Supporting Information

Exploitation of antibiotic resistance as a novel drug target: development of a β-lactamase-activated antibacterial prodrug

Lindsay E. Evans[†,‡], Aishwarya Krishna[†], Yajing Ma[‡], Thomas E. Webb[§], Dominic C. Marshall[§], Catherine L. Tooke[‖], James Spencer[‖], Thomas B. Clarke[†], Alan Armstrong[‡] and Andrew M. Edwards*[†]*

[†] MRC Centre for Molecular Bacteriology and Infection, Imperial College London, SW7 2AZ, United Kingdom.

[‡] Department of Chemistry, Molecular Sciences Research Hub, Imperial College London, W12 0BZ, United Kingdom.

[§] Department of Medicine, Imperial College London, SW7 2AZ, United Kingdom.

[I] School of Cellular and Molecular Medicine, University of Bristol, Biomedical Sciences Building, University Walk, Bristol, BS8 1TD, United Kingdom.

Table of Contents

Additional Data

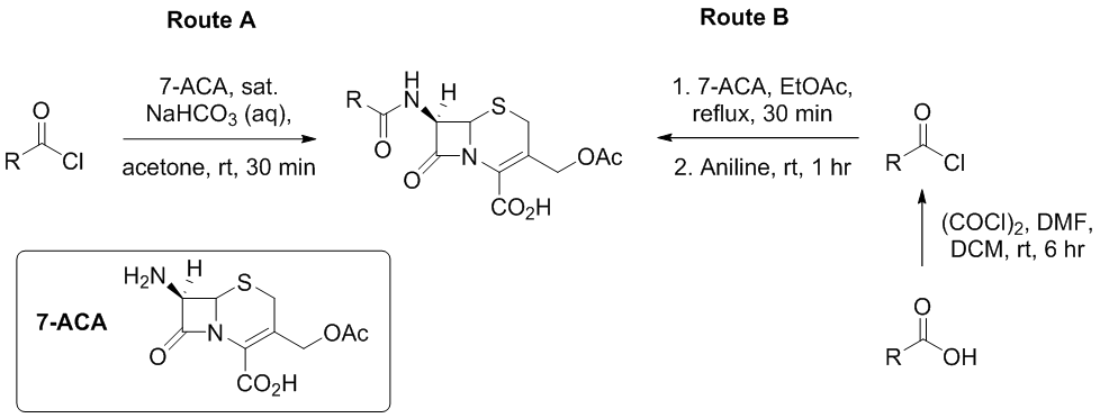


Figure S1. Summary of synthetic routes to β-lactam analogues **9** – **25**.[1,2]

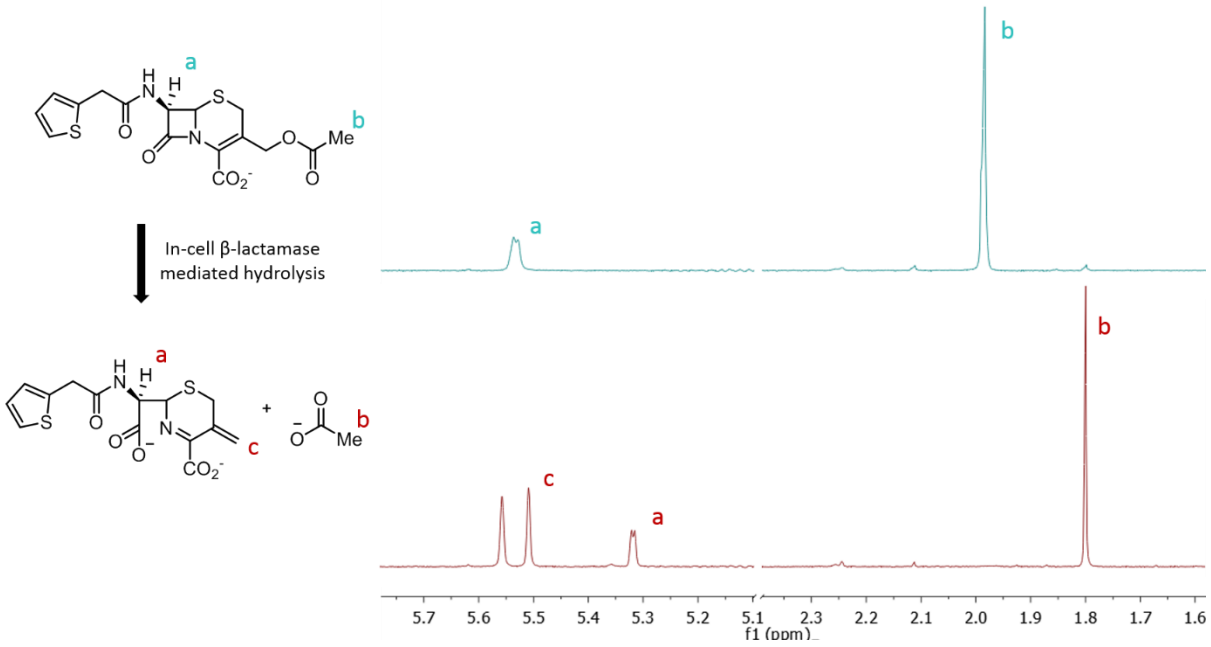


Figure S2. Example of ^{1}H NMR signals used to determine β-Lactamase hydrolytic activity in whole-cell NMR assay. Chemical structures and section of the ^{1}H NMR spectra of ciprofloxacin **31** (blue) and corresponding hydrolysis products (red).

Table S1. Summary of MIC values determined for ciprofloxacin **31** and pro-drug **35** against six uropathogenic *E. coli* clinical isolates.

Strain	MIC value in µM, n=3	
	Ciprofloxacin **31**	Pro-drug **35**
Ec11	0.63	0.63
Ec12	> 5	> 5
Ec13	> 5	> 5
Ec16	0.08	0.31
Ec17	2.5	2.5
Ec19	> 5	> 5
Ec11	0.63	0.63

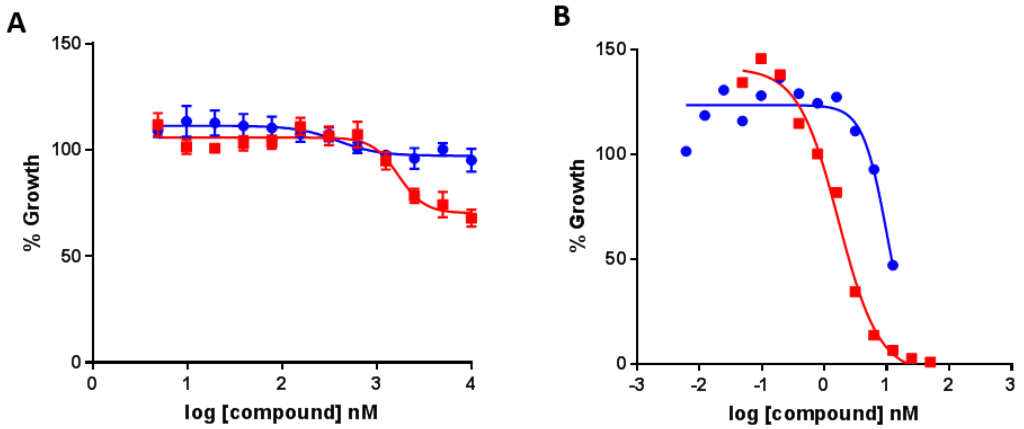


Figure S3. Antibacterial activities for pro-drug **35** (blue) and ciprofloxacin **31** (red) against *E. faecalis* (A) ATCC29212 and (B) GW01.

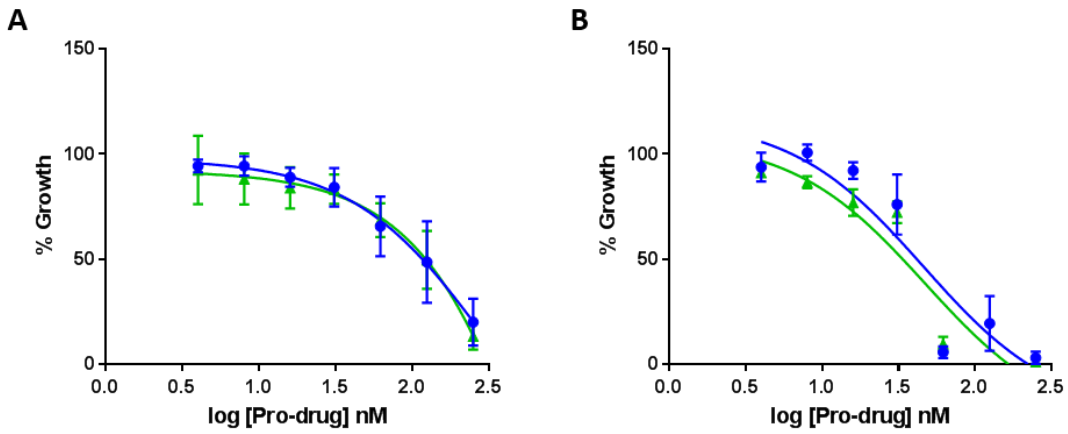


Figure S4. Antibacterial activities for pro-drug **35** against *E. coli* CFT073 expressing (A) empty plasmid (pEMP) and (B) CTX-M-15 (pCTX) with (green) and without (blue) serum.

Experimental Procedures (Biology)

Recombinant β-lactamase protein

Amp-C

Recombinant Amp-C protein was purchased from Abcam (ab104926) and used without further purification.

CTX-M-15

Recombinant CTX-M-15 was expressed with an *N*-terminal His6-tag in SoluBL21 (DE3) cells and purified as described previously.[1] Briefly, bacterial cells were grown to an OD_{600} of 0.6-0.8 in 2x YT media at 37 °C with shaking. CTX-M-15 expression was induced with 0.5 mM IPTG, after which cells were grown at 18 °C for a further 16 h. Cells were harvested by centrifugation at 6,500 xg for 10 mins at 4 °C before the pellet was resuspended in 50 mM Hepes (pH 7.5) with 400mM NaCl and complete EDTA-free protease inhibitor (Roche). Cells were homogenised and lysed via two passages through a cell disruptor (constant systems) at 30 kpsi. The resulting cell lysate was centrifuged at 100,000 xg for 1 hour at 4 °C before incubation with 4 ml of Ni-NTA bead slurry (Qiagen) for 1.5 hours with 10 mM imidazole to prevent non-specific binding. Ni-NTA resin bound to protein was washed with 50 mM Hepes pH 7.5, 400 mM NaCl,10 mM imidazole followed by 50 mM Hepes pH 7.5, 300 mM NaCl, 20 mM imidazole and eluted after 5 minute incubation with 50 mM Hepes pH 7.5, 200 mM NaCl, 400 mM imidazole. The His_6 tag was cleaved and removed as described previously using 3C protease.[3]

Bacterial Strains

Supplementary Table 1. Bacterial strains and characteristics relevant to this study

Bacterium/ plasmid	**Relevant characteristics**	**Source/Ref**
***E. coli* strains**		
DH5α	β-lactam and ciprofloxacin susceptible	
DH5α pQE80	Strain transformed with a multi-copy plasmid that contains a gene for a TEM116 β-lactamase, conferring resistance to ampicillin and cephalosporins	This study
DH5α pCTX	Strain transformed with a multi-copy plasmid that contains the gene for the CTX-M-1 β-lactamase, conferring resistance to ampicillin and cephalosporins	This study
CFT073	Isolated from a case of polynephritis (ATCC 700928). Ciprofloxacin susceptible.	Mobley et al., 1990[4]
CFT073 pEMP	Transformed with empty pSU18. Does not express β-lactamase.	This study
CFT073 pCTX	Expresses CTX-M-1 β-lactamase. Resistant to penicillins and cephalosporins	This study
CFT073 pNDM	Expresses NDM-1 β-lactamase Resistant to penicillins, cephalosporins and carbapenems	This study
CFT073 pKPC	Expresses KPC-3 β-lactamase. Resistant to penicillins, cephalosporins and carbapenems	This study
11	Isolated from a human UTI. Expresses CTX-M-15 β-lactamase. Ciprofloxacin susceptible.	This study
12	Isolated from a human UTI. Expresses CTX-M-15 β-lactamase. Ciprofloxacin resistant.	This study
13	Isolated from a human UTI. Expresses CTX-M-15 β-lactamase. Ciprofloxacin resistant.	This study
16	Isolated from a human UTI. Expresses CTX-M-15 β-lactamase. Ciprofloxacin susceptible.	This study
17	Isolated from a human UTI. Expresses CTX-M-15 β-lactamase. Ciprofloxacin susceptible.	This study
19	Isolated from a human UTI. Expresses CTX-M-15 β-lactamase. Ciprofloxacin resistant.	This study
***E. faecalis* strains**		
ATCC29212	Vancomycin-sensitive strain isolated from a human UTI.	American type culture collection
GW01	Vancomycin-sensitive strain isolated from a human UTI.	This study
Plasmids		
pEMP	pSU18 vector without coding sequences for beta-lactamases.	Bartolomé et al., 1991[5]
pCTX	pSU18 containing the native coding sequence for CTX-M1 under the control of the native promoter	Brem et al., 2016[6]

pNDM	pSU18 containing the native coding sequence for NDM-1 under the control of the native promoter	Jiménez-Castellanos et al., 2018[7]
pKPC	pSU18 containing the native coding sequence for KPC-3 under the control of the native promoter	Brem et al., 2016[6]

Transformation of *E. coli* with plasmids

Plasmids were introduced via heat shock into chemically competent DH5a and via electroporation into *E. coli* CFT073 following published protocols.[8]

Bacterial cultures

The bacterial strains and plasmids used in this study are detailed in Supplementary Table 1. *E. coli* was grown in Mueller-Hinton Broth (MHB) or Lennox Broth (LB) at 37 °C, with shaking (180 r.p.m.) overnight (16-18 h). Where required, culture medium was supplemented with Chloramphenicol (25 μg ml^{-1}). *E. faecalis* was grown statically in Brain-Heart Infusion Broth (BHI) at 37 °C in 5% CO_2 overnight (16-18 h).

Assay Procedures

Determination of MIC

MIC assays were performed in 96-well thermo scientific Nuclon Delta Surface plates and MICs were determined in accordance with the broth microdilution protocol.[7] *E. coli* grown to stationary-phase in MHB or LB were adjusted to OD_{600} nm of 0.05 in MHB and supplemented with a range of concentrations (two-fold dilution series) of ciprofloxacin or test compounds. For some assays with *E. coli*, MHB was supplemented with 10 % normal human serum (Sigma-Aldrich). For assays with *E. faecalis*, BHI was used in place of MHB.

After static incubation at 37 °C for 18 h in air (*E. coli*) or 5 % CO_2 (*E. faecalis*), the MIC was determined as the lowest concentration of antibiotic that inhibited visible bacterial growth. OD_{595} measurements were recorded on a BioRad 1Mark Microplate Reader. Measurements were background corrected against the no-inoculum control and normalized to the no-drug control.

Determination of bactericidal activity

Stationary-phase bacteria were inoculated into 3 ml MHB containing no antibiotic, ciprofloxacin (78 nM) or prodrug (78 nM), to give an OD_{600} nm of 0.1. Cultures were subsequently incubated at 37 °C with shaking (180 r.p.m.) and bacterial viability determined by c.f.u. counts. All compound concentrations were at 2.5 × MIC of ciprofloxacin, which has been shown previously to be bactericidal.

Recombinant β-lactamase assay

Each compound was incubated for 1 hour at room temperature in 100 mM $NaPO_4$ (pH 7) ± 0.5 M NaOH. The wavelength at which the difference between un-hydrolysed and hydrolysed compound was the greatest was selected (typically 263 nm) and a compound concentration vs. absorbance standard curve was generated. Compounds, typically 250 μM in assay buffer (100 mM $NaPO_4$ (pH 7)), were dispensed into a Corning 96-well UV transparent flat bottom plate. Recombinant Amp-C in assay buffer was added immediately before incubation in the microplate reader at 37 ºC. UV absorbance was measured every 20 s for 1 h on a BMG LABTECH SPECTROstarNano. Kinetic parameters were determined by half-life analysis.

NMR β-lactamase hydrolysis assay

Overnight bacterial cultures (5 – 25 ml) were pelleted by centrifugation at 3,200 x g for 20 min at 4 °C. Pellets were washed twice with 100 mM $NaPO_4$ (pH 7.0), 10 mM $MgCl_2$ and cultures were corrected to an OD_{600} nm of 2.5 in 100 mM $NaPO_4$ (pH 7.0), 10 mM $MgCl_2$,

10% (v/v) deuterated water. Compounds were prepared in deuterated DMSO and added to 700 µl of culture to give a final compound concentration of 100 µM and incubated at room temperature for 1 h.

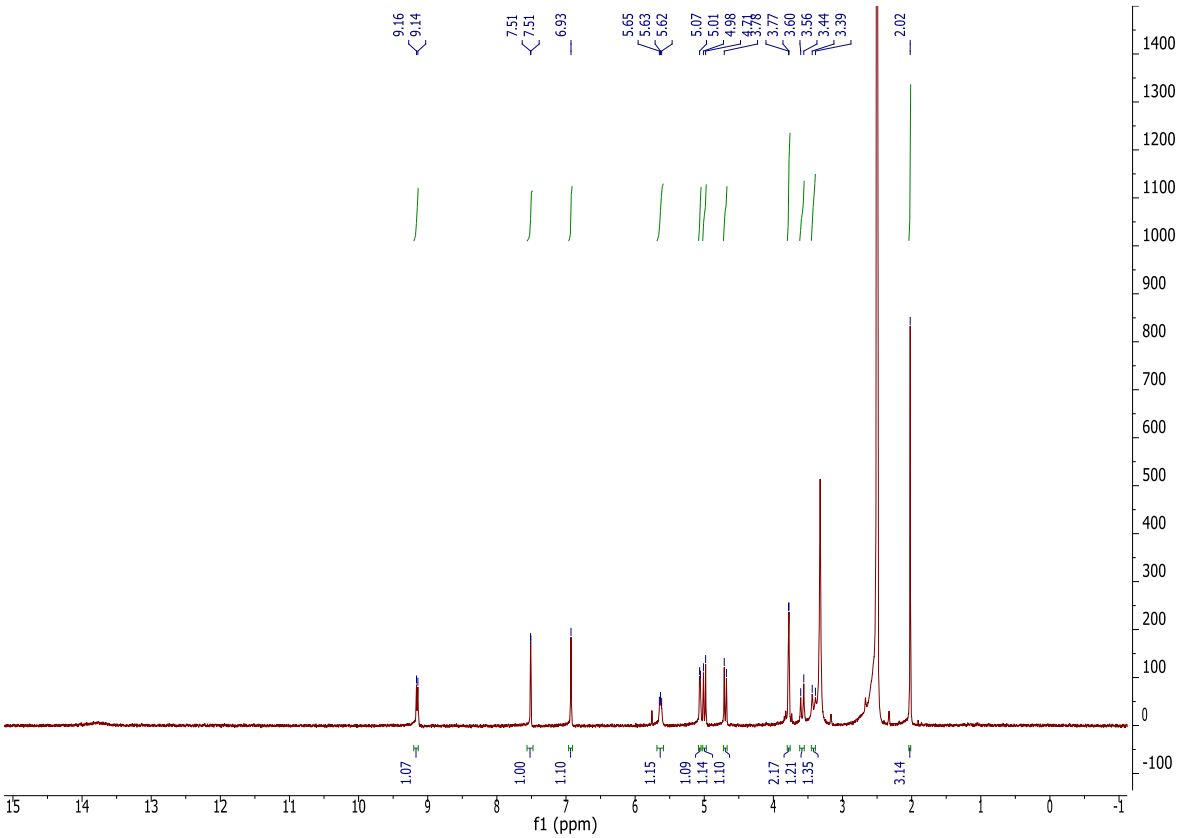
^{1}H NMR spectra were collected at 298 K on a Bruker 500MHz AVANCE III HD spectrometer running TopSpin3.2 and equipped with a z-gradient bbfo/5mm tuneable SmartProbe and a GRASP II gradient spectroscopy accessory providing a maximum gradient output (100%) of 53.5G/cm (5.35G/cmA). ^{1}H water suppression spectra[8] were collected using the Bruker pulse program zgesgp at a frequency of 500.13MHz with a spectral width of 10 kHz (centred on 4.705 ppm) and 65536 data points. A relaxation delay of 1s was employed along with square shaped 180o selective pulses of 2 ms (Squa100.1000 from Bruker library) and gradients pulses of 1ms. The strength of the first pair of gradient pulses was 31% and the second pair 11%. All gradient pulses were smoothed-square shaped (SMSQ10.100 from Bruker library) and after each application a recovery delay of 200us used. 64 transients were collected after 4 dummy scans. The data was processed using 65536 data points applying an exponential function with a line broadening of 0.3 Hz. Integration of peaks corresponding to the intact and hydrolysed products was performed using MestReNova 8.0 and used to determine the percentage hydrolysis.

