Contents

1. Materials and methods

1.1 Materials

Standard reagents and solvents were acquired from commercial sources and used without further purification. Carboxylic acid building blocks were purchased from commercial suppliers, including ABCR (Karlsruhe, Germany), ChemBridge (San Diego, CA), Sigma-Aldrich (St. Louis, MO), TCI Europe (Zwijndrecht, Belgium), Alfa Aesar (Ward Hill, MA), Matrix Scientific (Columbia, SC), and Acros Organics (Geel, Belgium). HATU, EDC-HCl, Biotin-LC-NHS were acquired from ChemPep (Wellington, FL) and all DNA-synthesis reagents from Glen Research (Sterling, VA). Protected DNA 41-mers with a terminal 5'-amino-C12 modifier (Glen Research) attached to a solid support (controlled pore glass, CPG) were synthesized on a MerMade DNA synthesizer. Other DNA-oligonucleotides were purchased from IBA-lifesciences (Goettingen, Germany). DEAE Sepharose was obtained from GE Healthcare Life Science (Piscataway, NJ). Amine-protected and azide-containing amino acids were purchased from Iris Biotech (Marktredwitz, Germany), with the exception of 3-azido-5-(phtalimidomethyl)benzoic acid. T4 DNA Ligase (M0202) and Klenow polymerase kit were purchased from New England Biolabs (Ipswitch, MA). Human serum albumin was acquired from Sigma Aldrich (HSA: catalogue number A3782, lyophilized powder, essential fatty acid free, 1 × crystallized). Fluorescein isothiocyanate (**FITC**, CAS: 3326-32-7) was supplied by Sigma Aldrich.

1.1.1 Instrumentation

HPLC analysis and purification was performed by reverse phase HPLC (Waters, Milford, CT) using a C18-XTerra column (5 μm, 10 on 10 x 150 mm, Waters, Milford, CT) and gradients of acetonitrile and water with 0.1 M TEAA buffer, pH 7 for oligonucleotide-conjugates purifications (**HPLC method 1**) either Synergi 4u Polar-RP 80A (4 μm, 10 x 150 mm), or gradients of acetonitrile and water with 0.1% of trifluoroacetic acid (TFA) for small-molecules purifications (**HPLC method 2**). Conjugates were characterized by LC-ESI-MS on an Agilent 6130 single quadrupole LC/MS system (Agilent Technologies, Santa Clara, CA) with electrospray ionization source. Affinity selections were performed robotically on a KingFisher Magnetic Particle Processor (Thermo Fisher, Waltham, MA) using C1 Dynabeads magnetic beads (Life

Technologies, Carlsbad, CA). Instant JChem (ChemAxon) was used for structure and data management. PCR purification and gel extraction kits were provided by QIAGEN and used according to the protocol provided by the supplier. The selection fingerprints were analysed using Matlab (MathWorks). Fluorescence polarization (FP) analysis were performed on Greiner bio-one 384-wells plates using Spectramax Paradigm (Molecular devices). SPR assays were performed using Biacore S200 (GE Healthcare Life Sciences).

1.2 Methods

1.2.1 Encoding strategy

Supplementary figure 1 provides an outline for the stepwise encoding of multiple chemical reactions. Individual molecules in the library consist of 3 building blocks and they are unambiguously identified by the corresponding DNA segments, termed *Code 1*, *Code 2* and *Code 3*.

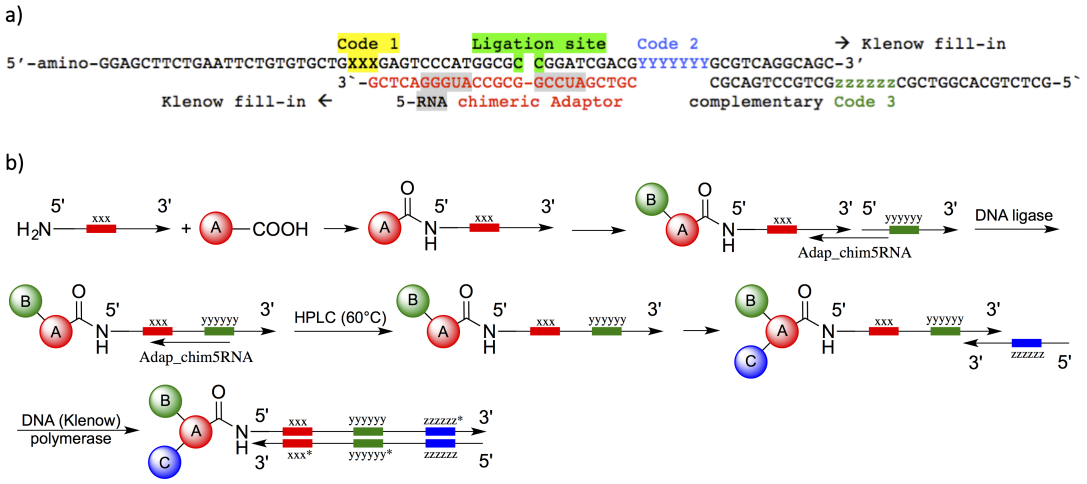


Figure 1.1: (a) Schematic representation of the key oligonucleotide building blocks, used for library construction and encoding. The "**xxx**", "**yyy yyy y**" and "**zzz zzz**" segments delineate portions of the DNA sequence with different codes for different library members. (b) Schematic representation of the encoding strategy. The seven scaffolds (building blocks "A") were encoded by chemical coupling with seven 5'-amino- C12 modifier oligonucleotides containing a 3-bases code (code 1, **xxx**). The first set of carboxylic acids connected to the scaffolds (building blocks "B") was encoded by including in the sequence a DNA fragment containing a 7-bases code (code 2, **yyy yyy y**). This encoding step was performed using DNA-ligase and a partially-complementary chimeric DNA/RNA adaptor (Adap-chim5RNA). The adaptor was removed by HPLC purification in denaturing conditions (60°C). The second set of carboxylic acids and alkynes (building blocks "C") was encoded by Klenow-polymerase fill-in reaction transferring a 6-bases reverse code (code 3, **zzz zzz**) from a complementary oligonucleotide. The result of the last encoding is a double-strand DNA containing the 3 codes unambiguously associated to the three sets of building blocks. The asterisk (∗) indicates that the codes were transferred as reverse and complementary sequence.

1.2.2 General procedure for library construction

Immobilization of DNA on DEAE anion exchange resin

DEAE sepharose (100 μL of slurry in 20 % EtOH; DEAE sepharose fast flow, GE healthcare, 17-0709-01) was transferred into a 96-wells filter plate (Unifilter 800 μL polystyrene, 25 μm

polypropylene filter, GE healthcare, 7700-2804) and the liquids removed by filtration in a multi-wells plate vacuum manifold (PALL Life Science). The sepharose was washed with water (2 × 500 μL) and 10 mM aq. AcOH (2 × 500 μL). The DNA solution (2 nmol of DNA in 100 μL of Milli-Q-purified water) was added to the resin and incubated for 10 min and dried under vacuum. The sepharose was washed with 10 mM aq. AcOH (2 × 500 μL) and DMSO (2 × 500 μL).

Elution from solid support

The DEAE sepharose was rinsed with DMSO (2 × 500 mL) and 10 mM aq. AcOH (2 × 500 mL). Elution buffer (3 M aq. AcOH/NaOAc, pH 4.75; 200 μL) was added to the sepharose and incubated for 5 min with eventual agitation. Vacuum was applied to the filter plates in a vacuum manifold collecting the eluate in a 96-well collector plate. Elution was repeated by application of additional elution buffer (3 M aq. AcOH/NaOAc, pH 4.75; 200 μL), incubation for 5 min and collection of eluate.

Ethanol precipitation

Samples were transferred into 2.0 mL Eppendorf tubes and 3M AcOH/AcONa buffer (pH=4.7, 10% of sample volume) was added. The samples were mixed and EtOH (300% of sample volume) was added. The mixtures were kept 1 hour at room temperature and 3 hours at -20°C. The tubes were centrifuged in a table-top centrifuge at 13.2 krpm (30 min at 4°C); the supernatant was removed and the pellet dispersed in cold (-20°C) 85 % EtOH and centrifugation repeated. The supernatant was removed, the pellet dried briefly under vacuum and dissolved in water for further analysis. Polyacrylamide gel electrophoresis. Ligation and RNA-digestion experiments were analyzed on denaturing polyacrylamide 15% TBE-Urea gels (EC68855BOX, 1.0 mm, 15 well, Invitrogen). A voltage of 180 V was applied on the electrophoresis box (Novex) for 1 hour. The gels were stained using SYBR Green I and imaged.

1.2.3 General procedure for "DNA-off" resynthesis

General procedure of amide bond formation

Carboxylic acids (1.2 eq.) were dissolved in dry DMF and activated (10 minutes at room temperature) adding a solution of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 1.3 eq.), HOAt (0.2 eq.) and DIPEA (3 eq.) in dry DMF. The amine (1 eq. in dry DMF) was added to the activated carboxylic acid solution and the resulting mixture was stirred overnight at room temperature. The reaction progress was monitored by UPLC-MS (TOF+).

General procedure of methyl ester hydrolysis

The methyl ester derivative were suspended in 3M LiOH (10 eq.) water : methanol = 1:1 solution and the suspension was stirred for 1 hour at room temperature. The reaction mixture was concentrated and neutralized with 2M HCl until reaching an acidic pH. The hydrolysed carboxylic acids were extracted from the aqueous phases with dichloromethane.

General procedure of Fmoc-deprotection

The Fmoc-protected compounds were dissolved in a Piperidine (10 eq., 20% in DMF) and DBU (0.05 eq.) solution. The mixture was stirred for 10 minutes at room temperature. The solution was concentrated and the de-protected amine was filtered on silica gel (Sigma Aldrich, pore size 60 Å, 230-240 mesh particle size), washed with ethyl acetate and eluted by ethyl acetate : methanol : TEA = 90 : 9 : 1.

General procedure of Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)

The reaction was carried out with degassed solvents in argon atmosphere. The reaction catalyst solution was prepared mixing in the order $CuSO_4 \cdot 5H_2O$ (0.2 eq, in Milli-Q-purified water), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 0.4 eq., in DMF) and Sodium ascorbate (0.6 eq., in Milli-Q-purified water) in DMF:water = 4:1. The catalyst solution was added to alkynes (1 eq.) dissolved in DMF. The reaction mixture was stirred overnight. The copper was removed washing the product with EDTA buffer (0.1 M, pH=9) and water.

2. Library synthesis

The following scheme depicts the iterative split&pool strategy, used for library synthesis. The details of the encoding procedure are not provided here, as they have been described in more detailed in a preceding section. The schematic representation, reported below, does not explicitly discriminates between single-stranded DNA and double-stranded DNA.

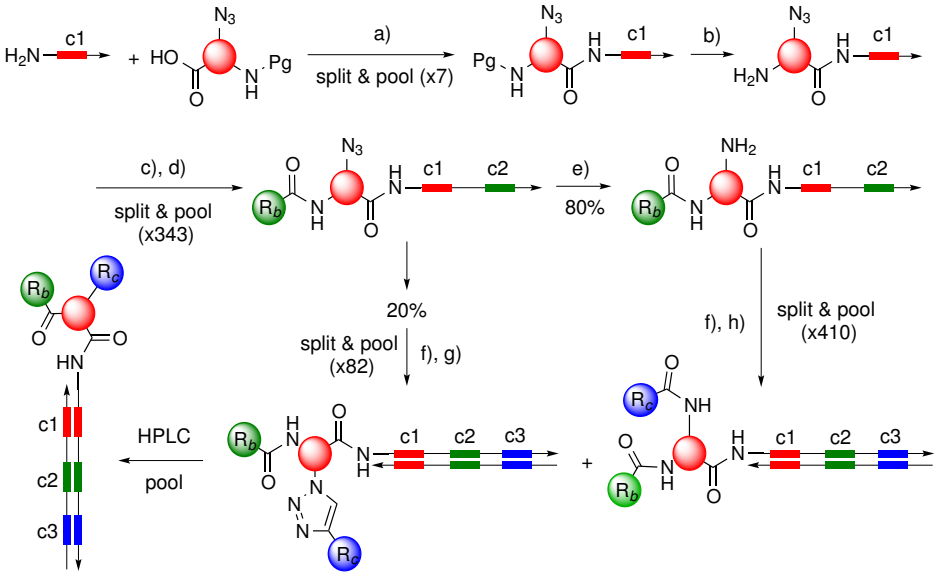


Figure 2.1: schematic representation of DECL synthesis. Reaction conditions: a) Pseudo-solid phase synthesis (on DEAE sepharose), HATU, DIPEA, DMF; b) NH_3/$MeNH_2$, H_2O; c) Pseudo-solid phase synthesis (on DEAE sepharose), $\mathbf{R_b}CO_2H$, EDC, HOAt, DMSO; d) code **c2**, Adap-chim5RNA, NEB buffer 1× (pH=7.9); e) TECP, Tris buffer (1M, pH=7.4), 14h, r.t. f) code c3, MOPS 10× (pH=7.9), DNA Polymerase I (Klenow), 5 mM dNTP; g) $\mathbf{R_c}C{\equiv}CH$, Cu(II)-TBTA, sodium ascorbate, DMSO:H2O = 2:1; h) $\mathbf{R_c}CO_2H$, EDC, HOAt, DIPEA, DMSO, 16h, r.t. Pg (protective group): Fmoc (scaffolds **1** - **6**) or phtalimido (scaffold **7**).

2.1 DNA-conjugation of variable scaffolds

Protected DNA 41-mers with a terminal 5'-amino- C12 modifier (Glen Research) attached to a solid support (controlled pore glass, CPG) were distributed into synthesis cartridges and further derivatized according to a literature protocol [1]. The supports were washed with MeCN and DCM (2 × 2 mL). For removal of the amine Mmt-protecting group, a solution of 3 % (m/v) trichloroacetic acid (TCA) in DCM (1 - 2 mL) was eluted dropwise from the cartridge followed by washing with DCM (2 mL) and these two steps were repeated 6 times. The solid support was washed with DCM (1 × 1 mL) and MeCN (2 × 1 mL) and dried under a stream of air.

For scaffold conjugation, a solution of the corresponding orthogonally protected azido-amino acid scaffold (50 mM, **Table 6.1**), DIPEA (150 mM) and HATU (50 mM) in 1 mL DMF was preincubated for 5 min, added to the immobilized DNA and agitated for 2 h at room temperature using an overhead-shaker. The solution was removed and the solid support rinsed with DMF, MeCN and DCM (2 × 1 mL) and dried under a stream of air. The DNA was cleaved from the solid support and deprotected by 2 h incubation in conc. aq. $NH_3/MeNH_2$ (AMA; 1 mL) at room temperature. The AMA solution was evaporated, the residue dissolved in water and the DNA conjugates purified by reverse-phase HPLC. Product containing fractions were combined, evaporated and analyzed by LC-ESI-MS measurement. Coupling of first set of carboxylic acids (B). Equimolar amounts of the 7 DNA-scaffold conjugates (125 nmol each) obtained as described above were combined and further derivatized according to a modified literature protocol [1, 2]. The combined conjugates (2 nmol) were immobilized on DEAE sepharose. A solution of the corresponding carboxylic acid building block (50 mM), EDC (HCl salt, 50 mM) and HOAt (5 mM) in DMSO (0.5 mL) was preincubated for 5 min, added to the resin-immobilized DNA and agitated for 2 h at room temperature using an overhead-shaker. The solution was removed and the resin washed with DMSO (2 × 0.5 mL) and treated with freshly activated reaction solution. These steps were repeated to reach three coupling steps of 2 h each. The solution was removed and the resin washed with DMSO (2 × 0.5 mL). To ensure a high degree of conversion at DE-2, only carboxylic acids with high conversion yields (typically >80%, **Table 6.2**) under the employed conditions were used for library synthesis [3].To cap unreacted amine functional groups, a solution of Acetyl-NHS (100 mM) and DMAP (20 mM) in DMSO (0.2 mL) was preincubated for 5 min, added to the resin- immobilized DNA and agitated for 3 h at room temperature on an orbital shaker. The solution was removed and the resin washed with DMSO (2 × 0.5 mL). The DNA was eluted from solid support, isolated by ethanol precipitation and the pellets redissolved in Milli-Q-purified water (50 μL). The aliquots were stored at -20 °C for the next step of encoding.

2.2 Encoding by Ligation

In PCR 96-wells plates (V=250 μL) the following components were pipetted: 343 "code 1" conjugates (170 μL, 1.5 μM, 0.255 nmol each), 343 "codes 2" (1.8 μL, 200 μM, 0.36 nmol, 1.4 eq. each), the chimeric DNA-RNA adaptor (Adap-chim5RNA, 1.5 μL, 500 μM, 0.75 nmol, 3 eq.), 25 μL of NEB buffer 10× (1X Buffer Components: 50mM NaCl , 10mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT, pH 7.9) and 48 μL of Milli-Q-purified water. The plates were heated at 65 °C for 5 minutes and then cooled to +25 °C. T4-DNA Ligase (1 μL, 400 units each reaction, Supplier: NEB) was added and the reactions were kept at room temperature overnight. The

enzyme was deactivated by heating at 65 °C for 20 minutes. After quenching, the reactions were pooled ad precipitated by ethanol. The DNA pellet was dissolved in 1.5 mL of Milli-Q-purified water and the adaptor was removed by HPLC purification (**HPLC method 1**, 60°C, Gradient (% of acetonitrile buffer): 5-20 in 9 min., 20-90 in 1 min., 90-100 in 8 min.). The purified "code1-code2" library was precipitated by ethanol and the pellet was dissolved in 2.00 mL of Milli-Q-purified water (c = 28.2 μM, n = 56.4 nmol). Staudinger reduction. The reaction was carried out with degassed solvents in argon atmosphere. The 28.2 μM "code1-code2" library solution (1.6 mL, 45 nmol) was treated for 14 hours at room temperature with 200 mg of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 0.7 mmol) in 2.8 mL of Tris-HCl buffer (1M, pH=7.4). The reaction was quenched by ethanol precipitation and the resulting crude product was purified by HPLC (**HPLC method 1**, 30°C, Gradient (% of acetonitrile buffer): 5-20 in 9 min., 20-90 in 1 min., 90-100 in 8 min.).

2.3 Encoding by Klenow polymerase

In PCR 96-wells plates (V=250 μL) were disposed the reduced (well 1-410, 50 pmol, 13 μL each) and the non-reduced "code1-code2" library (well 411-492, 50 pmol, 13 μL each). To each well was added: 10 μM "code 3" (7.5 μL, 75 pmol, 1.5 eq.), 5μL of MOPS buffer 10x (500 mM NaCl, 100 mM MOPS, 100 mM MgCl2 10 mM DTT, pH=7.9 at 25°C) and 22 μL of Milli-Q-purified water. The plates were heated for 10 minutes at 65°C and then cooled to +25°C. 10 units of DNA Polymerase I, Large (Klenow) fragment (NEB, 5'000 U/mL) dissolved in 10 μL of of MOPS buffer 1.3x and 5mM dNTP (3 μL) were added to each well. The reactions were kept at room temperature for 1 hour, quenched by freezing (-20°C) and dried overnight by speed-vacuum machine.

2.4 Coupling of third set of building blocks

The 492 building blocks consisting of 410 carboxylic acids (**Table 6.3**, C1-C410) and 82 alkynes ((**Table 6.3**, C411-C492) were selected according with their coupling yields. These results were obtained by screening one thousand compounds in different reaction conditions [4].

2.4.1 Coupling by amide bond formation

To each well corresponding to the first 410 "codes 3" primary-amino compounds dissolved in 50 mM MOPS buffer (30 μL each well, pH=7.9) were added the mixture of carboxylic acid (45 μL, 60 mM), EDC (4 μL, 300 mM), HOAt (4 μL, 60 mM) and DIPEA (4 μL, 300 mM) in dry DMSO previuosly activated for 15 minutes at room temperature. The reaction solutions were agitated for additional 16 hours. The reaction solutions were treated for a second addition

by freshly activated carboxylic acid in DMSO and they were agitated for further 6 hours at room temperature. The reactions were quenched by addition of ammonium acetate (NH4OAc, 500 mM, 25 μL each). The 410 reactions were pooled together, concentrated and the DNA conjugates were precipitated by Ethanol. The pellet was dissolved in Milli-Q-purified water and purified by HPLC (HPLC method 1, 30°C, Gradient (% of acetonitrile buffer): 10-40 in 15 min., 40-100 in 9 min.).

2.4.2 Coupling by Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)

The reaction was carried out with degassed solvents. The catalyst solution was prepared by separately mixing 1.0 mL of 20 mM TBTA in DMSO:tBuOH = 3:1, 500 μL of 20 mM Copper (II) Acetate in DMSO:H2O:tBuOH = 3:4:1 and 500 μL of 100 mM (+)-Sodium L-ascorbate in Milli-Q-purified water. The alkynes (100 mM in DMSO, 5μL each) were activated for 10 minutes at room temperature by adding 100 μL of DMSO:water = 2:1, 20 μL of 0.5M TEA buffer (pH=10) and 30 μL of catalyst solution (freshly prepared). To each well containing the "code 3" azido derivatives (411 - 492) in 10 μL of water:DMSO = 2:1 were added 6.5 μL of alkyne solution and the reactions were agitated overnight at room temperature. The reaction solutions were treated for a second addition of freshly activated alkynes in DMSO:water = 2:1 and agitated for 2 hours at room temperature. The 96 reactions were pooled and purified directly by HPLC (**HPLC method 1**, 30°C, Gradient (% of acetonitrile buffer): 10-40 in 15 min., 40-100 in 9 min.). The purified compounds synthesised by amide coupling and by CuAAC coupling were mixed in molar ratio 8:2, respectively. The final DECL was precipitated twice by ethanol and the obtained pellet (8 nmol) was dissolved in 8.0 mL of Milli-Q-purified water (DECL stock solution 1 μM).

3. Affinity selections

Affinity selection, PCR and high-throughput sequencing. The synthesised DNA-encoded chemical library (DECL), comprising 1.2 million members, was screened against biotinylated proteins. A target protein is immobilized on magnetic beads and subsequently incubated with an aliquot of DECL (2,5 μL of 200 nM). The non-binding library members were washed away with several washing steps whereas binding compounds were eluted and the DNA barcodes were amplified by PCR, using two Illumina primers containing also a selection identification code [5]. After each PCR step, the products were purified using a quick PCR purification kit (QIAGEN). The PCR2 products were pooled in equimolar ratio (0.35 pmol each selection), purified on Agarose / TBE gel and extracted by using a quick Gel Extraction Kit (QIAGEN). The purified DNA barcodes were sequenced by Illumina high-throughput sequencing (Functional Genomic Centre Zürich). Data analysis. DNA HT-sequencing generated raw data which were converted from the standard output $*$.fastq data files into $*$.fasta files. The generated $*$.fasta file was processed by C++ program as reported in a published procedure [5]. The final selection fingerprints were generated by analysing the output data with a Matlab code [5]. Examples of affinity selection results are reported in the appendix (6.2).

4. Hit Validation

All reactions using anhydrous conditions were carried out under an argon atmosphere in oven-dried glassware. Anhydrous solvents for reactions were purchased from Acros Organics or Sigma-Aldrich.

4.1 Synthesis of A7/B66/C292 (HSA binder)

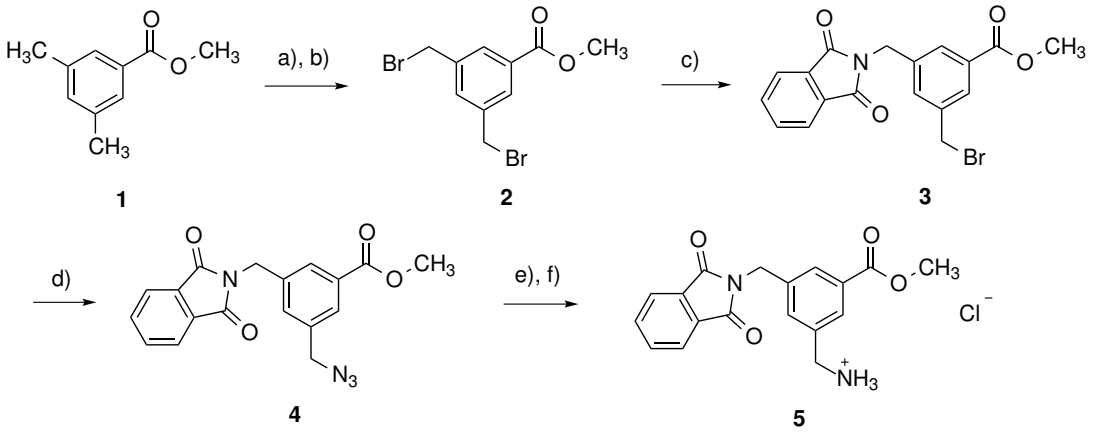


Figure 4.1: Synthesis of scaffold **A7**. Reaction conditions: a) NBS, benzoyl peroxide 5%, CCl_4 reflux 4h; b) diethylphosphite, TEA, THF, r.t. 48h; c) N-potassium phtalimide (1.1 eq), CH_3CN, reflux 4h; d) NaN_3 (10 eq), DMSO:Acetone = 1:1, 50°C, o.n.; e) PPh_3, THF, r.t., 2h; f) H_2O, 5% HCl, r.t., o.n.

4.1.1 Synthesis of compound 2

N-Bromosuccinimide (**NBS**, 12 g, 67 mmol, 2.05 eq.) was added in five equal portions for 12 h to a refluxing solution of 5.0 g of commercially available Methyl 3,5-dimethylbenzoate (33 mmol, Sigma-Aldrich, Mol ID: 370215-5G, CAS N°: 25081-39-4) in 100 mL of dry carbon tetrachloride. Each addition of NBS was followed by the addition few milligrams of benzoyl peroxide. After the completion of the reaction, the mixture was cooled and filtered to remove NBS. The filtrate was washed with 200 mL of water, then with 100 mL of aqueous sodium bicarbonate solution and 10 mL of brine followed by drying with anhydrous sodium sulfate. The solvent was removed under reduced pressure; the residue was dissolved in 100 mL of dry THF. To the resulting solution were added 8.6 mL (67 mmol) of diethyl phosphite and 11.2 mL (67 mmol) of N,N-di-isopropyl ethyl amine at 0°C under argon atmosphere. The mixture

was gradually warmed to room temperature and stirred for 2 days. The solvent was removed under vacuum; the residue was poured into ice water and extracted with diethyl ether (3 x 100 mL). The organic layer was washed with 100 mL of 1 N hydrochloric acid and brine, dried with anhydrous sodium sulfate, filtered and evaporation of the solvent gave the crude product. The pure product **2** was obtained by silica gel column chromatography with 4:1 hexane:ethylacetate as an eluent. The product was a white crystalline solid [6] (5.0 g = 47%.). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.00 (d, J = 1.8 Hz, 2H), 7.62 (t, J = 1.8 Hz, 1H), 4.50 (s, 4H), 3.93 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.93, 138.96, 133.85, 131.42, 130.03, 52.43, 32.05.

4.1.2 Synthesis of compound 3

The di-bromo derivative **2** (5.0 g, 15.5 mmol) and 2.9 g of N-potassium phtalimide (1.1 eq., 15.7 mmol) were refluxed in 100 mL of dry acetonitrile for 4 hours, yielding product **3**, the di-substituted derivative and the unreacted starting material **2** in ratio 2:1:1. After removing the solvent, the crude product was re-dissolved in dichloromethane and extracted with water. The organic phases were dried with Na_2SO_4 and concentrated. The pure product **3** was isolated from the mixture by silica gel column chromatography with 8:2 hexane:ethylacetate as an eluent (3.7 g = 61%). ^{1}H NMR(400 MHz, Chloroform-d) δ 8.00 (dt, J = 9.5, 1.7 Hz, 2H), 7.93 - 7.81 (m, 2H), 7.82 - 7.69 (m, 2H), 7.64 (t, J = 1.8 Hz, 1H), 4.88 (s, 2H), 4.48 (s, 2H), 3.91 (s, 3H). ^{13}C NMR - dept135 (101 MHz, $CDCl_3$) δ 134.18, 133.50, 129.59, 123.55, 52.34, 41.04, 32.06.

4.1.3 Synthesis of compound 4

Product **3** (3.7 g, 9.5 mmol) and sodium azide (10 eq., 95 mmol, 6.2 g) were stirred in 150 mL of DMSO:Acetone = 1:1 at 50 °C overnight. After the reaction was completed, the solvent was removed under reduced pressure. The reaction mixture was concentrated under vacuum and diluted with ethyl acetate. The organic solution was washed with water (x4) and dried with anhydrous sodium sulphate. The pure product **4** was obtained by silica gel column chromatography with 3:7 hexane:ethylacetate as an eluent (1.9 g = 58%). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.05 (s, 1H), 7.91 (s, 1H), 7.88 - 7.84 (m, 2H), 7.72 (dd, J = 5.5, 3.1 Hz, 2H), 7.56 (s, 1H), 4.89 (s, 2H), 4.39 (s, 2H), 3.90 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.88, 166.26, 137.47, 136.57, 134.17, 132.56, 131.97, 131.30, 129.47, 128.70, 123.52, 119.99, 54.09, 52.34, 41.11.

4.1.4 Synthesis of compound 5

1.5 g of azido derivative **4** (4.3 mmol) were dissolved in 50 mL of dry tetrahydrofuran (THF) and 2.3 g of triphenylphosphine (PPh_3, 2 eq, 8.6 mmol) were added. The reaction was stirred at

room temperature for 4 hours and the mixture was concentrated under vacuum. The triphenylphosphanimine adduct was hydrolysed by adding 10 mL of water and 2 mL of 5% HCl solution. The resulting solution was stirred overnight. After the completed hydrolysis, the mixture was diluted with water, filtered and washed many times with ethyl acetate. The aqueous phases containing the product **5** were frozen and lyophilised overnight. The pure product **5** was obtained by HPLC purification (570 mg = 41%.). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.27 (s, 3H), 8.01 (t, J = 1.6 Hz, 1H), 7.94 (t, J = 1.6 Hz, 1H), 7.92 - 7.83 (m, 4H), 7.64 (s, 1H), 4.84 (s, 2H), 4.08 (s, 2H), 3.86 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 168.13, 166.14, 138.36, 135.78, 135.12, 132.96, 132.05, 130.65, 129.40, 128.77, 123.79, 52.82, 42.14, 40.88.

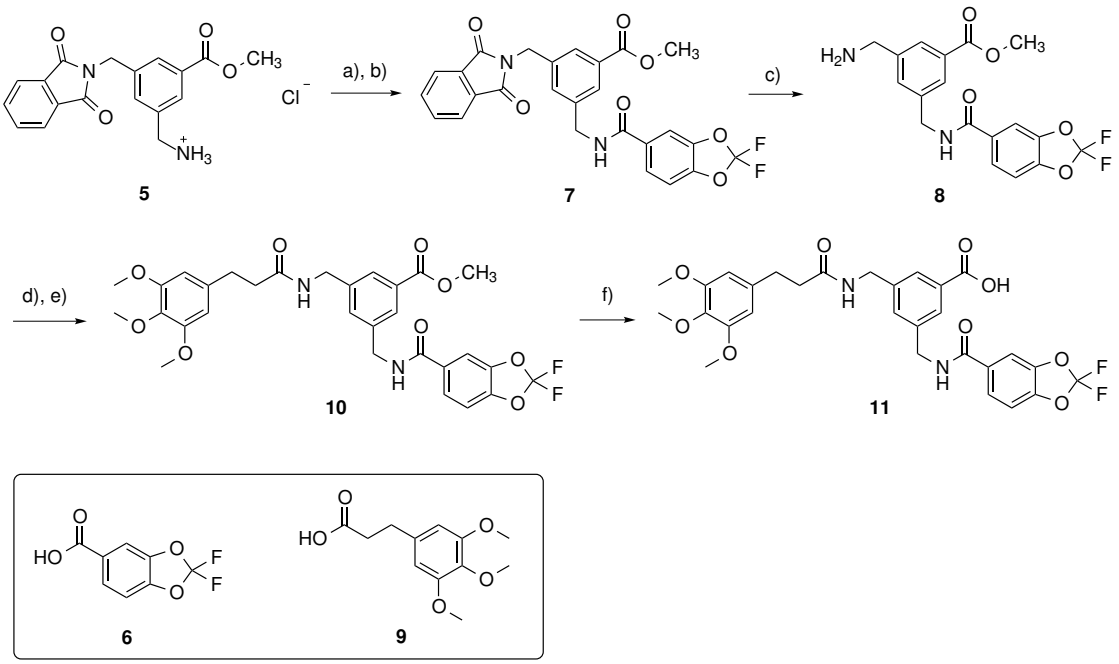


Figure 4.2: Synthesis of compound **A7/B66/C292-COOH**. Reaction conditions: a) **6**, HOAt, EDC, DIPEA, DMF, 15', r.t.; b) Activated solution of **6**, DMF:DCM, o.n., r.t.; c) H_2N-NH_2, Ethanol, reflux 1h; d) **9**, HOAt, EDC, DIPEA, DMF, 15', r.t.; e) Activated solution of **9**, DMF:DCM, o.n., r.t.; f) 3M LiOH, MeOH:H_2O = 1:1, 1h, r.t.

4.1.5 Synthesis of compound 7

The amino derivative **5** (0.462 mmol, 150 mg) was coupled with the commercially available carboxylic acid **6** (1.2 eq. 110 mg, 0.54 mmol, abcr GmbH, CAS: 656-46-2) according with the general procedure of amide bond formation (1.2.3). The obtained product **7** was dissolved in ethyl acetate and washed with $NaHCO_3$ saturated solution and water. The organic phases were dried and concentrated. The pure product **7** was obtained by silica gel column chromatography with 1:1 hexane:ethylacetate as an eluent (226 mg, 96%). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.97 (s, 1H), 7.90 (s, 1H), 7.83 (dd, J = 5.5, 3.1 Hz, 2H), 7.72 (dd, J = 5.5, 3.1 Hz, 2H), 7.60 - 7.49 (m, 3H), 7.08 (d, J = 8.9 Hz, 1H), 6.54 (t, J = 5.7 Hz, 1H), 4.86 (s, 2H), 4.63 (d, J = 5.7 Hz, 2H), 3.88 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$3) δ 163.17, 161.64, 161.02, 141.24,

139.19, 134.24, 132.63, 129.44, 127.51, 127.19, 126.49, 125.83, 123.98, 123.62, 118.75, 118.33, 104.47, 104.27, 47.55, 38.95, 36.36.

4.1.6 Synthesis of compound 8

The compound **7** (1 eq., 226 mg, 0.445 mmol) was suspended in ethanol and hydrazine monohydrate (4 eq., 1.78 mmol, 136 μL). The suspension was refluxed for 1 hour since the entire solid was dissolved. The solution was cooled in an ice bath and the precipitated was filtered out. The ethanol solution was concentrated and the crude product **8** was purified by HPLC (165 mg, 98%). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.26 (t, J = 5.9 Hz, 1H), 8.28 (s, 3H), 7.99 (s, 1H), 7.93 (s, 1H), 7.88 (d, J = 1.6 Hz, 1H), 7.81 (dd, J = 8.4, 1.6 Hz, 1H), 7.67 (s, 1H), 7.53 (d, J = 8.4 Hz, 1H), 4.53 (s, 2H), 4.10 (s, 2H), 3.85 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 166.33, 165.07, 145.24, 143.24, 141.17, 135.28, 133.22, 131.70, 131.32, 130.49, 128.79, 128.47, 124.92, 110.41, 109.65, 52.76, 42.88, 42.24.

4.1.7 Synthesis of compound 10

The amino derivative **8** (0.437 mmol, 165 mg) was coupled with the commercially available carboxylic acid **9** (1.3 eq. 137 mg, 0.57 mmol, Fluka, CAS: 25173-72-2) according with the general procedure of amide bond formation (1.2.3). The obtained product **10** was dissolved in ethyl acetate and washed with $NaHCO_3$ saturated solution and water. The organic phases were dried and concentrated. The pure product **10** was obtained by silica gel column chromatography with 1:1 hexane:ethylacetate as an eluent (200 mg, 78%). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.13 (t, J = 5.9 Hz, 1H), 8.39 (s, 1H), 7.80 (d, J = 1.6 Hz, 1H), 7.75 (d, J = 1.7 Hz, 1H), 7.73 (d, J = 1.7 Hz, 2H), 7.67 (s, 2H), 7.44 (d, J = 8.4 Hz, 1H), 7.39 (s, 1H), 6.41 (s, 2H), 4.44 (d, J = 5.9 Hz, 2H), 4.25 (d, J = 5.9 Hz, 2H), 3.77 (s, 3H), 3.64 (s, 6H), 3.54 (s, 3H), 2.73 - 2.63 (m, 3H), 2.40 - 2.32 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 171.96, 166.59, 164.99, 153.13, 145.20, 143.24, 141.06, 140.71, 137.42, 136.07, 131.54, 130.22, 127.03, 124.85, 110.35, 109.62, 105.77, 60.37, 56.14, 52.63, 42.95, 42.15, 37.53, 31.91.

4.1.8 Synthesis of compound 11

The methyl ester **10** (100 mg, 0.167 mmol) was hydrolysed to the carboxylic acid derivative **11** according with the reported general procedure (1.2.3). The pure product **11** was obtained by HPLC purification (60 mg = 60%.). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.81 (s, 1H), 7.74 (s, 1H), 7.66 (d, J = 1.8 Hz, 1H), 7.64 (d, J = 1.8 Hz, 1H), 7.61 (d, J = 1.5 Hz, 1H), 7.28 (s, 1H), 7.19 (s, 1H), 7.17 (s, 1H), 6.37 (s, 2H), 4.47 (s, 2H), 4.28 (s, 2H), 3.65 (s, 6H), 3.61 (s, 3H), 2.75 (t, J = 7.5 Hz, 2H), 2.42 (t, J = 7.6 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ 173.75,

168.11, 166.75, 152.96, 145.75, 143.60, 139.60, 136.98, 135.88, 131.21, 130.76, 130.61, 127.32, 127.25, 123.96, 109.16, 108.65, 105.20, 59.70, 55.06, 42.90, 42.25, 37.54, 31.82. 19F NMR (376 MHz, MeOD) δ -52.07. TOF-MS ES(-): 585.2118 m/z.

4.2 Synthesis of A6/B66/C292-COOH (HSA negative control)

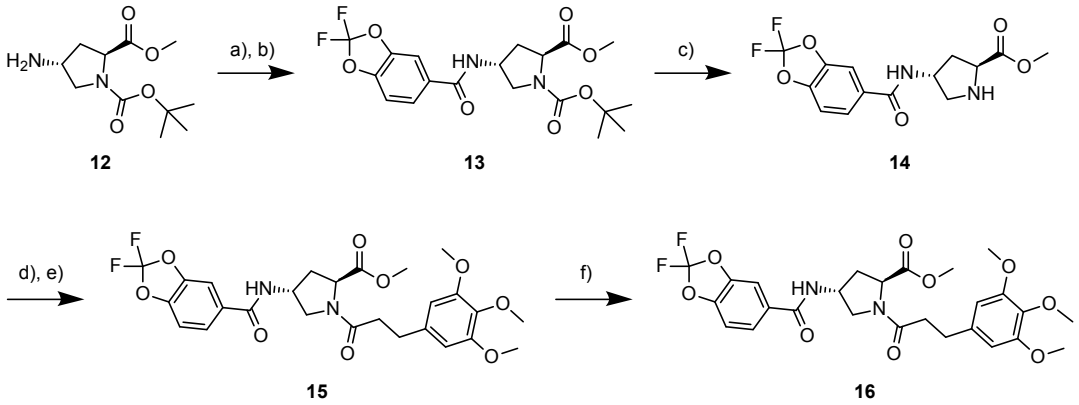


Figure 4.3: Synthesis of compound **A6/B66/C292**. Reaction conditions: a) **6**, EDC, HOAt 20%, DIPEA, DMF, 15' r.t.; b) solution (a), DMF, r.t. overnight; c) DCM:TFA=1:1, 1h, r.t.; d) **9**, EDC, HOAt 20%, DIPEA, DMF, 15' r.t.; e) solution (d), DMF, r.t. overnight; f) LiOH, H_2O:MeOH=1:1, 1h, r.t..

4.2.1 Synthesis of compound 14

The commercially available (2*S*,4*R*)-4-amino-Boc-L-pro-OMe (Bachem, 120 mg, 0.492 mmol) was dissolved in DMF and coupled to the commercially available carboxylic acid **6** (130 mg, 0.643 mmol, 1.3 eq.) according with the general procedure of amide bond formation (1.2.3). The crude product **13** was dissolved in DCM and washed with $NaHCO_3$ sat. water solution. The organic phases were collected and concentrated under reduced pressure. The resulting Boc-protected derivative was dissolved in DCM : trifluoroacetic acid (TFA) = 1:1 and the resulting mixture was stirred for 1 hour at room temperature.The reaction solution was concentrated and the pure compound **14** was obtained by silica gel column chromatography with dichloromethane:methanol 9:1 as an eluent (+1% triethylamine) (160 mg = 76%). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.87 - 7.64 (m, 2H), 7.31 (d, J = 8.4 Hz, 1H), 4.80 (t, J = 8.5 Hz, 1H), 4.67 (tt, J = 6.9, 4.3 Hz, 1H), 3.90 (s, 3H), 3.78 (dd, J = 12.3, 6.8 Hz, 1H), 3.55 (ddd, J = 12.3, 4.4, 0.8 Hz, 1H), 3.33 (p, J = 1.6 Hz, 1H), 2.69 - 2.49 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 168.70, 167.07, 145.95, 143.55, 131.67, 130.12, 124.21, 109.14, 108.80, 58.58, 52.66, 50.15, 49.45, 33.42.

4.2.2 Synthesis of compound 15

The amine **14** (40 mg, 0.12 mmol) was dissolved in DMF and coupled to the commercially available carboxylic acid **9** (36 mg, 0.15 mmol, 1.3 eq.) according with the general procedure

of amide bond formation (1.2.3). The crude product **15** was dissolved in DCM and washed with $NaHCO_3$ sat. water solution. The organic phases were collected and concentrated under reduced pressure. The pure product **15** was obtained by silica gel column chromatography with 1:1 hexane:ethylacetate as an eluent (55 mg = 83%). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.63 - 7.51 (m, 2H), 7.23 - 7.12 (m, 1H), 6.44 (s, 2H), 4.61 - 4.46 (m, 2H), 3.92 - 3.84 (m, 1H), 3.71 (d, J = 1.9 Hz, 6H), 3.64 - 3.59 (m, 6H), 3.21 (p, J = 1.6 Hz, 1H), 2.87 - 2.68 (m, 3H), 2.62 - 2.47 (m, 2H), 2.31 - 2.14 (m, 2H). ^{1}3C NMR (101 MHz, MeOD) δ 172.44, 167.00, 153.01, 145.79, 143.49, 137.10, 136.02, 134.20, 131.67, 130.45, 129.14, 128.53, 124.11, 109.11, 108.74, 105.36, 59.67, 57.65, 55.16, 51.60, 51.48, 49.41, 35.71, 33.65, 30.65, 19.59.

4.2.3 Synthesis of compound 16

The methyl ester **15** (55 mg, 0.10 mmol) was hydrolysed to the carboxylic acid derivative **16** according to the reported general procedure (1.2.3). The pure product **16** was obtained by HPLC purification (30 mg = 59%.). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.56 (dd, J = 15.6, 6.7 Hz, 2H), 7.83 - 7.74 (m, 3H), 7.69 (dd, J = 8.4, 1.8 Hz, 1H), 7.51 - 7.40 (m, 1H), 6.55 - 6.40 (m, 2H), 4.63 (dd, J = 7.8, 4.1 Hz, 0H), 4.51 (h, J = 6.5 Hz, 1H), 4.36 (dd, J = 8.6, 5.2 Hz, 1H), 3.88 - 3.76 (m, 1H), 3.67 (d, J = 2.7 Hz, 6H), 3.53 (d, J = 3.1 Hz, 3H), 3.40 (ddd, J = 20.4, 10.8, 5.8 Hz, 1H), 2.78 - 2.60 (m, 3H), 2.52 (ddd, J = 11.2, 6.6, 2.4 Hz, 2H), 2.37 - 1.98 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 174.10, 173.75, 170.97, 170.68, 165.27, 153.13, 145.18, 143.11, 137.48, 136.08, 131.68, 131.27, 125.05, 110.23, 109.75, 106.00, 60.37, 57.76, 56.21, 51.84, 50.94, 49.47, 35.81, 34.45, 30.86, 21.03. ^{19}F NMR (376 MHz, DMSO) δ -48.97.

4.3 Synthesis of A4/B66/C292-COOH (HSA negative control)

4.3.1 Synthesis of compound 20

Commercially available N-Fmoc-L-Azidolysine **17** (1.0 g., 2.5 mmol, IRIS biotech) was dissolved in dry Methanol (30 mL) and H_2SO_4 (1 eq., 118 mg., 140 μL) was added. The solution was refluxed for 4 hours and concentrated at reduced pressure. The methyl ester derivative was re-dissolved in ethyl acetate and washed with water and $NaHCO_3$ saturated solution. The organic phases were dried with H_2SO_4 and the solvent was removed. The crude product **18** was deprotected as reported in the general procedure of Fmoc deprotection (1.2.3), yielding the compound **19**. The amine **19** (1.3 mmol) was coupled with the carboxylic acid **9** (385 mg, 1.6 mmol) according with the general procedure of amide bond formation (1.2.3). The obtained product **20** was purified by silica gel column chromatography with ethyl acetate:hexane = 1:1 as an eluent (250 mg = 25%). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.25 (d, J = 7.6 Hz, 1H), 6.51 (s, 2H), 4.26 (ddd, J = 8.9, 7.5, 5.1 Hz, 1H), 3.76 (s, 6H), 3.62 (d, J = 1.0 Hz, 6H), 3.29

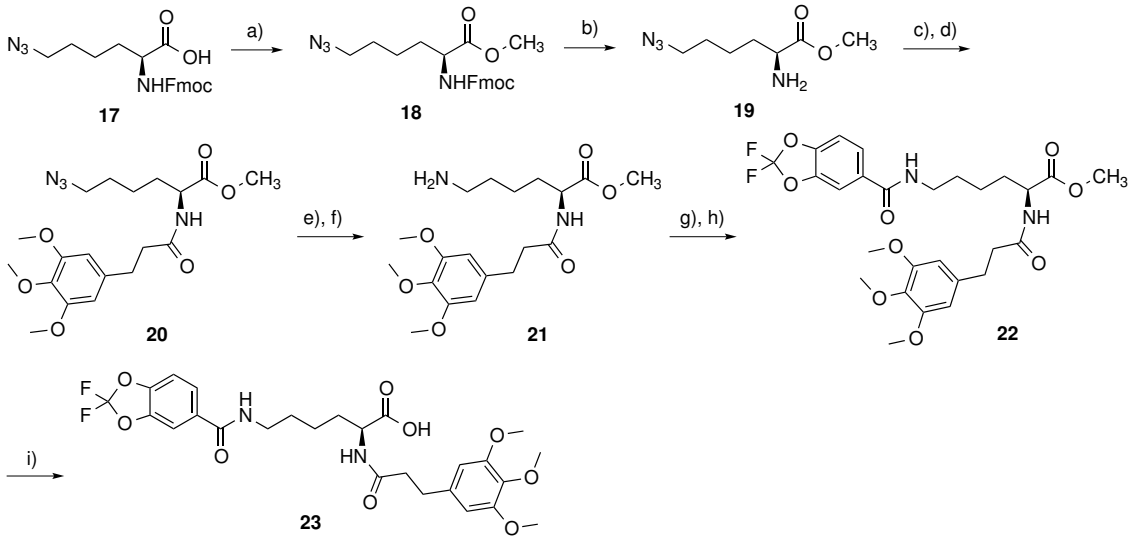


Figure 4.4: Synthesis of compound **A4/B66/C292**. Reaction conditions: a) MeOH, H_2SO_4, reflux, 4h; b) Piperidine:DMF=1:4, DBU 0.05 eq., 10', r.t.; c) **9**, EDC, HOAt, DIPEA, DMF, 15', r.t.; d) activated solution of **9**, DMF, o.n., r.t.; e) PPh_3, THF, o.n., r.t.; f) H_2O, 5%HCl, 2h, r.t.; g) 9, EDC, HOAt, DIPEA, DMF, r.t., o.n.; h) activated solution of 9, DMF, o.n., r.t.; i) 3M LiOH, H_2O:MeOH = 1:1, 1h, r.t.

(t, J = 6.8 Hz, 2H), 2.82 - 2.72 (m, 2H), 2.53 - 2.40 (m, 2H), 1.75 - 1.42 (m, 4H), 1.35 - 1.23 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 173.12, 172.10, 153.13, 137.30, 136.10, 105.83, 60.34, 56.14, 52.10, 50.87, 36.97, 31.75, 30.96, 28.24, 23.06.

4.3.2 Synthesis of compound 22

The azido derivative **20** (236 mg, 0.578 mmol) was dissolved in dry THF (5 mL) and triphenylphosphine (PPh_3, 2 eq., 303 mg) was added. The resulting solution was stirred overnight at room temperature. The resulting phosphamine was hydrolysed in 2 hours by adding 5% HCl solution (3 mL). The amine **21** was washed with diethyl ether and the aqueous phases were lyophilized overnight. the dry compound **21** was coupled with the carboxylic acid **6** (0.9 mmol, 174 mg) according with the general procedure of amide bond formation (1.2.3). The pure product **22** was obtained by silica gel column chromatography with ethyl acetate:hexane = 1:1 as an eluent (230 mg = 70%). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.75 - 7.58 (m, 3H), 7.10 (d, J = 8.8 Hz, 1H), 6.95 (d, J = 6.1 Hz, 1H), 6.41 (s, 3H), 6.18 (d, J = 7.7 Hz, 1H), 4.54 (td, J = 8.0, 4.3 Hz, 1H), 3.82 (s, 6H), 3.81 (s, 3H), 3.72 (s, 3H), 3.41 (dq, J = 12.5, 6.2 Hz, 1H), 3.21 (tq, J = 12.8, 7.0, 6.3 Hz, 1H), 2.98 - 2.80 (m, 2H), 2.67 - 2.42 (m, 2H), 1.75 (dtd, J = 13.8, 8.0, 4.3 Hz, 1H), 1.65 - 1.55 (m, 1H), 1.55 - 1.44 (m, 2H), 1.07 (p, J = 7.6 Hz, 2H). ^{13}C NMR (101 MHz, CDCl3) δ 172.59, 166.43, 153.25, 145.89, 143.85, 136.23, 136.07, 131.68, 130.63, 123.51, 109.15, 108.90, 105.30, 60.90, 56.01, 52.60, 51.80, 40.10, 38.28, 32.03, 28.46, 22.23.

