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Development of strategies for synthesis of branched Caenorhabditis elegans-associated oligosaccharides and of photo-activated lectin capture reagents

by

Randy A. Benedict

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Organic Chemistry

Program of Study Committee:
Nicola Pohl, Co-Major Professor
William Jenks, Co-Major Professor
Yan Zhao
Art Winter
Young-Jin Lee

Iowa State University
Ames, Iowa
2013

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LIST OF ABBREVIATIONS

Ac	Acetyl
AcOH	Acetic acid
ASW	Automated Synthesis Workstation
BSA	Bovine serum albumin
CAN	Ceric ammonium nitrate
CIP	Contact ion pair
ConA	Concanavalin A
COSY	Correlation spectroscopy
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DMAP	*N*,*N*-Dimethylaminopyridine
DMF	*N*,*N*,-Dimethylformamide
DMSO	Dimethylsulfoxide
EIC	Extracted Ion Chromatogram
ESI-MS	Electrospray ionization mass spectrometry
FimH	*E. coli* fimbria mannose recognizing cell adhesin
F-tag	*Cis*-3-(perfluorooctyl)propyloxy-2-butenyl
FSPE	Fluorous solid phase extraction
ΔH	Enthalpy change
HATU	2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate
HMQC	Heteronuclear multiple-quantum correlation spectroscopy
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single-quantum correlation spectroscopy
ITC	Isothermal titration calorimetry
K_d	Dissociate constant
Lev	Levulinate
MeCN	Acetonitrile
MeOH	Methanol
m/z	Mass/charge ratio
N	Binding stoichiometry
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
ΔS	Entropy change
SPE	Solid phase extraction
SSIP	Solvent-separated ion pair
TBAF	Tetrabutylammonium fluoride
TBS	*tert*-Butyldimethylsilyl
TBSCl	*tert*-Butyldimethylsilyl chloride
TBDPS	*tert*-Butyldiphenylsilyl
TBDPSCl	*tert*-Butyldiphenyl(chloro)silane
TEA	Triethylamine
THF	Tetrahydrofuran
TIC	Total Ion Current

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TLC	Thin layer chromatography
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TAPP	Trifunctional Affinity Proteomics Probe

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ABSTRACT

The development and investigation of a new affinity proteomics probe and methodology are presented in this dissertation. A trifunctional affinity proteomics probe (TAPP) was envisioned and prepared to serve as a lectin capture reagent. The TAPP consists of a solid support to serve as a handle for captured protein as well as scaffold for multivalency, a saccharide of interest to fish for an unknown or elicit a suspected specific interaction, and a photoactivated cross-linking moiety to covalently capture binding proteins. The perfluorinated phenylazide cross-linker was shown to exhibit better performance relative to the parent phenylazide with respect to sensitivity and selectively for specific and nonspecific interactions. The synthetic strategy included coupling of the saccharide signal as late as possible in the preparation to allow for ease of diversification of the saccharide signal. Such a strategy could be exploited with automated combinatorial chemical synthesis of oligosaccharides to quickly build a diverse series of probes for study. Synthesis of several galactose and galactosamine based monosaccharide donors are presented in this dissertation with the potential to be used to create an array of six oligosaccharides from the nematode *Caenorhabditis elegans*. Solution phase, automated synthesis was employed to test the building blocks, and to investigate current methods to accomplish the formation of a 3,4,6 glycosidic branching pattern on a singular monosaccharide unit. Results suggest such a glycosylation reaction is, indeed, possible.

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CHAPTER I

REVIEW OF GLYCAN FUNCTIONS AND LIMITATIONS OF THEIR STUDY

General Introduction

Very few roles of carbohydrates in biological systems are well understood or known (Varki 2009). General knowledge may consider them as being functionally insignificant structural feature of proteins and DNA, or as an energy source (Lee 2006, Lee 2009, Varki 2009). The roles of carbohydrates in biological systems are much broader and are in many cases, critical to the proper functions of these carbohydrate containing structures, or glycoconjugates (Figure 1.1) (Elbein 1991, Lee 2009, Öberg 2011). Glycans, the carbohydrate portion of a glycoconjugate, are found as constituents of all other well studied biological macromolecules: lipids, proteins, and nucleic acids (Varki 2009). While ubiquitous in biological systems, carbohydrate study lags significantly behind in understanding relevant or important structural and/or functional features compared to proteins and nucleic acids. Interest in glycomics is growing among the scientific community and considerable efforts have been made to elucidate the biological relevance of glycans (Lee 2006, Lee 2009) . Glycans have been implicated to contribute to molecular recognition, cell-cell recognition and adhesion, and cell signaling functions that are vital for cell proliferation as well as pathogen infection in hosts (Disney 2004, Gama 2006, Green 2007, Lee 2009). The study of this class of biomolecules remains a significant challenge for many reasons; methodologies for probing these interactions suffer many limitations and obtaining carbohydrates of interest is not a trivial task.

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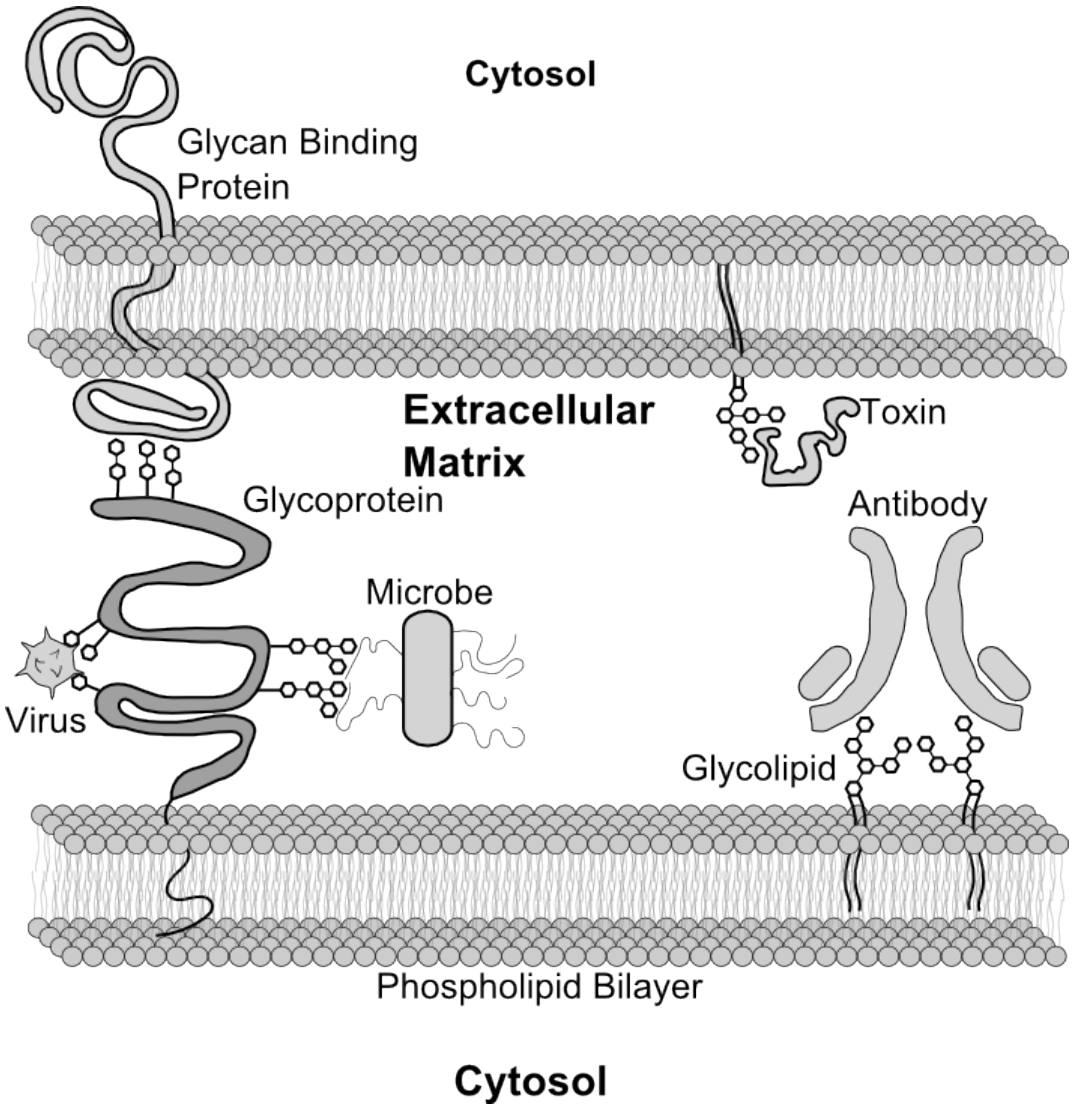

Figure 1.1 General overview of some glycan roles in living systems. Hexagons represent generic monosaccharide units, this figure does not represent any specific interaction. Figure adapted from www.biochem.emory.edu/glycomicscenter.

Current Methods and Limitations

Identification of carbohydrate roles in biological systems have shown that glycans frequently act as substrates for lectins or bacterial adhesins (Berthet 2012). Lectins are proteins

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that have a binding motif that binds selectively to specific carbohydrates and are considered to be the foremost interaction between cells with other cells directly or indirectly, such as interactions mediated by antibodies (Green 2007), . Similarly, bacterial adhesins are found on the surface of bacterial cells, and also are responsible for mediating cellular adhesion, which is regarded as the first essential step to pathogen infection (Rohde 2013). Identification of the binding pairs responsible for these interactions facilitates the development of therapeutics with applications ranging from treatment of autoimmune disorders (Green 2007) to attenuating pathogen infections (Pieters 2007). To this end, methods have been developed, however they are limited by several factors.

Many of the interactions of interest take place on the surface of a cell, and as a result, the protein or glycan involved in the interaction is usually tethered to the cell as part of a larger complex. Proteins are typically pili or fimbriae bound making isolation for identification problematic because these sorts of protein complexes do not resolve very well with methods like gel electrophoresis (Ilver 2003), the structural conformation may change in solution, and solubility issues can also occur (Smith 2011). Glycans involved in recognition events are typically glycolipids or glycoproteins. The sheer heterogeneity of glycolipids make them very difficult to analyze as mixtures, but methods have been used to do so. However, these methods are prone to miss identifications due to loss of multivalency or poor resolution and mis-identification of the signal (Ilver 2003). The glycans present on a glycoprotein are heterogenic, so after binding is shown, the binding glycan remains ambiguous. Subsequent fractionation of the glycoprotein to isolate the individual carbohydrates results in loss of multivalency and thus binding avidity (Ilver 2003).

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Multivalency has been described as the multiple interactions between two entities arising from an identical signal and receptor pair (Krishnamurthy 2006). Due to the cooperative effects

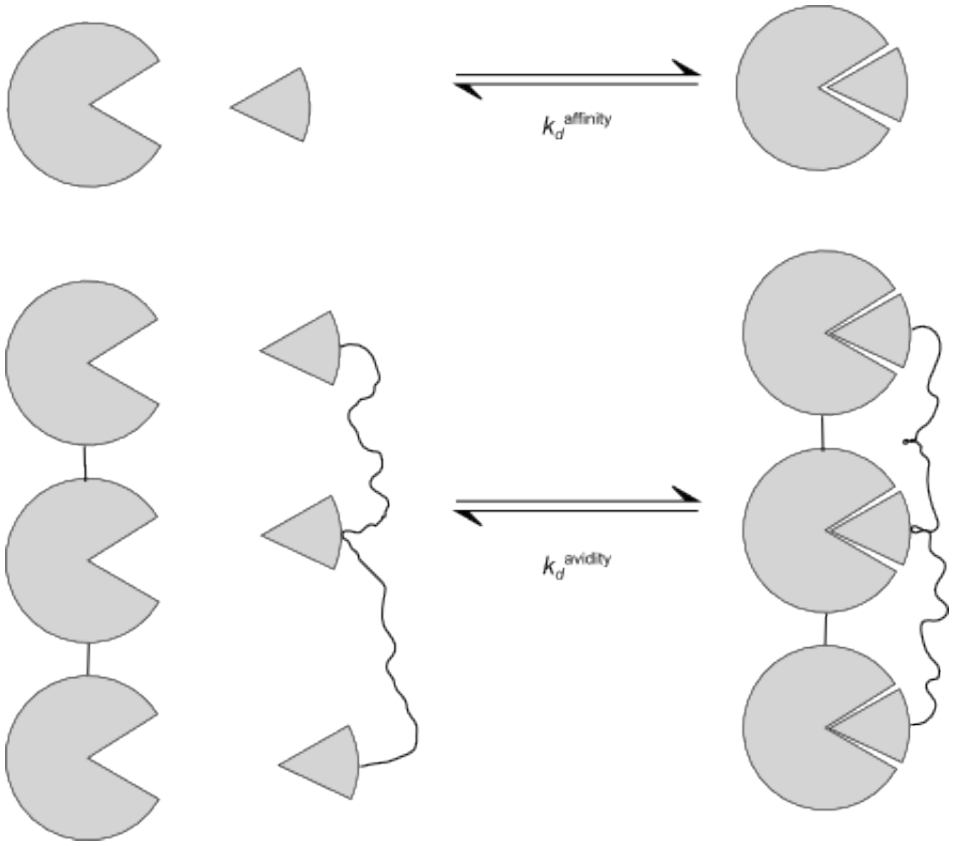

Figure 1.2 Comparison of monovalency to multivalency. Figure adapted from Krishnamurthy 2006.

of multiple binding events, the observed binding between two bodies is much greater than the individual affinity for each signal/receptor interaction. Literature uses the dissociation constant, k_d, as a measurement for host/guest binding (Figure 1.2)(Kitov 2003, Krishnamurthy 2006). A typical k_d for monovalent interactions, described as $k_d^{affinity}$, is on the order of mM to μM. The observed multivalent interaction, $k_d^{avidity}$, will typically be on the order of nM (Krishnamurthy 2006). Briefly, the enhancement of the binding affinity is thought to arise from the idea that each binding event will produce the nearly similar enthalpic stabilization while the entropic

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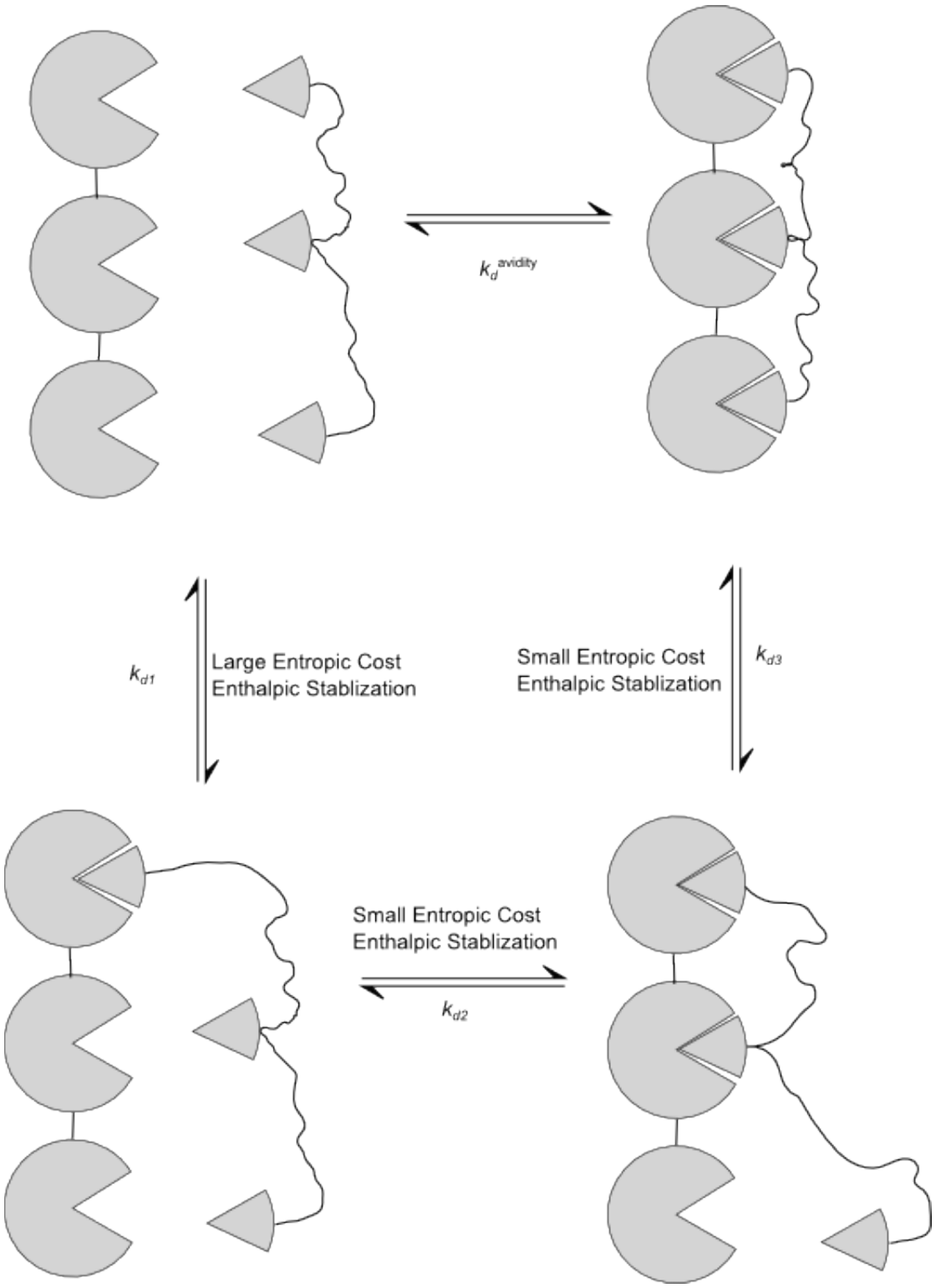

Figure 1.3 General explanation of enhanced binding in multivalent systems.

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penalty is greatly reduced after the initial binding event, and mildly reduced after each subsequent binding event for a multivalent host guest pair (Figure 1.3)(Krishnamurthy 2006). This principle explains why multivalent interactions drive Gibbs free energy of binding to be more and more negative (favorable).

Currently, very few methods currently exist that can identify a binding pair from its native environment (Ilver, 1998) but that method was extremely specific to those conditions, and multiple proteins were identified and further knockout experiments were necessary to identify the proteins (Ilver 2003). Identity of necessary binding proteins is typically confirmed through gene knockout experiments combined with some assay to study an interaction of interest (Dani 2012). Systematic knockouts of genes to identify essential binding proteins is not an effective method due to several reasons: knockouts can be deleterious to the organism, concomitantly giving no information about the gene's function; knockouts of similar or identical genes in one organism does not produce the same results in another; the background genotype of the organism may be responsible for the phenotype and not the induced mutation, causing misinterpretation of the results; and the gene may encode for a protein that is involved upstream of the phenotypically observed result giving more importance to that gene and ignoring the effects of others involved (Gerlai 1996). If there is some existing evidence that suggests a binding partner, more facile and robust methods are available . They usually require isolated carbohydrate (or a heterogeneous carbohydrate mixture) and binding protein with suspected specificity for the presented carbohydrate(s)(Chaubard 2012, Liu 2009, Martos-Maldanado 2013). Typically they require at least one of the binding pairs to be homogenous, if not both, and almost all methods cannot distinguish the active carbohydrate epitope or peptide responsible for binding from their

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respective mixtures. A high throughput method could help narrow potential genes for knockout studies if at least one binding partner was discernible from a heterogenic mixture.

It's common that following isolation and the subsequent experiments to elucidate structure, synthetic methods are often employed to verify the specific binding structure (Meloncelli 2011). These identification experiments take considerable time. Most of the rapid identification techniques used in proteomics or genomics make use of mass spectrometry, but due to the isobaric nature of carbohydrates, rapid identification techniques are not currently available. Thus is makes since that most of the known binding experiments frequently use purified carbohydrates with attempts to probe protein receptors.

Obtaining Carbohydrates

Isolation of glycoconjugates from natural sources is a very tedious process that often results in relatively small amounts of heterogenic mixtures of glycoconjugates. As a result, subsequent experiments will retain some level of ambiguity in important structure function relationships. There have been efforts in the analytical field to develop reliable methods to purify mixtures, but such separations are far from trivialized (Yu 2012). Well defined chemical structures can be obtained by synthetic methods but also suffers from significant challenges.

Carbohydrates are linked through glycoside bonds which incorporate the anomeric carbon of one monosaccharide with an alcohol of another monosaccharide. Many carbohydrates involved in glycoconjugates have, on average, five hydroxyl groups available for glycosidic bond formation, which give rise to five regioisomers possible for any given pair of monosaccharides. The stereochemistry at the anomeric carbon can also vary, further adding

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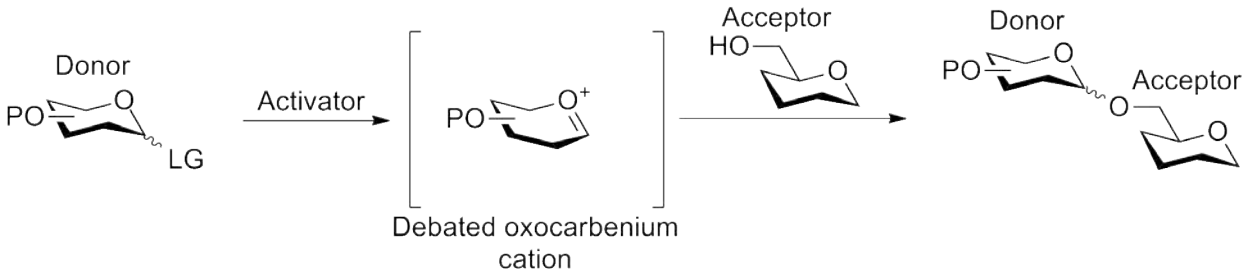
complexity to oligosaccharide structure. Typical synthetic routes to oligosaccharides originate from natural monosaccharides, provided they are available and economically feasible, which undergo a series of manipulations to protect hydroxyls to exclude them during the glycosylation of two monosaccharides. The synthetic routes can vary depending on monosaccharide and desired linkage, but it is not uncommon for 8 or more transformations to be necessary to obtain a suitable monosaccharide for glycosylation.



Scheme 1.1 Typical glycosylation between a donor and acceptor. The donor usually has some leaving group that can be activated, orthogonally to other protecting groups, that allows for the donation of the anomeric carbon to the hydroxyl nucleophile on the acceptor to form a glycosidic linkage.

Glycosylation reactions are also poorly understood; the mechanism of which is actively being explored and debated (Scheme 1.1) (Crich 2010). The typical glycosylation involves a pair of monosaccharides, the donor, which donates its anomeric carbon, and the acceptor, which bears an alcohol that accepts the anomeric carbon. The donor usually bears a leaving group that can be selectively activated to from a reactive species that will react with a nucleophile to form a covalent bond between the nucleophile and the anomeric center of the donor. The acceptor will bear a free hydroxyl group for formation of the glycosidic bond. Diastereomeric selectivity in a

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glycosylation reaction is empirically understood and seemingly is dependent upon substrate, protecting groups, leaving group, activation conditions, solvent, temperature, and concentration. Considerable efforts may be necessary to achieve a desired linkage after preparation of the donor or acceptor.

Oligosaccharide synthesis is, typically, iterative cycles of glycosylation followed by deprotection of a specific hydroxyl on the resulting glycoside that was protected with an orthogonal protecting group during the building block preparation. There are reported methods that try to tailor the reactivity of building blocks, through choice of protecting groups, such that they can do multiple glycosylation reactions in one pot without need for deprotection (Koeller 2000, Ye 2000). Despite the generation of a database through empirical testing of monosaccharide donors and acceptors (Zhang 1999, Sears 2001), this method suffers from not being able to have enough differentiation in reactivity between many of the acceptors to avoid mixtures of products.

Automated Synthesis of Oligosaccharides

Synthetic challenges due to the complexity of carbohydrate structure have hindered the development of automated synthetic methods that have already been successfully and efficiently employed for the corresponding biological macromolecules: DNA, RNA, and proteins. There are known examples of solid phase synthesis of oligosaccharides (Ranter 2002), despite the time between its initial report, there are very few reports of exploitation of the methodology to access novel and interesting carbohydrates. Typical conditions employing solid phase handles require extreme excess of solution reagents to effect complete coupling that overcome the biphasic

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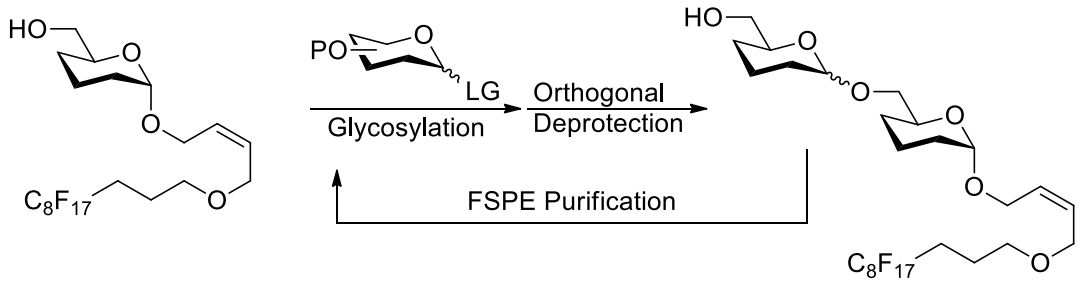
nature of the reaction. The preparation of reactive building is extremely limiting in oligosaccharide synthesis a contributing factor in the lack of development in solid phase approaches for oligosaccharide access. The optimization of reaction conditions to achieve specific linkages in solution does not necessarily translate to solid phase reaction conditions. As previously mentioned, the glycosylation reaction is dependent upon activation conditions, concentration, and inherent substrate reactivity. All of these conditions are hard to control for substrates on solid supports.



Scheme 1.2 General schematic for an iterative cycle of fluorous tag assisted automation solution phase synthesis of oligosaccharides.

Solution phase automated synthesis has been recently explored as an alternative to circumvent the troubles associated with solid phase automated synthesis (Scheme 1.2)(Pohl 2008). The method makes use of a perfluorinated alkane as a handle for isolation of synthetic intermediates after each couple cycle in the preparation of the oligosaccharide. This method allows for solution phase reaction conditions to translate well to automation as well limiting the need of excess donor due to the homogenous reaction conditions. Automation of carbohydrates would propel the field of glycomics forward into a perspective of better understanding of the roles of oligosaccharides in biological systems and the molecular basis for those interactions.

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Access to this class of biological molecules through automated methods would allow for systematic investigation of structure-function relationships and the interactions of these molecules in biological systems. It would also provide a means to construction of carbohydrate libraries to probe detailed structure-function relationships. Insights into the basis of these molecular functions could aid the development of therapeutics to treat a host of problems that are related to oligosaccharide behavior.

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CHAPTER II

TRIFUNCTIONAL AFFINITY PROTEOMICS PROBES AS LECTRIN CAPTURE REAGENTS

Introduction

The roles of many carbohydrates and glycoconjugates in biological systems remain ambiguous despite the rapidly developing field of glycomics (Ilver 2003). Glycoconjugates found on the surface of cells as abundant as they are diverse, owing that diversity to the innate chemistry of oligosaccharides, and exhibit extraordinary binding specificities (Larsson 2000). One immensely important function of glycoconjugates is to serve as a signal for many vital cellular functions, such as cell-cell recognition. The first essential step to pathogen infection is host cell recognition(Ilver 2003, Larsson 2000) which is mediated through the exploitation of intimate signal-receptor pairs (Nizet 2009). Identification of specific cell surface protein-saccharide pairs involved in such events can provide insight into the mechanism of infection and aid the development of therapeutic strategies (Ilver 2003). Herein we report the synthesis of trifunctional affinity proteomics probes (TAPPs) for the identification of specific binding proteins from protein solutions.

Efforts have been made towards developing methods to probe protein-saccharide specific interactions, but these methods still suffer from limitations that often make them unreliable or impractical (Ilver, 2003). For example, a thin layer chromatography (TLC) overlay assay has been employed to identify glycolipid binding proteins in bacteria such as *Shigella dysenteriae*

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(Lindberg 1987, Soltyk 2002).This assay employs thin layer chromatography to separate a heterogeneous mixture of carbohydrates, typically glycolipids, which are then stained with radiolabeled bacteria. This method, however, no longer presents the carbohydrates to binding proteins in the multivalent manner typical of cell surfaces (Ilver 2003). The dissociation constant (K_d) for monovalent specific interactions is usually in the range of mM whereas the K_d for multivalent specific interactions is in the nM range (Houk, 2003). This million-fold difference in binding is the fundamental reason methods such as TLC overlay assay are inadequate for detection of interactions dependent on multivalency, which is exemplified in the cases where the binding partner escaped detection or was misidentified (Ilver 2003). A similar assay using gel-electrophoresis to separate glycoproteins has been explored but faces many more shortcomings. Glycoproteins, unlike glycolipids, have a diverse set of glycosides per molecule. Thus, the exact identification of the carbohydrate is impossible without further fractionations and assays (Ilver 2003). Not only does this method suffer from being a lengthy procedure, once fractionated, loss of multivalency occurs, allowing binding partners to evade detection. An affinity tag approach has been used to capture lectins (Ilver 1998) and improved upon with a bottom-up affinity proteomics approach (Larsson 2000) to identify binding proteins. This method use a bovine serum albumin (BSA) core that has been covalently modified with oligosaccharides and a photoactivated cross linking reagent, therefore circumventing the multivalency issue, but still has its limitations. The protocols for preparing and employing the conjugated BSA core are not general enough to be efficient for the continual increase of new pathogen targets (Ilver 2003). Lectins are often found in low abundance and after the tagging require streptavidin enrichment prior to separation using gel electrophoresis. Adhesins are often membrane or pilus bound and do not typically resolve well with gel-electrophoresis (Ilver 2003). More recent efforts to solve

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these problems have been reported, but have yet to show diverse utility. One method involves using modified biological pathways to incorporate derivatives of carbohydrates to capture binding proteins *in vivo* (Yu 2012). Another employs photosensitizing nanoparticles conjugated with a purified saccharide and protein of interest (Chang 2011). These methods present significant constraints to throughput in detecting bacterial adhesins or lectins. The former method involves modifying molecular pathways to tolerate non-canonical saccharides and presumes the saccharide modification will not inhibit receptor recognition. The latter method requires purified saccharides and protein receptors, and would not be directly applicable to capturing unknown membrane-bound binding partners. A more versatile carrier capable of being applied to pilus and membrane bound adhesins coupled with the bottom-up proteomics detection would be a very powerful lectin capture reagent.

Results and Discussion

To achieve the goal of identifying carbohydrate-binding proteins with greater fidelity, ideally a trifunctional affinity proteomics probe would need to be employed (Figure 2.1). To meet the requirement of a bottom up proteomics approach a handle is required to purify captured proteins. A solid phase resin confers the necessary properties to support such a general and flexible lectin capture reagent.

The development of rinsing protocols that inhibit nonspecific interactions while leaving specific interactions intact can be tedious and irreproducible, thus if a covalent bond can be formed between the solid support and the binding protein, very stringent, denaturing, and reproducible washing conditions are needed to remove matrix proteins without concern of losing

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binding proteins. This can be achieved by using one of the many known protein cross-linking reagents in literature (Sinz 2006). Finally, the carbohydrate signal that is being used to probe for receptors is the third functionality.

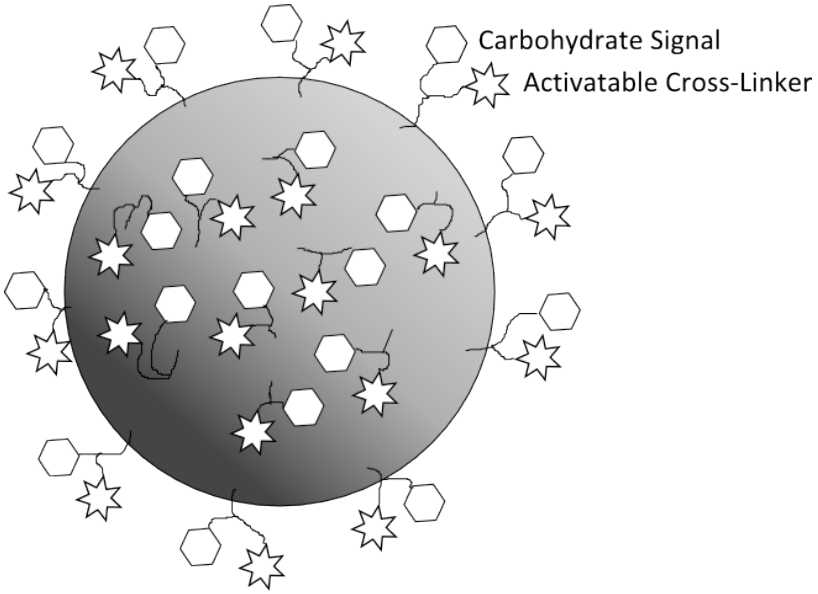

Figure 2.1 Conceptual design of a trifunctional affinity proteomics probe (TAPP) to capture lectins and adhesins

With this model in mind, two probes were devised, one bearing α-D-mannose (**1**) and the other β-D-galactose (**2**), intended to be used as positive and negative controls, respectively, in a simple model system to test the basic principle (Figure 2.2a). For characterization puprposes, the resin coupling will be the final step, which would make benzoic acids **3** and **4** the penultimate targets for **1** and **2**, respectively.

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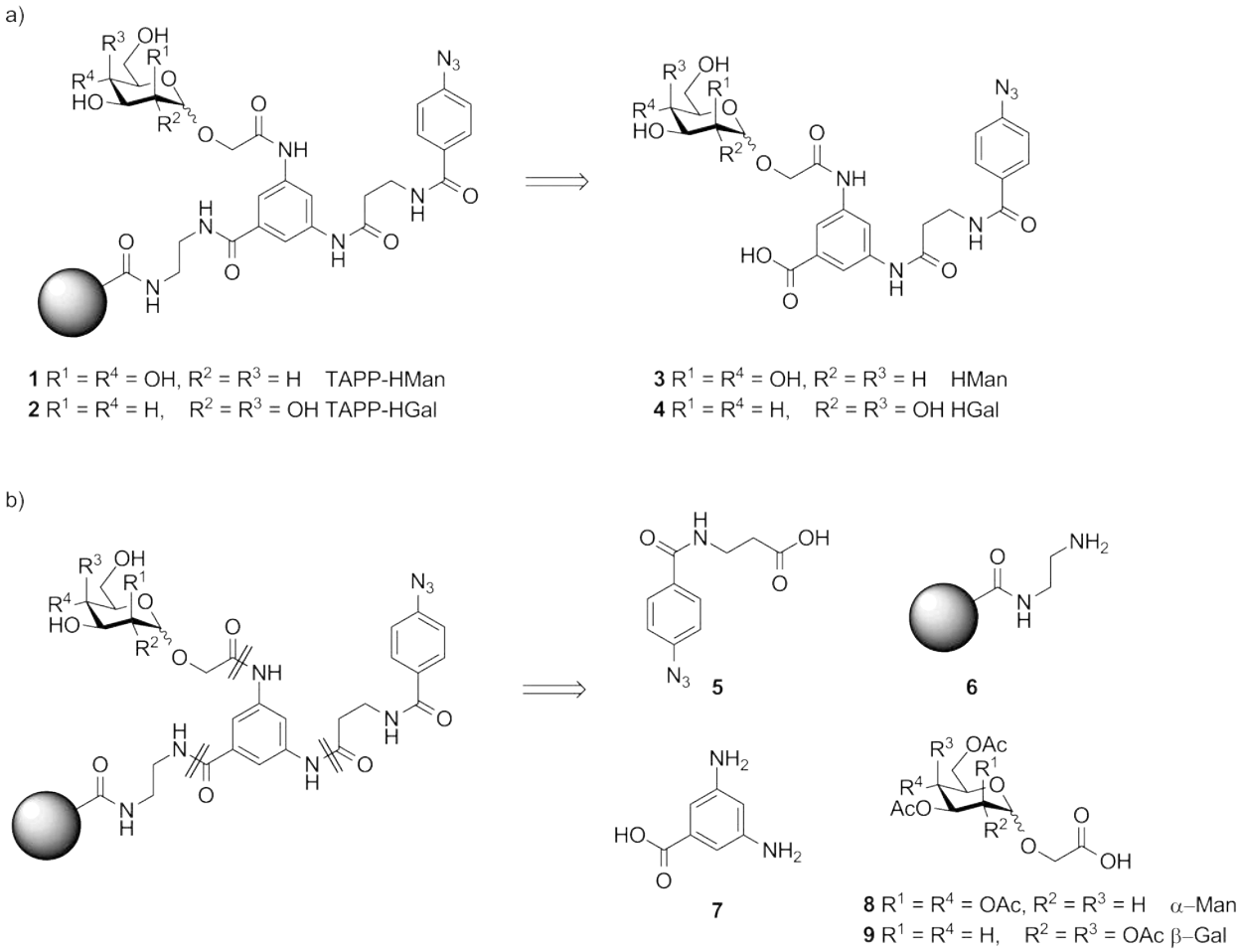

Figure 2.2 (a) Molecular design of TAPPs and (b) retrosynthetic analysis.

The two excitation maxima, of which the higher energy maxima is hypothesized to excite the probe to an intermediate that does not form cross-links adducts (Buchmueller 2003). To simplify characterization data of the probe, the central tethering moiety was chosen to be 3,5-diaminobenzoic acid (**7**), which allows for three unique branches to be coupled without creating regio- or stereoisomers, as well as being economically effective and providing convenient handles for peptide coupling chemistry. The amide linkages were exploited for coupling the three functional moieties together as the amide is a robust functional group and the final probe would then mimic proteins and be suitable for the aqueous experimental conditions.

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Retrosynthetic analysis of TAPPs leads to a convergent synthesis (Figure 2.2b) in which the saccharide coupling conditions can be effected as late in the synthetic scheme as possible to make it as general as possible as well as be amenable to combinatorial chemical methods.

Synthesis of the aryl azide branch started with the well known *para*-azidobenzoic acid **10** (Pinney 1991). The acid was converted to the activated acyl chloride with phosphorus pentachloride in anhydrous diethyl ether, the product of which was immediately subjected to a solution of β-alanine in 1N $NaOH_{(aq)}$ which afforded **5** in good yield. The carboxy methyl glycosides **8** (Cheaib 2008) and **9** (Listkowski 2007) are known compounds and were prepared as reported. Preparation of the amine terminated polystyrene resin was achieved with standard peptide coupling reagents, namely diispropylcarbodiimide (DIC) and ethylene diamine in *N*,*N*-dimethylformamide. To ensure good loading, the resin was again subjected to the reaction conditions. Verification of functional group transformation was done with Kaiser's test (Kaiser 1970) which resulted in a deep blue resin indicative of a successful coupling of the diamine to give the amine terminal resin (**6**), considered to be the result of the formation of a Schiff base between the reagents and the amine functionality on the surface of the resin. aforementioned saccharides were used in order to take advantage of well known interactions of α-D-mannose with plant. lectin concanavalin A from *C. ensiformis* and the bacterial adhesin, *FimH*, expressed by *E. coli* (Summer 1938). The cross-linking reagent used for this set of experiments belongs to a well studied class of photoactivated cross-linkers: aryl azides (Buchmueller 2003). The *para*-substituted phenylazide (**5**) was employed for its low cost and ease of synthetic preparation. A commercially available carboxy-modified polystyrene (**6**) solid support was utilized as it offers general coupling conditions, good loading capacity, and, critically, low wavelength protection of the photo cross-linker. Aryl azides are known to exhibit

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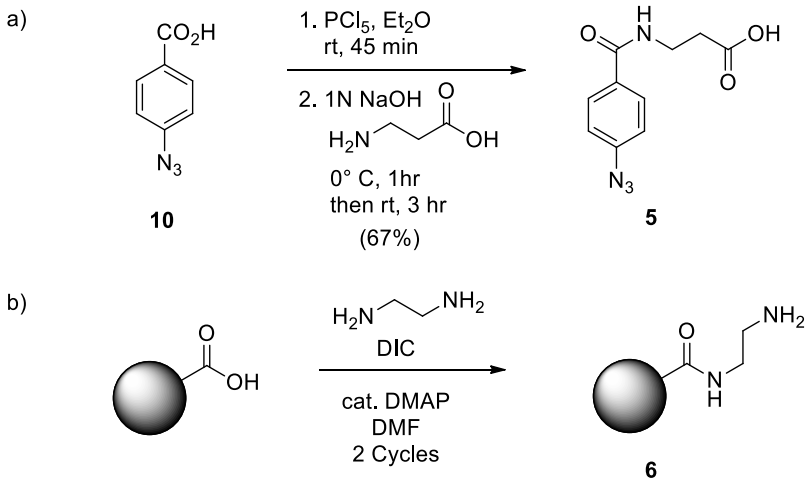

Scheme 2.1 Preparation of the three functional moieties of the TAPP reagents.

With all three functional moieties in hand, exploration of coupling conditions originated from the known ethyl 5-amino-2-(*t*-butoxycarbonyl)aminobenzoate **12** (Ishida 2001). The preparation of **13** was achieved in a slightly modified procedure than the reported method. Ester formation was accomplished with commercially available **7** and 3 eq. of conc. sulphuric acid in anhydrous ethanol (Scheme 2.2) to give diamine **11**. Selective mono-protection of **12** was achieved with di-*tert*-butyl-dicarbonate in methylene chloride in the presence of triethylamine to afford carbamate **12**. Peptide coupling reagents were explored to effect the formation of the desired amide bond. Initial efforts using DIC or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were unsuccessful (Table 2.1). Additives, such as *N'*,*N'*-dimethylaminopyridine (DMAP), are known to enhance the reactivity of the intermediate complex, but DMAP was ineffective in increasing the reactivity. Activation with *N*-hydroxybenzotriazole successfully completed the transformation to the desired amide **13**. However, later in the course of the project, these conditions were abandoned for 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl

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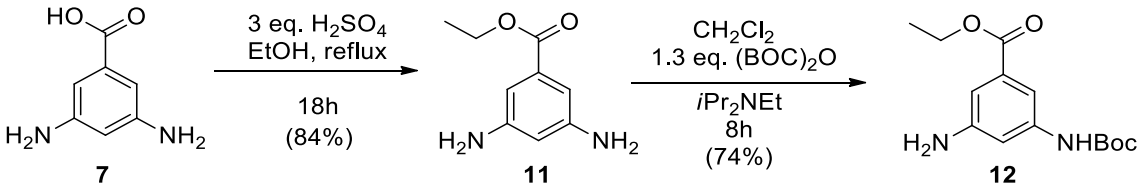
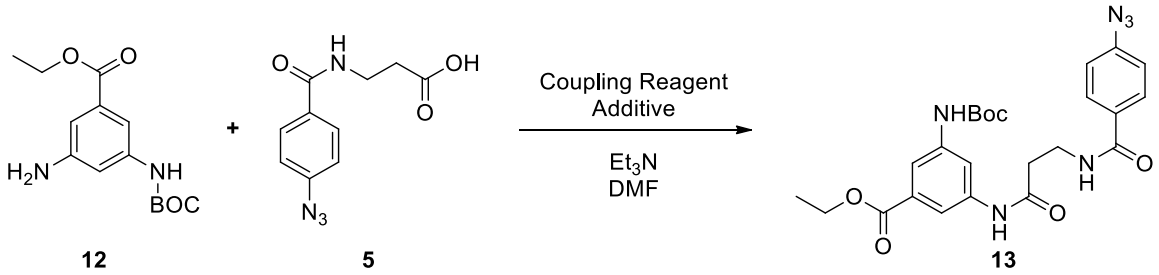
uranium hexafluorophosphate (HATU), which was able to perform the transformation in a considerably shorter reaction time.



Scheme 2.2 Synthetic strategy for the TAPP scaffold.

Table 2.1 Explored coupling conditions.



Entry	Coupling Reagent	Additive	Time (hr)	Temperature (°C)	Yield[a] (%)
1	DIC	DMAP	>96	25	N/A
2	DIC	DMAP	>96	60	N/A
3	EDC	HOBt	96	25	95
4	HATU	-	60	25	92
5	HATU	-	40	40	96

[a]N/A indicates no isolated product **13**.

Acid-mediated cleavage of the boc carbamate protecting using trifluoroacetic acid (TFA) and subsequent basic work up afforded amine **14** in good yield (Scheme 2.3). The previous

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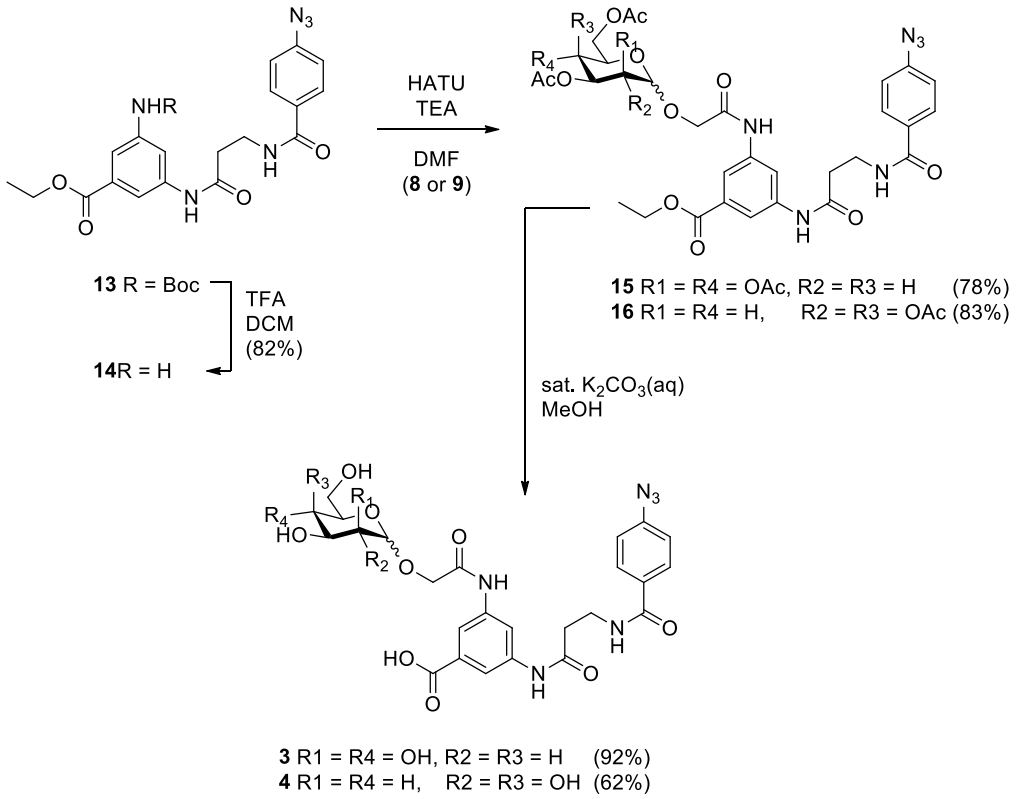
peptide coupling conditions were again employed for each of the carboxy methyl glycosides, α-D-mannoside **8** and β-D-galactoside **9,** to furnish the protected probes **15** and **16**, respectively, in good yields. The deprotected acids **3** and **4** were obtained by global hydrolysis of the ester protecting groups using saturated aqueous potassium carbonate and methanol. Solid phase functionalization was affected in two 24-hour coupling cycles, each using five equivalents of probe relative to the carboxylate sites on the resin and HATU in DMF. The extent of coupling was probed using the phenol sulphuric acid assay (Table 2.2) (Dubois 1956).



Scheme 2.3 Final synthetic procedure for TAPP-HMan and TAPP-HGal.

Cross-linking experiments were performed in glass test-tubes pre-treated with BSA to minimize interactions of ConA with the test tubes' glass surface (Varmette, 2010**).** One milligram of probe was suspended in PBS buffer. The test tubes were incubated for 16 hours in

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Table 2.2 Extent of resin loading.

Probe	Loading (μg probe/mg resin)	Error (μg probe/mg resin)
TAPP-HMan	70	3
TAPP-HGal	44	1
TAPP-FMan	52	1
TAPP-FGal	63	1

the dark. They were then irradiated with a 350 nm broadband source for 2 hours at 37 °C. The probes were then rinsed with a series of solutions of an aqueous DMSO gradient, three cycles each (10, 25, 50, 75% aq. DMSO). Each cycle consisting of vortexing with the rinsing solution then centrifugation at 12,000 RPM for 10 minutes followed by removing the supernatant. The resins were finally rinsed with pure DI water three times to remove any DMSO before the tryptic digest. The resulting peptide mixtures were then analyzed with MALDI-QTOF. The results from the MASCOT database search of the MS/MS data resulted in scores well above the 95% confidence level for the presence of ConA captured by both probes (Perkins 1999) (Appendix D). Initial results would suggest one of two things: either the employed analysis was not adequate for detecting the discrepancy in specific and non-specific interactions or the underlying chemistry in the capturing probe was not selective enough.

Fluorinated probes have shown to be more useful in such applications due to their difference in photochemistry (Schnapp 1993). The singlet nitrene lifetime in perfluorinated probes are sufficiently longer to form C-H insertion products that give rise to more useful cross-links relative to the reactive ketenimine of the parent aryl azide. To further improve upon the initial

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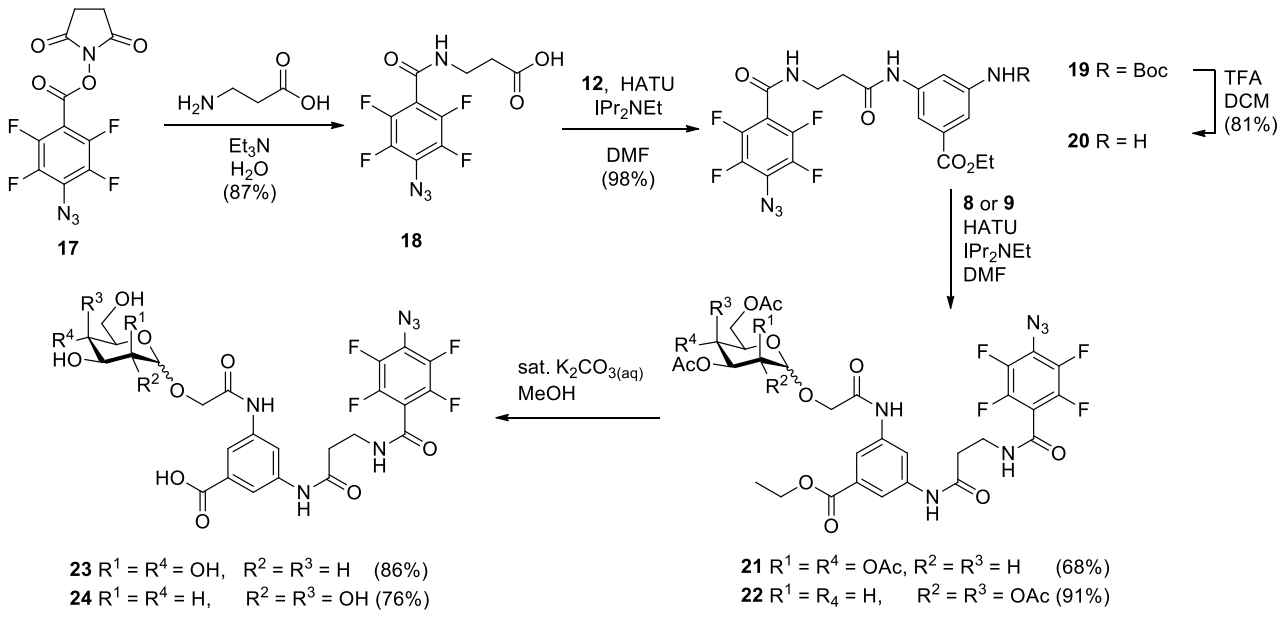
experiments, relative quantification methods should be employed to provide objective definition between specific and non-specific interactions (Zhu 2010).

Preparation of the fluorinated probes originated from the known NHS ester **17** (Norberg 2009). Displacement of the NSH ester with β-alanine was achieved in aqueous triethylamine (Scheme 2.4) with high yield of amide **18**. Synthesis of the fluorinated probes mimicked the parent aryl azide without any complications. After obtaining **23** and **24**, coupling to the resin was done with HATU to produce the final resin bound TAPP-FMan, **25**, and TAPP-FGal, **26** (Scheme 2.5). The extent of loading for each probe was quantified with the phenol sulphuric acid assay (Table 2.2).



Scheme 2.4 Synthesis of the fluorinated TAPPs

All four probes were subjected to the same incubation, cross-linking, rinsing, and digest protocol as described earlier. An aliquot of the samples obtained from the tryptic digestion were subjected to nano-LC ESI-Ion Trap. The obtained MS/MS spectra were analyzed using a

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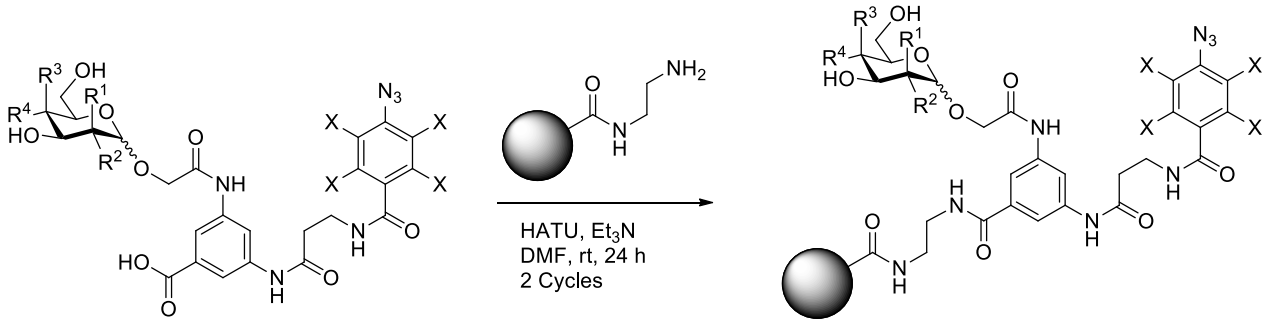
MASCOT database search. Several fragments were matched as belonging to the parent protein, concanavalin A from *C. ensiformis*. The peptide chosen for relative quantification using the extracted ion chromatogram (EIC) was selected due to its ubiquitous nature. The extracted ion chromatogram was obtained by using Thermo Scientific Xcalibur and extracting the chromatogram to a range ± 0.5 Da from the experimentally determined M/Z corresponding to ubiquitously matched fragment with sequence VGLSASTGLYK.



3 $R^1 = R^4 = OH$, $R^2 = R^3 = X = H$
4 $R^2 = R^3 = OH$, $R^1 = R^4 = X = H$
23 $R^1 = R^4 = OH$, $R^2 = R^3 = H$, X = F
24 $R^2 = R^3 = OH$, $R^1 = R^4 = H$, X = F

1 **TAPP-HMan** $R^1 = R^4 = OH$, $R^2 = R^3 = X = H$
2 **TAPP-HGal** $R^2 = R^3 = OH$, $R^1 = R^4 = X = H$
25 **TAPP-FMan** $R^1 = R^4 = OH$, $R^2 = R^3 = H$, X = F
26 **TAPP-FGal** $R^2 = R^3 = OH$, $R^1 = R^4 = H$, X = F

Scheme 2.5 Coupling of probes to solid phase resin.

The retention times were validated comparing the MASCOT database search matched peak list for the fragment to the individual MS/MS spectra obtained at given retention times and are in good agreement (Table 2.3). The EIC was then integrated over the area in which the parent ion's signal to noise ratio was at least 3:1 (Table 2.4) (Appendix C). The peak areas were normalized by dividing the peak area by the largest peak area in the data set.

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Table 2.3 Retention times (Min.) for peptide fragment VGLSASTGLYK for each sample.

Probe	123 nmol	24.6 nmol	12.3 nmol	2.5 nmol
TAPP-HMan	16.08	16.03	16.13	N/A
TAPP-HGal	16.01	16.00	16.01	N/A
TAPP-FMan	16.13	15.88	16.04	16.09
TAPP-FGal	16.10	16.16	16.19	N/A

Table 2.4 Normalized peak areas of the extracted ion chromatogram for the mass ±0.5 Da corresponding to matched peptide VGLSASTGLYK.

Probe	123 nmol	24.6 nmol	12.3 nmol	2.5 nmol
TAPP-HMan	0.35 ± 0.02	0.30 ± 0.01	0.10 ± 0.04	N/A
TAPP-HGal	0.17 ± 0.04	0.09 ± 0.01	0.04 ± 0.01	N/A
TAPP-FMan	1.00 ± 0.03	0.55 ± 0.01	0.38 ± 0.03	0.07 ± 0.03
TAPP-FGal	0.17 ± 0.03	0.12 ± 0.01	0.07 ± 0.01	N/A

The signal to noise ratio in the case of the minimal amount of probe used in the tryptic digest for TAPP-HMan, TAPP-HGal, and TAPP-FGal was so low for the matched peptide, no discernible peaks for integration were able to be identified from the background with confidence. The normalized peak areas suggest the fluorinated aryl azide out performs the parent aryl azide in selectivity, in all cases exhibiting a 5:1 ratio in peak area for the positive control to the

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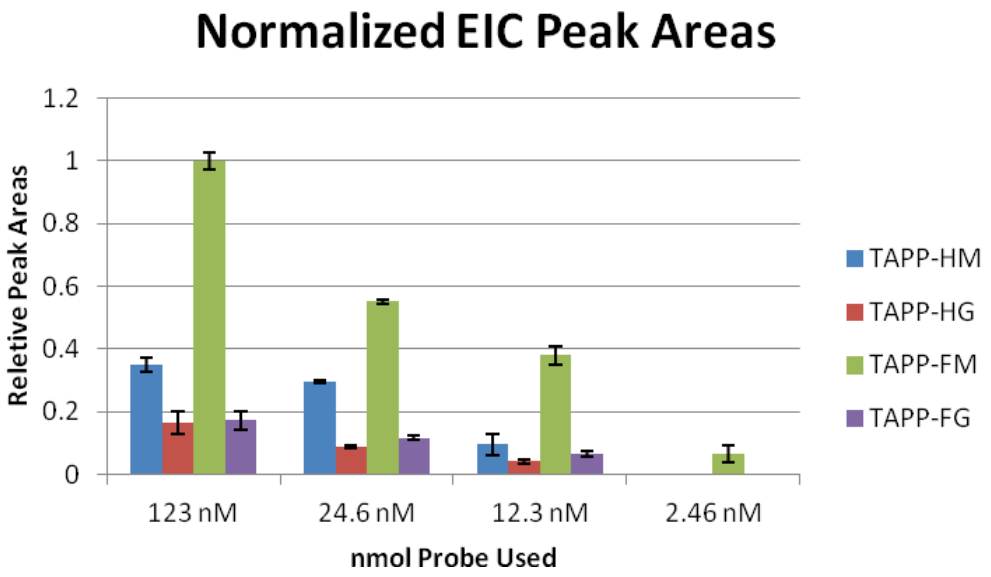

Figure 2.3 Normalized peak areas for TAPP probes are various amounts of resin used for the experiment.

negative control. The discrepancy between the positive and negative controls for the parent aryl azide for the maximum subjected probe was 3:2 and in the best case 9:2 for sample 3. The sensitivity difference is apparent as the only probe to give quantifiable signal for the matched peptide is TAPP-FMan at the lowest subjected probe amount.

Although no modeling was done to ensure capture by the probe is possible while con A was bound, the probe and carbohydrate had relatively similar linker length thus it seems reasonable that it is indeed possible. The difference in captured protein amounts should not be dependent on the probe if it were not involved. The results suggest the captured protein is dependent upon the probe chemistry, making it logical to implicate the photo probe in being intimately involved in capturing the protein.

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Conclusion

A solid phase platform was successfully able to capture lectin capture reagents in a selective manner for positive and negative controls. The method was shown to be sensitive and efficient with costly materials, needing only 100 nm of probe to obtain useful data, which can be achieved with 1 μmol of material. Trypsin was shown to be able to cleave proteins off the surface of the resin in adequate amounts for several nano-LC-ESI MS/MS runs for the case of con A. Such an experiment produces data that can be analyzed with existing tools to extract relative peptide concentrations across the samples.

The perfluorinated aryl azide probes were shown to have greater efficiency and selectivity for capturing specific interactions relative to probes with the parent aryl azide. One limitation of this method is that the proteins are only able to be relatively quantified across samples and thus, one negative control would always be necessary to put the interaction of interest in perspective. Sstudies involving multiple signals for comparison could prove to help define a specific interaction much like work with microarrays (Smith 2013).

The utility of this method is highly dependent on the ability to directly capture surface binding proteins. Combined with combinatorial chemical methods for carbohydrate production, it would certainly create a versatile and facile tool for identification of host and pathogen interactions.

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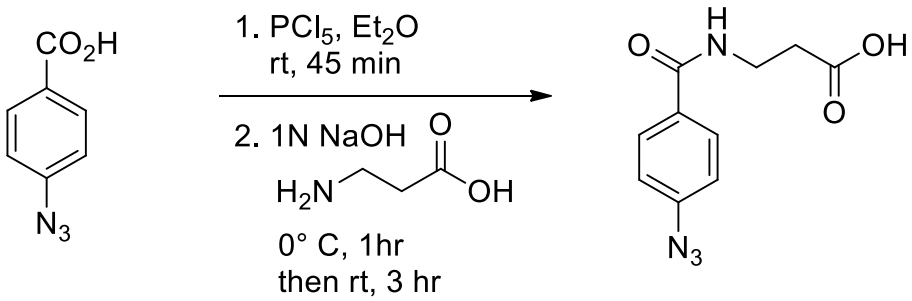
Experimental

General: All commercially available compounds were purchased from TCI, Sigma Aldrich, or Fisher Scientific; they were reagent grade and used without further purification. HATU was purchased from Accela ChemBio. For sensitive reactions, solvents were dried prior to use by distillation from a suitable drying agent. Column chromatography was performed with ZEOprep ECO silica gel 60 with 40-63 μm particle size (American International Chemical). Reactions were monitored using thin layer chromatography coated with a 0.25 mm layer of silica gel 60 F_{254} (Sorbent Technologies). Compounds were visualized with UV light and/or 5% *v:v* sulfuric acid in ethanol. ^{1}H NMR and ^{13}C NMR were performed with either a Bruker Avance DRX-400 MHz spectrometer or a Varian VXR-400 MHz spectrometer, or otherwise noted. All ^{1}H NMR peak assignments were made using solvent residual peaks as an internal standard (DMSO δ 2.50 ppm, $CDCl_3$ δ 7.26 ppm, and CD_3OD δ 3.31) as were ^{13}C NMR peak assignments (DMSO δ 39.51 ppm, $CDCl_3$ δ 77.31 ppm, and CD_3OD δ 49.00) and supported using ^{1}H-^{1}H COSY experiments as needed. High resolution mass spectra were obtained with a Finnigan TSQ700 for compound characterization. Mass spectroscopy data for protein identification was obtained with a Thermo Scientific LTQ Velos Pro, a nano-LC-ESI ion trap instrument.



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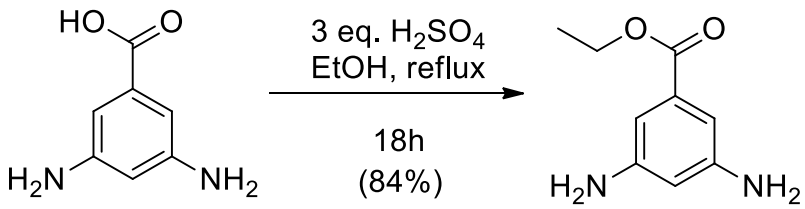
3-(4-azidobenzamido)propanoic acid (5): To a solution of *p*-azidobenzoic acid (2.06g, 12.6 mmol) in diethyl ether (60 mL) was added Phosphorus Pentachloride (2.68g, 12.9 mmol) and let stir for 1 hour. The ether was removed *in vacuo* to yield a yellow residue which was triturated with hexanes at 0° C to yield a pale yellow solid which was used directly in the next step. To a solution of β-alanine (1.00 g, 11.26 mmol) in 1 N NaOH (23 mL) was added *p*-azidobenzoyl chloride (1.13 g, 6.62 mmol) at 0 °C. The solution was let stir for one hour then removed from the ice bath and let warm slowly to room temp (2 hours). The reaction was brought to pH 3 using 1 N HCl in which a white precipitate formed. The solid was filtered and recrystallized from ethanol to yield **5** as a pale yellow needles (0.97 g, 67% over 2 steps).

^{1}H NMR (DMSO, 400 MHz): δ 8.54 (1H, t, J = 4.8 Hz, H_N), 7.88 (2H, d, J = 8.4 Hz, H_{Ar}), 7.19 (2H, d, J = 8.8 Hz, H_{Ar}), 3.47-3.42 (2H, m, H_2), 2.51 (2H, t, J = 7 Hz, H_1).

^{13}C NMR (DMSO, 400 MHz): δ 33.75, 35.60, 118.89 (x2), 129.07 (x2), 130.90, 142.24, 156.25, 172.92.

HRMS Calcd for $[M+H]^+$. 235.0825 Found: 235.0820.



Ethyl 3,5-Diaminobenzoate(11): To a solution of 3,5-Diaminobenzoic Acid (7.64 g, 50.21 mmol) in 200 proof Ethanol (300 ml) was added conc. Sulfuric Acid (8.37 mL, 150.6 mmol).

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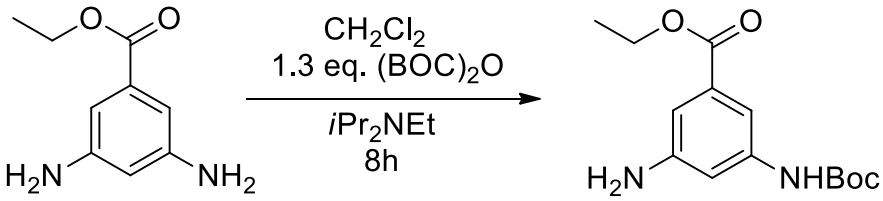
The reaction was refluxed for 24 hrs. The ethanol was removed *in vacuo.* Iced cold water was added (75 mL) to the residue and made slightly alkaline with the addition of solid sodium carbonate. The aqueous layer was extracted with ethyl acetate (4x100 mL), washed with sodium bicarbonate (3x250mL), water (3x250mL), and dried over sodium sulfate. The ethyl acetate was removed *in vacuo* to yield a dark amber residue. The residue was purified by column chromatography (EtOAc:Hexane, 1:1) to yield an amber residue (7.59 g, 84%). NMR Matched previously reported spectra (Ishida 2001).



Ethyl 5-amino-3-(*t*-butoxycarbonyl)aminobenzoate(12): To a solution of Ethyl 3,5-Diaminobenzoate (5.02 g, 5.66 mmol) and Diisopropylethyl Amine (2.5 mL, 15.1 mmol) in Dichloromethane at 0° C was added Ditertbutyldicarbonate (1.24 g, 5.68mmol) in 20 ml dichloromethane dropwise over 30 minutes. The reaction was let stir for 8 hours at room temperature and the solvent was removed *in vacuo* to yield a brown residue. The mono protected compound was obtained with flash chromatography (EtOAc:Hexane 1:1. The resulting fractions were pooled and concentrated *in vacuo* to yield a yellow oil which was triturated with hexanes to afford a white solid. The hexane was filtered off and the compound was dried under high vacuum to yield a white powder (0.932g, 74%). NMR Matched previously reported spectra (Ishida 2001).

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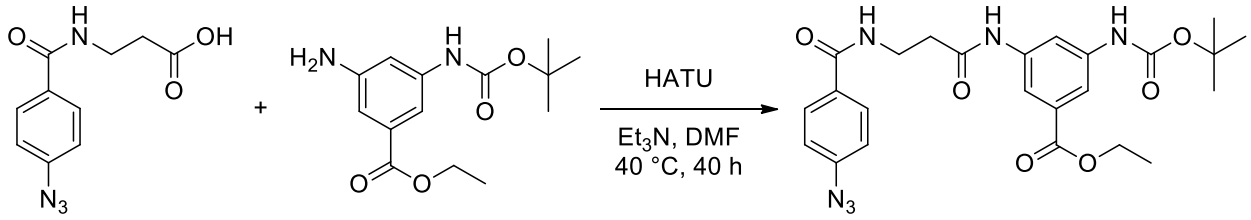

Ethyl 3-(*t*butoxycarbonyl)amino-5-(3-(4-azidobenamido) propanamidobenzoate(13): To a solution of **5** (2.19g, 9.37 mmol), **12** (2.63 g, 9.37 mmol), and HATU (3.92 g, 10.3 mmol) in DMF (20 mL) was added triethylamine (2.61 mL, 18.7 mmol) The reaction was warmed to 40 ℃ for 40 hours. The reaction was quenched by the addition of water (100 ml). Ethyl acetate (50 ml) was added and the reaction was let stir vigorously for 10 minutes. The layers were separated and the aqueous layer was extracted with ethyl acetate (3x100 ml) and the combined organic layer was washed with sat. sodium bicarbonate (3 x 300 ml), water (3 x 300 mL) and dried over sodium sulfate. The solvent was removed *in vacuo* and the crude product was recrystallized with a mixture of ethyl acetate and hexanes to furnish **13** (4.44 g, 8.94 mmol, 96%) as a white solid.

^{1}H NMR (DMSO, 400 MHz): δ 10.17 (1H, s, $H_{NH\text{-}Ar}$), 9.61 (1H, s, $H_{carbamate}$), 8.63 (1H, t, J = 4 Hz, $H_{NH\text{-}alk}$), 8.02 (1H, s, H_{Ar}), 7.96 (1H, s, H_{Ar}), 7.89 (2H, d, J = 12 Hz, H_{Ar}), 7.74 (1H, s, H_{Ar}), 7.19 (2H, d, J = 12 Hz, H_{Ar}), 4.29 (2H, q, J = 8 Hz, H_{OCH2}), 3.57-3.50 (2H, m, H_{CH2}), 2.61 (2H, t, J = 8 Hz, H_{CH2CON}), 1.47 (9H, s, H_{tBu}), 1.30 (3H, t, J = 8 Hz, H_{CH3}).

^{13}C NMR (DMSO, 100 MHz): 172.49, 169.45, 167.84, 155.16, 145.05, 141.66, 140.64, 132.69, 132.21, 130.36 (x2), 120.14 (x2), 116.34, 115.74, 81.30, 62.41, 42.84, 37.65, 28.81 (x3), 23.68, 14.74.

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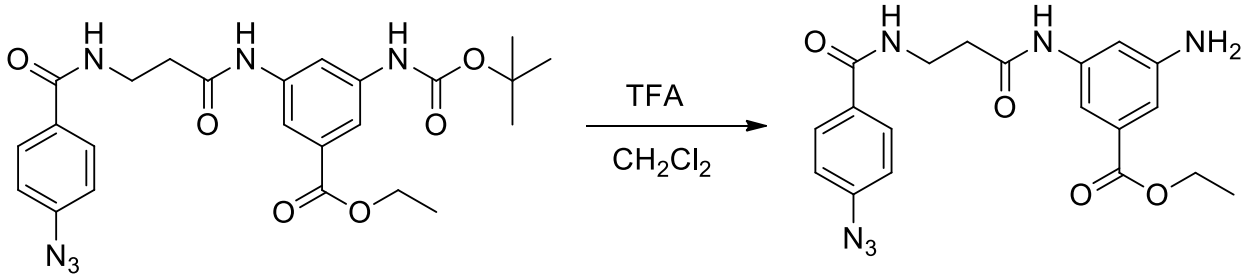
HRMS Calcd for $[M+H]^+$ 497.2143 Found: 497.2147.



Ethyl 5-amino-3-(3-(4-azidobenzamido)propanamido)benzoate(14): To **13** (4.19 g, 8.44 mmol) was added 1:1(v:v) mixture of methylene chloride and trifluoroacetic acid (20 ml). The reaction was let stir for 10 minutes at room temperature. The solvent was removed *in vacuo.* Iced water (20 ml) was added to the crude product and made slightly alkaline with the addition of sodium carbonate. The mixture was diluted with 200 ml DI water and the aqueous layer was extracted with ethyl acetate (3x 150 ml) and the combined organic layers were washed with sodium bicarbonate (3 x 400 ml), water (3 x 400 ml), and dried over sodium sulfate. The solvent was removed *in vacuo* and the crude product was recrystallized from an ethyl acetate and hexanes mixture to afford compound **14** (2.75 g, 6.94 mmol, 82%) as a light tan amorphous solid.

^{1}H NMR (DMSO, 400 MHz): δ 9.87 (1H, s, H_{NH}), 8.60 (1H, t, J = 5.4 Hz, $H_{N'H}$), 7.89 (2H, d, J = 8.4 Hz, H_{Ar}), 7.34 (1H, s, H_{Ar}).

^{13}C NMR (DMSO, 100 MHz) δ 169.69, 165.57, 165.30, 152.68, 142.16, 140.13, 139.71, 131.00, 130.45, 129.05, 118.84, 113.58, 113.03, 79.30, 60.72, 36.27, 35.88, 28.07, 14.18.