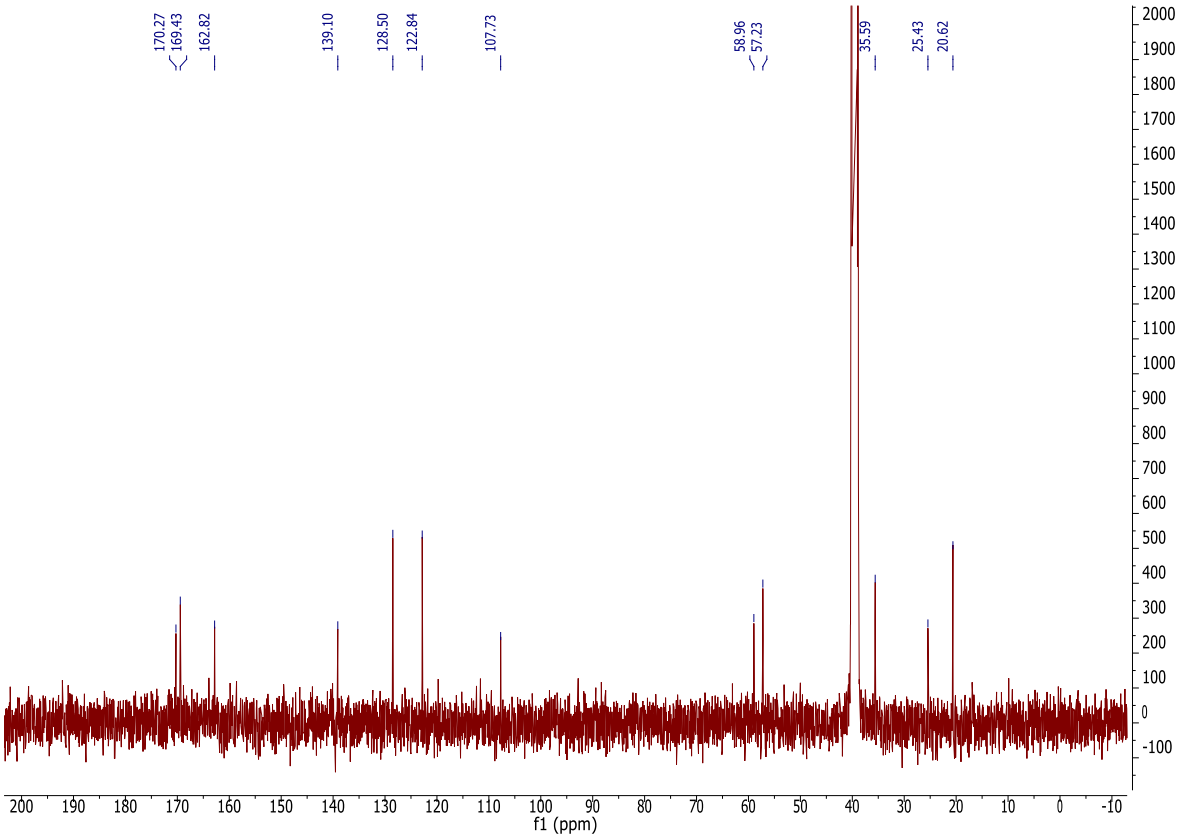
Recombinant DNA gyrase assay

The *E. coli* gyrase supercoiling assay (Inspiralis) was performed according to the manufacturer's instructions with the addition of 6 nM recombinant CTX-M-15 where required. Samples were loaded on a 1% (w/v) agarose gel prepared with Tris/Borate/EDTA (TBE) or Tris/Acetate/EDTA (TAE) buffer. Electrophorese was carried out at 100 V for 90 minutes. The gel was then stained with SYBR Safe DNA gel stain (Invitrogen) (1:1000 dilution in TBE) for 30 min. Visualization was performed using a Syngene Gel Doc system.

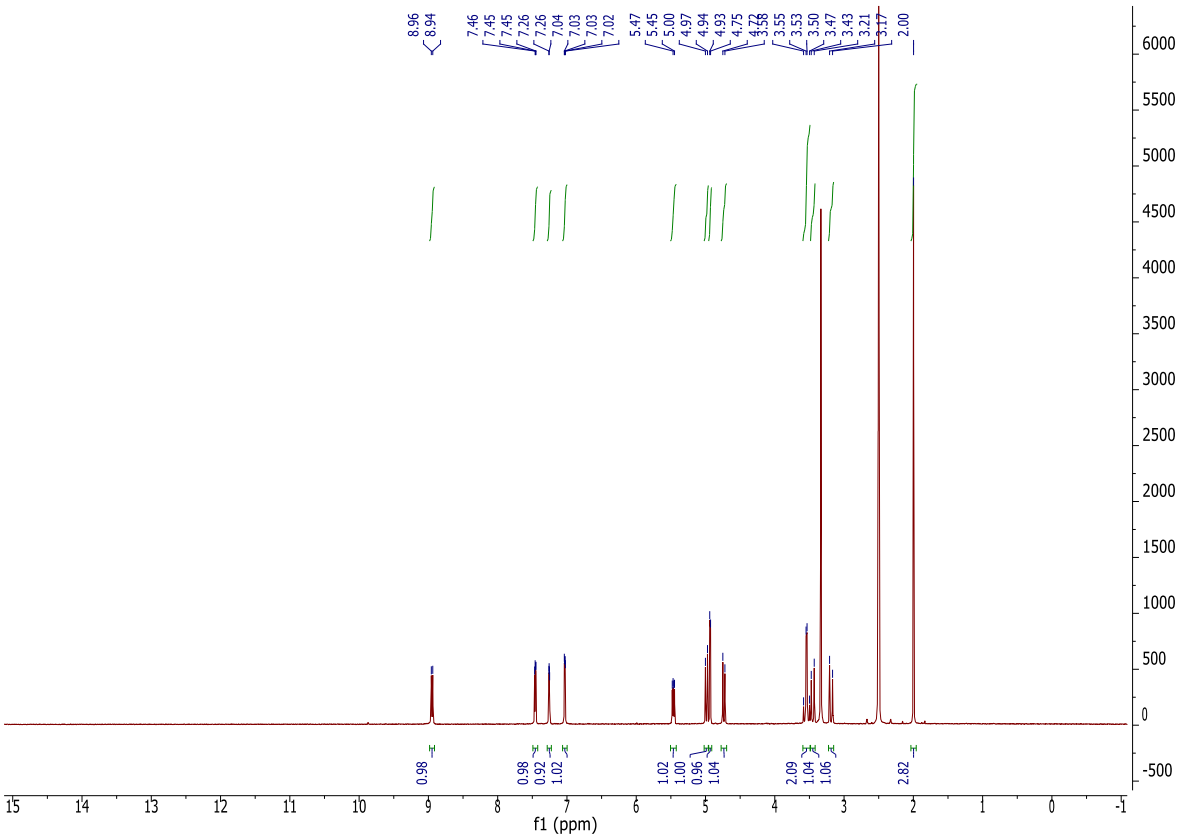
Quantification of gel bands corresponding to supercoiled DNA was performed using ImageJ 1.52a. Values were background corrected against the no-gyrase control and normalized to gyrase only activity. Gyrase activity values reflects the mean of three or four independent replicates ± SEM.

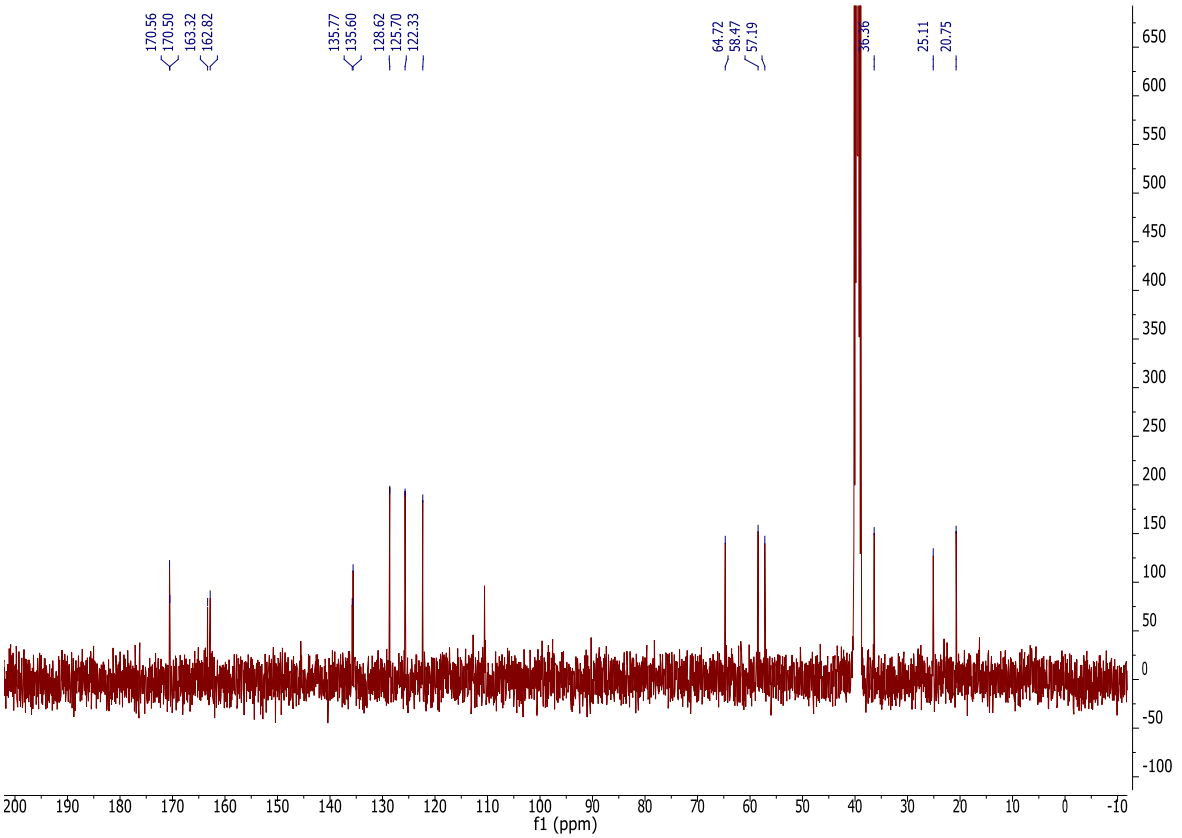
Compound **8** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



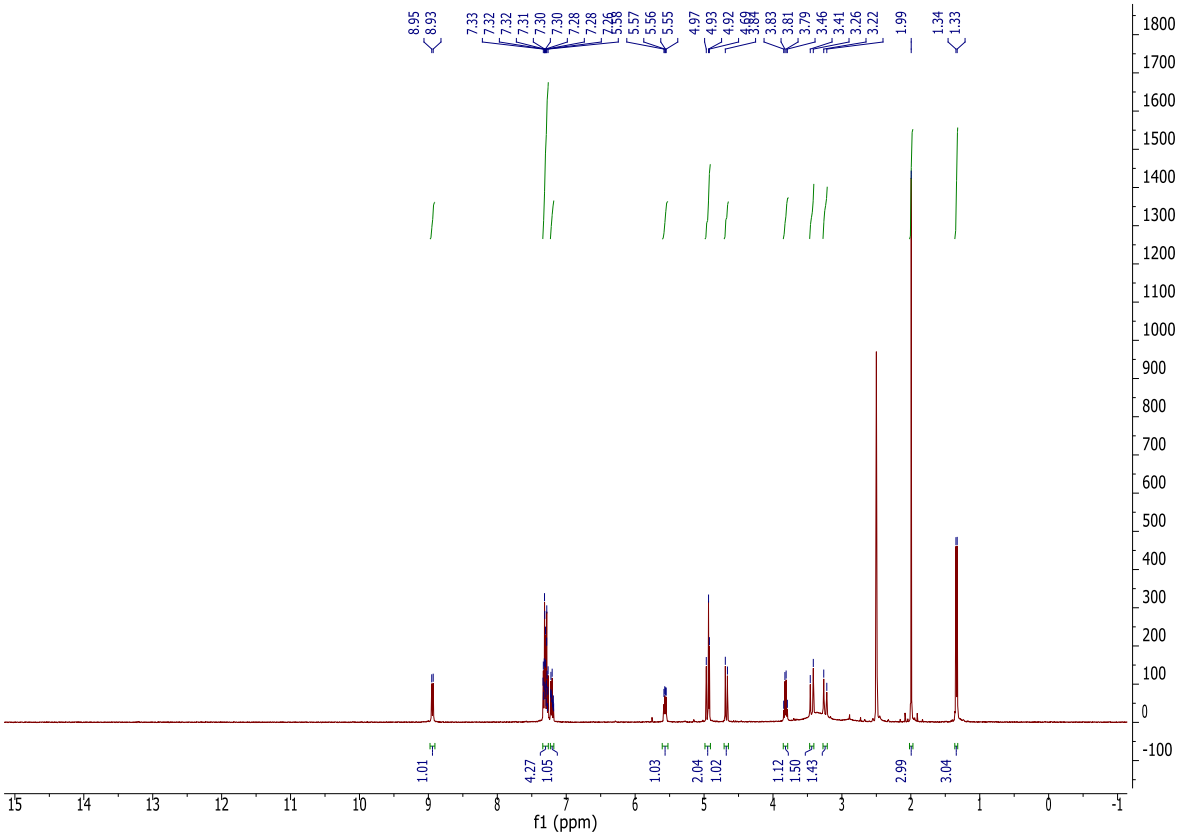


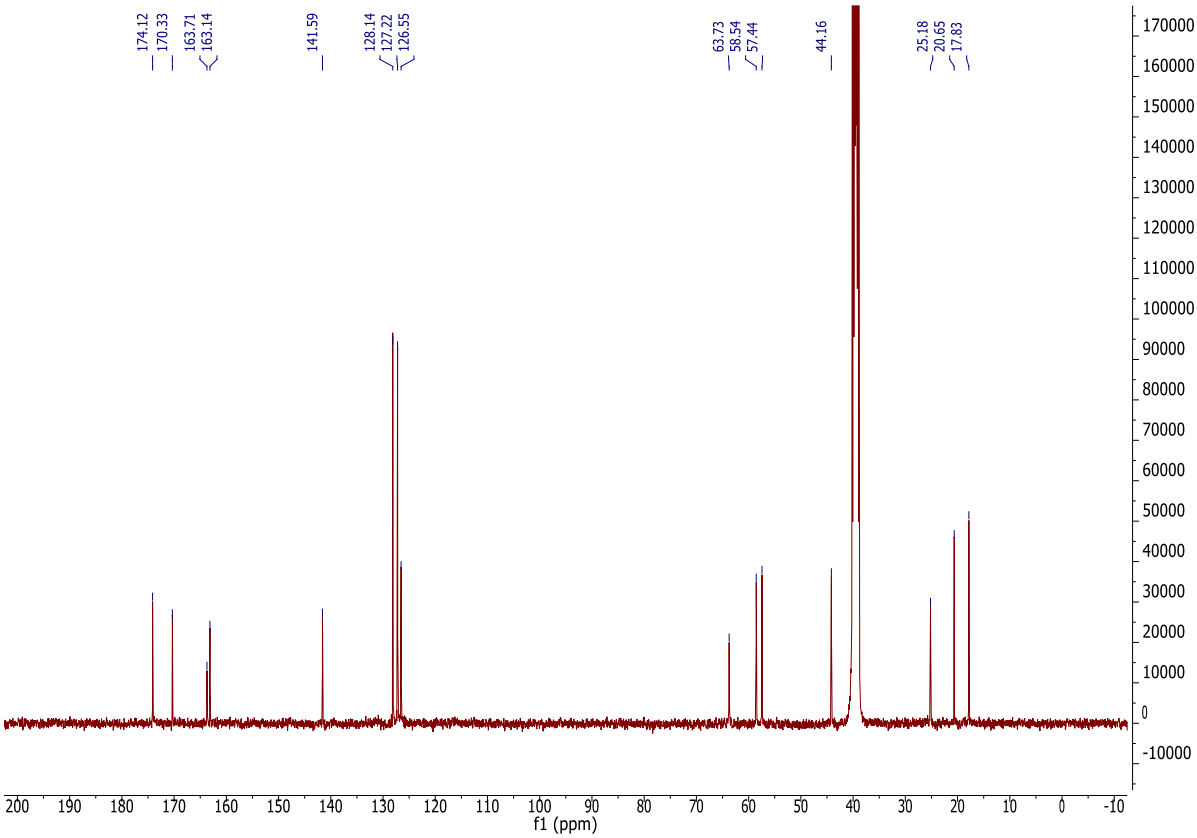
Compound **9** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



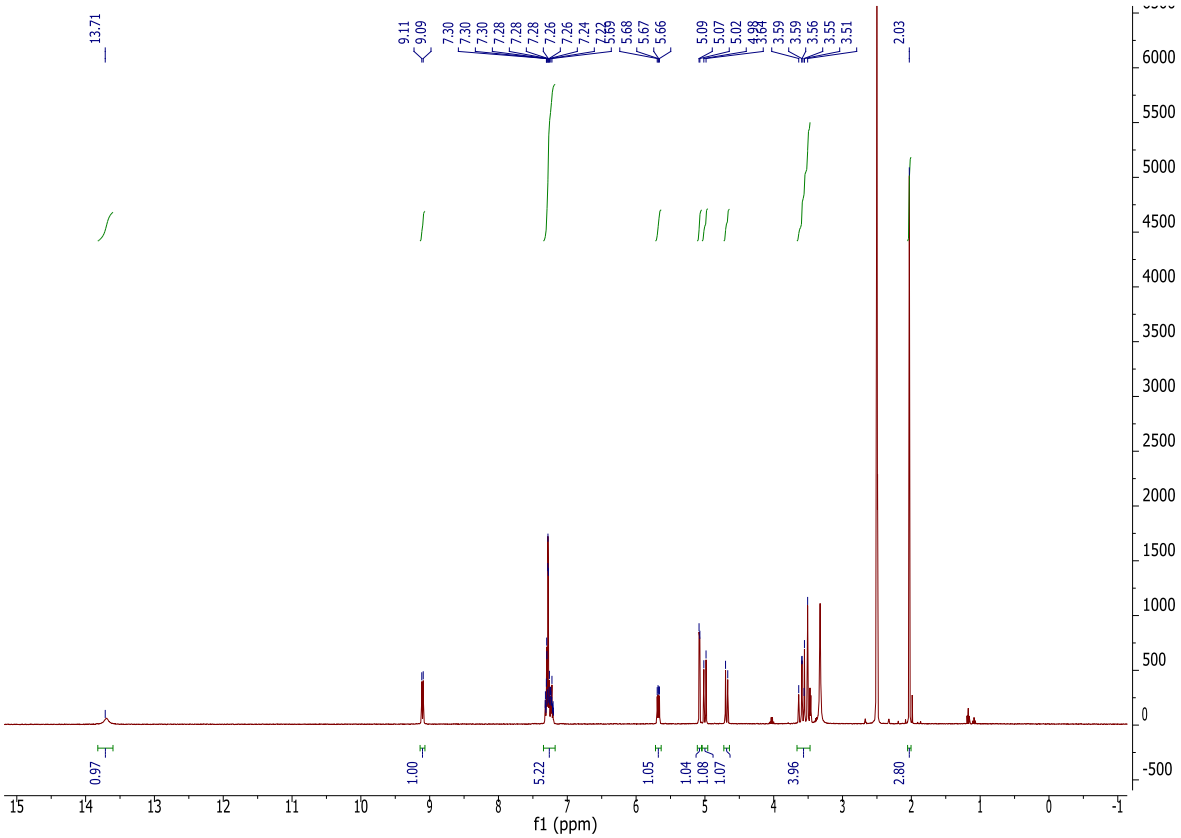


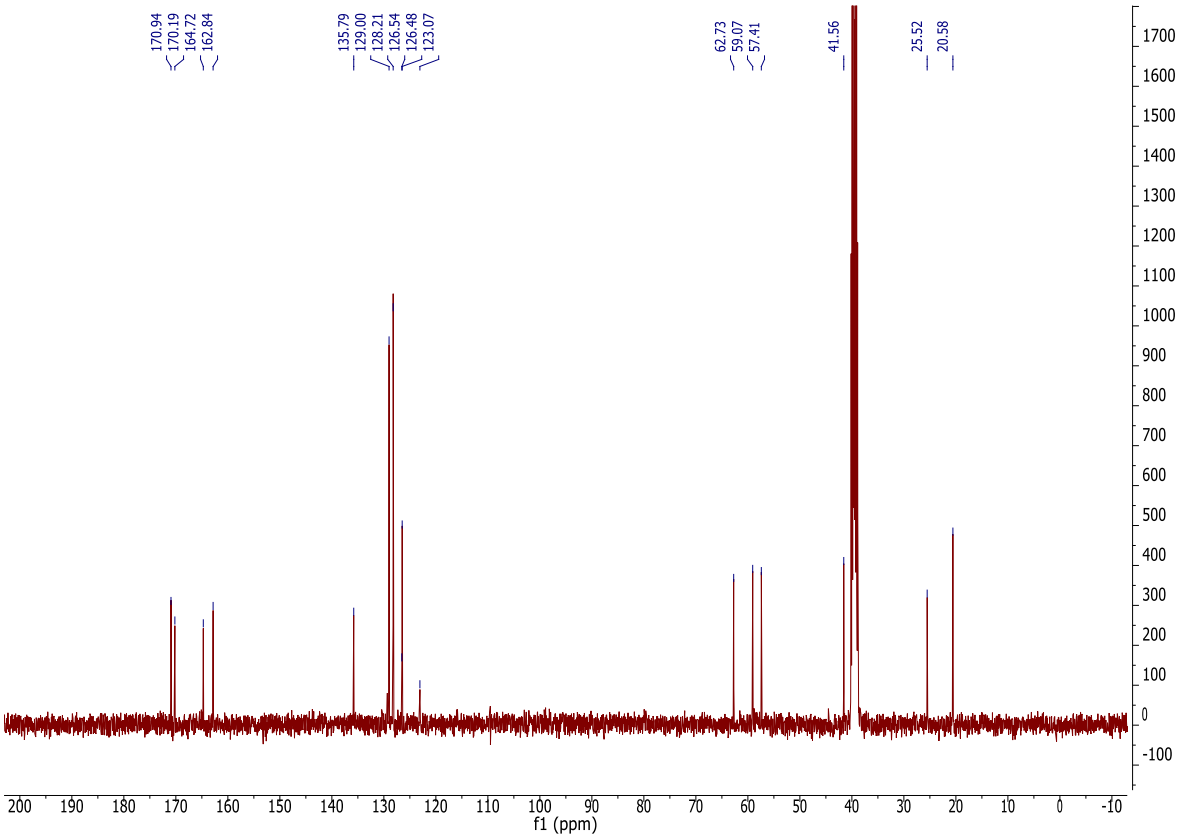
Compound **10** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



