I) SYNTHESIS OF PLASMINOGEN ACTIVATOR (Pla) INHIBITORS IN *YERSINIA PESTIS*

II) DEVELOPMENT OF SMALL MOLECULE INHIBITORS TO RESTORE NUCLEAR p27 AS A THERAPEUTIC METHOD FOR ENDOMETRIAL CANCER

A Dissertation Presented to

the Faculty of the Department of Chemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Pawinee Wichienukul

May 2017

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ABSTRACT

Plasminogen activator (Pla) is an outer membrane protease produced by *Yersinia Pestis* (*Y. pestis*), a pathogenic bacterium that causes plague. Pla helps spread the bacteria from the primary infection site by activating a change from plasminogen to plasmin which degrades fibrin in human hemostatic system. Pla plays a very important role in the invasion of *Y. Pestis* as evidence in the increase of the median lethal dose (LD_{50}) by a million-fold when mice were infected with bacteria in which the plasmid gene encoded Pla was inactivated. For this reason, Pla has become an interesting target in drug design and development. To develop an inhibitor for Pla, a tripeptide substrate DABCYL-Arg-Arg-Ile-EDANS has been synthesized. This fluorophore-containing substrate was used in a fluorogenic method to study Pla activity and inhibitory assay. Identification of a lead compound was conducted at Harvard Medical School. Derivatization of the lead gave analogs which will be tested for Pla inhibition to obtain IC_{50}. Structure-activity relationship (SAR) study of the lead analogs can provide useful information to develop small molecule organic inhibitors for Pla and *Y. pestis* infection.

Endometrial carcinoma (EndoCa) is the most-common gynecologic malignancy and the fourth most common cancer in women in the United State. Along with kidney, prostate and breast cancer, EndoCa frequently loses (or mislocalizes) a tumor suppressor nuclear p27. p27 is a cyclin-dependent kinase inhibitor (CKI) that inactivate cyclin A- and cyclin E-CDK2 complexes in the nucleus to block cell cycle progression and inhibit cell growth. SCF E3 ligase (also called ubiquitin ligase) targets p27 for ubiquitination and further degradation. Interrupting the binding between the ligase and p27 could restore

p27 activity specifically in the nucleus of the cells. High-throughput virtual screening (HTS) was performed by our collaborator at MD Anderson Institute for Advancing Clinical Studies to identify a lead compound, (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide. Analogs of the lead compound has been synthesized and sent for EndoCa inhibition testing. Hydrogenation of the double bond or removing the aromatic (benzylic) side chain of the amide section caused the loss of EndoCa inhibition activity. The results indicated that the double bond and the amide side chain are essential for inhibition. This information will be used to continue to develop a drug candidate, providing a plausible therapeutic method for EndoCa.

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LIST OF SCHEMES

ABBREVIATIONS

Å	angstrom
AcOH	acetic acid
aq	aqueous
Bn	benzyl
$BnNH_2$	benzylamine
Boc	*tert*-butyloxycarbonyl
BOP	(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
δ	chemical shift
ºC	degree Celsius centigrade (unit)
^{13}C NMR	carbon nuclear magnetic resonance
d	doublet (spectra)
DABCYL	4-(dimethylaminoazo)benzene-4-carboxylic acid
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMSO	*N*,*N*-dimethyl sulfoxide
EDANS	5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid
EDC·HCl	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
equiv	equivalent
ESI	electrospray ionization
Et	ethyl
Et_3N	triethylamine
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	fluorenylmethyloxycarbonyl
g	gram
h	hour

HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
^{1}H NMR	proton nuclear magnetic resonance
HOBt	1-hydroxybenzotriazole
H_2O	water
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
Hz	hert
J	coupling constant (NMR spectroscopy)
L	liter
LC	liquid chromatography
m	milli (10^{-3})
	multiplet (spectra)
	meter
m	meta
M	mega (10^6)
	molar (mol L^{-1})
μ	micro (10^{-6})
Me	methyl
MeCN	acetonitrile
MeOH	methanol
min	minute
mol	mole
m/*z*	mass-to-charge ratio
Ms	mesyl (methylsulfonyl)
MS	mass spectrometry
	mass spectrum
	molecular sieves
n	nano (10^{-9})
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance

%	percent
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
ppm	parts per million
q	quartet (spectra)
rt	room temperature
s	singlet (spectra)
t	triplet (spectra)
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
Tris	tris(hydroxymethyl)aminomethane
Trt	trityl (tripheylmethyl)
UV	ultraviolet

CHAPTER 1: SYNTHESIS OF PLASMINOGEN ACTIVATOR (Pla) INHIBITORS IN *YERSINIA PESTIS*

1.1 Introduction

Yersinia pestis (*Y. pestis*), a gram-negative coccobacillus, is a pathogenic bacterium that causes plague.[1-2] It is the agent that is responsible for three human pandemics, the Justinian plague in 6th–8th centuries, the Black Death in 14th–18th centuries and modern plague in 19th century to the present day. *Y. pestis* was classified by the Centers for Disease Control and Prevention (CDC) as a “category A” agent, which is the highest priority of agents that pose a risk to national security for three reasons (i) it can be easily disseminated or transmitted from person to person, (ii) it results in high mortality rates and has the potential for major public-health impact, and (iii) it may cause public panic and social disruption because it requires special action for public-health preparedness.

Y. pestis is typically transmitted from an infected rodent to humans *via* a bite of an infected flea. Incubation period of plague is usually 1–6 days. The infection can take three main forms in humans, these are bubonic, septicemic, and pneumonic. The bubonic form is the most common type of infection occurring in lymph nodes. Septicemic is another form of infection occurring when the bacteria are spread directly through the circulatory system without evidence of an infection in lymph nodes. This form of the infection will turn the skin to deep purple shade due to the disseminated intravascular

coagulation (leading to the name “Black Death” pandemic in 14th century). Untreated patients often die within 24 hours after symptoms first appear. The most virulent but least common form of infection is the pneumonic form where the infection occurs in the lungs. This form of plague can spread from human-to-human through respiratory droplets. The infected patients will usually experience “flu-like” symptoms at the beginning and can die if not treated.

Y. pestis uses several mechanisms to infect host bodies. One important feature in the invasion of *Y. pestis* is the production of plague plasminogen activator (Pla), a cell surface-located protease. [3-4] Its invasive properties come from the ability of this enzyme to induce fibrinolysis. Pla is an outer membrane protease that can convert human plasminogen to plasmin which will degrade fibrin in human hemostatic system. This process enhances the spread of the bacteria from the primary infection site. It also activates the plasminogen activator urokinase which assists in turning plasminogen to plasmin. Moreover, Pla can indirectly enhance fibrinolysis by inactivating plasminogen activator inhibitor 1 (PAI-1), α_2-antiplasmin and thrombin-activatable fibrinolysis inhibitor (TAFI) which are agents that prevent the fibrin degradation (Figure 1.1). Pla plays a very important role in the invasion of *Y. pestis* as evidence in the increase of the median lethal dose (LD_{50}) by a million-fold when mice were infected with bacteria in which the plasmid gene encoded Pla was inactivated.[5] For this reason, Pla has become an interesting target in drug design and development.

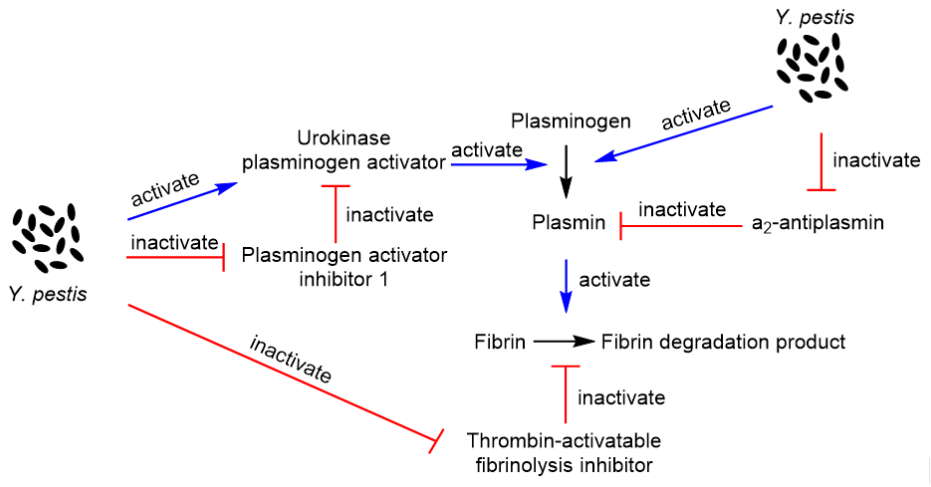


Figure 1.1 Interaction of plasminogen activator (Pla) from *Y. pestis* in the hemostatic system

The outer membrane protease Pla belongs to the omptin protease family. Omptins are found in many gram-negative pathogenic bacteria such as *Escherichia coli*, *Shigella flexneri*, and *Salmonella enterica*. Pla is encoded by a unique, 9.5-kb plasmid pPCP in *Y. pestis*. It is a 70 Å monomeric β-barrel protein with 10 antiparallel β-strands and its catalytic residues were identified as D84 (D=aspartic acid), D86, D206, and H208 (H=histidine). D84-D86 dyad is located on one side of the barrel while another D206-D208 dyad is on the opposite side.[4, 6-7] The catalytic residues of Pla are conserved within the omptin family. In addition, omptins share highly similar sequence and structure. So, it is hypothesized that if we can design a protease inhibitor for Pla, we should be able to use the information to design and develop inhibitors for other omptins as well.

Protease OmpT of *Escherichia coli*, a member of the omptin family was reported to hydrolyze proteins between two consecutive basic amino acids such as two

arginines.[6, 8] The same observation should also be applied for Pla since the active site is conserved within the omptin protease family.

To develop protease inhibitors, the process generally begins with the determination of the substrate specific to the protease of interest. One method generally used to study protease's enzyme activity is fluorometric assay. In the fluorometric assay of proteases, a peptide substrate is labeled with a fluorophore at one end and a quencher on the opposite end making the peptide non-fluorescent. In the present of a protease enzyme, the substrate is hydrolyzed at a cleavage site leaving half of the fluorescent-tagging peptide without the quencher. The peptide will then emit fluorescence which can be detected by a fluorescent plate reader.

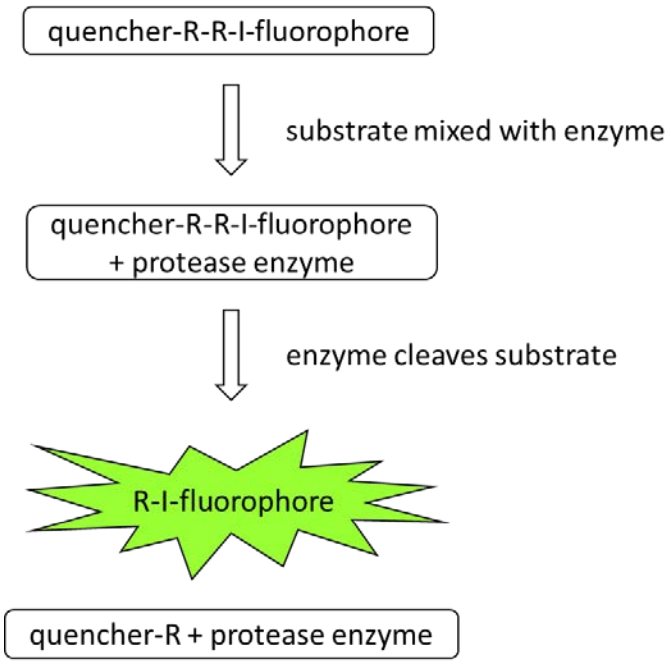
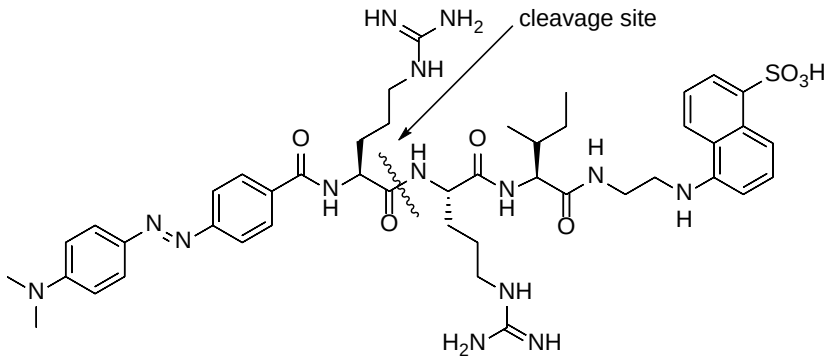


Figure 1.2 Fluorometric assay

Using a fluorometric assay, our group, in collaboration with Prof. Motin from UTMB, found that DABCYL-Arg-Arg-Ile-Asn-Arg-Glu-EDANS-NH_2 was a fluorogenic substrate for Pla.[8]

The cleavage site of the substrate was at a site between two basic arginine residues. The site of hydrolysis was determined by using LC-MS to analyze the reaction products. A shorter peptide with two arginines in the sequence, DABCYL-Arg-Arg-Ile-EDANS (Figure 1.3), was also found to be a substrate for Pla. Interestingly, at high concentration, this tripeptide acted as an inhibitor for Pla expressed by *Y. pestis* but did not inhibit mammalian plasminogen activator urokinase.[8]



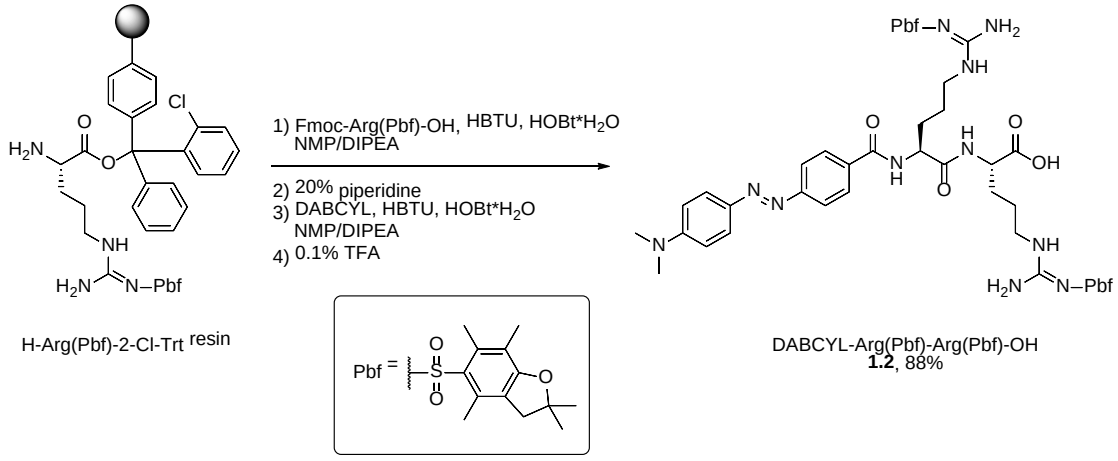
1.1

Figure 1.3 Structure of Pla's substrate DABCYL-Arg-Arg-Ile-EDANS (**1.1**)

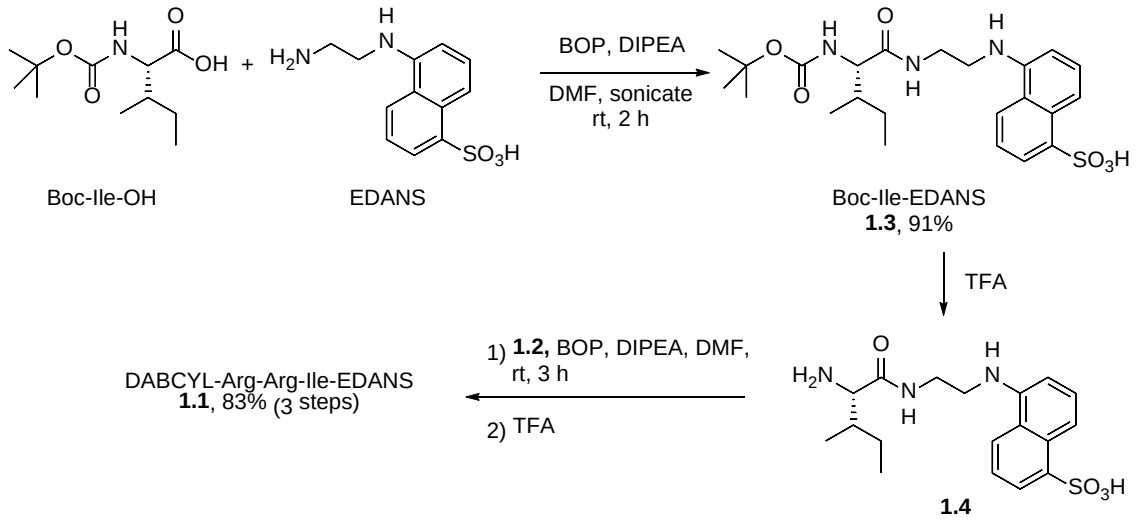
1.2 Results and Discussion

1.2.1 Synthesis of DABCYL-Arg-Arg-Ile-EDANS (1) Substrate

To study Pla inhibition, the substrate tripeptide DABCYL-Arg-Arg-Ile-EDANS (**1.1**) was synthesized as shown in Scheme 1.1. The Arg-Arg coupling step was done on a peptide synthesizer using commercially available H-Arg(Pbf)-2-Cl-Trt resin and Fmoc-Arg(Pbf)-OH, following DABCYL coupling with the dipeptide to give DABCYL-Arg(Pbf)-Arg(Pbf)-OH (**1.2**) after acid cleavage from the resin. The synthesis of Boc-Ile-EDANS (**1.3**) was carried out using commercially available Boc-Ile-OH. After Boc deprotection of **1.3**, followed by coupling with **1.2**, **1.1** was obtained after removal of the protecting groups.



Scheme 1.1 Synthesis of Pla's substrate DABCYL-Arg-Arg-Ile-EDANS (**1.1**)



Scheme 1.1 *Continued.*

1.2.2 Synthesis of Lead Analogs

To develop a small molecule organic inhibitor, a structure-activity relationship (SAR) study needs to be performed. To decide which compounds should be used as a lead compound in the study, high-throughput screening (HTS) was conducted at Harvard Medical School with about 54,000 compounds tested in 150 assays with one duplicate per assay. Each assay contained around 350 compounds. Around 150 hits that displayed high percent inhibition were considered as high-confident hits. The hits structures were identified and grouped based on similarity of their structures. The lead was chosen due to its availability and drug potential, as shown in Figure 1.4. Consider the structure of the tripeptide substrate, a structure of guanidine side chain of arginine is similar to isothiourea functional group in the lead compound. pKa value of isothiouronium group is around 10-11 which is less basic compare to arginine guanidinium group (pKa= 13.8 ± 0.1).

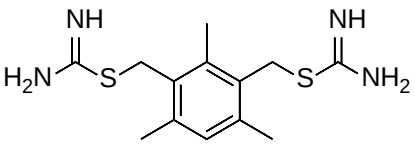
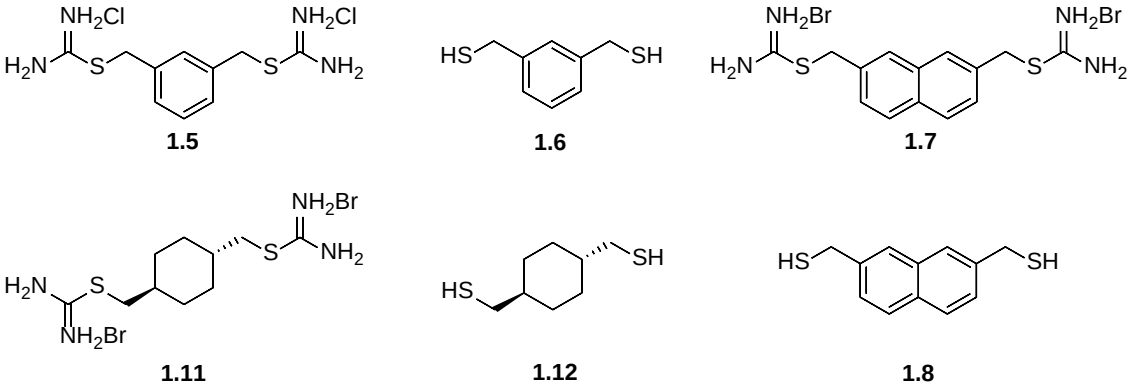


Figure 1.4 The lead compound chosen from HTS

After the lead was chosen, the structure was diversified. Based on a similarity of the lead and availability of starting materials, analogs of the leads were then synthesized (Figure 1.5). Structure of analog **1.5** was the closest to the lead with exception of trimethyl groups. It could be synthesized from commercially available 3-bis(chloromethyl)benzene. Analog **1.7** was synthesized to study an effect on an activity if the aromatic core structure was extended. Vice versa, analog **1.11** synthesized to see an affect if the aromatic ring was removed. Compounds **1.7** and **1.11** could be synthesized from 2,7-bis(bromomethyl)naphthalene and 1,4-cyclohexanedimethanol, respectively. Thiol versions of each analog (**1.6**, **1.8** and **1.12**) were also synthesized to see an effect on an activity if an isothiouronium group was removed (thiol pKa=10).

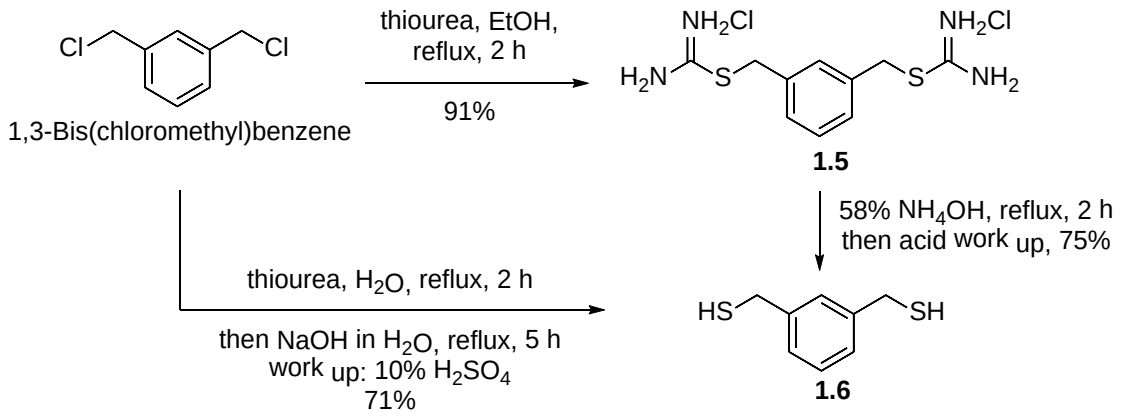


1.5 **1.6** **1.7**

1.11 **1.12** **1.8**

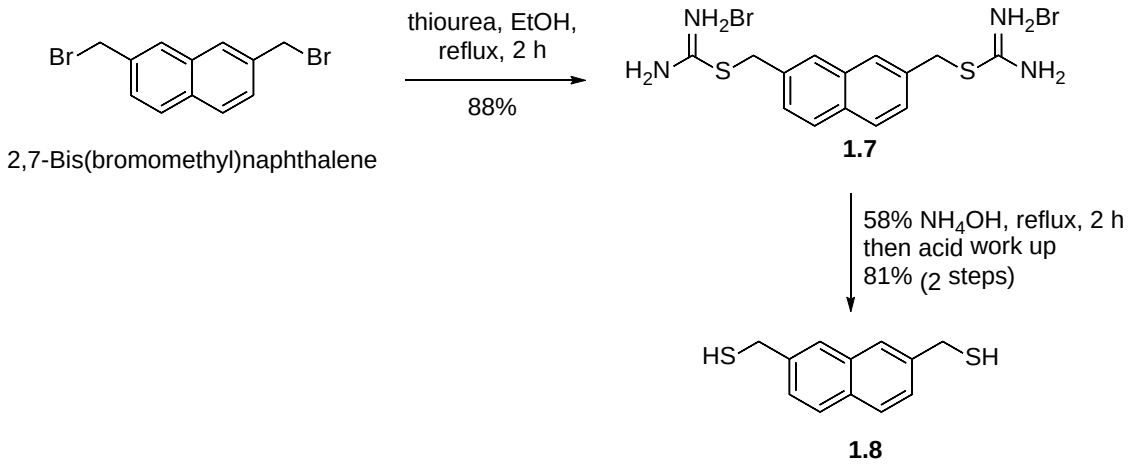
Figure 1.5 Analogs of the lead compound

The synthesis of compounds **1.5** and **1.6** is shown in Scheme 1.2. Having chloride as a leaving group, an S_N2 reaction was employed using thiourea as a nucleophile.[9-10] Treatment of commercially available 1,3-bis(chloromethyl)benzene with thiourea in EtOH under reflux, compound **1.5** was obtained in high yield as a white solid product that was easily separate by filtration. Basic hydrolysis of **1.5** using 58% NH_4OH under reflux followed by acid work up yielded thiol **1.6**. The thiol can also be made in one-pot conditions from 1,3-bis(chloromethyl)benzene by treatment with thiourea in water at reflux then the reflux was continued after an addition of aqueous NaOH followed by acid work up to give the product.



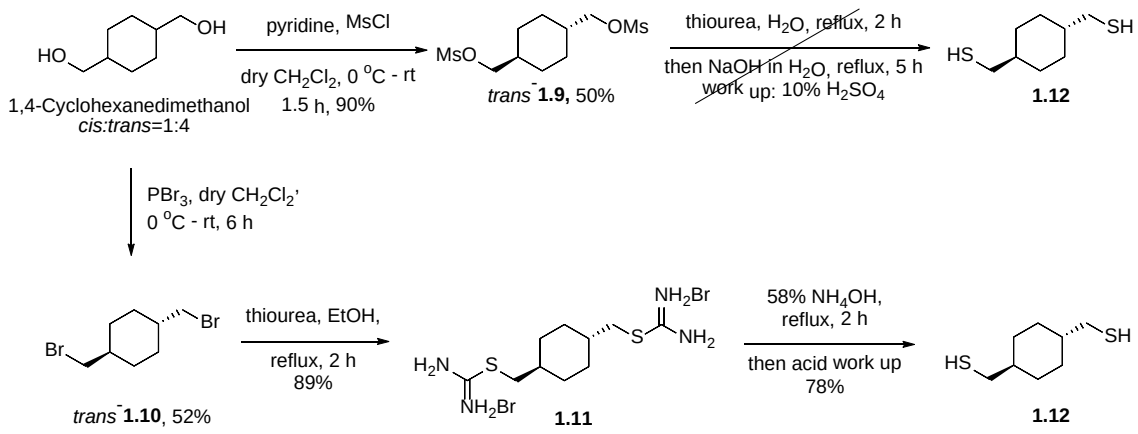
Scheme 1.2 Synthesis of compounds **1.5** and **1.6**

Compounds **1.7**, and **1.8** were synthesized from commercially available 2,7-bis(bromomethyl)naphthalene using a similar strategy to compounds **1.5** and **1.6**. (Scheme 1.3)



Scheme 1.3 Synthesis of compounds **1.7** and **1.8**

The synthesis of **1.10** and **1.11** started from a 1:4 *cis:trans* mixture of 1,4-cyclohexanedimethanol (the ratio of the mixture determine by ^{1}H NMR). To utilize the same strategy, the hydroxy groups of 1,4-cyclohexanedimethanol were converted to a good leaving group. The first attempt was to convert the hydroxy to OMs using MsCl and pyridine in CH_2Cl_2.[11] However, the OMs group was not a good enough leaving group in this case for the thiourea reaction. Thus, halide (in this case, bromide) was chosen to mimic the substrate of the reaction shown earlier. Bromination of 1,4-cyclohexanedimethanol using PBr_3 in CH_2Cl_2 gave pure *trans*-product (*trans*-**1.10**) in 52% after recrystallization from EtOH and CH_2Cl_2. The last two steps were similar to the synthesis of **1.5**, **1.6**, **1.7**, and **1.8** (Scheme 1.4).



Scheme 1.4 Synthesis of compounds **1.11** and **1.12**

1.3 Future Work and Conclusion

With the DABCYL-Arg-Arg-Ile-EDANS (**1.1**) substrate in hand, enzyme-substrate activity assay needs to be done. Unfortunately, a batch of 70 μg/mL Pla enzyme obtained from our collaborator Professor Motin from UTMB was denatured and lost its activity so the enzyme related assay has not been performed.[8]

For future work, Pla activity and inhibitory assays need to be optimized. The assay could be done using a condition similar to a previously study by our group in the screening of substrates in 10 mM Tris-HCl pH 8.0/150 nM NaCl (final concentration) buffer in 200 μL 96-well plate. Fluorescence could be detected using a plate reader (Synergy HY, Bio-Tek Instrument Inc., Vermont) at excitation and emission wavelengths of 360 and 460 nm, respectively.

Final concentration in 200µL 96-well plate
[S]=35µM
[Buffer]=10mM Tris-HCl/150nM NaCl buffer at pH=8.0

	1	2	3	4	5	6	7	8	9	10	11	12
A [E]µg/mL	35.00	17.50	8.75	4.38	2.19	1.09	0.55	0.27	0.14	0.07		control
B	35.00	17.50	8.75	4.38	2.19	1.09	0.55	0.27	0.14	0.07		control

Note: [E] in the control is zero.

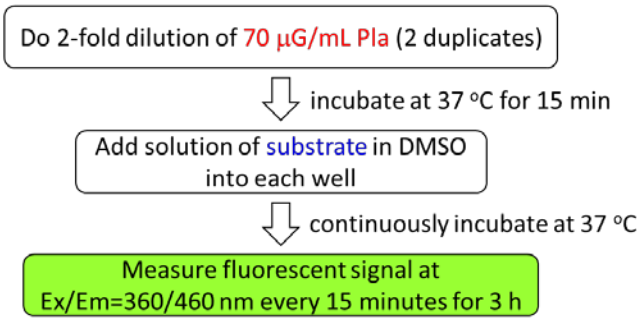


Figure 1.6 Procedure for Pla's activity measurement

To determine the quantity of active enzyme present, 2-fold dilution of the enzyme will be added 35 µM of the substrate (Figure 1.6). The fluorescence resulting from substrate cleavage will be measure every 15 minutes for 3 hours. The data will be used to determine rate of the reaction (Figure 1.7a). The active enzyme will cleavage the substrate making half of the fluorescent-tagging peptide fluoresce.

To determine *Vmax*, maximum reaction velocity, and *Km*, Michaelis–Menten constant, various substrate concentration will be mixed with the enzyme (Figure 1.8). Then, the fluorescence will be measure every 15 minutes for 3 hours. The data will be used to make a plot between velocity, obtained from Figure 1.7a graph, and substrate concentration (Figure 1.7b). This enzyme kinetic information (*Vmax* and *Km*) will be used to determine *Ki* (enzyme-inhibitor dissociation constant) afterward.

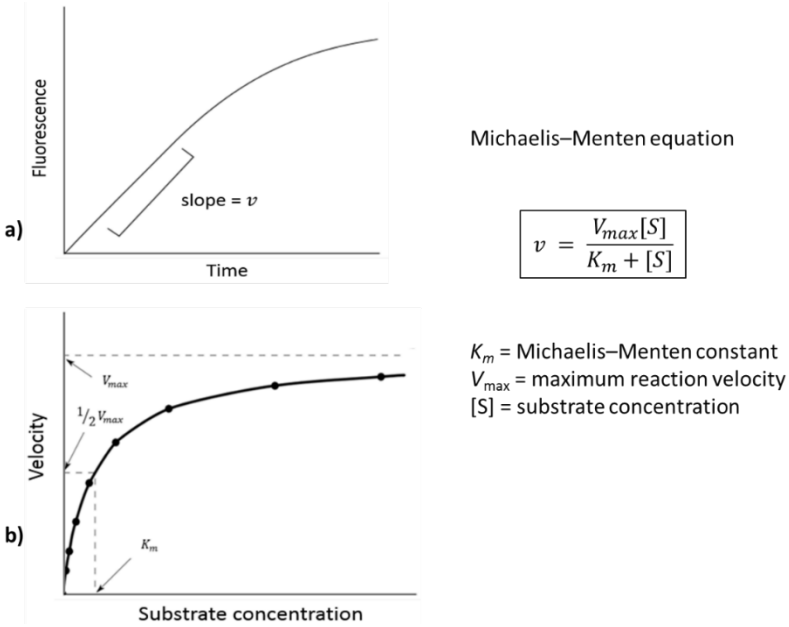


Figure 1.7 Enzyme kinetics **a)** substrate cleavage rate determined by a slop of a plot between fluorescence and time **b)** maximum reaction rate determined by a plot between velocity and substrate concentration

Final concentration in 200µL 96-well plate
[E]=35µg/mL
[Buffer]=10mM Tris-HCl/150nM NaCl buffer at pH=8.0

	1	2	3	4	5	6	7	8	9	10	11	12
A [S]µM	35.00	17.50	8.75	4.38								control
B	35.00	17.50	8.75	4.38								control

Note: [S] in the control is zero.

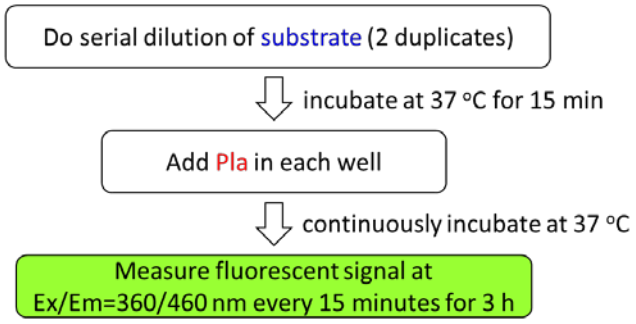
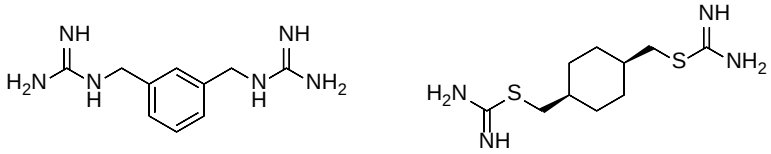


Figure 1.8 Procedure for substrate concentration optimization

SAR study could then be done by performing enzyme-inhibitor activity assay on each analog to determine the *Ki* value. The *Ki* is proportion to IC_{50} (the concentration of an inhibitor where the response is reduced by half). This information would lead to knowledge about the significant core structure for the inhibitor.



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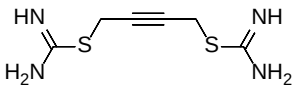

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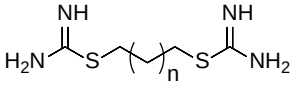


1.16

Figure 1.9 Proposed analogs for SAR study

Further derivatization of the lead needs to be carried out (Scheme 1.9). Since the cleavage site of the peptide substrate contain arginines, an analog with quinidine group (**1.13**) may be synthesized to mimic the substrate and tested for an inhibition. Diastereomer of analog **1.11** (**1.14**) (*cis*-isomer) may also be synthesized and tested to determine an activity. Analogs **1.15** and **1,16** with alkynyl and alkyl group between two isothiourea groups could be synthesized to study an effect of a flexible core structure to an activity.

In conclusion, DABCYL-Arg-Arg-Ile-EDANS (**1.1**) Pla substrate has been made. The substrate would then be use to optimize the assay for Pla activity and inhibitory assays. Various analogs of the lead compound obtained from high-throughput screening

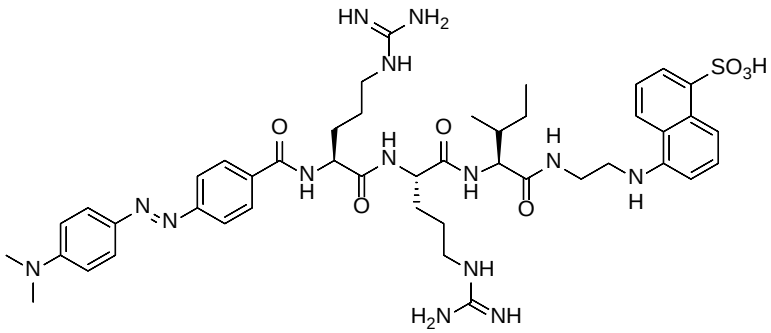
need to be synthesized and would be use to study a relation between analog structure and the inhibitory activity. This information can be used to develop a small-molecule organic inhibitor for Pla and other omptins and will be one of a therapeutic method for *Y. pestis* infection.

1.4 Experimental Procedures

General:

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich, Acros or AAPPTec and used without future purification. Analytical HPLC was carried out on Thermo Scientific™ BetaBasic™ 8 (5 μm, 4.6×100 mm) column with flow rate 0.8 mL/min. Preparative HPLC was performed on XBridge™ C_{18} (5 μm, 19×150 mm) column with flow rate 10 mL/min. Same solvent system was used for analytical and preparative HPLC, which is 0.1% TFA in H_2O and 0.1% TFA in MeCN. Liquid chromatography-mass spectrometry (LC-MS) was performed on a Thermo Scientific™ Finnigan Surveyor instrument equipped with MSQ Plus™ single quadrupole detector. Thin layer chromatography (TLC) was performed on silica gel 60 Å F-254 pre-coated plates (250 μm) from SiliCycle® and components were visualized by UV light (254 nm) and/or 10% phosphomolybdic acid in EtOH stain, anisaldehyde or $KMnO_4$ solution. Chromatography was performed using SiliCycle® silica gel 230-400 (particle size 40-63 μm) mesh. Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were obtained on JEOL ECX-400 NMR, ECA-500 or ECA-600II spectrometer and processed using software MestReNova 6.1.0. Chemical shifts are reported in parts per million (ppm, δ). Multiplicity is indicated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet. Coupling constants (*J*) are reported in Hz.

Procedure for peptide synthesis

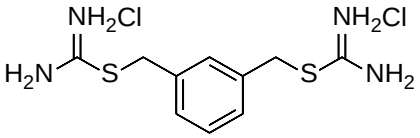


1.1

The Arg-Arg coupling step was done on solid phase using H-Arg(Pbf)-2-Cl-Trt resin. The resin was treated with 5 equiv of Fmoc-Arg(Pbf)-OH, HBTU, HOBt and 10 equiv of DIPEA in NMP for 1 h. The resin was treated twice with the same mixture for 30 min. Fmoc deprotection was done by treating the resin twice with 20% piperidine in DMF for 5 min. The resin was treated with 5 equiv of DABCYL, HBTU, HOBt and 10 equiv of DIPEA in NMP for 1 h and the resin was treated twice with the same mixture for 30 min. For the resin cleavage, 0.1% TFA in CH_2Cl_2 was used.

The Ile-EDANS coupling was done in solution phase using BOP as a coupling reagent. A mixture of 1 equiv of Boc-Ile-OH, 1 equiv of EDANS, 1 equiv of BOP and 2.5 equiv of DIPEA in DMF was sonicate at room temperature for 2 h to give Boc-Ile-EDANS. The Boc group was deprotected with TFA and the coupling between DABCYL-Arg-Arg with Ile-EDANS was done in solution phase using 1 eq of BOP and 2.5 eq of DIPEA in DMF as a coupling reagent. Pbf group was then deprotected with TFA. The purification was done by HPLC. ESI-MS (*m/z*) 942.87.

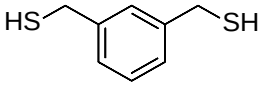
Preparation of 1,3-bis(isothiouroniummethyl)benzene hydrochloride (1.5)



1.5

A mixture of 1,3-bis(chloromethyl)benzene (8.75 g, 50 mmol) and thiourea (9.51 g, 125 mmol) in EtOH (30 mL) was refluxed for 2 h under a nitrogen atmosphere. The mixture was cooled to room temperature and a white solid was collected by vacuum filtration to give 1,3-bis(isothiouroniummethyl)benzene hydrochloride (**1.5**) (15 g, 91%). ^{1}H NMR (400 MHz, DMSO-D6) δ 9.39 (s, 8H), 7.49 (s, 1H), 7.40 (m, 3H), 4.56 (s, 4H); ^{13}C NMR (101 MHz, DMSO-D6) δ 141.5, 128.9, 127.7, 126.7, 101.5, 28.8. ESI-MS (*m/z*) 326.11.

Preparation of 1,3-phenylenedimethanethiol (1.6)



1.6

Method A:

A mixture of 1,3-bis(isothiouroniummethyl)benzene hydrochloride (**1.5**) (3.27 g, 10 mmol) and 58% NH_4OH (2.70 mL, 40 mmol) in H_2O (5 mL) was refluxed for 2 h. The reaction was cooled to room temperature and acidified with 10% aq H_2SO_4. The mixture was extracted with EtOAC (3x30 mL) and the combined EtOAc layer was washed with H_2O (1x30 mL) and brine (1x30 mL). EtOAc layer was dried over Na_2SO_4 and the solvent

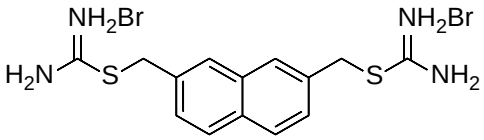
was removed under reduced pressure. The crude mixture was purified by flash column chromatography using CH_2Cl_2 as an eluent to provide 1,3-phenylenedimethanethiol (**1.6**) (1.27 g, 75%) as a colorless oil.

Method B:

A mixture of 1,3-bis(chloromethyl)benzene (350 mg, 2 mmol) and thiourea (380 mg, 5 mmol) in H_2O (4 mL) was refluxed for 2 h. The mixture was cooled to room temperature and a solution of NaOH (800 mg, 20 mmol) in H_2O (10 mL) was added. The reaction mixture was refluxed for 5 h and then cooled to room temperature. The mixture was acidified using 10% aq H_2SO_4 and extracted with EtOAc (3x10 mL). The organic layer was washed with with H_2O (1x10 mL) and brine (1x10 mL). EtOAc layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude mixture was purified by flash column chromatography using CH_2Cl_2 as an eluent to provide 1,3-phenylenedimethanethiol (**1.6**) (240 mg, 71%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (dd, *J* = 8.3, 6.4 Hz, 2H), 7.21 (d, *J* = 1.2 Hz, 1H), 7.20 (s, 1H), 3.73 (d, *J* = 7.6 Hz, 4H), 1.78 (t, *J* = 7.6 Hz, 2H).; ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.7, 129.2, 127.9, 126.9, 29.0. ESI-MS (*m/z*) 172.04.

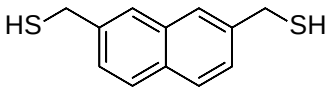
Preparation of 2,7-bis(isothiouroniummethyl)naphthalene hydrobromide (1.7)



1.7

A mixture of 2,7-bis(bromomethyl)naphthalene (157 mg, 0.50 mmol) and thiourea (95 mg, 1.25 mmol) in EtOH (4 mL) was refluxed for 2 h under a nitrogen atmosphere. The mixture was cooled to room temperature and solvent was removed under reduced pressure. The crude mixture was used in a next step without future purification. For characterization purposes, the crude mixture was recrystallized in EtOH to give 2,7-Bis(isothiouroniummethyl)naphthalene hydrobromide (**1.7**) (206 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s, 4H), 7.56 (s, 2H), 7.47 (d, *J* = 25.5 Hz, 4H), 4.50 (s, 4H), 3.07 (d, *J* = 19.0 Hz, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 136.5, 134.8, 133.6, 128.4, 127.9, 125.7, 101.5, 36.1. ESI-MS (*m/z*) 465.97.

Preparation of 2,7-naphthalenedimethanethiol (1.8)

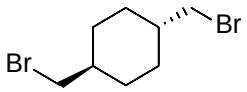


1.8

A crude 2,7-Bis(isothiouroniummethyl)naphthalene hydrobromide (**1.7**) and 58% NH_4OH (0.14 mL, 2 mmol) in H_2O (3 mL) were refluxed for 2 h. The reaction was cooled to room temperature and acidified with 10% aq H_2SO_4. The mixture was extracted with EtOAC (3x10 mL) and the combined EtOAc layer was washed with H_2O (1x10 mL) and

brine (1x10 mL). EtOAc layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude mixture was purified by flash column chromatography using CH_2Cl_2 as an eluent to provide 2,7-naphthalenedimethanethiol (**1.8**) (89 mg, 81% over 2 steps) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, *J* = 6.0 Hz, 2H), 7.33 (d, *J* = 6.7 Hz, 2H), 7.28 (d, *J* = 1.2 Hz, 2H), 3.80 (d, *J* = 7.6 Hz, 4H), 1.85 (t, *J* = 7.6 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.3, 135. 3, 133.2, 128.7, 127.5, 126.5, 28.6. ESI-MS (*m/z*) 222.01.

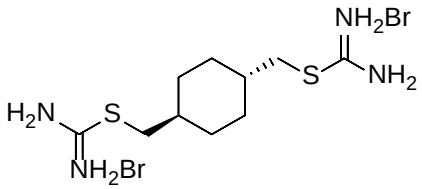
Preparation of *trans*-1,4-bis(bromomethyl)cyclohexane (*trans*-1.10)



***trans*-1.10**

To a cooled solution of PBr_3 (1.0 mL, 11 mmol) in dry CH_2Cl_2 (15 mL) was added dropwise 1,4-cyclohexanedimethanol (721 mg, 5 mmol). The reaction was allowed to warm to room temperature and stirred for 6 h under a nitrogen atmosphere. The reaction mixture was washed with $NaHCO_3$ (2x10 mL), H_2O (1x10 mL) and brine (1x10 mL). Organic layer was dried over $MgSO_4$ and solvent was removed under reduced pressure. The crude product was recrystallized in EtOH and CH_2Cl_2 to provide *trans*-1,4-bis(bromomethyl)cyclohexane (***trans*-1.10**) (703 mg, 52%) as a white crystal. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.30 (d, *J* = 6.3 Hz, 4H), 1.95 (d, *J* = 7.0 Hz, 4H), 1.63 (m, 2H), 1.07 (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 40.1, 39.9, 31.1. ESI-MS (*m/z*) 269.95.

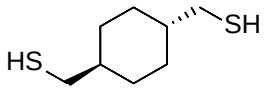
Preparation of *trans*-1,4-bis(isothiouroniummethyl)cyclohexane hydrobromide (1.11)



1.11

A mixture of *trans*-1,4-bis(bromomethyl)cyclohexane (***trans*-1.10**) (540 mg, 2 mmol) and thiourea (380 mg, 5 mmol) in EtOH (16 mL) was refluxed for 2 h under a nitrogen atmosphere. The mixture was cooled to room temperature and solvent was removed under reduced pressure. The crude mixture was recrystallized in EtOH to give *trans*-1,4-bis(isothiouroniummethyl)cyclohexane hydrobromide (**1.11**) (750 mg, 89%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.05 (d, *J* = 6.3 Hz, 4H), 3.01 (s, 6H), 1.90 (m, 4H), 1.73 (m, 2H), 1.08 (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 74.4, 37.4, 37.3, 28.2. ESI-MS (*m/z*) 422.03.

Preparation of *trans*-1,4-bis(mercaptomethyl)cyclohexane (1.12)



1.12

A mixture of *trans*-1,4-bis(isothiouroniummethyl)cyclohexane hydrobromide (**1.11**) (422 mg, 1 mmol) and 58% NH_4OH (0.27 mL, 4 mmol) in H_2O (3 mL) was refluxed for 2 h. The reaction was cooled to room temperature and acidified with 10% aq H_2SO_4. The mixture was extracted with EtOAc (3x10 mL) and the combined EtOAc layer was

washed with H_2O (1x10 mL) and brine (1x10 mL). EtOAc layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude mixture was purified by flash column chromatography using CH_2Cl_2 as an eluent to provide *trans*-1,4-bis(mercaptomethyl)cyclohexane (**1.12**) (137 mg, 78%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.84 (d, *J* = 6.9 Hz, 4H), 1.99 (m, 4H), 1.68 (m, 2H), 1.46 (m, 2H), 1.06 (td, *J* = 9.4, 3.3 Hz, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 37.4, 37.3, 28.2. ESI-MS (*m/z*) 178.34.