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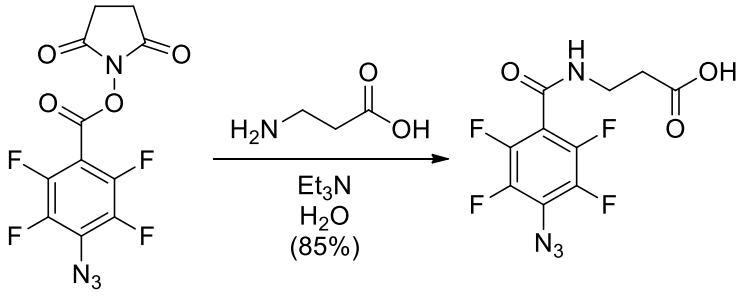
HRMS Calcd for $[M+H]^+$: 397.1619 Found: 397.1612.



3-(4-azido-2,3,5,6-tetrafluorobenzamido)propanoic acid(18): To a solution of β-alanine(0.644 g, 7.23 mmol) in DI water (5 ml) at 0 °C was added TEA (1.1 ml, 7.83 mmol)) was added **17** (2.00 g, 6.02 mmol). The reaction was let stir for 1 hr at 0 °C and then let warm up to room temperature and stirred 16 hours at rt. The reaction was acidified to pH 3 with dilute $HCl_{(aq)}$. The crude precipitate was filtered and recrystallized with a mixture of chloroform and hexane to yield a white, amorphous solid (1.60 g, 5.23 mmol, 87%).

^{1}H NMR (500 MHz, cdcl3) δ 6.58 (s, 1H), 3.74 (dd, J = 11.5, 5.7 Hz, 2H), 2.75 (dd, J = 10.8, 5.1 Hz, 2H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.54, 166.33, 158.05, 152.87, 139.60, 139.29, 131.64, 115.25, 115.03, 114.13, 80.76, 61.29.

HRMS Calcd for $[M+Na]^+$: 329.0268 Found: 329.0272.

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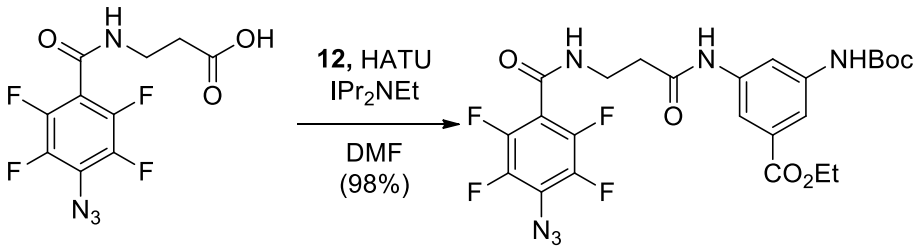

Ethyl 3-(*t*-butoxycarbonyl)amino-5-(3-(4-azido-2.3.5.6-tetrafluorobenzamido) propanamido)benzoate(19): To solution of **18** (1.58 g, 5.16 mmol) in , Ethyl 5-amino-3-(*t*-butoxycarbonyl)aminobenzoate (1.80 g, 5.67 mmol) and HATU (2.14 g, 5.67 mmol) in DMF (10 ml) under an inert atmosphere was added DIPEA (1.8 ml, 10.4 mmol). The reaction was let stir at room temperature for 60 hours. The reaction was quenched with DI water and extracted 3x75 ml Ethyl Acetate and the combined organic layer was washed 3x250 ml sat. $NaHCO_3$ and 3x250 ml DI water. The solvent was dried over Na_2CO_3 and then removed *in vacuo*. The crude product was recrystallized from a mixture of methylene chloride and hexane.

^{1}H NMR (400 MHz, CD_3OD) δ 7.47 (t, J = 1.6 Hz, 1H), 7.26 (t, J = 2.0 Hz, 1H), 7.14 – 6.96 (m, 1H), 5.52 (s, 2H), 4.31 (q, J = 7.1 Hz, 2H), 3.71 (t, J = 6.6 Hz, 2H), 2.71 (t, J = 6.7 Hz, 2H), 1.36 (t, J = 7.1 Hz, 3H).

HRMS Calcd for $[M+Na]^+$: 519.1962 Found: 519.1968.

Ethyl 5- amino-3-(3-(4-azido-2.3.5.6-tetrafluorobenzamido)propanamido) benzoate(20):

To **13** (4.19 g, 8.44 mmol) was added 1:1(v:v) mixture of methylene chloride and trifluoroacetic acid (20 ml). The reaction was let stir for 10 minutes at room temperature. The solvent was removed *in vacuo.* Iced water (20 ml) was added to the crude product and made slightly

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alkaline with the addition of sodium carbonate. The mixture was diluted with 200 ml DI water and the aqueous layer was extracted with ethyl acetate (3x 150 ml) and the combined organic layers were washed with sodium bicarbonate (3 x 400 ml), water (3 x 400 ml), and dried over sodium sulfate. The solvent was removed *in vacuo* and the crude product was recrystallized from an chloroform and hexanes mixture to afford compound **14** (2.75 g, 6.94 mmol, 82%) as a light tan amorphous solid.

^{1}H NMR (400 MHz, DMSO) δ 10.84 (s, 1H), 7.34 (s, 1H), 7.23 (s, 1H), 6.99 (s, 1H), 5.57 (s, 2H), 4.26 (q, J = 7.1 Hz, 2H), 1.29 (t, J = 7.1 Hz, 3H).

HRMS Calcd for$[M+H]^+$: 469.1241Found 469.1247.

General procedure for HATU mediated carboxymethyl glycoside and probe coupling: To aminoprobe (**14** or **20**) (1 eq.), carbohydrate (**8** or **9**) (1.2 eq), and HATU (1.2 eq) in a dry round bottom under an inert atmosphere was added DMF (5 ml) and DIPEA (2 eq). The reaction was stirred at room temperature for 60 hours or at 40 °C for 40 hours. The reaction was quenched with addition of 20 50 ml DI water and extracted 3x50 ml Ethyl Acetate. The combined organic layer was washed 3x200 ml DI Water and 2x200 ml Brine. The Organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The crude product was purified by column chromatography, ($MeOH/Me_2CO/CH_2Cl_2$ 1:20:79).

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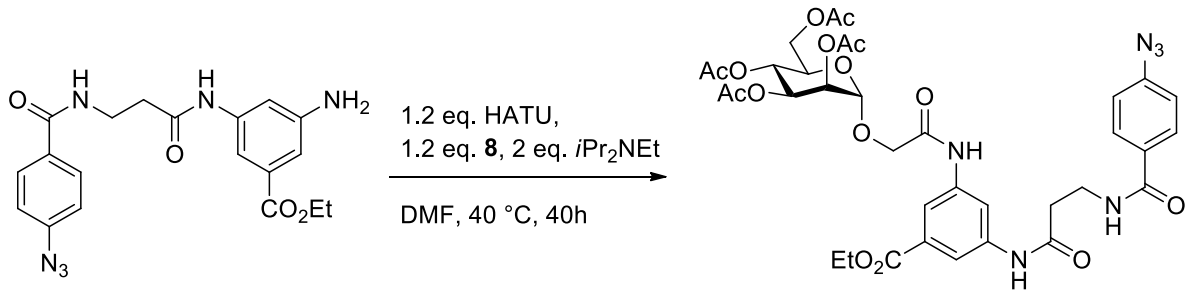

Ethyl 3-((4-azidobenzamido)propanamido)-5-(1-O-(2,3,4,6-tetra-O-acetyl-α-D-mannosyl)acetamido)benzoate(15): Compound **14** (1.00 g, 2.52 mmol) and **8** (1.23 g, 3.03 mmol) were coupled using the general coupling conditions described above to afford compound **15** (1.57 g, 1.97 mmol, 78%) as a pale yellow syrup.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 1H), 8.03 – 7.94 (m, 3H), 7.90 (s, 1H), 7.81 (d, J = 8.8 Hz, 2H), 7.18 (t, J = 6.3 Hz, 1H), 7.04 (d, J = 8.8 Hz, 2H), 5.46 (ddd, J = 13.3, 6.6, 2.6 Hz, 2H), 5.33 (t, J = 9.9 Hz, 1H), 4.98 (d, J = 1.8 Hz, 1H), 4.44 – 4.26 (m, 4H), 4.21 – 4.12 (m, 2H), 4.08 – 4.00 (m, 1H), 3.92 – 3.80 (m, 2H), 2.76 (t, J = 5.7 Hz, 2H), 2.19 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 1.39 (t, J = 7.1 Hz, 3H).

^{13}C NMR (100 MHz, CD3OD) δ 169.74, 167.94, 167.01, 165.33, 142.20, 139.58, 138.81, 131.43, 130.99, 129.07, 118.88, 115.45, 115.24, 114.36, 99.89, 74.49, 77.07, 69.82, 66.93, 65.29, 61.17, 36.32, 35.92.

HRMS Calcd for $[M+H]^+$: 785.2624 Found: 785.2632

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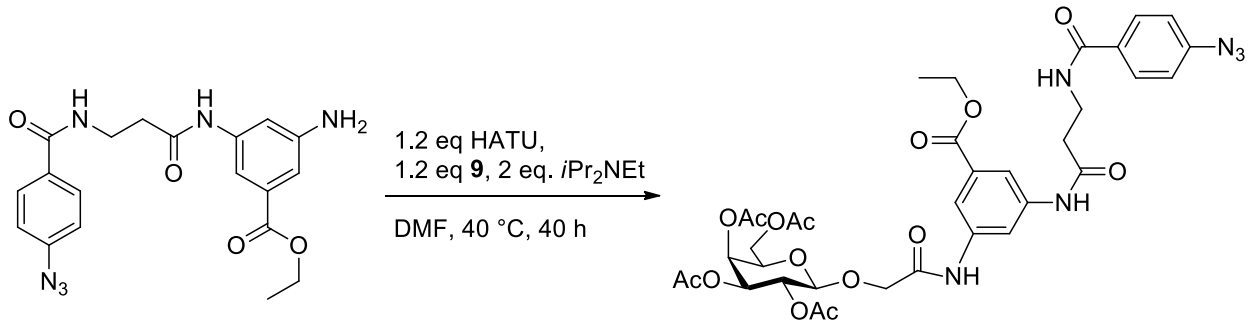

Ethyl 3-((4-azidobenzamido)propanamido)-5-(1-O-(2,3,4,6-tetra-O-acetyl-β-D-galactosyl)acetamido)benzoate(16): Compound **14** (530 mg, 1.3 mmol) and **9** (636 mg, 1.6 mmol) were coupled with the general procedure above. After column chromatography,**16** (854 mg, 1.09 mmol, 83%) was obtained as a pale yellow syrup.

^{1}H NMR (400 MHz, CDCl3) δ 8.53 (s, 1H), 8.26 (s, 1H), 7.97 (s, 1H), 7.91 (s, 1H), 7.80 (d, J = 8.7 Hz, 2H), 7.57 (s, 1H), 7.07 (dd, J = 15.6, 6.9 Hz, 3H), 5.44 (d, J = 2.8 Hz, 1H), 5.32 (dd, J = 10.5, 7.8 Hz, 1H), 5.09 (dd, J = 10.6, 3.4 Hz, 1H), 4.59 (d, J = 7.9 Hz, 1H), 4.44 (d, J = 15.2 Hz, 1H), 4.38 (q, J = 7.1 Hz, 2H), 4.17 (t, J = 6.9 Hz, 2H), 3.99 (t, J = 6.2 Hz, 1H), 3.89 – 3.76 (m, 2H), 2.74 (t, J = 5.5 Hz, 2H), 2.21 (s, 3H), 2.13 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.39 (t, J = 7.1 Hz, 4H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 207.10, 206.86, 206.52,172,71, 172.36, 171.52, 155.66, 155.55, 151.34, 151.24, 137.81, 137.61, 129.12, 128.23, 126.54, 126.46, 119.22, 119.17, 114.54, 102.74, 101.08, 100.99, 100.95, 77.55, 77.23, 76.91, 71.67, 69.01, 68.29, 66.54, 55.70, 38.11, 37.85, 29.80, 29.74, 28.30, 28.24, 27.96.

HRMS Calcd for $[M+H]^+$: 785.2624 Found: 785.2632

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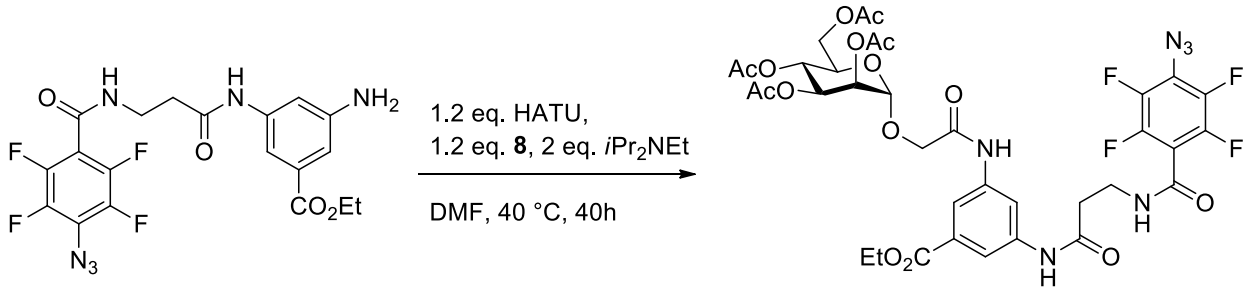
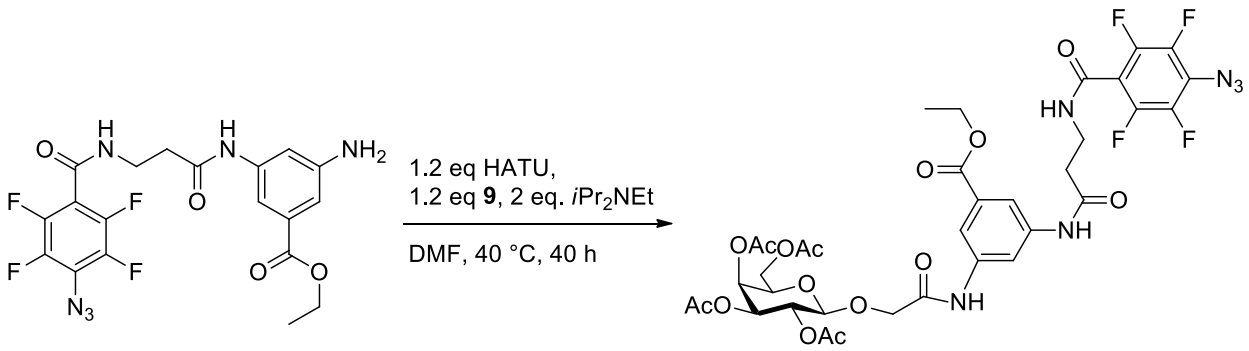


Ethyl 3-((4-azido-2,3,5,6-tetrafluorobenzamido)propanamido)-5-(1-O-(2,3,4,6-tetra-O-acetyl-α-D-mannosyl)acetamido)benzoate(21): Compound **20** (355 mg, 0.76 mmol) and **8** (370 mg, 0.91 mmol) were coupled using the general procedure above to provide **21** (443 mg, 0.52 mmol, 68%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H), 8.18 (s, 2H), 8.05 (s,1H), 7.94 (s, 1H), 6.51 (s, 1H), 5.42-5.40 (m, 2H) , 5.34- 5.32 (m, 2H), 4.97 (s, 1H), 4.37-4.29 (m, 4H), 4.25-4.03 (m, 3H), 3.81 (s, 4H), 2.19 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.39 (t, 3H, J = 6.8 Hz).

^{13}C NMR (150 MHz, $CDCl_3$) δ 171.05, 170.90, 170.53, 170.38, 169.86, 167.36, 166.92, 166.03, 143.57, 138.82, 137.57, 131.65, 130.82, 129.14, 119.11, 117.78, 117.41, 116.55, 98.29, 69.53, 69.36, 69.22, 67.33, 66.00, 62.45, 61.57, 36.90, 36.51, 21.06, 20.96, 20.94, 20.89, 14.44.

HRMS Calcd for $[M+Na]^+$: 879.2067 Found: 879.2070



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Ethyl 3-((4-azido-2,3,5,6-tetrafluorobenzamido)propanamido)-5-(1-O-(2,3,4,6-tetra-O-acetyl-β-D-galactosyl)acetamido)benzoate(22): Compound **20** (575 mg, 1.23 mmol) and **9** (599 mg, 1.47 mmol) were coupled using the general procedure above to provide **22** (771 mg, 0.90 mmol, 73%).

^{1}H NMR (400 MHz, cdcl3) δ 8.55 (s, 1H), 8.28 (s, 1H), 7.97 (s, 1H), 7.85 (s, 1H), 7.67 (s, 1H), 7.09 (s, 1H), 5.44 (d, J = 3.0 Hz, 1H), 5.32 (dd, J = 10.5, 7.9 Hz, 1H), 5.09 (dd, J = 10.6, 3.3 Hz, 1H), 4.59 (d, J = 7.8 Hz, 1H), 4.45 – 4.34 (m, 3H), 4.25 (d, J = 15.3 Hz, 1H), 4.20 – 4.11 (m, 2H), 3.98 (t, J = 6.5 Hz, 1H), 3.80 (d, J = 5.7 Hz, 2H), 2.74 (s, 2H), 2.21 (s, 3H), 2.13 (s, 3H), 2.03 (s, 4H), 1.95 (s, 3H), 1.39 (t, J = 7.1 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 170.96, 170.70, 170.65, 170.44, 170.27, 167.17, 167.15, 166.10, 143.60, 139.33, 138.06, 132.08, 130.92, 129.14, 119.19, 117.08, 116.49, 115.48, 101.71, 71.39, 70.49, 69.46, 67.07, 61.63, 61.53, 38.87, 36.83, 36.26, 31.19, 21.22, 20.89, 20.82, 14.52.

HRMS Calcd for $[M+Na]^+$: 879.2067 Found: 879.2070.

General procedure for the hydrolysis of ester protecting groups

To protected probe (**16**, **17**, **21**, or **22**) was added equal parts methanol and sat. $K_2CO_{3(aq.)}$ to make a final concentration of 0.1M probe. The reaction was let stir at room temperature for 12 hours. Two ml methanol was added to the reaction, and the potassium acetate was removed by filtration. The filtrate was acidified with DOWEX 50WX8 acidic resin to pH 3. The resin was filtered and rinsed with generous portions of methanol. The solvent was removed *in vacuo* to

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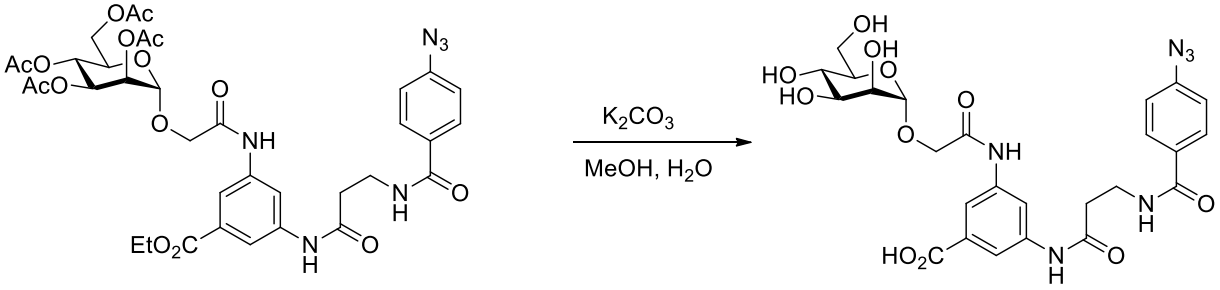
afford crude products (**3**, **4**, **5** or **6**). Probes **5** and **6** were used without further purification. Products **3** and **4** were triturated with 0.5 ml DI water and obtained as a white solid after filtration.



3-((4-azidobenzamido)propanamido)-5-((1O-α-D-mannosyl)acetamido)benzoic acid(3): Compound **15** (615 mg, 0.784 mmol) was deprotected according to the general procedure. The crude product was triturated with water to yield **3** as a white amorphous solid (425 mg, .723 mmol, 92%).

^{1}H NMR (DMSO, 400 MHz): δ 12.94 (1H, s, H-CO_2H), 10.19 (1H, s, H-NH_{amide}), 9.97 (1H, s, H-NH_{amide}), 8.62 (1H, t, J = 5.4 Hz, H-NH_{amide}), 8.21 (1H, s, H-Ar), 7.98 (1H, s, H-Ar), 7.90 (1H, s, H-Ar), 7.89 (2H, d, J = 8.4 Hz, H-Ar), 7.19 (2H, d, J = 8.8 Hz, H-Ar), 4.82 (2H, d, J = 4.4 Hz, H-OH_d), 4.75 (1H, s, H-H_α), 4.75 (1H, s, H-OH_b), 4.59 (1H, d, J = 5.2 Hz, H-OH_c), 4.49 (1H, t, J = 6 Hz, H-OH_f), 4.14 (2H, m, $H_{acetamido}$), 3.81 (1H, m, H-CH_d), 3.68-3.63 (1H, m, H-CH_f), 3.60-3.50 (1H, m, H-CH_c), 3.60-3.50 (2H, m, H-$CH_{2(3\text{-propanamide})}$), 3.55-3.36 (3H, m, H-$CH_{f',e,b}$), 2.63 (2H, t, J = 6.8 Hz, H-$CH_{2(2\text{-Propanamido})}$).

^{13}C NMR (DMSO, 100 MHz): δ 169.8, 168.0, 167.0, 165.3, 142.2, 139.6, 138.8, 131.4, 131.0, 129.1 (x2) 118.9 (x2), 115.5, 115.2, 114.4, 99.9, 74.5, 70.8, 69.8, 66.9, 65.3, 61.2, 36.3, 35.9.

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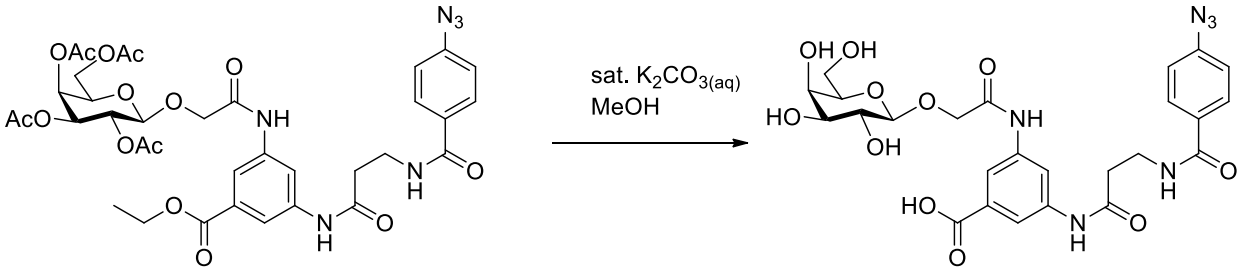
HRMS Calcd for $[M+H]^+$: 589.1889 Found 589.1890, Calcd for $[M+Na]^+$: 611.1708 Found 611.1708.



3-((4-azidobenzamido)propanamido)-5-((1O-β-D-galactosyl)acetamido)benzoic acid(4): Compound **15** (668 mg, 0.85 mmol) was deprotected according to the general proceudre. The crude product was triturated with water to yield **4** as a white amorphous solid (379 mg, 0.64 mmol, 76%).

^{1}H NMR (DMSO, 400 MHz): δ 12.94 (1H, s, H-CO_2H), 10.19 (1H, s, H-NH_{amide}), 9.97 (1H, s, H-NH_{amide}), 8.62 (1H, t, J = 5.4 Hz, H-NH_{amide}), 8.21 (1H, s, H-Ar), 7.98 (1H, s, H-Ar), 7.90 (1H, s, H-Ar), 7.89 (2H, d, J = 8.4 Hz, H-Ar), 7.19 (2H, d, J = 8.8 Hz, H-Ar), 4.82 (2H, d, J = 4.4 Hz, H-OH_d), 4.75 (1H, s, H-H_α), 4.75 (1H, s, H-OH_b), 4.59 (1H, d, J = 5.2 Hz, H-OH_c), 4.49 (1H, t, J = 6 Hz, H-OH_f), 4.14 (2H, m, $H_{acetamido}$), 3.81 (1H, m, H-CH_d), 3.68-3.63 (1H, m, H-CH_f), 3.60-3.50 (1H, m, H-CH_c), 3.60-3.50 (2H, m, H-$CH_{2(3\text{-propanamide})}$), 3.55-3.36 (3H, m, H-$CH_{f',e,b}$), 2.63 (2H, t, J = 6.8 Hz, H-$CH_{2(2\text{-Propanamido})}$).

^{13}C NMR (DMSO, 400 MHz): δ 169.8, 168.0, 167.0, 165.3, 142.2, 139.6, 138.8, 131.4, 131.0, 129.1 (x2) 118.9 (x2), 115.5, 115.2, 114.4, 99.9, 74.5, 70.8, 69.8, 66.9, 65.3, 61.2, 36.3, 35.9.

HRMS Calcd for $[M+H]^+$: 589.1816 Found: 589.1879.

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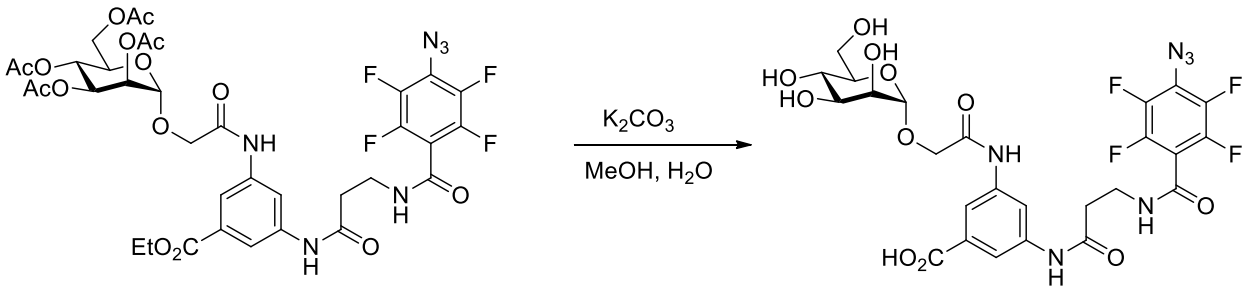
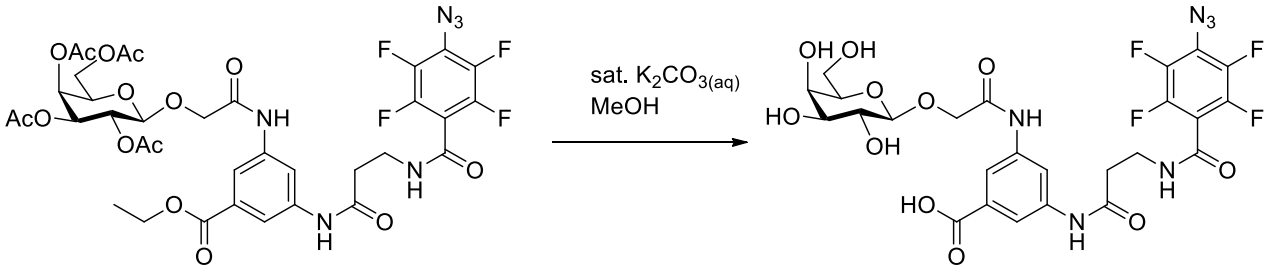


3-((4-azido-2,3,5,6-tetrafluorobenzamido)propanamido)-5-(1-O-(α-D-mannosyl)acetamido)benzoic acid(23): Compound **21** (599 mg, 0.700 mmol) was deprotected according to the general procedure which gave **23** as a a light yellow amorphous solid (396 mg, 599 µmol, 86%). ^{1}H NMR (400 MHz, CD3OD) δ 8.22 (d, J = 20.2 Hz, 1H), 7.96 (dd, J = 44.2, 18.4 Hz, 2H), 4.27 (dd, J = 33.3, 15.2 Hz, 2H), 4.03 (s, 1H), 3.86 (dd, J = 24.8, 13.5 Hz, 3H), 3.65 (ddd, J = 65.7, 35.0, 26.2 Hz, 5H), 2.74 (t, J = 6.5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.89, 170.50, 169.96, 166.27, 138.69, 137.66, 132.05, 117.67, 114.92, 98.23, 69.83, 69.44, 67.45, 65.98, 62.61, 61.71, 53.72, 37.05, 31.23, 30.01, 21.16, 21.02, 20.94, 14.55.



3-((4-azido-2,3,5,6-tetrafluorobenzamido)propanamido)-5-(1-O-(β-D-galactosyl)acetamido)benzoic acid(24): Compound **22** (707 mg, 0.83 mmol) was deprotected according to the general procedure to give **24** (399 mg, 0.60 mmol, 73%) as a pale yellow syrup that turned to a pale yellow foam on under vacuum.

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Bead Preperation(6): To a suspension of Carboxy Polystyrene HL (100-200 mesh) 1%Divinyl Benzene (250 mg) in dichloromethane (25 mL) was added Ethylene Diamine (25 mg, 0.42 mmol), N,N-Dimethyl-4-Amino-Pyridine (15 mg, 0.12 mmol) and N,N'-Diisopropylcarbodiimide (50.6 mg, 0.40 mmol). The reaction was let stir over night. The bead was filtered and washed with ethanol (3x100 mL). The resin was immediately subjected to the same conditions for a second cycle. The Kaiser test was positive for free amine on the surface of the bead.

General conditions for functionalizing resin surface: The resin was allowed to swell in DMF for 6 hours prior to starting the cycle. To the resin (~10 mg, 1.00 mmol/g loading capactiy) in DMF (1 ml) was added **3**, **4**, **23**, or **24** (5 mol equivalents), HATU (5 eq) and *i*Pr_2NEt (10 eq). The resin was stirred for 24 h at rt. The resin was filtered off and subjected to these conditions to ensure complete coupling. The phenol sulfuric acid assay was used to measure the loading of the resins.

Phenol sulfuric acid assay: Calibration curves were prepared as described (Dubois 1956) for D-mannose and D-galactose. For resin containing samples, the procedure was slightly modified. A one mg/ml resin suspension was prepared by ultrasonicating 1 mg of resin with 1 ml of DI water. 100 µl of this suspension was transferred to a clean test tube and diluted with 300 µl of DI water. 100 µl of an 80% phenol (*v/v*) solution was added followed by slow addition of 1 ml conc. H_2SO_4. The test tube was vortexed (being careful, this tube gets really hot!) and let cool back to rt over 20 minutes. One ml of this solution was carefully syphoned off to prevent the udpate of flocculated resin, and it's absorbance measured at the reported wavelengths.

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Cross Linking Experiments: Test tubes were charged with 3 ml of 1 mg/ml BSA solution and let incubate at 37 °C for one hour. The solutions were poured out and 1 mg of resin was added to individual test tube, one TAPP per test tube. To all test tubes was added 0.5 ml of 1 ng/ml Concanavalin A. The solutions were let incubate at 37 °C for 12 hours, in the dark, with gentle shaking. The test tubes were transferred to the photoreactor, a rayonet RPR-100 from Southern New Englang Ultraviolet, and irradiated with a 350 nm broadband light source for two hours. The resins were filtered and collected into 1.5 ml microfuge tubes for rinsing.

Rinsing Protocol: For all rinsing solutions: 1 ml of solution was added and the tubes were vortexed for 20 seconds. The resulting suspension was centrifuged at 12,000 rpm for 10 minutes. The supernatant was carefully removed. The rinsing solutions are 1 x DI water, 3 x 10% aqueous DMSO, 3 x 25% aqueous DMSO, 3 x 75% aqueous DMSO, and 3 x DI water. The final rinsing solutions are to ensure a safe concentration of DMSO for the enzymatic liberation and cleavage of the bound proteins.

Sample Preparation: After cross-linking and rinsing, the resins were centrifuged at 12,000 rpm for 10 minutes, and the supernatant was removed. The resins were rinsed twice with 20 mM NH_4HCO_3, pH 7.4, buffer and resuspended by ultrasonication to give a final 1 mg/ml suspension in NH4HCO3. Aliquots corresponding to 500 nm, 125 nm, 25 nm, and 12.5 nm of bound probe, were transferred to new microfuge tubes. The tubes were centrifuged again and the supernatent removed in preparation for tryptic digest.

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Trypsin Digestion: To each sample was added 5 µl 20 mM NH_4HCO_3 and 5 µl 10x trypsin (0.1 mg/ml trypsin in 50 mM aqueous acetic acid). The tubes were pulsed in a centrifuge to pull the contents to the tip of the microfuge tube and they were incubated for 2 hours at 37 °C with gentle shaking. The tubes were centrifuged and the digest supernatant was removed to clean, labeled microfuge tubes. To the tubes with the resins was added 20 µl 20 mM NH_4HCO_3 buffer and the tubes were vortexed for 20 seconds and centrifuged at 12,000 rpm for 1 minute. The supernatant was combined with each samples respective digest solution. This process was repeated one more time for microfuges tubes containing resin. For each sample, both 2 hour digests and rinses were combined for 60 µl of peptide solution. These solutions were allowed to incubate for 8 more hours at 37 °C before being subjected to mass spectrometric analysis.

Mass spectrometry and analysis: The peptide solution was analyzed by nano-LC-ESI Ion Trap with the intent of using Mascot (Perkins 1999) to search the NCBI database, limited to the peptide source organism, in the case of ConA, that is *C. ensiformis* (Jack Bean). Analysis was performed by the Laboratory for Biological Mass Spectrometry, Department of Chemistry, Indiana University, Simon Hall 120B, 212 S. Hawthorne Drive, Bloomington, IN, 47405.

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CHAPTER III

INVESTIGATION OF SYNTHESIS AND AUTOMATED SYNTHESIS OF HIGHLY BRANCHED OLIGOSACCHARIDES

Introduction

One major bottleneck to the study of carbohydrates, namely glycans, are the problematic routes to access homogenous samples for study. There are three routes in which these biologically relevant compounds can be obtained. Isolation from natural products is a widely shared endeavor of groups who are studying glycans (Yu 2012) and efforts have been made to simplify such tasks (Trader 2011). Material throughput is a large limitation to this method due to the expensive and time consuming separations of complex mixtures. Genetic engineering of metabolic pathways have been employed produce glycan substrates and intermediates (Mizanur 2009). This method also involves isolation from a heterogenic mixture, but in these cases, the target compounds have been amplified and obtaining homogeneous samples is possible. The final route to accessing these compounds is through chemical synthetic methods.

Synthetic strategies are routinely carried out to produce biologically active glycan and glycoconjugates (Lakshminarayanan 2012, Boltje 2012). Despite efforts, access to carbohydrates is still highly limited. Many of these reasons can be attributed to one underlying feature of carbohydrates, their structural diversity (Chang 2011). In brief, combining the number of regio- and stereoisomers for a particular pair of monosaccharides, with the number of possible monosaccharides, and with the number of linkages in oligosaccharides, the number of possible

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compounds grows quickly, on the order of 10^3 for a trisaccharide library arbitrarily limited to 8 monosaccharide building blocks just to give perspective.

Another challenge facing carbohydrate chemistry is the lack of understanding and predictability in the outcome of the glycosylation reaction. There has been several efforts to probe these reactions (Crich 2004) but in spite of these efforts, no precise mechanism has been elucidated (Crich 2010). Much effort can be invested in the preparation of a monosaccharide donor for glycosylation, and failure at this step is very costly.

The preparation of these monosaccharide donors has also not been trivialized. Many donors can take six to seven synthetic steps to prepare, and in some cases upwards of 15 steps have been reported. These steps involve differentiating the reactivity of hydroxyl groups in these polyols, which can lead to difficult to separate regioisomers and sensitive reactions. Development of synthetic routes to building blocks with similar protecting group features would allow for the use of general preparation techniques for donors as well as reproducible and predictable orthogonal deprotection of hydroxyl groups for chain elongation.

Automated chemical methods for carbohydrates would certainly benefit from having predictable deprotection conditions by allowing for the facile production of small arrays of glycans for study. Routine scripts and standard reagent stocks could be used without concern for specific substrate. This would be limited to cases where the glycosylation is easily controlled, such as in cases where the 2 position protecting group will anchimerically participate during glycosylation.

The nematode, *Caenorhabditis elegans*, has been shown to produce a series of oligosaccharides, **1**-**6** (Figure 3.1) (Guerardel 2001). The structurally interesting feature of these saccharides is the heavily branched pattern on the galactose amine residue (**4**) as well as the

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galactose residue (**6**). The similarity in these structures would suggest the enzymes that are responsible for the production of these saccharides is most likey highly erroneous due the steric hindrance, and is likely the cause of the identification of such a structurally related set of oligosaccharides. This highly branched pattern also poses a challenge to current synthetic methods and warrants investigation into the synthetic capabilities to produce such branched oligosaccharides.

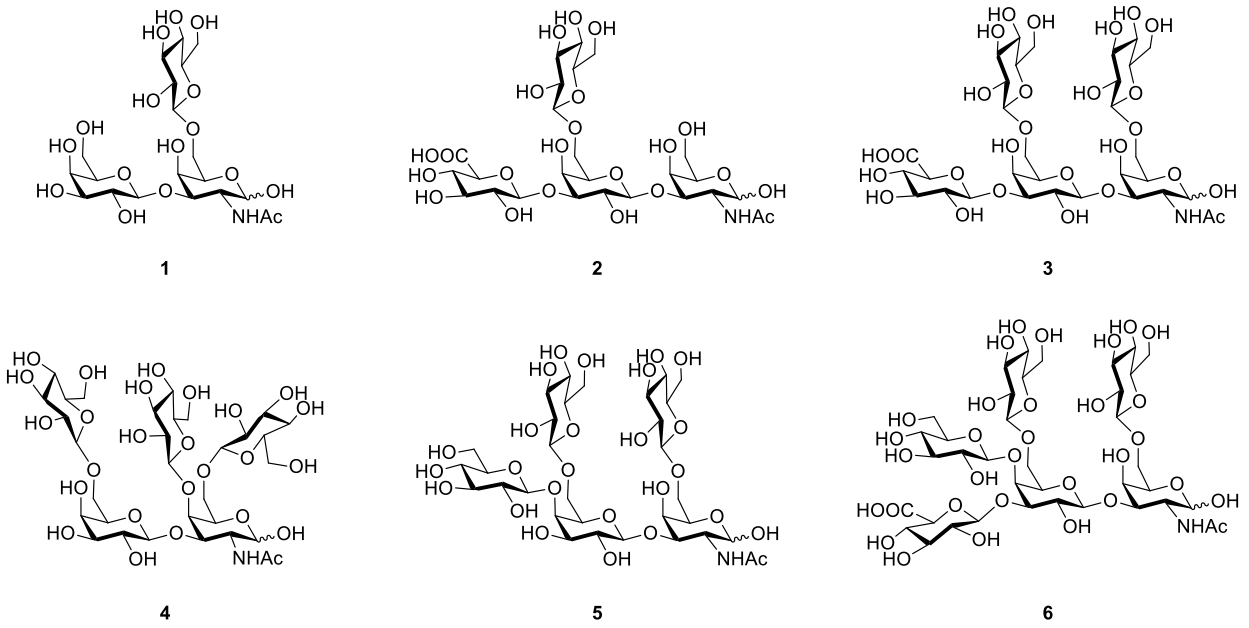

Figure 3.1 Structurally similar oligosaccharides isolated from the nematode *C. elegans*.

A specific *C. elegans* mutant, srf-3 (Cipollo 2004), was found to produce these carbohydrates in considerably reduced quantities. This mutant variant of the nematode was also was found to display immunity to pathogen infection by *Y. pestis*, *Y. pseudotuberculosis*, and *M. nematophilum*. It's logical to implicate these saccharides as being key components in pathogen recognition of host cells. One significant structural feature of this set of carbohydrates is the

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core disaccharide unit Gal-β(1→3)-GalNAc. This core saccharide is the well known TF antigen (Imamura 2005, Ju 2013) and is a conserved feature of mucin type I proteins (Therkildsen 1995). Thus, it's valid to assert a hypothesis that implicates the binding proteins that recognize these carbohydrates in *C. elegans* as well as recognize the TF antigen in mammals.

To probe the ability of creating a donor library for facile production of a series of oligosaccharides, concurrently investigating current glycosylation methods to achieve multiple, sterically hindered glycosyations, and providing synthetic access to an interestingly relevant set of oligosaccharides, pursuit of these targets seemed worthwhile. Here in we report the synthesis of donors to investigate current methods to synthesize heavily branched and sterically hindered glycosylation reactions with standardized deprotection conditions and preliminary results of automation using fluorous tag assisted automated solution phase synthesis of heavily branched oligosaccharides.

Results and Discussion

A protecting group strategy was devised keeping two ideas in mind, the first being minimal steps required for complete deprotection, and the second to be applicable to biological experimental platforms for studies that target the biological significance of such glycans. Protecting groups that are linked through an ester linkage were chosen to be the protecting groups for hydroxyl groups that are not incorporated into glycosidic bonds as the hydrolysis of these functional groups occurring under quite mild alkaline conditions. The envisioned protected carbohydrates (Figure 3.2) meeting those requirements exhibit acetyl, levulinoyl, and methyl esters as hydroxyl and carboxylate esters. The reducing end of the sugar is modified with a

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fluorous alkene, *cis*-3-(perfluorooctyl)propyloxy-2-butenol (**Ftag**), that serves as both a protecting group for the anomeric carbon at the reducing end as well as a handle for purification that is critical for automated synthetic methods. This paritcular handle works in combination with fluorous silica gel and is known as fluorous solid phase extraction (FSPE).

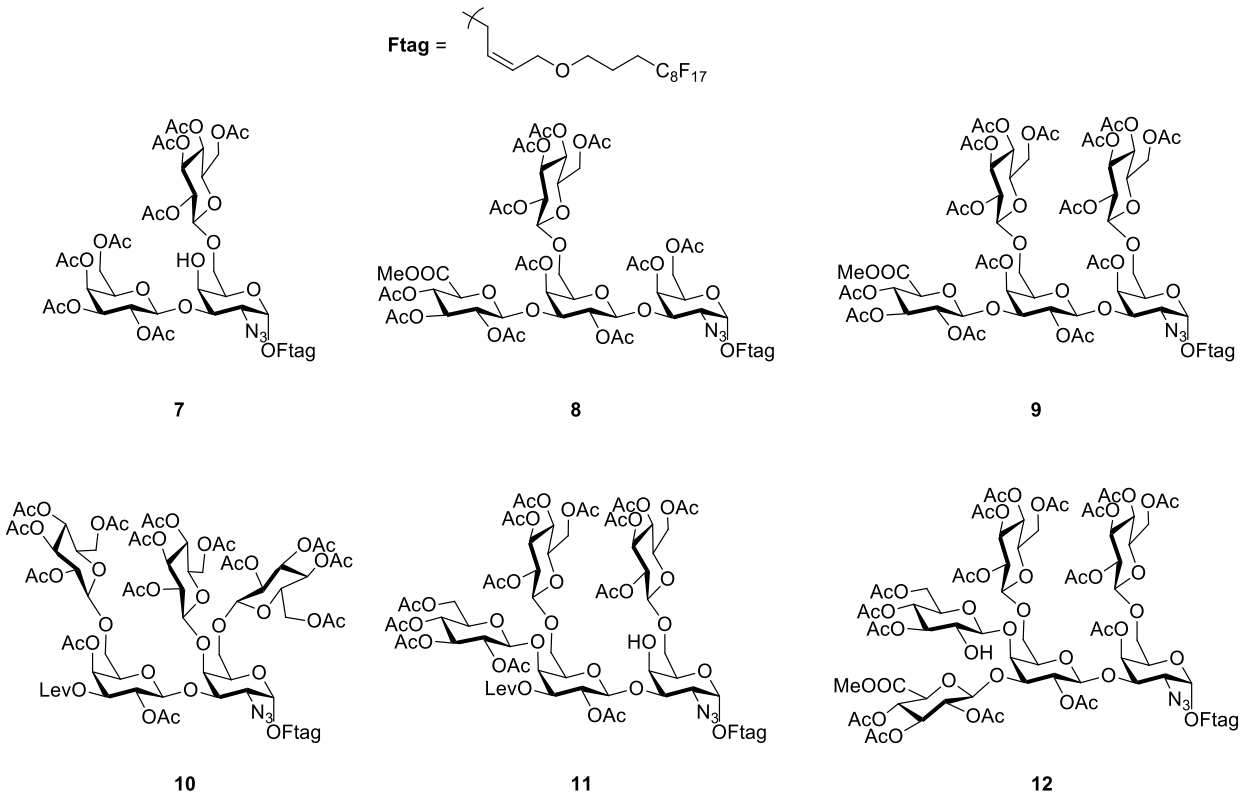

Figure 3.2 Protected saccharide targets from *C. elegan*.

The six target oligosaccharides, all containing the core disaccharide Gal(β1→3)GalNAc, can be prepared from four protected disaccharides, **13-16**, (Figure 3.3) that each have a unique protecting group pattern to allow for orthoganol deprotection conditions to selectively free hydroxyls for glycosylation. Disaccharide **13** would be an intermediate for target trisaccharide **1**, disaccharide **14** would give rise to pentasaccharide **3**, core **15** would be employed to access target pentasaccharide and hexasaccharide **5** and **6**, and **16** would be used for the preparation of

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tetrasaccharide and pentasaccharide **2** and **4**. Retrosynthetic analysis of the general disaccharides results in a need for four donor monosaccharides, two galactose amine monosaccharides for the reducing end carbohydrate, and two galactose monosaccharides for the nonreducing end of the disaccharide , **17-20**.

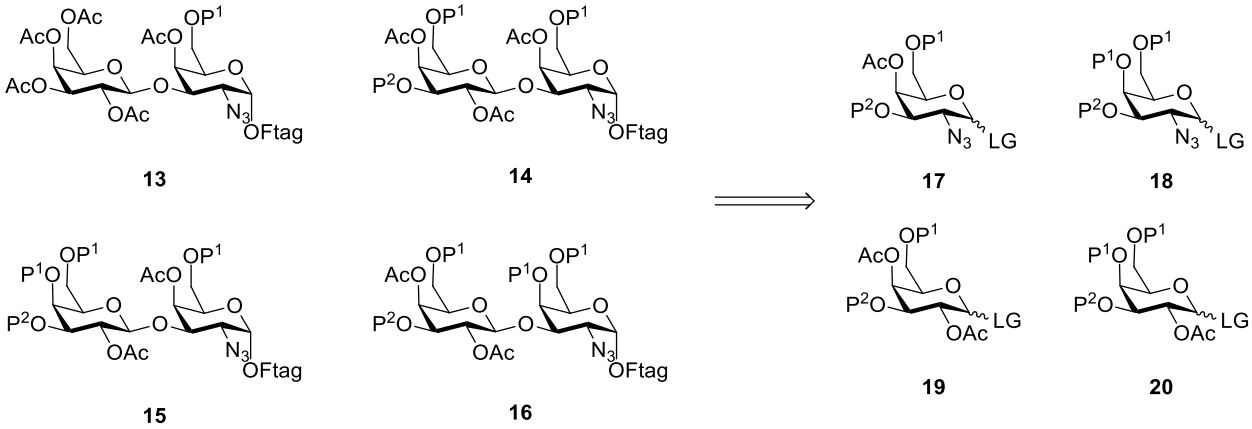

Figure 3.3 Retrosynthetic analysis of core disaccharide to access the six oligosaccharide targets.

The branching pattern of all *C. elegans* saccharides requires two orthogonal protecting groups, one to protect hydroxyls for glycosylation a glucose donor, P^1 (**17-20**), and one for glycosylation with a galactose or glucuronic acid donor, P^2 (**17-20**). The desired ester groups and the azide group in the protected products, **7-12**, excludes protecting groups that require strongly basic or reductive conditions for liberation of the hydroxyl group. The acidic glycosylation conditions exclude acid sensitive groups like methoxy benzyl ethers or trimethylsilyl ethers. However, other silyl ethers are known to have longer half lives in acidic conditions and have many known cleavage conditions across a range of solution pH, allowing for mild, orthogonal deprotection of silyl ethers in presence of esters. Specifically, *tert*-butyldiphenylsilyl (TBDPS) ethers are well known and extensively used in oligosaccharide

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synthesis. One convenient feature of this protecting group is its selective protection for primary hydroxyls, making it ideal for use in building blocks **17** and **19**. Currently, there is no evidence of being able to protect four position hydroxyls of carbohydrates with the TBDPS ether, and many of the other silyl groups might be too labile under glycosylation conditions, another protecting group will need to be employed for donors, **18** and **20**. To avoid unnecessary deprotection steps, a strategy to deprotect both four and six positions of **18** and **20** would be ideal. Benzylidene acetals have been shown to be stable to the acidic conditions of glycosylation and glycosidic bonds are not susceptible to solvolysis under these conditions. Preferential acetal formation with 1,3 diols over 1,2 diols have been applied frequently to carbohydrates to protect the four and six hydroxyl groups and would be a suitable protecting group for both donors, **18** and **20**. The final protecting group needed to protect the three position hydroxyl of the four donor compounds ought to cleave under relatively nuetral conditions to make it orthogonal to the basic cleavage conditions of ester groups and the acidic cleavage conditions of acetals. One protecting group that can conform to these requirements is the levulinate protecting group. This ester will selectively cleave under very mild acidic conditions in the presence of hydrazine.

With this strategy in mind, seven donor compounds, **21-28** (figure 3.4), were envisioned to be necessary for the preparation of all six targets (table 3.1). Trichloroacetimidate (TCA) donors (shmidt 1980) were chosen for ease of preparation, robust reactivity, mild activation conditions, and solubility of byproducts. TCA donors have been employed in numerous glycosylation reactions, in various solvents, under a wide range of temperatures, for a large variety of substrates . Soluble byproducts are desired to make this synthetic route amenable to automated solution phase chemical synthesis of oligosaccharides . One extra galactosamine

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donor was prepared to simplify deprotection of the protected saccharide to obtain target saccharide **2**.

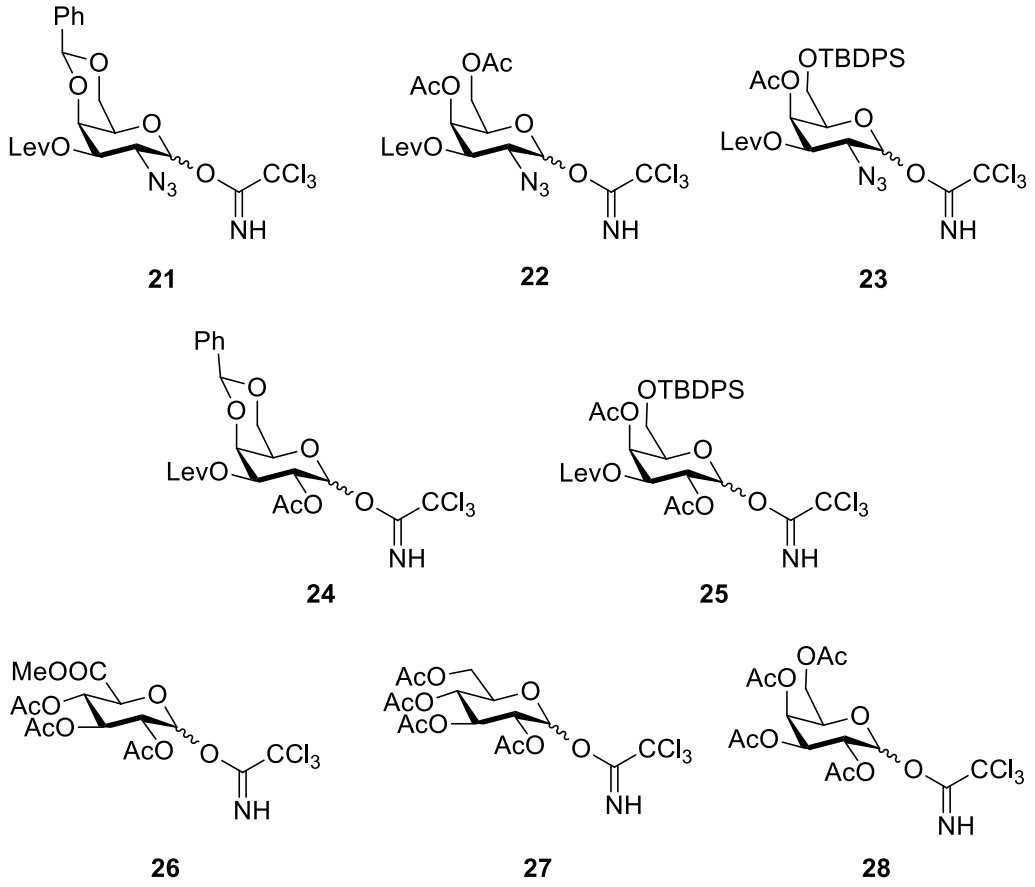

Figure 3.4 Glycosyl donors needed to synthesize all six oligosaccharide targets.

Table 3.1 Donors required by target

Target	Donor 1	Donor 2	Donor 3	Donor 4
1	**23**	**28**	**27**	**-**
2	**22**	**25**	**27**	**26**
3	**23**	**25**	**27**	**26**
4	**23**	**24**	**27**	**-**
5	**21**	**23**	**27**	**-**
6	**23**	**24**	**27**	**26**

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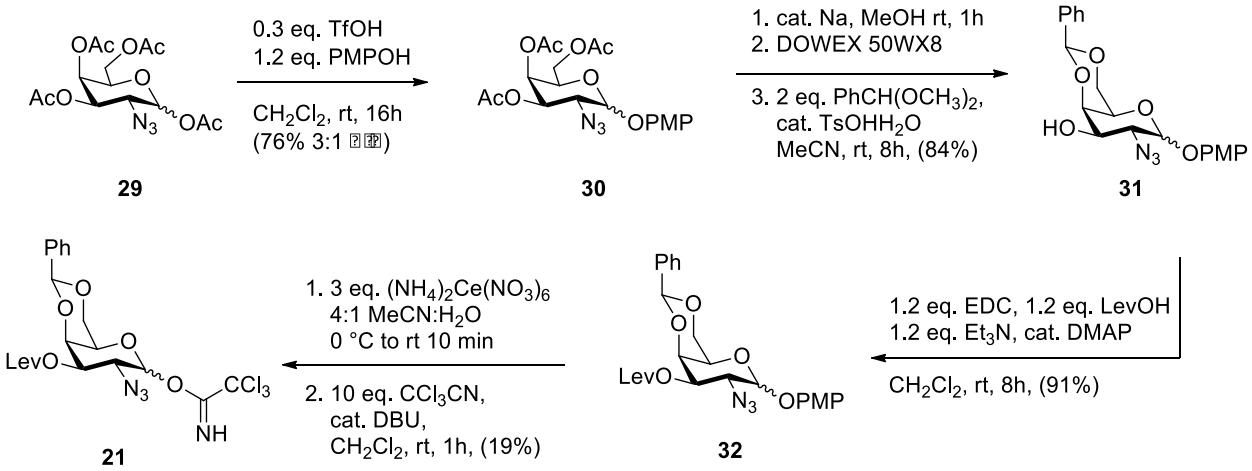
Donors **26**, **27**, and **28** have been prepared previously (Wayne 2010). Preparation of compound **21** (scheme 3.1) originated from the known 1,3,4,6-tetra-O-acetyl-2-deoxy-2-azido-α/β-D-galactoside, **29** (Mukherjee 2008). The glycosidation with *p*-methoxyphenol was achieved with catalytic triflic acid to produce acetal **30** in good yield. Basic solvolysis of the three esters with sodium in methanol, subsequent protonation with DOWEX 50WX8 cation exchange resin, followed by selective acid catalyzed acetal formation with benzaldehyde dimthyl acetal to transform triester **30** to the 4,6-O-benzylidene acetal **31** in 84% yield over three steps. Acylation of the sole alcohol with levulinic acid was effected under standard carbodiimide coupling condition to provide **32** in excellent yield, 91%. Oxidative cleavage of the *p*-methoxyphenyl protecting group with ceric ammonium nitrate (CAN) gave poor yield, but enough material to carry onto TCA formation with catalytic DBU and CCl_3CN, well known conditions, to obtain target donor **21**, in 11% over 7 steps from known compound **29**.



Scheme 3.1 Synthetic route to donor **21**.

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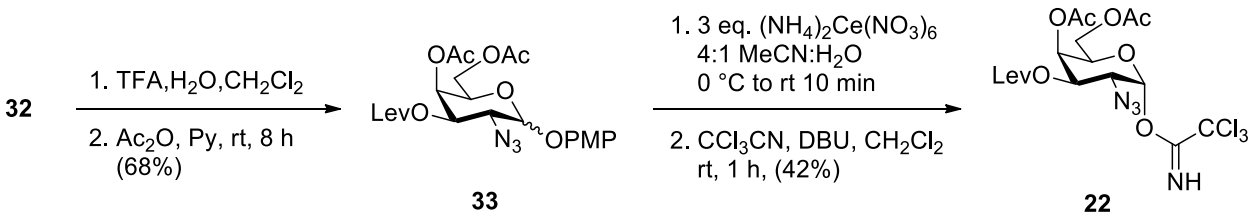
From compound **32** (scheme 3.2), hydrolysis of the benzylidene acetal was accomplished with aq. trifluoroacetic acid and the free hydroxyl groups were acylated with acetic anhydride to afford compound **33** in 68% yield over both steps. Cleavage of the phenolic ether with CAN and then formation of TCA donor **22** with standard conditions provided in modest yield, 17% over 9 steps from known compound **29**.



Scheme 3.2 Preparation of donor **22**.

Access to donor **23** originated from compound **30** (scheme 3.3). Basic hydrolysis of the esters, acidification, and regioselective silylation of the primary hydroxyl group furnished silyl ether **34** in 73% yield over all three steps. Trimethyl orthoacetate and cat. tosylic acid was employed to form an orthoester with the cis diol of **34**, which was regioselectively opened to the axial ester. The final hydroxyl group was converted to the levulinate ester with levulinic acid and EDC to provide compound **35** in 85% yield over all three steps. Cleavage of the *p*-methoxyphenyl ether was achieved with CAN and the hemiacetal was transformed to TCA donor, **23** in total of 29% over 9 steps from **29**.

With all necessary galactosamine donors prepared, attention was turned to the two necessary galactose donors, **24** and **25**. The route to **24** initially started from known *p*-methoxyphenyl 4,6-O-benzylidene-β-D-galactopyrannoside, **36** (scheme 3.4). Regioselective protection of the three

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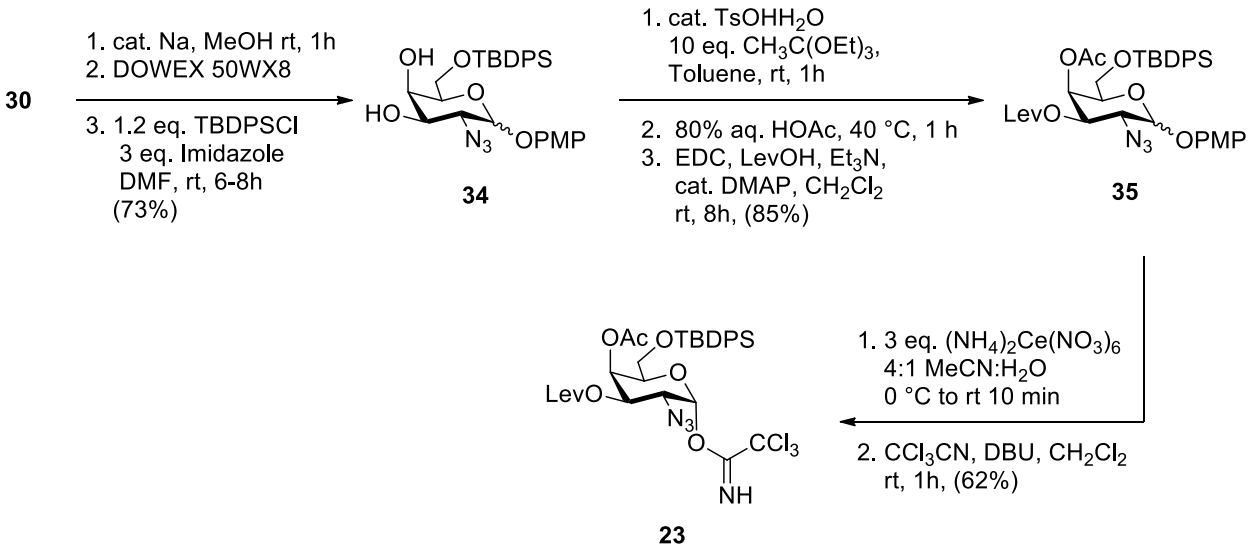
position hydroxyl with *tert*-butylchlorodimethylsilyane was achieved after 24 hours in DMF to give isomer **37** in 66% yield, which was easily separated from the 2-TBS regioisomer on silica gel. The acylation of alcohol **37** initially proved unreactive towards standard conditions until catalytic DMAP was added. The reaction then proceeded, slowly, over



Scheme 3.3 Synthetic scheme for donor **23**.

8 hours, which was then directly subjected to desilylation conditions. Standard conditions which employ TBAF as the fluoride donor was presumed to be provoking migration of the two position acetate to the three position acetate. Buffered conditions with acetic acid didn't prevent migration completely, but switching fluoride source to thriethylamine trihydrogen fluoride complex, buffered with Et_3N, provided compound **38** in 68% yield over both steps. Alcohol **38** was converted to levulinate ester **39** with standard carbodiimide coupling in great yield. Oxidative cleavage of the *p*-methoxyphenyl ether was completed with CAN and followed by TCA formation at the hemiacetal to furnish donor **24** in 29% yield over 6 steps.

The final galactose building block needed for the preparation of the oligosaccharide series began with known *p*-methoxyphenyl 6-O-*tert*-butyldiphenylsilyl-β-D-galactopyrannoside,

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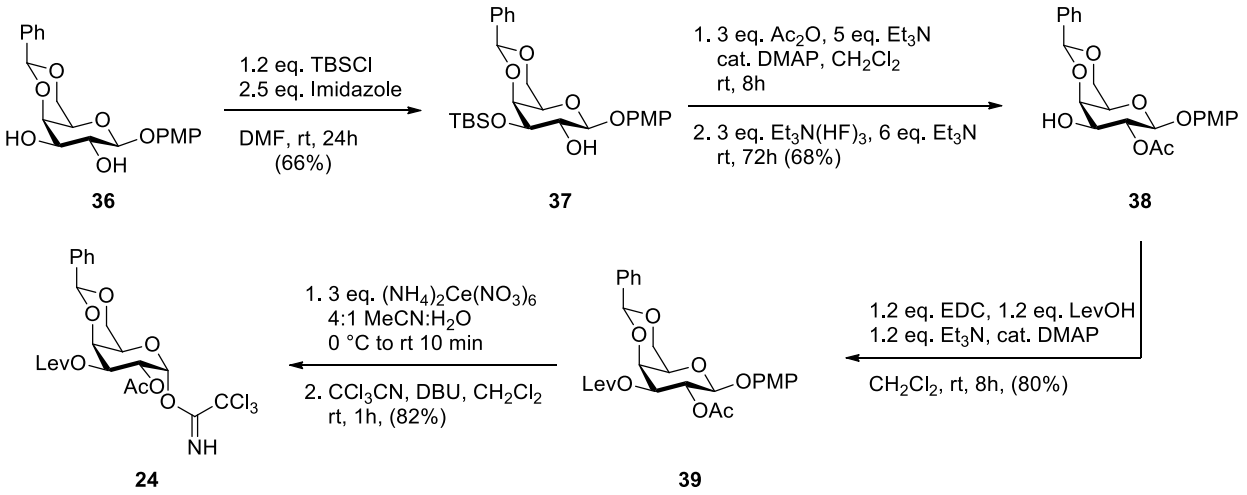
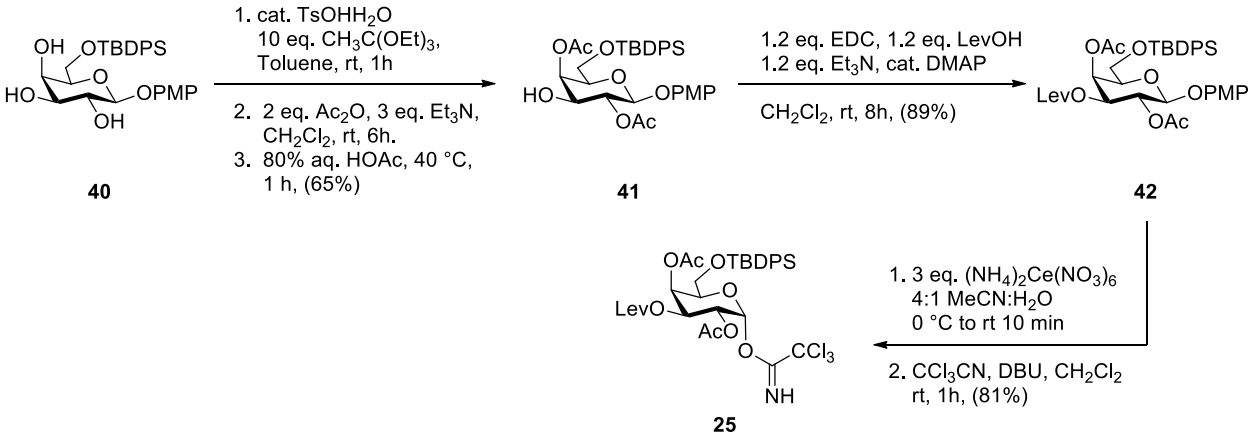
40 (scheme 3.5). The *cis* diol was first converted to an orthoester using trimethyl orthoacetate, the remaining hydroxyl was acylated with acetic anhydride under basic conditions, and then the orthoester was regioselectively opened to the axial ester to give alochol **41** in 65% yield over all three steps. The remaining three position hydroxyl group was converted to the levulinate ester under standard coupling conditions to access compound **42** in high yield. The anomeric ether was oxidatively cleaved with aqueous CAN and converted to TCA donor **25**, in 47% yield over 6 steps.



Scheme 3.4 Preparation of donor **24**.



Scheme 3.5 Synthesis of donor **25**.

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With all the necessary intermediate donor targets prepared, efforts were directed towards the glycosylation and deprotection conditions to finish preparing the six target oligosaccharides. The main question in this study is if it is possible to prepare the highly branched oligosaccharide structures that feature the 3,4, and 6 glycosylation pattern (**4** and **6**) with current chemical methods. Thus, target **4** was chosen for preliminary experiments. The first glycosidation reaction will install the fluorous tag compound at the reducing end of target **4** prior to saccharide extension glycosylations. The initial glycosidation reaction, (Table 3.2, entry 1) was surprisingly selective for formation of the β anomer. Literature precedent had given the hypothesis that this building block would give good selectivity for the α anomer (Zhitao Li, Seeberger). Further discussion with Zhitao Li indicated that he only observed his reported selectivity with deactivated acceptors and the selectivities were substrate (acceptor) controlled. Work published later (Zhitao Li) indicated that with simple nucleophiles give excellent β selectivities with 2-deoxy-2-azido TCA donors. The strereochemistry of the linkage of these carbohydrates *in vivo* is unknown but are presumed to be α due to core structural similarities of these carbohydrates and the mucin glycans, which are found as α O-linked glycosides.

There is evidence in literature (Park 2007) that suggests using additives during glycosylation can influence stereoselectivity of the resulting glycosidic bond. Specifically, the addition of 10 equivalents of thiophene could lead to the formation of α glucosides presumably through coordination of the sulfur atom of thiophene to the oxocarbenium intermediate (Scheme 3.6) in the β conformation, which is assumed to be due to steric effects based on computational studies. Investigation of this additive did have a large effect on the reaction outcome, shifting the results to about a 1:1 ratio of α to β. After thiophene was shown to be only mildly able to

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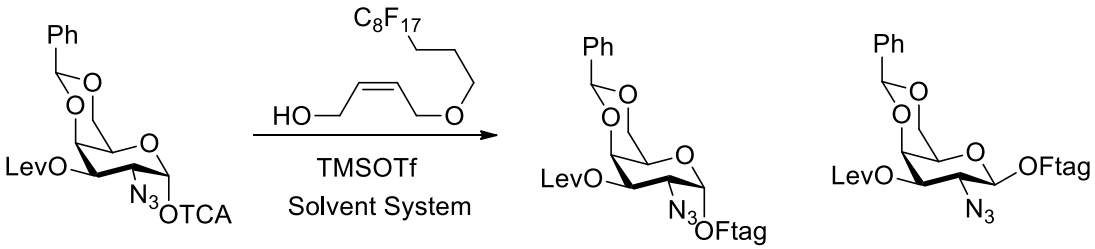
controlling the selectivity of the glycosidation completely, it was hypothesized that a participating solvent might have a more drastic effect in the outcome of the reaction.

Table 3.2 Glysoylation of donor **21** with acceptor **Ftag**. Selectivities were determined on by isolated mass. All glycosylations were carried out using 3 eq. of **21**, 0.3 eq. TMSOTf, 1 eq. **Ftag** alcohol, and 10 eq. thiophene when used. The reaction was run at a concentration of 0.4M with respect to donor **21**. [a]Concentration of donor **21** was 0.01M with all other equivalents held constant.



21 **43** **44**

Entry	Solvent	Thiophene	Temperature	Yield	Selectivity
1	CH_2Cl_2	N/A	25 °C	80	All β
2	CH_2Cl_2	10 eq.	0 °C	70	1.2:1 α/β
3	CH_2Cl_2	10 eq.	25 °C	73	1.1:1 α/β
4	Et_2O	N/A	0 °C	30	2.5:1 α/β
[a]5	Et_2O	N/A	0 °C	82	3.5:1 α/β

The reaction with thiophene suggested that cooridanation of the intermediate can be a solution to the stereochemical outcome of the glycosidation and thus, solvents effects were explored. Participating solvents are well known in organic synthesis, but solvents of particular

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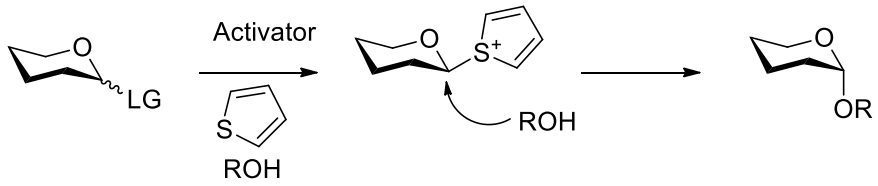

Scheme 3.6 Hypothesized role of thiophene in generation of α glycosides.

interest in glycosylations are ether, acetonitrile, and toluene. All three of these solvents posses either lone pairs or enough electron density to donate to the oxocarbenium cation intermediate during glycosyalation. Acetonirtrile has been shown to primarly prefer to coordinate to donors from the α face of the cation, giving rise to β anomer products while conversely to that, ethers, notable diethyl ether and dioxane, have been used due to their preferential coordination to the carbocation via the β face, giving rise to favored α glycosides. Diethyl ether was then investigated as the solvent during glycosylations with the fluorous tag acceptor. In diethyl ether, the reaction provided a 5:2 mixture in favor of the α anomer but the yield of the reaction fell to only 30%. While the selectivity for the α anomer did improve, the magnitude was not adequate when considering the cost of all the subsequent donors and poor yield, other solutions were investigated.

Di-*tert*-butylsilylene protected carbohydrates have started appearing frequently in oligosaccharide synthesis in recent literature. Similar to many reactions involving carbohydrates, the effect of these groups is heavily dependent on the carbohydrate. These groups have been shown to invoke a ring flip of the carbohydrate during glycosylations in some cases, or just prevent the formation of one anomer through steric blockage of a particular face of the carbohydrate. The use of these with 2-deoxy-2-galactose derivatives have now appeared, studies into their mechanistic effects have not yet been pursued to my knowledge. However, in one

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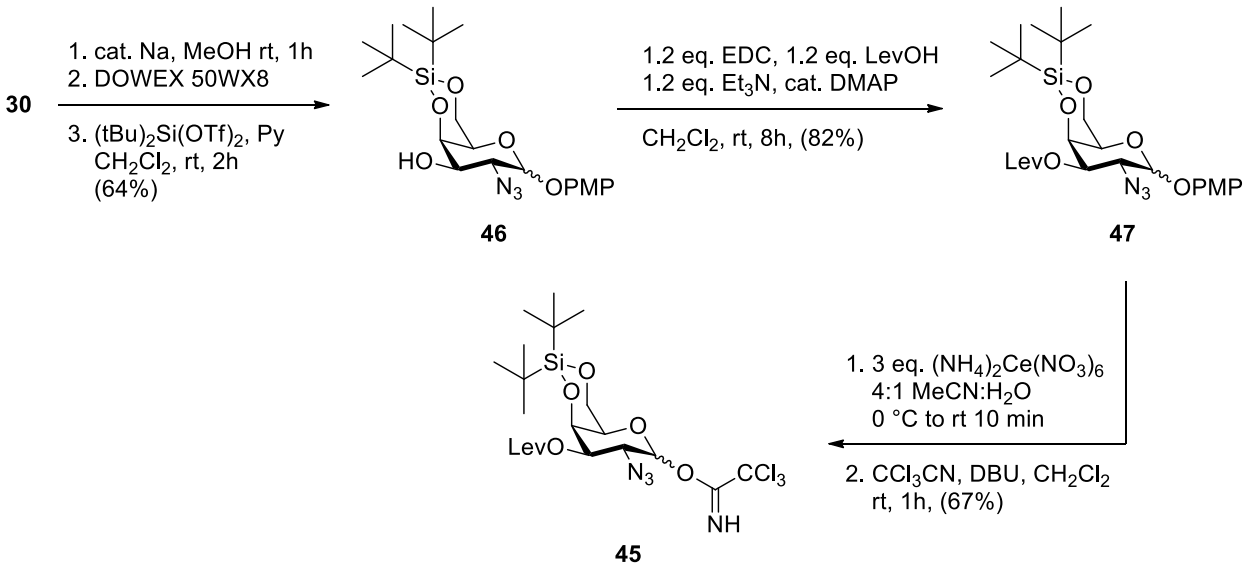
case, it was shown that such a silylene based 2-deoxy-2-azido galactose based derivative gave excellent α selectivity. Thus donor **45** was envisioned to provide good α selectivities while still allowing for a simultaneous deprotection of both the four and six position hydroxyls (Scheme 3.7).



