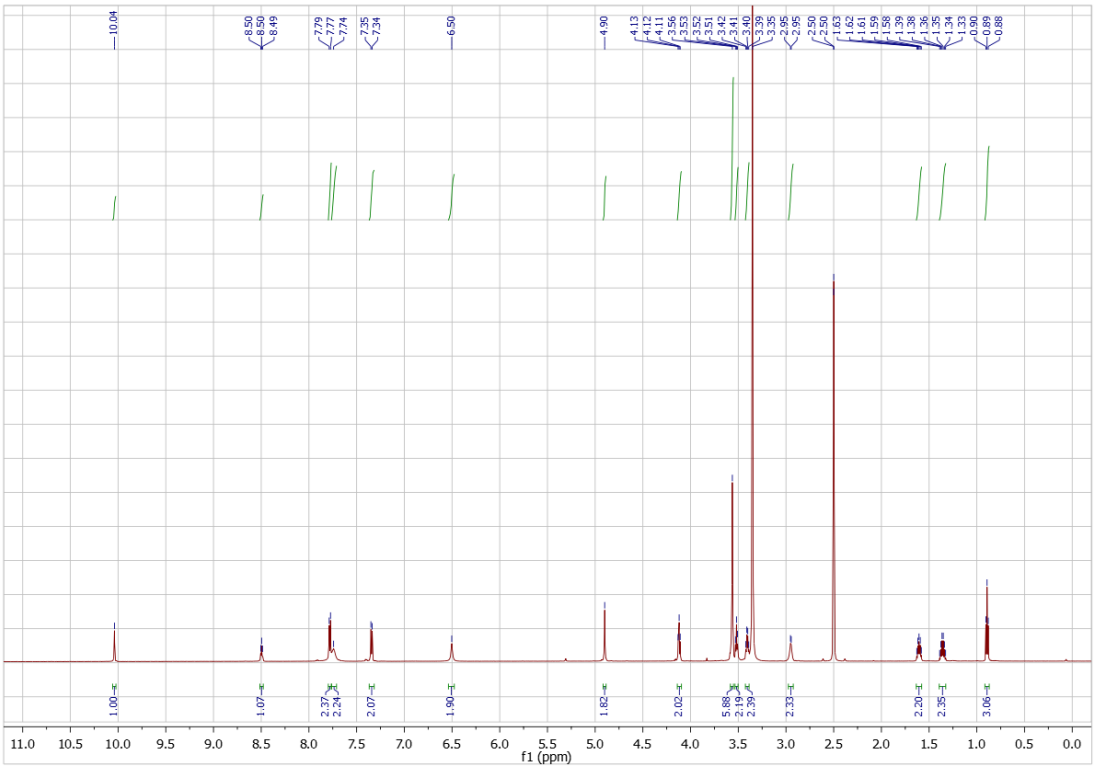
Spectra 40. ^{13}C NMR of **25**



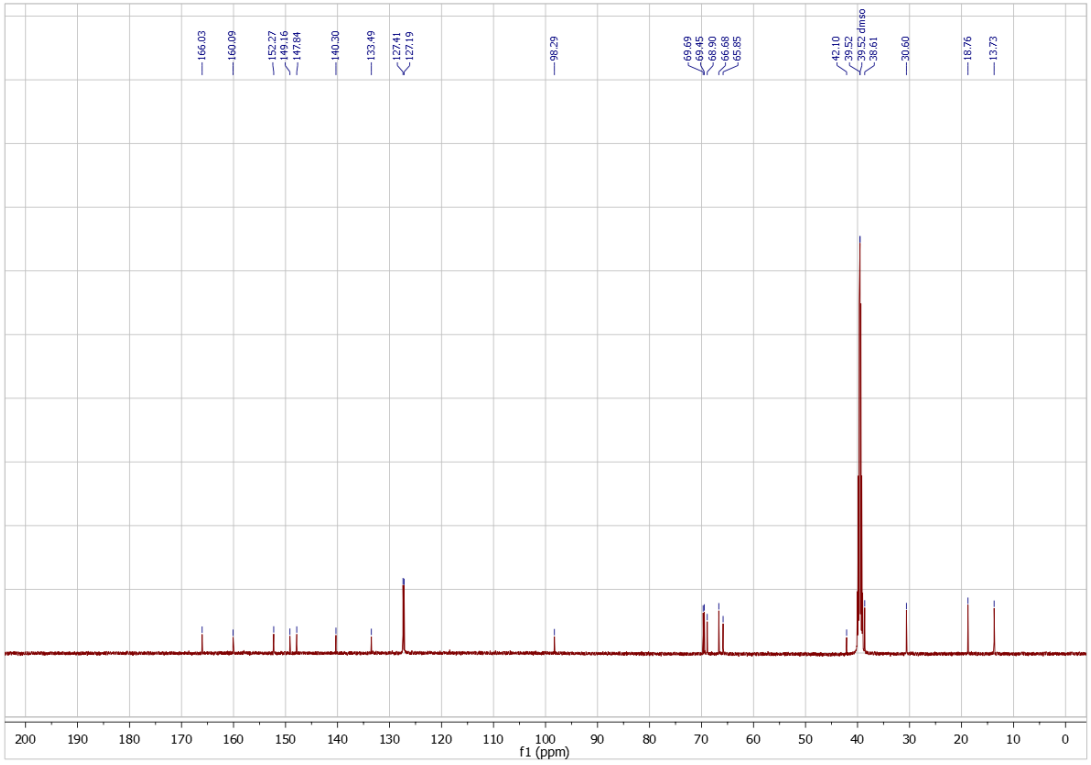
Spectra 41. ^{1}H NMR of **26**



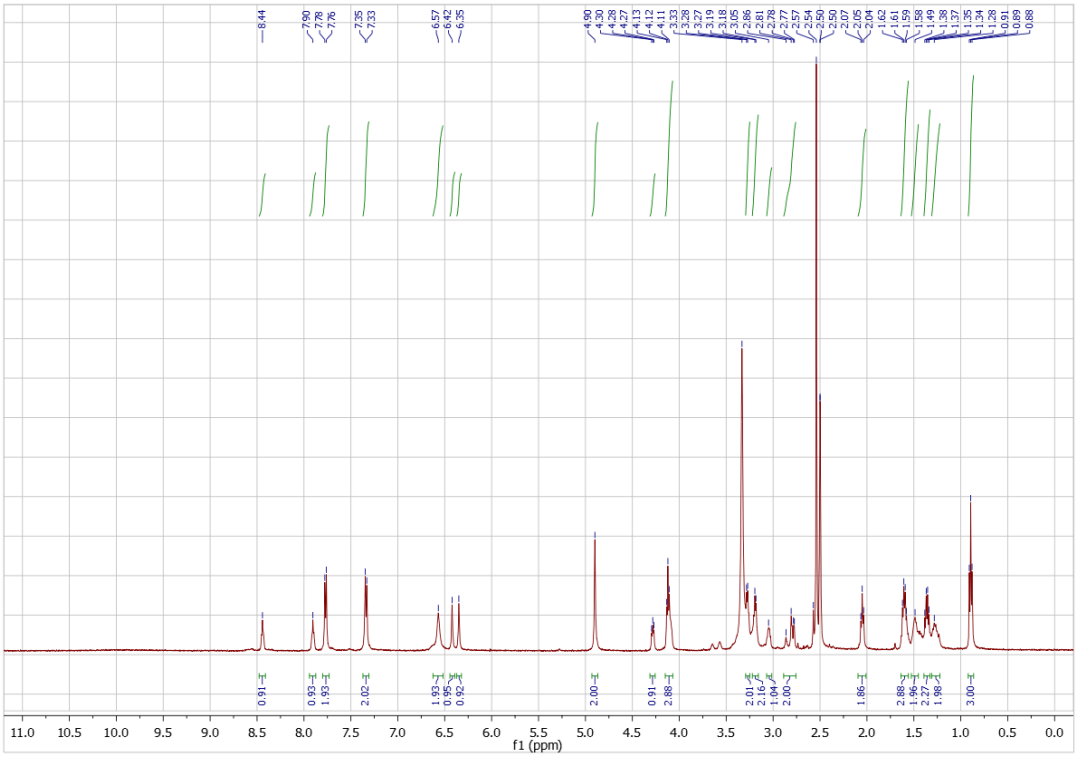
Spectra 42. ^{13}C NMR of **26**



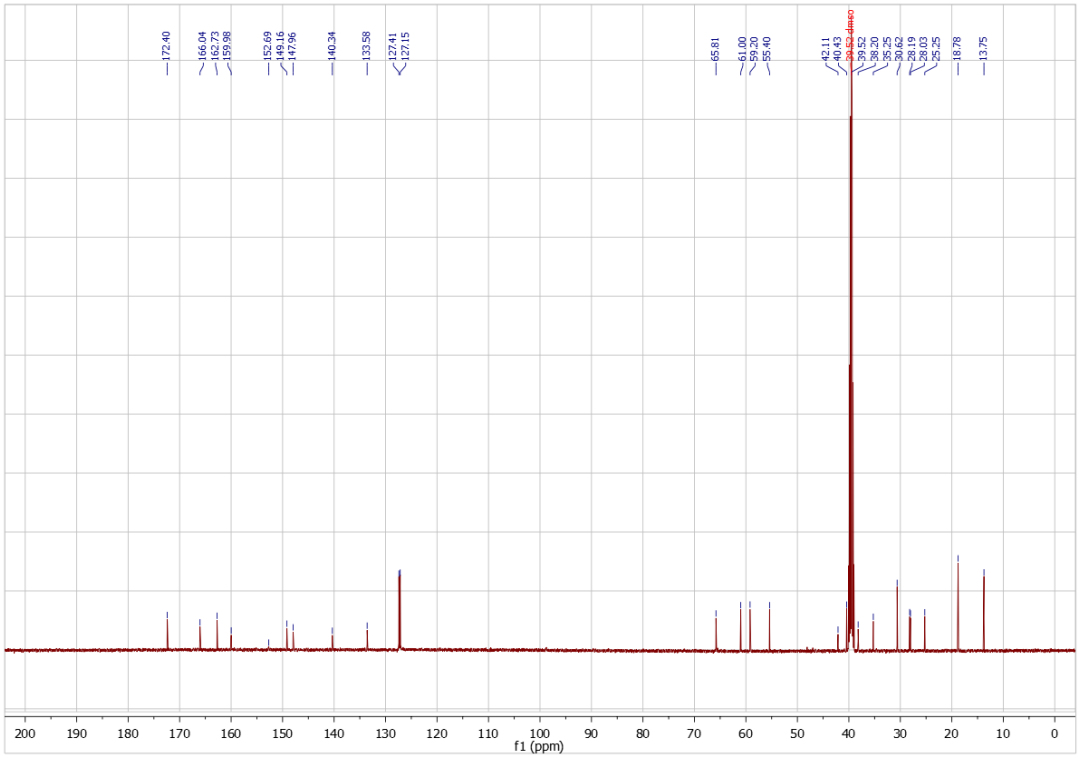
Spectra 43. ^{1}H NMR of **27**



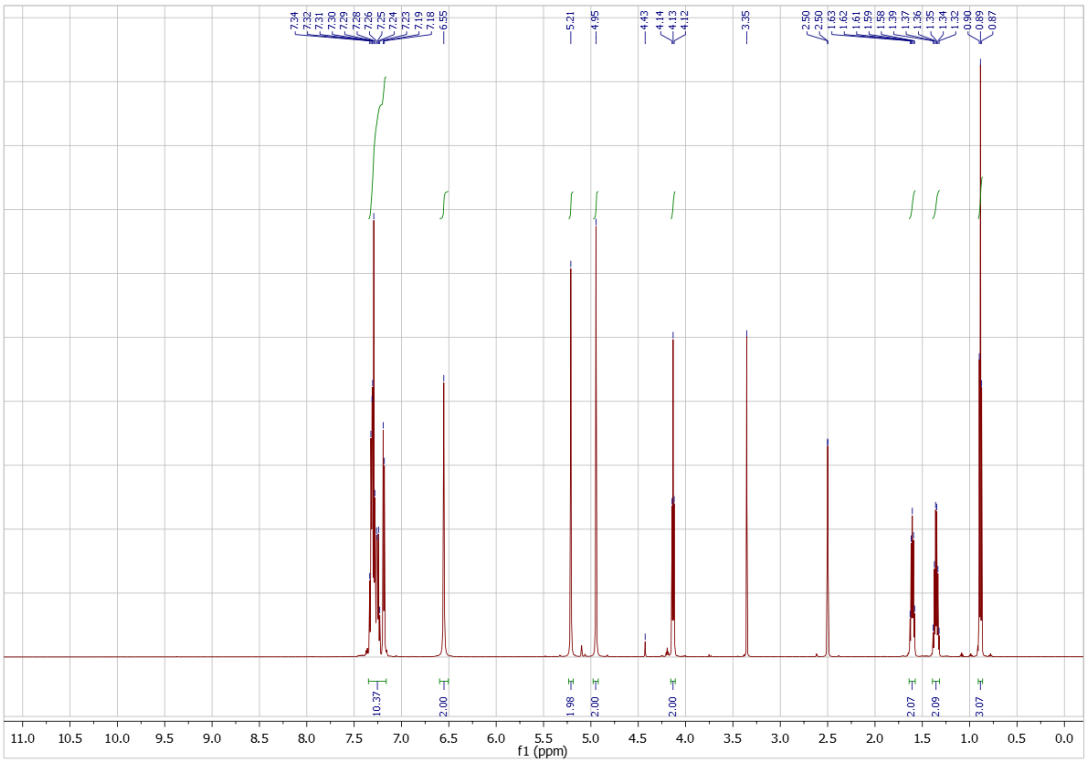
Spectra 44. ^{13}C NMR of **27**



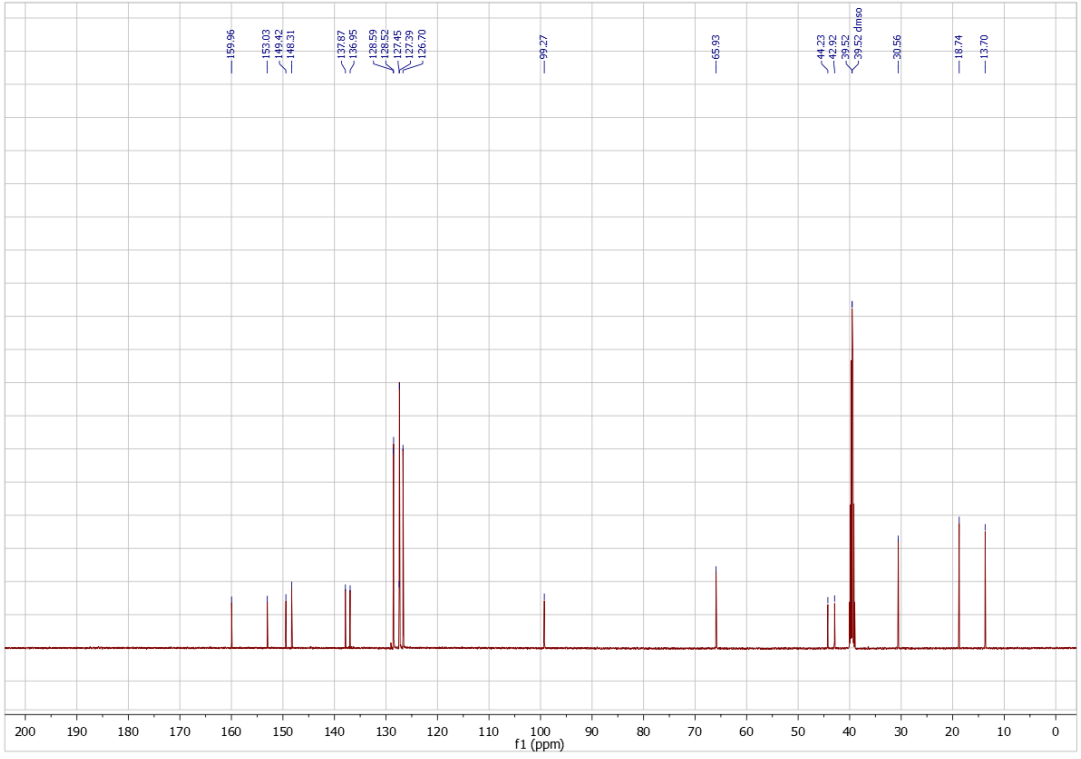
Spectra 45. ^{1}H NMR of **28**



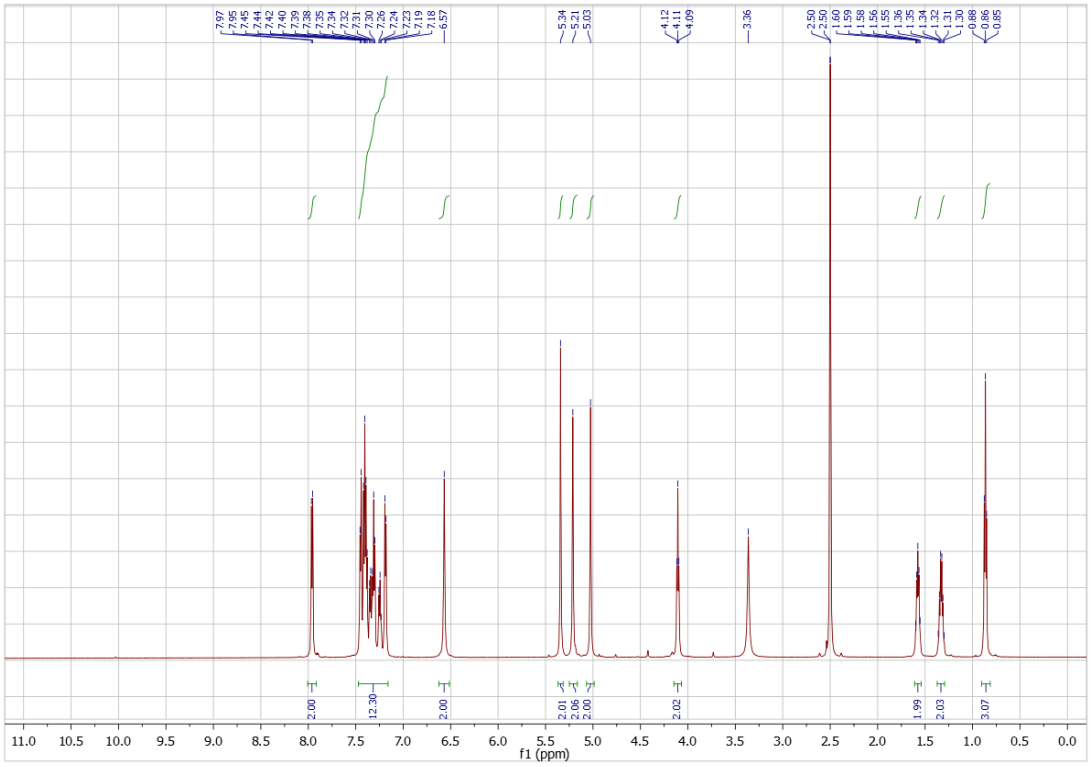
Spectra 46. ^{13}C NMR of **28**



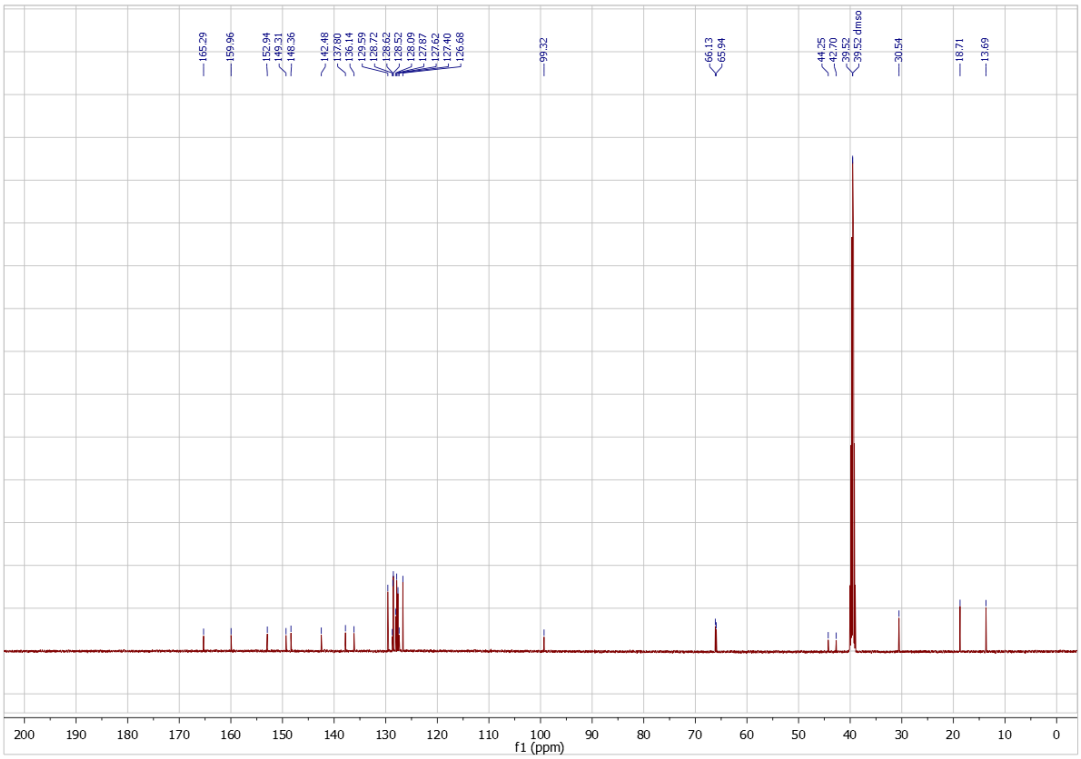
Spectra 47. ^{1}H NMR of **29**



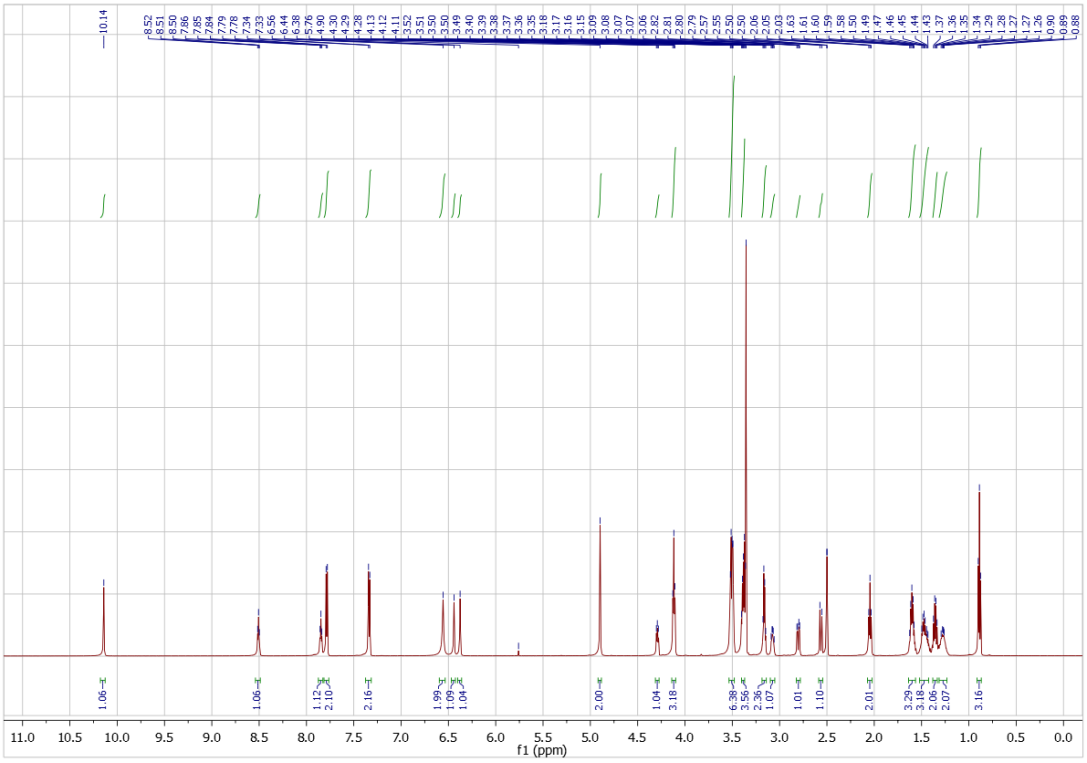
