

I. Preparation of β -amino sulfoxides as potential selective connexin inhibitors

II. Synthesis of Isoxylitone analogues for the treatment of epilepsy

Amanda Saikaley
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Candidate

Supervisor

Amanda Saikaley

Dr. Tony Durst

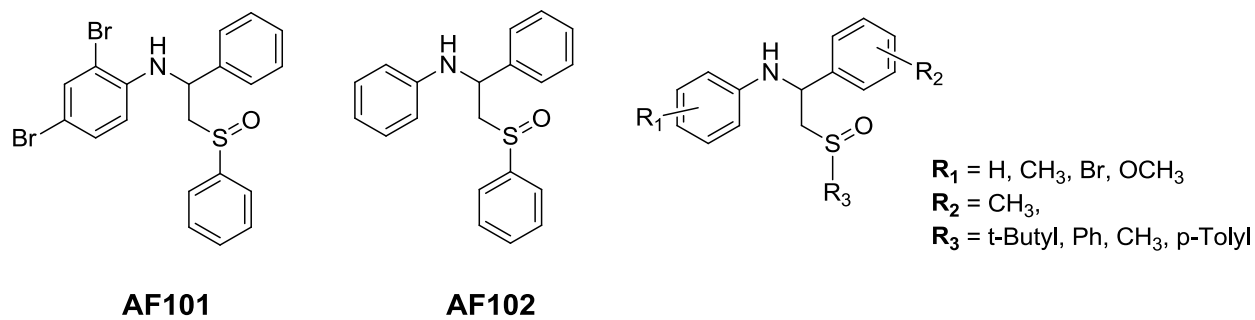
Dedication

To my Family

Abstract

Part I. Preparation of β -amino sulfoxides as potential selective connexin inhibitors

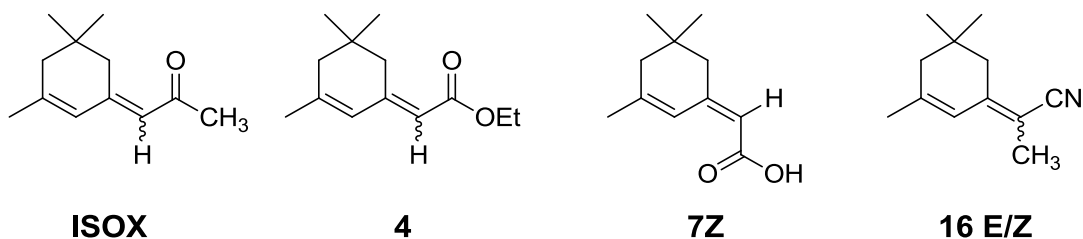
Intercellular communication is the basis for the development and homeostasis of tissues and multicellular organisms, influencing functions such as regulation of growth, cell differentiation, and developmental signalling. The connexins are a family of integral membrane proteins that oligomerize into clusters of intercellular channels that connect the cytoplasm of neighbouring cells, allowing gap junction intercellular communication (GJIC). In particular, hemi-channels remain closed to avoid dissolution of ionic gradients and leakage of cytoplasmic constituents, whereas conditions of anoxia provoke the opening of these hemi-channels, often leading to cell death. Thus far, a compound code named **AF101** possesses the greatest amount of selectivity and inhibition of GJIC. A series of nearly twenty β -amino sulfoxides using **AF102** as the lead structure were prepared by introducing various new substituents to the three aromatic rings on the molecule and by changing the aryl substituents at the sulfoxide moiety. In each example, the diastereomers were separated and the relative configuration at carbon and sulfur was assigned via ^1H NMR. The amino sulfoxides were subsequently transformed into amino sulfones and amino sulfides via oxidation and reduction reactions, respectively. We are awaiting the results of the bioassays of all of these compounds. The results will determine if any of them show selective altering of connexin activity.



Part II. Synthesis of Isoxylitone analogues for the treatment of epilepsy.

Epilepsy is a chronic disorder of the brain that affects roughly 50 million people worldwide. Extracts of a variety of plants have been used for many years to treat a number of neurological disorders. *Delphinium denudatum* Wall has been used to treat seizures by local folk medicine practitioners and a bioassay guided fractionation identified the active component as isoxylitone, also referred to as ISOX. Thirty five compound analogues were prepared starting from readily available isophorone [3,5,5, trimethylcyclohex – 2- ene -1one] and other cyclohexenones. Compound **16** possesses the highest activity by inhibiting epileptic seizures in rats by 65% at a concentration of 200 nM. This compound has been further investigated and widely studied in a variety of other in vitro and in vivo models. The *E/Z* mixture of the ethyl ester **4** also showed promising activity. The *Z* isomer of **4** and its methyl ester analogue **8**, were prepared by reacting the potassium salt of the pure *Z* acid **7Z** with Et₃I and CH₃I respectively. These *Z* isomers were less potent than the mixture, indicating that most of the activity resides in the *E* isomer. Recrystallization of **16** from hexanes afforded the *E* isomer in 92% purity. Surprisingly, this isomer was less active than the *E/Z* mixture of **16**.

Esterification of **7Z** in acidic MeOH or EtOH afforded the same *E/Z* mixture of esters obtained in the initial synthesis. This indicates that a rapid isomerization under acidic conditions. In contrast, **16E** remained unchanged upon heating in MeOH containing a catalytic amount of HCl or PTSA over a period of 8 hours.



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Abbreviations

°C	Degrees Celsius
¹³ C NMR	Carbon 13 NMR
¹ H NMR	Proton NMR
ACN	Acetonitrile
AD	After discharge
AED	Anti Epileptic Drug
BDNF	Brain Derived Neurotropic Factor
CBZ	Carbamazepine
CDCl ₃	Deuterated Chloroform
CNS	Central Nervous System
Conc.	Concentrated
Cx	Connexin
DCM	Dichloromethane
dd	Doublet of doublets
DMSO	Dimethyl sulfoxide
dt	Doublet of triplets
Equiv.	Equivalents
g	Gram
GABA	γ-Aminobutyric acid
GJIC	Gap Junction Intercellular Communication
h	Hour
HRMS	High Resolution Mass Spectroscopy
HWE	Horner Wadworth Emons
Hz	Hertz
ISOX	Isoxylitone
J	Coupling Constant
LDA	Lithium Diisopropylamine
LogP	Octanol/Water partition coefficient
LY	Lucifer Yellow
m	Multiplet
M	Molar
mCPBA	meta- Chloroperoxybenzoic acid
MEST	Maximal electric shock test
mins	Minutes
mL	Millilitres
mmol	Millimoles
MW	Molecular Weight
NBS	N-Bromosuccinamide
nBuLi	nButyl Lithium
NCS	N-Chlorosuccinamide
NMR	Nuclear Magnetic Resonance
Panx	Pannexin
Ph	Phenyl
ppm	Parts per million

PTSA / TsOH

q

R_f

rt

RT- PCR

s

scPTZ

THF

TLC

VGSC

Para-Tolylsulfonic acid

Quartet

Retention Factor

Room Temperature

Real-time polimerase chain reaction

Singlet

Subcutaneous penylenetrazole

Tetra Hydrofuran

Thin Layer Chromatography

Voltage Gated Sodium Channels

**PART I. Preparation of β -amino sulfoxides as potential selective
connexin inhibitors**

1.1 Introduction

1.1.1. Intercellular Communication

Intercellular communication is part of a complex system of communication that governs basic cellular activities and coordinates cell actions. It is an essential process used for the basis of development and homeostasis of tissues and multicellular organisms. Such communication influences important functions including regulation of growth, cell differentiation, survival and apoptosis as well as developmental signalling within cells¹.

The connexins are a family of integral membrane proteins that oligomerize into clusters of intercellular channels that connect the cytoplasm of neighbouring cells, allowing intercellular communication. These channels are also known as gap junctions. Communication between cells is mediated by the intercellular transfer of various ions and molecules with a mass up to 1000 Da, such as nutrients, secondary messengers (eg. inositol -1,4,5-triphosphate), metabolites, cations, anions, water and electrical impulses².

1.1.2. Connexins

The connexin subunit is a four transmembrane spanning protein, bearing two extracellular loops, one cytoplasmic loop and one cytoplasmic N- as well as C-terminal region. Connexins range in size from 26 to 62 kDa and are named using the prefix "Cx" followed by its predicted molecular weight. Six connexin subunits can assemble to form a hemi-channel, also known as a connexon, in the plasma membrane that can dock to another hemi-channel in the plasma membrane of an adjacent cell. The docking of two connexons forms an intercellular gap junction channel that enables adjacent cells to exchange ions and small metabolites².

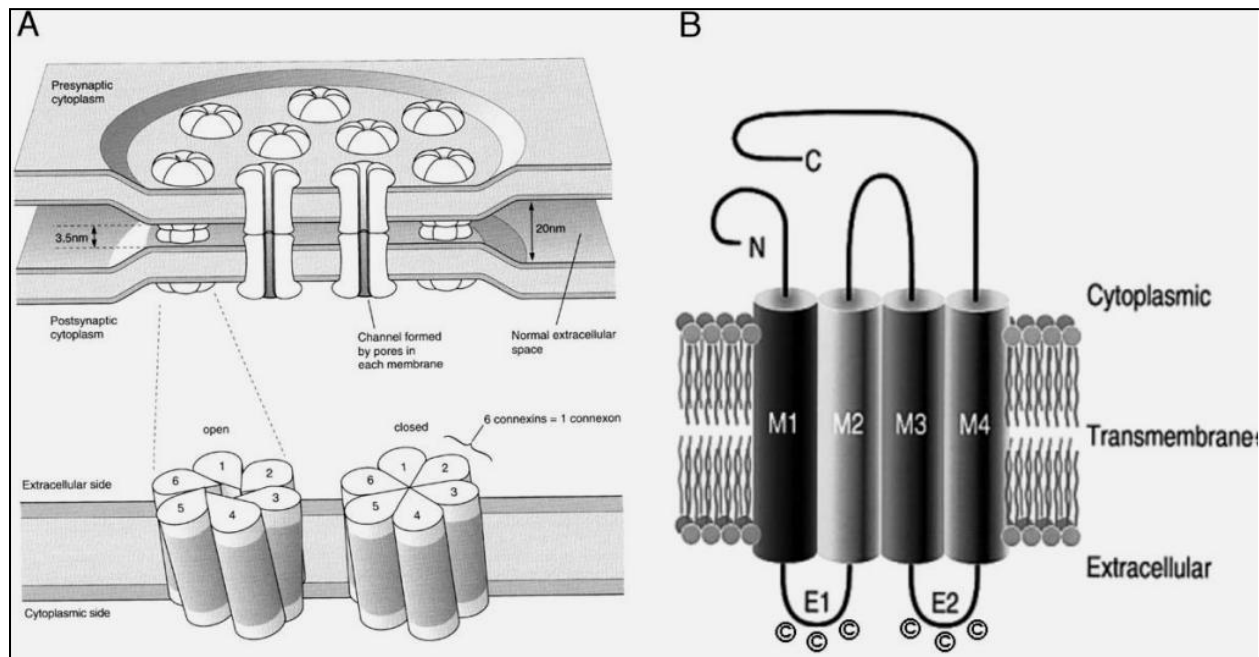


Figure 1.1.2.1.: A. Schematic representation of the gap junction channels between adjacent cells. Each opposed cell contributes one hemi channel to complete the gap junction. The six connexins forming a hemi-channel are able to coordinately change configuration to open and close the hemi- channel. **B.** Topological model of a connexin. Cylinders represent transmembrane domains. Loops indicated by E1 and E2 are predicted to be extracellular, each comprising three conserved cysteine residues (indicated by the letter C)².

Connexins are present in many different cells in the body and each have different physiological characteristics where the properties of the resulting hemi channels are determined by the combination of connexins that form them. Connexons formed by a singly type of connexin are referred to as homomeric, while gap junctions formed by identical homomeric connexons are referred to as homotypic. Alternatively, when two homomeric connexons composed of different connexins dock, they form a heterotypic gap junction. If the hemi channels do not dock to one another, they remain as hemi-channels and have similar transporting properties as ones observed for gap junction channels.

Gap junctions and hemi channels composed of different connexins have different physiological properties where tissue and cell type specific connexin expression determine which

cell types are capable of functional intercellular communication. These channels play diverse roles in the tissues they are present in, including the brain, liver and kidneys, just to name a few. There are 21 genes in the human genome that code for different connexins³. At least 12 different connexins including Cx 26, 29, 30, 31.9, 32, 36, 37, 40, 43, 45, 47 and 57 are expressed in the central nervous system (CNS), each having a unique expression pattern during differentiation.

1.1.3. Pannexins

Pannexins (Panxs), a family of connexin-like proteins have also been identified. In humans, 3 different pannexins are expressed, Panx1, Panx2 and Panx3. Of the pannexin family, Panx1 and Panx2 are expressed in the CNS. Similar to connexin hemi channels, pannexin channels are large, single membrane pores that are permeable to ions, secondary messengers and fluorescent dyes weighing up to approximately 1.5 kDa. Although it has recognized that pannexins and connexins evolved independently to each other, their basic topology has striking similarities⁴.

1.1.4. Connexins and Neurological disease

Connexins and pannexins play many important roles in the CNS and the peripheral nervous system (PNS). Gap junction intercellular communication (GJIC) maintains tissue homeostasis and enables coordination of cellular activities. For example: in a healthy CNS, connexins play an important roles in electrochemical and metabolic coupling between cells. More specifically, hemi-channels remain closed to avoid dissolution of ionic gradients and leakage of cytoplasmic constituents. Nevertheless, physiological changes caused by anoxia conditions and ischemia provoke the opening of these hemi channels, often leading to swelling

and cell death⁵. Connexins and pannexins are also important for regulation of postnatal neural progenitor cell proliferation and differentiation. Intercellular and single membrane channels are required to maintain myelin, the lipid rich sheath produced by oligodendrocytes in the CNS and Schwann cells in the PNS. Furthermore, mutations of Cx32 are the genetic determinants of Charcot-Marie-Tooth Disease, a peripheral demyelinating neuropathy. Genetic deletion of both oligodendroglial connexins or pairs of oligodendrocyte and astrocyte connexins results in a dramatic perinatal loss of myelinating oligodendrocytes, demyelination of the major fibre tracts, axonal damage and lethal epileptogenesis⁴.

In addition, connexins have been implicated in neuronal survival decisions following injury. It has been well documented that connexin expression is spatially and temporally regulated following stroke, spinal cord injury and epilepsy. Activation of both Panx1 and Panx2 in neurons have shown to contribute to ischemic brain damage. Excessive opening of Panx1 pores in mature oligodendrocytes, neurons, and microglia releases ATP activating P2X7 receptors that exacerbate excitotoxic injury. ATP release acts as a phagocytic "find me signal", therefore Panx1 channel activity also increases inflammatory white matter lesions in experimental autoimmune encephalomyelitis (EAE), an experimental model of MS⁴.

1.1.5. Connexins and pannexins as therapeutic targets

It is for these reasons that connexins and pannexins have been identified as important therapeutic targets to promote neural repair and regeneration following neural injury. It is currently not possible to selectively target specific gap junctions and hemichannels, nor is it possible to selectively modulate specific connexins and pannexins. The majority of the existing connexin natural toxins modulate all connexin channels regardless of composition. Not only are

they not specific, but they are toxic at dosages required to cross the blood brain barrier and often exhibit secondary effects that are predicted to intensify demyelinating conditions. Of these natural toxins, synthetic inhibitors and/or modulators, only a few are able to distinguish between connexon hemi channels and gap junction channels. The gold-standard inhibitor, carbenoxolone, non-selectively alters all connexin and pannexin functions and is too toxic for therapeutic use.

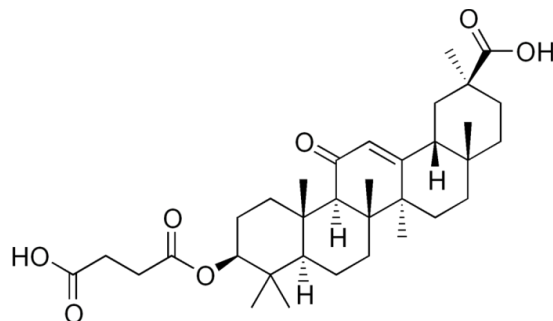


Figure 1.1.5.1. Structure of the gold standard connexin and pannexin inhibitor, carbenoxolone.

1.1.6. Objective

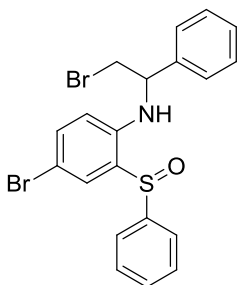
Therefore, there is a need for therapeutic compounds that can modulate connexin and pannexin channel function and/or expression in the nervous system with some specificity. Pharmacological modulators and inhibitors that target the right connexin or pannexin and promote the right type of communication (GJIC or single membrane channel activity) are urgently needed for use in treating neural injury, reducing neural damage and promoting neural protection and regeneration after injury.

Some time ago, the Bennett group selected compound at random from the Durst library code named **187**. It had been assigned the structure shown below. This compound was shown to decrease GJIC activity in a bioassay. From this, it was concluded that the compound had inhibitory effects on specific connexins. This finding was intriguing due to the fact that the

majority of other toxins against connexins are non-selective, in which case they inhibit all connexins to the same degree.

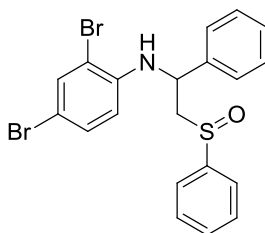
Subsequently, Dr. Ana Francis Carballo-Arce resynthesized this compound as part of her doctoral work and attempted to prepare analogs based on the **187** structure. None of the obvious derivation attempts were successful, casting doubt on the original structure assignment. An x-ray structure determination confirmed that suspicion. The corrected structure was assigned the code **AF101**. Dr. Carballo-Arce synthesized a number of analogs of **AF101** including **AF102**, which is considered the parent compound in this series. Several of these derivatives showed some selectivity in the three connexin assays carried out by the Bennett group.

The objective of the work presented in this part of this thesis was to expand the compound library by preparing additional analogues of **AF102** by changing the substituent's on the three aromatic groups present in **AF102** or replacing the aryl group joined to the sulfoxide function by non aromatic groups. It was hoped that one or more of these changes would result in a molecule that would target selectivity of one or more of the various connexins by either opening or closing the transport of positive or negative ions and increasing or decreasing gap junction intercellular communication (GJIC).



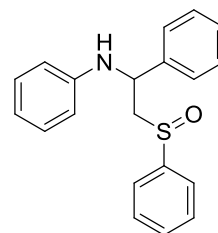
Chemical Formula: $C_{20}H_{17}Br_2NOS$
Molecular Weight: 479.23

187



Chemical Formula: $C_{20}H_{17}Br_2NOS$
Molecular Weight: 479.23

AF101



Chemical Formula: $C_{20}H_{19}NOS$
Molecular Weight: 321.44

AF102

1.2. Discussion and Results

1.2.1. Introduction

In 2011, a member of Dr. Steffany Bennett's research group, in the Faculty of Medicine, University of Ottawa, requested compounds from the Durst group compound library for testing in a new bioassay that targets inhibition of connexins in the CNS. Amongst the chosen compounds, a compound code-named **187** displayed intriguing activity in the assay.

Activity is determined by the permeability of fluorescent tracers throughout gap junctions to determine intercellular communication. In **Figure 1.2.1.3**, panel A shows that compound **187** decreases gap junction activity and this can be analyzed through the amount of calcein that is present in the image in comparison to the vehicle (DMSO). As shown, the amount of calcein passing through the gap junction is greater with the vehicle compared to the sample containing **187**.

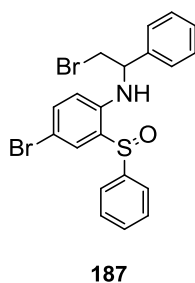


Figure 1.2.1.1 Chemical structure drawn on sample vial **187**.

Calcein is a fluorescent "tracer" that is membrane impermeable but has the ability to pass through gap junctions. Fluorescent dyes are commonly used to assess which molecules or ions can pass through gap junctions. In this bioassay, the amount of calcein dye that transfers to

neighbouring cells is quantified and is used as a measurement of gap junction function. The Bennett group concluded that **187** had an inhibitory effect on connexins.

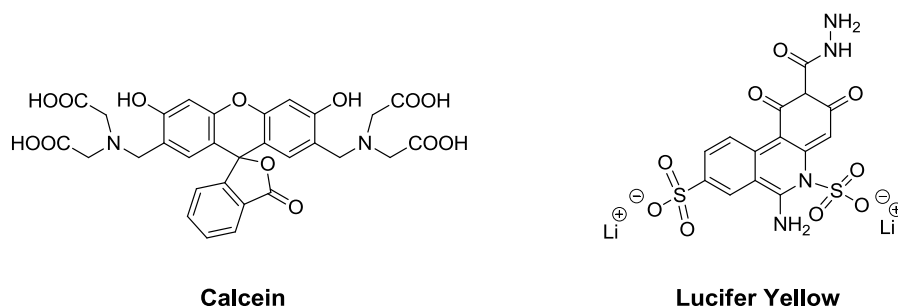
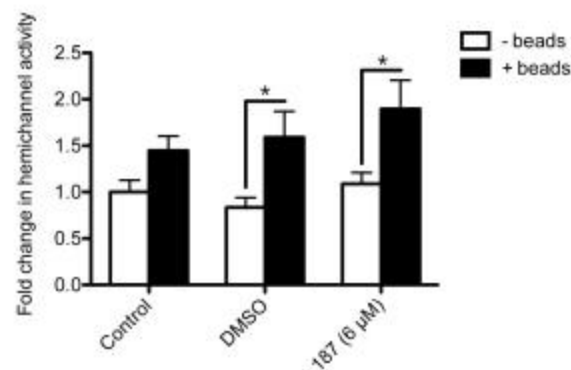
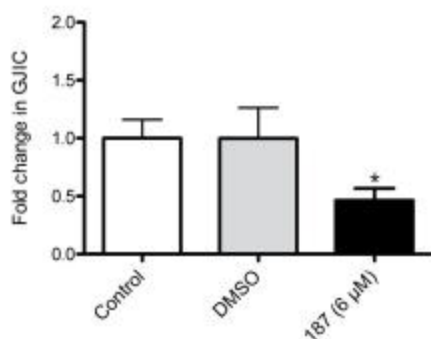


Figure 1.2.1.2. Chemical structures of fluorescent dyes Calcein and Lucifer Yellow.

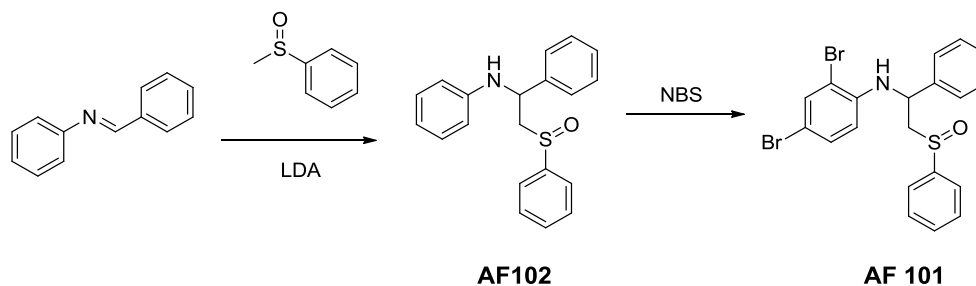
Panel B in **Figure 1.2.1.3.** evaluates the connexin hemi-channel as a single membrane channel. Hemi-channels are normally closed to promote homeostasis, but can open as a consequence of an injury, providing an open channel from the cell to the extra cellular space. In this particular assay, cells are incubated in a solution with Lucifer yellow (LY), which is a fluorescent compound that is able to pass through connexin channels. The cells are then washed and viewed under a microscope to observe how many cells took up the LY from the extracellular space through the hemi channels. Cells with a higher intake of LY, have greater hemi-channel activity. As a part of this assay, glass beads are used to provoke the mechanism opening stimulation of the hemi-channels and carbenoxolone is used as a positive control. The results observed with compound **187** show that this compound does not affect the opening of the hemi channels but instead displays solely GJIC activity. These data show that compound **187** reduces connexin mediated intercellular channel function but not single membrane hemi-channels. Data sets and a detailed outline of the bioassays are described in **Appendix 1A**.



As mentioned earlier, the majority of compounds that are used to inhibit connexin function are non specific, and act on all connexins to the same degree, which makes them unsuitable for therapeutic use. The indication that **187** acts solely on one channel was exciting and led to a decision to synthesize analogues that might show both greater selectivity and higher potency. The initial plan was to re-synthesize **187** and from it, prepare analogues. None of the proposed derivations, such as displacing the supposed aliphatic bromine with thiophenolate

anion was successful. The inability to replace a bromine from a primary sp^3 carbon casted doubt on the correctness of the structure drawn on the sample vial. The correct structure of **187** was determined by x-ray crystallography, and the compound was renamed **AF101**. Once the correct structure was known, the mechanism of its formation and its NMR properties were easily understood.

Compound **AF101** is prepared by reaction of the lithio derivative of methyl phenyl sulfoxide with benzalaniline, derived from aniline condensed with benzaldehyde. This sequence results in the formation of the core structure, **AF102**. This compound when exposed to two equivalents of NBS undergoes two consecutive electrophilic aromatic substitution reactions on the aromatic ring activated by the amine function to yield **AF101**⁵. These compounds can be described as β -amino sulfoxides; often times in this thesis, they will be referred to simply as amino sulfoxides.



Scheme 1.2.1.1. Preparation of **AF 101**.

Dr. Ana Francis Carballo Arce, a Ph.D. graduate from Dr. Durst's research group began working on this project. She synthesized analogues of compound **AF101**, focusing mainly on the introduction of various substituents in the aniline ring including chloro groups and methoxy groups; one example in which the benzene ring attached to the sulfoxide group was replaced by a methyl group was also prepared. The compounds synthesized by Dr. Ana Francis Carballo Arce are shown in **Figure 1.2.4**.

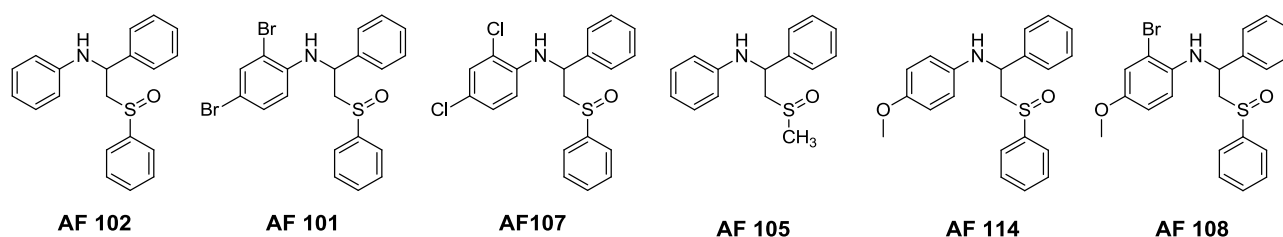


Figure 1.2.1.4. AF102 and analogues synthesized by Dr. Ana Francis Carballo Arce.

These compounds were subjected to the bioassays described above. Compound **AF102** in which the bromine atoms were missing from the aniline ring, showed greatly reduced GJIC activity, but it also showed activity in the hemi-channel transport bioassay. Surprisingly, the replacement of the bromine by chlorine atoms gave **AF107**, which showed only minor non-statistically significant changes. **AF108** reduced anionic hemi channel activity in the assay, while **AF102** reduced both anionic and cationic channel activity. Replacement of the phenyl sulfoxide moiety by a methyl sulfoxide unit gave a product that showed no activity in any of the bioassays. Amongst all of these newly synthesized compounds, **AF101** continued to demonstrated the highest inhibition of GJIC⁵. Results are shown in **Figure 1.2.1.5**.

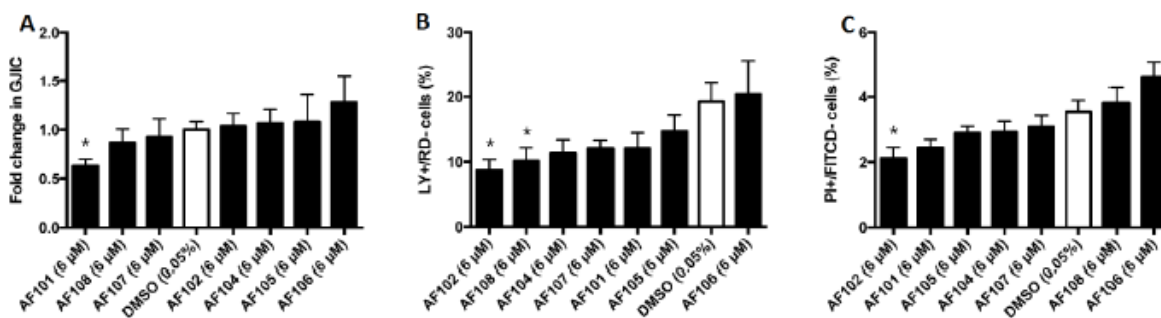
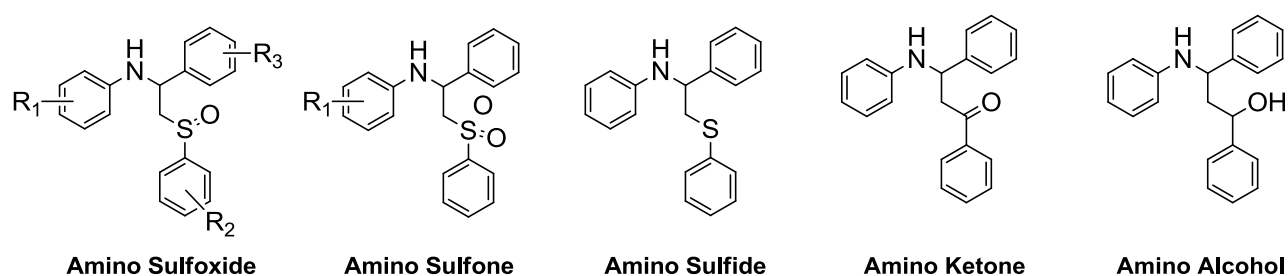


Figure 1.2.1.5. Summary of bioassay results. **A.** Change in GJIC. **B.** Anionic activity in hemi-channels. **C.** Cationic activity in hemi-channels. Compounds with an asterisk are indicative of statistically significant results. This figure was taken from Dr. Ana Francis Carballo Arce's Ph.D thesis⁵.

Compounds that specifically affect different connexins are potentially very valuable. Preliminary results demonstrated that some of the new compounds show this desirable behaviour to some extent. Although results were encouraging, too few compounds were available to draw conclusions concerning which structural features were necessary and important for activity. The objective of the work presented here was to expand the available compound library by preparing additional analogues of **AF102**. Additionally, except for structures of **AF101**, **102** and **108**, the relative and absolute configuration of the compounds prepared by Dr. Carballo Arce were not unambiguously known. Indeed in the case of the methyl sulfoxide **AF105**, a 3:1 mixture of diastereomers were sent for bioassays. Thus, it also became important to not only prepare additional analogues, but to also separate diastereomers and assure their relative configuration. The author was closely involved and supervised an undergraduate thesis in which the preparation of the individual enantiomers in one series of amino sulfoxides and the related amino sulfones and amino sulfides is described. The availability of pure diastereomers, and in one case, pure enantiomers, should facilitate the development of a Structure – Activity – Relationship (SAR).

Dr. Ana Francis Carballo Arce used primarily recrystallization as a purification method. This was sometimes only partially successful in preparing diastereomerically pure samples. Also, since it depended on solubility differences between diastereomers, it was not always assured that the same diastereomer would crystallize preferentially for this series of compounds. Although it was much more time consuming and difficult due to the minimal polarity difference of the isomers, the work presented here describes purification via column chromatography, which resulted in the complete separation of diastereoisomers in almost all sets of amino sulfoxides that were prepared in this thesis.

The parent β -amino structure (**AF102**) for this series of compounds has three main aromatic rings that can all be altered either by adding substituents at various positions in any one of the three phenyl rings or replacing the phenyl ring by another aromatic ring or by an aliphatic substituent. One can also suggest replacing the β -amino sulfoxide unit by a β -amino ketone or β -amino alcohol unit. Finally, the β -amino sulfoxide can be oxidized to the corresponding β -amino sulfone or reduced to the β -amino sulfide. To a considerable extent, examples of all the above variations are described in this thesis.



1.2.2. Synthesis and Structure Identification

1.2.2.1. Synthesis of β -Amino Ketone Analogues

One can readily imagine that due to intramolecular hydrogen bonding, the β -amino sulfoxide **AF102** and the β -amino ketone **2** should have very similar conformations in solution. Therefore, if shape is the key in determining connexin activity then the amino ketone **2** should show activity similar to that of **AF102**. Interestingly, the calculated LogP value [ChemDraw] is higher for the amino ketone than the corresponding amino sulfoxide.

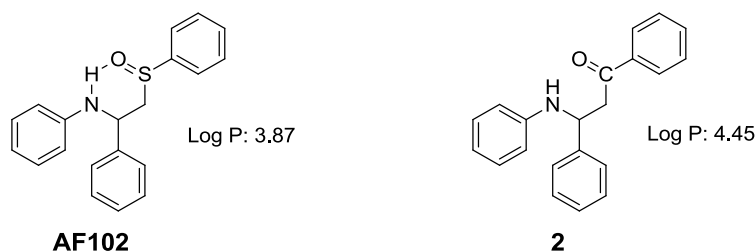
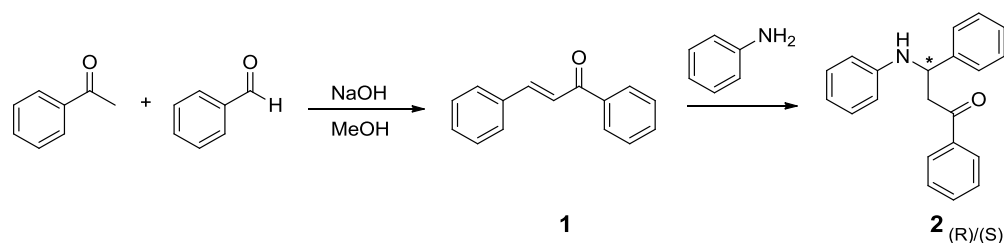


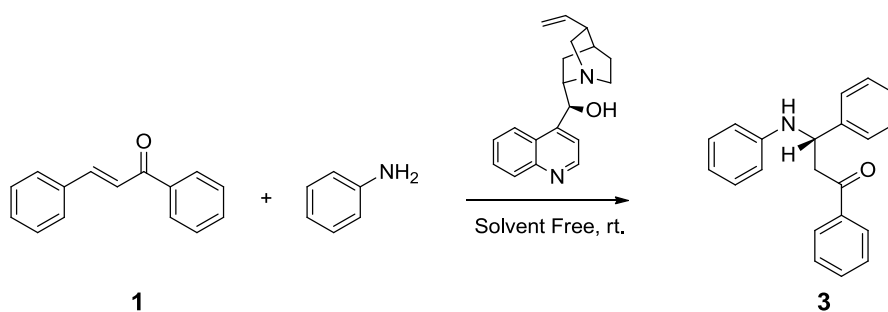
Figure 1.2.2.1. Comparison of β amino sulfoxide, **AF102** and β -amino ketone, **2**.

The racemic β -amino ketone **2** was obtained as a yellow solid in 47% yield via Michael addition of aniline to chalcone **1**.



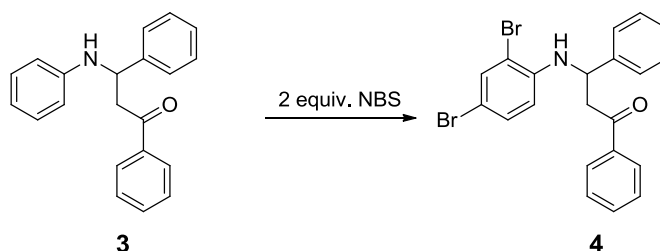
Scheme 1.2.2.1. Synthesis pathway of compound **2**.

The Michael addition of aniline to **1** has been studied in detail including enantioselective addition using a variety of chiral catalysts. The enantioselective Michael addition of aniline to chalcone **1** was promoted by an inexpensive and commercially available cinchona alkaloid under solvent free conditions. This simple experimental procedure required no work-up and a short reaction time, which facilitated the preparation of these compounds. The use of cinchonine (10 mol %) produced **3** in 99% yield with a reported 58% of enantiomeric excess favouring the *R* enantiomer⁷. This indicated a ratio of **3R** to **3S** of approximately 4:1.



Scheme 1.2.2.2. Preparation of **3** using cinchonine as a chiral catalyst.

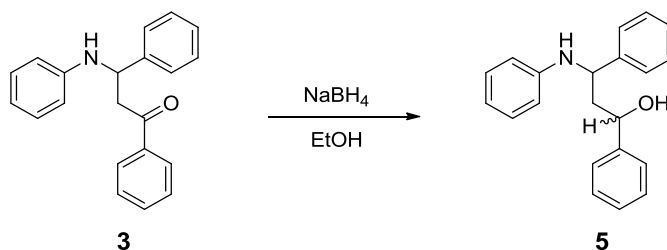
Once the racemic carbonyl mimic of **AF102** was available, it was decided to brominate as was done for **AF101**. Aromatic electrophilic substitution of **3**, using 2 equiv. of NBS led to the bromination at the ortho and para positions on the aniline ring affording **4** in 65% yield as a white solid.



Scheme 1.2.2.3. Synthesis of compound **4**.

1.2.2.2. Synthesis of β -amino alcohols

Compound **3** was reduced using NaBH_4 resulting in a diastereomeric mixture of amino alcohol analogues. The product was purified via gradient column chromatography, followed by recrystallization from MeOH affording compound **5** as a white solid in 84% yield.



Scheme 1.2.2.4. Synthesis of compound **5**.

The ^1H NMR of **5** showed the two ABX patterns clearly indicating the presence of both diastereomers. Attempts at separating the diastereomers via column chromatography were unsuccessful since the isomers had very similar polarities. Compound **5** was successfully separated into its two isomers via preparative TLC. The plate was developed three consecutive times in 1:4 EtOAc: hexanes, where the two isomers displayed minor R_f differences. The more

polar top spot, compound **6**, had an R_f value of 0.63 whereas the less polar bottom spot, compound **7**, had an R_f of 0.59. Only 0.03 g of compound **5** could be loaded onto a standard commercially available plate in order to achieve ~0.01 g of each isomer to send for bioassay testing. The ^1H NMR spectra of both isomers show clean ABX patterns in comparison to the mixture, with compound **6** displaying a cluster of doublet of doublet's (dd's) more down field compared to compound **7**. The ^1H NMR spectra of compound **6** and **7** are shown below in **Figures 1.2.2.2** and **1.2.2.3**, respectively. At this point, we have not determined the relative configuration of the two isomers. This will be done once the bioassays have been completed and it has been shown that there is a significant difference in activity.

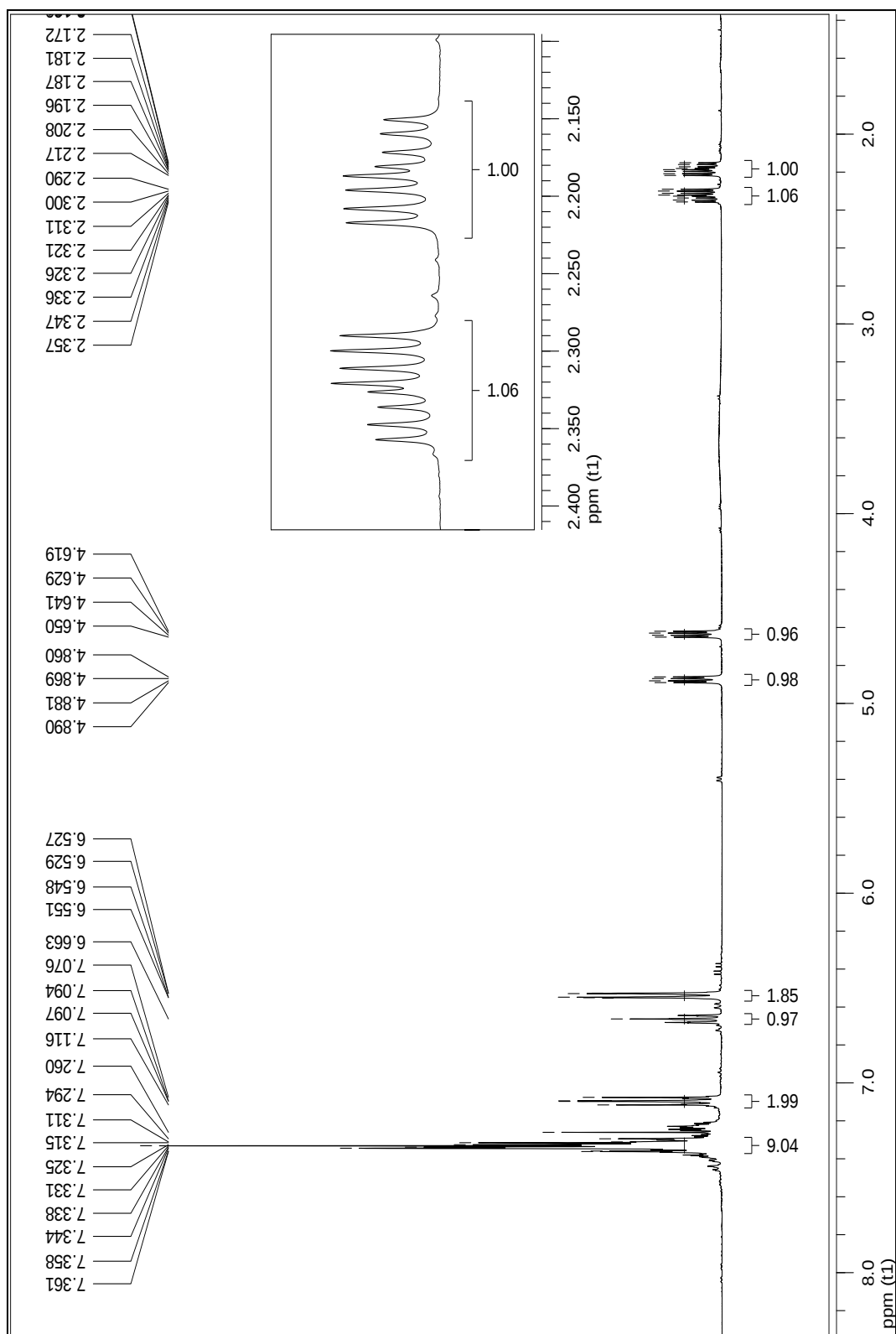


Figure 1.2.2.2. 400 MHz ^1H NMR spectrum of compound **6** in CDCl_3 .

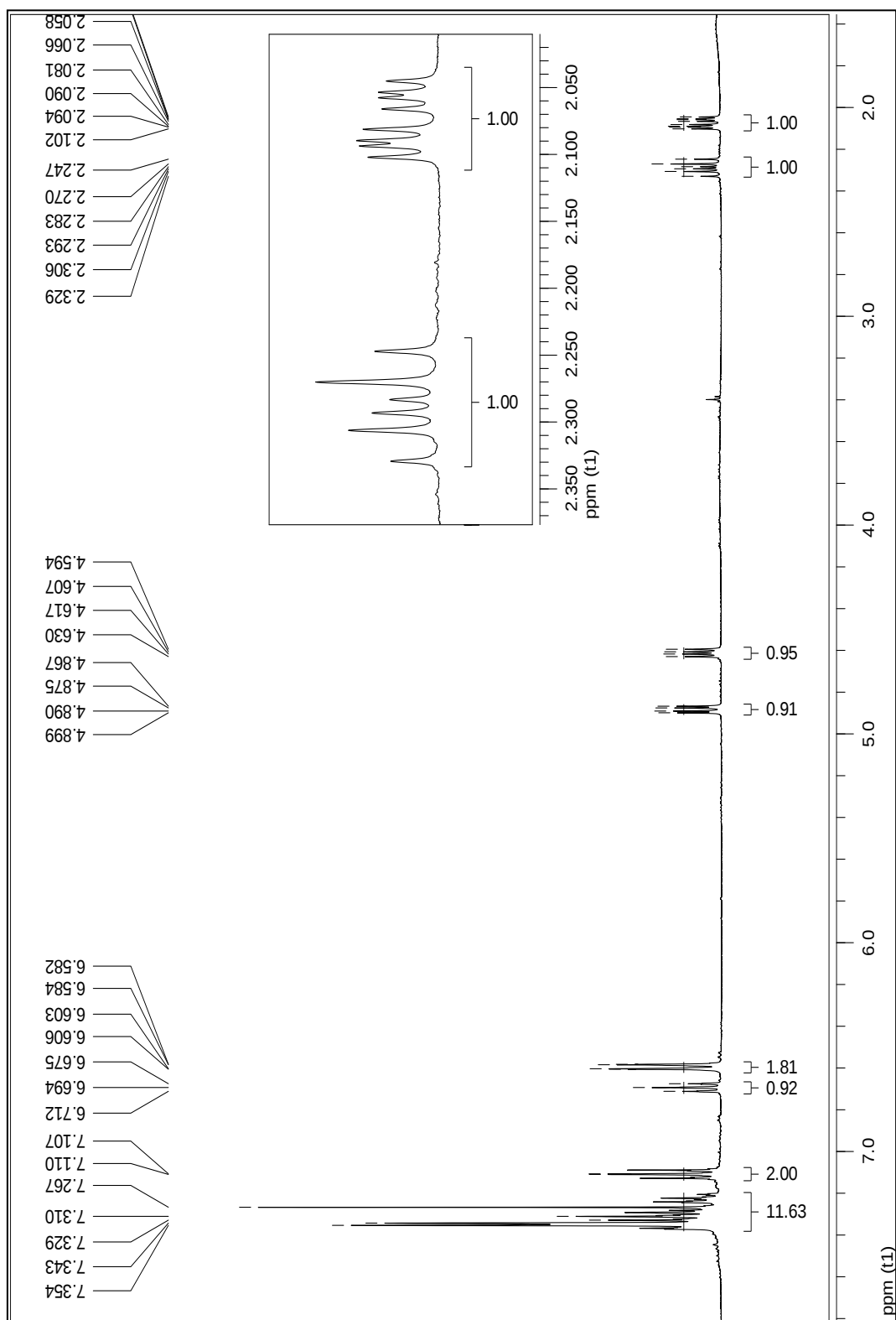


Figure 1.2.2.3. 400 MHz ^1H NMR spectrum of compound 7 in CDCl_3 .

1.2.2.3. Synthesis of β -amino sulfone analogues

It is known that sulfoxides show very strong hydrogen bonding with OH and NH groups. In contrast, hydrogen bonding involving the oxygen atoms in sulfones is very weak. Thus, it seems reasonable to suggest that the β -amino sulfoxides **AF101** and **AF102** could have rather different conformations in solution and thus might interact differently with the connexin receptors.

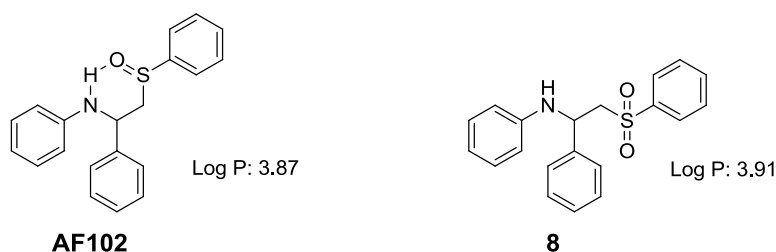
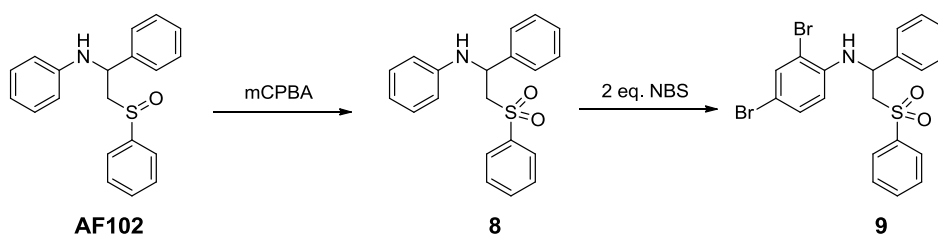


Figure 1.2.2.4. Comparison of β -amino sulfoxide, **AF102** and β -amino sulfone, **8**.

AF102 was oxidized using mCPBA to produce the corresponding β -amino sulfone derivative, **8**, as a yellow solid in 74% yield. Since **AF101** displayed excellent activity in the bioassays, it was decided to brominate the sulfone **8**. The use of 2 equiv. of NBS, resulted in a 89% yield of bromo-sulfone, **9**, as a yellow solid. In both cases, the R_f values of the sulfones on analytical silica gel plates were significantly larger than those of the corresponding sulfoxides.



Scheme 1.2.2.5. Synthesis of compounds **8** and **9**.

1.2.2.4. Preliminary screening and results

Compounds were synthesized by making minor changes to the molecule in order to optimize activity. Six compounds were sent to be tested in the three bioassays against the compounds previously synthesized by Dr. Ana Francis Carballo Arce. Replacement of the sulfoxide group for a carbonyl group, compound **3**, resulted in no activity in all three of the assays. Addition of bromine atoms at the ortho and para positions on the amino ketone, compound **4**, displayed statistically significant activity in the inhibition of anionic hemi channels as well as activity in the cationic hemi channels. Oxidation of the sulfoxide resulting in sulfone, compound **8**, showed no activity in any of the bioassays. In the GJIC assay, **AF101** still remained the best compound in terms of inhibition activity. **AF102** also displayed excellent activity in the anionic and cationic hemi channel assays. These results are shown in **Figure**

1.2.2.5.

The bromo sulfone analogue, compound **9**, showed outstanding activity in the GJIC assay, comparable to **AF101**. Surprisingly, **9** also showed an enhancement in anionic hemi channels in comparison to the gold standard carbenoxolone; however, showed no significant activity in the cationic hemi channel assay. This was an intriguing result as it is difficult to selectivity target one channel over the other. These results are shown in **Figure 1.2.2.6**.

Replacing the sulfoxide by a hydroxyl group, compound **6** (**AF115t**=top isomer) and **7** (**AF115b** = bottom isomer) were sent individually to be tested; however, they displayed no activity in the GJIC bioassay. These results are shown in **Figure 1.2.2.7**.

All other compounds tested in the assay represented by AF codes were either fractions of *Piperacae* plant material and/or betulinic acid derivatives, which have both been known to inhibit intercellular communication.

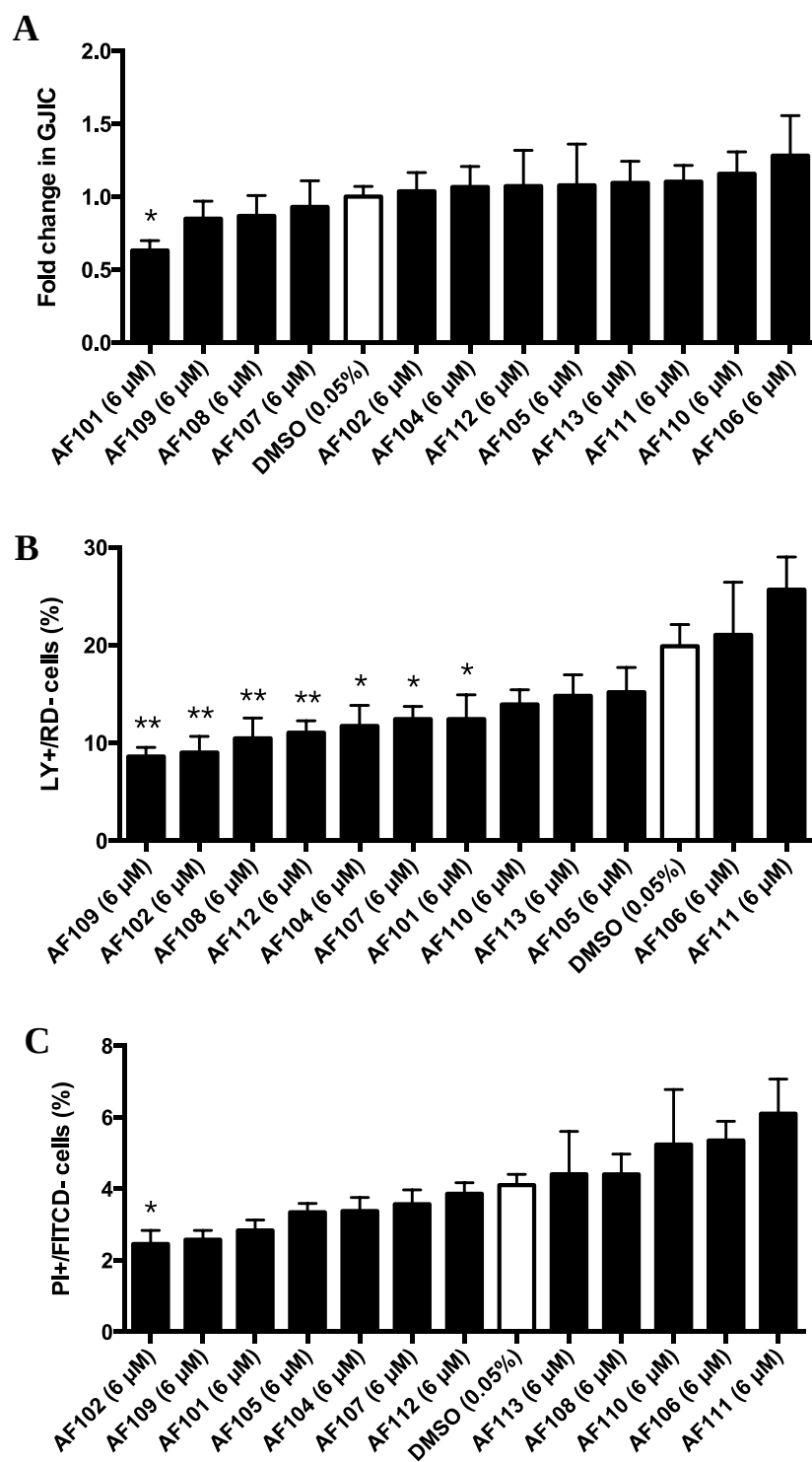


Figure 1.2.2.5. Summary of bioassay results. **A.** Change in GJIC activity. **B.** Anionic change in hemi channels. **C.** Cationic change in hemi channels. Compounds with an asterisk are indicative of statistically significant results. Compound Legend: AF109 = **4**, AF110= **3**. AF111= **8**.

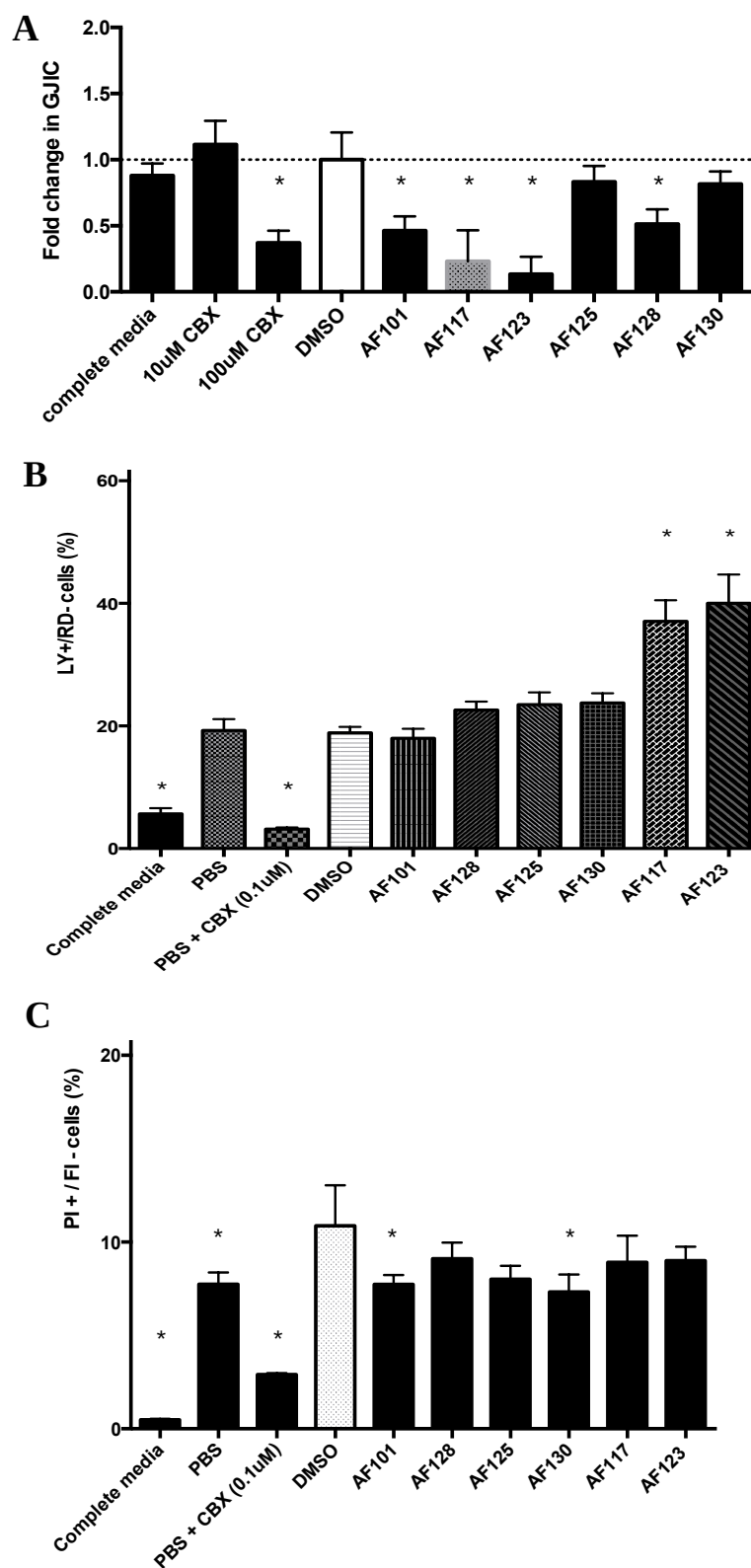


Figure 1.2.2.6. Summary of bioassay results. **A.** Change in GJIC activity. **B.** Anionic change in hemi channels. **C.** Cationic change in hemi channels. Compounds with an asterisk are indicative of statistically significant results. Compound Legend: **AF123= 9**.

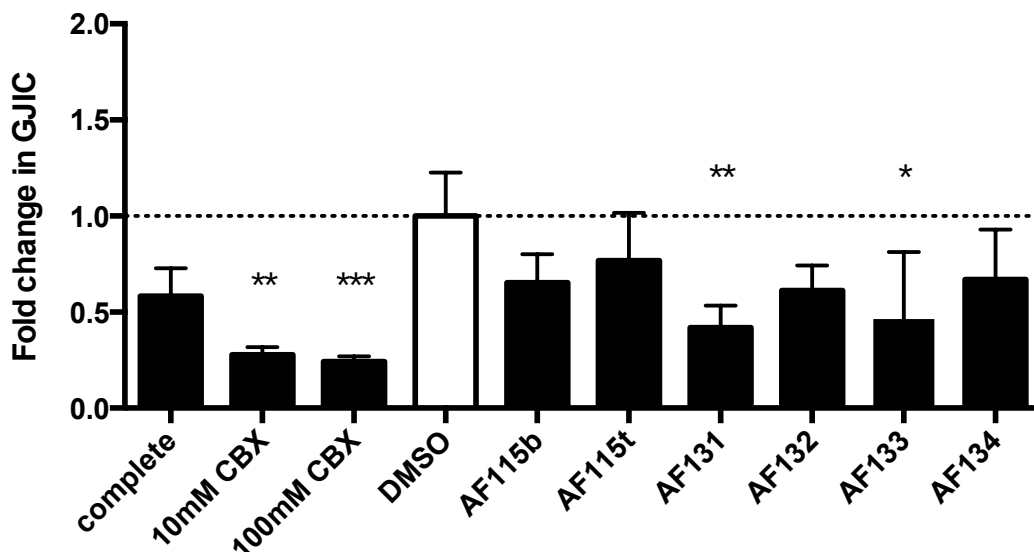


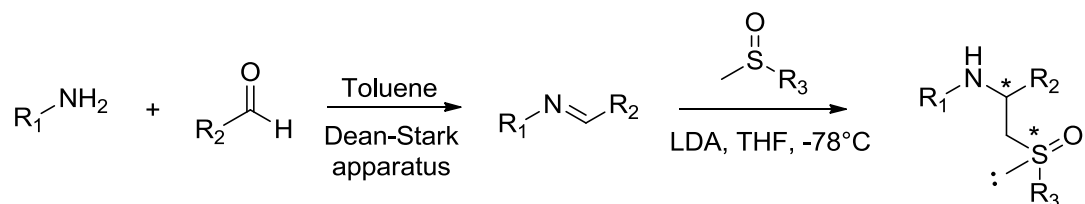
Figure 1.2.2.7. Summary of bioassay results displaying change in GJIC activity.

1.2.2.5. General approach to synthesis and structure identification of amino sulfoxide analogues

The replacement of the sulfoxide function in the amino sulfoxides by a ketone or hydroxyl group or its oxidation to a sulfone, resulted typically in compounds with lower or statistically insignificant activity. All compounds containing two bromine atoms in the aniline ring demonstrated some activity, but much lower than **AF101**. Interestingly, the bromo sulfone, **9**, was selective in the hemi channel assays. Based on these somewhat preliminary results, it was decided to refocus on the sulfoxide analogues in hopes of providing greater possibilities to enhance the desired activity and selectivity.

All sulfoxide analogues were prepared using the approach described in Dr. Carballo Arce's Ph.D thesis, **Scheme 1.2.2.6**. The requisite imines were obtained via a condensation reaction between an amine and an aldehyde in refluxing toluene using a Dean-Stark apparatus. Following this, the desired methyl sulfoxides were converted into their reactive lithio species by reaction with LDA in THF at -78°C. Addition of the lithio sulfoxides to the polarized C=N bond

of the imine resulted in a diastereomeric mixture of β -amino sulfoxides. All of these molecules contain two chiral centres, one at carbon and one at sulfur, resulting in the possibility of four stereoisomers comprising two pairs of enantiomers: $R_S S_C / S_S R_C$ and $R_S R_C / S_S S_C$.



Scheme 1.2.2.6. General scheme for synthesis of β - amino sulfoxide analogues. Chiral centres indicated by asterisks.

The crude coupling products were usually purified by gradient column chromatography yielding both diastereomers of the desired product. The polarity difference between both diastereomers as determined by R_f values was typically quite small, which made the complete separation long and tedious. Elutions were carried out with mixtures of hexanes and EtOAc, beginning with 100% hexanes and solvent gradients being incrementally changed by adding an additional 5% of EtOAc. The first few product fractions contained one pure diastereomer, with the bulk of the fractions containing mixtures and ending with the second pure isomer. Two or more chromatographs were carried out in order to obtain a workable amount of each pure product.

One of the major constraints experienced with this compound series was the synthesis of the imines. These compounds decompose quickly under acidic conditions, which made it impossible to purify these compounds by column chromatography using silica gel. Therefore, recrystallization was the most effective way to purify these compounds. Often, the imines were not crystalline. In such cases, the imine was kept as a crude product in the toluene solution in the

freezer until needed. A downside to this approach was that reactions with these crude imines led to several by-products, making purification of the final product even more challenging.

1.2.2.5.1. Diastereoselectivity under kinetic conditions

The addition of the α -lithio derivative of methyl phenyl sulfoxide to imines having at least one aryl substituent, under kinetically controlled conditions, gives β -amino sulfoxides with good to modest diastereoselection. In 1990, Pyne and Dikic⁶ reported conditions resulting in the diastereoselective addition of methyl phenyl sulfoxide and *p*-tolyl sulfoxide to imines. Reaction time and temperature contribute to the diastereoselectivity of these addition reactions. It was noticed that a reaction kept at $-78\text{ }^{\circ}\text{C}$ for 5 h gave modest diastereoselection of 82:18 $R_S S_C/S_S R_C$: $R_S R_C/S_S S_C$ isomers. Longer reaction times (5 h-12 h) at $0\text{ }^{\circ}\text{C}$ resulted in poorer diastereoselection, suggesting that equilibration had occurred most likely via reversion of addition reaction. All amino sulfoxide reactions in this thesis were carried out at -78°C for 1.5h which favours the $R_S S_C/S_S R_C$ diastereomer 75:25 as reported in literature⁶.

1.2.2.5.2. Diastereomer Structure Identification

The configuration assigned to the amino sulfoxides throughout this thesis are based on comparison of their ^1H NMR spectra with those reported by Pyne and Dikic for the condensation products obtained from methyl phenyl and *p*-tolyl amino sulfoxides⁶.

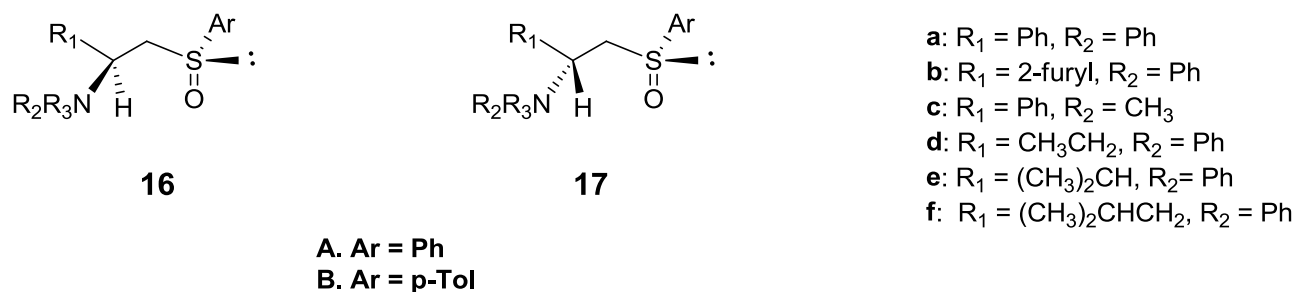
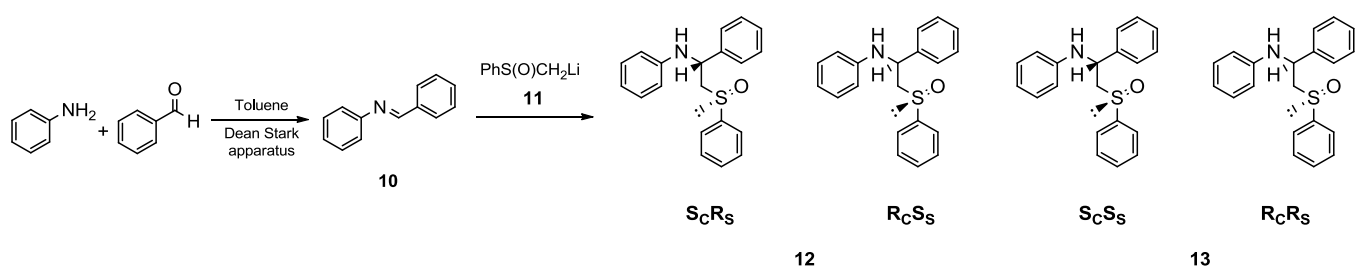


Figure 1.2.2.8. Stereoisomers produced by Pyne and Dikic⁶.

Table 1.2.2.1. Partial ¹H NMR spectral data for AB pattern of compounds synthesized by Pyne and Dikic⁶.

Compound 16.A.a			
Shift (ppm)	Peak Pattern	Coupling constants (J)	Integration
3.18	dd	3.7, 13.7	1H
3.12	dd	8.7, 13.7	1H
Compound 17.A.a			
3.28	dd	9.8, 13.8	1H
2.98	dd	4.9, 13.6	1H

In order to verify these findings, commercially available methyl phenyl sulfoxide was coupled to *N*-benzylideneaniline in the presence of LDA in THF at -78 °C. Purification by gradient column chromatography afforded two separate isomers as white solids. The ¹H NMR spectra of each diastereomer were compared to the spectra reported in literature. It was noticed that the more polar top spot correlated to the *RsSc/SsRc* isomer, compound **12**, whereas the slightly less polar bottom spot corresponded to the *RsRc/SsSc* isomer, compound **13**.



Scheme 1.2.2.7. Synthesis of compound 10, 12 and 13.

The pattern of the ABX system in the ^1H NMR spectrum of each diastereomer was used as a guide to identify all diastereomers in this series. The ABX pattern for the $\text{R}_\text{S}\text{S}_\text{C}/\text{S}_\text{S}\text{R}_\text{C}$ isomer is extremely narrow, often times having the dd patterns overlap one another. On the other hand, the ABX pattern for the $\text{R}_\text{S}\text{R}_\text{C}/\text{S}_\text{S}\text{S}_\text{C}$ isomer is much more broad and is shifted downfield compared to its diastereomer counterpart. This is illustrated for the diastereomer pair 12 and 13 in **Figure 1.2.2.9.** and **1.2.2.10.** The same features were used to analyse all future amino sulfoxide spectra and aided in assigning the diastereostereomers where applicable.

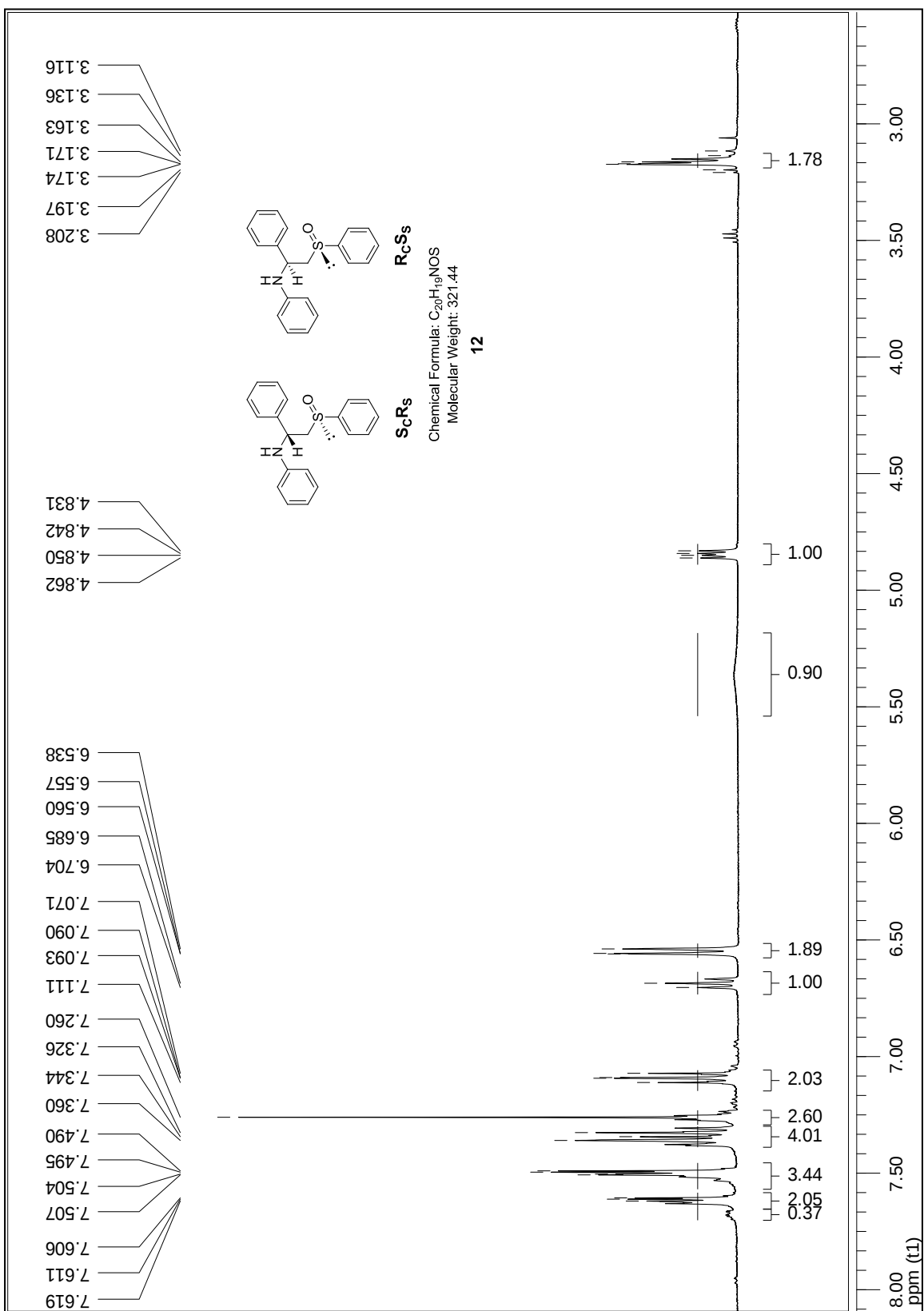


Figure 1.2.2.9. 400 MHz ^1H NMR spectrum of compound **12** in CDCl_3 .

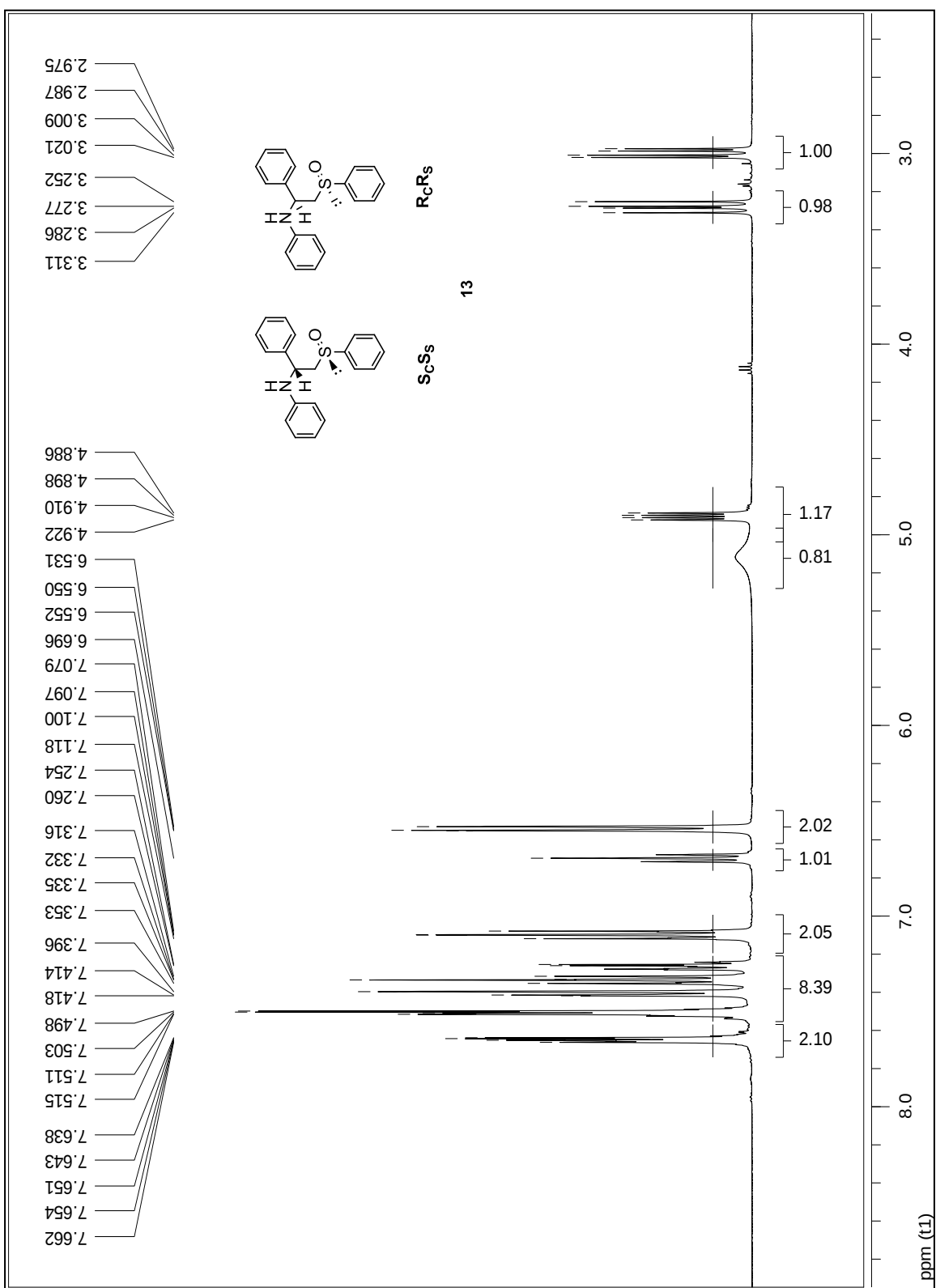
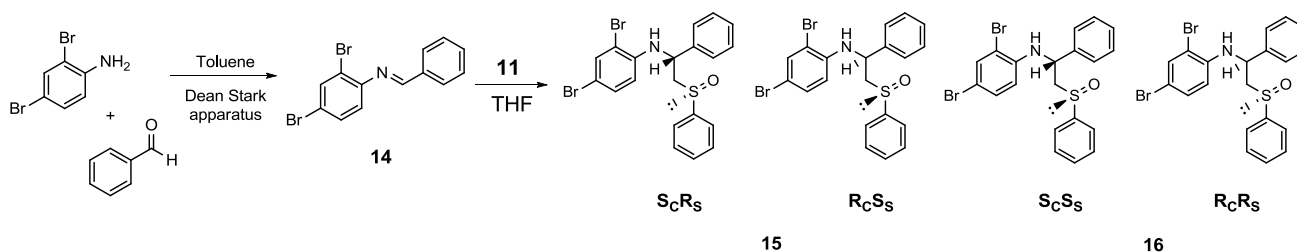


Figure 1.2.2.10. 400 MHz ^1H NMR spectrum of compound **13** in CDCl_3 .

1.2.2.6. Synthesis of amino sulfoxide analogues

Analogues of **12** and **13** were prepared and identified as described above. The most interesting compound based on the bioassays was the dibromo derivative **AF101**. It was available only as the single *RsRc/SsSc* isomer. In order to obtain unambiguously both diastereomers, it was decided to couple the imine **14** with the lithio sulfoxide **11**. Unfortunately, this imine, prepared by heating 2,4-dibromo aniline and benzaldehyde in toluene, did not crystallize and therefore, was kept as a crude preparation in the toluene solution. The coupling product between crude **14** and lithio derivative **11** when carried out in THF -78°C afforded a mixture of isomers as a yellow solid in 50% yield.



Scheme 1.2.2.8. Synthesis of compounds **14**, **15** and **16**.

Separation of isomers via gradient column chromatography was unsuccessful. Attempts at separating these compounds by preparative TLC seemed promising as a clean separation of isomers was observed with three consecutive elutions at 20% EtOAc in hexanes on an analytical TLC plate. However, once larger amounts of material was subjected to a preparative TLC plate, the compounds began to crystallize on the plate during development which resulted in no migration or separation whatsoever. Separation of the isomers by reverse phase HPLC using 80% ACN in H₂O resulted in the isolation of the pure diastereomers **15** and **16**.

^1H NMR spectra of **15** and **16** showed the same differences in the ABX patterns, (**Figure 1.2.2.11**), as was seen in the parent sulfoxides **12** and **13**. Again, the ABX system for diastereomer **15** was narrower than that of **16**; its relative configuration was therefore assigned as *RsSc/SsRc*.

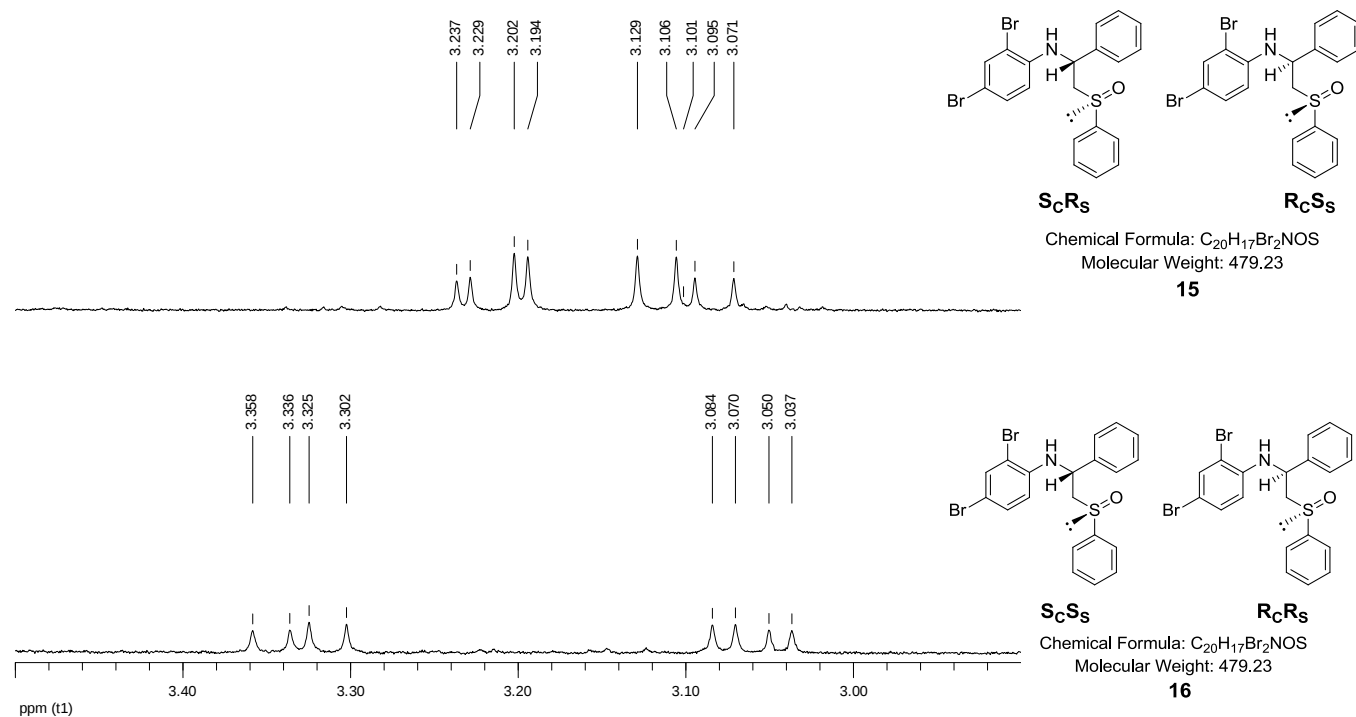


Figure 1.2.2.11. ^1H NMR spectra AB peak pattern comparison of compound **15** and **16** in CDCl_3 .

The above results gave us confidence that the use of the ABX patterns to assign the diastereomer structure of the other pairs of diastereomers below was valid. In order to make the reading of this portion of the thesis less repetitive, the structure assignments of diastereomers will generally not be described, unless it had unusual features or the structures were significantly different from the above two sets of compounds.

Since the bromine substituent's in the aniline ring increased the activity significantly in the GJIC assay, as shown by the difference between **AF101** and **AF102**, it was decided to prepare both sets of the ortho and para mono-brominated isomers. We were intrigued by the

notion that the bromine substituent in the ortho position might be more important than the one at the para position since the former could affect significantly the shape of the molecule by interaction with the other substituents on the aniline nitrogen, thereby causing the phenyl ring to rotate relative to its position in **AF102** or in the para bromo compounds. This is shown below in **Figure 1.2.2.12**.

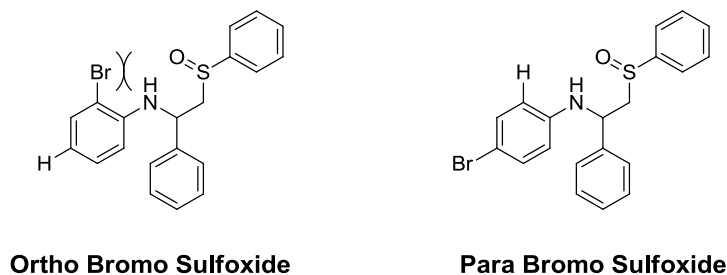


Figure 1.2.2.12. Structures of ortho and para monobrominated amino sulfoxides.

These desired compounds were obtained, as expected by reaction of the imines obtained from ortho and para bromoaniline and benzaldehyde with the lithio sulfoxide **11**. Purification via column chromatography was successful, affording compounds **17** and **18** as off-white solids in 21% and 4% yield, respectively. Unfortunately, separation of the ortho bromo sulfoxide diastereomers via column chromatography was unsuccessful due to the diastereomers having essentially the same polarity on silica gel. The reverse phase HPLC method also failed. Two consecutive recrystallizations from DCM and hexanes gave the pure the *RsRc/SsSc* diastereomer, compound **19**, as verified by ^1H NMR spectral analysis. Disappointingly, we have not had any success at obtaining the *RsSc/SsRc* isomer.

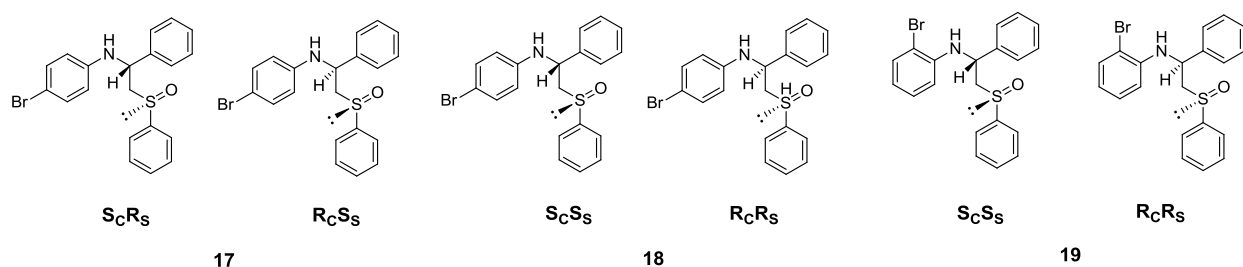


Figure 1.2.2.13. Chemical structures of compounds **17**, **18** and **19**, with associated stereochemistry.

With the focus still on the variations of the aniline ring, it was decided to introduce other substituent's such as a methoxy group. Compound **20** and **21** were obtained from the reaction of *p*-anisidine and benzaldehyde with lithio sulfoxide **11**. Purification of the crude reaction product via column chromatography was successful affording white solids in 32% and 11% yields, respectively. Again, since the *o,p*-dibrominated product, **AF101**, showed an increase in activity in the GJIC assay, it was decided to brominate **21** using 1 equiv. of NBS. This resulted in the introduction of a bromine at the ortho position on the ring, affording **22** as a white solid in 41% yield. This reaction was only performed on *R_sR_c/S_sS_c* isomer, **21**, since Dr. Carballo Arce had previously synthesized the *R_sS_c/S_sR_c* counterpart, **AF108**.

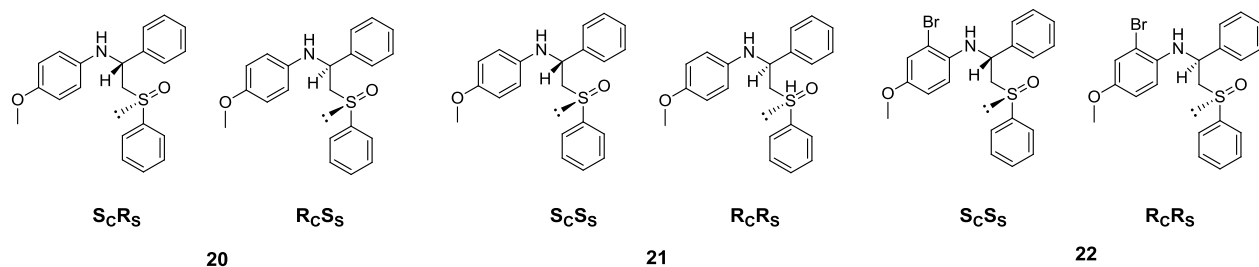


Figure 1.2.2.14. Structures of compounds **20**, **21** and **22**.

The analogues prepared thus far have all focused on changes to the aniline ring. Since changes to the other aromatic rings in the system have not been made and tested, it was decided to focus on variations of the aromatic ring derived from the aldehyde precursor. Only two compounds were prepared in this series, the first to test the effect of an ortho methyl group onto benzene ring B, since such a change, as was argued above, could affect the shape of the molecule by causing its ring to twist relative to its position in the parent compound, **AF102**. For the second compound the B phenyl ring was replaced by a 2-furyl substituent, additionally an ortho methyl group was introduced into ring A.

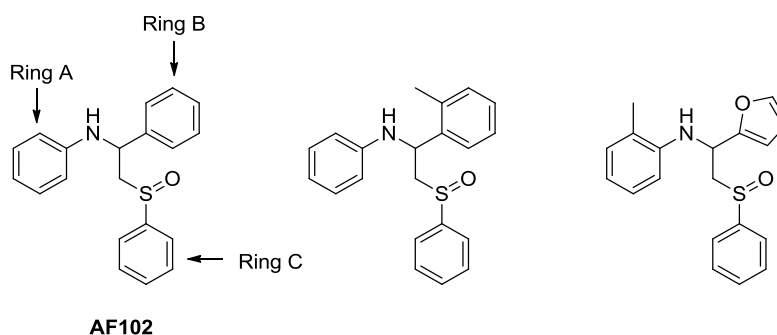
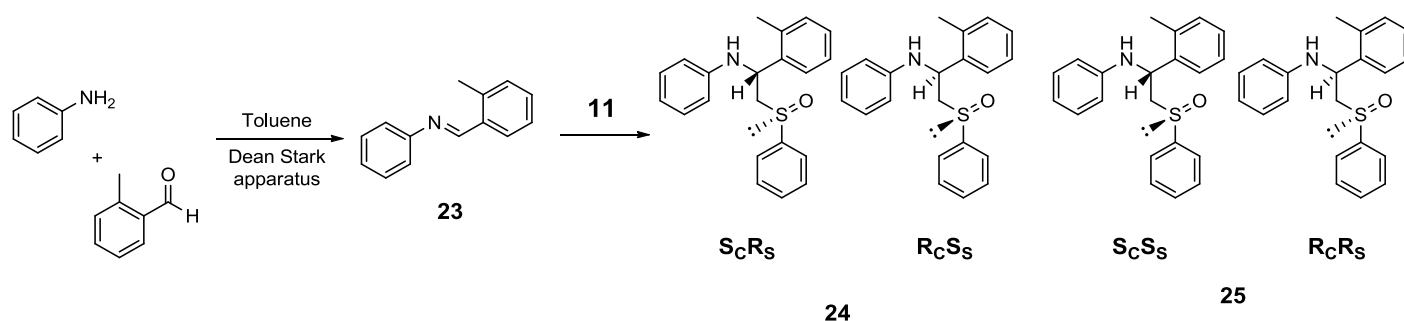


Figure 1.2.2.15. Proposed changes to ring B.

A condensation reaction of aniline with 2-methylbenzaldehyde produced imine **23**. The crude imine was coupled with the lithio sulfoxide **11** affording compound **24** and **25** as off white solids in 17% and 8% yields isolated after isomer separation. Again, comparison of the shape and position of the AB part of the ABX pattern with those of known structures were crucial in making the assignments.



Scheme 1.2.2.9. Synthesis of compound **23**, **24** and **25**.

The reaction of o-toluidine and furfural gave a new and interesting imine that included a methyl substituent on the aniline ring and a furan ring in place of the existing phenyl group. The usual reaction sequence followed by careful column chromatography gave compound **26** and **27** in 15% and 12% yields, respectively.

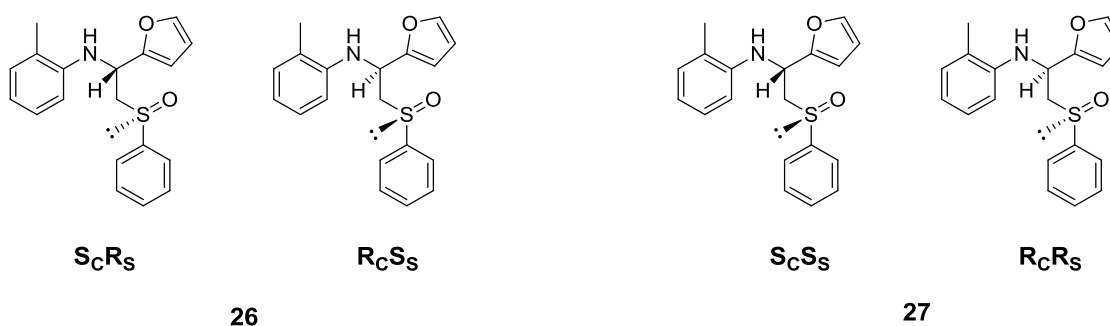
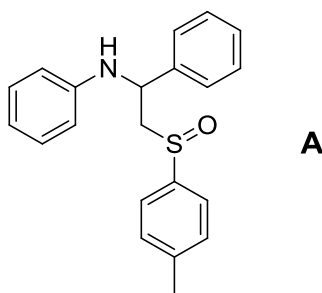


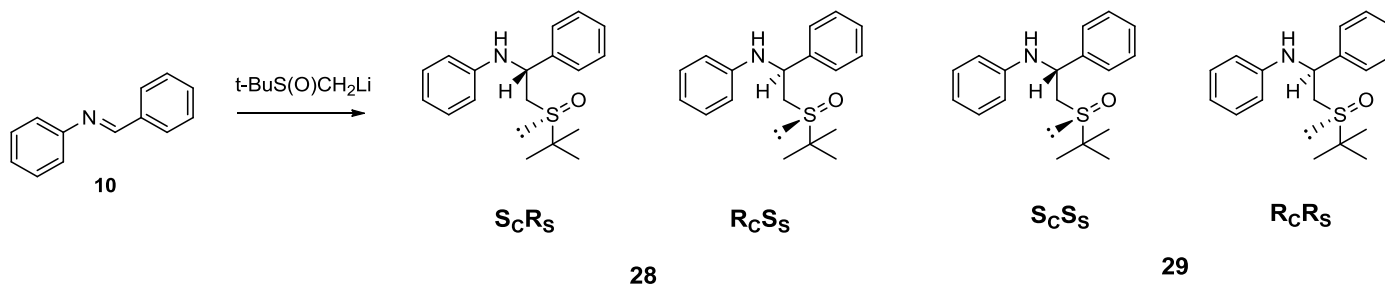
Figure 1.2.2.16. Chemical structure and relative stereochemistry of compound **26** and **27**.

An obvious change in the parent structure considering the method of synthesis, is to use a methyl sulfoxide other than methyl phenyl sulfoxide. Two changes can be imagined: a) replacement of the phenyl group by an alkyl group using for example dimethyl and methyl t-butyl sulfoxide and b) adding a substituent to the phenyl ring. For this case we chose methyl p-

tolyl sulfoxide mainly because both the *R* and the *S* isomer of this sulfoxide are commercially available, with the former being less expensive. The availability of enantiomerically pure sulfoxides would allow us to prepare all four isomers of the adduct (**A**) below. Although not definitive, we do not expect the para methyl group to effect the activity significantly of **A** vs. **AF102**. Thus, if one finds that one of the four diastereomers of **A** shows significantly more potency or selectivity towards individual connexins, one might reasonably decide whether it is worthwhile to prepare optically pure isomers of other analogues.



Commercially available methyl *t*-butyl sulfoxide was coupled with benzalaniline **10**. The crude mixture was chromatographed to give the pure diastereomers **28** and **29** as off white solids in 12 and 6% yield respectively. The ^1H NMR spectral pattern and chemical shifts of the ABX pattern of each isomer was compared to the reference spectra, and the diastereomers were identified accordingly.



Scheme 1.2.2.10. Synthesis of compound **28** and **29**.

Reaction using a four-fold excess of DMSO and LDA (2 M) in THF at -78 °C relative to imine resulted in a reasonable amount of product formation and barely any imine as starting material remaining. Purification via column chromatography afforded **30** as an orange solid in 17% yield. The ^1H NMR spectra of compound **30** (**Figure 1.2.2.17.**) was expected to have two ABX systems as observed for all other compounds in this series. Two ABX systems were indeed present; however, they were completely overlapped, which was not observed in the other compounds. The expected remaining methyl group from DMSO was not observed in the spectra; thus this compound could not be the expected product.