1.5 References

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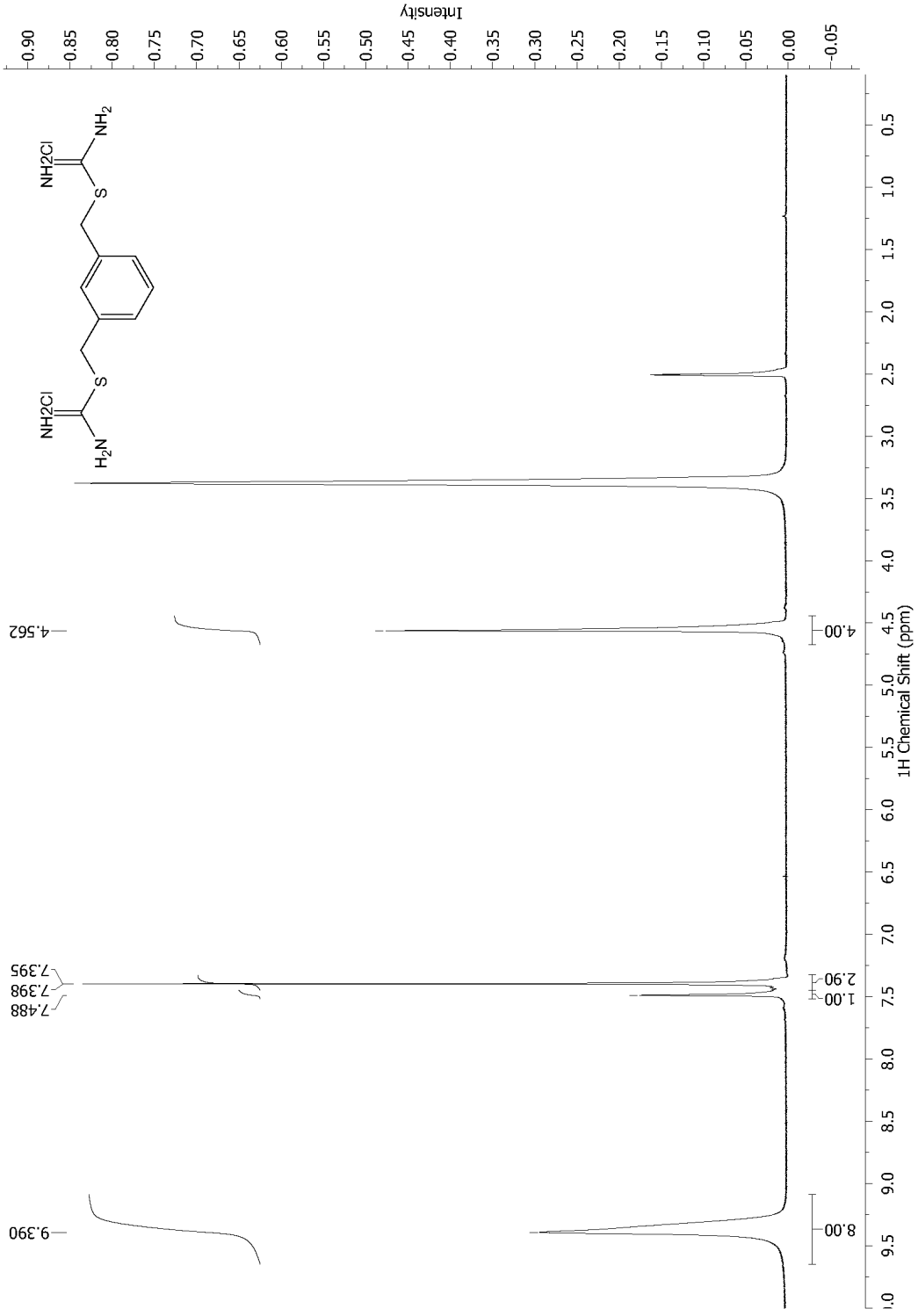
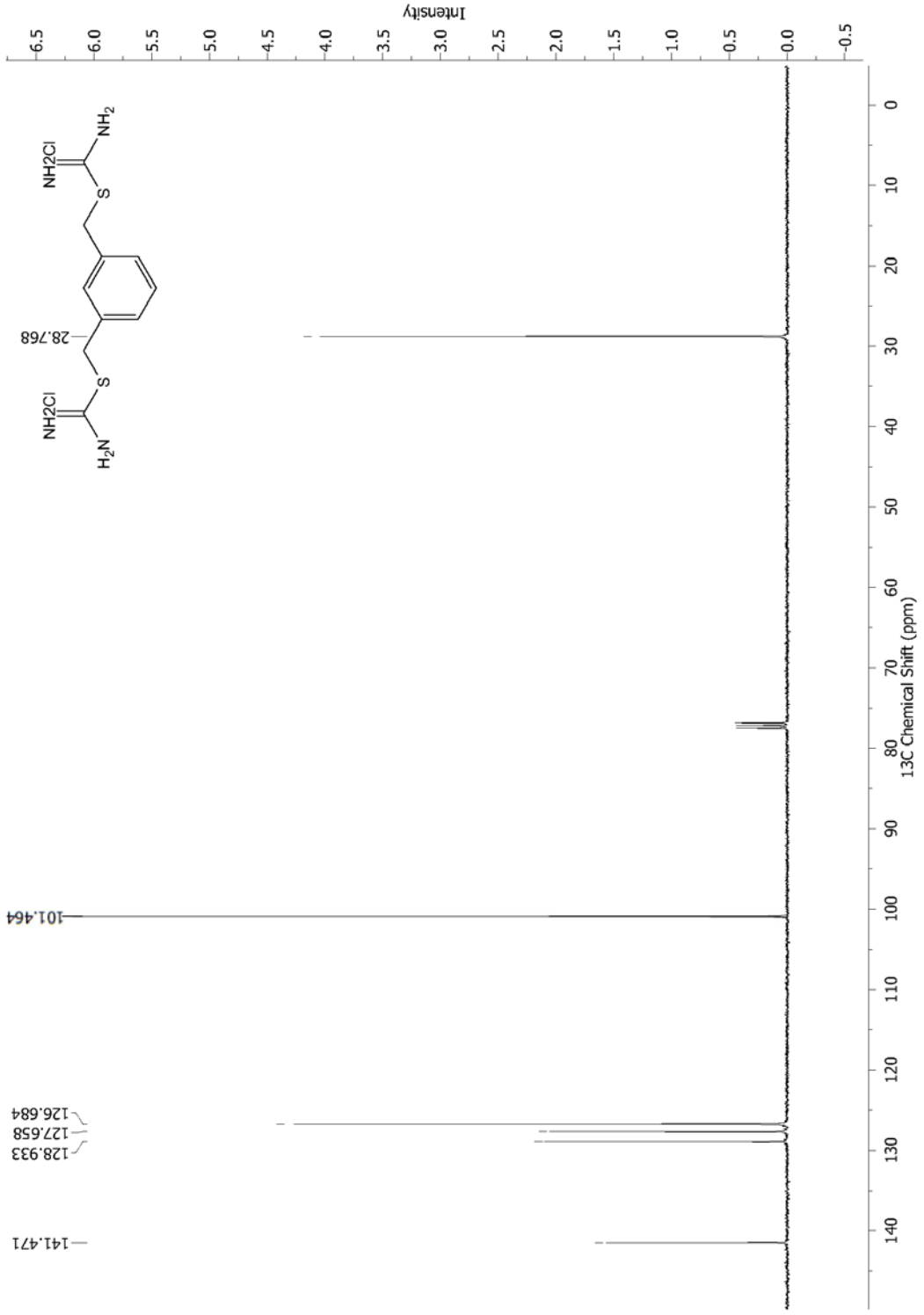
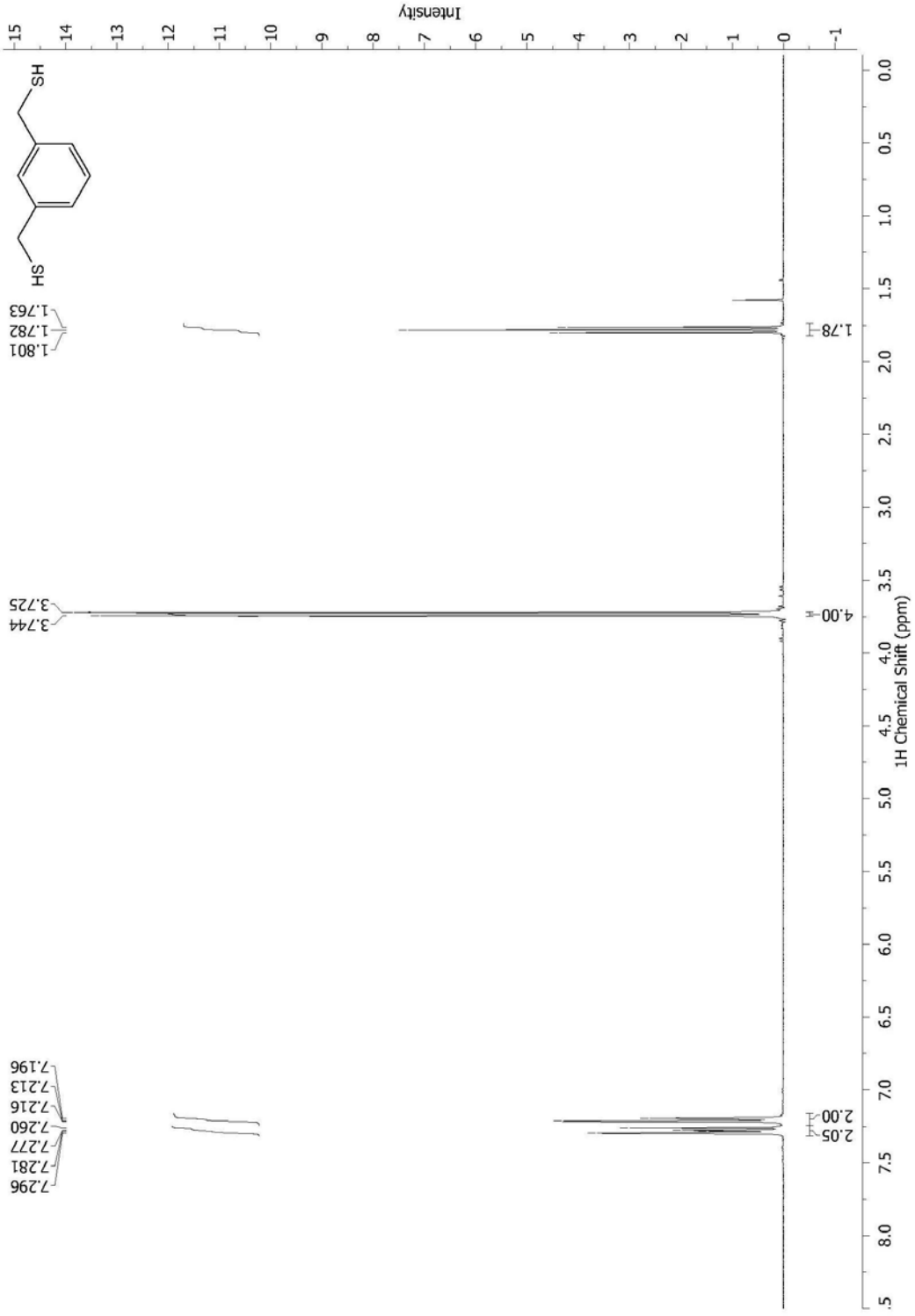
SUPPORTING INFORMATION



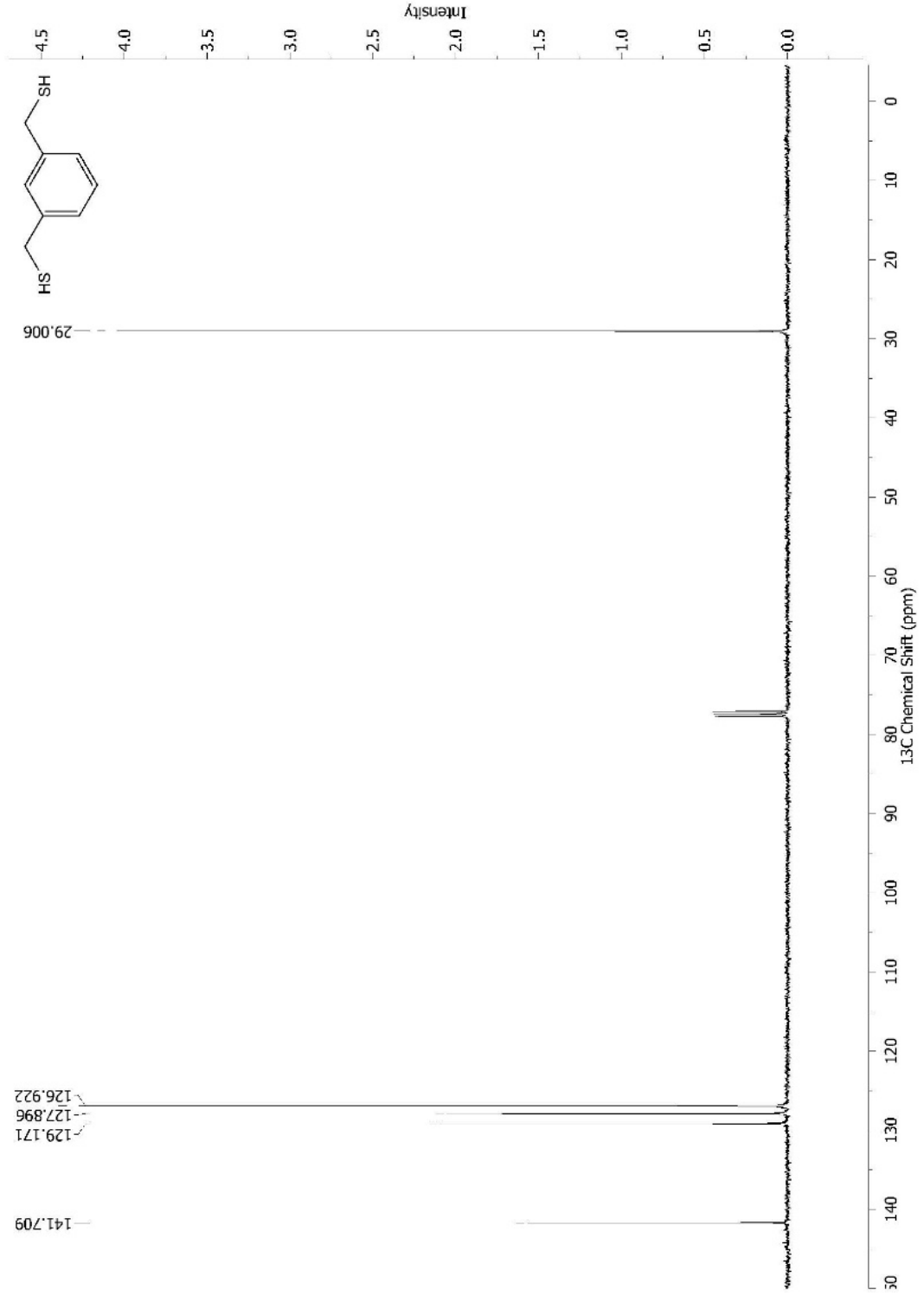
^{1}H NMR spectrum (400 MHz, DMSO-D6) of compound **1.5**



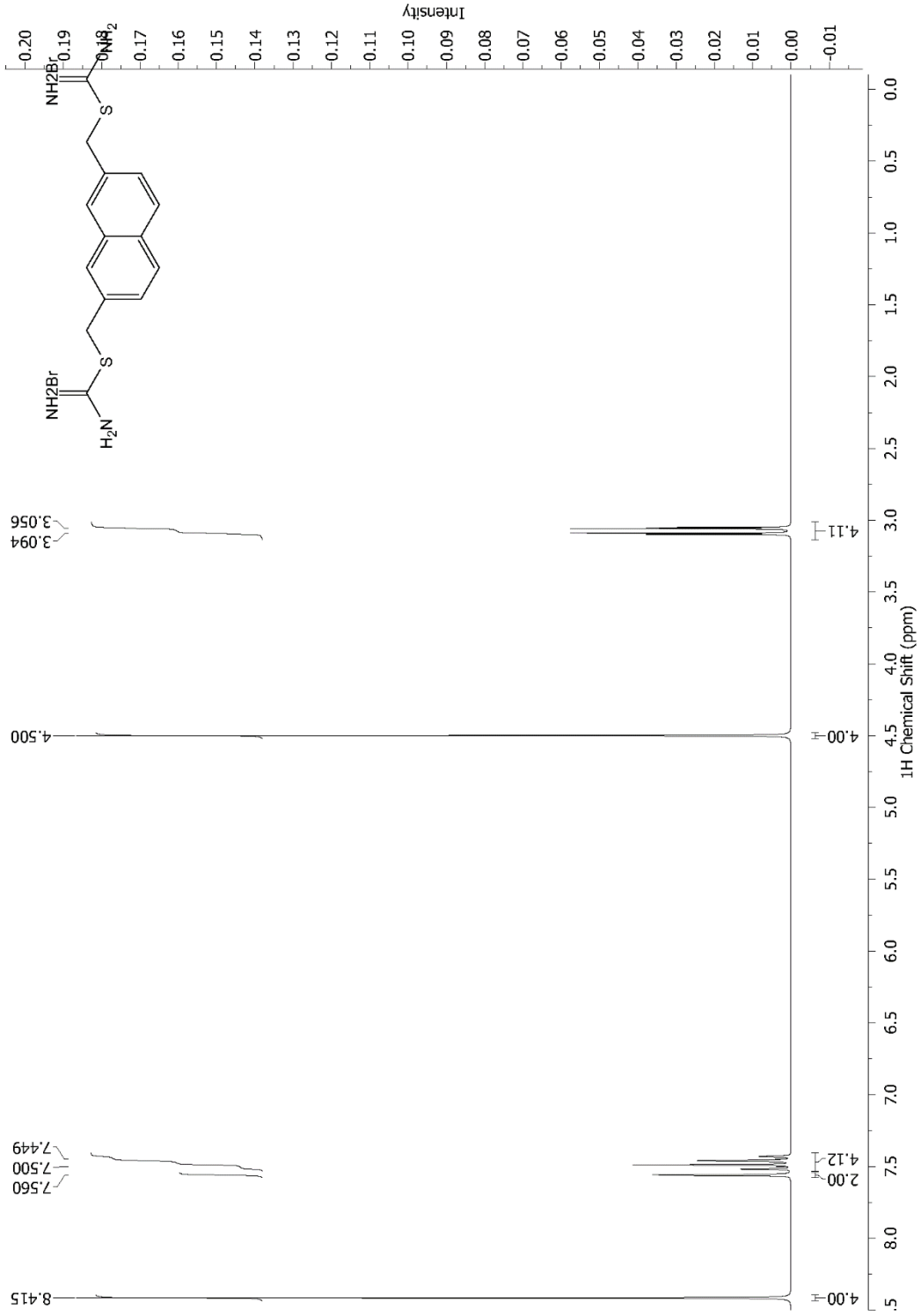
^{13}C NMR spectrum (101 MHz, DMSO-D6) of compound **1.5**



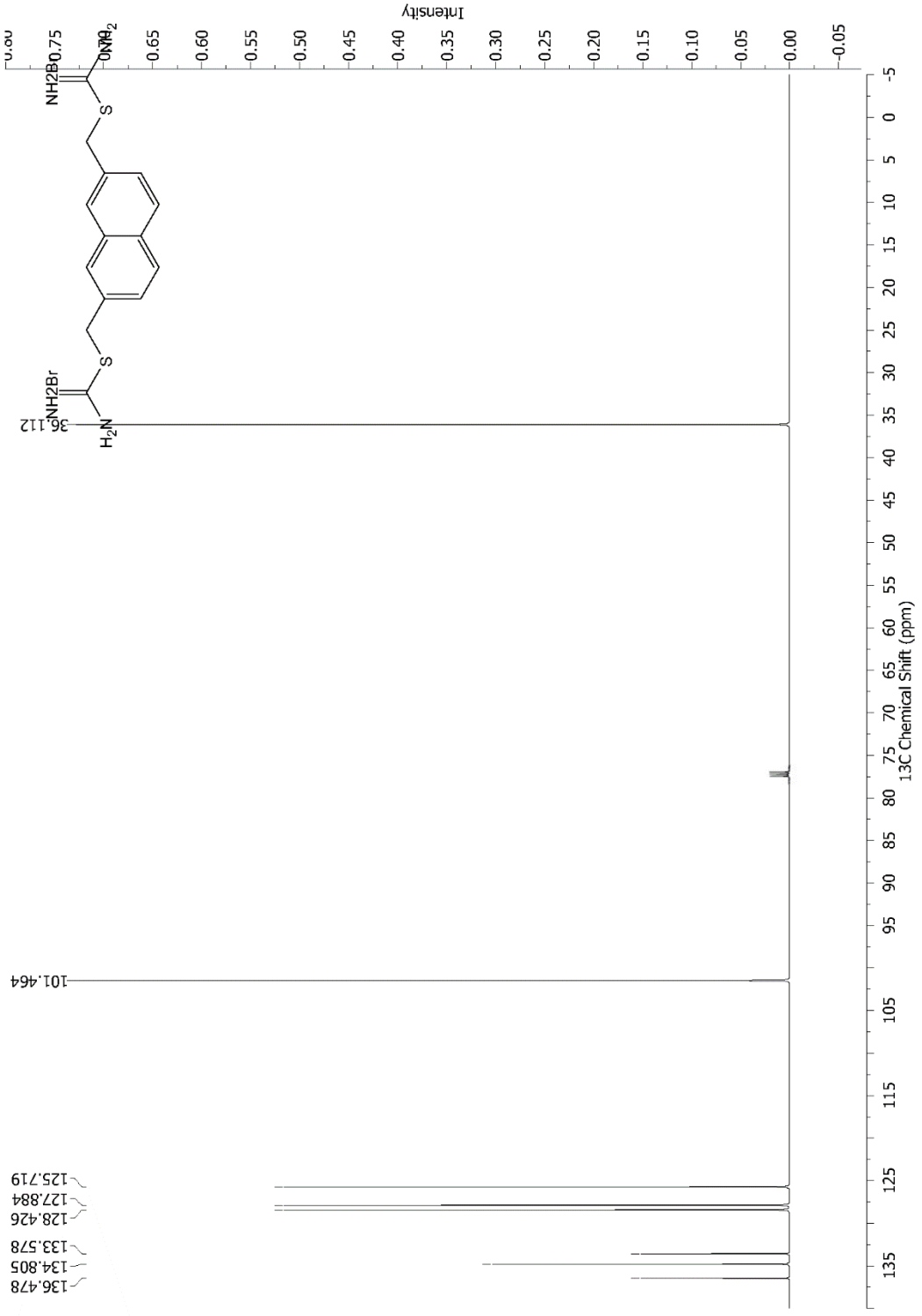
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1.6**



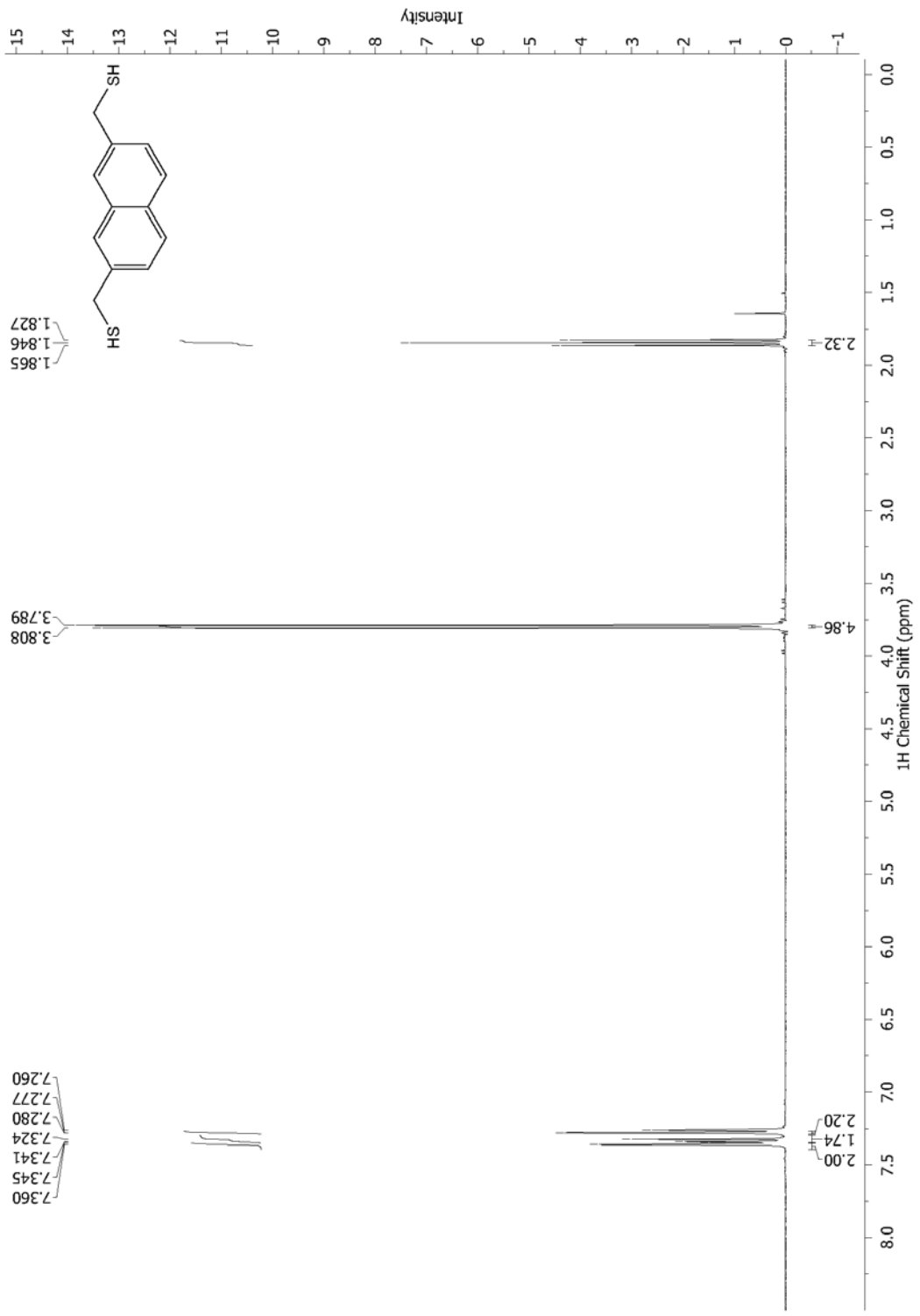
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **1.6**



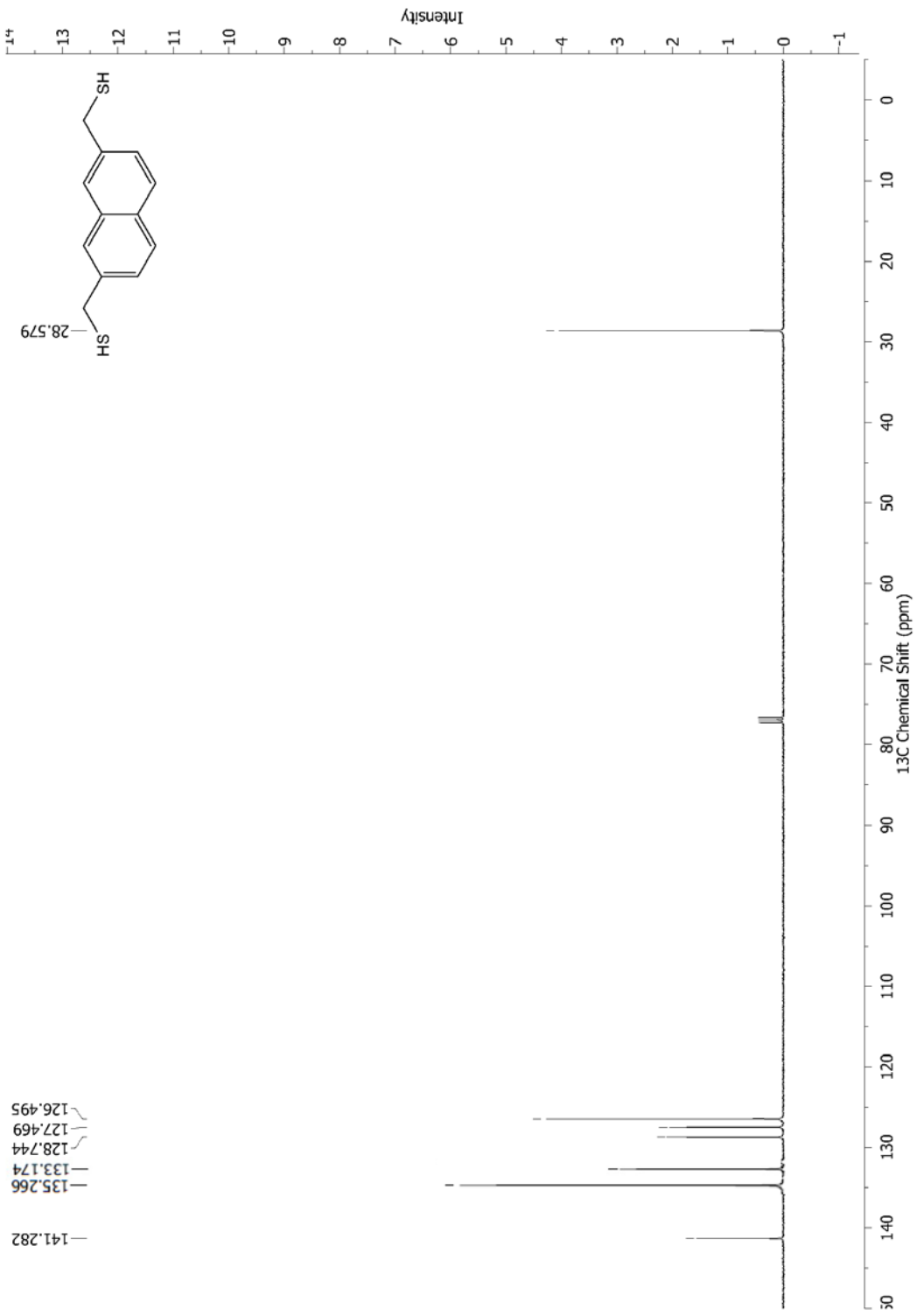
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1.7**



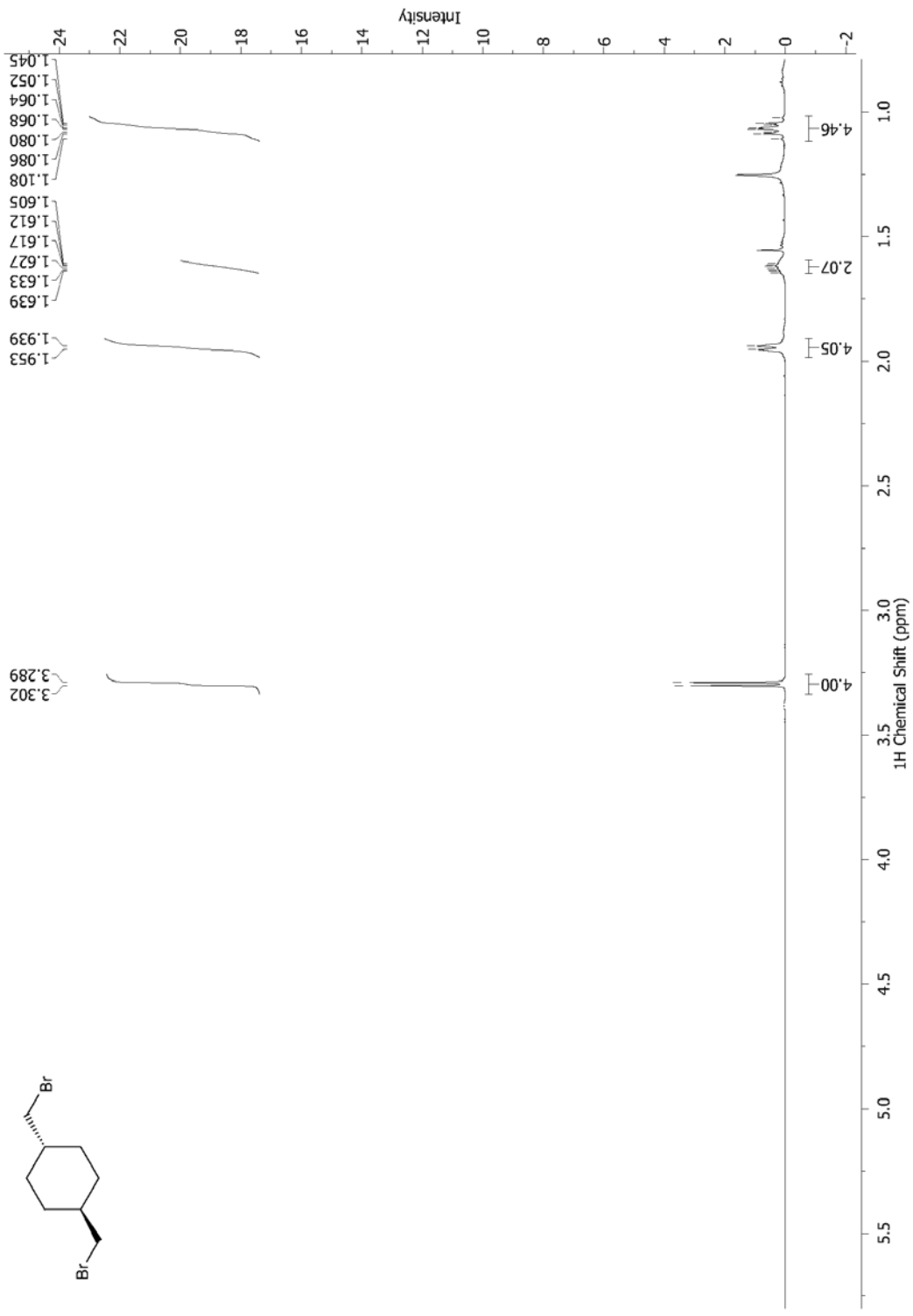
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **1.7**



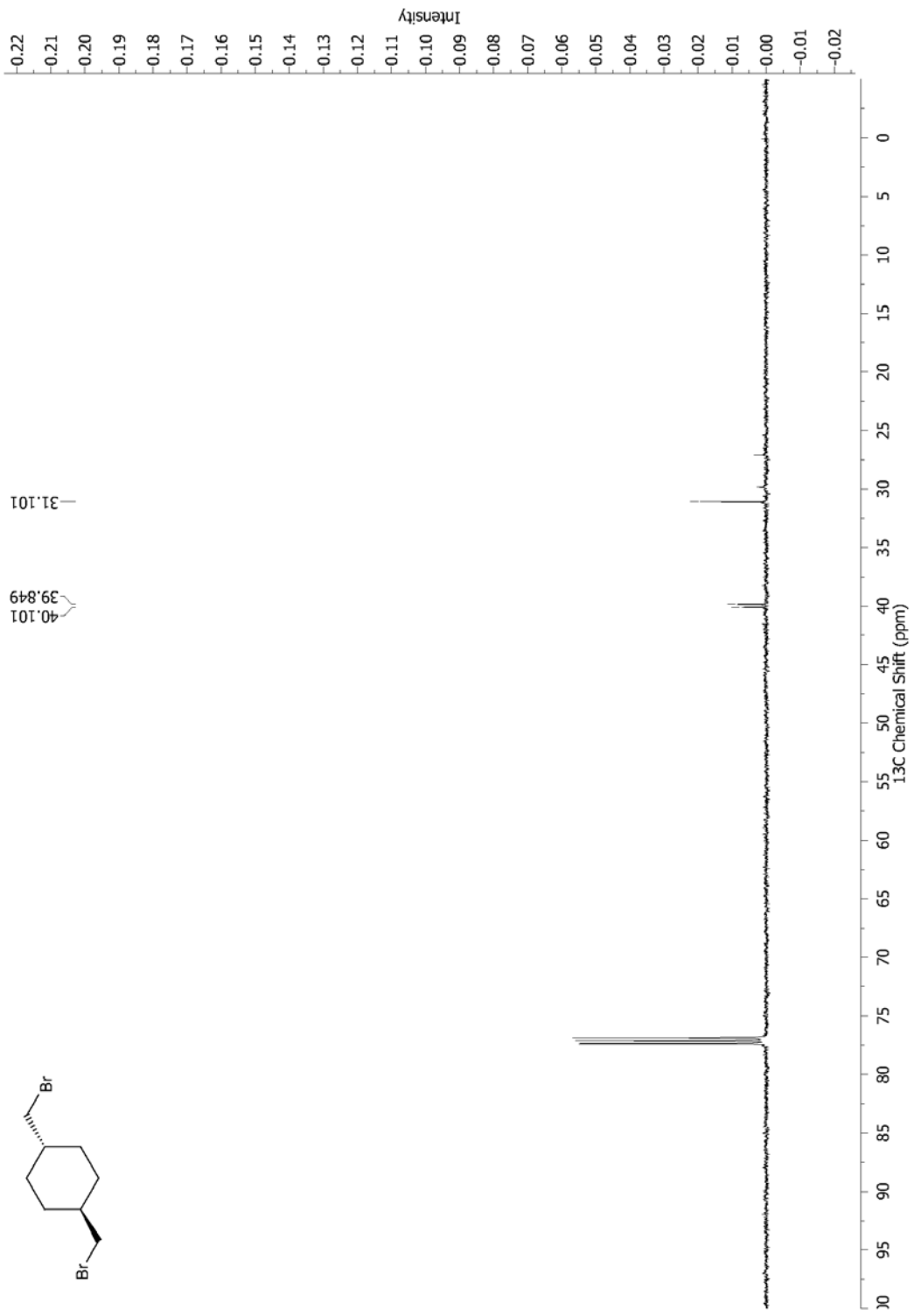
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1.8**



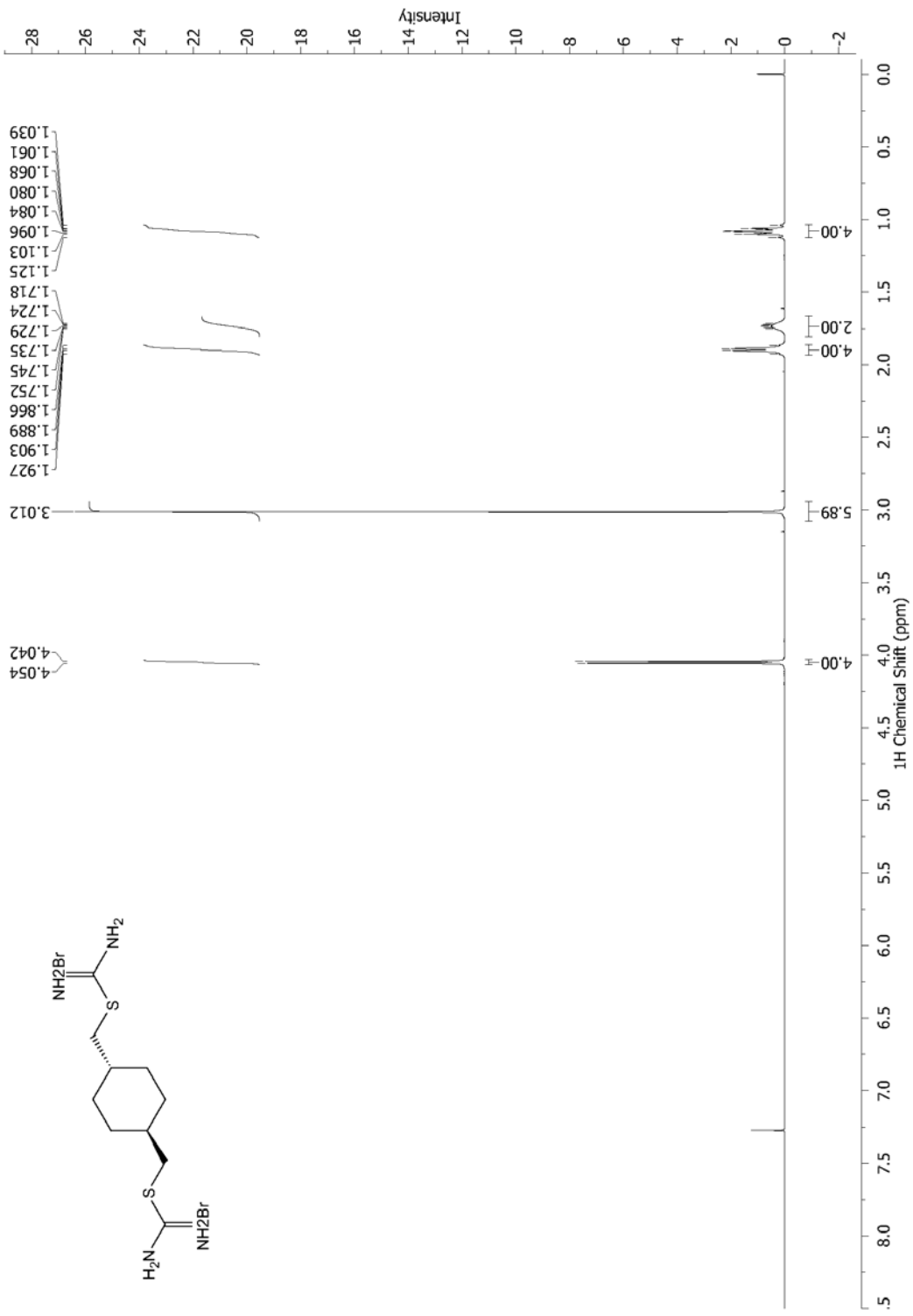
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **1.8**



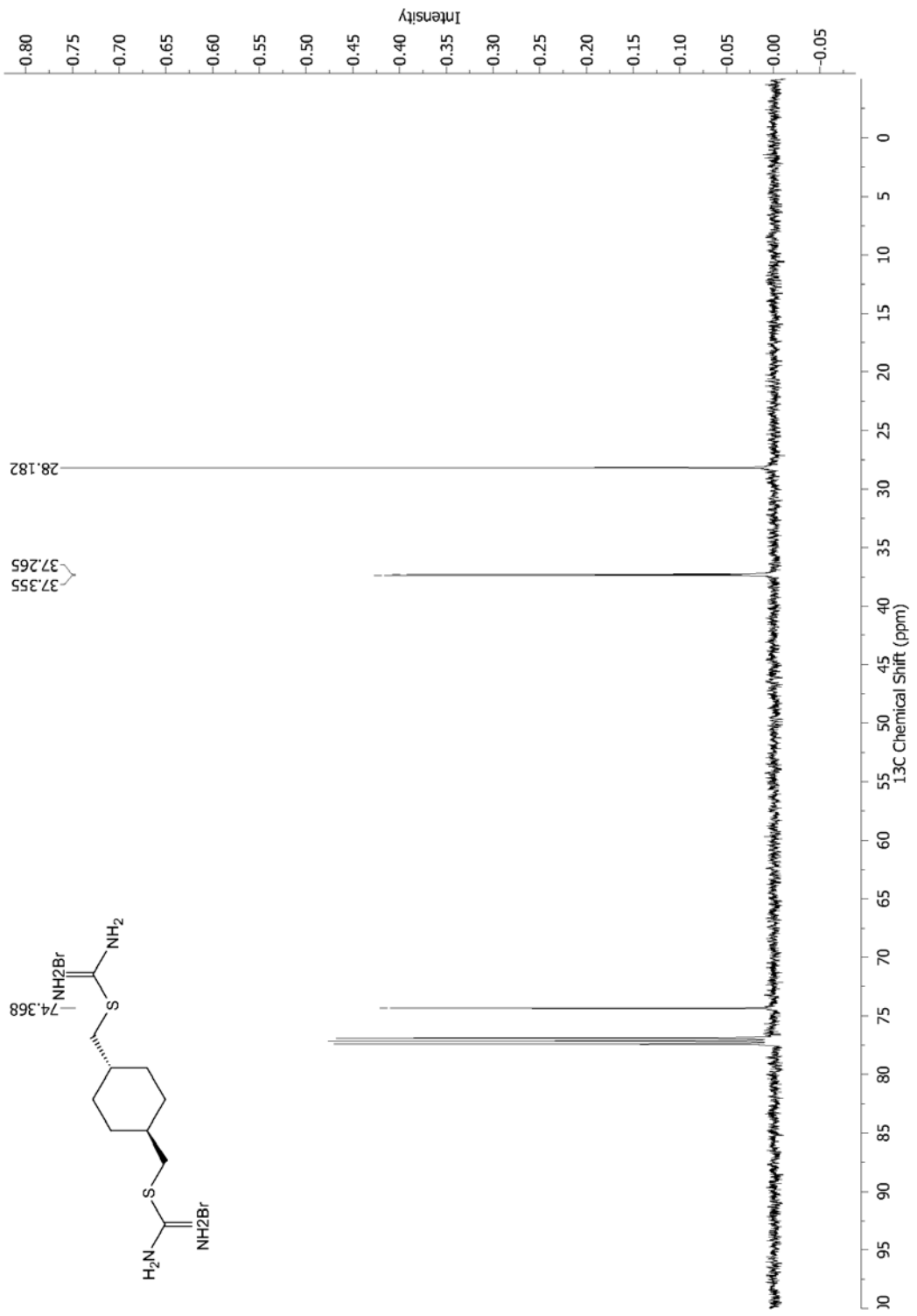
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound ***trans*-1.10**



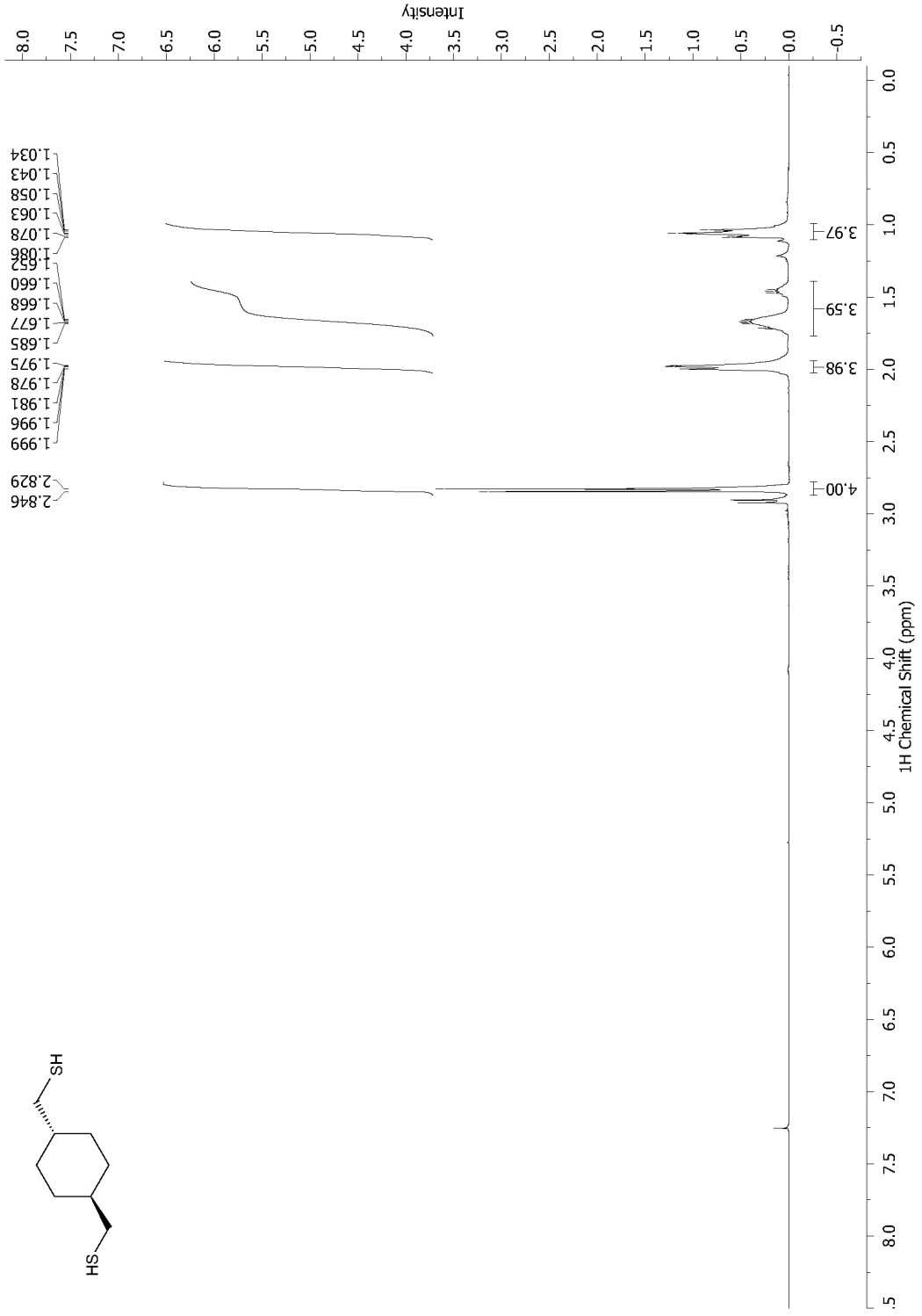
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound ***trans*-1.10**



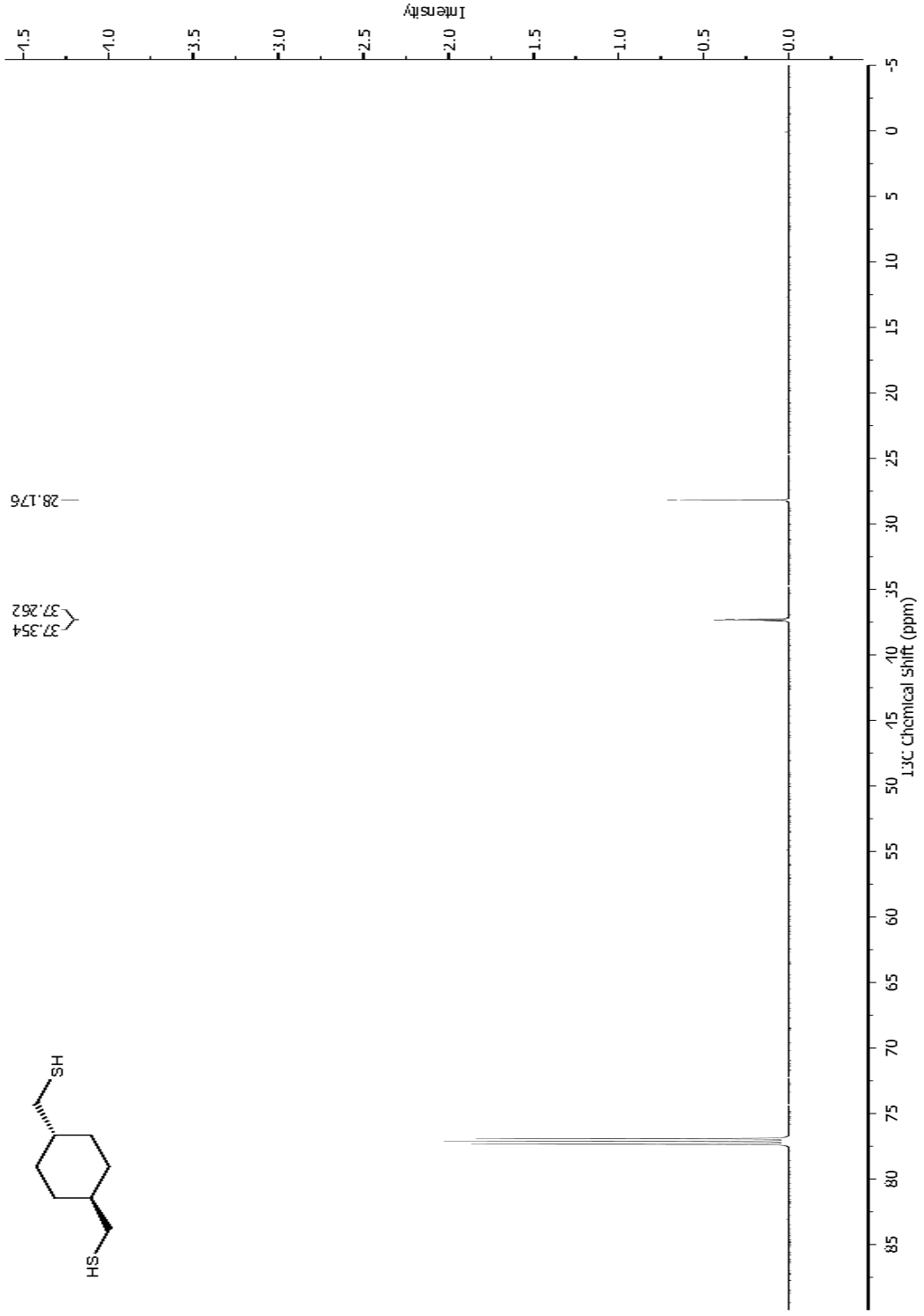
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **1.11**



^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **1.11**



^{1}H NMR spectrum (600 MHz, $CDCl_3$) of compound **1.12**



^{13}C NMR spectrum (151 MHz, $CDCl_3$) of compound **1.12**

CHAPTER 2: DEVELOPMENT OF SMALL MOLECULE INHIBITORS TO RESTORE NUCLEAR p27 AS A THERAPEUTIC METHOD FOR ENDOMETRIAL CANCER

2.1 Introduction

Endometrial carcinoma (EndoCa), also referred to as corpus uterine cancer or corpus cancer, is a major type of uterine cancer in which the cancer cells form in the tissues of the inner layer of the uterus. It is the most-common cancer of the female reproductive system and the fourth most common female cancer in North America and Europe.[1-4]

A hysterectomy (an operation in which the uterus is removed) is currently a primary treatment for EndoCa. For early diagnosed cancer, a hysterectomy in combination with hormone, radiation or chemotherapy may be used. These therapeutic methods are effective with stage I where the cancer is localized and stage II where the malignant cell has only spread within the uterus. For stage III and IV where the cancer has spread beyond the uterus, patients may also be candidates for clinical trials of new treatment options along with a hysterectomy, hormone, radiation and chemotherapy.