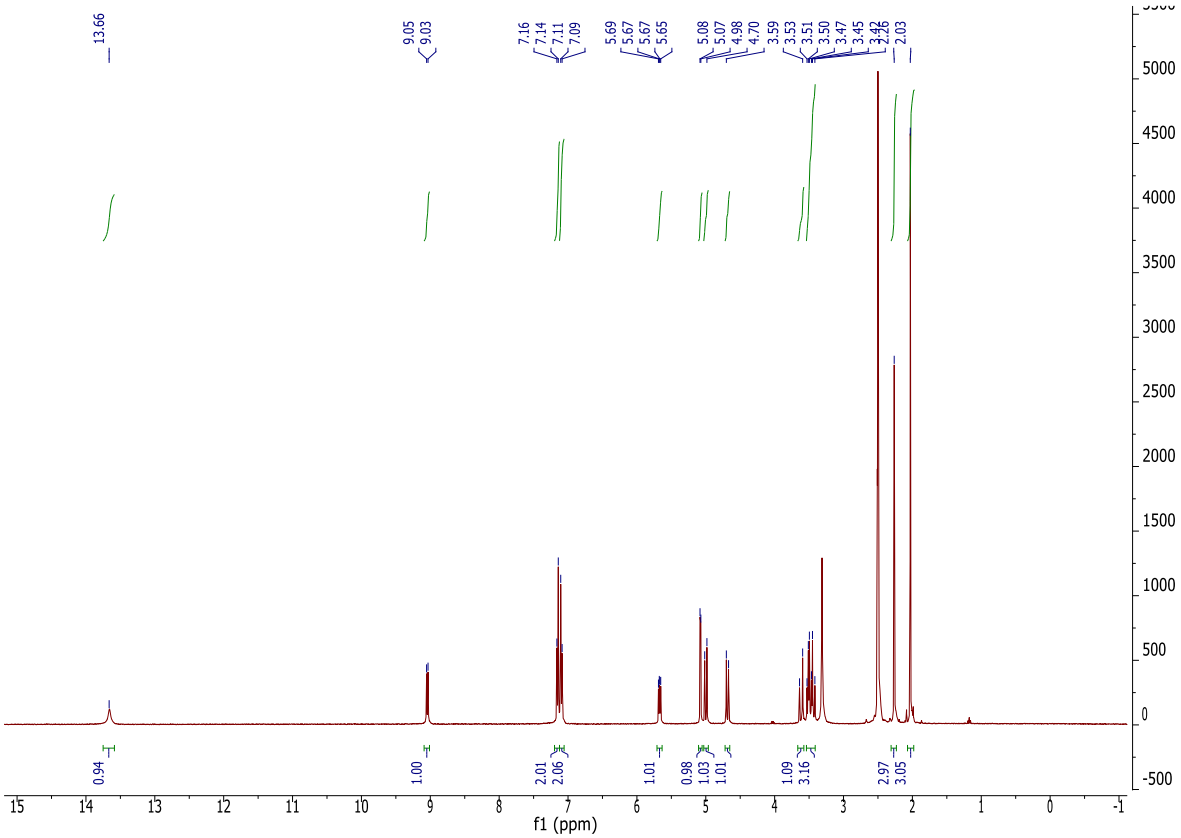


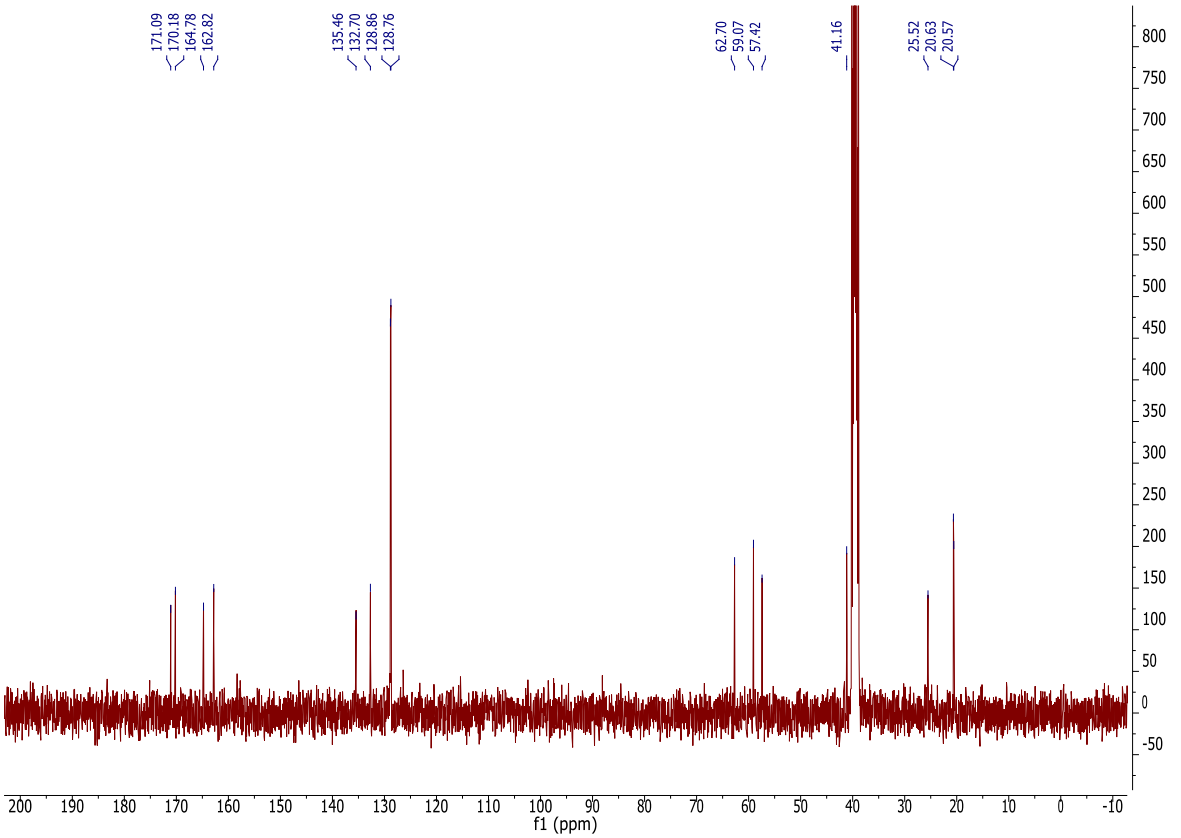
Compound **11** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



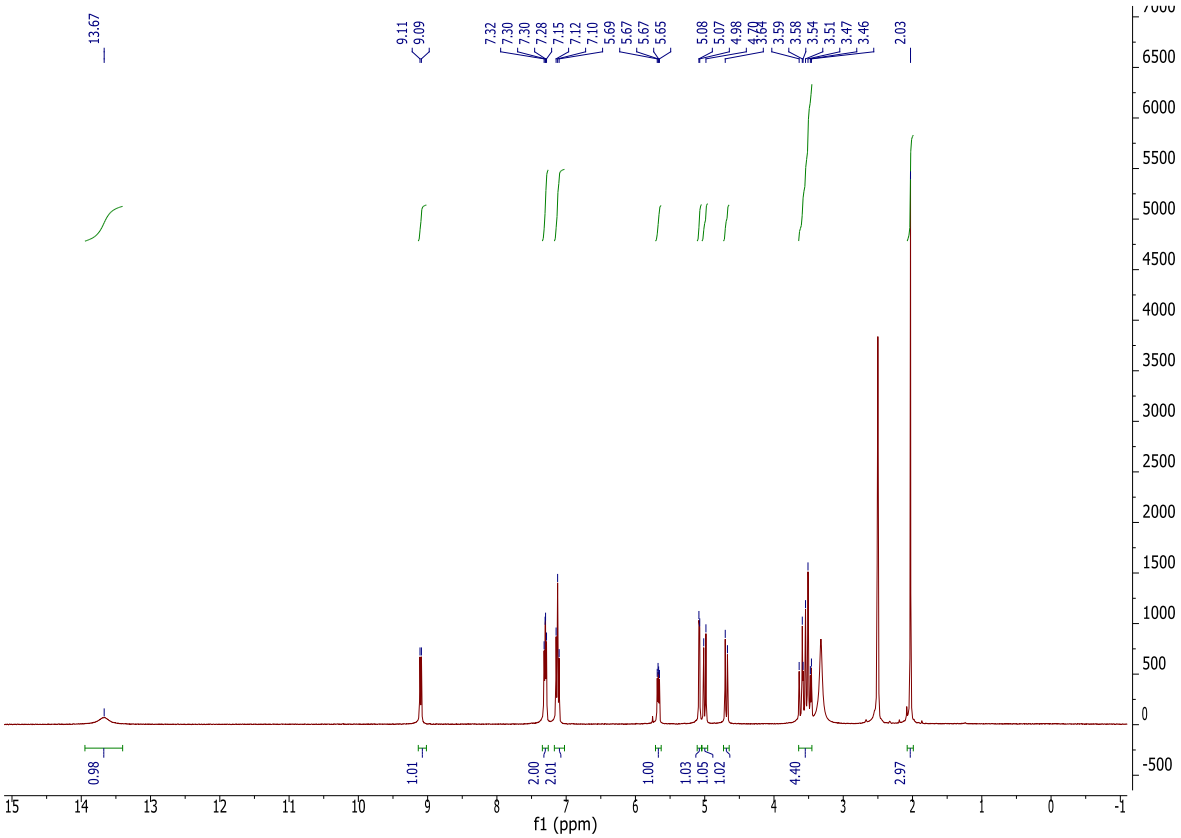


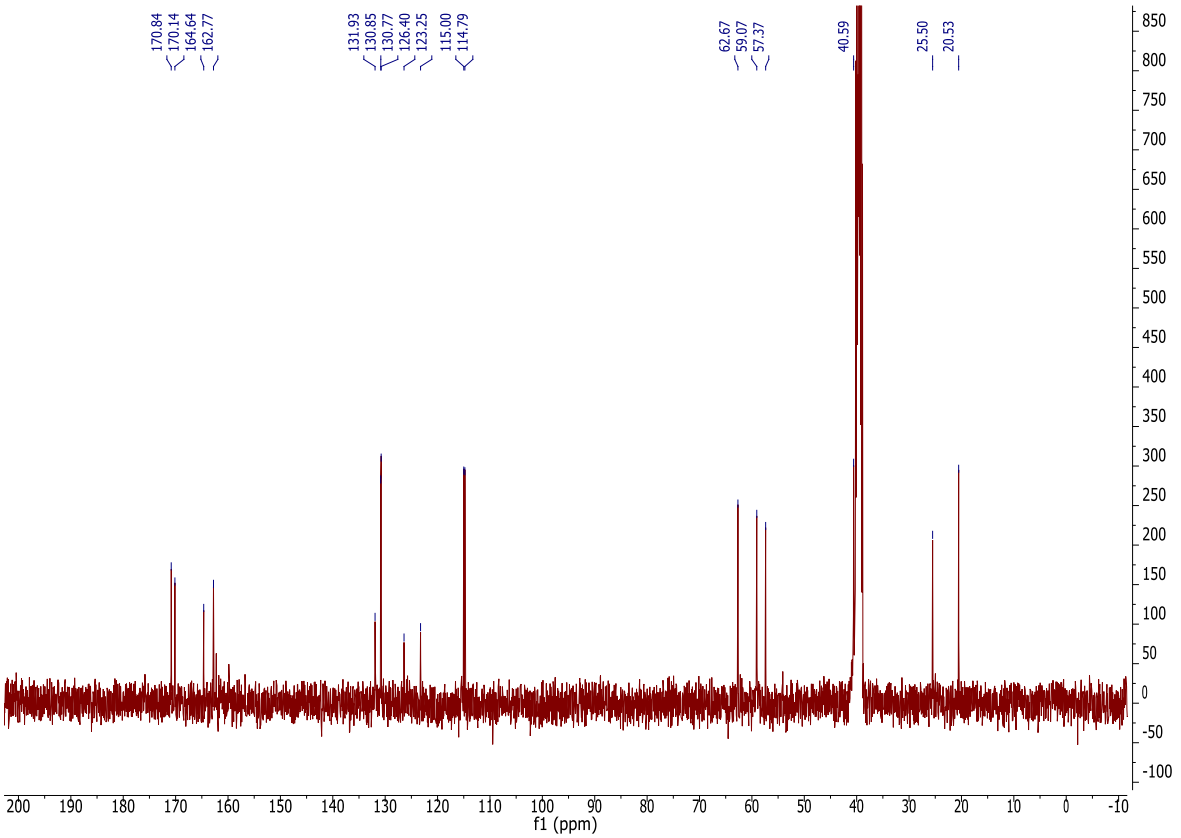
Compound **12** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



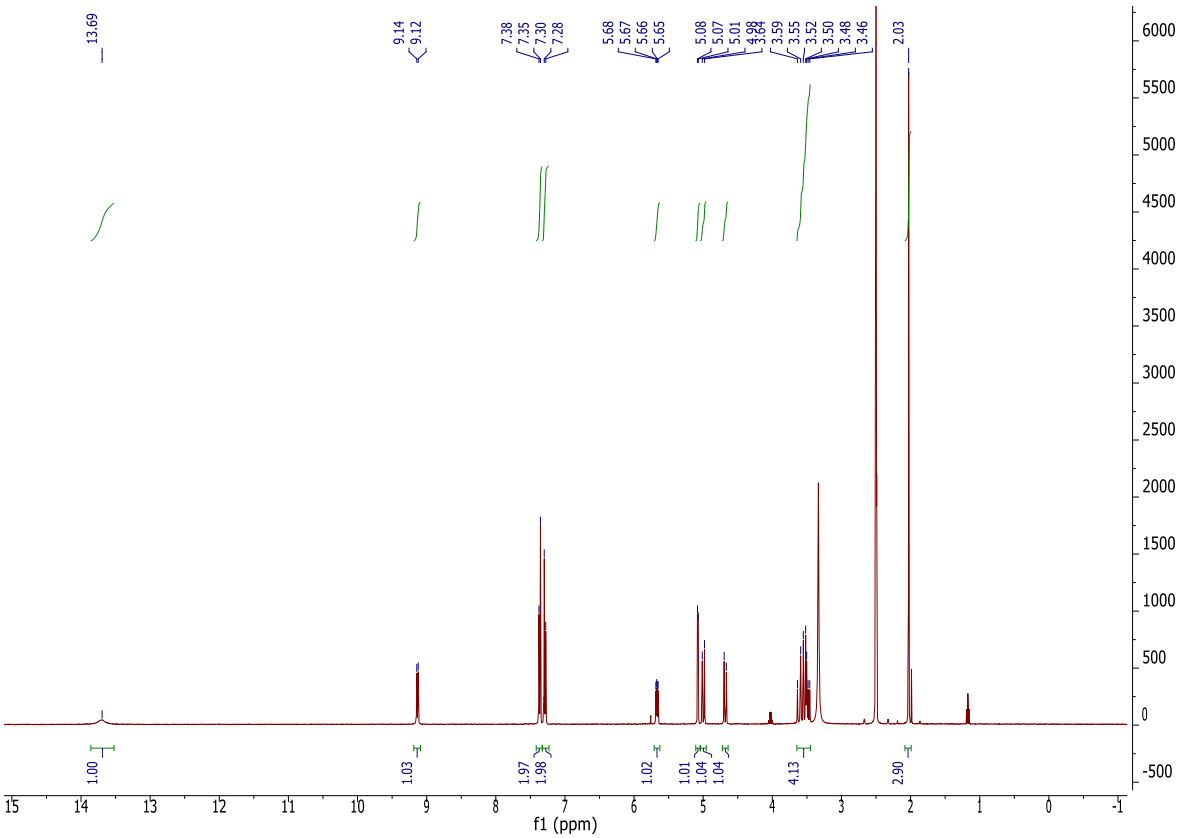


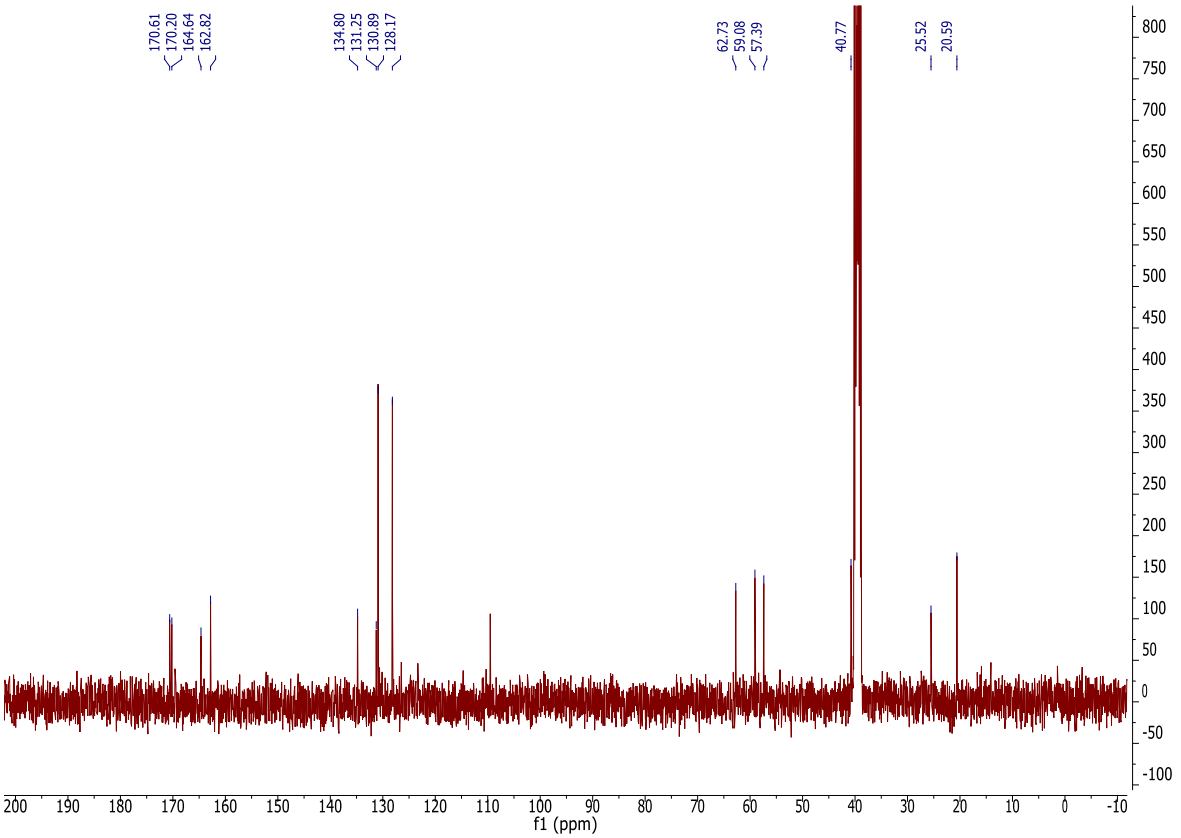
Compound **13** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



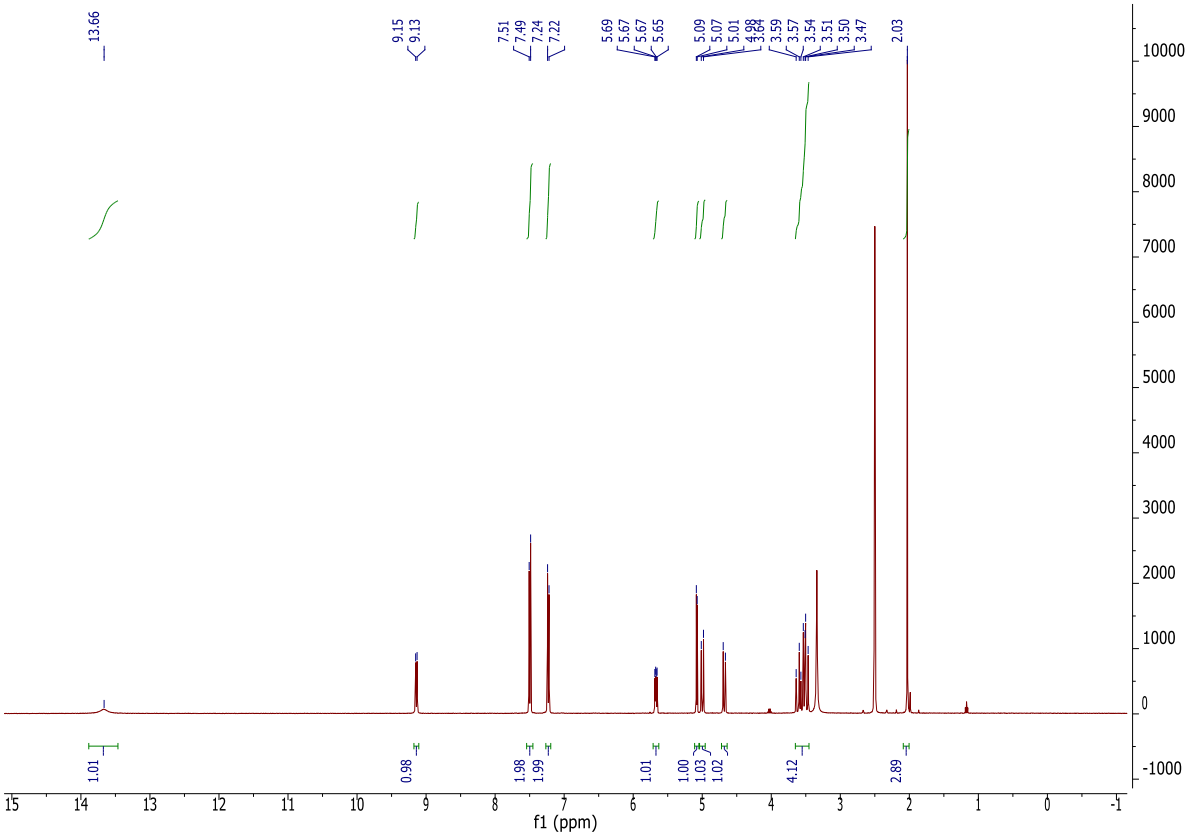


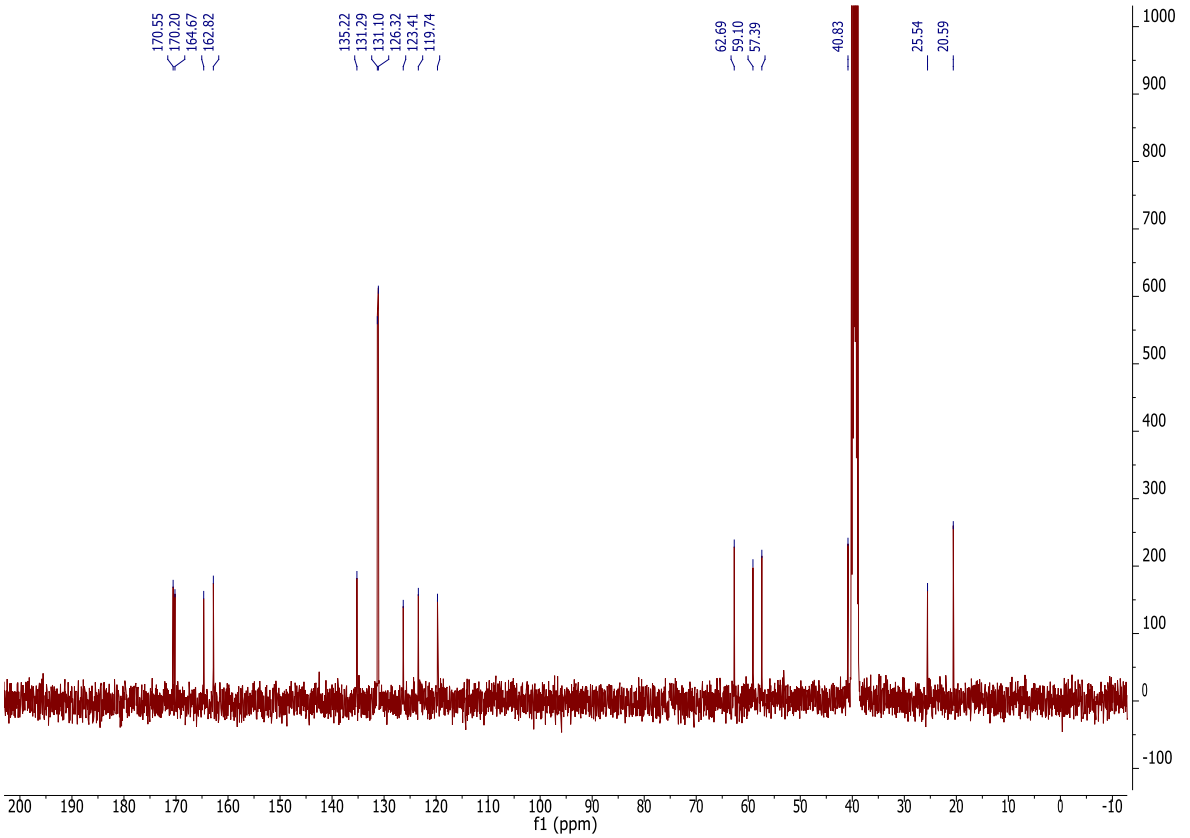
Compound **14** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