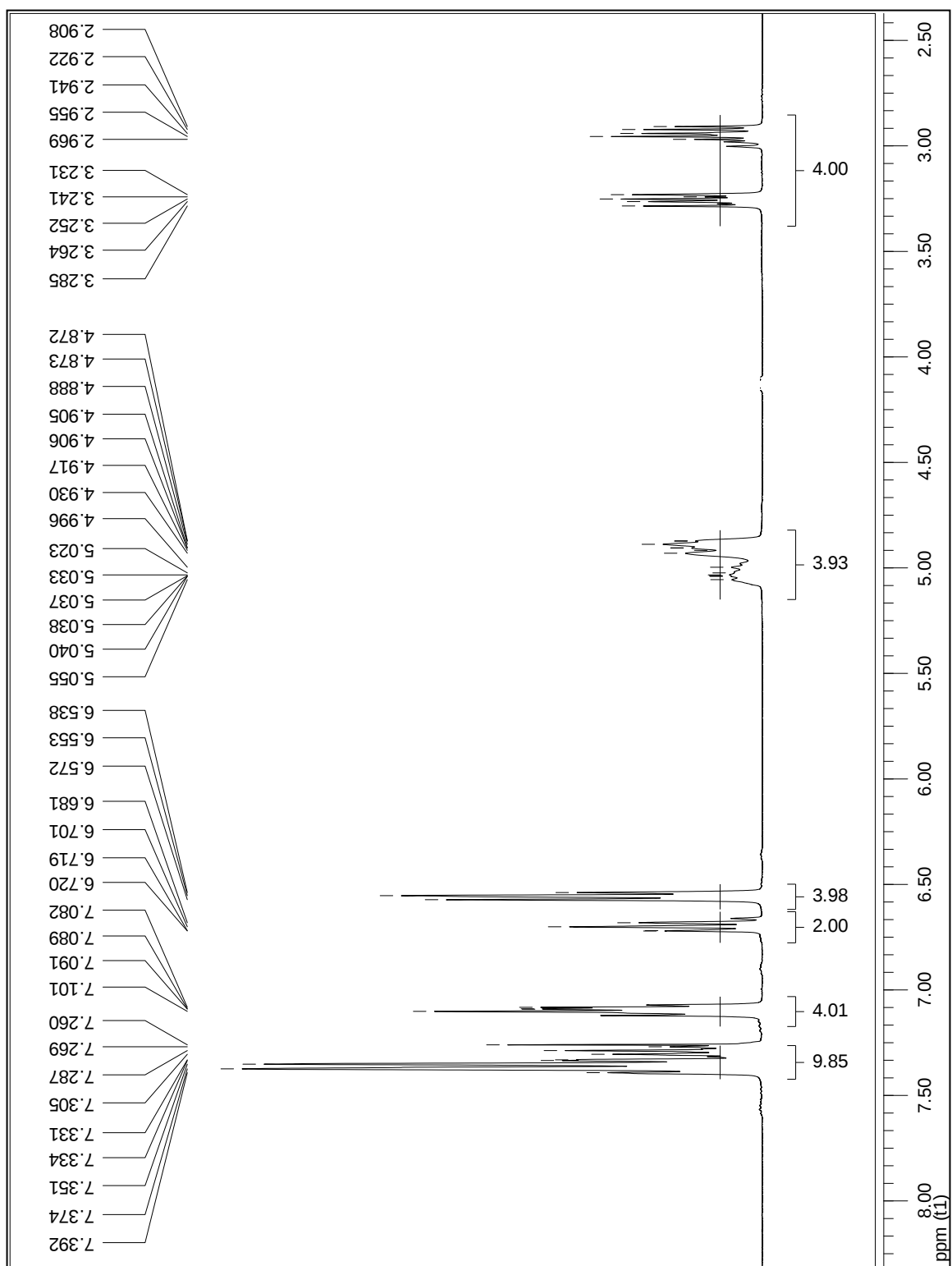
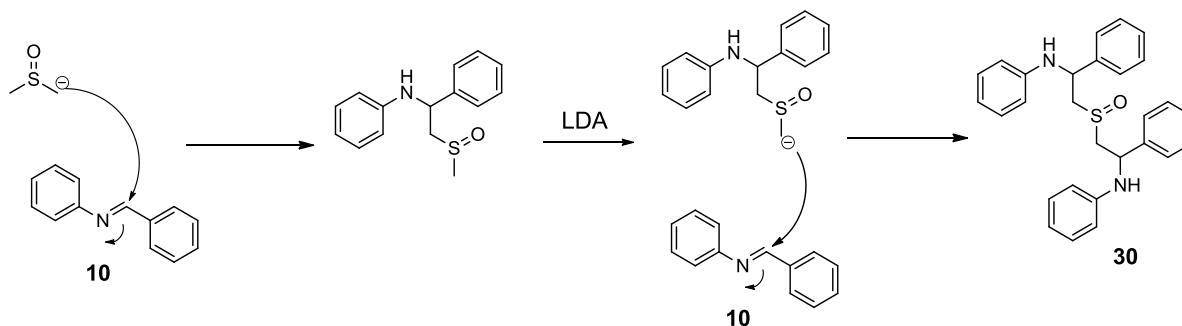


Figure 1.2.2.17. 400 MHz ^1H NMR spectrum of compound **30** in CDCl_3 .

The MS data confirmed that this product was in fact a "dimerized" structure as shown in **Scheme 1.2.2.11**. A possible explanation for the formation of **30** is that excess LDA was available and that the initial adduct was converted into its lithio derivative which reacted with a second equivalent of imine. Although this was not the desired expected product, it was sent for bioassay testing regardless.



Scheme 1.2.2.11. Synthesis of compound **30**.

In order to obtain the methyl sulfoxide compound, a third synthesis was performed; this time using 0.8 equiv. LDA vs DMSO. Product formation was visible by TLC and ^1H NMR of the crude product confirmed the presence of both diastereomers. Purification by column chromatography was unsuccessful at separating the diastereomers from one another; however, the mixture was obtained in 28% yield as a white solid. Separation of isomers by preparative TLC afforded compound **31** and **32** after being developed ten consecutive times in 40% EtOAc in hexanes.

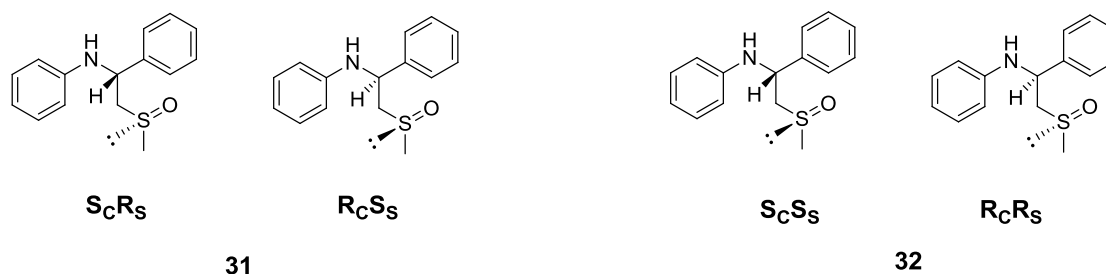


Figure 1.2.2.18. Structure of compound **31** and **32** with relative configuration.

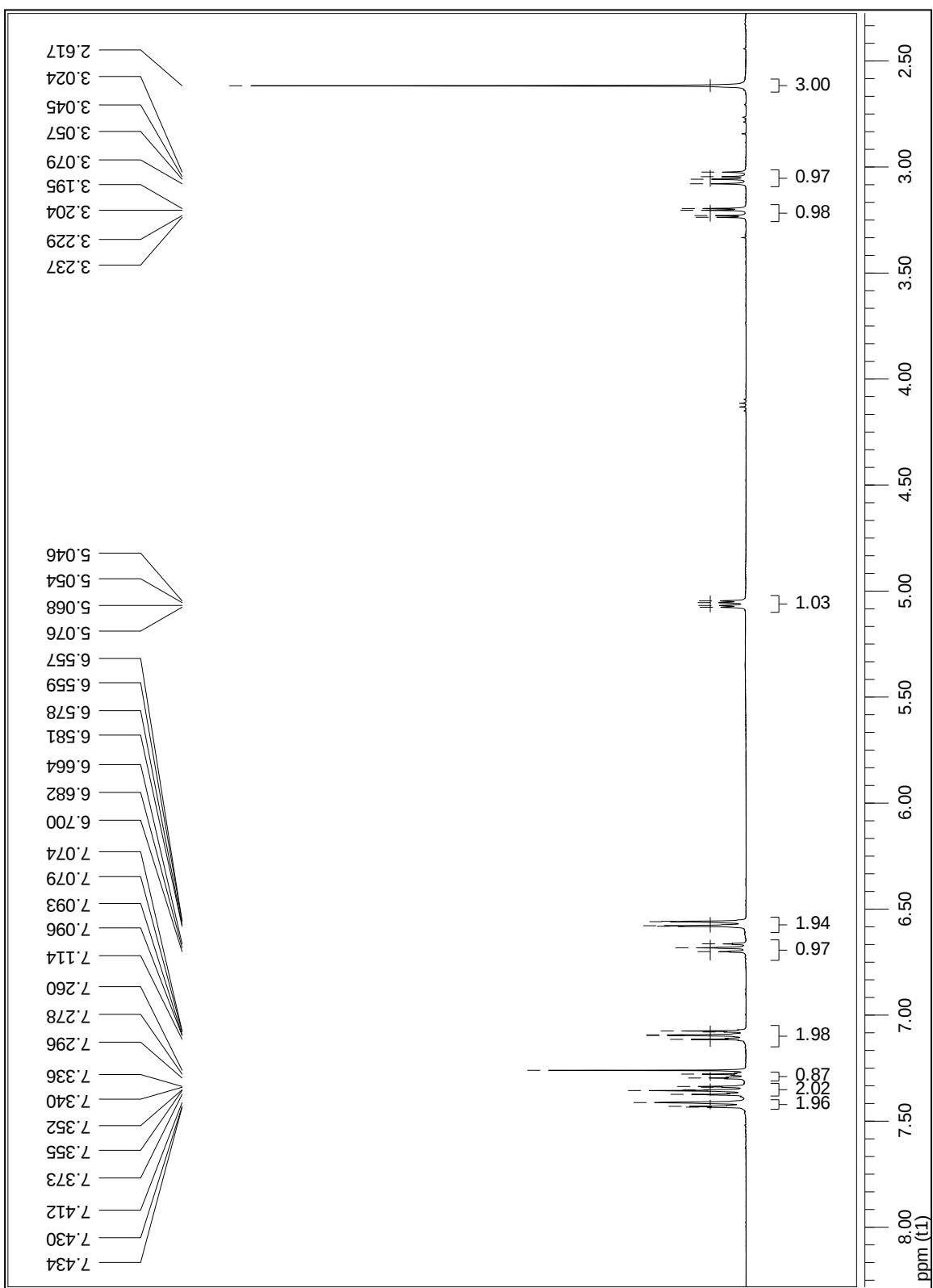


Figure 1.2.2.19. 400 MHz ^1H NMR spectrum of compound **32** in CDCl_3 .

The final pair of diastereomers was prepared by reaction of lithio p-tolyl sulfoxide and imine **11**. Purification via gradient column chromatography was a long and tedious process as the compounds began to crystallize on the silica gel column partway through chromatography. Excess amounts of 100% EtOAc was used to elute the products and unexpectedly yielded a crude separation of each isomer. Separate recrystallizations of each product fraction were performed using DCM and MeOH, which afforded compound **33** and **34** as off white solids. The assignment of the relative stereochemistry for these compound was done by comparison of the ^1H NMR spectra to those reported by Pyne and Dikic⁶.

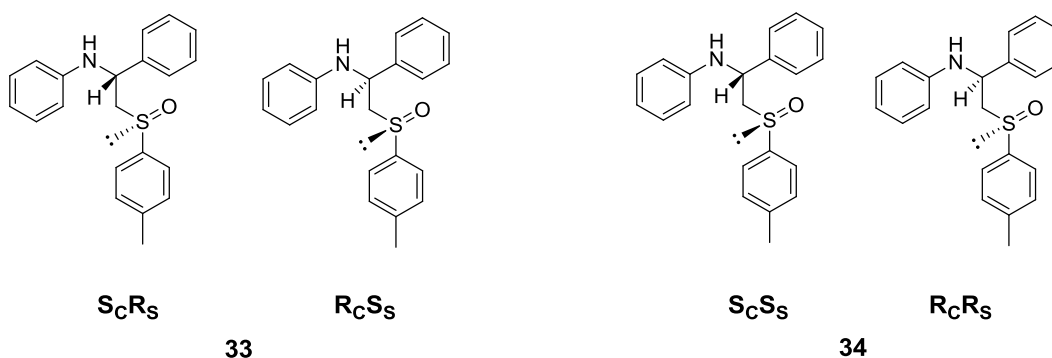
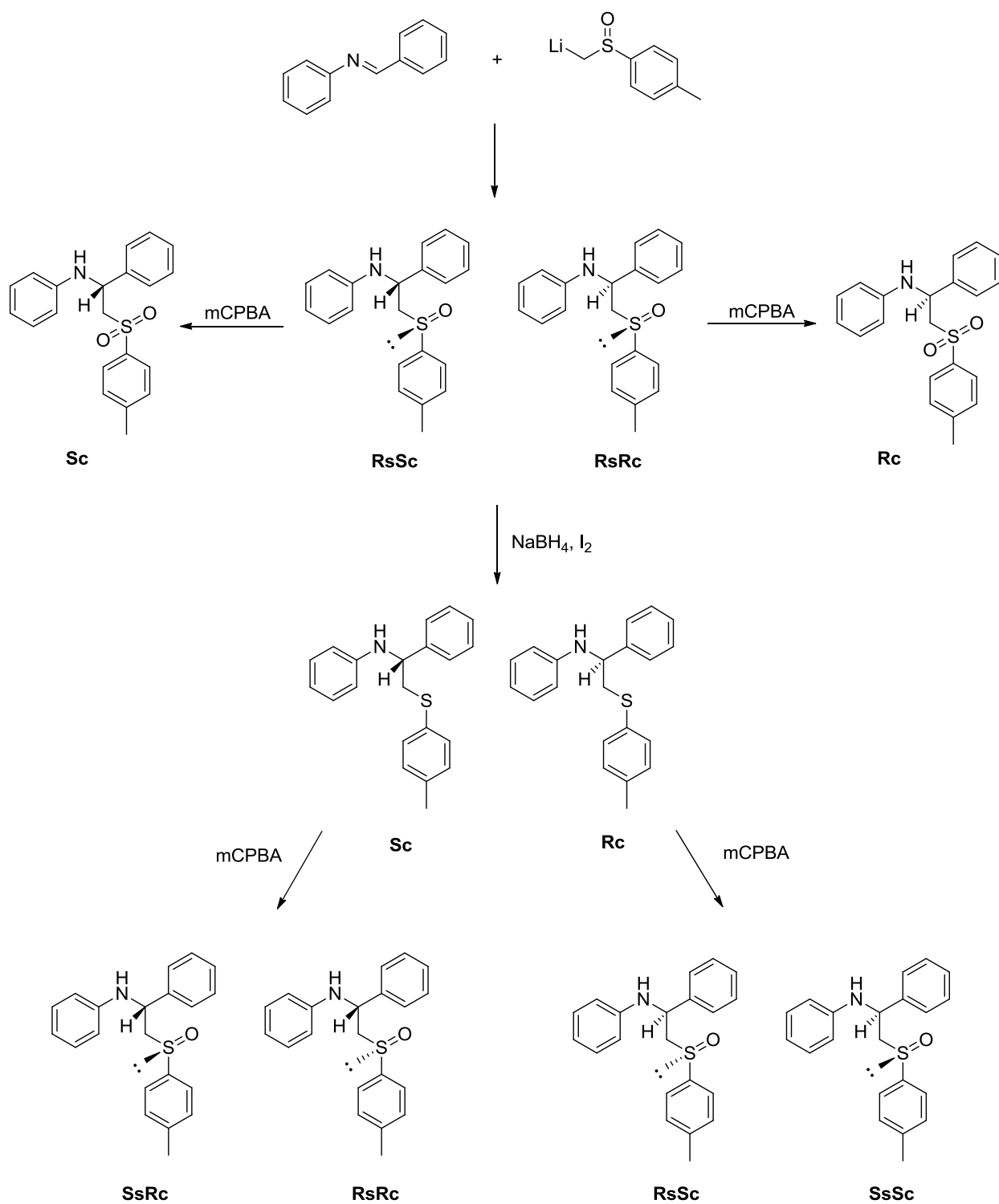


Figure 1.2.2.20. Chemical structure and stereochemistry of compound **33** and **34**.

It is well known that the desired biological activity is typically due mainly, or exclusively to one of the two enantiomers. I acted as the key mentor to Adrien Fluet-Chouinard, whose honors thesis was to prepare enantiomerically pure versions of the sulfoxides **33** and **34**.

A scheme was produced whereby use of the commercially available, less expensive *R*(+)-methyl p-tolyl sulfoxide would lead to the four possible enantiomerically pure β-amino sulfoxides, two enantioerically pure β-amino sulfones and two β-amino sulfides. This is shown in the scheme below.



Scheme 1.2.2.12. Preparation of enantiomerically pure β -amino sulfoxides.

The lithio derivative of *R*-methyl *p*-tolyl sulfoxide was coupled to **11** under the same conditions presented in this thesis. Purification via gradient column chromatography afforded the *R*_s*R*_c and the *R*_s*S*_c isomers. The key to success was the use of a combination of DCM and EtOAc, since this combination of solvent overcame the solubility issue. Each of these enantiomerically pure diastereomeric compounds (their optical rotations were compared to literature values and showed indeed that they were essentially enantiomerically pure) were subjected separately to oxidation using mCPBA, affording the *R*_c and *S*_c β-amino sulfones. Reduction of each sulfoxide yielding the *R*_c and *S*_c sulfides. Again, dibromination of the *R*_s*S*_c sulfoxide enantiomer was carried out in order to mimic the structure of **AF101**. The initial plan was to re-oxidize the sulfide isomers to obtain a mixture of the *S*_s and *R*_s configuration in each case, however due to time constraints this was not performed. These compounds are currently undergoing bioassays. Depending on the connexin activity of the *R*_s enantiomers, it may not be worthwhile to further investigate and synthesize the *S*_s enantiomers.

1.2.2.7. Synthesis of β-amino sulfide analogues

Once the ketone, sulfoxide and sulfone analogues were prepared, it was decided to reduce the parent sulfoxide structure, **AF102**, to the corresponding β-amino sulfide. The change from a sulfoxide to sulfide eliminates the possibility of significant intramolecular hydrogen bonding and thus the two compounds should have different conformations in solution and thus might interact differently with the connexin receptors.

Reducing the sulfoxide to sulfide removes a stereocentre on the molecule at the sulfur which leaves the possibility for only two enantiomers. The parent sulfoxide was reduced to its amino sulfide **35** in 49% yield using NaBH₄ and I₂. Purification by gradient column

chromatography afforded what was expected to be the desired product; however, ^1H NMR analysis indicated that there were two ABX systems in the spectra, as can be seen in **Figure 1.2.2.21**.

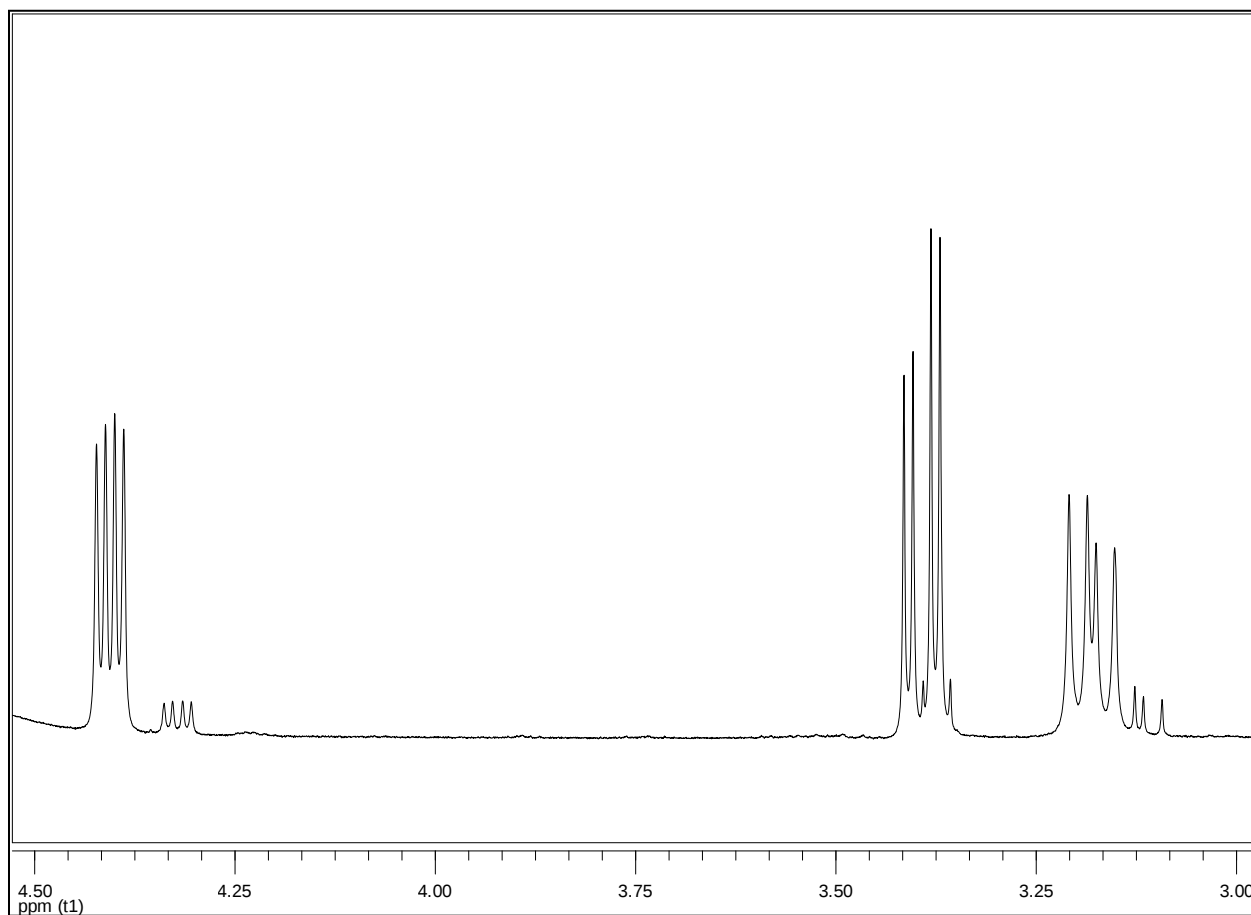


Figure 1.2.2.21. ^1H NMR spectra of amino sulfide before purification displaying two ABX patterns.

Separation of these two products was successful via preparative TLC with three consecutive elutions in 10% EtOAc in hexanes. Surprisingly, the by-product also contained an ABX system with the same peak patterns as was expected for the amino sulfide. Additionally, examination of the aromatic region showed that the signal for the para hydrogen of the aniline ring was absent, indicating that a substituent had been introduced at this position. Since we are introducing an electrophilic source of iodine into the reaction mixture, one can assume that an

aromatic substitution at the para position of the activated aniline ring had occurred. The same chemistry occurs with the bromination reactions. Mass spectroscopy analysis showed the presence of one iodine atom in the molecule, in agreement with the proposed structure.

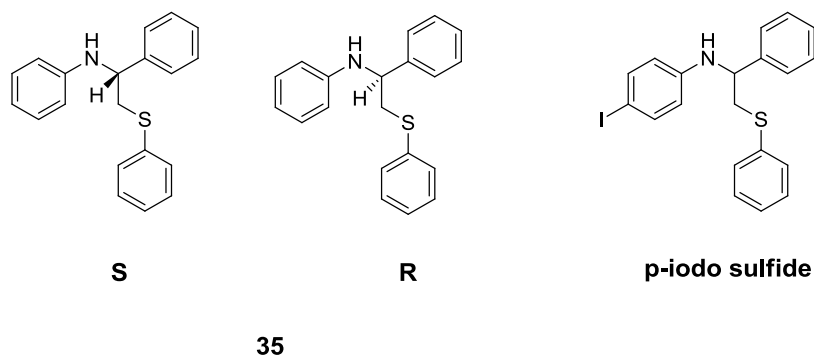
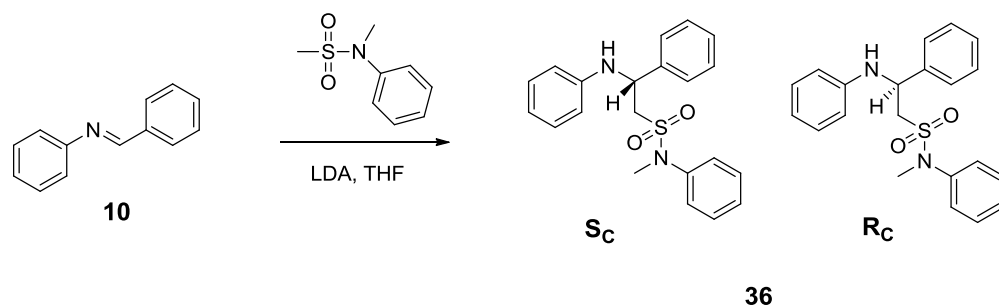


Figure 1.2.2.22. Compound **35** and p-iodo sulfide by-product.

1.2.2.8. Synthesis of β -amino sulfonamide analogues

As mentioned earlier, a sulfone functional group in the molecule would have very weak intramolecular hydrogen bonding to OH and NH groups. Not only would this affect its conformation in solution, but it also could affect how this compound interacts with the connexin channels. The introduction of a sulfonamide group could in fact change the conformation even more, possibly either enhancing or inhibiting its interaction with the channels. The lithio derivative of N-methyl-N-phenyl methanesulfonamide, a compound available in our laboratory, was coupled to **11**, affording compound racemic **36** as a yellow solid in 29% yield.



Scheme 1.2.2.13. Synthesis of compound **36**.

1.2.3. Bioassays Results

All newly synthesized compounds were sent to the Bennett group at the University of Ottawa, Faculty of Medicine, to assess their GJIC activity in connexin and pannexin channels. The cell model chosen for use in the bioassays was the NT2/D1 cell line. These cells are derived from a human teratocarcinoma, have a multipotent neural progenitor cell phenotype, are metabolically coupled by connexin gap junctions and exhibit functional connexin hemichannel activity⁴. To establish the complete repertoire of connexins and pannexins expressed by NT2/D1 cells, these cells were profiled at the mRNA level by reverse transcriptase polymerase chain reaction (RT-PCR). To assess intercellular connexin gap junction activity, a parachute assay was performed, which measures the intercellular flux of fluorescent calcein between coupled cells through gap junction channels. To assess single membrane hemichannel activity, the uptake of both anionic lucifer yellow (YL) and cationic propidium iodide (PI) in a calcium free medium are measured^{4,5}. These bioassays are described in detail in **Appendix 1A**.

AF101 was identified as the potential lead compound for the design of a selective connexin blockers as a result of its ability to inhibit GJIC. Compounds are tested in two different assays testing for 1) connexin-mediated gap junctional intercellular communication (GJIC) and 2) pannexin and connexin mediated single membrane channel (hemichannel) communication (anionic or cationic). All compounds are tested at 6 μ M. As mentioned earlier, the bioassays are

difficult and long to perform and for this reason, only a number of compounds have been assayed thus far.

Table 1.2.3.1. Percent inhibition of connexin mediated GJIC and pannexin and connexin mediated single membrane channel (hemichannel) communication.

Compound	% Inhibition (CBX 100 μ M =100%)	
	GJIC 1) Docking and anionic dye transfer through Cx43 and Cx45 GJ channels. 2) Anionic dye transfer through pre-docked Cx43 and Cx45 GJ channels.	Hemichannel 1) Channels assayed: Panx1,Panx2, Cx45, Cx43, Cx36 for anionic dye transfer 2) Channels assayed Panx1,Panx2, Cx45, Cx43, Cx36 for cationic dye transfer
12	1) TBD* 2) 52.4%	1) 56.9% 2) 91.0%
13	1) 42.4% 2) 47.9%	1) 15.2% 2) 18.3%
15	1) TBD 2) TBD	1) 86.8% 2) TBD
16	1) 54.0% 2) 31.7%	1) 80.9% 2) 59.0%
28	1) TBD 2) 26.9%	1) 43.9% 2) 38.6%
29	1) TBD 2) 28.1%	1) 31.5% 2) 32.2%

* TBD = To Be Determined

Although there is some data present in **Table 1.2.3.1**, it is difficult to draw conclusions on which compounds optimize GJIC activity due to the fact that all assays need to be performed in order to determine compound selectivity. The remaining newly synthesized compounds are currently undergoing bioassays.

1.2.4. Conclusions and Future Directions

As mentioned throughout the discussion of the synthesis of these compounds, one of the major constraints of this project was the synthesis of the imines. These compounds decompose quickly under acidic conditions and therefore purification of these products by column chromatography was virtually impossible. Unless crystalline, the material was left as a crude solution in the toluene which produced a number of by-products when preparing the sulfoxide analogues. This made purification of the final products even more challenging.

Where applicable, separation of diastereomers was done in order to test each diastereomer of the product mixture. For one set of sulfoxides, enantiomers were prepared from enantiomerically pure commercially available starting material. The results from these compounds will determine the significance of diastereomers and enantiomers on GJIC activity.

A variety of different sulfoxide, sulfide and sulfone analogues have been submitted in hopes to optimize GJIC inhibitory activity. Unfortunately, there are a number of assays that need to be conducted in order to determine compound selectivity on different channels. These assays are not only time consuming but are difficult to perform and throughout the course of the project, very little feedback was obtained. At this stage, all newly synthesized are currently undergoing bioassays. The project therefore continues hopefully with pleasant surprises.

1.3. Experimental Data

1.3.1. General Methods

Reactions

All reactions were monitored by TLC using aluminum backed TLC plates (EMD Chemicals, TLC Silica Gel 60 F₂₅₄), which were visualized by UV lamp (254 nm – fixed wavelength). Plates were then permanently stained with Hannessian's stain. All moisture sensitive reactions were carried out under nitrogen or argon atmosphere. Anhydrous THF was prepared by distillation from sodium and benzophenone and DCM from calcium hydride.

Purification

Purification by column chromatography was performed using SiliCycle SiliFlash ® F60 silica gel of 230-400 mesh and glass columns fitted with a cotton piece and sand or fritted glass filter. Elutions were carried out with mixtures of hexanes and ethyl acetate. Solvent gradients were incrementally changed by adding an additional 5% of ethyl acetate. Purification by preparative HPLC was performed using a reverse phase C18 preparative HPLC column (10µm particle size, 21.2 x 250 mm).

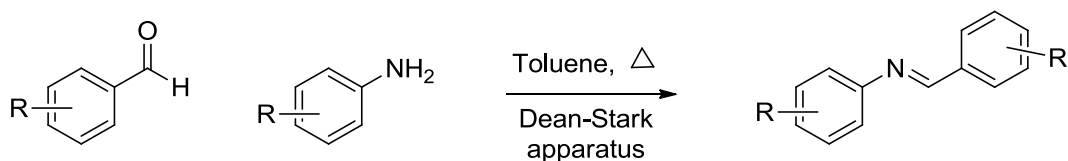
NMR

¹H NMR and ¹³C NMR were recorded on Bruker Avance 400 and 300 spectrometers. Samples were dissolved in deuterated chloroform, methanol or acetone as indicated. All chemical shifts are reported in parts per million (ppm) and are referenced accordingly to the deuterated solvent used. Integrations are listed in parentheses along with coupling constants which are reported in Hz, where applicable.

1.3.2. General Procedures:

General Procedure #1:

Imine Synthesis

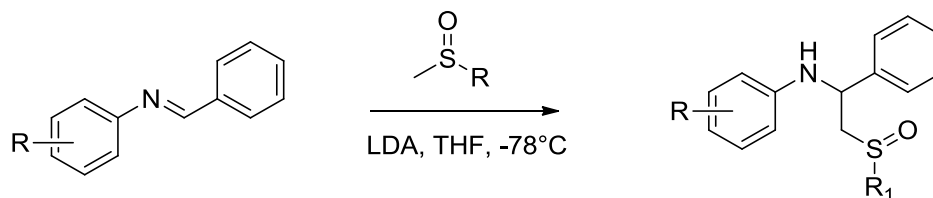


Selected amine (1 equiv.) and corresponding aldehyde (1 equiv.) were dissolved together in toluene and placed in a round bottomed flask equipped with a Dean-Stark trap and reflux condenser. The mixture was set to reflux until all of the water was removed through azeotrope formation. The resulting solution was then permitted to cool and was concentrated in vacuo to afford an oil⁸.

Note: Spectroscopic data for imines are not shown in this section due to the fact that these compounds are well known. The spectral data correspond to those reported previously in the literature⁸. Crystalline imines were used as such in the synthesis as described. Non crystalline imines were kept as crude imine solution in toluene in the freezer (-20°C) and was used when needed.

General Procedure #2:

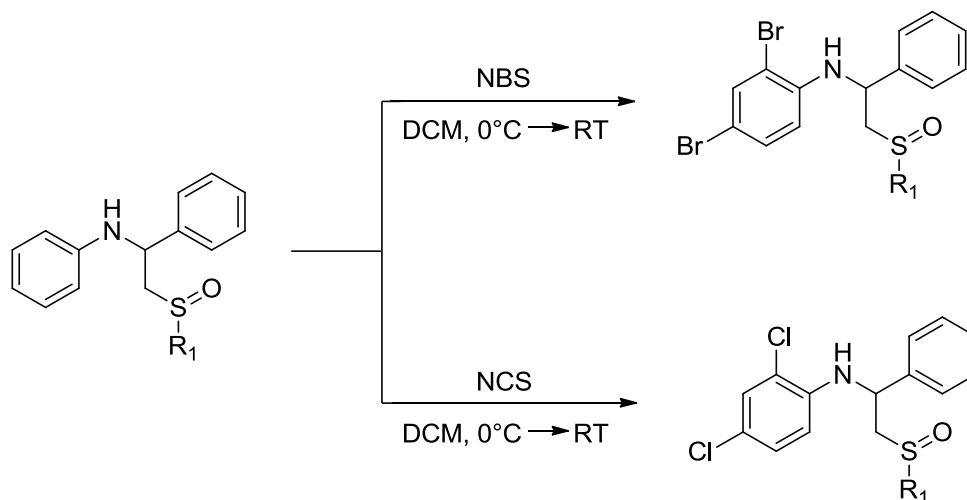
Imine-Sulfoxide Coupling



Lithium diisopropylamide (LDA) (2.0 M equiv.) was added drop wise to a $-78^\circ C$ solution of the corresponding sulfoxide (2.0 M equiv.) in 10 mL THF. The resulting mixture was kept under these conditions for 15 min and the imine (1.0 M equiv), dissolved in 5 mL of THF was added via syringe. The progress of the reaction was monitored by TLC. Workup of the reaction involved quenching with 10% aqueous NH_4Cl solution followed by extraction with EtOAc (3 x 10mL). The combined organic extracts were washed with water, dried ($MgSO_4$), filtered and concentrated in vacuo. The product was purified by gradient column chromatography using a gradient solvent system of EtOAc and hexanes.

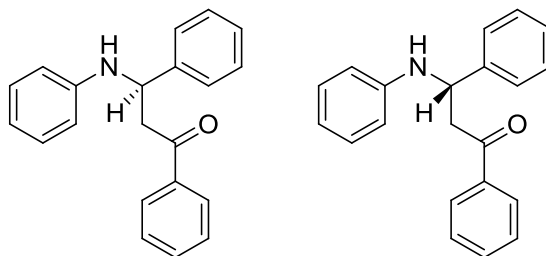
General Procedure #3:

Halogenation



Aminosulfoxide (1.0 M equivalent) was dissolved in 5 mL of anhydrous DCM at 0°C. A catalytic amount of PTSA was added, followed by drop wise addition of NBS or NCS (2.0 M equivalents) in a minimal amount of DCM. The resulting solution was stirred at 0°C for 10 min, after which the ice bath was removed and the reaction was left to warm to RT. Upon completion of the reaction as monitored by TLC, the solution was diluted with water and NaHCO₃ solution (3 mL, 10%) was added. The aqueous phase was extracted three times with DCM (3 x 10mL) and the combined organic extracts were dried (MgSO₄), filtered and concentrated in vacuo. The resulting solid material was purified by column chromatography.

Preparation of 1,3-diphenyl-3-(phenylamino)propan-1-one



Chemical Formula: C₂₁H₁₉NO
Molecular Weight: 301.38

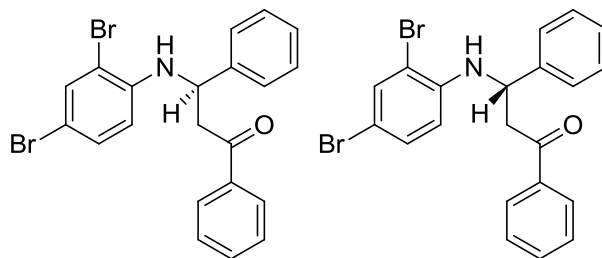
2

Aniline (0.5 g, 4.8 mmol) was added to a round bottom flask containing chalcone (1 g, 4.8 mmol) in toluene (10 mL). The solution was set to reflux for 4 hs. Excess solvent was evaporated in vacuo and H₂O was added. The mixture was extracted with EtOAc (3 x 10mL) and purified via gradient column chromatography (30-35% EtOAc in hexanes) affording **2** as a yellow solid (0.68 g, 47%, R_f=0.3 in 1:4 EtOAc: hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.93 – 7.84 (m, 2H), 7.54 (s, 1H) 7.42 (t, *J* = 7.9 Hz, 4H), 7.37 – 7.17 (m, 4H), 7.08 (dd, *J* = 8.5, 7.4 Hz, 2H), 6.73 – 6.55 (m, 3H), 4.99 (dd, *J* = 7.2, 5.6 Hz, 1H), 3.51 (dd, *J* = 12.8, 6.4 Hz, 2H).

HRMS: Calculated for C₂₁H₁₉NO = 301.14666; Found = 301.14449

Preparation of 3-((2,4-dibromophenyl)amino)-1,3-diphenylpropan-1-one



Chemical Formula: $C_{21}H_{17}Br_2NO$
Molecular Weight: 459.17

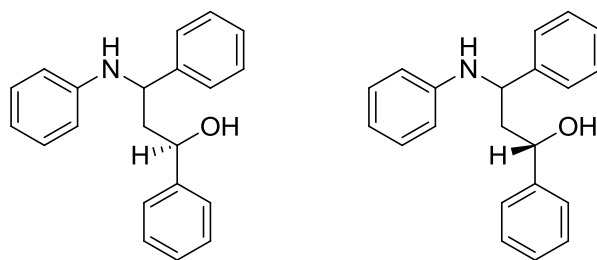
4

This compound was prepared following general procedure 3 using **2** (0.18 g, 0.59 mmol), NBS (0.21 g, 1.19 mmol) and a catalytic amount of PTSA in DCM (10 mL). Crude product was purified via gradient column chromatography (25-30% EtOAc in hexanes) to afford **4** as a white solid (0.176 g, 65%).

1H NMR (400 MHz, Acetone D_6) δ 7.98 (dd, J = 8.4, 1.3 Hz, 2H), 7.67 – 7.44 (m, 8H), 7.23 (dt, J = 24.5, 7.3 Hz, 3H), 5.67 – 5.59 (m, 1H), 4.96 (d, J = 10.6 Hz, 1H), 3.80 (dd, J = 6.5, 2.2 Hz, 2H).

HRMS: Calculated for $C_{21}H_{17}Br_2NO$ = 456.96769; Found = 456.96400

Preparation of 1,3-diphenyl-3-(phenylamino)propan-1-ol



Chemical Formula: $C_{21}H_{21}NO$
Molecular Weight: 303.40

5

To a solution of **2** (0.8 g, 2.7 mmol) in EtOH (5 mL) at 0°C, was slowly added NaBH₄ (0.11 g, 2.9 mmol). The mixture was kept stirring under these conditions for 15 mins and then warmed to room temperature. Reaction mixture was quenched with NH₄Cl solution (5 mL) and extracted with DCM (3 x 10mL). The combined organic extracts were dried (MgSO₄), filtered and concentrated in vacuo. Crude product was purified via column chromatography (40% EtOAc in hexanes) affording **5** as a white solid (0.69 g, 84%).

¹H NMR (400 MHz, CDCl₃) δ 7.59 – 7.05 (m, 12H), 6.74 – 6.48 (m, 3H), 4.85 (dd, *J* = 8.9, 3.5 Hz, 1H), 4.68 – 4.56 (m, 1H), 3.72 – 3.22 (m, 1H), 2.38 – 2.01 (m, 2H).

¹³C NMR (100 MHz, CDCl₃) δ 147.1, 147.1, 144.4, 144.2, 143.6, 143.2, 129.0, 129.0, 128.6, 128.6, 128.5, 128.5, 127.7, 127.6, 127.1, 126.9, 126.2, 126.1, 125.7, 125.6, 117.9, 117.4, 114.2, 113.6, 73.7, 71.8, 57.9, 55.3, 47.4, 46.7.

HRMS: Calculated for C₂₁H₂₁NO = 303.16231; Found = 303.16004

In order to separate both isomers, compound 5 was purified further by preparative TLC plate. The plate was developed three consecutive times at 20% EtOAc in hexanes in order to isolate two separate isomers.

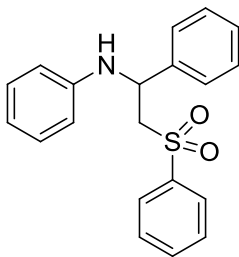
TOP ISOMER (6):

¹H NMR (400 MHz, CDCl₃) δ 7.37 – 7.29 (m, 9H), 7.13 – 7.06 (m, 2H), 6.69 – 6.63 (m, 1H), 6.54 (dd, J = 8.6, 1.0 Hz, 2H), 4.87 (dd, J = 8.4, 3.6 Hz, 1H), 4.63 (dd, J = 8.4, 3.9 Hz, 1H), 2.32 (ddd, J = 14.5, 8.4, 3.9 Hz, 1H), 2.18 (ddd, J = 14.5, 8.4, 3.6 Hz, 1H).

BOTTOM ISOMER (7):

¹H NMR (400 MHz, CDCl₃) δ 7.36 – 7.17 (m, 11H), 7.11 – 7.05 (m, 2H), 6.67 (t, J = 7.3 Hz, 1H), 6.57 (dd, J = 8.6, 1.0 Hz, 2H), 4.86 (dd, J = 9.3, 3.3 Hz, 1H), 4.59 (dd, J = 9.1, 5.0 Hz, 1H), 2.26 (dt, J = 14.5, 9.2 Hz, 1H), 2.05 (ddd, J = 14.5, 5.0, 3.4 Hz, 1H).

Preparation of N-(1-phenyl-2-(phenylsulfonyl)ethyl)aniline



Chemical Formula: $C_{20}H_{19}NO_2S$

Molecular Weight: 337.44

8

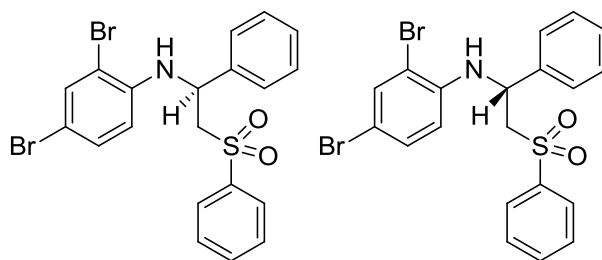
To a solution of **12** (1.5 g, 1.6 mmol) in DCM (10 mL) at 0°C, was slowly added mCPBA (2.4 g, 13.9 mmol). The mixture was set to warm to room temperature over 2 hours, at which time the reaction was quenched with 10% $NaHCO_3$ solution in H_2O and extracted with DCM (3 x 10 mL). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated in vacuo. Crude product was purified via gradient column chromatography (20-25% EtOAc in hexanes) yielding **8** as a yellow solid (0.4 g, 74%).

1H NMR (400 MHz, $CDCl_3$) δ 8.08 (t, J = 1.8 Hz, 1H), 8.01 – 7.97 (m, 1H), 7.85 (dd, J = 8.4, 1.2 Hz, 2H), 7.65 – 7.56 (m, 2H), 7.49 (dd, J = 10.7, 4.9 Hz, 2H), 7.42 (t, J = 7.9 Hz, 1H), 7.28 (dd, J = 6.8, 2.0 Hz, 3H), 7.10 – 7.05 (m, 2H), 6.71 (t, J = 7.3 Hz, 1H), 6.46 (d, J = 7.7 Hz, 2H), 4.72 (dd, J = 9.9, 3.6 Hz, 1H), 3.65 – 3.40 (m, 2H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 169.7, 140.5, 138.8, 134.7, 134.0, 133.8, 130.9, 130.2, 129.8, 129.4, 129.2, 129.1, 128.6, 128.3, 128.1, 128.0, 126.2, 118.6, 114.2, 62.2, 54.5.

HRMS: Calculated for $C_{20}H_{19}NO_2S$ = 337.11365; Found = 337.11249

Preparation of 2,4-dibromo-N-(1-phenyl-2-(phenylsulfonyl)ethyl)aniline



Chemical Formula: $C_{20}H_{17}Br_2NO_2S$
Molecular Weight: 495.23

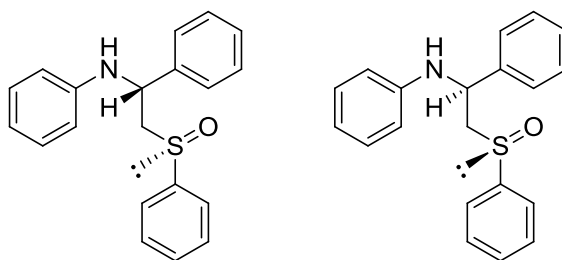
9

To a solution of **15** (0.06 g, 0.125 mmol) in DCM (5 mL) at 0°C, was slowly added mCPBA (0.06 g, 0.35 mmol). The mixture was set to warm to room temperature over 2 hours, at which time the reaction was quenched with 10% $NaHCO_3$ solution in H_2O and extracted with DCM (3 x 10 mL). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated in vacuo. Crude product was purified via gradient column chromatography (20-25% EtOAc in hexanes) yielding **9** as a yellow solid (0.055 g, 89%).

1H NMR (400 MHz, $CDCl_3$) δ 7.89 – 7.00 (m, 13H), 6.16 (t, $J = 19.2$ Hz, 1H), 4.80 (dd, $J = 10.0, 3.4$ Hz, 1H), 3.53 (ddd, $J = 18.1, 14.7, 6.7$ Hz, 2H).

HRMS: Calculated for $C_{20}H_{18}Br_2NO_2S = 492.93467$; Found = 492.93511

Preparation of N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: C₂₀H₁₉NOS

Molecular Weight: 321.44

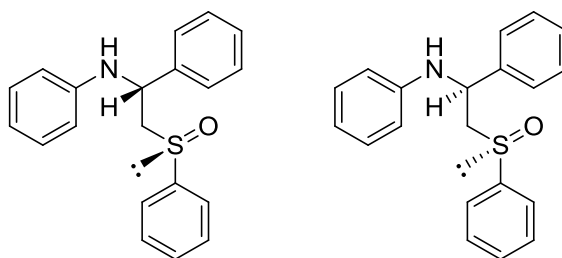
12

This compound was prepared following general procedure 2 using methyl phenyl sulfoxide (2.32 g, 16.55 mmol) in anhydrous THF (10 mL), LDA (8.28 mL, 16.55 mmol) and benzalaniline (1.5 g, 8.27 mmol) in anhydrous THF (5 mL). Crude product (0.92 g) was purified by gradient column chromatography (35-40% EtOAc in hexanes) resulting in **12** as an off white solid (0.46 g, 17.3%, R_f = 0.45 in 2:3 EtOAc : Hexanes) The obtained spectra matches those report in literature⁶.

¹H NMR (400 MHz, CDCl₃) δ 7.64 – 7.57 (m, 2H), 7.53 – 7.44 (m, 3H), 7.42 – 7.18 (m, 6H), 7.07 (dd, *J* = 8.5, 7.4 Hz, 2H), 6.67 (t, *J* = 7.3 Hz, 1H), 6.54 (d, *J* = 7.7 Hz, 2H), 4.83 (t, *J* = 6.2 Hz, 1H), 3.16 (d, *J* = 6.1 Hz, 2H).

¹³C NMR (100 MHz, CDCl₃) δ 146.2, 143.1, 140.9, 131.2, 129.4, 129.0, 127.8, 126.4, 124.0, 118.2, 114.1, 63.8, 54.9

Preparation of N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: C₂₀H₁₉NOS
Molecular Weight: 321.44

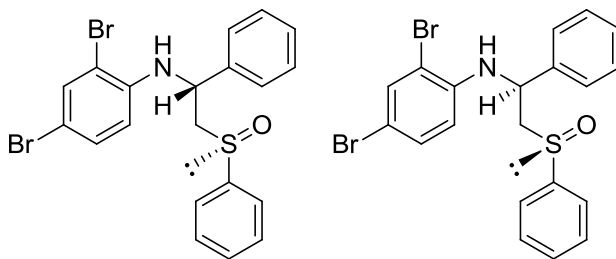
13

The procedure yielding **12** also gave **13** as an off white solid (0.19 g, 7.15%, R_f = 0.36 in 2:3 EtOAc : Hexanes) after purification by column chromatography (35-40% EtOAc in hexanes). The obtained spectra matches those reported in literature⁶.

¹H NMR (400 MHz, CDCl₃) δ 7.73 – 7.55 (m, 2H), 7.54 – 7.19 (m, 8H), 7.18 – 6.98 (m, 2H), 6.68 (t, *J* = 7.3 Hz, 1H), 6.61 – 6.43 (m, 2H), 5.10 (s, 5H), 5.02 – 4.74 (m, 1H), 3.27 (dd, *J* = 13.5, 9.7 Hz, 1H), 3.07 – 2.90 (m, 1H).

¹³C NMR (100 MHz, CDCl₃) δ 147.6, 145.7, 143.5, 131.1, 129.8, 129.5, 128.5, 126.7, 124.1, 120.8, 113.5, 62.5, 60.9.

Preparation of 2,4-dibromo-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: $C_{20}H_{17}Br_2NOS$
Molecular Weight: 479.23

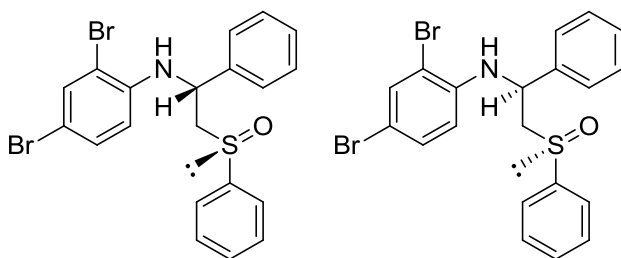
15

This compound was prepared following general produce 2 using methyl phenyl sulfoxide (1g, 7.13 mmol) in anhydrous THF (10 mL), LDA (3.21 mL, 6.42 mmol) and N-benzylidene-2,4-dibromoaniline (1.21 g, 3.57 mmol) in anhydrous THF (5 mL). Crude product (1.47 g) was purified by column chromatography (50% EtOAc in hexanes), affording a mixture of two isomers as a yellow solid (0.85 g, 50%). Separation of isomers was carried out using reverse phase HPLC (80% CAN in H_2O) affording compound **15** as a white solid (R_f =0.63 in 2:3 EtOAc : Hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.66 – 7.59 (m, 3H), 7.56 – 7.45 (m, 6H), 7.35 – 7.30 (m, 5H), 7.06 (dd, J = 8.7, 2.1 Hz, 1H), 6.22 (d, J = 8.8 Hz, 1H), 4.90 – 4.85 (m, 1H), 3.22 (dd, J = 13.8, 3.4 Hz, 1H), 3.10 (dd, J = 13.7, 9.4 Hz, 1H).

HRMS: Calculated for $C_{20}H_{18}Br_2NOS$ = 476.939476; Found = 476.94151

Preparation of 2,4-dibromo-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: $C_{20}H_{17}Br_2NOS$

Molecular Weight: 479.23

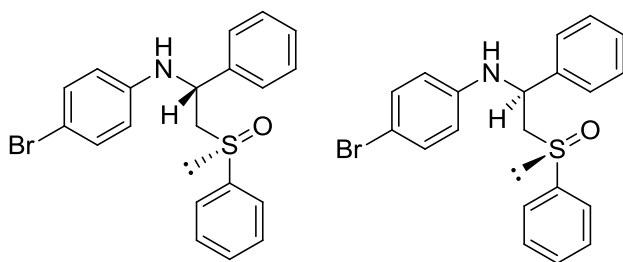
16

The procedure yielding **15** also gave **16** as a white solid ($R_f = 0.51$ in 2:3 EtOAc : Hexanes) after purification by reverse phase HPLC (80% ACN in H_2O).

1H NMR (400 MHz, $CDCl_3$) δ 7.64 (dd, $J = 6.6, 3.0$ Hz, 2H), 7.55 – 7.49 (m, 4H), 7.35 (t, $J = 6.1$ Hz, 4H), 7.30 (dd, $J = 13.2, 9.0$ Hz, 2H), 7.09 (dd, $J = 8.7, 2.3$ Hz, 1H), 6.26 (d, $J = 8.8$ Hz, 1H), 4.90 (d, $J = 8.9$ Hz, 1H), 3.33 (dd, $J = 13.5, 8.9$ Hz, 1H), 3.06 (dd, $J = 13.5, 5.5$ Hz, 1H).

HRMS: Calculated for $C_{20}H_{18}Br_2NOS = 476.939476$; Found = 476.94162

Preparation of 4-bromo-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: C₂₀H₁₈BrNOS

Molecular Weight: 400.33

17

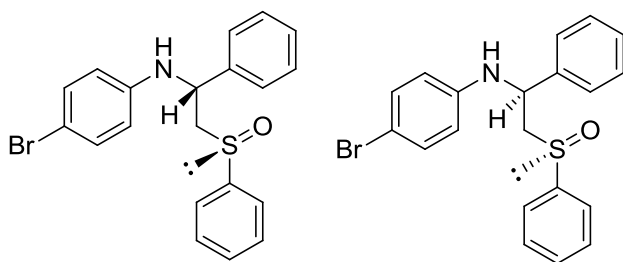
This compound was prepared following general procedure 2 using methyl phenyl sulfoxide (1.08 g, 7.69 mmol), in anhydrous THF (5 mL), LDA (3.46 mL, 6.92 mmol) and N-benzylidene-4-bromoaniline (1.0 g, 3.84 mmol) in anhydrous THF (5 mL). Crude product (1.72 g) was purified using gradient column chromatography (30-40% EtOAc in hexanes) yielding **17** as an off white solid (0.32 g, 20.8%, R_f = 0.58 in 2:3 EtOAc: hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.60 (ddd, *J* = 8.7, 3.9, 2.2 Hz, 2H), 7.51 – 7.45 (m, 3H), 7.39 – 7.27 (m, 4H), 7.12 – 7.04 (m, 2H), 6.67 (t, *J* = 7.3 Hz, 1H), 6.54 (d, *J* = 7.7 Hz, 2H), 4.82 (t, *J* = 6.2 Hz, 1H), 3.16 (d, *J* = 6.1 Hz, 2H).

¹³C NMR (100 MHz, CDCl₃) δ 146.1, 143.0, 140.8, 131.2, 129.3, 129.0, 129.0, 127.8, 126.3, 123.9, 118.3, 114.1, 63.7, 54.9, 30.9.

HRMS: Calculated for C₂₀H₁₈BrNOS = 399.02925; found = 399.02903

Preparation of 4-bromo-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: C₂₀H₁₈BrNOS

Molecular Weight: 400.33

18

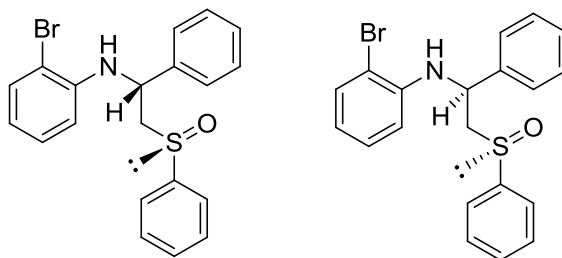
The procedure yielding **17** also gave **18** as an off white solid (0.04 g, 3%, R_f = 0.52 in 2:3 EtOAc : Hexanes) after purification by column chromatography (30-40% EtOAc in hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.70 – 7.59 (m, 2H), 7.49 (dd, *J* = 5.0, 1.9 Hz, 3H), 7.45 – 7.21 (m, 4H), 7.16 – 7.03 (m, 2H), 6.68 (s, 1H), 6.60 – 6.48 (m, 2H), 5.07 (s, 1H), 4.89 (dd, *J* = 9.7, 4.8 Hz, 1H), 3.26 (dd, *J* = 13.5, 9.7 Hz, 1H), 2.98 (dd, *J* = 13.5, 4.8 Hz, 1H).

¹³C NMR (100 MHz, CDCl₃) δ 146.4, 143.7, 141.6, 131.3, 129.4, 129.0, 129.0, 127.9, 126.1, 123.9, 118.0, 113.9, 65.02, 55.9.

HRMS: Calculated for C₂₀H₁₈BrNOS = 399.02925; found = 399.02912

Preparation of 2-bromo-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: $C_{20}H_{18}BrNOS$

Molecular Weight: 400.33

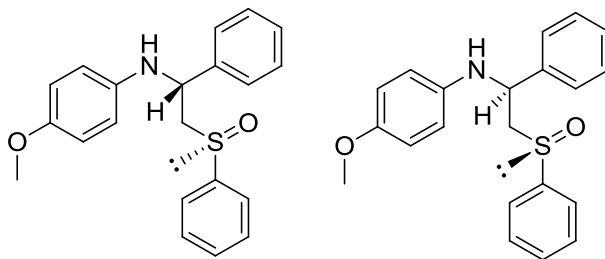
19

This compound was prepared following general procedure 2 using methyl phenyl sulfoxide (1.0 g, 7.13 mmol), in anhydrous THF (5 mL), LDA (3.21 mL, 6.42 mmol) and N-benzylidene-2-bromoaniline (0.93 g, 3.57 mmol) in anhydrous THF (5 mL). Crude product (1.35 g) was purified by gradient column chromatography (35% EtOAc in hexanes) yielding a mixture of two isomers (0.2 g, 14%, R_f = 0.47 in 2:3 EtOAc: hexanes). Compound **19** was recrystallized twice consecutively from DCM and hexanes.

1H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.60 (m, 2H), 7.56 – 7.17 (m, 10H), 7.03 – 6.93 (m, 1H), 6.54 (td, J = 7.8, 1.5 Hz, 1H), 6.41 (dd, J = 8.2, 1.3 Hz, 1H), 4.98 (dd, J = 9.6, 3.2 Hz, 1H), 3.23 (dd, J = 13.7, 3.3 Hz, 1H), 3.11 (dd, J = 13.7, 9.6 Hz, 1H).

HRMS: Calculated for $C_{20}H_{18}BrNOS$ = 399.02925; found = 399.03004

Preparation of 4-methoxy-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: $C_{21}H_{21}NO_2S$
Molecular Weight: 351.46

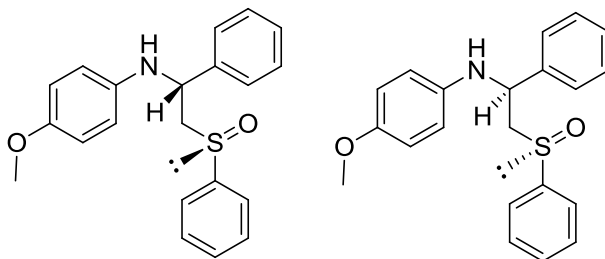
20

This compound was prepared following general procedure 2 using methyl phenyl sulfoxide (0.774 g, 5.52 mmol), in anhydrous THF (8 mL), LDA (2.48 mL, 4.96 mmol) and N-benzylidene-4-methoxyaniline (0.583 g, 2.75 mmol) in anhydrous THF (5 mL). Crude product (1.04 g) was purified by gradient column chromatography (45-50% EtOAc in hexanes) yielding **20** as a white solid (0.554 g, 32%, R_f = 0.39 in 2:3 EtOAc: hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, J = 1.9 Hz, 2H), 7.50 (dd, J = 5.0, 1.9 Hz, 3H), 7.36 – 7.27 (m, 4H), 7.26 – 7.21 (m, 1H), 6.72 – 6.66 (m, 2H), 6.53 (d, J = 8.8 Hz, 2H), 4.78 (dd, J = 8.3, 4.1 Hz, 1H), 3.69 (s, 3H), 3.15 (dd, J = 6.3, 5.0 Hz, 2H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 143.1, 141.1, 140.2, 131.1, 129.3, 128.9, 127.7, 126.3, 123.9, 115.6, 114.6, 63.9, 55.6, 55.4.

Preparation of 4-methoxy-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: C₂₁H₂₁NO₂S

Molecular Weight: 351.46

21

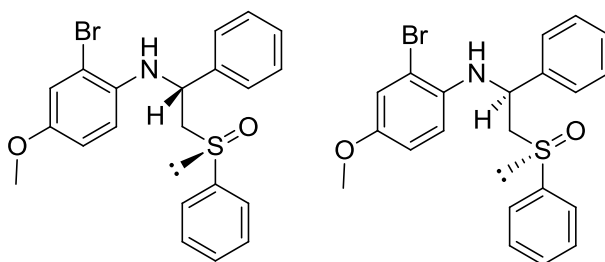
The procedure yielding **20** also gave **21** as an off white solid (0.191 g, 11%, R_f = 0.36 in 2:3

EtOAc : Hexanes) after purification by column chromatography (40-50% EtOAc in hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.68 – 7.61 (m, 2H), 7.55 – 7.27 (m, 9H), 7.24 (dd, *J* = 3.7, 2.5 Hz, 1H), 6.72 – 6.66 (m, 2H), 6.54 – 6.46 (m, 2H), 4.82 (dd, *J* = 9.6, 4.8 Hz, 1H), 3.70 (s, 3H), 3.28 (dd, *J* = 13.5, 9.6 Hz, 1H), 2.97 (dd, *J* = 13.5, 4.8 Hz, 1H).

¹³C NMR (100 MHz, CDCl₃) δ 152.5, 143.8, 141.8, 140.5, 131.3, 129.4, 129.1, 127.9, 126.3, 123.9, 115.4, 114.7, 65.0, 56.8, 55.6.

Preparation of 2-bromo-4-methoxy-N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline



Chemical Formula: $C_{21}H_{20}BrNO_2S$

Molecular Weight: 430.36

22

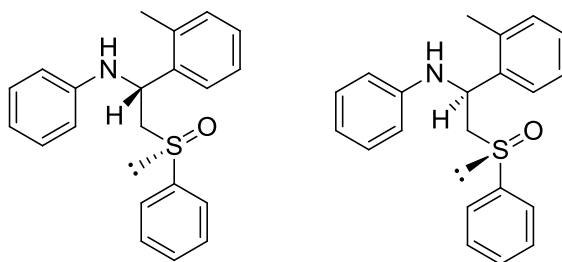
This compound was prepared following general procedure 3 using **1** (0.06 g, 0.171 mmol), NBS (0.03 g, 0.171 mmol) and a catalytic amount of PTSA in DCM (10 mL). Crude product was purified via gradient column chromatography (35-40% EtOAc in hexanes) to afford **22** as a white solid (0.03 g, 41%, R_f = 0.43 in 2:3 EtOAc : hexanes).

1H NMR (400 MHz, $CDCl_3$) δ ppm 7.71-7.45 (m, 5H), 7.38-7.18 (m, 5H), 7.04 (d, J = 2.81 Hz, 1H), 6.60 (dd, J = 8.94, 2.83 Hz, 1H), 6.39 (d, J = 8.93 Hz, 1H), 5.23 (d, J = 6.82 Hz, 1H), 4.91 (ddd, J = 9.61, 6.67, 2.94 Hz, 1H), 3.13 (ddd, J = 23.50, 13.58, 6.55 Hz, 2H), 3.67 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 152.1, 143.67, 140.7, 137.8, 131.2, 129.4, 129.0, 127.8, 126.2, 123.9, 118.2, 114.3, 114.1, 110.6, 65.4, 55.8, 54.6.

HRMS: Calculated for $C_{21}H_{20}BrNO_2S$ = 429.03981; found = 429.04655

Preparation of N-(2-(phenylsulfinyl)-1-(o-tolyl)ethyl)aniline



Chemical Formula: C₂₁H₂₁NOS

Molecular Weight: 335.46

24

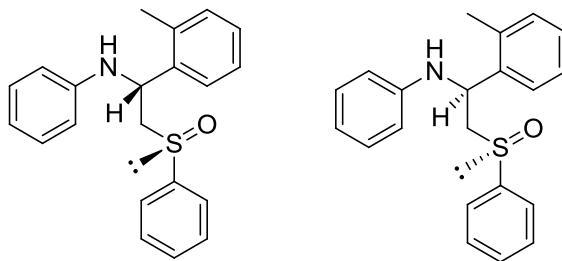
This compound was prepared following general procedure 2 using methyl phenyl sulfoxide (1.0 g, 7.13 mmol), in anhydrous THF (5 mL), LDA (3.21 mL, 6.42 mmol) and N-(2-methylbenzylidene)aniline (0.7 g, 3.57 mmol) in anhydrous THF (5 mL). Crude product was purified by gradient column chromatography (35-40% EtOAc in hexanes) yielding **24** as an off white solid (0.2 g, 17%, R_f = 0.43 in 2:3 EtOAc: hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.67 – 7.46 (m, 7H), 7.14 (s, 3H), 7.08 (dd, *J* = 8.5, 7.4 Hz, 2H), 6.69 (s, 1H), 6.48 (d, *J* = 7.8 Hz, 2H), 4.94 (dd, *J* = 9.4, 2.9 Hz, 1H), 3.23 – 3.13 (m, 1H), 3.01 (dd, *J* = 14.0, 2.9 Hz, 1H), 2.21 (s, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 146.3, 142.8, 138.6, 134.2, 131.1, 130.8, 129.3, 129.0, 127.5, 126.8, 125.8, 123.9, 118.1, 113.8, 61.3, 51.2, 18.6.

HRMS: Calculated for C₂₁H₂₁NOS = 335.13438; found = 335.13607

Preparation of N-(2-(phenylsulfinyl)-1-(o-tolyl)ethyl)aniline



Chemical Formula: $C_{21}H_{21}NOS$

Molecular Weight: 335.46

25

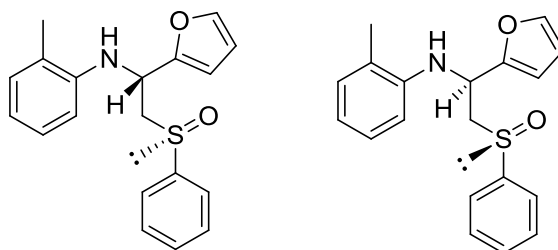
The procedure yielding **24** also gave **25** as an off white solid (0.09 g, 8%, $R_f = 0.31$ in 2:3 EtOAc : Hexanes) after purification by column chromatography (35-40% EtOAc in hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.64 (m, 2H), 7.52 (dd, $J = 4.9, 1.8$ Hz, 3H), 7.47 – 7.44 (m, 1H), 7.16 (d, $J = 2.7$ Hz, 3H), 7.09 (d, $J = 1.1$ Hz, 2H), 6.69 (dd, $J = 10.5, 4.2$ Hz, 1H), 6.46 (dd, $J = 8.6, 1.0$ Hz, 2H), 5.03 (dd, $J = 9.8, 4.4$ Hz, 1H), 3.24 (dd, $J = 13.7, 9.8$ Hz, 1H), 2.97 (dd, $J = 13.7, 4.4$ Hz, 1H), 2.34 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 146.3, 143.6, 139.0, 134.3, 131.3, 130.9, 129.3, 129.0, 127.5, 126.9, 125.3, 123.9, 117.9, 113.5, 63.3, 51.8, 18.8.

HRMS: Calculated for $C_{21}H_{21}NOS = 335.13438$; found = 335.13472

Preparation of N-(1-(furan-2-yl)-2-(phenylsulfinyl)ethyl)-2-methylaniline



Chemical Formula: C₁₉H₁₉NO₂S

Molecular Weight: 325.42

26

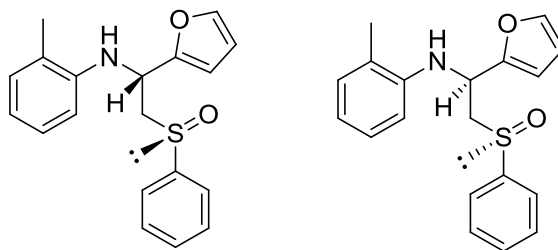
This compound was prepared following general procedure 2 using methyl phenyl sulfoxide (1.0 g, 7.13 mmol), in anhydrous THF (5 mL), LDA (3.21 mL, 6.42 mmol) and N-(furan-2-ylmethylene)-2-methylaniline (0.66 g, 3.57 mmol) in anhydrous THF (5 mL). Crude product (1.44 g) was purified by gradient column chromatography (40% EtOAc in hexanes) yielding **26** as a white solid (0.17 g, 15%, R_f = 0.49 in 2:3 EtOAc: hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.67 – 7.62 (m, 2H), 7.51 (dd, *J* = 5.1, 2.0 Hz, 3H), 7.37 – 7.34 (m, 1H), 7.11 – 7.01 (m, 2H), 6.69 (t, *J* = 7.4 Hz, 1H), 6.55 (d, *J* = 8.0 Hz, 1H), 6.33 – 6.23 (m, 2H), 5.08 (t, *J* = 5.9 Hz, 1H), 3.31 (d, *J* = 5.9 Hz, 2H), 2.26 (s, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 153.3, 144.3, 143.5, 142.1, 131.1, 130.1, 129.3, 126.8, 123.8, 123.0, 118.0, 111.2, 110.5, 107.5, 61.3, 48.9, 17.5.

HRMS: Calculated for C₁₉H₁₉NO₂S = 325.11365; found = 325.11560

Preparation of N-(1-(furan-2-yl)-2-(phenylsulfinyl)ethyl)-2-methylaniline



Chemical Formula: $C_{19}H_{19}NO_2S$
Molecular Weight: 325.42

27

The procedure yielding **26** also gave **27** as an off white solid (0.13 g, 12%, $R_f = 0.47$ in 2:3

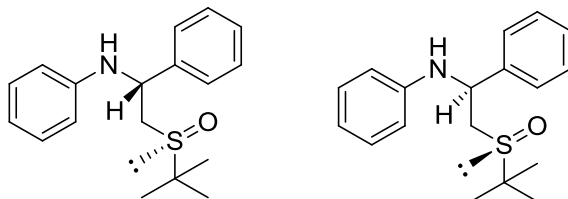
EtOAc : Hexanes) after purification by column chromatography (40% EtOAc in hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.66 – 7.61 (m, 2H), 7.50 (dd, $J = 5.1, 1.8$ Hz, 3H), 7.37 (t, $J = 1.3$ Hz, 1H), 7.07 (t, $J = 8.0$ Hz, 2H), 6.70 (dd, $J = 7.3, 6.8$ Hz, 1H), 6.60 (d, $J = 7.9$ Hz, 1H), 6.32 (d, $J = 1.3$ Hz, 2H), 5.11 (t, $J = 7.3$ Hz, 1H), 3.41 (dd, $J = 13.3, 7.8$ Hz, 1H), 3.22 (dd, $J = 13.3, 6.7$ Hz, 1H), 2.18 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 153.0, 143.9, 143.7, 142.2, 131.2, 130.2, 129.3, 126.9, 123.9, 123.0, 118.1, 110.9, 110.5, 107.5, 61.8, 48.9, 17.4.

HRMS: Calculated for $C_{19}H_{19}NO_2S = 325.11365$; found = 325.11573

Preparation of N-(2-(tert-butylsulfinyl)-1-phenylethyl)aniline



Chemical Formula: C₁₈H₂₃NOS

Molecular Weight: 301.45

28

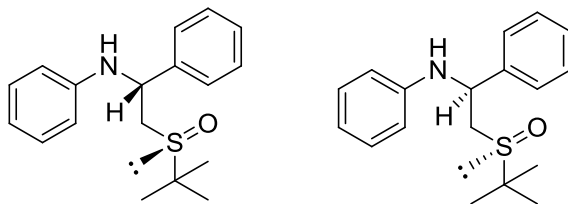
This compound was prepared following general procedure 2 using t-butyl methyl sulfoxide (0.4 g, 3.33 mmol) in anhydrous THF (5 mL), LDA (1.5 mL, 2.99 mL) and benzalaniline (0.3 g, 1.66 mmol) in anhydrous THF (5 mL). Crude product was purified by gradient column chromatography (35% EtOAc in hexanes) yielding **28** as a beige powder (0.03 g, 6%, R_f = 0.33 in 2:3 EtOAc: hexanes).

¹H NMR (400 MHz, CDCl₃) δ 8.26 (dd, *J* = 7.5, 2.0 Hz, 2H), 7.46 (t, *J* = 7.2 Hz, 2H), 7.38 (t, *J* = 7.5 Hz, 1H), 7.30 (t, *J* = 7.3 Hz, 2H), 7.09 (t, *J* = 7.9 Hz, 2H), 6.68 (t, *J* = 7.3 Hz, 2H), 6.57 (d, *J* = 7.9 Hz, 1H), 5.01 – 4.77 (m, 1H), 2.93 – 2.75 (m, 2H), 1.26 (s, 9H).

¹³C NMR (100 MHz, CDCl₃) δ 147.0, 142.5, 129.2, 128.0, 126.3, 118.0, 114.1, 57.0, 53.8, 52.9, 22.8.

HRMS: Calculated for C₁₈H₂₃NOS = 301.15004; Found = 301.15449

Preparation of N-(2-(tert-butylsulfinyl)-1-phenylethyl)aniline



Chemical Formula: $C_{18}H_{23}NO$

Molecular Weight: 301.45

29

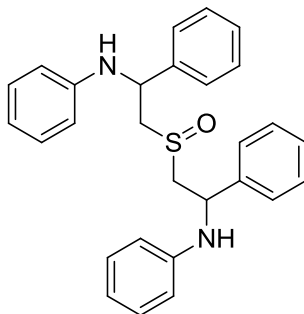
The procedure yielding **28** also gave **29** as a beige solid (0.06 g, 12%, R_f = 0.21 in 2:3 EtOAc : Hexanes) after purification by column chromatography (35% EtOAc in hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.40 (m, 2H), 7.35 (dd, J = 8.1, 6.8 Hz, 2H), 7.27 (dd, J = 8.7, 5.8 Hz, 1H), 7.13 – 7.04 (m, 2H), 6.66 (t, J = 7.3 Hz, 1H), 6.61 – 6.53 (m, 2H), 5.08 (dd, J = 8.4, 3.0 Hz, 1H), 3.00 (dd, J = 12.9, 3.0 Hz, 1H), 2.82 (dd, J = 12.9, 8.4 Hz, 1H), 1.24 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 146.4, 141.3, 129.1, 128.9, 127.6, 126.4, 117.8, 113.8, 53.9, 53.2, 53.1, 22.8.

HRMS: Calculated for $C_{18}H_{23}NO$ = 301.15004; Found = 301.15324

Preparation of N,N'-(sulfinylbis(1-phenylethane-2,1-diyl))dianiline



Chemical Formula: $C_{28}H_{28}N_2OS$

Molecular Weight: 440.60

30

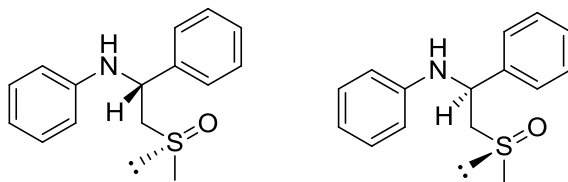
This compound was prepared following general procedure 2 using dimethyl sulfoxide (0.86 g, 11.04 mmol), in anhydrous THF (10 mL), LDA (11 mL, 22.07 mmol) and N-benzylideneaniline (1.0 g, 5.52 mmol) in anhydrous THF (5 mL). Crude product was purified by gradient column chromatography (40-45% EtOAc in hexanes) yielding **30** as an orange solid (0.41 g, 17%, R_f = 0.3 in 2:3 EtOAc: hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.26 (m, 10H), 7.10 (dt, J = 5.4, 4.8 Hz, 4H), 6.71 (t, J = 7.7 Hz, 2H), 6.57 (d, J = 7.7 Hz, 4H), 4.96 (ddd, J = 14.5, 8.7, 6.3 Hz, 4H), 3.09 (ddd, J = 18.8, 13.2, 7.2 Hz, 4H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 147.6, 143.5, 129.5, 128.5, 126.9, 126.7, 120.8, 113.5, 61.3, 60.6.

HRMS: Calculated for $C_{28}H_{28}N_2OS$ = 440.19223; found = 440.19249

Preparation of N-(2-(methylsulfinyl)-1-phenylethyl)aniline



Chemical Formula: C₁₅H₁₇NOS
Molecular Weight: 259.37

31

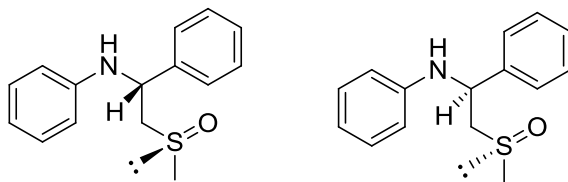
This compound was prepared following general procedure 2 using dimethyl sulfoxide (0.4 mL, 5.52 mmol) in anhydrous THF (5 mL), LDA (1.4 mL, 2.76 mmol) and benzalaniline (0.5 g, 2.76 mmol) in anhydrous THF (5 mL). Crude product (0.52 g) was purified by gradient column chromatography (40-50% EtOAc in hexanes) resulting in two isomers as an off white solid (0.20g, 27.9%). Separation of isomers by preparative TLC (Developed 10 consecutive times in 40% EtOAc in hexanes), gave final desired product (RcSs/ScRs isomer: R_f= 0.36 in 2:3 EtOAc:hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.47 – 7.42 (m, 2H), 7.37 (dd, *J* = 10.1, 4.8 Hz, 2H), 7.33 – 7.27 (m, 1H), 7.16 – 7.07 (m, 2H), 6.70 (t, *J* = 7.3 Hz, 1H), 6.58 (d, *J* = 7.7 Hz, 2H), 4.91 (dd, *J* = 9.2, 5.5 Hz, 1H), 3.24 (dd, *J* = 13.3, 9.2 Hz, 1H), 2.97 (dd, *J* = 13.3, 5.5 Hz, 1H), 2.61 (s, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 146.0, 141.3, 129.1, 129.1, 128.1, 126.3, 118.5, 114.4, 61.4, 56.4, 39.5.

HRMS: Calculated for C₁₅H₁₇NOS = 259.10308; found = 259.10487

Preparation of N-(2-(methylsulfinyl)-1-phenylethyl)aniline



Chemical Formula: $C_{15}H_{17}NOS$

Molecular Weight: 259.37

32

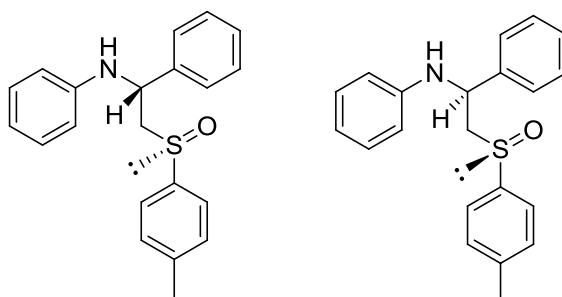
The procedure yielding **31** also gave **32** as a white solid (RcRs/ScSs isomer: $R_f = 0.19$ in 2:3 EtOAc : Hexanes) after purification by preparative TLC plate (10 consecutive elutions in 40% EtOAc in hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.40 (m, 2H), 7.38 – 7.32 (m, 2H), 7.29 (d, $J = 7.2$ Hz, 1H), 7.15 – 7.05 (m, 2H), 6.68 (t, $J = 7.3$ Hz, 1H), 6.57 (dd, $J = 8.6, 0.9$ Hz, 2H), 5.06 (dd, $J = 8.6, 3.3$ Hz, 1H), 3.22 (dd, $J = 13.4, 3.3$ Hz, 1H), 3.05 (dd, $J = 13.4, 8.6$ Hz, 1H), 2.62 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 145.7, 140.6, 129.1, 129.0, 127.9, 126.4, 118.5, 114.3, 61.2, 54.6, 38.7.

HRMS: Calculated for $C_{15}H_{17}NOS$ = 259.10308; found = 259.10493

Preparation of N-(1-phenyl-2-(p-tolylsulfinyl)ethyl)aniline



Chemical Formula: C₂₁H₂₁NOS
Molecular Weight: 335.46

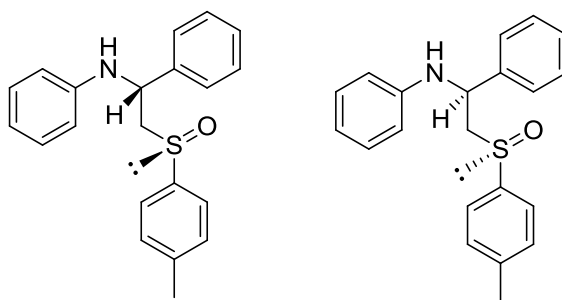
33

This compound was prepared following general procedure 2 using p-tolyl methyl sulfoxide (1.7 g, 11.04 mmol), in anhydrous THF (10 mL), LDA (4.97 mL, 9.94 mmol) and benzalaniline (1 g, 5.52 mmol) in anhydrous THF (5 mL). Crude product (2.21 g) was purified by gradient column chromatography (30-35% EtOAc in hexanes) yielding a crude separation of both isomers. Isomers were further purified by recrystallization using DCM and MeOH. The obtained spectra match those reported in literature⁶.

¹H NMR (400 MHz, CDCl₃) δ 7.54 – 7.49 (m, 2H), 7.37 – 7.27 (m, 6H), 7.27 – 7.21 (m, 2H), 7.17 – 7.11 (m, 2H), 6.45 – 6.27 (m, 2H), 4.81 (dd, *J* = 10.0, 4.4 Hz, 1H), 3.20 (dd, *J* = 13.6, 10.1 Hz, 1H), 2.94 (dd, *J* = 13.6, 4.5 Hz, 1H), 2.39 (s, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 145.4, 142.0, 141.2, 140.3, 131.7, 130.1, 129.1, 128.0, 126.0, 123.9, 115.6, 109.9, 64.7, 56.2, 21.4.

Preparation of N-(1-phenyl-2-(p-tolylsulfinyl)ethyl)aniline



Chemical Formula: C₂₁H₂₁NOS
Molecular Weight: 335.46

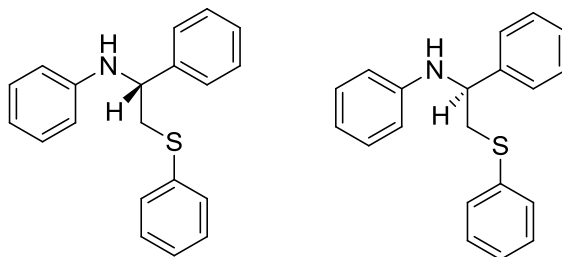
34

The procedure yielding **33** also gave **34** as a white solid, after purification by recrystallization (DCM/MeOH). The obtained spectra match those reported in the literature⁶.

¹H NMR (400 MHz, CDCl₃) δ 7.46 (d, *J* = 8.1 Hz, 2H), 7.35 – 7.21 (m, 8H), 7.13 (d, *J* = 8.7 Hz, 2H), 6.38 (d, *J* = 8.7 Hz, 2H), 4.71 (dd, *J* = 7.3, 4.6 Hz, 1H), 3.22 – 3.02 (m, 2H), 2.39 (s, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 145.3, 141.9, 140.5, 139.3, 131.8, 130.1, 129.0, 127.9, 126.3, 124.0, 115.6, 109.9, 62.9, 55.2, 21.4.

Preparation of N-(1-phenyl-2-(phenylthio)ethyl)aniline



Chemical Formula: C₂₀H₁₉NS
Molecular Weight: 305.44

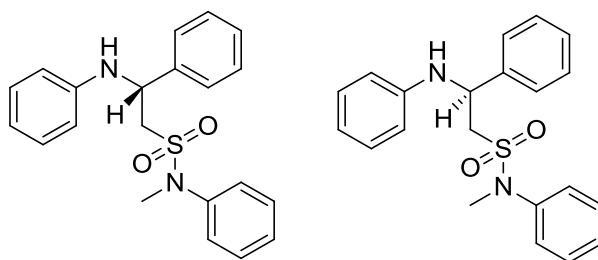
35

To a solution of **12** (0.15 g, 0.47 mmol) and I₂ (0.24 g, 0.94 mmol) in THF (10 mL) was slowly added NaBH₄ (0.018 g, 0.47 mmol). The mixture was stirred at room temperature and the progress of the reaction was followed by TLC. After stirring for 2 hours, the reaction was quenched with a 10% solution of NaOH. The aqueous layer was extracted with Et₂O (3 x 20 mL) and the combined organic extracts were washed successively with 5% aqueous Na₂S₂O₃ and H₂O. The resulting organic layer was dried (MgSO₄) and concentrated in vacuo. Purification of crude product (0.21 g) was done via gradient column chromatography (15-20% EtOAc in Hexanes) affording the final product (0.07g, 49%, R_f = 0.76 in 2:3 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.44 – 7.19 (m, 9H), 7.06 (dd, *J* = 8.5, 7.4 Hz, 2H), 6.66 (t, *J* = 7.3 Hz, 1H), 6.46 (d, *J* = 7.7 Hz, 2H), 4.39 (dd, *J* = 9.1, 4.5 Hz, 1H), 3.38 (dd, *J* = 13.5, 4.5 Hz, 1H), 3.18 (d, *J* = 9.2 Hz, 1H).

¹³C NMR (100 MHz, CDCl₃) δ 147.1, 142.3, 135.0, 130.6, 129.1, 129.0, 128.8, 127.6, 126.9, 126.3, 117.9, 113.8, 57.3, 42.6.

Preparation of N-methyl-N,2-diphenyl-2-(phenylamino)ethanesulfonamide



Chemical Formula: $C_{21}H_{22}N_2O_2S$
Molecular Weight: 366.48

36

This compound was prepared following general procedure 2 using N-methyl-N-phenylmethane sulfonamide (0.25g, 1.35mmol) in anhydrous THF (3mL), LDA (0.61mL, 1.21mmol) and benzalaniline (0.122g, 0.67mmol) in anhydrous THF (3mL). Crude product (0.27g) was purified by gradient column chromatography (30-35% EtOAc in hexanes) yielding **36** as a yellow solid (0.07g, 28.5%, R_f = 0.39 in 2:3 EtOAc: hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.23 (m, 11H), 7.09 – 7.02 (m, 2H), 6.71 – 6.64 (m, 1H), 6.49 (dd, J = 8.6, 1.0 Hz, 2H), 4.94 – 4.79 (m, 1H), 3.34 – 3.29 (m, 5H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 146.4, 141.3, 140.8, 129.4, 129.1, 129.0, 127.9, 127.5, 126.2, 126.1, 118.3, 114.1, 55.5, 54.6, 38.4.

HRMS: Calculated for $C_{21}H_{22}N_2O_2S$ = 366.14020; found = 366.14385

1.3.3. NMR Spectra

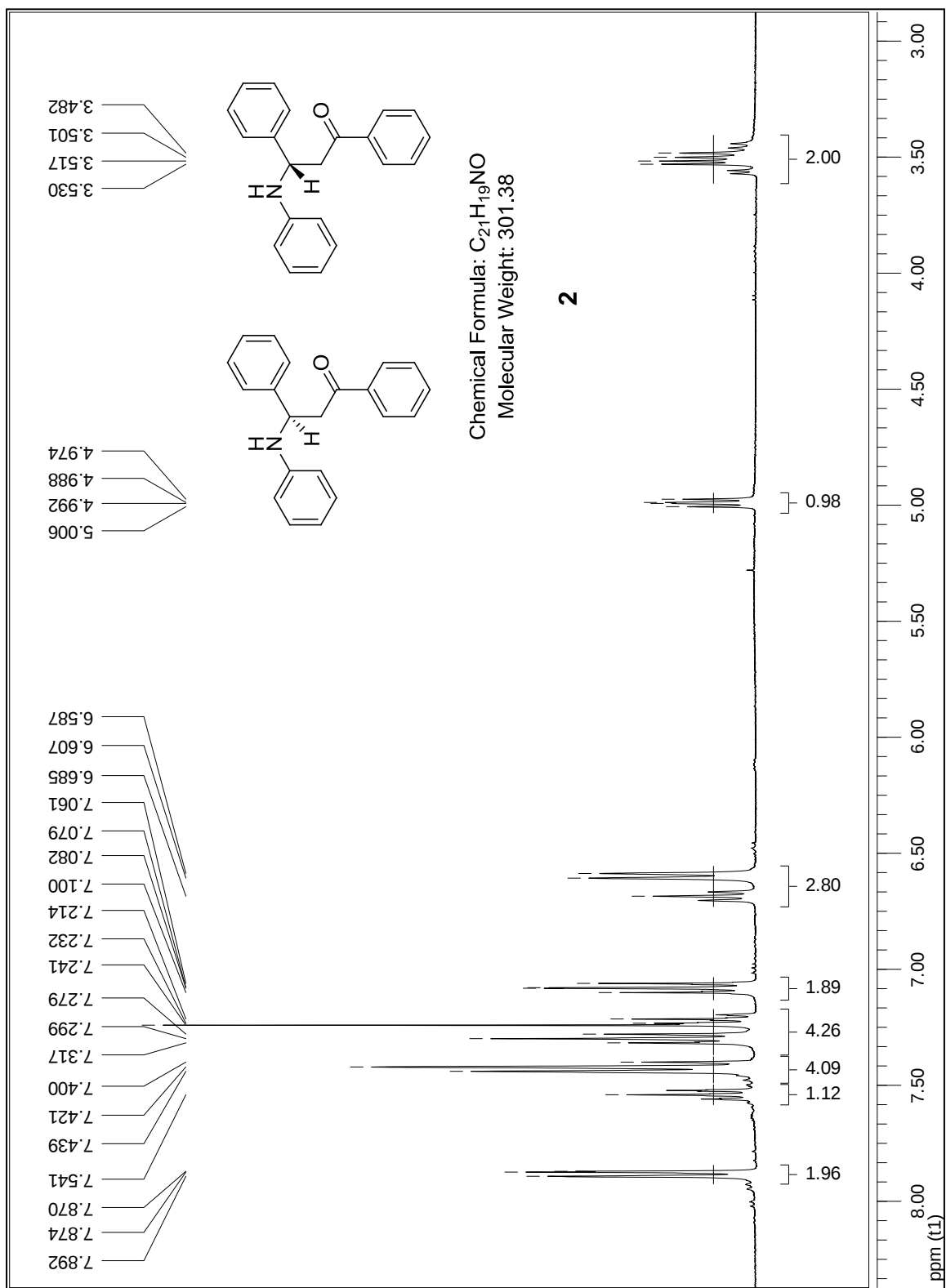


Figure 1.3.3.1. 400 MHz 1H NMR spectrum of compound **2** in $CDCl_3$.

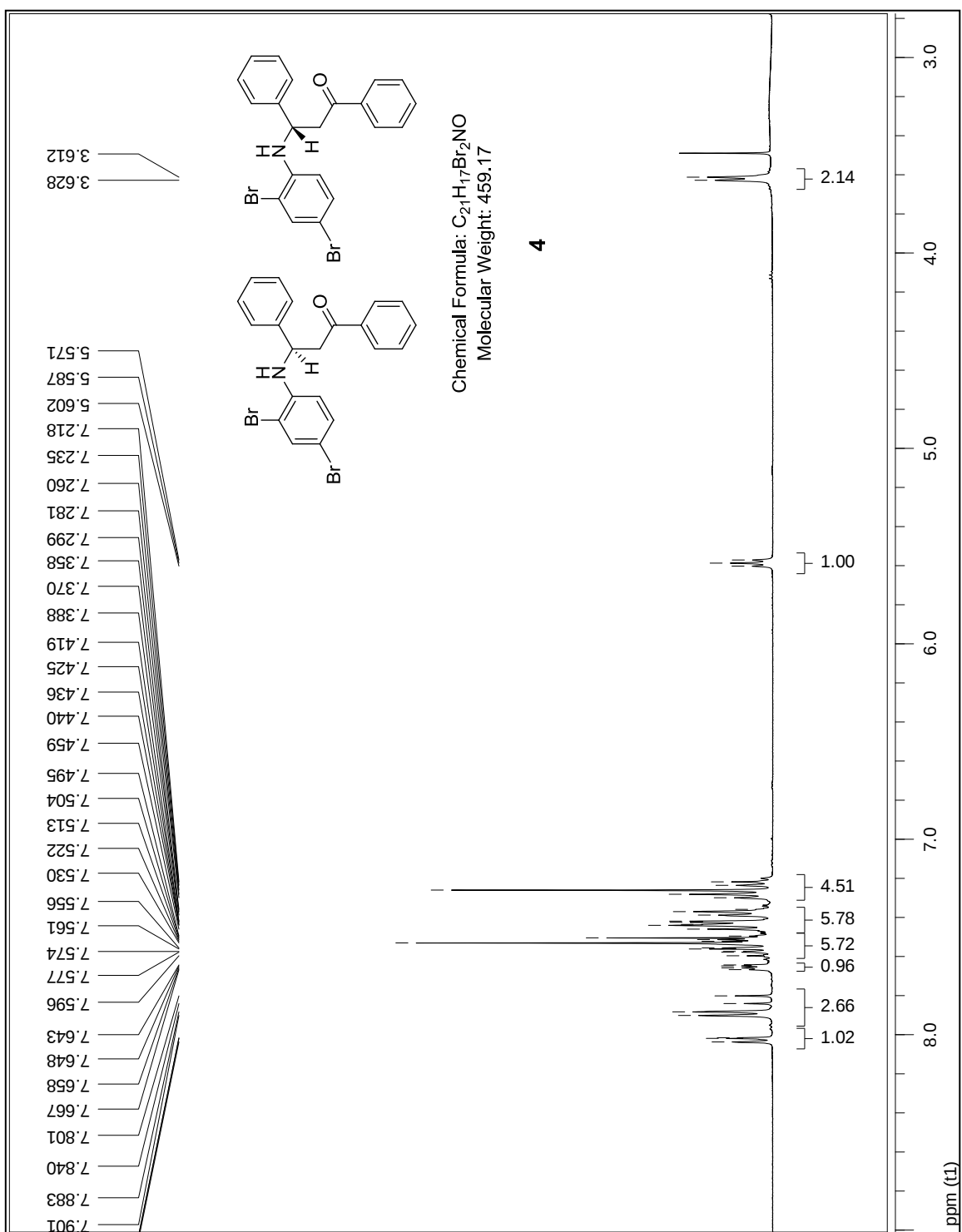


Figure 1.3.3.2. 400 MHz 1H NMR spectrum of compound **4** in $CDCl_3$.