4.3.3 Synthesis of compound 23

The methyl ester **22** (60 mg, 0.11 mmol) was hydrolysed to the carboxylic acid derivative **23** according with the reported general procedure (1.2.3). The obtained compound **24** was purified by HPLC (33 mg = 54%). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.51 (t, J = 5.6 Hz, 1H), 8.11 (d, J = 7.8 Hz, 1H), 7.82 (t, J = 1.6 Hz, 1H), 7.75 (dt, J = 8.4, 1.7 Hz, 1H), 7.49 (dd, J = 8.4, 1.6 Hz, 1H), 6.50 (d, J = 1.6 Hz, 2H), 4.26 - 4.14 (m, 1H), 3.75 (d, J = 1.6 Hz, 6H), 3.62 (d, J = 1.6 Hz, 3H), 3.24 (q, J = 6.9 Hz, 2H), 2.74 (td, J = 7.4, 4.5 Hz, 2H), 2.43 (ddt, J = 11.2, 8.2, 5.6 Hz, 2H), 1.80 - 1.19 (m, 6H). ^{13}C NMR NMR (101 MHz, DMSO) δ 174.27, 172.00, 164.83, 153.12, 144.95, 143.14, 137.42, 136.05, 131.90, 124.66, 110.19, 109.49, 105.78, 60.37, 56.16, 52.12, 37.15, 31.84, 31.32, 29.04, 23.36.

4.4 Synthesis of fluorescently labelled compounds

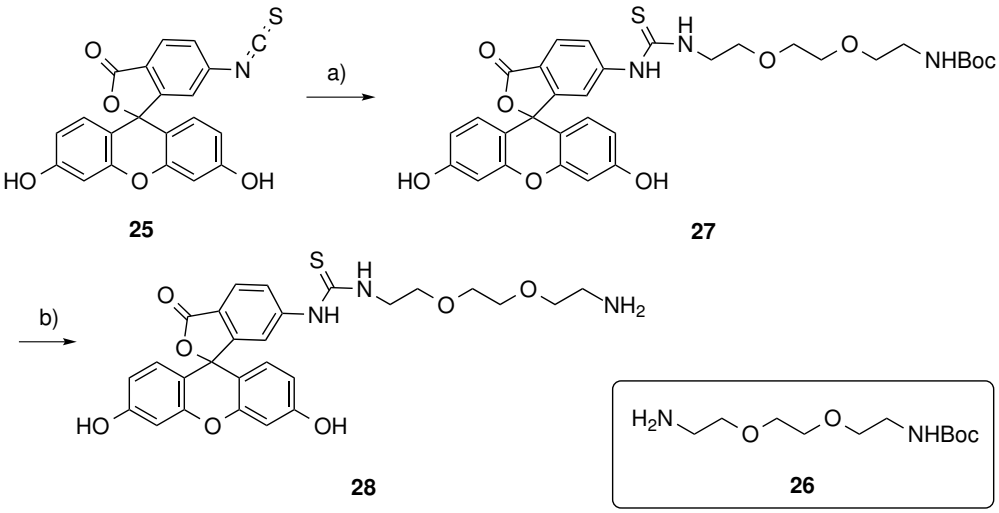


Figure 4.5: Synthesis of FITC-PEG2-aminolinker. Reaction conditions: a) **26**, TEA, DCM:DMF=3:1, 18h, r.t., in the dark; b) TFA:DCM=1:1, 1h, r.t..

4.4.1 Synthesis of compound 28

. To a solution of mono-Boc PEG-2 diaminolinker **26** (240 mg, 0.967 mmol) in dry DCM (15 mL), Fluorescein isothiocyanate (FITC, 250 mg, 0.643 mmol) in dry DMF (5 mL) and TEA (250 μL) were added. The reaction mixture was stirred at room temperature, in the dark, for 18 hours. The reaction mixture was diluted with DCM and washed with 0.5 M HCl water solution. The dried product **27** was stirred in 50% TFA (trifluoroacetic acid) in DCM for 1 hour at room temperature. The resulting solution was concentrated and the product **28** was purified by HPLC (412mg = 80%). ^{1}H NMR (400 MHz, DMSO-d6) δ 10.33 (s, 2H), 8.48 - 8.29 (m, 2H), 8.03 - 7.72 (m, 4H), 7.24 (d, J = 8.3 Hz, 1H), 6.73 (d, J = 2.2 Hz, 2H), 6.67 - 6.59 (m, 4H), 3.74 (h, J = 4.1, 3.2 Hz, 2H), 3.69 - 3.62 (m, 8H), 3.03 (h, J = 5.6 Hz, 2H).

^{13}C NMR (101 MHz, DMSO) δ 181.11, 169.01, 160.00, 152.36, 147.54, 141.90, 129.88, 129.47, 126.97, 124.50, 116.84, 113.06, 110.16, 102.71, 83.71, 70.16, 69.95, 68.83, 67.17, 44.09, 39.09.

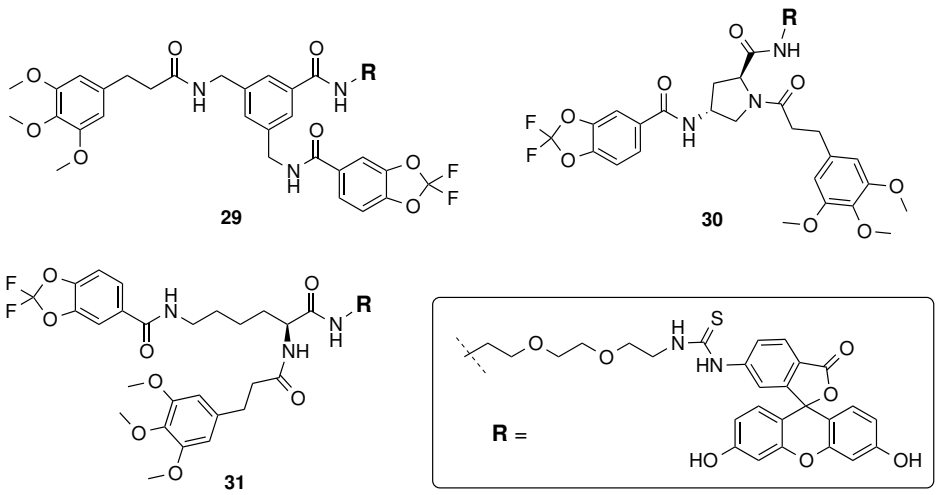


Figure 4.6: Structure of the fluorescent derivatives **29**, **30**, **31**.

4.4.2 Synthesis of compounds 29, 30 and 31

The synthesised compounds **11** (0.04 mmol, 1 eq., 23 mg), **16** (0.04 mmol, 1 eq., 21mg), **23** (0.04 mmol, 1 eq., 22mg) were coupled in the dark with Fluorescein-PEG2 amino linker **28** (24 mg, 0.044 mmol, 1.1 eq. in dry DMF) according to the general procedure of amide bond formation (1.2.3). The obtained conjugates **29**, **30** and **31** were purified by HPLC and lyophilized overnight (15mg = 34%; 20 mg = 47%, 13 mg = 30%). Compound **29**: ^{1}H NMR (500 MHz, DMSO-d6) δ 10.18 (s, 1H), 9.16 (t, J = 5.9 Hz, 1H), 8.53 (t, J = 5.6 Hz, 1H), 8.43 (t, J = 5.8 Hz, 1H), 8.36 - 8.30 (m, 1H), 8.23 (d, J = 6.2 Hz, 1H), 7.88 (d, J = 1.7 Hz, 1H), 7.82 (dd, J = 8.5, 1.7 Hz, 1H), 7.76 (d, J = 8.2 Hz, 1H), 7.69 (t, J = 1.7 Hz, 1H), 7.64 (t, J = 1.6 Hz, 1H), 7.48 (dd, J = 8.5, 1.9 Hz, 1H), 7.33 (d, J = 1.7 Hz, 1H), 7.28 (d, J = 5.0 Hz, 1H), 7.18 (d, J = 8.2 Hz, 2H), 7.08 (d, J = 4.9 Hz, 1H), 6.69 (d, J = 2.3 Hz, 2H), 6.65 - 6.54 (m, 4H), 6.48 (s, 2H), 4.50 (d, J = 5.8 Hz, 2H), 4.31 (d, J = 5.9 Hz, 2H), 3.71 (s, 8H), 3.63 - 3.50 (m, 11H), 3.42 (q, J = 6.0 Hz, 2H), 2.80 - 2.71 (m, 3H), 2.46 - 2.35 (m, 3H), 1.30- 1.12 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 181.07, 171.95, 168.99, 166.77, 165.00, 159.99, 153.13, 152.37, 147.51, 145.15, 143.20, 141.88, 140.33, 140.09, 137.47, 136.09, 135.07, 133.68, 131.66, 131.45, 129.46, 129.15, 126.98, 125.29, 125.16, 124.86, 124.47, 113.06, 110.23, 109.61, 105.77, 102.70, 70.02, 69.38, 68.88, 60.35, 56.13, 44.12, 43.15, 42.40, 37.61, 31.96. TOF-MS ES(-): 1106.3220 m/z. Compound **30**: ^{1}H NMR (600 MHz, DMSO-d6) δ 10.05 (s, 2H), 8.58 (dd, J = 24.7, 6.8 Hz, 1H), 8.28 (dd, J = 13.9, 8.6 Hz, 1H), 8.07 (s, 1H), 7.98 (t, J = 5.7 Hz, 1H), 7.87 - 7.80 (m, 1H), 7.78 - 7.70 (m, 2H), 7.56 - 7.48 (m, 1H), 7.22 - 7.15 (m, 1H), 6.68 (dd, J = 2.4, 0.7 Hz, 2H), 6.63 - 6.48 (m, 6H), 4.63 - 4.48 (m, 1H), 4.43 (dd, J = 8.6, 3.8 Hz, 1H), 3.92 (dd, J = 10.1,

7.1 Hz, 1H), 3.75 (d, J = 1.1 Hz, 6H), 3.71 (ddd, J = 13.0, 6.2, 3.6 Hz, 1H), 3.65 - 3.55 (m, 6H), 3.54 - 3.37 (m, 4H), 3.35 - 3.05 (m, 1H), 2.81 - 2.69 (m, 2H), 2.66 - 2.53 (m, 1H), 2.32 - 2.04 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 181.01, 172.05, 170.81, 168.95, 165.23, 159.94, 158.79, 158.55, 153.14, 152.34, 147.60, 145.15, 143.11, 141.80, 137.57, 136.08, 133.36, 131.68, 131.35, 129.48, 126.99, 125.00, 124.52, 113.03, 110.25, 109.74, 106.01, 102.69, 70.07, 69.47, 68.90, 60.39, 59.36, 58.75, 56.23, 51.77, 51.27, 49.15, 47.59, 46.23, 44.18, 39.05, 37.34, 35.98, 35.16, 30.90, 9.11. TOF-MS ES(-): 1056.3063 m/z. Compound **31**: ^{1}H NMR (600 MHz, DMSO-d6) δ 10.04 (s, 1H), 8.49 (dt, J = 15.8, 5.6 Hz, 1H), 8.29 (s, 1H), 8.15 - 8.05 (m, 1H), 7.99 - 7.93 (m, 2H), 7.82 (ddd, J = 3.3, 1.7, 0.5 Hz, 1H), 7.75 (ddd, J = 8.4, 2.9, 1.7 Hz, 2H), 7.48 (td, J = 8.3, 0.4 Hz, 1H), 7.18 (dd, J = 8.3, 0.6 Hz, 1H), 6.68 (dd, J = 2.3, 0.4 Hz, 2H), 6.64 - 6.54 (m, 4H), 6.49 (d, J = 7.1 Hz, 3H), 4.24 (td, J = 8.4, 5.4 Hz, 1H), 3.74 (d, J = 4.6 Hz, 7H), 3.61 (d, J = 3.2 Hz, 6H), 3.59 - 3.53 (m, 4H), 3.41 (t, J = 6.1 Hz, 2H), 3.26 - 3.16 (m, 5H), 2.76 - 2.70 (m, 2H), 2.49 - 2.37 (m, 2H), 1.74 - 1.15 (m, 6H). ^{13}C NMR (151 MHz, DMSO) δ 181.02, 174.26, 172.36, 171.80, 168.95, 164.79, 159.95, 158.58, 153.11, 152.34, 144.94, 143.13, 141.79, 137.44, 136.04, 133.35, 131.90, 131.67, 129.48, 126.99, 124.65, 116.87, 113.04, 110.18, 109.48, 105.78, 102.69, 70.09, 69.42, 68.90, 60.37, 56.16, 52.79, 44.15, 38.94, 37.25, 32.51, 31.91, 29.17, 23.29. TOF-MS ES(-): 1072.3369 m/z.

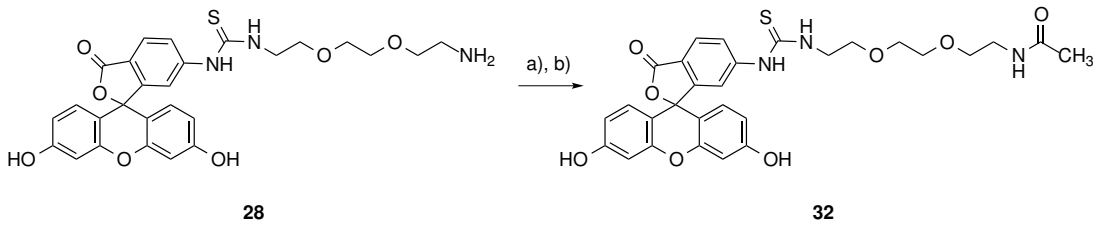


Figure 4.7: Synthesis of negative control for fluorescence polarization assays (**RNHAc**). Reaction conditions: a) Ac_2O:Py = 1:2, 10', r.t.; b) NaH 1M in MeOH, 2h, 0°C - r.t.

4.4.3 Synthesis of compound 32

Fluorescein-PEG2 amino linker **28** (30 mg, 0.056 mmol) was dissolved in Acetic anhydride : Piridine = 1:2 solution (1.5 mL) and stirred for 10 minutes at room temperature. The reaction mixture was concentrated and the acetylated intermediate was dissolved in dry methanol (20 mL). The solution was cooled at 0°C and NaH (20 mmol, 480 mg) was slowly added obtaining a 1M sodium methoxide solution. The resulting mixture was stirred for 2 hours at room temperature and finally quenched with 2M HCl. The obtained product **32** was purified by HPLC (12 mg = 37%). ^{1}H NMR (500 MHz, DMSO-d6) δ 10.11 (s, 1H), 8.31 (s, 1H), 8.14 (s, 1H), 7.92 (t, J = 5.6 Hz, 1H), 7.76 (d, J = 8.3 Hz, 1H), 7.20 (d, J = 8.3 Hz, 1H), 6.75 - 6.54 (m, 7H), 3.71 (d, J = 6.5 Hz, 2H), 3.64 - 3.53 (m, 6H), 3.42 (t, J = 5.9 Hz, 2H), 3.20 (q, J = 5.8 Hz, 2H), 1.81 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 181.04, 169.83, 169.76, 168.95, 160.17, 160.06, 152.42, 141.83, 129.51, 127.03, 124.56, 116.91, 113.11, 110.26, 102.79, 102.71, 70.09, 70.00, 69.61, 68.89, 44.16, 39.03, 22.98, 22.97. TOF-MS ES(-): 579.1692 m/z.

4.4.4 Synthesis of "A7" negative control

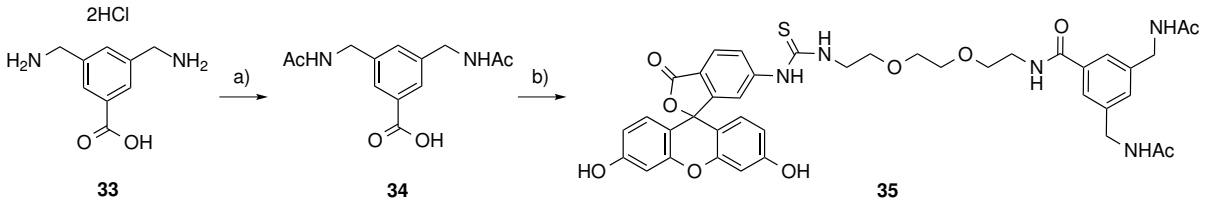


Figure 4.8: Synthesis of **R-A7/Ac/Ac**: a) Pyridine:Ac_2O = 3:1, 4h, r.t.; b) **28**, EDC, DIPEA, HOAt, dry DMF, o.n., r.t..

Synthesis of compound 34

100 mg (0.556 mmol) of commercially available 3,5-bis(aminomethyl)benzoic acid dihydrochloride (**33**, Fluorochem, CAS:185963-32-0) were dissolved in 3 mL of dry pyridine and 1 mL of acetic anhydride was added. The resulting solution was stirred 4 hours at room temperature. The solution was concentrated under vacuum and the compound **34** was purified by HPLC (90 mg = 61%). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.43 (t, J = 6.0 Hz, 1H), 7.79 - 7.63 (m, 1H),

7.37 (d, J = 1.6 Hz, 1H), 4.28 (d, J = 6.0 Hz, 2H), 1.89 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.68, 167.69, 140.72, 131.17, 127.06, 42.24, 23.03.

Synthesis of compound 35

The carboxylic acid **34** (23 mg, 0.09 mmol) was coupled overnight in the dark with Fluorescein-PEG2-NH_2 (**28**, 1eq., 47 mg) using the general procedure for amide bond formation (1.2.3). The resulting amide **35** was purified by HPLC (21 mg = 30%). ^{1}H NMR (600 MHz, DMSO-d6) δ 10.04 (s, 2H), 8.49 (t, J = 5.6 Hz, 1H), 8.37 (t, J = 5.9 Hz, 2H), 8.31 - 8.24 (m, 1H), 8.11 (d, J = 24.7 Hz, 1H), 7.79 - 7.70 (m, 1H), 7.63 - 7.57 (m, 2H), 7.26 (dq, J = 1.7, 0.8 Hz, 1H), 7.18 (dd, J = 8.3, 0.6 Hz, 1H), 6.69 - 6.65 (m, 3H), 6.61 (s, 1H), 6.59 (s, 1H), 6.56 (dd, J = 8.7, 2.3 Hz, 3H), 4.26 (d, J = 6.0 Hz, 5H), 3.71 - 3.66 (m, 3H), 3.60 (d, J = 8.6 Hz, 7H), 3.54 (t, J = 6.1 Hz, 2H), 3.46 - 3.39 (m, 3H), 1.87 (s, 6H). ^{13}C NMR (151 MHz, DMSO) δ 181.02, 169.62, 168.96, 166.75, 161.67, 159.95, 152.34, 147.59, 141.78, 140.28, 135.04, 129.50, 126.99, 125.14, 124.53, 113.05, 110.18, 102.69, 70.10, 70.03, 69.39, 68.90, 44.14, 42.44, 23.06. TOF-MS ES(-): 783.2534 m/z.

4.5 Synthesis of TNKS1 binders

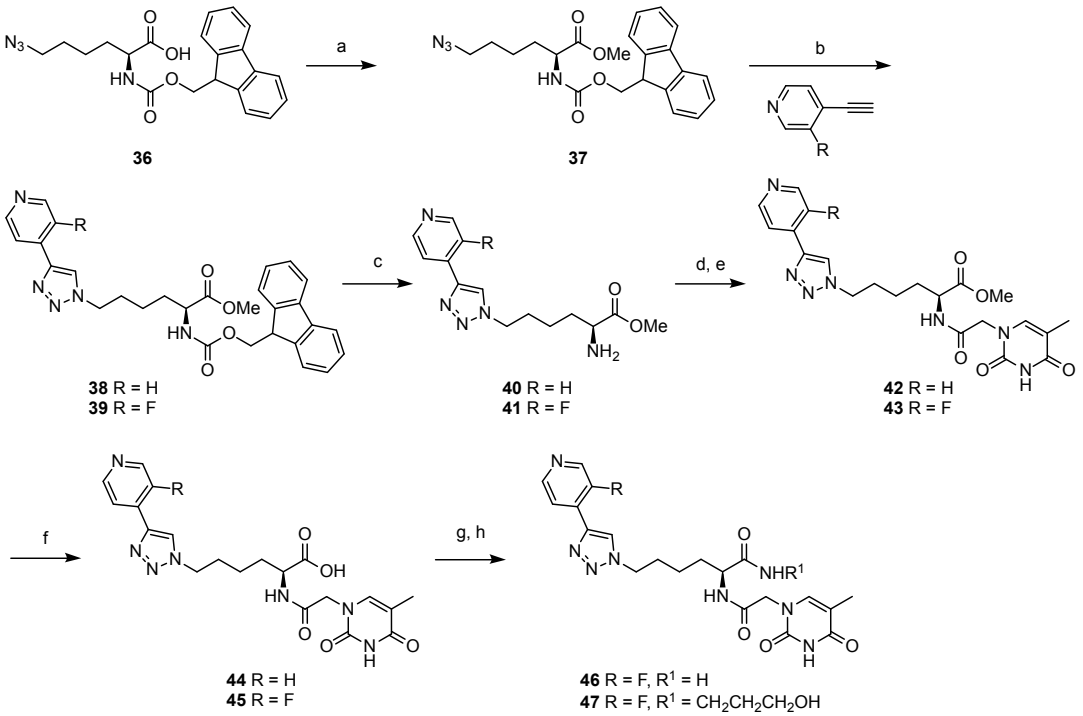


Figure 4.9: Synthesis of **46** and **47**: a) HCl 4M in dioxane, MeOH, reflux 4h; b) CuI 20%, TBTA 30%, DMF:THF=1:1 degassed, RT o.n.; c) DBU 10%, Piperidine:DMF = 1:4, RT 10 min; d) Thymine-1-acetic acid 1.1 eq., EDC 1.2 eq., HOAt 0.6M in DMF 20%, DIPEA 3 eq., RT 10 min; e) mixture d, RT o.n.; f) LiOH, H_2O:MeOH=1:1, RT 1h; g) HATU 1.1 eq., HOAt 1.1 eq., DIPEA 3 eq., DMF, 0°C 10 min; R^1NH_2 10 eq., DMF, RT o.n.

4.5.1 Synthesis of compound 37

The commercially available Fmoc-azido-L-lysine (**36**, IRIS biotech, 1.6g, 4.1 mmol) was dissolved in dry methanol and 1 eq. of HCl (4M in dioxane) was added. The solution was refluxed for 4 hours, then the solvent was removed under reduced pressure. The pure compound **37** was obtained by silica gel column chromatography with 1:1 hexane:ethylacetate as an eluent (1.5 = 90%). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.68 (d, J = 7.6 Hz, 2H), 7.55 - 7.47 (m, 2H), 7.35 - 7.27 (m, 3H), 7.23 (tt, J = 7.5, 1.1 Hz, 2H), 5.30 (d, J = 8.3 Hz, 1H), 4.32 (t, J = 6.0 Hz, 3H), 4.14 (t, J = 7.0 Hz, 1H), 3.67 (s, 2H), 3.18 (t, J = 6.7 Hz, 2H), 1.87 - 1.12 (m, 6H). ^{13}C NMR (101 MHz, CDCl3) δ 172.83, 155.91, 143.75, 141.34, 127.75, 127.09, 125.08, 120.01, 67.01, 53.64, 52.51, 51.08, 47.20, 32.19, 28.39, 22.43.

4.5.2 Synthesis of compounds 40 and 41

The compound **37** (580 mg, 1.42 mmol) and 1.2 eq. (1.6 mmol) of the respective alkyne (4-ethynylpyridine or 4-ethynyl-3-fluoropyridine - commercially available by Enamine) was dissolved in dry and degassed DMF:THF=1:1 solution and CuI (0.2 eq, 60 mg) and TBTA (0.3 eq., 220 mg) were added. The solutions were stirred overnight at room temperature and then

diluted with ethyl acetate. The products **38** and **39** were washed with EDTA 0.1 M solution (pH=10) and water, then the organic phases were collected and concentrated. The obtained compounds **38** and **39** were dissolved in DMF:piperidine=4:1 solution and 0.1 eq. of DBU were added. The reaction mixtures were stirred for 10 minutes at room temperature and then the solvent was removed under reduced pressure. The products **40** and **41** were purified by silica gel column chromatography with 9:1 dichloromethane:methanol as an eluent (368 mg = 85%; 342 mg = 83%). Compound **40** ^{1}H NMR (400 MHz, DMSO-d6) δ 8.85 (s, 1H), 8.70 - 8.54 (m, 2H), 7.89 - 7.73 (m, 2H), 4.43 (t, J = 7.0 Hz, 2H), 3.60 (s, 3H), 3.36 (dd, J = 7.5, 5.5 Hz, 1H), 2.02 - 1.75 (m, 2H), 1.74 - 1.43 (m, 2H), 1.41 - 1.25 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 176.06, 150.79, 144.40, 138.44, 123.78, 119.89, 101.69, 54.00, 51.97, 50.01, 33.81, 29.76, 22.54. Compound **41** ^{1}H NMR (400 MHz, DMSO-d6) δ 8.75 (dt, J = 4.6, 2.5 Hz, 2H), 8.57 (d, J = 5.1 Hz, 1H), 8.23 - 8.09 (m, 1H), 4.62 - 4.44 (m, 2H), 3.68 (d, J = 1.9 Hz, 3H), 3.57 - 3.49 (m, 1H), 1.94 (p, J = 7.5 Hz, 2H), 1.81 - 1.52 (m, 2H), 1.38 (p, J = 7.6 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 175.05, 156.69, 154.13, 146.85, 138.94, 137.83, 126.34, 121.00, 53.67, 52.19, 49.95, 33.10, 29.78, 22.37.

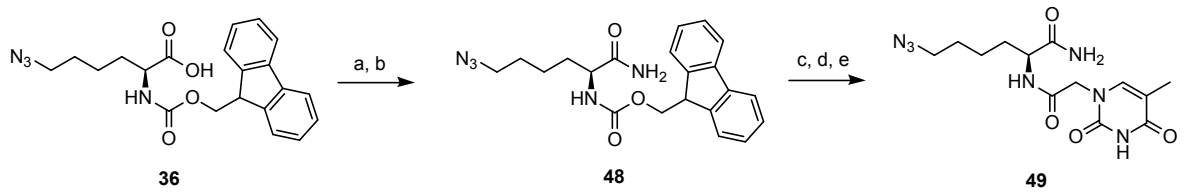
4.5.3 Synthesis of compounds 44 and 45

The commercially available Thymine-1-acetic acid (Sigma aldrich, 1.2 eq., 1.44 mmol, 265 mg) was dissolved in dry DMF and activated by adding EDC (1.1 eq.), HOAt (0.2 eq.) and DIPEA (3 eq.). The resulting mixture was stirred for 10 minutes at room temperature and then added to the amide **40** and **41** solutions (1.2 mmol). The reactions were kept at room temperature overnight and then the products were purified by silica gel column chromatography with ethyl acetate as an eluent. The obtained methyl esters **42** and **43** were hydrolysed according with the reported general procedure (1.2.3). The obtained carboxylic acid derivatives **44** and **45** were purified by HPLC (205 mg = 39%, 240 mg = 44%). Compound **44** Compound **44** ^{1}H NMR (400 MHz, DMSO-d6) δ 11.27 (d, J = 3.1 Hz, 1H), 9.10 (s, 1H), 8.94 - 8.82 (m, 1H), 8.70 (dd, J = 4.4, 2.8 Hz, 1H), 8.57 - 8.46 (m, 1H), 8.32 - 8.23 (m, 1H), 8.10 (dd, J = 6.5, 5.0 Hz, 1H), 7.41 (t, J = 1.5 Hz, 1H), 4.47 (td, J = 7.1, 3.7 Hz, 2H), 4.33 (d, J = 2.9 Hz, 2H), 4.22 (tt, J = 8.3, 5.1 Hz, 1H), 1.90 (pd, J = 6.6, 3.7 Hz, 2H), 1.83 - 1.56 (m, 5H), 1.34 (h, J = 7.0, 6.5 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 173.63, 167.44, 164.89, 151.42, 146.71, 145.12, 144.69, 142.86, 126.24, 121.60, 108.32, 52.10, 50.23, 49.97, 49.53, 30.94, 29.42, 22.65, 12.37, 12.33. TOF-MS ES(-): 440.1686 m/z. Compound **45** ^{1}H NMR (400 MHz, DMSO-d6) δ 11.26 (s, 1H), 8.72 (d, J = 3.0 Hz, 2H), 8.61 - 8.40 (m, 2H), 8.12 (dd, J = 6.5, 5.0 Hz, 1H), 7.41 (d, J = 1.3 Hz, 1H), 4.47 (t, J = 7.1 Hz, 2H), 4.21 (td, J = 8.3, 5.1 Hz, 1H), 1.96 - 1.84 (m, 2H), 1.82 - 1.56 (m, 5H), 1.33 (p, J = 7.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 173.63,

167.44, 164.89, 164.88, 156.71, 156.70, 154.16, 151.42, 146.48, 142.85, 138.88, 138.88, 138.64, 137.80, 126.45, 126.37, 121.16, 120.02, 108.32, 52.18, 49.98, 49.48, 31.02, 29.66, 22.66, 12.35. TOF-MS ES(-): 459.1679 m/z.

4.5.4 Synthesis of compounds 46 and 47

The carboxylic acid **45** (34 mg, 0.075 mmol) was dissolved in DMF and the solution was cooled at 0 °C and then HATU (1.1 eq.), HOAt (1.1 eq.) and DIPEA (3 eq.) were added to the carboxylic acid solution. The resulting mixture was stirred at 0 °C for 10 minutes, then added to NH_4OAc (10 eq.) DMF solution and to 3-aminopropan-1-ol ($NH_2CH_2CH_2CH_2OH$, 10 eq.) DFM solutions. The reactions were stirred overnight at room temperature. The obtained amides were purified by HPLC (17 mg = 40%; 21 mg = 54%). Compound **46** ^{1}H NMR (400 MHz, DMSO-d6) δ 11.27 (s, 1H), 8.70 (d, J = 3.2 Hz, 2H), 8.52 (d, J = 5.0 Hz, 1H), 8.32 (d, J = 8.2 Hz, 1H), 8.11 (dd, J = 6.5, 4.9 Hz, 1H), 7.39 (dd, J = 16.1, 1.8 Hz, 2H), 7.17 - 6.99 (m, 1H), 4.46 (t, J = 7.1 Hz, 2H), 4.32 (s, 2H), 4.20 (td, J = 8.4, 5.2 Hz, 1H), 1.88 (p, J = 7.4 Hz, 2H), 1.74 (d, J = 1.2 Hz, 4H), 1.55 (ddd, J = 13.9, 8.8, 5.8 Hz, 1H), 1.27 (dd, J = 19.2, 11.7 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 173.63, 167.25, 164.88, 151.51, 146.69, 140.97, 139.07, 138.84, 137.83, 126.24, 122.78, 121.09, 108.35, 52.57, 50.04, 49.85, 31.87, 29.73, 22.62, 12.37. TOF-MS ES(+): 442.1620 (-F), 460.2047 m/z. Compound **47** ^{1}H NMR (400 MHz, DMSO-d6) δ 11.33 (s, 1H), 8.76 (dd, J = 3.0, 1.9 Hz, 3H), 8.58 (dd, J = 5.0, 1.2 Hz, 1H), 8.43 (d, J = 8.2 Hz, 1H), 8.17 (td, J = 5.7, 5.0, 1.4 Hz, 1H), 8.00 (t, J = 5.6 Hz, 1H), 7.48 (d, J = 1.4 Hz, 1H), 4.53 (q, J = 7.4 Hz, 2H), 4.33 (s, 2H), 4.28 (td, J = 8.2, 5.7 Hz, 1H), 3.54 (t, J = 6.0 Hz, 1H), 3.43 (t, J = 6.3 Hz, 2H), 3.16 (ddp, J = 19.2, 13.0, 6.3 Hz, 2H), 2.93 (dddd, J = 11.7, 7.6, 5.3, 2.5 Hz, 1H), 1.95 (p, J = 7.2 Hz, 2H), 1.81 (d, J = 1.2 Hz, 3H), 1.79 - 1.51 (m, 4H), 1.41 - 1.26 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 171.42, 167.21, 156.71, 154.17, 151.59, 146.78, 142.92, 139.13, 137.86, 126.33, 126.22, 121.06, 108.34, 58.79, 52.82, 50.01, 37.25, 36.24, 32.70, 32.16, 30.52, 29.73, 22.59. TOF-MS ES(+): 518.2464 m/z.



36 **48** **49**

Figure 4.10: Synthesis of **49**: a) EDC 1.1 eq., HOAt 20% 0.6M in DMF 20%, DIPEA 3 eq., RT 10 min; b) NH_4OAc 1.5 eq., RT o.n.; c) DBU 10%, Piperidine:DMF = 1:4, RT 10 min; d) Thymine-1-acetic acid 1.1 eq., EDC 1.2 eq., HOAt 0.6M in DMF 20%, DIPEA 3 eq., RT 10 min; e) mixture d, RT o.n.

4.5.5 Synthesis of compound 49

The commercially available Fmoc-azido-L-lysine **36** (IRIS biotech, 400 mg, 1.02 mmol) was coupled with NH_4OAc (117 mg, 1.5 eq. in DMF) according with the general procedure of amide bond formation (1.2.3). The obtained product was dissolved in DCM and washed with $NaHCO_3$ saturated solution and water. The organic phases were dried with dry Na_2SO_4 and concentrated under reduced pressure. The Fmoc-protected compound **48** was dissolved in DMF:piperidine = 1:4 solution and 10% of DBU was added. The solution was stirred at room temperature for 10 minutes and then concentrated under reduced pressure. The obtained product was filtered on silca gel and coupled overnight with Thymine-1-acetic acid (1.2 eq., 220 mg) according with the general procedure of amide bond formation (1.2.3). The obtained product was purified by HPLC (172 mg = 50%). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.27 (s, 1H), 8.31 (d, J = 8.2 Hz, 1H), 7.39 (dd, J = 15.7, 1.8 Hz, 2H), 7.18 - 7.02 (m, 1H), 4.33 (d, J = 3.2 Hz, 2H), 4.20 (td, J = 8.4, 5.1 Hz, 1H), 3.31 (t, J = 6.9 Hz, 2H), 1.74 (d, J = 1.2 Hz, 3H), 1.72 - 1.25 (m, 6H). ^{13}C NMR (101 MHz, DMSO) δ 173.70, 167.25, 164.90, 151.51, 142.91, 108.33, 52.63, 50.99, 49.86, 32.04, 28.35, 22.96, 12.37. TOF-MS ES(+): 337.1468 m/z.

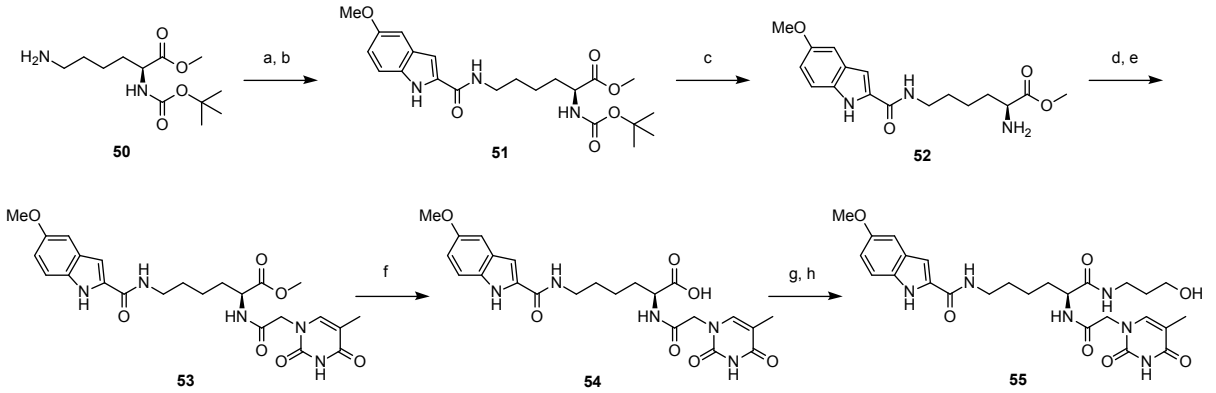


Figure 4.11: Synthesis of **55**: a) 5-methoxy-1H-indole-2-carboxylic acid, EDC, HOAt, DIPEA, DCM, 10', r.t.; b) DMF, solution(a), o.n., r.t.; c) TFA:DCM = 1:1, 1h, r.t.; d) Thymine-1-acetic acid, EDC, HOAt, DIPEA, DMF, 10', r.t.; e) DMF, solution(d), o.n., r.t.; f) LiOH, MeOH:H_2O = 1:1, 1h, r.t.; g) HATU, HOAt, DIPEA, DMF, 10', 0 °C; h) 3-aminopropan-1-ol, DMF, o.n., r.t..

4.5.6 Synthesis of compound 51

The commercially available Boc-Lys-OMe acetate salt (A-1925, Bachem, 520 mg, 2 mmol) was dissolved in Na_2CO_3 5% water solution and extracted many times by DCM. The organic phases were dried and concentrated, then the "free" amine **50** was coupled with 5-methoxy-1H-indole-2-carboxylic acid (420 mg, 2.2 mmol, 1.1 eq.) according with the general procedure of amide bond formation (1.2.3). The crude product **51** was dissolved in DCM and washed with water and $NaHCO_3$ saturated solution. The pure product **51** was obtained by silica gel column chromatography with ethylacetate:hexane = 1:1 as an eluent (810 mg = 94%). ^{1}H NMR (400 MHz, Chloroform-d) δ 9.77 (s, 1H), 7.34 (s, 1H), 7.31 (s, 1H), 7.01 (s, 1H), 6.94 (dd, J = 8.9, 2.4 Hz, 1H), 6.81 (s, 1H), 6.52 (t, J = 5.0 Hz, 1H), 4.31 - 4.29 (m, 2H), 3.83 (s, 3H), 3.71 (s, 3H), 3.47 (q, J = 6.3 Hz, 3H), 1.90 - 1.45 (m, 4H), 1.42 (s, 9H), 1.31 - 1.21 (m, 2H). ^{13}C NMR (101 MHz, CDCl3) δ 173.33, 161.95, 155.61, 154.57, 131.83, 131.16, 127.96, 115.63, 112.94, 102.29, 101.91, 79.99, 55.71, 53.21, 52.34, 39.28, 32.43, 29.06, 28.31, 22.66.

4.5.7 Synthesis of compound 52

The Boc-protected amine **51** (510 mg, 1.18 mmol) was dissolved in DCM:TFA = 1:1 and the solution was stirred at room temperature for 1 hour. The mixture was concentrated under reduced pressure and the crude product **52** was purified by silica gel column chromatography with DCM:Methanol = 9:1 (+1% triethylamine) as an eluent (360 mg = 92%). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.38 (d, J = 2.3 Hz, 1H), 11.28 (s, 1H), 8.61 (d, J = 7.4 Hz, 1H), 8.41 (t, J = 5.7 Hz, 1H), 7.44 - 7.37 (m, 1H), 7.30 (d, J = 8.9 Hz, 1H), 7.06 (d, J = 2.5 Hz, 1H), 7.01 (d, J = 2.3 Hz, 1H), 6.82 (dd, J = 8.9, 2.4 Hz, 1H), 4.35 (s, 2H), 4.27 (td, J = 8.1, 5.4 Hz, 1H), 3.75 (s, 3H), 3.62 (s, 3H), 3.26 (q, J = 6.7 Hz, 2H), 1.76 - 1.71 (m, 3H), 1.73 - 1.29 (m, 6H). ^{13C} NMR (101 MHz, DMSO) δ 170.48, 161.46, 154.16, 132.65, 132.09, 127.85, 114.73, 113.52,

102.41, 55.71, 53.21, 52.35, 38.73, 30.25, 29.13, 22.18.

4.5.8 Synthesis of compound 53

The compound **52** (340 mg, 1.02 mmol) was dissolved in DMF and then coupled with Thymine-1-acetic acid acid (1.4 eq., 265 mg) according with the general procedure of amide bond formation (1.2.3). The crude product **53** was dissolved in DCM and washed with water and $NaHCO_3$ saturated solution. The pure product **53** was obtained by silica gel column chromatography with DCM:Methanol = 9:1 as an eluent (437 mg = 86%). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.38 (d, J = 2.3 Hz, 1H), 11.28 (s, 1H), 8.61 (d, J = 7.4 Hz, 1H), 8.41 (t, J = 5.7 Hz, 1H), 7.44 - 7.37 (m, 1H), 7.30 (d, J = 8.9 Hz, 1H), 7.06 (d, J = 2.5 Hz, 1H), 7.01 (d, J = 2.3 Hz, 1H), 6.82 (dd, J = 8.9, 2.4 Hz, 1H), 4.27 (td, J = 8.1, 5.4 Hz, 1H), 3.75 (s, 2H), 3.62 (s, 3H), 3.35 (s, 3H), 3.26 (q, J = 6.7 Hz, 2H), 1.75 - 1.72 (m, 3H), 1.73 - 1.22 (m, 6H). ^{13C} NMR (101 MHz, DMSO) δ 172.81, 167.65, 164.88, 161.44, 154.15, 151.42, 142.83, 132.65, 132.06, 127.86, 114.72, 113.51, 108.36, 102.43, 55.70, 52.34, 49.44, 49.07, 38.92, 31.24, 30.48, 29.27, 23.19, 12.36.

4.5.9 Synthesis of compound 54

The methyl ester **53** (400 mg, 0.802 mmol) was hydrolysed according with the reported general procedure (1.2.3). The obtained carboxylic acid derivative **54** was purified by HPLC (175 mg = 45%). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.37 (d, J = 2.2 Hz, 1H), 11.27 (s, 1H), 9.05 (s, 1H), 8.84 (d, J = 5.8 Hz, 1H), 8.55 - 8.36 (m, 2H), 8.27 - 8.16 (m, 1H), 7.41 (dd, J = 3.1, 1.4 Hz, 1H), 7.30 (d, J = 8.8 Hz, 1H), 7.06 (d, J = 2.4 Hz, 1H), 7.01 (d, J = 2.1 Hz, 1H), 6.81 (dd, J = 8.9, 2.5 Hz, 1H), 4.47 (t, J = 7.0 Hz, 1H), 4.35 (s, 2H), 4.22 (qd, J = 7.9, 5.0 Hz, 1H), 3.34 - 3.19 (m, 2H), 1.99 - 1.84 (m, 1H), 1.74 (t, J = 1.5 Hz, 3H), 1.71 - 1.23 (m, 5H). ^{13}C NMR (101 MHz, DMSO) δ 173.79, 167.46, 164.91, 161.43, 154.14, 151.43, 145.86, 142.88, 132.67, 132.06, 127.86, 125.92, 121.40, 114.71, 113.51, 108.32, 102.44, 55.70, 52.43, 50.19, 49.47, 29.35, 23.30, 22.65, 12.36. TOF-MS ES(-): 485.1973 m/z

4.5.10 Synthesis of compound 55

The carboxylic acid **55** (36 mg, 0.075 mmol) was dissolved in DMF and the solution was cooled to 0°C and HATU (1.1 eq.), HOAt (1.1 eq.) and DIPEA (3 eq.) were added in the order. The resulting mixture was stirred for 10 minutes at 0°C and then 3-aminopropan-1-ol (10 eq. in DMF) was added to the solution. The reaction was kept at room temperature overnight and then the solution was concentrated under reduced pressure. The final product **55** was purified by HPLC (20 mg = 49%). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.37 (d, J = 2.3 Hz, 1H), 11.28 (s, 1H), 8.46 - 8.27 (m, 2H), 7.91 (t, J = 5.6 Hz, 1H), 7.40 (d, J = 1.5 Hz, 1H), 7.30 (d, J = 8.9

Hz, 1H), 7.06 (d, J = 2.5 Hz, 1H), 7.00 (d, J = 2.1 Hz, 1H), 6.81 (dd, J = 8.9, 2.4 Hz, 1H), 4.33 (d, J = 2.8 Hz, 2H), 4.21 (td, J = 8.3, 5.4 Hz, 1H), 3.75 (s, 3H), 3.38 (t, J = 6.3 Hz, 2H), 3.25 (dq, J = 12.2, 6.9, 6.4 Hz, 2H), 3.19 - 2.98 (m, 2H), 1.83 - 1.18 (m, 11H). ^{13}C NMR (101 MHz, DMSO) δ 171.62, 167.22, 164.91, 161.43, 154.16, 151.53, 142.93, 132.69, 132.07, 127.87, 114.71, 113.52, 108.35, 102.43, 58.85, 55.72, 53.15, 49.82, 36.27, 32.74, 29.46, 23.31, 12.36. TOF-MS ES(+): 544.2717 m/z.

4.6 Synthesis of TNKS1 negative controls

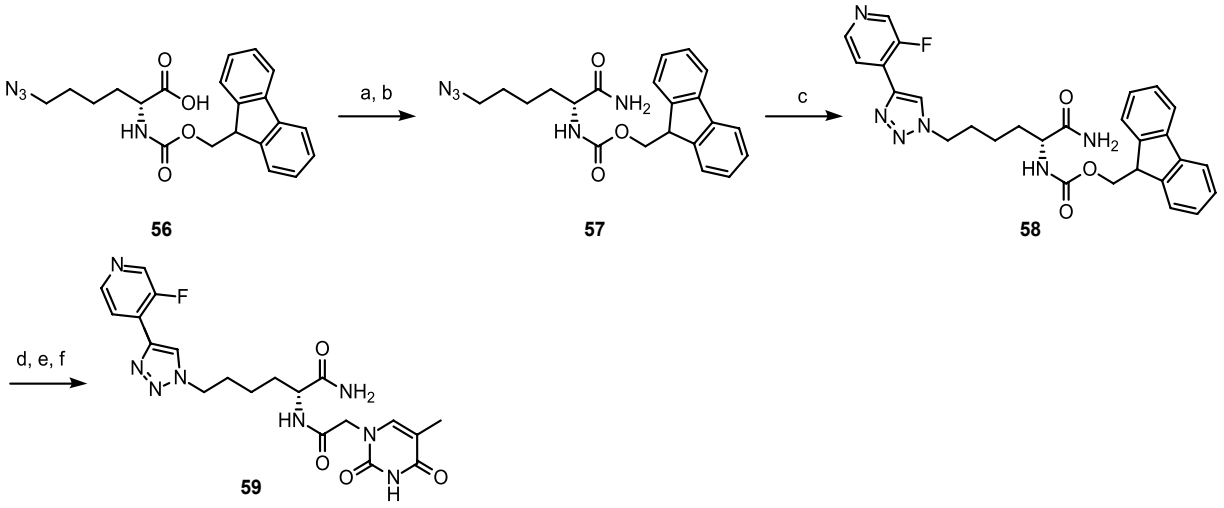


Figure 4.12: Synthesis of **59**: a) EDC 1.1 eq., HOAt 20% 0.6M in DMF 20%, DIPEA 3 eq., RT 10 min; b) NH_4OAc 1.5 eq., RT o.n.; c) 4-ethynyl-3-fluoropyridine 1.2 eq., CuI 20%, TBTA 30%, DMF:THF=1:1 degassed, RT o.n.; d) DBU 10%, Piperidine:DMF = 1:4, RT 10 min; e) Thymine-1-acetic acid 1.1 eq., EDC 1.2 eq., HOAt 0.6M in DMF 20%, DIPEA 3 eq., RT 10 min; f) mixture d, RT o.n..

4.6.1 Synthesis of compounds 59

The commercially available protected D-Lys-N_3 **56** (IRIS biotech, 394 mg, 1.0 mmol) was activated by EDC (1.1 eq.), HOAt (0.2 eq.), DIPEA (3 eq.) for 10 minutes at room temperature. To the reaction solution, 1.5 eq. of NH_4OAc were added and the reaction was kept at room temperature overnight. The reaction mixture was concentrated under vacuum and the pure compound **57** was obtained by silica gel column chromatography with ethyl acetate:Hexane = 1:1 as an eluent. The azido derivative **62** was dissolved in DMF:THF = 1:1 (degassed) under argon atmosphere and 4-ethynyl-3-fluoropyridine (1.2 eq.), CuI (0.2 eq.) and TBTA (0.3 eq.) were added in the order. The reaction was kept under argon atmosphere overnight and then the solvent was removed under reduced pressure. The crude product was dissolved in DCM and washed with EDTA 0.1M in water and water. The organic phases were collected and concentrated. The pure product **58** was obtained by silica gel column chromatography with ethyl acetate as an eluent. Compound **58** was deprotected as reported in the general

procedure of Fmoc-deprotection (1.2.3). The product was filtered on silca gel and washed with DCM:MeOH=8:2. The intermediate was coupled wit Thymine-1-acetic acid as described in the standard procedure (1.2.3). The obtained product **59** was purified by HPLC (135 mg = 29%). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.27 (s, 2H), 8.71 (t, J = 3.1 Hz, 2H), 8.53 (d, J = 5.0 Hz, 1H), 8.32 (d, J = 8.2 Hz, 1H), 8.12 (dd, J = 6.5, 5.0 Hz, 1H), 7.39 (dd, J = 14.9, 1.7 Hz, 2H), 7.10 - 7.05 (m, 1H), 4.46 (s, 1H), 4.33 (s, 2H), 4.21 (td, J = 8.4, 5.2 Hz, 1H), 1.88 (p, J = 7.4 Hz, 2H), 1.74 (s, 3H), 1.73 - 1.50 (m, 2H), 1.38 - 1.21 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 173.65, 167.26, 164.89, 151.52, 146.48, 142.88, 138.88, 138.65, 137.79, 126.42, 121.16, 108.36, 52.57, 50.05, 40.16, 31.86, 29.76, 22.62, 12.36. TOF-MS ES(+): 442.1707 (-F), 460.2047 m/z.

