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Synthesis of bioconjugatable furimazine analogues

Master's thesis

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Bioluminescence resonance energy transfer (BRET) has extensive applications in the studies of dynamic processes in various biological systems such as protein-protein interactions, signaling events, metabolites and drug concentrations. Recently it was found that BRET can not only act as a reporter of an event, but an effector which turns on functions in livings systems. Herein, we propose the synthesis of furimazine analogues, which are common substrates for luciferases in BRET studies, with a novel modification allowing the bioconjugation of small-molecules.

Key words: Bioluminescence, BRET, biological systems, living systems, synthesis, furimazine, luciferases, bioconjugation

Table of contents

Abbreviations

BRET = Bioluminescence resonance energy transfer

CMF = Chloromethylfurfural

cpDHFR = Circularly permutated bacterial dihydrofolate reductase

Cy5 = Cyanine5 far-red-fluorescent dye

ErbB2 = Erythroblastic oncogene B2

HME = Horner–Wadsworth–Emmons

HMF = Hydroxymethylfurfural

LUCID = Luciferase-based indicator of drugs

LUPIN = Luciferase-based photocatalysis induced via nucleic acid template

MTX = Methotrexate

NLuc = NanoLuc luciferase

PNA = Peptide nucleic acid

SNAP-Pro30 = Synaptosomal-associated protein, 30 kDa

SKBR3 = Human breast cancer cell line

TBAF = Tetra-*n*-butylammonium fluoride

TBDMS = tert-Butyldimethylsilyl

TMG = Tetramethylguanidine

TFA = Trifluoroacetic acid

VH = Vilsmeier-Haack

1 Introduction

Bioluminescent enzymes, also referred to as luciferases, have been widely used for reporting biological events all the way from cell-based assays to *in vivo* imaging applications.[1-5] Luciferases are enzymes catalyzing the oxidation of suitable chemical substrates to generate photons. They overcome some shortcomings perceived to conventional fluorescence imaging.[6] One of the most common ones being phototoxicity. Moreover, such approach yields ultrasensitive imaging data with high signal-to-noise ratio. Therefore, it is not a surprise that bioluminescence resonance energy transfer (BRET) has gained a lot of attraction in the field of biological systems, in order to study dynamic processes, such as monitoring of protein-protein proximity, signaling events, metabolites and they can even be used as drug sensors.[7-13] Commonly BRET applications rely on the principle of photon generation by a luciferase as a signal reporting event. However recent studies have shown the utility of BRET as an effector rather than solely a reporter function. Such BRET applications have demonstrated an ability to control gene expression, neuronal activity and a photo-switchable fluorescent protein.[14-17] Initial research has led to the development of BRET-induced ruthenium-photocatalyzed reactions name LUPIN (luciferase-based photocatalysis induced via nucleic acid template), wherein the photocatalyst gets conjugated to a suitable peptide nucleic acid (PNA) which acts as a barcode, in turn creating high effective concentration of substrate and taking advantage of the fast chemical transformation of nucleic acid-templated reactions at low substrate concentrations (Figure 1).[18]

1.1 Luciferase-based systems

Herein, used NanoLuc (NLuc) is one of the brightest luciferase originally developed by Promega from *Oplophorus* luciferase and it was paired with a ruthenium-based photocatalyst $Ru(bpy)_2phen$.[19] Such donor (NLuc) and acceptor ($Ru(bpy)_2phen$) pair was selected on the basis of good overlap between the absorption spectra of the catalyst with the emission spectra of the enzyme. Moreover, the ruthenium photocatalyst has been successfully used in the past to perform templated reactions in live cells and live vertebrates.[20,21] However, in order to facilitate the initiation of the energy transfer process, all of the systems components have to be in close proximity to each other. Previously the Johnsson lab has reported a luciferase-based indicator of drugs (LUCID), which is a system consisting of semi-synthetic sensors intended for drug concentration monitoring.[22,23] LUCID has three main components at its core: SNAP protein is used to conjugate a dye-drug complex through a suitable synthetic linker, NLuc is responsible for bioluminescence and a receptor which specifically binds the selected drug.[22,23] Inspired by this

luciferase based construct the idea was then transposed to our own photoswitch system, and later LUPIN.[18] Herein, a fusion protein SNAP-Pro30-NanoLuc (NLuc)-cpDHFR was at the core of the whole system, consisting of the SNAP protein conjugated to NLuc as a BRET donor, a circularly permutated bacterial dihydrofolate reductase (cpDHFR) with methotrexate (MTX) as a bound ligand and a ruthenium photocatalyst as a BRET acceptor molecule. Upon achieving a proximal position of a donor-acceptor pair the energy transfer starts driving the conversion of a substrate into a product leading to an open conformation of the fusion protein. This event was evident by a new band at 610 nm in the luminescence emission spectrum, which corresponds to the emission wavelength of $Ru(bpy)_2$phen complex without MTX.[18]

Harnessing the power of a controllable photo-switch system lead to investigations of small molecule unmasking. Previously described photoswitch system was modified by introducing a 5-mer PNA conjugated to a small molecule. This modification allowed to us transform a regular photoswitch system to a LUPIN sensor. The single stranded PNA is strategically preinstalled to be in close proximity to the ruthenium photocatalyst. This PNA can serve as a barcode to which only the complementary strand can bind. Then a complementary strand bearing a desired effector molecule is added to the system. This binding results in the favorable placement of the small molecule close to the ruthenium catalyst. The BRET initiated electron transfer process promotes a photoreductive cleavage of a small molecule. One such molecule is rhodamine with fluorescent properties, which can emit light once the molecule is released. The experimental conditions used 100 μM furimazine added to 50 nM of the LUPIN construct with 0.5 μM PNA-PyRho-substrate and with 10 mM sodium ascorbate serving as a reducing agent for ruthenium catalyst. An observed increase in fluorescence was attributed to the release of rhodamine. The concertation of the fluorescent molecule reached 65 nM (1.3 turnovers), which is calculated to be in slight excess relative to the LUPIN construct. It was found that the reactions tested on this system were effective over a broad range of substrate concentrations (0.2-5 μM). At low concentrations of LUPIN (2–10 nM), the concentration of product (small molecule) obtained in the reactions was 100–600 nM. These concentrations sit well in the effective concentration range of many drugs, thus suggesting that the system has potential in the therapeutics field of research. [18]

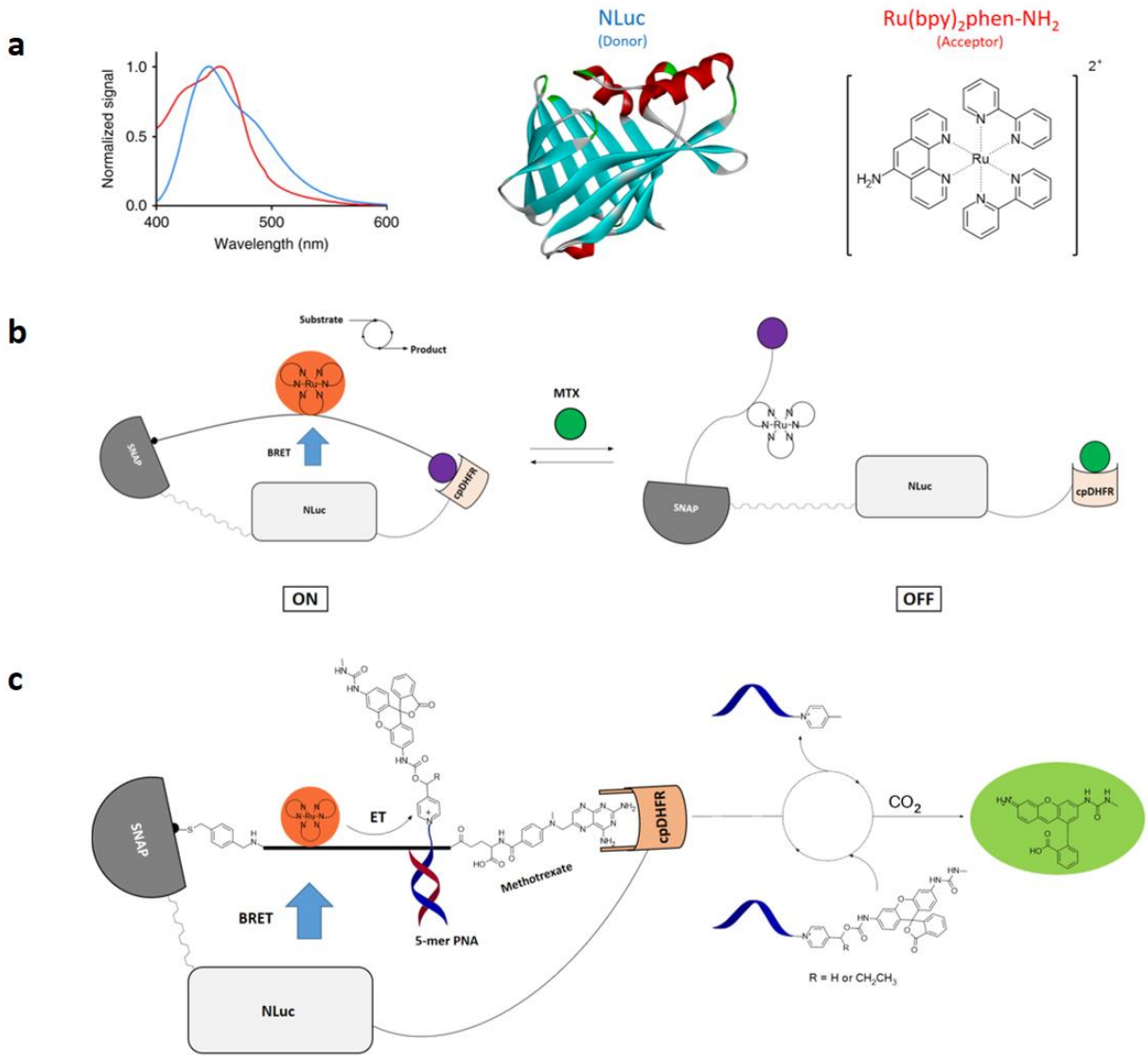


Figure 1. Concept and design of LUPIN. **a** The emission spectrum of NLuc (blue) and the absorption spectrum of the ruthenium photocatalyst (red); Snapshot of NLuc (PDB: 5B0U)[**50**]. **b** Photoswitch construct SNAP-Pro30-NanoLuc (NLuc)-cpDHFR promotes the BRET process as a result of close proximity between the NLuc and the ruthenium catalyst. The proximity for efficient BRET is enabled through SNAP and a DHFR ligand (methotrexate, purple ball) bound to the cpDHFR. Upon interaction with free methotrexate (green ball), the conformation is switched to an open one and BRET can no longer occur. **c** Single stranded PNA preinstalled to be in close proximity with the ruthenium catalyst, interacts with a complementary strand bearing an effector molecule. The proximal placement of the effector molecule to the photocatalyst allows for an efficient electron transfer to occur as a result of BRET, unmasking the small molecule. (adapted from [**18**]).

1.1.1 Bioluminolysis in cells

The successful implementations of a photoswitch chemistry have created a solid ground for further investigations and improvements of the LUPIN concept. Therefore, not so long after a direct BRET-induced photocatalyzed reaction for photouncaging of small molecules in living cells or process referred to as bioluminolysis (Figure 2) was established.[1] Herein, a chimera protein Nanoluc-Halotag (H-Luc), developed by the Johnsson, group was a BRET donor and a 7-diethylaminocoumarin organic molecule, developed by Ellis-Davies group, was used as a BRET acceptor.[24,25] The H-Luc protein has significantly better BRET efficiency compared to a regular Nanoluc-Halotag. Therefore, a selection of a photolabile chromophore had to be strict and synergize well with the chosen fusion protein. The DEAC450 was perfectly suitable for this role. This coumarin molecule has an absorbance maximum of 450 nm, whereas an emission maximum of H-Luc is close to 460 nm. Previously reported photouncaging experiments on various small molecules like cGMP, Glutamate and GABA have presented a rather neat range for quantum yields 0.39-0.78.[26-29] Consequently, the DEAC450 can be dually-modified for both photouncaging and conjugation, which is exactly what is required for bioluminolysis experiments.[1,26-29] The photouncaging in these experiments occurs as follows; upon addition of the H-Luc's substrate furimazine, the BRET process from the donor to the coumarin acceptor occurs leading to photoreductive cleavage of a small molecule. The proposed construct is substantially less complex compared to the LUPIN, all of the components are tethered in an easy manner and are not as proximity dependent. Once the construct is pushed into the open conformation, BRET process can no longer take place due to lack of sufficient distance for an effective energy transfer between the donor-acceptor pair. However, in the case of bioluminolysis experiments the system is not subjected to conformational changes, that makes it solely dependent on availability of the substrate, like furimazine in the system. Thus, the energy transfer induced photolysis to release a small molecule can only occur in the presence of H-Luc substrate.

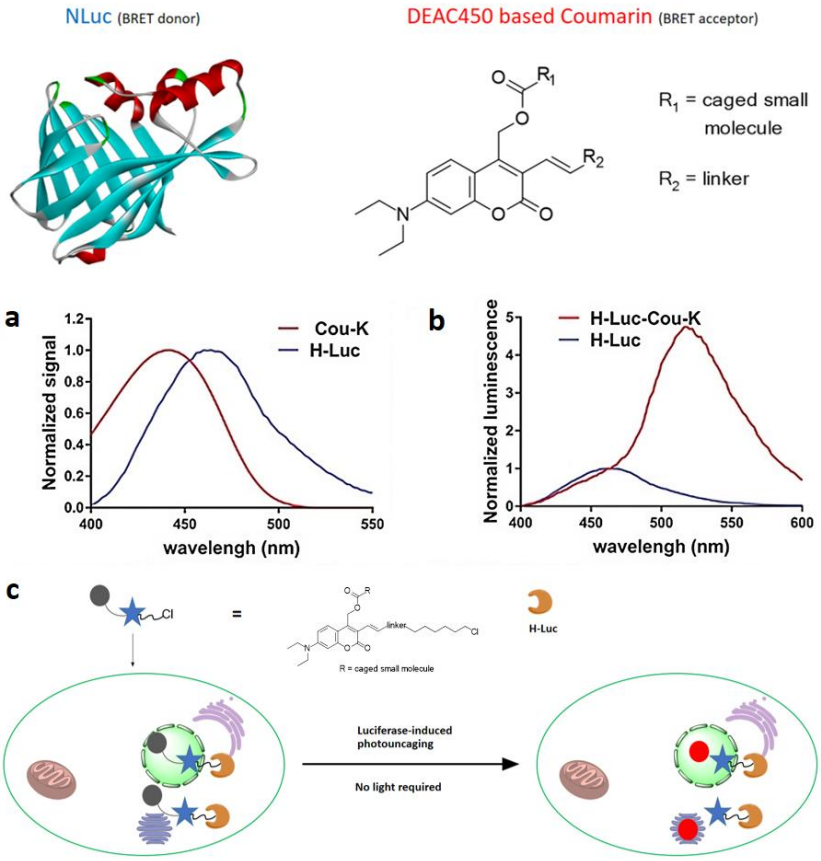


Figure 2. Bioluminolysis principle. **a)** Bioluminescence spectra of **H-Luc** (blue) and absorption spectra of DEAC450, **Cou-K** (red) have good overlap **b)** BRET spectra of H-Luc and **Cou-K** conjugate normalized to H-Luc emission. **c)** Schematic representation of bioluminolysis principle (H-Luc = orange; coumarin = blue, small molecule = grey, uncaged molecule = red). Upon addition of substrate the luciferase-induced photouncaging of small molecule can initiate (adapted from [**1**]).

The use of bioluminolysis concept was investigated through conjugation of bioactive molecule **ibrutinib** to a coumarin with a Halo-tag linker (**Cou-ibrutinib**, Figure 3). **Ibrutinib** is an irreversibly binding covalent inhibitor of the B-cell receptor pathway (strongly binds to Bruton's tyrosine kinase protein). While the synthesized **Cou-ibrutinib** was designed specifically to prevent the interaction between the inhibitor and kinase, through a presence of bulky group in this coumarin-caged molecule. Therefore only the uncaged **ibrutinib** can have an effect in the photouncaging process in living cells.[1,30] During the experiment it was established that the caged **Cou-ibrutinib** did not possess any biological activity as expected. Then in order to obtain quantitative data on target engagement via drug release in cells the target kinase was labelled with **ibrutinib-Cy5**. The treatment of SKBR3 (breast cancer cell line) with **ibrutinib-Cy5**, which is overexpressing ErbB2 followed by a subsequent wash yielded a very strong stain, which occurs from a covalent interaction of kinase-drug complex (subsequent treatment of **Cou-ibrutinib** did

not have any effect of **ibrutinib-Cy5** labelling). The inference was checked by running a control experiment with just a furimazine treatment followed by **ibrutinib-Cy5** and a wash. As predicted no interference was observed and the data obtained from the Cy5 channel was comparable to the experiments without furimazine. The main experiment revolved around the addition of furimazine after the **Cou-ibrutinib** to initiate the release of a small molecule, followed by addition of **ibrutinib-Cy5.** It very clearly illustrated in (Figure 3B) that there is no signal in both the coumarin channel (since the drug is released and is no longer visible) and no signal on the Cy5 channel, since there is no more complex binding. Therefore, this data suggests that BRET uncaging experiments can be effectively conducted in cells and do not suffer from penetrability of light or phototoxicity like conventional external light experiments.[1]

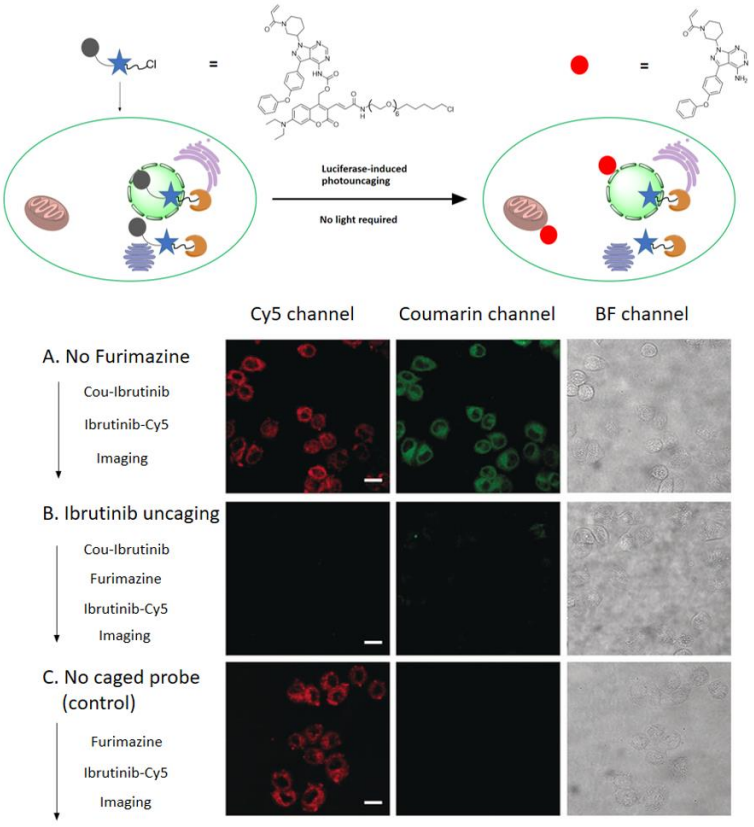
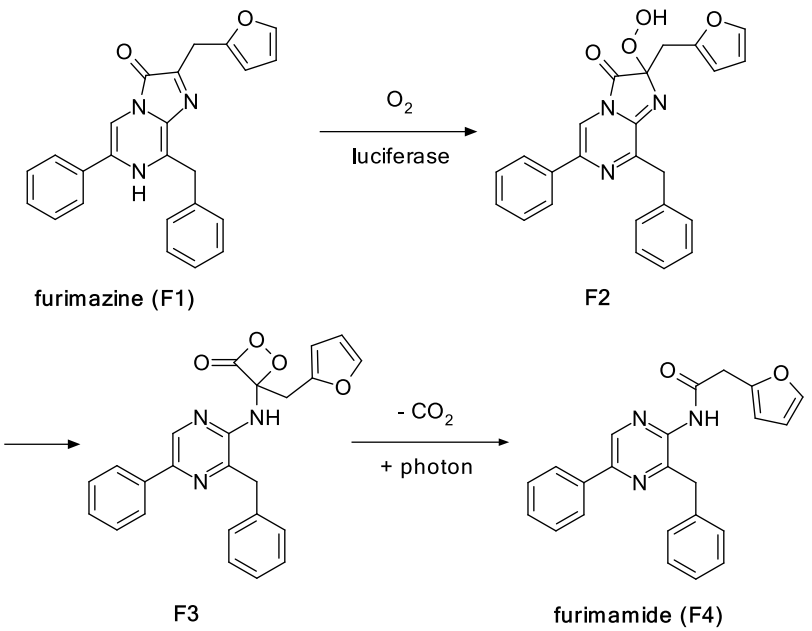


Figure 3. Uncaging of ibrutinib in live SKBR3 cell via BRET. **Cou-ibrutinib** (2 μm) was incubated with H-Luc expressing SKBR3 cells for 15 mins, then furimazine (50 μm), incubated for 30 min, washed with PBS 3x times, incubated with **ibrutinib-Cy5** (50 nm) in HBSS for 30 min followed by a final wash with HBSS, A) Caged probe with no furimazine (Visible signal in both Cy5 and Coumarin channels); B) Release of caged ibrutinib with furimazine (50 μm) (No visible signal from both Cy5 and Coumarin channels); C) Control (furimazine with no caged probe) (Only the coumarin channel does not produce any visible signal) (adapted from [**1**]).

1.2 Novel modification of existing an bioluminolysis system

Despite aforementioned advantages, the discussed bioluminescence platform has some limitations, one of them being the distance for energy transfer and as a result, quantum yield.[1] This drawback is less prominent in bioluminolysis set-ups contrary to external light systems as the process occurs inside the cell, thus in greater proximity. However, the rate of electron transfer relies on the strength of electronic coupling between the donor and the acceptor, with strength decreasing exponentially with the increasing distance.[31] In other words, if the distance is minimized, one can expect an overall increase in quantum yields and overall efficiency of the process. With this goal in mind we have considered to tether the furimazine molecule as a substrate for the H-Luc enzyme (BRET donor) to the DEAC450 coumarin as an efficient BRET acceptor, which could then initiate desired processes in cells through photouncaging of a designated small-molecule. Such an approach besides the decreased distance of the components, also excludes the necessity of furimazine addition separately. As described previously, the bioluminolysis can not take place without the consumption of substrate by H-Luc. In other words, without sufficient amount of the substrate in the system or without continuous addition of the substrate to the system the bioluminolysis can not occur. On the other hand, if the furimazine is already tethered to the BRET acceptor molecule like DEAC450 or to its analogues, not only is the necessary concentration of substrate is minimized, as a result of strictly one to one equivalent of the substrate per luciferase, but also it is no longer necessary to add external furimazine, potentially opening up new possibilities for *in vivo* experiments. Unfortunately, the substrate can not be recycled in a catalytic manner (Scheme 1). In the presence of oxygen and by catalytic activity of luciferase, furimazine gets converted into a corresponding furimamide, which is no longer active, and is not recyclable.



Scheme 1. Proposed mechanism for furimazine (**F1**) bioluminescence.

1.2.1 Biorthogonal luciferase substrates

With this in mind, we have set out on endeavor for designing molecules that could improve current bioluminolysis experiments. These compounds had to meet a few criteria to be viable for such purpose. First, the site of modification should not severely impact the efficacy of the substrate. The site of modification should be accessible, thus there must be the necessary space to allocate a linker or a molecule directly without hindering the binding mode of the substrate. And lastly the furimazine analogues conjugation chemistry should be compatible with living systems (biorthogonal chemistry), or in other words, coupling should proceed under mild and biologically friendly conditions.

With these goals in mind, the four molecules illustrated in (Figure 4) were proposed. According to the previously published research modifications at the 5' position of the furan with alkyl chains were well tolerated.[32] Then through visual inspection of the furimazine cocrystallized with the enzyme (Figure 5, PDB: 7SNT[33]) we observed that there is unoccupied space emerging from the 5' position of the furan which could allocate a linker, while benzylic and phenylic groups were inaccessible. Lastly, the criteria of bio-compatible groups and chemistry was also met, since click-chemistry has become a new staple in medicinal chemistry and biological systems chemistry.[34] Acetyl-protected furimazines **H1** and **H2**, also referred to as hikarazines, have recently been discovered as more stable precursors to their corresponding furimazine derivatives (in our case **H3** and **H4** are deprotected derivatives of **H1** and **H2** respectively).[32] Moreover, these molecules can

also be used as substrates by the H-Luc enzyme.[35] Thus, initial efforts have been aimed at the synthesis of suitable hikarazines as more stable and easily handled precursor molecules.

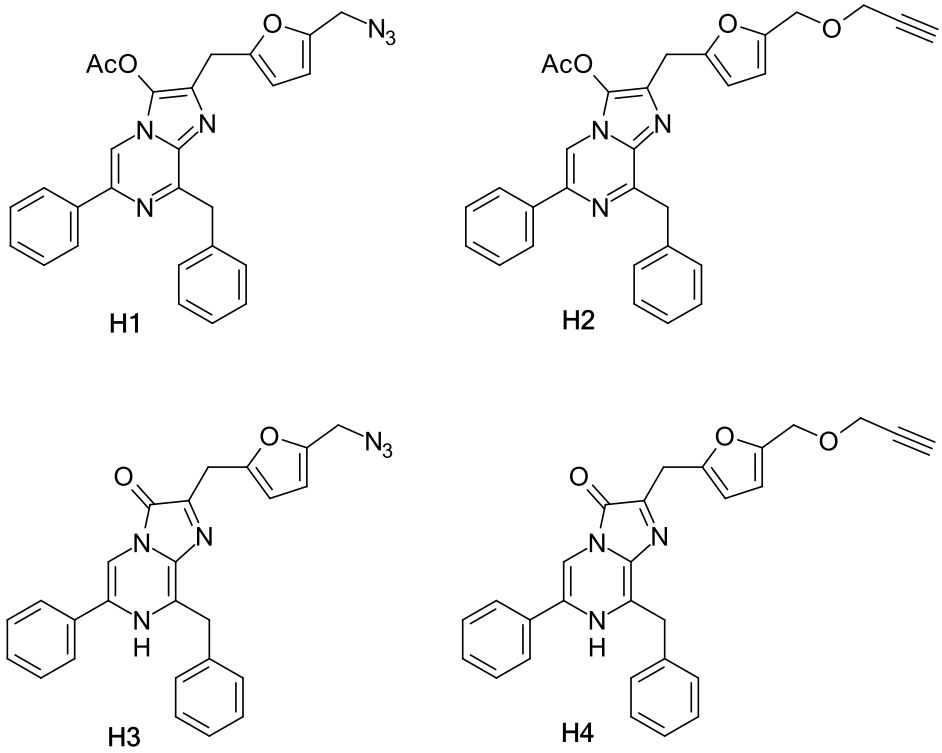


Figure 4. Proposed hikarazine analogues with access to click chemistry **H1** and **H2**, with their corresponding de-acetylated furimazine forms **H3** and **H4**.

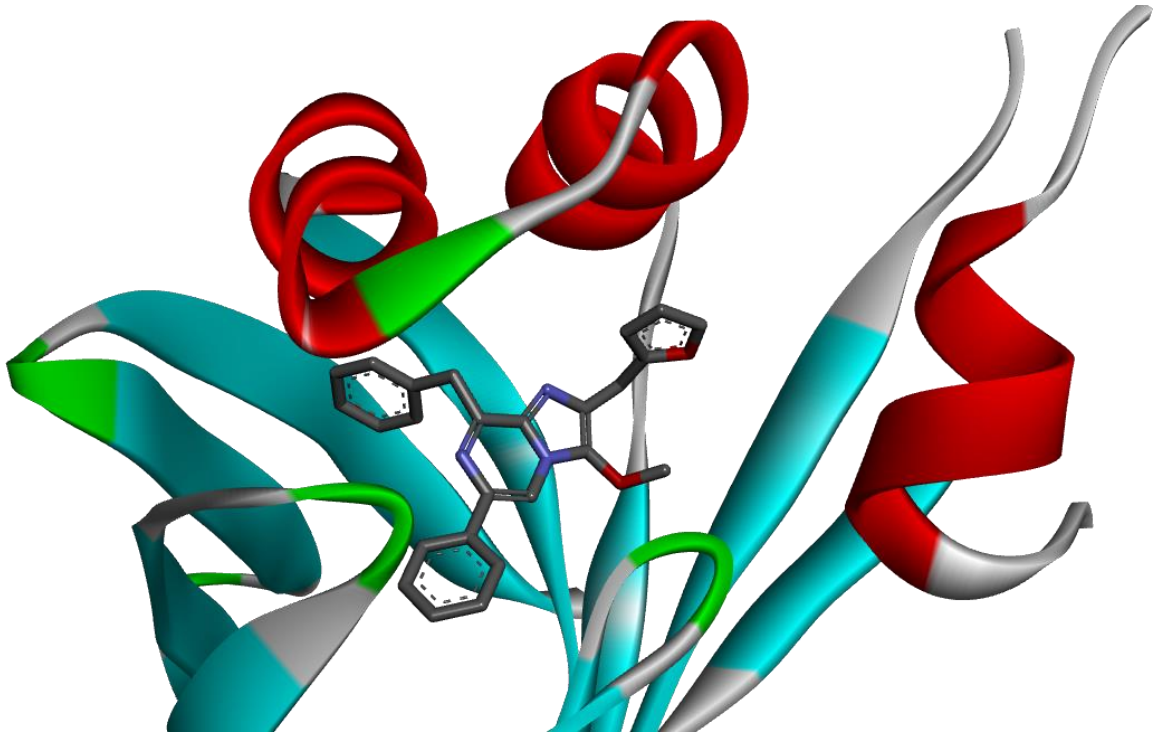
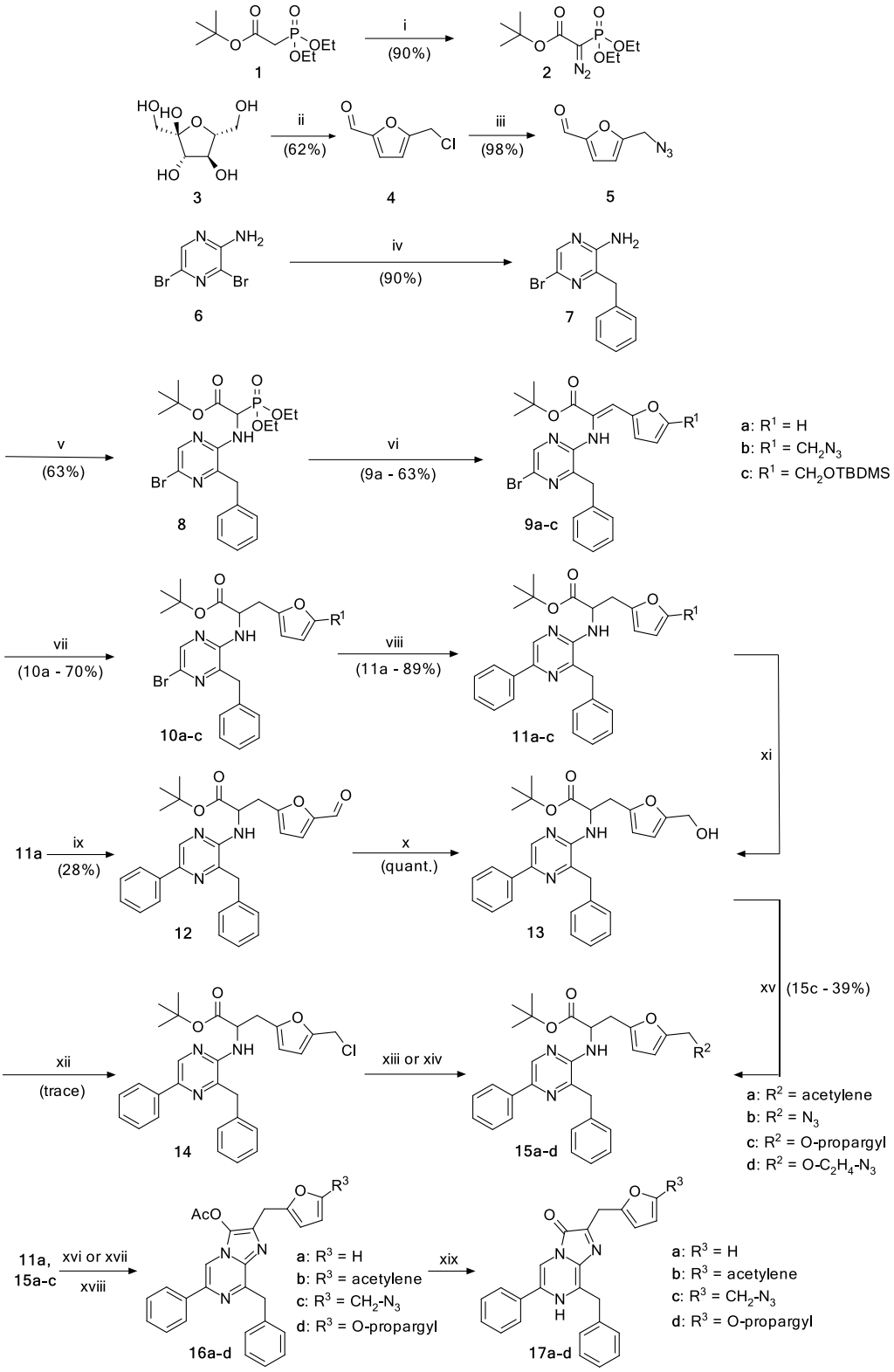


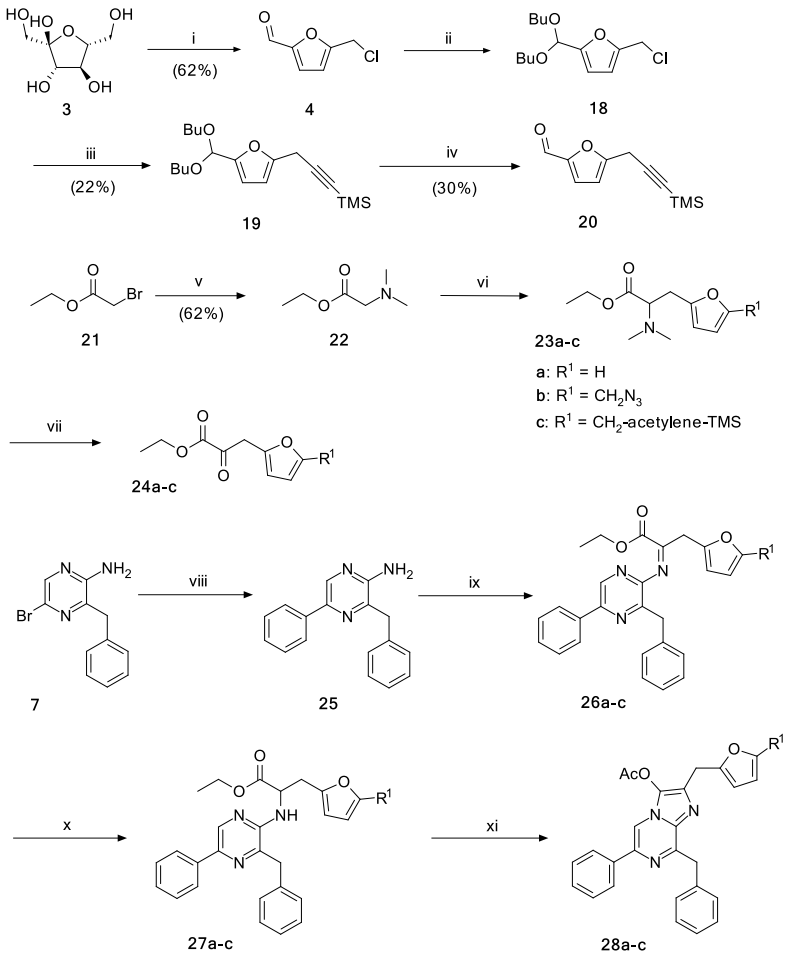
Figure 5. Snapshot of NanoLuc Luciferase with cocrystallized 3-methoxy-furimazine (PDB: 7SNT) [**33**].

1.3 Synthetic routes of newly proposed substrates



Scheme 2. i) Tosyl azide, NaH, THF, 0 °C for 1h, rt for 2h; ii) HCl 37% *aq.*, 1,2-DCE, MW, 75 °C for 20 min; iii) NaN_3, acetonitrile, reflux for 24h; iv) Benzylbromide, Zn, I_2, $(PPh_3)_2PdCl2$, THF/DMF, rt 30 min, 85 °C for 3h; v) diazo **2**, $Rh_2(OAc)_4$, chlorobenzene, 105 °C for 24h; vi) Tetramethylguanidine, furfural or **5** or HMF-TBDMS, MeOH, rt, 30min; vii) Parr-Shaker, H_2 at 45 PSI, $(PPh_3)_3RhCl$, EtOH; viii) $PhB(OH)_2$, Cs_2CO_3,

$(PPh_3)_2PdCl_2$, dioxane, 85 °C for 16 h; ix) $POCl_3$, DMF, 1,2-DCE, 0 °C for 1h, rt for 24h; x) $NaBH_4$, EtOH, 0 °C for 30 min; xi) TBAF, THF, 0 °C; xii) cyanuric acid (TCT), DMF, DCM, rt for 4h; xiii) TMS-acetylene, CuI, K_2CO_3, acetonitrile 55 °C for 24h, then TBAF, THF; xiv) NaN_3, acetonitrile, reflux for 24h; xv) N_3-C_2H_4-OTs or propargyl bromide, NaH, THF, 0 °C for 30 min, rt for 24h; xvi) TFA, DCM, rt for 24h; xvii) NaOH, THF, rt for 12h; xviii) Ac_2O, THF, rt for 2h; xix) 37% HCl *aq.*, DMSO, EtOH, 50 °C, 2 h.



Scheme 3. i) HCl 37% *aq.*, 1,2-DCE, MW, 75 °C for 20 min; ii) *n*-BuOH, *cat.* HCL 37% *aq.*, high vacuum (10.0 mmHg); iii) TMS-acetylene, CuI, K_2CO_3, acetonitrile, 55 °C overnight; iv) 1M HCL *aq.*, 2h; v) Et_3N, $(CH_2)_2NH$, acetonitrile, 0 °C for 10 min, rt for 16h; vi) NaH, furfural or **5** or **20**, *cat.* MeOH, Et_2O, 0 °C for 10 min, rt for 12h; vii) 1M HCL *aq.*, 3h; viii) $PhB(OH)_2$, $Pd(dppb)Cl_2$, $(C_6H_5CN)_2PdCl_2$, EtOH, toluene, 1M Na_2CO_3 *aq.*, 105 °C for 5h; ix) TsOH, **24a-c**, toluene, 110 °C for 12h; x) $NaBH_4$, DCM, MeOH, 0 °C for 30 min.; xi) a. NaOH, THF, rt for 12h; b. Ac_2O, rt for 2h.

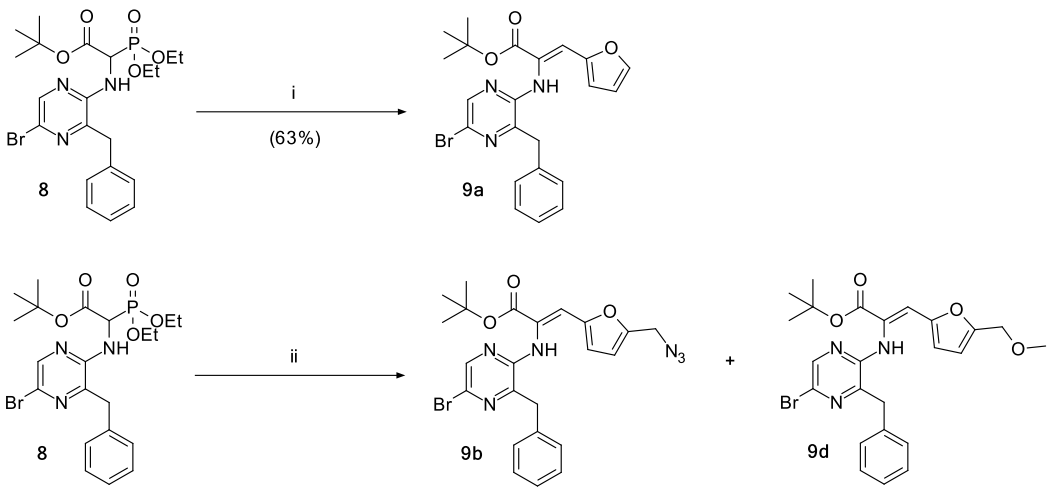
2 Discussion and results

Current work presents several routes investigated and tested for the synthesis of the proposed furimazine analogues (Schemes 2,3).

2.1 Synthesis of furimazine precursor suitable for modification

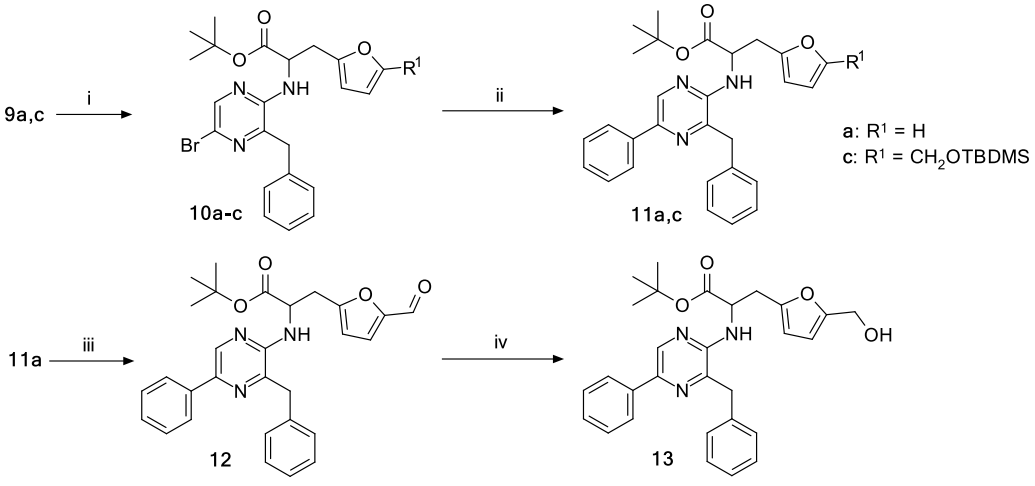
The first step is a simple Regitz diazo transfer reaction with Tosyl azide which yields an α-diazocarbonyl **2** and serves as the first building block containing the crucial carboxyl moiety.[36] The second building block containing a furyl structural motif was obtained from fructose by simple microwave reaction in acidic conditions of hydrochloric acid to afford compound chloromethylfurfural (CMF) **4**.[37] Part of this product was converted to an azide derivative while the other fraction was protected by butanol to yield an acetal **18** (Scheme 3). **18** was then successfully subjected to Sonogashira coupling with acetylene-TMS and deprotected to afford an aldehyde **20** which could be coupled to the other building blocks of furimazine.[38]

The pyrazine core was assembled by a carbon-carbon coupling between dibromopyrazine **6** and the bromobenzyl in the presence of a palladium catalyst, to afford compound **7** (Scheme 2)[36]. **7** can then be either subjected to an early Suzuki coupling to form **25** as a complete pyrazine building block (Scheme 3)[39], or functionalized with the phenyl moiety at a later stage.[36] Throughout the majority of research publications, the latter strategy was commonly used.[36,40,41] The benzylpyrazine **7** was coupled to previously synthesized α-diazocarbonyl **2** building block with assistance of a rhodium catalyst commonly used in these coupling reactions. The obtained compound **8** can then be coupled with various aldehyde building blocks containing a furyl structural motif.[40,41] Precisely, a regular furfural and the synthesized furfural **5** were used in the Horner–Wadsworth–Emmons (HME) type of reaction to form **9a** and **9b** (Scheme 4).[40,41] Due to inability to synthesize **9b** as a result of Tetramethylguanidine's (TMG) basicity (Appendices; **A1**), a small detour was made, and we have shifted our attention to further synthesis of **9a**.



Scheme 4. i) Tetramethylguanidine, furfural, MeOH, rt, 30min; i) Tetramethylguanidine, furfural **5**, MeOH, rt, 30min.

As a result, the hikarazine with a methylazide functionality was considered unattainable via proposed reaction pathway, and the focus was shifted to other functional groups. The reduction of **9a,c** to **10a,c** was performed under the hydrogen atmosphere and in presence of Wilkinson catalyst, since the reduction by an established sodium borohydride-treatment was not successful.[40] This step was followed up by a classical Suzuki reaction to yield expected products **11a,c**.[40,41] Consequently, the synthesized **11a** was functionalized by a classical Vilsmeier-Haack (VH) reaction conditions to yield a corresponding aldehyde **12**, which was then reduced with sodium borohydride to an alcohol **13** (Scheme 5).[42,43]



Scheme 5. i) Parr-Shaker, H_2 at 45 PSI, $(PPh_3)_3RhCl$, EtOH; ii) $PhB(OH)_2$, Cs_2CO_3, $(PPh_3)_2PdCl_2$, dioxane, 85 °C for 16 h; iii) $POCl_3$, DMF, 1,2-DCE, 0 °C for 1h, rt for 24h; iv) $NaBH_4$, EtOH, 0 °C for 30 min.

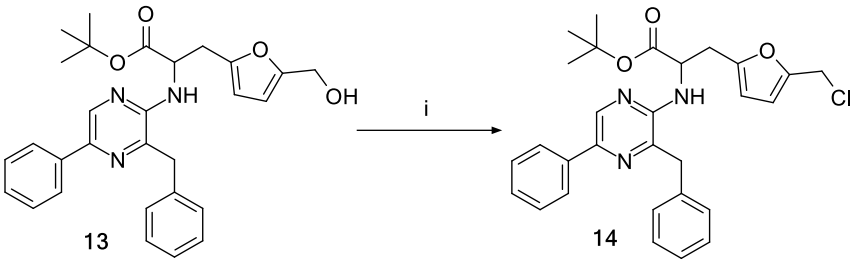
To the best of our knowledge, both of these reactions were previously unexplored approaches of functionalizing and modifying furan motif of the potential furimazine analogues. The compound

13 was also then synthesized in our laboratory by a post-doc researcher with a different reaction pathway (Scheme 1). This synthetic route started from a common biomass recycling product hydroxymethylfurfural (HMF)[44], which was then protected with tert-Butyldimethylsilyl (TBDMS) group, yielding a desirable building block. This furfural was then subjected to the standard procedure of the furimazine synthesis involving the repetition of the HME reaction and the same steps as described for compounds **9a-11a.** The only difference is that the resulting compound **11c** required a subsequent protecting group removal step with tetra-*n*-butylammonium fluoride (TBAF) leading up to the same product **13** (Appendices; **A3**) obtained previously in the project.

2.2 Investigation into linker conjugation to a synthesized precursor

Then we attempted to couple two linkers to **13**, an azide L2 and propargyl (Appendices; **A2**). Our attempts to attach linker **L2**, though a common sodium hydride chemistry were not successful. However, the reaction with the propargyl bromide and sodium hydride proved to be reproducible, albeit the conversion of the formed **15c** into its corresponding furimazine or hikarazine product proved to be unachievable as a result of degradation under hydrolytic conditions (Supplementary; **S1**).

Therefore, we took a few steps back and returned to the compound **13** and evaluated a possibility of substituting a hydroxyl group with halogen. This approach should exclude the labile ester bridge connection between the furimazine precursor and the linker. Due to the change from a C-O bond to a purely C-C bond, the compound is expected to be unaffected by linker cleavage under classical hydrolysis conditions. First trial was conducted with classical Appel reaction conditions. Unfortunately, no formation of the desired product could be observed (Appendices; **A1**). Then **13** was then directly subjected to new conditions[45] involving dimethylformamide (DMF) and cyanuric acid (TCT) which together form a positively charged intermediate, thus corroborating to the formation of desired product **14** (Scheme 6). The reaction was monitored by LC-MS (Appendices; **A3**). However, the desired product could not be isolated for NMR analysis to confirm the findings. Only trace amounts of compound were observed. Change in retention time of the compound on LC, and a correct mass of the analyte, suggest that product **14** was in fact present. However, the reaction needs further optimization.