The 5-year relative survival rate of EndoCa is 82% which means that out of every 100 women diagnosed with EndoCa about 82 survive for at least 5 years after being diagnosed. However, this statistic does not reflex the poor prognosis for late stage

cancer. While the 5-year relative survival rate of stage I and II are high (75–90%), the survival rates for stage III and IV are low (60% and 15–26%, respectively).[2] This statistic indicates that current therapeutic methods, though effective for the early state cancer, are insufficient for late stage cancer. Thus, new and effective therapies are in great need.

In the cell cycle, cells stay in G1 phase (also called the growth phase) to synthesize mRNA and proteins until they gain enough nutrition and size. In this phase, the presence of p27, a cyclin-dependent kinase inhibitor (CKI), in the nucleus inactivating cyclin A- and cyclin E-CDK2 complexes to block cell cycle progression and inhibit cell growth.[5] For a cell that passes the G1 checkpoint, nuclear p27 is targeted by SCF E3 ligase (also called ubiquitin ligase) for degradation allowing the cycle to proceed to S phase. Phosphorylation of p27 can stabilized and sequester it to the cytoplasm separating p27 from its cyclin-CDK targets in the nucleus. This process prevents p27 from functioning as a tumor suppressor.[5-8] Moreover, cytosol p27 can inhibit apoptosis and increase autophagy. Thus, while p27 acts as a tumor suppressor in the nucleus, it acts as an oncogene in the cytoplasm.

In the degradation of nuclear p27, SCF E3 ligase recognizes phosphorylated p27 via a pocket formed by the interaction of S-phase kinase-associated protein 2 (Skp2) with the cyclin-dependent kinase subunit 1 (Cks1). SCF E3 ligase or ubiquitin ligase (**a** in Figure 2.1) will transfer ubiquitin protein onto p27. The ubiquitinated p27 (**c** in Figure 2.1) will then be targeted by other enzymes for further degradation.[9] If the SCF E3 ligase is inactivated, nuclear p27 is increased leading to massive cellular senescence, a state of irreversible growth arrest, and/or apoptosis.[10-11]

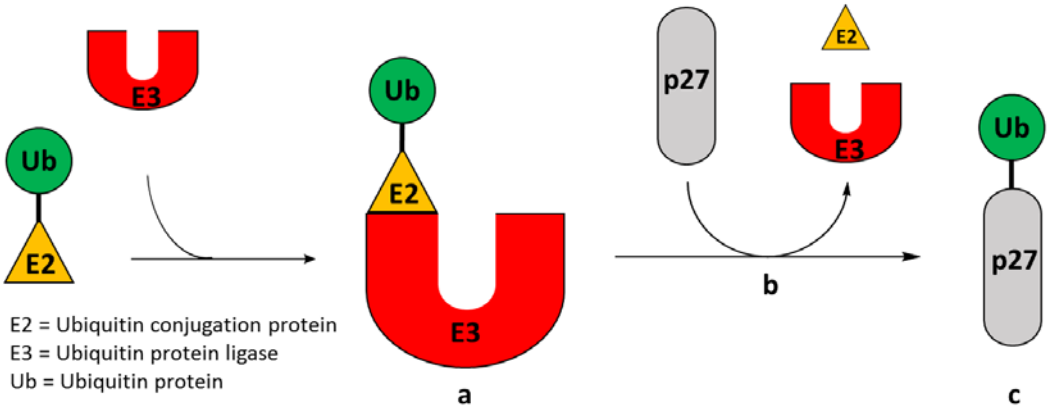


Figure 2.1 Ubiquitination of p27

In EndoCa, the tumor suppressor p27 is frequently lost or mislocalized to the cytoplasm.[12-13] The loss of nuclear p27 is also found in breast, kidney and prostate cancer.[6, 8, 11, 14-17] Thus, restoring the tumor suppressor nuclear p27 by interrupting the binding between p27 and SCF E3 ligase (**b** in Figure 2.1) would provide a plausible new therapeutic method for EndoCa and other cancers in which p27 is lost or mislocalized to the cytoplasm.

To develop a new therapeutic method for EndoCa by restoring nuclear p27, lead compounds need to be identified. High-throughput virtual screening (HTS) of about 52,000 compounds was performed by our collaborator at MD Anderson Institute for Advancing Clinical Studies. Using HiPCDock *in silico* predictive modeling, each compound was docked into the Skp2-Cks1 pockets to block SCF binding to p27.[18] The top 500 potentially active hits were selected to move into primary cell-based screens. The top 30 scaffolds that specifically restore nuclear p27 without increasing cytoplasmic p27 were selected for secondary biochemical screens. After performing secondary screens, a lead compound **2.1** was chosen as shown in Figure 2.2.

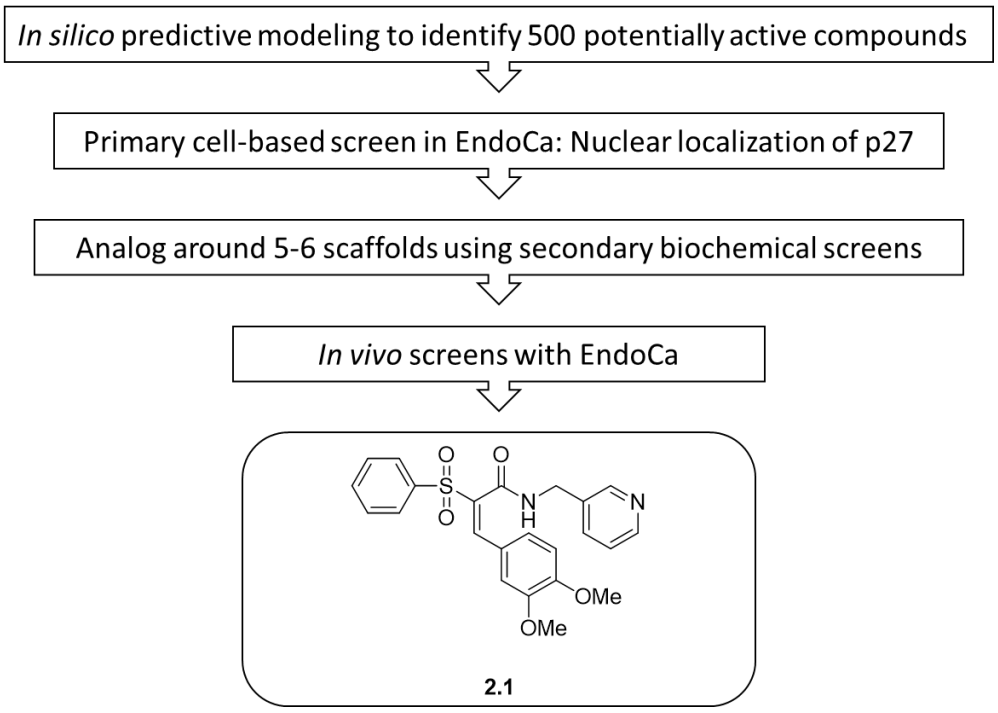


Figure 2.2 Lead compound identification process

2.2 Results and Discussion

To study the structure-activity relationship, the lead compound **2.1** and its analogs were synthesized. Considering the structure of lead compound **2.1**, there are four parts in the molecule that could be modified (Figure 2.3). They are a substituent on the nitrogen of the amide (a), a substituent on the sulfone (b), a substituent on the double bond (c) and the double bond itself.

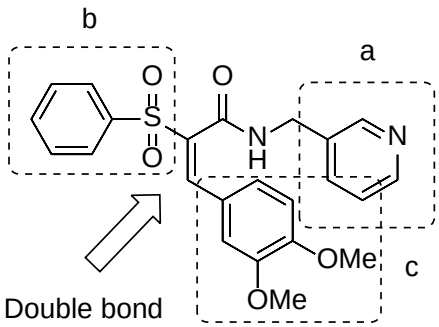


Figure 2.3 Lead compound **2.1**

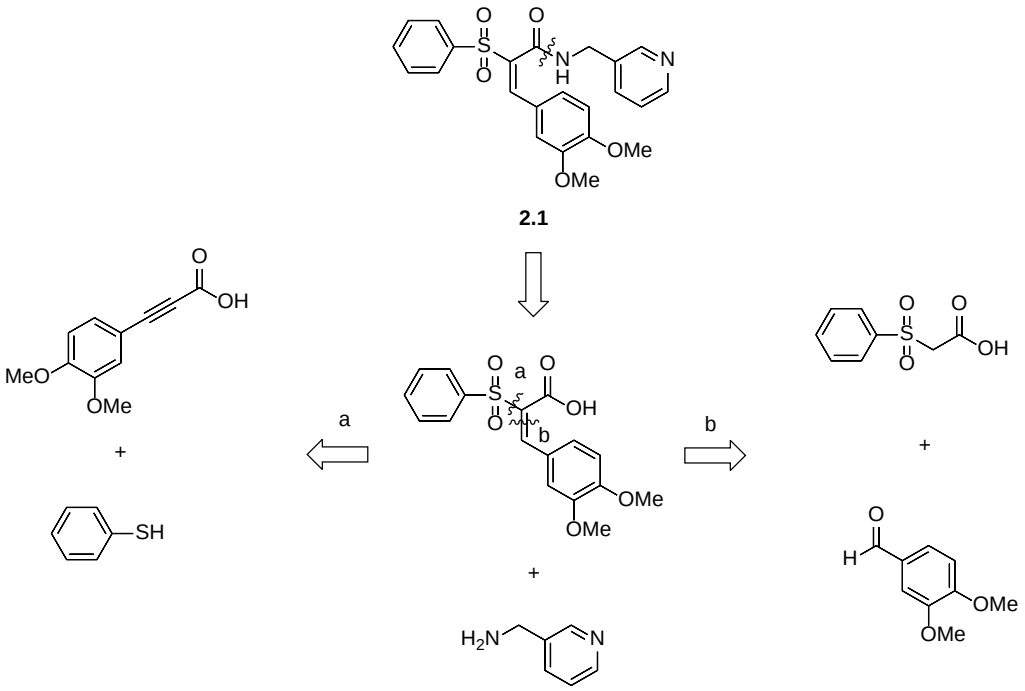
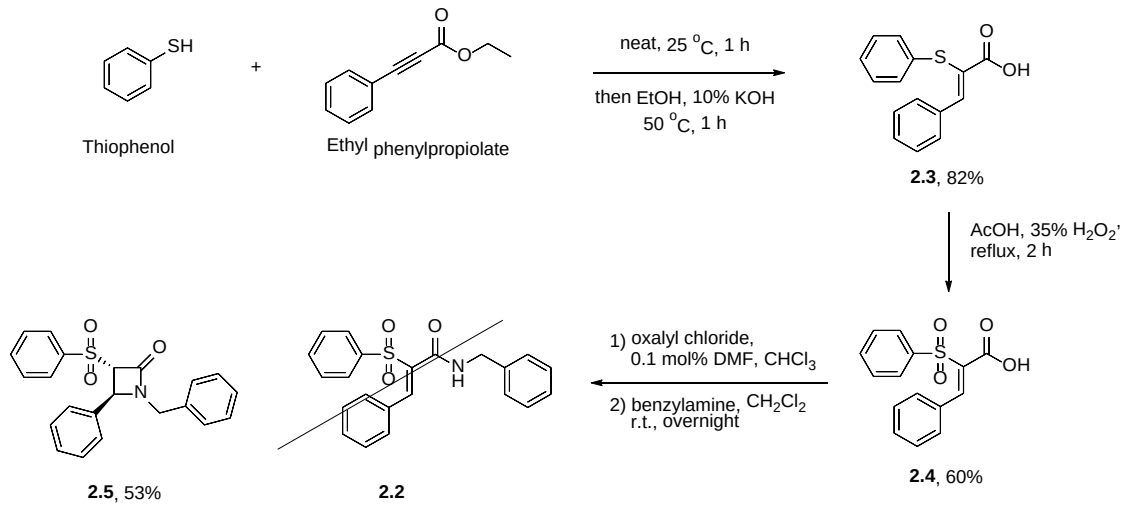


Figure 2.4 Retrosynthetic analysis of lead compound **2.1**

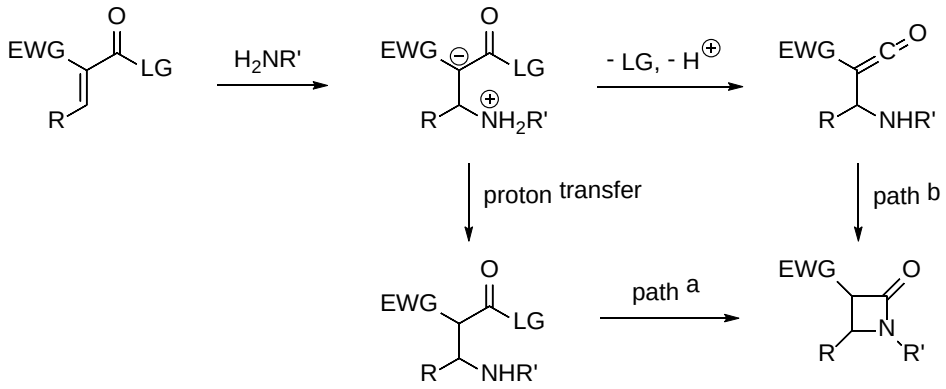
Synthesis of compound **2.1** could be accomplished by two different strategies as shown in Figure 2.4. Amide formation of carboxylic acid intermediate with 3-picolylamine could be done early in the first step or later in the last step of the synthesis depending

on an availability of starting material. Substituted acrylic acid intermediate could be prepared by thiophenol and propiolic acid derivative (pathway a) or Knoevenagel condensation between 2-(phenylsulfonyl)acetic acid and 3,4dimethoxy benzaldehyde (pathway b).

Due to availability of the starting materials, thiophenol and ethyl phenylpropiolate were used as a model study to synthesize analog **2.2** (Scheme 2.1). The reaction between thiophenol and ethyl phenylpropiolate followed by basic hydrolysis gave compound **2.3**.[19-21] Addition of thiophenol to ethyl phenylpropiolate presumably involved a radical.[20] Addition of thiophenol radical to α-position of ethyl phenylpropiolate gave vinylic radical which was stabilized by an adjacent aromatic ring. However, the geometry of the double bond observed in compound **2.3** was *Z*- which is different from compound **2.1** (stereochemistry assignment was based on the literatures).[21] Oxidation of thioether **2.3** using acetic acid and hydrogen peroxide provided sulfone **2.4**. Activation of carboxylic acid in **2.4** to acid chloride with oxalyl chloride followed by amide formation with $BnNH_2$ gave β-lactam **2.5** instead of analog **2.2**.



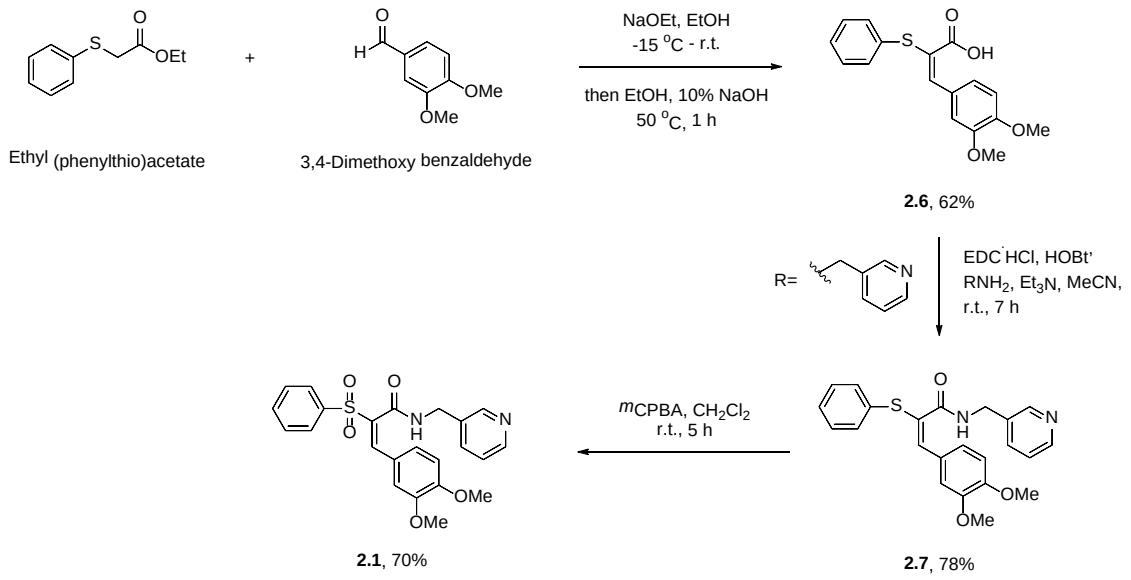
Scheme 2.1 Synthesis of compound **2.5**



Scheme 2.2 β-Lactam formation

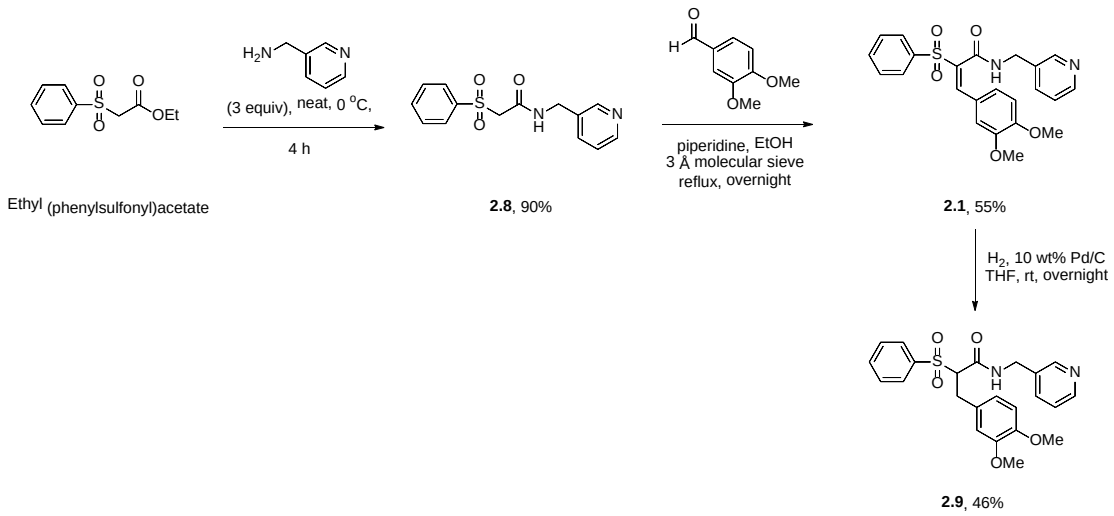
Formation of β-lactam **2.5** occurred as a result of 1,4-addition of the $BnNH_2$ to the α,β-unsaturated carboxylic acid derivative to form a zwitterionic intermediate (Scheme 2.2).[21] The zwitterionic intermediate then either undergo proton transfer followed by 1,2-addition of the amine to the carbonyl (path a) or eliminate the leaving group to form ketene intermediate followed by cyclization to form β-lactam (path b).

To avoid the cyclization, Knoevenagel condensation between ethyl (phenylthio)acetate and 3,4-dimethoxy benzaldehyde was employed to synthesize lead compound **2.1** (Scheme 2.3). The condensation was done by using NaOEt in EtOH. The ester functional group was hydrolyzed with 10% NaOH in one-pot manner at 50 ^{o}C to give intermediate **2.6**. The carboxylic acid was allowed to react with 3-picolylamine using EDC, HOBt and Et_3N in MeCN to provide intermediate **2.7**. Oxidation of thioether intermediate **2.7** with *m*CPBA gave the lead compound **2.1**.



Scheme 2.3 Synthesis of compound **2.1** from ethyl (phenylthio)acetate

Starting from ethyl (phenylsulfonyl)acetate, the lead compound **2.1** was synthesized using difference strategy as shown in Scheme 2.4. Amide formation between ethyl (phenylsulfonyl)acetate and 3-picolylamine was performed in the first step under solvent-free conditions to obtain intermediate **2.8**. Knoevenagel condensation of compound **2.8** with 3,4-dimethoxy benzaldehyde using piperidine in EtOH gave compound **2.1**. To study a significance of the double bond in the activity of compound **2.1**, it was hydrogenated using 10 wt% Pd/C as a catalyst.



Scheme 2.4 Synthesis of compounds **2.1** and **2.9** from ethyl (phenylsulfonyl)acetate

To confirm the geometry of the double bond in lead compound **2.1**, x-ray crystallography was performed. Crystal structure of compound **2.1** (Figure 2.5) illustrates that the geometry of the double bond is *E*-.

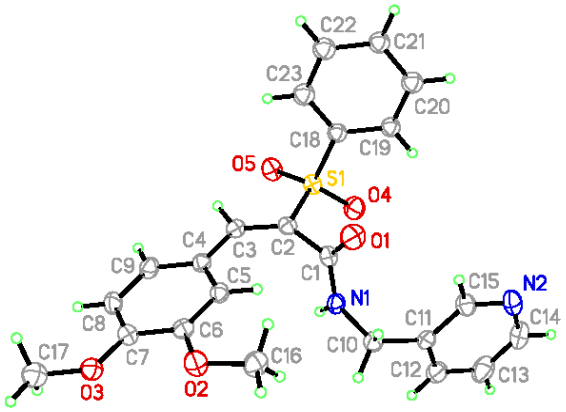
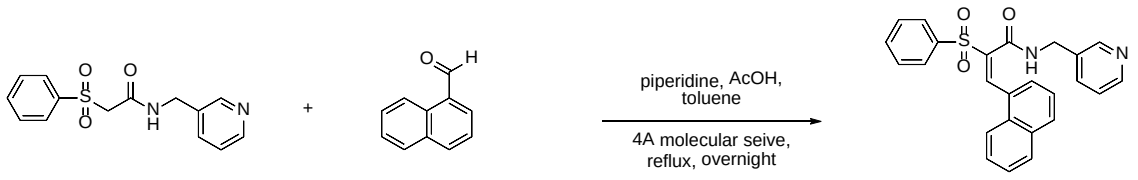


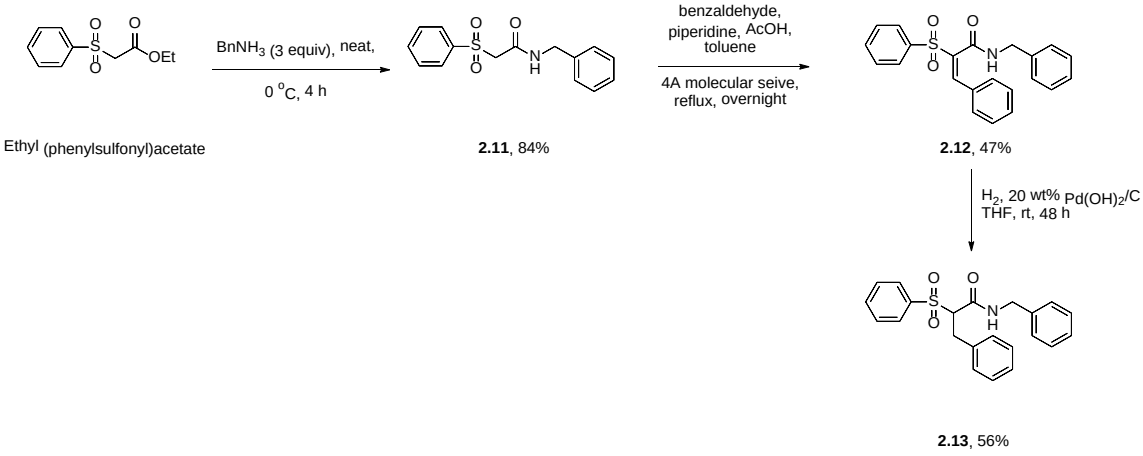
Figure 2.5 Crystal structure of lead compound **2.1**

Analogs of lead compound **2.1** were prepared using ethyl (phenylsulfonyl)aceate as a starting material. Extended 3,4-dimethoxyphenyl substituent to naphthalene by condensation of intermediate **2.8** with 1-naphthaldehyde gave compound **2.10** (Scheme 2.5).



2.8 1-Naphthaldehyde **2.10**, 30%

Scheme 2.5 Synthesis of compound **2.10**



Ethyl (phenylsulfonyl)acetate **2.11**, 84% **2.12**, 47%

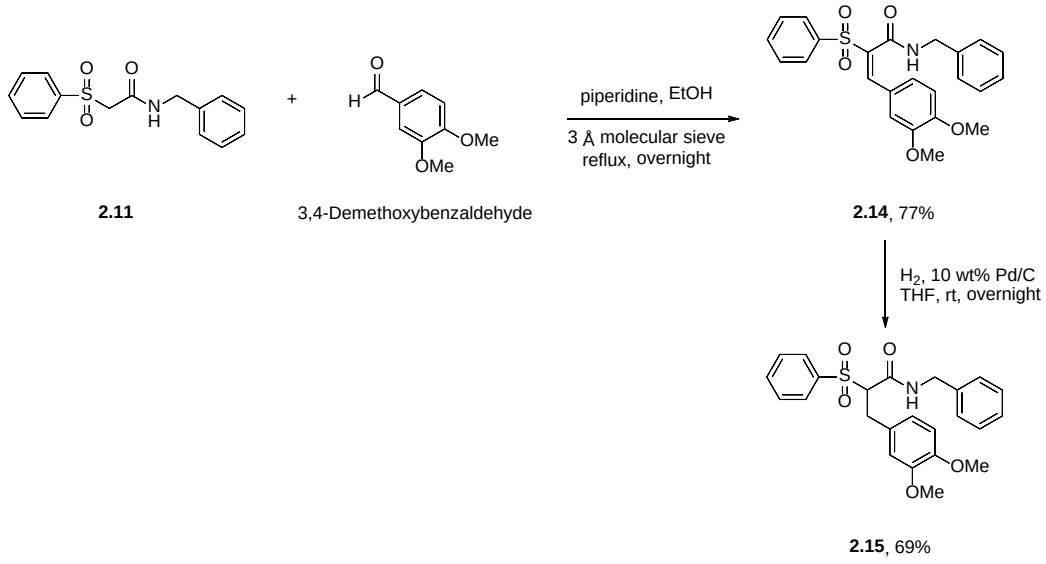
2.13, 56%

Scheme 2.6 Synthesis of compounds **2.12** and **2.13**

Analogs in which the substituent on *N* of the amide was changed from 3-methylpyridinyl to a benzyl group were also synthesized (Scheme 2.6). Ethyl (phenylsulfonyl)acetate was treated with $BnNH_2$ to give intermediate **2.11**. Knoevenagel

condensation of compound **2.11** and benzaldehyde with piperidine, acetic acid in toluene provided analog **2.12**. Hydrogenation of the double bond in compound **2.12** gave compound **2.13**.

Intermediate **2.11** was also used to synthesize analogs **2.14** and **2.15** (Scheme 2.7). Knoevenagel condensation between intermediate **2.11** with 3,4-dimethoxy benzaldehyde to give compound **2.14**. Double-bond hydrogenation of compound **2.14** gave compound **2.15**.

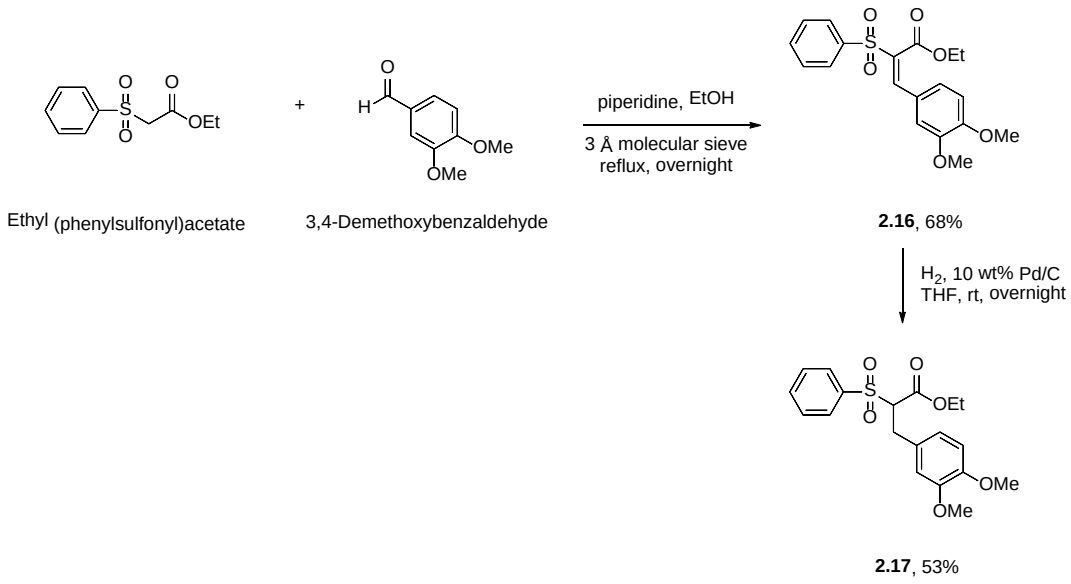


2.11 3,4-Demethoxybenzaldehyde **2.14**, 77%

2.15, 69%

Scheme 2.7 Synthesis of compounds **2.14** and **2.15**

To determine the importance of the amide side chain, compound **2.16** was synthesized by employing Knoevenagel condensation between ethyl (phenylsulfonyl)acetate and 3,4-dimethoxybenzaldehyde (Scheme 2.8). Hydrogenation of compound **2.16** gave compound **2.17**.



Ethyl (phenylsulfonyl)acetate

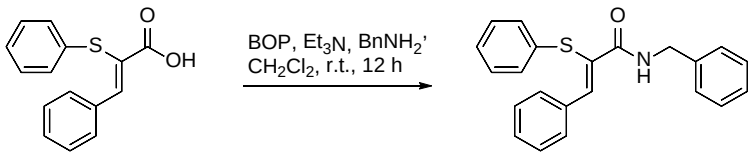
3,4-Demethoxybenzaldehyde

2.16, 68%

2.17, 53%

Scheme 2.8 Synthesis of compounds **2.16** and **2.17**

Beside the modification on the amide side chain and the double bond substituent, the sulfone moiety was also derivatized. To determine if the sulfone is important for the binding, the thioether analog **2.18** was made (Scheme 2.9). Amide coupling between carboxylic acid **2.3** and $BnNH_2$ by using BOP and Et_3N in CH_2Cl_2 gave compound **2.18**.



2.3

2.18, 50%

Scheme 2.9 Synthesis of compound **2.18**

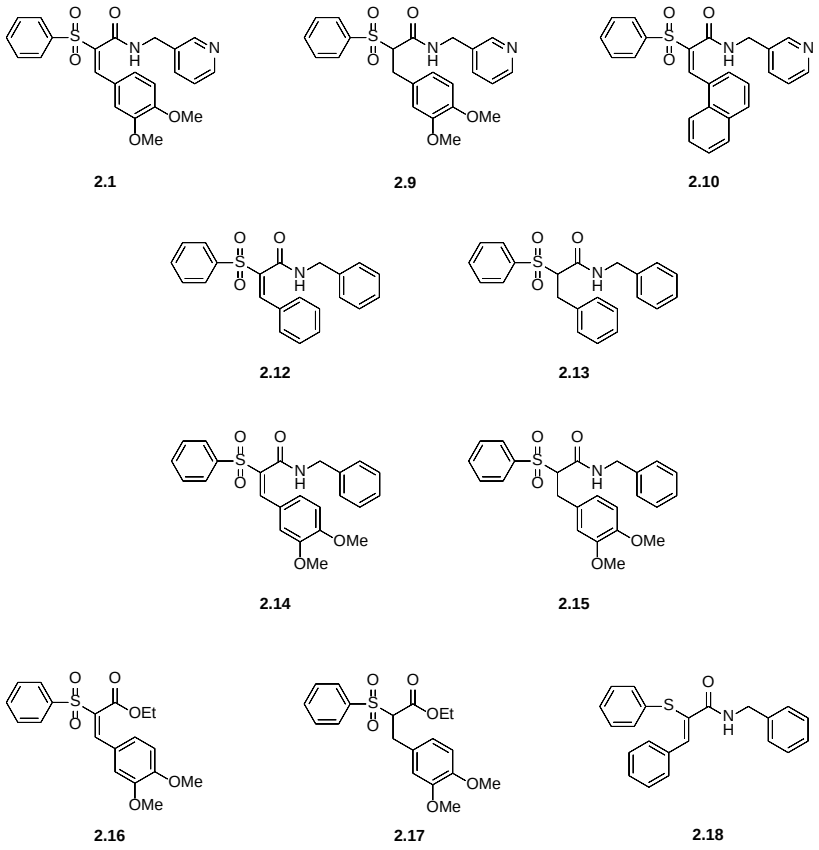


Figure 2.6 Enzyme-activity testing analogs

Synthesized lead compound **2.1** and its analog (Figure 2.6) were sent for testing in an EndoCa cell inhibition assay. Results of the test was shown in Figure 2.7 and 2.8. The NCI growth index measures the percentage of cell growth relative to the number of cells in the well-plate at the time the test compound was added. A growth index value of +100 implies the cells grew as well as the controls. A value of -100 implies there are no cells remaining in the well (complete cytotoxicity). An index value equal to zero implies the cells exhibited no growth in the presence of the compound. Compound is cytostatic.

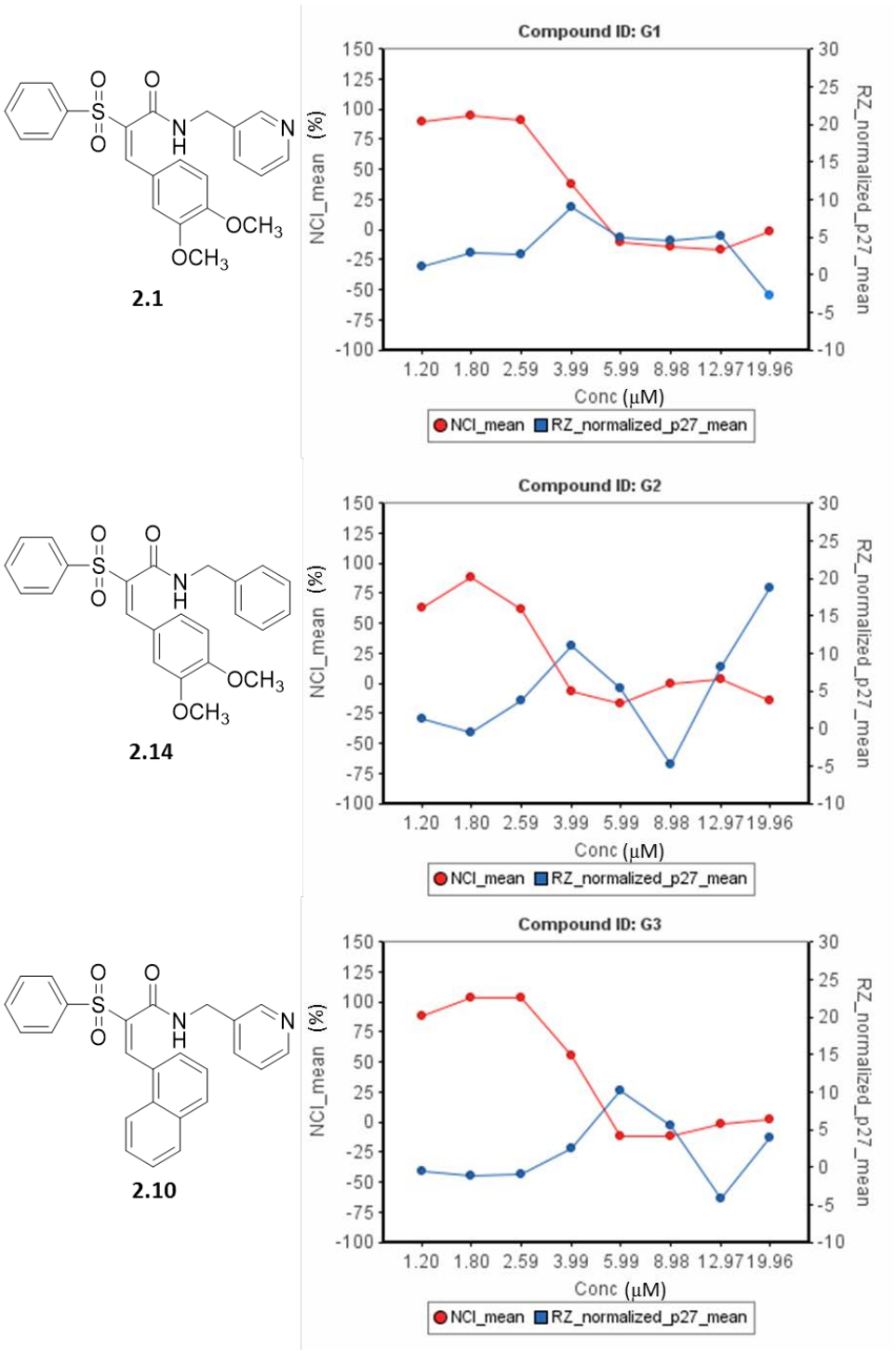


Figure 2.7 Dose respond curve of compounds **2.1**, **2.10**, **2.12**, **2.14** and **2.18**

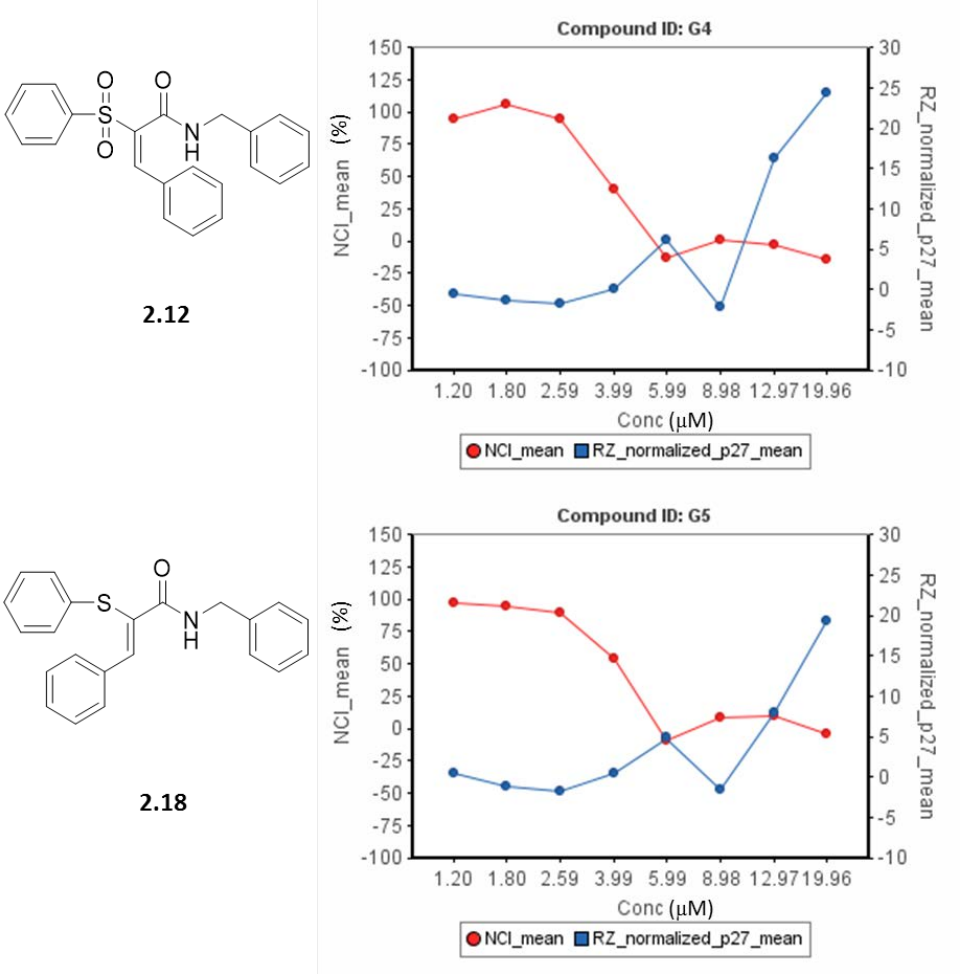


Figure 2.7 *Continued.*

Analog **2.1**, **2.10**, **2.12**, **2.14** and **2.18** showed the decrease of the malignant cells as the concentration of the tested compound increase (Figure 2.7). The results indicated that the nitrogen on the pyridine ring was not important in inhibition (compound **2.14**). The size of aromatic ring on double bond substituent (compound **2.10**) and the methoxy groups (compound **2.12**) did not affect the activity. The result of compound **2.18** also indicated that the oxygen of the sulfone and geometry of the alkene were not significant for the activity.