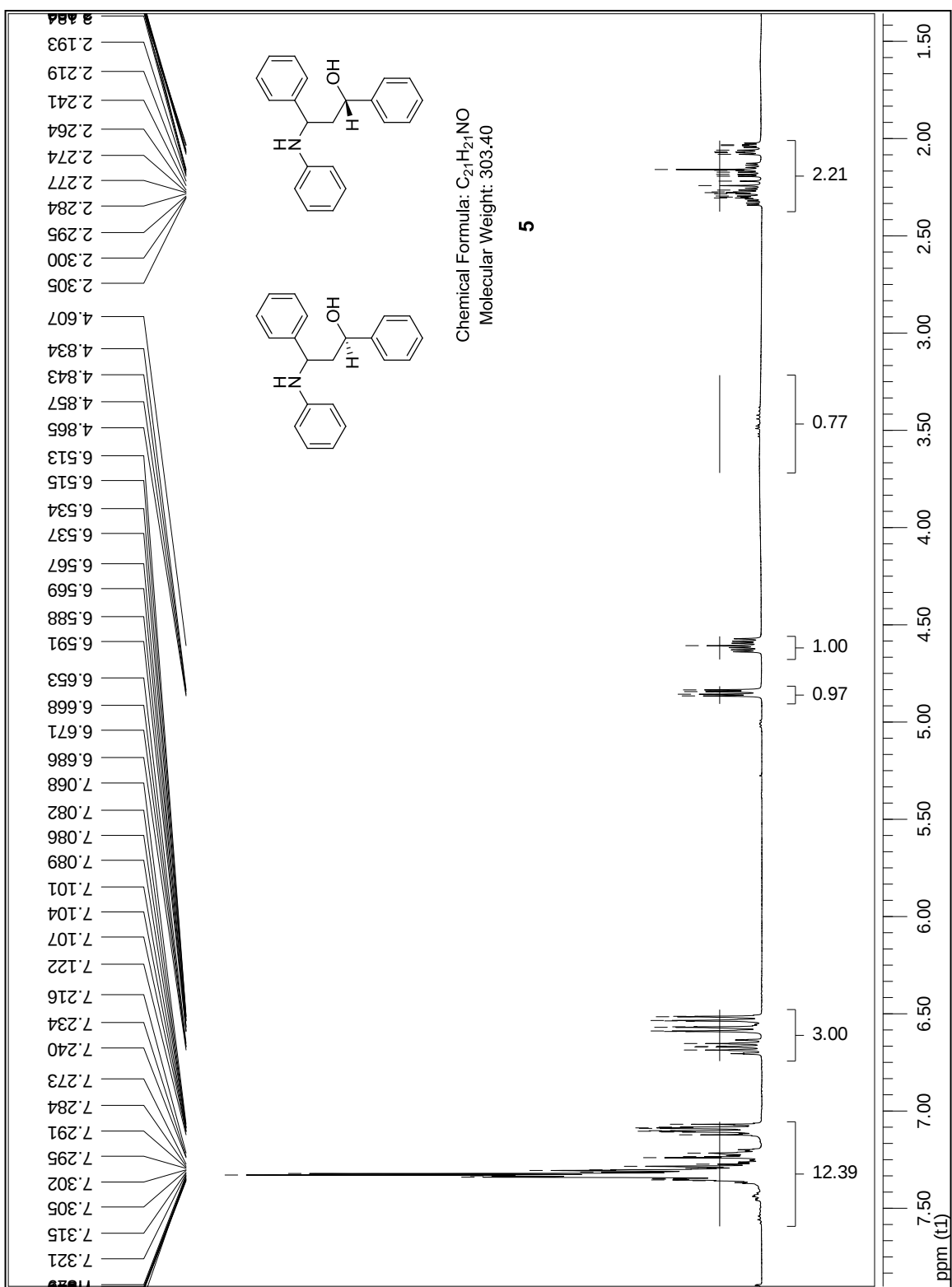


Figure 1.3.3.3. 400 MHz ^1H NMR spectrum of compound 5 in CDCl_3 .

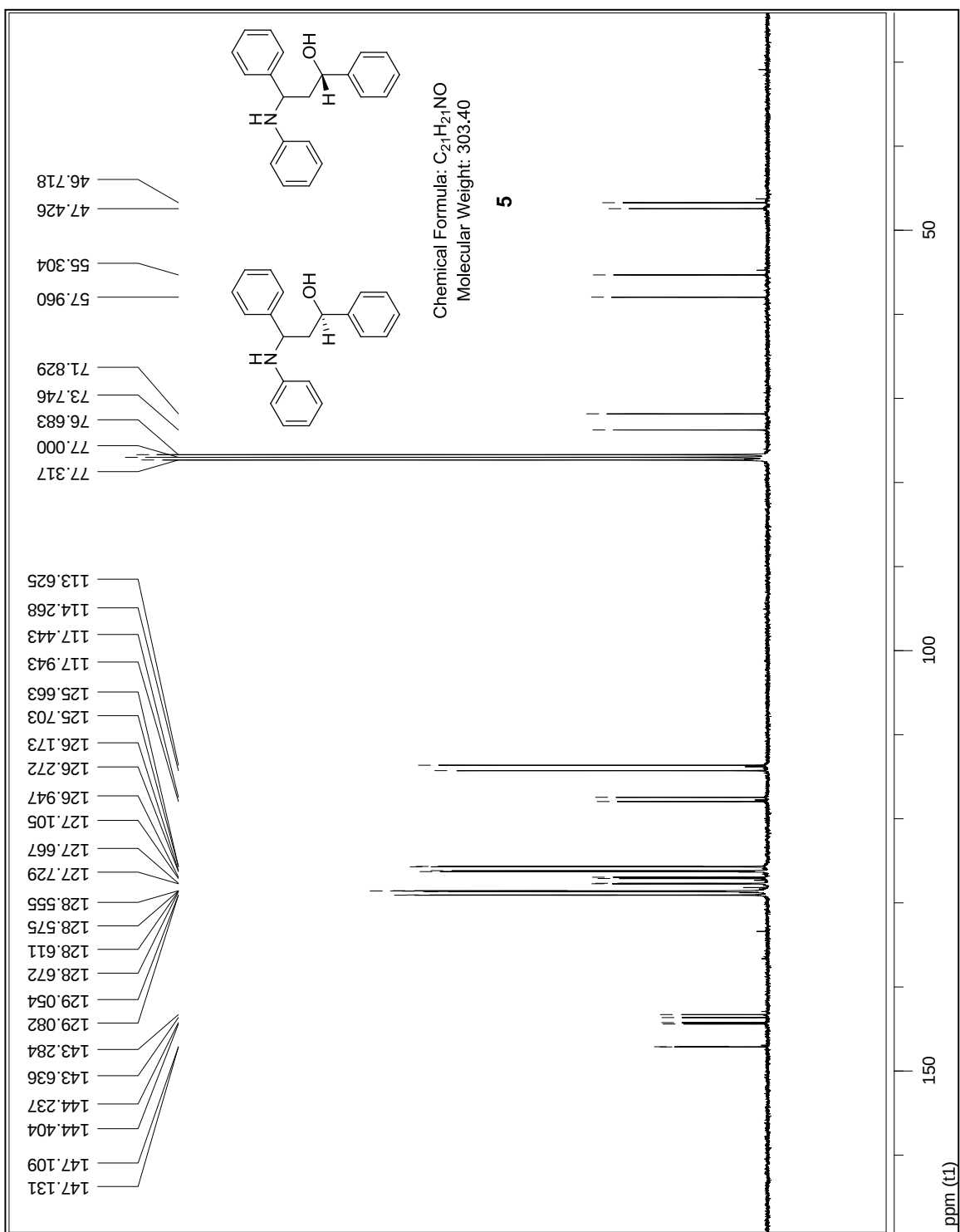


Figure 1.3.3.4. 100 MHz ^{13}C NMR spectrum of compound **5** in $CDCl_3$.

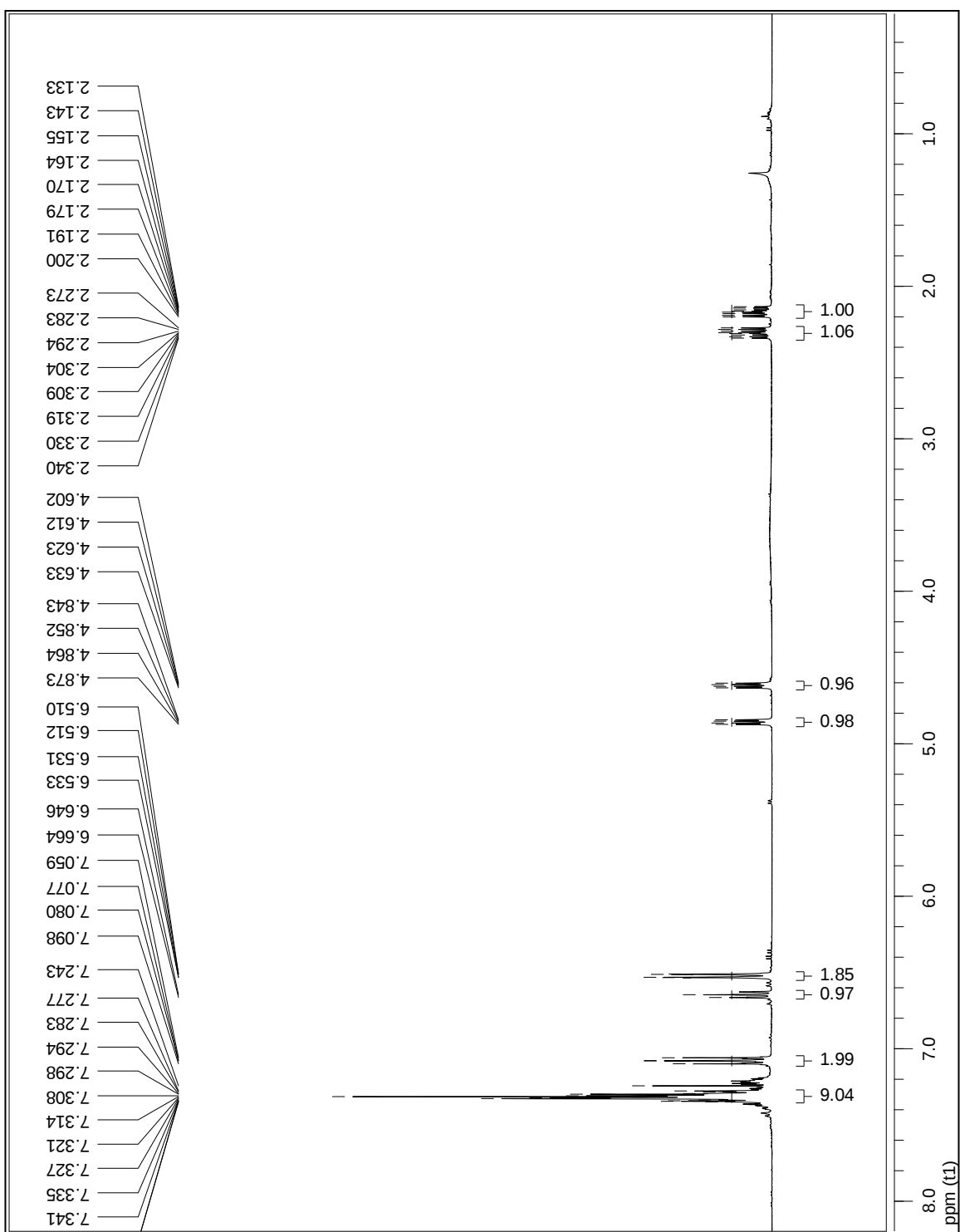


Figure 1.3.3.5. 400 MHz ^1H NMR spectrum of compound **6** in CDCl_3 .

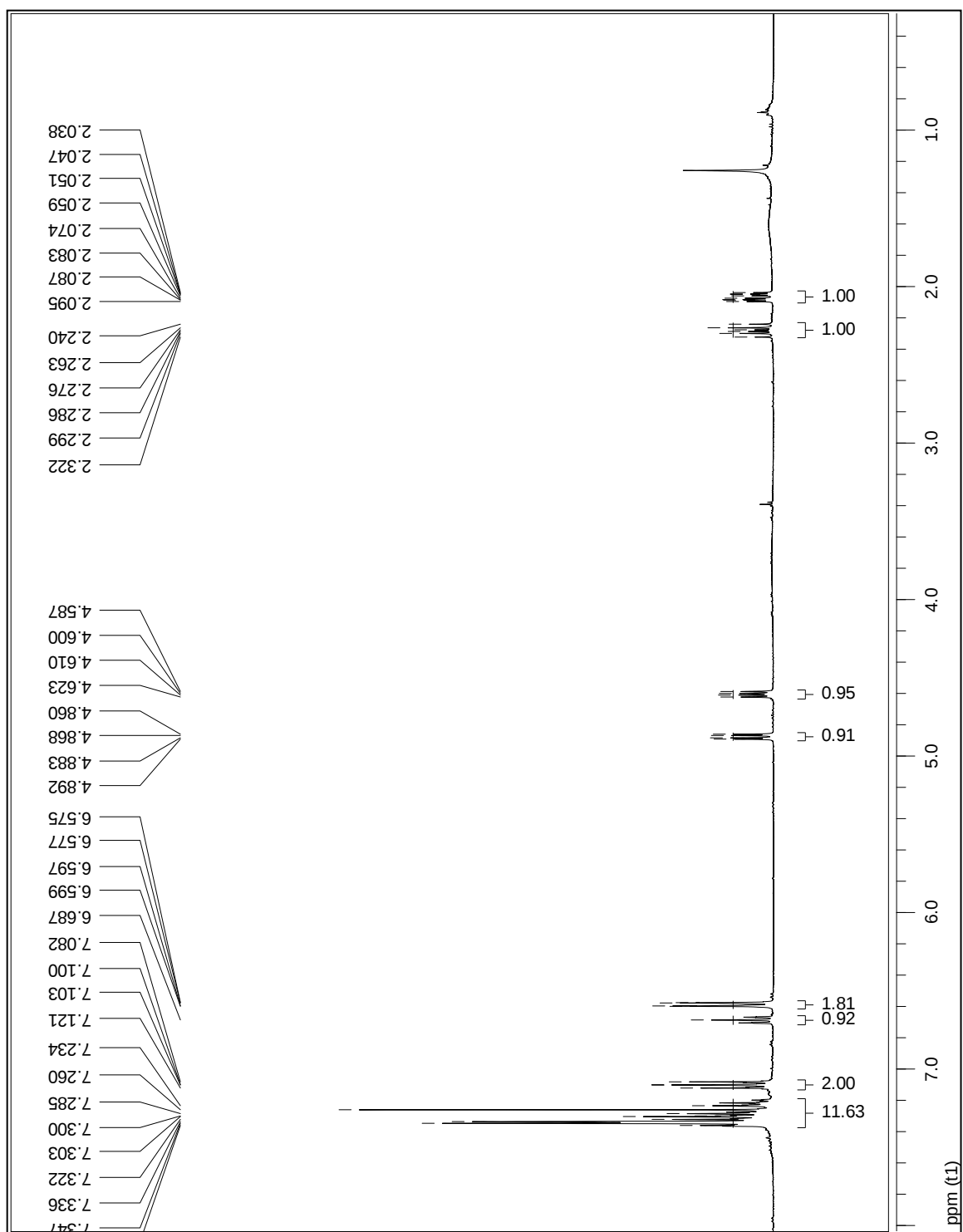
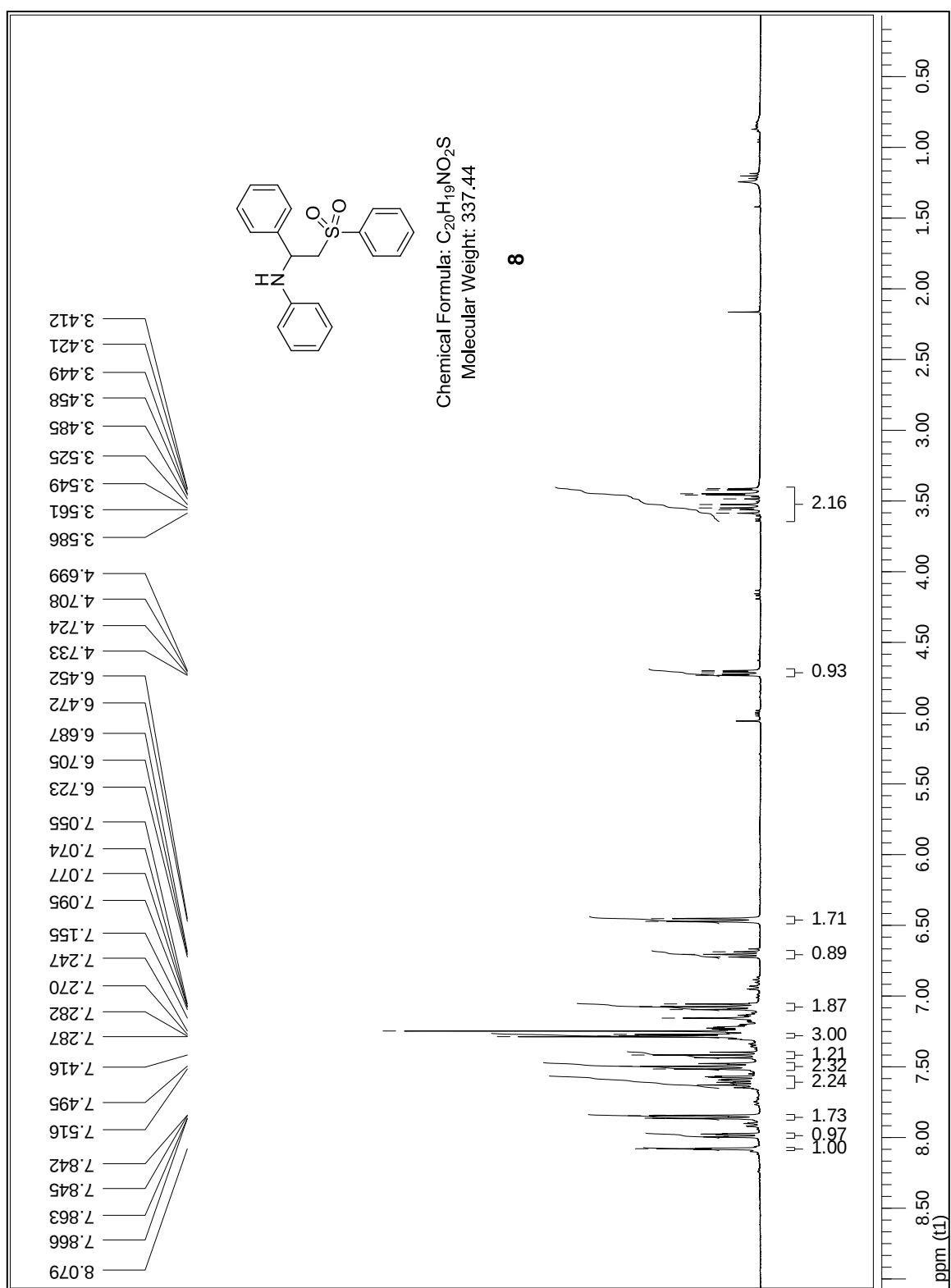


Figure 1.3.3.6. 400 MHz ^1H NMR spectrum of compound **7** in CDCl_3 .



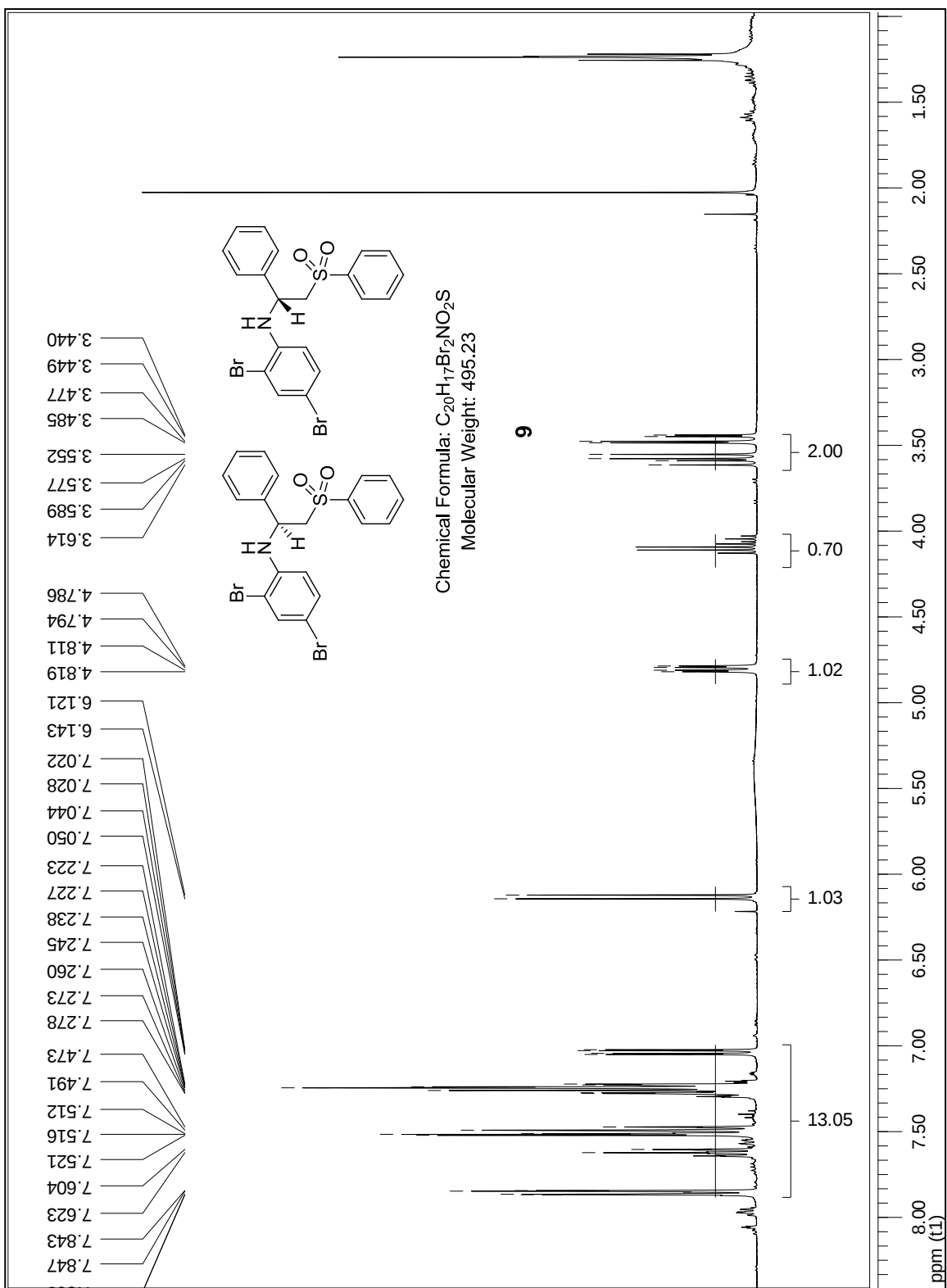


Figure 1.3.3.8. 400 MHz 1H NMR spectrum of compound **9** in $CDCl_3$.

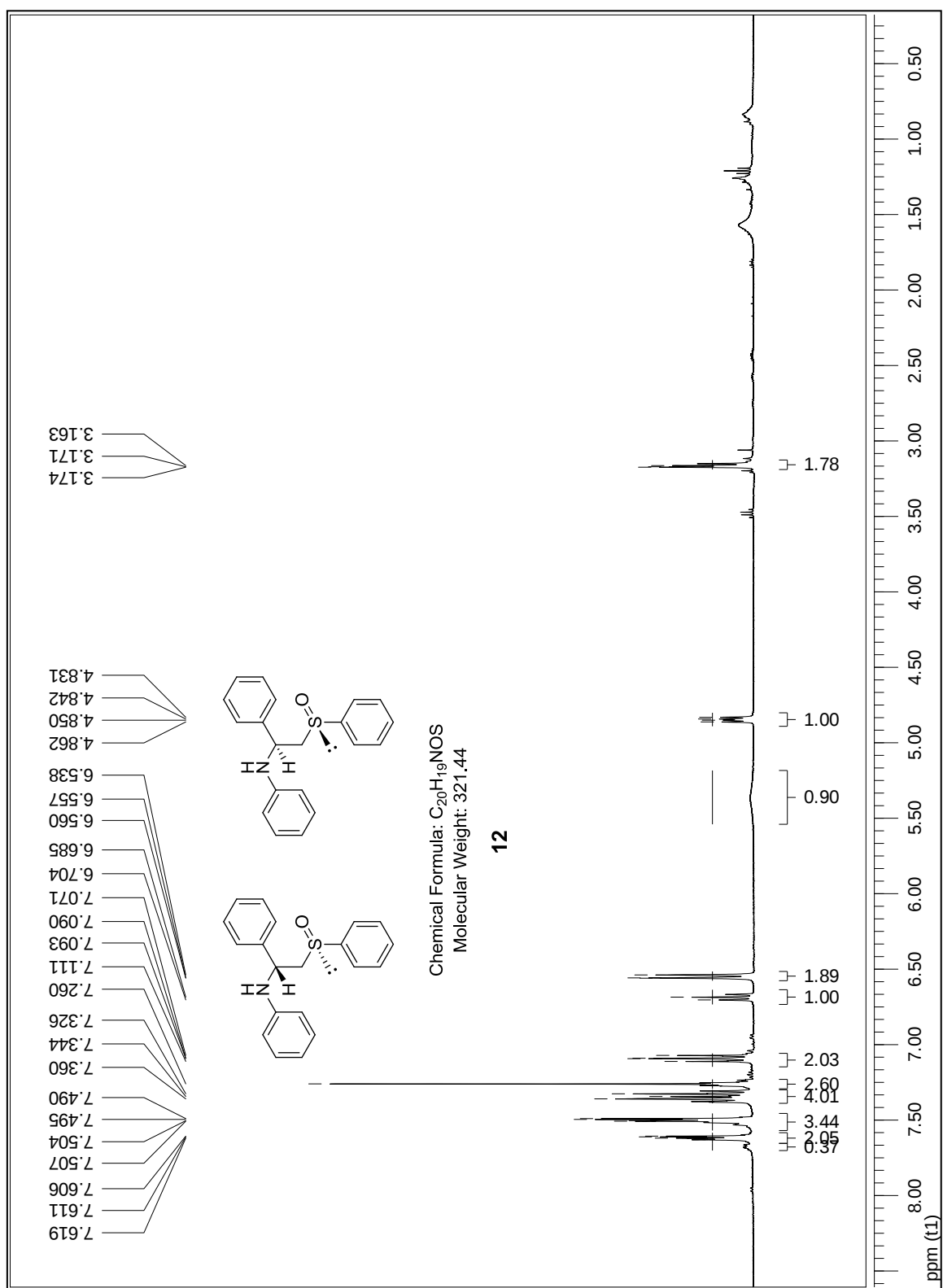


Figure 1.3.3.9. 400 MHz ¹H NMR spectrum of compound **12** in CDCl₃.

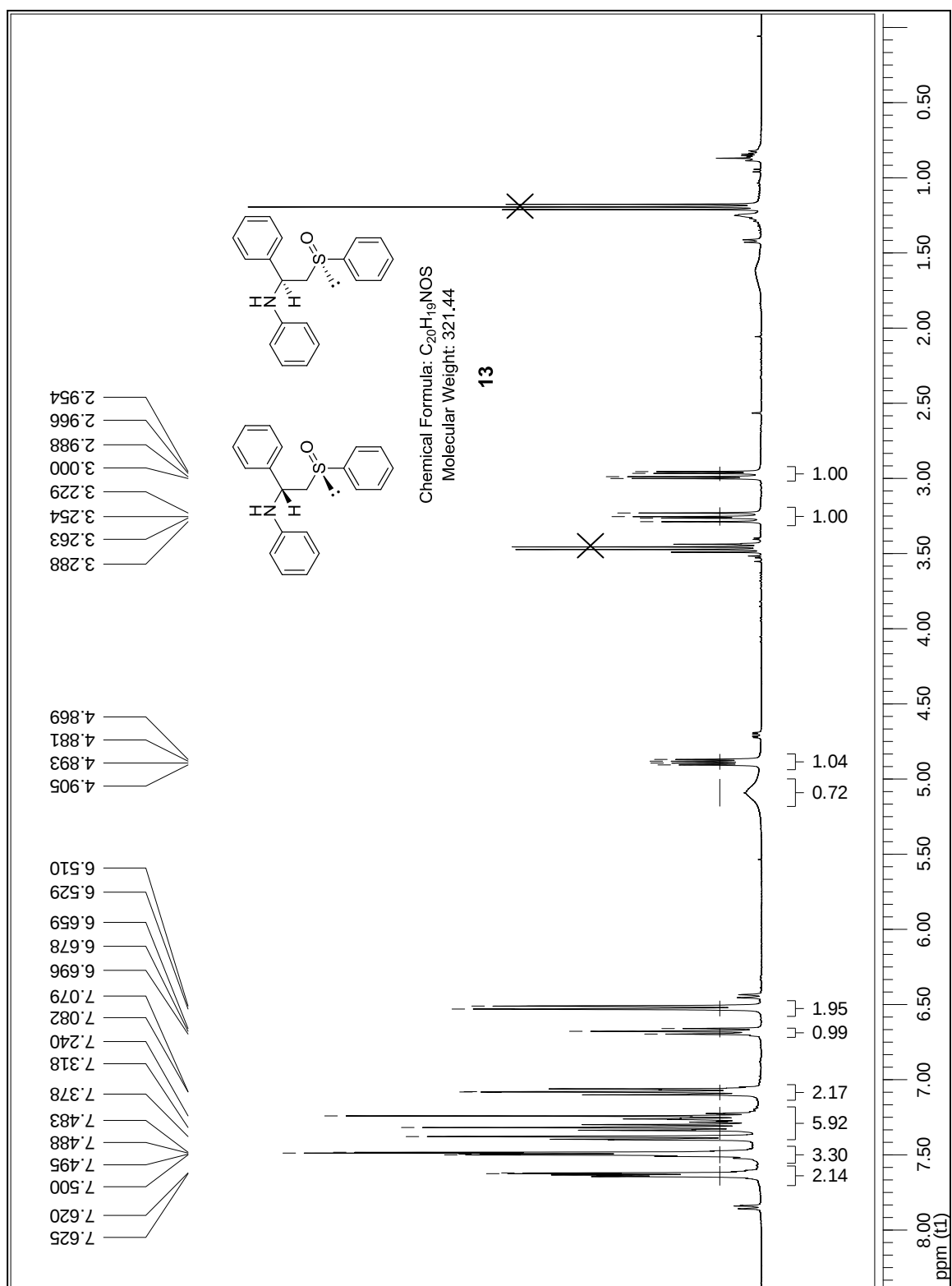


Figure 1.3.3.10. 400 MHz 1H NMR spectrum of compound **13** in $CDCl_3$.

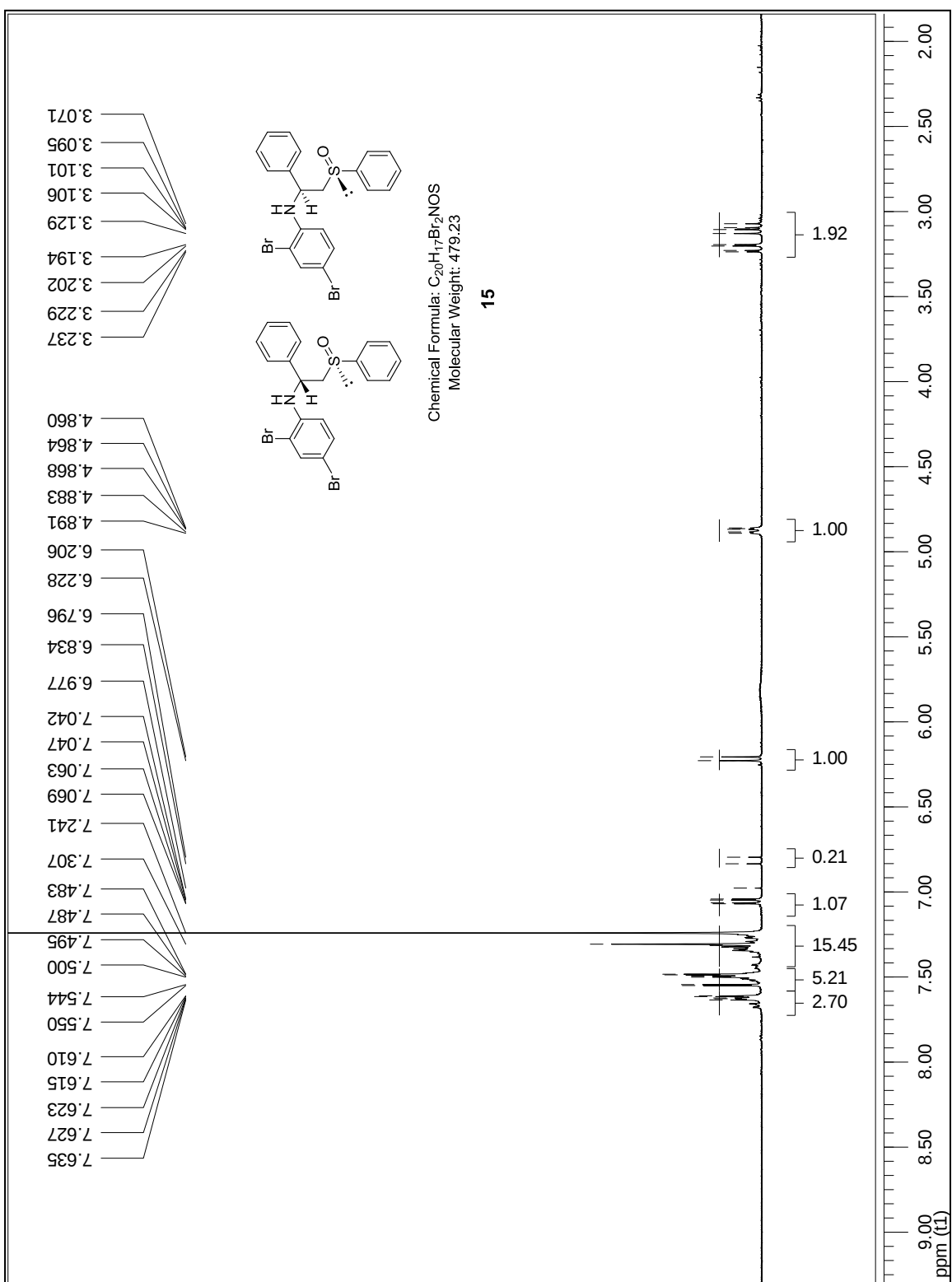


Figure 1.3.3.11. 400 MHz ^1H NMR spectrum of compound **15** in CDCl_3 .

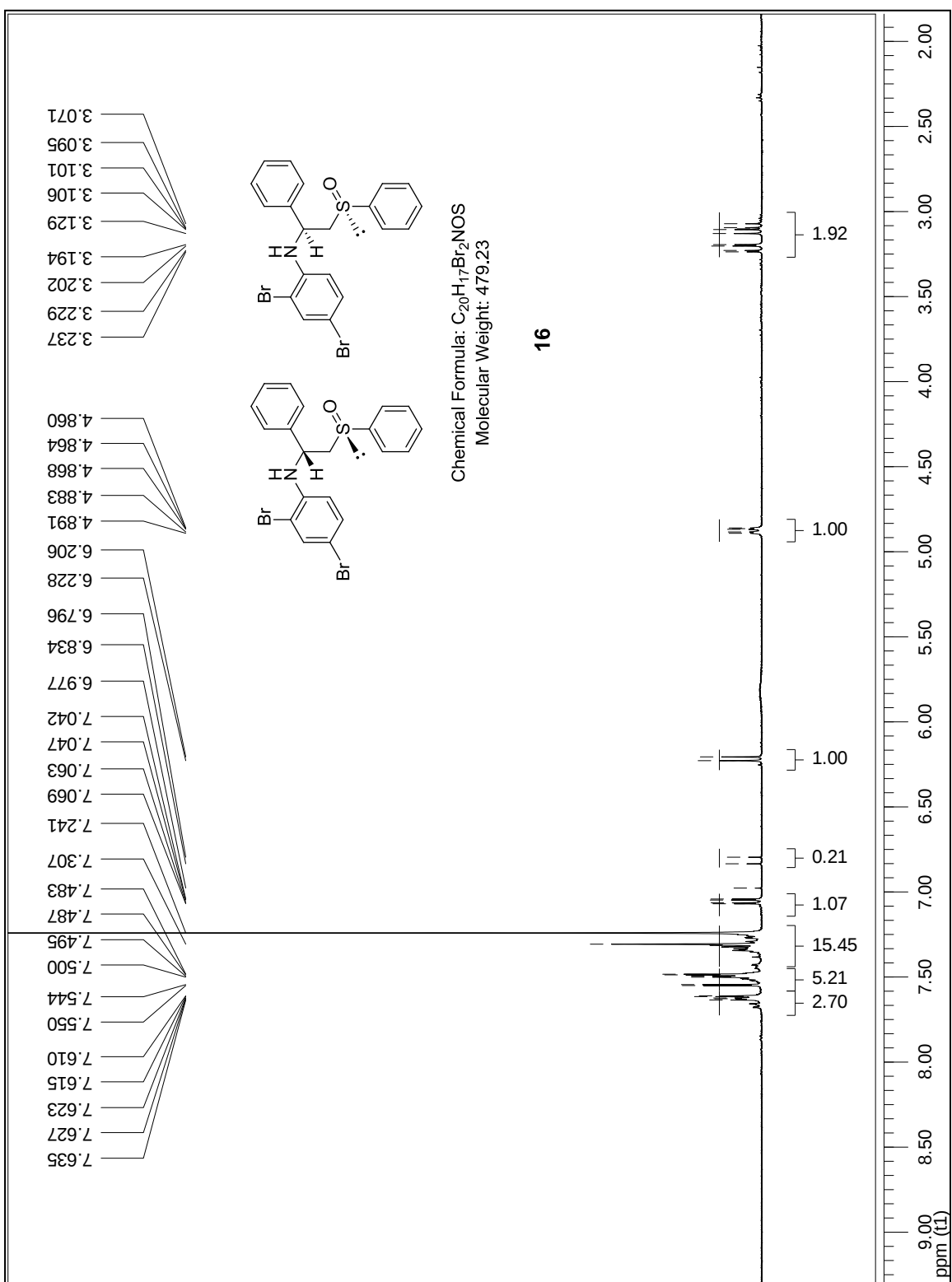


Figure 1.3.3.12. 400 MHz 1H NMR spectrum of compound **16** in $CDCl_3$.

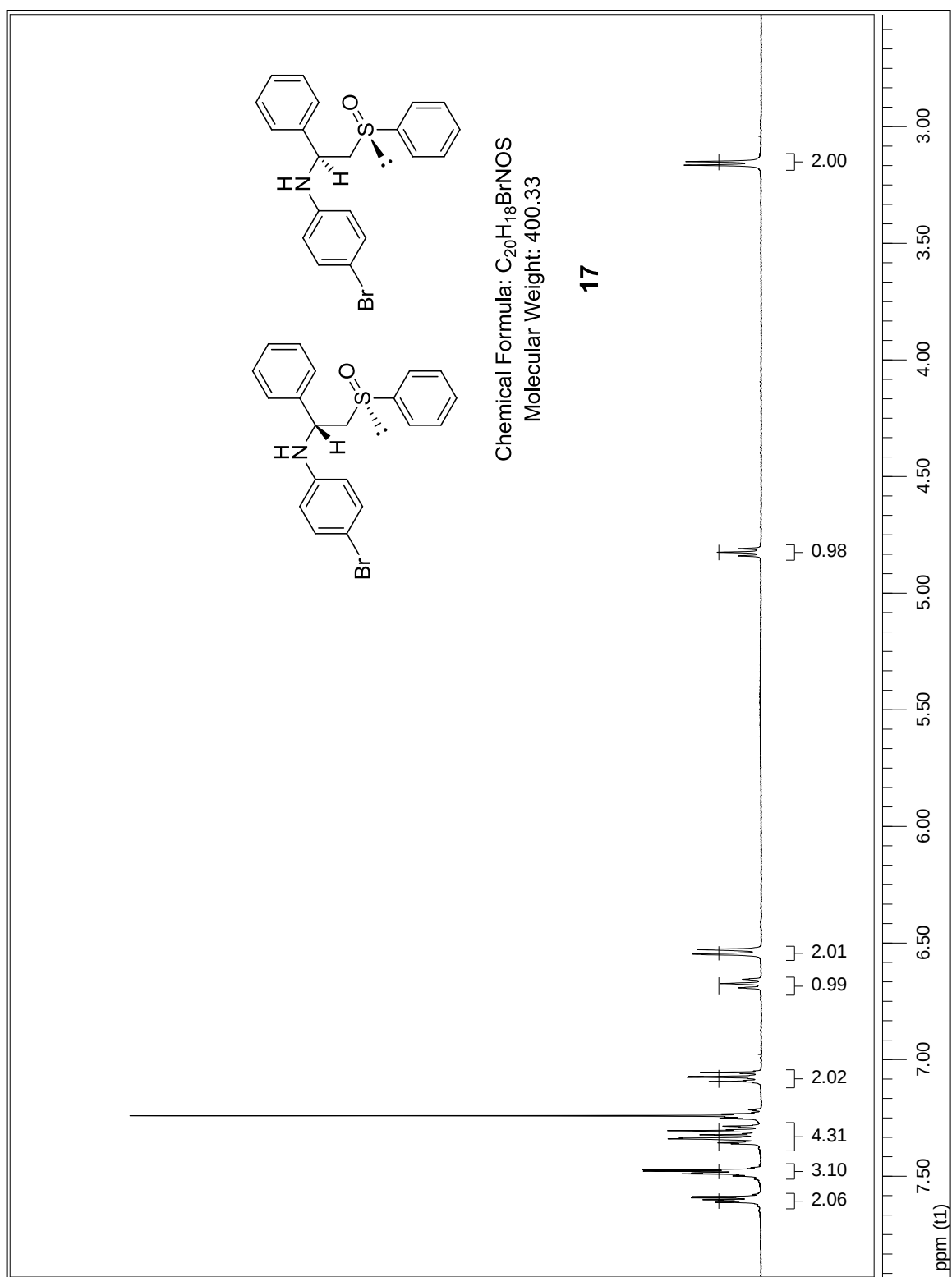


Figure 1.3.3.13. 400 MHz ¹H NMR spectrum of compound **17** in CDCl₃.

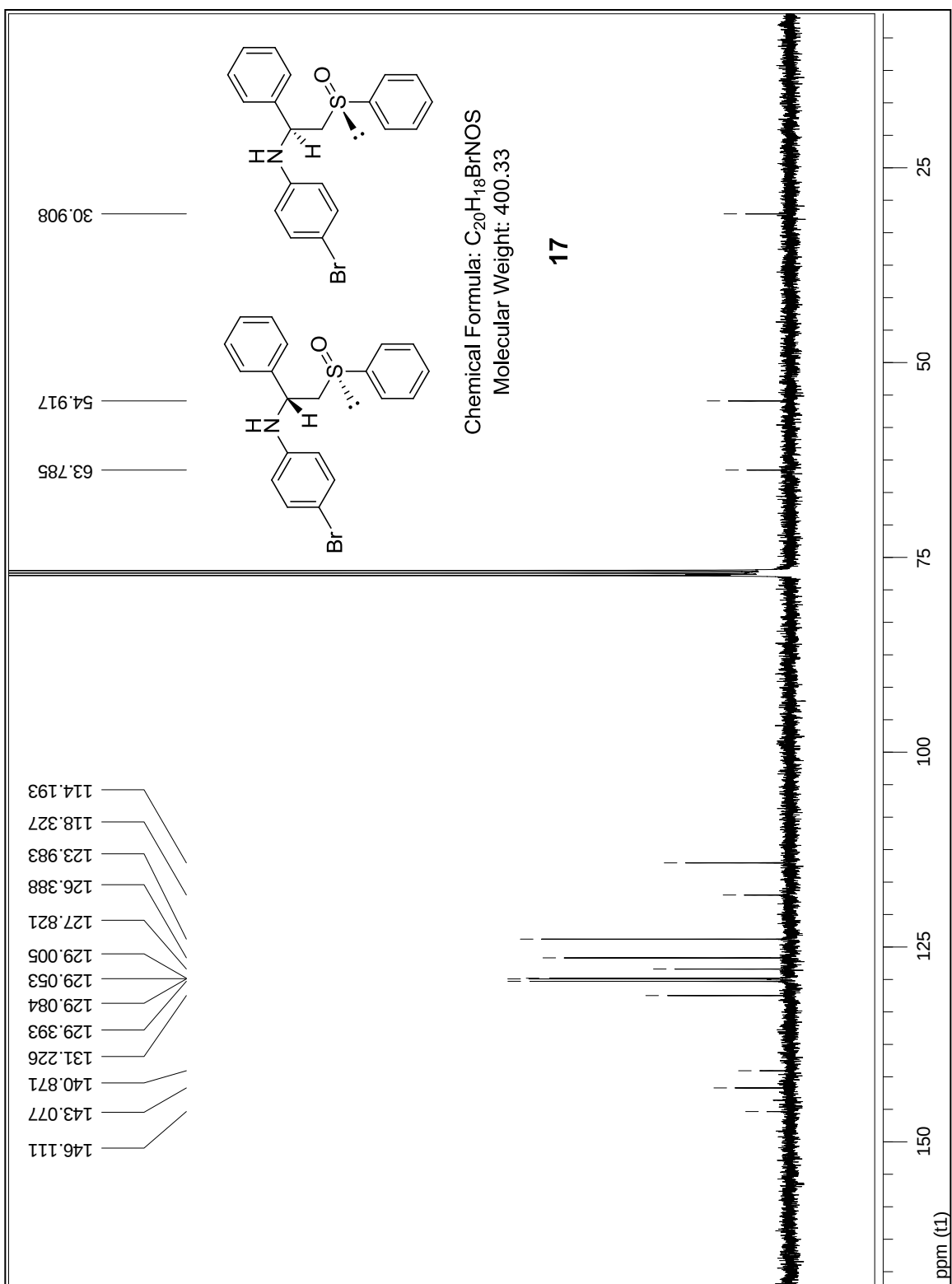


Figure 1.3.3.14. 100 MHz ^{13}C NMR spectrum of compound **17** in $CDCl_3$.

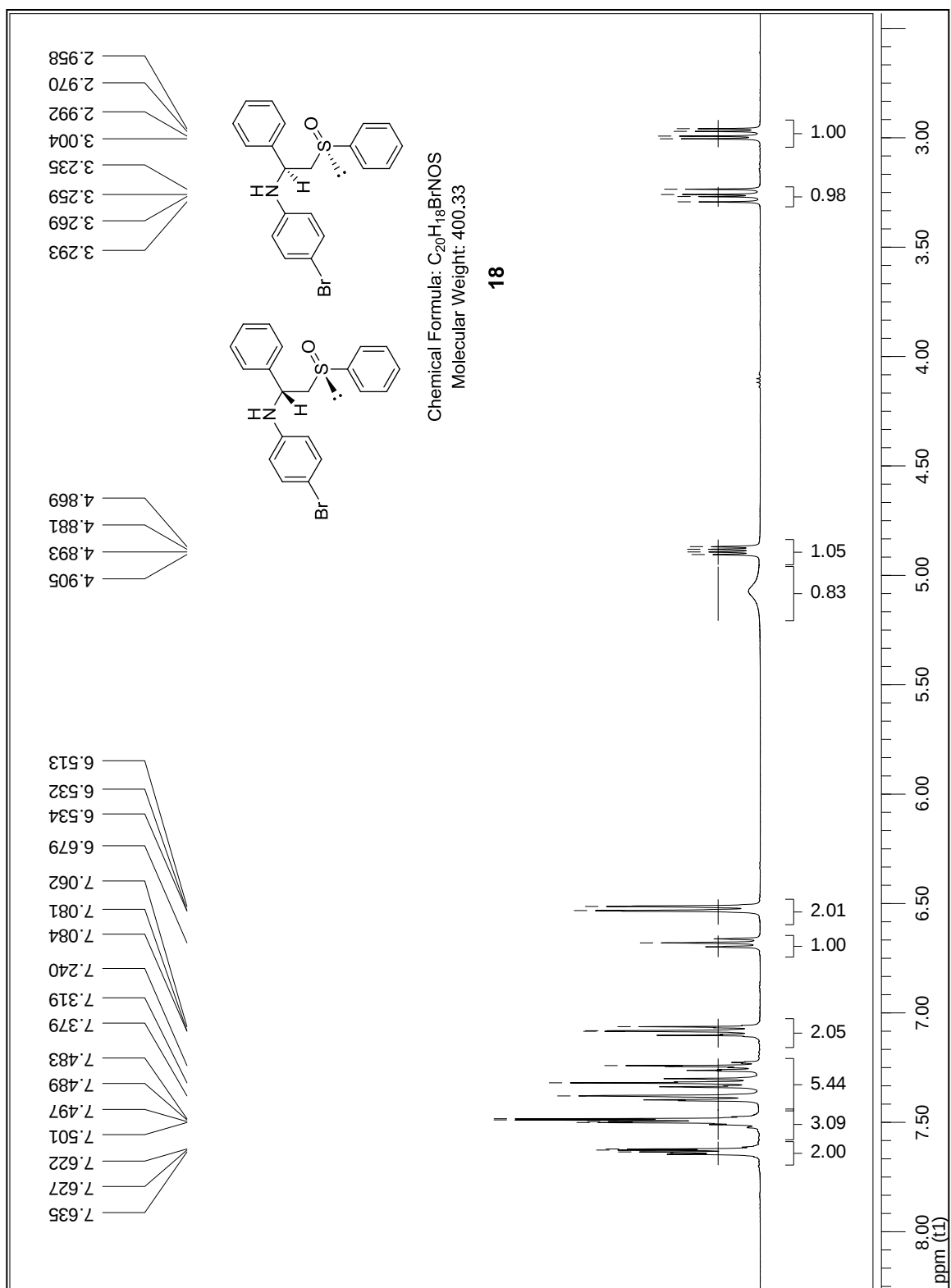


Figure 1.3.3.15. 400 MHz 1H NMR spectrum of compound **18** in $CDCl_3$.

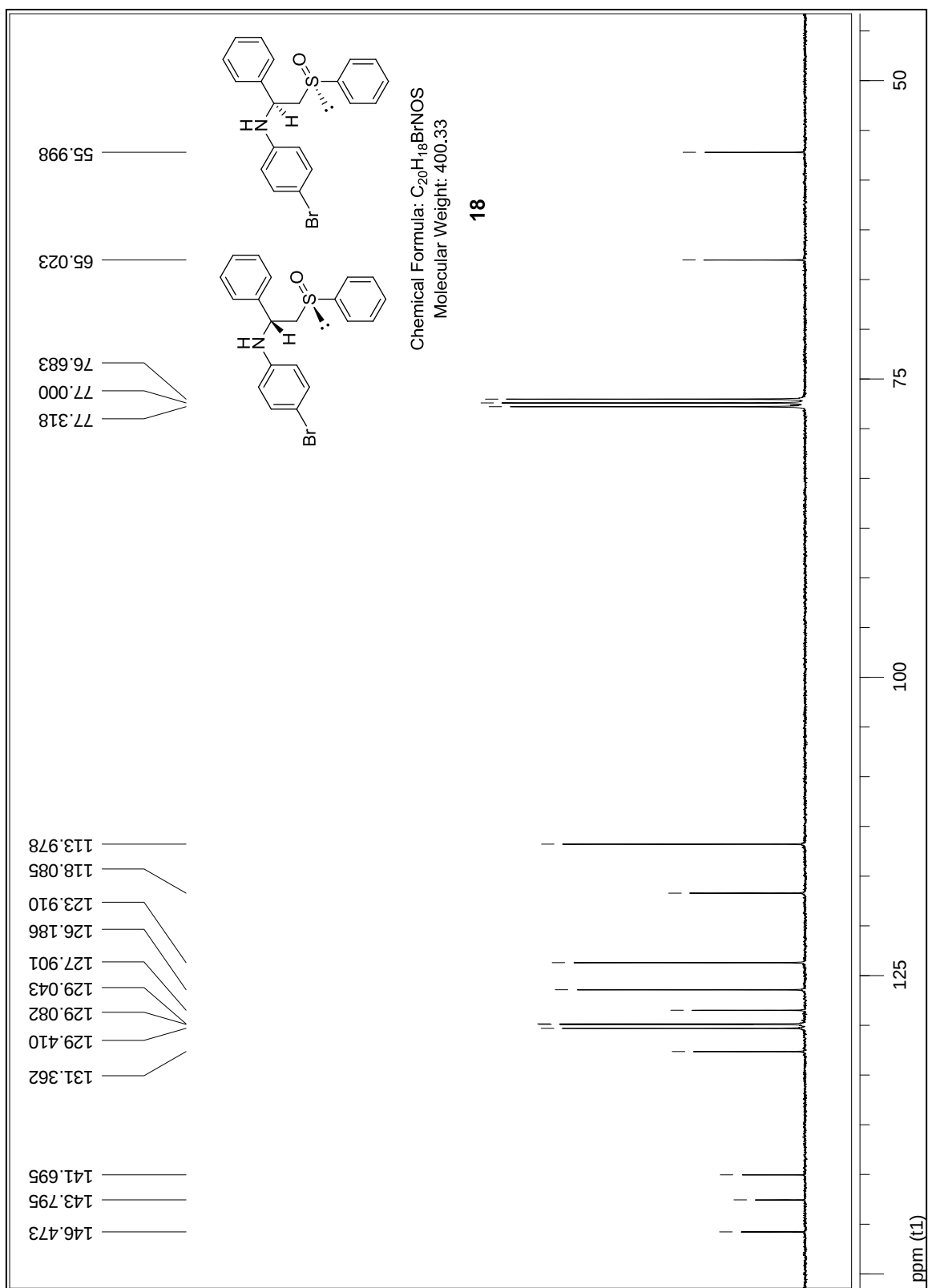


Figure 1.3.3.16. 100 MHz ^{13}C NMR spectrum of compound **18** in $CDCl_3$.

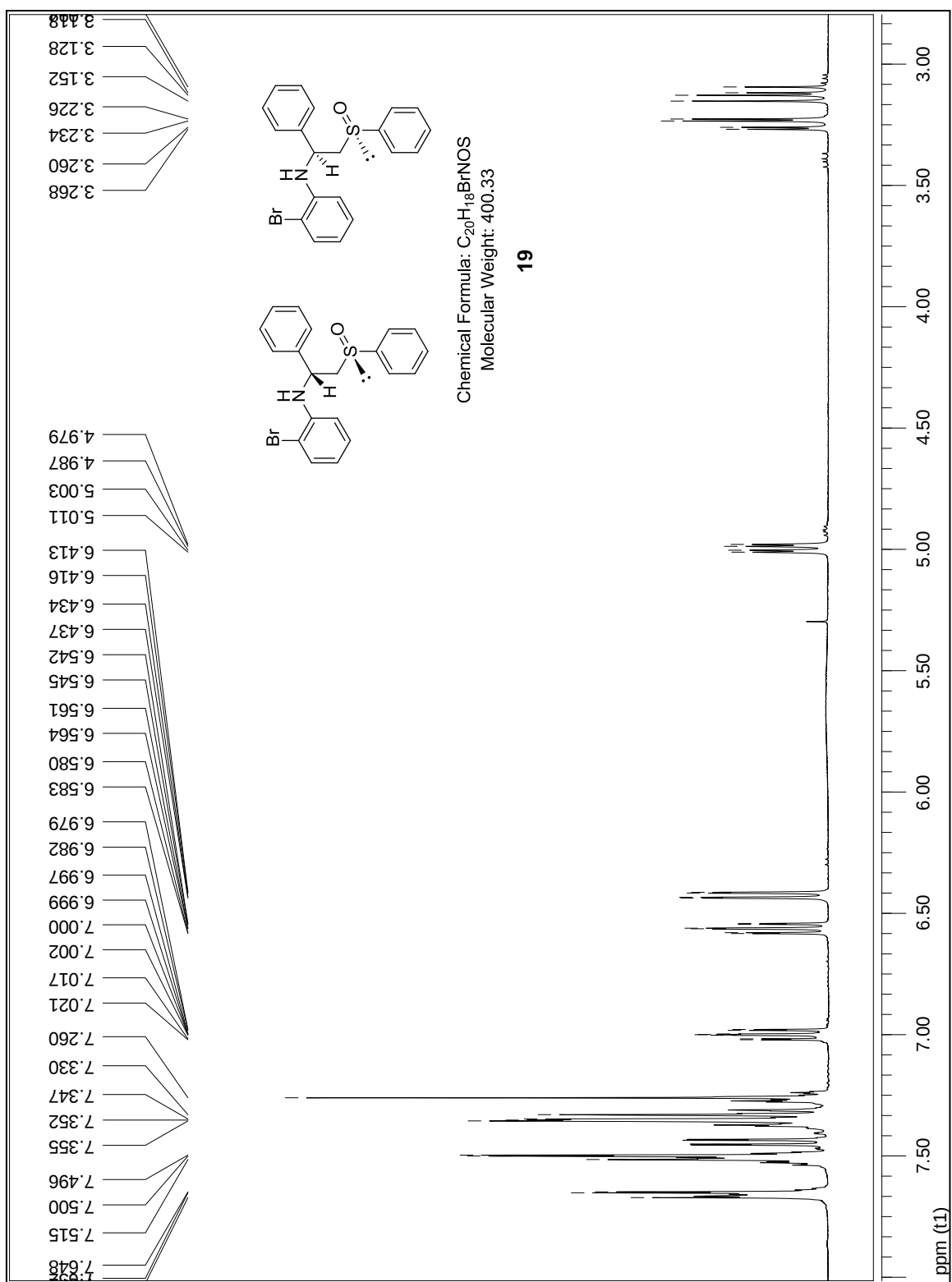


Figure 1.3.3.17. 400 MHz ¹H NMR spectrum of compound **19** in CDCl₃.

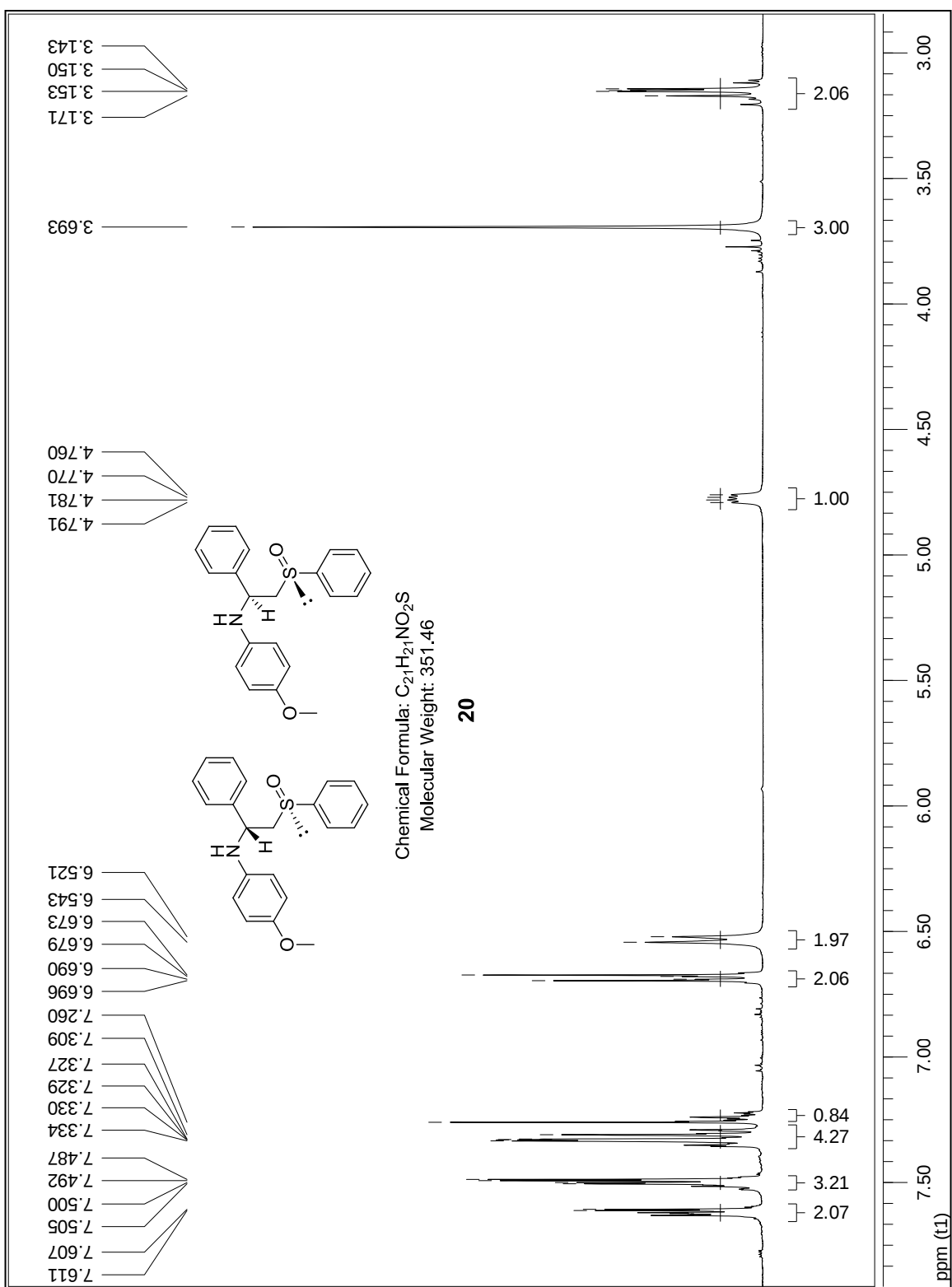


Figure 1.3.3.18. 400 MHz 1H NMR spectrum of compound **20** in $CDCl_3$.



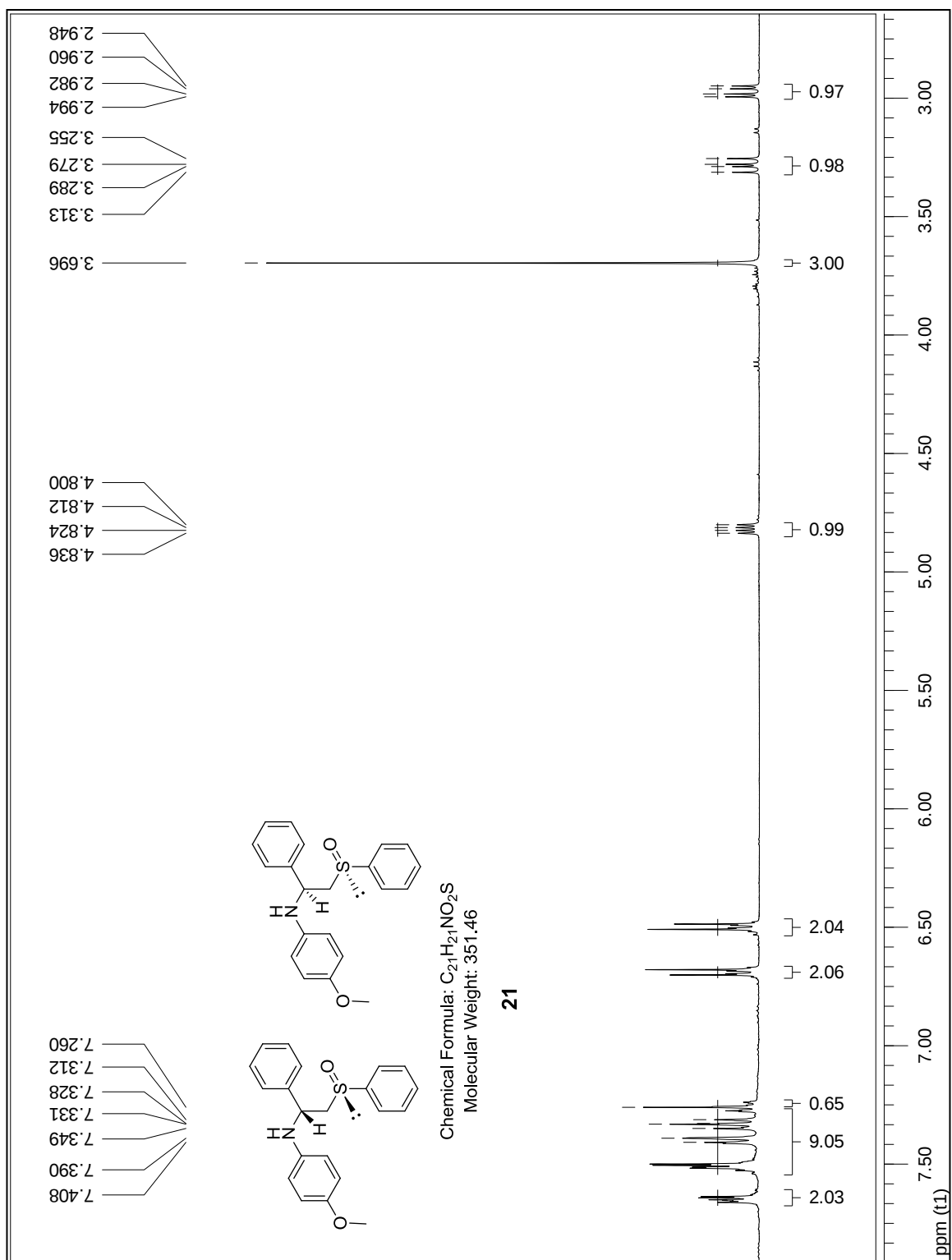


Figure 1.3.3.20. 400 MHz 1H NMR spectrum of compound **21** in $CDCl_3$.

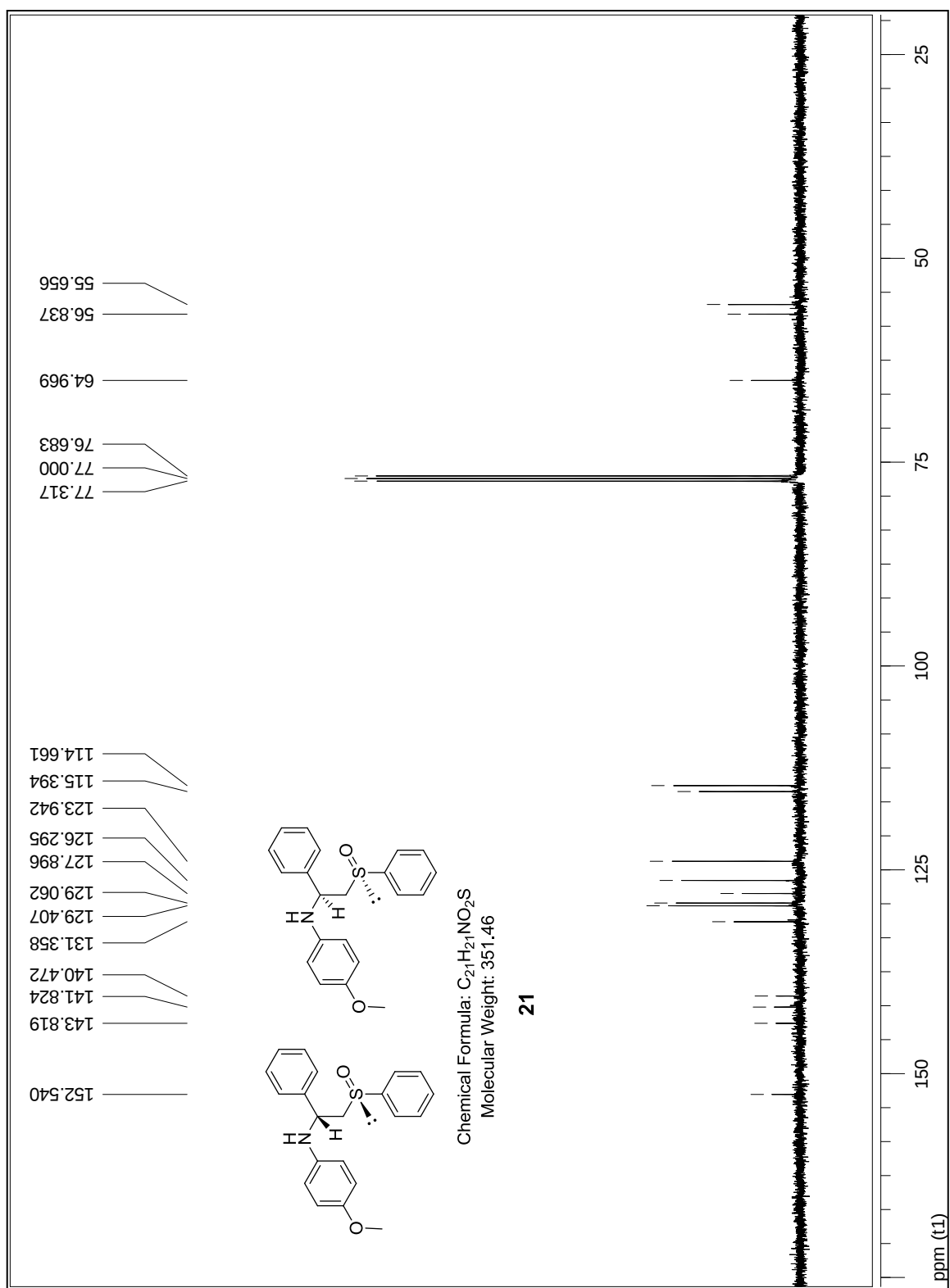


Figure 1.3.3.21. 100 MHz ¹³C NMR spectrum of compound **21** in CDCl₃.

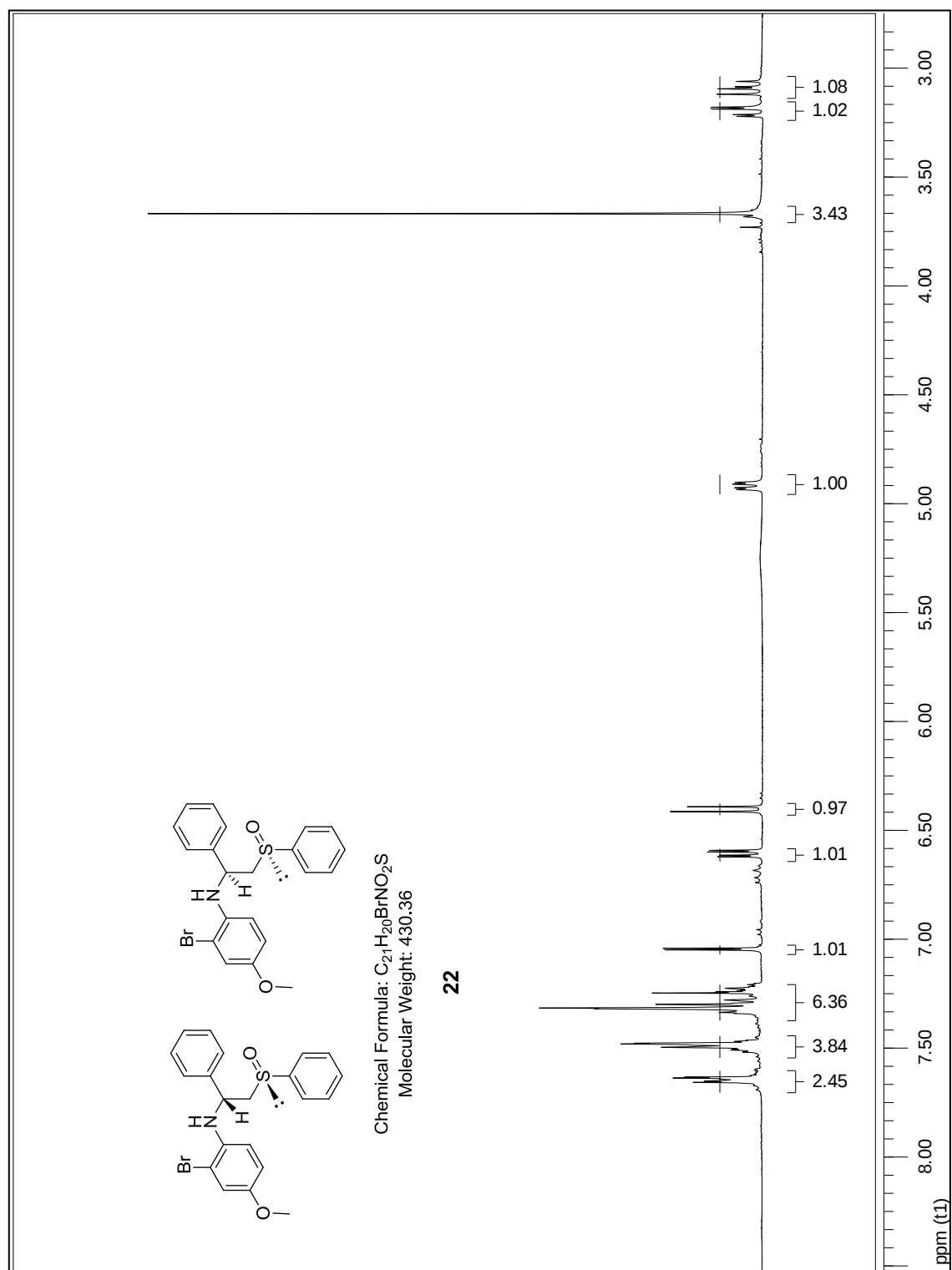


Figure 1.3.3.22. 400 MHz 1H NMR spectrum of compound **22** in $CDCl_3$.

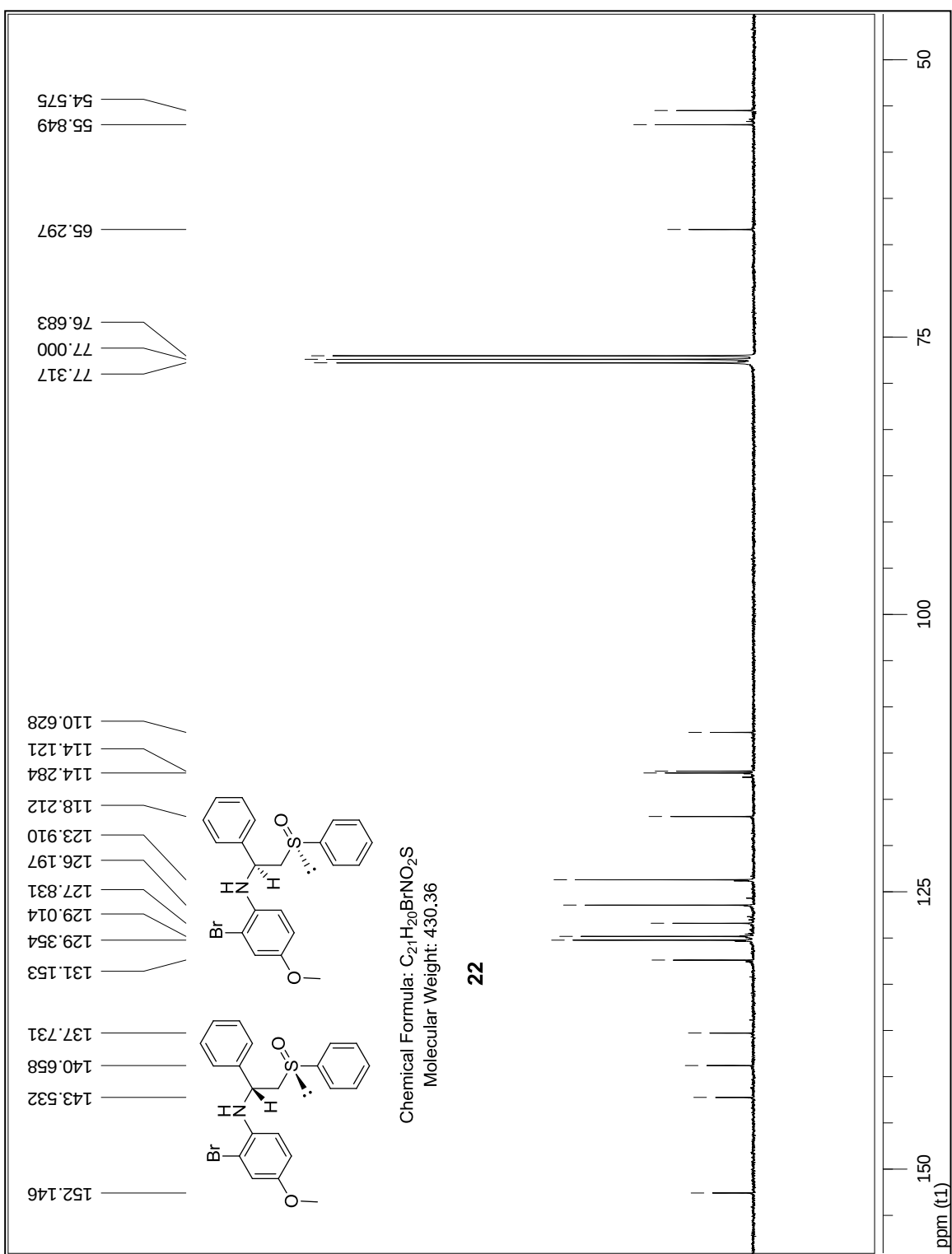


Figure 1.3.3.23. 100 MHz ¹³C NMR spectrum of compound **22** in CDCl₃.

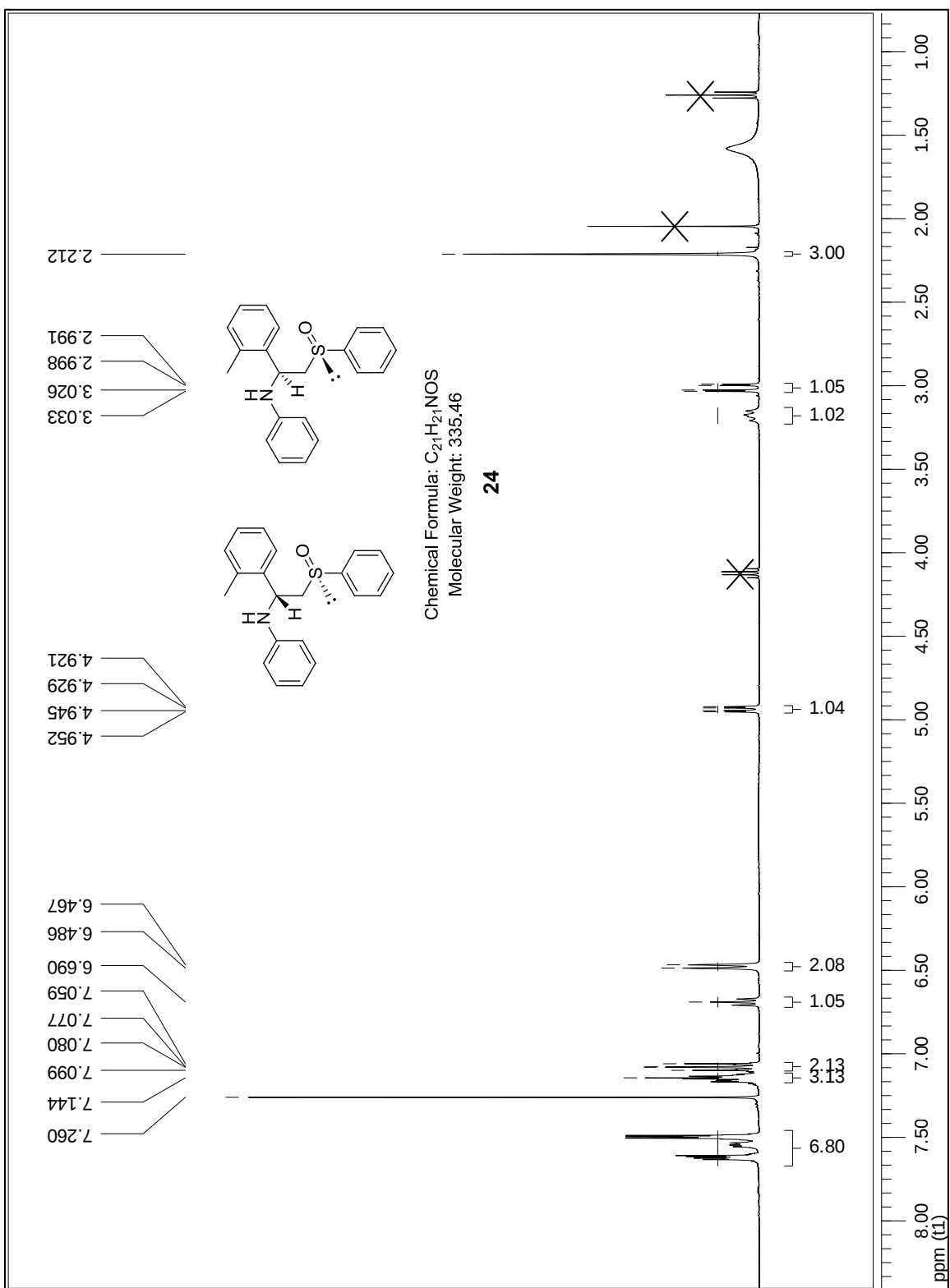


Figure 1.3.3.24. 400 MHz 1H NMR spectrum of compound **24** in $CDCl_3$.

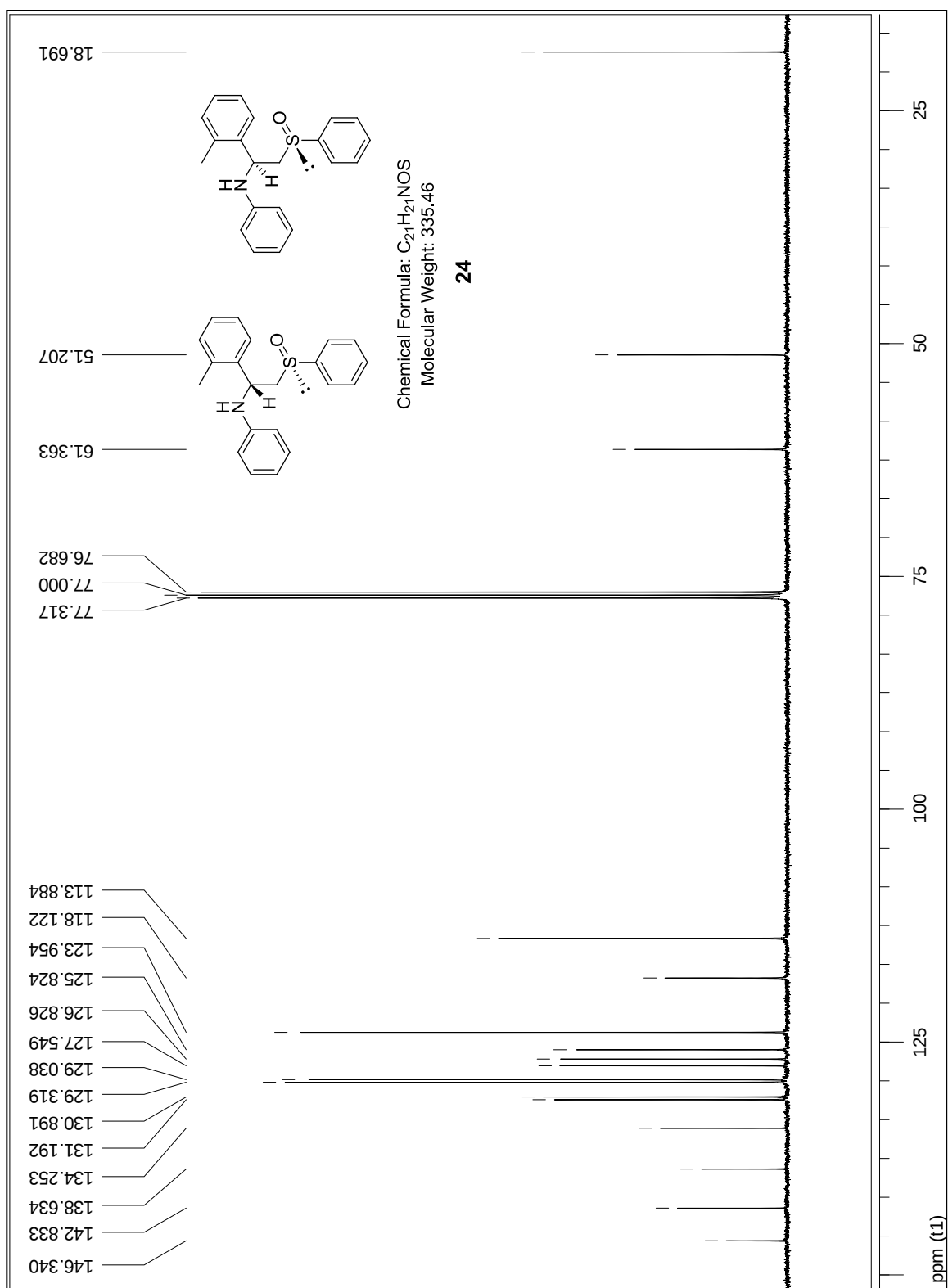


Figure 1.3.3.25. 100 MHz ¹³C NMR spectrum of compound **24** in CDCl₃.

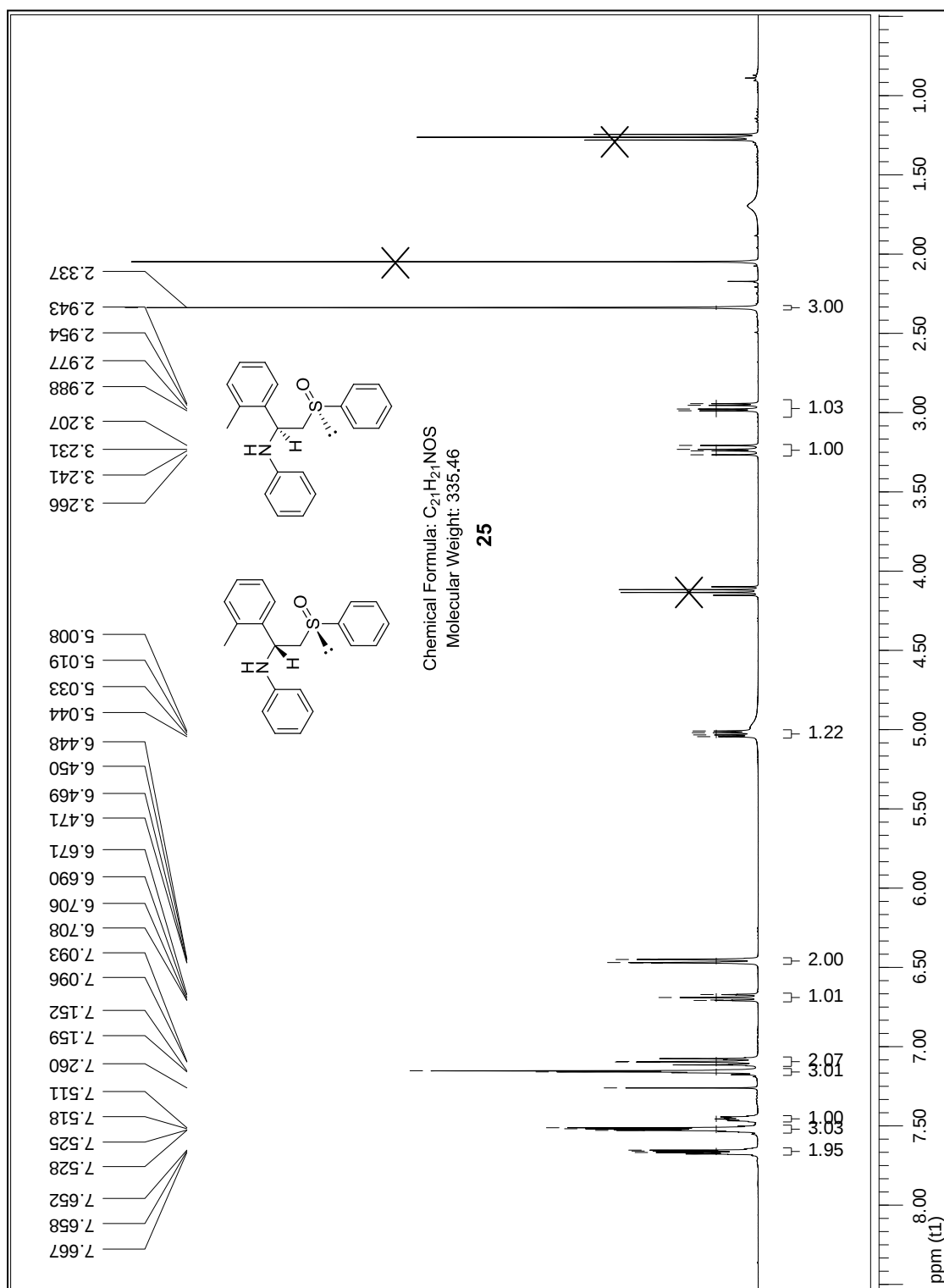


Figure 1.3.3.26. 400 MHz 1H NMR spectrum of compound **25** in $CDCl_3$.

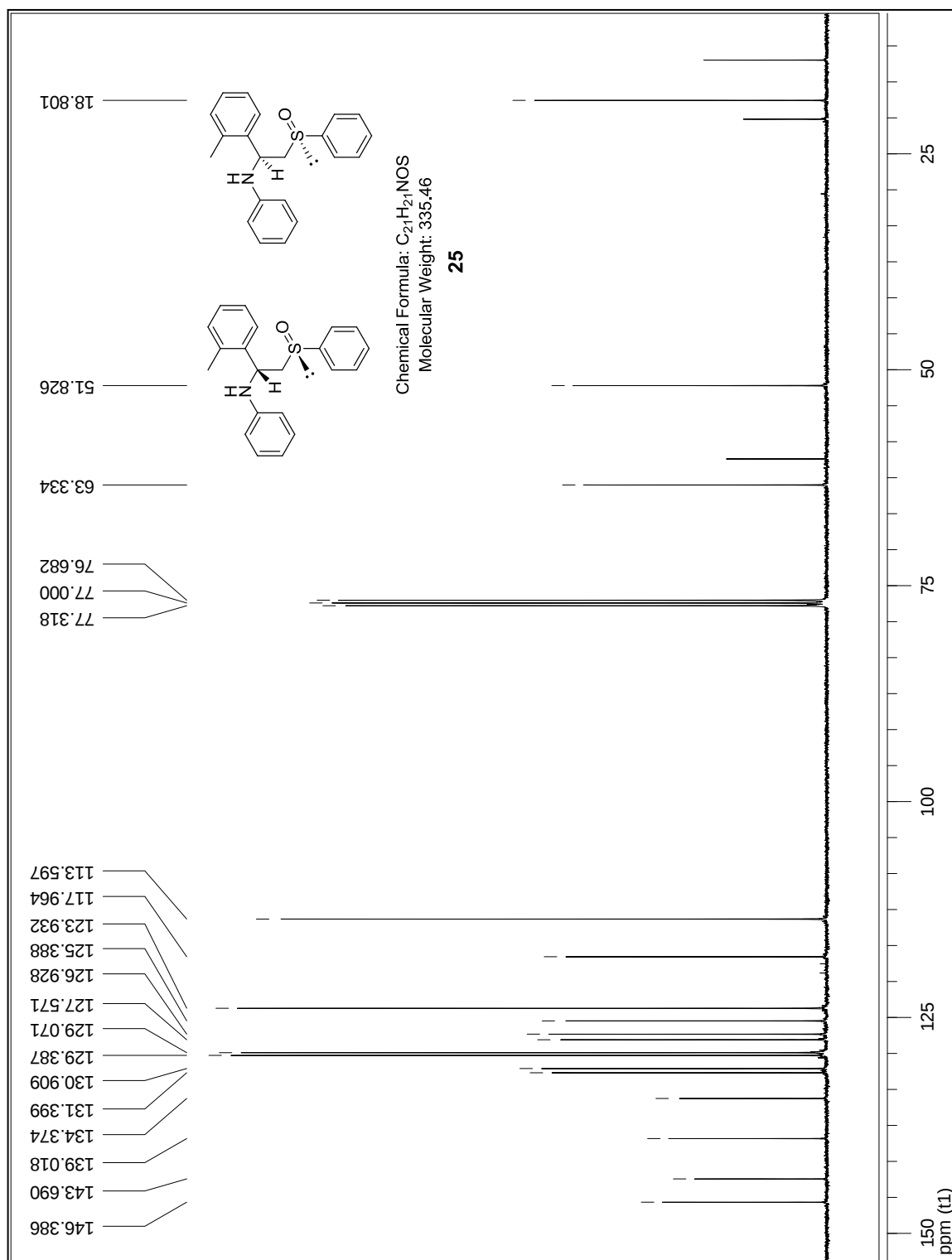


Figure 1.3.3.27. 100 MHz ^{13}C NMR spectrum of compound 25 in $CDCl_3$.

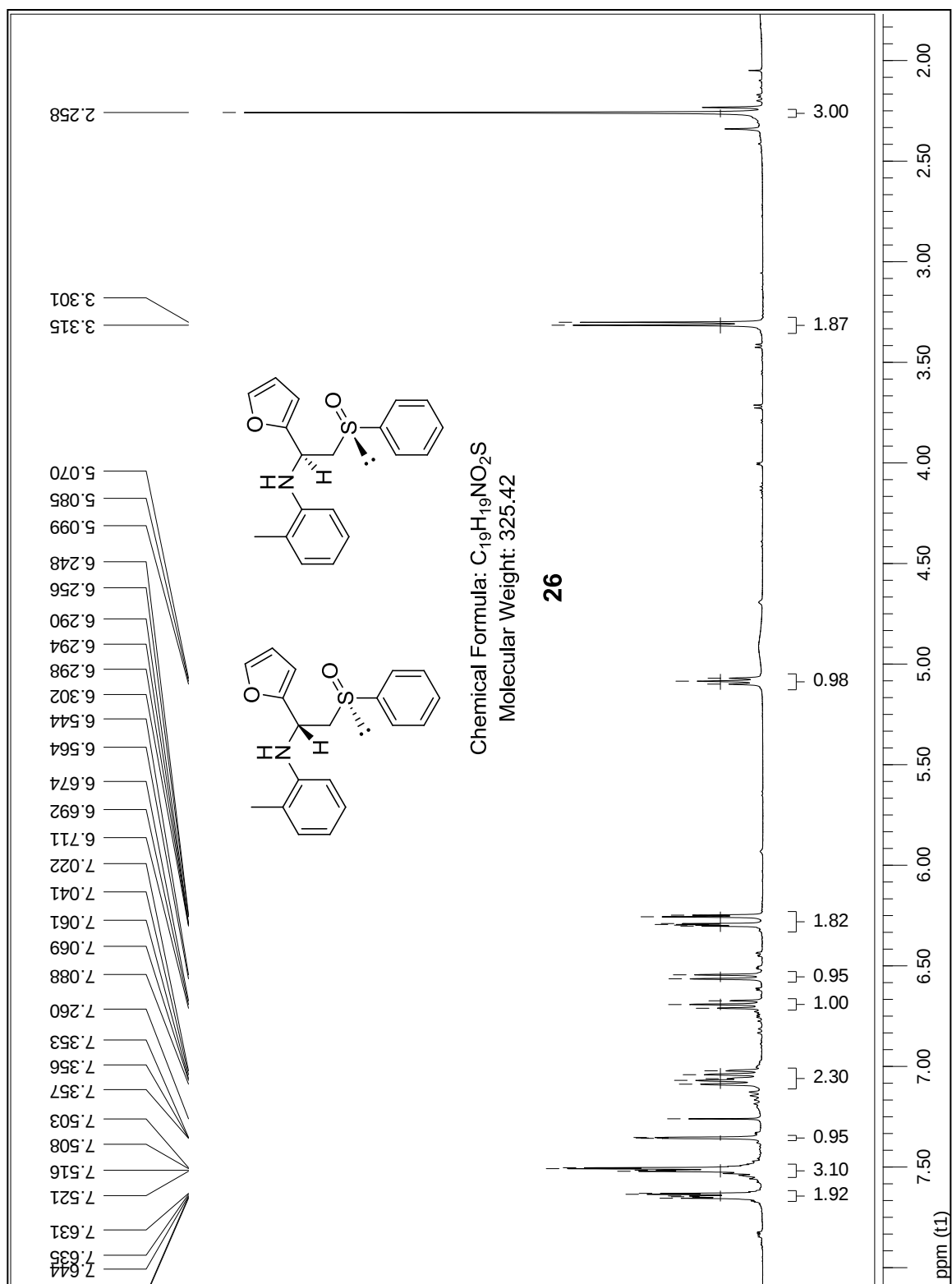


Figure 1.3.3.28. 400 MHz ^1H NMR spectrum of compound **26** in CDCl_3 .