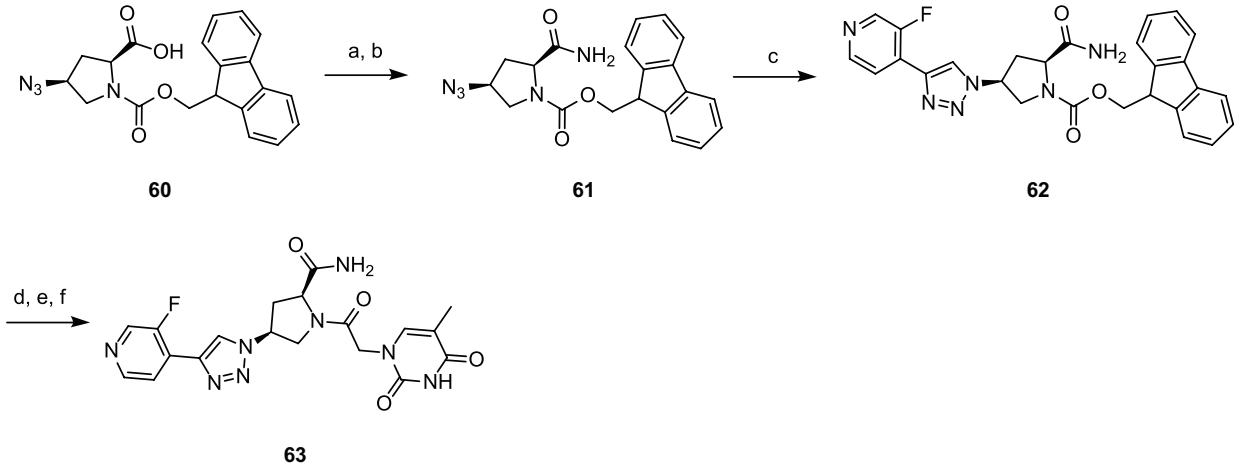


Figure 4.13: Synthesis of **63**: a) EDC 1.1 eq., HOAt 20% 0.6M in DMF 20%, DIPEA 3 eq., RT 10 min; b) NH_4OAc 1.5 eq., RT o.n.; c) 4-ethynyl-3-fluoropyridine 1.2 eq., CuI 20%, TBTA 30%, DMF:THF=1:1 degassed, RT o.n.; d) DBU 10%, Piperidine:DMF = 1:4, RT 10 min; e) Thymine-1-acetic acid 1.1 eq., EDC 1.2 eq., HOAt 0.6M in DMF 20%, DIPEA 3 eq., RT 10 min; f) mixture d, RT o.n.

4.6.2 Synthesis of compounds 63

The commercially available protected (2*S*,4*S*)-L-pro-N_3 **60** (IRIS biotech, 380 mg, 1.0 mmol) was activated by EDC (1.1 eq.), HOAt (0.2 eq.), DIPEA (3 eq.) for 10 minutes at room temperature. To the reaction solution, 1.5 eq. of NH_4OAc were added and the reaction was kept at room temperature overnight. The reaction mixture was concentrated under vacuum and the pure compound **61** was obtained by silica gel column chromatography with ethyl acetate:Hexane = 1:1 as an eluent. The azido derivative **61** was dissolved in DMF:THF = 1:1 (degassed) under argon atmosphere and 4-ethynyl-3-fluoropyridine (1.2 eq.), CuI (0.2 eq.) and TBTA (0.3 eq.) were added in the order. The reaction was kept under argon atmosphere overnight and then the solvent was removed under reduced pressure. The crude product was dissolved in DCM and washed with EDTA 0.1M in water and water. The organic phases were collected and concentrated. The pure product **62** was obtained by silica gel column chromatography with ethyl acetate as an eluent. Compound **62** was deprotected as reported in the general procedure of Fmoc-deprotection (1.2.3). The product was filtered on silca gel and

washed with DCM:MeOH=8:2. The intermediate was coupled wit Thymine-1-acetic acid as described in the standard procedure (1.2.3). The obtained product **63** was purified by HPLC (116 mg = 26%). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.34 (d, J = 2.1 Hz, 1H), 8.88 - 8.69 (m, 2H), 8.55 (d, J = 4.8 Hz, 1H), 8.12 (dt, J = 6.0, 4.4 Hz, 1H), 7.42 - 7.33 (m, 2H), 7.11 - 7.05 (m, 1H), 5.38 (dp, J = 42.8, 6.9 Hz, 1H), 4.79 - 4.48 (m, 2H), 4.46 - 4.29 (m, 2H), 4.27 - 3.87 (m, 1H), 3.15 - 2.53 (m, 2H), 1.83 - 1.69 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 172.63, 166.90, 166.29, 164.82, 151.53, 146.67, 142.58, 142.42, 139.07, 138.84, 138.10, 125.95, 121.19, 108.62, 59.17, 58.59, 57.08, 51.62, 50.99, 49.00, 48.36, 37.28, 34.82, 12.42. TOF-MS ES(+): 444.1768 m/z.

5. Affinity assays

5.1 Fluorescence Polarization (FP)

FITC conjugates **29** (R-A7/B66/C292), **30** (R-A6/B66/C292), **31** (R-A4/B66/C292) and **32** (RAc) were dissolved in DMSO obtaining 1 mM stock solutions. Stocks were diluted in two steps with PBS buffer pH=7.4 yielding 100 nM, 50 nM and 25 nM solutions. A serial dilution (1×10^{-3}M - 1×10^{-13}M in PBS) of protein was prepared in a 384-wells plate and the ligand solution was added reaching the same ligand concentration in each well. The plate was incubated for 30 minutes and the fluorescence was read by SpectraMax Paradigm instrument. The raw data was exported as anisotropy values and plotted against the protein concentration. The data were fitted using equation (**1**) by KaleidaGraph.

$$A = \alpha \cdot L_0 + (\beta - \alpha) \cdot \frac{1}{2} \cdot \left[(P_0 + L_0 + K_d) - \sqrt{(P_0 + L_0 + K_d)^2 + 4 \cdot P_0 \cdot L_0}\right] \tag{5.1}$$

Equation 5.1: A = measured anisotropy, [P] = protein concentration, L0 = initial ligand concentration, α and β = coefficients.

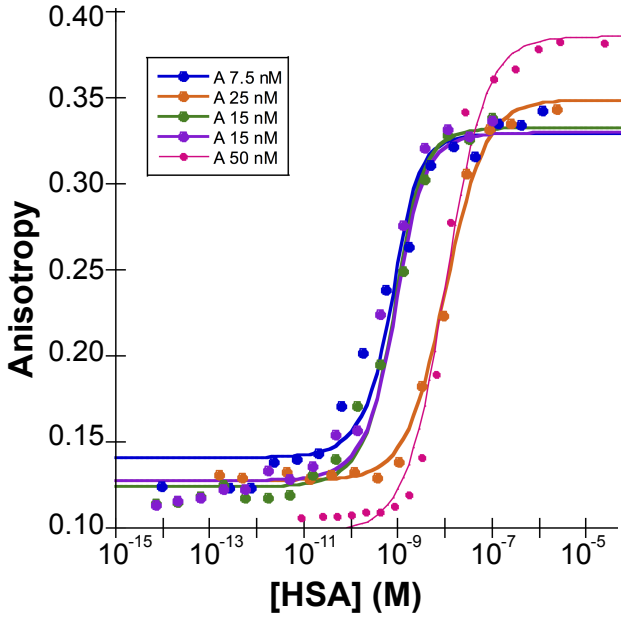


Figure 5.1: Fluorescence polarisation of compound **29** in different concentrations (from 7.5 nM to 50 nM) against HSA.

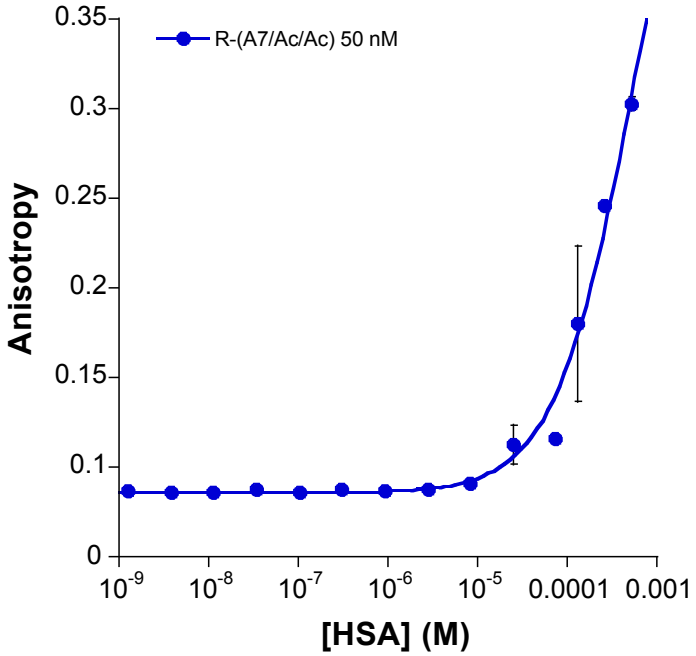


Figure 5.2: Fluorescence polarisation of compound **35** 50 nM against HSA. This compound represents the preferred scaffold **A7** without any additional building block (the amino functions were acetylated). The fitting reveals a mM dissociation constant (K_d = 0.5 mM)

5.2 Surface plasmon resonance (SPR)

5.2.1 HSA hit validation

The affinity of compounds **R-A7/B66/C292** (**29**), **R-A6/B66/C292** (**30**), **R-A4/B66/C292** (**31**) and the negative control RNHAc (**32**) against Human Serum Albumin (HSA) was confirmed by Surface plasmon resonance (SPR) assay carried out by Biacore S200. 20 μM HSA solution was prepared by dissolving lyophilised HSA in PBS 1× buffer (pH = 7.4). The resulting HSA solution was diluted to 200 nM by coating buffer (HBS-EP, pH=4.5). 5770 RU of protein were coated on Biacore Sensor Chip CM5 (Series S) using the 200 nM HSA solution, 400 mM EDC-HCl, 100 mM N-hydroxysucinimmide (NHS) and 1M Ethanolamine (pH=8.5). FITC conjugates **29**, **30**, **31** and **32** were dissolved first in DMSO (100 mM solutions) and then diluted by PBS 1× buffer (pH = 7.4) reaching the final concentration of 5 μM (sample **S1**, 0.1% DMSO in PBS buffer). Four serial dilutions (dil. 1:4, **S2** = 1.25 μM, **S3** = 312.5 nM, **S4** = 78.13 nM, **S5** = 19.53 nM) of 5 μM **S1** stock solution were prepared for each compound. Solutions from **S1** to **S5** were used in order to perform SPR experiments.

5.2.2 TNKS1 hit validation

Human tankyrase-1 was immobilised by "Biotin - Streptavidin" interactions on Biacore SA chip by flowing a 0.4 mL of 500 nM biotinylated TNKS solution on the cell. After the coating the Biacore response was equal to 3000 RU. Compounds **44**, **45**, **46** (NH_2(A4/B101/C491)),

47, **49**, **54**, **55**, **59** and **63** were first dissolved in DMSO (100 mM stock solutions) and then diluted in HEPES buffer (pH=7.52) to the final solutions (**S1** = 10 μM - **S6** = 156 nM). These solution were used for the SPR experiment carried out by Biacore S200 as described in the paragraph 5.2.1. The compound **46** (NH_2(A4/B101/C491)) was also tested against HSA in order to confirm specificity for TNKS1. The solutions of **46** (**S1** = 10 μM - **S6** = 156 nM) were tested on HSA immobilised on CM5 chip (5.2.1).

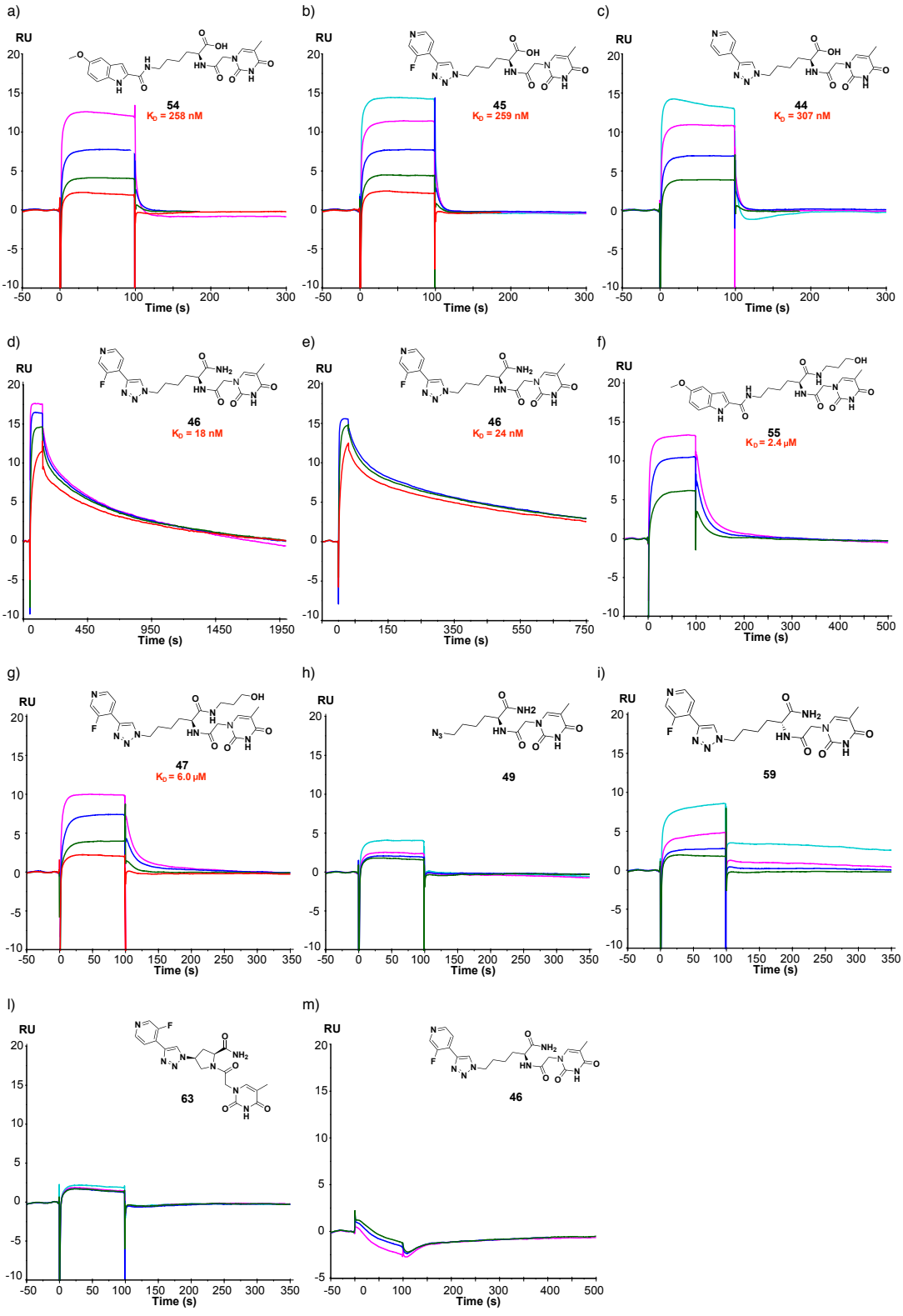


Figure 5.3: Binding properties of different compounds in different concentrations from 10 μM to 156 nM (in PBS pH=7.4) analyzed by Surface Plasmon Resonance (SPR) against Human Tankyrase-1 (TNKS1) and Human serum albumin (HSA) immobilized on a BIAcore chip (SA and CM5). The dissociation constants (K_d were calculated by fitting the sensograms with Biacore S200 software: (a) **55** against TNKS1, K_d=258 nM; (b) **45** against TNKS1, K_d=259 nM; (c) **44** against TNKS1, K_d=307 nM; **46** against TNKS1, (d) K_d=24 nM and (e)18 nM; (f) **55** against TNKS1, K_d=2.4 μM and (g)**47** against TNKS1, K_d=6.0 μM. For compounds **49** (h), **59** (i) and **63** (l) against TNKS1 and for the compound **46** against HSA (m) were not found any residual affinity in order to fit the sensograms and calculate the dissociation constants.

6. Appendix

6.1 Fingerprints

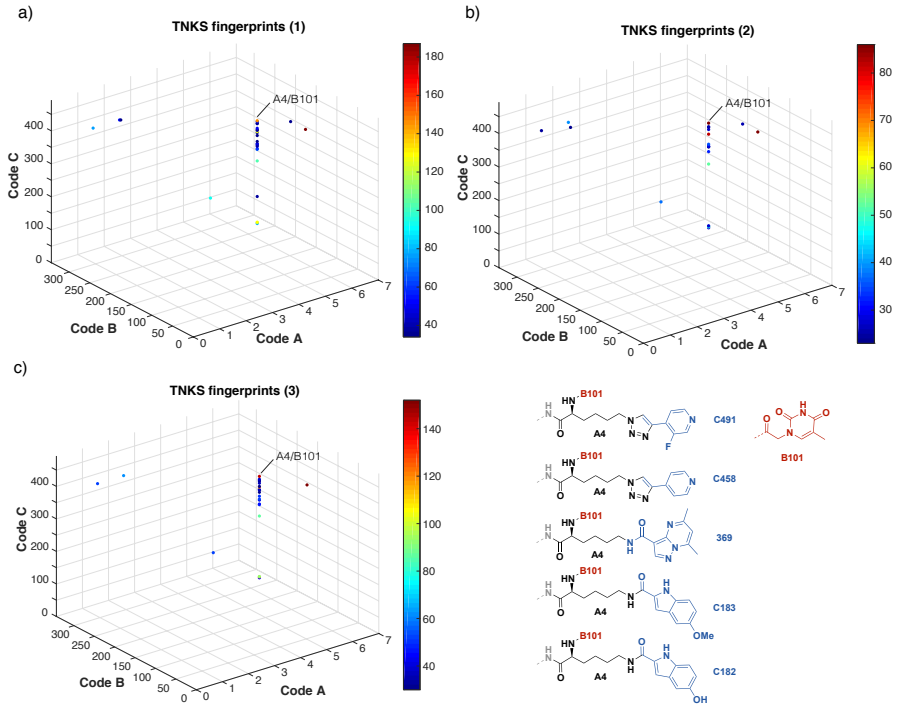


Figure 6.1: Results of library selections against human tankyrase 1 (TNKS) carried out using different concentration of tween-20 in the washing steps: (a) tween-20 = 0.1% in HEPES buffer, (b) tween-20 = 0.05% in HEPES buffer and (c) tween-20 = 0.01% in HEPES buffer. In the last panel are reported the five most enriched compounds in each selections.

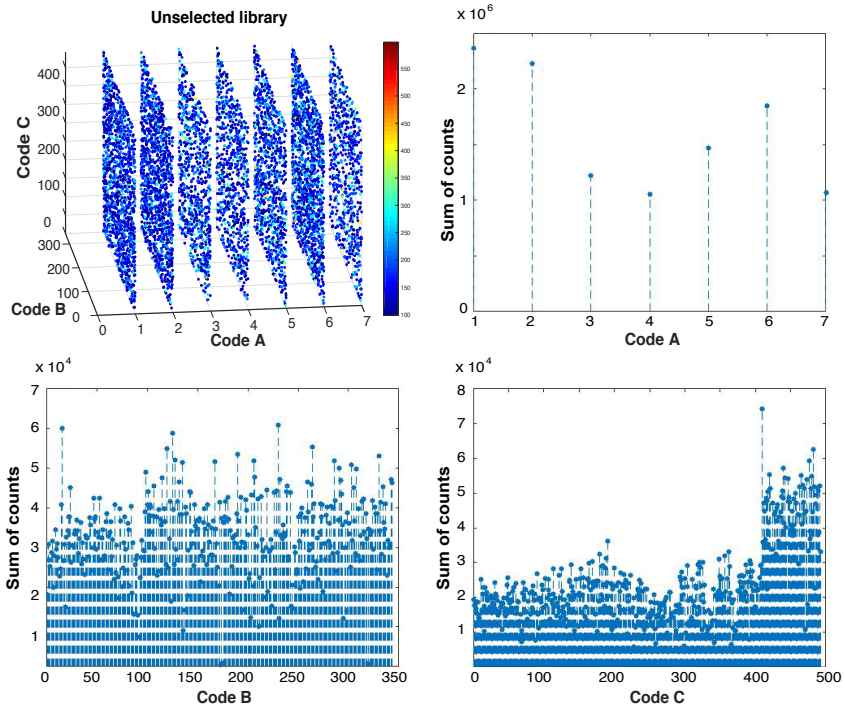


Figure 6.2: Unselected library. The individual library members are unambiguously identified by their code A (ranging between 1 and 7), B (ranging between 1 and 343) and C (ranging between 1 and 492). The number of sequence counts for each compound is displayed as spheres of a different color, with a cut-off threshold set at 100 counts. The amplification and the HTS analysis of the unselected library is useful in order to understand its quality before selections. In this case we obtained difference of counts as factor 2 for codes A, a factor 3 for codes B and a factor 4 for the codes C. Particularly, code C corresponding to alkynes (C411-C492) were 2 times more enriched then the code C corresponding to carboxylic acids (C1-C410).

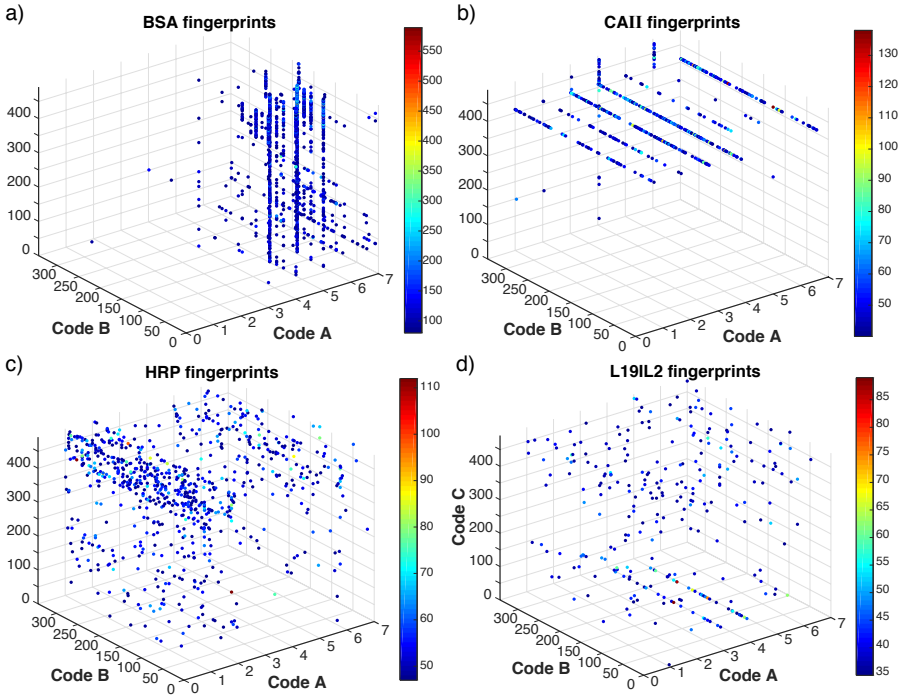


Figure 6.3: Results of library selections against (a) bovin serum albumin (BSA), (b) Carbonic anhydrase II (CAII), (c) Horseradish peroxidase (HRP) and (d) L19IL2.

6.2 NMR

6.2.1 Compound 2

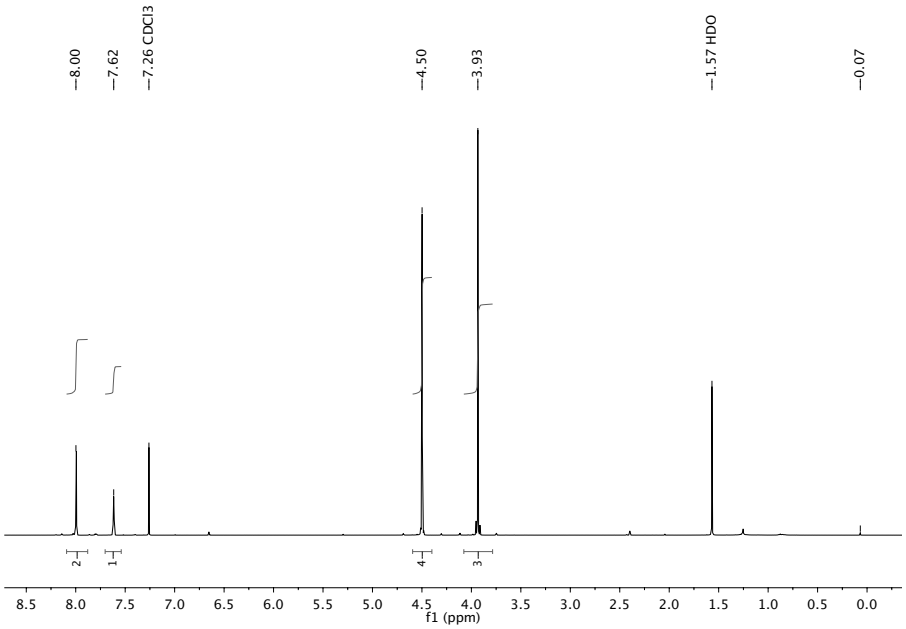


Figure 6.4: ^{1}H-NMR of compound **2** (400 MHz, Chloroform-d).

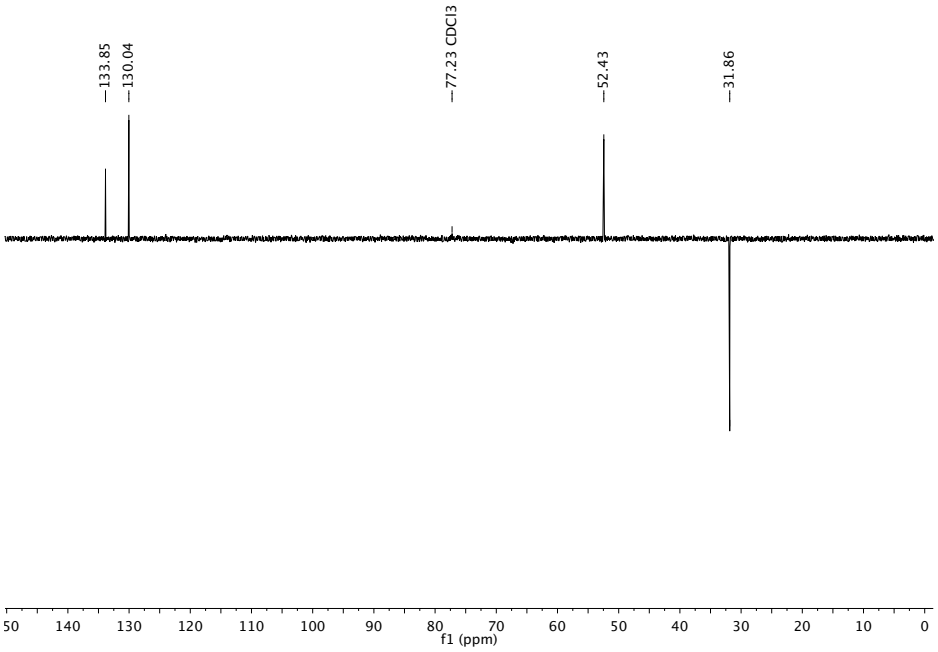


Figure 6.5: ^{13}C-NMR (dept-135) of compound **2** (101 MHz, Chloroform-d).

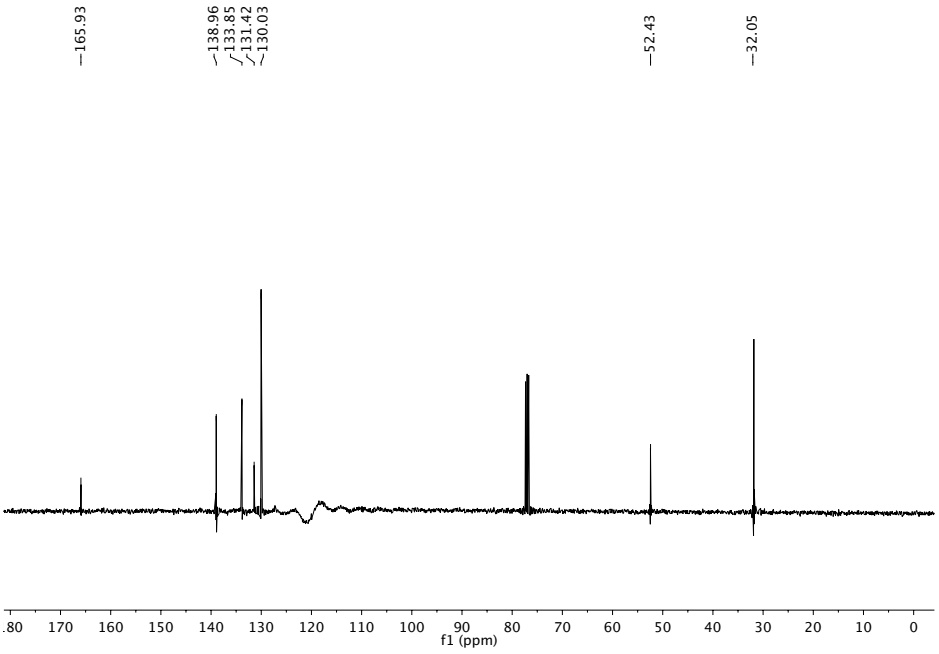


Figure 6.6: ^{13}C-NMR of compound **2** (101 MHz, Chloroform-d).

6.2.2 Compound 3

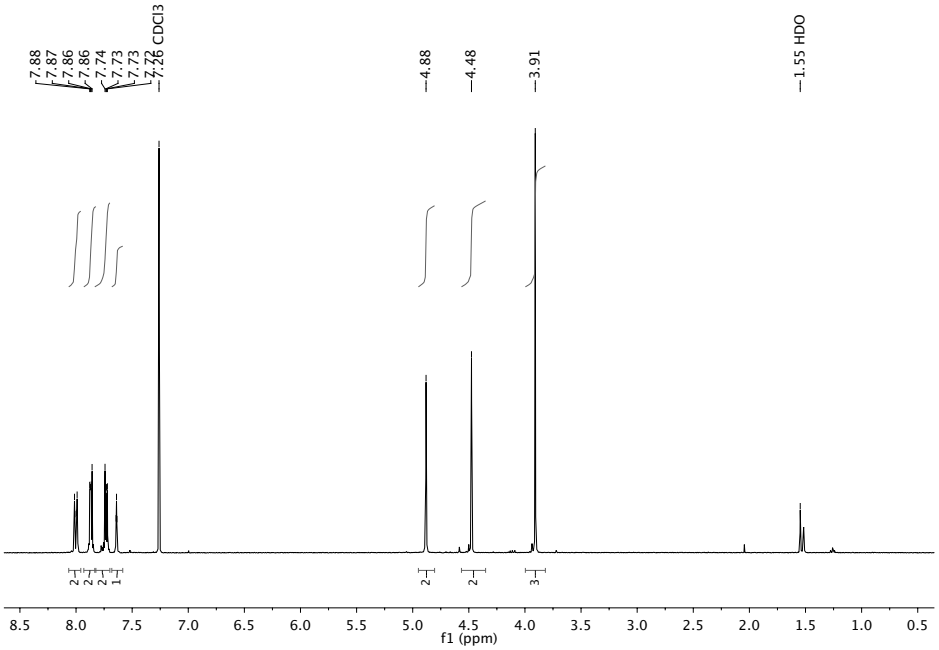


Figure 6.7: ^{1}H-NMR of compound **3** (400 MHz, Chloroform-d).

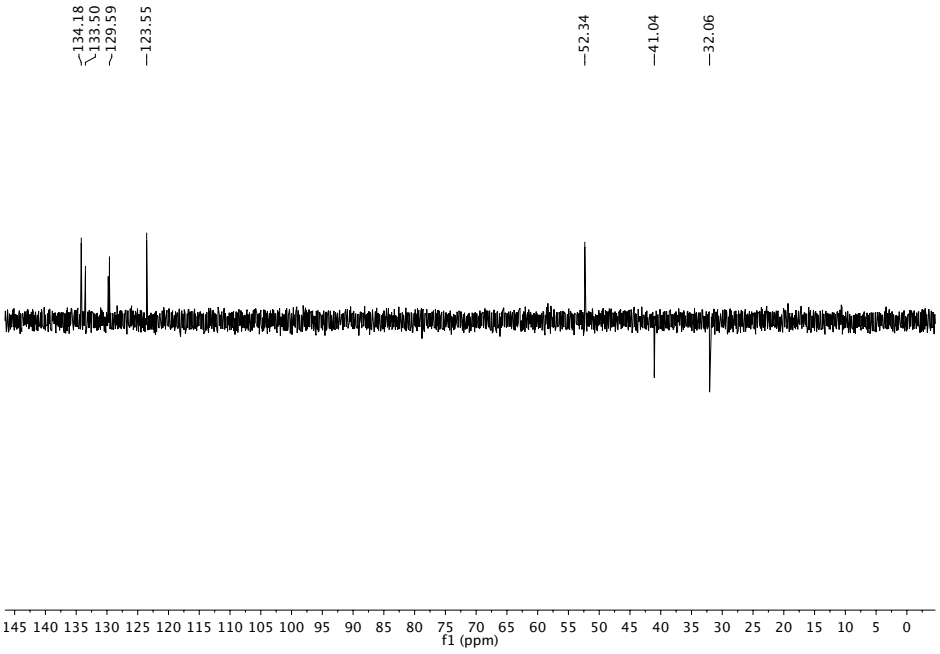


Figure 6.8: ^{13}C-NMR (dept-135) of compound **3** (101 MHz, Chloroform-d).

6.2.3 Compound 4

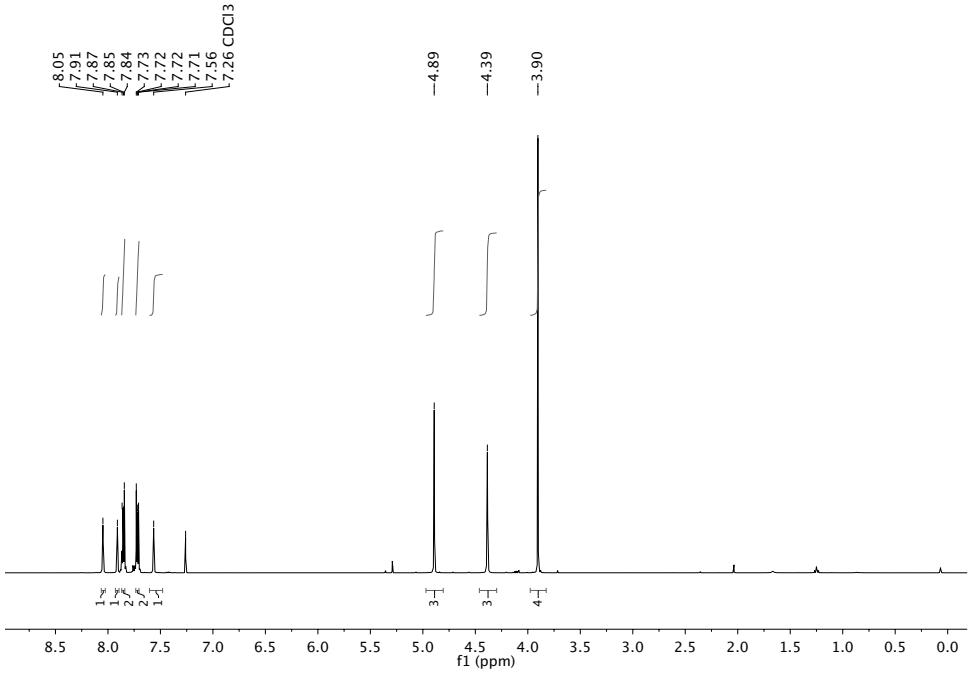


Figure 6.9: ^{1}H-NMR of compound **4** (400 MHz, Chloroform-d).

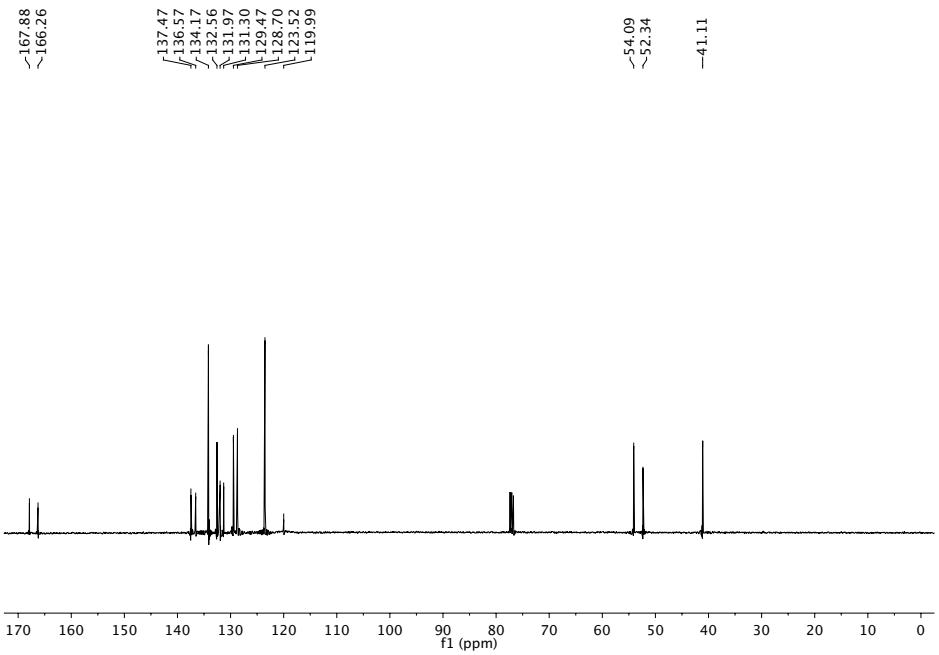


Figure 6.10: ^{13}C-NMR of compound **4** (101 MHz, Chloroform-d).

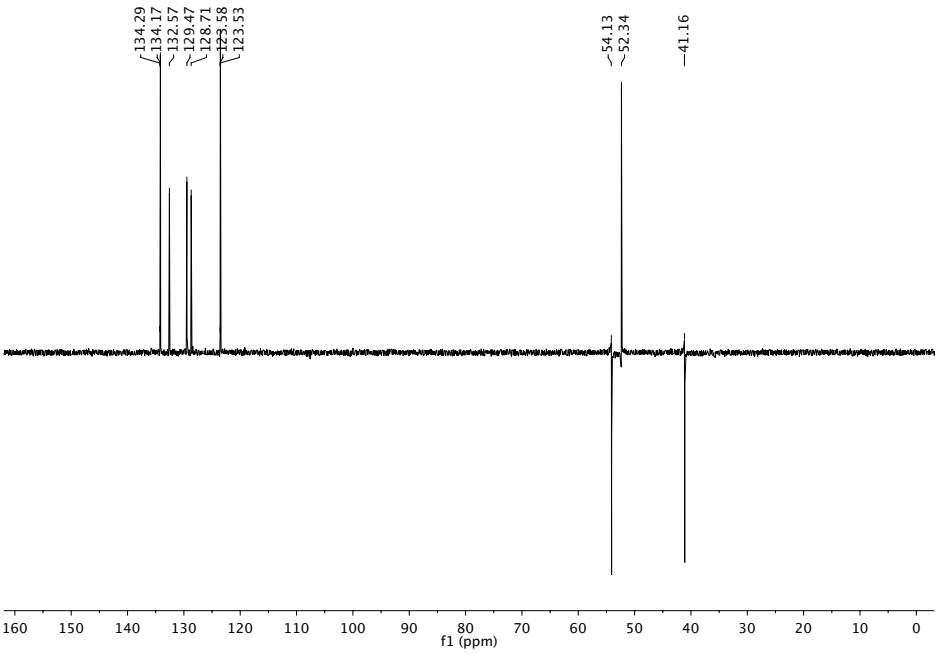


Figure 6.11: ^{13}C-NMR (dept-135) of compound **4** (101 MHz, Chloroform-d).

6.2.4 Compound 5

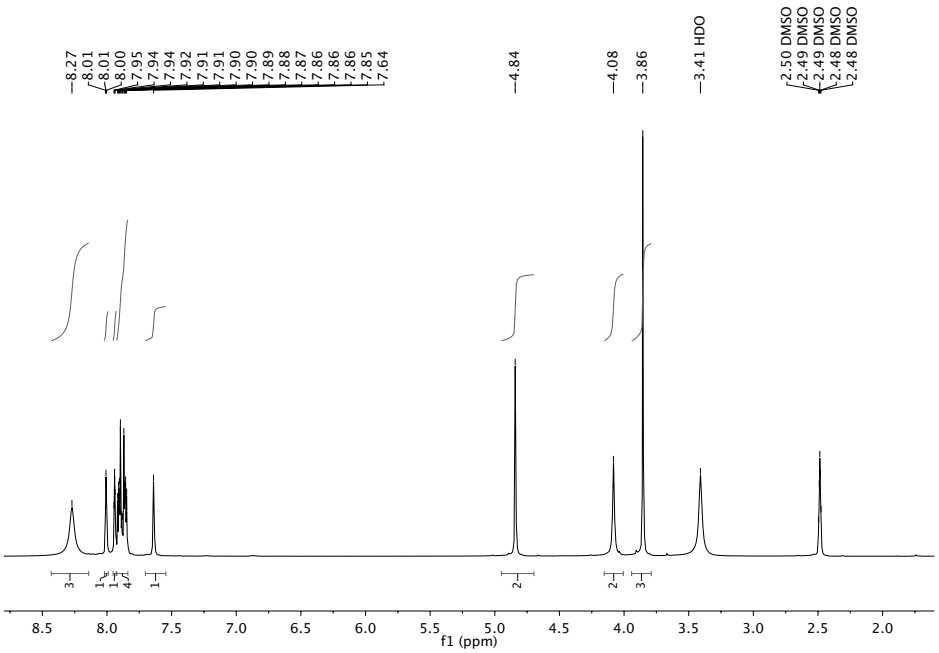


Figure 6.12: ^{1}H-NMR of compound **5** (400 MHz, DMSO-d6).

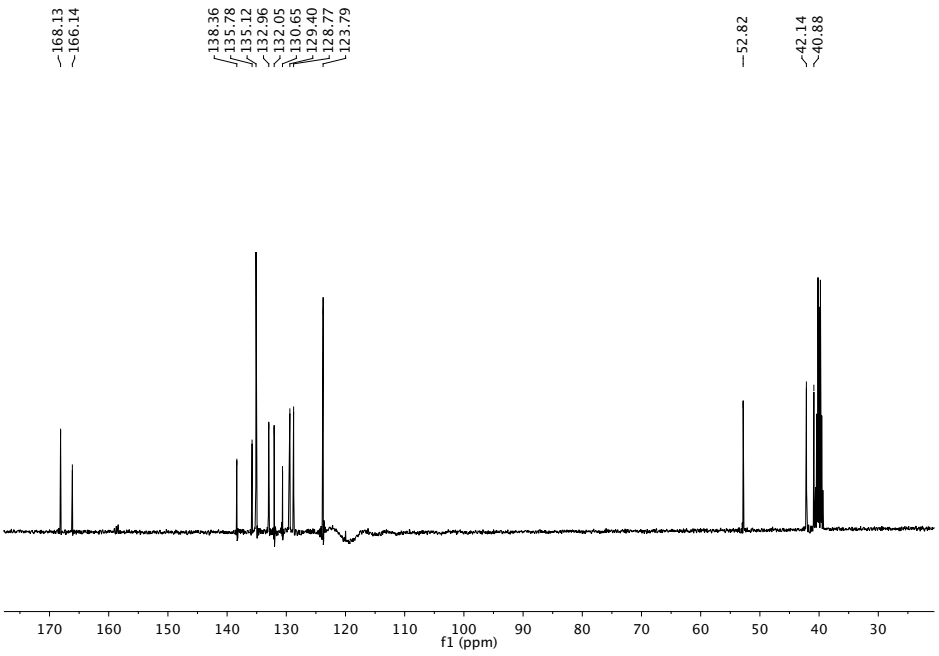


Figure 6.13: ^{13}C-NMR of compound **5** (101 MHz, DMSO-d6).

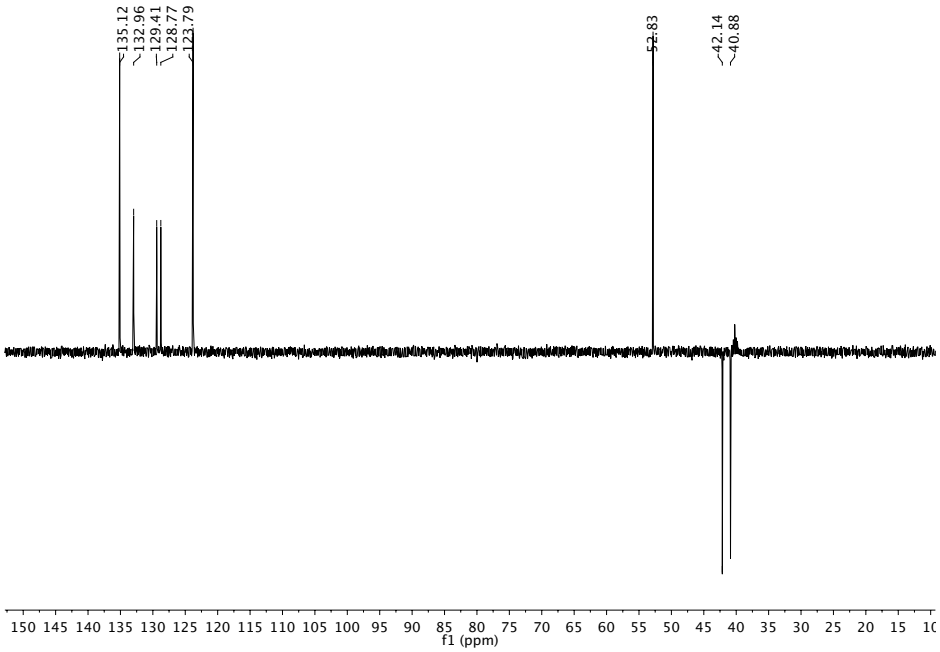


Figure 6.14: ^{13}C-NMR (dept-135) of compound **5** (101 MHz, DMSO-d6).

6.2.5 Compound 7

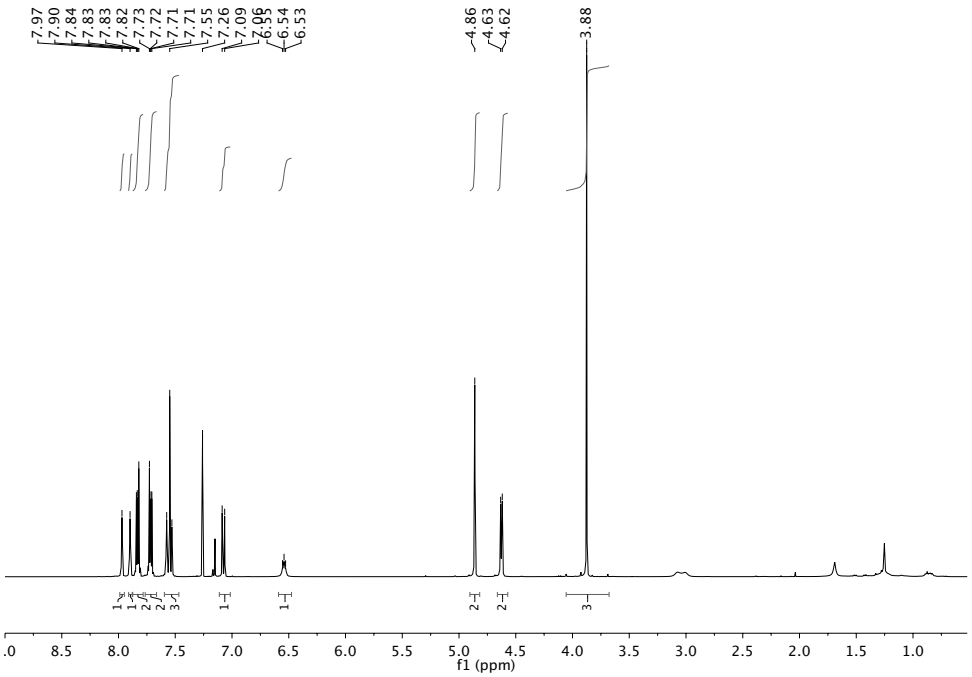


Figure 6.15: ^{1}H-NMR of compound **7** (400 MHz, Chloroform-d).

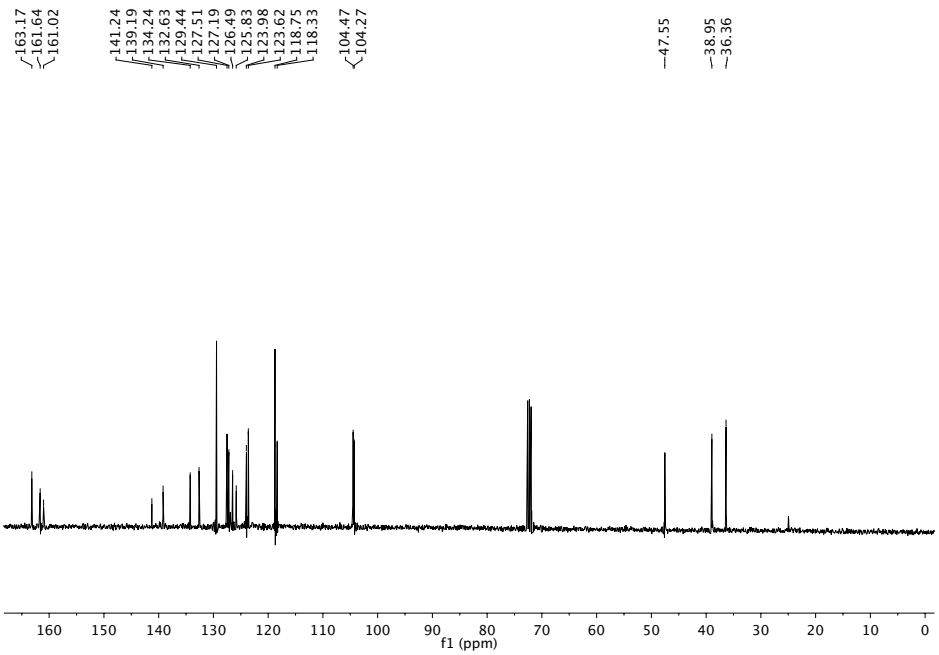


Figure 6.16: ^{13}C-NMR of compound **7** (101 MHz, Chloroform-d).

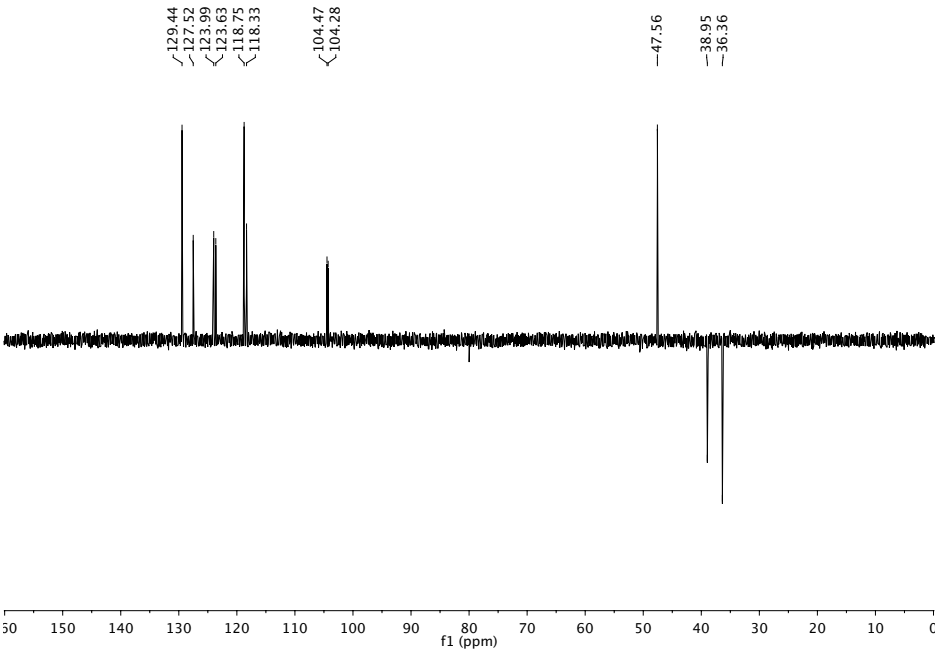


Figure 6.17: ^{13}C-NMR (dept-135) of compound **7** (101 MHz, Chloroform-d).