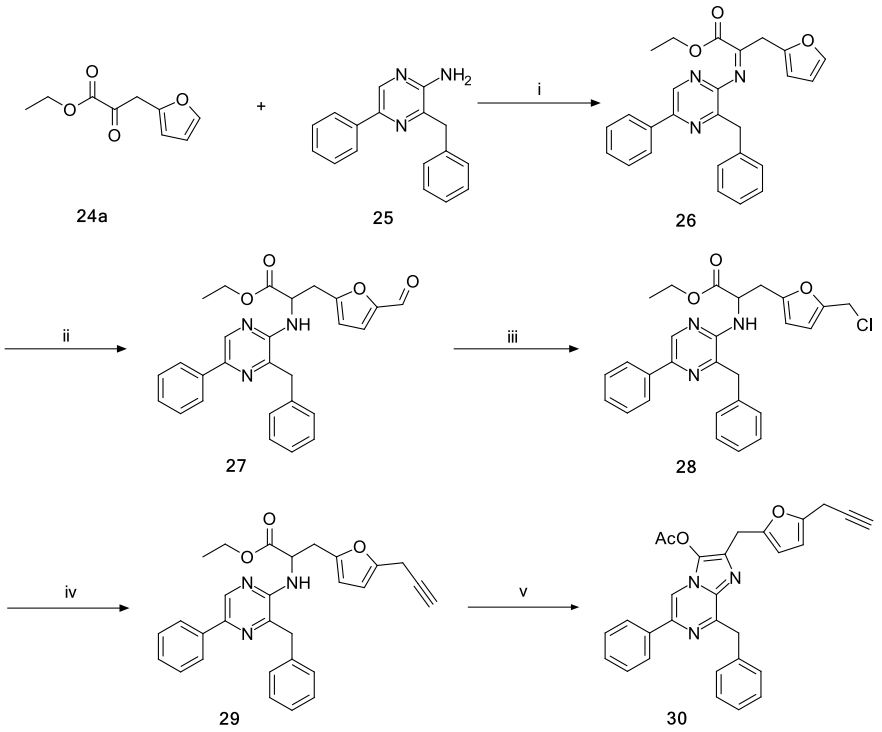


Scheme 6. i) cyanuric acid (TCT), DMF, DCM, rt for 4h.

2.3 Proposed synthetic route for the desired substrate molecule

Due to insufficient amount of products for further experiments, a new synthetic pathway depicted in the (Scheme 3) was proposed and carried out.[46] Herein, the furfural is modified at an early synthetic stage. Such strategy did not yield the desired compounds **24a-c**. The coupling step of the furfurals **5** and **20** to the glycine moiety of **22** led to degradation of product (Appendices; **A1**). the This finding suggests, that the furan moiety should be decorated at a later stage in the synthesis, once all of the building blocks have been conjugated together. Alternatively, the VH reaction can be attempted with **23a** to see if consecutive synthetic steps will work.

The accumulated learnings from previous attempts led to the proposed design illustrated in (Scheme 7). This strategy relies on the assembly of **26** which is described in the patent.[46] According to it, compound **23a** is subjected to an overnight acidic conditions to form on oxalic like motif in the compound **24a** which is coupled to **25** (product of Suzuki coupling of desired aryl with **7**) in presence of a lewis acid (*p*-toluenesulfonic acid) resulting in product **26a** (Scheme 7). Then we propose a sodium borohydride reduction, or a reduction in hydrogen atmosphere if the first strategy will not work, to get compound **27a**. The formation of a fully assembled uncyclized intermediate **27a**, which has an ethyl ester contrary to the **11a** with a *tert*-butyl ester, can be performed without the use of highly expensive rhodium catalyst.[47]



Scheme 7. i) TsOH, **24a-c**, toluene, 110 °C for 12h; ii) a. $NaBH_4$, DCM, MeOH, 0 °C for 30 min.; b. $POCl_3$, DMF, 1,2-DCE, 0 °C for 1h, rt for 24h; iii) a. $NaBH_4$, EtOH, 0 °C for 30 min; b. cyanuric acid (TCT), DMF, DCM, rt for 4h; iv) a. TMS-acetylene, CuI, K_2CO_3, acetonitrile, 55 °C overnight; b. TBAF, THF; v) a. TFA, DCM, rt for 24h; b. Ac_2O, THF, rt for 2h.

At this point, further synthetic steps should be identical to compound **11a.** First the compound is subjected to VH conditions, followed by a subsequent reduction and halogen substitution to from a **14**-like product. Then a Sonogashira reaction can be performed. According to the success of this reaction for the compound **19** which is sharing the same chloromethylfurfural structural motif, such approach is expected to proceed well. Then if the used alkyne is bearing a protective group, it can be removed. The formed product can then be cyclized under general furimazine/hikarazine conditions, of an ester deprotection in TFA and subsequent cyclization in acetic acid.

In case the compound will not be attainable via this pathway, it may maybe worthwhile to investigate the use of alternative conjugation points, like amine groups which form very stable amides or use of aldehyde for conversion to alkynes with Bestmann-Ohira reagents.[48,49] In particular, the latter option seems very interesting as throughout the proposed pathway we already form compounds with an aldehyde group, like **12** or **27**. Therefore investigation into this approach is definitely worthwhile to test, as it will giver direct access to furimazine precursors with the linkers of interest.

The focus of this work revolved around modifications of the methyl furfuryl part of the furimazine, due to easy accessibility of the compounds and also higher flexibility of the adducts, which may in turn result in better fit in the pocket of the luciferase's active site, thus decrease in the activity might not be very significant. On the other hand, extending the methyl moiety carbon chain length or removing it completely might result in greater compound stability, while not affecting the activity of luciferase drastically.

Several generations of executed synthetic routes highlighted the main problems of our previous attempts and presented new ways to overcome them. It was established that addition of linker to our furimazine core should occur only after the assembly of our uncyclized-furimazine structural motif. This decision is dictated by both the degradation of the linker moiety throughout the route and also inability to join two building block together in some cases. The other stepping stone on our way to achieve decorated furimazine was labile ester bond susceptible to hydrolysis. The use of well-established carbon-carbon bond forming chemistry is expected to yield the cyclized furimazines and hikarazines with various set of linkers. However, in order to prove our theory, the synthesis of this compound will need to be performed in the future.

3 Conclusion

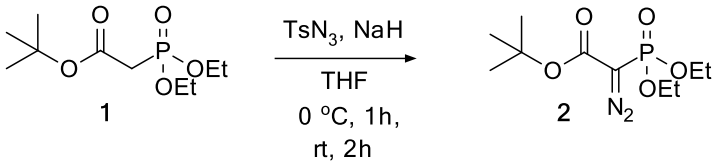
In summary, we have investigated several synthetic pathways to generate suitable precursors for bioconjugatable furimazine analogs. Out of them two novel compounds **12** and **13** were synthesized, characterized and they have a great potential in further modifications and optimizations as valuable precursors to the desired hikarazines and furimazines. Unfortunately, current attempts to synthesize desired luciferase substrates were not successful. However, the encountered problems and pitfalls are valuable experience which will greatly assist in future research with hopes of synthesizing molecules with good BRET quantum yields for bioluminolysis experiments.

4 Acknowledgements

I would like to thank the whole Winssinger group for providing valuable insights and assistance in the project. Jose Laxio-Arenas for additional tests and experiments conducted in parallel and Nicolas Winssinger for accepting me into the group during my exchange, guidance and evaluation of the proposed synthetic strategies.

5 Experimental

***Tert*-Butyl 2-diazo-2-(diethoxyphosphoryl)acetate (2)**

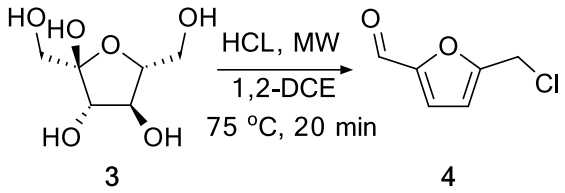


To a solution of 4-methylbenzenesulfonyl azide (8.0 g, 40.5 mmol) in THF (85ml) at 0 °C, sodium hydride was added (1.6 g, 40.5 mmol, 60% oil dispersion) in three batches. Then to this mixture *tert*-butyl 2-(diethoxyphosphoryl)acetate (8.5 g, 33.8 mmol) was added dropwise over 30 min. The mixture was stirred for 1 h at 0 °C and 2 h at rt. After completion, water was added and the reaction was extracted with ether. The two layers were separated and the aqueous layer was extracted with ether two times. The organic layers were combined, dried with sodium sulfate, filtered, concentrated *in vacuo* and purified with silica gel chromatography (EtOAc/pentane, 90/10, v/v) to afford the desired product (8.04 g, 90%) as a yellow mobile oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.26 – 4.06 (m, 4H), 1.48 (s, 9H), 1.34 (td, *J* = 7.1, 0.8 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 162.60, 162.48, 129.59, 126.40, 83.02, 63.47, 63.41, 28.23, 21.48, 16.17, 16.10.

5-(Chloromethyl)furfural (4)



To a 35 mL CEM microwave reactor vessel were added fructose (1.0 g, 5.6 mmol), 37 % *aq.* HCl (6.5 mL), 1,2-Dichloroethane (6.5 mL) and mixed for 2 minutes before sealing the vessel. Same procedure was repeated for seven other vessels. Then the reaction mixtures were placed in a Microwave Synthesizer for 20 minutes at 75 °C one at a time. Upon completion the reaction content of the vessels was filtered over Celite® and transferred to a separator funnel. The lower organic layer was collected and the aqueous layer was washed three times with 1,2-Dichloroethane. The organic layers were combined and the solvent was removed *in vacuo* to yield

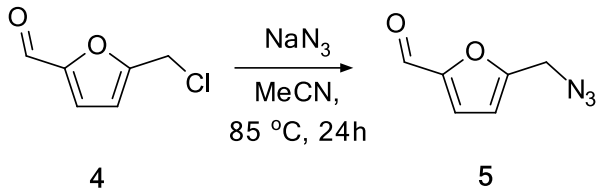
a dark brown oil (4.73 g, 74%), which was already pure for most reactions. Additionally, the product was purified via column chromatography to yield a yellow oil (4.00 g, 62%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.64 (s, 1H), 7.20 (d, *J* = 3.6 Hz, 1H), 6.59 (d, *J* = 3.6 Hz, 1H), 4.61 (s, 2H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 177.75, 156.06, 152.90, 121.61, 111.91, 36.51.

FT-IR: 3124, 1672, 1519, 1400, 1261, 1198, 1021, 969, 806, 770, 754, 720 cm^{-1}

5-(Azidomethyl)furfural (5)



4 5

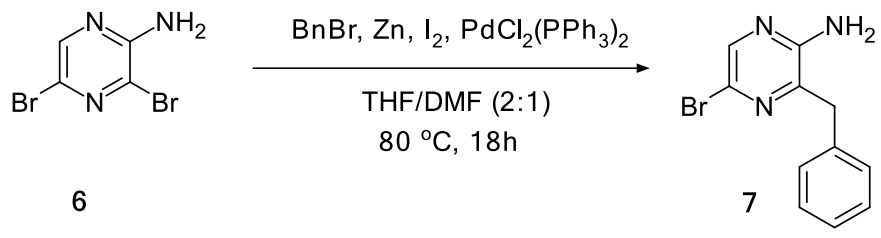
To a solution of **4** (2.0 g, 13.9 mmol) in acetonitrile (40 mL), sodium azide (1.8 g, 27.8 mmol) was added. The mixture was heated to 85 °C and stirred for 24 h. The mixture was then filtered through Celite and the solvent was evaporated under reduced pressure. The residue was dissolved in diethyl ether, filtered through Celite and the solvent was evaporated under reduced pressure. The product was obtained as an orange oil (2.14 g, 98%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.62 (s, 1H), 7.21 (d, *J* = 3.5 Hz, 1H), 6.55 (dd, *J* = 3.5, 0.7 Hz, 1H), 4.41 (s, 2H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 177.64, 155.35, 152.97, 121.80, 111.45, 46.99.

FT-IR: 3123, 2838, 2094, 1671, 1519, 1400, 1270, 1021, 807 cm^{-1}

3-Benzyl-5-bromopyrazin-2-amine (7)



6 7

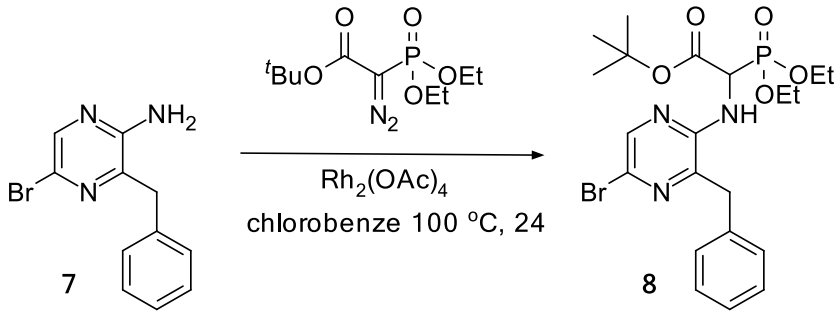
To a mixture of dry THF/DMF (2:1) 0.3 M, Zn dust (5.0 g, 76.5 mmol) and I_2 (0.5 g, 10 % of Zn) under protective atmosphere of N_2 were added and stirred until the brown color disappeared. Then benzylbromide (3.75 mL, 30 mmol) was added dropwise, and the reaction mixture was refluxed at 85 ºC for 3 h. Then to the resulting mixture a suspension of 2-amino-3,5-dibromopyrazine (5.0 g, 20.0 mmol) and $PdCl_2(PPh_3)_2$ (0,70 g, 1.0 mmol) in dry THF/DMF (2:1) were added. The reaction was stirred overnight and then filtered through celite. The filtrate was poured in water and extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered and the solvent was removed *in vacuo*. The residue was dissolved in DCM and loaded onto silica and purified by chromatography on silica gel (EtOAc/pentane, 30/70, v/v) to yield a viscous yellow solid (4.75 g, 90%).

LC-MS-ESI (m/z): Calcd for $C_{11}H_{10}BrN_3$ $[M+H]^+$: 264.01, Found 264.05

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.40 – 7.18 (m, 5H), 4.50 (br s, 2H), 4.09 (s, 2H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 151.78, 142.34, 141.53, 135.56, 129.23, 128.49, 127.47, 126.21, 40.85.

***Tert*-butyl 2-[(3-benzyl-5-bromopyrazin-2-yl)amino]-2-(diethoxyphosphoryl)acetate (8)**



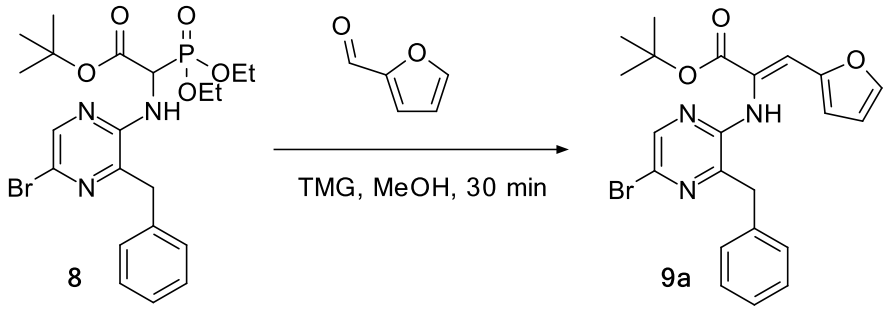
To a 50 mL roudbottom flask were added 3-benzyl-5-bromopyrazine-2-amine (0.93 g, 3.5 mmol), *tert*-butyl 2-diazo-2-(diethoxyphosphoryl) acetate (1.36 g, 5.2 mmol), $Rh_2(OAc)_4$ (144 mg, 10 mol%) and 15 mL of chlorobenzene. The reaction was refluxed for 24h and then extracted with ethyl acetate and washed with brine. After being dried over anhydrous sodium sulfate the solvent was removed *in vacuo* and the crude product was dissolved in DCM, loaded onto silica and purified by chromatography on silica gel (EtOAc/pentane, 30/70, v/v) to give an off-white solid (1.14 g, 63%).

LC-MS-ESI (m/z): Calcd for $C_{17}H_{22}BrN_3O_5P$ $[M+H]^+$: 460.05, Found 459.81

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (s, 1H), 7.33 – 7.15 (m, 5H), 5.17 (dd, *J* = 8.0, 5.3 Hz, 1H), 4.95 (dd, *J* = 21.6, 8.0 Hz, 1H), 4.15 – 3.78 (m, 6H), 1.38 (s, 9H), 1.18 (dt, *J* = 20.9, 7.4 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 166.14, 150.28, 142.92, 141.59, 135.51, 129.01, 128.73, 127.18, 126.31, 83.11, 63.50, 63.43, 63.37, 63.31, 54.27, 52.83, 40.38, 27.83, 21.05, 16.33, 16.27.

***Tert*-butyl (2Z)-2-[(3-benzyl-5-bromopyrazin-2-yl)amino]-3-(furan-2-yl)prop-2-enoate (9a)**



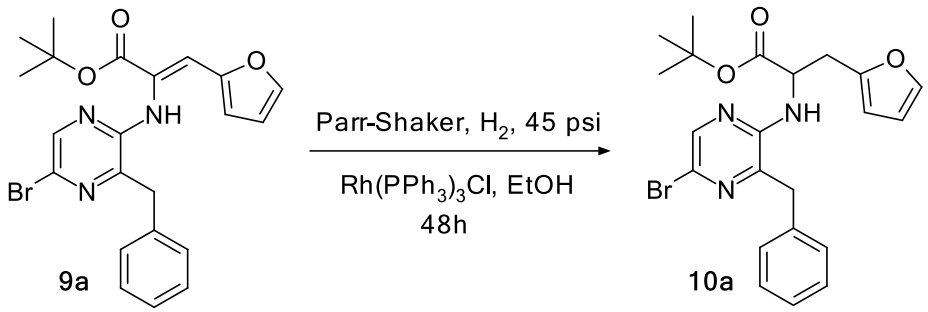
To a solution of **8** (806 mg, 1.6 mmol) in MeOH (30 mL), furfural (226 mg, 2.4 mmol) was added. Tetramethylguanidine (0.59 mL, 4,7 mmol) was added dropwise and the reaction stirred at rt for 30 min. Then DCM was added and transferred to the separatory funnel. The organic layer was extracted with 0.1 M HCl, and the lower organic layer was drained. The aqueous layer was extracted two times with DCM. Collected organic layers were dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The resulting crude product was purified by silica gel chromatography (EtOAc/pentane, 30/70, v/v) to give an off-white solid (450 mg, 63%).

LC-MS-ESI (m/z): Calcd for $C_{18}H_{15}BrN_3O_3$ $[M+H]^+$: 400.03, Found 399.92

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (s, 1H), 7.42 – 7.24 (m, 5H), 6.94 (d, *J* = 1.8 Hz, 1H), 6.88 (s, 1H), 6.67 (s, 1H), 6.27 (dd, *J* = 3.5, 1.8 Hz, 1H), 6.14 (d, *J* = 3.4 Hz, 1H), 4.25 (s, 2H), 1.43 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 164.01, 150.37, 149.59, 143.84, 143.25, 142.11, 129.22, 128.81, 128.33, 127.53, 127.30, 112.70, 111.69, 110.83, 81.71, 40.49, 27.90.

***Tert*-butyl 2-[(3-benzyl-5-bromopyrazin-2-yl)amino]-3-(furan-2-yl)propanoate (10a)**



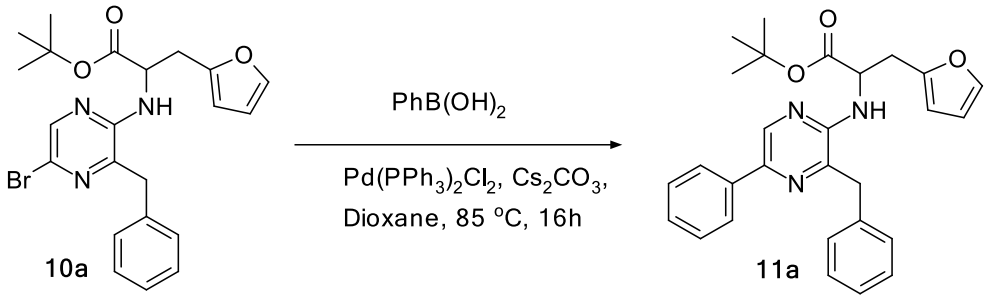
To a Parr-Shaker flask **9a** (440 mg, 0.96 mmol) and $Rh(PPh_3)_3Cl$ (92 mg, 0.1 mmol) were added followed by EtOH (30 mL). The flask was sealed and atmosphere was exchanged to hydrogen. The pressure was kept above 40 psi at all times never exceeding 45 psi, and the flask was kept shaking for 48 h. Then the volatiles were evaporated under reduced pressure, the residue was dissolved in DCM, loaded onto silica and purified by silica gel chromatography (EtOAc/pentane, 10/90, v/v) to give a yellow solid (309 mg, 70%).

LC-MS-ESI (m/z): Calcd for $C_{18}H_{17}BrN_3O_3$ $[M+H]^+$: 402.04, Found 401.92

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.34 – 7.17 (m, 5H), 6.18 (dd, *J* = 3.2, 1.9 Hz, 1H), 5.75 (dd, *J* = 3.2, 0.8 Hz, 1H), 5.13 (d, *J* = 7.4 Hz, 1H), 4.69 (dt, *J* = 7.4, 5.2 Hz, 1H), 4.03 (d, *J* = 4.4 Hz, 2H), 3.11 (d, *J* = 5.2 Hz, 2H), 1.36 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 170.52, 150.49, 142.53, 141.73, 141.49, 135.59, 128.98, 128.63, 127.13, 125.15, 110.28, 107.76, 82.08, 53.82, 40.36, 30.08, 27.85.

***Tert*-butyl 2-[(3-benzyl-5-phenylpyrazin-2-yl)amino]-3-(furan-2-yl)propanoate (11a)**



To a flask with dioxane (20 mL), under protective atmosphere of nitrogen were added **10a** (291 mg, 0.60 mmol), phenylboronic acid (81mg, 0.65 mmol), $Pd(PPh_3)_2(Cl)_2$ (44 mg, 10 mol%), Cs_2CO_3 (587 mg, 1.80 mmol) and stirred for 16h at 85 °C. Then the volatiles were evaporated

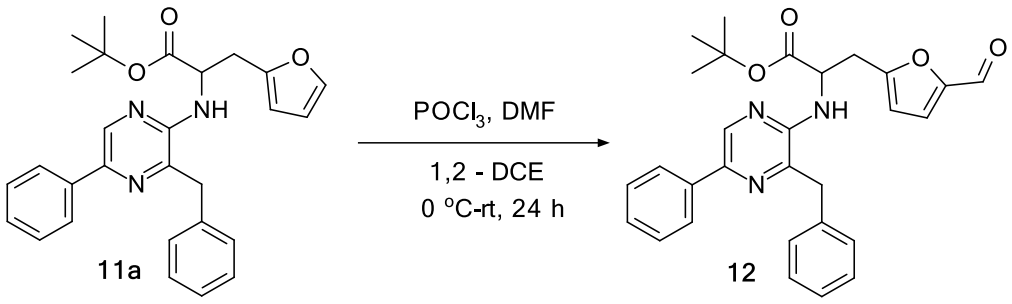
under reduced pressure, the residue was dissolved in DCM, absorbed on silica and purified by silica gel column chromatography (EtOAc/pentane, 10/90, v/v) to give a yellow solid (257 mg, 89%).

LC-MS-ESI (m/z): Calcd for $C_{24}H_{22}N_3O_3$ $[M+H]^+$: 400.17, Found 400.08

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.38 (s, 1H), 7.97 – 7.89 (m, 2H), 7.50 – 7.41 (m, 2H), 7.40 – 7.19 (m, 6H), 6.19 (dd, *J* = 3.2, 1.9 Hz, 1H), 5.79 (dd, *J* = 3.2, 0.8 Hz, 1H), 5.26 (d, *J* = 7.5 Hz, 1H), 4.87 (dt, *J* = 7.5, 5.2 Hz, 1H), 4.17 (d, *J* = 5.4 Hz, 2H), 3.18 (d, *J* = 5.1 Hz, 2H), 1.38 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 170.69, 150.61, 141.72, 140.84, 136.26, 128.88, 128.82, 128.02, 126.95, 125.68, 110.30, 107.80, 82.11, 53.91, 40.69, 30.94, 30.29, 27.87.

***Tert*-butyl-2-[(3-benzyl-5-phenylpyrazin-2-yl)amino]-3-(5-formylfuran-2-yl)propanoate(12)**



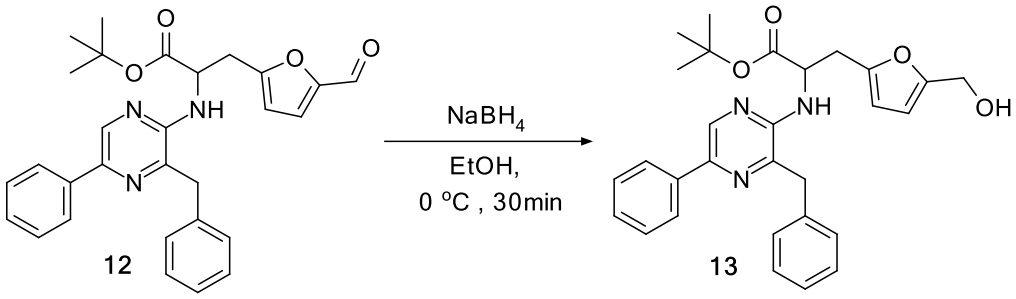
A flask with 1,2 - DCE (15mL), was placed on an ice bath and kept at 0 ºC. Then under protective atmosphere of nitrogen were added DMF (0.694 mL, 8.5 mmol), $POCl_3$ (0.714 mL, 7.7 mmol) and stirred for 15min. To the mixture dropwise **11a** (257 mg, 0.6 mmol) was added, stirred for 15 minutes, brought to rt and stirred overnight. The reaction was washed with saturated sodium carbonate and extracted with ether three times. The collected organic layers were washed three times with water, then brine. The organic layer was dried over anhydrous sodium sulfate, filtered and reduced *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc/pentane, 20/80, v/v) to give a grey-purple solid (76.6 mg, 28%).

LC-MS-ESI (m/z): Calcd for $C_{29}H_{30}N_3O_4$$[M+H]^+$: 484.22, Found 484.08

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.48 (s, 1H), 8.38 (s, 1H), 7.97 – 7.89 (m, 2H), 7.49 – 7.40 (m, 2H), 7.39 – 7.30 (m, 1H), 7.29 – 7.19 (m, 4H), 6.98 (d, *J* = 3.5 Hz, 1H), 5.79 (d, *J* = 3.5 Hz, 1H), 5.18 (d, *J* = 6.8 Hz, 1H), 4.88 (dt, *J* = 6.8, 5.3 Hz, 1H), 4.22 (d, *J* = 15.2 Hz, 1H), 4.11 (d, *J* = 15.3 Hz, 1H), 3.34 (dd, *J* = 15.3, 5.4 Hz, 1H), 3.21 (dd, *J* = 15.2, 5.2 Hz, 1H), 1.41 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 177.01, 170.40, 158.26, 152.13, 149.86, 141.26, 141.20, 137.40, 136.80, 136.63, 128.89, 128.80, 128.74, 127.90, 126.91, 125.68, 122.61, 110.80, 82.72, 53.17, 40.92, 30.79, 27.89.

***Tert*-butyl-2-[(3-benzyl-5-phenylpyrazin-2-yl)amino]-3-[5-(hydroxymethyl)furan-2-yl]propanoate (13)**



To a solution of **12** (61.7 mg, 0.13 mmol) in absolute ethanol (7 mL) cooled at 0 °C, $NaBH_4$ (1.69 mg, 0.05 mmol) was added. The reaction was stirred at 0 °C for 4 hours and then washed with saturated solution of NH_4Cl and extracted 5 times with DCM. The combined organic phases were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to afford a yellow solid (61.1 mg, 99%).

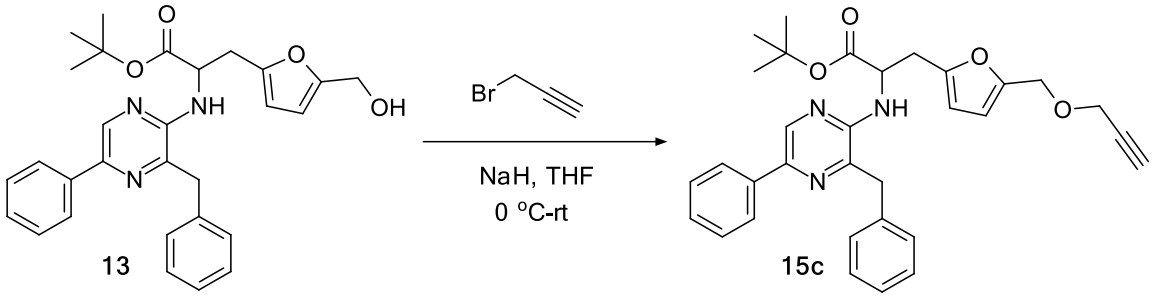
LC-MS-ESI (m/z): Calcd for $C_{29}H_{32}N_3O_4[M+H]^+$: 486.24, Found 486.13

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 (s, 1H), 7.96 – 7.89 (m, 2H), 7.49 – 7.40 (m, 2H), 7.39 – 7.18 (m, 5H), 6.08 (d, *J* = 3.1 Hz, 1H), 5.71 (d, *J* = 3.1 Hz, 1H), 5.12 (d, *J* = 7.4 Hz, 1H), 4.82 (dt, *J* = 7.4, 5.3 Hz, 1H), 4.48 (s, 2H), 4.22 – 4.07 (m, 2H), 3.22 – 3.07 (m, 2H), 2.00 (br s, 1H), 1.41 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 171.07, 153.18, 150.96, 150.27, 141.12, 140.95, 137.52, 136.80, 136.70, 128.80, 128.76, 127.79, 126.79, 125.64, 108.65, 108.57, 108.54, 81.97, 57.49, 53.50, 40.69, 30.57, 27.92.

FT-IR: 3422, 2920, 2851, 1727, 1513, 1486, 1369, 1152, 1014, 786, 695 cm^{-1}

***Tert*-butyl-2-[(3-benzyl-5-phenylpyrazin-2-yl)amino]-3-{5-[(prop-2-yn-1-yloxy)methyl]furan-2-yl}propanoate (15c)**



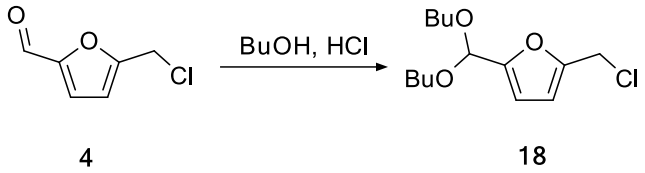
To a solution of **13** (27.7 mg, 57 µmol) in dry THF (5 mL) cooled at 0 ºC, sodium hydride (4.6 mg, 114 µmol, 60% oil dispersion) was added. Then dropwise propargyl bromide (17.0 mg, 114 µmol, 80% in Toluene) was added and stirred for 3 h. The reaction was brought to rt and stirred overnight, then quenched with water and extracted three times with DCM. The combined organic layers were dried over anhydrous sodium sulfate, filtered, concentrated *in vacuo* and purified by silica gel column chromatography (EtOAc/pentane, 10/90, v/v) to give a yellow solid (11.5 mg, 39%).

LC-MS-ESI (m/z): Calcd for $C_{32}H_{34}N_3O_4[M+H]^+$: 524.25, Found 524.13

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.38 (s, 1H), 7.96 – 7.89 (m, 2H), 7.48 – 7.40 (m, 2H), 7.37 – 7.20 (m, 7H), 6.23 – 6.14 (m, 1H), 5.70 (d, *J* = 3.2 Hz, 1H), 5.35 (s, 1H), 5.13 (d, *J* = 7.4 Hz, 1H), 4.81 (dt, *J* = 7.4, 5.2 Hz, 1H), 4.45 (s, 2H), 4.10 (d, *J* = 2.4 Hz, 2H), 3.23 – 3.07 (m, 2H), 1.40 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 170.94, 151.57, 150.26, 141.16, 137.56, 136.80, 128.79, 127.76, 126.80, 125.64, 111.12, 108.51, 81.96, 79.36, 74.80, 63.06, 56.54, 53.50, 40.71, 30.51, 27.93.

2-(Chloromethyl)-5-(dibutoxymethyl)furan (18)

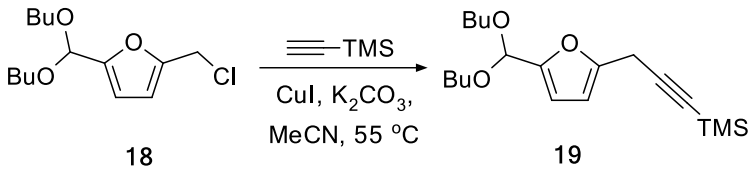


4 18

To a solution of **4** (4.0 g, 27.7 mmol) in BuOH (80 mL), was added 37 % *aq.* HCl (0.4 mL) and the solvent was removed on rotary evaporator under high vacuum (10.0 mmHg). The crude product was obtained as yellow oil (5.0 g, 52%) and used further without purification.

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.39 – 6.30 (m, 2H), 5.50 (d, *J* = 0.8 Hz, 1H), 4.57 (s, 2H), 3.58 – 3.48 (m, 3H), 1.60 – 1.52 (m, 4H), 1.44 – 1.31 (m, 4H), 0.91 (t, *J* = 7.3 Hz, 6H).

{3-[5-(Dibutoxymethyl)furan-2-yl]prop-1-yn-1-yl}trimethylsilane (19)



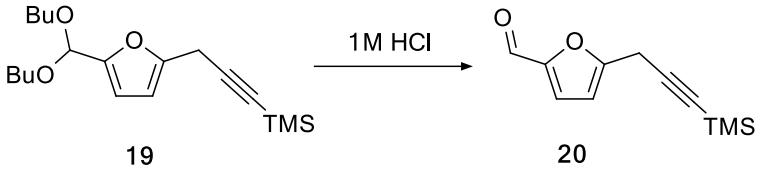
18 19

To a solution of **18** (5.0 g, 18.2 mmol) in dry acetonitrile (150 mL) under protective atmosphere of N_2, CuI (4.2 g, 22.1 mmol) and K_2CO_3 (5.1 g, 36.9 mmol) were added. To this mixture (trimethylsilyl)acetylene (2.3 g, 23.3 mmol) was added and the reaction was stirred overnight at 55 °C. The solvent was removed under reduced pressure. The residue was washed with $NaHCO_3$ and extracted three times with DCM. The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (EtOAc/pentane, 10/90, v/v) to give a yellow oil (1.37 g, 22%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.38 – 6.24 (m, 2H), 5.52 – 5.43 (m, 1H), 3.65 (t, *J* = 6.6 Hz, 2H), 3.59 – 3.43 (m, 4H), 1.63 – 1.50 (m, 4H), 1.44 – 1.32 (m, 4H), 0.92 – 0.88 (m, 6H), 0.19 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 151.00, 150.12, 109.04, 106.70, 100.97, 96.49, 86.51, 64.79, 31.74, 20.03, 19.36, 13.87, -0.05.

5-[3-(Trimethylsilyl)prop-2-yn-1-yl]furan-2-carbaldehyde (20)

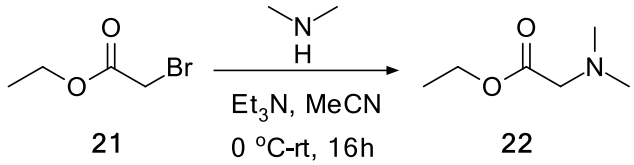


Compound **20** (1.37 g, 4.1 mmol) was dissolved in *aq.* HCl (1M, 100 mL) and stirred for 2h. Then the mixture was extracted with DCM three times, combined organic layer were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (EtOAc/pentane, 10/90, v/v) to give a yellow oil (0.25 g, 30%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.56 (s, 1H), 7.20 (d, *J* = 3.5 Hz, 1H), 6.50 (d, *J* = 3.5 Hz, 1H), 3.73 (br s, 2H), 0.19 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 177.41, 157.73, 152.58, 123.07, 109.98, 98.96, 88.11, 20.61, 0.00.

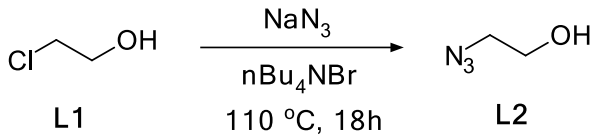
Ethyl 2-(dimethylamino)acetate (22)



To a solution of ethylbromoacetate (3 g, 20 mmol) in acetonitrile (30 mL) cooled at 0 °C, Et_3N (3.61 g, 30 mmol) was added. The suspension was stirred for 10 min and dimethylamine (0.96 g, 21 mmol, 40% *aq.*) was added dropwise. The resulting solution was stirred at rt for 16h. Then the reaction was extracted with DCM five times, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give a yellow oil (1.3 g, 62%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.20 (q, *J* = 7.1 Hz, 2H), 3.19 (s, 2H), 2.39 (s, 6H), 1.28 (t, *J* = 7.2 Hz, 3H).

2-Azidoethan-1-ol (L2)



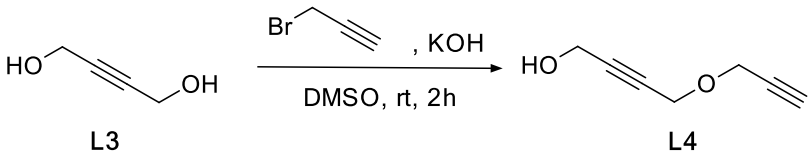
A mixture of 2-chloroethanol (2 g, 24.8 mmol, 1 eq.), NaN3 (2.1 g, 32.2 mmol, 1.3 eq.) and *n*Bu_4NBr (0.2 g, 0.62 mmol, 2.5 mol%) was stirred for 16 h at 110 °C. Then the mixture was cooled, dispersed in Et_2O and filtered. The filtrate was reduced *in vacuo* to afford a colorless oil (2.14 g, 99%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.75 (t, *J* = 5.0 Hz, 2H), 3.42 (t, 2H), 2.55 (s, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 61.41, 53.50.

FT-IR: 3381, 2932, 2878, 2095 cm^{-1}

4-(Prop-2-yn-1-yloxy)but-2-yn-1-ol (L4)

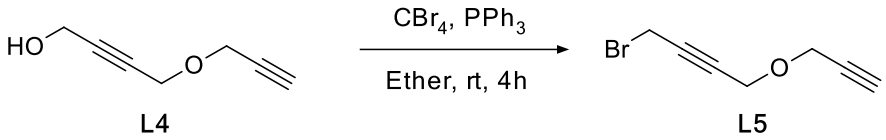


To a mixture of KOH (7.07 g, 126 mmol) in DMSO (91 mL) was added propargyl bromide (5g, 42 mmol, 80% in Toluene) and 2-butyne-1,4-diol (10.7 g, 123 mmol). The mixture was stirred for 3h at rt, then quenched with 6M of *aq.* HCL. The aqueous layer was extracted with DCM three times. The combined organic layers were washed with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (EtOAc/pentane, 25/75, v/v) to give a yellow oil (2.18 g, 42%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.30 – 4.28 (m, 4H), 4.24 (d, *J* = 2.6 Hz, 2H), 2.45 (t, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 85.35, 80.69, 78.78, 75.17, 56.78, 56.55, 50.95.

1-Bromo-4-(prop-2-yn-1-yloxy)but-2-yne (L5)



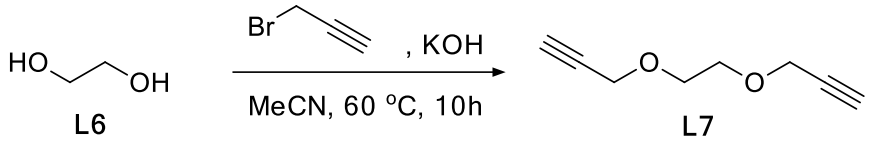
To a solution of **L4** (1.0 g, 8.1 mmol) in ether (25 mL) were added CBr_4 (5.4 g, 16.3 mmol) and PPh_3 (4.27g, 16.3 mmol). After 4h, the mixture was filtered through Celite and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (Et_2O/Pentane, 0/100 → 10/90, v/v) to give a colorless oil (1.0 g, 66%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.32 (t, *J* = 2.0 Hz, 2H), 4.24 (d, *J* = 2.4 Hz, 2H), 3.94 (t, *J* = 2.1 Hz, 2H), 2.46 (t, *J* = 2.4 Hz, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 81.97, 81.90, 78.70, 75.20, 56.77, 56.65, 13.97.

FT-IR: 3297, 3021, 2854, 2117, 650 cm^{-1}

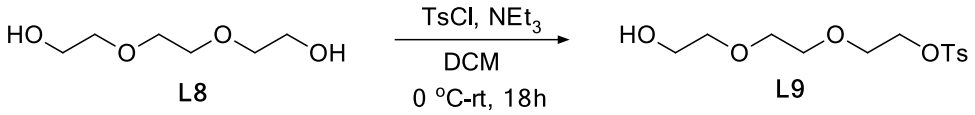
3-[2-(Prop-2-yn-1-yloxy)ethoxy]prop-1-yne (L7)



To a solution of ethylene glycol (1g, 16.1 mmol) in acetonitrile (12 mL) under protective atmosphere of N_2, KOH (2.5g, 44.8 mmol) was added and heated to 60 °C. Propargyl bromide (5.33 g, 44.8 mmol, 80% in Toluene) was added dropwise and stirred for 10 h. Then reaction was cooled, filtered and reduced *in vacuo*. The crude product was purified by silica gel chromatography (Acetone/Pentane, 10/90, v/v) to give a yellow oil (0.65 g, 29%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.21 (d, *J* = 2.4 Hz, 4H), 3.73 (s, 4H), 2.43 (t, *J* = 2.4 Hz, 2H).

2-(2-{2-[(4-Methylbenzenesulfonyl)oxy]ethoxy}ethoxy)ethan-1-ol (L9)

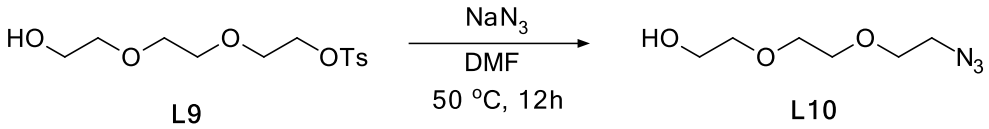


To a solution of triethylene glycol (33.5mL, 250 mmol) in dry DCM (80 mL) cooled at 0 °C, NEt_3 (10.5 mL, 75 mmol) was added, followed by portion wise addition of p-toluenesulfonyl chloride (TsCl, 9.55g, 50mmol). Then solution was brought to rt and stirred for 18h. The solution was washed with water three times, and the aqueous layer was washed with DCM. The combined organic layers were washed with 5% citric acid three times, dried over anhydrous sodium sulfate, filtered and reduced *in vacuo* to give a pale yellow oil (15.1 g, 99%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 – 7.75 (m, 2H), 7.34 (ddt, *J* = 8.1, 2.2, 1.1 Hz, 2H), 4.19 – 4.08 (m, 3H), 3.74 – 3.68 (m, 4H), 3.60 (s, 4H), 3.58 – 3.55 (m, 2H), 2.44 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 144.88, 132.96, 129.85, 127.98, 72.46, 70.79, 70.69, 70.31, 69.21, 69.17, 68.72, 61.76, 21.64.

2-[2-(2-Azidoethoxy)ethoxy]ethan-1-ol (L10)



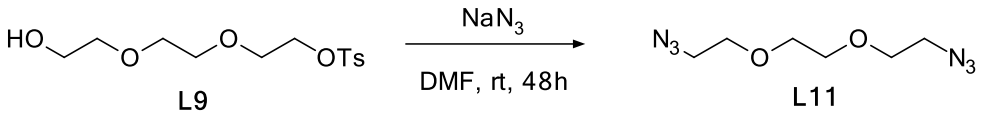
To a solution of **L9** (2.2 g, 7.1 mmol) in DMF (15mL), NaN_3 (0.7 g, 10.7mmol) was added and the mixture was heated to 50 °C and stirred for 12h. Then the mixture was washed with water and extracted with DCM three times. The combined organic layers were washed water six times, dried over anhydrous sodium sulfate and reduced *in vacuo* to give a light yellow oil (0.38g, 31%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.78 – 3.72 (m, 2H), 3.71 – 3.66 (m, 6H), 3.64 – 3.59 (m, 2H), 3.43 – 3.36 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 72.48, 72.47, 70.76, 70.68, 70.42, 70.16, 70.08, 61.80, 50.70, 50.67.

FT-IR: 3432, 2868, 2100, 1665 cm^{-1}

1-Azido-2-[2-(2-azidoethoxy)ethoxy]ethane (L11)

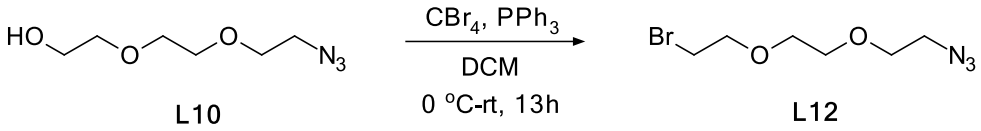


To a solution of **L9** (1.64 g, 5.4 mmol) in DMF (15mL), NaN_3 (0.7 g, 10.7mmol) was added and the mixture was stirred at rt for 48h. Then the mixture was washed with water and extracted with DCM three times. The combined organic layers were washed water six times, dried over anhydrous sodium sulfate and reduced *in vacuo* to give a light yellow oil (0.43g, 40%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.73 – 3.64 (m, 8H), 3.39 (t, *J* = 5.0 Hz, 4H).

FT-IR: 2869, 2092, 1727б 1284, 1118 cm^{-1}

1-Azido-2-[2-(2-bromoethoxy)ethoxy]ethane (L12)



To a solution of **L10** (0.35g, 2.0 mmol) in DCM (5mL), cooled at 0 °C, under protective atmosphere of N_2, CBr_4 (0.72g, 2.4 mmol) was added, followed by portion wise addition of PPh_3 (0.57g, 2.4 mmol). Then the mixture was brought to rt and stirred for 13h. The mixture was washed with water and extracted with DCM three times. The combined organic layers were dried over anhydrous sodium sulfate and reduced *in vacuo*. The crude product was purified by silica gel chromatography (Et_2O/Pentane, 0/100 → 10/90, v/v) to give a yellow oil (0.39 g, 83%).

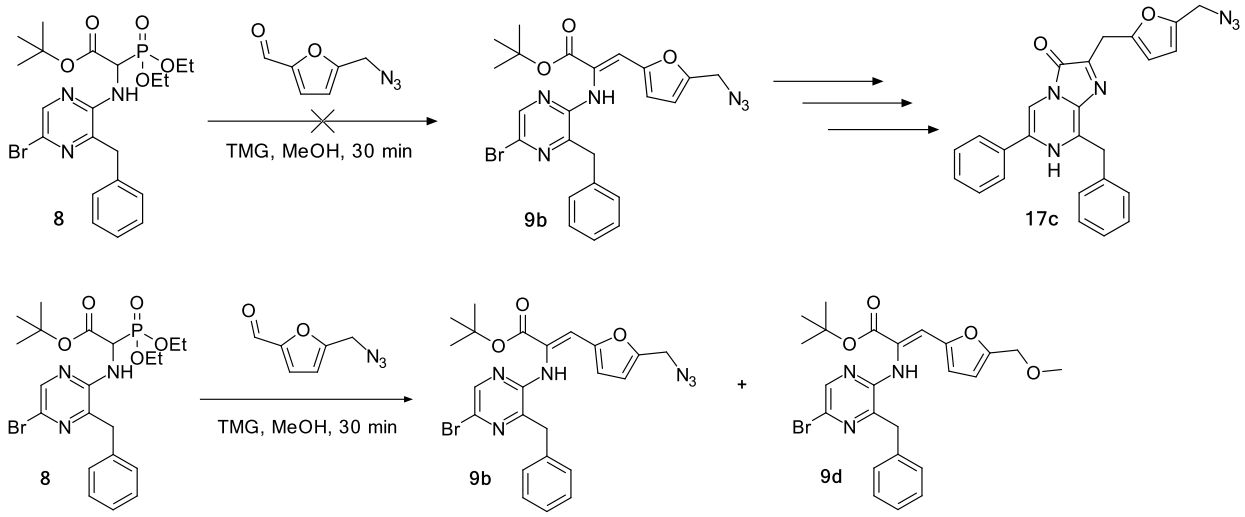
^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (t, *J* = 6.3 Hz, 2H), 3.73 – 3.66 (m, 6H), 3.48 (t, *J* = 6.2 Hz, 2H), 3.39 (t, *J* = 5.0 Hz, 2H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 71.29, 70.77, 70.70, 70.60, 70.16, 50.72, 30.34.