Spectra 48. ^{13}C NMR of **29**



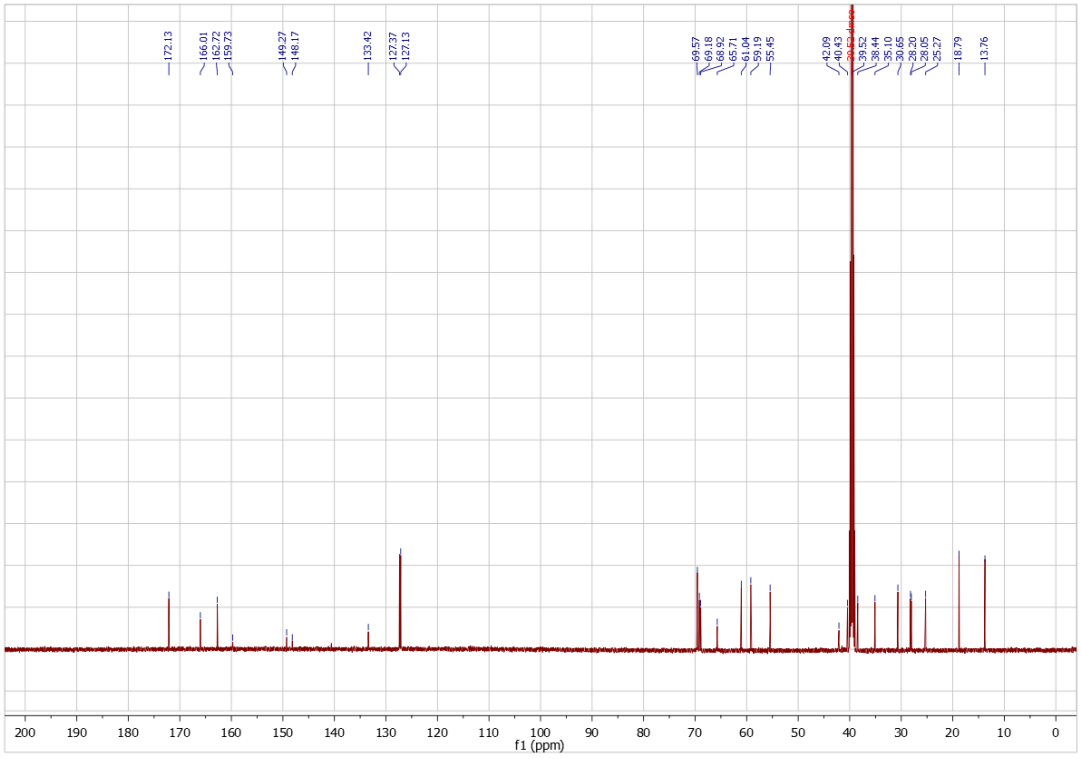
Spectra 49. ^{1}H NMR of **30**



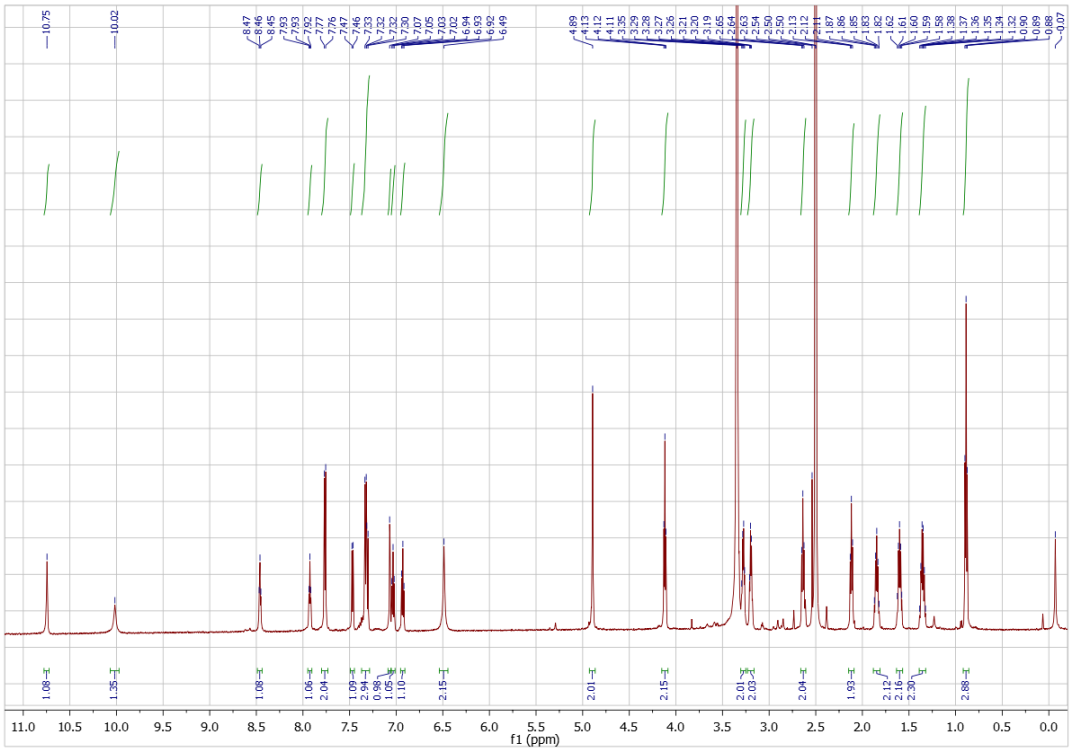
Spectra 50. ^{13}C NMR of **30**



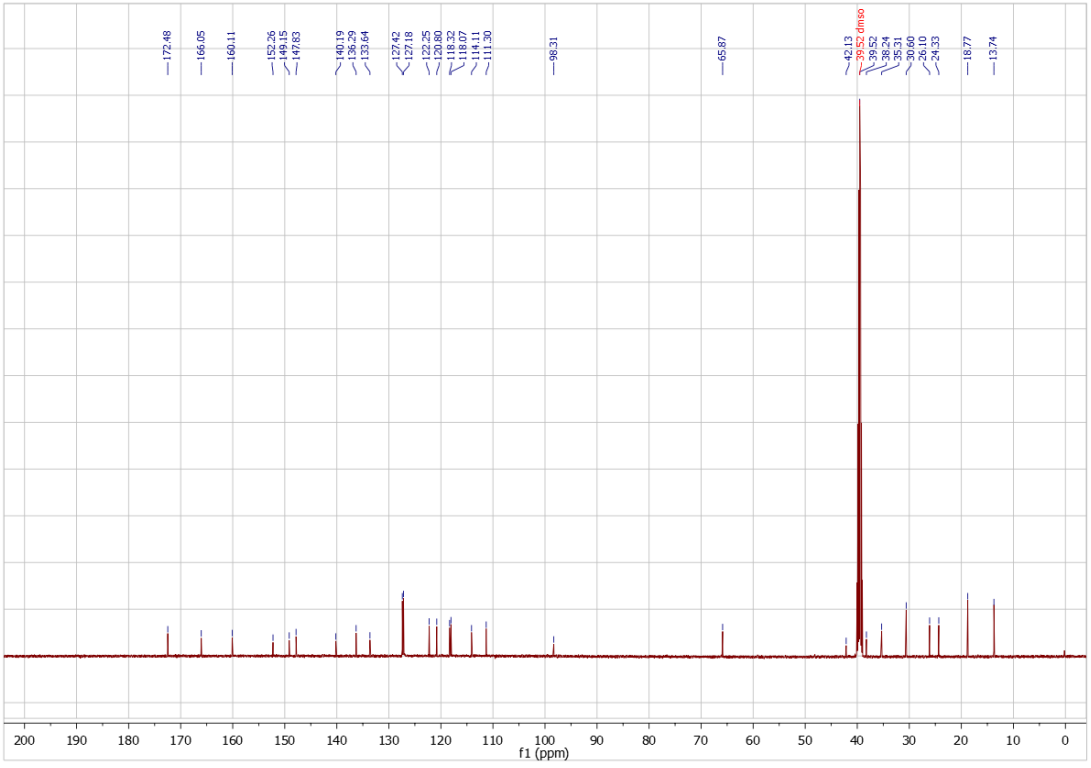
Spectra 51. ^{1}H NMR of **31**



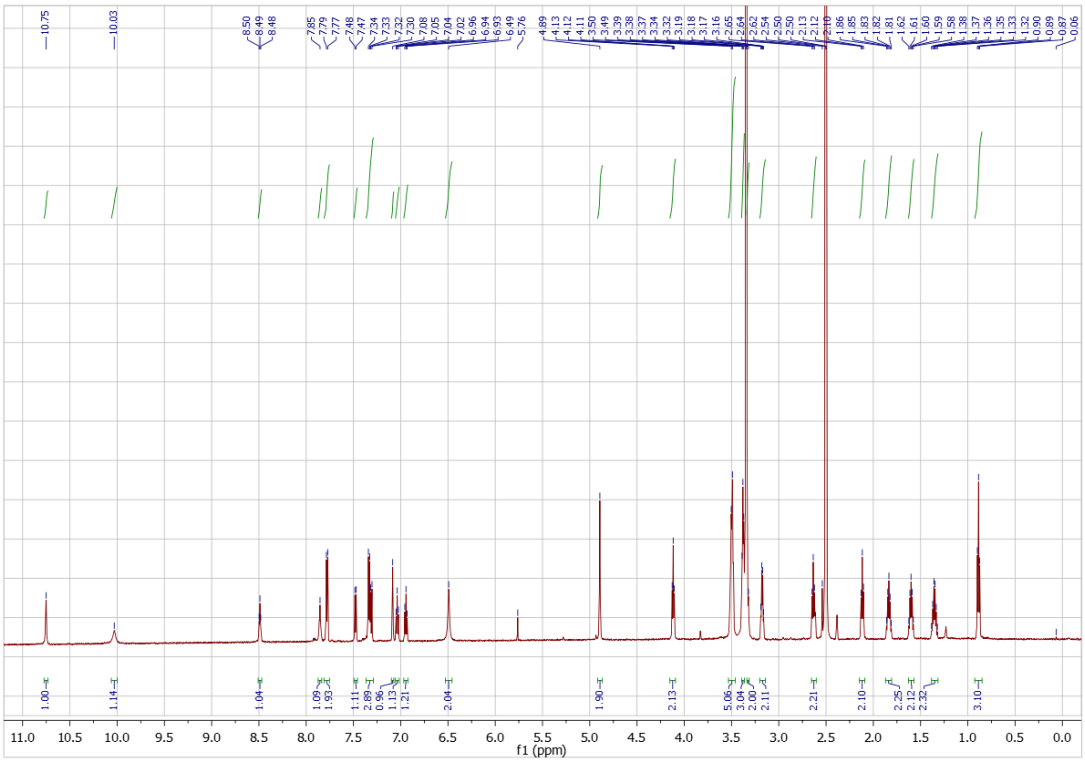
Spectra 52. ^{13}C NMR of **31**



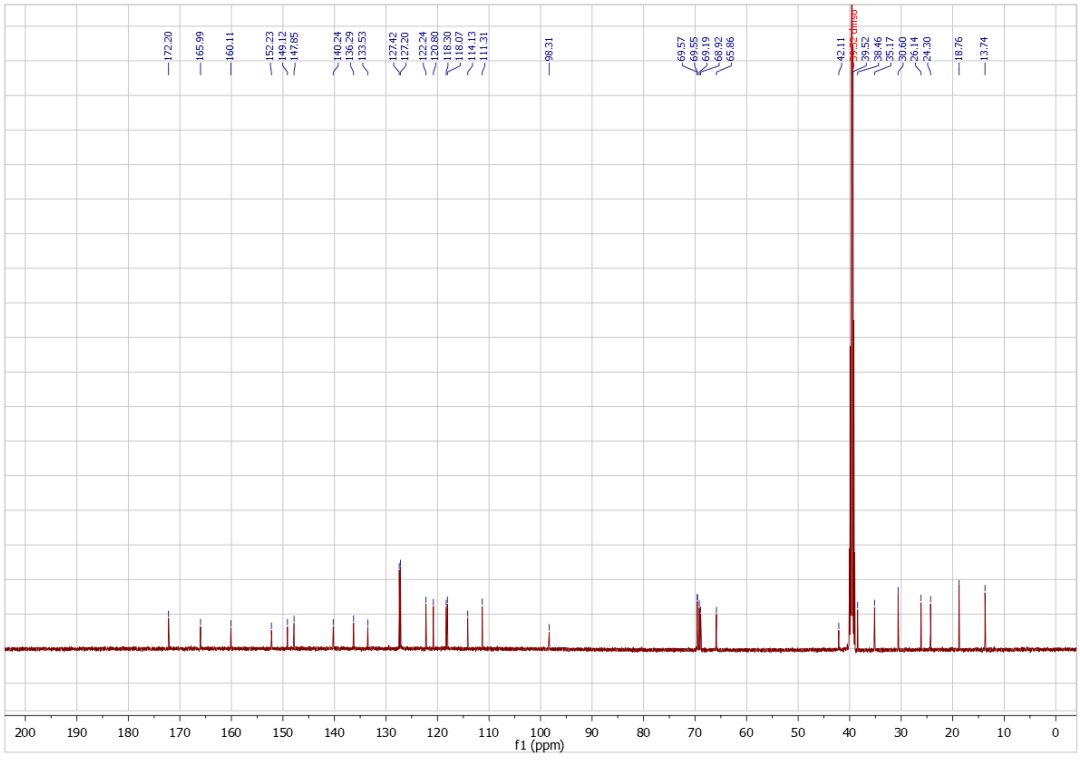
Spectra 53. ^{1}H NMR of **32**



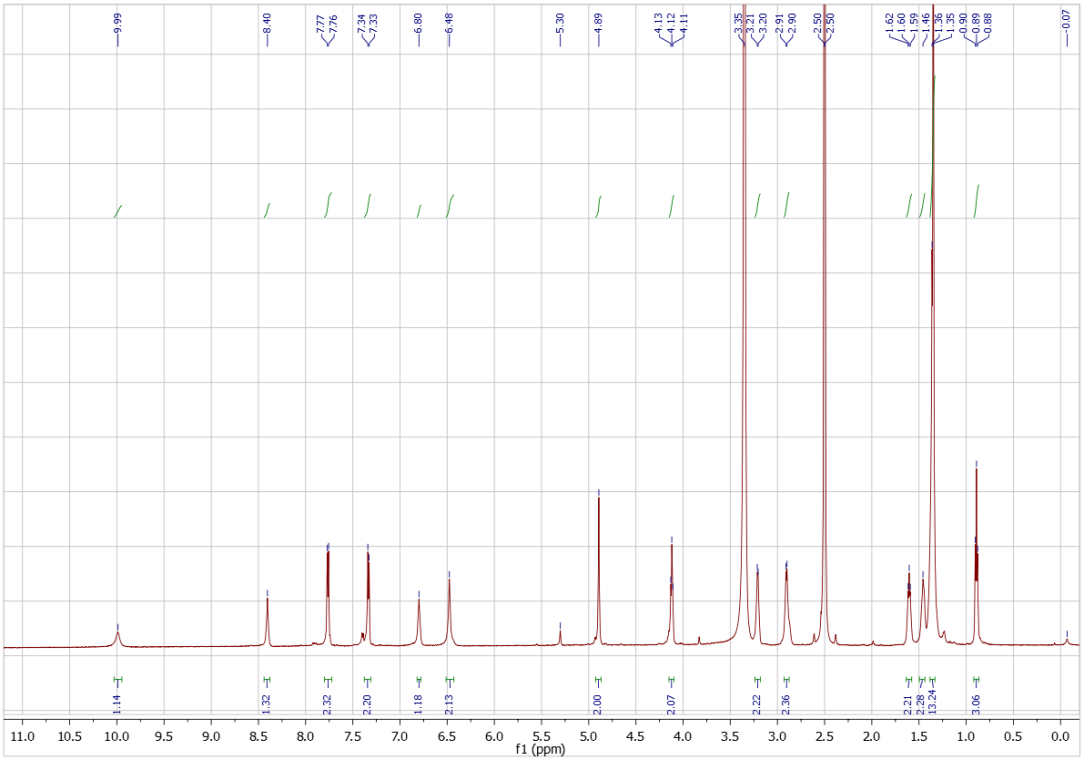
Spectra 54. ^{13}C NMR of **32**



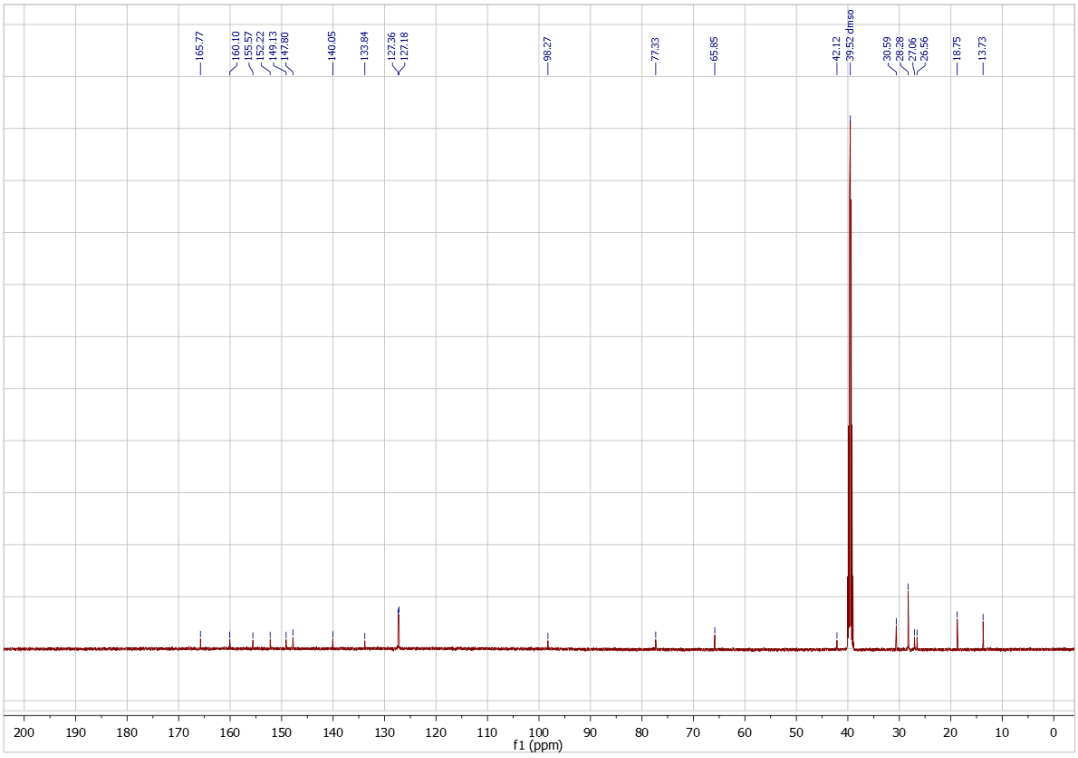
Spectra 55. ^{1}H NMR of **33**



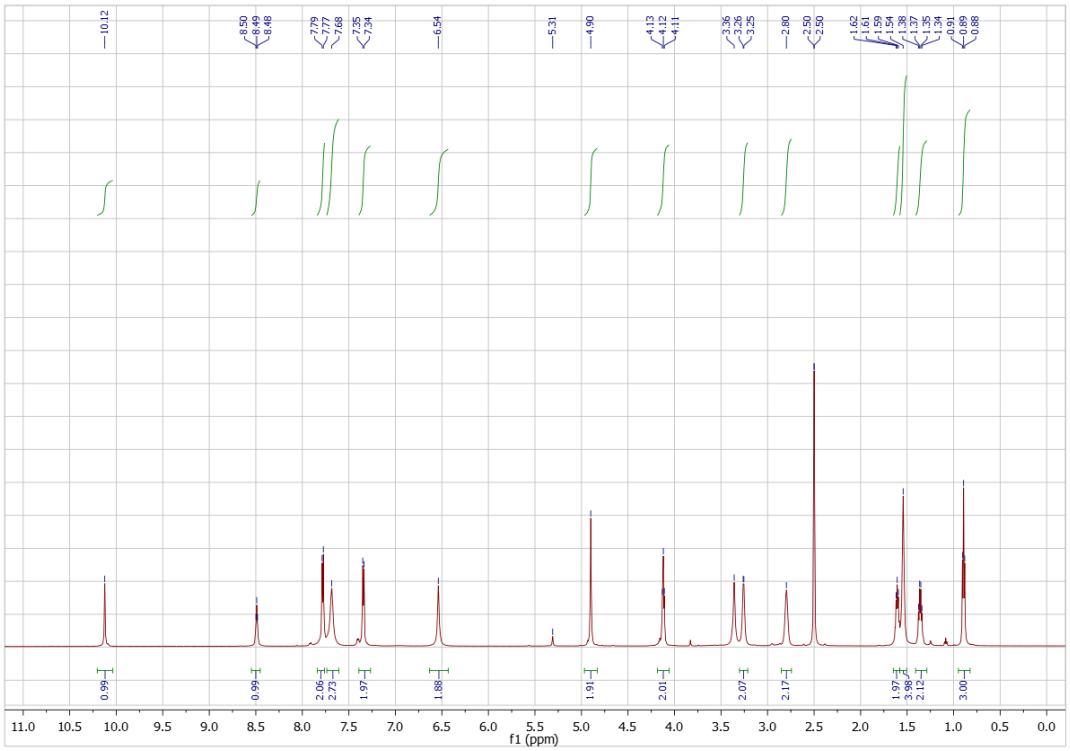
Spectra 56. ^{13}C NMR of **33**



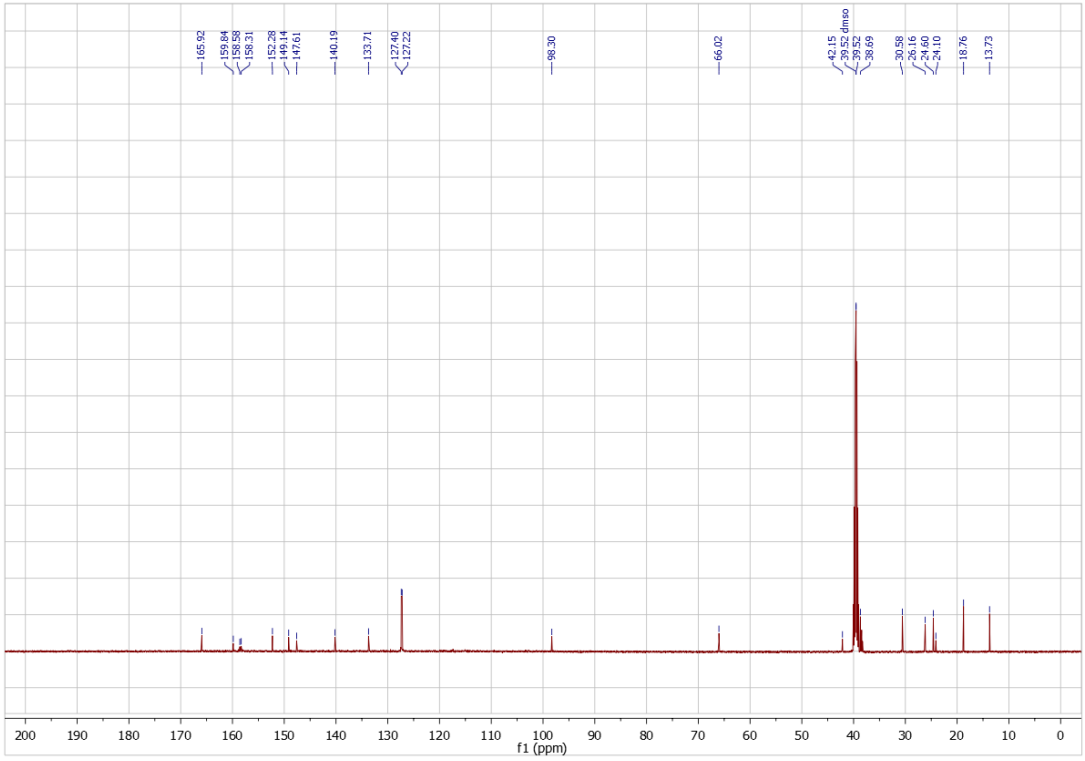