6.2.6 Compound 8

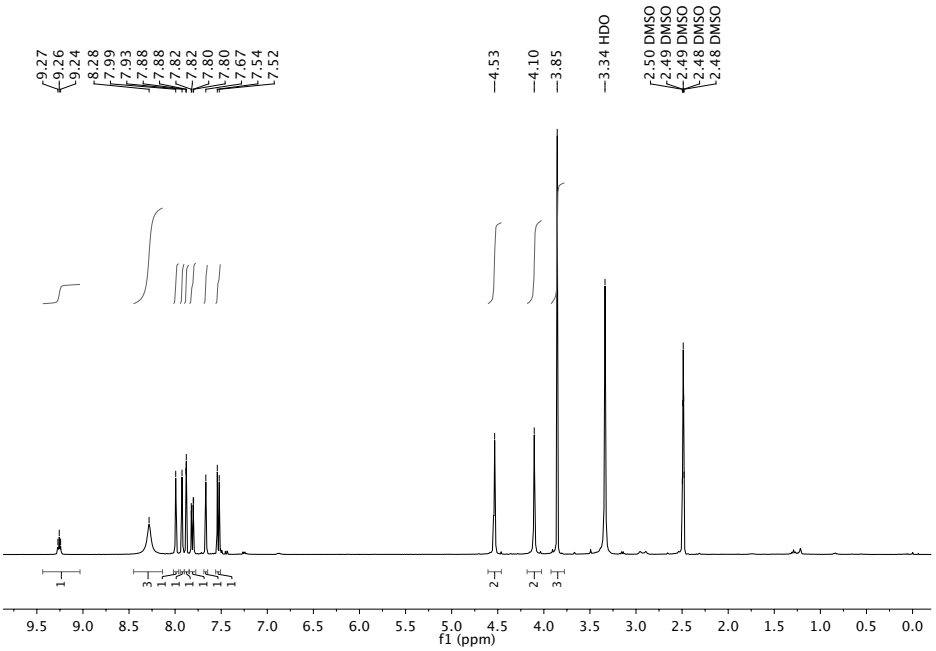


Figure 6.18: ^{1}H-NMR of compound **8** (400 MHz, DMSO-d6).

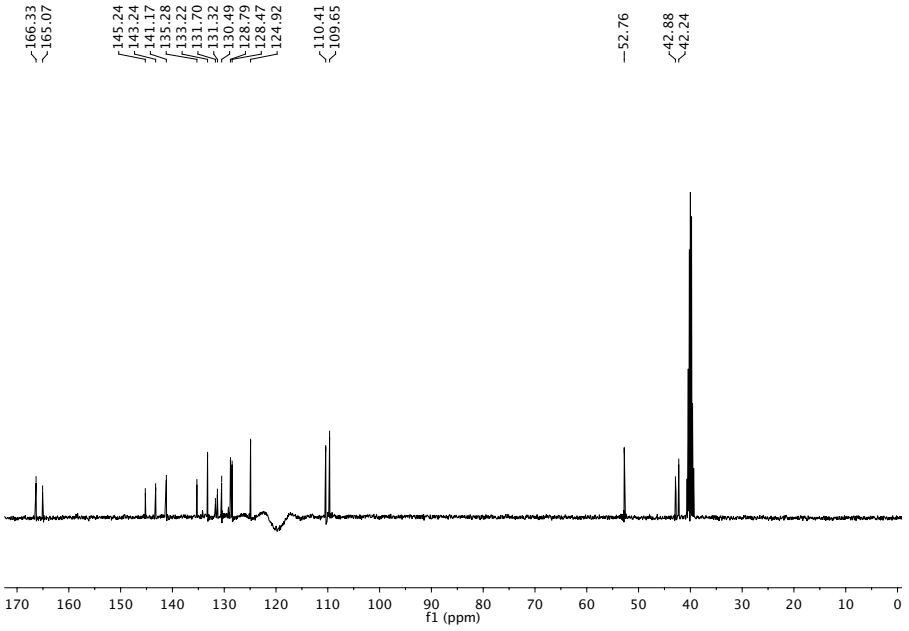


Figure 6.19: ^{13}C-NMR of compound **8** (101 MHz, DMSO-d6).

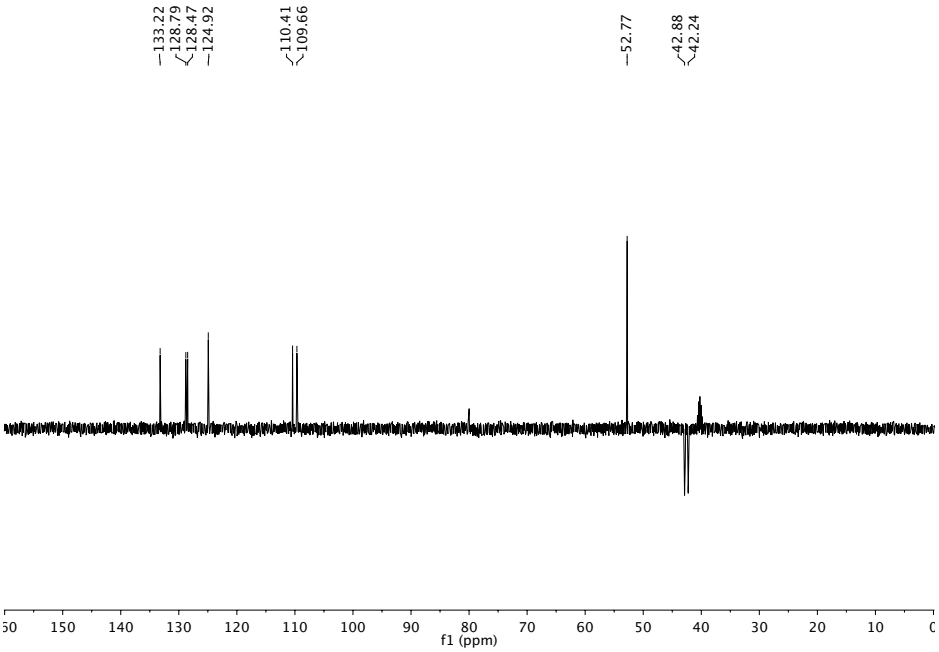


Figure 6.20: ^{13}C-NMR (dept-135) of compound **8** (101 MHz, DMSO-d6).

6.2.7 Compound 10

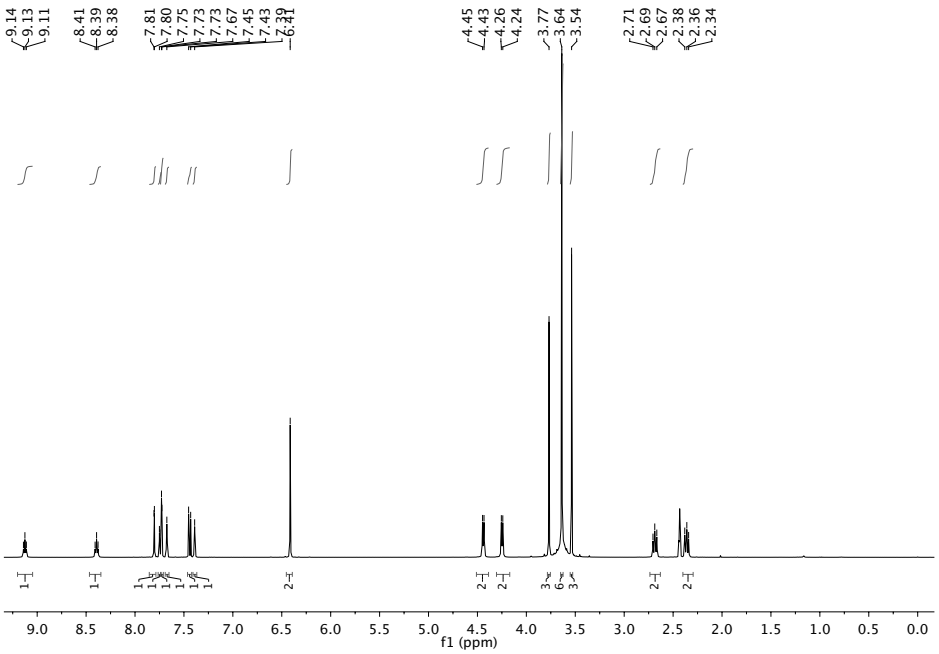


Figure 6.21: ^{1}H-NMR of compound **10** (400 MHz, DMSO-d6).

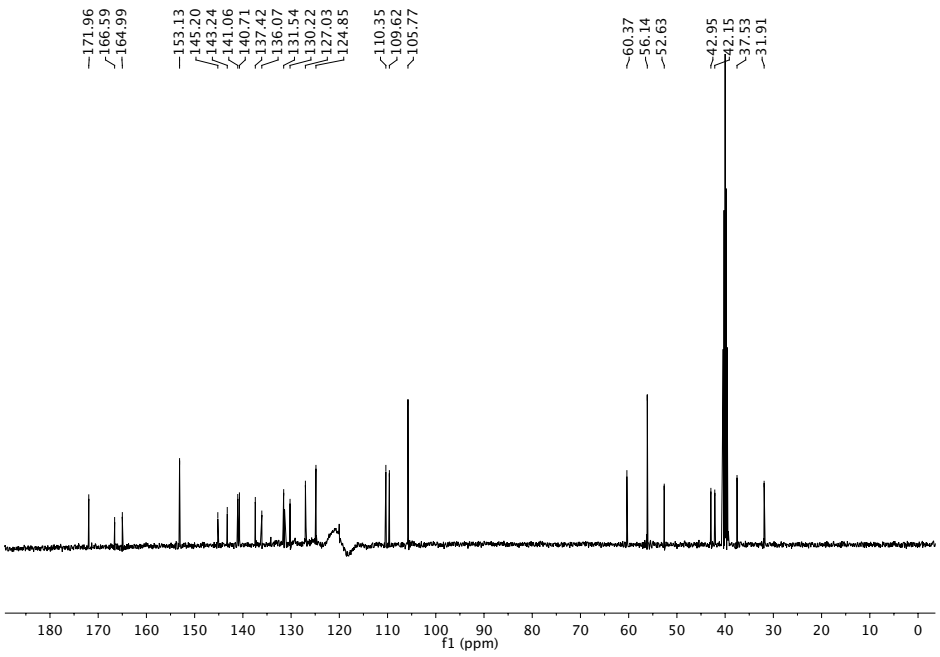


Figure 6.22: ^{13}C-NMR of compound **10** (101 MHz, DMSO-d6).

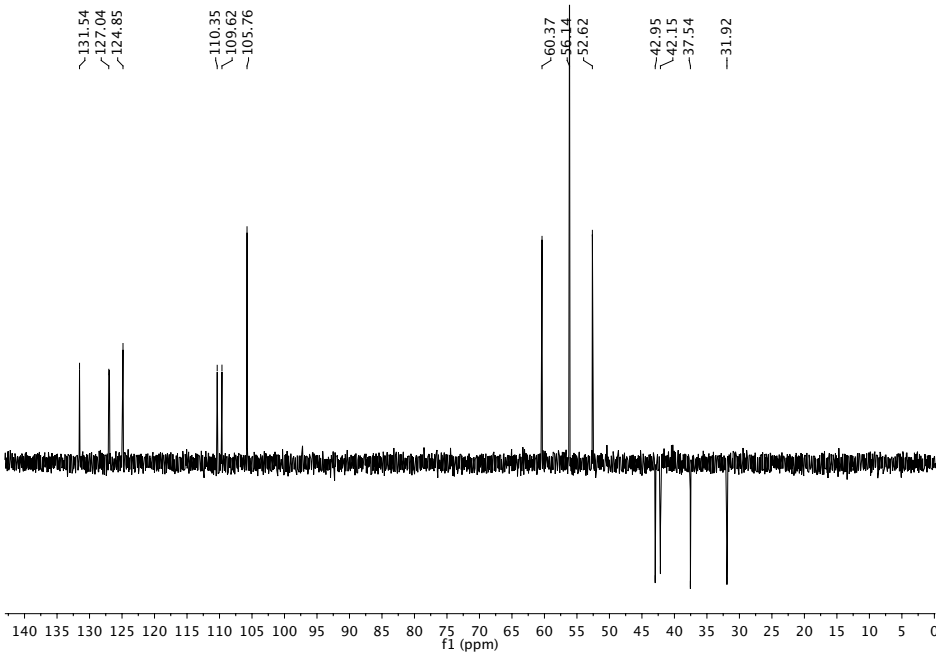


Figure 6.23: ^{13}C-NMR (dept-135) of compound **10** (101 MHz, DMSO-d6).

6.2.8 Compound 11

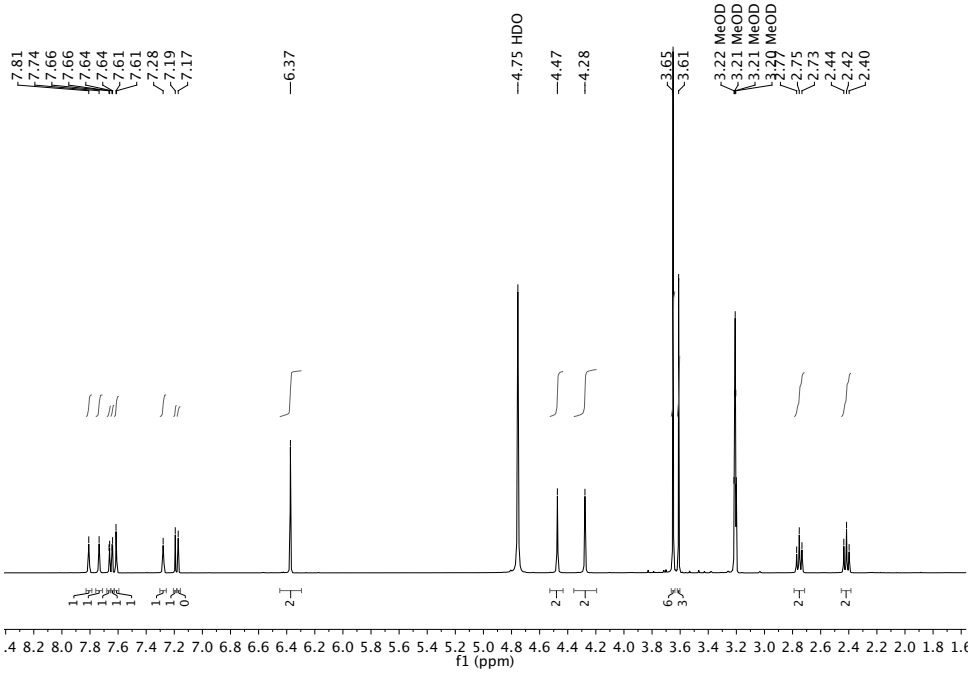


Figure 6.24: ^{1}H-NMR of compound **11** (400 MHz, methanol-d4).

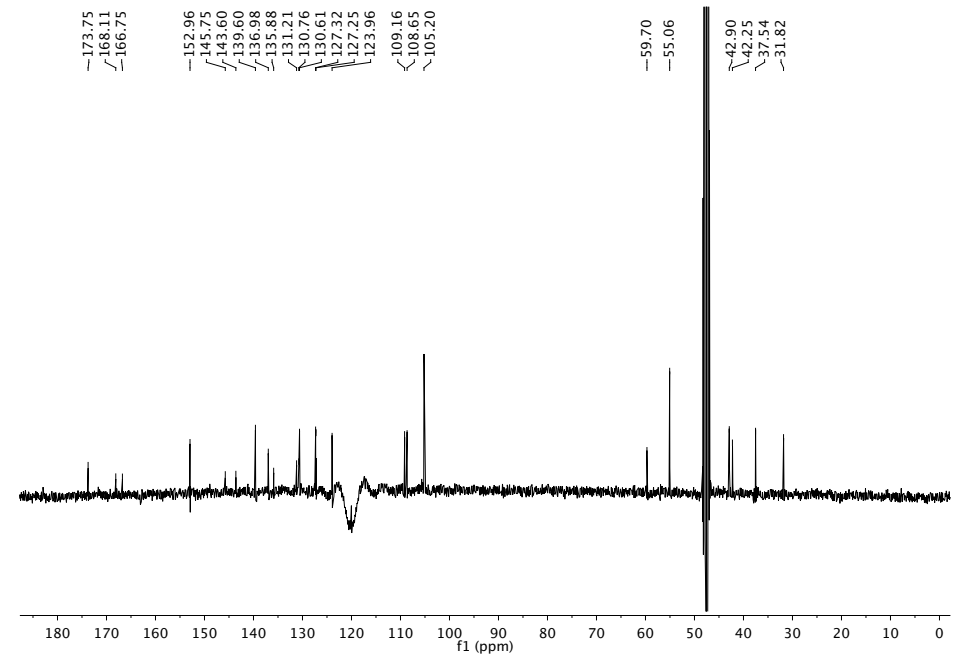


Figure 6.25: ^{13}C-NMR of compound **11** (101 MHz, methanol-d4).

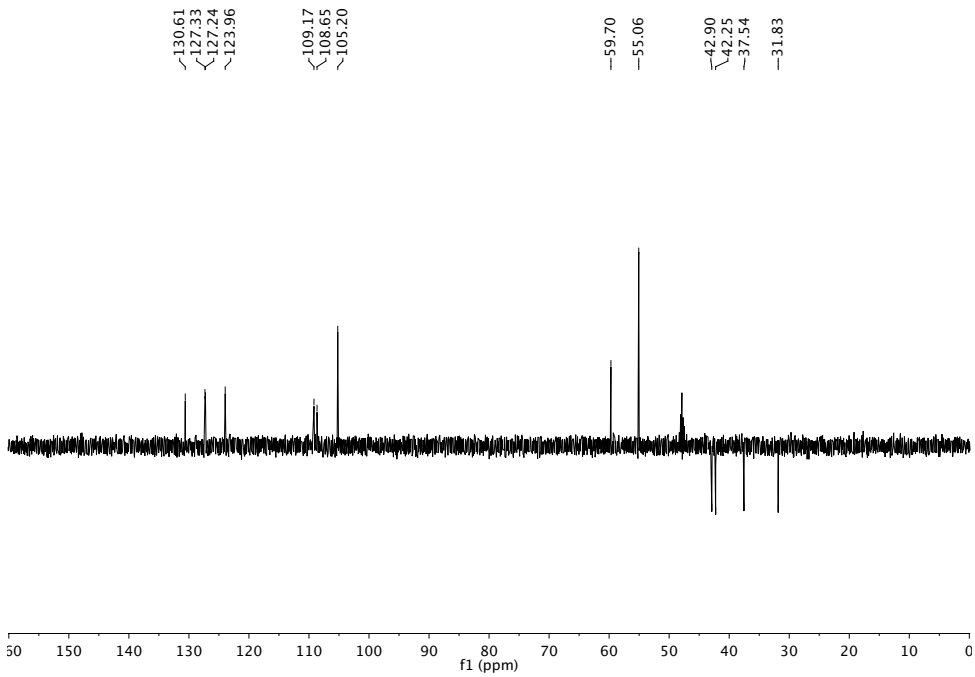


Figure 6.26: ^{13}C-NMR (dept-135) of compound **11** (101 MHz, methanol-d4).

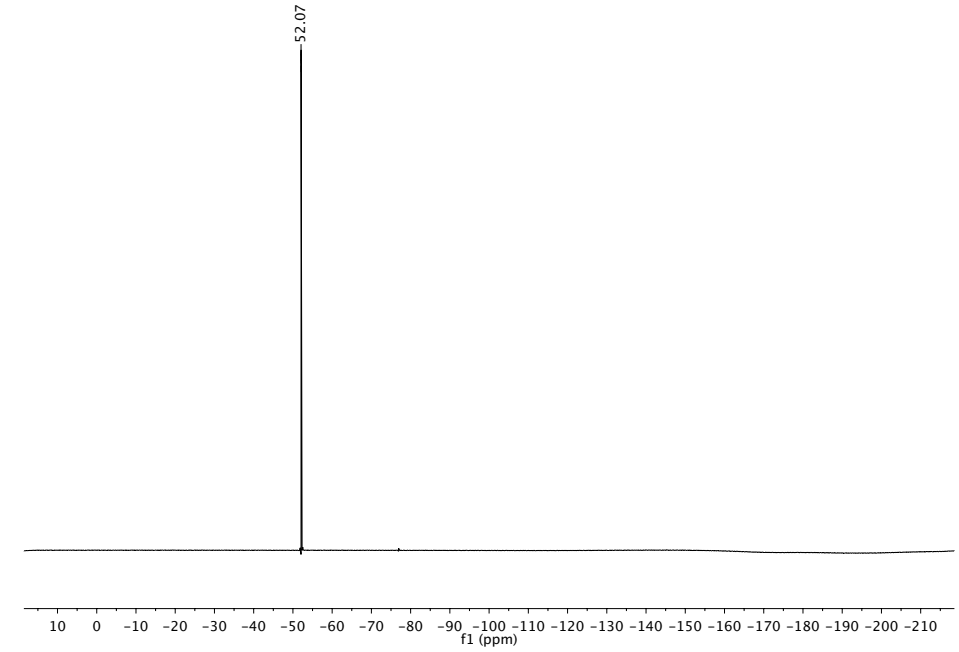


Figure 6.27: ^{14}F-NMR of compound **11** (376 MHz, methanol-d4).

6.2.9 Compound 14

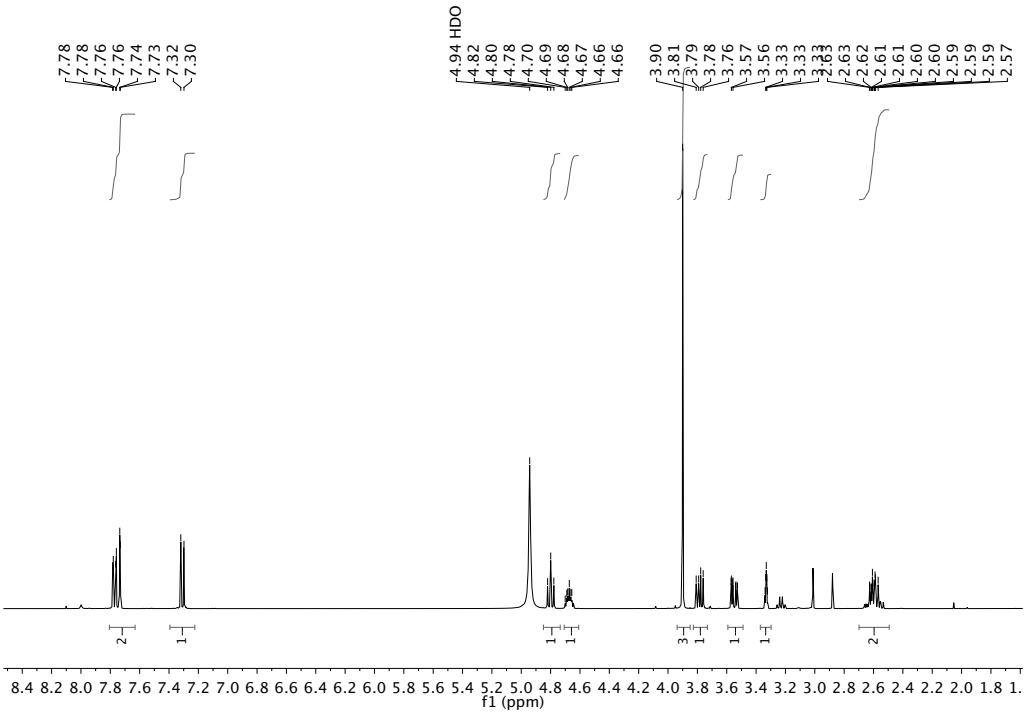


Figure 6.28: ^{1}H-NMR of compound **14** (400 MHz, Methanol-d4).

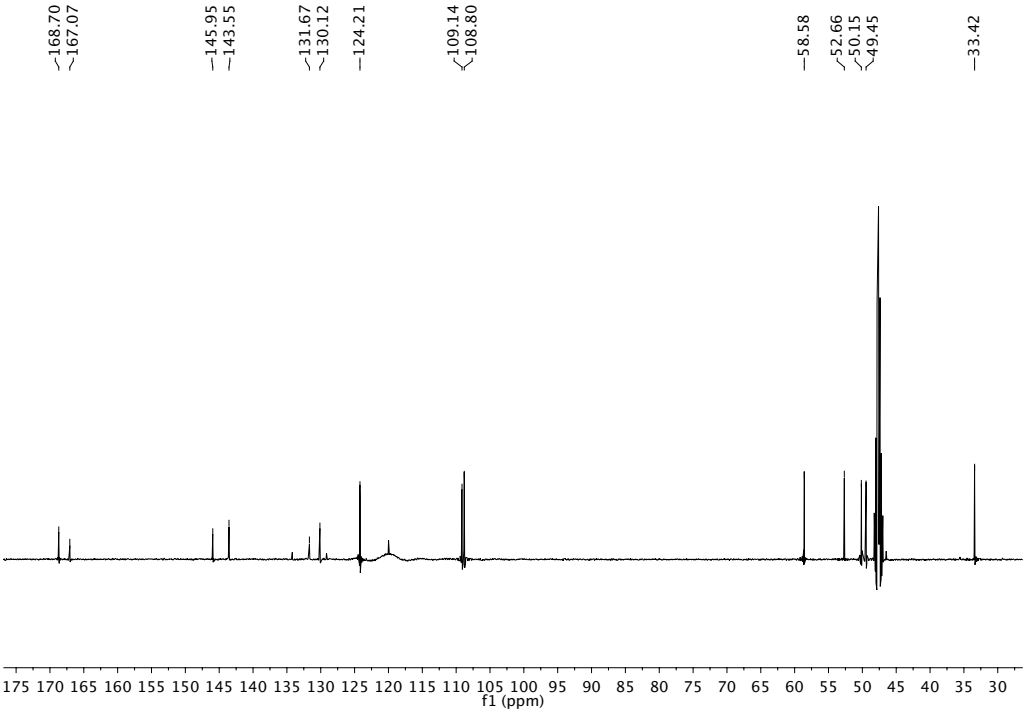


Figure 6.29: ^{13}C-NMR of compound **14** (101 MHz, Methanol-d4).

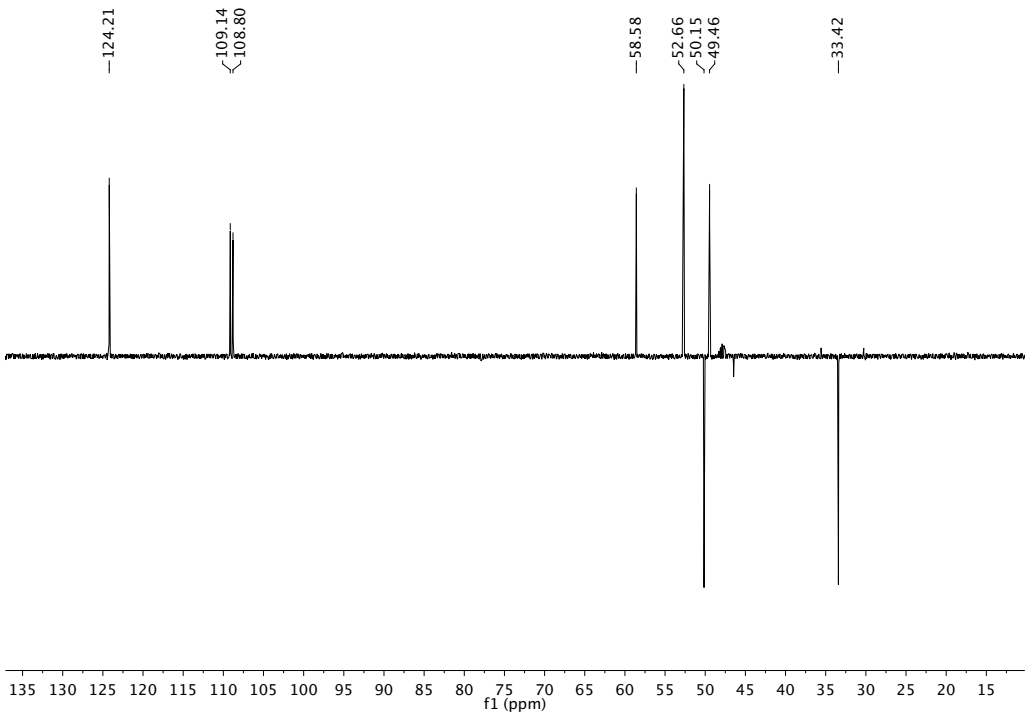


Figure 6.30: ^{13}C-NMR (dept-135) of compound **14** (101 MHz, Methanol-d4).

6.2.10 Compound 15

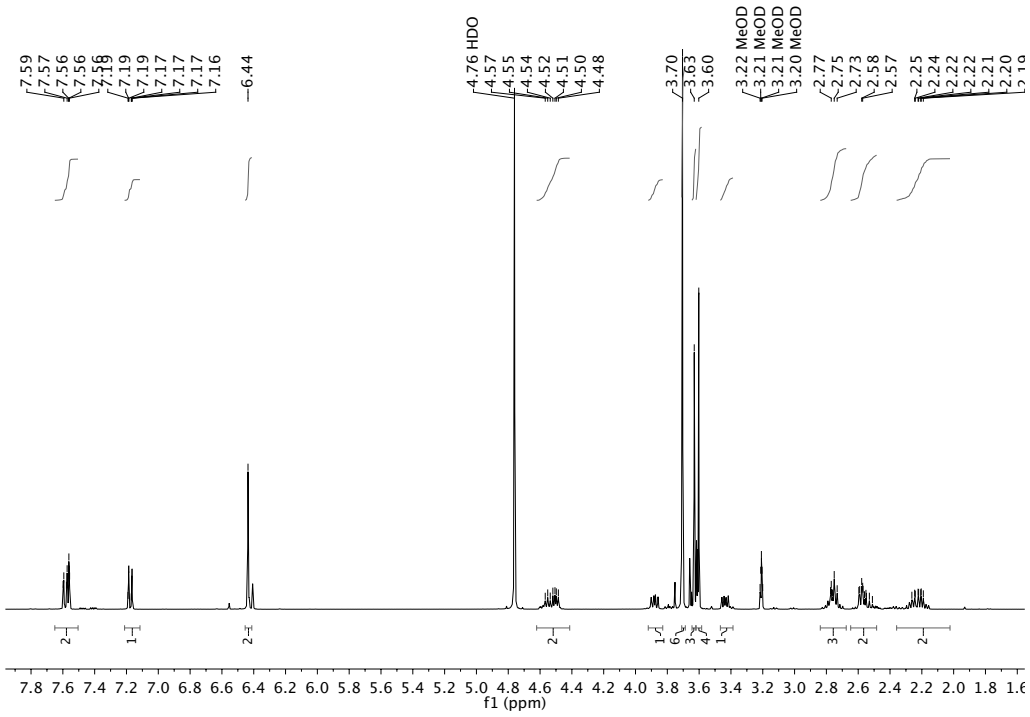


Figure 6.31: ^{1}H-NMR of compound **15** (400 MHz, Methanol-d4).

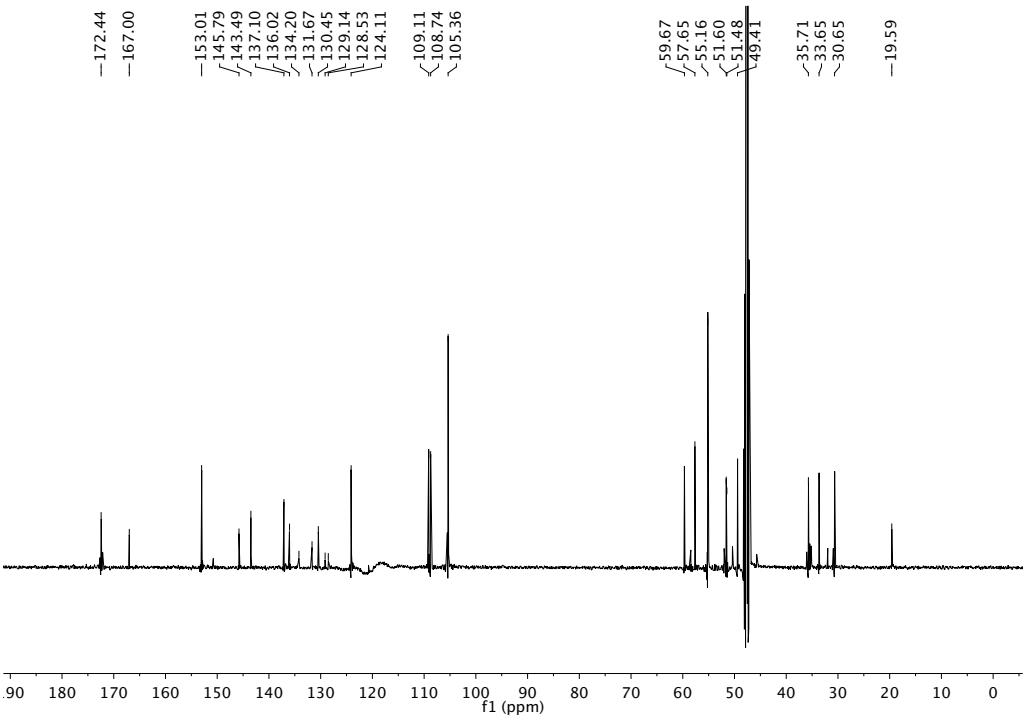


Figure 6.32: ^{13}C-NMR of compound **15** (101 MHz, Methanol-d4).

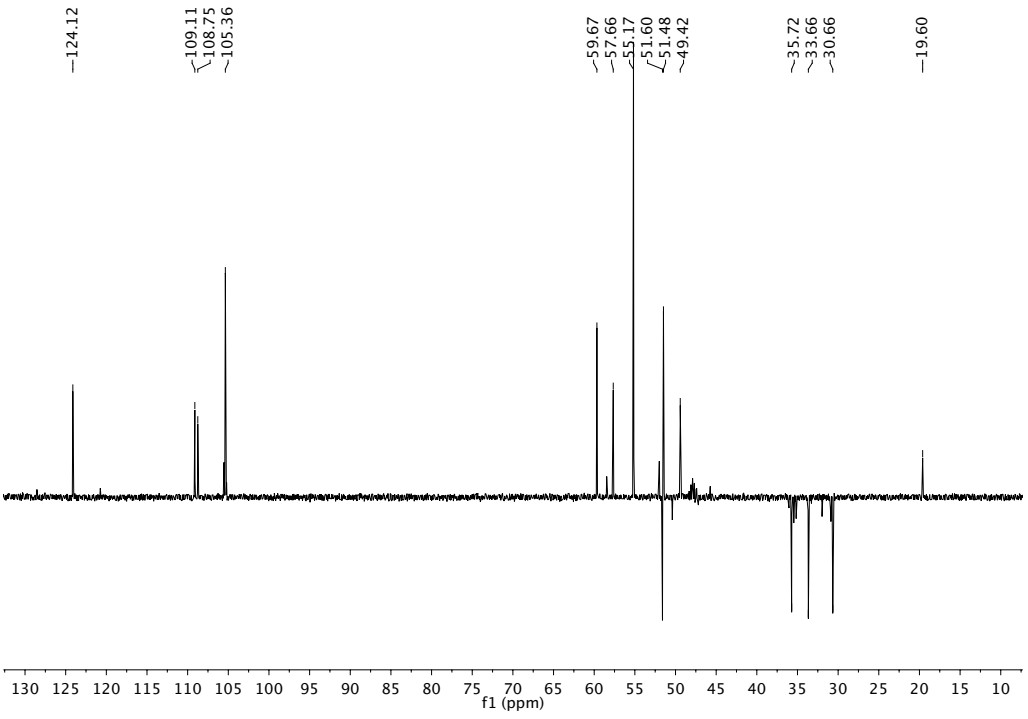


Figure 6.33: ^{13}C-NMR (dept-135) of compound **15** (101 MHz, Methanol-d4).

6.2.11 Compound 16

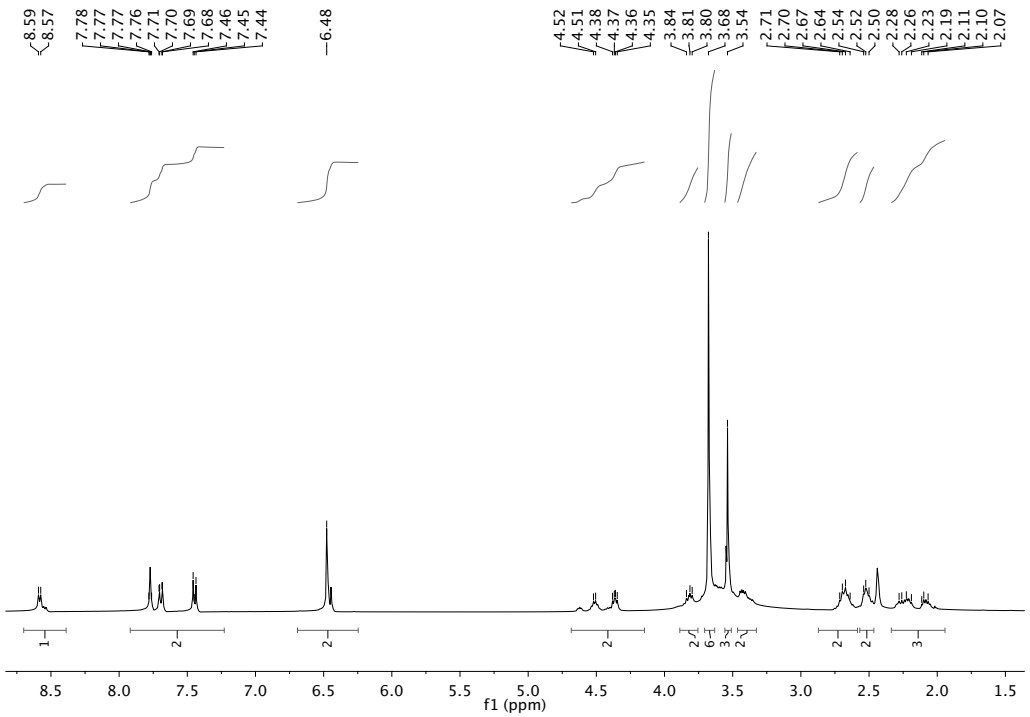


Figure 6.34: ^{1}H-NMR of compound **16** (400 MHz, DMSO-d6).

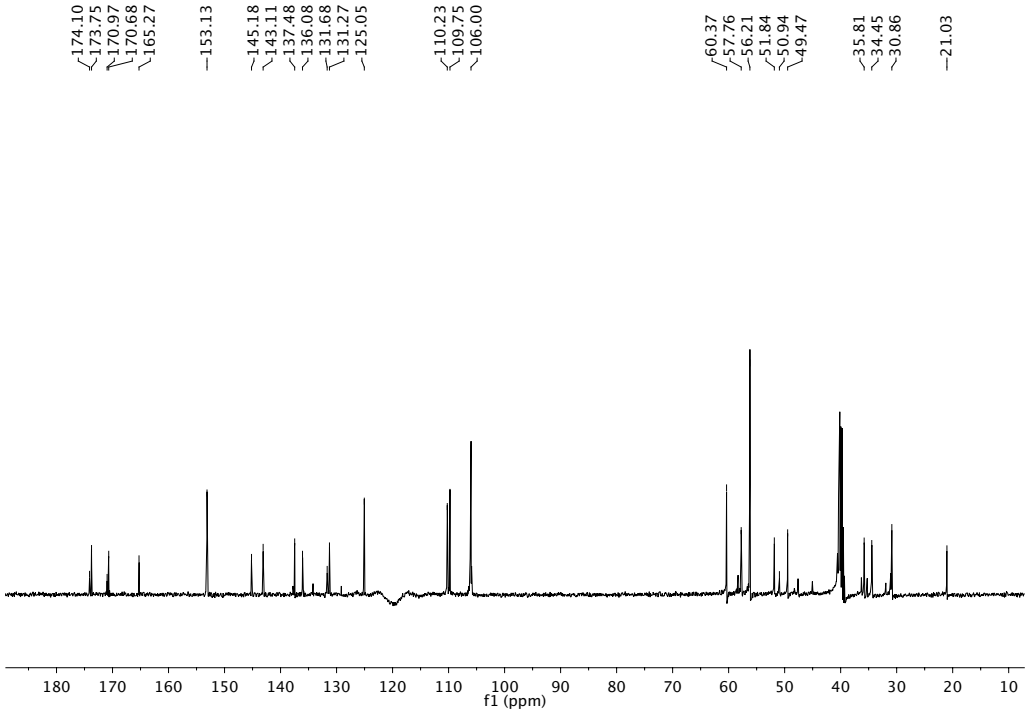


Figure 6.35: ^{13}C-NMR of compound **16** (101 MHz, DMSO-d6).

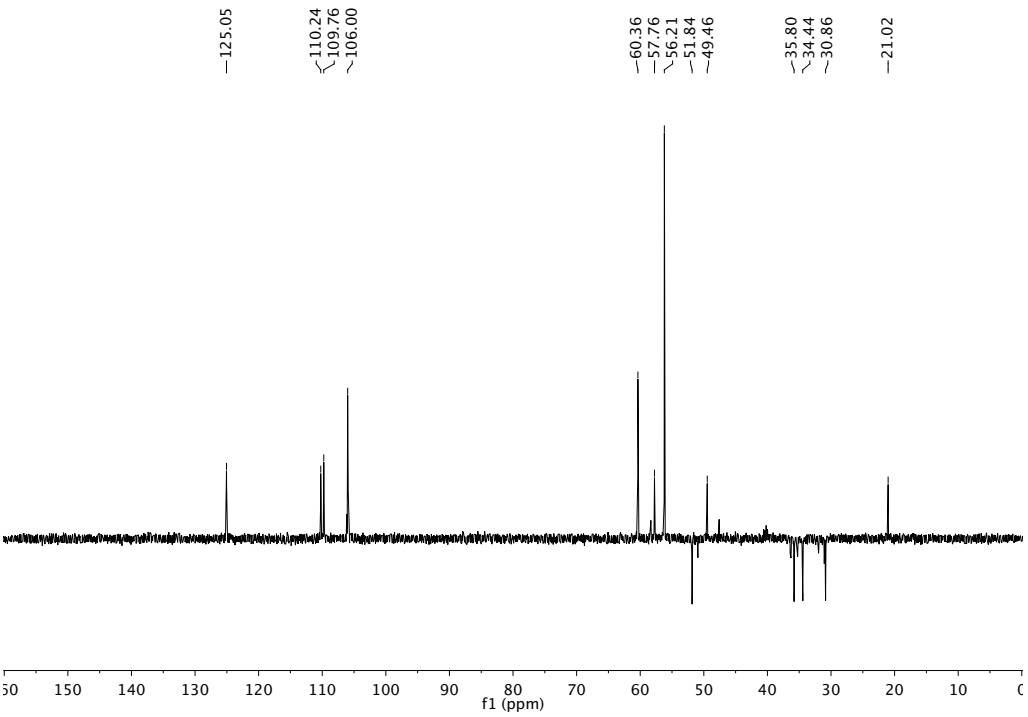


Figure 6.36: ^{13}C-NMR (dept-135) of compound **16** (101 MHz, DMSO-d6).

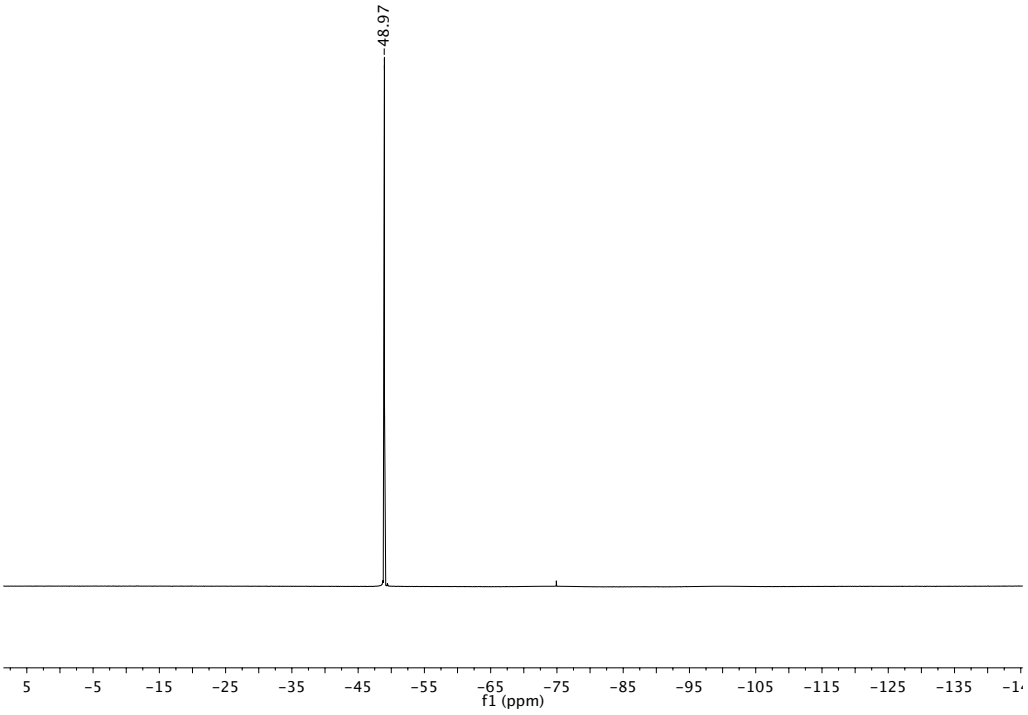


Figure 6.37: ^{19}F-NMR of compound **16** (376 MHz, DMSO-d6).

6.2.12 Compound 20

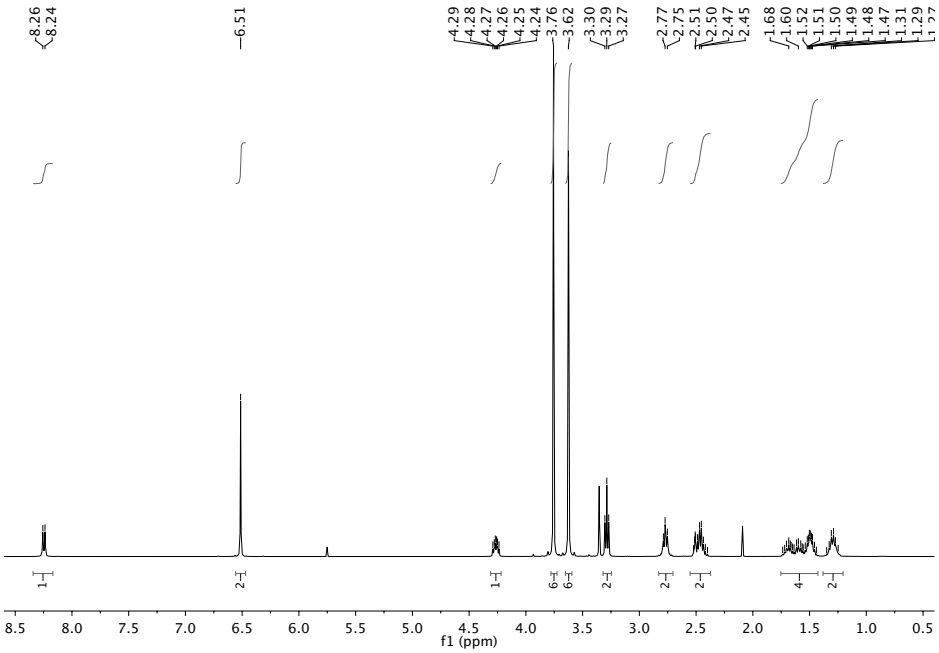


Figure 6.38: ^{1}H-NMR of compound **20** (400 MHz, DMSO-d6).

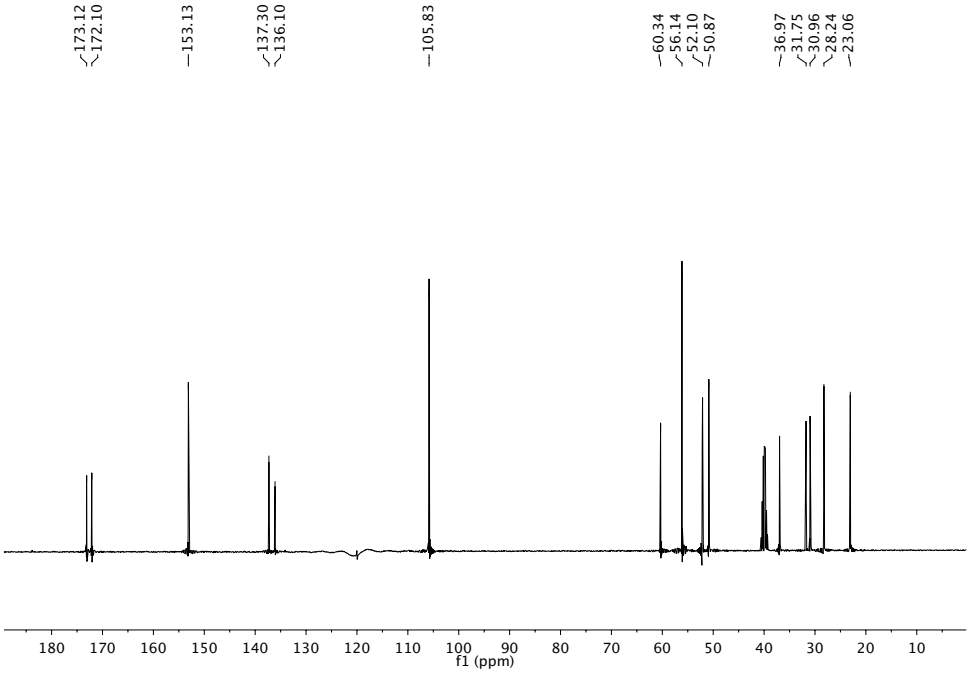


Figure 6.39: ^{13}C-NMR of compound **20** (101 MHz, DMSO-d6).

