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Structure activity investigations of XTH inhibitors

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I. Abstract

XTHs (xyloglucan endotransglucosylase/hydrolases) are a group of plant enzymes that regulate xyloglucan crosslinking in cell walls and play an essential role in regulation of plant growth. It is thought that XTHs play an integral role in the way the parasitic vine plant *Cuscuta* is able to penetrate host plant cell walls. Parasitic higher plants are a formidable threat to agricultural production around the globe, and finding new ways to fight these is essential for sustaining the world population's need for food.

Through recent research it was discovered that the triphenylmethyl food colorant Brilliant Blue R250 inhibits XTHs and the development of *Cuscuta* haustorium, specialized organs by which the parasite absorbs host nutrients. In this master's thesis, the structure of Brilliant Blue R250 was used as a starting point for synthesis of potential inhibitors of XTHs. The aim was to synthesize compounds with increased activity and water solubility.

A range of Brilliant Blue derivatives were synthesized and their ability to bind proteins and inhibit the endotransglucosylation activity of XTHs was tested at the Department of Arctic and Marine Biology. The project is a collaboration between Organic Chemistry and Arctic and Marine Biology at UiT.

II. Acknowledgements

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III. Symbols and abbreviations

¹³ C NMR	Carbon-13 nuclear magnetic resonance
¹ H NMR	Proton nuclear magnetic resonance
AB	Aniline Blue
BB	Brilliant Blue
BB-FCF	Brilliant Blue FCF
BB-R250	Brilliant Blue R250
BB-G250	Brilliant Blue G250
BBD	Brilliant Blue derivative
DCM	Dichloromethane
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DIPEA	N,N-Diisopropylethylamine
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide
GGB	Guinea Green B
H	Proton
HRMS	High-resolution mass spectrometry/spectrometer
m/z	Mass to charge ratio
MDA	4,4'-methylenedianiline
MW	Microwave
Mw	Molecular weight
MS	Mass spectrometry/spectrometer
NMR	Nuclear magnetic resonance
PMDA	4,4'-(phenylmethylene)dianiline
ppm	Parts per million
R _f	Retention
rt.	Room temperature
S _N 1	Unimolecular nucleophilic substitution
S _N 2	Bimolecular nucleophilic substitution
THF	Tetrahydrofuran
TLC	Thin layer chromatography
V-200	Coomassie Violet R200
XEH	Xyloglucan endohydrolase
XET	Xyloglucan endotransglucosylation
XTH	Xyloglucan endotransglucosylase/hydrolase

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1. Introduction

1.1 *Cuscuta*

Parasitic weeds are one of the largest challenges facing agricultural production in both developed and developing countries on every continent, and can cause 30-80 % loss in the production of staple food and industrial crops¹. Controlling the growth and spread of such parasitic plants can be difficult due to several factors. The parasitic plant is intimately involved with the host, and there's a high degree of metabolic overlap between the parasite and its host, making it hard to develop treatments that differentiate between the two. The parasites produce vast amounts of seeds that may be capable of surviving in soil for more than 15 years. Additionally, some parasitic plants attach to the roots of the host and are concealed underground for most of their life cycle¹.

There is a total of 20 families (3000-5000 species) of parasitic higher plants¹. One of these is the *Cuscuta* family: obligate parasites that lack both leaves and roots, and that twirl around the stem of the host plant, relying entirely on the host for sustenance. *Cuscuta* are defined as stem holoparasites: holoparasites lack chlorophyll and are unable to photosynthesize, and stem parasites absorb nutrients and water from host stems⁴⁺¹¹. Due to the low or totally absent photosynthesis in *Cuscuta* in addition to its lack of roots, it gains all its water, carbohydrates and minerals from the host¹⁰. Because of this, *Cuscuta* must attack its host rapidly and penetrate into its vascular system before the parasite's seed reserves deplete. This requires the plant to overcome the mechanical barriers of the host plant (mainly the cuticle and the cell walls) without doing too much damage¹¹⁺¹³. Aggressively damaging the host plant could not only initiate host defense mechanisms, but may also damage the host's ability to nurture the *Cuscuta*. The parasite must therefore have developed a mechanism of infection which is both aggressive, but not excessively intrusive¹¹.

After attaching to the host, all *Cuscuta* develop haustorium, a special tissue which sucks nutrients out of the host plant. The parasite penetrates the host tissue by either mechanical or enzymatic means, and research seems to indicate that the penetration mechanism involves a combination of mechanical pressure applied to host tissue and secretion of enzymes that degrade the host cell wall¹¹⁺¹³. Electron microscopic examination shows that the *Cuscuta* minimizes stress to the host by "stretching" the host wall around the haustoria instead of simply penetrating it¹¹.

1.2 Xyloglucan endotransglucosylases/hydrolases (XTHs)

Xyloglucan is a major structural polysaccharide which plays an integral role in cell wall architecture by cross-linking the linear cellulose microfibrils³⁺⁸. Regulation of xyloglucan crosslinking is done by

enzymes called xyloglucan endotransglucosylases/hydrolases (XTHs), which catalyze both the cutting and pasting of xyloglucan⁷. The predominant activity of a majority of XTHs is xyloglucan endotransglucosylation (XET), where the reducing end of cleaved xyloglucan is attached to an acceptor xyloglucan chain. The other mechanism of XTHs, xyloglucan endohydrolysis (XEH), where the acceptor is water, is the predominant activity in only a minority of XTHs²⁺⁸. XET activity can both loosen and strengthen the cell wall, while XEH activity is only able to do the former. Commonly XTHs are associated with cell growth, as the cutting of xyloglucan allows for lengthening of the cell wall, but XTHs can also inhibit plant cell growth, possibly by increasing the xyloglucan cross-linking of cellulose microfibrils⁸.

Research done by Olsen⁷⁺⁸ seems to indicate that XTHs play a significant role in the infection mechanism of *Cuscuta*. This mechanism can be divided into three stages: the initial swelling of the parasitic stem, the haustorium penetrating the host tissue, and the final stage where the parasite is fully able to feed on its host⁷. Several genes encoding XTHs in *Cuscuta reflexa* were observed to be highly expressed during the initial swelling stage⁷. Research into *C.reflexa* XET activity during the different stages of infection revealed the activity to be highest during the second stage where the haustoria penetrate the cell wall of the host plant⁷. It is possible that loosening of the cell walls of the host plant caused by *Cuscuta* XTHs is the reason why the haustorium can “glide” between the cell walls and relatively unobtrusively penetrate the host plant⁸.

Since XET activity is related to plant cell growth, and the haustorium is a growing organ, the increase in XET activity is at least partly related to the growth of the parasite. However, Olsen⁷ makes several arguments for why XTHs do not exclusively affect parasite cells. XTHs were secreted where the haustoria were developing on the host, and in addition the abundance of xyloglucan was found to be reduced in both the tissue of the haustoria and the host. There was also no clear band of XET activity observed at the interface between parasite and host at the final stage of invasion, implying that the role of the XTHs is most important during the penetration stage where the need for cell wall loosening is greatest. Research done into the defense mechanism of cultivated tomato *Solanum lycopersicum* shows an increase in the expression of an XTH gene¹², possibly to combat this cell wall loosening initiated by *Cuscuta* XTHs. Finally, with a few exceptions, grasses with lower concentrations of xyloglucan are immune to *Cuscuta*¹⁴.

1.3 Brilliant Blue

Chormova² developed a method for large scale screening of inhibitors of XET activity of all XTHs. After screening a wide variety of xenobiotics, they ended up with 30 main xenobiotics which inhibited XET activity. While the compounds vary wildly in structure, the most promising inhibitor seemed to be the

triphenylmethane dye Brilliant Blue R250 (BB-R250). The structure of the compound is shown in Figure 1.

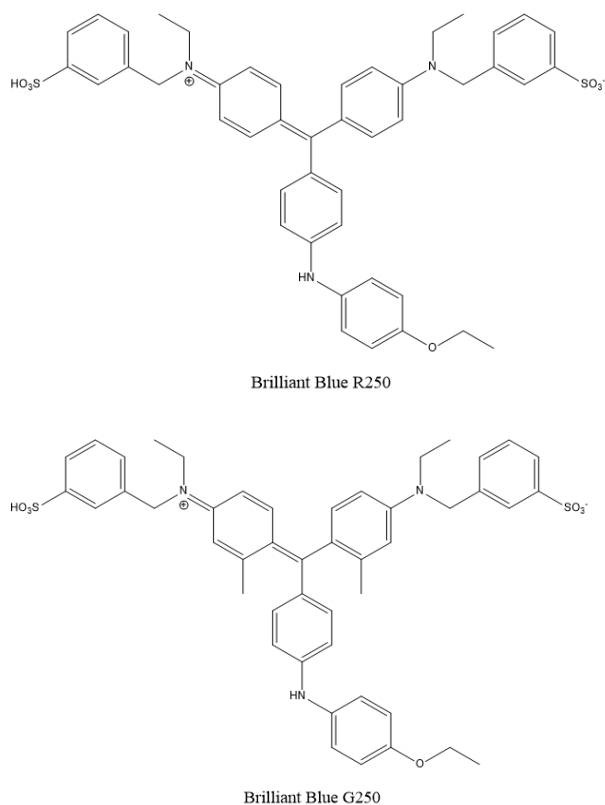


Figure 1. Structures of Brilliant Blue R250 and Brilliant Blue G250

BB-R250 was also employed as a potential inhibitor of *Cuscuta* XET activity in tests performed by Olsen⁷. XET activity in extracts prepared from host-invading haustoria of *C.reflexa* was reduced by BB-R250, and the inhibiting effect was dependent on the concentration. Additionally, BB-R250 also hindered approx. 1/3 of *C.reflexa* haustoria from successfully growing into host tissue (the tests were performed on *P.zonale*). It is noted in the article that while BB-R250 has an inhibiting effect, the compound is known for binding proteins in general, so the observed effect may be caused by BB-R250 binding to other proteins than XTHs.

Through x-ray crystallography Li et al.²³ reveal how Brilliant Blue G250 (BB-G250, shown in Figure 1), which is structurally similar to BB-R250, binds to human serum albumin. The main form of interaction seems to be hydrogen bonding between the protein and the sulfonic acid groups present in BB-G250.

1.4 Biological testing

Any compounds of interest will be screened for their general ability to bind proteins, and their effect on XET activity. This initial screening will serve as a way to find promising candidates for further testing on live plants.

1.4.1 Protein binding

The protein binding abilities of different compounds were determined by staining polyvinylidene difluoride membranes with compound solutions. Before staining, the membranes were wetted with methanol and water-equilibrated. 5 μ L bovine serum albumin standards or protein extract from *Cuscuta reflexa* were spotted onto the membranes, which were then dried. The protocol for Coomassie

Blue R-250 staining described by Goldman⁵ was used for staining and destaining. First, membranes with bound proteins were again wetted with methanol and equilibrated with water. The membranes were then stained for 5 min with a 300 μ M compound solution with 40 % methanol and 7 % acetic acid. Finally, the membranes were rinsed with water for a few minutes and dried afterwards. Changes in the visibility of colors on the protein spots were used to determine the protein binding ability of compounds. Because of this, the method is limited to compounds that have visible colors in solution.

1.4.1 XET inhibition

The effect compounds have on XET activity was tested by spotting compound solutions on XET test papers coated with xyloglucan. Xyloglucan oligosaccharides on the test papers were labeled with sulforhodamine according to the procedure described by Kosik and Farkas⁶, while the preparation of the test papers was done according to the procedure described by Fry³.

To *C.reflexa* extracts, 0.1X volumes of 10 mM or 50 mM compounds solutions were added, while only solvent was added for no inhibition control. At 4°C, 3 μ L of the mixtures were spotted onto XET test papers or control papers only coated with xyloglucan. The papers were incubated for 1 hour at 21°C. To wash away background fluorescence, the papers were gently agitated in a 1:1:1 mix of ethanol, formic acid and water for at least 2 hours, and at most overnight. After destaining, the papers were rinsed in water and dried. Images of the papers were taken with a ChemiDoc MP imaging system (Bio-Rad) by using the Cy3 application with an exposure time of 400 ms. Stronger fluorescence in a spot would correlate with stronger XET activity. The Image Lab software (Bio-Rad) was used to calculate global background-adjusted volumes of fluorescent spots, which represent relative XET activity.

2. Aim of project

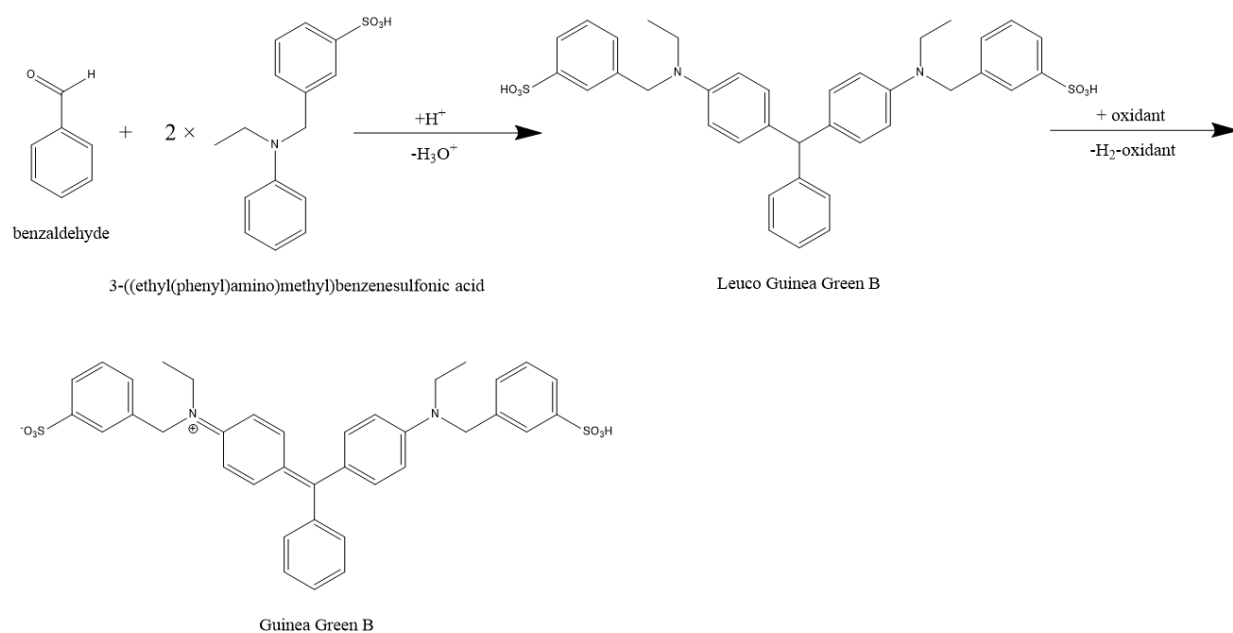
The general aim was to use Brilliant Blue (BB) as a jumping-off point for synthesis of potentially better XTH-inhibitors, both in terms of solubility and activity, the latter being the primary focus. Due to the wide scope of the project, there was a focus on developing methods for synthesis of a wide variety of molecules to gain as much information as possible, and to lay a solid foundation for future research. Synthesis of a variety of structurally similar molecules was not prioritized. At this early stage the more interesting information can be gained from somewhat larger changes in molecular structure (i.e. substitution/removal of functional groups and/or entire parts of the molecule). In this way a wider range of information can be gained before focusing on more specific properties like electronic/solvation effects of substituent groups. Yield was only a priority only if there wasn't enough compound for biological testing.

3. Synthetic strategy

The first part of this section is dedicated to the discussion of a general synthesis for molecules structurally similar to Brilliant Blue, both with and without sulfonic acid groups. This synthesis consists of two steps: the formation of the central triphenylmethane system through a triaryl condensation reaction, and the oxidation of the compound to create the central conjugated system. In the latter part, pathways that offer greater flexibility for synthesis of Brilliant Blue derivatives (BBDs), are introduced. These pathways utilize smaller building blocks, and consequently offer some important advantages over the general synthesis of BBDs.

3.1 The two-step synthesis of Brilliant Blue derivatives

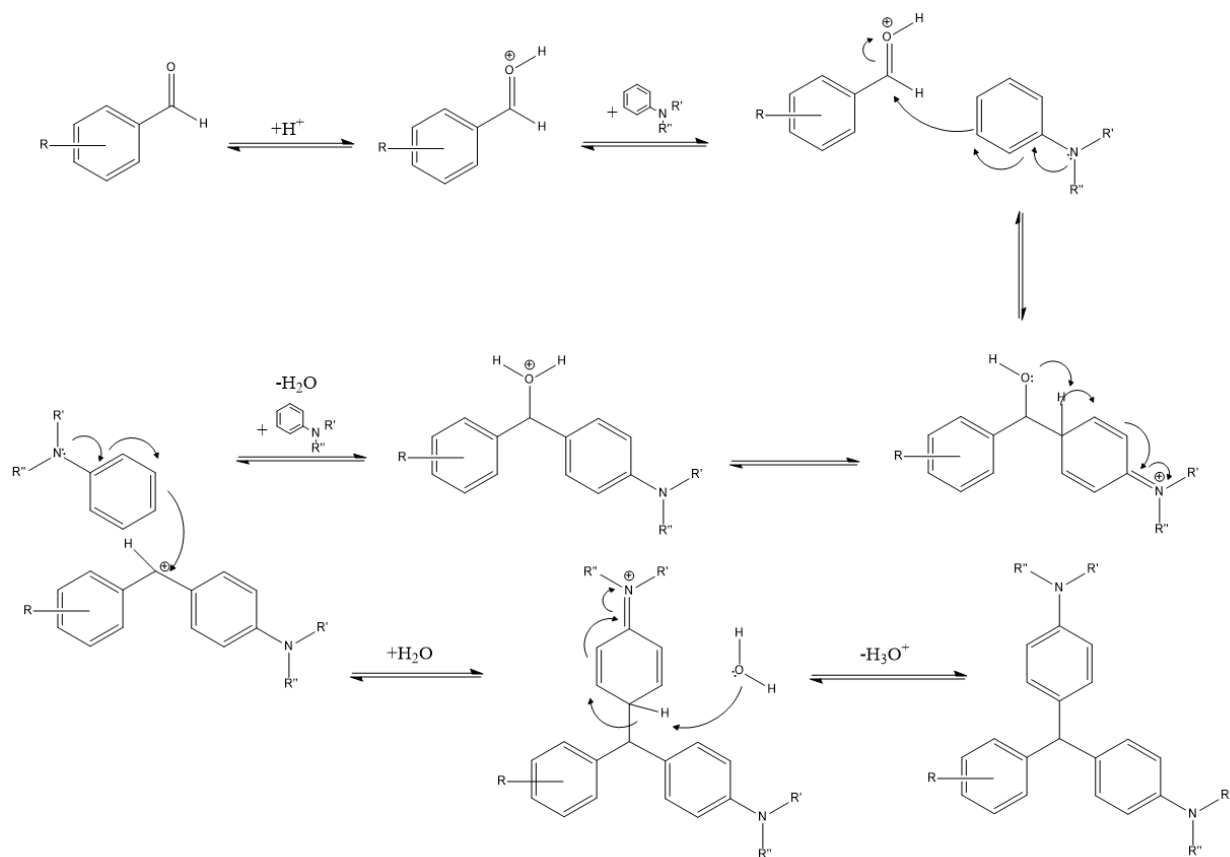
The first step was to find a way to synthesize different kinds of Brilliant Blue derivatives (BBDs). The article by Wang²⁴ was used as a starting point. The two-step synthesis of Guinea Green B (GGB) is shown in Scheme 1 based on the reaction steps from the aforementioned article. GGB was chosen as an example due to it being the product with the simplest structure (no substituents on the benzaldehyde).



Scheme 1. Two-step synthesis of BBDs. The reduced BBD is formed in an acid catalyzed triaryl condensation reaction and is then oxidized in the second step.

The first step is a condensation reaction in which two equivalents of 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid attack the carbonyl carbon of the benzaldehyde, resulting in water and a triphenylmethane compound. The reaction mechanism is thought to be similar to the general

mechanism for the formation of 4,4'-diaminotriphenylmethane (PMDA) compounds¹⁵⁺¹⁶, which is shown in Scheme 2.



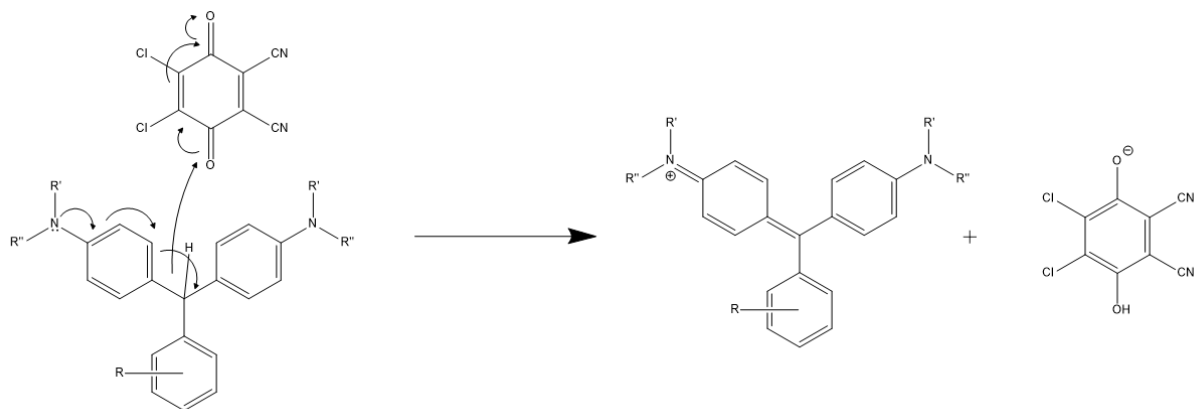
Scheme 2. General triaryl condensation mechanism.