Spectra 57. ^{1}H NMR of **34**



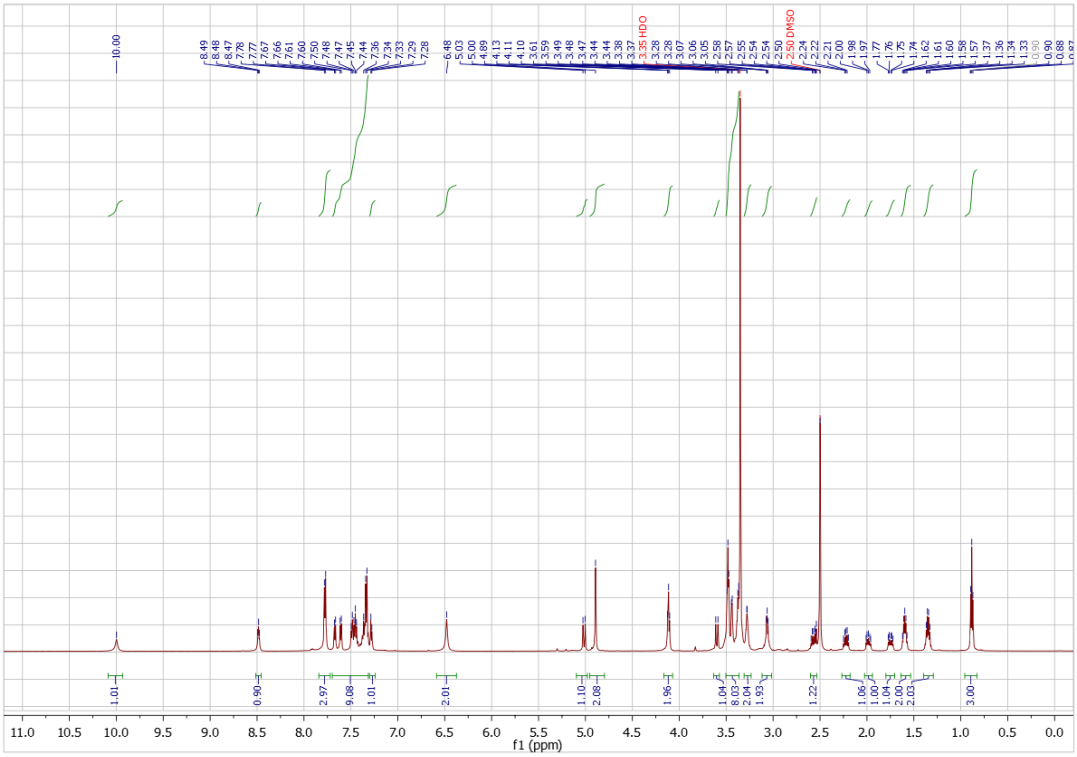
Spectra 58. ^{13}C NMR of **34**



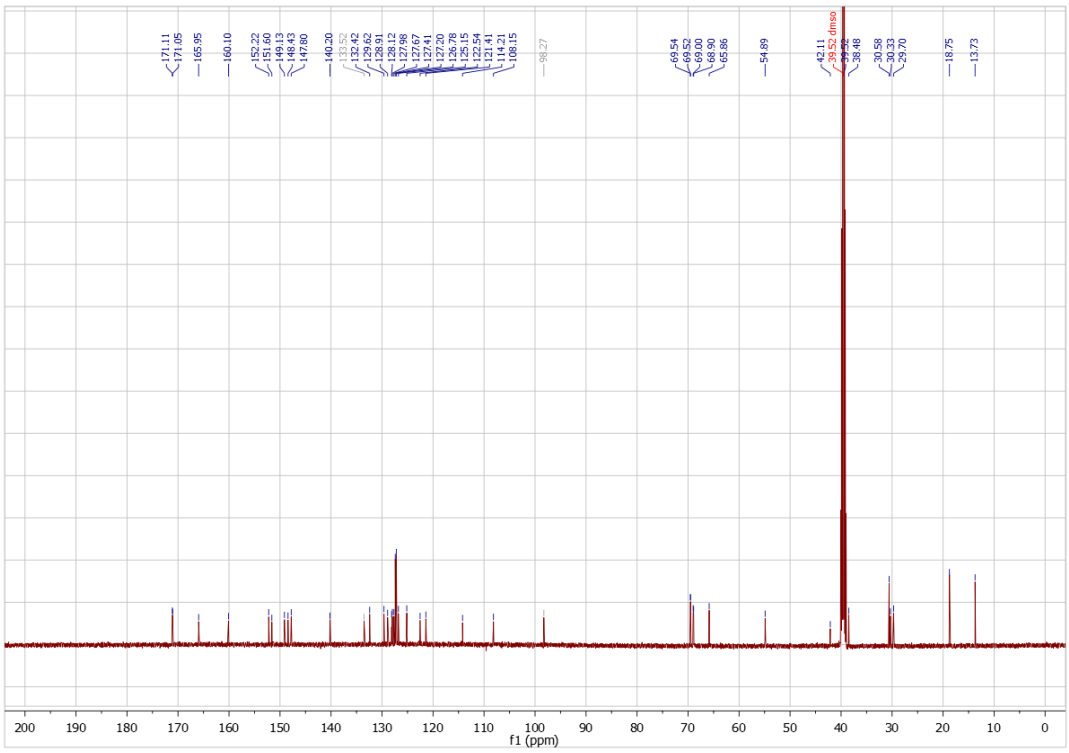
Spectra 59. ^{1}H NMR of **35**



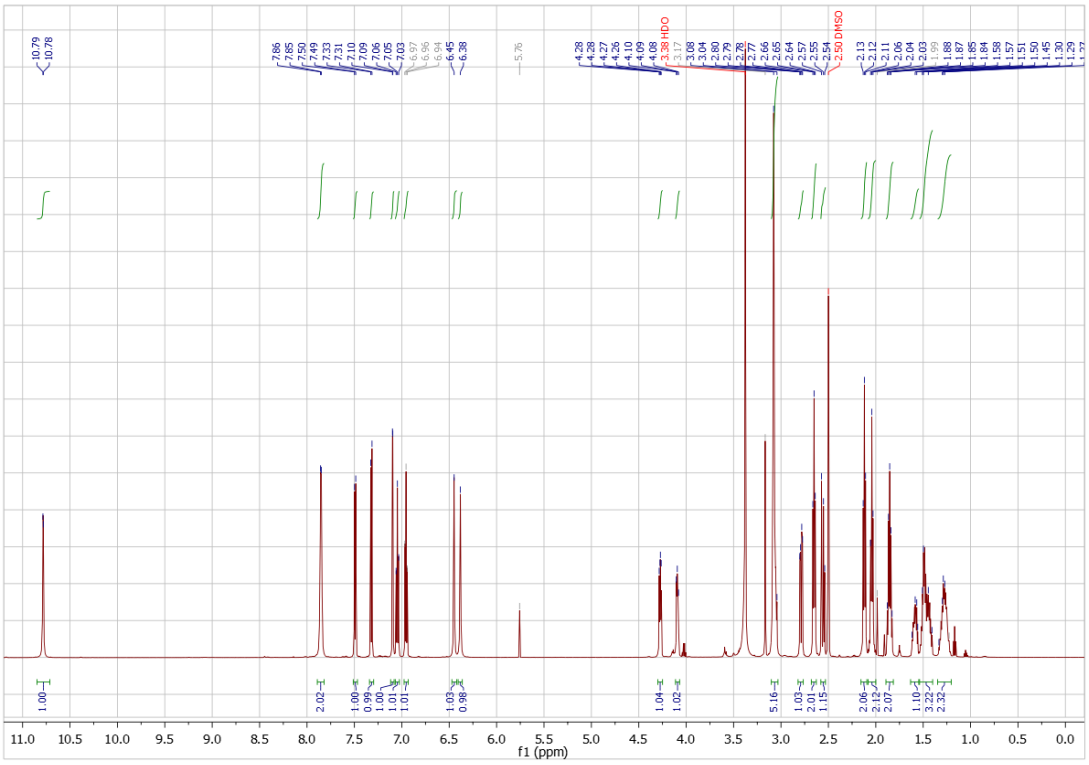
Spectra 60. ^{13}C NMR of **35**



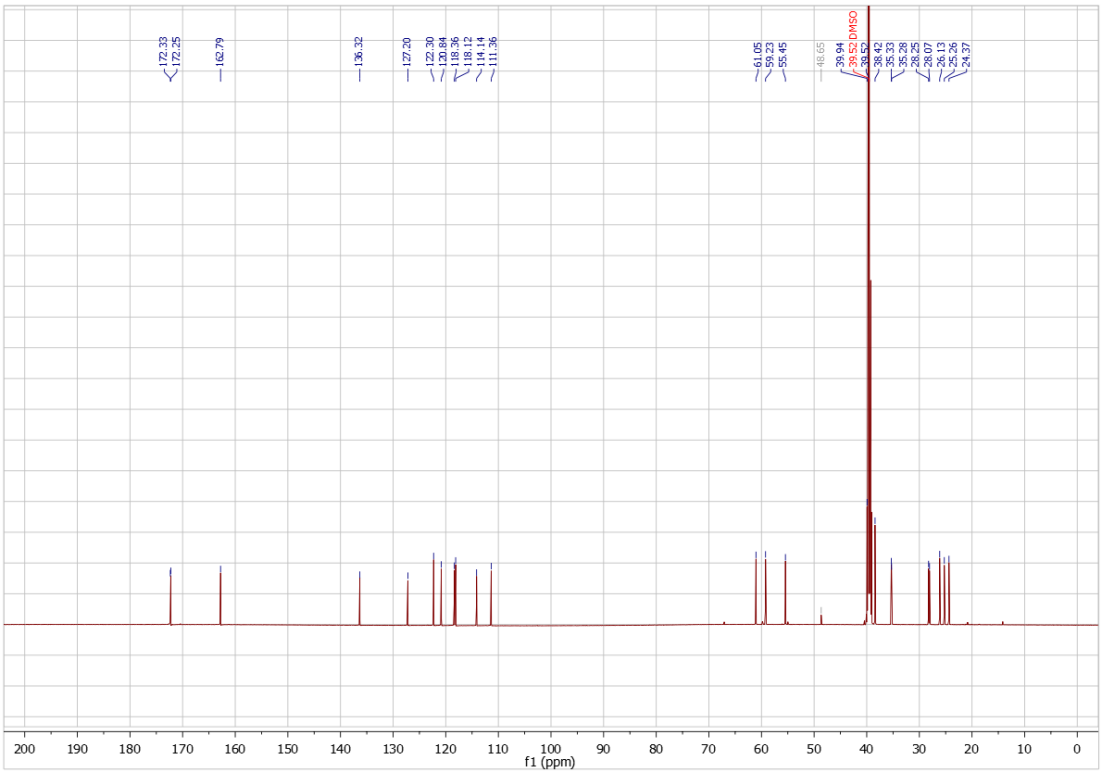
Spectra 61. ^{1}H NMR of **36**



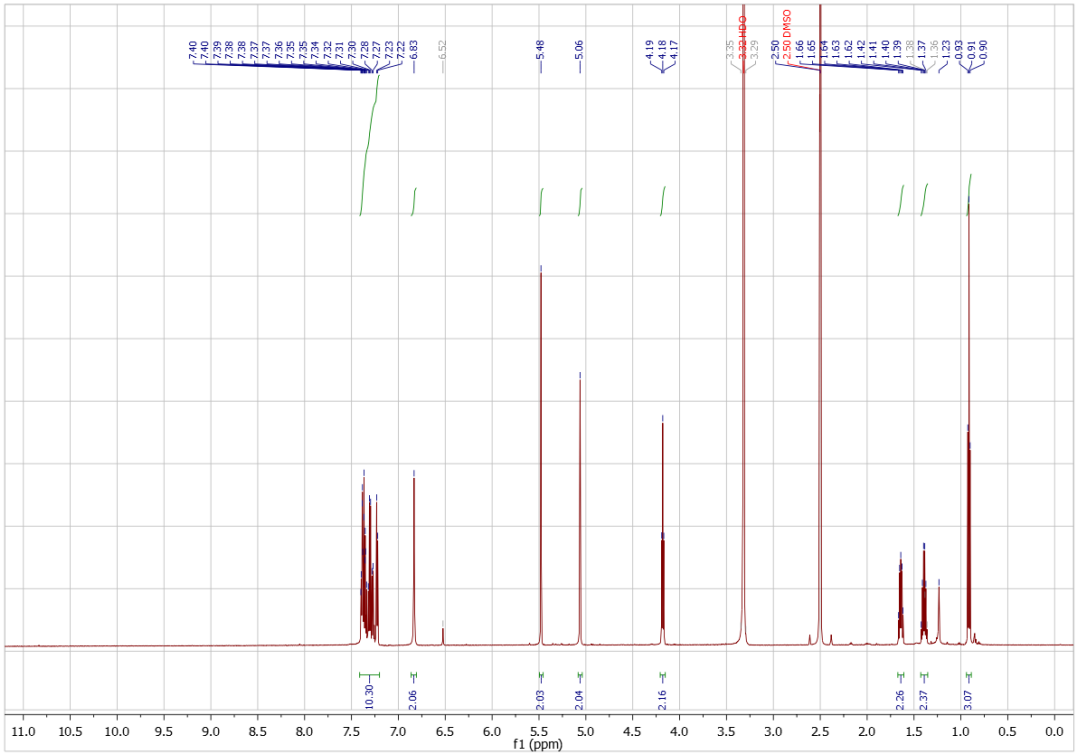
Spectra 62. ^{13}C NMR of **36**



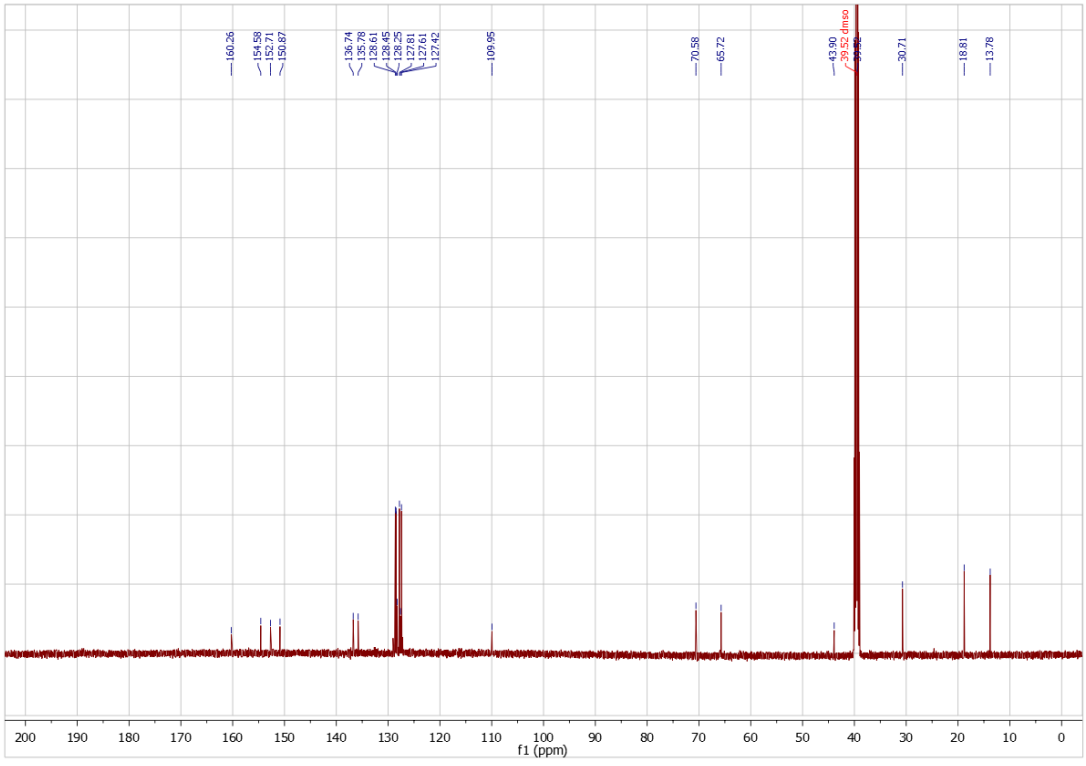
Spectra 63. ^{1}H NMR of **37**



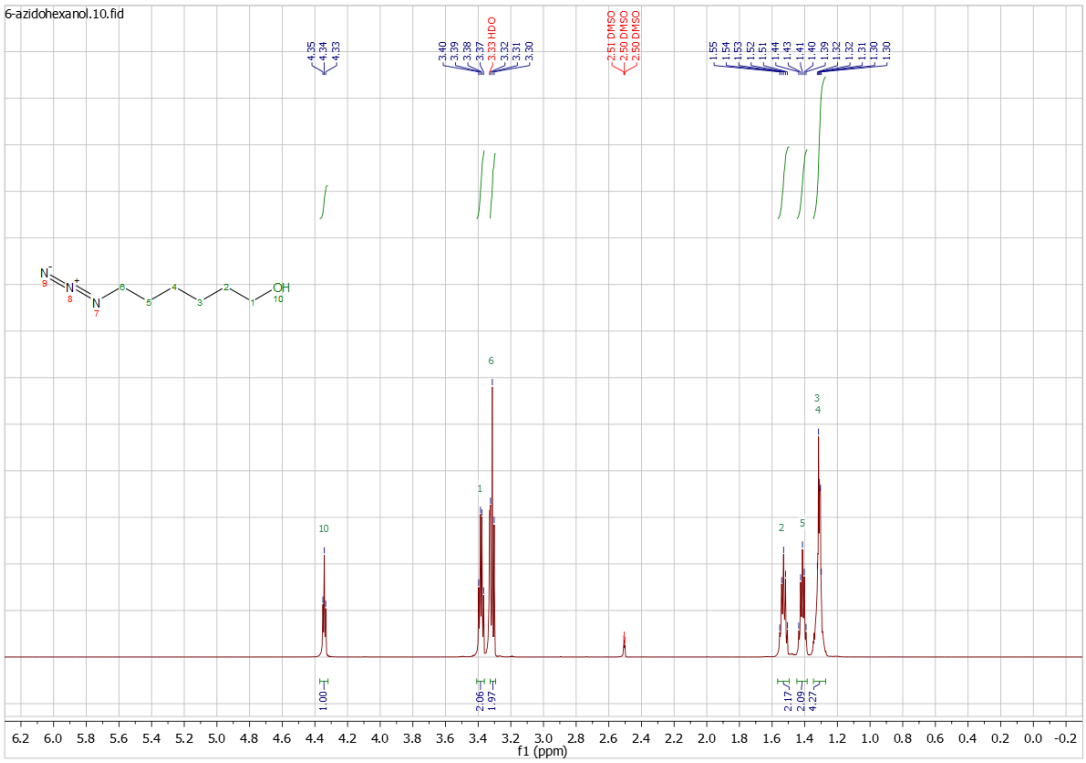
Spectra 64. ^{13}C NMR of **37**



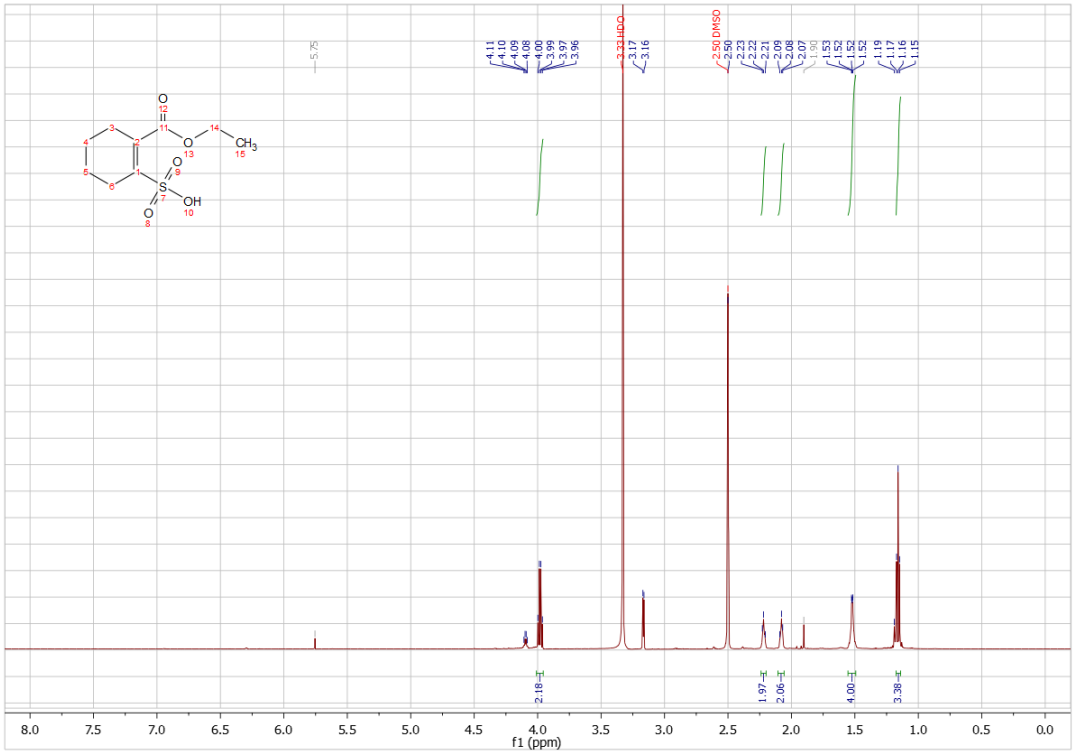
Spectra 65. ^{1}H NMR of **38**