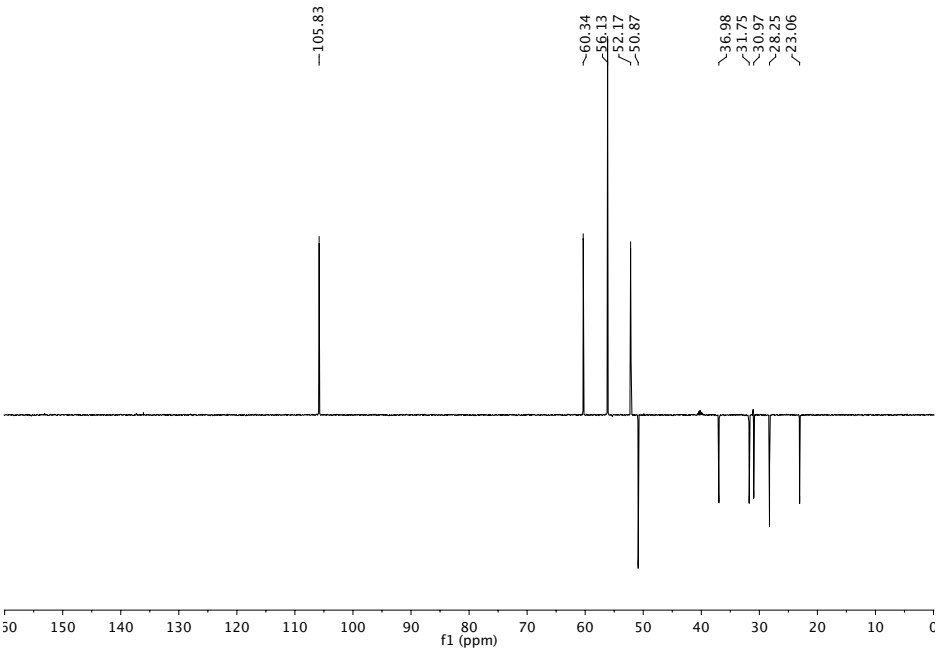


Figure 6.40: ^{13}C-NMR (dept-135) of compound **20** (101 MHz, DMSO-d6).

6.2.13 Compound 22

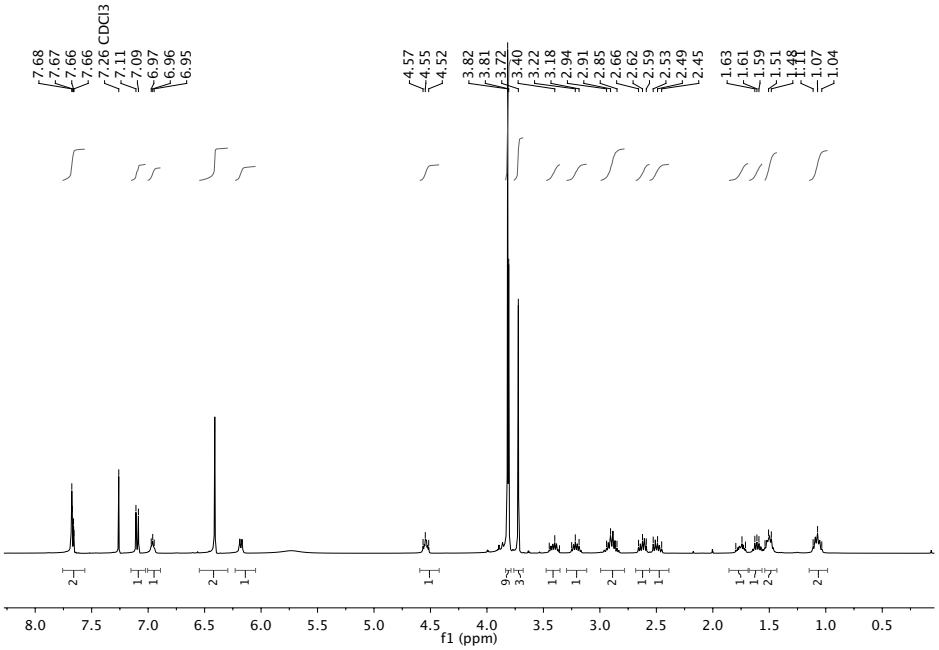


Figure 6.41: ^{1}H-NMR of compound **22** (400 MHz, Chloroform-d).

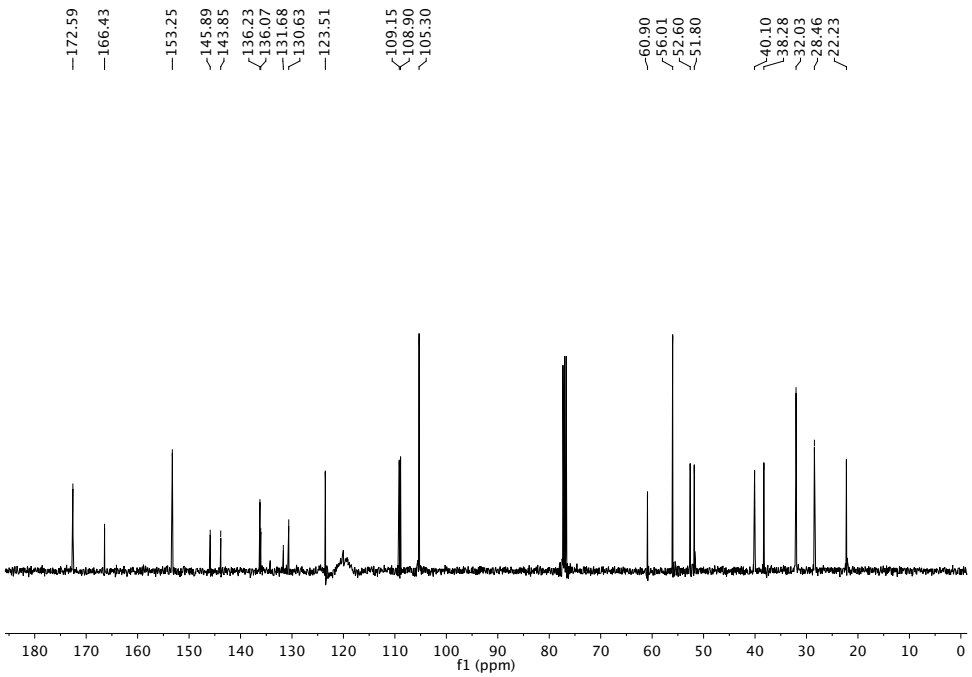


Figure 6.42: ^{13}C-NMR of compound **22** (101 MHz, Chloroform-d).

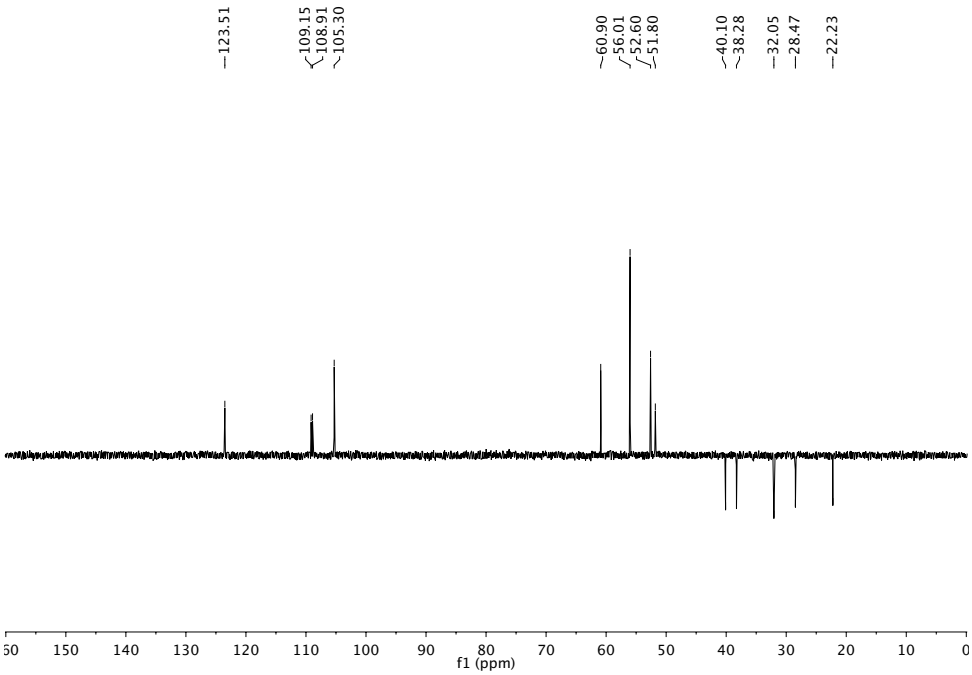


Figure 6.43: ^{13}C-NMR (dept-135) of compound **22** (101 MHz, Chloroform-d).

6.2.14 Compound 23

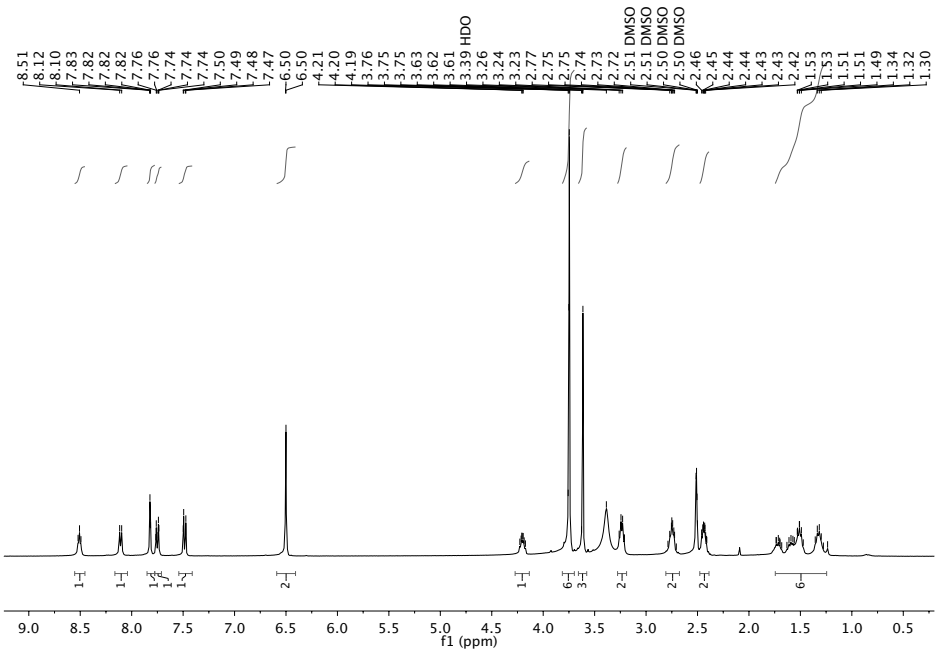


Figure 6.44: ^{1}H-NMR of compound **23** (400 MHz, Chloroform-d).

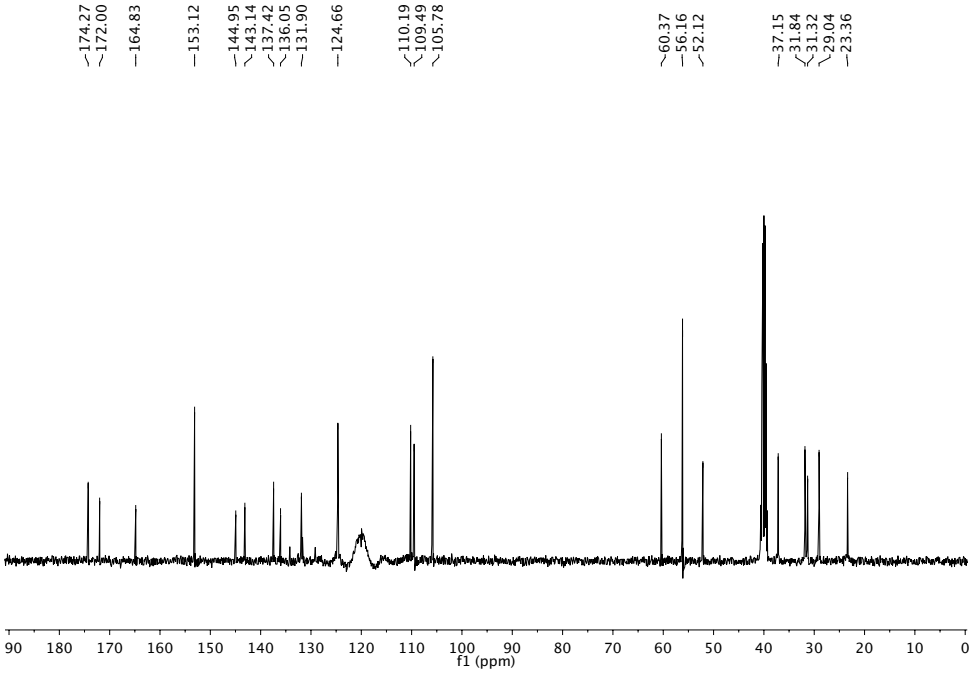


Figure 6.45: ^{13}C-NMR of compound **23** (101 MHz, Chloroform-d).

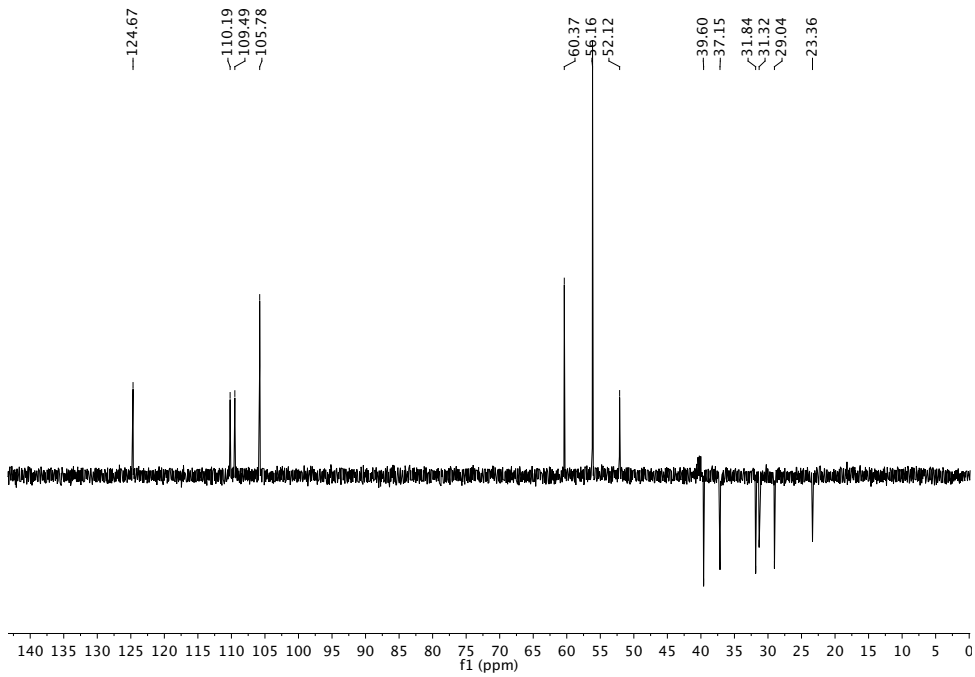


Figure 6.46: ^{13}C-NMR (dept-135) of compound **23** (101 MHz, Chloroform-d).

6.2.15 Compound 28

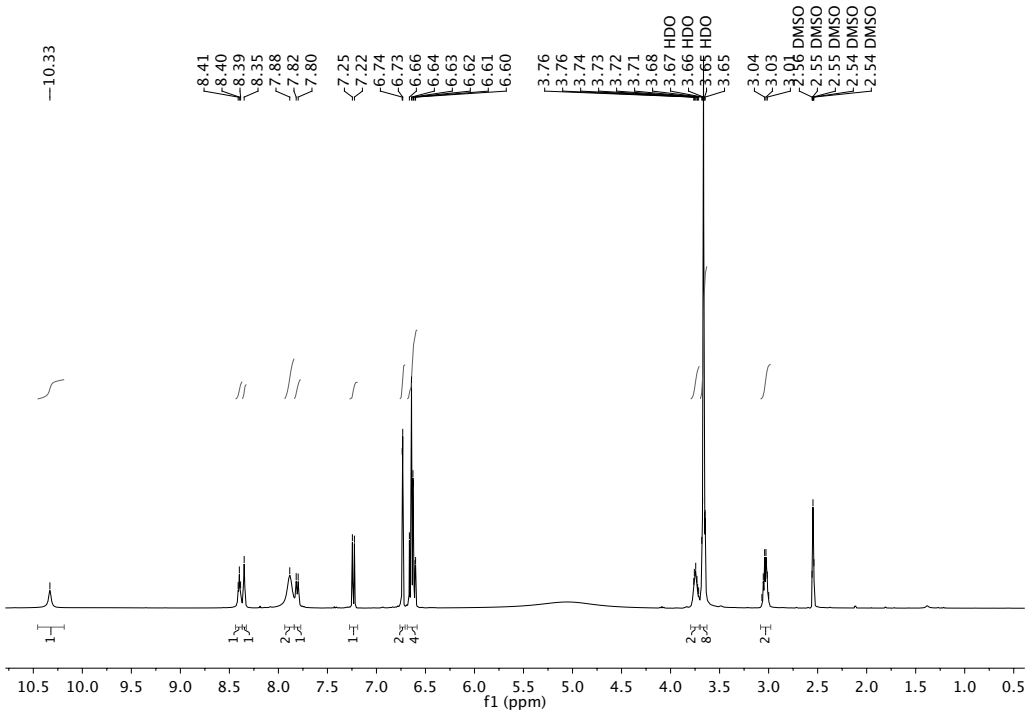


Figure 6.47: ^{1}H-NMR of compound **28** (400 MHz, DMSO-d6).

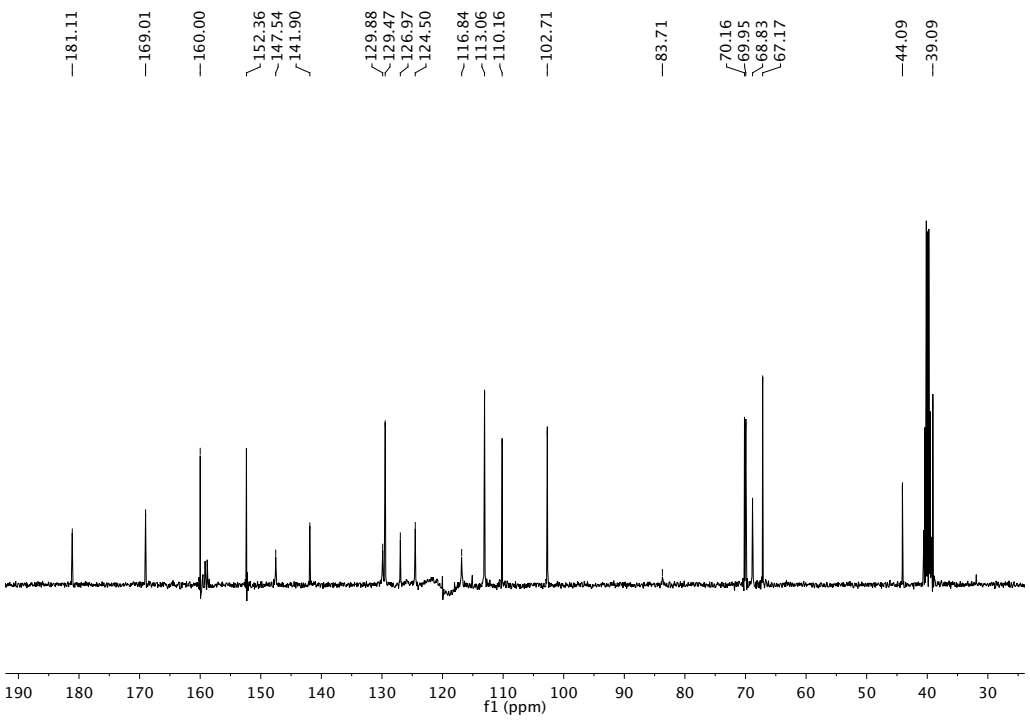


Figure 6.48: ^{13}C-NMR of compound **28** (101 MHz, DMSO-d6).

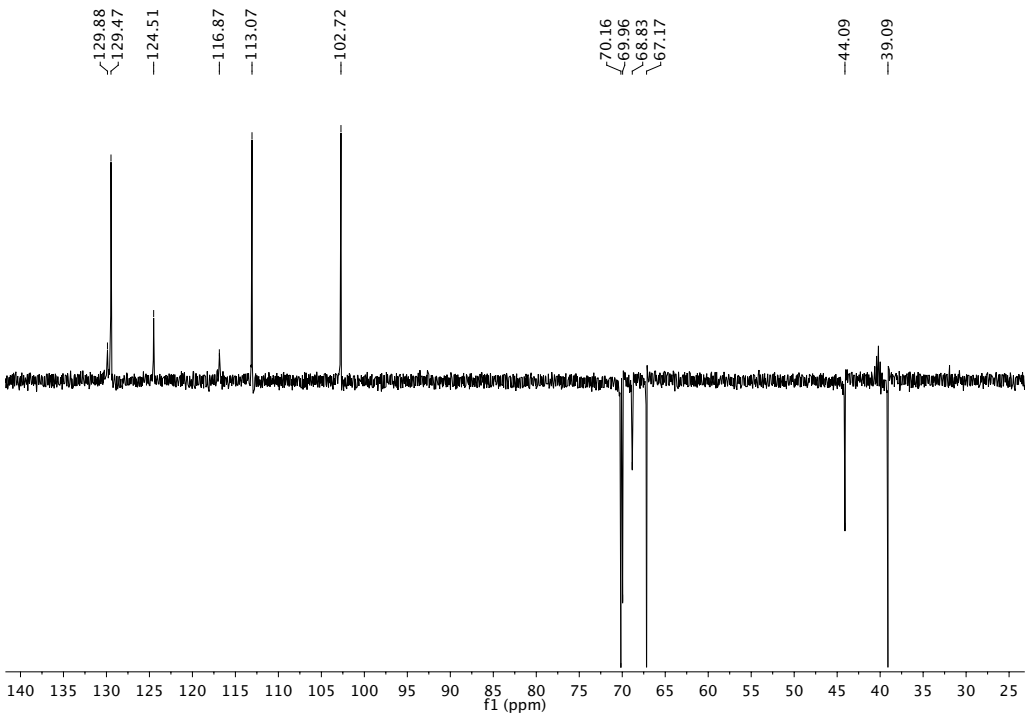


Figure 6.49: ^{13}C-NMR (dept-135) of compound **28** (101 MHz, DMSO-d6).

6.2.16 Compound 29

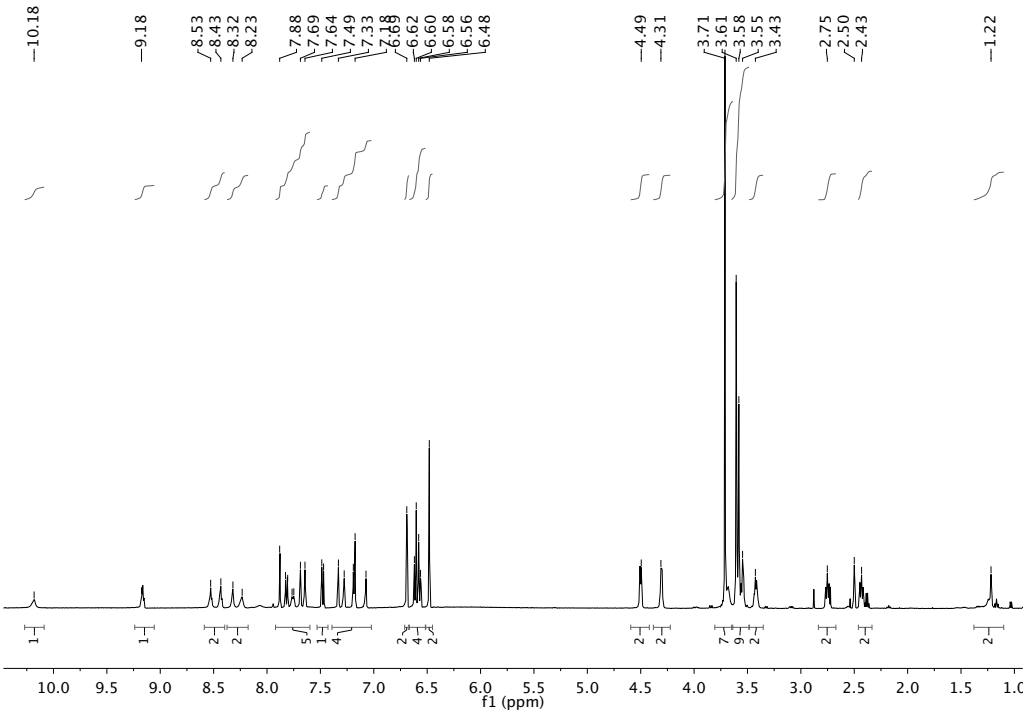


Figure 6.50: ^{1}H-NMR of compound **29** (500 MHz, DMSO-d6).

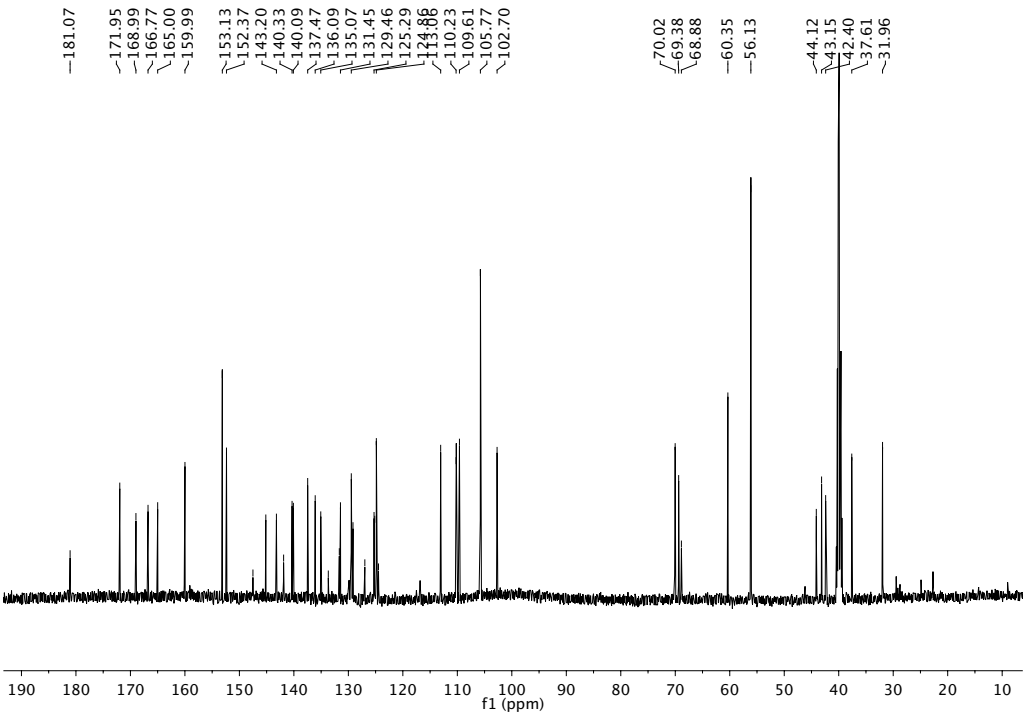


Figure 6.51: ^{13}C-NMR of compound **29** (126 MHz, DMSO-d6).

6.2.17 Compound 30

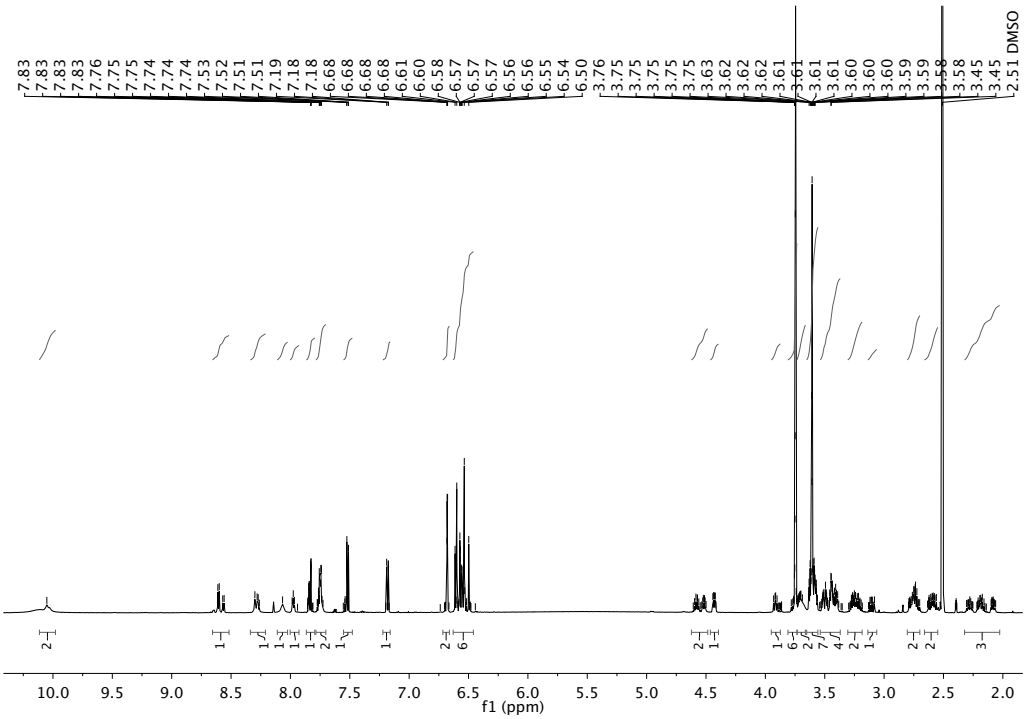


Figure 6.52: ^{1}H-NMR of compound **30** (600 MHz, DMSO-d6).

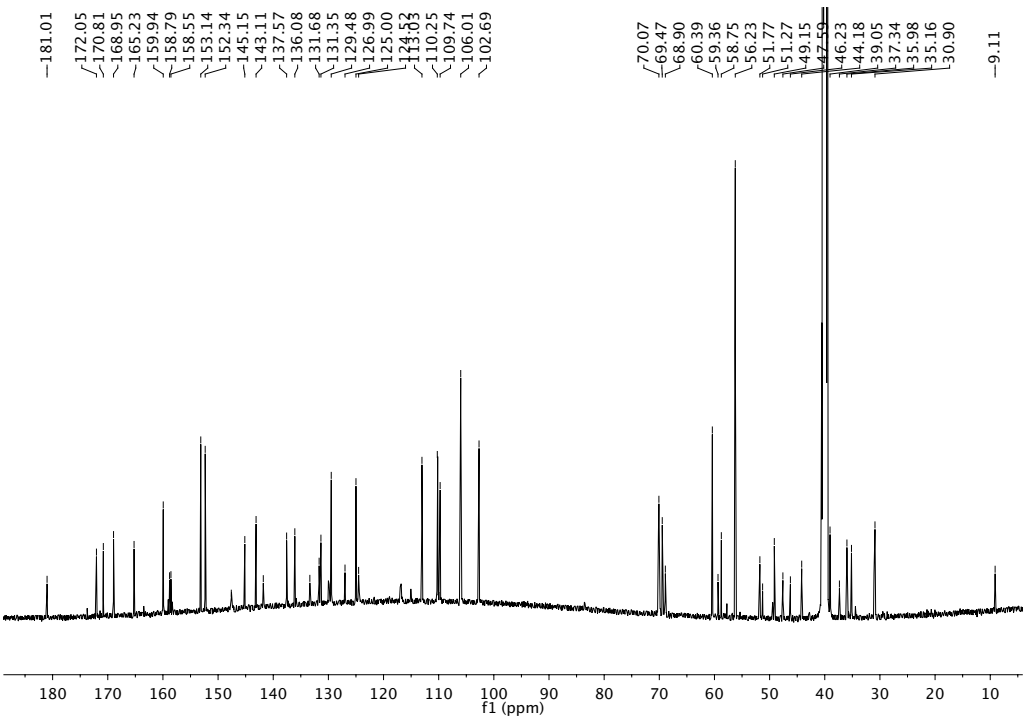


Figure 6.53: ^{13}C-NMR of compound **30** (151 MHz, DMSO-d6).

6.2.18 Compound 31

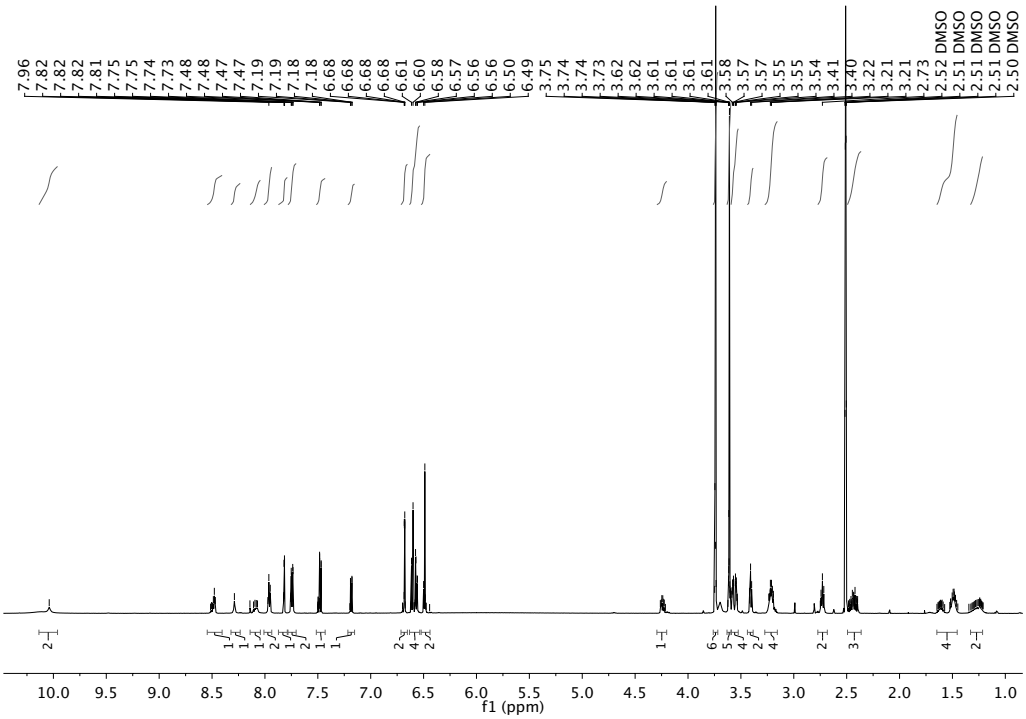


Figure 6.54: ^{1}H-NMR of compound **31** (600 MHz, DMSO-d6).

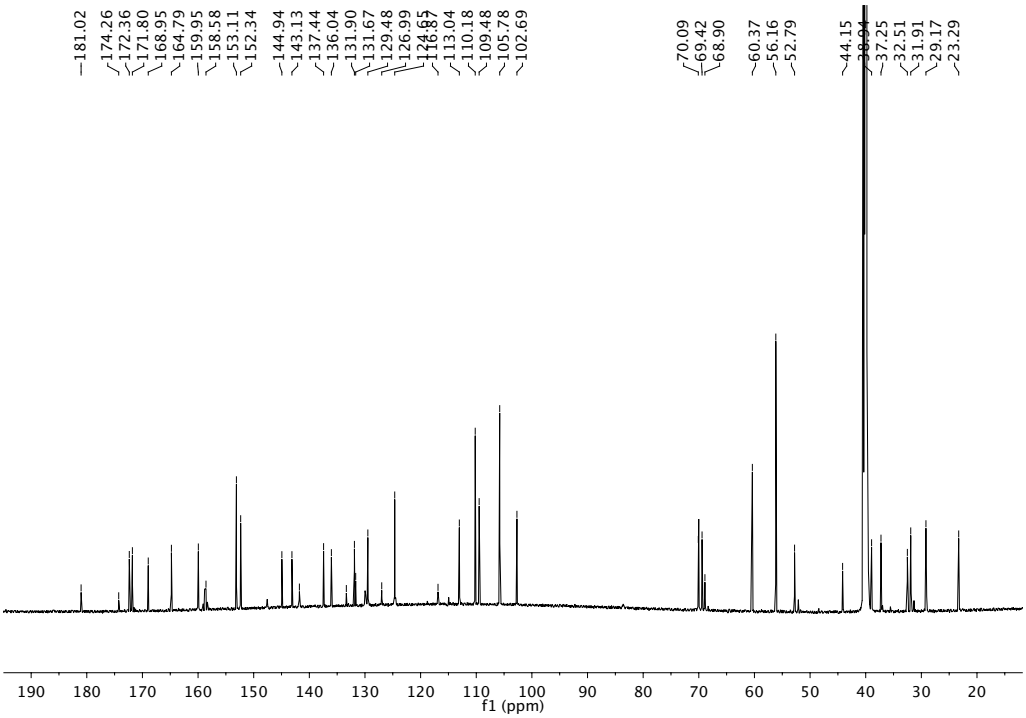


Figure 6.55: ^{13}C-NMR of compound **31** (151 MHz, DMSO-d6).

6.2.19 Compound 32

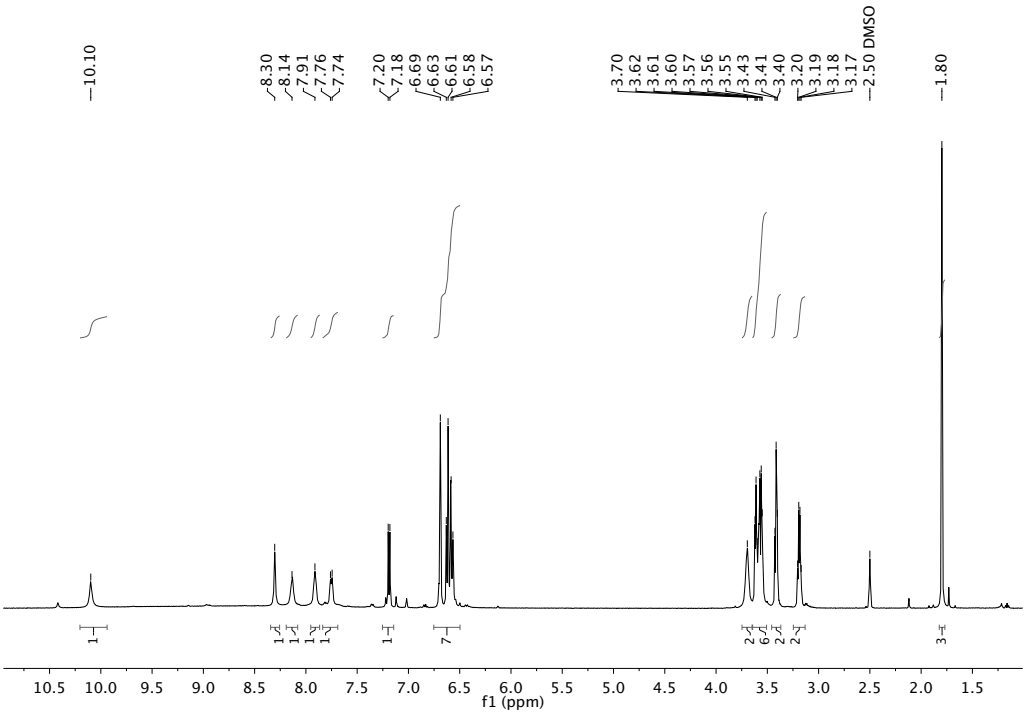


Figure 6.56: ^{1}H-NMR of compound **32** (500 MHz, DMSO-d6).

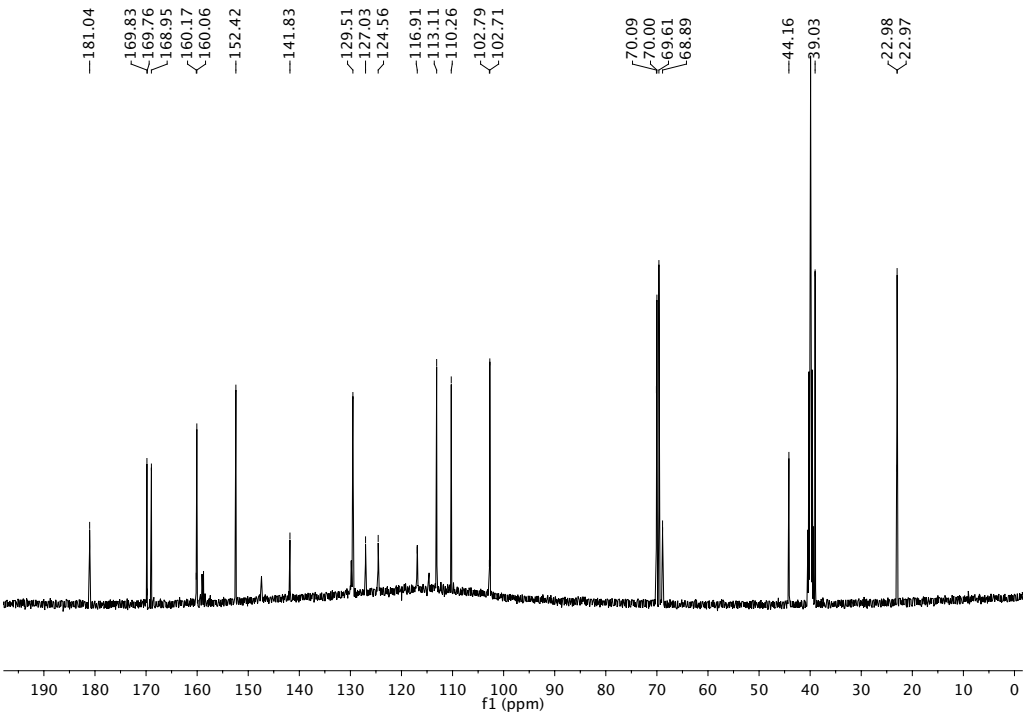


Figure 6.57: ^{13}C-NMR of compound **32** (126 MHz, DMSO-d6).

6.2.20 Compound 35

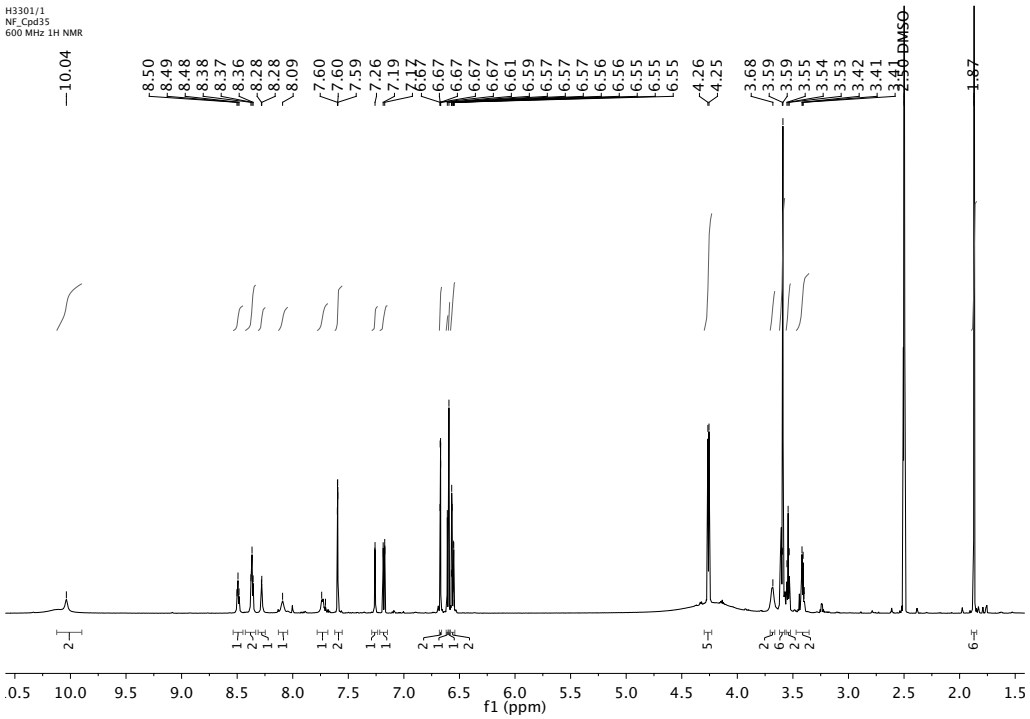


Figure 6.58: ^{1}H-NMR of compound **35** (600 MHz, DMSO-d6).

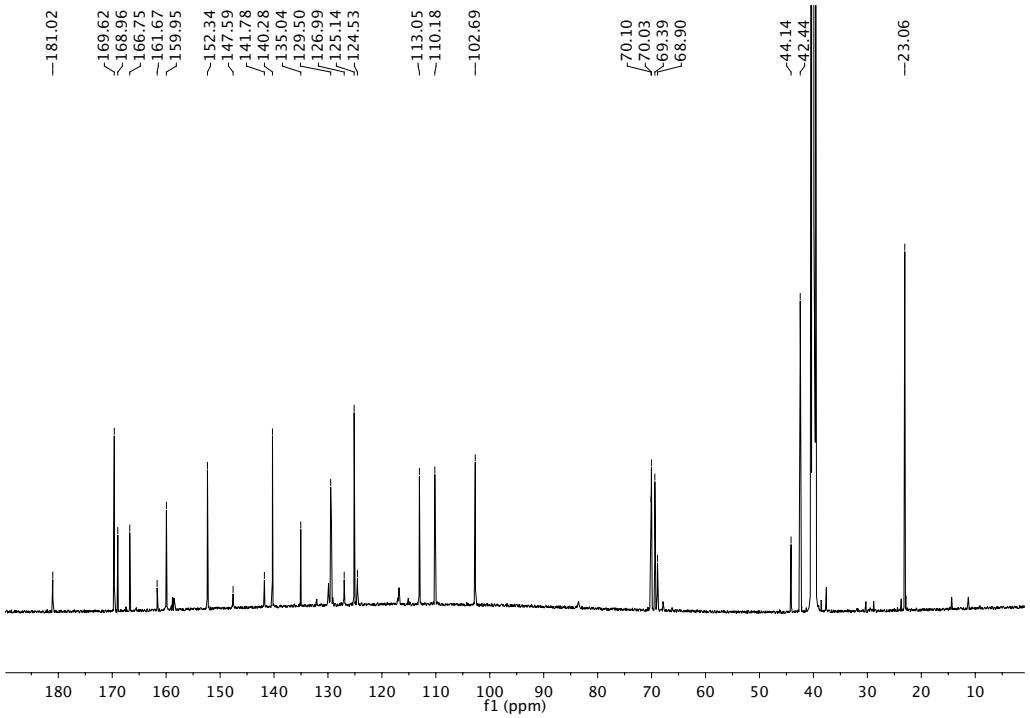


Figure 6.59: ^{13}C-NMR of compound **35** (151 MHz, DMSO-d6).

6.2.21 Compound 37

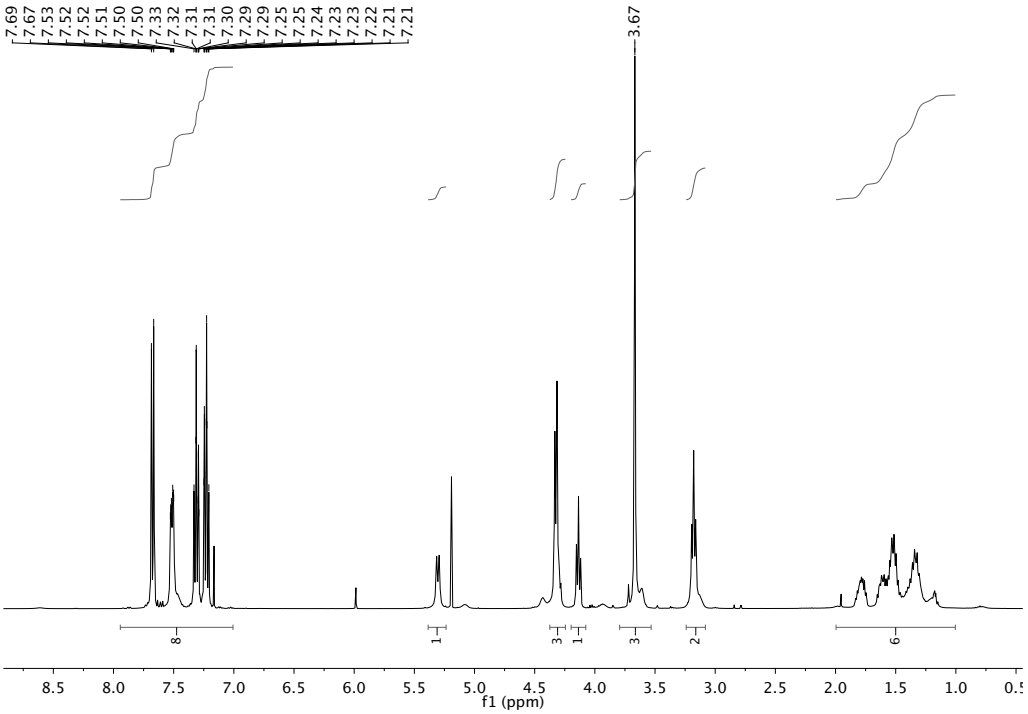


Figure 6.60: ^{1}H-NMR of compound **37** (400 MHz, Chloroform-d).

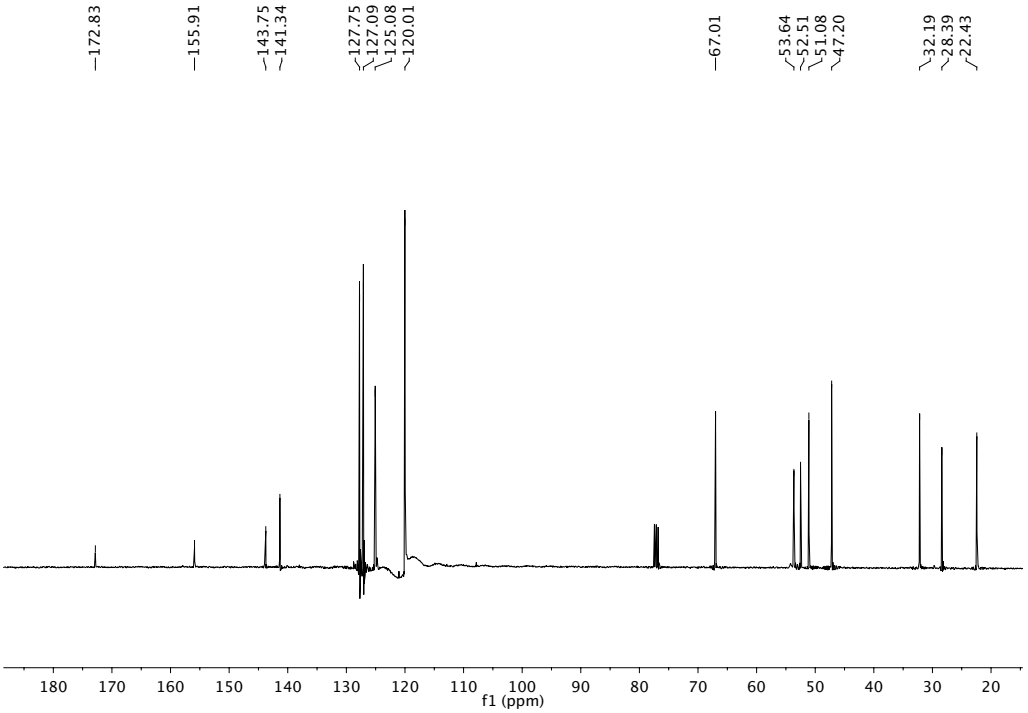


Figure 6.61: ^{13}C-NMR of compound **37** (400 MHz, Chloroform-d).

