Electronic Supplementary Material (ESI) for Chemical Communications.

Nitroisobenzofuranone, a small molecule inhibitor of multidrug-resistant *Staphylococcus aureus* targets peptidoglycan biosynthesis

Viral Rawat,[a#] Sona Tiwari,[a#] Shweta Khanna,[a#] Umang Gupta,[b] S. N. C. Sridhar,[a] Dharmendra K. Yadav,[a] Grace Kaul,[c,d] Abdul Akhir,[c] Deepanshi Saxena,[c] Saravanan Matheshwaran,[b] Sidharth Chopra,[c,d] and Dharmaraja Allimuthu[a]*

[a]Department of Chemistry, Indian Institute of Technology Kanpur, Uttar Pradesh India.

[b]Department of Biological Sciences and Bioengineering, Indian Institute of Technology Kanpur, Uttar Pradesh India.

[c]Division of Molecular Microbiology and Immunology, CSIR-Central Drug Research Institute Sector 10, Jankipuram Extension, Sitapur Road, Lucknow 226031 Uttar Pradesh, India.

[d]AcSIR: Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India

[#]These authors contributed equally

Table of Contents:

1. **General.** All chemical reactions were conducted under a argon atmosphere. All chemicals were purchased from commercial sources viz. Sigma, Avra, Spectrochem, TCI, GLR etc. and used as received unless stated otherwise. Dichloromethane (DCM), methanol, carbontetrachloride, tetrahydrofuran (THF) for reaction were used as dried as specified, and petroleum ether and ethyl acetate (EtOAc) for chromatography were distilled before use. *N*-bromosuccinimide was recrystallized in water to obtain a white crystalline solid before use. Column chromatography was performed on Merck silica gel (60-120 and 100–200 mesh). ^{1}H and ^{13}C spectra were recorded on JEOL ECS-400 MHz or ECX-500 MHz spectrometers using either residual solvent signals as an internal reference ($CDCl_3$ δH, 7.24 ppm, δC 77.1 ppm) or an internal tetramethylsilane (δH = 0.00, δC = 0.0). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. The following abbreviations are used: m (multiplet), s (singlet), d (doublet), t (triplet) dd (doublet of doublet) and dt (doublet of triplet). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS or APCI. High-performance liquid chromatography (HPLC) was performed on an Agilent 1260 infinity machine model with a ZORBAX SB-C-18 reverse phase column (150 × 4.6 mm, 5 µm). Fluorimetric, luminometric and spectrophotometric measurements were performed using Promegapc-GloMax® explorer reader. Homology models of the *S. aureus* MurA and MurZ were generated using the SWISS-MODEL Interactive Workspace developed by the Swiss Institute of Bioinformatics, Switzerland.[1] Molecular docking was performed using Schrodinger Drug Discovery Suite v2020-1. Gels of in-gel fluorescence analysis experiment were visualized using Alexa 448 filter in Chemidoc (ChemiDoc™ Touch Imaging System from Bio-Rad). The purity of all compounds synthesized in this study was ≥ 95% as determined by HPLC. Compounds IITK2001,[2] IITK2003,[3] IITK2004,[4] IITK2013,[5] IITK2014,[6] IITK2015,[7] IITK2016,[8] IITK2012,[6] IITK2020,[9] IITK2021,[10] IITK2022,[11] IITK2024,[12] IITK2025,[13] IITK2026,[10] IITK2027,[14] IITK2032,[15] IITK2035,[16] IITK2036,[16] have been previously reported and analytical data that we collected were consistent with the reported values.

2. Supporting Figures:

Figure S1. Whole-cell bacterial viability screening against *S. aureus* and synthetic analogues: The screen identified IITK2001 as top hit. (Minimum Inhibitory concentration (MIC): minimum concentration at which no visible growth of *S. aureus* was recorded). Small molecules classes (N = 205): Michael acceptors (α,β-unsaturated ketone/amides), N = 59; Electrophiles (Chloroacetamides, benzyl bromides), N = 25; β-lactams, N = 18; Amino acid analogues, N = 52; Bicyclic/ Heterocyclic systems, N = 34; a, β -Epoxyketone/ester/acids, N = 17.

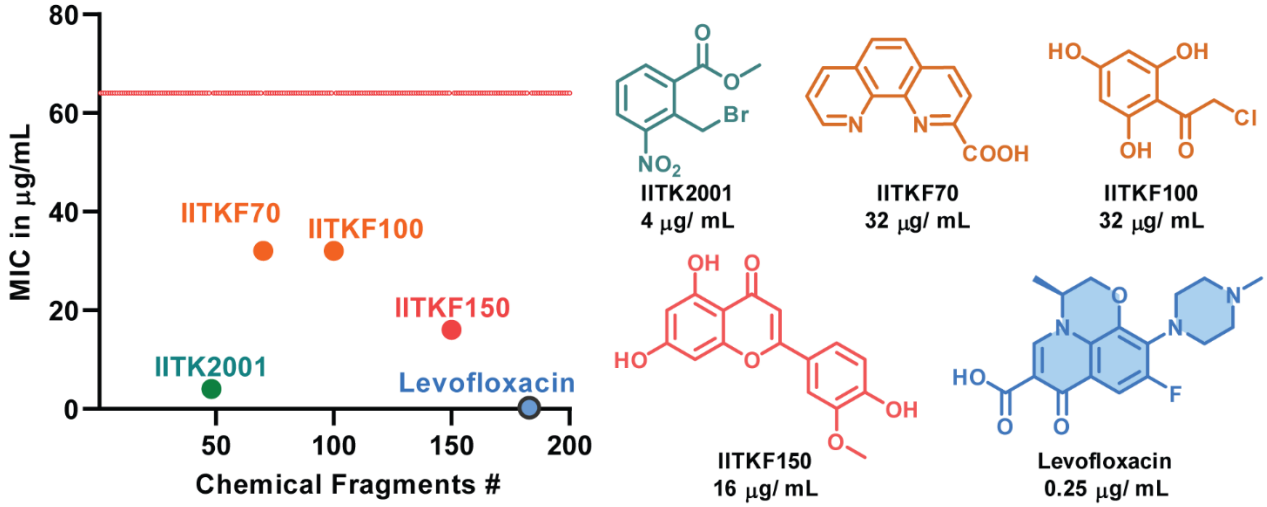
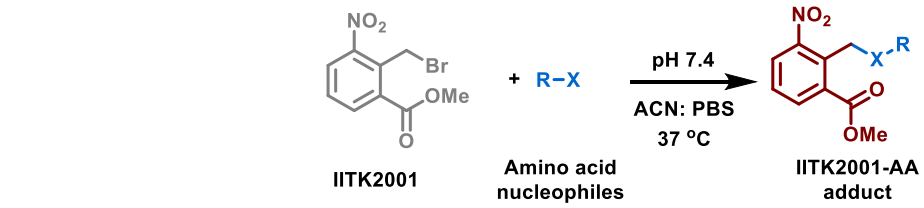
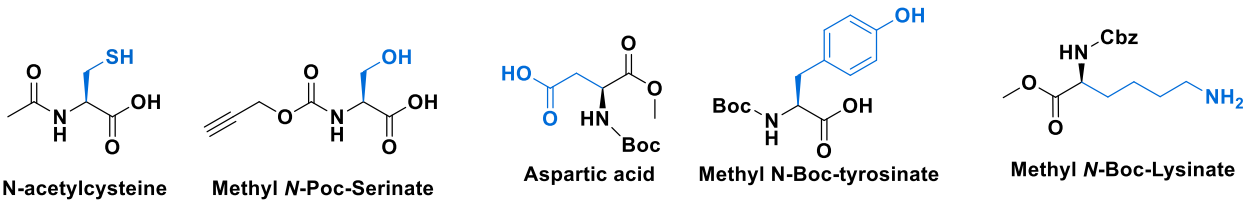


Figure S2 HPLC traces of IITK2001 combined with nucleophilic amino acids:

In 1:1 acetonitrile: PBS at 37 °C after 1 h.





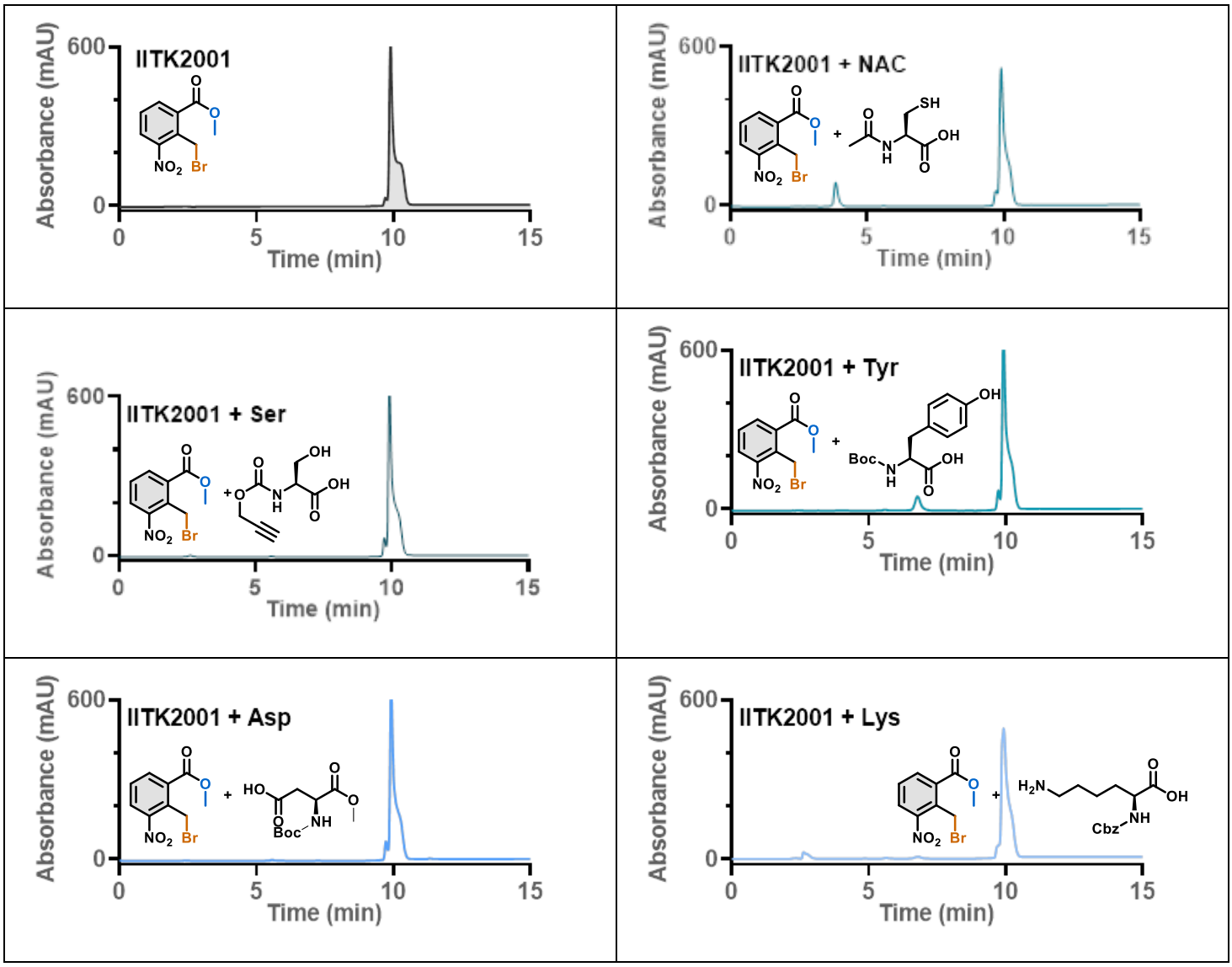
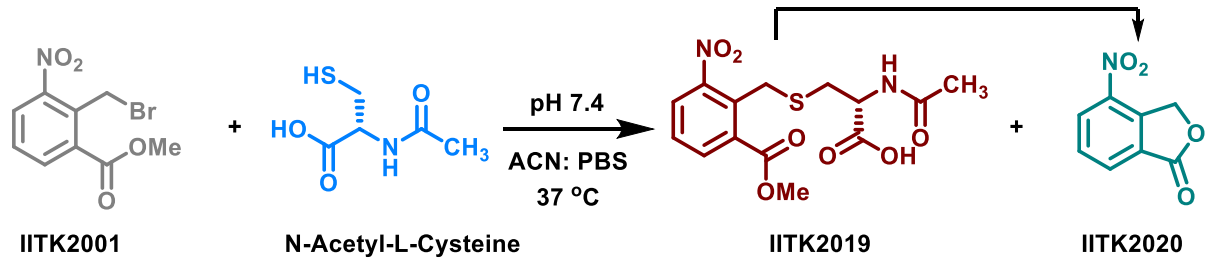


Figure S3. Overlapped HPLC traces of IITK2001 combined with N-Acetylcysteine (NAC):

In 1:1 acetonitrile: PBS at 37 °C for 24 h at various time points.



IITK2001 N-Acetyl-L-Cysteine IITK2019 IITK2020

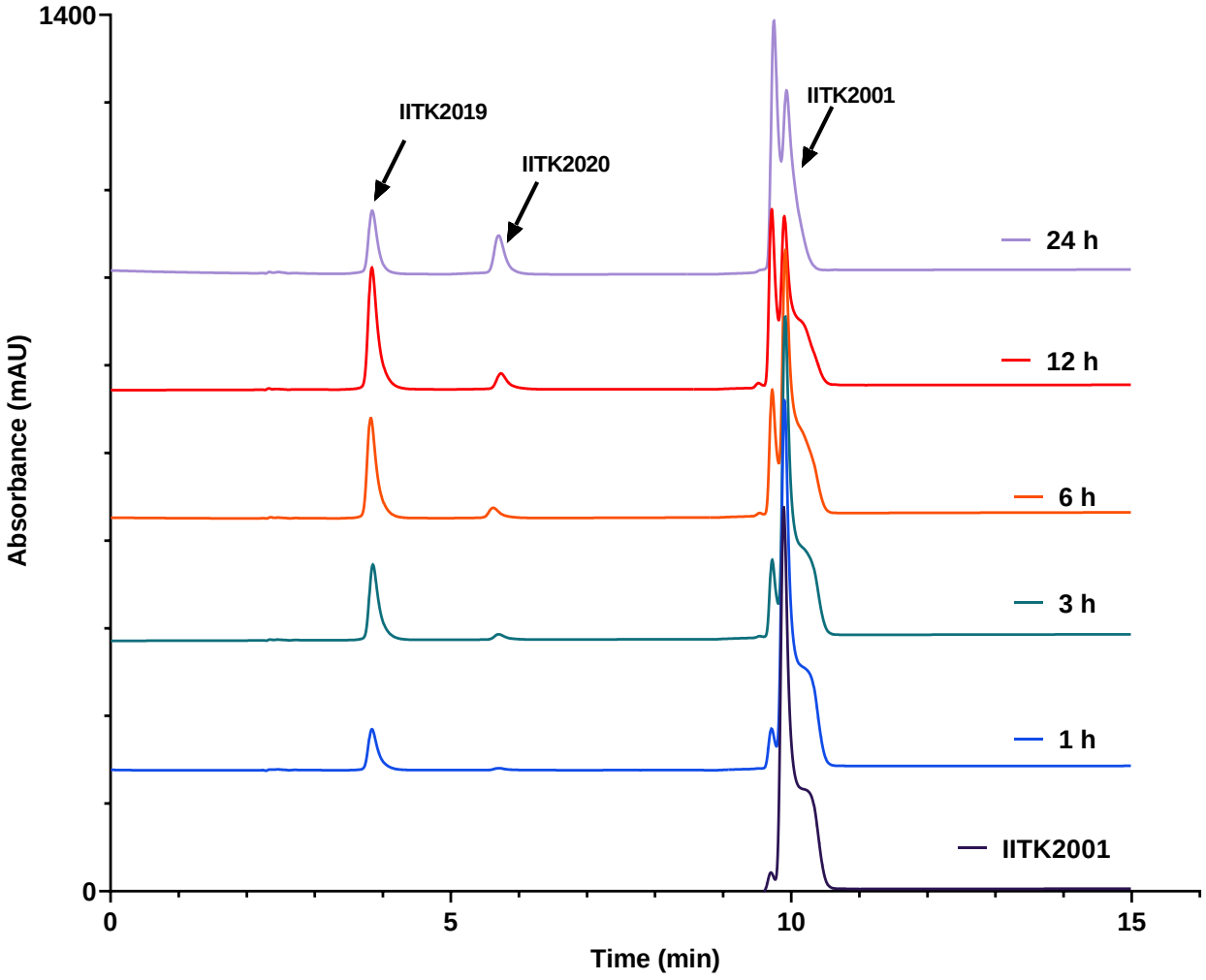
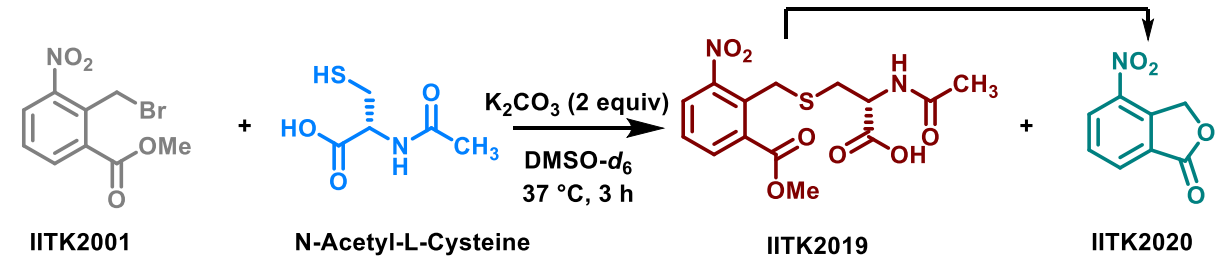
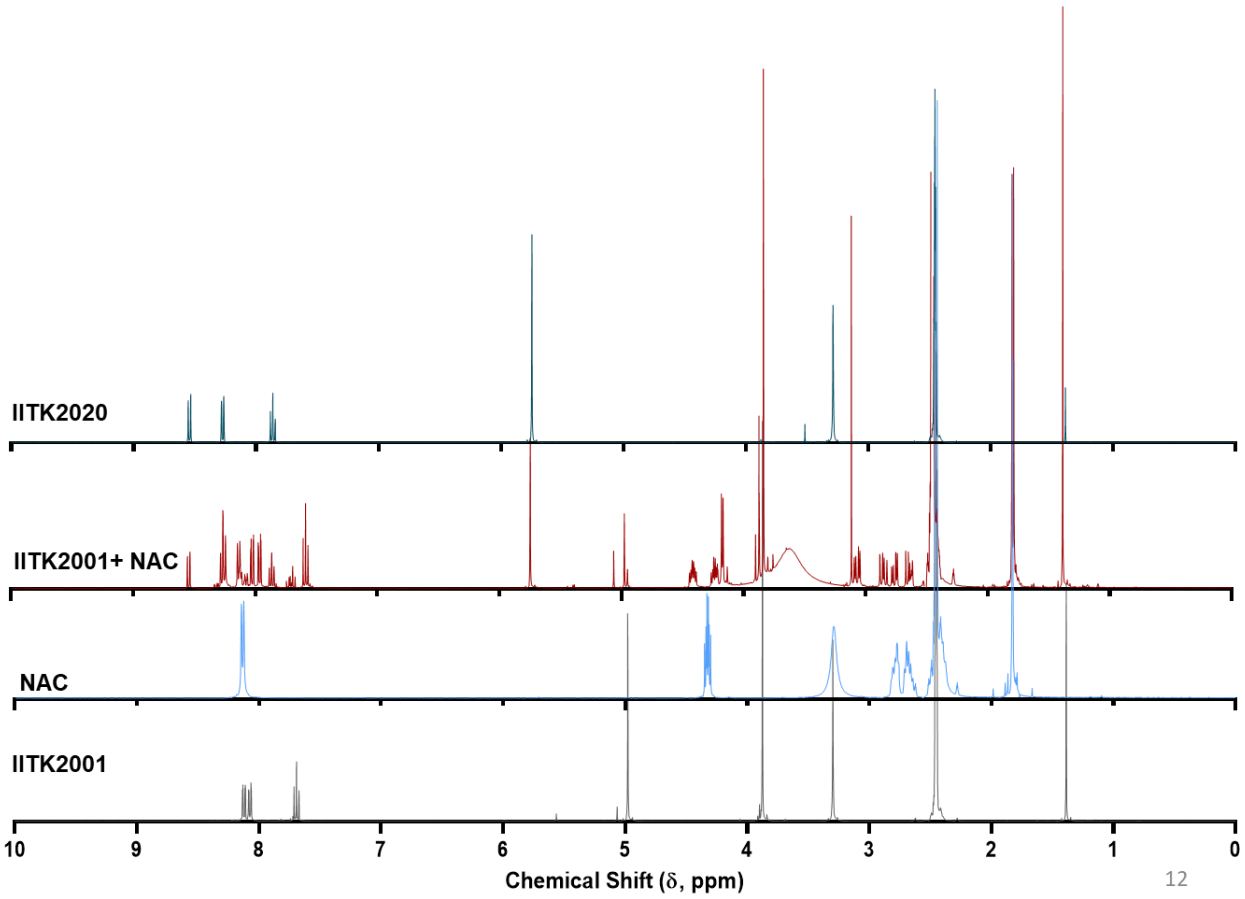


Figure S4. ^{1}H NMR spectra of IITK2001, NAC, and their combination:

In DMSO-d_6 at 37 °C after 3 h is compared with IITK2020.

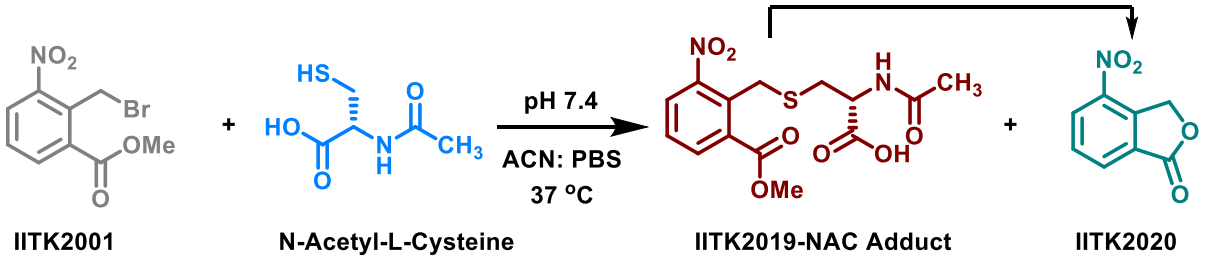




12

Figure S5. HPLC traces of IITK2001, IITK2001+NAC combination:

In 1:1 acetonitrile: PBS at 37 °C after 24 h is compared with NMR reaction mixture.



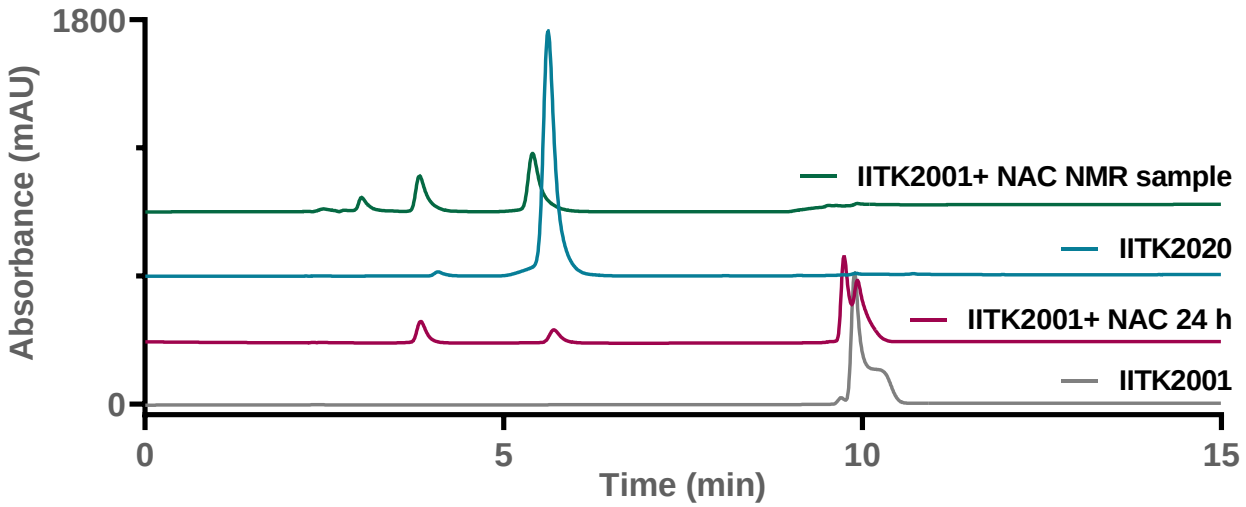
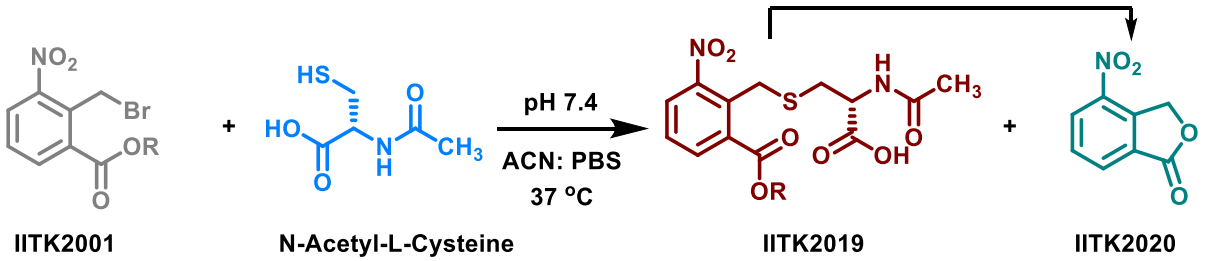


Figure S6. Overlapped HPLC traces of IITK2001, IITK2008, IITK2010 and IITK2011 combined with *N*-Acetylcysteine (NAC):

In 1:1 acetonitrile: PBS at 37 °C after 24 h.



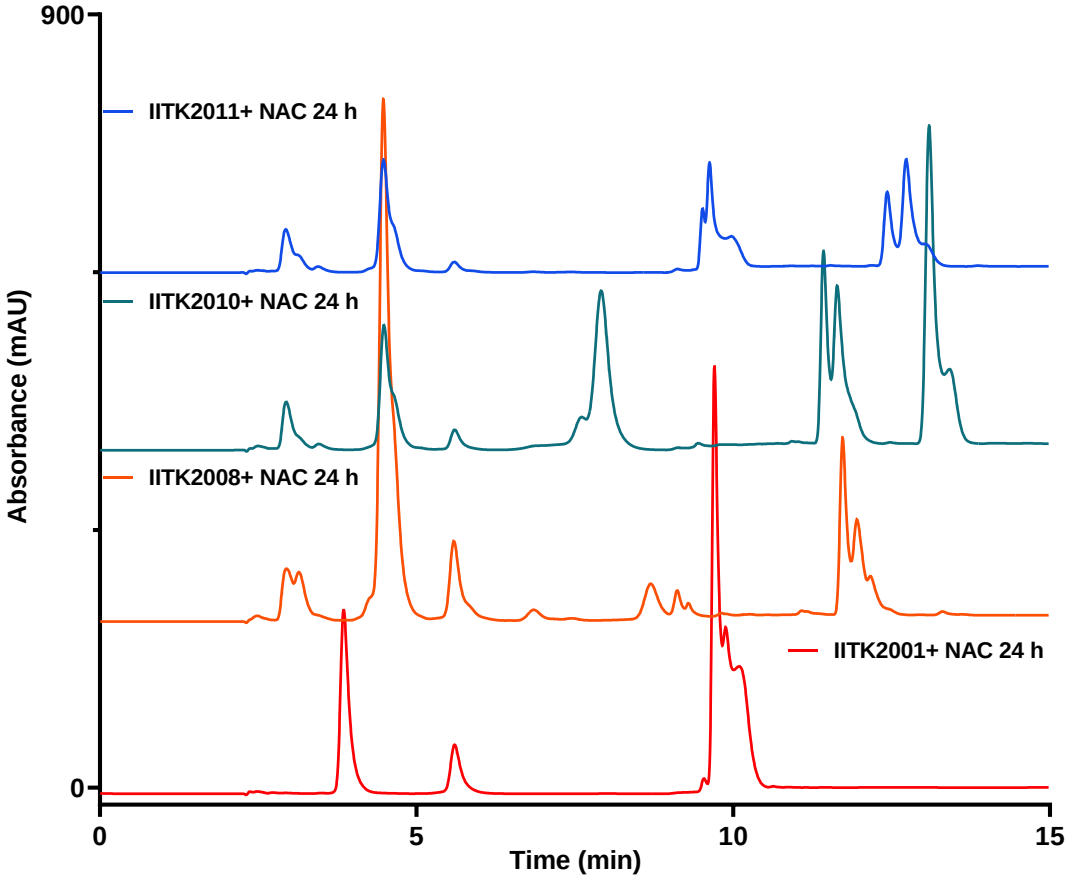


Figure S7. HPLC traces of IITK2008, IITK2010 and IITK2011 alone and combined with *N*-Acetylcysteine (NAC):

In 1:1 acetonitrile: PBS at 37 °C after 24 h.

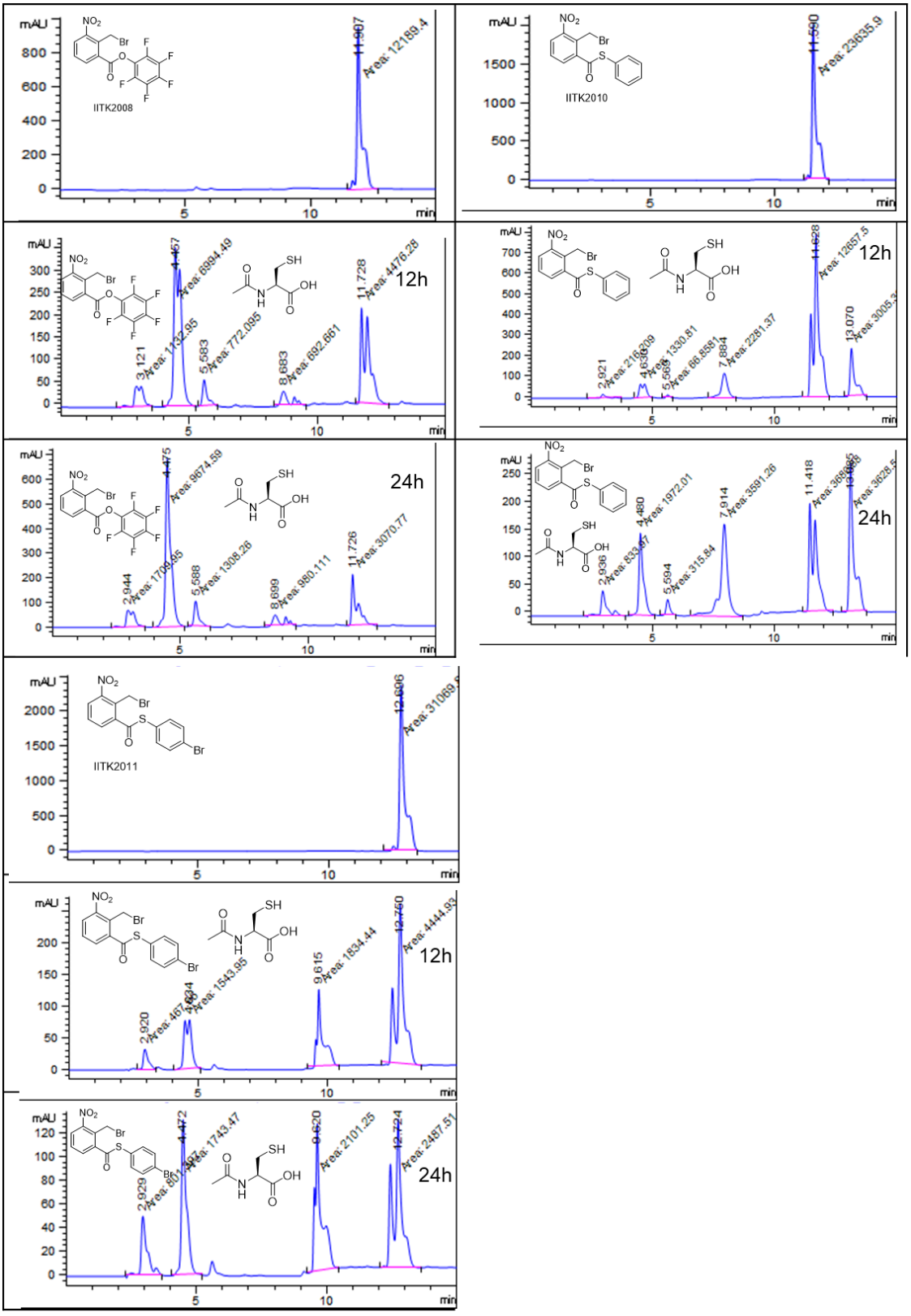


Figure S8. HPLC traces of IITK2020 alone and combined with nucleophilic amino acids.

In 1:1 acetonitrile: PBS at 37 °C after 1 h

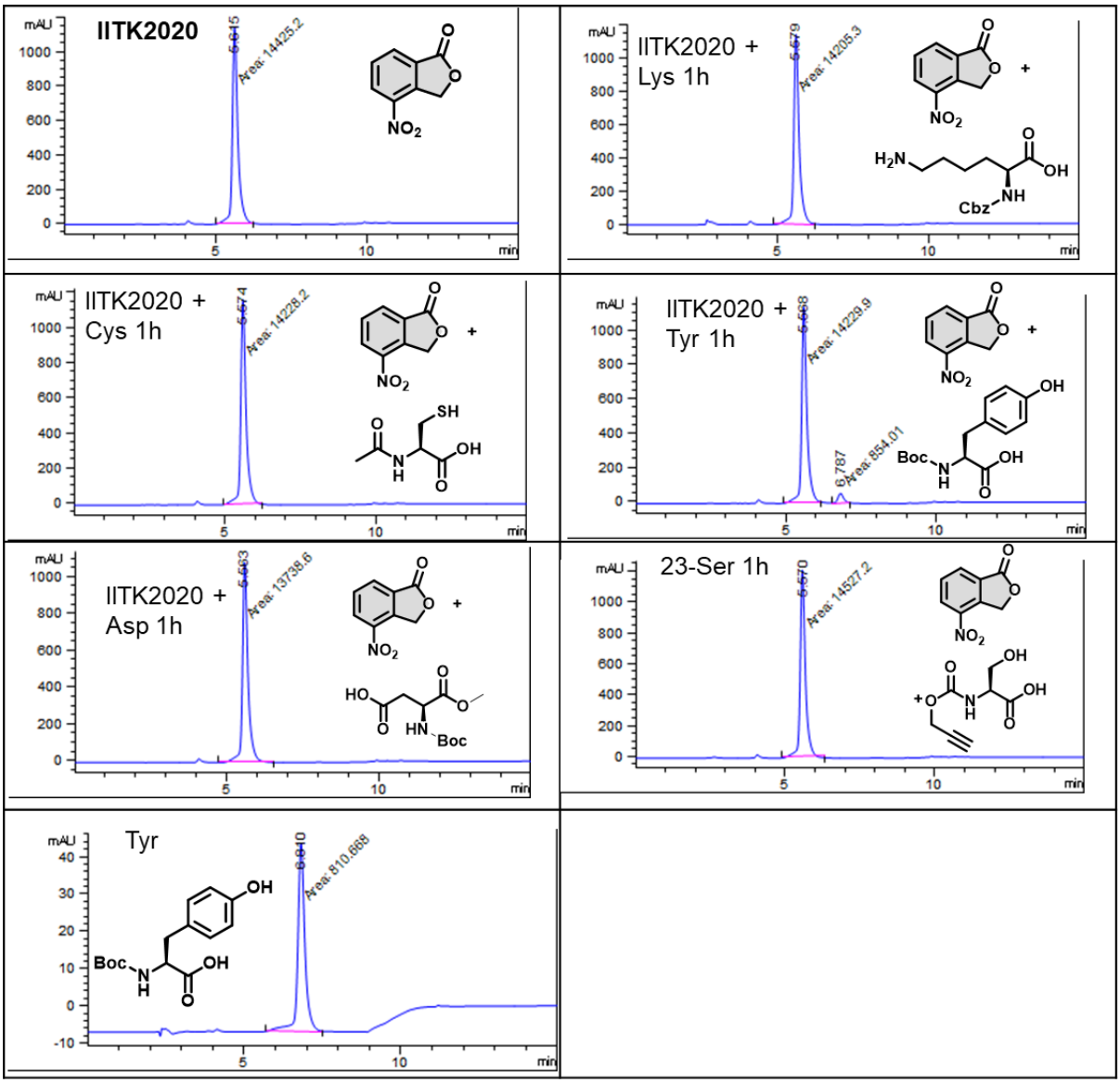
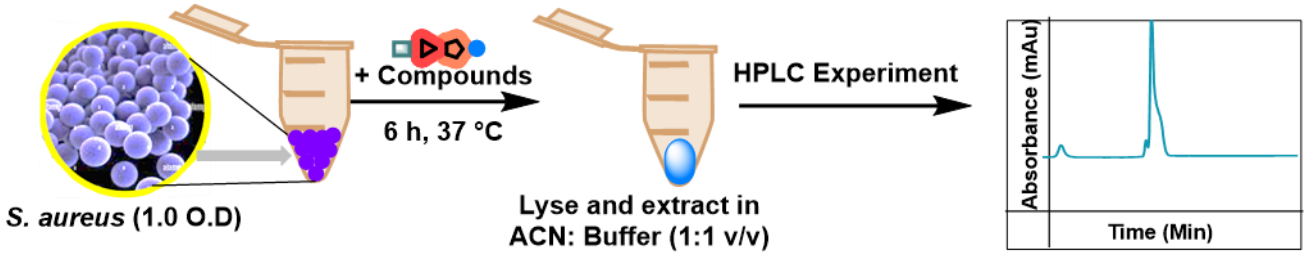


Figure S9. HPLC traces of metabolites extracted from *S. aureus* after treatment with IITK2001, IITK2008, IITK2010 and IITK2011 at 37 °C after 1 h.