Scheme 3.7 Preparation of donor **45**.

Synthesis of donor **45** started from previously prepared **30** (Scheme 3.7). The acetate protecting groups were removed by basic solvolysis with sodium in methanol, which was acidified with DOWEX acidic resin, and the resulting triol was converted to the 4,6 silylene **46** in modest yield. Alcohol **46** was transformed to levulinate ester **47** with standard carbodiimide coupling conditions in good yield. The *p*-methoxyphenyl ether of **47** was oxidatively cleaved with CAN, and the resulting hemiacetal was transformed to the TCA donor, **45**, with base and CCl_3CN in 27% yield over 8 steps from **29**.

Donor **45** was explored as a glycosyl donor with the fluorous tag alcohol acceptor (Table 3.3). The reaction in methylene chloride at room temperature afforded only α anomer in

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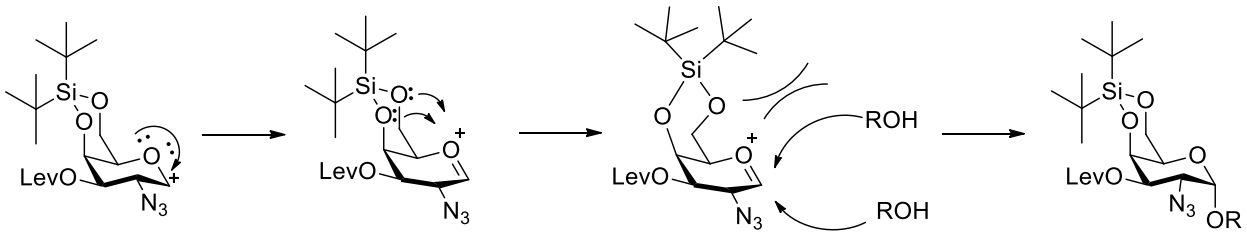

Scheme 3.8 Proposed silyl group influence with stereoselectivity.

good yields. The selectivity for formation of the α anomer is thought to arise out of steric hinderance of the α face (scheme 3.8). Persumable, after formation of the carbocation, the oxygen will donate its electrons into the vacant carbocation orbital, and the resulting planarization puts structural strain on the 4,6 fused ring system. In turn, this causes the secondary ring structure to pucker. It's hypothesized (Imamura 2005) that the loan pair on the oxygens in the silylene donating towards the carbocation will influence the tilt of the *tert*-butyl groups to be over the carbocation, successfully hindering the β face of donor **45**.

With glycosylation conditions suitable for automation elucidated, the fluorous tag assisted solution phase automated synthesis and investigation of these glycosylations commenced. Automation was performed on a Chemspeed Automation Synthesis Workstation 1000 robotics platform (ASW). A series of macrotasks were designed (Table 3.3) that would be amenable to any of the protected targets (**7**-**12**). The macro tasks were created such that when setting up new programs for a particular target, minimal changes would be necessary. The only changes would be adjusting zones (specific vial locations), times and volumes.

Once the variable macro tasks were established, a program was designed to prepare **10** and investigate current chemical methods' to achieve a heavily branched 3,4 and 6 position glycosylations (figure 3.5). The envisioned route started with TMSOTf catalyzed glycosidation

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Table 3.3 Program flow for ASW protocol for **10**. [a]Only if task includes intentional waits or time consuming tasks. Where A, D, RV, RT, TLC, FSPE, WASTE, COLLECT, DIRECT, MeOH, 80MeOH, DS are variables that are set for each macro task prior to running.

Macro Task	Step	Sub Tasks	Operational Time[a]
Evaporation	1 2 3	Heat/Cool Apply vacuum Heat/Cool	45 minutes
Glycosylation	1 2 3 4 5 6 7 8	Transfer A to RV Transfer D to RV Heat/Cool Transfer TMSOTf to RV Wait for RT Transfer TLC aliquot Transfer Et_3N Evaporation	Variable
Drying Cycle	1 2	Transfer toluene to RV Evaporation Repeat from 1 twice	2 hours
FSPE	1 2 3 4 5 6 7 8 9 10 11 12	Set FPSE to WASTE Transfer RV to FSPE Transfer MeOH to RV Transfer H_2O to RV Transfer RV to FSPE Transfer 80MeOH to FSPE Set FSPE to COLLECT Transfer MeOH to FSPE Set FSPE to DIRECT Transfer FSPE to next RV Evaporate Repeat from step 8	About 2 hours
Deprotection	1 2 3 4	Transfer DS to RV Heat/Cool Wait for RT Transfer TLC aliquot	Variable

of **45** with **Ftag** followed by cleavage of the levulinic ester with hydrazine hydrate. The reaction was quenched with water and loaded directly on to FSPE for purification from the reaction mixture. The resulting acceptor was then subjected to glycosylation with donor **25**.

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Directly after the reaction, desilylation was performed with TBAF and HOAc in DMF. The reaction was again loaded directly onto FSPE for purification of the fluorous containing material. The glycosylation of the presumed triol intermediate was attempted using six equivalents of donor **27**. The program (Table 3.4) made use of the previously described macro tasks and included the removal of aliquots for TLC.

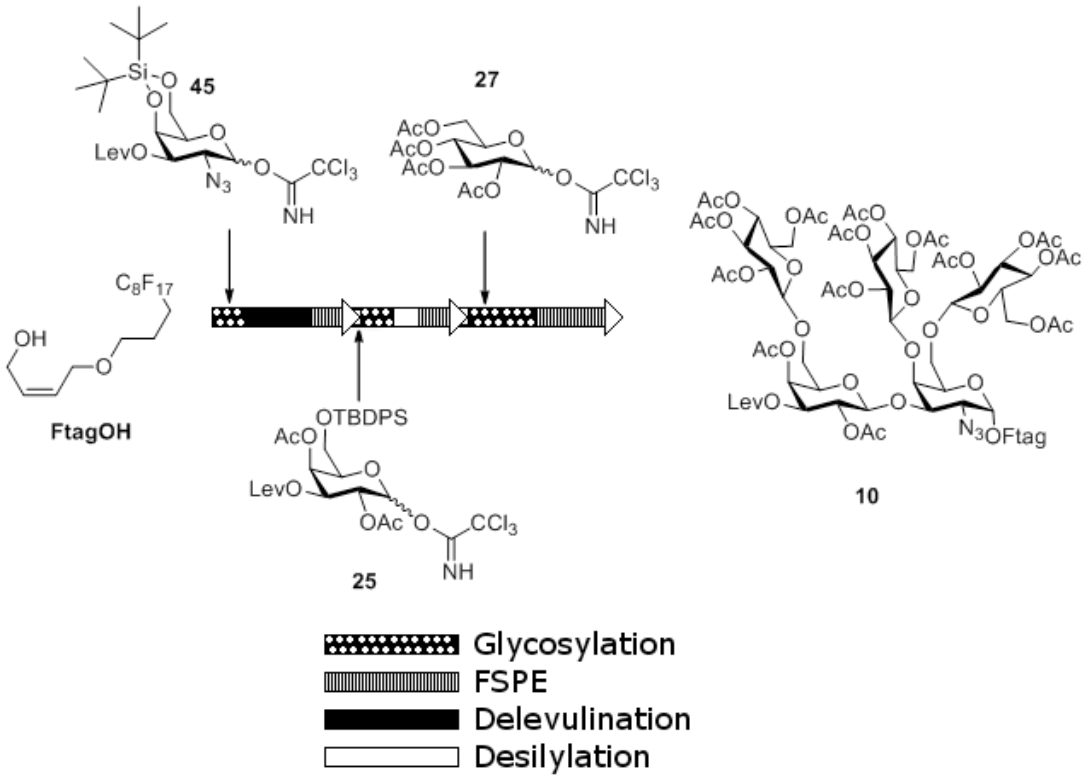

Figure 3.5 Visual diagram for automated synthetic protocol.

After the program was run, the obtained fluorous compounds were analyzed and program and reaction conditions produced a highly impure sample. No product was readably discernible from the mixture, but one convenient aspect of solution phase automation synthesis is the ability

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Table 3.4 Program for ASW to obtain target **10**. Macro tasks and simple tasks included.

Step	Macro Task	Variables
1	Drying Cycle	All Reactor Vials
2	Glycosylation	Reactor Vial 1 0.45 ml **Ftag** 0.45 ml **45** 25 °C 0.1 ml TMSOTf 45 minutes
3	Deprotection	0.4 ml Hydrazine Solution in DMF 25 °C 180 minutes
4	Liquid Transfer	0.1 ml H_2O to Reactor Vial 1
5	FSPE	Source: Reactor Vial 1 0.7 ml Crude Volume FSPE station 1 Destination: Reactor Vial 2
6	Drying Cycle	
7	Glyosylation	Reactor Vial 2 No Acceptor Transfer 0.9 ml **25** 0 °C 0.1 ml TMSOTf 45 minutes
8	Deprotection	0.5 ml TBAF/HOAc in DMF 25 °C 60 minutes
9	FSPE	Source: Reactor Vial 2 0.7 ml Crude Volume FSPE station 2 Destination: Reactor Vial 3
10	Drying Cycle	
11	Glycosylation	Reactor Vial 3 No Acceptor Transfer 1.8 ml **27** 0 °C 0.1 ml TMSOTf 45 minutes
13	Liquid Transfer	0.5 ml DMF to Reactor Vial 3
14	FSPE	Source: Reactor Vial 3 0.7 ml Crude Volume FSPE station 3 Destination: Reactor Vial 4

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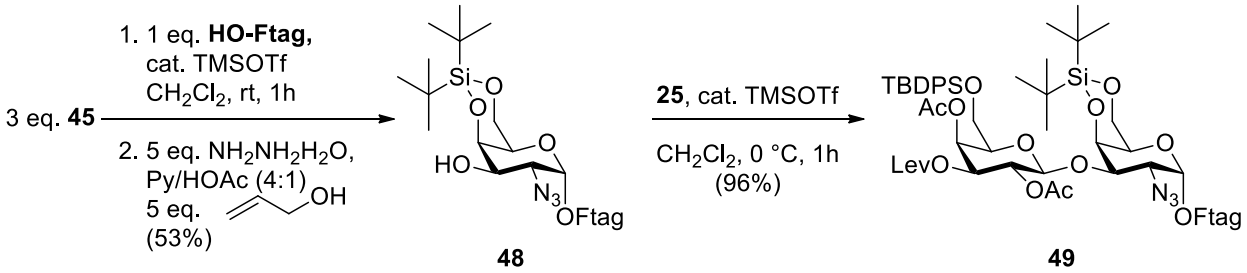
to take TLC aliquots. TLC analysis of the aliquots quickly identified the problem step as being related to the desilylation conditions. To verify that this was indeed the problem step, bench top methods were employed. This can be done without needing to worry about later optimization on the platform as optimization on the bench top translates directly to solution phase automated synthesis.



Scheme 3.9

Starting with donor **45**, glycosidation with the fluorous tag was performed in methylene chloride at room temperature (Scheme 3.9). The crude was directly subjected to hydrazine under mild acidic conditions to cleave the levulinate ester to provide acceptor **48** in 53% yield over both steps. Donor **25** was subjected to glycosylations conditions with acceptor **49** to produce disaccharide **50** in excellent yield, 96%. Desilylation with TBAF and acetic acid conditions (as used on the ASW platform) and TBAF resulted in several decomposed products as indicated by TLC analysis of the reaction mixture (Scheme 3.10). Triethylamine trihydrogen fluoride complex was one condition that seemed to provide only one product as seen by TLC. This condition was persued and **49** was subjected to triehtlymine trihydrogen fluoride buffered with triethylamine, which was completely consumed after 3 days. The reaction produced what is presumed to be an inseparable mixture of **50** and **51** based on ^{1}H NMR and ^{13}C NMR, in good yield. The mixture was subjected to glycosylations conditions (Scheme 3.11) with donor **27** to

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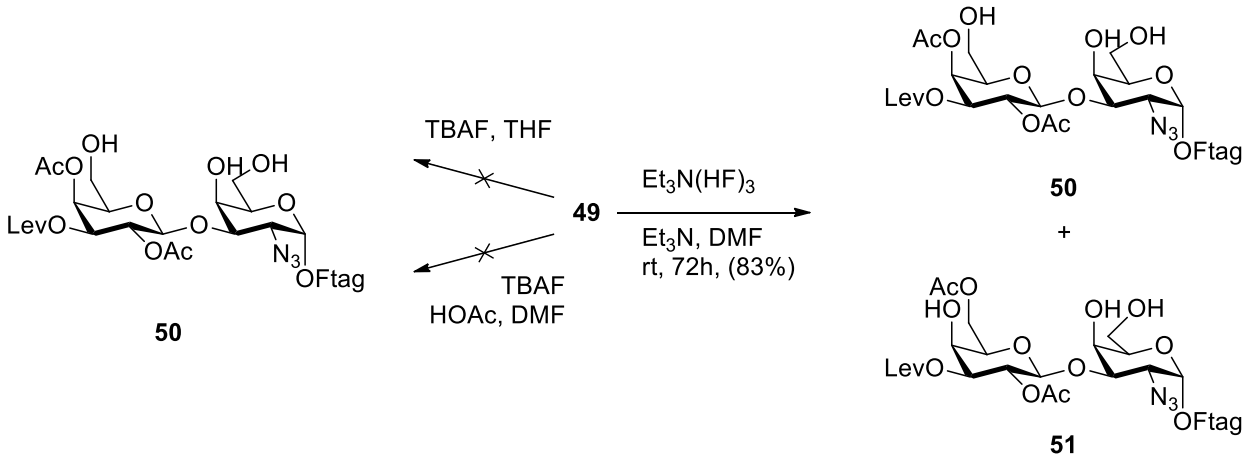
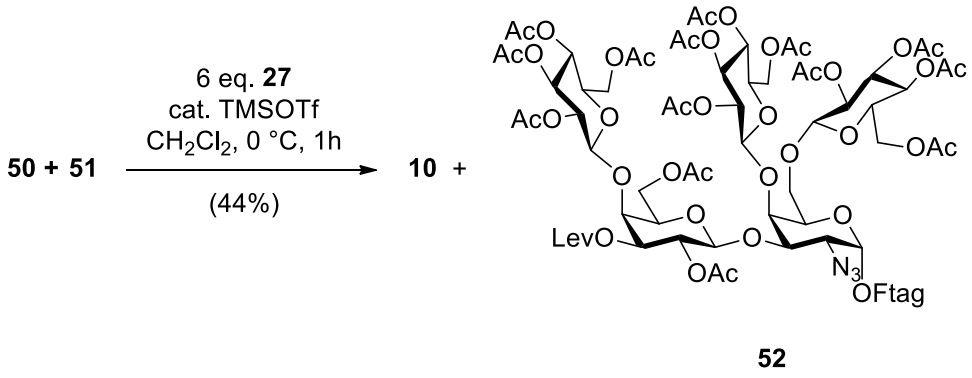


Scheme 3.10 Desilylation conditions investigated for **49**.

investigate the possibility of forming multiple glycosidic bonds in one pot of a 4,6 diol after the three position has already been incorporated into a glycosidic bond. Six equivalents of donor **27** were initially used but after all the donor had decomposed, several compounds were present by TLC analysis. Another six equivalents of **27** were added which resulted in several spots on TLC to vanish. Separation of the material is possible, but currently, only HRMS has been successful in characterizing the compound. The hypothesized products are compounds **10** and **52,** which were obtained in 44% yield, both of which feature the 3,4,6 branching pattern.



Scheme 3.11 Triple glycosylation on 6' and 4,6 diols of mixture of **51** and **52**.

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Conclusion

Many methods are available for obtaining glycans but chemical synythetic methods are highly attractive for obtaining chemically well defined structures. The attactiveness will further grow as combinatorial chemical methods continue to improve. Reported here in this project is the rational design of monosaccharide building blocks that could be employed for the rapid production of an array of related oligosaccharides. One interesting challenge of this carbohydrates investigated was the possibility to perform multiple glycoslyations in one step as well as investigate the capabilities of current methods to affect a 3,4, and 6 glycosylation pattern on a singular monosaccharide unit. Despite poor desilylating conditions, it has been shown here that such reactions are possible with current methods.

To expand on this, it has been shown here how solution phase automated methods can be used to probe glycosylation and deprotection conditions by allowing for stepwise monitoring of the performed reactions. This is unique only to solution phase automated methods, other methods such as those making use of solid supports do not have this utility. Another issue with solid supports is the optimization conditions necessary for each step do not simply translate due the solid support. One major concern is that concentration is hard to control in biphasic reactions which can have huge impacts on glycosylation outcomes. Deprotection reagents and solvents can be adsorbed onto the resin which can have detrimental effects on subsequent reactions. One other large concern I would have is that solid phase typically calls for 10 equivalents of donor for each glycosylation. If trying to do such a glycosylation as shown here, the number of equivalents needed could be in incredibly wasteful excess, even if possible. Solution phase methods combined with a good rational design of building blocks allows for minimal reaction

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optimization and programming in attempts to construct arrays of oligosaccharides that would be useful in biological studies.

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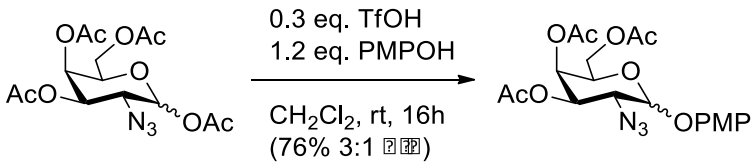
Experimental Section

General: All commercially available compounds were purchased from TCI, Sigma Aldrich, or Fisher Scientific; they were reagent grade and used without further purification. For sensitive reactions, solvents were dried prior to use by distillation from a suitable drying agent. Column chromatography was performed with ZEOprep ECO silica gel 60 with 40-63 μm particle size (American International Chemical). Reactions were monitored using thin layer chromatography coated with a 0.25 mm layer of silica gel 60 F_{254} (Sorbent Technologies). Compounds were visualized with UV light and/or 5% *v:v* sulfuric acid in ethanol. ^{1}H NMR and ^{13}C NMR were performed with either a Varian i400 MHz or Varian i500 spectrometer, or otherwise noted. All ^{1}H NMR peak assignments were made using solvent residual peaks as an internal standard (DMSO δ 2.50 ppm, $CDCl_3$ δ 7.26 ppm, and CD_3OD δ 3.31) as were ^{13}C NMR peak assignments (DMSO δ 39.51 ppm, $CDCl_3$ δ 77.31 ppm, and CD_3OD δ 49.00) and supported using ^{1}H-^{1}H COSY experiments as needed. High resolution mass spectra were obtained with a Thermo Electron Corporation MAT 95XP-Trap for compound characterization. Automation was performed on a modified Chemspeed ASW-1000 robotics platform.



Synthesis of *p*-methoxyphenyl 3,4,6-tri-O-acetyl-2-deoxy-2-azido-α/β-D-galactoside (30). A solution of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-azido-α/β-D-galactoside (7.26 g, 19.4 mmol) and 4-methoxyphenol (2.9 g, 23 mmol) in methylene chloride (50 ml) under argon was cooled to 0 °C in an ice bath while stirring. Trifluoromethanesulfonic acid (0.52 ml, 5.8 mmol) was added dropwise (0.52 ml in 5 minutes). The reaction was removed from the ice

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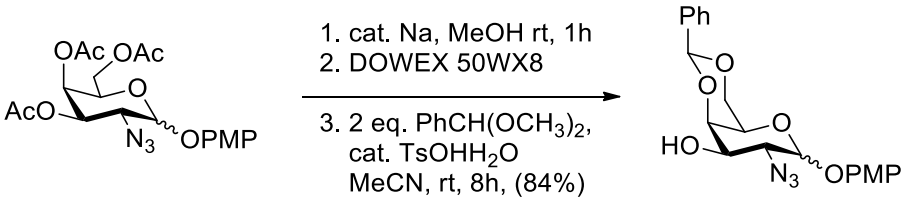
bath and let stir for 16 hours at room temperature. The reaction was diluted with methylene chloride (300 ml) and the organic layer was washed with DI water (3x250 ml), sat. sodium bicarbonate (3x250 ml) and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was purified by column chromatography on silica gel (30% EtOAc in Hexanes) to afford **30** (6.49 g, 14.83 mmol, 76%) as a light yellow oil which turned to a white foam under high vac.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.03 (d, *J* = 9.1 Hz, 2H), 6.83 (d, *J* = 9.1 Hz, 2H), 5.56 (dd, *J* = 11.1, 3.2 Hz, 0.75H), 5.51 (d, *J* = 3.2 Hz, 1.5H), 5.37 (d, *J* = 2.8 Hz, 0.25H), 4.84 (dd, *J* = 10.9, 3.3 Hz, 0.25H), 4.79 (d, *J* = 8.0 Hz, 0.25H), 4.39 (t, *J* = 6.6 Hz, 0.75H), 4.21 (dd, *J* = 11.3, 6.9 Hz, 0.25H), 4.17 – 4.05 (m, 2H), 3.99 – 3.90 (m, 0.5H), 3.82 – 3.72 (m, 3.75H), 2.16 (d, *J* = 5.0 Hz, 3H), 2.11 – 1.95 (m, 6H);

^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.54, 170.25, 170.06, 170.01, 156.18, 155.90, 151.05, 150.47, 118.98, 118.49, 114.95, 114.86, 102.16, 98.21, 71.20, 68.41, 67.76, 67.65, 66.47, 61.78, 61.54, 60.91, 57.63, 55.90, 20.92, 20.86.;

HRMS Calcd for $[M+Na]^+$: 460.1332 Found: 460.1333.



Synthesis of *p*-methoxyphenyl 2-deoxy-2-azido-4,6-O-benzylidene-α/β-D-galactoside (31): To a solution of compound **30** (1.07g, 2.45 mmol) in methanol (5 ml) was

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added solid sodium (0.056 g, 2.45 mmol). The reaction was let stir for 1 hour at room temperature. The base was neutralized with DOWEX 50WX8 (H^+) cation exchange resin. The resin was filtered and rinsed with methanol. The methanol was removed *in vacuo* and trace methanol or water was removed by co-evaporation with toluene (3 x 5 ml). The crude product was used directly in the next step. A flask containing the crude product was flushed with argon, cat. *p*-toluenesulfonic acid monohydrate (0.045 g, 0.25 mmol) was added and the flask equipped with a stir bar. To the solids was added acetonitrile (5 ml), followed by benzaldehyde dimethyl acetal (0.55 ml, 3.67 mmol) while stirring. The reaction was let stir for 8 hours at room temperature. The reaction was quenched with triethylamine (0.1 ml, 1.3 mmol) and the solvent was removed *in vacuo*. The crude was purified by flash column chromatography on silica silica gel (20% to 30% EtOAc gradient in hexanes) to afford **31** (0.82 g, 2.05 mmol, 84%) as a pale yellow oil that turned into a white foam under high vac.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.51 (dd, *J* = 6.6, 3.2 Hz, 2H), 7.46 – 7.32 (m, 3H), 7.07 (dd, *J* = 12.0, 5.2 Hz, 2H), 6.92 – 6.78 (m, 2H), 5.67 – 5.51 (m, 1.75H), 4.74 (d, *J* = 8.1 Hz, 0.25H), 4.44 – 4.31 (m, 1.75H), 4.26 (dd, *J* = 12.7, 1.3 Hz, 0.75H), 4.17 (d, *J* = 3.3 Hz, 0.25H), 4.09 – 4.00 (m, 1H), 3.87 (s, 1H), 3.77 (s, 3H), 3.69 (dd, *J* = 10.4, 3.3 Hz, 0.75H), 3.65 – 3.55 (m, 0.25H), 3.49 (s, 0.25H), 2.72 (dd, *J* = 32.3, 10.0 Hz, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.9, 155.5, 151.3, 150.7, 137.4, 129.6, 128.5, 128.5, 126.6, 126.4, 119.1, 117.8, 115.0, 114.8, 102.0, 101.7, 101.5, 98.4, 75.6, 74.6, 71.6, 69.4, 69.2, 67.6, 66.9, 64.0, 63.6, 60.7, 55.9, 55.9;

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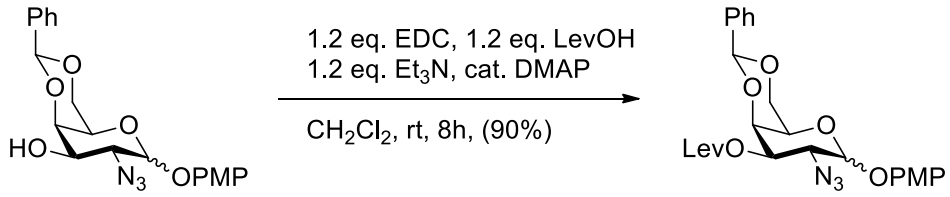
HRMS Calcd for $[M+Na]^+$: 422.1328 Found: 422.1341.



Synthesis of *p*-methoxyphenyl 2-deoxy-2-azido-4,6-O-benzylidene-3-O-Levulinoyl-α/β-D-galactoside (32): To a solution of compound **31** (0.68 g, 1.71 mmol), EDC (0.49 g, 2.56 mmol), and DMAP (21 mg, 0.17 mmol) in methylene chloride (20 ml) was added levulinic acid (0.26 ml, 2.56 mmol) and triethylamine (0.36 ml, 2.56 mmol) while stirring. The reaction was let stir for 8 hours at room temperature. The reaction was diluted with methylene chloride (100 ml), the organic layer was washed with 5% $CuSO_{4(aq)}$ (2 x 150 ml), DI water (2 x 150 ml), sat. sodium bicarbonate (3 x 150 ml) and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was purified by column chromatography on silica gel (20% to 40% EtOAc in hexanes) to afford compound **32** (0.77 g, 1.54 mmol, 90%) as a pale yellow oil that turned to a white foam under high vac.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.61 – 7.47 (m, 2H), 7.38 (dd, *J* = 4.7, 2.4 Hz, 3H), 7.13 – 7.02 (m, 2H), 6.91 – 6.78 (m, 2H), 5.65 (d, *J* = 3.3 Hz, 0.75H), 5.61 – 5.52 (m, 1.75H), 4.87 – 4.77 (m, 0.5H), 4.51 (d, *J* = 3.0 Hz, 0.75H), 4.39 – 4.20 (m, 1.25H), 4.15 (dd, *J* = 10.8, 8.1 Hz, 0.25H), 4.05 (dd, *J* = 11.1, 3.3 Hz, 1.75H), 3.90 (s, 0.75H), 3.77 (s, 3H), 3.57 (d, *J* = 23.8 Hz, 0.25H), 2.88 – 2.61 (m, 4H), 2.12 (d, *J* = 7.3 Hz, 3H);

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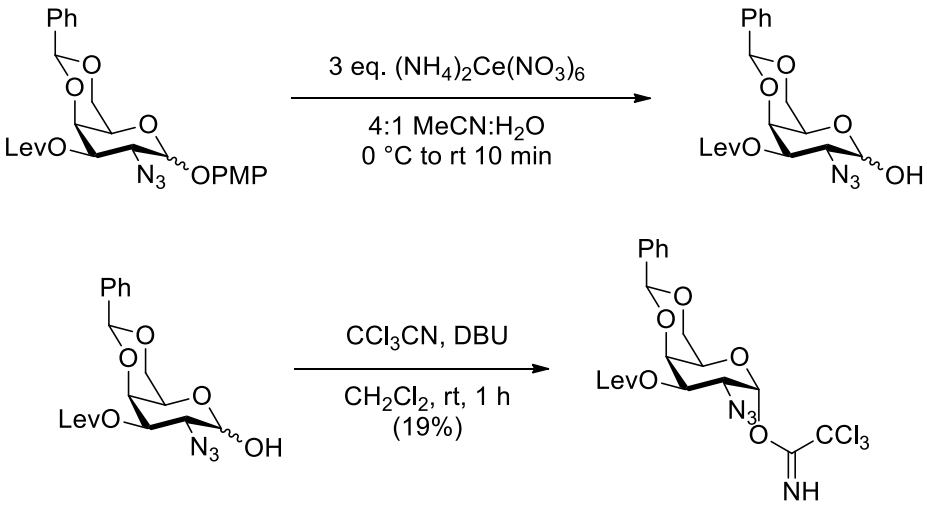
^{13}C NMR ($CDCl_3$, 100 MHz) δ 206.33, 172.42, 172.31, 156.01, 155.57, 151.22, 150.62, 137.71, 129.35, 128.46, 126.60, 126.40, 119.33, 117.74, 114.99, 114.79, 102.19, 101.23, 100.99, 98.16, 73.60, 72.74, 72.27, 69.60, 69.30, 69.11, 66.70, 63.44, 60.35, 57.40, 55.92, 55.88, 38.15, 29.94, 28.43, 28.36.

HRMS Calcd for $[M+Na]^+$: 520.1696 Found: 520.1679.



Synthesis of 2-deoxy-2-azido-4,6-O-benzylidene-3-O-Levulinoyl-α/β-D-galactopyranose trichloroacetimidate (21): To a solution of compound **32** (149 mg, 299 μmol) in a mixture of acetonitrile and water (1 ml, 4:1 acetonitrile/water) stirring at 0 °C in an ice bath was added solid ceric ammonium nitrate (492 mg, 897 μmol). The reaction was removed from the ice bath and let warm to room temperature. The reaction was let stir for 10 minutes at room temperature. The reaction was diluted with DI water (5 ml) and extracted with methylene chloride (3 x 15 ml). The organic fractions were pooled and washed with DI water (3 x 50 ml), sat. sodium bicarbonate (3 x 50 ml), and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was filtered over a plug of silica gel (20% to 50% EtOAc in

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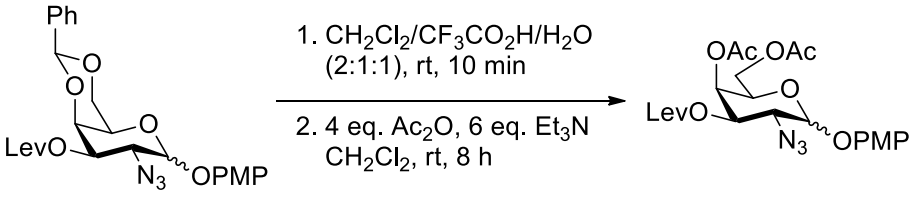
hexanes) to afford the impure hemiacetal as an orange oil. The compound was pure enough to be used directly in the next step. To the crude in methylene chloride (1 ml) was added trichloroacetonitrile (0.36 ml, 2.99 mmol) and DBU (3 µl, 15 mmol). The reaction was let stir for 1 hour at room temperature. The solvent was removed *in vacuo* and purified by flash column chromatography on silica gel (30% EtOAc and 1% triethylamine in hexanes) to afford compound **21** (30.3 mg, 57 µmol, 19%) as a pale yellow oil which turned to a white foam under high vac.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.76 (s, 1H), 7.52 (dd, J = 7.5, 1.9 Hz, 2H), 7.46 – 7.31 (m, 3H), 5.56 (s, 1H), 5.37 (dd, J = 11.0, 3.3 Hz, 1H), 4.55 (d, J = 3.0 Hz, 1H), 4.30 (dt, J = 10.9, 2.4 Hz, 2H), 4.05 (dd, J = 12.8, 1.5 Hz, 1H), 3.97 (s, 1H), 2.85 – 2.60 (m, 4H), 2.12 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$) δ 206.45, 172.39, 163.88, 160.93, 137.60, 129.50, 128.54, 126.48, 101.11, 95.66, 92.11, 91.12, 73.14, 70.19, 69.04, 65.13, 57.19, 38.11, 29.99, 28.40.

HRMS Calcd for $[M+Na]^+$: 557.0374 Found: 557.0385.



Synthesis of *p*-methoxyphenyl 4,6-O-diacetyl-2-deoxy-2-azido-3-O-Levulinoyl-α/β-D-galactopyranoside (33): To a solution of compound **32** (0.58 g, 1.17 mmol) in methylene chloride (5 ml) was added aqueous trifluoroacetic acid (50% v/v, 10 ml). The reaction was vigorously swirled at room temperature for 5-10 minutes until the starting material was

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completely consumed by TLC. The layers were separated and the water layer extracted twice with methylene chloride (2 x 20 ml). The combined organic layer was washed with water (3 x 50 ml), sat. sodium bicarbonate (3 x 50 ml), and dried over Na_2SO_4. The organic solvent was removed *in vacuo* to afford a yellow oil and trace water was removed by coevaporation with toluene (3 x 5 ml). To a solution of the crude product in methylene chloride (5 ml) was added triethylamine (0.98 ml, 7.01 mmol) and acetic anhydride (0.45 ml, 4.68 mmol). The reaction was let stir for 8 hours at room temperature. The reaction was diluted with methylene chloride (50 ml), washed with water (3 x 50 ml), washed with sat. sodium bicarbonate (3 x 50 ml), and dried over Na_2SO_4. The organic layer was removed *in vacuo* and the crude syrup was purified by flash column chromatography to afford **33** as a yellow syrup (0.39 g, 391 µmol, 68%).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.04 (d, *J* = 9.1 Hz, 2H), 6.83 (d, *J* = 9.1 Hz, 2H), 5.63 – 5.47 (m, 2.25H), 5.38 (dd, *J* = 9.9, 2.7 Hz, 0.25H), 4.87 – 4.76 (m, 0.5H), 4.39 (t, *J* = 6.5 Hz, 0.75H), 4.21 (dd, *J* = 11.2, 7.0 Hz, 0.25H), 4.12 – 4.03 (m, 1.5H), 3.99 – 3.89 (m, 0.5H), 3.77 (d, *J* = 2.5 Hz, 3.75H), 2.92 – 2.43 (m, 4.25H), 2.25 – 2.06 (m, 6H), 2.06 – 1.94 (m, 3H);

^{13}C NMR ($CDCl_3$, 100 MHz) δ 206.27, 172.06, 170.58, 170.30, 155.93, 150.52, 119.02, 118.51, 114.97, 114.89, 102.17, 98.28, 71.53, 71.24, 68.74, 67.74, 67.69, 66.44, 61.85, 60.98, 57.71, 55.94, 38.09, 30.03, 28.08, 20.92, 20.90;

HRMS Calcd for $[M+Na]^+$: 516.1594 Found $[M+Na]^+$: 516.1597, $[2M+Na]^+$: 1009.3298.

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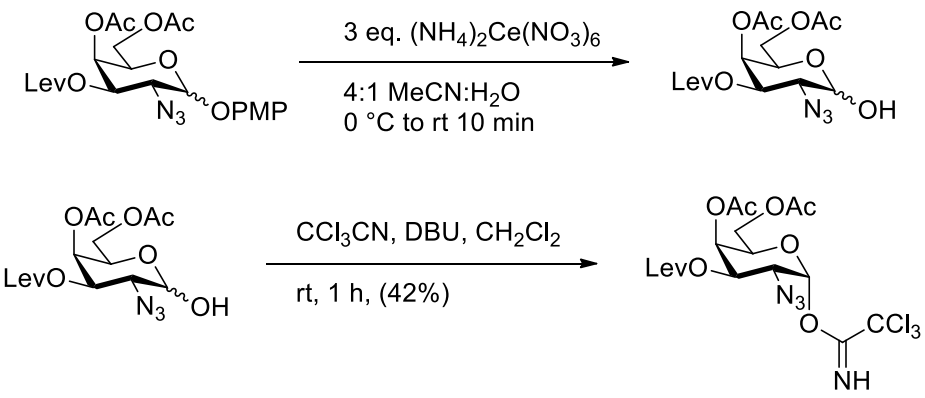

Synthesis of 4,6-O-diacetyl-2-deoxy-2-azido-3-O-Levulinoyl-α/β-D-galactopyranose trichloroacetimidate (22): To a solution of compound **33** (371 mg, 752 µmol) in a mixture of acetonitrile and water (2.2 ml, 4:1 acetonitrile/water) stirring at 0 °C in an ice bath was added solid ceric ammonium nitrate (1.24 g, 2.26 mmol). The reaction was removed from the ice bath and stir for 10 minutes. The reaction was diluted with DI water (10 ml) and extracted with methylene chloride (3 x 35 ml). The organic fractions were pooled and washed with sat. sodium bicarbonate (3 x 100 ml), Brine (3 x 100 ml), and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was filtered over a plug of silica gel (20% to 50% EtOAc in hexanes) to afford a crude orange oil. The compound was pure enough to be used in the next step. To a solution of the crude product in methylene chloride (2.2 ml) was added trichloroacetonitrile (0.75 ml,7.52 mmol) and DBU (11 µl, 80 µmol). The reaction was let stir for 1 hour at room temperature. The solvent was removed *in vacuo* and purified by flash column chromatography on silica gel (30% EtOAc and 1% triethylamine in hexanes) to afford compound **22** (0.17 g, .32 mmol, 42%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.79 (s, 1H), 6.47 (d, J = 3.5 Hz, 1H), 5.67 (d, J = 8.5 Hz, 1H), 5.49 (d, J = 2.2 Hz, 1H), 5.40 – 5.27 (m, 1H), 4.89 (dd, J = 10.8, 3.4 Hz, 1H), 4.37 (t, J = 6.4 Hz,

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1H), 4.17 – 3.98 (m, 3H), 3.92 (dd, J = 10.7, 8.5 Hz, 1H), 2.86 – 2.41 (m, 4H), 2.21 – 1.90 (m, 9H);

^{13}C NMR (125 MHz, $CDCl_3$) δ 206.50, 172.12, 172.05, 170.75, 170.72, 170.53, 170.42, 163.96, 161.13, 161.03, 97.08, 94.94, 91.04, 90.69, 72.09, 71.93, 69.58, 69.39, 67.33, 66.46, 61.70, 61.29, 60.89, 57.52, 38.18, 38.14, 30.18, 28.19, 28.13, 21.08, 21.05.

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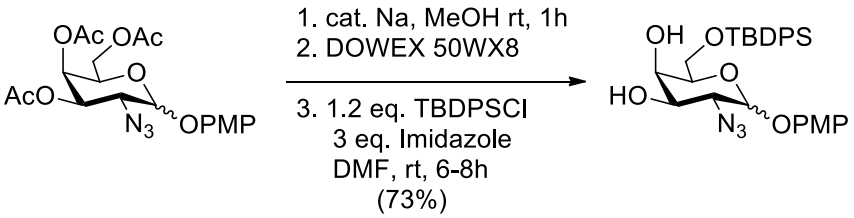

Synthesis of *p*-methoxyphenyl 2-deoxy-2-azido-6-O-t-butyldiphenylsilyl-α/β-D-galactoside (34): To a solution of compound **30** (2.29 g, 5.24 mmol) in methanol (15 ml) was added solid sodium (121 mg, 5.24 mmol). The reaction was let stir for 1 hour at room temperature. The base was neutralized with DOWEX 50WX8 (H^+) cation exchange resin until neutral pH (~7) was achieved. The resin was filtered and rinsed with methanol. The methanol was removed *in vacuo* and trace methanol or water was removed by co-evaporation with toluene (3 x 10 ml). The crude product was used directly in the next step. To flask containing the crude product was added imidazole (1.04 g, 15.3 mmol), then the flask was purged with argon, and the solids were dissolved in DMF (10 ml). To the reaction was added t-butylchloro(diphenyl)silane (1.59 ml, 6.1 mmol) and the reaction was let stir for 8 hours at room temperature. The reaction

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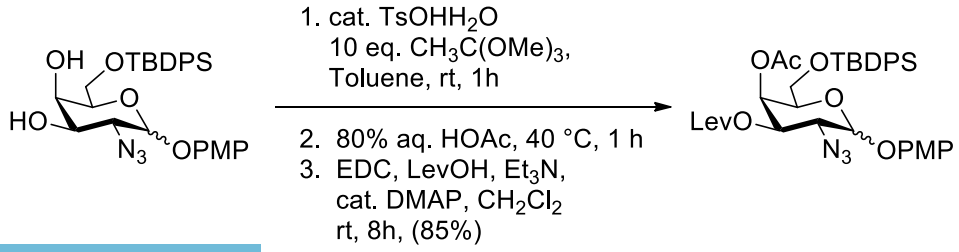
was quenched with the addition of DI water (40 ml), EtOAc (30 ml) and allowing to stir for an additional 2 hours. The layers were separated and the aqueous layer was extracted with EtOAc (2 x 30 ml). The combined organic layers were washed with DI water (3 x 100 ml), brine (3 x 100 ml) and dried over Na_2SO_4. The organic solvent was removed *in vacuo* and the crude was purified by flash column chromatography on silica gel (20% to 50% EtOAc gradient in hexanes) to afford **34** (2.1 g, 3.82 mmol, 73%) as a pale yellow oil that turned into a white foam under high vac.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.76 – 7.61 (m, 4H), 7.50 – 7.30 (m, 6H), 7.02 (d, *J* = 9.1 Hz, 2H), 6.86 – 6.76 (m, 2H), 5.47 (d, *J* = 3.5 Hz, 0.75H), 4.73 (d, *J* = 8.1 Hz, .25H), 4.24 (s, 1.5H), 4.09 – 4.05 (m, 0.25H), 4.05 – 3.89 (m, 2.75H), 3.85 (dd, *J* = 10.0, 8.1 Hz, 0.25H), 3.77 (s, 3H), 3.66 (dd, *J* = 10.0, 3.4 Hz, 0.75H), 3.57 (dd, *J* = 9.1, 3.6 Hz, 0.25H), 3.48 (s, 0.75H), 3.15 (d, *J* = 3.3 Hz, 0.25H), 2.92 (d, *J* = 6.9 Hz, 0.75H), 1.08 (d, *J* = 5.6 Hz, 9H);

^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.79, 155.55, 151.41, 150.98, 135.96, 135.90, 135.76, 132.90, 132.74, 130.24, 128.12, 128.07, 118.77, 118.47, 114.90, 114.82, 102.13, 98.54, 74.71, 72.59, 70.35, 70.23, 68.85, 68.77, 64.50, 64.27, 63.89, 60.73, 60.57, 55.91, 27.03, 21.31, 19.39, 14.45.

HRMS Calcd for $[M+Na]^+$: 572.2193 Found: 572.2198.



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Synthesis of *p*-methoxyphenyl 4-O-acetyl-2-deoxy-2-azido-6-O-t-butyldiphenylsilyl-3-O-Levulinoyl-α/β-D-galactoside (35): To a solution of compound **34** (1.78 g, 3.34 mmol) and *p*-toluenesulfonic acid monohydrate (63 mg, 0.33 mmol) in toluene (10 ml) was added trimethylorthoacetate (4.24 ml, 33.34 mmol). The reaction was let stir for 1 hour, until compound **34** was completely consumed by TLC, at room temperature. The solvent was removed *in* vacuo to afford a pale yellow oil that was used directly in the next step. To the crude was added aqueous 80% acetic acid (10 ml) and the reaction was let stir for 1-2 hour, until the orthoester intermediate was completely consumed by TLC, at 40 °C. The reaction was diluted with water (40 ml) and extracted with methylene chloride (3 x 50 ml). The combined organic layer was washed with DI water (3 x 150 ml), sat. sodium bicarbonate (3 x 150 ml), and dried over Na_2SO_4. The organic layer was removed *in vacuo* to afford a yellow oil. Trace amounts of water or acetic acid were removed by coevaporating with toluene (3 x 10 ml). The crude intermediate, EDC (0.96 g, 5.0 mmol), and DMAP (40 mg, 0.3 mmol) were dissolved in methylene chloride (10 ml). To the reaction was added levulinic acid (0.51 ml, 5.0 mmol) and triethylamine (0.7 ml, 5.0 mmol). The reaction was let stir for 8 hours at room temperature. The reaction was diluted with methylene chloride (100 ml) and washed with 2N HCl (2 x 200 ml), DI water (2 x 200 ml), sat. sodium bicarbonate (2 x 200 ml), and dried over Na_2SO_4. The organic solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica gel (20% to 30% EtOAc in hexanes) to afford **35** (1.89 g, 2.74 mmol, 85%) as a pale yellow syrup that turned to a white foam under high vac.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.68 – 7.50 (m, 4H), 7.48 – 7.30 (m, 6H), 7.03 (d, J = 9.1 Hz, 2H), 6.79 (d, J = 9.1 Hz, 2H), 5.66 – 5.53 (m, 1.6H), 5.46 (d, J = 3.4 Hz, 1H), 4.90 – 4.73 (m,

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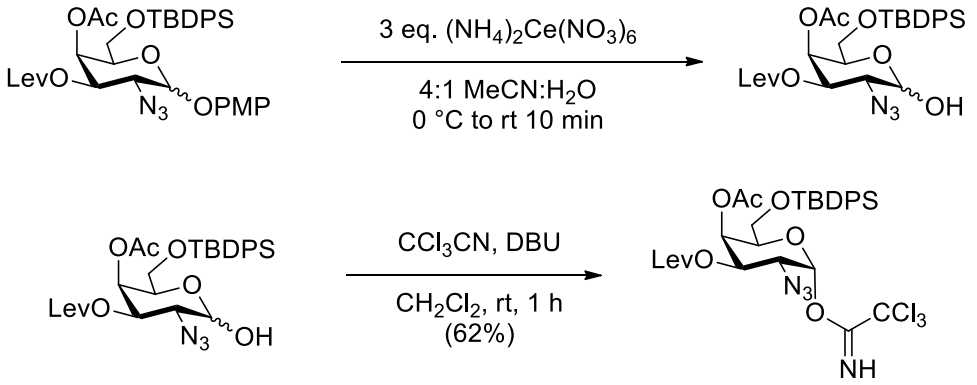
0.4H), 4.28 (t, *J* = 6.7 Hz, 0.8H), 3.98 – 3.50 (m, 6H), 2.68 (m, 4H), 2.20 (d, *J* = 1.8 Hz, 3H), 2.03 (d, *J* = 3.3 Hz, 3H), 1.01 (d, *J* = 15.0 Hz, 9H);

^{13}C NMR (125 MHz, $CDCl_3$) δ 205.92, 171.71, 171.61, 169.93, 169.83, 155.74, 155.58, 150.93, 150.49, 135.57, 135.53, 135.49, 132.85, 132.79, 132.68, 129.97, 129.88, 129.84, 127.86, 127.82, 127.80, 127.79, 118.54, 118.49, 114.70, 114.60, 101.76, 98.33, 73.84, 71.54, 69.91, 68.69, 67.62, 66.43, 61.71, 61.03, 57.68, 55.51, 37.80, 37.76, 29.64, 27.88, 27.80, 26.73, 26.71, 20.53, 19.03.

HRMS Calcd for $[M+Na]^+$: 712.2666 Found: 712.2639.



Synthesis of 4-O-acetyl-2-deoxy-2-azido-6-O-t-butyldiphenylsilyl-3-O-Levulinoyl-α/β-D-galactopyranose trichloroacetimidate (23): To a solution of compound **35** (1.88 g, 2.72 mmol) in a mixture of acetonitrile and water (90 ml, 4:1 acetonitrile/water) stirring at 0 °C in an ice bath was added solid ceric ammonium nitrate (4.47 g, 8.16 mmol). The reaction was removed from the ice bath and let warm to room temperature. The reaction was let stir for 10 minutes at room temperature. The reaction was diluted with DI water (200 ml) and extracted with ethyl acetate (3 x 100 ml). The organic fractions were pooled and washed with DI water (3 x 200 ml), sat. sodium bicarbonate (3 x 200 ml) and dried over Na_2SO_4. The solvent was

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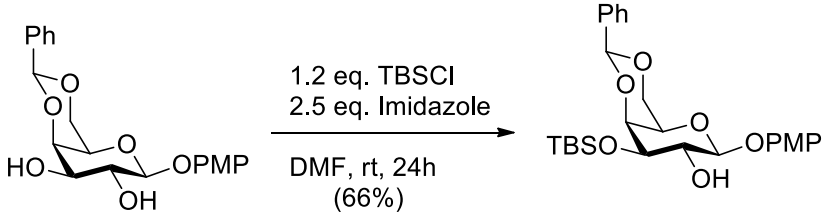
removed *in vacuo* and the crude oil was filtered over a plug of silica gel (20% to 50% EtOAc in hexanes) to afford an impure orange oil. The compound was pure enough to be used in the next step. To a solution of the crude product in methylene chloride (22 ml) was added trichloroacetonitrile (2.73 ml, 27.2 mmol) and DBU (20 µl, 0.03 mmol). The reaction was let stir for 1 hour at room temperature. The solvent was removed *in vacuo* and purified by flash column chromatography on silica gel (20% EtOAc and 1% triethylamine in hexanes) to afford compound **23** (1.23 g, 1.69 mmol, 63%) as a pale yellow oil which turned to a white foam under high vac.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (d, J = 7.3 Hz, 1H), 7.72 – 7.52 (m, 4H), 7.51 – 7.31 (m, 6H), 6.46 (d, J = 3.5 Hz, 0.5H), 5.75 – 5.66 (m, 1H), 5.56 (d, J = 2.9 Hz, 0.5H), 5.43 (dd, J = 11.1, 3.1 Hz, 0.5H), 4.94 (dd, J = 10.7, 3.3 Hz, 0.5H), 4.29 (t, J = 6.9 Hz, 0.5H), 4.02 – 3.86 (m, 1H), 3.68 (dddd, J = 17.8, 15.0, 10.1, 6.8 Hz, 2H), 2.95 – 2.41 (m, 4H), 2.28 – 1.95 (m, 6H), 1.00 (d, J = 6.2 Hz, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 206.35, 171.89, 171.81, 170.18, 170.06, 161.02, 160.94, 135.87, 135.78, 133.08, 130.14, 130.10, 128.03, 128.01, 97.03, 94.90, 91.01, 74.47, 72.10, 71.84, 69.46, 67.23, 66.31, 61.52, 61.16, 60.99, 57.58, 38.10, 38.07, 30.04, 28.09, 28.02, 26.95, 26.91, 20.90, 20.84, 19.32, 19.29.

HRMS Calcd for $[M+Na]^+$: 751.1322 Found: 751.1292.



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Synthesis of *p*-methoxyphenyl 4,6-O-benzylidene-3-O-t-butyldimethylsilyl-β-D-galactoside (37): To a solution of *p*-methoxyphenyl 4,6-O-benzylidene-β-D-galactoside (7.45 g, 19.9 mmol) and imidazole (3.39 g, 50 mmol) in DMF (50 ml) was added t-butylchlorodimethylsilane (3.9 g, 26 mmol). The reaction was let stir for 24 hours at room temperature. The reaction was quenched with addition of DI water (300 ml) and EtOAc (200 ml) and the reaction was let stir for an additional 2 hours at room temperature. The layers were separated and the aqueous layer was extracted one time with EtOAc (200 ml). The combined organic layers were washed with DI water (3 x 400 ml) and dried over Na_2SO_4. The crude product was purified by column chromatography on silica gel (50% EtOAc in hexanes) to afford **37** (6.43 g, 13.2 mmol, 66%) as a colorless oil that turned to a white foam under high vac.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.60 – 7.49 (m, 2H), 7.40 – 7.30 (m, 3H), 7.06 (d, *J* = 9.0 Hz, 2H), 6.80 (d, *J* = 9.0 Hz, 2H), 5.53 (s, 1H), 4.79 (d, *J* = 7.8 Hz, 1H), 4.35 (d, *J* = 12.3 Hz, 1H), 4.06 (dt, *J* = 9.8, 7.0 Hz, 3H), 3.83 – 3.74 (m, 4H), 3.49 (s, 1H), 0.93 (s, 9H), 0.15 (d, *J* = 4.6 Hz, 6H);

^{13}C NMR ($CDCl_3$, 100 MHz) δ ^{13}C NMR (101 MHz, cdcl$_3$) δ 155.62, 151.62, 138.16, 128.97, 128.27, 126.43, 119.41, 114.62, 102.93, 101.08, 76.57, 74.35, 70.81, 69.44, 67.04, 55.86, 26.01, 18.51, -4.08, -4.37;

HRMS Calcd for $[M+Na]^+$: 511.2128 Found: 511.2123.

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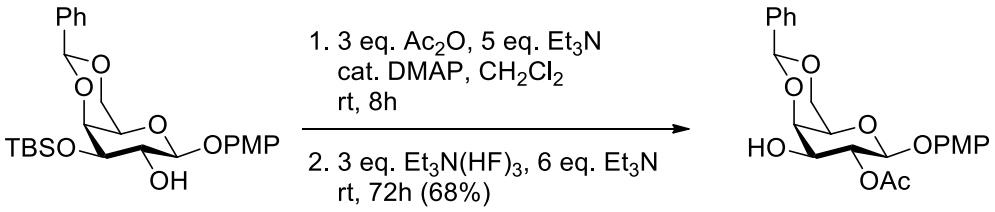

Synthesis of *p*-methoxyphenyl 2-O-acetyl-4,6-O-benzylidene-β-D-galactoside (38):

To a solution of **37** (6.33 g, 13.0 mmol) and DMAP (159 mg, 1.3 mmol) in methylene chloride (40 ml) was added triethylamine (10.8 ml, 77.7 mmol) and acetic anhydride (4.9 ml, 52 mmol). The reaction was let stir for 8 hours at room temperature. The reaction was diluted with methylene chloride (260 ml) and washed with DI water(3 x 300 ml), sat. sodium bicarbonate(3 x 300 ml), and dried over Na_2SO_4. The solvent was removed *in vacuo* and any trace water was coevaporated with toluene (3 x 25 ml) to afford a crude white solid. To crude product in DMF (20 ml) was added triethylamine (7.22 ml, 51.8 mmol) and triethylamine trihydrogen fluoride complex (4.22 ml, 25.9 mmol). The reaction was let stir for 72 hours at room temperature. The reaction was quenched with the addition of DI water (200 ml). The precipitate was filtered and recrystallized from $CHCl_3$/hexanes mixture to afford **38** (3.68 g, 8.84 mmol, 68%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53-7.50 (m, 2H), 7.37-7.36 (m, 3H), 6.99 (d, J = 9.1 Hz, 2H), 6.80 (d, J = 9.1 Hz, 2H), 5.54 (s, 1H), 5.30 (dd, J = 9.9, 8.1 Hz, 1H), 4.87 (d, J = 8.0 Hz, 1H), 4.40 – 4.30 (m, 1H), 4.23 (d, J = 3.5 Hz, 1H), 4.07 (dd, J = 12.5, 1.5 Hz, 1H), 3.89 – 3.67 (m, 4H), 3.54 (s, 1H), 2.88 (d, J = 24.9 Hz, 1H), 2.13 (d, J = 3.3 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.42, 155.49, 151.22, 137.23, 129.27, 128.21, 126.42, 118.91, 114.41, 101.46, 100.55, 75.34, 71.96, 71.55, 68.81, 66.59, 55.58, 25.44, 20.94.

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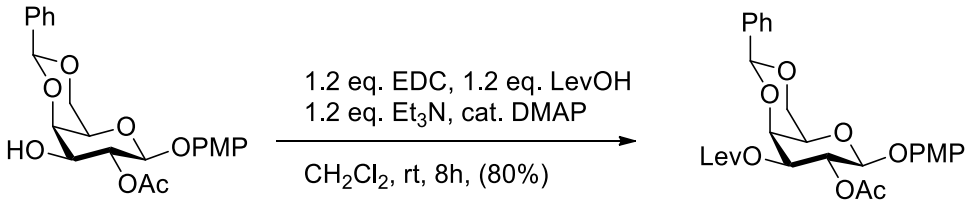
HRMS Calcd for $[M+Na]^+$: 439.1369 Found: 439.1353.



Synthesis of *p*-methoxyphenyl 2-O-acetyl-4,6-O-benzylidene-3-O-levulinoyl-β-D-galactoside (39): To a solution of compound **23** (3.56 g, 8.55 mmol), EDC (2.46 g, 12.8 mmol), and DMAP (104 mg, 0.86 mmol) in methylene chloride (20 ml) was added levulinic acid (1.31 ml, 12.8 mmol) and triethylamine (1.8 ml, 12.8 mmol). The reaction was let stir for 8 hours at room temperature. The reaction was diluted with methylene chloride (280 ml), the organic layer was washed with 5% $CuSO_{4(aq)}$ (2 x 200 ml), DI water (2 x 200 ml), sat. sodium bicarbonate (2 x 200 ml) and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was purified by column chromatography on silica gel (20% to 30% EtOAc in hexanes) to afford compound **39** (3.56 g, 6.80 mmol, 80%) as a pale yellow oil that turned to a white foam under high vac. ^1H

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (dd, J = 7.5, 2.0 Hz, 2H), 7.38 (dd, J = 4.8, 2.4 Hz, 3H), 7.06 – 6.94 (m, 2H), 6.87 – 6.76 (m, 2H), 5.60 (dd, J = 10.4, 8.0 Hz, 1H), 5.52 (s, 1H), 5.05 (dd, J = 10.4, 3.6 Hz, 1H), 4.96 (d, J = 8.0 Hz, 1H), 4.36 (dd, J = 13.2, 2.2 Hz, 2H), 4.08 (dd, J = 12.5, 1.7 Hz, 1H), 3.77 (s, 3H), 3.59 (d, J = 0.9 Hz, 1H), 2.80 – 2.50 (m, 4H), 2.09 (d, J = 13.4 Hz, 6H).

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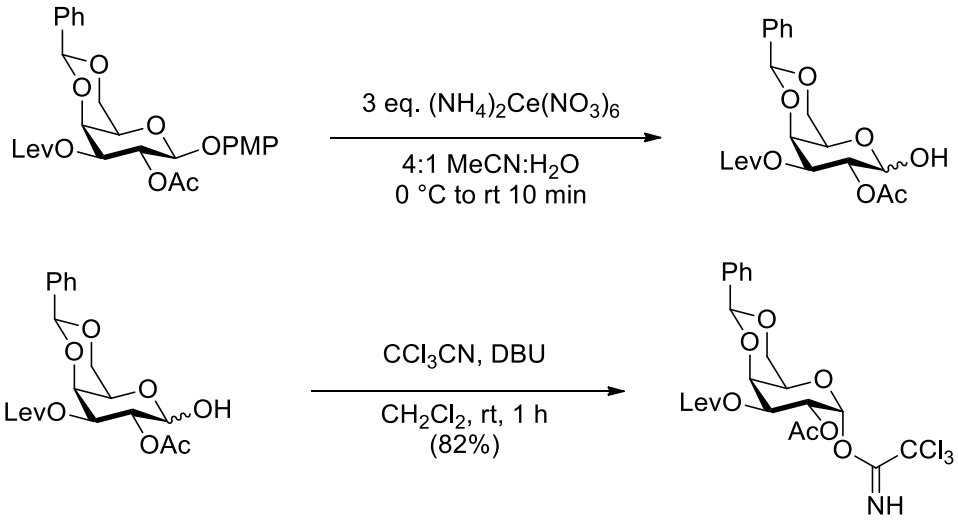
^{13}C NMR (100 MHz, cdcl3) δ 206.56, 172.39, 169.79, 155.91, 151.53, 137.75, 129.41, 128.46, 126.77, 119.40, 114.74, 101.45, 101.30, 73.53, 72.26, 69.12, 68.68, 66.79, 55.93, 38.03, 29.92, 28.50, 21.16.

HRMS Calcd for $[M+Na]^+$: 537.1736 Found $[M+Na]^+$: 537.1732, $[2M+Na]^+$: 1051.3625.



Synthesis of 2-O-acetyl-4,6-O-benzylidene-3-O-levulinoyl-β-D-galactopyranose trichloroacetimidate (24): To a solution of compound **39** (3.46 g, 6.73 mmol) in a mixture of acetonitrile and water (225 ml, 4:1 acetonitrile/water) stirring at 0 °C in an ice bath was added solid ceric ammonium nitrate (11.1 g, 20.2 mmol). The reaction was removed from the ice bath and was let stir for 10 minutes.. The reaction was diluted with DI water (500 ml) and extracted with ethyl acetate (3 x 150 ml). The organic fractions were pooled and washed with sat. sodium bicarbonate (3 x 500 ml) and brine (3 x 500 ml) dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was purified by column chromatography on silica gel (20% to 30% EtOAc in hexanes) to afford impure compound an orange oil. The compound was pure enough to be used in the next step. To a solution of the crude intermediate in methylene chloride (60 ml) was added trichloroacetonitrile (6.75 ml, 67.3 mmol) and DBU (50 µl, 0.33 mmol). The reaction

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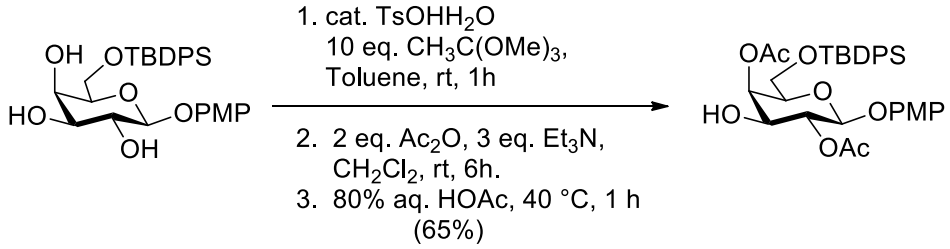
was let stir for 1 hour at room temperature. The solvent was removed *in vacuo* and purified by flash column chromatography on silica gel (20% EtOAc and 1% triethylamine in hexanes) to afford compound **24** (3.86 g, 6.99 mmol, 82%) as a pale yellow oil which turned to a white foam under high vac.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.79 (s, 1H), 6.47 (d, J = 3.5 Hz, 1H), 5.67 (d, J = 8.5 Hz, 1H), 5.49 (d, J = 2.2 Hz, 1H), 5.40 – 5.27 (m, 1H), 4.89 (dd, J = 10.8, 3.4 Hz, 1H), 4.37 (t, J = 6.4 Hz, 1H), 4.17 – 3.98 (m, 3H), 3.92 (dd, J = 10.7, 8.5 Hz, 1H), 2.86 – 2.41 (m, 4H), 2.21 – 1.90 (m, 9H).

^{13}C NMR (100 MHz, cdcl3) δ 206.44, 172.33, 170.33, 161.21, 137.64, 129.42, 128.50, 126.55, 101.22, 94.83, 91.26, 73.63, 69.02, 68.78, 67.10, 65.00, 38.02, 29.96, 28.39, 20.87.

HRMS Calcd for $[M+Na]^+$: 574.0414 Found:574.0427.



Synthesis of *p*-methoxyphenyl 2,4-O-diacetyl-6-O-t-butyldiphenylsilyl-β-D-galactoside (41): To a solution of compound *p*-methoxyphenyl 6-O-t-butyldiphenylsilyl-β-D-galactoside (16.7 g, 32.8 mmol) and *p*-toluenesulfonic acid monohydrate (0.63 g, 3.28 mmol) in toluene (100 ml) was added trimethylorthoacetate (42 ml, 3.28 mmol). The reaction was let stir for 1 hour, until the starting material was completely consumed by TLC analysis, at room temperature. The solvent was removed *in* vacuo to afford a pale yellow oil that was used directly

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in the next step. Trace methanol and water was removed by coevaporation with toluene (3 x 25 ml). To the crude oil was added methylene chloride (100 ml), triethylamine (27 ml, 197 mmol), and acetic anhydride (9.31 ml, 98.5 mmol). The reaction was let stir for 6 hours at room temperature. The reaction was diluted with methylene chloride (300 ml), washed with sat. sodium bicarbonate (3 x 400 ml), and dried over Na_2SO_4. The solvent was removed *in vacuo* to afford a pale yellow oil which was used directly in the next step. To the crude oil was added aqueous 80% acetic acid (75 ml) and the reaction was let stir for 1-2 hour, until the intermediate was completely consumed, at 40 °C. The reaction was diluted with water (350 ml) and extracted with methylene chloride (3 x 200 ml). The combined organic layer was washed with DI water (3 x 400 ml), sat. sodium bicarbonate (3 x 400 ml), and dried over Na_2SO_4. The organic layer was removed *in vacuo* to afford a light yellow oil that turned to a white solid if let sit over night. The crude product was purified by recrystallization from a $CHCl_3$/hexanes mixture to provide **41** (12.6 g, 51.26 mmol, 65%) as a white amorphous solid.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.69 – 7.58 (m, 4H), 7.40 (ddd, *J* = 9.3, 7.6, 2.1 Hz, 6H), 7.02 – 6.91 (m, 2H), 6.81 – 6.72 (m, 2H), 5.45 (d, *J* = 3.5 Hz, 1H), 5.21 (dd, *J* = 10.0, 8.0 Hz, 1H), 4.87 (d, *J* = 7.9 Hz, 1H), 3.96 – 3.88 (m, 1H), 3.83 – 3.72 (m, 6H), 2.69 (d, *J* = 5.5 Hz, 1H), 2.15 (s, 3H), 2.07 (s, 3H), 1.06 (s, 9H) ;

^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.43, 171.29, 155.74, 151.57, 135.86, 135.84, 133.21, 133.11, 130.19, 130.09, 128.09, 128.04, 118.71, 114.79, 100.74, 74.42, 72.97, 72.05, 70.05, 62.18, 55.92, 27.04, 21.26, 21.06, 19.39;.

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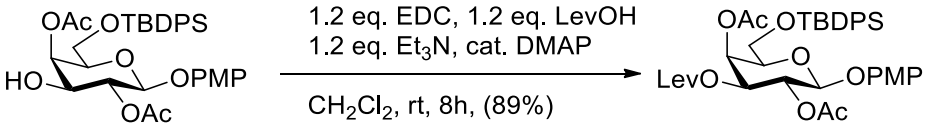
HRMS Calcd for $[M+Na]^+$: 631.2339 Found $[M+Na]^+$: 631.2348, $[2M+Na]^+$: 1239.4775.



Synthesis of *p*-methoxyphenyl 2,4-O-diacetyl-6-O-t-butyldiphenylsilyl-3-O-Levulinoyl-β-D-galactoside (42): To a solution of compound **41** (12.28 g, 20.21 mmol), EDC (5.81 g, 30.3 mmol), and DMAP (247 mg, 2.02 mmol) in methylene chloride (100 ml) was added levulinic acid (3.1 ml, 30.3 mmol) and triethylamine (4.22 ml, 30.3 mmol). The reaction was let stir for 8 hours at room temperature. The reaction was diluted with methylene chloride (300 ml), the organic layer was washed with 2N HCl (2 x 400 ml), DI water (2 x 400 ml), sat. sodium bicarbonate (2 x 400 ml), and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was purified by column chromatography on silica gel (20% to 50% EtOAc in hexanes) to afford compound **42** (12.71 g, 18.0 mmol, 89%) as a pale yellow oil that turned to a white foam under high vac.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 – 7.51 (m, 4H), 7.37 (dt, J = 14.3, 7.0 Hz, 6H), 6.93 (d, J = 8.8 Hz, 2H), 6.74 (d, J = 8.8 Hz, 2H), 5.49 (s, 1H), 5.47 – 5.32 (m, 1H), 5.08 (dd, J = 10.4, 2.8 Hz, 1H), 4.88 (d, J = 8.0 Hz, 1H), 3.84 (t, J = 6.4 Hz, 1H), 3.81 – 3.73 (m, 1H), 3.72 (s, 3H), 3.69 – 3.63 (m, 1H), 2.80 (d, J = 7.6 Hz, 4H), 2.17 – 1.98 (m, 9H), 1.01 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 206.14, 171.68, 170.13, 169.63, 155.51, 151.15, 135.54, 135.53, 132.78, 132.66, 129.88, 129.79, 127.78, 127.74, 118.46, 114.48, 100.73, 73.76, 71.37, 68.78, 67.09, 61.49, 55.59, 37.64, 29.64, 27.80, 26.67, 20.76, 20.65, 19.01.

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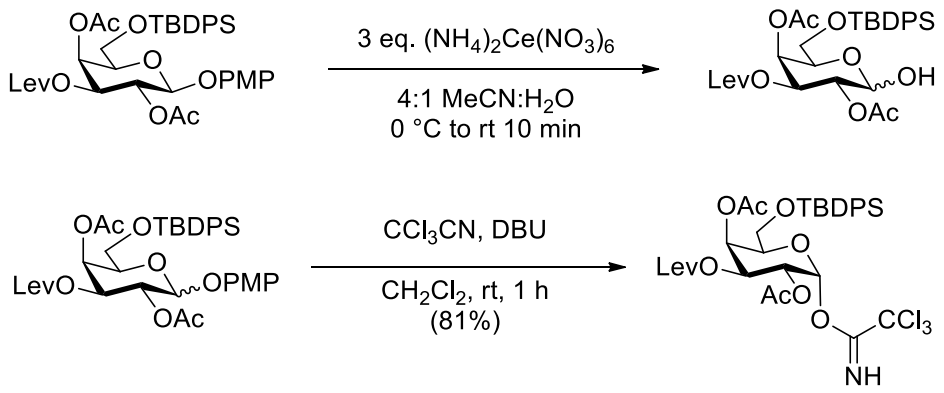
HRMS Calcd for $[M+Na]^+$: 729.2707 Found: 729.2691.



Synthesis of 2,4-O-diacetyl-6-O-t-butyldiphenylsilyl-3-O-Levulinoyl-β-D-galactopyranose trichloroacetimidate (25): To a solution of compound **42** (2.91 g, 4.22 mmol) in a mixture of acetonitrile and water (137 ml, 4:1 acetonitrile/water) stirring at 0 °C in an ice bath was added solid ceric ammonium nitrate (5.59 g, 12.35 mmol). The reaction was removed from the ice bath and was let stir for 10 minutes. The reaction was diluted with DI water (300 ml) and extracted with ethyl acetate (3 x 100 ml). The organic fractions were pooled and washed with sat. sodium bicarbonate (3 x 200 ml), brine (3 x 200 ml) and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was filtered through a plug of silica gel (20% to 50% EtOAc in hexanes) to give an impure orange oil. The compound was pure enough to be used in the next step. To a the crude product in methylene chloride (20 ml) was added trichloroacetonitrile (4.13 ml, 41.2 mmol) and DBU (30 µl, 0.41 mmol). The reaction was let stir for 1 hour at room temperature. The solvent was removed *in vacuo* and purified by flash column chromatography on silica gel (20% EtOAc and 1% triethylamine in hexanes) to afford compound **25** (2.48 g, 3.32 mmol, 81%) as a pale yellow oil which turned to a white foam under high vac.

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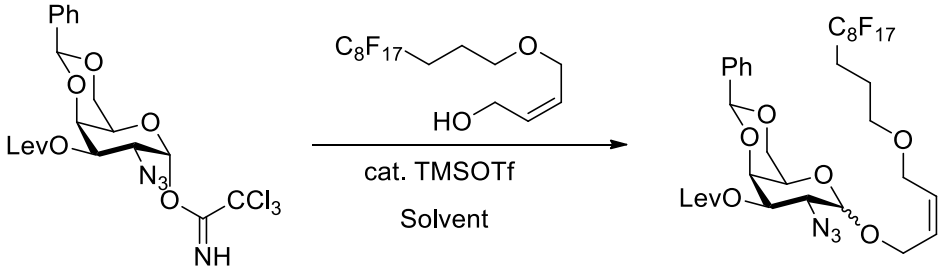
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (d, J = 23.8 Hz, 1H), 7.58 (d, J = 6.9 Hz, 4H), 7.38 (tdd, J = 14.6, 6.2, 2.4 Hz, 6H), 6.53 (d, J = 3.7 Hz, 1H), 5.84 (d, J = 8.3 Hz, 1H), 5.64 (dd, J = 37.5, 2.4 Hz, 1H), 5.47 (dd, J = 10.8, 3.2 Hz, 1H), 5.34 (dd, J = 10.8, 3.7 Hz, 1H), 5.16 (dd, J = 10.5, 3.4 Hz, 1H), 4.30 (t, J = 6.9 Hz, 1H), 4.02 (dt, J = 13.8, 6.5 Hz, 1H), 3.82 – 3.56 (m, 2H), 2.90 – 2.36 (m, 4H), 2.17 (d, J = 6.5 Hz, 3H), 2.08 – 2.00 (m, 6H), 1.01 (d, J = 5.9 Hz, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 206.55, 171.90, 170.56, 170.27, 161.22, 135.89, 135.80, 133.12, 130.13, 130.09, 128.04, 128.00, 110.30, 96.38, 93.92, 91.17, 71.73, 68.34, 67.73, 67.27, 61.51, 37.97, 30.05, 28.06, 26.96, 26.93, 20.95, 20.88, 20.85, 19.33.

HRMS Calcd for $[M+Na]^+$: 766.1385 Found: 766.1368.