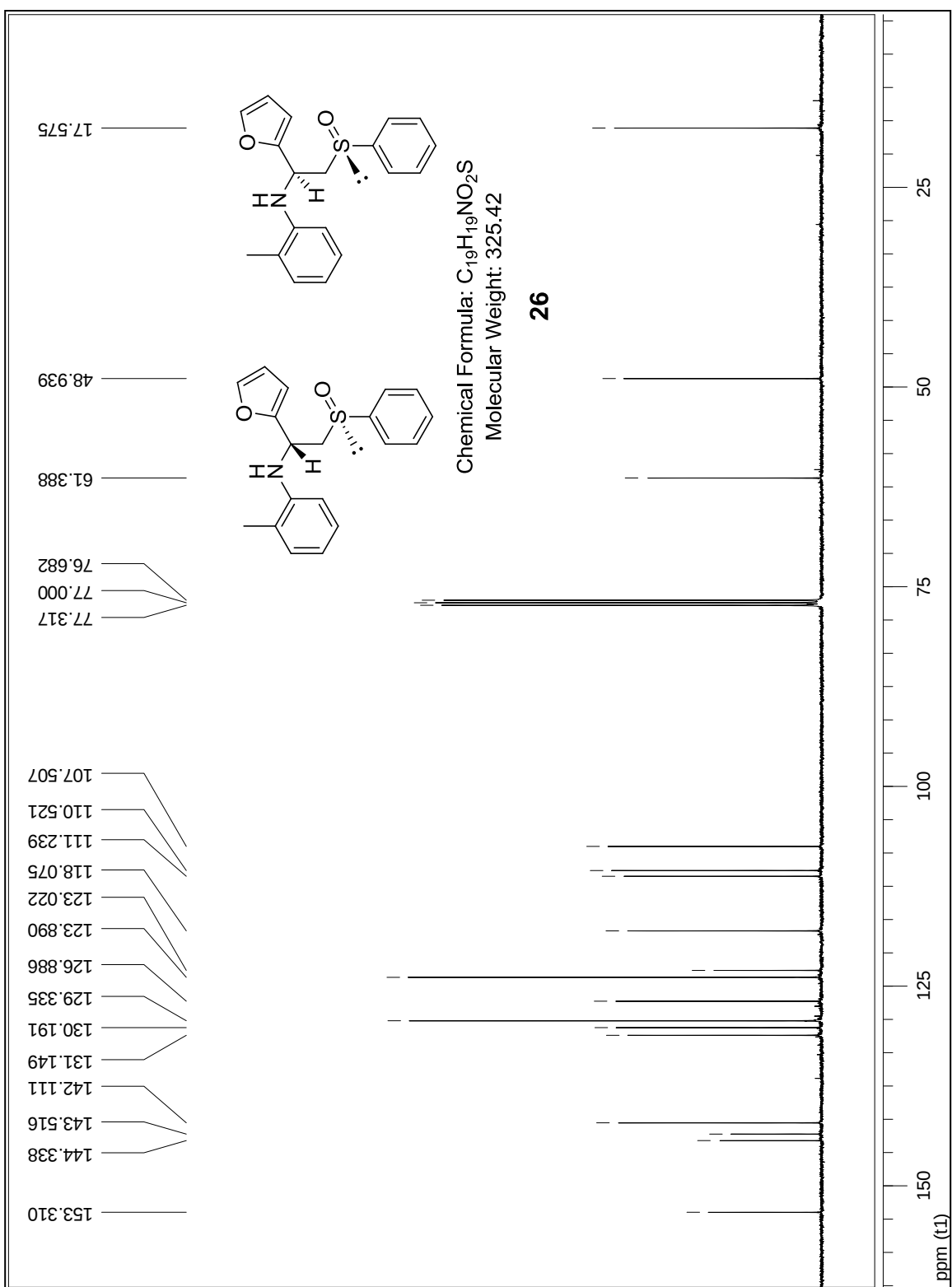


Figure 1.3.3.29. 100 MHz ^{13}C NMR spectrum of compound **26** in $CDCl_3$.

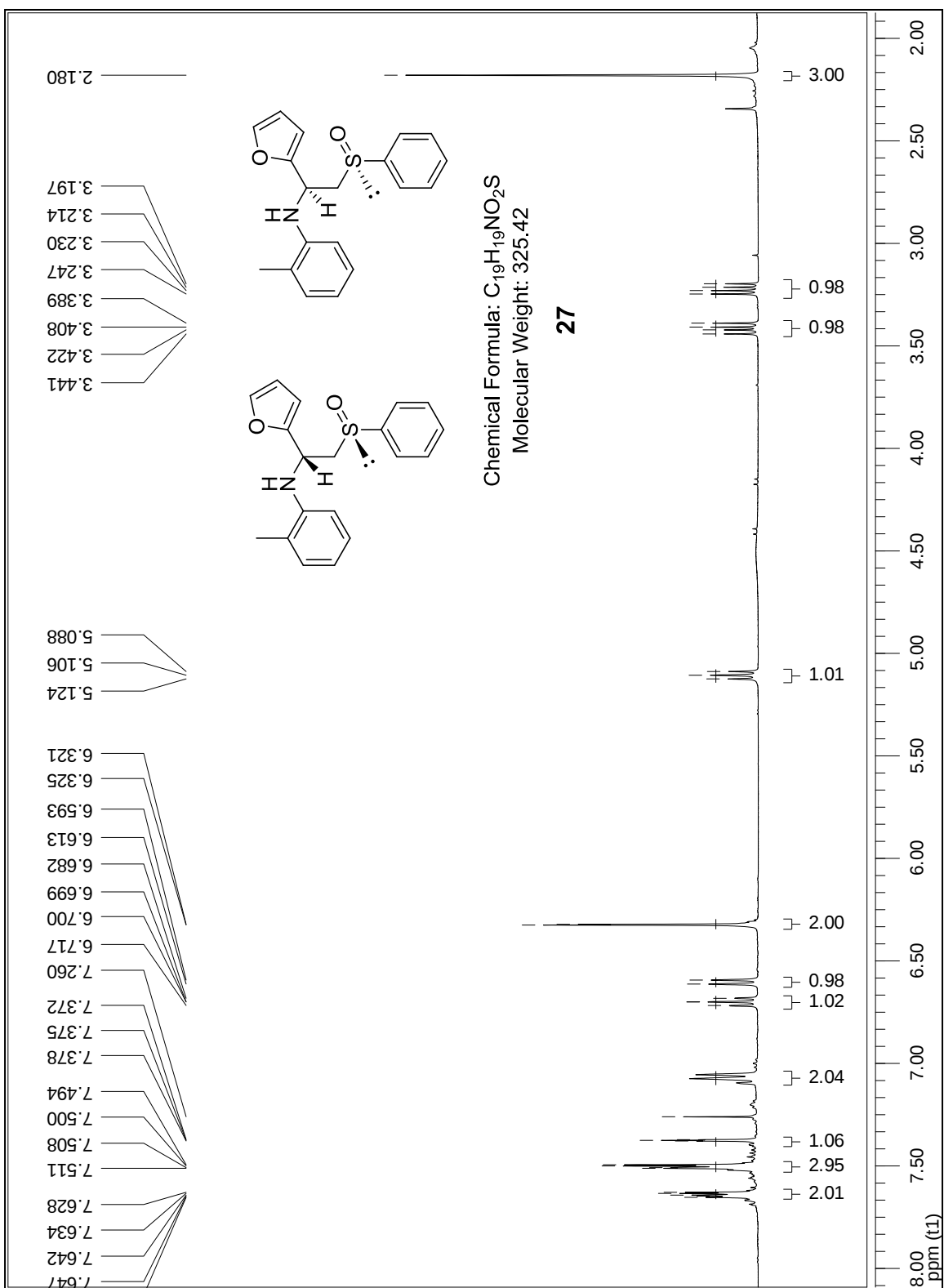


Figure 1.3.3.30. 400 MHz 1H NMR spectrum of compound 27 in $CDCl_3$.

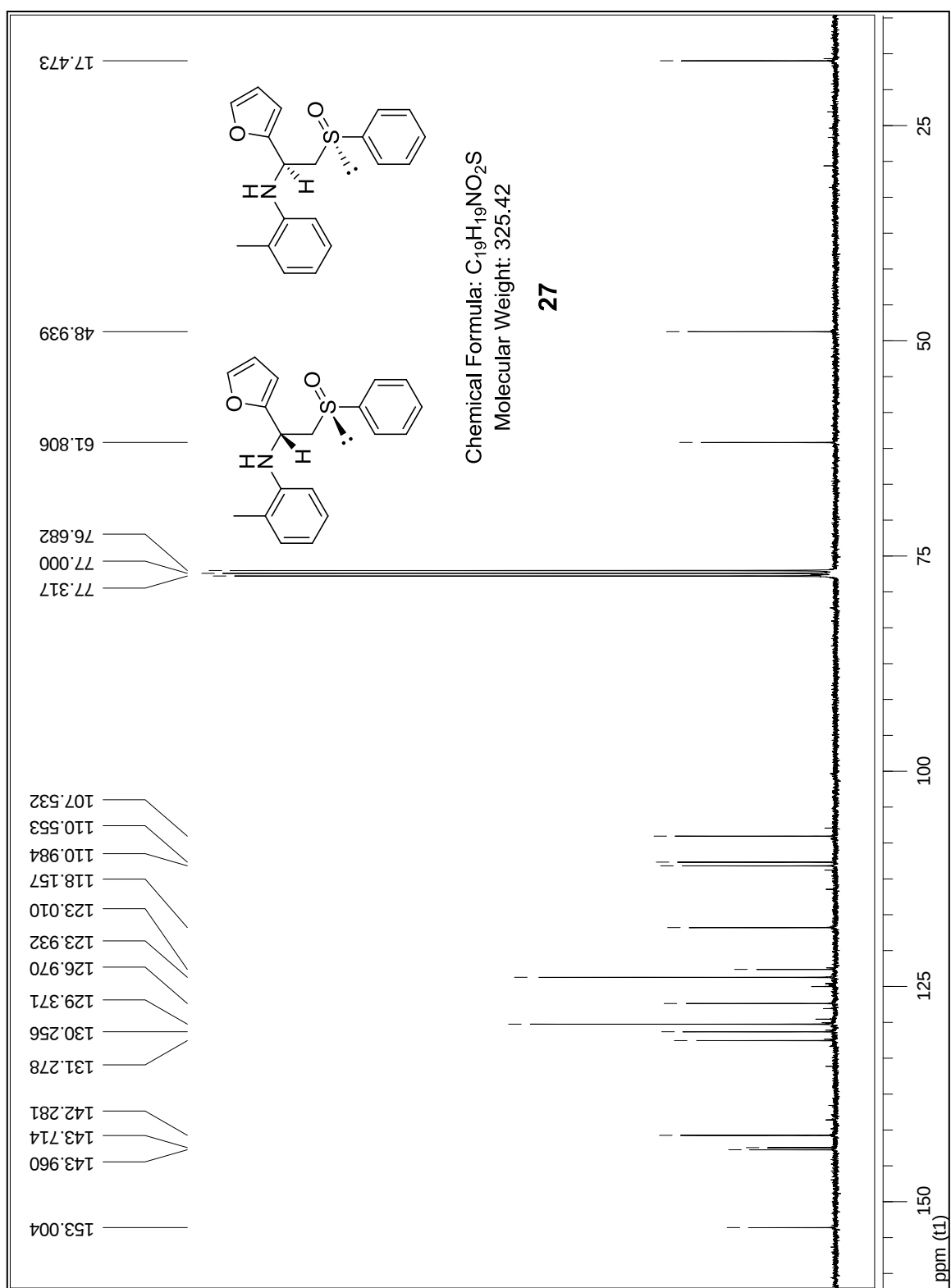


Figure 1.3.3.31. 100 MHz ^{13}C NMR spectrum of compound 27 in $CDCl_3$.

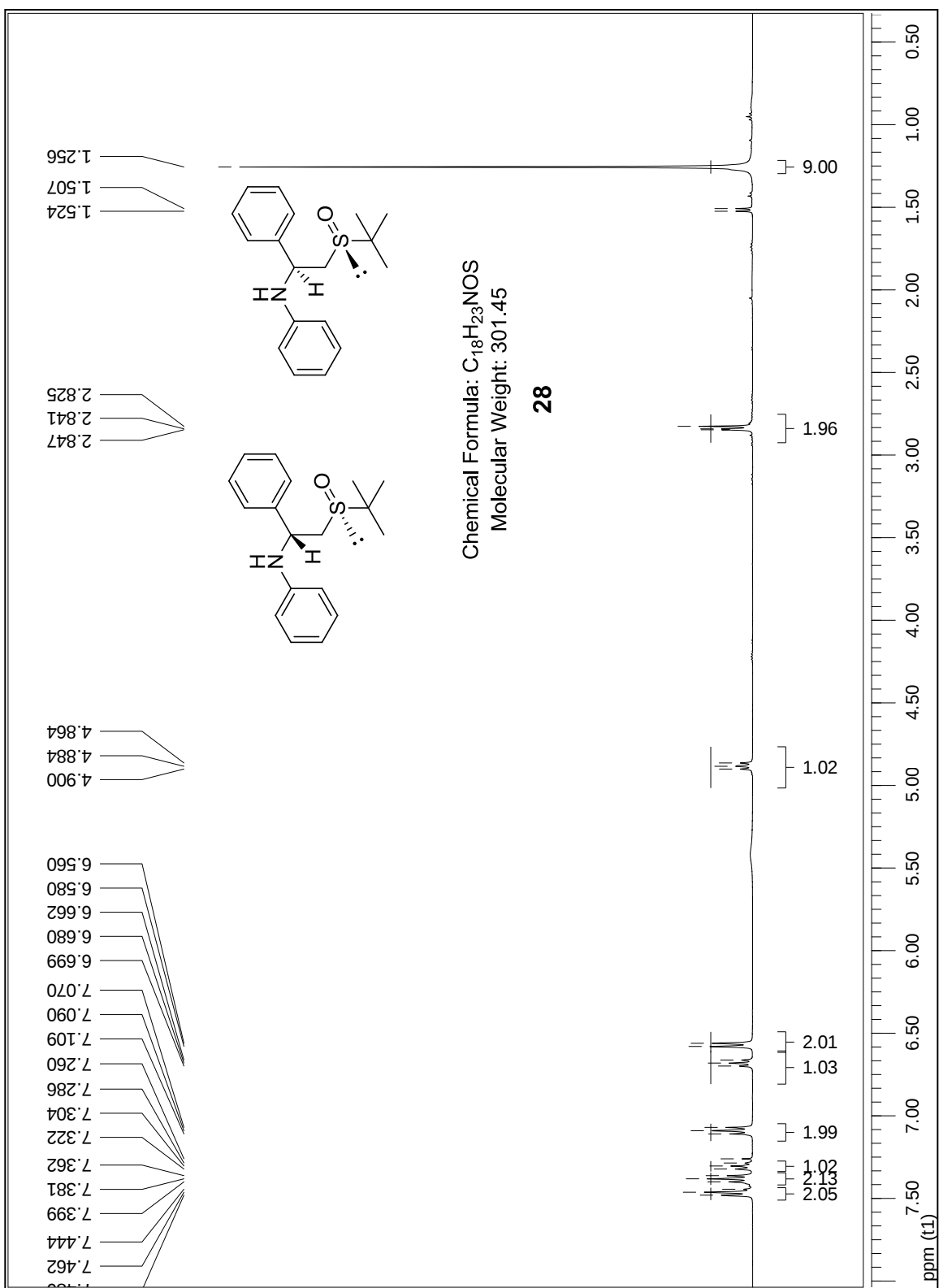


Figure 1.3.3.32. 400 MHz ¹H NMR spectrum of compound **28** in CDCl₃.

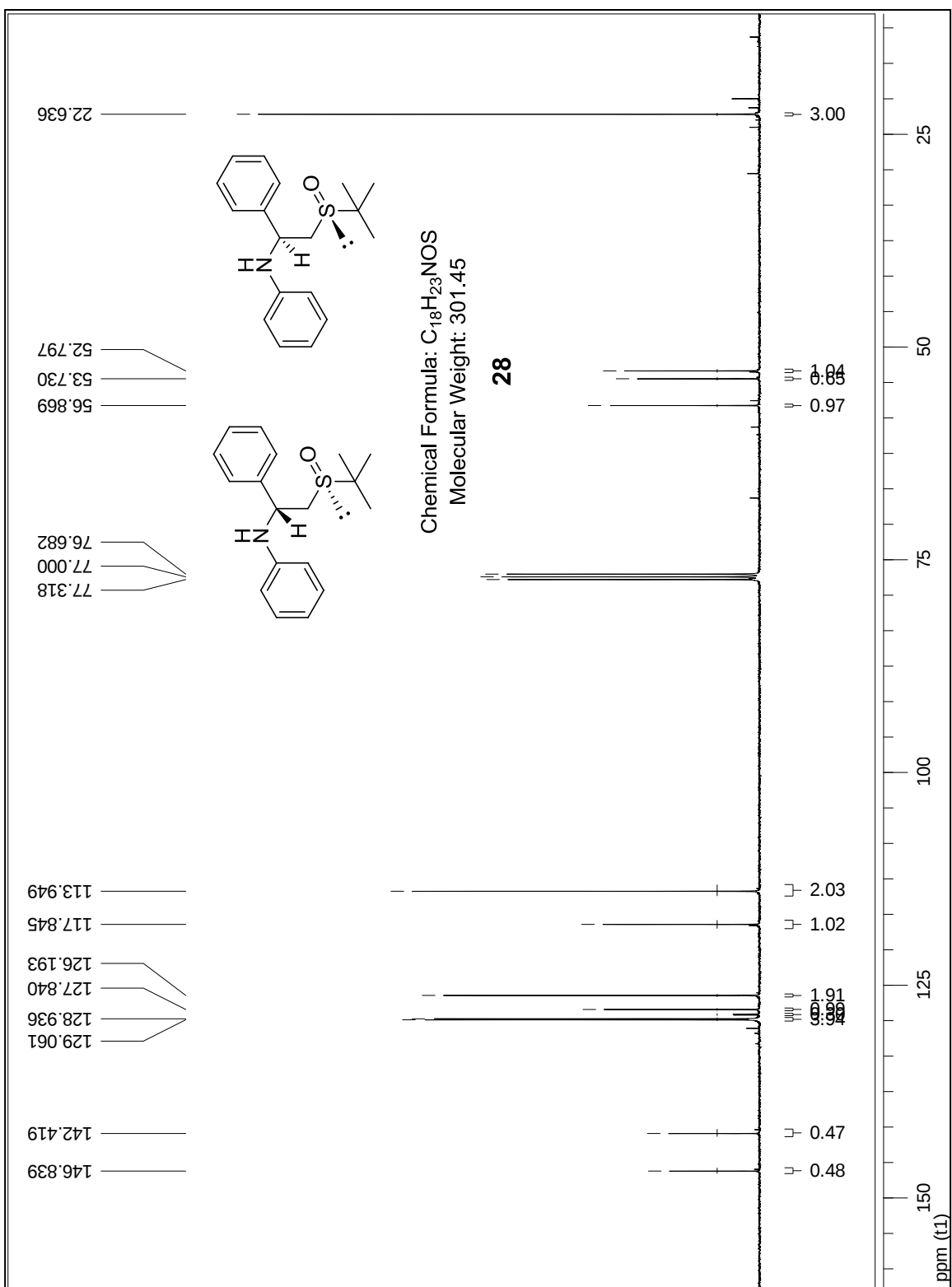


Figure 1.3.3.33. 100 MHz ^{13}C NMR spectrum of compound **28** in $CDCl_3$.

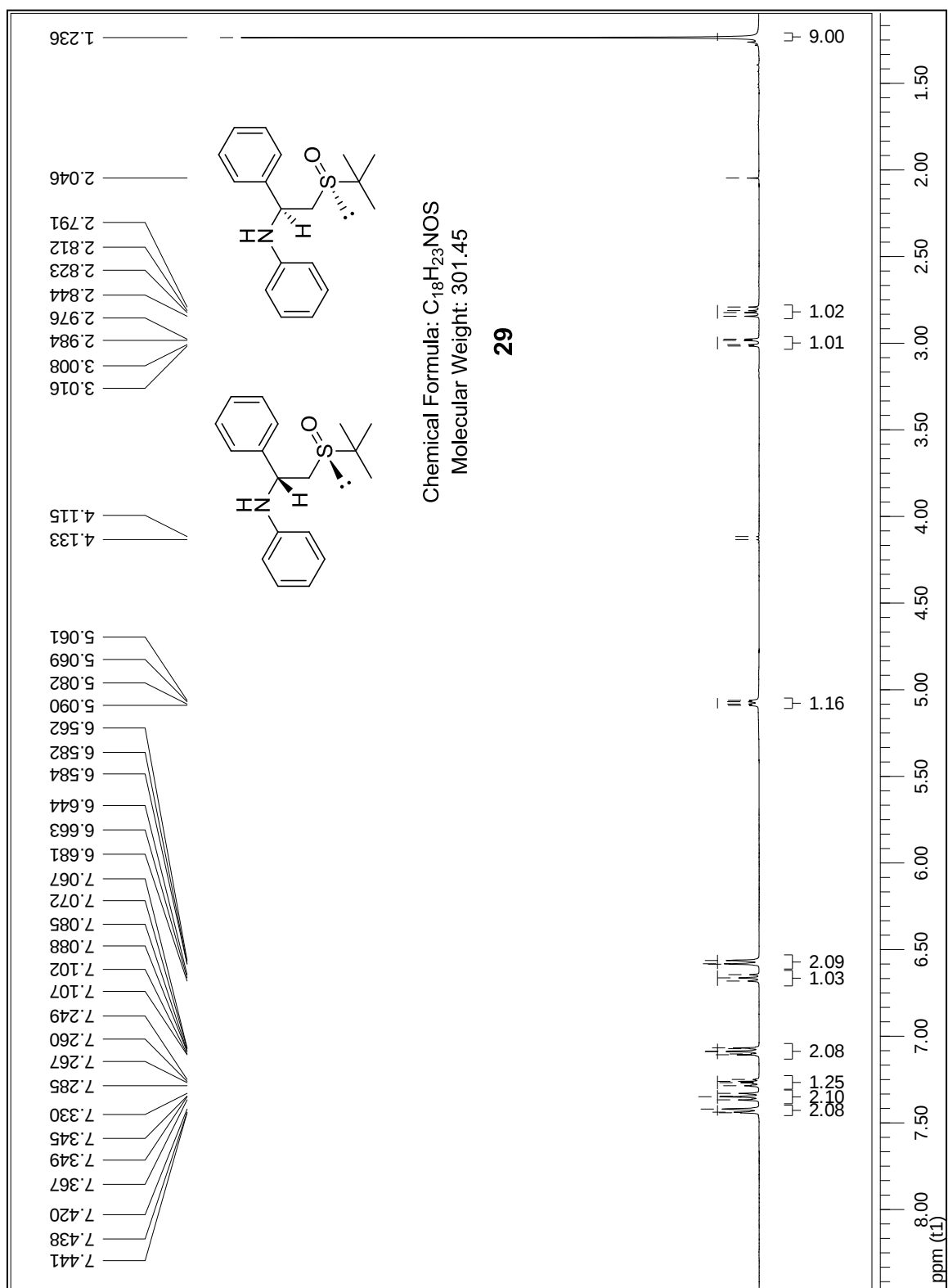


Figure 1.3.3.34. 400 MHz 1H NMR spectrum of compound **29** in $CDCl_3$.

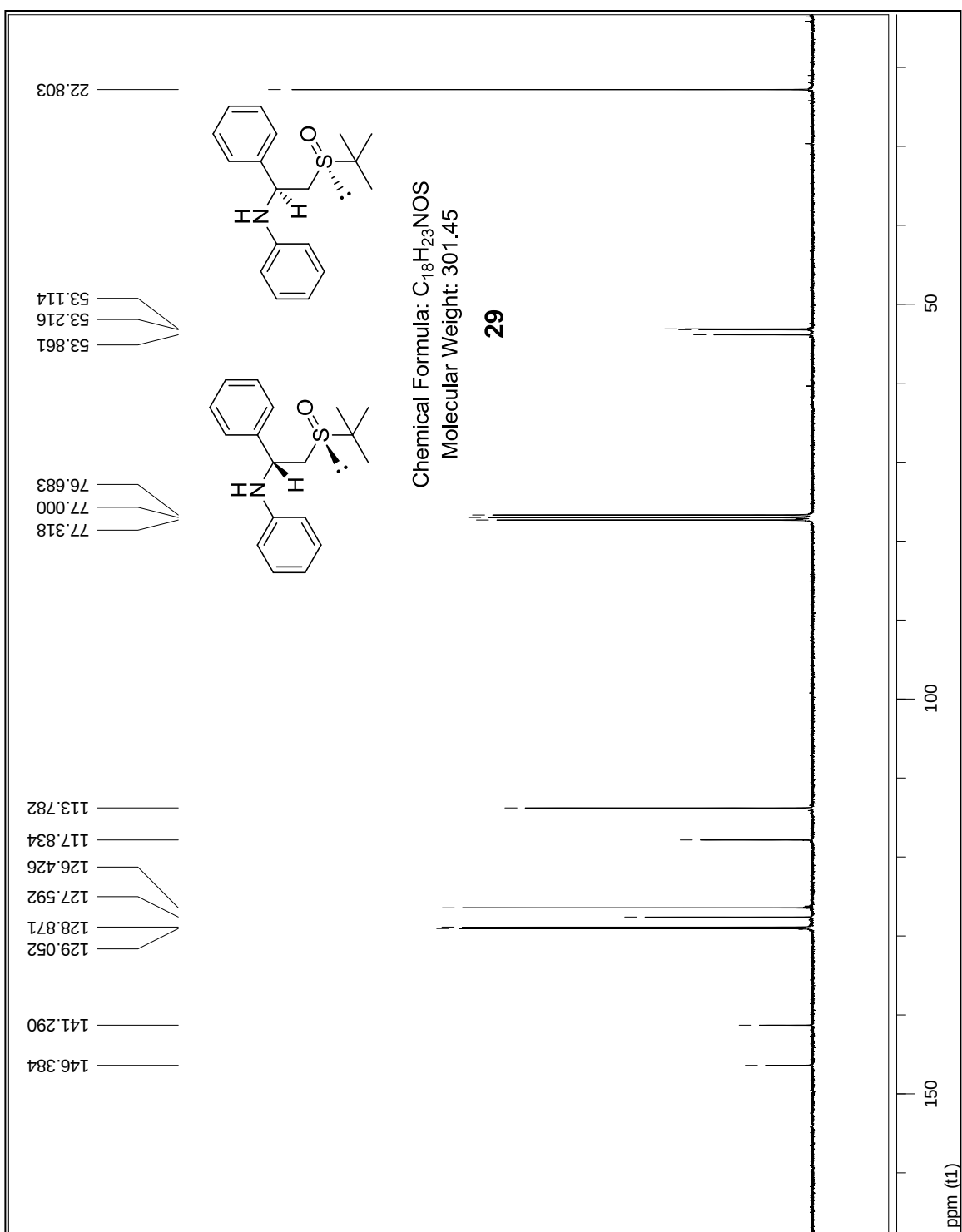


Figure 1.3.35. 100 MHz ^{13}C NMR spectrum of compound **29** in $CDCl_3$.

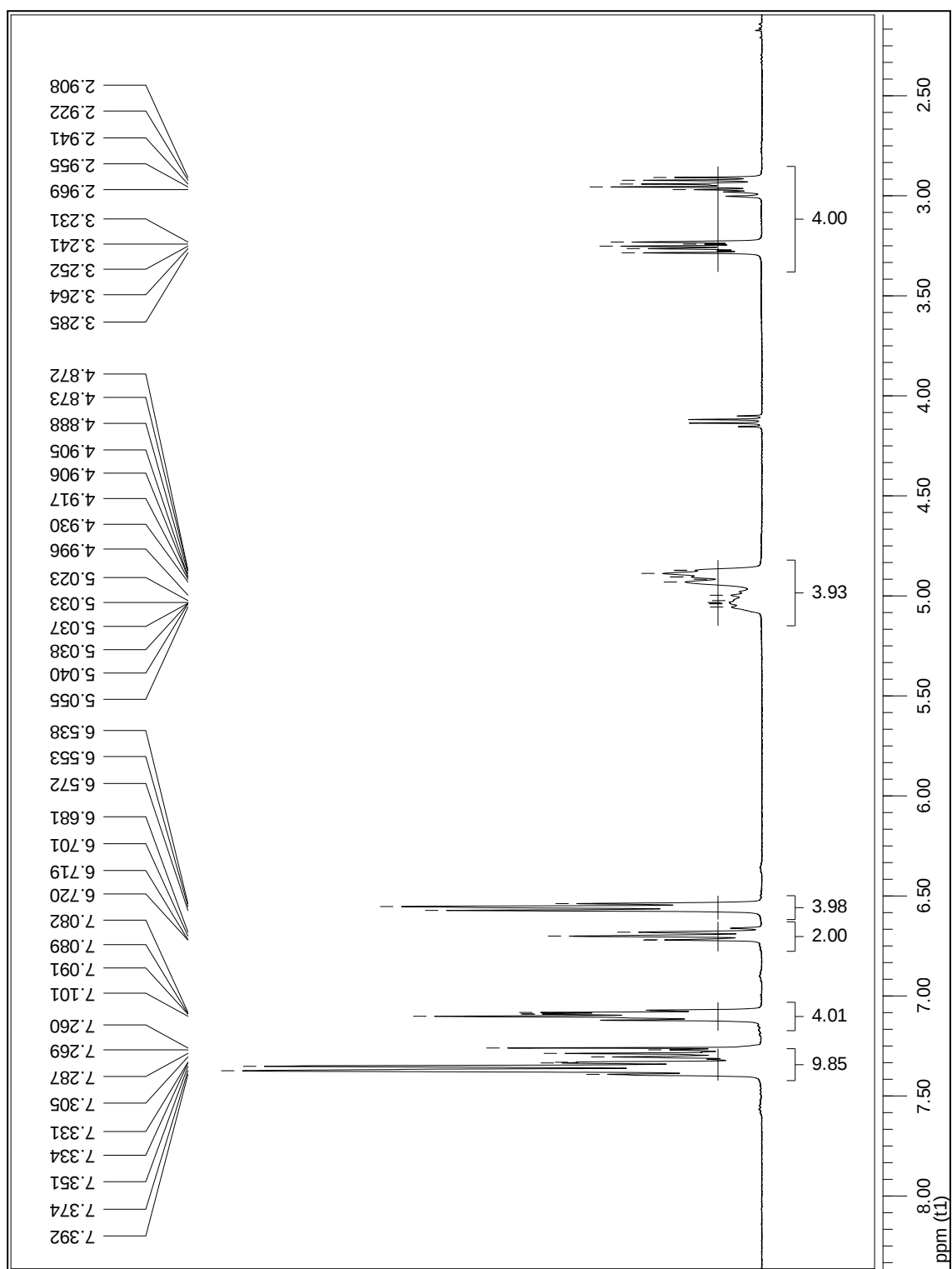


Figure 1.3.36. 400 MHz ^1H NMR spectrum of compound **30** in CDCl_3 .

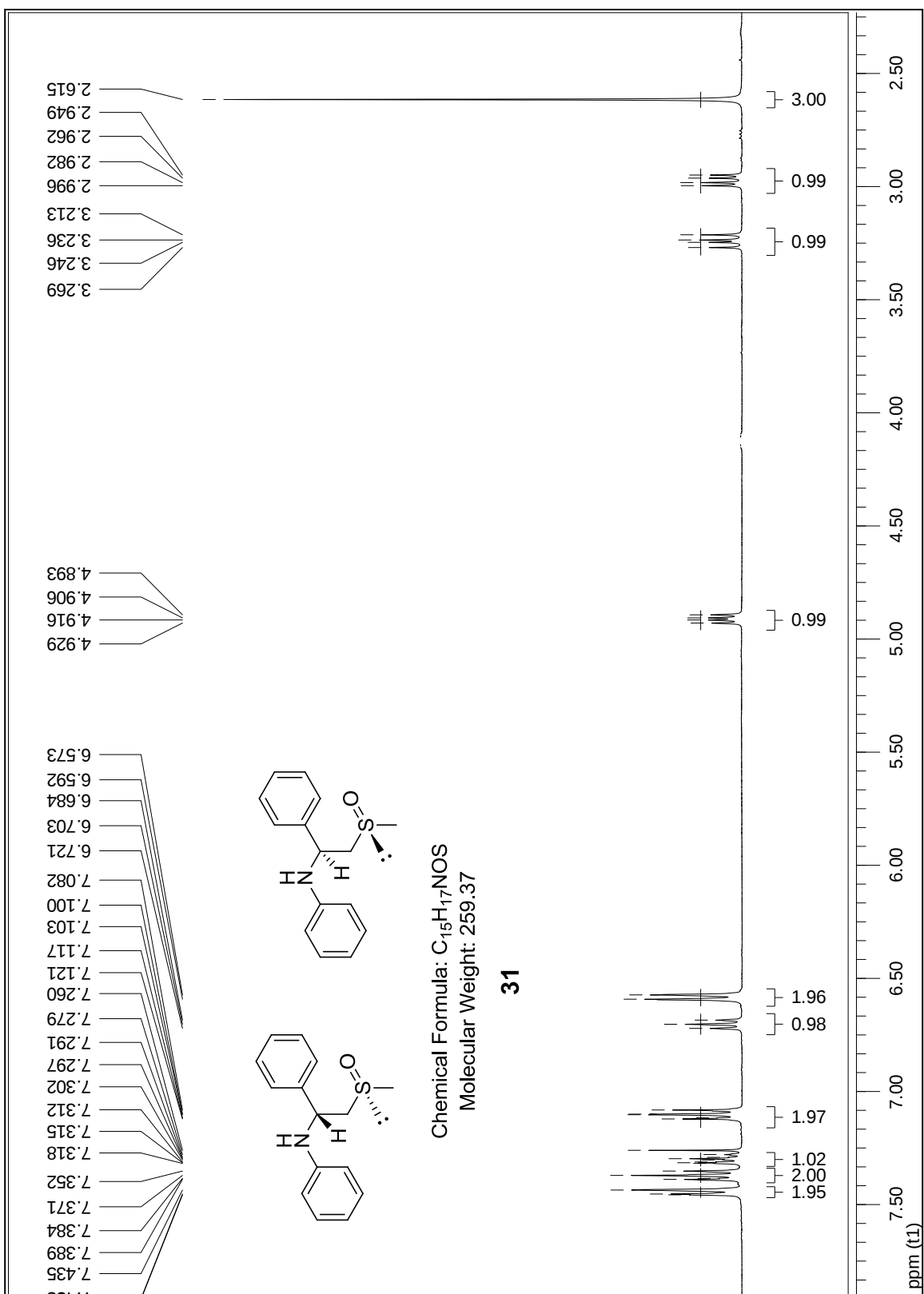


Figure 1.3.3.37. 400 MHz 1H NMR spectrum of compound **31** in $CDCl_3$.

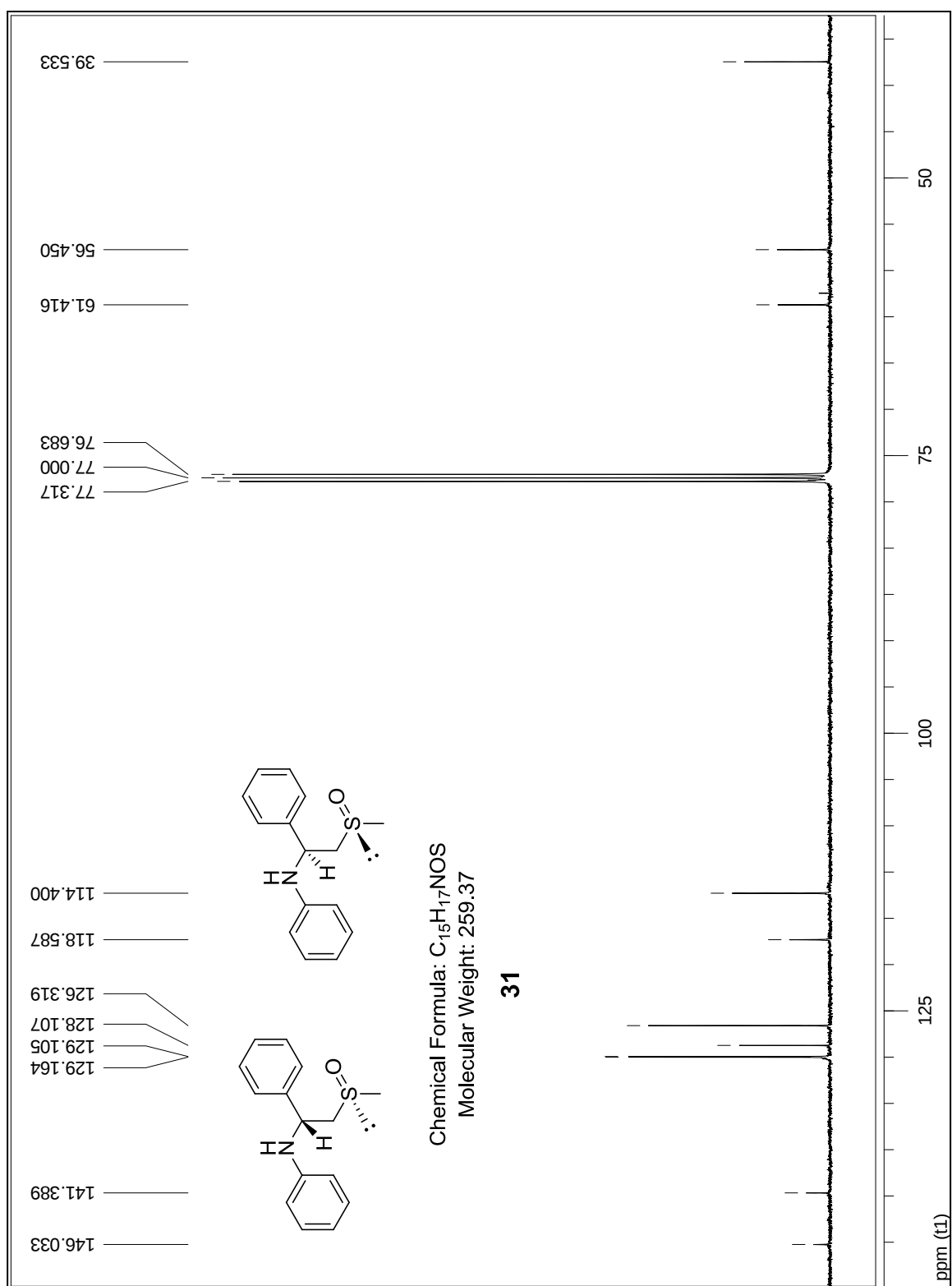


Figure 1.3.3.38. 100 MHz ^{13}C NMR spectrum of compound **31** in $CDCl_3$.

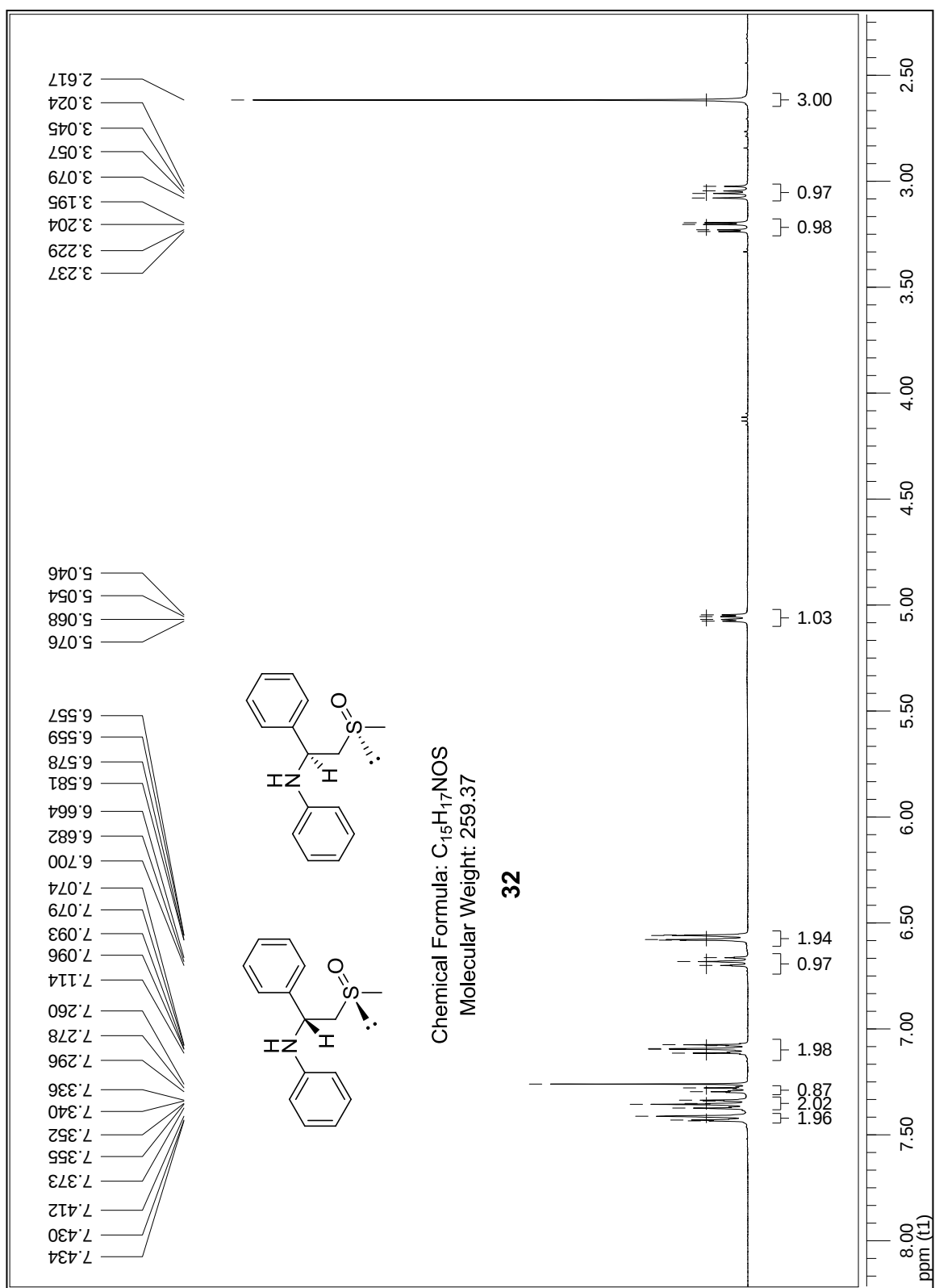


Figure 1.3.3.39. 400 MHz ¹H NMR spectrum of compound **32** in CDCl₃.

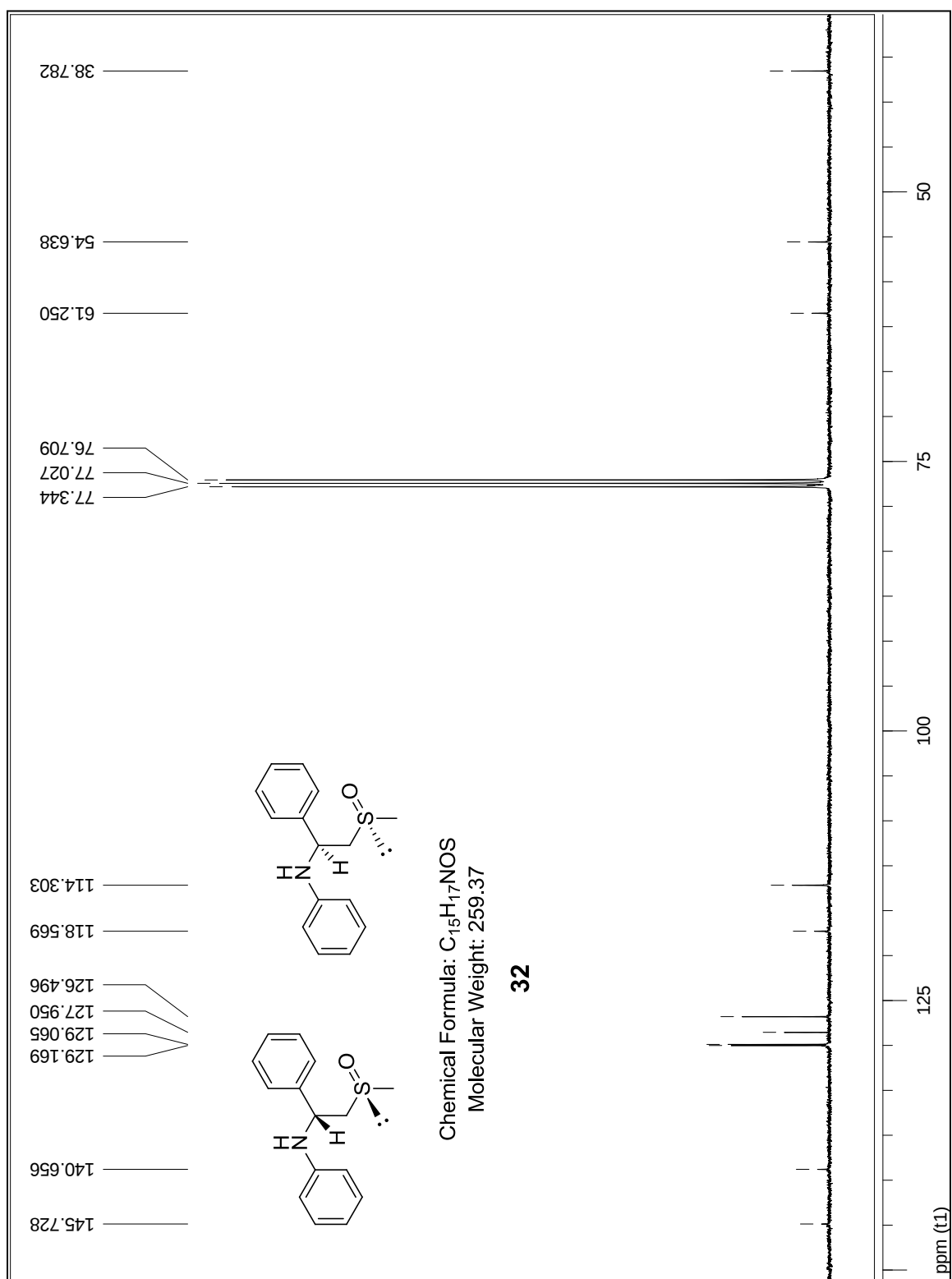


Figure 1.3.3.40. 100 MHz ¹³C NMR spectrum of compound **32** in CDCl₃.

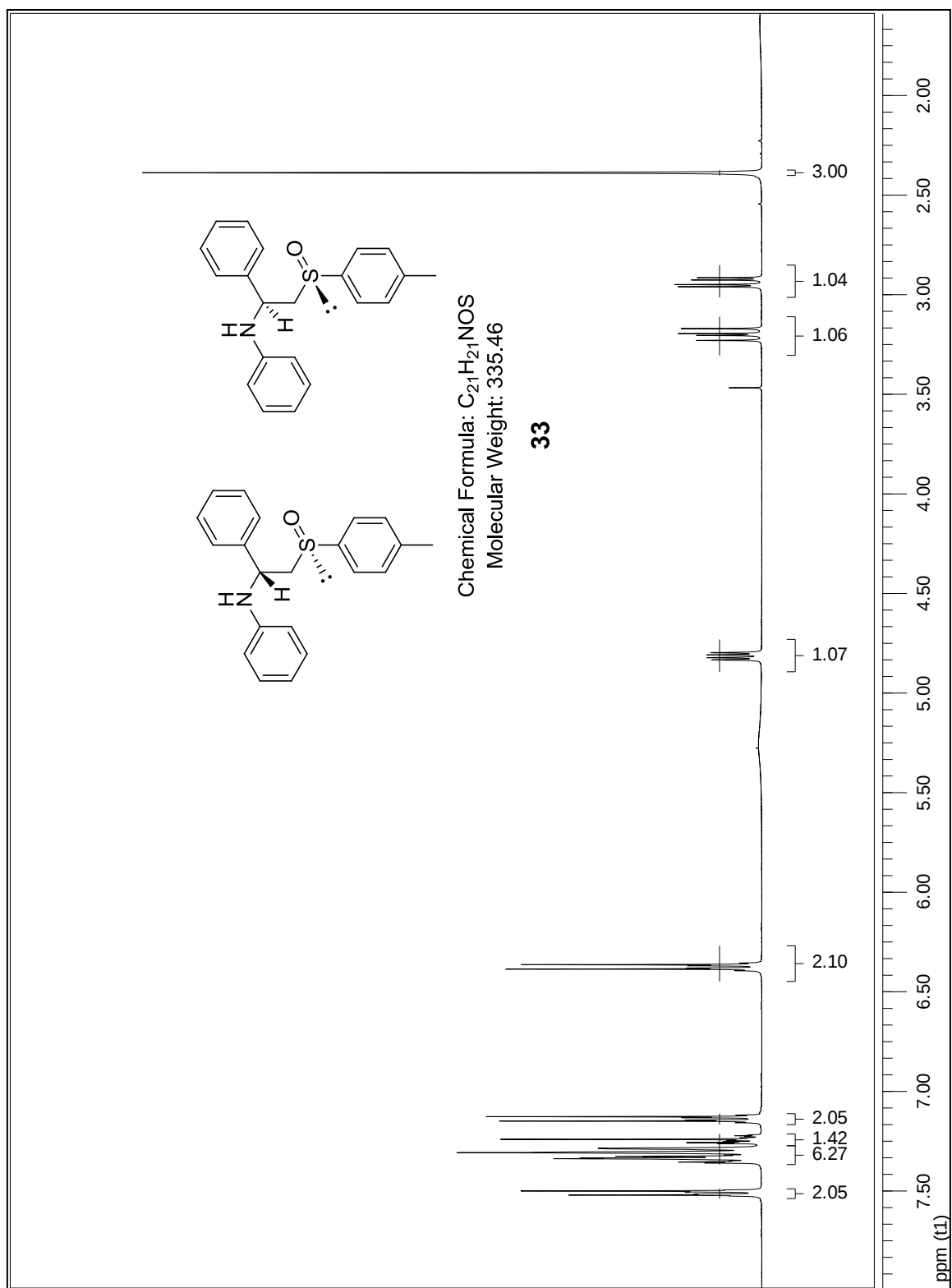


Figure 1.3.3.41. 400 MHz ^1H NMR spectrum of compound **33** in CDCl_3 .

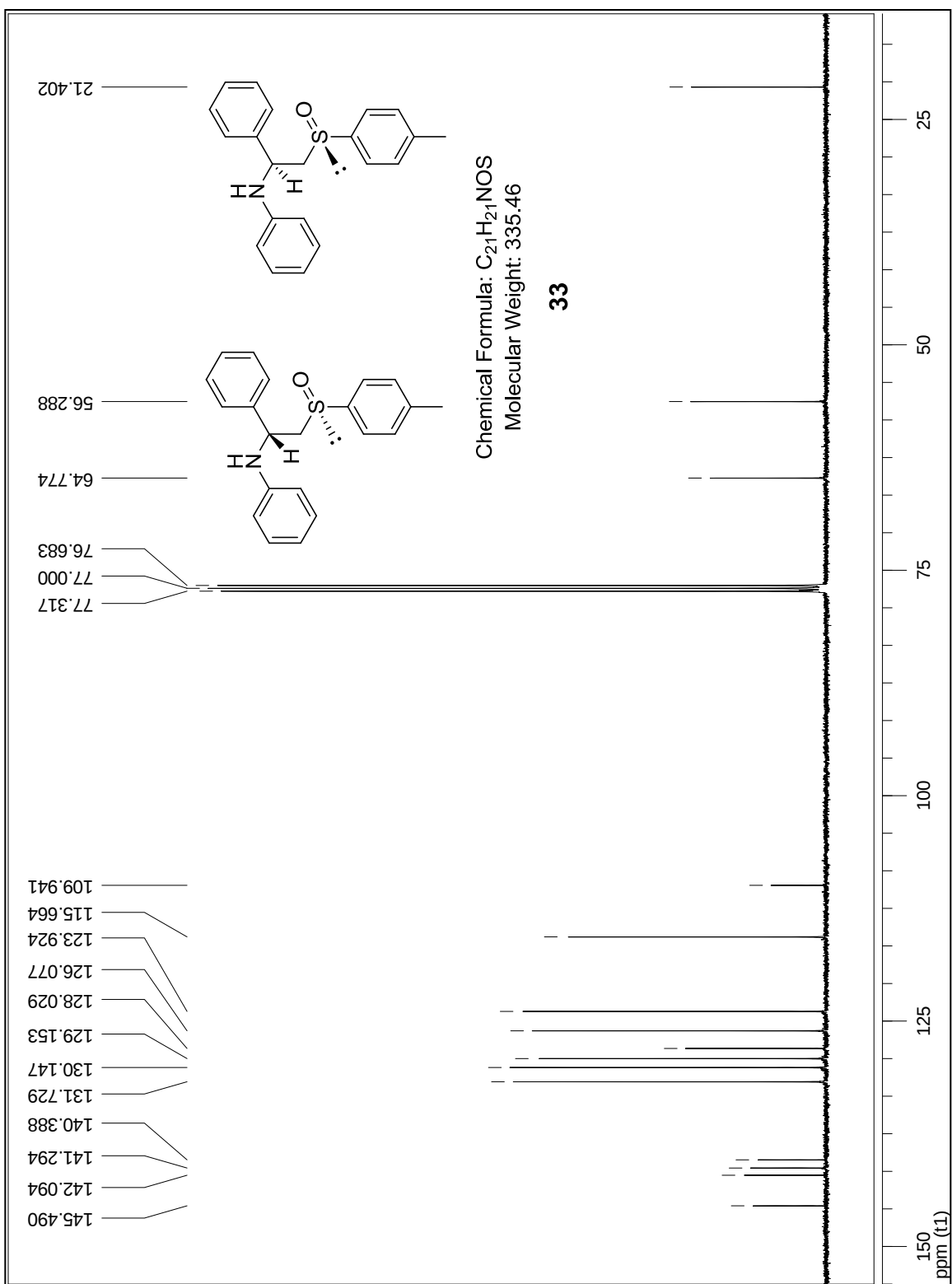


Figure 1.3.3.42. 100 MHz ^{13}C NMR spectrum of compound **33** in $CDCl_3$.

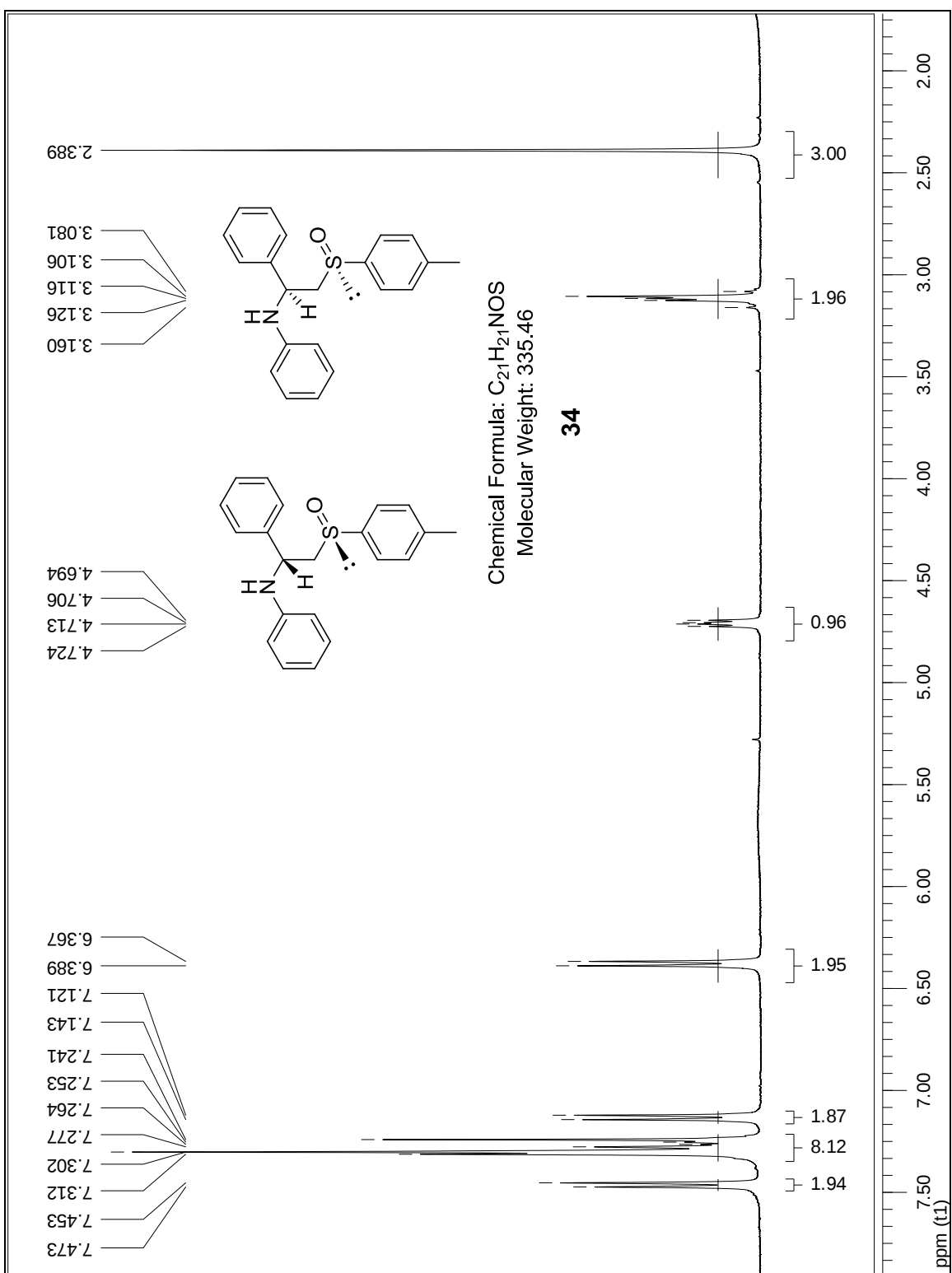


Figure 1.3.3.43. 400 MHz 1H NMR spectrum of compound **34** in $CDCl_3$.

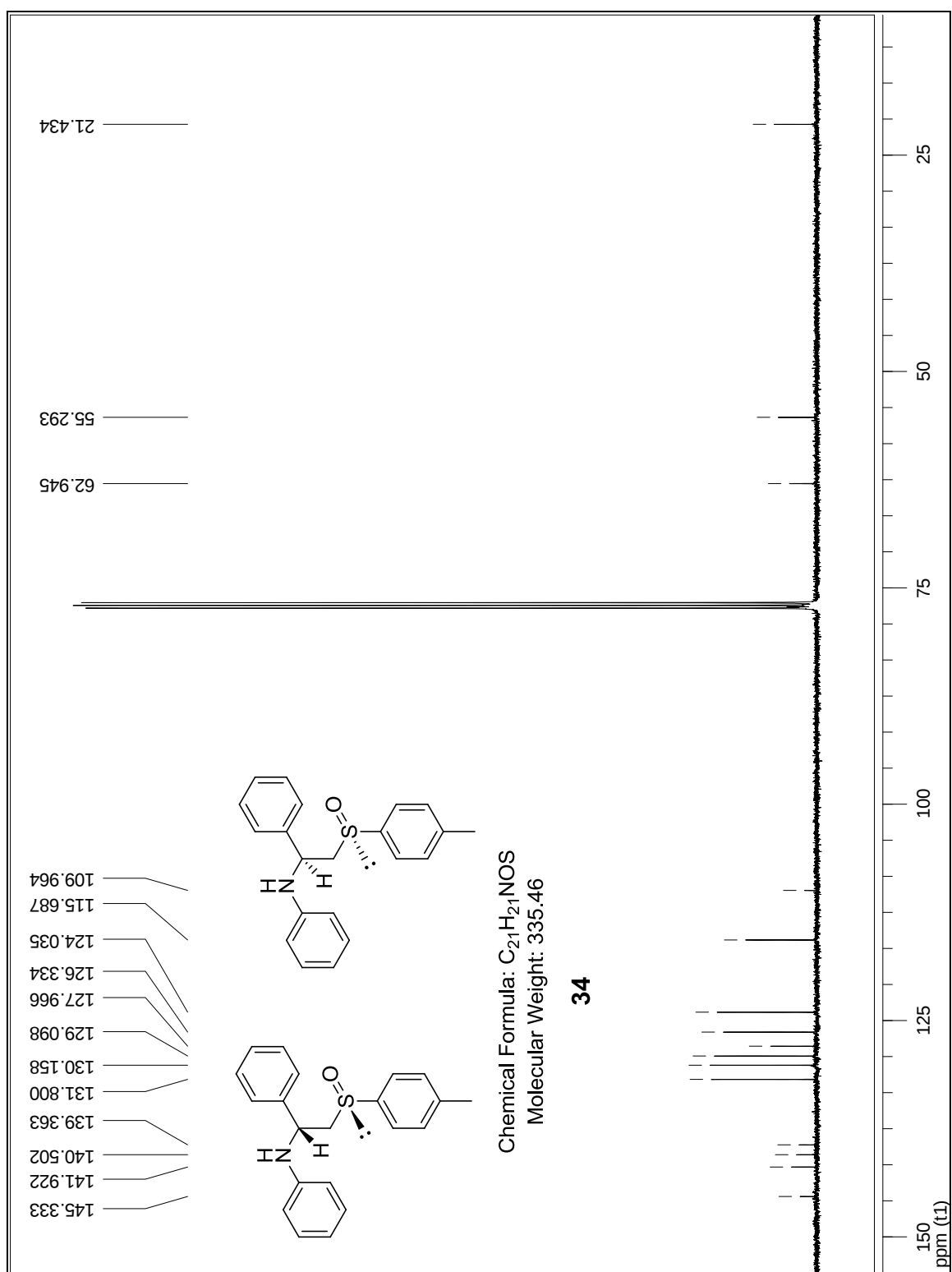


Figure 1.3.3.44. 100 MHz ^{13}C NMR spectrum of compound **34** in $CDCl_3$.

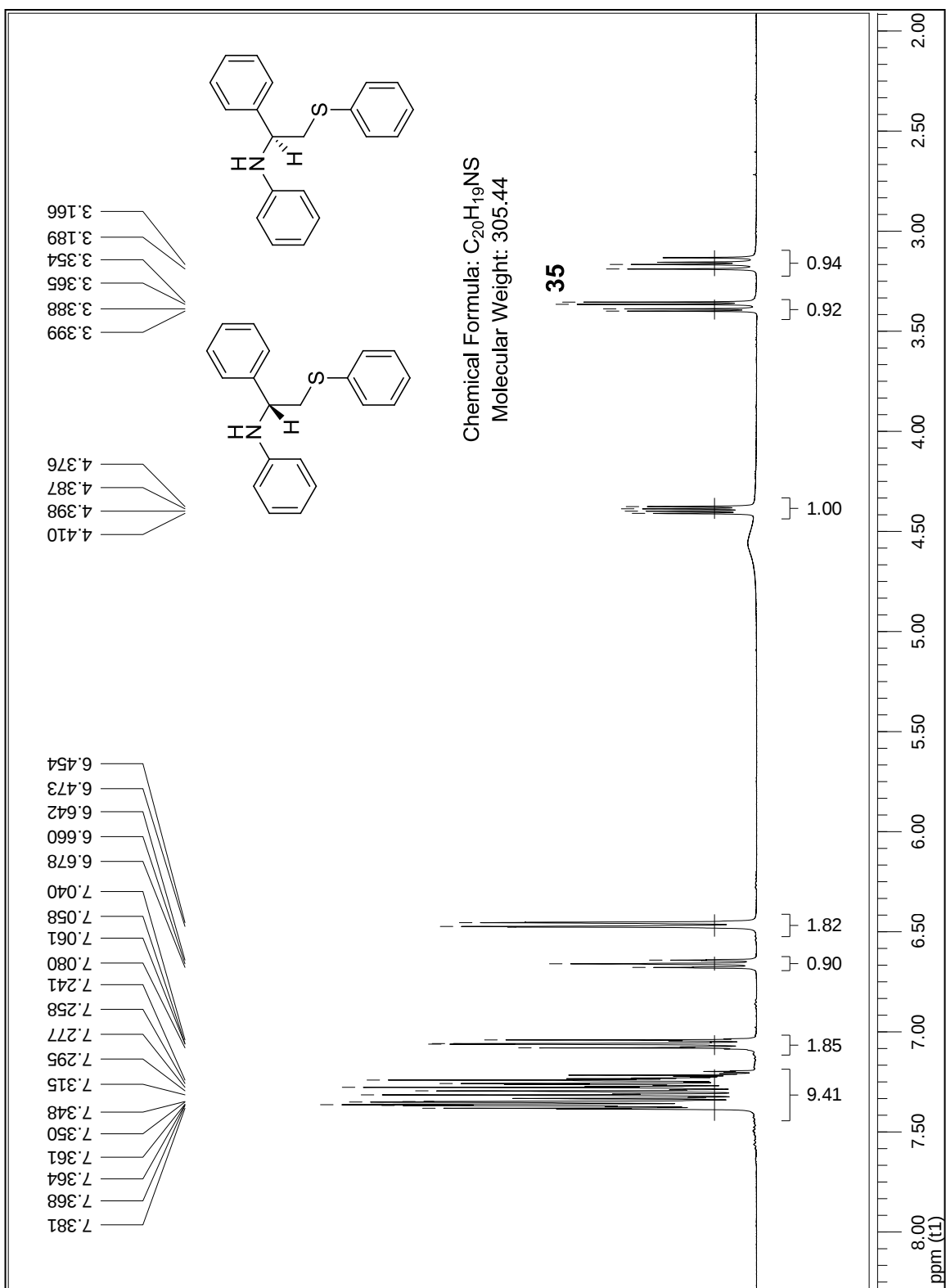


Figure 1.3.3.45. 400 MHz ¹H NMR spectrum of compound 35 in CDCl₃.

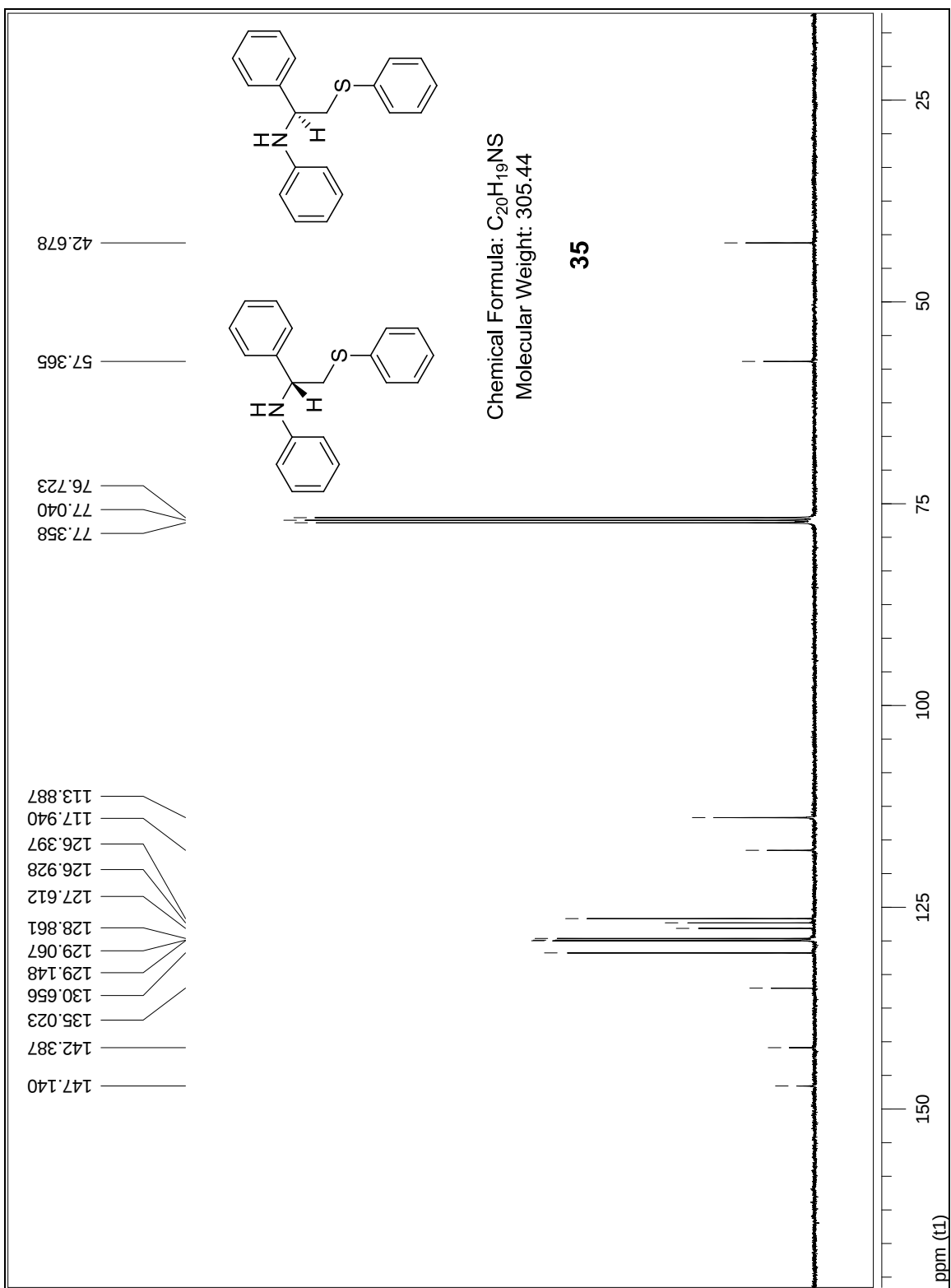


Figure 1.3.3.46. 100 MHz ^{13}C NMR spectrum of compound **35** in $CDCl_3$.

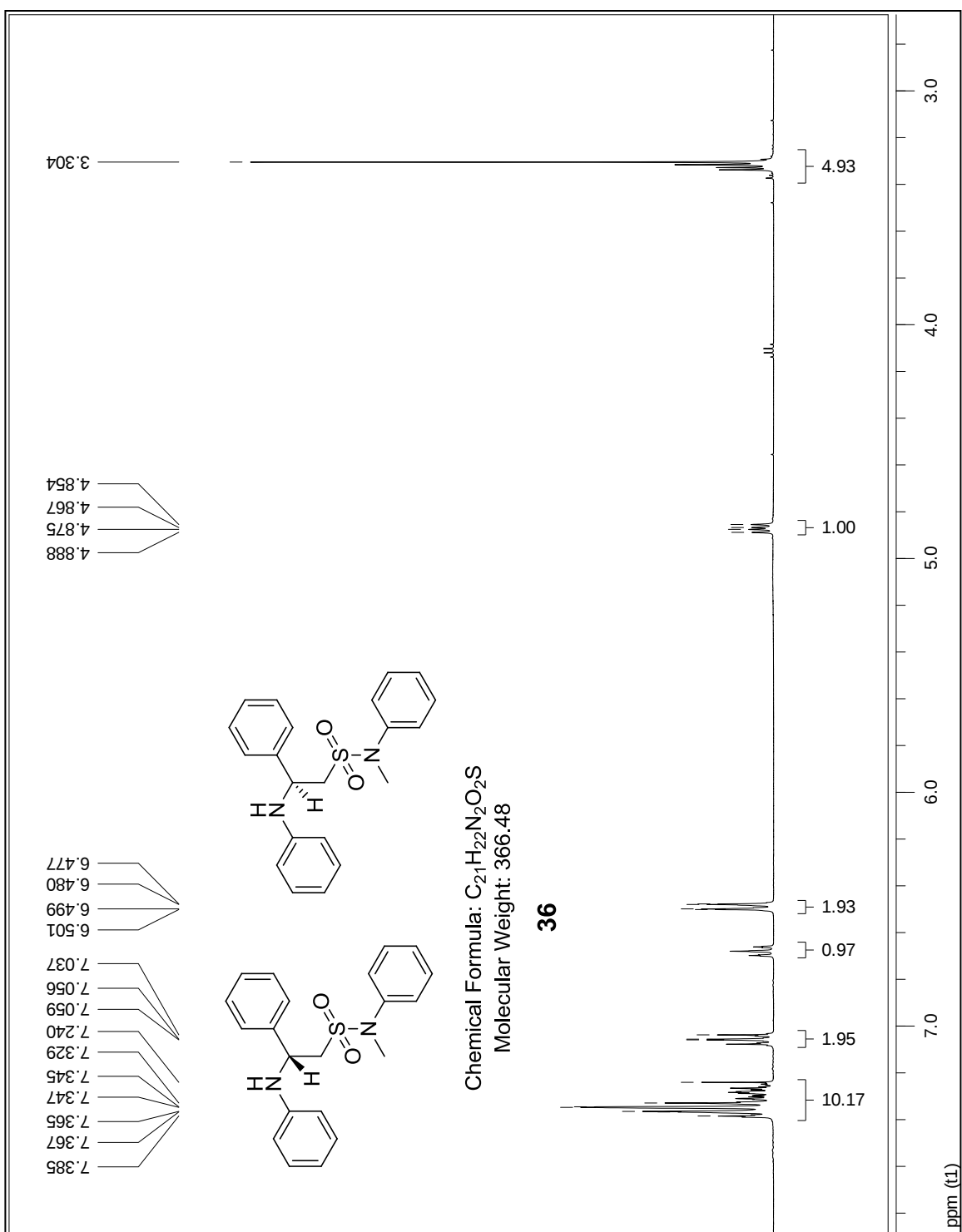


Figure 1.3.3.47. 400 MHz 1H NMR spectrum of compound **36** in $CDCl_3$.

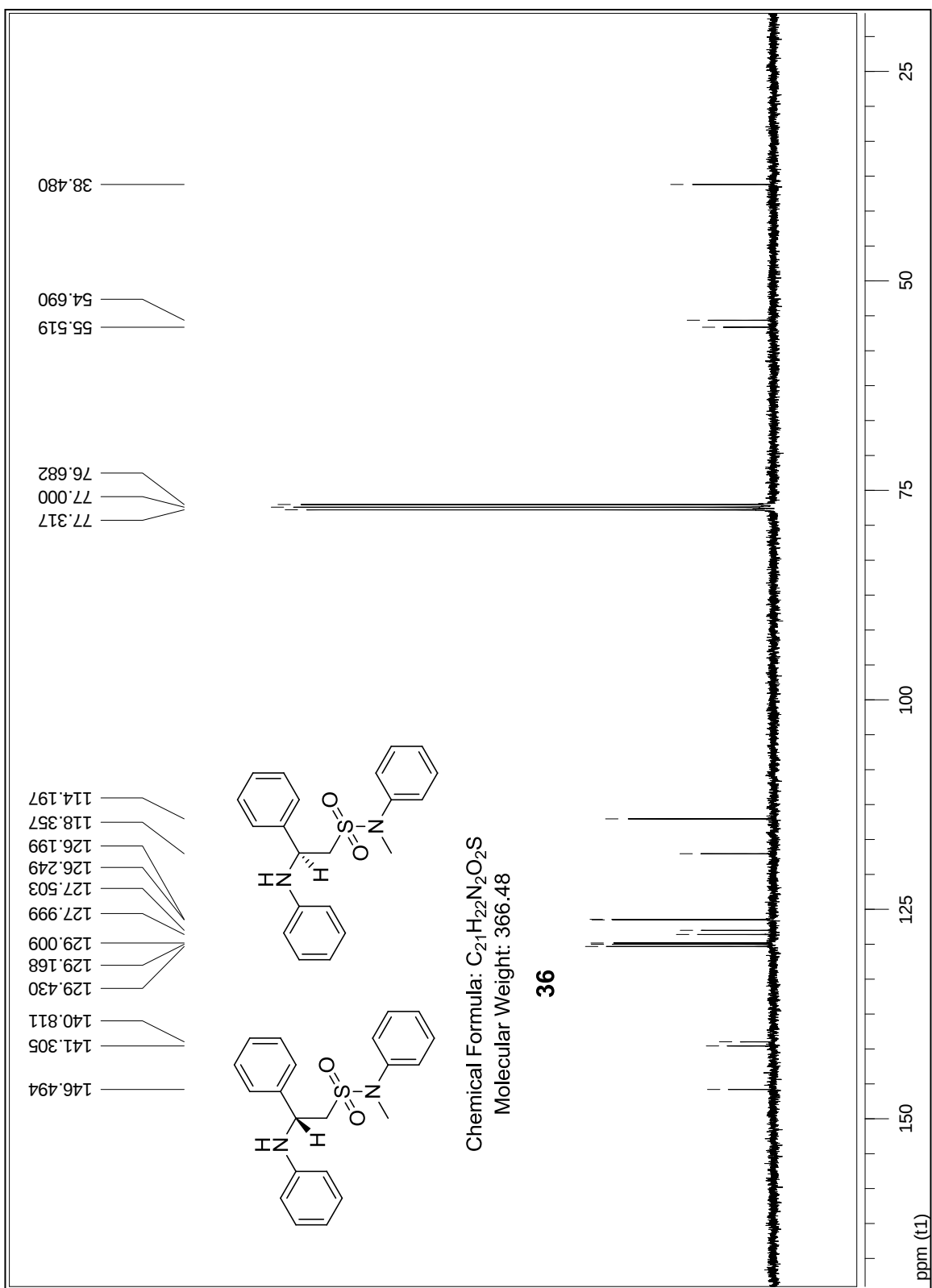


Figure 1.3.3.48. 100 MHz ^{13}C NMR spectrum of compound **36** in $CDCl_3$.

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PART II. Synthesis of Isoxylitane analogues for the treatment of epilepsy

2.1 Introduction

2.1.1. Epilepsy

Epilepsy is a chronic disorder of the brain that affects roughly 50 million people worldwide. It is characterized by recurrent seizures, which are brief episodes of involuntary shaking, involving a part of the body (partial) or the entire body (generalized), sometimes accompanied by loss of consciousness. Generalized seizures are produced by electrical impulses from in the entire brain whereas partial seizures are produced by electrical impulses in a relatively small part of the brain. There are six types of generalized seizures, with the most common and dramatic being the "Grand Mal" seizure. Partial seizures are divided into simple, complex and those that evolve into secondary generalized seizures. The differences between simple and complex seizures is that during simple partial seizures, patients retain awareness whereas during complex partial seizures, they lose awareness. The episodes are a result of excessive electrical discharges in a group of brain cells which disrupt normal brain function. Seizures can vary from the briefest lapses of attention or muscle jerks, to severe prolonged convulsions. Epilepsy is one of the world's oldest recognized conditions where fear, misunderstanding, discrimination and social stigma have surrounded the disorder for centuries¹. A summary of the most common types of seizures are listed in **Table 2.1.1.**, together with their associated symptoms.¹⁶

Table 2.1.1.1. Types of Seizures and Symptoms

Type of Seizure	Symptoms
Generalized Seizures	
1. "Grand Mal" or generalized tonic clonic	Unconsciousness, convulsions, muscle rigidity
2. Absence	Brief loss of consciousness
3. Myoclonic	Sporadic (isolated), jerking movements
4. Clonic	Repetitive, jerking movements
5. Tonic	Muscle stiffness, rigidity
6. Atonic	Loss of muscle tone
Partial Seizures	
1. Simple (awareness is retained) A. Simple Motor B. Simple Sensory C. Simple Psychological	A. Jerking, muscle rigidity, spasms, head-turning B. Unusual sensations affecting either the vision, hearing, smell, taste or touch C. Memory or emotional disturbances
2. Complex (Impairment of awareness)	Automatisms such as lip smacking, chewing, fidgeting, walking and other repetitive, involuntary but coordinated movements
3. Partial seizure with secondary generalization	Symptoms that are initially associated with a preservation of consciousness that then evolves into a loss of consciousness and convulsions.

Epilepsy is one of the more common neurological problems worldwide. In developed countries, annual new epilepsy cases range between 40 to 70 per 100 000 people. In developing countries, however, this figure is almost doubled, due to higher risk of people having experienced previous conditions that may have resulted in permanent brain damage⁵. Thus, close to 90% of epilepsy cases worldwide are found in developing regions.

In recent years, important advances have been made in the diagnosis and treatment of seizure disorders. However, the cellular and molecular mechanisms by which epilepsy develops, are still unknown⁴.

2.1.2. Anti-epileptic drug development

2.1.2.1 Bromides

Sir Charles Locock, working at the Medical and Chirurgical Society of London during the mid-nineteenth century, is credited with the introduction of bromides for the treatment of epilepsy¹⁴. He successfully used potassium bromide to treat women with what he called hysterical epilepsy (a term used widely until two decades ago). Following this discovery, subsequent reports of its efficacy eventually led to the widespread use of bromides for the treatment of seizures, often in combination with other agents including digitalis, belladonna, zinc and iron. The use of bromides remained the foundation of epilepsy treatment for many years, however their use was associated with dermatological conditions, such as severe rash, and psychological symptoms, including irritability, emotional instability and schizophrenic-like psychosis in many patients⁹.

2.1.2.2. Barbiturates

In the early twentieth century, serendipity played a role in the discovery of the antiepileptic efficacy of phenobarbital. At this time, barbiturates were widely used as hypnotics and sedatives. With this knowledge, a clinical assistant in Germany gave phenobarbital to epilepsy patients as a tranquilizer and it was observed that the frequency of their seizures was greatly reduced. Furthermore, studies revealed that phenobarbital was more efficacious than the bromides. Importantly, phenobarbital had none of the physical or emotional side effects that

accompanied the bromides and therefore eventually became a widely used anti-epileptic drug (AED)¹⁰.

2.1.2.3. AED's in animal model

The next major advancement in AED discovery was the introduction of epilepsy in animal models. The importance of this was the ability to test and screen multiple AED candidates in animals for antiepileptic activity prior to testing in humans. One of the first pieces of work for screening anti-seizure activity was using electrically induced seizures in cats. The anticonvulsant properties of diphenylhydantoin was discovered using this model. Diphenylhydantoin, known today as phenytoin, demonstrated significant activity in various ways^{11, 14}. Firstly, phenytoin was shown to be beneficial for a large number of epileptic patients for whom bromides and barbiturates were ineffective. Secondly, clinical doses of phenytoin were not associated with any sedation effects seen with other agents. Lastly, the success of using an animal model for testing demonstrated that this approach could be utilized to discover additional families of compounds with clinical efficacy. Phenytoin proved its clinical value, showing greater activity and efficacy than phenobarbital in reducing various seizure types without having the associated sedation effects and it became the first choice AED for partial onset seizures; it is still used today. Although, phenytoin demonstrated its great efficacy in multiple ways, it was also found to have major limitations with respect to pharmacokinetics, extensive drug interactions and chronic toxicities. Other unwanted side effects that are associated with this drug include, carcinogenicity and teratogenicity, which are serious health risks that are delayed effects of the drug. The discovery of phenytoin led to the identification and introduction of several new drugs produced by simple alterations of its structure. These include analogues of phenytoin such as mephenytoin, and those of phenobarbital including metharbital and primidone¹².

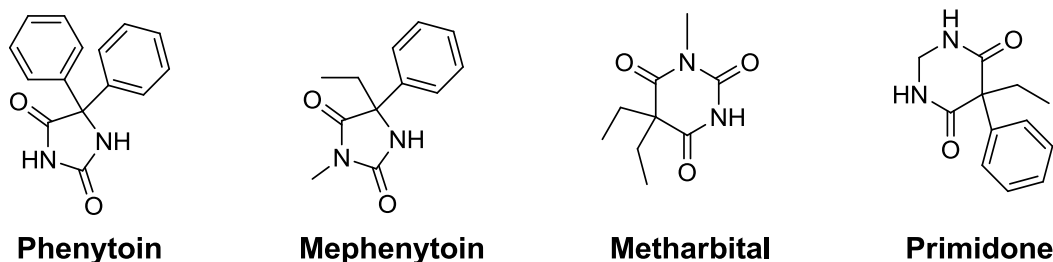
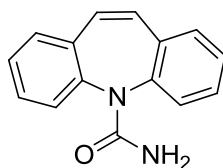


Figure 2.1.2.3. Structures of early AED's based on phenytoin and phenobarbital.

2.1.2.4. New generation AED's

The next major advancement in AED development was carbamazepine (CBZ) which remains to this day, remains the gold standard for treatment of partial onset epilepsy in Europe. This compound was the first of the new generation of AED's with a tricyclic structure and was discovered by two small open label studies to possess antiepileptic effects. It was first approved in the mid 1960's in the majority of European countries; however, its approval in the USA was delayed until 1974 due to multiple reports of aplastic anemia. Although CBZ was found to be one of the drugs that is most effective in retention and seizure control, there are a number of additional serious side effects associated with it. For example, CBZ has also been associated with idiosyncratic rash, hyponatraemia, hematological toxicity and hepatic dysfunction, as well as rare adverse events including thrombocytopenia and Stevens-Johnson syndrome. There is an obvious need for new effective AED's with improved safety profiles¹³.



Carbamazepine

Figure 2.1.2.4. Structure of CBZ.

2.1.2.5. Improved AED's

An increasing number of new AED's have become available during the recent years that provide both patients and physicians with a wealth of alternative treatment options. Despite this development, the newer AED's have not resulted in significant improvement of patient outcomes. The rate of seizure freedom achieved long ago was approximately 30% and unfortunately this number has not changed much over the years, despite the new treatments¹⁵. The older drugs share a 5 or 6 membered heterocyclic ring which includes one or two nitrogen atoms and a variety of side chains, some containing other ring structures. It is speculated that the common heterocyclic ring structure may underlie the allergic reactions in some patients to more than one drug. The structures of the newer drugs possess few similarities to the older generations resulting in different activity and mechanisms of action¹⁴.

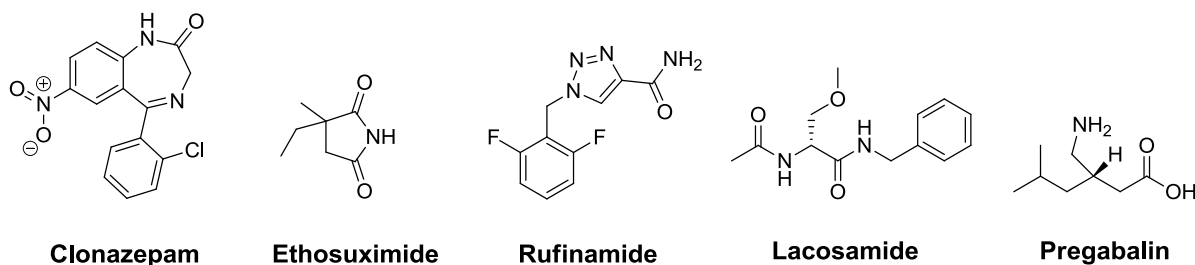


Figure 2.1.2.5. Structures of old (Clonazepam and Ethosuximide) and new (Rufinamide, Lacosamide and Pregabalin) generation AED's.

2.1.2.6. Mechanisms of AED activity

Although the underlying mechanisms for how epilepsy arises is still unknown, there are known ways by which AED's act in order to lower the frequency of seizures. Seizures as we know, are hyper-excitable neuronal networks, where the balance of normal neuronal activity is altered. This leads to a predominate excited state rather than an inhibitory one, which leads to convulsions and seizures. Therefore, a typical AED treatment should effectively augment the

inhibitory process or oppose excitatory processes in the brain to rebalance neuronal function. While trying to restore normal resting neuronal potential, hyperpolarizing or depolarizing the cell controls electrical current through the membrane. Drugs can direct specific ions within channels or influence synthesis, metabolism or function of neurotransmitters indirectly or control opening and closing of channels when needed¹⁷. The majority of AED's, both old and new generations, act either by blocking voltage gated sodium channels (VGSC), or by prolonging/inhibiting GABA mediated transmissions¹⁸. Other molecular targets include voltage gated potassium channels, glutamate receptors and acting upon carbonic anhydrase.

2.1.2.6.1. Voltage gated sodium channels and its role in epilepsy

Voltage gated sodium channels are responsible for depolarisation of the nerve cell membrane and conduction of action potentials across the surface of neuronal cells. Depolarisation refers to the sudden dramatic electrical change in which the negative internal charge of the cell becomes positive for a brief period of time. This brief shift in electrical environment allows for the transmission of electrical impulses both within and between cells. The bulk expression of these channels is on the axon initial segment where action potentials are generated¹⁷. Sodium channels belong to a super family of voltage gated channels that are composed of multiple protein subunits and form ion selective pores in the membrane. There are four predominant sodium channel subunits expressed in the mammalian brain and these channels are expressed differently in the nervous system¹⁸. Blockade of VGSC's is the most common mechanism of action among currently available AED's. VGSC's exist in one of three basic conformational states; resting, open and inactivated. During a single round of depolarisation, channels will cycle between these three states, from resting to open, open to inactivated and inactivated back to resting, and are unable to respond to further depolarisations until sufficient

numbers have returned from inactivated state to resting state¹⁷. AED's with sodium channel blocking abilities have the highest affinity for the channel protein in the inactivated state and binding slows the conformational recycling process between states. Such drugs produce a characteristic voltage and frequency dependent reduction in channel conductance, which in turn limits the repetitive neuronal firing. Although details of these mechanisms are still unclear, it has been shown that slow inactivation of this pathway and prolonged depolarisation of cells result in retention of epileptic seizures¹⁸.

2.1.2.6.2. AED's and GABA inhibitory neurotransmission

γ -Aminobutyric acid (GABA), the predominant inhibitory neurotransmitter in the CNS is released at up to 40% of all synapses in the brain. GABA is synthesized from glutamate by the action of the enzyme glutamic acid decarboxylase and consists of two types of receptors. Barbiturates and benzodiazepenes share this effect, but they bind to distinct sites on the receptor complex and differentially influence the opening of the chloride ion pore¹⁷. GABA is localized primarily in short axon interneurons that synapse on cell bodies and proximal axons and serves to maintain an inhibitory tone that counterbalances neuronal excitation. When this balance is perturbed, seizures may develop²⁰. Abnormalities of GABAergic function have been observed in genetic and various animal models of epilepsy. Reductions of GABA-mediated inhibition, activity of glutamate decarboxylase and GABA in cerebrospinal fluid and brain tissue have all been reported in the studies of human epileptic brain tissues¹⁹. It has been shown that GABA agonists suppress seizures while GABA antagonists produce them. Essentially, AED's would act by either increasing the duration of the chloride channel opening by increasing the frequency by which the channel opens, or by modulating GABA responses at the GABA_A receptor to suppress the frequency of seizures.²⁰

2.1.2.7 Epilepsy today and expectations for the future

Today, there are approximately 25 AED's used for the treatment of epilepsy and different AED's work to control different types of seizures. AED's are neither preventative nor curative and are employed to the patient solely as a means of controlling symptoms and to suppress the occurrence of seizures. There have been multiple improvements over the past few decades that have led to minor success in overall treatment. It is important to recognize that epilepsy is a chronic condition, which in most cases requires long term and maybe even life-long treatment. The key to effective management is to provide treatment options that patients are able and willing to continue taking long term without any adverse side effects associated with them.

Recent studies, in both developed and developing countries, have shown that a small portion of individuals are able to control seizures with anti-epileptic drugs (AED's), while others may experience drug resistance or may require large dosages. Although there are AEDs that have been successful in treatment, there is a low availability of these drugs today and the majority of them are not affordable, which may act as a barrier to accessing treatment. Response to drug treatment is different for each individual. Surgical therapy may be beneficial to patients who respond poorly to drug treatments². About 30% of the epileptic population have seizures that are non-responsive to the presently available medical therapies. Regardless of the available treatments, none of them are capable of completely controlling seizures and the majority of drugs have severe side effects. Considering the large percentage of uncontrolled epileptics alongside the severe side effects experienced by patients with existing medication, there is a critical need for more selective and less toxic antiepileptic medications³.

2.1.3. *Delphinium denudatum*

Extracts of a variety of plants have been used for many years to treat a number of neurological disorders. Delphinium is a genus comprising annual or perennial ornamental herbs. There are roughly 270 species of Delphinium found worldwide, *Delphinium denudatum* Wall grows extensively in the Western Himalayas and in Kashmir. The roots of this plant, known locally as Jadwar, have been used to treat seizures by local folk medicine practitioners⁶.

Bio-assay guided isolation was carried out by a team of researchers at the University of Karachi, to isolate the anticonvulsant components of this plant. The crude ethanolic extracts of this plant were subjected to bioassay guided fractionation, which revealed that chloroform extracts containing diterpenoid alkaloids were highly toxic to the neuromuscular system of mice. Further fractionation led to the conclusion that the anticonvulsant constituents were found in the least toxic of all extracts, the non-alkaloidal aqueous extracts of the plant. This extract was subjected to vacuum liquid chromatography, which afforded an oily fraction. Further purification of this oily material led to the isolation and structure identification of a strongly anticonvulsant isomeric mixture of *E/Z* isoxylitane. The structures of both isomers, also referred to as ISOX is shown below⁶.

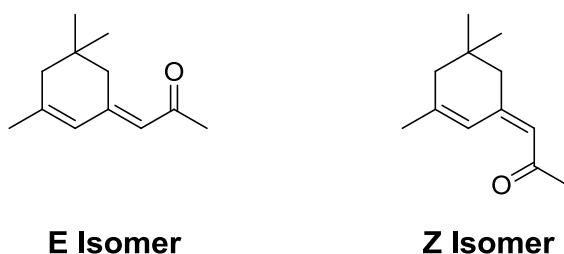


Figure 2.1.3.1. Isomers of *E/Z* isoxylitane

2.1.4. Screening for potential anticonvulsant molecules

The screening of potential anticonvulsant molecules is based mainly on two types of tests; the acute seizure model and the chronic seizure model. The acute seizure program uses maximal electroshock tests (MEST) and subcutaneous penylenetrazole (scPTZ) programs to monitor anticonvulsant activity, whereas the kindling model of epilepsy is considered to be a chronic model of epilepsy, which is primarily used for evaluating test drugs for anti-epileptogenic activity³.

The scPTZ test is widely used in AED discovery as it evaluates the ability of the test substance to raise the seizure threshold for excitation of neural tissues. This test is most commonly used for the preliminary screening for new AED's. Almost all of the drugs that are active in this scPTZ test demonstrate some clinical efficacy against myoclonic seizures that are shock-like jerks of a muscle or a group of muscles. The kindling is the model of epileptogenesis and was described by Graham Goddard in 1967³. Kindling is a process in which regular sub-convulsant stimulation of the brain, elicited by chemical convulsant or electrical current, gradually results in the development of generalized seizure pattern which then leads to convulsions. This model is a very good screening tool for developing new AED's due to its universality across species and parameters throughout the brain⁷. Repeated daily stimulation results in an increase in the duration and complexity of excitable stimulus also known as afterdischarge (AD) . The growth of the AD is accompanied by the development and behavioral convulsions that can be scored on a scale from 1-5 according to severity. Seizures with a score between 1-2 are usually equivalent to partial seizures, while behavioral seizure scores at a stage of 3-5 are generalized. Once an animal exhibits three consecutive stage 5 seizures, the animal is considered to be fully kindled and the kindling process is completed²¹.

2.1.5. ISOX Studies

In order to better understand the ISOX activity, the University of Karachi team conducted multiple different neurological studies in order to validate its anti-epileptic properties³. Effects of the isomeric isoxylitane compounds were evaluated on the c-Fos protein and mRNA expression in the brain samples of kindled mice compared with normal and untreated kindled groups. c-Fos protein is a proto oncogene, which is a normal gene that becomes an oncogene due to mutations and is expressed in some neurons following depolarization. Throughout this study, kindling was induced in male mice by repeated administration of sub-convulsive dose of PTZ until a seizure score of 4-5 was achieved. c-Fos expression levels were quantified via RT-PCR analysis, where a high expression of c-fos mRNA and protein were found in the brain regions of the PTZ-kindled group. In contrast, treatment with ISOX significantly reduced c-Fos expression compared to the control group. Based on these observations, it was suggested that isoxylitones have the capacity to control seizure patterns by mechanism of c-Fos mRNA and protein level suppression in different regions of the brain⁷.

The effect of ISOX on brain derived neurotrophic factor (BDNF), which is the most potent factor required for neurogenesis and is necessary for peripheral and central nervous system development, maintenance and response to injury was investigated⁶. Levels of both BDNF mRNA and BDNF protein are known to be up-regulated in epilepsy. BDNF modulates excitatory and inhibitory synaptic transmissions by inhibiting GABA receptor mediated post synaptic currents; this provides a potential mechanism for up regulation. Treatment of kindled mice with ISOX was able to reduce the levels of BDNF expression, thereby significantly reducing the occurrence of seizures³.