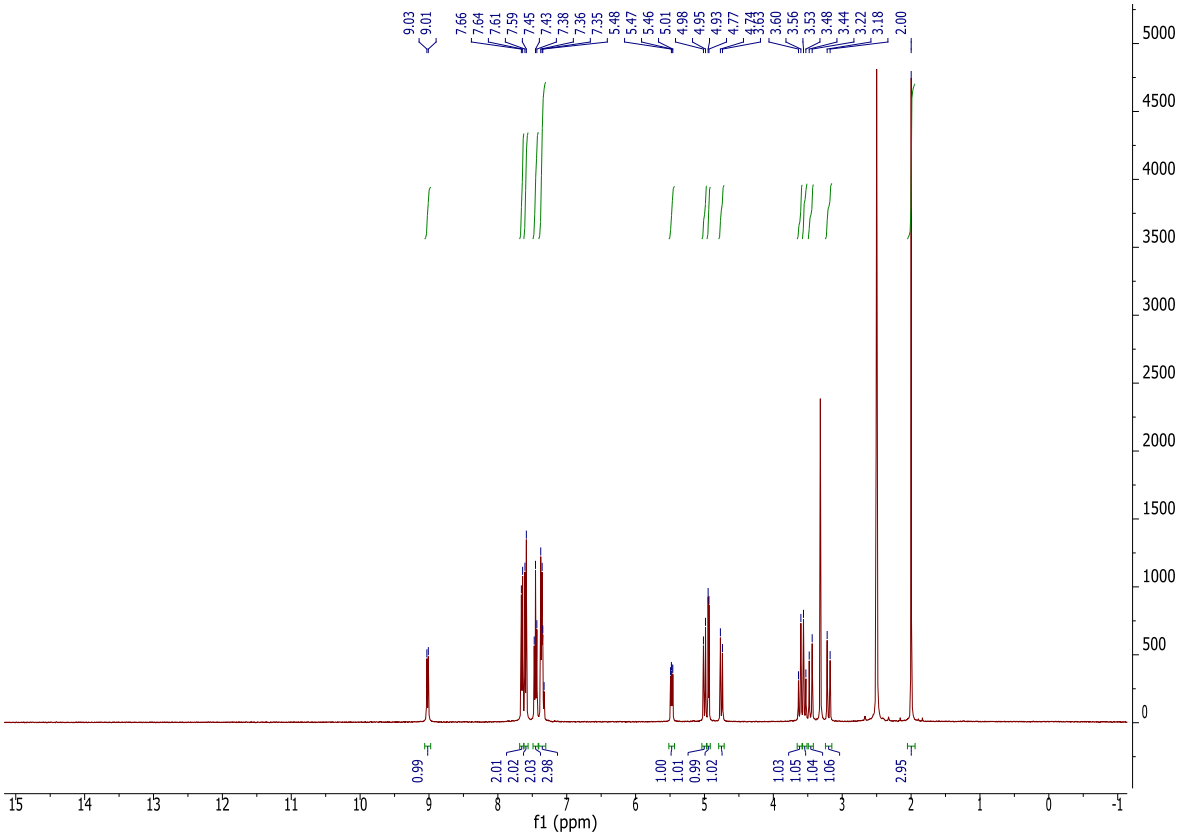


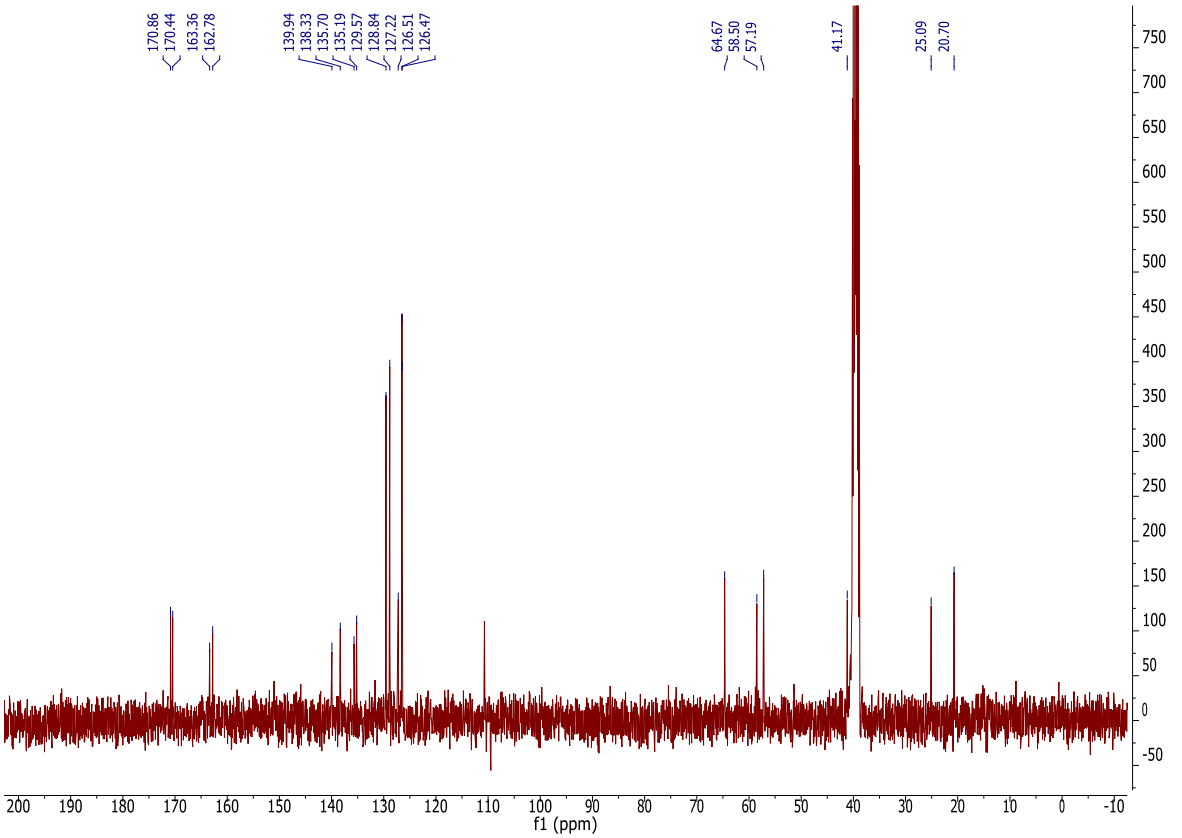
Compound **15** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



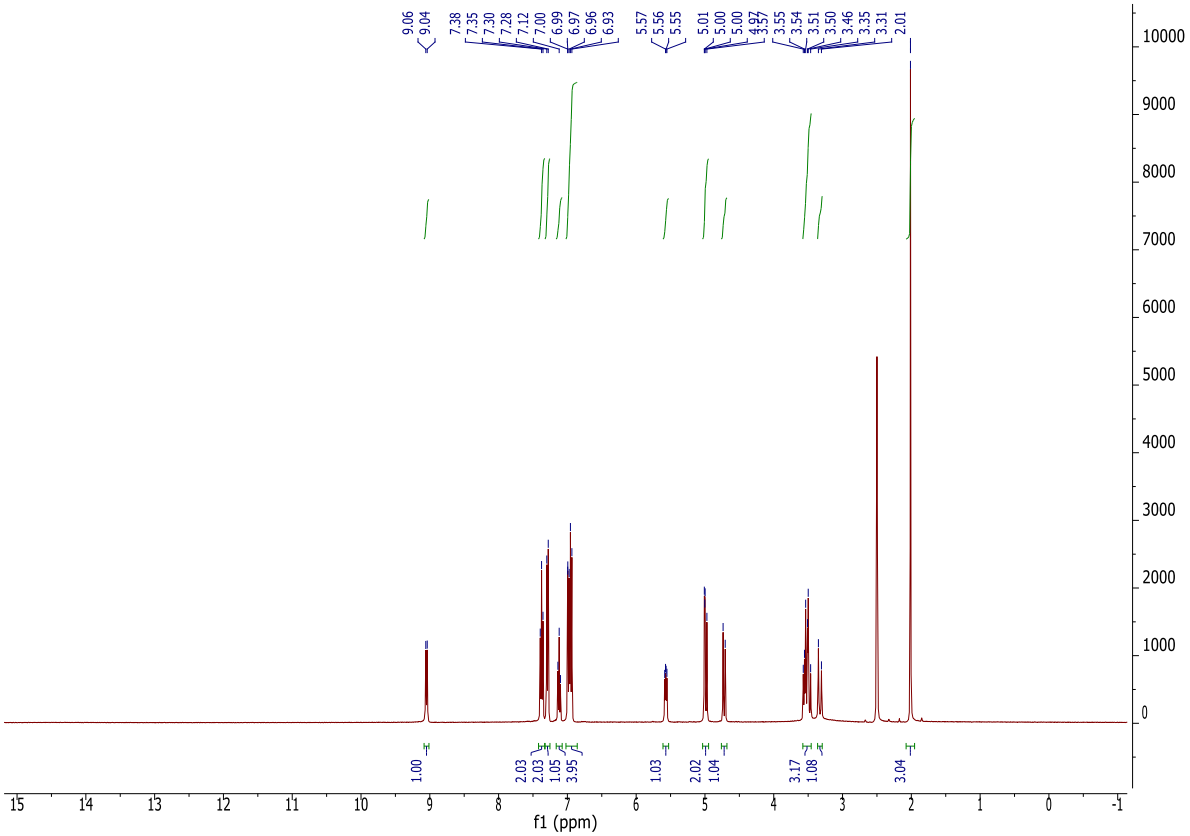


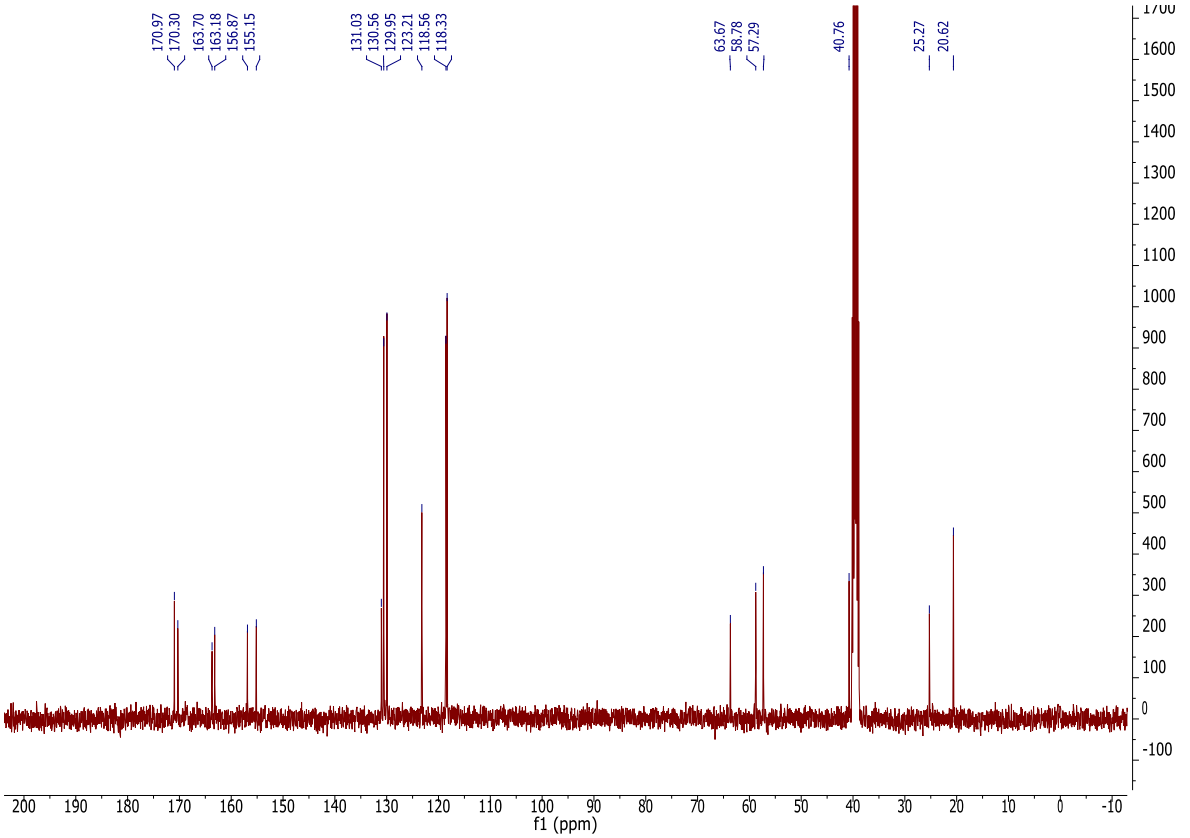
Compound **16** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



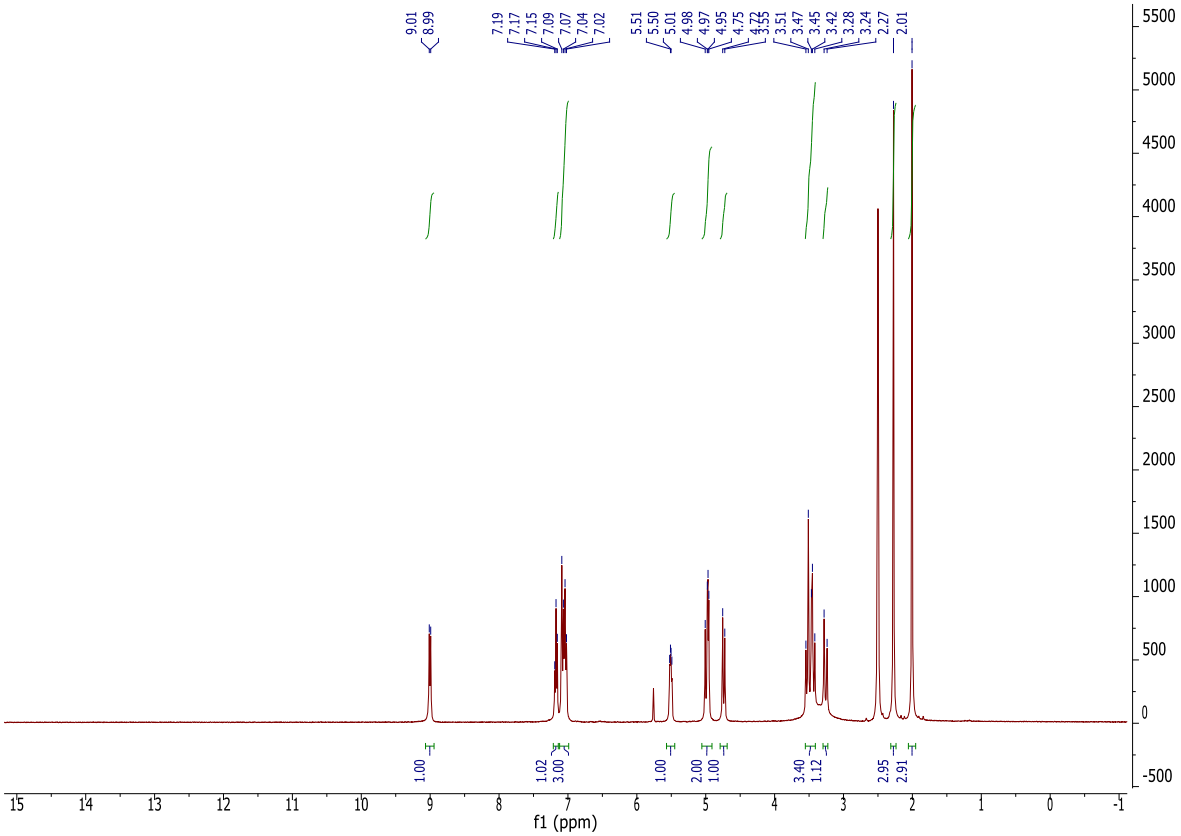


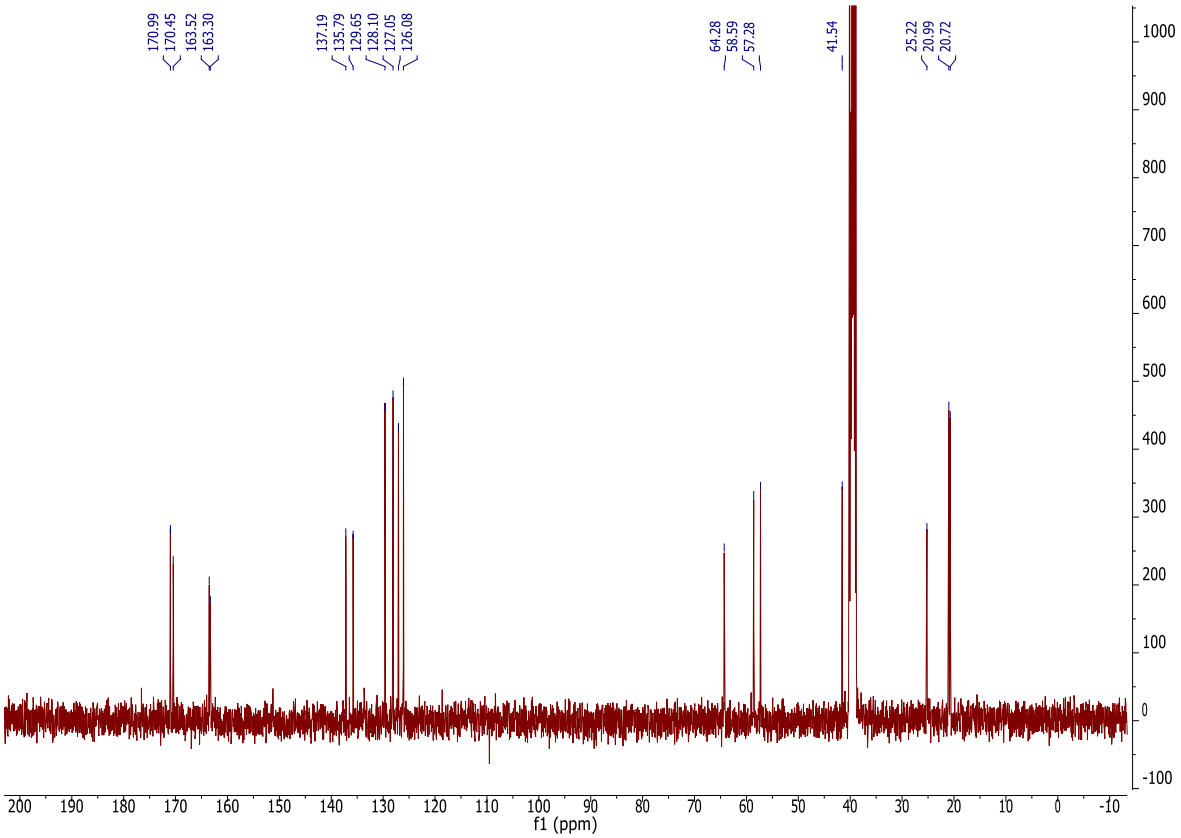
Compound **17** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