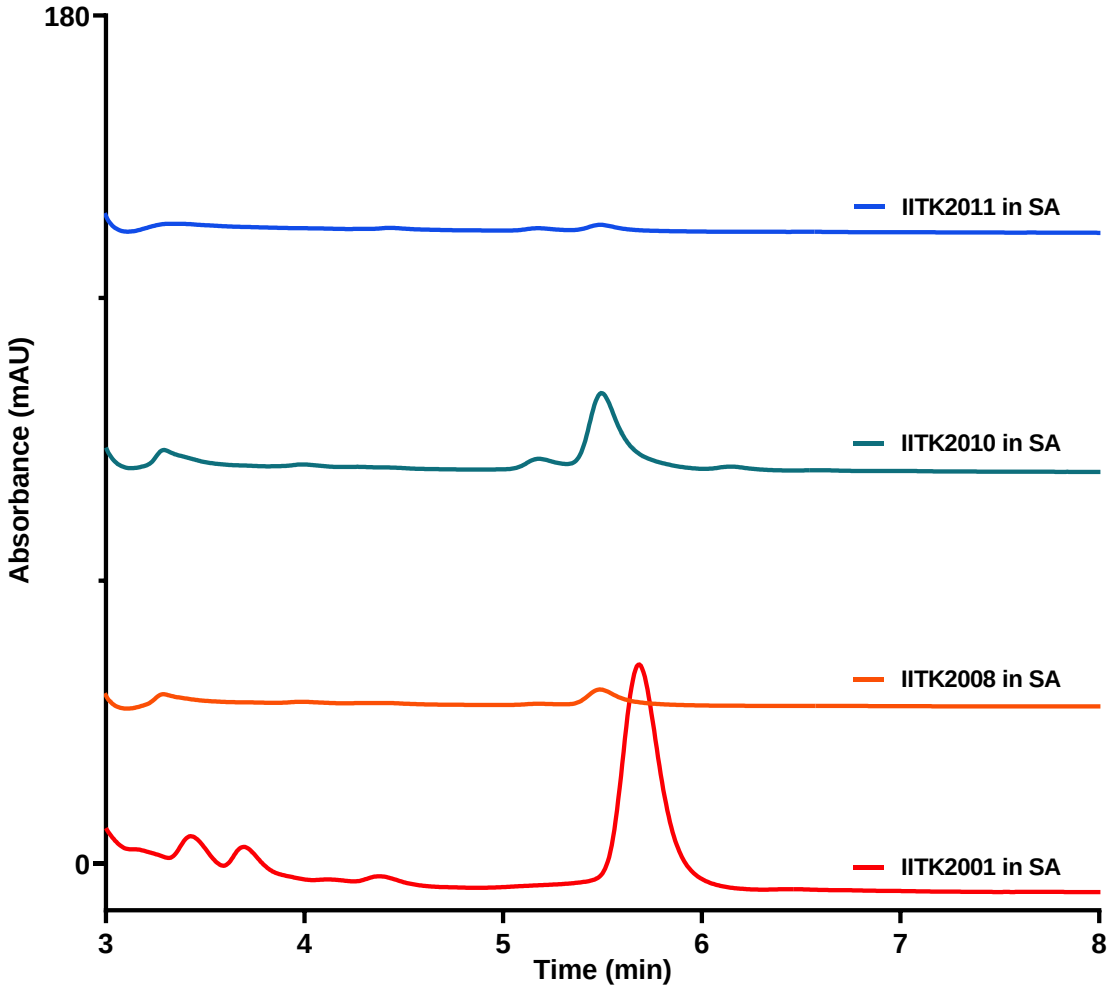
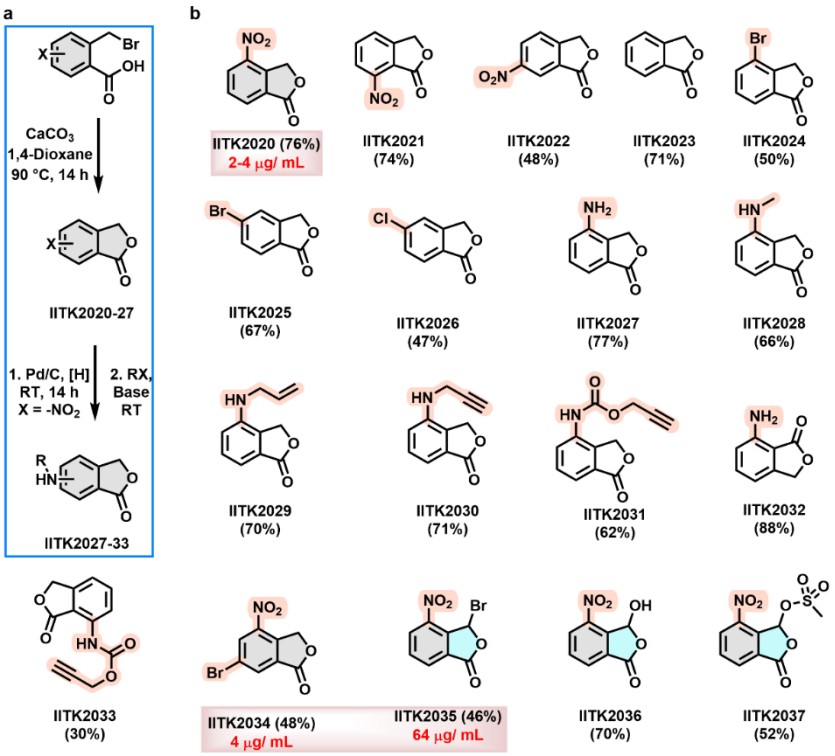


Figure S10. (A). Synthesis scheme and (B). structures of IITK2020 analogues (IITK2021 – IITK2037) and their MIC's against *S. aureus.*

MIC is not mentioned for inactive molecules (>64 mg/mL. Practical yield is given in parenthesis

.



Supporting text: Lead molecule is cell permeable and conserved its antibacterial property against Multidrug-resistant clinical strains of *S. aureus*.

The challenges associated with small molecules permeability in Gram-negative bacteria (GNB) is a major roadblock for antibiotic development. Therefore, we investigated the antibiotic activity of IITK2020 in presence of outer membrane permeabilizer of GNB, polymyxin B nonapeptide (PMBN, Fig. 4D).[17] We validated the experimental design using Fosfomycin, levofloxacin and vancomycin. Fosfomycin uptake is facilitated by glucose-6-phosphate (G-6-P)-inducible hexose phosphate transport system.[18] We observed a sharp increase in antibiotic activity of Fosfomycin in presence of G-6-P (32 μg/mL to 1 μg/mL, 32-fold rise) in *E. coli*, while this was unaffected upon addition of PMBN or in *A. baumannii* (Fig. 4D). Levofloxacin freely diffuses through the bacterial membrane, which could be observed from our studies as well that the addition of PMBN does not affect its antibacterial activity. In parallel, the effect of PMBN on the potency of rifampicin and vancomycin is well captured in both *E. coli* and *A. baumannii* (Fig. 4D). Under identical conditions, IITK2020 was found to be inactive and unperturbed by the addition of G-6-P or PMBN, demonstrating the molecule's permeability is unlikely to be the reason for its weak potency (Fig. 4D). Collectively, this detailed SAR analysis revolving around the concept of lactonization to induce antibacterial activity resulted in identification of IITK2020, a chemical fragment (>180 Da molecular weight) with 2-4 μg/mL MIC acting exclusively against *S. aureus*

Figure S11. Lead molecule is cell permeable and conserved its antibacterial property against Multidrug-resistant clinical strains of *S. aureus*.

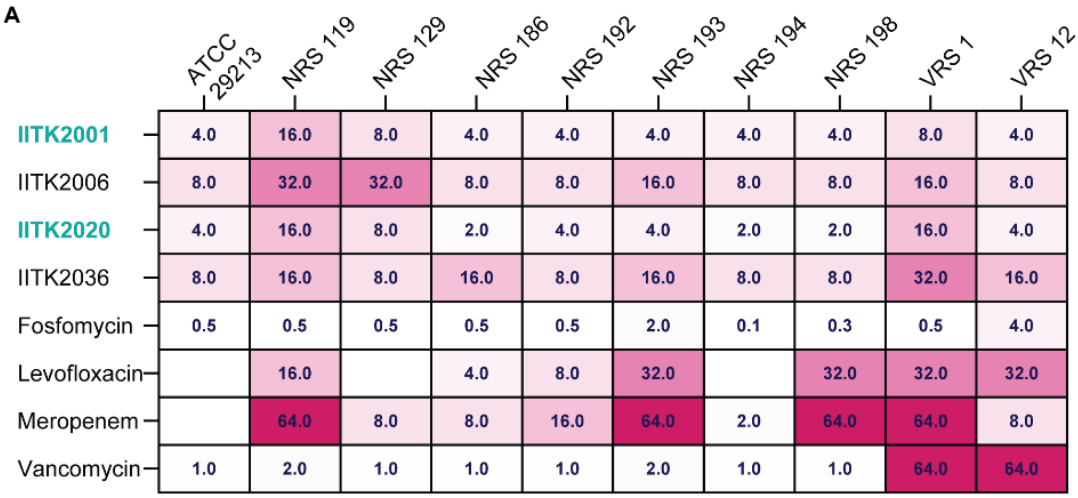
(A) MIC of lead molecules against methicillin-susceptible *S. aureus* (MSSA), MRSA and VRSA;

(B) MIC of IITK2020 against ESKAPE bacterial strains;

(C) Cytotoxicity analysis of IITK2020 and other antibiotics against Vero cells;

(D) Effect of outer membrane permeabilizer (PMBN) on MIC of given antibiotics against E. coli and A. baumannii. Data presented here is a representative of two or three independent experiments performed in triplicate.

A

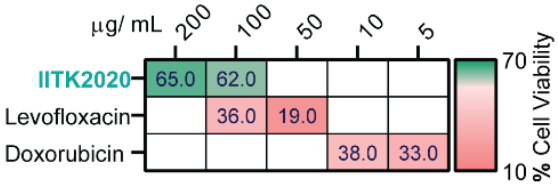


B

IITK2020, MIC in μg/ mL

E. coli	>64	*P. aeruginosa*	>64
K. pneumoniae	>64	*E. faecalis*	>64
A. baumannii	>64	*S. aureus*	2 -4

C



D

S. No.	Compound code	MIC (μg/mL)							
		E. coli ATCC 25922				*A. baumannii* BAA 1605			
		Alone	PMBN	PMBN + G-6-P	G-6-P	Alone	PMBN	PMBN + G-6-P	G-6-P
1	IITK2020	>64	>64	>64	>64	>64	>64	>64	>64
2	Fosfomycin	32	32	1	1	64	64	64	64
3	Rifampicin	8	0.0625	0.0625	8	4	0.0625	0.0625	4
4	Levofloxacin	0.0156	0.0156	0.0156	0.0156	4	4	4	4
5	Vancomycin	512	64	64	512	128	32	32	128

PMBN- Polymyxin B Nonapeptide Hydrochloride at 10 μg/ml

G-6-P- Glucose-6-Phosphate at 25 μg/ml

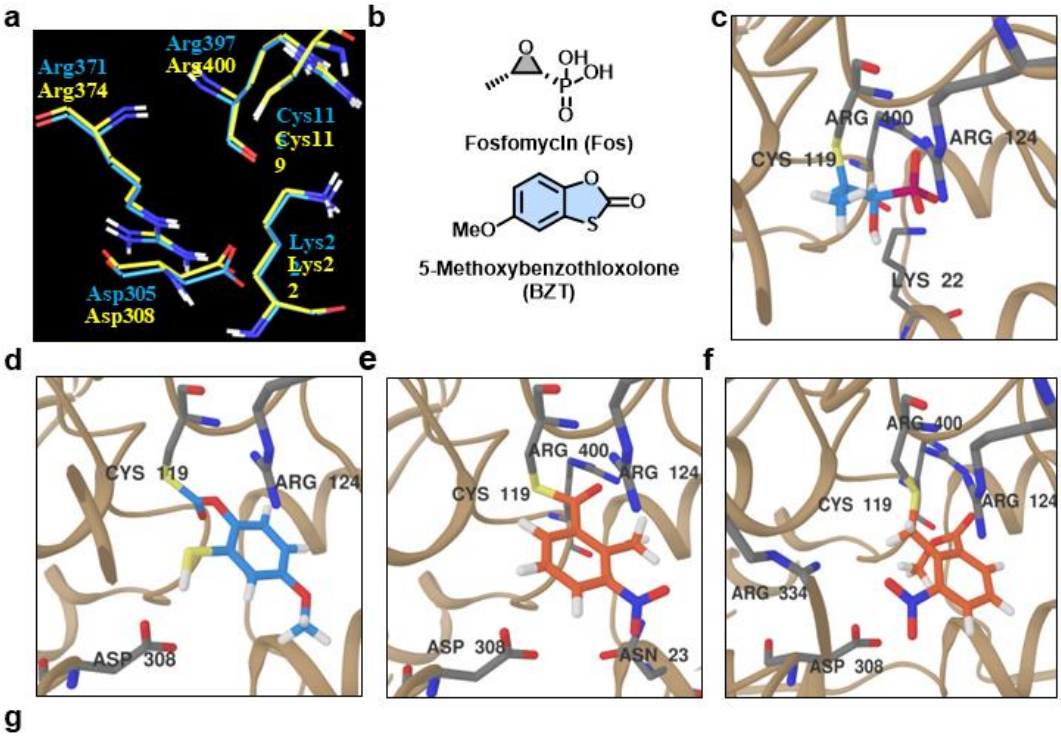
Figure S12. Homology modelling cell wall biosynthetic enzymes MurA and molecular covalent docking studies with Fosfomycin, BZT, IITK2001 and IITK2020.

(a). Superimposition of homology modelled S. aureus MurA structure (given in yellow) with X-ray crystal structure of E. coli MurA (Given in blue).

(b) Structures of Fosfomycin and BZT;

(c-f). Three-dimensional representation of MurA docked with Fosfomycin (c), BZT (d), IITK2001 (e) and IITK2020 (f);

(g). Molecular docking energy values (kcal/mol) for indicated molecules with optimized MurA and MurZ



g

Docking energies (ΔG, Kcal/mol) of lead molecules with MurA and MurZ				
S. aureus	Fosfomycin	BZT	IITK2001	IITK2020
MurA	-6.545	-4.140	-3.196	-3.796
MurZ	-6.725	-4.130	-3.487	-3.817

Figure S13. Homology modelling and molecular covalent docking studies of Fosfomycin, BZT, IITK2001 and IITK2020 with cell wall biosynthetic enzymes MurZ.

a). Superimposition of homology modelled *S. aureus* MurZ structure (given in yellow) with X-ray crystal structure of *E. coli* MurA (Given in blue). (b) Structures of Fosfomycin and BZT;

(c-f). Three-dimensional representation of MurZ docked with Fosfomycin (c), BZT (d), IITK2001 (e) and IITK2020 (f). g). 3D-Representation of MurA reversible covalent docking with IITK2020 (XP score: -4.675 kcal/mol).

(h, i) Three-dimensional representation of MurZ docked with IITK2001 and IITK2008 in Molegro Virtual Docker (XP Score for IITK2001: 48.79 and IITK2008: 30.3 kcal/mol)

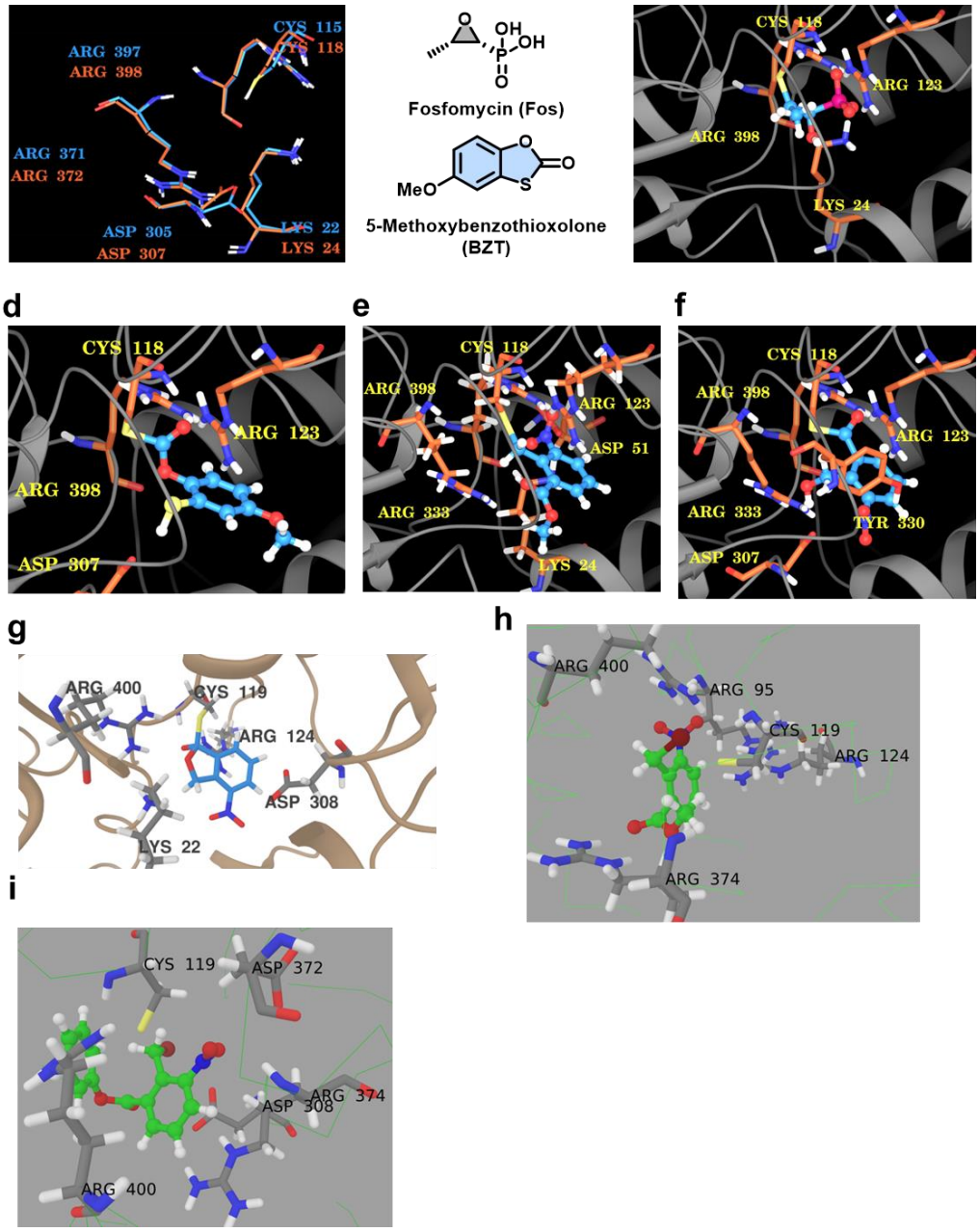


Figure S14. IITK2001 and IITK2020 interacts with S. aureus MurA and MurZ.

(A) Schematic representation of competitive in-gel fluorescence assay;

(B-G). Labelling (B-E) and quantification (F, G) of recombinant MurA (B, C, F) and MurZ (D, E, G) by CAA probe with or without pretreatment of IITK2001 (B, D, F) and IITK2020 (C, E, G);

(H, J). Click-ABPP in S. aureus lysate pretreated 6 h with the indicated concentrations of lead molecules, then subjected to reaction with CAA for 1 h (H) and its quantification (J);

(I, K). Coomassie stained gel image (I) and quantification (K).

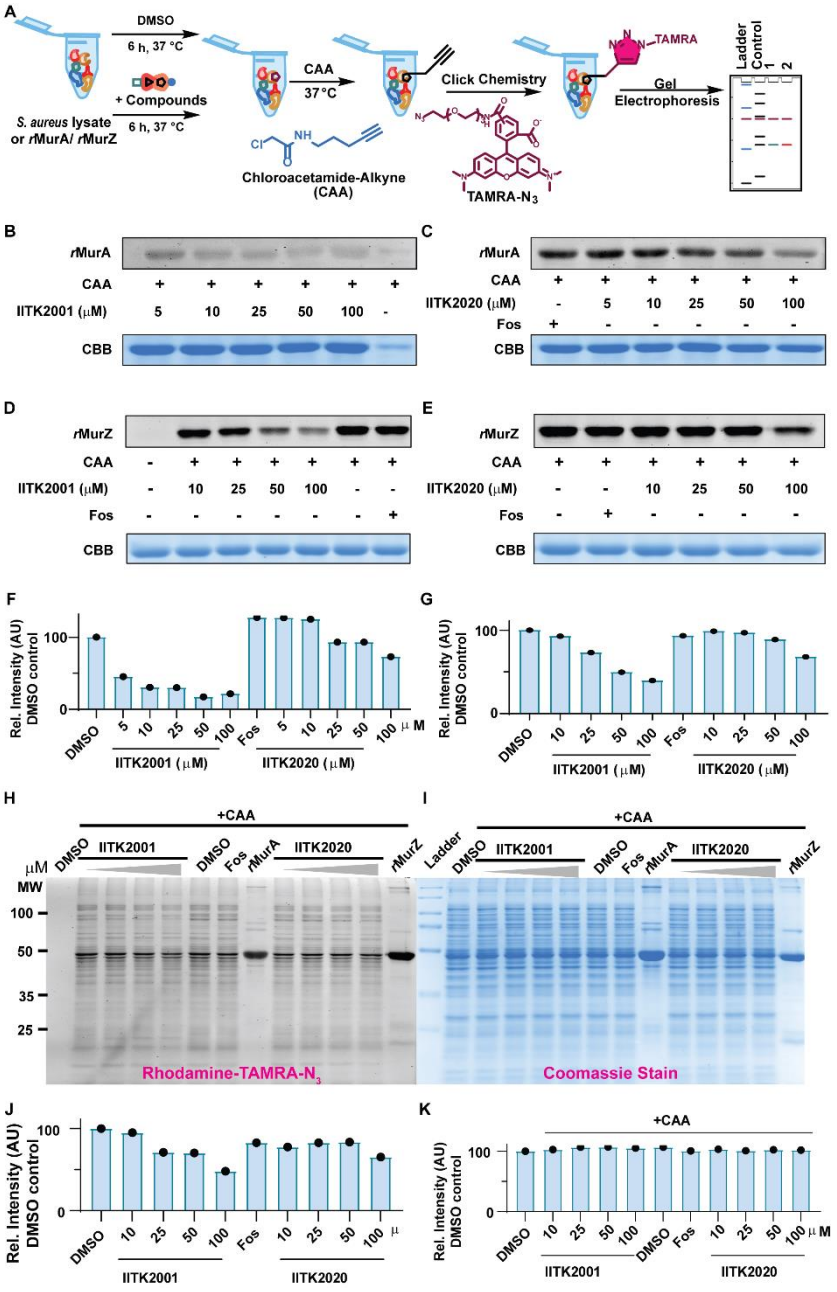


Figure S15. IITK2001 and IITK2020 induce membrane depolarization and disrupt the cell wall architecture in *S. aureus*.

(A) Structures of IITK2001, IITK2020 and DISC3-(5) potentiometric fluorophore;

(B). Measurement of membrane depolarization by IITK2001 and IITK2020 using fluorescence-based DISC3-(5) leakage assay;

(C-E). Representation of *S. aureus* morphological structures upon treatment with DMSO (C), IITK2001 (D) and IITK2020 (E) determined using AFM experiments.

Two-dimensional and three-dimensional representation of *S. aureus* bacterial surface (C-H) and their height distribution profiles (I-K) are shown.

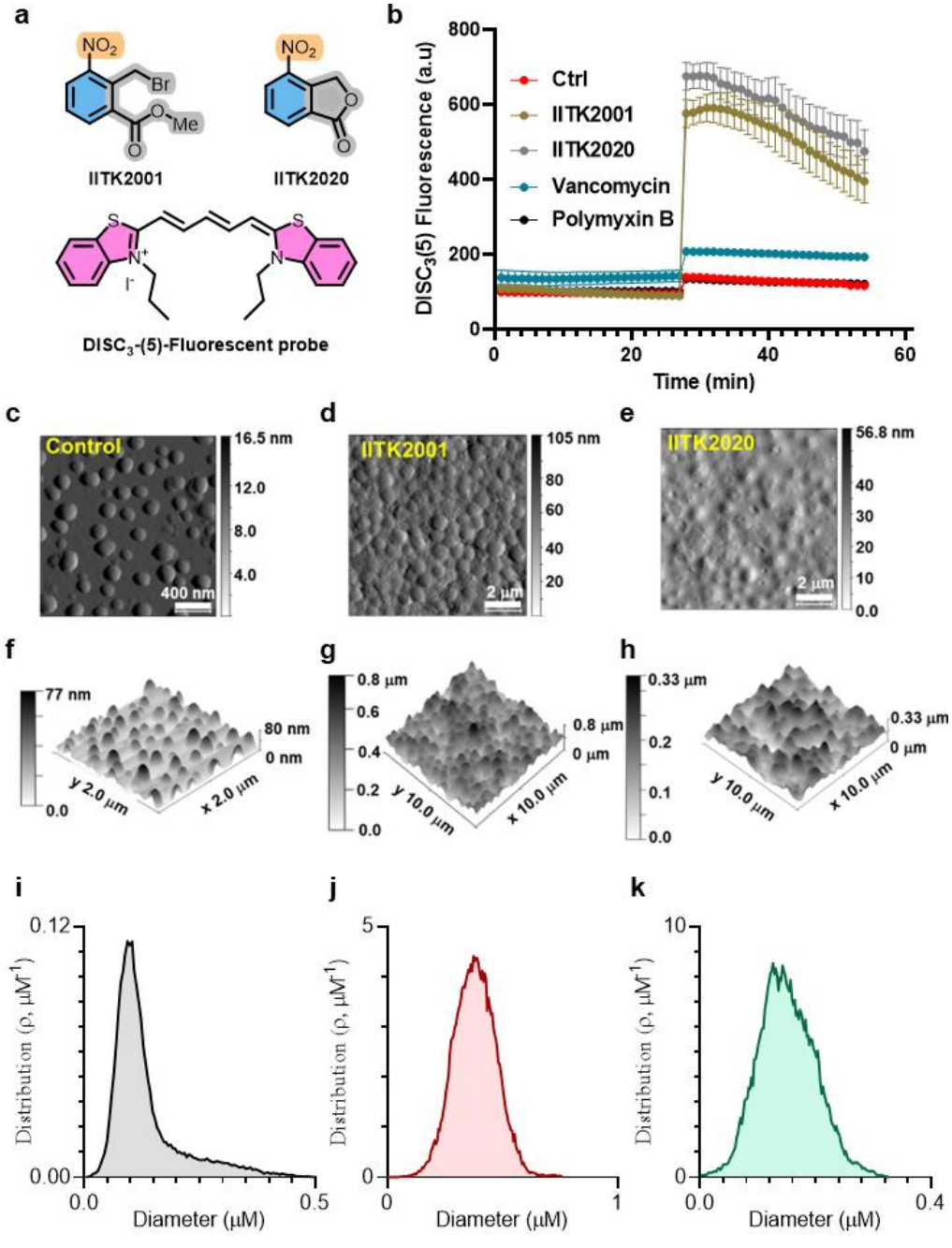
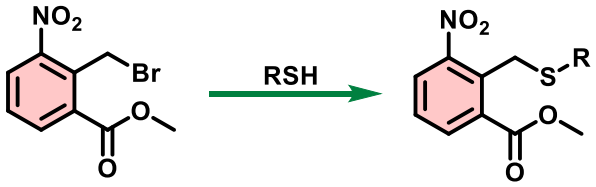
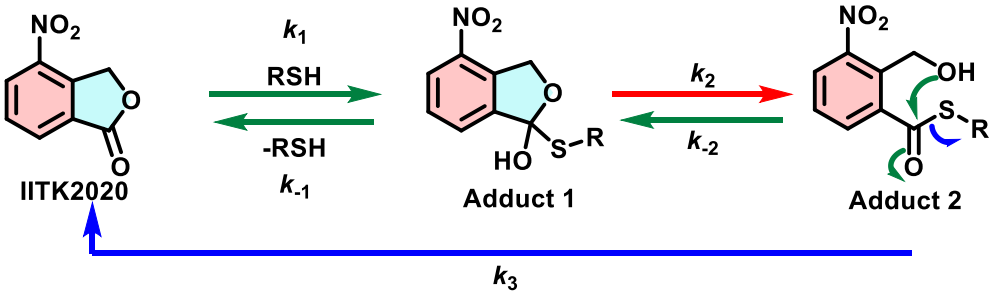


Figure S16. Schematics representing mechanism of action of our lead molecules

(A) **Scheme 1.** Thiol reaction with IITK2001 producing the irrerversibly alkylated modified thiol

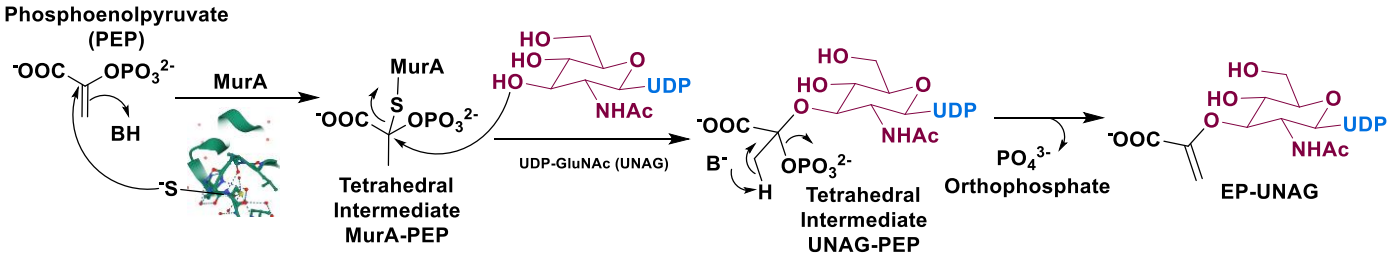


(B) **Scheme 2.** Thiol reaction with IITK2020 representing a reversible covalent modification mimicking tetrahedral intermediate, MurA-PEP

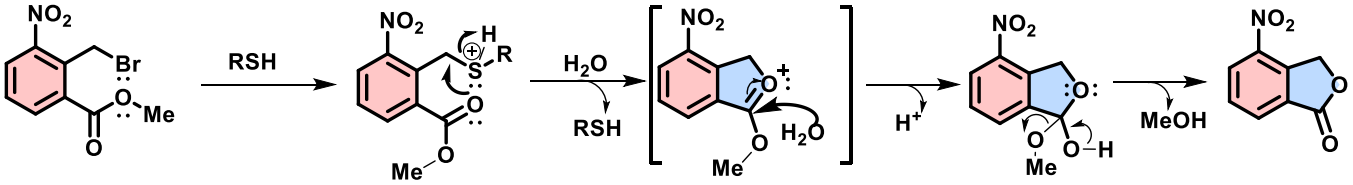


The molecule IITK2020 is stable with *N*-acetylcysteine (NAC) even after 24 h of exposure, understandably the lactone is quite stable, and we do not have a strong leaving group to make an irreversible covalent modification as in IITK2001. Instead, there is an electrophilic carbonyl-group in the lactone which has the potential to undergo an addition reaction with a nucleophile (k_1) to generate adduct 1, subsequent ring opening could generate adduct 2 (k_2) and produce a covalent modification. Due to the high reactivity of thioester, a reversible reaction to produce either the adduct 1 (k_{-2}) or IITK2020 (k_3) is very much possible.

(C) **Scheme 3.** Mechanism of MurA facilitating the synthesis of peptidoglycan precursor, UNAG-EP



(D) **Scheme 4.** Proposed mechanism of thiol-reactivity-driven lactonization of IITK2001 to IITK2020:



While IITK2001 underwent lactonization to produce IITK2020 (confirmed using ^{1}H-NMR, HPLC and mass spectrometry studies). We believe that the reactivity with thiol displacing bromide could make it a better leaving group, which facilitates the attack of lone pair from the methoxy-oxygen. Upon cyclization, the byproduct expected is methanol and we observed the peak at 3.12 ppm for CH_3- of methanol in the ^{1}H NMR when IITK2001 was exposed to NAC in DMSO-d_6 (Fig. S4).

Experimental Section: *In vitro* reactivity studies, cell biology experiments and molecular docking protocols:

Materials and Methods:

Growth media and Reagents

All bacterial media and supplements including Mueller-Hinton cation supplemented broth II (MHBII), Mueller-Hinton agar (MHA) and Tryptic soy broth (TSB) were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). All other chemicals and antibiotics were procured from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute Medium (RPMI) and Fetal Bovine Serum (FBS) were purchased from Lonza (Lonza, USA). All methods were performed in accordance with the relevant guidelines and regulations.

Bacterial strains

DSF was screened against a bacterial panel consisting of ESKAPE pathogens, namely Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 29213), Klebsiella pneumoniae (BAA-1705), Acinetobacter baumannii (BAA-1605), Pseudomonas aeruginosa (ATCC 27853) and Enterococcus sp. The panel was further expanded to include drug-resistant clinical S. aureus and Enterococci strains including those resistant to Vancomycin and other clinically-utilized antibiotics. These strains were procured from Biodefense and Emerging Infections Research Resources Repository/Network on Antimicrobial Resistance in Staphylococcus aureus/American Type Culture Collection (BEI/NARSA/ATCC, USA) and routinely cultivated on MHA and MHBII. Before starting the experiment, a single colony was picked from MHA plate, inoculated in MHBII and incubated overnight at 37 °C with shaking for 18–24 h to get the starter culture.

Experimental protocol for chemical and biological experiments:

Whole-cell bacterial viability screening for testing antibiotic susceptibility:

Antibiotic susceptibility testing of DSF was conducted according to the CLSI guidelines using the broth microdilution assay. 10 mg/mL stock solutions of test compounds were prepared in DMSO. Bacterial cultures were inoculated in MHBII and optical density (OD) was measured at 600nm, followed by dilution to achieve ~10^6 CFU/mL. The compounds were tested from 64–0.5 mg/L in two-fold serial diluted fashion with 2.5 µL of each concentration added to well of a 96-well round bottom microtiter plate. Later, 97.5 µL of bacterial suspension was added to each well containing either test compound or appropriate controls. The plates were incubated at 37°C for 18-24 h following which the MIC was determined. The MIC is defined as the lowest concentration of the compound at which there is absence of visible growth. For each test compound, MIC determinations were carried out independently three times using duplicate samples.[19]

Cell cytotoxicity

Cell toxicity was performed against Vero cells using the MTT as-say. ~10^3 cells/well were seeded in 96 well plate and incubated at 37°C in an 5% CO_2 atmosphere. After 24 h, compound was added ranging from 100-12.5 µg/mL concentration and incubated for 72 h. After the incubation was over, MTT was added in each well, incubated at 37°C for further 4 h, residual medium was discarded, 0.1 mL of DMSO was added to solubilize the formazan crystals and OD was taken at 540 nm for the calculation of CC_{50}. CC_{50} is de-fined as the lowest concentration of compound which leads to a 50% reduction in cell viability. Doxorubicin was used as positive control and each experiment was repeated in triplicate.[20]

Outer membrane (OM) permeability Assay

The MIC of compounds against E. coli ATCC 25922 and A. baumannii BAA-1605 was determined in presence of 10 mg/L Polymyxin N nonapeptide (PMBN).47

Reactivity of IITK2001 with amino acid nucleophiles using HPLC:

The stock solution of IITK2001 in DMSO and amino acid nucleophiles (*N*-acetylcysteine (NAC), *N*-propargyloxycarbonylserine (Ser), *N*-Cbz-lysinine (Lys), Methyl *N*-Boc-aspartiate (Asp) and *N*-Boc-tyrosine (Tyr)) in phosphate buffer-saline (PBS pH 7.4 buffer) or acetonitrile mixture (100 mM each) was prepared at RT. Individual purity of the sample was analysed prior to reactivity studies using HPLC experiments with the following method: A gradient mixture of water and acetonitrile possessing 0.1% trifluoroacetic acid was used as mobile phase. Method: 0 min to 15 min, 0.5 mL per min flow rate.

Time (minutes)	Acetonitrile (0.1% trifluoroacetic acid)	Water (0.1% trifluoroacetic acid)
5	50%	50%
5.10	10%	90%
8.00	10%	90%
8.10	10%	90%
12.00	10%	90%
12.10	50%	50%
15.00	50%	50%

The reactivity of IITK2001 with indicated amino acids for various time points were measured by mixing IITK2001 and amino acids at equal ratio (4 mM) in 1 :1 acetonitrile: buffer medium, then placed at 37 °C for mentioned time duration in duplicate. Area under the curve measured at 315 nm in HPLC was used as a measure of % IITK2001 remained unreacted with respect to the control conditions. The reaction mixture was analysed using High-resolution mass spectrometry. A similar protocol using the same method was used for assessing the reactivity of IITK2001a, IITK2008, IITK2010, IITK2011, IITK2018 and IITK2020 with cysteine nucleophile.