The formation of the triaryl compound is catalyzed by the protonation of the carbonyl. This promotes nucleophilic attack at the carbonyl carbon. Attack from the para-position on the aromatic ring in the aniline is activated by the free nitrogen electron pair. Deprotonation reforms the aromatic ring, and the hydroxy group is protonated. The proton transfer is shown in one intramolecular step in the scheme, though the transfer can happen through the solvent. In the first step of an S_N1 mechanism, water leaves, leading to a carbocation intermediate that is stabilized by the aromatic rings. In the second step, another equivalent of aniline attacks the positively charged carbon. The aromatic ring is reformed, here shown by water that deprotonates the intermediate, resulting in product.

The resulting product is a reduced BBD: the central carbon has a hydrogen, meaning that the conjugated system that gives the BBDs their characteristic strong colors, is lacking. The color is not the only difference, as the reduced compounds have a different 3D structure. For example, the central carbon in BBDs is sp^2 hybridized, while in the reduced compounds it is sp^3 hybridized. Additionally, BBDs have a positive charge distributed through the central conjugated system, which affects solubility. Therefore, the biological screening of reduced compounds (called leuco compounds²⁴) is of

interest. It is possible that the flexibility gained from the non-conjugated system makes the leuco compounds better inhibitors than the oxidized BBDs, or, on the contrary, some property of the conjugated system is essential or beneficial for activity.

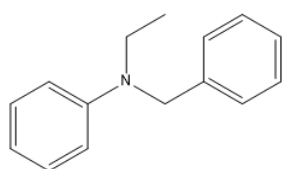
The second step in the reaction pathway shown in Scheme 1 is the oxidation of the leuco compound. 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was considered as an oxidizing agent. Scheme 3 shows a proposed mechanism for the DDQ oxidation of PMDA compounds¹⁹⁺²⁰.



Scheme 3. DDQ oxidation mechanism.

Here, it is shown that the hydride is transferred to one of the carbonyl oxygens in DDQ. The hydride transfer is promoted by the formation of an aromatic ring in DDQ, and the formation of a large conjugated system in the PMDA compound.

This pathway consisting of the formation and oxidation of the leuco compound, allows for a potentially quick synthesis of BBDs with different substituents on the third phenyl ring in the PMDA core, but is restricted by the other starting material. The reaction pathway could be potentially lacking when changes in other parts of the structure are desired. One such change that is important for biological screening, is the replacement of the sulfonic acid groups with other functional groups.



N-benzyl-*N*-ethylaniline

Figure 2. Structure of N-benzyl-N-ethylaniline

N-benzyl-*N*-ethylaniline (shown in Figure 2), which is structurally similar to 3-((ethyl(phenyl)amino)methyl)-benzenesulfonic acid, but lacks the sulfonic acid group, was commercially available. This opens up the possibility to synthesize BBDs without sulfonic acid groups utilizing the pathway from Scheme 1. The main reason to synthesize compounds lacking sulfonic acid groups is to better understand what effect the sulfonic acid groups have on biological activity. Additionally, it might be

possible to create BBDs with only one sulfonic acid group and substitute different functional groups on BBDs lacking one or both sulfonic acid group(s).

Aniline Blue (AB), shown in Figure 3, is a dye with the same central PMDA core as BBDs, but an overall different structure. A noticeable difference between AB and sulfonic acid BBDs (ignoring any substituents on the third phenyl ring in the core), are the missing CH₂-groups between the amine and the outermost phenyl

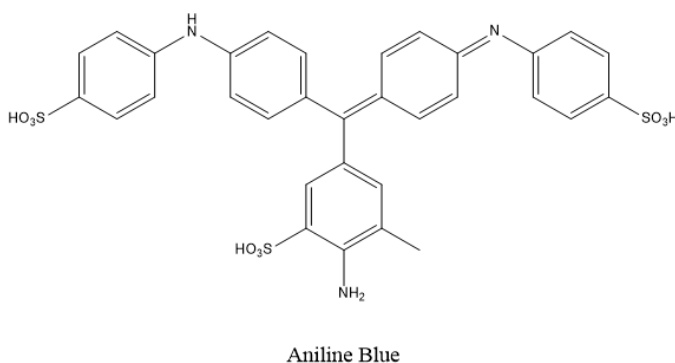


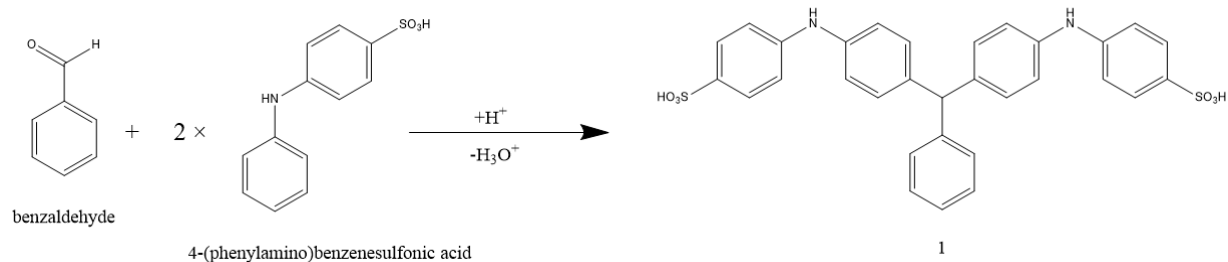
Figure 3. Structure of Aniline Blue.

rings, making the overall structure shorter. Additionally, the sulfonic acid groups in Aniline Blue are in the para-positions, as opposed to the meta-positions in the previously discussed compounds. Aniline Blue also lacks the ethyl groups on the amines.

In the XET inhibition tests performed by Chormova², AB was determined to be an inhibitor, though not as effective as BB-R250. Despite being a poorer inhibitor than BB-R250, AB derivatives might be of interest. AB has a significantly more rigid structure than BBDs due to the lack of the CH₂-groups, but leuco AB derivatives may be flexible enough in their 3D structures to be able to compete with BBDs. Additionally, AB has a third sulfonic acid group, which might interfere with binding.

Biological testing of AB derivatives would give more information about the mechanism by which XET activity is inhibited. If AB derivatives prove to be bad inhibitors, it would indicate that some difference between BBDs and AB derivatives is crucial for activity.

Since the PMDA core is the same as in BBDs, the synthesis of AB derivatives should be possible by utilizing the same two-step pathway as for BBDs. The synthesis of a leuco AB derivative from benzaldehyde and 4-(phenylamino)benzenesulfonic acid is shown in Scheme x.

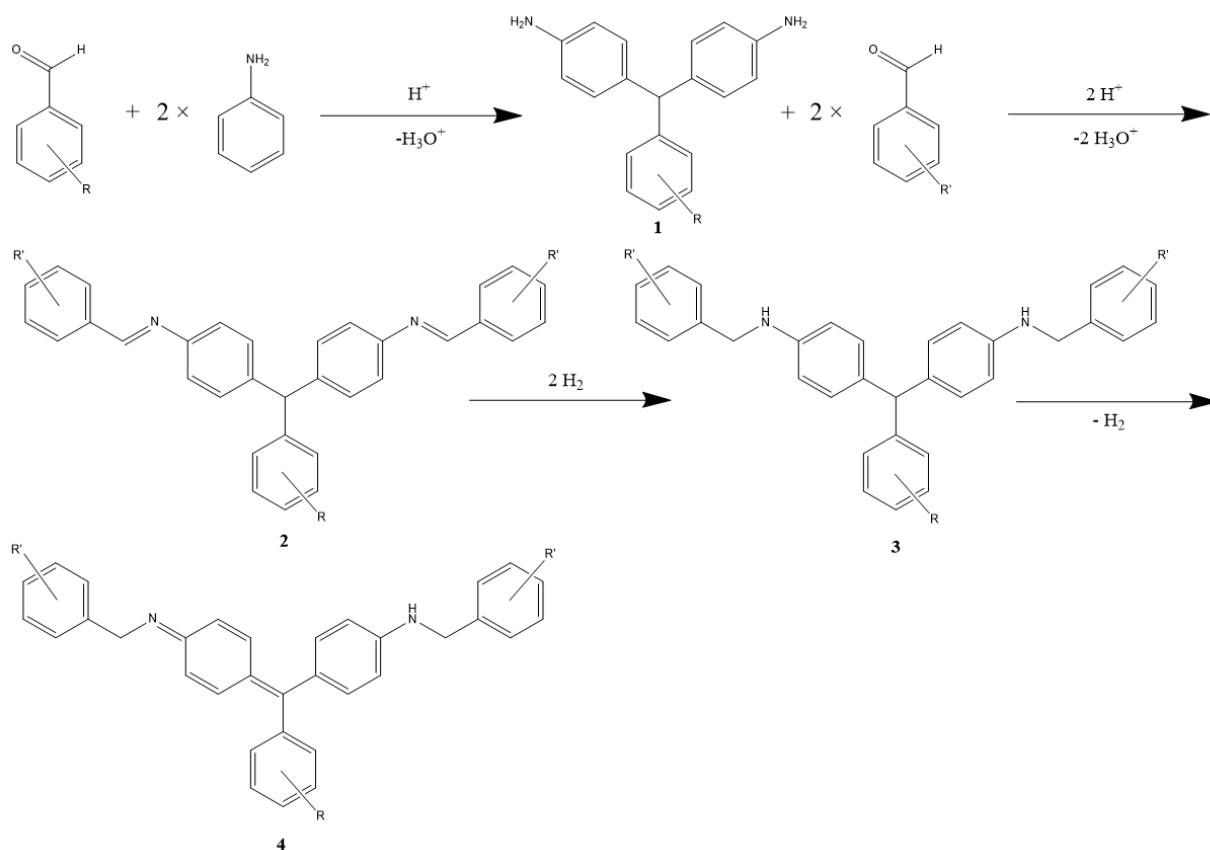


Scheme 4. Synthesis of a leuco Aniline Blue derivative 1.

3.2 The 4,4'-diaminotriphenylmethane (DPTM) and 4,4'-diaminodiphenylmethane (MDA) pathways

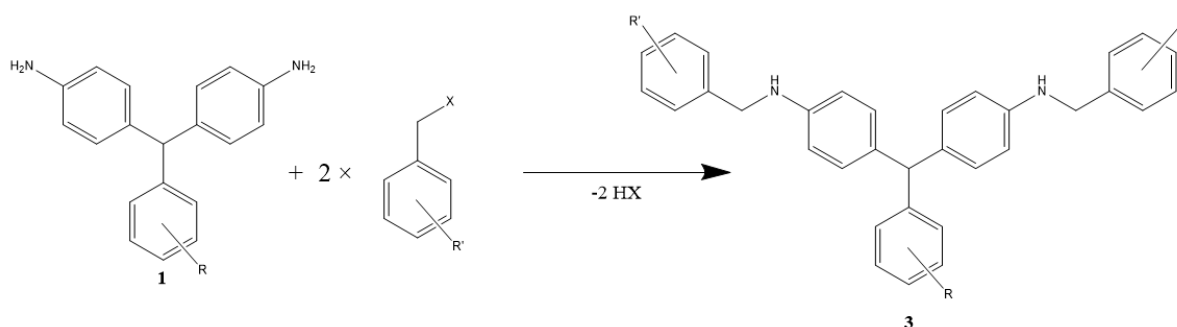
In order to synthesize biologically interesting compounds with a greater range of variety, alternative pathways to the one described in the previous section, were considered. One such pathway is to synthesize the core 4,4'-diaminotriphenylmethane (PMDA) body first, for then to substitute different groups on the amines, This not only allows for flexibility in the construction of the central PMDA body, but also allows for flexibility when constructing the outer ends of the molecule.

The proposed pathway for the synthesis of BBDs from benzaldehydes and aniline is shown in Scheme 5. For synthesis of PMDA (**1**), a benzaldehyde and two equivalents of aniline are used¹⁵⁺¹⁶⁺¹⁷⁺¹⁸. The triaryl condensation mechanism is the same as the one shown in Scheme 2. The PMDA reacts with two equivalents of a benzaldehyde to form **2**, a double-imine compound with a carbon skeleton reminiscent of a BBD. The imine double bonds are reduced to gain the leuco Compound **3**, and this compound is then oxidized to get the final compound **4**. Compound **2** is drawn as the double trans (*E,E*) conformer in all schemes and figures as it should be the most stable conformation. Should double imine compounds such as **2** prove to be of interest as XET inhibitors, conformational analysis should be considered.



Scheme 5. The 4,4'-diaminotriphenylmethane and benzaldehyde pathway for synthesis of BBDs.

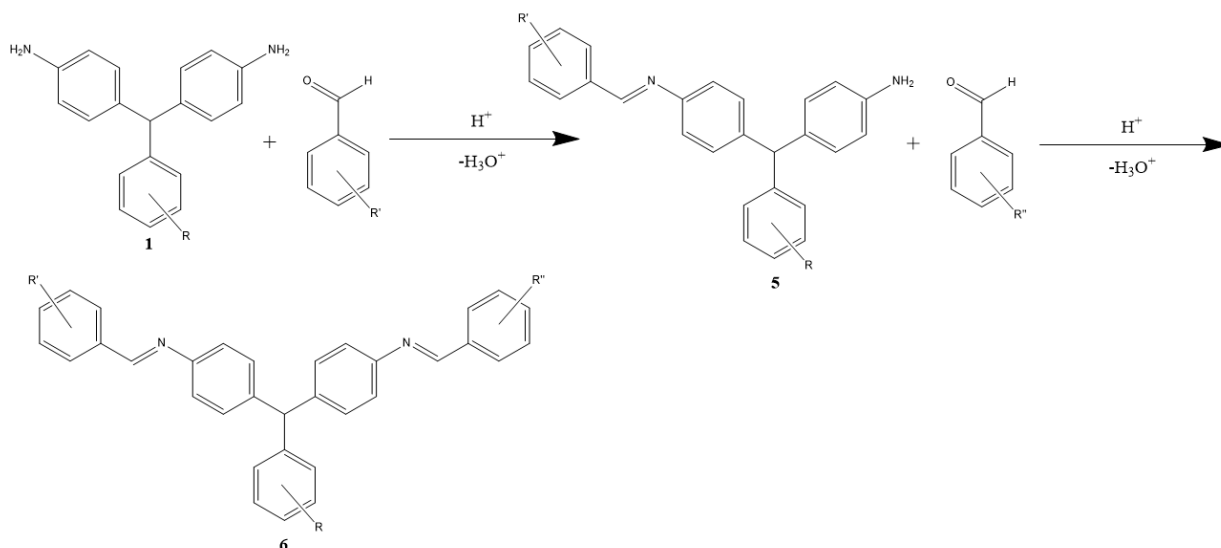
Reacting PMDA with benzaldehydes is not the only option. An alternative is the S_N2 reaction shown in Scheme 6, in which two equivalents of a benzyl halide react with PMDA to form the leuco compound **3**. Using benzyl halides instead of benzaldehydes, skips the synthesis of **2**, but the range of commercially available benzyl halides and their cost compared to benzaldehydes potentially makes this pathway more limiting.



Scheme 6. Synthesis of leuco compound 3 from PMDA and two equivalents of benzyl halide.

Perhaps one of the biggest advantages of the PMDA pathway is that it may allow for controlled synthesis of asymmetric compounds. Instead of reacting the PMDA with two equivalents of a

benzaldehyde or benzyl halide, it could be possible to do this with only one equivalent and obtain the monosubstituted compound. The monosubstituted compound can then react further to create an asymmetric disubstituted compound. Scheme 7 shows the formation of monosubstituted compound **5** from PMDA and a benzaldehyde, which then reacts with a different benzaldehyde to form compound **6**.



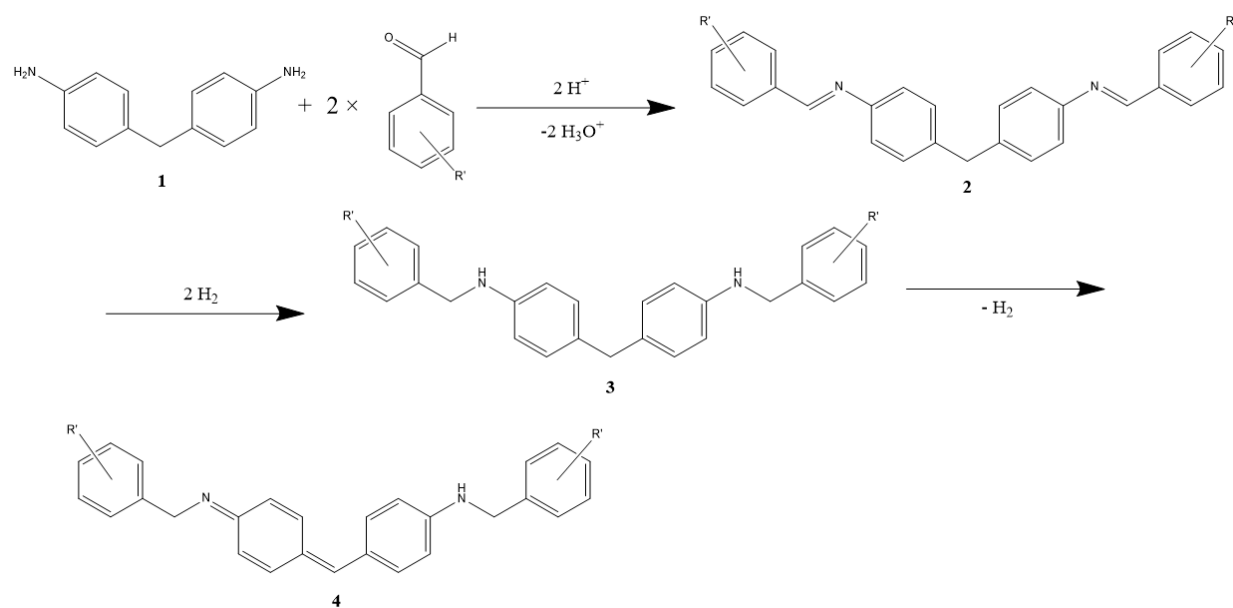
*Scheme 7. Synthesis of the asymmetric compound **6** from PMDA and two different benzaldehydes.*

Something that may limit the usefulness of the benzaldehyde pathway, is the reduction of the two imine groups in **2**. Strong reduction agents may be required, which in turn can reduce certain substituent groups (e.g. nitro groups may be reduced to amines if the reduction agent is too strong).

For maximum flexibility and the possibility to react PMDA with benzaldehydes, the aniline used to form PMDA should be a primary amine. This means that the aniline is free to react with benzaldehyde and form the imine (Schiff base) instead of the desired PMDA. In order to promote the formation of PMDA, specific catalysts may be required¹⁵⁺¹⁷. Ahmadu¹⁶ describes the synthesis PMDAs in near-critical and supercritical water using an autoclave. Guzmán-Lucero¹⁸ synthesized a wide variety of PMDAs in high yields using microwave irradiation for heating, and the aniline hydrochloride salt as a catalyst. This seemed like the best place to start.

If the third phenyl ring in the PMDA core is not required for inhibition of XET activity, then the synthesis of PMDA could be skipped entirely. The replacement for PMDA would be the diphenyl compound 4,4'-diaminodiphenylmethane (MDA). Scheme 8 shows the synthesis of compounds lacking the third phenyl ring from MDA (**1**) and benzaldehydes. The steps are the same as for PMDA in Scheme 5, except that this pathway starts with the synthesis of the di-imine compound **2** due to MDA being commercially available. **3** can either be synthesized from **2**, as shown in Scheme 8, or directly by reacting MDA with

benzyl halides. The final step would then be the oxidation of **3** to form **4**. Just as with PMDA compounds, it should be possible to synthesize asymmetric compounds from different benzaldehydes and benzyl halides.



Scheme 8. The 4,4'-diaminodiphenylmethane and benzaldehyde pathway for synthesis of BBDs.

Other than skipping the possibly challenging synthesis of PMDA, the MDA compounds might be easier to dissolve in polar solvents due to the absence of an entire phenyl ring. What is lost, is the flexibility gained from the range of benzaldehydes and anilines that can be used in the synthesis of PMDA. However, commercially available alternatives to MDA do exist, e.g. 4,4'-oxydianiline, in which an oxygen replaces the central $-\text{CH}_2-$.

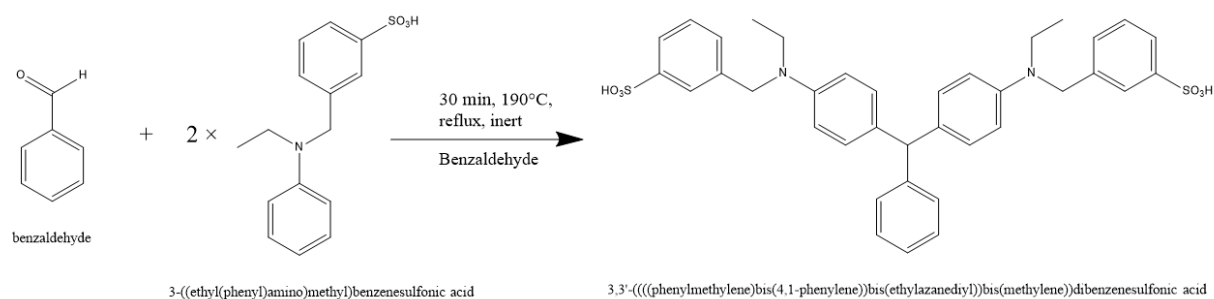
4. Results and discussion

In the first part of this chapter, the synthesis of different molecules and the results from these syntheses are discussed. The experimental section, and the appendices that correspond to each reaction, end with the same number as the section in question.