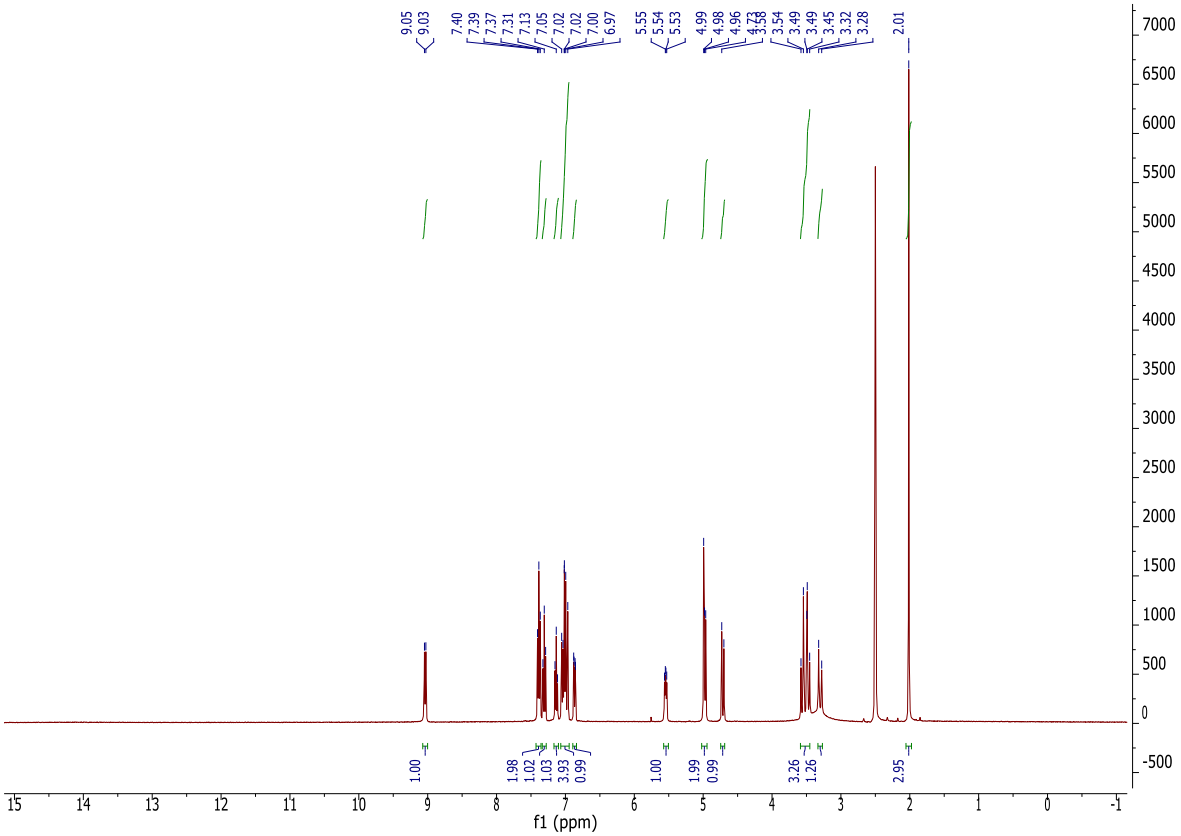


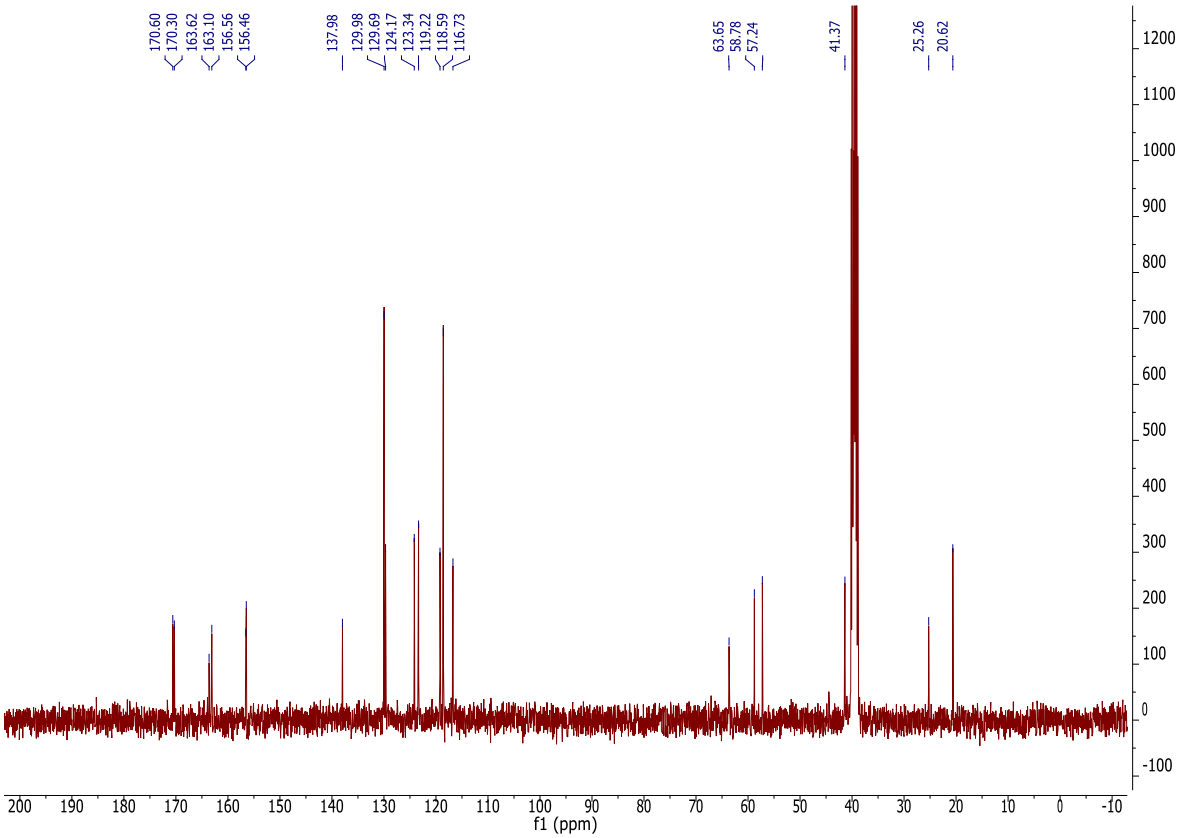
Compound **18** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



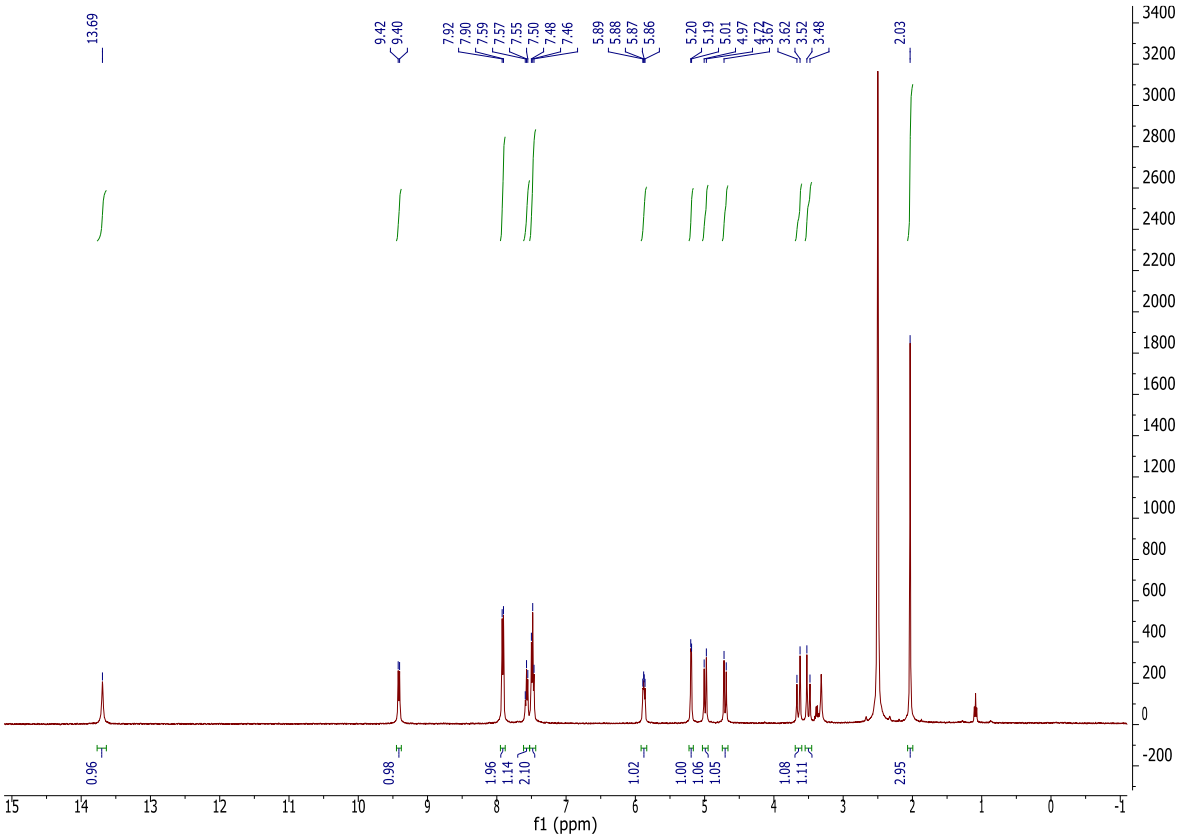


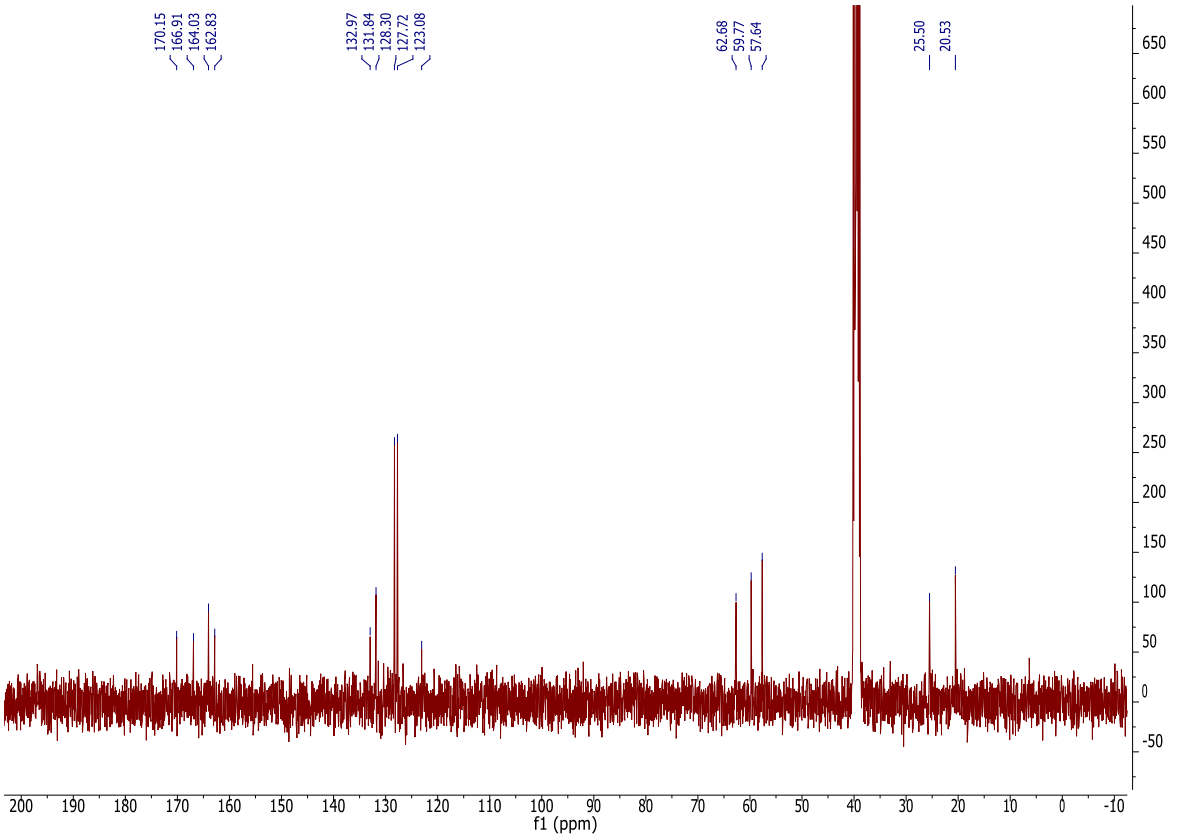
Compound **19** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



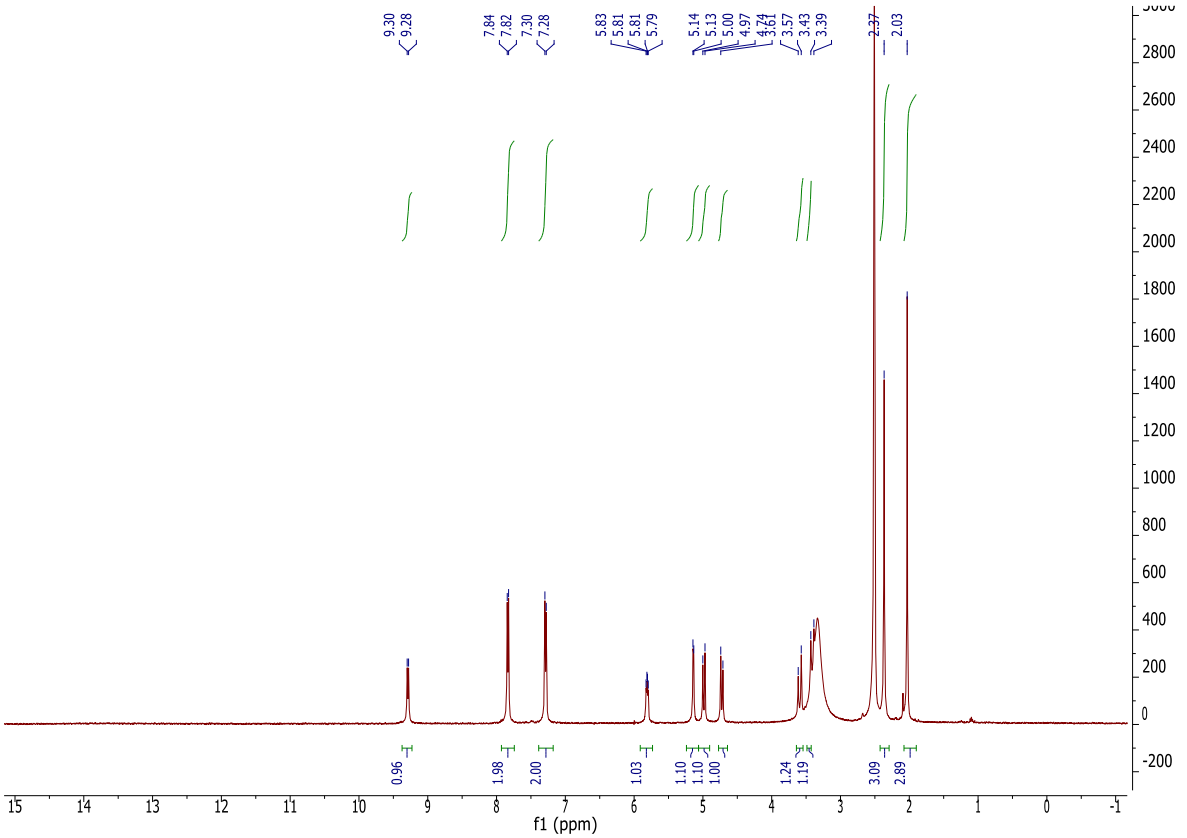


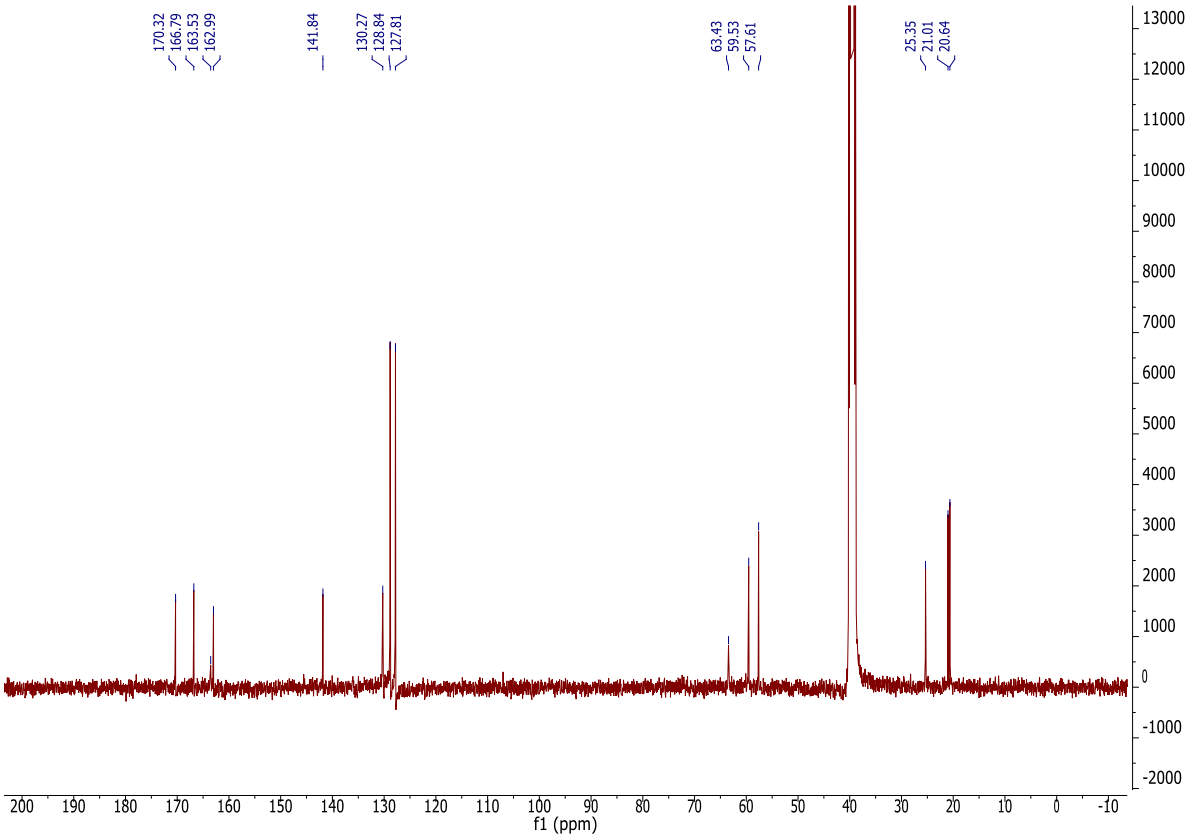
Compound **20** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



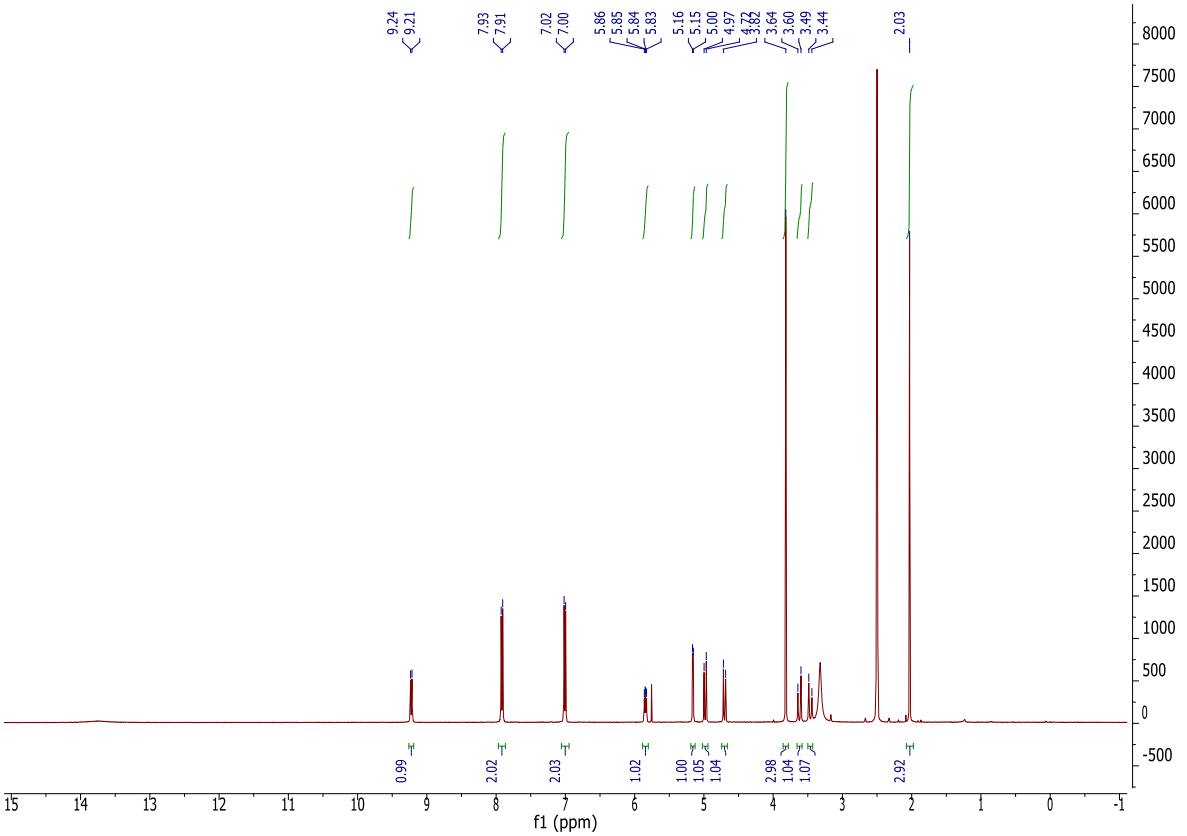


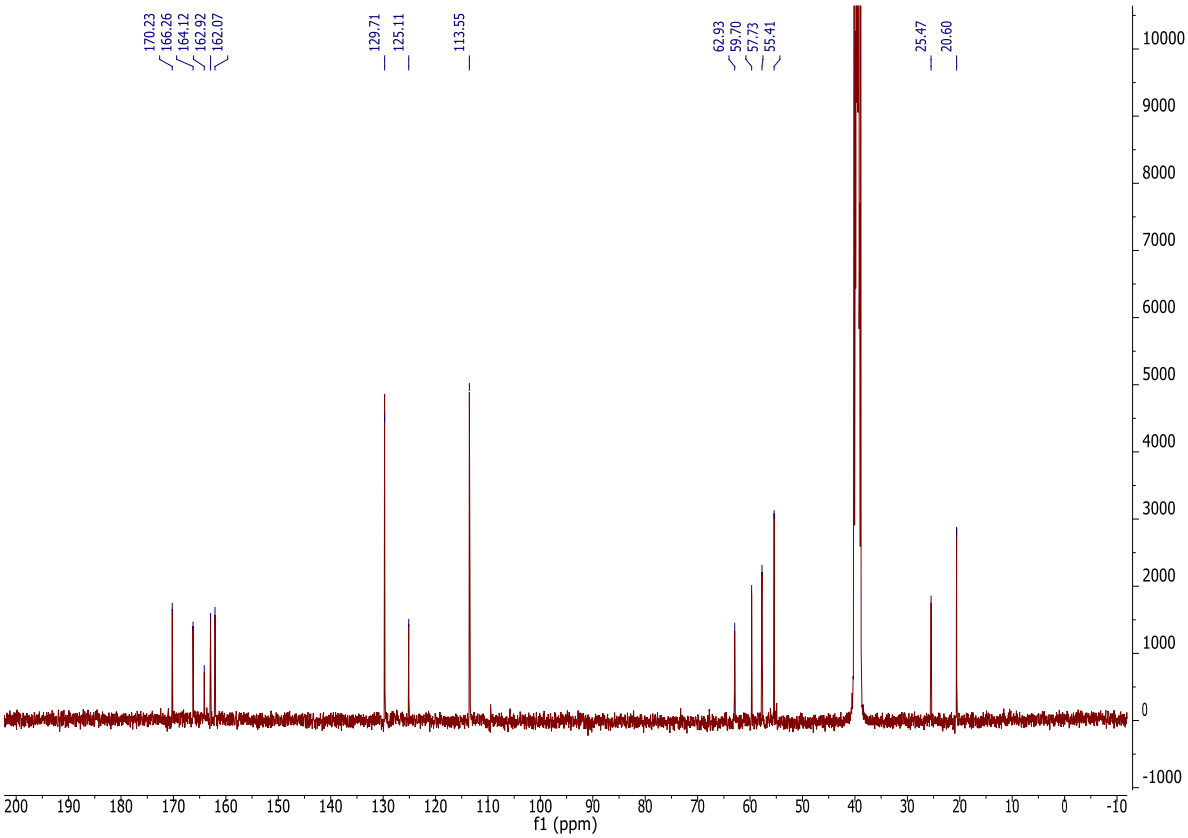
Compound **21** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