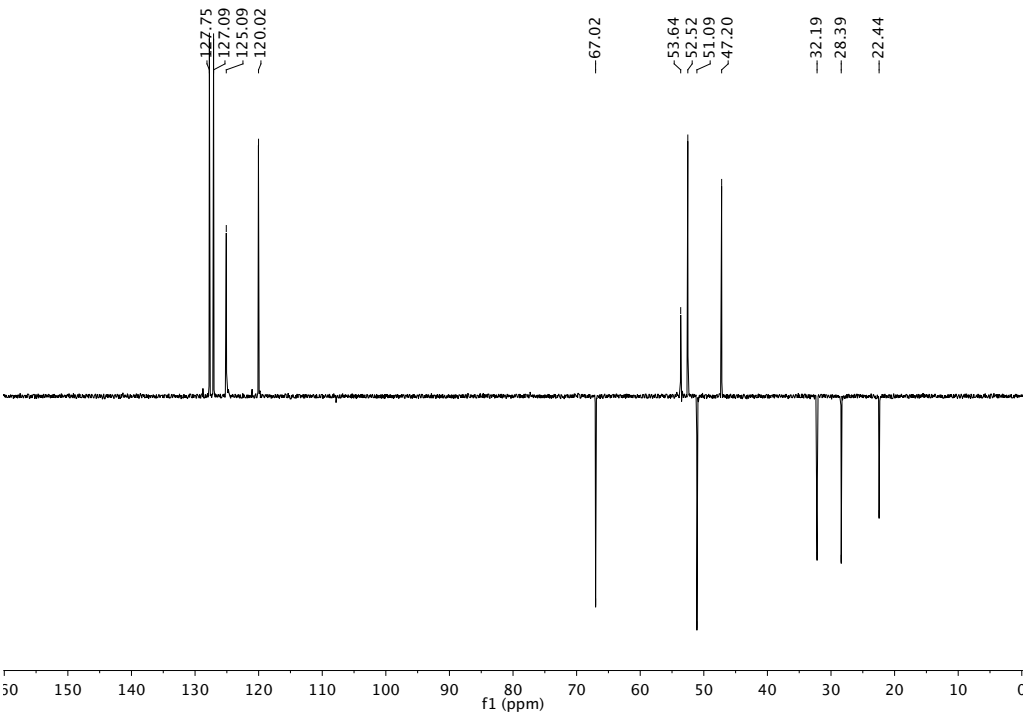


Figure 6.62: ^{13}C-NMR (dept-135) of compound **37** (400 MHz, Chloroform-d).

6.2.22 Compound 40

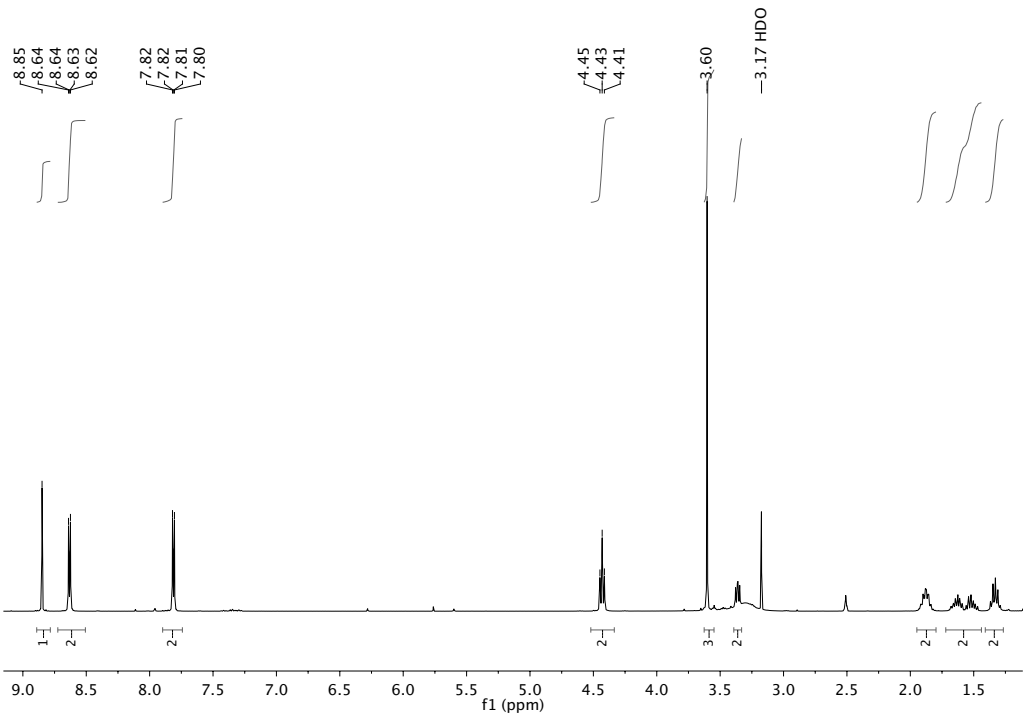


Figure 6.63: ^{1}H-NMR of compound **40** (400 MHz, DMSO-d6).

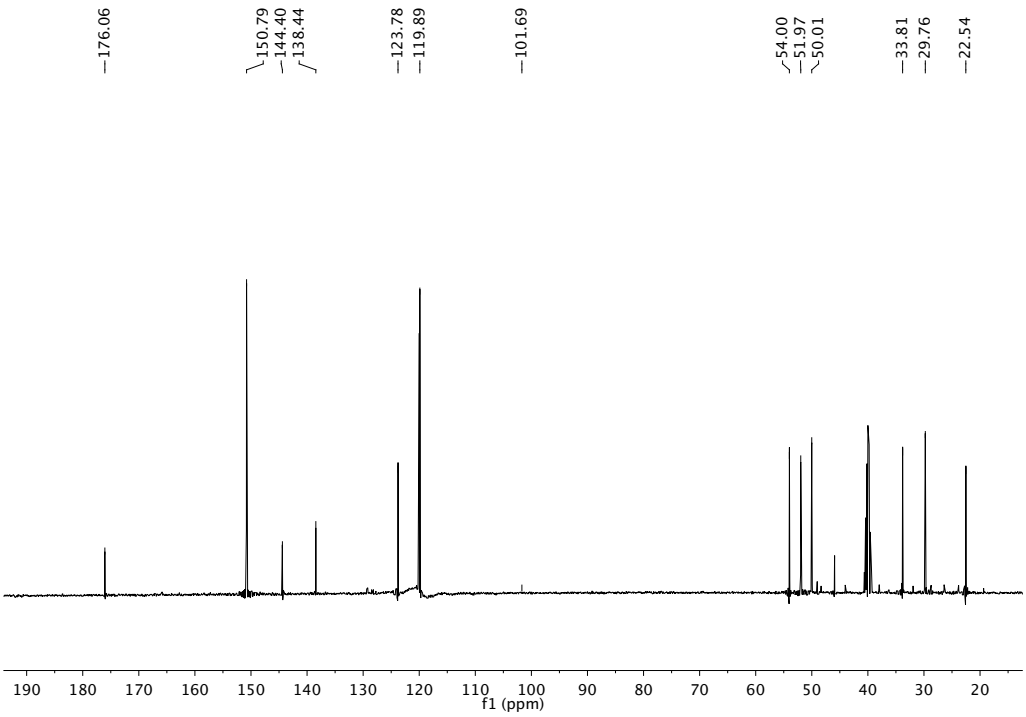


Figure 6.64: ^{13}C-NMR of compound **40** (400 MHz, DMSO-d6).

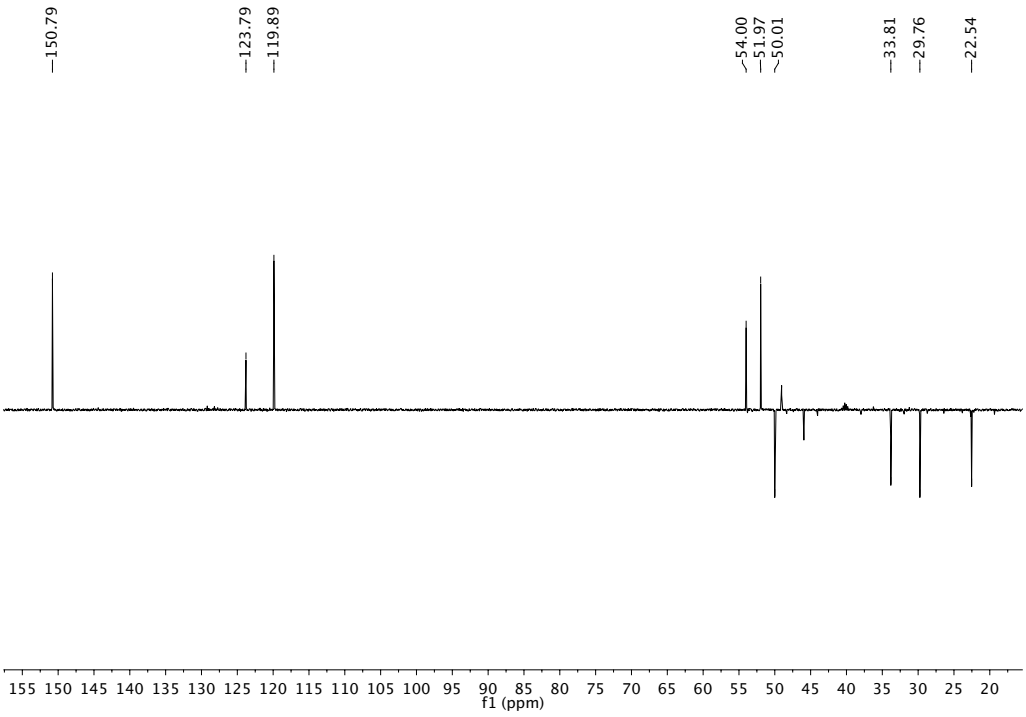


Figure 6.65: ^{13}C-NMR (dept-135) of compound **40** (400 MHz, DMSO-d6).

6.2.23 Compound 41

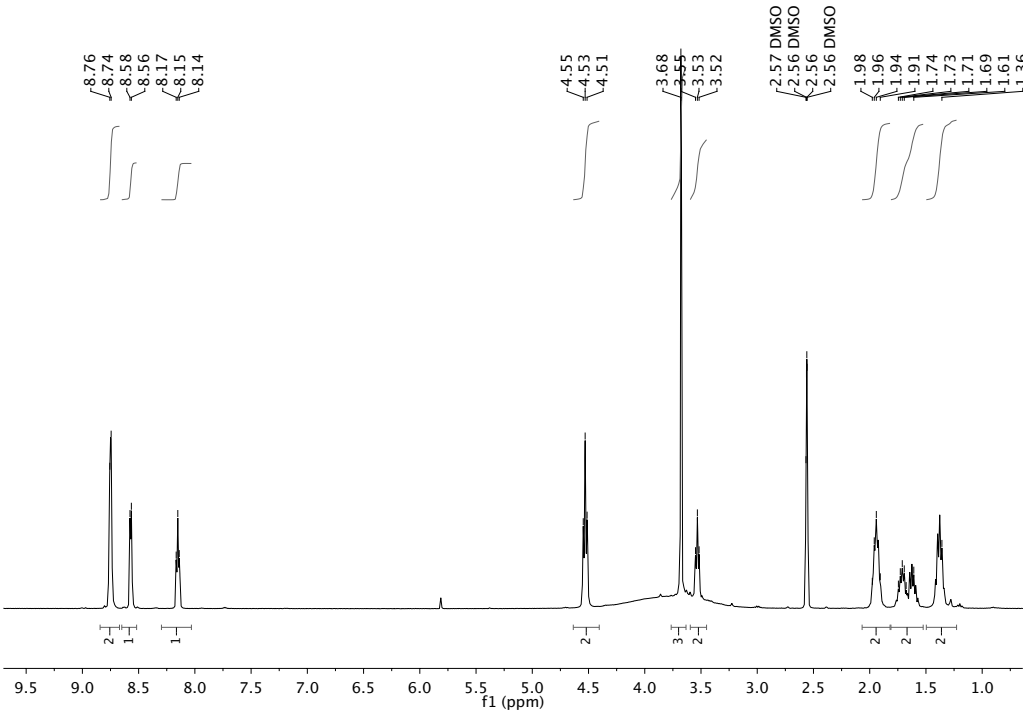


Figure 6.66: ^{1}H-NMR of compound **41** (400 MHz, DMSO-d6).

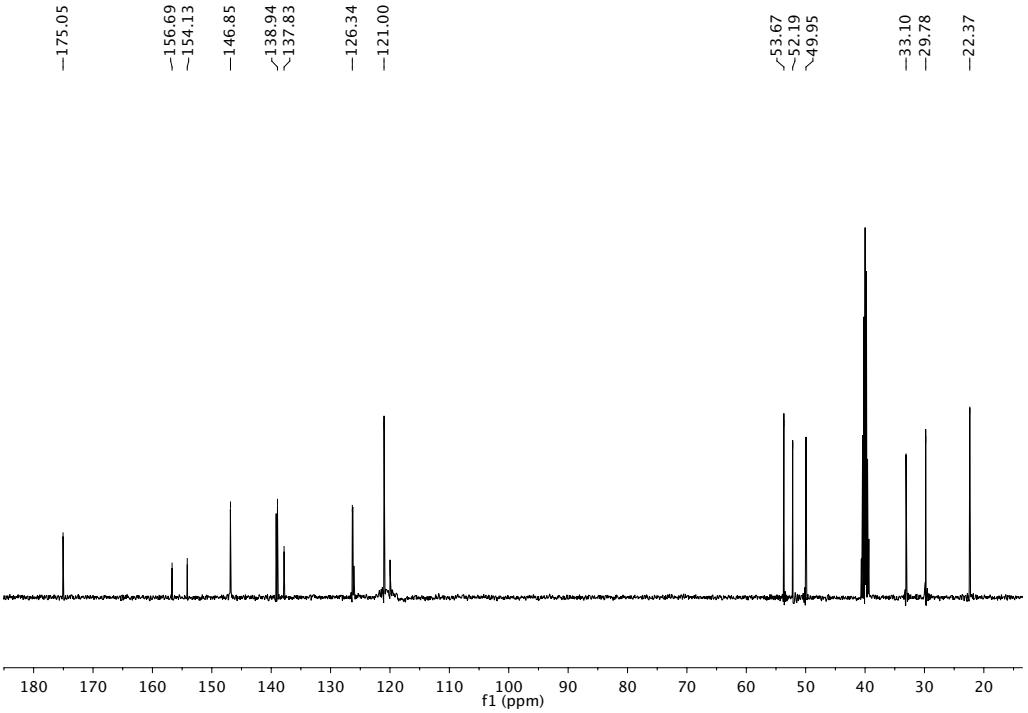


Figure 6.67: ^{13}C-NMR of compound **41** (400 MHz, DMSO-d6).

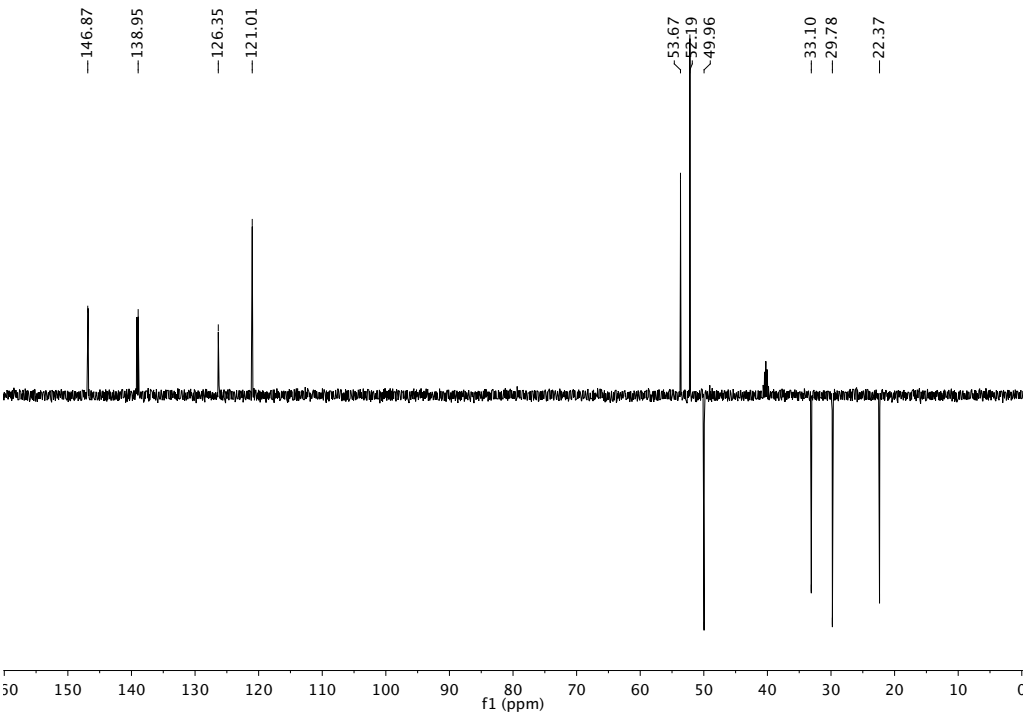


Figure 6.68: ^{13}C-NMR (dept-135) of compound **41** (400 MHz, DMSO-d6).

6.2.24 Compound 44

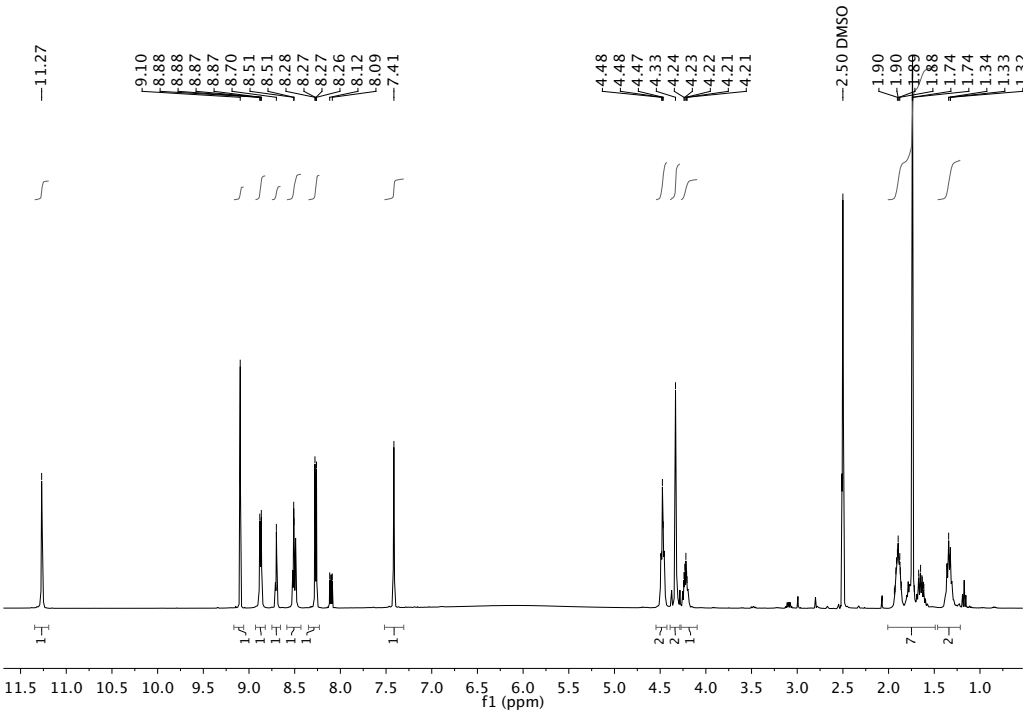


Figure 6.69: ^{1}H-NMR of compound **44** (400 MHz, DMSO-d6).

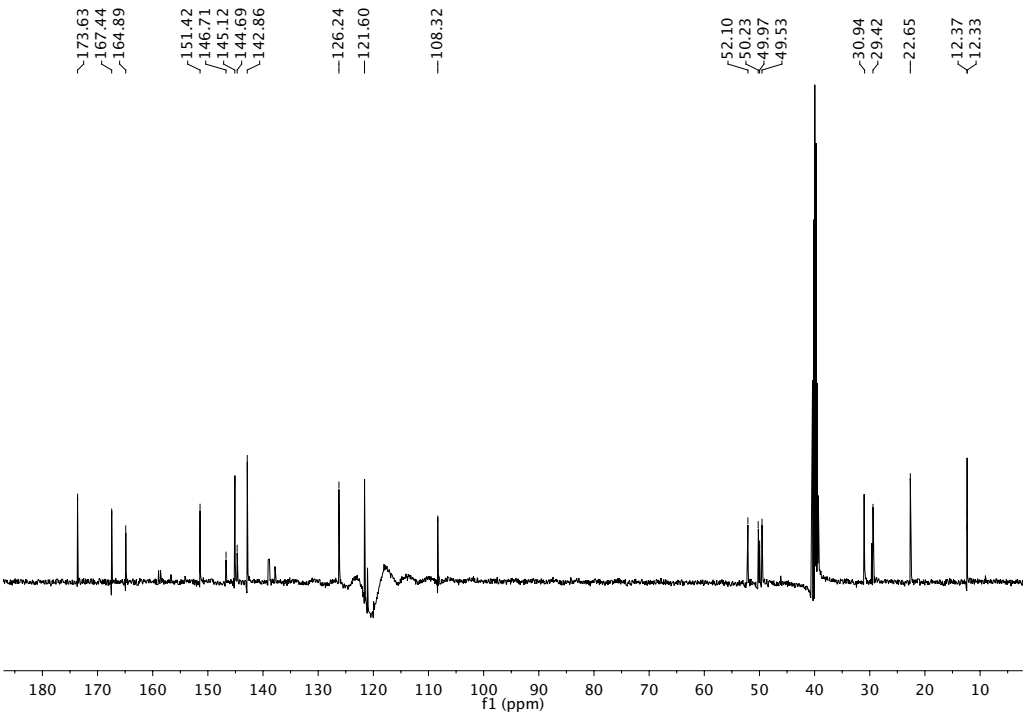


Figure 6.70: ^{13}C-NMR of compound **44** (400 MHz, DMSO-d6).

6.2.25 Compound 45

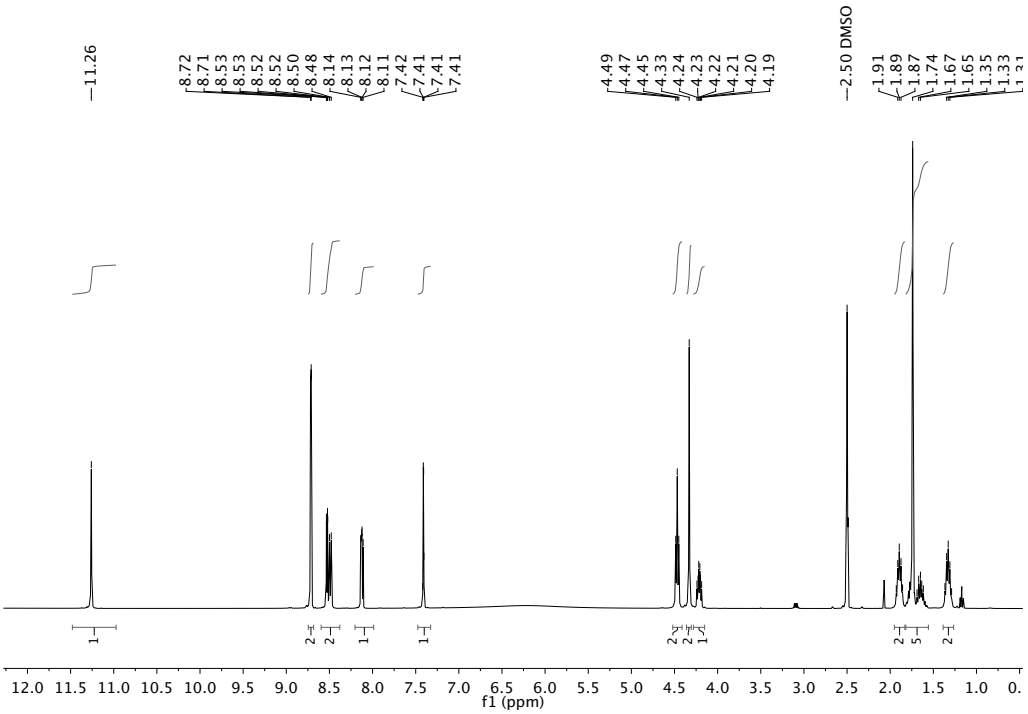


Figure 6.71: ^{1}H-NMR of compound **45** (400 MHz, DMSO-d6).

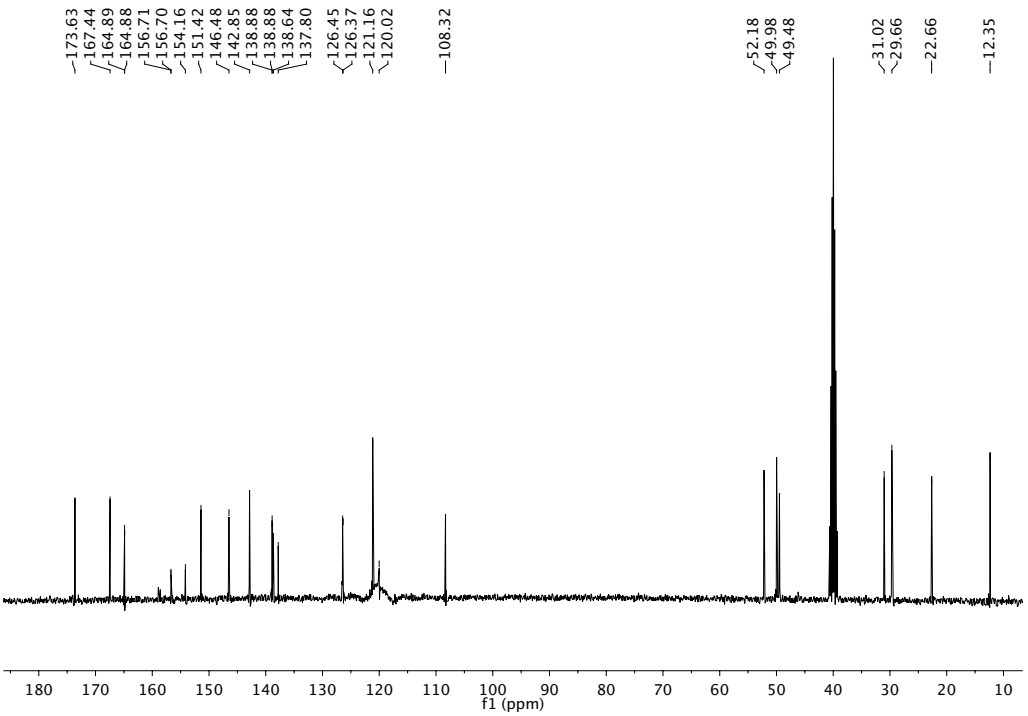


Figure 6.72: ^{13}C-NMR of compound **45** (400 MHz, DMSO-d6).

6.2.26 Compound 46

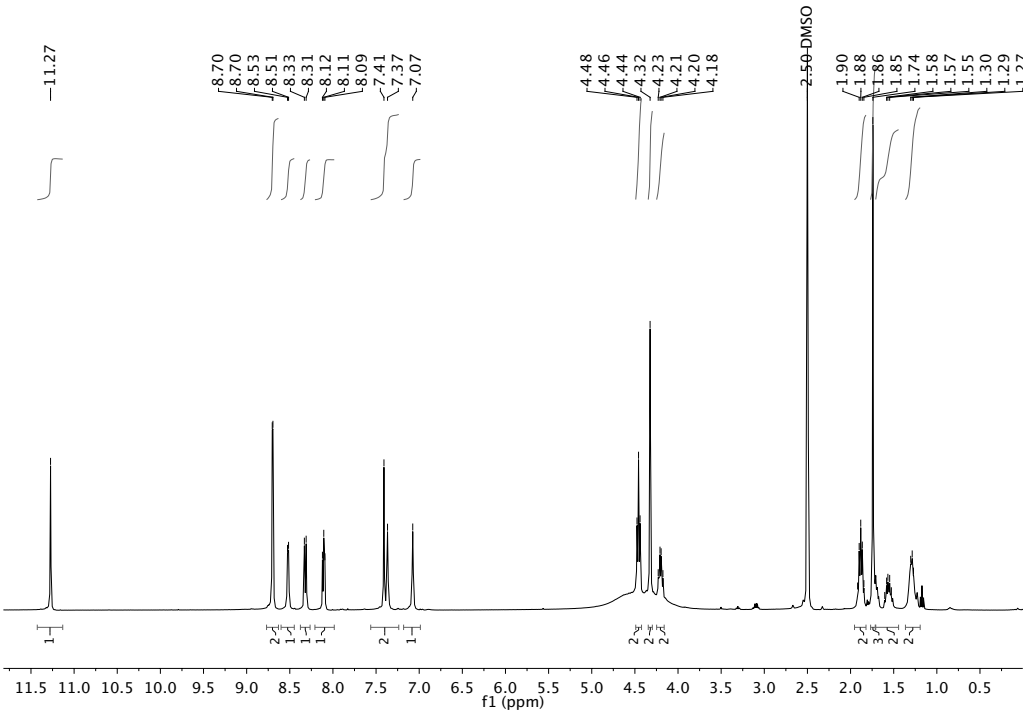


Figure 6.73: ^{1}H-NMR of compound **46** (400 MHz, DMSO-d6).

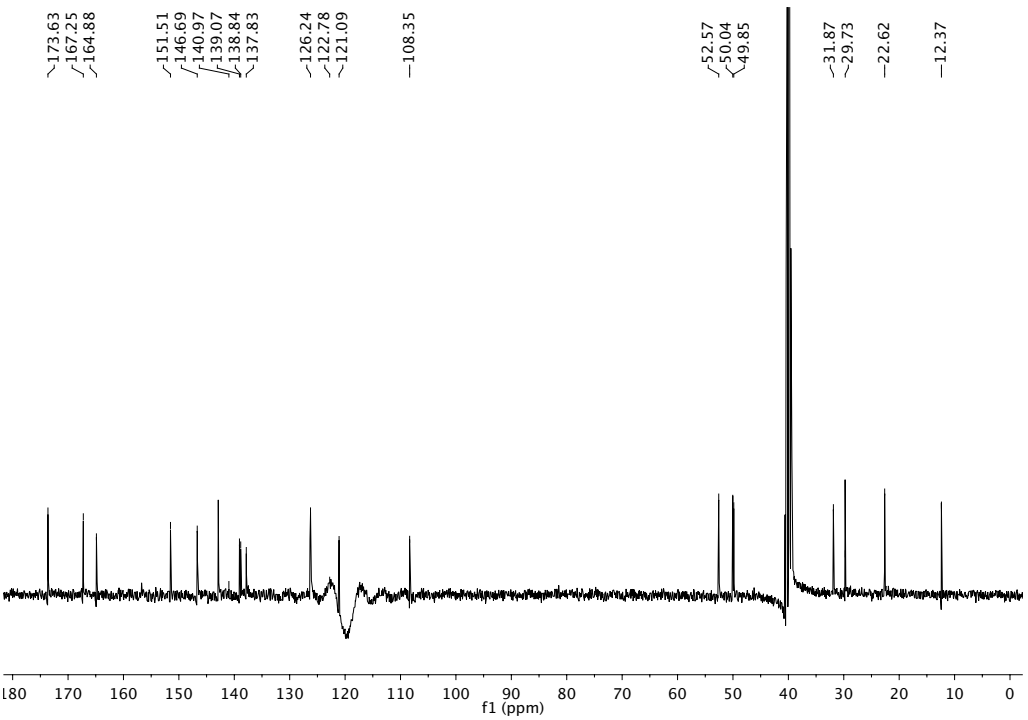


Figure 6.74: ^{13}C-NMR of compound **46** (400 MHz, DMSO-d6).

6.2.27 Compound 47

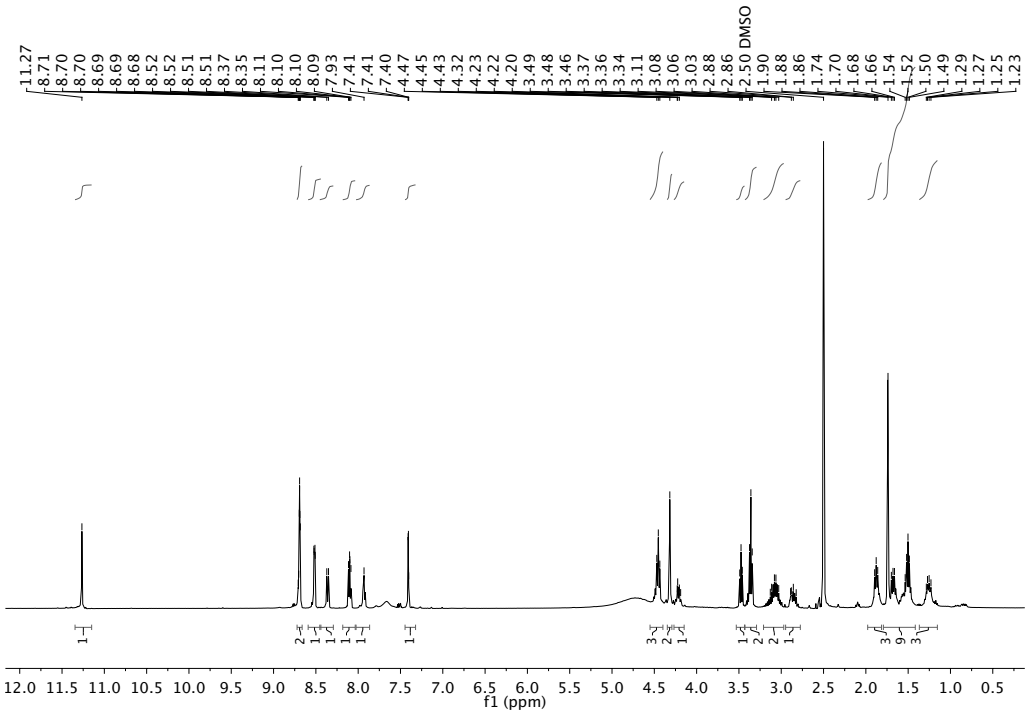


Figure 6.75: ^{1}H-NMR of compound **47** (400 MHz, DMSO-d6).

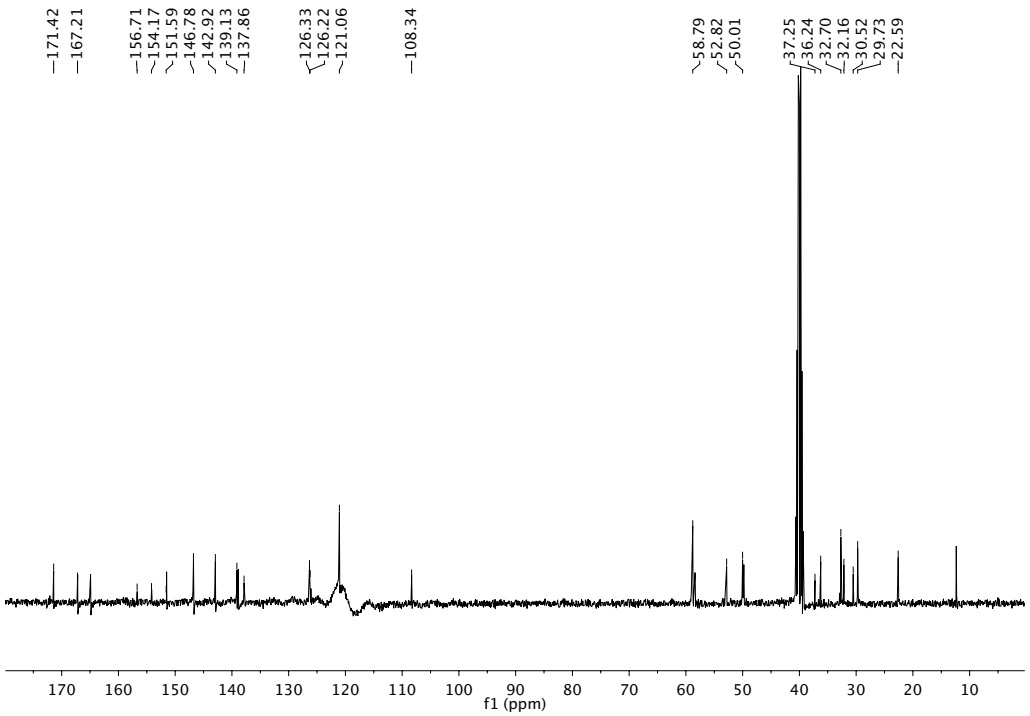


Figure 6.76: ^{13}C-NMR of compound **47** (400 MHz, DMSO-d6).

6.2.28 Compound 49

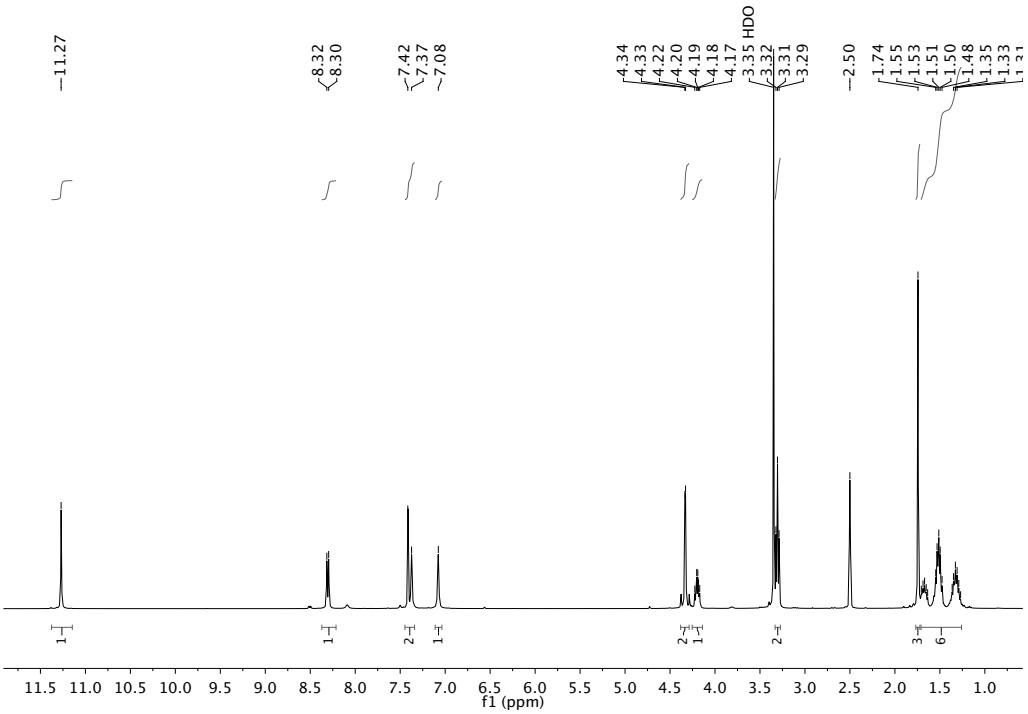


Figure 6.77: ^{1}H-NMR of compound **49** (400 MHz, DMSO-d6).

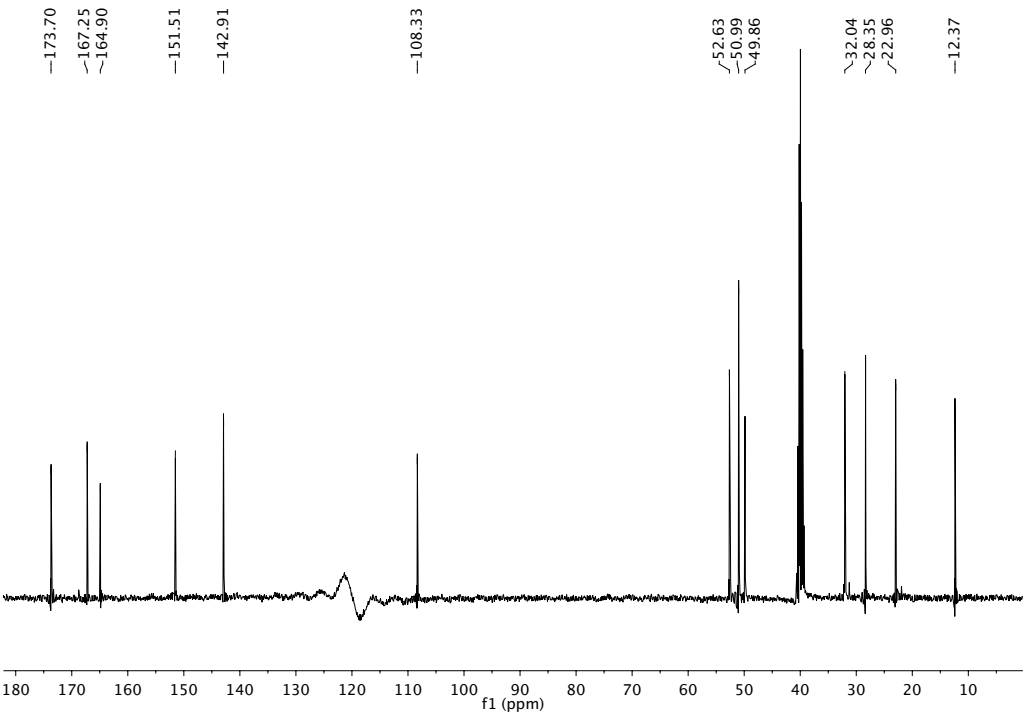


Figure 6.78: ^{13}C-NMR of compound **49** (400 MHz, DMSO-d6).

6.2.29 Compound 51

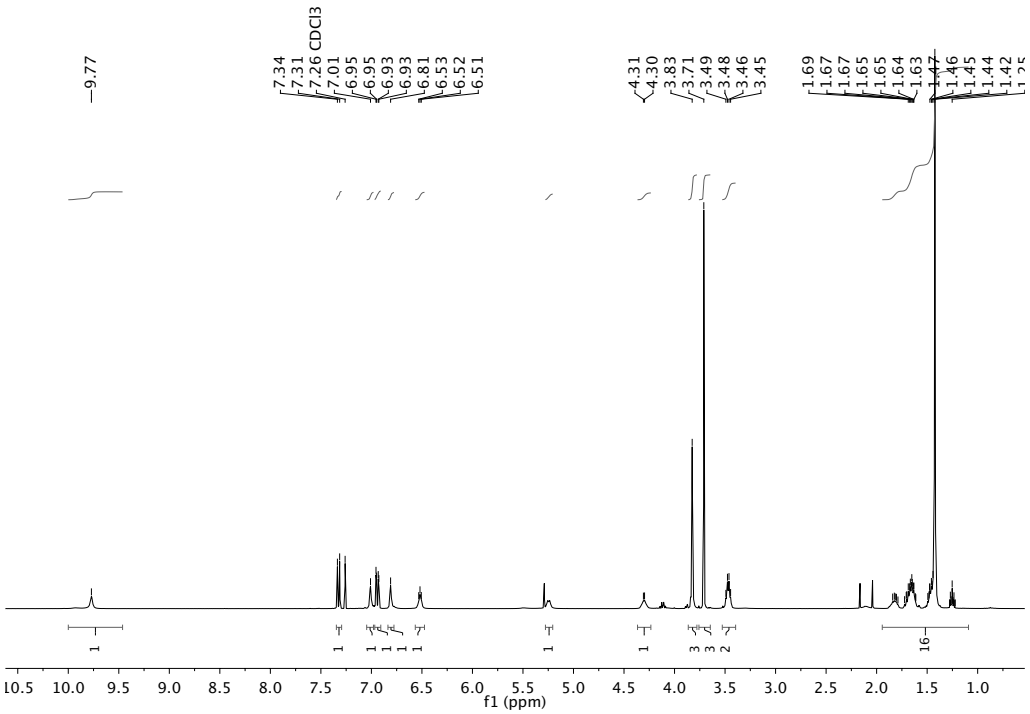


Figure 6.79: ^{1}H-NMR of compound **51** (400 MHz, DMSO-d6).

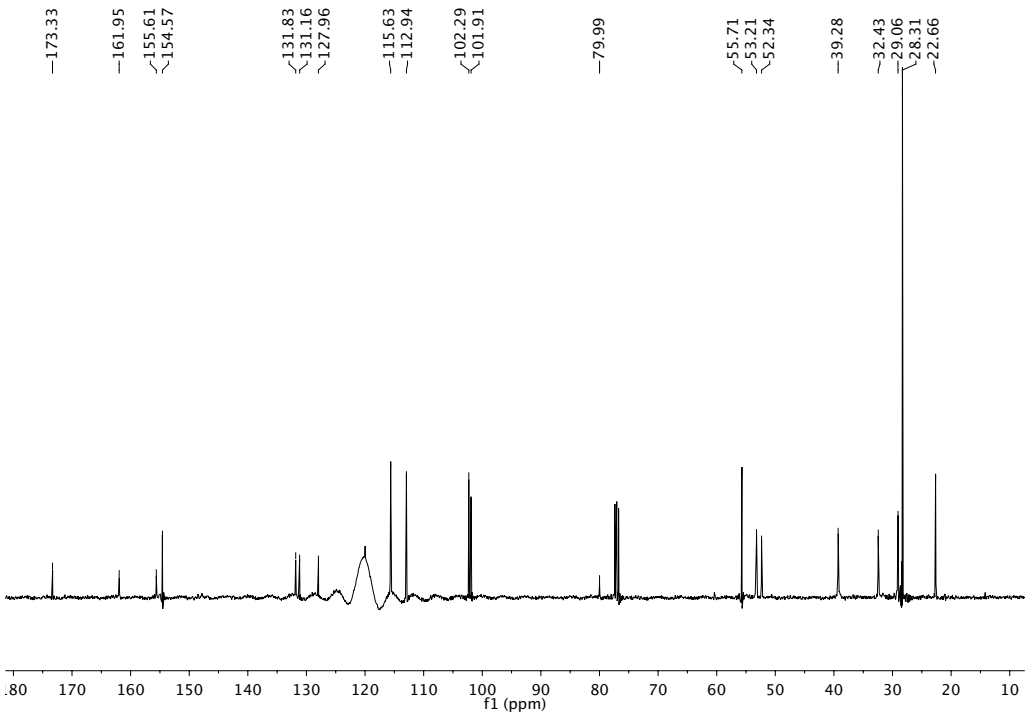


Figure 6.80: ^{13}C-NMR of compound **51** (400 MHz, DMSO-d6).

6.2.30 Compound 53

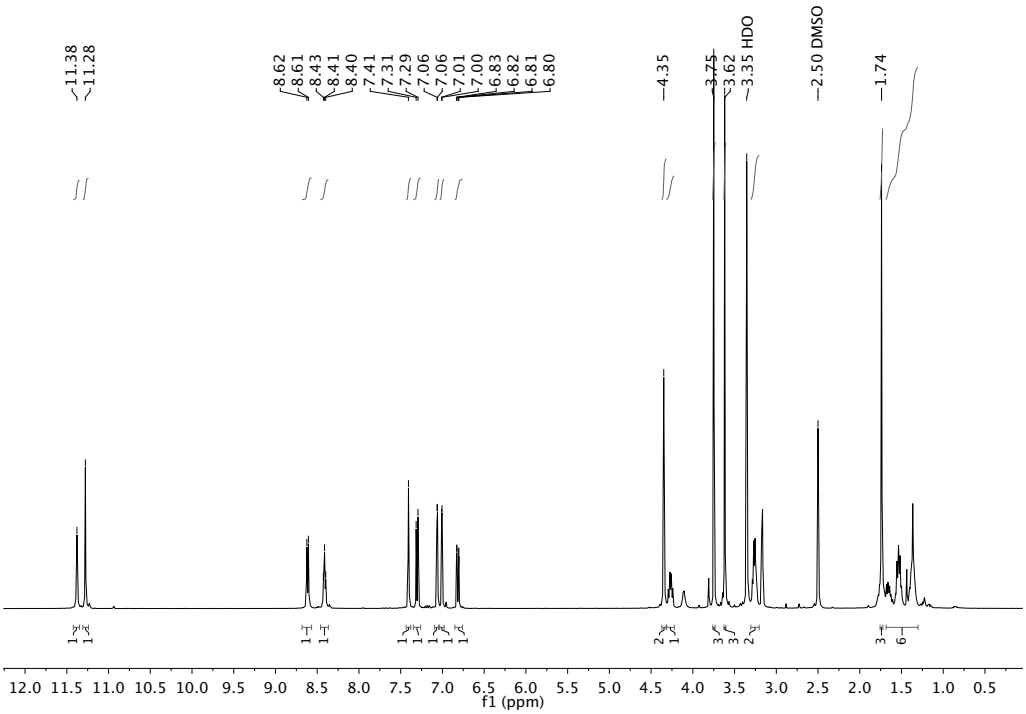


Figure 6.81: ^{1}H-NMR of compound **53** (400 MHz, DMSO-d6).