In the second part the results from biological screening of commercially bought BBDs, are presented and discussed. In the third section, this is done for the biological screening results of synthesized compounds. All biological screening was done by Stian Olsen, PhD candidate in Arctic and Marine Biology at UiT. Only the results from biological screening will be presented and discussed.

4.1 Synthesis of potential inhibitors of XET activity

4.1.1 Triaryl condensation synthesis of 3,3'-((((phenylmethylene)bis(4,1-phenylene))bis(ethylazanediyl))bis(methylene))dibzenesulfonic acid from excess benzaldehyde



Scheme 9. Excess benzaldehyde synthesis of 3,3'-((((phenylmethylene)bis(4,1-phenylene))bis(ethylazanediyl))bis(methylene))dibzenesulfonic acid.

One way to create reduced BBDs is by reacting two equivalents of 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid with a benzaldehyde (section 3.1) in a condensation reaction where the central triphenylmethane is formed. Benzaldehyde was used for synthesis development, the thought being that it is the simplest benzaldehyde with no substituent groups that may complicate matters. The article by Guzmán-Lucero¹⁸ was chosen as a starting point, as the synthesis of triphenylmethanes using microwave irradiation looked promising.

The neat reaction conditions described in the article proved a challenge since the benzenesulfonic acid is a solid at rt. The total volume of benzaldehyde in a 2:1 eq. reaction was too small to successfully mix with the solid powder, and as a result initial attempts at neat MW reactions proved unsuccessful. The acid catalyst used by Guzmán-Lucero¹⁸ is the hydrochloric aniline salt, but early on it was observed that the formation of blue or green color took place in the absence of any acid catalyst. In some cases,

it even seemed like the addition of an acid catalyst (e.g. 0.1 eq. hydrochloric acid) worked to the detriment of any product formation (this is only based on how much colored compound was formed). It was suspected that acidic conditions would lead to the protonation of the nitrogen in the benzenesulfonic acid and hinder its ability to attack the benzaldehyde in the desired way.

As the neat MW reaction didn't look promising, the conditions used for heat reactions described by Guzmán-Lucero¹⁸ were tested. The benzenesulfonic acid proved poorly soluble in almost all solvents that were tested. The best solvents were DMSO and DMF, in which relatively large amounts of the compound could be dissolved by heating the solvents. However, while the compound dissolved much more poorly in methanol, it was considered the most promising solvent due to DMSO and DMF workup potentially being challenging due to their high boiling points. Wang²⁴ used water as a solvent, and consequently both MW and heat reactions where either methanol or water was the solvent, were tested to little success. Another potential solution was to run the reaction at a high enough temperature to melt the benzenesulfonic acid, therefore eliminating the need for any solvent. However, heating the compound did not melt it, but instead, when high enough temperatures were reached, the compound would turn orange and rock-solid under inert conditions, and green under air, potentially being oxidized in some manner. This is another reason for why neat reactions proved challenging, because at high enough temperatures it seemed likely that the benzenesulfonic acid would react with itself due to poor contact with the liquid benzaldehyde.

In a reaction with excess benzaldehyde the reactant itself would act as solvent, and the benzenesulfonic acid would constantly be in contact with benzaldehyde. Excess benzaldehyde could mean the possible formation of a monosubstituted product instead of the desired disubstituted one, but a monosubstituted compound was considered of interest for both biological testing and further synthesis. The reaction was attempted with 5 equivalents of benzaldehyde and 1 equivalent of the benzenesulfonic acid using an oil bath for heating. In contrast to any of the previous attempts at synthesis, here the reaction mixture turned almost immediately green when the round-bottom flask containing the mix was immersed into the preheated oil bath. Workup resulted in a fine green powder.

The expected m/z of the negatively charged compound is present in the **HRMS** spectrum.

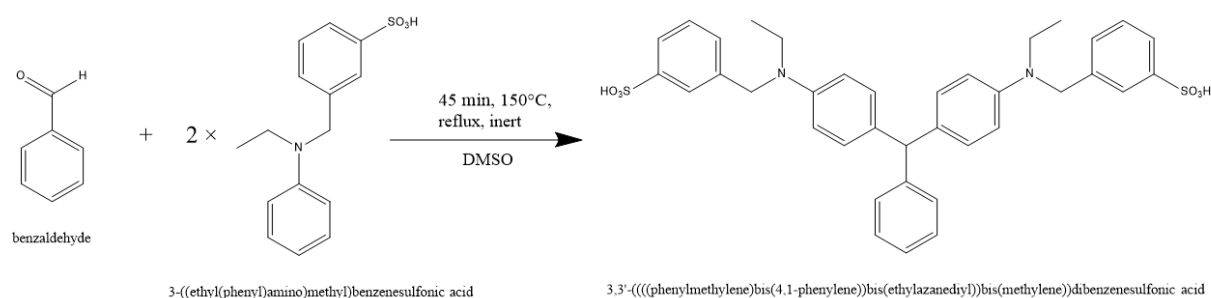
The **NMR** solvent was DMSO- d_6 . There appear to be some impurities present. The most notable impurity peak in the ^1H NMR spectrum is a singlet at 3.2 ppm.

The best indicator of the product being present in the ^1H **NMR** spectrum, is the presence of a singlet corresponding to the central proton in the triphenylmethane body. Considering the shift of protons next to aromatic groups, it seems likely that the singlet at 5.6 ppm belongs to this proton. Using this as a reference, the integrals of the singlet at 4.7 ppm and the triplet at 1.1 ppm match up almost

perfectly with the target structure. The broad water peak likely eclipses the expected 4H quartet that connects to the 6H triplet. The COSY spectrum of the product in the next section confirms that the triplet connects to something obscured by the water peak. The total amount of aromatic protons should be 21, while the integral of the aromatic area is approx. 25H, likely due to some impurities. The aromatic area is not clear enough for any in-depth analysis.

Based on the specter data, this seemed to be the first time a reduced BBD was successfully isolated, although with some unknown impurities present. No acid catalyst nor MW irradiation was needed. It is possible that the benzenesulfonic acid was strong enough to catalyze the reaction by itself. However, this synthesis is restricted to benzaldehydes that are liquid or melt relatively easily, and, of course, it requires significant amounts of excess benzaldehyde. The development of a synthesis with different reaction conditions was therefore of interest.

4.1.2 Triaryl condensation synthesis of 3,3'-((((phenylmethylene)bis(4,1-phenylene))bis(ethylazanediyl))bis(methylene))dibenzenesulfonic acid



Scheme 10. Synthesis of 3,3'-((((phenylmethylene)bis(4,1-phenylene))bis(ethylazanediyl))bis(methylene))dibenzenesulfonic acid with DMSO as solvent.

While searching for another way to synthesize leuco GGB, DMSO was reconsidered as a solvent. Considering the high temperature of the previous reaction, it seemed likely that for a reaction with a solvent, one with a high boiling point would be beneficial. Initial reactions with DMSO as solvent looked promising due to the reaction mix turning almost immediately green, but evaporating the solvent proved challenging. High temperatures were required to evaporate all traces of DMSO even when using a high-vacuum pump, and it was suspected that when there was little solvent left, potential side reactions took place, making workup harder and potentially even degrading any formed product. Considering how the solid benzenesulfonic acid starting material changes properties such as color and consistency when exposed to high temperatures, this did not seem unlikely. However, by adding water to the reaction mixture, a turquoise solid crashed out. When water was added to concentrated DMSO

solutions containing only the benzenesulfonic acid, the compound did not crash out. This seemed like a pretty good indicator that not only had some new compound formed, but that the starting material remained in the solution. The precipitate from the reaction was washed with water and warm methanol and analyzed.

In the **HRMS** spectrum there are two peaks corresponding to the expected m/z of the desired compound. The first peak at approx. 669 corresponds to the compound minus one proton, while the peak at approx. 334 corresponds to the compound minus two protons. The doubly negatively charged compound is no surprise considering that the expected product has two sulfonic acid groups.

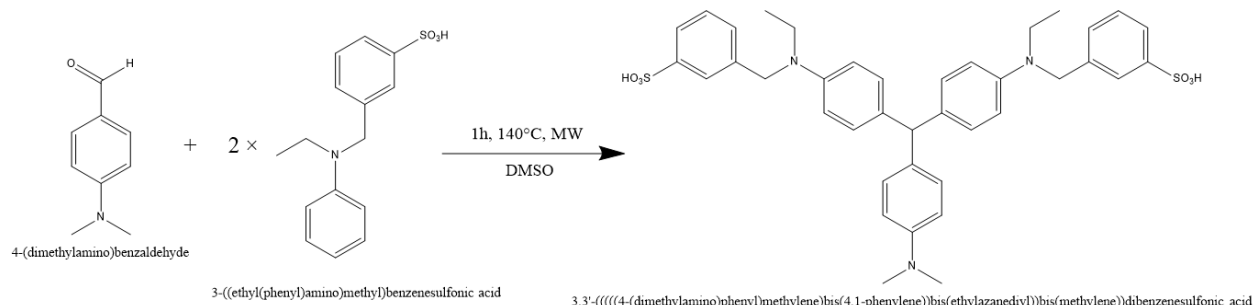
The **NMR** solvent was DMSO- d_6 . The singlets between approx. 3.2 ppm and 2.0 ppm likely belong to small amounts of impurities/an impurity. The singlet 3.2 ppm was also present in the ^1H NMR spectrum of the product from the previous section, but here it is significantly smaller.

The ^1H **NMR** spectrum looks better here than in the previous section. Once again, the singlet at 5.6 ppm is a good indicator that the reaction has been a success. With this as a reference, the singlet at 4.7 ppm and the triplet at 1.1 ppm match with the expected structure. The COSY spectrum shows that the triplet connects to an unknown peak that is eclipsed by the broad water peak. This is likely the 4H quartet belonging to the two ethyl groups. The total size of the aromatic area is 24H, closer to the expected value of 21H than in the previous section. The 2H singlet at 7.7 ppm very likely belongs to the isolated protons next to the sulfonic acid groups. The peak at 7.5 ppm having an integral of 3H seems to indicate that the para-proton of what was originally the benzaldehyde, is part of this multiplet. The rest of the aromatic area is hard to analyze.

The sample was not concentrated enough to get a decent ^{13}C NMR spectrum. The solubility of the compound is poor in most solvents. Even with DMSO- d_6 getting a high enough concentration for a decent ^1H NMR spectrum required heating the solvent.

This reaction was considered a significantly better alternative to the previous one, as it doesn't require excessive amounts of benzaldehyde, it isn't restricted to benzaldehydes with lower melting points. It also has the same advantage as the previous one in that no acid catalyst is needed, as it seems likely that the benzenesulfonic acid itself acts as a catalyst. Both successful syntheses were done with an oil bath as a heating source, but MW irradiation was still considered as an alternative.

4.1.3 Triaryl condensation synthesis of 3,3'-((((4-(dimethylamino)phenyl)methylene)bis(4,1-phenylene))bis(ethylazanediy))bis(methylene))dibzenesulfonic acid



Scheme 11. Synthesis of 3,3'-((((4-(dimethylamino)phenyl)methylene)bis(4,1-phenylene))bis(ethylazanediy))bis(methylene))dibzenesulfonic acid.

The synthesis of product was attempted using the same reaction mix composition as in the previous section, but with N-benzaldehyde instead of normal benzaldehyde. This reaction was run in the microwave for 1 hour at a slightly lower temperature. The reaction mixture turned a strong purple color, and addition of water crashed out a strongly colored purple compound. While the precipitate from the previous section was washed with small amounts of boiling methanol, this precipitate was washed with cold methanol. The reason for this was that while the warm methanol wash did seem to remove some impurity, it also washed away significant amounts of product. Additionally, due to the substituent group in the desired product of this reaction, it was thought that it would be more soluble in methanol than the product from the two previous reactions, meaning that a hot methanol wash would wash away even more product in this case.

In the **HRMS** spectrum, although peaks for the expected compound with both one and two negative charges, are present, there are multiple other peaks present. Most notable is the large peak at approx. 290, which likely belongs to the benzenesulfonic acid starting material (Mw: 291.4). THE CALCULATED Z=2 IS WRONG

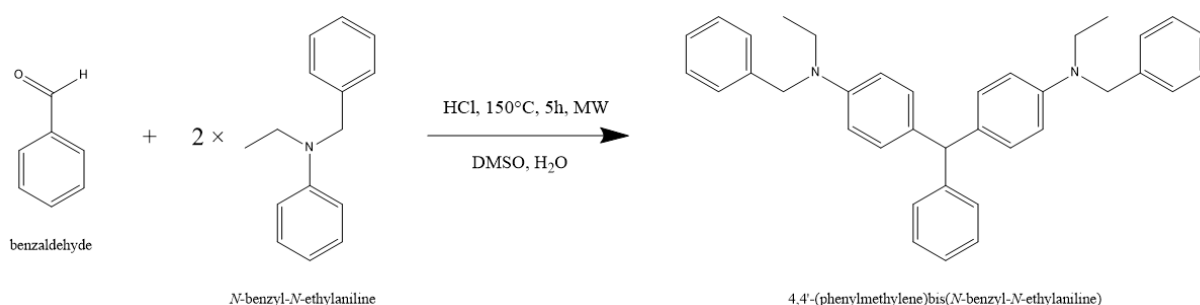
The **NMR** solvent was DMSO-d₆. It was hard to obtain a decent ¹H NMR spectrum. From the obtained ¹H NMR spectrum there is nothing that implies that the expected product is present. The most damning indicator is the lack of the singlet stemming from the central proton, which is expected to have a shift around 5.5 ppm. Based on analysis of similar compounds, the singlet at 4.8 ppm should belong to the protons between the nitrogen and the meta-substituted aromatic ring. Even when using this as a reference, there is little information to be gained from the integrals, in large part due to the poor quality of the spectrum. From the COSY spectrum, it appears that the large water peak obscures a peak

at approx. 3.6 ppm, which is in accordance with the ethyl groups present in both starting material and product. The aromatic area differs a great deal from the aromatic area of starting material [appendix], so it seems likely that some reaction has taken place. It could be possible that there is some amount of oxidized product present in the mix, and that this dissolved in the DMSO. This would explain the missing singlet. However, it is best not to draw any conclusions from this ^1H NMR spectrum alone.

While it seems likely that some amount of product was formed, this could not be confirmed by analysis. Further purification would have been necessary, but purification of leuco BBDs had already proved challenging due to their poor solubility. This is a major reason for why the method developed in the previous section was so promising.

The exact reason for why this MW synthesis was unsuccessful was not explored in any significant detail. This was mainly due to the fact that at the time the more interesting information could be gained from compounds which varied much more significantly in structure, and synthesis of these compounds was therefore a priority.

4.1.4 Triaryl condensation synthesis of 4,4'-(phenylmethylene)bis(*N*-benzyl-*N*-ethylaniline)



Scheme 12. Synthesis of 4,4'-(phenylmethylene)bis(N-benzyl-N-ethylaniline).

Since it was suspected that the two sulfonic acid groups present in all tested BBDs played a significant role in the inhibition of XET activity, it was of great interest to synthesize a BBD which lacked these two functional groups. The starting material for the initial condensation reaction in which the central triphenylmethane body is created, was *N*-benzyl-*N*-ethylaniline. For the development of a synthesis of BBDs lacking sulfonic acid groups, benzaldehyde was chosen due to it being the simplest benzaldehyde. Additionally, both starting materials are liquids at rt., meaning that neat reactions would not encounter some of the problems that were encountered in the synthesis of the compound in sections 4.1.1 and 4.1.2. Due to this, neat reactions were tested first.

While both starting materials mixed with each other, no product was formed in reactions without any acid catalyst. This seemed to confirm the previous suspicion that the benzenesulfonic acid starting

material acted as the catalyst. Several acids were tested, and most of them encountered some problems. Acid solutions containing water, such as hydrochloric acid, would not mix with the two starting materials and TLC screening showed that no reaction took place.

A solid acid catalyst was tested in the form of DOWEX pellets, but these reactions were also inefficient. It seemed likely that the pellets were covered by a layer of formed product which prevented further catalysis. The compound which was considered to be product, turned green when exposed to air. This was observed with TLC, as the spot formed during a reaction would initially be colorless, only to turn green after a while. 2D TLC revealed that a green colored spot would remain in place, while a new colorless spot with the same R_f value as the initial spot would again turn green after a while. In DOWEX reactions the pellets would turn a strong green color, while analysis of the reaction mixture showed little to no formation of product. Additionally, DOWEX reactions were temperature restricted due to the degradation of the pellets at higher temperatures²⁵, in which case they would turn black.

At one point Lewis acid catalysis was attempted by using Titanium tetraisopropoxide. This reaction did not look promising, as it resulted in a strongly yellow compound which did not change color when exposed to air. TLC and HRMS [appendix] analysis appeared to confirm that no product had been formed. This path was not explored further due to the development of the method described below.

A reaction in which 1 eq. benzaldehyde was mixed with approx. 0.4 eq. hydrochloric acid in methanol solution, was one of the first reactions which looked promising. Benzaldehyde dissolved in the methanol hydrochloric acid solution, and once the other reactant was added and the mixture was immersed into a preheated oil bath, it turned green after a while. This resulted in a green oil, but both TLC and HRMS analysis indicated that there was a significant amount of starting material left. After attempts at extraction and crystallization of the oil, column chromatography seemed like the best purification option. However, on both silica and aluminum oxide TLC plates, the R_f values of the suspected product and *N*-benzyl-*N*-ethylaniline was almost identical no matter which solvents/solvent mixes were used. Only on reverse-phase C13 plates did the two separate. However, before the crude was purified by reverse-phase column chromatography, another attempt at synthesis proved more promising.

In this reaction a 0.1 M HCl solution was used, but as mentioned before, *N*-benzyl-*N*-ethylaniline is very poorly soluble in water, and in an attempt to somewhat mitigate this problem, DMSO was added into the mix. In this mix the starting material would remain as a separate layer on top of the water/DMSO phase, while in a water-only reaction the compound would be the bottom layer. The density of *N*-benzyl-*N*-ethylaniline is lower than that of the 1:1 DMSO/water mix, which turned out to

be important. The reaction was run in the microwave with rigorous stirring in order to mix the two layers as much as possible.

In many of the previous attempts at synthesis the amount of acid was usually not much more than 0.1 eq., which was also the case for this reaction. Considering that TLC showed poor conversion to product after 2 hours in the microwave, the option was either to run the reaction for much longer, or tweak something to see if the conversion would happen faster. The latter option was chosen, and some drops of concentrated HCl were added before the reaction was run for a total of 3 hours more. Here the higher density of the DMSO/water solution showed its advantage, as the starting material layer on top was almost gone, while a denser, brown compound had gathered at the bottom of the MW vial. After workup the compound was still oily, and vacuum drying, freezing with dry ice, and a combination of the two were tested in an attempt to solidify the compound. When exposed to air, the initially brown oil would turn green.

The **HRMS** spectrum clearly shows a peak with the expected m/z of product.

The **NMR** solvent was Chloroform-d. The most noticeable impurity peaks in the ^1H NMR spectrum are the multiplets at 0.8 ppm and 1.2 ppm. This is likely heptane²⁶. In the ^{13}C NMR spectrum there are 4 peaks that correspond to the ^{13}C NMR shifts of the four unique carbons in heptane²⁶. The quartet at 4.1 ppm and the singlet at 2.0 ppm likely belong to traces of ethyl acetate²⁷. The singlet at 2.1 ppm likely belongs to some unknown impurity.

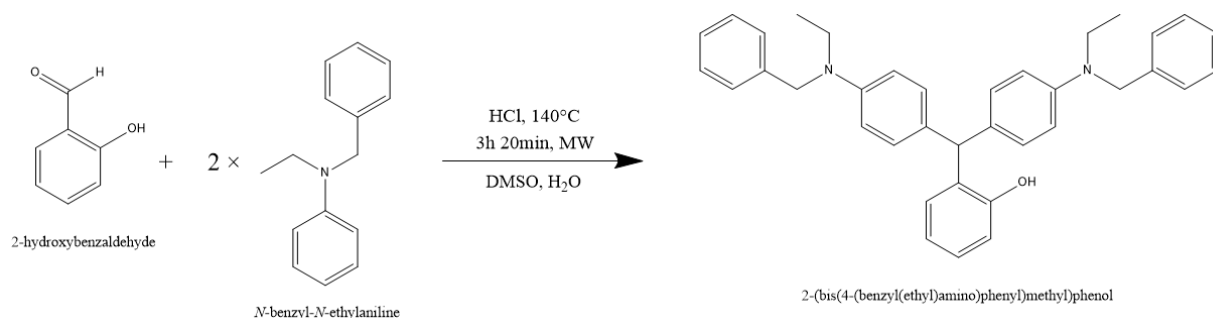
In the ^1H NMR spectrum the singlet at 5.2 ppm is a great indicator of the synthesis being successful, at this peak likely belongs to the proton at the center of the expected molecule. Using this as a reference, all non-aromatic protons are accounted for. The quartet at 3.3 ppm is closer to 5H than the expected 4H, but this is not considered a major deviation. The total size of the aromatic area is approx. 28H, which is off from the expected value of 23H. This is likely due to some impurities. Two 4H duplets in the aromatic area connect to each other. This is confirmed by the COSY spectrum. These two duplets match with the protons on the amine aromatic rings. The rest of the aromatic area is difficult to analyze.

In the ^{13}C NMR spectrum, if the four heptane peaks are ignored, the amount of visible peaks are 15, while the expected structure has 16 unique carbons. There are 4 non-aromatic peaks, which is in accordance with the expected structure. The missing peak should therefore belong to an aromatic carbon. It is likely that this peak is either too small to be observed, or that it is obscured by one of the other peaks.