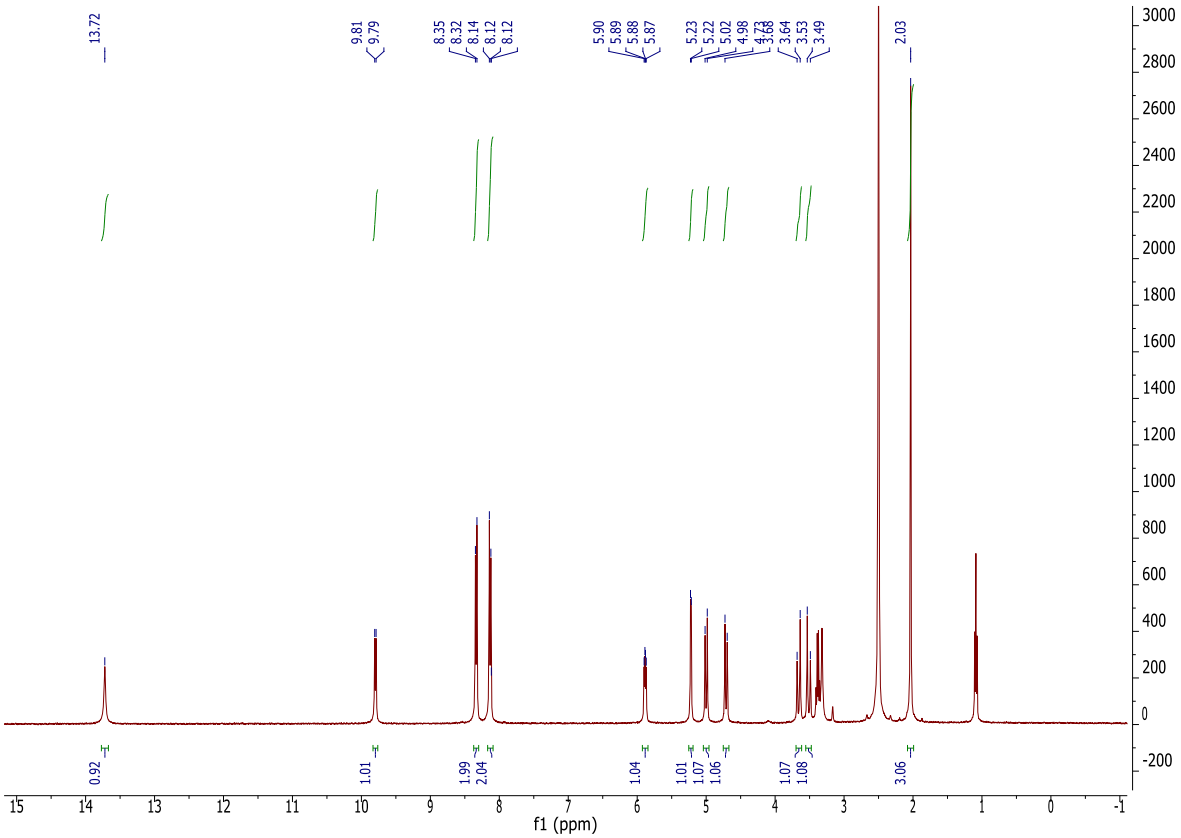


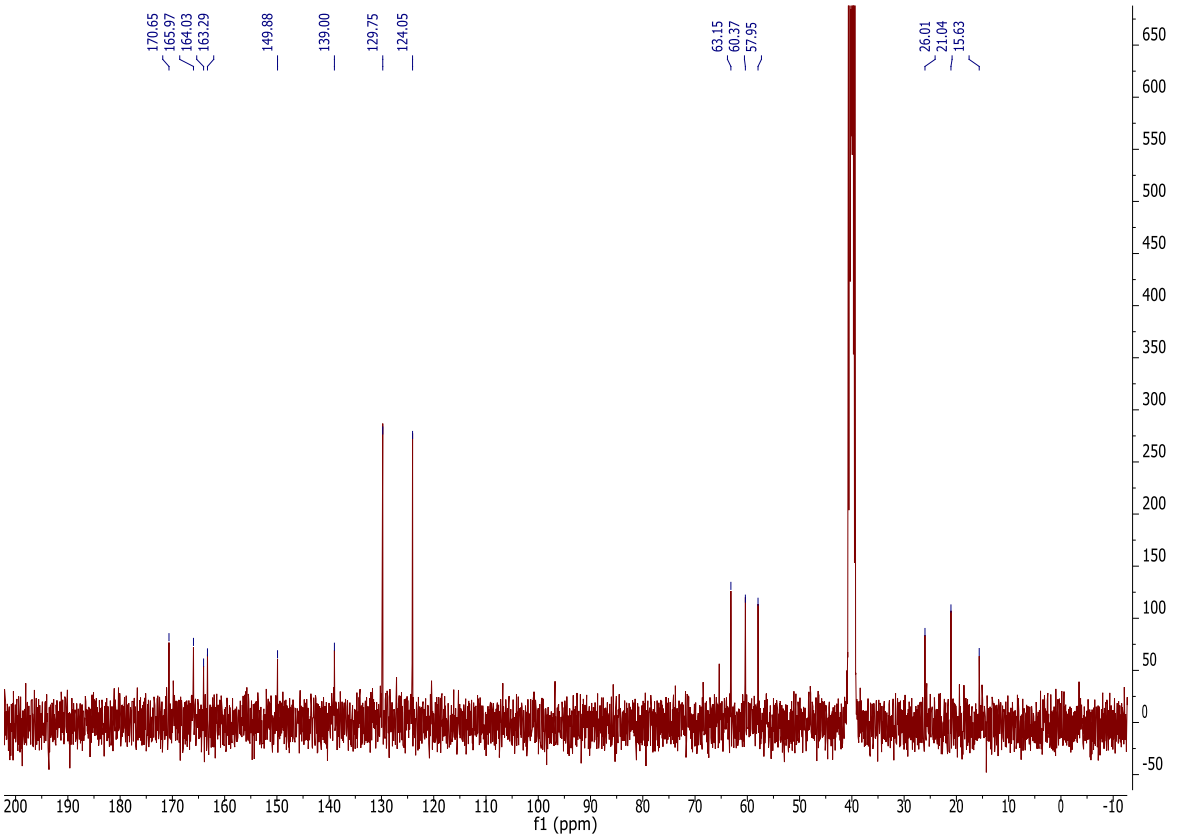
Compound **22** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



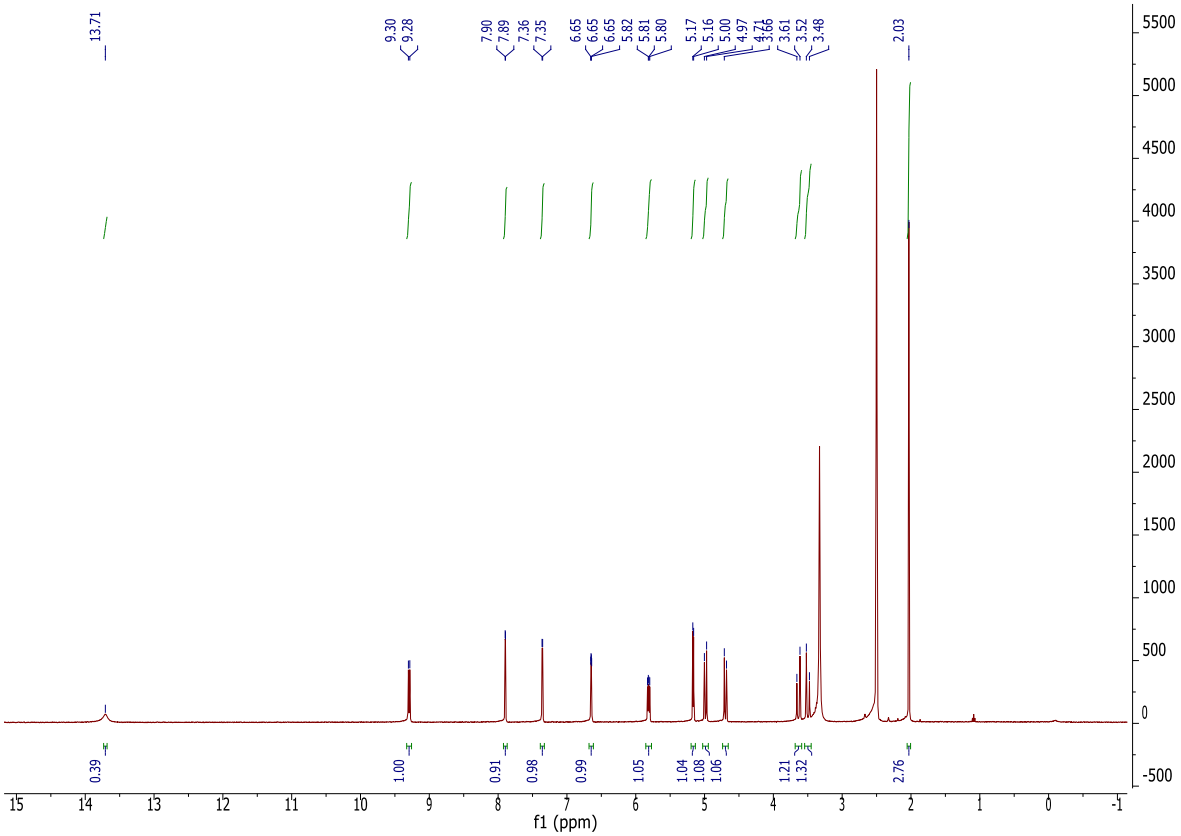


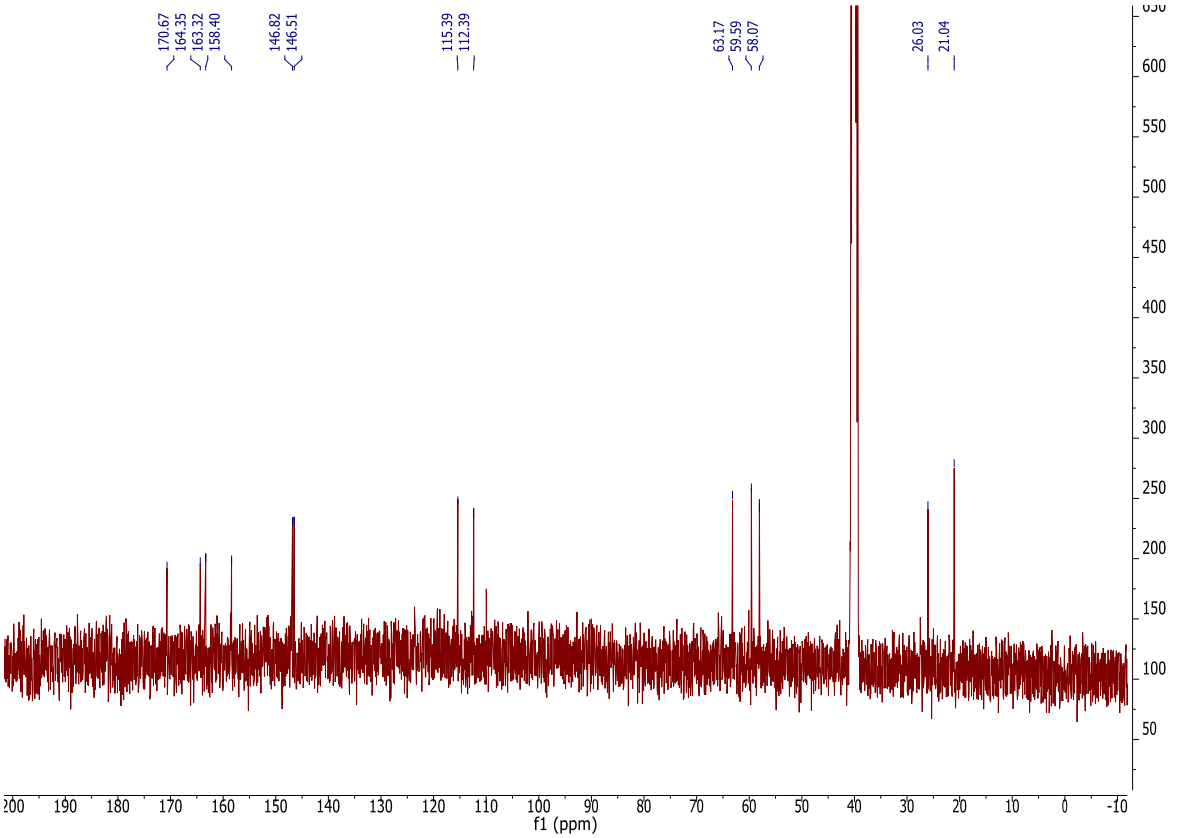
Compound **23** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



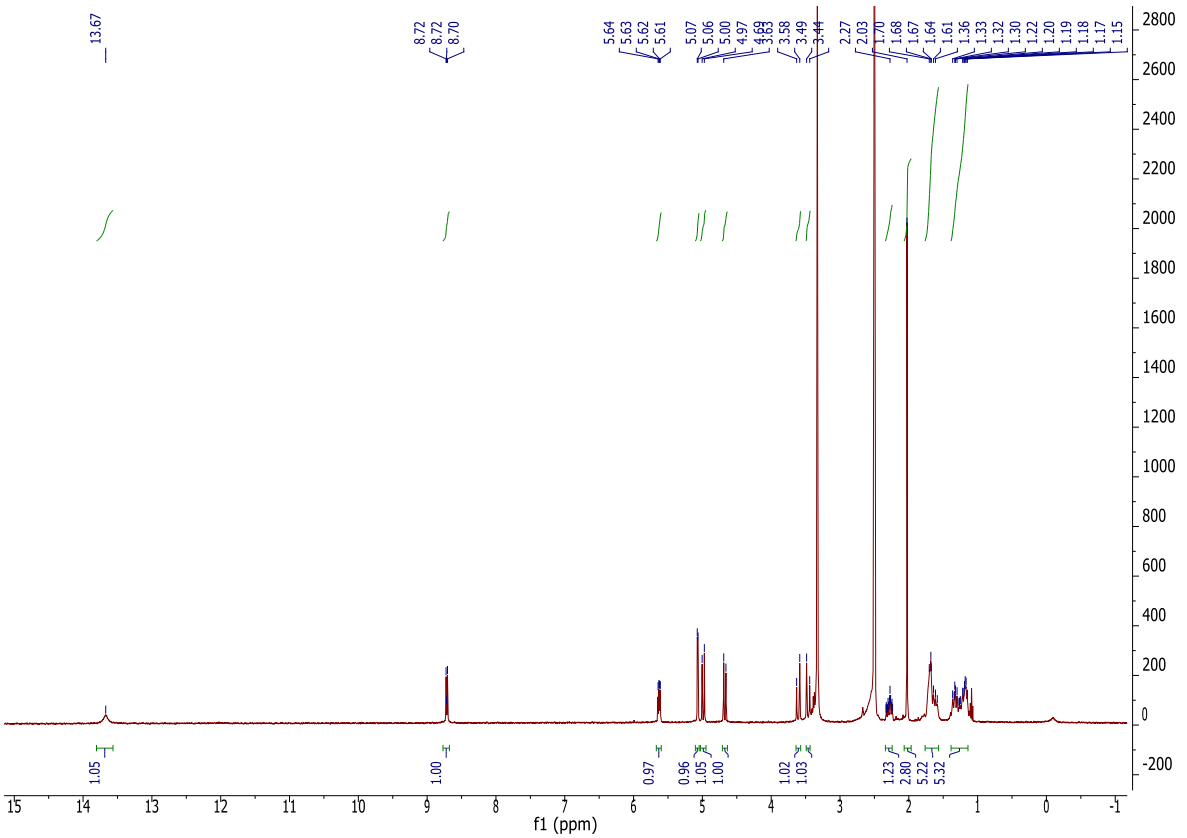


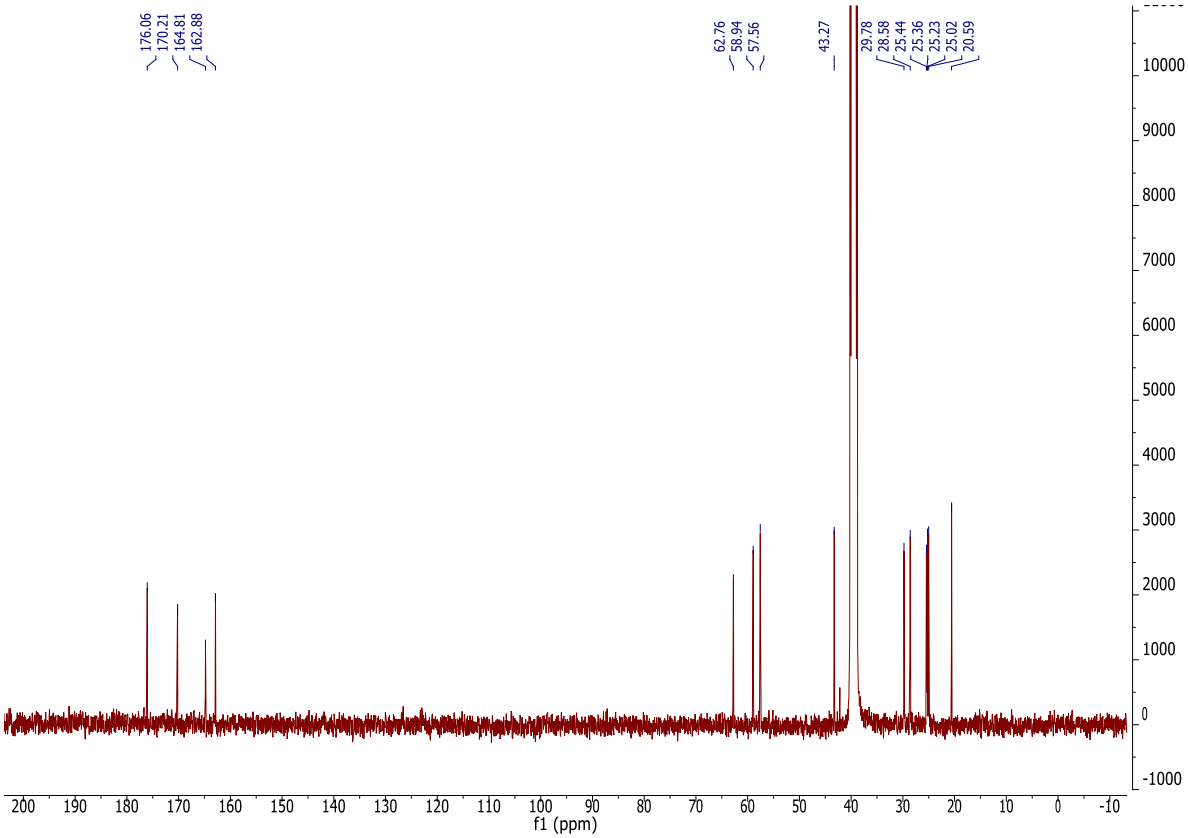
Compound **24** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