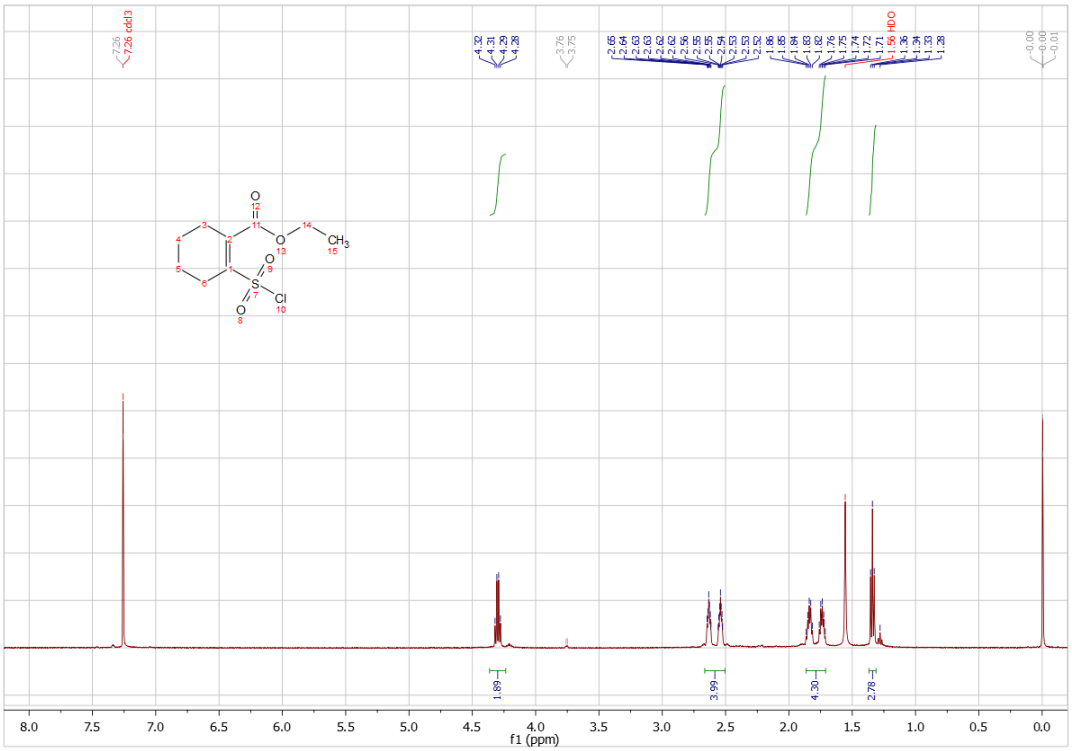
Spectra 66. ^{13}C NMR of **38**



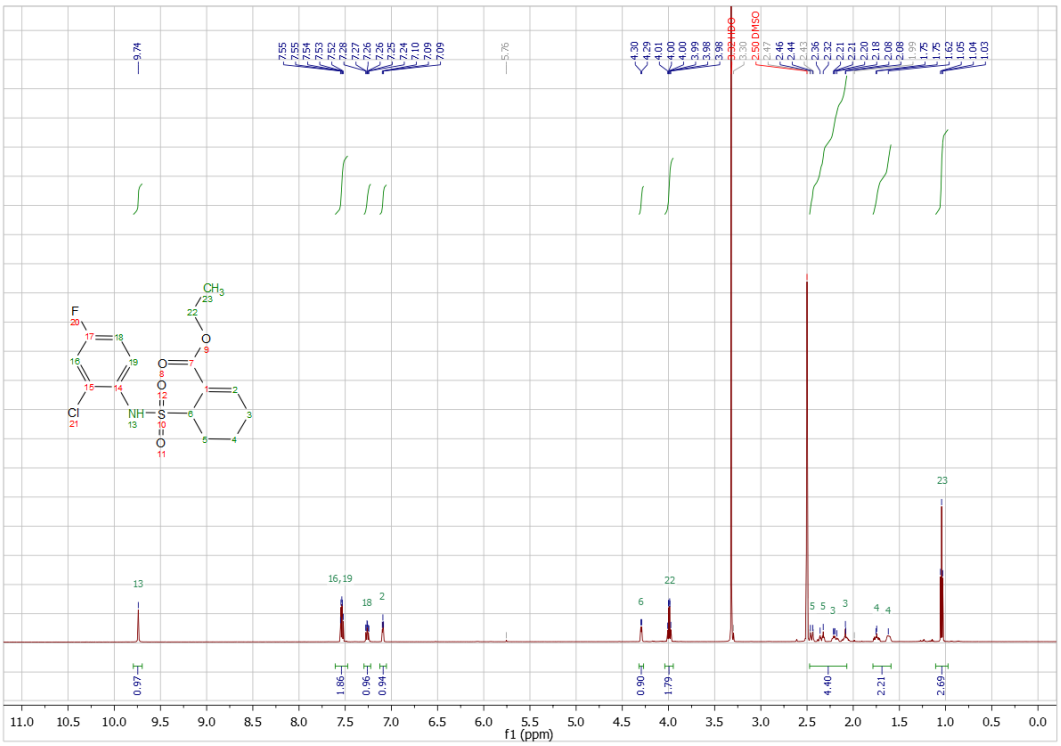
Spectra 67. ^{1}H NMR of 6-Azidohexan-1-ol



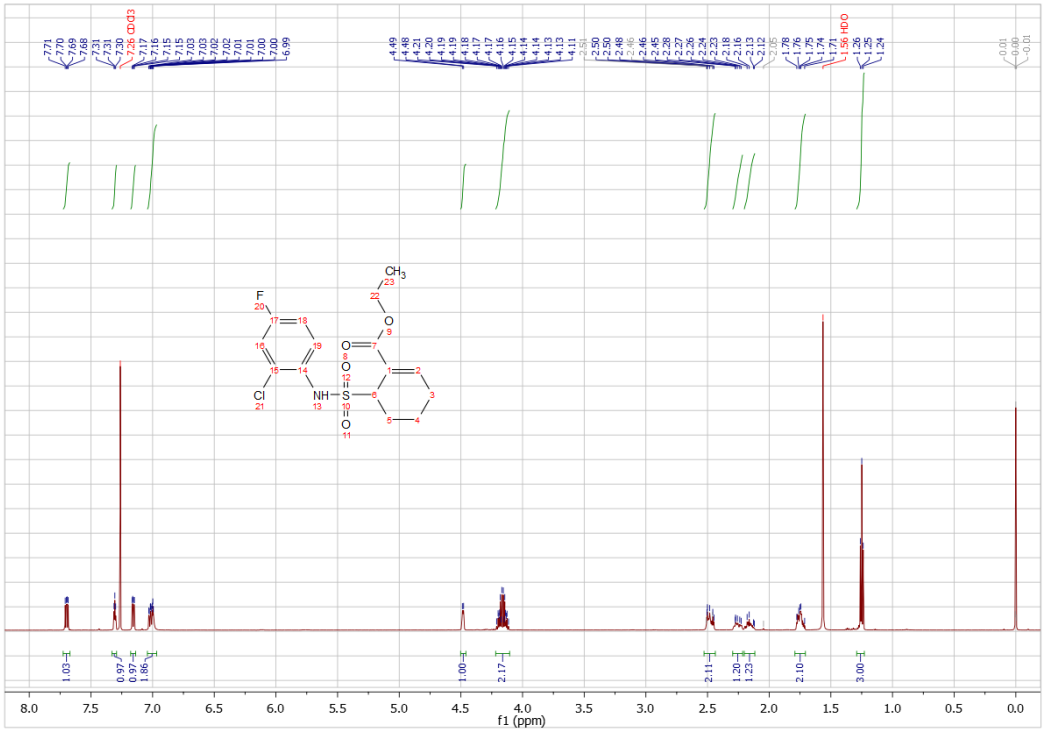
Spectra 68. ^{1}H NMR of 2-(Ethoxycarbonyl)cyclohex-1-ene-1-sulfonic acid



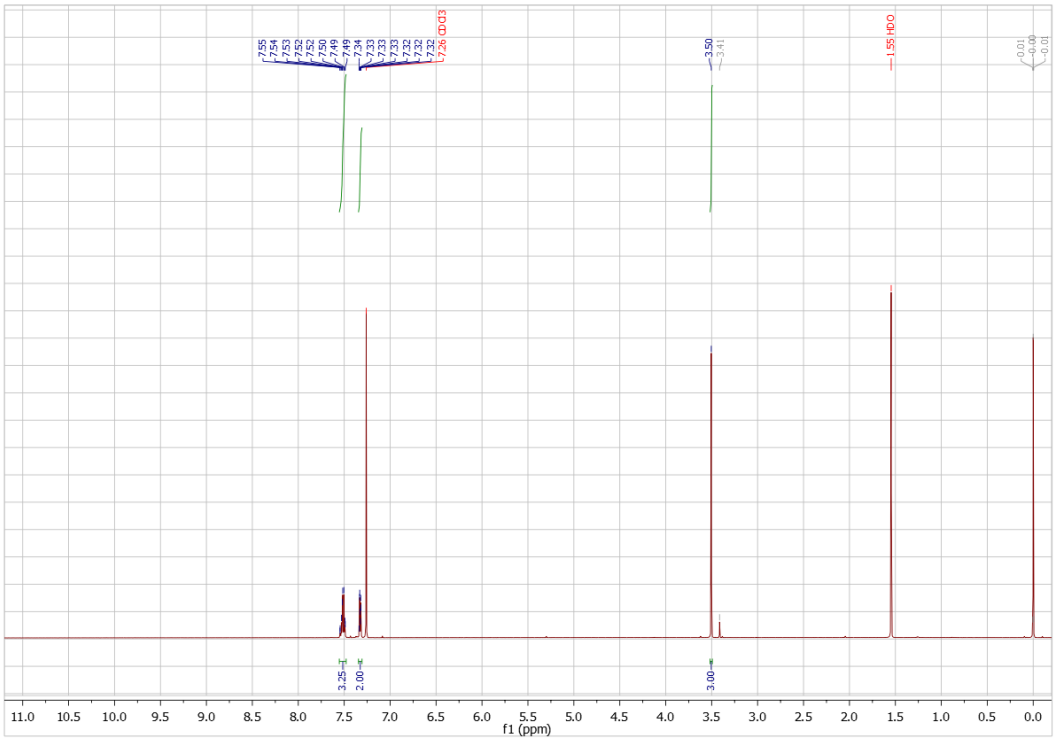
Spectra 69. ^{1}H NMR of Ethyl 2-(chlorosulfonyl)cyclohex-1-ene-1-carboxylate



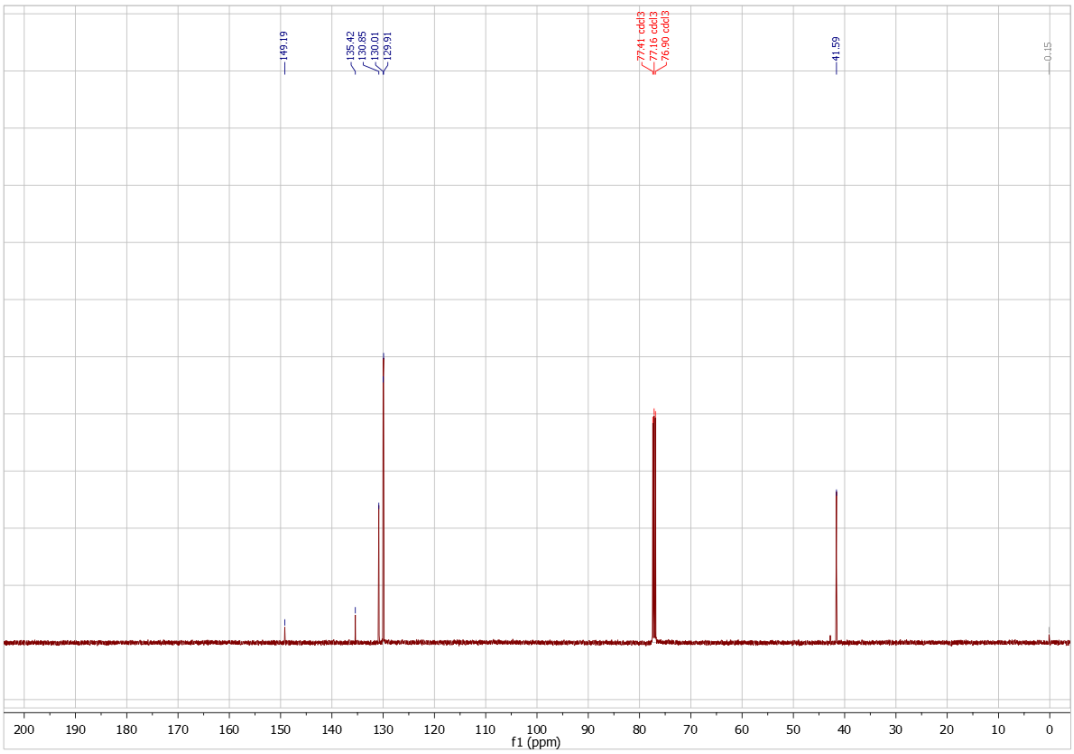
Spectra 70. ^{1}H NMR of Ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate (DMSO-d_6)



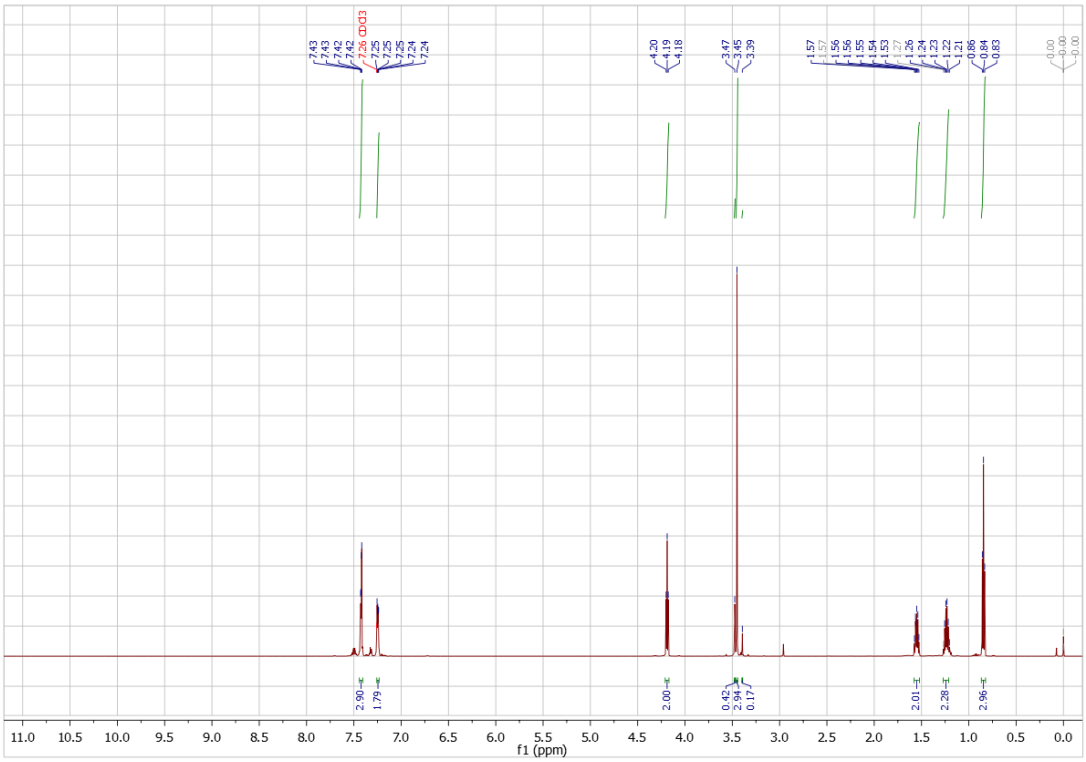
Spectra 71. ^{1}H NMR of Ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate ($CDCl_3$)