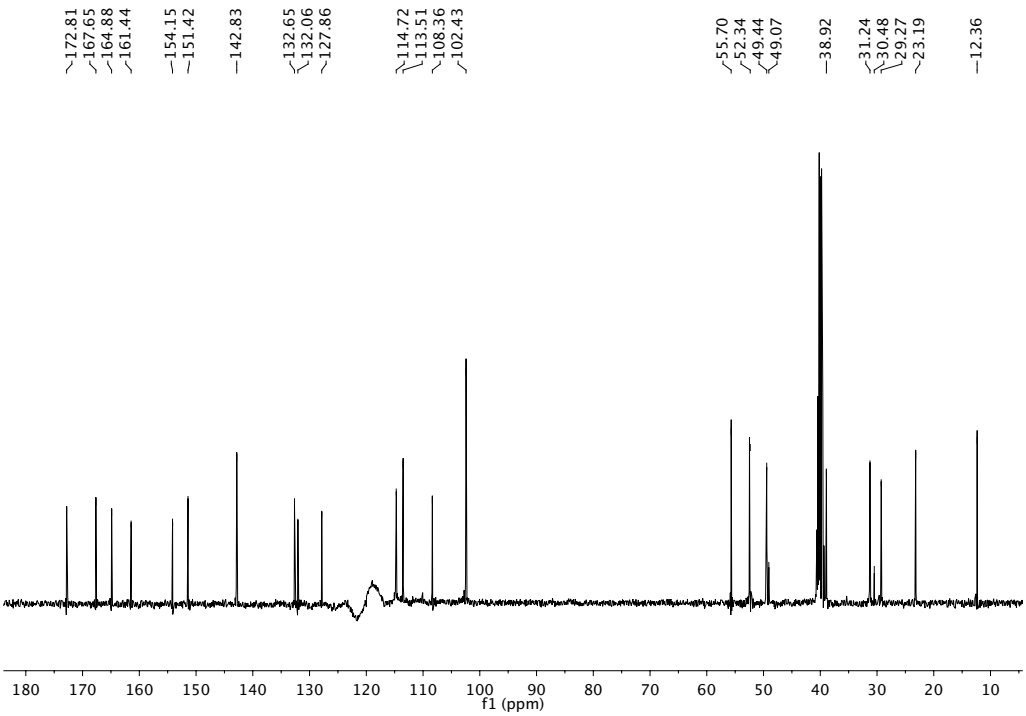


Figure 6.82: ^{13}C-NMR of compound **53** (400 MHz, DMSO-d6).

6.2.31 Compound 54

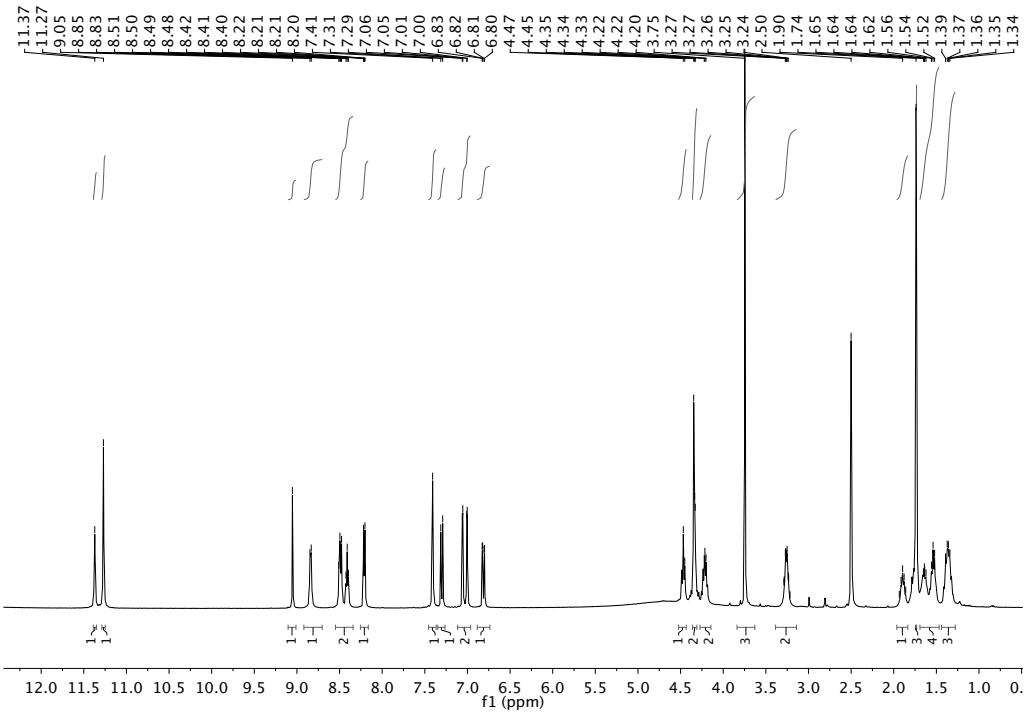


Figure 6.83: ^{1}H-NMR of compound **54** (400 MHz, DMSO-d6).

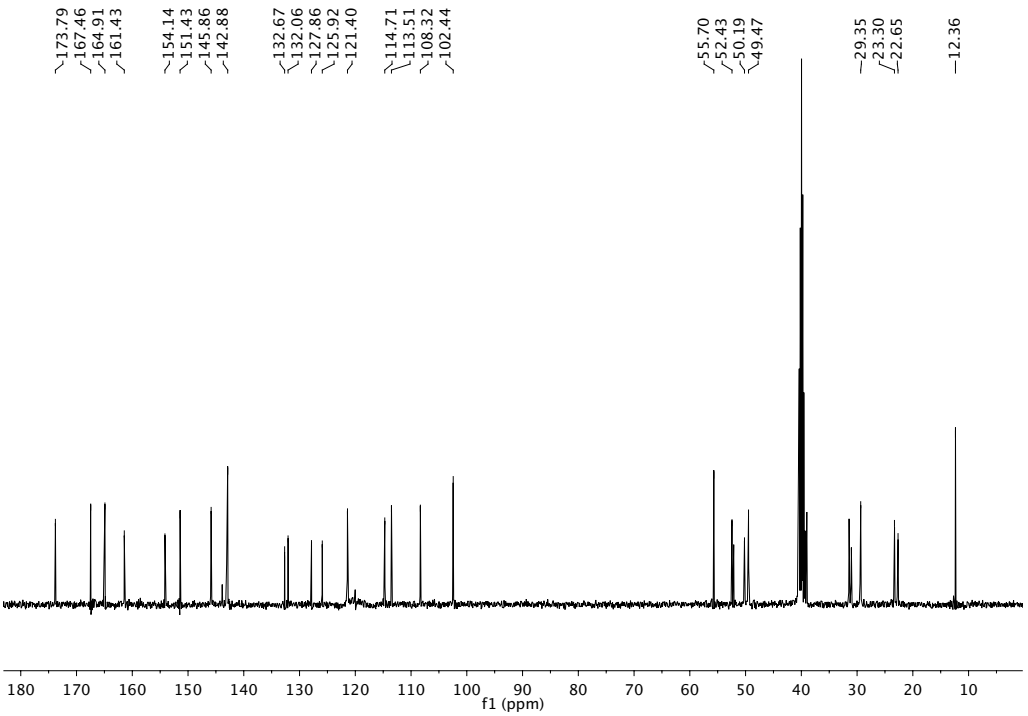


Figure 6.84: ^{13}C-NMR of compound **54** (400 MHz, DMSO-d6).

6.2.32 Compound 55

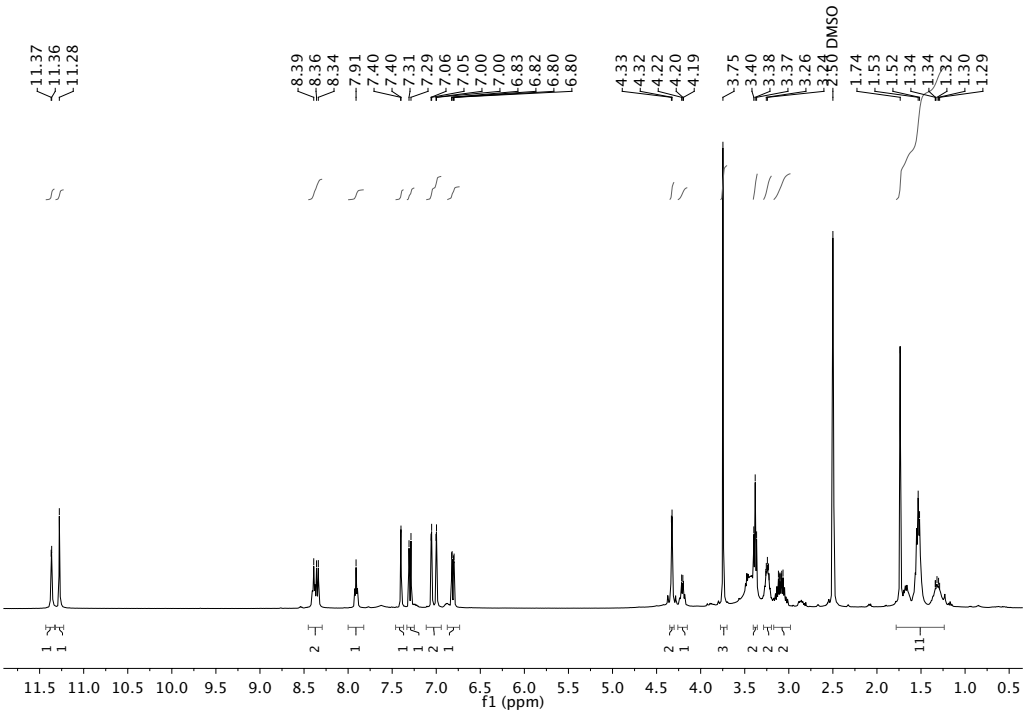


Figure 6.85: ^{1}H-NMR of compound **55** (400 MHz, DMSO-d6).

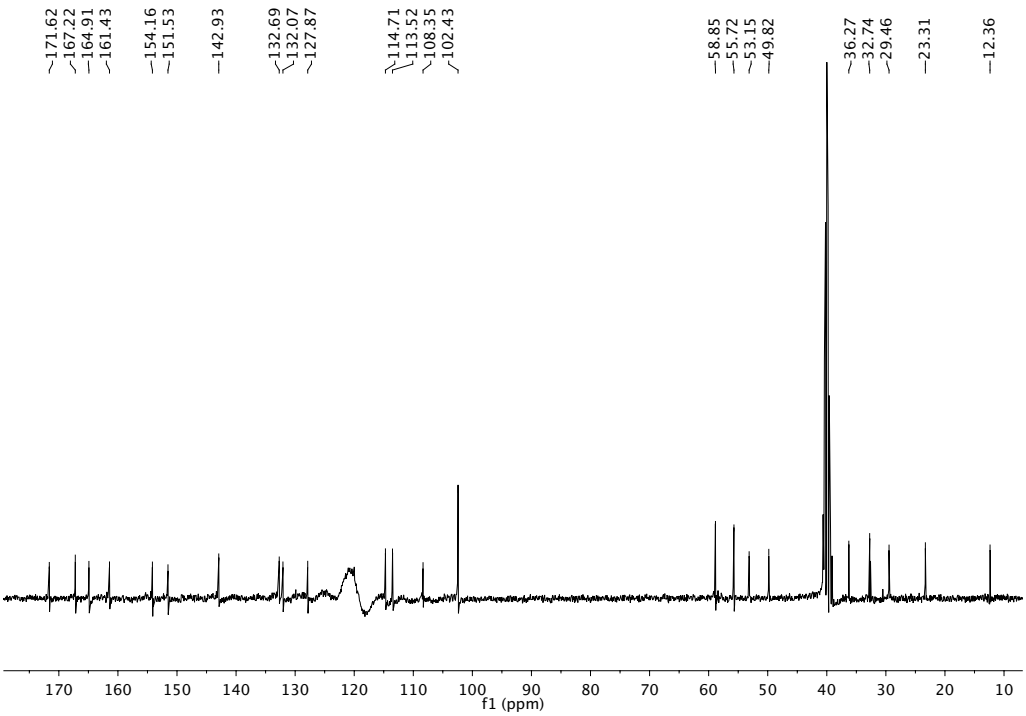


Figure 6.86: ^{13}C-NMR of compound **55** (400 MHz, DMSO-d6).

6.2.33 Compound 59

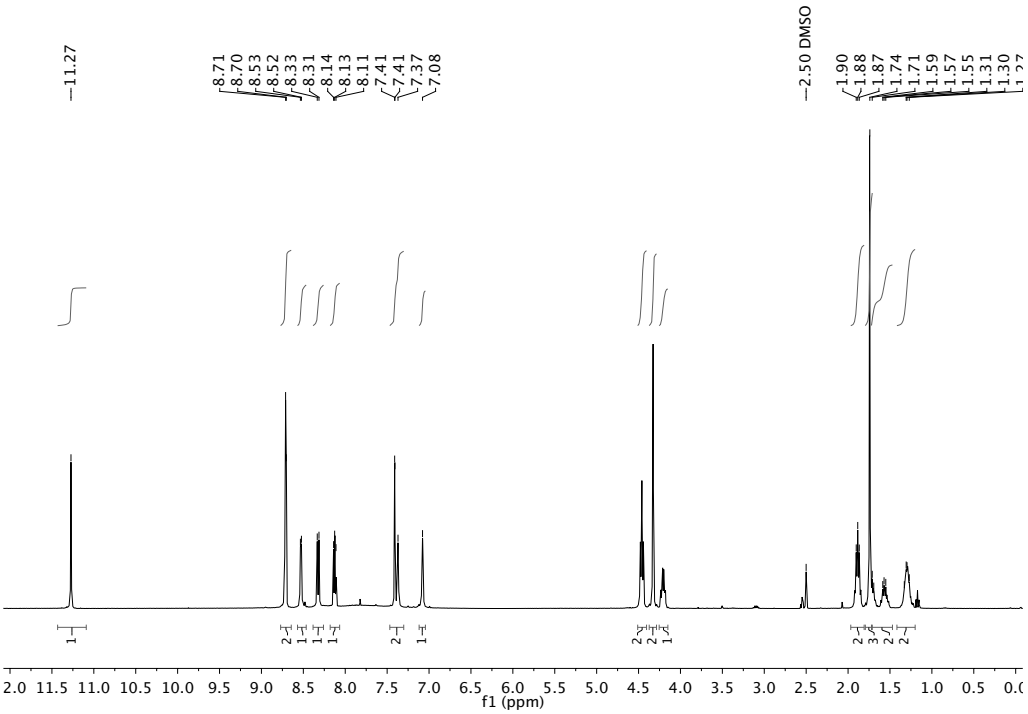


Figure 6.87: ^{1}H-NMR of compound **59** (400 MHz, DMSO-d6).

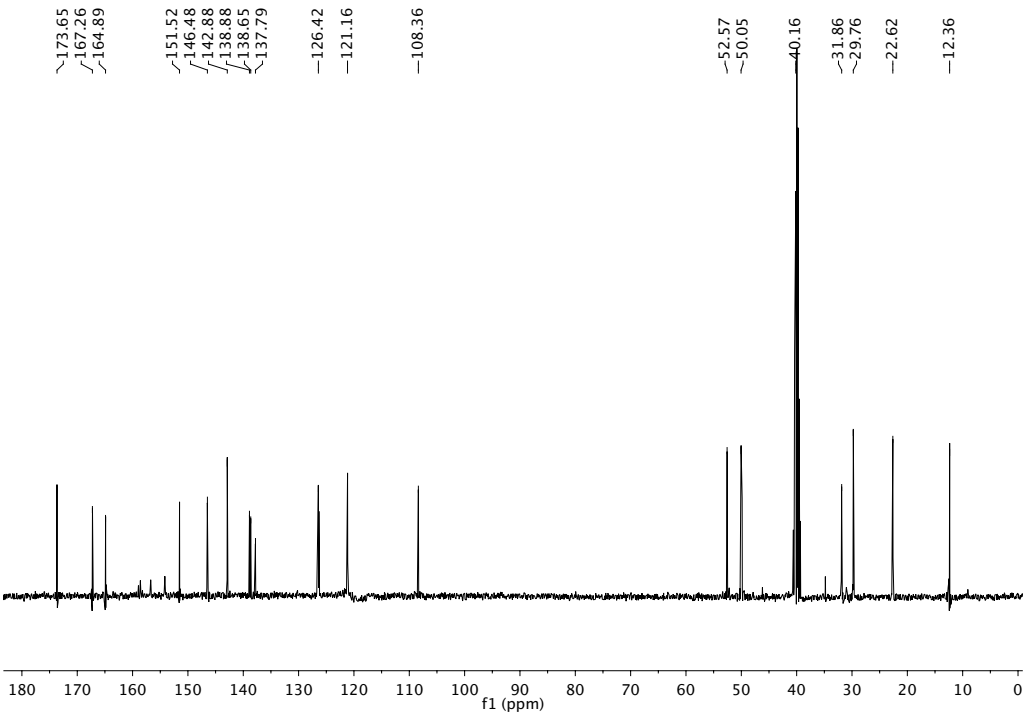


Figure 6.88: ^{13}C-NMR of compound **59** (400 MHz, DMSO-d6).

6.2.34 Compound 63

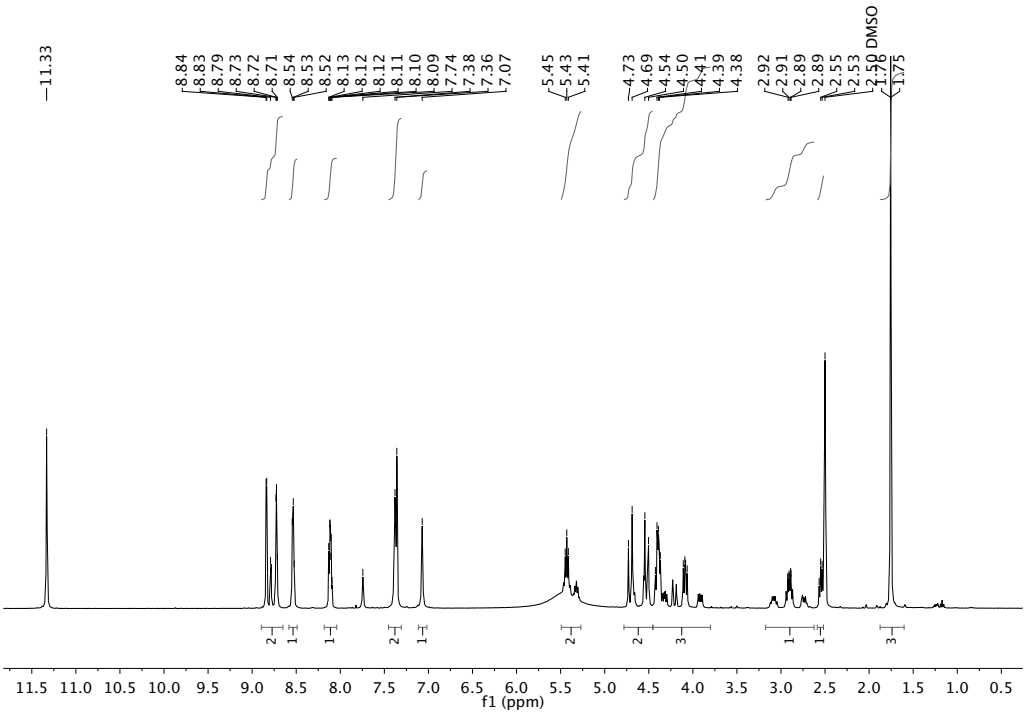


Figure 6.89: ^{1}H-NMR of compound **63** (400 MHz, DMSO-d6).

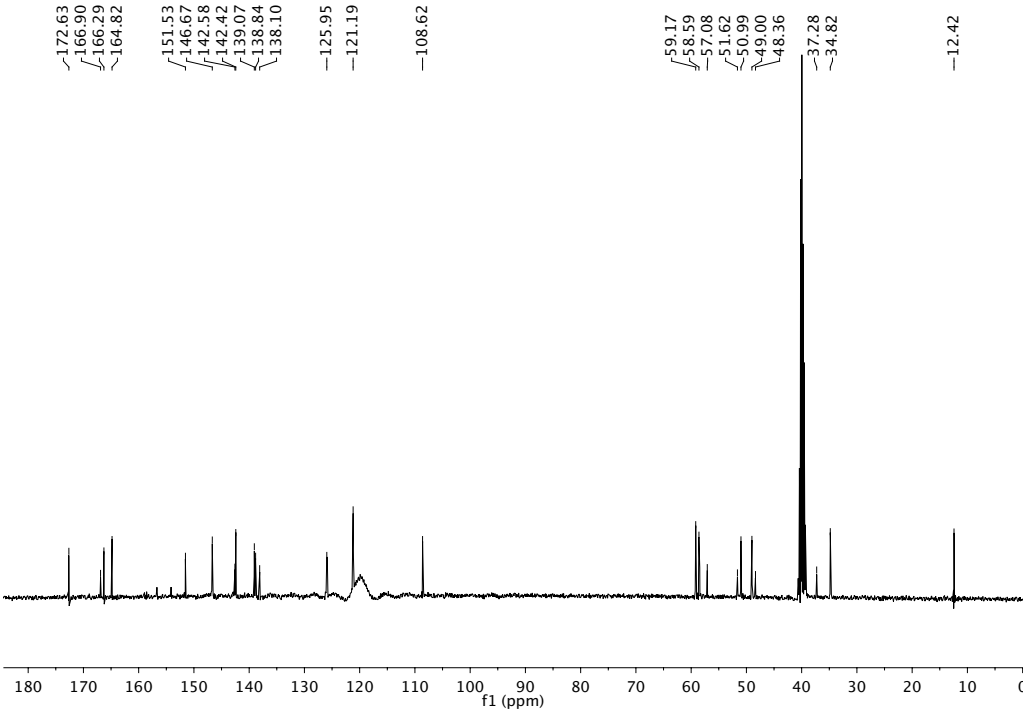


Figure 6.90: ^{13}C-NMR of compound **63** (400 MHz, DMSO-d6).

6.3 UPLC-MS analysis

6.3.1 HSA Hits

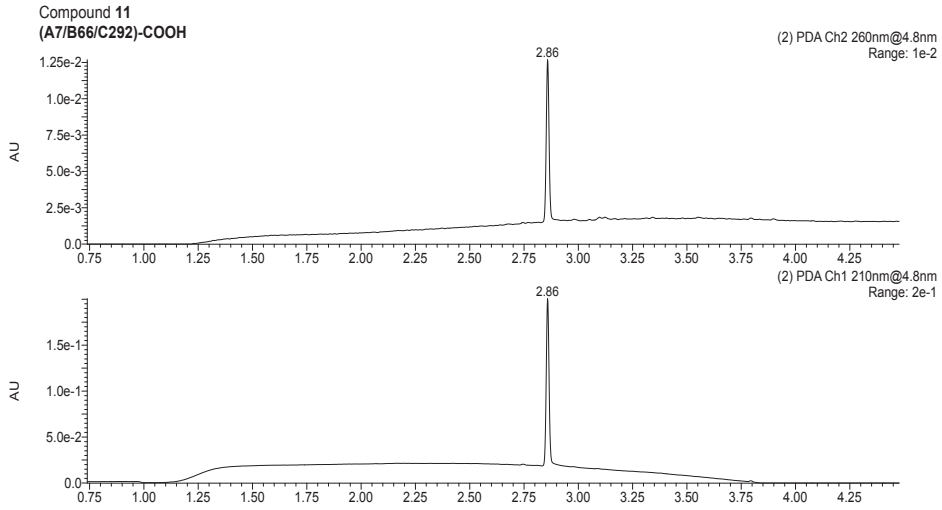


Figure 6.91: UPLC of compound **11**

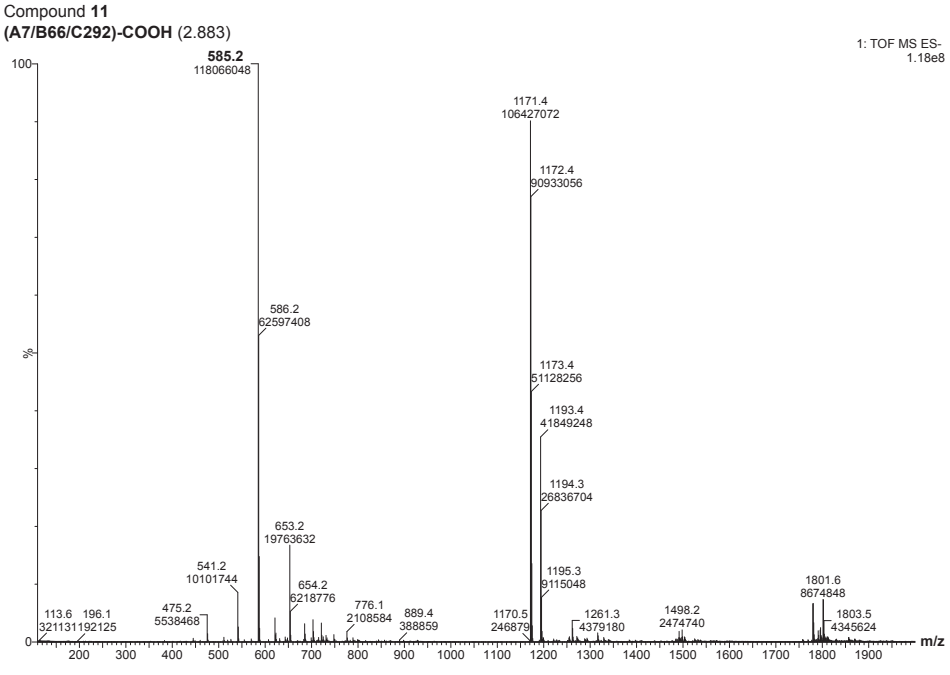


Figure 6.92: TOF MS ES (negative ionisation) of compound **11**.

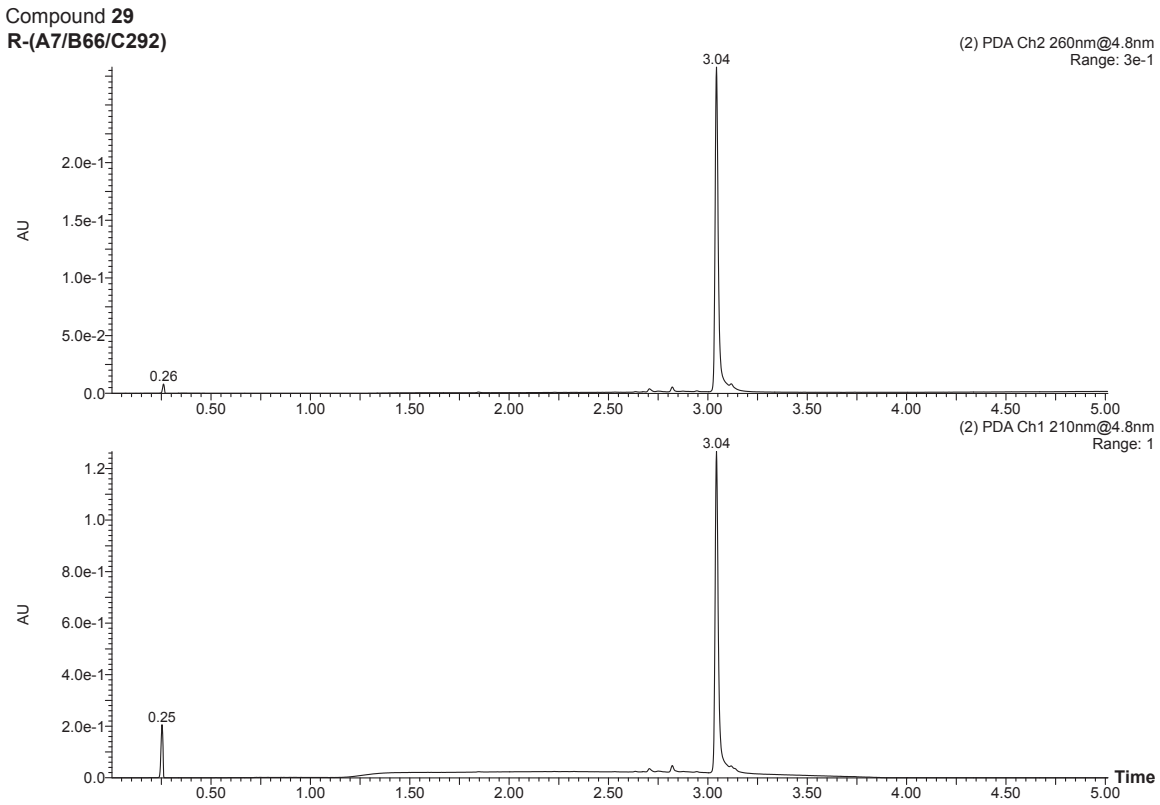


Figure 6.93: UPLC of compound **29**

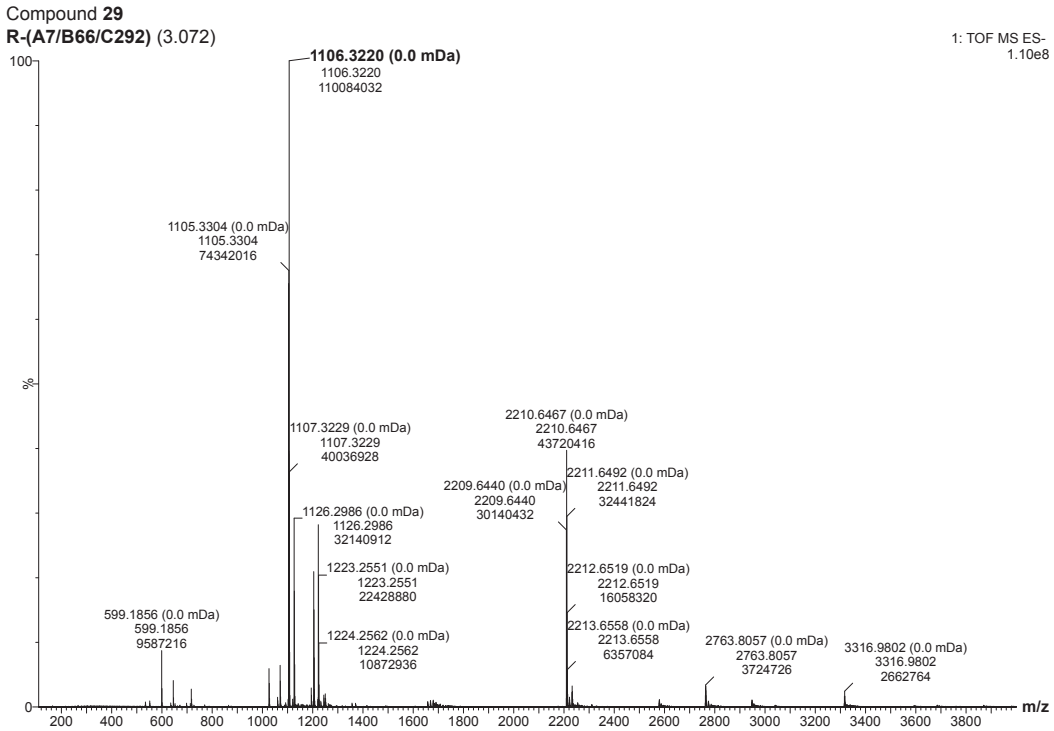


Figure 6.94: TOF MS ES (negative ionisation) of compound **29**.

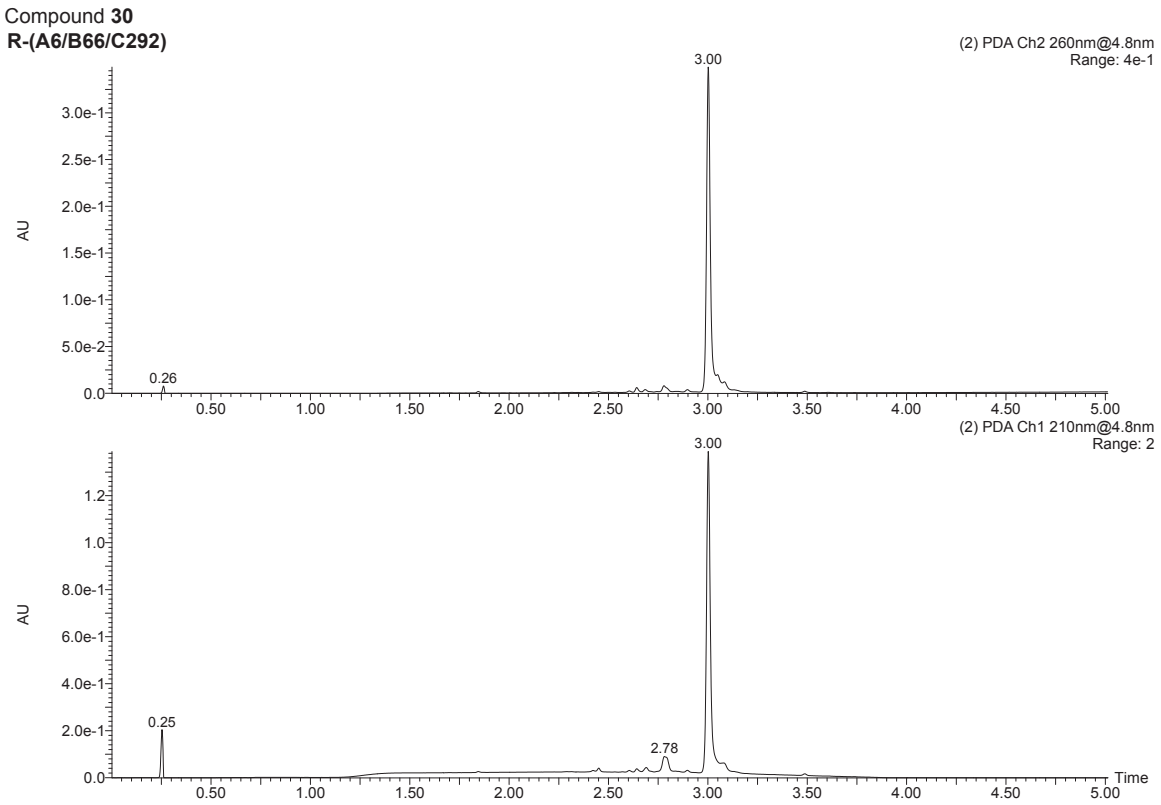


Figure 6.95: UPLC of compound **30**

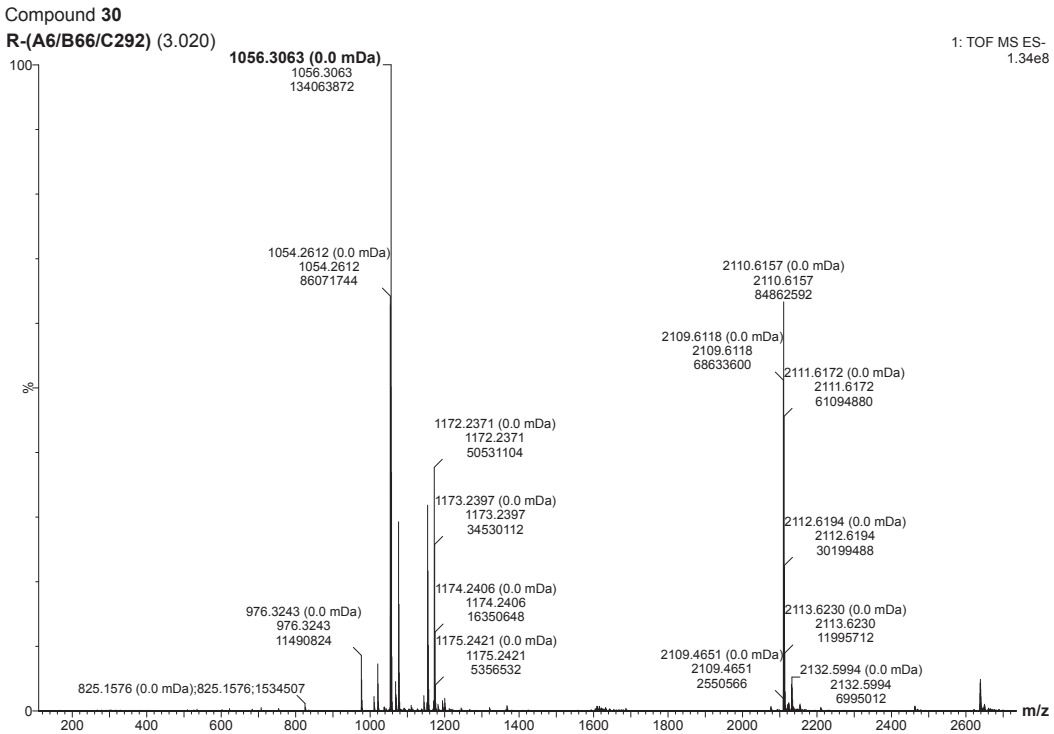


Figure 6.96: TOF MS ES (negative ionisation) of compound **30**.

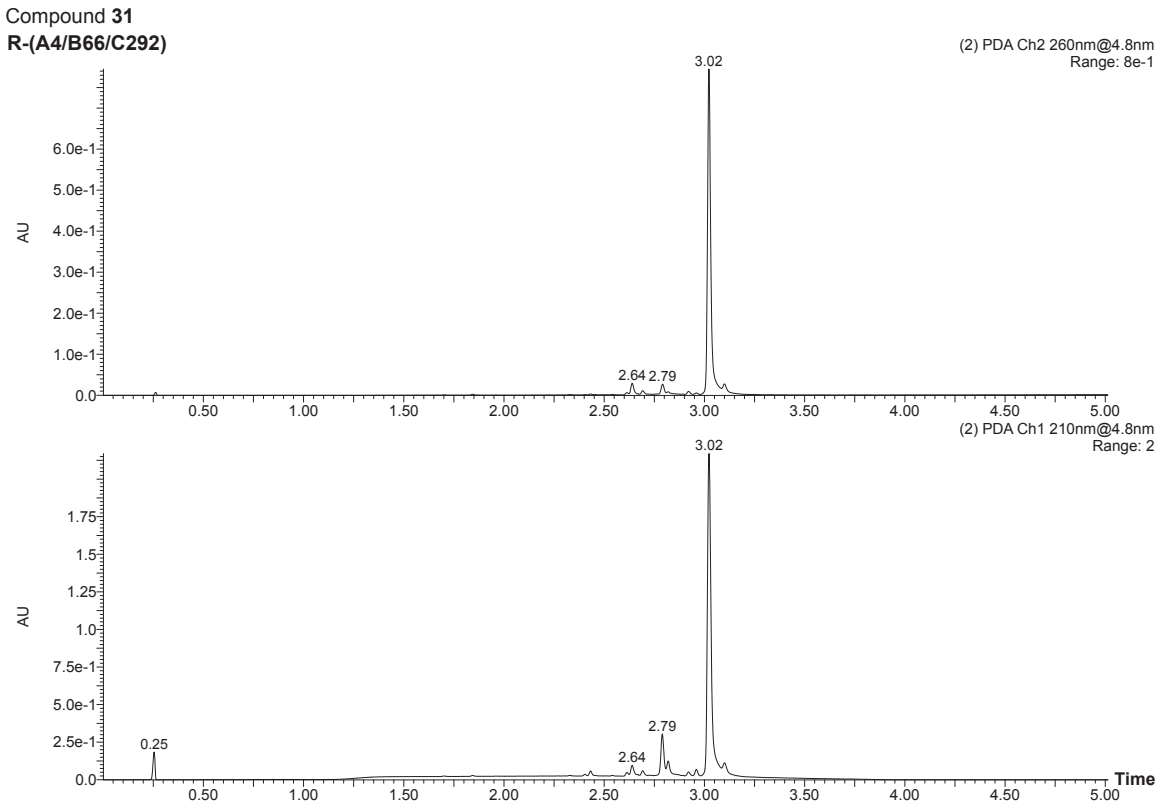


Figure 6.97: UPLC of compound **31**

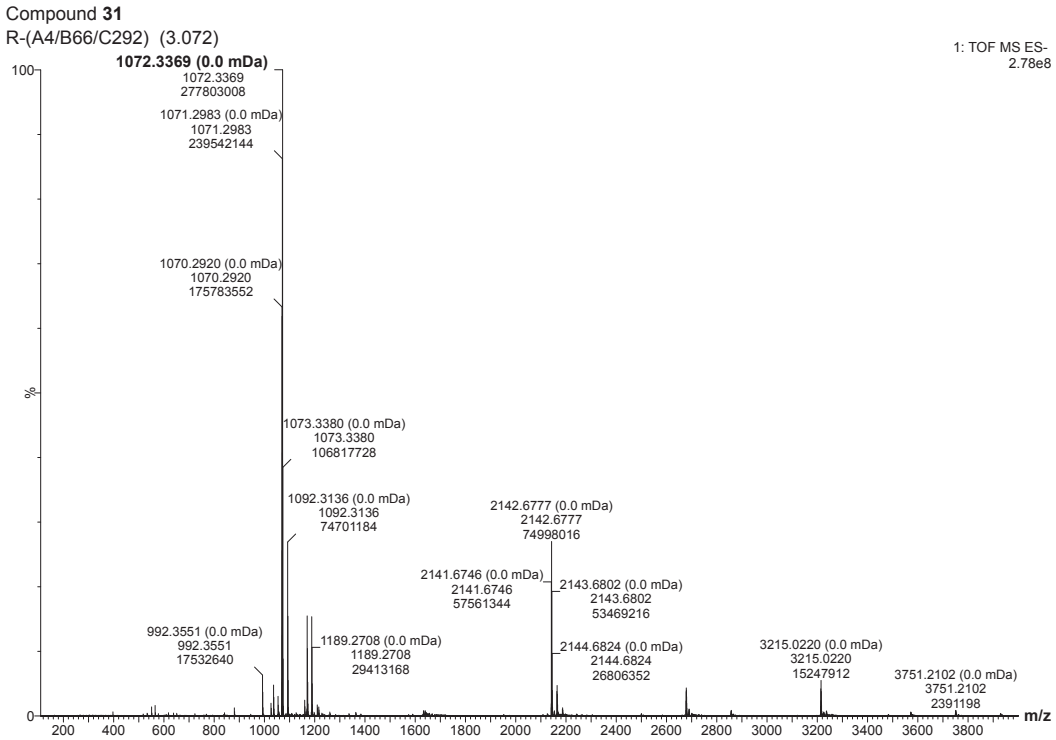


Figure 6.98: TOF MS ES (negative ionisation) of compound **31**.

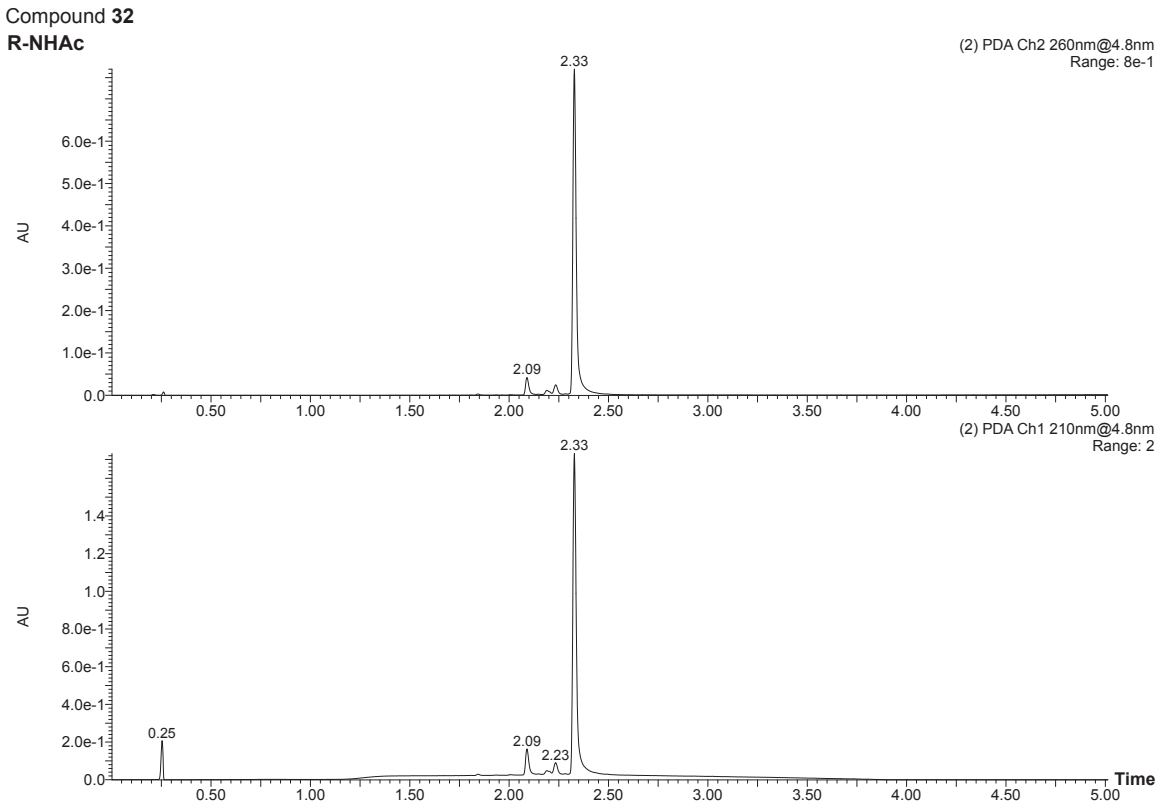


Figure 6.99: UPLC of compound **32**

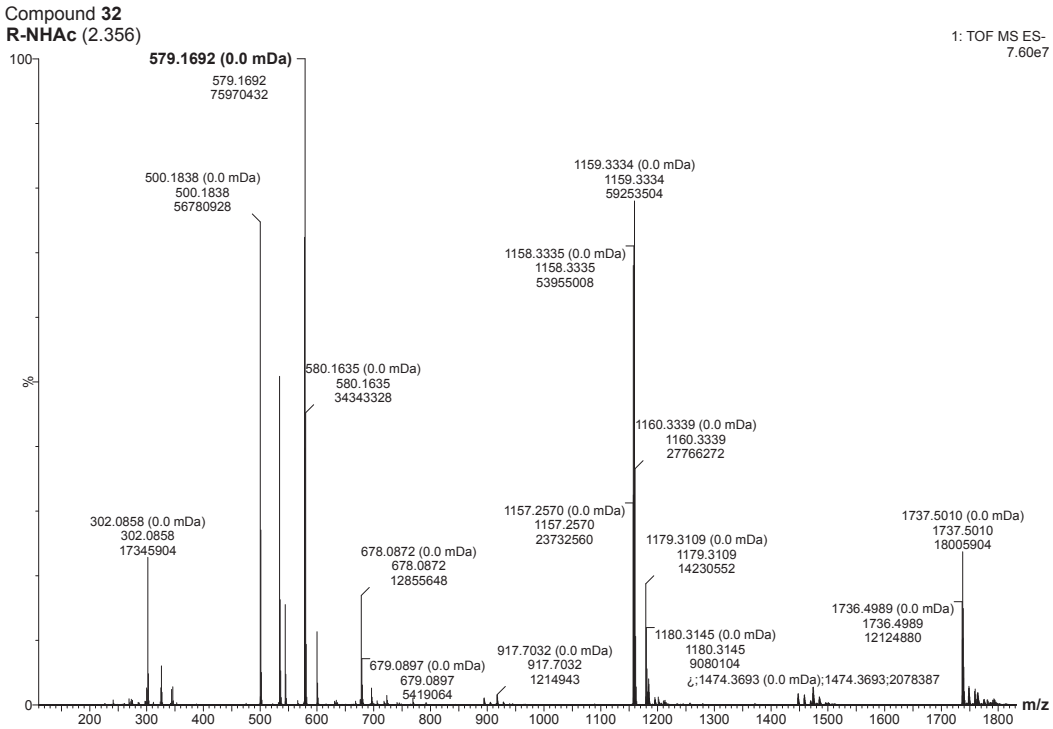


Figure 6.100: TOF MS ES (negative ionisation) of compound **32**.

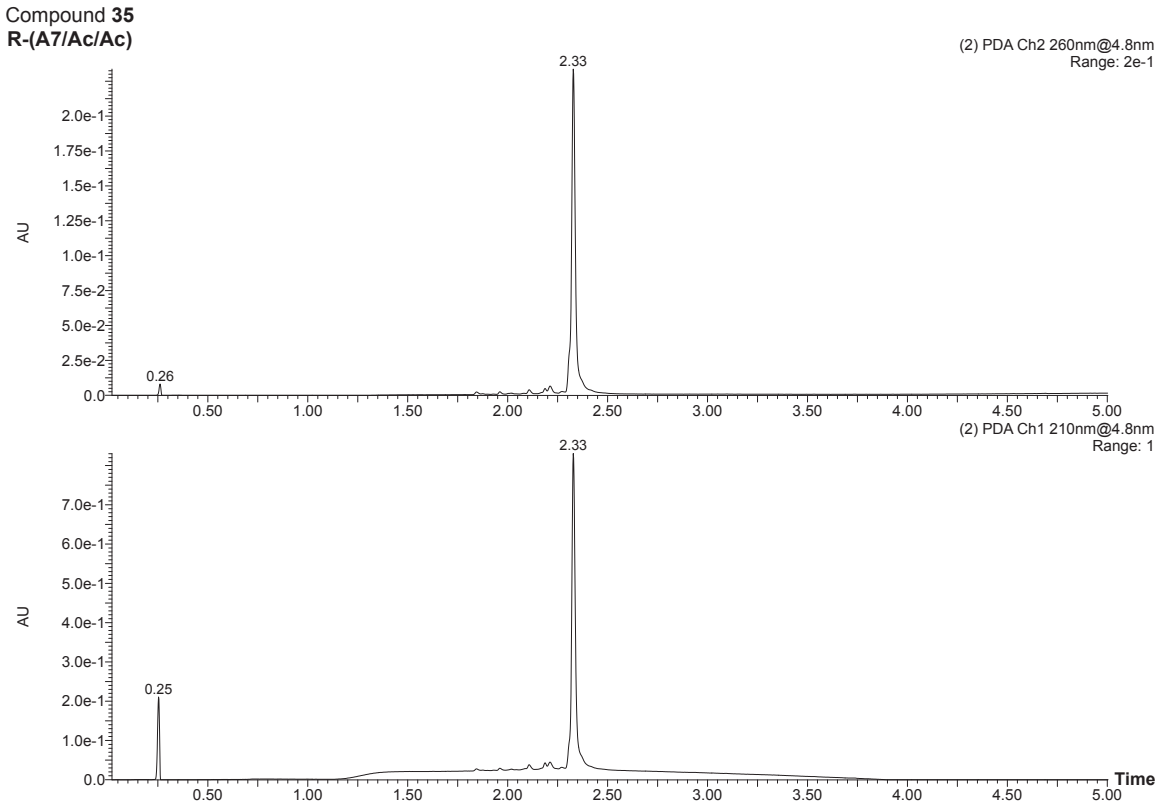


Figure 6.101: UPLC of compound **35**

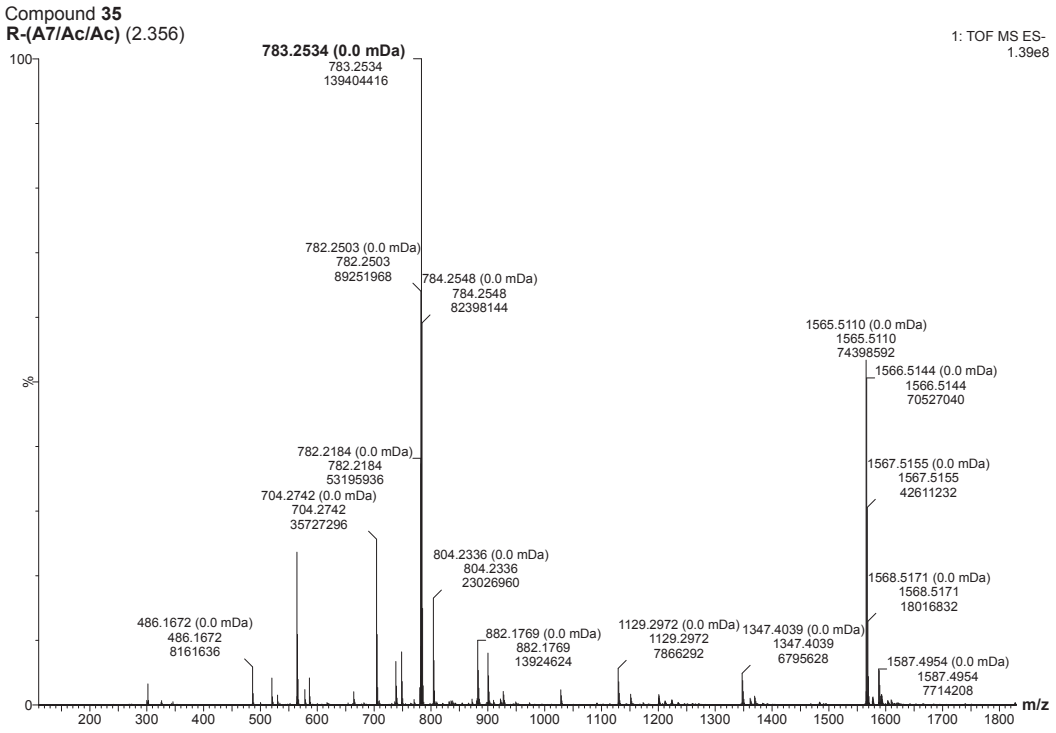


Figure 6.102: TOF MS ES (negative ionisation) of compound **35**.