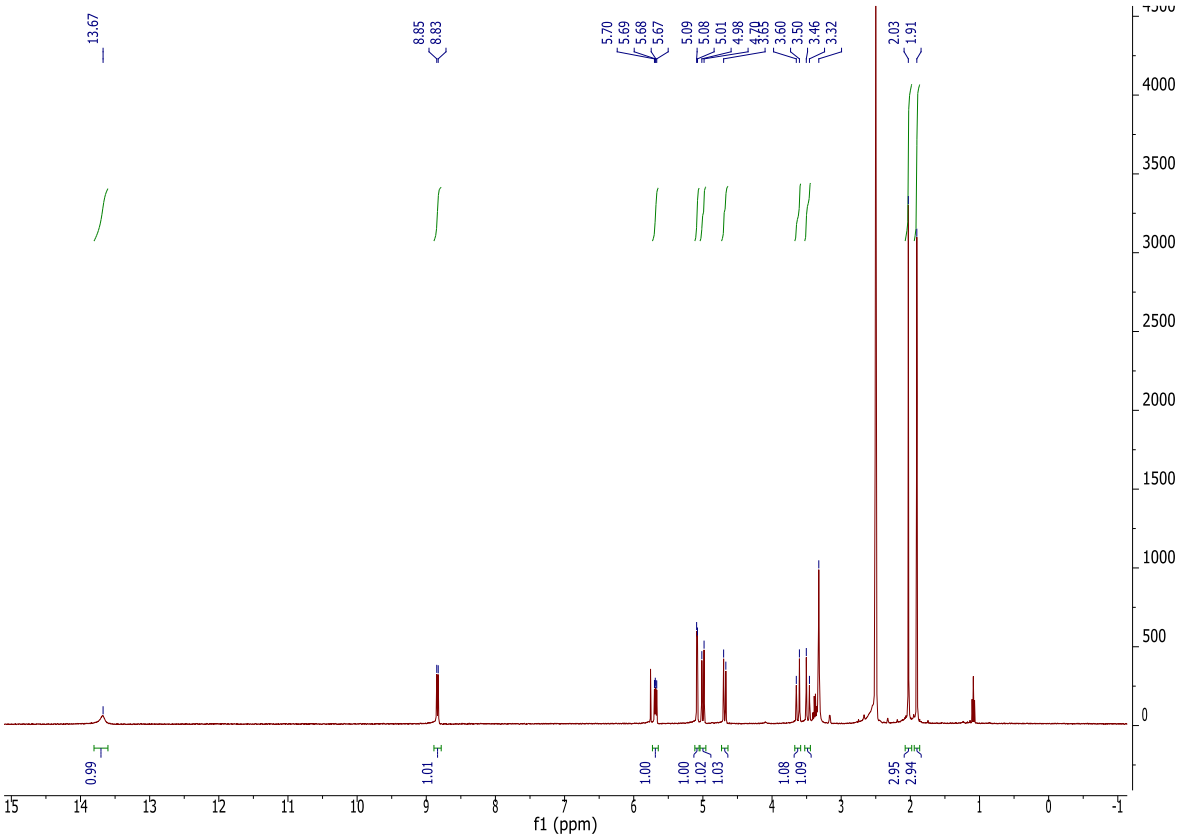


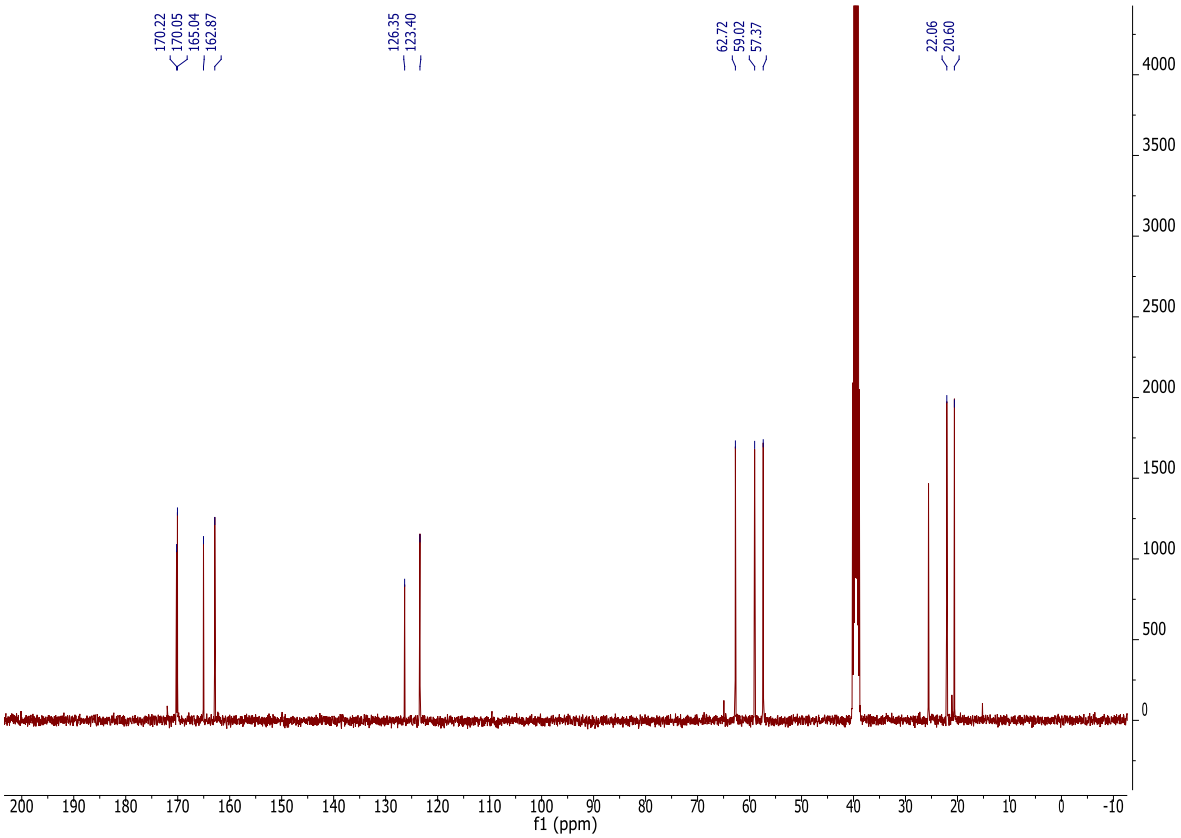
Compound **25** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



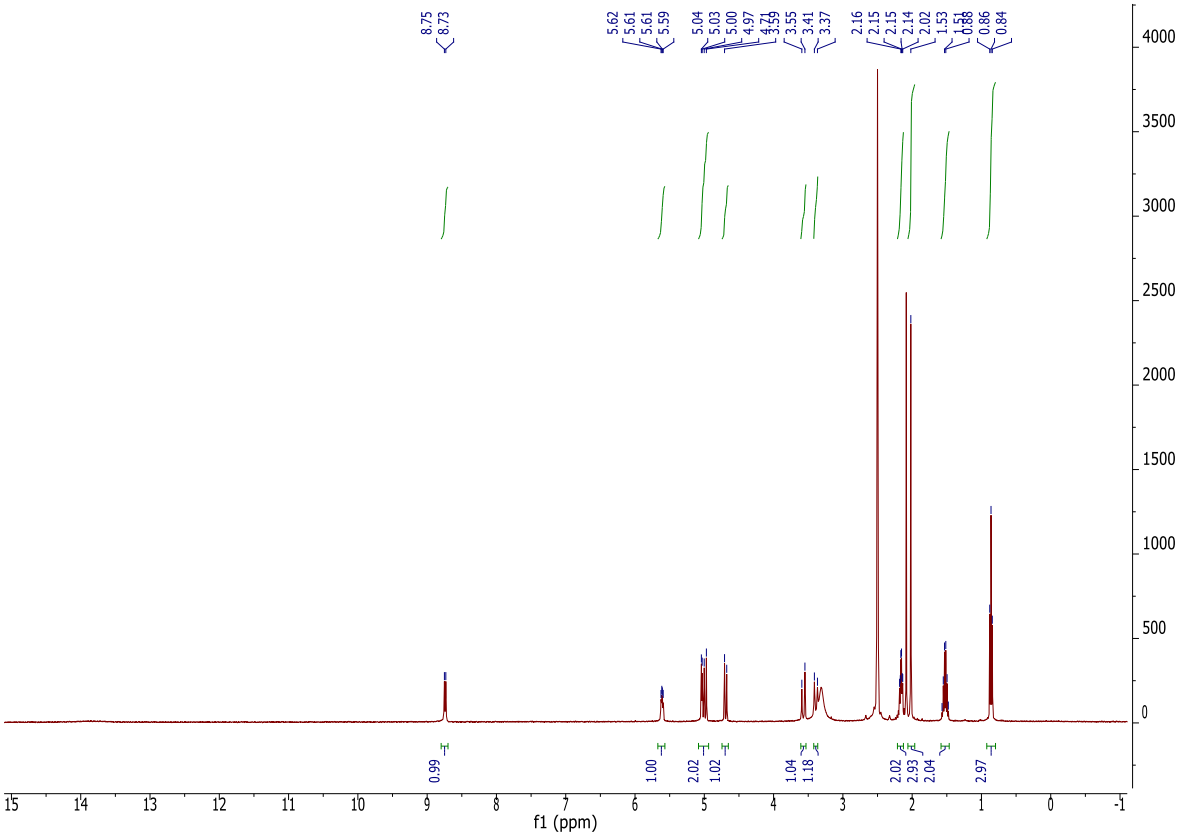


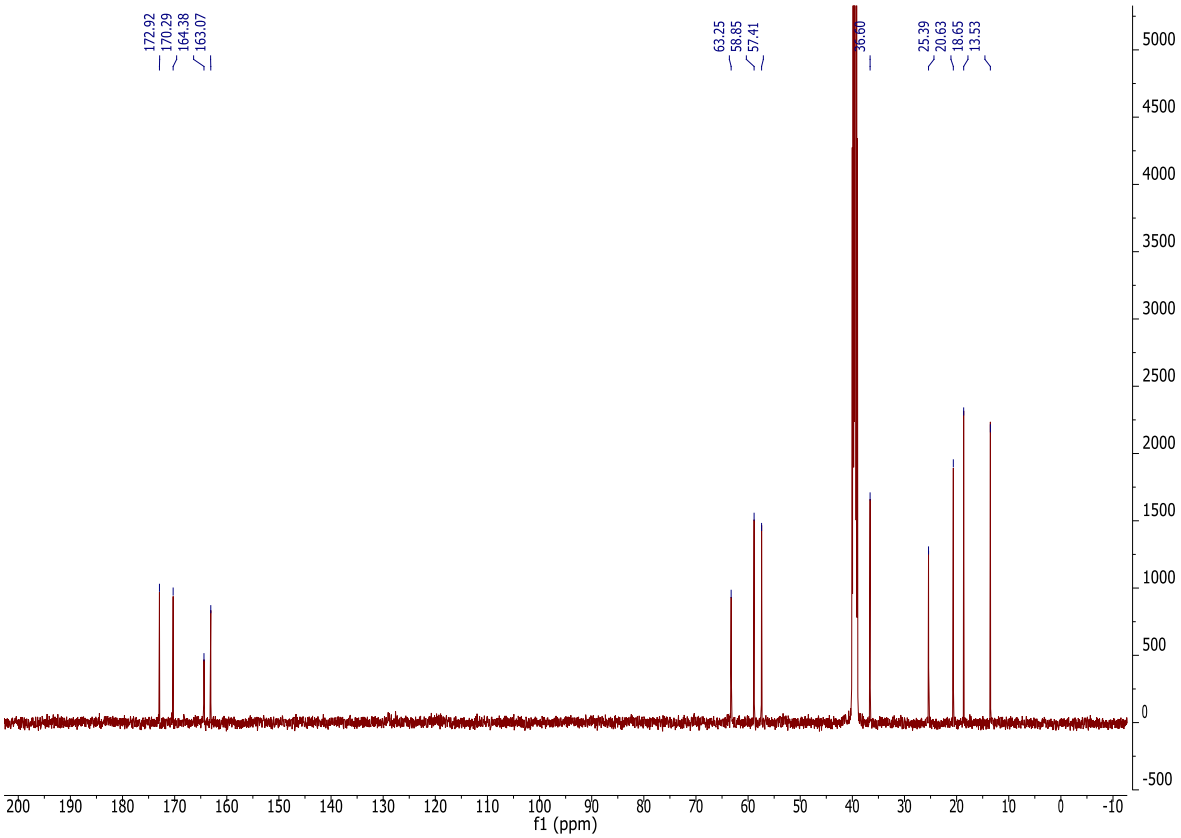
Compound **26** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



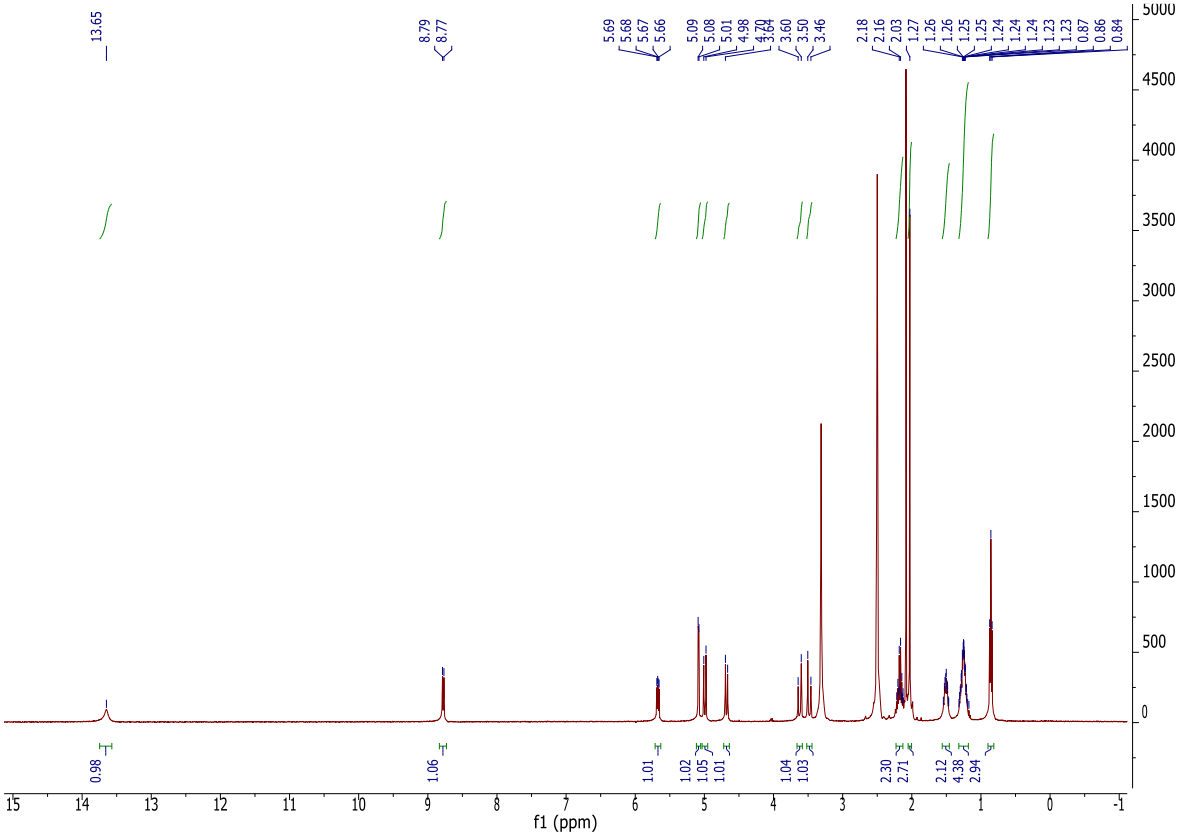


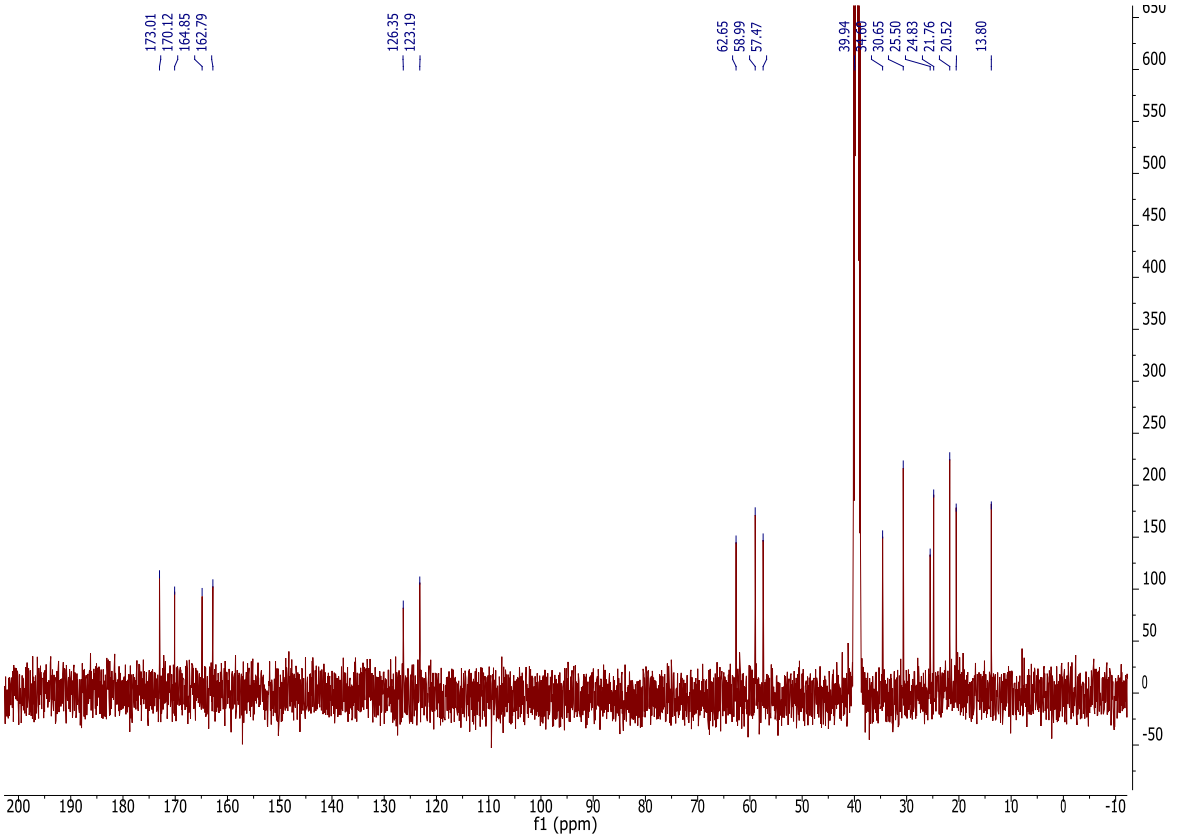
Compound **27** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



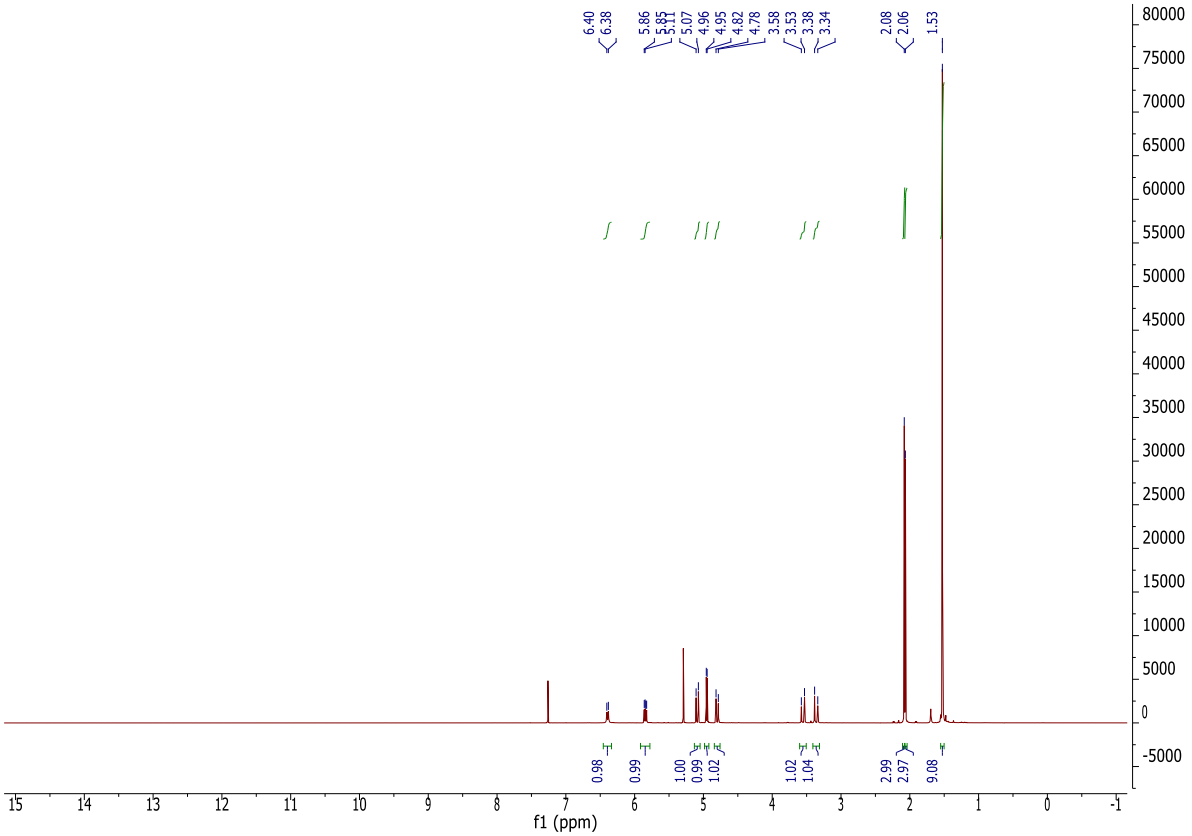


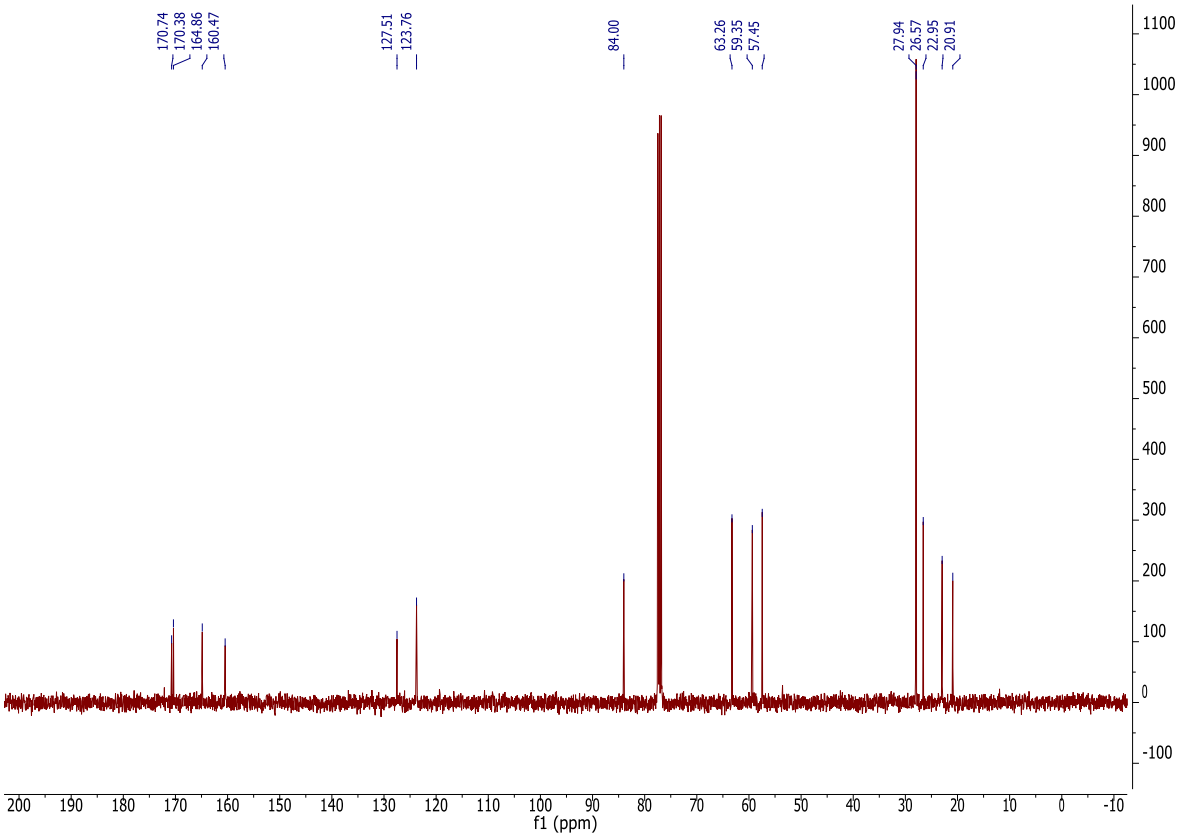
Compound **28** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)