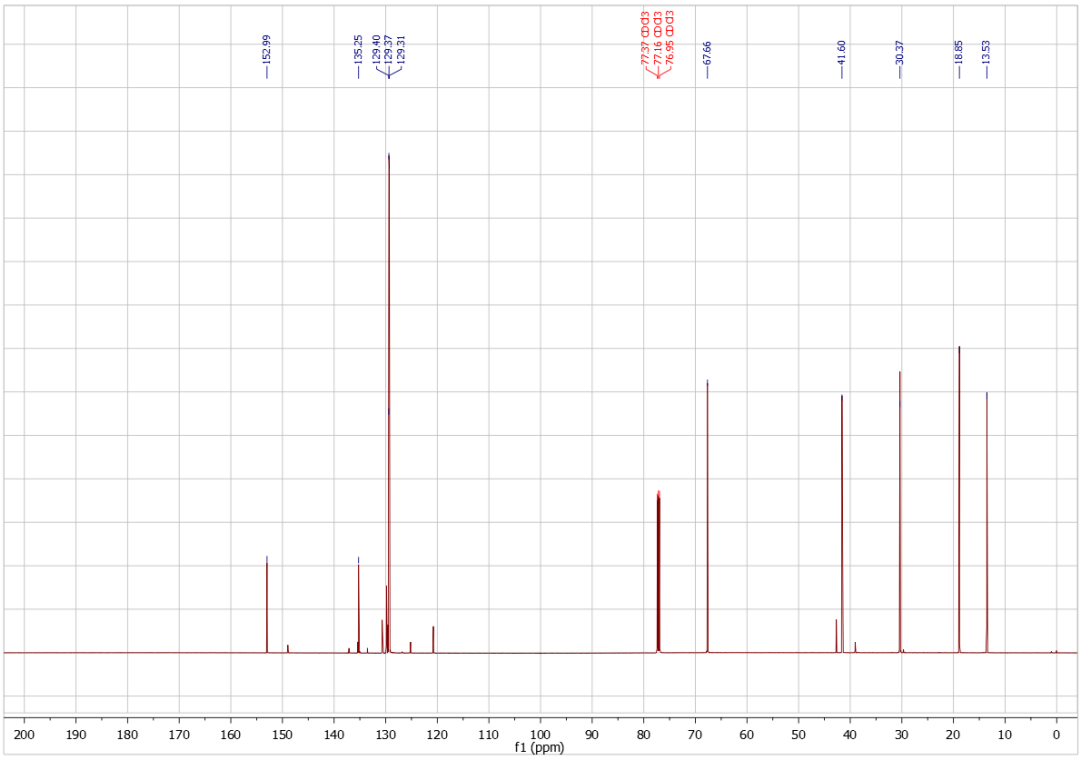
Spectra 72. ^{1}H NMR of **39**



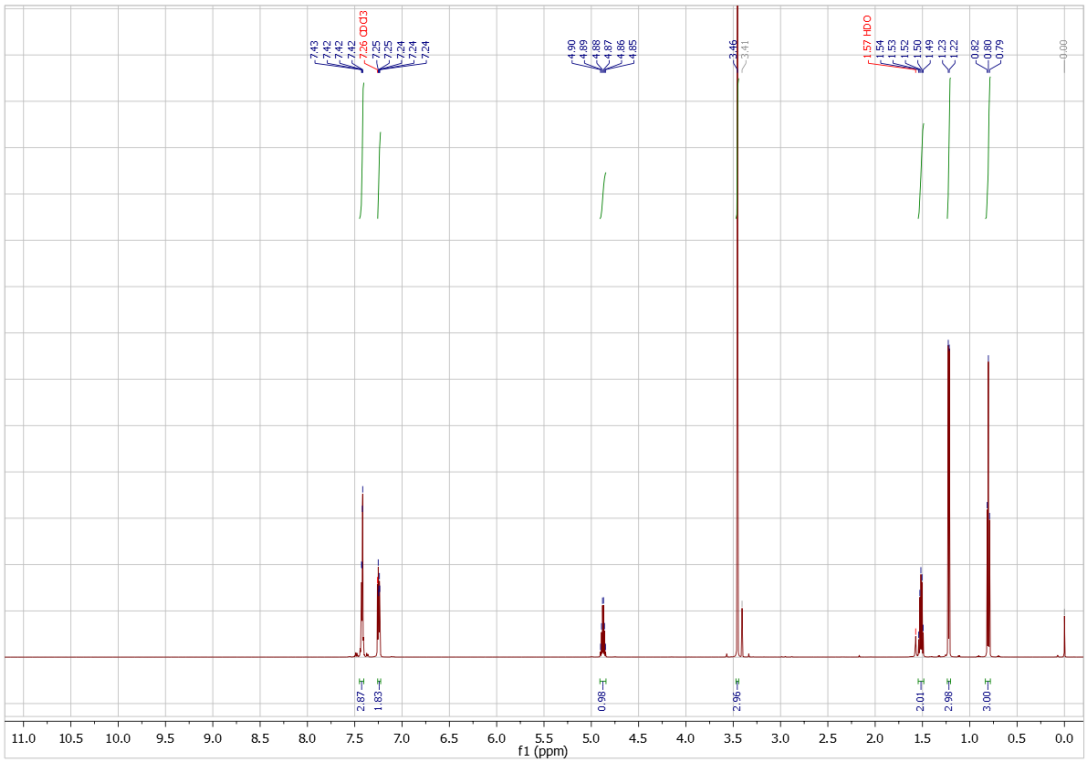
Spectra 73. ^{13}C NMR of **39**



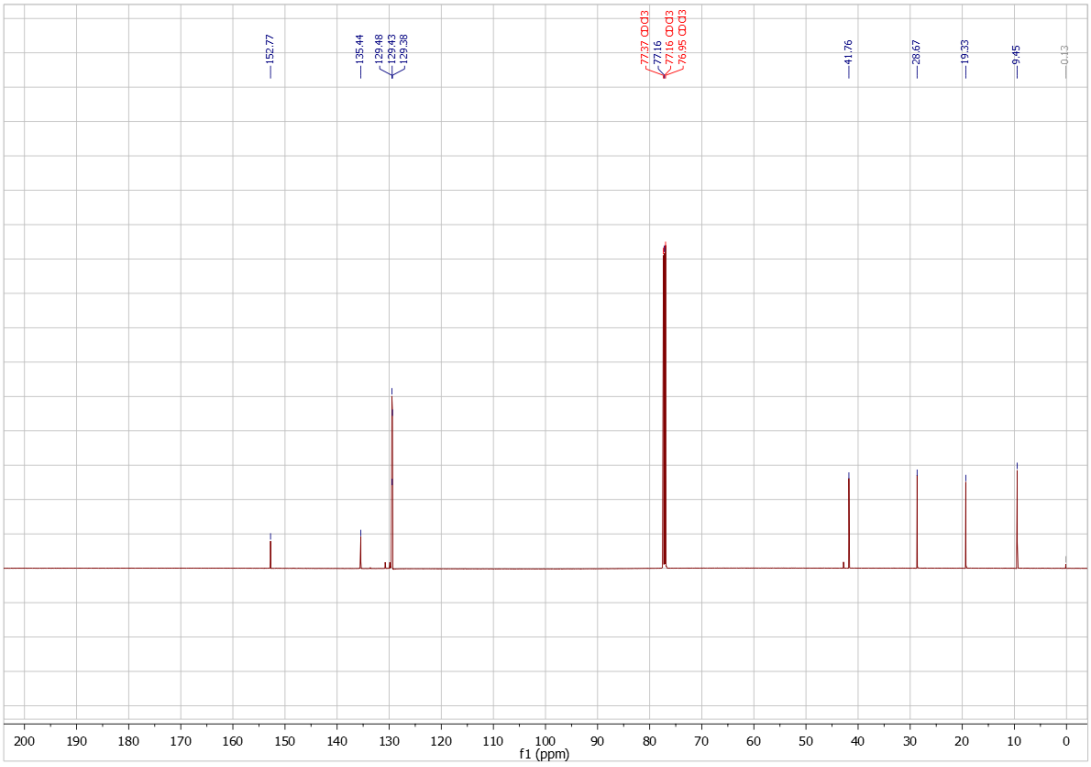
Spectra 74. ^{1}H NMR of **40**



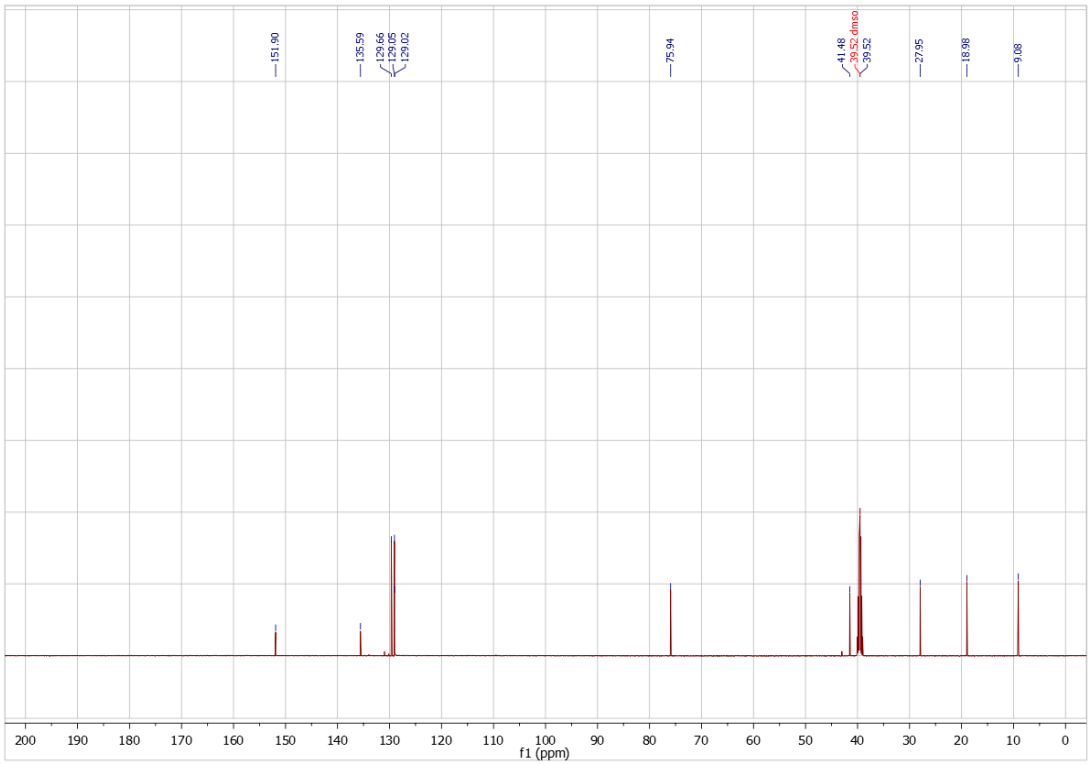
Spectra 75. ^{13}C NMR of **40**



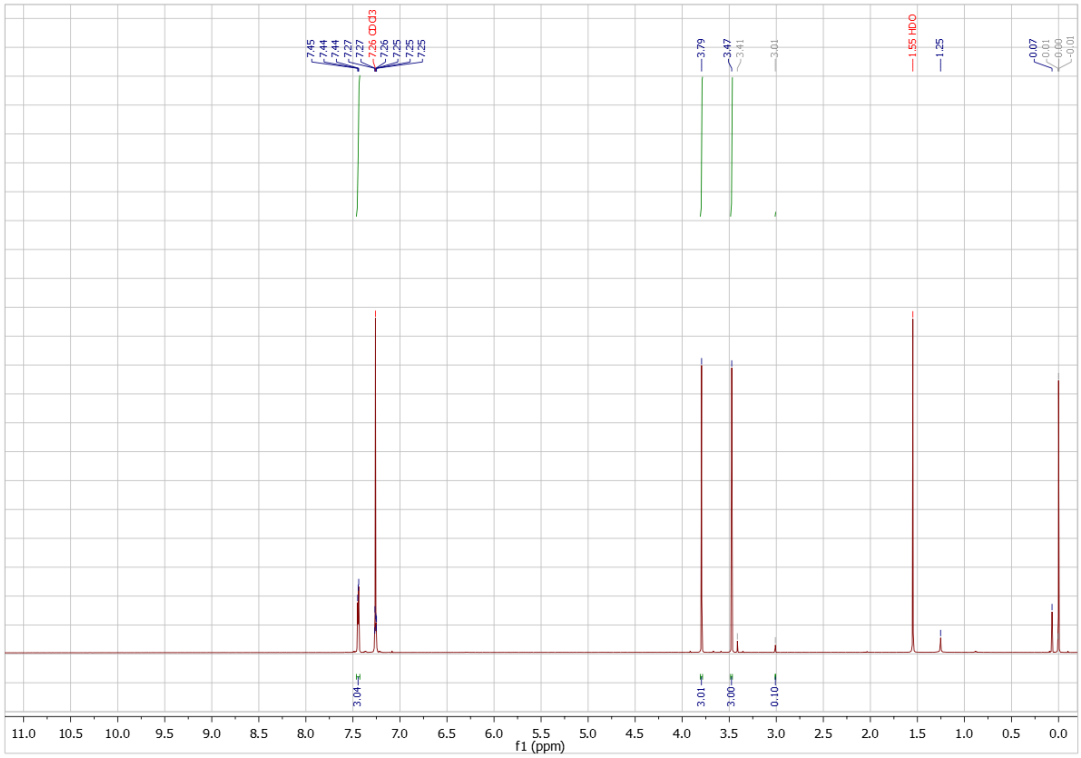
Spectra 76. ^{1}H NMR of **41**



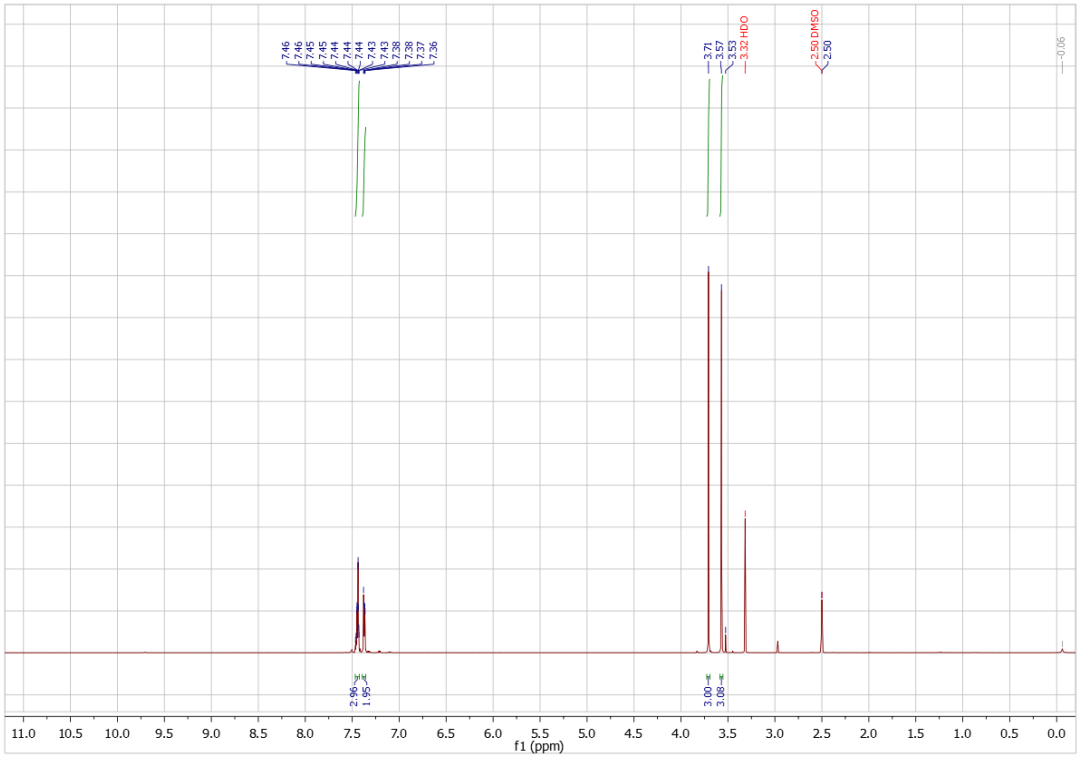
Spectra 77. ^{13}C NMR of **41** ($CDCl_3$)



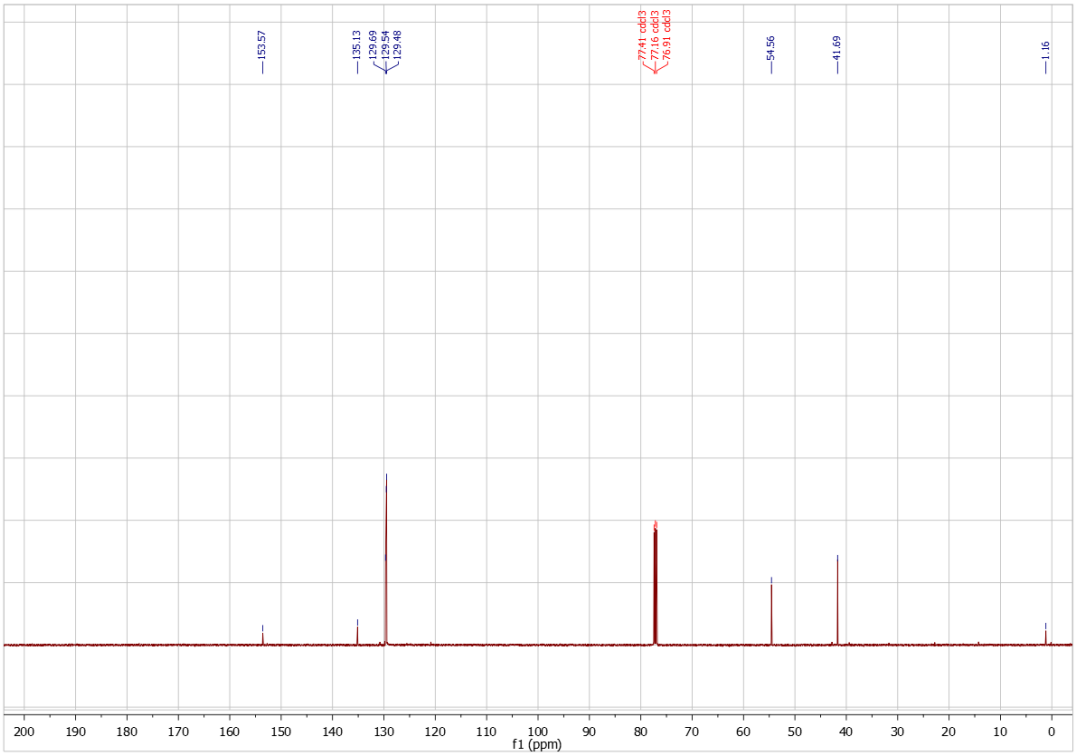
Spectra 78. ^{13}C NMR of **41** (DMSO-d_6)



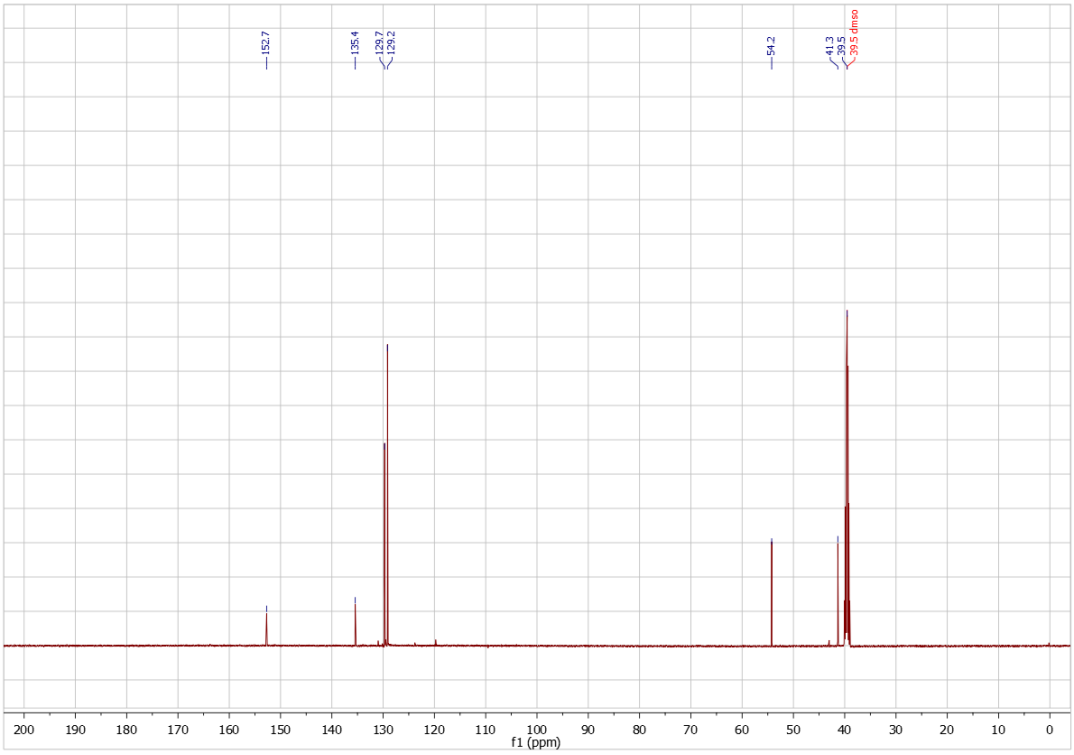
Spectra 79. ^{1}H NMR of **42** ($CDCl_3$)



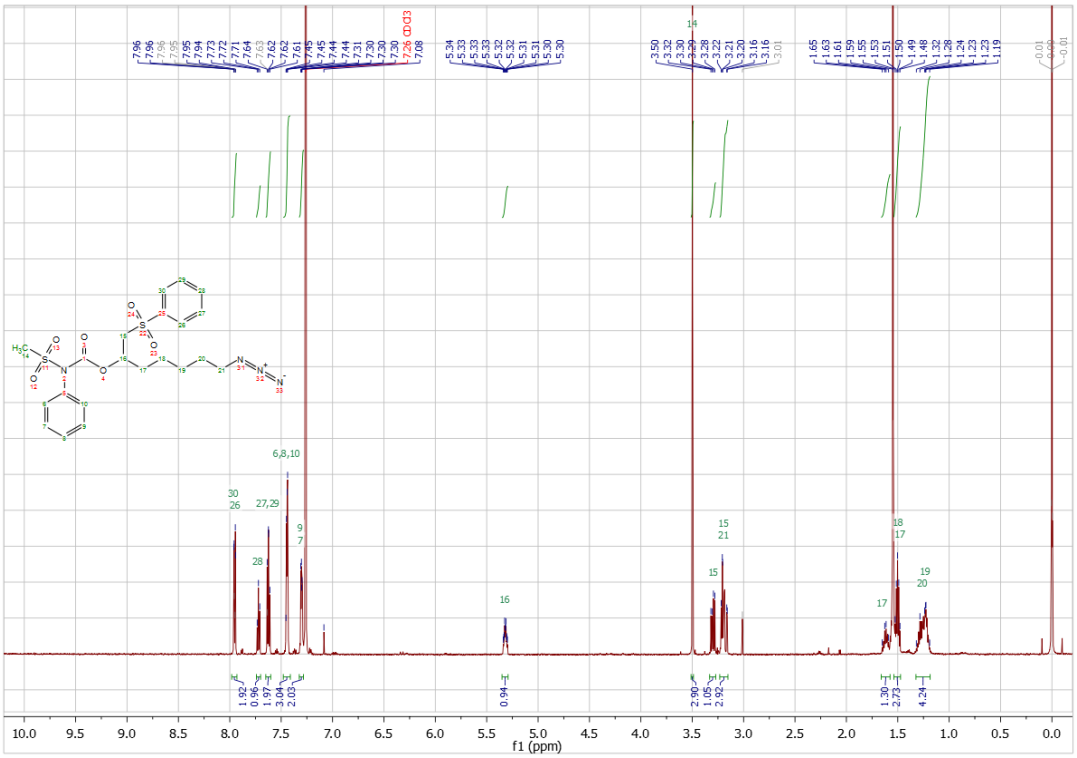
Spectra 80. ^{1}H NMR of **42** (DMSO-d_6)



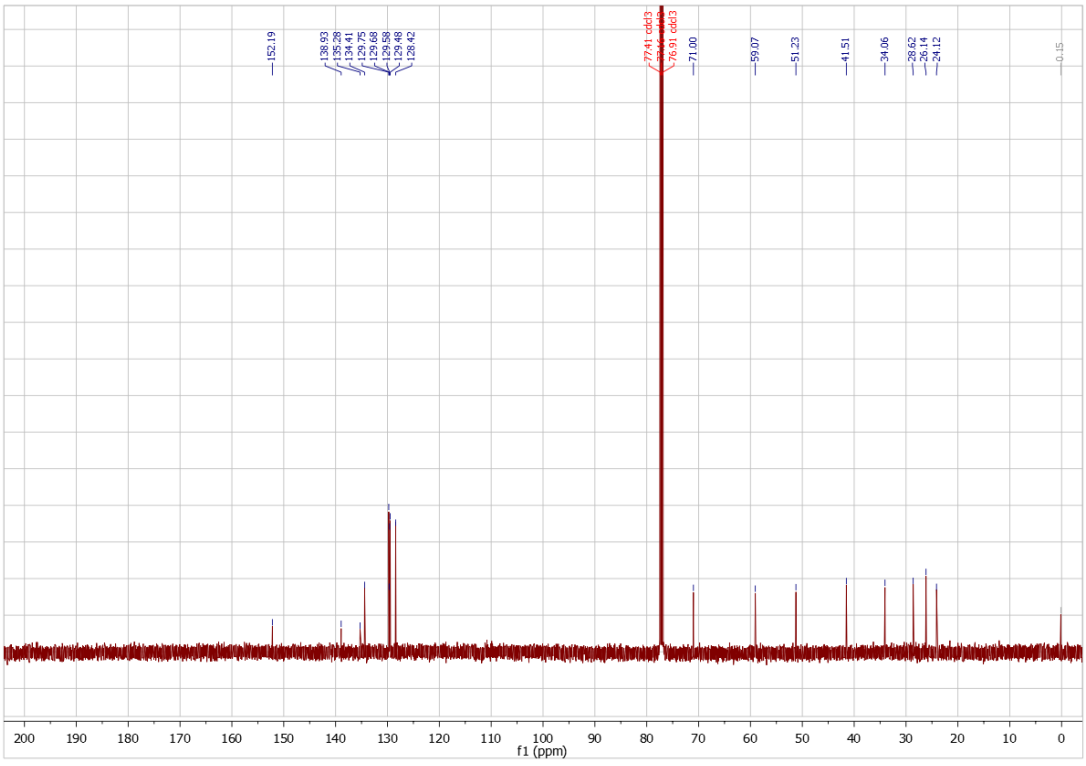
Spectra 81. ^{13}C NMR of **42** ($CDCl_3$)



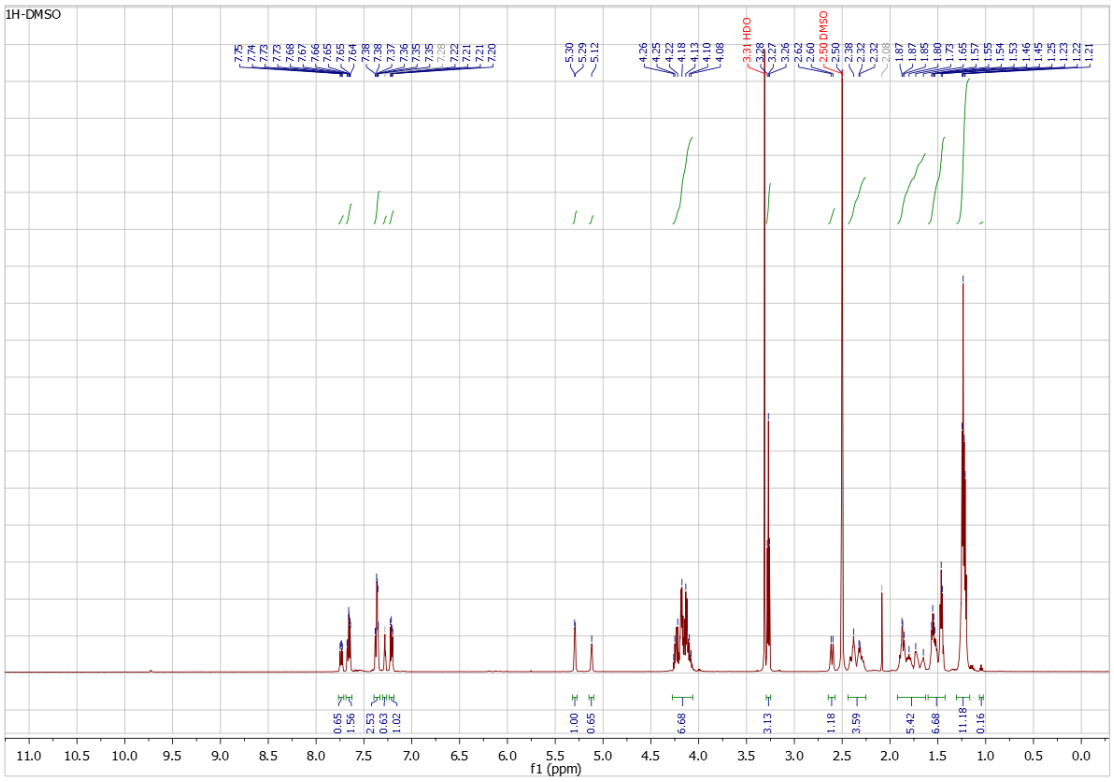
Spectra 82. ^{13}C NMR of **42** (DMSO-d_6)