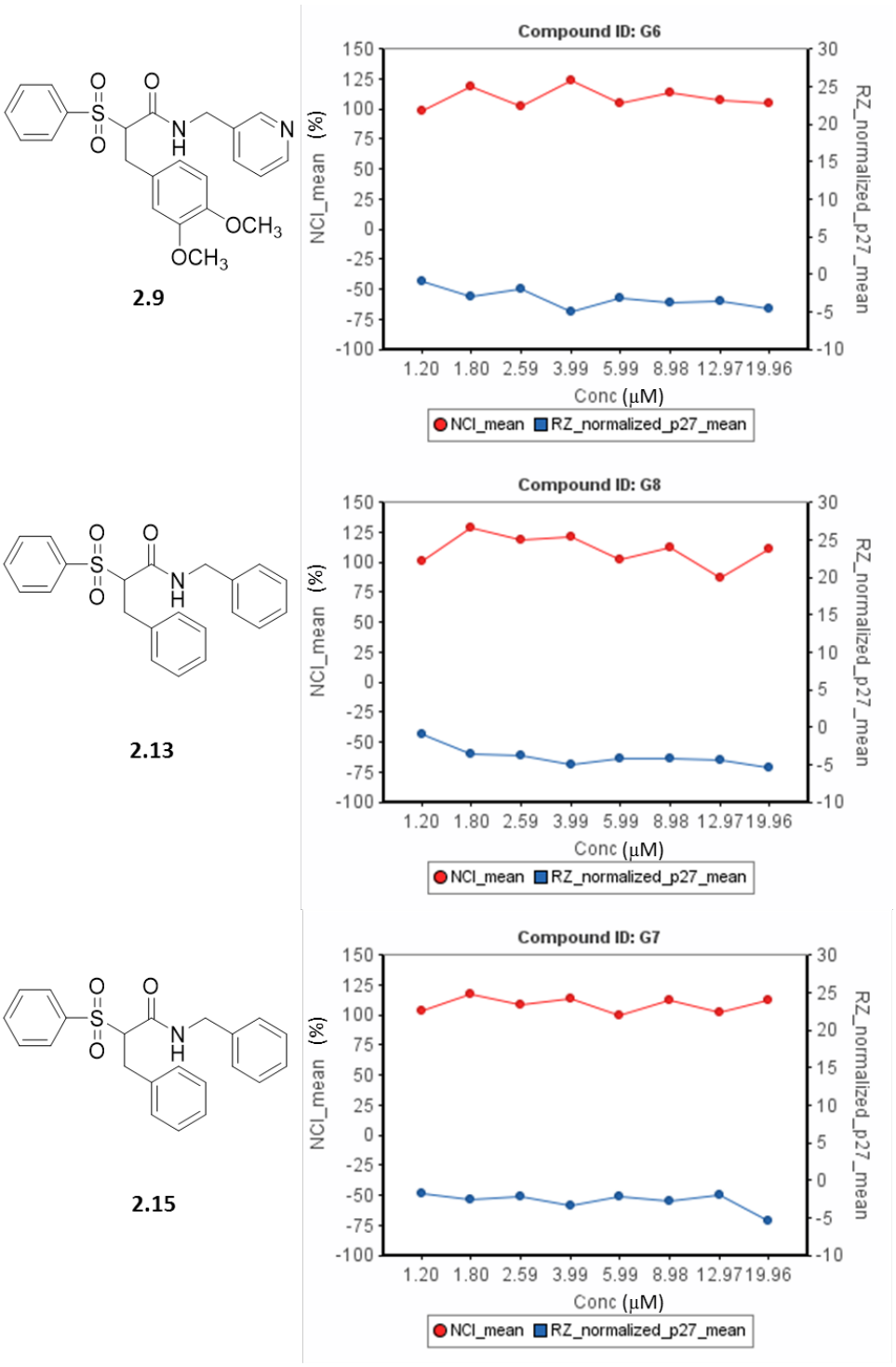


Figure 2.8 Dose respond curve of compounds **2.9**, **2.13**, **2.15**, **2.16** and **2.17**

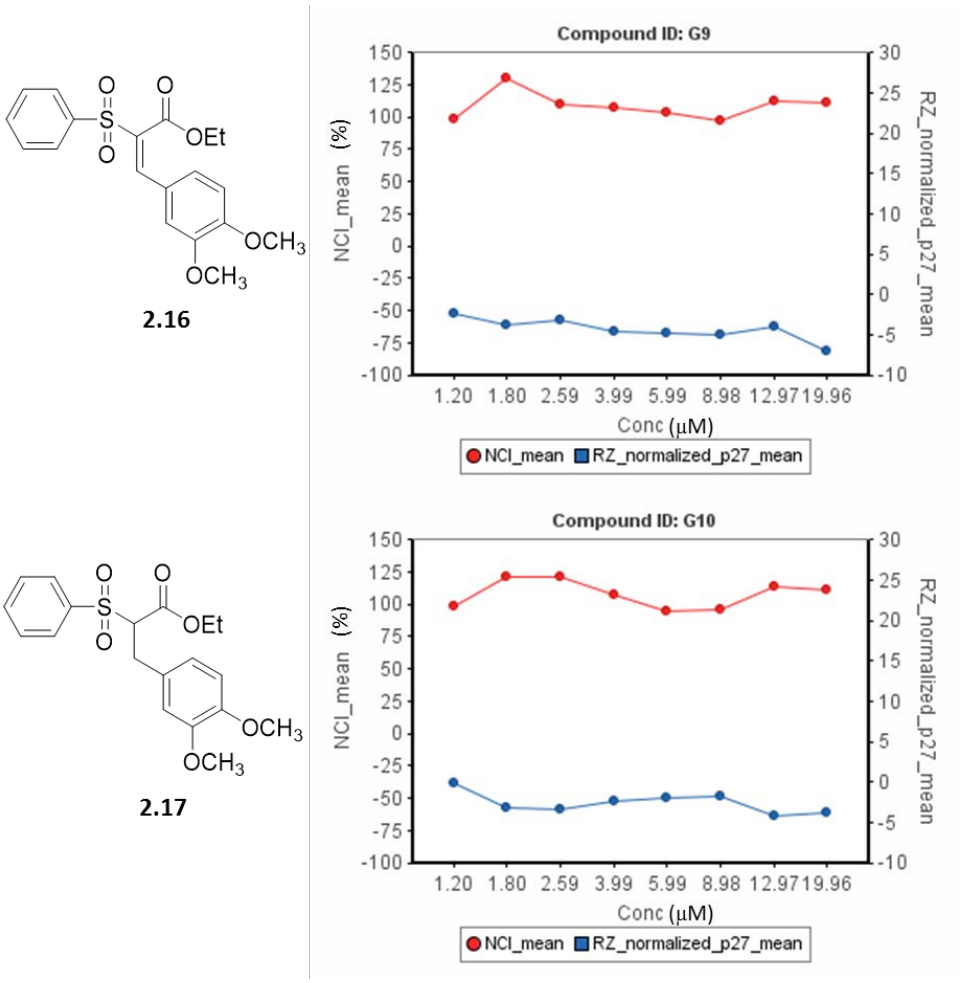
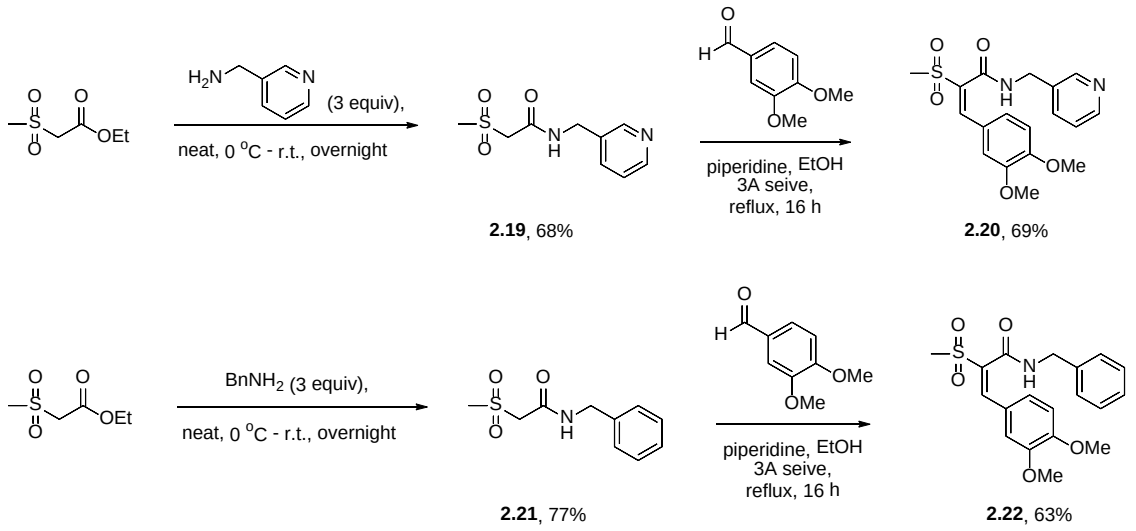


Figure 2.8 *Continued.*

Although changing the pyridine ring did not affect inhibitory activity, complete removal of the amide side chain resulted in loss of activity (compound **2.16**) (Figure 2.8). The most notable results shown here are from the hydrogenation of the double bond. As shown in Figure 2.8, hydrogenated analogs (compound **2.9**, **2.13**, **2.15** and **2.17**) did not inhibit the growth of the cancer cells. These results indicated that the double bond is essential for the activity.

Further modification of different sections of the lead compound **2.1** needed to be carried out. This is important to better understand and direct the derivatization to a correct part of the molecule. An aromatic side chain on the sulfone was replace with methyl to determine the effect on cancer cell inhibition. Compound **2.20** and **2.22** were synthesized from 2-(methylsulfonyl)acetate with 3-picolylamine and $BnNH_2$, respectively, followed by Knoevenagel condensation with 3,4-dimethoxybenzaldezyde and piperidine in EtOH (Scheme 2.10). Compounds **2.20** and **2.22** were submitted to inhibitory activity test and we are waiting for results.



Scheme 2.10 Synthesis of compounds **2.20** and **2.22**

2.3 Future Work and Conclusion

To develop a new therapeutic approach for EndoCa, tumor suppressor p27 has been identified as a promising target. In endometrial cancer as well as kidney, prostate and breast cancer, tumor suppressor p27 is usually found lost or mislocated from the nucleus to the cytoplasm of the cell. SFC E3 ligase or ubiquitin ligase decrease nuclear p27 by targeting it for ubiquitination. The ubiquitinated p27 will then be degraded by other enzymes. By interrupting the interaction with SFC E3 ligase, nuclear p27 is not ubiquitinated and further degraded.

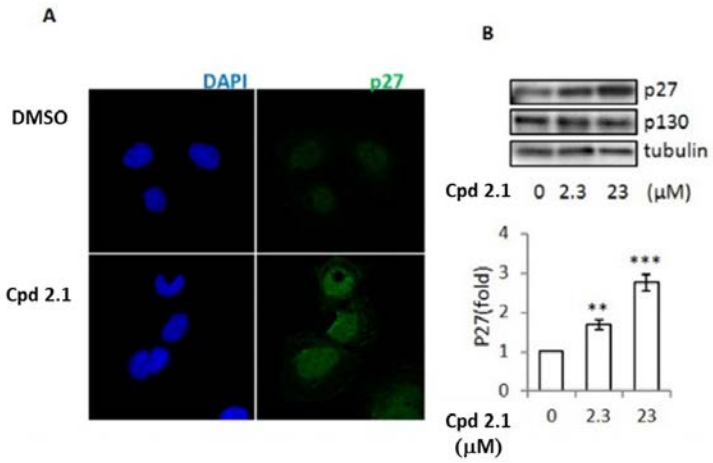


Figure 2.9 A) Compound **2.1** increases nuclear p27. **B**) Western analysis showing p27 increased levels.

Using western analysis, an immunoprecipitation method to detect a specific target protein, compound **2.1** showed ability to restore nuclear p27 (Figure 2.9). Compound **2.1** also inhibit EndoCa cell proliferation (Figure 2.10). EndoCa inhibitory assay is used to determine activity of the lead compound and analogs.

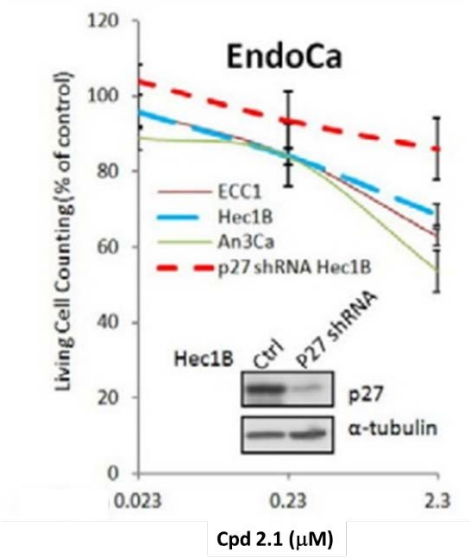


Figure 2.10 Compound **2.1** inhibits cell proliferation. (A 24 h treatment at low μM concentrations significantly inhibited cell proliferation and the shRNA knockdown (inset) of p27 significantly attenuated inhibition by **2.1**)

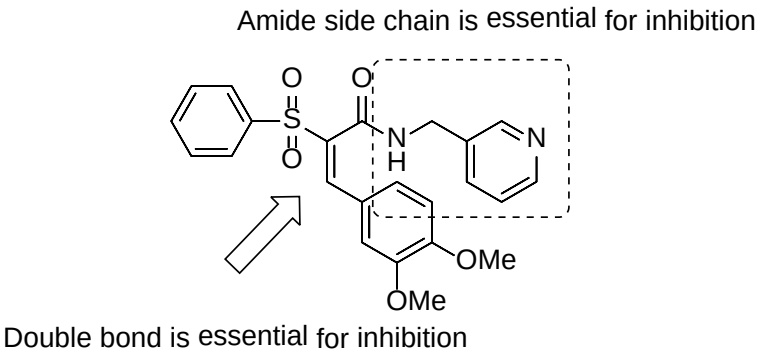


Figure 2.11 Structure of compound **2.1** showing essential parts for inhibition

A lead-compound optimization for SAR study has been done. Modification on the double bond suggested that the double bond in the molecule is importance for the compound to exhibit inhibitory activity. Changing from benzylamide to ethyl ester also

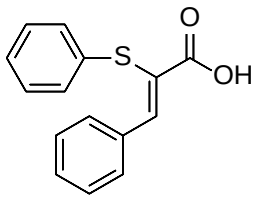
resulted in loss of activity. Slight change to the substituent on the olefin (aromaticity was maintained) did not affect activity. The results on the sulfone modification part need to be investigated in the future and the structure-activity relationship needs to be further studied.

2.4 Experimental Procedures

General:

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich, Acros or Oakwood and used without future purification. Analytical HPLC was carried out on Thermo Scientific™ BetaBasic™ 8 (5 μm, 4.6×100 mm) column with flow rate 0.8 mL/min. Preparative HPLC was performed on XBridge™ C_{18} (5 μm, 19×150 mm) column with flow rate 10 mL/min. Same solvent system was used for analytical and preparative HPLC, which is 0.1% TFA in H_2O and 0.1% TFA in MeCN. Liquid chromatography-mass spectrometry (LC-MS) was performed on a Thermo Scientific™ Finnigan Surveyor instrument equipped with MSQ Plus™ single quadrupole detector. Thin layer chromatography (TLC) was performed on silica gel 60 Å F-254 pre-coated plates (250 μm) from SiliCycle® and components were visualized by UV light (254 nm) and/or 10% phosphomolybdic acid in ethanol stain, anisaldehyde or $KMnO_4$ solution. Column chromatography was performed using SiliCycle® silica gel 230-400 (particle size 40-63 μm) mesh. Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were obtained on JEOL ECX-400 NMR, ECA-500 or ECA-600II spectrometer and processed using software MestReNova 6.1.0. Chemical shifts are reported in parts per million (ppm, δ). Multiplicity is indicated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet. Coupling constants (*J*) are reported in Hz.

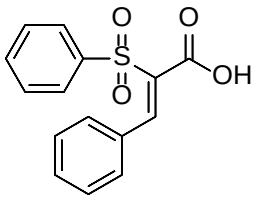
Preparation of (*Z*)-3-phenyl-2-(phenylthio)propenoic acid (2.3)



2.3

A mixture of thiophenol (2.05 mL, 20 mmol) and ethyl phenylpropiolate (3.30 mL, 20 mmol) was stirred at room temperature for 1 h under a nitrogen atmosphere. The mixture was diluted with EtOH (50 mL) and 10% KOH (30 mL) was then added slowly over 15 minutes. The resulting mixture was stirred at 50 ^{o}C for 1 h under a nitrogen atmosphere. The reaction mixture was then diluted with cold water (150 mL). The aqueous solution was washed with Et_2O (2 x 50 mL). The aqueous layer was acidified with 10% HCl. A yellow solid was collected by vacuum filtration, washed with cold water and air-dried. The crude product was recrystallized from EtOH to give (*Z*)-3-phenyl-2-(phenylthio)propenoic acid (**2.3**) (4.2 g, 94%) as a yellow crystal. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.59 (s, 1H), 8.30 (s, 1H), 7.88 (m, 2H), 7.39 (m, 3H), 7.24 (m, 4H), 7.16 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.6, 149.7, 135.2, 134.0, 131.3, 130.9, 129.3, 128.6, 128.1, 126.5, 123.6. ESI-MS (*m/z*) 256.99.

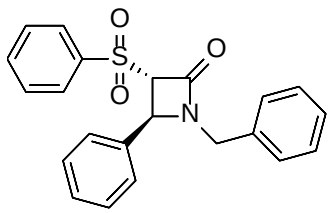
Preparation of (*Z*)-3-phenyl-2-(phenylsulfonyl)propenoic acid (2.4)



2.4

(*Z*)-3-Phenyl-2-(phenylthio)propenoic acid (**2.3**) (1.0 g, 3.90 mmol) was dissolved in glacial acetic acid (3.90 mL) with mild heating. Hydrogen peroxide (1.33 mL of a 35% solution, 13.65 mmol) was added to the mixture. The reaction was refluxed for 2 h. The mixture was diluted with Et_2O (10 mL), washed with water (2 x 10 mL) and brine (10 mL) and dried over $MgSO_4$. The solvent was removed under reduce pressure. A solid crude mixture was recrystallized from EtOAc to give (*Z*)-3-phenyl-2-(phenylsulfonyl)propenoic acid (**2.4**) (671 mg, 60%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (s, 1H), 8.08 (s, 1H), 7.95 (m, 2H), 7.63 (m, 1H), 7.53 (m, 4H), 7.41 (m, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.6, 146.1, 139.6, 134.0, 132.0, 131.2, 130.4, 129.3, 129.1, 128.8, 128.6. ESI-MS (*m/z*) 289.03.

Preparation of *N*-benzyl-trans-4-phenyl-3(phenylsulfonyl)-2-azetidinone (2.5)

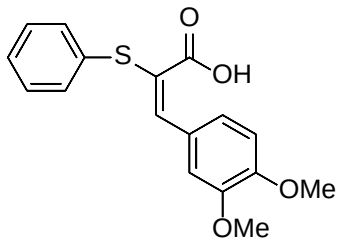


2.5

To a solution of (*Z*)-3-phenyl-2-(phenylsulfonyl)propenoic acid (**2.4**) (218 mg, 0.76 mmol) in dry $CHCl_3$ (3.78 mL) was added oxalyl chloride (0.19 mL, 2.27 mmol) followed by DMF (10 μL). The reaction was stirred at room temperature for 1 h under a nitrogen atmosphere. The mixture was then concentrated under reduce pressure and was used in the next step without further purification.

To a solution of the crude mixture in dry CH_2Cl_2 (3.78 mL) was added Et_3N (0.32 mL, 2.27 mmol) followed by $BnNH_2$ (0.12 mL, 1.13 mmol). The reaction was stirred at room temperature overnight under a nitrogen atmosphere. The mixture was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (CH_2Cl_2) to provide *N*-benzyl-*trans*-4-phenyl-3(phenylsulfonyl)-2-azetidinone (**2.5**) (150 mg, 53%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.01 (d, *J* = 7.5 Hz, 1H), 7.69 (t, *J* = 7.5 Hz, 1H), 7.56 (t, *J* = 7.8 Hz, 1H), 7.35 (m, 2H), 7.26 (m, 2H), 7.20 (dd, *J* = 6.5, 3.0 Hz, 1H), 7.04 (dd, *J* = 6.5, 2.7 Hz, 1H), 4.87 (d, *J* = 15.4 Hz, 1H), 4.84 (d, *J* = 2.3 Hz, 1H), 4.43 (d, *J* = 2.1 Hz, 1H), 3.82 (d, *J* = 15.3 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.0, 137.6, 134.7, 134.5, 133.8, 129.6, 129.5, 129.5, 129.1, 129.0, 128.3, 128.1, 126.7, 77.9, 55.9, 45.2. ESI-MS (*m/z*) 378.09.

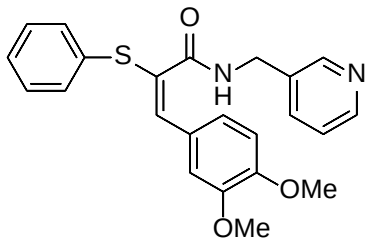
Preparation of (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylthio)propenoic acid (2.6)



2.6

To a cooled suspension of ethyl 2-(phenylthio)acetate (5.0 g, 25 mmol), 3,4-dimethoxybenzaldehyde (4.65 g, 28 mmol) in EtOH (76 mL) was added NaOEt (2.08 g, 30 mmol). The reaction mixture was allowed to warm to room temperature and stirred overnight under a nitrogen atmosphere. To a mixture was added 10% NaOH (38 mL). The reaction mixture was stirred at 50 ^{o}C for 1 h. The solution was cooled in an ice-bath and made acidic with 10% HCl. A solid crude product was collected by vacuum filtration and recrystallized in EtOH to give (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylthio)propenoic acid (**2.6**) (4.96 g, 62%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.68 (s, 1H), 8.34 (s, 1H), 7.83 (d, *J* = 2.0 Hz, 1H), 7.46 (dt, *J* = 6.4, 3.3 Hz, 1H), 7.29 (m, 5H), 6.86 (d, *J* = 8.5 Hz, 1H), 3.89 (s, 3H), 3.77 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2, 151.7, 150.6, 148.6, 135.5, 134.7, 130.0, 129.3, 126.8, 126.3, 119.1, 113.4, 110.7, 56.1, 55.9. ESI-MS (*m/z*) 317.25.

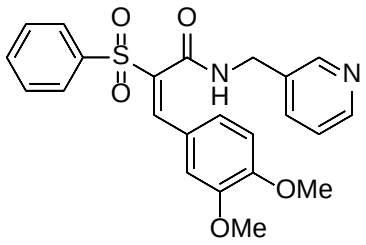
Preparation of (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylthio)-*N*-(pyridin-3-ylmethyl)acrylamide (2.7)



2.7

To a suspension of (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylthio)propenoic acid (**2.6**) (500 mg, 1.58 mmol), HOBt (64 mg, 0.47 mmol), EDC·HCl (364 mg, 1.90 mmol) and $BnNH_2$ (0.17 mL, 1.66 mmol) in MeCN (3.16 mL) was added Et_3N (0.23 mL, 1.66 mmol). The mixture was stirred at room temperature for 7 h under a nitrogen atmosphere. Water (10 mL) was then added and the mixture was extracted with EtOAc (2 x 15 mL). The organic phase was washed with brine (10 mL) and dried over Na2SO4. The solvent was removed under reduce pressure and the crude product was purified by flash column chromatography (1% MeOH in CH_2Cl_2) to provide (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylthio)-*N*-(pyridin-3-ylmethyl)acrylamide (**2.7**) (464 mg, 78%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.45 (dd, *J* = 4.8, 1.5 Hz, 1H), 8.43 (s, 1H), 8.33 (d, *J* = 2.1 Hz, 1H), 7.64 (d, *J* = 2.0 Hz, 1H), 7.58 (t, *J* = 5.9 Hz, 1H), 7.44 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.22 (m, 6H), 7.10 (dd, *J* = 7.8, 4.8 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 1H), 4.49 (d, *J* = 6.0 Hz, 2H), 3.90 (s, 3H), 3.77 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.5, 152.0, 149.0, 141.7, 139.3, 137.1, 133.8, 133.6, 129.3, 128.9, 128.2, 128.01, 127.8, 125.9, 124.1, 112.4, 110.9, 56.1, 55.9, 44.1. ESI-MS (*m/z*) 407.13, 229.07.

Preparation of (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (2.1)



2.1

Method A:

To a solution of (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylthio)-*N*-(pyridin-3-ylmethyl)acrylamide (**2.7**) in dry CH_2Cl_2 (3.2 mL) was added a solution of *m*CPBA (128 mg, 0.74 mmol) in dry CH_2Cl_2 (9.6 mL). The reaction was stirred at room temperature for 5 h under a nitrogen atmosphere. The reaction mixture was washed with 40% $NaHSO_3$ (2 x 10 mL) and saturated $NaHCO_3$ (10 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduce pressure. The crude product was purified by flash column chromatography (3% MeOH in CH_2Cl_2) to provide (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (**2.1**) (76 mg, 70%) as a white solid.

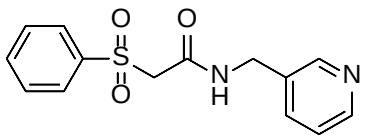
Method B:

To a suspension of 2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acetamide (**2.8**) (500 mg, 1.72 mmol), 3,4-dimethoxybenzaldehyde (301 mg, 1.81 mmol) and 3 Å molecular

sieves in EtOH (22.39 mL) was added piperidine (0.17 mL, 1.72 mmol). The reaction was refluxed overnight under a nitrogen atmosphere. The mixture was filtered through Celite® and washed with CH_2Cl_2. The filtrate was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (3% MeOH in CH_2Cl_2) to provide (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (**2.1**) (415 mg, 55%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.52 (d, *J* = 1.9 Hz, 1H), 7.84 (m, 1H), 7.81 (s, 1H), 7.62 (dddd, *J* = 9.9, 6.0, 2.3, 1.5 Hz, 2H), 7.51 (m, 2H), 7.26 (ddd, *J* = 7.8, 4.8, 0.7 Hz, 1H), 7.15 (dt, *J* = 8.4, 2.0 Hz, 1H), 7.09 (t, *J* = 6.1 Hz, 1H), 6.81 (d, *J* = 8.3 Hz, 1H), 4.53 (d, *J* = 6.2 Hz, 2H), 3.93 (s, 3H), 3.77 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.5, 152.0, 149.0, 141.7, 139.3, 137.1, 133.8, 133.6, 129.3, 128.9, 128.2, 128.1, 127.8, 125.9, 124.1, 112.4, 110.9, 56.1, 55.9, 44.1. ESI-MS (*m/z*) 439.10, 331.06.

Preparation of 2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acetamide (2.8)

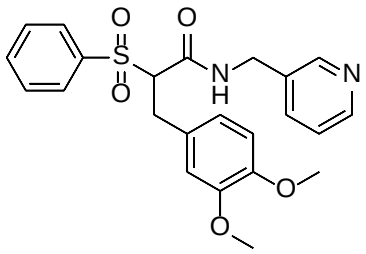


2.8

A mixture of ethyl 2-(phenylsulfonyl)acetate (1.00 g, 4.38 mmol) and 3-picolylamine (1.34 mL, 13.14 mmol) was stirred at 0 ^{0}C for 3 h under a nitrogen atmosphere. White precipitate which formed in a reaction flask was recrystallized in

EtOH to give 2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acetamide (**2.8**) (1.14 g, 90%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.54 (dd, *J* = 4.9, 1.2 Hz, 1H), 8.52 (d, *J* = 1.6 Hz, 1H), 7.81 (d, *J* = 7.3 Hz, 1H), 7.68 (t, *J* = 7.5 Hz, 1H), 7.63 (d, *J* = 7.8 Hz, 1H), 7.53 (t, *J* = 7.9 Hz, 1H), 7.47 (s, 1H), 7.27 (t, 1H), 4.46 (d, *J* = 6.0 Hz, 1H), 4.08 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.8, 149.4, 149.1, 138.0, 135.9, 134.7, 133.3, 129.9, 128.2, 123.8, 61.9, 41.5. ESI-MS (*m/z*) 291.05.

Preparation of 3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)propenamide (2.9)

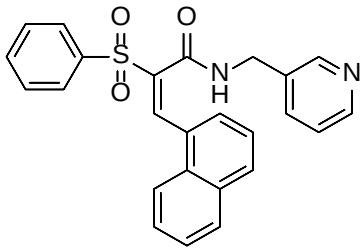


2.9

To a solution of (*E*)-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (**2.1**) (150 mg, 0.34 mmol) in THF (2.7 mL) was added 10 wt% Pd/C (73 mg). The reaction mixture was stirred at room temperature overnight under a hydrogen atmosphere. A second portion of 10 wt% Pd/C (73 mg) was added to the reaction mixture and stirred for 3 h at room temperature under a hydrogen atmosphere. The mixture was then filtered through Celite® and washed with EtOAc. The filtrate was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to give 3-(3,4-dimethoxyphenyl)-2-

(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)propanamide (**2.9**) (69 mg, 46%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, *J* = 3.8 Hz, 1H), 8.31 (d, *J* = 1.1 Hz, 1H), 7.80 (d, *J* = 7.4 Hz, 2H), 7.70 (t, *J* = 7.5 Hz, 1H), 7.55 (t, *J* = 7.8 Hz, 2H), 7.35 (d, *J* = 7.9 Hz, 1H), 7.17 (dd, *J* = 7.8, 4.8 Hz, 1H), 7.12 (t, *J* = 6.0 Hz, 1H), 6.69 (d, *J* = 8.8 Hz, 1H), 6.64 (dd, *J* = 6.4, 1.9 Hz, 2H), 6.63 (s, 1H), 4.33 (qd, *J* = 15.1, 6.0 Hz, 2H), 4.11 (dd, *J* = 11.6, 3.1 Hz, 1H), 3.82 (s, 3H), 3.72 (s, 3H), 3.28 (dd, *J* = 13.3, 3.1 Hz, 1H), 3.08 (dd, *J* = 13.1, 11.8 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.0, 149.1, 149.0, 148.8, 148.1, 136.1, 135.7, 134.6, 133.4, 129.6, 129.2, 128.2, 123.7, 121.2, 112.2, 111.3, 77.5, 77.2, 76.8, 73.2, 55.9, 41.4, 32.8. ESI-MS (*m/z*) 441.13.

Preparation of (*E*)-3-(naphthalen-1-yl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (2.10)

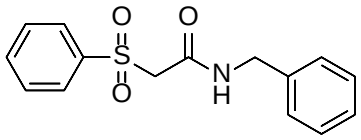


2.10

To a suspension of 2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acetamide (**2.8**) (500 mg, 1.72 mmol), 1-naphthaldehyde (0.25 mL, 1.81 mmol) and 4 Å molecular sieves in toluene (5.17 mL) were added piperidine (0.09 mL, 0.86 mmol) and AcOH (0.10 mL, 1.81 mmol). The reaction was refluxed overnight under a nitrogen atmosphere. The mixture was filtered through Celite® and washed with EtOAc. The organic layer was washed with

water (2 x 15 mL), brine (15 mL) and dried over $MgSO_4$. The solvent was removed under reduce pressure and the crude mixture was purified by flash column chromatography (1% MeOH in CH_2Cl_2) to give (*E*)-3-(naphthalen-1-yl)-2-(phenylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (**2.10**) (224 mg, 30%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.43 (dd, *J* = 4.8, 1.5 Hz, 1H), 8.18 (d, *J* = 1.9 Hz, 1H), 7.96 (dd, *J* = 13.1, 5.0 Hz, 3H), 7.88 (m, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.55 (m, 5H), 7.30 (m, 2H), 7.11 (dd, *J* = 7.8, 4.8 Hz, 1H), 6.69 (t, *J* = 5.9 Hz, 1H), 4.32 (d, *J* = 6.2 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.1, 151.7, 150.6, 148.6, 135.5, 134.7, 130.0, 129.3, 129.3, 127.4, 127.3, 126.8, 126.3, 119.1, 113.4, 110.7, 36.7. ESI-MS (*m/z*) 429.10, 321.06.

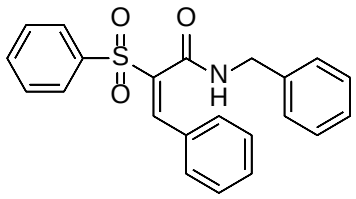
Preparation of *N*-benzyl-2-(phenylsulfonyl)acetamide (2.11)



2.11

A mixture of ethyl 2-(phenylsulfonyl)acetate (1.00 g, 4.38 mmol) and $BnNH_2$ (1.44 mL, 13.14 mmol) was stirred at 0 ^{o}C for 2 h under a nitrogen atmosphere. White precipitates which formed in a reaction flask was recrystallized in EtOH to give *N*-benzyl-2-(phenylsulfonyl)acetamide (**2.11**) (1.07 g, 84%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (dd, *J* = 8.2, 1.1 Hz, 1H), 7.67 (t, *J* = 7.6 Hz, 1H), 7.51 (t, *J* = 7.8 Hz, 1H), 7.37–7.27 (m, 2H), 7.09 (s, 1H), 4.44 (d, *J* = 5.9 Hz, 1H), 4.03 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.4, 138.1, 137.3, 134.6, 129.6, 128.9, 128.2, 128.1, 127.9, 61.9, 44.2. ESI-MS (*m/z*) 290.07.

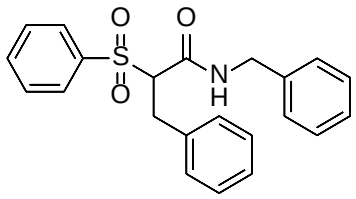
Preparation of (*E*)-*N*-Benzyl-3-phenyl-2-(phenylsulfonyl)acrylamide (2.12)



2.12

To a suspension of *N*-benzyl-2-(phenylsulfonyl)acetamide (**2.11**) (500 mg, 1.73 mmol), benzaldehyde (0.18 mL, 1.81 mmol) and 4 Å molecular sieves in toluene (5.18 mL) were added piperidine (0.09 mL, 0.86 mmol) and AcOH (0.10 mL, 1.81 mmol). The reaction was refluxed overnight under a nitrogen atmosphere. The mixture was filtered through Celite® and washed with EtOAc. The organic layer was washed with water (2 x 15 mL), brine (15 mL) and dried over $MgSO_4$. The solvent was removed under reduce pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to give (*E*)-*N*-benzyl-3-phenyl-2-(phenylsulfonyl)acrylamide (**2.12**) (356 mg, 47%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (s, 1H), 7.85 (s, 2H), 7.62 (dd, *J* = 10.8, 3.9 Hz, 1H), 7.50 (t, *J* = 7.1 Hz, 4H), 7.40 (t, *J* = 7.1 Hz, 1H), 7.32 (m, 5H), 7.23 (dd, *J* = 6.9, 2.1 Hz, 2H), 6.64 (s, 1H), 4.49 (d, *J* = 6.0 Hz, 2H); ^{13}C NMR (101 MHz,) δ 172.1, 151.9, 150.8, 148.8, 135.7, 134.9, 130.2, 129.5, 129.5, 127.6, 127.4, 127.0, 126.4, 119.3, 113.6, 110.9, 36.8. ESI-MS (*m/z*) 338.11, 271.04.

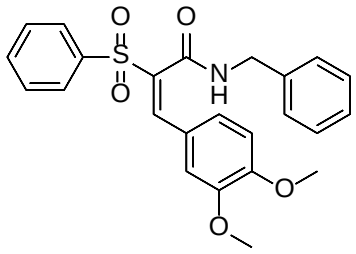
Preparation of *N*-benzyl-3-phenyl-2-(phenylsulfonyl)propenamide (2.13)



2.13

To a solution of (*E*)-*N*-benzyl-3-phenyl-2-(phenylsulfonyl)acrylamide (**2.12**) (350 mg, 0.93 mmol) in THF (7.4 mL) was added 20 wt% $Pd(OH)_2/C$ (260 mg). The reaction mixture was stirred for 48 h at room temperature under a hydrogen atmosphere. The mixture was then filtered through Celite® and washed with EtOAc. The filtrate was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to give *N*-benzyl-3-phenyl-2-(phenylsulfonyl)propenamide (**2.13**) (198 mg, 56%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (dd, *J* = 7.1, 1.1 Hz, 1H), 7.68 (dd, *J* = 7.5, 1.1 Hz, 1H), 7.52 (t, *J* = 7.9 Hz, 1H), 7.31–7.21 (m, 4H), 7.14 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.09 (dd, *J* = 6.9, 2.4 Hz, 1H), 6.35 (s, 1H), 4.34 (qd, *J* = 14.8, 5.9 Hz, 1H), 3.96 (dd, *J* = 11.3, 3.1 Hz, 1H), 3.36 (dd, *J* = 13.4, 3.1 Hz, 1H), 3.21 (dd, *J* = 13.5, 11.4 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.3, 137.3, 136.3, 136.0, 134.5, 129.5, 129.2, 129.2, 128.9, 128.7, 128.0, 127.7, 127.3, 77.5, 77.2, 76.8, 73.1, 44.1, 32.9. ESI-MS (*m/z*) 380.09.

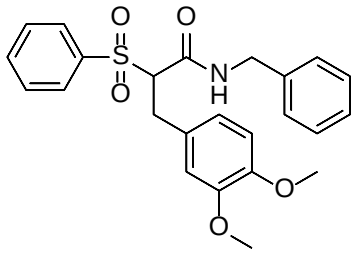
Preparation of (*E*)-*N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl) acrylamide (2.14)



2.14

To a suspension of *N*-benzyl-2-(phenylsulfonyl)acetamide (**2.11**) (500 mg, 1.73 mmol), 3,4-dimethoxybenzaldehyde (302 mg, 1.81 mmol) and 3 Å molecular sieves in EtOH (22.46 mL) was added piperidine (0.17 mL, 1.73 mmol). The reaction was refluxed overnight under a nitrogen atmosphere. The mixture was filtered through Celite® and washed with CH_2Cl_2. The filtrate was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to provide (*E*)-*N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl)acrylamide (**2.14**) (582 mg, 77%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84 (dd, *J* = 7.5, 1.2 Hz, 1H), 7.78 (s, 1H), 7.60 (dd, *J* = 7.5, 1.1 Hz, 1H), 7.48 (t, *J* = 7.9 Hz, 1H), 7.34–7.27 (m, 2H), 7.24 (dd, *J* = 7.5, 1.7 Hz, 1H), 7.17 (d, *J* = 2.0 Hz, 1H), 7.14 (dd, *J* = 8.4, 2.1 Hz, 1H), 6.87 (t, *J* = 6.0 Hz, 1H), 6.79 (d, *J* = 8.4 Hz, 1H), 4.50 (d, *J* = 6.1 Hz, 1H), 3.90 (s, 2H), 3.71 (s, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.5, 152.0, 149.0, 141.7, 139.3, 137.1, 133.8, 133.6, 129.3, 128.9, 128.2, 128.1, 127.8, 125.9, 124.1, 112.4, 110.9, 56.1, 55.9, 44.1. ESI-MS (*m/z*) 438.10, 331.05.

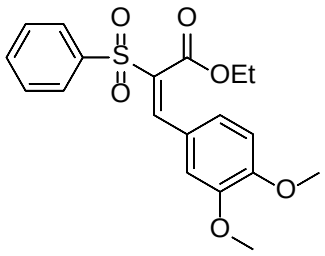
Preparation of *N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl) propenamide (2.15)



2.15

To a solution of (*E*)-*N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl) acrylamide (**2.14**) (500 mg, 0.34 mmol) in THF (9.14 mL) was added 10 wt% Pd/C (243 mg). The reaction mixture was stirred at room temperature overnight under a hydrogen atmosphere. A second portion of 10 wt% Pd/C (243 mg) was added to the reaction mixture and stirred for 3 h at room temperature under a hydrogen atmosphere. The mixture was then filtered through Celite® and washed with EtOAc. The filtrate was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to give *N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl) propanamide (**2.15**) (348 mg, 69%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 7.4 Hz, 1H), 7.69 (t, *J* = 7.4 Hz, 1H), 7.53 (t, *J* = 7.9 Hz, 1H), 7.29 (dd, *J* = 5.1, 1.9 Hz, 1H), 7.10 (dd, *J* = 6.8, 2.3 Hz, 1H), 6.73 (d, *J* = 8.1 Hz, 1H), 6.71–6.66 (m, 1H), 6.35 (t, *J* = 5.6 Hz, 1H), 4.36 (qd, *J* = 14.8, 5.9 Hz, 1H), 3.93 (dd, *J* = 11.3, 3.0 Hz, 1H), 3.85 (s, 2H), 3.77 (s, 2H), 3.33 (dd, *J* = 13.4, 3.2 Hz, 1H), 3.15 (dd, *J* = 13.4, 11.4 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.5, 149.0, 148.2, 137.2, 136.2, 134.5, 129.5, 129.2, 128.8, 128.4, 127.9, 127.7, 121.2, 112.2, 111.3, 73.5, 55.9, 44.2, 32.6. ESI-MS (*m/z*) 440.19.

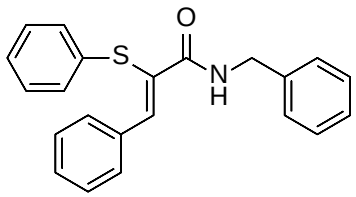
Preparation of (*E*)-ethyl 3-(3,4-dimethoxyphenyl)-2-(phenylsulfonyl) acrylate (2.16)



2.16

A suspension of ethyl 2-(phenylsulfonyl)acetate (250 mg, 1.10 mmol), 3,4-dimethoxybenzaldehyde (191 mg, 1.15 mmol) and 3 Å molecular sieves in EtOH (2.19 mL) was added piperidine (0.11 mL, 0.093 mmol). The reaction was stirred for 48 H overnight under a nitrogen atmosphere. The mixture was filtered through Celite® and washed with CH_2Cl_2. The filtrate was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to provide (*E*)-*N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(methylsulfonyl)acrylamide (**2.16**) (281 mg, 68%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.95 (d, *J* = 7.9 Hz, 1H), 7.91 (s, 1H), 7.63 (t, *J* = 7.4 Hz, 1H), 7.55 (t, *J* = 7.7 Hz, 1H), 7.15 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.09 (d, *J* = 1.8 Hz, 1H), 6.87 (d, *J* = 8.4 Hz, 1H), 4.24 (q, *J* = 7.1 Hz, 1H), 3.93 (s, 2H), 3.85 (s, 2H), 1.18 (t, *J* = 7.1 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.6, 152.3, 149.0, 144.3, 140.5, 133.5, 131.8, 129.1, 128.4, 125.7, 124.2, 112.5, 110.9, 62.4, 56.1, 56.0, 13.9. ESI-MS (*m/z*) 376.99, 331.04.

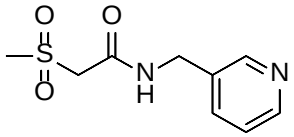
Preparation of (*Z*)-*N*-benzyl-3-phenyl-2-(phenylthio)acrylamide (2.18)



2.18

To a suspension of (*Z*)-3-phenyl-2-(phenylthio)propenoic acid (**2.3**) (1.0 g, 3.90 mmol), BOP (2.0 g, 4.68 mmol) and $BnNH_2$ (0.51 mL, 4.68 mmol) in dry CH_2Cl_2 (19.51 mL) was added Et_3N (1.36 mL, 9.75 mmol). The mixture was stirred at room temperature for 12 h under a nitrogen atmosphere. Water (20 mL) was then added and the mixture was extracted with CH_2Cl_2 (2 x 15 mL). The organic phase was washed with brine (20 mL) and dried over Na_2SO_4. The solvent was removed under reduce pressure and the crude product was purified by flash column chromatography (30% EtOAc in hexane) to provide (*Z*)-*N*-benzyl-3-phenyl-2-(phenylthio)acrylamide (**2.18**) (673 mg, 50%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8.28 (s, 1H), 7.54 (dd, *J* = 8.4, 1.0 Hz, 2H), 7.48 (t, *J* = 7.5 Hz, 1H), 7.31 (m, 12H), 4.55 (d, *J* = 5.7 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.2, 151.0, 140.2, 137.5, 136.2, 133.6, 132.6, 129.9, 129.7, 129.0, 128.9, 128.0, 127.9, 127.8, 127.4, 77.4, 77.2, 76.9, 44.6. ESI-MS (*m/z*) 346.02.

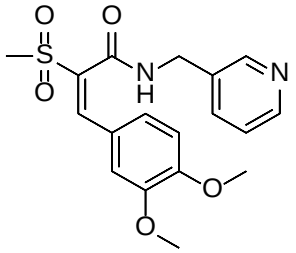
Preparation of 2-(methylsulfonyl)-*N*-(pyridin-3-ylmethyl)acetamide (2.19)



2.19

To a cooled solution of ethyl 2-(methylsulfonyl)acetate (0.81 mL, 6.02 mmol) was added 3-picolylamine (1.84 mL, 18.05 mmol). The reaction was stirred at room temperature overnight under a nitrogen atmosphere. To the mixture was added EtOH (3 mL), cooled in ice-bath and stirred with glass rod. White precipitate was collected by vacuum filtration and recrystallized in EtOH to give 2-(methylsulfonyl)-*N*-(pyridin-3-ylmethyl)acetamide (**2.19**) (928 mg, 68%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, *J* = 2.1 Hz, 1H), 8.52 (dd, *J* = 4.8, 1.4 Hz, 1H), 7.67 (d, *J* = 7.8 Hz, 1H), 7.29 (dd, *J* = 7.8, 4.9 Hz, 1H), 7.24 (s, 1H), 4.51 (d, *J* = 6.0 Hz, 2H), 3.93 (s, 2H), 3.09 (s, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.6, 149.2, 149.2, 135.7, 133.0, 123.8, 60.8, 41.7, 41.4. ESI-MS (*m/z*) 229.03.

Preparation of (*E*)-3-(3,4-dimethoxyphenyl)-2-(methylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (2.20)

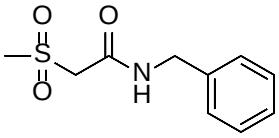


2.20

To a suspension of 2-(methylsulfonyl)-*N*-(pyridin-3-ylmethyl)acetamide (**2.19**) (500 mg, 2.19 mmol), 3,4-dimethoxybenzaldehyde (383 mg, 2.30 mmol) and 3 Å molecular sieves in EtOH (28.5 mL) was added piperidine (0.65 mL, 6.58 mmol). The reaction was refluxed overnight under a nitrogen atmosphere. The mixture was filtered through Celite® and washed with CH_2Cl_2. The filtrate was concentrated under reduce

pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to provide (*E*)-3-(3,4-dimethoxyphenyl)-2-(methylsulfonyl)-*N*-(pyridin-3-ylmethyl)acrylamide (**2.20**) (566 mg, 69%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.46 (s, 1H), 8.44 (d, *J* = 4.0 Hz, 1H), 7.65 (d, *J* = 7.5 Hz, 1H), 7.54 (s, 1H), 7.32 (s, 1H), 7.24 (dd, *J* = 7.3, 4.9 Hz, 1H), 7.04 (d, *J* = 8.3 Hz, 1H), 7.01 (s, 1H), 6.76 (d, *J* = 8.3 Hz, 1H), 4.55 (d, *J* = 5.9 Hz, 2H), 3.91 (s, 3H), 3.76 (s, 3H), 3.16 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.9, 152.0, 149.3, 149.0, 141.6, 135.7, 133.5, 132.8, 124.7, 123.8, 112.4, 111.1, 56.1, 55.9, 43.1, 41.5. ESI-MS (*m/z*) 377.03, 269.00.

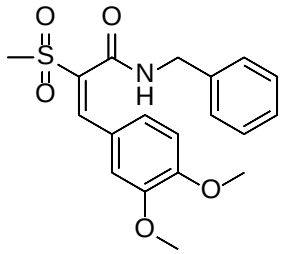
Preparation of *N*-benzyl-2-(methylsulfonyl)acetamide (2.21)



2.21

To a cooled solution of ethyl 2-(methylsulfonyl)acetate (0.81 mL, 6.02 mmol) was added $BnNH_2$ (1.97 mL, 18.05 mmol). The reaction was stirred at room temperature overnight under a nitrogen atmosphere. White precipitate was collected by vacuum filtration and recrystallized in EtOH to give *N*-benzyl-2-(methylsulfonyl) acetamide (**2.21**) (1.06 g, 77%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, *J* = 2.1 Hz, 1H), 8.52 (dd, *J* = 4.8, 1.4 Hz, 1H), 7.67 (d, *J* = 7.8 Hz, 1H), 7.29 (dd, *J* = 7.8, 4.9 Hz, 1H), 7.24 (s, 1H), 4.51 (d, *J* = 6.0 Hz, 2H), 3.93 (s, 2H), 3.09 (s, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.6, 149.2, 149.2, 135.7, 133.0, 123.8, 60.8, 41.7, 41.4. ESI-MS (*m/z*) 228.05.

Preparation of (*E*)-*N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(methylsulfonyl) acrylamide (2.22)



2.22

To a suspension of *N*-benzyl-2-(methylsulfonyl)acetamide (**2.21**) (500 mg, 2.20 mmol), 3,4-dimethoxybenzaldehyde (383 mg, 2.31 mmol) and 3 Å molecular sieves in EtOH (28.6 mL) was added piperidine (0.22 mL, 2.20 mmol). The reaction was refluxed overnight under a nitrogen atmosphere. The mixture was filtered through Celite® and washed with CH_2Cl_2. The filtrate was concentrated under reduce pressure and the crude mixture was purified by flash column chromatography (30% EtOAc in hexane) to provide (*E*)-*N*-benzyl-3-(3,4-dimethoxyphenyl)-2-(methylsulfonyl)acrylamide (**2.22**) (520 mg, 63%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, *J* = 2.1 Hz, 1H), 8.52 (dd, *J* = 4.8, 1.4 Hz, 1H), 7.67 (d, *J* = 7.8 Hz, 1H), 7.29 (dd, *J* = 7.8, 4.9 Hz, 1H), 7.24 (s, 1H), 4.51 (d, *J* = 6.0 Hz, 2H), 3.93 (s, 2H), 3.09 (s, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.5, 152.0, 149.1, 141.6, 136.8, 133.6, 128.9, 127.9, 125.1, 123.8, 112.2, 111.0, 77.4, 77.1, 76.9, 56.1, 55.9, 44.2, 42.9. ESI-MS (*m/z*) 376.21, 269.02.