Monitoring the reactivity of IITK2001 with N-acetylcysteine using ^{1}H NMR experiments:

Stocks of the small molecules IITK2001 (10 mg in 0.6 mL DMSO-d_6), IITK2021 (10 mg in 0.6 mL DMSO-d6), and N-acetylcysteine (1 equivalent of IITK2001, 5.95 mg in 0.6 mL DMSO-d6) was prepared and ^{1}H NMR spectra (JEOL ECH-400 MHz) were recorded for all of them. From those NMR solutions, 0.3 mL of IITK2001, 0.3 mL of N-acetylcysteine mixed and exposed to oven dried potassium carbonate (2 equivalents of IITK2001, 10.1 mg) in a

microcentrifuge tube and vortexed till 15 counts before incubating at 37 °C in water bath. After 3 hours of incubation ^{1}H NMR was recorded for the reaction mixture, in parallel, HRMS data was recorded for the same reaction mixture. The NMR sample also analyzed using HPLC using the method described above.

Metabolism of selected bromomethylbenzoate esters in *Staphylococcus aureus* using HPLC:

To a 990 µL of Staphylococcus aureus bacteria (1.0 OD in nutrient broth) in a 1.5 mL microcentrifuge tube, 10 µL of small molecule (IITK2001, IITK2008, IITK2010 and IITK2011; 100 mM stock in molecular biology grade DMSO) was added to get a final concentration of 1 mM concentration. Then, the bacterial suspension was incubated at 37 °C in water bath for 60 minutes and centrifuged to collect the bacterial pellet (for 10 minutes at 20,000g, Thermo Scientific legend 21R centrifuge). The cell pellet was resuspended in 150 µL of 1X phosphate buffer saline (pH = 7.4), lysed using a probe sonicator (Labman PRO-650; (1 minute at 5% power rate with 2 sec pulse on and 1 sec pulse off time) and centrifuged for 10 minutes at 20,000g to collect the clarified cell lysate. The lysate collected was half diluted with acetonitrile and directly taken for HPLC analysis using the method discussed above.

^{31}P-NMR studies for MurA activity with PEP, UNAG, IITK2001 and IITK2020:

Solutions of Phosphoenolpyruvate sodium salt (PEP, 1.6 mM), potassium phosphate (1.6 mM, K_3PO_4), UDP-N-acetylglucosamine (UNAG, 1.2 mM) were prepared in HEPES buffer (50 mM in water, pH 7.4). ^{31}P-NMR of individual components in the presence of MurA (20 µL of 0.5 mg/mL solution in ddH_2O) was recorded. PEP solution was also spiked with 1 equiv. of K_3PO_4 to mark the chemical shift of monophosphate in ^{31}P-NMR spectra. To a mixture of UNAG (250 µL) and PEP (250 µL), MurA (20 µL of 0.5 mg/mL solution) was added at RT and incubated for 1 h at 37 °C and ^{31}P-NMR spectrum was taken. Under comparable conditions, MurA pretreated with IITK2001 or IITK2020 for 1 h was incubated with PEP+UNAG mixture for 1 h as mentioned above and 31P-NMR spectra were recorded.

In-gel fluorescence assay protocol for experiments with recombinant proteins:

For identifying the interaction between MurA or MurZ with compounds, 0.23 μg MurA or 0.22 μg MurZ protein (20 μL protein + 5μL 1X PBS) was aliquoted in micro centrifuge tubes followed by addition of IITK2001 or IITK2020 at 10 μM, 25 μM, 50 μM and 100 μM or DMSO (1%) as negative control or Fosfomycin (170 μM) final concentration as positive control and 400 μM TCEP was added to all samples. Reaction was performed for 6 hours at 37 °C in water bath, then, 10 μM CAA was added and incubated for 1 hour at 37 °C in water bath. Following that, a click reaction mixture of 4.25 μL consisting of 1.25 μL TBTA (1 mM in 1:4 DMSO: tert-Butyl alcohol), 1.5 μL SDS (20% in dH2O), 0.5 μL TCEP (50 mM in dH2O), 0.5 μL CuSO4 (50 mM in dH_2O) and 0.5 μL Aide fluor 545 (5 mM in DMSO) and reaction was incubated for 1 hour at RT in dark. The reactions were quenched by adding 12.6 μL of 5X protein loading dye and tubes were incubated for 10 minutes at RT in dark. Samples were resolved on a 4-12% SDS-PAGE gel at 145 V for 80 minutes. Gel was visualized using Alexa 448 filter in chemidoc (ChemiDoc™ Touch Imaging System from Bio-Rad). Gels were stained with gel staining solution (0.5% w/v brilliant blue R-250 dissolved in 45% v/v ethanol and 10% v/v glacial acetic acid in dH2O) and destained with destaining solution (10% glacial acetic acid in dH2O). Gel image was captured using chemidoc instrument.

In-gel fluorescence analysis experiment with *S. aureus* lysate:

Staphylococcus aureus culture at OD ~2 (5mL in 2 falcon tubes) was centrifuged at 10,000g for 10 minutes at RT and 500 μL 1X protease phosphatase inhibitor (Thermo Scientific) was added to pellet in each tube. Samples were sonicated (2 seconds on and 1 second off; 5% power and 20 cycles 4 times) on ice using probe sonicator (Labman). Samples were centrifuged at 20,000g for 10 minutes at 4 °C and lysates were collected in fresh microcentrifuge tube. Protein concentration was quantified with Bradford assay using Bradford reagent (SRL) and BSA as standard. Protein lysate (3.12 μL) corresponding to (0.6 mg) protein was incubated with IITK2001 or IITK2020 at 10 □M, 25 μM, 50 μM and 100 μM or DMSO (1%) as negative control or Fosfomycin (170 μM) final concentration as positive control and 400 μM TCEP was added to all samples. To SA samples 100 μM CAA and to MurZ samples 10 μM CAA was added SA samples were incubated for 3 hours in a water bath. Following that, a click reaction as described for 1 hour at RT in dark. The reactions were quenched by adding 12.6 μL of 5X protein loading dye and tubes were

incubated for 10 minutes at RT in dark. Samples were resolved on a 4-12% SDS-PAGE gel at 145 V for 80 minutes. Gel was visualized using Alexa 448 filter in chemidoc (ChemiDoc™ Touch Imaging System from Bio-Rad). Gels were stained with gel staining solution (0.5% w/v brilliant blue R-250 dissolved in 45% v/v ethanol and 10% v/v glacial acetic acid in dH_2O) and destained with destaining solution (10% glacial acetic acid in dH_2O). Gel image was captured using chemidoc in-strument.

Membrane depolarization assay in *S. aureus* after treatment with IITK2001 and IITK2020:

In a microcentrifuge tube, 1 mL bacterial solution of *Staphylococcus aureus* (1 OD) was centrifuged for 10 minutes at 20,000g (Thermo Scientific legend 21R centrifuge). The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL of 10 mM phosphate buffer saline (pH=7.4). From this, 800 µL of the bacterial suspension was taken and added with EDTA (pH 8.0) at a final concentration of 0.25 mM. After 10 minutes of incubation at RT, a final concentration of 0.4 µM of $DISC_3$-(5) dye was added to the solution and 160 µL/well was distributed in a 96 well plate. Fluorescence was recorded with 1 minute interval for 30 iterations (λ_{ex} 627 nm, λ_{em} 660-720 nm) using microplate reader (Promega GloMax Explorer). To the 96-well plate, 40 µl/ well of potassium chloride (200 µM) was added, followed by. 1 µL of small molecules from 10 mM stock solution was added to each condition (a final concentration of 50 µM) and the fluorescence emission was recorded for 30 min with 1 minute time (λ_{ex} 627 nm, λ_{em} 660-720 nm) using a microplate reader.

Atomic Force Microscopy Imaging of *S. aureus* treated with IITK2001/IITK2020:

For bacterial suspension (control): In a microcentrifuge tube, 1 mL bacterial solution of Staphylococcus aureus (1 OD) was centrifuged for 10 minutes at 20,000g. The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL of deionized distilled water. 100 µL droplet of the bacterial suspension was applied on a clean glass slide and dried in vacuum oven for 45 minutes at 25 °C. The control was imaged just after drying the samples. For bacterial suspension with small molecule incubation: To the S. aureus suspension (1 OD) in nutrient broth, 5 µL of small molecule (10 mM stock) to get 50 µM final concentration was added and incubated at 37 °C in water bath for 12 h. The cells were

centrifuged for 10 minutes at 20,000g. The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL of deionized water. 100 µL droplet of the suspension was applied on a clean glass slide and dried in vacuum oven for 45 minutes at 25 °C. The AFM images were taken just after drying the samples.

2.3: Molecular docking experiments for IITK2001, IITK2020 and benzothioxazolinone (Bzt) with S. aureus Mura and MurZ:

2.3.1: Homology Modelling:

Homology models of the *S. aureus* MurA and MurZ were generated using the SWISS-MODEL Interactive Workspace developed by the Swiss Institute of Bioinformatics, Switzerland (10.1038/nprot.2008.197). The target protein sequences of *S. aureus* MurA and MurZ were retrieved from UniProt KB (A0A0E8J2T6_STAAU for MurA with 421 aa and W8TUM1_STAAU for MurZ with 419 aa). For the model generation, crystal structure of *E. coli* MurA (PDB Code: 1UAE) was used as the template which had 49.40% and 42.03% similarity with *S. aureus* MurA and MurZ respectively.

2.3.2: Molecular Docking

Molecular docking studies of the ligands **IITK2001**, **IITK2020** and Bzt (Ref ligand) alongside Fosfomycin were performed using Schrodinger Drug Discovery Suite v2020-1. The modelled protein structures of S. aureus MurA and MurZ as well as E. coli MurA (PDB Code: 1UAE) were pre-processed using the Protein Preparation Wizard and optimized to pH 7.4, followed by minimization. Prior to molecular docking, all the ligands were subjected to energy minimization using LigPrep module, while also generating their possible states at pH 7.4. These molecules were then covalently docked into the active sites using the CovDock module of Schrodinger.[21] For receptor grid generation, active site Cysteines (Cys115 for E. coli MurA, Cys119 and Cys118 for S. aureus MurA and MurZ, respectively) were picked as the reactive residues, while a ligand centroid grid was generated with grid radius of 15 Å. The reaction types for covalent docking varied with the ligand i.e., Nucleophilic substitution for IITK2001 (Lactone_Open) and Epoxide Opening for Fosfomycin, while custom cdock files were generated to define the reaction types for IITK2020 and Bzt (Supplementary info, given below)

For IITK2020,

RECEPTOR_SMARTS_PATTERN 2,[C,c]-[S,O;H1,-1] (denotes Cys Sulfur as the nucleophilic reactive centre)

LIGAND_SMARTS_PATTERN 2,O=C1ccCO1 (denotes lactone's carbonyl carbon as electrophilic reactive centre)

CUSTOM_CHEMISTRY ("<1>|<2>",("bond",1,(1,2))) (to make bond between between the reactive centres of protein and ligand)

CUSTOM_CHEMISTRY ("<2>[O]",("bond",-1,(1,2))) (to break bond between electrophilic carbon and its adjacent sp3 oxygen)

For Bzt,

RECEPTOR_SMARTS_PATTERN 2,[C,c]-[S,O;H1,-1] (denotes Cys Sulfur as the nucleophilic reactive centre)

LIGAND_SMARTS_PATTERN 2,[s]c=O (denotes carbonyl carbon as electrophilic reactive centre)

CUSTOM_CHEMISTRY ("<2>[s]",("bond",-1,(1,2))) (to break C-S bond in the ligand)

CUSTOM_CHEMISTRY ("<1>|<2>",("bond",1,(1,2))) (to make C-S bond between ligand and Cysteine).

2.4: Biochemical experiments to assess the interaction of IITK2001 and IITK2020 with *S. aureus* MurA and MurZ:

2.4.1: Expression and purification of recombinant *S. aureus* MurA and MurZ:

Purification of MurA protein:

The gene encoding MurA and MurZ protein was amplified from the DNA of *S. aureus* (ATCC 29213). The gene-specific primers with unique restriction sites (NcoI and HindIII highlighted in bold) were used to amplify the target gene:

Primers for MurA:

Forward: 5'- accCCATGGCCGATAAAATAGTAATCAAAGG 3'

Reverse: 5'- accCTCGAGATCGTTAATACGTTCAATGTCTG 3')

Primers for MurZ:

Forward: 5'- accCCATGGCCGCTCAAGAGGTAATAAAAATAAG 3'

Reverse: 5'- accCTCGAGTACAGTTTCCGTCCAAATATC 3'

The amplicon was ligated into pET28a vector and transformed into *E.coli* DH5α competent cells. The clone was confirmed by restriction digestion and sequencing. The confirmed clone was transformed into *E.coli* Rosetta cells. The recombinant protein (MurA with C-terminal 6X His tagged) expression in *E.coli* cells were induced by 0.8mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) when the OD of the culture was OD600~0.6-0.7 for 4 h at 37 °C. 2L culture of cells were pelleted and lysed in lysis buffer (25 mM Tris-Cl, 150 mM NaCl, 10 mM Imidazole, pH-8) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by sonication (15 sec on, 30 sec off, 35% amplitude 24 °C). The cell debris were separated by centrifugation. The supernatant was filtered and loaded on to pre-equilibrated 5mL Hi-Trap Ni-NTA column (Cytiva). The elution was done in imidazole gradient from 20-500 mM in 20 m. The protein containing fractions were pooled and loaded into pre-equilibrated (Buffer- 25 mM Tris-Cl, 100 mM NaCl, 1 mM DTT, pH-8) 5 mL Fast Flow Q sepharose anion exchange column (Cytiva). The elution was done in NaCl gradient from 100 mM-1 M NaCl in 30 mL. The fractions containing protein were pooled and concentrated and loaded onto Superdex 200 10/300 size exclusion column (GE Healthcare). The purified protein was stored in 25 mM Tris-Cl, 100 mM NaCl, 5% glycerol, pH-8 at -80 °C until further use. The proteins were analysed on 12% SDS-PAGE gels at each step for purity.

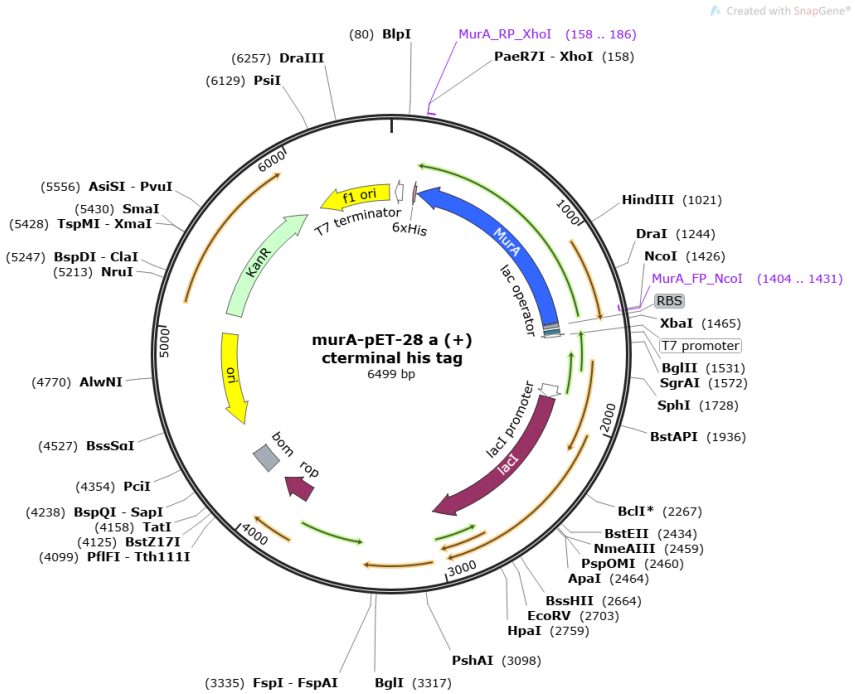
Table 2.4.1a: Bacterial strains and plasmid used in the study

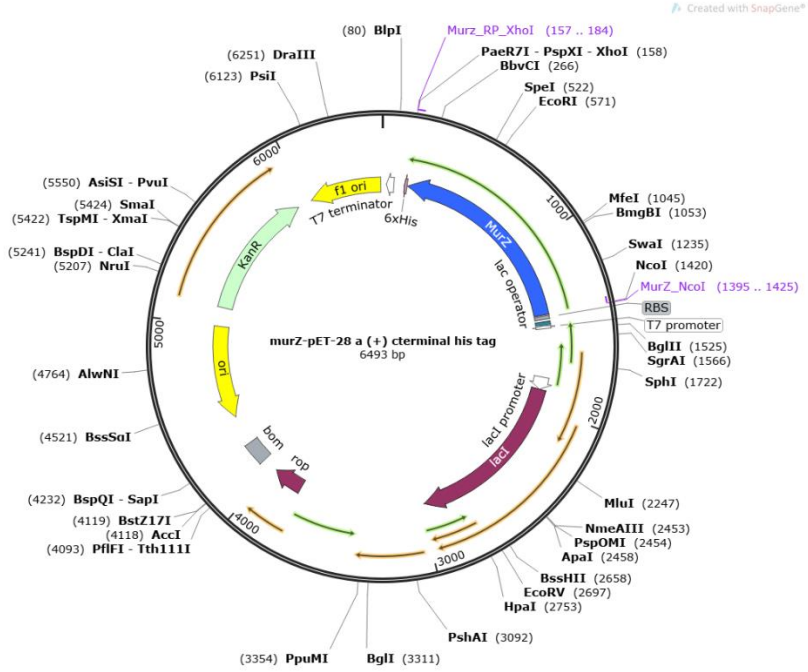
Name	**Characteristics**
Bacterial Strains	
E.coli DH5α	F– φ80lacZΔ M15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK– mK+) phoA supE44 λ- thi–1 gyrA96 relA1
E.coli Rosetta	F- ompT hsdSB(rB- mB-) gal dcm pRARE (CamR))
Plasmids	
pET28a(+)	Expression vector for low copy number (pBR322 ori), strong phage promoter (T7), IPTG induction (lac operon), and Kanamycin selection (Kan r)

Table 2.4.1b: Primers used in the study

S.No.	Protein	Primer used	Tag
1	MurA	FP- 5' acc**CCATGG**CCGATAAAATAGTAATCAAAGG 3'	6X-His Tag (C-terminal)
		RP- 5' acc**CTCGAG**ATCGTTAATACGTTCAATGTCTG 3'	
2	MurZ	FP-5' acc**CCATGG**CCGCTCAAGAGGTAATAAAAATAAG 3'	6X-His Tag (C-terminal)
		RP- 5' acc**CTCGAG**TACAGTTTCCGTCCAAATATC 3'	

2.4.1c: Vector Maps:

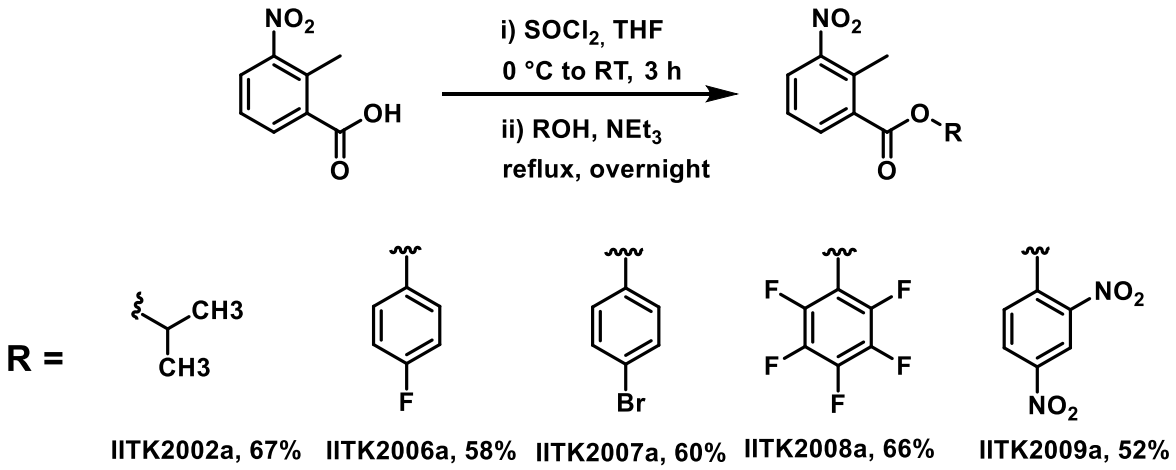




4. Experimental Section: Synthesis and characterization

3. 1: General protocol for phenolic and allylic ester derivative preparation:

Reaction Scheme:

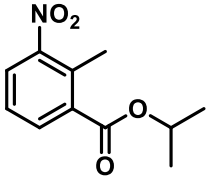


General protocol:

To an ice-cold solution of 2-methyl-3-nitrobenzoic acid (3 g) in dry THF (30 mL), thionyl chloride (1.5 equivalent, 1.85 mL) was added dropwise. The reaction mixture was warmed to RT and stirred for 3 h. Consumption of the starting material was followed by thin layer chromatography (TLC). From the reaction mixture, 4 mL/ vial (2 mmol, 100 mg/mL) was distributed into oven-dried glass vials, then 3 equiv. of (6 mmol) alcohols/phenols and triethylamine (3 equiv, 6 mmol) were added at 0 °C. Then the reaction mixture was stirred overnight at 70 °C. After complete consumption of the starting material recorded by TLC, the reaction mixture was evaporated to dryness and the crude material was directly taken for purification by silica gel column chromatography.

Experimental data:

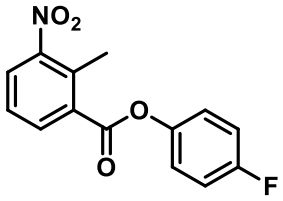
Isopropyl 2-methyl-3-nitrobenzoate (IITK2002a).



Following the general protocol IITK2010a was synthesized and purified using silica gel column chromatography (Solvent System: 10% ethyl acetate in Hexane) in 67% yield (328 mg, R_f = 0.7

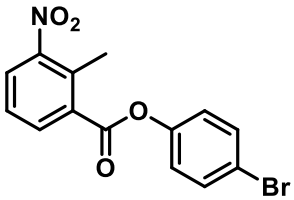
(20% EtOAc: Hexane)) as transparent liquid**.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, *J* = 7.6 Hz, 1H), 7.83 – 7.77 (m, 1H), 7.35 (t, *J* = 7.9 Hz, 1H), 5.25 (dt, *J* = 12.7, 6.3 Hz, 1H), 2.59 (s, 3H), 1.36 (d, *J* = 6.2 Hz, 6H), ^{13}C NMR (100 MHz, CHLOROFORM-D) δ 165.2, 150.5, 134.6, 133.7, 132.4, 129.1, 127.6, 70.4, 29.8, 22.8, 21.8. GCMS(APCI) for $[C_{11}H_{13}NO_4+H]^+$: calcd.,224.0917. Found: 224.0907.

4-Fluorophenyl 2-methyl-3-nitrobenzoate (IITK2006a).



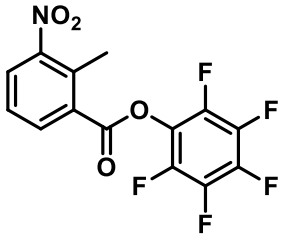
Following the general protocol IITK2013a was synthesized and purified using silica gel column chromatography (Solvent System: 2-3% ethyl acetate in Hexane) in 58% yield (352 mg, R_f = 0.7 (20% EtOAc: Hexane)) as white solid**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.91 (dd, *J* = 8.0, 1.1 Hz, 1H), 7.47 (t, *J* = 8.0 Hz, 1H), 7.18 (ddd, *J* = 10.3, 5.1, 2.9 Hz, 2H), 7.15 – 7.10 (m, 2H), 2.70 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164, 155, 150, 138, 146, 134, 133, 131, 129, 128, 123, 117, 22. GCMS(APCI) for $[C_{14}H_{10}FNO_4+H]^+$: calcd.,276.0667. Found: 276.0660.

4-Bromophenyl 2-methyl-3-nitrobenzoate (IITK2007a).



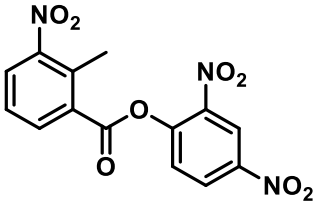
Following the general protocol IITK2013a was synthesized and purified using silica gel column chromatography (Solvent System: 2% ethyl acetate in Hexane) in 60% yield (445 mg, R_f = 0.8 (20% EtOAc: Hexane)) as white solid**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (dd, *J* = 7.9, 1.0 Hz, 1H), 7.91 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.59 – 7.53 (m, 2H), 7.47 (t, *J* = 7.9 Hz, 1H), 7.13 – 7.09 (m, 2H), 2.70 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.3, 152.4, 149.6, 134.2, 132.8, 131.9, 127.6, 126.7, 123.4, 119.6, 16.3. GCMS(APCI) for $[C_{14}H_{10}BrNO_4+H]^+$: calcd.,335.9866. Found: 335.9865.

Pentafluorophenyl 2-methyl-3-nitrobenzoate (IITK2008a).



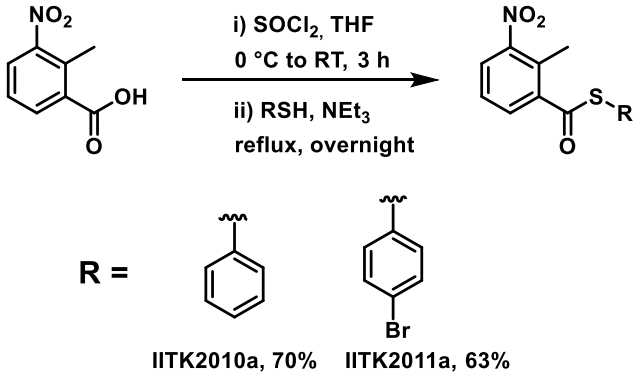
Following the general protocol IITK2010a was synthesized and purified using silica gel column chromatography (Solvent System: 2% ethyl acetate in Hexane) in 66% yield (352 mg, R_f = 0.8 (20% EtOAc: Hexane)) as white solid**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.28 (dd, *J* = 7.6, 0.8 Hz, 1H), 7.97 (dd, *J* = 8.0, 0.8 Hz, 1H), 7.52 (t, *J* = 8.0 Hz, 1H), 2.70 (s, 3H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.9, 152.4, 142.6, 141.1, 139.3, 138.6, 136.7, 134.9, 134.5, 129.8, 128.5, 127.0, 16.3. GCMS(APCI) for $[C_{14}H_6F_5NO_4+H]^+$: calcd.,348.0290. Found: 348.0298.

2,4-Dinitrophenyl 2-methyl-3-nitrobenzoate (IITK2009a).



Following the general protocol IITK2010a was synthesized and purified using silica gel column chromatography (Solvent System: 10% ethyl acetate in Hexane) in 52% yield (402 mg, R_f = 0.4 (20% EtOAc: Hexane)) as yellow solid**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.05 (d, *J* = 2.7 Hz, 1H), 8.60 (dd, *J* = 8.9, 2.7 Hz, 1H), 8.36 (d, *J* = 7.9 Hz, 1H), 7.98 (d, *J* = 8.0 Hz, 1H), 7.62 (d, *J* = 8.9 Hz, 1H), 7.54 (t, *J* = 8.0 Hz, 1H), 2.70 (s, 3H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.5, 152.5, 148.4, 145.5, 141.7, 135.2, 134.8, 129.8, 129.4, 128.6, 127.1, 126.8, 122.1, 16.2. GCMS(APCI) for $[C_{14}H_9N_3O_8+H]^+$: calcd.,348.0462. Found: 348.0458.

General procedure for thiophenol ester derivatives preparation:

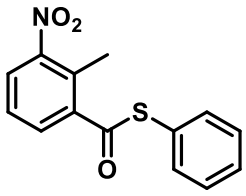


General protocol:

In an oven-dried glass vial, 2-(bromomethyl)-3-nitrobenzoyl chloride (4 mL of 100 mg/mL, 2 mmol) solution in THF was taken, then thiophenol (3 equivalent, 6.01 mmol) was added at ice-cold condition. After 2 minutes over ice, triethylamine (3 equivalent, 6.01 mmol) was added dropwise, then the reaction mixture moved to 70 °C-oil bath and the stirring was continued overnight. Consumption of the starting material was followed by TLC, upon near-complete consumption of the starting material, the reaction mixture was evaporated to dryness and the crude material was directly taken for purification by silica gel column chromatography. A similar protocol was followed for 4-bropmothiophenol substrate.

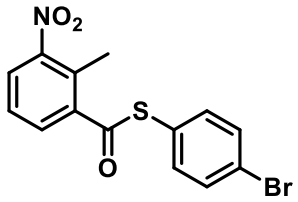
Experimental data:

S-phenyl 2-methyl-3-nitrobenzothioate (IITK2010a).



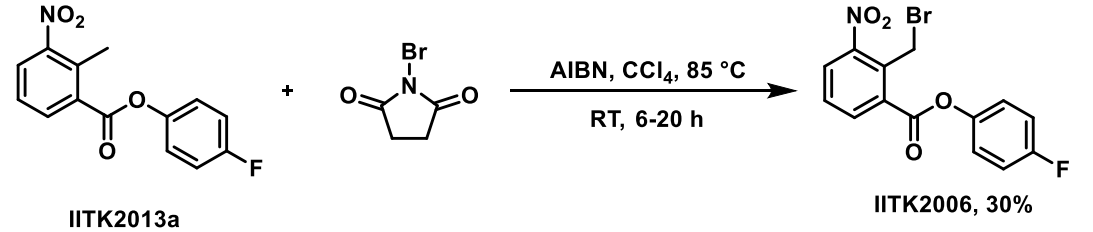
Following the general protocol IITK2010a was synthesized and purified using silica gel column chromatography (Solvent System: 1% ethyl acetate in Hexane) in 70% yield (423 mg, R_f = 0.8 (20% EtOAc: Hexane)) as white solid**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.97 – 7.88 (m, 4H), 7.55 – 7.41 (m, 13H), 2.55 (s, 7H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 151.5, 140.8, 134.7, 131.4, 130.8, 130.1, 129.6, 127.1, 126.8, 126.7, 15.9. GCMS(APCI) for $[C_{14}H_{11}NO_3S+H]^+$: calcd.,274.0532. Found: 274.0539.

S-(4-bromophenyl) 2-methyl-3-nitrobenzothioate (IITK2011a).



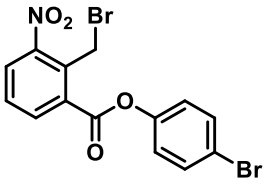
Following the general protocol IITK2010a was synthesized and purified using silica gel column chromatography (Solvent System: 2% ethyl acetate in Hexane) in 63% yield (488 mg, R_f = 0.7 (20% EtOAc: Hexane)) as off-white solid**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92 (t, *J* = 8.3 Hz, 2H), 7.64 – 7.59 (m, 2H), 7.45 (t, *J* = 8.0 Hz, 1H), 7.40 – 7.35 (m, 2H), 2.54 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.2, 151.6, 140.4, 136.1, 132.8, 131.4, 130.9, 126.9, 126.2, 124.9, 15.9. GCMS(APCI) for $[C_{14}H_{10}BrNO_3S+H]^+$: calcd.,351.9638. Found: 351.9640.

General protocol for bromination of 2-methyl-3-nitrobenzoate ester derivatives:

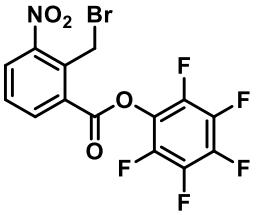


IITK2013a

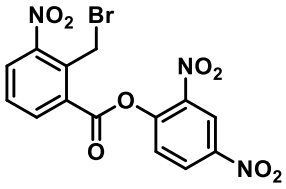
IITK2006, 30%



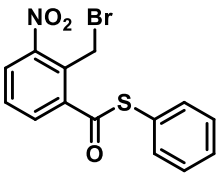
IITK2007, 25%



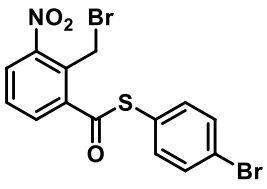
IITK2008, 30%



IITK2009, 21%



IITK2010, 27%



IITK2011, 26%

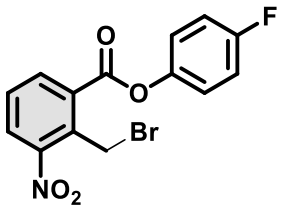
General protocol:

A solution of ester precursors (1. equiv) in dry CCl_4 was taken in oven dried glass vials, to them *N*-bromosuccinimide (1.1 equivalent) and Azobisisobutyronitrile (AIBN) (0.5 equivalent) were added at room temperature under inert atmosphere. Then the vials were moved to 85 °C oil bath

and stirred for 6 h, TLC analysis showed the remaining of ester starting materials, therefore, an additional equivalent of NBS and 25 mol% of AIBN were added to the reaction mixture and the stirring continued overnight. Although the conversion of starting material improved, a significant amount (~50%) of starting material remained unreacted in each case. Therefore, the crude reaction mixtures were evaporated and taken for column purification.

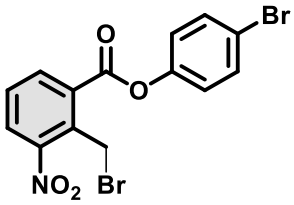
Experimental data:

4-fluorophenyl 2-(bromomethyl)-3-nitrobenzoate (IITK2006).



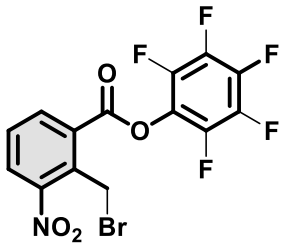
Following the general protocol IITK2006 was synthesized and purified using silica gel column chromatography (Solvent System: 3% ethyl acetate in Hexane) in 30% yield (32 mg, R_f = 0.6 (20% EtOAc: Hexane)) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (dd, *J* = 7.7, 1.1 Hz, 1H), 8.01 (dd, *J* = 8.2, 1.2 Hz, 1H), 7.61 (t, *J* = 8.0 Hz, 1H), 7.26 – 7.18 (m, 2H), 7.14 (t, *J* = 8.6 Hz, 2H), 5.17 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.1, 161.7, 159.8, 150.9, 146.2, 135.1, 133.4, 131.7, 129.4, 128.5, 123.1, 123.0, 116.6, 116.4, 22.6. HRMS(ESI) for $[C_{14}H_9BrFNO_4+Na]^+$: calcd.,375.9591. Found: 375.0829.

4-bromophenyl 2-(bromomethyl)-3-nitrobenzoate (IITK2007).



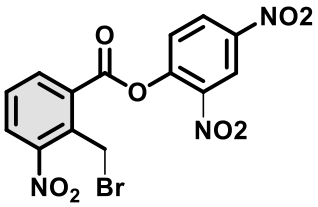
Following the general protocol IITK2007 was synthesized and purified using silica gel column chromatography (Solvent System: 3% ethyl acetate in Hexane) in 25% yield (33 mg, R_f = 0.7 (20% EtOAc: Hexane)) as white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.30 (dd, *J* = 7.9, 1.0 Hz, 1H), 8.01 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.67 – 7.52 (m, 3H), 7.19 – 7.11 (m, 2H), 5.17 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.7, 150.8, 149.4, 135.1, 133.4, 132.9, 131.5, 129.5, 128.6, 123.4, 119.9, 22.5. HRMS(ESI) for $[C_{14}H_9Br_2NO_4+H]^+$: calcd.,415.8951. Found: 415.2107

Pentafluorophenyl 2-(bromomethyl)-3-nitrobenzoate (IITK2008).



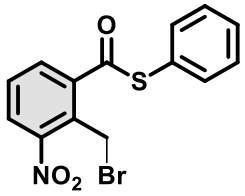
Following the general protocol IITK2008 was synthesized and purified using silica gel column chromatography (Solvent System: 2% ethyl acetate in Hexane) in 30% yield (37 mg, R_f = 0.7 (20% EtOAc: Hexane)) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (dd, *J* = 7.9, 1.0 Hz, 1H), 8.09 (dd, *J* = 8.4, 0.9 Hz, 1H), 7.67 (t, *J* = 8.0 Hz, 1H), 5.12 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161, 150, 135, 134, 129, 129, 128, 21. ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.2, 150.9, 142.3, 140.3, 139.0, 135.5, 134.4, 133.7, 129.7, 129.6, 128.9, 21.8. HRMS(ESI) for $[C_{14}H_5BrF_5NO_4+Na]^+$: calcd.,447.9214. Found: 447.0831.

2,4-dinitrophenyl 2-(bromomethyl)-3-nitrobenzoate (IITK2009).



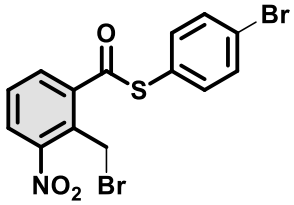
Following the general protocol IITK2009 was synthesized and purified using silica gel column chromatography (Solvent System: 6-15% ethyl acetate in Hexane) in 21% yield (26 mg, R_f = 0.3 (20% EtOAc: Hexane)) as yellow solid. ^{1}H NMR (500 MHz) δ 9.02 (d, J = 2.7 Hz), 8.57 (dd, J = 8.9, 2.7 Hz), 8.37 (dd, J = 7.9, 1.3 Hz), 8.04 (dd, J = 8.2, 1.3 Hz), 7.64 (dd, J = 12.6, 4.7 Hz), 5.08 (s). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.5, 152.5, 148.4, 145.5, 141.7, 135.2, 134.8, 129.8, 129.4, 128.6, 127.1, 126.8, 122.1, 16.1. GCMS(APCI) for $[C_{14}H_8BrN_3O_8+H]^+$: calcd.,425.9568. Found: 425.9561.