General Synthesis of 1H,1H,2H,2H,3H,3H-perfluoroundecyloxybutenyl 2-deoxy-2-azido-4,6-O-benzylidene-3-O-Levulinoyl-α/β-D-galactopyranoside (43/44): To a solution of 1H,2H,2H,3H,3H-perfluoroundecyloxybutenyl alcohol (~67 µmol, 1 eq.) in solvent was added thiophene (~670 µmol, 10 eq.), if used, and donor **17** (~200 µmol, 3 eq.). The reaction was let stir for 30 minutes while allowing for the mixture to reach the desired temperature. A solution of TMSOTf in methylene chloride (0.9 ml of 230 mmol, ~20 µmol, 0.3 eq.) was added to the reaction while stirring. The products were easily separable by column

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chromatography on silica gel (20% EtOAc in hexanes) to give compounds **43** and **44** as colorless oils. Anomeric assignments were based on COSY experiments to identify $H_{anomeric}$ and ^{1}H to determine coupling constant.

Table III. Glycosylation conditions. All reactions were run at 0.4M with respect to donor **17**. *Reaction was run at 0.01M with respect to donor **17**.

Entry	Solvent	Thiophene	Temperature	Yield	Selectivity
1	CH_2Cl_2	N/A	25 °C	80	All β
2	CH_2Cl_2	10 eq.	0 °C	70	1.2:1 α/β
3	CH_2Cl_2	10 eq.	25 °C	73	1.1:1 α/β
4	Et_2O	N/A	0 °C	30	2.5:1 α/β
5*	Et_2O	N/A	0 °C	82	3.5:1 α/β

Compound **43** (α): $H_{anomeric}$ at 5.04 (d, J = 3.2 Hz)

^{1}H NMR (400 MHz, cdcl3) δ 7.49 (dd, J = 6.7, 2.9 Hz, 1H), 7.45 – 7.33 (m, 1H), 5.76 (q, J = 5.7 Hz, 1H), 5.59 (s, 1H), 5.04 (d, J = 3.4 Hz, 1H), 4.44 – 3.96 (m, 4H), 3.77 (s, 1H), 3.61 (dd, J = 10.5, 3.4 Hz, 1H), 3.50 (t, J = 6.0 Hz, 1H), 2.43 (d, J = 10.8 Hz, 1H), 2.30 – 2.08 (m, 1H), 1.89 (dd, J = 10.1, 5.7 Hz, 1H).

Compound **44** (β): $H_{anomeric}$ at 4.33 (d, J = 8 Hz)

^{1}H NMR (400 MHz, cdcl3) δ 7.45 (dd, J = 7.3, 2.1 Hz, 2H), 7.30 (dd, J = 4.9, 2.3 Hz, 3H), 5.80 – 5.63 (m, 2H), 5.46 (s, 1H), 4.68 (dd, J = 10.8, 3.5 Hz, 1H), 4.44 (dd, J = 12.5, 5.6 Hz, 1H), 4.33 (d, J = 8.0 Hz, 1H), 4.29 – 4.13 (m, 3H), 3.98 (dd, J = 8.7, 3.7 Hz, 3H), 3.84 (dd, J = 10.8, 8.0 Hz, 1H), 3.41 (dd, J = 14.3, 8.3 Hz, 3H), 2.63 (dtd, J = 10.8, 7.1, 2.9 Hz, 4H), 2.22 – 2.00 (m, 5H), 1.86 – 1.74 (m, 2H).

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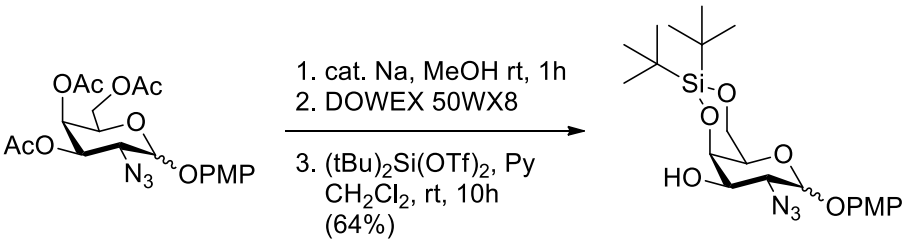

Synthesis of *p*-methoxyphenyl 2-deoxy-2-azido-4,6-O-[bis(*t*-Butyl)silylene]-α-D-galactopyranoside (46): To a solution of compound **30** (4.14 g, 9.46 mmol) in methanol (25 ml) was added solid sodium (220 mg, 9.46 mmol). The reaction was let stir for 1 hour at room temperature. The base was neutralized with DOWEX 50WX8 (H^+) cation exchange resin until neutral pH (~7) was achieved. The resin was filtered and rinsed with methanol. The methanol was removed *in vacuo* and trace methanol or water was removed by co-evaporation with toluene (3 x 10 ml). The crude product was used directly in the next step. To flask containing the crude product was purged with argon and the material was dissolved in methylene chloride (20 ml). To the reaction was added pyridine (2.3 ml, 28.4 mmol) followed by the dropwise addition (~.8 ml/min) of di-*tert*-butylsilyl bus(trifluoromethanesulfonate) (3.7 ml, 11.35 mmol). The reaction was let stir at rt for 10 hours. The reaction was diluted with methylene chloride the organic layer was washed with DI water (3 x 100 ml), brine (3 x 100 ml) and dried over Na_2SO_4. The organic solvent was removed *in vacuo* and the crude was purified by flash column chromatography on silica gel (20% to 30% EtOAc gradient in hexanes) to afford **46** (2.74 g, 6.07 mmol, 64%) as a pale yellow oil that turned into a white foam under high vac.

^{1}H NMR (400 MHz, CDCl3) δ 7.04 (d, J = 9.0 Hz, 2H), 6.83 (d, J = 9.0 Hz, 2H), 4.69 (dd, J = 21.1, 8.1 Hz, 1H), 4.42 (dd, J = 30.3, 3.0 Hz, 1H), 4.33 – 4.18 (m, 2H), 4.18 – 3.83 (m, 1H), 3.83 – 3.69 (m, 4H), 3.61 – 3.36 (m, 2H), 2.79 (t, J = 18.1 Hz, 1H), 1.07 (d, J = 9.3, Hz, 18H).

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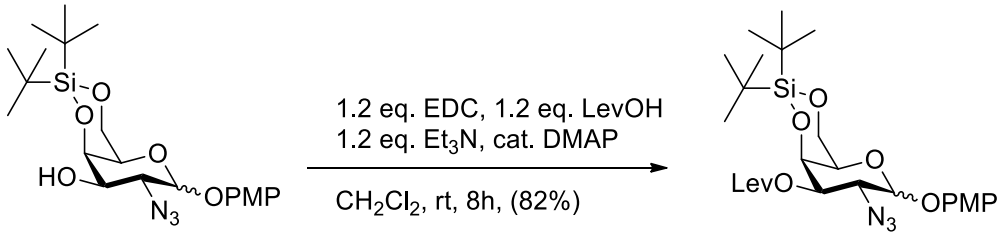
^{13}C NMR (100 MHz, CDCl3) δ 156.07, 151.41, 119.70, 119.66, 114.79, 102.56, 102.28, 76.99, 73.86, 72.94, 72.65, 71.94, 71.72, 67.08, 64.49, 55.94, 27.77, 27.69, 27.61, 27.52, 23.69, 23.63, 21.10, 20.98, 20.84.

HMRS Calcd for $[M+Na]^+$: 474.2036 Found: 474.2026.



Synthesis of *p*-methoxyphenyl 2-deoxy-2-azido-3-O-Levulinoyl-4,6-O-[bis(*t*-Butyl)silylene]-α-D-galactopyranoside(47): To a solution of compound **46** (1.5 g, 3.32 mmol), EDC (0.76 g, 3.99 mmol), and DMAP (41 mg, 0.33 mmol) in methylene chloride (12 ml) was added levulinic acid (0.41 ml, 3.99 mmol) and triethylamine (0.56 ml, 3.99 mmol). The reaction was let stir for 8 hours at room temperature. The reaction was diluted with methylene chloride (280 ml), the organic layer was washed with 2N HCl (2 x 200 ml), DI water (2 x 200 ml), sat. sodium bicarbonate (2 x 200 ml) and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was purified by column chromatography on silica gel (20% to 30% EtOAc in hexanes) to afford compound **47** (1.5 g, 2.73 mmol, 82%) as a pale yellow oil that turned to a white foam under high vac.

^{1}H NMR (500 MHz, cdcl3) δ 7.13 – 6.98 (m, 2H), 6.92 – 6.72 (m, 2H), 4.72 (d, J = 8.0 Hz, 1H), 4.62 – 4.55 (m, 2H), 4.26 – 4.17 (m, 2H), 4.04 (dd, J = 10.4, 8.0 Hz, 1H), 3.73 (d, J = 3.2 Hz, 3H), 3.43 (s, 1H), 2.80 – 2.65 (m, 4H), 2.17 (s, 3H), 1.05 (d, J = 32.6 Hz, 18H).

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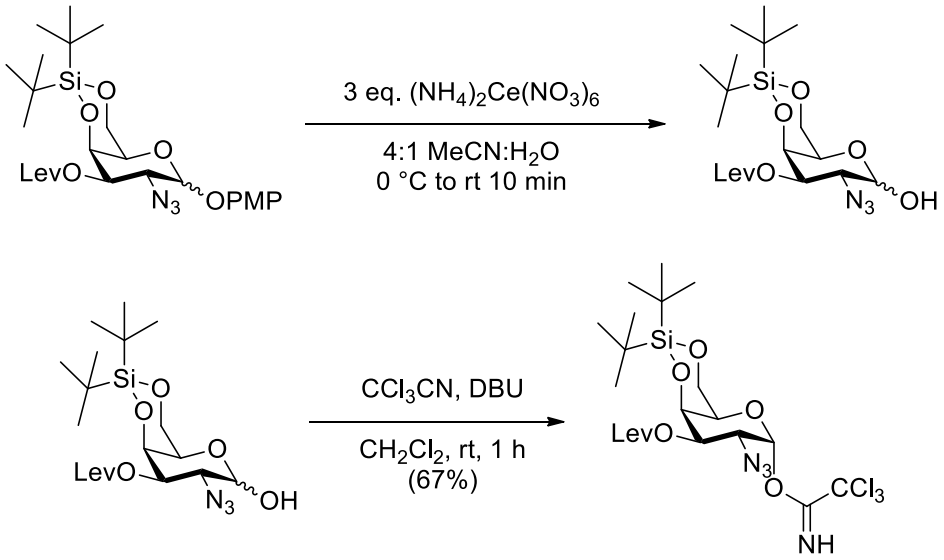
^{13}C NMR (125 MHz, CDCl3) δ 206.02, 172.20, 155.99, 151.20, 119.60, 114.68, 102.22, 74.25, 71.32, 69.40, 67.01, 60.47, 55.74, 38.02, 29.90, 28.27, 27.65, 27.59, 23.41, 20.93.

HRMS Calcd for $[M+Na]^+$: 537.1736 Found $[M+Na]^+$: 537.1732, $[2M+Na]^+$: 1051.3625.



Synthesis of 2-deoxy-2-azido-3-O-Levulinoyl-4,6-O-[bis(*t*-Butyl)silylene]-α-D-galactose trichloroacetimidate(45): To a solution of compound **47** (1.38 g, 2.56 mmol) in a mixture of acetonitrile and water (75 ml, 4:1 acetonitrile/water) stirring at 0 °C in an ice bath was added solid ceric ammonium nitrate (4.13 g, 8.08 mmol). The reaction was removed from the ice bath and let stir for 10 minutes. The reaction was diluted with DI water (150 ml) and extracted with ethyl acetate (3 x 75 ml). The organic fractions were pooled and washed with sat. sodium bicarbonate (3 x 200 ml), brine (3 x 200 ml) and dried over Na_2SO_4. The solvent was removed *in vacuo* and the crude oil was filtered over a plug of silica gel (20% to 50% EtOAc in hexanes) to afford the impure hemiacetal as an orange oil. The compound was pure

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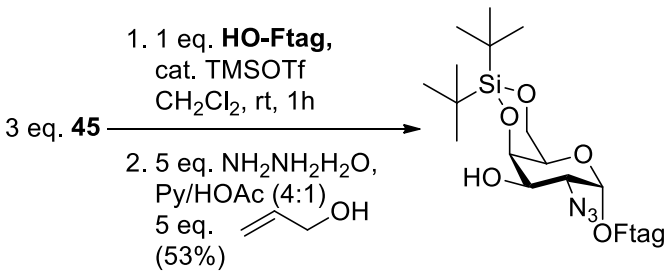
enough to be used directly in the next step. To the crude in methylene chloride (5 ml) was added trichloroacetonitrile (0.60 ml, 6.03 mmol) and DBU (30 µl, 0.2 mmol). The reaction was let stir for 1 hour at room temperature. The solvent was removed *in vacuo* and purified by flash column chromatography on silica gel (10% EtOAc and 1% triethylamine in hexanes) to afford compound **45** (1.01 g, 1.72 mmol, 67%) as a pale yellow oil which turned to a white foam under high vac.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H), 6.49 (d, J = 3.5 Hz, 1H), 5.18 (dd, J = 10.9, 2.9 Hz, 1H), 4.81 (d, J = 2.7 Hz, 1H), 4.30 – 4.12 (m, 3H), 3.97 (s, 1H), 2.88 – 2.64 (m, 4H), 2.20 (s, 3H), 1.04 (d, J = 8.4 Hz, 18H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 206.08, 172.29, 160.96, 95.54, 91.03, 71.99, 69.92, 69.80, 66.68, 56.85, 38.03, 29.99, 28.30, 27.73, 27.43, 23.44, 20.94.

HRMS Calcd for $[M+Na]^+$: 609.1082 Found: 609.1081.



Synthesis of *cis*-3-(perfluorooctyl)propyloxy-2-butenyl 2-deoxy-2-azido-4,6-O-[bis(*t*-Butyl)silylene]-α-D-galactoside(48): To **FtagOH** (149 mg, 0.27 mmol) and **45** (400 mg, 0.82 mmol) in methylene chloride (2.4 ml) was added TMSOTf (0.36 ml of 230 mM, 81 µmol) at room temperature. The reaction was quenched with 0.1 ml Et_3N and the solvent was

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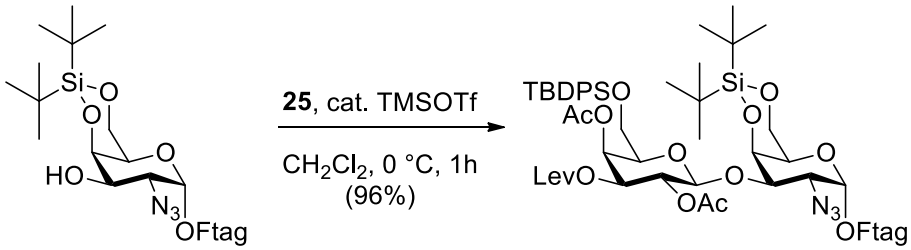
removed *in vacuo*. The crude was taken up in a mixture of pyridine and acetic acid (1 ml, 4:1 Py/HOAc) to which was added allyl alcohol (0.14 ml, 2.04 mmol) and hydrizine hydrate (0.1 ml, 2.04 mmol). The reaction was let stir for 3 hours at room temperature. To the reaction was added 0.2 ml water and the reaction mixture was loaded directly onto fluorofllash silica gel. Non-fluorous compounds were eluted with 80% $MeOH_{(aq)}$ and fluorous compounds were eluted with MeOH. The fluorous containing fractions were concentrated and further purified by flash column chromatography to provide compound **48** (127 mg, 145 µmol, 53%) as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74 (q, J = 4.8 Hz, 2H), 4.95 (d, J = 3.5 Hz, $1H_{anomeric}$), 4.46 (d, J = 2.9 Hz, 1H), 4.33 – 4.26 (m, 1H), 4.23 – 4.12 (m, 3H), 4.02 (dd, J = 24.1, 7.9 Hz, 3H), 3.77 (s, 1H), 3.52 – 3.43 (m, 3H), 2.63 (d, J = 10.4 Hz, 1H), 2.17 (td, J = 18.8, 9.8 Hz, 2H), 1.87 (td, J = 11.8, 6.0 Hz, 2H), 1.04 (d, J = 13.9 Hz, 18H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 130.60, 128.18, 97.84, 73.14, 69.13, 68.98, 67.65, 67.07, 66.79, 63.84, 60.91, 30.01, 27.83, 27.48, 23.65, 21.01.

HRMS Calcd for $[M+Na]^+$: 898.2156 Found: 898.2137.



Synthesis of *cis*-3-(perfluorooctyl)propyloxy-2-butenyl 2,4-O-diacetyl-6-O-t-butyldiphenylsilyl-3-O-Levulinoyl-β-D-galactopyranosyl-(1→3)-2-deoxy-2-azido-4,6-O-[bis(*t*-Butyl)silylene]-α-D-galactoside(49): A solution of **48** (38.8 mg, 49 µmol)

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and **25** (99.0 mg, 133 µmol) in methylene chloride (0.5 ml) was cooled to 0 °C in an ice bath. To the reaction was added TMSOTf (60 µl of a 230 mM solution in CH_2Cl_2, 5 µmol) and the reaction was let stir for 1 h. The reaction was qunched with 50 µl of Et_3N and the solvent was removed *in vacuo*. The product was obtained after column chromatography on silica gel (25% EtOAc in hexanes) to give disaccharide **49** (62 mg, 42.5 mmol, 96%) as a colorless oil that turned into a white foam under vacuum.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, J = 7.4 Hz, 4H), 7.47 – 7.35 (m, 6H), 5.80 – 5.63 (m, 2H), 5.48 (d, J = 2.8 Hz, 1H), 5.27 (dd, J = 10.2, 7.9 Hz, 1H), 5.02 (dd, J = 10.3, 3.2 Hz, 1H), 4.92 (d, J = 3.3 Hz, 1H), 4.70 (d, J = 7.7 Hz, 1H), 4.63 (s, 1H), 4.27 – 3.95 (m, 6H), 3.86 (dd, J = 10.6, 2.3 Hz, 1H), 3.67 (ddd, J = 26.4, 17.2, 7.7 Hz, 5H), 3.47 (t, J = 5.4 Hz, 2H), 2.74 (ddd, J = 35.6, 21.6, 6.4 Hz, 4H), 2.28 – 1.96 (m, 11H), 1.94 – 1.75 (m, 2H), 1.04 – 0.85 (m, 27H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 206.48, 172.01, 170.35, 170.10, 135.84, 135.79, 132.99, 130.56, 130.29, 130.20, 128.26, 128.15, 128.12, 102.92, 97.79, 78.59, 73.29, 72.87, 72.02, 69.41, 69.12, 67.90, 67.22, 67.05, 66.74, 63.55, 61.57, 58.76, 37.97, 29.92, 28.19, 27.69, 27.44, 26.97, 23.50, 21.08, 20.88, 20.84, 19.31.

HRMS Cacld for $[M+Na]^+$: 1480.4441 Found: 1480.4481.

Synthesis of *cis*-3-(perfluorooctyl)propyloxy-2-butenyl 2,4-O-diacetyl-3-O-Levulinoyl-β-D-galactopyranosyl-(1→3)-2-deoxy-2-azido-α-D-galactoside(50) and *cis*-3-(perfluorooctyl)propyloxy-2-butenyl 2,6-O-diacetyl-3-O-Levulinoyl-β-D-

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galactopyranosyl-(1→3)-2-deoxy-2-azido-α-D-galactoside(51): To solution of **49** (37.5 mg, 25.7 µmol) in DMF (0.5 ml) was added Et_3N (24 µl, 170 µmol) and $Et_3N(HF)_3$ (14 µl, 85 µmol). The reaction was let stir at room temperature for 72 hours. The reaction was diluted with 5 ml water and extracted with methylene chloride (3 x 5 ml) and washed with DI water (3 x 5 ml) and brine (3 x 5 ml). The solvent was removed *in vacuo* and the crude product was purified by column chromatography (3% to 5% MeOH in CH_2Cl_2) to give an inseparable mixture of compounds **50** and **51** (23 mg, 21.3 µmol, 83%).

^{1}H NMR (400 MHz, cdcl3) δ 5.84 – 5.67 (m, 2H), 5.33 (ddd, J = 27.9, 10.4, 7.8 Hz, 2H), 5.01 (ddd, J = 9.8, 8.4, 3.5 Hz, 2H), 4.84 (dd, J = 10.3, 3.0 Hz, 1H), 4.71 (dd, J = 18.0, 8.0 Hz, 1H), 4.39 (dd, J = 11.6, 7.5 Hz, 1H), 4.33 – 4.11 (m, 4H), 4.09 – 3.96 (m, 3H), 3.84 (ddd, J = 17.3, 15.9, 8.1 Hz, 4H), 3.74 – 3.61 (m, 2H), 3.60 – 3.42 (m, 3H), 3.11 (s, 1H), 2.92 (d, J = 30.2 Hz, 1H), 2.84 – 2.33 (m, 6H), 2.27 – 2.00 (m, 12H), 1.87 (td, J = 11.8, 6.1 Hz, 2H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 208.71, 206.32, 172.15, 171.98, 171.38, 171.10, 170.29, 170.24, 130.78, 130.72, 128.13, 128.06, 102.36, 102.21, 97.76, 97.66, 78.66, 78.59, 77.63, 74.28, 74.13, 72.78, 71.36, 69.80, 69.75, 69.58, 69.20, 68.85, 68.78, 67.97, 66.70, 63.68, 63.63, 63.09, 62.86, 62.76, 61.25, 60.86, 58.95, 58.88, 38.46, 37.90, 30.00, 28.07, 21.02.

HRMS Calcd for $[M+Na]^+$: 1102.2242 Found: 1102.2212.

Synthesis of *cis*-3-(perfluorooctyl)propyloxy-2-butenyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-(1→6)-2,4-O-diacetyl-3-O-Levulinoyl-β-D-galactopyranosyl-(1→3)-(

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2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-(1→4))-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-(1→6))- (2-deoxy-2-azido-3-O-Levulinoyl-α-D-galactoside(10): To a solution of **50** and **51** (18.7 mg, 17 µmol) and **27** (51 mg, 104 µmol) in methylene chloride (0.1 ml) at 0 °C was added TMSOTf(0.1 ml of a 500 µM solution, 500 nm). The reaction was let stir for 1 hour. The reaction was quenched with the addition of 50 µl Et_3N and the solvent was removed *in vacuo*. The residue was purified by column chromatography (3% to 5% MeOH in CH_2Cl_2) to produce either compound **10** or it's 4-O glycoylated regioisomer product (35 mg, 17 µmol, 44%) as a colorless oil.

HRMS Calcd for $[M+Na]^+$: 2092.5059 Found: 2092.5139.

NMR data is inconclusive, see appendex B.

Automation of pentasaccharide 10

Prepared stock solutions for glycosylation:

Compound	Concentration M	Solvent
45	0.8	CH_2Cl_2
Ftag	0.26	CH_2Cl_2
25	0.4	CH_2Cl_2
27	0.4	CH_2Cl_2
TMSOTf	0.23	CH_2Cl_2

Prepared stock solutions for hydrolysis of levulinoyl ester was 0.25 M hydrazine hydrate and 0.25 M allyl alcohol in 4:1 pyridine/acetic acid.

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Prepared stock solution for cleavage of the silyl groups was 0.1M TBAF and 0.1M AcOH in DMF.

Necessary reagents: methanol, 80% aqueous methanol, water, DMF, Et_3N and toluene. The platform was set up according to the figure **3.7**.

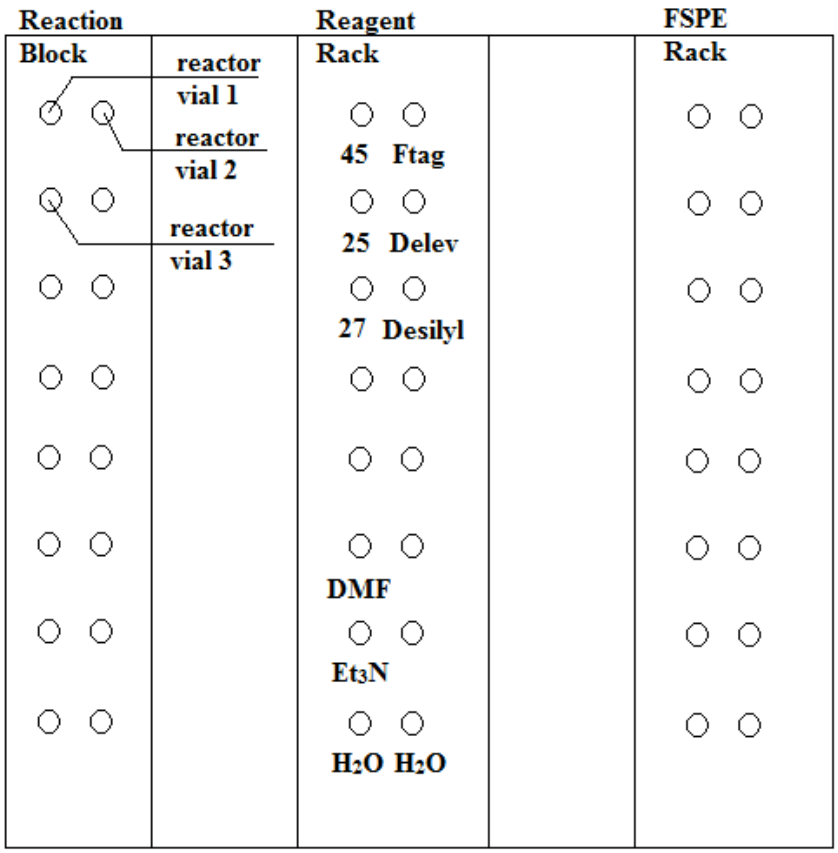

Figure 3.7 Chemspeed platform set up. Not pictured are toluene, methanol, %80 methanol and water, and TMSOTf. Those are contained in larger reservoirs, except for TMSOTf, which is kept in a special location for moisture sensitive reagents.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Conclusions

Trifunctional affinity proteomics probes (TAPPs) featuring a signal to elicit binding interactions, a photoactivatable cross-linker to capture those proteins, and a solid resin exploited for captured protein purification have been synthesized. Initial experiments were to develop a protocol to use these TAPPs as well as to test the ability of TAPPs to selectively capture known binding lectins with specificity for control glycans. Incubation, cross-linking, and rinsing protocols were optimized to selectively capture purified proteins. TAPPs with the perfluorinated phenylazide photo cross-linker exhibited greater sensitivity, about 3 fold increase in ion current for an ubiquitous peptide fragment. Furthermore, the fluorinated TAPPs displayed greater selectivity when comparing ion current of positive controls to negative controls of fluorinated phenyl azide to phenyl azide. The synthetic route was designed to make use of combinatorial chemical methods , such as automated synthetic methods of carbohydrates.

Reported in this dissertation is the development of synthetic routes to a series of monosaccharide donors that could be used on an automated platform to rapidly produce an array of related oligosaccharides from *C. elegans*. Attempts to automate, while unsuccessful in producing the target compound, did show the usefulness of a solution phase platform by allowing for easy determination of the problem step by TLC analysis, something that is not possible with solid phase methodology.

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One interesting feature of these oligosaccharides is the heavily branched singular saccharide units that feature a 3,4, and 6 glycosidic linkage. Investigation of known chemical methods to affect such a coupling, as well as accomplishing multiple glycoslyations was achieved.

Future Directions

Investigation into linker effects and resin supports might confer better behavior, in terms of senstivity and selectivity, of the TAPPs reported in this dissertation. A host of questions can be raised about the surface density and display of the carbohydrates on the surface of the resin. The surface of the resin support is considered to be a highly amorphous surface. It's possible the display of carbohydrates is not very conducive to binding. Considering the results with capturing purified proteins, maybe the soluble protein has more freedom to distort and find a conformation for favorable binding. It is also known that conA (the protein used in these experiments) exhibits shallow binding pockets (Kiessling 1996). Possibly the shallow pockets on conA make it possible to still bind to mannose that is tethered very near to the surface of the resin. Potentially, there is some aryl aryl interaction between the probe and the surface of the resin that could causes the probe to not present well in solution. It's also possible the polystyrene based probe contracts in the aqueous environment, distorting, collapsing, or in some way having adverse effects on the display of the carbohydrate on the surface of the resin. A switch to a differently designed probe that uses latex particles that are known to swell in aqueous systems

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and to linkers that are more hydrophilic (ether based), presentation of the carbohydrates will improve and the overall outcome of the cross-linking will improve.

The utility of this probe to be create array-like carbohydrate screening with the added benefit of being able to capture that interaction from live bacteria or a protein mixture would be a great advancement to current methodology. Another challenge for the TAPPs that would be interesting to exploit is to use libraries of compounds such as the saccharides from *C. elegans* and try to capture surface binding proteins directly from cell culture.

The saccharides from the *C. elegans* are accessible with current methodolgy. The donors here should be able to complete the synthesis of the six oligosaccharides. If there was need to circumvent the acyl migration of the acetate during desilylation because no condition is able to avoid it, a benzoyl ester could be employed. These esters are well known to resist migration. The routes described here could easily be modified to do so. Using trimethyl orthobenzoate instead of trimethyl orthoacetate during orthoester formation of the *cis* diols in the galactose and galactosamine based donors, then the corresponding ester after ring opening would be the desired benzoyl ester.

The automated synthesis of carbohydrates is severally lagged by the access to donor saccharides in large scale. If convenient routes were available to crystallizable intermediates, as well as developing methods to access the more rare carbohydrates, then automation could be realize its full potential. A lot of outlook on automation is focused on the idea that it is to produce materials. It's possible that automation can be much more useful than as a manufacturing platform, it could be used to systematically explore carbohydrate chemistry. It could be employed to study concentration effects, temperature effects, and solvent effects rapidly if there is access to a handful of carbohydrates on large scale. If better protecting group

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strategies are employed, the ASW could also be used to probe the effects of protecting groups as well. Currently, the literature is limited to small subsets of reactions with little systematic, or contiguous, data that allows for complete understanding of the glycosylation reaction. The reactions are also spread out over the hands of hundreds of chemists who do things just a 'little different' then the other guy. Automation would be better suited for such data acquisition by minimizing random error in the glycosylation outcomes as well.

Once a thorough understanding of carbohydrate chemistry is realized, and as chemical biological methodology develops, probing the functions of carbohydrates in biological systems will become more rapid and easier. Developing our understanding of disease, treatments, and generally rounding out the scientific paradigm.

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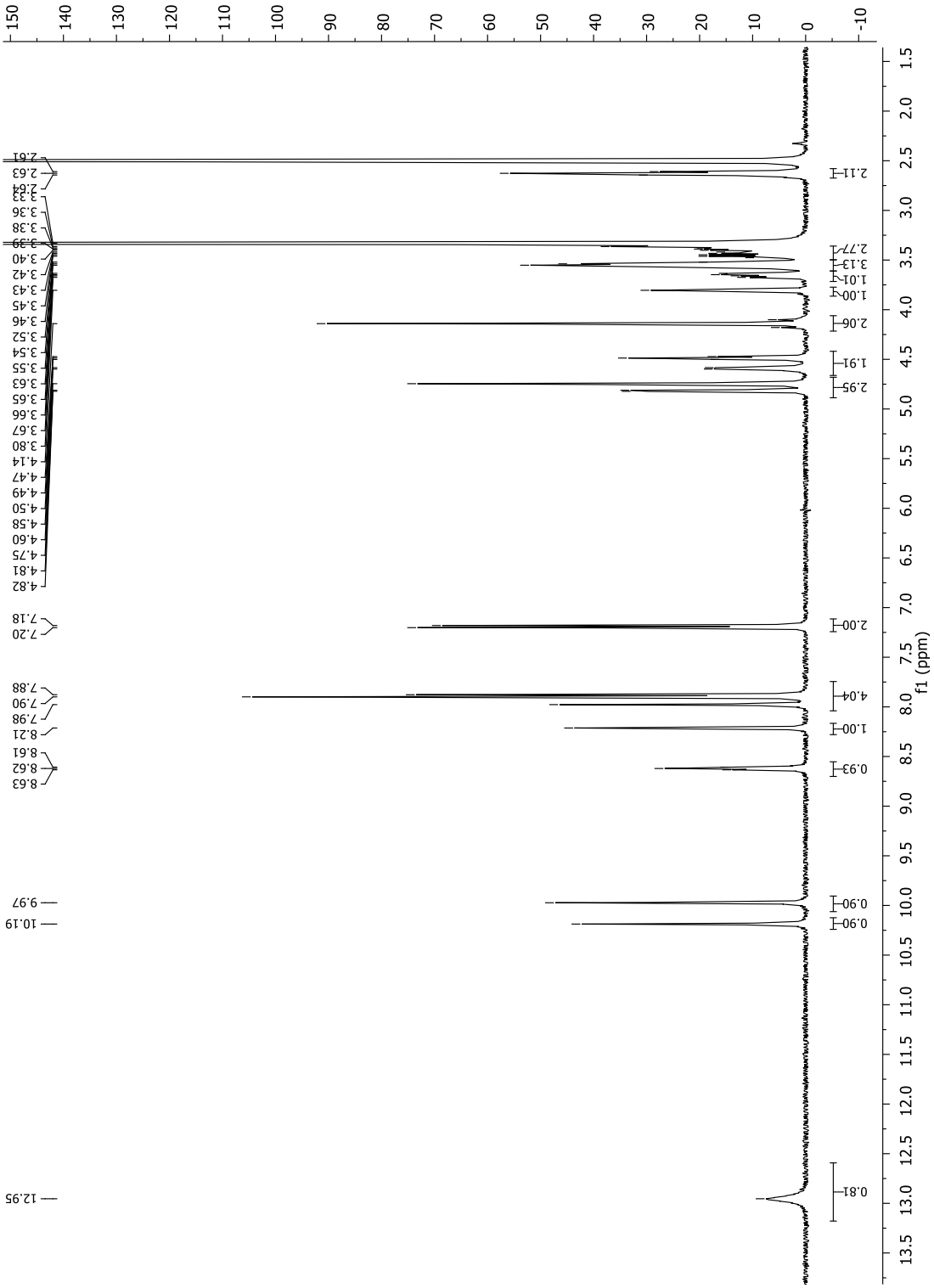
APPENDIX A. CHAPTER II ^{1}H NMR AND ^{13}C NMR



^{1}H NMR spectrum of compound **3** in d_6-DMSO at 400 Mhz

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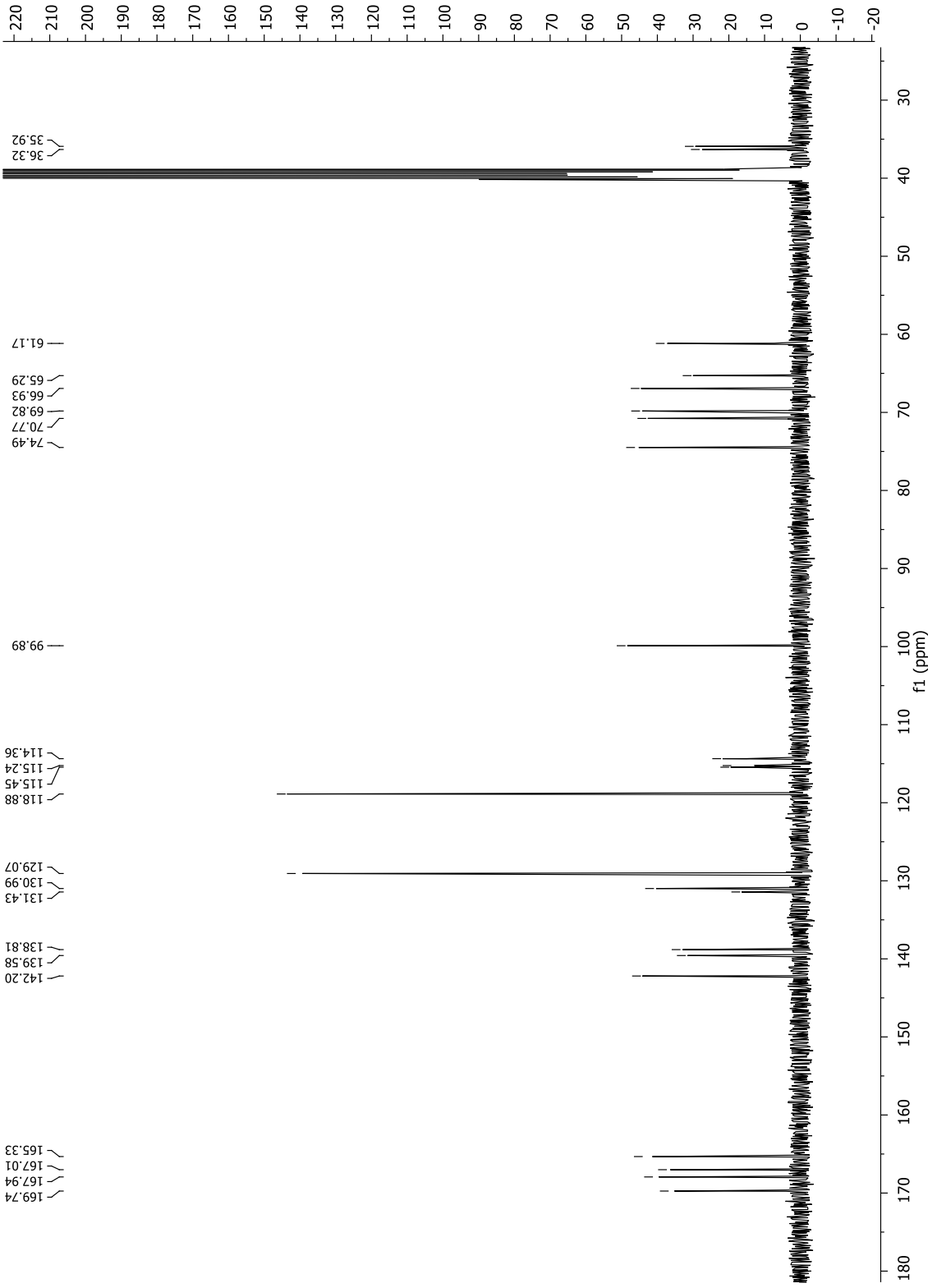

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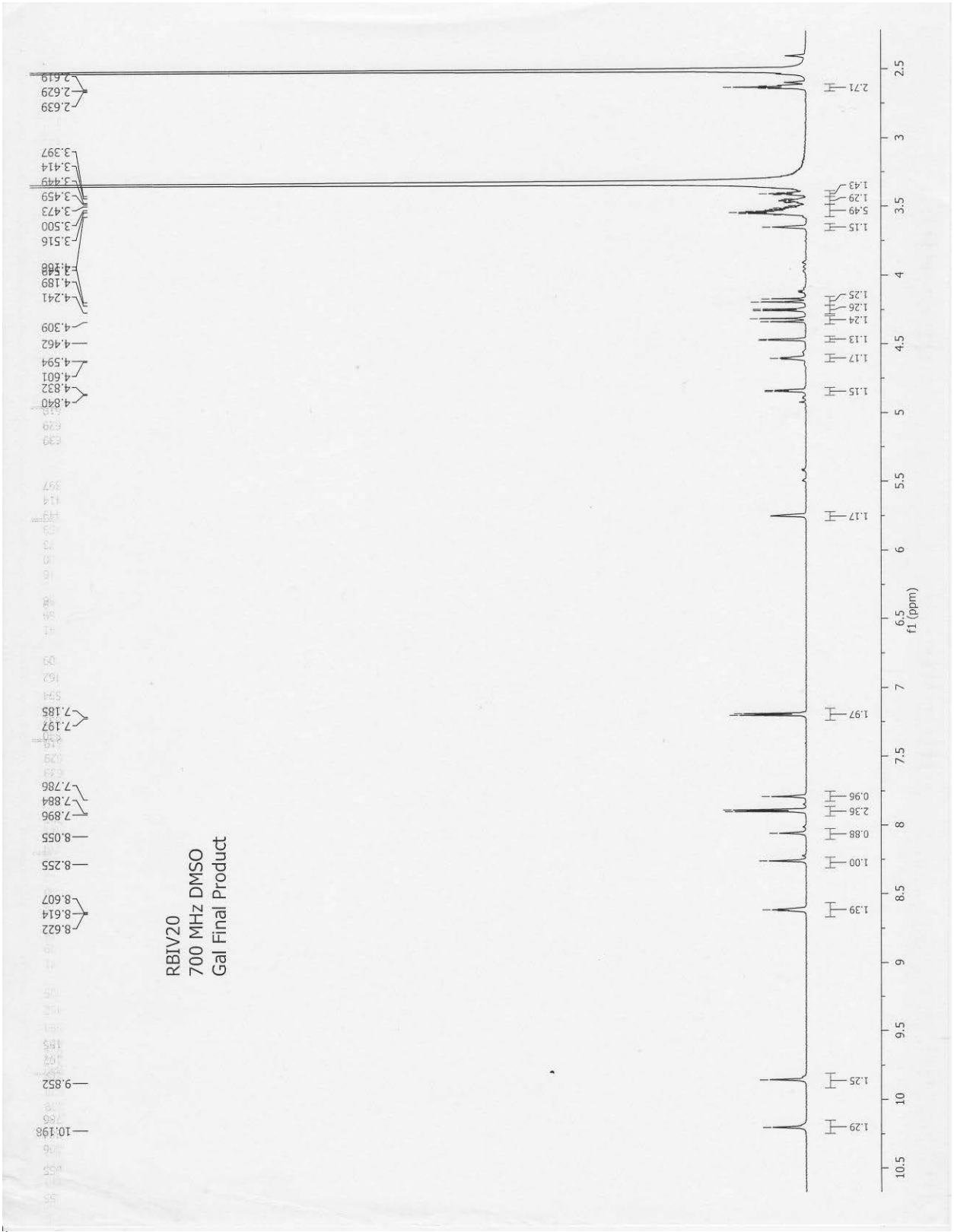

^{1}H NMR spectrum of compound **4** on 700 MHz Varian NMR in d_6-DMSO at 400 Mhz.

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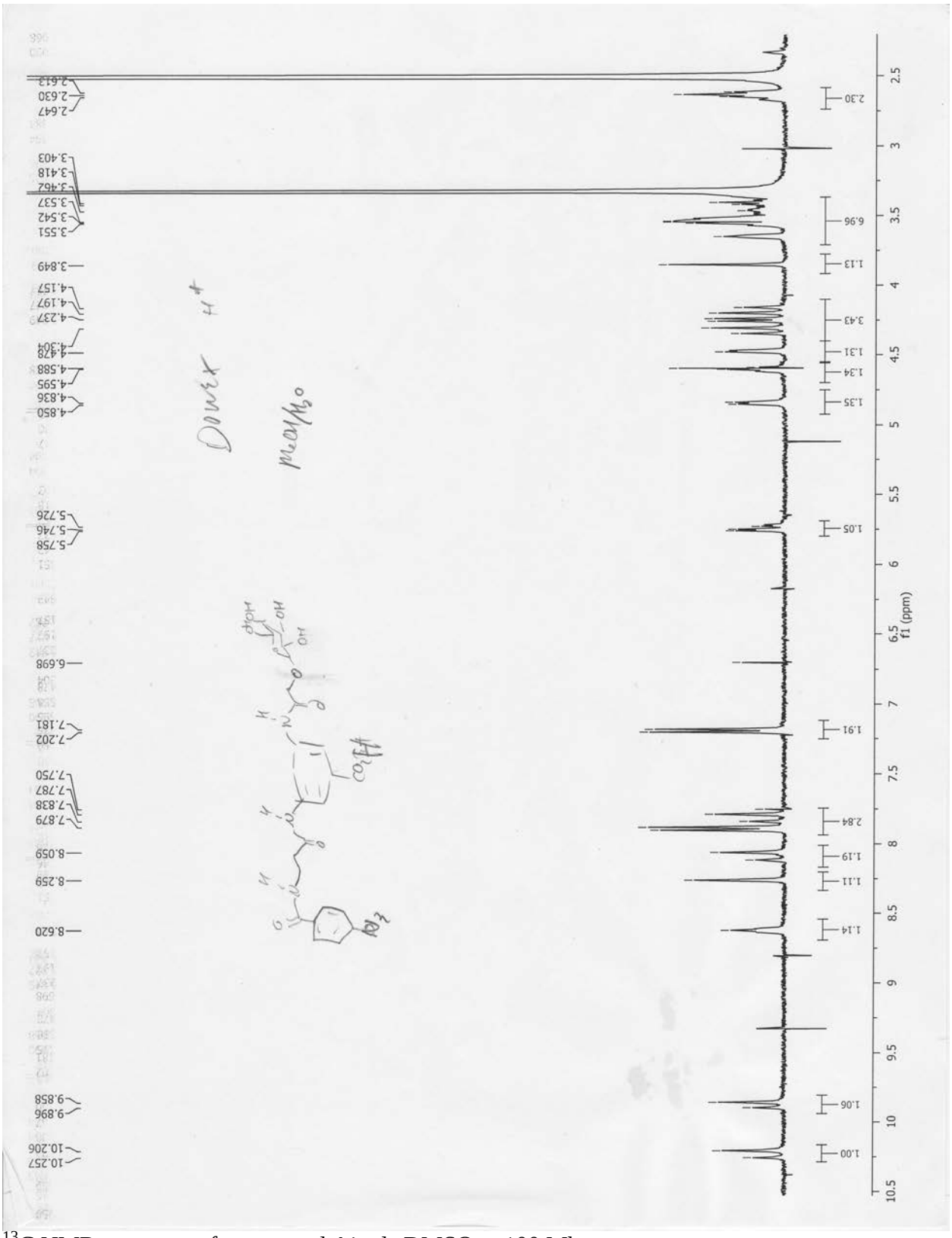

^{13}C NMR spectrum of compound **4** in d_6-DMSO at 100 Mhz.

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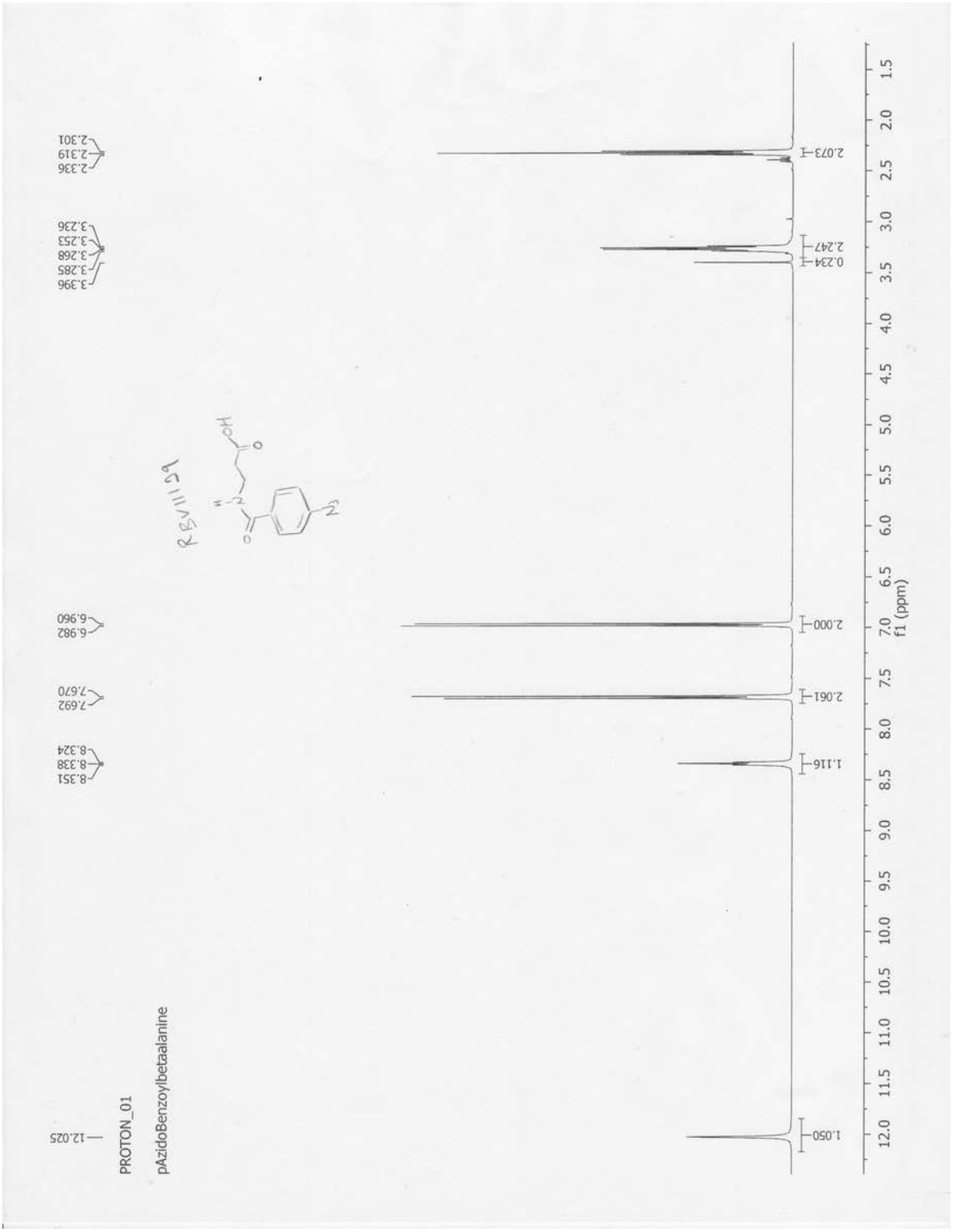

^{1}H NMR spectrum of compound **5** in d_6-DMSO at 400 Mhz.

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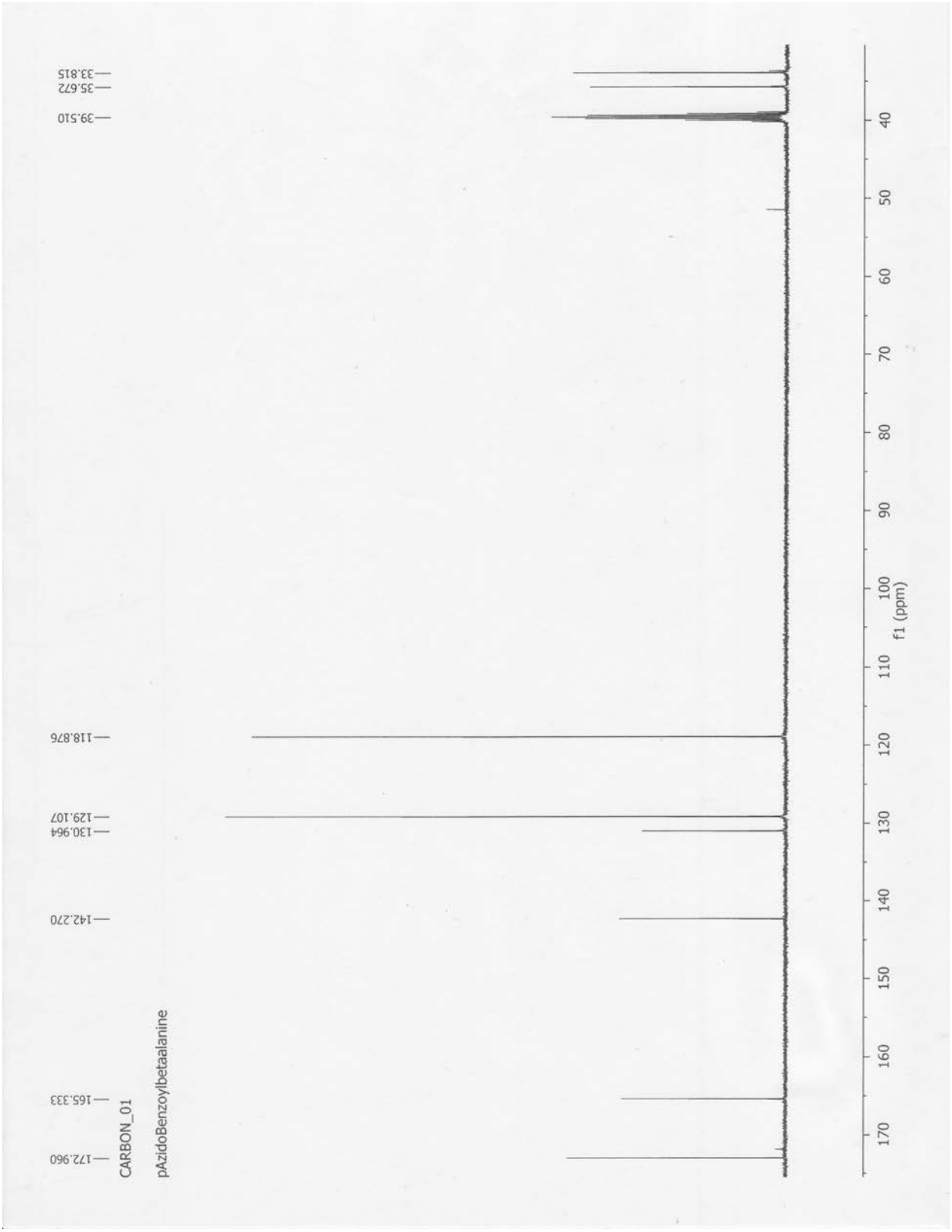

^{13}C NMR spectrum of compound **5** in d_6-DMSO at 100 Mhz.

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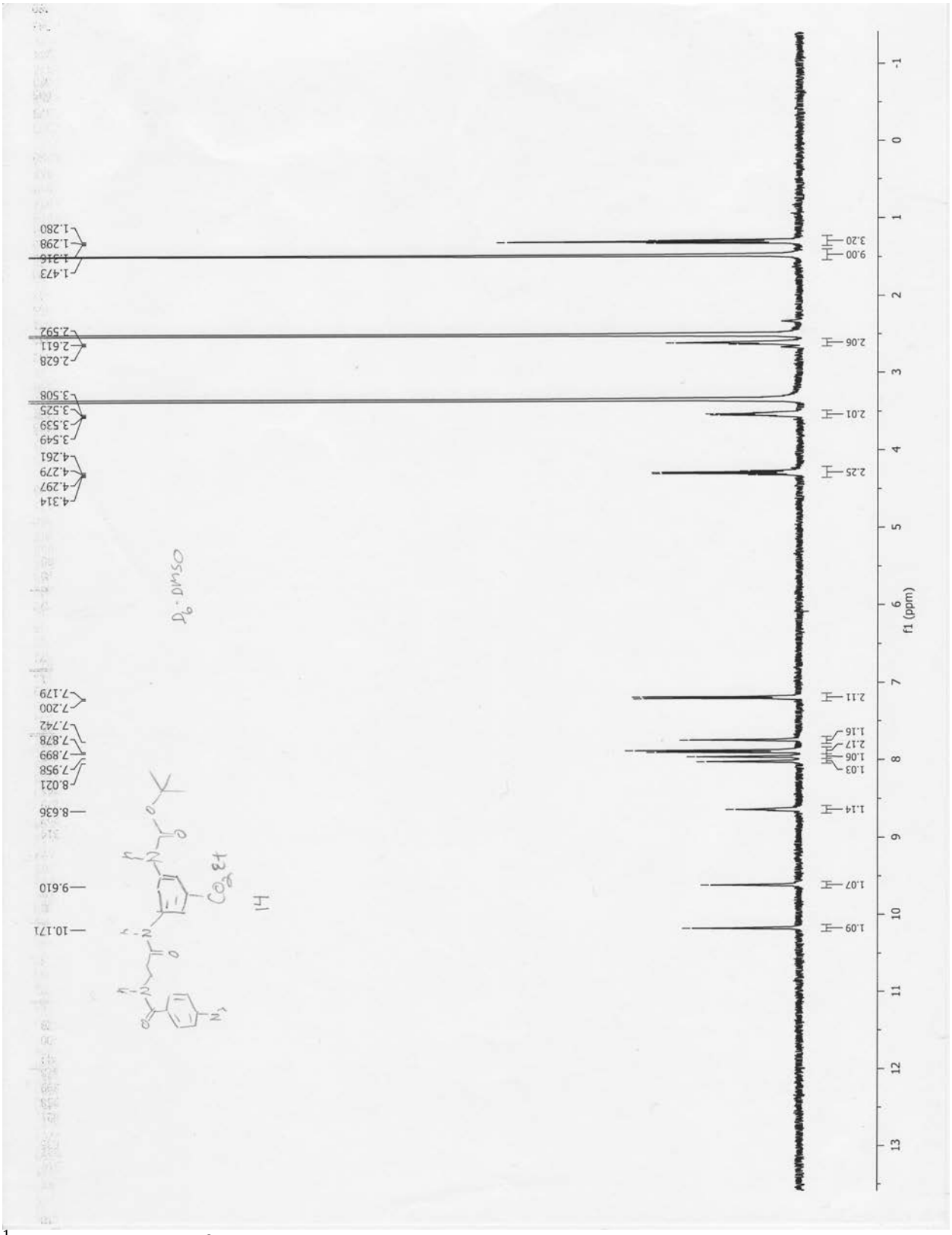

^{1}H NMR spectrum of compound in d_6-DMSO at 400 Mhz.

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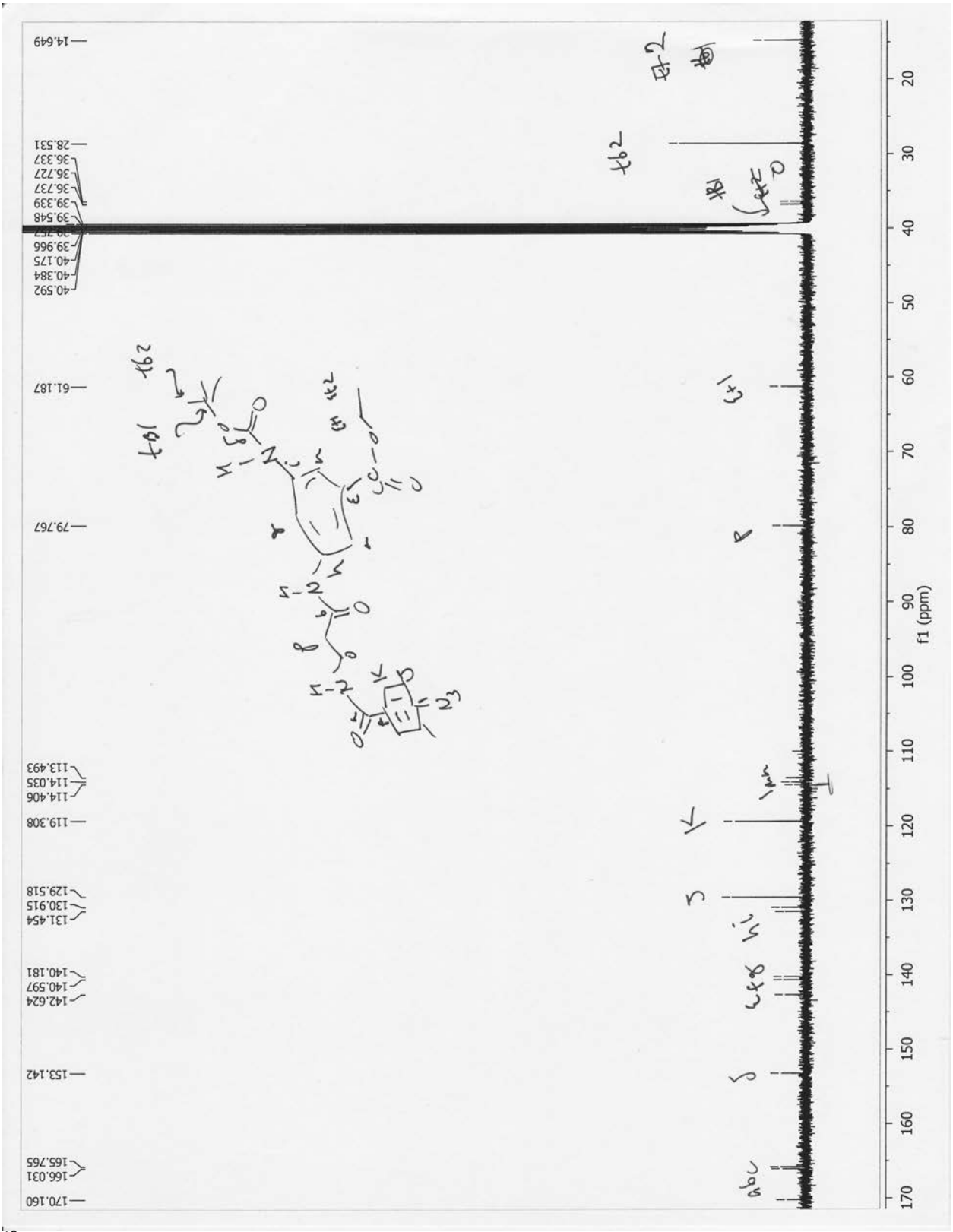

^{13}C NMR spectrum of compound **13** in d_6-DMSO at 100 Mhz.

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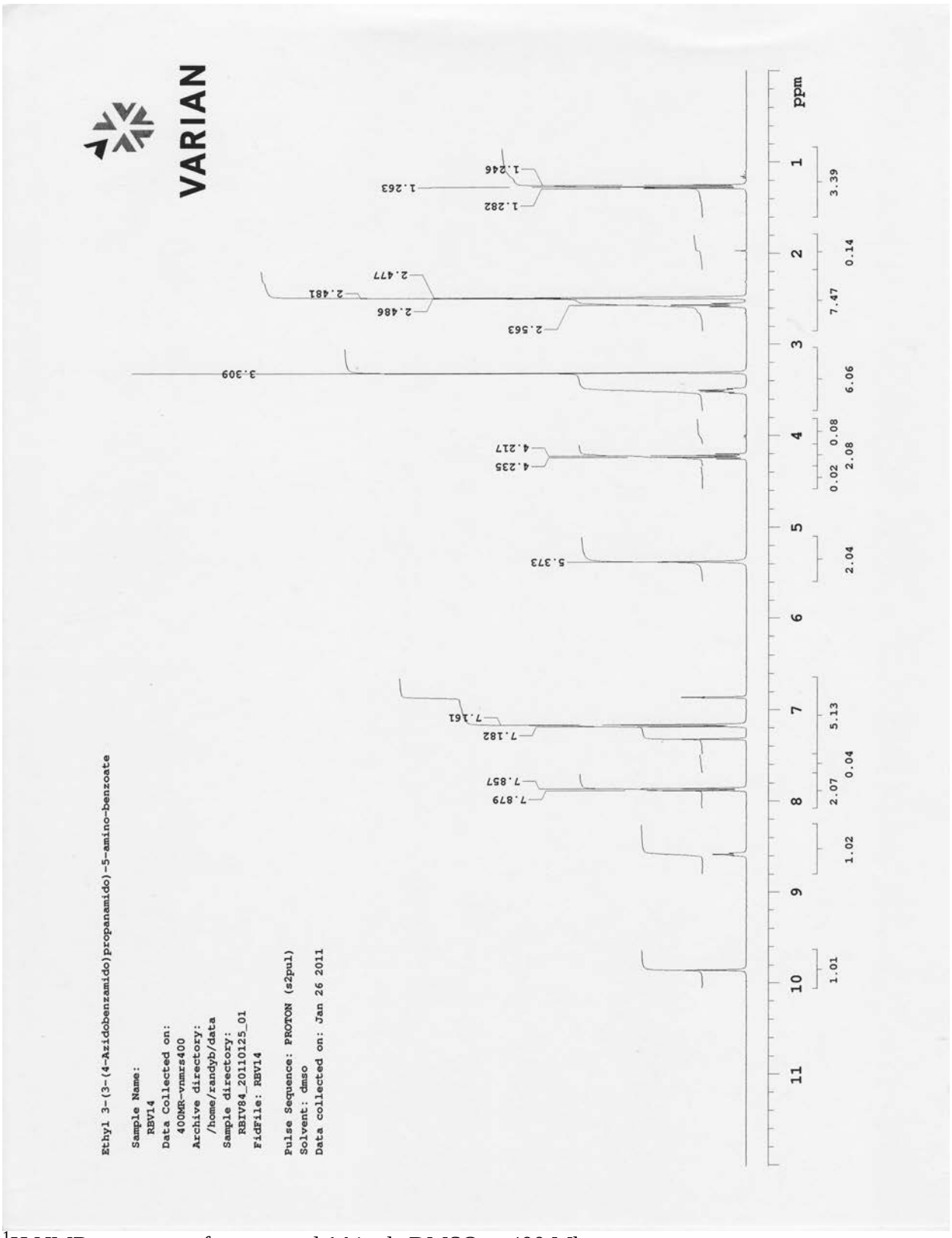

[1]H NMR spectrum of compound **14** in d_6-DMSO at 400 Mhz.

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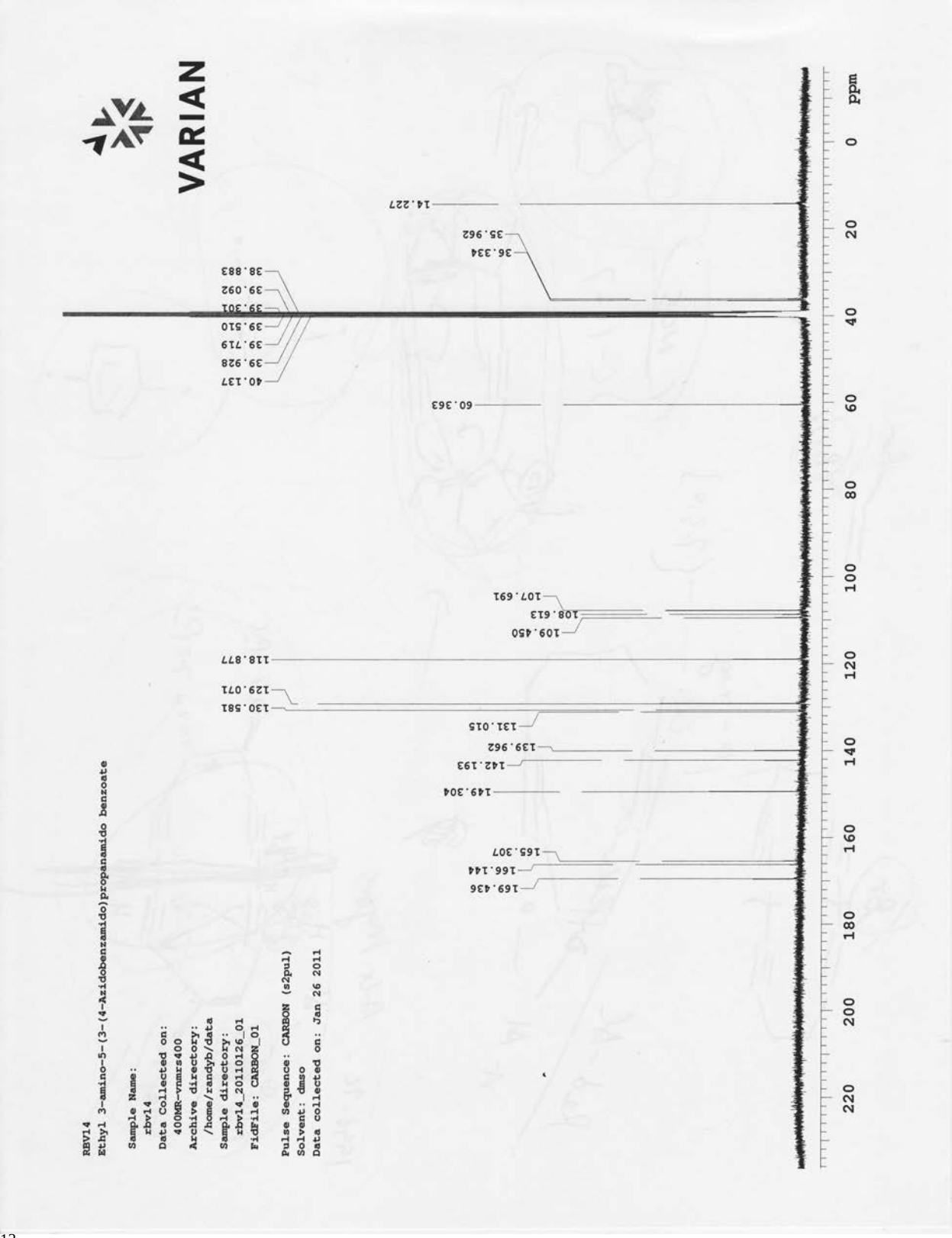

^{13}C NMR spectrum of compound **14** in d_6-DMSO at 100 Mhz.

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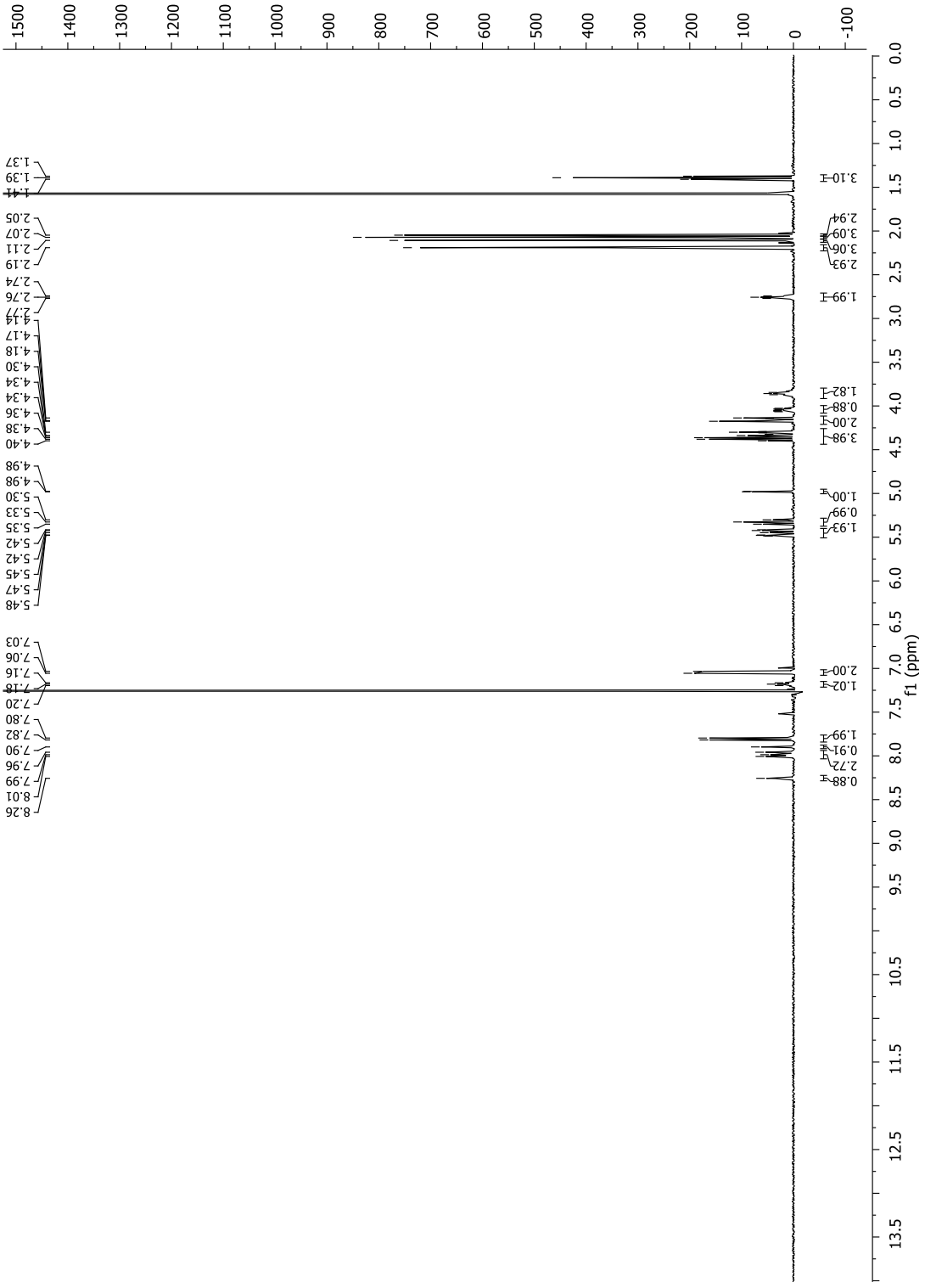

^{1}H NMR spectrum of compound **15** in CDCl3 at 400 MHz.