As mentioned earlier, the factors contributing to epilepsy remain unknown, in which case the information behind the mechanism of action of current AED's is incomplete. In order to better understand the mechanism of action of ISOX activity, the group in Pakistan conducted a study on the effects of ISOX on voltage gated sodium channels (VGSC) to establish its ability to reduce electrically kindled seizures. Patch clamp electrophysiology assayed the activity of ISOX on VGSC's in both cultured neurons and brain slices isolated from controls and rats with experimental epilepsy. Studies indicated that ISOX inhibits the function of VSGC's in two ways. Firstly, by causing a tonic block on the VGSC that cannot be altered by voltage, and secondly, it affects the speed of recovery from the inactivation and causes a hyperpolarizing shift in the voltage dependence of the entrance into the inactivation state. Treatment with ISOX prevented kindled stage 5 seizures and decreased the enhanced BDNF mRNA expression that is normally associated with kindling. Overall, the results of the study concluded that ISOX is a potent inhibitor of VGSC's that stabilized the steady state inactivation while slowing recovery and enhancing inactivation development. The majority of AED's impair the access of sodium through VGSC's which make them effective sodium channel modifiers. The IC_{50} values for most modifiers is correlated to the lipophilicity, that being, the more lipophilic the drug, the more it blocks the sodium current. Studies demonstrate that ISOX is more lipophilic in comparison to other AED's on the market, thus making it a stronger VGSC blocker. This lipophilic character allows for a smaller IC_{50} value, thereby making it more selective to the lipid membrane sites on the sodium channel. These data show a new class of anti-seizure compounds that inhibits sodium channel function and prevents the development of epileptic seizures, being more selective and less toxic in nature⁸. The results of these studies are the basis of two approved US patents.^{3,6}

2.1.6. Objective

Intrigued by the reported activity of ISOX, Professor Michael Poulter, Department of Pharmacology at Western University in London, Ontario, and also a member of the Ontario Brain Institute, set out to confirm these results in his own laboratory. Poulter's research group has been focusing on epilepsy for two decades and claims not to have seen any other molecules having remotely close to the activity of ISOX. This group encouraged the Durst lab to prepare a variety of analogues of this ISOX compound, to build a library, in hopes of optimizing epileptic activity.

2.2 Discussion and Results

2.2.1. Introduction

In the fall of 2013, Dr. Michael Poulter at Western University, who is also a member of the Ontario Brain Institute, became aware that healers in the Himalayan region of Pakistan had used extracts from *Delphinium denudatum* for the treatment of epilepsy and seizures and that researchers in Pakistan had identified isoxylitone (ISOX) **Figure 2.2.1.1.** as the active component. After thorough preliminary studies, Dr. Poulter who has been working on anti-epilepsy agents for years, could not believe the high level of activity of this compound. In fact, he was so impressed with the potential of this compound for the treatment of epilepsy and seizures that he convinced his brother to join with him in founding a biotech start-up company, called OB Pharma, to develop this compound either itself or as a lead compound for the treatment of epilepsy and seizures.

The Poulter group approached the Durst lab to build a compound library by preparing a variety of analogues of ISOX, in hopes of optimizing epileptic activity. This compound had previously been described in the literature and given the trivial name "isoxylitone".

In the beginning, it was impossible to decide which features of the molecule contribute to the activity due to the fact that only one compound, ISOX, had been tested previously. Therefore, the initial plan was to prepare a small set of compounds (keeping the original trimethylcyclohexenone core structure) by introducing various new functional groups in place of the methyl ketone. Bioassay feedback from this preliminary set of compounds would allow us to understand the features contributing to the activity. Obvious functional group variations included replacing the ketone in ISOX by ester, acid, amide, nitrile, sulfoxide and sulfone functionalities. (**Structure A**) Except for the sulfoxide and sulfones, each of these groups like

the ketone in ISOX represents an electron withdrawing group via π -conjugation. Thus, such compounds should have shapes very similar to the lead structure. We also considered changing the methyl ketone group with larger substituents, both aliphatic and aromatic (**Structure B**) and finally the replacement of the vinylic hydrogen alpha to these functional groups by other alkyl and even eventually aryl substituents was also planned (**Structure C**). These proposals are shown as the general structures A, B and C, respectively in **Figure 2.2.1.1.** below.

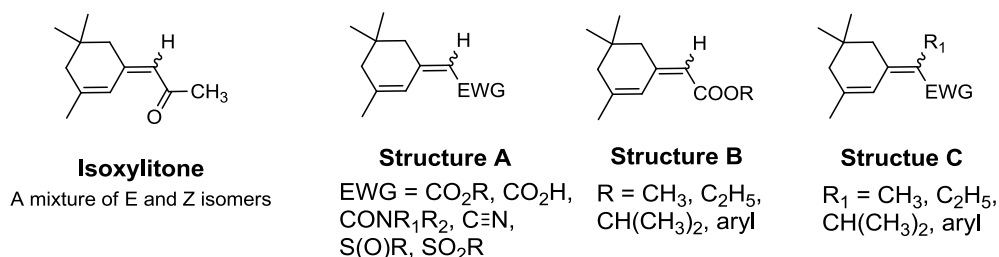


Figure 2.2.1.1. Isoxylitone and general structures A, B and C.

The obvious retrosynthetic disconnection for isoxylitone and all of the proposed analogues involved breaking of the exocyclic double bond. The reconnection is most readily envisaged via a Horner-Wadsworth-Emmons (HWE) reaction. The required enone 3,5,5-trimethylcyclohex-2-en-1-one, also known by the trivial name isophorone is an inexpensive commercially available material.

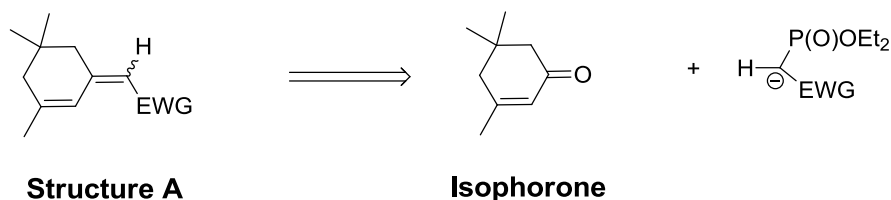


Figure 2.2.1.2. Retrosynthesis of isoxylitone analogues via HWE reaction.

Alternatively, since it is known that HWE reactions are often unsuccessful with ketones when the phosphonate reagent is highly stabilized, one can envisage reacting stabilized α -lithio

derivatives (LiCH_2EWG) with isophorone. Such a reaction would result in the formation of a tertiary alcohol dehydration of which should lead to the desired analogues. **Figure 2.2.1.3.** Indeed, the necessity of this approach became quite obvious when examining the reported synthesis of isoxylitone by the Pakistani researchers⁶. These authors resorted to a lengthy procedure involving an initial HWE reaction between isophorone and the triethylphosphonoacetate followed by conversion of the ester to the Weinreb amide and a final reaction with methyl magnesium bromide (**Scheme 2.2.2.1**).

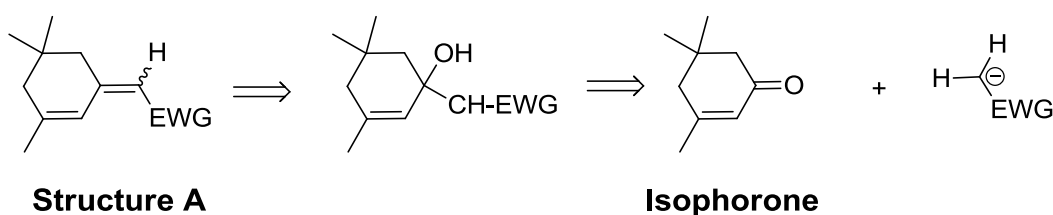
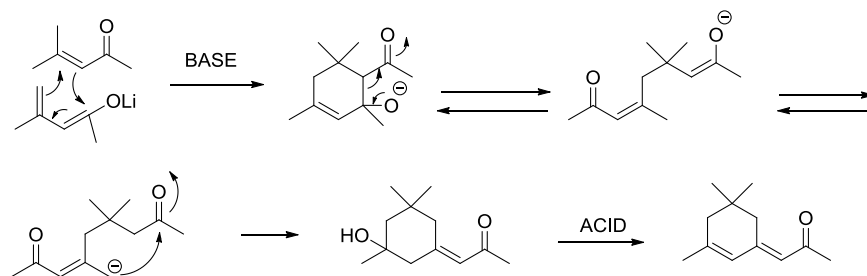


Figure 2.2.1.3. Retrosynthesis of isoxylitone derivatives via a carbanion coupling reaction.

2.2.2. Synthesis of the parent isoxylitone (ISOX)

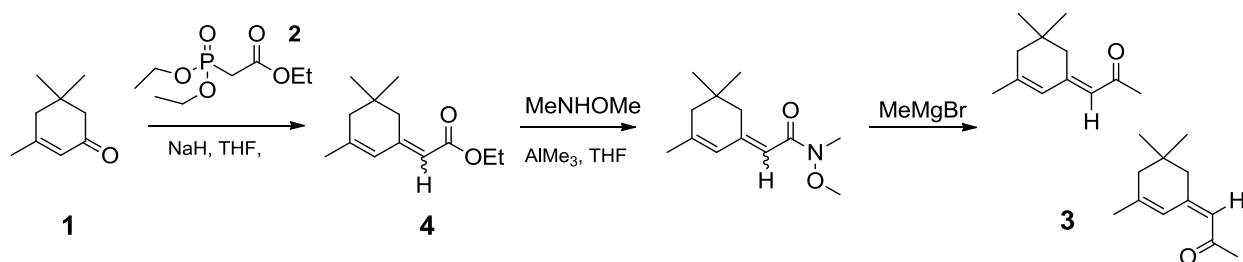
It was decided first to prepare ISOX for comparison with the natural material in the bioassays. Dimerization of mesityl oxide under basic conditions has been reported to yield isoxylitone accompanied by a number of other compounds. The dimerization of mesityl oxide followed by the loss of water can be explained in a many ways. One can theorize the preparation of ISOX via a series of 1,2 and 1,4 Michael addition reactions. In 1965, Furth and Weimann suggested the possibility of a Diels Alder reaction (**Scheme 2.2.2.2**) between the dienolate of mesityl oxide with some un-reacted starting material followed by a ring opening reaction under basic conditions and a final dehydration reaction yielding the ISOX product²². Under these same conditions, the authors claimed to observe nine different isomers and after tremendous effort were able to isolate isoxylitone via distillation. Although this appears to be a simple synthesis,

the isolation of isoxylitone from the mixture is difficult and it is likely for this reason the Pakistani group approached the preparation of ISOX via a different synthetic route.



Scheme 2.2.2.2. Schematic representation of Diels Alder reaction proposed by Furth and Weimann²².

The first patent, filed in 2008 by the Pakistan group, outlines a chemical procedure to prepare ISOX that describes first, the synthesis of an ester via a HWE reaction with ethyl diethylphosphonoacetate, **2**, using NaH in THF and isophorone **1**. Next, the ester is converted to a Weinreb amide using N,O-dimethyl hydroxylamine hydrochloride with AlMe₃ and lastly, the prepared Weinreb amide undergoes a Grignard reaction, affording the parent ISOX compound⁶ as shown in **Scheme 2.2.2.3**.

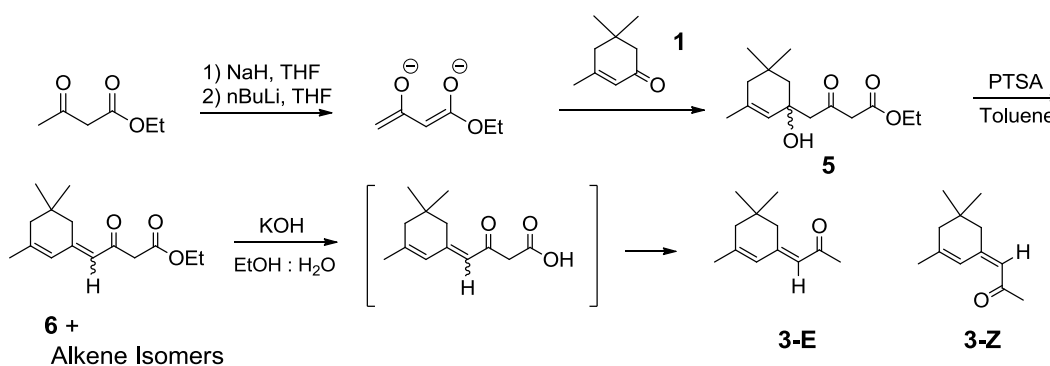


Scheme 2.2.2.3. Preparation of ISOX reported by Pakistan group⁶.

Because the mesityl oxide route gave mixtures which were difficult to separate, we decided to investigate variations of the scheme described by the Pakistan group. Our first attempt at the preparation of **3**, involved an aldol condensation between the lithium enolate of acetone and isophorone, **1**, in anhydrous THF at -78°C. No product formation was observed by

TLC. Typically the reaction between enones and enolates is reversible at higher temperatures and unfortunately even at low temperatures, the starting materials were favoured in this reaction. An attempt to prepare ISOX in one step via the HWE reaction, using commercially available diethyl (2-oxopropyl)phosphonate and NaH or NaHMDS in THF was also not successful. From this point onward, it became clear that the starting enone, **1**, was unreactive under a variety of different conditions towards relatively stable nucleophiles such as ketone enolates and ketone stabilized HWE reagents.

The 1,3-dianion species generated from ethyl acetoacetate by sequential treatment with NaH in THF and nBuLi is a potent nucleophile at the CH₂ terminus. It was reacted with isophorone to afford the coupled product **5**, as a colourless oil in 28% yield after gradient column chromatography. Compound **5** was subjected to a dehydration reaction, using a catalytic amount of PTSA in toluene at rt, affording **6**, as a colourless oil in 76% yield. It was noted that the NMR spectrum of **6**, showed multiple olefin peaks between 4.9 and 6.1 ppm, indicating that a complex mixture of alkenes had been formed. The formation of multiple alkenes in similar dehydration reactions is discussed in more detail later in this chapter. The mixture **6** underwent an ester hydrolysis reaction using KOH in a mixture of 3:1 EtOH: H₂O. The resultant β -keto acid decarboxylated under the reaction conditions to yield the desired product **3** as a pale yellow oil in 50% yield after purification by gradient column chromatography. The ¹H and ¹³C NMR spectra of **3** corresponded with those reported in the patent⁶. Compound **3** was obtained as a 5:4 mixture of *E* and *Z* isomers based on integration of the alkene peaks at 7.53 and 5.93 ppm due to the *E* and *Z* isomers, respectively.



Scheme 2.2.2.4. Synthesis of compounds **3-E** and **3-Z**.

The assignment of the peaks due to each isomer has been reported. Indeed, the Pakistani researchers claimed to have successfully separated the two isomers on a small scale via careful column chromatography. Despite considerable efforts, we were not successful in repeating the separation.

As shown in **Figure 2.2.2.1**, the *Z* isomer of ISOX has its carbonyl group in close proximity to this hydrogen resulting in a downfield shift (7.53ppm) in the NMR spectra relative to the same hydrogen of the *E* isomer which is found at 5.93ppm. The integration of these peaks gives the relative ratios of each isomer in the mixture. For compound **3**, the integration comparison depicts a 5:4 ratio of *E*:*Z* isomers. This analysis was used to identify the isomers and the isomer ratios in the product mixtures for all compounds synthesized in this series.

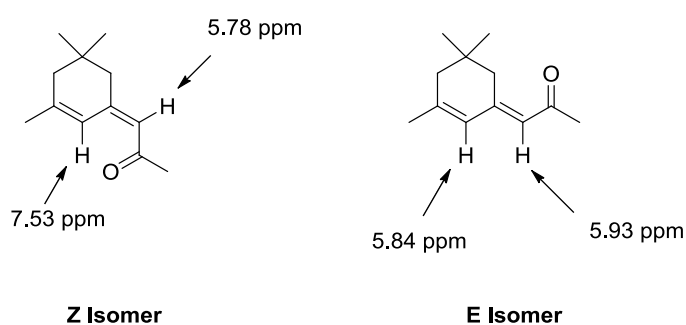


Figure 2.2.2.1. Structures of *Z* and *E* isomers with their relative olefin peak chemical shifts.

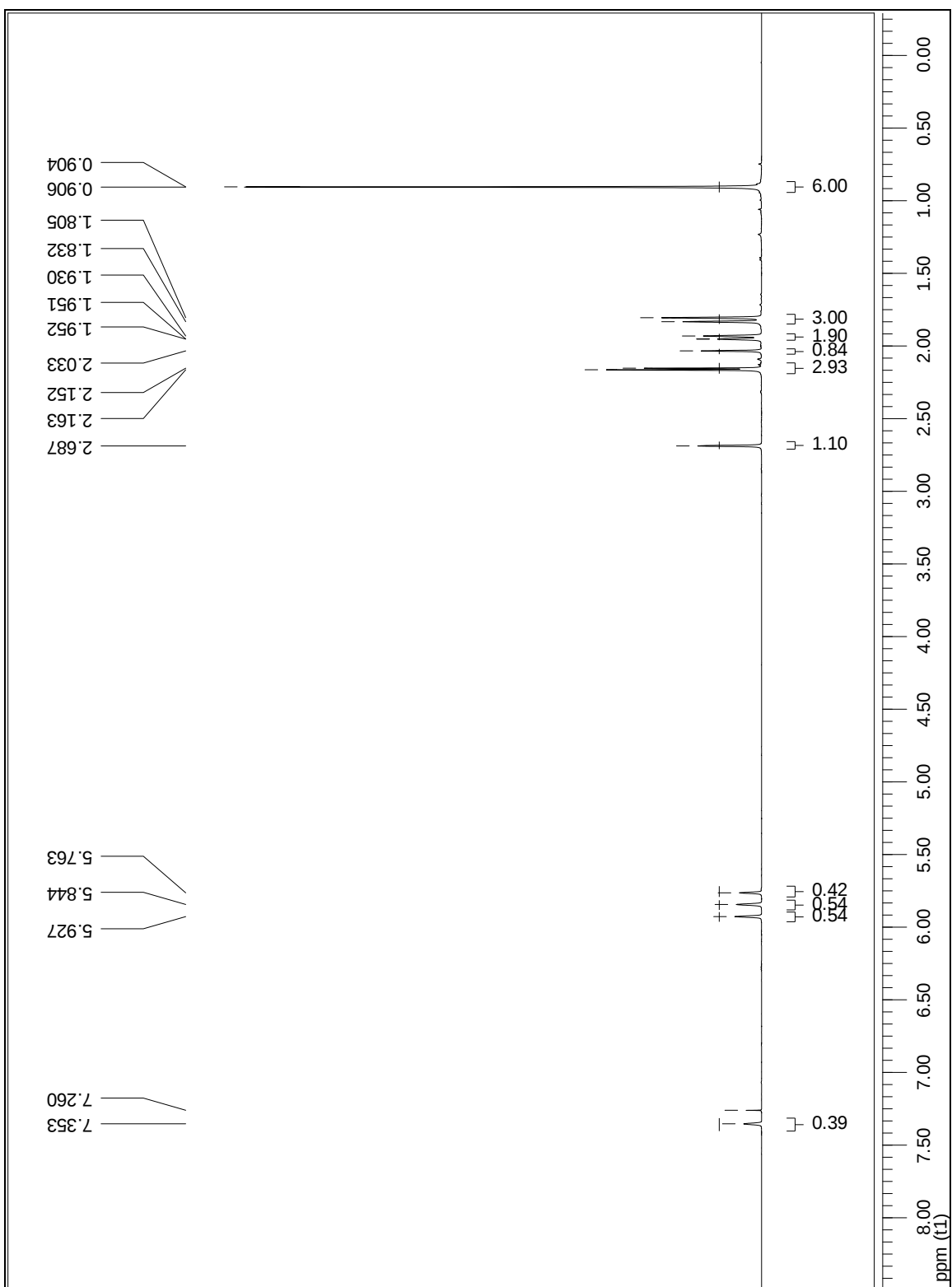
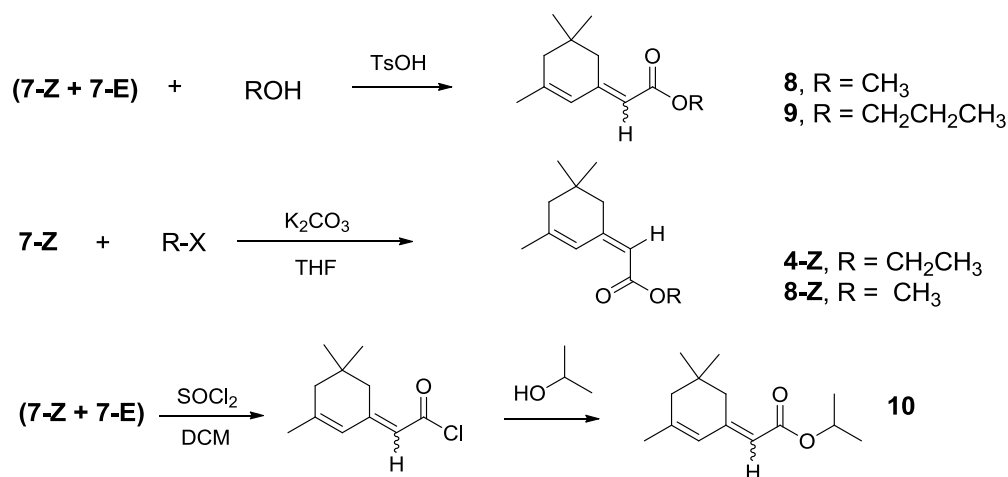


Figure 2.2.2.2. 400 MHz ^1H NMR spectrum of compound **3** in CDCl_3 .

2.2.3. Synthesis of ISOX analogues

As mentioned in the introduction of this section, it was not possible to predict which functional groups could replace the methyl ketone moiety in ISOX in order to obtain compounds that might improve its potency. Thus, we undertook the general approach of synthesizing a variety of compounds as suggested in **Figure 2.2.1.1.** via the general methods outlined in **Figures 2.2.1.2.** and **2.2.1.3.** We anticipated that we would be able to prepare efficiently a number of analogues and that timely feedback from the bioassays would then allow us to focus on groups which had the desired activity.

The synthesis of ISOX as presented in the 2008 patent, described a protocol for preparing the ethyl ester **4**, using a HWE reaction. This ester was prepared on a large scale, converted into its carboxylic acid, and from there transformed into multiple other compounds including other esters and an amide. Except for several specific cases, the products were obtained as mixtures of *E* and *Z* isomers. Since these transformations are quite straightforward, they will not be discussed in detail. The compounds prepared are shown in **Scheme 2.2.3.1.** below.



Scheme 2.2.3.1. Preparation of acid and ester analogues of ISOX.

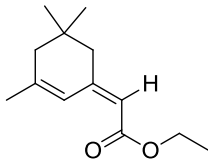
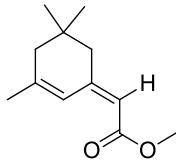
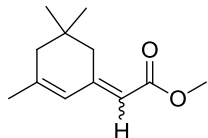
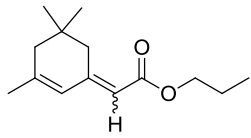
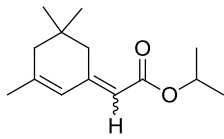
The parent ester **4**, was prepared as described in the patent⁶. **Scheme 2.2.2.3** The product was purified via column chromatography affording a mixture of *E* and *Z* isomers as a colourless oil in 69% yield. The ratio of isomers as determined by the relative peak size of the olefin peaks at 5.42 ppm and 7.24 ppm was 2:1 *E*:*Z*. Compound **4** was subjected to hydrolysis in a mixture of KOH in 3:1 EtOH:H₂O, affording the acid **7** as a white solid in 60% yield. The isomer ratio of *E*:*Z* did not change during the conversion of ester to acid and remained at 2:1. Compound **7** was recrystallized two consecutive times from DCM and MeOH, affording enriched *Z* isomer (**7-Z**) in ~ 92% purity.

This pure isomer was used to generate the *Z* isomer analogues of the methyl and ethyl esters. Preparation of compounds **8-Z** and **4-Z** was performed via reaction with CH₃I and EtI, respectively, using K₂CO₃ in THF. The pure *Z* isomers of these esters were subjected to acidic ethanol conditions, and after an hour, these pure *Z* isomers were readily converted into the earlier obtained *E* and *Z* isomer mixtures of the ethyl ester, **4**. This confirmed that the *E* and *Z* ISOX esters are readily isomerized under acidic conditions. These experiments suggest that stomach acidity is probably sufficient enough to isomerize these compounds and thus working with pure *E* and *Z* isomers, despite the fact that they may be active in the assays, would be irrelevant.

It was decided to synthesize other esters which were expected to show comparable activity to the ethyl ester. These analogues included a propyl, isopropyl and methyl ester. The methyl and propyl esters were synthesized by subjecting the acid **7** in refluxing MeOH and propanol, respectively in the presence of acid. The crude products were purified via gradient column chromatography, affording **8** and **9** as colourless oils in 40% and 74% yield, respectively. The isopropyl ester was synthesized via addition of isopropanol to an acid chloride

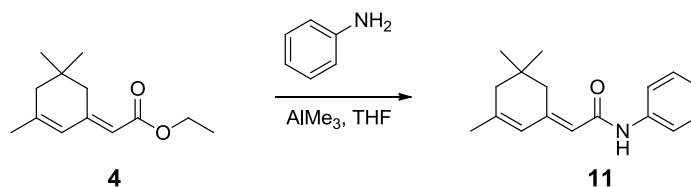
generated from compound **7** with SOCl_2 in DCM. Purification by gradient column chromatography afforded compound **10** as an orange oil in 40% yield.

Table 2.2.3.1. Formation of ester analogues.

Compound	% Yield	E:Z isomer ratio
 4Z	52% ^a	---
 8Z	58% ^a	---
 8	40% ^b	2:1
 9	74% ^b	5:4
 10	40% ^c	5:4

a) Via alkylation of the carboxylate; **b)** acid catalyzed esterification; **c)** via the acid chloride.

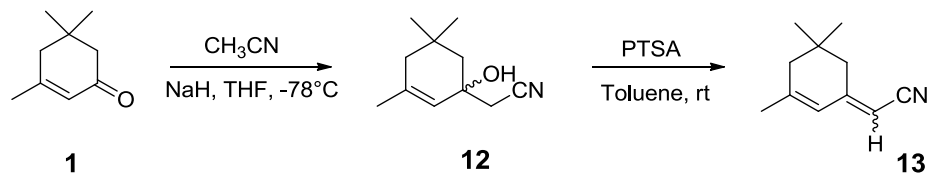
An amide was synthesized by the addition of aniline to compound **4** in a mixture of AlMe₃ in refluxing THF. Purification by column chromatography afforded compound **11** as a yellow oil in 37% yield, resulting in a 2:1 ratio of *E*:*Z* isomers as verified by ¹HNMR.



Scheme 2.2.3.2. Synthesis of compound **11**.

Although the aldol condensation reaction was unsuccessful when preparing the parent ISOX compound **3**; it was decided to use this method of preparing compounds by generating a more reactive carbanion species.

Acetonitrile was treated with LDA in THF at -78 °C and coupled to isophorone, **1**, generating the coupled product, **8**, in 50% yield. Subsequent dehydration using a catalytic amount of PTSA in toluene at rt, afforded compound **9** as a pale yellow oil in 89% yield. Analysis of the ¹H NMR spectra suggested a mixture of *E* and *Z* isomers in a ratio of 5:4 respectively, by observation of the relative size of the peaks at 5.92 ppm and 6.36 ppm.

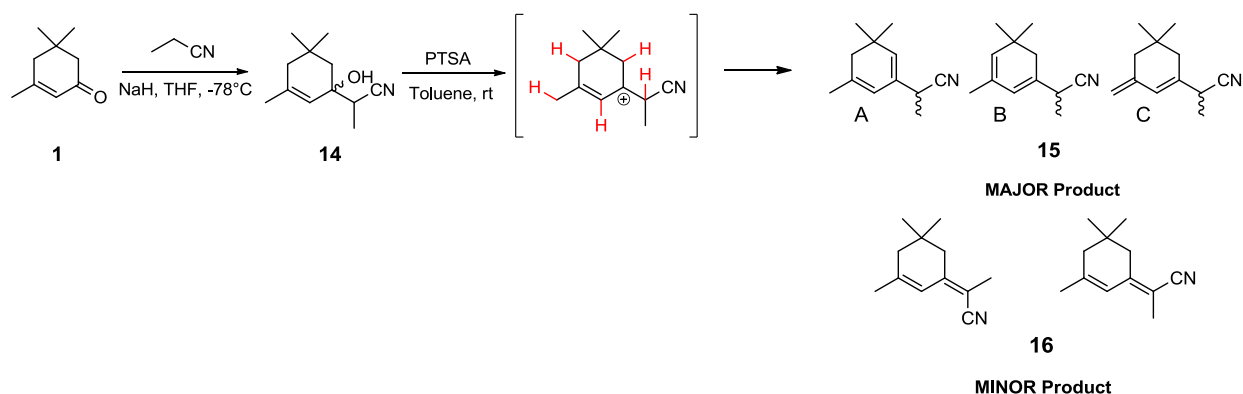


Scheme 2.2.3.3. Preparation of compound **12** and **13**.

Propionitrile was coupled to **1** using LDA in THF at -78 °C, affording **14** as a colourless oil in 49% yield after purification by column chromatography. Dehydration at room temperature

afforded **15** as a pale yellow oil in 36% yield. The ^1H NMR spectra of the dehydrated material showed seven (two of which overlapped) olefin peaks in the spectrum, which suggested that three alkene species were generated. Additionally, there was a cluster of quartet peaks between 3.24 and 3.29 ppm suggesting the presence of a hydrogen alpha to the nitrile functional group.

These data are in agreement with the formation of three dienes that were not conjugated to the nitrile functional group. **Scheme 2.2.2.6**. The formation of the three dienes is readily rationalized as resulting from the loss of a β - or vinylogously β -hydrogen from the carbocation intermediate. It is surprising that the expected thermodynamically more stable products, the *E* and *Z* conjugated isomers **16E** and **16Z** were not formed as the major products under these reaction conditions. However, when amplitude of the spectra was increased tremendously, we are able to see two additional peaks at 5.34 and 5.55 ppm, indicating the presence of *E* and *Z* of **16** isomers in extremely minor amounts. We assigned the doublet at 4.9 ppm to isomer C since vinyl geminal hydrogens are typically up field in chemical shift compared to those of simple alkenes. It is difficult to assign which olefin peaks correspond to isomer A and B because of their structural similarity. The isomer ratio within this mixture as indicated by the integration of each of these peaks is approximately 1:1:1 of A:B:C.



Scheme 2.2.3.4. Synthesis of compound **14** and **15**.

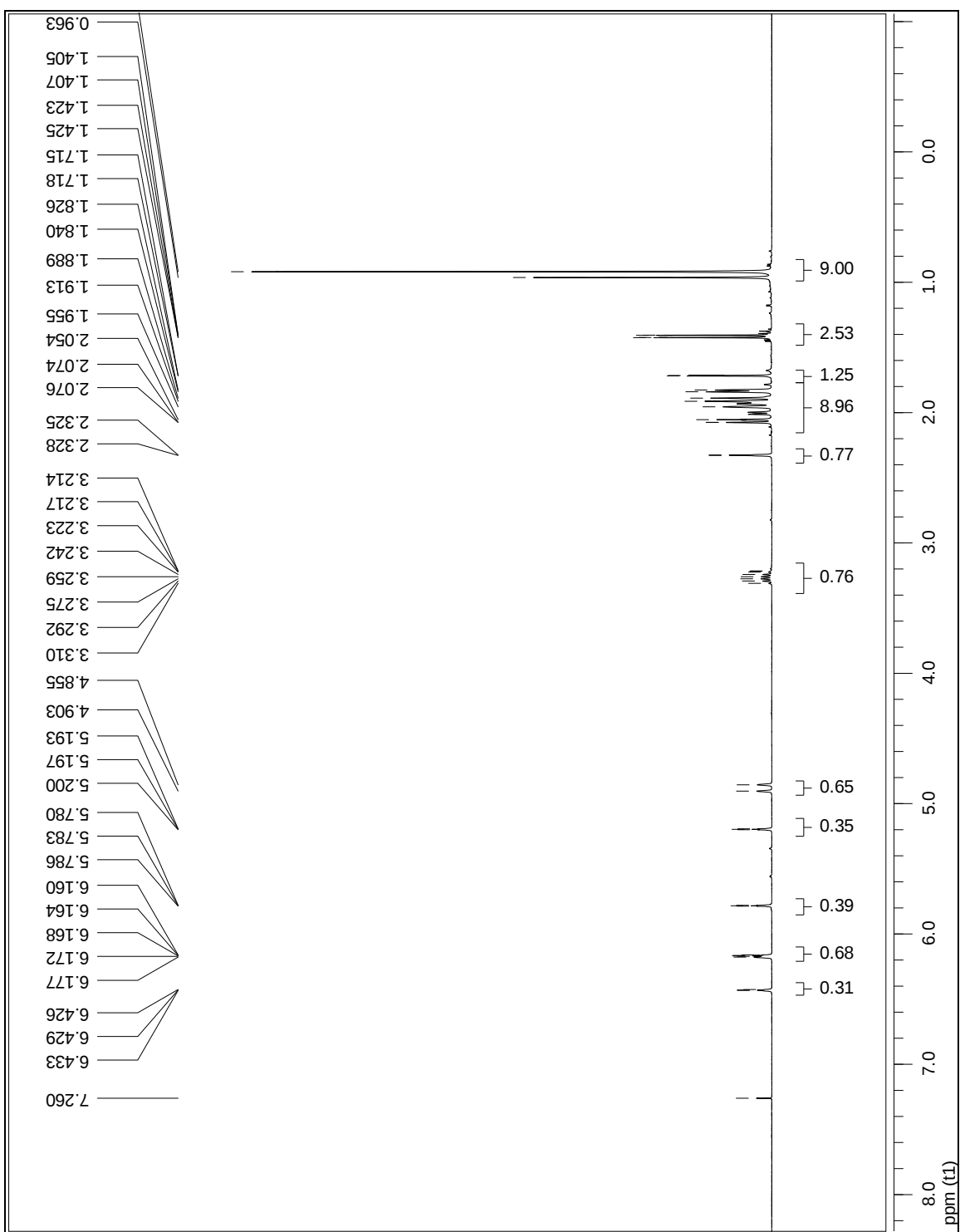
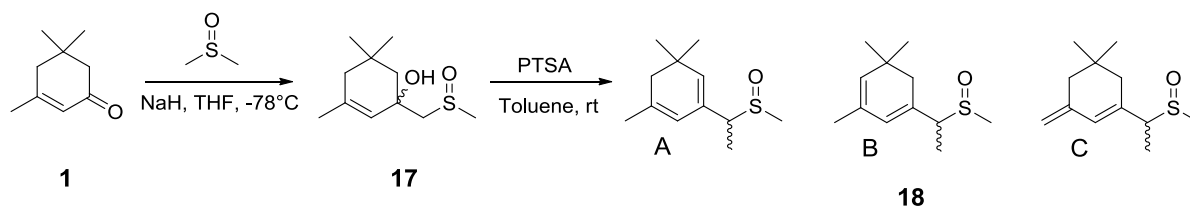


Figure 2.2.3.1. 400 MHz ^1H NMR spectrum of compound **15** in CDCl_3 .

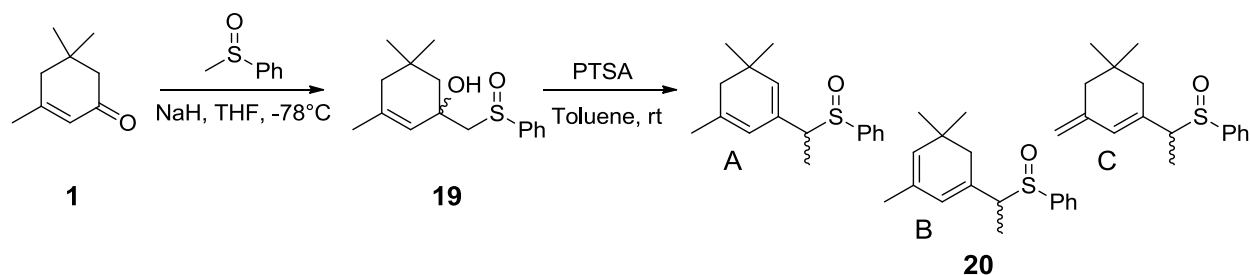
Due to the availability of many sulfoxides and sulfones in the laboratory, it was decided to prepare the sulfoxide equivalent of ISOX. The α -lithio derivative of DMSO obtained upon treatment with LDA in THF at -78°C was reacted with **1** affording the coupled product **17**, as a yellow oil in 47% yield after purification by column chromatography. Compound **17** was dehydrated using PTSA in toluene at rt, giving **18**, as a mixture of isomers in 45% yield as shown in **Scheme 2.2.3.5**. Again, the same olefin peak pattern was observed with compound **18** as with compound **15**, suggesting a mixture of three isomers of alkenes with the olefins within the isophorone ring. In this case, the formation of these three alkenes was not a surprise since it is known that β,γ -unsaturated sulfoxides are preferred over their α,β -unsaturated analogues.



Scheme 2.2.3.5. Preparation of compound **17** and **18**.

Reaction of methyl phenyl sulfoxide with LDA in THF followed by addition of **1** afforded after purification by column chromatography compound **19** as a white solid in 30% yield. Dehydration at room temperature yielded again a mixture of three isomers, **20**, as a yellow oil in 49% yield. Interestingly, one of the three isomers was slightly less polar than the bulk of the mixture, which afforded a pure isomer via chromatography, either **20A** or **20B**. The three AB quartets found at 3.3 to 3.6 ppm were assigned to the diastereotopic CH_2 group α to the chiral sulfoxide carbon. This, together with the seven peaks from 4.7 to 5.9 ppm was consistent with the presence of the three isomeric structures, **20**. The three compounds appeared to have been formed in a 1:2:1 or a 2:1:1 ratio of A:B:C based off the integration of different peaks due to the

alkene hydrogens. The structure of the pure isomer cannot be assigned with certainty. It is either **20A** or **20B** since the peaks representing the geminal vinyl hydrogens are absent in its ^1H NMR.



Scheme 2.2.3.6. Preparation of compound **19** and **20**.

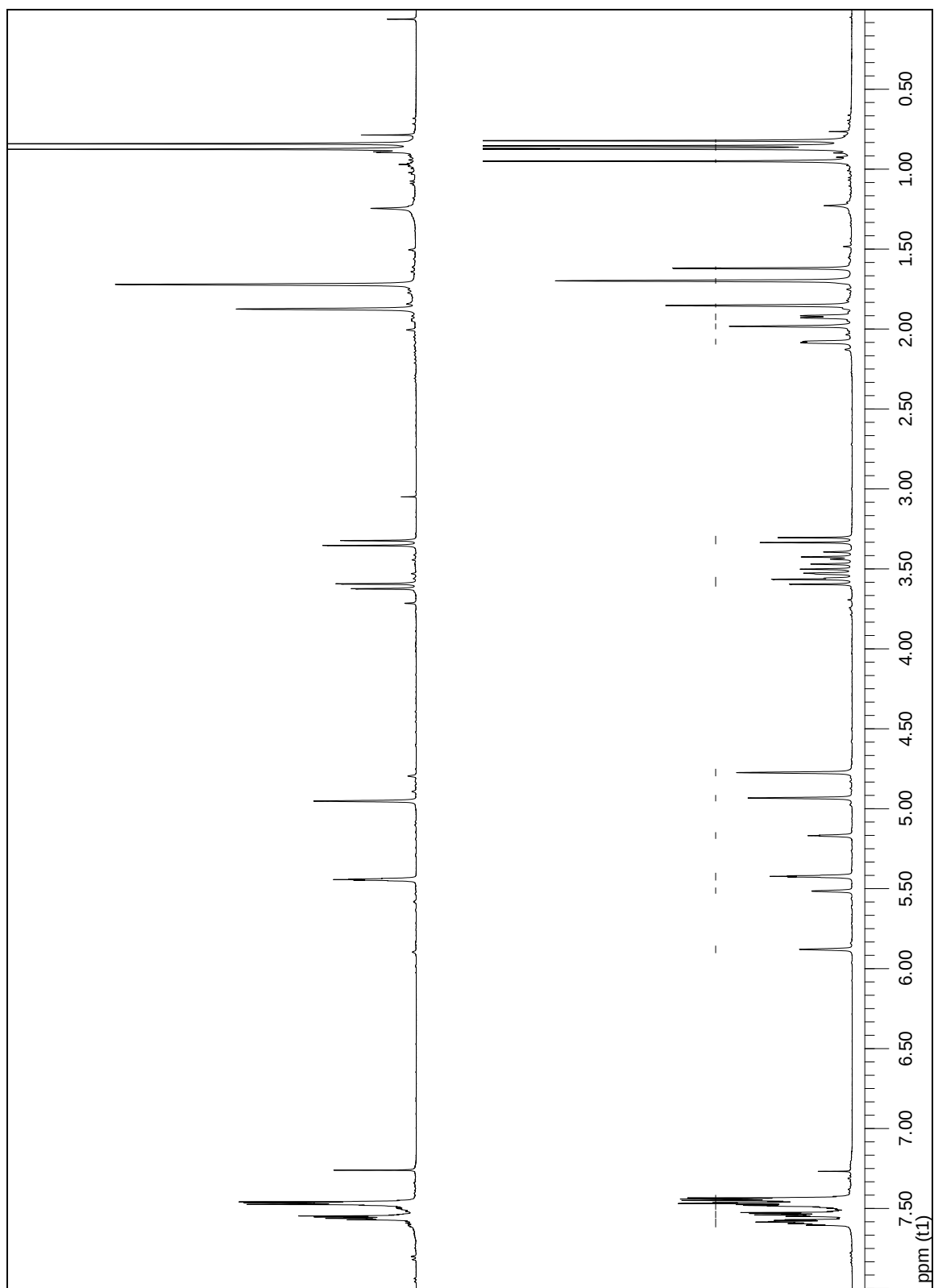


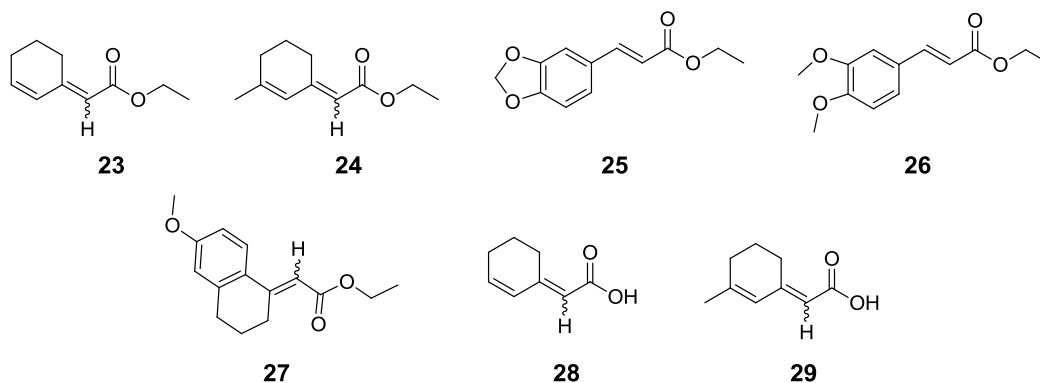
Figure 2.2.3.2. ^1H NMR comparison of mixture **20** (bottom) and pure isomer **20A** or **20B** (top).

The sulfone, **21** corresponding to the phenyl sulfoxide was prepared via the reaction of methyl phenyl sulfone with LDA in THF at -78°C, followed by the addition of **1**. Purification via column chromatography afforded compound **21** as a viscous oil in only 13% yield. The very low yield is presumably due to the unreactivity of the relatively stable α -sulfonyl carbanion towards isophorone. Again, dehydration of this material at room temperature using PTSA in toluene afforded the mixture **22** as a yellow oil in 55% yield. The ^1H NMR spectrum indicated a 1:1:2 ratio of the same three isomers A,B and C.

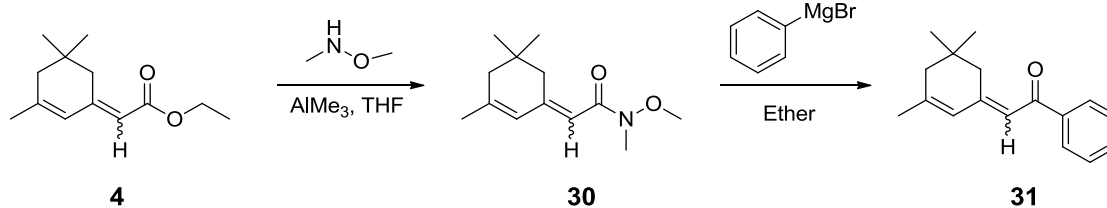
Since the ethyl esters were the simplest to synthesize, it was decided to prepare a number of ethyl ester analogues by changing the enone component of the structure. For example, instead of using isophorone, it was suggested we used cyclohex-2-en-1-one to test the importance of the three methyl groups on the cyclohexenone ring. Secondly, it was decided to use 3-methyl cyclohexenone to test the activity of a compound lacking the 5,5 di-methyl groups. Lastly, it was decided to change the enone component entirely, by introducing a random core structure to see if we could optimize activity. Examples of these starting materials include piperonal, 3,4-dimethoxybenzaldehyde and 6-methoxy-3,4-dihydronaphthalen-1-one. These desired compounds were obtained, as expected by reaction of the starting ketone or aldehyde with triethylphosphonoacetate and NaH in refluxing THF as described above. The compound number, % yield of the reaction and the *E:Z* isomer ratio are presented in **Table 2.2.3.2**. Compound **23** and **24** were converted into their respective carboxylic acid derivatives obtaining the desired product as expected by reaction with KOH in 3:1 EtOH:H₂O. The structure assignments were based on an examination of the ^1H NMR spectra of these compounds.

Table 2.2.3.2. Synthesis of ethyl ester analogues.

Compound #	Starting Material	% yield	E:Z isomer ratio
23	Cyclohexenone	16	4:1
24	3-methylcyclohexenone	33	2:1
25	piperonal	35	1:1
26	3,4-dimethoxybenzaldehyde	75	1:1
27	6-methoxy-3,4-dihydronaphthalen-1-one	55	2:1

**Figure 2.2.3.3.** Structures of ethyl ester and carboxylic acid analogues.

Lastly, we prepared one ketone analogue of the parent ISOX compound **1**. In place of the methyl group, it was decided to replace it with a phenyl substituent. The choice of the phenyl group was based on the premise that if this compound were to show activity in the bioassay, it would lead to the possibility of synthesizing a new set of compounds. The ester **4** was converted into its Weinreb amide using N,O- dimethylhydroxylamine hydrochloride with AlMe_3 in THF. Purification by column chromatography afforded compound **30** as a viscous oil in 46% yield. This material was subjected to a Grignard reagent using phenyl magnesium bromide in ether. The crude material was purified by column chromatography affording compound **31** as a colourless oil in 55% yield.



Scheme 2.2.3.7. Preparation of compound **30** and **31**.

2.2.4. Bioassays and preliminary results

At this point in time, the available compounds were sent to the Poulter group at Western University to assess their activity in appropriate in vitro epilepsy models.

To test for activity, a first assay is done using voltage sensitive dye imaging on isolated rat brain slices that are kept viable in artificial cerebral spinal fluid (ACSF). The neural assemblies are activated by electrical stimulation. A voltage sensitive dye, such as, di-4-ANEPPS, is incubated with a brain slice for 1h in a suitable solution that enhances the dye penetration into the tissues. The dye reacts to the changes in voltage across the cell membrane of the neurons in the brain slices. Compounds are added to the ACSF at known concentrations between 200 nM and 1 μM . The brain slices are then subjected to an electrical stimulus that activates the neurons in the slice. As the dye reacts to the change in voltage, which can be observed and quantified, the degree to which activation of the brain is dampened by the presence of the compound, is evaluated. In a second assay, the inhibition of the compounds on the activity of voltage gated sodium channels is determined using patch clamp electrophysiology. This analysis is done on cultured cortical neurons isolated from rats²³. A complete description of the bioassays is found in **Appendix 2A**.

Not surprisingly, the synthetic ISOX compound **3** displayed comparable activity to that isolated from the plant. The ethyl ester, **4**, was the more efficacious than the parent compound

and indeed among all the compounds tested. Compound **4** reduced activity by more than 80% at 200 nM and has undergone testing in two other animal seizure models. The models are done in rats in the kindling model at 2 mg/kg, 20 mg/kg and 100 mg/kg. After 5-10 days of stimulation, little or no progression to seizures had been observed. Concentrations in fully kindled rat models (stage 5) are currently being evaluated. Compound **24**, which is closely related to **4**, but is missing the geminal dimethyl group on the ring reduces activity by 40% at 200 nM. Compound **23**, devoid of all methyl substituents on the cyclohexenone ring was not active at 200 nM or at 1 μ M. Compounds **25**, **26** and **27**, all ethyl esters with different enone starting materials show no activity in the assay. These results were not overly surprising since the core structures were quite dissimilar to that of ISOX itself. Any surprises in activity would have constituted an important lead for further synthetic efforts.

Table 2.2.4.1. Summary of bioassay results for the esters compounds.

Compound	Average % reduction at 200 nM	Average % reduction at 1 μM
4	80%	Not Tested
4Z	Inactive	26%
8	Inactive	31%
8Z	Inactive	20%
9	Inactive	Inactive
10	Inactive	20%
23	Inactive	30%
24	Inactive	Inactive
25	Inactive	Inactive
26	Inactive	Inactive
27	Inactive	Inactive

The reported activity of the four esters: methyl, **8**, ethyl, **4**, propyl, **9**, and isopropyl, **10**, is quite baffling. The ethyl shows a strong reduction in the firing of brain neurons. The activity is greatly reduced both in going to the smaller methyl and the larger propyl and isopropyl esters **Table 2.2.4.1**. This leads to the conclusion that the ethyl ester has the "ideal size" and that replacement of the ethyl group by both larger or smaller alkyl groups is unfavourable.

We were pleased that we were able to isolate via recrystallization an almost pure sample of the *Z* isomer of the acid **7** and from it prepared the pure *Z* isomer of both the methyl and ethyl esters **8Z** and **4Z**, respectively. Both the *Z* acid, **7Z** and the *Z* esters, **8Z** and **4Z** showed little to no activity. This suggests that the *E* isomer configuration is largely responsible for the observed activity assuming that there is no isomerization during the bioassay conditions.

The mixture of the acid **7** showed activity comparable to that of the parent compound **3**; it was somewhat less potent than the ethyl ester **4**. The carboxylic acid derivatives **29** and **30**, lacking all three methyl substituents and geminal dimethyl substituents respectively, show no activity in the assay. As mentioned above, the 92% pure *Z* acid, **7Z** was not active.

Table 2.2.4.2. Summary of bioassay results for the carboxylic acid derivatives.

Compound	Average % reduction at 200 nM	Average % reduction at 1 μ M
7	50%	Not Tested
7Z	Inactive	Inactive
29	Inactive	Inactive
30	Inactive	30%

The methyl sulfoxide, compound **18**, resulted in 50% reduction of brain neuron activity at 200 nM; this is comparable to the parent compound **3**. The question remains: Are the three isomers that constituted the mixture sent for the bio-assay equally potent or is one more potent? This question would be difficult to address since separation of the isomers is not possible using silica gel chromatography and does not even appear feasible via preparative HPLC. The notion of developing a drug which is a mixture of three isomers is not particularly appealing; it would likely be challenged by regulatory agencies that prefer new drugs to be single entities include single enantiomers.

Replacement of the methyl group for a phenyl group resulted in a compound having no activity. The corresponding phenyl sulfone was also not active. These results agree in general with the observations reported in the second Pakistani patent³. The inventors focused on a series of ketones and reported that replacement of methyl ketone present in ISOX by larger groups resulted in a steady decrease in activity with the phenyl ketone being essentially inactive. Thus it is not surprising that the phenyl ketone **31**, the phenyl sulfoxide **20**, the phenyl sulfone **22** and the N-phenyl amide **11**, were all essentially inactive in this assay.

The complex mixture of nitriles **15**, a mixture of three isomers, displayed almost the same activity as the parent. Quite surprisingly, the lower homologous compound **13**, lacking the methyl substituent α to the nitrile functionality, compound **13**, was not active.

Table 2.2.4.3. Summary of bioassay results for the nitriles, sulfoxides, sulfones, ketone and amide compounds.

Compound	Average % reduction at 200 nM	Average % reduction at 1 μ M
11	Inactive	Inactive
13	Inactive	Inactive
15	50%	Not Tested
18	50%	Not Tested
20	Inactive	Inactive
22	Inactive	Inactive
31	Inactive	Inactive

2.2.5. Optimizing compound activity

The structure of the most active compounds are shown in **Figure 2.2.5.1**.

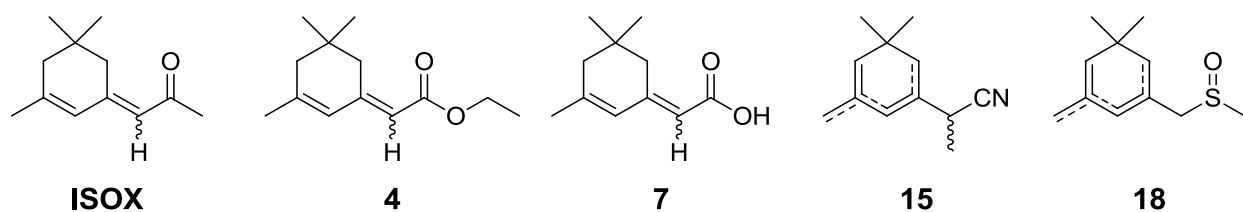
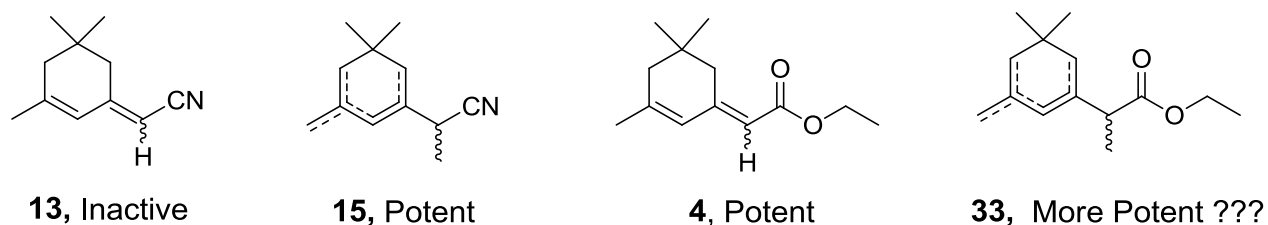


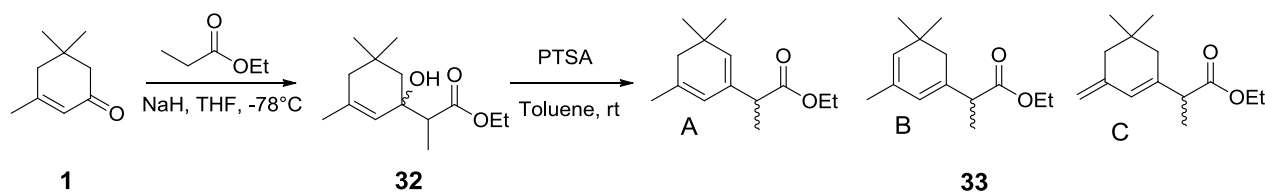
Figure 2.2.5.1. Isoxylitone and highly active analogues.

Since the introduction of a methyl group α -to CN in going from **13** to **15** resulted in converting an essentially inactive material into a highly active one, we anticipated that a similar change in the ester **4**, a highly active compound, would produce an even more potent **33**.



Ethyl propionate was coupled to **1**, via an aldol condensation reaction in the presence of LDA and THF. The crude product was purified via gradient column chromatography affording **32** as a viscous oil in 46% yield. Dehydration using a catalytic amount of PTSA in toluene at rt, afforded compound **33** as a colorless oil in 99% yield. The ^1H NMR spectra showed the presence of the three alkene isomers as seen with compound **15**. Integration of relative peak size suggested a 2:2:1 ratio of A:B:C. Minor amounts of *E* and *Z* isomers were also present.

Disappointingly, **33** only reduced neuronal firing by 24% at 200 nM and 22% at 1 μM , which was low in comparison to **4** which reduced activity by approximately 80%.

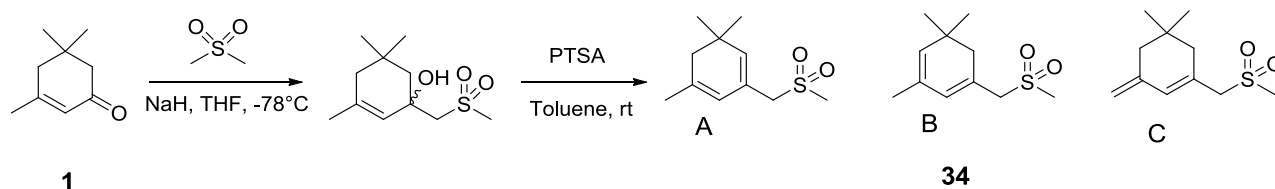


Scheme 2.2.5.1. Synthesis of compound **32** and **33**.

The observed activity resulting from the methyl sulfoxide, compound **18**, was an unexpected surprise when compared to the original structure of ISOX. The structural and physical properties including polarity of sulfoxides are very different compared to those of ketones, esters and nitriles. The replacement of the methyl group in **18** by a phenyl group resulted in a loss of activity, the only plausible change with the potential of leading to an active compound was the conversion of the sulfoxide functional group into a sulfone.

Compound **34** was synthesized by coupling the α lithio derivative of dimethyl sulfone to compound **1** and further dehydrating this material using a catalytic amount of PTSA in toluene. Again, the three component isomer mixture was present in a 1:1:1 ratio of A:B:C, along with the presence of *E* and *Z* isomers.

The methyl sulfone, compound **34**, which was expected to show activity was in fact not active and only reduced epileptic activity by 5% and 6% at 200 nM and 1 μ M, respectively. This was extremely low compared to its sulfoxide adduct, **18**, which reduced epileptic activity by 50% at 200 nM.

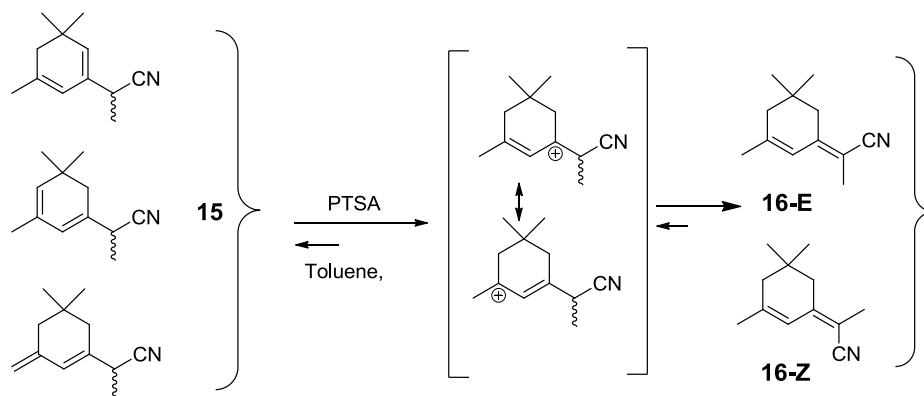


Scheme 2.2.5.2. Preparation of compound **34**.

The acid catalyzed room temperature dehydration of the initial condensation product between the lithio derivative of propionitrile and isophorone leads to a mixture of three alkene isomers, with a minor amount of *E* and *Z* isomers. Surprisingly the mixture of isomers, compound **15**, showed significant suppression of seizure activity.

We were asked by the Poulter groups to prepare additional quantities of **15** for additional biological evaluations.. In order to speed up the dehydration of the initial alcohol this reaction was carried out with a catalytic amount of PTSA in refluxing toluene for 3h. We were surprised that a different set of products were obtained. Analysis of the ^1H NMR allowed us to assign the structure of this material as a mixture of the *E* and *Z* alkenes, compound **16**, the *E*:*Z* ratio was 5:4. **Scheme 2.2.5.3.** This material was initially isolated as a pale yellowish oil that solidified when placed in the freezer at -20°C . We were able to show that the mixture of alkenes **15**, was also converted into the *E*/*Z* mixture **16**, upon refluxing with PTSA. The mechanism of isomerization involves reversible protonation of the individual isomers of **15** to form the allylic carbocation intermediate followed by loss of the hydrogen α to the nitrile function. The *E* and *Z*

isomers of **16** are thermodynamically preferred, most likely due to the additional π -conjugation of the diene to the CN group.



Scheme 2.2.5.3. Isomerization of compound **15** to compound **16E/Z**.

Interestingly, the *E*-isomer of **16** crystallized preferentially from hexanes and MeOH, two consecutive recrystallizations gave a sample of containing approximately 91% of **16-E**. NOE experiments of confirmed that the more up field alkene peak (6.17ppm) correlated with the α -methyl peak indicating that the two groups were in close proximity to each other. It is also reasonable to expect that the electronegative CN group can cause a downfield shift of the nearby alkene hydrogen as it is in the *Z* isomer compared to the *E* isomer. The NOE studies carried out on the *E/Z* mixture, **16**, showed no correlation between the methyl group and the more downfield alkene peak (6.44 ppm), confirming our initial isomer assignment.

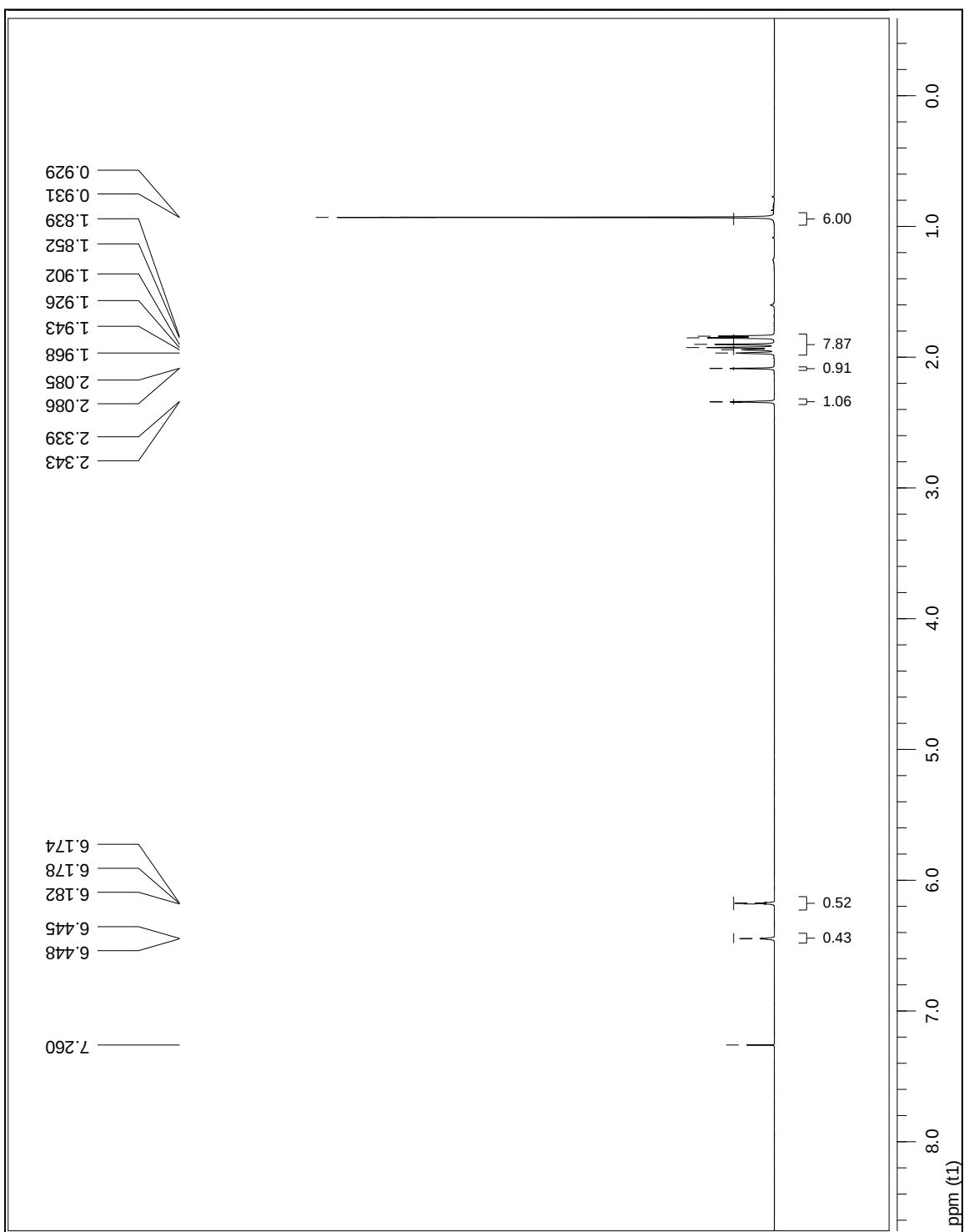
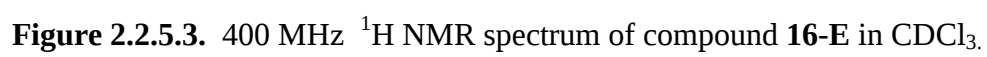


Figure 2.2.5.2. 400 MHz ^1H NMR spectrum of compound **16E/Z** in CDCl_3 .



Attempts at converting the nitrile **16** to its ethyl ester via HCl gas and EtOH was unsuccessful. The almost pure *E* isomer, **16-E** was also subjected to a heating with a catalytic amount of PTSA in refluxing toluene to see whether the pure isomer reverts back to its *E* and *Z* isomer mixture. Only a minor amount of additional *Z* isomer formed based on ¹HNMR analysis.

We were pleasantly surprised when the *E/Z* mixture **16** was found to be 4 times more potent than the parent compound **3**. Based on the earlier observations that the *Z* isomer of both the acid and the methyl and ethyl esters, we anticipated that activity of the mixture would be mainly due to the crystalline **16-E** and indeed that this isomers would be up to two times more potent than the thermodynamic mixture. Unfortunately, the pure *E* isomer, **16-E** showed barely any suppression of epilepsy in the assays and thus we are forced to conclude that in this case, the *Z* isomer was the more active one. We were keenly disappointed by this result since we could imagine the possibility of producing large amounts of the *E* isomers by first crystallizing out the *E* isomer and then isomerizing the remaining mixture enriched in the *Z* isomer back to the original *E/Z* ratio and then obtaining additional *E* isomer by another crystallization procedure.

Based on the promising initial bio assay results, the *E/Z* mixture **16** was further evaluated and has shown that it is able to suppress seizure development in rats. Contract research done by Intervivo has also shown that **16** is able to suppress seizures in another mice model. Intervivo has also evaluated side effects such as sedation or changes in movement, in which no obvious changes were observed even at the highest dosages. More excitingly, in three evaluations on human drug resistant brains, **16** has shown that is able to suppress brain activity. In two cases, this was compared to a widely used antiepileptic drug (CBZ). CBZ was ineffective while **16** (at a dose 50x smaller) suppressed activity by about 50%. The work presented here has been filed in a patent to claim these unique findings.

OB Pharma contracted Axion Biosystems to test compound **16** in their model of seizure activity. Axion has technology that permits the assessment of drug activity on neurons that are maintained in culture in plates having 24 separate compartments or well. The excitement of this technology is that the neurones in each well are growing on an array of microelectrodes that permit the operator to record the electrical activity of all 24 wells simultaneously. Seizure inducing chemicals and compound **16** at differing concentrations can be introduced into the wells and **16** can be evaluated across many different scenarios to get a better understanding of its effectiveness. Results from this evaluation are expected to be completed in late summer 2015.

Evaluations on how the mixture **16** suppresses seizures must be determined and will be done in Dr. Poulter's in house model of epilepsy. Dose regimens must also be evaluated. Preliminary findings suggest that the effects of **16** take about 2 h to become fully developed. If so, this implies high stability once in the blood stream which is important as rapid degradation of the drug reduces its potency.

The synthesis of **16-E** and **16-Z** has been carried out a number of times in order to produce the additional quantities needed to conduct the additional bioassays. Currently, the best overall yield of **16E/Z** starting with 5 g of isophorone is 93%. It appears to be advantageous to carry out a preliminary purification of the coupling product prior to the high temperature dehydration.

The discovery of the important activity of **16** and its potential in being developed as a drug candidate for the treatment of epilepsy and seizures suggested strongly that analogues of this compound should be prepared and evaluated.

During the past year, I supervised Nadia Ayoub Jami, whose honours thesis describes the preparation of nitrile analogues of compound **16**. Two approaches were taken when synthesizing

these compounds; 1) Reaction of nitriles with various alkyl and aryl groups, including valeronitrile, isovaleronitrile and cyclopropyl nitrile with isophorone ((3,5,5-trimethylcyclohex-3-en-1-one) and 2) Reaction of the enolate of propionitrile with enone analogues to isophorone for examples with enones such as 2-methylcyclohex-2-en-1-one, 3-methylcyclohex-2-en-1-one and 4,4-dimethylcyclohex-2-en-1-one.

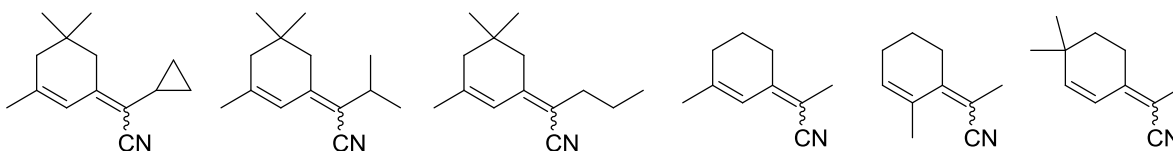
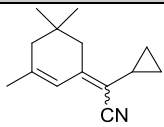
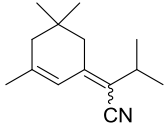
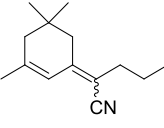
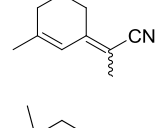
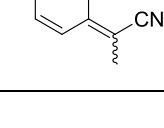


Figure 2.2.5.4. Compounds prepared by Nadia Ayoub Jami.

These compounds were sent for bioassay testing as described above. For these compounds, the room temperature dehydration with PTSA and toluene, generated the three alkene mixture accompanied by small amounts of the *E* and *Z* isomers in relatively equal amounts. Direct formation of the *E* and *Z* isomers occurred when the dehydration reaction was performed under reflux. In several examples, both sets of these mixture were sent for bioassays as separate samples. None of the newly synthesized nitrile analogues were as active as **16** in the assay. This confirms once again that substituents larger than methyl when attached to the side chain carrying the function group whether alkyl or aryl, are detrimental in the suppression of epileptic activity. Based on these results it continues to surprise us that alpha methylated nitrile **16** is more active than either parent nitrile **13** or any of the derivatives shown in **Table 2.2.5.1**.

Table 2.2.5.1. Summary of bioassay results for compounds prepared by Nadia Ayoub Jami.

Compound	Average % reduction at 200 nM	Average % reduction at 1 μ M
	6.0% $\pm$ 0.05	19.7% $\pm$ 0.08
	27.4% $\pm$ 0.08	46.4% $\pm$ 0.08
	18.6% $\pm$ 0.04	21.9% $\pm$ 0.05
	16.8% $\pm$ 0.06	27.7% $\pm$ 0.04
	20.7% $\pm$ 0.12	35.9% $\pm$ 0.10

As mentioned above, the introduction of a methyl group α -to CN in going from **13** to **15** resulted in converting an inactive material into an active one. The preparation of compound **33**, was carried out in hopes to optimize the activity of the ethyl ester, **4**, which demonstrated high potency compared to the parent ISOX compound **3**. Unfortunately, compound **33** showed significantly lower activity in the assays. The isomerization of the five component mixture **15** into a mixture containing essentially only the *E* and *Z* isomers **16** which is currently the lead structure in the development of an epilepsy treatment. The α -methyl ester mixture **33** was also isomerized upon treatment with PTSA in refluxing toluene to give solely the *E* and *Z* isomer **33E/Z**. The figure below compares the known activity of the two series of compounds. It is tempting to use the feedback from the previous bioassay results, and predict that compound **33E/Z** should exhibit higher activity than compound **4** and indeed even **16**.

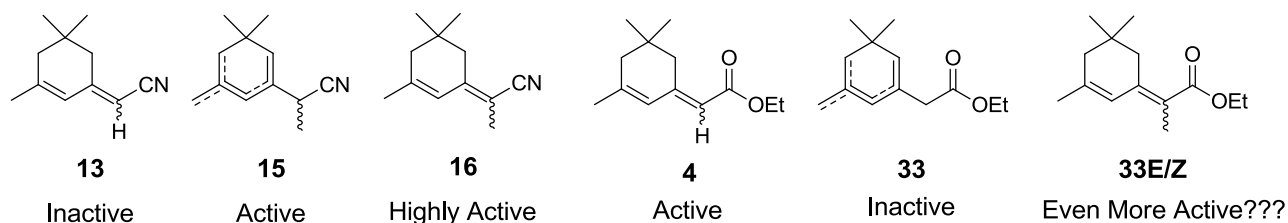
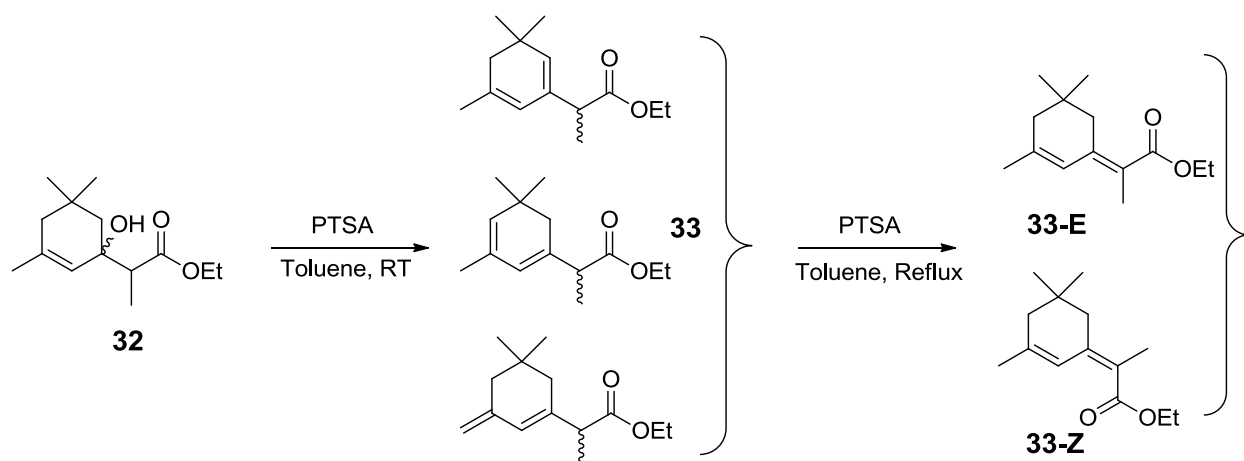


Figure 2.2.5.5. Summary of active and inactive compounds.

The formation of isomers in the synthesis of **33E/Z** from isophorone and α -lithiated ethyl propionate is readily followed by ^1H NMR by focusing on the alkene region. The initial coupling product shows two olefinic hydrogens due each of the possible diastereomers. The room temperature dehydration reaction gives a product in which nine alkene possible peaks can be observed. Four of the nine peaks are due to the loss of a hydrogen from the cyclohexene ring which generated two cyclohexadienes, three are the result of the loss of a hydrogen from the methyl group and two each are due to the *E* and *Z* isomers. The latter two represent minor components in this mixture. Except for the assignment of the two equal size peaks at 4.66 and 4.67 ppm which are assigned to the exo methylene group, it is not possible to assign the peaks due to the cyclohexadiene isomers. The isomerization reaction in refluxing toluene converts the five component mixture to essentially only the thermodynamically more stable *E* and *Z* isomers of **33** where we observe two peaks in the olefinic region, one for each isomer. As in the case of the ISOX itself, the lower field peak at 6.89ppm is assigned to the *Z* isomer and the higher field peak at 6.22ppm to the *E*-isomer. The ratio of *E* to *Z* in this case is approximately 1:1. **Scheme 2.2.5.4** shows the conversion of the coupled product **32** into **33E/Z**. **Figure 2.2.5.6** illustrates this with emphasis on the peaks in the alkene region of the ^1H NMR.



Scheme 2.2.5.4. Representation of the preparation of compounds **32**, **33** and **33E/Z**.

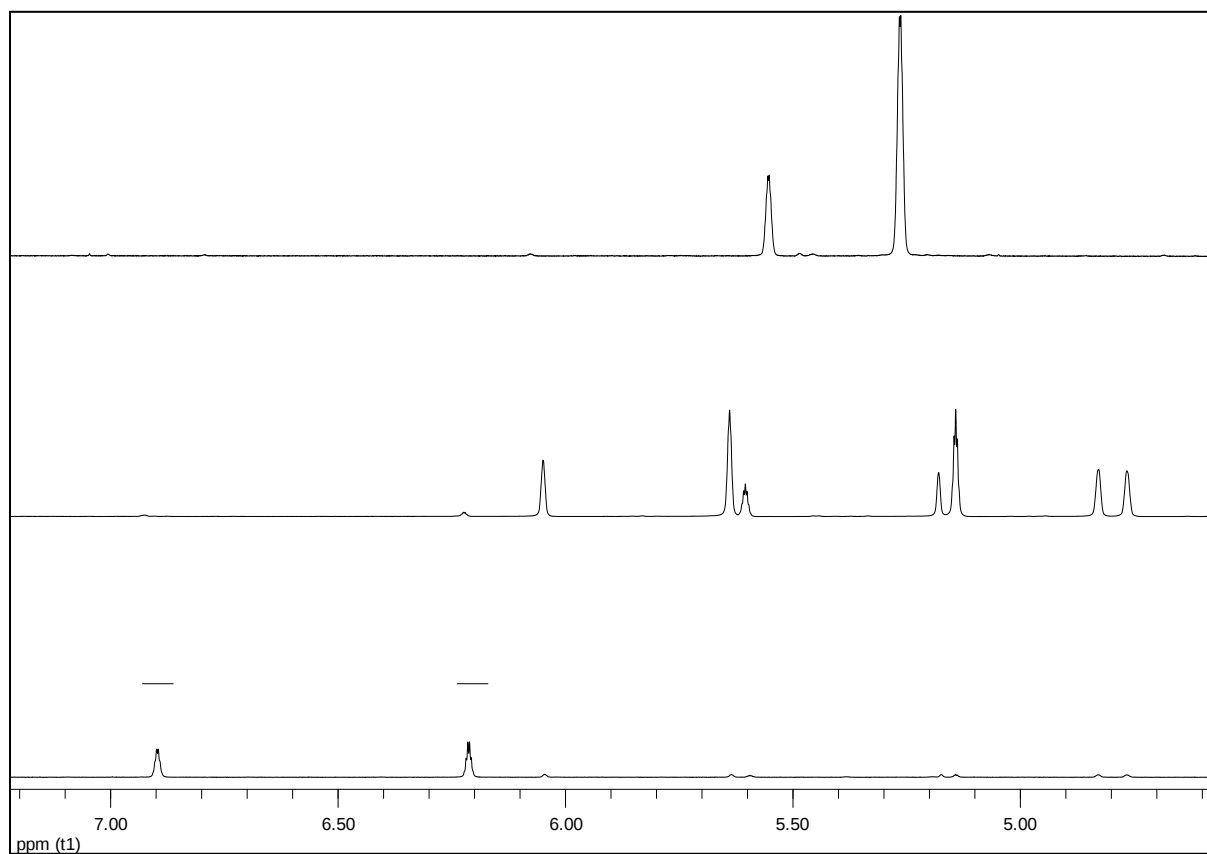


Figure 2.2.5.6. Olefinic region of compound **32** (top), **33** (middle) and **33E/Z** (bottom).

2.2.6. Conclusions and Future Work

The available bio assay data for the esters and acids indicates strongly that the *E*-isomers are more potent than the *Z* isomers. For example, the three pure *Z* isomers, the acid **7Z**, the methyl ester **8Z** and the ethyl ester **4Z** all appear to be significantly less effective than their mixture which contain more or less equal amounts of the *E* and *Z* isomers. We recognize that efforts to isolate the *E* isomers is not likely worth the effort since these compounds are readily interconverted under even relatively mildly acidic conditions. An alternate approach would be to prepare structures in which that stereochemistry is maintained by incorporation into a five and six membered ring respectively. This would result in a series of bicyclic enones, lactones and lactams in which the second ring is either a five or six membered ring with a fixed *E* or *Z* geometry. We have already noted that the activity of the ISOX type compounds is highly sensitive to steric effects. It is by no means certain that these proposed compounds would show significant activity in the Poulter group assays.

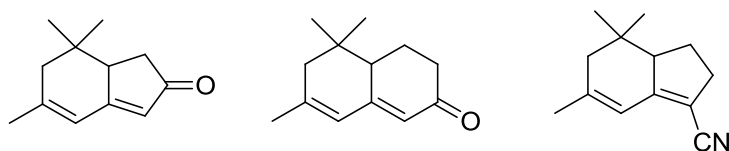
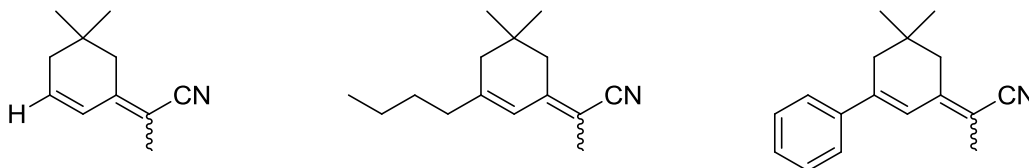


Figure 2.2.6.1. Possible structures of bicyclic enones.

Surprisingly, in the case of the propionitrile derivative, compound **16**, the *E* isomer was inactive. In this case, the isomers are quite resistant to isomerization under acidic conditions and thus the testing of a sample of the pure *Z* isomer becomes an important goal. Attempts at separating these *E* and *Z* isomers via column chromatography has been unsuccessful thus far. The use of a preparative recycling HPLC to separate these isomers has been investigated;

however no success was achieved. The E and Z mixture **16** remains the gold standard out of all 35 compounds synthesized and tested in the assay. Future work on this compound includes determining its mode of action, identifying the correct dosage regimens as well as further toxicity studies.

Although the preliminary results from compounds derived from enones other than isophorone, for example 2-methyl cyclohex-2-ene-1-one, 3-methylcyclohex-2-ene-1-one and 4,4-dimethylcyclohex-2-en-1-one are not exciting, further exploration in this direction could be considered. Indeed a current student in our lab has been preparing different variations of isophorone, by introducing various groups at the 3 position on the ring, using dimedone as a starting material. These will eventually be coupled to propionitrile and bioassay feedback from these compounds will indicate which groups on the enone component contribute optimally to activity. The following three structures have been prepared and sent for bioassays; the results will be compared against **16E/Z**.



The activity of the sulfoxide mixture **18** continues to be intriguing. It is difficult to imagine additional analogues using sulfoxides other than dimethyl sulfoxide and isophorone since in each case the sulfoxide would have more bulky substituents and thus likely to be less active. A possibility avenue is to change the enone component as was done in the preparation of the nitriles by Nadia Ayoub Jami and those prepared starting with dimedone.



2.3. Experimental Data

2.3.1. General Methods

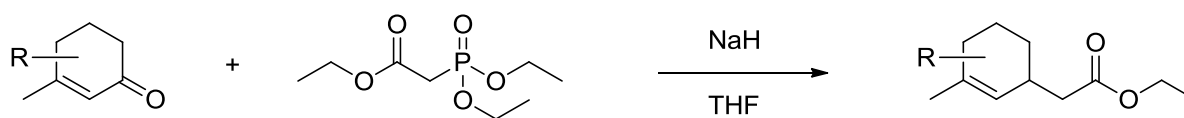
Please refer to general methods in **Section 1.3.1.**

Note: Integration for peaks in the ^1H NMR spectra of the compound mixtures are not indicated due to the fact that often times these mixtures produced extremely complex peak patterns which were difficult to identify. In many cases, ^{13}C NMR spectra of intermediates have been omitted due to complexity arising from having multiple isomers in compound mixtures. The work presented in this chapter was done based on a contract with OB Pharma. It was decided that only compounds that showed promise would be fully characterized and that such characterization would be the minimum that is required to support a patent application should one eventually be made.

2.3.2. General Procedures

General Procedure 1:

Preparation of ethyl esters

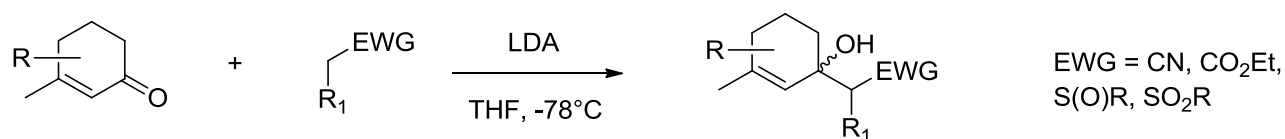


Triethylphosphonoacetate (2 equiv.) was slowly added drop wise to a slurry of 60% NaH (2 equiv.) in anhydrous THF (10-20 mL). After addition, the reaction mixture was stirred for 15 mins. The desired enone (1 equiv.) in anhydrous THF (10-20 mL) was added drop wise via syringe to the reaction mixture and was refluxed for 20 hours. The progress of the reaction was monitored through TLC. After 20 hours, the mixture was dissolved in water and extracted with EtOAc (3 x 20 mL). The organic extracts were dried (MgSO_4), filtered and concentrated in

vacuo. Crude product was purified by gradient column chromatography using EtOAc and hexanes⁶.

General Procedure 2:

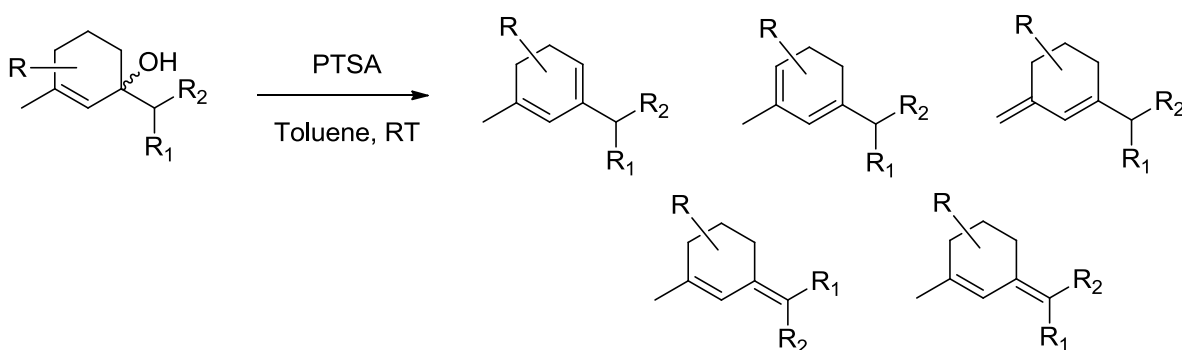
Coupling Reaction



The desired reagent (R_1CH_2EWG) (2 equiv.) was added drop wise via syringe to a -78°C solution of LDA (2 M) in anhydrous THF (5-10 mL) under nitrogen atmosphere. The solution was stirred under these conditions for 5 mins before adding the enone drop wise. The reaction mixture was kept at low temperature and quenched after 1 hour with 10% aqueous NH_4Cl solution. Workup involved extraction with EtOAc (3x 10 mL). The combined organic extracts were washed with water, dried over $MgSO_4$, filtered and concentrated in vacuo. The product was purified by gradient column chromatography using a gradient solvent system of EtOAc and hexanes.

General Procedure 3:

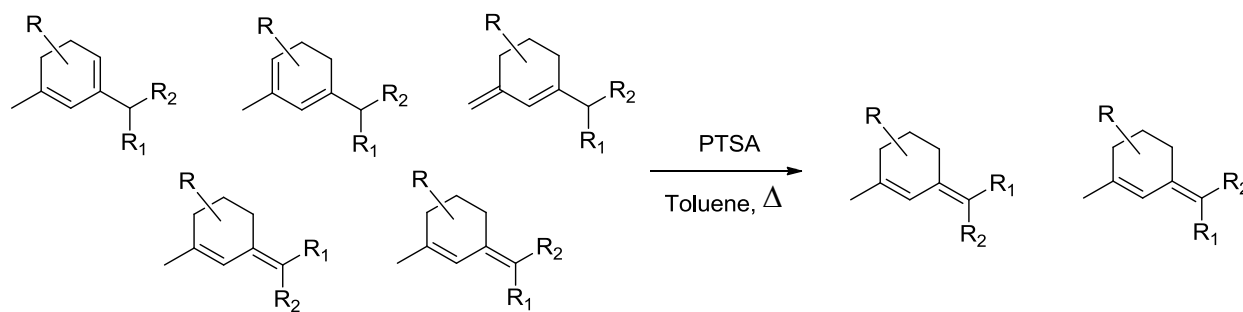
Room temperature dehydration



The coupled material obtained via General Procedure 2 was stirred at room temperature in toluene (10-15 mL) containing a catalytic amount of PTSA until product formation was observed and the starting material had been consumed, as shown by TLC. The reaction mixture was quenched after 1 h with 10% aqueous NaHCO_3 and extracted with EtOAc (3 x10 mL). The combined extracts were washed with water, dried (MgSO_4), filtered and concentrated in vacuo. Purification by column chromatography afforded a mixture of up to five isomers.

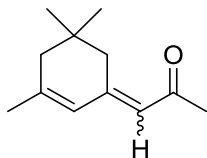
General Procedure 4:

Isomerization to the E/Z isomers



The mixture from General Procedure 3 was dissolved in toluene containing a catalytic amount of PTSA and refluxed for 3 hours, then quenched with 10% aqueous NaHCO_3 and finally extracted with EtOAc (3x10 mL). The combined extracts were washed with water, dried (MgSO_4), filtered and concentrated in vacuo. Purification by column chromatography afforded the E/Z isomers of the desired compound.

Preparation of 1-(3,5,5-trimethylcyclohex-2-en-1-ylidene)propan-2-one



Chemical Formula: C₁₂H₁₈O

Molecular Weight: 178.27

3

Compound **6** (0.25 g, 1 mmol) and KOH (0.07 g, 1.75 mmol) in a mixture of EtOH : H₂O (3:1 – 20 mL) was refluxed for 2 h. After this time, the solution was stirred to rt, concentrated in vacuo and the resulting residue was dissolved in H₂O (5 mL) . The solution was than acidified with concentrated HCl and extracted with EtOAc (3 x 20 mL). The crude product (0.16 g) was purified by gradient column chromatography yielding **3** as a pale yellow oil (0.09 g, 50%, R_f = 0.40 in 1:9 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.35 (s), 5.93 (s), 5.84 (s), 5.76 (s), 2.69 (s), 2.16 (d, *J* = 4.3 Hz), 2.03 (s), 1.96 – 1.92 (m), 1.82 (d, *J* = 10.8 Hz), 0.90 (d, *J* = 0.8 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 199.0, 198.4, 153.1, 151.4, 148.5, 147.6, 125.2, 121.8, 121.0, 119.7, 45.8, 45.6, 45.0, 39.3, 31.9, 31.8, 31.5, 30.9, 28.2, 28.0, 24.6, 24.5.

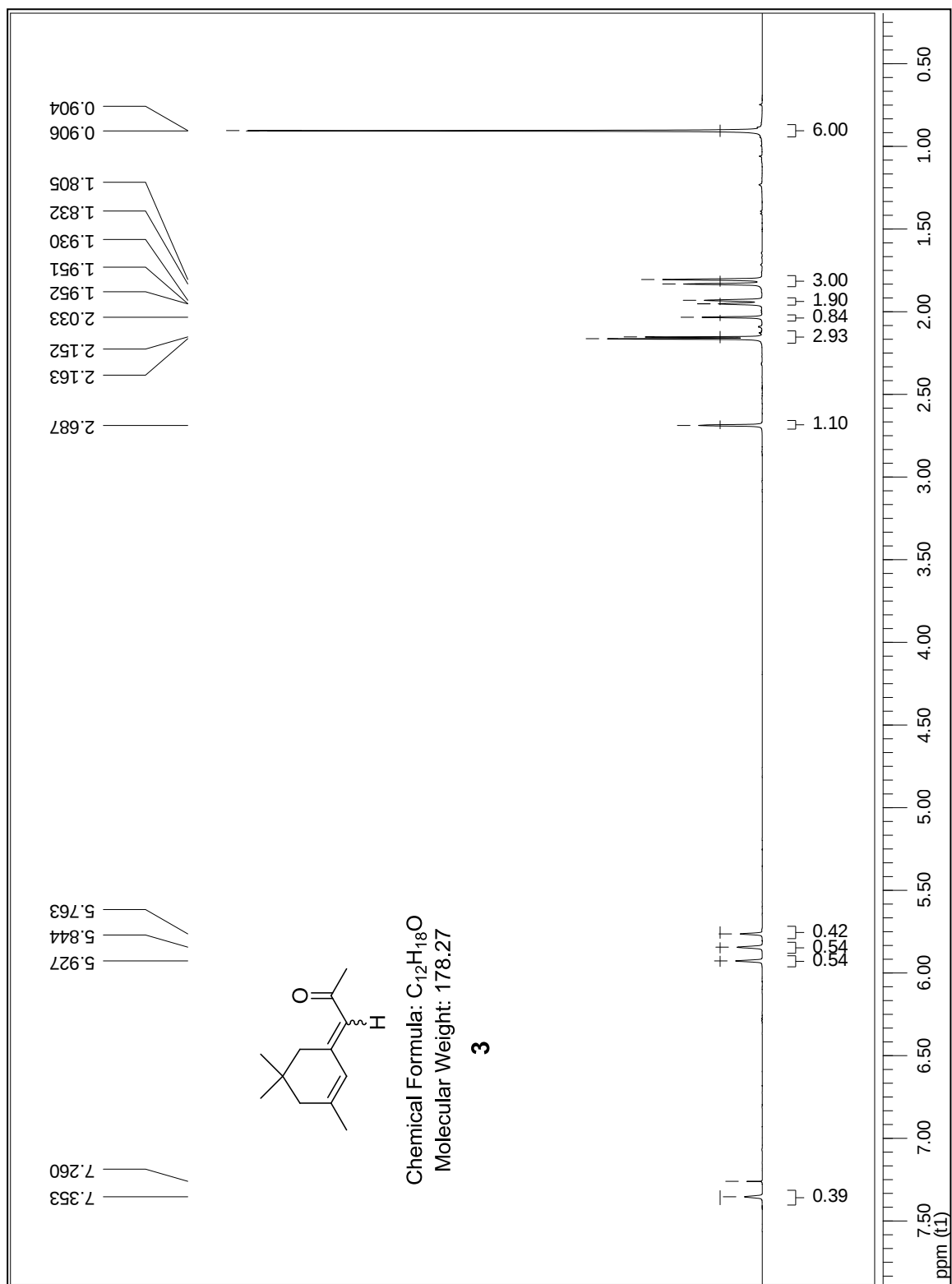


Figure 2.3.2.1. 400 MHz ¹H NMR spectrum of compound **3** in CDCl₃.