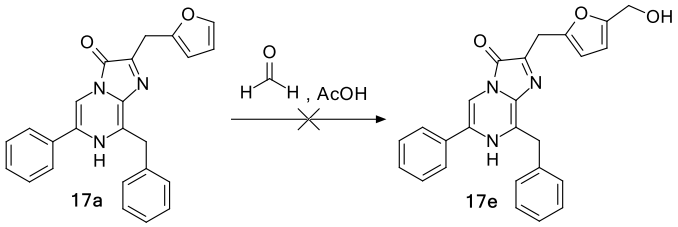
FT-IR: 2867, 2094, 1279, 1115, 665, 556 cm^{-1}

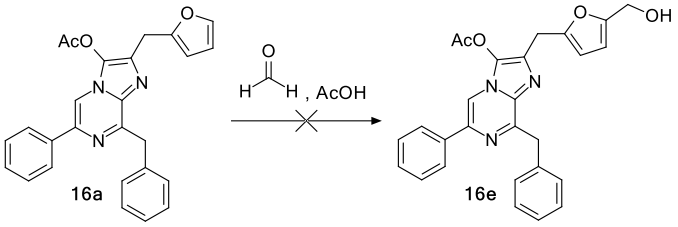
6 Appendices

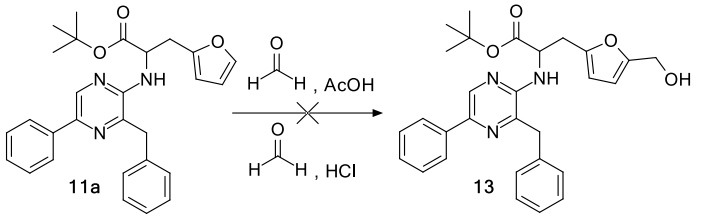
A1. Brief summary of failed routes.



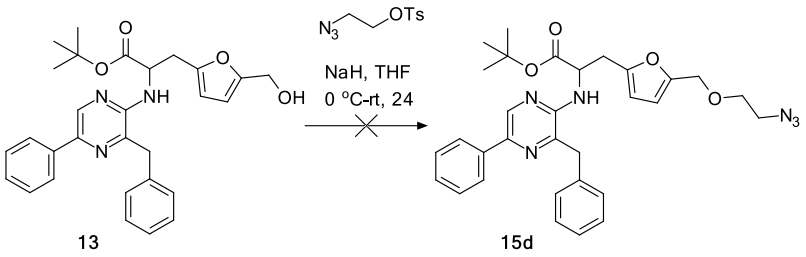
A) The main idea behind this strategy was to build an already functionalized furfural structural motif and then used it directly in the established synthetic route without any changes to the general furimazine synthesis procedures. The synthesized furfural **5** used in the Horner–Wadsworth–Emmons (HME) type of reaction yielded a mixture of **9b** and **9d**. Tetramethylguanidine (TMG) made the reaction environment basic. Consequently, partial methanolysis occurred. The unwanted methoxide ion-promoted side product was not possible to isolate form the desired product by column chromatography (Scheme 4). The exact ratio of compounds was not determined, however FT-IR shows that **9d** is the major product, since the intense peak of azide around 2100 cm^{-1} had lower than expected signal intensity due to signal masking (*S.I.*; FT-IR spectra). Consequent synthetic steps like Suzuki coupling or protecting group hydrolysis were also proceeding under basic conditions, therefore this strategy was not pursued further.





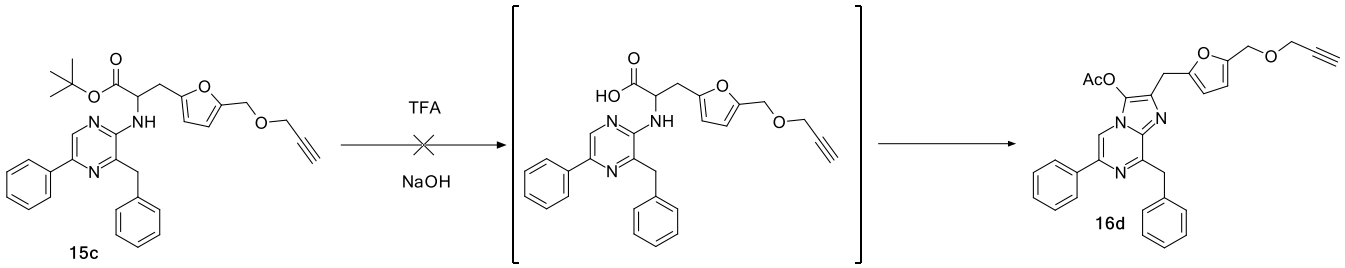


B) Second strategy presumed late functionalization of our target molecule. This approach was attempted at three stages, first directly on the furimazine molecule. Due to presence of water and weekly acidic conditions, target molecule along with the starting product decomposed over time. Second approach was to try functionalization of the hikarazine, this molecule was described as less prone to degradation. Moreover, the acidic hydrolysis product of the reaction is the furimazine molecule. It was observed that several products were formed, the target molecule being functionalized hikarazine, the functionalized furimazine, regular hikarazine, regular furimazine along with decomposed side-products. Lastly, in the third approach the molecule was functionalized prior to cyclization in attempts to avoid product decomposition. The acetic acid was not strong enough to initiate the reaction, even in large excess of 10 equivalents relative to the **11a**. The 2 equivalents of concentrated hydrochloric acid managed to start the reaction, however too many side products were formed, and the target compound could not be isolated by means of preparative TLC.

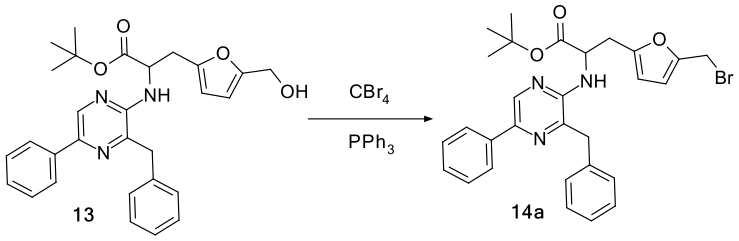


C) Once compound **13** was synthesized a suitable linker had to be attached to it. Two linker molecule were selected for this purpose, tosylated **L2** and a propargyl bromide which were available in the lab. Our attempts to attach linker **L2**, though a common sodium hydride chemistry

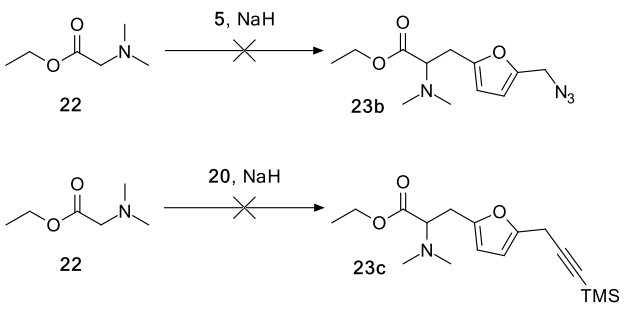
were not successful, and we did not observe the formation of the desired product. Therefore, the compound with the propargyl linker was prioritized. It was proposed that propargyl bromide will not be prone to degradation and formation of many side products under basic conditions of sodium hydride.



D) Both acidic and basic treatments of compound **15c** showed signs of linker hydrolysis, thus intermediate product of hikarazine formation step was unattainable. Removal of *t*BuO protecting group takes around 12-24 hours, thus as long as there is another ester bond present in the molecule it will get affected.



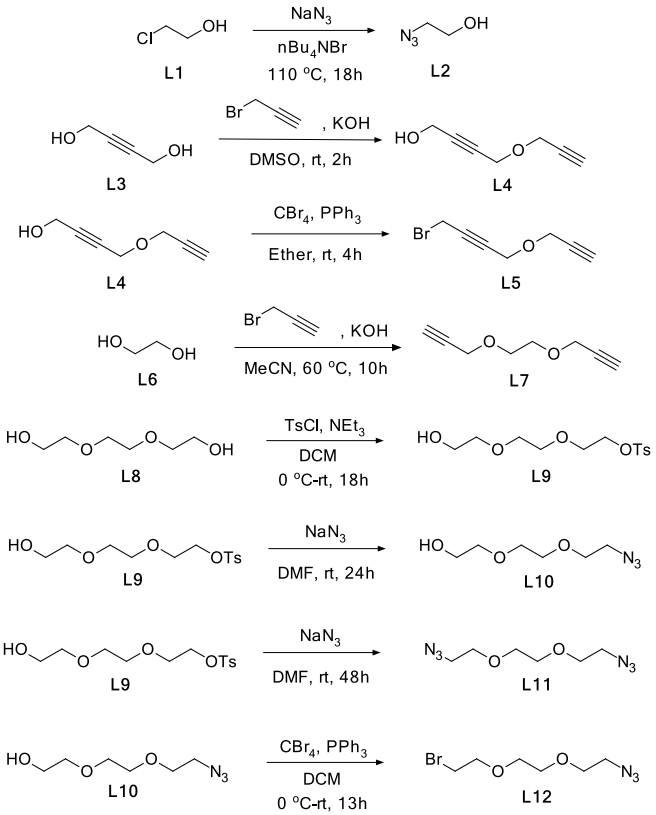
E) After failed attempts of compound cyclization we have decided to remove the ester bond between the linker and the furimazine precursor in our new strategy. Instead we decided to use C-C bond, which should withstand both strong acidic and basic conditions required in deprotection step prior to cyclization. For carbon-carbon bond formation chemistry, the hydroxyl moiety had to be changed to a halogen group. Classical Appel conditions did not contribute to product formation, the starting material showed signs of degradation (very polar fraction observed on both TLC and LC-MS). Remaining starting material was partially recovered via preparative TLC.



F) Due to insufficient amount of compound **14** and lack of suitable catalyst for scaling up previous reactions we decided to draw a new route. Herein, we proposed to decorate the furfural moiety

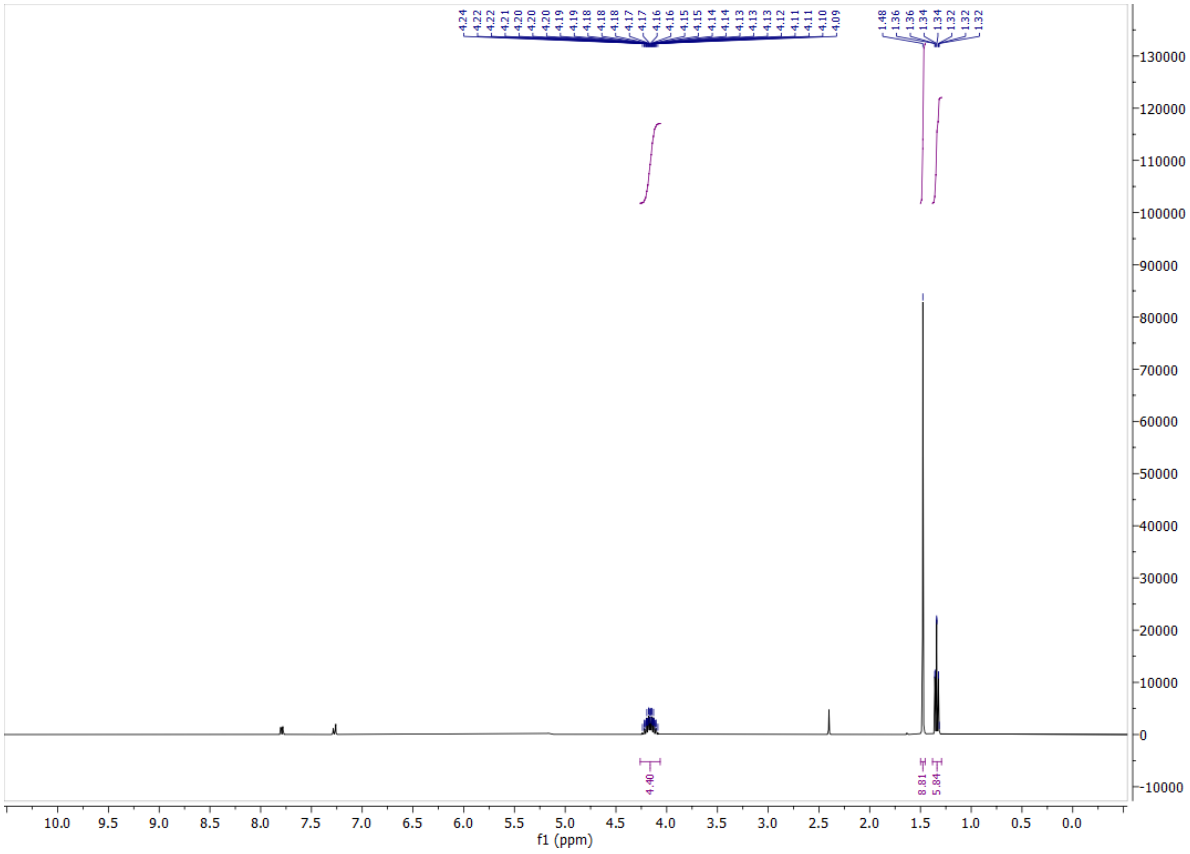
first and then use it as a building block. Both the synthesis of **20** and the approach of coupling of furfural to the **22** were already described in literature, and we combined both routes together. Unfortunately, attempts to couple **20** to **22** through sodium hydride chemistry used were not successful and led to degradation. Similar trend was observed for furfural **5**.

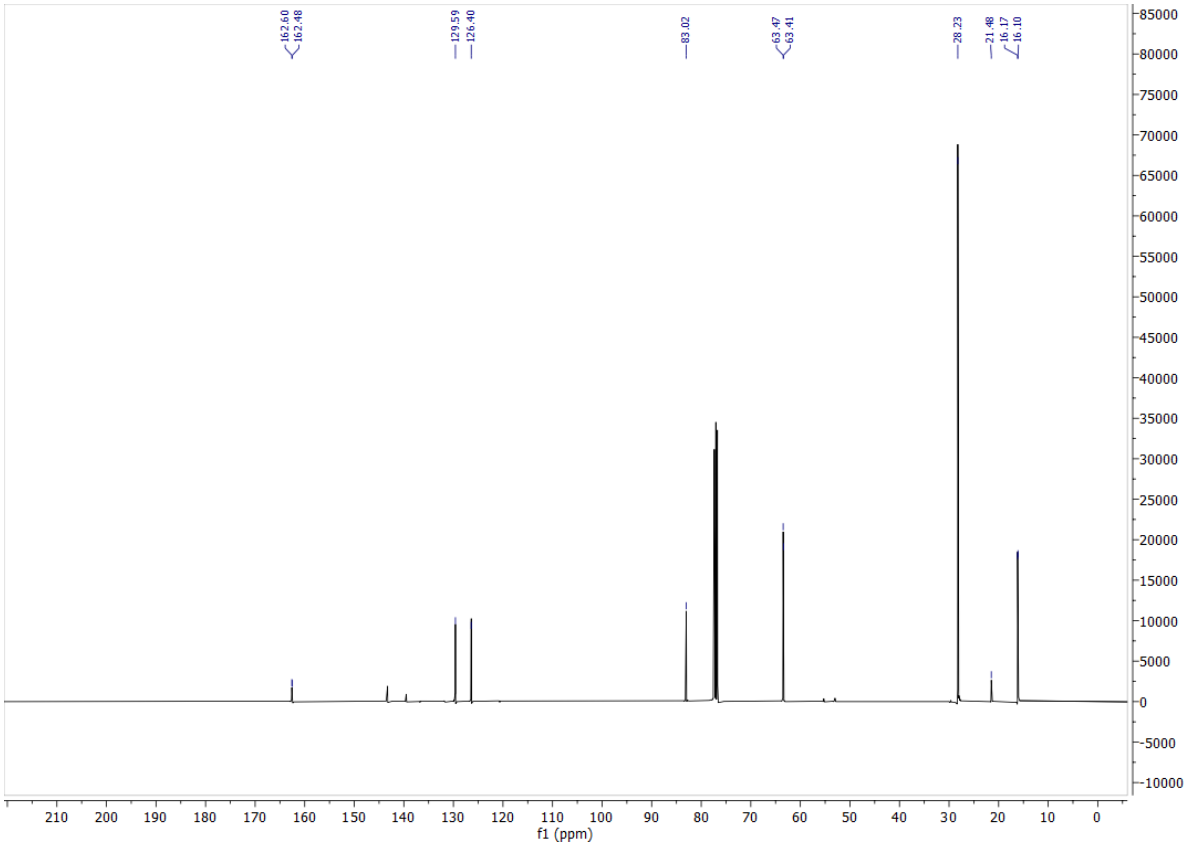
A2. Synthesized linkers.