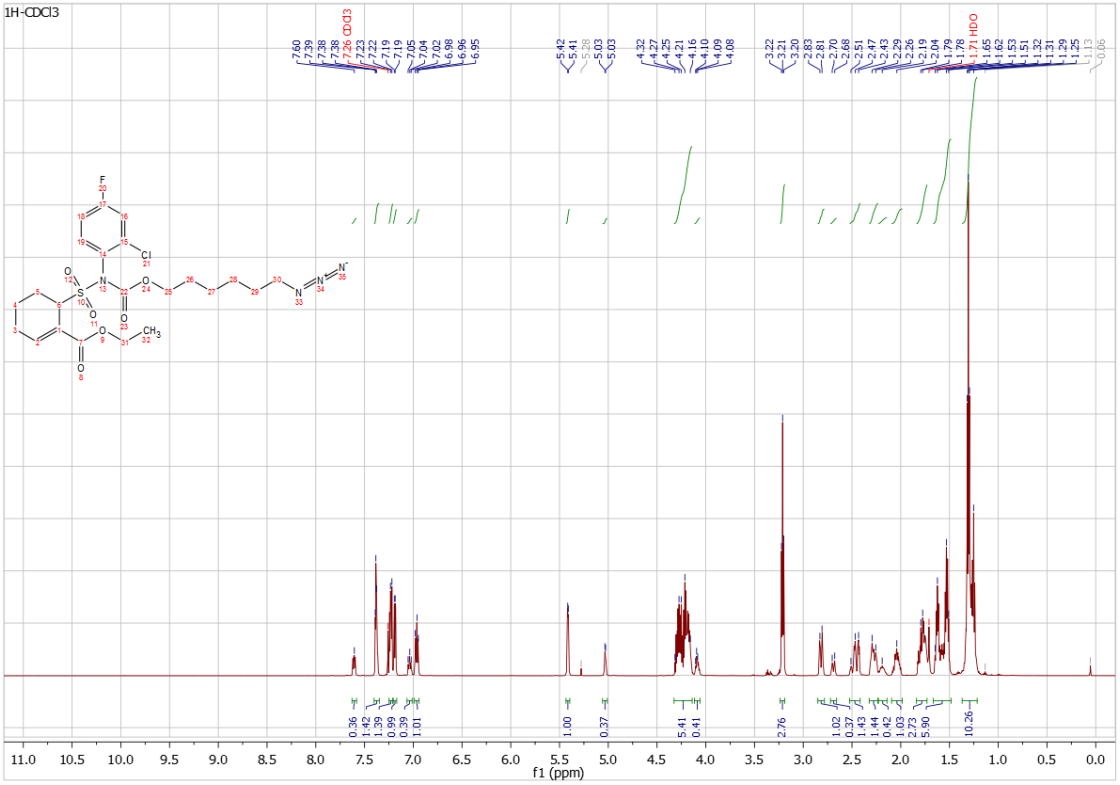
Spectra 83. ^{1}H NMR of **43**



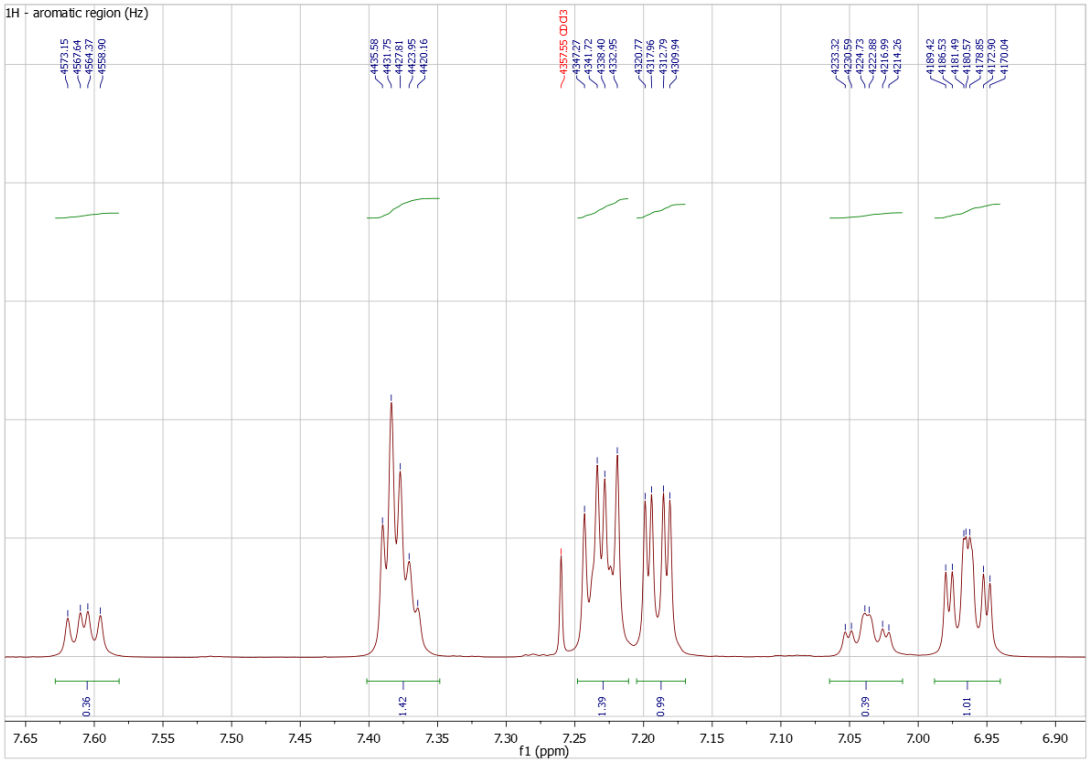
Spectra 84. ^{13}C NMR of **43**



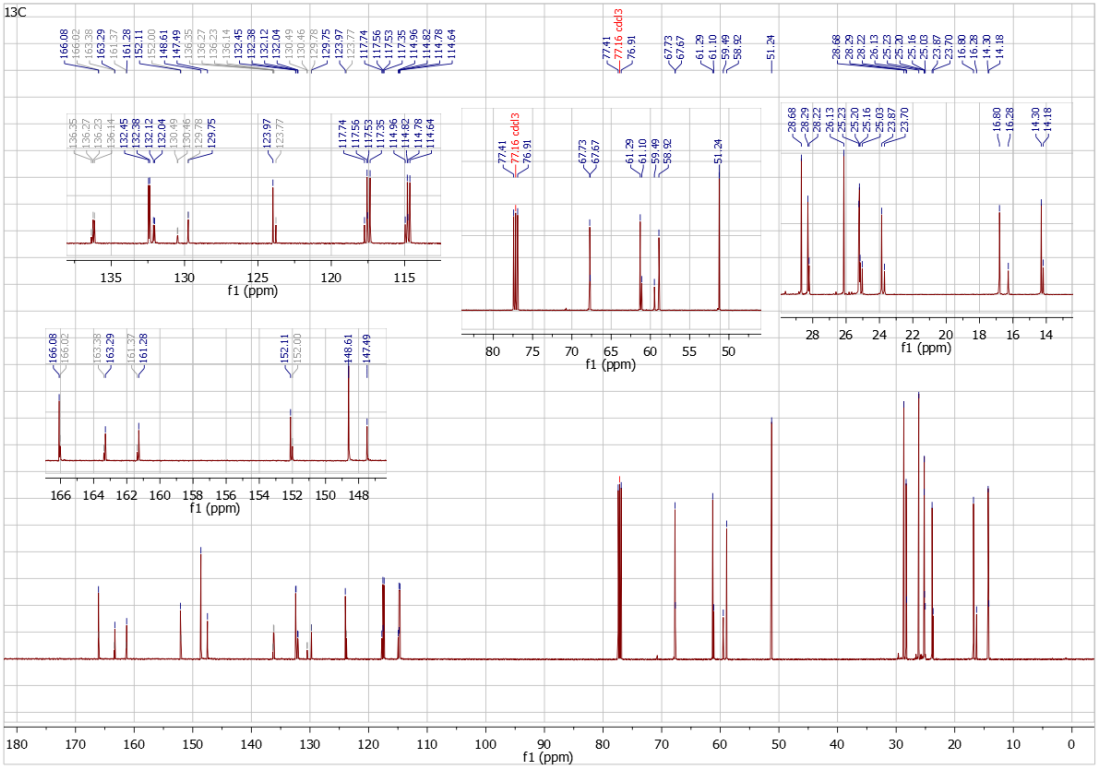
Spectra 85. ^{1}H NMR of **44** (DMSO-d_6)



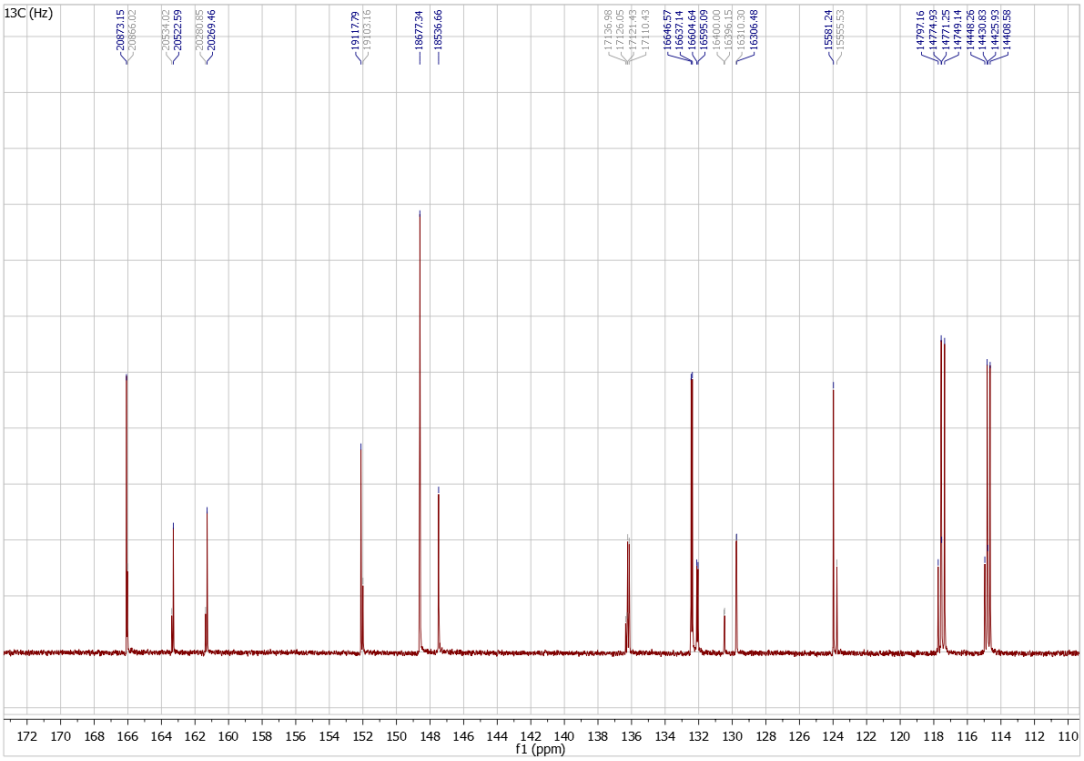
Spectra 86. ^{1}H NMR of **44** ($CDCl_3$)



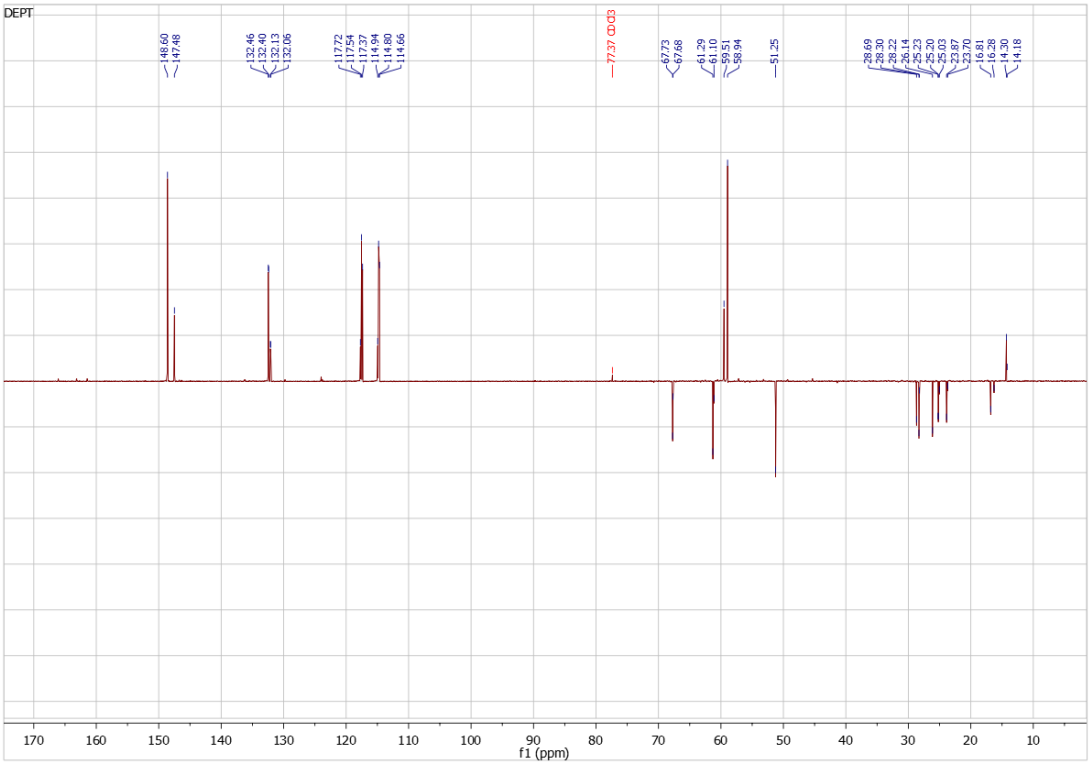
Spectra 87. ^{1}H NMR of **44** ($CDCl_3$, Hz)



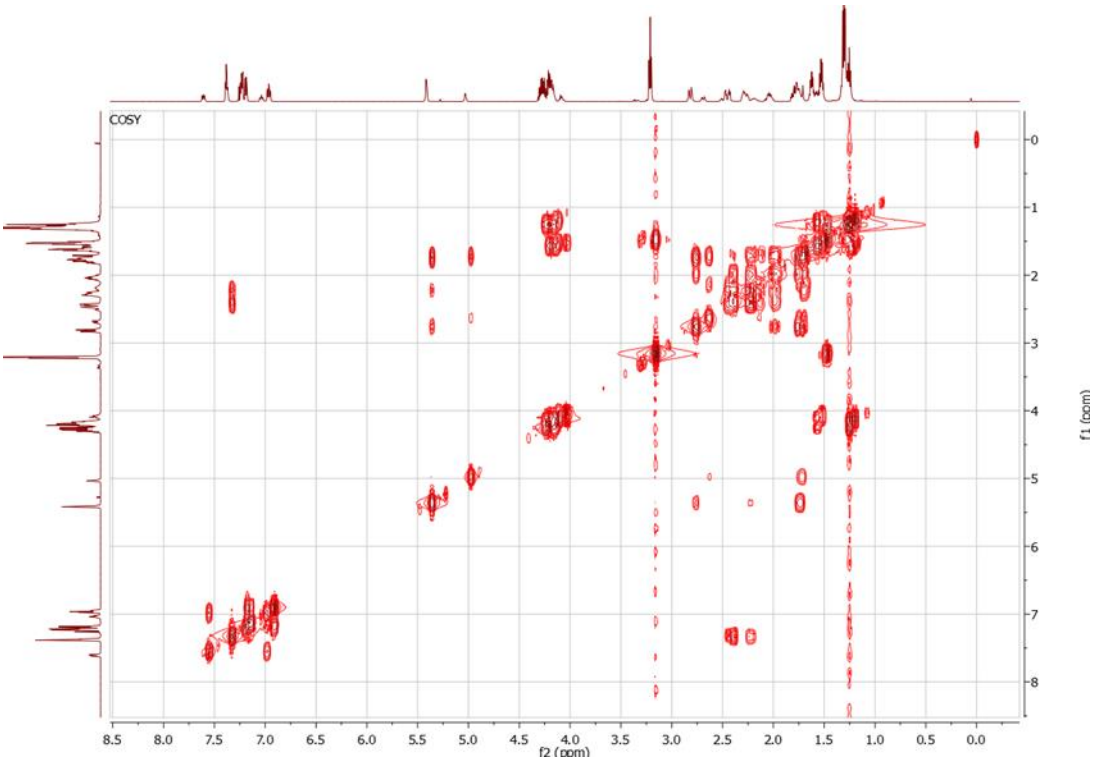
Spectra 88. ^{13}C NMR of **44** (full spectrum with zoomed-in inserts)



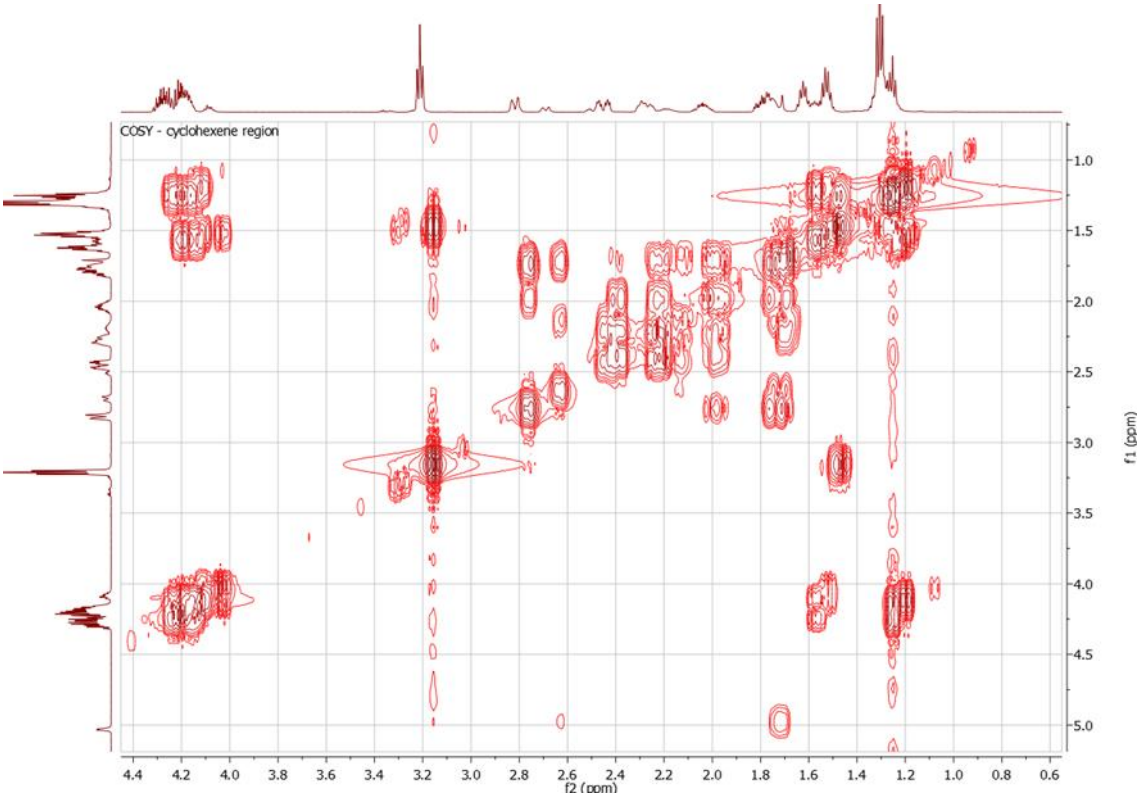
Spectra 89. ^{13}C NMR of **44** (Hz)