S-phenyl 2-(bromomethyl)-3-nitrobenzothioate (IITK2010).



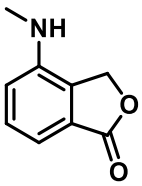
Following the general protocol IITK2009 was synthesized and purified using silica gel column chromatography (Solvent System: 3-4% ethyl acetate in Hexane) in 27% yield (35 mg, R_f = 0.6 (20% EtOAc: Hexane)) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 – 7.96 (m, 2H), 7.64 – 7.42 (m, 6H), 4.93 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.7, 150.3, 140.0, 134.8, 132.4, 130.4, 130.3, 129.7, 129.5, 127.7, 126.8, 22.3. GCMS(APCI) for $[C_{14}H_{10}BrNO_3S+H]^+$: calcd.,351.9638. Found: 351.9617.

S-(4-bromophenyl) 2-(bromomethyl)-3-nitrobenzothioate (IITK2011).



Following the general protocol IITK2009 was synthesized and purified using silica gel column chromatography (Solvent System: 3% ethyl acetate in Hexane) in 26% yield (32 mg, R_f = 0.6 (20% EtOAc: Hexane)) as off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (dd, *J* = 11.1, 8.1 Hz, 2H), 7.60 (dd, *J* = 16.3, 8.2 Hz, 3H), 7.40 (d, *J* = 8.4 Hz, 2H), 4.92 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.0, 150.3, 139.6, 136.2, 132.9, 132.3, 130.5, 129.6, 127.9, 125.8, 125.2, 22.2. GCMS(APCI) for $[C_{14}H_9Br_2NO_3S+H]^+$: calcd.,431.8722. Found: 431.8659.

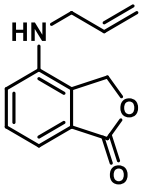
Synthesis of 4-(methylamino)isobenzofuran-1(3H)-one (IITK2028).



To a solution of 4-aminoisobenzofuran-1(3H)-one (737.5 µmol, 110 mg) in dry DMF, potassium carbonate (4.43 mmol, 6 equiv., 612 mg) and methyl iodide (1.11 mmol, 1.5 equiv., 70 µL) was added and the mixture was left for 18 h stirring at 80°C. After 18 h TLC showed complete

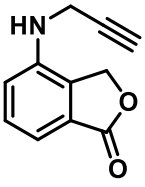
conversion of starting material. Reaction mixture was concentrated under reduced pressure to remove maximum amount of DMF and then compound was purified by column chromatography (Solvent System: 16-18% ethyl acetate in hexane) in 66% yield (75 mg, R_f = 0.4 (40% EtOAc: Hexane)) as white solid**.** ^{1}H NMR (500 MHz) δ 7.39 (d, *J* = 7.7 Hz), 7.26 (d, *J* = 7.5 Hz), 6.82 (d, *J* = 7.9 Hz), 5.14 (s), 2.94 (s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.0, 143.7, 131.5, 130.1, 126.1, 114.0, 113.8, 68.0, 30.6. GCMS(APCI) for $[C_9H_9NO_2+H]^+$: calcd.,164.0706. Found: 164.0709.

Synthesis of 4-(allylamino)isobenzofuran-1(3H)-one (IITK2029).



To a solution of 4-aminoisobenzofuran-1(3H)-one (737.5 µmol, 110 mg) in dry DMF, potassium carbonate (4.43 mmol, 6 equiv., 612 mg) and allyl bromide (1.11 mmol, 1.5 equiv., 100 µL) was added and the mixture was left for 18 h stirring at 80°C. After 18 h TLC showed complete conversion of starting material. Reaction mixture was concentrated under reduced pressure to remove maximum amount of DMF and then compound was purified by column chromatography (Solvent System: 3-4% ethyl acetate in hexane) in 70% yield (102 mg, R_f = 0.6 (20% EtOAc: Hexane)) as white solid**.** ^{1}H NMR (500 MHz) δ 7.36 (t, *J* = 7.8 Hz), 7.26 (d, *J* = 7.3 Hz), 6.83 (d, *J* = 7.9 Hz), 5.93 (m), 5.29 (dd, *J* = 17.2, 1.4 Hz), 5.21 (dd, *J* = 10.3, 1.3 Hz), 5.16 (s), 3.87 (dt, *J* = 5.3, 1.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9, 142.5, 134.3, 131.5, 130.6, 126.3, 117.2, 115.0, 114.2, 68.0, 46.3. GCMS(APCI) for $[C_{11}H_{11}NO_2+H]^+$: calcd.,190.0863. Found: 190.0863.

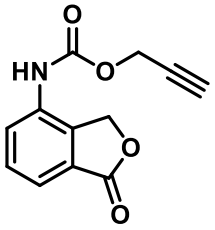
Synthesis of 4-(prop-2-yn-1-ylamino)isobenzofuran-1(3H)-one (IITK2030).



To a solution of 4-aminoisobenzofuran-1(3H)-one (737.5 µmol, 110 mg) in dry DMF, potassium carbonate (4.43 mmol, 6 equiv., 612 mg) and propargyl bromide (1.11 mmol, 1.5 equiv., 90 µL) was added and the mixture was left for 18 h stirring at 80°C. After 18 h TLC showed complete conversion of starting material. Reaction mixture was concentrated under reduced pressure to remove maximum amount of DMF and then compound was purified by column chromatography (Solvent System: 3-4% ethyl acetate in hexane) in 71% yield (82 mg, R_f = 0.6 (20% EtOAc: Hexane)) as off-white solid**.** ^{1}H NMR (500 MHz) δ 7.36 (t, *J* = 7.6 Hz), 7.05 (d, *J* = 7.4 Hz), 6.91

(d, *J* = 7.9 Hz), 6.36 (s), 5.18 (s), 3.96 (d, *J* = 3.7 Hz), 3.29 (s), 3.10 (s). ^{13}C NMR (100 MHz, DMSO-D6) δ 142.8, 133.0, 130.7, 126.0, 115.0, 112.7, 81.9, 74.1, 69.2, 32.3. GCMS(APCI) for $[C_{11}H_9NO_2+H]^+$: calcd.,188.0706. Found: 188.0711.

Synthesis of prop-2-yn-1-yl (1-oxo-1,3-dihydroisobenzofuran-4-yl)carbamate (IITK2031).



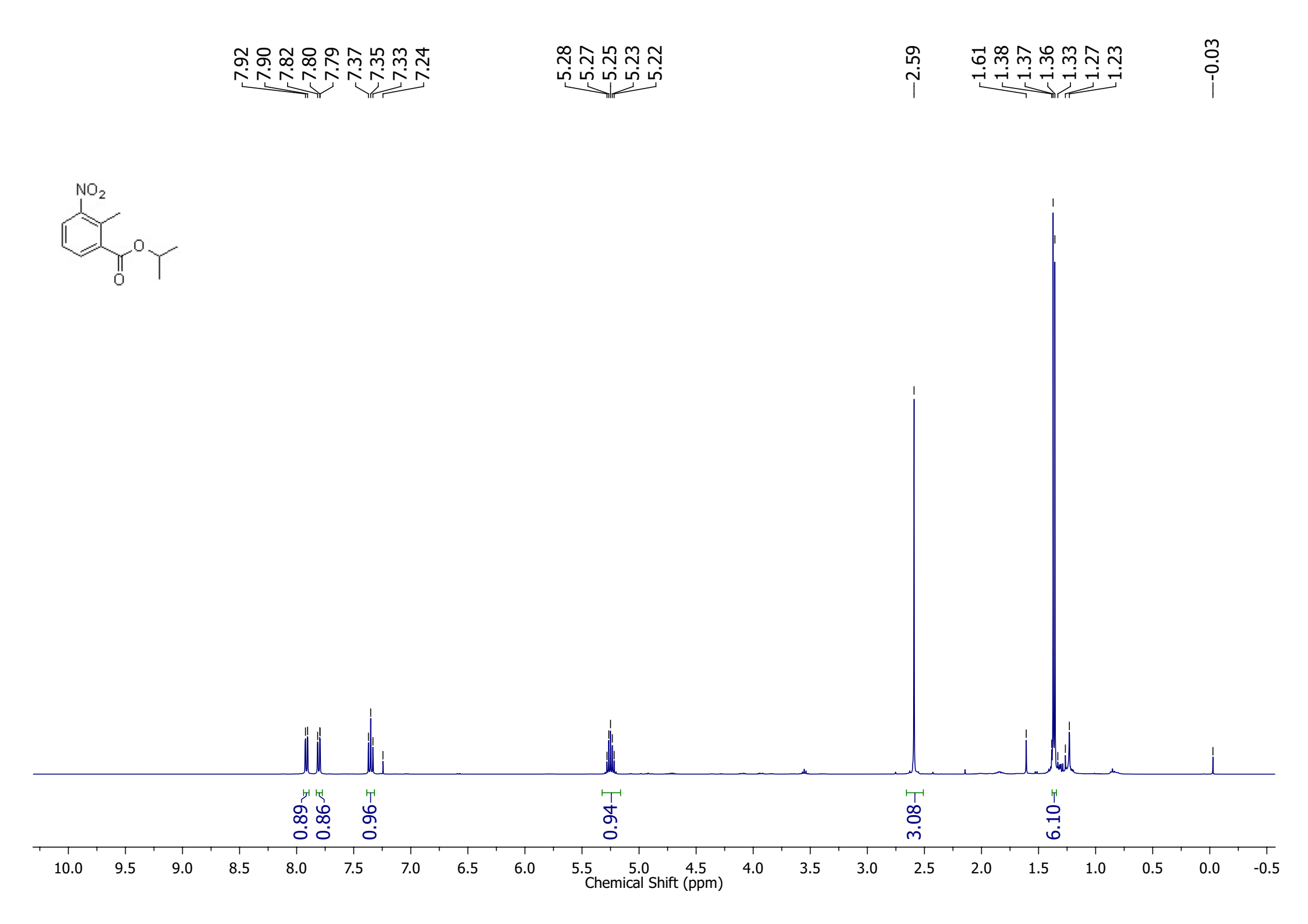
To a solution of 4-aminoisobenzofuran-1(3H)-one (670.74 µmol, 100 mg) in dry THF, triethylamine (1.34 mmol, 2 equiv., 187µL) and prop-2-yn-1-yl carbonochloridate (871.61 µmol, 1.3 equiv., 85 µL) was added under ice cold condition and the mixture was left for 6 h stirring at RT. After 6 h TLC showed complete conversion of starting material. Reaction mixture was concentrated under reduced pressure to remove maximum amount of DMF and then compound was purified by column chromatography (Solvent System: 50% ethyl acetate in hexane) in 62% yield (96 mg, R_f = 0.6 (40% EtOAc: Hexane)) as white solid**.** ^{1}H NMR (400 MHz, DMSO-D6) δ 9.96 (s, 1H), 7.86 (dd, *J* = 5.9, 2.8 Hz, 1H), 7.61 – 7.42 (m, 2H), 5.34 (s, 2H), 4.76 (d, *J* = 2.3 Hz, 2H), 3.55 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-D6) δ 170.9, 153.3, 137.8, 133.9, 130.4, 126.8, 125.8, 120.6, 79.1, 78.5, 69.7, 53.0. GCMS(APCI) for $[C_{12}H_9NO_4+H]^+$: calcd.,232.0604. Found: 232.0610.

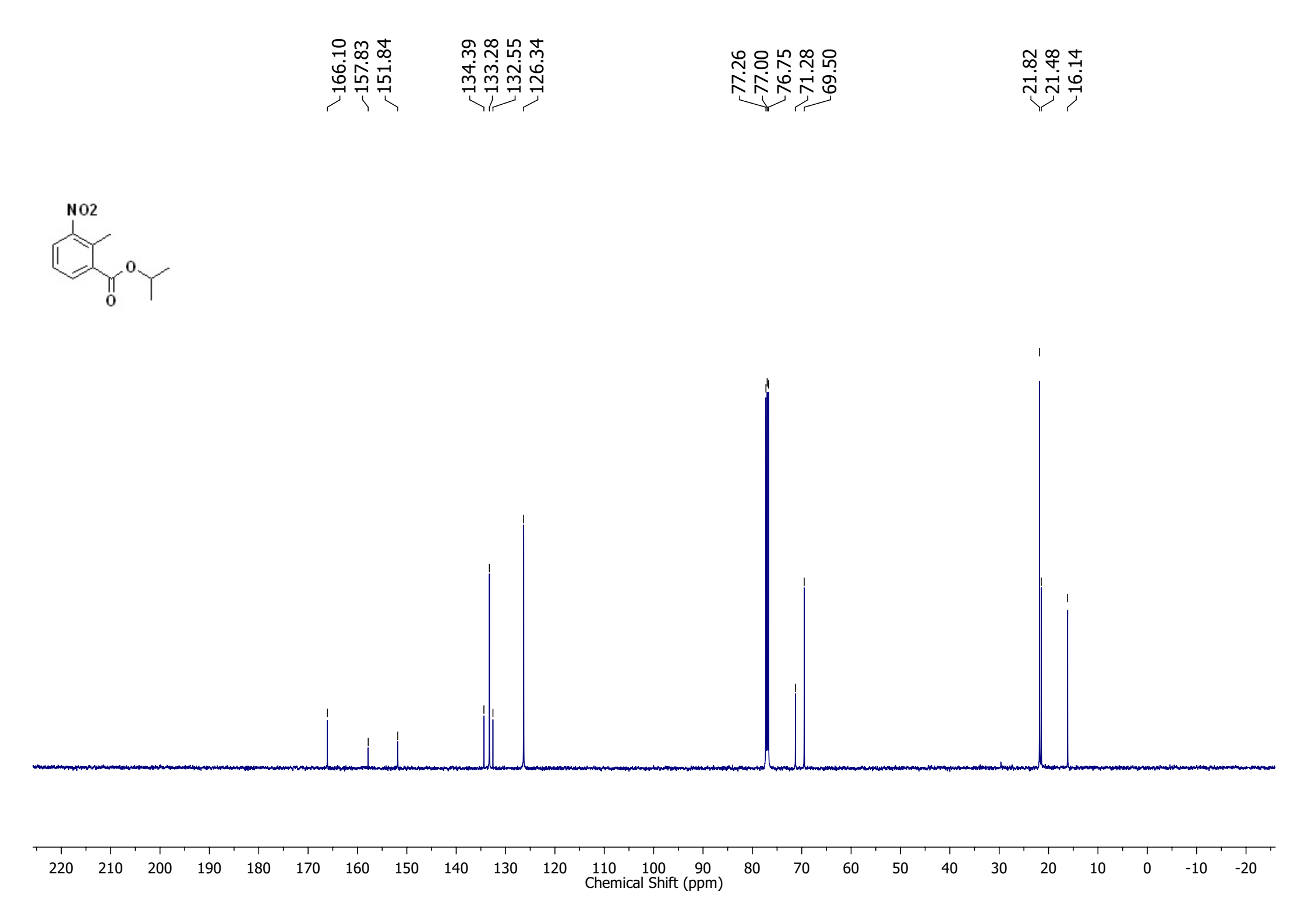
5. References:

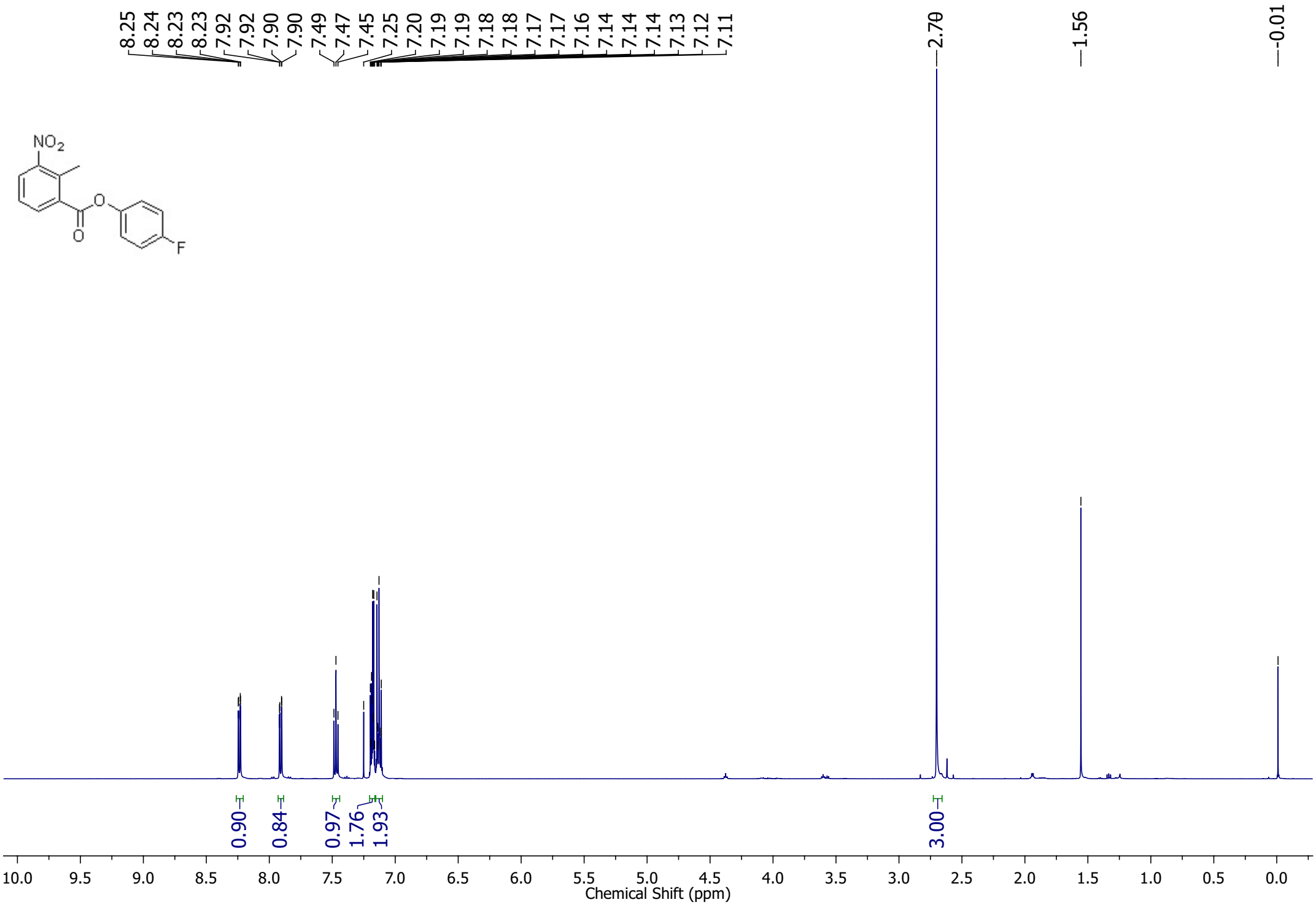
1. L. Bordoli, F. Kiefer, K. Arnold, P. Benkert, J. Battey and T. Schwede, *Nat Protoc*, 2009, **4**, 1-13.
2. H. Wang, Z. Xie, B. Lu, K. Zhong, J. Lu and J. Liu, *Tet Lett*, 2021, **74**, 153152.
3. J. Xi, S. Xu, L. Zhang, X. Bi, Y. Ren, Y. C. Liu, Y. Gu, Y. Xu, F. Lan and X. Zha, *Bioorganic chemistry*, 2018, **78**, 7-16.
4. T. H. Pillow, P. Adhikari, R. A. Blake, J. Chen, G. Del Rosario, G. Deshmukh, I. Figueroa, K. E. Gascoigne, A. V. Kamath, S. Kaufman, T. Kleinheinz, K. R. Kozak, B. Latifi, D. D. Leipold, C. Sing Li, R. Li, M. M. Mulvihill, A. O'Donohue, R. K. Rowntree, J. D. Sadowsky, J. Wai, X. Wang, C. Wu, Z. Xu, H. Yao, S. F. Yu, D. Zhang, R. Zang, H. Zhang, H. Zhou, X. Zhu and P. S. Dragovich, *ChemMedChem*, 2020, **15**, 17-25.
5. Y. Tanaka, M. Seto, K. Kakegawa, K. Takami, F. Kikuchi, T. Yamamoto, M. Nakamura, M. Daini, M. Murakami, T. Ohashi, T. Kasahara, J. Wang, Z. Ikeda, Y. Wada, F. Puenner, T. Fujii, M. Inazuka, S. Sato, T. Suzaki, J.-H. Oak, Y. Takai, H. Kohara, K. Kimoto, H. Oki, S. Mikami, M. Sasaki and Y. Tanaka, *J Med Chem*, 2022, **65**, 4270-4290.
6. P.-F. Chen, B. Zhou, P. Wu, B. Wang and L.-W. Ye, *Angew Chem Int Ed*, 2021, **60**, 27164-27170.
7. P. Zhang, J. Pan, W. Duan, X. Li, Y. Zhang, Y. Zhou, Q. Jiang, Z. Mao and P. Yu, *Molecules*, 2011, **16**.
8. Y. R. Hong, H. T. Kim, S. C. Lee, S. Ro, J. M. Cho, I. S. Kim and Y. H. Jung, *Bioorg Med Chem Lett*, 2013, **23**, 5953-5957.
9. S. Li, G. Luan, X. Ren, W. Song, L. Xu, M. Xu, J. Zhu, D. Dong, Y. Diao, X. Liu, L. Zhu, R. Wang, Z. Zhao, Y. Xu and H. Li, *Sci Rep*, 2015, **5**, 14836.
10. P. Nandhikonda and M. D. Heagy, *Org Lett*, 2010, **12**, 4796-4799.
11. T. Christine, A. Tabey, T. Cornilleau, E. Fouquet and P. Hermange, *Tetrahedron*, 2019, **75**, 130765.
12. E. C. Son, S. Y. Kim and S.-G. Kim, *J Org Chem*, 2021, **86**, 6826-6839.
13. S. Li, M. Su, J. Sun, K. Hu and J. Jin, *Org Lett*, 2021, **23**, 5842-5847.
14. B. Wang, D. Chu, Y. Feng, Y. Shen, M. Aoyagi-Scharber and L. E. Post, *J Med Chem*, 2016, **59**, 335-357.

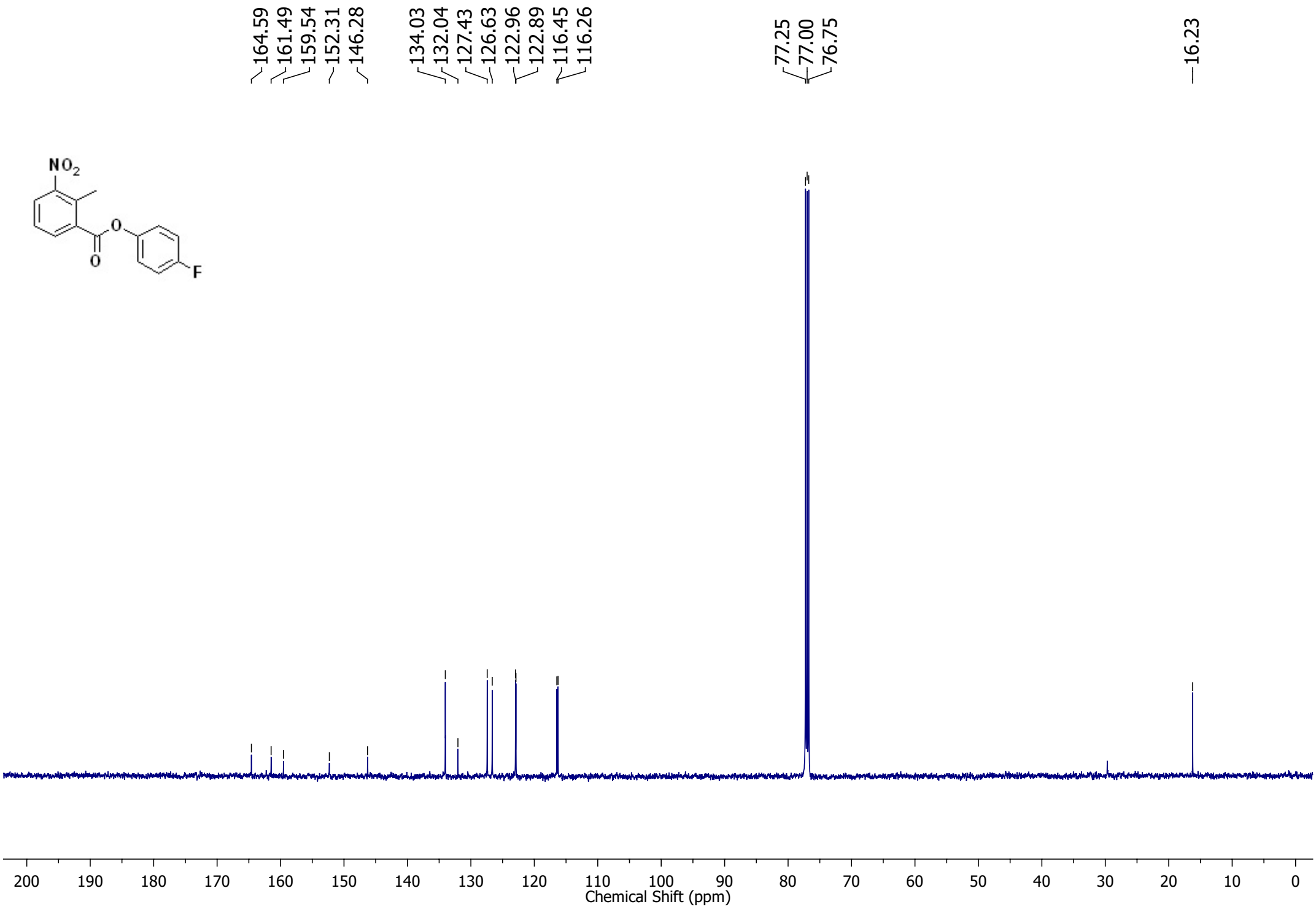
15. K. Mohammed Khan, S. Hayat, U. Zia, R. Atta ur, M. Iqbal Choudhary, G. M. Maharvi and E. Bayer, *Synth Commun*, 2003, **33**, 3435-3453.
16. Y.-L. Pan, H.-L. Zheng, J. Wang, C. Yang, X. Li and J.-P. Cheng, *ACS Catal*, 2020, **10**, 8069-8076.
17. H. Tsubery, H. Yaakov, S. Cohen, T. Giterman, A. Matityahou, M. Fridkin and I. Ofek, *Antimicrob Agents Chemother*, 2005, **49**, 3122-3128.
18. H. H. Winkler, *J bacteriol*, 1973, **116**, 1079-1081.
19. *CLSI. Performance Standards for Antimicrobial Susceptibility Testing*, 2012, **Twenty-First Edition**, M100.
20. R. Thakare, M. Shukla, G. Kaul, A. Dasgupta and S. Chopra, *International journal of antimicrobial agents*, 2019, **53**, 709-715.
21. K. Zhu, K. W. Borrelli, J. R. Greenwood, T. Day, R. Abel, R. S. Farid and E. Harder, *J Chem Inf Model*, 2014, **54**, 1932-1940.

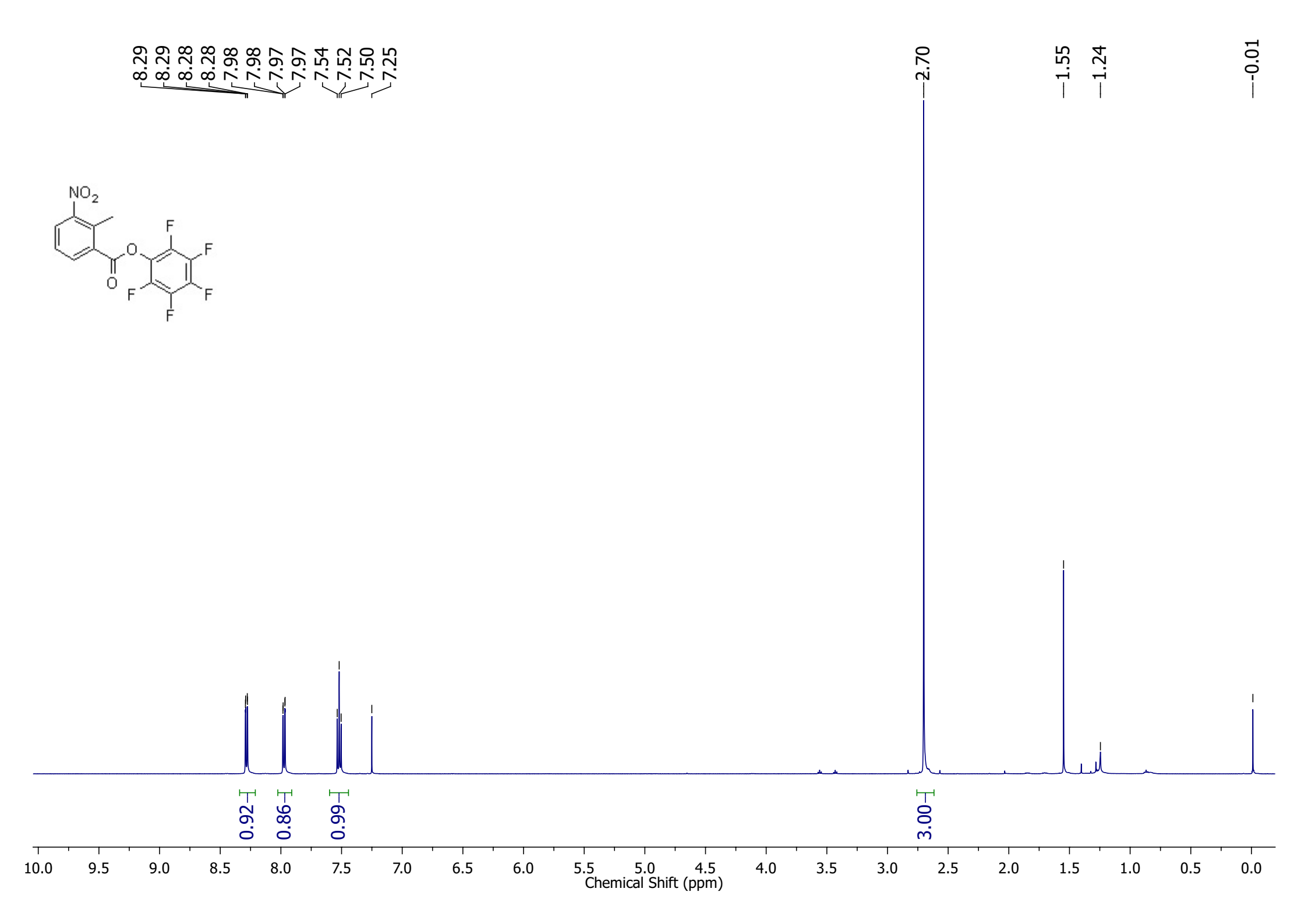
NMR Spectra

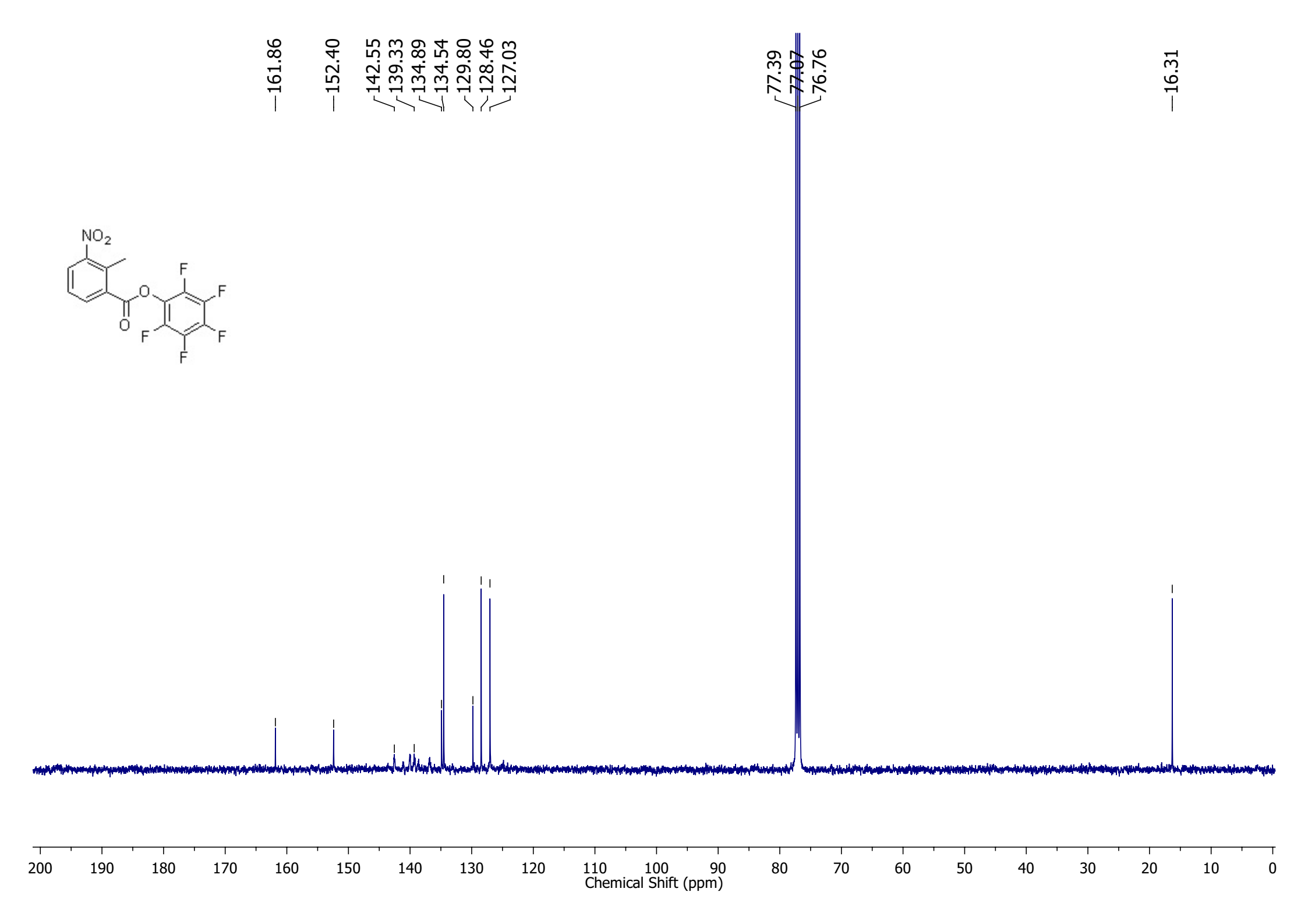

7.92
7.90
7.82
7.80
7.79
7.37
7.35
7.33
7.24
5.28
5.27
5.25
5.23
5.22
2.59
1.61
1.38
1.37
1.36
1.33
1.27
1.23
-0.03
NO2
O
O
0.89
0.86
0.96
0.94
3.08
6.10
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Chemical Shift (ppm)

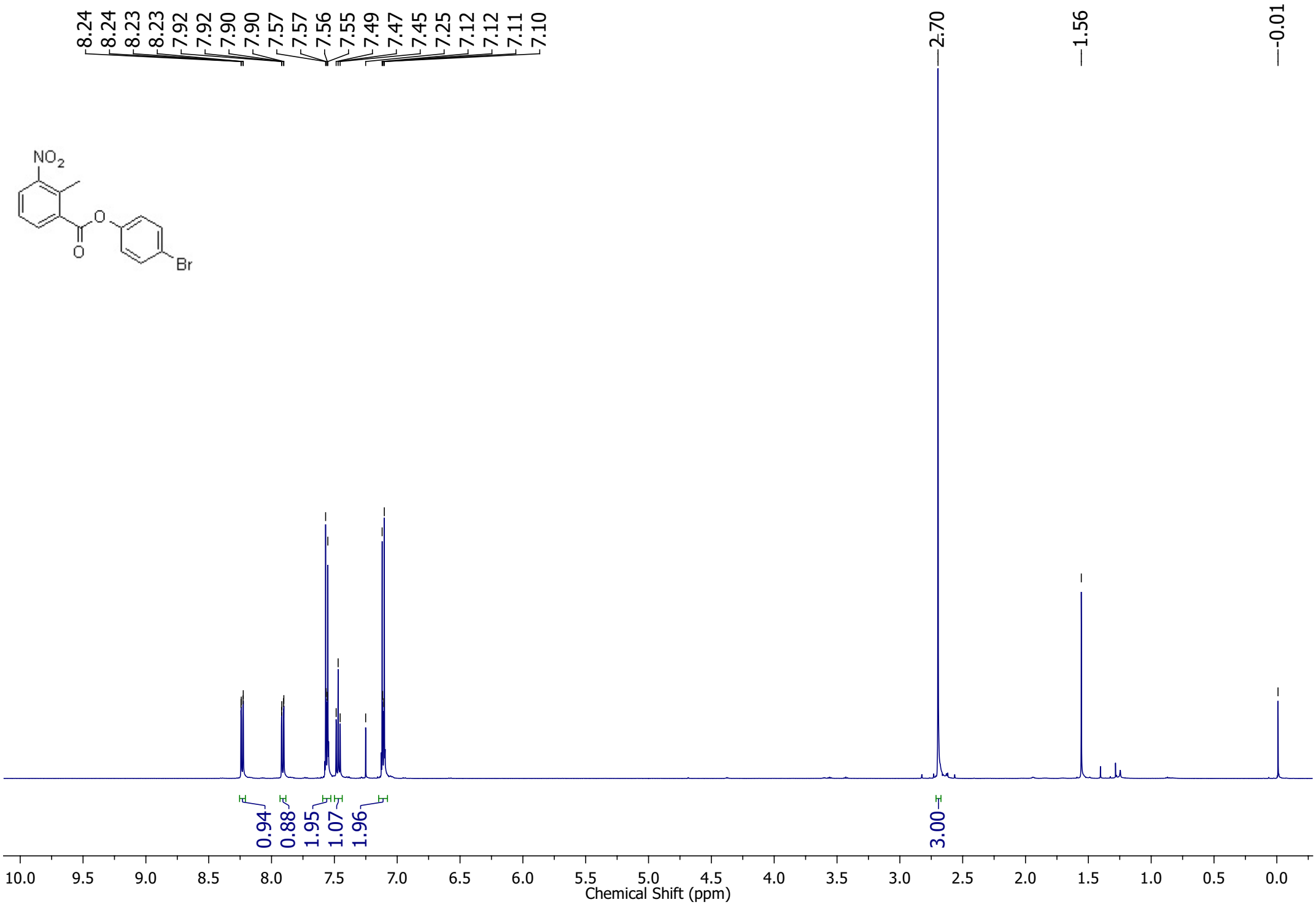

166.10
157.83
151.84
134.39
133.28
132.55
126.34
77.26
77.00
76.75
71.28
69.50
21.82
21.48
16.14
NO2
O
O
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
Chemical Shift (ppm)