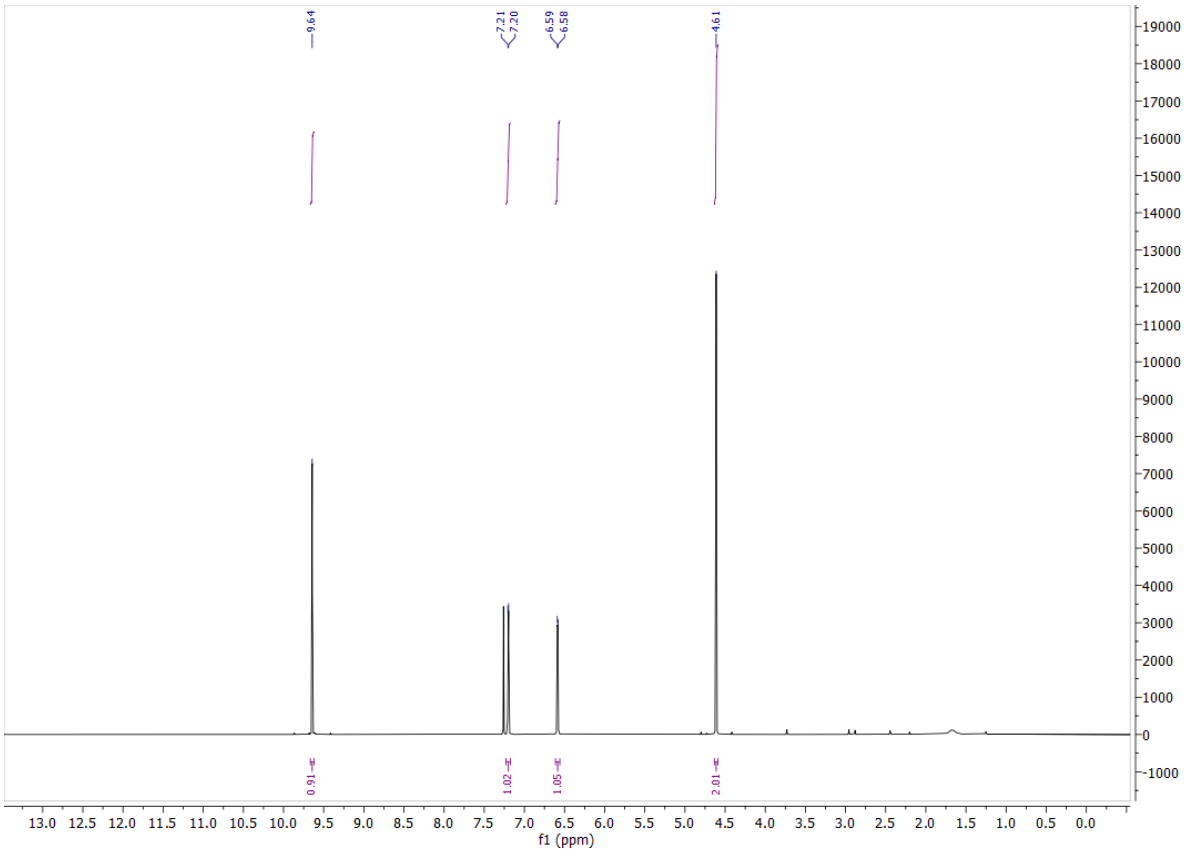
A3. NMR, LC-MS-ESI and IR spectra

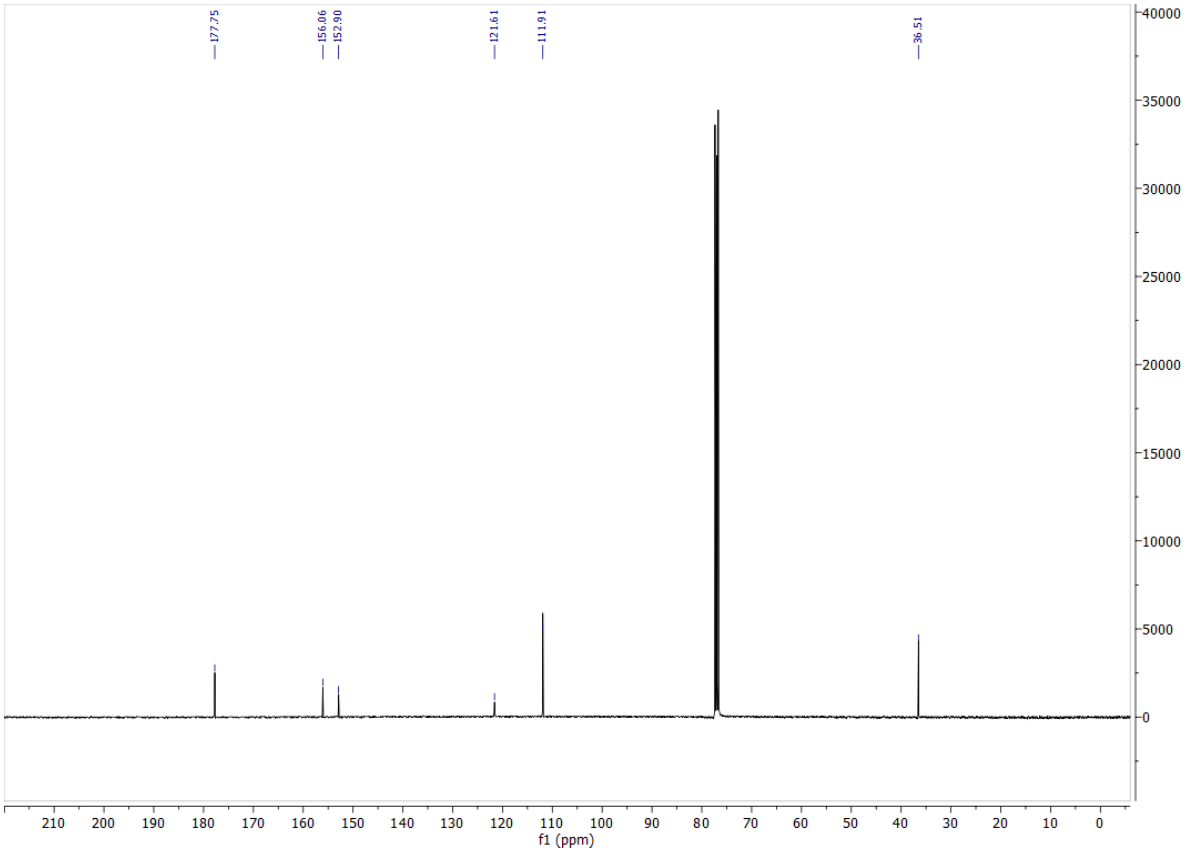
Spectra ^{1}H and ^{13}C compound 2



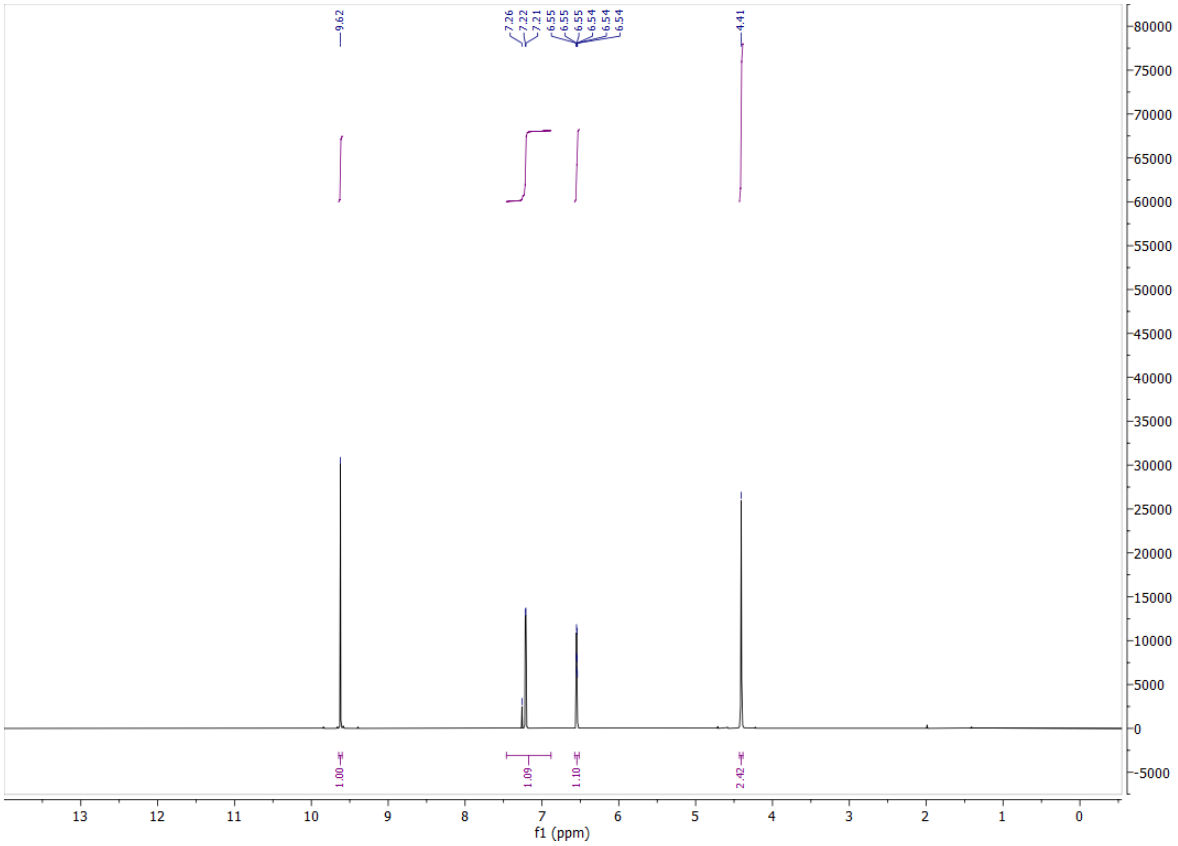


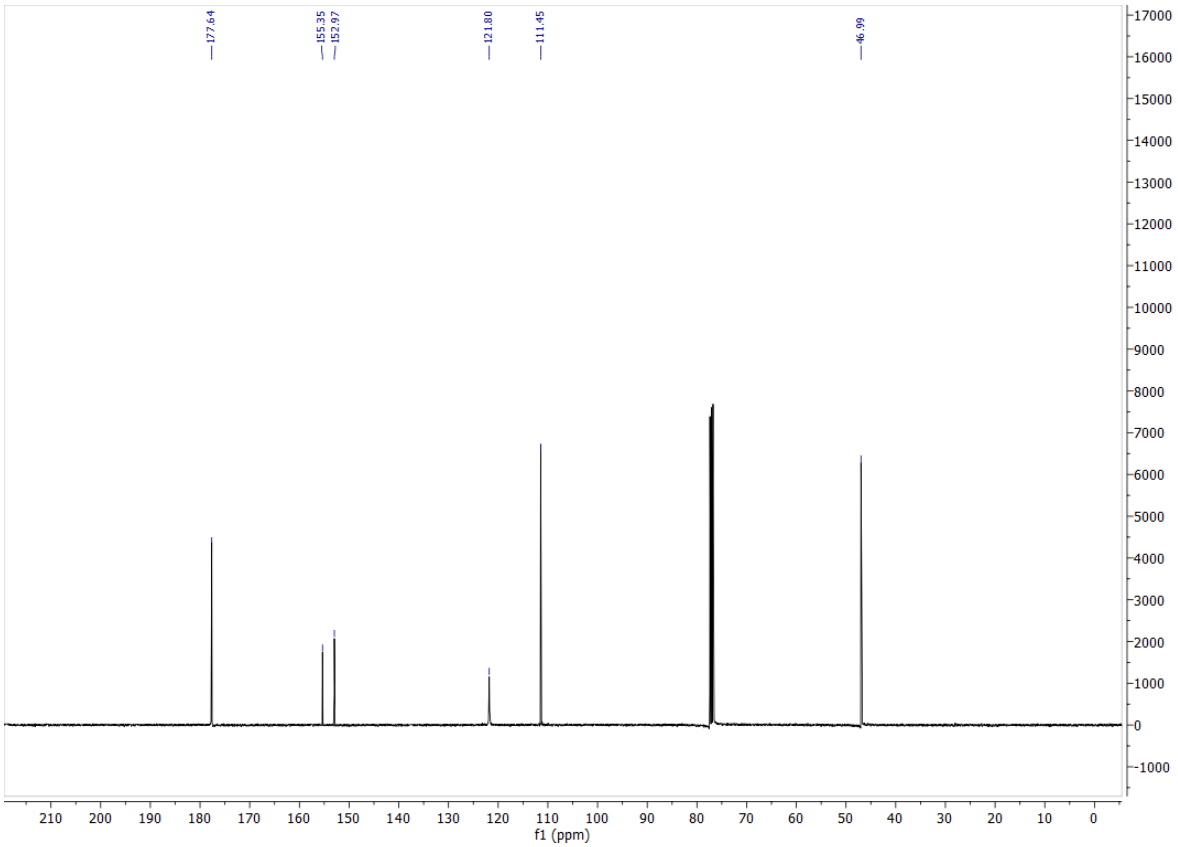
Spectra ^{1}H and ^{13}C compound 4



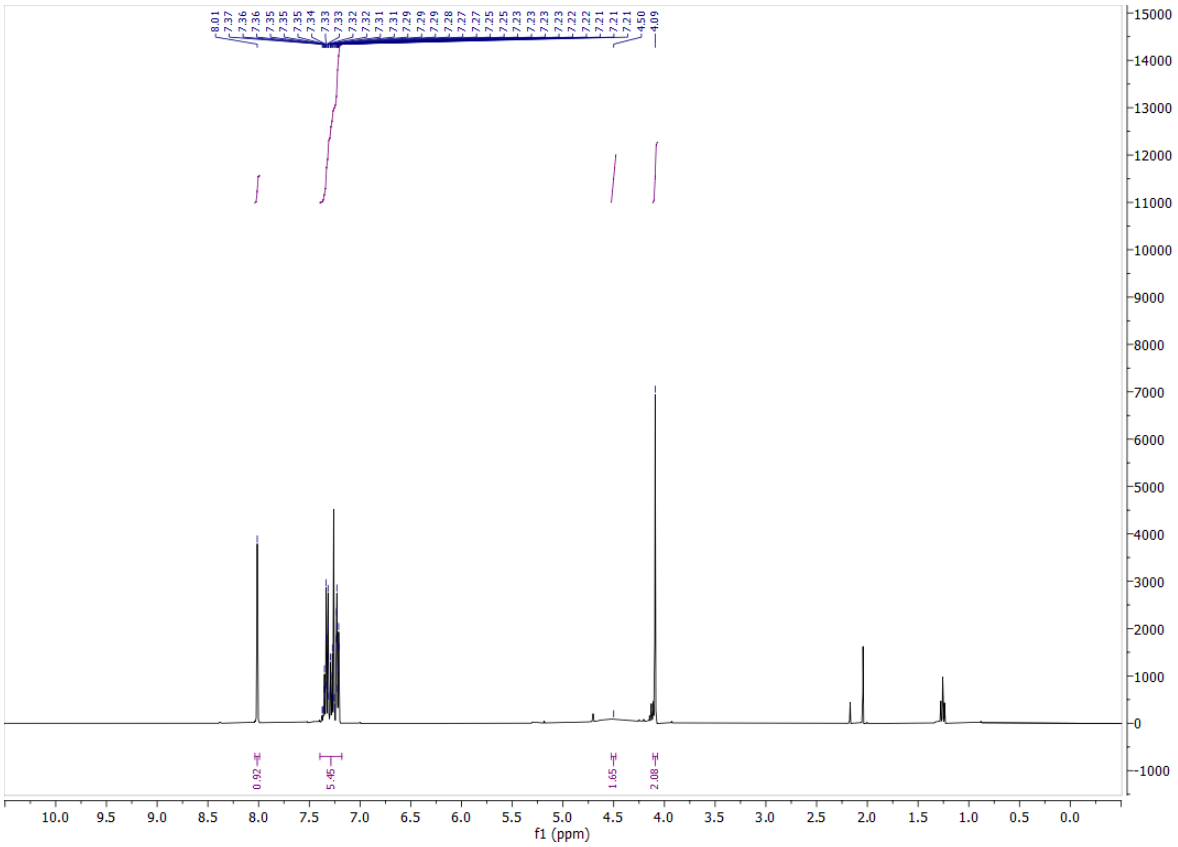


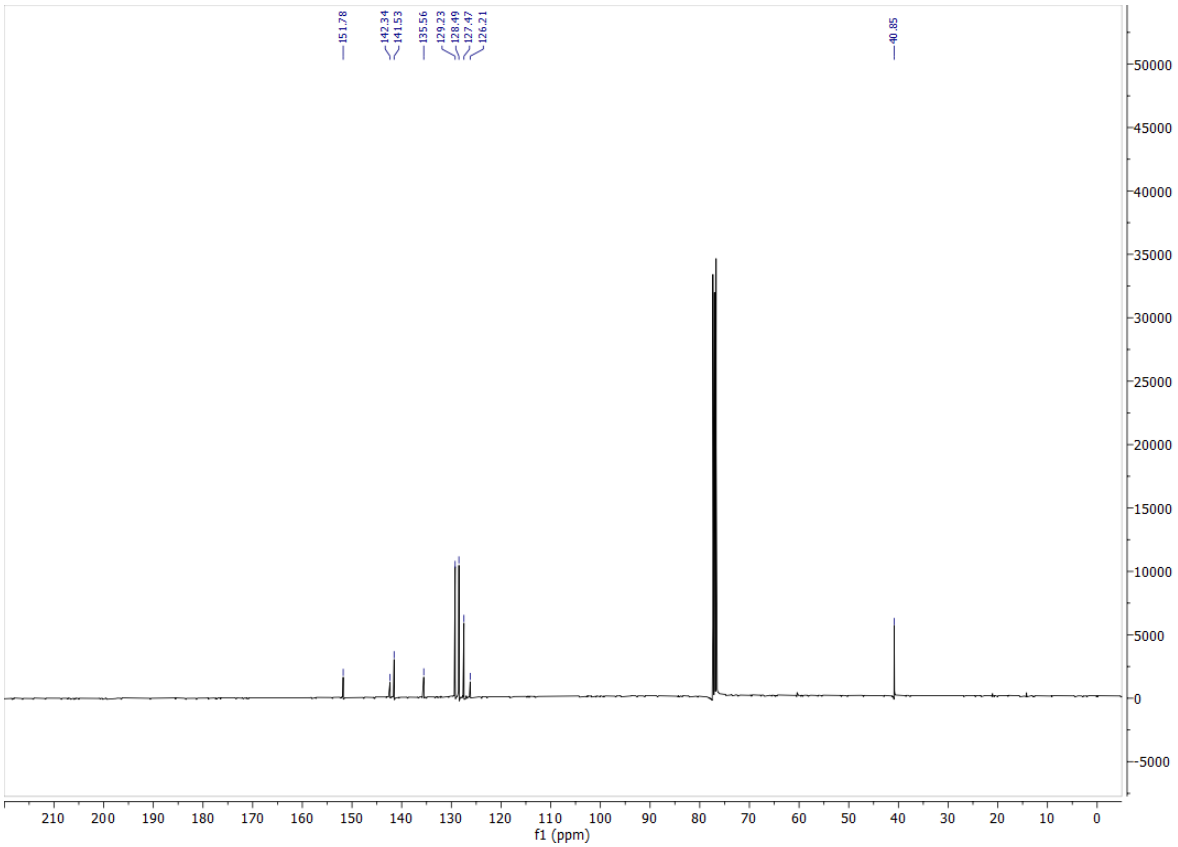
Spectra ^{1}H and ^{13}C compound 5



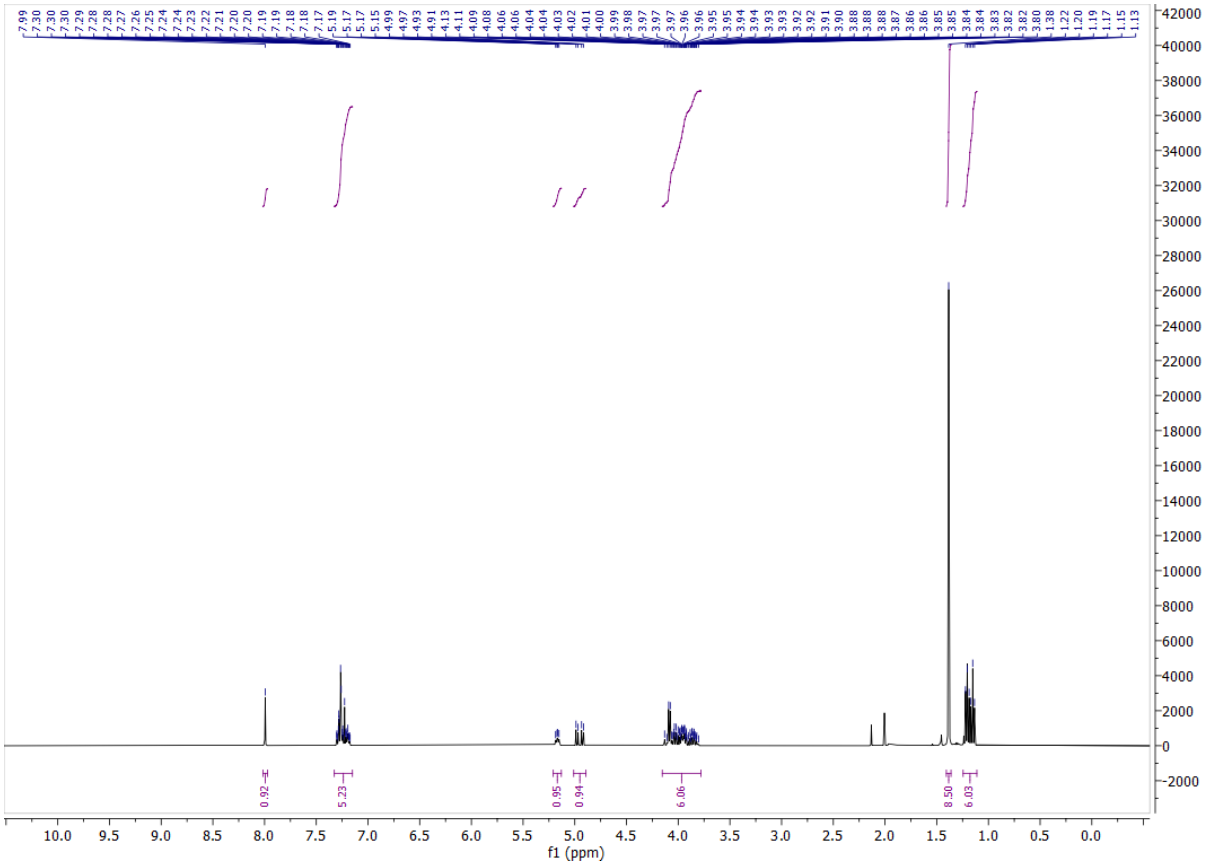


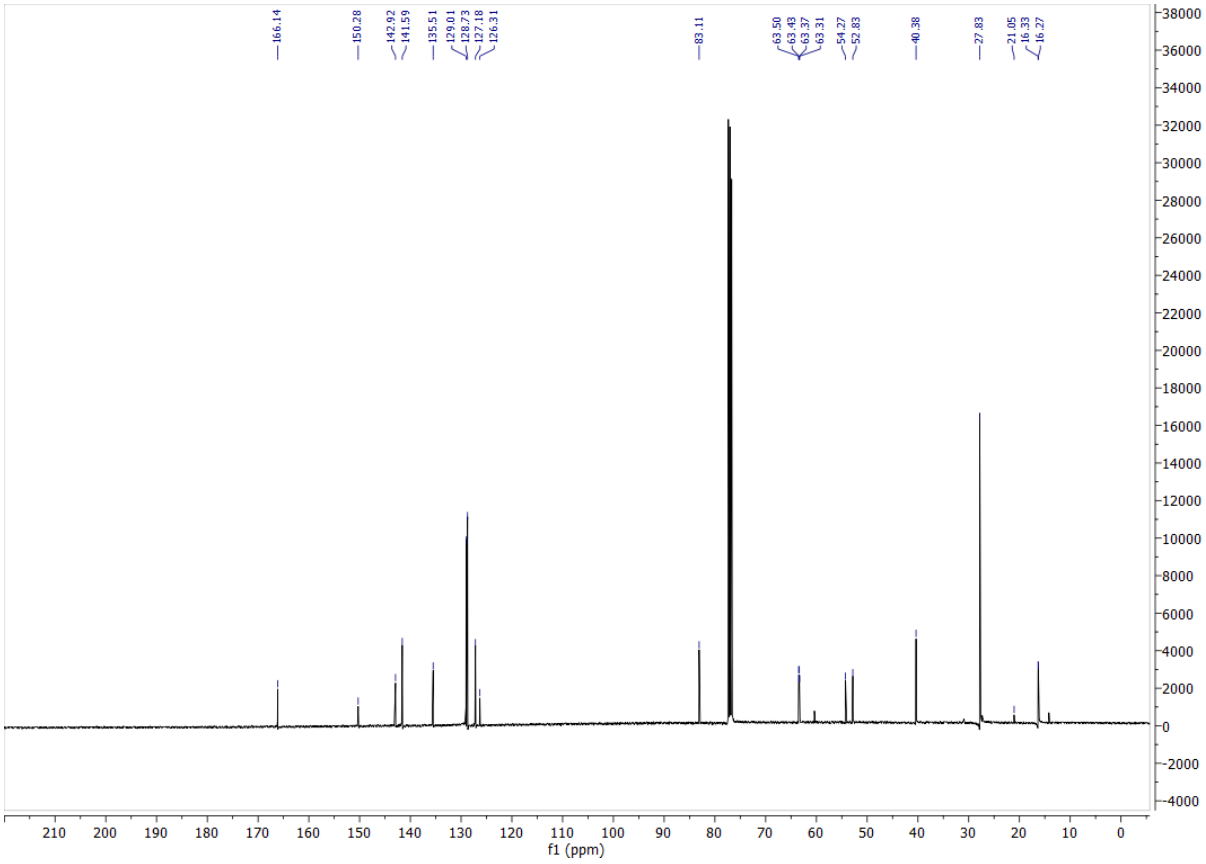
Spectra ^{1}H and ^{13}C compound 7



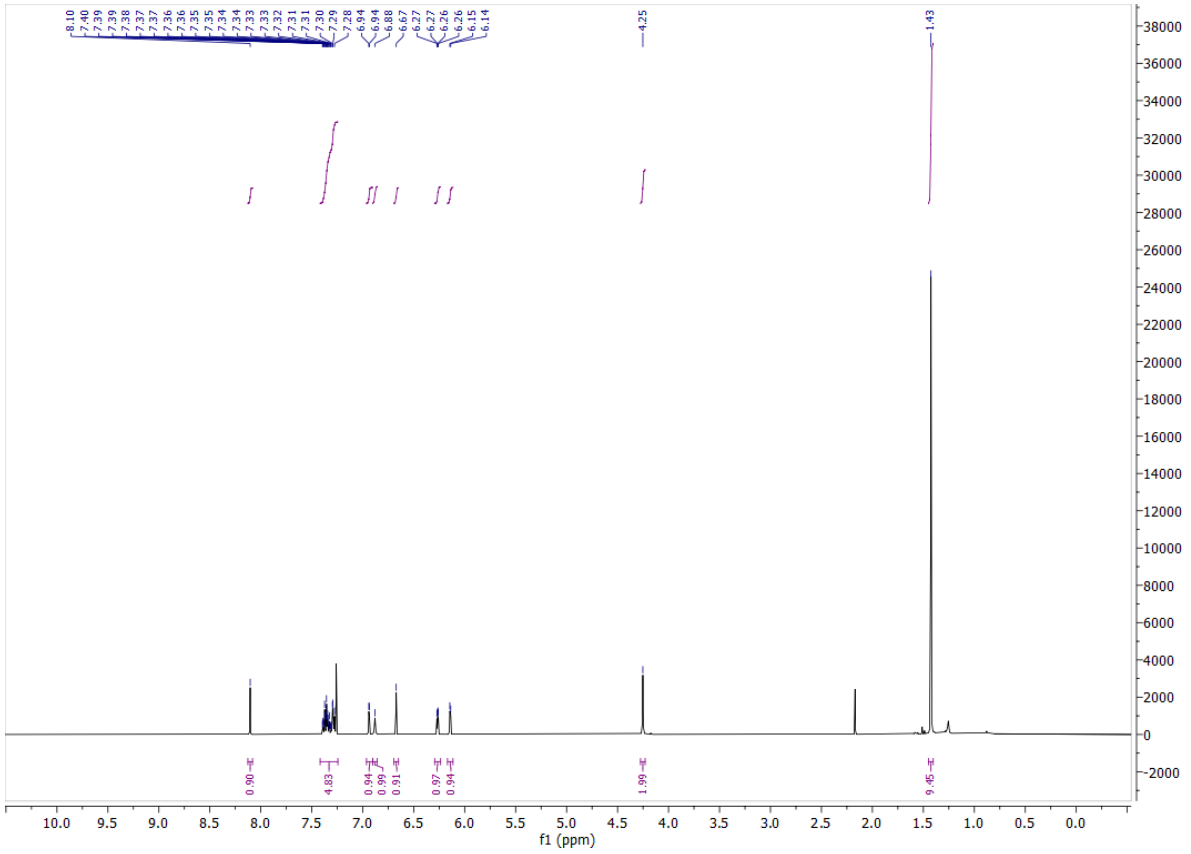


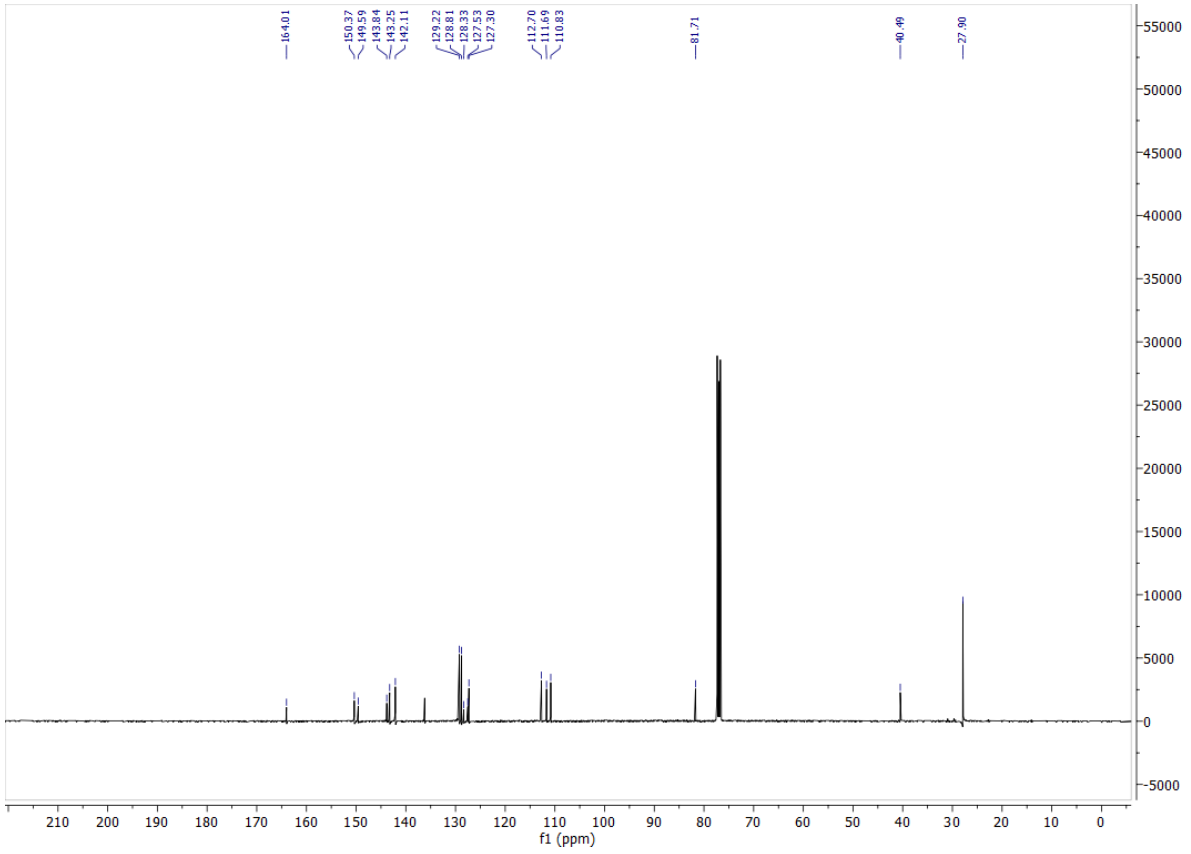
Spectra ^{1}H and ^{13}C compound 8



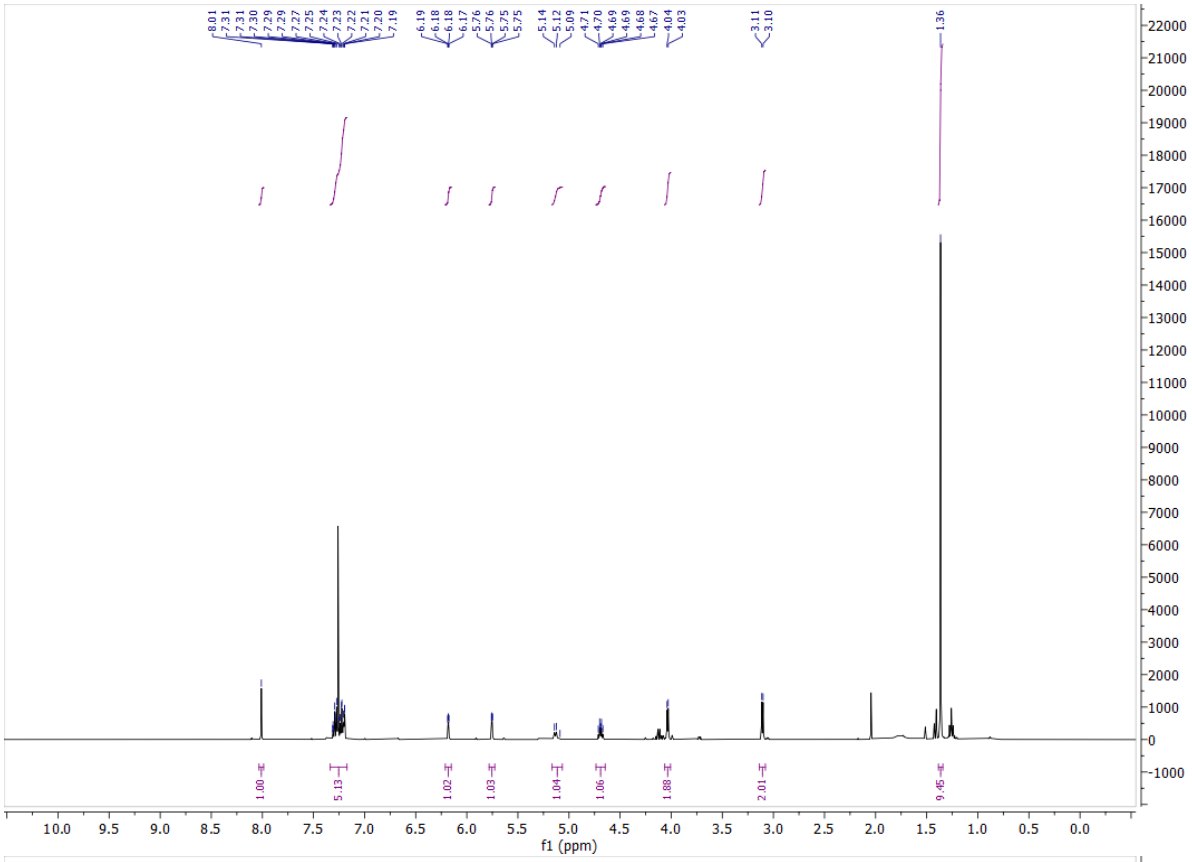


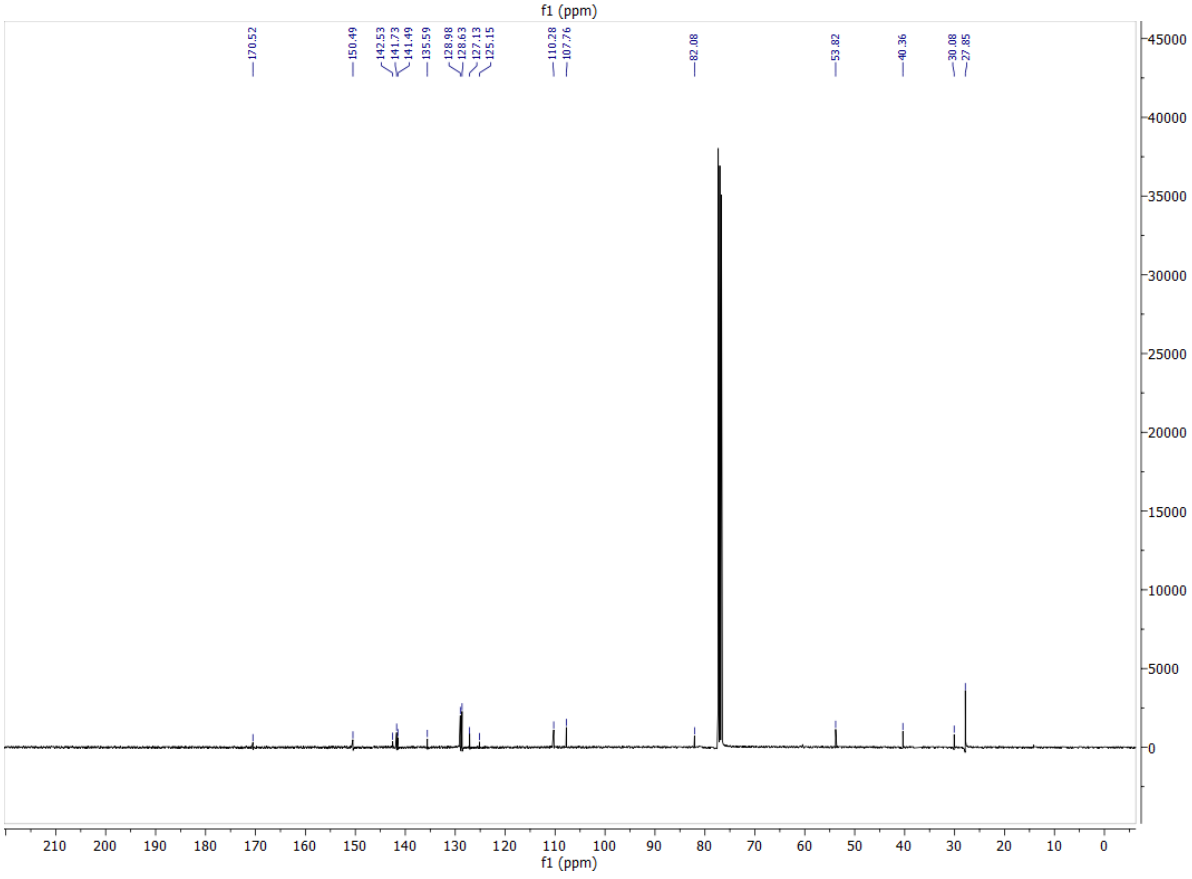
Spectra ^{1}H and ^{13}C compound 9a



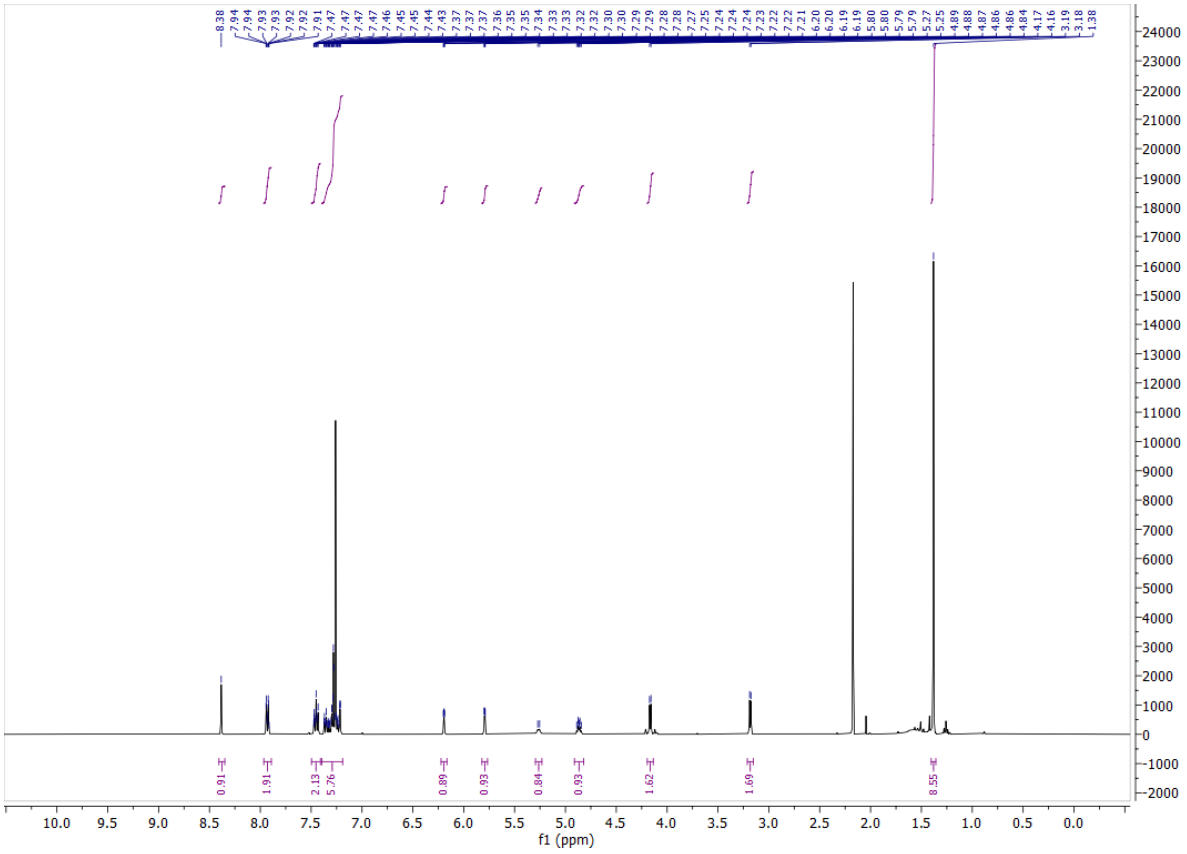


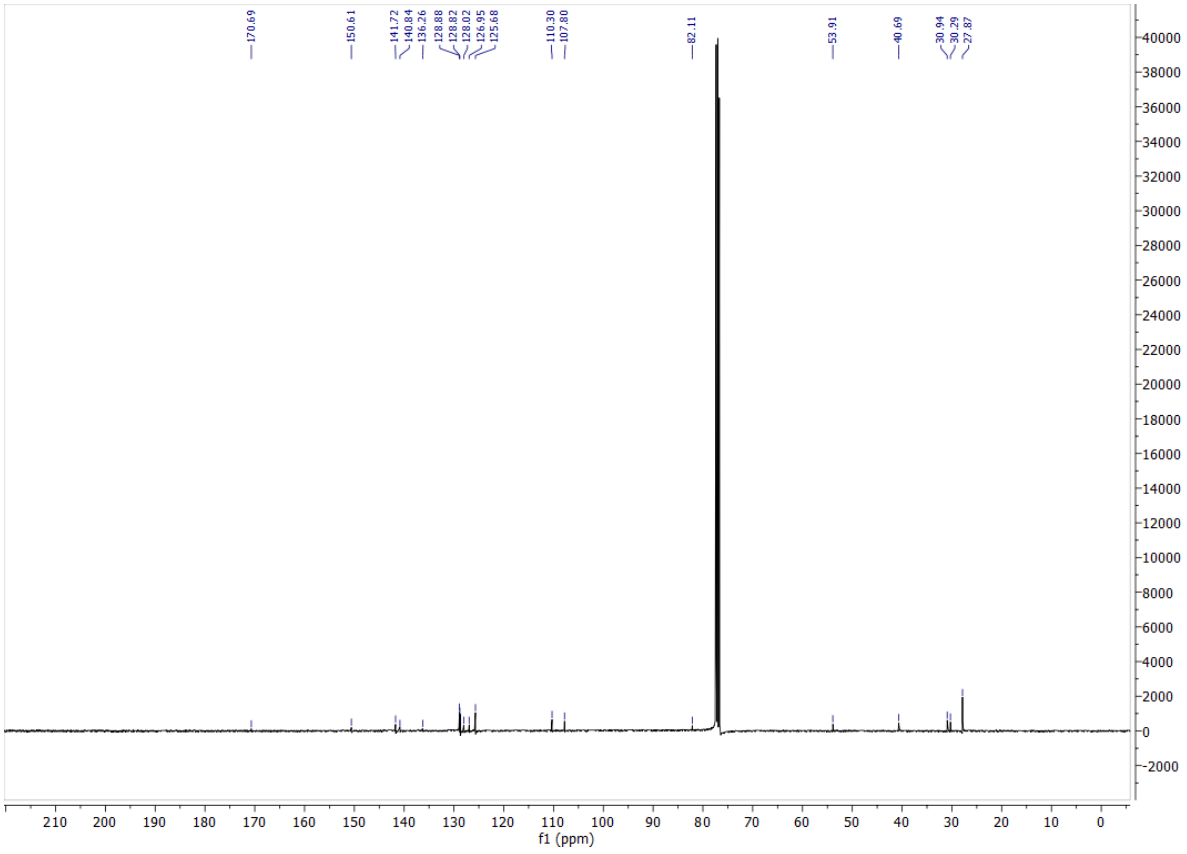
Spectra ^{1}H and ^{13}C compound 10a



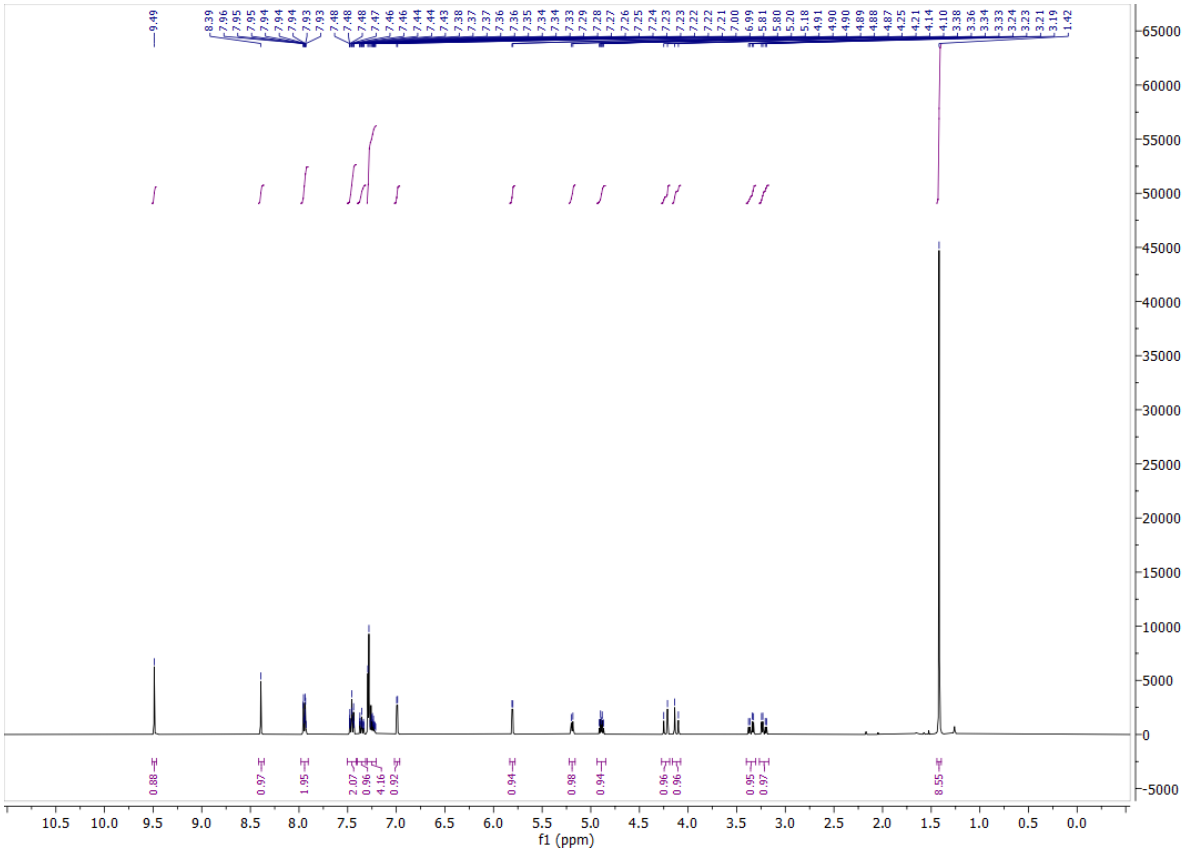


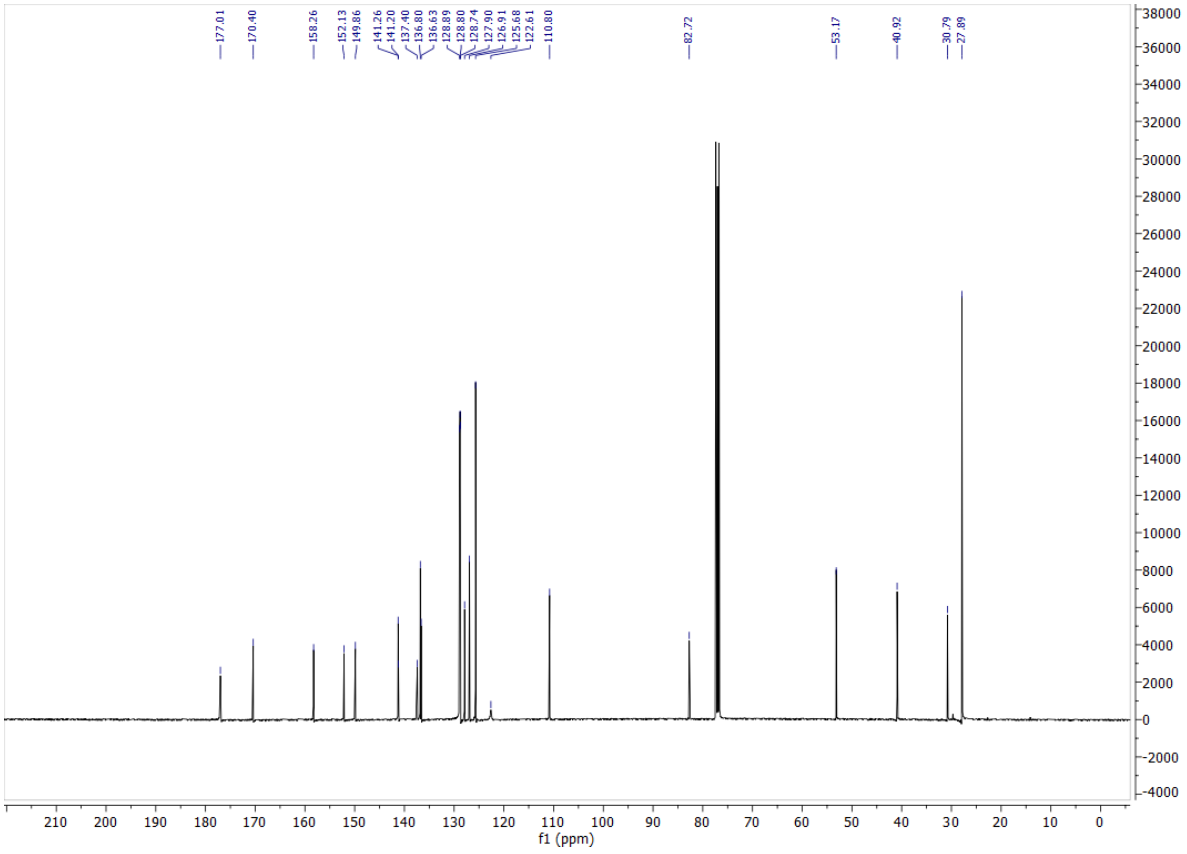
Spectra ^{1}H and ^{13}C compound 11a

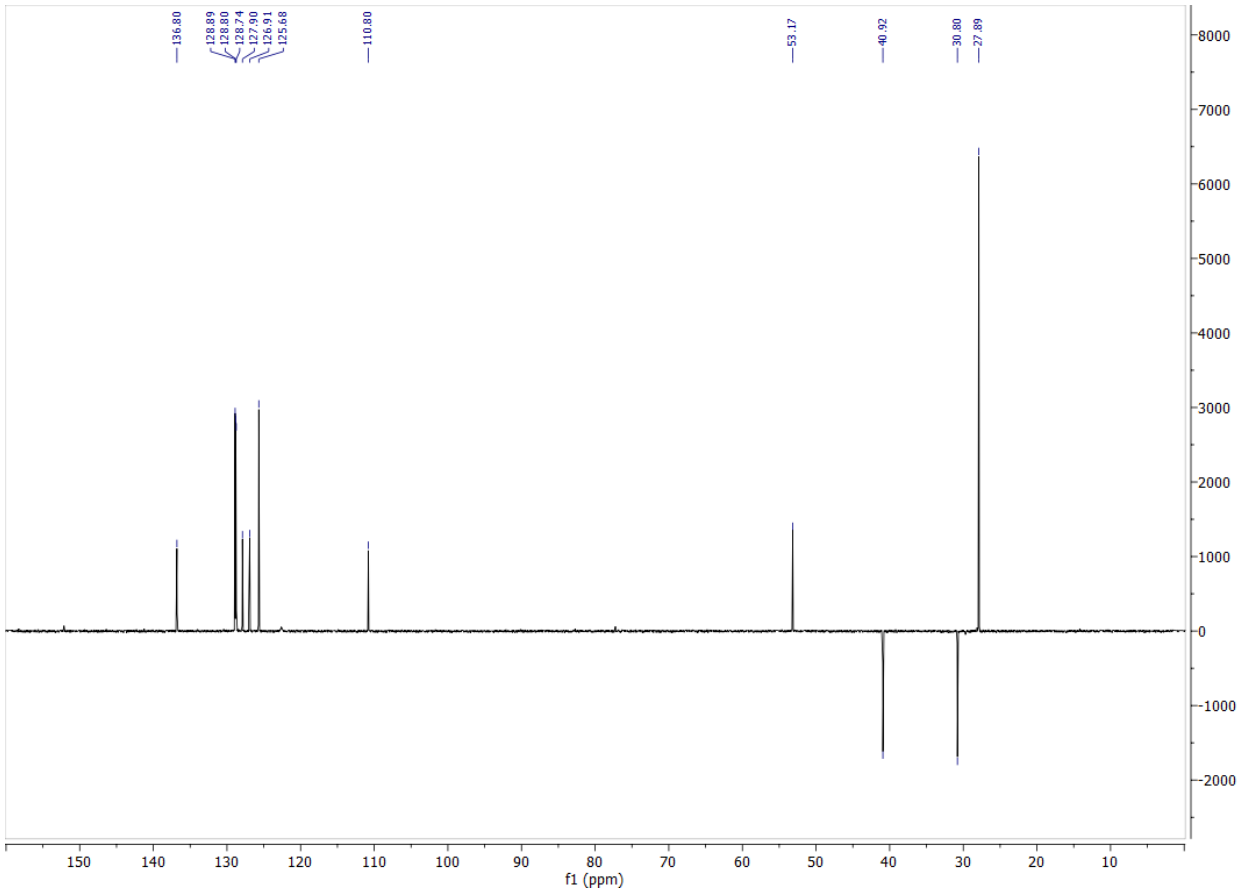


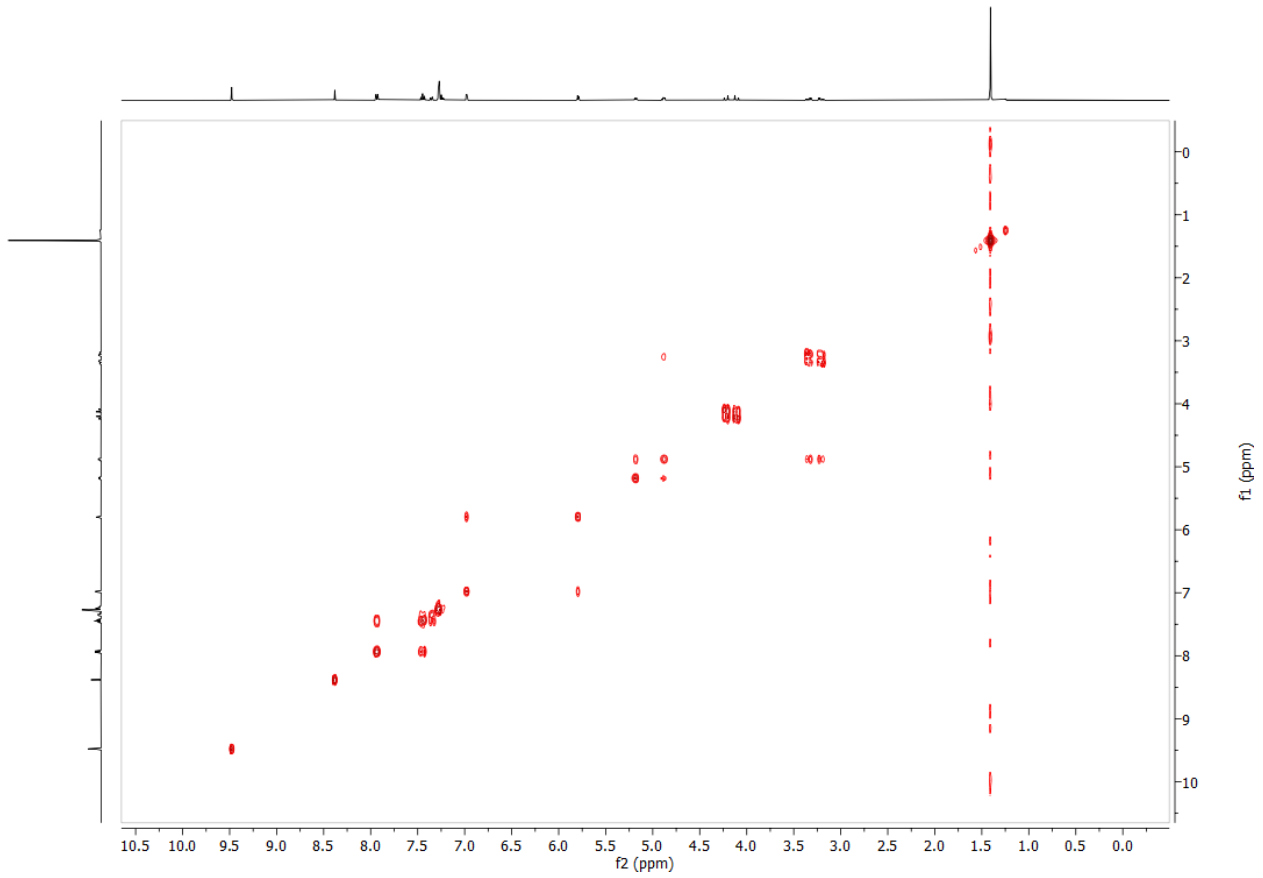


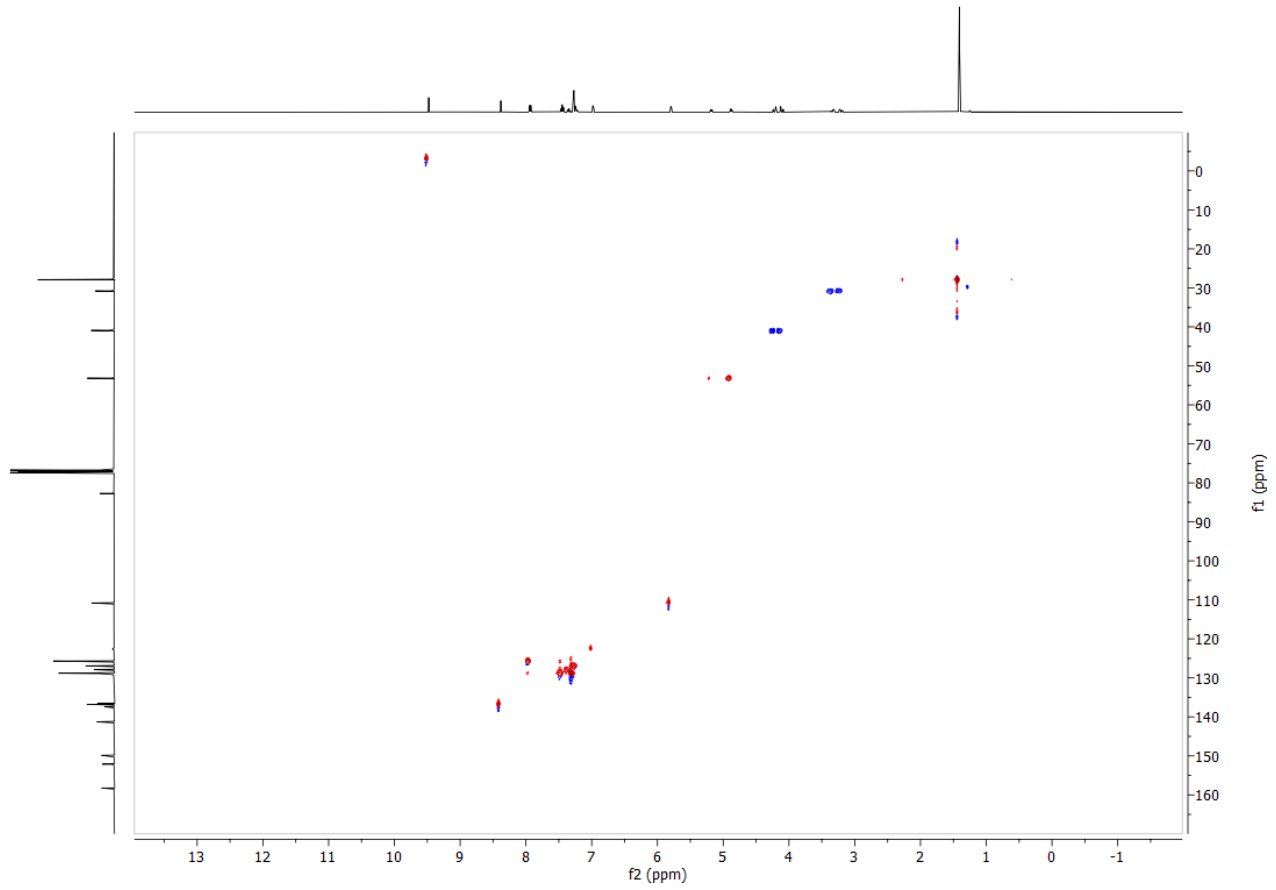
Spectra ^{1}H, ^{13}C, ^{13}C-DEPT135, COSY, HSQC and HMBC compound 12

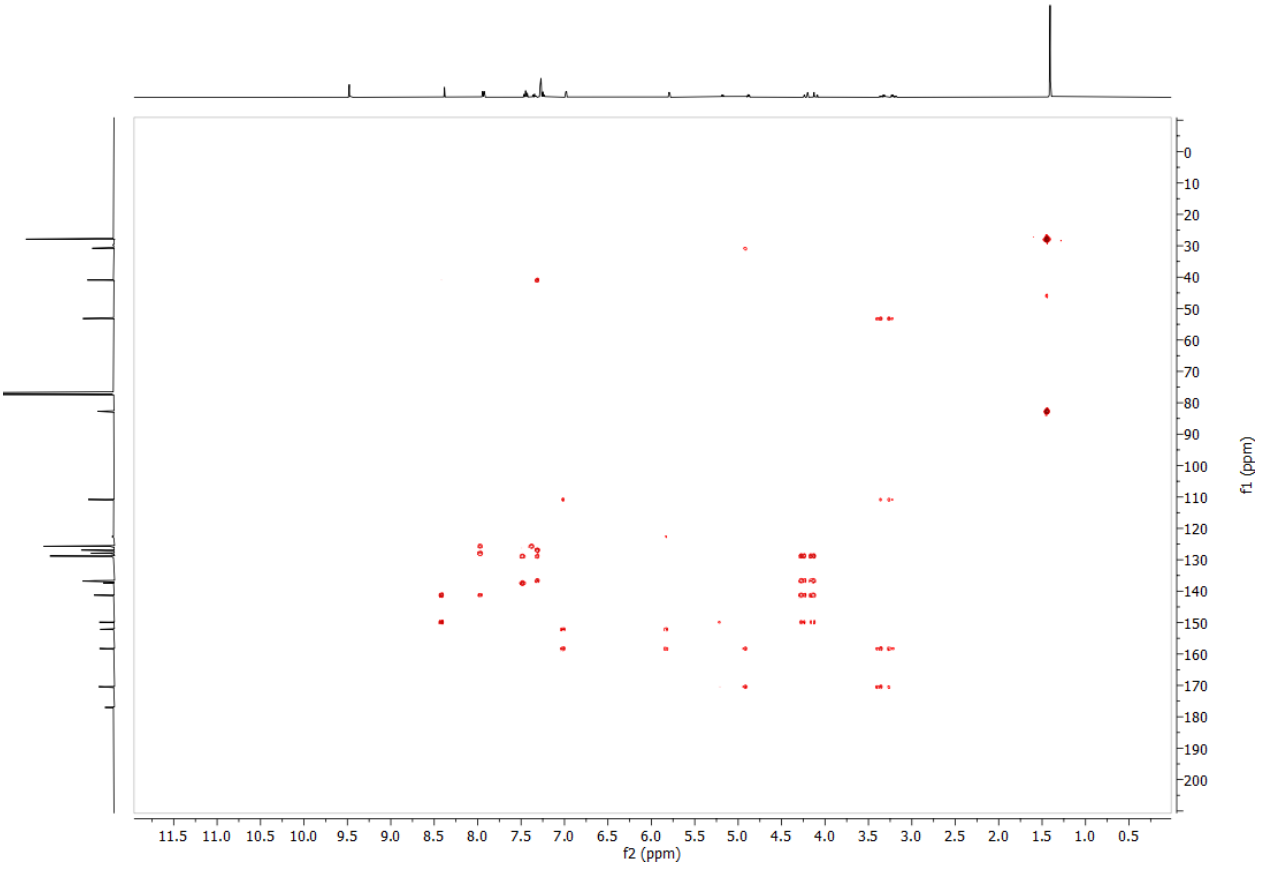




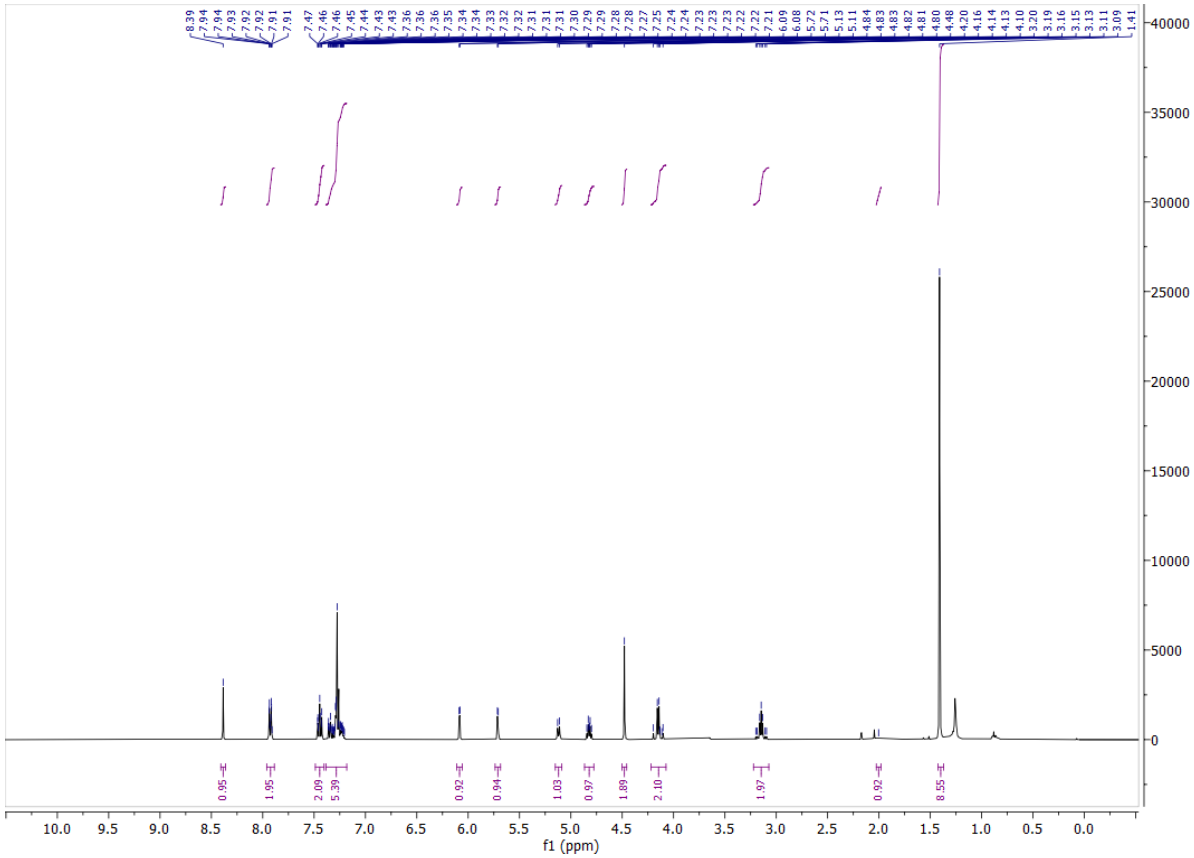

136.80
128.89
128.80
128.74
127.90
126.91
125.68
110.80
53.17
40.92
30.80
27.89
f1 (ppm)

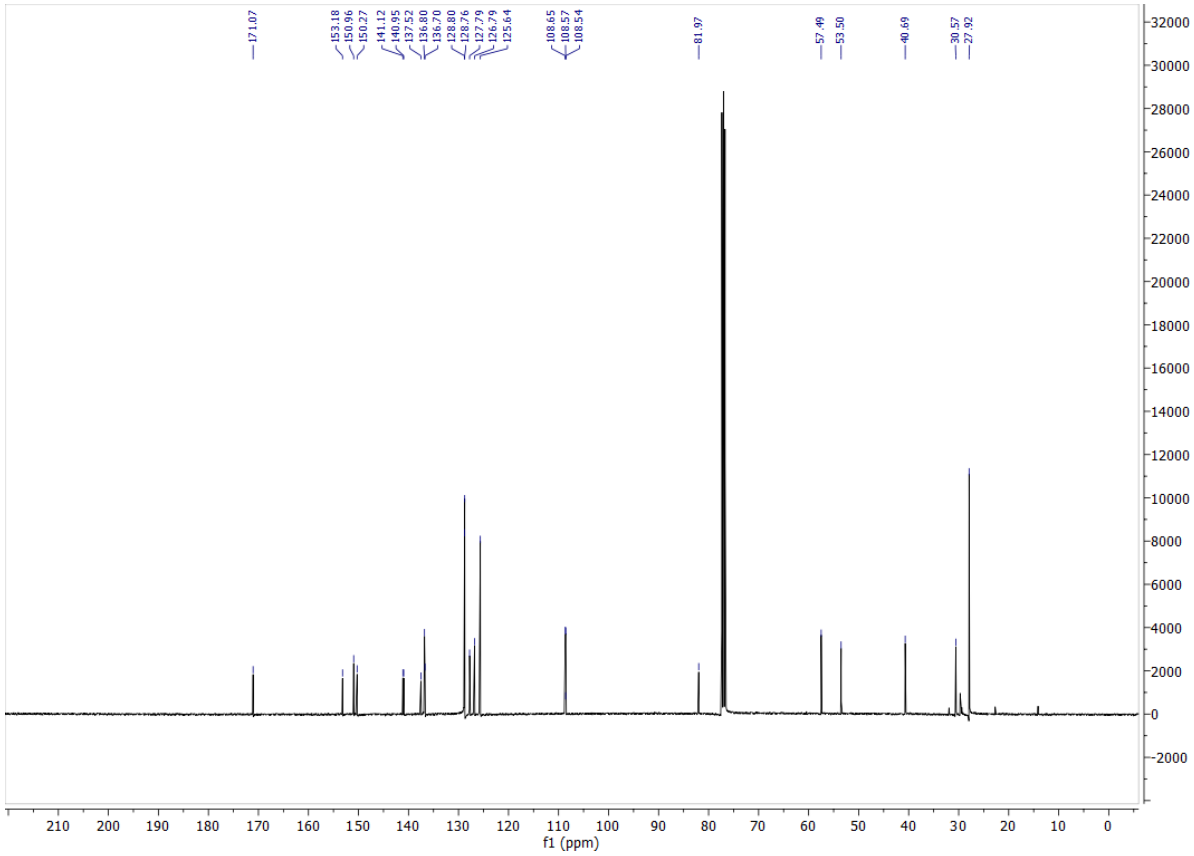

f2 (ppm)
f1 (ppm)

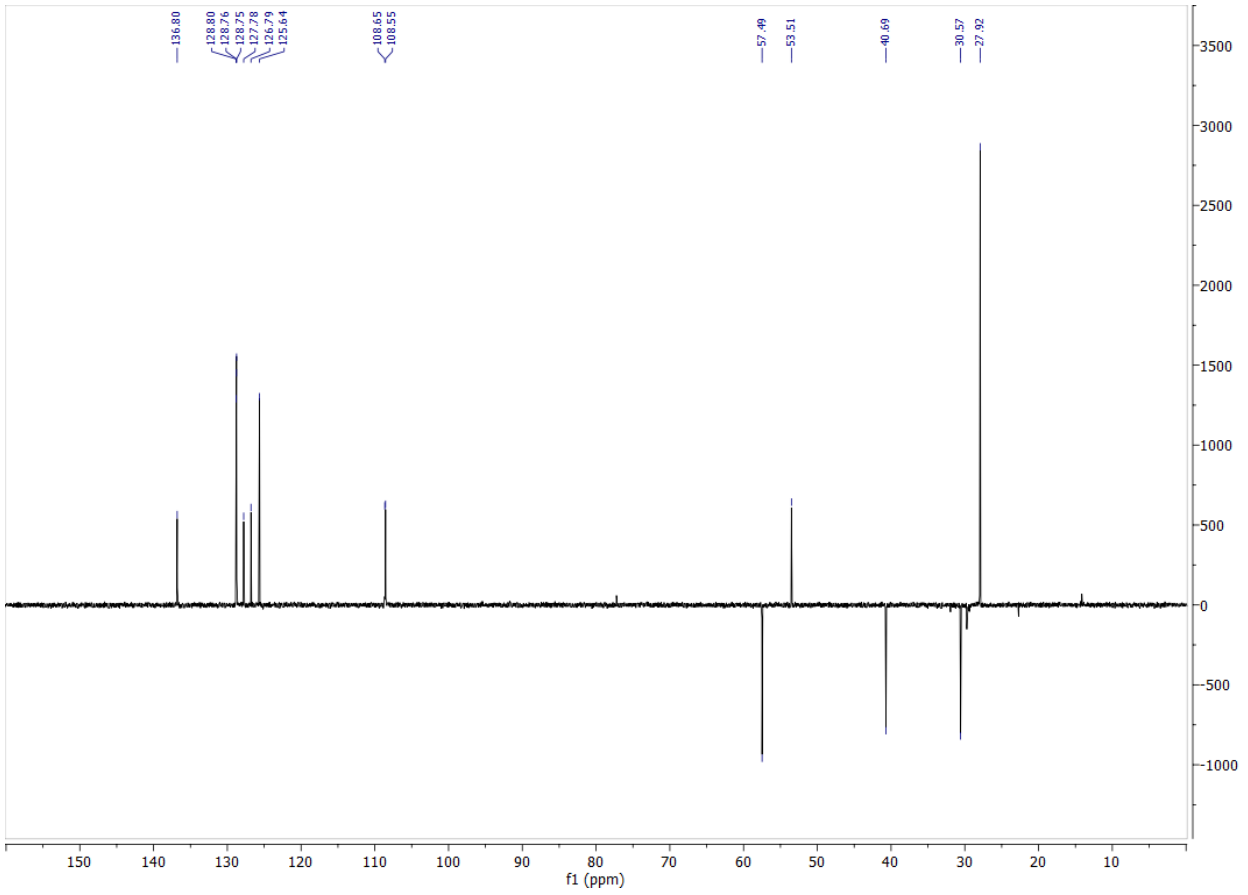

13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
f2 (ppm)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
f1 (ppm)

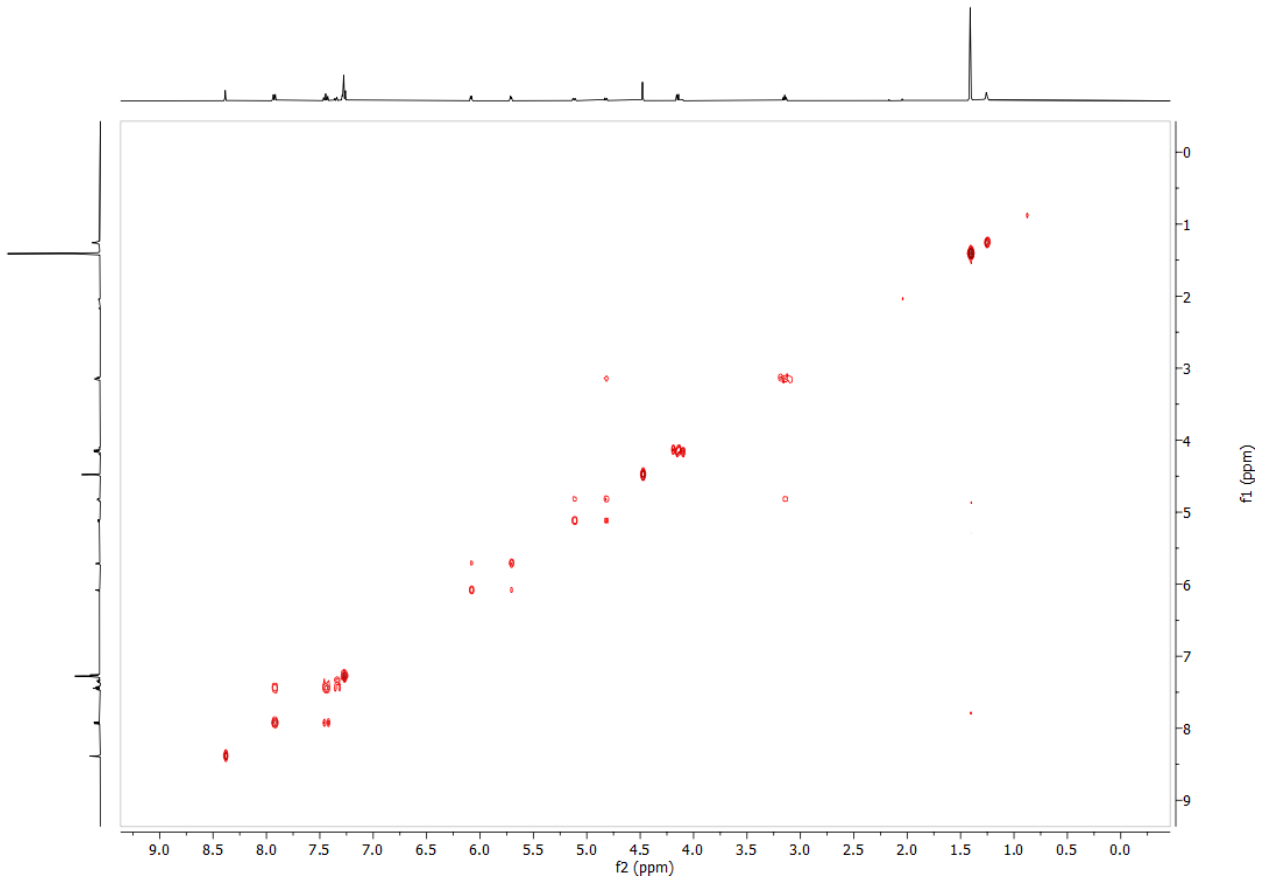

11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f2 (ppm)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
f1 (ppm)