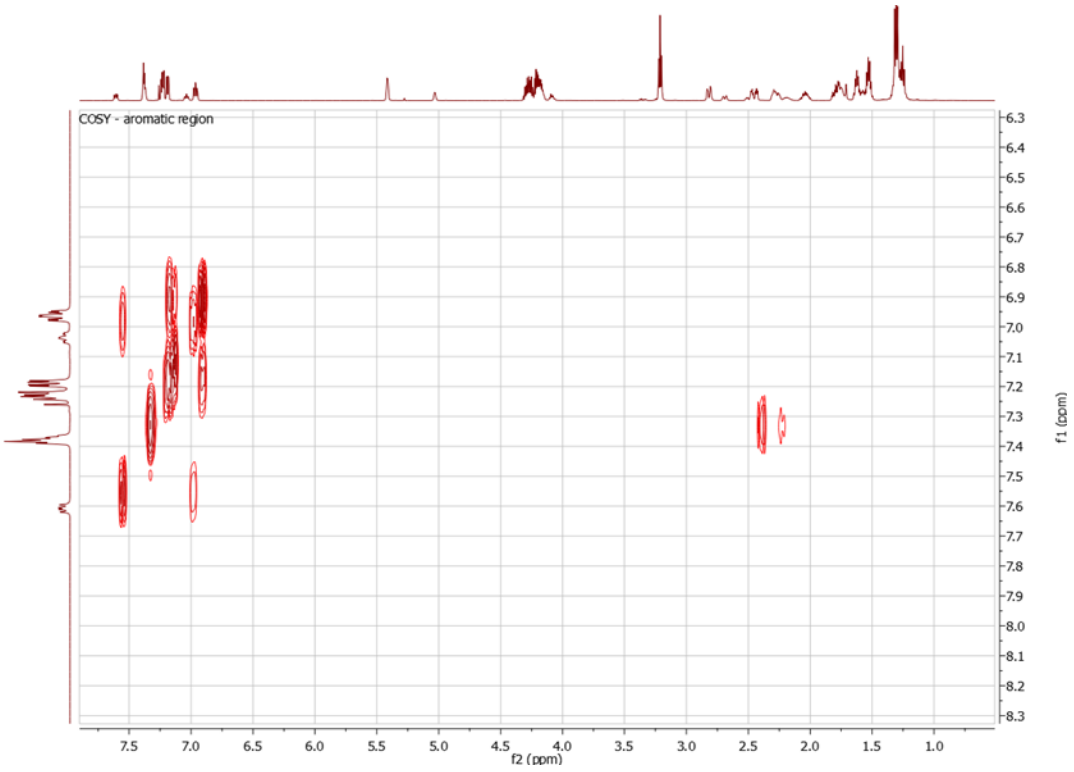
Spectra 90. DEPT-135 of **44**



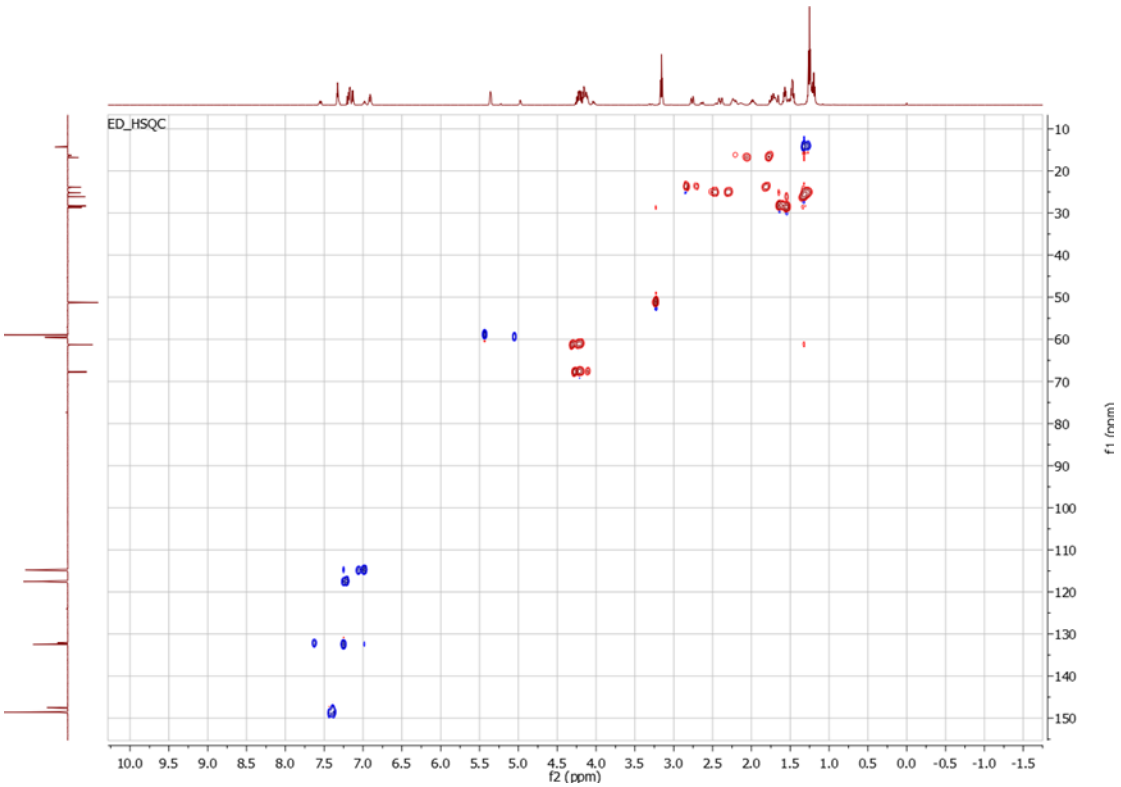
Spectra 91. COSY of **44**



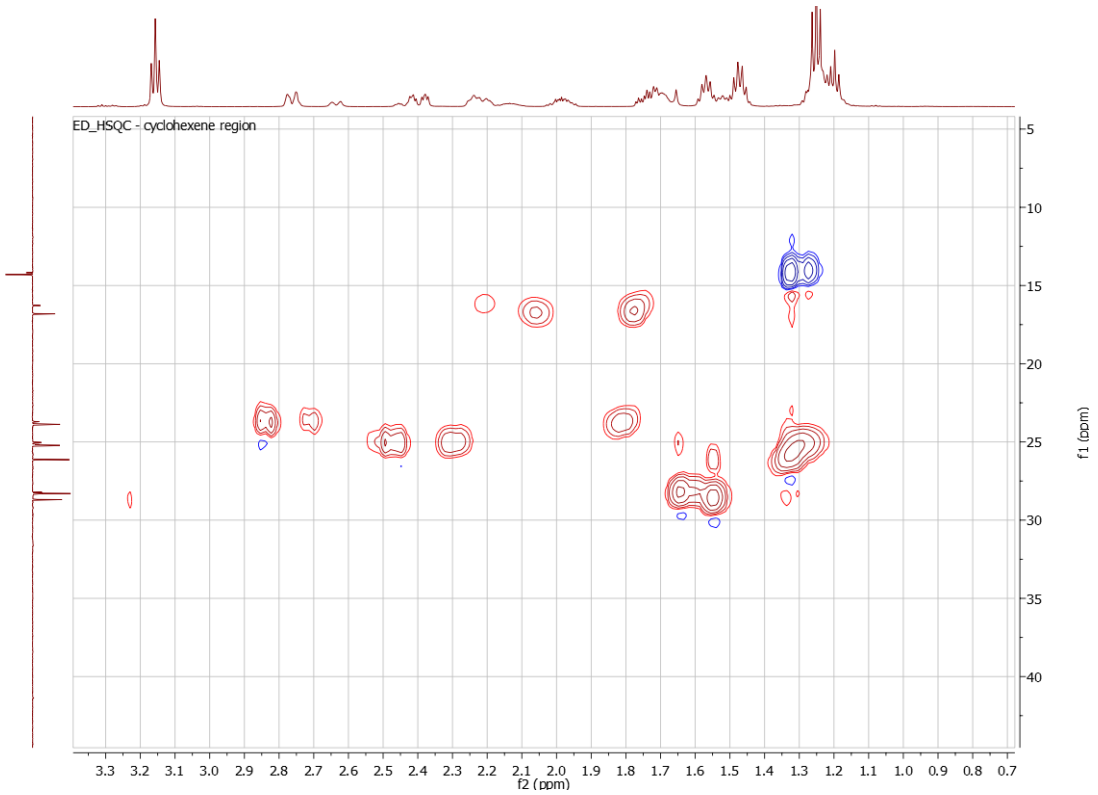
Spectra 92. COSY of **44** (cyclohexene region)



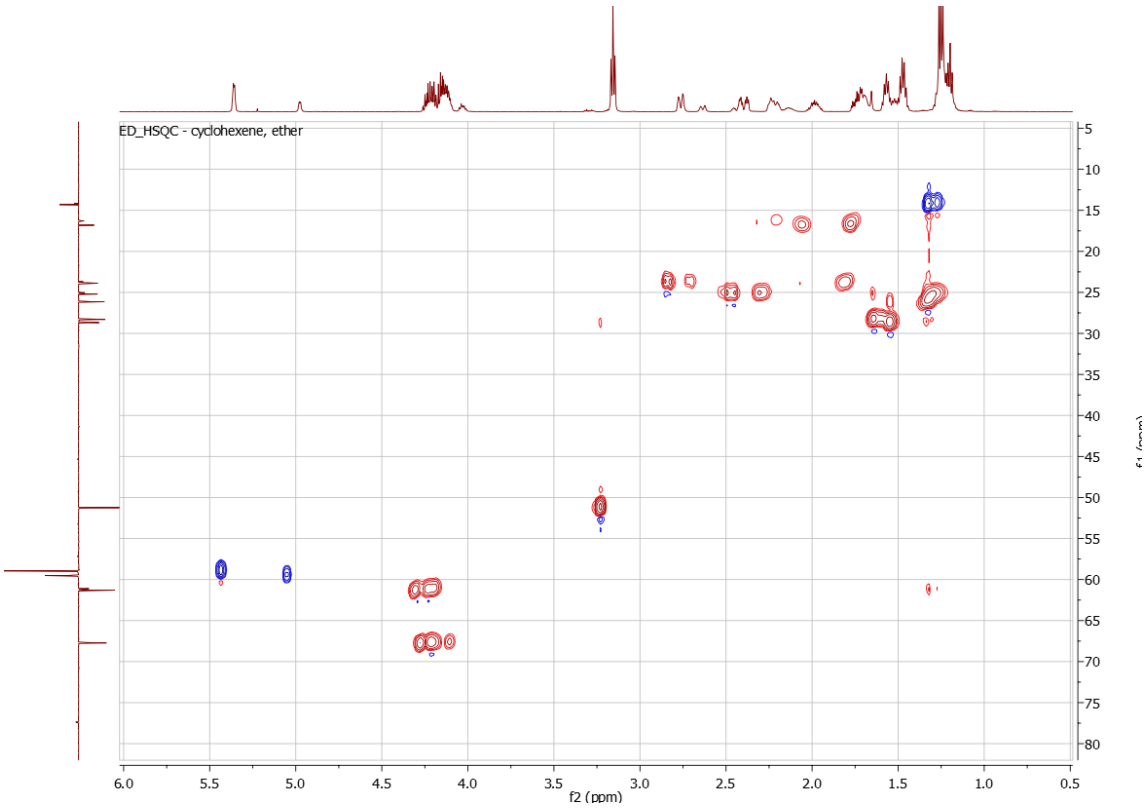
Spectra 93. COSY of **44** (aromatic region)



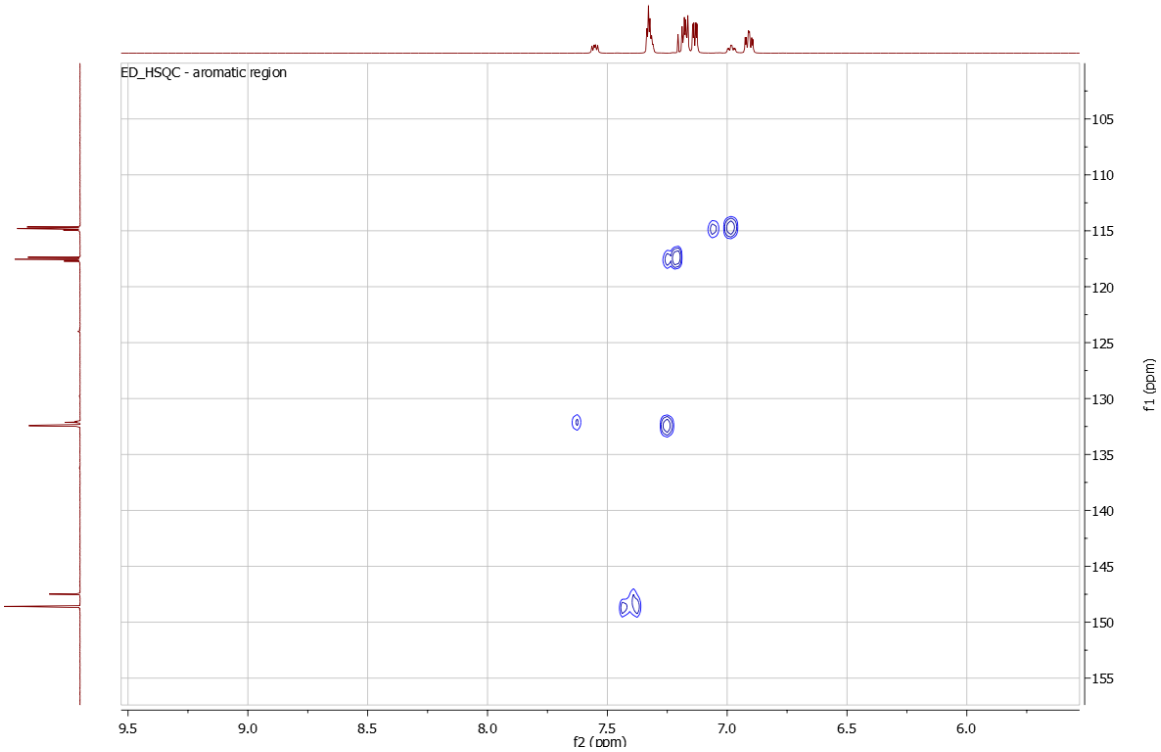
Spectra 94. HSQC of **44**



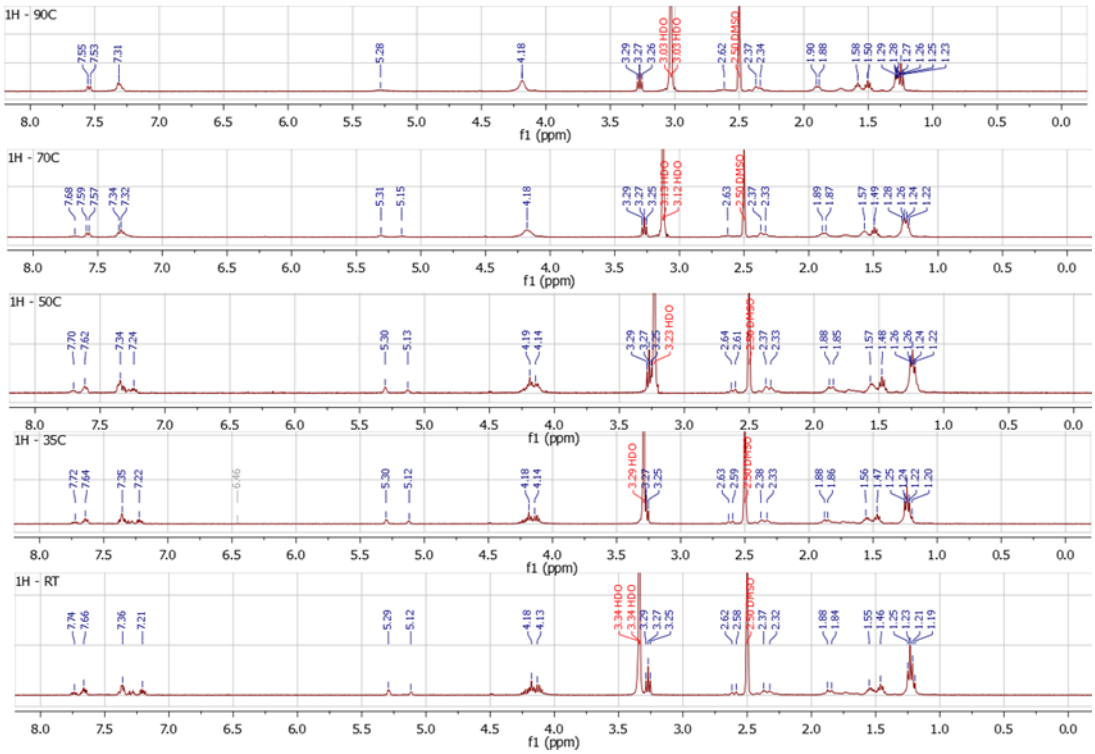
Spectra 95. HSQC of **44** (cyclohexene region)



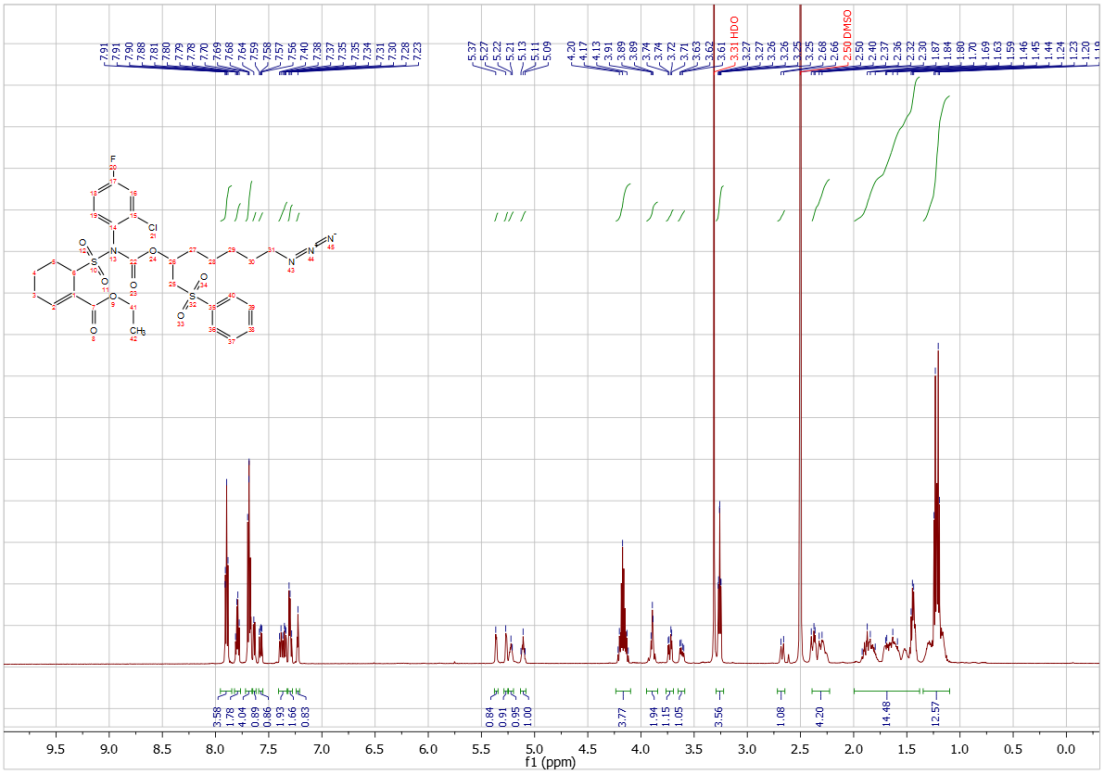
Spectra 96. HSQC of **44** (cyclohexene and ether-ester regions)



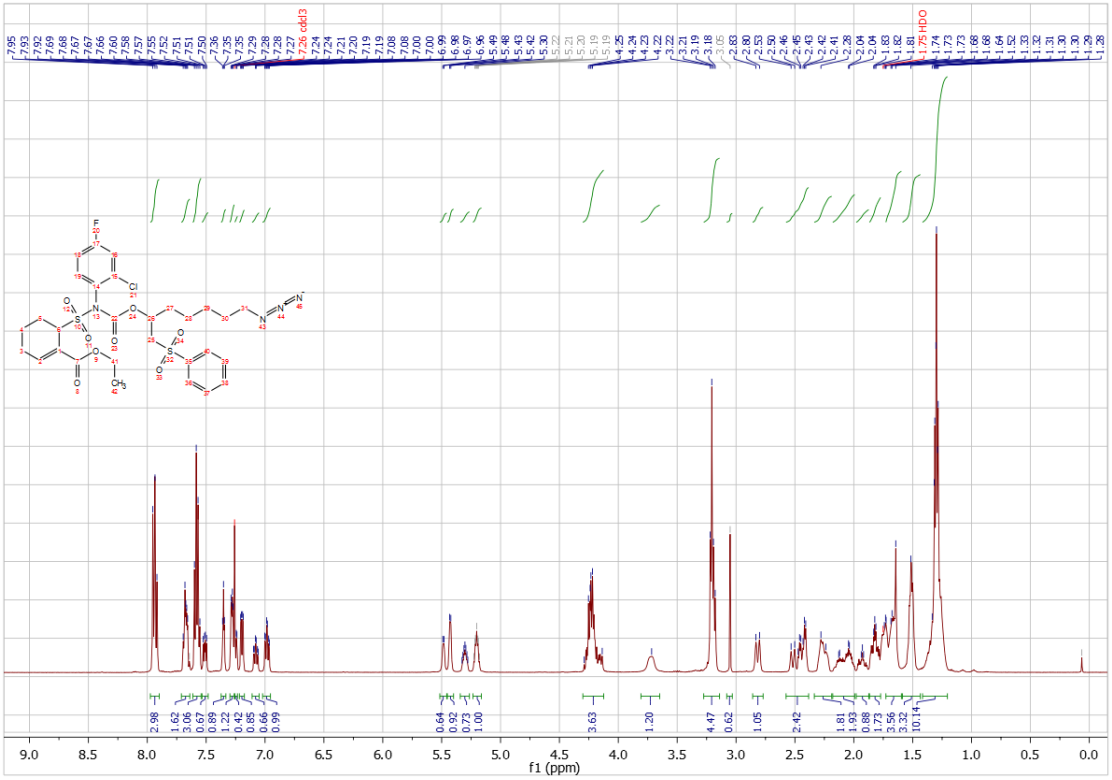
Spectra 97. HSQC of **44** (aromatic region)



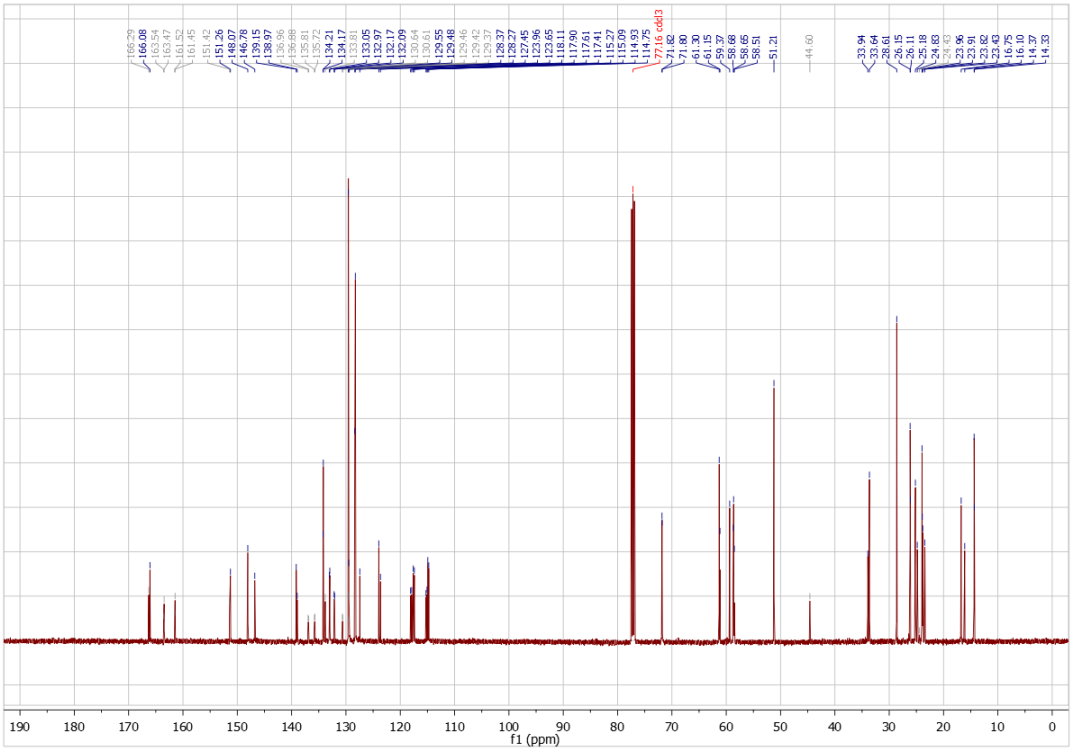
Spectra 98. Variable Temperature ^{1}H NMR of **44**



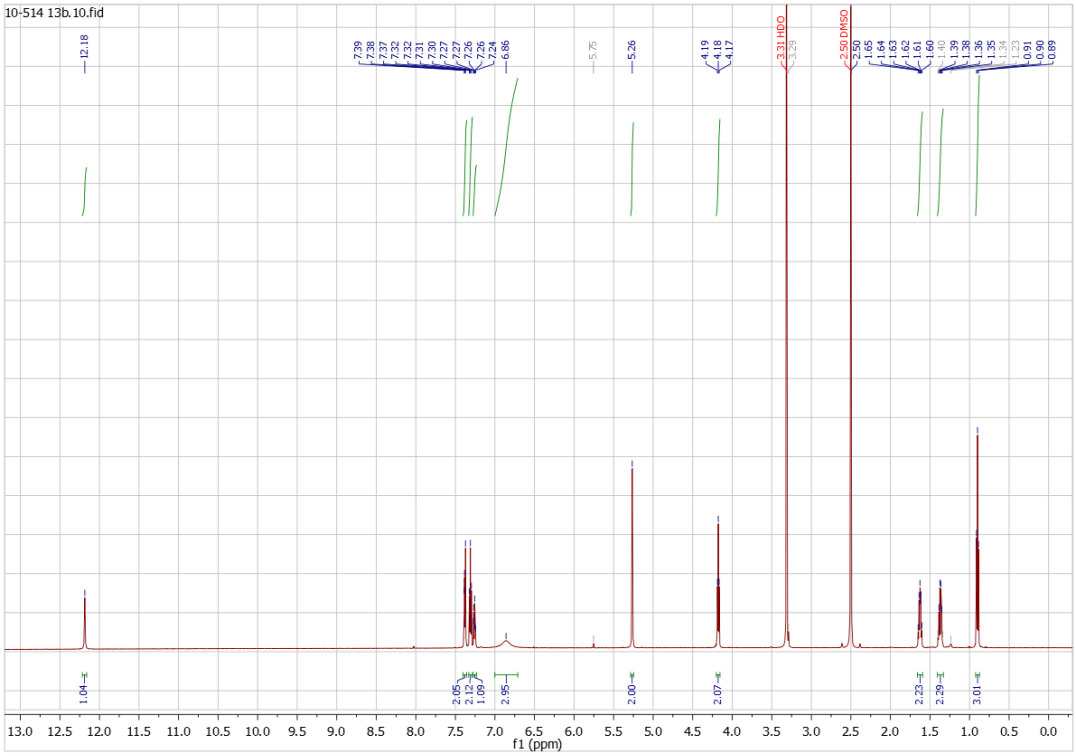
Spectra 99. ^{1}H NMR of **45** (DMSO-d_6)