2.5 References

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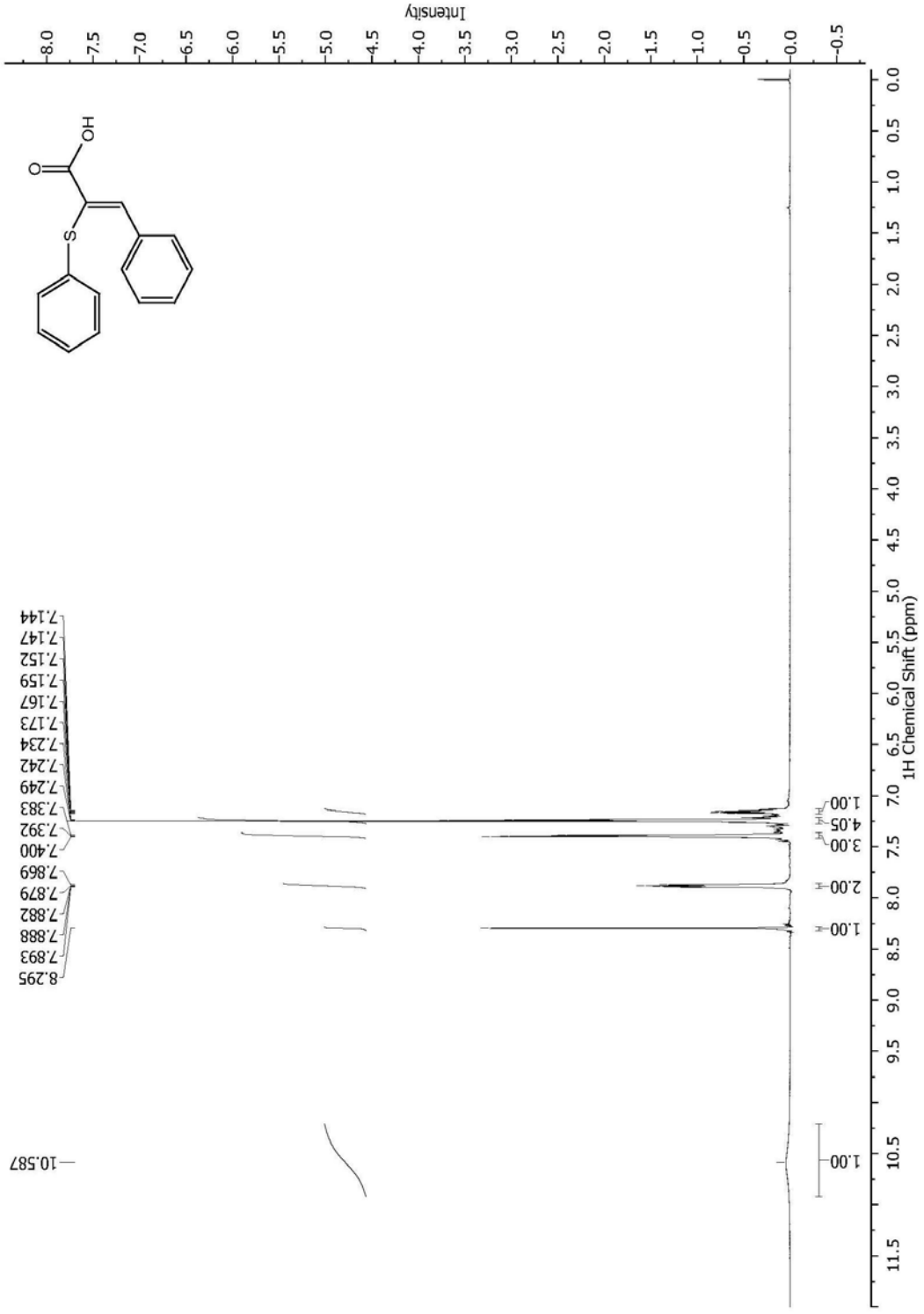
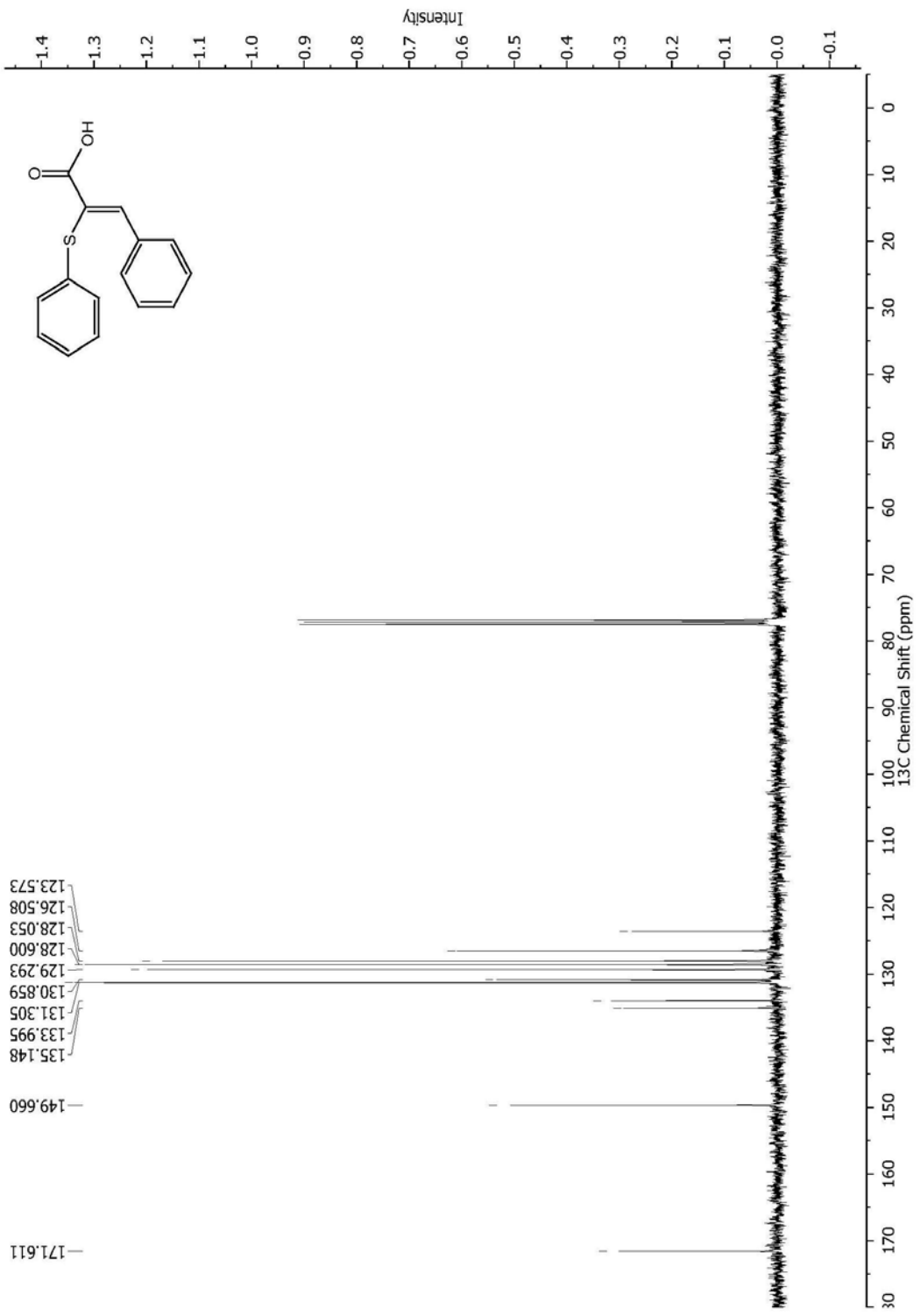
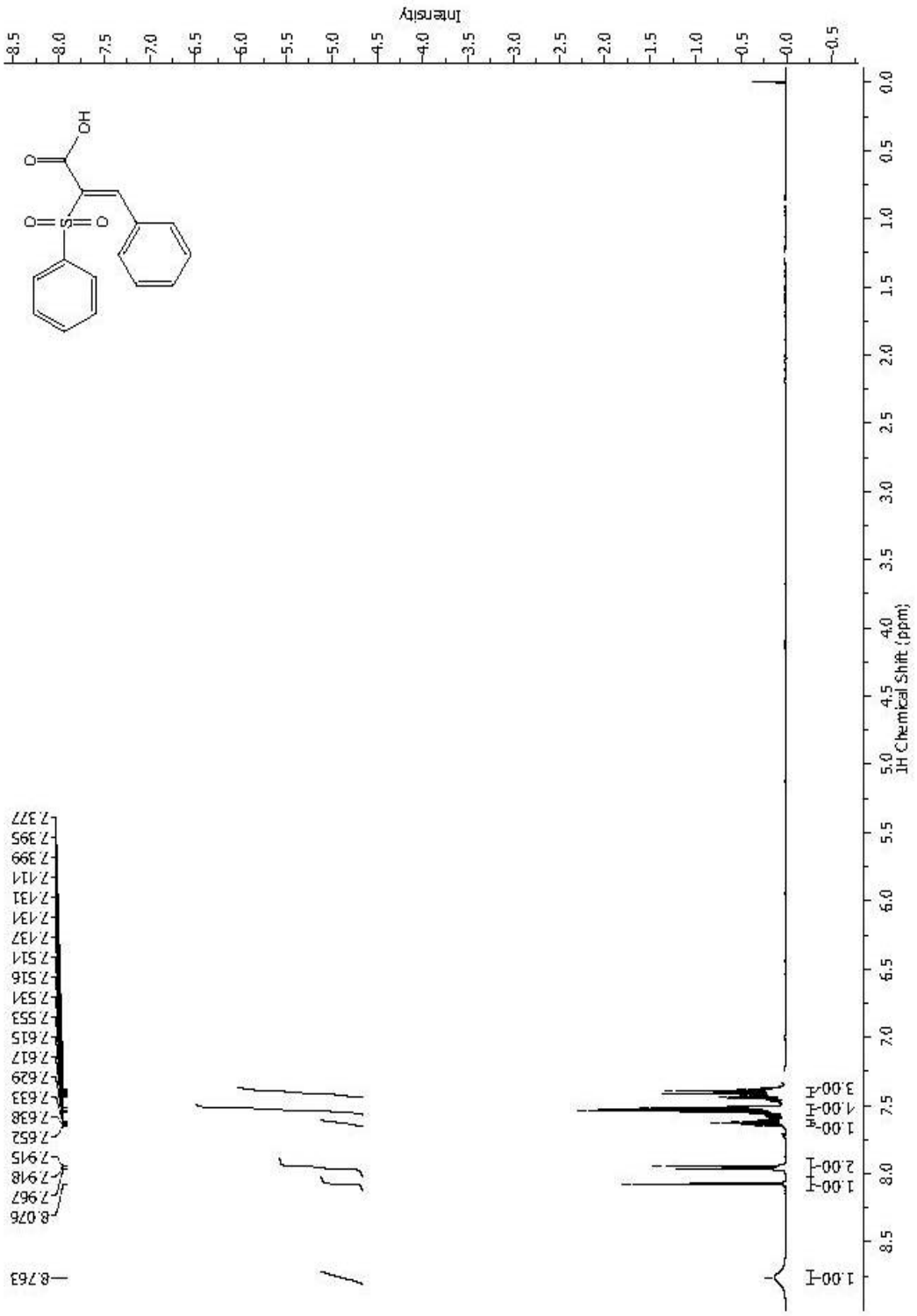
SUPPORTING INFORMATION



^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2.3**



^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **2.3**



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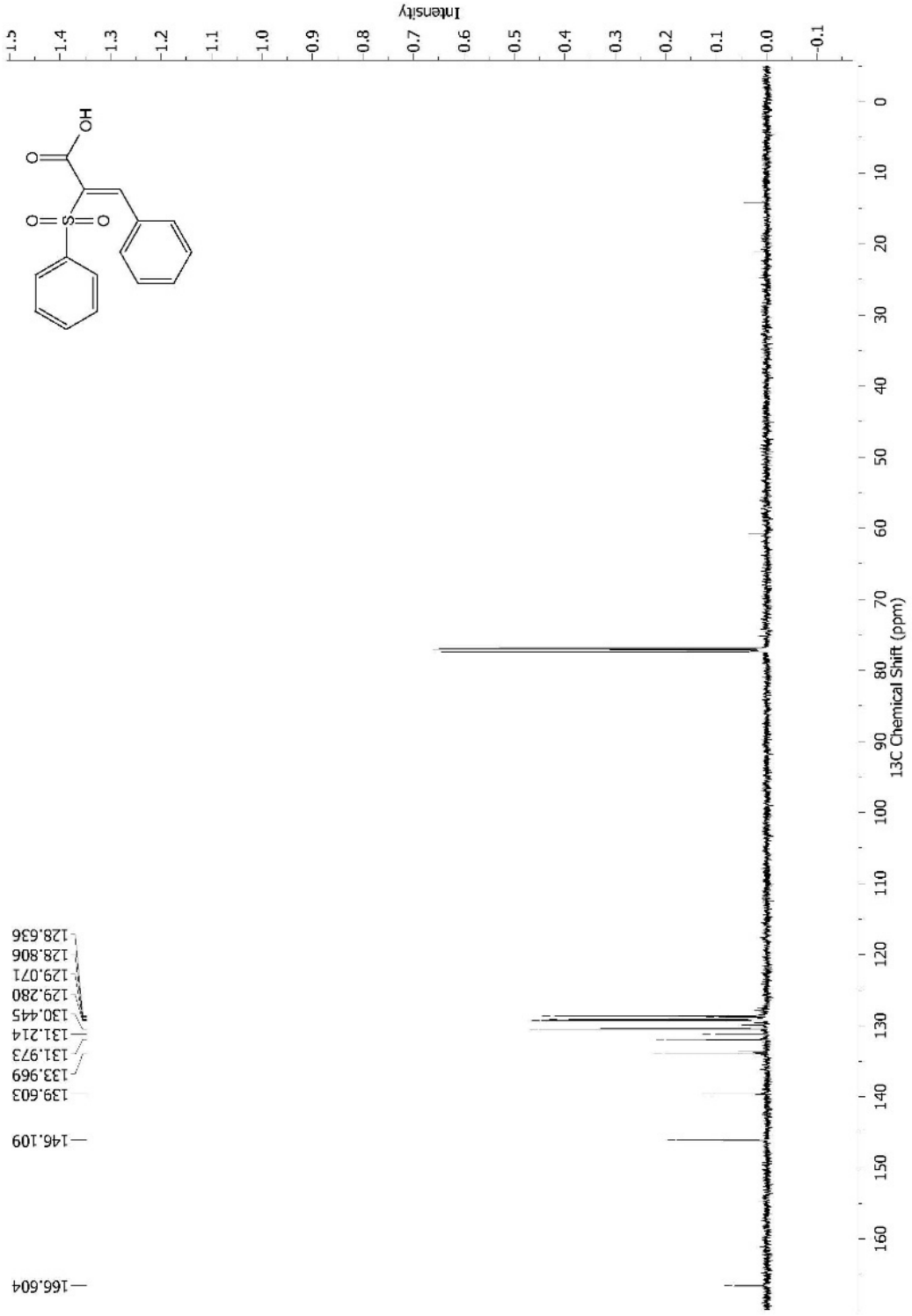

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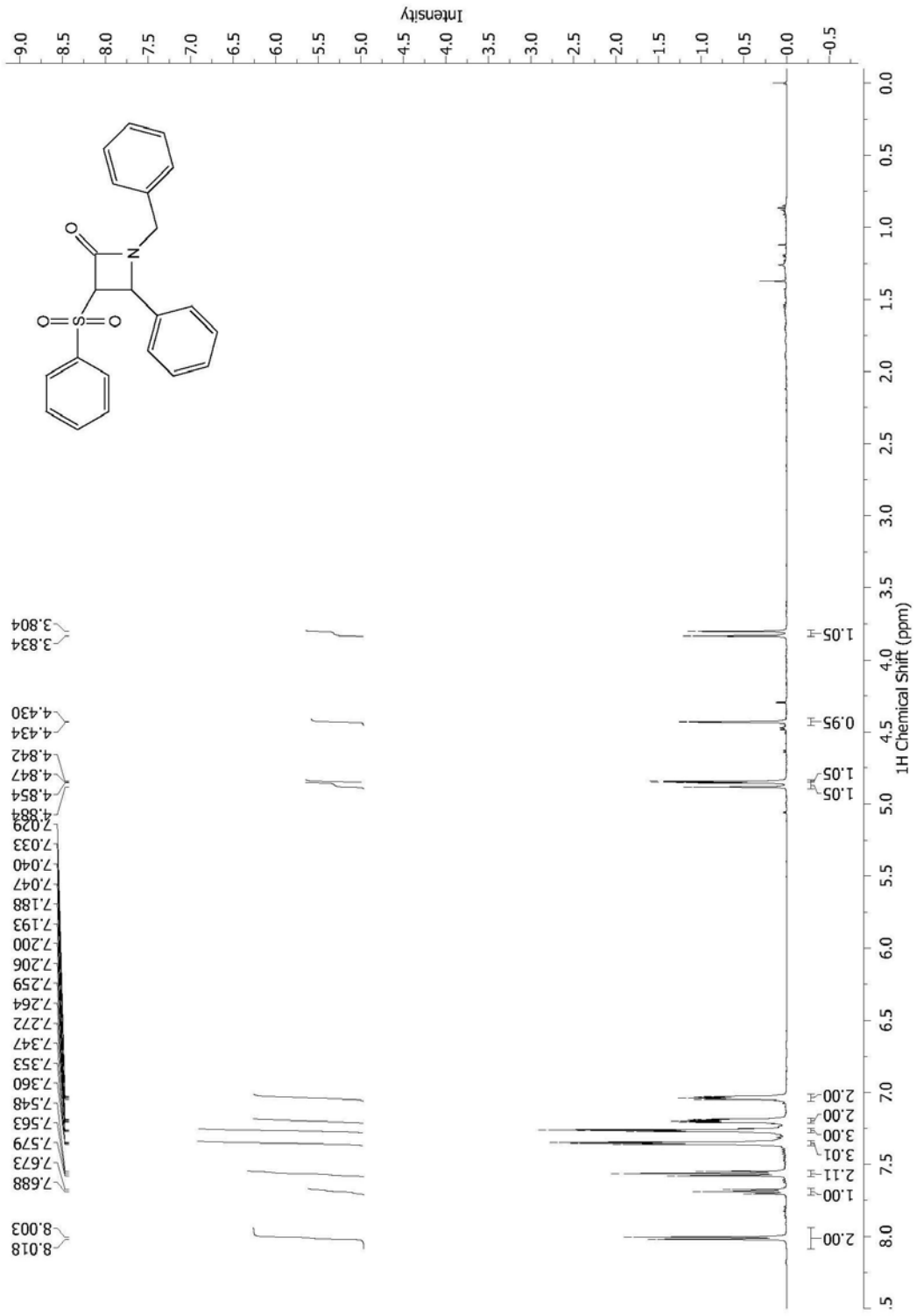
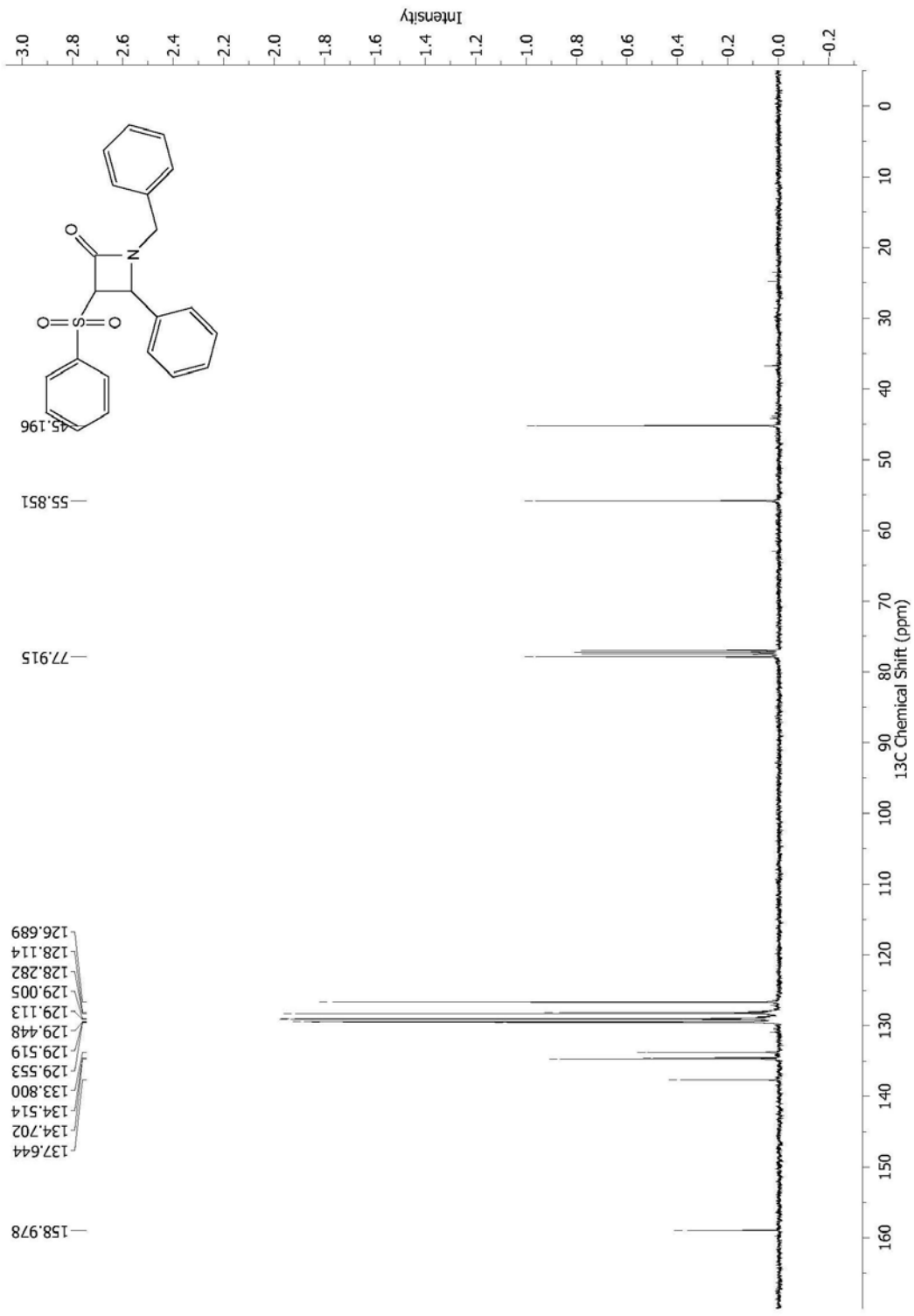


^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.5**



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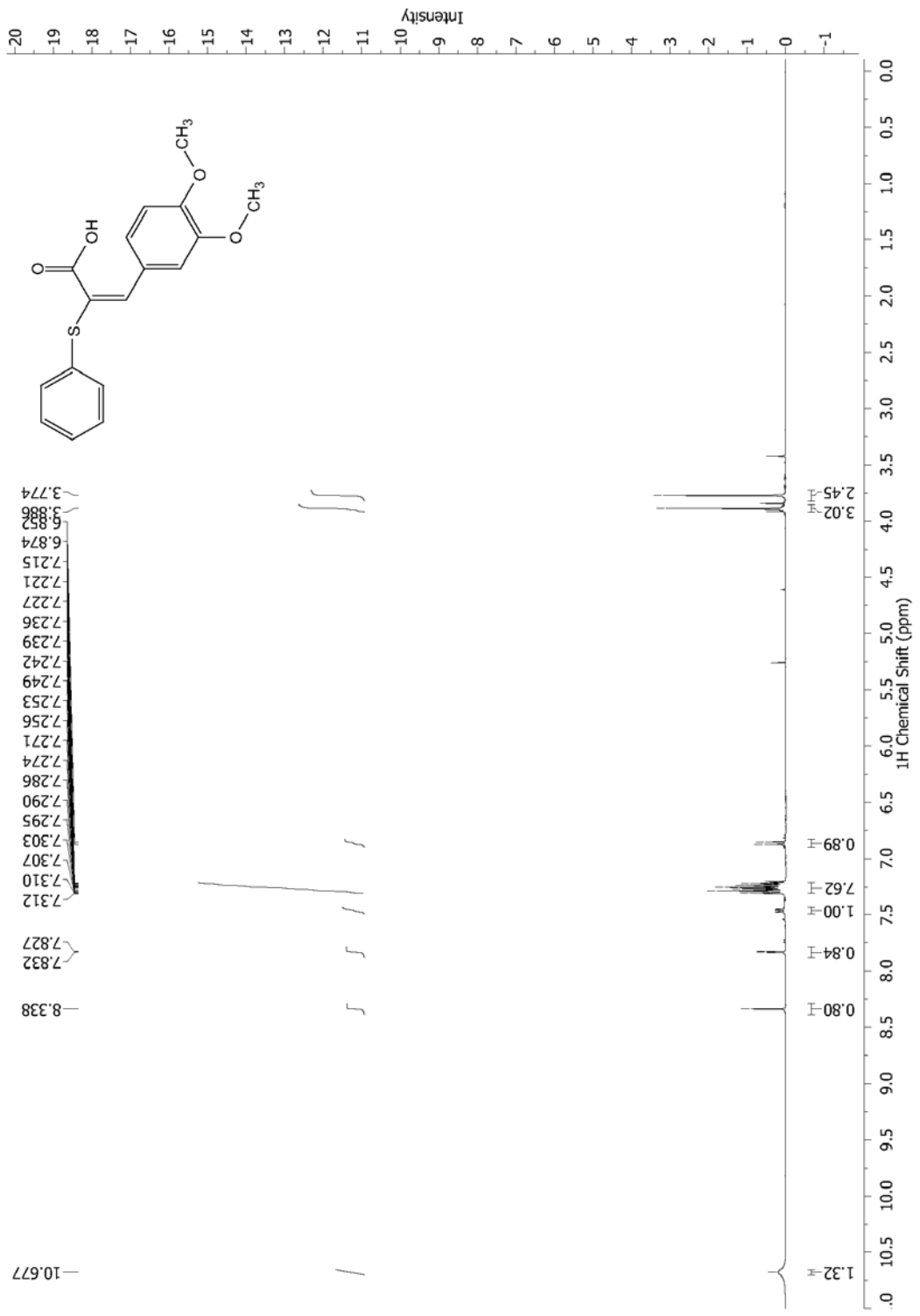

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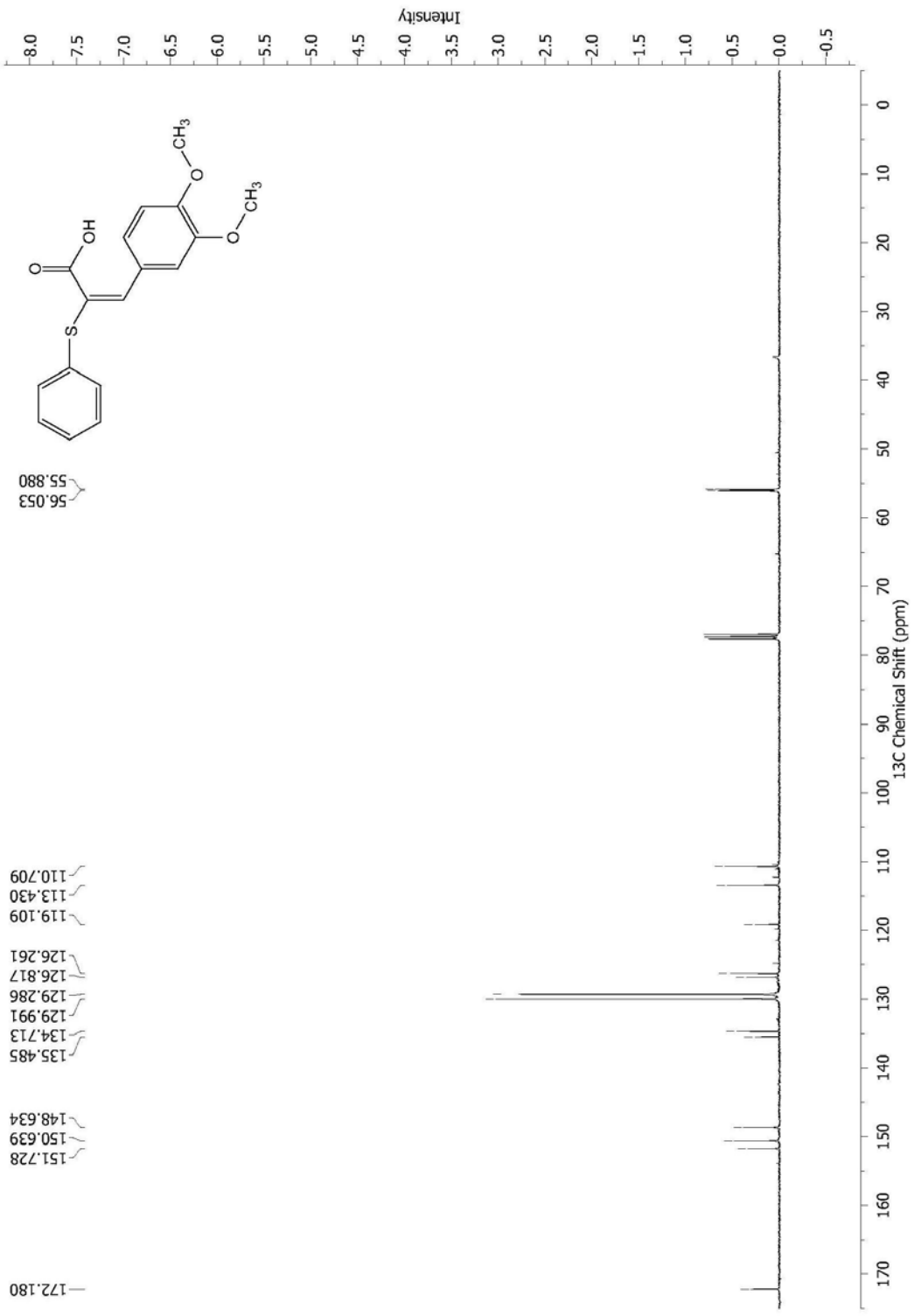

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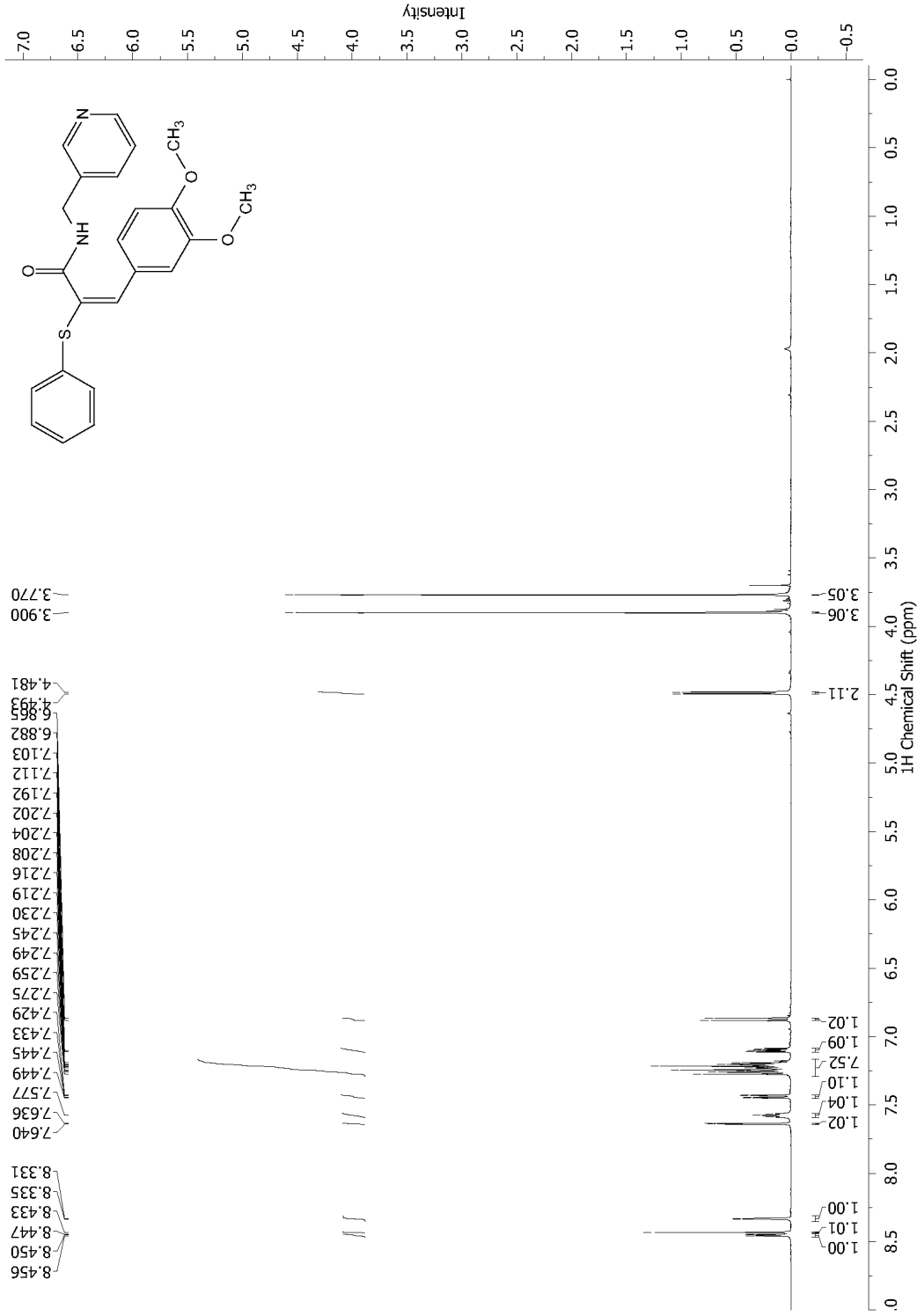

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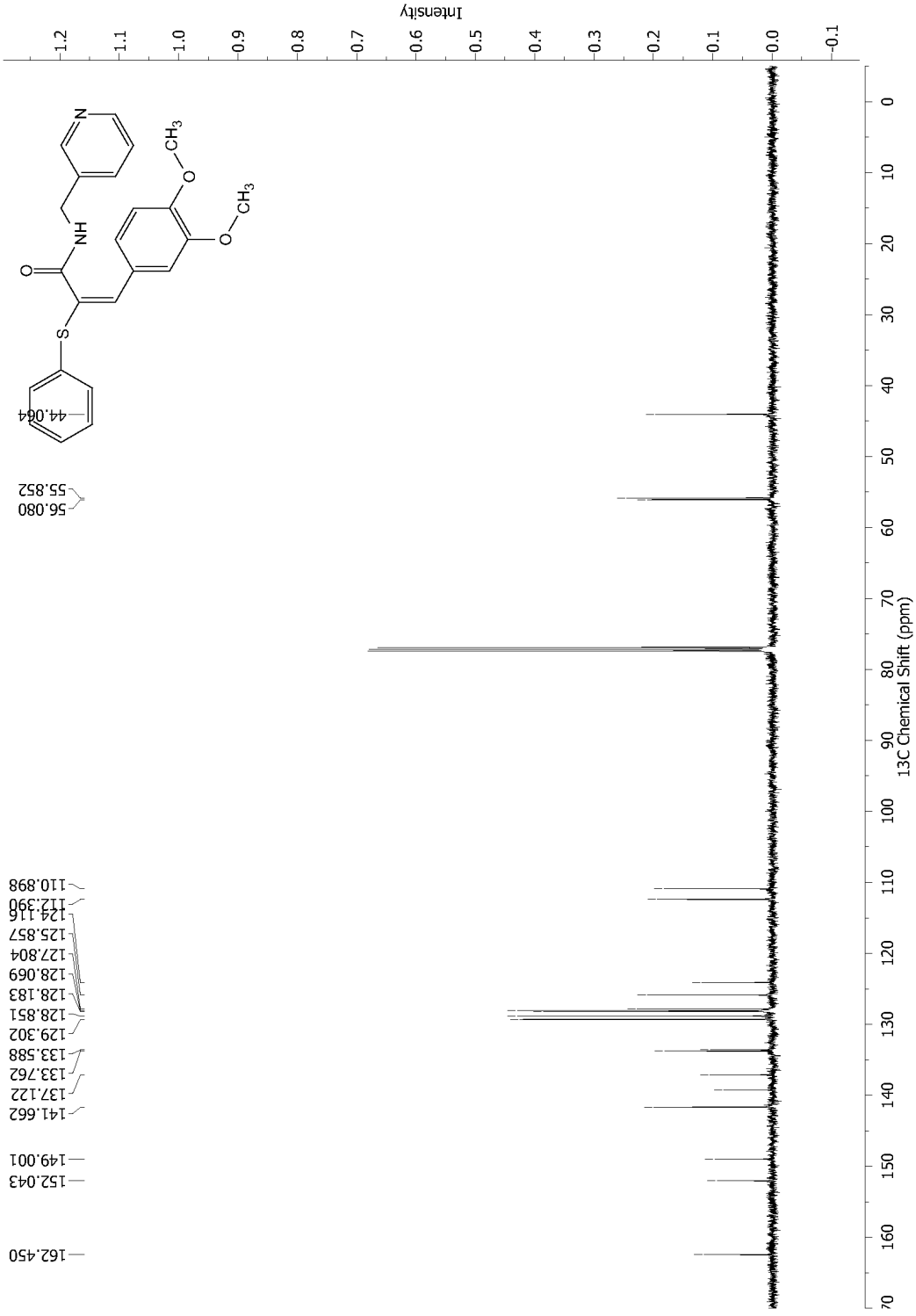
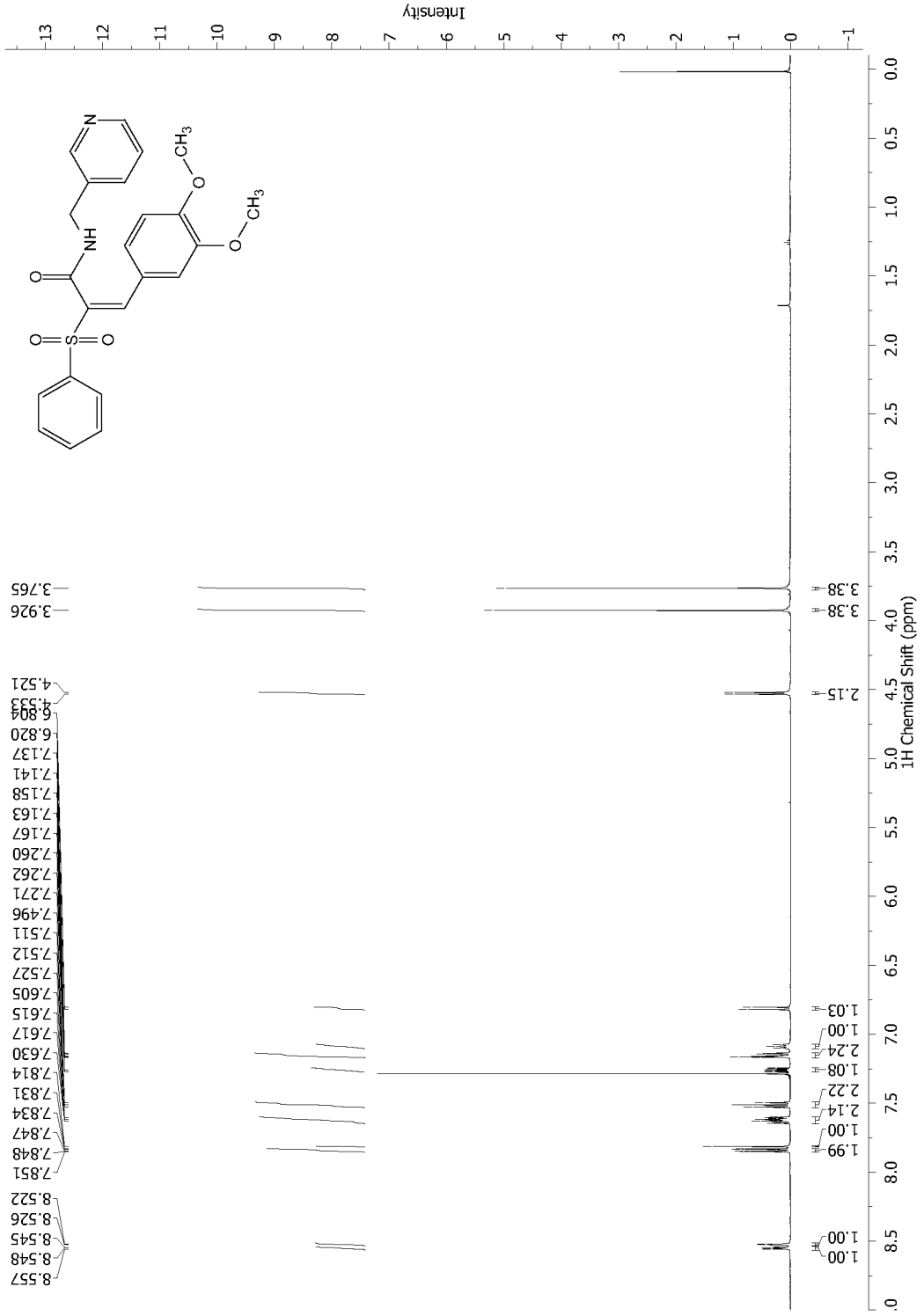
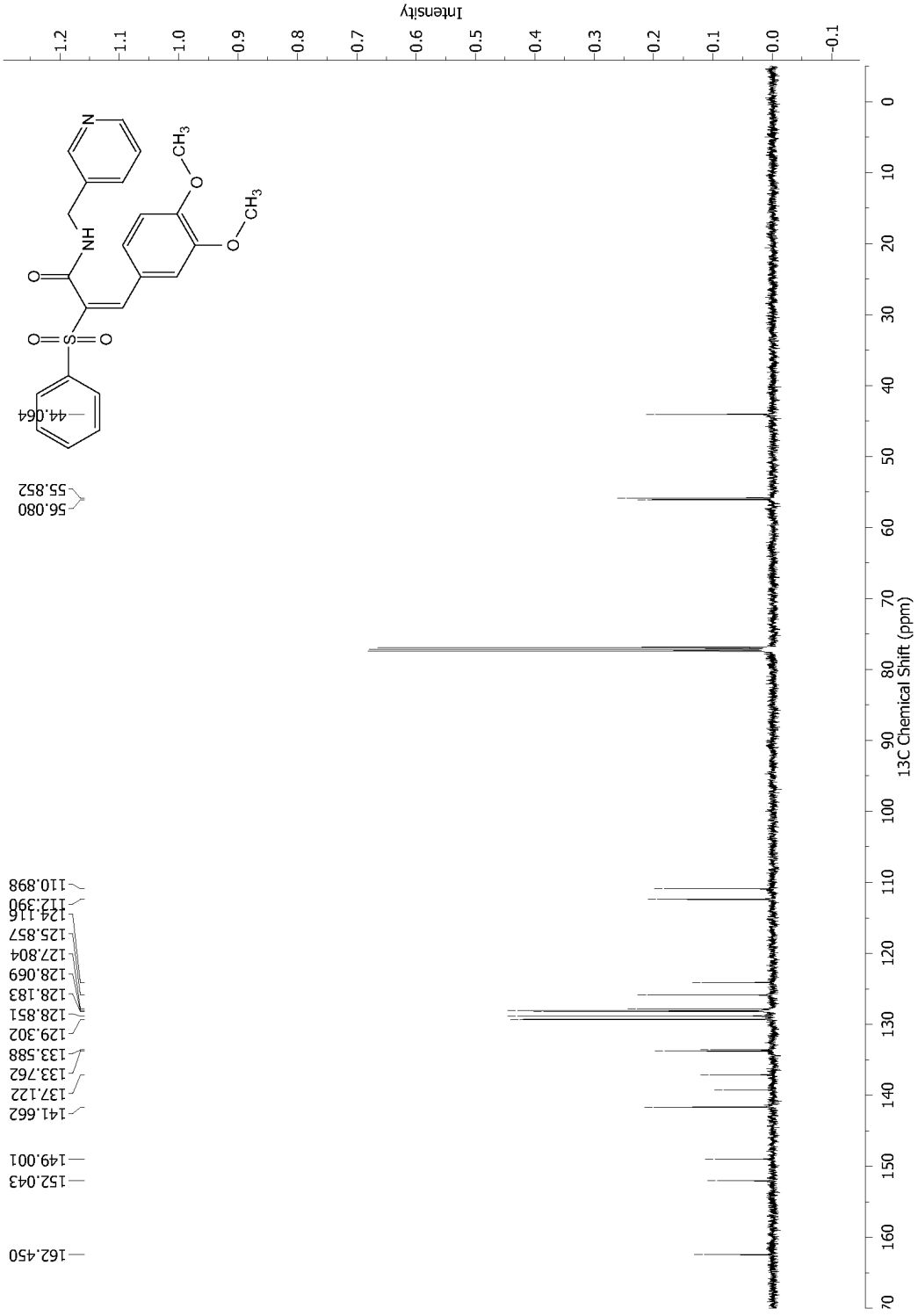


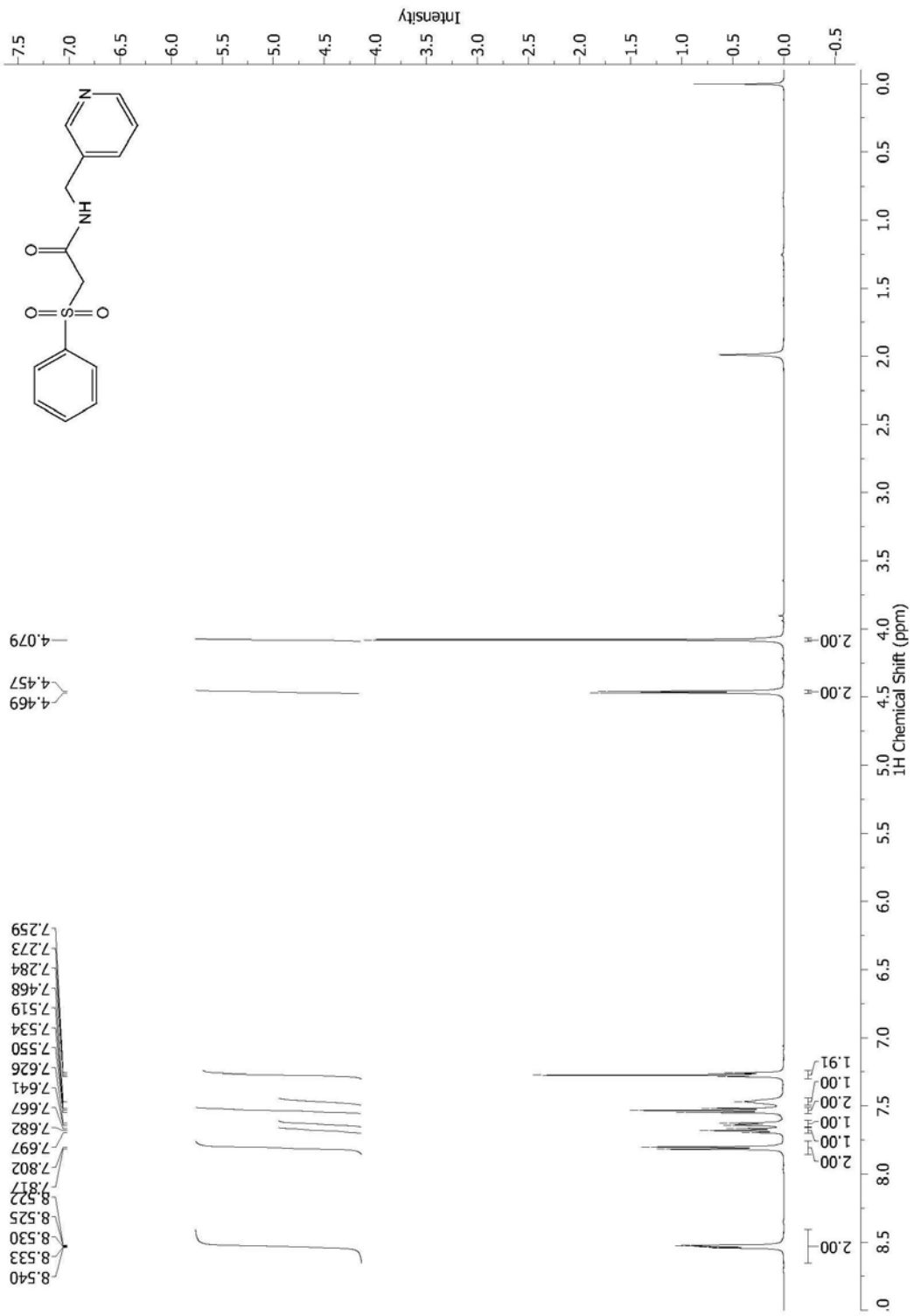
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.7**