Spectra ^{1}H, ^{13}C, ^{13}C-DEPT135 and COSY compound 13

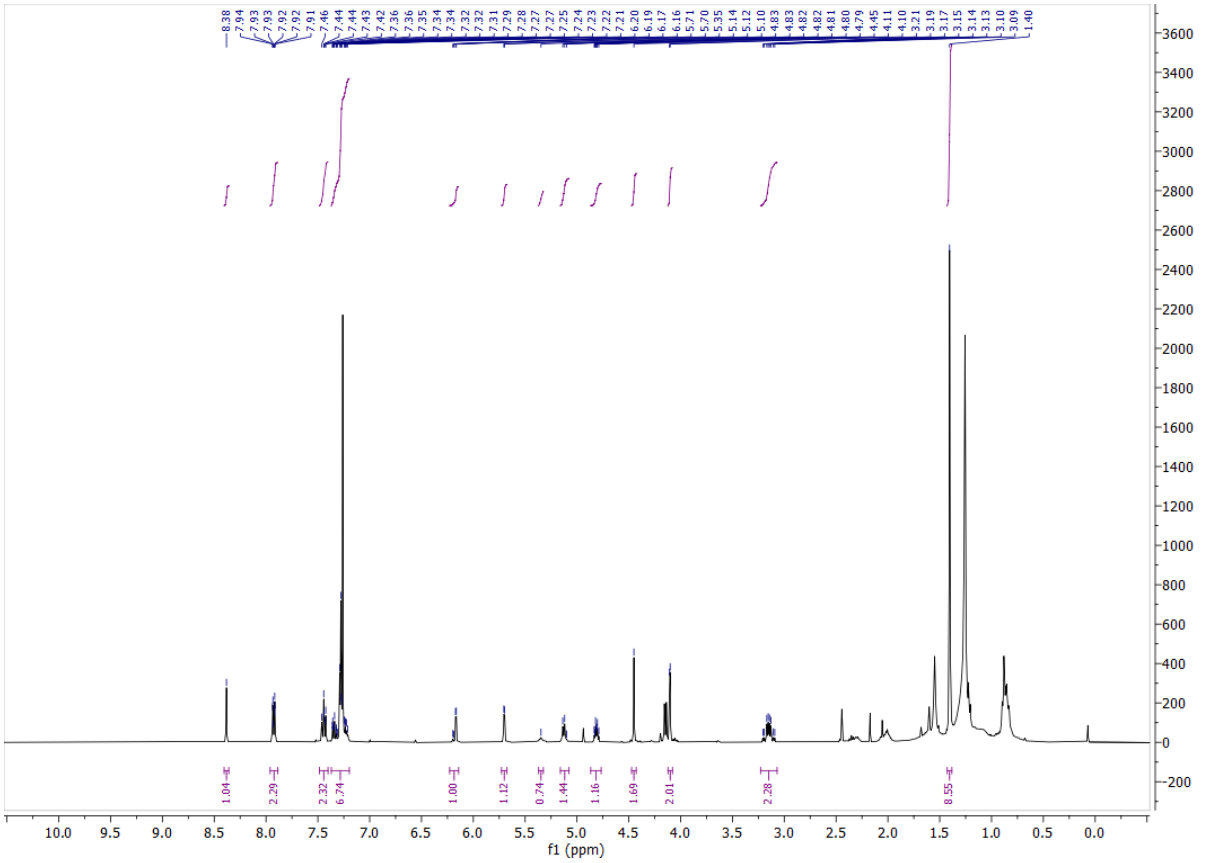


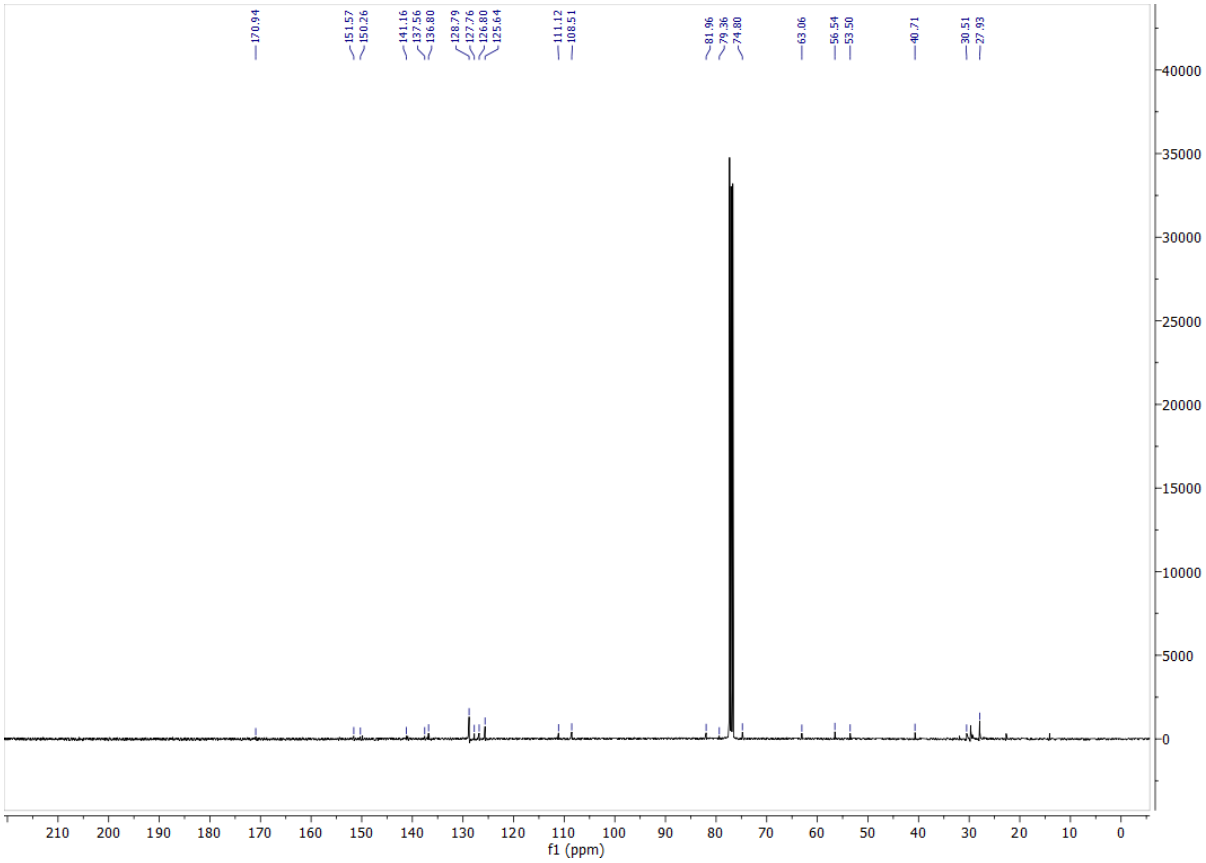


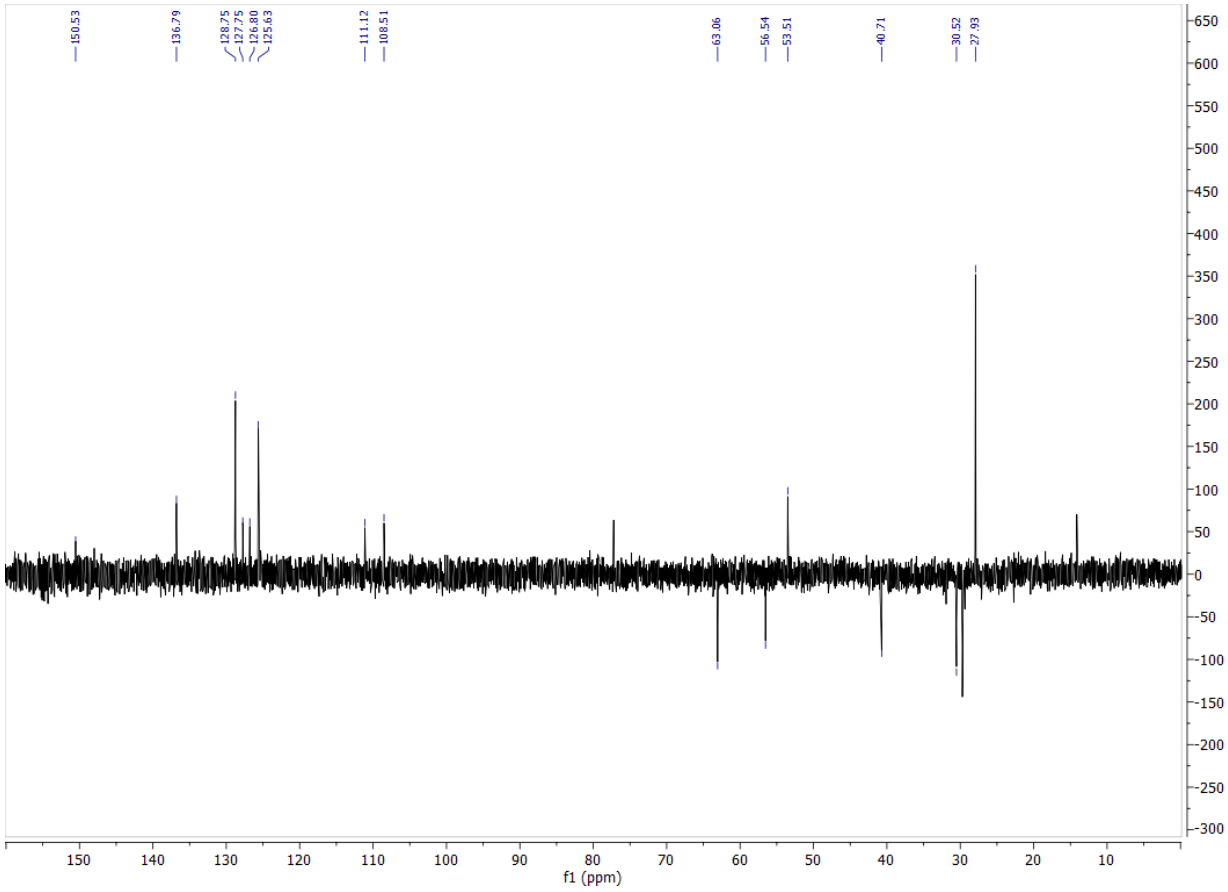

136.80
128.80
128.76
128.75
127.78
126.79
125.64
108.65
108.55
57.49
53.51
40.69
30.57
27.92
f1 (ppm)

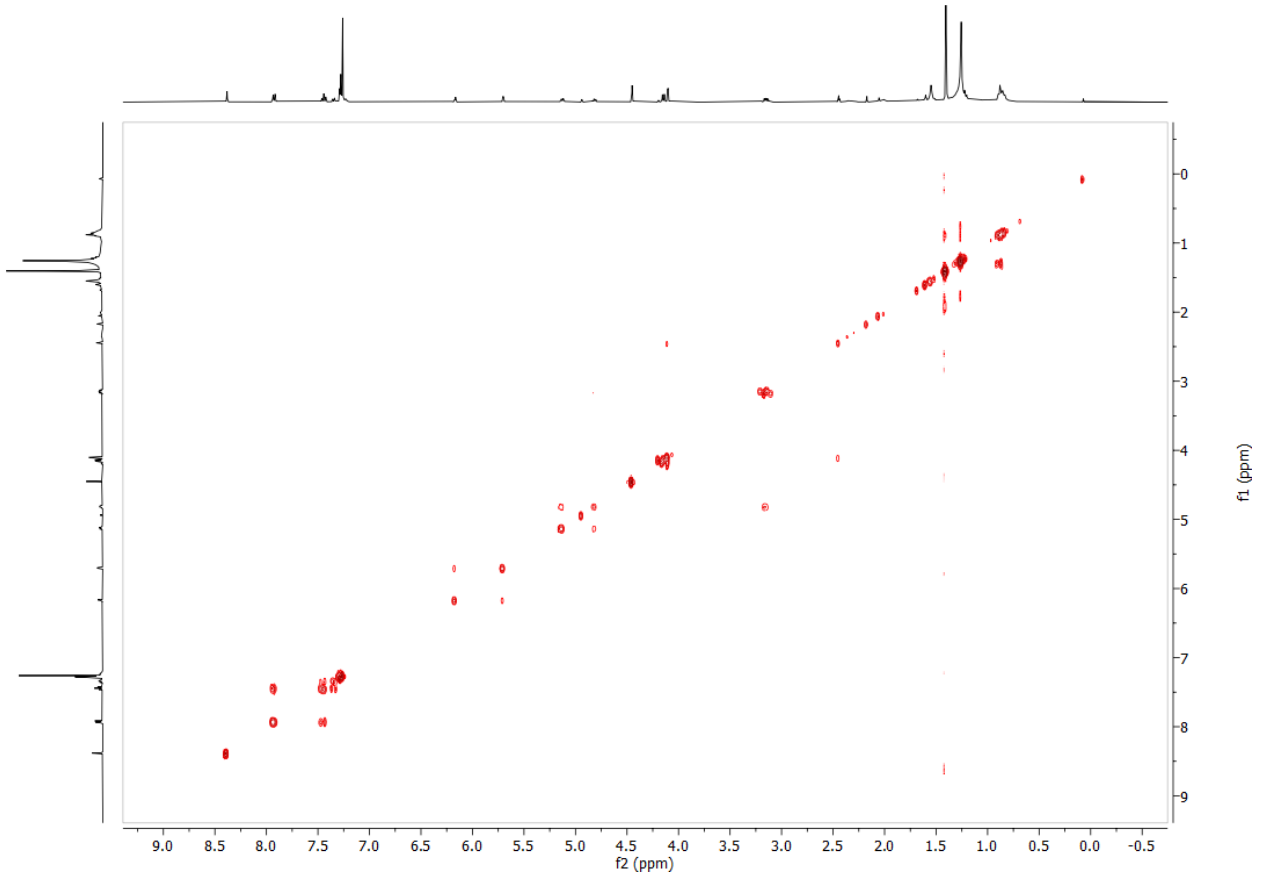

f2 (ppm)
f1 (ppm)

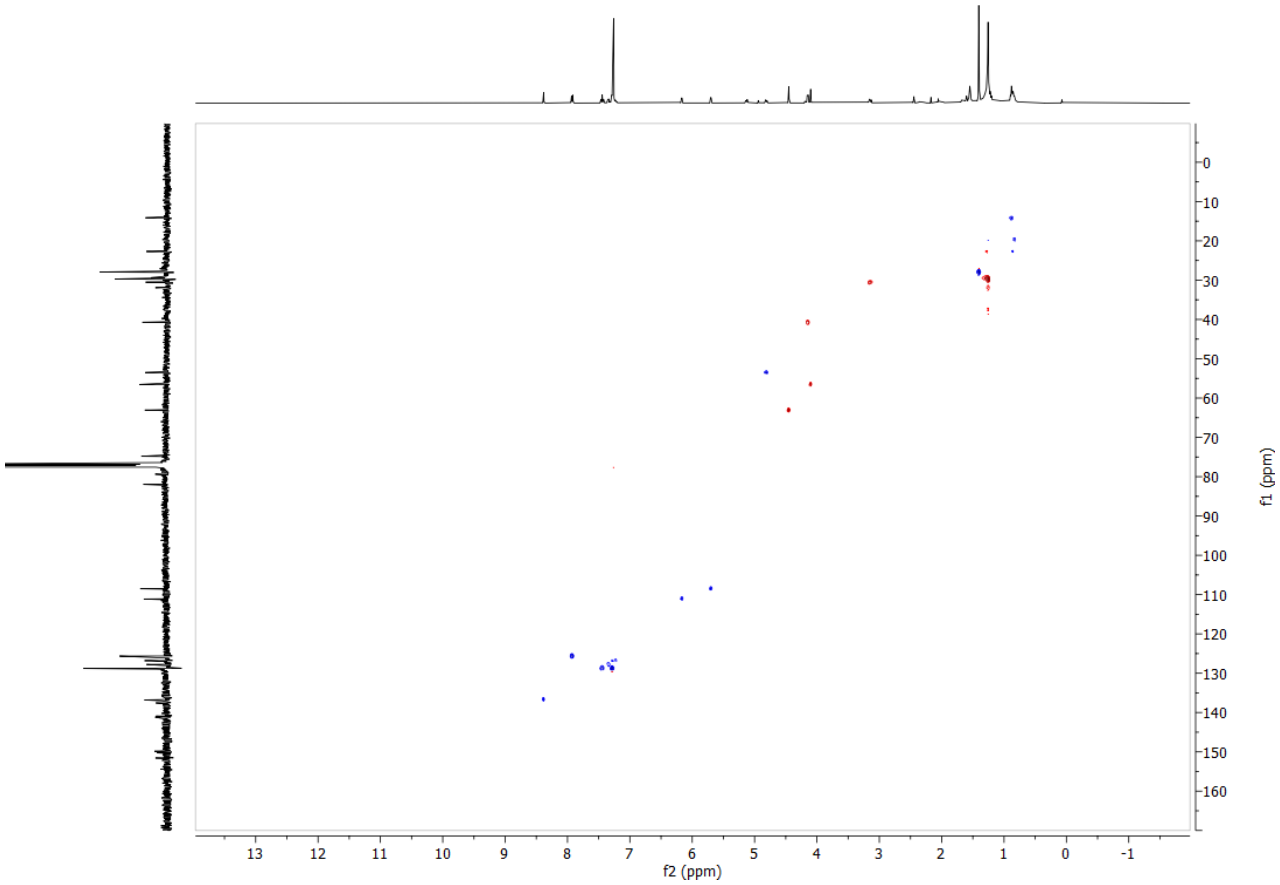
Spectra ^{1}H, ^{13}C, ^{13}C-DEPT135, COSY, HSQC and HMBC compound 15c

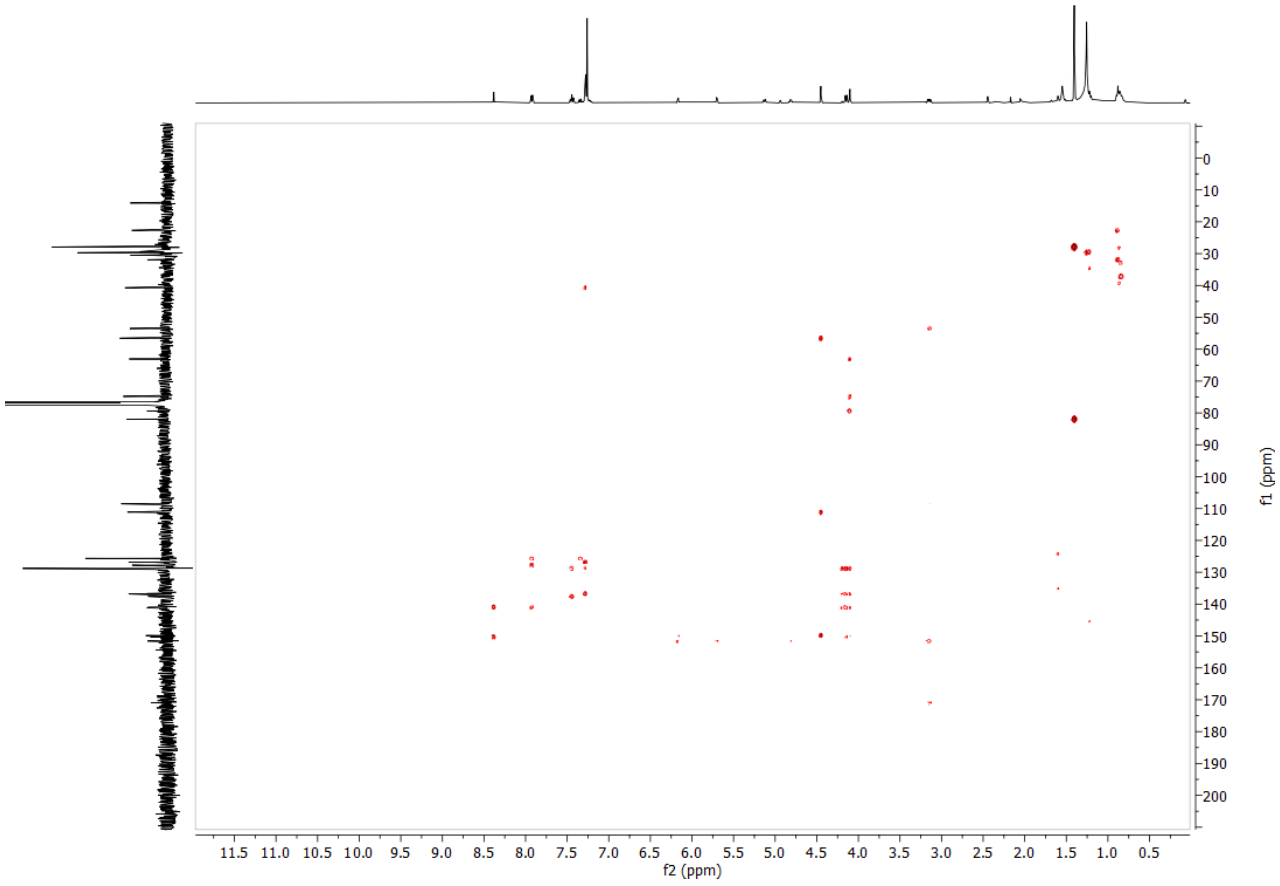




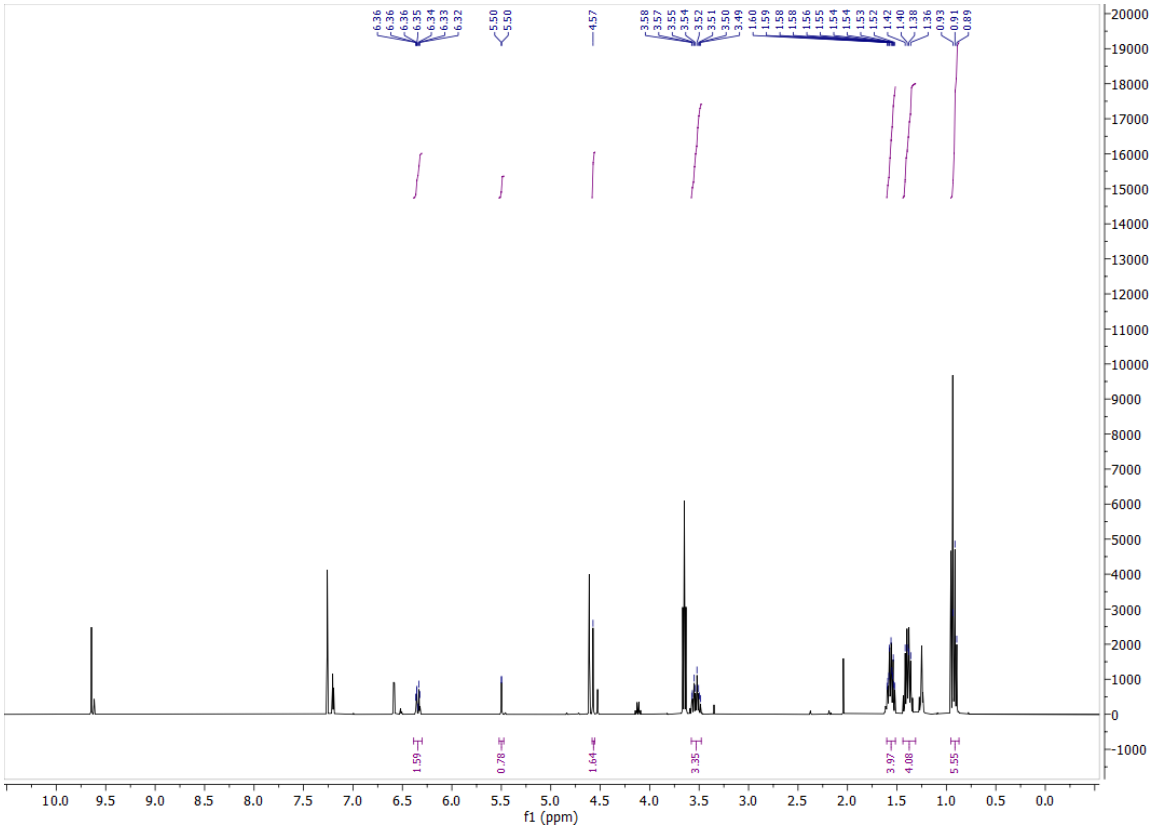

150.53
136.79
128.75
127.75
126.80
125.63
111.12
108.51
63.06
56.54
53.51
40.71
30.52
27.93
f1 (ppm)


f2 (ppm)
f1 (ppm)

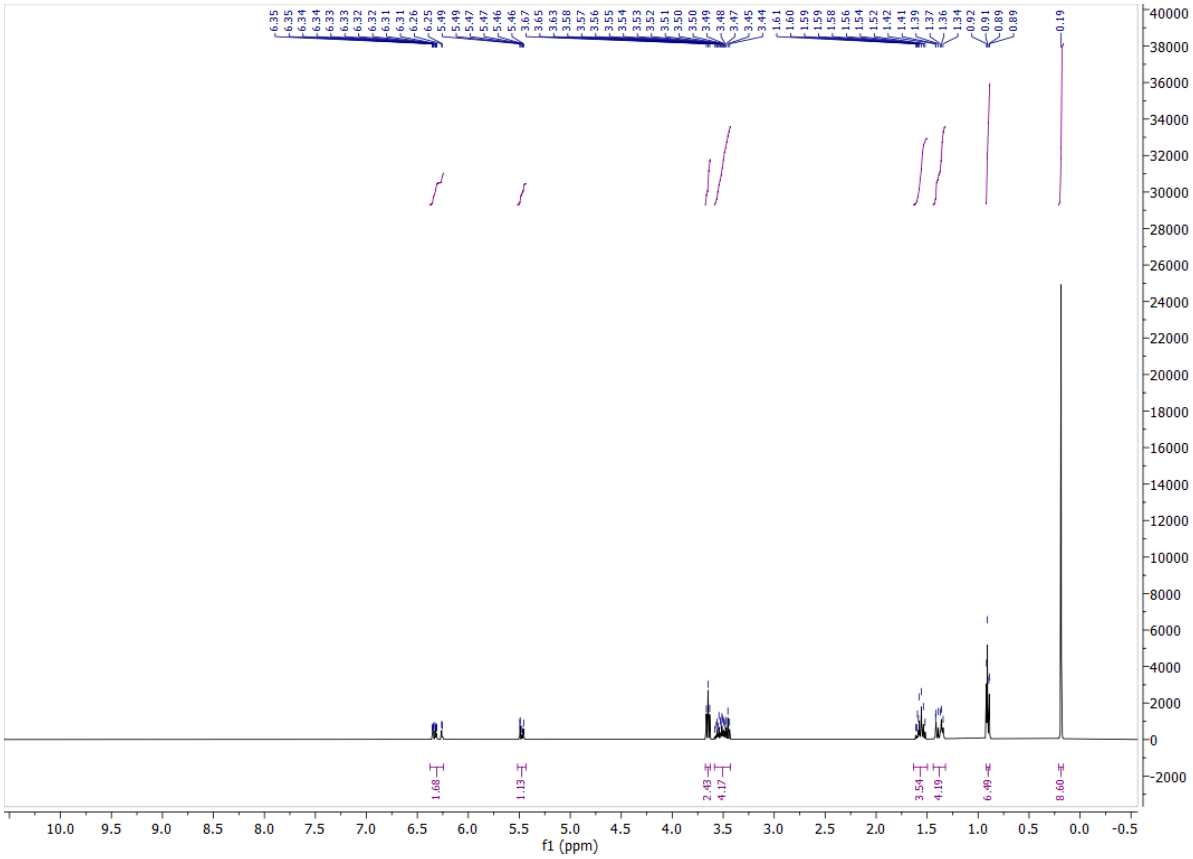

13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1
f2 (ppm)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
f1 (ppm)

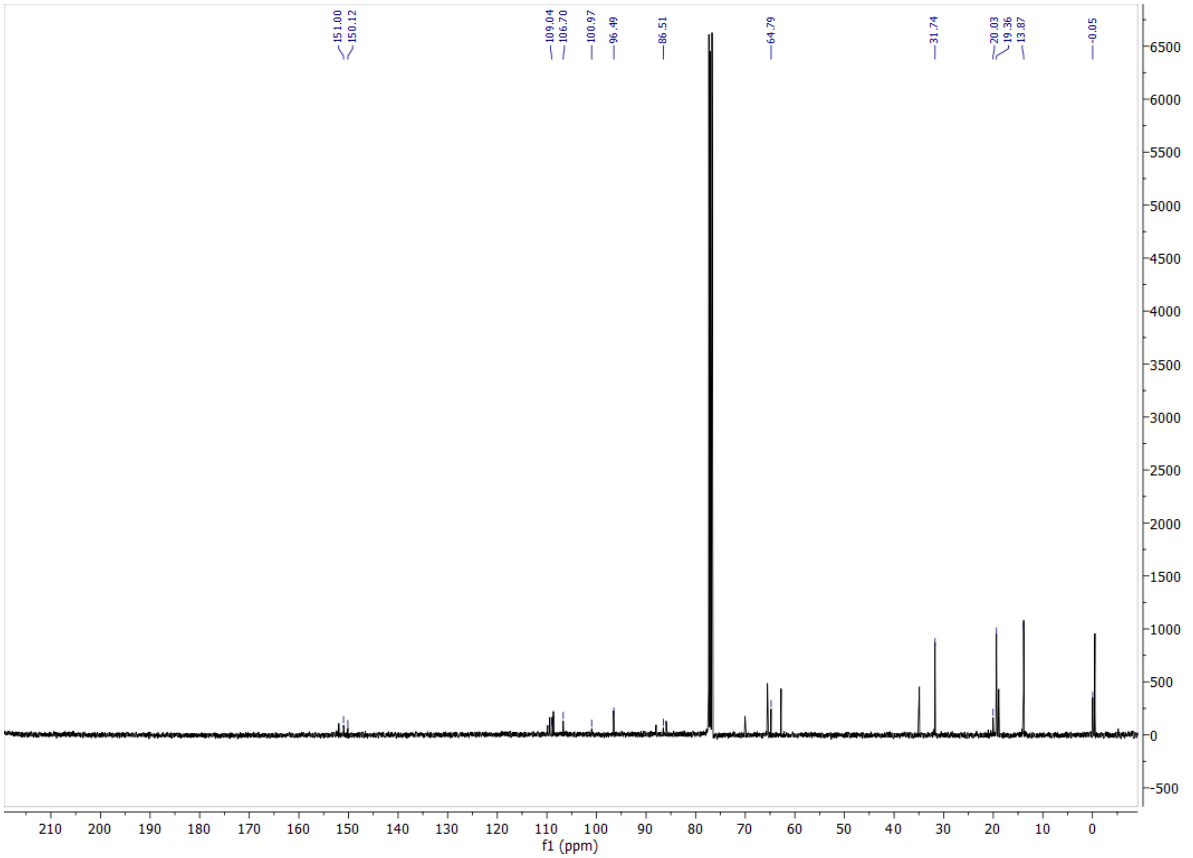

11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f2 (ppm)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
f1 (ppm)