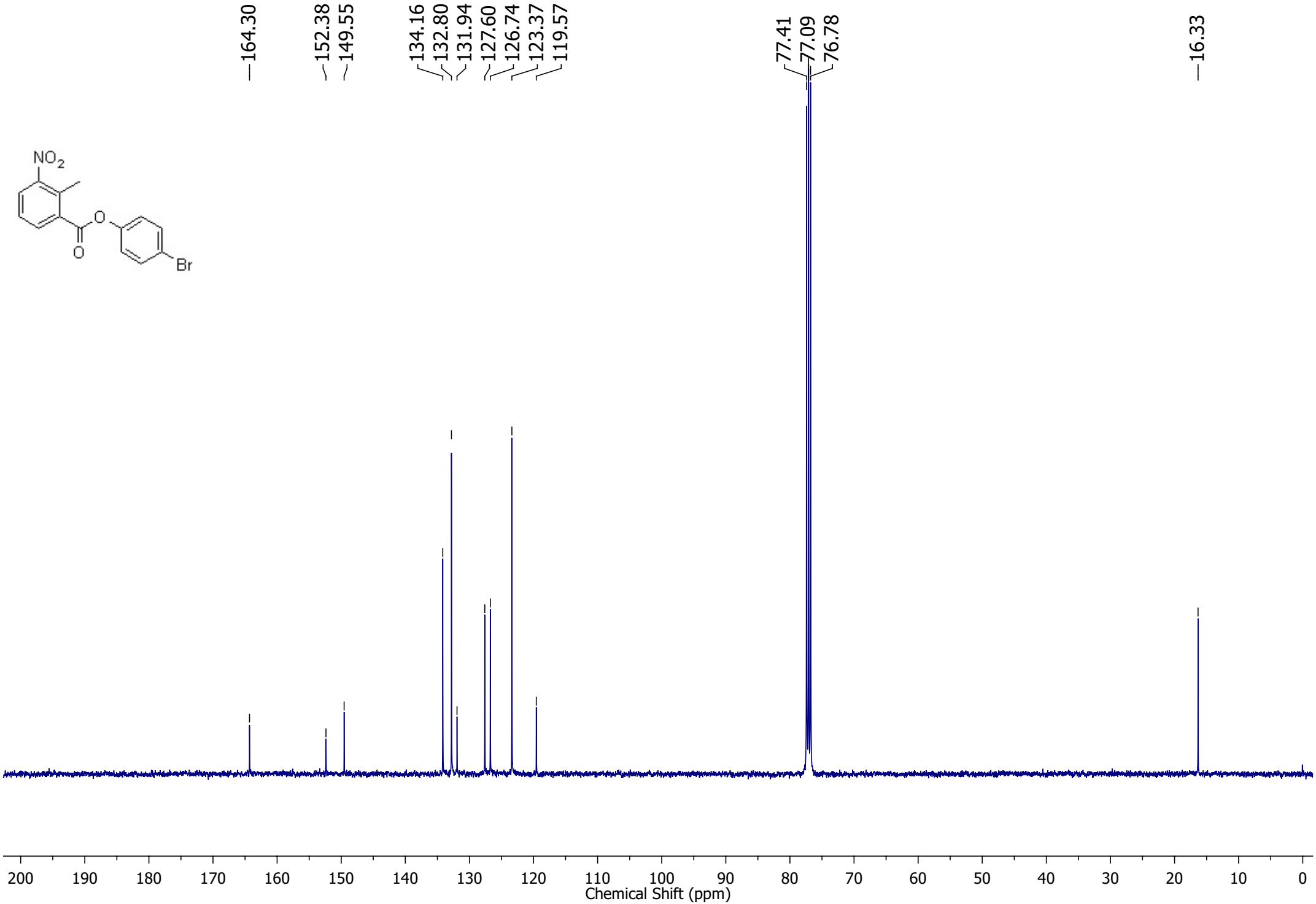

8.25
8.24
8.23
8.23
7.92
7.92
7.90
7.90
7.49
7.47
7.45
7.25
7.20
7.19
7.19
7.18
7.18
7.17
7.17
7.16
7.14
7.14
7.14
7.13
7.12
7.11
2.70
1.56
-0.01
NO2
O
O
F
0.90
0.84
0.97
1.76
1.93
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

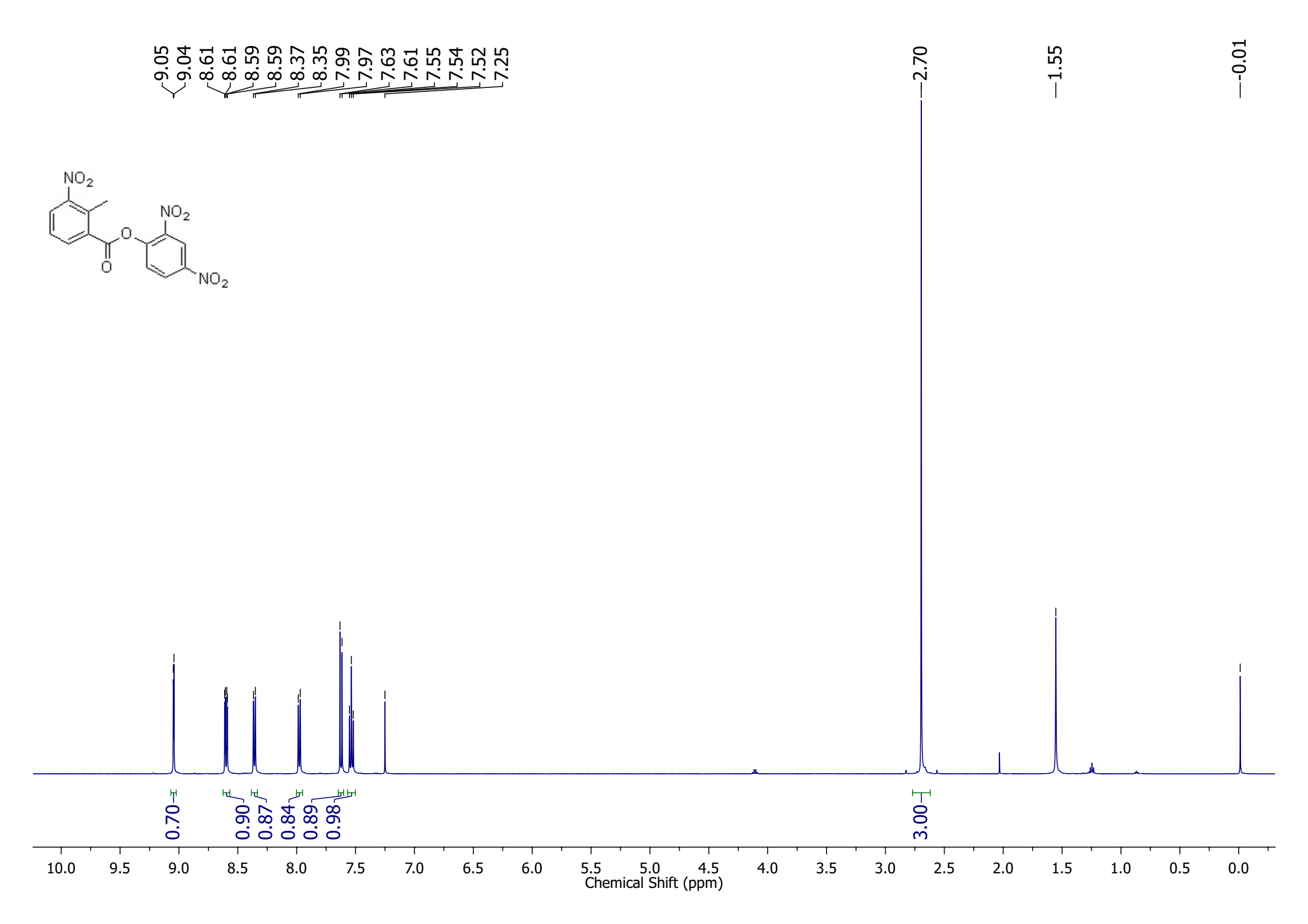

164.59
161.49
159.54
152.31
146.28
134.03
132.04
127.43
126.63
122.96
122.89
116.45
116.26
77.25
77.00
76.75
16.23
NO2
O
O
F
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

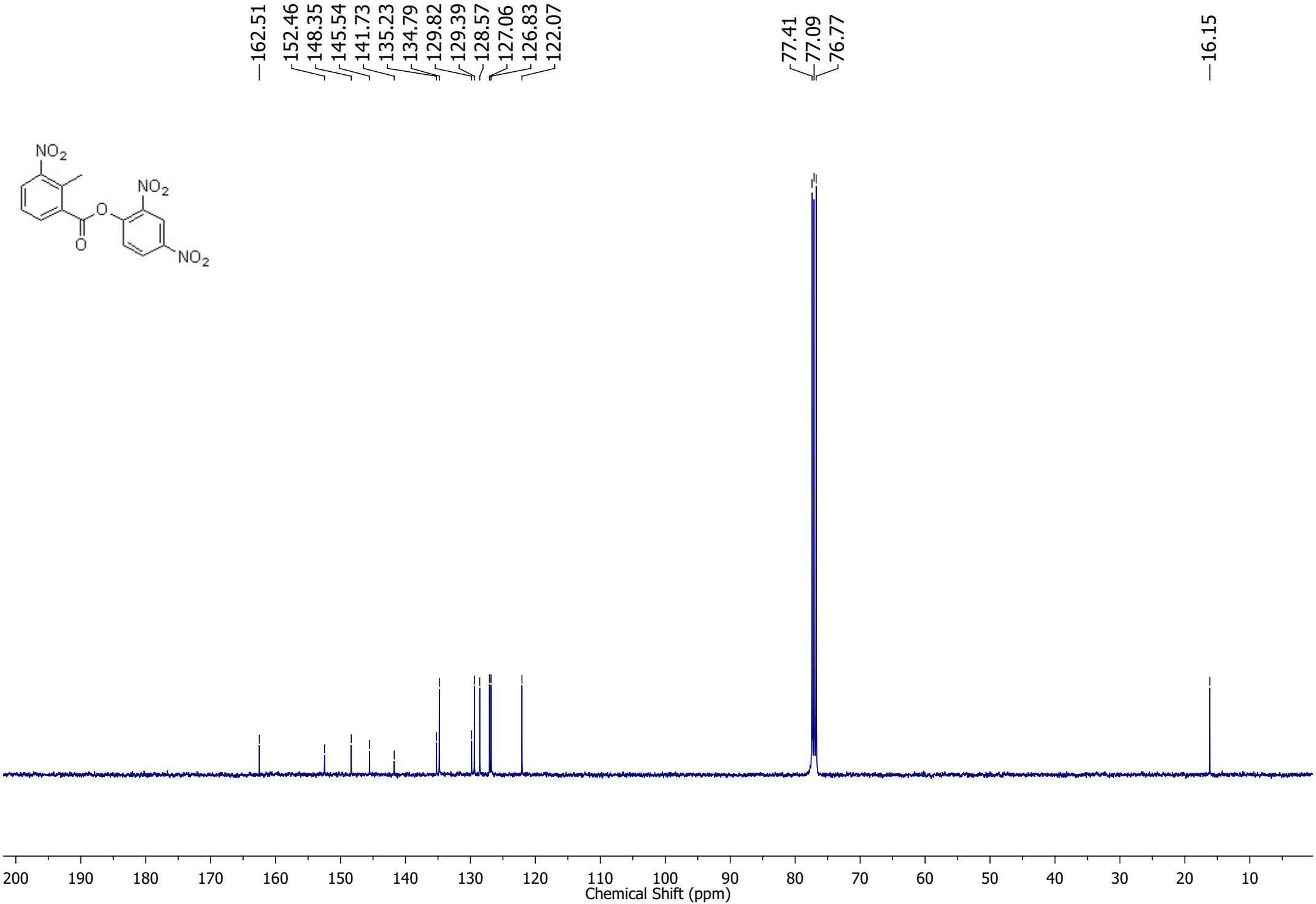

8.29
8.29
8.28
8.28
7.98
7.98
7.97
7.97
7.54
7.52
7.50
7.25
2.70
1.55
1.24
-0.01
NO2
O
O
F
F
F
F
F
0.92
0.86
0.99
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

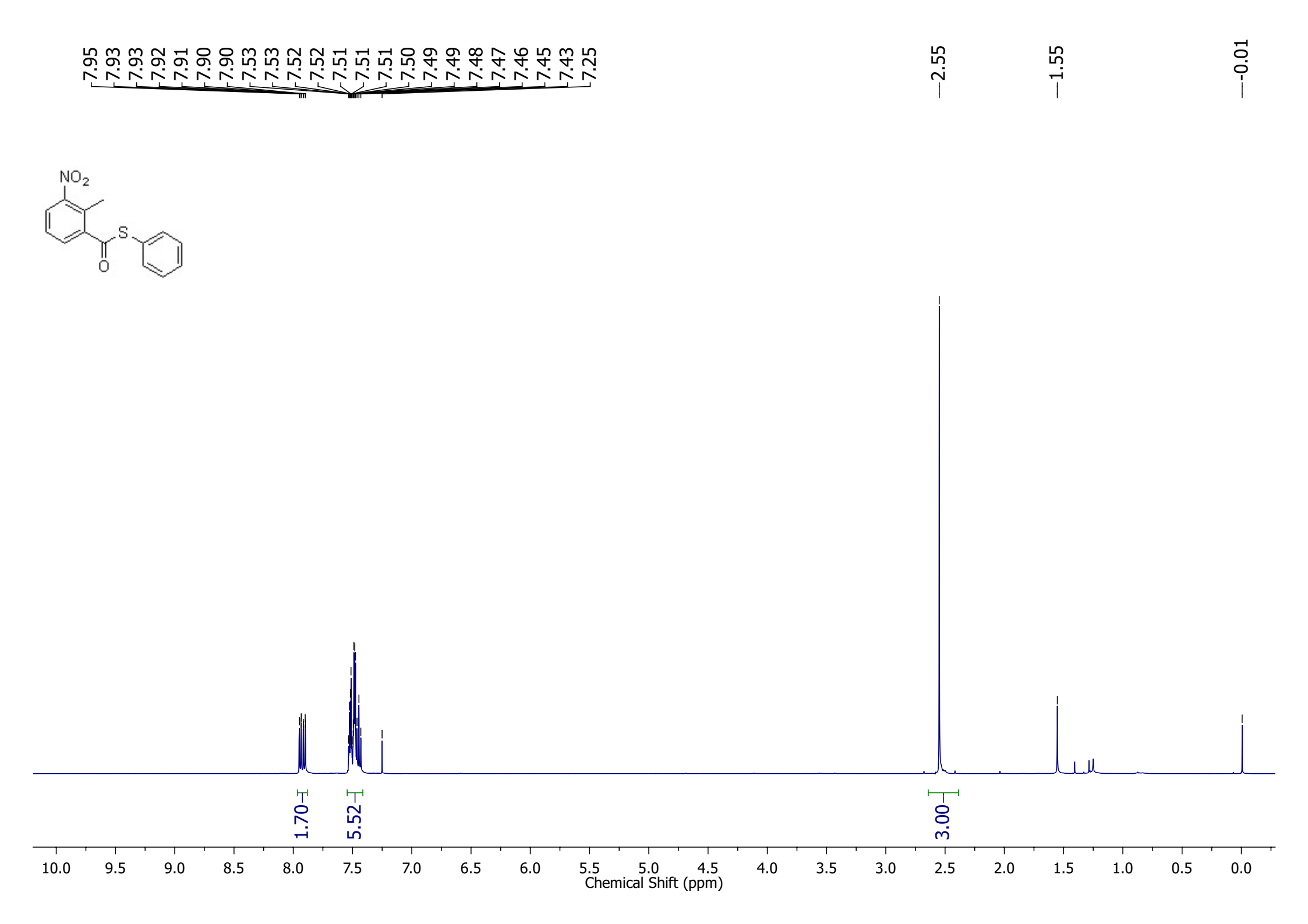

161.86
152.40
142.55
139.33
134.89
134.54
129.80
128.46
127.03
77.39
77.07
76.76
16.31
NO2
O
O
F
F
F
F
F
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

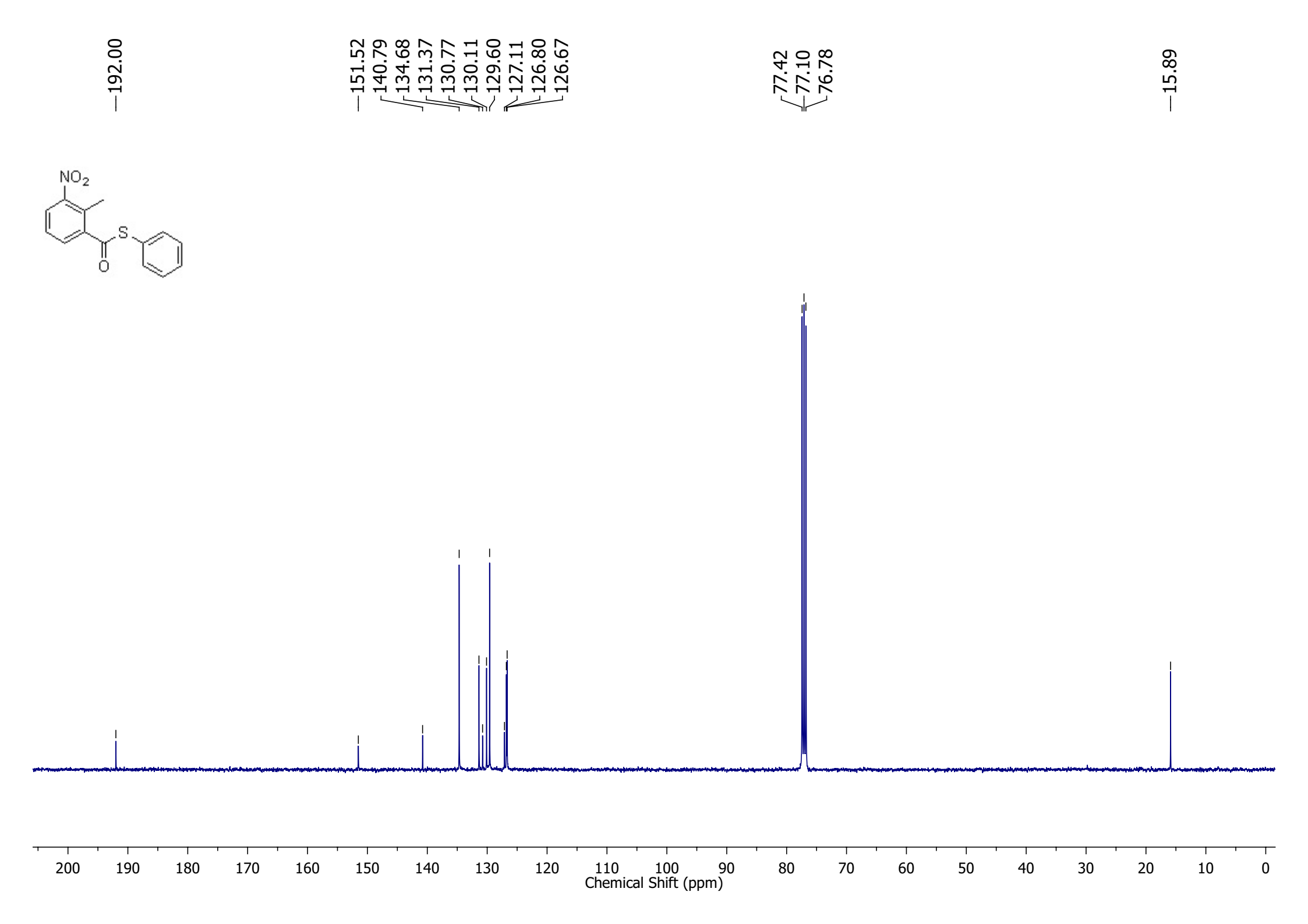

8.24
8.24
8.23
8.23
7.92
7.92
7.90
7.90
7.57
7.57
7.56
7.55
7.49
7.47
7.45
7.25
7.12
7.12
7.11
7.10
2.70
1.56
-0.01
NO2
O
O
Br
0.94
0.88
1.95
1.07
1.96
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

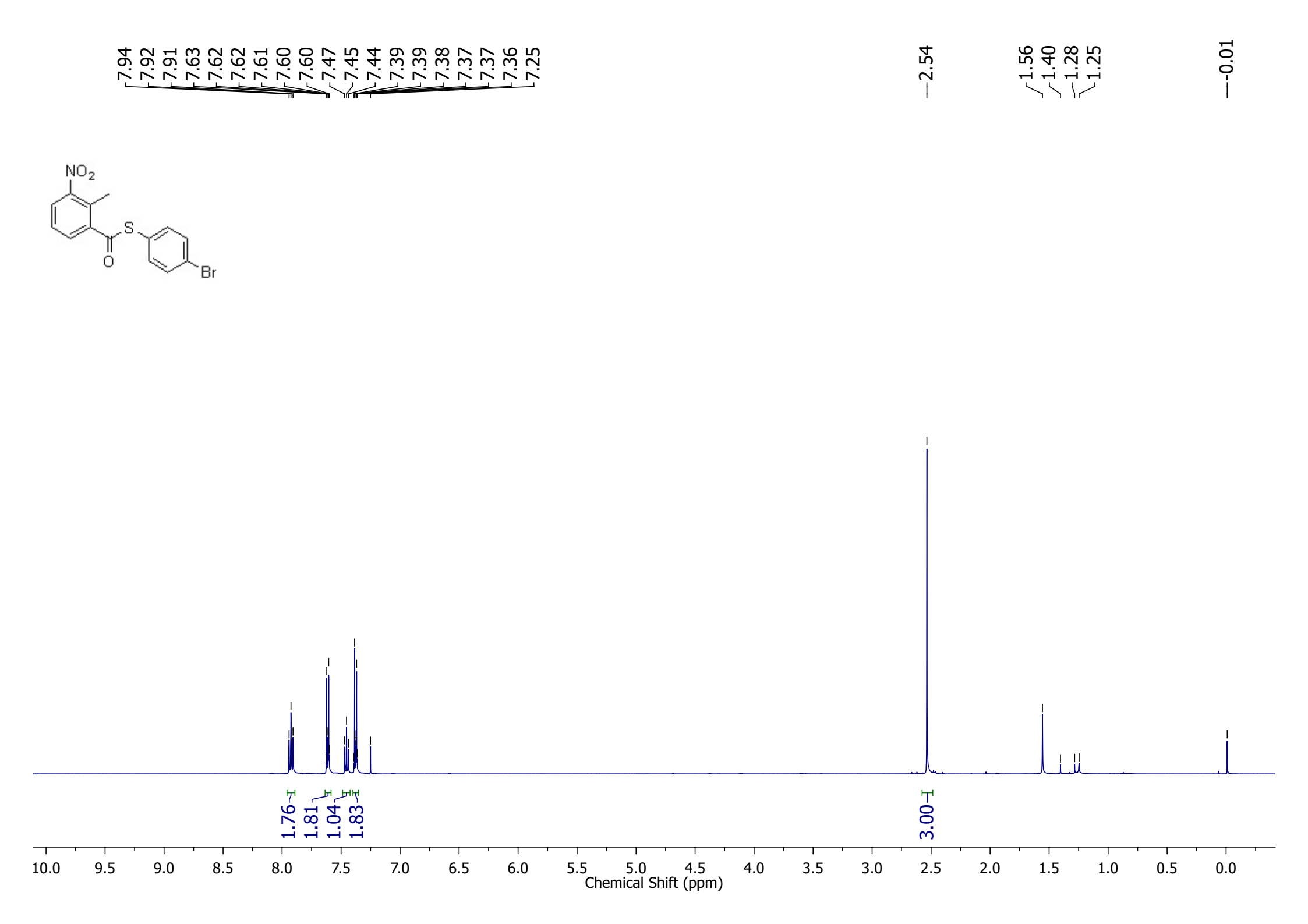

164.30
152.38
149.55
134.16
132.80
131.94
127.60
126.74
123.37
119.57
77.41
77.09
76.78
16.33
NO2
O
O
Br
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

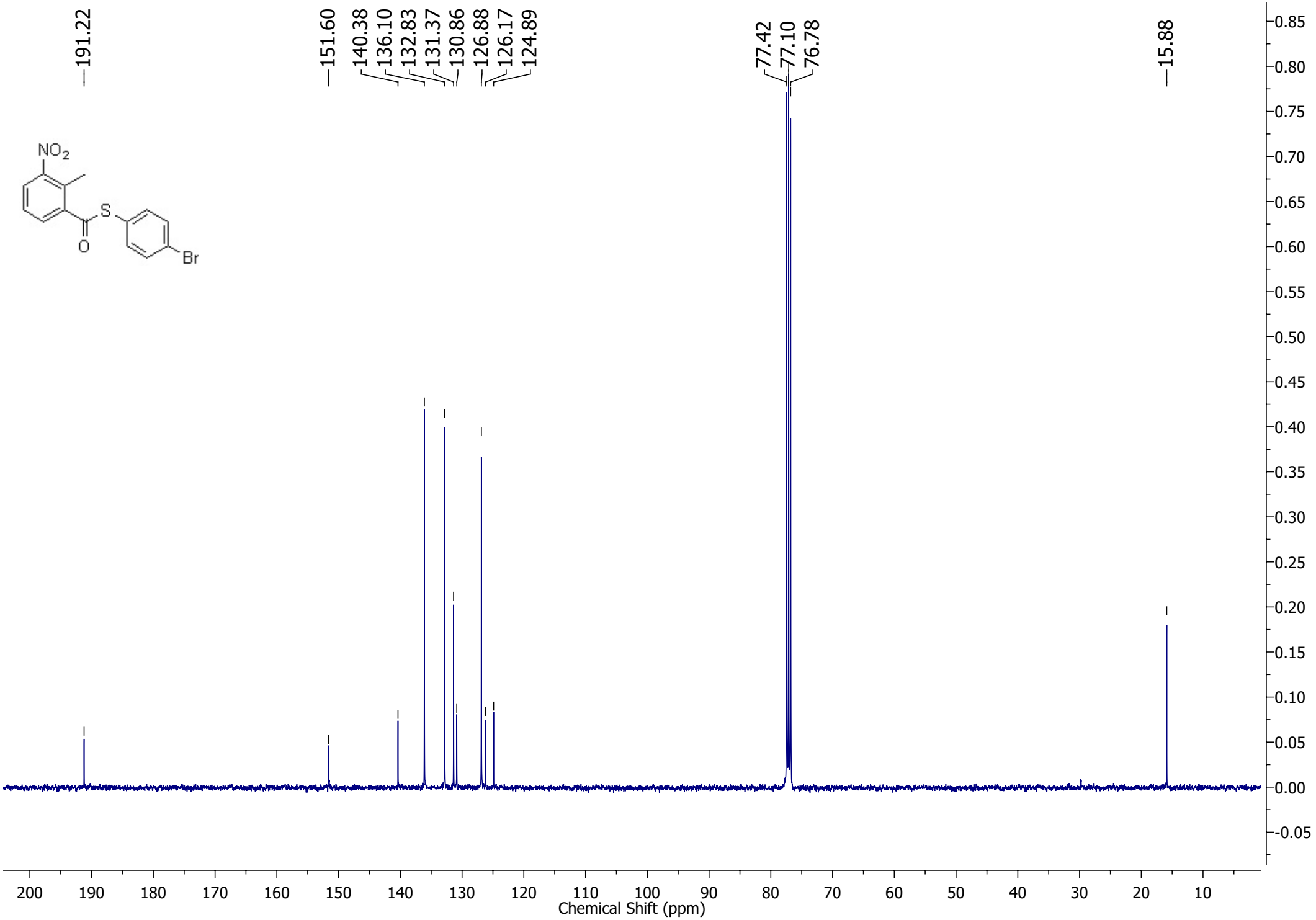

9.05
9.04
8.61
8.61
8.59
8.59
8.37
8.35
7.99
7.97
7.63
7.61
7.55
7.54
7.52
7.25
2.70
1.55
-0.01
NO2
NO2
NO2
O
O
0.70
0.90
0.87
0.84
0.89
0.98
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

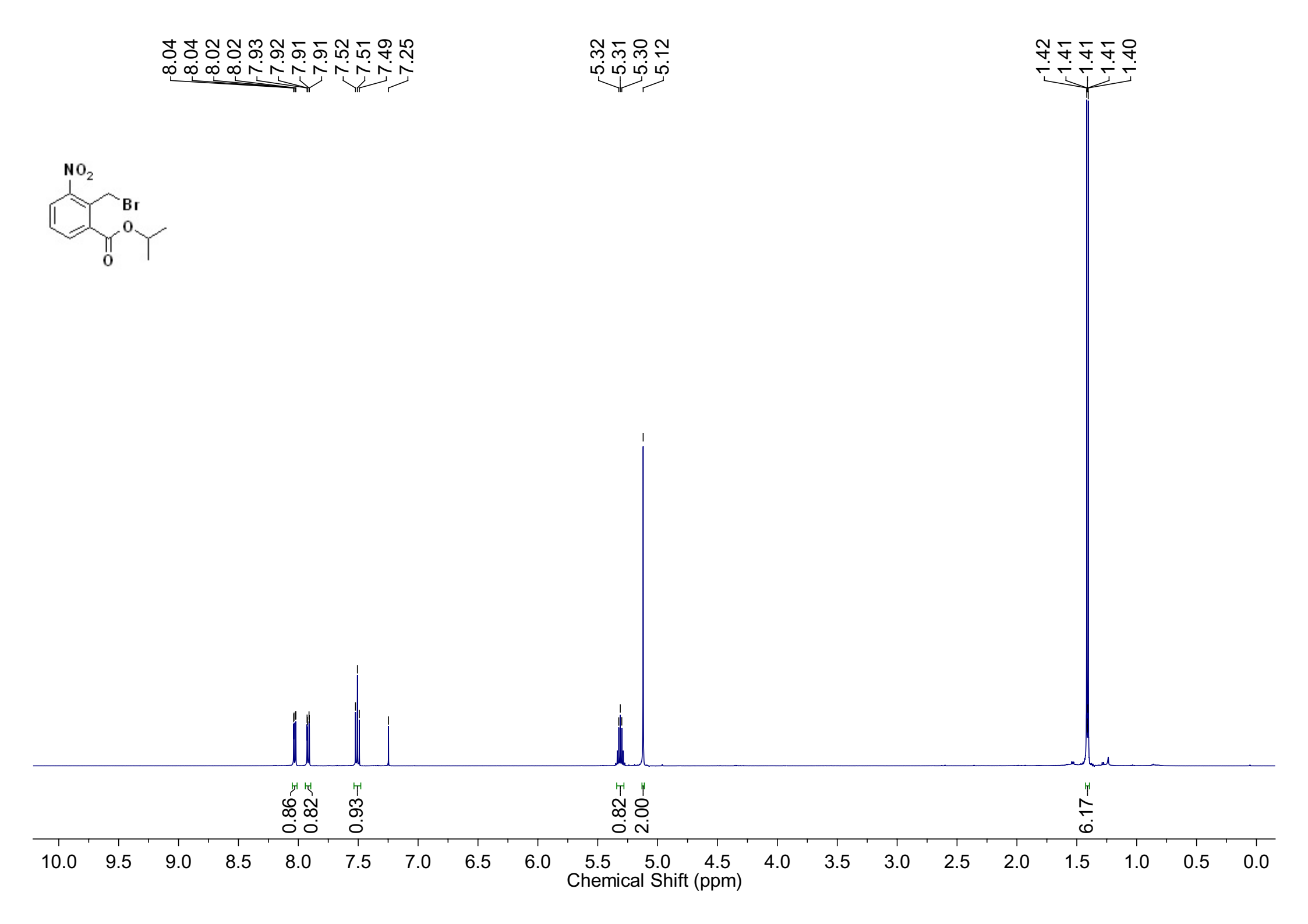

162.51
152.46
148.35
145.54
141.73
135.23
134.79
129.82
129.39
128.57
127.06
126.83
122.07
77.41
77.09
76.77
16.15
NO2
NO2
NO2
O
O
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Chemical Shift (ppm)

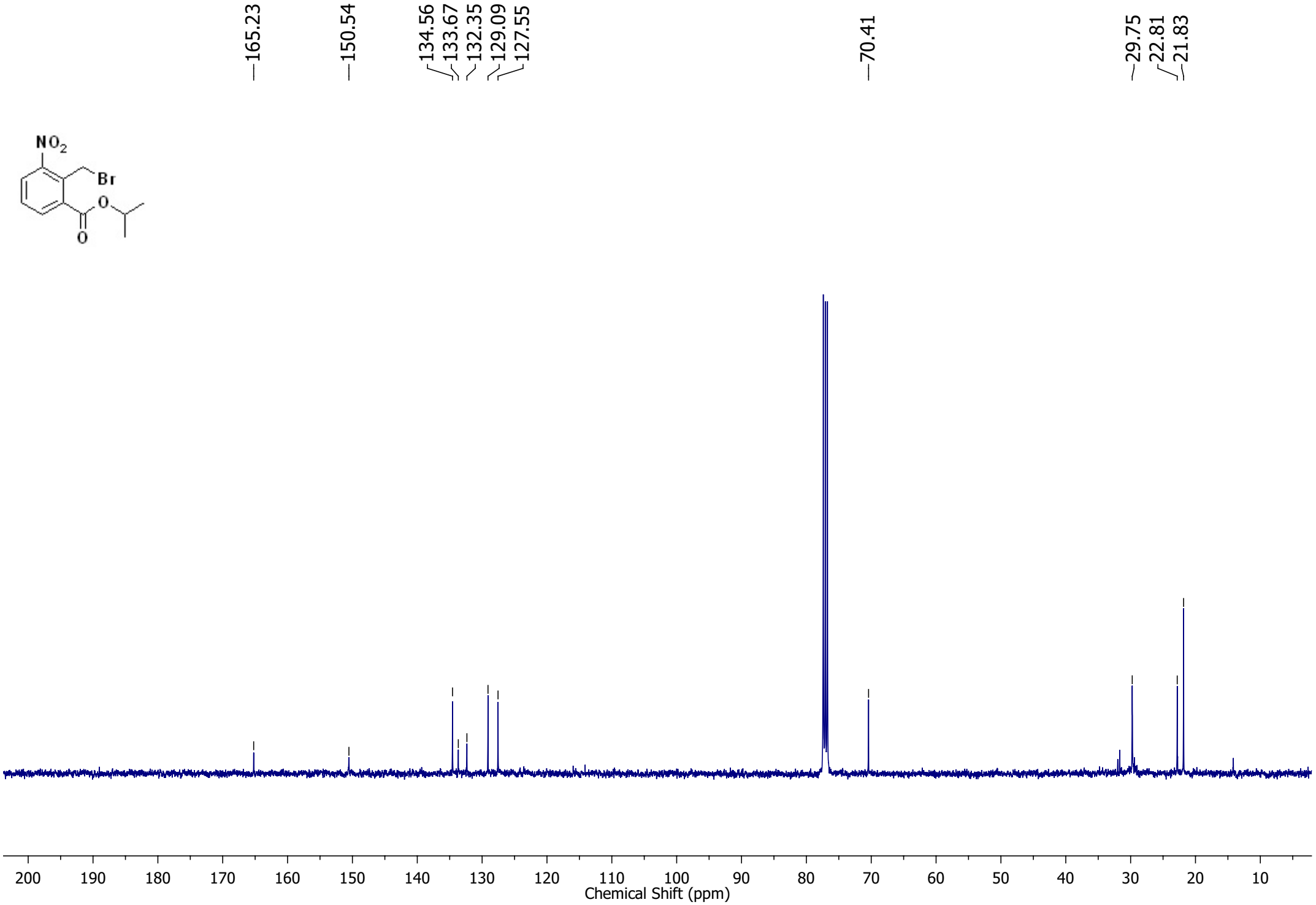

7.95
7.93
7.93
7.92
7.91
7.90
7.90
7.53
7.53
7.52
7.52
7.51
7.51
7.51
7.50
7.49
7.49
7.48
7.47
7.46
7.45
7.43
7.25
2.55
1.55
-0.01
NO2
S
O
1.70
5.52
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