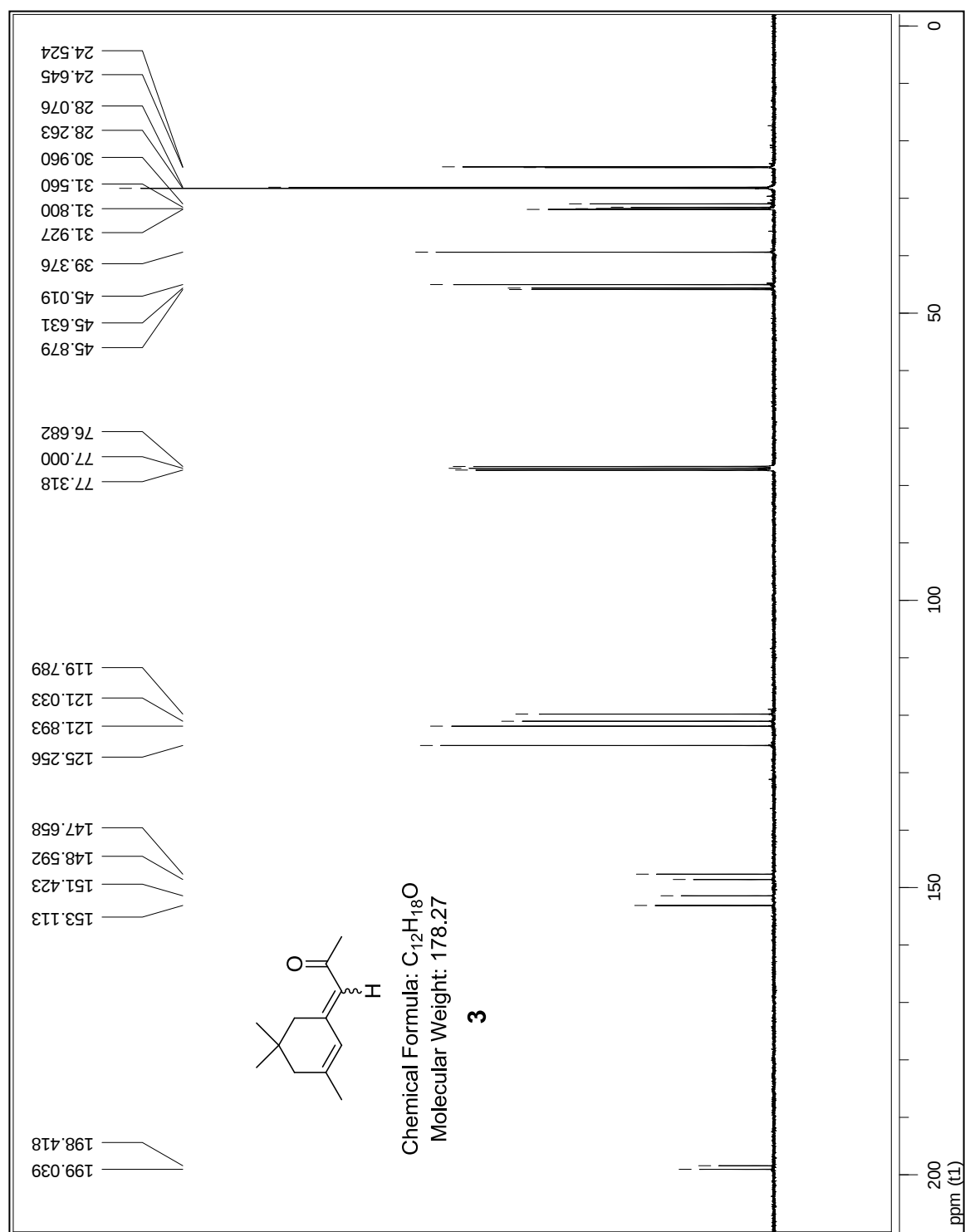
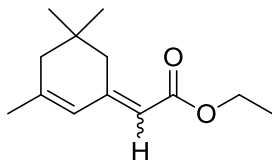


Figure 2.3.2.2. 100 MHz ¹³C NMR spectrum of compound 3 in CDCl₃.

Preparation of Ethyl 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetate



Chemical Formula: C₁₃H₂₀O₂

Molecular Weight: 208.30

4

This compound was prepared following general procedure 1 using triethylphosphonoacetate (12.97 g, 57.9 mmol) in anhydrous THF (10 mL), NaH (1.38 g, 57.9 mmol) and isophorone (4 g, 28.9 mmol) in anhydrous THF (10 mL). The crude product was purified via gradient column chromatography (10-20% EtOAc in hexanes) resulting in **4** as a colorless oil (3.98 g, 69%, R_f = 0.73 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.24 (s), 5.77 (d, *J* = 0.7 Hz), 5.42 (s), 5.27 (s), 4.00 (qd, *J* = 7.1, 2.3 Hz), 2.61 (d, *J* = 1.7 Hz), 1.95 (d, *J* = 1.3 Hz), 1.82 (d, *J* = 9.4 Hz), 1.72 (s), 1.14 (dd, *J* = 7.8, 6.4 Hz), 0.80 (d, *J* = 7.1 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 166.9, 166.1, 154.1, 152.6, 145.9, 145.0, 130.8, 130.1, 128.6, 124.7, 120.1, 113.6, 111.4, 58.8, 45.6, 45.1, 44.7, 42.5, 41.2, 38.7, 31.0, 30.6, 27.9, 27.7, 24.2, 24.0, 14.0.

HRMS: Calculated for C₁₃H₂₀O = 208.14633; found = 208.14596

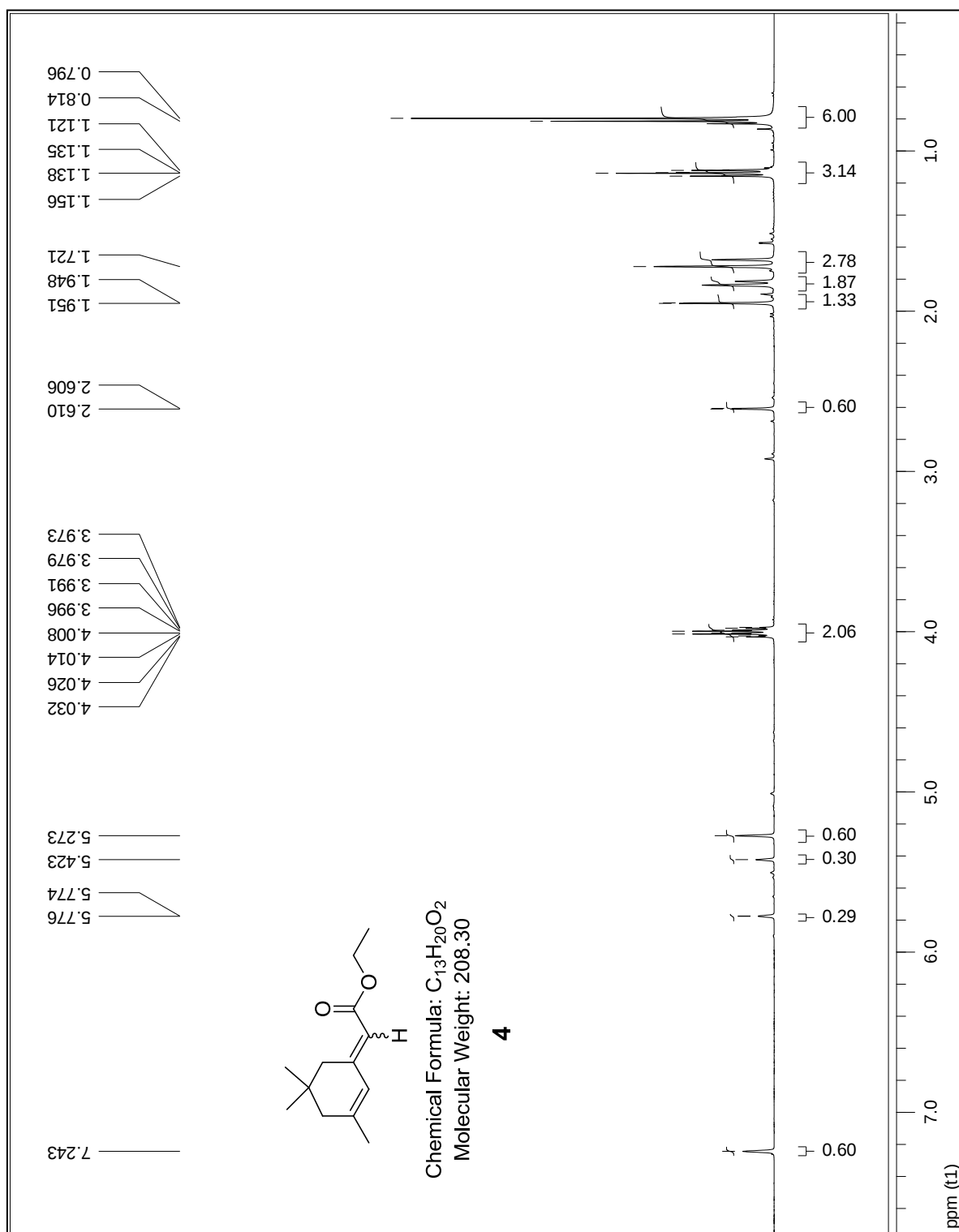


Figure 2.3.2.3. 400 MHz 1H NMR spectrum of compound **4** in $CDCl_3$.

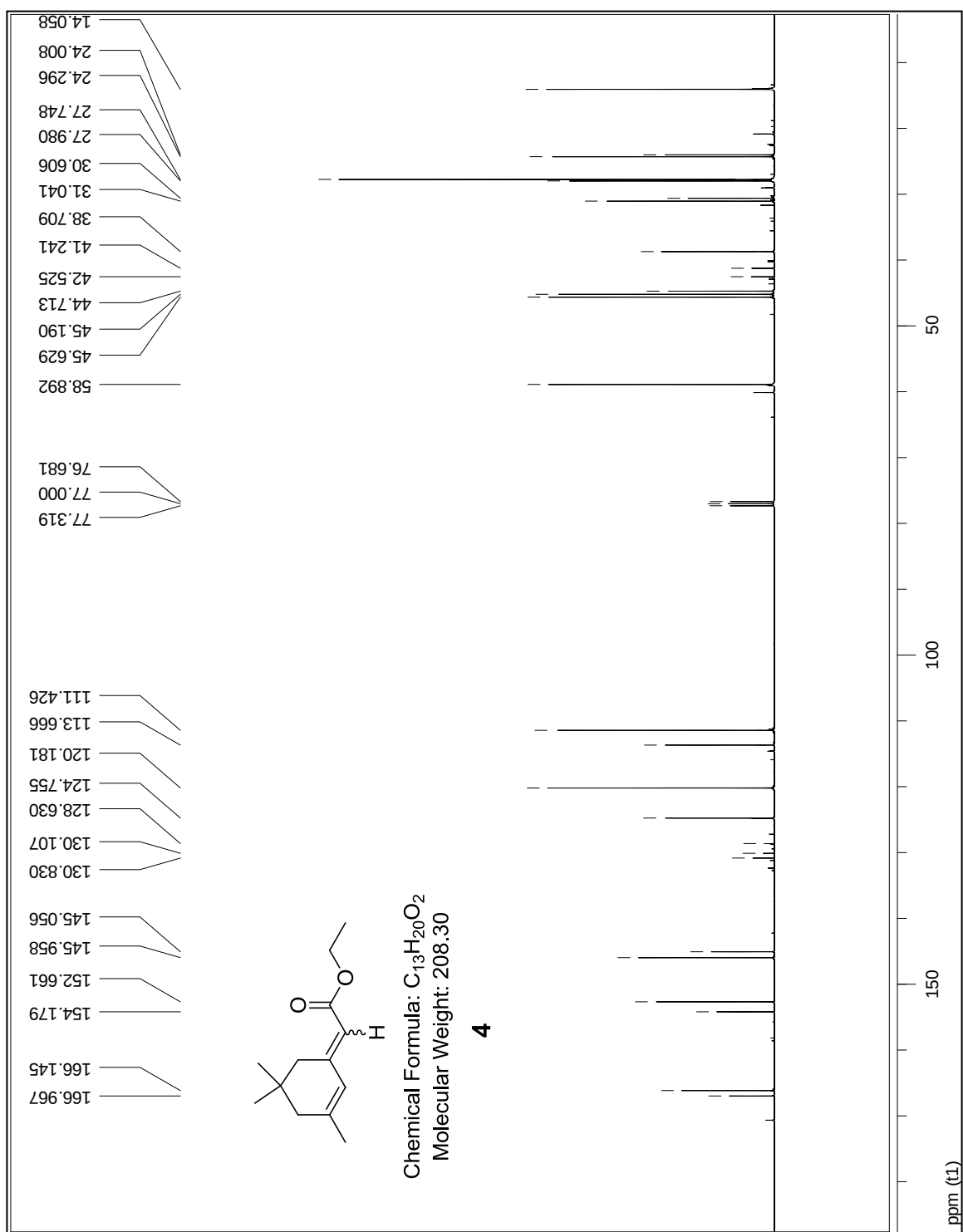
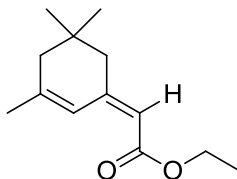


Figure 2.3.2.4. 100 MHz ^{13}C NMR spectrum of compound **4** in $CDCl_3$.

Preparation of (Z)-ethyl 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetate



Chemical Formula: C₁₃H₂₀O₂

Molecular Weight: 208.30

4-Z

To a stirred solution of **7-Z** (0.025 g, 0.138 mmol) in THF (5 mL), was added K₂CO₃ (0.192 g, 1.38 mmol) and EtI. The mixture was refluxed for 3 h, then cooled to room temperature. The solvent was evaporated in vacuo and water was added. The mixture was extracted with EtOAc (3 x 10 mL), dried (MgSO₄), filtered and concentrated, yielding **4-Z** (0.0148 g, 52%, R_f = 0.58 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.34 (s, 1H), 5.40 (s, 1H), 4.14 (dt, *J* = 7.1, 5.6 Hz, 2H), 2.08 (d, *J* = 0.9 Hz, 2H), 1.97 (s, 2H), 1.85 (s, 3H), 1.28 (t, *J* = 7.1 Hz, 3H), 0.92 (s, 6H).

¹³C NMR (100 MHz, CDCl₃) δ 166.7, 153.2, 146.7, 120.32, 111.5, 59.3, 45.9, 45.5, 39.0, 31.4, 30.2, 28.0, 24.6, 14.3.

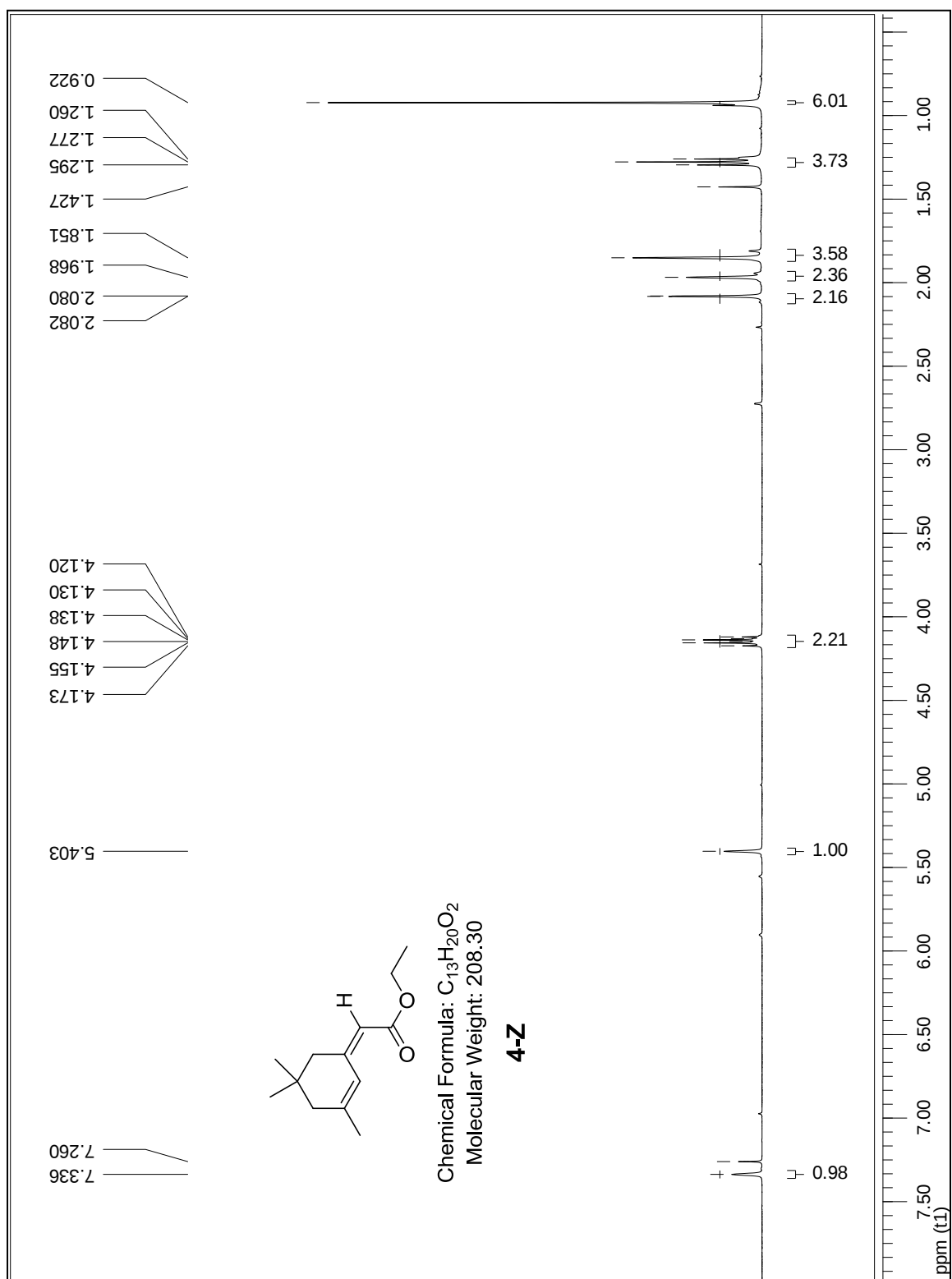


Figure 2.3.2.5. 400 MHz 1H NMR spectrum of compound **4Z** in $CDCl_3$.

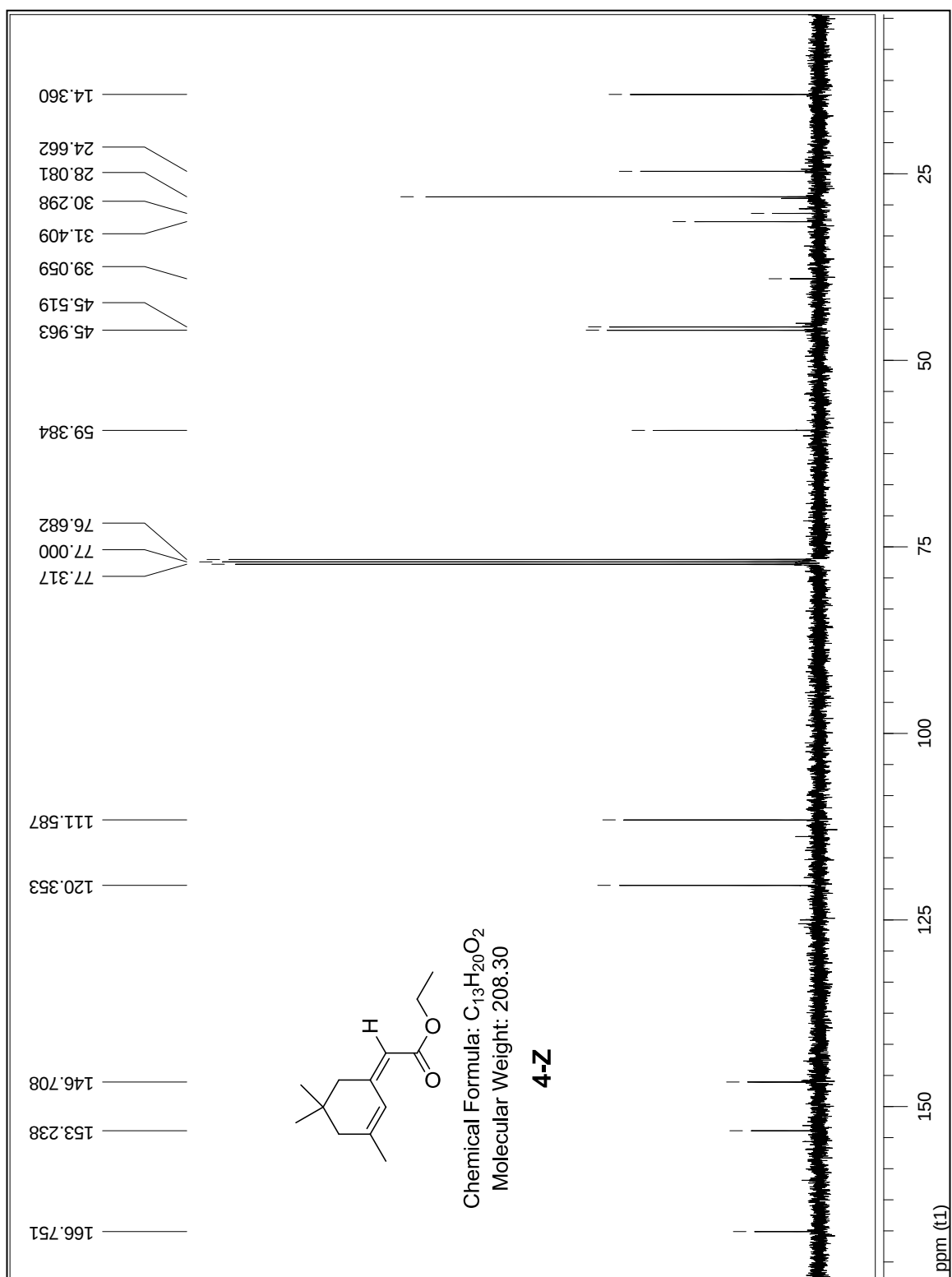
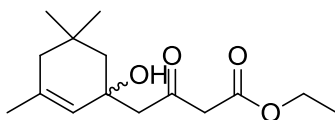


Figure 2.3.2.6. 100 MHz ^{13}C NMR spectrum of compound **4Z** in $CDCl_3$.

Preparation of ethyl 4-(1-hydroxy-3,5,5-trimethylcyclohex-2-en-1-yl)-3-oxobutanoate



Chemical Formula: C₁₅H₂₄O₄
Molecular Weight: 268.35

5

NaH (0.88 g, 22 mmol) was added to a round bottom flask containing anhydrous THF (25 mL). The flask was kept under a nitrogen atmosphere and put in an ice bath (0°C). Ethyl acetoacetate (2.6 mL, 20 mmol) was added drop wise via syringe and the solution was stirred at 0°C for 10 min. To this solution was added nBuLi (8.4 mL, 21 mmol) and the solution was kept at 0°C for an additional 10 min. To this dianion solution was added isophorone (1.52 g, 11 mmol). The solution was allowed to warm to room temperature and after 15 min, the reaction was quenched with 2mL of conc. HCl in 5 mL of H₂O and extracted with ether (3 x 15 mL). The crude product was purified by gradient column chromatography (30% EtOAc in hexanes) affording **5** as a colorless oil (0.780 g, 28%, R_f = 0.35 in 2:3 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 5.34 (s), 4.20 – 4.06 (m), 3.44 (d, *J* = 1.5 Hz), 3.13 (d, *J* = 1.0 Hz), 2.66 (d, *J* = 1.7 Hz), 1.77 – 1.54 (m), 1.37 (d, *J* = 14.1 Hz), 1.22 (td, *J* = 7.1, 2.0 Hz), 0.93 (dd, *J* = 32.1, 1.7 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 203.9, 174.9, 166.8, 136.5, 124.3, 123.7, 92.2, 70.3, 61.2, 54.6, 50.7, 47.8, 44.0, 31.0, 29.8, 27.5, 23.8, 13.9.

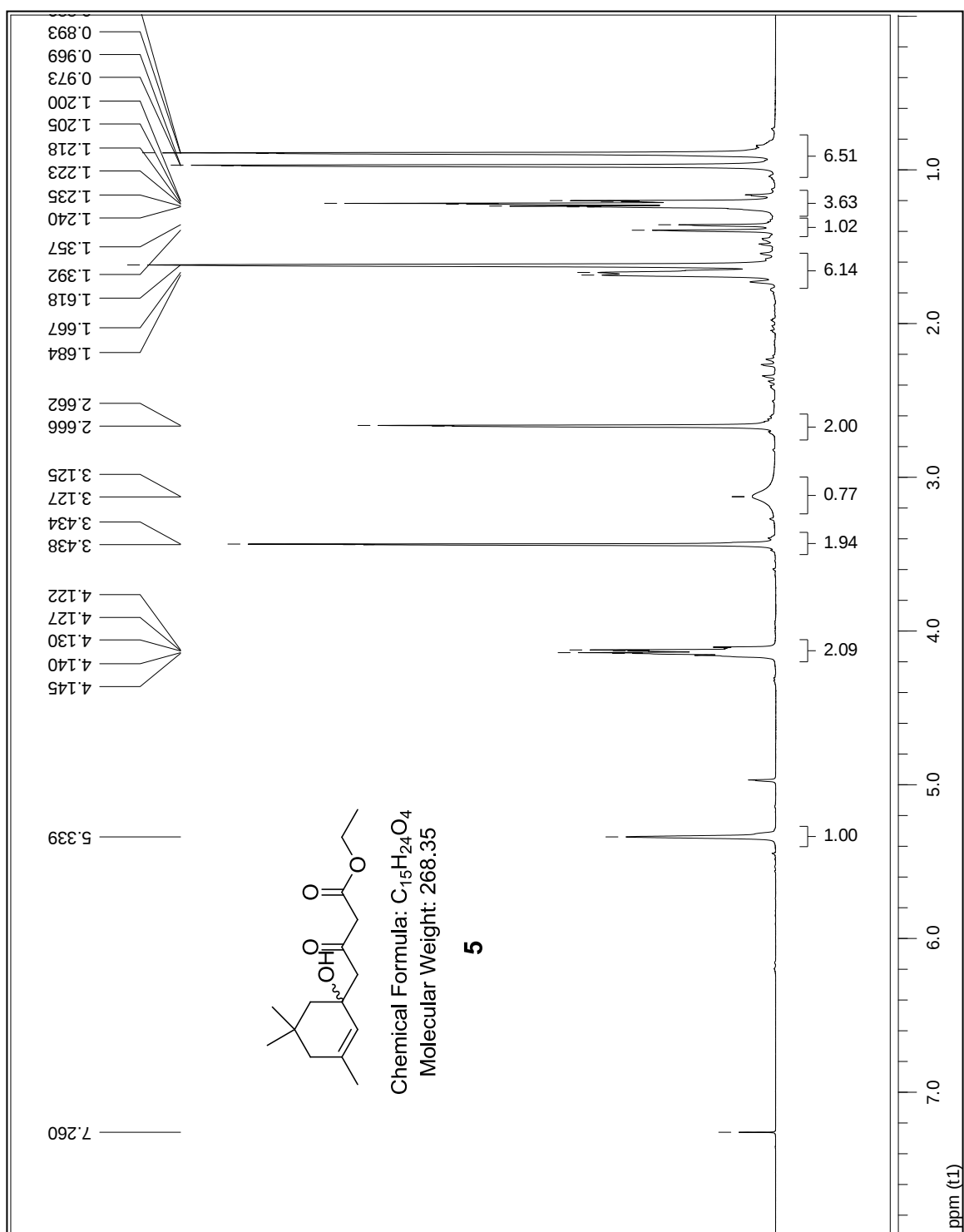


Figure 2.3.2.7. 400 MHz 1H NMR spectrum of compound **5** in $CDCl_3$.

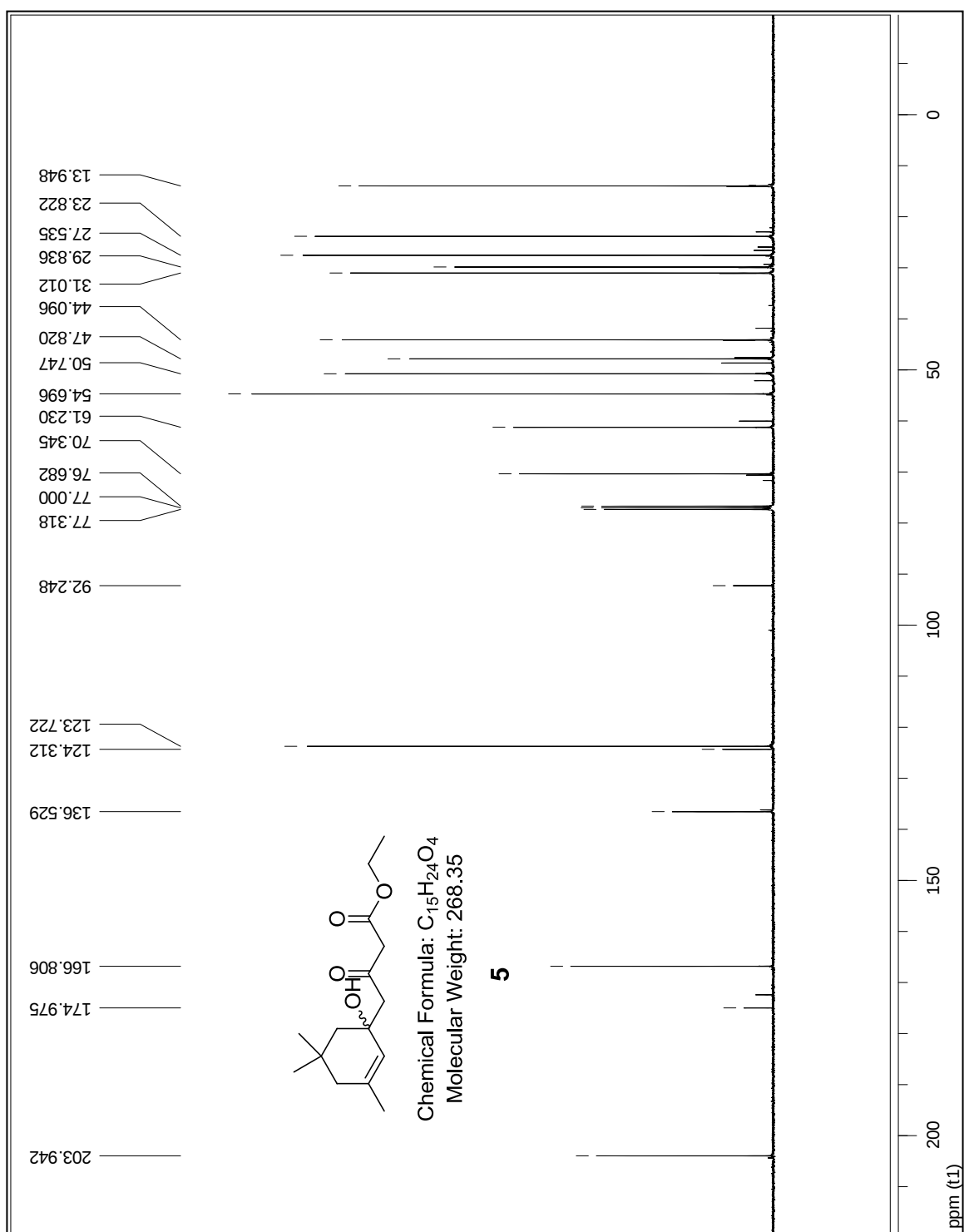
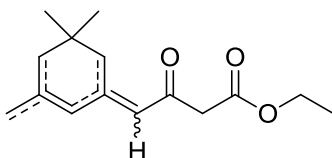


Figure 2.3.2.8. 100 MHz ^{13}C NMR spectrum of compound 5 in CDCl_3 .

Preparation of Ethyl 3-oxo-4-(3,5,5-trimethylcyclohex-2-en-1-ylidene)butanoate



Chemical Formula: C₁₅H₁₈O₃

Molecular Weight: 246.30

6

This compound was prepared using general procedure 3 using compound 5 (0.78 g, 2.9 mmol), in toluene (10 mL) containing a catalytic amount of PTSA. The crude product was purified by gradient column chromatography (20-25% EtOAc in hexanes) affording **6** as a colorless oil (0.549 g, 76%, R_f=0.45 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.40 (d, *J* = 0.8 Hz), 6.03 (s), 5.94 (s), 5.90 – 5.86 (m), 5.77 (s), 5.64 (d, *J* = 1.2 Hz), 4.97 (dd, *J* = 12.8, 7.5 Hz), 4.80 (d, *J* = 17.3 Hz), 4.17 (q, *J* = 7.1 Hz), 3.44 (d, *J* = 5.9 Hz), 3.29 – 3.10 (m), 2.72 (d, *J* = 1.7 Hz), 2.15 – 1.61 (m), 1.25 (dd, *J* = 7.6, 6.7 Hz), 0.91 (t, *J* = 8.2 Hz).

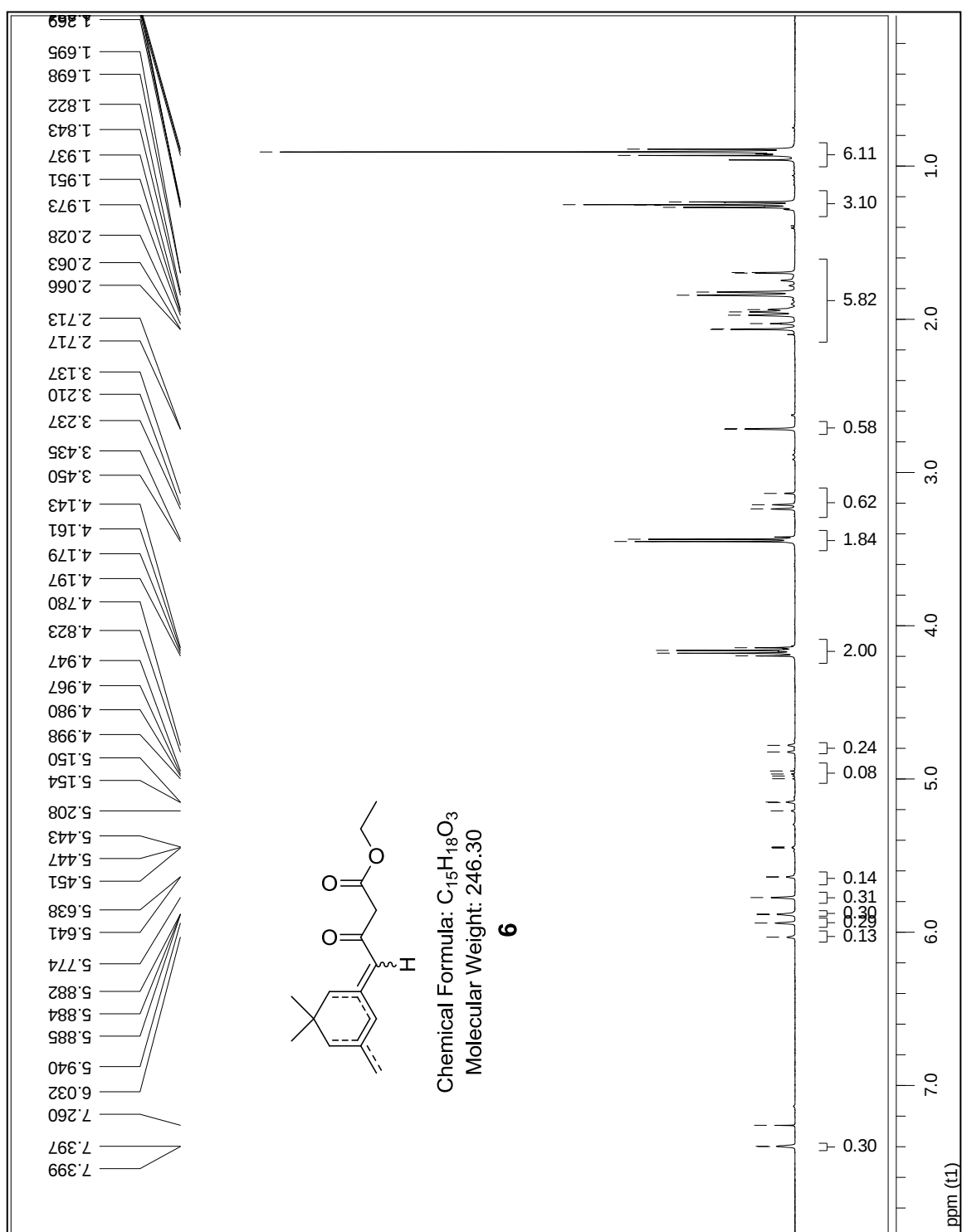
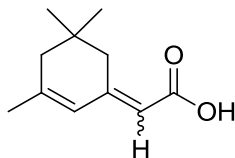


Figure 2.3.2.9. 400 MHz 1H NMR spectrum of compound **6** in $CDCl_3$.

Preparation of 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetic acid



Chemical Formula: C₁₁H₁₆O₂

Molecular Weight: 180.24

7

Compound **4** (1 g, 4.8 mmol) and KOH (0.539 g, 9.6 mmol) in a mixture of EtOH : H₂O (3:1 – 20mL) were refluxed for 2 h. After this time, the solution was stirred to rt, concentrated in vacuo and the resulting residue was dissolved in H₂O (5 mL) . The solution was then acidified with concentrated HCl. Precipitate was filtered by suction filtration affording **7** as a white solid (0.52 g, 60%, R_f = 0.2 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.30 (s), 5.94 (d, *J* = 0.6 Hz), 5.57 (s), 5.42 (s), 2.71 (d, *J* = 1.6 Hz, 1H), 2.11 (d, *J* = 1.1 Hz, 1H), 1.97 (d, *J* = 10.4 Hz, 2H), 1.84 (d, *J* = 16.0 Hz, 3H), 0.92 (s, 6H).

¹³C NMR (100 MHz, CDCl₃) δ 171.7, 170.8, 157.1, 155.7, 147.9, 147.0, 125.0, 120.4, 112.9, 110.6, 46.1, 45.5, 45.0, 39.2, 31.5, 31.0, 28.2, 28.0, 24.7, 24.4.

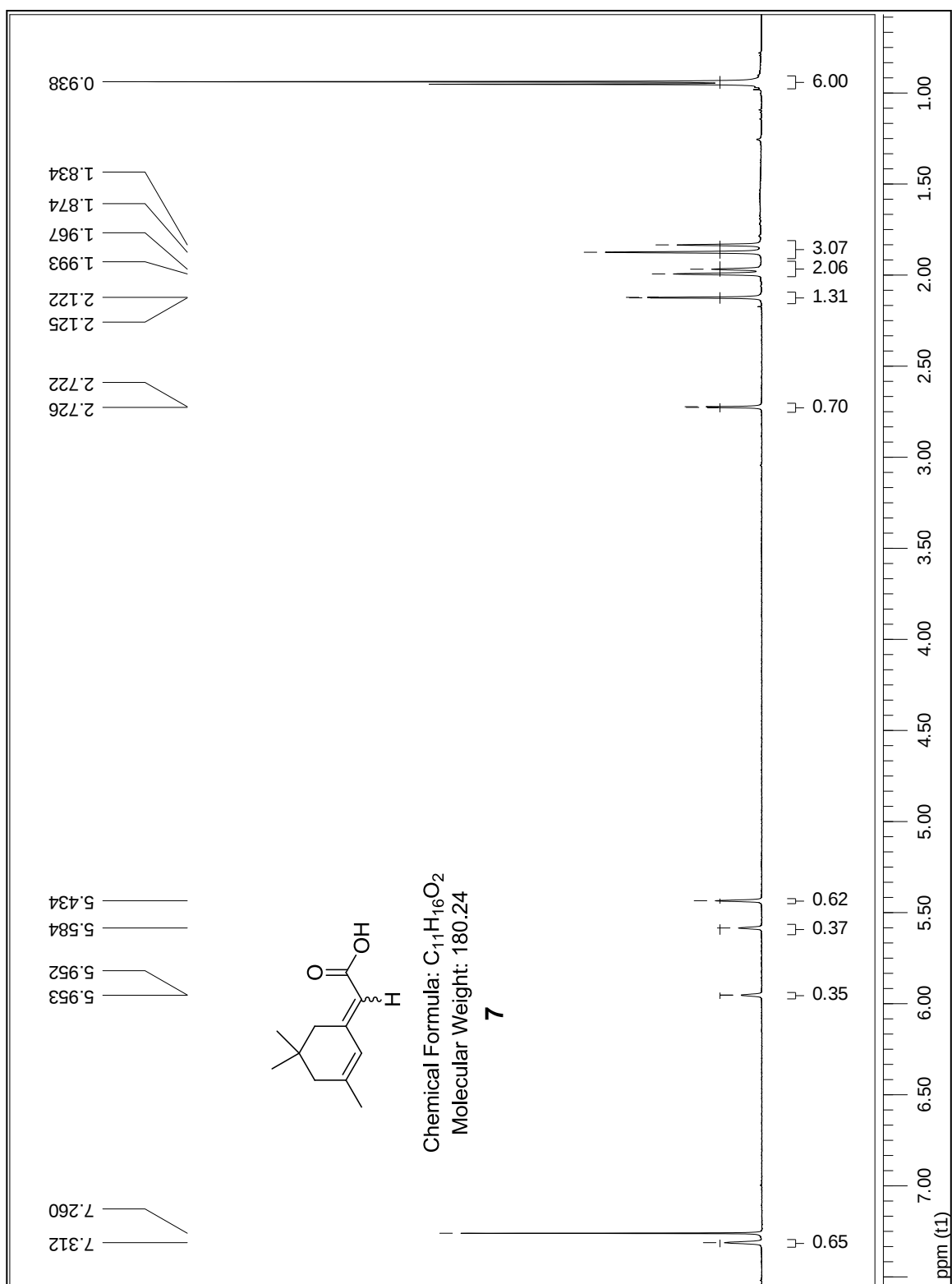
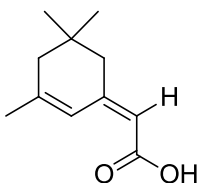


Figure 2.3.2.10. 400 MHz 1H NMR spectrum of compound 7 in $CDCl_3$.

Preparation of (Z)-2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetic acid



Chemical Formula: C₁₁H₁₆O₂
Molecular Weight: 180.24

7-Z

Compound **7** was subjected to two consecutive recrystallizations using MeOH at rt. Solid material was filtered by suction filtrated affording **7-Z** as a white solid.

¹H NMR (400 MHz, CDCl₃) δ 7.31 (s, 1H), 5.43 (s, 1H), 2.12 (s, 2H), 1.99 (s, 2H), 1.87 (s, 3H), 0.94 (s, 6H).

¹³C NMR (100 MHz, CDCl₃) δ 171.2, 155.7, 147.9, 120.4, 110.8, 46.1, 45.5, 31.5, 28.0, 24.7.

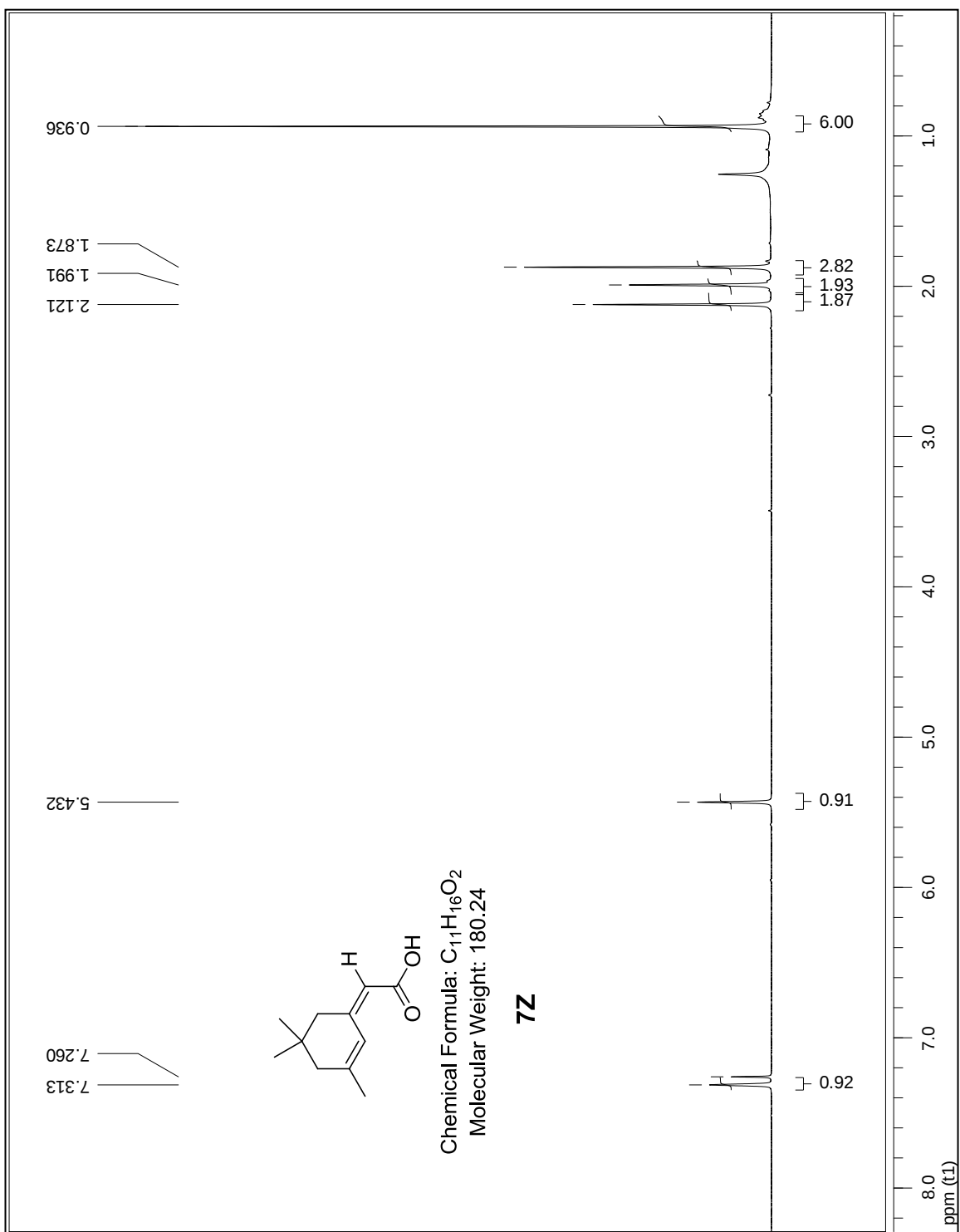
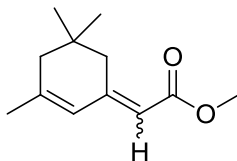


Figure 2.3.2.11. 400 MHz 1H NMR spectrum of compound **7Z** in $CDCl_3$.

Preparation of methyl 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetate



Chemical Formula: C₁₂H₁₈O₂
Molecular Weight: 194.27

8

In a round bottomed flask containing compound **7** (0.01 g, 0.55 mmol) in MeOH (10 mL) was added a few drops of conc. HCl. The reaction was set to reflux for 1 h. The progress of the reaction was monitored via TLC analysis. The reaction was quenched with 1mL NaHCO₃ in H₂O solution and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried (MgSO₄), filtered, and concentrated in vacuo. The crude product was purified via gradient column chromatography (10-15% EtOAc in hexanes) affording **8** as a colorless oil (0.043 g, 40%, R_f=0.71 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.36 – 7.30 (m), 6.97 (s), 5.91 (d, *J* = 0.8 Hz), 5.56 (s), 5.41 (s), 5.00 (s), 3.68 (s), 2.72 (d, *J* = 1.7 Hz), 2.31 – 2.20 (m), 2.08 (d, *J* = 1.4 Hz), 1.97 (s), 1.83 (d, *J* = 17.2 Hz), 1.42 (s), 1.25 (s), 0.92 (s).

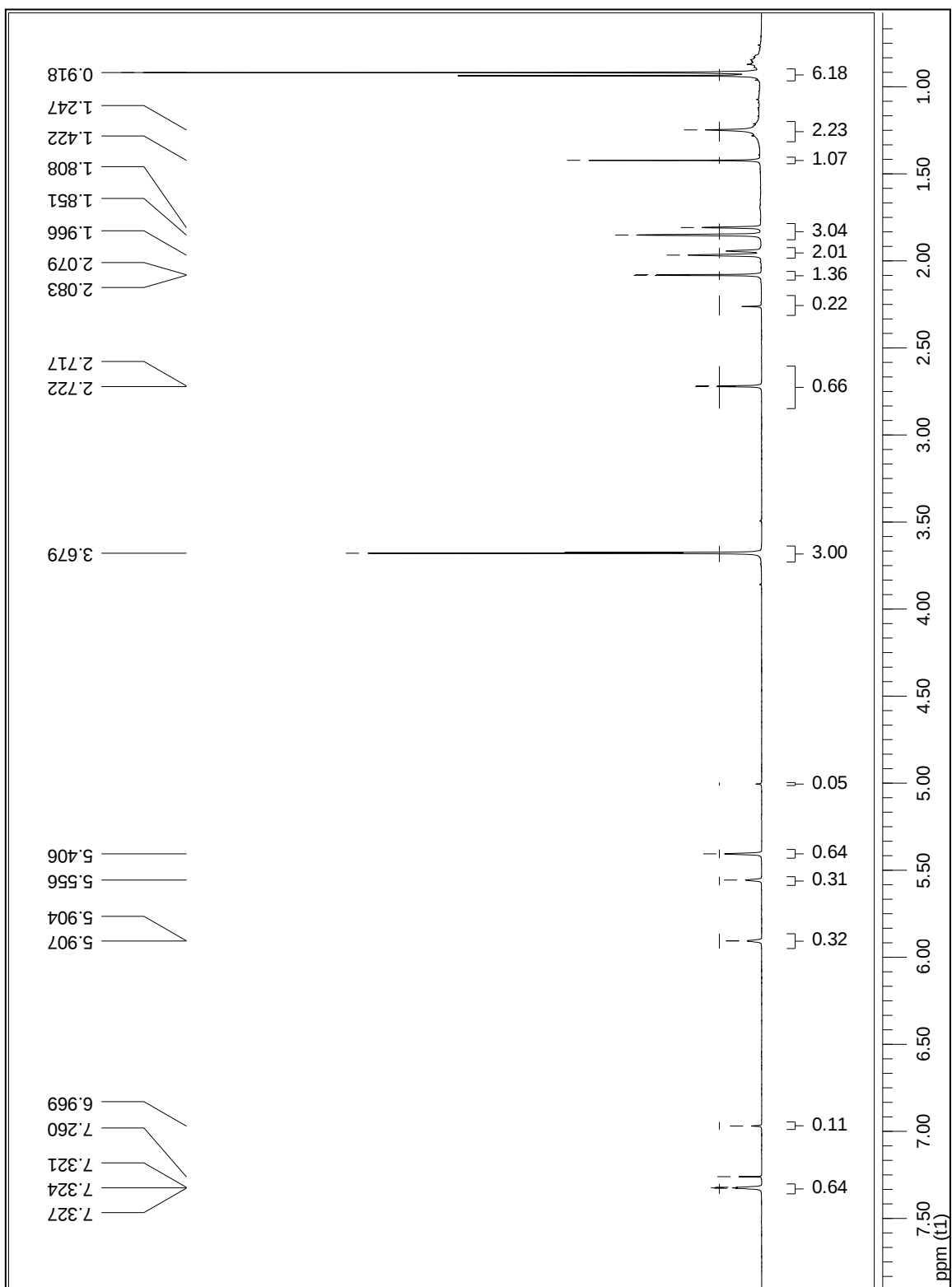
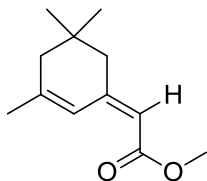


Figure 2.3.2.13. 400 MHz ^1H NMR spectrum of compound **8** in CDCl_3 .

Preparation of (Z)-methyl 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetate



Chemical Formula: C₁₂H₁₈O₂

Molecular Weight: 194.27

8-Z

To a stirred solution of **7-Z** (0.017 g, 0.09 mmol) in THF (5 mL), was added K₂CO₃ (0.124 g, 0.9 mmol) and CH₃I. The mixture was refluxed for 2 h, than cooled to room temperature. The solvent was evaporated in vacuo and water was added. The mixture was extracted with EtOAc (3 x 10 mL), dried (MgSO₄), filtered and concentrated, yielding **8-Z** (0.01 g, 58%, R_f = 0.58 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.31 (s, 1H), 5.39 (s, 1H), 3.67 (s, 3H), 2.07 (d, *J* = 1.1 Hz, 2H), 1.94 (d, *J* = 9.5 Hz, 2H), 1.84 (s, 3H), 0.91 (s, 6H).

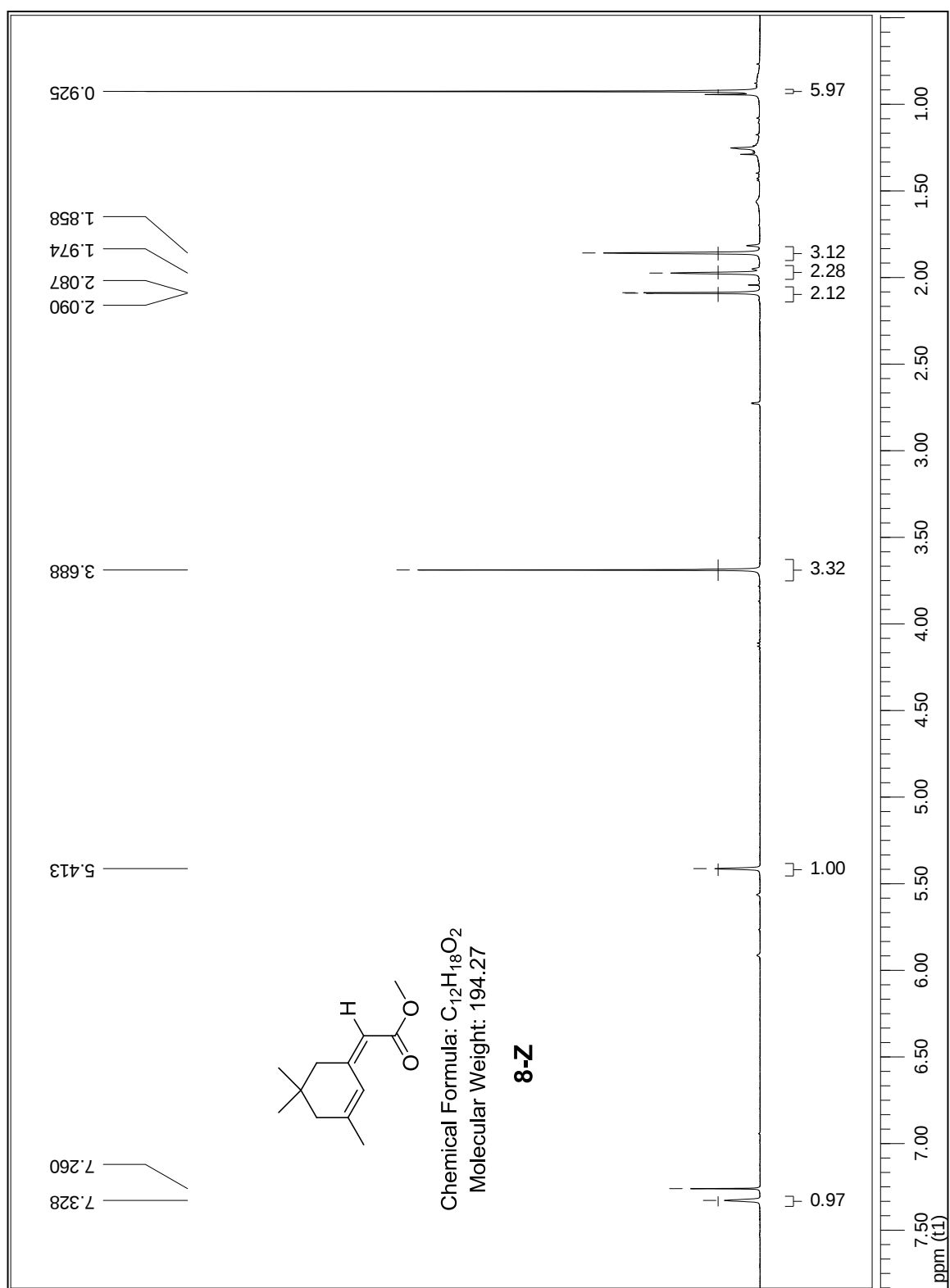
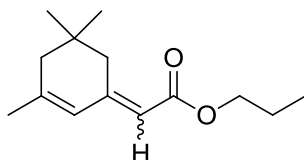


Figure 2.3.2.14. 400 MHz ^1H NMR spectrum of compound **8Z** in CDCl_3 .

Preparation of propyl 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetate



Chemical Formula: C₁₄H₂₂O₂
Molecular Weight: 222.32

9

In a round bottomed flask containing compound **7** (0.1 g, 0.55 mmol) in propanol (10 mL) was added a few drops of conc. HCl. The reaction was set to reflux for 1 h. The progress of the reaction was monitored via TLC analysis. The reaction was quenched with 2 mL NaHCO₃ in H₂O solution and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried (MgSO₄), filtered, and concentrated in vacuo. The crude product was purified via gradient column chromatography (10-15% EtOAc in hexanes) affording **9** as a colorless oil (0.091 g, 74%, R_f=0.78 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.33 (s), 5.90 (s), 5.56 (s), 5.41 (s), 4.04 (td, *J* = 6.7, 2.6 Hz), 3.61 (t, *J* = 6.7 Hz), 2.71 (d, *J* = 1.6 Hz), 2.10 – 2.05 (m), 1.95 (d, *J* = 9.5 Hz), 1.82 (d, *J* = 15.9 Hz), 1.71 – 1.54 (m), 0.93 (s).

¹³C NMR (100 MHz, CDCl₃) δ 167.6, 166.8, 154.6, 153.1, 146.6, 145.6, 124.9, 120.3, 113.8, 111.6, 65.1, 65.1, 45.9, 45.5, 45.0, 39.0, 31.3, 30.9, 28.2, 28.0, 24.6, 24.3, 22.1, 10.4.

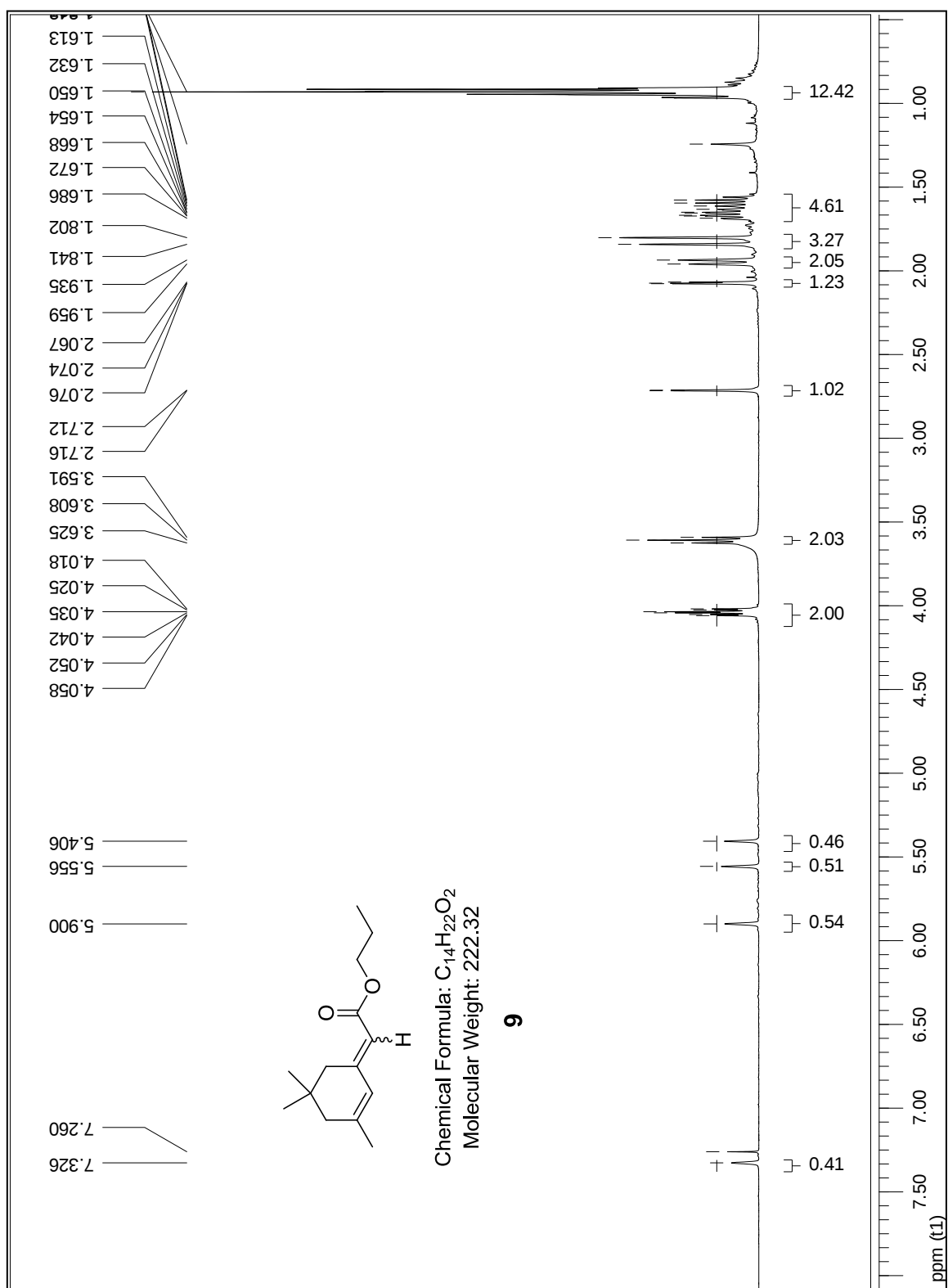


Figure 2.3.2.15. 400 MHz 1H NMR spectrum of compound **9** in $CDCl_3$.

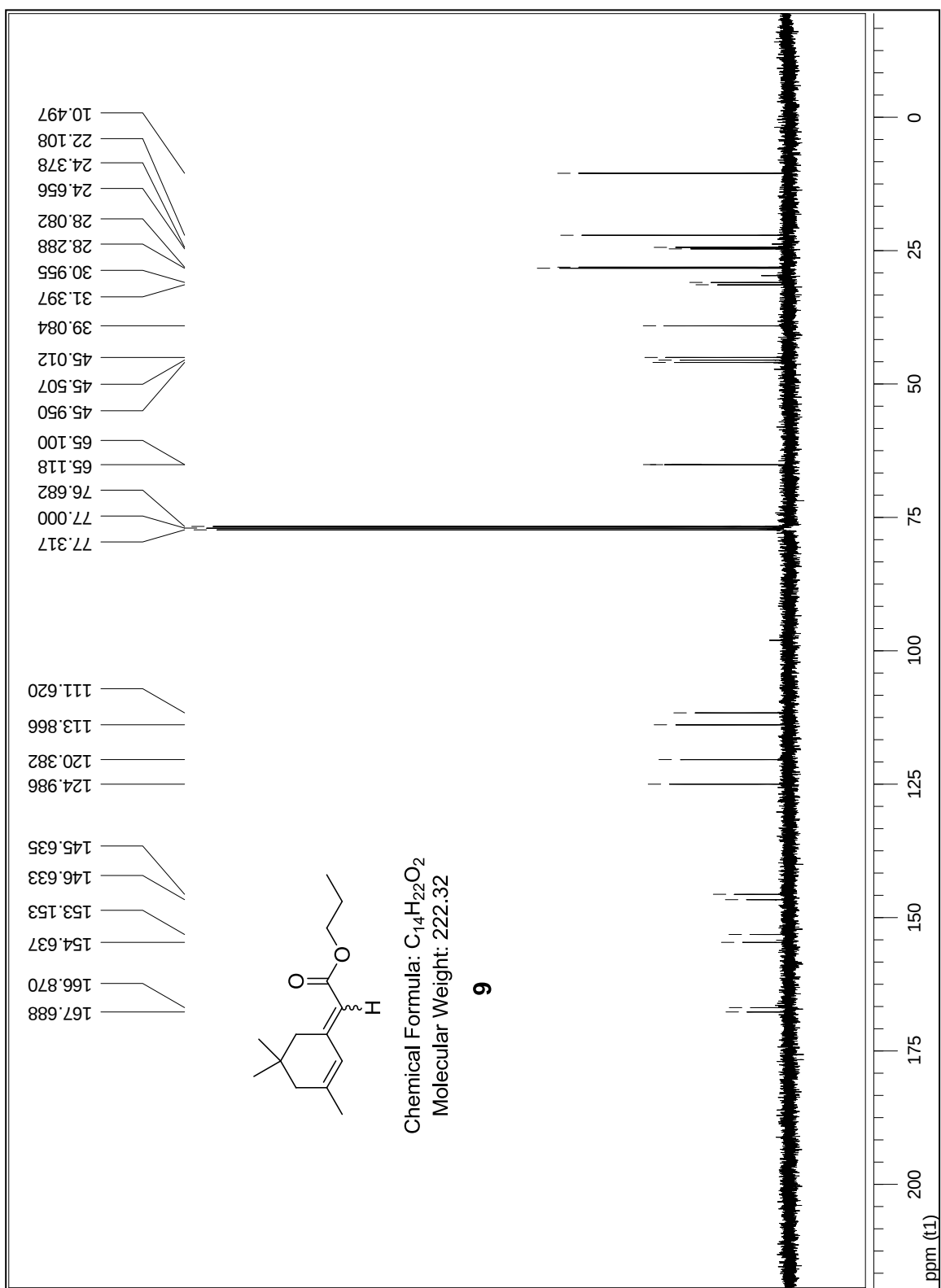
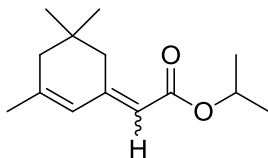


Figure 2.3.2.16. 100 MHz ^{13}C NMR spectrum of compound **9** in $CDCl_3$.

Preparation of Isopropyl 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetate



Chemical Formula: C₁₄H₂₂O₂

Molecular Weight: 222.32

10

To a mixture of compound **7** (0.15 g, 1.1 mmol) in DCM (5 mL) was added SOCl₂ (1 mL) and stirred under reflux for 2 h. Solution was further concentrated in vacuo and diluted with DCM (5 mL). Isopropanol (0.1 g, 1.5 mmol) was added simultaneously with Et₃N (0.21 mL, 1.5 mmol) drop wise via syringe. The crude product was purified by gradient column chromatography (20-25% EtOAc in hexanes) yielding **10** as an orange oil (0.09 g, 40%, R_f = 0.76 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.35 (s), 5.89 (d, *J* = 0.8 Hz), 5.53 (s), 5.38 (s), 5.08 – 4.96 (m), 2.73 (d, *J* = 1.7 Hz), 2.07 (d, *J* = 1.3 Hz), 1.95 (d, *J* = 10.0 Hz), 1.83 (d, *J* = 17.1 Hz), 1.25 (dd, *J* = 6.3, 2.1 Hz), 0.93 (d, *J* = 6.9 Hz).

HRMS: Calculated for C₁₄H₂₂O₂ = 222.16198; found = 222.16078

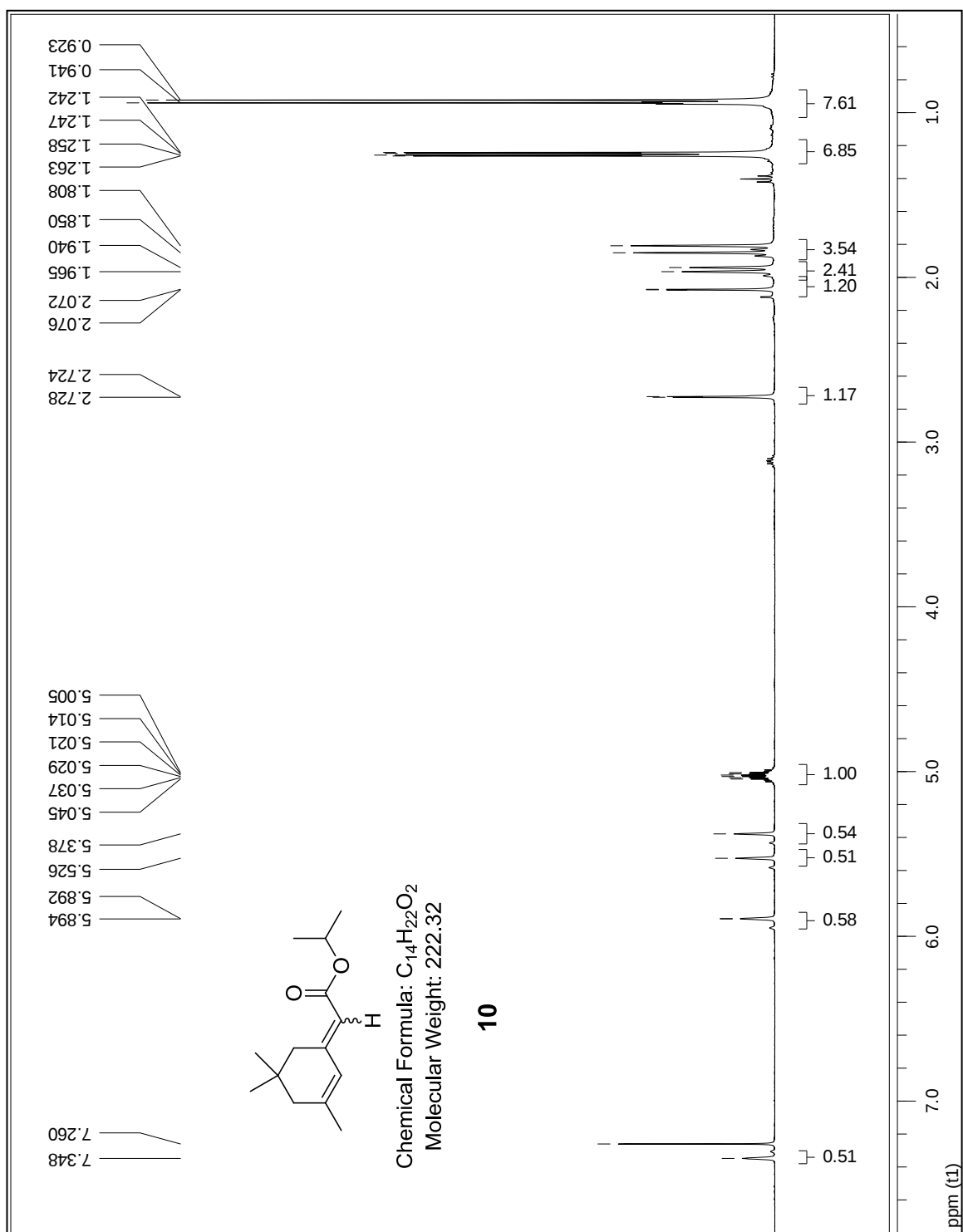
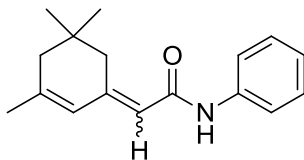


Figure 2.3.2.17. 400 MHz ¹H NMR spectrum of compound **10** CDCl₃.

Preparation of N-phenyl-2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetamide



Chemical Formula: C₁₇H₂₁NO

Molecular Weight: 255.35

11

To a solution of **4** (0.3 g, 1.7 mmol) in toluene (2 mL), was added aniline (0.16 mL, 1.7 mmol). To this mixture was then added Me₃Al/toluene solution (0.85 mL, 1.7 mmol). The resulting mixture was refluxed for 3 h. The reaction was then diluted with DCM (20 mL) and the organic extract was washed with 20% NH₄OH solution (20 mL). The combined organic extracts were dried (MgSO₄), filtered and concentrated in vacuo. The crude product was purified by gradient column chromatography (35-40% EtOAc in hexanes) affording **11** as a yellow oil (0.16 g, 37%, R_f=0.74 in 2:3 EtOAc: hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.56 (d, *J* = 7.7 Hz), 7.47 (s), 7.32 – 7.21 (m), 7.04 (t, *J* = 7.4 Hz), 5.83 (d, *J* = 0.6 Hz), 5.62 (s), 5.44 (s), 2.81 (d, *J* = 1.5 Hz), 2.03 (d, *J* = 1.0 Hz), 1.92 (d, *J* = 9.1 Hz), 1.79 (s), 0.91 (s).

¹³C NMR (400 MHz, CDCl₃) δ 152.3, 150.6, 145.7, 144.7, 138.4, 128.7, 124.9, 123.6, 120.3, 119.7, 119.6, 119.6, 114.4, 45.9, 45.4, 44.9, 38.8, 31.2, 28.2, 28.0, 24.4.

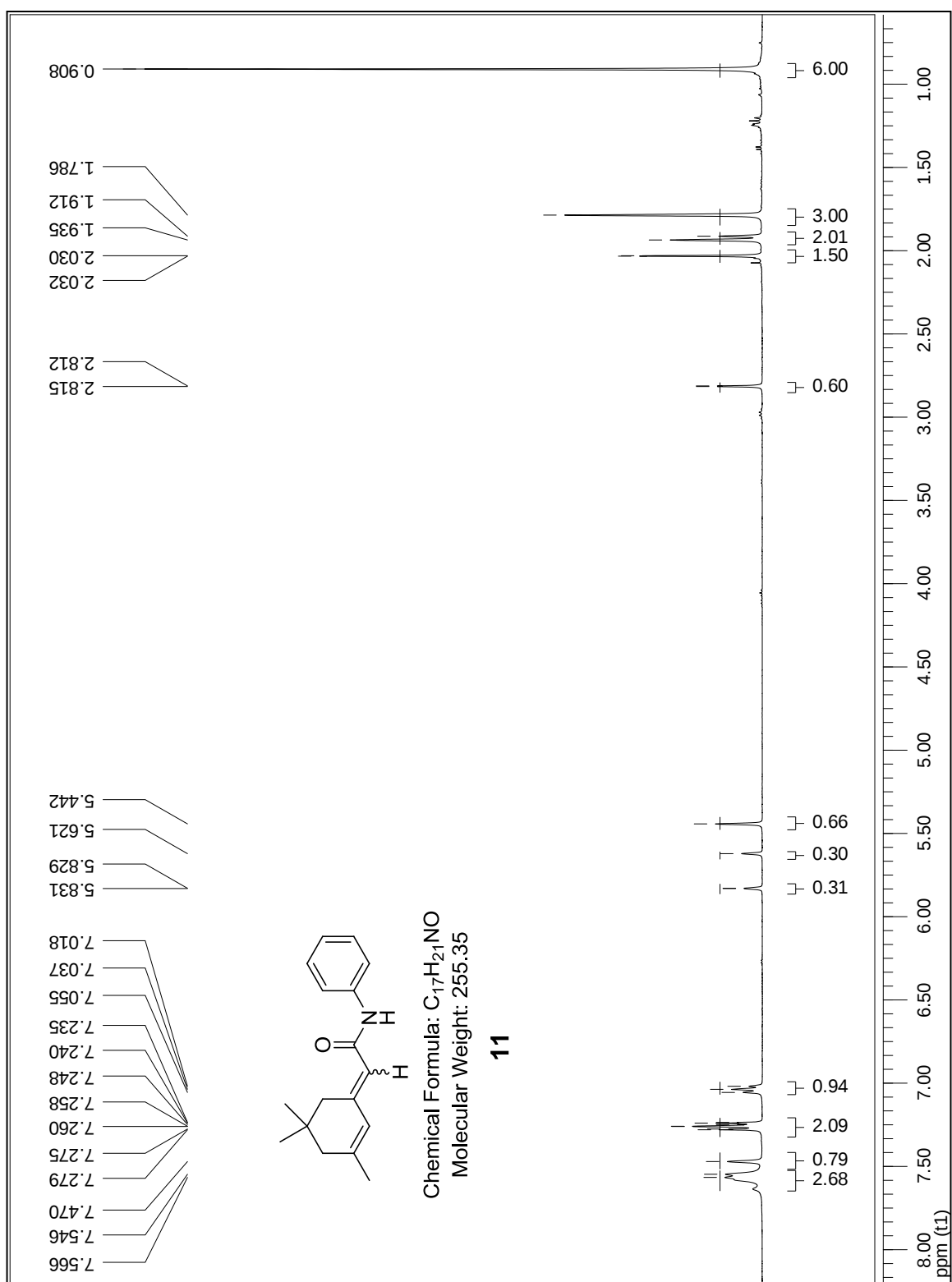


Figure 2.3.2.18. 400 MHz 1H NMR spectrum of compound **11** in $CDCl_3$.

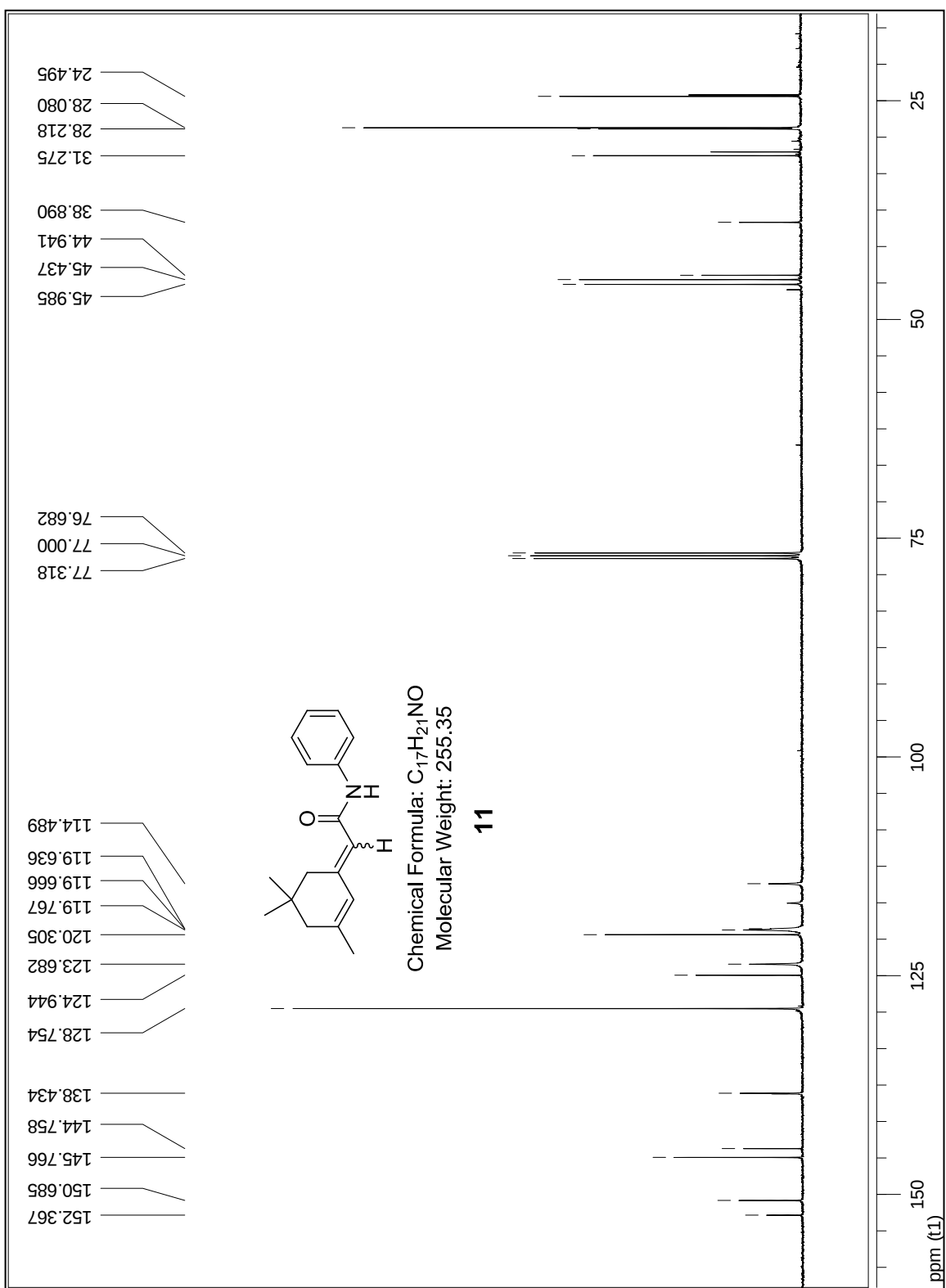
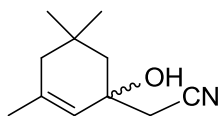


Figure 2.3.2.19. 100 MHz ¹³C NMR spectrum of compound **11** in CDCl₃.

Preparation of 2-(1-hydroxy-3,5,5-trimethylcyclohex-2-en-1-yl)acetonitrile



Chemical Formula: $C_{11}H_{17}NO$

Molecular Weight: 179.26

12

This compound was prepared following general procedure 2 using acetonitrile (0.59 g, 14.48 mmol) in anhydrous THF (10 mL), LDA (7.24 mL, 14.48 mmol) and isophorone (1 g, 7.24 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (45-50% EtOAc in hexanes) yielding **12** as a colorless oil (0.65 g, 50%, $R_f=0.29$ in 1:4 EtOAc : hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 5.44 (s, 1H), 2.51 (d, $J = 1.5$ Hz, 2H), 1.87 (d, $J = 10.4$ Hz, 1H), 1.83 – 1.75 (m, 2H), 1.73 (s, 3H), 1.57 (d, $J = 14.0$ Hz, 2H), 1.02 (d, $J = 19.0$ Hz, 6H).

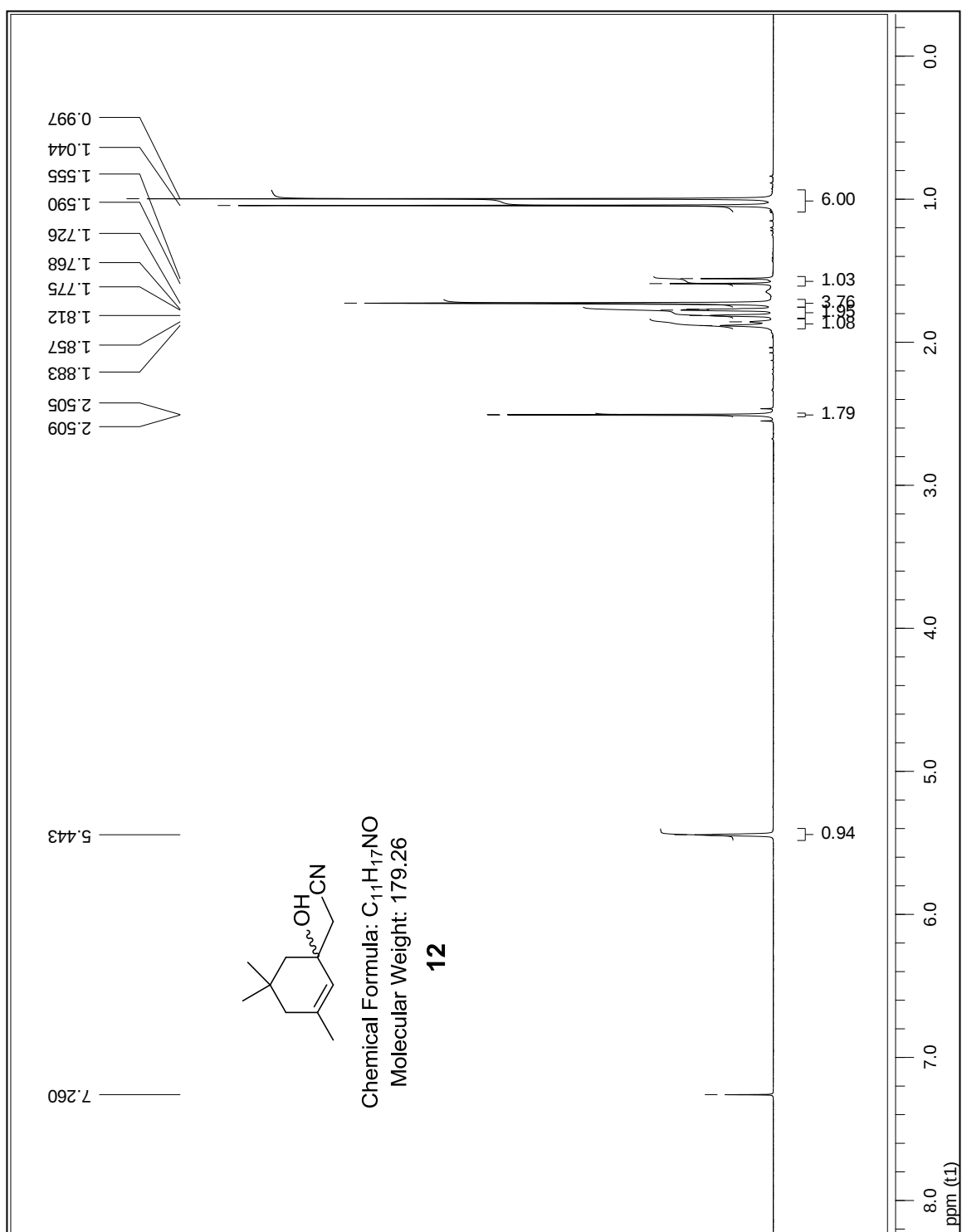
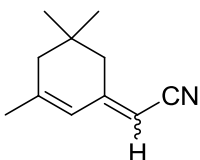


Figure 2.3.2.20. 400 MHz ¹H NMR spectrum of compound **12** in CDCl₃.

Preparation of 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetonitrile



Chemical Formula: C₁₁H₁₅N
Molecular Weight: 161.24

13

This compound was prepared using general procedure 3 using compound **12** (0.62 g, 3.46 mmol), in toluene (5 mL) containing a catalytic amount of PTSA. The crude product was purified by gradient column chromatography (20% EtOAc in hexanes) affording **13** as a pale yellow oil (0.5 g, 89%, R_f=0.7 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 6.39 – 6.33 (m), 5.92 (d, *J* = 1.4 Hz), 4.90 (s), 4.78 (s), 2.27 (d, *J* = 1.5 Hz), 2.05 (d, *J* = 1.5 Hz), 1.92 (s), 1.78 (d, *J* = 16.0 Hz), 0.87 (d, *J* = 11.8 Hz).

HRMS: Calculated for C₁₁H₁₅N = 161.12045; found = 161.12054

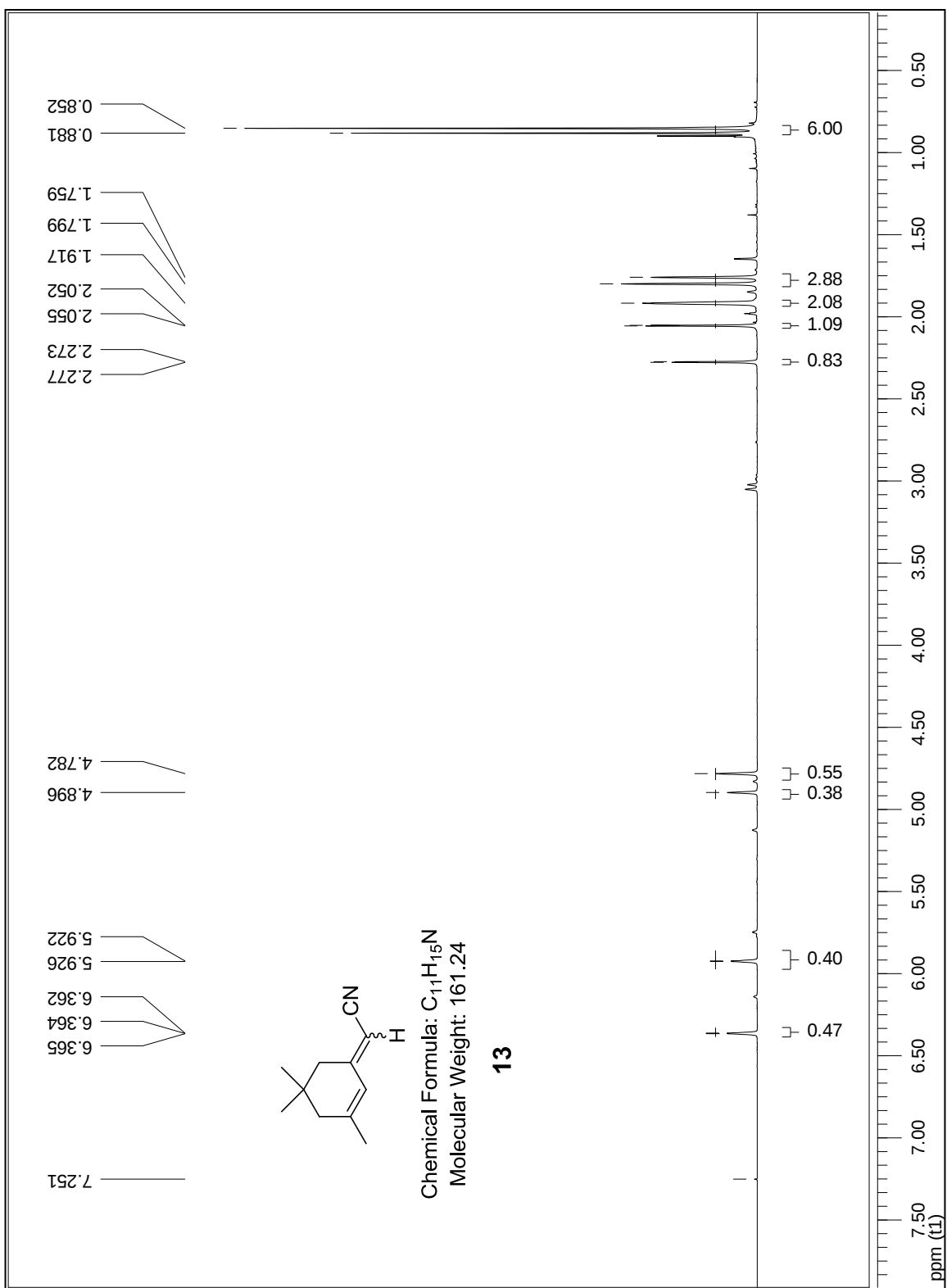
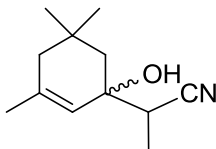


Figure 2.3.2.21. 400 MHz 1H NMR spectrum of compound **13** in $CDCl_3$.

Preparation of 2-(1-hydroxy-3,5,5-trimethylcyclohex-2-en-1-yl)propanenitrile

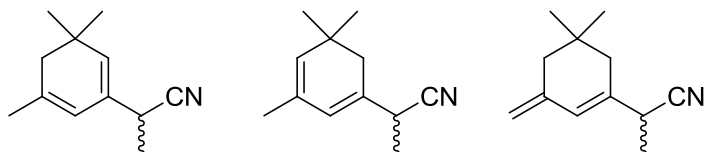


Chemical Formula: C₁₂H₁₉NO
Molecular Weight: 193.29

14

This compound was prepared following general procedure 2 using propionitrile (3.98 g, 72.35 mmol) in anhydrous THF (10 mL), LDA (36.2 mL, 72.35 mmol) and isophorone (5 g, 36.17 mmol) in anhydrous THF (5 mL). The crude product (6.50 g) was purified via gradient column chromatography (35-40% EtOAc in hexanes) yielding **14** as a colorless oil (5.53 g, 78%, R_f=0.33 in 1:4 EtOAc : hexanes).

Preparation of 2-(3,3,5-trimethylcyclohexa-dien-1-yl)propanenitrile



Chemical Formula: C₁₂H₁₇N
Molecular Weight: 175.27

15

This compound was prepared using general procedure 3 using compound **14** (5.53 g), in toluene (15mL) containing a catalytic amount of PTSA. The crude product was purified by gradient column chromatography (20% EtOAc in hexanes) affording **15** as a pale yellow oil (5.20 g, 94%, $R_f = 0.81$ in 3:2 Hexanes : EtOAc).

¹H NMR (400 MHz, CDCl₃) δ 6.46 – 6.37 (m), 6.21 – 6.13 (m), 5.84 – 5.74 (m), 5.25 – 5.15 (m), 4.88 (d, $J = 19.2$ Hz), 3.27 (dd, $J = 13.3, 6.8$ Hz), 2.33 (d, $J = 1.4$ Hz), 2.11 – 1.65 (m), 1.41 (dd, $J = 7.2, 0.7$ Hz), 0.96 (s), 0.92 (s).

¹³C NMR (100 MHz, CDCl₃) δ 151.3, 150.0, 145.9, 144.1, 121.7, 118.4, 99.3, 45.2, 44.7, 43.2, 39.4, 31.2, 31.0, 28.2, 27.9, 24.6, 24.2, 15.5, 14.8.

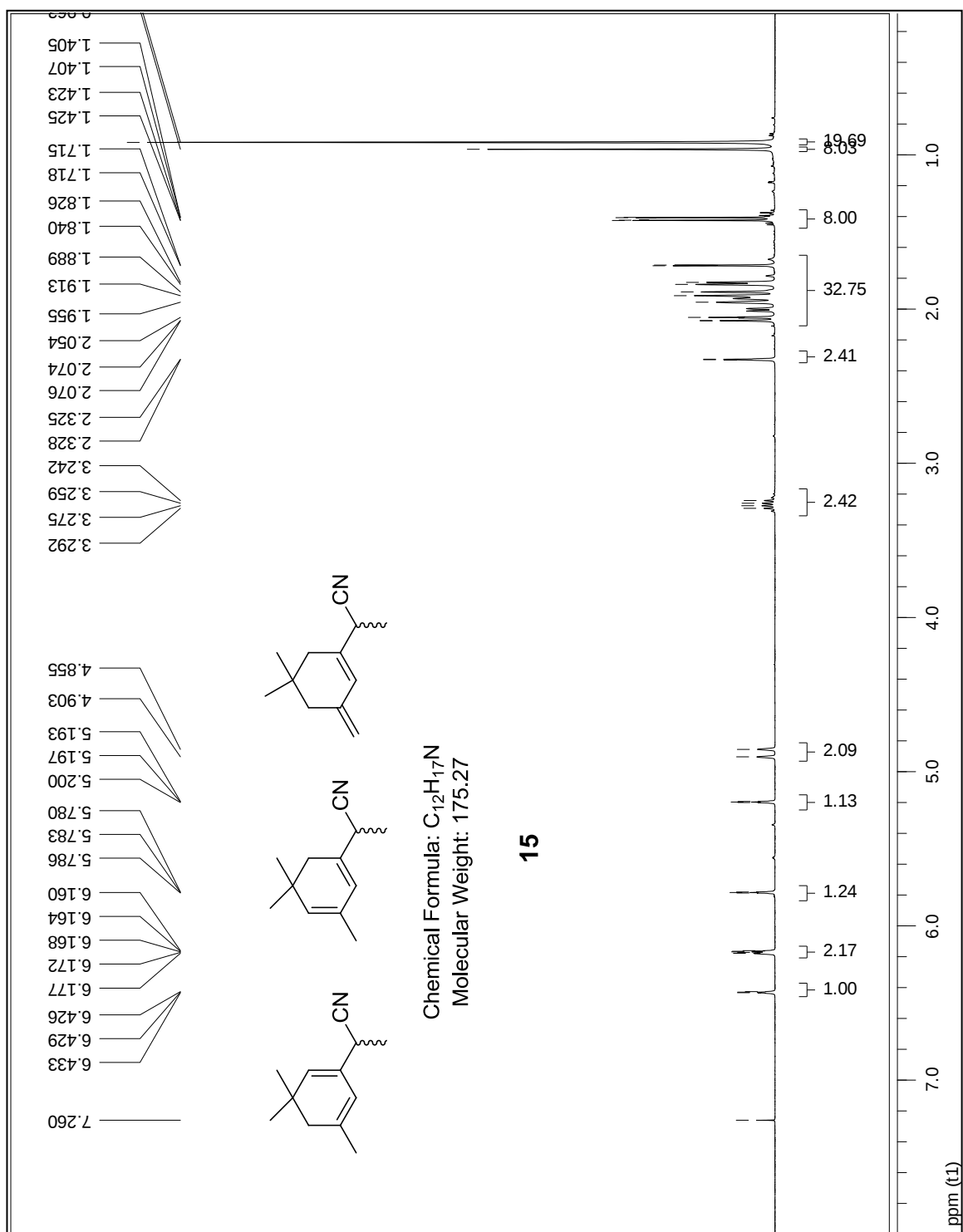


Figure 2.3.2.22. 400 MHz 1H NMR spectrum of compound **15** in $CDCl_3$.