Not only did it seem like almost all the starting material reacted, but even if that hadn't been the case, the rest would most likely have remained as the top layer, separated from the product. The starting material layer on top of the DMSO/water solution does therefore not only serve as an indicator for how much of it has reacted, but also aids significantly in workup and circumvents the problem of separating the starting material from the product.

This reaction was used as a basis for the synthesis of two other compounds lacking sulfonic acid groups. These are discussed in the two upcoming sections: 4.2.5 and 4.2.6.

4.1.5 Triaryl condensation synthesis of 2-(bis(4-(benzyl(ethyl)amino)phenyl)methyl)phenol



Scheme 13. Synthesis of 2-(bis(4-(benzyl(ethyl)amino)phenyl)methyl)phenol.

This synthesis was similar to the one discussed in the previous section, the main difference of note being that benzaldehyde was replaced by OH-benzaldehyde. In this case the reaction was run from the very start with a stronger HCl solution (approx. 1 pH solution), and consequently the reaction was run for a shorter amount of time. Just as with the previous reaction, the top layer of starting material disappeared during the reaction, while a denser compound gathered at the bottom. In this case the compound was dark green from the very beginning but remained oily after workup.

The expected m/z for product is clearly seen in the **HRMS** spectrum. There is also a smaller peak with a m/z of 525.2904, likely belonging to the oxidized compound.

The **NMR** solvent was DMSO- d_6 . There appear to be no impurities of note.

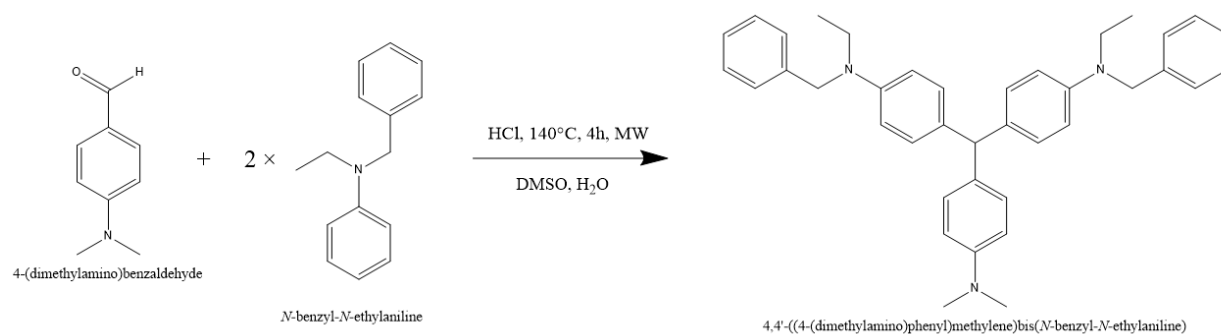
In the ^1H NMR spectrum, the singlet at 5.3 ppm that likely belongs to the central proton was used as a reference. For some reason the spectrum was somewhat unclear, and the expected 4H quartet and 6H triplet from the ethyl groups appear as singlets. The peaks in the aromatic area have the same problem. From the integrals alone, the spectrum is in accordance with the expected structure. The broad 1H singlet at 4.8 ppm likely belongs to the hydroxy-group. The aromatic area is approx. 23H,

while the expected amount of aromatic protons is 22. Since the solvent peak overlaps with the aromatic area, and since the integrals are not exact, this is hardly a noteworthy deviation.

The ^{13}C NMR spectrum also looks excellent. The amount of unique carbons in the expected structure is 18, and there are exactly 18 peaks, ignoring the solvent peak. There are four peaks outside of the aromatic area, which is in accordance with the expected structure.

While the reaction seemed like a success and worked the same way as the one discussed in the previous section, there does not exist enough analytical data to plausibly confirm that product was actually formed, nor can its purity be approximated. Further analysis and biological testing of the compound was not deemed important due to results discussed in section 4.3.

4.1.6 Triaryl condensation synthesis of 4,4'-((4-(dimethylamino)phenyl)methylene)bis(*N*-benzyl-*N*-ethylaniline)



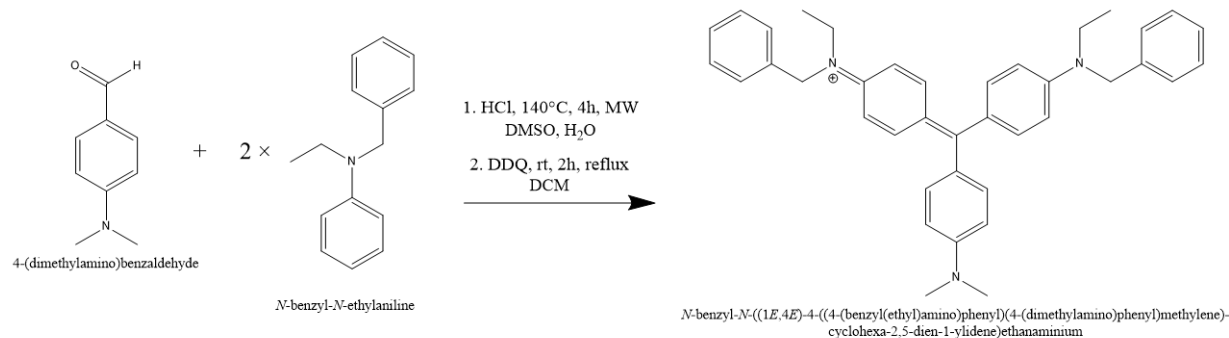
*Scheme 14. Synthesis of 4,4'-((4-(dimethylamino)phenyl)methylene)bis(N-benzyl-*N*-ethylaniline).*

This synthesis is similar to the previous two. Here the reaction time was 4 hours. The top layer consisting of starting material was used as an indicator for when the reaction was finished. Here the compound gathering at the bottom of the MW vial had a strong purple color. This one, too, remained an oil after workup.

The expected m/z peak is clearly seen in the HRMS spectrum. The m/z peak that likely belongs to the oxidized compound is also clearly visible.

As with the compound from the previous section, there is not enough analytical data to draw any plausible conclusions. Once again, a colored compound was formed, and the HRMS shows the m/z of the expected product, but without NMR data the structure can hardly be confirmed. As with the product from the previous section, this one was also abandoned.

4.1.7 Two-step synthesis of *N*-benzyl-*N*-((1*E*,4*E*)-4-((4-(benzyl(ethyl)amino)phenyl)(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)ethanaminium



Scheme 15. Synthesis of N-benzyl-*N*-((1*E*,4*E*)-4-((4-(benzyl(ethyl)amino)phenyl)(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)ethanaminium.

This was the final attempt at synthesizing a BBD lacking sulfonic acid groups. The main purpose of this synthesis was threefold: to test if the reduced compound could be oxidized with DDQ, to see if the workup of the oxidized compound circumvented many of the challenges present when purifying the reduced compounds, and to see if some property of an oxidized BBD lacking sulfonic acid groups inhibited XET activity. Oxidation would put a positive charge on the product molecule, meaning that it could potentially be dissolved in more polar solvents, opening up the possibility to separate it from any nonpolar starting materials or byproducts.

A 0.1 M sodium carbonate solution was used for DDQ quenching. Most of the steps in the workup were screened with TLC and HRMS, and analysis of the organic phase after washing with the carbonate solution showed that a spot on the TLC silica plate with the same *R_f* value as DDQ had been removed. The solid gained at the end of workup was shiny and bronze-colored, and just trace amounts gave strongly colored solutions, indicating that the oxidation had been successful.

The *m/z* of the oxidized compound can be seen in the **HRMS** spectrum. The *m/z* for starting material is not observable.

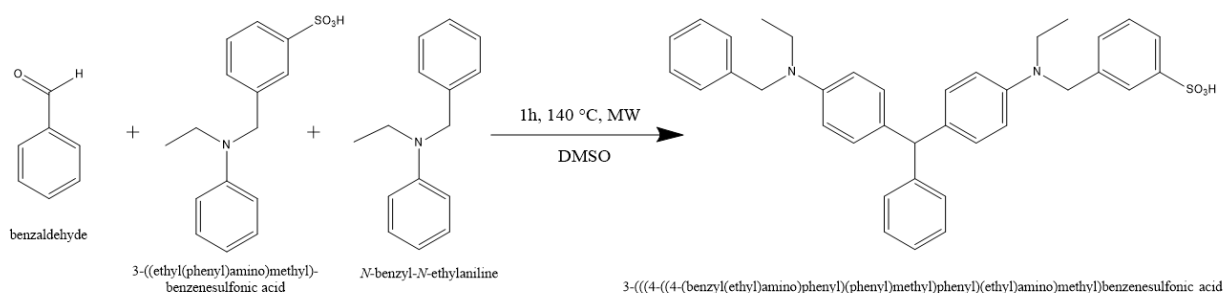
The **NMR** solvent was Chloroform-*d*. In the ¹H NMR spectrum there seems to be heptane present (peaks at 0.8 and 1.2)²⁶ in addition to many other impurity peaks.

The ¹H NMR spectrum does not look as expected. If the peak at 5.2 ppm is assumed to be the central proton in the starting material, the other peaks and their integrals match up with starting material. The integral for the aromatic area is 29H, while the total amount of aromatic protons in both starting material and oxidized product should be 22. The discrepancy is at least in part due to the large solvent peak, which overlaps with the aromatic peaks.

In an attempt to get better spectra, a small amount (less than 1 drop) of DIPEA was added to make the solution basic to avoid potential charge-related complications. This did not change the spectra in any significant way. It should however be noted that the oxidized compound does have a positive charge, and this charge may complicate matters when trying to obtain NMR spectra. However, the presence of a peak that most likely belongs to the methane proton in the triphenylmethane core, strongly indicates that the solid consists at least partly of the reduced compound. It is possible that the heptane wash did not successfully remove it, as at this point the reduced form was possibly protonated due to the initially acidic reaction conditions, and consequently could have been more soluble in DCM than heptane. The best option would either have been to wash a second time with a nonpolar solvent after washing with base, or to neutralize the reaction mix before the DDQ oxidation step.

The presence of the oxidized compound does not, however, seem unlikely, as the expected m/z is observed in the HRMS spectra, and the obtained solid did not only look significantly different than any previously obtained reduced BBDs, but also had a much stronger color in solution, comparable to the commercially bought BBDs discussed in section 4.2. Unfortunately, the structure cannot be plausibly confirmed by specter analysis.

4.1.8 Triaryl condensation synthesis of 3-(((4-((4-(benzyl(ethyl)amino)phenyl)(phenyl)-methyl)phenyl)(ethyl)amino)methyl)benzenesulfonic acid



Scheme 16. Synthesis of 3-(((4-((4-(benzyl(ethyl)amino)phenyl)(phenyl)-methyl)phenyl)(ethyl)amino)methyl)benzenesulfonic acid.

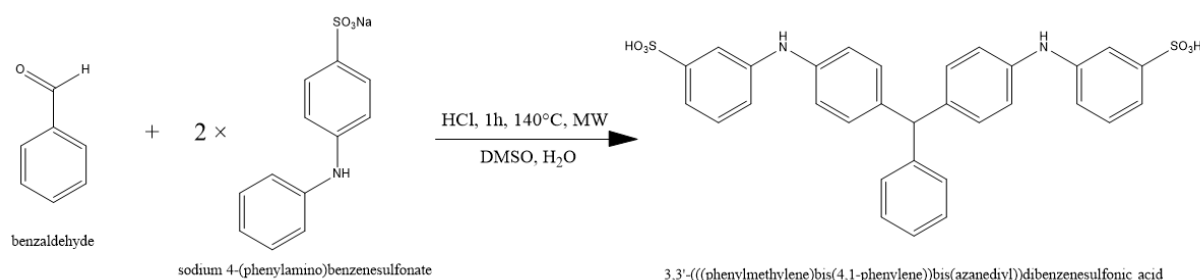
A microwave synthesis of a compound using 1 eq. of both 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid and N-benzyl-N-ethylaniline was done. This was done in hopes of synthesizing a reduced BBD with only one sulfonic acid group. This eventually resulted in small amounts of a brown solid crashing out when distilled water was added to the DMSO/water solution.

The expected m/z for product is clearly visible in the **HRMS** spectrum. The peak at approx. 290 likely belongs to benzenesulfonic acid starting material.

The **NMR** solvent was DMSO-d₆. Not much can be determined from the ¹H **NMR** spectrum. The most significant indicator of what compounds are present, are the three apparent singlets at around 5.5 ppm with integrals of approx. 1H, 1.5H and 1H from left to right. The singlet belonging to the central proton in similar compounds usually ends up in this area, which could mean that these singlets belong to three different triphenylmethane compounds. Compared to the combined singlets, the multiplet at 4.6 ppm, which would also usually be a singlet, is 4H, as expected. The multiplet at 1.1 ppm, which would usually be a triplet, is 6H, as expected. There is a strong indication that there are three compounds present.

While it seems likely that the compound with one sulfonic acid group was successfully synthesized, the obtained solid seems to be a mixture of three different compounds containing two, one and no sulfonic acid group(s). Considering that the mass of the crude was less than 100 mg, further purification was not considered. The crude was not biologically tested as the results would have been largely uninteresting due to the likely presence of the reduced BBD containing two sulfonic acid groups.

4.1.9 Triaryl condensation synthesis of 3,3'-(((phenylmethylene)bis(4,1-phenylene))bis-(azanediyl))dibenzenesulfonic acid



Scheme 17. Synthesis of 3,3'-(((phenylmethylene)bis(4,1-phenylene))bis-(azanediyl))dibenzenesulfonic acid.

As discussed in section 3.1, three different starting materials were considered for the synthesis of BBDs using the two-step pathway. The third of these was sodium 4-(phenylamino)benzenesulfonate, which differs from the benzenesulfonic acid starting material in several ways discussed in that section. This compound would also contain two sulfonic acid groups and potentially bind in the same way as the other sulfonic acid BBDs.

Initially the reaction was run without any added acid. No observable reaction took place, which was expected due to the starting material being a disodium salt. Hydrochloric acid was added as a catalyst, and the reaction was run again, resulting in a green solution. Interestingly, while some colored compound had obviously been formed, the expected m/z was not observed in the HRMS spectra. TLC

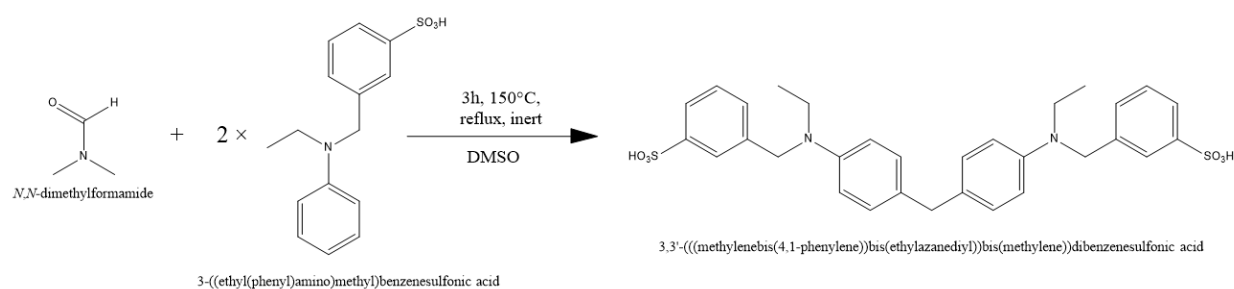
showed several spots, and it was of interest to separate the compounds to see if any of those was the product. To do this the compound was run through a silica column. While this led to the separation of the compounds initially observed in the TLC, a wide range of new spots appeared in the TLCs of the column fractions. A potential reason for this is that those compounds were present in small enough concentrations in the crude solution that was used for TLC, to be invisible. Once their concentrations were higher in the column fractions, they were visible on the TLC plates. Despite this, fractions of interest were combined, and the resulting solid was analyzed.

The largest peak in the **HRMS** spectrum corresponds to the doubly deprotonated product. There is also a peak that corresponds to the doubly deprotonated product that has been oxidized (missing one proton).

The **NMR** solvent was Methanol-d₄. The solvent peaks are not shown in their entirety due to their size. There seems to be significant amounts of ethyl acetate present [source]. Not much can be said about the ¹H **NMR** spectrum, as it looks bad. If the peak at 5.5 ppm is assumed to belong to the central proton, the aromatic area is roughly 101H, which is not remotely close to the expected 20H.

While the expected m/z was now visible in the HRMS spectra, little could be determined from the ¹H NMR spectra. A small amount of DIPEA was added to the NMR sample in an attempt to obtain better spectra, but nothing of interest changed. Considering the TLCs of the combined fractions, the best explanation for the poor NMR specter is the presence of different impurities. While there seems to be a peak that could belong to the proton in the middle of the triphenylmethane system, its integral is way too small compared to the integrals of other peaks of interest. It is likely that product was formed considering the HSMR spectra, but in small amounts.

4.1.10 Synthesis of 3,3'-(((methylenebis(4,1-phenylene))bis(ethylazanediy))bis(methylene))-dibzenesulfonic acid from 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid and *N,N*-dimethylformamide



Scheme 18. Synthesis of 3,3'-(((methylenebis(4,1-phenylene))bis(ethylazanediy))bis(methylene))-dibzenesulfonic acid.

In section 4.2.1 it was mentioned that the solubility of S1 was best in DMSO and DMF of all tested solvents. While DMSO was used as a solvent, DMF was not because it seemed like the benzenesulfonic acid reacted with the solvent. A solution of the compound dissolved in DMF turned purple over time. This was easily replicated, and heating led to a quicker formation of color. A controlled synthesis with 1 eq. of DMF was done with DMSO as the solvent.

In the **HRMS** spectrum there are two peaks that correspond to the product in which a carbon has been inserted between two equivalents of the benzenesulfonic acid. THE OTHER CALCULATED PEAK IS WRONG

The **NMR** solvent was Methanol-d4. There appears to be a multitude of impurities present. The quartet at 4.1 ppm, singlet at 2.0 ppm, and triplet at 1.2 ppm likely belong to ethyl acetate [source].

In the **¹H NMR** spectrum, the quartet is used as a reference peak. A singlet that could belong to the central protons, is approx. 1H, which is not in accordance with the expected structure. The water peak likely obscures the singlet at 4H. The expected size of the aromatic area would be 16H, while the calculated integral is 19H.

A good enough **¹³C NMR** spectrum was not obtained.

The exact mechanism by which the carbon could be inserted, is not completely understood. The most likely alternative seems to be that the starting material attacks DMF in the same manner it would attack other aldehydes. However, even if two equivalents should attack, it is uncertain how the dimethylamine leaves.

Literature research shows that it is possible for one of the amine methyl groups to be inserted between compounds²¹⁺²². However, this would need a catalyst which oxidizes the amine.

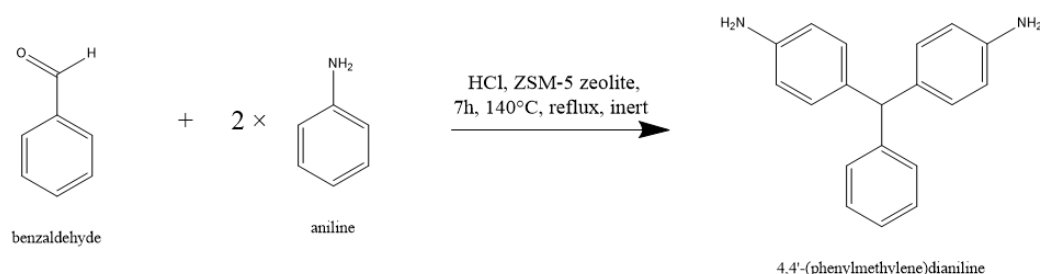
Either way, the reaction is of interest, as a lot of information can be gained from biological tests performed with a BBD lacking the third phenyl ring in the core. The structure has at minimum 6 less carbons than any of the previous BBDs, which can have a significant effect on solubility. This reaction also opens the possibility to create other similar molecules with only a carbon inserted between them.

Attempts at doing this reaction with the other starting materials for 3.1 reactions were made. The N-benzyl-N-ethylaniline reaction was done mostly in the same manner as the successful reaction described in 4.1.2, with benzaldehyde replaced with DMF. The reaction was run for a total of 3 hours. No observable reaction took place, and TLC and HRMS analysis seemed to confirm this. It is possible that the sulfonic acid groups are somehow integral to catalyzing this reaction, but this reaction alone

is not enough to support this. It is very much likely that some other factor did not work in favor of the DMF reaction.

The sodium 4-(phenylamino)benzenesulfonate reaction was done in the MW in mostly the same manner as the reaction discussed in section 4.1.9. In this case a visible reaction took place; the solution turned purple. The m/z could not be observed in the HRMS spectra, which did not exclude the fact that the desired compound was present, considering that the m/z of the desired compound in section 4.1.9 was initially elusive. However, mostly due to time restraints, further purification of was not a priority.

4.1.11 Synthesis of 4,4'-(phenylmethylene)dianiline from benzaldehyde and aniline



Scheme 19. Synthesis of 4,4'-(phenylmethylene)dianiline.

Of all the syntheses discussed so far, this proved the most challenging mainly due to the fact that benzaldehyde and aniline not only form the imine spontaneously, but also can react in several other ways, and these compounds can in turn react further. As discussed in section 3.2, the synthesis of PMDA usually seems to need a specific catalyst or unusual reaction conditions. This is the reason for why the article by Guzmán-Lucero¹⁸ was chosen as the starting point, as here the catalysator was the aniline hydrochloride salt, and the synthesis was done quickly by using microwave irradiation. However, attempts at recreating this reaction using hydrochloric acid were unsuccessful. It is mentioned in the article that less than 0.1 eq. of the catalyst results in the imine, and this is true. Unfortunately, when using hydrochloric acid instead of the aniline hydrochloric acid salt, the imine seemed to form no matter what, and the m/z of the desired compound was never observed in HRMS spectra. Appendix 8.2.1 is an example HRMS spectra from a failed HCl catalyzed MW synthesis, showing a multitude of peaks, the desired m/z of the desired product being absent.