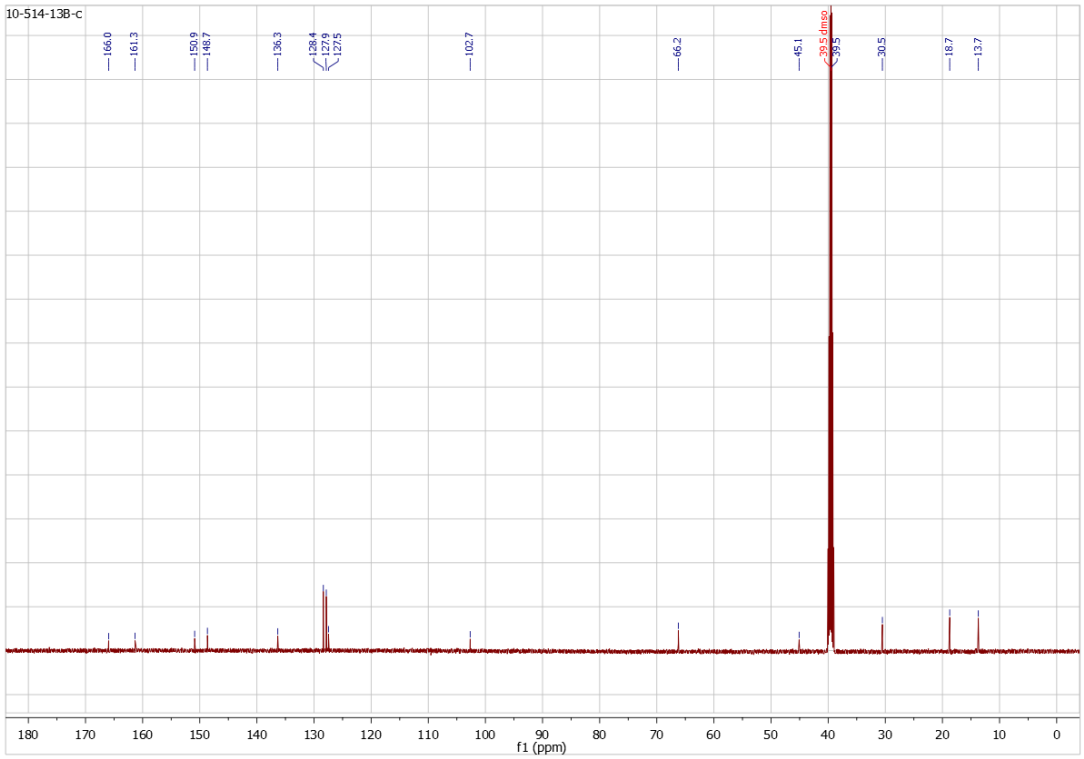
Spectra 100. ^{1}H NMR of **45** ($CDCl_3$)



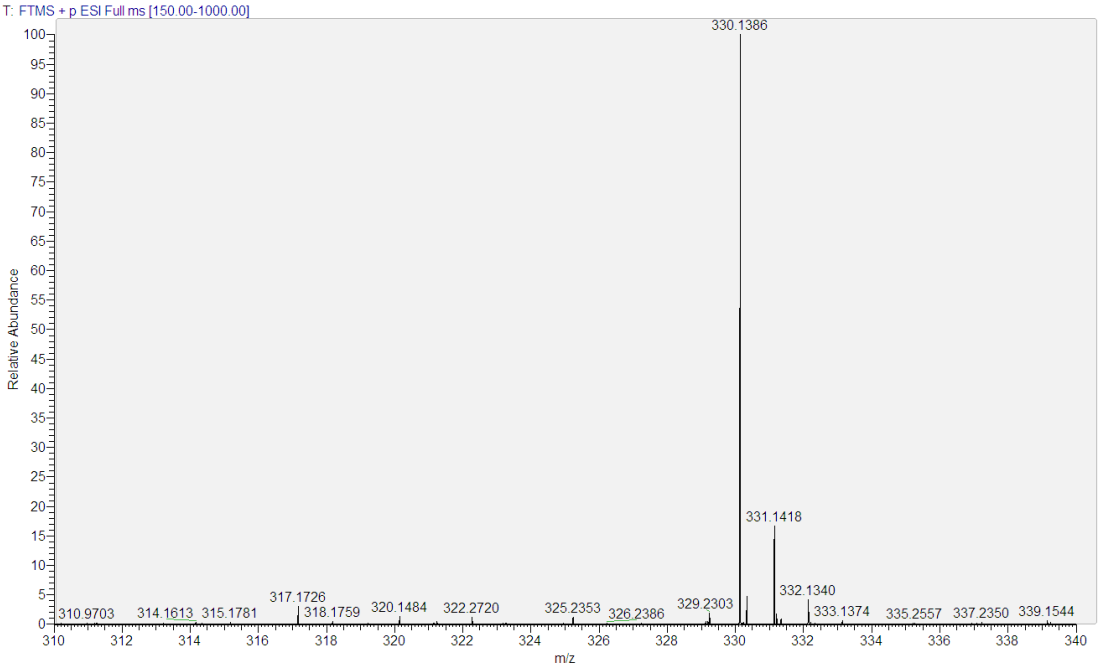
Spectra 101. ^{13}C NMR of **45**



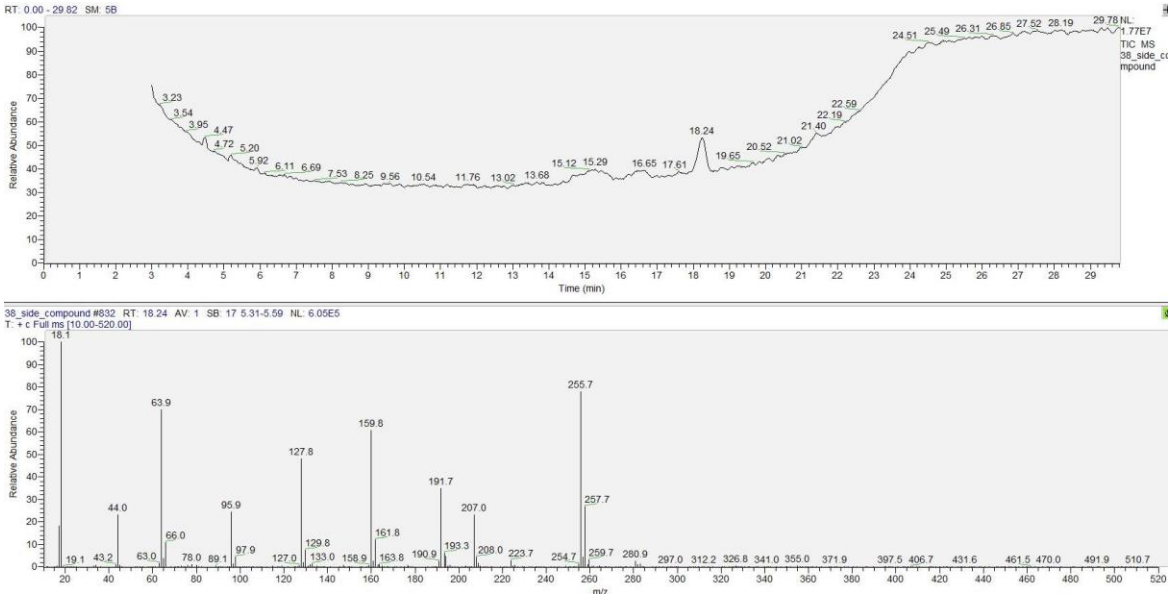
Spectra 102. ^{1}H NMR of **46**



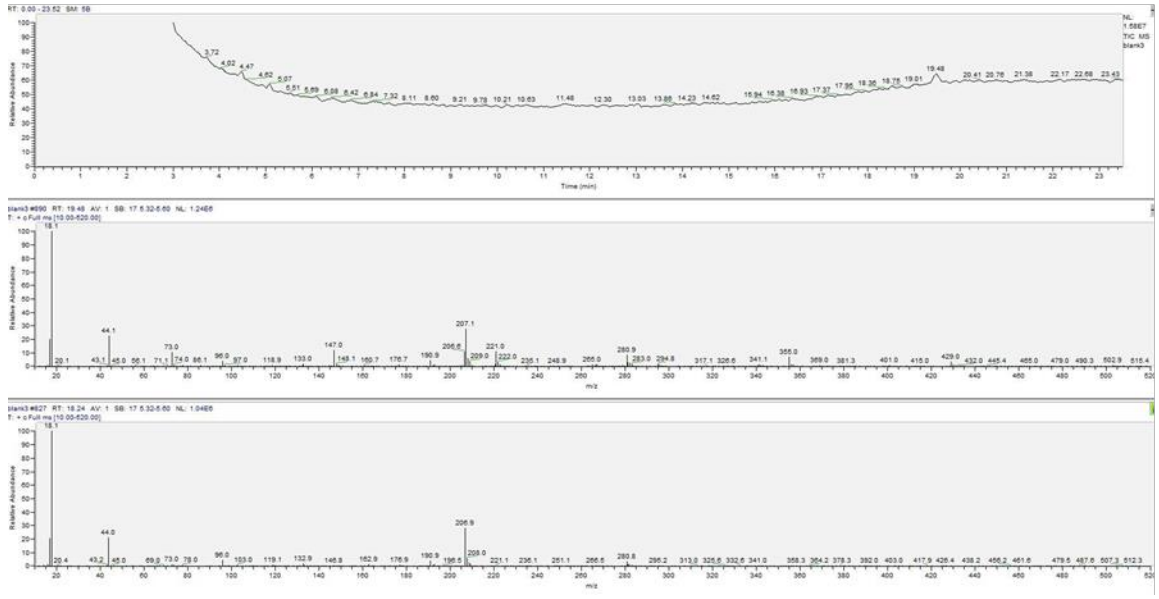
Spectra 103. ^{13}C NMR of **46**



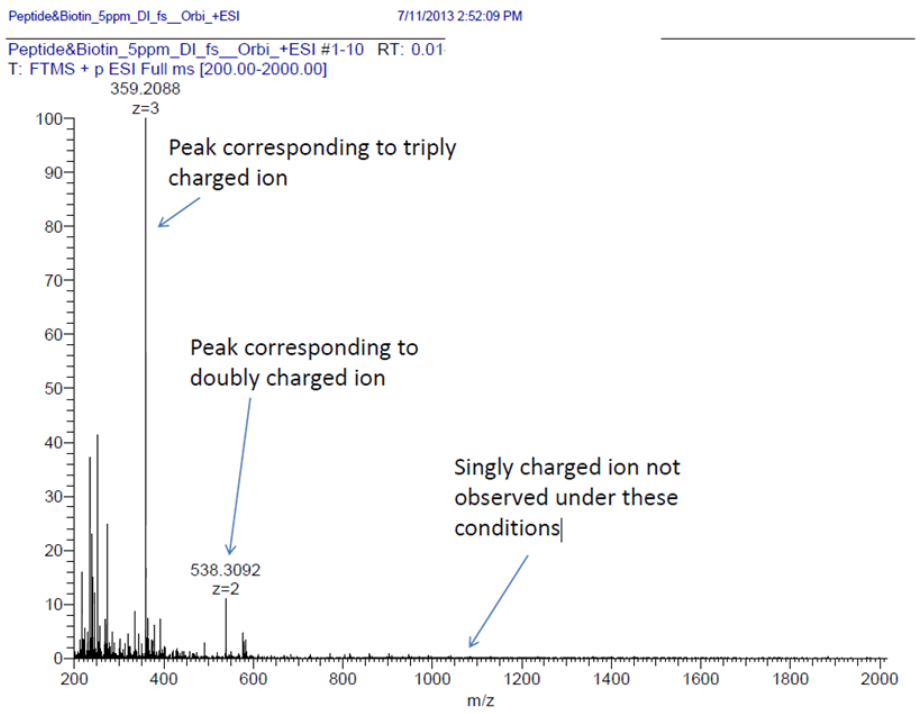
Spectra 104. HRMS (+ESI) of **46**



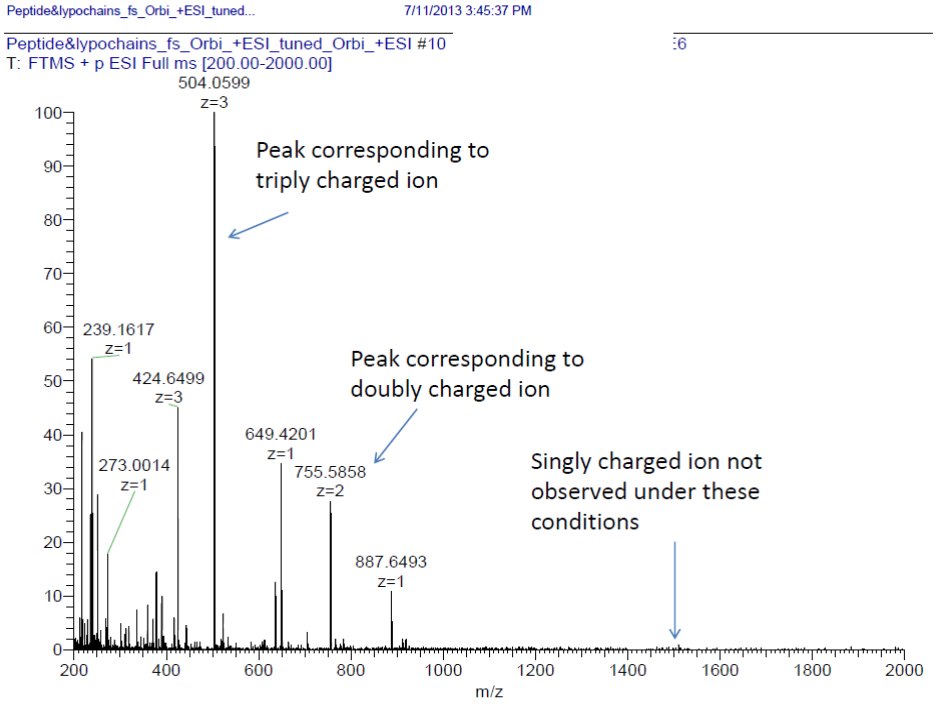
Spectra 105. GC-MS of yellow material present in crude **13**



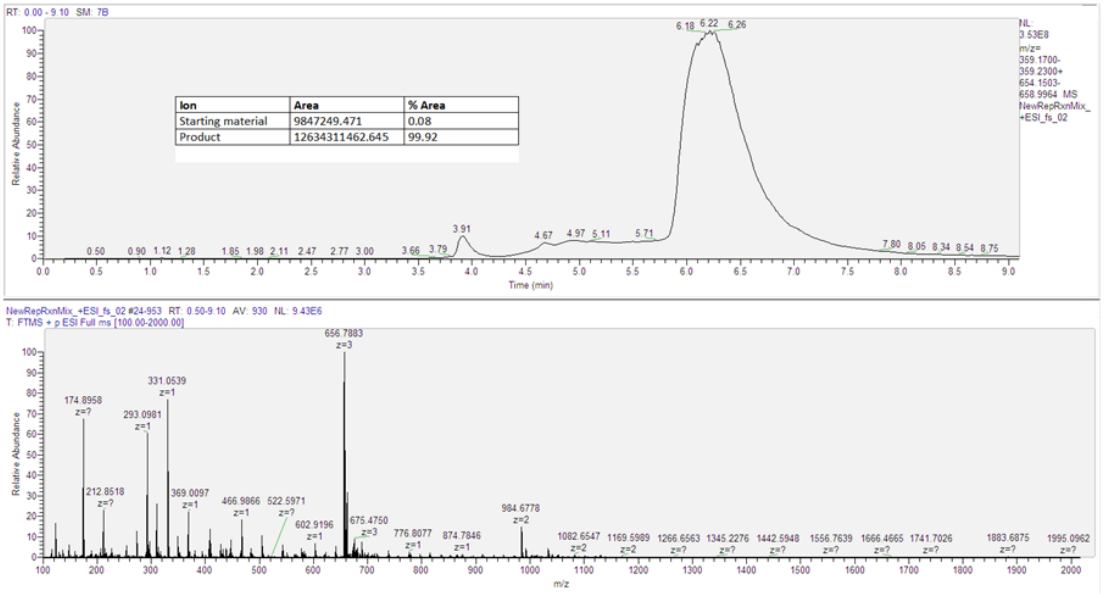
Spectra 106. GC-MS of yellow material present in crude **13** (blank spectrum)



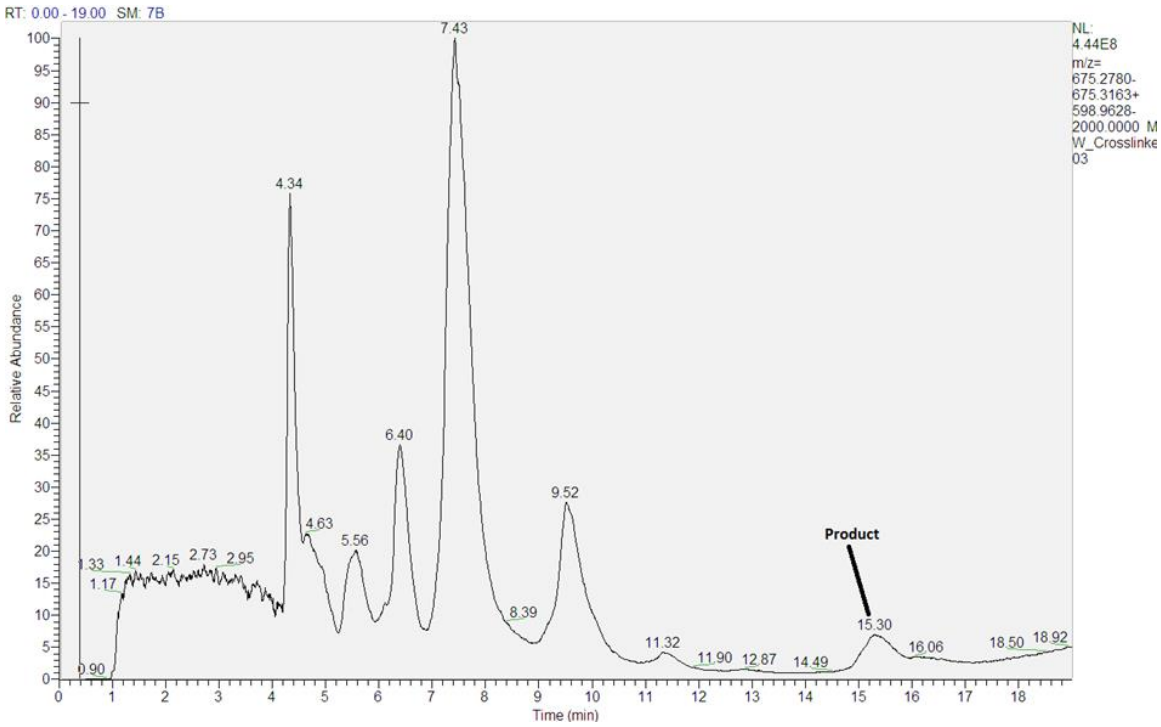
Spectra 107. HRMS of H_2NSK_4CK-biotin peptide (after cleavage and deprotection)



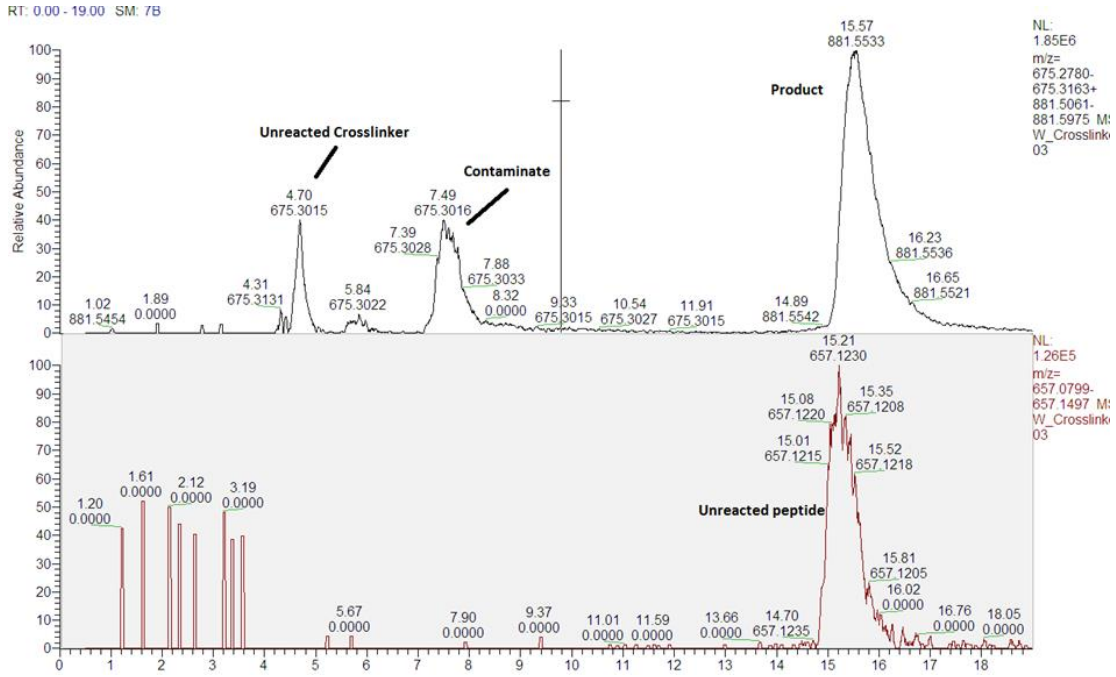
Spectra 108. HRMS of Pam3CSK4



Spectra 109. LC-MS for Pam3CSK4CK-biotin peptide (**22**)



Spectra 110. LC-MS for Pam3-biotin-DBCO **- 23** (total ion chromatogram)



Spectra 111. LC-MS for Pam3-biotin-DBCO - **23** (extracted chromatogram)

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