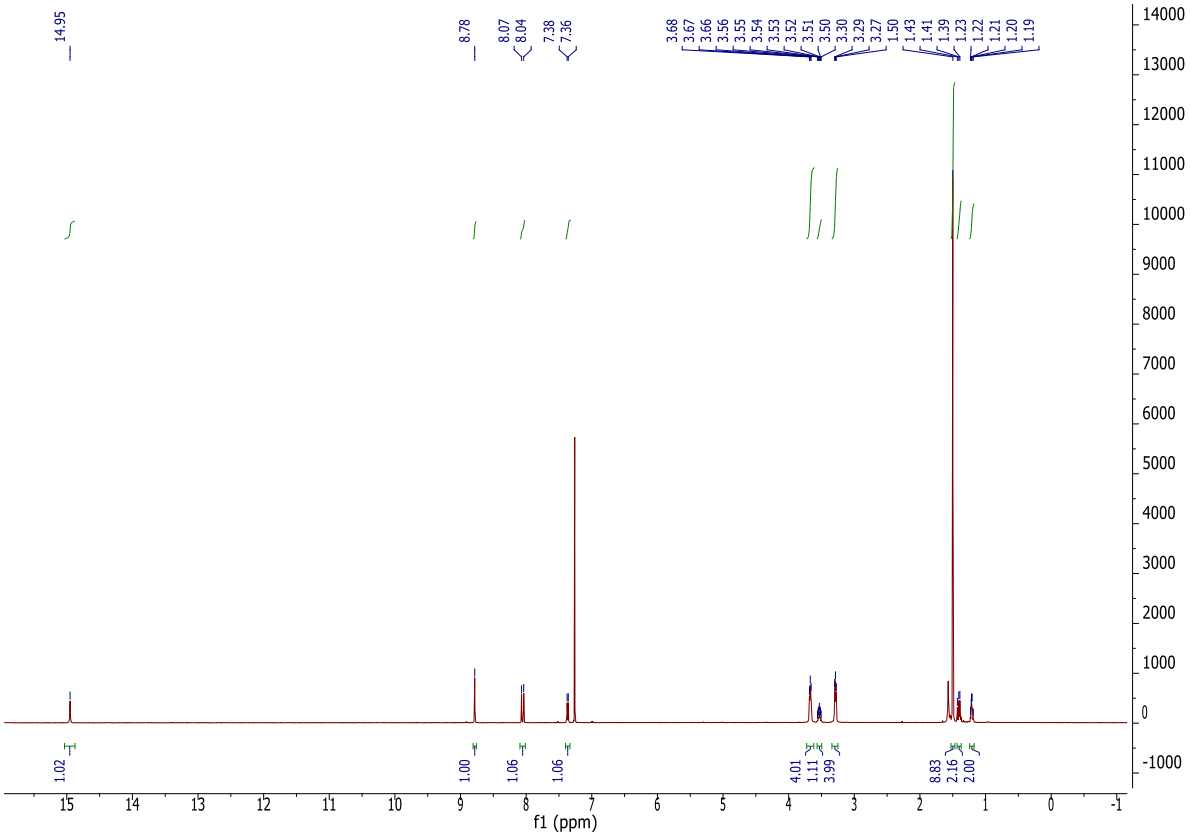


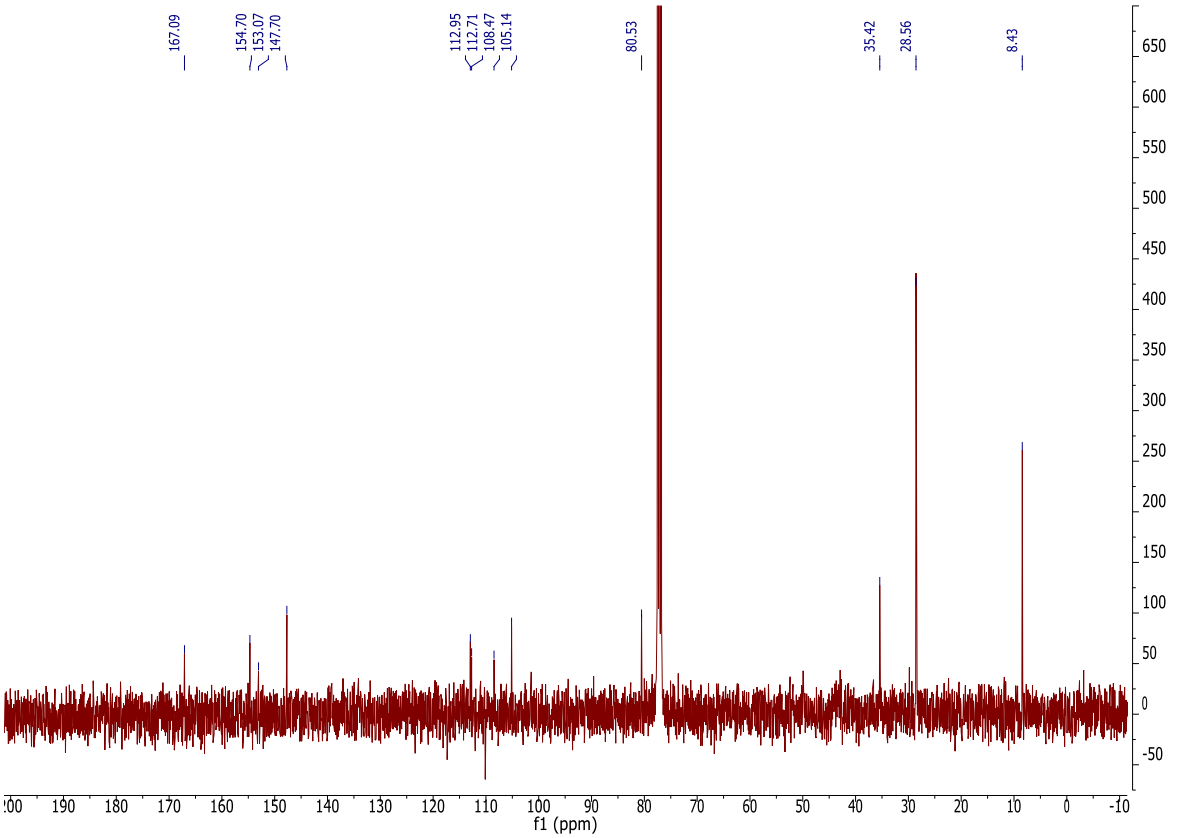
Compound **29** - ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 101 MHz)



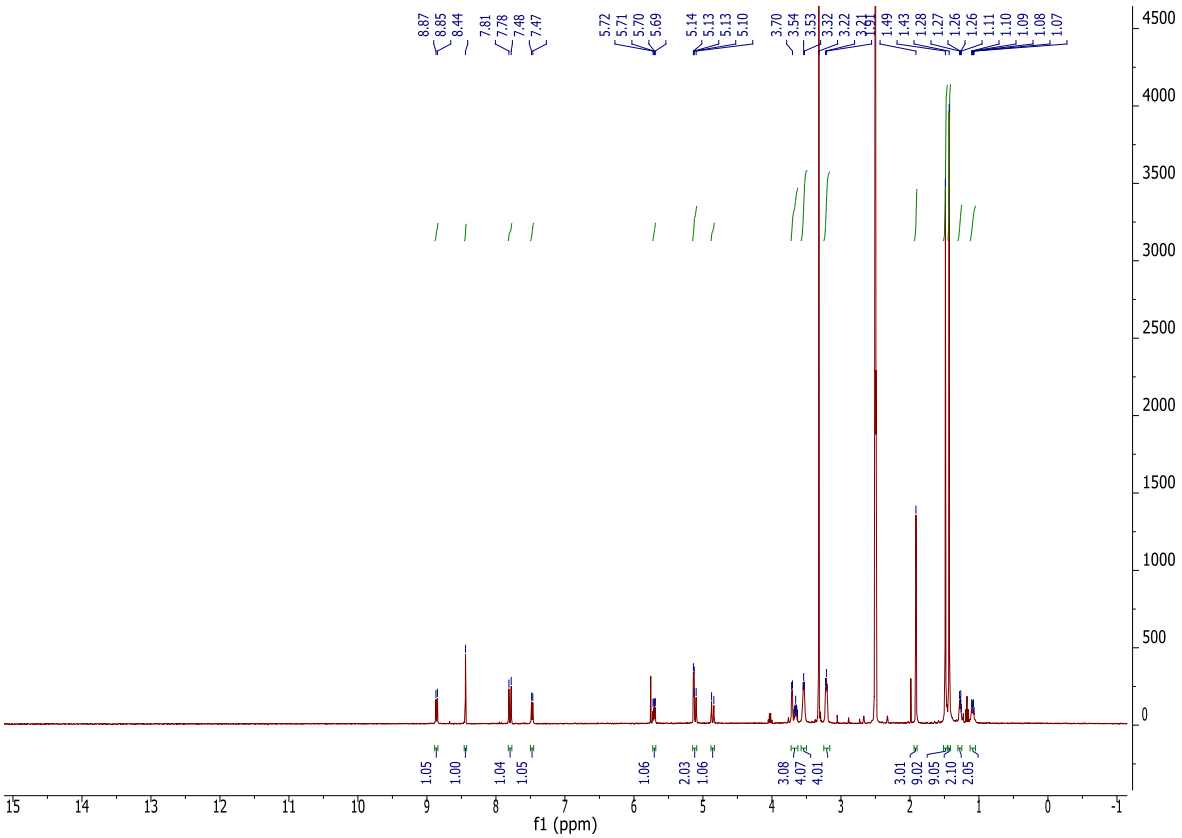


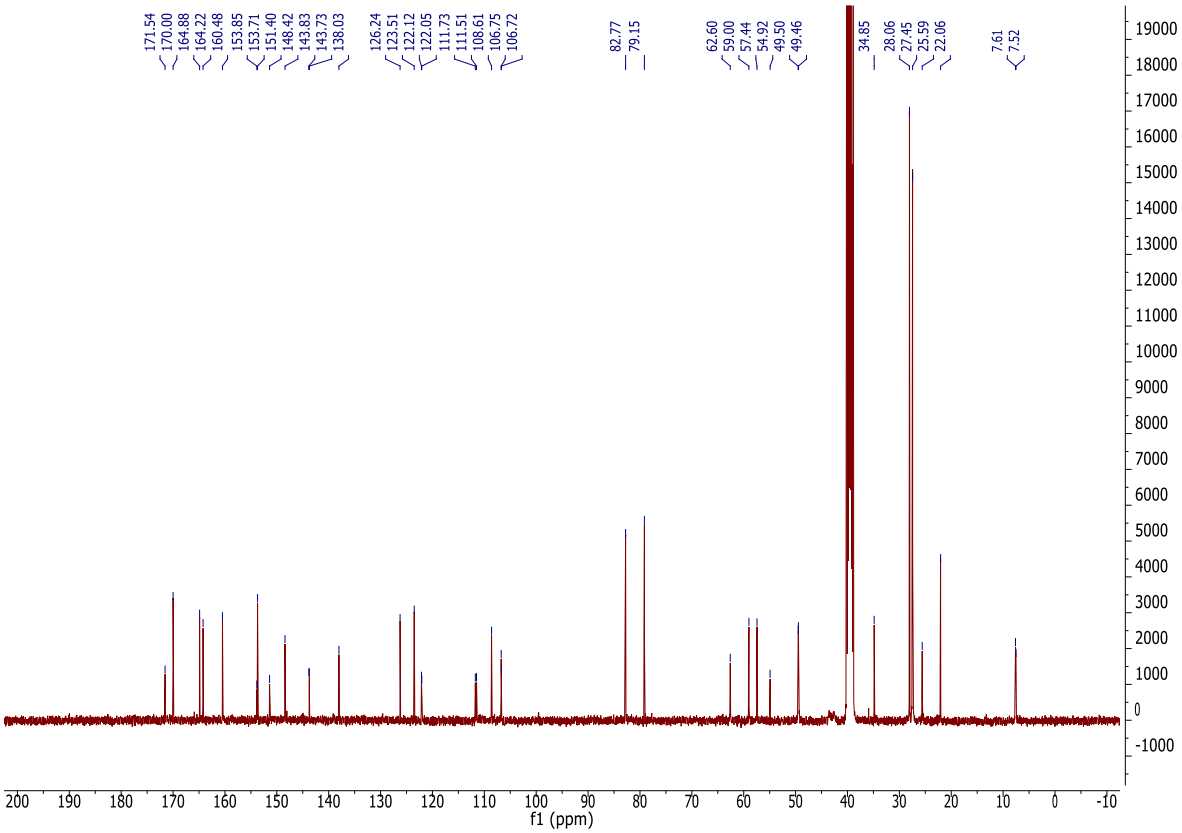
Compound **32** - ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 101 MHz)



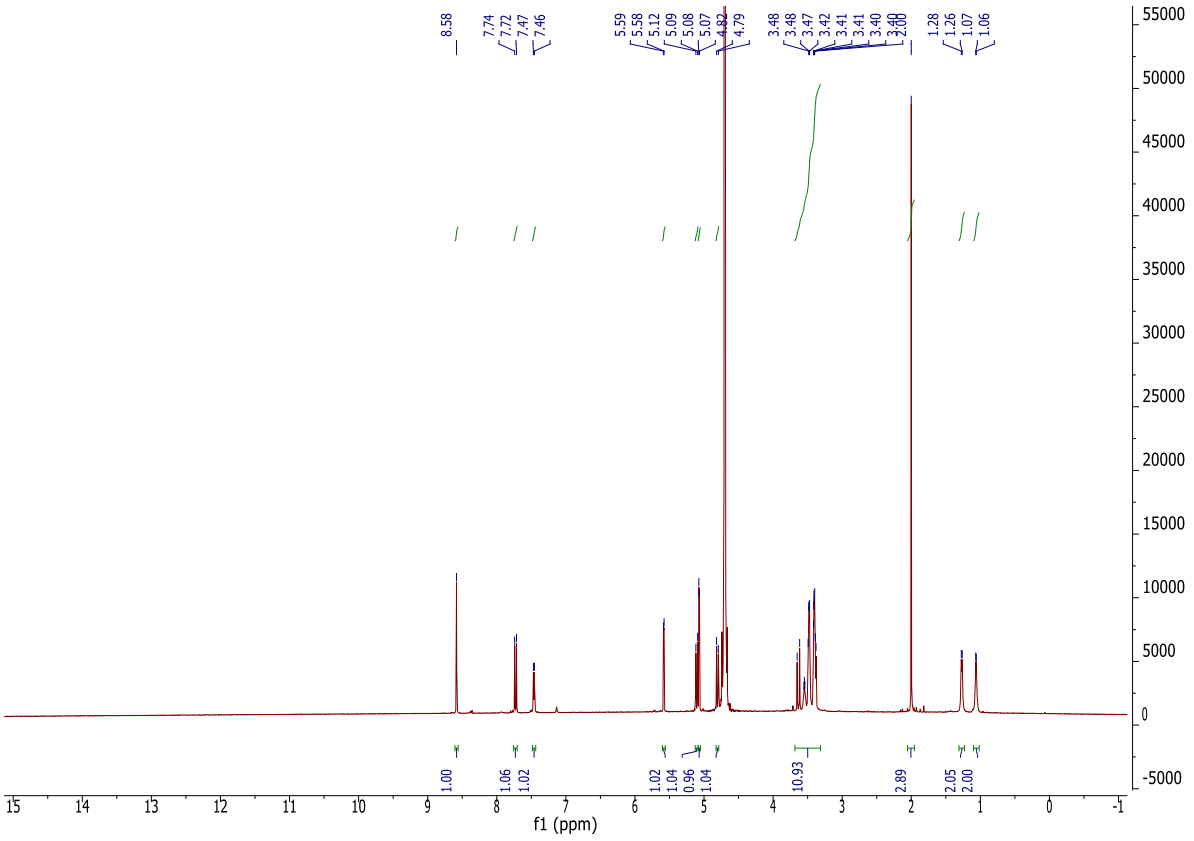


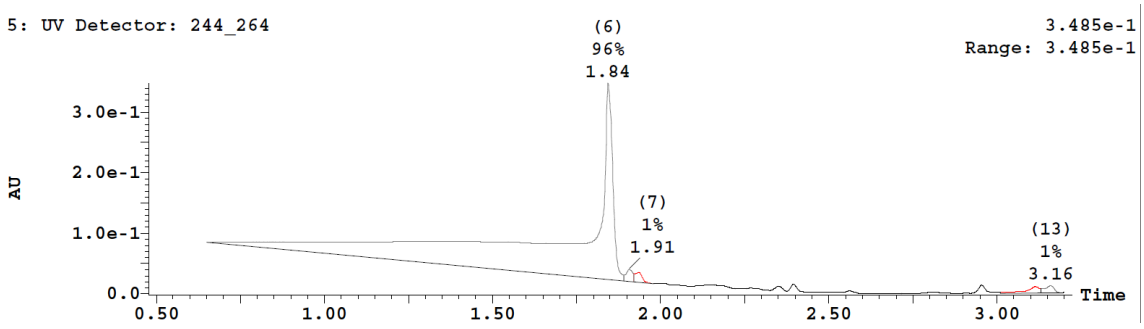
Compound **34** - ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 101 MHz)





Compound **35** - ^{1}H NMR (D_2O, 400 MHz) and LCMS UV trace





(1) Buckwell, S. C.; Page, M. I.; Waley, S. G.; Longridge, J. L. Hydrolysis of 7-Substituted Cephalosporins Catalysed by β-Lactamases I and II from Bacillus Cereus and by Hydroxide Ion. J. Chem. Soc., Perkin Trans. 2 1988, 0 (10), 1815–1821.

(2) Rao, W.-H.; Zhan, B.-B.; Chen, K.; Ling, P.-X.; Zhang, Z.-Z.; Shi, B.-F. Pd(II)-Catalyzed Direct Sulfonylation of Unactivated C(Sp3)–H Bonds with Sodium Sulfinates. Org. Lett. 2015, 17 (14), 3552–3555.

(3) Cahill, S. T.; Cain, R.; Wang, D. Y.; Lohans, C. T.; Wareham, D. W.; Oswin, H. P.; Mohammed, J.; Spencer, J.; Fishwick, C. W. G.; McDonough, M. A.; et al. Cyclic Boronates Inhibit All Classes of β-Lactamases. *Antimicrob. Agents Chemother.* **2017**, *61* (4), e02260-16.

(4) Mobley, H. L.; Green, D. M.; Trifillis, A. L.; Johnson, D. E.; Chippendale, G. R.; Lockatell, C. V; Jones, B. D.; Warren, J. W. Pyelonephritogenic Escherichia Coli and Killing of Cultured Human Renal Proximal Tubular Epithelial Cells: Role of Hemolysin in Some Strains. *Infect. Immun.* **1990**, *58* (5), 1281–1289.

(5) Bartolomé, B.; Jubete, Y.; Martínez, E.; de la Cruz, F. Construction and Properties of a Family of PACYC184-Derived Cloning Vectors Compatible with PBR322 and Its Derivatives. *Gene* **1991**, *102* (1), 75–78.

(6) Brem, J.; Cain, R.; Cahill, S.; McDonough, M. A.; Clifton, I. J.; Jiménez-Castellanos, J.-C.; Avison, M. B.; Spencer, J.; Fishwick, C. W. G.; Schofield, C. J. Structural Basis of Metallo-β-Lactamase, Serine-β-Lactamase and Penicillin-Binding Protein Inhibition by Cyclic Boronates. *Nat. Commun.* **2016**, *7*, 12406.

(7) Jiménez-Castellanos, J.-C.; Wan Nur Ismah, W. A. K.; Takebayashi, Y.; Findlay, J.; Schneiders, T.; Heesom, K. J.; Avison, M. B. Envelope Proteome Changes Driven by RamA Overproduction in Klebsiella Pneumoniae That Enhance Acquired β-Lactam Resistance. *J. Antimicrob. Chemother.* **2018**, *73* (1), 88–94.

(8) Sambrook, J.; Russell, D. W. *Molecular Cloning a Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press, 2001.

(9) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3* (2), 163–

175.

(10) Hwang, T. L.; Shaka, A. J. Water Suppression That Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. *J. Magn. Reson. Ser. A* **1995**, *112* (2), 275–279.