Spectra ^{1}H compound 18

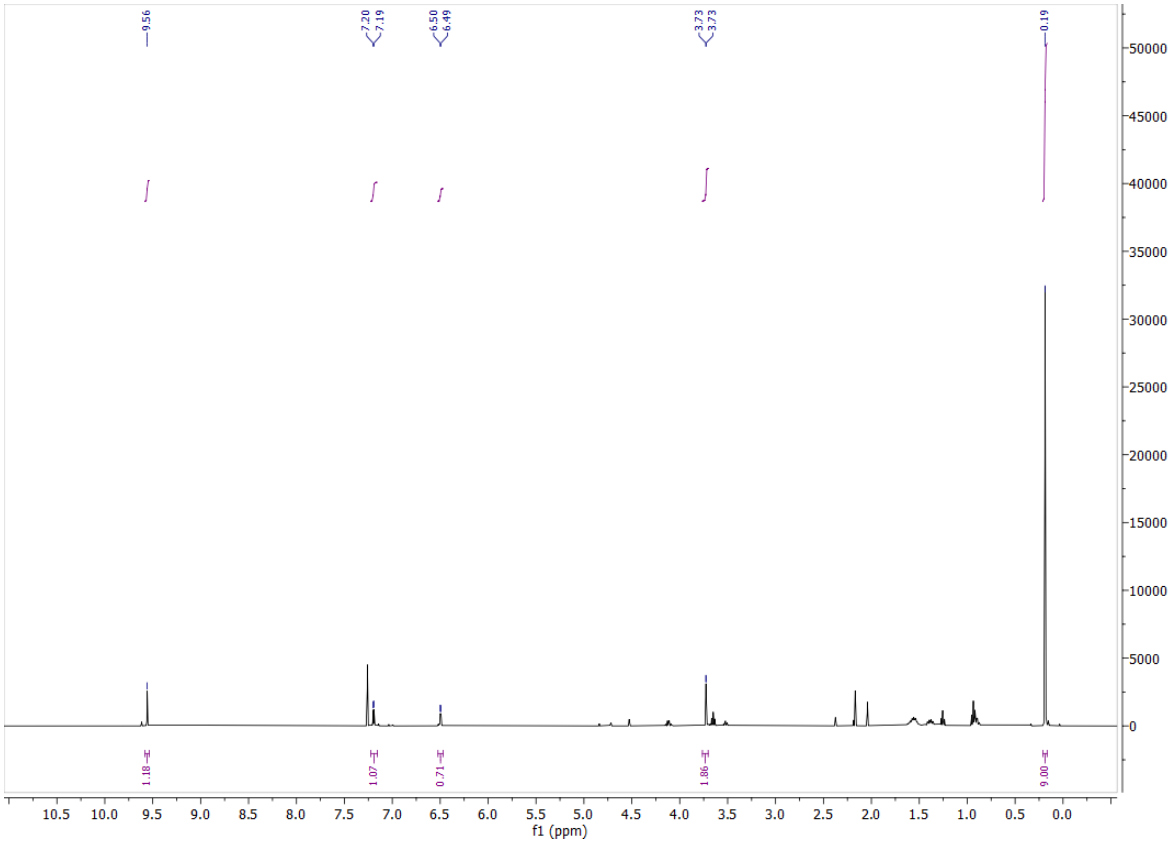


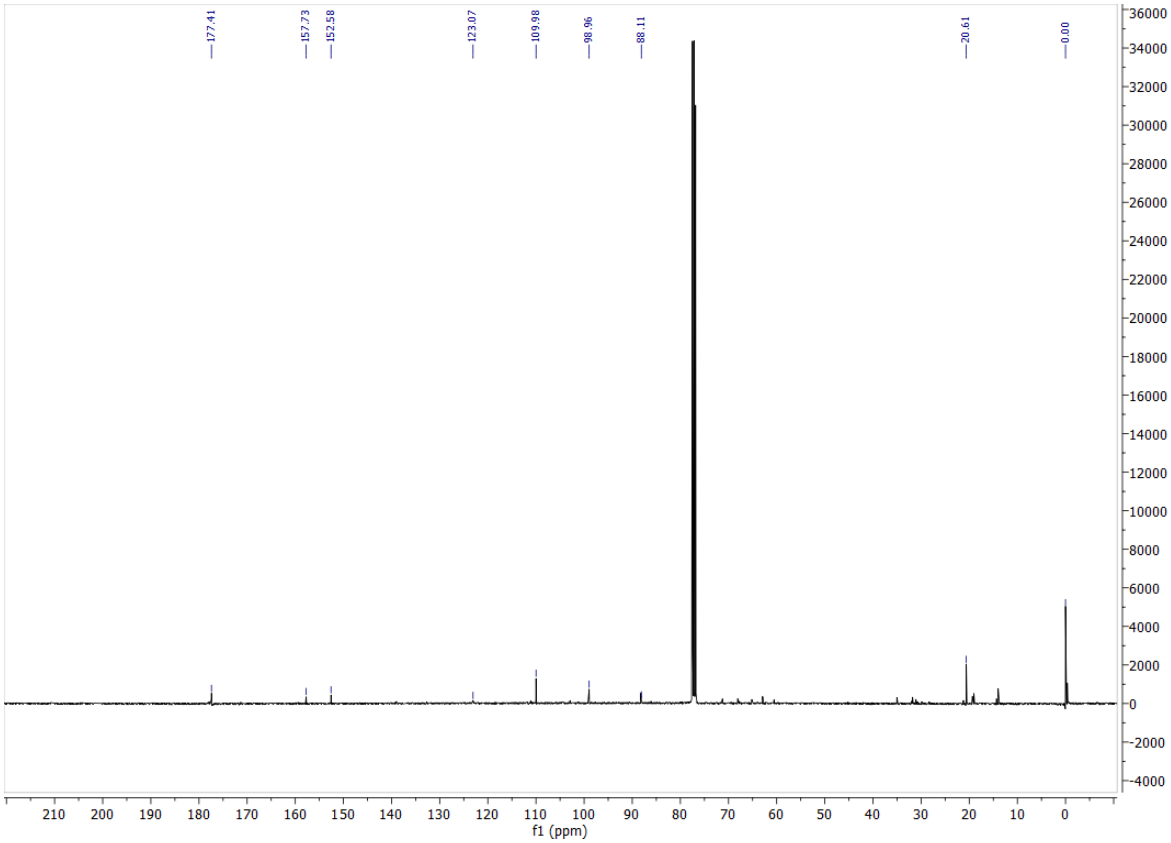
Spectra ^{1}H and ^{13}C compound 19



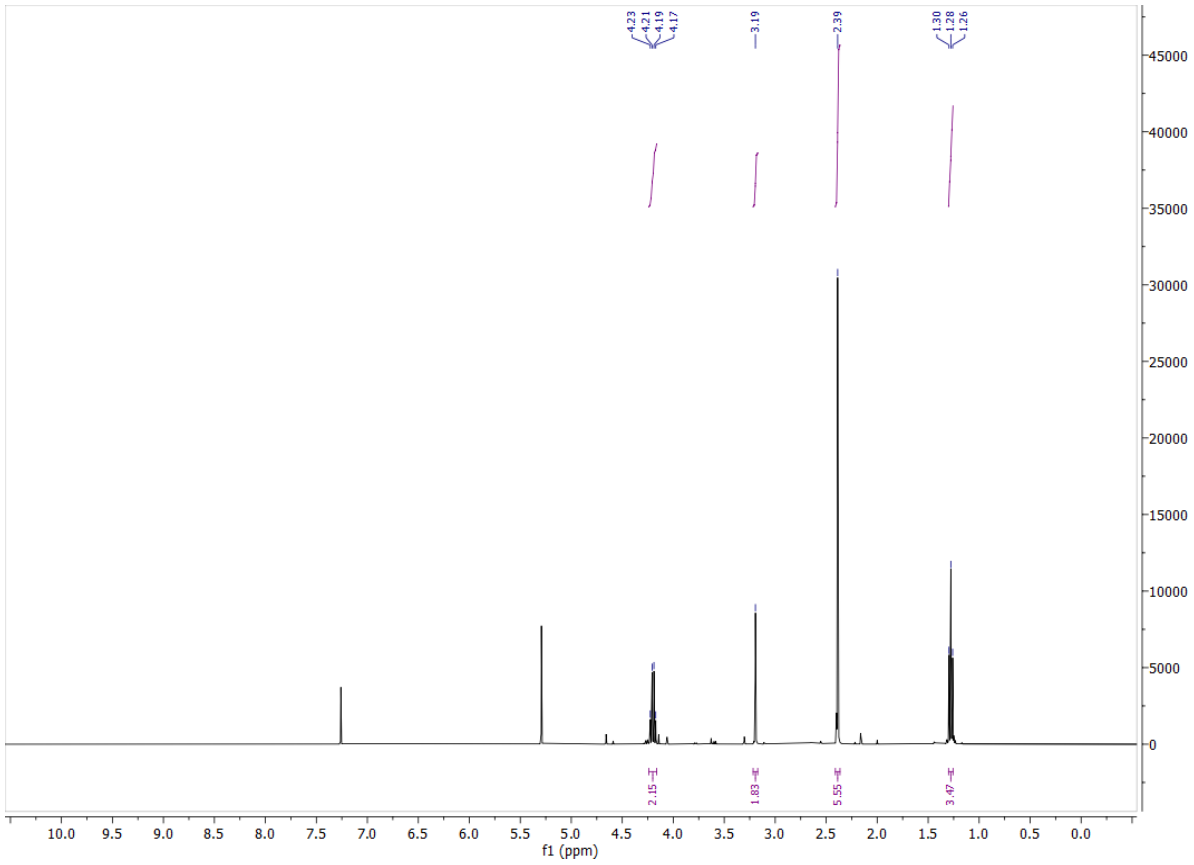


Spectra ^{1}H and ^{13}C compound 20

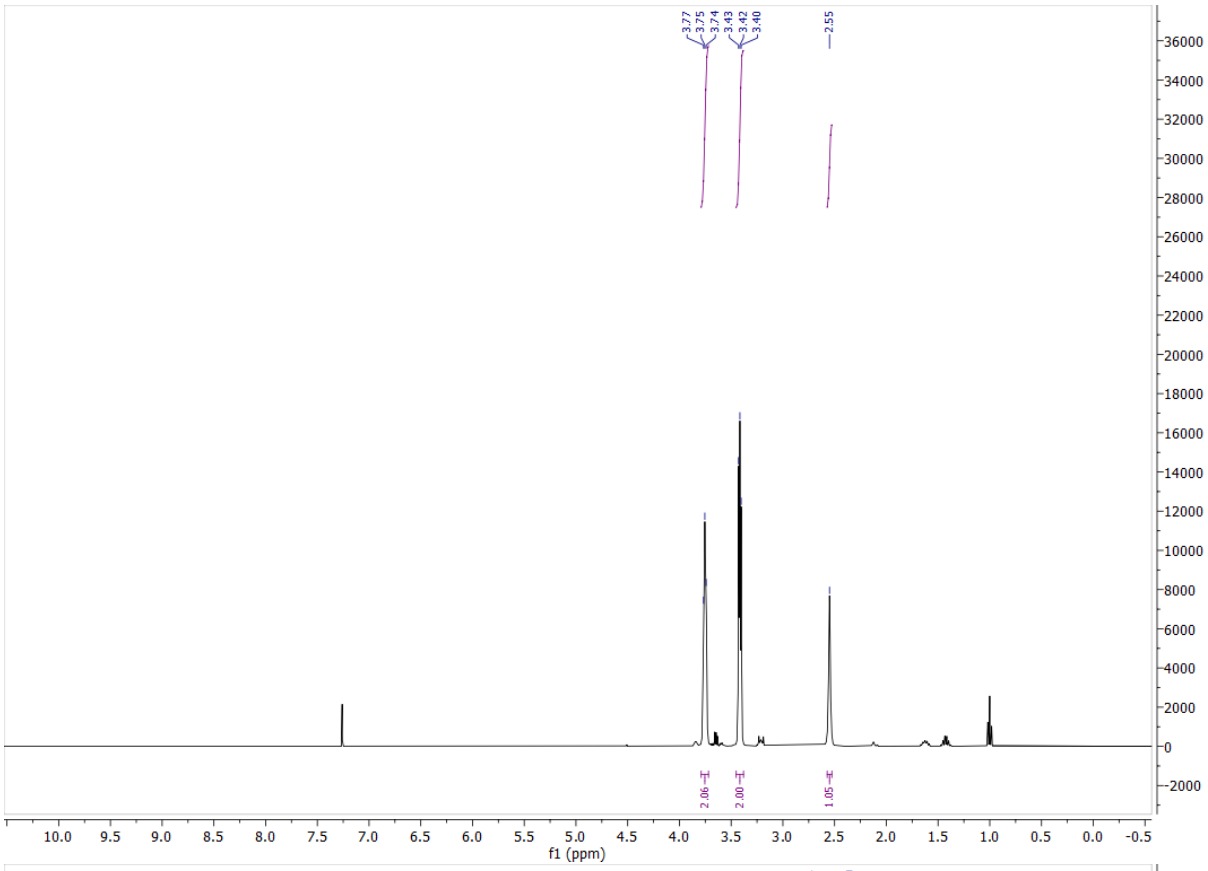


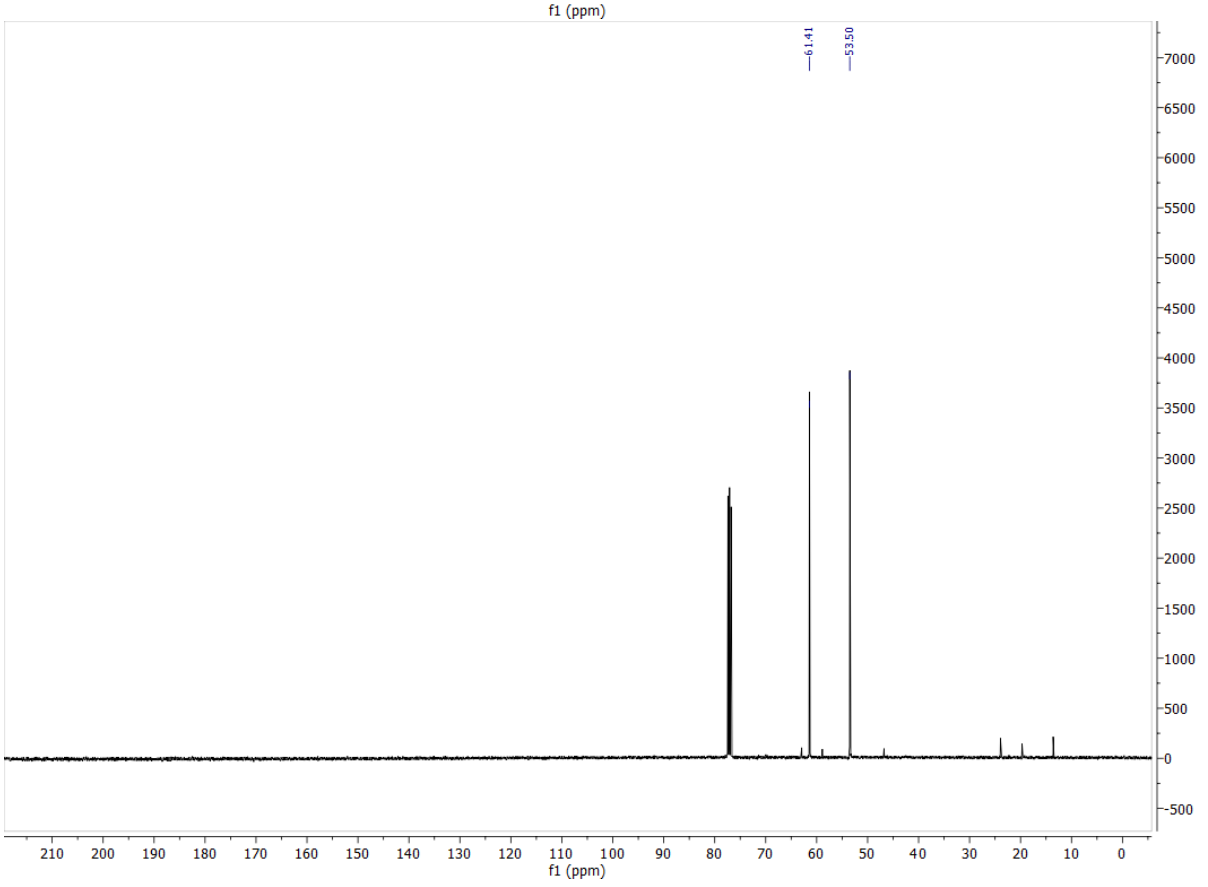


Spectra ^{1}H compound 22

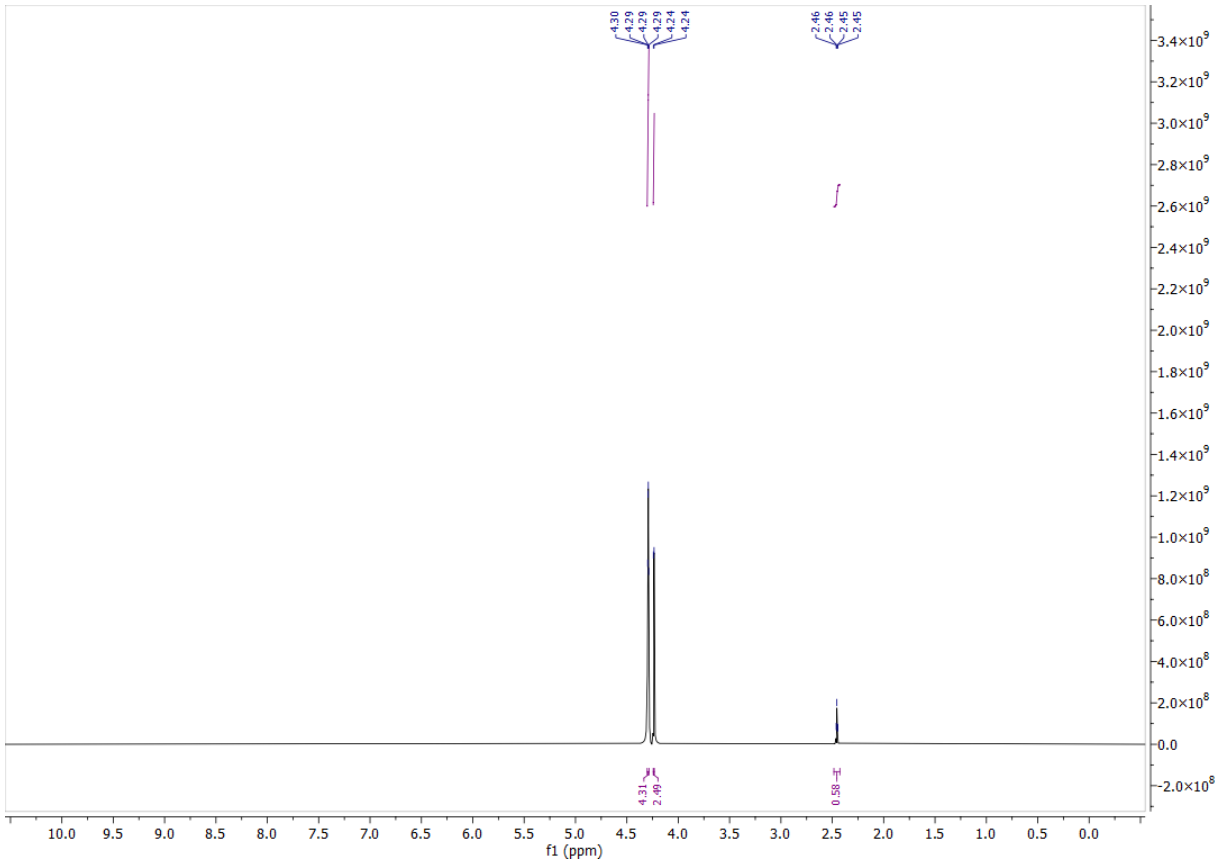


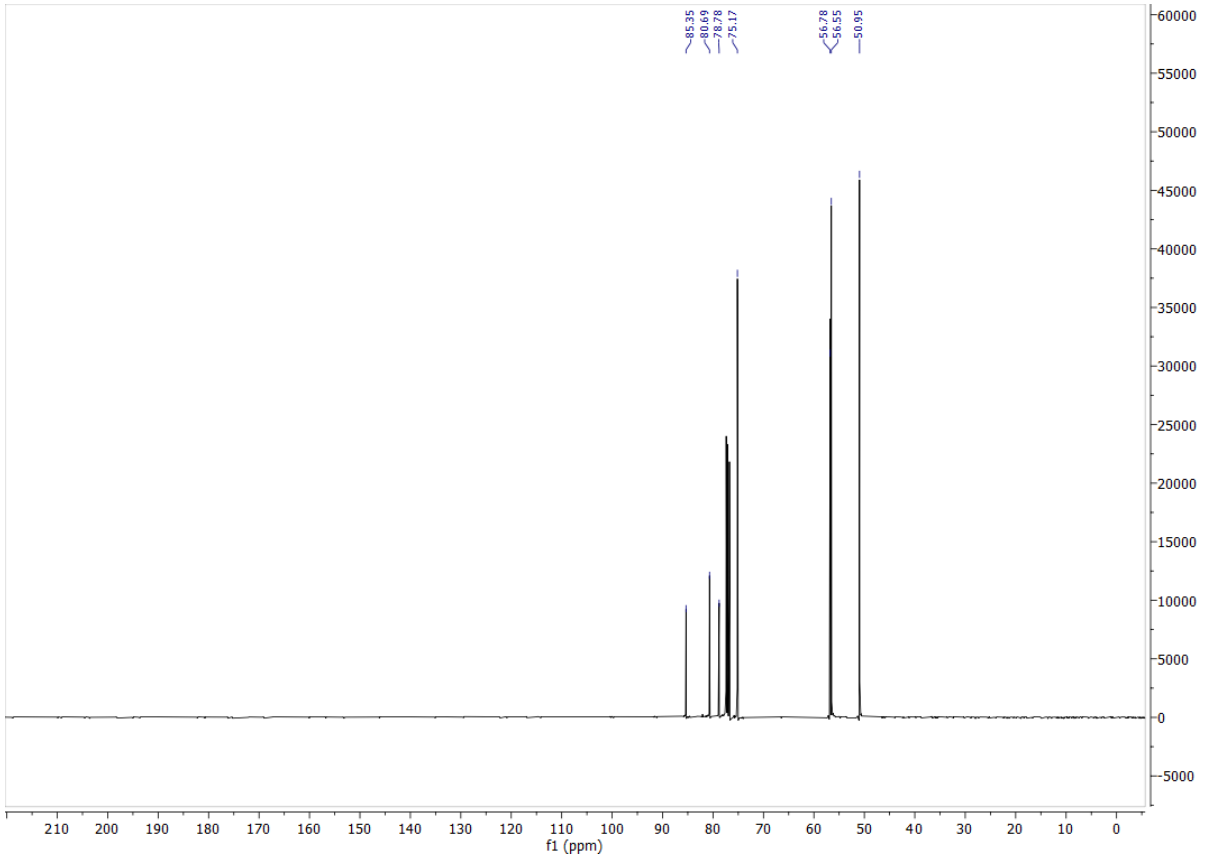
Spectra ^{1}H and ^{13}C compound L2



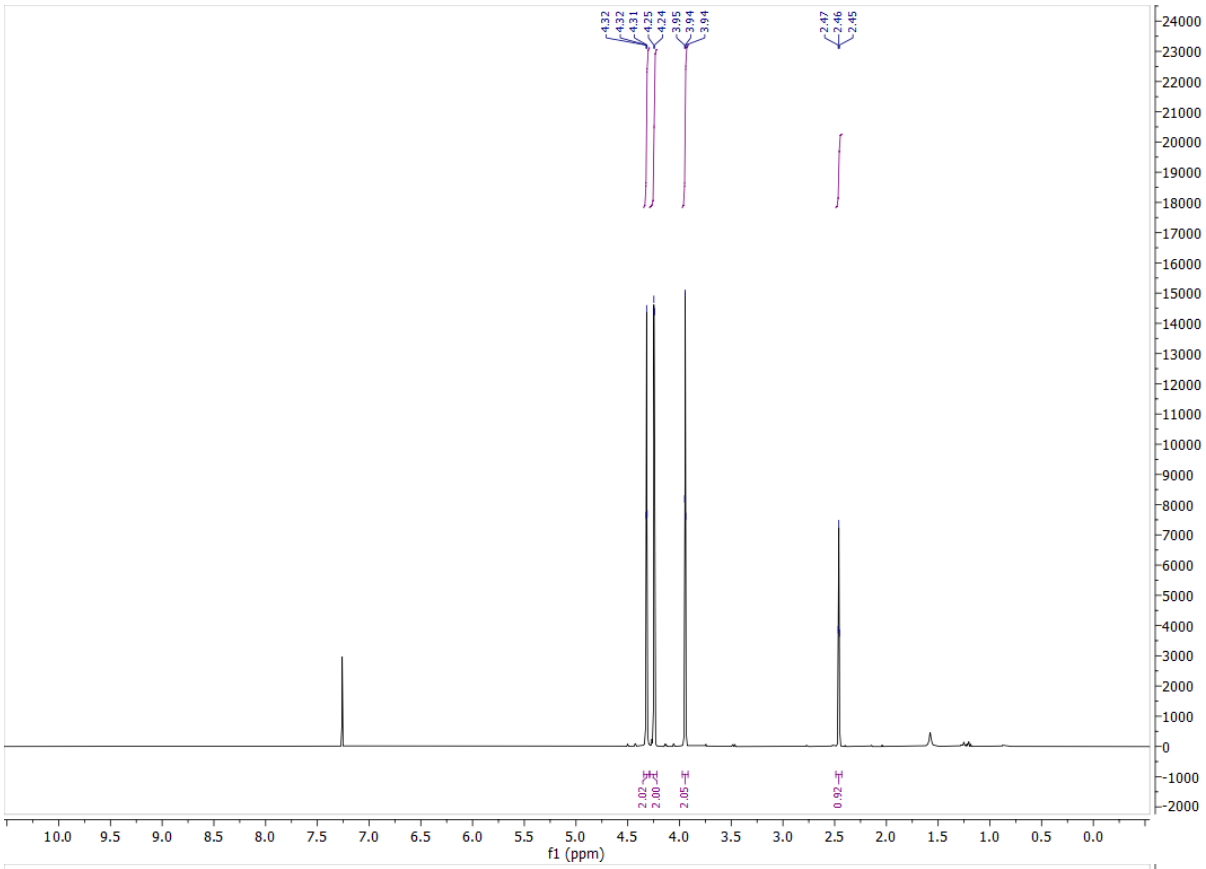


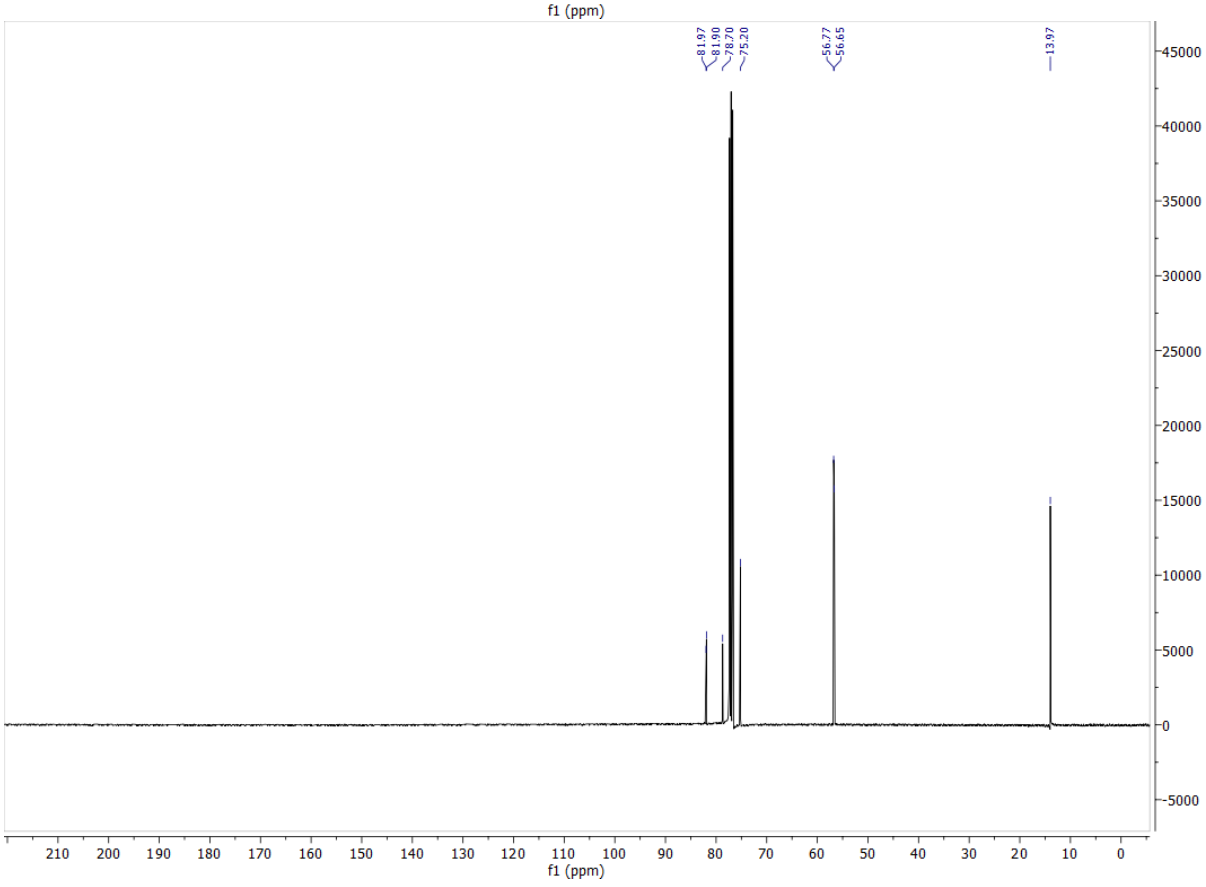
Spectra ^{1}H and ^{13}C compound L4



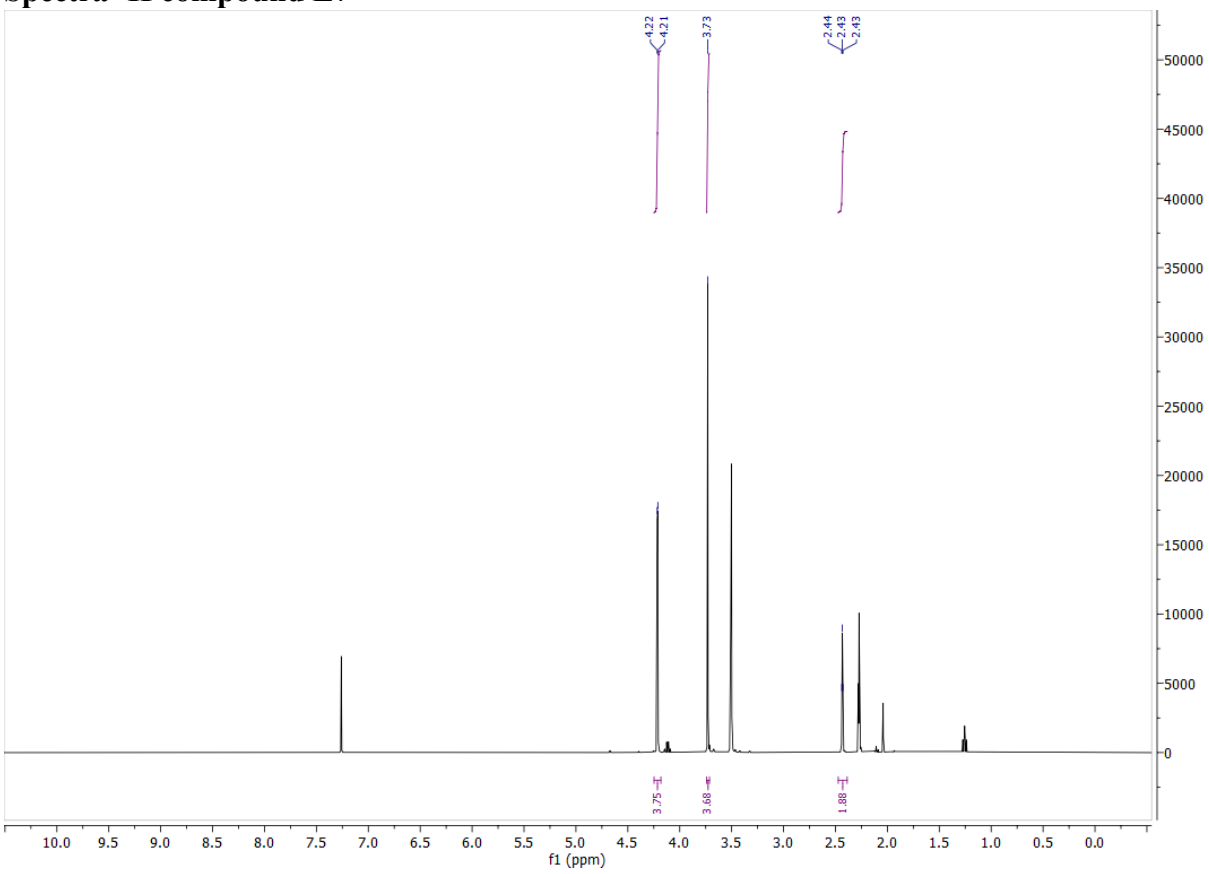


Spectra ^{1}H and ^{13}C compound L5

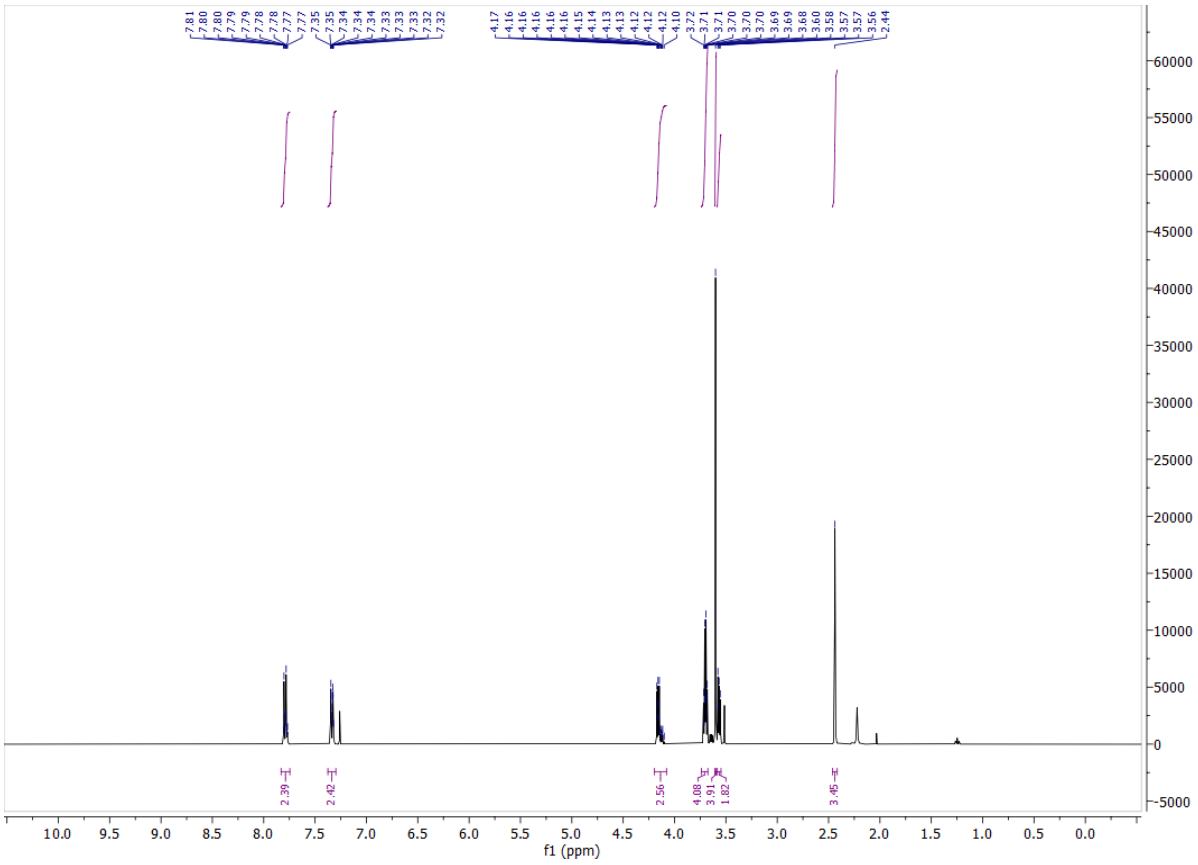


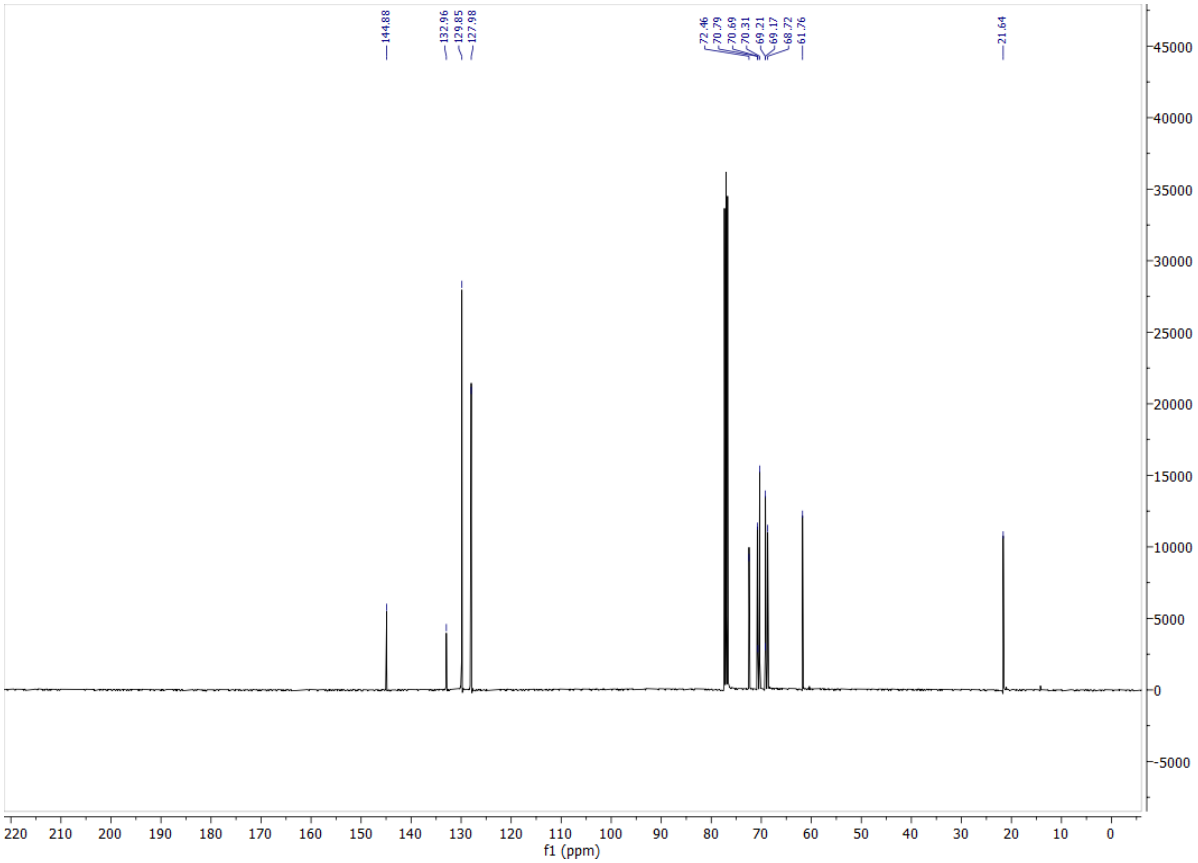


Spectra ^{1}H compound L7

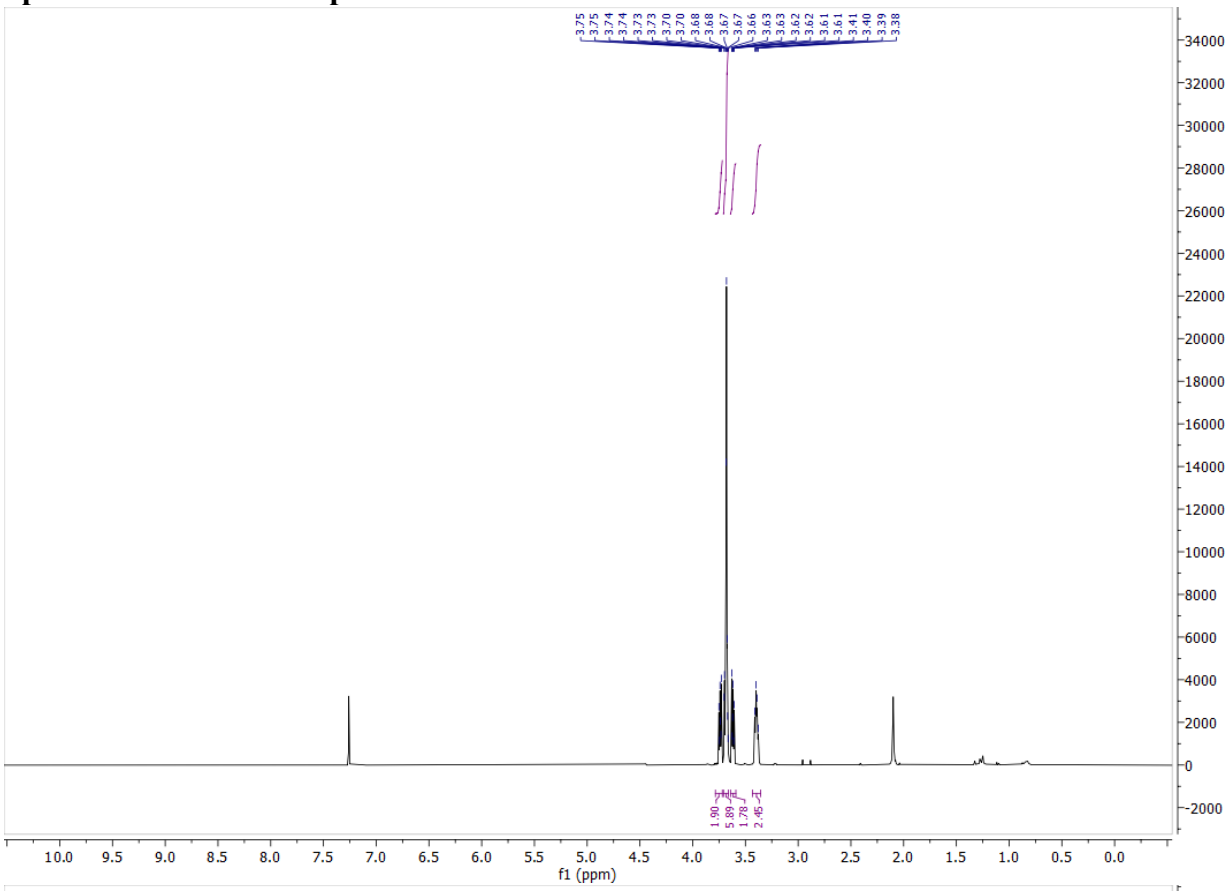


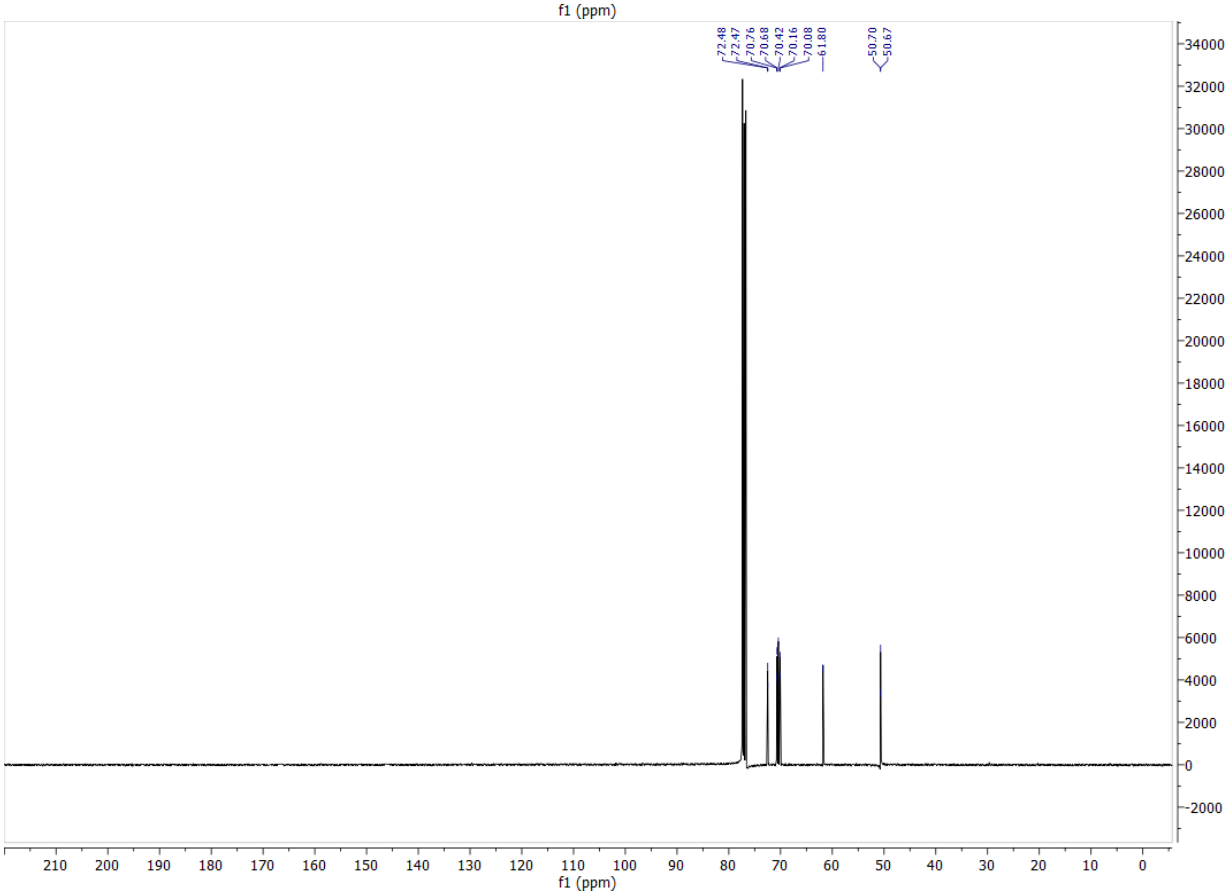
Spectra ^{1}H and ^{13}C compound L9



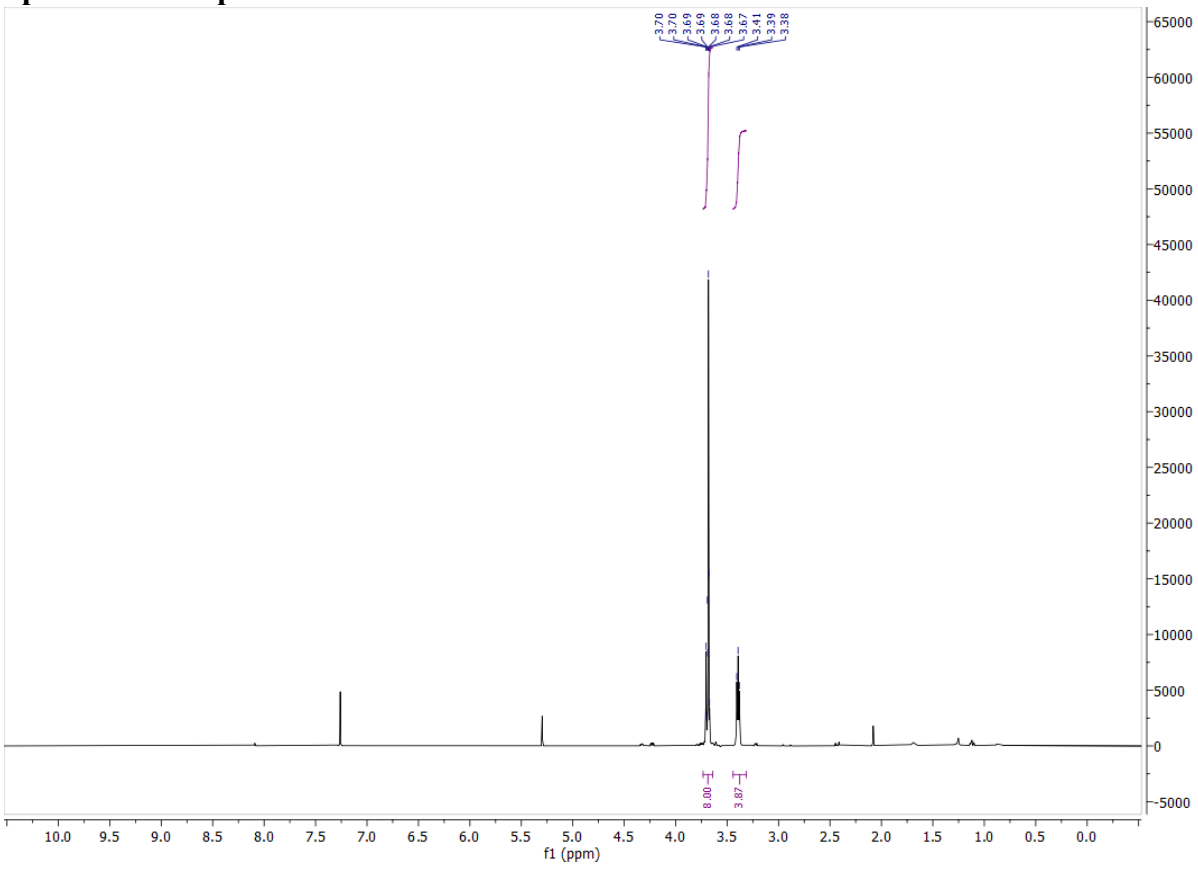


Spectra ^{1}H and ^{13}C compound L10

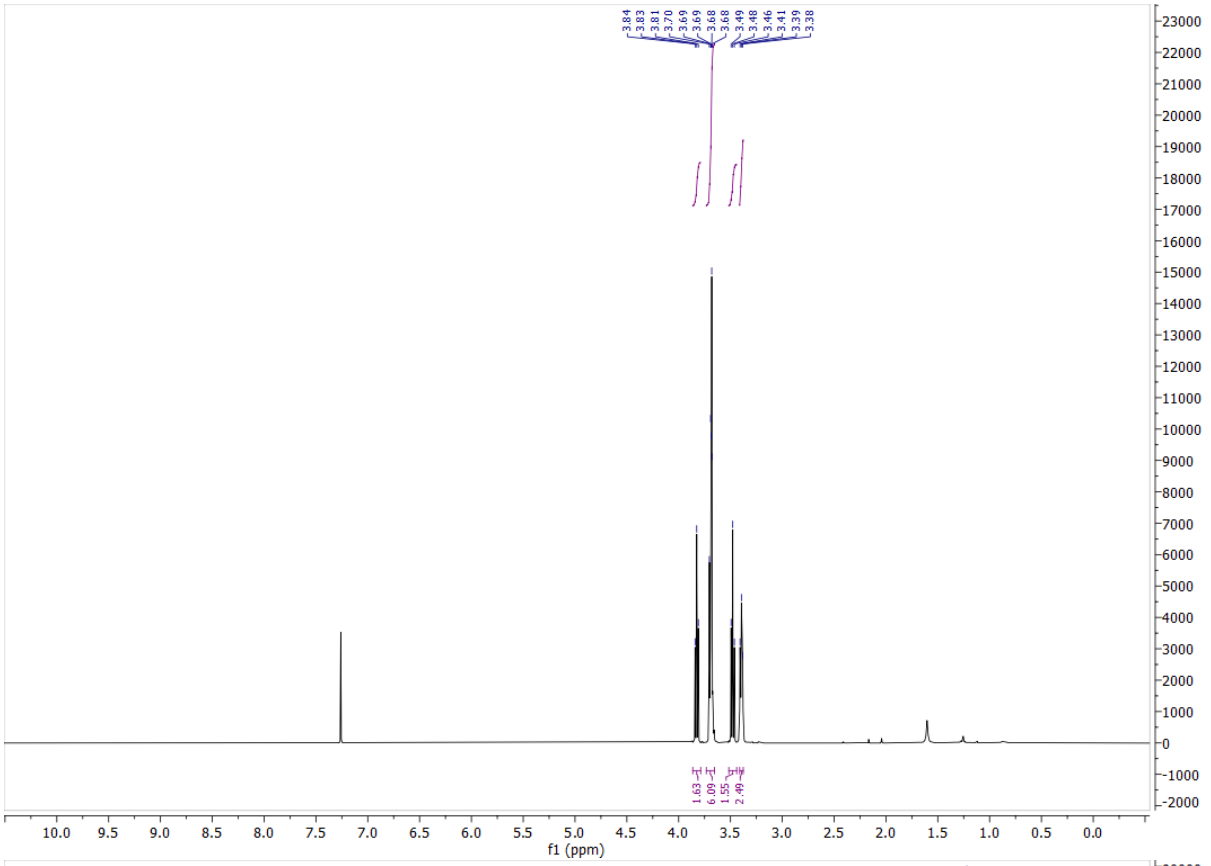


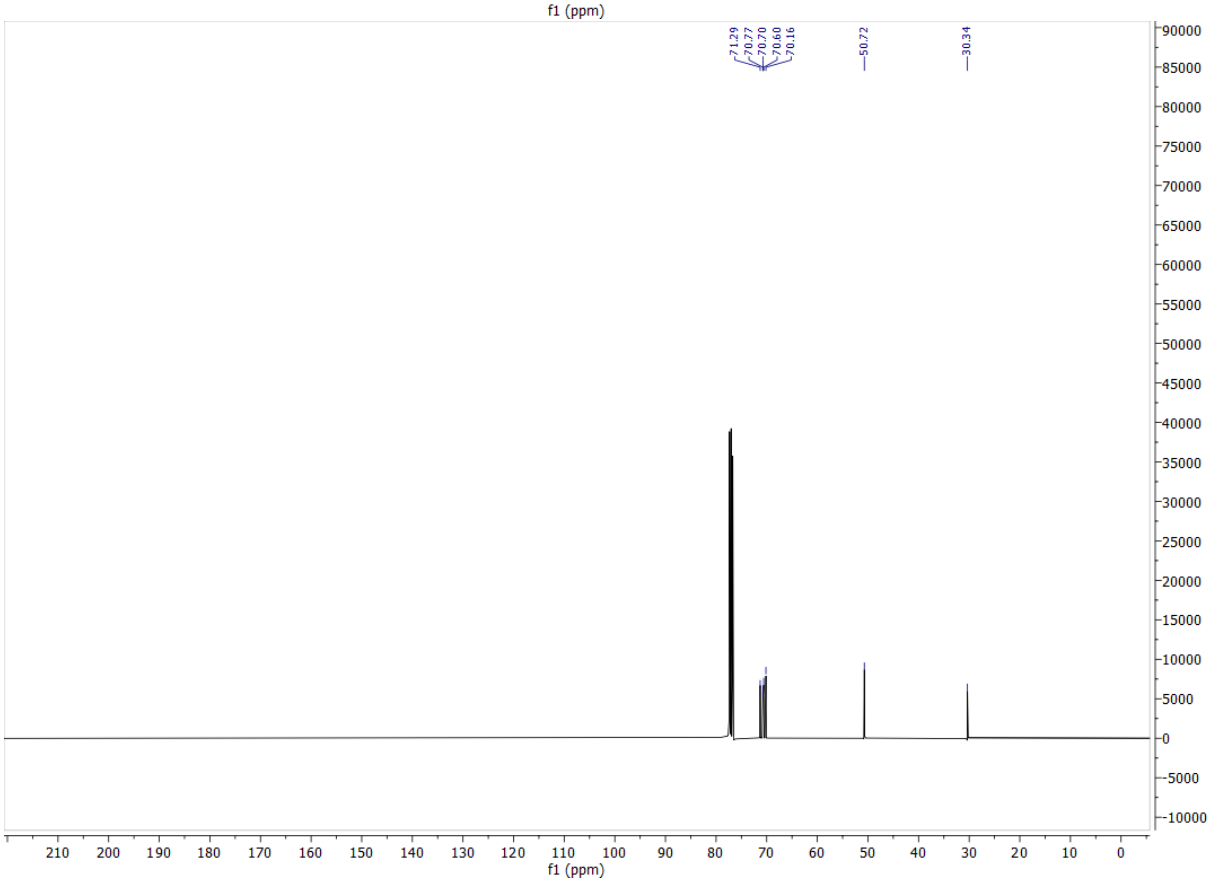


Spectra ^{1}H compound L11

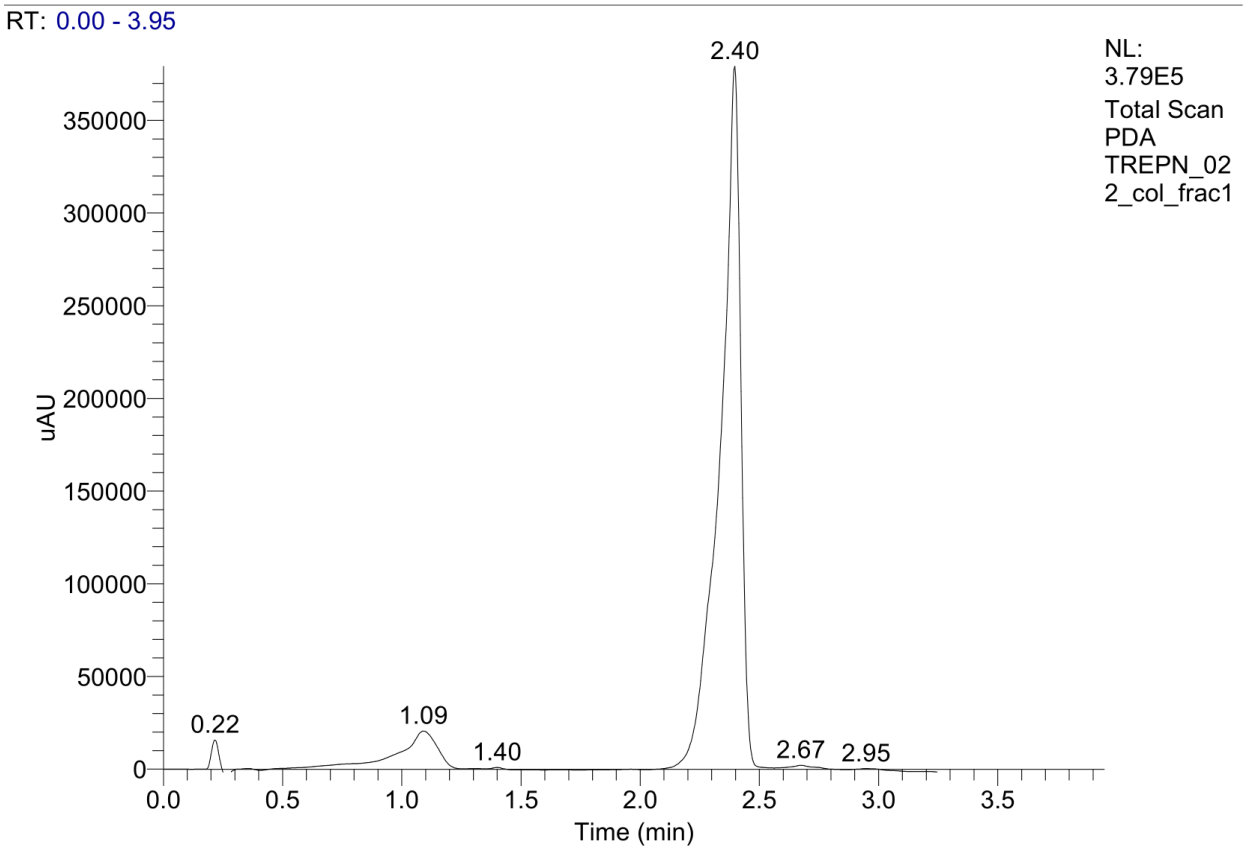


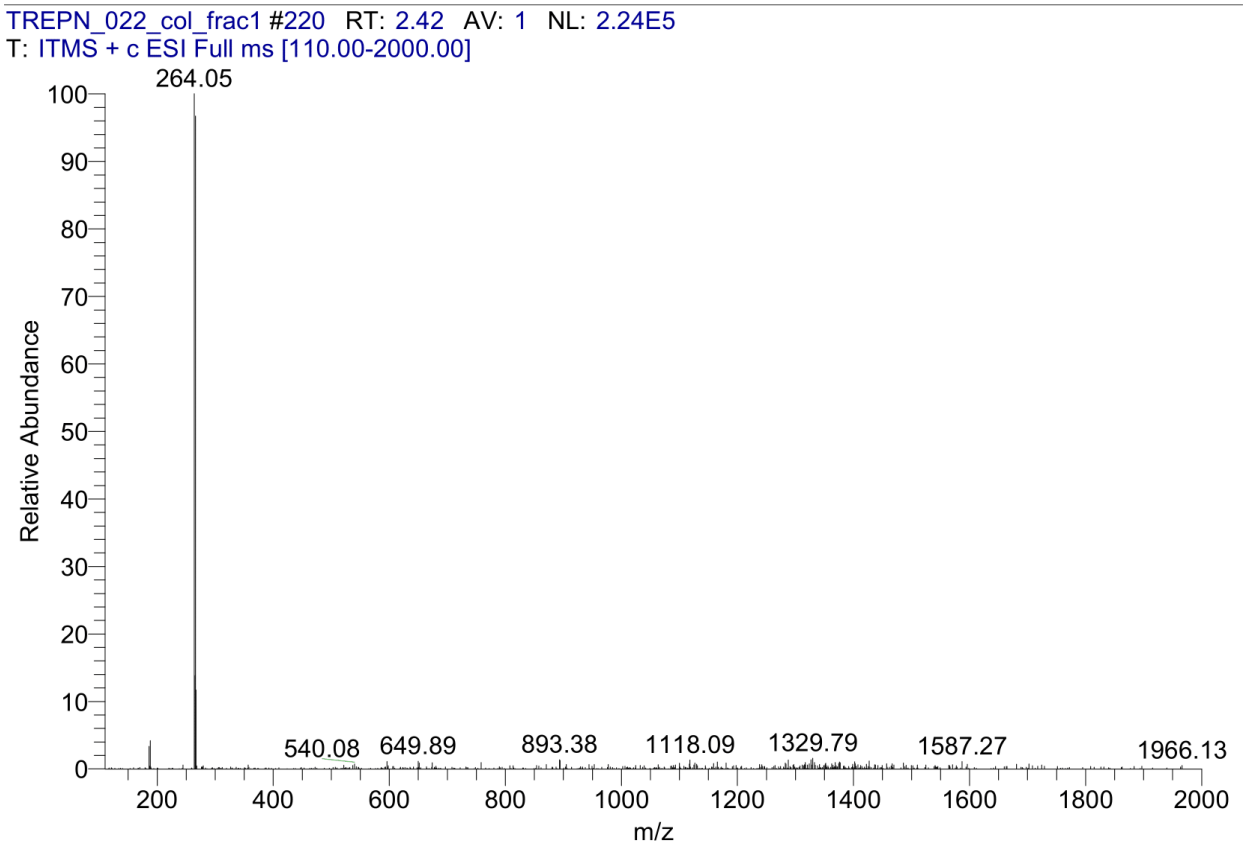
Spectra ^{1}H and ^{13}C compound L12



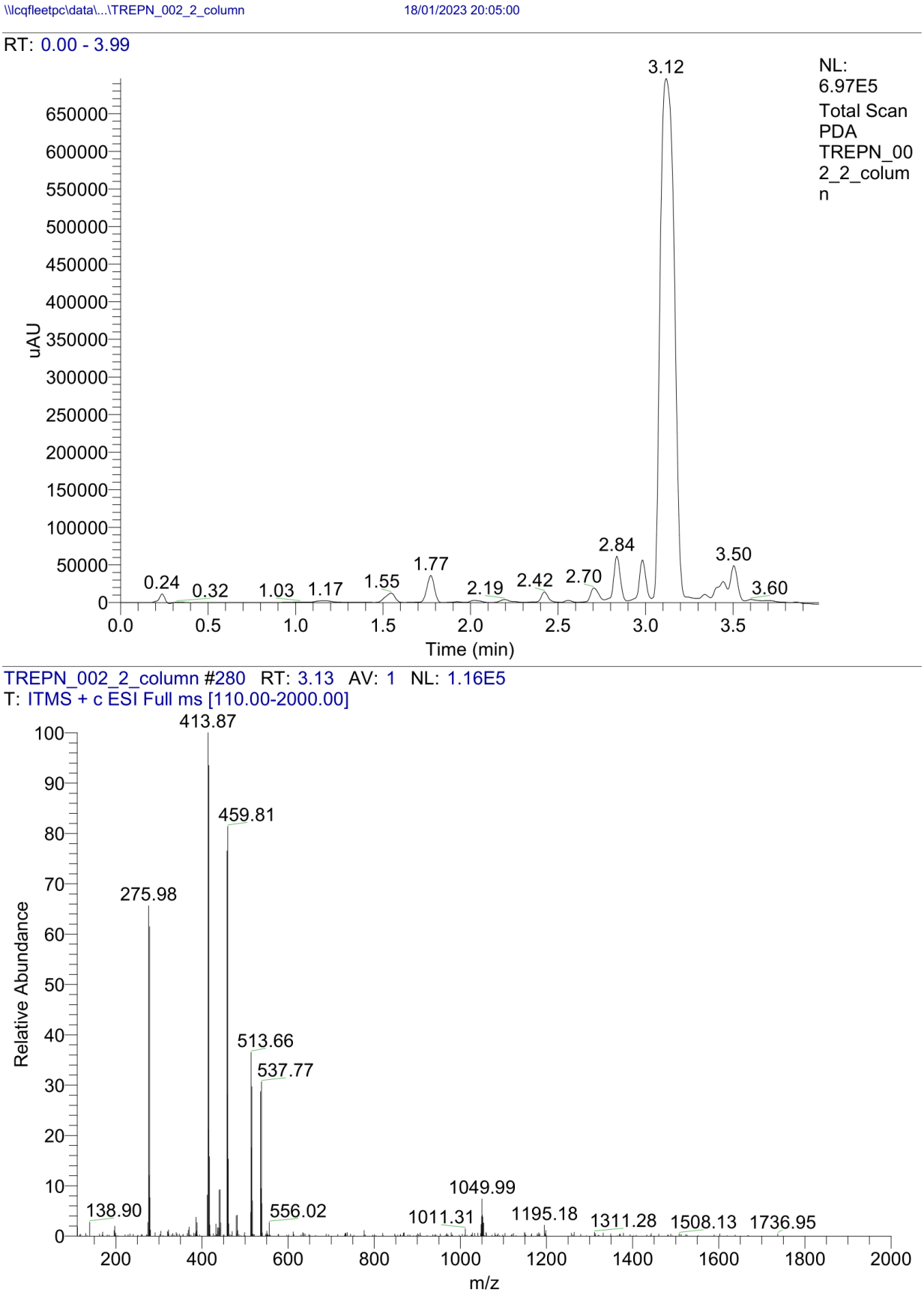


Screenshot of spectra LC-MS-ESI (m/z) compound 7





Screenshot of spectra LC-MS-ESI (m/z) compound 8



Screenshot of spectra LC-MS-ESI (m/z) compound 9a

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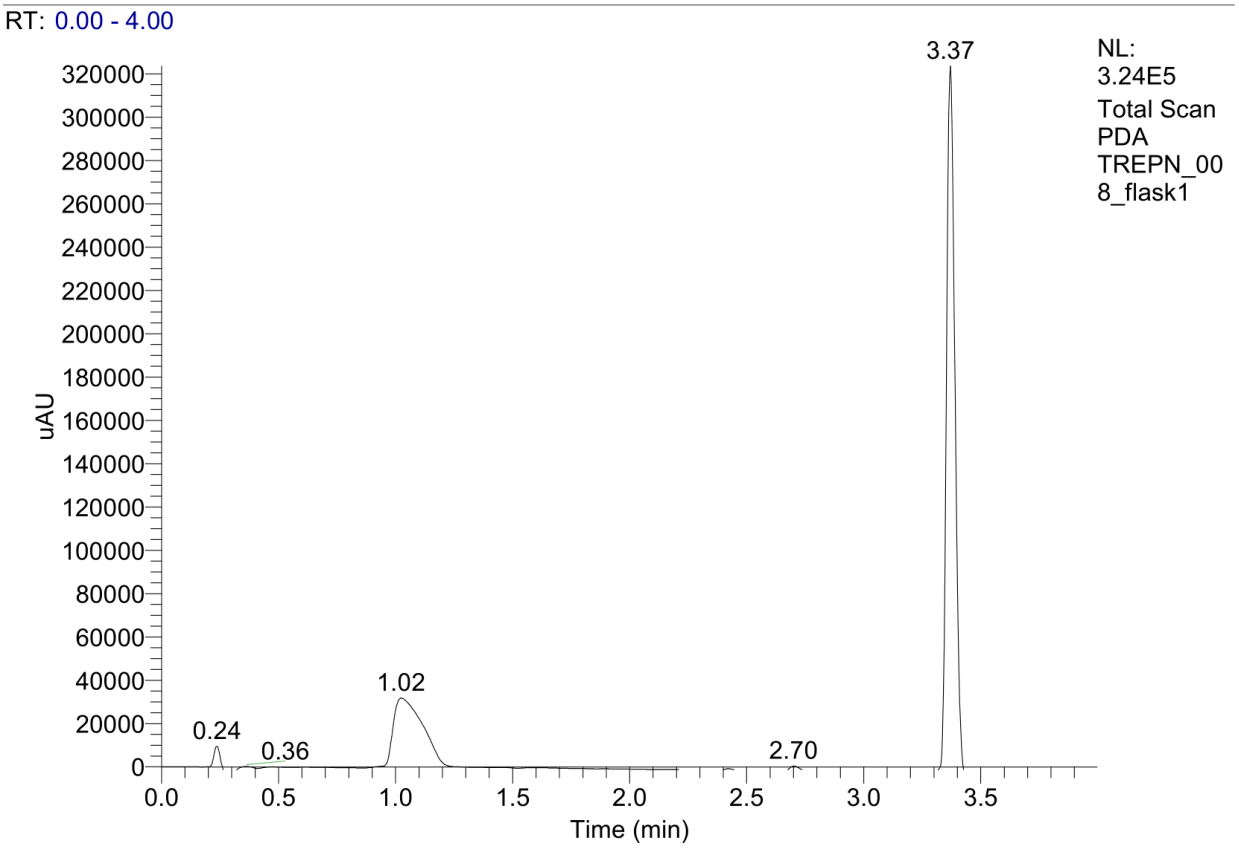