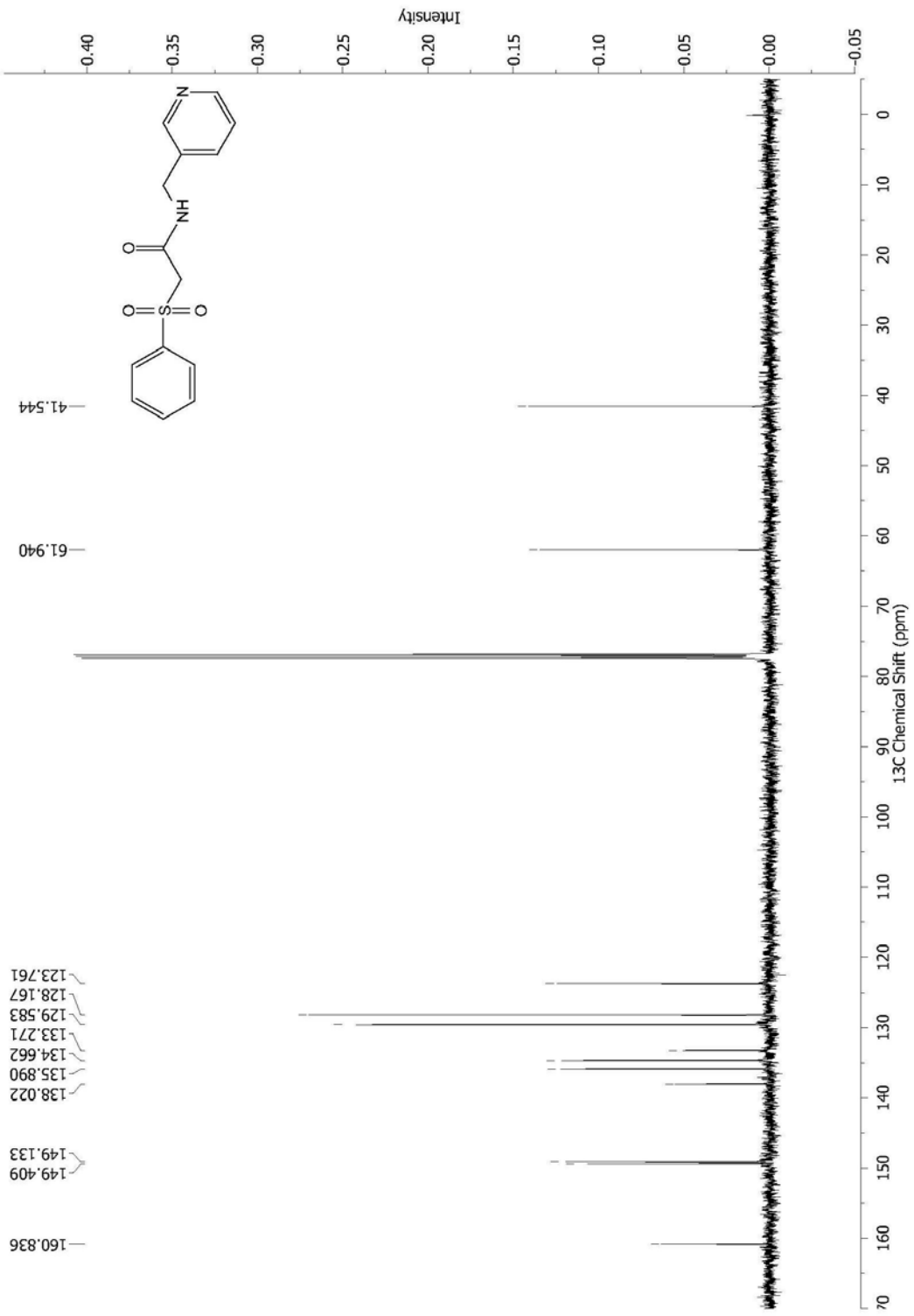
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.1**



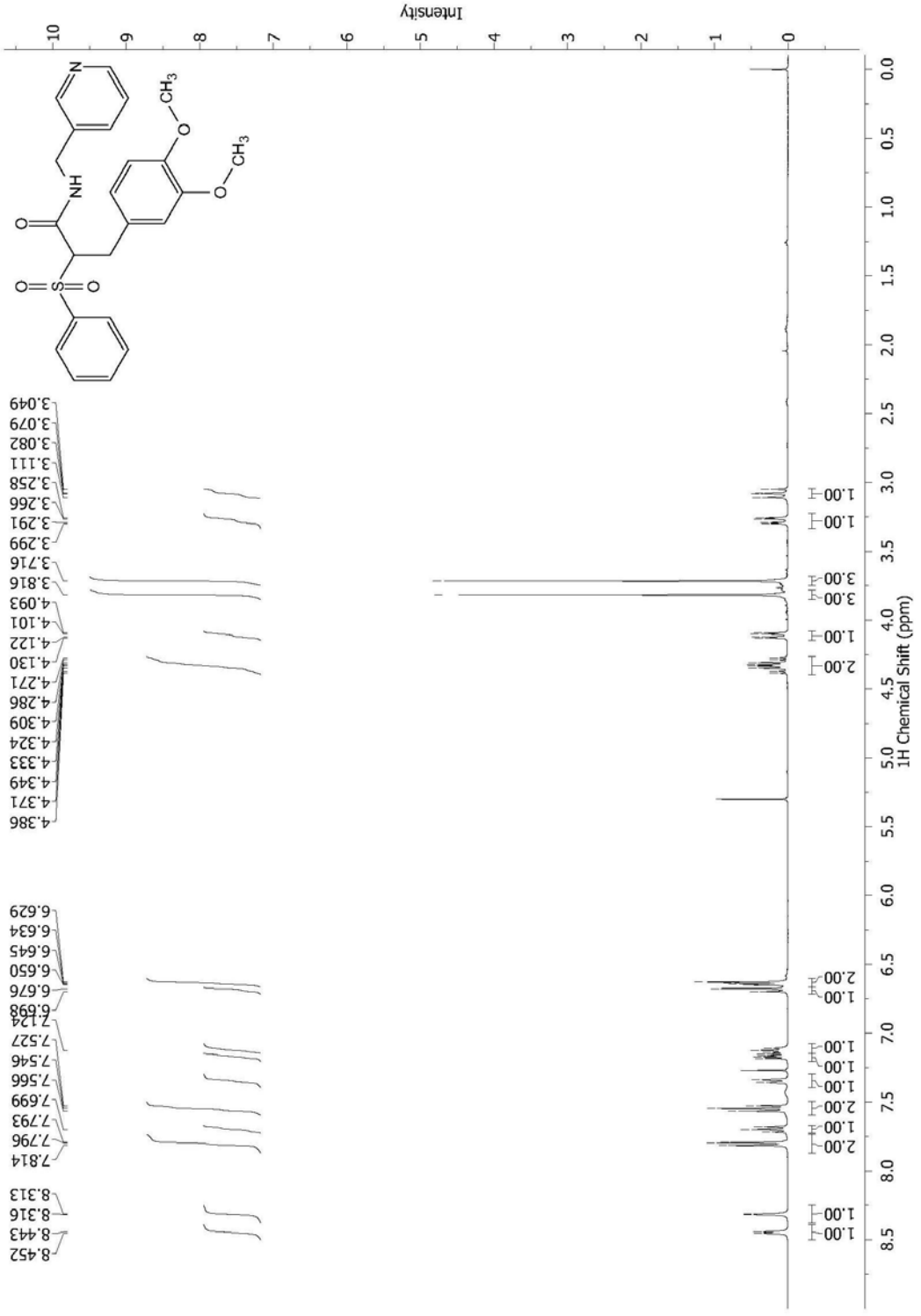
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.1**



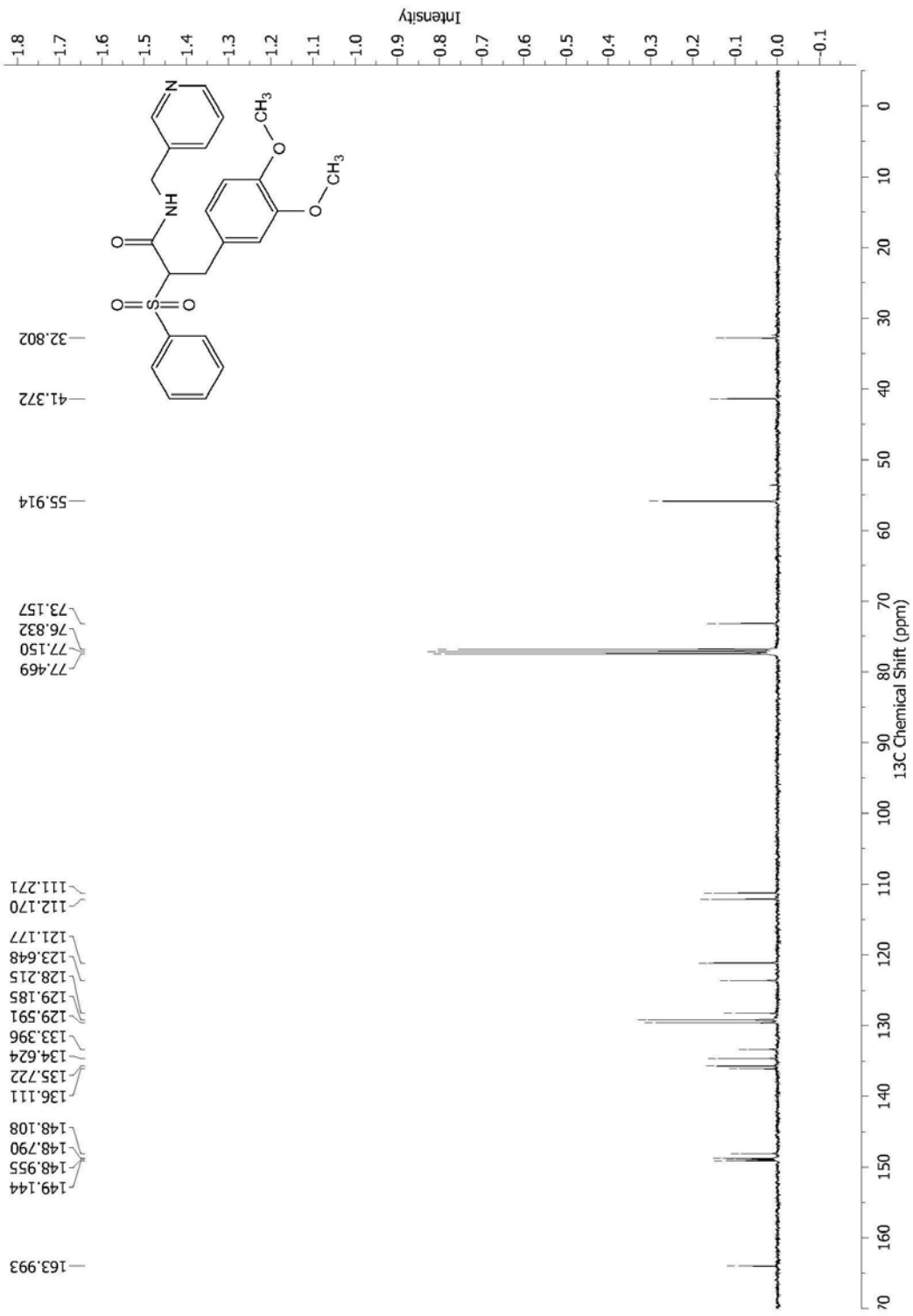
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.8**



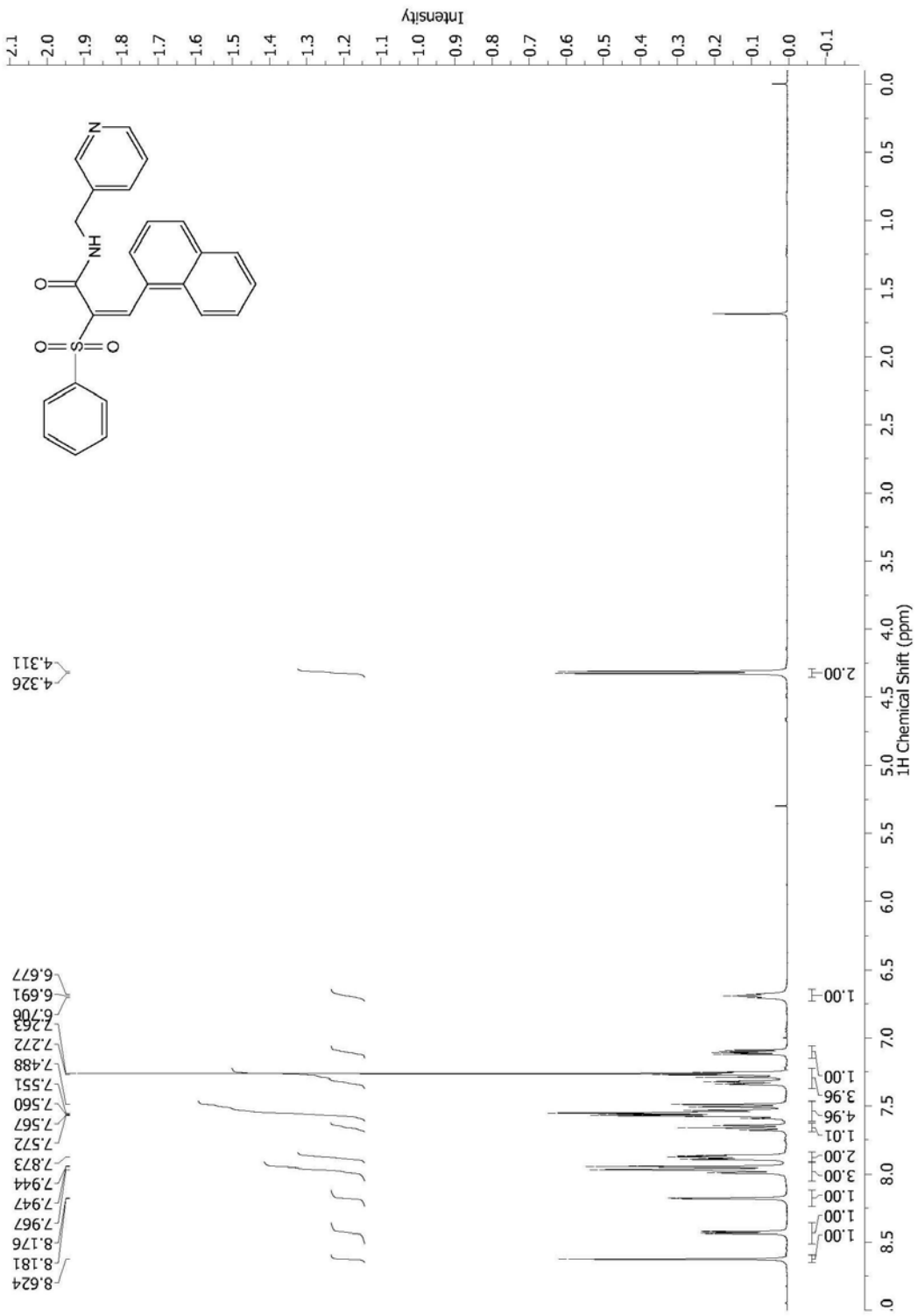
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.8**



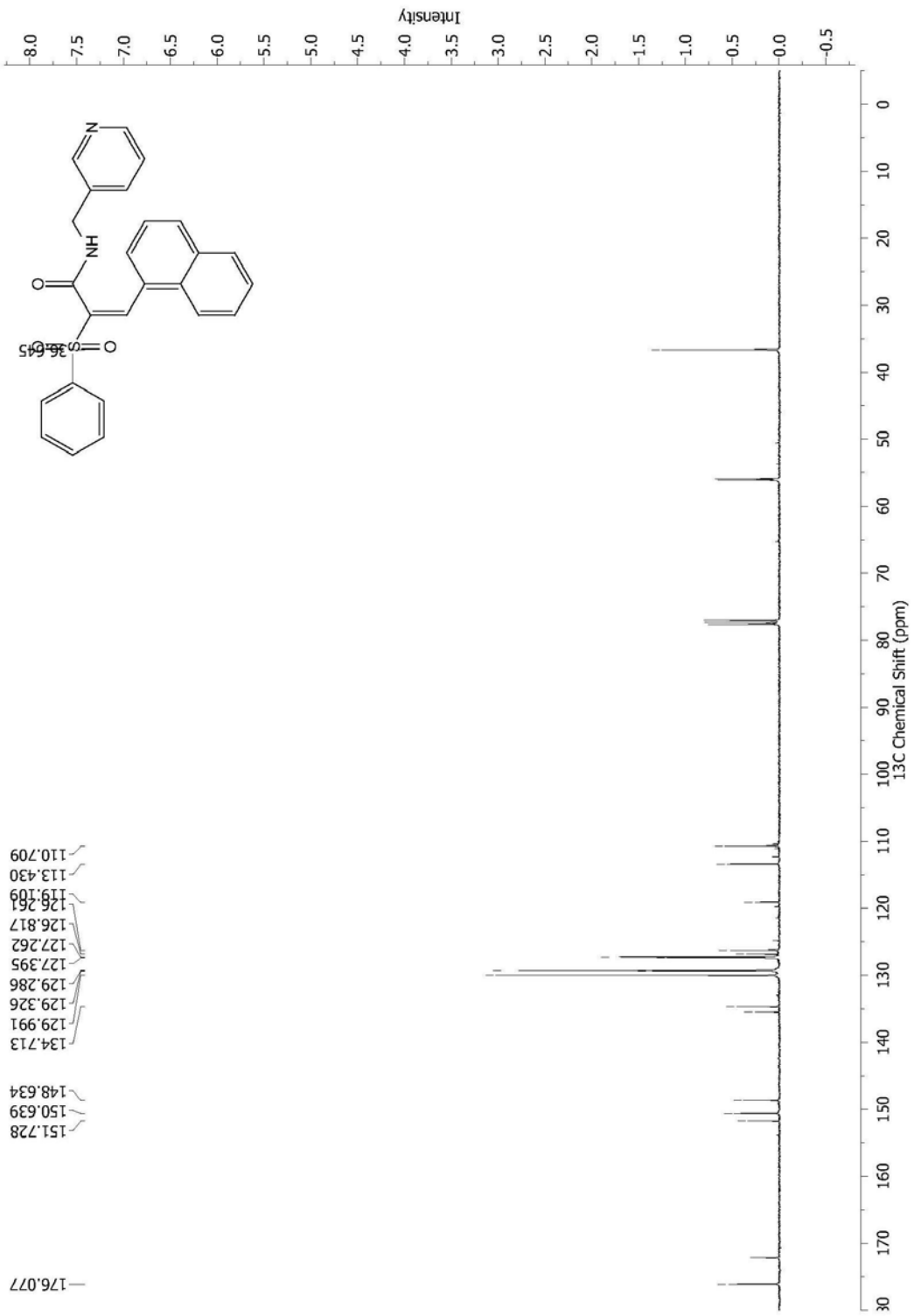
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2.9**



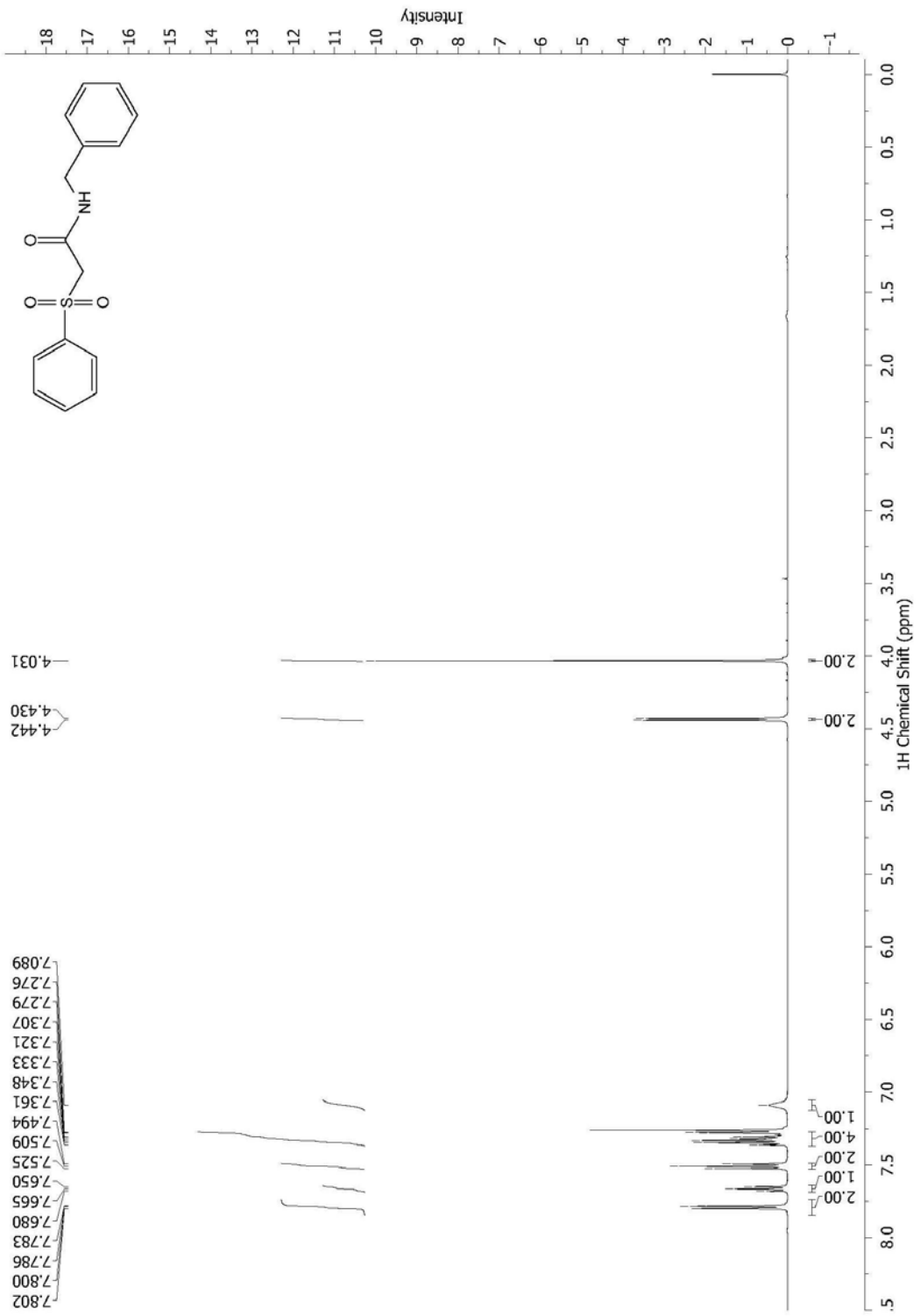
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **2.9**



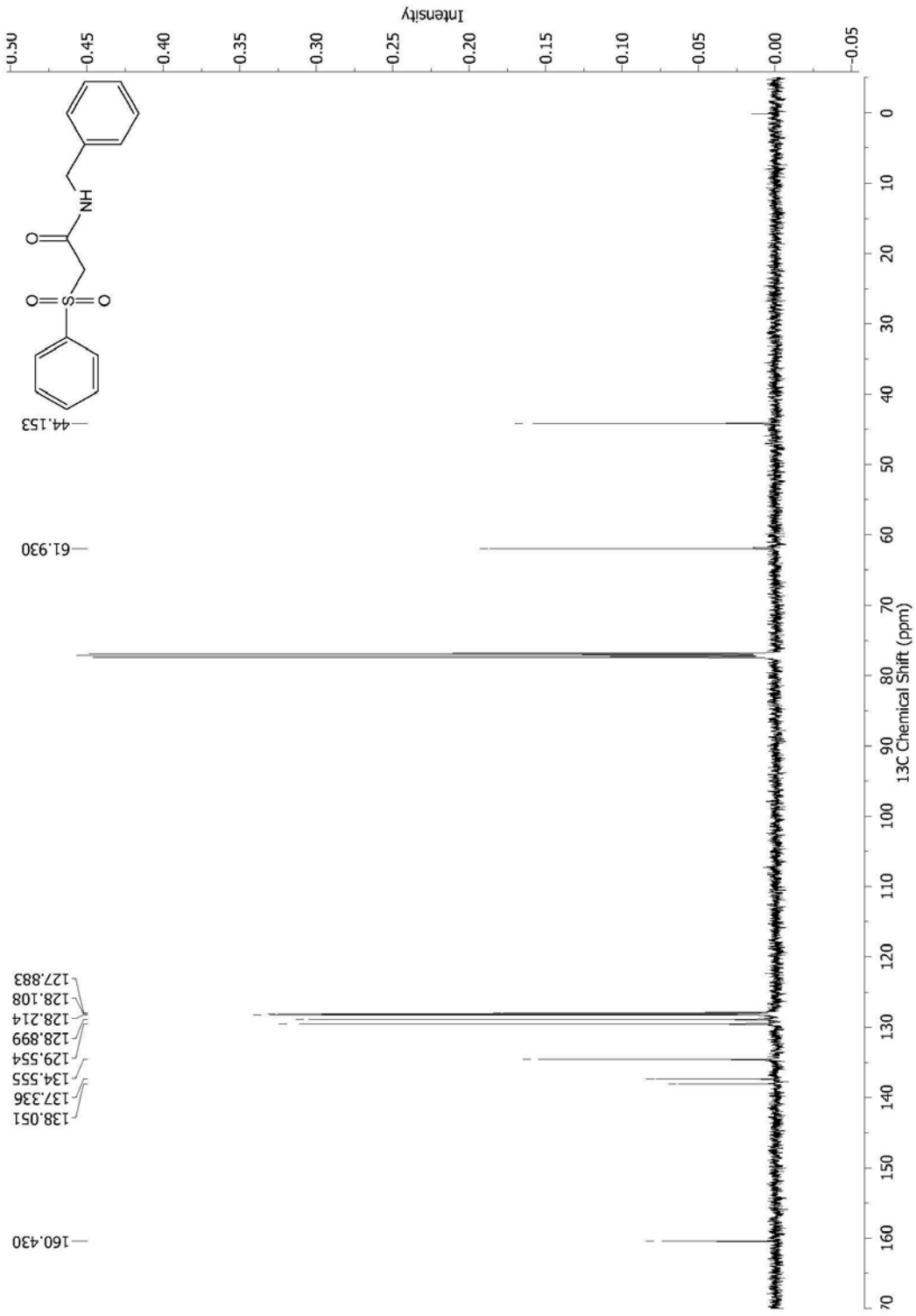
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2.10**



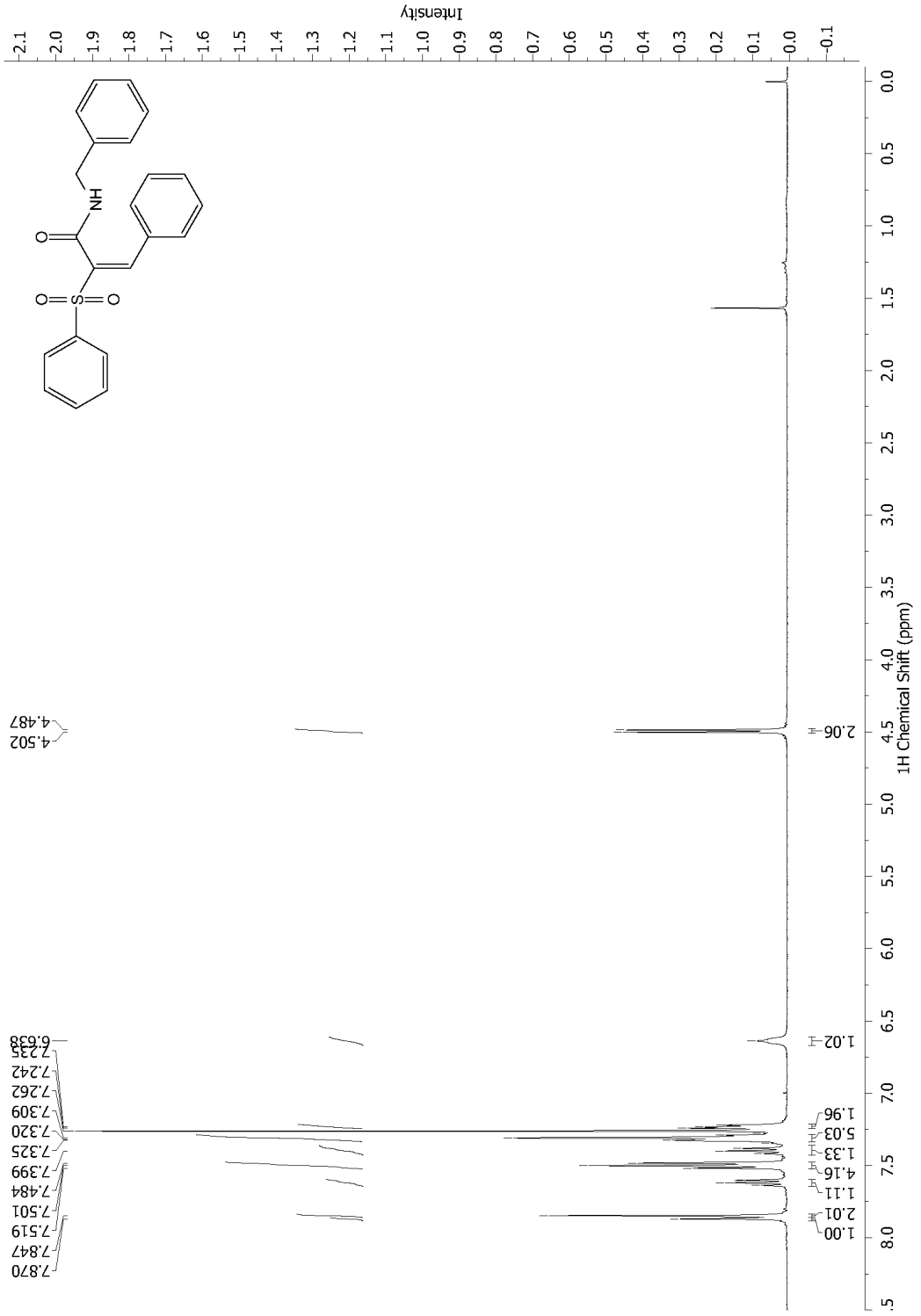
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **2.10**



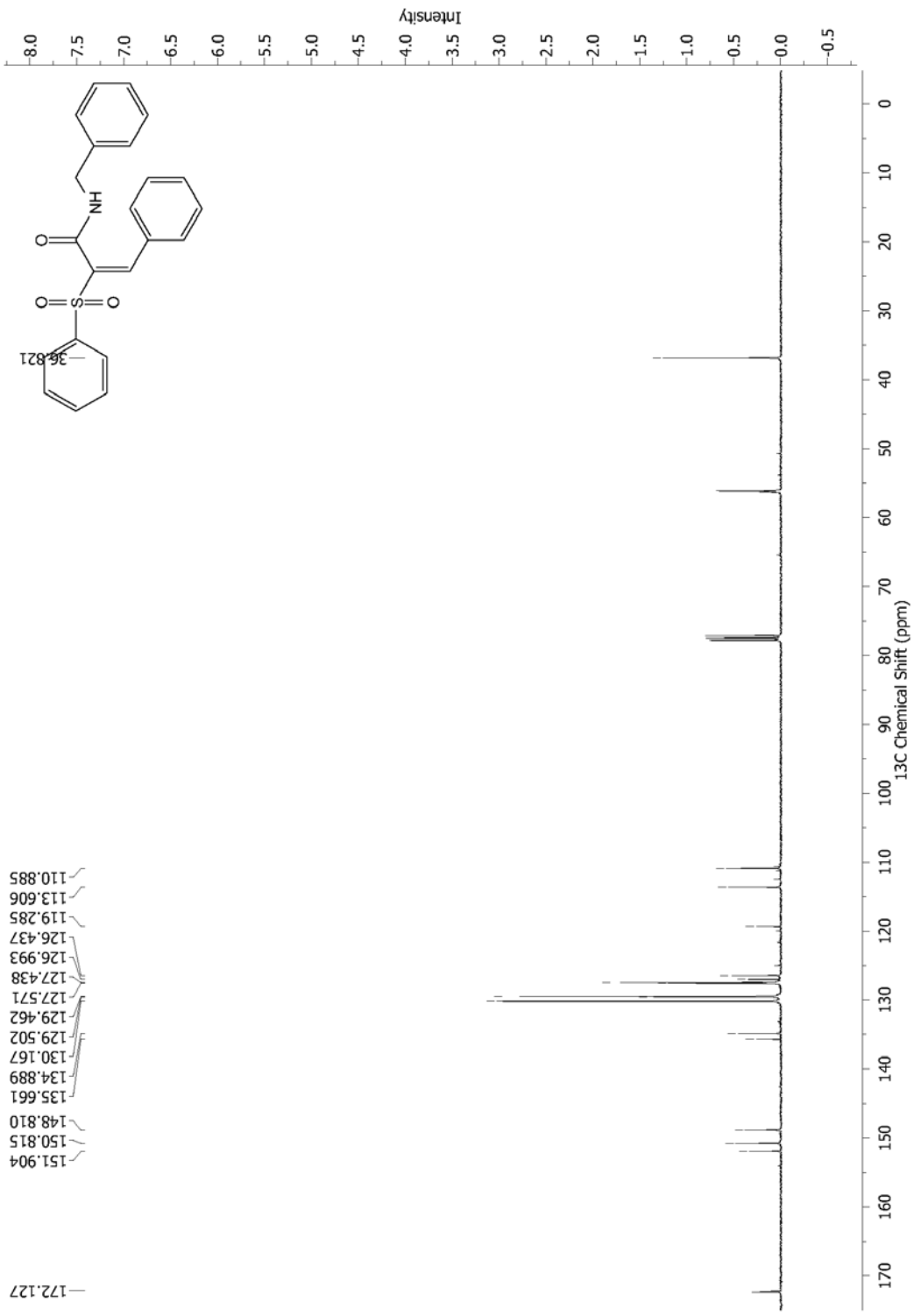
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.11**



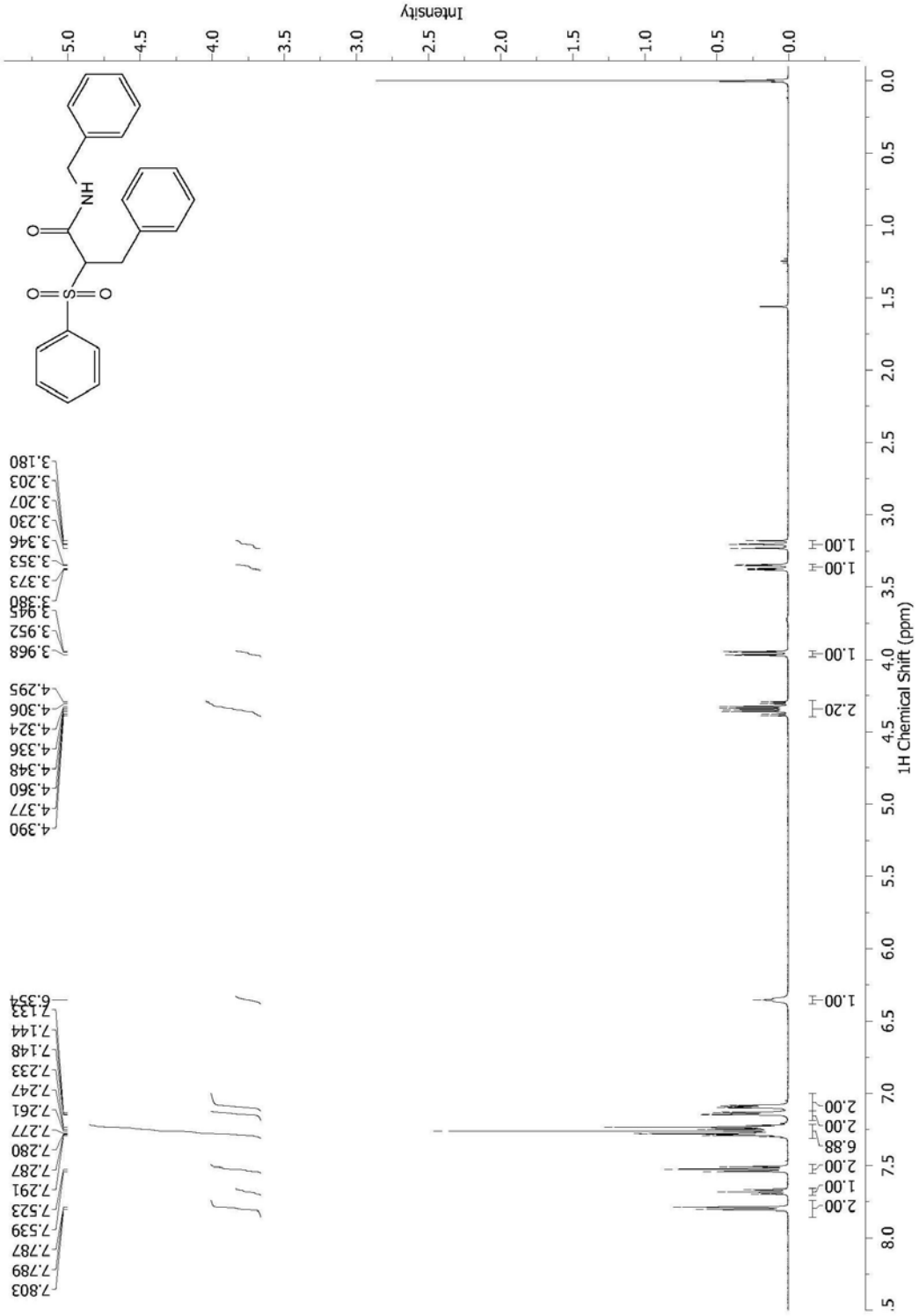
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.11**



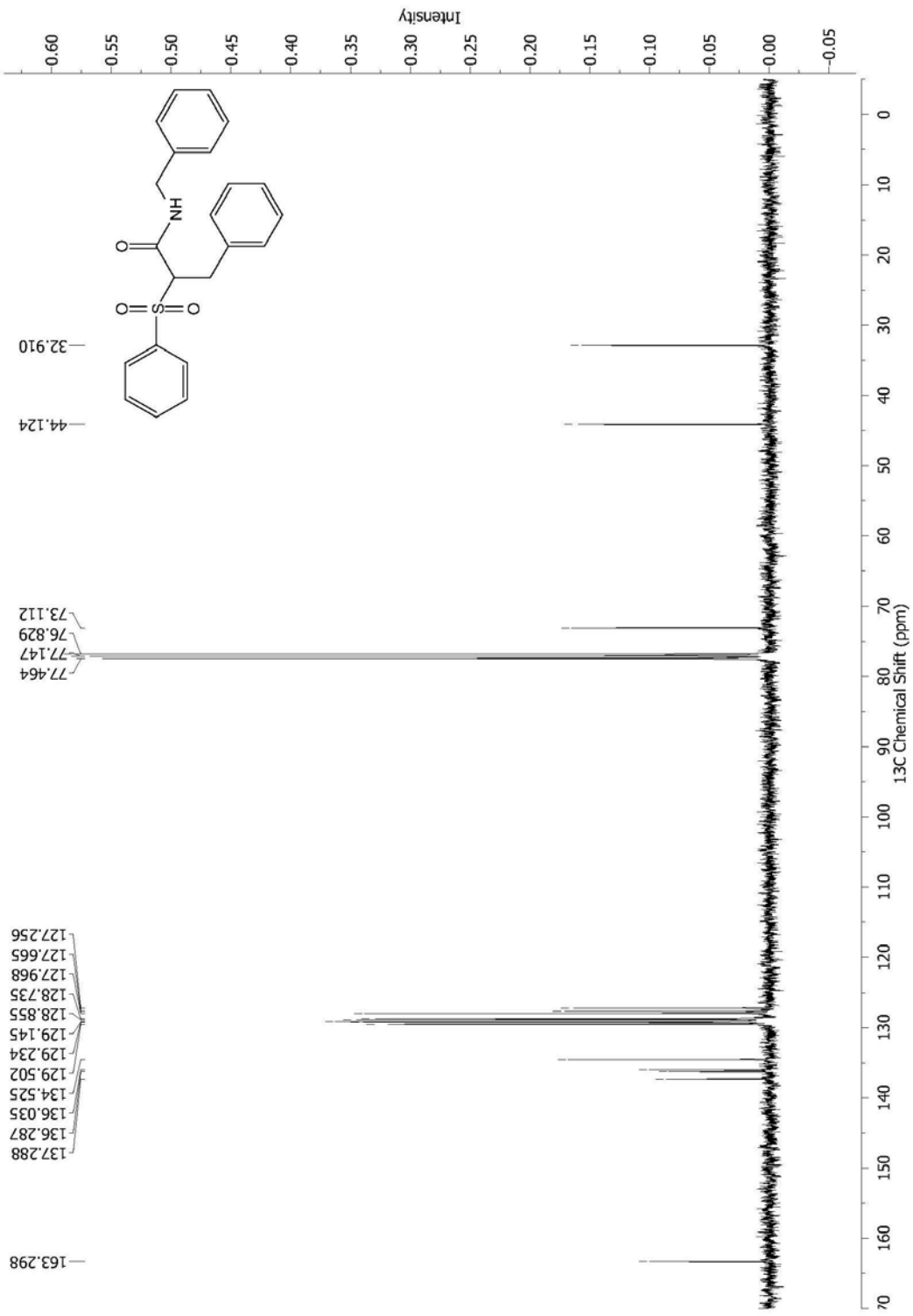
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2.12**



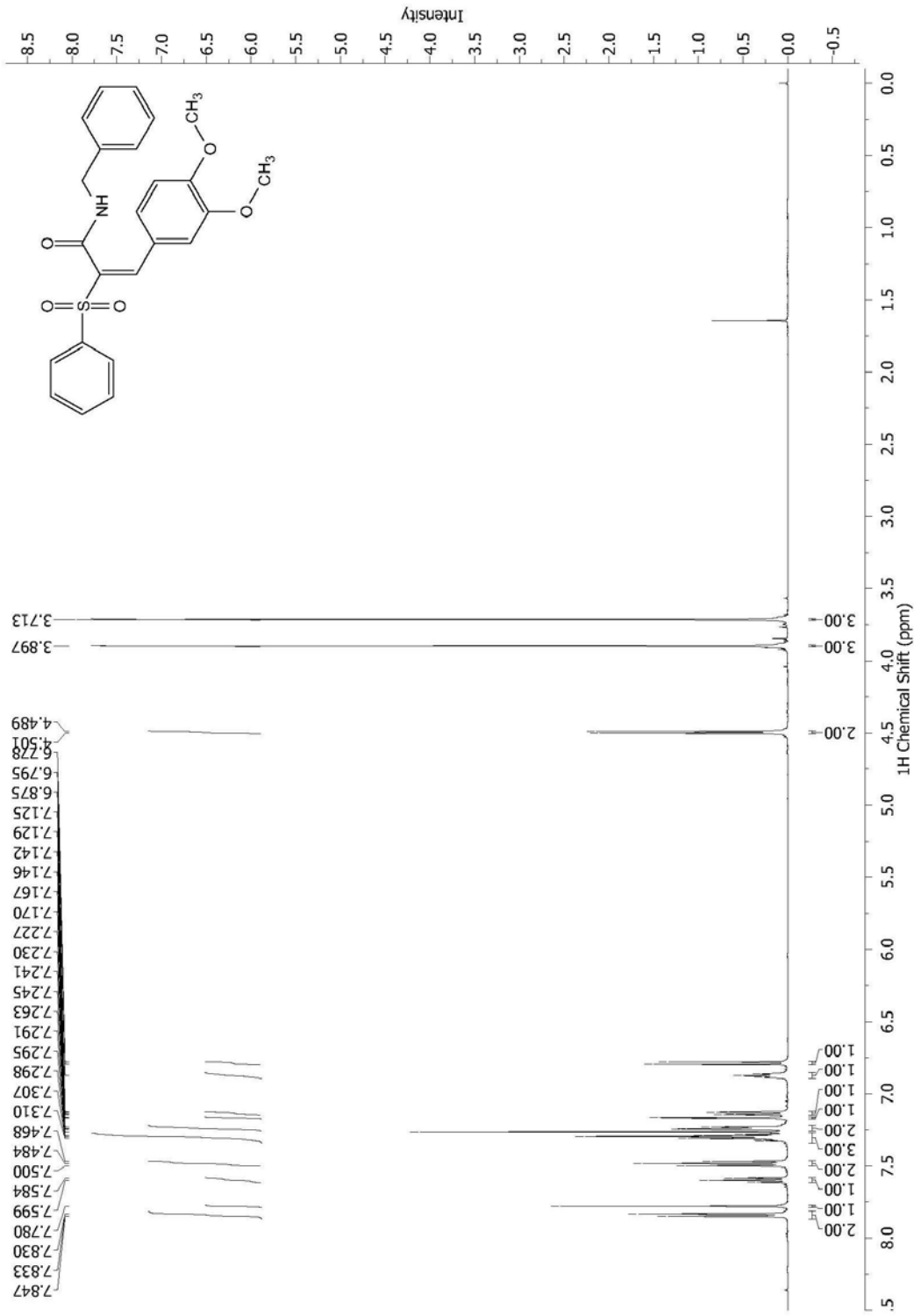
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **2.12**



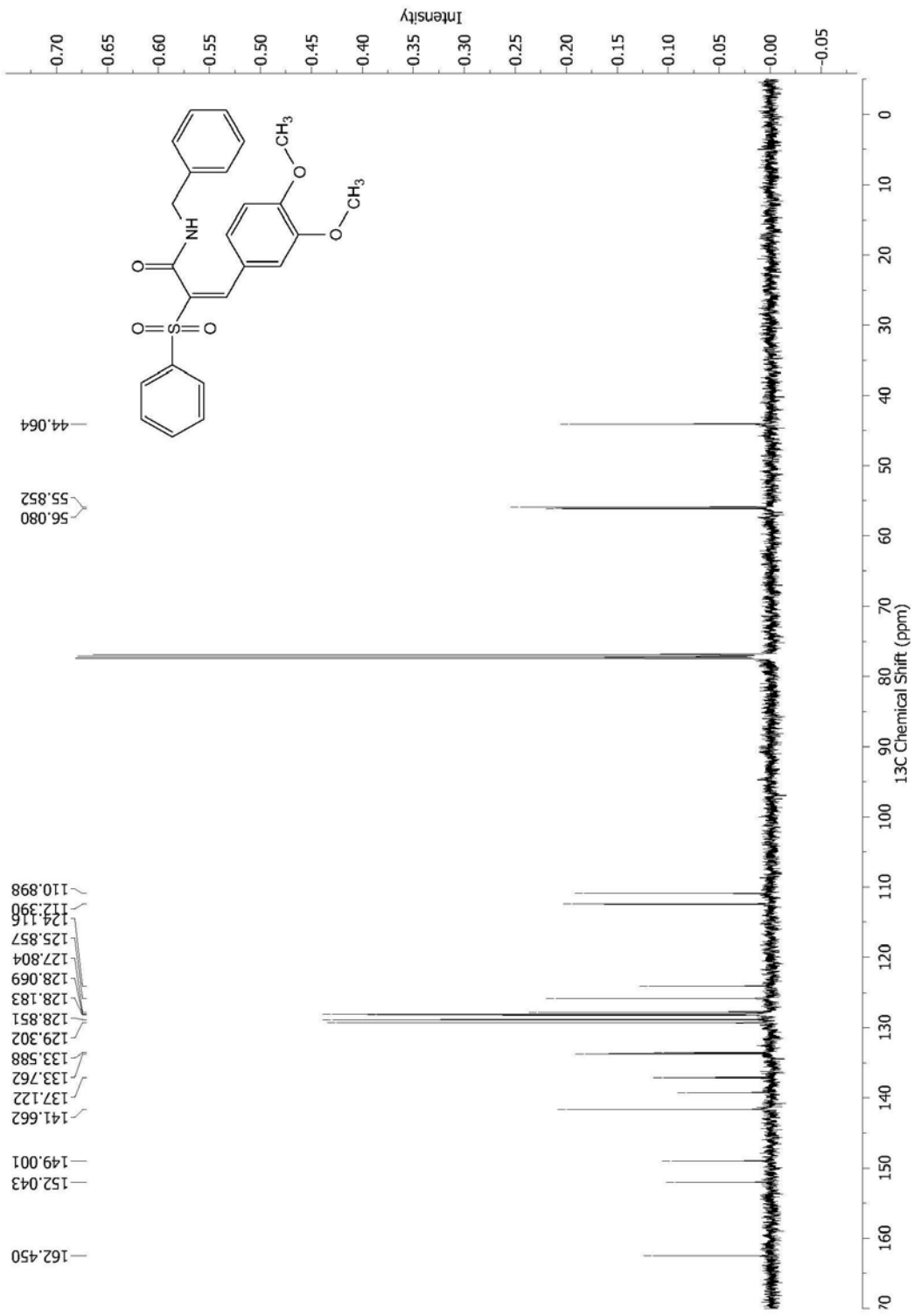
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.13**