A method from another source was adopted, in which a zeolite was used as a catalyst¹⁷. It must be noted that the role of the zeolite described in the article is questionable, as here it is thought to act a solid acid which catalyzes the formation of imine, which in turn reacts with another equivalent of aniline, leading to a rearrangement that results in PMDA. Not only does this proposed mechanism

contradict the triaryl condensation mechanism described in section 3.1, but it can be interpreted in a way in which imine formation is desired. Despite this, if the zeolite catalyzed the formation of PMDA, then its exact role as a catalyst and how the reaction mechanism works, was of secondary interest.

The exact zeolite was not available at the university. Test reactions done with a ZSM-5 zeolite looked somewhat promising, and a reaction where the zeolite was mixed with hydrochloric acid was attempted. This was the first time the m/z of the desired compound could clearly be seen in the HRMS spectra.

The most important step of purification was the neutralization of the ethyl acetate/methanol solution of the crude with 0.1 M sodium carbonate solution. When a pH value of approx. 6-7 was reached, a pale brown compound crashed out. The pH value was important, because if excessive amounts of base solution was added, the compound would dissolve again. Additionally, using HCl solution to get the pH value down, crashed out other compounds in addition to the one that crashed out initially. This was likely due to either the sodium from the base solution and chloride from the acid solution resulting in large enough amounts of sodium chloride in the mix to crash out otherwise soluble compounds, or the added water volume crashing out these compounds. Likely it could have been a combination of the two. The solution was therefore made acidic again and extracted with ethyl acetate. To a new ethyl acetate/methanol solution 0.1 M sodium carbonate was added until the pale brown compound crashed out at the same approximate pH value as before. This compound was recrystallized from toluene based on the purification of PMDAs described by Guzmán-Lucero¹⁸.

A peak corresponding to the m/z of the desired compound can be seen in the **HRMS** spectrum. The approx. 182 m/z peak likely belongs to the imine.

The **NMR** solvent was Chloroform-d. There are two singlets in the ¹H NMR spectrum that are likely impurity peaks. The peak at 6.6 ppm is approx. 4H, while the one at 2.4 ppm is approx. 2.5-3H. The latter peak likely belongs to toluene²⁸, in which case approx. 4-5H in the aromatic area should belong to toluene. ¹³C NMR also has peaks that could belong to toluene, the most noticeable at approx. 22 ppm.

Except for the impurity peaks, the ¹H **NMR** spectrum looks promising. The most important peak is the singlet at 5.4 ppm, which should belong to the central proton. The total integral of the aromatic area is approx. 20H, while the expected amount of protons is only 13. Considering that 5H is likely accounted for by toluene, and that the solvent peak overlaps with the aromatic peaks, it seems more likely that the desired compound is present. The two 4H duplets correspond to the protons on the two symmetric aromatic rings.

In the ^{13}C NMR spectrum, there are a total of 14 peaks, while the expected structure has only 9 unique carbons. However, if there is toluene present, the 5 unexpected peaks are accounted for. Ignoring the potential toluene peaks, the peak at 55 ppm should belong to the central carbon, while the remaining 8 peaks in the aromatic area correspond to the 8 unique aromatic carbons in the structure. It is not completely certain which peaks most likely belong to toluene, so all 14 peaks are listed.

While the yield was not exactly great, it seems like the expected compound was obtained, although there are some odd peaks in the ^1H NMR spectra. It is possible some impurity remains, but the obtained compound was considered good enough for any further synthesis.

It should be noted that the zeolite reaction mixtures should not be allowed to cool down without stirring, as the zeolite could turn rock hard. As a result, getting the zeolite out of some glassware proved difficult. The most effective way for cleaning was to let a mix of nitric acid and sulfuric acid mingle with the zeolite, for then to use an ultrasound bath to crush it. Only the top layer could then be mechanically removed, which meant that this had to be done several times. Finally, using glass filters for the filtration of these zeolite mixtures is not recommended, as the zeolite can not only clog the filters, but also make it almost impossible to clean them.

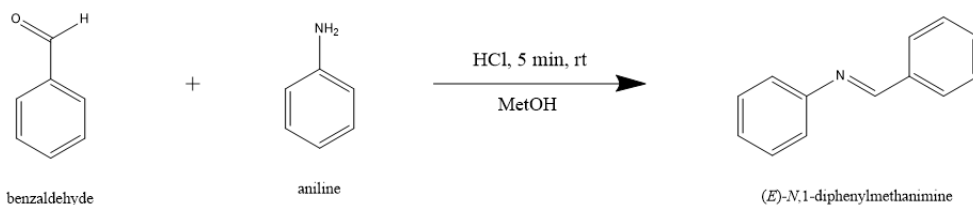
A reaction with zeolite that was run in the microwave, was run at 250 °C. Interestingly, the HRMS spectra showed an m/z corresponding to the compound shown in Figure x in addition to imine. This reaction was duplicated with and without the zeolite with 1.5 eq. benzaldehyde, 1 eq. aniline and a couple of drops of acetic acid. In the reaction without zeolite, the peak could not be observed. It is possible that the imine was formed first, and due to the high temperature, two equivalents of the imine reacted fast enough with benzaldehyde to form the triphenylmethane center, a step which possibly was catalyzed by the zeolite. Exploring this reaction further could have been of interest, as this could be a potential one-pot synthesis of compounds like Compound 4 in Scheme 5. It might also be possible that zeolite can catalyze the triphenyl condensation of the reactions described in 3.1, increasing yields. However, this is all speculation, as it is entirely possible that the m/z observed in the HRMS spectra belonged to something entirely different than the proposed compound.

Attempts at synthesizing a PMDA with a substituent group were made to little success. N-benzaldehyde was chosen for these attempts. In the final attempt at synthesis the benzaldehyde solution was added very slowly to a concentrated aniline solution with zeolite in hopes of it reacting with two equivalents of aniline to form the desired product. However, only imine was isolated from this reaction (8.2.3)

The synthesized PMDA was reacted with 3-nitrobenzaldehyde under acidic conditions. While the disubstituted product was observed in the HRMS spectrum (8.2.4), the compound eventually

decomposed due to too acidic conditions. At this point, the MDA pathway was prioritized, as it would allow for better understanding reactions of this type without the risk of losing synthesized PMDA in the process.

4.1.12 Synthesis of (*E*)-*N*,1-diphenylmethanimine



*Scheme 20. Synthesis of (*E*)-*N*,1-diphenylmethanimine.*

When finding a way to synthesize PMDA, a simple synthesis of the benzaldehyde and aniline imine was developed. Several different attempts at synthesis were made, but the most efficient one was with a concentrated methanol solution to which some drops of HCl was added. No heating was required.

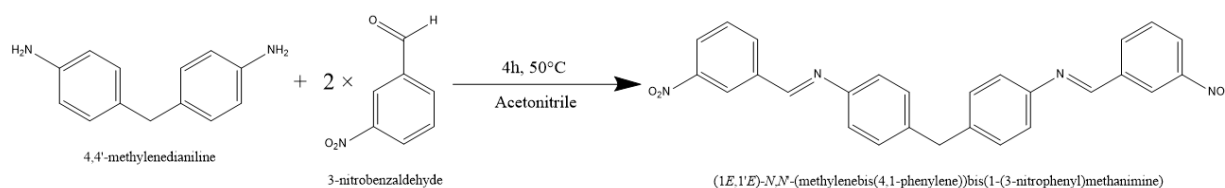
A peak with the *m/z* value of the desired compound is clearly visible in the **HRMS** spectrum.

The **NMR** solvent was Chloroform-*d*. Aside from some minor impurities, ¹H NMR the spectrum looks as expected with only small amounts of impurities present.

During the earlier attempts at synthesis, a method for purifying an imine crude was developed by recrystallization from water/methanol. Recrystallization proved surprisingly difficult due to the compound's low melting point, and due to it readily dissolving in all tested solvents, while being insoluble or near insoluble in water. Recrystallization from acidic water was attempted, but this resulted in the degradation of the imine. In the case of the reaction described, the purity was acceptable based on the ¹H NMR spectra.

The imine was used further for reactions which were unsuccessful. An attempt was made at reacting the imine with benzaldehyde in a triphenyl condensation reaction but proved unsuccessful. Additionally, the imine was mixed with 1.5 eq. of aniline, zeolite and HCl to see if this would result in PMDA, which should have been the case based on the mechanism proposed by Alinezhad¹⁷. However, no visible reaction took place even when the reaction was run overnight, and quick analysis showed only unreacted imine.

4.1.13 Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine) from 4,4'-methylenedianiline and 3-nitrobenzaldehyde



*Scheme 21. Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine).*

3-nitrobenzaldehyde was the first benzaldehyde tested with MDA. To promote the formation of disubstituted compound, a 1 eq. solution of MDA was gradually added to a solution of excess benzaldehyde (3 eq.).

While an acid catalyst was initially considered, the first test reaction was run without any. Imine formation seemed to be spontaneous without any catalyst based on the immediate formation of yellow compound when the two reactants were mixed. While methanol was initially used as a solvent, it was replaced by acetonitrile, which seemed to dissolve both starting materials and the monosubstituted compound. The monosubstituted compound crashing out was unfavorable, as it would have a harder time reacting further to create the disubstituted compound. On the other hand, the disubstituted compound crashing out would be ideal, as this would make workup significantly easier and at least partly prevent the compound from reacting further or going back to monosubstituted compound. Considering this, acetonitrile seemed like the perfect solvent, as the disubstituted product eventually crashed out. Not much experimenting with temperature was done. Heating of the solution was partly done to increase solubility during the reaction to avoid anything crashing out too early. It is very likely that the reaction time is very short, as just mixing the starting materials leads to a visible reaction, but there didn't seem to be any disadvantages to running the reaction for longer. It seemed unlikely that the benzaldehyde and MDA could react in any unexpected ways, especially when the temperature wasn't that high.

The expected *m/z* value for disubstituted product can clearly be seen in the **HRMS** spectrum.

The **NMR** solvent was DMSO-*d*₆. In addition to the DMSO and water peaks in the ¹H NMR spectrum, there is a peak at 2.2 ppm, which most likely does not belong to the compound. This peak likely stems from washing acetone from the NMR tube²⁹. The peak at approx. 32 ppm in the ¹³C NMR spectrum also likely belongs to acetone.

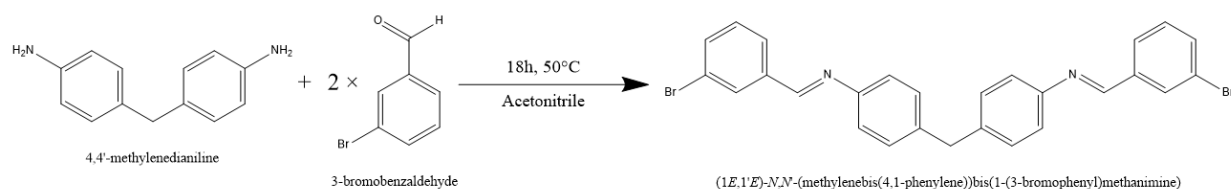
In the ¹H NMR spectrum, there is a singlet at 4.0 ppm that should belong to the two protons at the center of the molecule, and was used as a reference. The integrals total to 20 protons, which is in

accordance with the expected product. One of the 2H singlets in the aromatic area likely belongs to the imine carbon protons.

In the ^{13}C NMR spectrum, excluding the solvent peak and the peak likely belonging to acetone at 31 ppm, there are 11 peaks, while the expected product has 12 unique carbons. The central carbon appears to be missing. Considering the carbon spectra of similar compounds (discussed in the upcoming sections), it seems likely that the solvent peak at 40 ppm eclipses the missing peak.

Now, as the synthesis of a disubstituted compound had been a success, a variety of disubstituted MDA and benzaldehyde products were synthesized. These will be briefly discussed in sections 4.2.16-4.2.23.

4.1.14 Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-bromophenyl)-methanimine) from 4,4'-methylenedianiline and 3-bromobenzaldehyde



*Scheme 22. Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-bromophenyl)-methanimine).*

For MDA reactions, immediately available benzaldehydes were tested based on how potentially interesting their substituent groups were. 3-bromobenzaldehyde seemed like an interesting candidate due to the bromine being in the meta-position, the same position as the sulfonic acid groups in BBDs. While it seemed doubtful that the product would inhibit XET activity, the bromines could possibly act as leaving groups in nucleophilic aromatic substitution reactions, opening up the possibility to replace them with other functional groups.

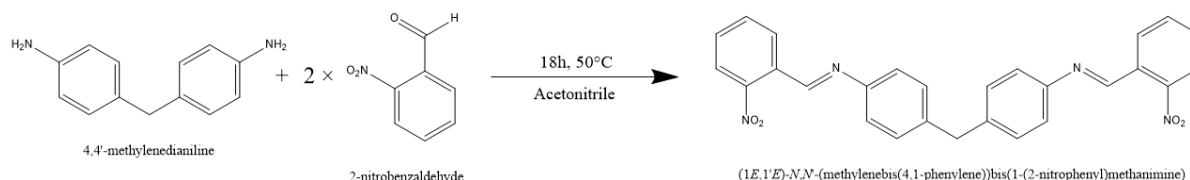
In the HRMS spectrum, *m/z* values corresponding to both mono- and disubstituted compounds can be seen. The peak at approx. 199 likely belongs to starting material.

The NMR solvent was Chloroform-*d*. Small traces of starting materials seem to be present.

In the ^1H NMR spectrum, with the singlet at 4.0 ppm as reference, the integrals make perfect sense. The only deviation is the multiplet at 7.2 ppm being 5H instead of 4H, but this is because of the solvent peak overlapping with the expected doublet. The singlet for the imine proton is present, so is the expected meta-pattern for the nitro-rings, and the expected para-pattern for the amine-rings.

All 12 unique carbons are accounted for in the ^{13}C NMR spectrum, with the central carbon having a chemical shift of 41 ppm.

4.1.15 Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(2-nitrophenyl)-methanimine) from 4,4'-methylenedianiline and 2-nitrobenzaldehyde



*Scheme 23. Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(2-nitrophenyl)-methanimine).*

If the disubstituted nitro-compound from section 4.1.13 or its reduced version ended up looking promising as an XET inhibitor, it could be of interest to study the effects the positions of the nitro-groups have an inhibition. Therefore, 2-nitrobenzaldehyde was chosen.

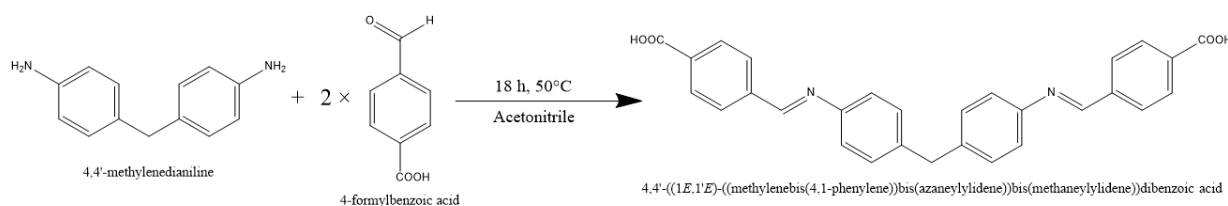
In the HRMS spectrum the expected m/z for product is seen.

The NMR solvent was Chloroform- d .

Using the 4.0 ppm singlet in the ^1H NMR spectrum as reference, the integrals match perfectly with the exception of the 9H multiplet, which overlaps with the solvent peak. This multiplet is likely two doublets, both at 4H, that couple with each other. Otherwise, the imine protons are accounted for, as are the protons in the ortho-substituted rings, which couple in a quite nice pattern.

All 12 unique carbons are accounted for in the ^{13}C NMR spectrum, with the central carbon having a chemical shift of 41 ppm.

4.1.16 Synthesis of 4,4'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis-(methaneylylidene)dibenzoic acid from 4,4'-methylenedianiline and 4-formylbenzoic acid



*Scheme 24. Synthesis of 4,4'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis-(methaneylylidene)dibenzoic acid.*

Carboxylic acid groups would be interesting due to them not being too dissimilar from sulfonic acid groups, potentially being able to bind in the same manner to inhibit XET activity. Since the benzaldehyde with the substituent group in the meta-position was not available, the para-benzaldehyde was chosen.

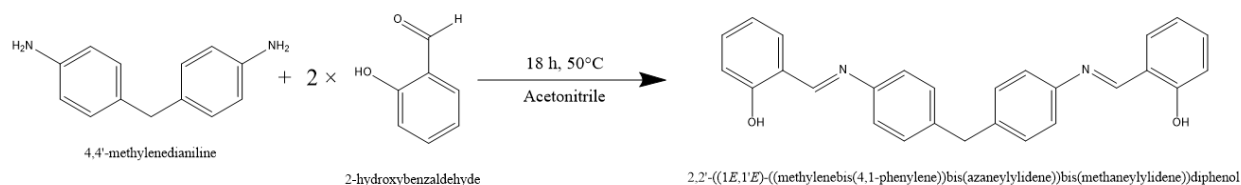
In the **HRMS** spectrum, peaks corresponding to mono- and disubstituted compounds can be seen.

The **NMR** solvent was DMSO-d₆. Unlike the other MDA products so far, this compound was poorly soluble in chloroform.

With the 4.0 ppm singlet in the ¹H **NMR** spectrum as reference, all protons are accounted for. The singlet at 8.7 should belong to the imine protons, while there are two pairs of 4H doublets that pair, which is in accordance with the two unique para-systems present in the expected product.

In the ¹³C **NMR** spectrum, only 12 unique carbons are accounted for. The central carbon peak is very likely obscured by the solvent peak at 40 ppm, considering the shift it has in similar compounds.

4.1.17 Synthesis of 2,2'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))diphenol from 4,4'-methylenedianiline and 2-hydroxybenzaldehyde



Scheme 25. Synthesis of 2,2'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))diphenol.

Of the tested benzaldehydes, this was probably the least interesting. The hydroxy-groups are able to hydrogen bond, which might be necessary for inhibition, and their small size may be an unforeseen advantage.

In the **HRMS** spectrum there are peaks corresponding to both mono- and disubstituted compound.

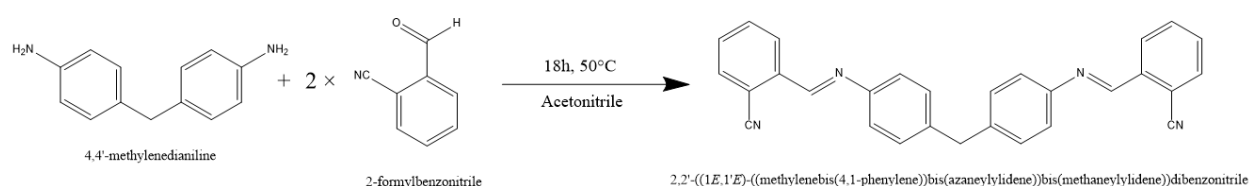
The **NMR** solvent was Chloroform-d.

Using the peak at 4.0 ppm in the ¹H **NMR** spectrum as reference, all protons are accounted for. The 10H multiplet at 7.2 ppm is larger than expected due to the overlapping solvent peak. This multiplet is likely the two doublets stemming from the protons in the para-substituted rings, totaling 8H. The broad approx. 2H peak at 13.2 ppm likely belongs to the two hydroxy-protons. From the COSY

spectrum, it looks like the peaks at 7.0 and 6.9 ppm couple with the multiplet at 7.3 ppm, which likely consists of two types of unique protons. These peaks should correspond to the protons in the ortho-substituted rings.

In the ^{13}C NMR spectrum there is a total of 13 peaks contra the expected 12 unique carbons in the expected structure. It is uncertain where this extra peak stems from. The central carbon has a shift of 41 ppm. All the other peaks lie between 165 and 100 ppm.

4.1.18 Synthesis of 2,2'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))dibenzonitrile from 4,4'-methylenedianiline and 2-formylbenzonitrile



Scheme 26. Synthesis of 2,2'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))dibenzonitrile.

Cyano-groups were considered potentially interesting in a large part because the groups can be modified, widening the range of compounds that can be synthesized.

The product did not crash out during or after the reaction, which lead to a more complicated workup procedure. It was thought likely that the crude remained oily after solvent was evaporated due to remaining water. In an attempt to remove the water, the crude was dissolved in toluene in hopes of creating a water-toluene azeotrope. The crude remained an oil after this. It was possible that the toluene successfully removed water, but that some toluene now remained and made the crude oily. If this was the case, the toluene could be removed by using pure methanol, as toluene and methanol create an azeotrope.

In the HRMS spectrum, peaks corresponding to both product and product + sodium are present.

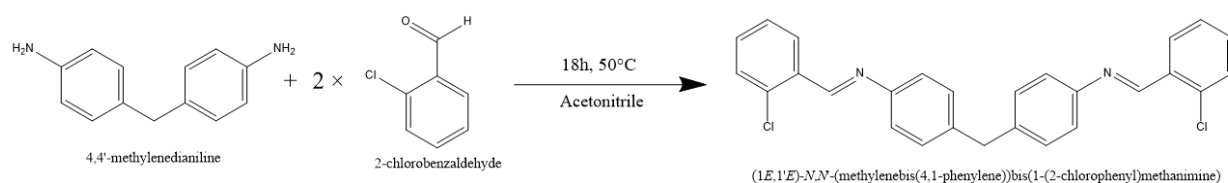
The NMR solvent was Chloroform-d. The peak at 2.0 ppm likely belongs to acetonitrile³⁰.