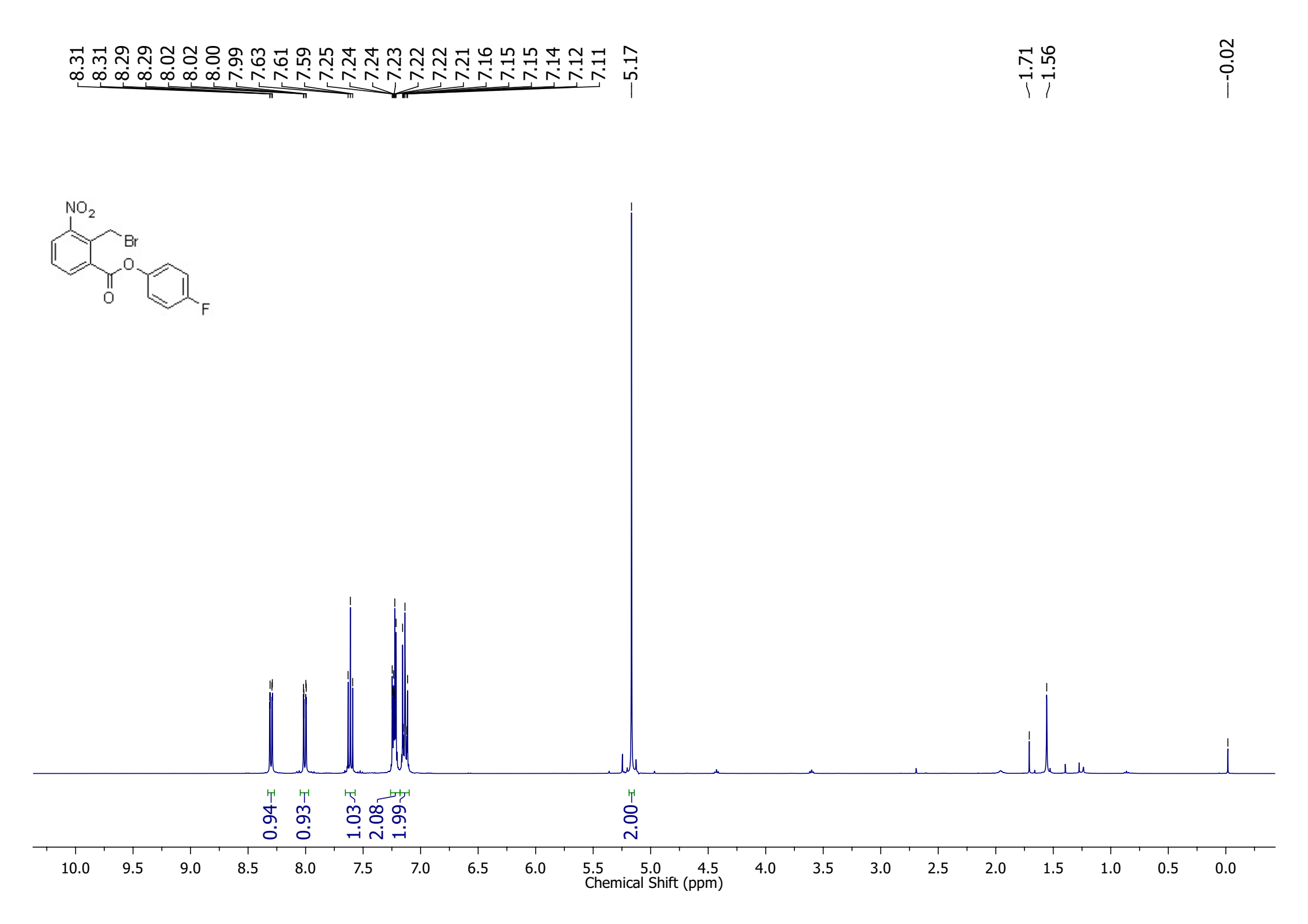

192.00
151.52
140.79
134.68
131.37
130.77
130.11
129.60
127.11
126.80
126.67
77.42
77.10
76.78
15.89
NO2
S
O
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

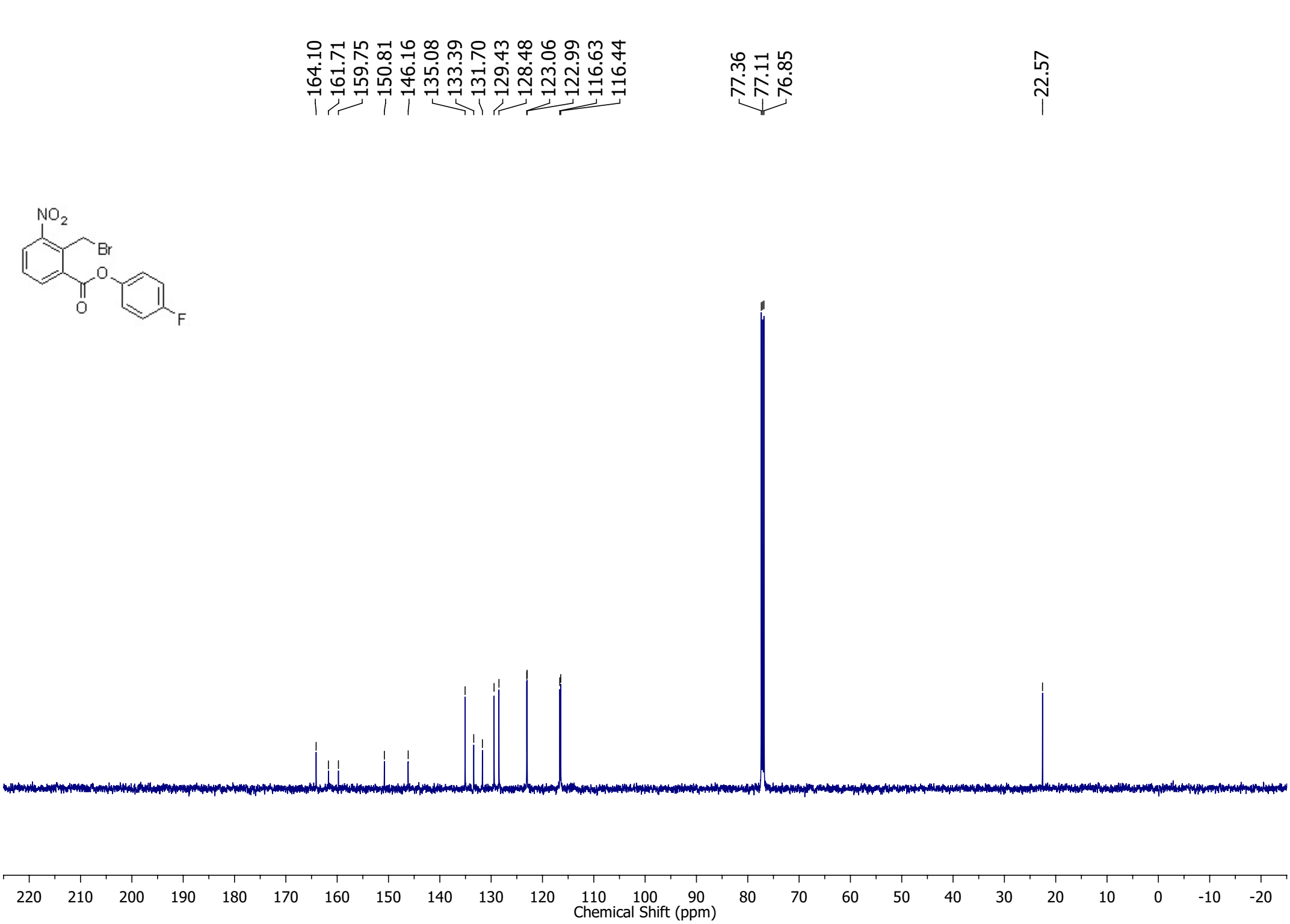

7.94
7.92
7.91
7.63
7.62
7.62
7.61
7.60
7.60
7.47
7.45
7.44
7.39
7.39
7.38
7.37
7.37
7.36
7.25
2.54
1.56
1.40
1.28
1.25
-0.01
NO2
S
O
Br
1.76
1.81
1.04
1.83
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

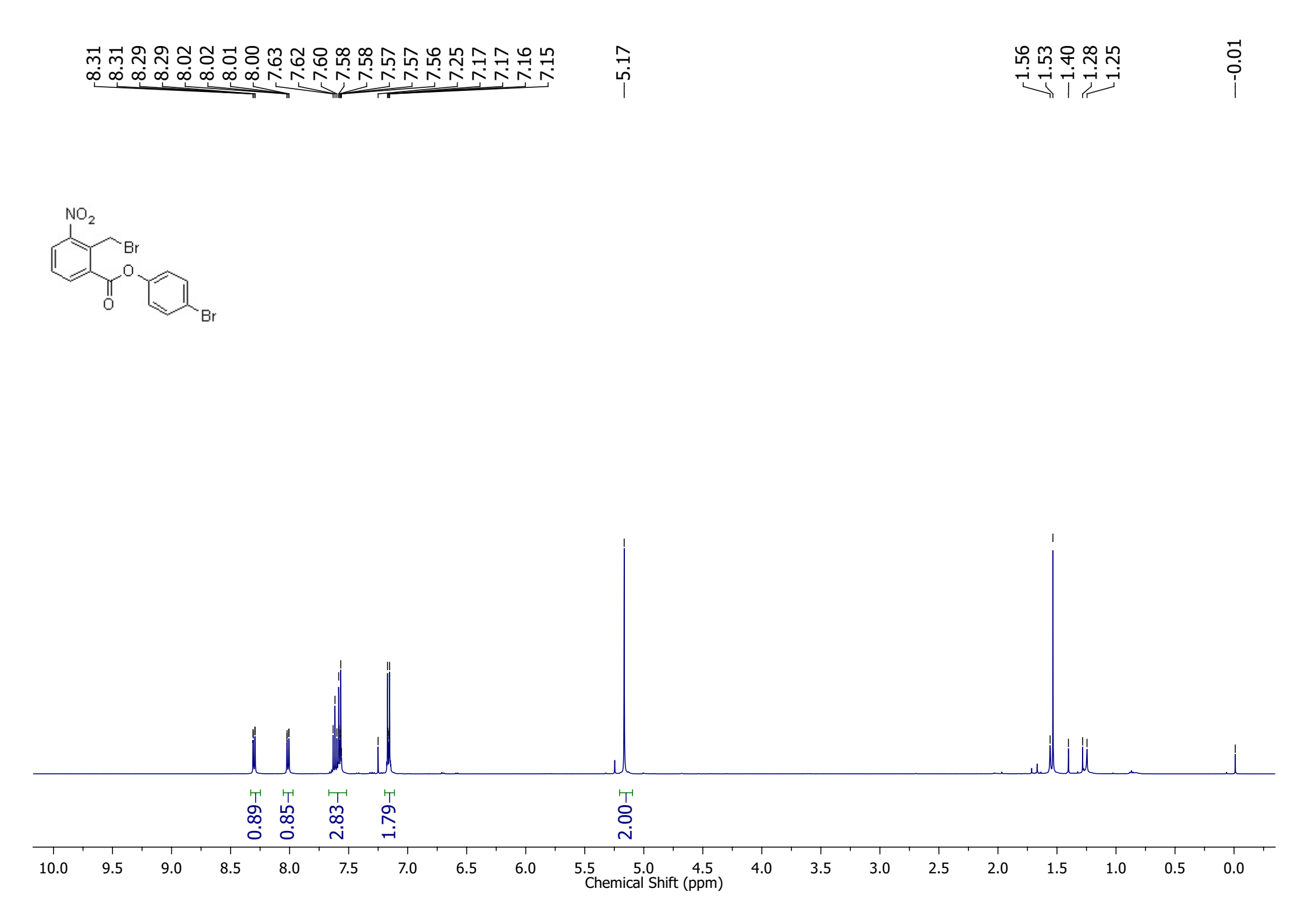

191.22
151.60
140.38
136.10
132.83
131.37
130.86
126.88
126.17
124.89
77.42
77.10
76.78
15.88
NO2
S
O
Br
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Chemical Shift (ppm)

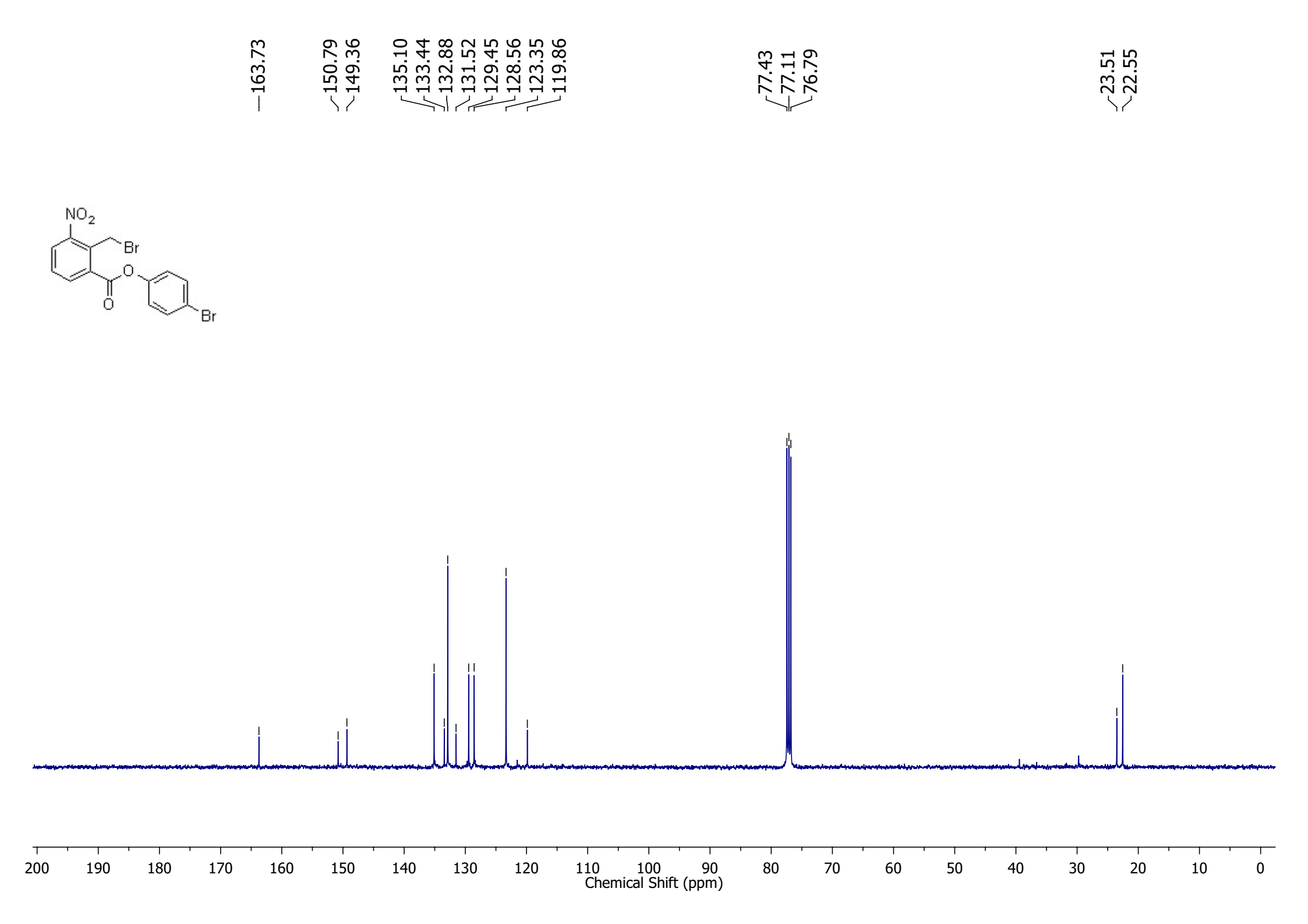

8.04
8.04
8.02
8.02
7.93
7.92
7.91
7.91
7.52
7.51
7.49
7.25
5.32
5.31
5.30
5.12
1.42
1.41
1.41
1.41
1.40
NO2
Br
O
O
0.86
0.82
0.93
0.82
2.00
6.17
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

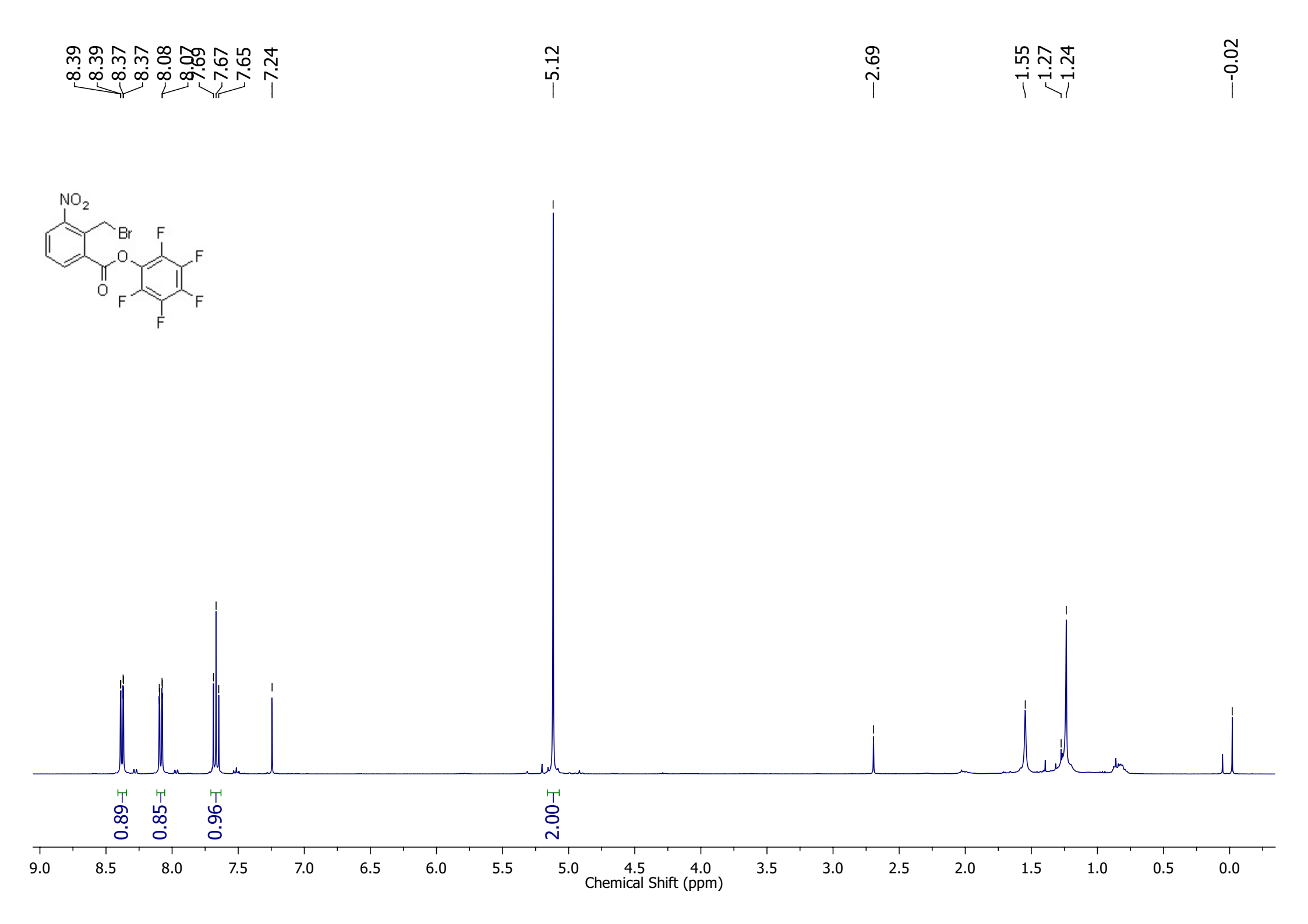

165.23
150.54
134.56
133.67
132.35
129.09
127.55
70.41
29.75
22.81
21.83
NO2
Br
O
O
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Chemical Shift (ppm)

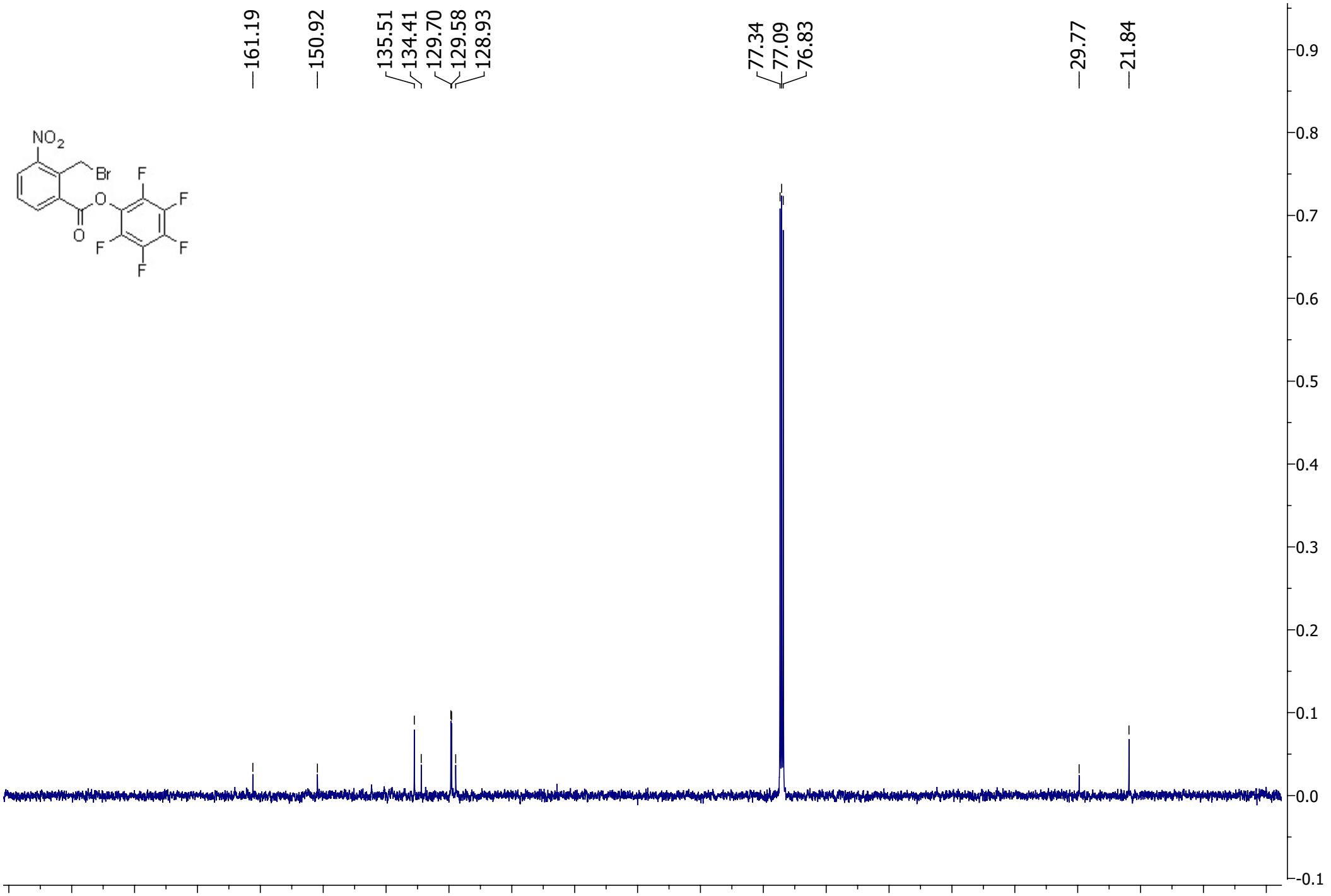

8.31
8.31
8.29
8.29
8.02
8.02
8.00
7.99
7.63
7.61
7.59
7.25
7.24
7.24
7.23
7.22
7.22
7.21
7.16
7.15
7.15
7.14
7.12
7.11
5.17
1.71
1.56
-0.02
NO2
Br
O
O
F
0.94
0.93
1.03
2.08
1.99
2.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

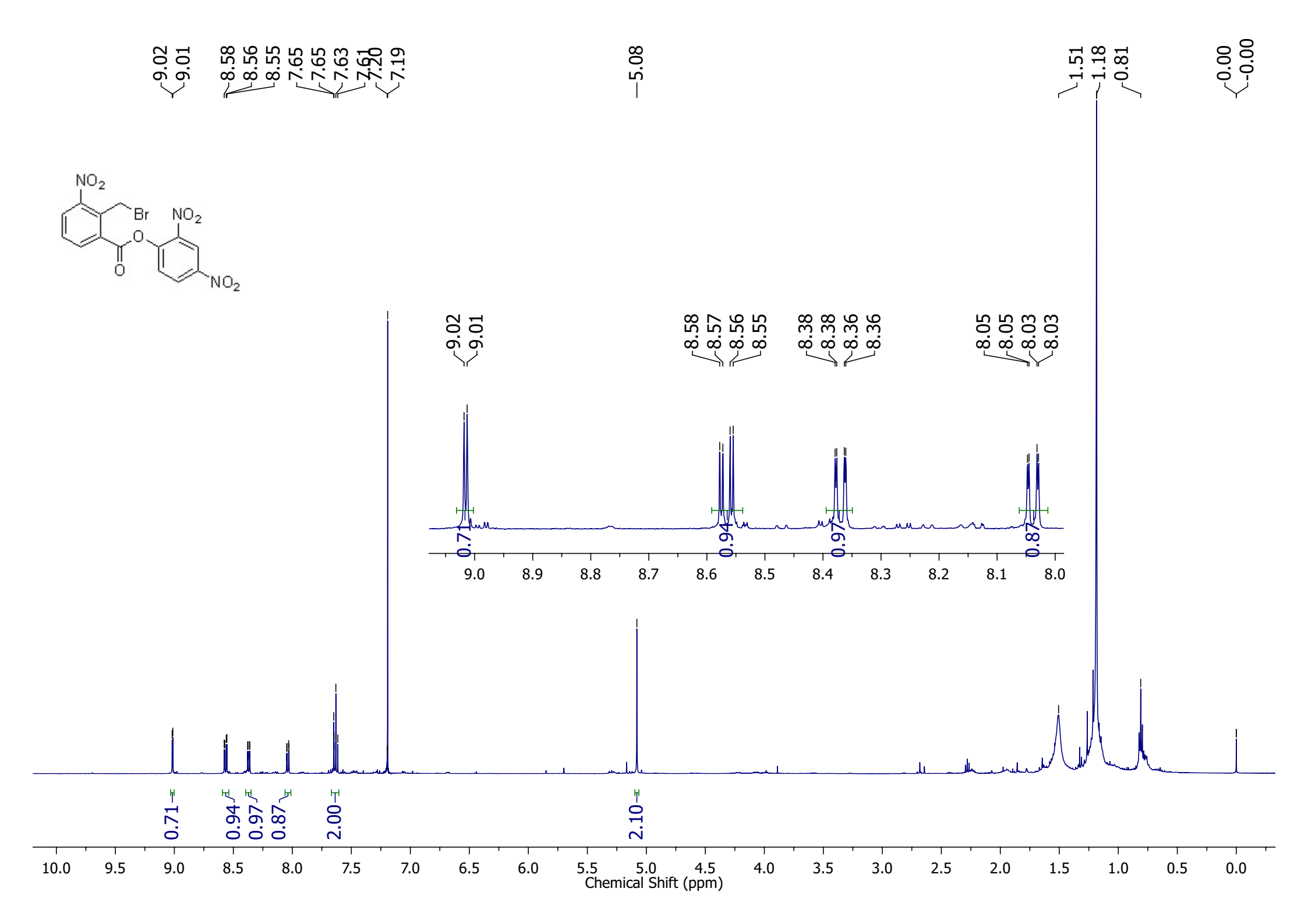

164.10
161.71
159.75
150.81
146.16
135.08
133.39
131.70
129.43
128.48
123.06
122.99
116.63
116.44
77.36
77.11
76.85
22.57
NO2
Br
O
O
F
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
Chemical Shift (ppm)

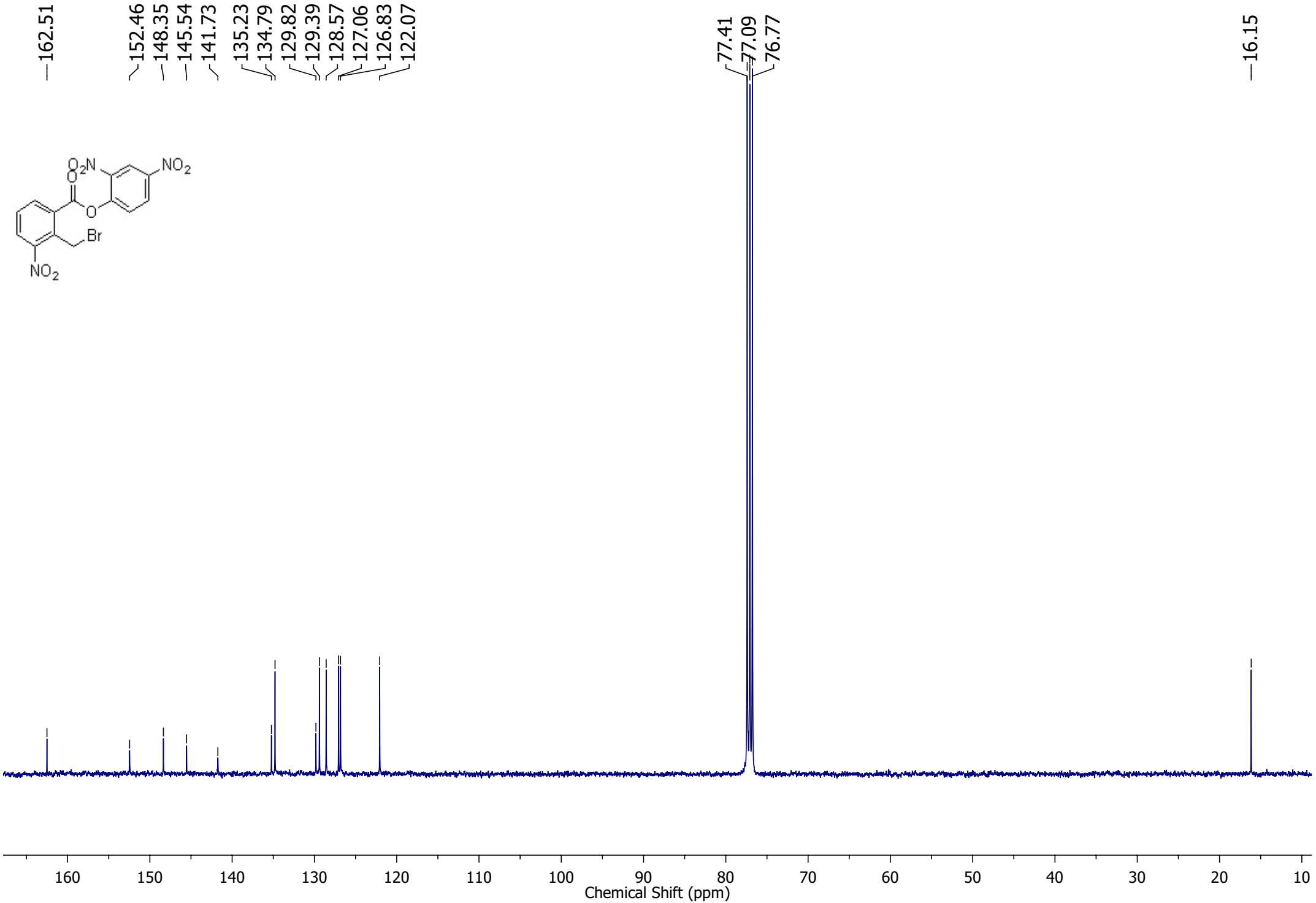

8.31
8.31
8.29
8.29
8.02
8.02
8.01
8.00
7.63
7.62
7.60
7.58
7.58
7.57
7.57
7.56
7.25
7.17
7.17
7.16
7.15
5.17
1.56
1.53
1.40
1.28
1.25
-0.01
NO2
Br
O
O
Br
0.89
0.85
2.83
1.79
2.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

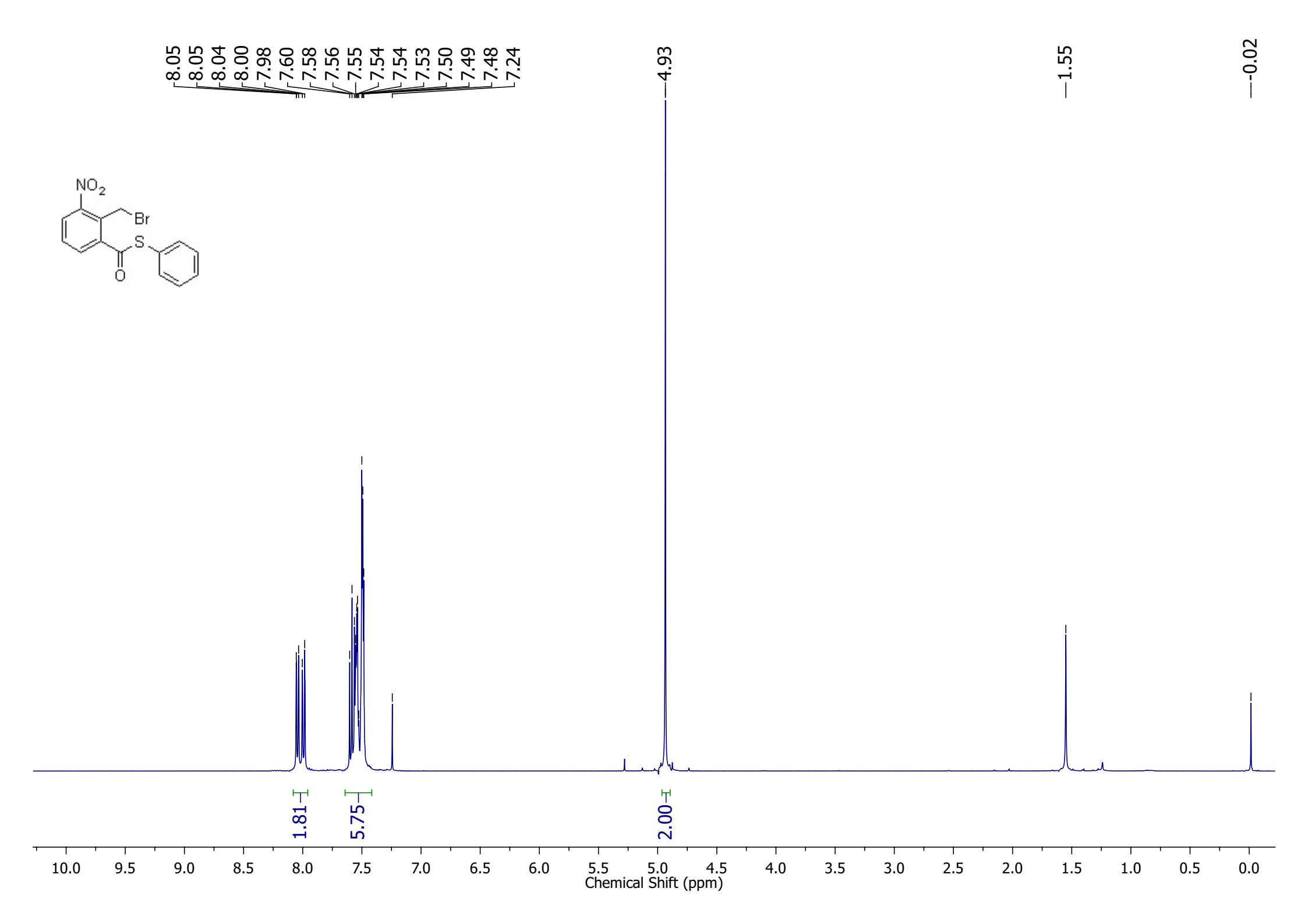

163.73
150.79
149.36
135.10
133.44
132.88
131.52
129.45
128.56
123.35
119.86
77.43
77.11
76.79
23.51
22.55
NO2
Br
O
O
Br
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