6.3.2 TNKS-1 Hits

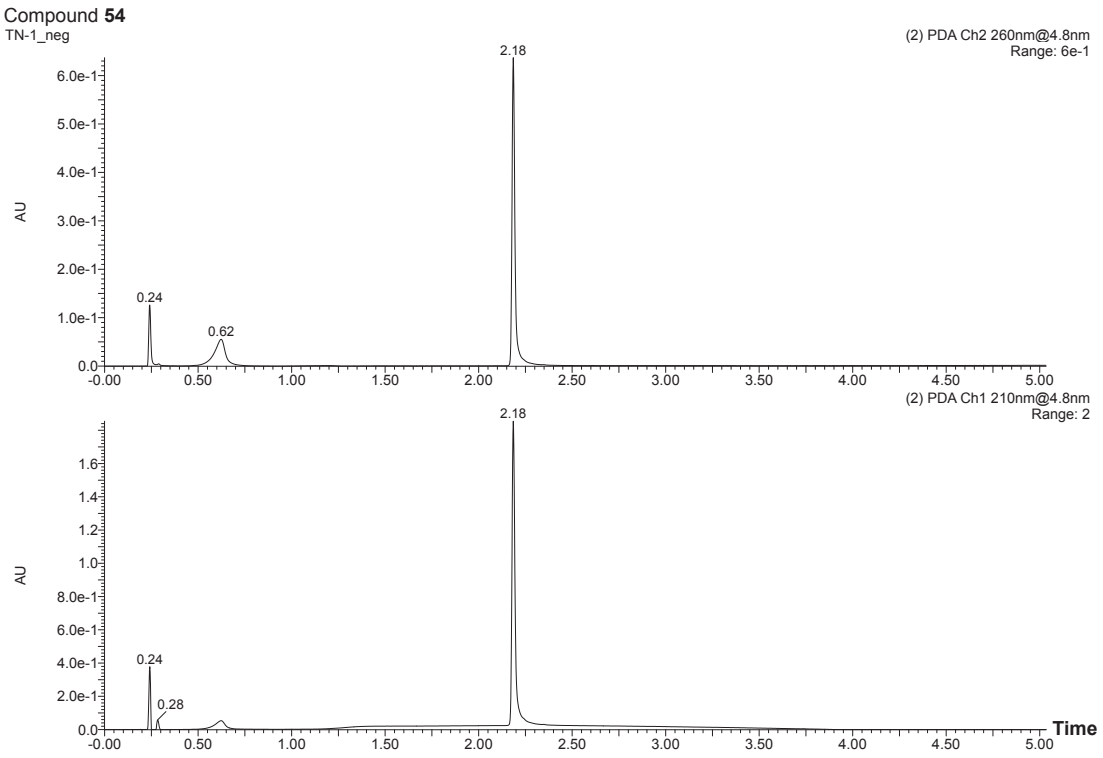


Figure 6.103: UPLC of compound **54**

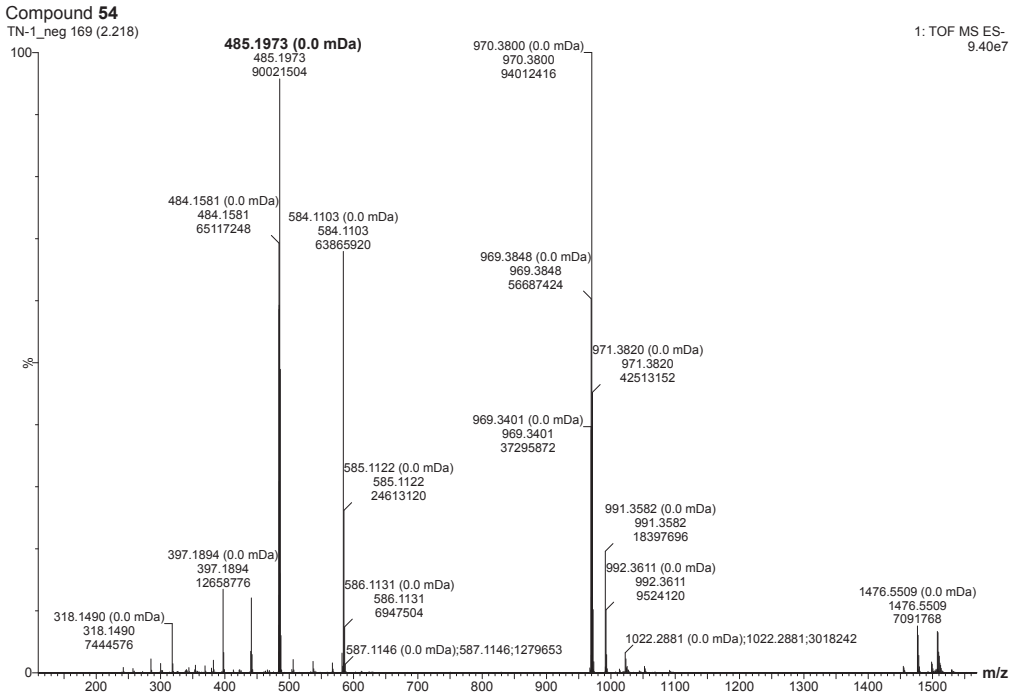


Figure 6.104: TOF MS ES (negative ionisation) of compound **54**.

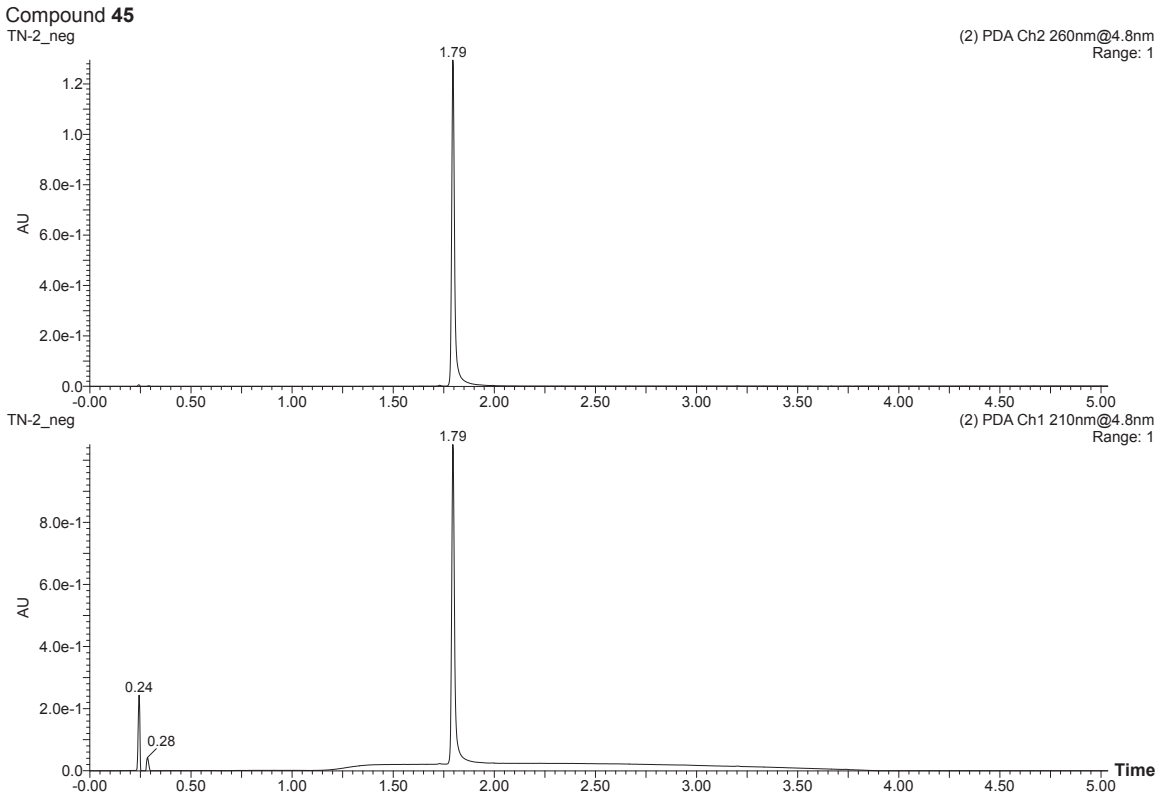


Figure 6.105: UPLC of compound **45**

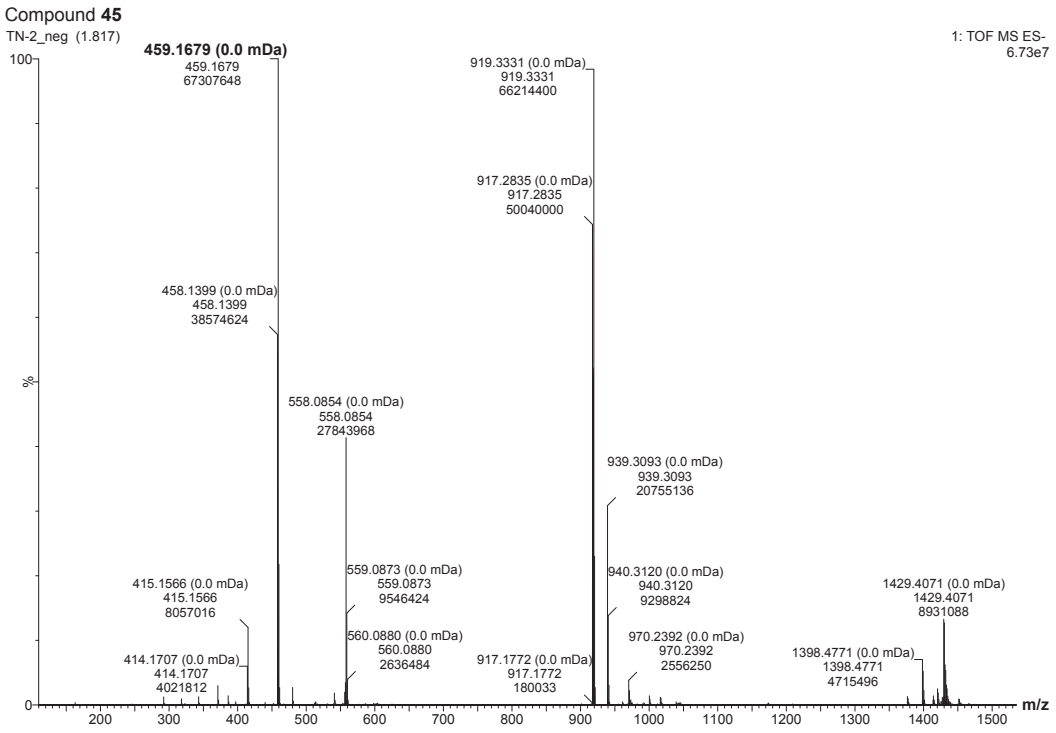


Figure 6.106: TOF MS ES (negative ionisation) of compound **45**.

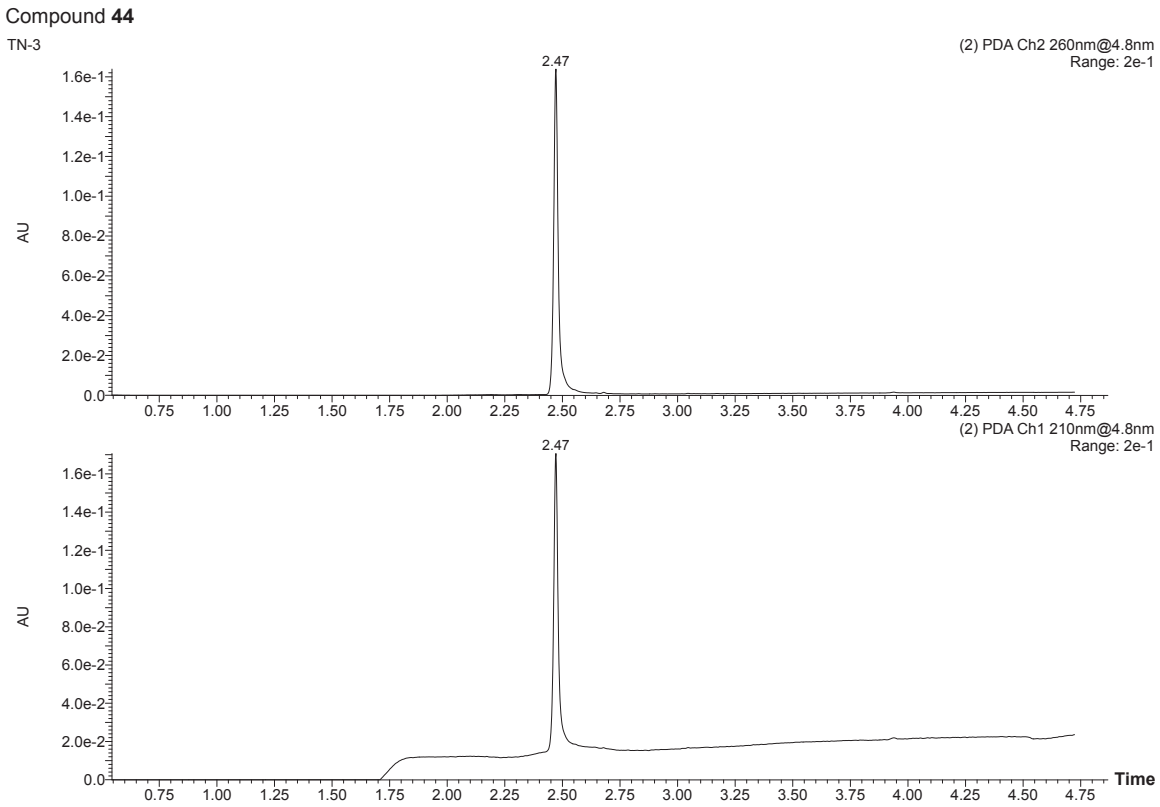


Figure 6.107: UPLC of compound **44**

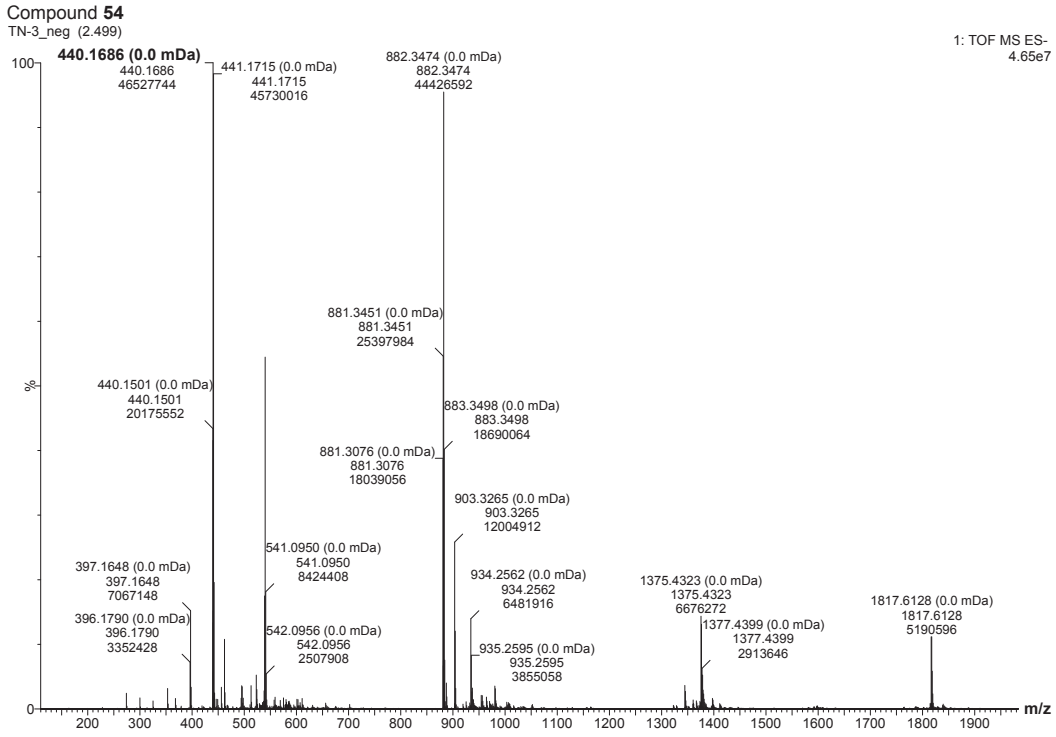


Figure 6.108: TOF MS ES (positive ionisation) of compound **44**.

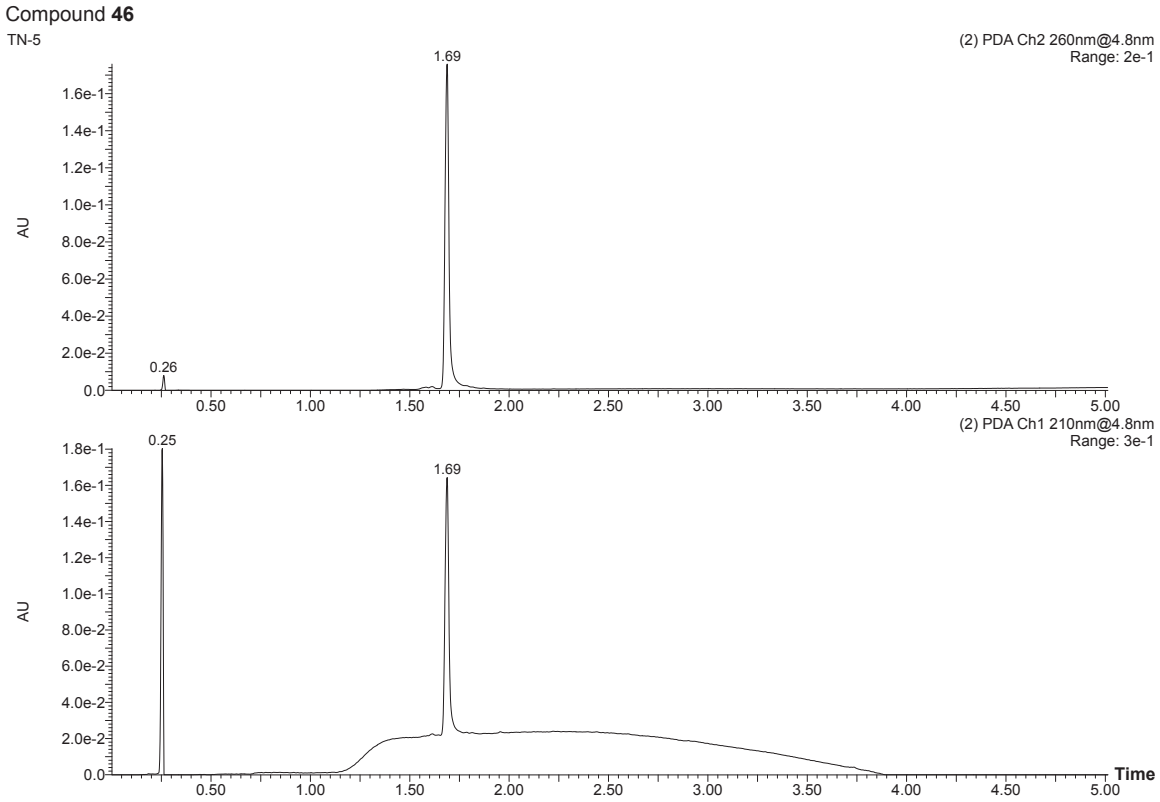


Figure 6.109: UPLC of compound **46**

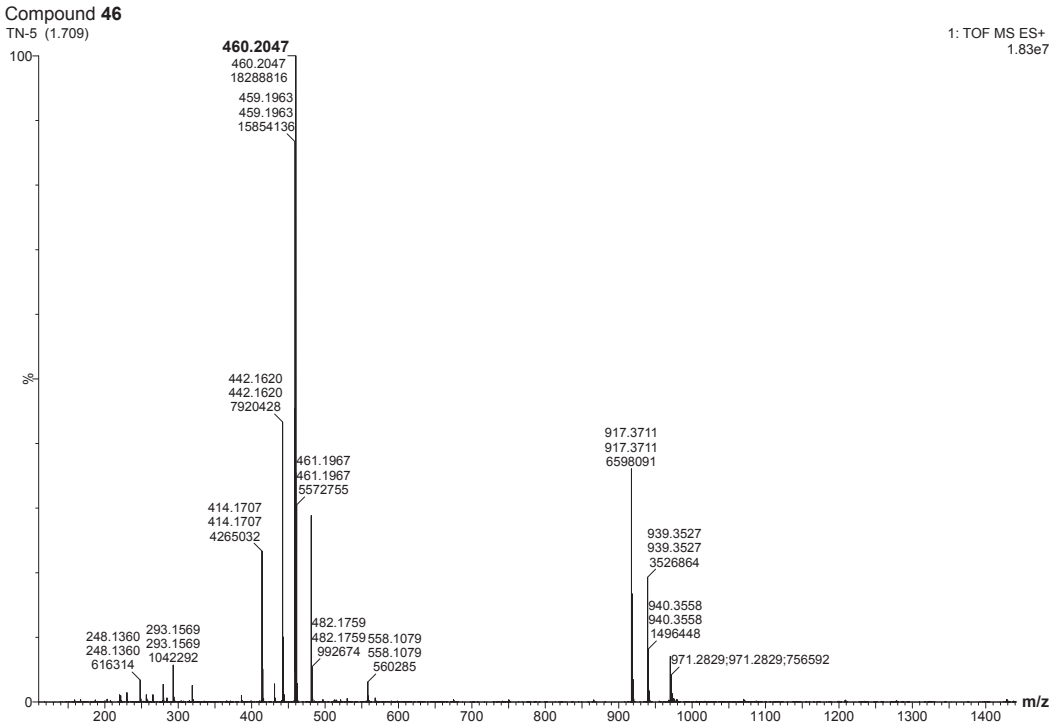


Figure 6.110: TOF MS ES (positive ionisation) of compound **46**.

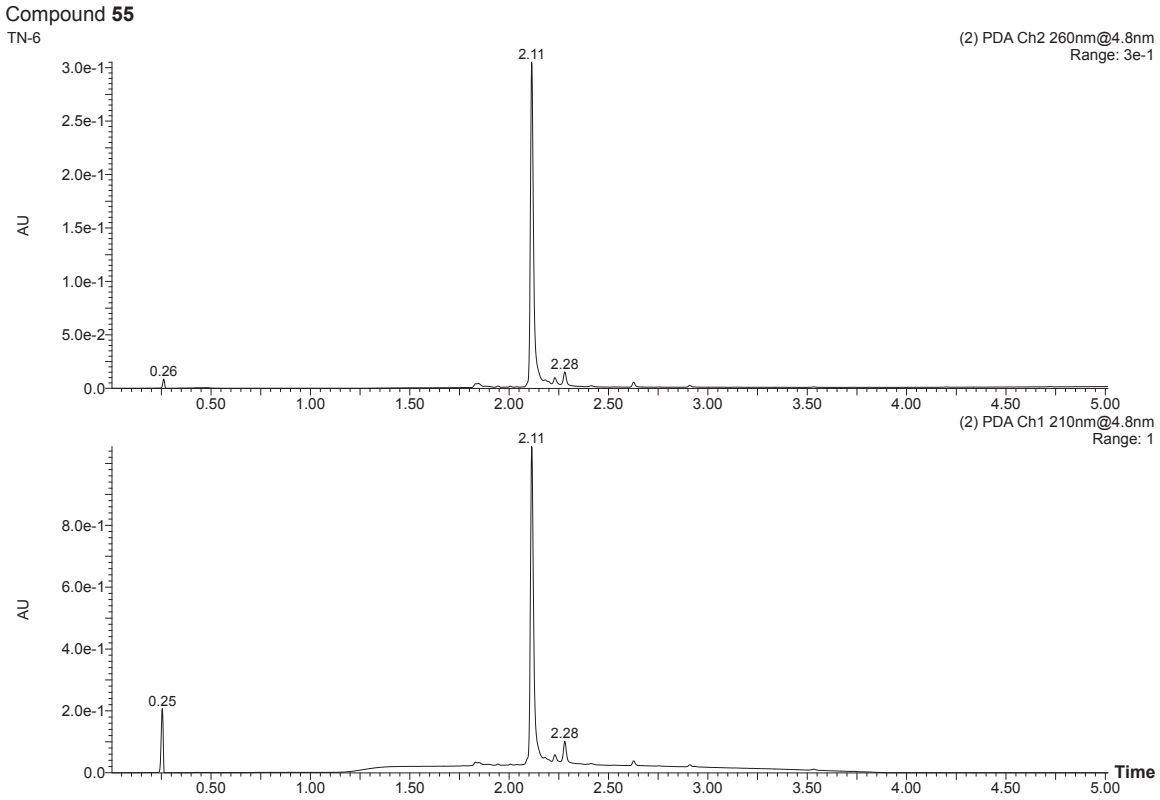


Figure 6.111: UPLC of compound **55**

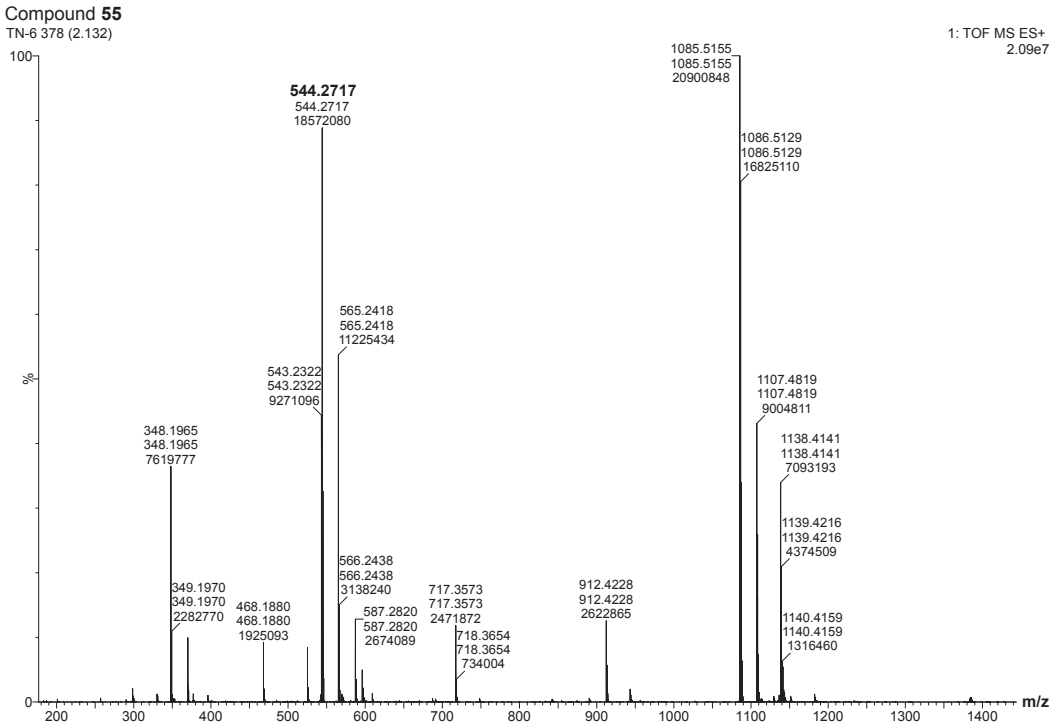


Figure 6.112: TOF MS ES (positive ionisation) of compound **55**.

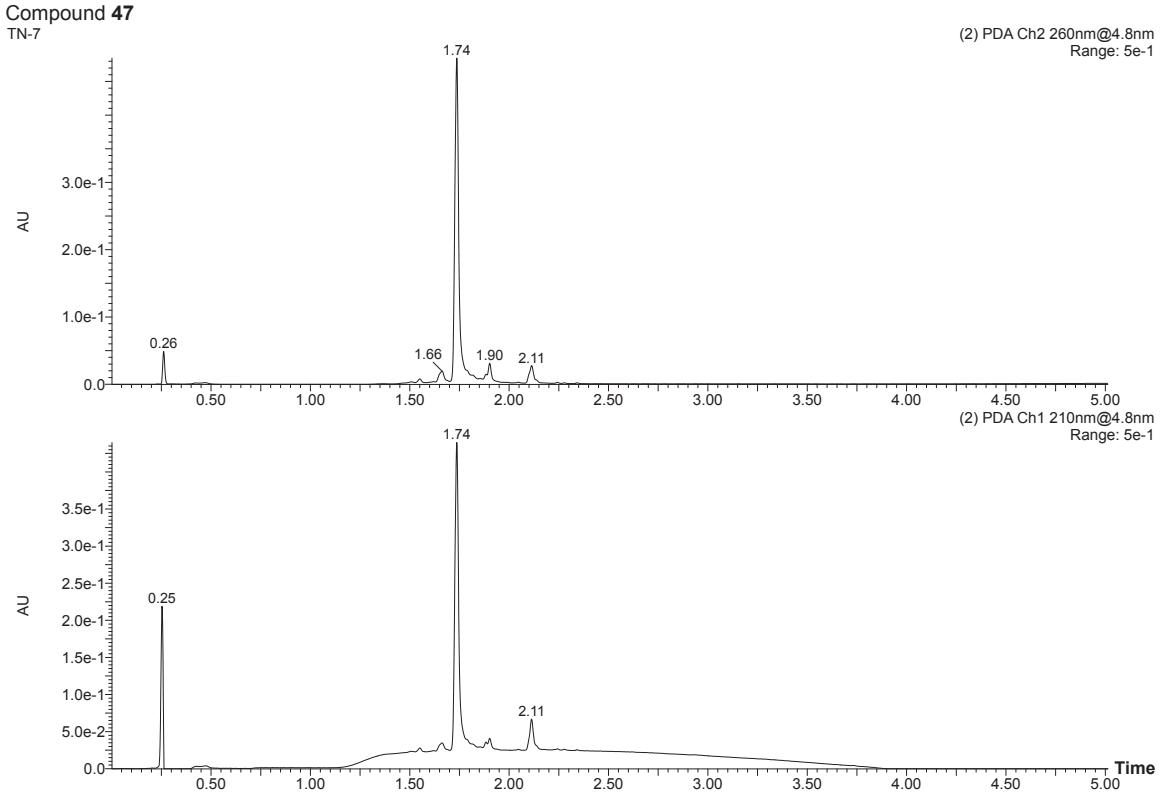


Figure 6.113: UPLC of compound **47**

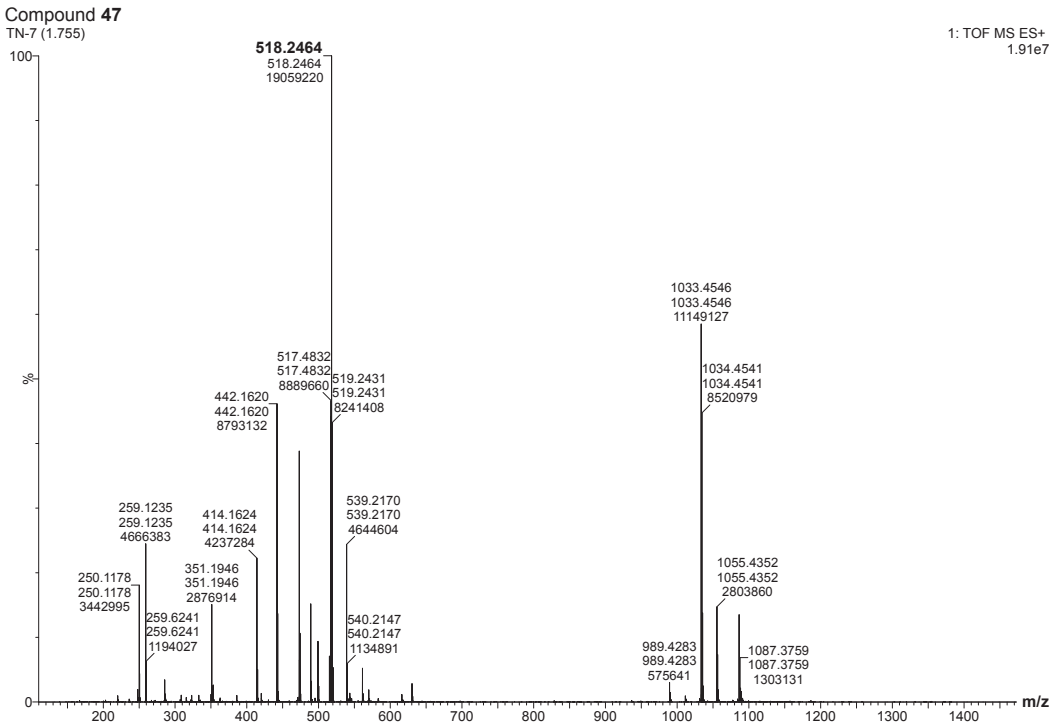


Figure 6.114: TOF MS ES (positive ionisation) of compound **47**.

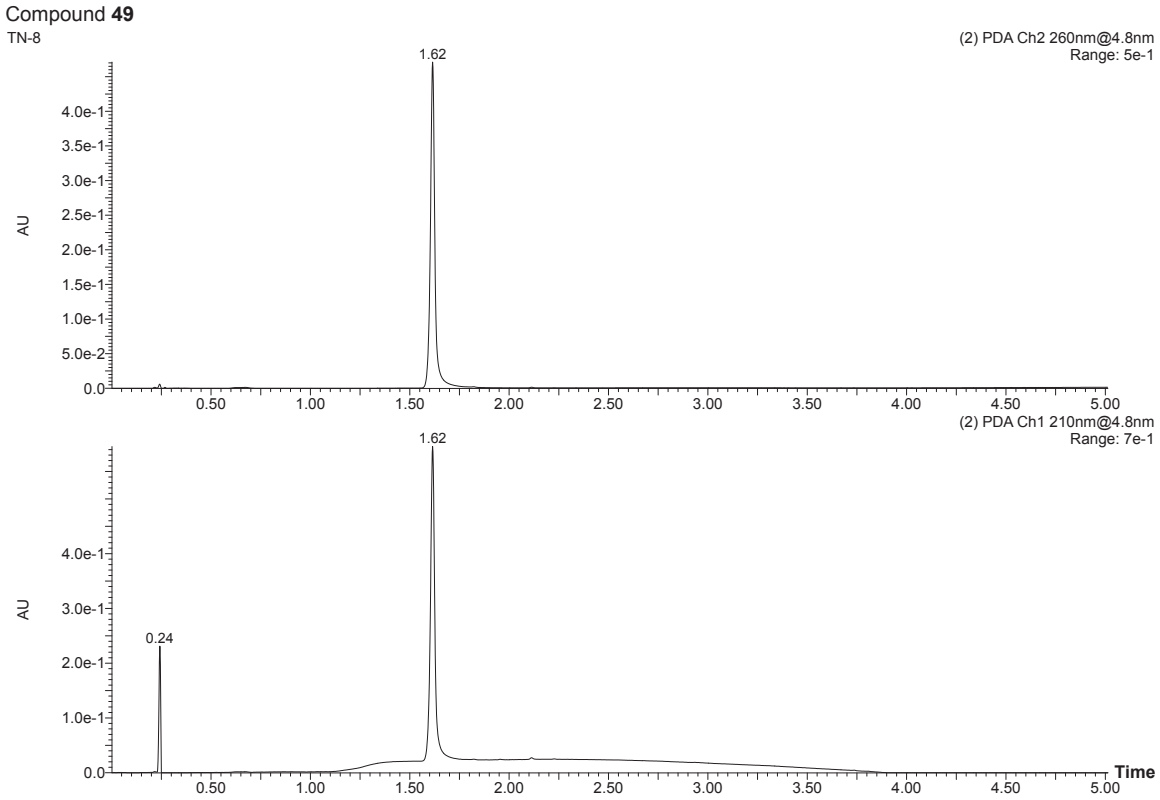


Figure 6.115: UPLC of compound **49**

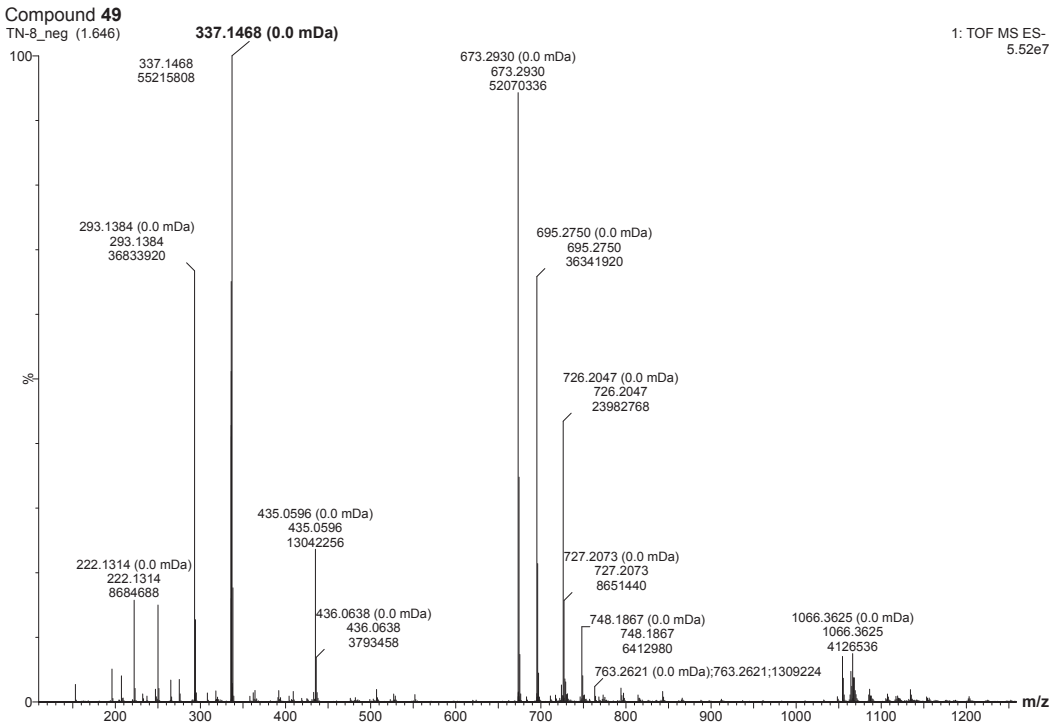


Figure 6.116: TOF MS ES (negative ionisation) of compound **49**.

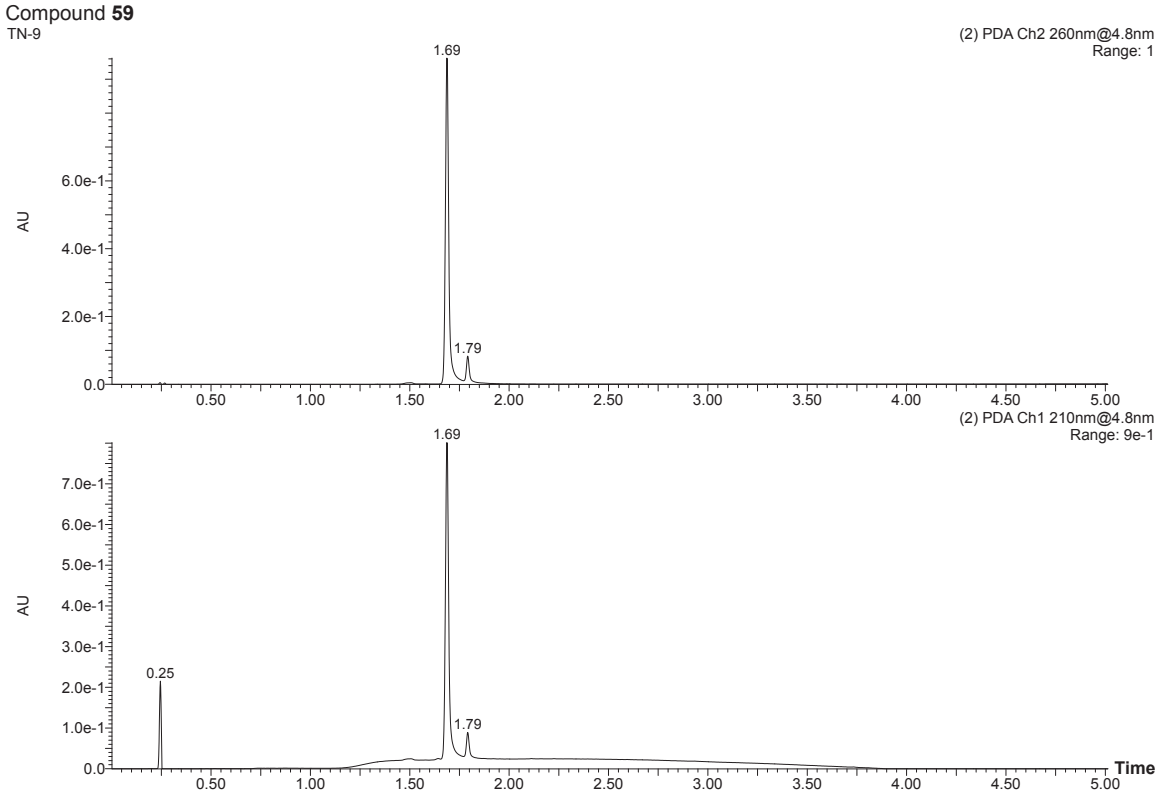


Figure 6.117: UPLC of compound **59**

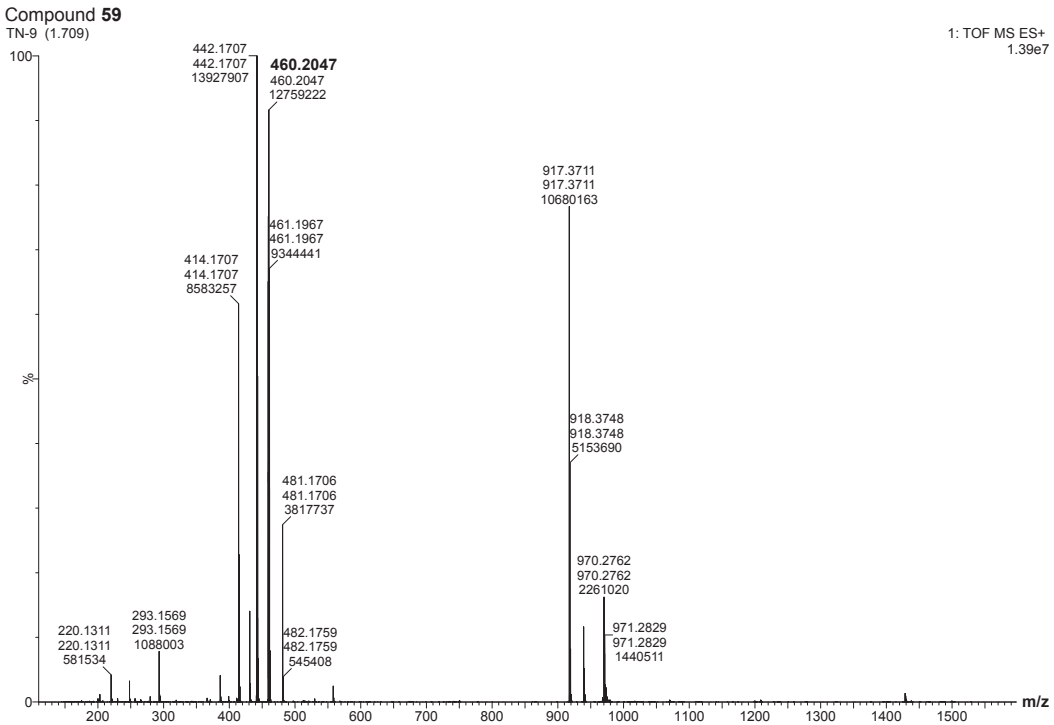


Figure 6.118: TOF MS ES (positive ionisation) of compound **59**.

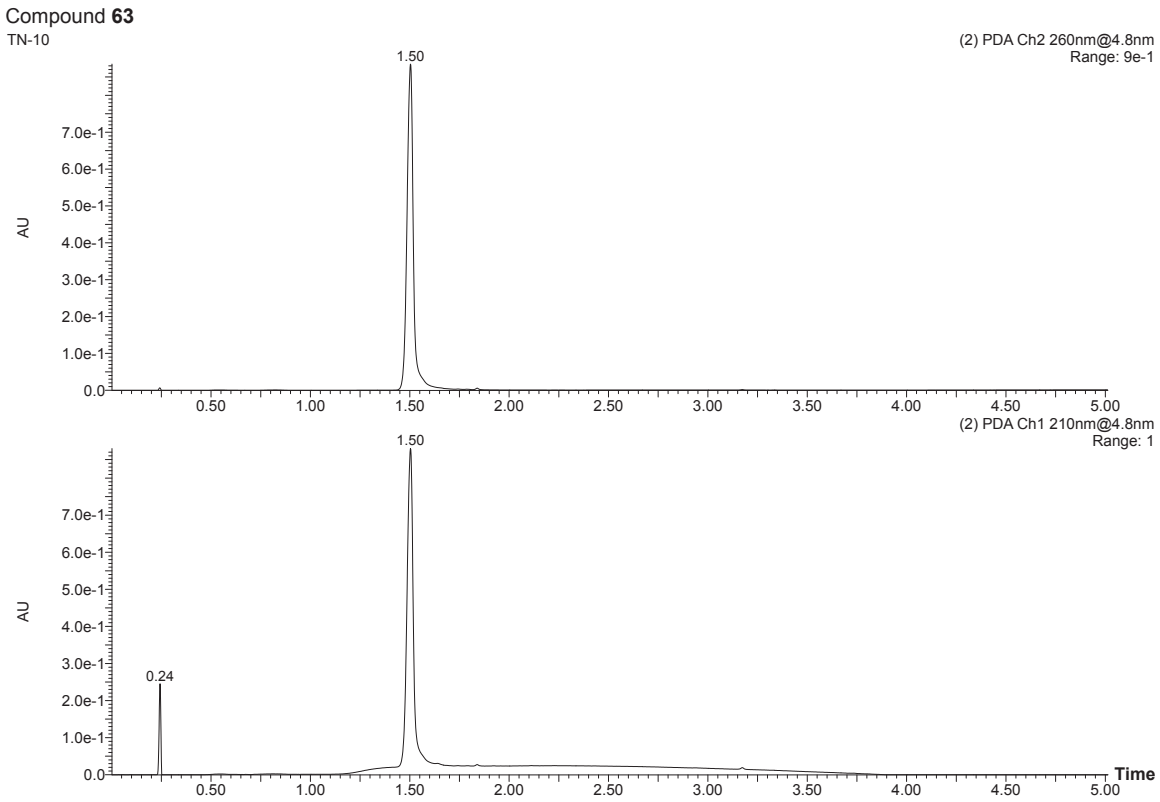


Figure 6.119: UPLC of compound **63**

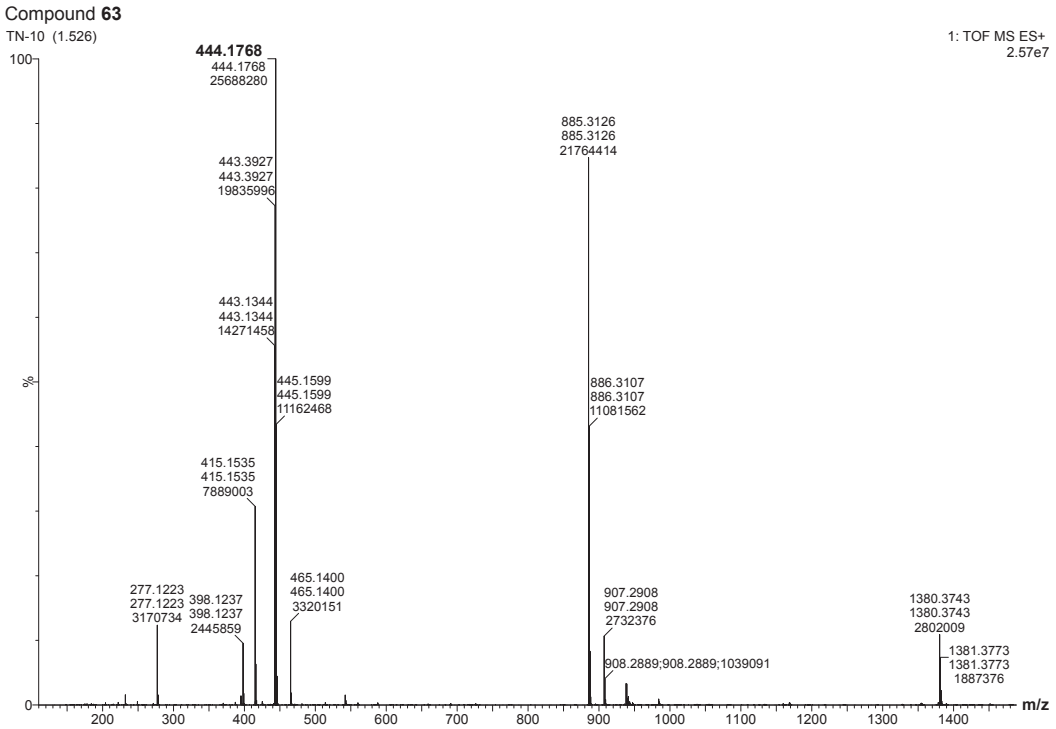


Figure 6.120: TOF MS ES (positive ionisation) of compound **63**.

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