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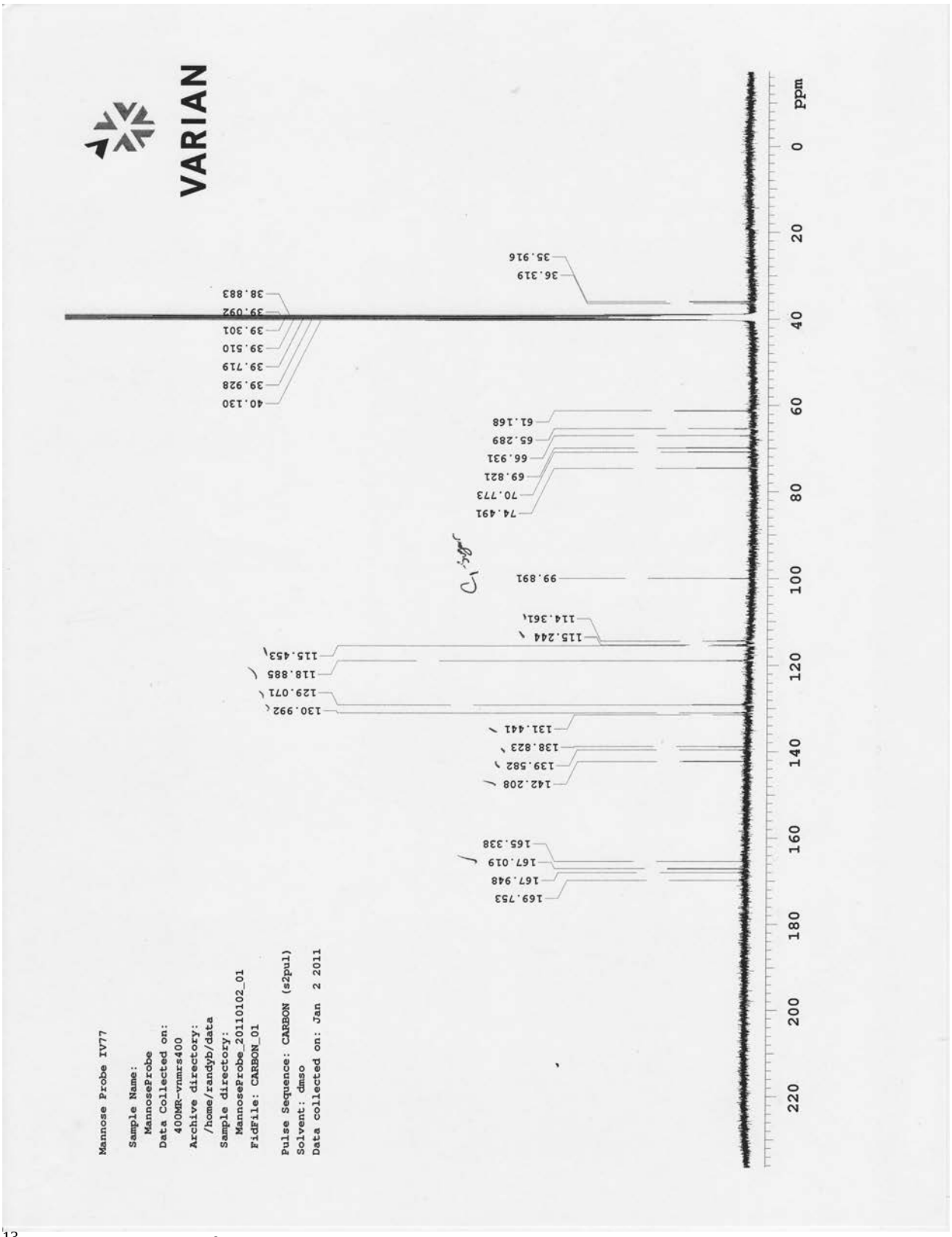

^{13}C NMR spectrum of compound **15** in d_6-DMSO at 100 MHz.

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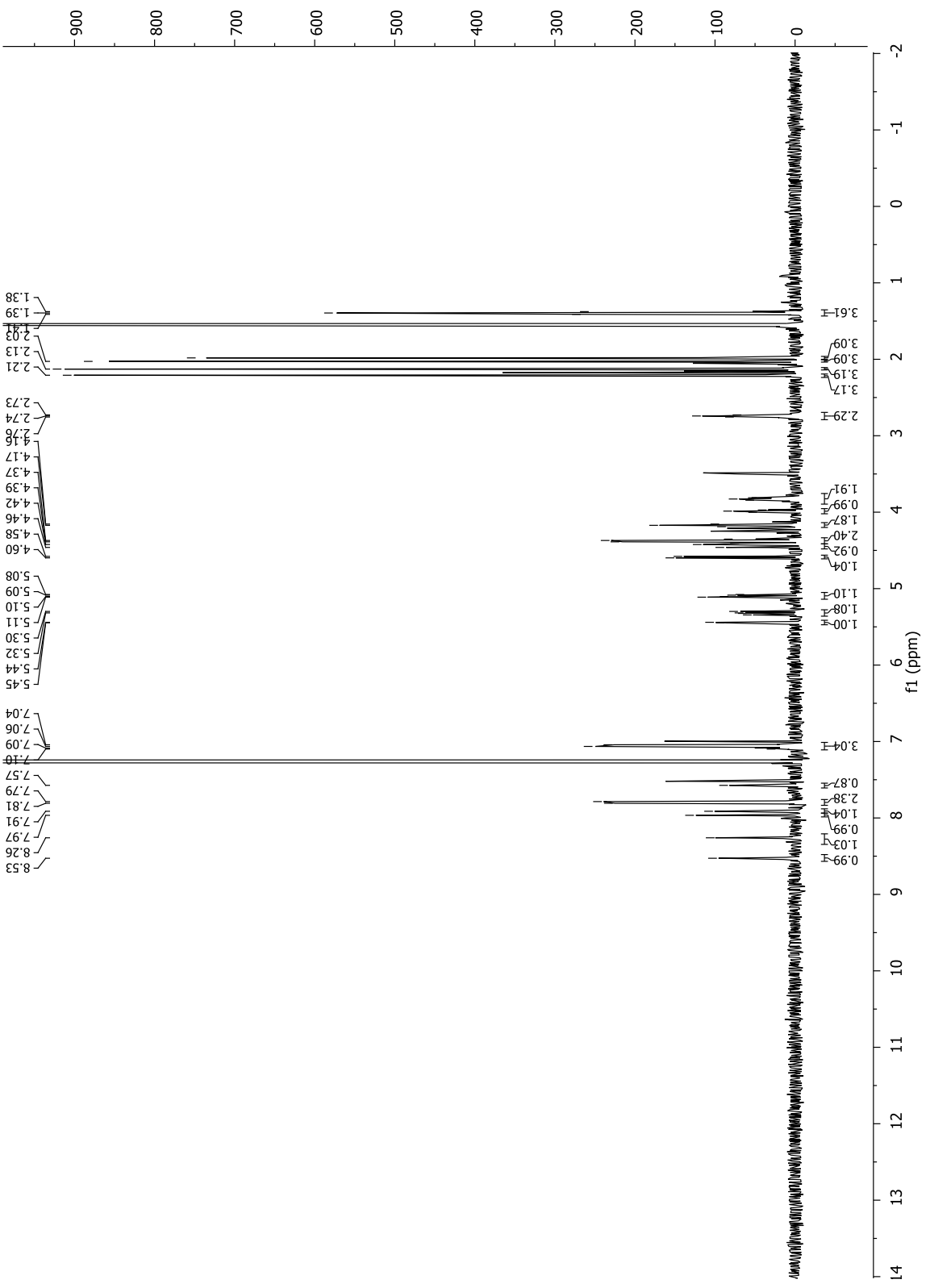

^{1}H NMR spectrum of compound **16** in CDCl3 at 400 MHz.

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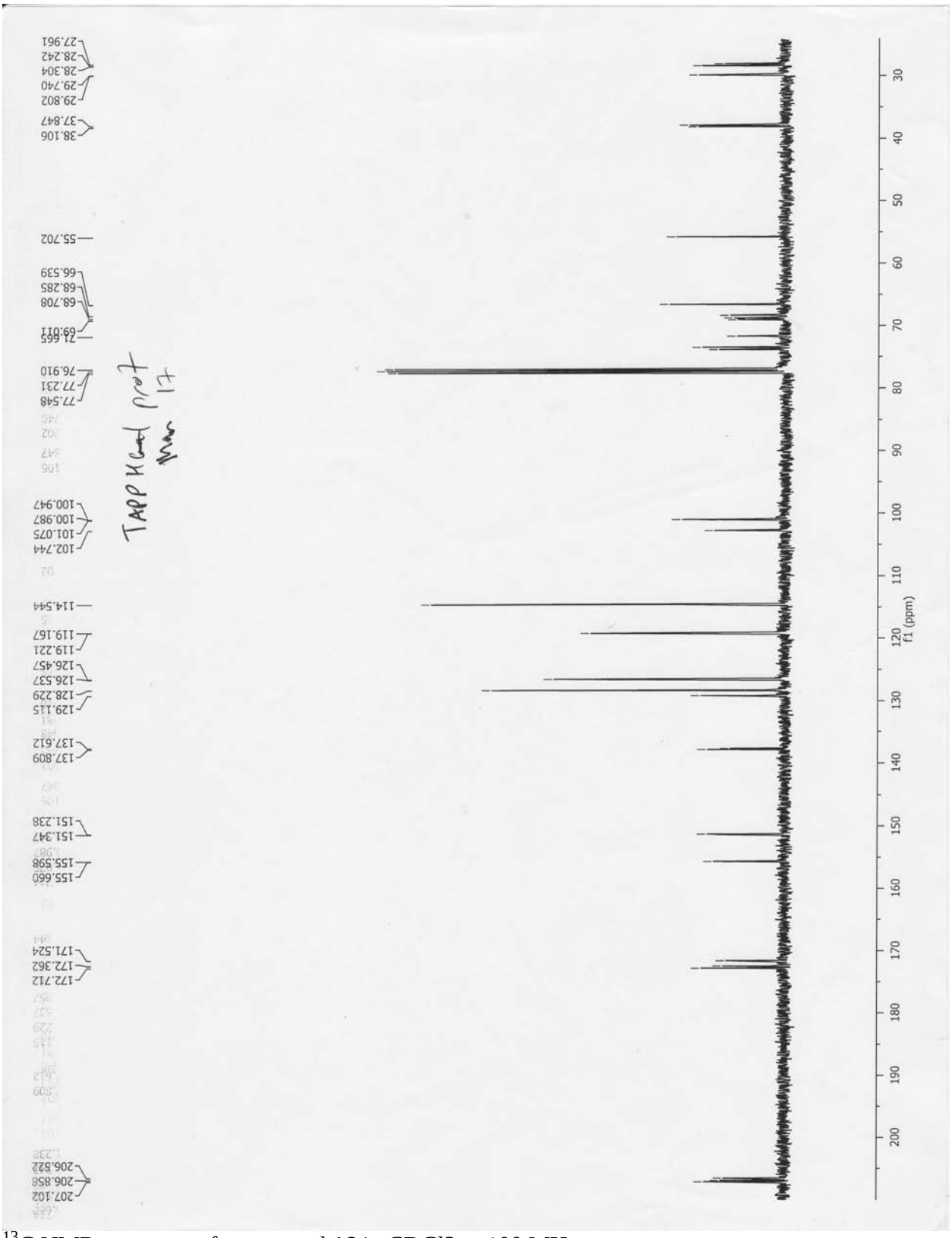

^{13}C NMR spectrum of compound **16** in CDCl3 at 100 MHz.

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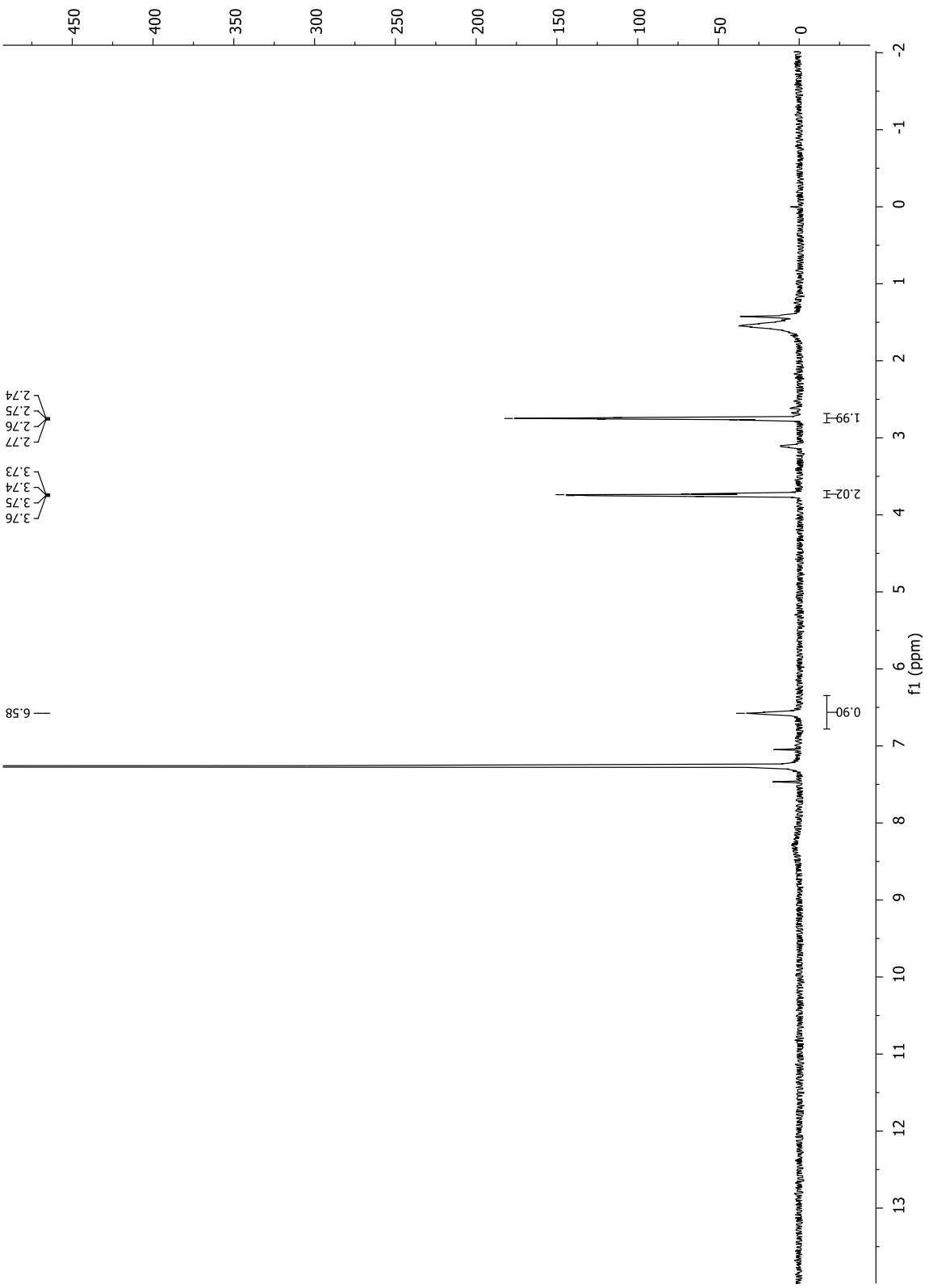

^{1}H NMR spectrum of compound **18** in CDCl3 at 400 MHz.

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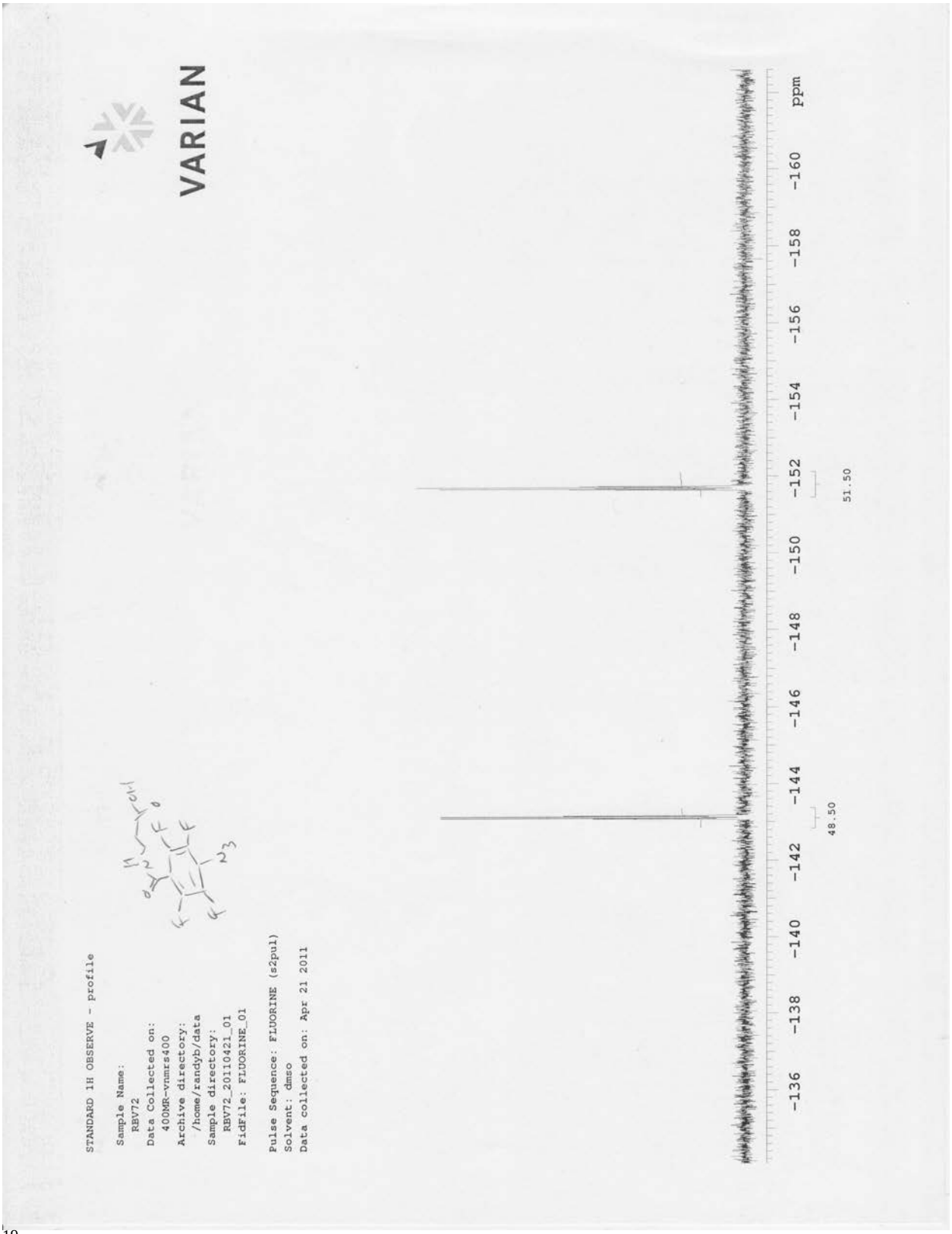

^{19}F NMR spectrum of compound **18** in d_6-DMSO at 400 Mhz.

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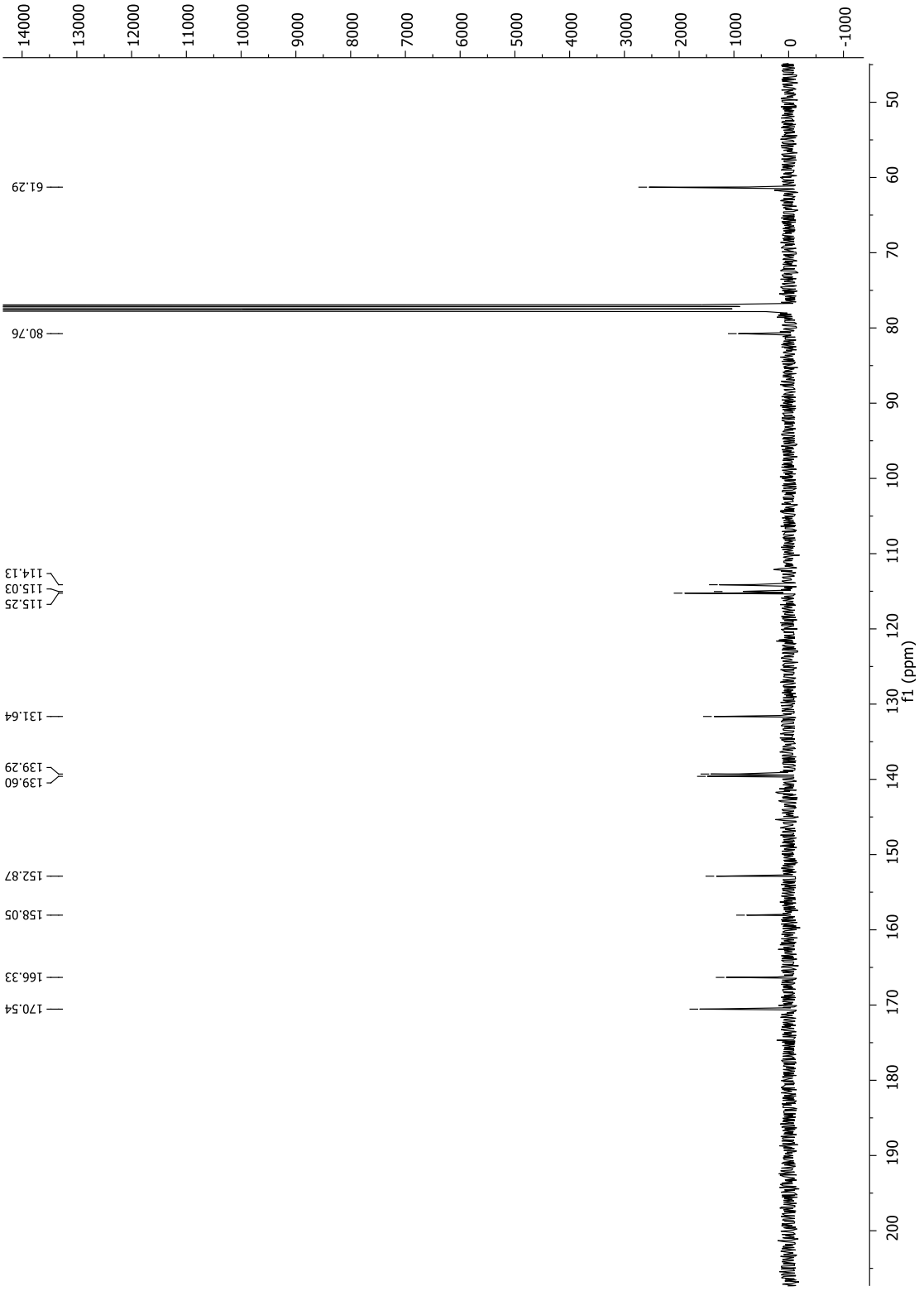

^{13}C NMR spectrum of compound **18** in d_6-DMSO at 100 MHz.

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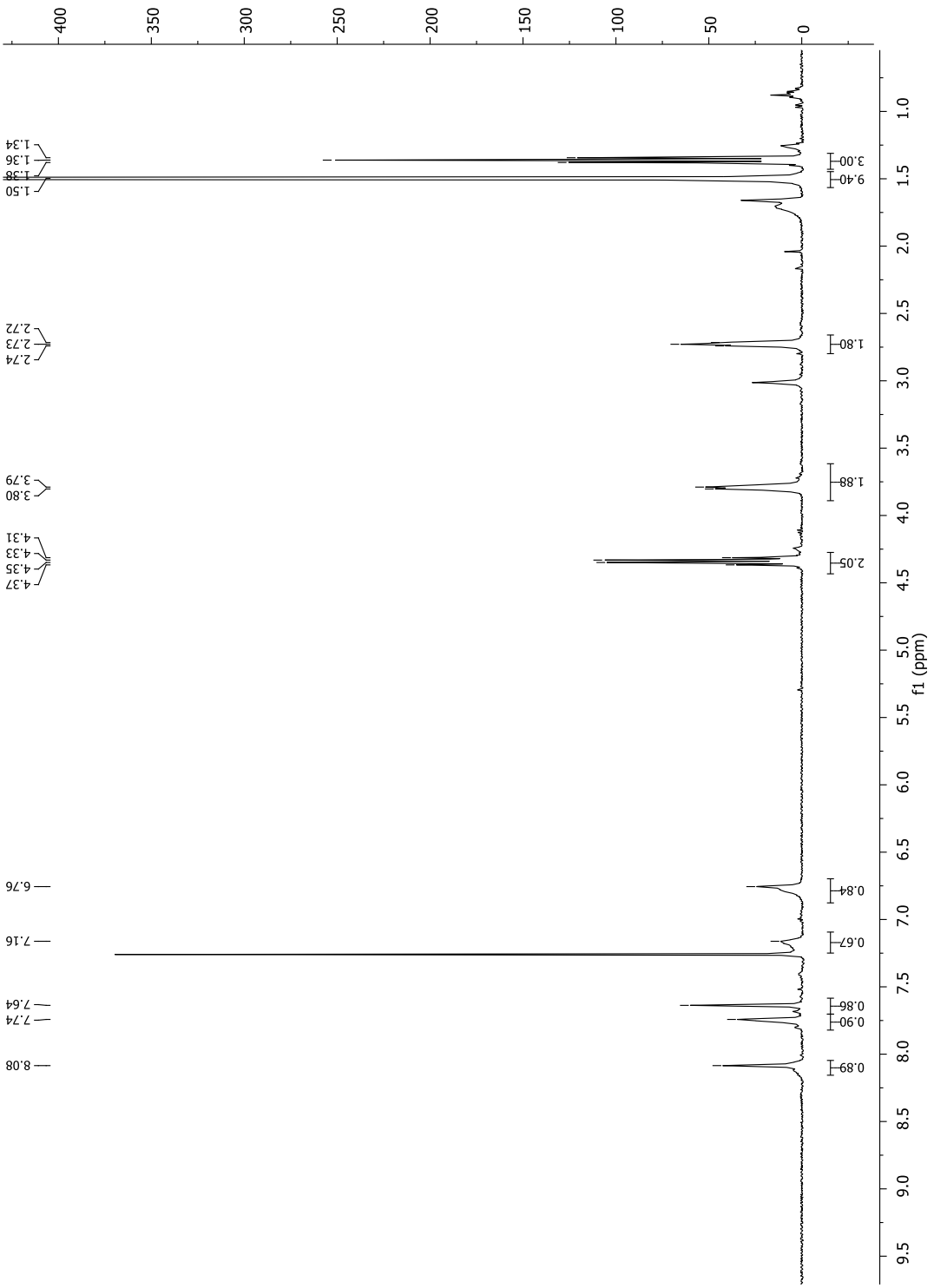

^{1}H NMR spectrum of compound **19** Needed a couple drops of d_6-DMSO to dissolve in CDCl3 at 400 MHz

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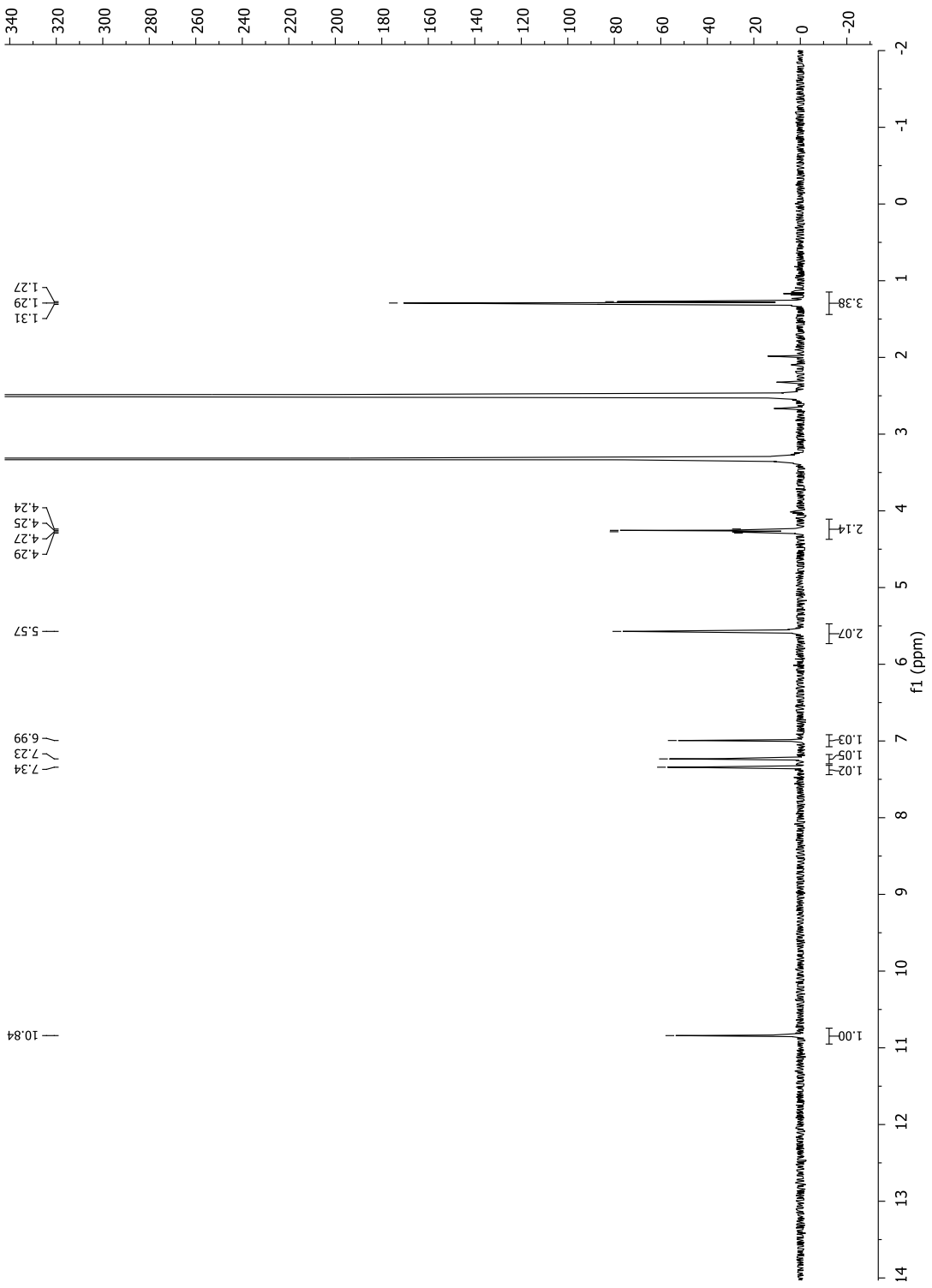

^{1}H NMR spectrum of compound **20** in CDCl3 at 400 MHz

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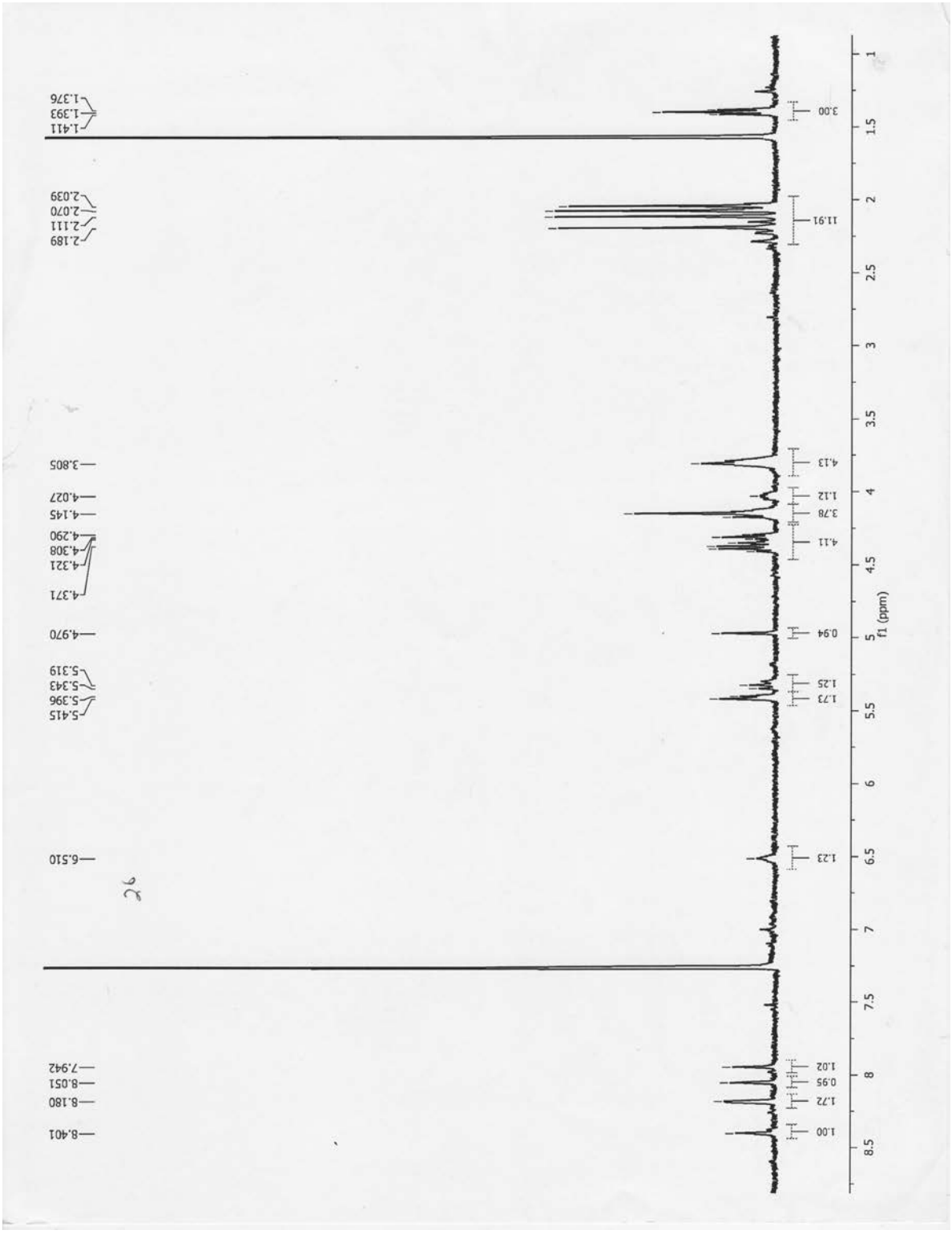

^{1}H NMR spectrum of compound **21** $CDCl_3$ at 400 Mhz.

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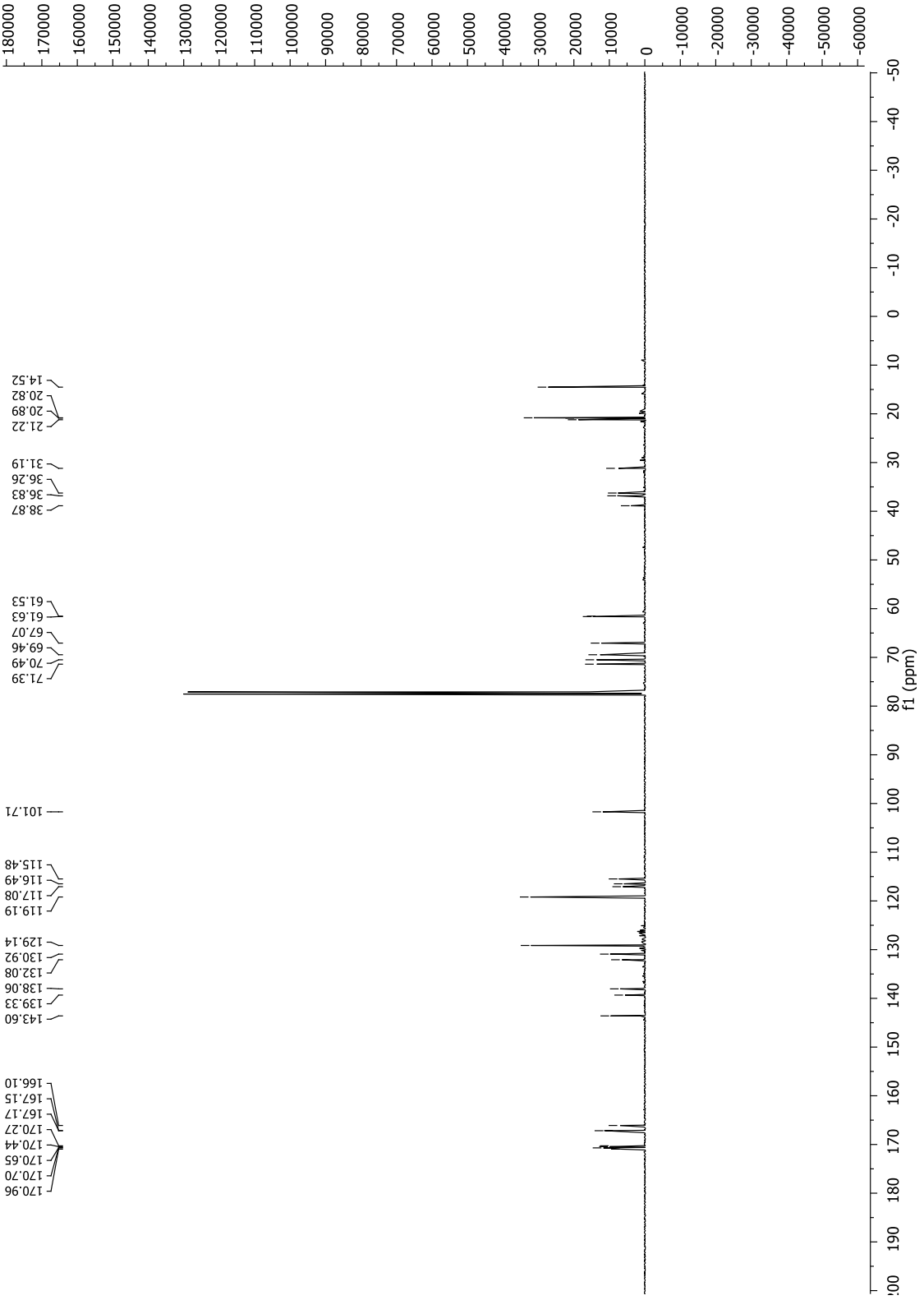

^{13}C NMR spectrum of compound **21** $CDCl_3$ at 100 Mhz.

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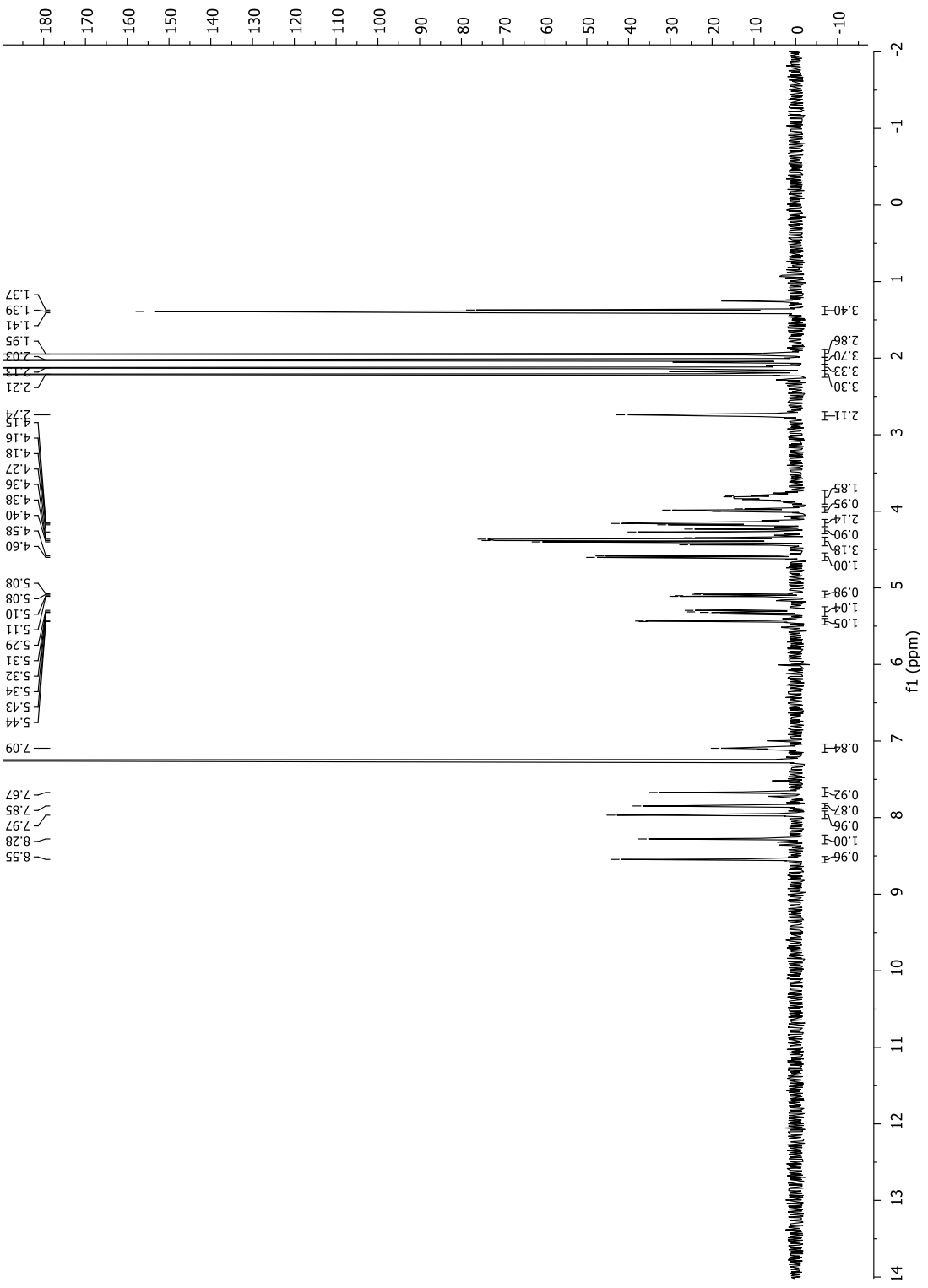

^{1}H NMR spectrum of compound **22** in CDCl3 at 400 MHz.

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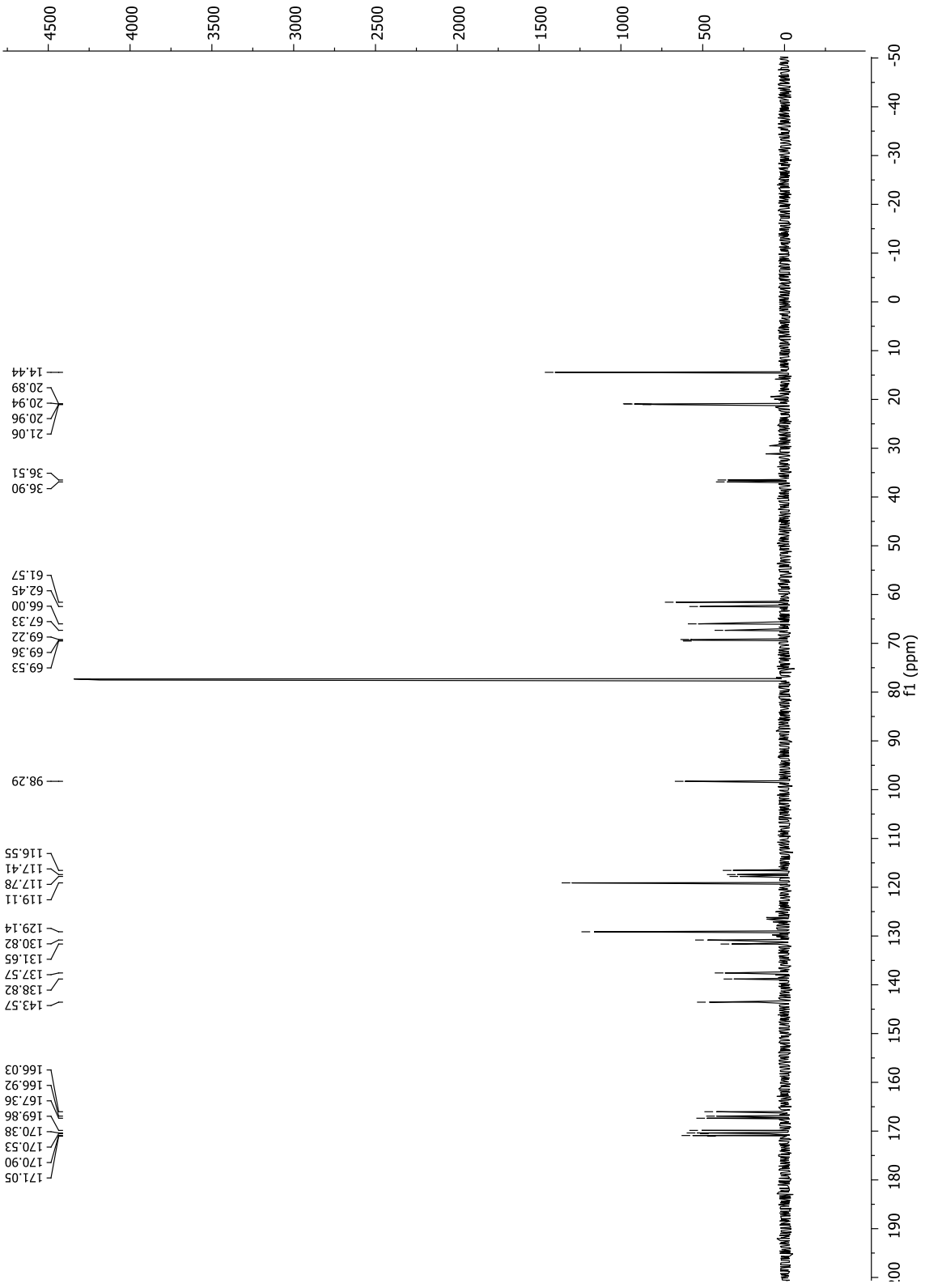

^{13}C NMR spectrum of compound **22** in CDCl3 at 100 MHz.

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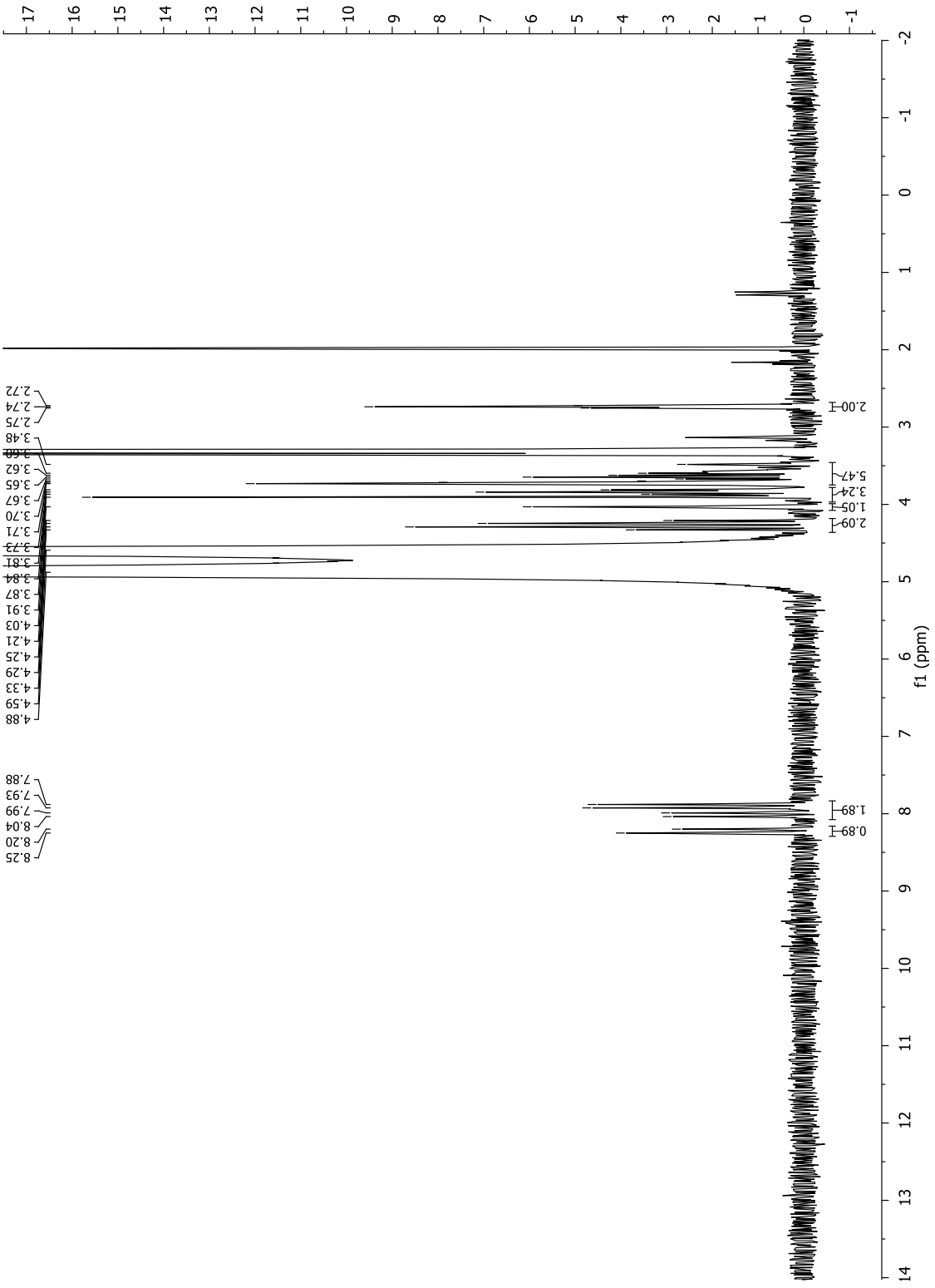
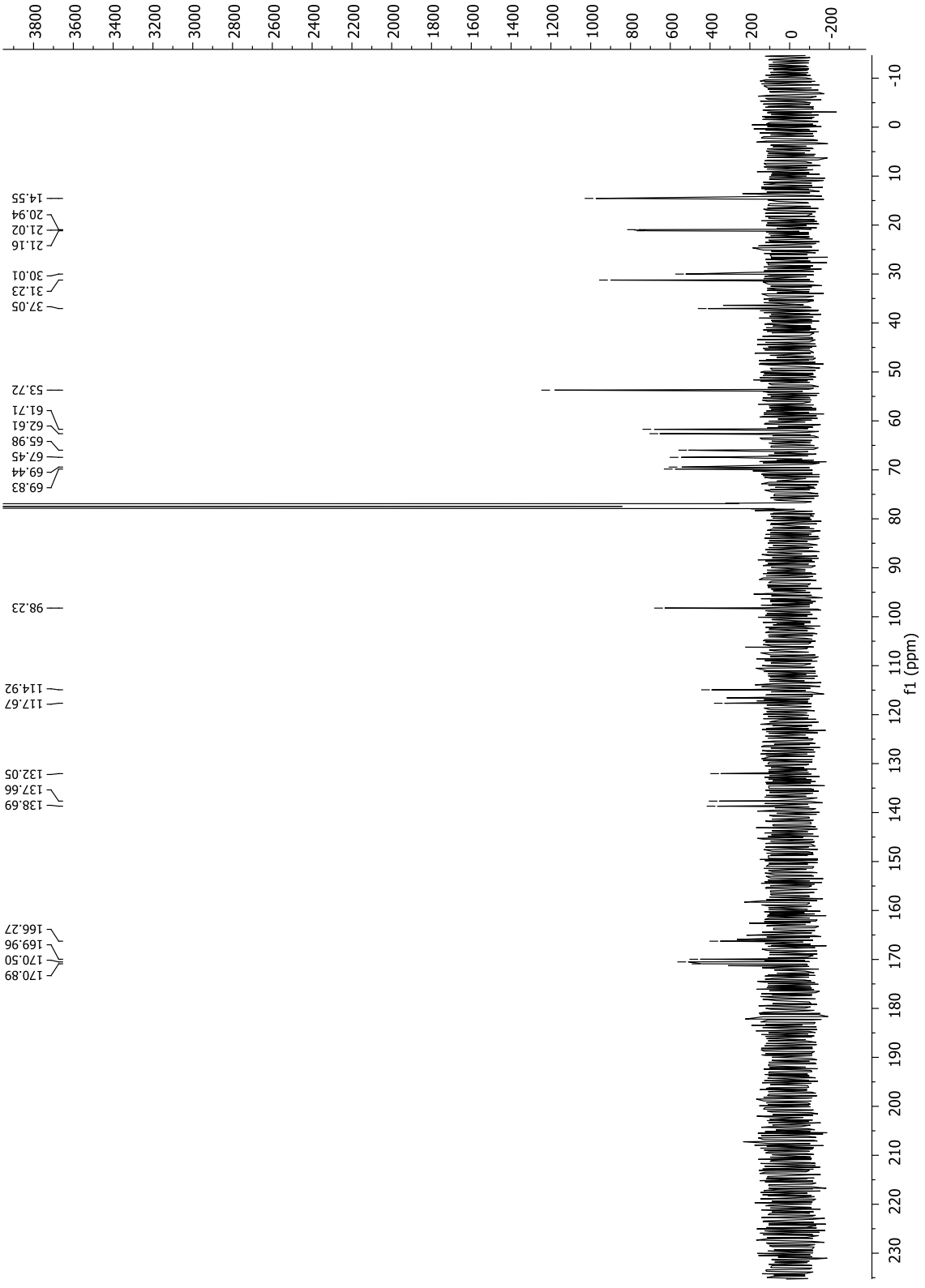


^{1}H NMR spectrum of compound **23** in CD_3OD at 400 MHz.

www.manaraa.com



^{13}C NMR spectrum of compound **23** in CD_3OD at 100 MHz

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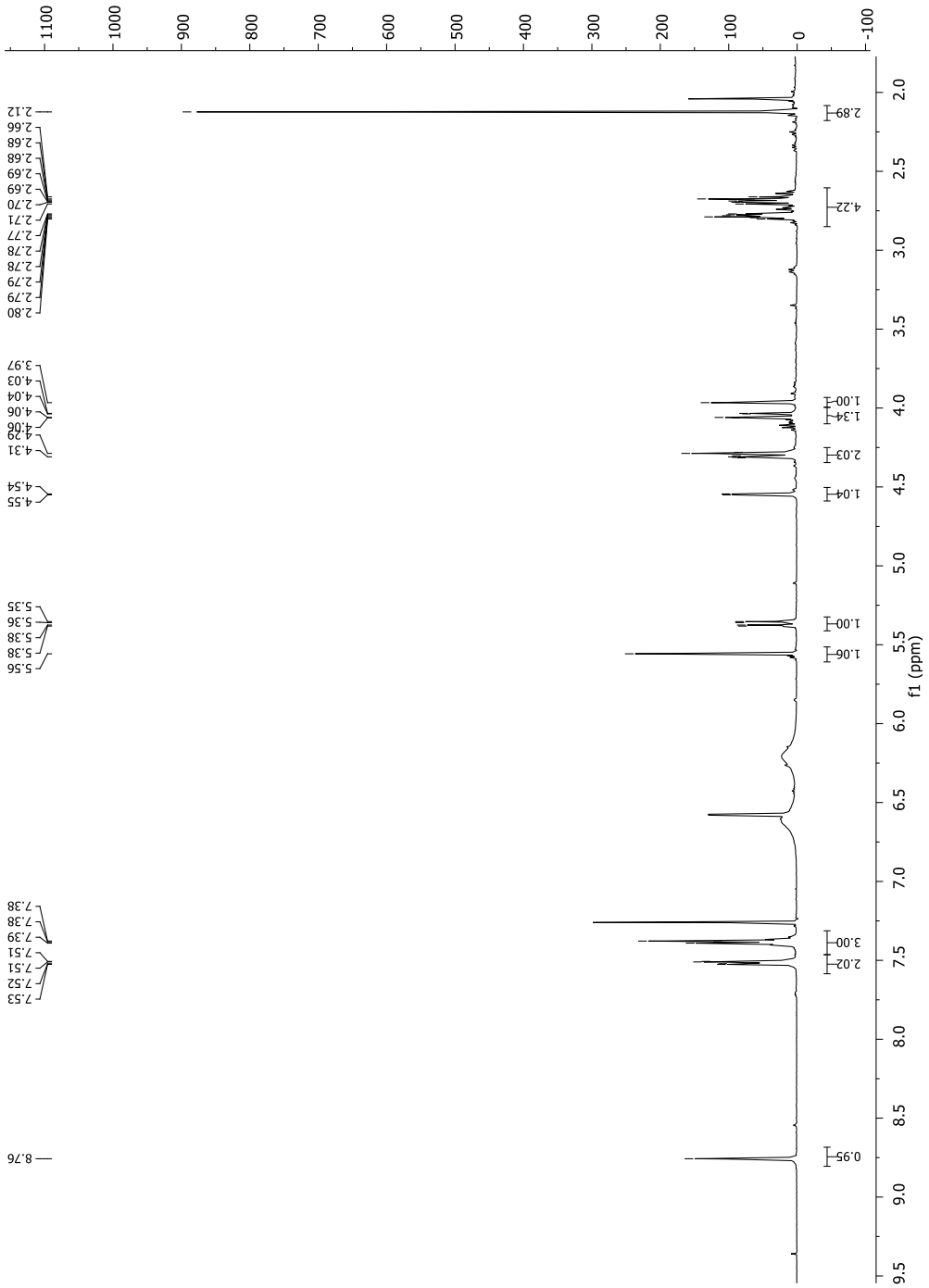
APPENDIX B. CHAPTER III ^{1}H NMR AND ^{13}C NMR



^{1}H NMR spectrum of compound **21** in CDCl3 at 400 MHz.

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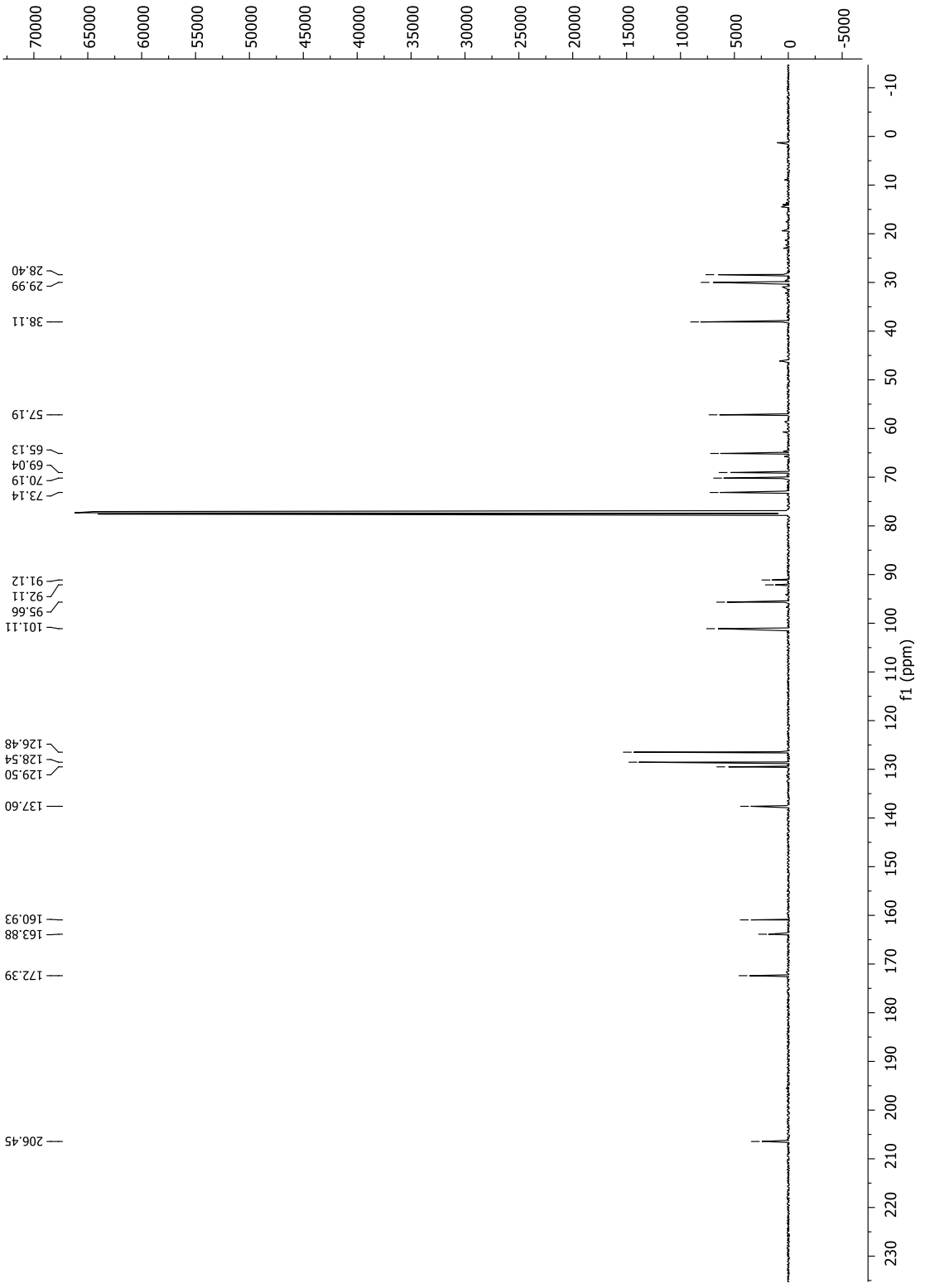
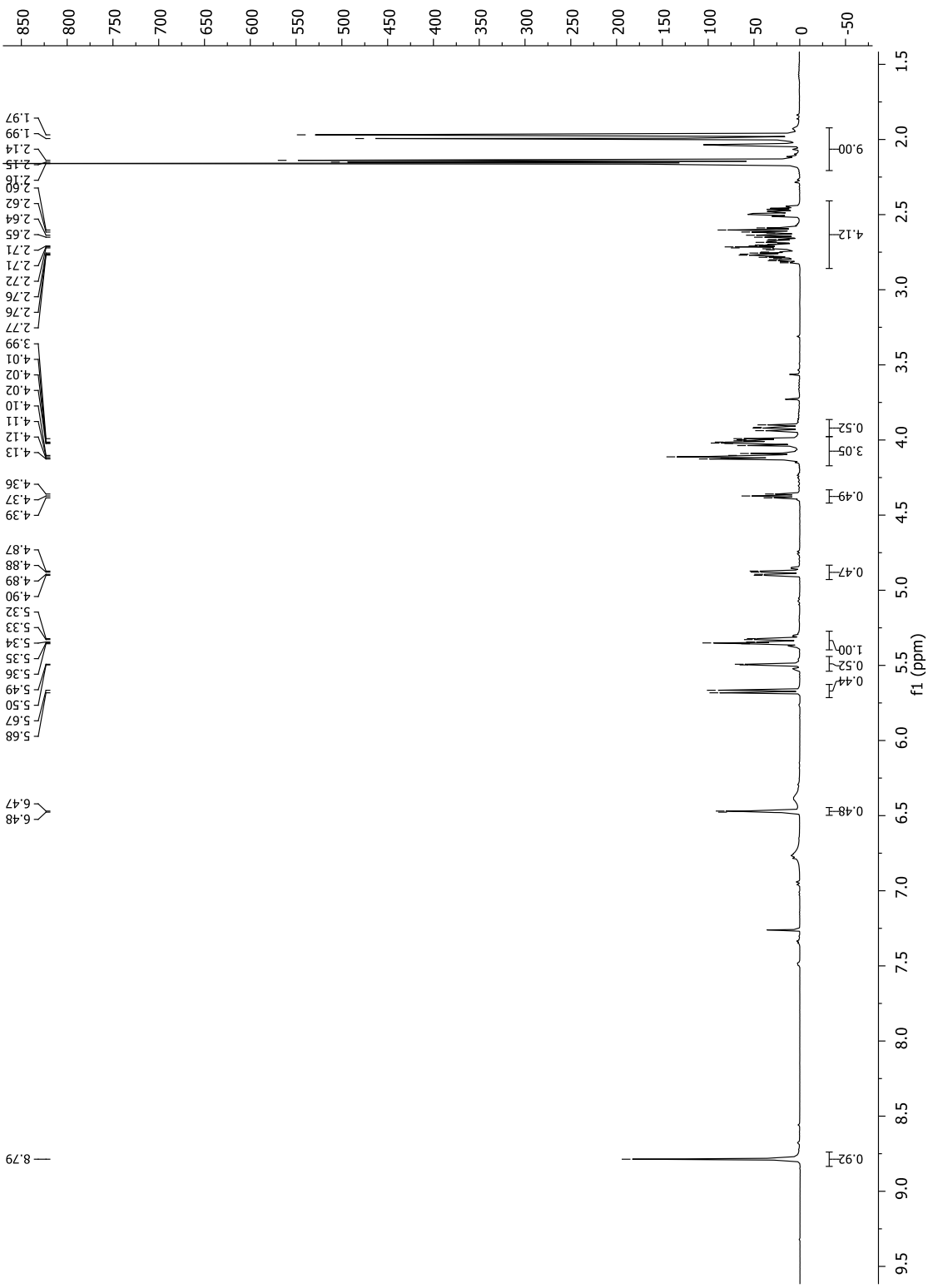


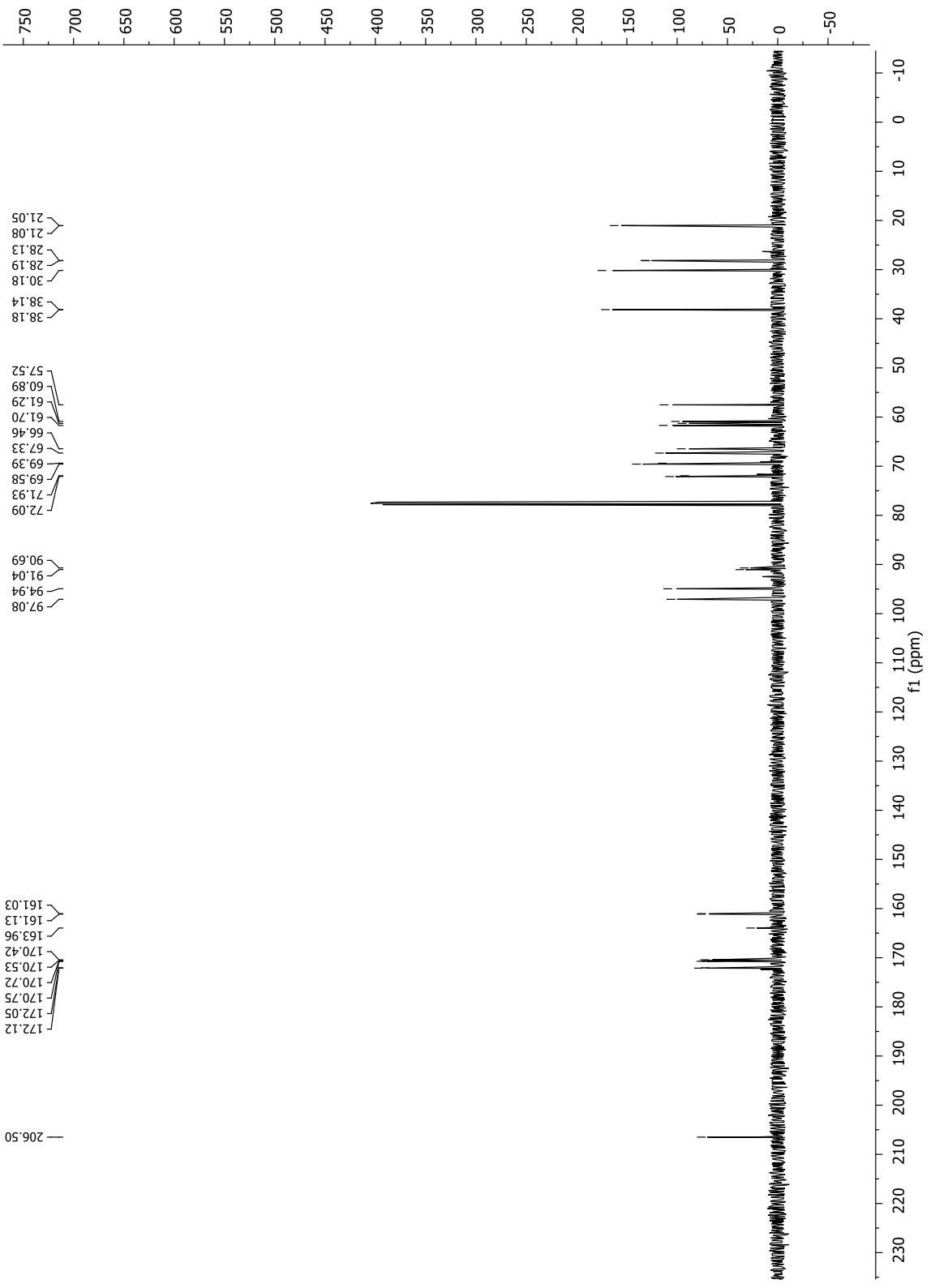
^{13}C NMR spectrum of compound **21** in CDCl3 at 100 MHz.

www.manaraa.com



^{1}H NMR spectrum of compound **22** in CDCl3 at 400 MHz.

www.manaraa.com



^{13}C NMR spectrum of compound **22** in CDCl3 at 100 MHz.

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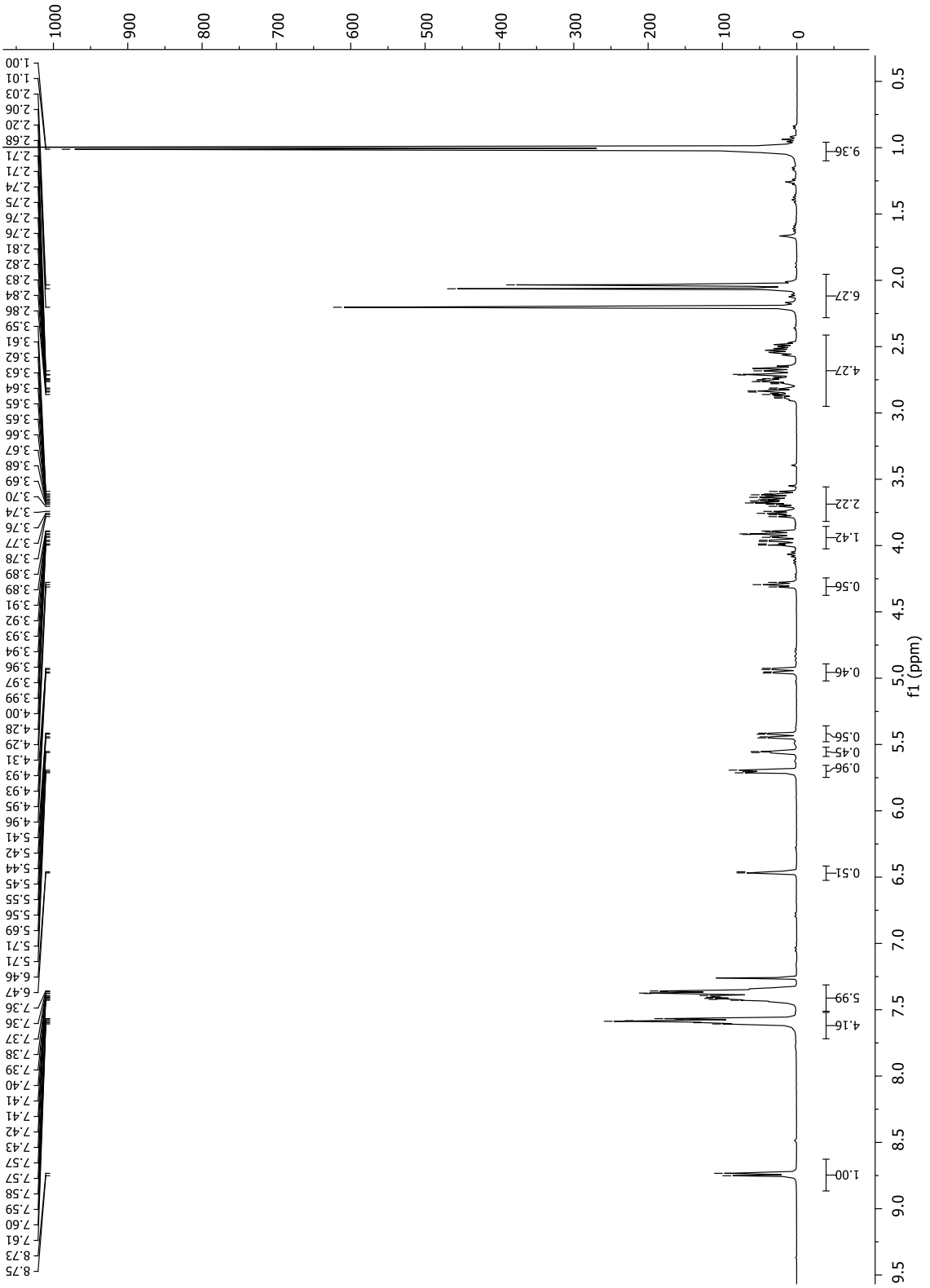

^{1}H NMR spectrum of compound **23** in CDCl3 at 400 MHz.