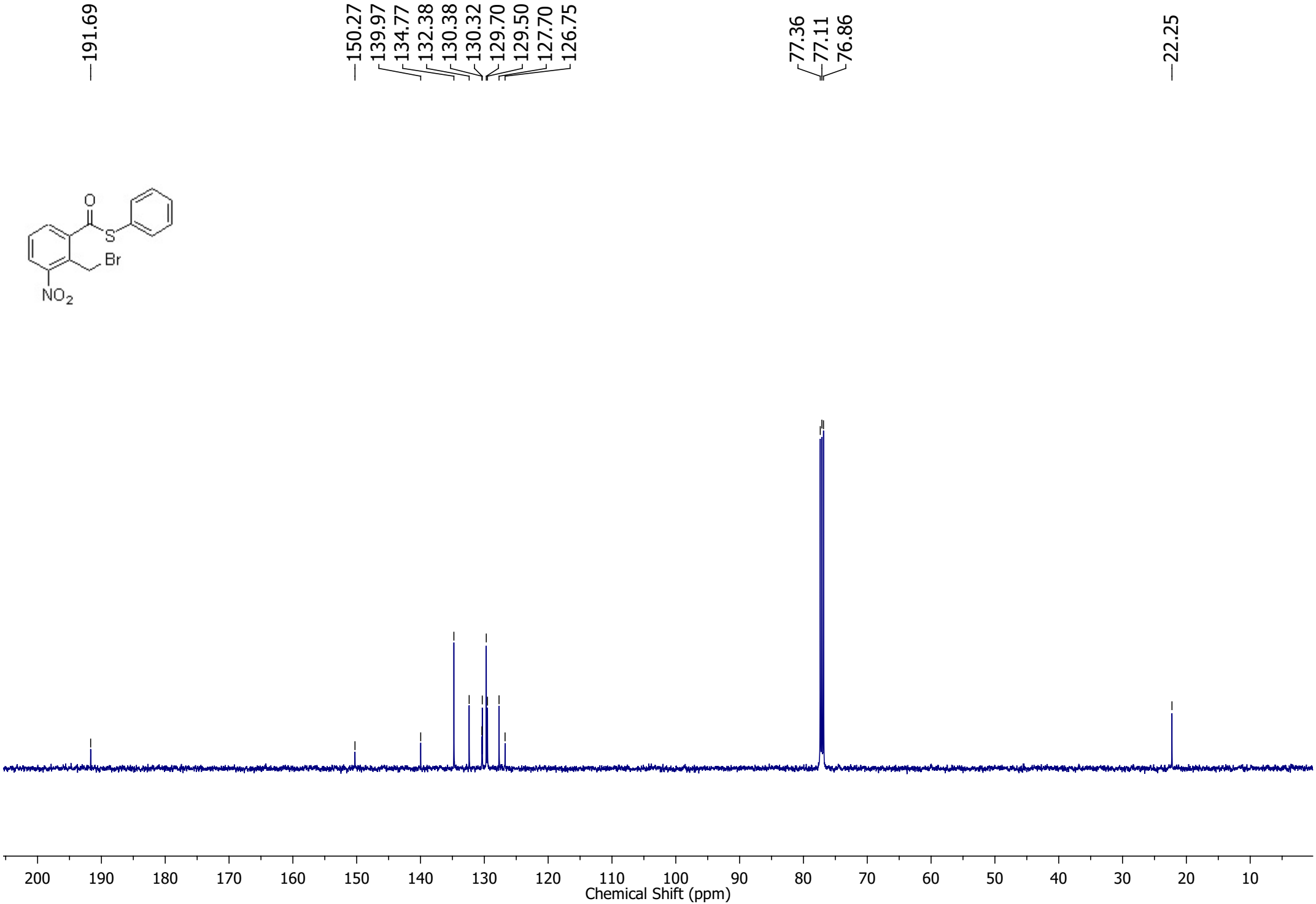

8.39
8.39
8.37
8.37
8.08
8.07
7.69
7.67
7.65
7.24
5.12
2.69
1.55
1.27
1.24
-0.02
NO2
Br
F
O
O
F
F
F
F
0.89
0.85
0.96
2.00
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

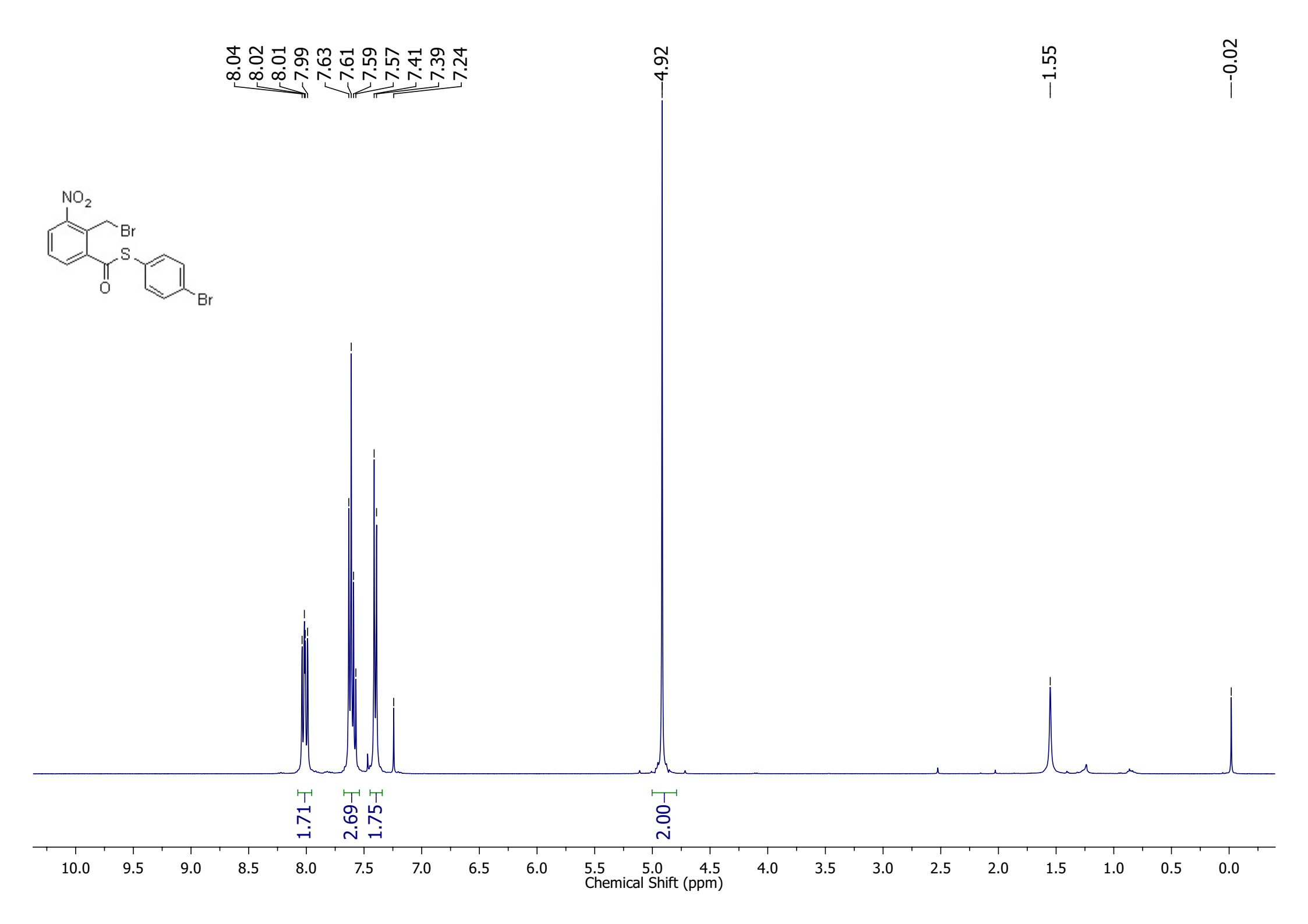

161.19
150.92
135.51
134.41
129.70
129.58
128.93
77.34
77.09
76.83
29.77
21.84
NO2
Br
F
F
F
F
F
O
O
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1

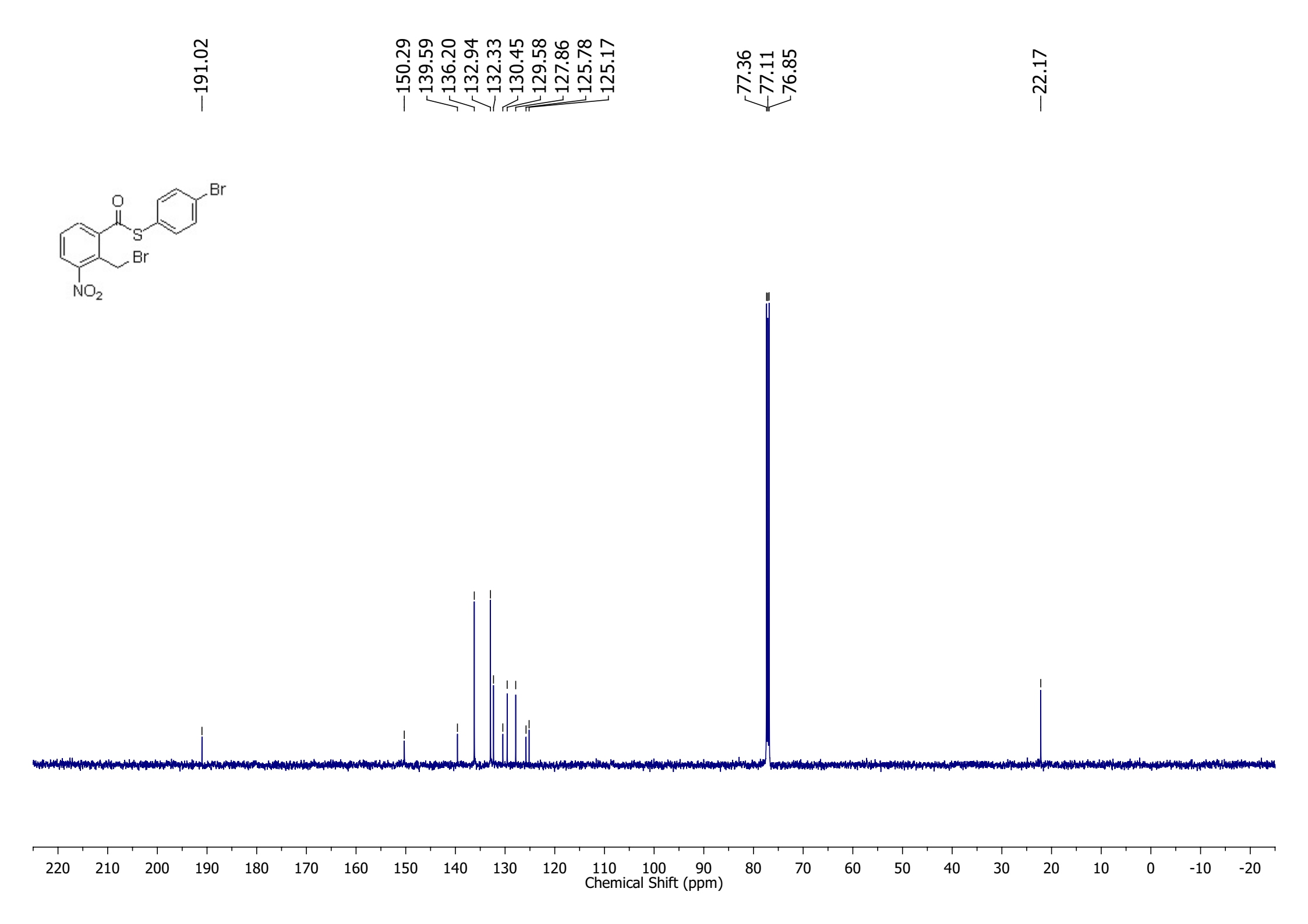

9.02
9.01
8.58
8.56
8.55
7.65
7.65
7.63
7.61
7.20
7.19
5.08
1.51
1.18
0.81
0.00
-0.00
NO2
Br
NO2
O
O
NO2
0.71
0.94
0.97
0.87
2.00
2.10
Chemical Shift (ppm)

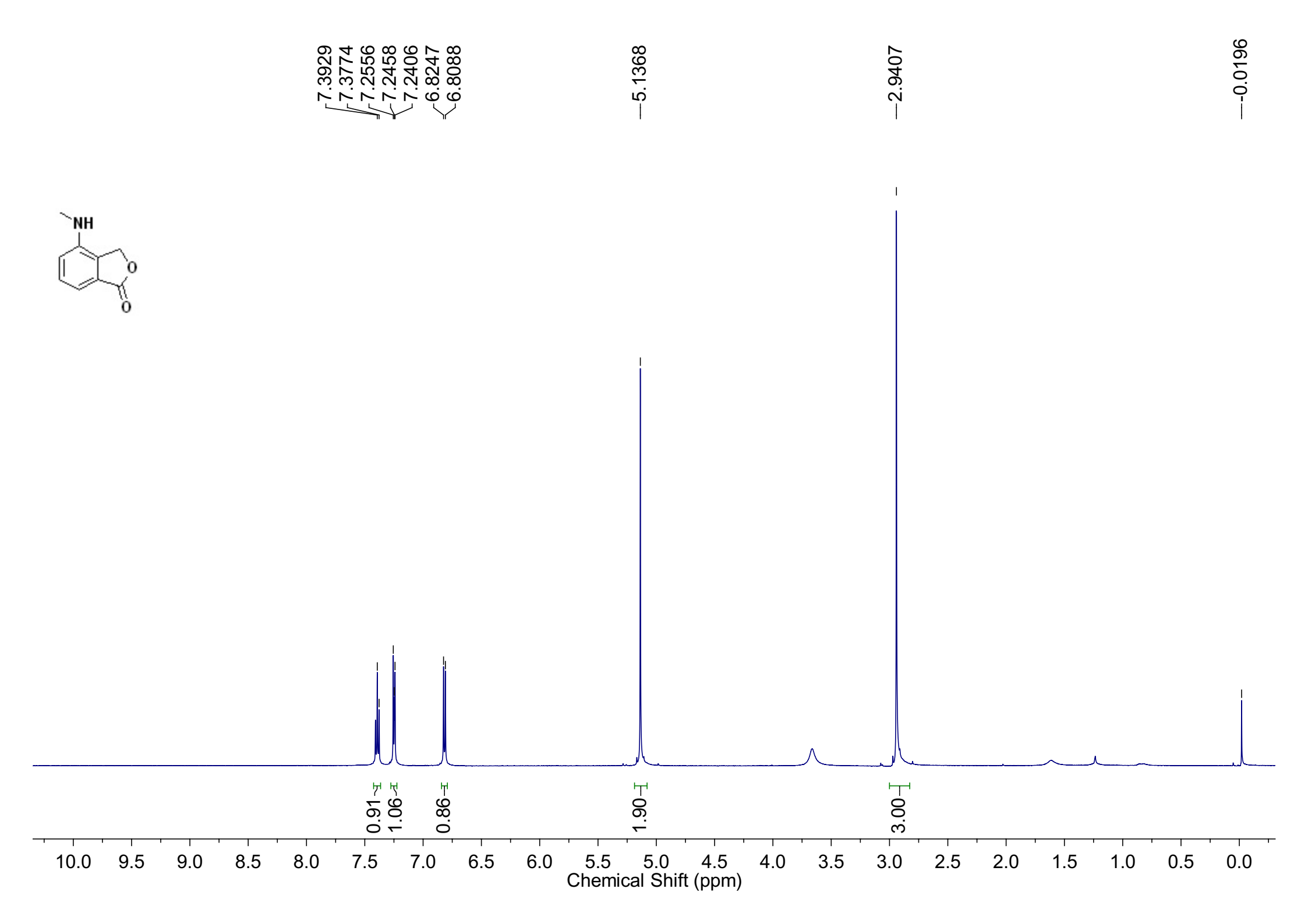

162.51
152.46
148.35
145.54
141.73
135.23
134.79
129.82
129.39
128.57
127.06
126.83
122.07
77.41
77.09
76.77
16.15
O2N
NO2
O
O
Br
NO2
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Chemical Shift (ppm)

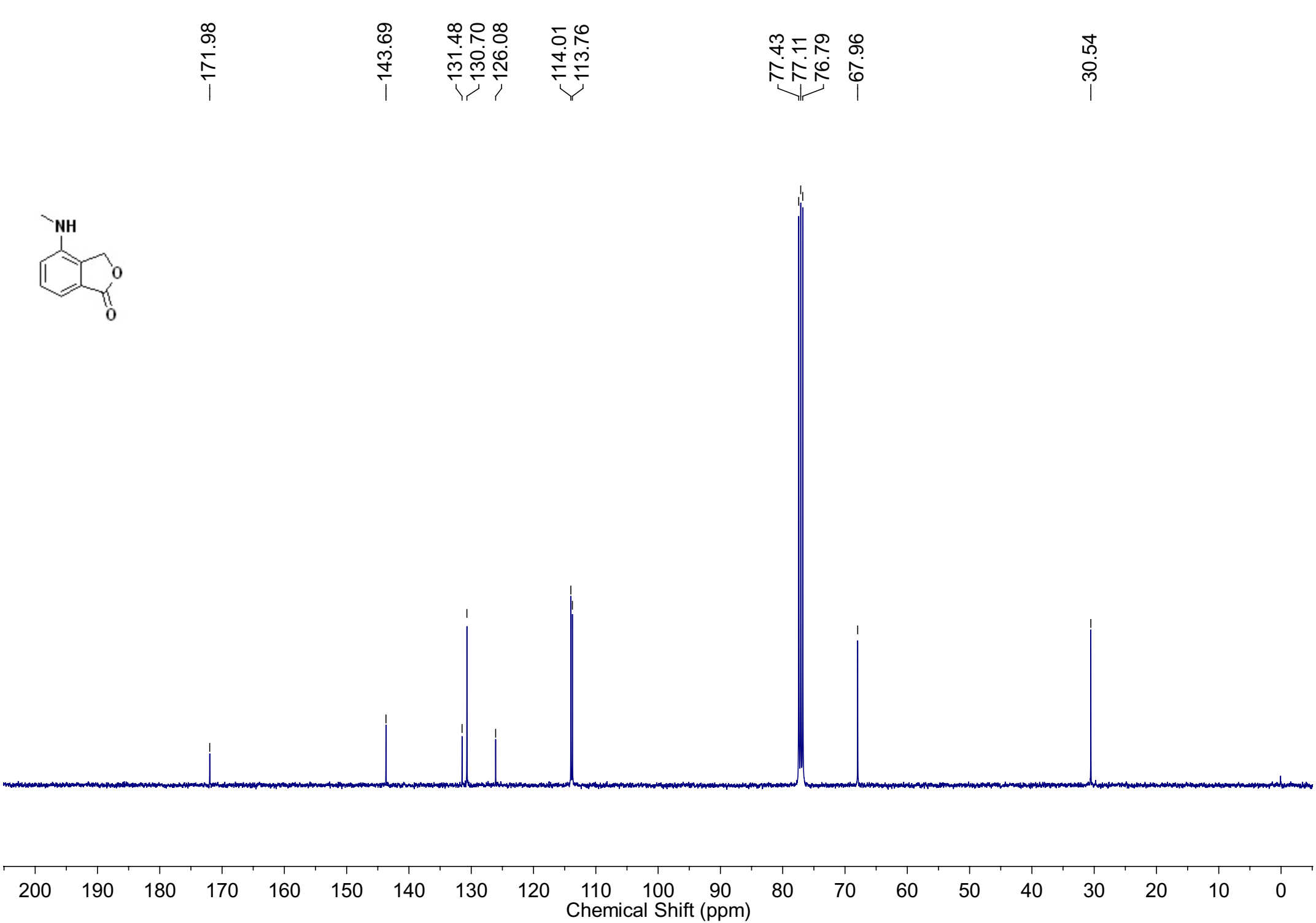

8.05
8.05
8.04
8.00
7.98
7.60
7.58
7.56
7.55
7.54
7.54
7.53
7.50
7.49
7.48
7.24
4.93
1.55
-0.02
NO2
Br
S
O
1.81
5.75
2.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

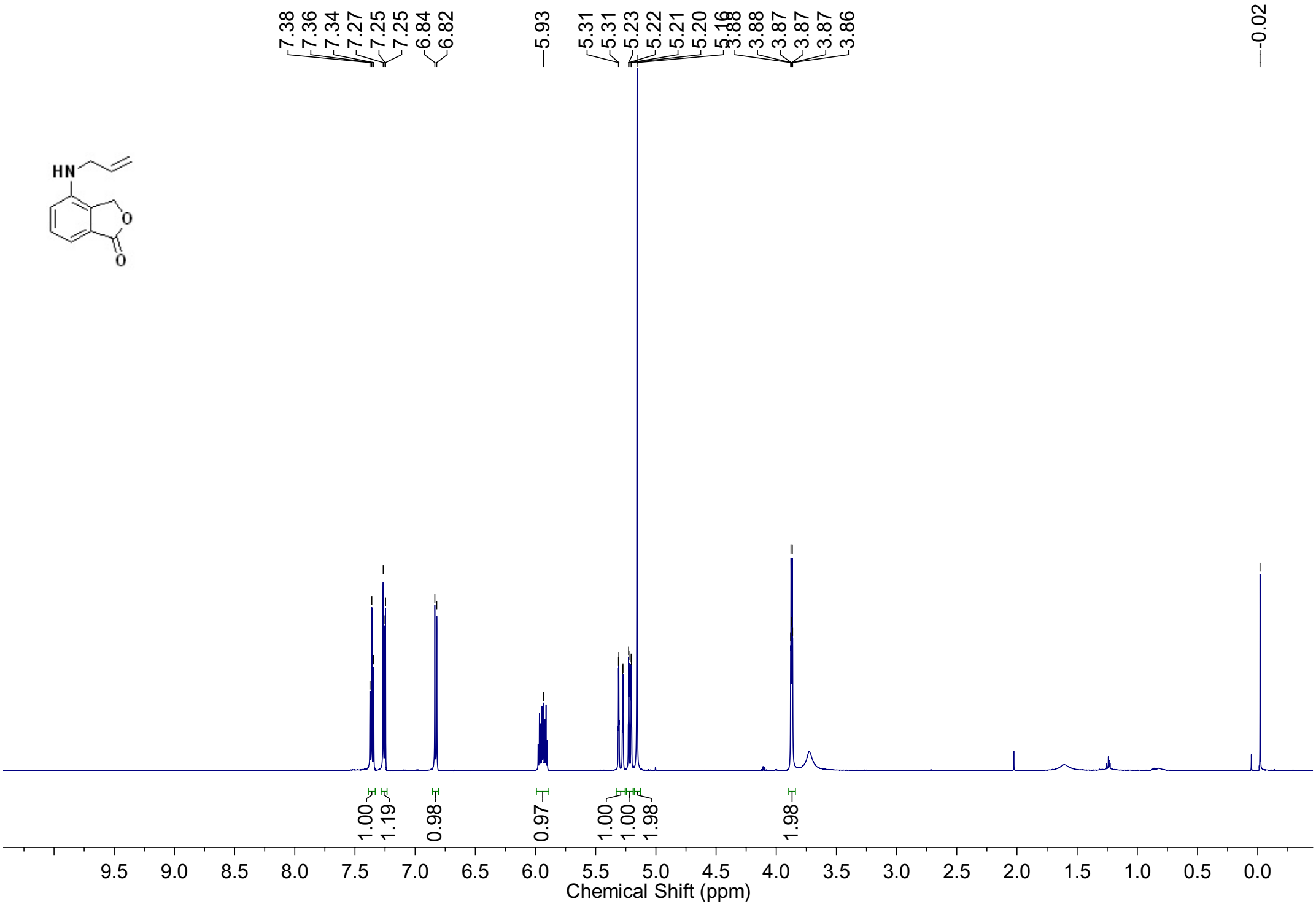

191.69
150.27
139.97
134.77
132.38
130.38
130.32
129.70
129.50
127.70
126.75
77.36
77.11
76.86
22.25
O
S
Br
NO2
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Chemical Shift (ppm)

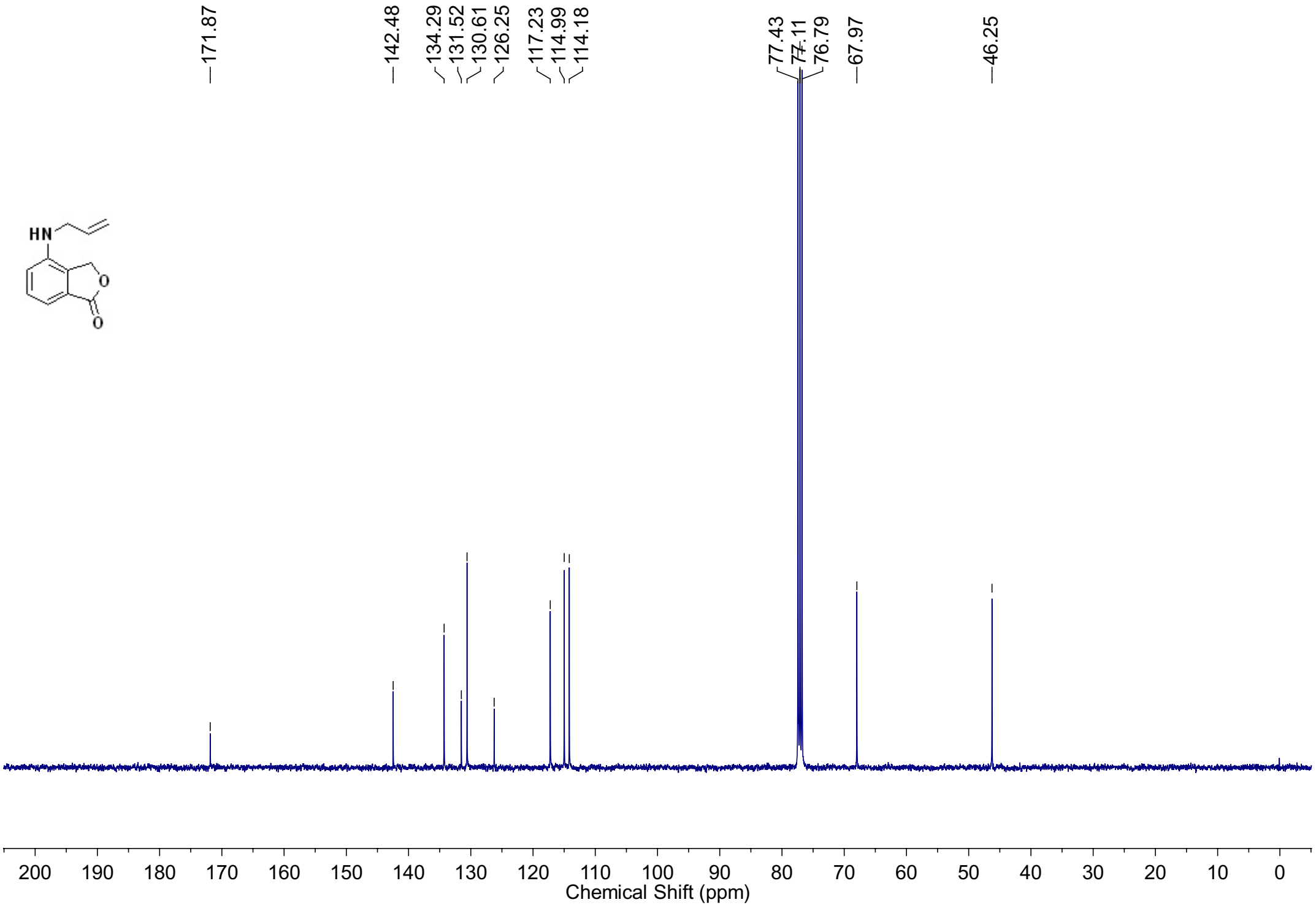

8.04
8.02
8.01
7.99
7.63
7.61
7.59
7.57
7.41
7.39
7.24
4.92
1.55
-0.02
NO2
Br
S
O
Br
1.71
2.69
1.75
2.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

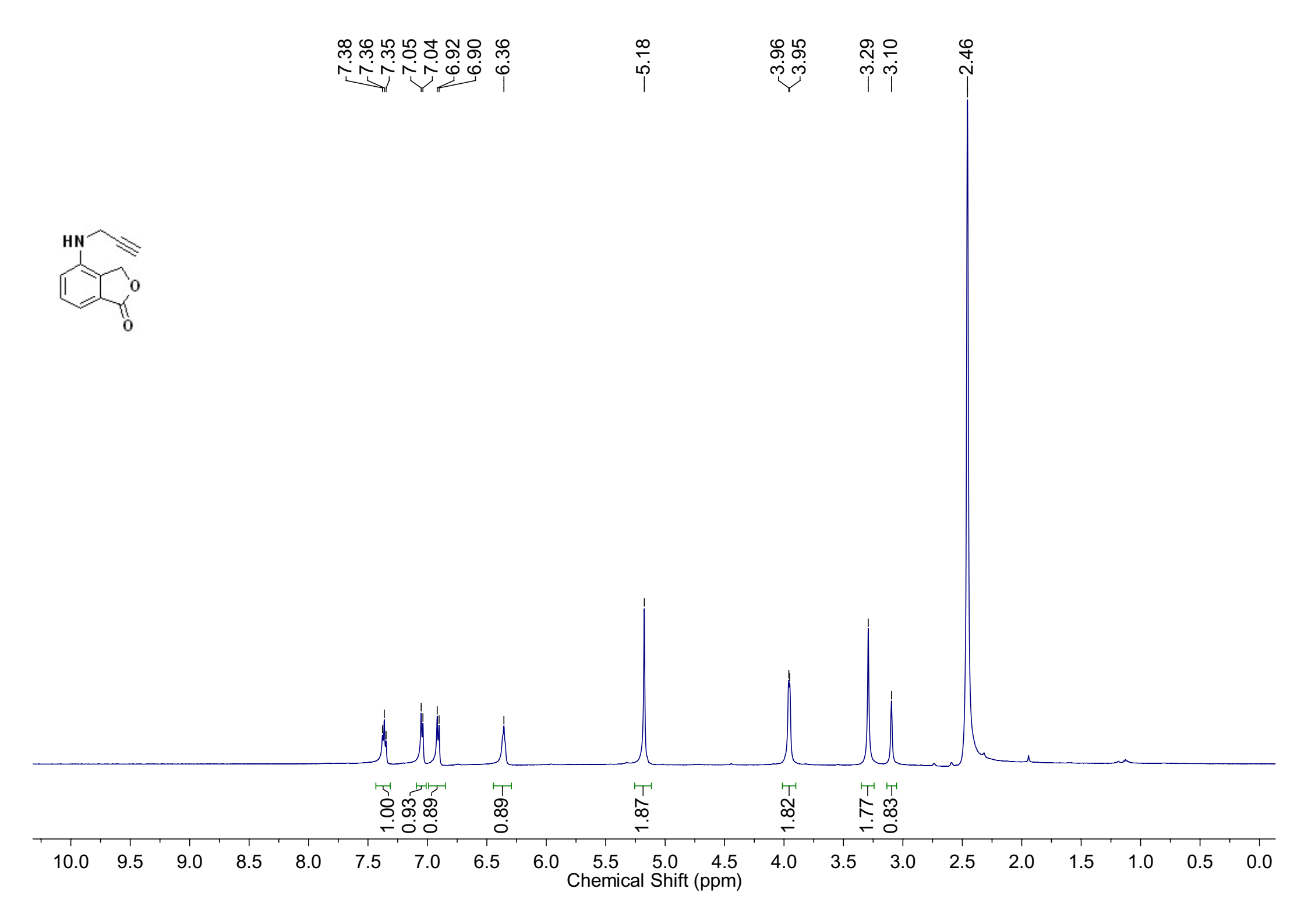

191.02
150.29
139.59
136.20
132.94
132.33
130.45
129.58
127.86
125.78
125.17
77.36
77.11
76.85
22.17
O
S
Br
Br
NO2
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
Chemical Shift (ppm)

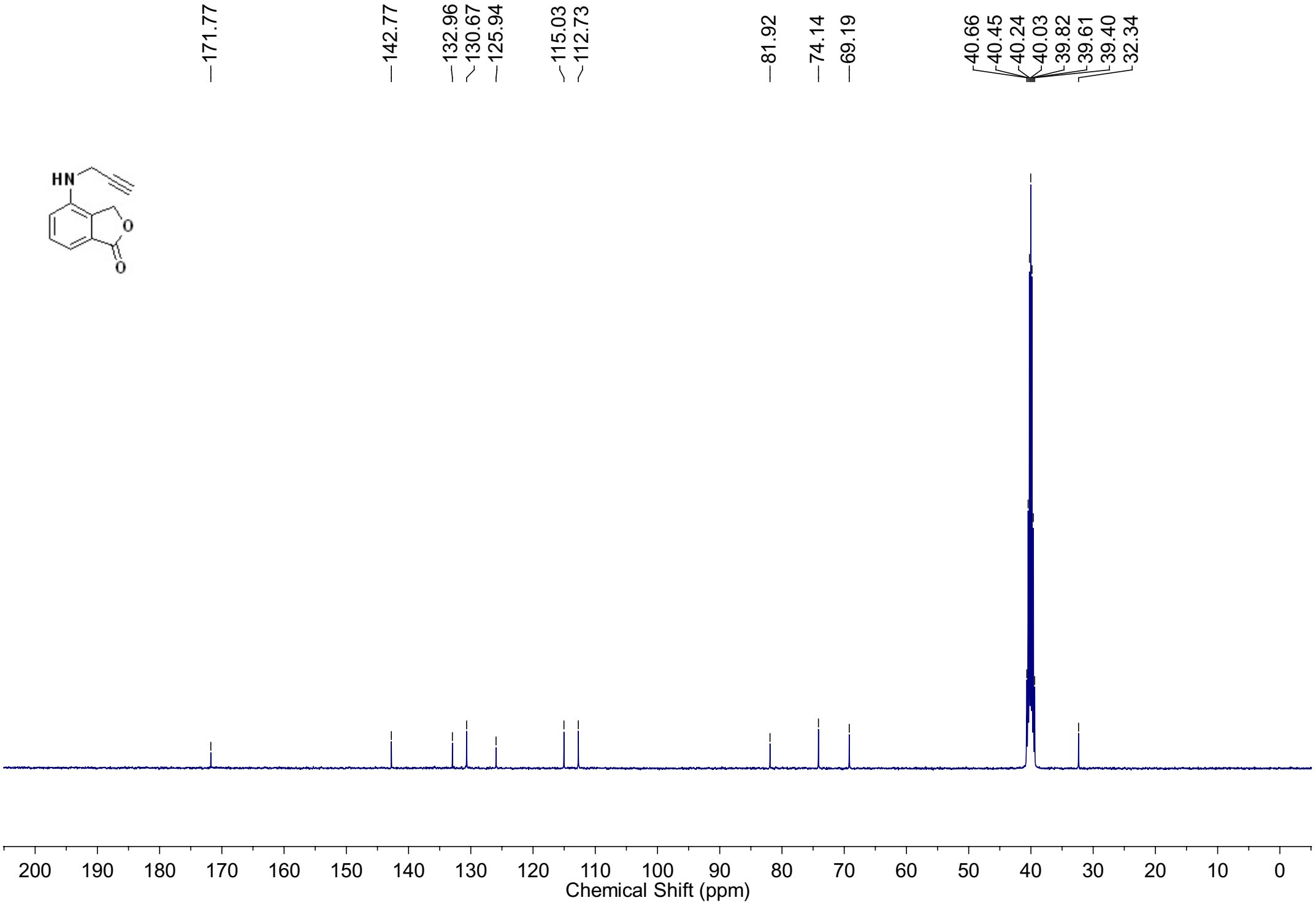

7.3929
7.3774
7.2556
7.2458
7.2406
6.8247
6.8088
5.1368
2.9407
-0.0196
NH
O
O
0.91
1.06
0.86
1.90
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

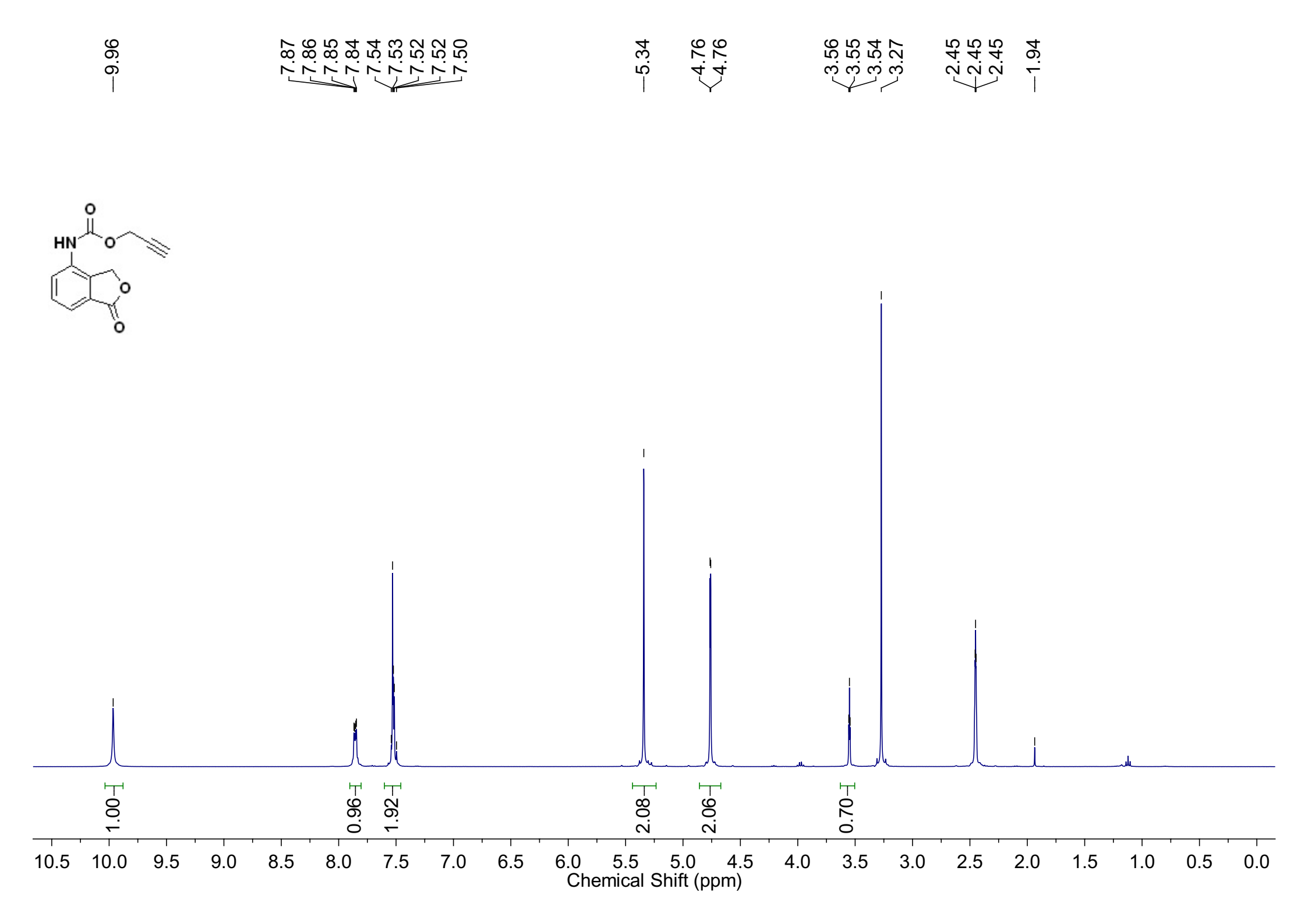

171.98
143.69
131.48
130.70
126.08
114.01
113.76
77.43
77.11
76.79
67.96
30.54
NH
O
O
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