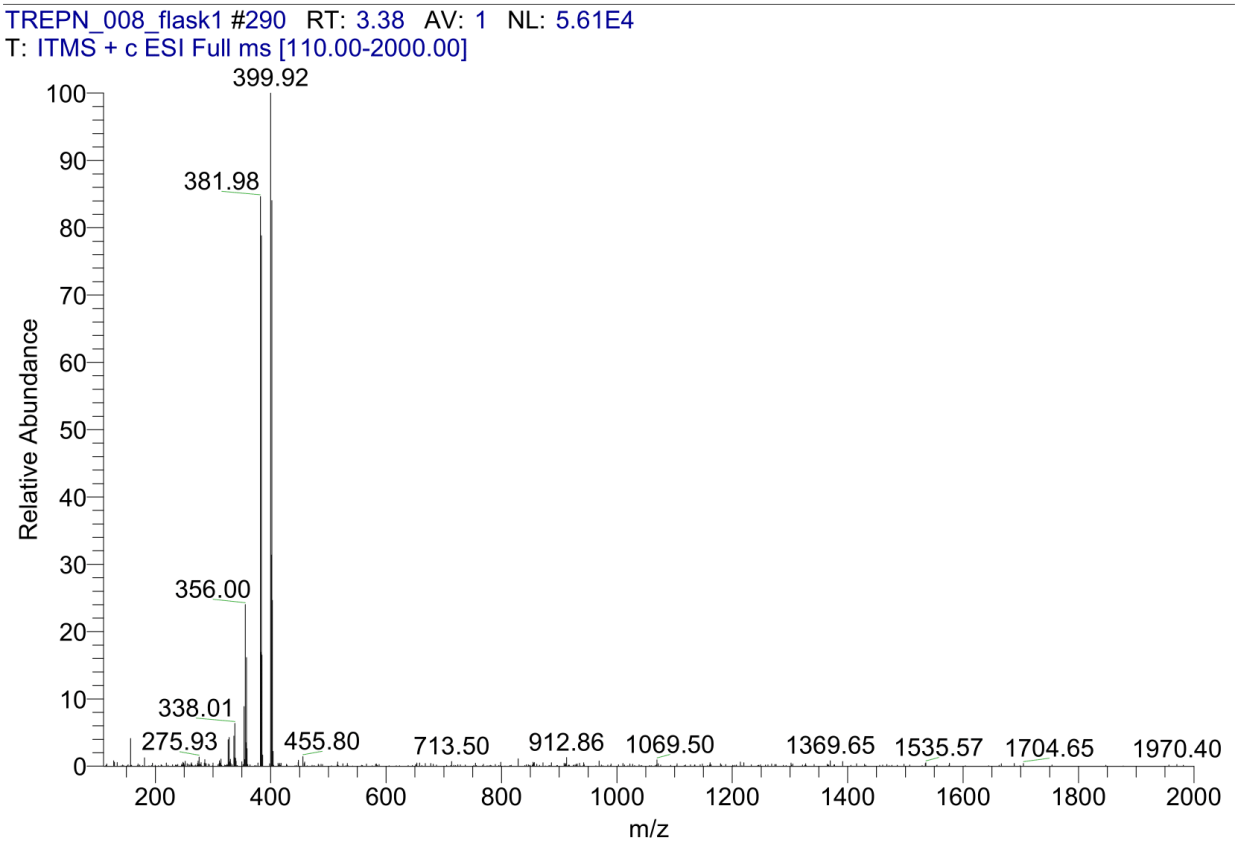

Screenshot of spectra LC-MS-ESI (m/z) compound 9b, 9d

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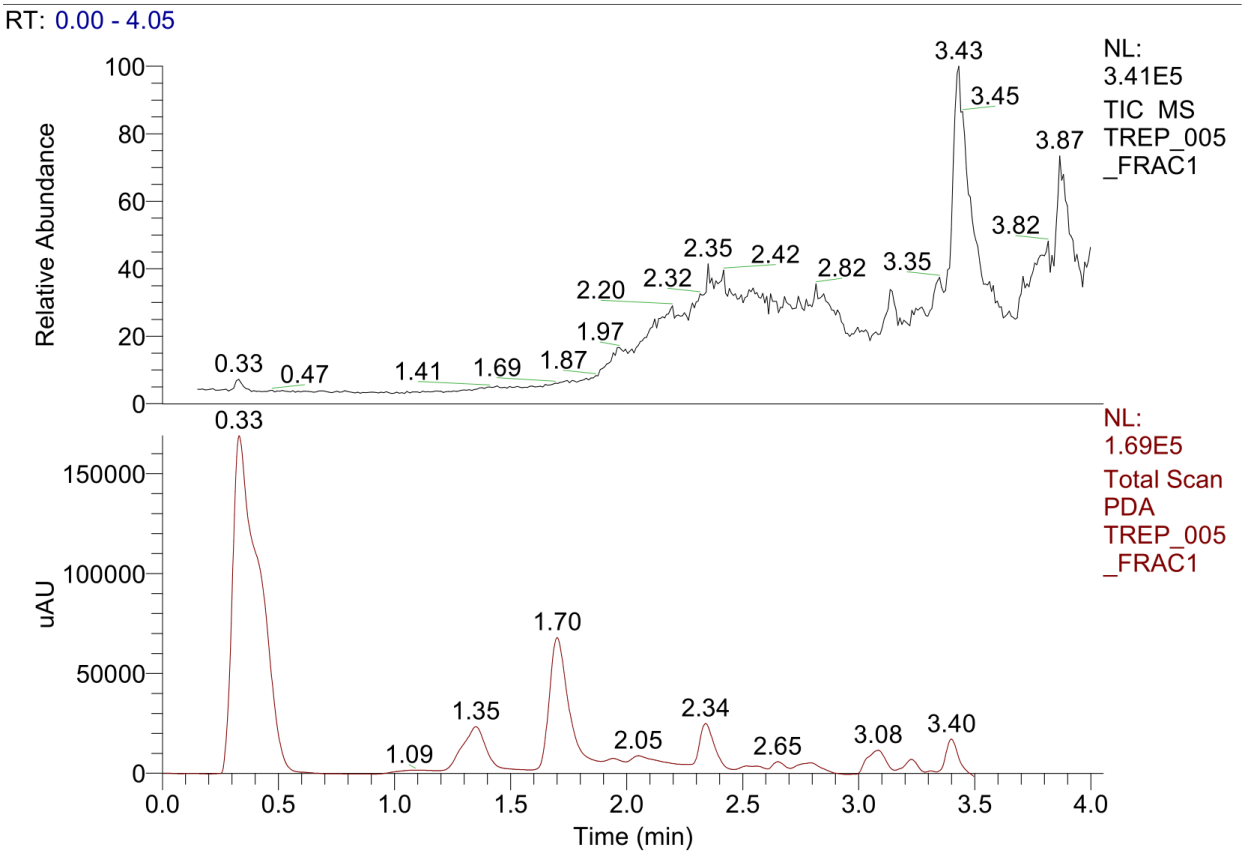

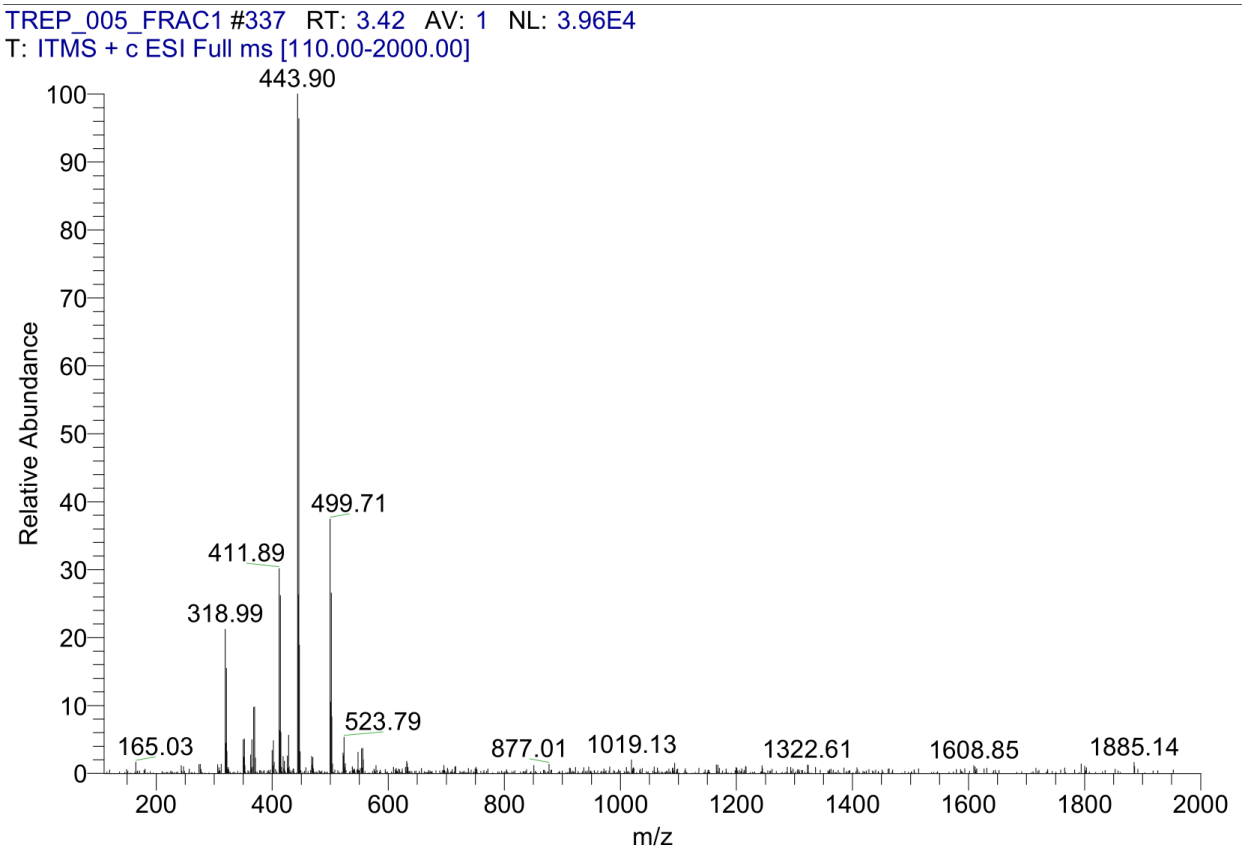

Screenshot of spectra LC-MS-ESI (m/z) compound 10a

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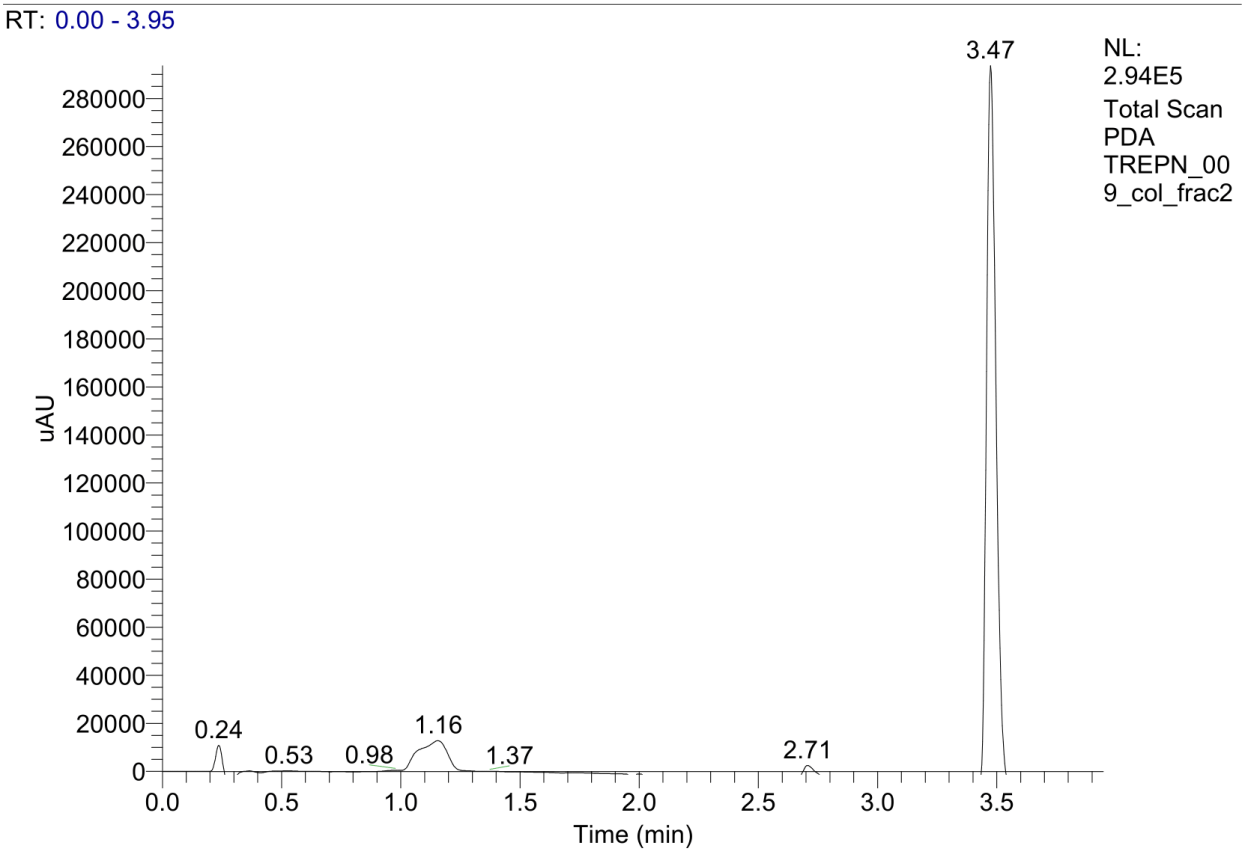

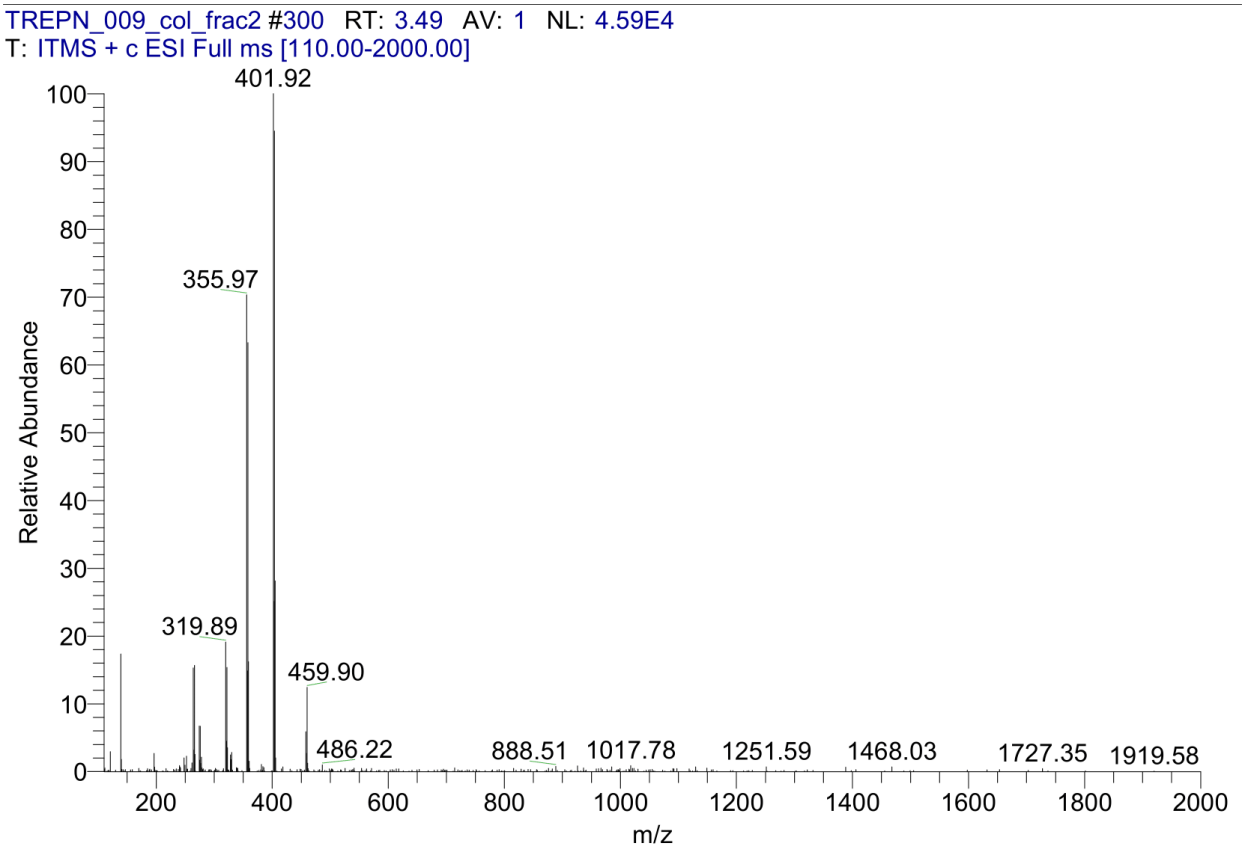

Screenshot of spectra LC-MS-ESI (m/z) compound 11a

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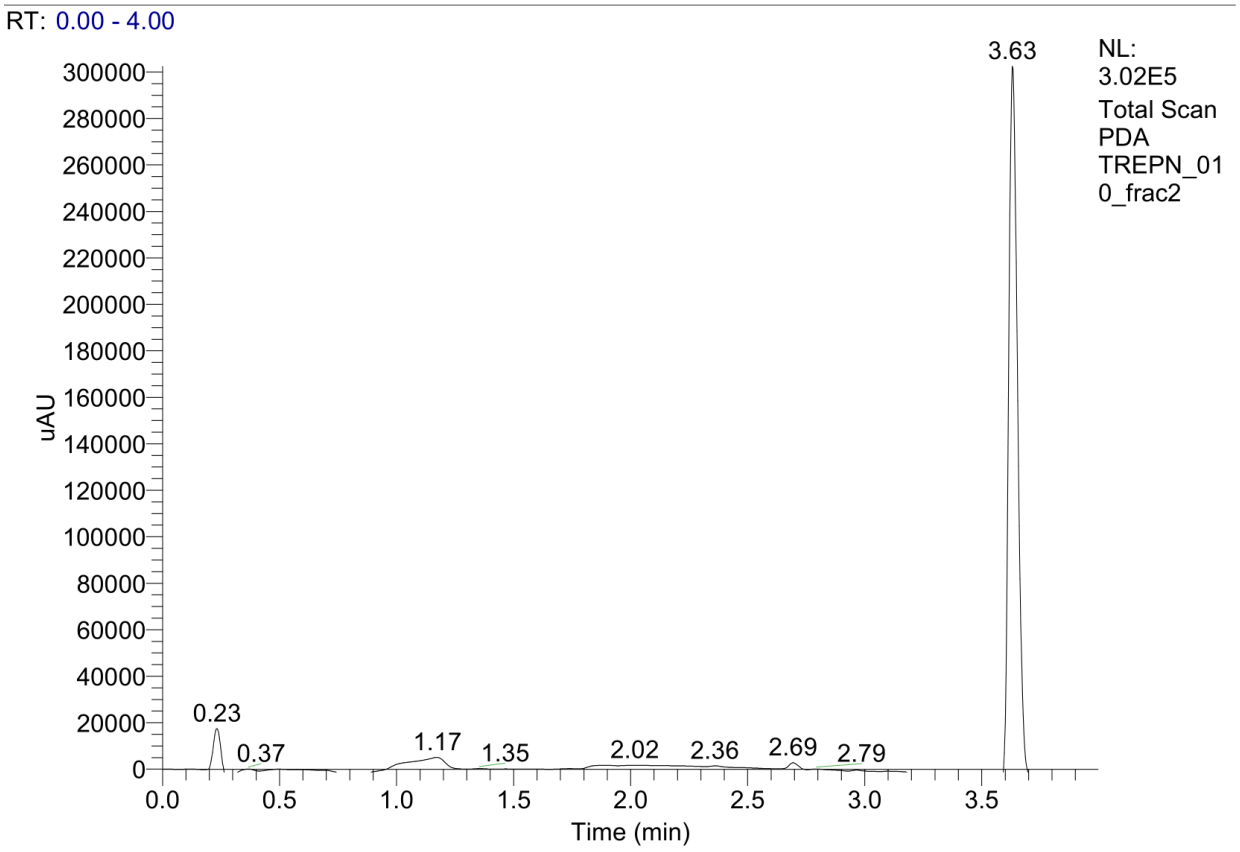
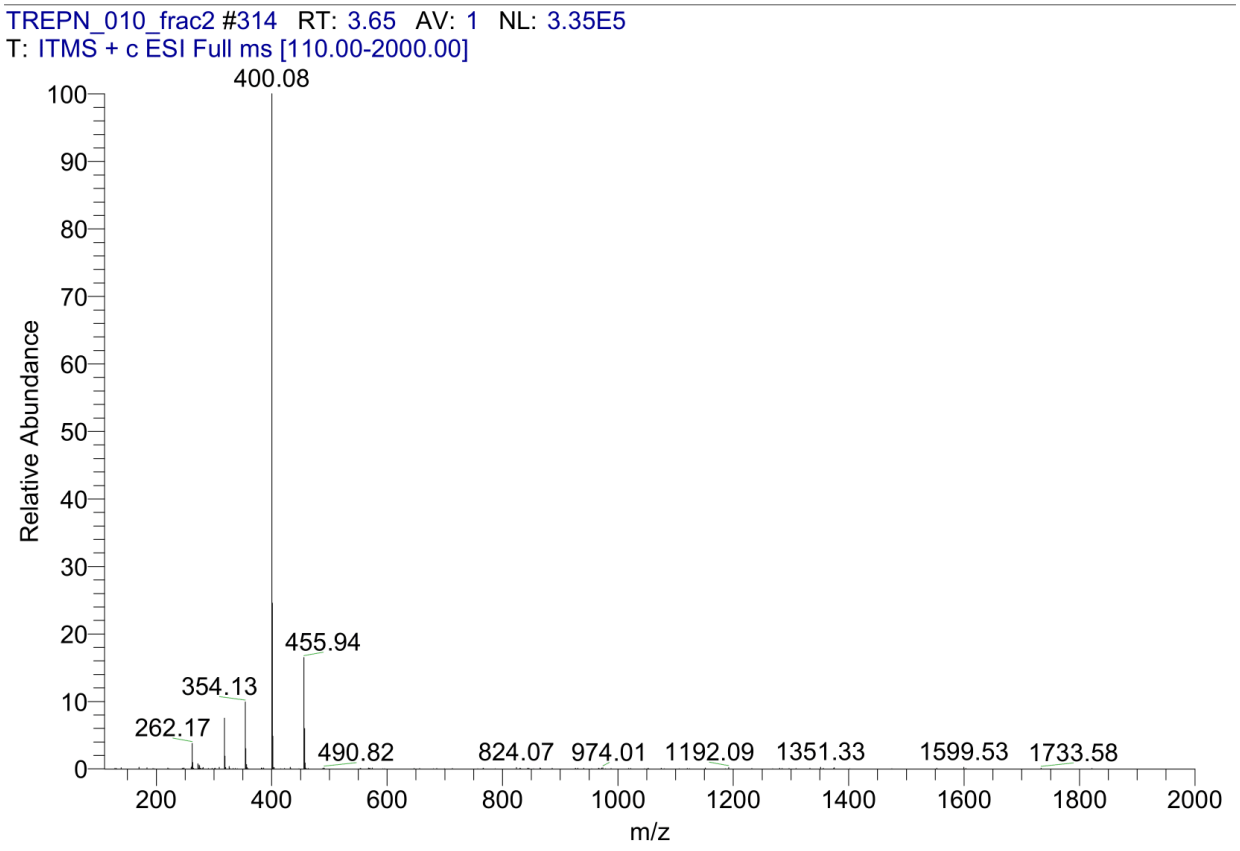
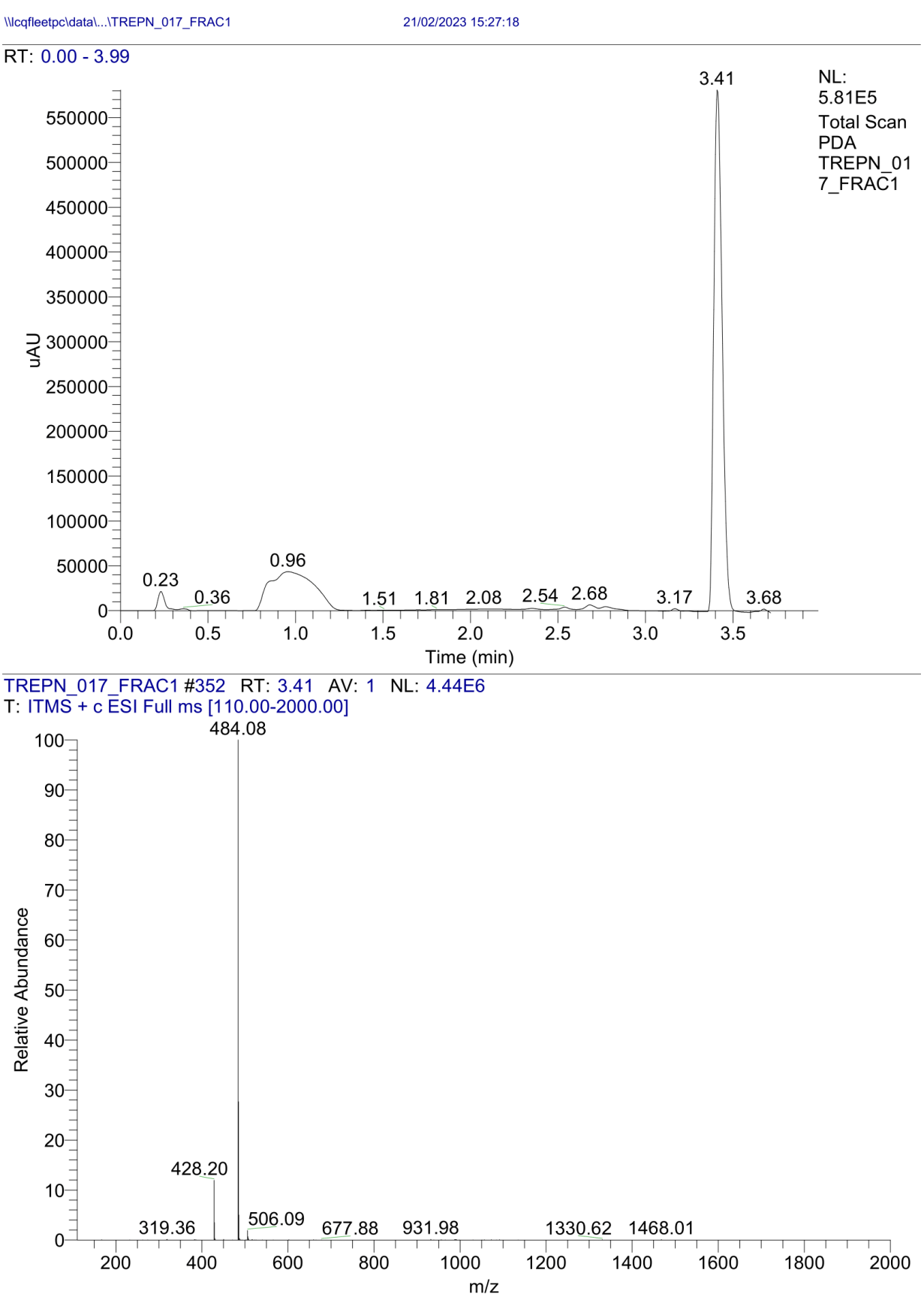




Screenshot of spectra LC-MS-ESI (m/z) compound 12



Screenshot of spectra LC-MS-ESI (m/z) compound 13

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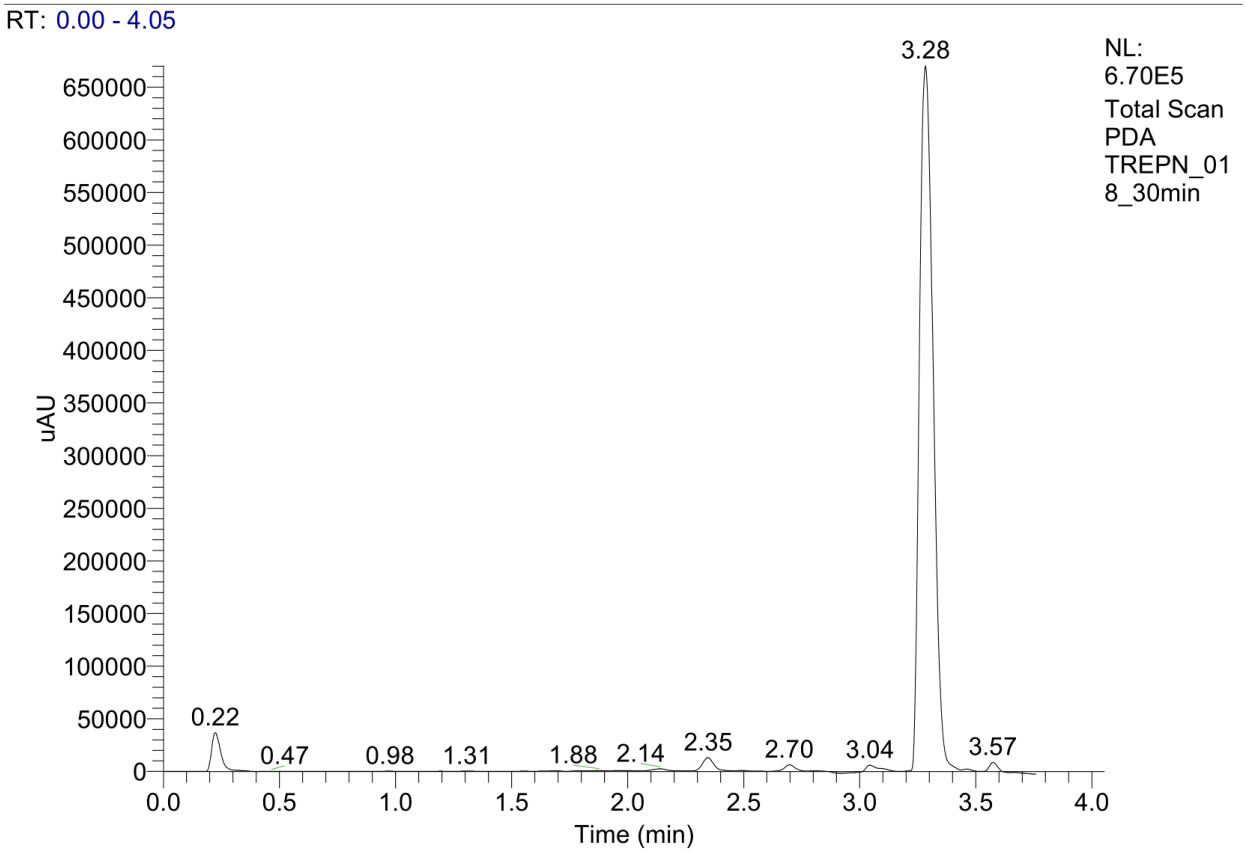

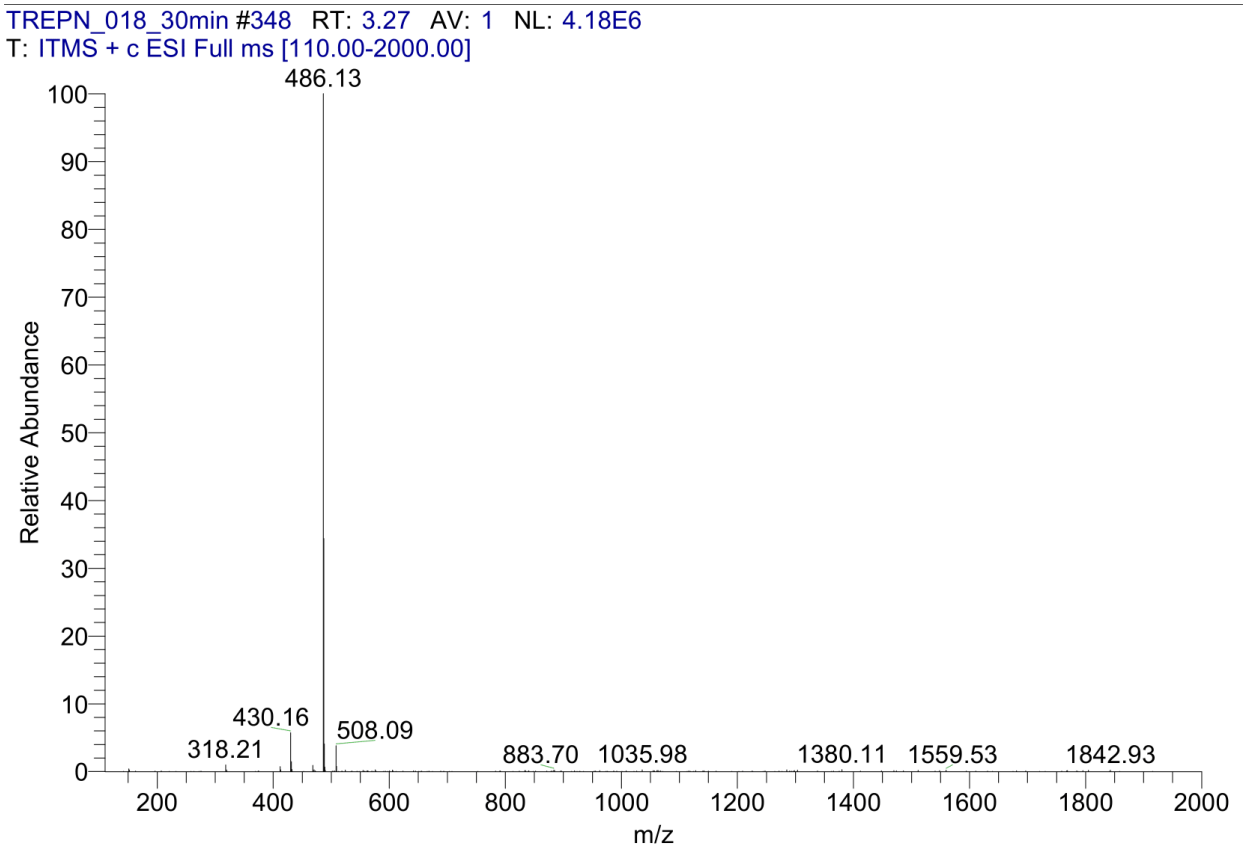

Screenshot of spectra LC-MS-ESI (m/z) compound 14

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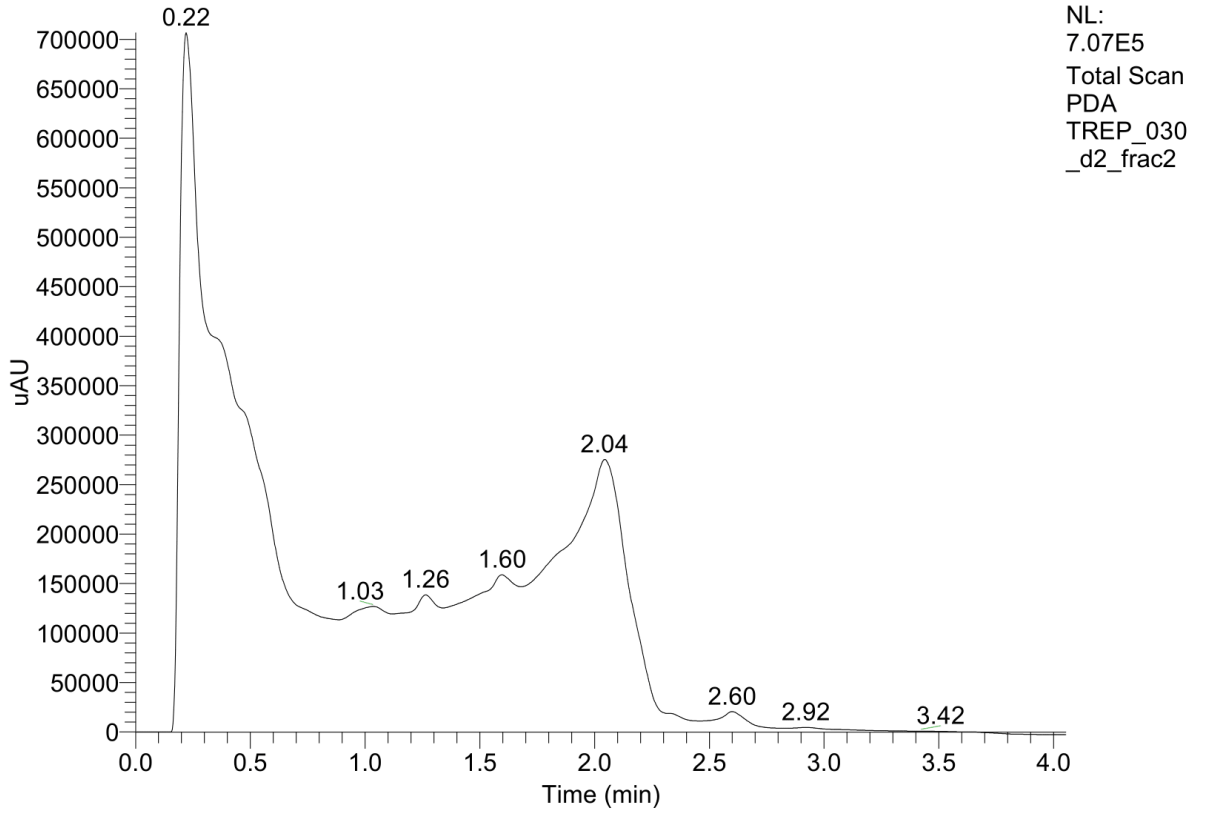
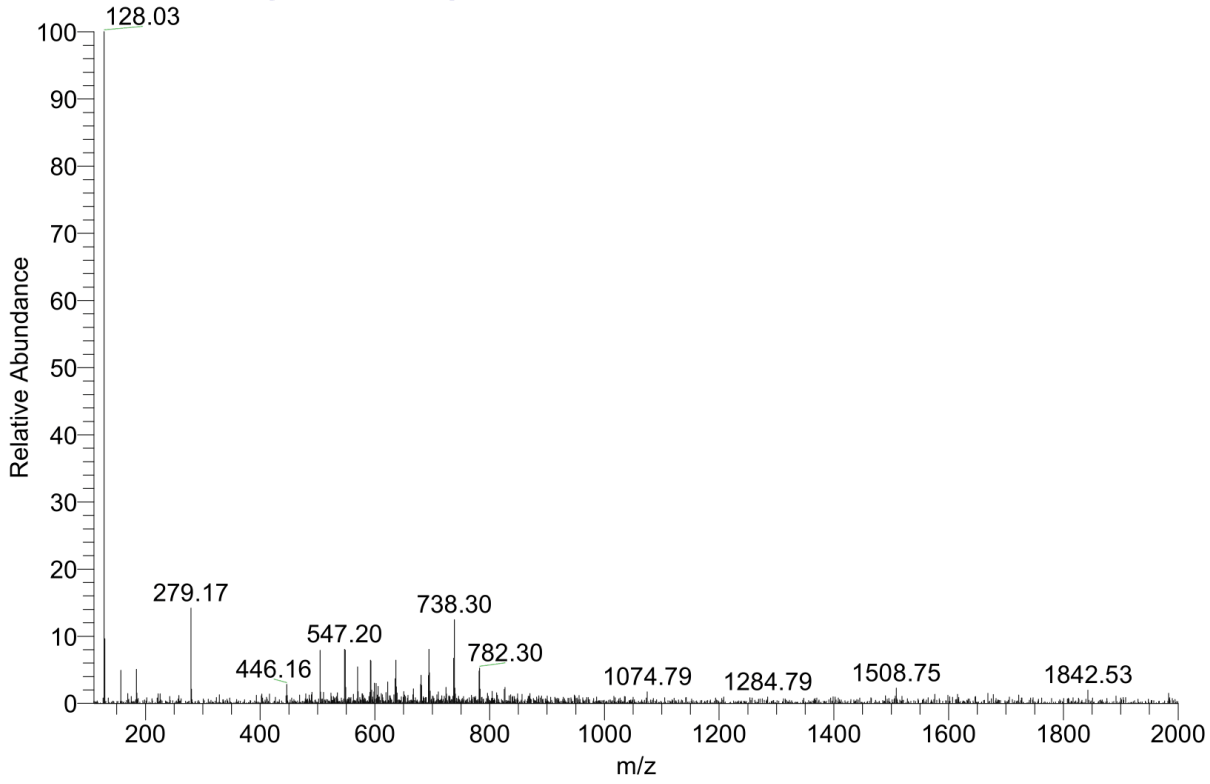


TREP_030_d2_frac2 #345 RT: 3.29 AV: 1 NL: 1.20E4

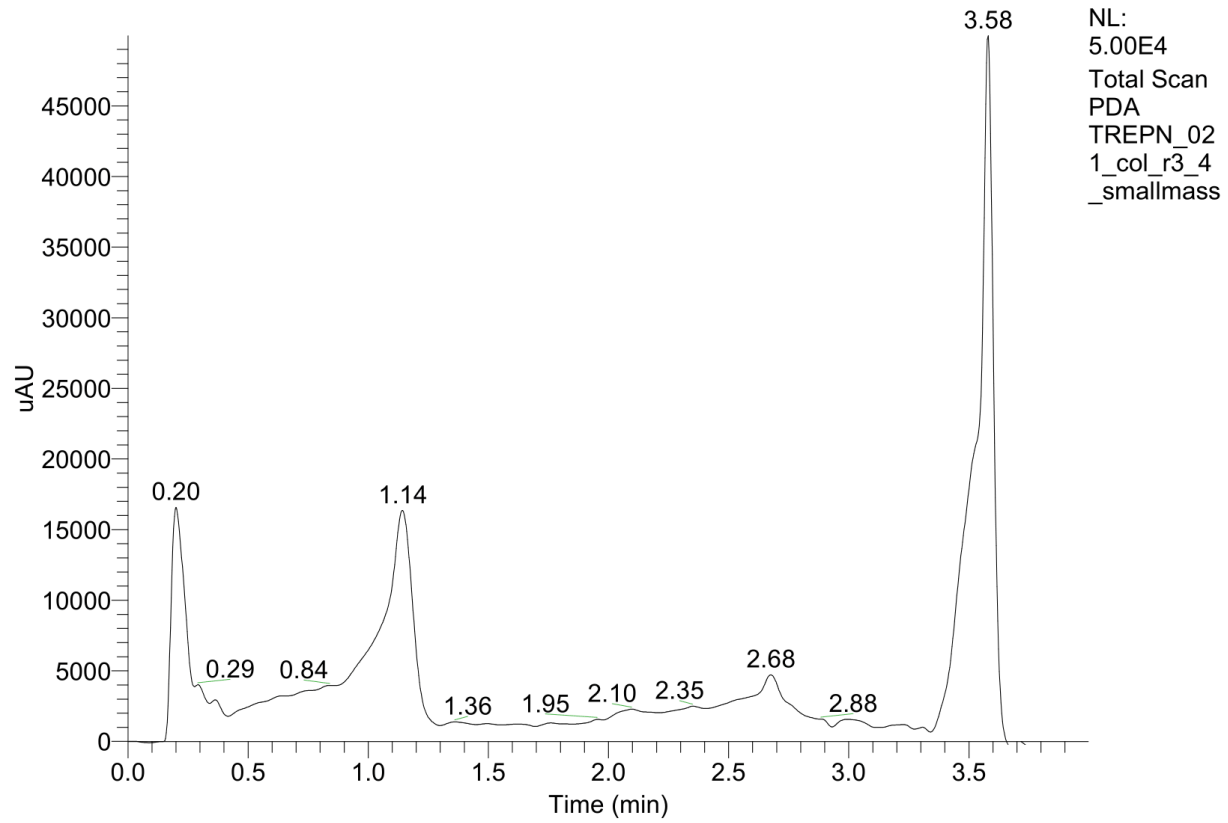
T: ITMS + c ESI Full ms [110.00-2000.00]



Screenshot of spectra LC-MS-ESI (m/z) compound 15c

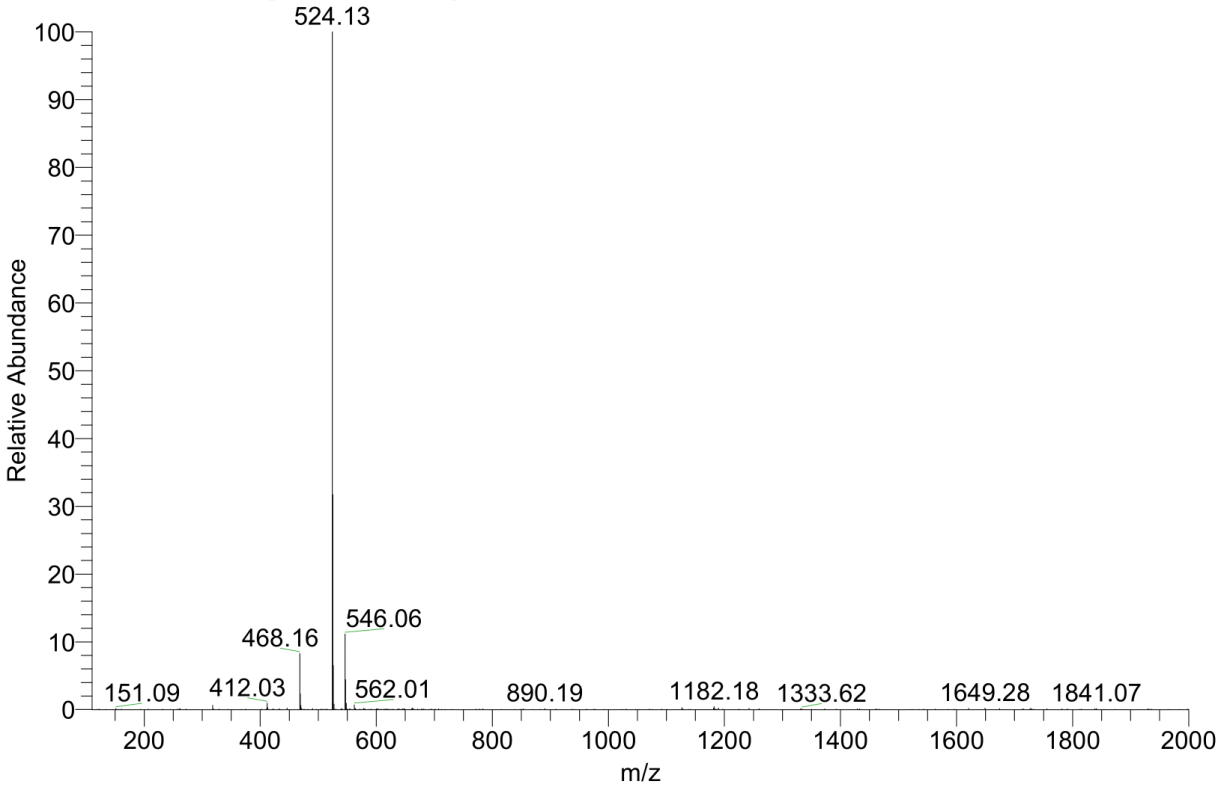
TREPN_021_col_r3_4_smallmass 01/03/2023 14:58:39