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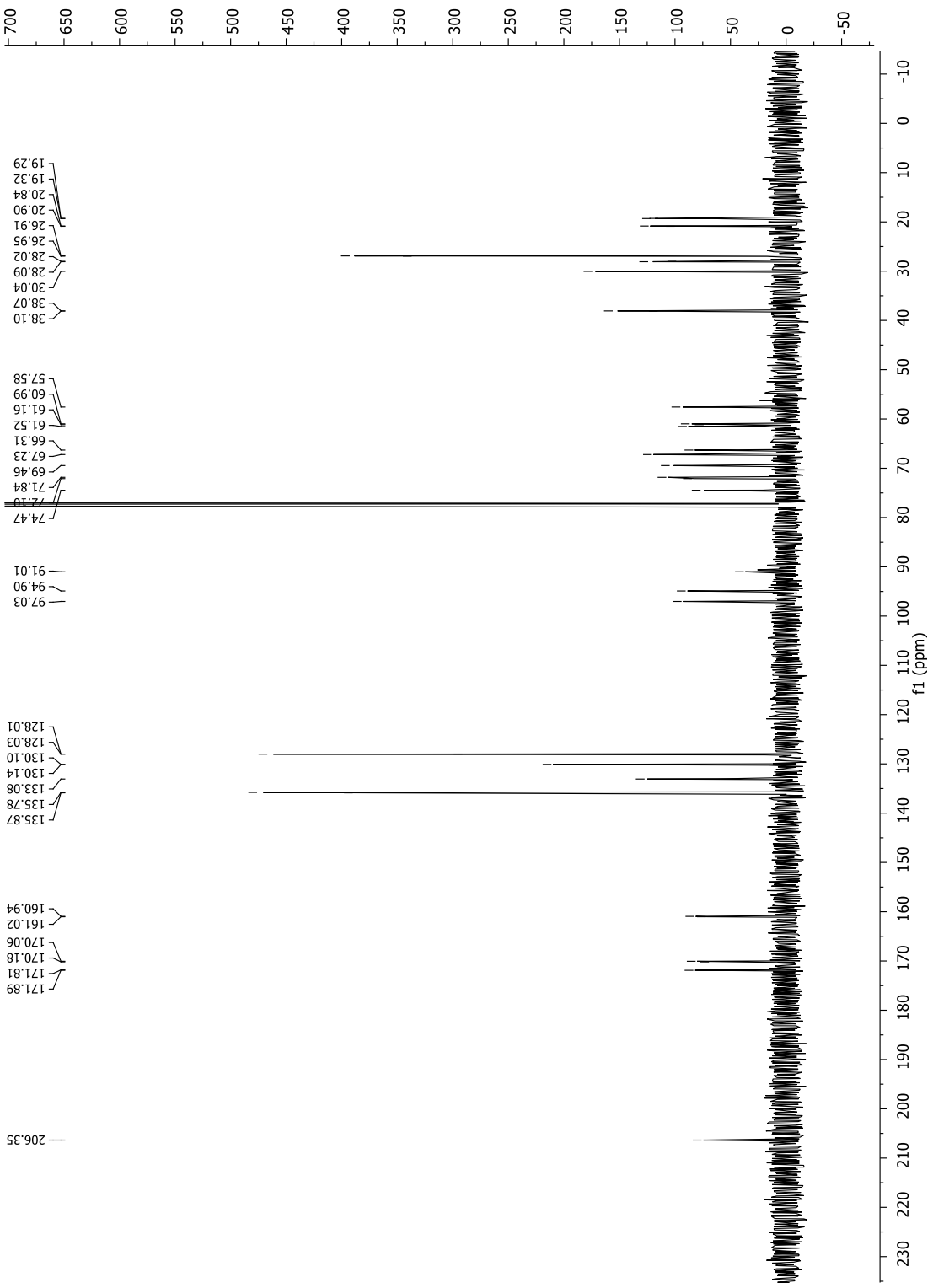
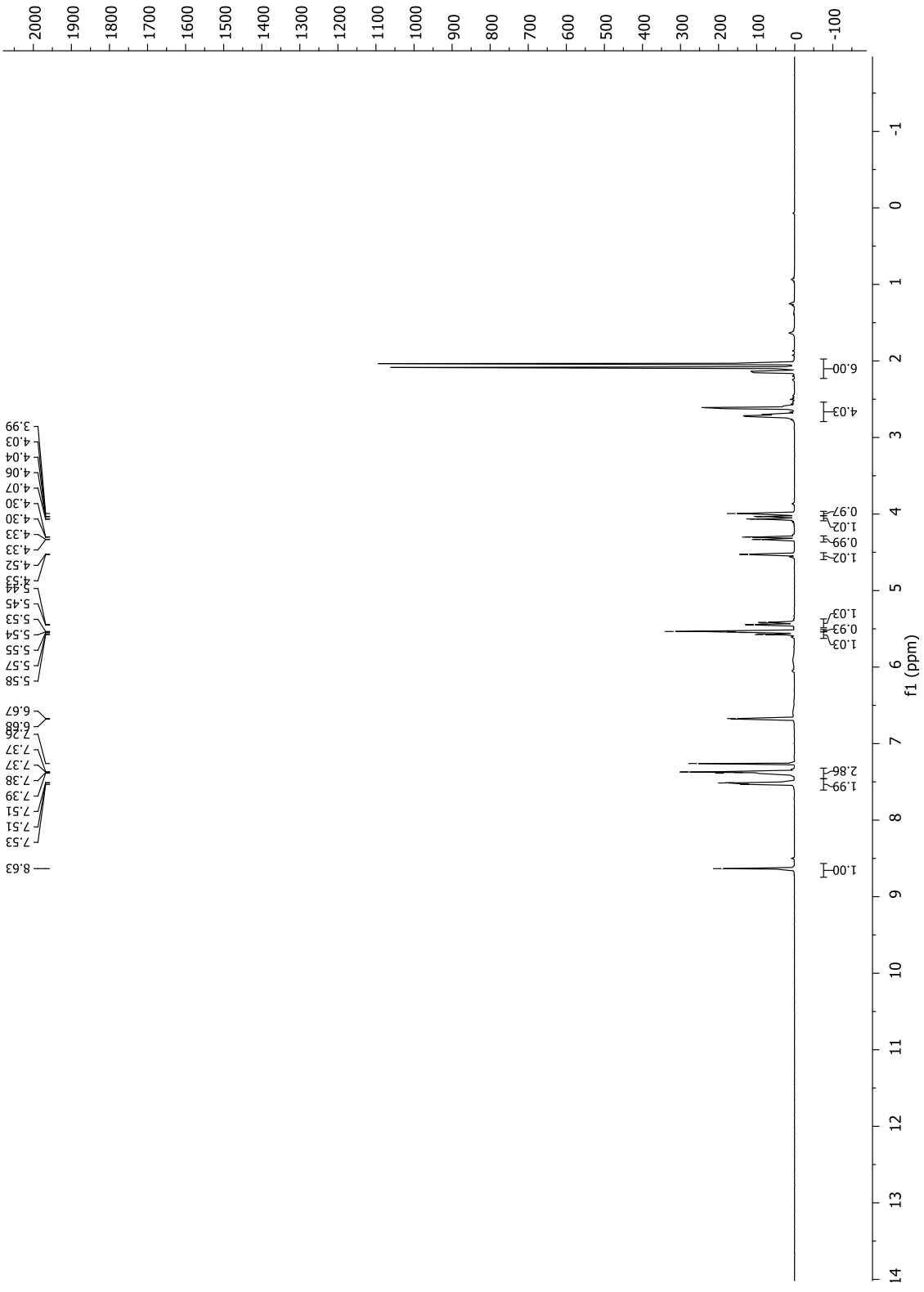


^{13}C NMR spectrum of compound **23** in CDCl3 at 100 MHz.

www.manaraa.com



^{1}H NMR spectrum of compound **24** in CDCl3 at 400 MHz.

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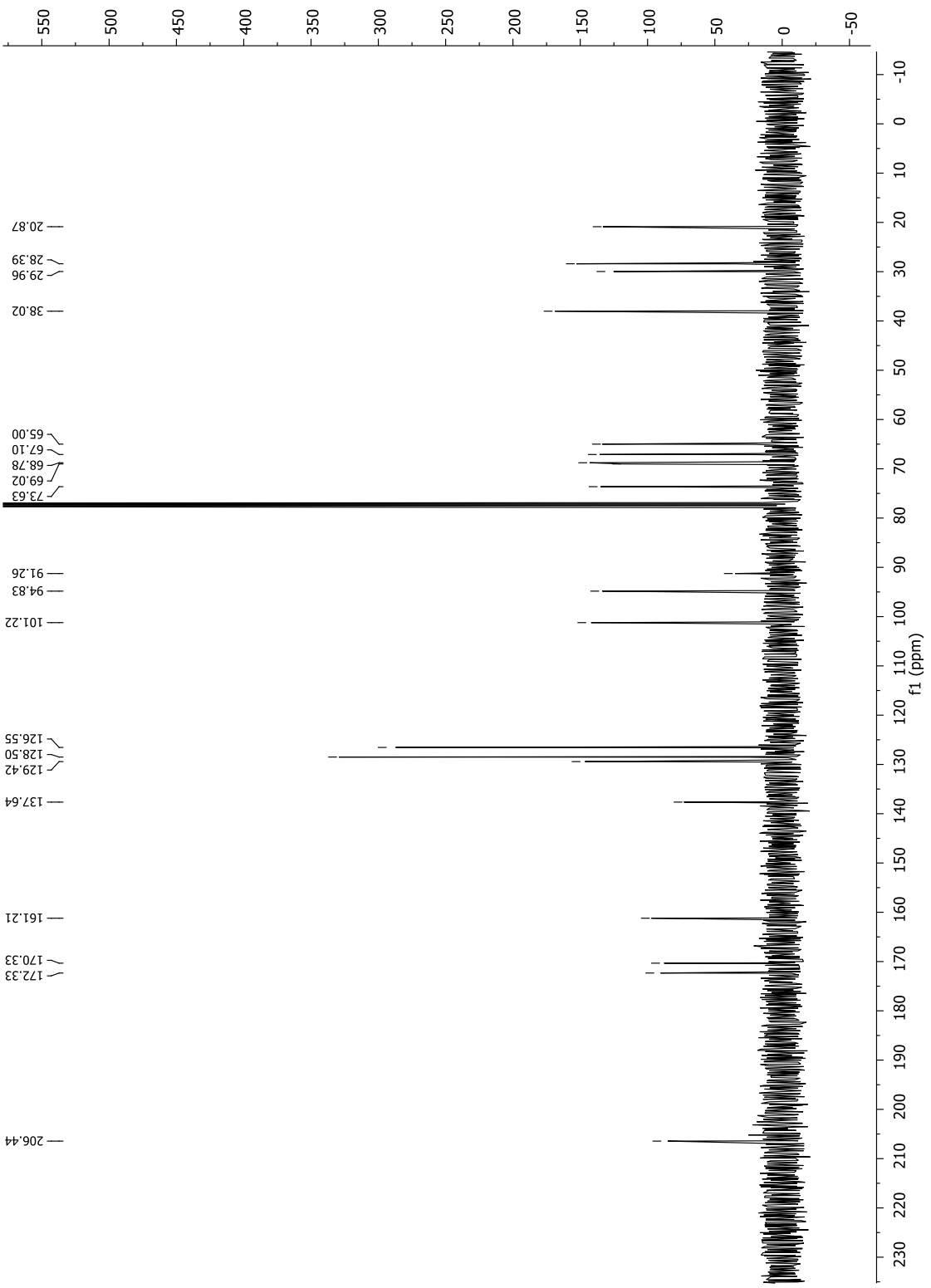

^{13}C NMR spectrum of compound **24** in CDCl3 at 100 MHz.

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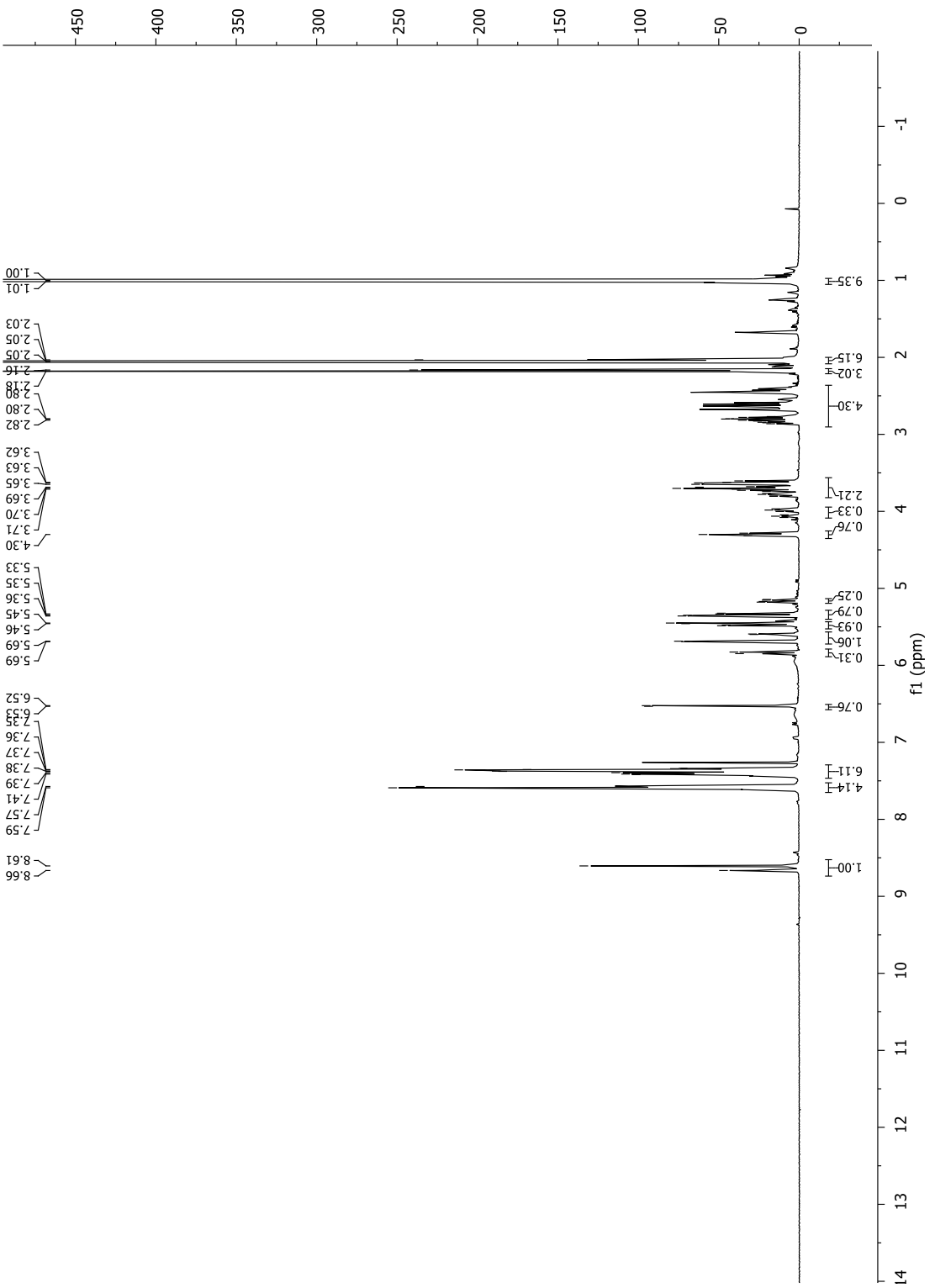

^{1}H NMR spectrum of compound **25** in CDCl3 at 400 MHz.

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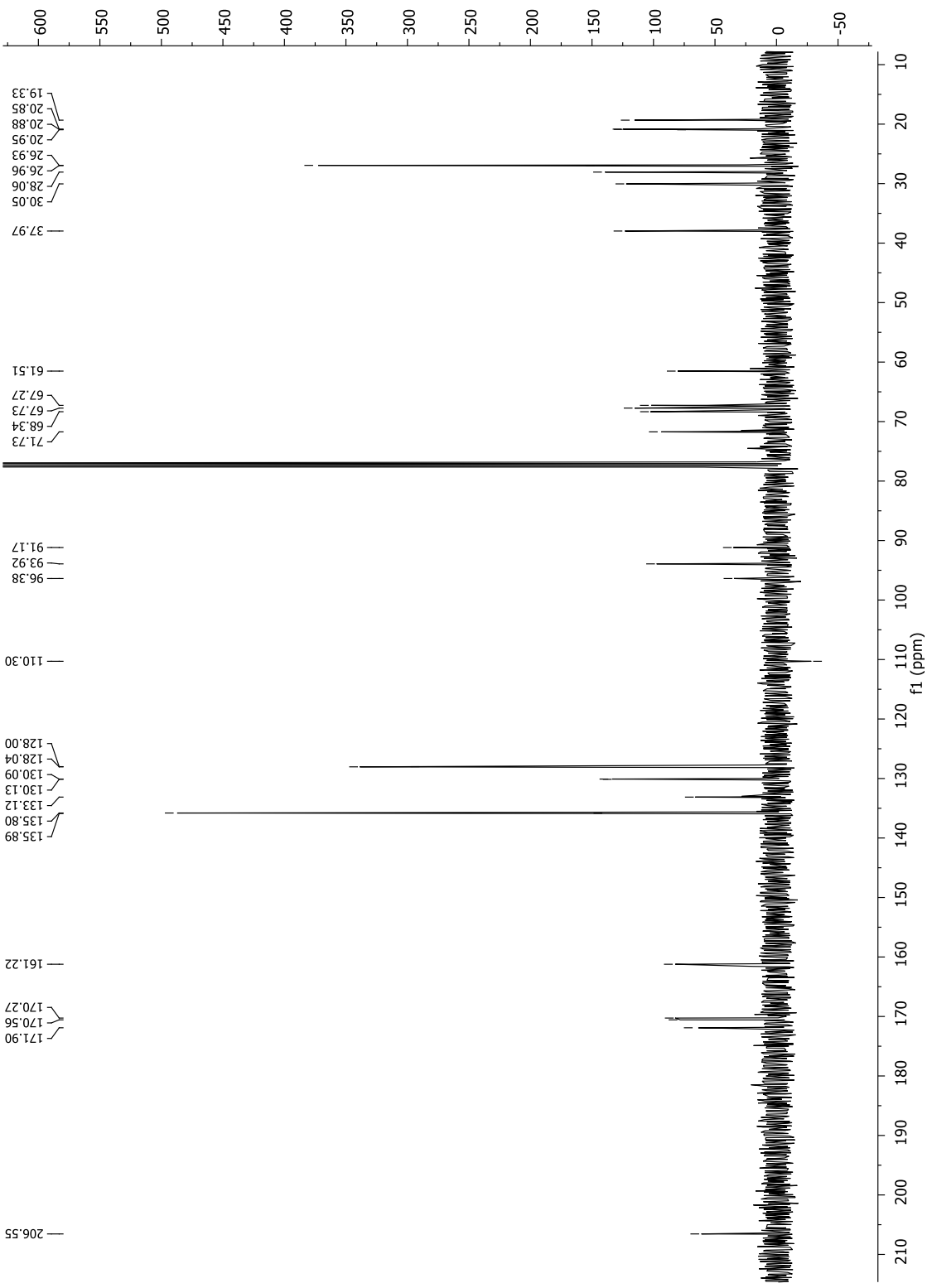

^{13}C NMR spectrum of compound **25** in CDCl3 at 100 MHz.

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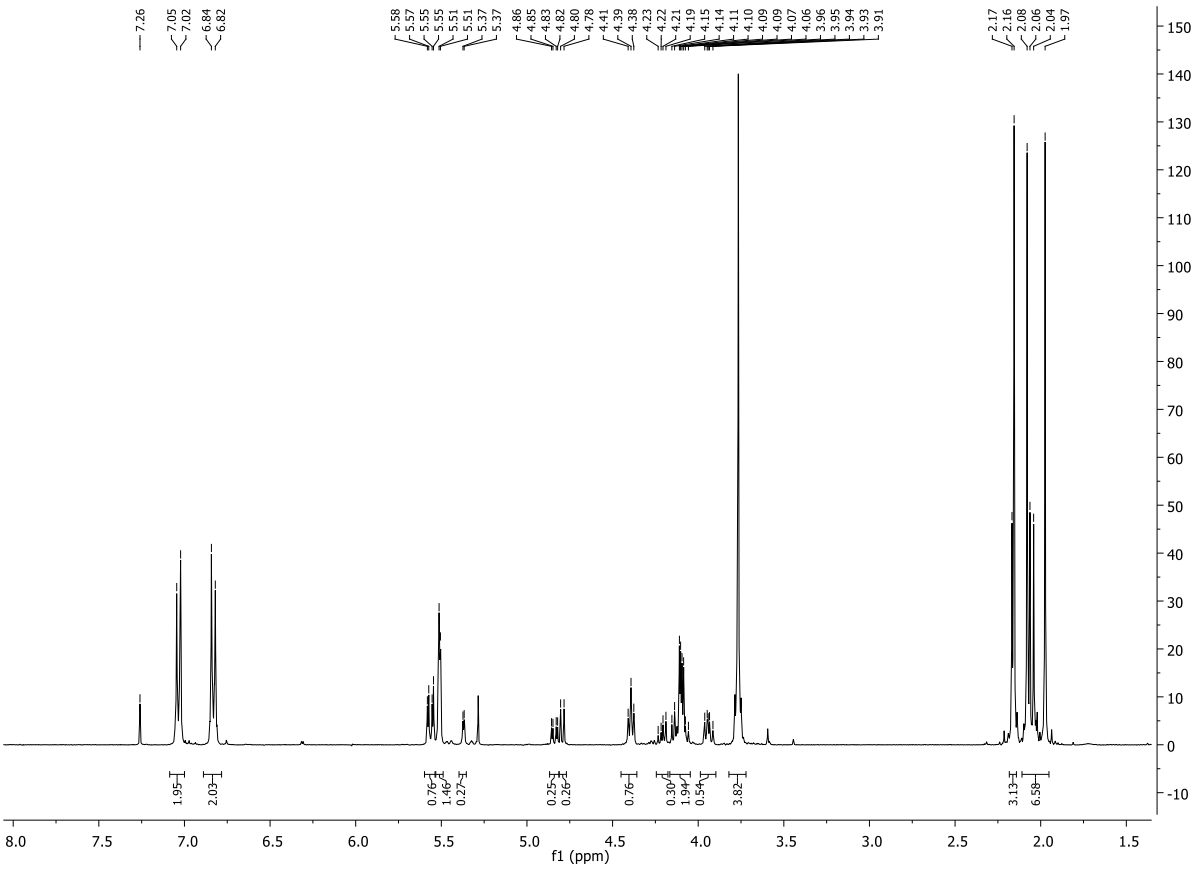

^{1}H NMR spectrum of compound **30** in CDCl3 at 400 MHz.

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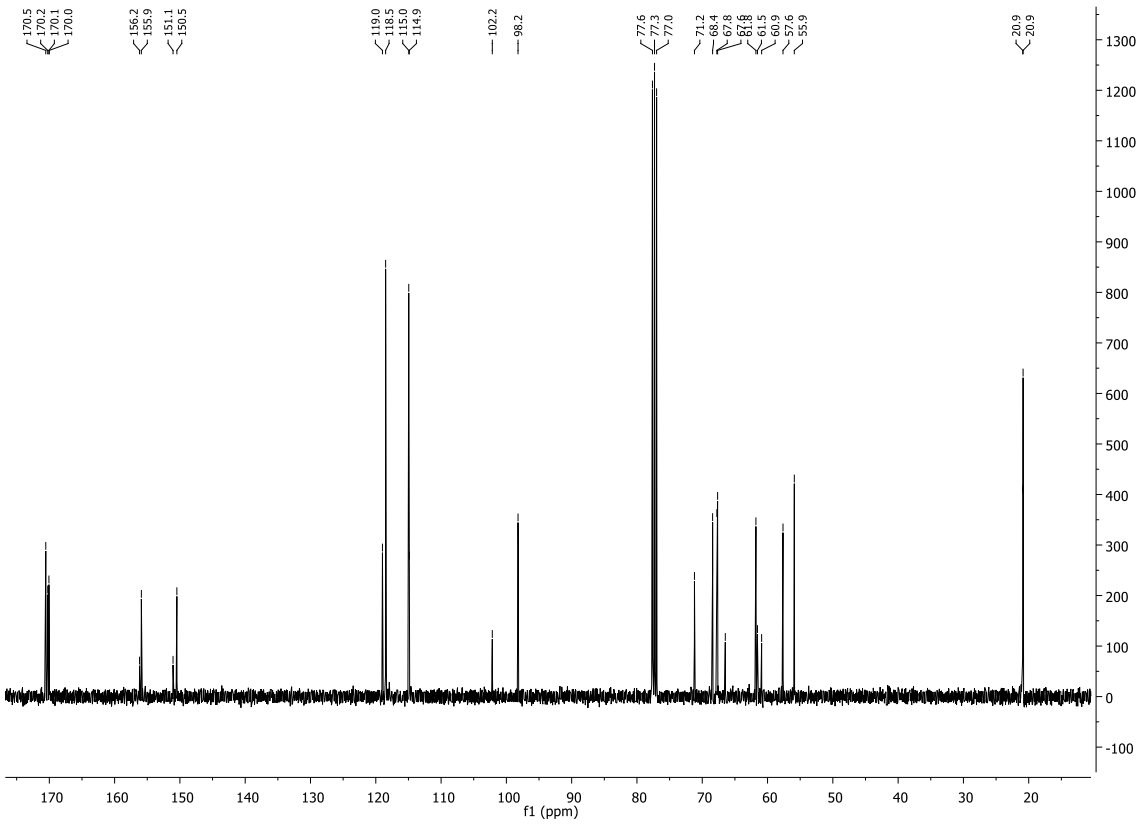
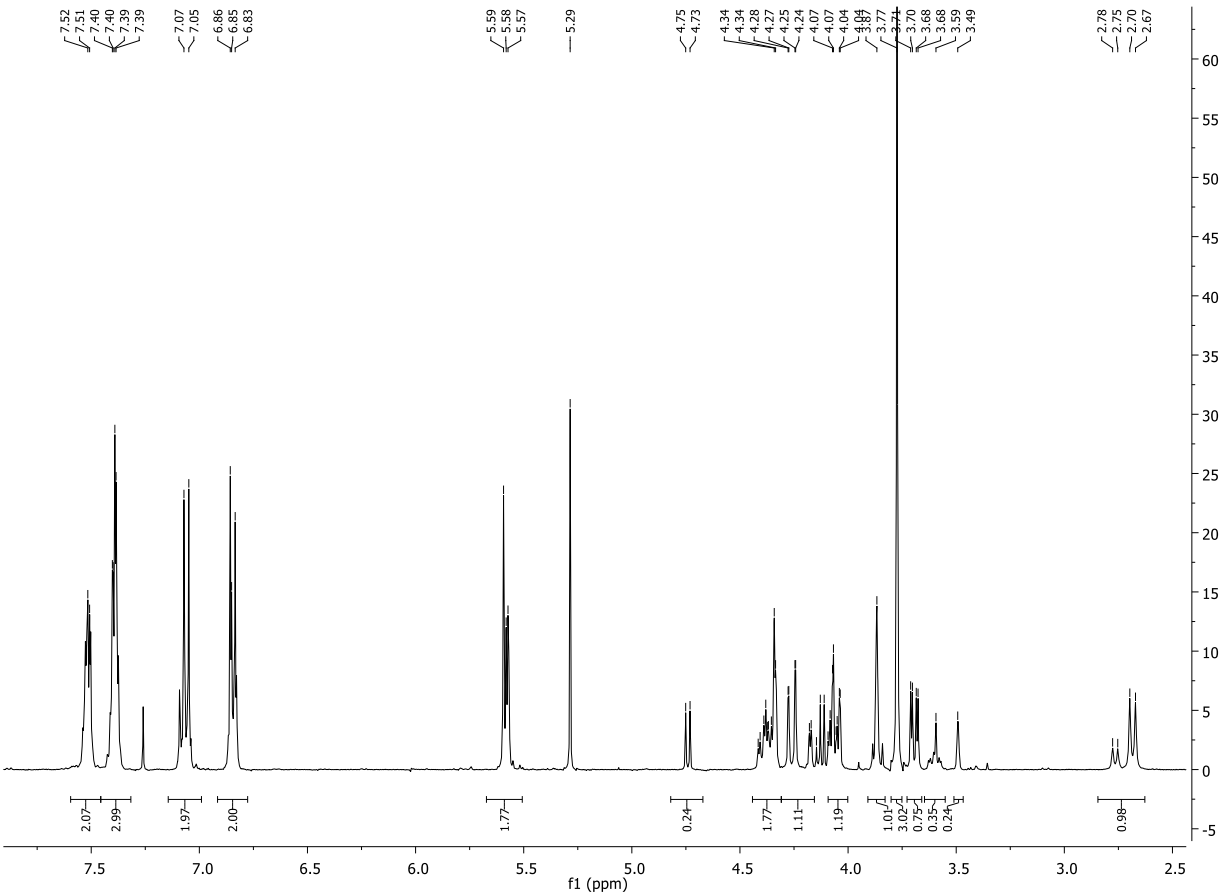


^{13}C NMR spectrum of compound **30** in CDCl3 at 100 MHz.

www.manaraa.com



^{1}H NMR spectrum of compound **31** in CDCl3 at 400 MHz.

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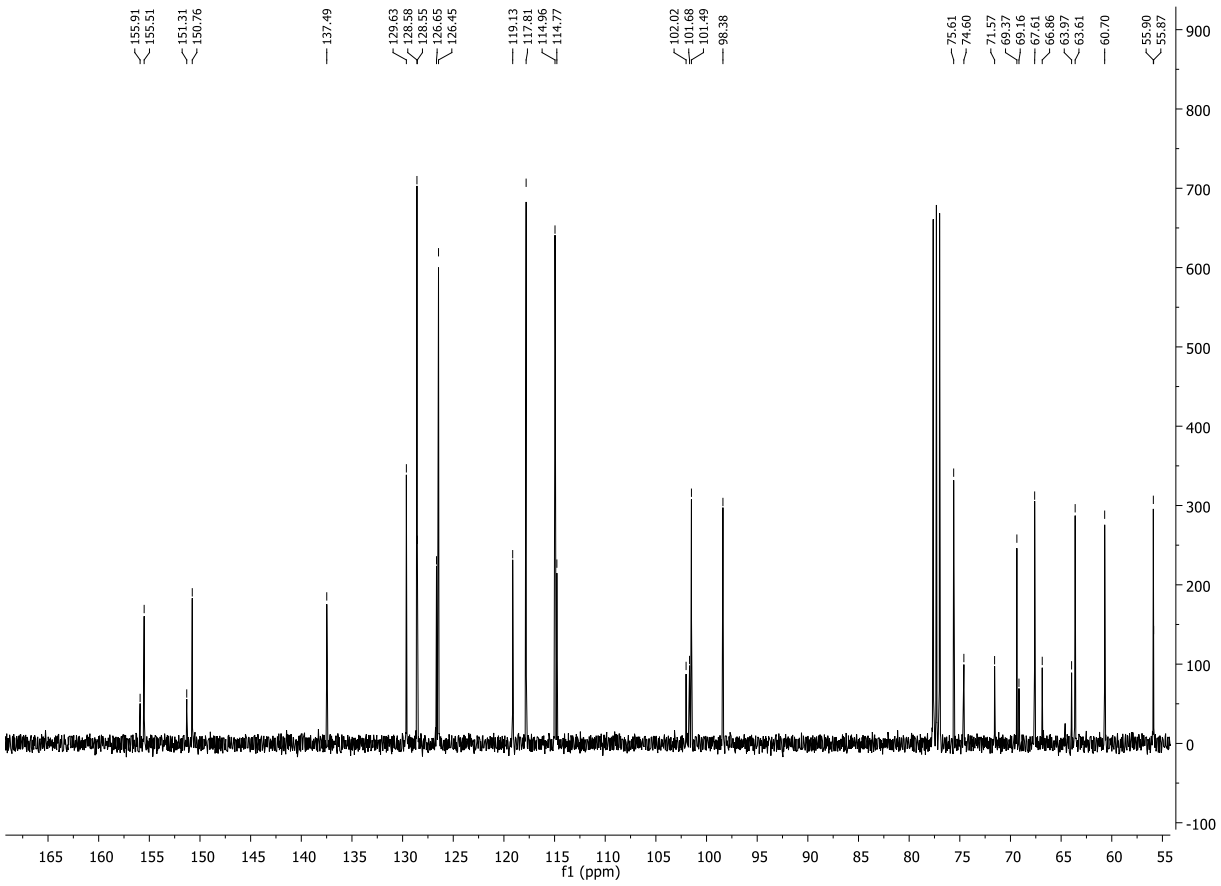

^{13}C NMR spectrum of compound **31** in CDCl3 at 100 MHz.

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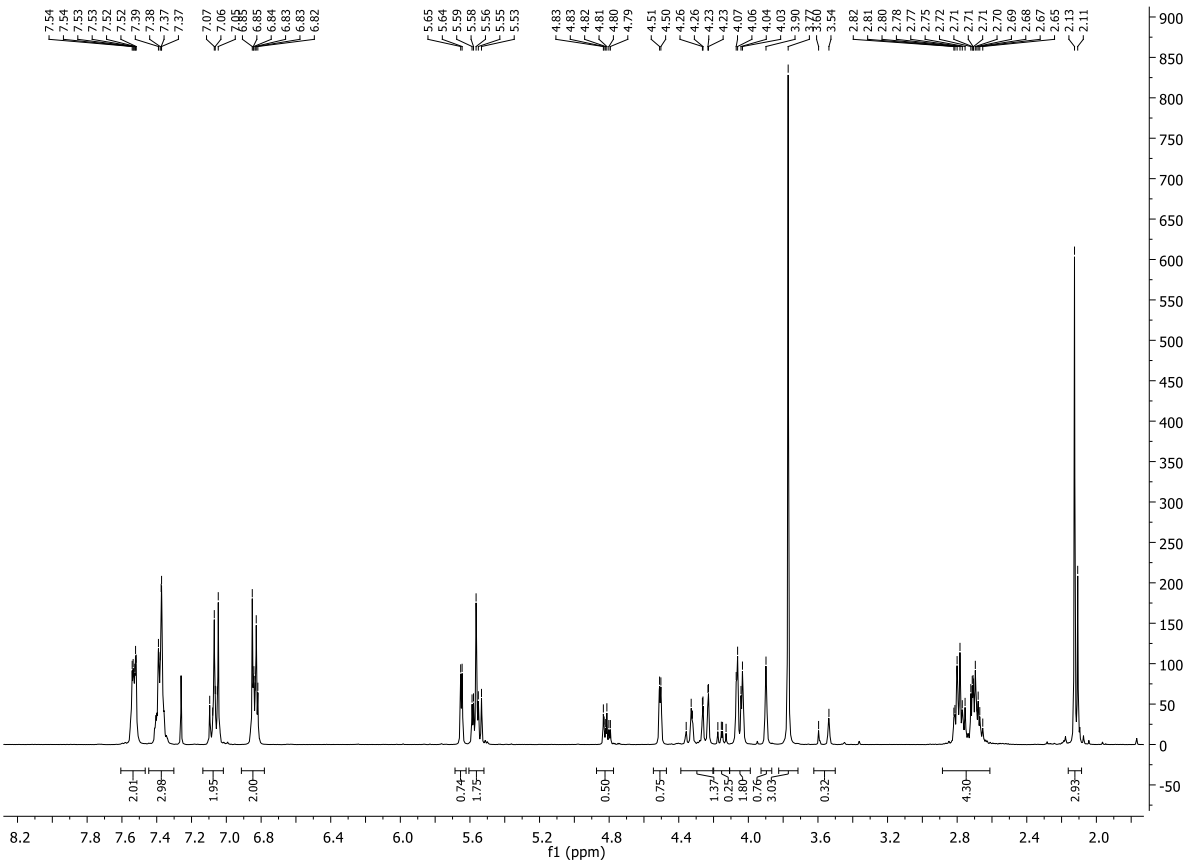

^{1}H NMR spectrum of compound **32** in CDCl3 at 400 MHz.

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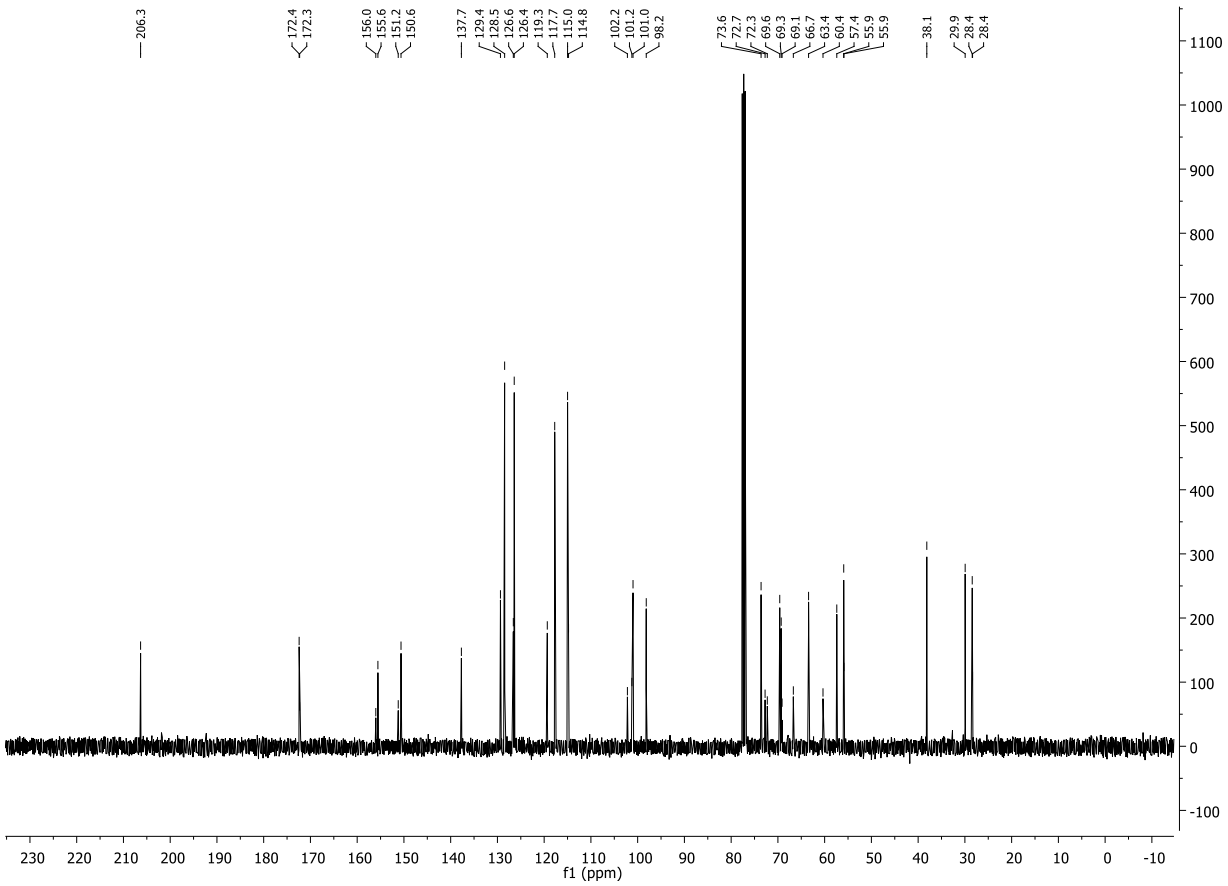

^{13}C NMR spectrum of compound **32** in CDCl3 at 100 MHz.

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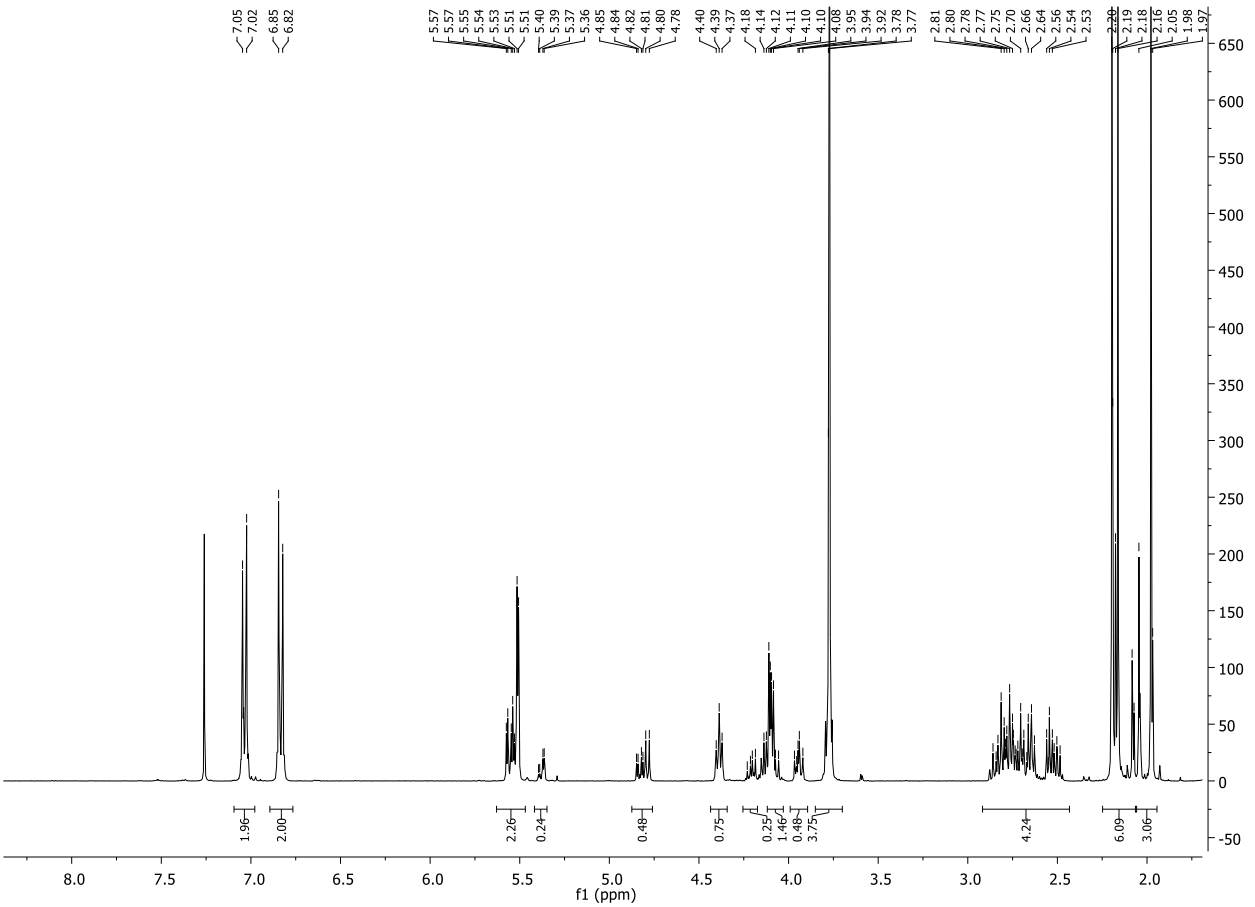

^{1}H NMR spectrum of compound **33** in CDCl3 at 400 MHz.

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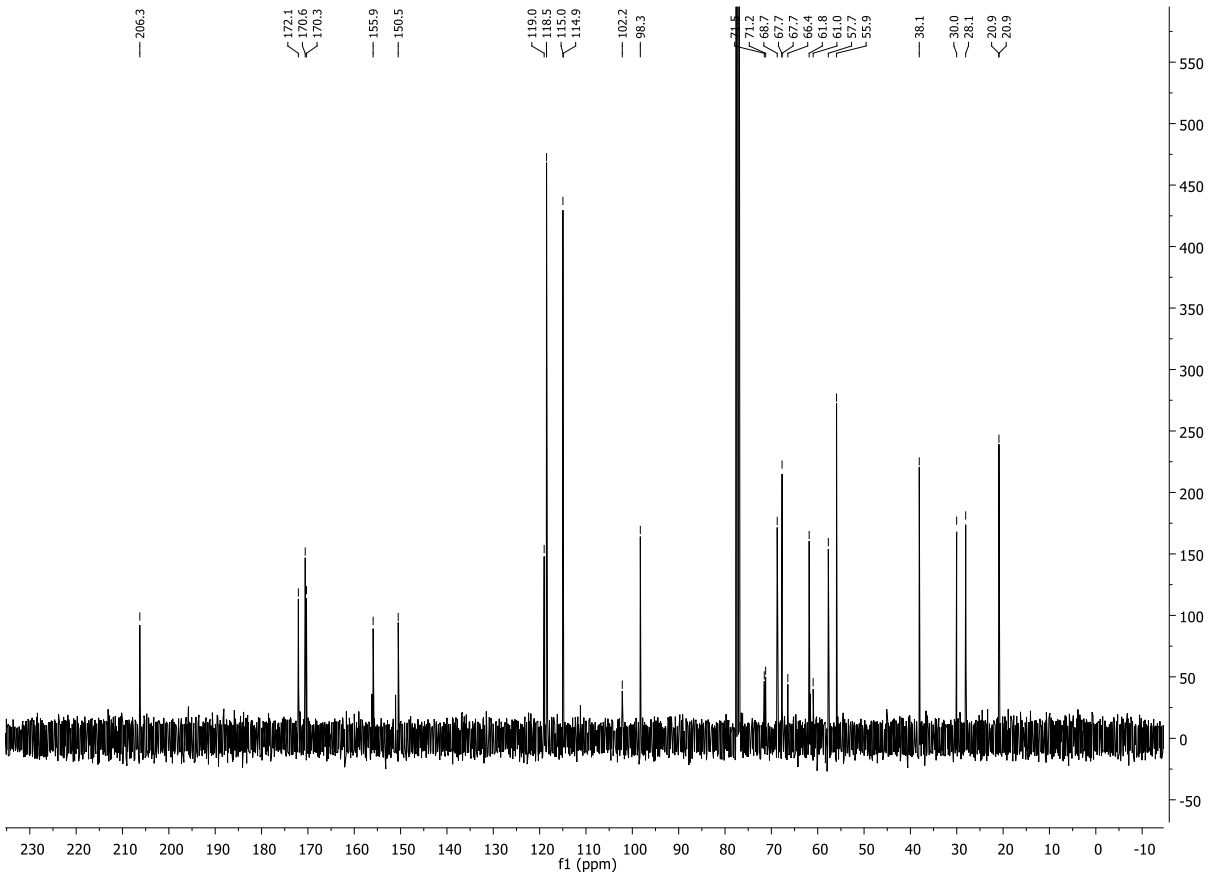

^{13}C NMR spectrum of compound **33** in CDCl3 at 100 MHz.

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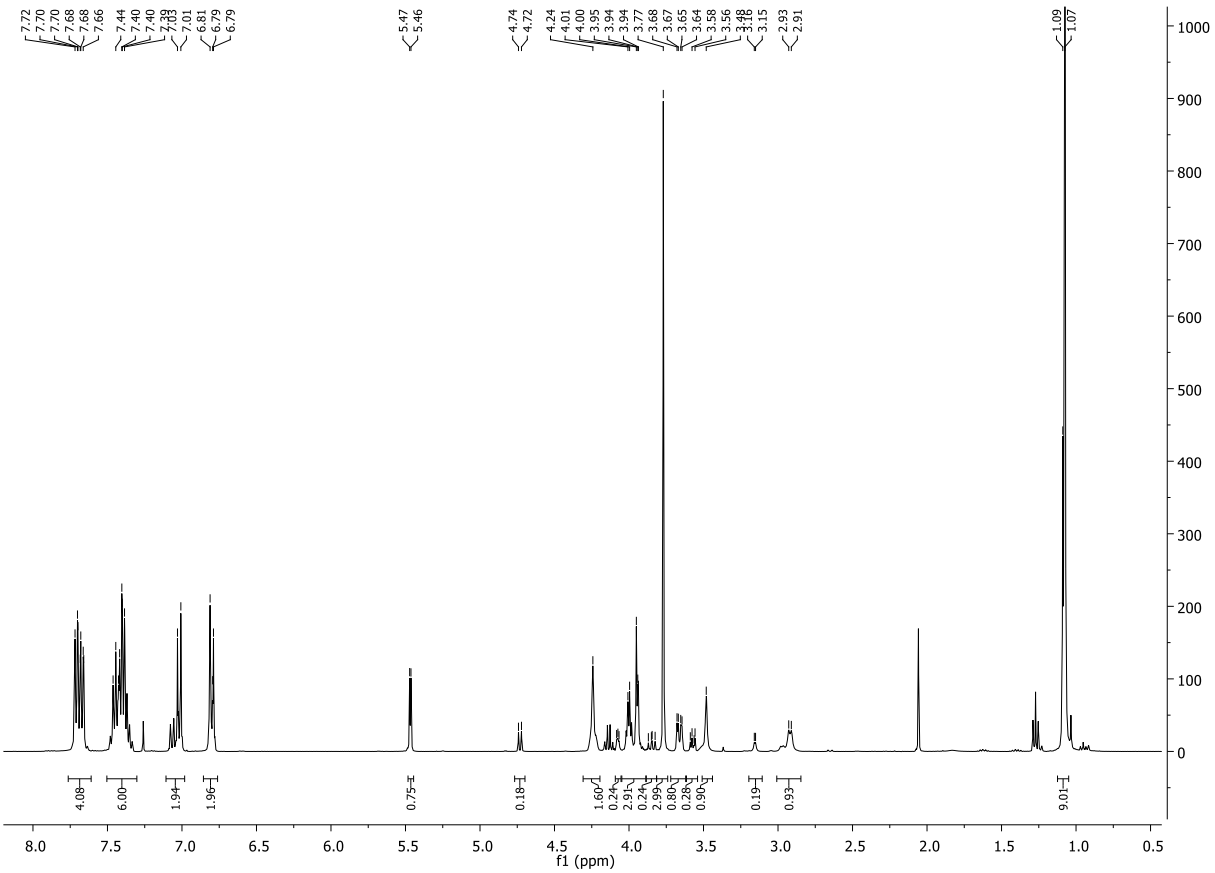

^{1}H NMR spectrum of compound **34** in CDCl3 at 400 MHz.

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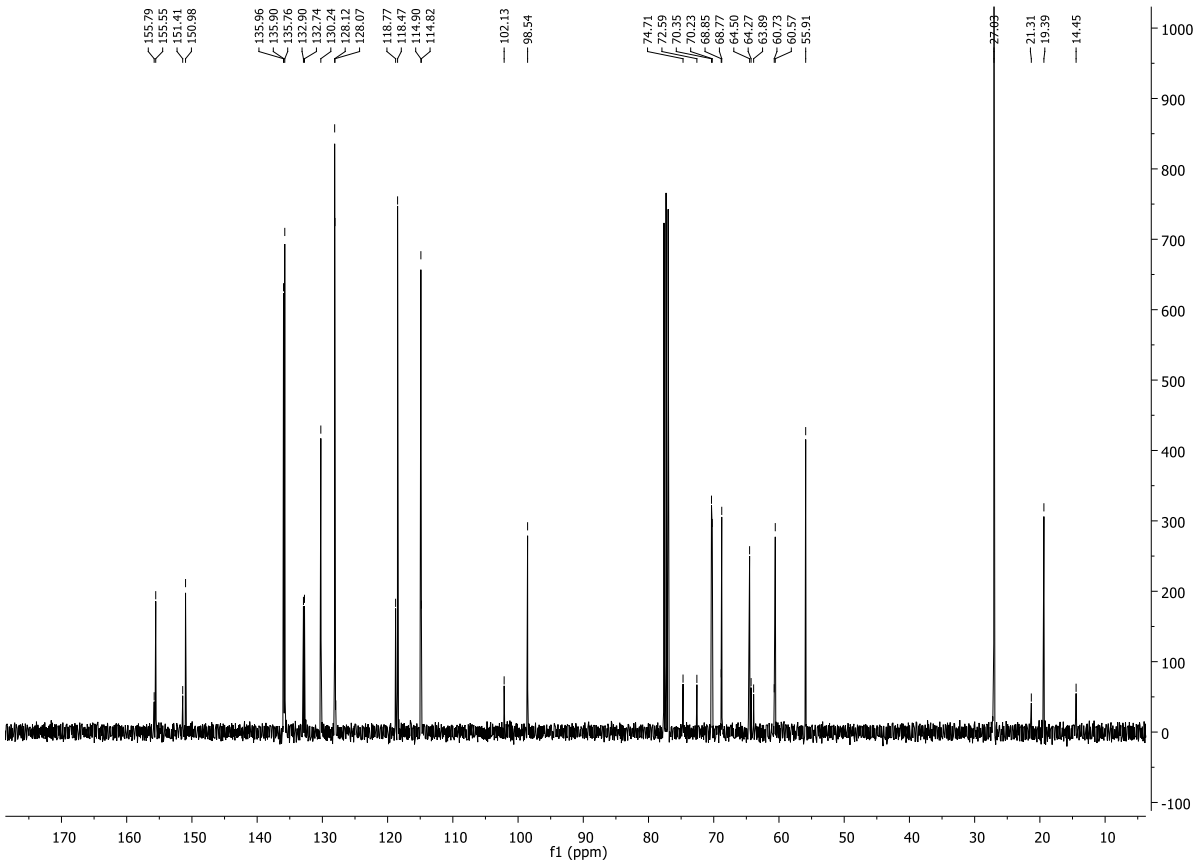

^{13}C NMR spectrum of compound **34** in CDCl3 at 100 MHz.

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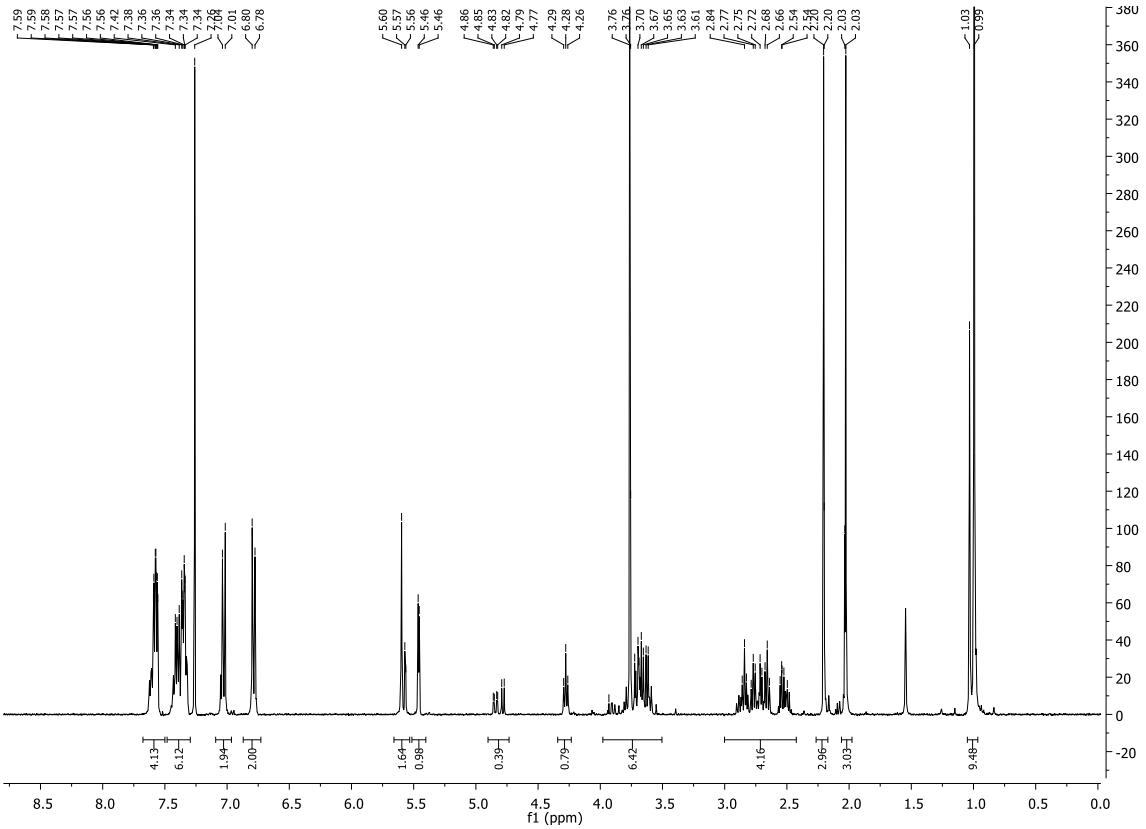

^{1}H NMR spectrum of compound **35** in CDCl3 at 400 MHz.

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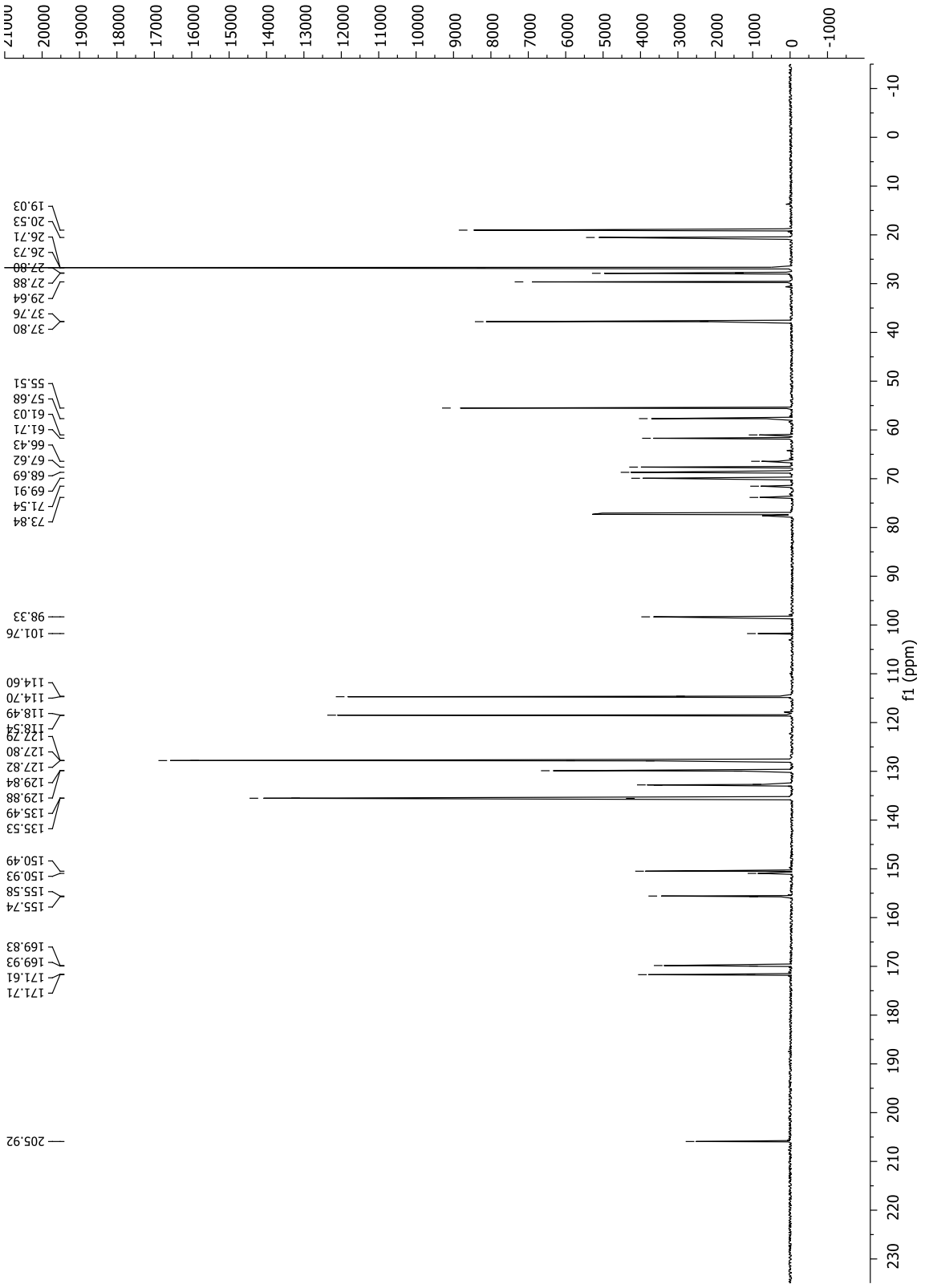

^{13}C NMR spectrum of compound **35** in CDCl3 at 100 MHz.

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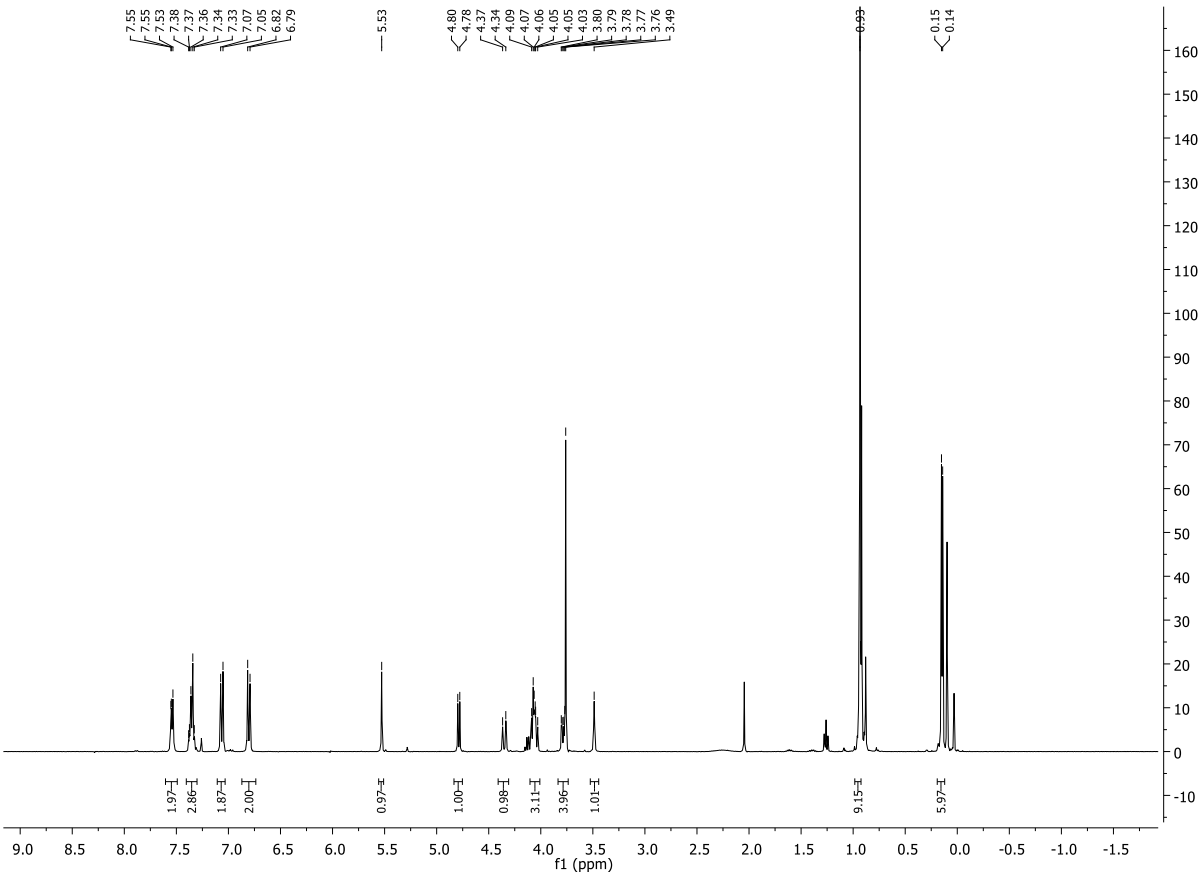

^{1}H NMR spectrum of compound **37** in CDCl3 at 400 MHz.

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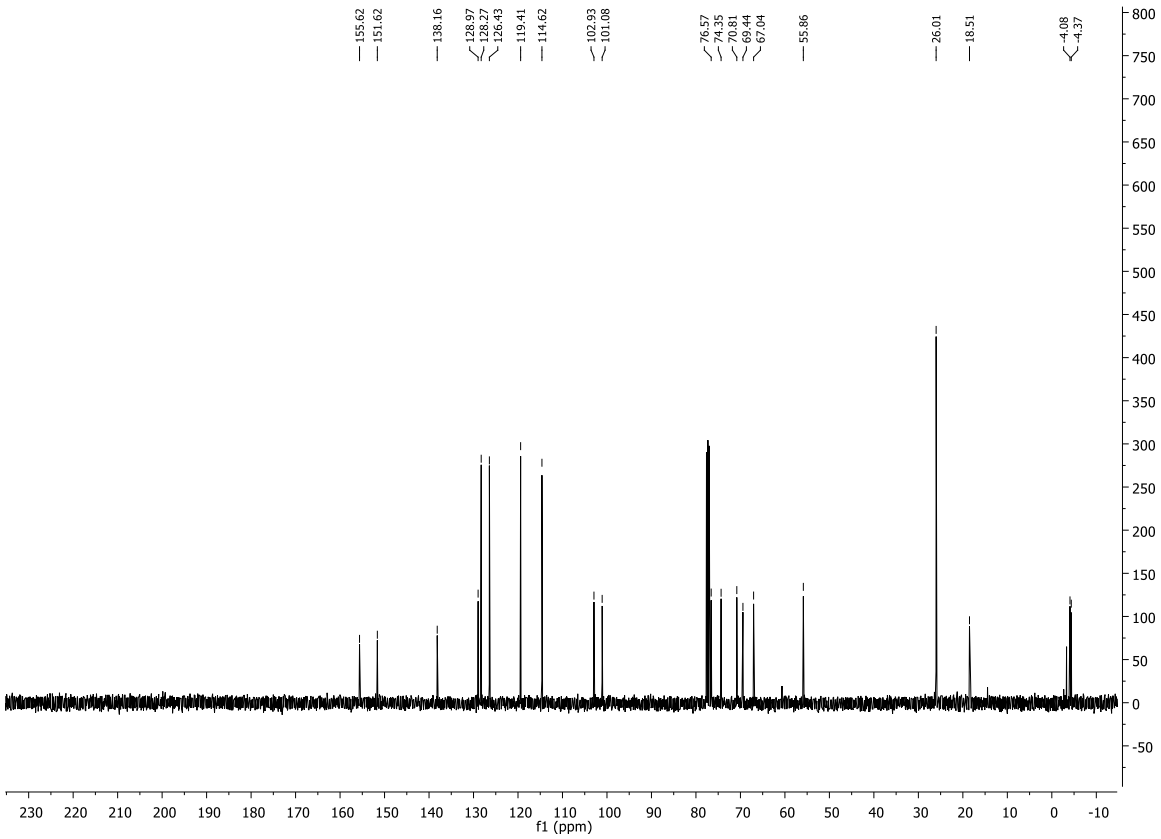

^{13}C NMR spectrum of compound **37** in CDCl3 at 100 MHz.

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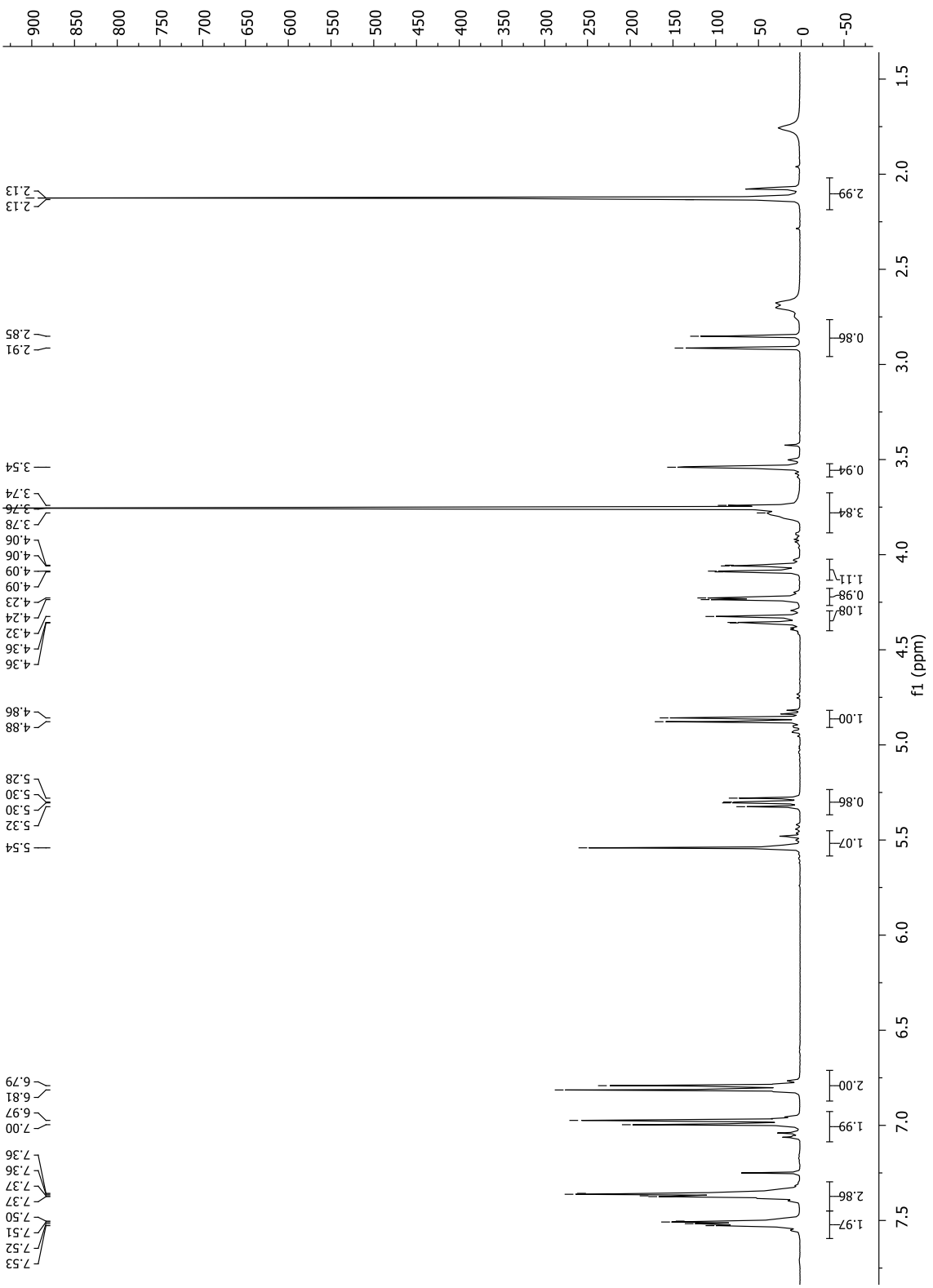

^{1}H NMR spectrum of compound **38** in CDCl3 at 400 MHz.

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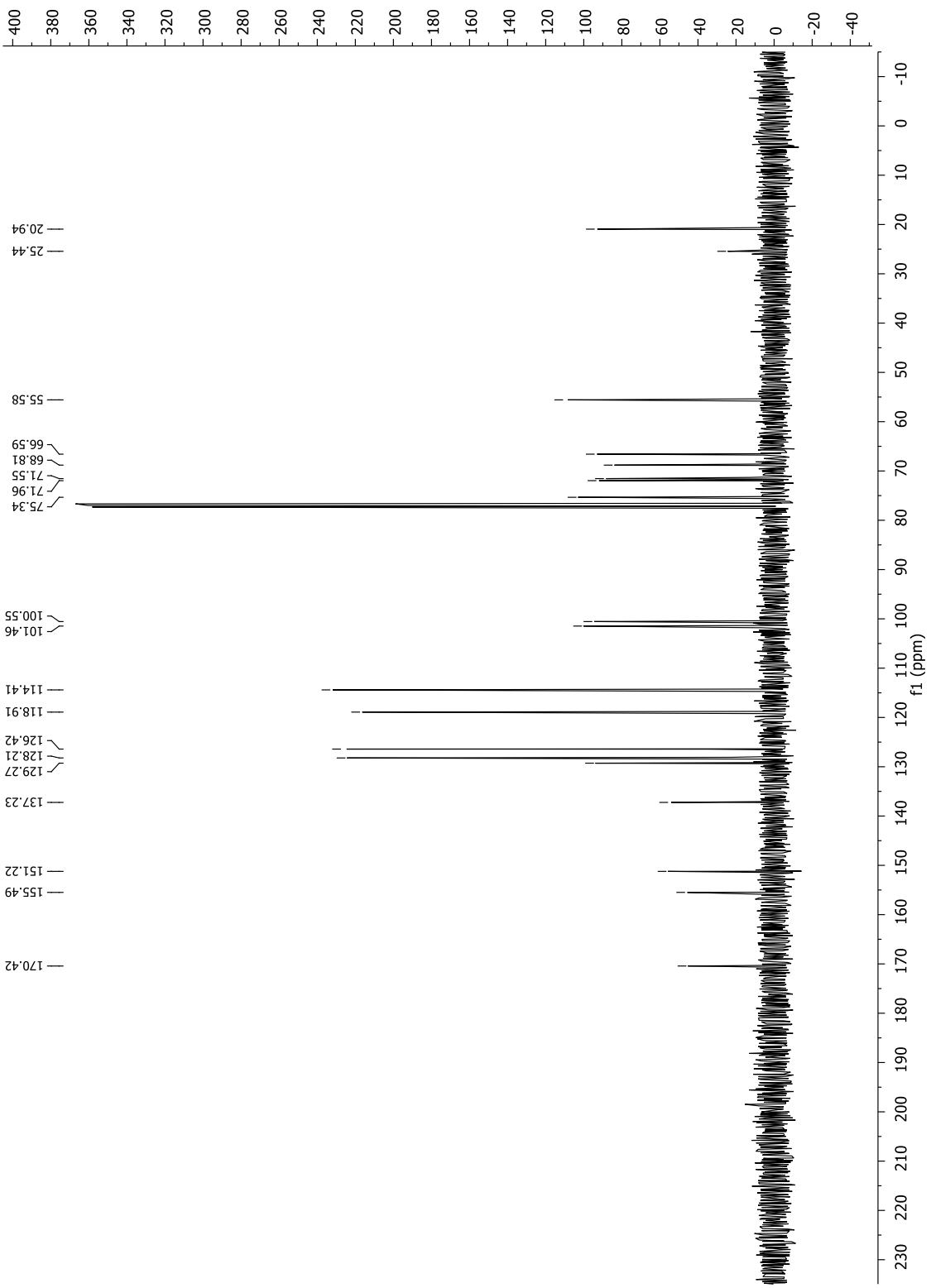

^{13}C NMR spectrum of compound **38** in CDCl3 at 100 MHz.