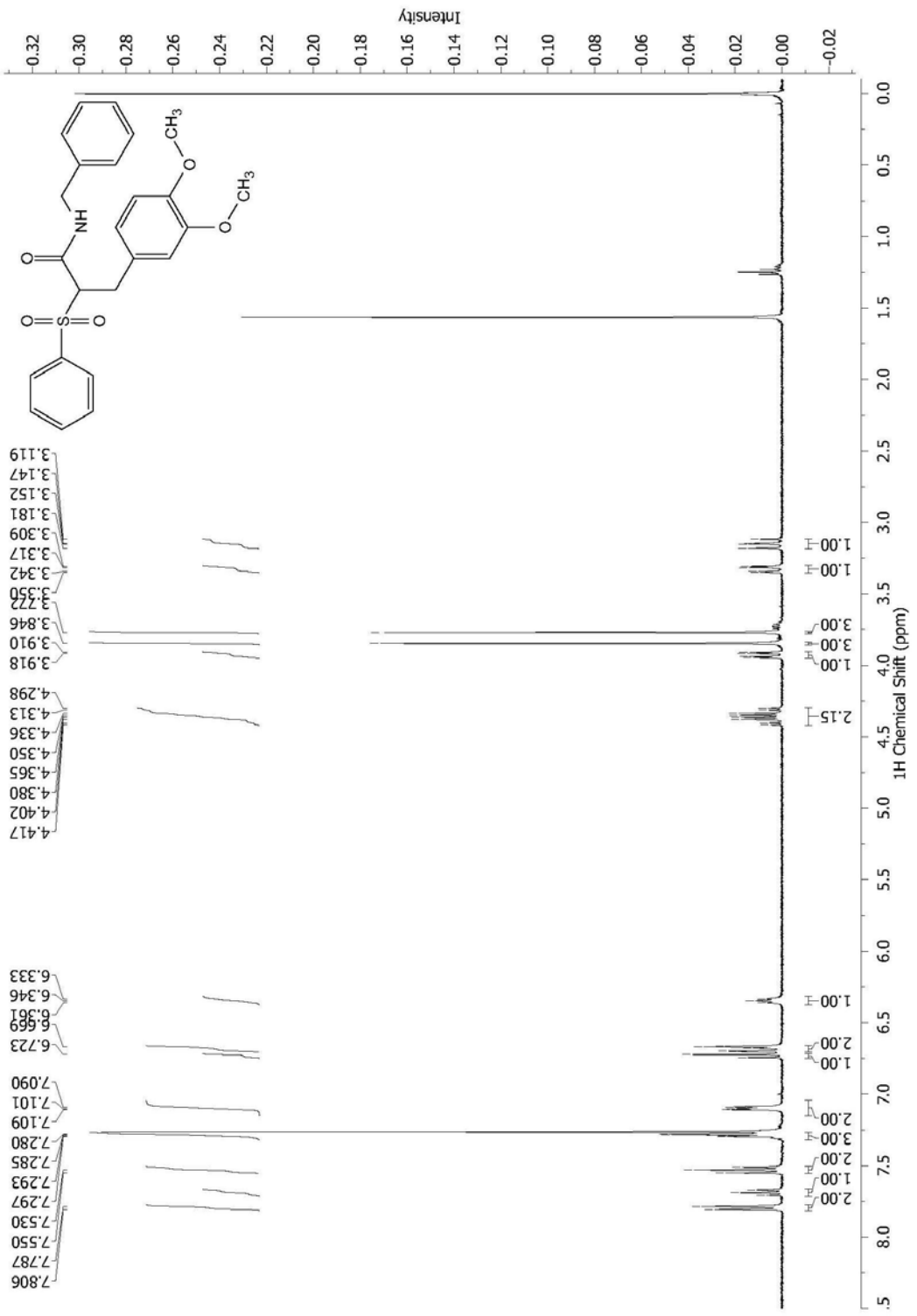
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **2.13**



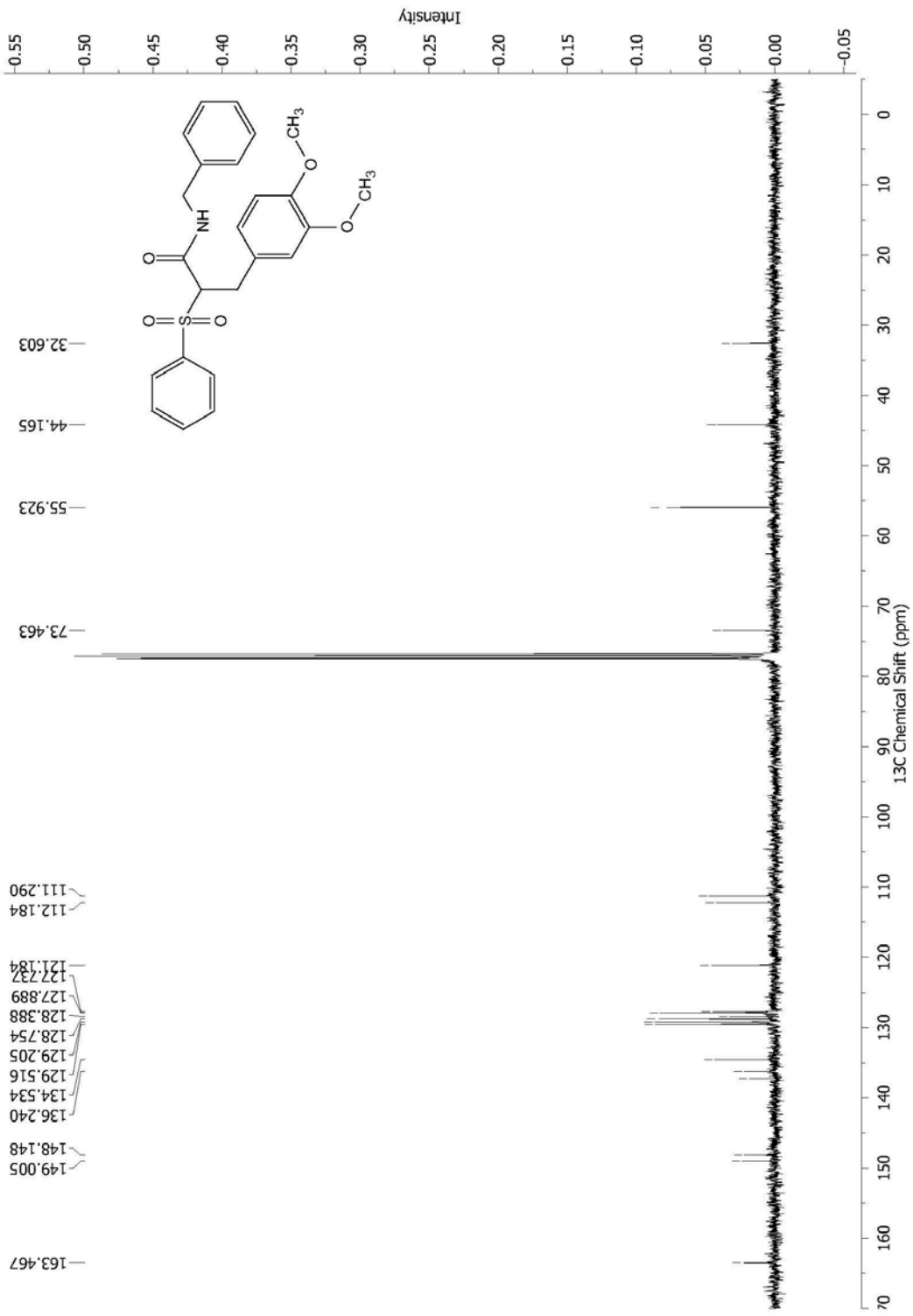
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.14**



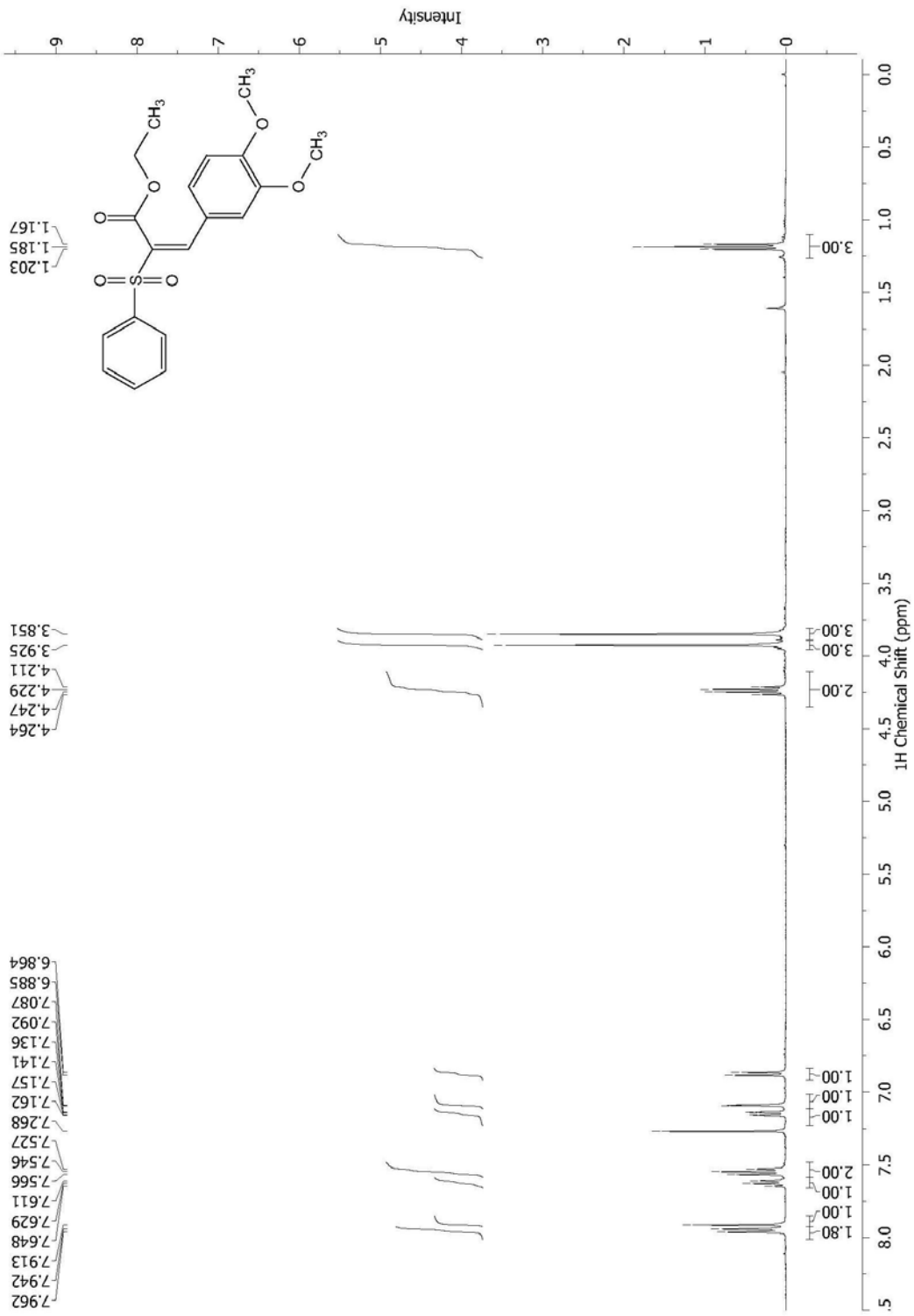
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.14**



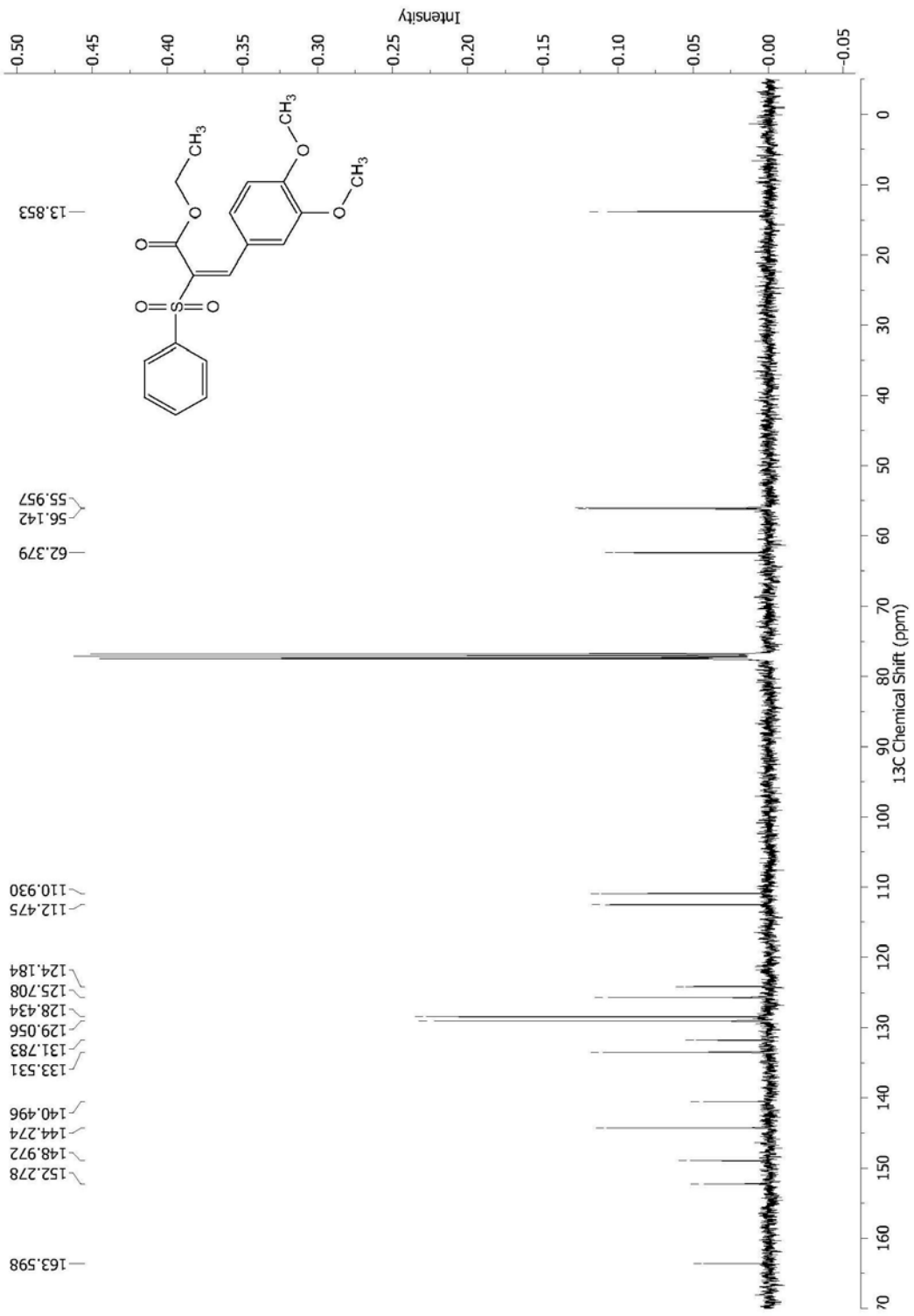
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2.15**



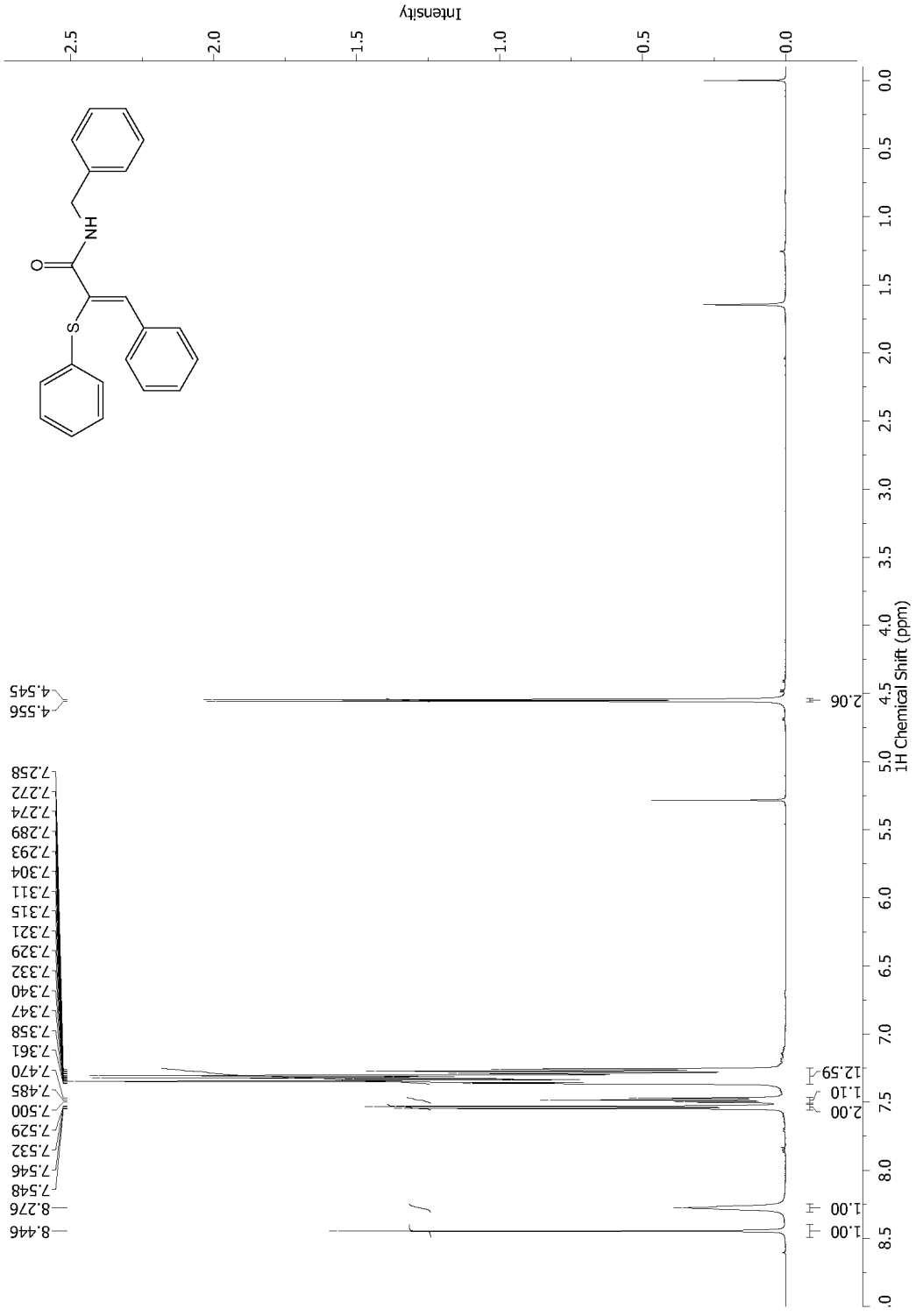
^{13}C NMR spectrum (101 MHz, $CDCl_3$) of compound **2.15**



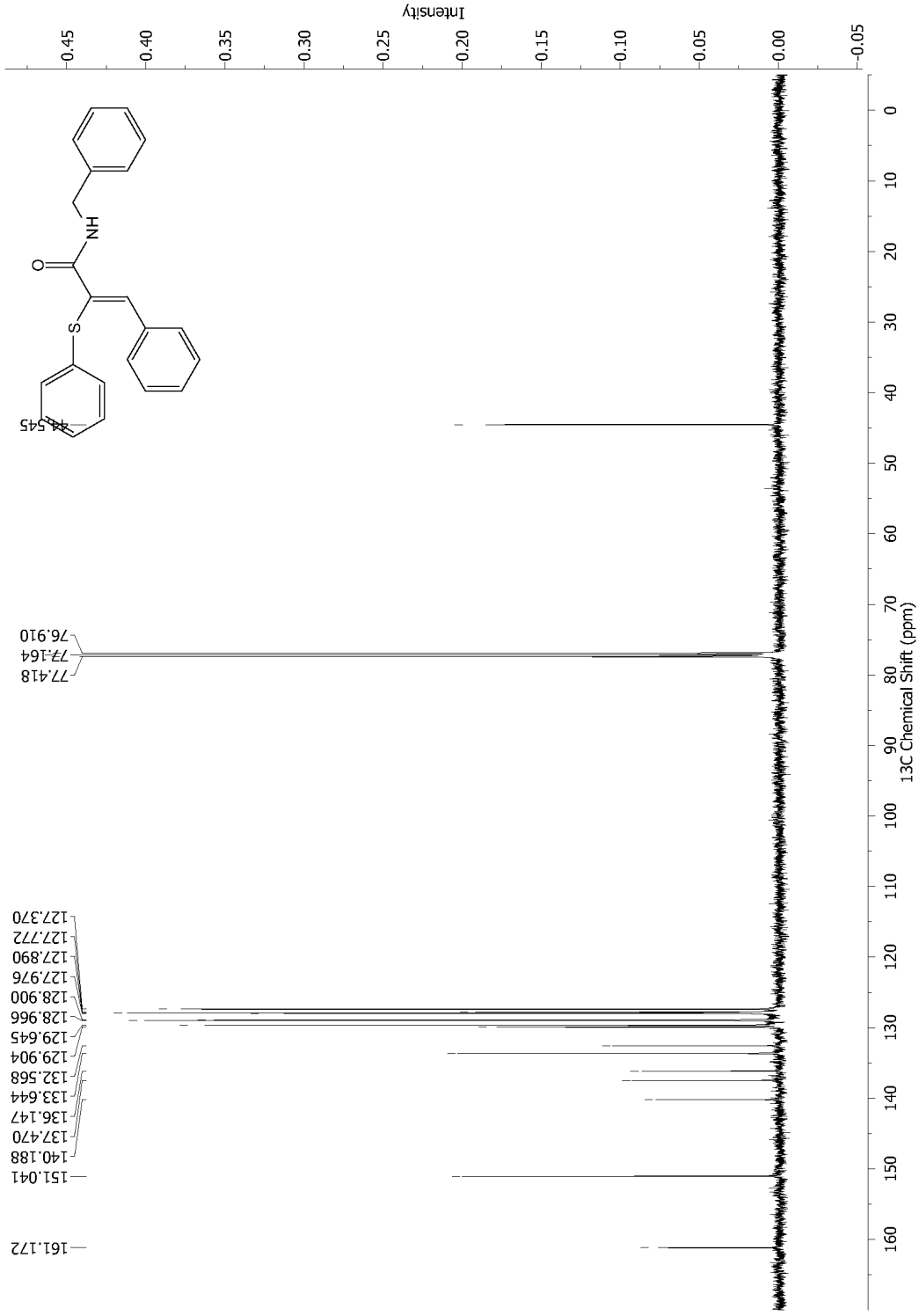
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.16**



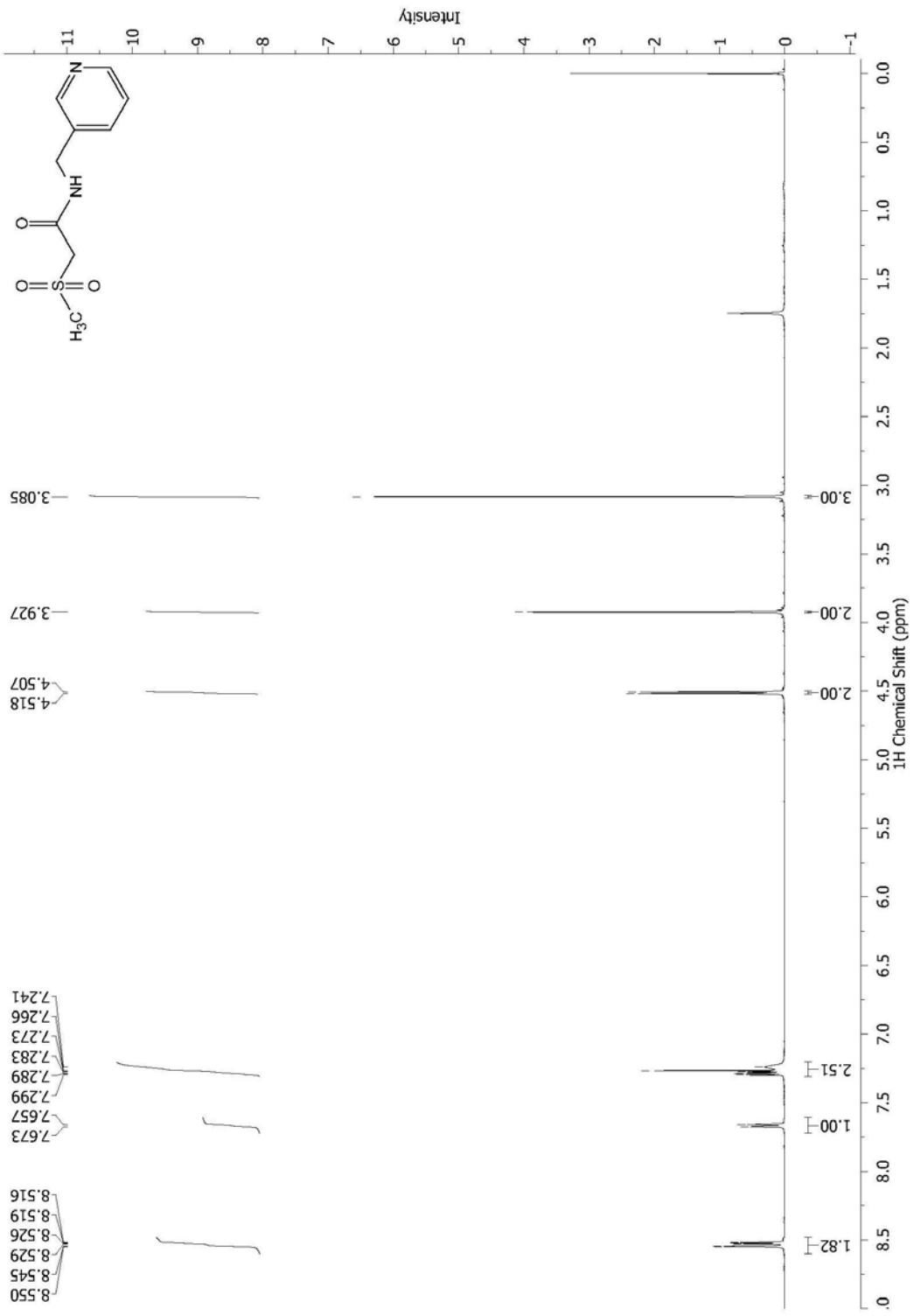
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.16**



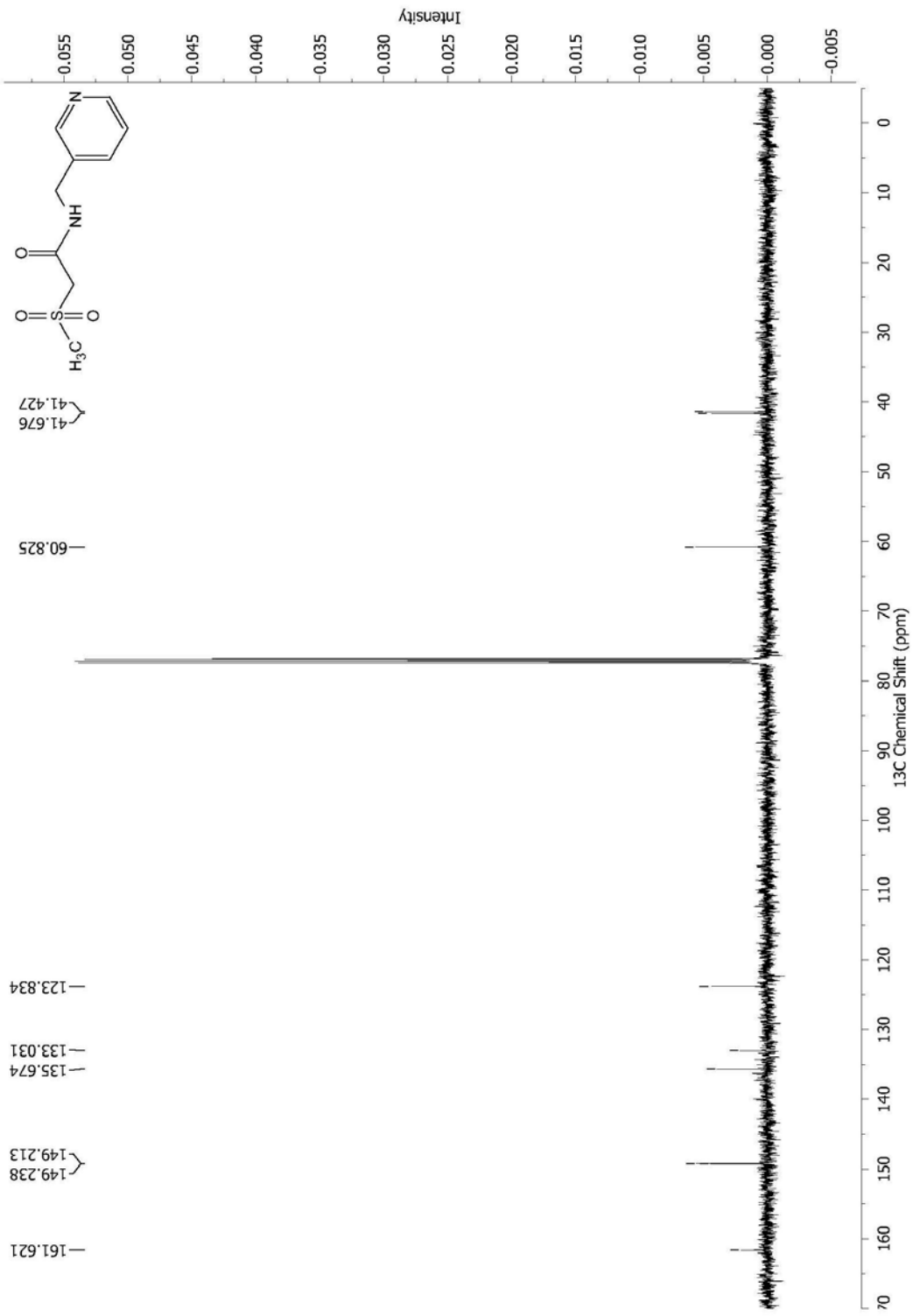
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.18**



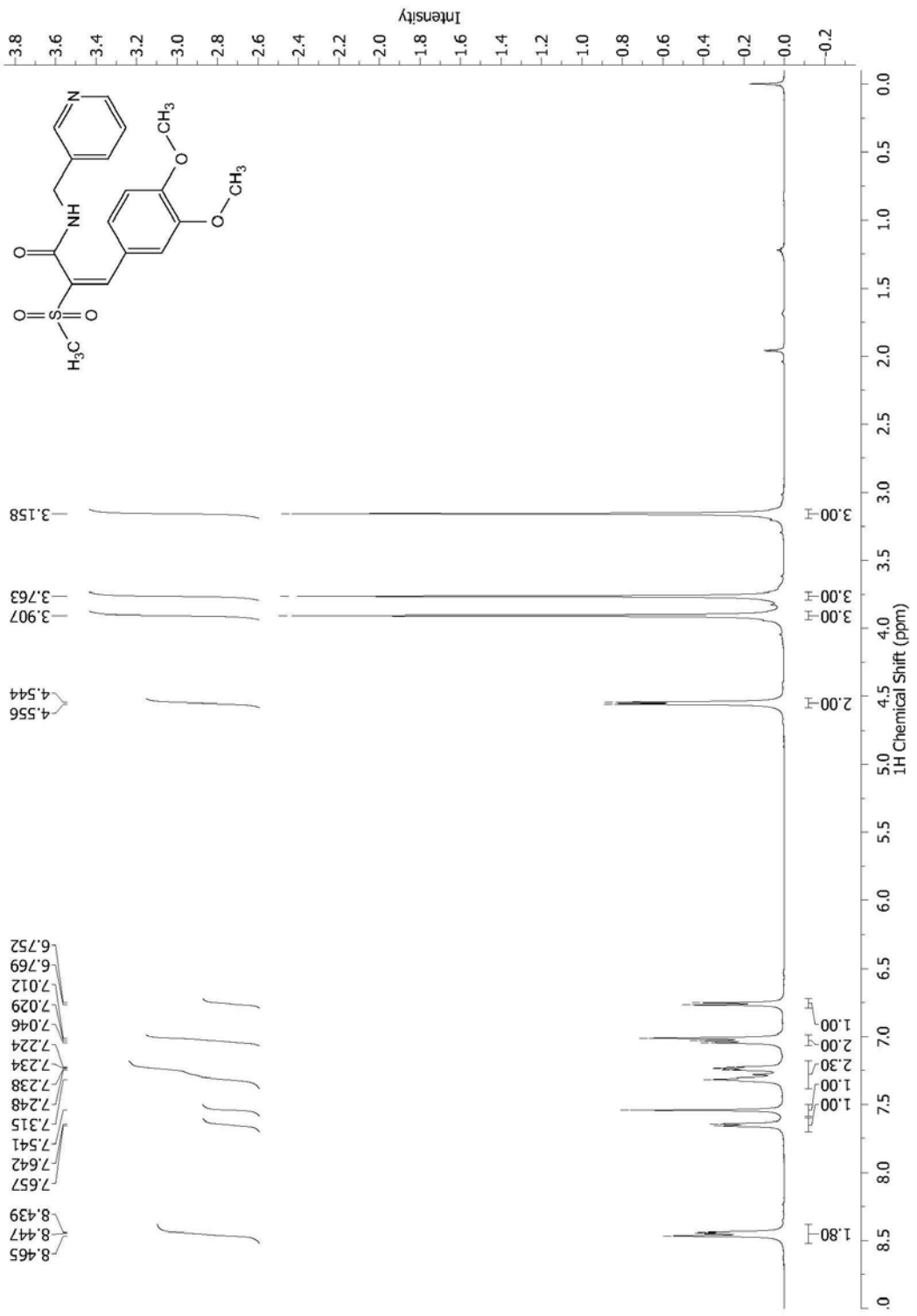
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.18**



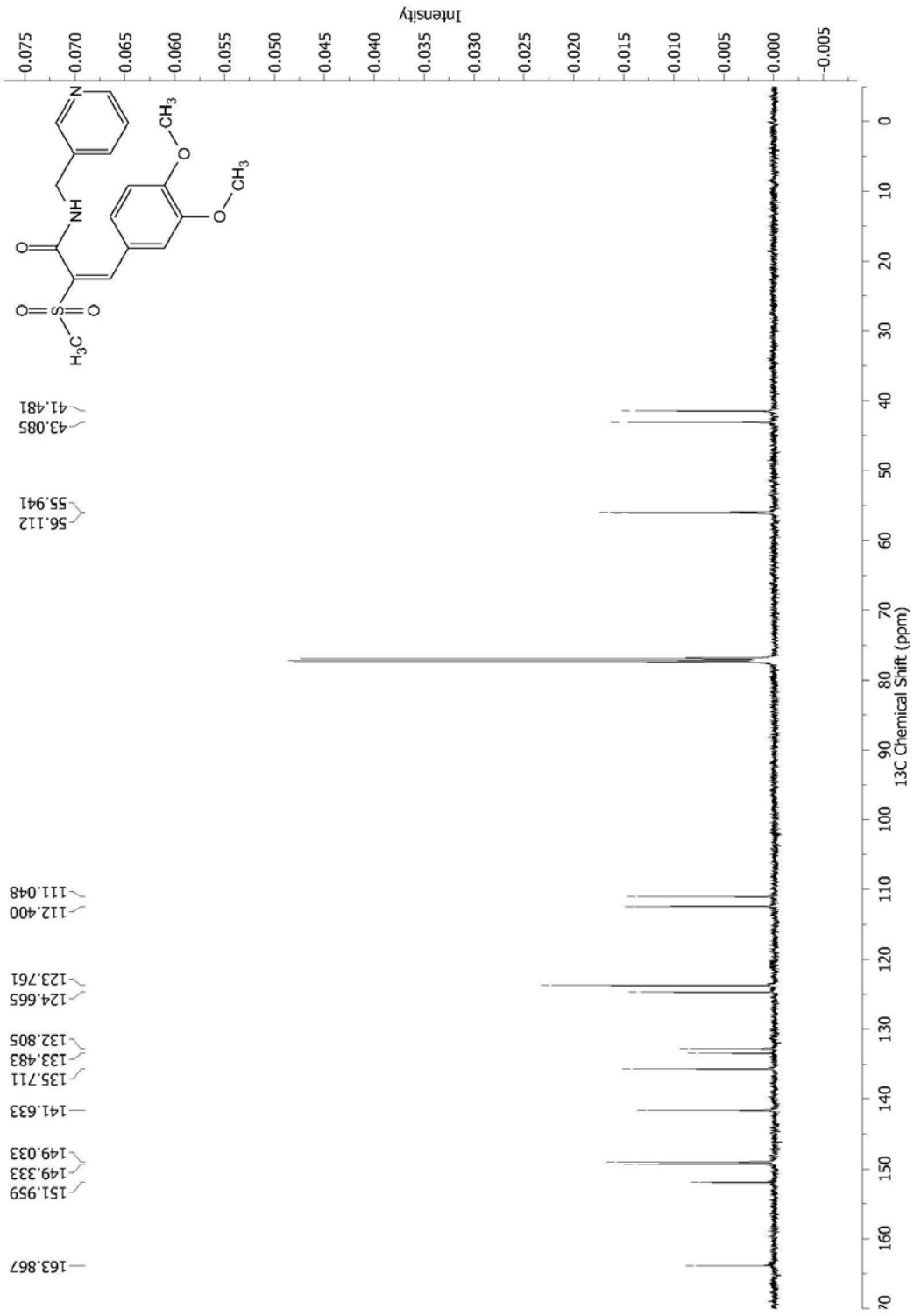
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.19**



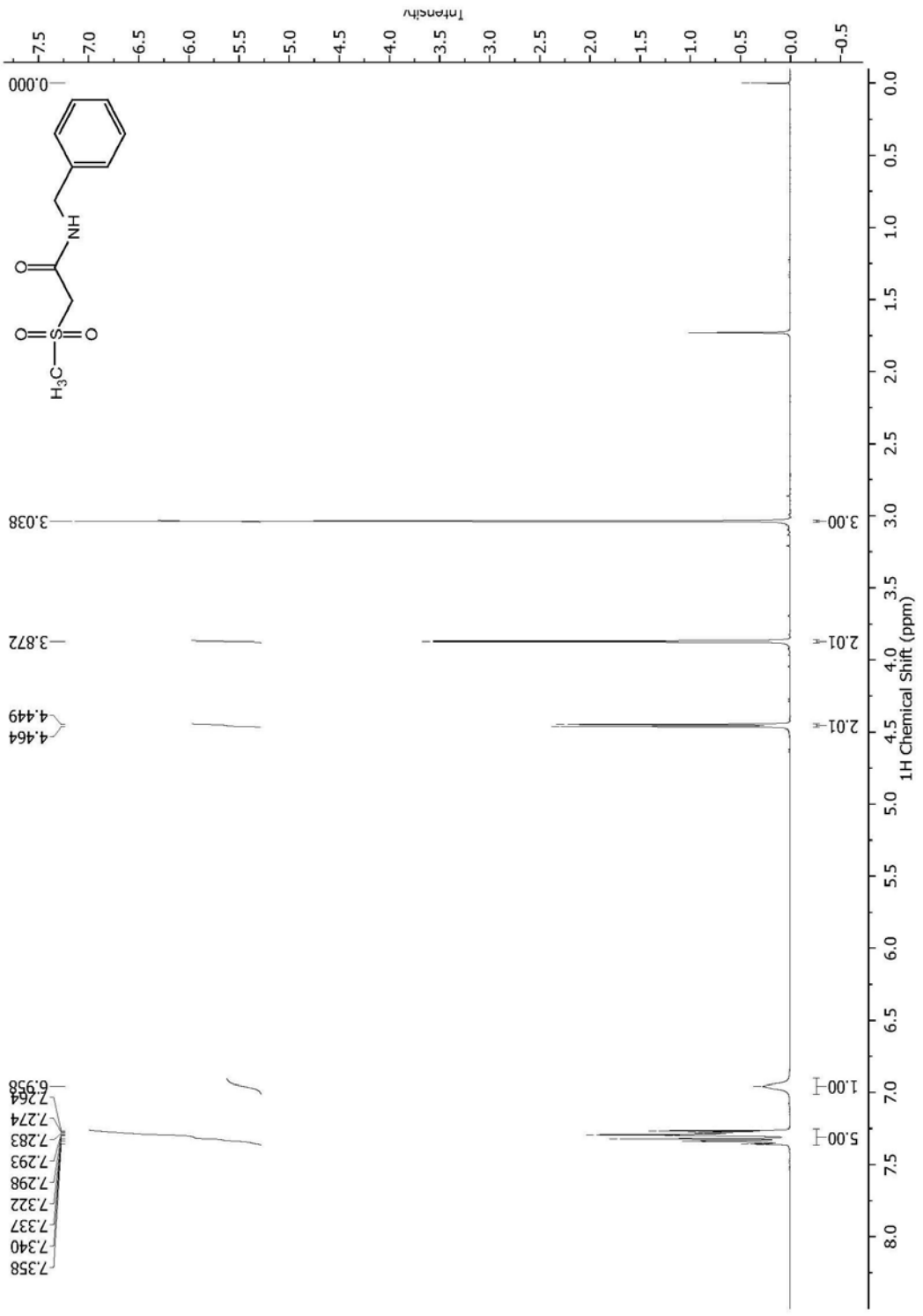
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.19**



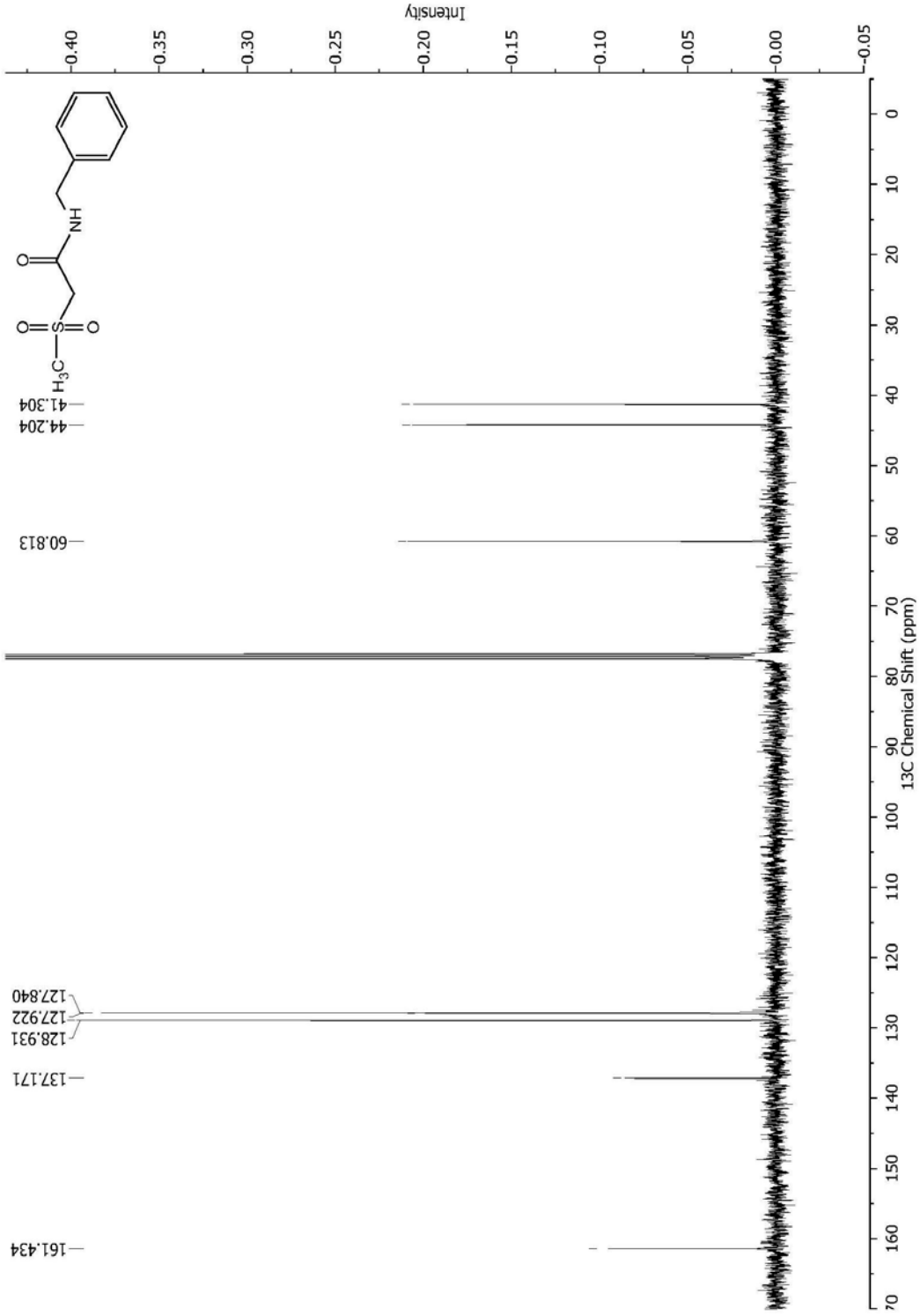
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.20**



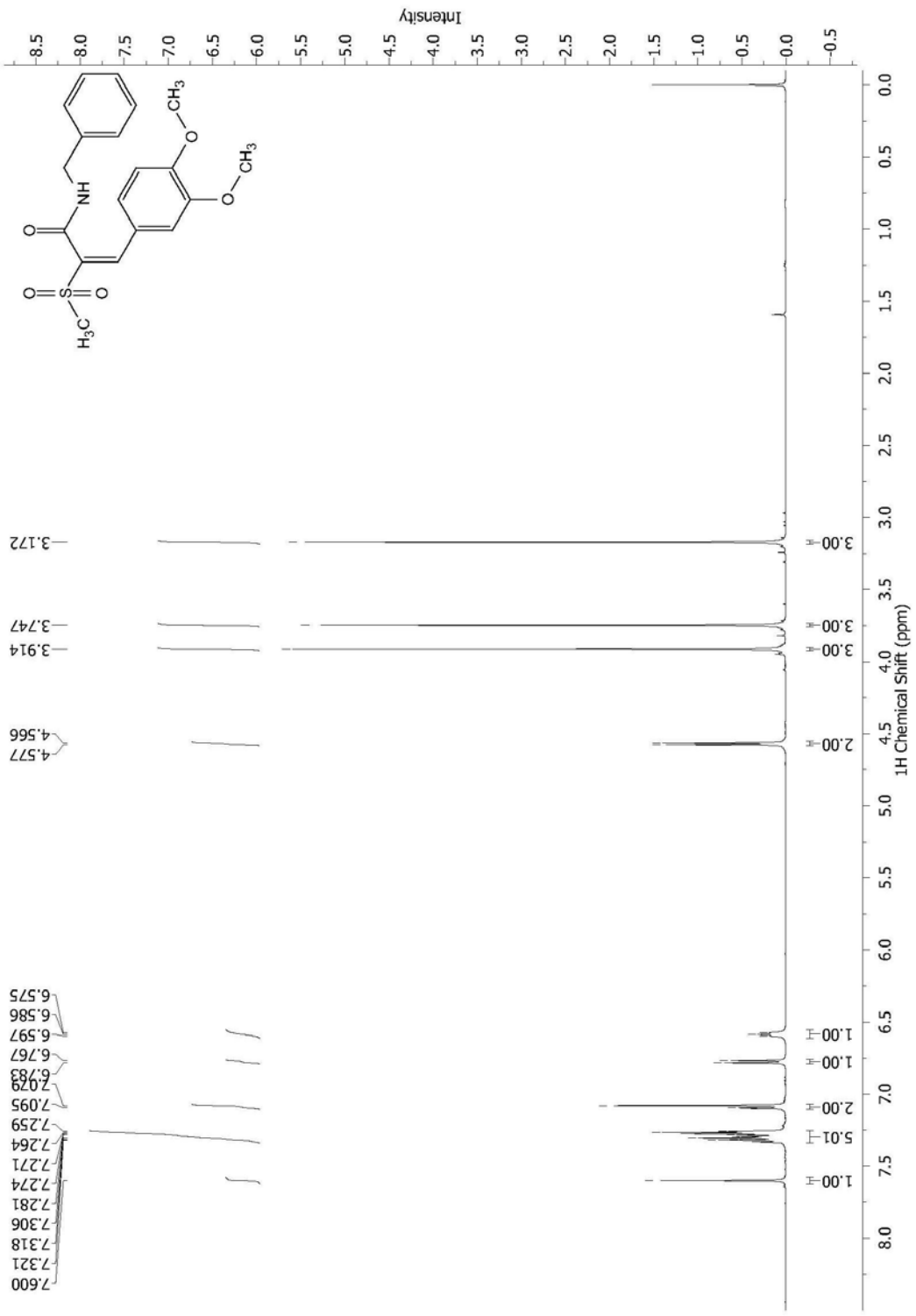
^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.20**



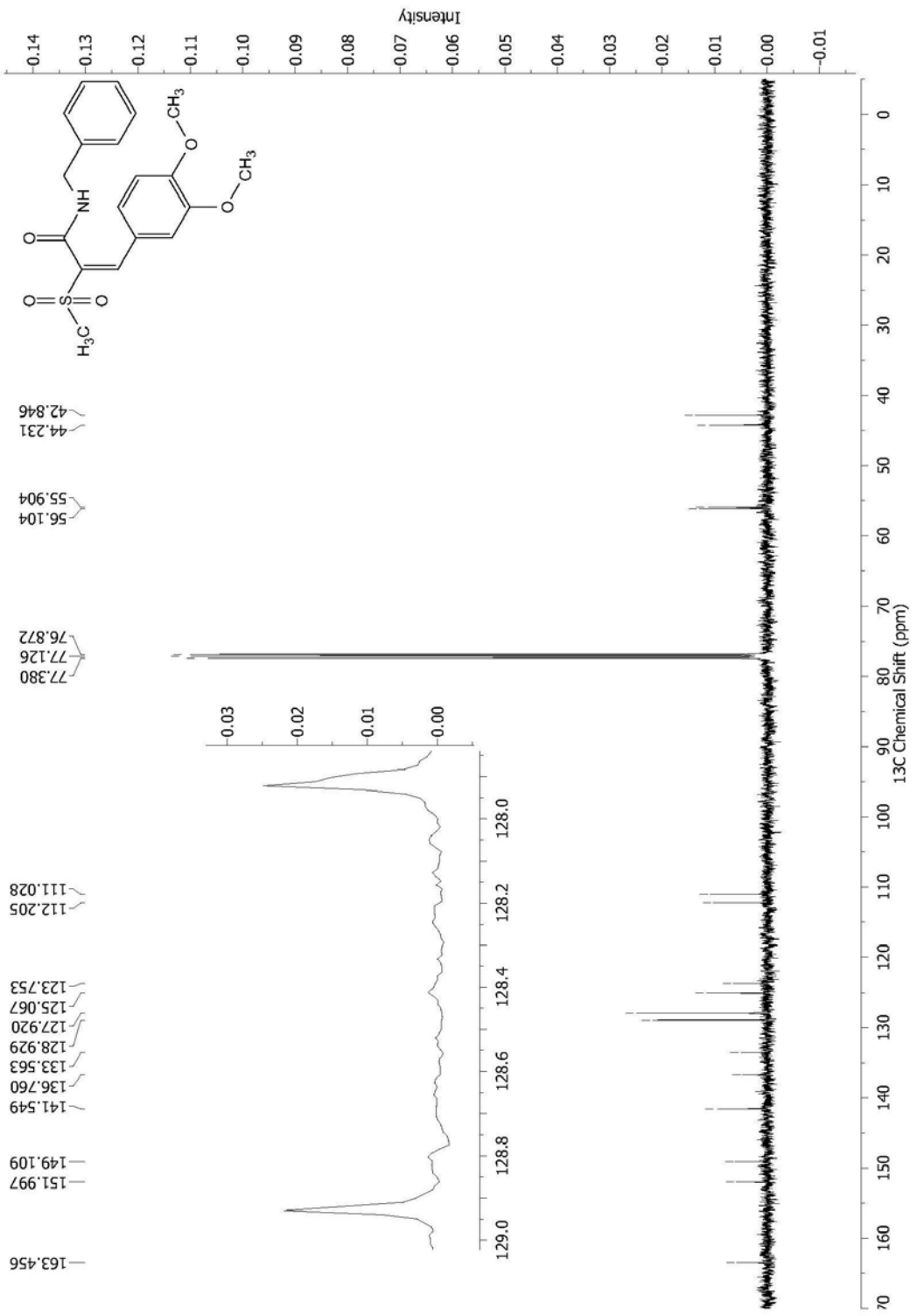
^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.21**



^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.21**



^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2.22**



^{13}C NMR spectrum (126 MHz, $CDCl_3$) of compound **2.22**

APPENDIX

Biological Data

Biological data in this appendix were obtain from Professor Clifford Stephen from Texas A&M University-Institute of Bioscience and Technology. Label and structure of compounds in the data was showed here.