RT: 0.00 - 4.00



TREPN_021_col_r3_4_smallmass #377 RT: 3.60 AV: 1 NL: 1.75E6

T: ITMS + c ESI Full ms [110.00-2000.00]



Screenshot of spectra LC-MS-ESI (m/z) compound 9c [Data from Jose Laxio-Arenas]

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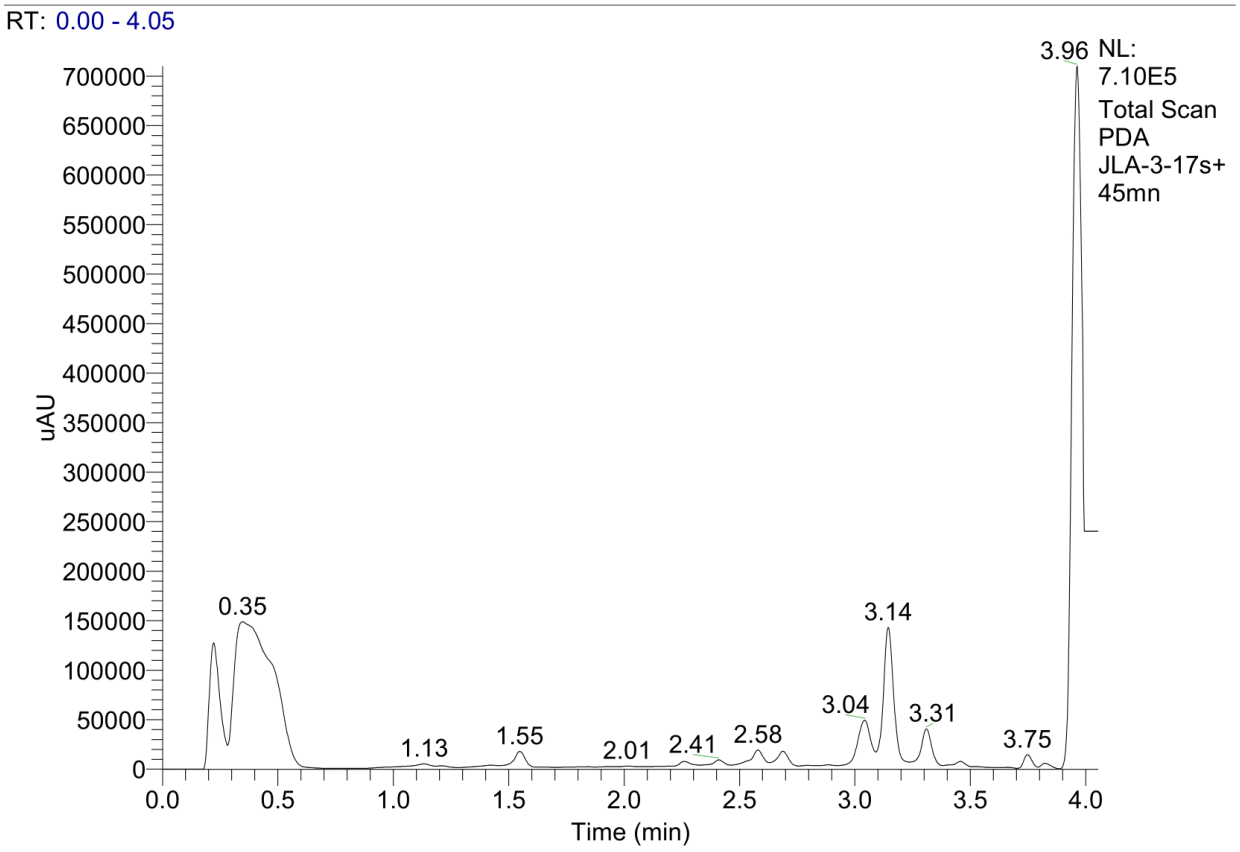
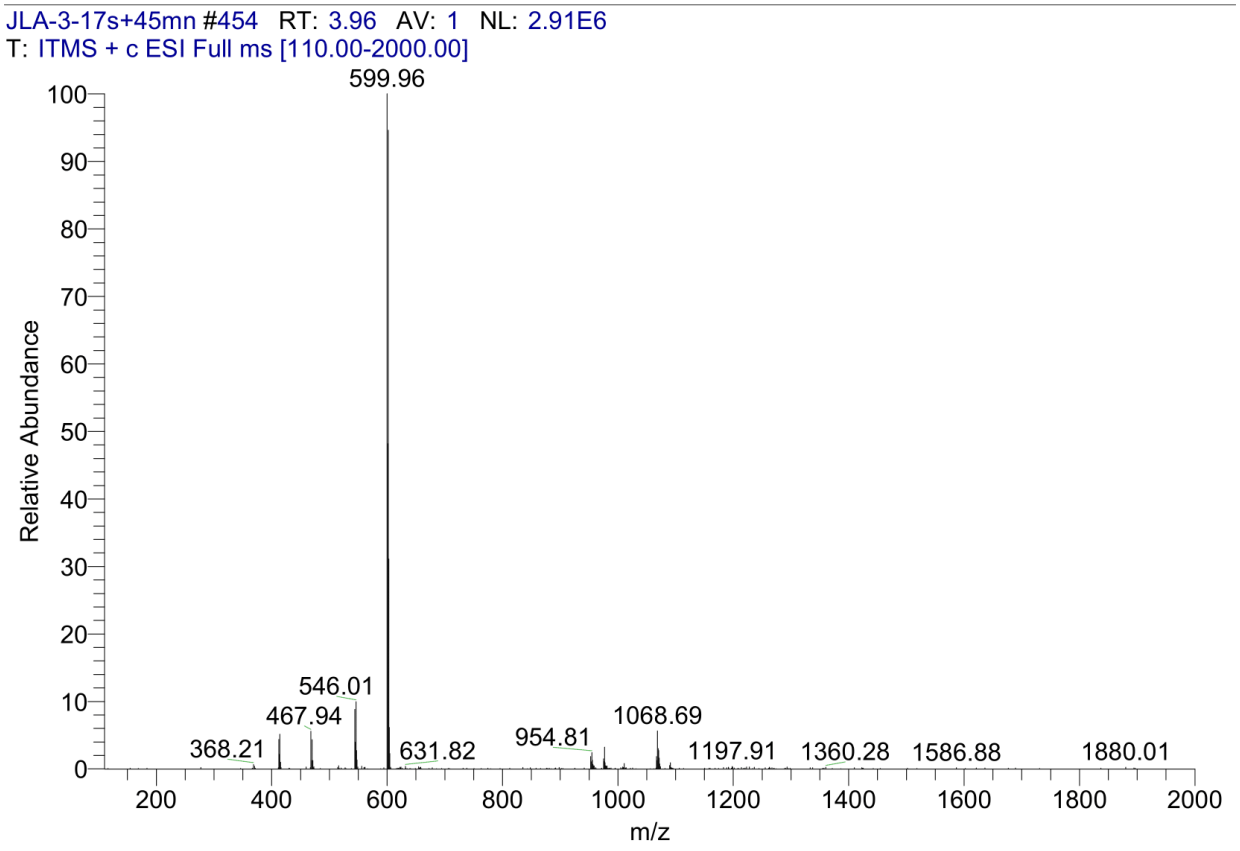
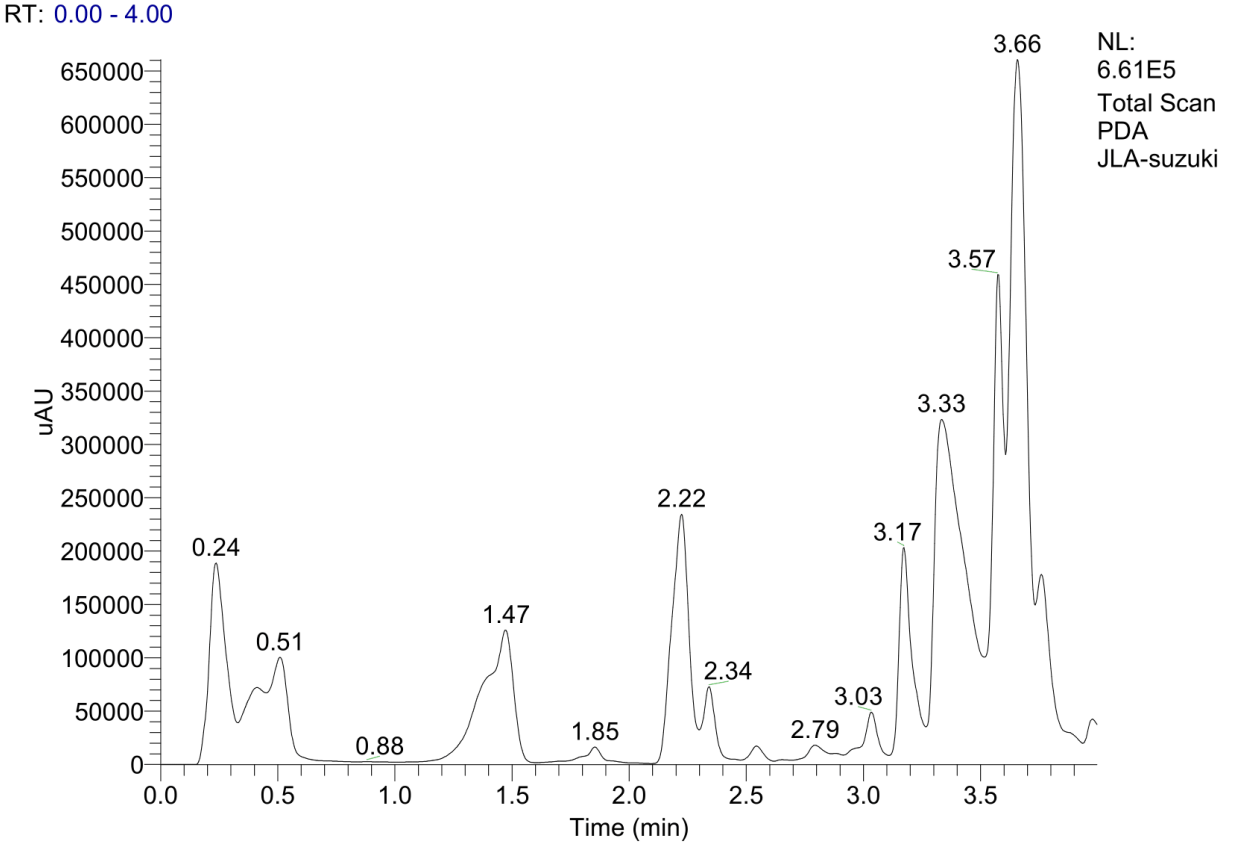




Screenshot of spectra LC-MS-ESI (m/z) compound 11c [Data from Jose Laxio-Arenas]

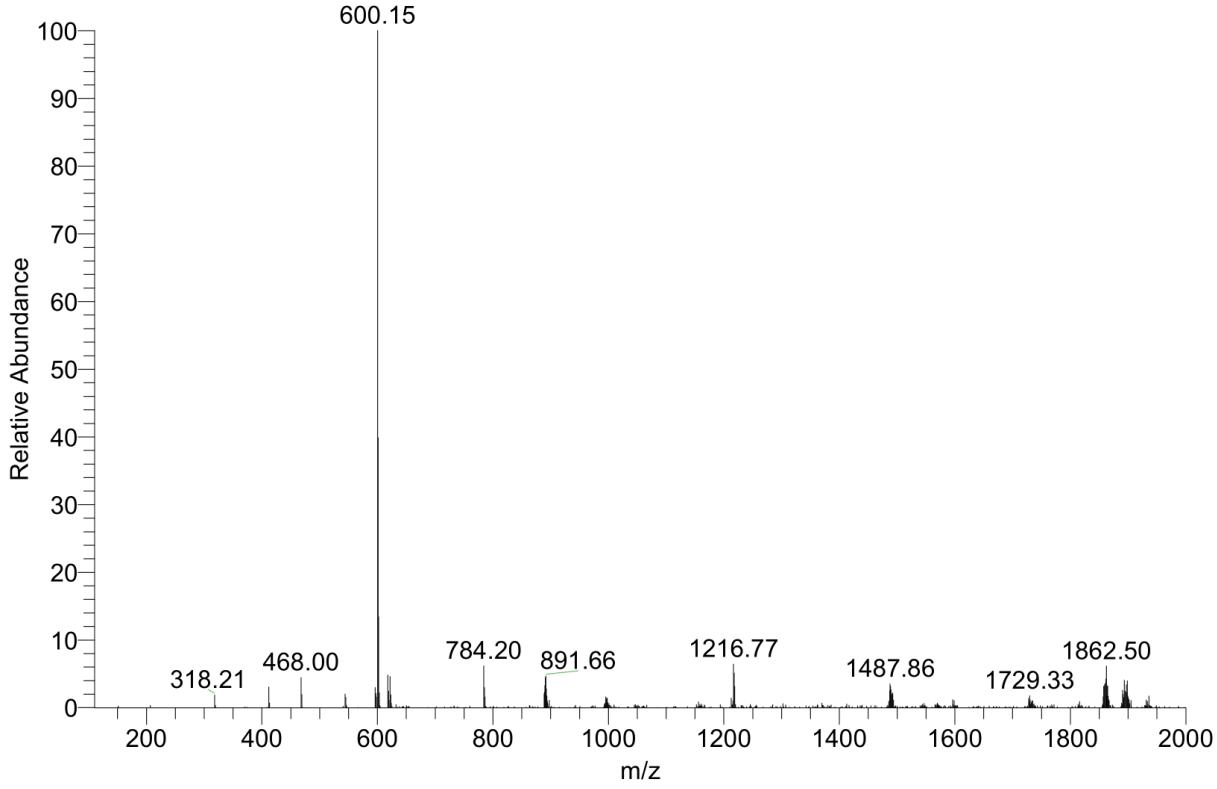
\\lcqfleetpc\data\...\José\JLA-suzuki 07/03/2023 15:27:24

RT: 0.00 - 4.00



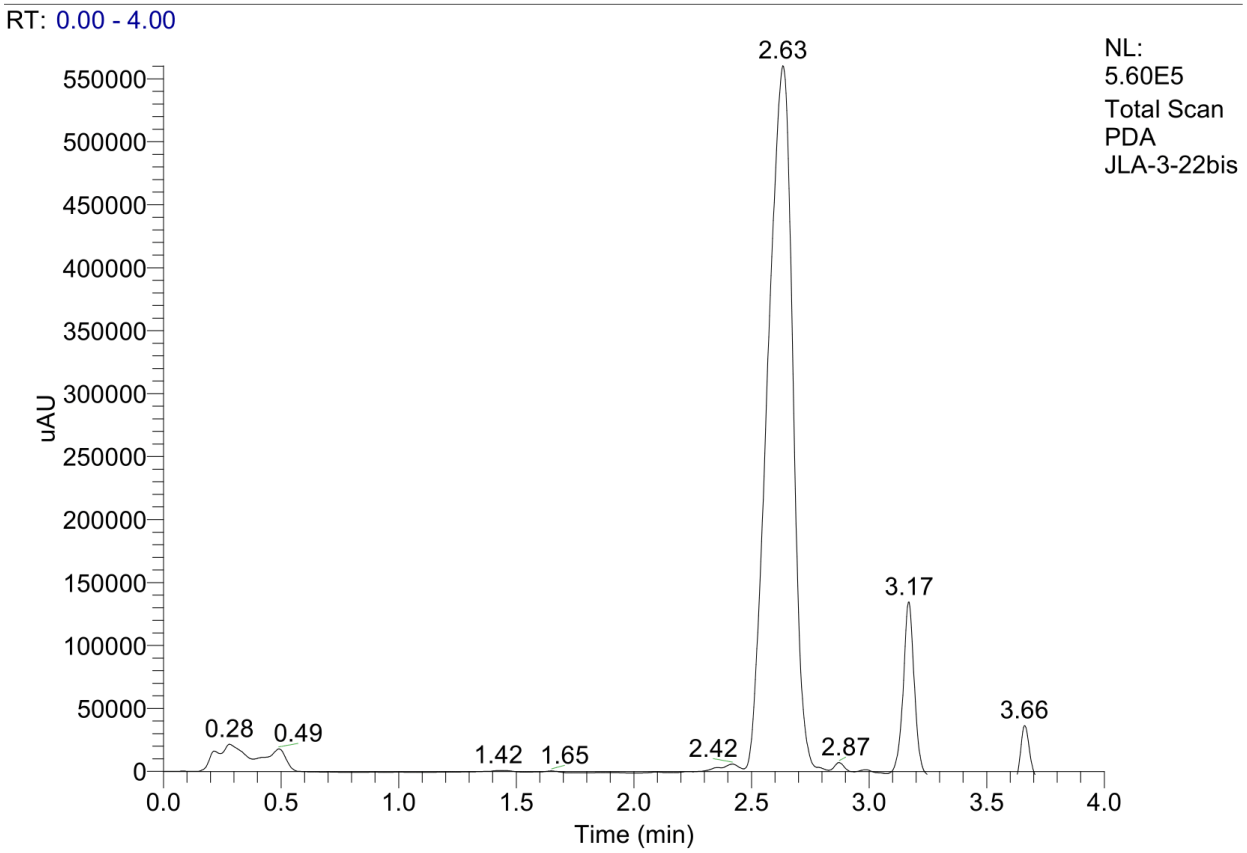
JLA-suzuki #440 RT: 3.66 AV: 1 NL: 2.15E6

T: ITMS + c ESI Full ms [110.00-2000.00]

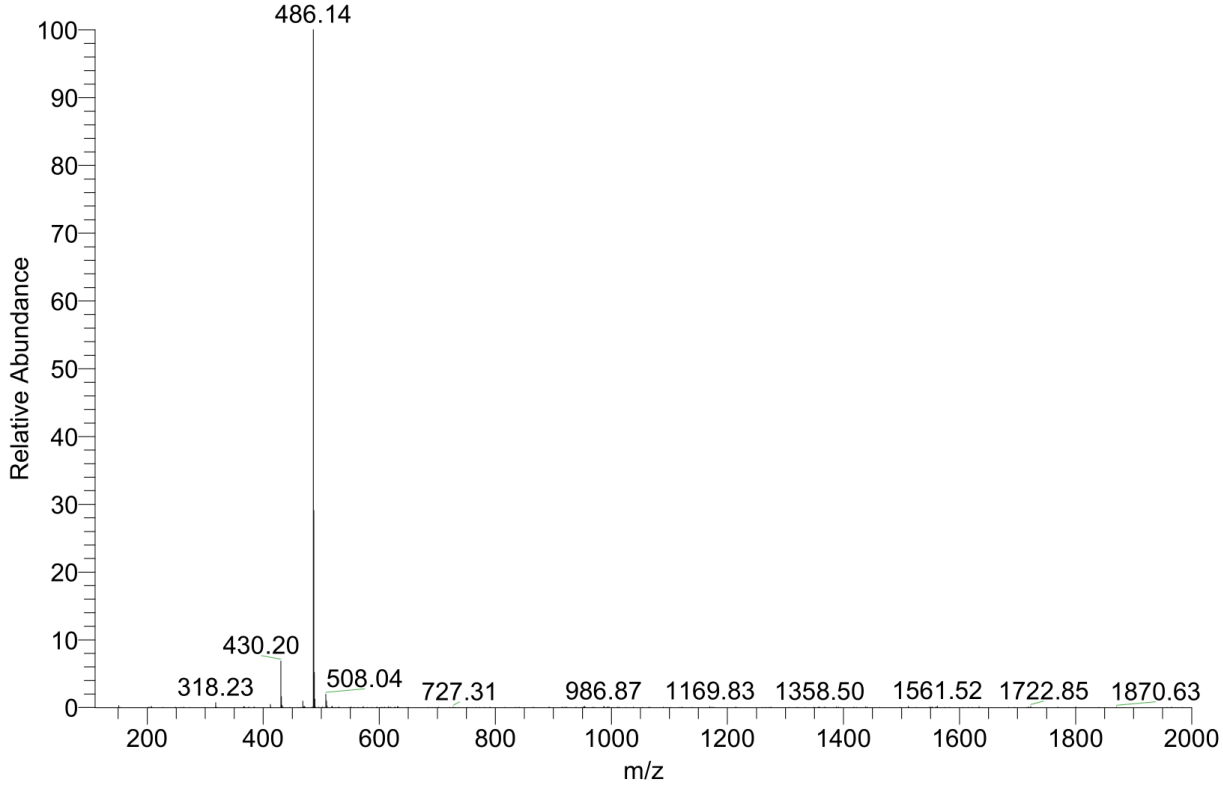


Screenshot of spectra LC-MS-ESI (m/z) compound 13 [Data from Jose Laxio-Arenas]

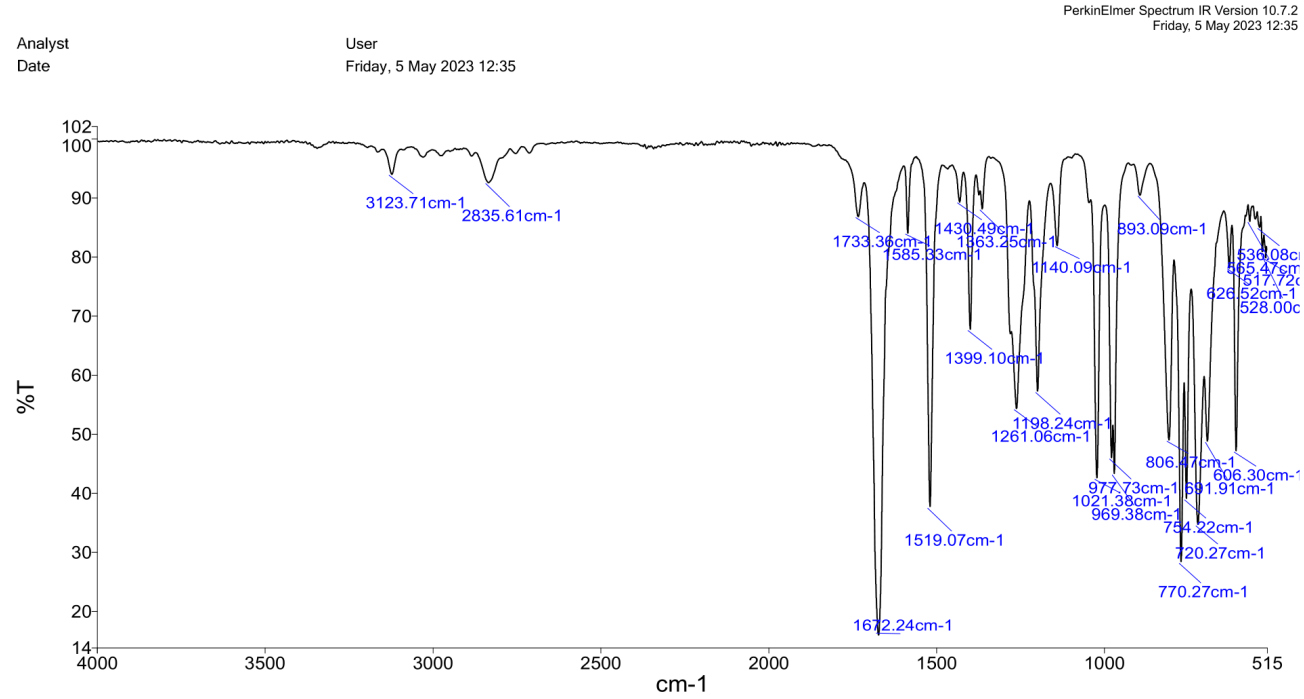
\\lcqfleetpc\data\...\José\JLA-3-22bis 22/02/2023 11:29:34



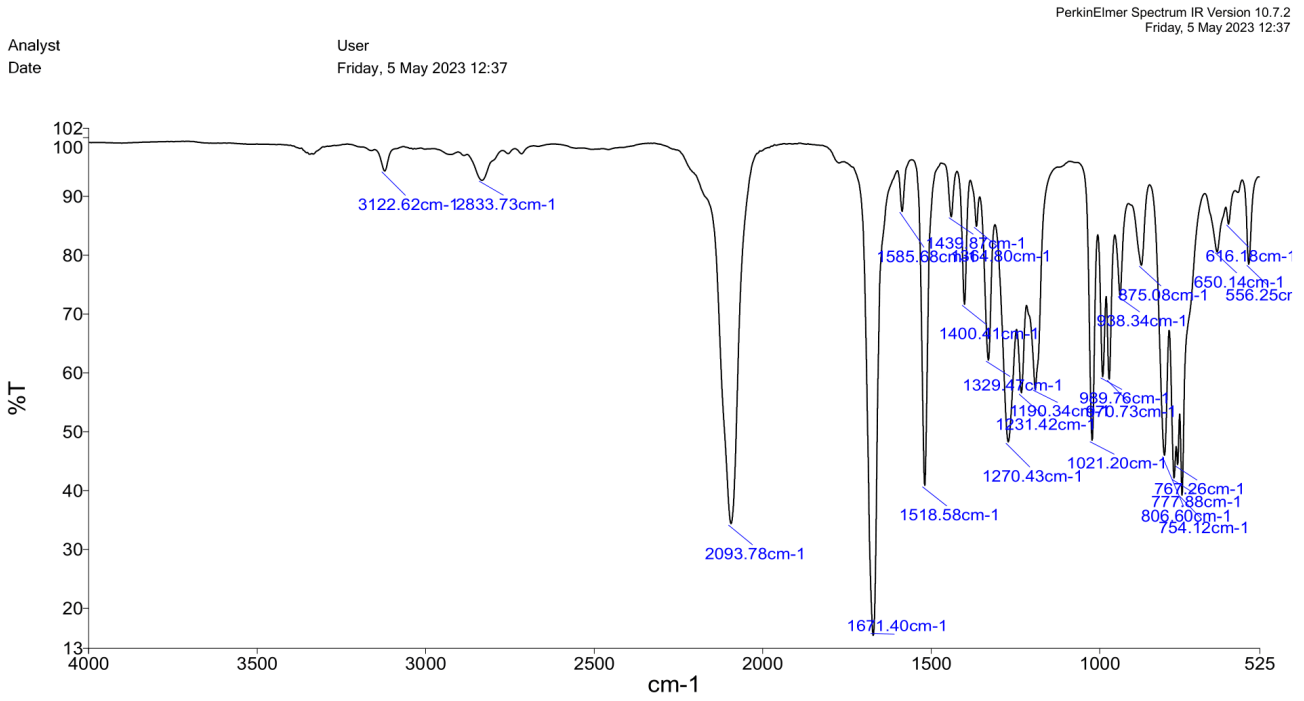
JLA-3-22bis #316 RT: 2.62 AV: 1 NL: 4.72E6
T: ITMS + c ESI Full ms [110.00-2000.00]



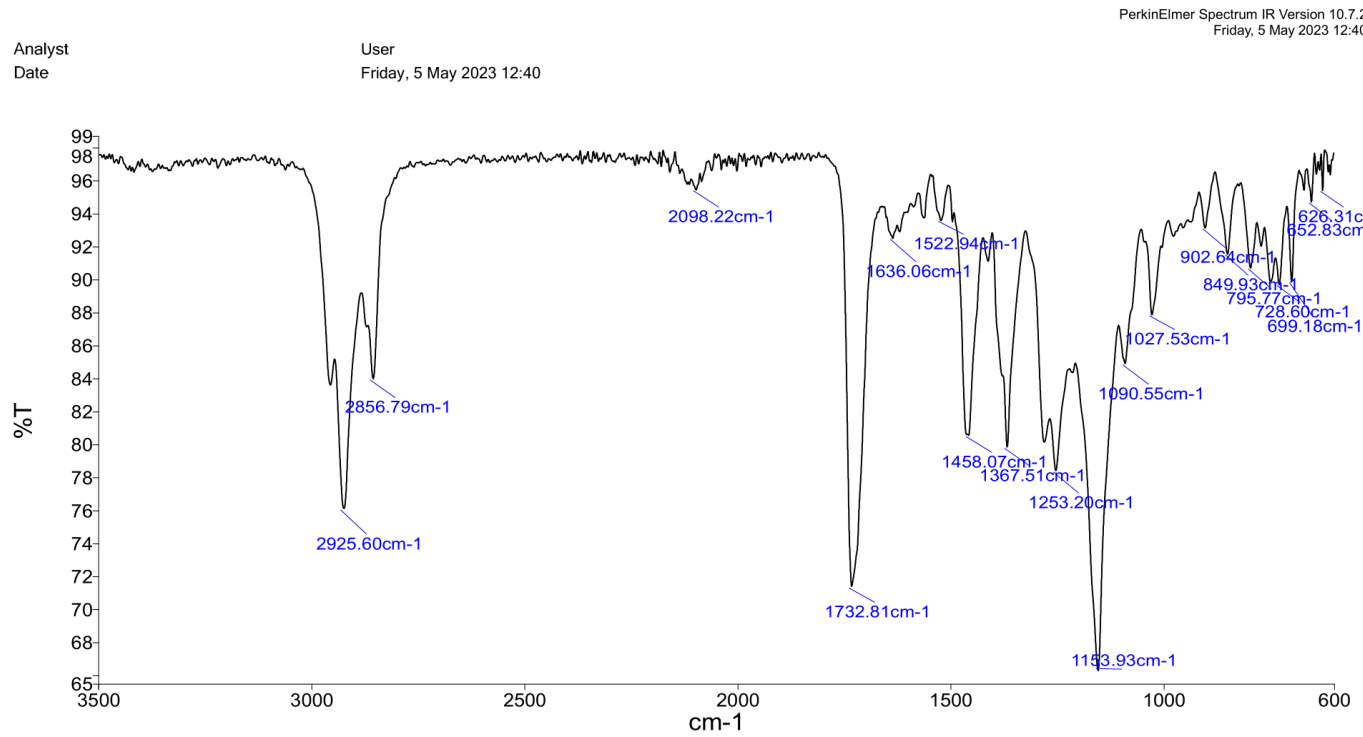
IR spectra compound 4



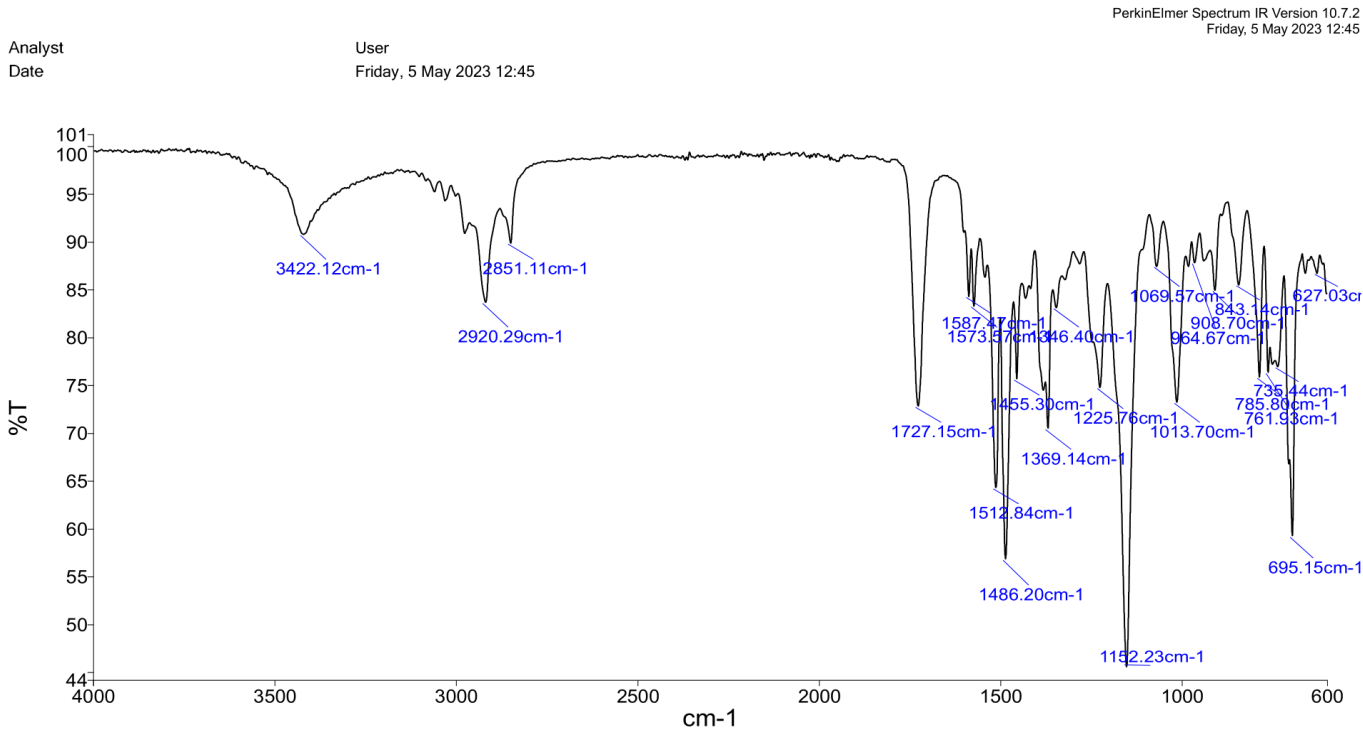
IR spectra compound 5



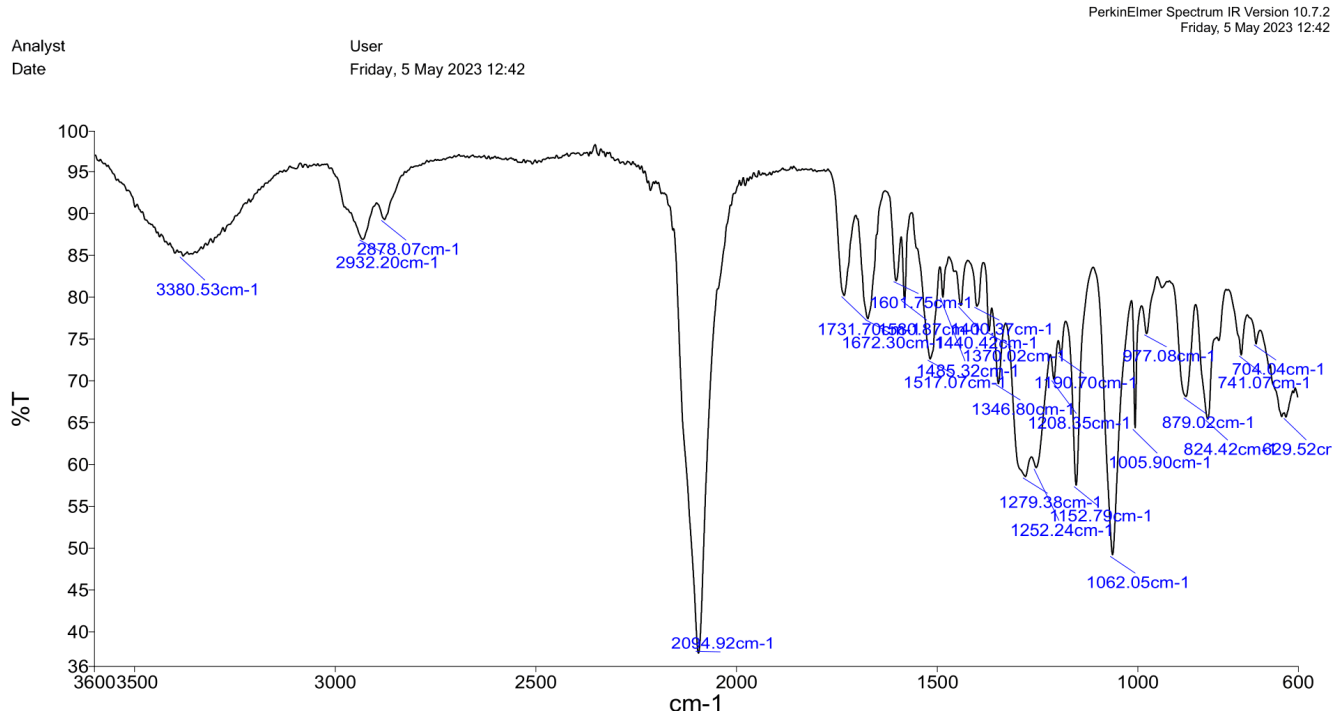
IR spectra compound 9b, 9d



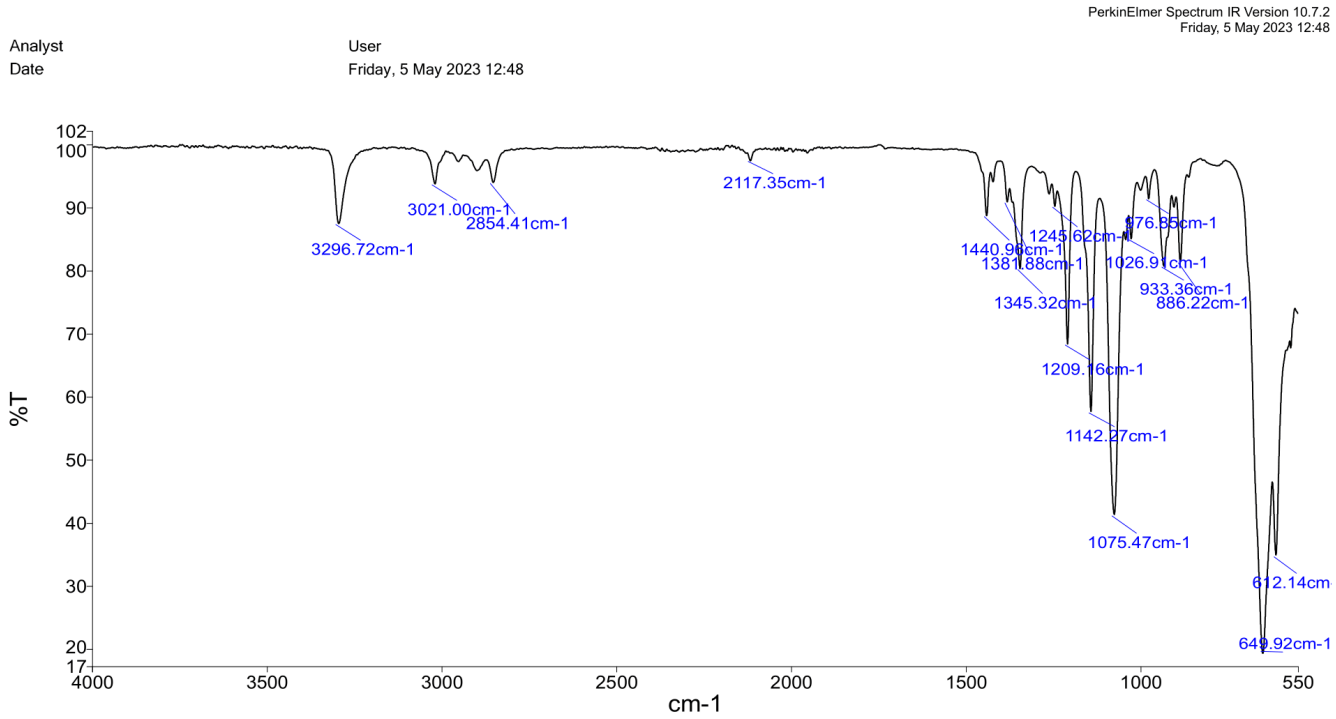
IR spectra compound 13



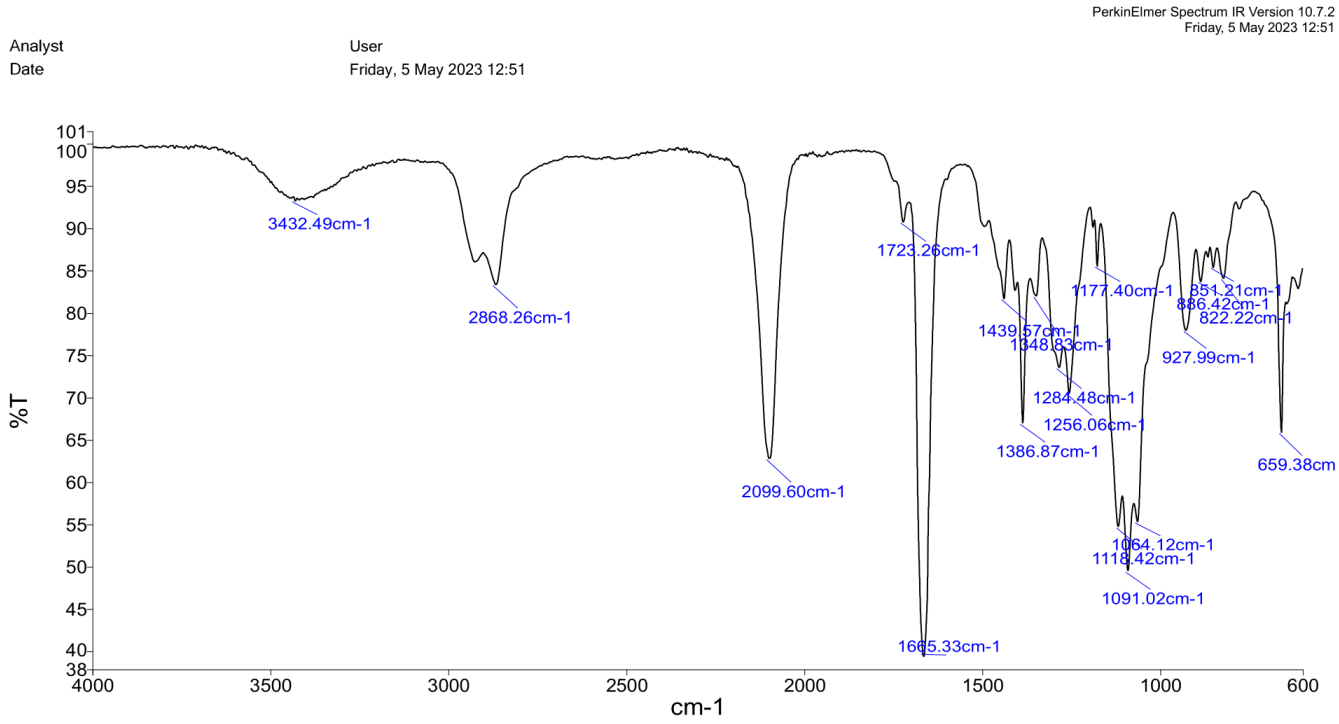
IR spectra compound L2



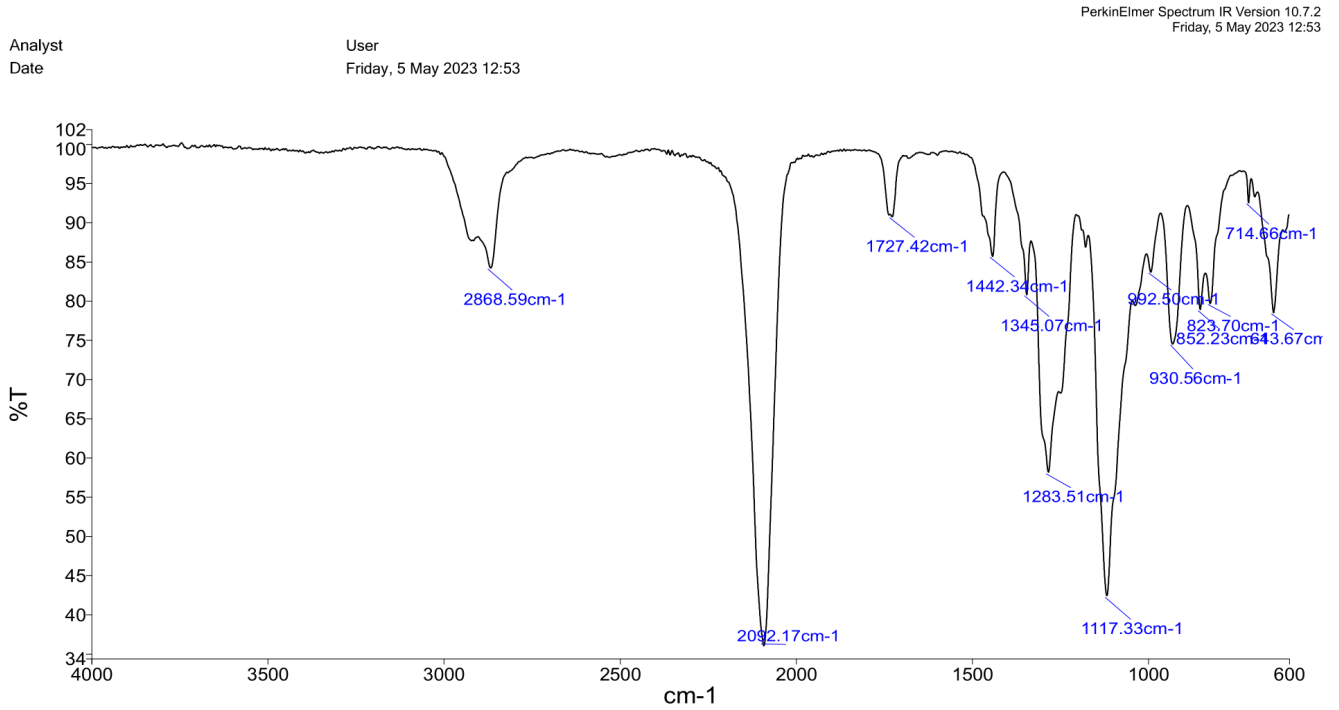
IR spectra compound L5



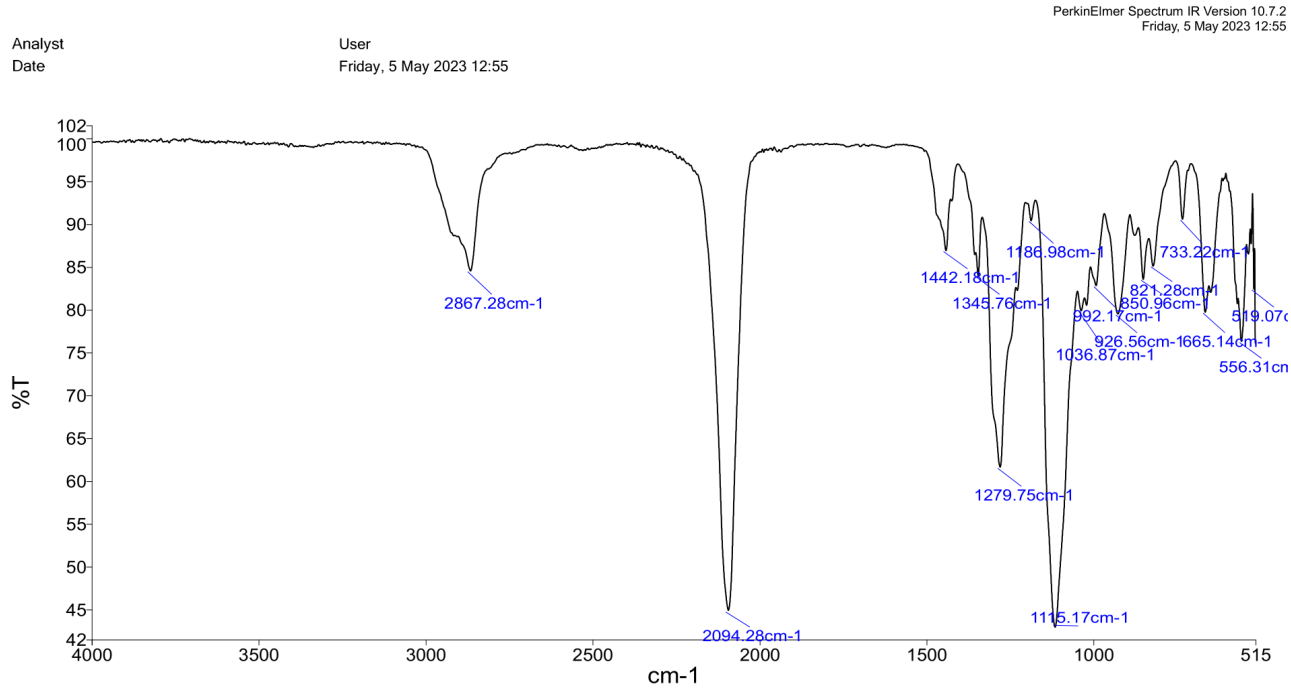
IR spectra compound L10



IR spectra compound L11



IR spectra compound L12



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