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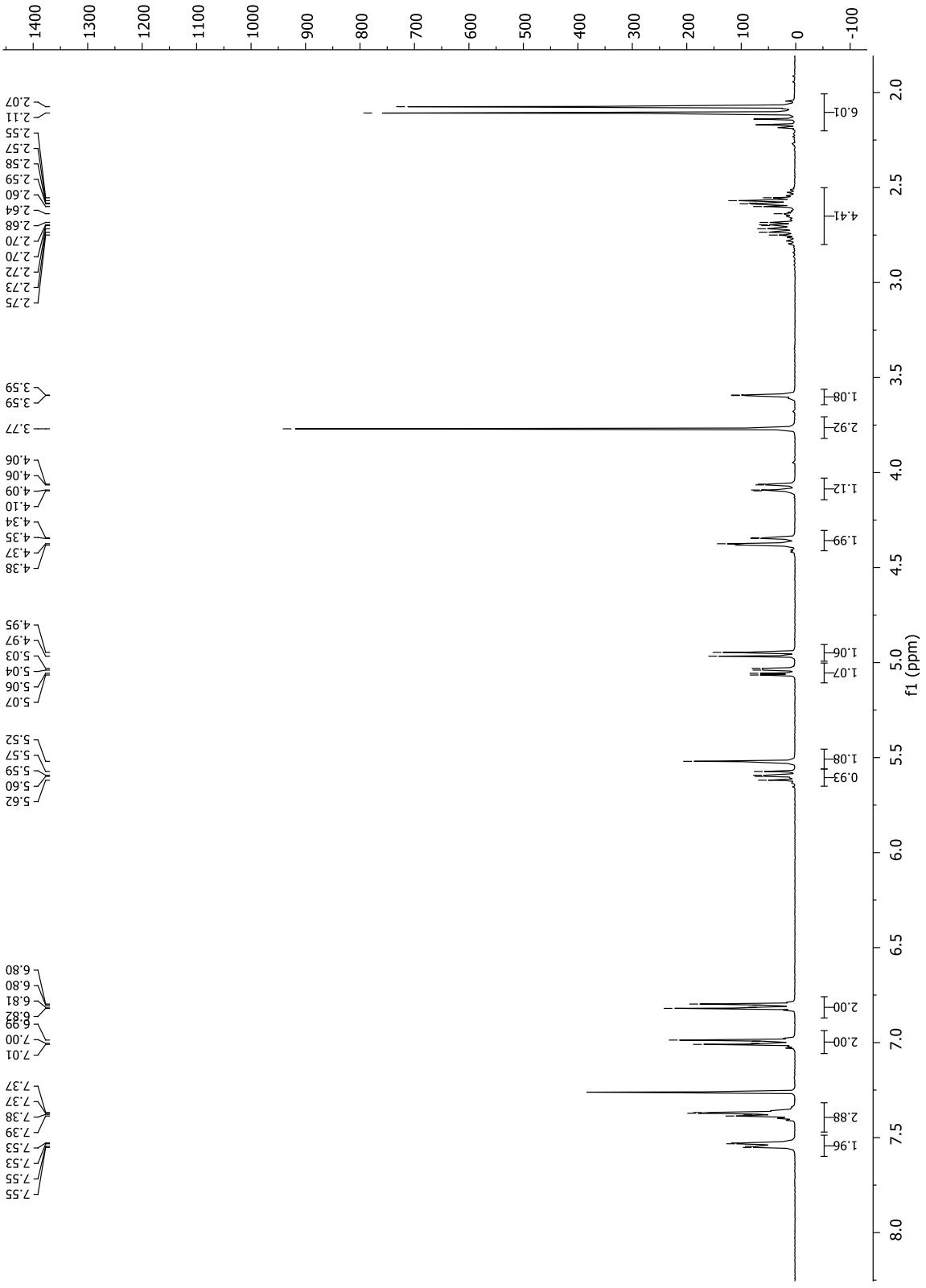

^{1}H NMR spectrum of compound **39** in CDCl3 at 400 MHz.

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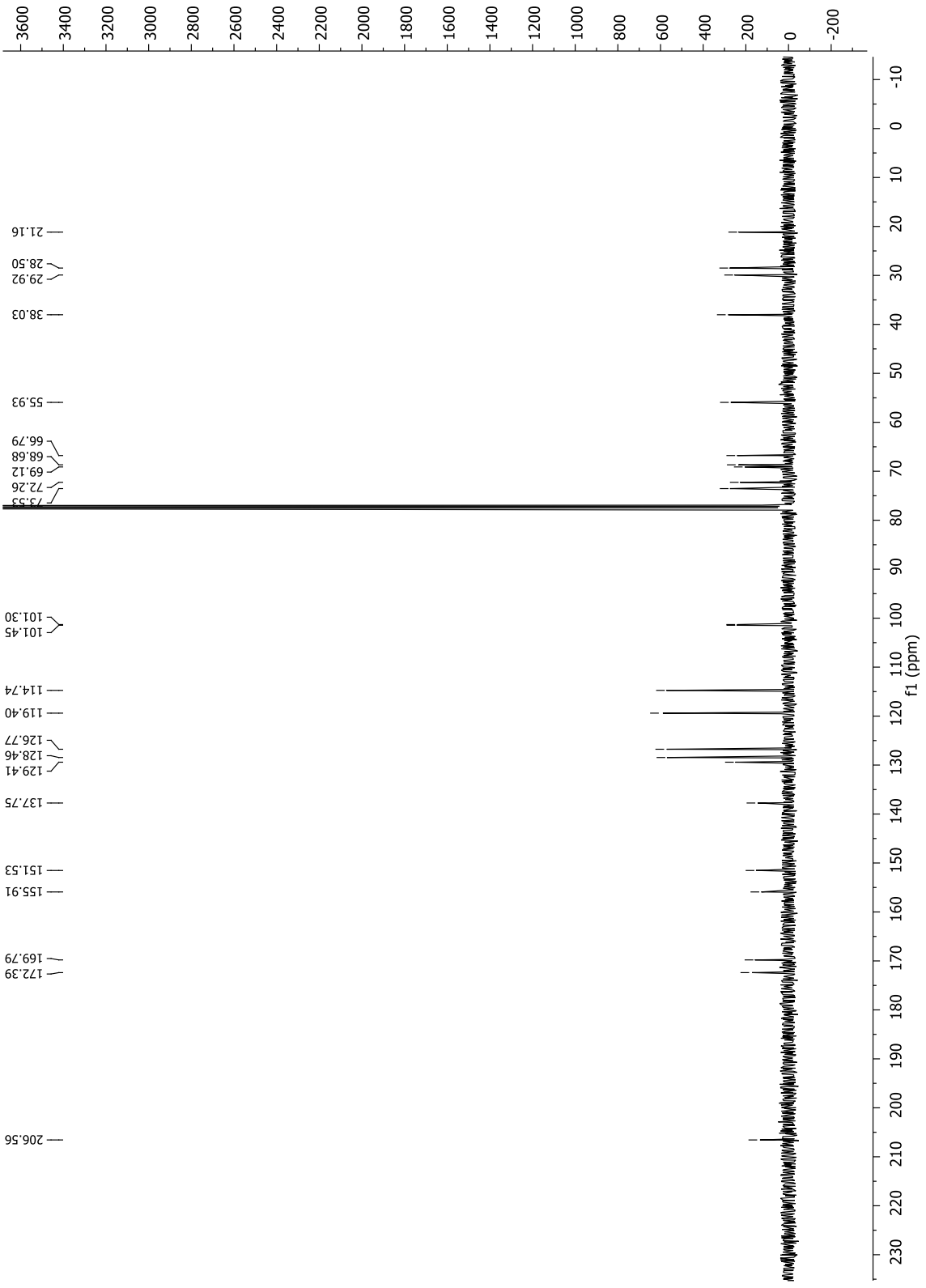

^{13}C NMR spectrum of compound **39** in CDCl3 at 100 MHz.

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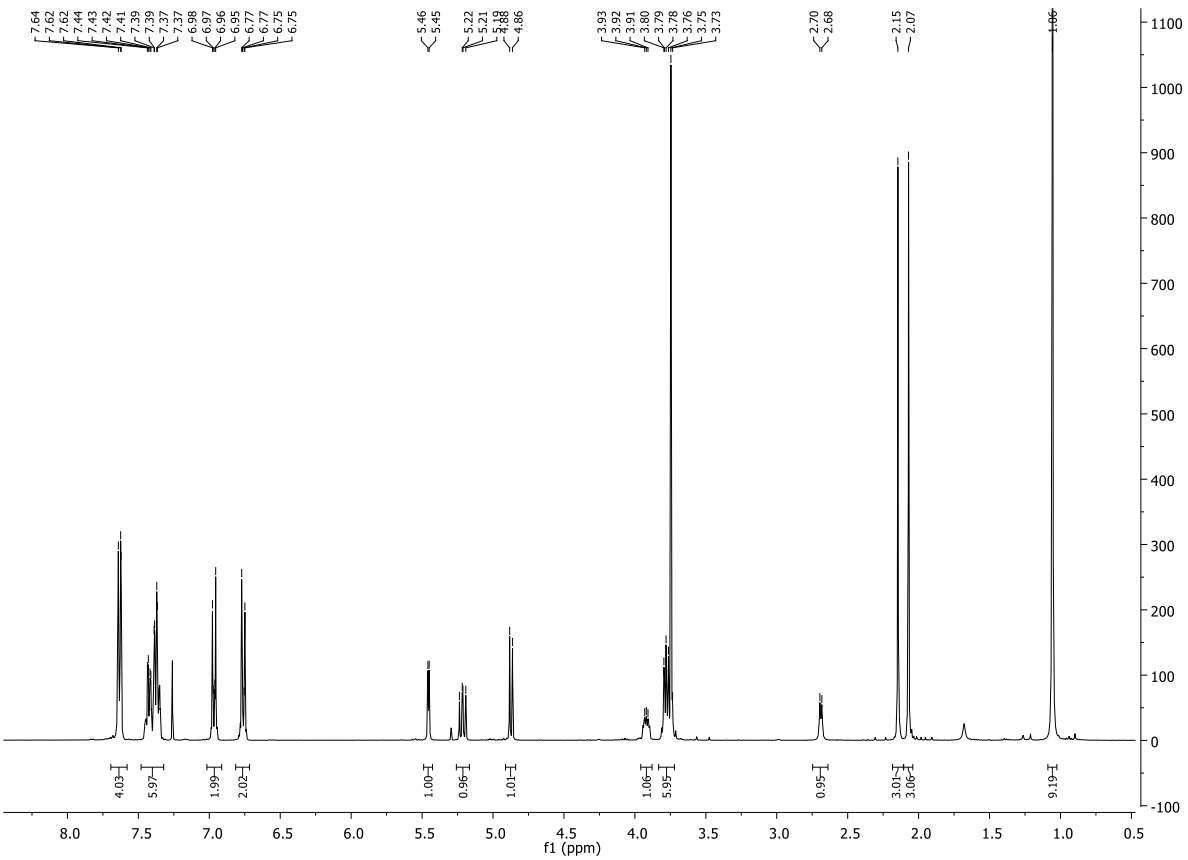

^{1}H NMR spectrum of compound **41** in CDCl3 at 400 MHz.

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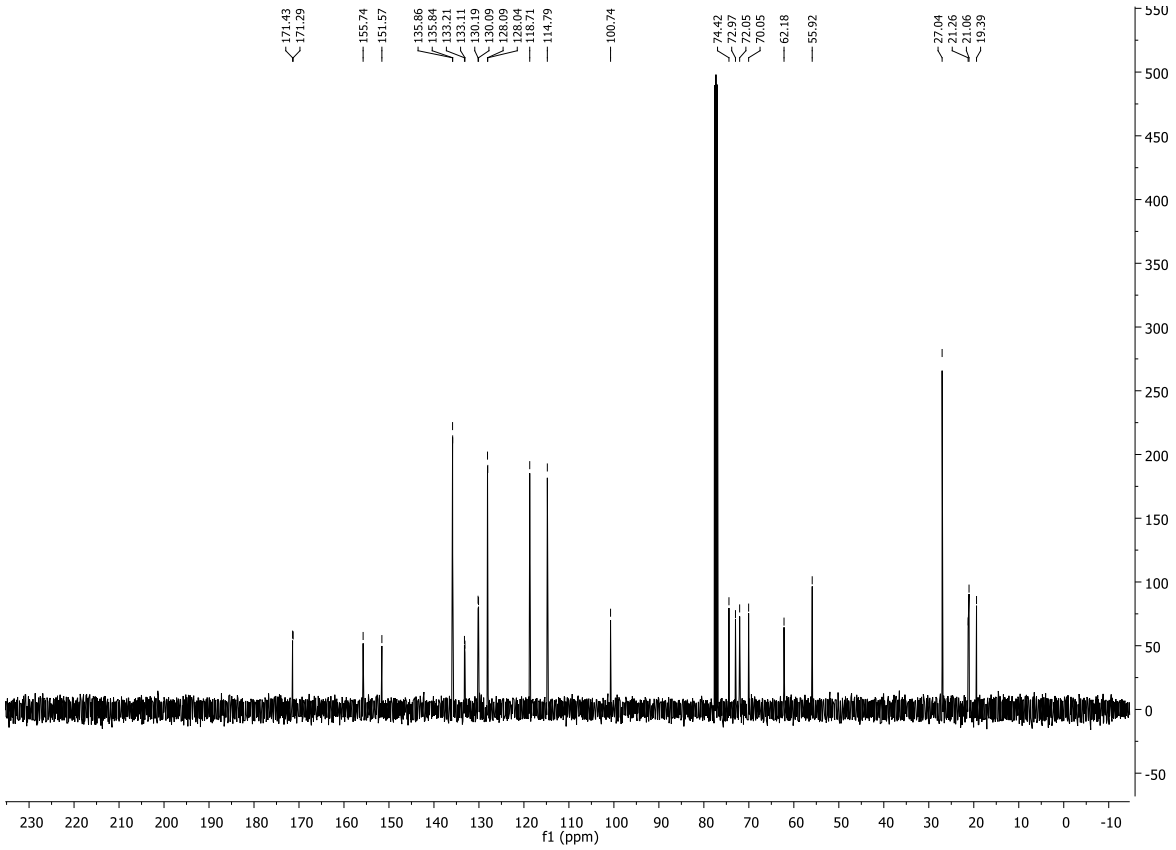

^{13}C NMR spectrum of compound **41** in CDCl3 at 100 MHz.

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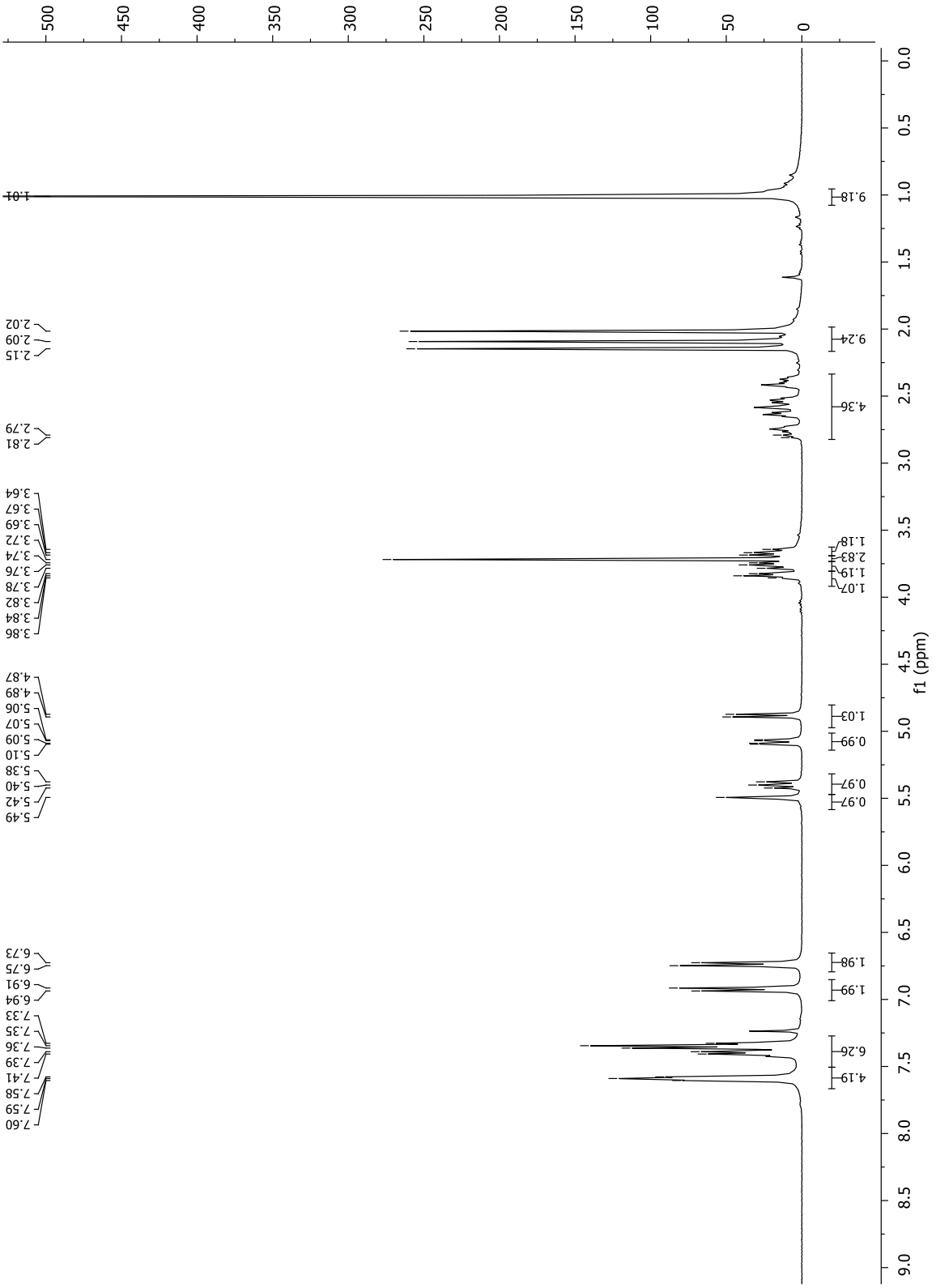

^{1}H NMR spectrum of compound **42** in CDCl3 at 400 MHz.

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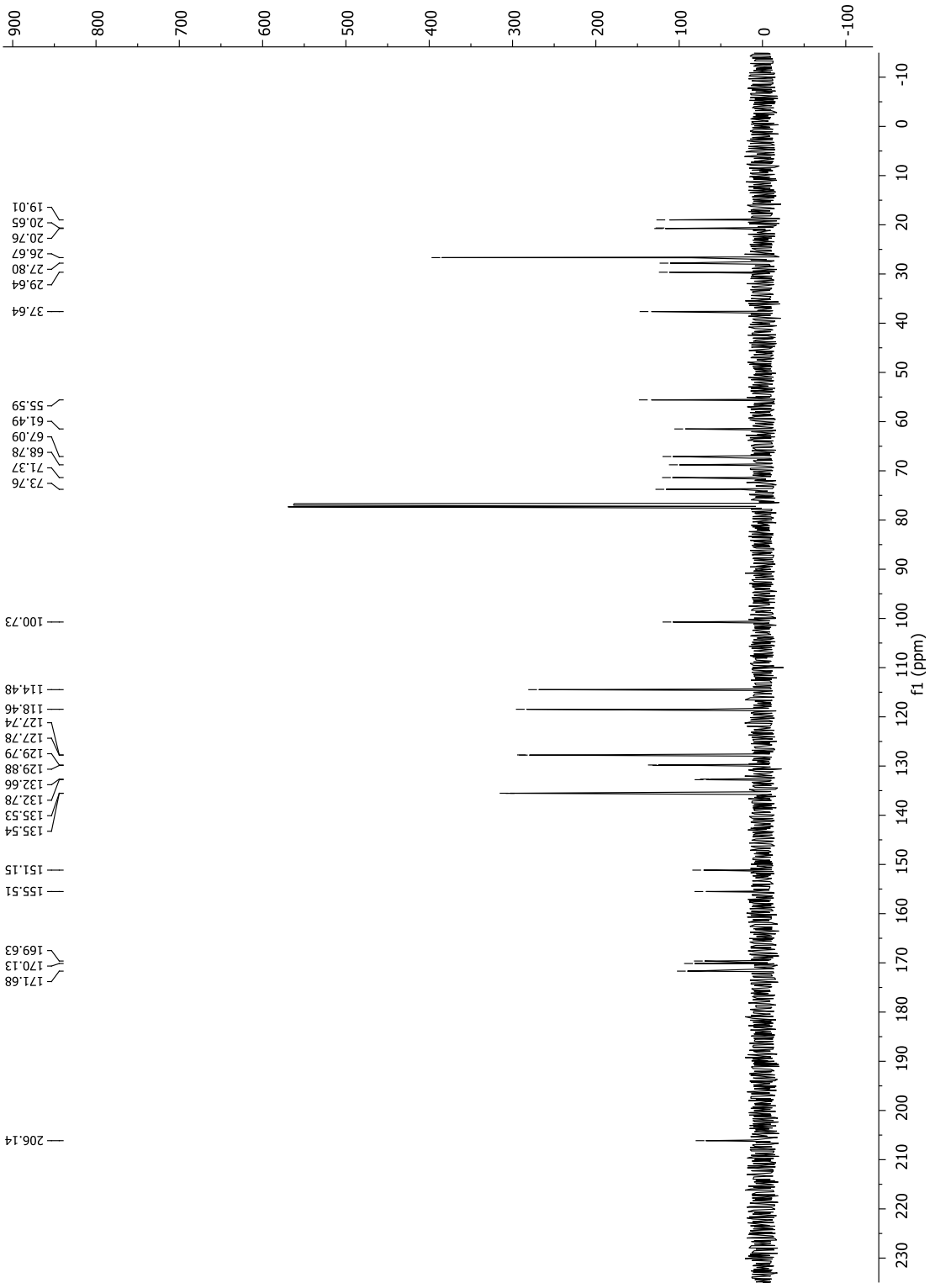

^{13}C NMR spectrum of compound **42** in CDCl3 at 100 MHz.

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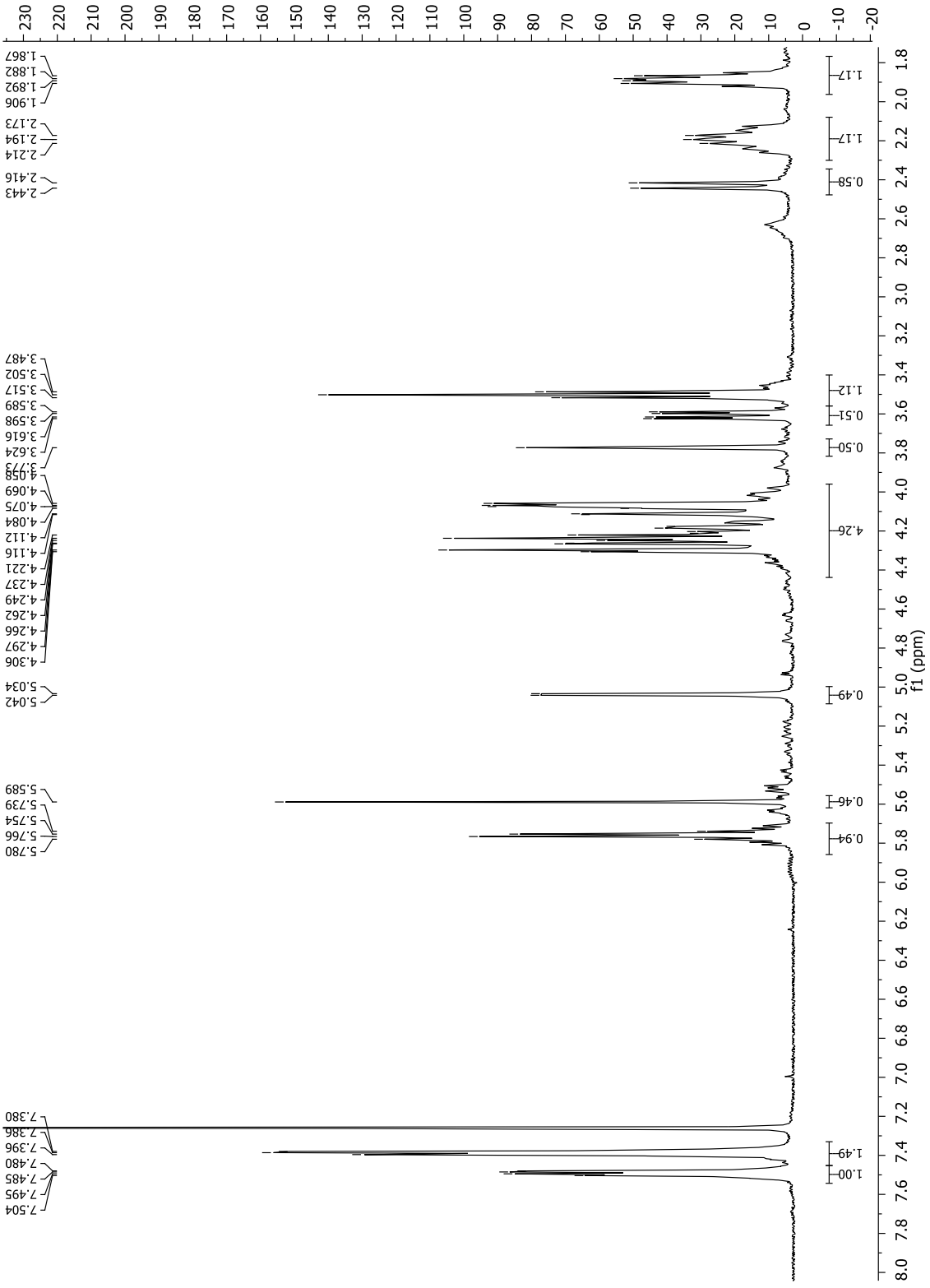

^{1}H NMR spectrum of compound **43** in CDCl3 at 400 MHz.

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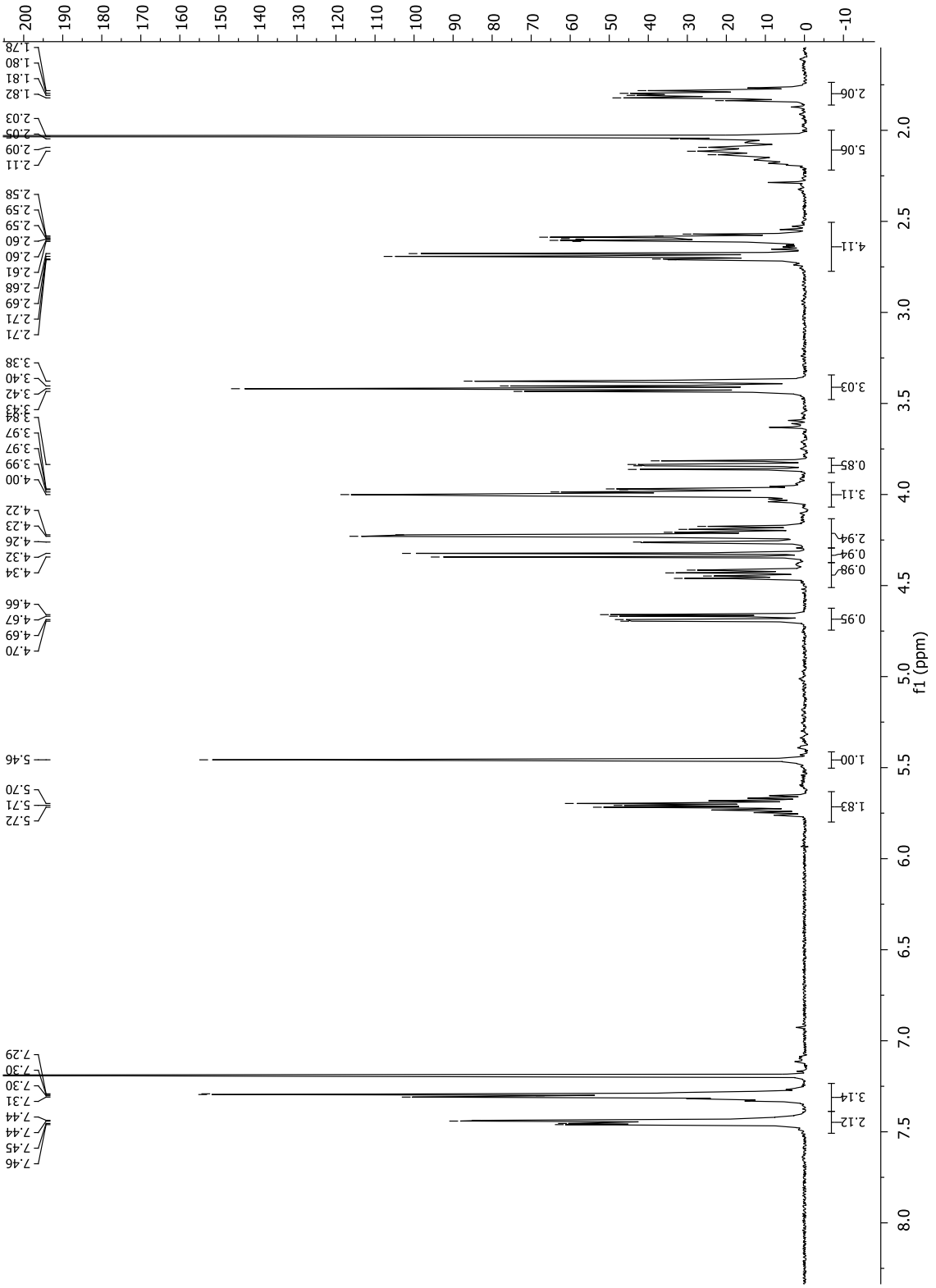

^{1}H NMR spectrum of compound **44** in CDCl3 at 100 MHz.

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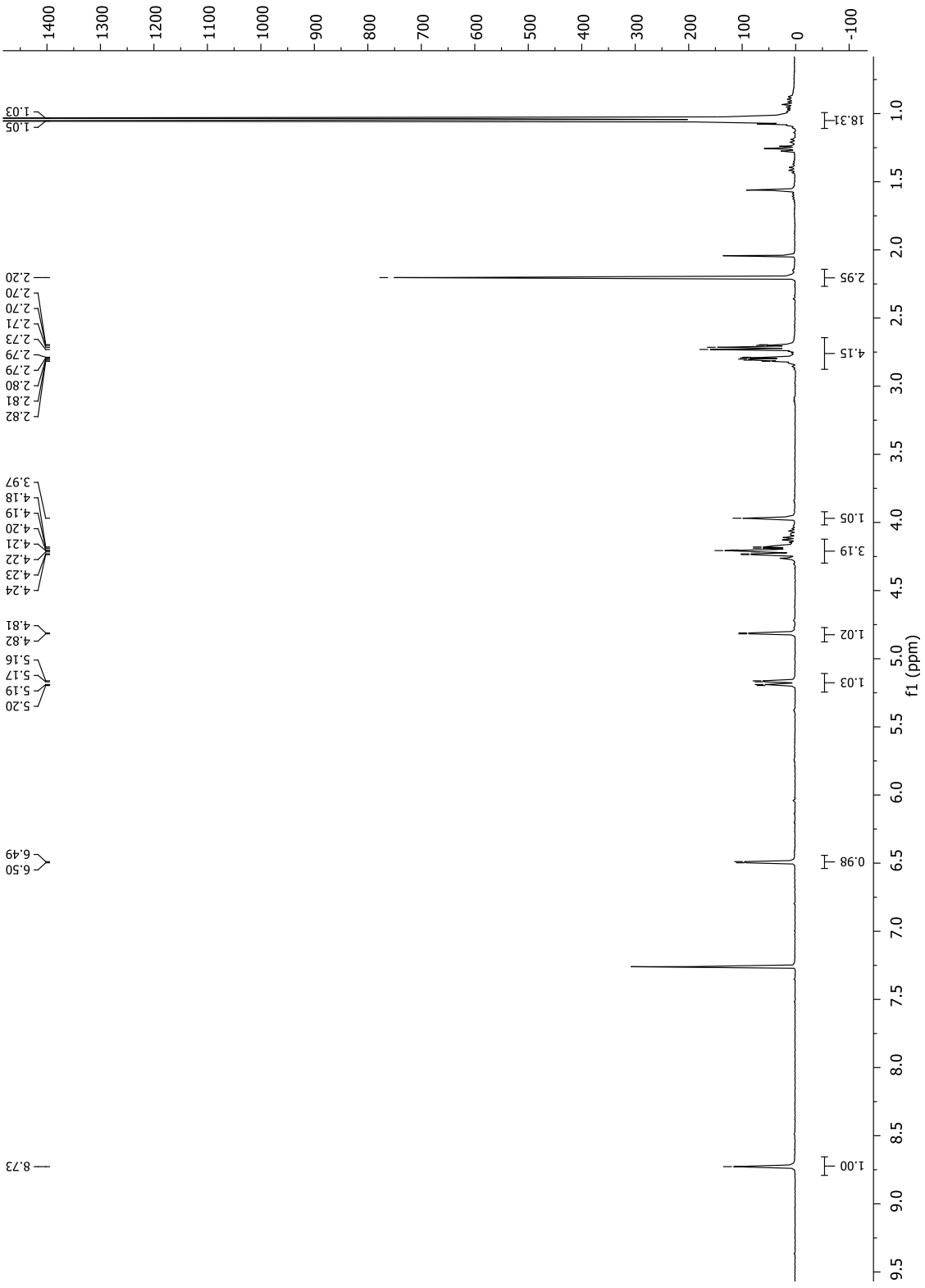

^{1}H NMR spectrum of compound **45** in CDCl3 at 400 MHz.

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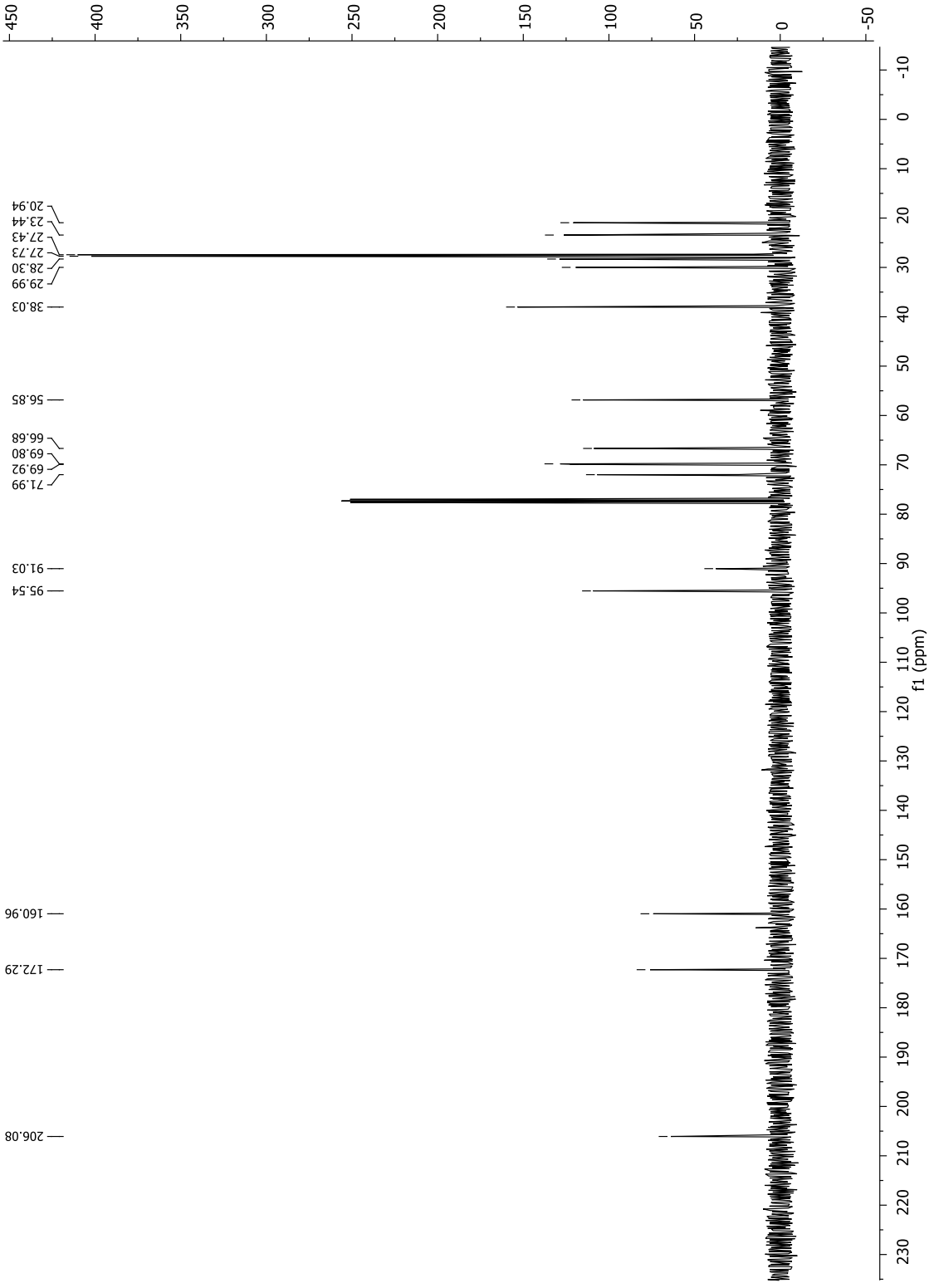

^{13}C NMR spectrum of compound **45** in CDCl3 at 100 MHz.

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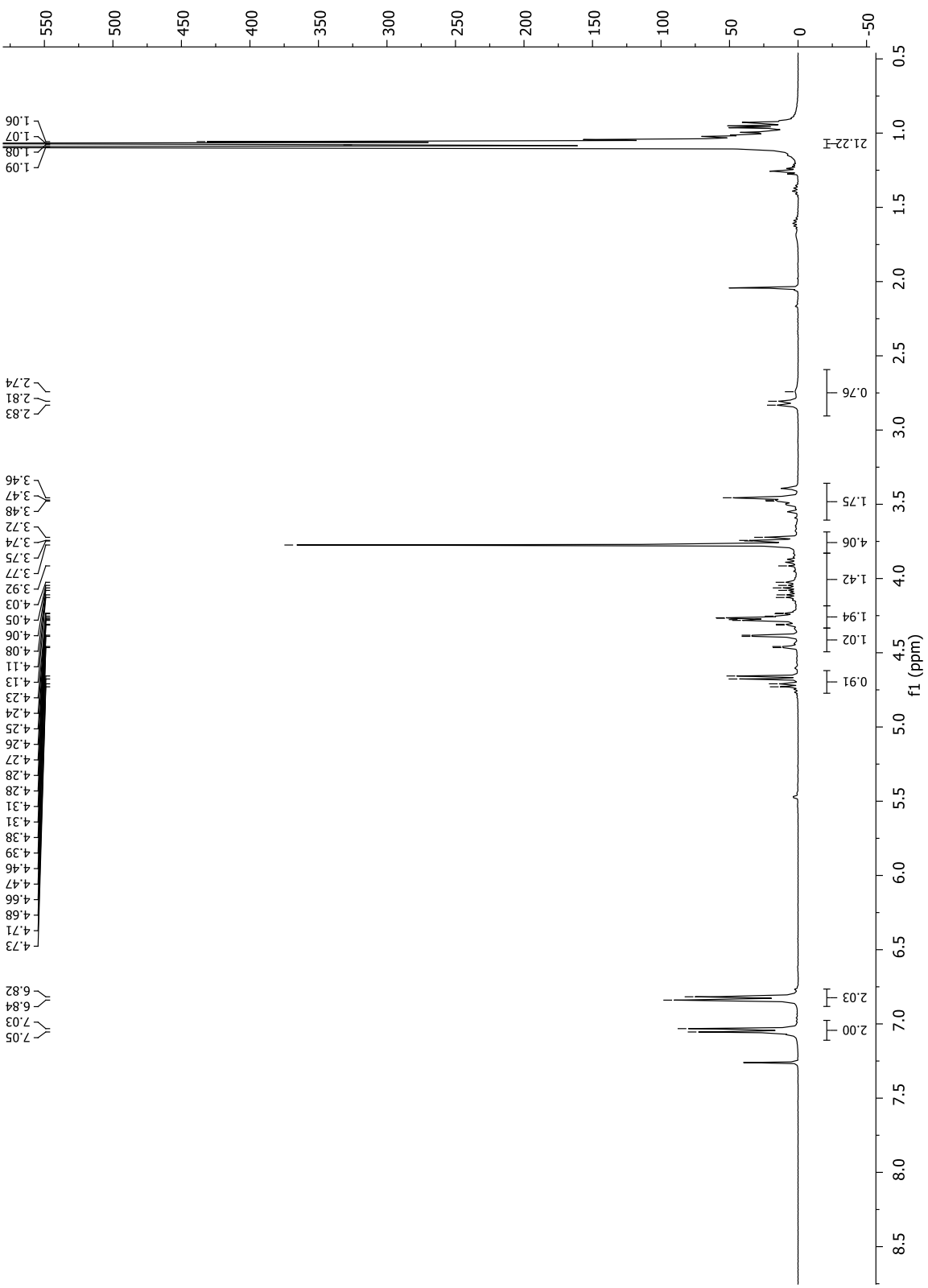

^{1}H NMR spectrum of compound **46** in CDCl3 at 400 MHz.

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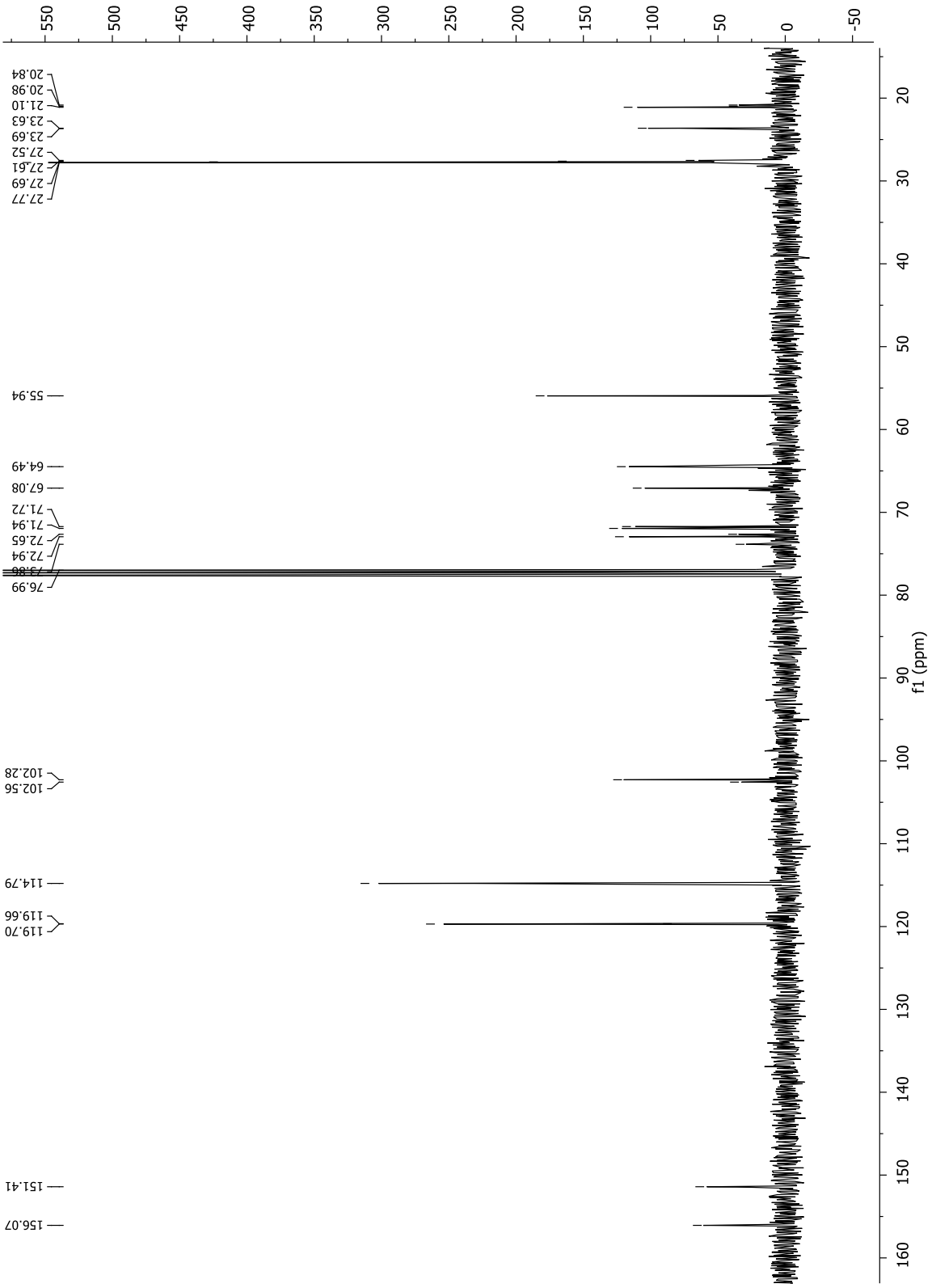

^{13}C NMR spectrum of compound **46** in CDCl3 at 100 MHz.

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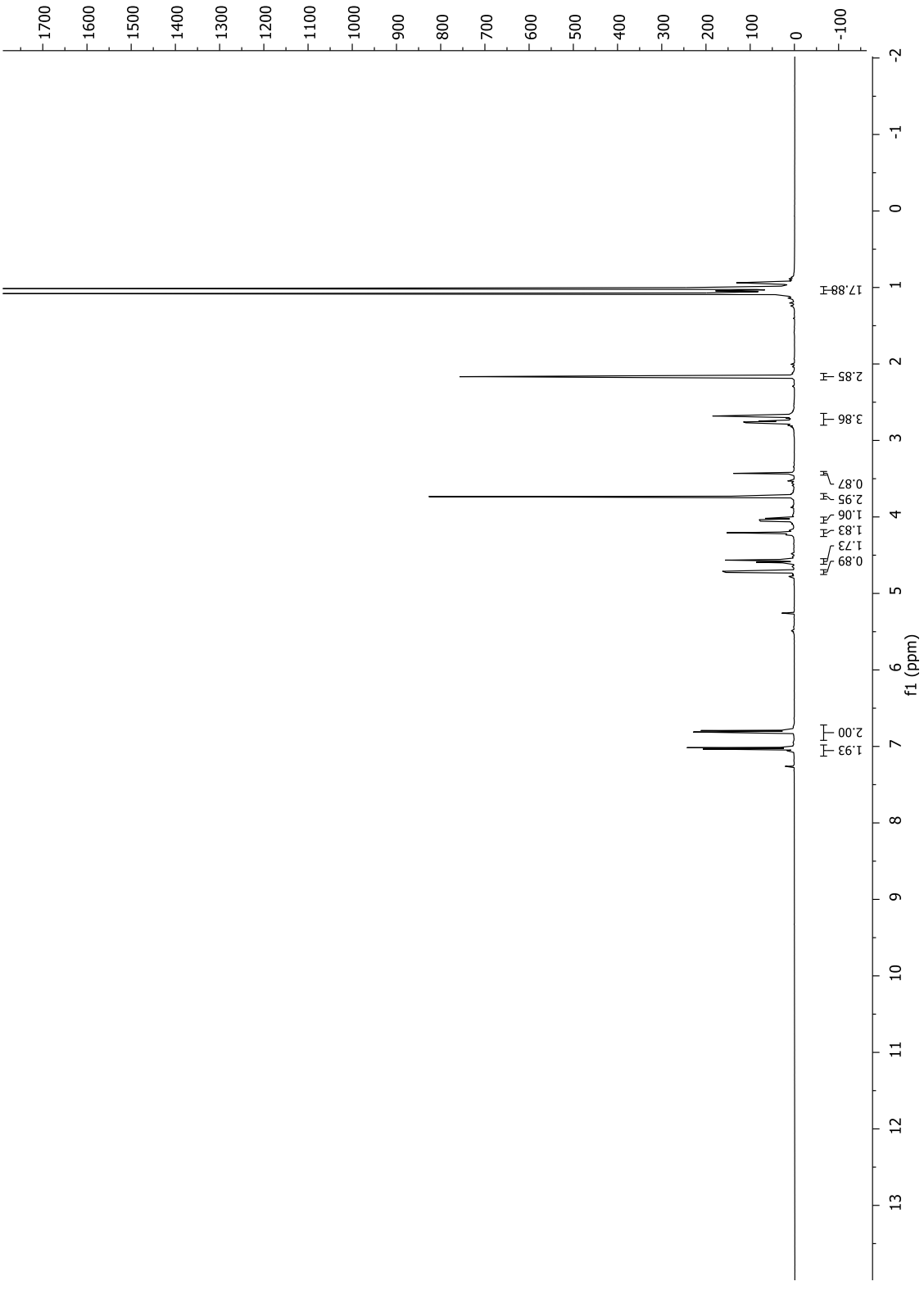

^{1}H NMR spectrum of compound **47** in CDCl3 at 400 MHz.

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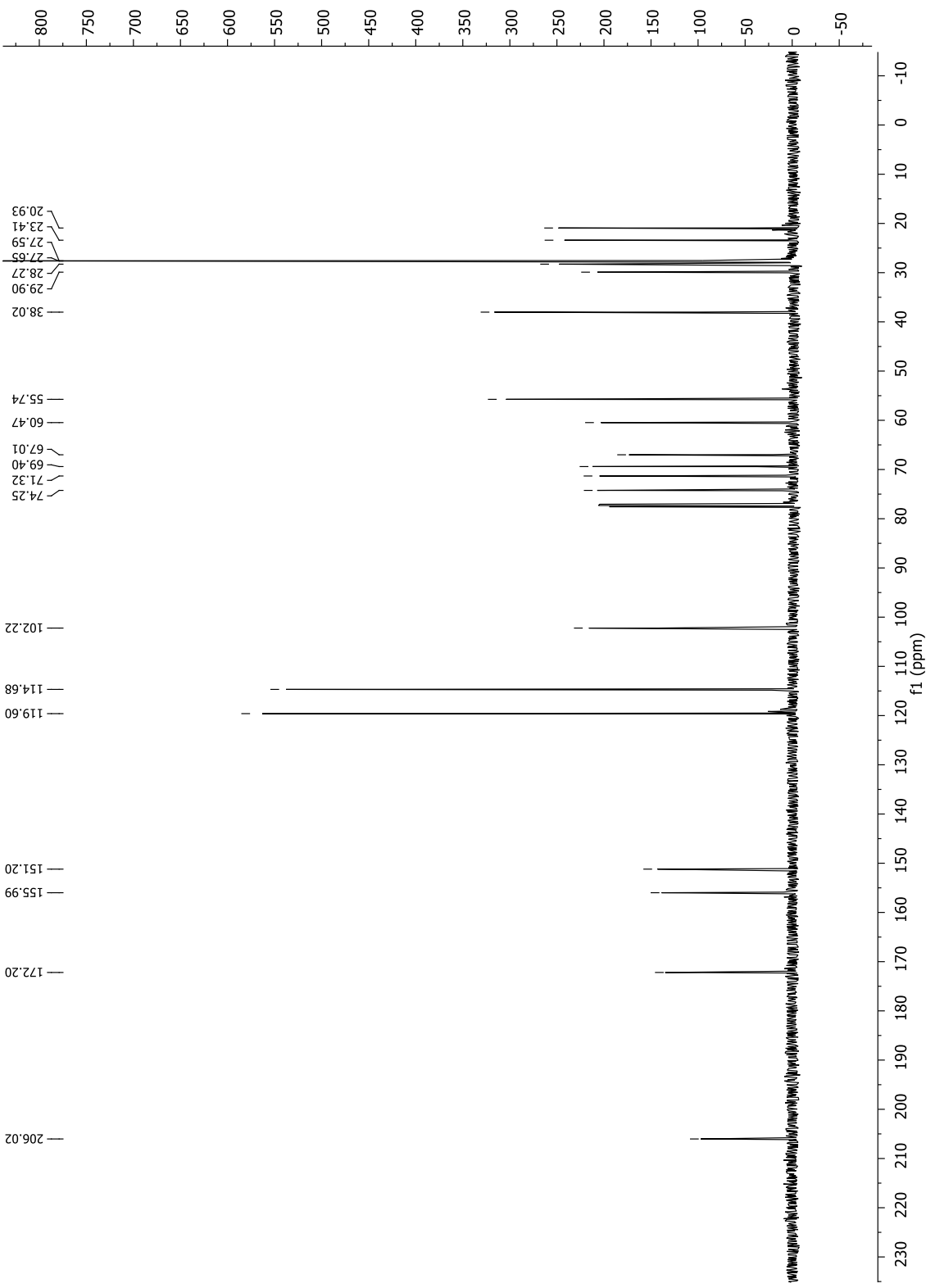

^{13}C NMR spectrum of compound **47** in CDCl3 at 100 MHz.

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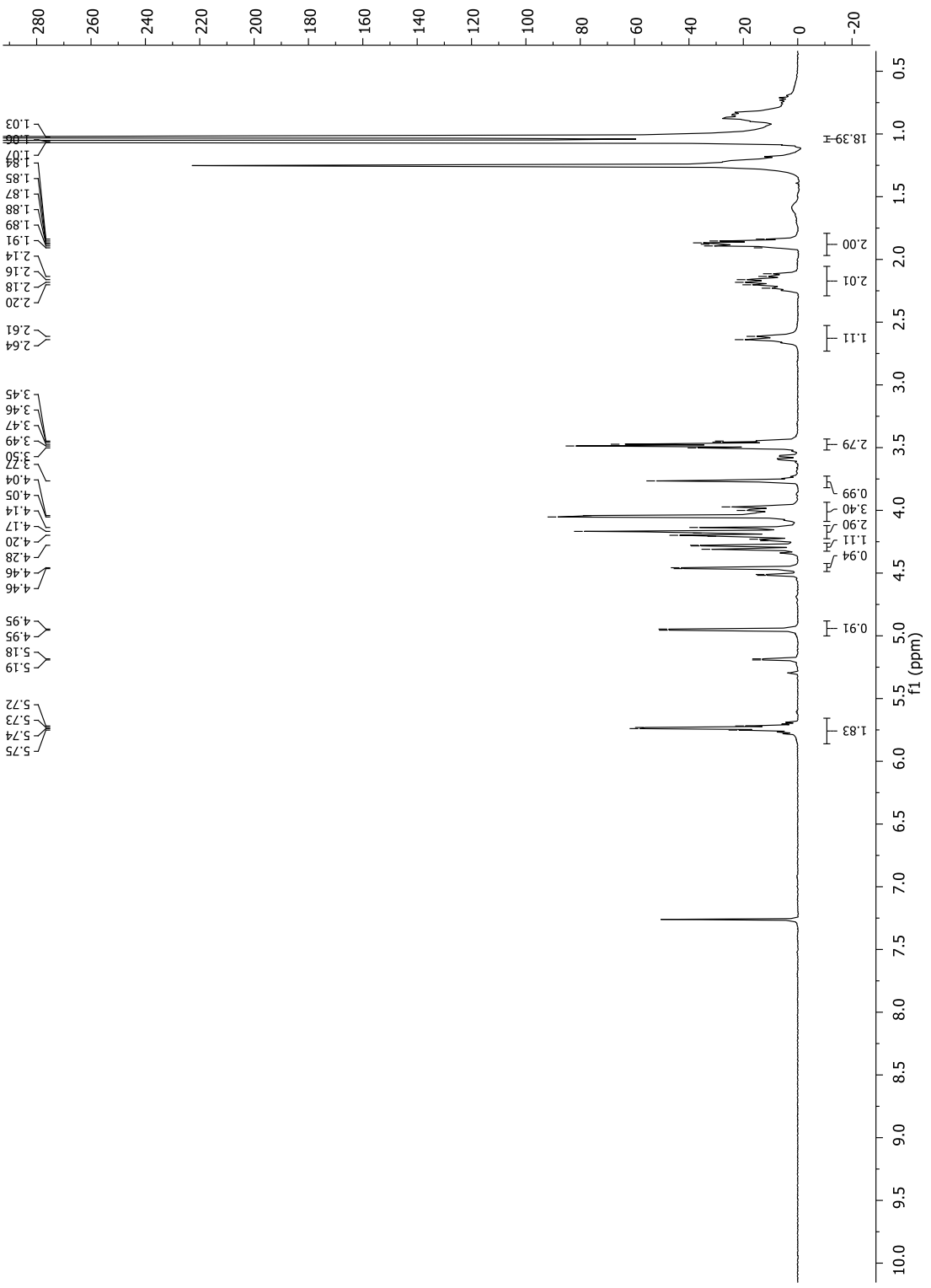

^{1}H NMR spectrum of compound **48** in CDCl3 at 400 MHz.

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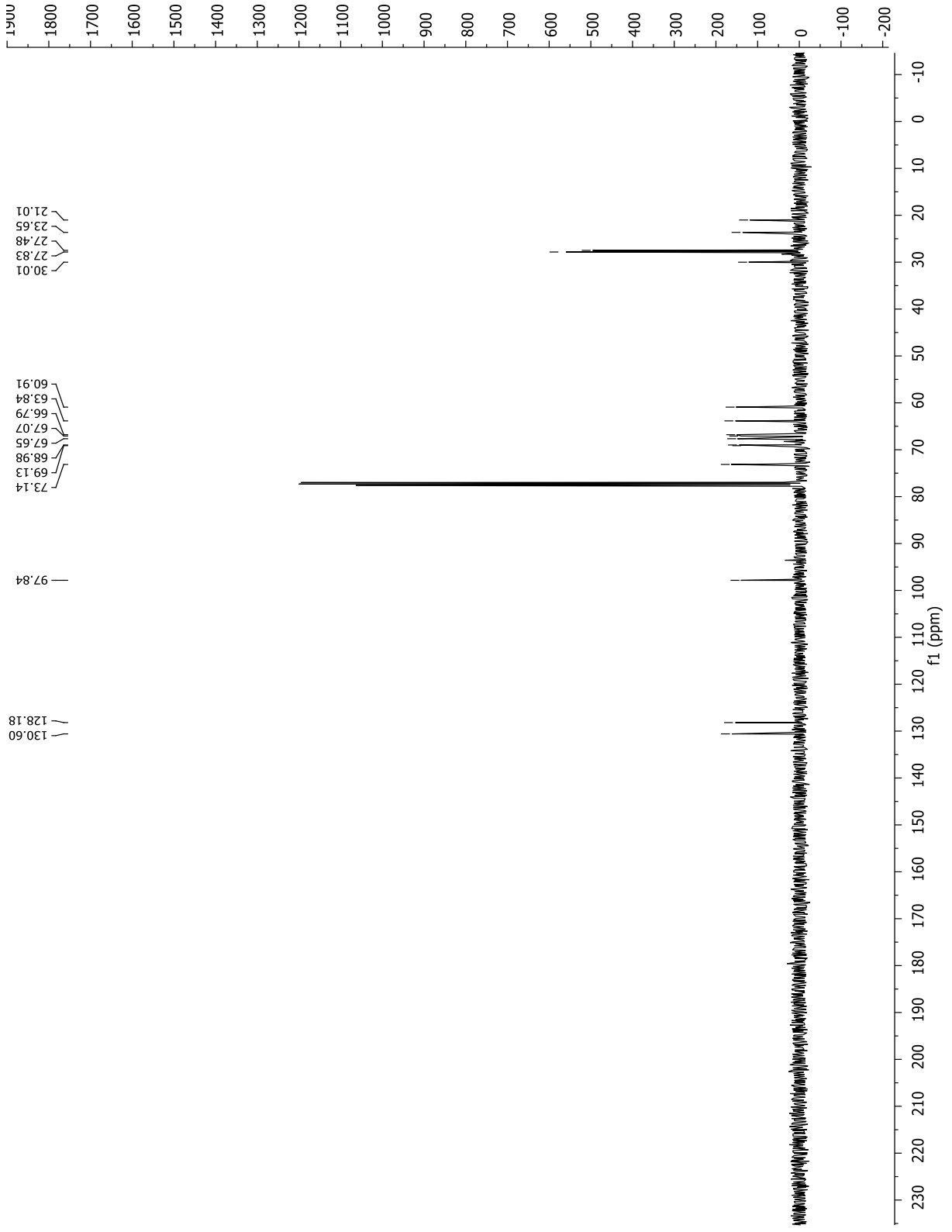

^{13}C NMR spectrum of compound **48** in CDCl3 at 100 MHz.

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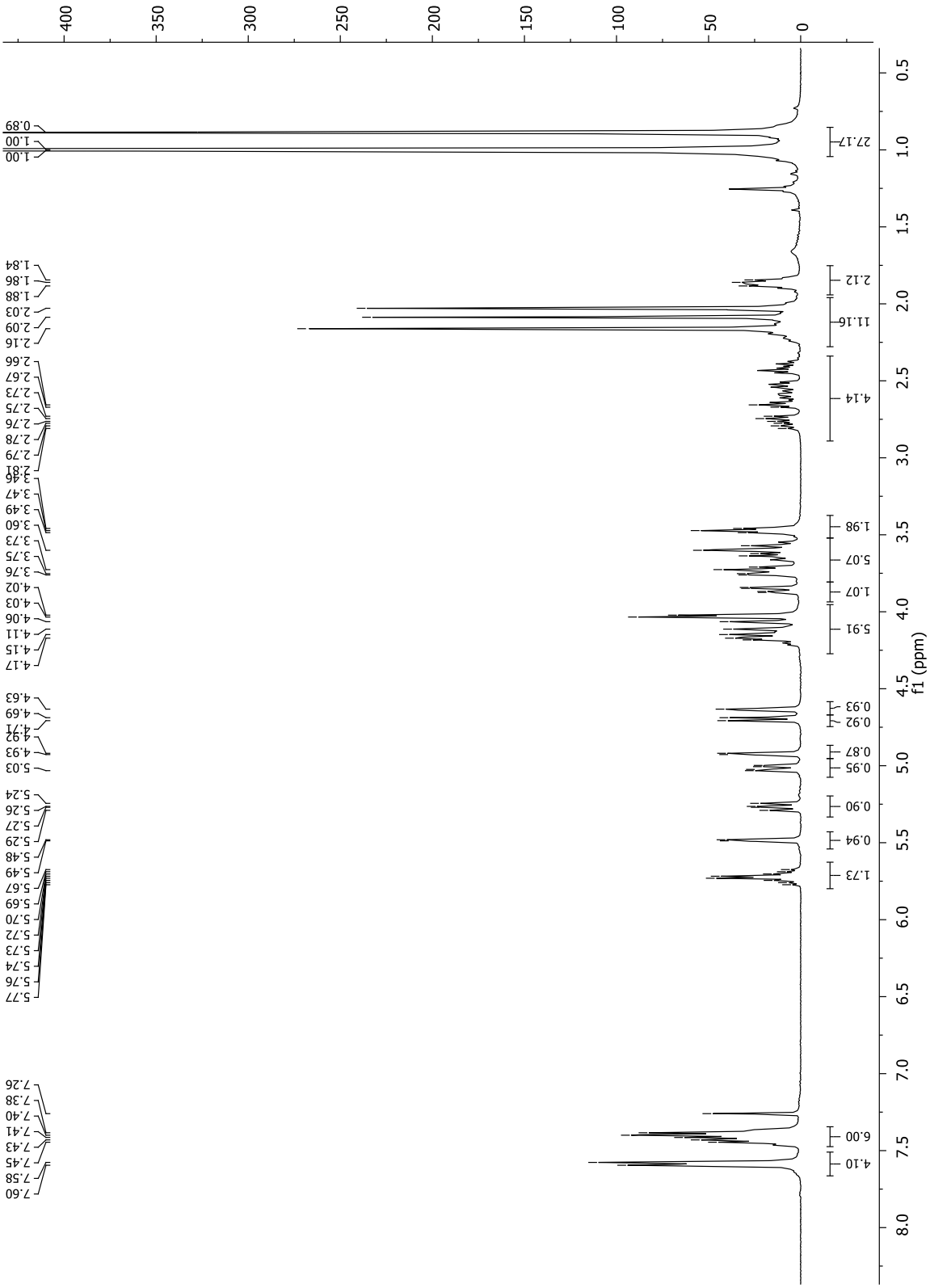

^{1}H NMR spectrum of compound **49** in CDCl3 at 400 MHz.

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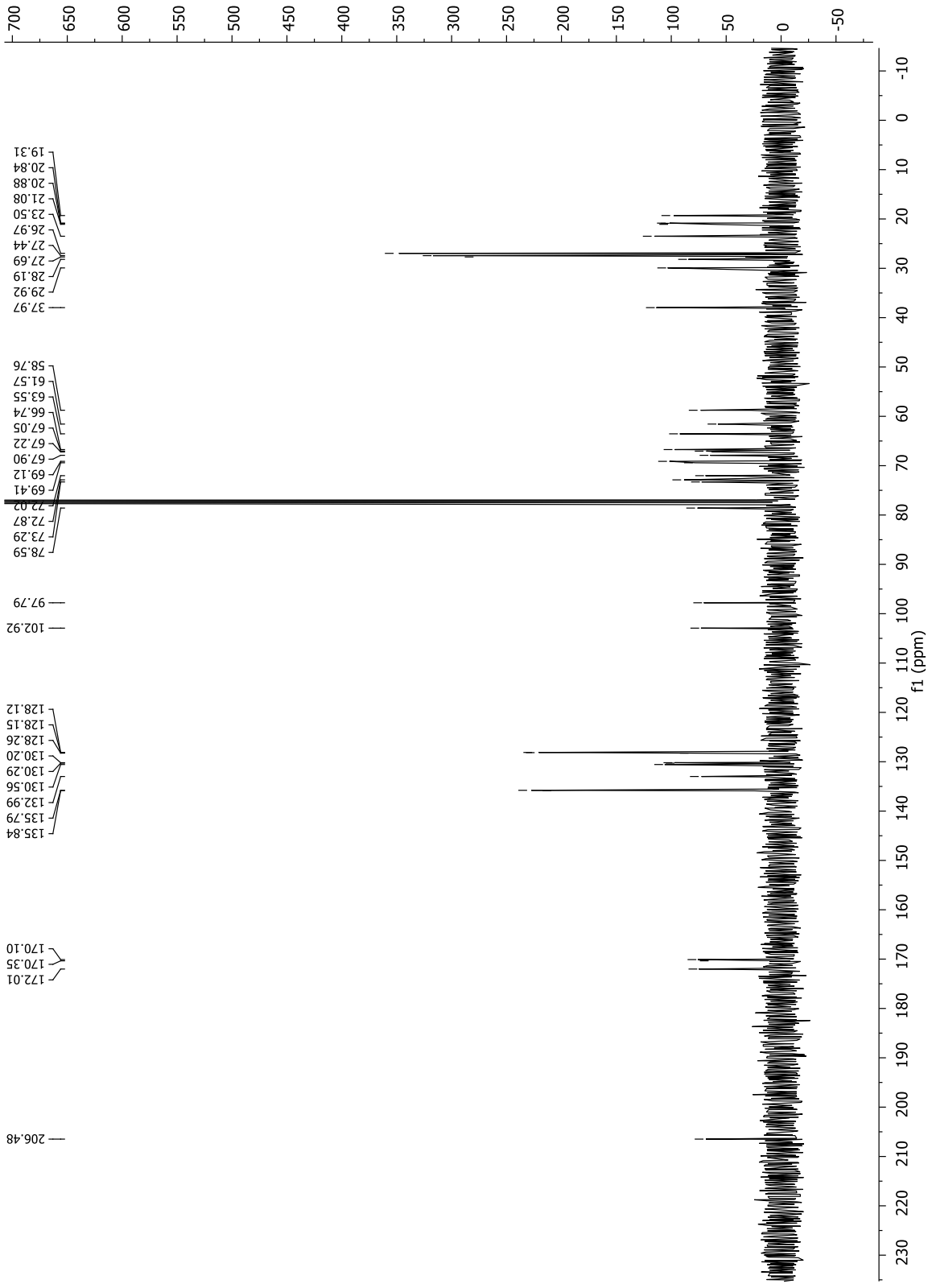

^{13}C NMR spectrum of compound **49** in CDCl3 at 100 MHz.

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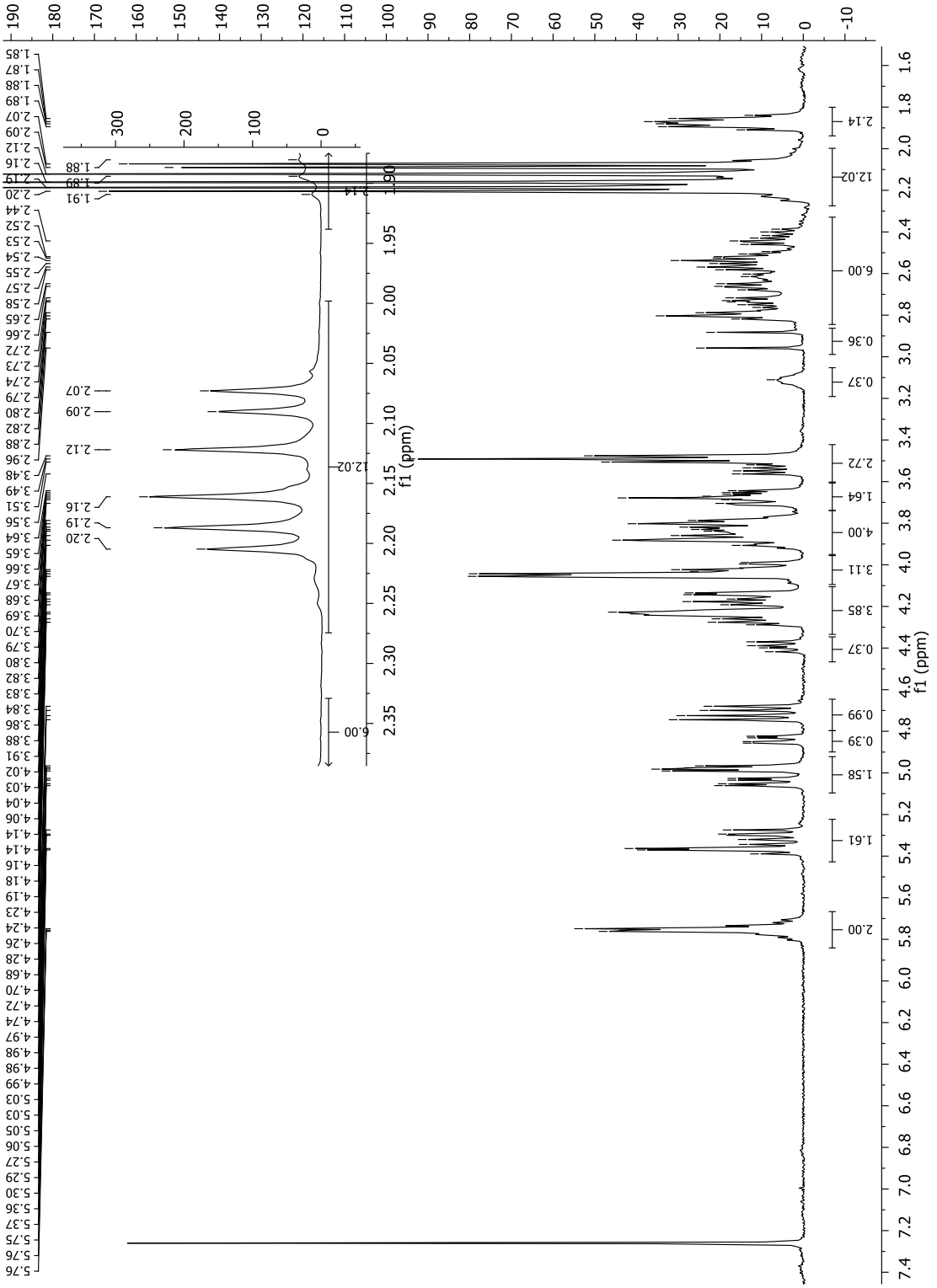

^{1}H NMR Mixture of **50** and **51** in CDCl3 at 400 MHz.