In the ^1H NMR spectrum, the singlet at 4.0 ppm was used as a reference. All other protons are accounted for in the aromatic area. The solvent peak overlaps with the multiplet at 7.2 ppm, hence it being 9H instead of the expected 8. This peak likely belongs to the protons on the two para-substituted rings. The imine peak is present at 8.8 ppm, while the remaining aromatic peaks account for the protons on the ortho-substituted rings.

In the ^{13}C NMR spectrum, there is a total 13 peaks, while the expected amount is 12. The 117 ppm peak could likely belong to acetonitrile³⁰.

It is uncertain why the yield of this reaction was so poor. It is possible that since the product did not crash out during the reaction, it was able to decompose. Or, possibly, some property of the cyano-group makes it react poorly. The most likely possibility is that a lot was lost during recrystallization.

4.1.19 Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(2-chlorophenyl)methanimine) from 4,4'-methylenedianiline and 2-chlorobenzaldehyde



*Scheme 27. Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(2-chlorophenyl)methanimine).*

The 2-chlorobenzaldehyde was interesting for the same reason as the 3-bromobenzaldehyde (4.2.16). The chlorines open up the possibility for nucleophilic aromatic substitution.

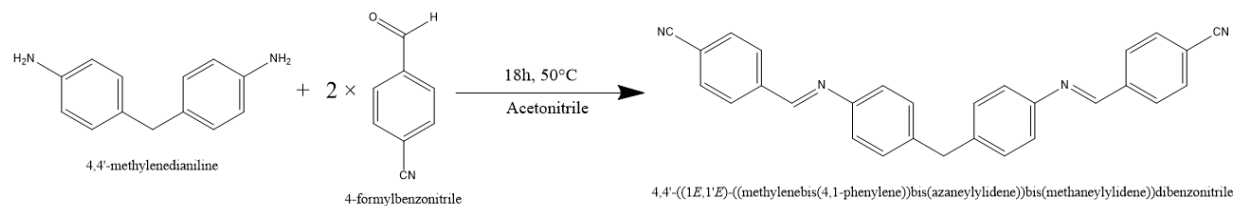
In the HRMS spectrum, a peak corresponding to the product can be seen.

The NMR solvent was Chloroform-d.

In the ^1H NMR spectrum, the peak at 4.0 ppm was used as a reference. All other protons are accounted for in the aromatic area. The solvent peak overlapping with what should be two 4H doublets explains the 9H multiplet. The peak at 8.9 ppm stems from the imine protons. The remaining peaks stem from the protons on the ortho-substituted rings. 6 of the protons overlapping result in the multiplet at 7.3 ppm.

In the ^{13}C NMR spectrum there are 12 unique carbons, which is in accordance with the expected structure. The central carbon has a shift of 41 ppm.

4.1.20 Synthesis of 4,4'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))dibenzonitrile from 4,4'-methylenedianiline and 4-formylbenzonitrile



*Scheme 28. Synthesis of 4,4'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))dibenzonitrile.*

The benzaldehyde was chosen for the same reason as in section 4.2.20.

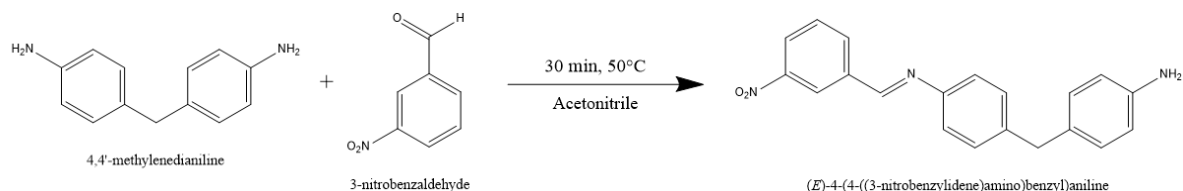
Peaks corresponding to both mono- and disubstituted compound are clearly visible in the **HRMS** spectrum. The peak at approx. 447 likely belongs to product + sodium.

The **NMR** solvent was chloroform-*d*.

In the **¹H NMR** spectrum, the singlet at 4.0 ppm was used as a reference. The rest of the protons are accounted for in the aromatic area. The peak at 8.4 belongs to the imine protons. There are two pairs of coupling 4H doublets, which is confirmed by the COSY spectrum. This is in accordance with the two different para-substituted systems present in the expected structure. The peak at 7.2 ppm is larger than expected and has an odd shape due to the overlapping solvent peak.

In the **¹³C NMR** spectrum, there are 8 peaks, meaning that 4 of the unique carbons are missing. There is a small peak at 41 ppm that should stem from the central carbon. When zooming in on the area with the other peaks, two of the missing peaks appear to have shifts of 118 and 114 ppm.

4.1.21 Synthesis of (*E*)-4-(4-((3-nitrobenzylidene)amino)benzyl)aniline from 4,4'-methylenedianiline and 3-nitrobenzaldehyde



*Scheme 29. Synthesis of (*E*)-4-(4-((3-nitrobenzylidene)amino)benzyl)aniline.*

Here, a 1 eq. solution of 3-nitrobenzaldehyde was added to a 1 eq. solution of MDA in hopes of promoting the formation of the monosubstituted compound. Initial analysis of the precipitate did not

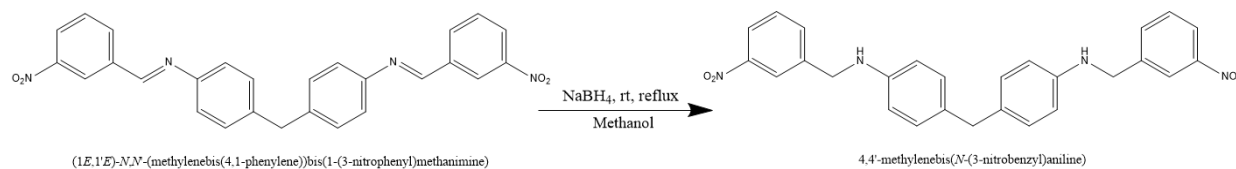
look too promising, but after recrystallization it looked like the monosubstituted compound had been successfully synthesized and isolated.

In the **HRMS** spectrum, there is a peak that corresponds to the expected monosubstituted product. The peak at 199 likely stems from starting material.

The **NMR** solvent was Chloroform-d. The small singlet at 2.0 ppm likely belongs to traces of acetone [source], and the singlet at 1.1 ppm is an indication that small traces of benzaldehyde remain. For the sake of comparison, the disubstituted product from section 4.1.13 was run in Chloroform-d (both DMSO and chloroform spectra in section 8.1.13). When comparing the spectra of this compound with those of the disubstituted compound, it is clear that the compound that was isolated here, was the disubstituted one. The ¹H NMR spectrum is clearer in chloroform, and here it possible to see the two 2H doublets that couple with the 2H triplet.

It is likely that the monosubstituted compound is ionized to a much larger degree in the HRMS. The disubstituted compound could be favored over the disubstituted due to the activation energy for the second condensation being lower than for the first.

4.1.22 Reduction of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine) to form 4,4'-methylenebis(*N*-(3-nitrobenzyl)aniline)



*Scheme 30. Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine).*

Synthesis of starting material is discussed in 4.1.13. An attempt at reducing the compound was made using sodium borohydride. The reducing agent was chosen due to it likely being able to reduce the imine without reducing the nitro groups. Borohydride was added to the reaction mix until no visible reaction took place (the formation of hydrogen gas). Additionally, TLC was used to screen the reaction, and the final TLC seemed to indicate that the starting material had been fully converted.

Peaks corresponding to both product and product + sodium are clearly visible in the **HRMS** spectrum. There is a small peak at approx. 465 that likely stems from starting material, and a small peak at approx. 467 which likely belongs to the compound in which only one double bond has been reduced.

The **NMR** solvent was DMSO-d₆. The DMSO and water peaks in the ¹H NMR spectrum are not shown in their entirety due to their size compared to the peaks of interest. The same goes for the singlet at

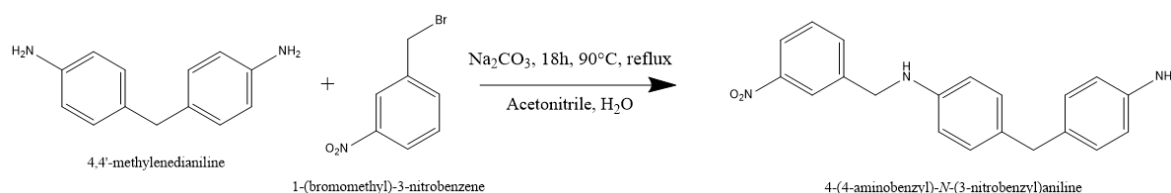
approx. 2.2 ppm, which most likely belongs to washing acetone²⁹. This is further confirmed by the peak for the carbonyl carbon in acetone being present at approx. 206 ppm in the ¹³C NMR spectrum.

The ¹H NMR spectrum appears to be nearly identical to the spectrum for the starting material, but with a variety of smaller peaks present, possible from traces of reduced compound. Using the singlet at approx. 4.1 ppm in the ¹H NMR spectrum as a reference, the integrals in the aromatic area total to almost exactly 16H, making the total amount of protons 18 instead of the desired 20. The desired compound should have a singlet at 4H, but instead the singlet at 8.7 with an integral of 2H persists. As discussed for the starting material (section x), this peak should belong to the two imine carbon protons, and serves as a great indicator for the success of the reduction. In this case, the reduction seems to have been a failure with possibly only trace amounts of reduced product present in the NMR solution.

In the ¹³C NMR spectrum, excluding the solvent peak and the peaks at approx. 206 and 31 ppm likely belonging to acetone, the same 11 peaks that were present in the starting material spectrum (appendix), are also present here.

It is possible that the small peaks present in the aromatic area belong to the reduced compound. If the reduction was unsuccessful, it is uncertain why the borohydride stopped reacting, and why TLC seemed to show good conversion. It might be possible that the methanol used for TLC and MS dissolved only the reduced compound, though if remaining starting material did not dissolve, this would likely have been observed. Conversely, if the reduced compound did not dissolve in the NMR solvent, this would also have been observed. The NMR solvent was DMSO-d₆, so it seems likely that everything dissolved in the NMR sample. To err on the side of caution, it would be safest to assume that the NMR spectra are more representative of the actual results.

4.1.23 Synthesis of 4-(4-aminobenzyl)-N-(3-nitrobenzyl)aniline from 4,4'-methylenedianiline and 1-(bromomethyl)-3-nitrobenzene



Scheme 31. Synthesis of 4-(4-aminobenzyl)-N-(3-nitrobenzyl)aniline.

As described in section 3.2, both PMDA and MDA can react with benzyl halides in addition to benzaldehydes. The benzyl halide reaction has the advantage of skipping directly to the double amine, instead of having to first synthesize the double imine, for then to reduce both imine double bonds. In

order to synthesize compounds with nitro-groups in the meta position(s), 1-(bromomethyl)-3-nitrobenzene was reacted with MDA in an S_N2 substitution reaction.

In order to favor the formation of the monosubstituted compound, the benzyl bromide was added dropwise to a fairly concentrated solution of MDA. Approx. 1/3 of the total crude mix was used to test different workup procedures with little success. Eventually, column chromatography seemed like the safest option, and it appears both the disubstituted and monosubstituted compounds were successfully separated. In order to get the monosubstituted compound as a solid, the same azeotrope-based procedure that was described in section 4.1.18 was utilized, only with benzene instead of toluene. Benzene is known to create an azeotrope with water. Only a small amount of benzene was used, and exposure was minimized as much as possible.

In the **HRMS** spectrum for the sample thought to contain **monosubstituted** product, clear peaks corresponding to both product and product + sodium can be seen. Small peaks corresponding to disubstituted compound (approx. 469) and disubstituted compound + sodium (approx. 491) are also present.

The **NMR** solvent for the **monosubstituted** compound was Chloroform-d.

The singlet at 4.4 ppm in the ^1H **NMR** spectrum was used as a reference. There being two H2 singlets at 4.4 and 3.7 ppm is a strong indicator of this being the monosubstituted compound. Additionally, excluding the solvent peak at 7.2 ppm, the aromatic area accounts perfectly for the remaining 12 protons. The four 1H peaks account for the protons in the meta-substituted ring with a coupling pattern matching the substitution. The two unique para-substituted rings are accounted for what appears to be two pairs of doublets that couple with each other, with two of the doublets overlapping to form what looks like a triplet. The COSY spectrum confirms the coupling pattern.

In the ^{13}C **NMR** spectrum there appears to be 15 peaks, meaning that one peak is missing. The non-aromatic carbons are accounted for by the 48 and 40 ppm peaks, so one of the aromatic peaks appears to be missing. This might be due to the peak being too small to observe, or due to it overlapping with one of the other peaks.

In the **HRMS** spectrum for what was thought to be **disubstituted** compound, there are clear peaks corresponding to both the compound and compound + sodium, the latter peak being significantly larger. It is possible that the peak at approx. 626 belongs to a possible trisubstituted compound + sodium.

The **NMR** solvent for the **disubstituted** compound was Chloroform-d.

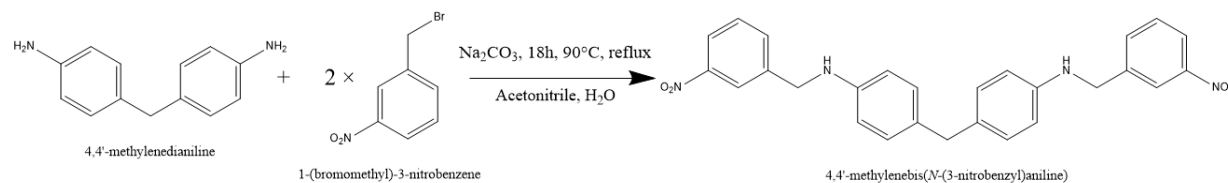
The singlet at 4.4 ppm in the $^1\text{H NMR}$ spectrum was used as a reference. There being two H2 singlets at 4.4 and 3.7 ppm is a strong indicator of this being the monosubstituted compound. Additionally, excluding the solvent peak at 7.2 ppm, the aromatic area accounts perfectly for the remaining 12 protons. The four 1H peaks account for the protons in the meta-substituted ring with a coupling pattern matching the substitution. The two unique para-substituted rings are accounted for what appears to be two pairs of doublets that couple with each other, with two of the doublets overlapping to form what looks like a triplet. The COSY spectrum confirms the coupling pattern.

In the $^{13}\text{C NMR}$ spectrum there appears to be 15 peaks, meaning that one peak is missing. The non-aromatic carbons are accounted for by the 48 and 40 ppm peaks, so one of the aromatic peaks appears to be missing. This might be due to the peak being too small to observe, or due to it overlapping with one of the other peaks.

As both the monosubstituted and disubstituted compounds were isolated, the reaction was considered a success despite the somewhat low yields. There was enough of both compounds for biological testing, meaning that a synthesis of disubstituted compound was not necessary. It could be beneficial to remove the base to push the reaction further towards monosubstituted compound, since the base deprotonates the monosubstituted compound after nucleophilic attack, making it more prone to further substitution.

An attempted synthesis of the disubstituted compound was done in parallel with the synthesis of the monosubstituted compound. This synthesis is presented in the next section, although it was of little interest after the disubstituted compound had already been successfully isolated.

4.1.24 Synthesis of 4,4'-methylenebis(*N*-(3-nitrobenzyl)aniline) from 4,4'-methylenedianiline and 1-(bromomethyl)-3-nitrobenzene



Scheme 32. Synthesis of 4,4'-methylenebis(N-(3-nitrobenzyl)aniline).

This reaction is mostly similar to the one discussed in the previous section, only that the aim was to synthesize the disubstituted compound. HRMS analysis done on the crude mixture looked promising (appendices), and TLC analysis showed only two spots, which corresponded with the earlier obtained

R_f values for mono- and disubstituted compounds. Therefore, it seems likely that both compounds could have been isolated from this reaction, too. This was not done in large part due to time restraints.

The **HRMS** spectrum of the crude is interesting. The expected peaks for both mono- and disubstituted compound are present, but the peaks with higher m/z values are also interesting. The peaks at approx. 604 and 626 could certainly belong to trisubstituted compound, and the peak at approx. 761 corresponds to the expected m/z for MDA which has reacted with four equivalents of the benzyl bromide. Considering the TLC analysis, it is likely these compounds are present in very small amounts, but are easily ionized in the MS.

Both reactions show the promise of the benzyl halide pathway. Being able to skip the reduction of the double imine might be important, as this step can potentially be restricting. Additionally, the synthesis of tertiary amine compounds can be of interest.

4.2 Biological testing of commercially available Coomassie Brilliant Blue derivatives

In addition to Brilliant Blue R250 and Brilliant Blue G250, three commercially available BBDs, Guinea Green B, Brilliant Blue FCF and Coomassie Violet R200, were biologically tested using the methods described in sections 1.4.1 and 1.4.2. Cas-101-11-1 is 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid (starting material for the two-step synthesis of BBDs containing sulfonic acid groups) was also tested for its ability to inhibit XET activity. Due to the compound being colorless its ability to bind proteins could not be tested. The general protein binding ability of the compounds is shown in Figure 4. The results of the XET tests are shown in Figure 5.

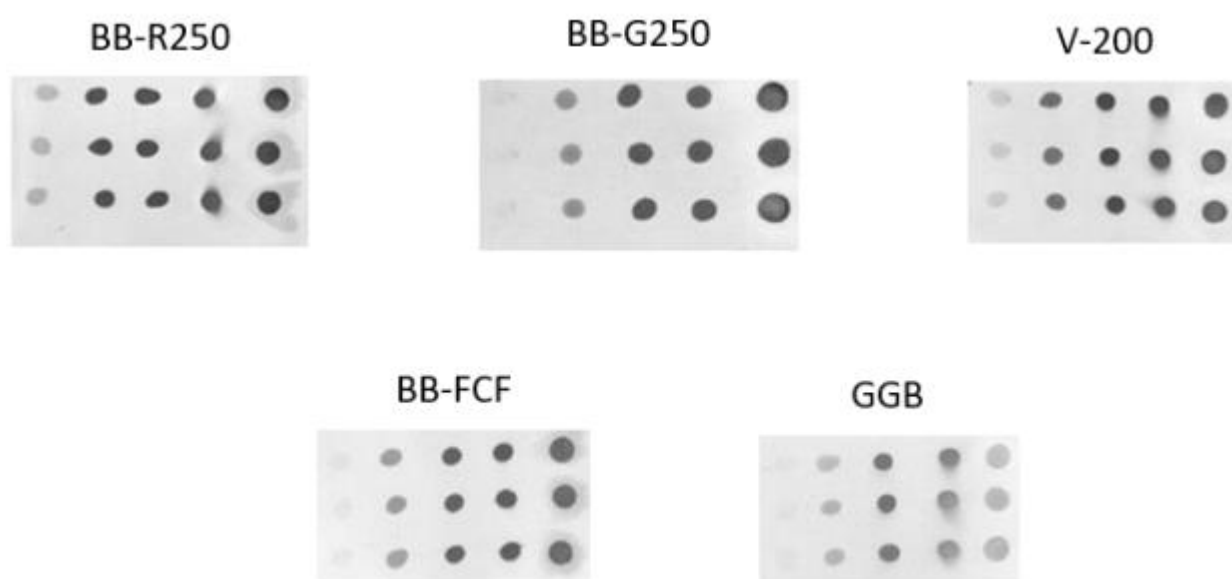


Figure 4. Protein binding results for commercially available BBDs.

As seen in Figure 4, all tested BBDs bound to proteins, and from Figure 5 it appears that these compounds also inhibit XET activity, though Coomassie Violet R200 appears to be a clearly weaker inhibitor than the other BBDs. x seems to have no or negligible inhibiting ability.

It appears that some similarity/similarities in the structures of the BBDs make them inhibit XET activity. GGB, BB-FCF and V-200 all lack the large substituent group present in BB-R250 and -G250, which indicates that this part of the molecule is not necessary for activity. V-200 does not inhibit to the same degree as the other compounds, which can likely be due to the substituent group on the third phenyl ring in the TPM core of the molecule. In V-200 this is a sulfonic acid group, which is significant considering that the XET inhibition may in part be caused by the two sulfonic acid groups present in all tested BBDs. V-200 may therefore bind differently to the relevant part of the enzyme or bind to a completely different part of the enzyme. The electron withdrawing effect of the sulfonic acid group can also be a reason for why V-200 does not inhibit to the same degree as the other BBDs considering

that BB-R250, -G250 and -FCF all have electron donating groups in the para-position. GGB, however, does not have any substituent groups on this phenyl ring, which indicates that substituents are not required for BBDs to inhibit XET activity. However, substituents may have positive effects on inhibition, or conversely – what seems to be the case with V-200 – negatively interfere with it.

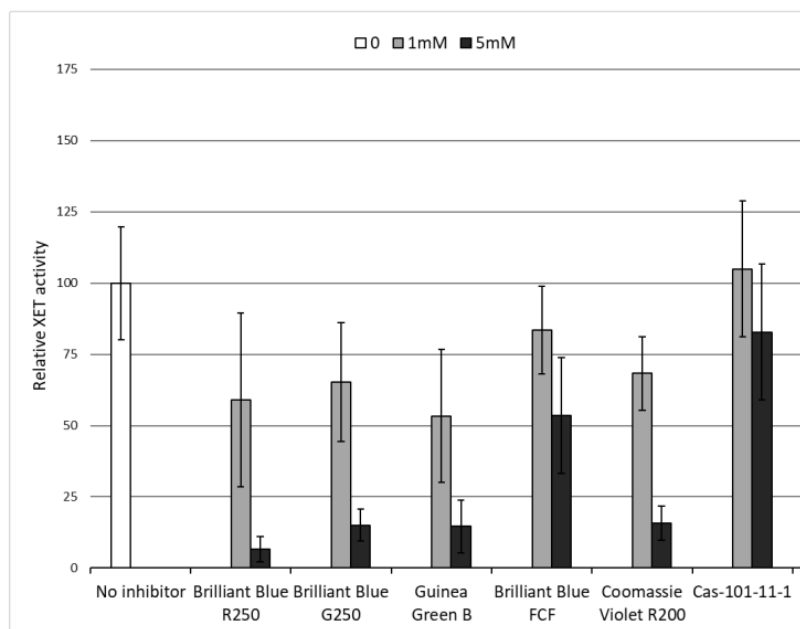


Figure 5. XET inhibition results for commercially available BBDs and benzenesulfonic starting material (Cas-101-11-1).