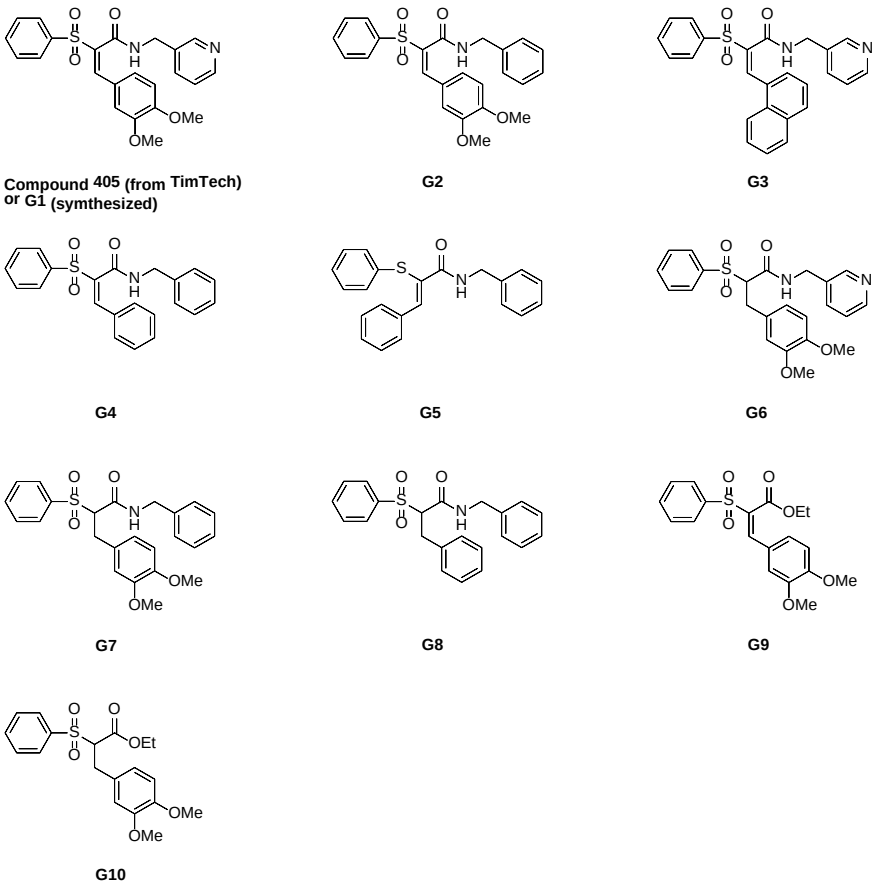


Figure A1 Label and structure of compounds in the appendix

Figure A2 Assay workflow/time line

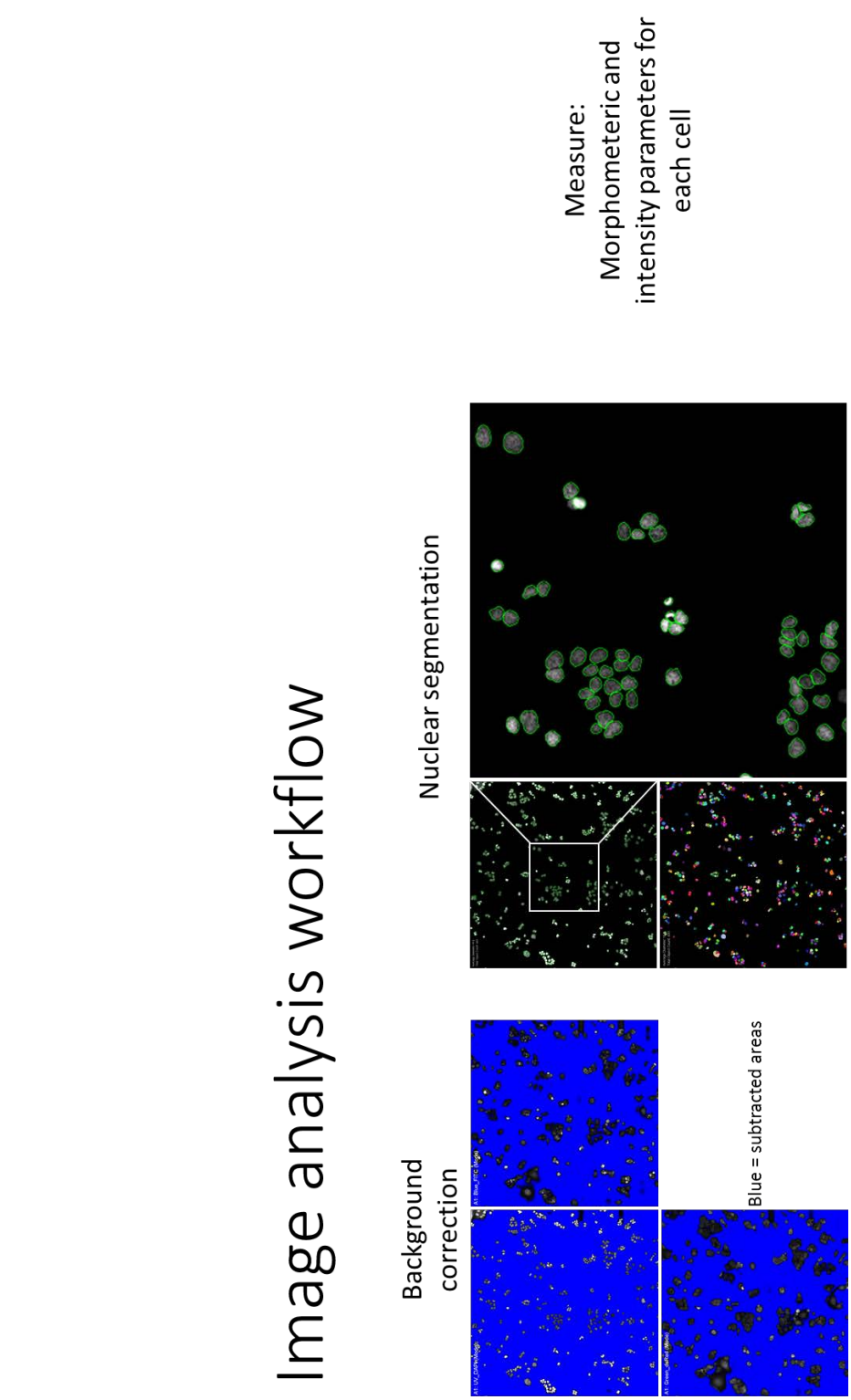


Figure A3 Image analysis workflow

Figure A4 Plate map of preliminary experiment

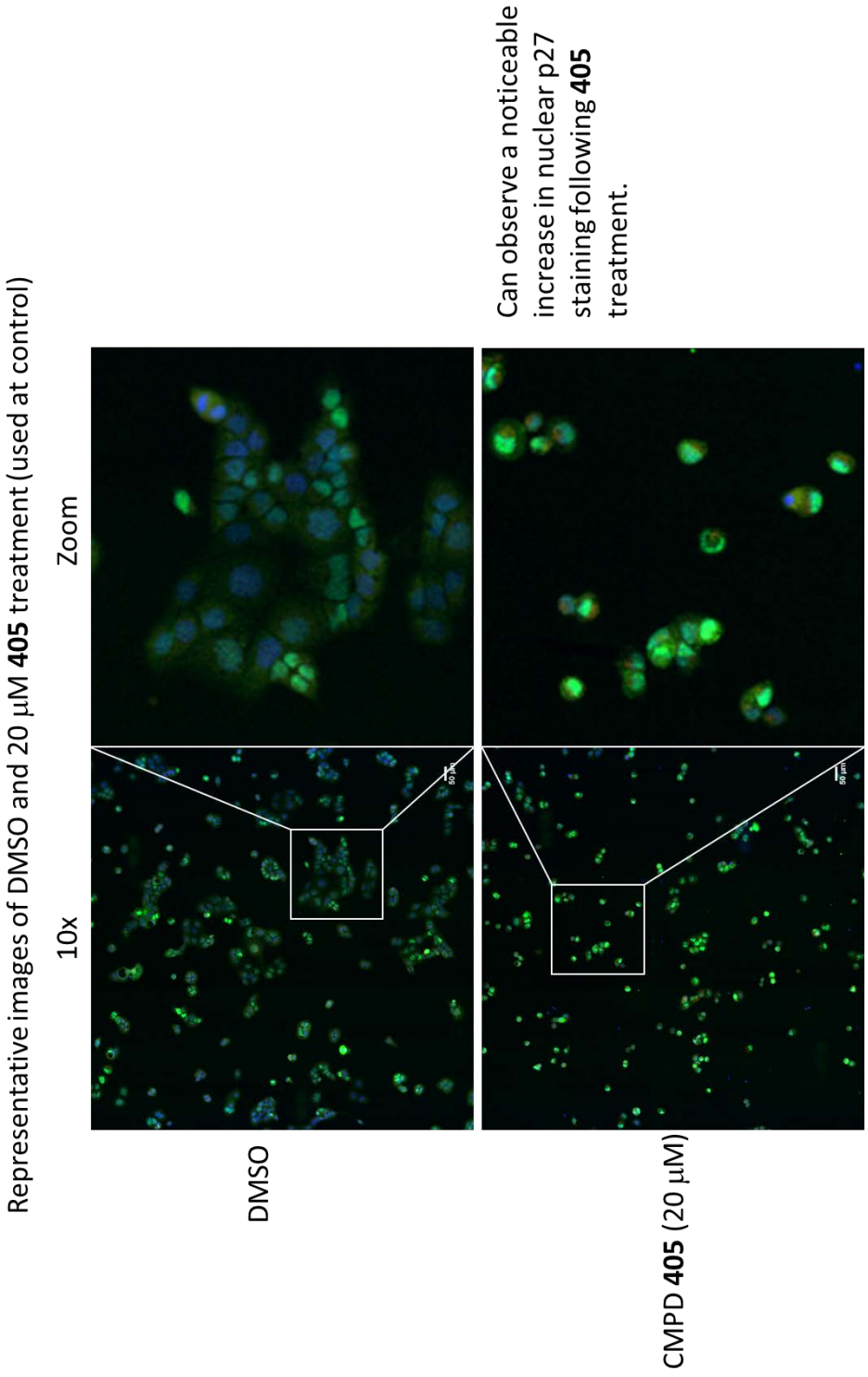


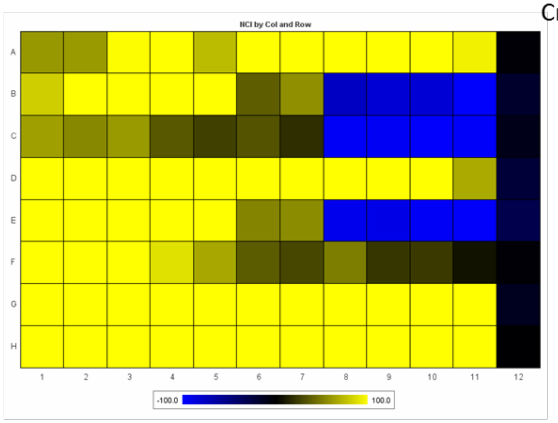
Figure A5 Representative images of DMSO and 20 μM **405** treatment (used at control)

Figure A6 Response for preliminary experiment

Figure A7 Compound **405** produces arrested cell growth at 20 μM

405 produces arrested cell growth at 20 μM

405 dose curve



CmpdId
115
243
276
314
331
405
442
838

$$Growth = ((T_i - T_0)/(C_{DMSO} - T_0))*100 \quad if\ T_i >= T_0$$
$$= ((T_i - T_0)/T_0)*100 \quad if\ T_i < T_0$$

T_i = *Cell count per well at time of imaging*
T_0 = *Mean cell count at time of treatment*
C_{DMSO} = *Mean cell count of DMSO control wells at time of imaging*

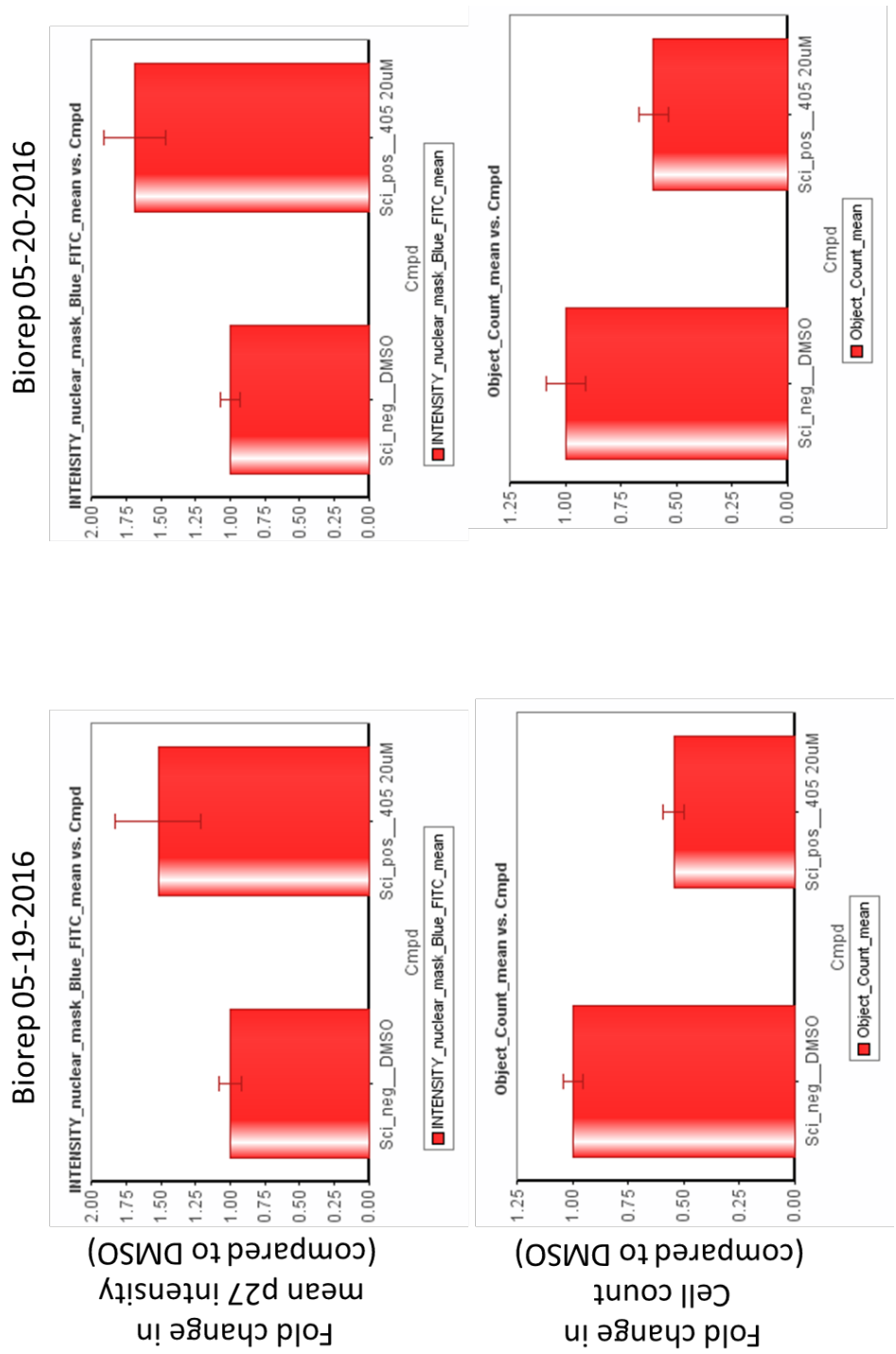


Figure A8 Graphs of the data contained in the response of preliminary experiment for compound **405** at 20 μM

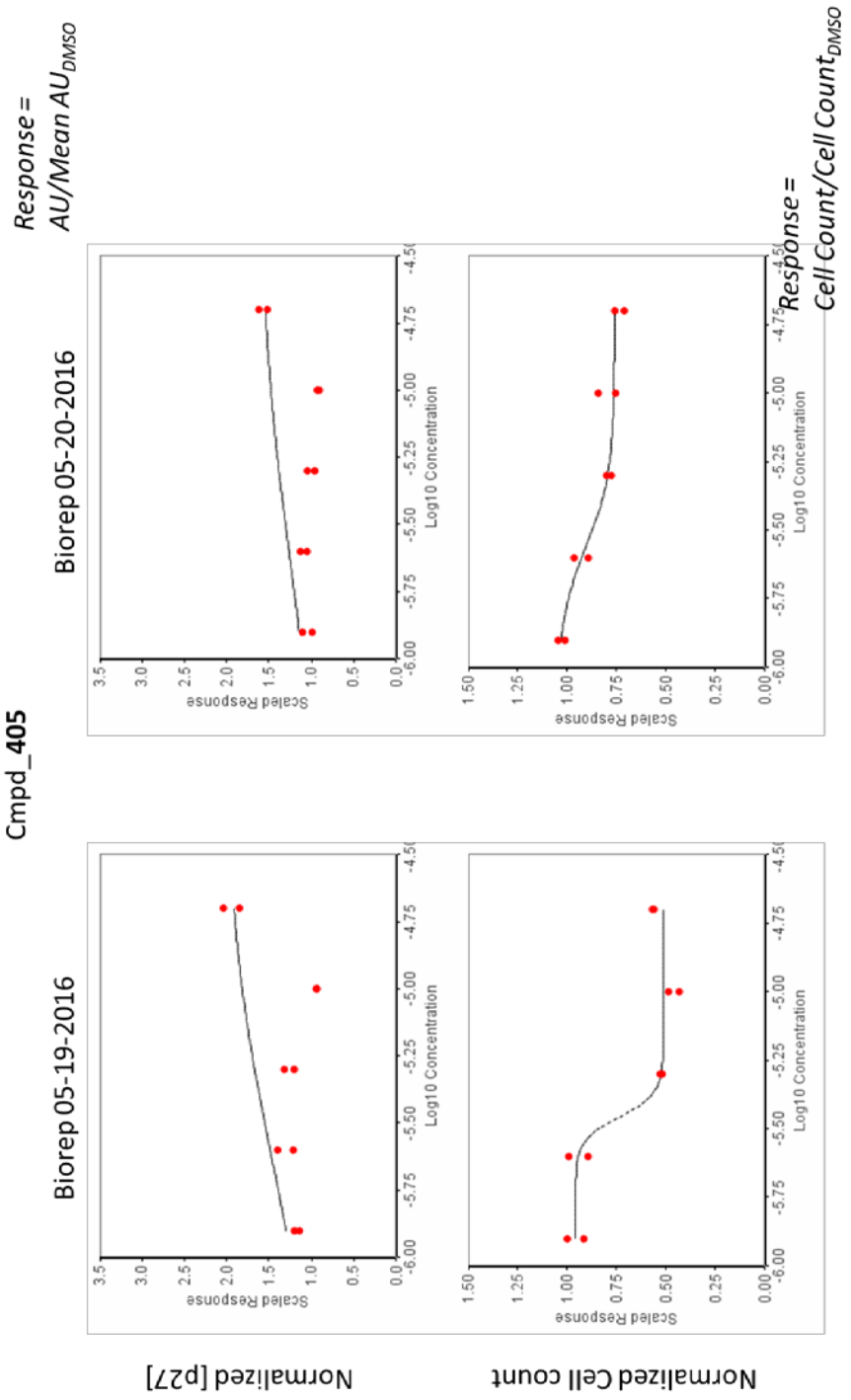


Figure A9 Graphs of the data contained in the response of preliminary experiment for compound **405**

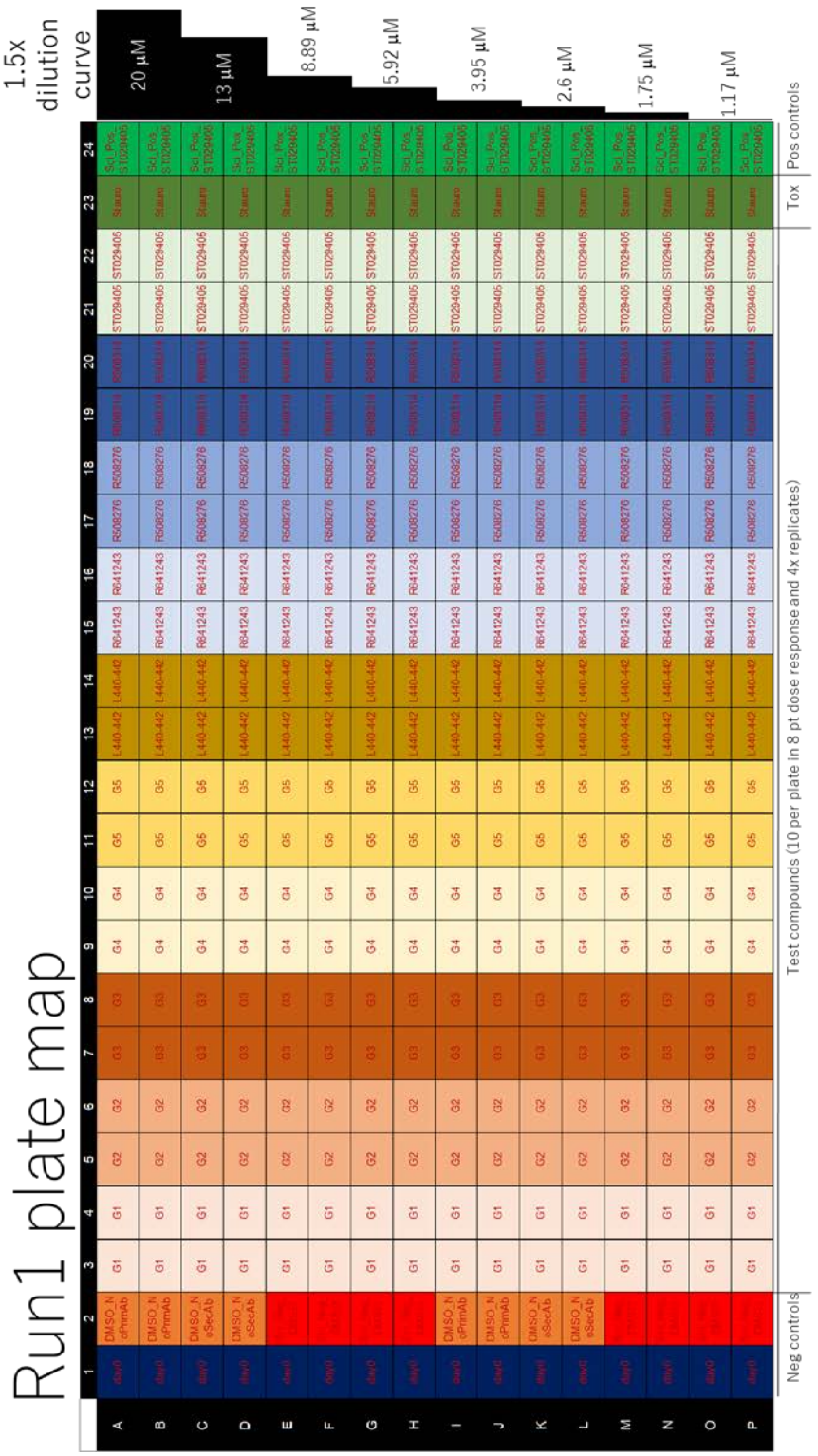


Figure A10 Plate map (run 1) used for the analysis of the UofH **405**-analogs (**G1**-**G5**)

Figure A11 Response (run 1) for the analysis of the UofH **405**-analogs (**G1**-**G5**)

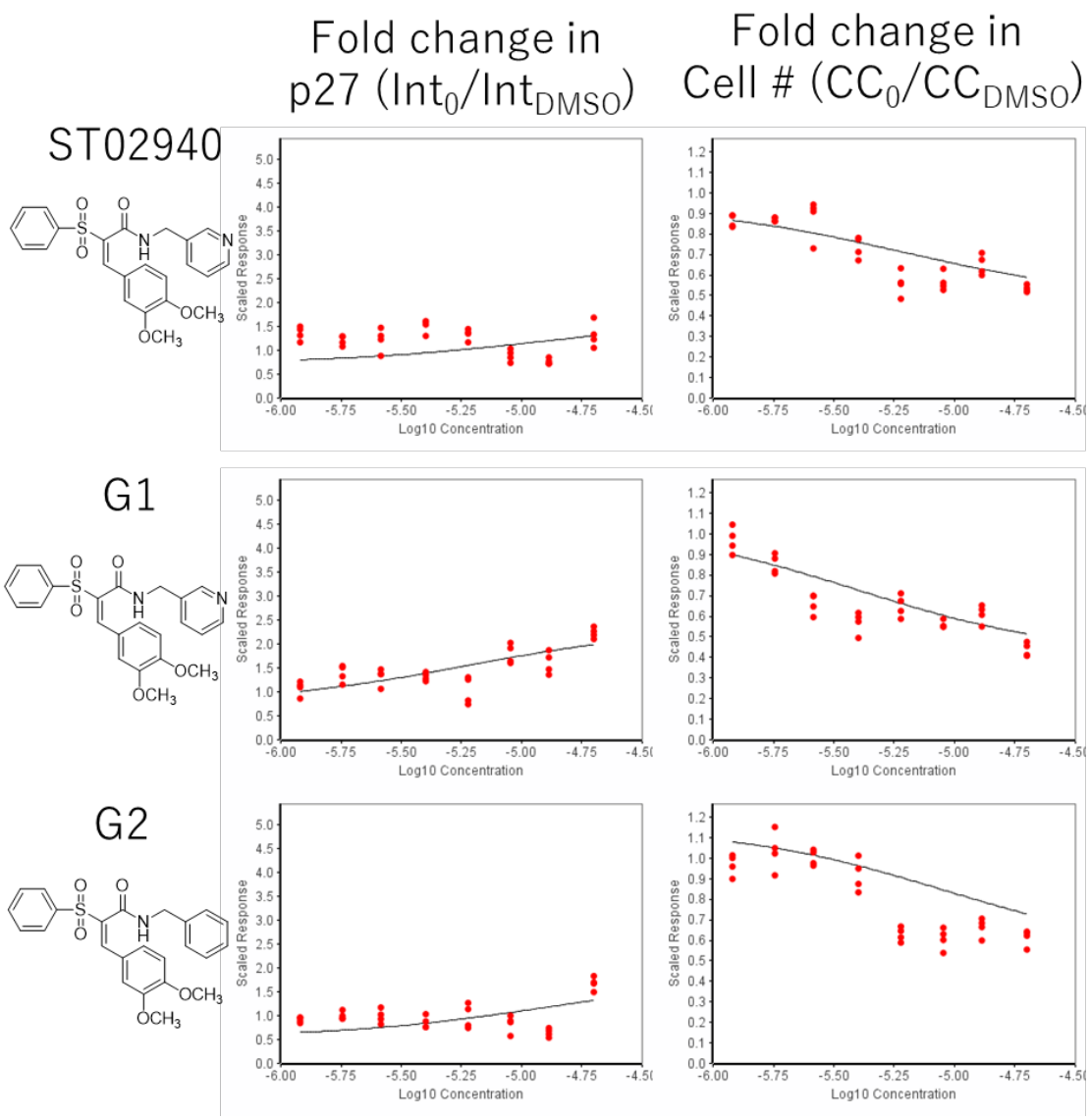


Figure A12 Graphs of the data contained in the run 1 heatmaps

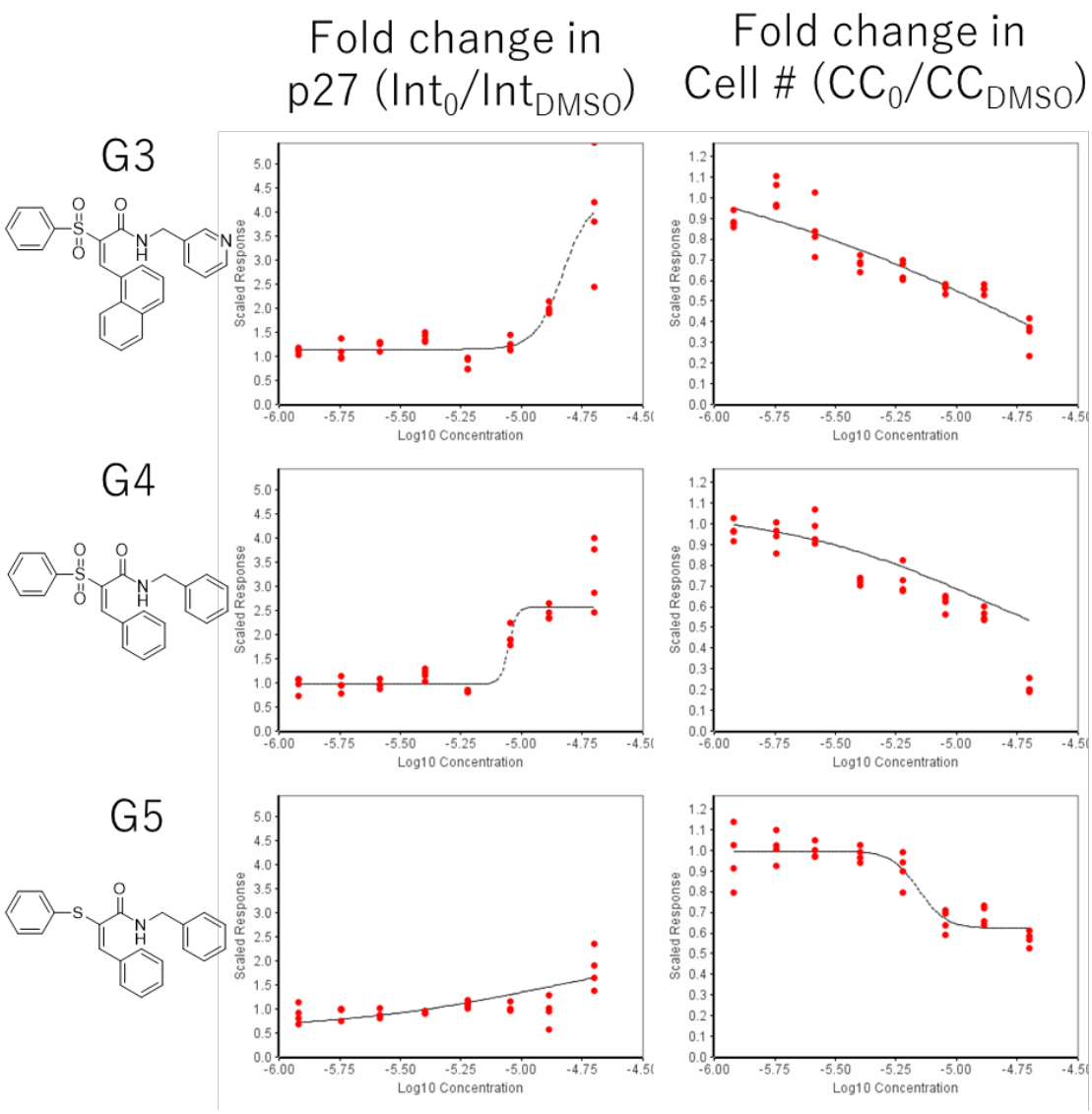


Figure A12 *Continued.*

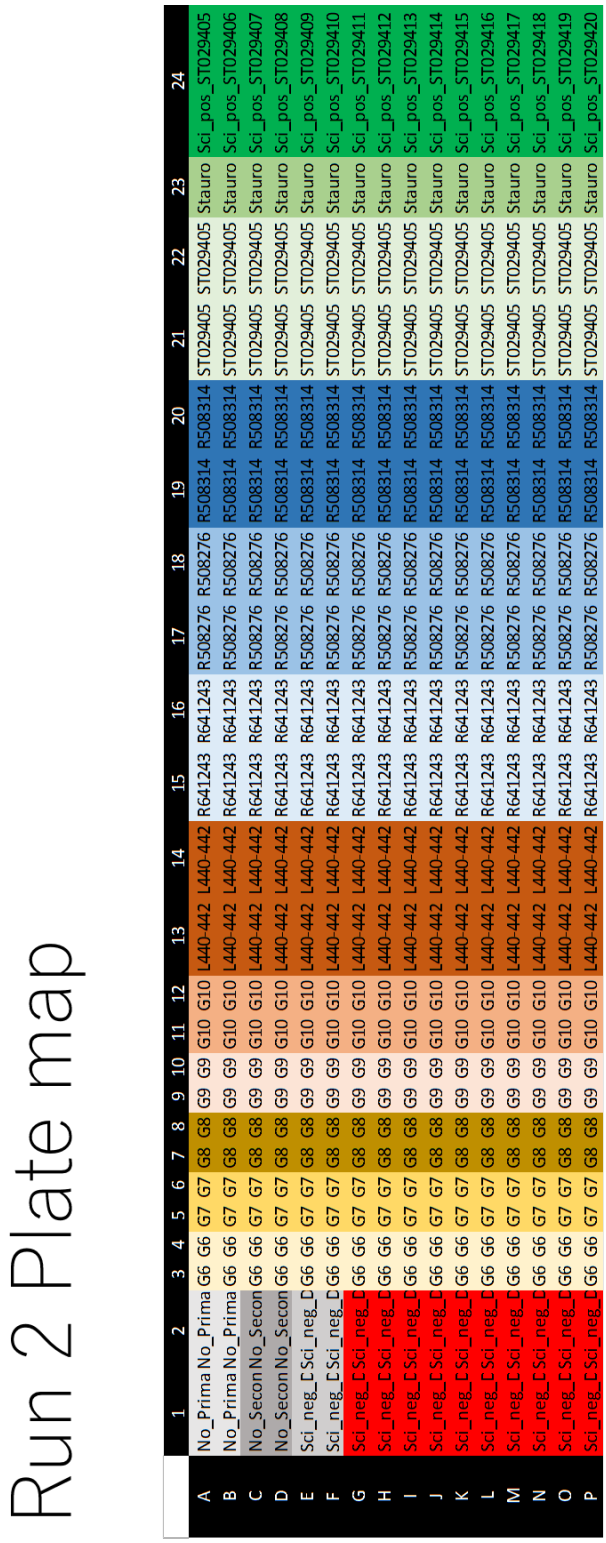


Figure A13 Plate map (run 2) used for the analysis of the UofH **405**-analogs (**G6**-**G10**)

Figure A14 Response (run 2) for the analysis of the UofH **405**-analogs (**G6**-**G10**)

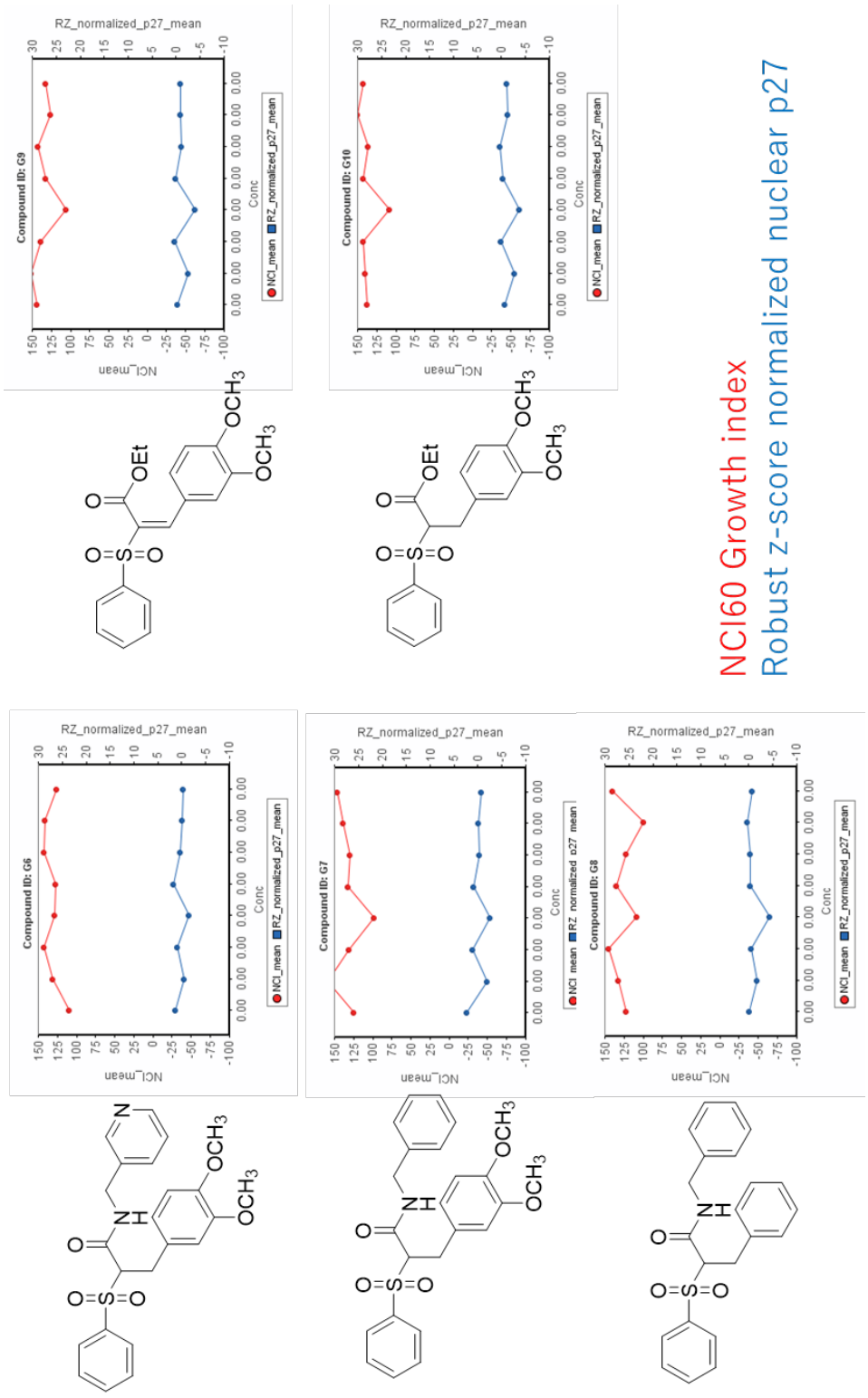


Figure A15 Run 2 dose response curves (**G6**-**G10**)

Run 3 Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	No_1-2An	No_1-2An	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
B	No_1-2An	No_1-2An	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
C	No_1Antil	No_1Antil	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
D	No_1Antil	No_1Antil	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
E	No_2Antil	No_2Antil	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
F	No_2Antil	No_2Antil	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
G	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
H	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
I	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
J	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
K	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
L	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
M	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
N	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
O	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405
P	DMSO	DMSO	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	Stau	ST029405

Figure A16 Plate map (run 3) used for the analysis of the UofH **405**-analogs (**G1-G10**)

Figure A17 Response (run 3) for the analysis of the UofH **405**-analogs (**G1-G10**)

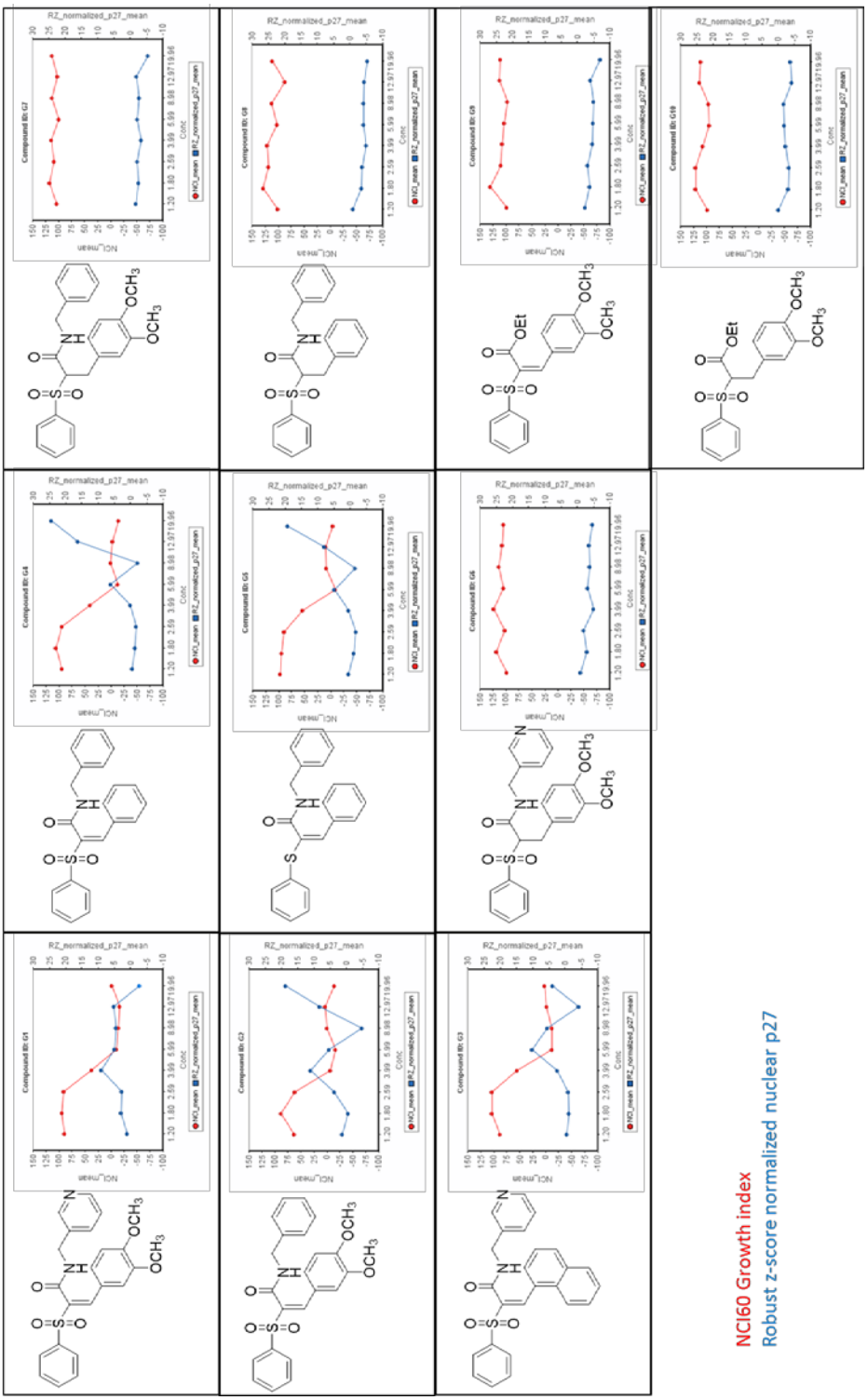


Figure A18 Run 3 dose response curves for **G1**-**G10**