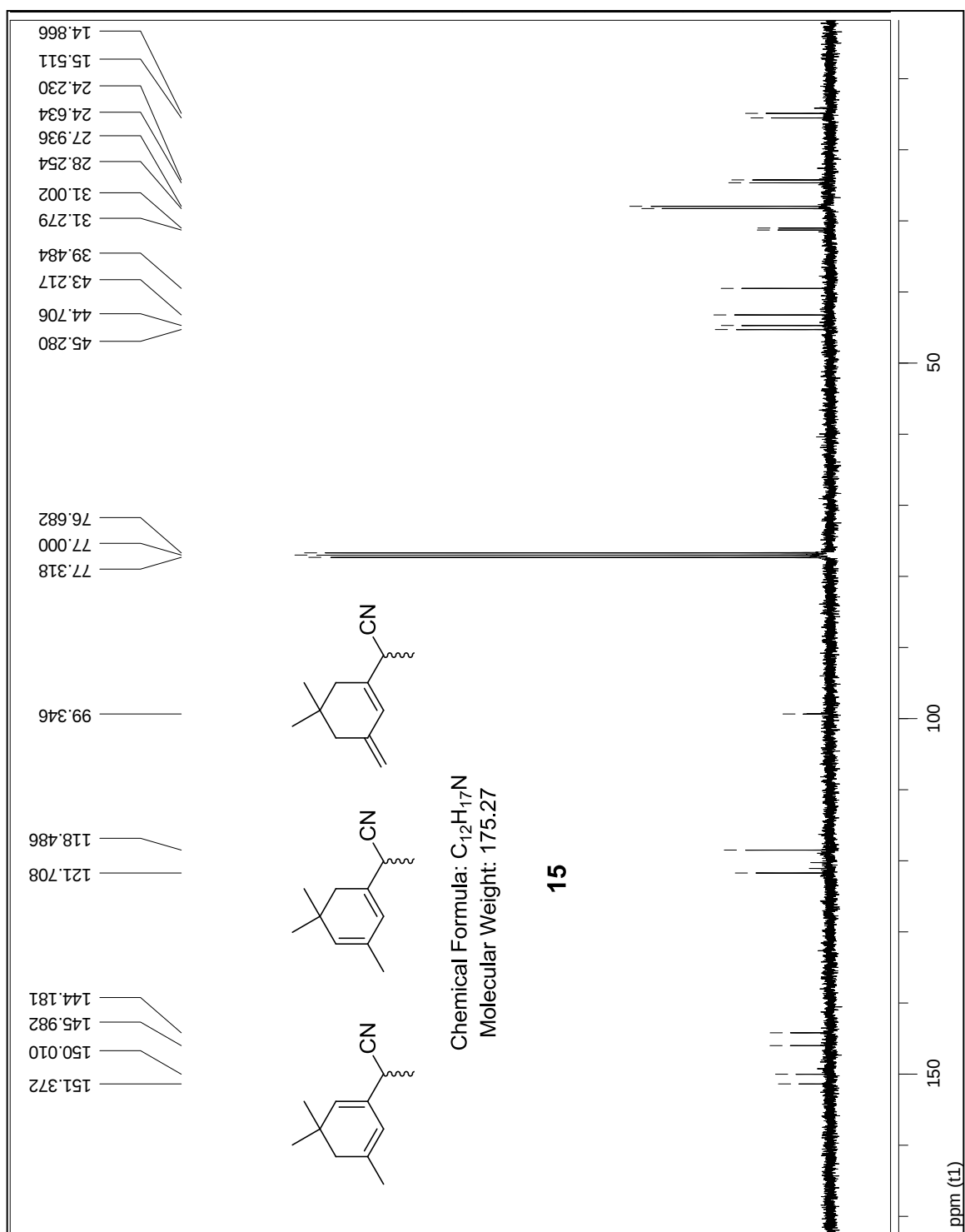
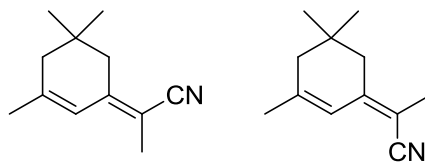


Figure 2.3.2.23. 100 MHz ^{13}C NMR spectrum of compound **15** in $CDCl_3$.

Preparation of 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)propanenitrile



Chemical Formula: $C_{12}H_{17}N$
Molecular Weight: 175.27

16

This compound was prepared following general procedure 4 using compound **15** (5.53g, 31.55mmol) and a catalytic amount of PTSA in refluxing toluene. Purification via gradient column chromatography afforded **16** as a pale yellow oil (5.20 g, 94%, R_f = 0.81 in 3:2 Hexanes : EtOAc) This material solidified when stored in a freezer at $-20^{\circ}C$.

1H NMR (400 MHz, $CDCl_3$) δ 6.45 (d, J = 1.4 Hz), 6.18 (dd, J = 3.0, 1.5 Hz), 2.34 (d, J = 1.4 Hz), 2.09 (d, J = 0.7 Hz), 1.98 – 1.89 (m), 1.84 (d, J = 5.1 Hz), 0.93 (s).

^{13}C NMR (100 MHz, $CDCl_3$) δ 151.2, 149.9, 145.9, 144.0, 121.7, 121.0, 118.4, 99.3, 99.3, 45.2, 44.6, 43.2, 39.4, 31.2, 30.9, 28.2, 27.9, 24.6, 24.2, 15.5, 14.8.

HRMS: Calculated for $C_{12}H_{17}N$ = 175.13610; found = 175.13623

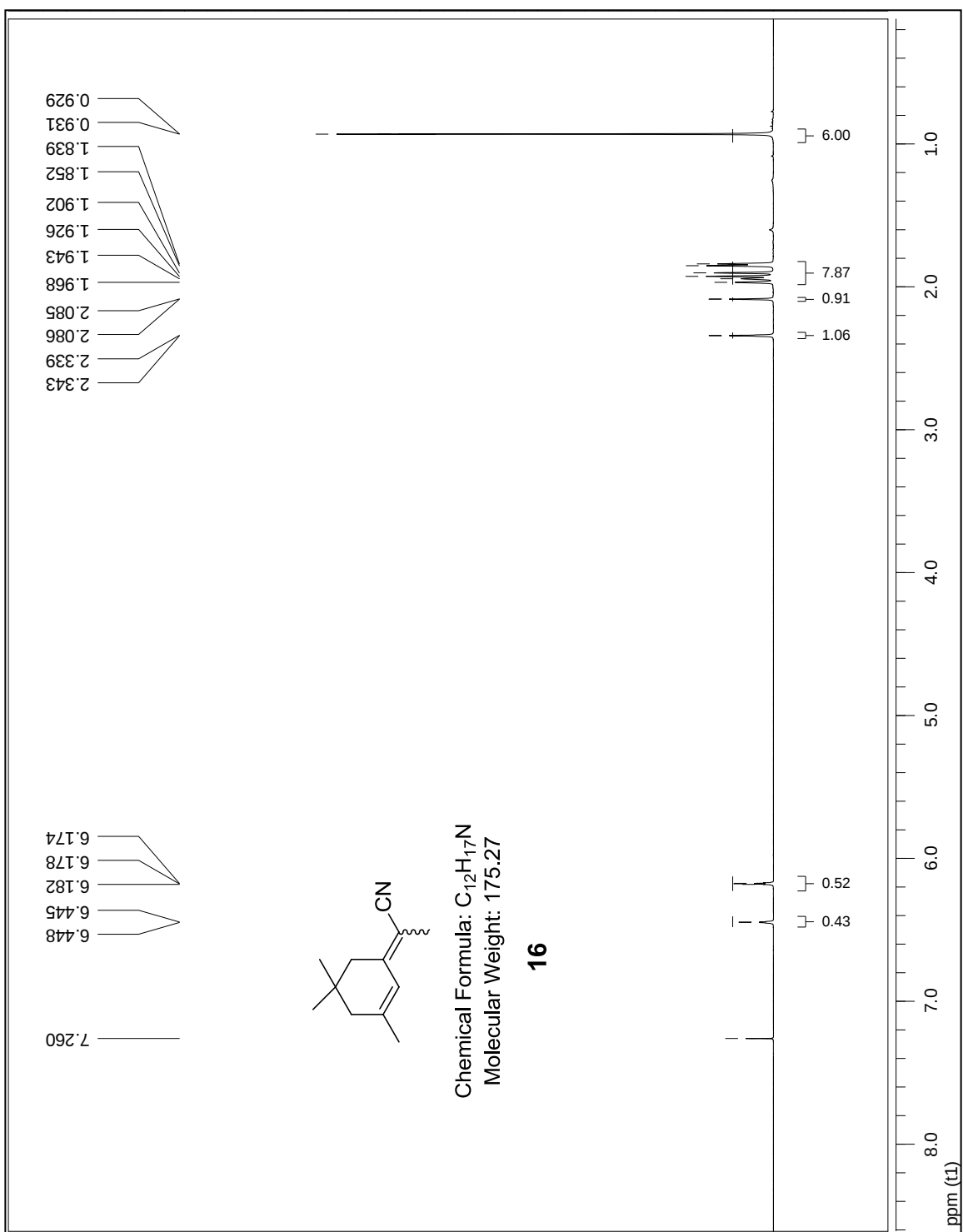


Figure 2.3.2.24. 400 MHz ¹H NMR spectrum of compound **16** in CDCl₃.

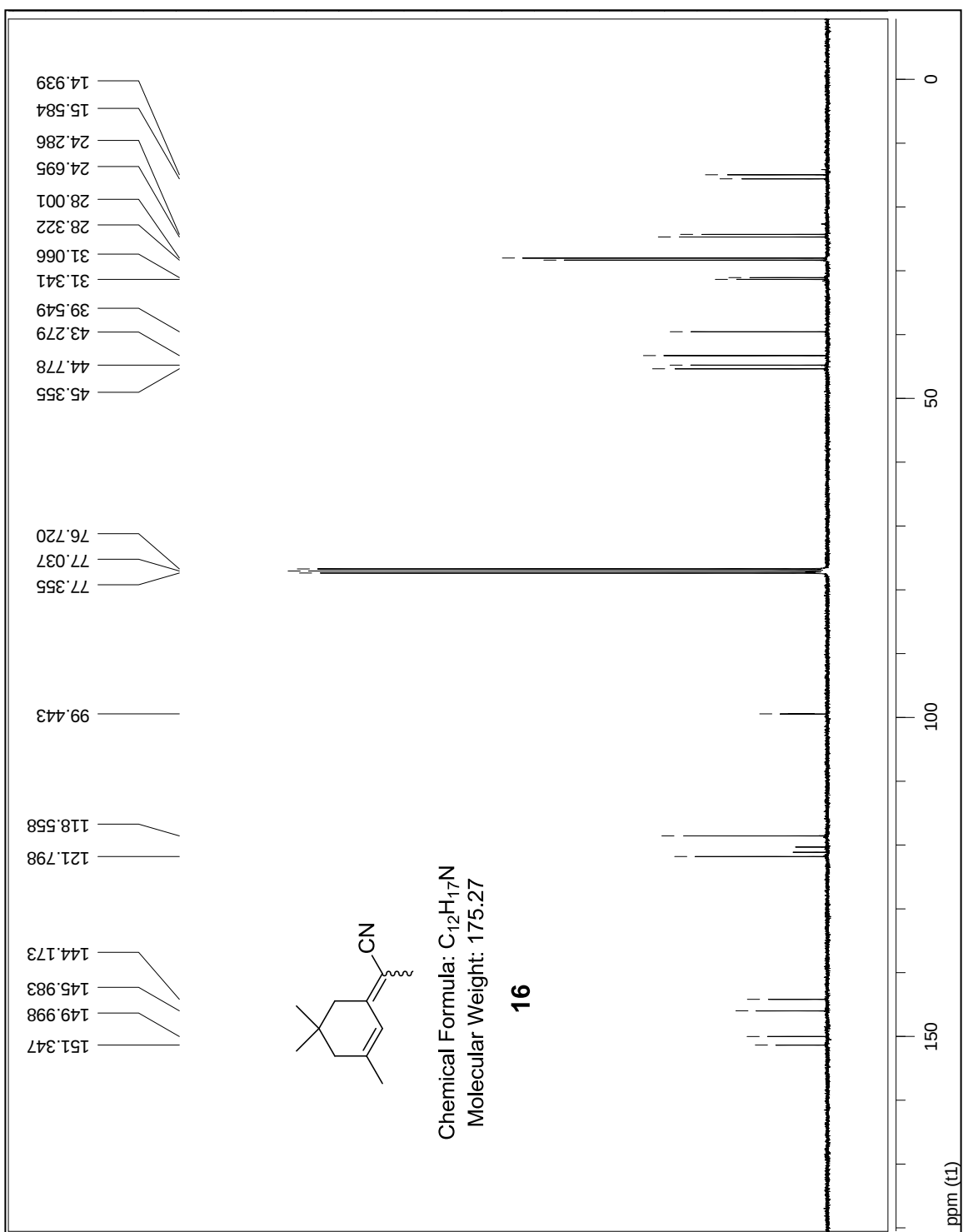
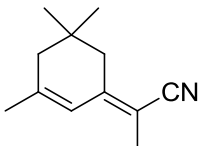


Figure 2.3.2.25. 100 MHz ¹³C NMR spectrum of compound **16** in CDCl₃.

Preparation of 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)propanenitrile



Chemical Formula: C₁₂H₁₇N
Molecular Weight: 175.27008

16-E

The 5:4 E:Z mixture **16** was recrystallized two consecutive times from hexanes at room temperature to yield 91% pure E isomer **16-E**.

¹H NMR (400 MHz, CDCl₃) δ 6.16 (dd, *J* = 2.9, 1.4 Hz, 1H), 2.33 (d, *J* = 1.3 Hz, 2H), 1.95 (s, 2H), 1.91 (s, 3H), 1.84 (s, 3H), 0.92 (s, 6H).

¹³C NMR (100 MHz, CDCl₃) δ 149.9, 145.9, 121.0, 118.5, 99.4, 45.3, 43.2, 31.3, 27.9, 24.6, 14.9.

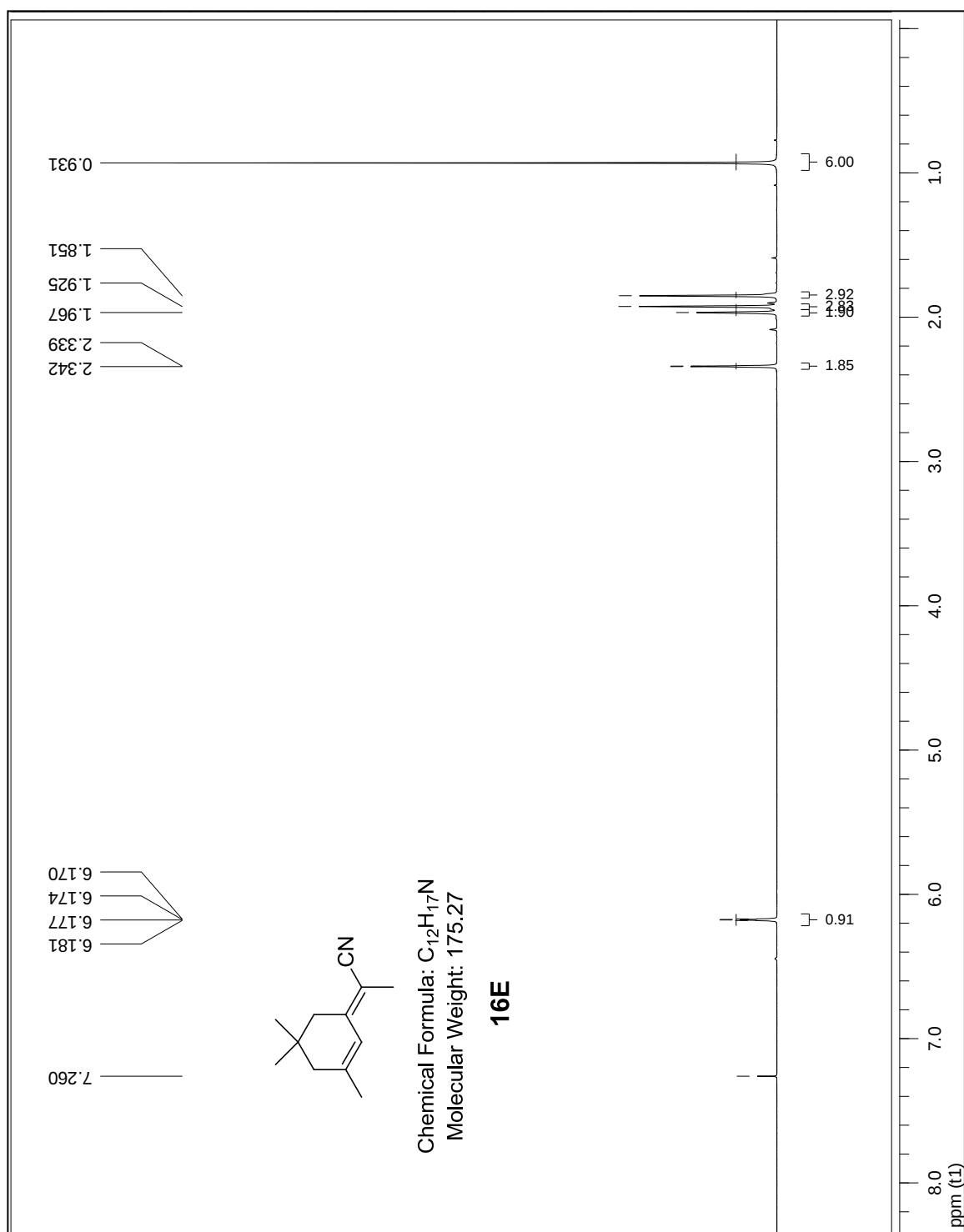


Figure 2.3.2.26. 400 MHz ¹H NMR spectrum of compound **16E** in CDCl₃.

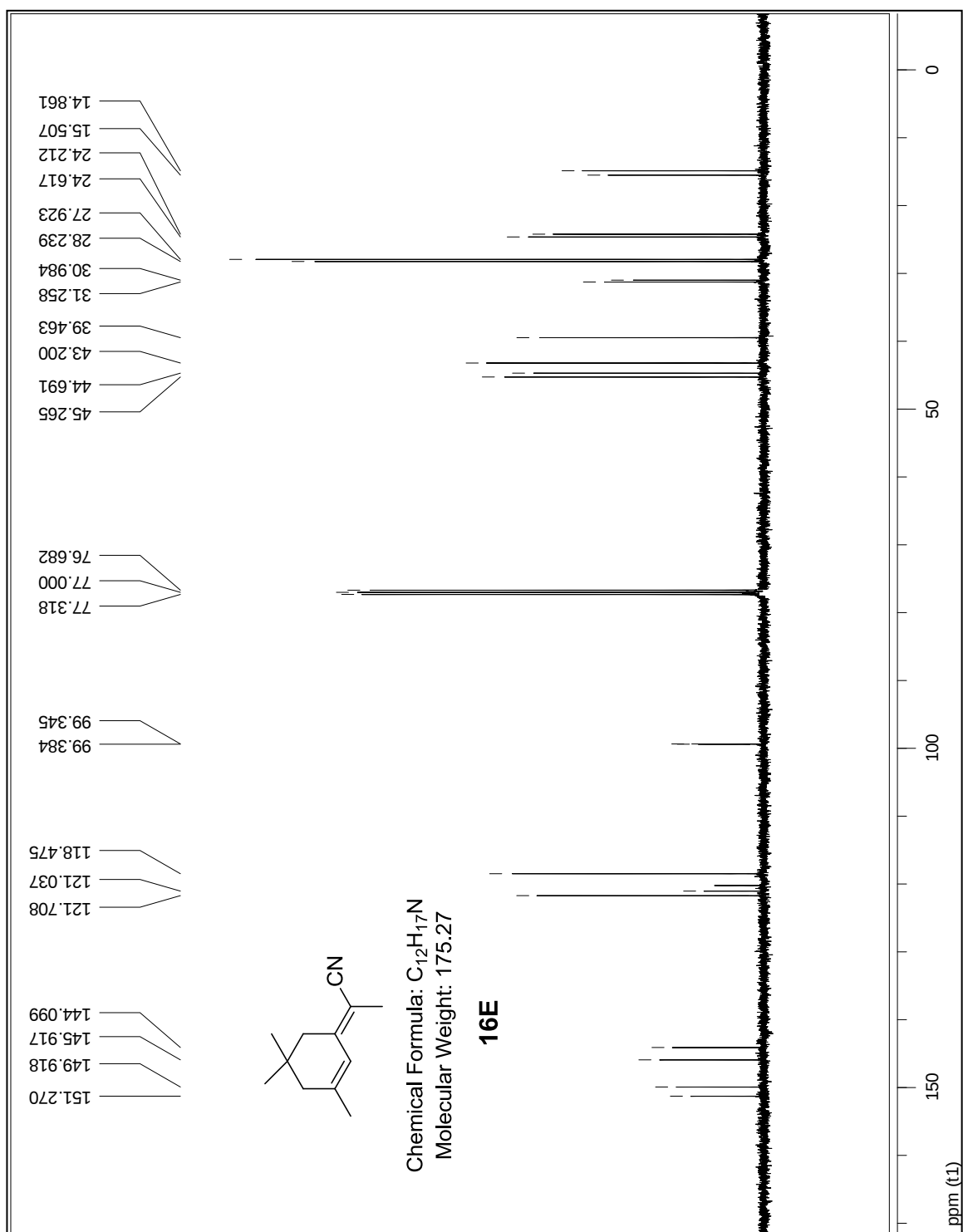
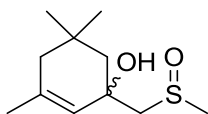


Figure 2.3.2.27. 100 MHz ^{13}C NMR spectrum of compound **16E** in $CDCl_3$.

Preparation of 3,5,5-trimethyl-1-((methylsulfinyl)methyl)cyclohex-2-enol



Chemical Formula: C₁₁H₂₀O₂S

Molecular Weight: 216.34

17

This compound was prepared following general procedure 2 using anhydrous DMSO (2.83 g, 36.2 mmol) in anhydrous THF (10 mL), LDA (18 mL, 36.2 mmol) and isophorone (1 g, 7.24 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (35-40% EtOAc in hexanes) yielding **17** as a yellow oil (0.73 g, 47%).

¹H NMR (400 MHz, CDCl₃) δ 5.33 (d, J = 8.0 Hz, 1H), 2.95 – 2.62 (m, 2H), 2.53 (d, J = 5.4 Hz, 3H), 1.57 (dd, J = 15.7, 13.2 Hz, 7H), 0.86 (dd, J = 24.6, 4.5 Hz, 6H).

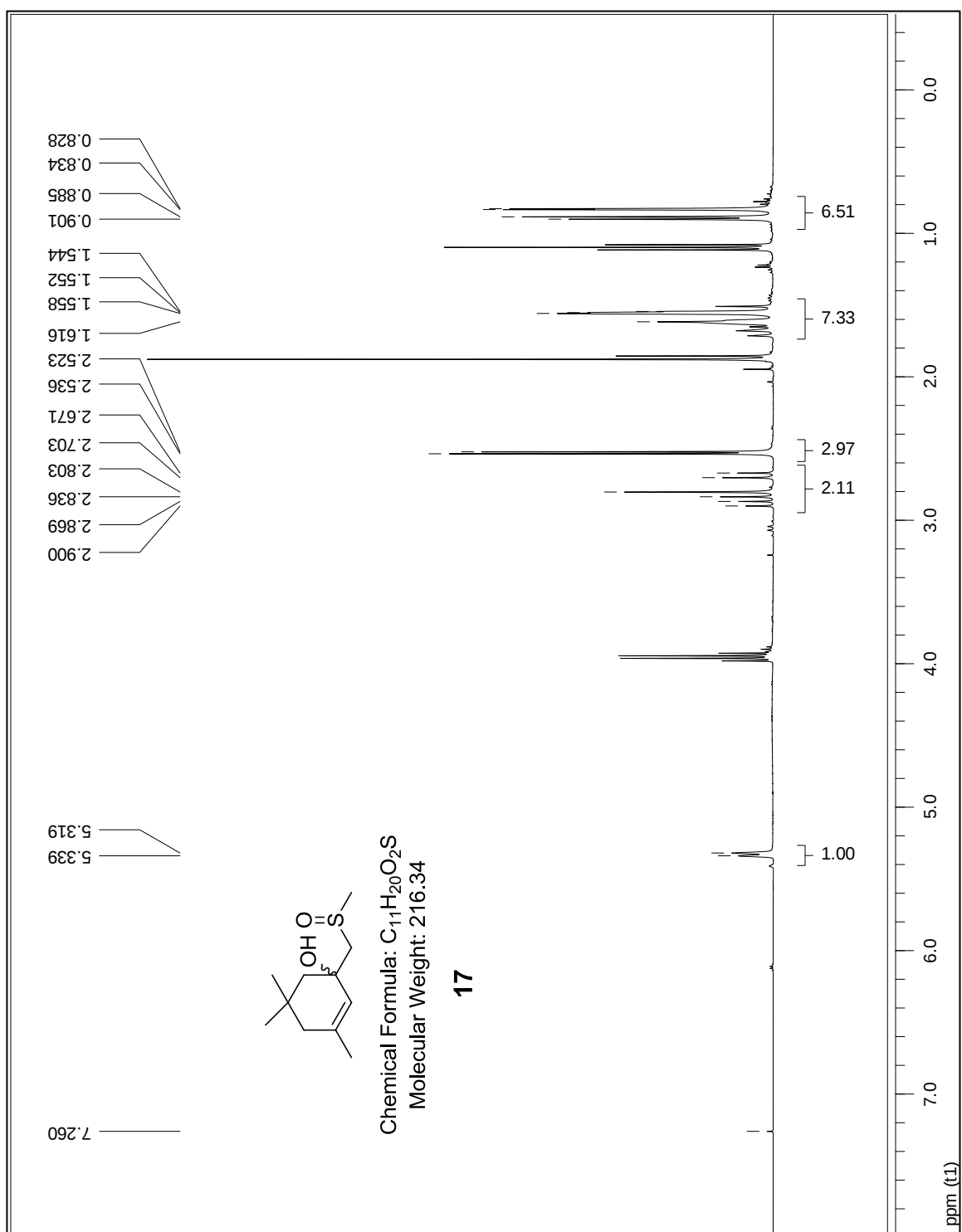
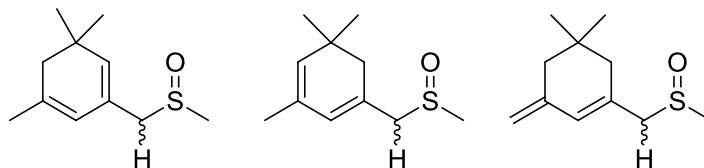


Figure 2.3.2.28. 400 MHz ¹H NMR spectrum of compound **17** in CDCl₃.

Preparation of 1,5,5-trimethyl-3-((methylsulfinyl)methyl)cyclohexa-diene



Chemical Formula: $C_{11}H_{18}OS$

Molecular Weight: 198.33

18

This compound was prepared using general procedure 3 using compound **17** (0.73 g, 3.37 mmol), in toluene (10 mL) containing a catalytic amount of PTSA. The crude product was purified by gradient column chromatography (50-60% EtOAc in hexanes) affording **18** as a pale yellow oil (0.3 g, 45%).

1H NMR (400 MHz, $CDCl_3$) δ 6.06 (s), 5.69 (d, $J = 0.9$ Hz), 5.47 (dt, $J = 2.9, 1.4$ Hz), 5.25 (s), 5.16 – 5.04 (m), 4.76 (d, $J = 21.8$ Hz), 3.46 (t, $J = 12.4$ Hz), 3.33 (t, $J = 13.4$ Hz), 3.22 (d, $J = 12.4$ Hz), 2.52 (d, $J = 2.1$ Hz), 2.48 (t, $J = 4.5$ Hz), 2.41 (s), 1.89 (s), 1.84 (d, $J = 1.1$ Hz), 1.66 (s), 0.84 (dt, $J = 18.9, 9.8$ Hz).

HRMS: Calculated for $C_{11}H_{18}OS$ = 198.10784; found = 198.11005

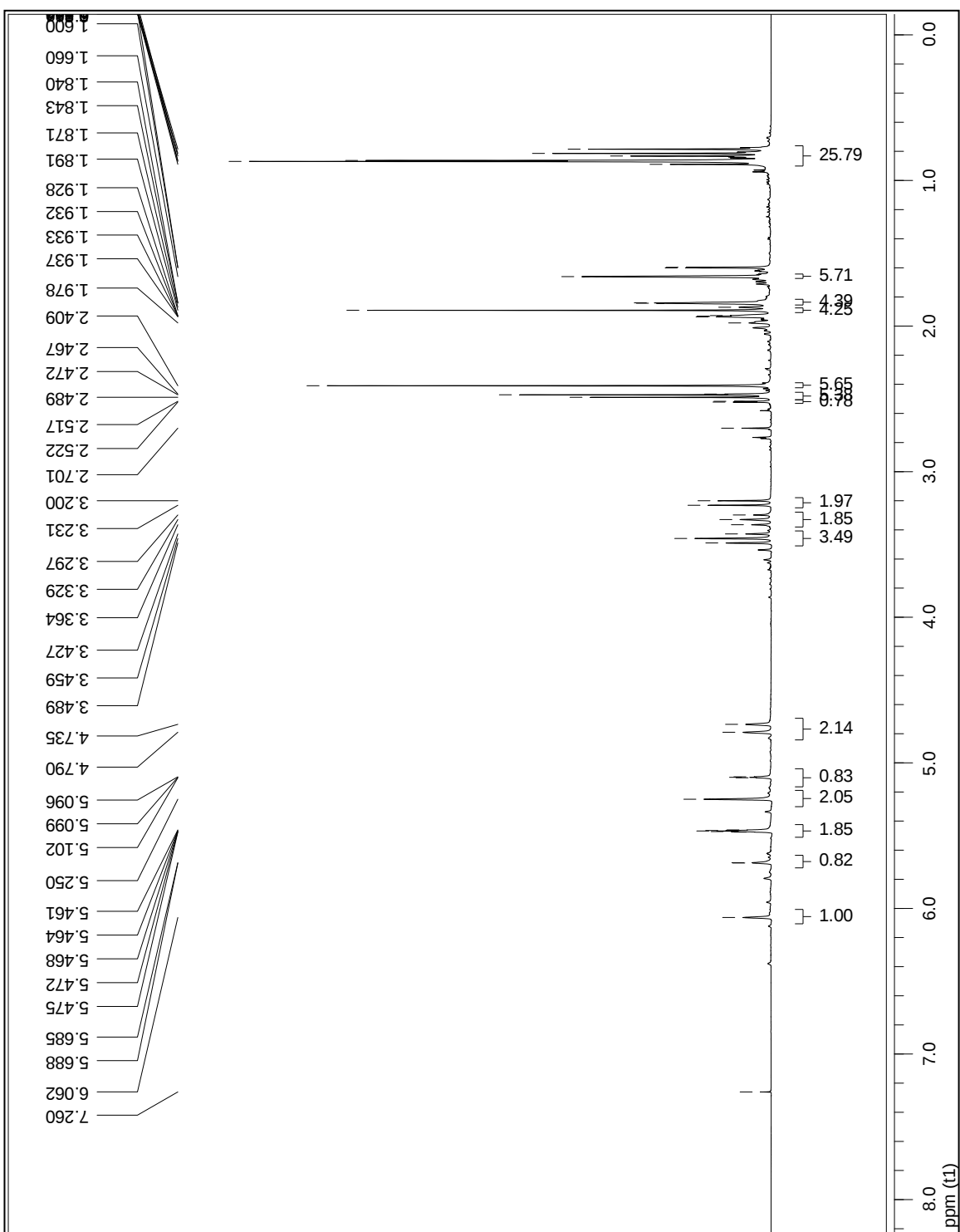
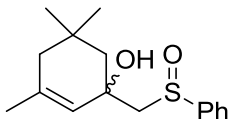


Figure 2.3.2.29. 400 MHz ^1H NMR spectrum of compound **18** in CDCl_3 .

Preparation of 3,5,5-trimethyl-1-((phenylsulfinyl)methyl)cyclohex-2-enol



Chemical Formula: C₁₆H₂₂O₂S

Molecular Weight: 278.41

19

This compound was prepared following general procedure 2 using methyl phenyl sulfoxide (0.7 g, 5 mmol) in anhydrous THF (10 mL), LDA (3 mL, 6 mmol) and isophorone (0.69 g, 5 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (45-50% EtOAc in hexanes) yielding **19** as a white solid (0.42 g, 30%, R_f=0.29 in 2:3 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.64 (d, *J* = 1.4 Hz), 7.51 (dd, *J* = 7.2, 1.2 Hz), 5.71 (s), 5.39 (d, *J* = 1.3 Hz), 3.77 (s), 3.60 (s), 3.03 (dd, *J* = 13.2, 8.2 Hz), 2.84 (d, *J* = 13.3 Hz), 2.76 – 2.65 (m), 2.16 (s), 2.12 – 2.02 (m), 1.96 – 1.57 (m), 1.01 (d, *J* = 36.5 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 144.6, 137.7, 137.3, 131.1, 131.0, 129.3, 129.3, 123.8, 123.8, 123.7, 123.0, 71.4, 69.9, 69.2, 48.9, 47.8, 44.2, 44.2, 31.2, 30.4, 30.3, 28.2, 27.4, 24.0, 23.9.

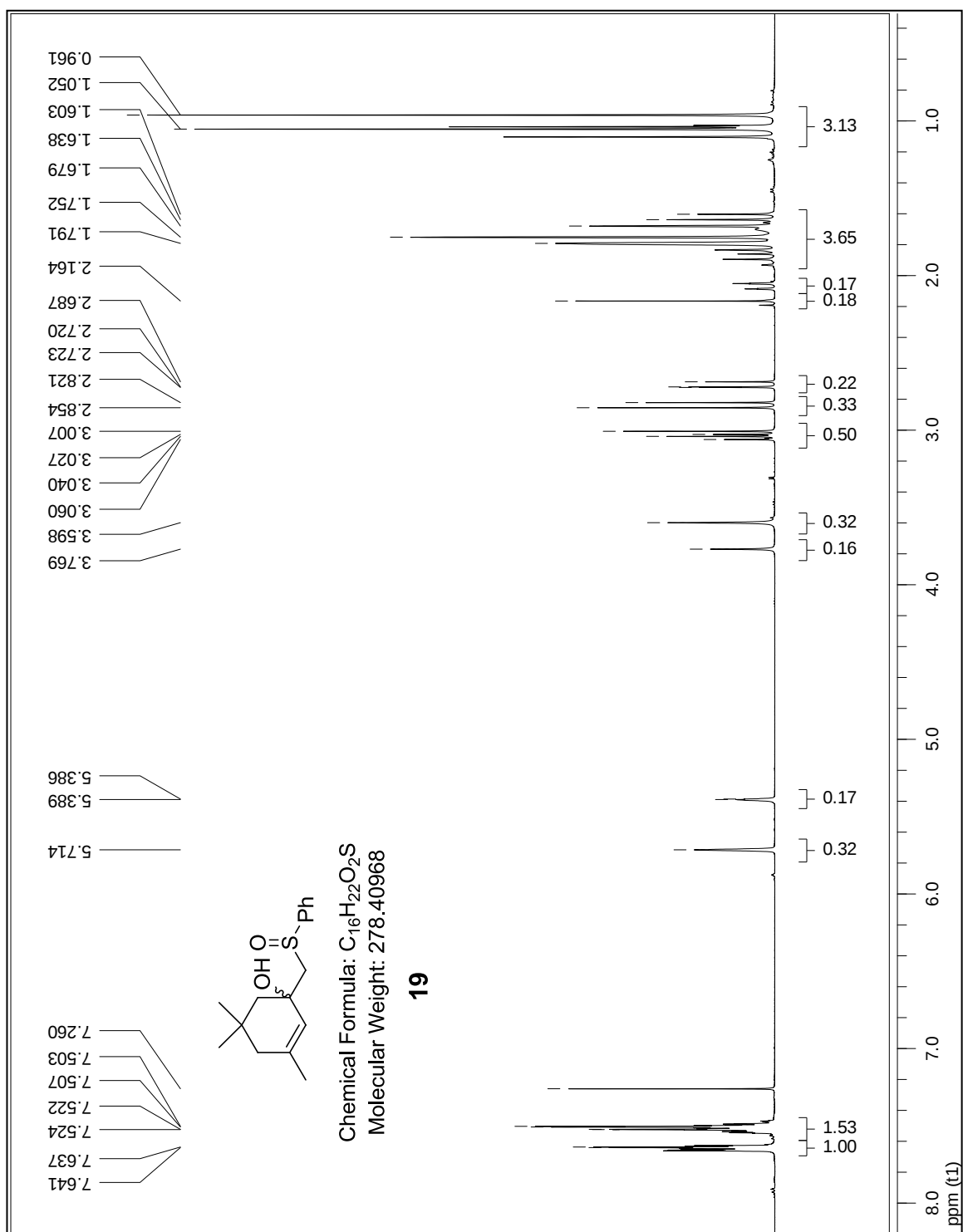


Figure 2.3.2.30. 400 MHz ¹H NMR spectrum of compound **19** in CDCl₃.

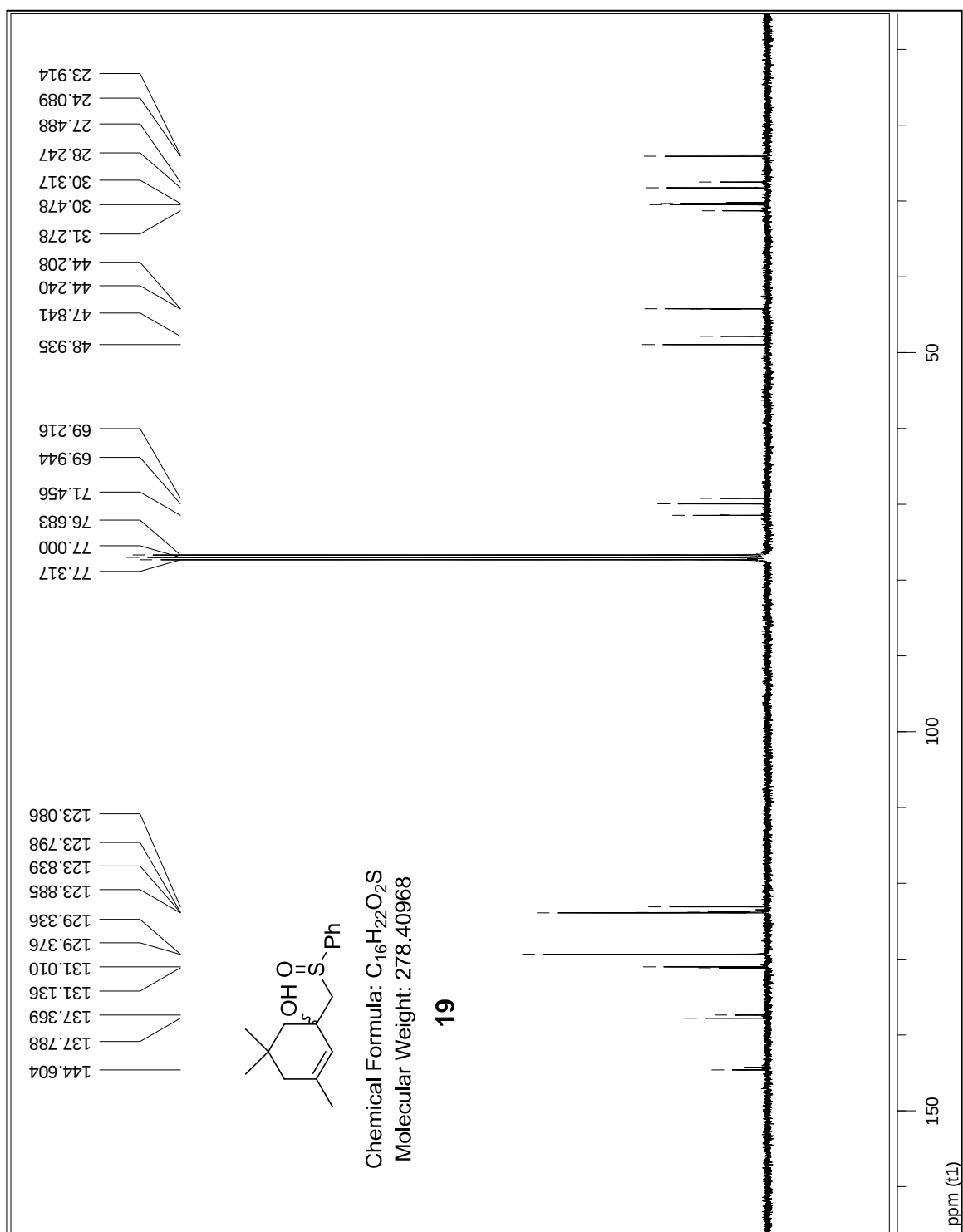
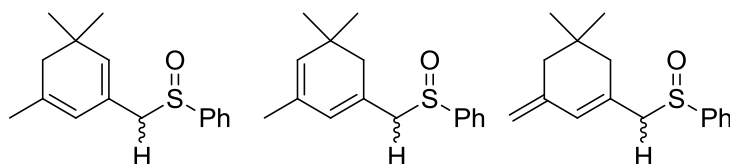


Figure 2.3.2.31. 100 MHz ^{13}C NMR spectrum of compound **19** in $CDCl_3$.

Preparation of (((3,3,5-trimethylcyclohexa-dien-1-yl)methyl)sulfinyl)benzene



Chemical Formula: C₁₆H₂₀OS

Molecular Weight: 260.39

20

This compound was prepared using general procedure 3 using compound **19** (0.2 g, 0.71 mmol), in toluene (5 mL) containing a catalytic amount of PTSA. The crude product was purified by gradient column chromatography (20-25% EtOAc in hexanes) affording **20** as a yellow oil (0.09 g, 49%, R_f=0.45 in 2:3 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.62 – 7.56 (m), 7.56 – 7.51 (m), 7.51 – 7.41 (m), 5.88 (s), 5.51 (d, *J* = 0.8 Hz), 5.45 – 5.40 (m), 5.19 – 5.15 (m), 4.93 (s), 4.77 (s), 3.58 (dd, *J* = 12.3, 0.8 Hz), 3.32 (d, *J* = 12.3 Hz), 2.08 (d, *J* = 2.4 Hz), 1.98 (s), 1.92 (d, *J* = 5.1 Hz), 1.85 (s), 1.70 (s), 1.62 (d, *J* = 1.5 Hz), 0.95 (s), 0.87 (d, *J* = 1.7 Hz), 0.85 (s), 0.82 (s).

¹³C NMR (100 MHz, CDCl₃) δ 143.1, 141.8, 136.7, 136.1, 132.5, 131.1, 130.9, 130.8, 130.8, 128.9, 128.6, 124.5, 119.4, 113.2, 67.2, 66.9, 64.1, 43.6, 43.4, 43.0, 42.1, 32.0, 30.4, 27.7, 27.6, 23.2, 20.8.

PURE ISOMER 20A or 20B:

¹H NMR (400 MHz, CDCl₃) δ 7.60 – 7.52 (m, 2H), 7.47 (dd, *J* = 6.2, 2.7 Hz, 3H), 5.44 (dt, *J* = 2.9, 1.4 Hz, 1H), 4.95 (s, 1H), 3.61 (dd, *J* = 12.3, 0.9 Hz, 1H), 3.34 (d, *J* = 12.3 Hz, 1H), 1.87 (s, 2H), 1.72 (s, 3H), 0.86 (d, *J* = 13.8 Hz, 6H).

¹³C NMR (100 MHz, CDCl₃) δ 143.2, 136.8, 136.3, 130.9, 128.7, 124.6, 123.4, 119.5, 64.2, 43.1, 32.1, 27.7, 27.6, 23.3.

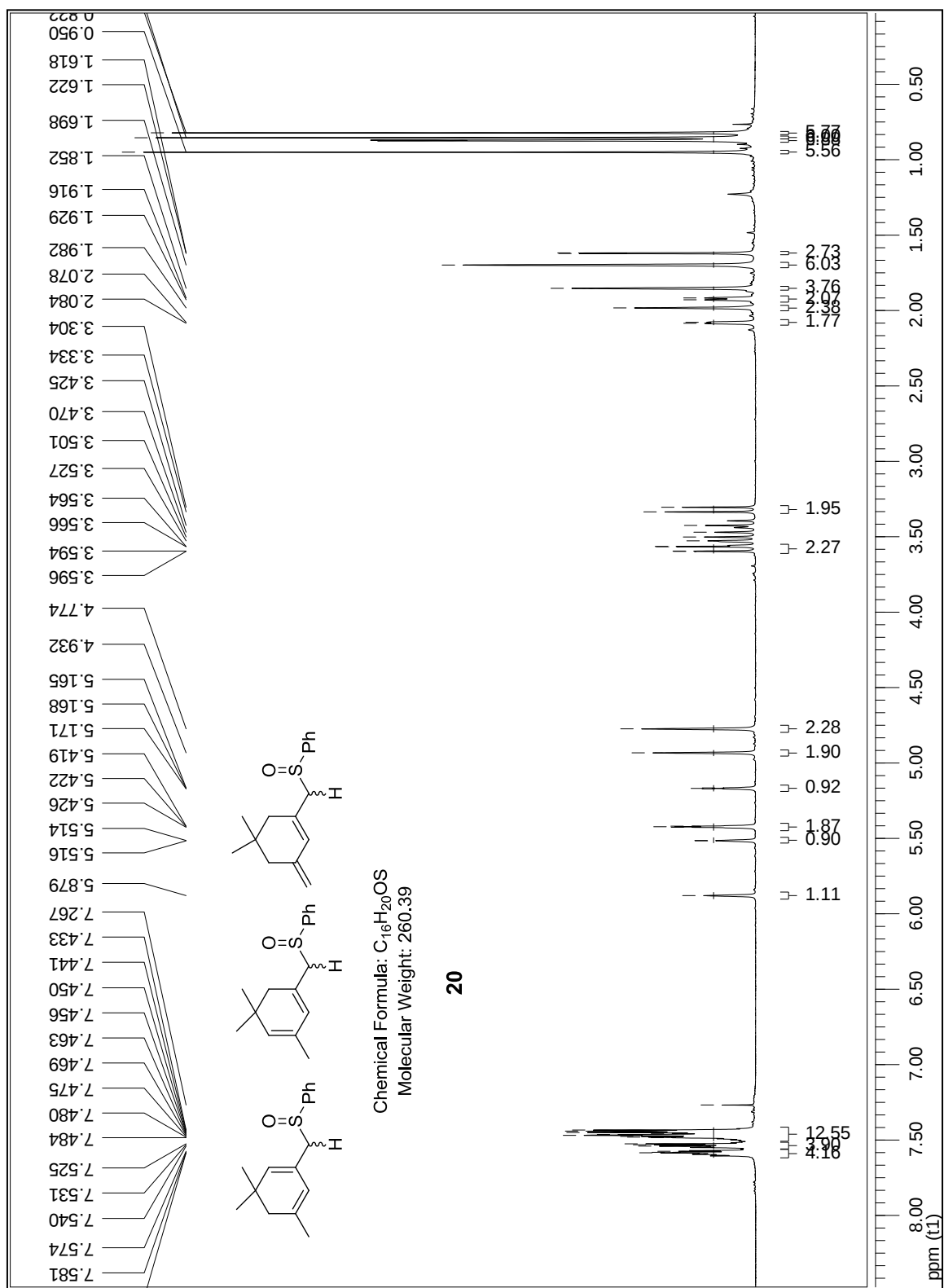


Figure 2.3.2.32. 400 MHz ¹H NMR spectrum of compound **20** in CDCl₃.

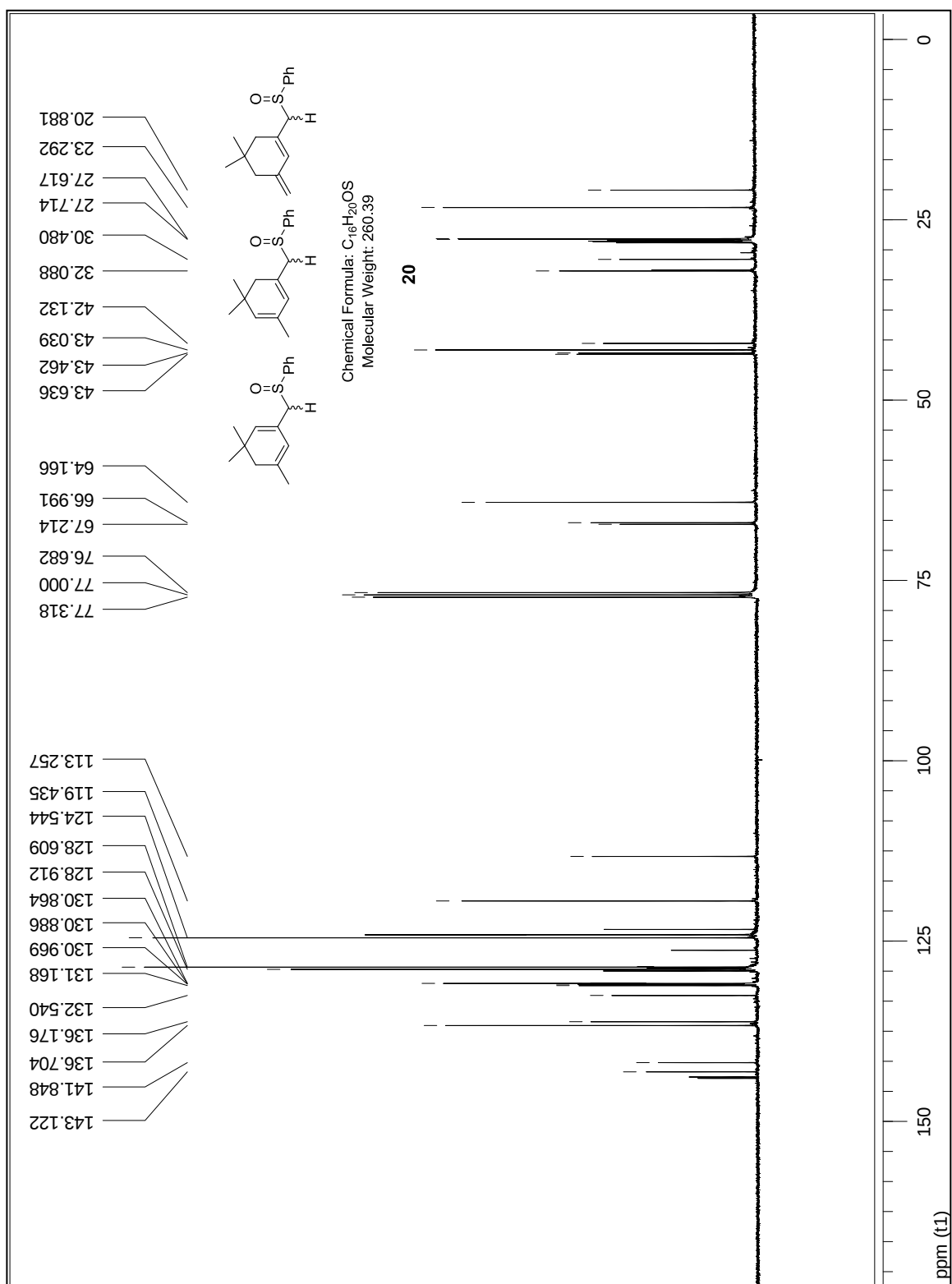


Figure 2.3.2.33. 100 MHz ¹³C NMR spectrum of compound **20** in CDCl₃.

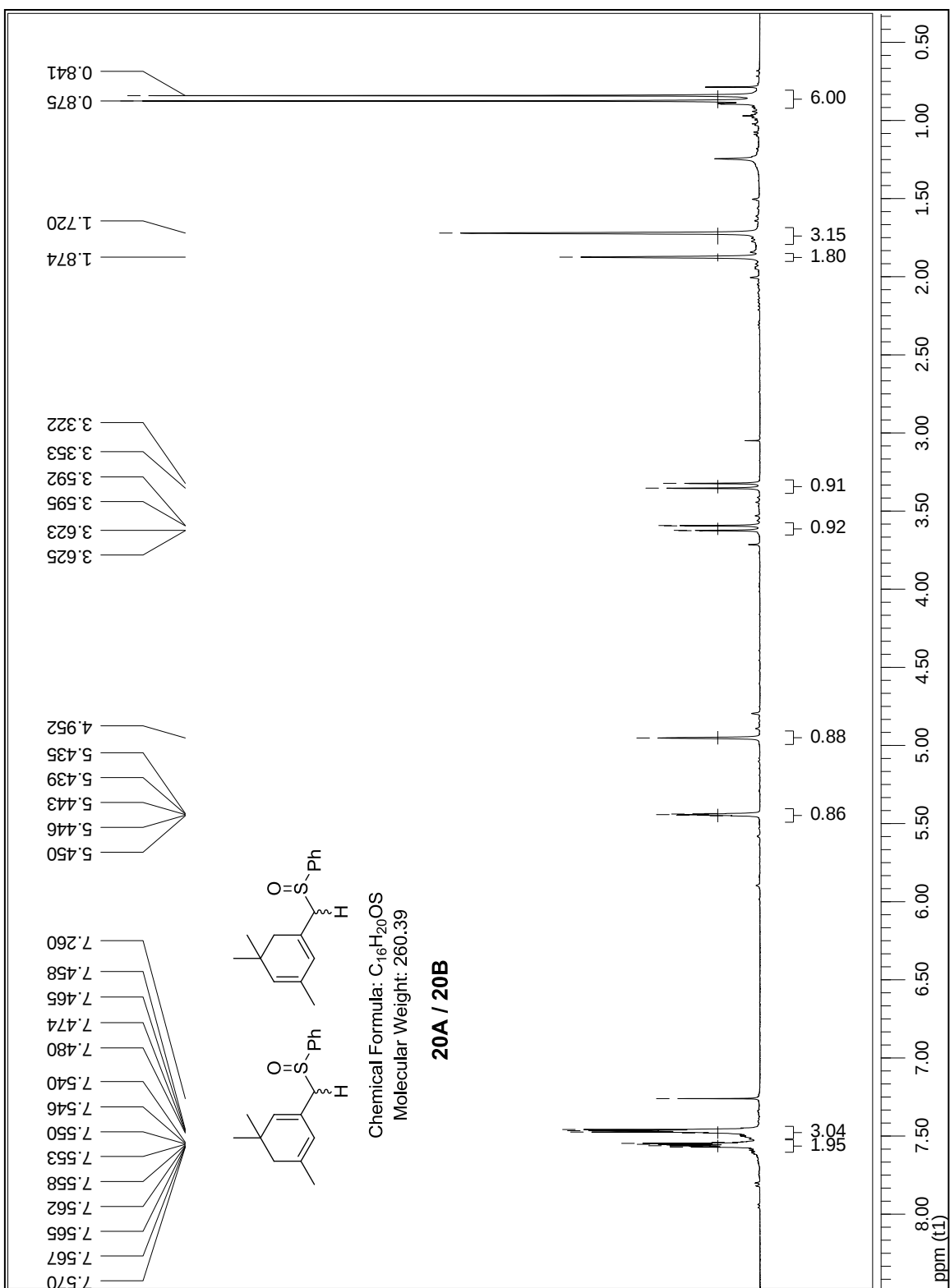


Figure 2.3.2.34. 400 MHz 1H NMR spectrum of compound **20A** or **20B** in $CDCl_3$.

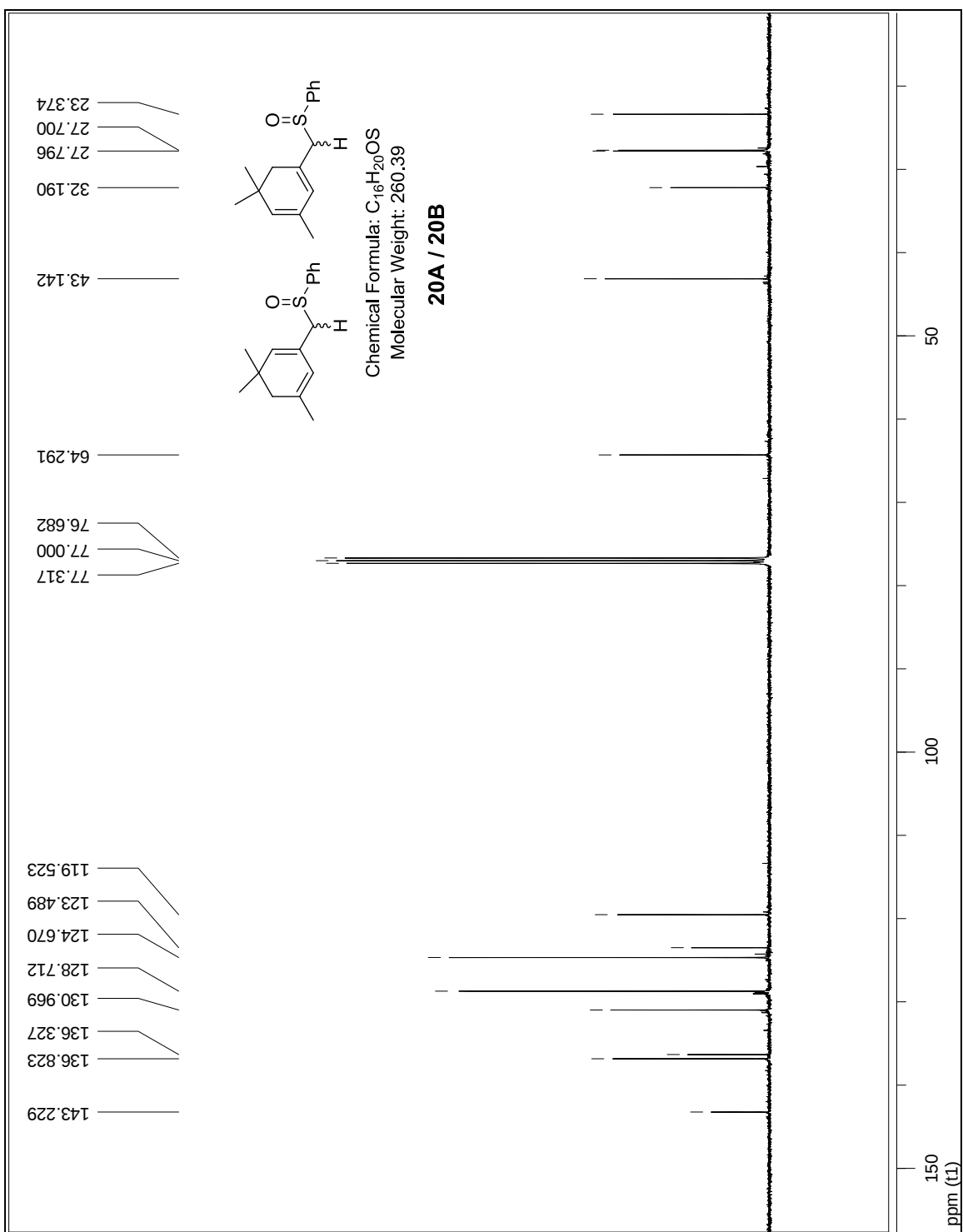
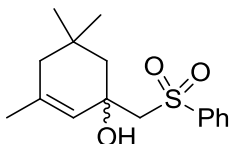


Figure 2.3.2.35. 100 MHz ¹³C NMR spectrum of compound **20A** or **20B** in CDCl₃.

Preparation of 3,5,5-trimethyl-1-((phenylsulfonyl)methyl)cyclohex-2-enol



Chemical Formula: C₁₆H₂₂O₃S

Molecular Weight: 294.41

21

This compound was prepared following general procedure 2 using methyl phenyl sulfone (2.3 g, 14.48 mmol) in anhydrous THF (10 mL), LDA (7.24 mL, 14.48 mmol) and isophorone (1 g, 7.24 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (45-50% EtOAc in hexanes) yielding **21** as a viscous oil (0.27 g, 13%, R_f=0.42 in 2:3 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.92 – 7.81 (m), 7.60 (d, *J* = 7.5 Hz), 7.52 (t, *J* = 7.6 Hz), 5.36 (s), 3.55 (s), 3.28 (d, *J* = 2.6 Hz), 1.99 (s), 1.83 (s), 1.73 (s), 1.72 – 1.67 (m), 1.61 (s), 0.96 (d, *J* = 30.6 Hz).

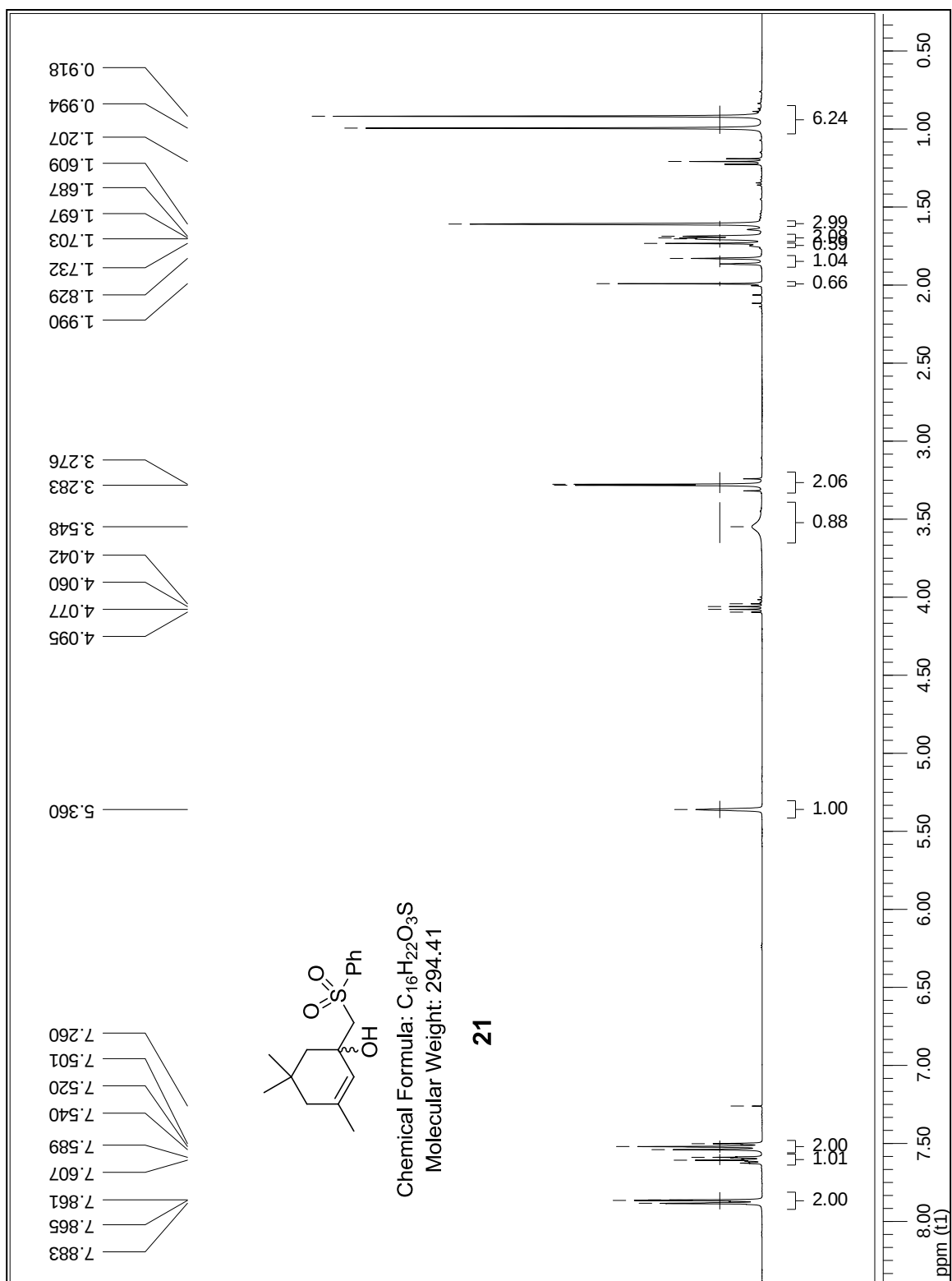
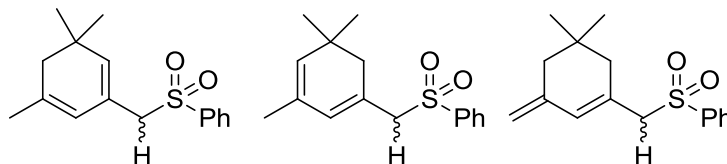


Figure 2.3.2.36. 400 MHz 1H NMR spectrum of compound **21** in $CDCl_3$.

Preparation of (((3,3,5-trimethylcyclohexa-dien-1-yl)methyl)sulfonyl)benzene



Chemical Formula: C₁₆H₂₀O₂S

Molecular Weight: 276.39

22

This compound was prepared using general procedure 3 using the coupled product (0.27 g, 0.92 mmol), in toluene (5 mL) containing a catalytic amount of PTSA. The crude product was purified by gradient column chromatography (30-35% EtOAc in hexanes) affording **22** as a yellow oil (0.14 g, 55%, R_f=0.51 in 3:7 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.91 – 7.82 (m), 7.66 – 7.60 (m), 7.53 (ddd, *J* = 8.2, 4.1, 0.8 Hz), 5.73 (s), 5.38 (d, *J* = 0.9 Hz), 5.24 – 5.15 (m), 4.77 (s), 4.70 (s), 3.80 (s), 3.76 (s), 2.14 (d, *J* = 0.7 Hz), 2.02 (s), 1.96 (t, *J* = 1.4 Hz), 1.56 (d, *J* = 1.6 Hz), 0.93 (s), 0.86 (s).

¹³C NMR (100 MHz, CDCl₃) δ 141.8, 138.7, 138.4, 133.6, 133.5, 133.3, 133.1, 131.1, 128.9, 128.9, 128.6, 128.4, 128.3, 126.9, 124.1, 113.9, 64.4, 64.2, 43.3, 43.1, 41.5, 32.0, 30.5, 28.1, 27.9, 20.8.

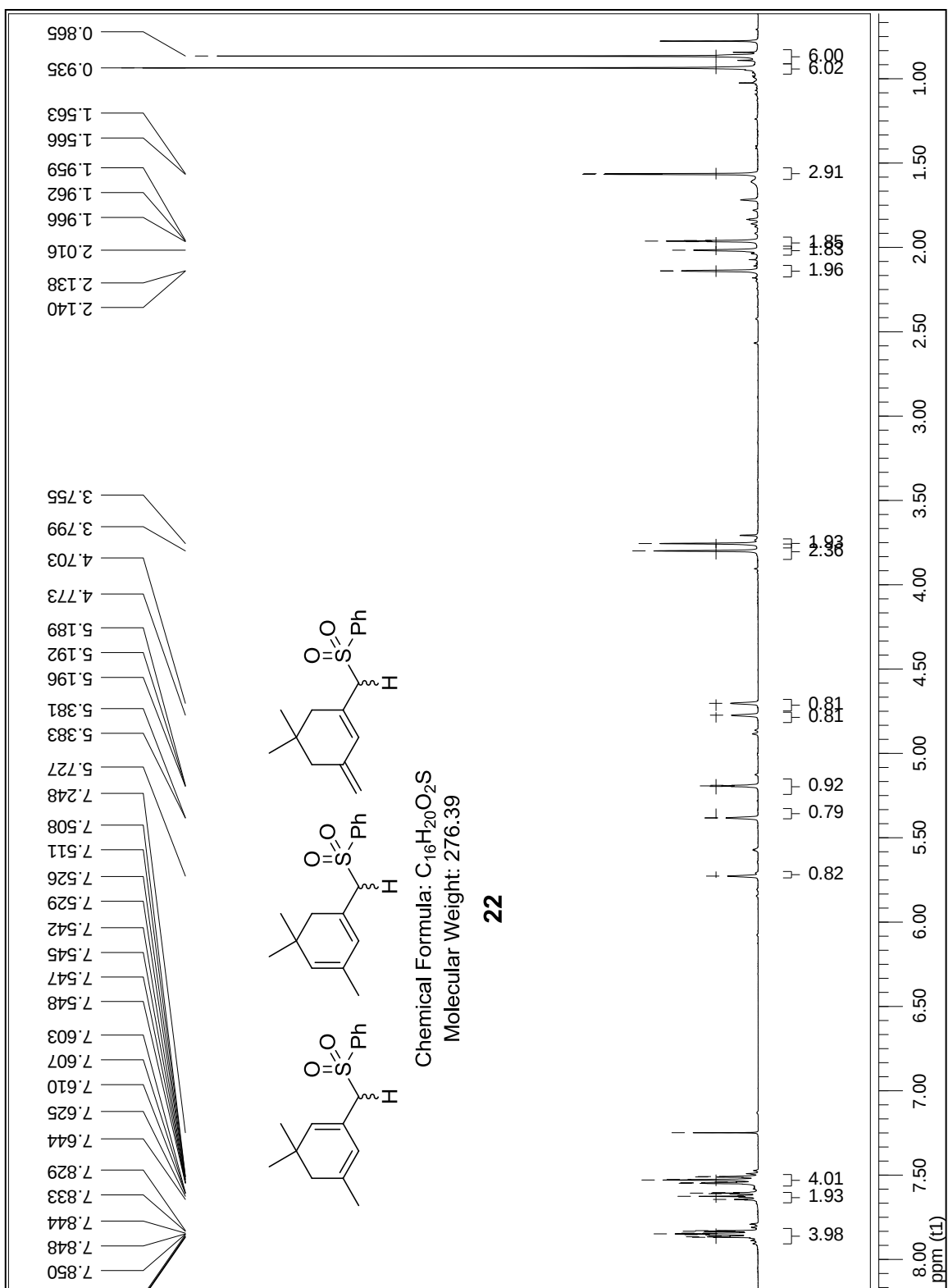


Figure 2.3.2.37. 400 MHz 1H NMR spectrum of compound **22** in $CDCl_3$.

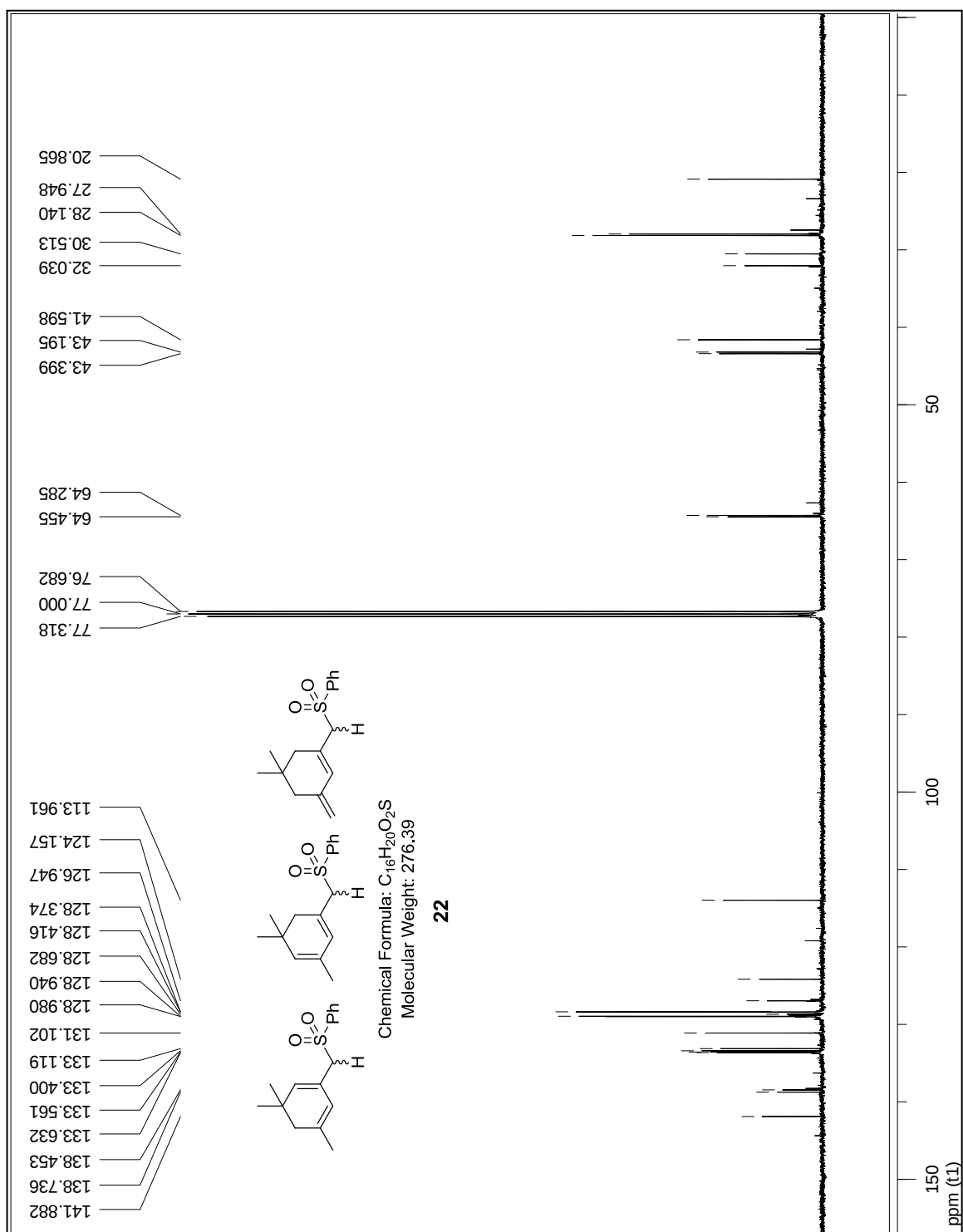
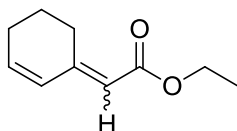


Figure 2.3.2.38. 100 MHz ^{13}C NMR spectrum of compound 22 in $CDCl_3$.

Preparation of Ethyl 2-(cyclohex-2-en-1-ylidene)acetate



Chemical Formula: $C_{10}H_{14}O_2$

Molecular Weight: 166.22

23

This compound was prepared following general procedure 1 using triethylphosphonoacetate (4.7 g, 20.8 mmol) in anhydrous THF (10 mL), NaH (0.5 g, 20.8 mmol) and 2-cyclohexen-1-one (1 g, 10.4 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (10-20% EtOAc in hexanes) resulting in **23** as a pale yellow oil (0.27 g, 16%, R_f = 0.29 in 1:4 EtOAc : hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.44 (ddd, J = 10.2, 2.0, 1.2 Hz), 6.27 – 6.13 (m), 6.08 (dt, J = 9.8, 1.6 Hz), 5.53 (s), 5.44 (s), 4.12 (q, J = 7.1 Hz), 2.97 – 2.88 (m), 2.42 – 2.31 (m), 2.22 – 2.11 (m), 1.82 – 1.65 (m), 1.29 – 1.19 (m).

^{13}C NMR (100 MHz, $CDCl_3$) δ 167.1, 166.4, 153.8, 152.3, 138.1, 130.2, 125.1, 114.9, 113.0, 59.5, 32.5, 26.1, 22.6, 21.8, 14.3.

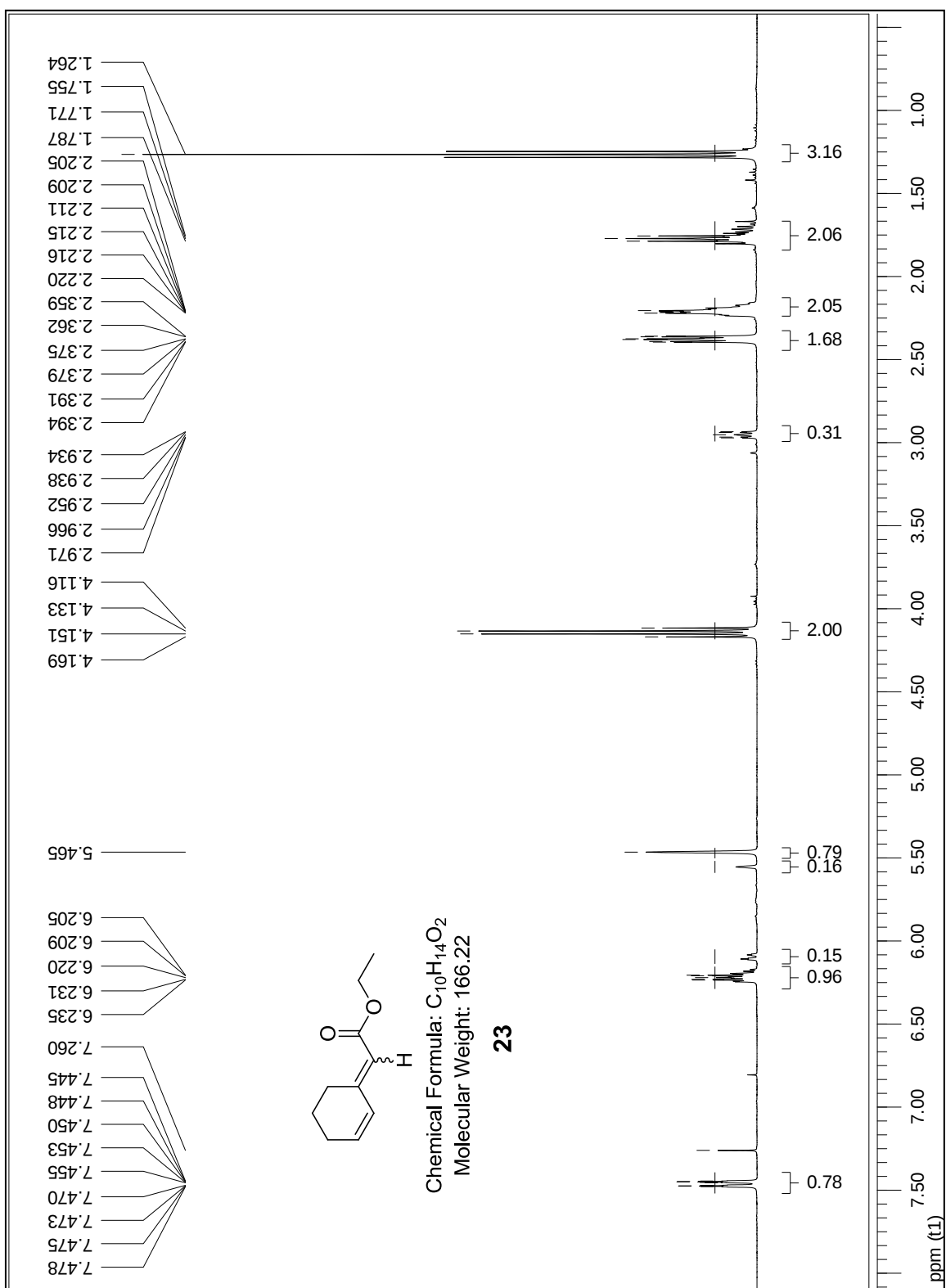


Figure 2.3.2.39. 400 MHz ¹H NMR spectrum of compound **23** in CDCl₃.

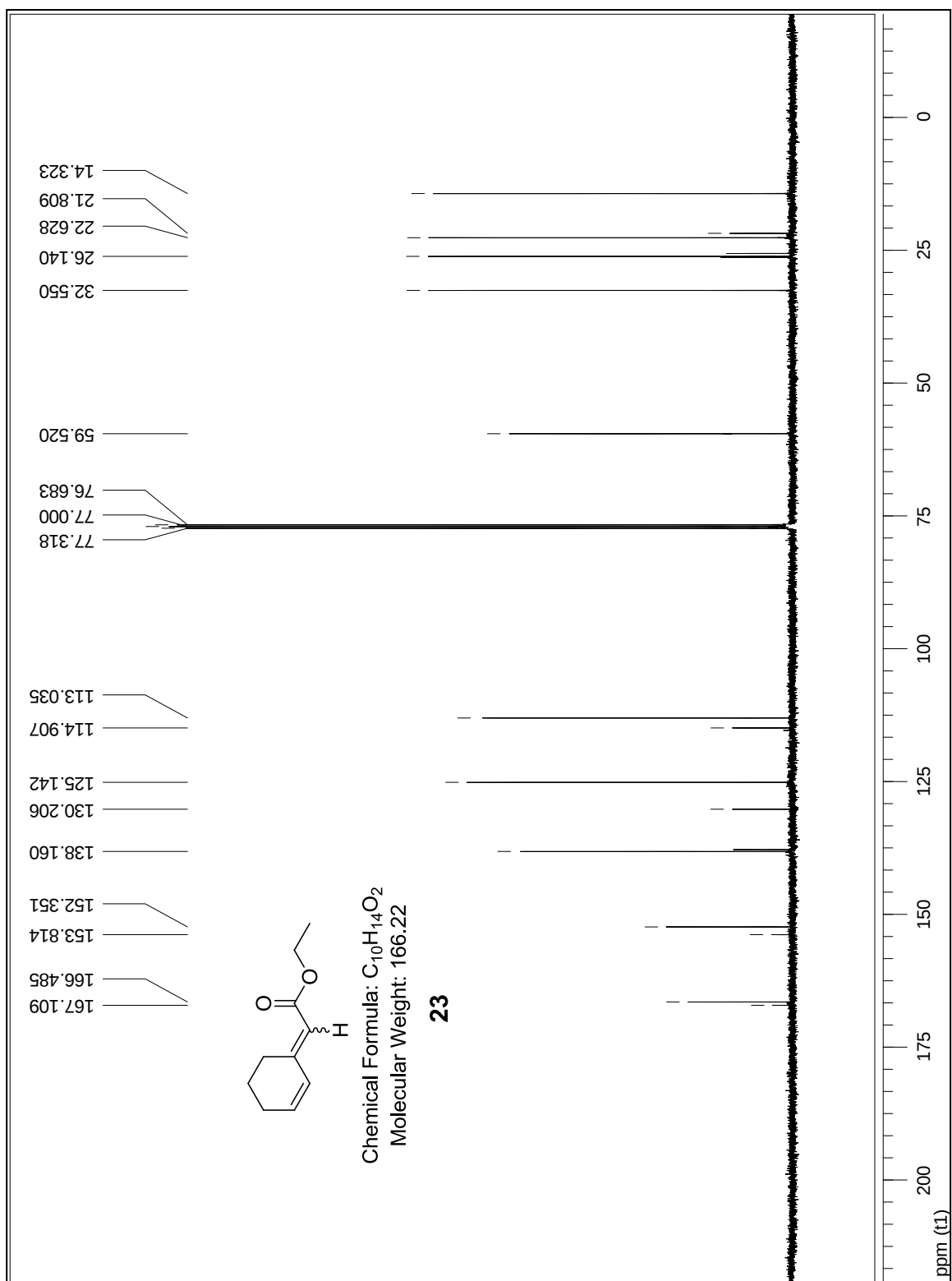
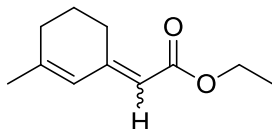


Figure 2.3.2.40. 100 MHz ¹³C NMR spectrum of compound **23** in CDCl₃.

Preparation of ethyl 2-(3-methylcyclohex-2-en-1-ylidene)acetate



Chemical Formula: C₁₁H₁₆O₂

Molecular Weight: 180.24

24

This compound was prepared following general procedure 1 using triethylphosphonoacetate (3.39 g, 15.13 mmol) in anhydrous THF (10 mL), NaH (0.36 g, 15.13 mmol) and 3-methylcyclohex-2-enone (0.833 g, 7.56 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (10-20% EtOAc in hexanes) resulting in **24** as a colorless oil (0.45 g, 33%, R_f = 0.66 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.30 (s), 5.87 (dd, *J* = 1.4, 0.6 Hz), 5.62 (d, *J* = 1.2 Hz), 5.44 (s), 5.35 (s), 4.18 – 4.01 (m), 3.03 (s), 2.91 – 2.81 (m), 2.34 – 2.20 (m), 2.08 (dd, *J* = 9.1, 6.5 Hz), 1.89 – 1.58 (m), 1.27 – 1.15 (m).

¹³C NMR (100 MHz, CDCl₃) δ 167.2, 166.6, 154.8, 153.4, 148.4, 147.6, 125.9, 121.2, 112.5, 110.4, 59.1, 59.1, 32.0, 31.1, 30.5, 25.6, 24.5, 24.2, 22.6, 22.0, 14.2.

HRMS: Calculated for C₁₁H₁₆O₂ = 180.11503; found = 180.11655

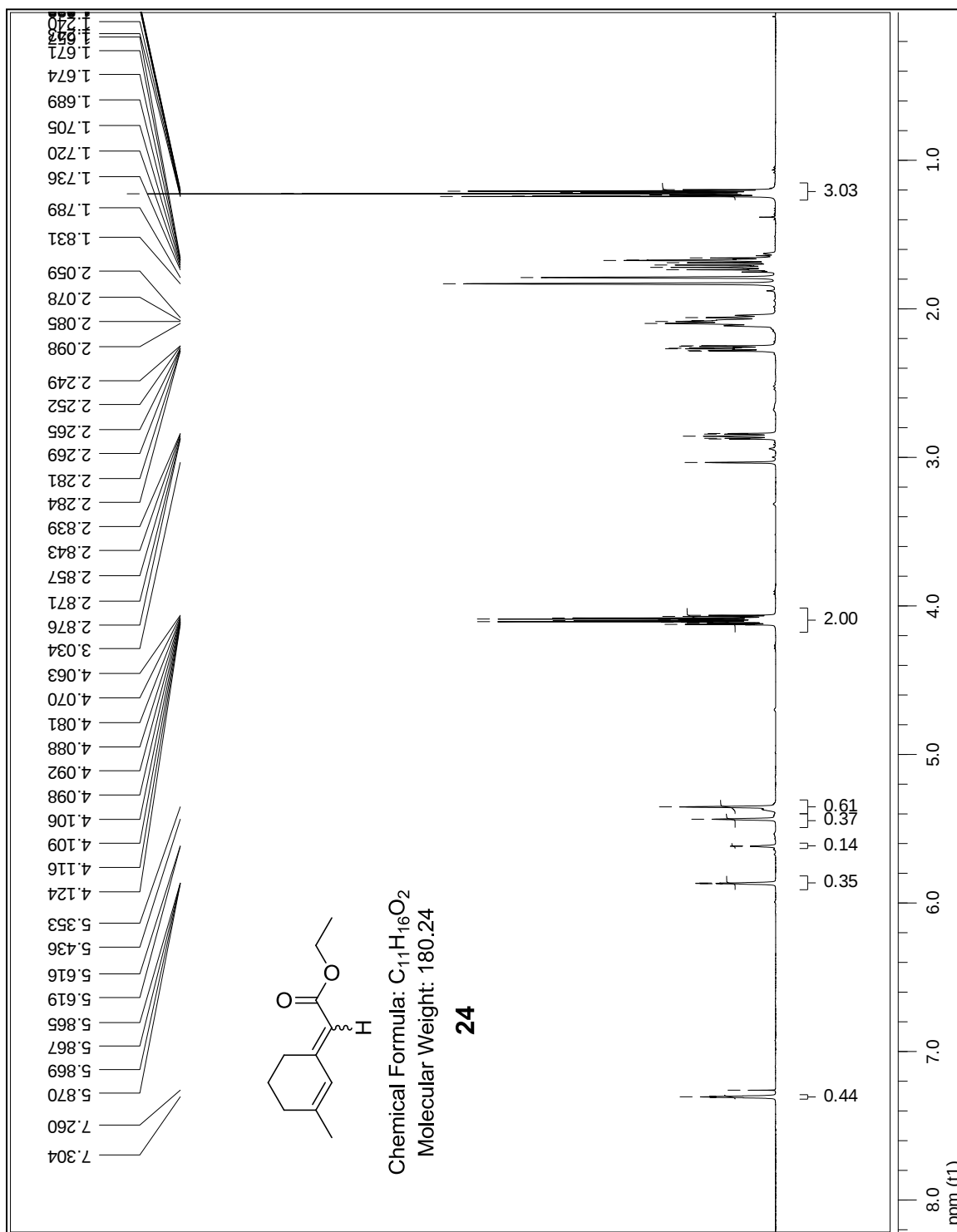


Figure 2.3.2.41. 400 MHz ^1H NMR spectrum of compound **24** in CDCl_3 .

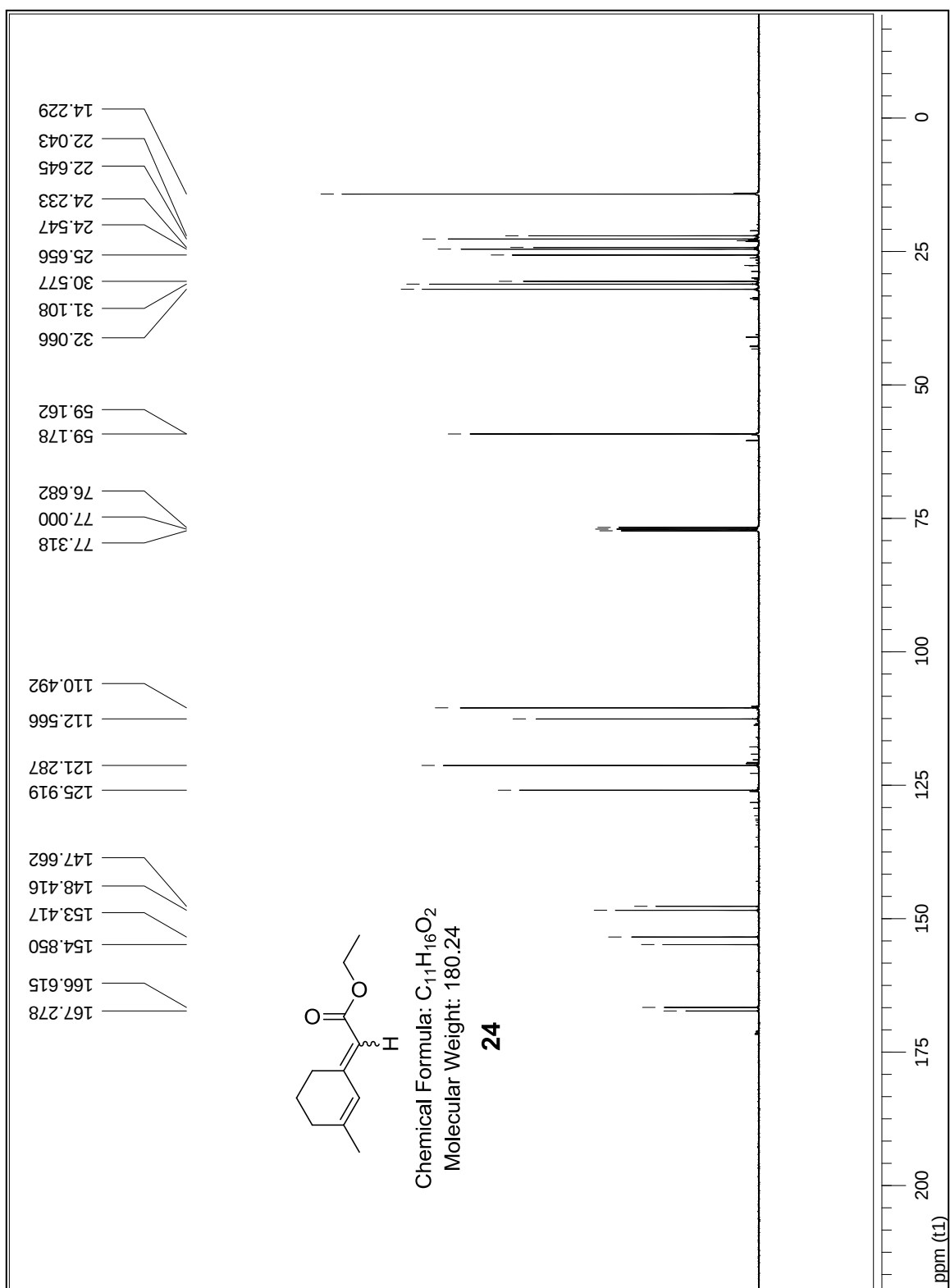
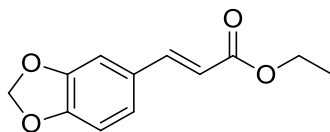


Figure 2.3.2.42. 100 MHz ^{13}C NMR spectrum of compound **24** in $CDCl_3$.

Preparation of ethyl 3-(benzo[d][1,3]dioxol-5-yl)acrylate



Chemical Formula: C₁₂H₁₂O₄

Molecular Weight: 220.22

25

This compound was prepared following general procedure 1 using triethylphosphonoacetate (8.96 g, 40 mmol) in anhydrous THF (10 mL), NaH (1.6 g, 40 mmol) and piperonal (3 g, 20 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (10-20% EtOAc in hexanes) resulting in **25** as an off white solid (1.54 g, 35%, R_f = 0.5 in 3:7 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.57 (d, *J* = 15.9 Hz, 1H), 6.99 (dd, *J* = 10.9, 4.9 Hz, 2H), 6.78 (d, *J* = 8.0 Hz, 1H), 6.24 (d, *J* = 15.9 Hz, 1H), 5.98 (s, 2H), 4.23 (q, *J* = 7.1 Hz, 2H), 1.30 (t, *J* = 7.1 Hz, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 167.1, 149.5, 148.3, 144.2, 128.8, 124.3, 116.2, 108.5, 106.4, 101.5, 60.3, 14.3.

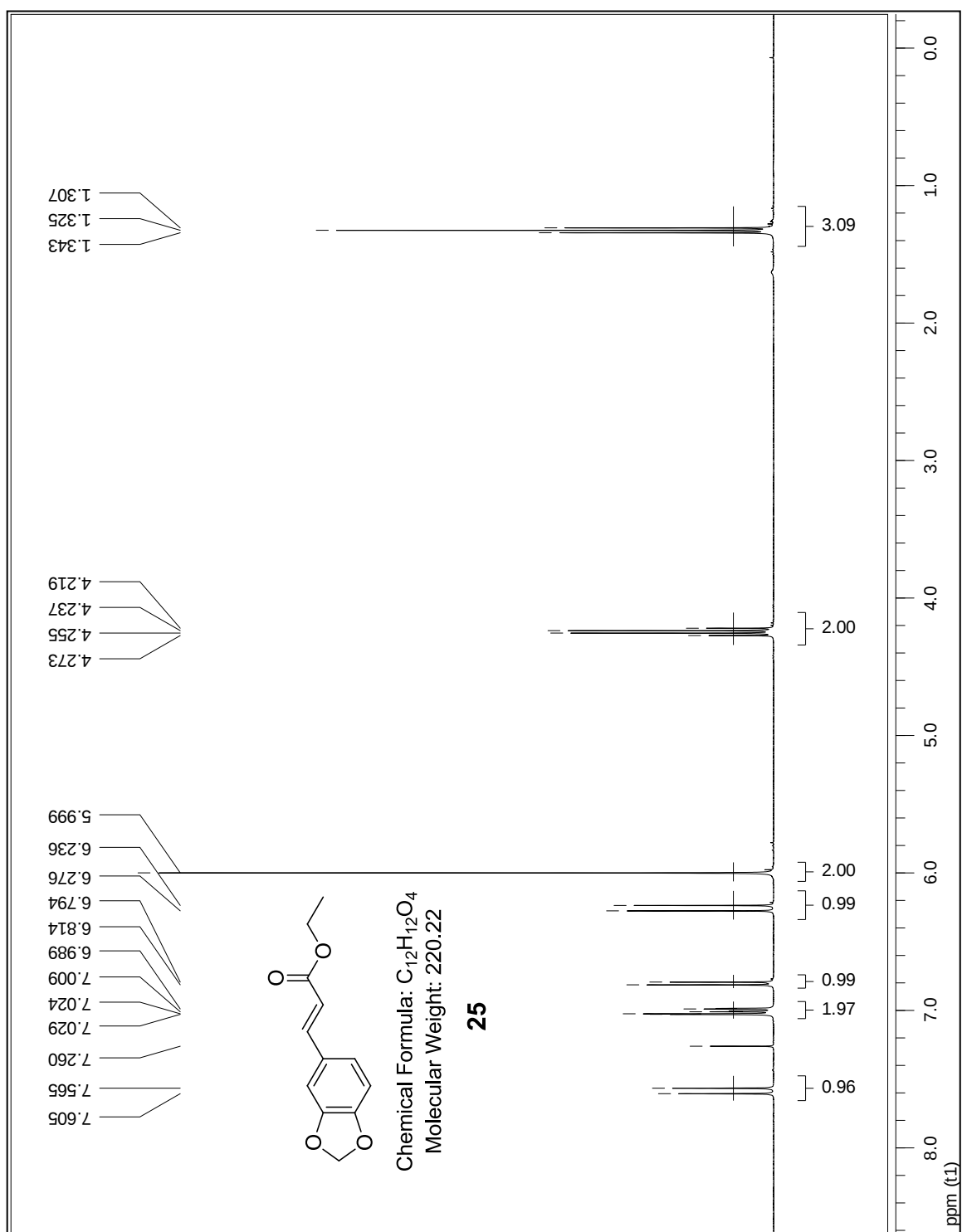


Figure 2.3.2.43. 400 MHz ^1H NMR spectrum of compound **25** in CDCl_3 .