The main reason for testing the benzenesulfonic starting material was to avoid potential false positives. If the compound did inhibit XET activity, traces of it could result in false positive results when testing synthesized BBDs. While the compound does not appear to inhibit XET activity by itself, and while this is reassuring when testing compounds synthesized from it, its effects on XET inhibition can

never be ruled out entirely. Traces of the benzenesulfonic acid starting material in a test compound solution may still interact with both the compound and enzymes in unforeseen ways and affect results.

As for the testing of the BBDs, it should be noted that the purities of the dyes vary significantly. For example, the dye content of Guinea Green B was listed as 50 %. There was no information to be gained on the other 50 %, but it was assumed that this was mostly the reduced form of the compound. Any impurities present in the commercially bought compounds may affect the results presented in this section.

4.3 Biological testing of synthesized compounds

All synthesized compounds which were biologically tested are shown in Figure 6. For some of the compounds the purity can be poor, and in some cases, it could not be entirely confirmed that the target molecule had been successfully synthesized. The results from biological screening of the compounds are presented in Figure 7 (protein binding) and Figure 8 (XET inhibition).

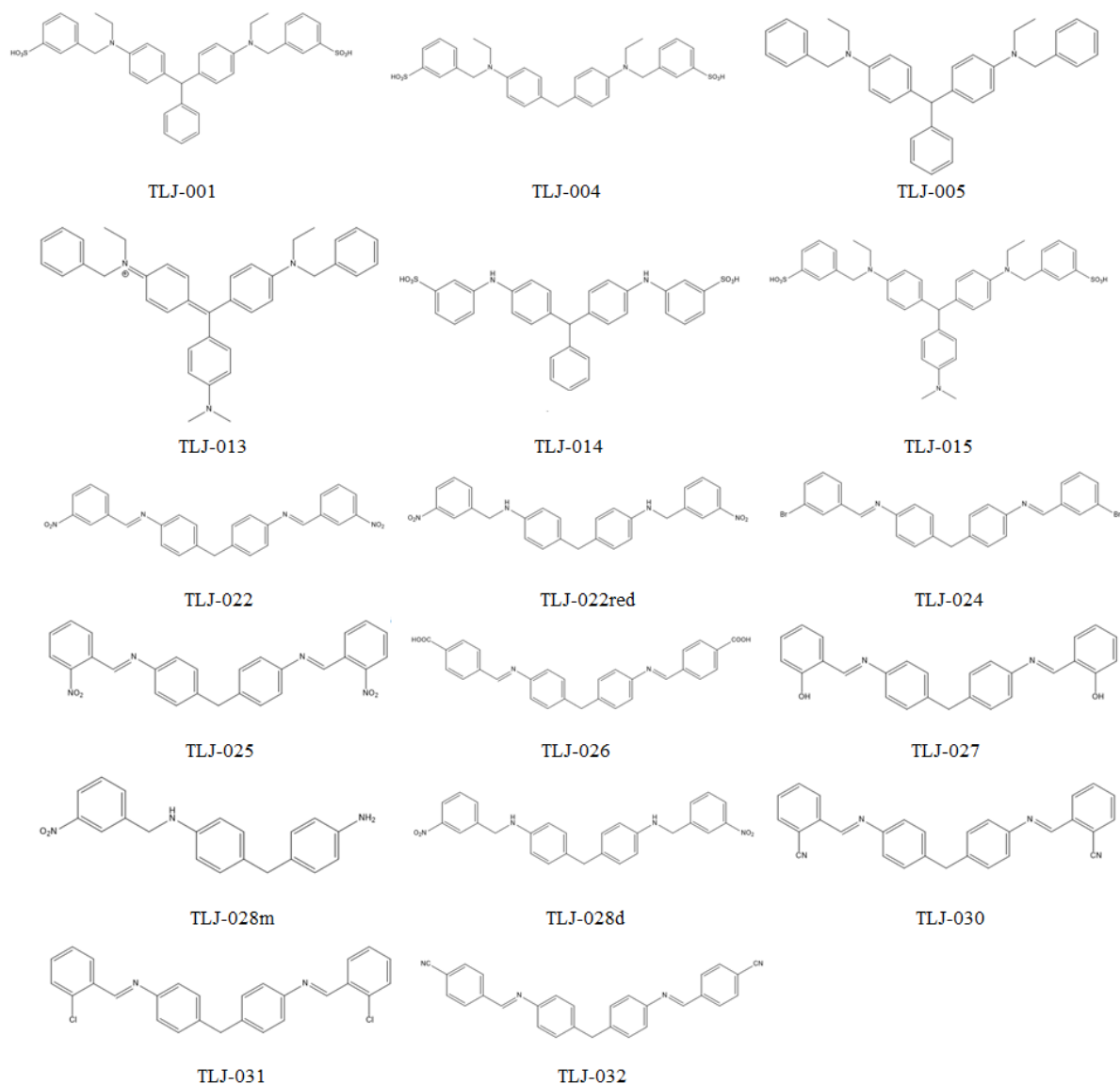


Figure 6. Synthesized compounds that were screened for biological activity.

The protein binding of only 6 of the compounds (TLJ-001, -004, -005, -013, -014, -015) was tested due to the method being dependent on the compounds having strong colors in solution. Of these compounds, only the two lacking the sulfonic acid groups (TLJ-005 and -013) did not bind proteins. This is not surprising, since it was a suspicion since the beginning that the sulfonic acid groups were integral to protein binding.

When discussing XET inhibition, the test solutions in question are the 5 mM ones unless specified otherwise, as in most cases the 1 mM solutions displayed little to no inhibition.

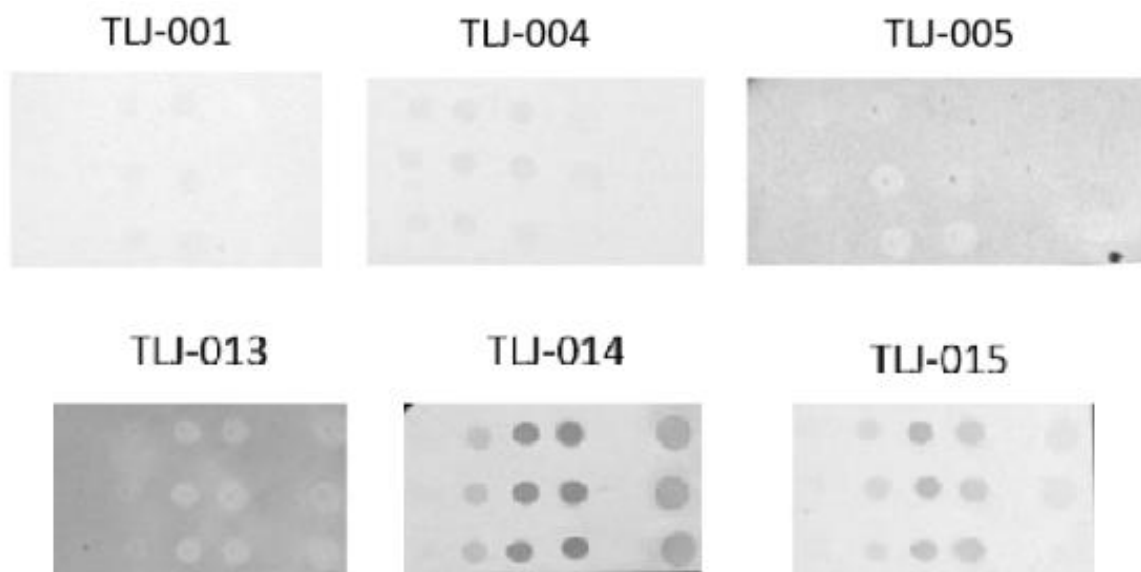


Figure 7. Protein binding results for TLJ-001, -004, -005, -013, -014 and -015.

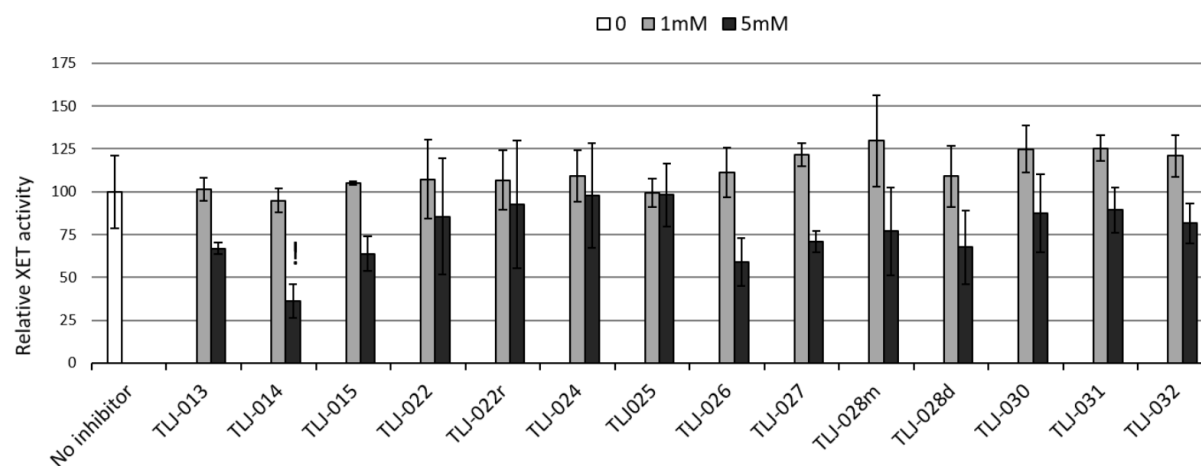


Figure 9. XET inhibition results for the remaining TLJ-compounds.

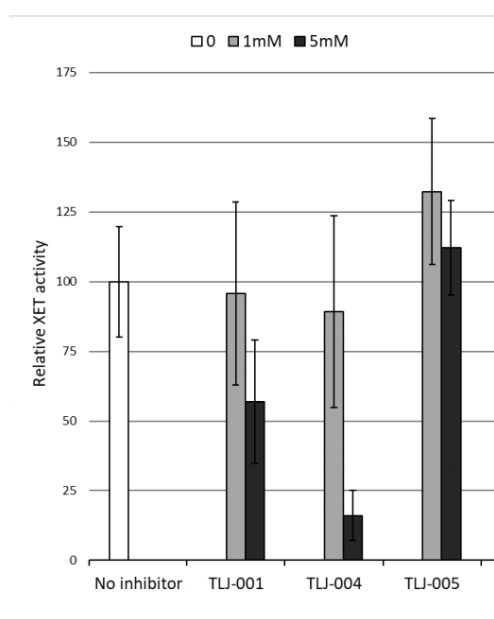


Figure 8. XET inhibition results for compounds TLJ-001, -004 and -005.

TLJ-001 is the reduced version of Guinea Green B, which also was tested (Figures 4 and 5, section 4.2). Considering that TLJ-001 lacks the conjugated system, but still inhibits to a decent degree, oxidizing the leuco compounds does not seem essential for XET inhibition. It is important to note that dissolving several of the compounds in the solvents used for the XET inhibition tests proved difficult, and it is likely that the actual concentrations of the 5 mM samples are lower than displayed in the figures. This was the case for TLJ-001. The TLJ-001 solution likely had a lower concentration than the GGB solution, meaning that the results cannot be directly compared. Considering this, the inhibiting activity of reduced GGB can be comparable or possibly even better than that GGB,

but the poorer solubility means that in praxis it is not as viable.

The other promising compound, TLJ-004, is the product formed from reacting DMF with 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid (4.1.10). While its exact structure could not be confirmed, it seemed likely that carbon had been inserted between two equivalents of the benzenesulfonic acid, most likely in the para-positions. Of the tested compounds, this one seemed to have the greatest inhibiting ability, which was interesting. If the compound had the expected diphenylmethane center instead of the triphenylmethane center present in all tested BBDs, it would appear that the third phenyl ring in the center complex is extraneous. This is possibly extremely advantageous, as removal of this third phenyl ring could increase solubility in polar solvents. It also narrows down which parts of the molecule are necessary for XET inhibition.

TLJ-005 did not bind proteins nor inhibit XET activity. It should be noted that this nonpolar molecule had to be dissolved in a different solvent than other samples. Considering that the target molecule is reduced GGB (TLJ-001), but without the two sulfonic acid groups, it seems undisputable that the sulfonic acid groups play an integral role.

TLJ-013 is the only tested compound with a conjugated triphenylmethane system. It is also, in addition to TLJ-014, the only compound which dissolved to a concentration of 50 mM in methanol. This compound lacks sulfonic acid groups and does not bind proteins but appears to inhibit XET activity to some degree (approx. 30% inhibition). Since the structure could not be exactly confirmed, it is possible

that the inhibition stems from some unknown impurities. However, it could also be possible that the inhibition either stems from some property of the conjugated system, or even the dimethylamine-group. The XET inhibition does seem to be in opposition with the negative protein binding results, so the observed inhibition may just be an unexpected error.

Interestingly, TLJ-014, which is the only tested compound synthesized from sodium 4-(phenylamino)benzenesulfonate, appeared to inhibit XET activity to a decent degree. The compound did not however wash off the XET test papers or control paper, leaving a light blue spot that resulted in a reduced fluorescence signal. Therefore, it seems likely that this was, at least partly, the reason for the observed reduction in XET activity. In addition to the fact that the structure of the compound and its purity could not be plausibly determined, no conclusions can be drawn. This does not mean that the synthesis of other similar structures is not of interest.

TLJ-015 did not inhibit XET activity close to the same degree as TLJ-001, although the structures are pretty similar. The purity of TLJ-015 could not be plausibly confirmed by analysis, although the expected m/z being present in the HRMS spectra was a strong indicator of the target molecule being present. It is likely that the observed reduction of XET activity stems from the expected compound, but the effect is not larger due to impurities. The compound did also dissolve poorly in methanol, so the concentration is lower than 5 mM.

Of the remaining compounds, only TLJ-026, -027, -028m (monosubstituted) and -028d (disubstituted) reduced XET activity to approx. 75% or below. Of these, TLJ-026 was most effective, reducing activity to approx. 60%.

TLJ-028d was the reduced version of TLJ-022, which at best had dubious effect on XET activity. The difference in inhibition is certainly interesting, as it seems very likely that the flexibility the double amine compound has contra the double imine compound, can aid the compound in binding to the enzymes. Interestingly, TLJ-028m reduced XET activity to almost the same degree as TLJ-028d. The inhibition is still low enough that drawing any conclusions with the present information seems unwise. Further testing of reduced MDA compounds does however seem of interest.

The XET inhibition results from testing TLJ-022 reduced (TLJ-022r) seem to confirm that the sodium borohydride reduction was a failure. The results are comparable to TLJ-022 and not TLJ-028d. It should also be noted that while the expected molecules for TLJ-022r and TLJ-028d were the same, the compounds looked completely different: the former being yellow and powdery, the latter being red and crystalline.

TLJ-026 is a compound with carboxylic acid groups in the para-positions. Of all the tested MDA products, this one seems the most promising, and testing a reduced version of the compound would have been a priority in addition to testing compounds with the carboxylic acid groups in the meta- and ortho-positions. It is uncertain if the observed reduction in XET activity stems from the compound binding to the enzymes, or from the acidity of the compound. It can be possible that the compound is acidic enough to degrade the enzymes, and that the observed inhibition stems from this. Further testing would certainly have been of interest.

TLJ-027 was the final compound that inhibited XET activity to a promising degree. This compound had hydroxy-groups in the ortho-positions on the two outermost rings. Testing other compounds with hydroxy-groups could have been of interest.

Overall, of the tested double imine MDA compounds, the ones with carboxylic acid and hydroxy-groups seemed most promising, despite none of the functional groups being in the meta-positions, in which the sulfonic acid groups in BBDs are found. It is possible that compounds with those functional groups in the meta-positions would inhibit to an even greater degree, and that reducing the imine double bonds would also aid with inhibition. Exploring this further would have been a priority moving on.

5. Conclusions

A major problem with synthesizing Brilliant Blue derivatives from 3-((ethyl(phenyl)amino)methyl)-benzenesulfonic acid and benzaldehydes is the solubility of the benzenesulfonic acid, and the solubility of the resulting product. Due to its solubility, the range of options available for workup are limited. The purity problem was avoided when synthesizing BBDs lacking sulfonic acid groups, as a MW synthesis in which unreacted starting material and product separated into different layers, was developed. A variety of BBDs that varied noticeable in structure were synthesized and screened for biological activity. Compounds lacking sulfonic acid groups showed little to no activity. The most promising compound was synthesized from 3-((ethyl(phenyl)amino)methyl)-benzenesulfonic acid and DMF. Its exact structure could not be confirmed, but it seems likely that carbon was inserted between two equivalents of sulfonic acid starting material. This is certainly of interest for future synthesis of XTH inhibitors, as it seems likely that one of the central phenyl rings is not essential for activity. Removing this ring completely should greatly increase the water-solubility of compounds.

Two other pathways, in which BBDs were synthesized from smaller building blocks, were proposed. For the 4,4'-(phenylmethylene)dianiline pathway, the greatest challenge arose from trying to synthesize PMDA itself. While the compound was successfully isolated, the other alternate pathway looked more promising. In this pathway, 4,4'-methylenedianiline serves as the base building-block. Compounds that were biologically screened, were synthesized by reacting 4,4'-methylenedianiline with both benzaldehydes and a benzyl bromide. Of these compounds, the product formed from 4,4'-methylenedianiline and 4-formylbenzoic acid seemed to inhibit XET activity the most. It is possible that the sulfonic acid groups in BBDs can be replaced with carboxylic acid groups to create even better inhibitors. Further synthesis would have been focused around the synthesis of BBDs containing carboxylic acid groups.

6. Experimental section

All chemicals were bought from Sigma Aldrich. Microwave reactions were run in an Anton Paar Monowave 300 Microwave Synthesis Reactor. All NMR samples were analyzed by using an Agilent (Varian) Mercury Plus 400 MHz NMR spectrometer. Processing of NMR spectra was done in Mest.ReNova. HRMS samples were analyzed by using an LTQ Orbitrap XL, in either positive or negative electrospray ionization (ESI) mode. HRMS spectra are provided for all reactions, while some lack NMR spectra, mainly due to solubility issues.

6.1 Triaryl condensation synthesis of 3,3'-((((phenylmethylene)bis(4,1-phenylene))bis-(ethylazanediyl))bis(methylene))dibenzenesulfonic acid from excess benzaldehyde

- 5 eq. benzaldehyde (1.00 g, ca. 1.0 mL, 9.43 mmol) and 1 eq. 3-((ethyl(phenyl)amino)methyl)-benzenesulfonic acid were mixed in a round-bottom flask.
- An oil bath was heated to 190 °C and the round-bottom flask containing the reaction mixture was immersed into the bath (immediate strong green color). The mixture was vigorously stirred for 30 minutes under inert atmosphere and with reflux.
- The reaction mix was taken off the oil bath and cooled down to room temperature. The mixture was filtrated, and the resulting solid was washed with small amounts of boiling methanol. The resulting compound weighed 0.249 g (39.6 %)

Specter data:

^1H NMR (400 MHz, DMSO- d_6) δ 7.82 – 6.84 (m, 25H), 5.58 (s, 1H), 4.72 (s, 4H), 1.05 (t, $J = 7.0$ Hz, 6H).

HRMS (ESI) m/z : $[\text{M-H}]^-$ Calculated for $\text{C}_{37}\text{H}_{37}\text{N}_2\text{S}_2\text{O}_6$ 669.2088; found 669.2055.

6.2 Triaryl condensation synthesis of 3,3'-((((phenylmethylene)bis(4,1-phenylene))bis-(ethylazanediyl))bis(methylene))dibenzenesulfonic acid

- 1 eq. Benzaldehyde (0.10 g, 0.10 mL, 0.94 mmol) and 2 eq. 3-((ethyl(phenyl)amino)methyl)-benzenesulfonic acid (0.551 g, 1.89 mmol) were added to approx. 1 mL DMSO in a round-bottom flask with a magnetic stirrer.

- The round-bottom flask was filled with argon gas and attached to a reflux condenser connected to a Schlenk line providing a constant stream of argon.
- An oil bath was heated to 150 °C before the round-bottom flask containing the reaction mixture was immersed into the bath (almost immediate green color observed). The mix was vigorously stirred for 45 minutes before taken off the oil bath and cooled down to room temperature.
- Ice-cold distilled water was added dropwise to almost immediately crash out turquoise powder. The powder was filtrated and washed with distilled water and small amounts of warm methanol to yield 0.291 g (46.2%) green powder.

Specter data:

¹H NMR (400 MHz, DMSO-*d*₆) δ 7.68 (s, 2H), 7.62 – 7.48 (m, 3H), 7.46 – 6.88 (m, 19H), 5.56 (s, 1H), 4.71 (s, 4H), 1.06 (t, *J* = 7.2, 5.5 Hz, 7H).

HRMS (ESI) *m/z*: [M-H]⁻ Calculated for C₃₇H₃₇N₂S₂O₆ 669.2088; found 669.2089.