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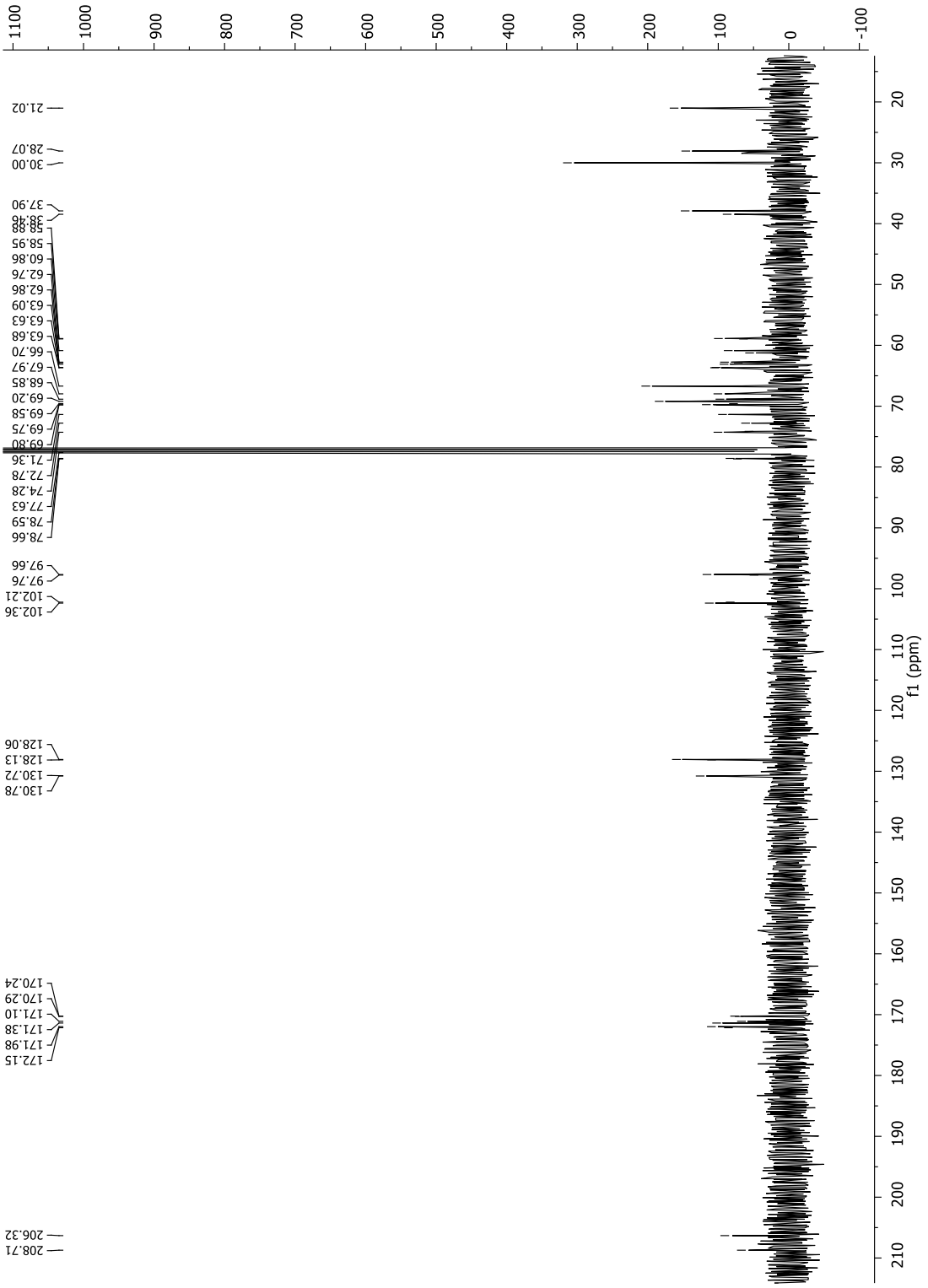

^{13}C NMR spectrum of compounds **50** and **51** in CDCl3 at 100 MHz.

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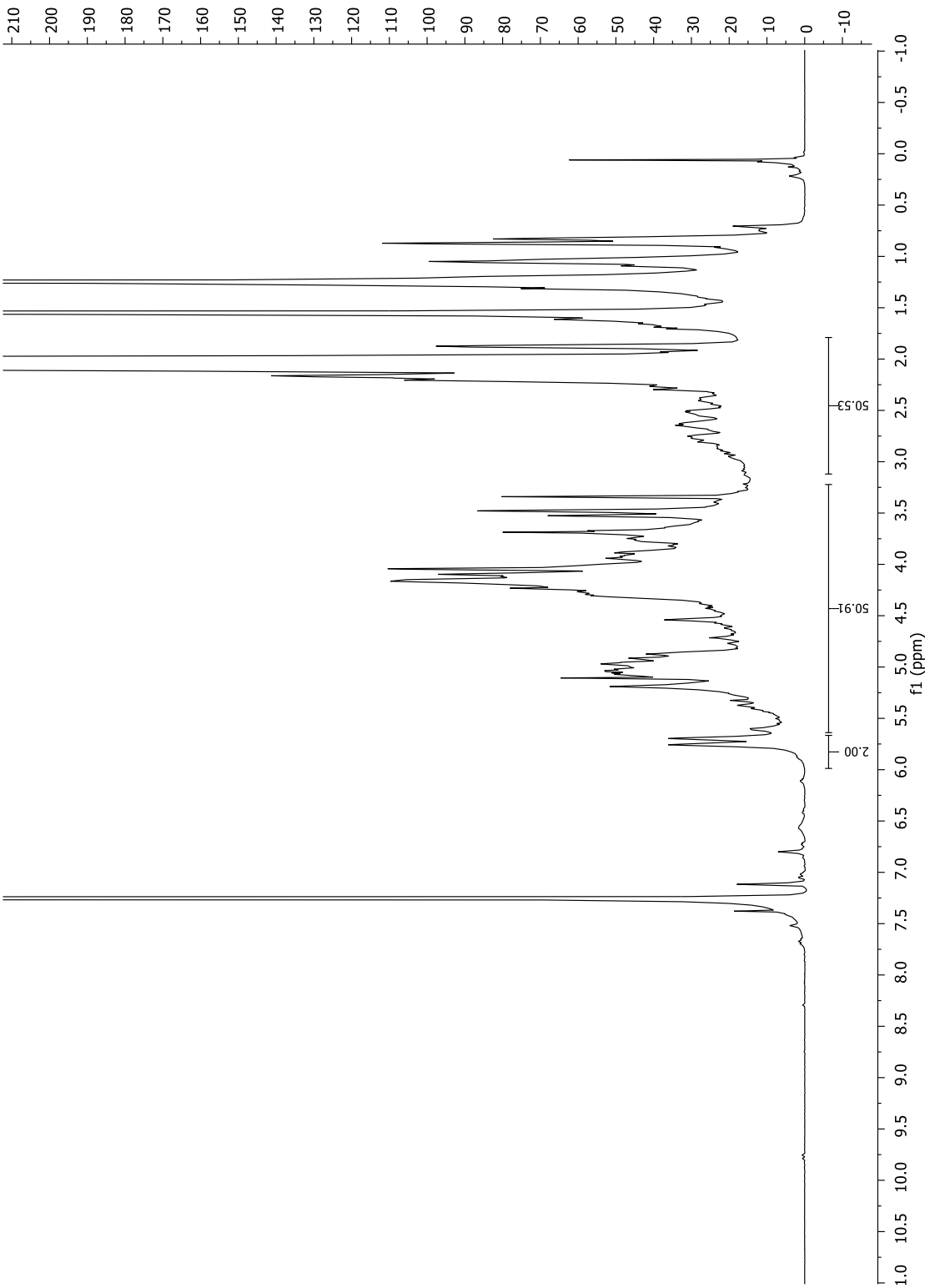

^{1}H NMR at 800 MHz to try improve the resolution of the NMR of target **10** in $CDCl_3$.

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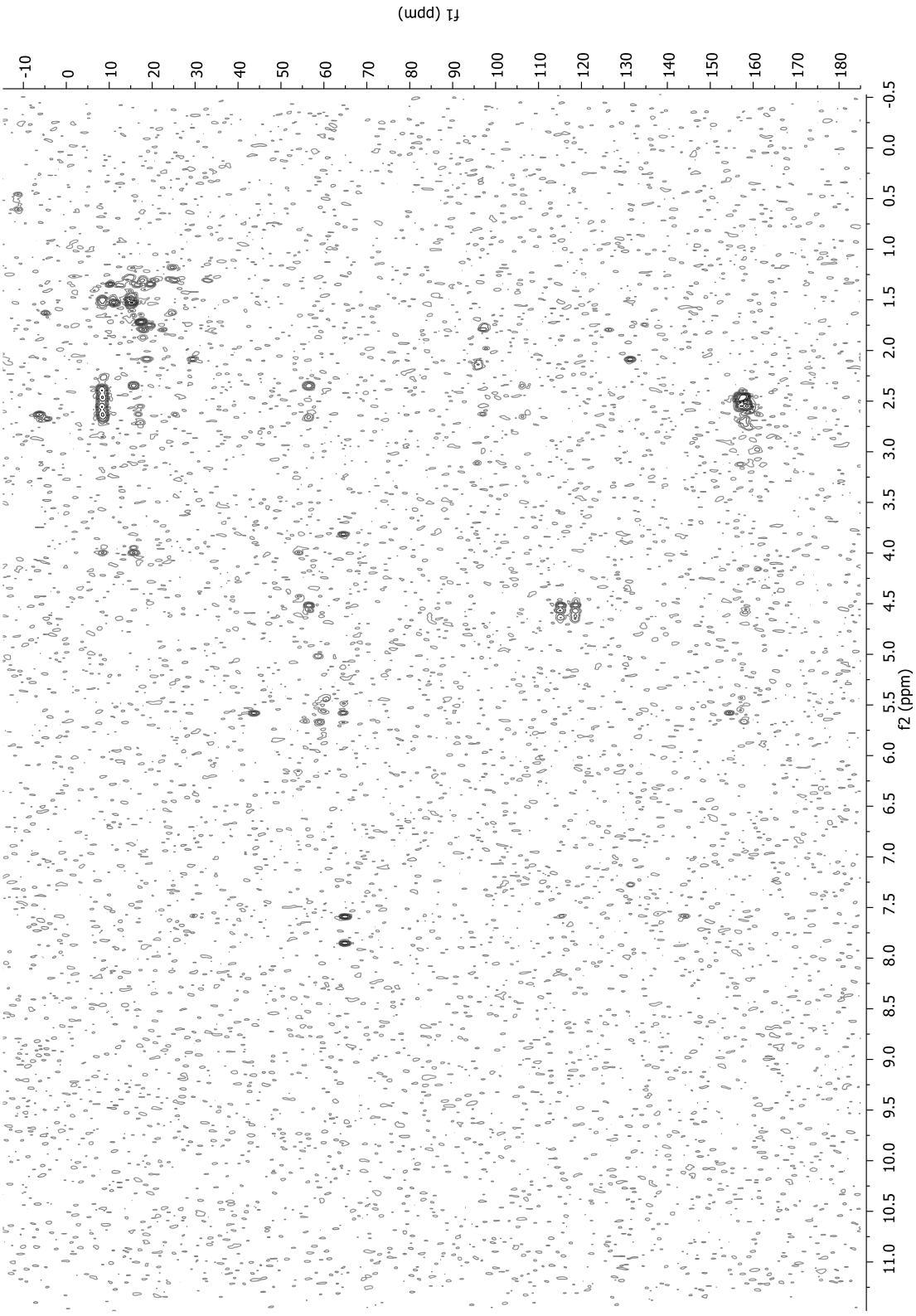

HMBC at 800 MHz for possible **10** in $CDCl_3$.

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APPENDIX C. CHAPTER 2 LC-ESI CHROMATOGRAMS

This section contains the extracted ion chromatograms for the cross linking experiments of chapter II. The top plot is the integrated peak that corresponds to matched peptide fragment from Con A. The bottom is the parent mass spectra for the middle of the integrated region. The matched peptide is VGLSASTGLYK.

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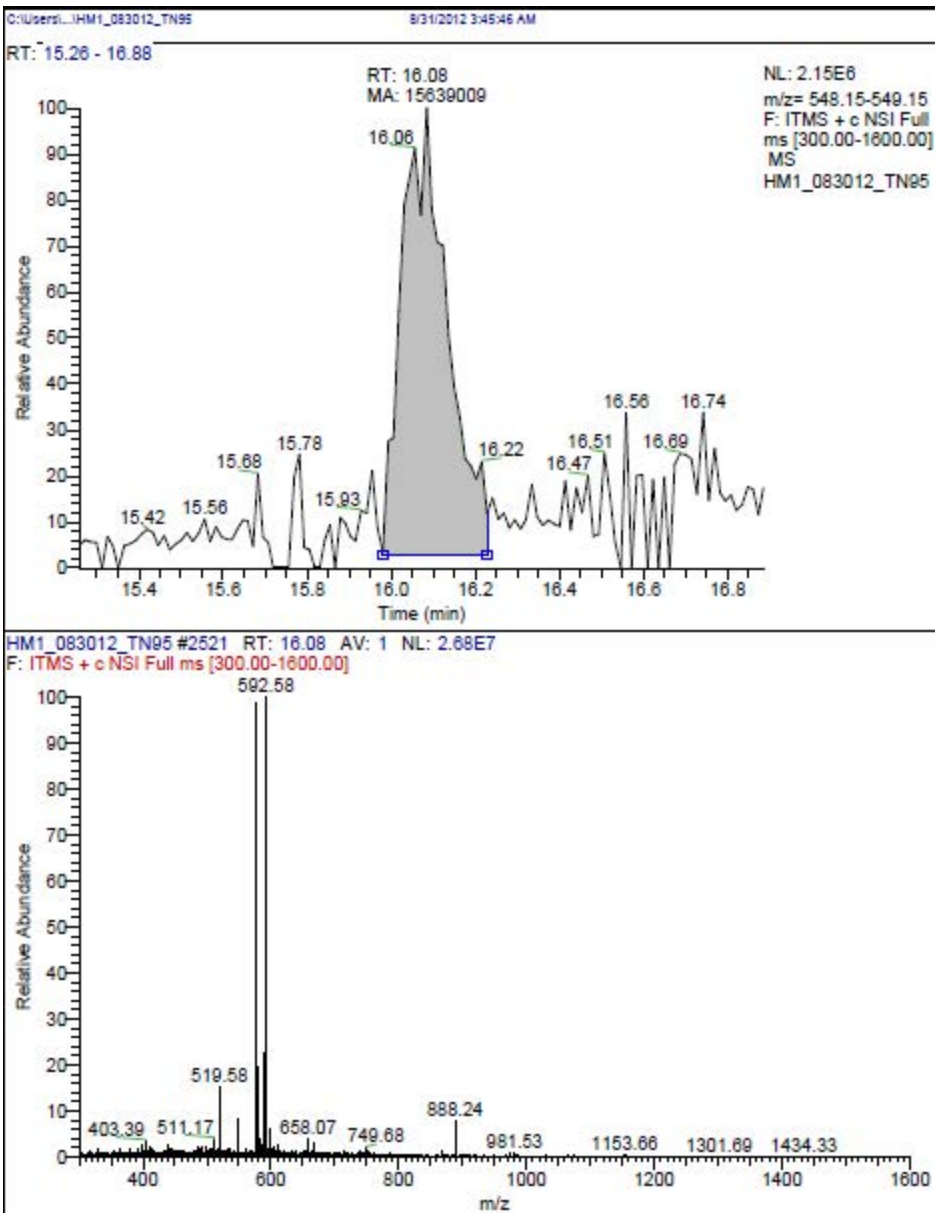

HM1

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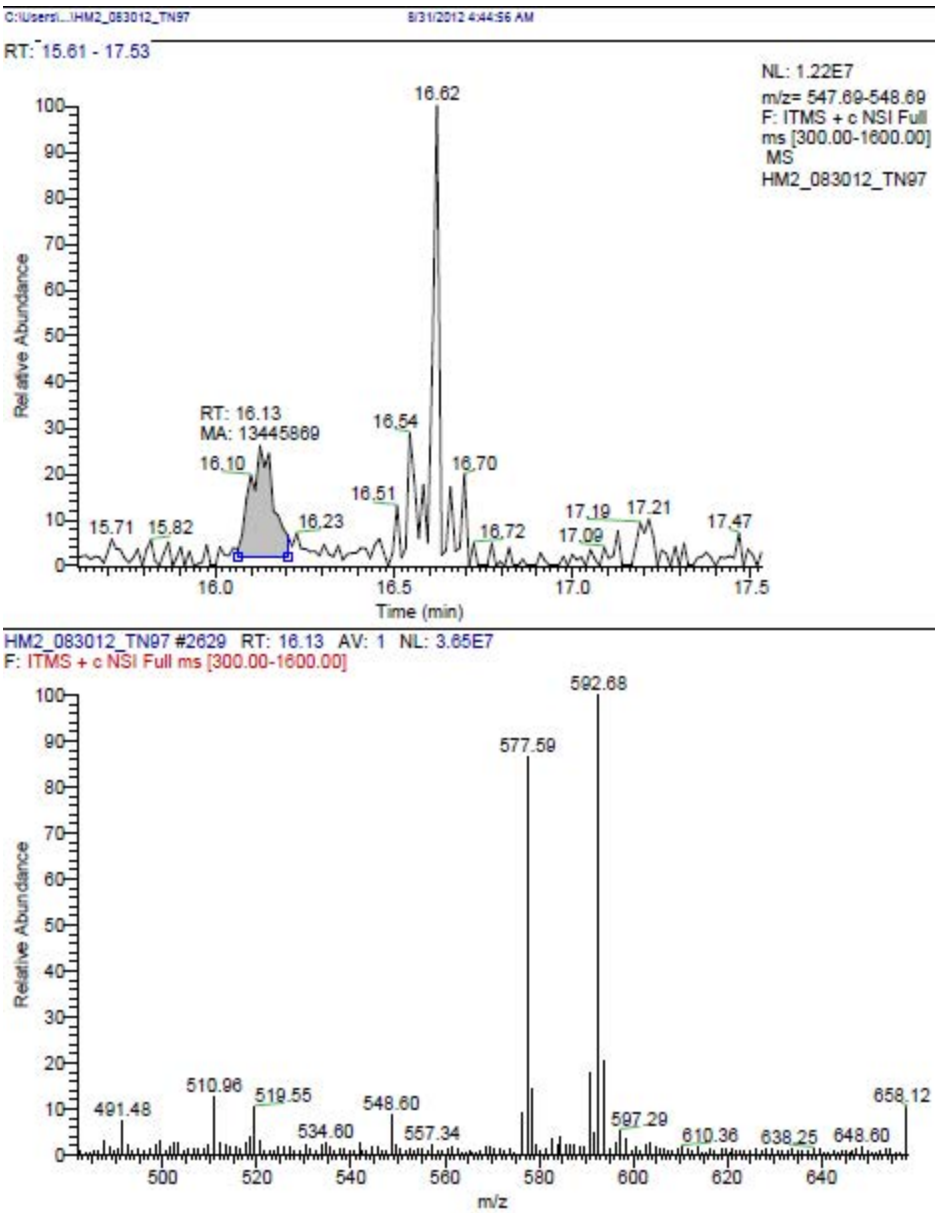

HM2

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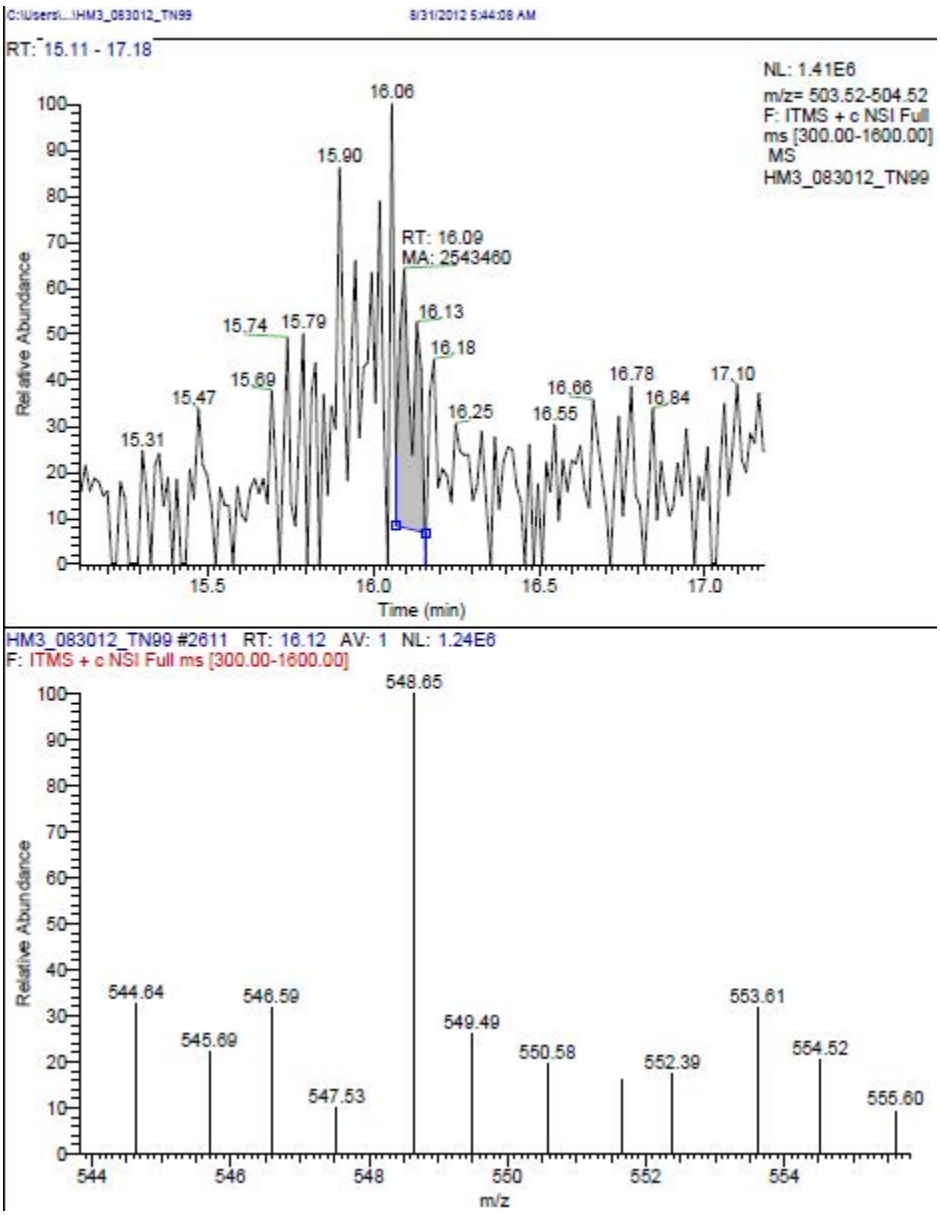

HM3

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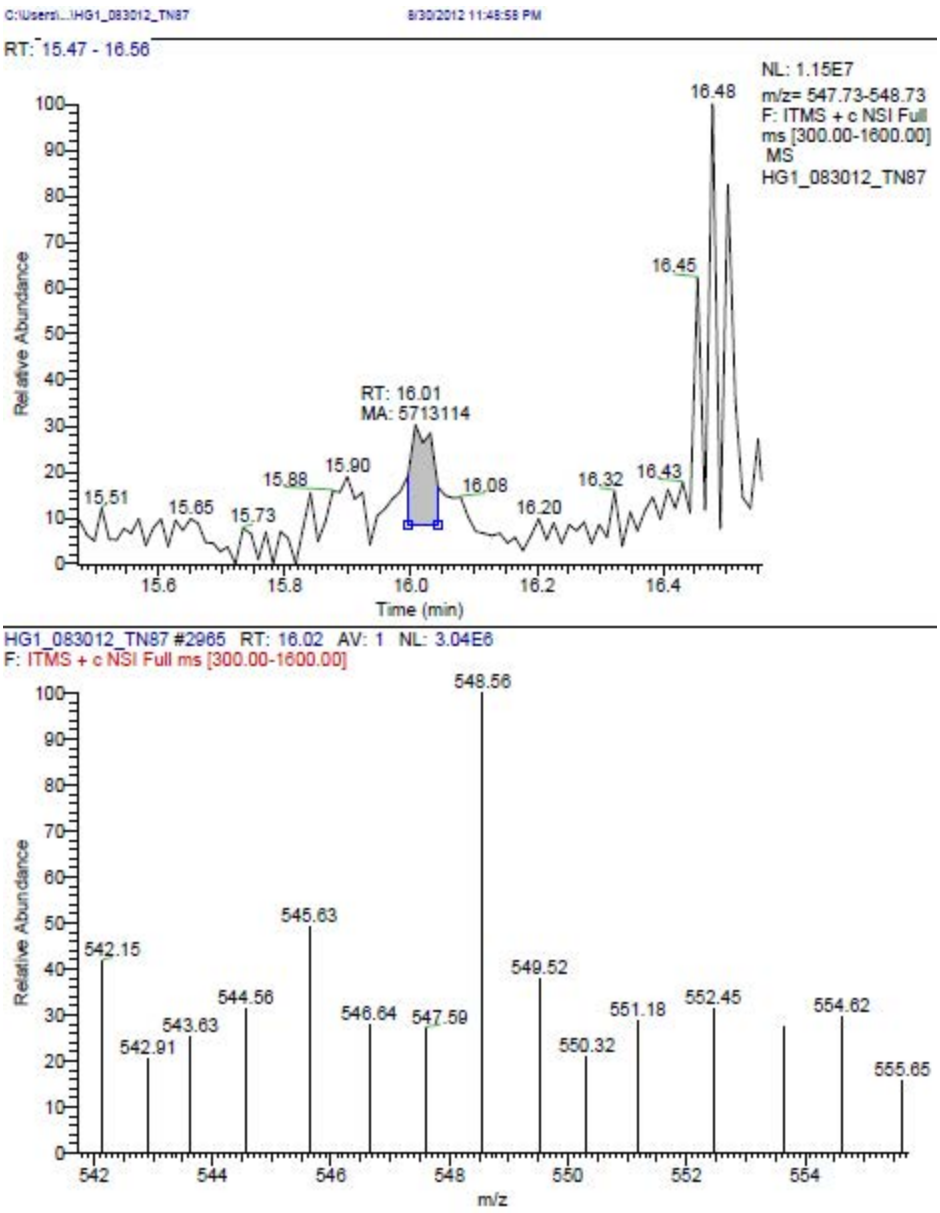

HG1

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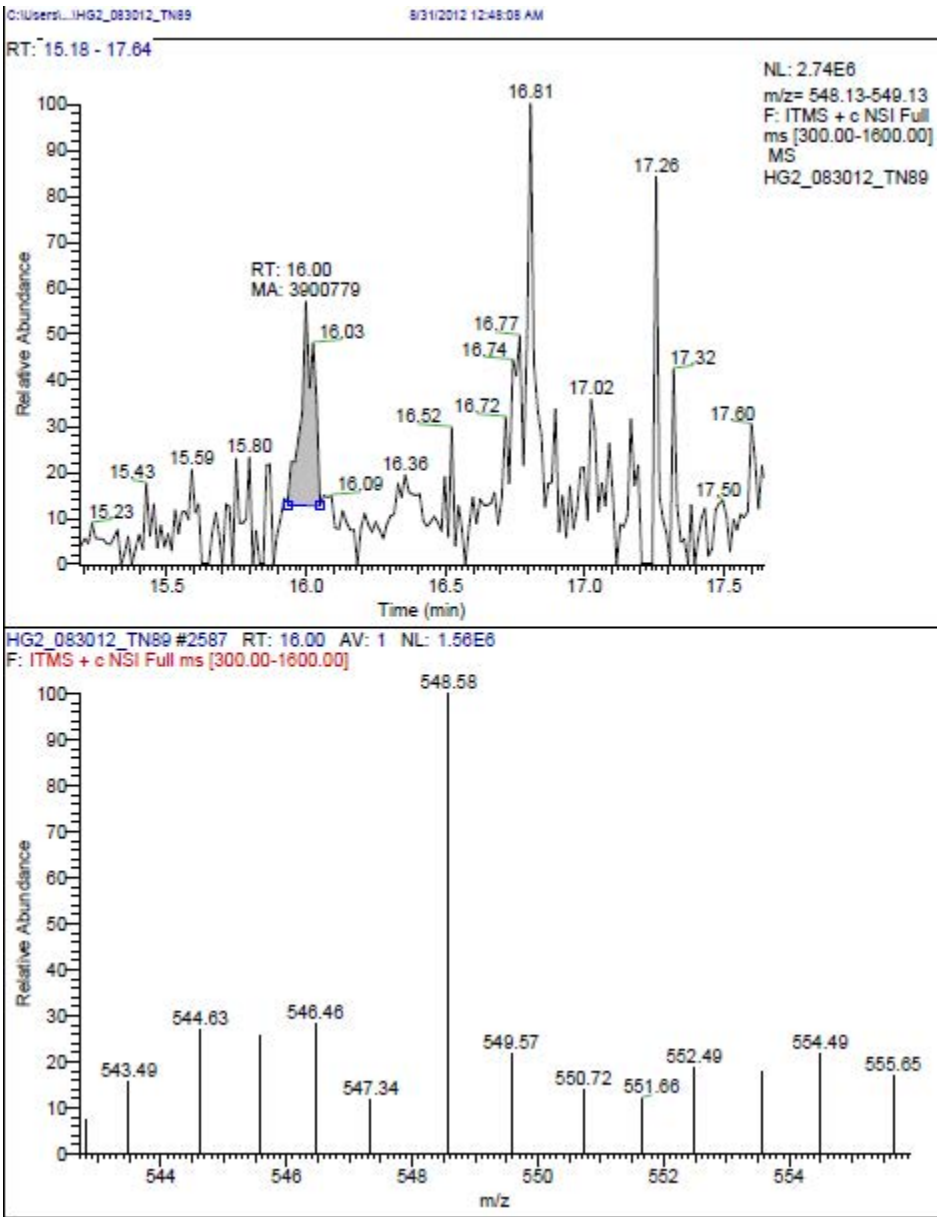

HG2

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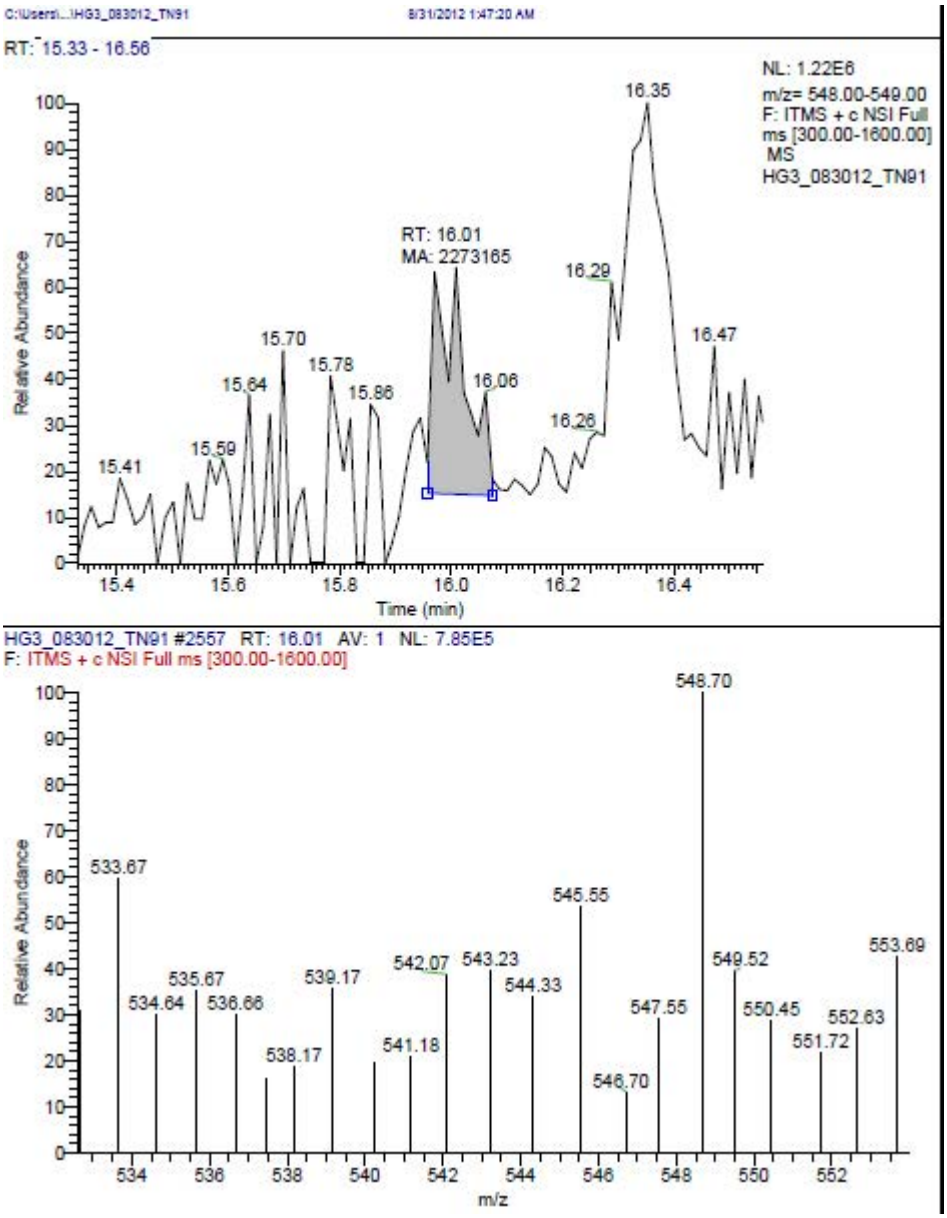

HG3

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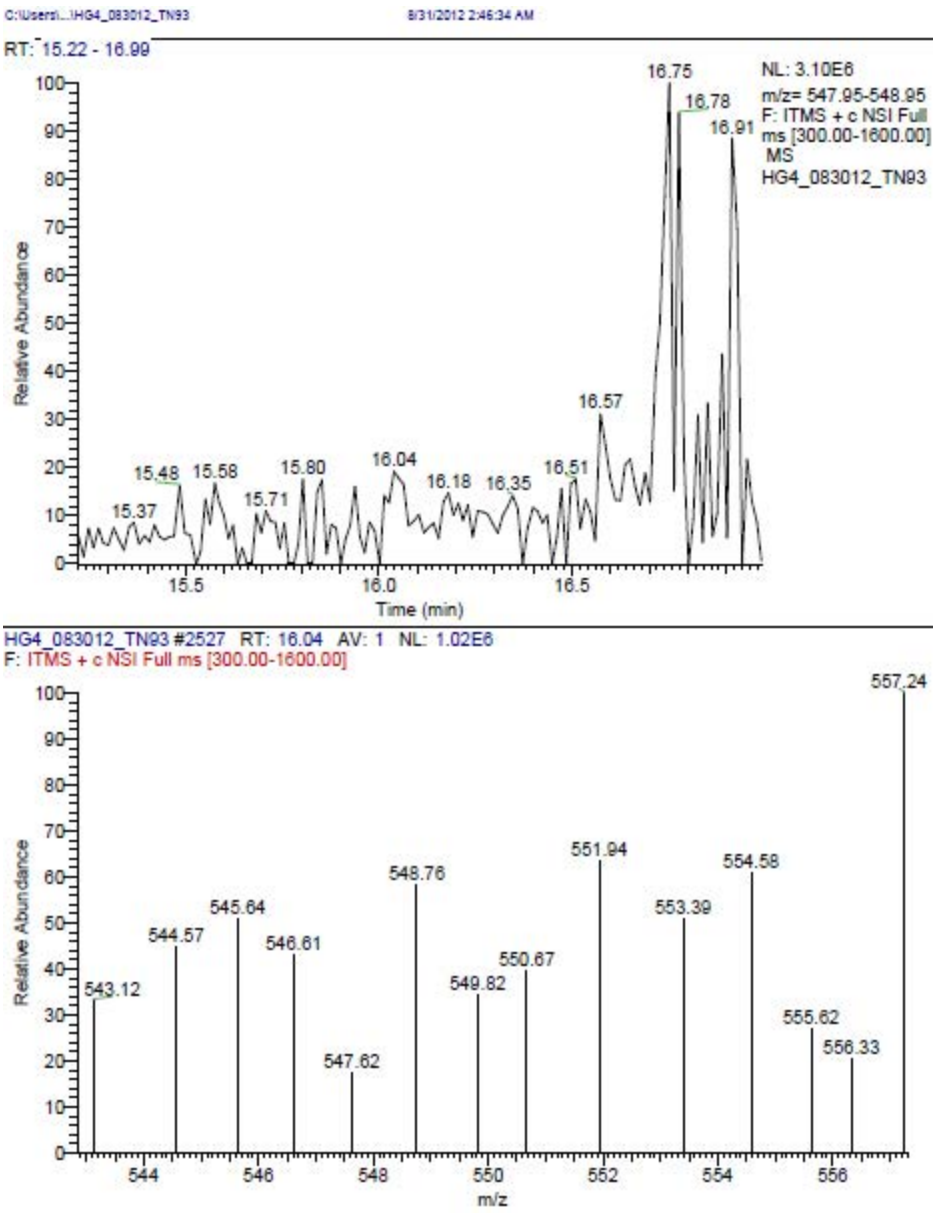

HG4

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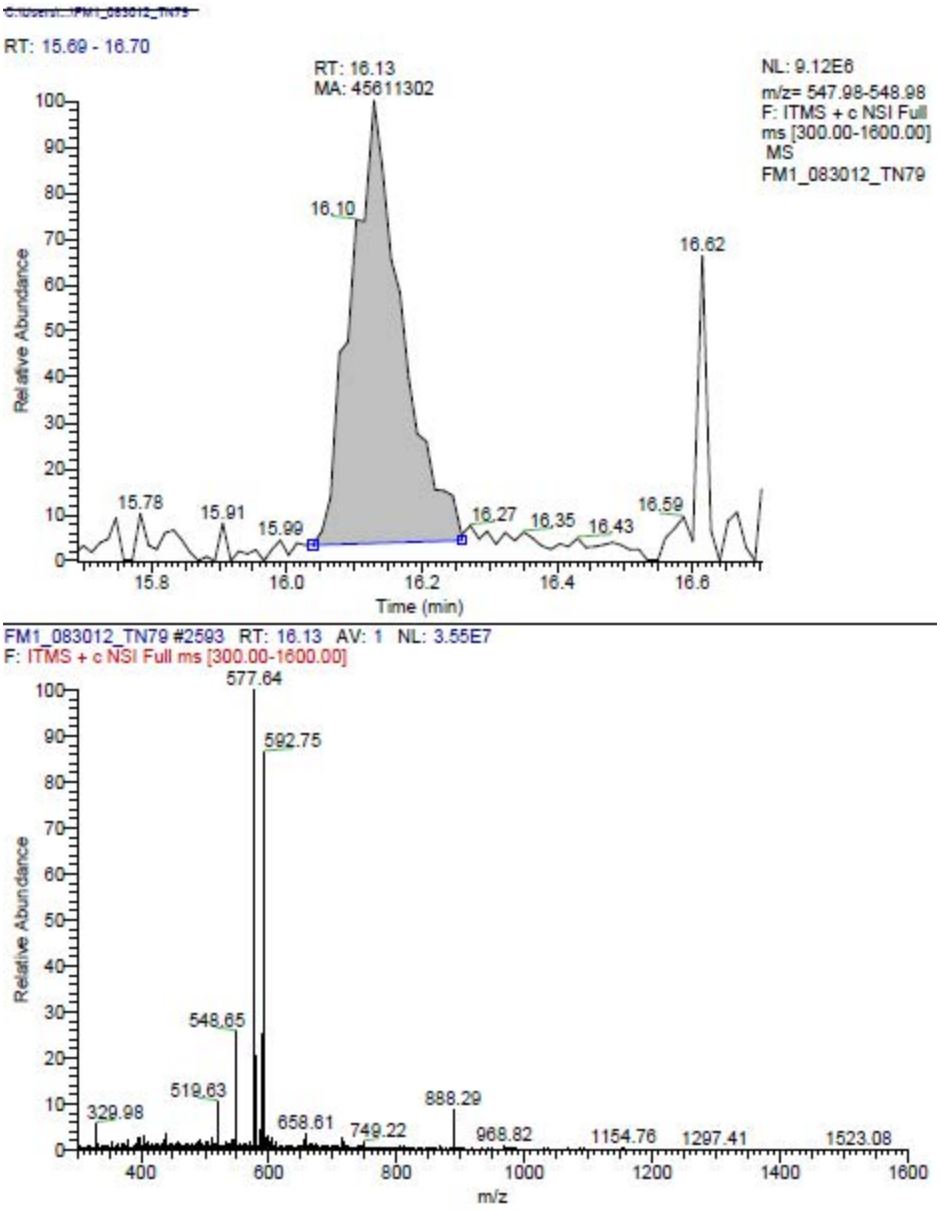

FM1

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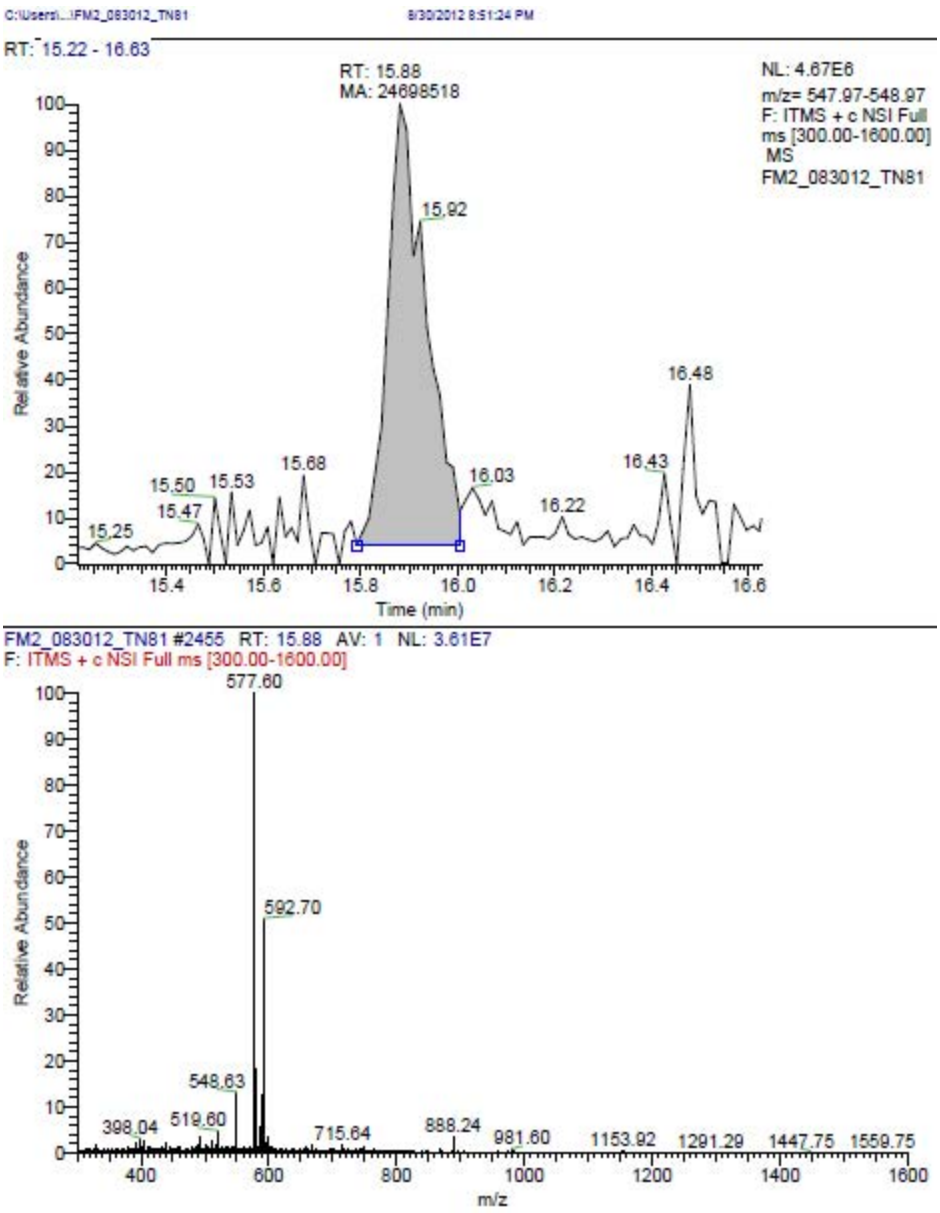

FM2

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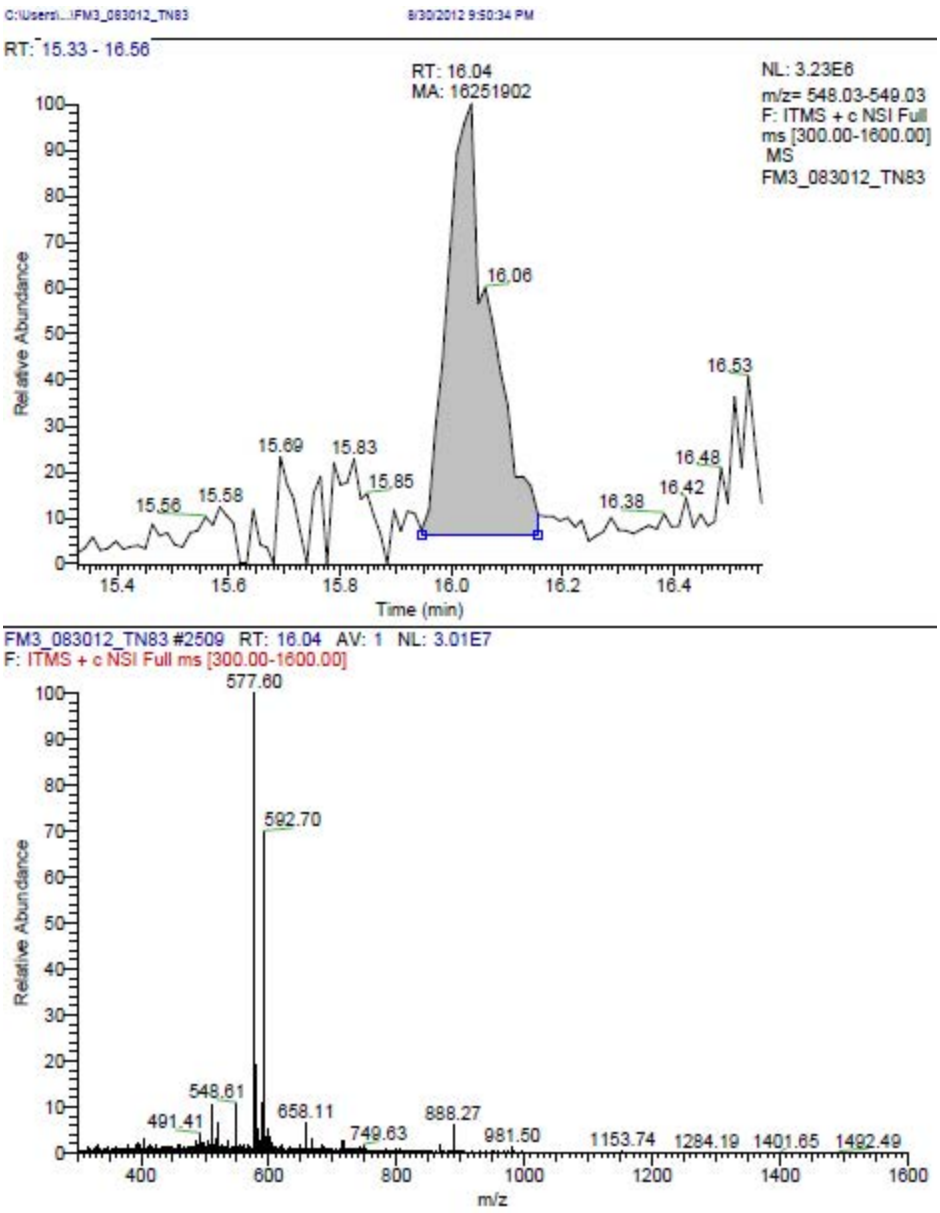
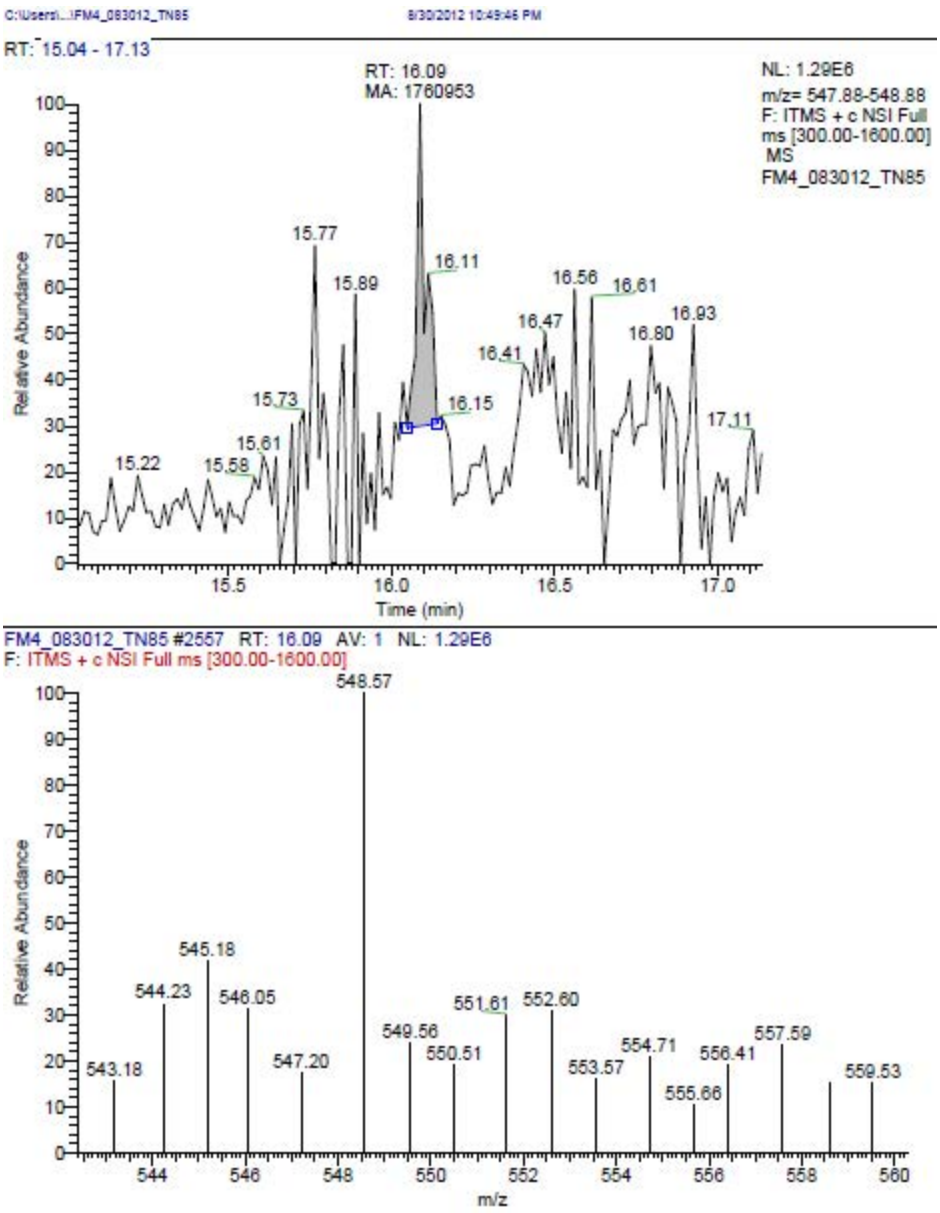


FM3

www.manaraa.com



FM4

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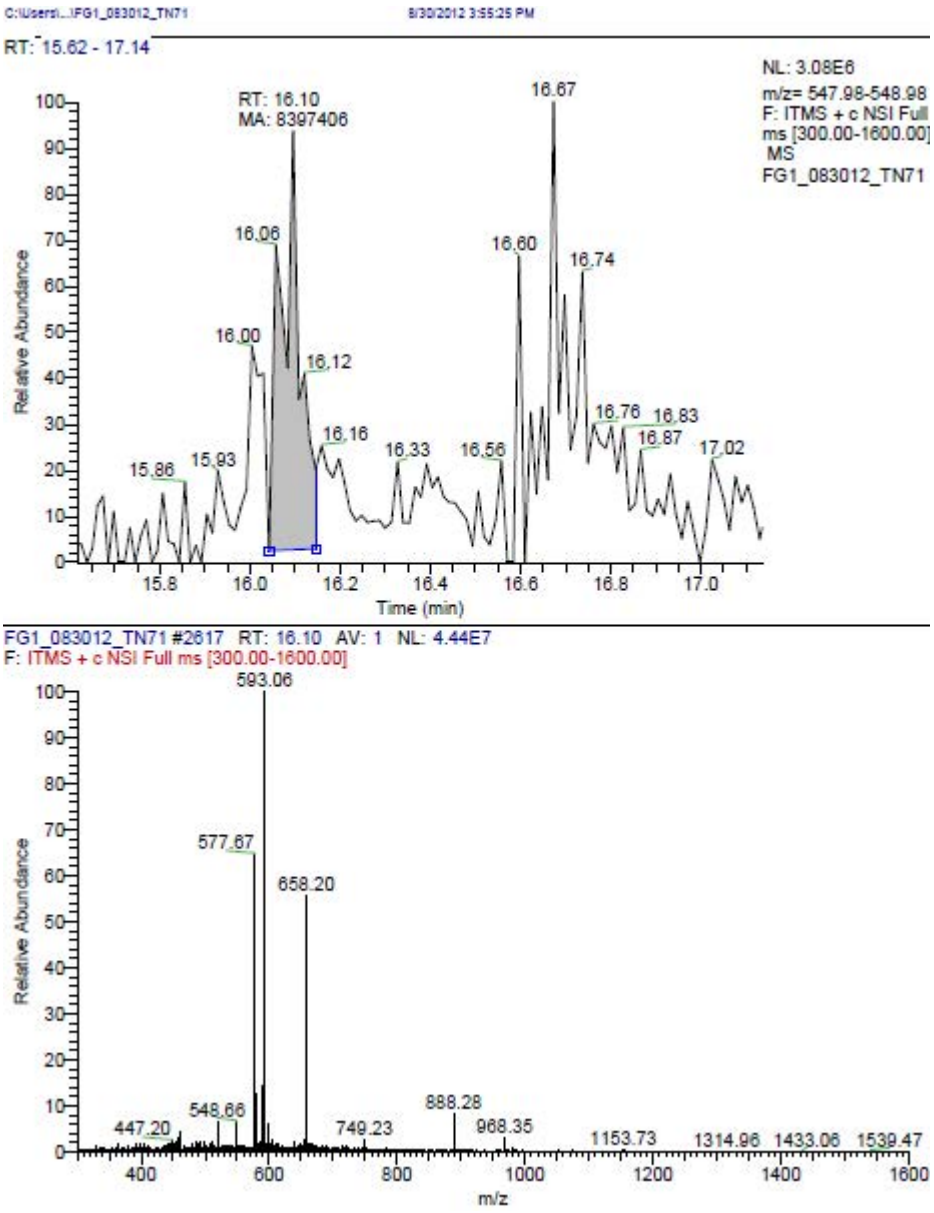

FG1

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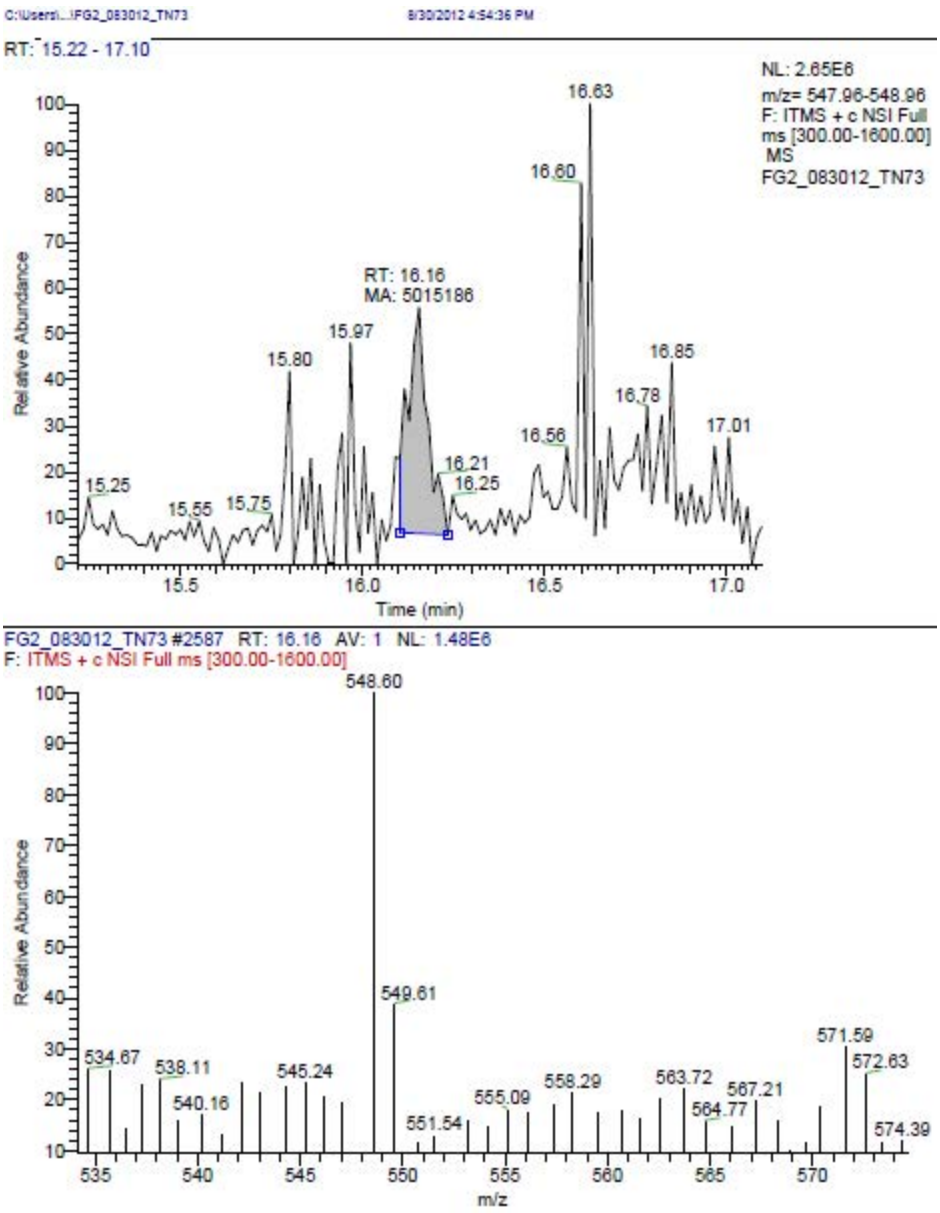

FG2

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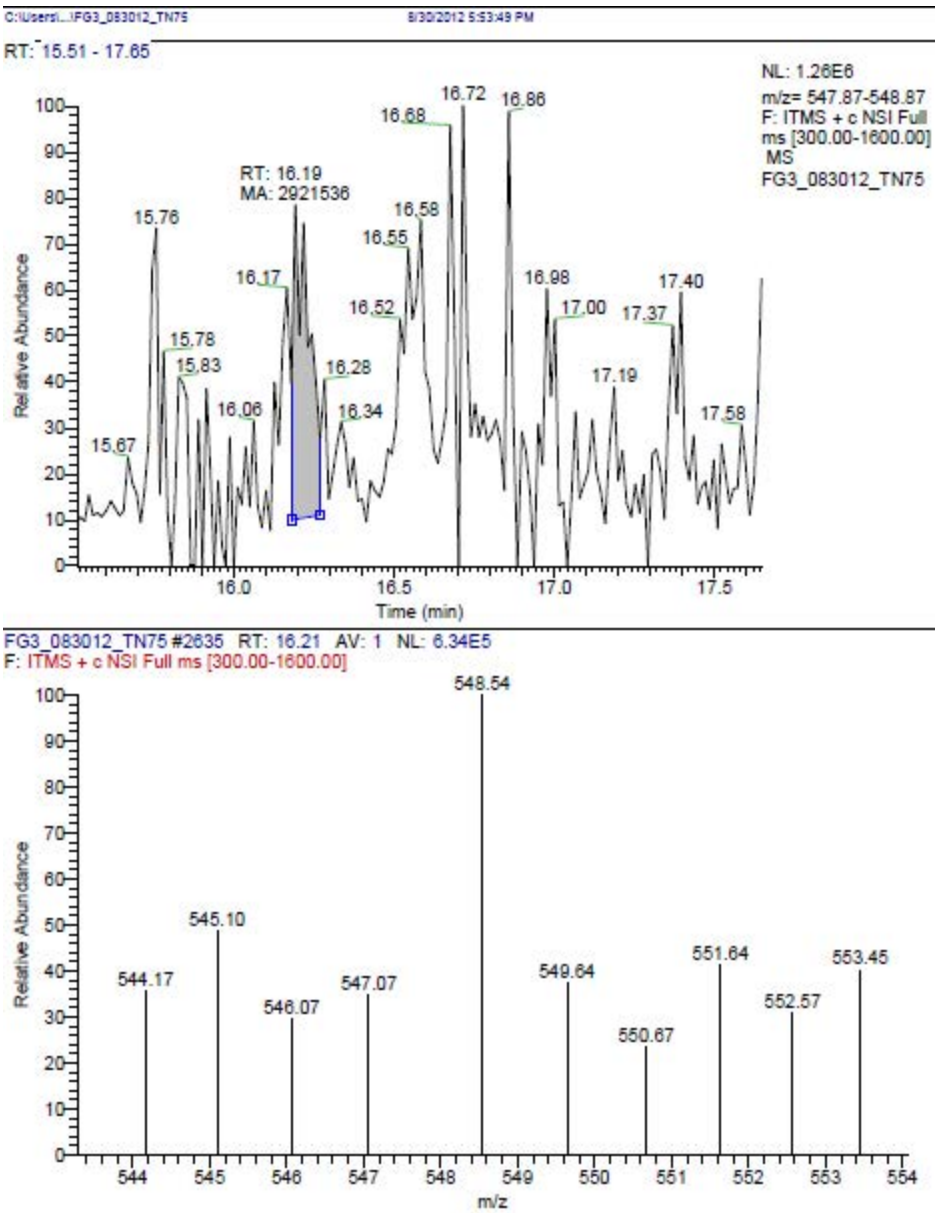

FG3

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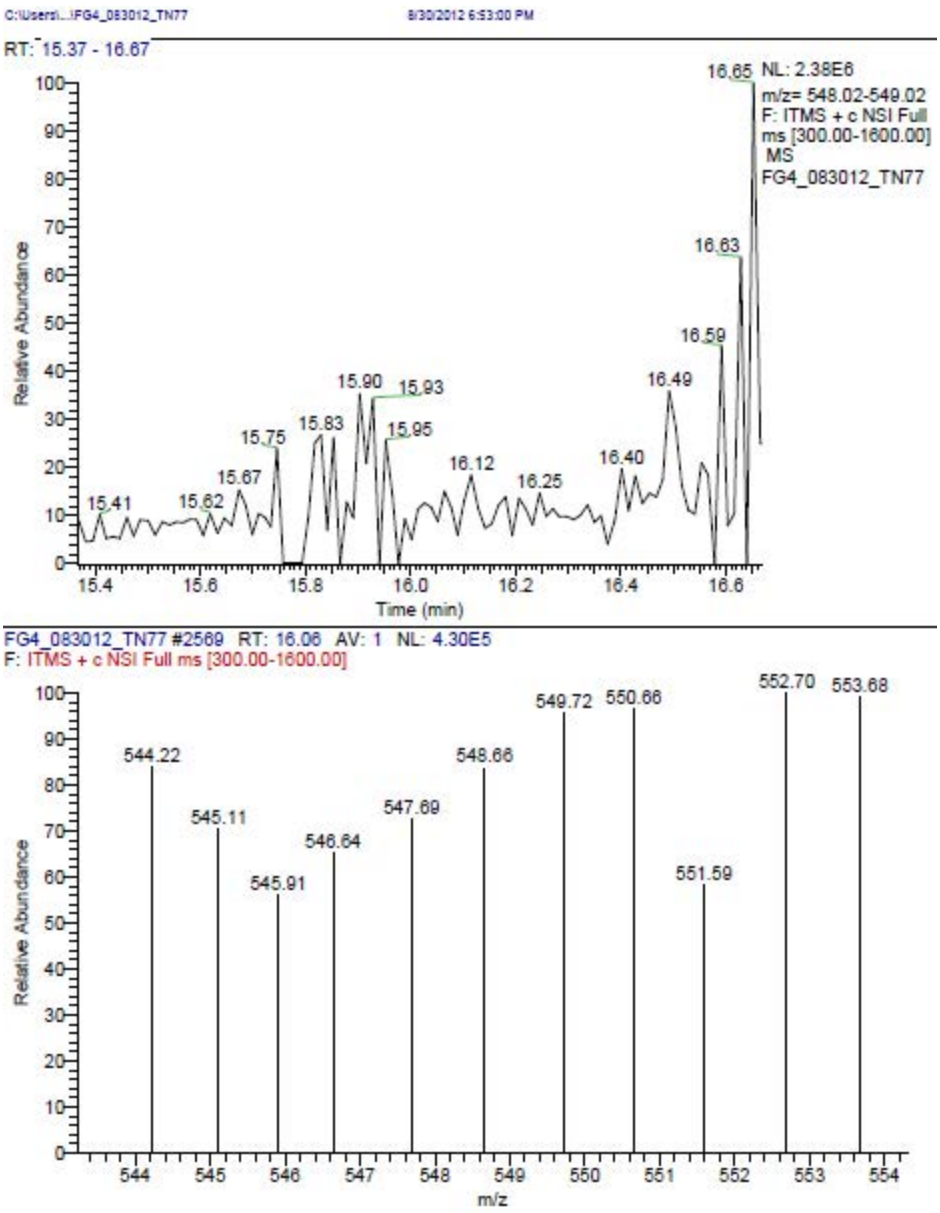

FG4

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Representative Matched Peptide Report

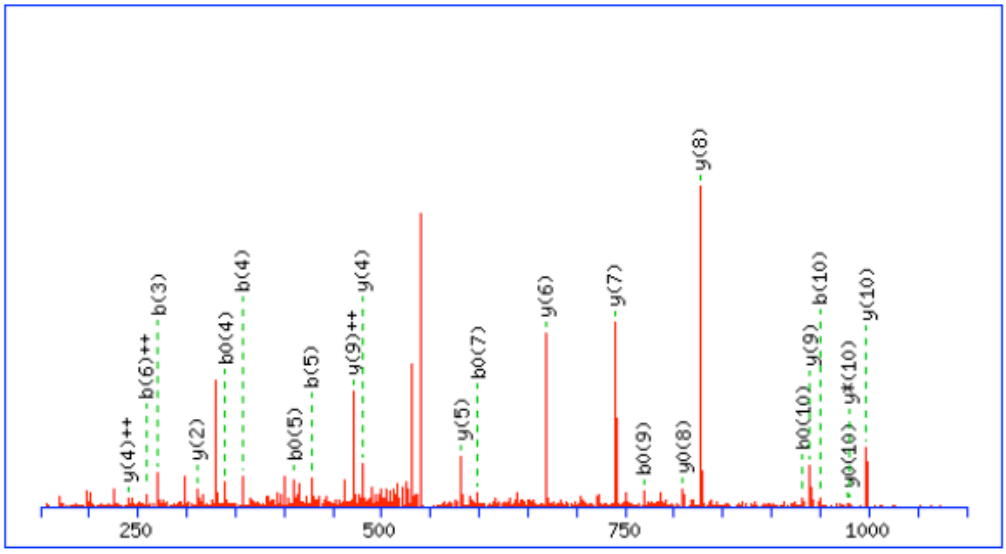
Mascot Search Results

Peptide View

MS/MS Fragmentation of **VGLSASTGLYK**
Found in **gi|72333|pir||CVJB**, concanavalin A - jack bean

Match to Query 6692 (548.60,2+) Cmpd 2672, +MSn(548.6), 16.30 min
From data file C:\Xcalibur\data\Randy\Pohl\083012_rb\mgf\FM1_083012_TN79.raw.mgf

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from 150 to 1100 Da



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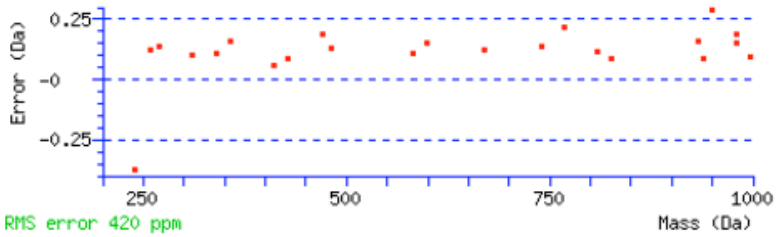
Monoisotopic mass of neutral peptide (Mr): 1094.60
Fixed modifications: Carbamidomethyl (C)
Ions Score: 45 **Matches (Bold Red):** 23/86 fragment ions using 52 most intense peaks

#	b	b^{++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y^{*}	y^{*++}	y^{0}	y^{0++}	#
1	100.08	50.54			V							11
2	157.10	79.05			G	**996.54**	498.77	**979.51**	490.26	**978.53**	489.77	10
3	**270.18**	135.59			L	**939.52**	**470.26**	922.49	461.75	921.50	461.26	9
4	**357.21**	179.11	**339.20**	170.11	S	**826.43**	413.72	809.40	405.21	**808.42**	404.71	8
5	**428.25**	214.63	**410.24**	205.62	A	**739.40**	370.20	722.37	361.69	721.39	361.20	7
6	515.28	**258.15**	497.27	249.14	S	**668.36**	334.68	651.34	326.17	650.35	325.68	6
7	616.33	308.67	**598.32**	299.66	T	**581.33**	291.17	564.30	282.66	563.32	282.16	5
8	673.35	337.18	655.34	328.17	G	**480.28**	**240.65**	463.26	232.13			4
9	786.44	393.72	**768.43**	384.72	L	423.26	212.13	406.23	203.62			3
10	**949.50**	475.25	**931.49**	466.25	Y	**310.18**	155.59	293.15	147.08			2
11					K	147.11	74.06	130.09	65.55			1



NCBI **BLAST** search of VGLSASTGLYK
(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)
Other BLAST web gateways

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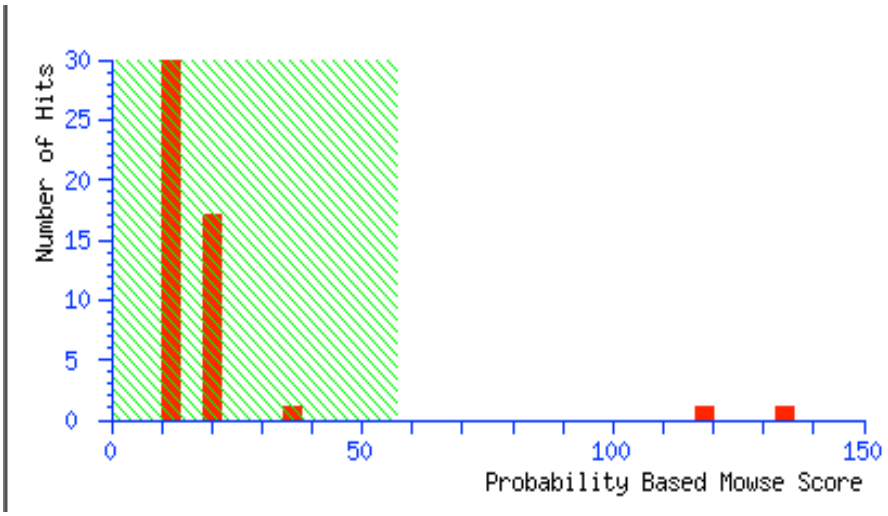

Figure D.1 MASCOT results for TAPP-HMan MALDI-QTOF experiments. Score for 95% confidence cutoff is 57. Matched proteins at 116 and 134 correspond to two different concanavalin A entries in the database. The score at 134 matched fragment 'R.VSSNGSP**Q**GSSVGR.A' while this same short sequence in the match 116 had 'R.VSSNGSP**E**GSSVGR.A' so this fragment didn't match, lowering the score.

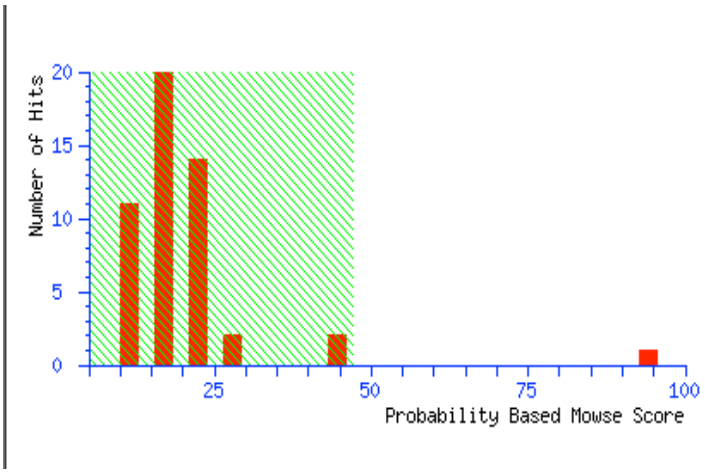

Figure D.2 MASCOT results for TAPP-HGal MALDI-QTOF experiments. Score for 95% confidence cutoff is 47. Score for matched protein is 94 and belongs to concanavalin A.

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