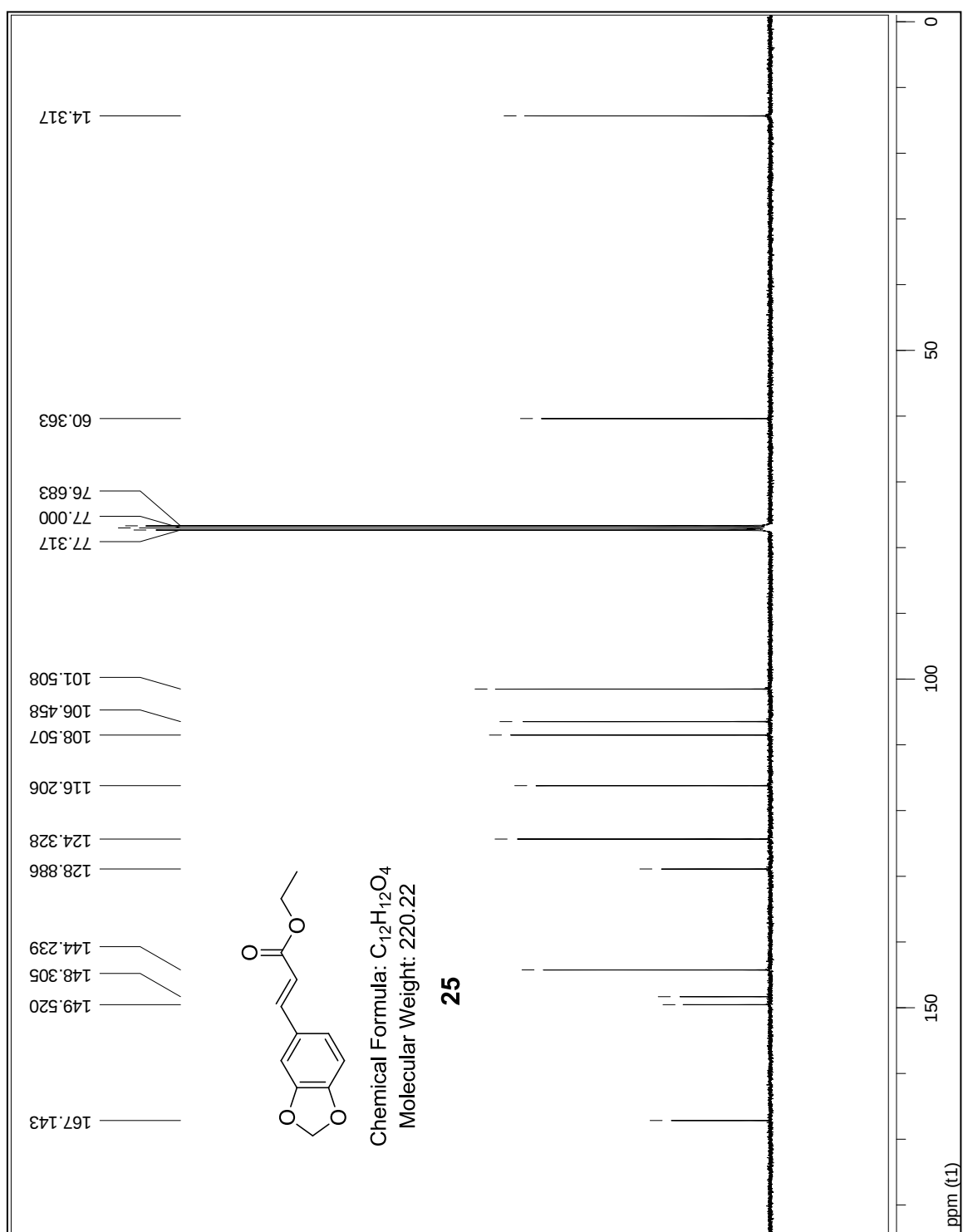
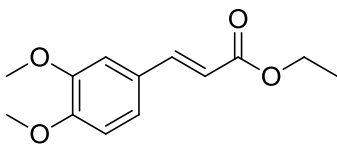


Figure 2.3.2.44. 100 MHz ^{13}C NMR spectrum of compound 25 in $CDCl_3$.

Preparation of ethyl 3-(3,4-dimethoxyphenyl)acrylate



Chemical Formula: C₁₃H₁₆O₄

Molecular Weight: 236.26

26

This compound was prepared following general procedure 1 using triethylphosphonoacetate (4.0 g, 18 mmol) in anhydrous THF (10 mL), NaH (0.72 g, 18 mmol) and 3,4-dimethoxy benzaldehyde (1.5 g, 9 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (10-20% EtOAc in hexanes) resulting in **26** as a white solid (1.6 g, 75%, R_f = 0.49 in 3:7 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.62 (d, *J* = 15.9 Hz, 1H), 7.10 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.05 (d, *J* = 1.9 Hz, 1H), 6.86 (d, *J* = 8.3 Hz, 1H), 6.31 (d, *J* = 15.9 Hz, 1H), 4.25 (q, *J* = 7.1 Hz, 2H), 3.91 (s, 6H), 1.33 (t, *J* = 7.1 Hz, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 167.1, 151.0, 149.1, 144.4, 127.4, 122.5, 115.9, 110.9, 109.5, 60.3, 55.9, 55.8, 14.3.

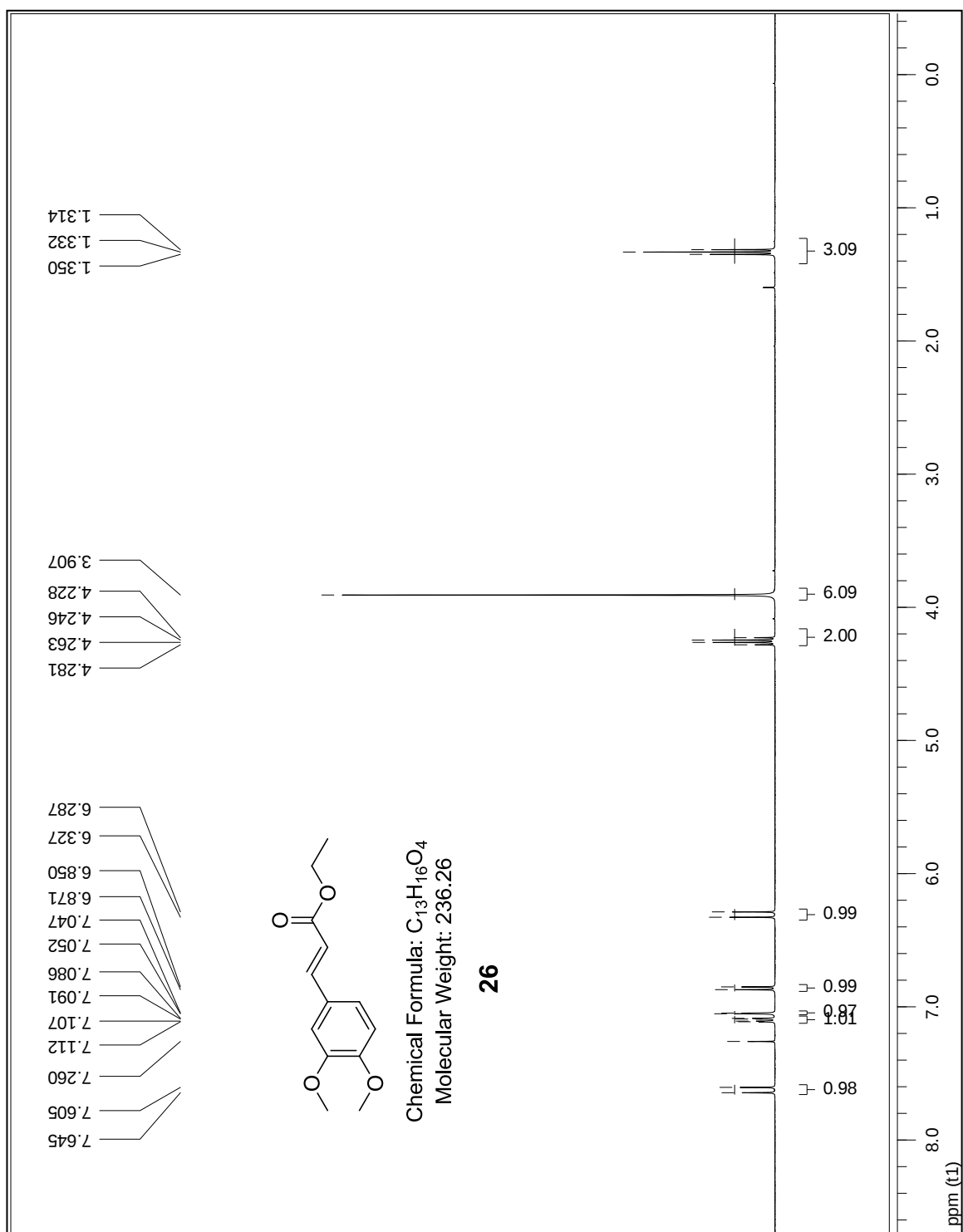


Figure 2.3.2.45. 400 MHz ^1H NMR spectrum of compound **26** in CDCl_3 .

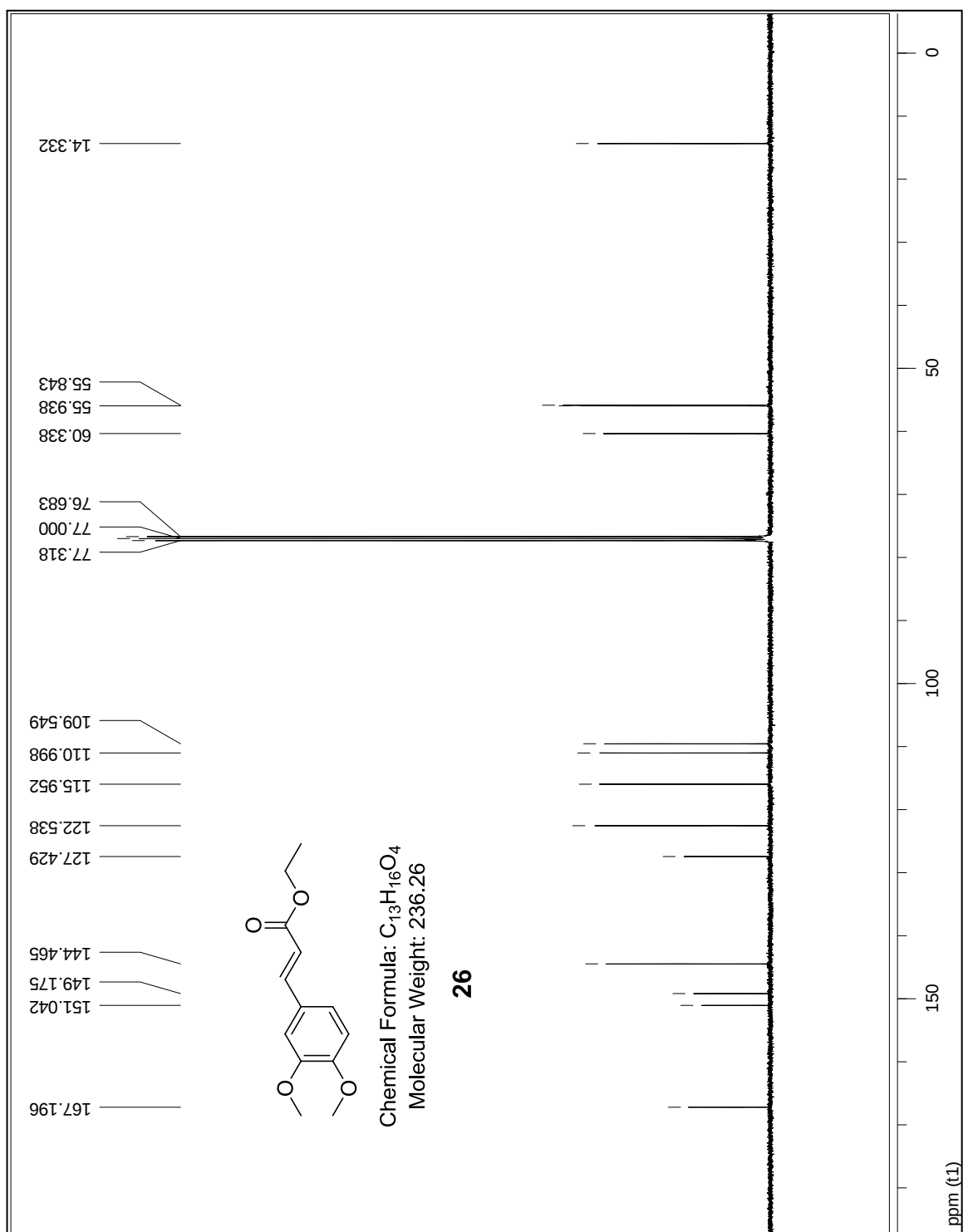
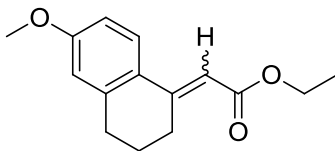


Figure 2.3.2.46. 100 MHz ^{13}C NMR spectrum of compound **26** in $CDCl_3$.

Preparation of 2-(6-methoxy-3,4-dihydronaphthalen-1-ylidene)acetate



Chemical Formula: C₁₅H₁₈O₃

Molecular Weight: 246.30

27

This compound was prepared following general procedure 1 using triethylphosphonoacetate (6.3 g, 28.38 mmol) in anhydrous THF (10 mL), NaH (1.13 g, 28.38 mmol) and 6-methoxy-3,4-dihydronaphthalen-1-one (2.5 g, 14.19 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (10-20% EtOAc in hexanes) resulting in **27** as an off white solid (1.92 g, 55%, R_f = 0.68 in 3:7 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.69 (d, *J* = 8.7 Hz), 7.61 (d, *J* = 8.9 Hz), 7.12 (d, *J* = 9.2 Hz), 6.76 – 6.57 (m), 6.25 (s), 5.71 (t, *J* = 1.2 Hz), 4.15 (dd, *J* = 10.9, 7.1 Hz), 3.77 (d, *J* = 4.7 Hz), 3.39 (d, *J* = 1.1 Hz), 3.20 (dt, *J* = 7.3, 4.7 Hz), 2.79 (d, *J* = 28.0 Hz), 2.47 (ddd, *J* = 6.4, 4.6, 1.2 Hz), 2.28 (d, *J* = 4.5 Hz), 2.03 (d, *J* = 1.6 Hz), 1.97 – 1.88 (m), 1.87 – 1.79 (m), 1.24 (d, *J* = 13.7 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 171.5, 166.9, 166.8, 160.4, 160.2, 158.3, 154.3, 152.7, 141.8, 140.7, 137.8, 131.2, 129.6, 127.1, 126.5, 126.1, 126.1, 125.4, 123.5, 113.6, 113.0, 112.6, 112.4, 110.6, 110.5, 109.9, 60.38, 59.5, 54.9, 54.8, 39.0, 35.3, 30.3, 29.5, 28.2, 27.9, 23.0, 22.9, 22.4, 14.1, 13.9, 13.9.

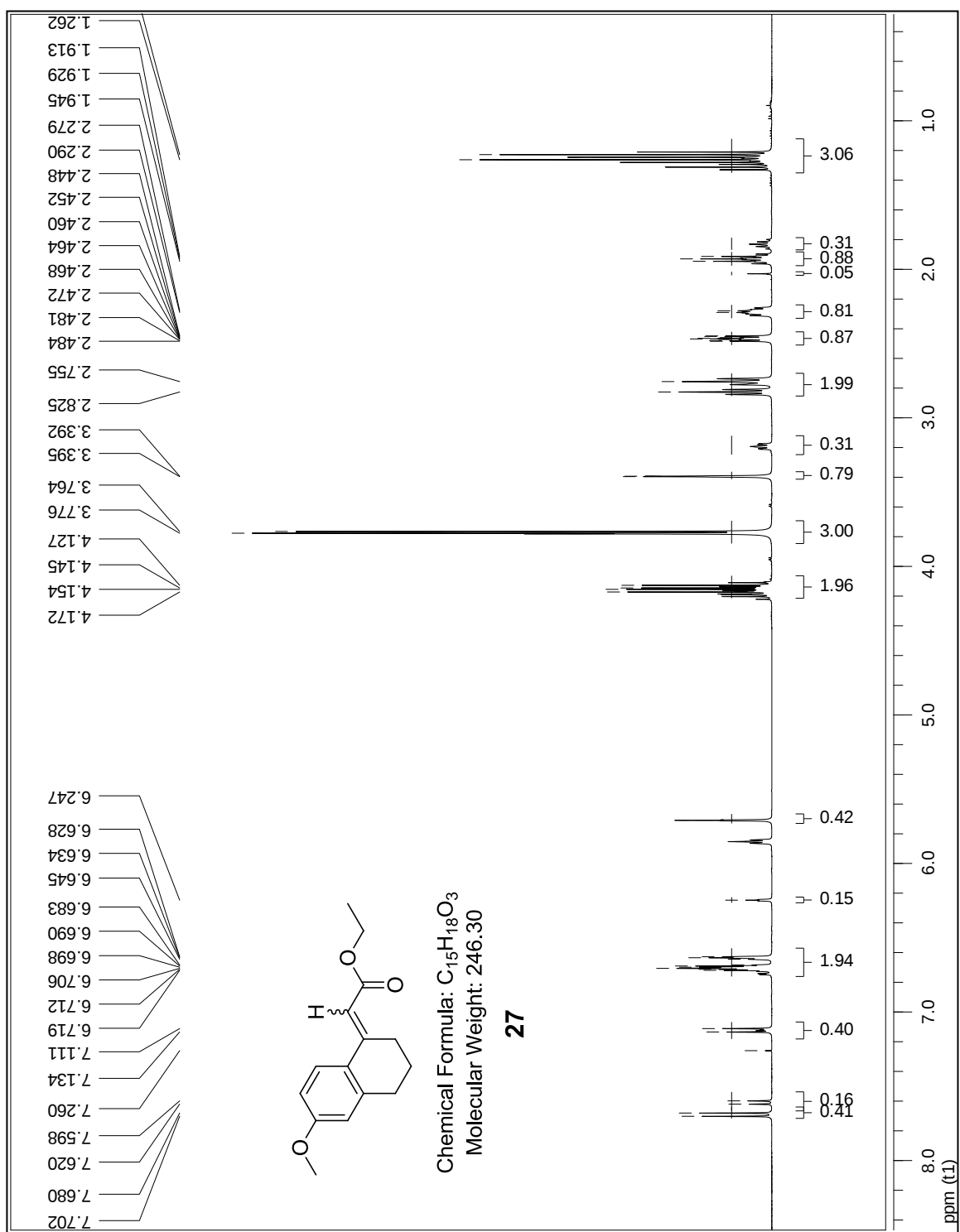
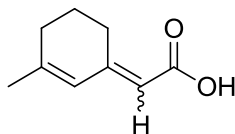


Figure 2.3.2.47. 400 MHz ^1H NMR spectrum of compound 27 in CDCl_3 .

Preparation of 2-(cyclohex-2-en-1-ylidene)acetic acid



Chemical Formula: C₉H₁₂O₂

Molecular Weight: 152.19

29

Compound **24** (0.3 g, 1.66 mmol) and KOH (0.28 g, 4.99 mmol) in a mixture of EtOH : H₂O (3:1 – 20mL) were refluxed for 2 h. After this time, the solution was stirred to rt, concentrated in vacuo and the resulting residue was dissolved in H₂O (5 mL) . The solution was then acidified with concentrated HCl and extracted with EtOAc (3 x 10 mL) affording **29** as a viscous oil (0.22 g, 87%, R_f = 0.23 in 1:4 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 7.33 – 7.30 (m), 5.98 – 5.94 (m), 5.51 (s), 5.43 (s), 2.93 – 2.88 (m), 2.38 – 2.32 (m), 2.14 (dt, *J* = 12.9, 6.1 Hz), 1.90 (s), 1.86 (s), 1.82 – 1.69 (m).

¹³C NMR (100 MHz, CDCl₃) δ 171.6, 171.3, 153.7, 153.5, 132.7, 131.0, 122.9, 115.1, 114.6, 31.4, 33.0, 27.0, 25.1, 22.6,

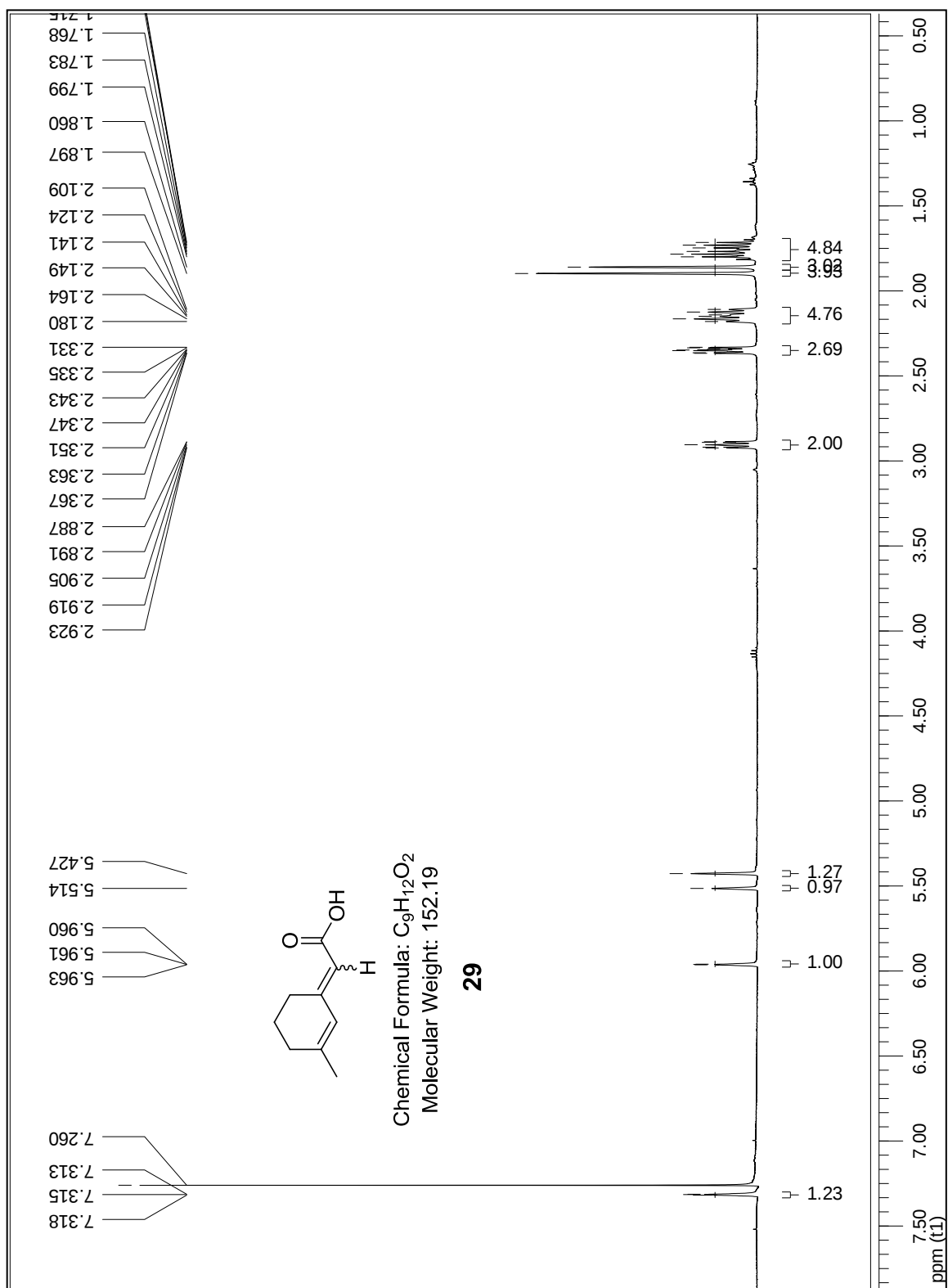
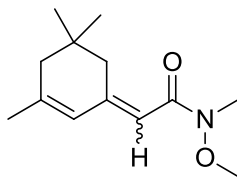


Figure 2.3.2.49. 400 MHz ¹H NMR spectrum of compound **29** in CDCl₃.

Preparation of N-methoxy-N-methyl-2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)acetamide



Chemical Formula: $C_{13}H_{21}NO_2$

Molecular Weight: 223.31

30

Me_3Al (2 M in toluene, 9.6 mL, 19.2 mmol) was added to a flask containing N,O-dimethylhydroxylamine hydrochloride (1.4 g, 14.4 mmol) in dry DCM (20 mL) under nitrogen atmosphere. The reaction mixture was left to stir for 30 min. Compound **4** (2 g, 9.6 mmol) was taken in anhydrous DCM (20 mL) and was added drop wise to the mixture via syringe. The mixture was set to reflux for 28 h. Excess Me_3Al was quenched by adding 0.5 M HCl under N_2 at 0°C and the reaction mixture was neutralized by adding 10% $NaHCO_3$ solution (5 mL). The organic layer was washed with brine and dried over $MgSO_4$. The crude organic extracts were purified by column chromatography (40% EtOAc in hexanes) affording **30** as a viscous oil (0.98 g, 46%, R_f = 0.19 in 1:4 EtOAc : hexanes).

1H NMR (400 MHz, $CDCl_3$) δ 7.32 (s), 6.01 (s), 5.91 (d, J = 0.8 Hz), 5.86 (s), 3.64 (s), 3.17 (s), 2.70 (d, J = 1.7 Hz), 2.08 (d, J = 1.3 Hz), 1.92 (s), 1.78 (d, J = 8.0 Hz), 0.90 (s).

^{13}C NMR (100 MHz, $CDCl_3$) δ 167.8, 152.3, 150.7, 145.2, 144.1, 125.4, 120.6, 112.1, 109.7, 61.2, 46.3, 45.4, 44.9, 39.1, 31.2, 28.3, 28.0, 24.4, 24.2.

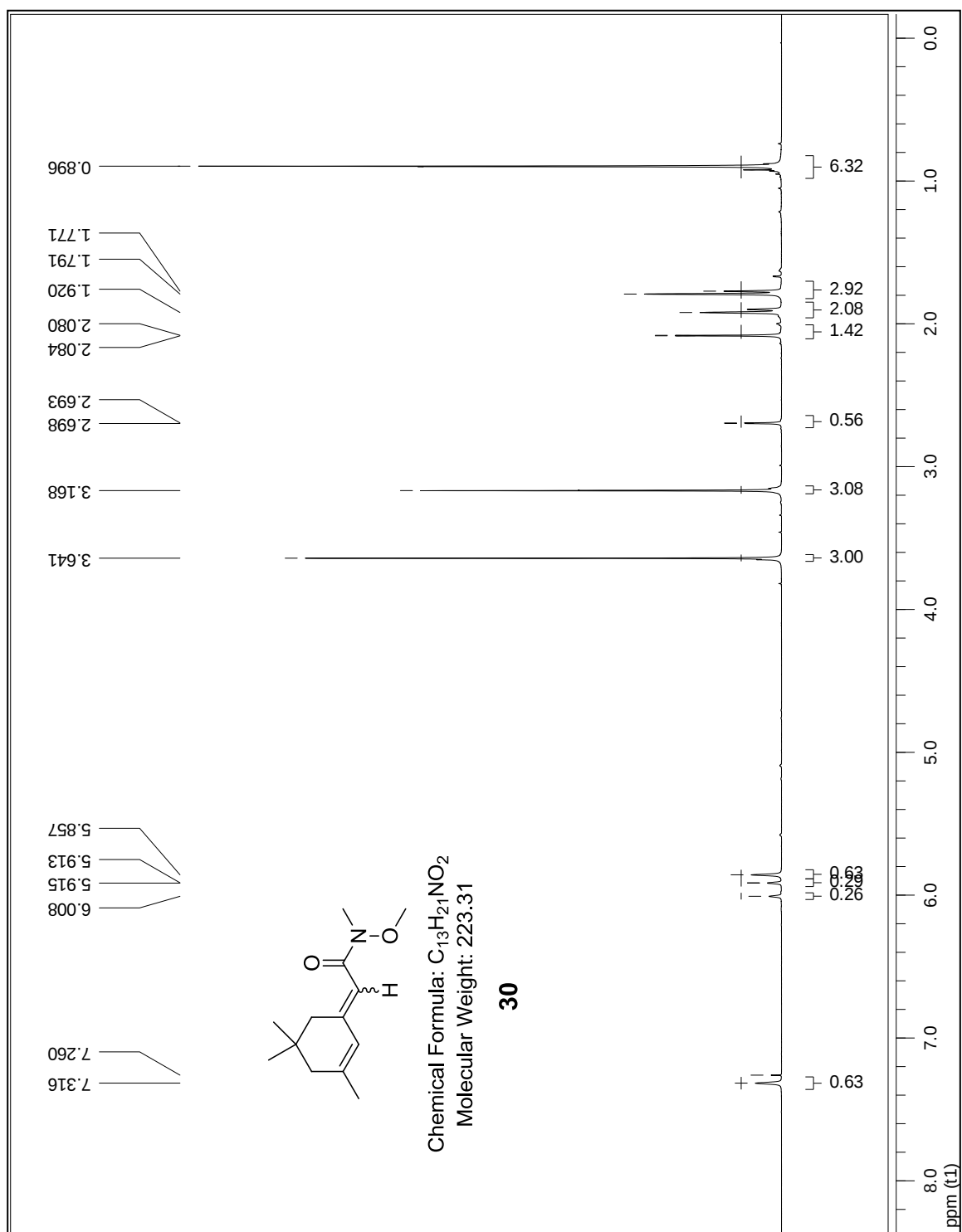


Figure 2.3.2.50. 400 MHz 1H NMR spectrum of compound **30** in $CDCl_3$.

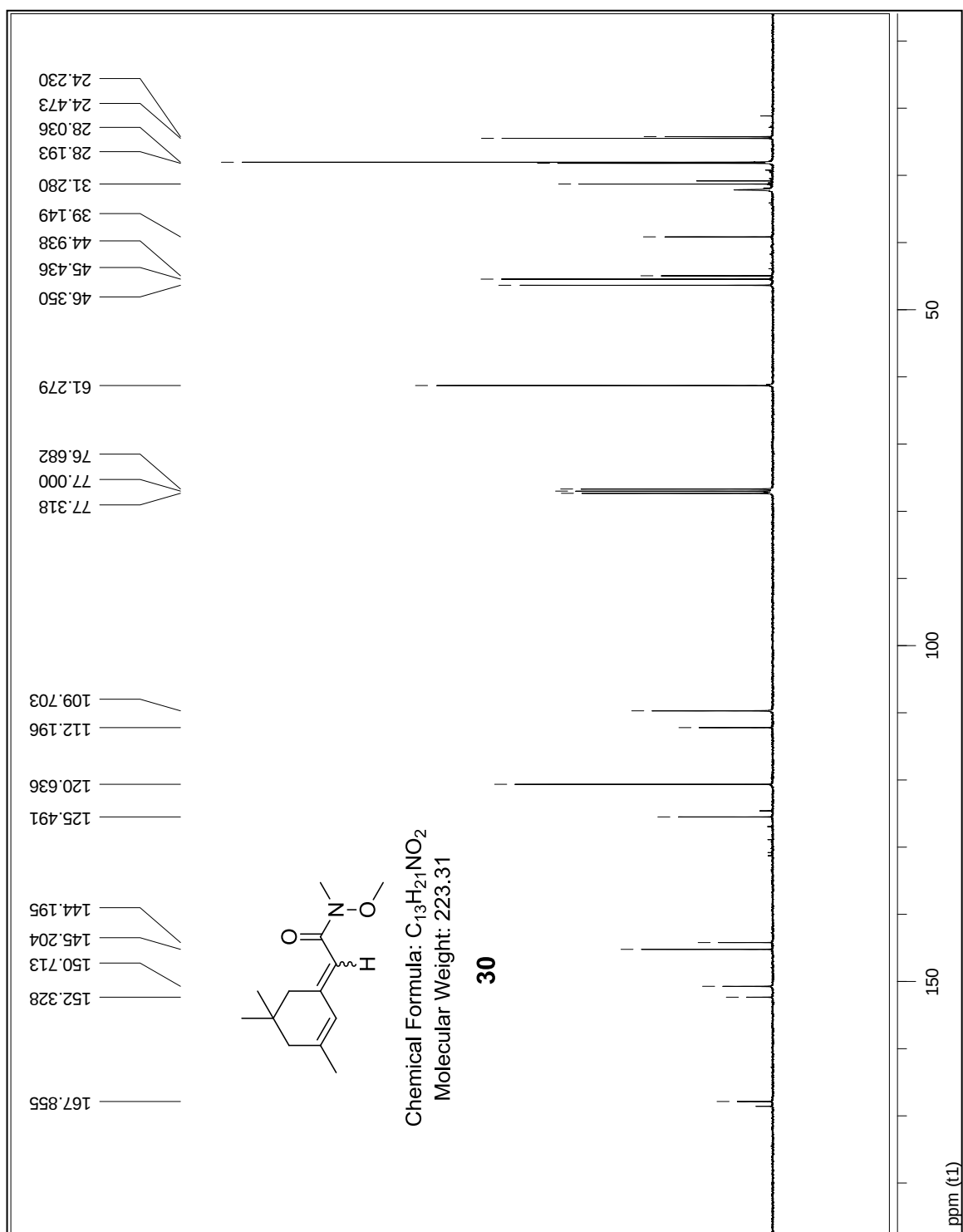
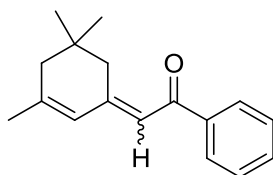


Figure 2.3.2.51. 100 MHz ^{13}C NMR spectrum of compound **30** in $CDCl_3$.

Preparation of 1-phenyl-2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)ethanone



Chemical Formula: C₁₇H₂₀O

Molecular Weight: 240.34

31

Compound **30** (0.14 g, 0.6 mmol) was taken in anhydrous ether (5 mL) and stirred under N₂ atmosphere at -78°C. Phenyl magnesium bromide in 1M ether (0.9 mL, 0.9 mmol) was added and the reaction mixture was stirred at rt. The progress of the reaction was monitored by TLC and the reaction mixture was quenched with saturated NH₄Cl solution. The reaction mixture was extracted with DCM (3 x 10 mL), dried (MgSO₄), filtered and concentrated in vacuo. The crude product was purified by gradient column chromatography (20% EtOAc in hexanes) affording **31** as a viscous oil (0.08 g, 55%).

¹H NMR (400 MHz, CDCl₃) δ 7.95 – 7.87 (m), 7.51 – 7.36 (m), 6.64 (s), 6.48 (s), 6.03 (d, *J* = 0.7 Hz), 2.81 (d, *J* = 1.6 Hz), 2.19 (d, *J* = 1.2 Hz), 1.98 (d, *J* = 8.3 Hz), 1.85 (d, *J* = 9.1 Hz), 1.23 (s), 0.94 (d, *J* = 4.2 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 191.4, 190.8, 155.2, 153.58, 148.9, 147.6, 139.9, 139.8, 131.9, 131.8, 128.2, 128.2, 127.9, 127.9, 125.8, 121.5, 118.8, 116.5, 46.4, 45.7, 45.1, 40.0, 31.6, 31.0, 29.6, 28.2, 28.1, 24.7, 24.5.

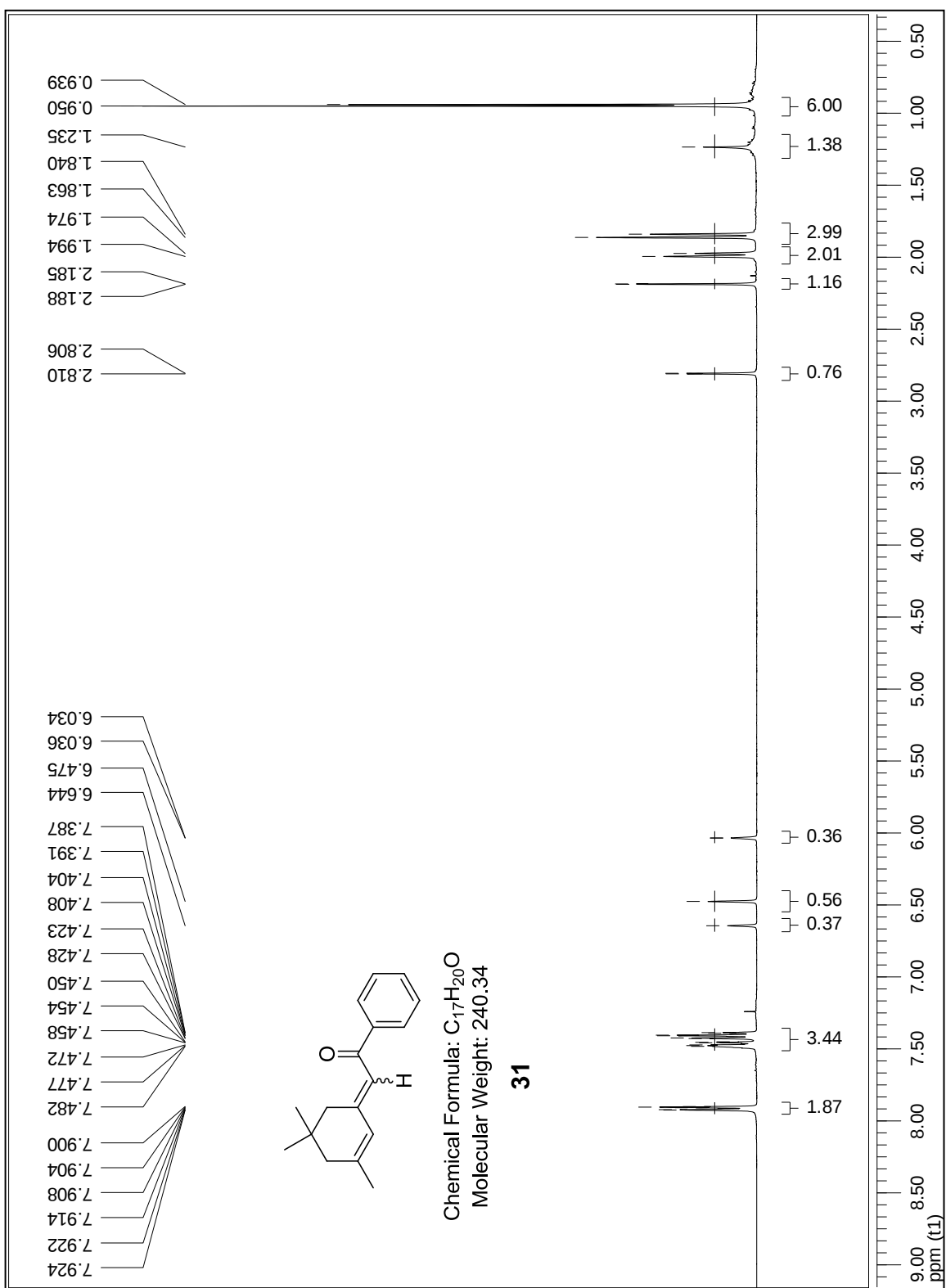


Figure 2.3.2.52. 400 MHz ^1H NMR spectrum of compound **31** in CDCl_3 .

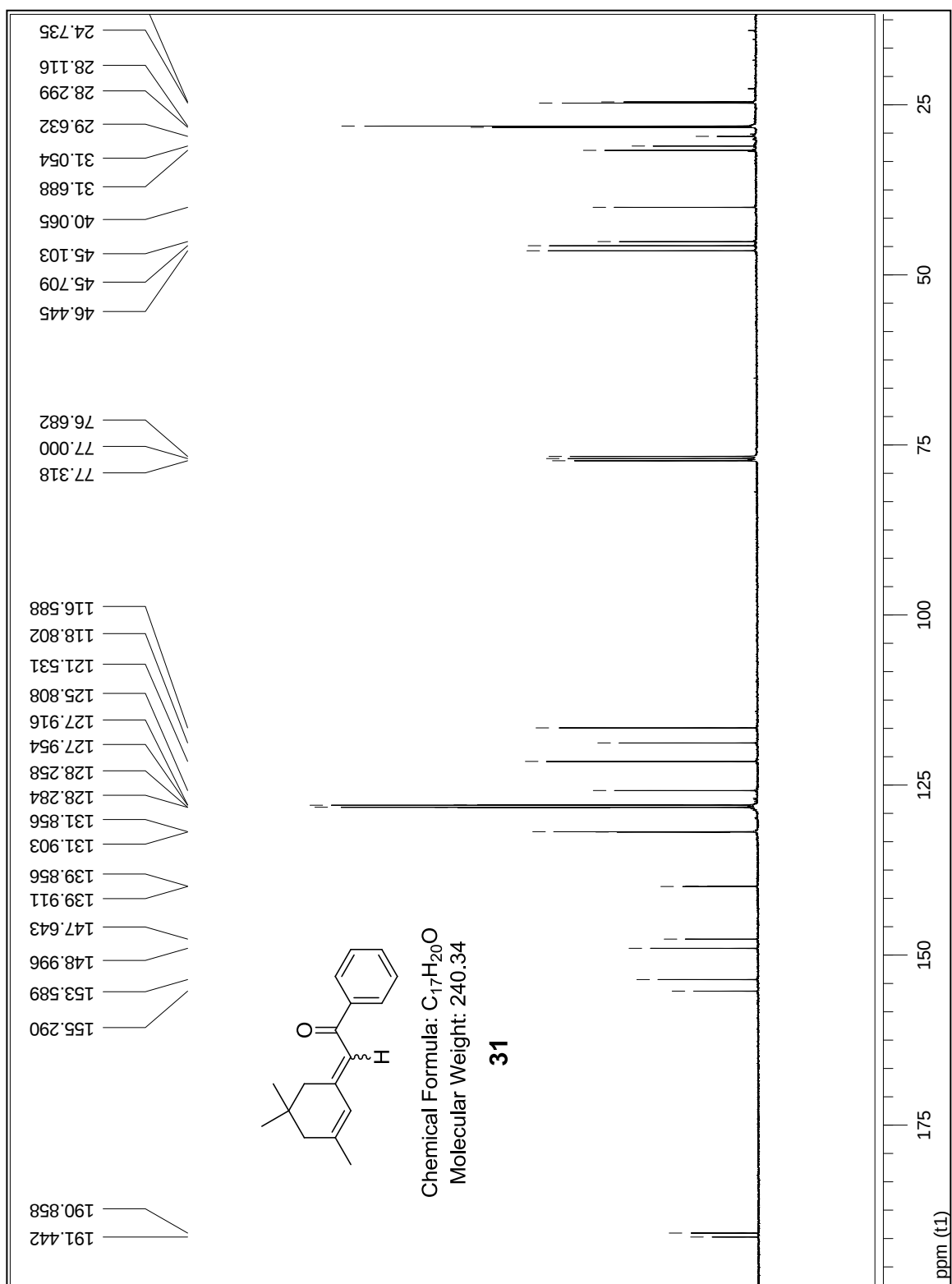
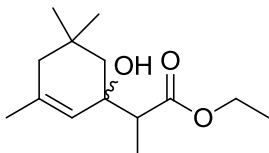


Figure 2.3.2.53. 100 MHz ¹³C NMR spectrum of compound **31** in CDCl₃.

Preparation of ethyl 2-(1-hydroxy-3,5,5-trimethylcyclohex-2-en-1-yl)propanoate



Chemical Formula: C₁₄H₂₄O₃

Molecular Weight: 240.34

32

This compound was prepared following general procedure 2 using ethyl propionate (1.48 g, 14.47 mmol) in anhydrous THF (10 mL), LDA (7.24 mL, 14.48 mmol) and isophorone (1 g, 7.24 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography (15-20% EtOAc in hexanes) yielding **32** as a viscous oil (0.8 g, 46%, R_f=0.77 in 2:3 EtOAc : hexanes).

¹H NMR (400 MHz, CDCl₃) δ 5.48 (d, *J* = 1.1 Hz), 5.19 (d, *J* = 1.2 Hz), 4.15 (dt, *J* = 11.0, 5.2 Hz), 3.48 (q, *J* = 7.2 Hz), 2.64 – 2.36 (m), 1.79 – 1.58 (m), 1.34 – 0.89 (m).

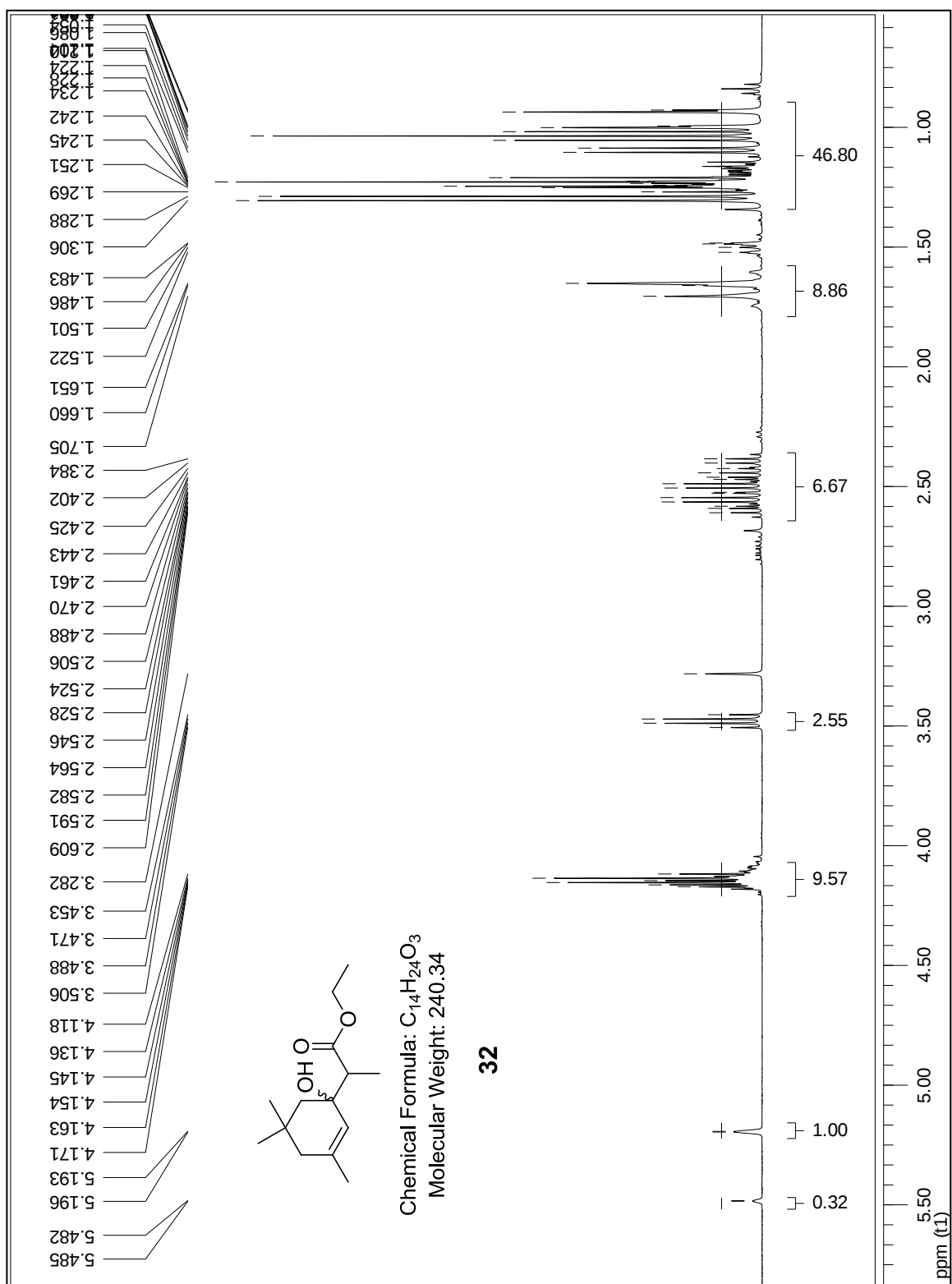
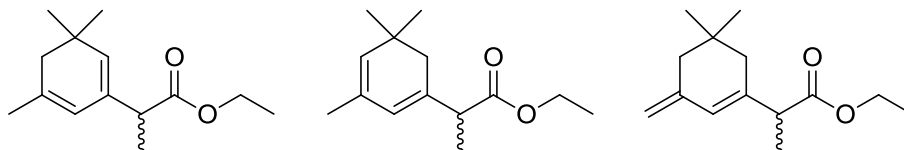


Figure 2.3.2.54. 400 MHz 1H NMR spectrum of compound **32** in $CDCl_3$.

Preparation of ethyl 2-(3,3,5-trimethylcyclohexa-dien-1-yl)propanoate



Chemical Formula: C₁₄H₂₂O₂

Molecular Weight: 222.32

33

This compound was prepared using general procedure 3 using compound **32** (0.75 g, 3.12 mmol), in toluene (10 mL) containing a catalytic amount of PTSA. Reaction mixture was set to reflux for an additional 2 h to ensure reaction completion. The crude product was purified by gradient column chromatography (10-15% EtOAc in hexanes) affording **33** as a colorless oil (0.69 g, 99%).

¹H NMR (400 MHz, CDCl₃) δ 6.00 – 5.80 (m), 5.63 – 5.48 (m), 5.05 (dd, *J* = 8.7, 7.3 Hz), 4.70 (dd, *J* = 24.7, 0.6 Hz), 4.16 – 3.93 (m), 3.13 – 2.89 (m), 2.14 (s), 2.01 – 1.56 (m), 1.36 – 0.67 (m).

¹³C NMR (100 MHz, CDCl₃) δ 174.5, 173.9, 173.8, 173.7, 172.1, 160.1, 145.1, 145.1, 142.5, 138.3, 138.1, 135.7, 135.2, 132.2, 131.1, 130.1, 129.0, 125.7, 125.4, 123.8, 123.7, 123.0, 120.5, 118.3, 112.5, 111.4, 60.3, 60.1, 46.6, 46.2, 44.6, 43.9, 43.3, 40.5, 39.3, 31.6, 30.2, 28.0, 27.9, 27.8, 27.7, 27.5, 27.4, 23.4, 21.0, 16.0, 15.2, 14.9, 14.1, 14.0, 13.9.

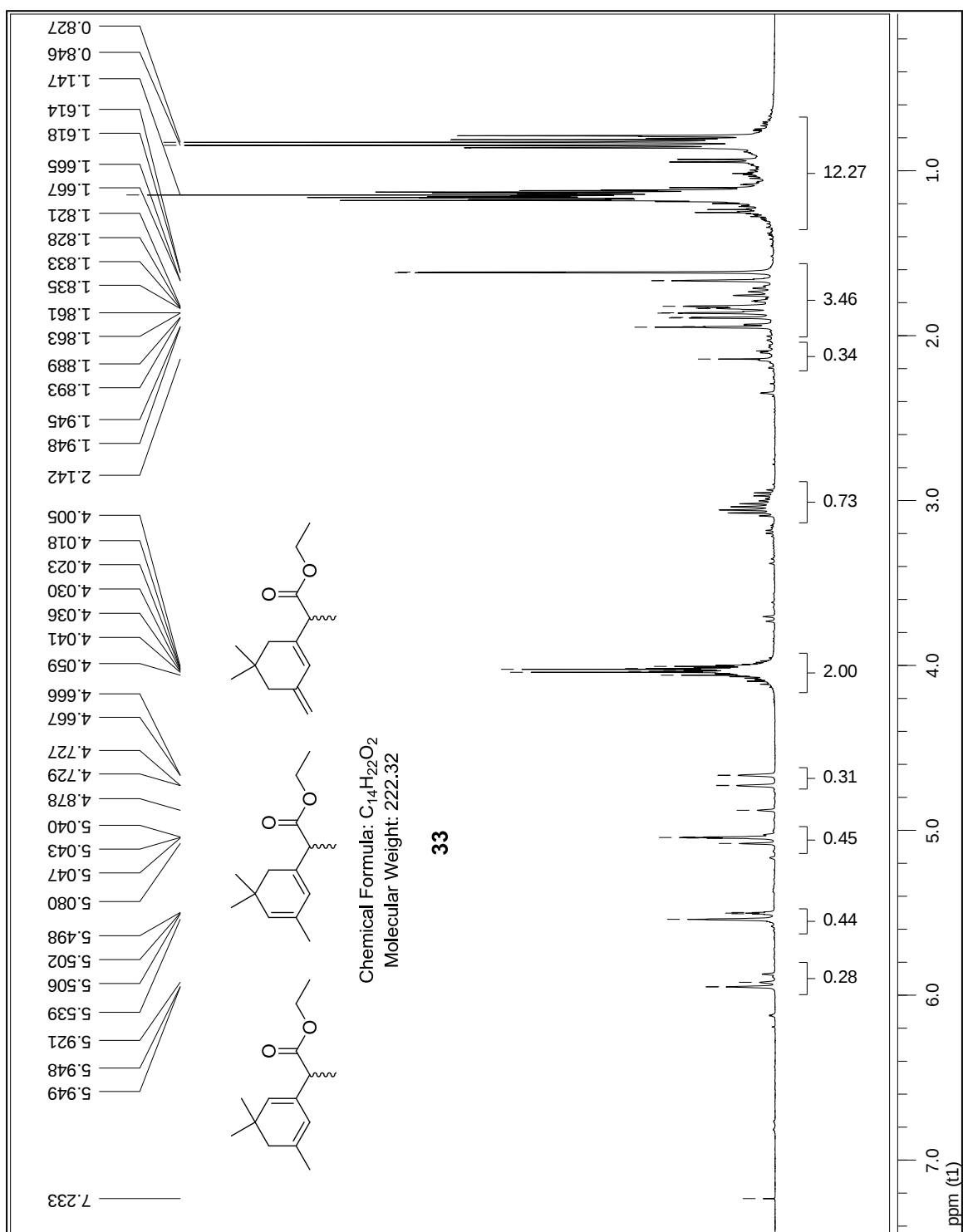


Figure 2.3.2.55. 400 MHz 1H NMR spectrum of compound 33 in $CDCl_3$.

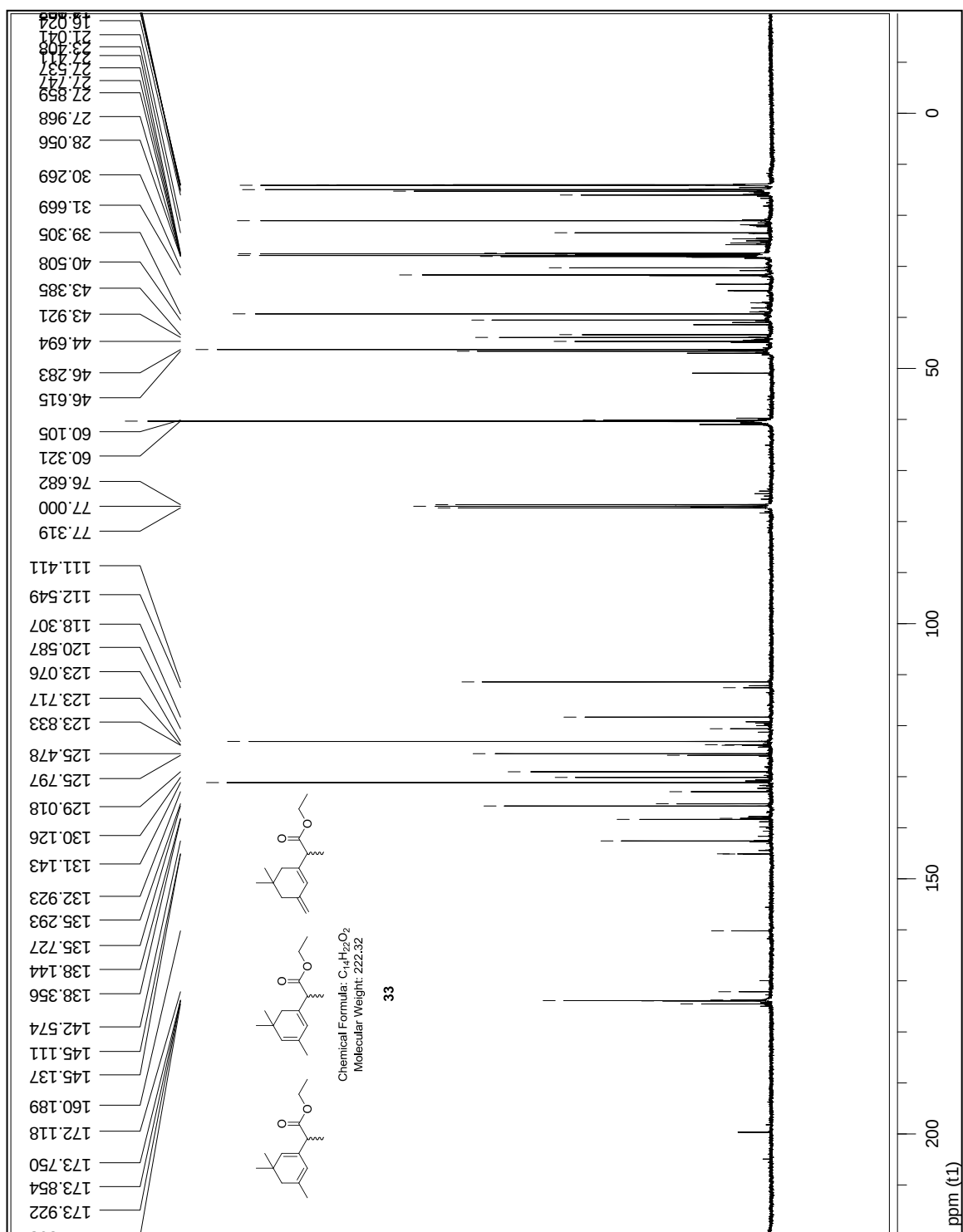
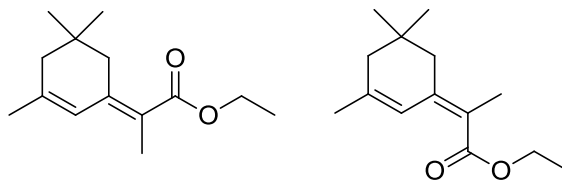


Figure 2.3.2.56. 100 MHz ^{13}C NMR spectrum of compound 33 in $CDCl_3$.

Preparation of Ethyl 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)propanoate



Chemical Formula: C₁₄H₂₂O₂

Molecular Weight: 222.32

33E/Z

This compound was prepared following general procedure 4 using compound **33** (0.7 g, 3.14 mmol) and a catalytic amount of PTSA in refluxing toluene. Purification via gradient column chromatography afforded **33 E/Z** as a yellow oil (0.6 g, 86%).

¹H NMR (400 MHz, CDCl₃) δ 6.90 (d, *J* = 1.4 Hz), 6.21 (d, *J* = 1.5 Hz), 4.20 (dd, *J* = 8.0, 7.1 Hz), 2.44 (d, *J* = 1.3 Hz), 2.09 (s), 1.93 (d, *J* = 14.6 Hz), 1.81 (d, *J* = 13.3 Hz), 1.30 (td, *J* = 7.1, 4.9 Hz), 0.91 (d, *J* = 15.0 Hz).

¹³C NMR (100 MHz, CDCl₃) δ 170.1, 169.5, 143.2, 142.9, 142.6, 140.9, 121.3, 120.7, 120.1, 119.9, 60.0, 59.9, 45.0, 44.6, 41.1, 40.8, 30.9, 30.9, 28.5, 28.3, 24.7, 24.6, 15.5, 14.7, 14.3, 14.3.

HRMS: Calculated for C₁₄H₂₂O₂ = 222.16198; found = 222.16376

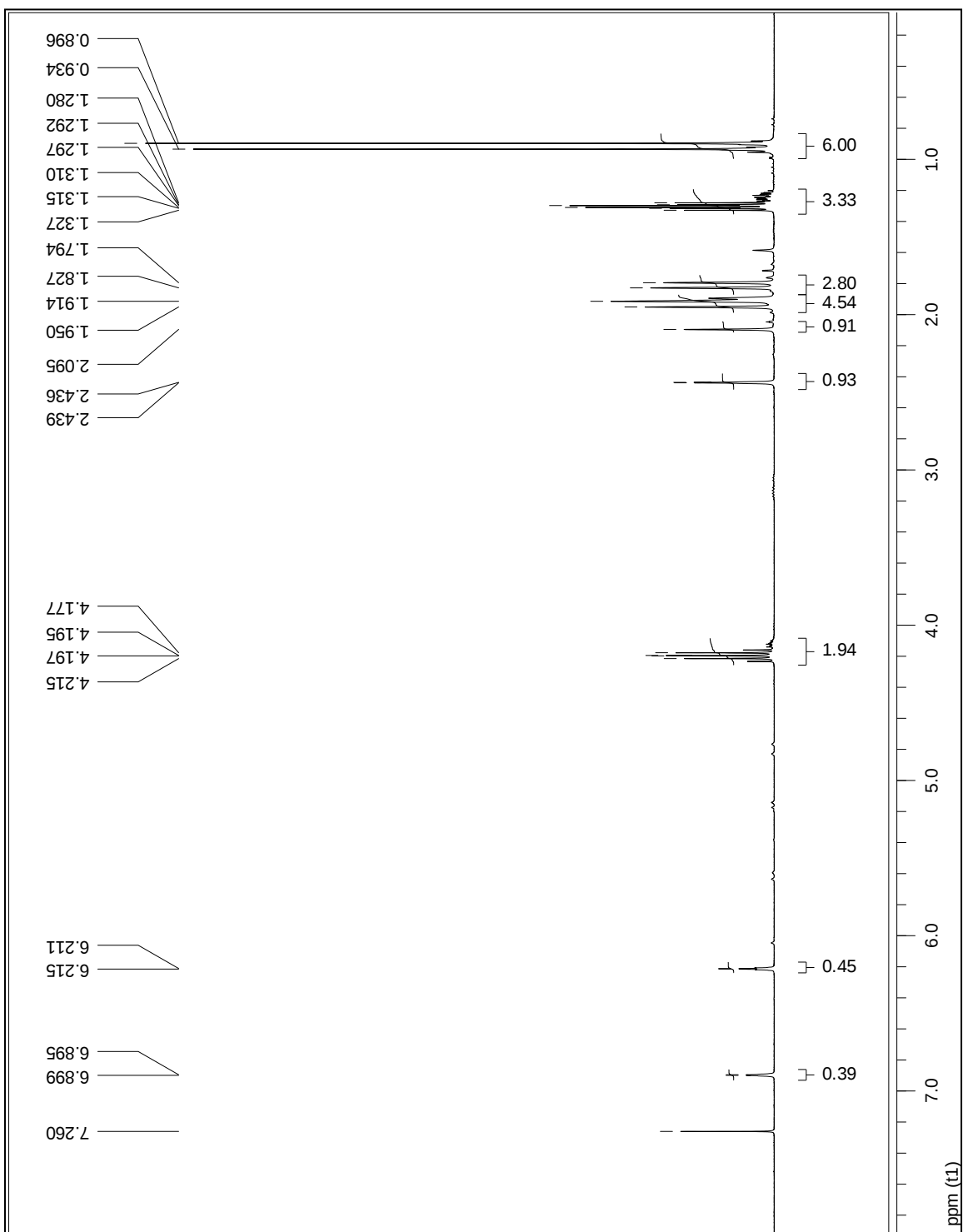


Figure 2.3.2.57. 400 MHz ^1H NMR spectrum of compound **33E/Z** in CDCl_3 .

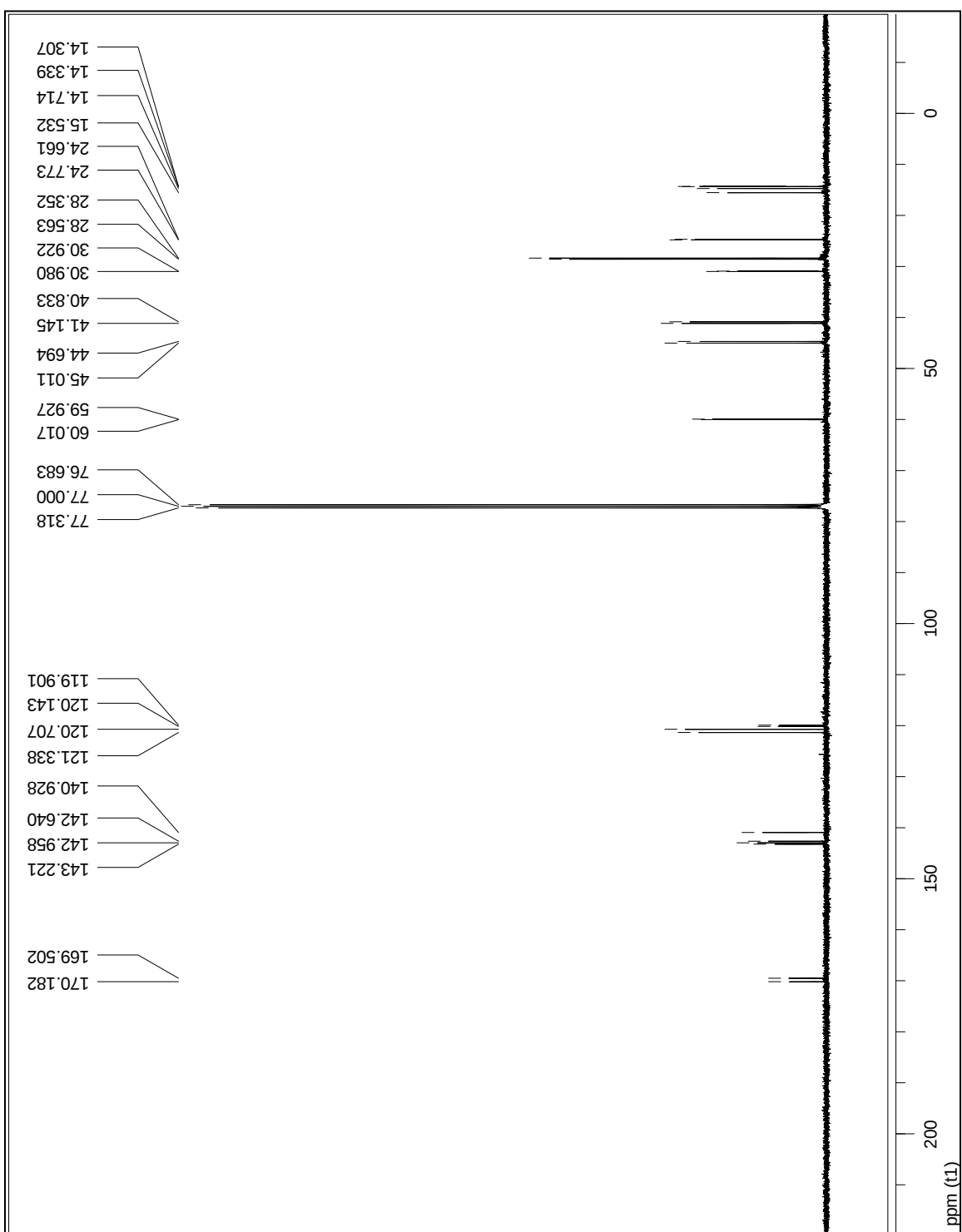
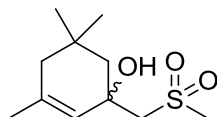


Figure 2.3.2.58. 100 MHz ^{13}C NMR spectrum of compound **33E/Z** in CDCl_3 .

Preparation of 3,5,5-trimethyl-1-((methylsulfonyl)methyl)cyclohex-2-enol



Chemical Formula: C₁₁H₂₀O₃S

Molecular Weight: 232.33

This compound was prepared following general procedure 2 using dimethyl sulfone (1.02 g, 10.85 mmol) in anhydrous THF (10 mL), LDA (5.4 mL, 10.85 mmol) and isophorone (0.5 g, 3.62 mmol) in anhydrous THF (5 mL). The crude product was purified via gradient column chromatography affording the product as a yellow oil (0.08 g, 9.5%)

¹H NMR (400 MHz, CDCl₃) δ 5.47 (s, 1H), 3.11 (t, *J* = 10.9 Hz, 2H), 2.96 (s, 3H), 1.81 – 1.60 (m, 7H), 0.93 (d, *J* = 18.5 Hz, 6H).

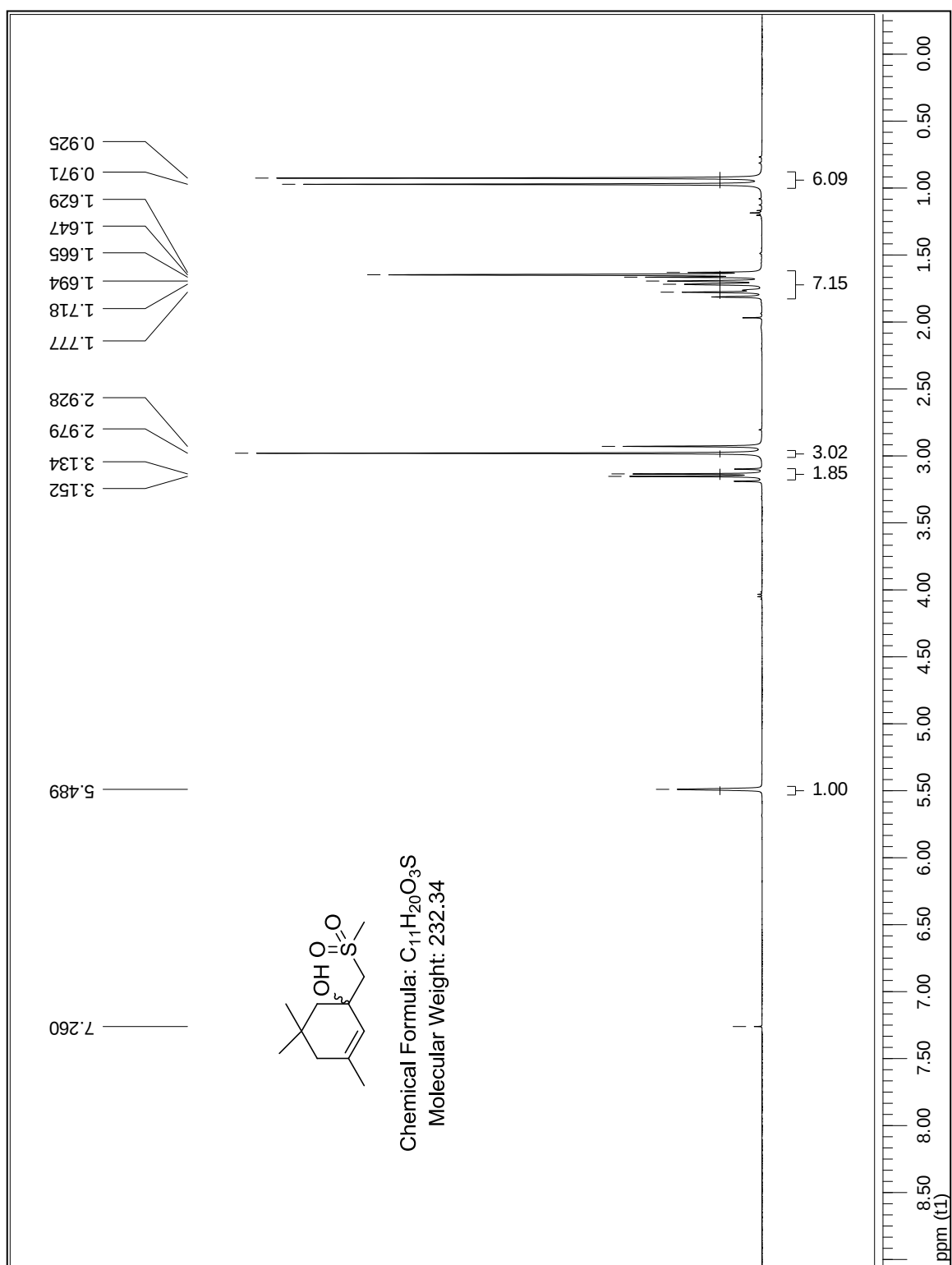
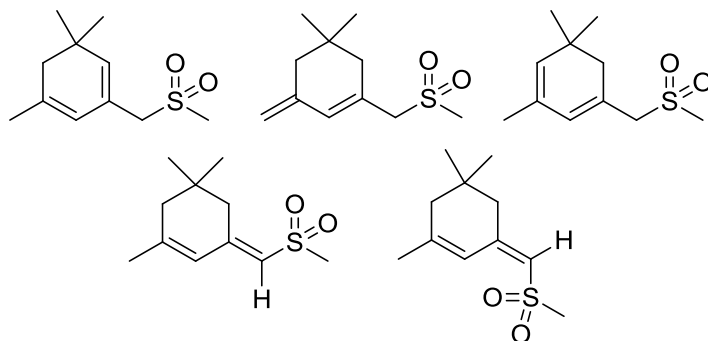


Figure 2.3.2.59. 400 MHz 1H NMR spectrum of methyl sulfone coupled product in $CDCl_3$.

Preparation of 1,5,5-trimethyl-3-((methylsulfonyl)methyl)cyclohexa-diene



Chemical Formula: C₁₁H₁₈O₂S
Molecular Weight: 214.32

34

This compound was prepared following general procedure 3 using the dimethyl sulfone coupled product (0.08 g, 0.34 mmol), in toluene (8 mL) containing a catalytic amount of PTSA. Reaction mixture was set to reflux for an additional 2 h to ensure reaction completion. The crude product was purified by gradient column chromatography (10-15% EtOAc in hexanes) affording **34** as a yellow oil (0.01 g, 14%).

¹H NMR (400 MHz, CDCl₃) δ 6.20 (s), 5.82 (d, *J* = 8.1 Hz), 5.76 – 5.67 (m), 5.41 (s), 5.25 (s), 4.89 (d, *J* = 16.2 Hz), 3.68 (d, *J* = 8.6 Hz), 3.61 (s), 2.93 (s), 2.84 (t, *J* = 3.2 Hz), 2.78 (s), 2.20 – 1.92 (m), 1.88 – 1.63 (m), 1.01 – 0.88 (m).

¹³C NMR (100 MHz, CDCl₃) δ 152.5, 151.3, 149.6, 148.3, 141.7, 140.1, 137.8, 136.9, 133.7, 132.6, 130.6, 128.6, 128.2, 127.8, 125.0, 123.3, 122.7, 122.4, 120.0, 118.7, 117.1, 114.3, 63.0, 62.5, 61.1, 43.2, 42.6, 42.7, 40.8, 39.2, 39.2, 38.6, 32.2, 31.9, 30.4, 27.8, 27.7, 27.7, 27.4, 23.3, 20.8.

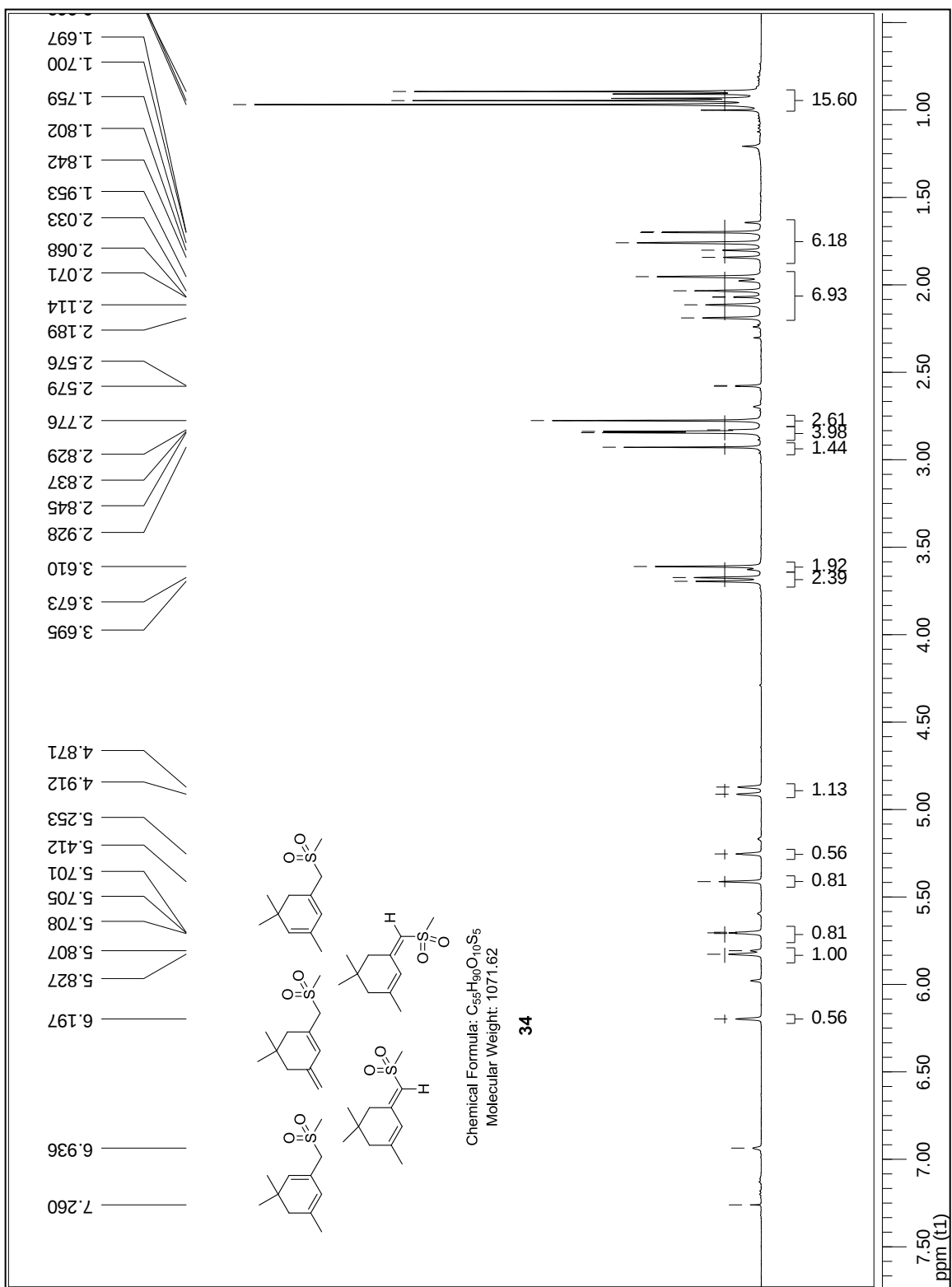


Figure 2.3.2.60. 400 MHz ^1H NMR spectrum of compound **34** in CDCl_3 .

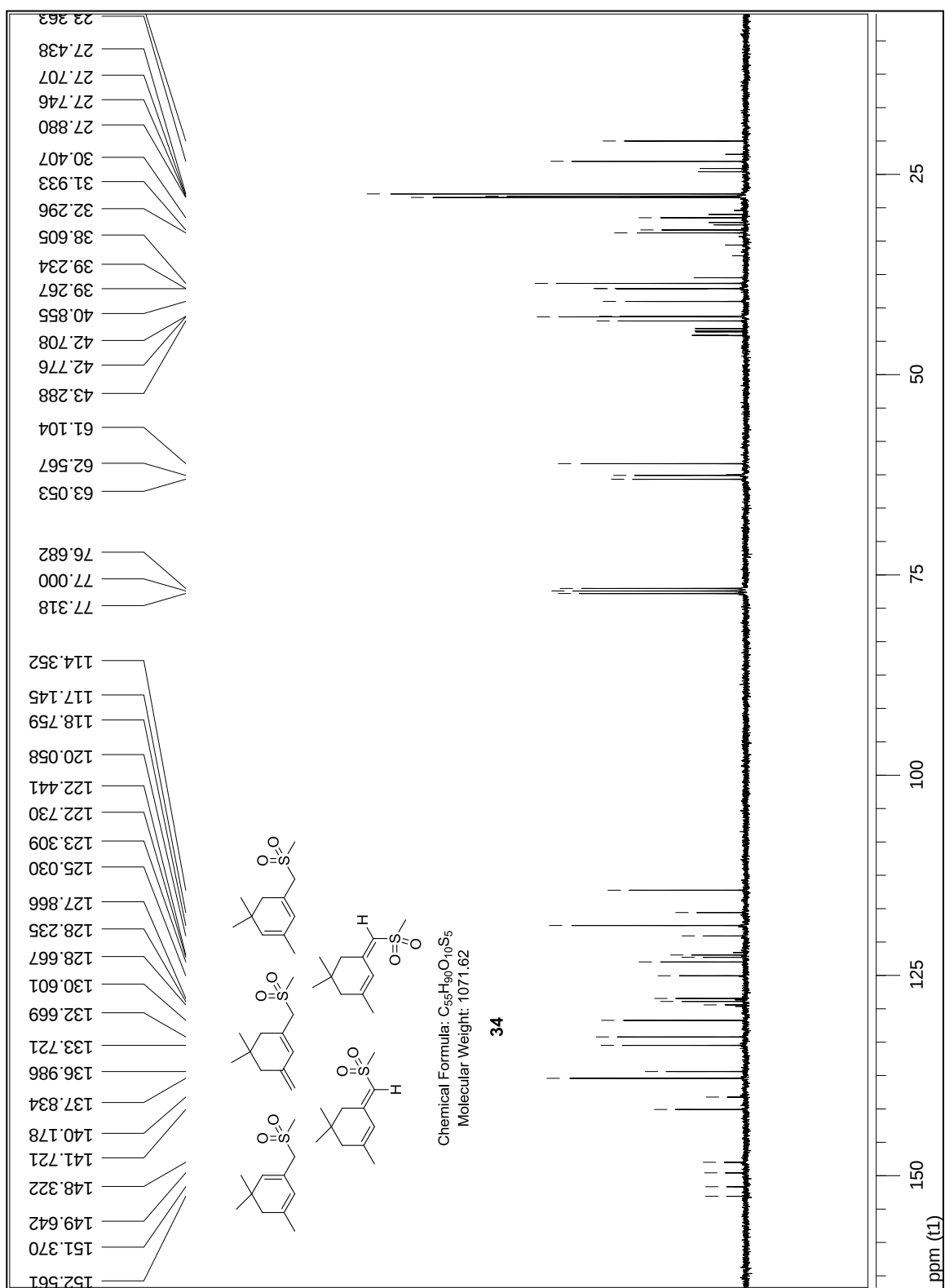


Figure 2.3.2.61. 100 MHz ^{13}C NMR spectrum of compound **34** in $CDCl_3$.

2.4. References

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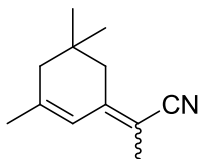
Claims to Original Research

1. The synthesis of 22 β -amino ketone, sulfoxide, sulfide and sulfone analogues for the use of potential selective connexin inhibitors. In the sulfoxide series, both possible diastereomers were prepared and identified via comparison of their ^1H NMR with the known parent compound [N-(1-phenyl-2-(phenylsulfinyl)ethyl)aniline]. These compounds are based on several lead structures identified in the Ph.D. thesis of Ana Francis Carballo Arce (University of Ottawa, 2013). The bioassay data for this set of compounds is not available at the time of the submission of the thesis.

A patent applicaion claiming these compounds has been filed in November 2014.

US 6218481 Neural Connexin Inhibitor Compounds and Methods of use Thereof.
Dr. Steffany Bennett, Graeme Taylor, Matthew Cooke, Dr. Tony Durst, Amanda Saikaley, Dr. Ana Francis Carballo Arce and Adrien Fluet-Chouinard.

2. The synthesis of 35 new ISOX [(E/Z)-1-(3,5,5-trimethylcyclohex-2-en-1-ylidene)propan-2-one] analogues for the treatment of epilepsy and seizures. Compounds **4**, **7**, **15**, **16** and **18** all showed significant activity in the bioassay developed by the Poutler group. Compound **16** is currently the gold standard amongst the newly synthesized compounds.



16 E/Z

A patent describing the synthesis and biological activity of the compounds described in this thesis is reported by Dr. Poulter to have been filed.

APPENDIX

1A. Bioassay summary for connexin blocker analogues

Materials and Methods

Cell culture

NT2/D1 cells (passage number 68-76) were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F12) (Invitrogen 12400, lot 1124811) supplemented with 10% Fetal Bovine Serum (Invitrogen 12483, lot 1129906), 1% Penicillin Streptomycin (Invitrogen 15140, lot 980006) and 1% L-Glutamine (Invitrogen 25030, lot 767886). Cells were maintained at 37°C and 5% CO₂ in 100 mm tissue culture dishes (BD Falcon 353003, lot 2062663).

RNA isolation

Total RNA from NT2/D1 cells, human hippocampus, QPCR Human Reference Total RNA (Stratagene 750500, lot 0006153539), and HeLa cells was isolated using TRIzol reagent (Invitrogen 15596018, lot 28120) following the manufacturer's recommended protocol. Briefly, cultures were washed with ice cold PBS and lysed with 1 ml of TRIzol reagent per 100 mm dish. Following a 5 minute incubation at room temperature, 200 µl of chloroform per 1 ml of TRIzol reagent was added to the homogenized cell lysates. Tubes were capped and shaken vigorously for 15 seconds and incubated at room temperature for an additional 3 minutes before being centrifuged at 12,000 g for 15 minutes at 4°C. Following centrifugation, the upper aqueous phase was transferred to a new 1.5 ml tube and 500 µl isopropanol per 1 ml of TRIzol reagent was added. Samples were then incubated for 10 minutes at room temperature to precipitate RNA and centrifuged at 12,000 g for 10 minutes at 4°C to pellet the RNA. Following centrifugation, the supernatant was removed and the pellet was washed with 1 ml 75% DEPC-ethanol per 1 ml TRIzol reagent and centrifuged at 7,400 g for 5 minutes at 4°C. The supernatant was then removed and RNA pellets were allowed to dry before being resuspended in nuclease-free water (Invitrogen 10977-015, lot 1237837).

DNase treatment

To eliminate genomic DNA contamination, RNA samples were treated with DNase-1 (Sigma-Aldrich D5307, lot SLBD2329V). 10 µg RNA was added to 10 µl of DNase-1, 4 µl 10X buffer (Sigma-Aldrich R6273, lot SLBC7096V) and nuclease-free water to a final reaction volume of 40 µl and incubated for 30 minutes at 37°C. After incubation, 160 µl nuclease-free water and 200 µl phenol/chloroform were added to each sample to precipitate RNA. Samples were then vortexed and centrifuged at 16,100 g for 1 minute at 4°C. The upper aqueous phase was transferred to a new tube, to which 22 µl 3 M sodium acetate and 900 µl ethanol were added. Samples were gently mixed and incubated overnight at -80°C. The following day, samples were centrifuged at 16,100 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed twice with 500 µl 75% DEPC-ethanol and spun at 16,100 g for 1 minute at 4°C. The supernatant was then removed and RNA pellets were allowed to dry before being resuspended in nuclease-free water.

Reverse transcription

Following DNase-1 treatment, RNA samples were reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen 18064-014, lot 924845C). For RT+ reactions, 2 µg DNase-1-treated RNA was added to 1 µl pdN6 random primers (Promega C1181, lot 0000017994) and nuclease-free water to a final volume of 12 µl. For reactions processed in the absence of RT reactions, 2 µg DNase-1-treated RNA was added to 1 µl pdN6 random primers and nuclease-free water to a final volume of 13 µl. For no template controls, 1 µl pdN6 random primers was added to 12 µl nuclease-free water. Samples were incubated at 70°C for 10 minutes and then 4°C for 2 minutes, at which point 4 µl 1st strand buffer, 2 µl DTT and 1 µl dNTPs (Fermentas 0181) were added to all reactions. Samples were then incubated at 37°C for 2 minutes and brought to 25°C, at which point 1 µl of reverse transcriptase was added to the RT reactions. Samples were then incubated at 25°C for 10 minutes, 42°C for 60 minutes, 50°C for 30 minutes, and brought to 4°C.

WST assay

Compound and extract toxicity in NT2/D1 cultures was tested using the WST-1 formazan dye assay (Roche 11644807001, lot 12426400). On day 0, 10,000 cells per well were plated in 96-well plates (BD Falcon 353072). On day 1, cells were treated in replicates of five for 24 hours with five concentrations of each compound or extract in regular growth medium. Vehicle concentration was held constant at 0.1% total medium. On day 2, cells were treated for 45 minutes at 37°C with WST-1 at a final concentration of 10% in regular growth medium. Absorbances were read at a test wavelength of 450 nm and a reference wavelength of 655 nm using an iMark Microplate Reader (Bio Rad). To determine cell numbers, a standard curve of untreated cells was also treated with WST-1. Compounds and extracts were treated at concentrations 10-fold lower than the median lethal concentration (LC₅₀) in each cell line. Compounds that did not exhibit any toxicity were treated at a maximal concentration of 100 µM.

Parachute Assay

The parachute assay was used to assess gap junctional intercellular communication as has been previously described (Goldberg, G. S. et al., BioTechniques 18: 490-497, 1995). On day 0, 2x10⁴ recipient NT2/D1 cells were plated in uncoated optical 96 well plates (Greiner Bio-One 655090). At this time, 5x10⁵ donor cells were also plated in a 60 mm tissue culture dish (Corning 430196). On day 1, donor cells were treated for 30 minutes with 4 µM calcein AM (Sigma-Aldrich C1359) and 10 µM 1,1'-Diocadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich 468495, lot 13021HO) in normal growth medium. Donor cells were then washed with PBS, trypsinized for 4 minutes with 0.05% trypsin-EDTA (Invitrogen 25300, lot 688247) and counted. Donor cells were then seeded on top of recipient cells at a density of 200 cells per well (1:100 donor to recipient ratio) and incubated with treatments and 0.1 µg/ml Hoechst 33342 trihydrochloride trihydrate (Invitrogen H1399, lot 908746) for 75 minutes in normal growth medium at 37°C prior to imaging. Vehicle concentration was held constant and did not exceed 0.1% total medium. Experiments were imaged on an Opera High Content Screening System (Perkin Elmer) at 20X magnification using the 20xW_UAPO20xW3/340_NA=0.7 objective. Hoechst was excited at a wavelength of 405 nm and its emission was detected at a wavelength of 450 nm. Calcein was excited at a wavelength of

488 nm and its emission was detected at a wavelength of 540 nm. DiI was excited at a wavelength of 561 nm and its emission was detected at a wavelength of 690 nm. Image analysis was performed using Columbus Image Analysis System version 2.3.0 (Perkin Elmer). Replicate experiments were analyzed with each treatment in replicates of 12 wells and 20 images per well. All plates were controlled with untreated cells, vehicle-treated cells, and cells treated with 10 and 100 μ M carbenoxolone disodium salt (C4790, lot 020M1257V).

Zero-calcium dye uptake assay

Uptake of Lucifer Yellow CH dilithium salt (Sigma L0259, lot MKBJ5898V) and Propidium Iodide (Invitrogen P1304MP, lot 890805) in the absence of extracellular calcium was used to assess hemichannel activity in NT2/D1 cells. On day 0, 2×10^4 NT2/D1 cells were plated in optical 96 well plates (Greiner Bio-One 655090, lot E1110CV) coated with 100 μ g/ml poly-L-lysine hydrobromide (Sigma-Aldrich P1274, lot 030K5101). On day 1, cells were incubated for 5 minutes in either 250 μ g/ml LY or 50 μ g/ml PI in PBS containing 1 mM EGTA. Cells were then washed with PBS and fixed with 3.7% formaldehyde in PBS for 10 minutes. Before imaging, cells were washed and incubated in 0.1 μ g/ml Hoechst 33342 in PBS for 30 minutes at room temperature. LY assays were controlled for dye uptake nonspecific to hemichannels using rhodamine B isothiocyanate dextran, 10,000 MW (RD) (Sigma R8881, lot 090M5307V). PI assays were controlled for dye uptake nonspecific to hemichannels using fluorescein isothiocyanate dextran, 10,000 MW (FITCD) (Sigma FD10S, lot 021M5302V). Experiments were imaged on an Opera High Content Screening System (Perkin Elmer) at 20X magnification using the 20xW_UAPO20xW3/340_NA=0.7 objective. Hoechst 33342 was excited at a wavelength of 405 nm and its emission was detected at a wavelength of 450 nm. LY was excited at a wavelength of 405 nm and its emission was detected at a wavelength of 600 nm. RD was excited at a wavelength of 561 nm and its emission was detected at a wavelength of 690 nm. PI was excited at a wavelength of 561 nm and its emission was detected at a wavelength of 690 nm. FITCD was excited at a wavelength of 488 nm and its emission was detected at a wavelength of 540 nm. Image analysis was performed using Columbus Image Analysis System version 2.3.0 (Perkin Elmer). Replicate experiments were analyzed with each treatment in replicates of 12 wells and 20 images per well. Controls included assays performed in normal growth medium and medium containing 100 μ M carbenoxolone.

2A. Bioassay summary for epileptic inhibitors

Materials and Methods

Slice Preparation and Staining

All animals used in these studies were adult male Sprague-Dawley rats aged 20–45 days. The preparation of brain slices and kindling methodology have been described in detail elsewhere (Gavrilovici et al., 2006). Slices prepared from kindled rats were usually about 45 days old. Control rats for these experiments were age matched but no electrode was implanted. Brain slices were incubated for 30 min in a solution that contained 0.6m Mofdyedi-4- ANEPPS (D-1199, Invitrogen Molecular Probes Inc., OR, USA). After washing for 10 min with ACSF slices were transferred to the recording chamber. During all recordings the slices were maintained at 32°C and continuously perfused with ACSF bubbled with a mixture of 95/5% oxygen and carbon dioxide. The slices were stimulated with a platinum/iridium electrode (MicroProbes, Inc., MD, USA) with a tip diameter of 200–300 µm at the border of the lateral olfactory tract (LOT) and layer I of the PCtx. The stimulation of each slice was in the range of 160–200 µA, each square pulse was 2.0ms in length. The electrode was connected to a stimulator (S88X dual output square pulse stimulator, Grass Technologies, Anastro-Med, Inc., QC, Canada), which controlled the pulse frequency and train duration.

Patch Clamp Recording

The whole cell patch clamp recording technique used and the preparation of brain slices from adult rats have been both described in detail elsewhere (McIntyre et al. 2002; Gavrilovici et al., 2012). The internal solution used in these experiments was in mM; K gluconate, 140; MgCl₂, 2; CaCl₂, 1; MgATP, 2; NaGTP, 0.2; EGTA, 1.1 and HEPES, 10. A multiclamp 700B amplifier was used to record from neurons located in layers II and III.

Optical Recording

The composition of ACSF used for optical recordings was the same composition used in the patch clamp recordings. Each recording was about 20s in length and consisted of two *époques*. The first was a 2s recording of background activity before the stimulus followed by the stimulus application for 1s with frequencies differing from 5 to 100Hz. The acquisition rate was between 3 and 10ms/frame. For each recording minimum camera saturation was set around 50% while the maximum was about 80%. Optical recording was conducted using a CMOS camera (MicamUltima, BrainVision, Inc., Tokyo, Japan) mounted on top of an upright microscope (FixedStageUprightMicroscope BX51WI, Olympus). The light from a 100 W halogen lamp source (HLX 64625, Microlites Scientific, Corp.) passed through an excitation filter ($\lambda = 530 \pm 10$ nm). The fluorescent signals were collected and projected onto the CMOS sensor through a long pass emission filter ($\lambda > 590$ nm). A long distance objective was used in these experiments (XLFluor4XN.A.0.28, Olympus). The movies were recorded and analyzed using Brain Vision Analyzer (Tokyo, Japan) software. The acquisition settings were: 100×100 pixels framesize, after magnification each represented $25\mu\text{m} \times 25\mu\text{m}$ space on the brain slice. The dye signal intensity decreases as the membrane depolarizes. However, to better match conventional recordings the signals all have been converted so that the excitatory and inhibitory signals were shown as positive and negative values. As bleaching can strongly affect the data, all recordings were corrected by subtracting the change in fluorescence that occurred in a region of the slice that was unresponsive to the stimulus. The fractional change in fluorescence signal relative to background signal ($\Delta F/F$) was calculated for each frame of each recording. For all the recordings, we binned 3×3 pixels into one representative signal. As there was considerable variability in the magnitude of the responses from slice to slice due to differences in loading of the dye, we normalized the recordings by dividing all signals by the response to the 20Hz stimuli. This permitted us to average the normalized responses between recordings. Thus, the input/output relationships shown are the normalized $\Delta F/F$. The lag time was calculated by measuring the time between the stimulus on set and the time for the signal to be 20% above baseline. Instead of using pixels bins, we measured the $\Delta F/F$ along a “stripe” that could be precisely placed along a group of pixels before and after the cut. Each stripe consisted of 10 pixels and covered $250\mu\text{m}$ length. The data derived from each stripe was the average $\Delta F/F$ of 10 pixels.