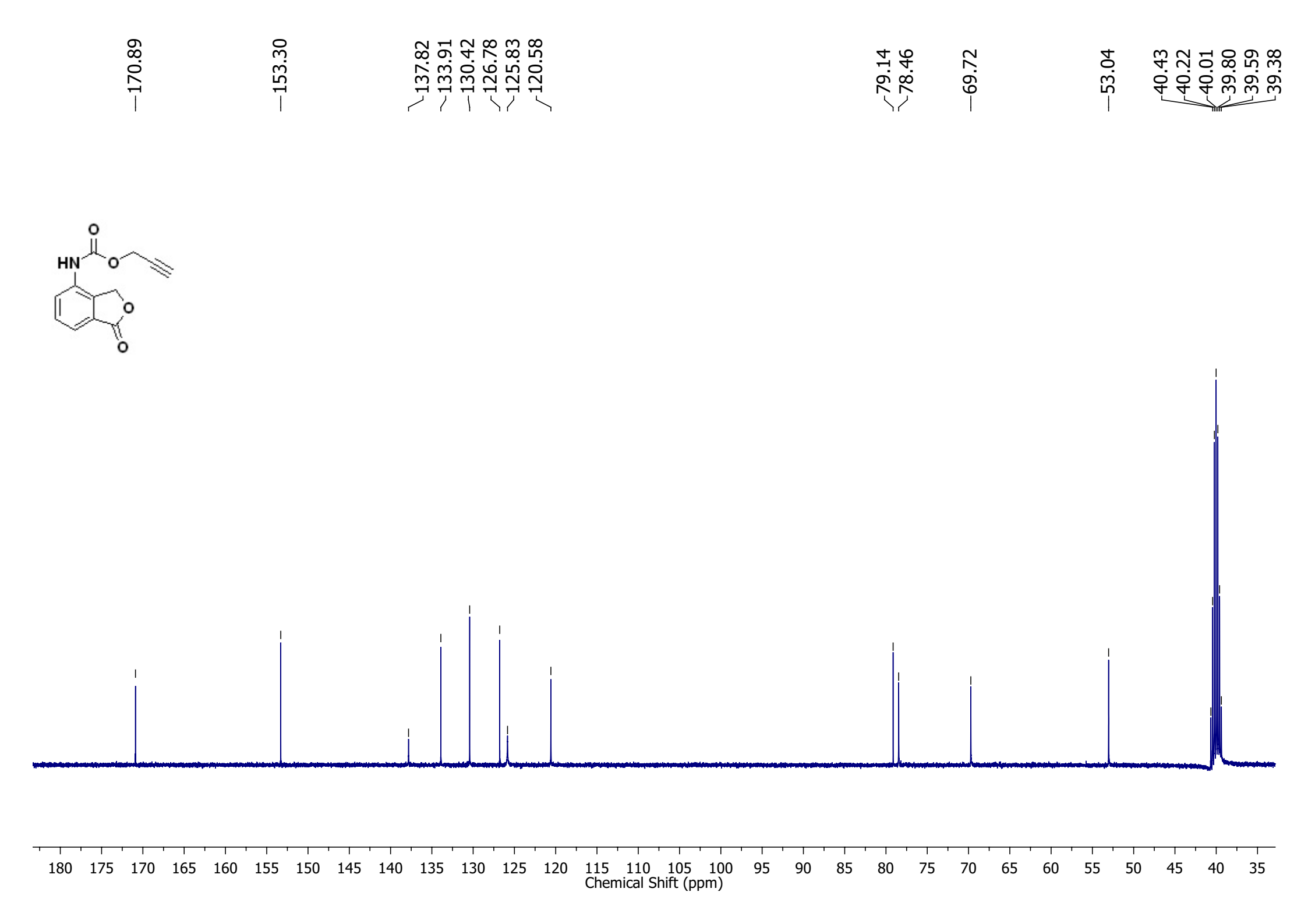

7.38
7.36
7.34
7.27
7.25
7.25
6.84
6.82
5.93
5.31
5.31
5.23
5.22
5.21
5.20
5.16
3.88
3.88
3.87
3.87
3.87
3.86
-0.02
HN
O
O
1.00
1.19
0.98
0.97
1.00
1.00
1.98
1.98
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

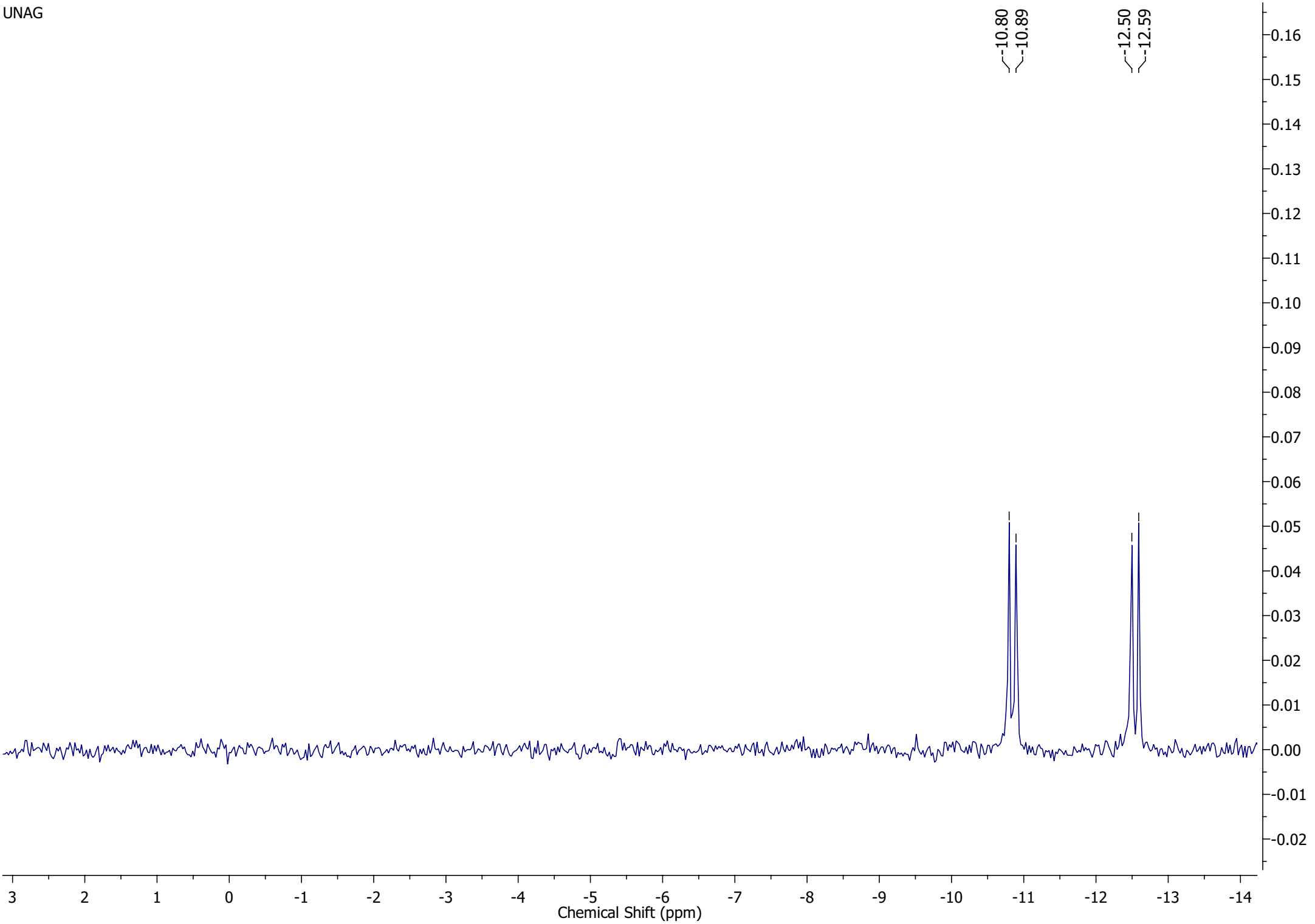

171.87
142.48
134.29
131.52
130.61
126.25
117.23
114.99
114.18
77.43
77.11
76.79
67.97
46.25
HN
O
O
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

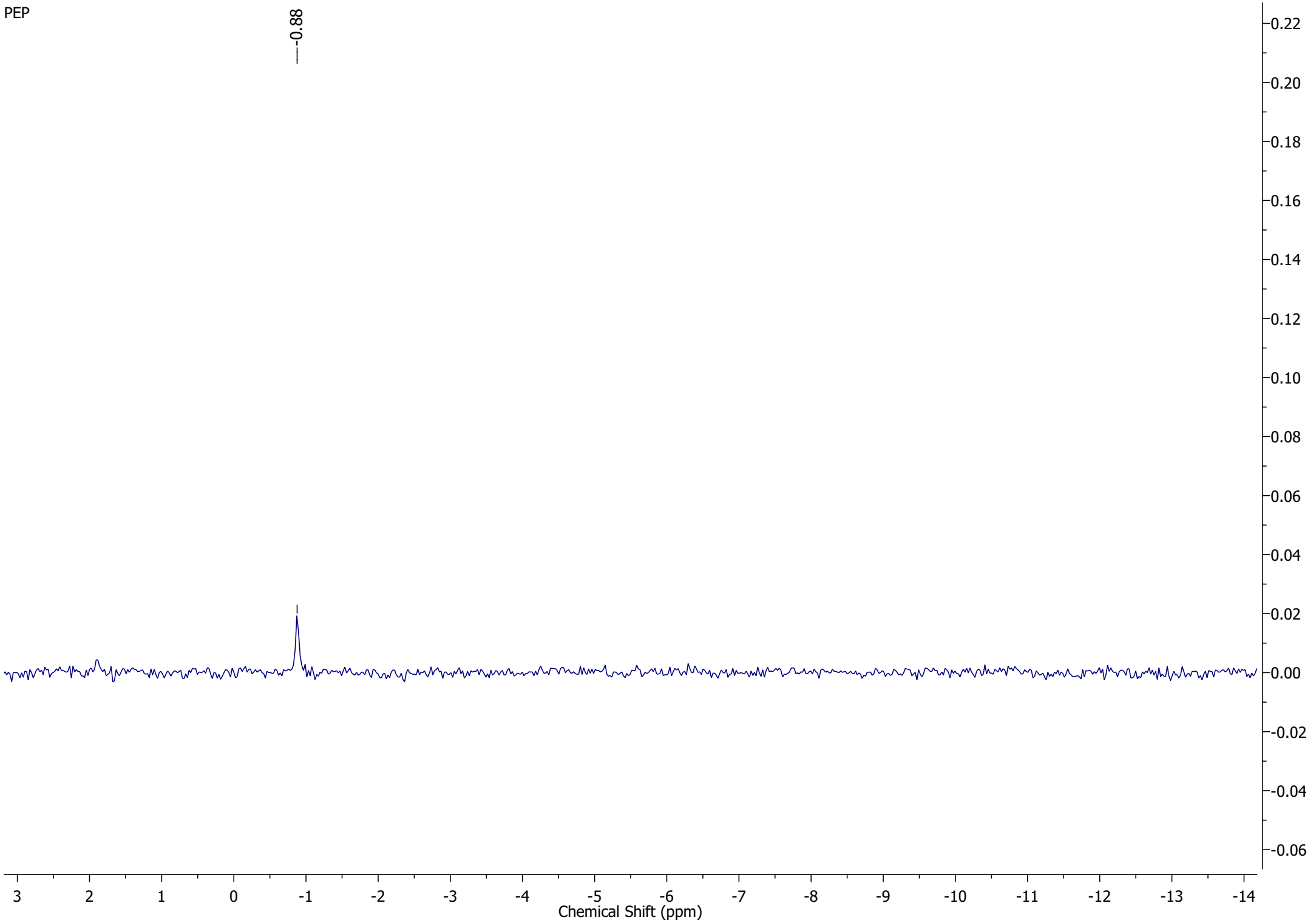

7.38
7.36
7.35
7.05
7.04
6.92
6.90
6.36
5.18
3.96
3.95
3.29
3.10
2.46
HN
O
O
1.00
0.93
0.89
0.89
1.87
1.82
1.77
0.83
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

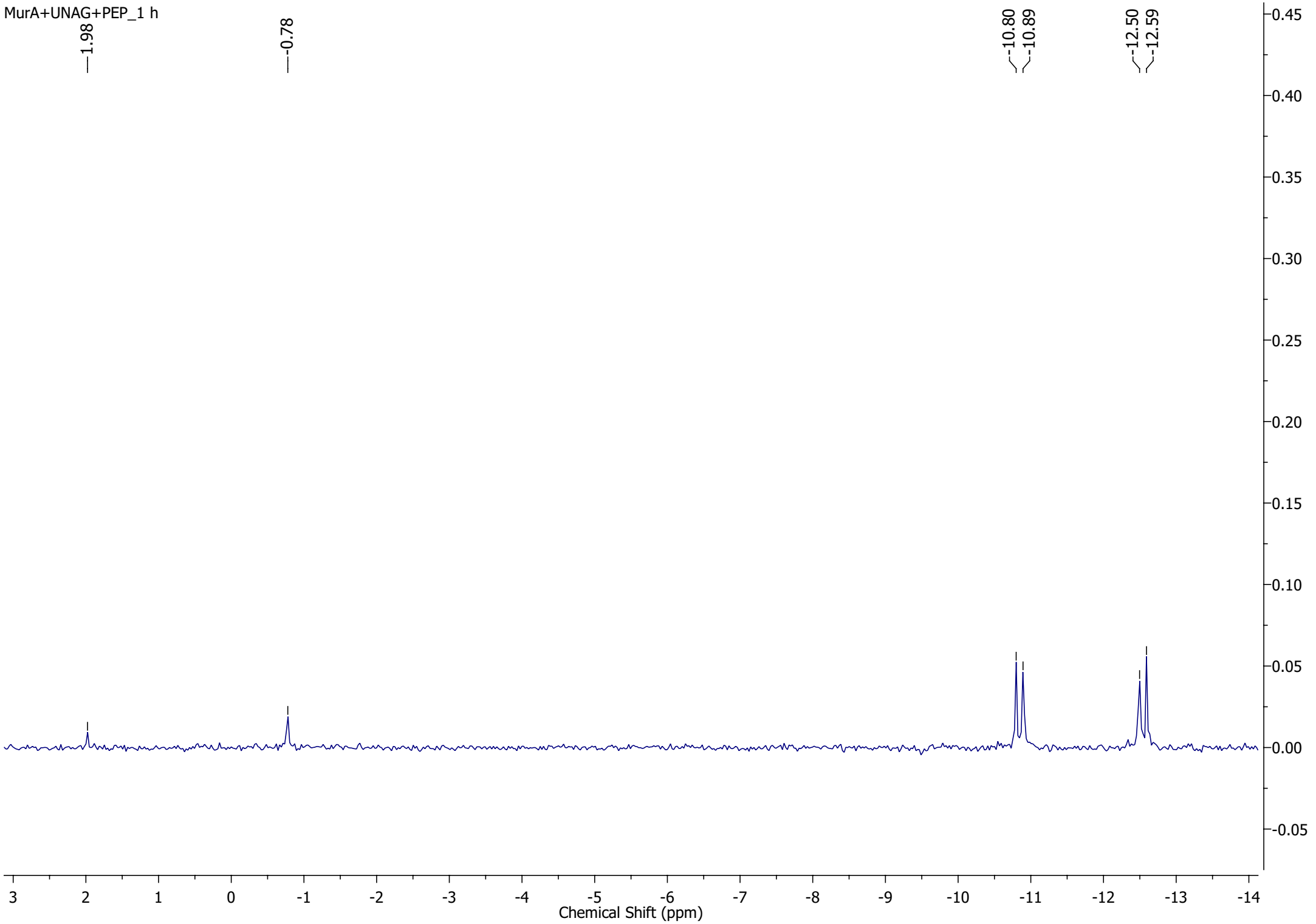

171.77
142.77
132.96
130.67
125.94
115.03
112.73
81.92
74.14
69.19
40.66
40.45
40.24
40.03
39.82
39.61
39.40
32.34
HN
O
O
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Chemical Shift (ppm)

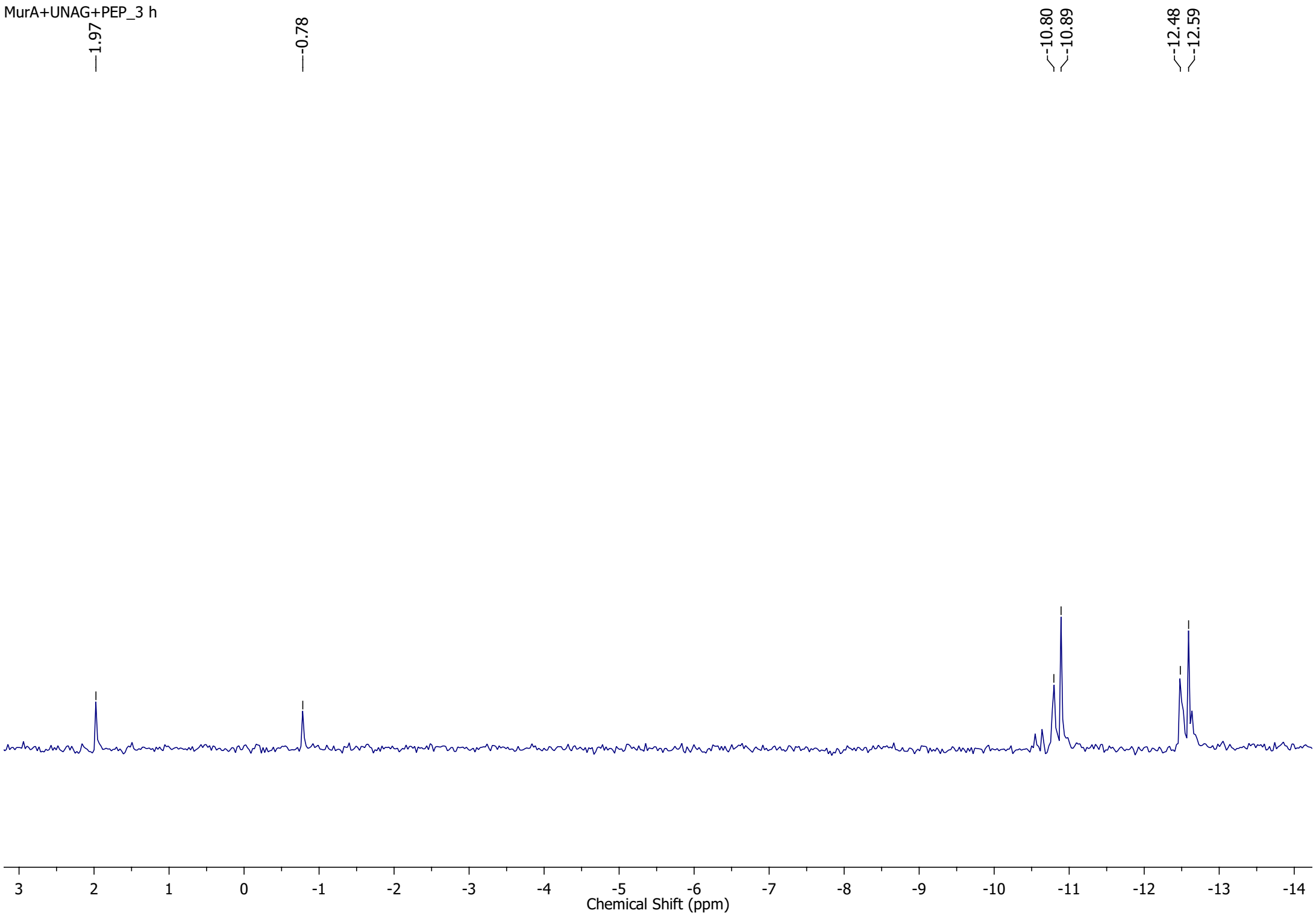

9.96
7.87
7.86
7.85
7.84
7.54
7.53
7.52
7.52
7.50
5.34
4.76
4.76
3.56
3.55
3.54
3.27
2.45
2.45
2.45
1.94
HN
O
O
O
O
1.00
0.96
1.92
2.08
2.06
0.70
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chemical Shift (ppm)

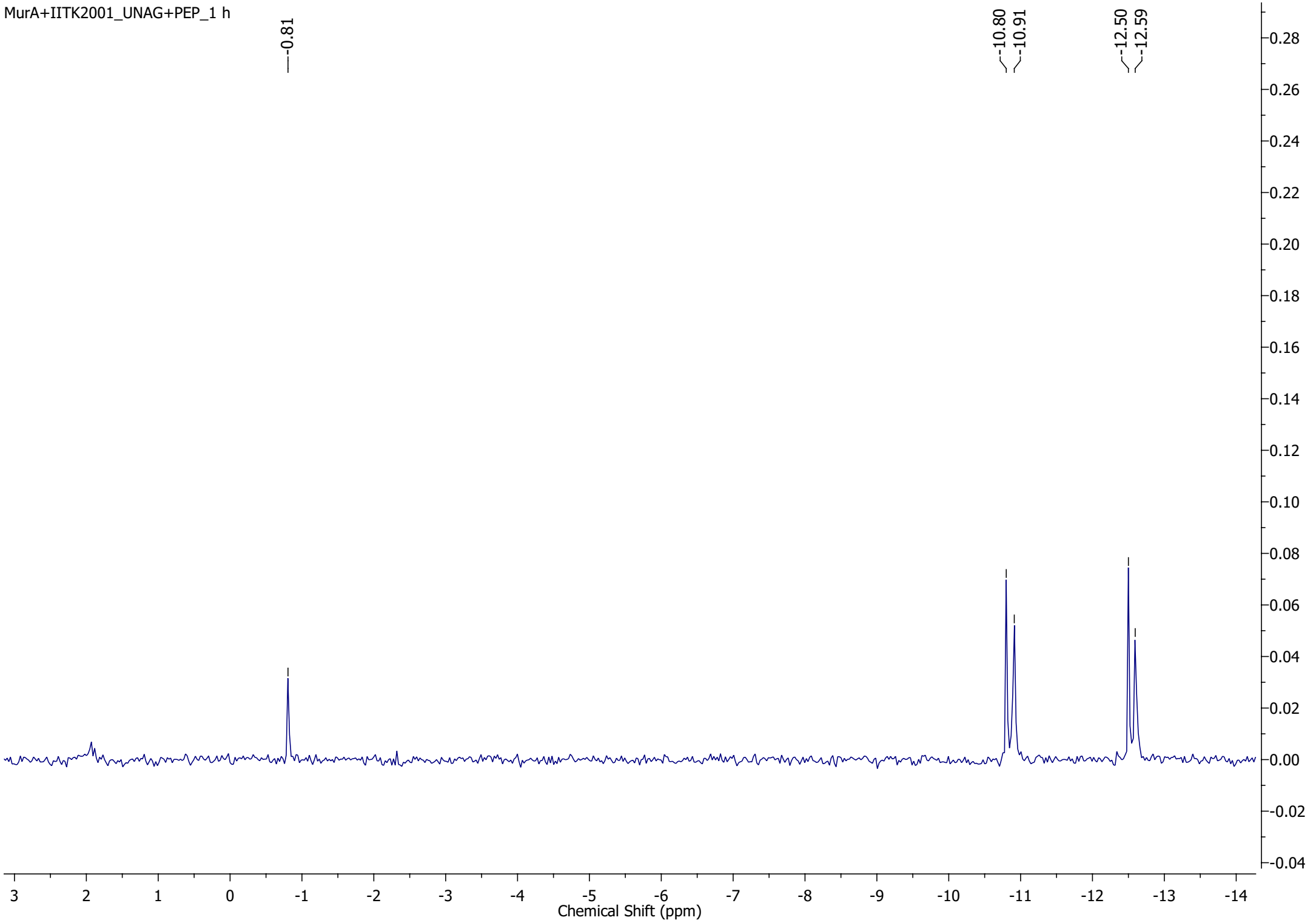

170.89
153.30
137.82
133.91
130.42
126.78
125.83
120.58
79.14
78.46
69.72
53.04
40.43
40.22
40.01
39.80
39.59
39.38
HN
O
O
O
O
180 175 170 165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35
Chemical Shift (ppm)

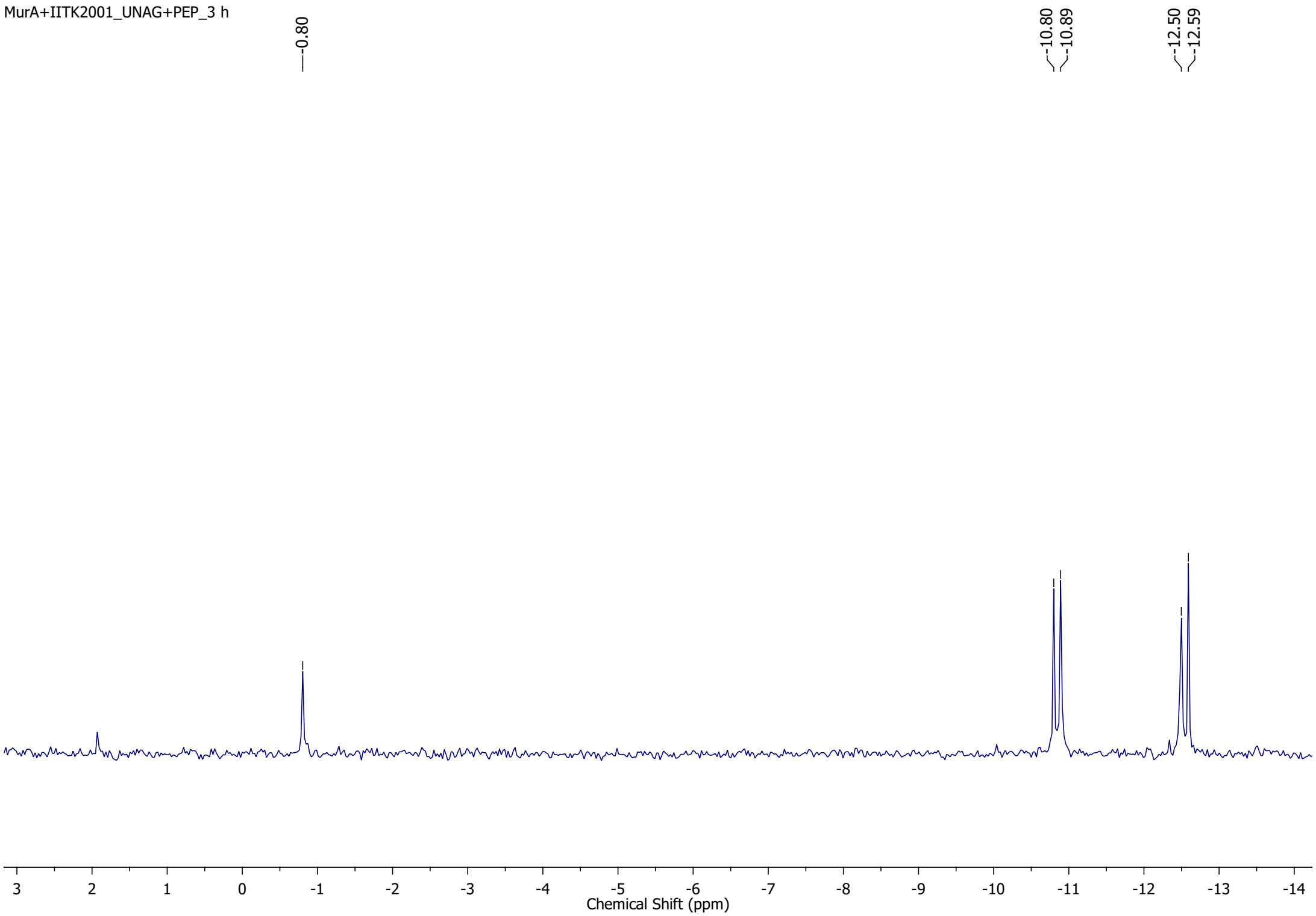

UNAG
-10.80
-10.89
-12.50
-12.59
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)

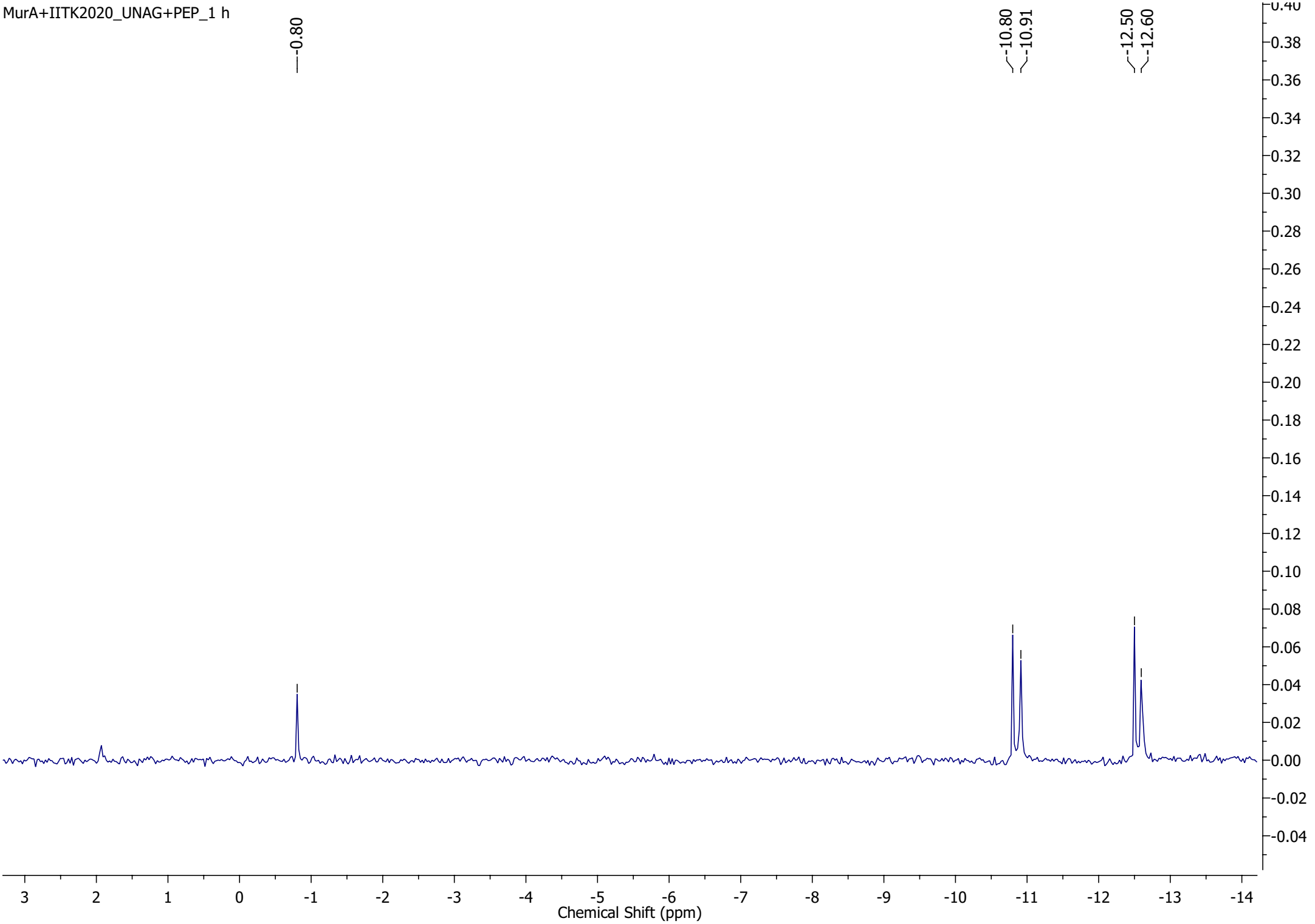

PEP
-0.88
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)

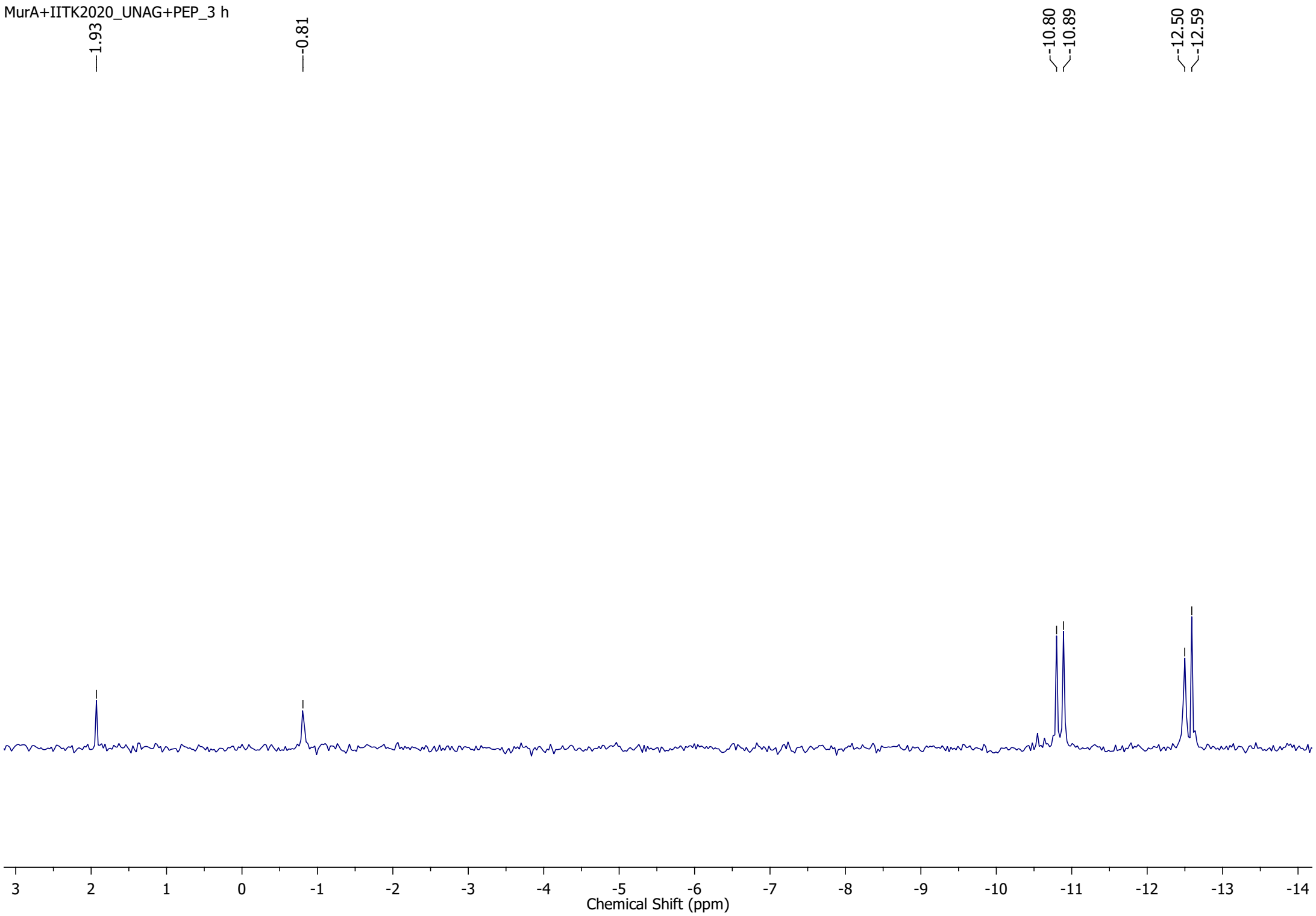

MurA+UNAG+PEP_1 h
1.98
-0.78
-10.80
-10.89
-12.50
-12.59
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)


MurA+UNAG+PEP_3 h
1.97
-0.78
-10.80
-10.89
-12.48
-12.59
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)


MurA+IITK2001_UNAG+PEP_1 h
-0.81
-10.80
-10.91
-12.50
-12.59
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)


MurA+IITK2001_UNAG+PEP_3 h
-0.80
-10.80
-10.89
-12.50
-12.59
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)


MurA+IITK2020_UNAG+PEP_1 h
-0.80
-10.80
-10.91
-12.50
-12.60
0.40
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)


MurA+IITK2020_UNAG+PEP_3 h
1.93
0.81
-10.80
-10.89
-12.50
-12.59
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Chemical Shift (ppm)