6.3 Triaryl condensation synthesis of 3,3'-((((4-(dimethylamino)phenyl)-methylene)bis(4,1-phenylene))bis(ethylazanediy))bis(methylene))dibenzenesulfonic acid

- 2 eq. 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid (0.551 g, 1.89 mmol) and approx. 1 mL DMSO was added to a G10 MW vial with a magnetic stirring bar. The mix was heated to dissolve all the reactant before 1 eq. of 4-(dimethylamino)benzaldehyde (0.141 g 0.94 mmol) was added.
- The vial was filled with argon and the cap was put on. The reaction was run for 1 hour at 140 °C in the microwave.
- The strongly purple solution was washed out with some DMSO into a beaker. Distilled water was added dropwise to the beaker until a purple powder crashed out. The powder was filtrated and washed with some distilled water and cold methanol to yield 0.232 g (34.7%) purple powder.

Specter data:

HRMS (ESI) *m/z*: [M-H]⁻ Calculated for C₃₉H₄₂N₃S₂O₆ 712.2521; found 712.2511.

6.4 Triaryl condensation synthesis of 4,4'-(phenylmethylene)bis(*N*-benzyl-*N*-ethylaniline)

- 1 eq. benzaldehyde (0.10 g, 0.10 mL, 0.94 mmol) and 2 eq. 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid (0.40 g, 0.40 mL 1.89 mmol) were mixed in a G10 microwave vial with 1 mL DMSO and 1 mL 0.1 M HCl solution (approx. 0.1 eq. HCl). X will mainly remain as a yellow oil on top of the DMSO-water mix (if there is no DMSO, X will be at the bottom of the vial).
- The reaction was run in the microwave at 150 °C for 2 hours with very strong stirring in order to mix the two layers as well as possible. Not full conversion according to TLC analysis.
- A couple of drops of concentrated HCl was added and the reaction was run for an additional 3 hours at 150 °C. Almost the entire top layer of X had disappeared, while a yellow oily substance had formed in the bottom of the vial.
- The DMSO-water layer and the remaining starting material was decanted out, and the remaining yellow compound was washed with distilled water (the dense oil started turning green after being exposed to air). The oily compound was dissolved in a mix of heptane and ethyl acetate, and the solution was washed once with distilled water. The solution was dried with magnesium sulfate, filtrated, and solvents were evaporated.
- 0.1 M Na₂CO₃ solution was added dropwise to the DMSO-water solution. The solution turned milky white in addition to a yellow oil crashing out. The DMSO-water was decanted out and the oil washed with distilled water.
- TLC and MS analysis of both yellow oils showed both product and some amount of byproduct. The oils were combined to net 0.311 g (64.8%) product.

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 7.34 – 6.99 (m, 20H), 6.84 (d, *J* = 8.3 Hz, 4H), 6.52 (d, *J* = 8.2 Hz, 4H), 5.22 (s, 1H), 4.39 (s, 4H), 3.35 (q, *J* = 7.0 Hz, 5H), 1.09 (t, *J* = 7.0 Hz, 6H).

¹³C NMR (101 MHz, Chloroform-*d*) δ 146.83, 139.58, 132.22, 130.06, 129.38, 128.51, 128.05, 126.71, 126.64, 125.76, 111.95, 55.05, 54.10, 45.14, 12.17.

HRMS (ESI) *m/z*: [M+H]⁺ Calculated for C₃₇H₃₉N₂ 511.3108; found 511.3093.

6.5 Triaryl condensation synthesis of 2-(bis(4-(benzyl(ethyl)amino)phenyl)methyl)-phenol

- 1 eq. of OH-benzaldehyde (0.115 g, 0.10 mL, 0.94 mmol) and 2 eq. of *N*-benzyl-*N*-ethylaniline (0.40 g, 0.40 mL, 1.89 mmol) was mixed in a G10 microwave vial with 1 mL approx. 1 pH HCl solution.
- The reaction was run for 3 hours and 20 minutes at 140 °C with strong stirring. Solution turned a strong green color with dark green oily compound at the bottom of the vial.
- The solution was decanted, and the compound was washed with distilled water. The compound was dissolved in ethyl acetate and the ethyl acetate solution was washed once with water.
- The solution was dried with magnesium sulfate, filtrated and ethyl acetate evaporated. The oily substance was dissolved in small amounts of boiling heptane. As the heptane cooled down, an oily substance separated from the solution. Analysis showed that some impurities remained in the heptane.
- The heptane was decanted, and the oily substance was washed with small amounts of pure heptane and dried.

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 7.26 – 7.05 (m, 11H), 7.06 – 6.93 (m, 1H), 6.93 – 6.80 (m, 4H), 6.80 – 6.62 (m, 3H), 6.59 – 6.43 (m, 4H), 5.27 (s, 1H), 4.78 (s, 1H), 4.36 (s, 4H), 3.41 – 3.21 (m, 4H), 1.15 – 0.97 (m, 6H).

¹³C NMR (101 MHz, Chloroform-*d*) δ 153.93, 147.37, 139.42, 131.49, 130.38, 130.05, 129.65, 128.60, 127.68, 126.82, 126.65, 120.54, 116.40, 112.34, 54.09, 49.92, 45.21, 12.23.

HRMS (ESI) *m/z*: [M+H]⁺ Calculated for C₃₇H₃₉N₂O 527.3057; found 527.3056.

6.6 Triaryl condensation synthesis of 4,4'-((4-(dimethylamino)phenyl)methylene)-bis(*N*-benzyl-*N*-ethylaniline)

- 1 eq. of *N*-benzaldehyde (0.141 g, 0.94 mmol) and 2 eq. of *N*-benzyl-*N*-ethylaniline (0.40 g, 0.40 mL, 1.89 mmol) were mixed in a G10 microwave vial with 1 mL approx. 1 pH HCl solution.

- The reaction was run for 4 hours at 140 °C with strong stirring. The solution turned a strong purple color with purple oily compound at the bottom of the vial.
- The solution was decanted, and the compound washed with some distilled water. The compound was dissolved in ethyl acetate, and the ethyl acetate solution was washed once with distilled water.
- The solution was dried with magnesium sulfate, filtrated, and the ethyl acetate was evaporated. The oily substance was dissolved in small amounts of boiling heptane. An oily substance separated out of the solution as the heptane cooled down.
- The heptane was decanted, and the oil was washed with small amounts of heptane and dried.

Specter data:

HRMS (ESI) m/z: [M+H]⁺ Calculated for C₃₉H₄₄N₃ 554.3530; found 554.3527.

6.7 Two-step synthesis of *N*-benzyl-*N*-((1*E*,4*E*)-4-((4-(benzyl(ethyl)amino)phenyl)(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)ethanaminium

- 1 eq. of *N*-benzaldehyde (0.141 g, 0.94 mmol) and 2 eq. of *N*-benzyl-*N*-ethylaniline (0.40 g, 0.40 mL, 1.89 mmol) were mixed in a G10 microwave vial with 1 mL approx. 1 pH HCl solution and 6 drops of concentrated HCl.
- The reaction was run in the microwave for 4 hours at 140 °C with strong stirring.
- The mix was added some 0.1 M HCl solution to increase volume, and was extracted 3 times with a mix of heptane and ethyl acetate. The combined fractions were washed once with brine. Anything that crashed out during the extractions/washing was dissolved in acetone and methanol and added to the heptane-ethyl acetate fractions.
- Solvents were evaporated, and the crude was dissolved in DCM (some small amounts of a dark brown powder did not dissolve). The DCM solution was moved to a 100 mL round-bottom flask and put under argon. 0.200 g DDQ was added.
- The reaction was run at room temperature for 2 hours.
- DCM was evaporated, and the crude was dissolved in methanol and washed three times with heptane. The combined heptane fractions were extracted once with methanol, and the methanol extract was combined with the original methanol solution.

- The methanol was evaporated to gain the crude as a bronze-colored powder. The crude was dissolved in 30 mL DCM and washed three times with 0.1 M Na₂CO₃ solution. The combined base solution fractions were extracted once with 20 mL DCM.
- The combined DCM fractions were washed three times with 0.1 M HCl. The combined aqueous fractions were extracted once with 20 mL DCM.
- A blue powder crashed out in the DCM. The DCM was decanted, and the powder washed a couple of times with small amounts of DCM, which were added to the original DCM solution. The unknown blue powder was weighed to be approx. 3.0 g.
- The DCM was evaporated to yield 0.248 g bronze-colored compound (47.9% yield).

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 7.45 – 6.31 (m, 29H), 5.18 (s, 1H), 4.39 (s, 4H), 3.51 – 3.25 (m, 4H), 2.90 (s, 6H), 1.16 – 1.04 (m, 7H).

HRMS (ESI) m/z: [M+H]⁺ Calculated for C₃₉H₄₂N₃ 552.3373; found 552.3371.

6.8 Triaryl condensation synthesis of 3-(((4-((4-(benzyl(ethyl)amino)phenyl)-(phenyl)-methyl)phenyl)(ethyl)amino)methyl)benzenesulfonic acid

- 1 eq. 3-((ethyl(phenyl)amino)methyl)-benzenesulfonic acid (0.275 g, 0.94 mmol) and 1 mL DMSO were added to a G10 microwave vial. The mix was stirred and heated until all of the compound dissolved.
- 1 eq. *N*-benzyl-*N*-ethylaniline (0.20 g, 0.20 mL, 0.94 mmol) and 1 eq. Benzaldehyde (0.10 g, 0.10 mL, 0.94 mmol) were added to the vial. 1 mL 0.1 M HCl solution and 5 drops of concentrated HCl were added and the solution was stirred thoroughly.
- The reaction was run in the microwave for 1 hour at 140 °C. Strong green color observed.
- Distilled water was added to crash out a green compound. The mix was washed with ethyl acetate. The solid was filtrated out and dried. MS analysis showed both disubstituted compound lacking sulfonic acid groups and with one sulfonic acid group.
- The solid was washed again with ethyl acetate and dried to gain a crude weighing 0.098 g.

Specter data:

HRMS (ESI) m/z: [M-H]⁻ Calculated for C₃₇H₃₇N₂SO₃ 589.2519; found 589.2489.

6.9 Triaryl condensation synthesis of 3,3'-(((phenylmethylene)bis(4,1-phenylene))bis-(azanediyl))dibenzenesulfonic acid

- 1 eq. Benzaldehyde (0.10 g, 0.10 mL, 0.94 mmol) and 2 eq. sodium 4-(phenylamino)-benzenesulfonate were mixed in 1 mL DMSO in a G10 microwave vial. The reaction was run in the microwave for 1 hour at 140 °C. No observable reaction took place, and TLC seemed to confirm this.
- 1 mL 0.1 M HCl solution and 3 drops of HCl were added. The reaction was run again with the same settings as before. The mixture turned green.
- Small amounts of methanol was used to transfer the mixture into a beaker. Ethyl acetate and heptane was added until a dark green solid crashed out.
- The solid was filtrated and dried. The crude weighed 0.368 g. TLC showed several spots, while a peak corresponding to the molecular weight of the product could not be observed using MS.
- The crude was purified using column chromatography, with a starting liquid phase consisting of 1:1:1 ratio of heptane, ethyl acetate and methanol. Polarity was gradually increased by adding methanol.
- Fractions of interest were combined and the solvents evaporated. The final product weighed 0.190 g (34.5% yield).

Specter data:

HRMS (ESI) m/z: [M-2H]²⁻ Calculated for C₃₁H₂₄N₂S₂O₆ 292.0543; found 292.0548.

6.10 Synthesis of 3,3'-(((methylenebis(4,1-phenylene))bis(ethylazanediyl))bis-(methylene))dibenzenesulfonic acid from 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid and *N,N*-dimethylformamide

- 1 eq. DMF (0.07 g, 0.075 mL, 0.94 mmol) and 2 eq. 3-((ethyl(phenyl)amino)methyl)benzenesulfonic acid (0.551 g, 1.89 mmol) were added to 1 mL DMSO in a round-bottom flask.

- The flask was put on a 130°C oil bath under inert conditions. After no visible change, the temperature was increased to 150 °C. The reaction was run for 3 hours at the final temperature.
- The reaction mixture was cooled down to room temperature. A mix of methanol and ethyl acetate was added to the mix until a blue powder crashed out. The powder was filtrated and washed with ice cold ethyl acetate.
- The blue powder was dried and weighed to be 0.271 g (48.5% yield).

Specter data:

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.99 – 6.94 (m, 19H), 5.49 (s, 1H), 3.82 (q, *J* = 7.1 Hz, 4H), 1.18 (t, *J* = 7.2, 2.4 Hz, 6H).

HRMS (ESI) *m/z*: [M-H]⁻ Calculated for C₃₁H₃₃N₂S₂O₆ 593.1786; found 593.1777.

6.11 Synthesis of 4,4'-(phenylmethylene)dianiline from benzaldehyde and aniline

- Several drops of hydrochloric acid was added to 1.2 g of ZSM-5 zeolite in a round-bottom flask. 1 eq. benzaldehyde (1.0 g, 1.0 mL, 9.43 mmol) and 3 eq. aniline (2.6 g, 2.7 mL, 28.1 mmol) was added to the flask.
- The reaction was run for 7 at 140°C hours under inert conditions with reflux. The mix got a strong purple color.
- Since the mixture was allowed to cool down without stirring, the zeolite turned rock hard. The oily top layer was flushed away with methanol, while the zeolite was crushed with a spatula and gradually flushed out with ethyl acetate.
- The zeolite was filtrated out of the ethyl acetate solution. Most of the ethyl acetate was evaporated. Some methanol was added.
- 0.1 M Na₂CO₃ solution was added dropwise. When pH 6-7 was reached, a pale brown compound crashed out. Further addition of the base solution dissolved the compound.
- In an attempt to crash out the compound, a 0.1 M HCl solution was used for neutralization. A variety of compounds crashed out, and the solution was made acidic and extracted 3 times with ethyl acetate.
- Once again, a concentrated ethyl acetate and methanol solution was made. The base solution was added until compound crashed out again.

- The compound was filtrated and washed with distilled water and dried. The final pale brown powder weighed 0.388 g (15.0% yield).

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 7.41 (s, 1H), 7.34 – 7.25 (m, 5H), 7.25 – 7.11 (m, 6H), 6.93 (d, *J* = 8.1 Hz, 4H), 6.65 (d, *J* = 8.1 Hz, 4H), 5.38 (s, 1H).

¹³C NMR (101 MHz, Chloroform-*d*) δ 145.12, 144.45, 134.72, 130.20, 129.35, 129.06, 128.36, 128.25, 128.13, 125.92, 125.32, 115.04, 55.24, 21.49.

HRMS (ESI) *m/z*: [M+H]⁺ Calculated for C₁₉H₁₉N₂ 275.1543; found 275.1550.

6.12 Synthesis of (*E*)-*N*,1-diphenylmethanimine

- 1 eq. of benzaldehyde (18.8 mmol, 2.12 g, 2.0 mL) and 1 eq. of aniline (18.8 mmol, 18.6 g, 1.8 mL) were dissolved in a couple of mL of methanol in a round bottom flask.
- 2 drops of concentrated HCl added. The reaction mixture was stirred for 5 minutes. The round bottom flask was put on ice, and after some time the product crashed out.
- The product was filtrated and washed with some distilled water. The final dried product weighed 3.34 g (98.1% yield) with small amounts of impurities.

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 8.51 (s, 1H), 8.03 – 7.90 (m, 2H), 7.61 – 7.48 (m, 3H), 7.48 – 7.38 (m, 2H), 7.35 – 7.24 (m, 3H).

HRMS (ESI) *m/z*: [M+H]⁺ Calculated for C₁₃H₁₂N 182.0964; found 182.0964.

6.13 Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine) from 4,4'-methylenedianiline and 3-nitrobenzaldehyde

- 3 eq. of 3-Nitrobenzaldehyde (11.0 mmol, 1.65 g) was dissolved in 5 mL of acetonitrile in a small round bottom flask. 1 eq. 4,4'-methylenedianiline (MDA) (3.7 mmol, 0.72 g) was dissolved in 25 mL of acetonitrile in a small Erlenmeyer flask.

- The benzaldehyde solution was heated to 50-60 °C and stirred. The diamine solution was added dropwise. Immediate formation of yellow color observed.
- The reaction was run for 4 hours. During the reaction, a yellow solid crashed out as a capillary tube was put into the solution.
- The solid was filtrated and washed with acetonitrile to yield a yellow powder.
- MS analysis showed some amount of impurity. The compound was recrystallized from acetonitrile (approx. 500 mL of solvent was needed). The final product was dried and weighed to be 1.36 g (79.2 % yield).

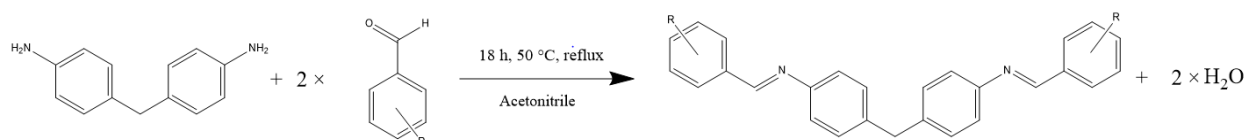
Specter data:

^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.83 (s, 2H), 8.73 (s, 2H), 8.41 – 8.32 (m, 4H), 7.83 (t, 2H), 7.33 (dd, 8H), 4.03 (s, 2H).

^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ 158.65, 148.98, 148.68, 140.48, 138.13, 134.98, 130.98, 130.06, 126.02, 123.13, 121.92.

HRMS (ESI) m/z: $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{27}\text{H}_{21}\text{N}_4\text{O}_4$ 465.1557; found 465.1554.

6.14-20 General procedure for synthesis



Scheme 33. The general procedure for synthesis of di-imine compounds from 4,4'-methylenedianiline (MDA) and benzaldehydes.

As the aim was to synthesize several disubstituted compounds from diamine and different benzaldehydes, the general procedure is similar to the one described in section 6.13). Diversions from the general procedure will be mentioned in the descriptions of the specific reactions.

- For the majority of reactions 1 eq. (1 mmol) MDA was dissolved in 10 mL acetonitrile in an Erlenmeyer flask. 3 eq. (3 mmol) of benzaldehyde was dissolved in 5 mL acetonitrile in a round-bottom flask.
- The benzaldehyde solution was heated to 50 °C and stirred. The diamine solution was added dropwise (small amounts of acetonitrile was used to get out remaining diamine solution).

- The reaction was run overnight (equating to 18-20 hours) with a stopper on the round-bottom flask. The disubstituted product would most often crash out during the reaction.
- The reaction mix was cooled down before the precipitate was filtrated and washed with acetonitrile. Then the product was dried and weighed.
- If necessary, the crude product was recrystallized from acetonitrile.

6.14 Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-bromophenyl)-methanimine) from 4,4'-methylenedianiline and 3-bromobenzaldehyde

- The reaction was run over the weekend (approx. 72 hours).
- The final product was weighed to be 0.435 g (81.7% yield).

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 8.33 (s, 2H), 8.02 (s, 2H), 7.71 (d, *J* = 7.7, 1.3 Hz, 2H), 7.52 (d, *J* = 8.0, 2.1, 1.1 Hz, 2H), 7.26 (t, *J* = 7.8 Hz, 2H), 7.20 – 7.13 (m, 5H), 7.10 (d, 4H), 3.96 (s, 2H).

¹³C NMR (101 MHz, Chloroform-*d*) δ 158.01, 149.53, 139.40, 138.26, 134.08, 131.24, 130.26, 129.74, 127.55, 123.07, 121.11, 41.03.

HRMS (ESI) *m/z*: [M+H]⁺ Calculated for C₂₇H₂₁N₂Br₂ 533.0045; found 533.0043.

6.15 Synthesis of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(2-nitrophenyl)-methanimine) from 4,4'-methylenedianiline and 2-nitrobenzaldehyde

- The reaction was run over the weekend (approx. 72 hours).
- The crude product was recrystallized from acetonitrile and dried.
- The final product weighed 0.272 g (58.6% yield).

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 8.88 (s, 2H), 8.24 (d, *J* = 7.8, 1.5 Hz, 2H), 8.00 (d, *J* = 8.2, 1.3 Hz, 2H), 7.66 (t, 2H), 7.54 (t, *J* = 8.6, 7.5, 1.5 Hz, 2H), 7.23 – 7.11 (m, 9H), 3.98 (s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 155.29, 149.31, 149.21, 139.94, 133.57, 131.20, 131.10, 129.81, 129.73, 124.55, 121.48, 41.08.

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{27}\text{H}_{21}\text{N}_4\text{O}_4$ 465.1557; found 465.1544.

6.16 Synthesis of 4,4'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis-(methaneylylidene))dibenzoic acid from 4,4'-methylenedianiline and 4-formylbenzoic acid

- Due to poor solubility, Benzaldehyde was dissolved in 30 mL of acetonitrile. A couple of mL of 0.1 M HCl solution was added and the mixture was heated until the benzaldehyde dissolved.
- The product was weighed to be 0.354 g (76.5% yield).

Specter data:

^1H NMR (400 MHz, DMSO-*d*₆) δ 8.73 (s, 2H), 8.05 (dd, 8H), 7.31 (dd, 8H), 4.02 (s, 2H).

^{13}C NMR (101 MHz, DMSO-*d*₆) δ 167.35, 159.73, 149.44, 140.24, 140.20, 133.31, 130.18, 130.02, 129.07, 121.80, 99.99.

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{29}\text{H}_{23}\text{N}_2\text{O}_4$ 463.1652; found 463.1652.

6.17 Synthesis of 2,2'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis-(methaneylylidene))diphenol from 4,4'-methylenedianiline and 2-hydroxybenzaldehyde

- The product was weighed to be 0.335 g (82.4% yield).

Specter data:

^1H NMR (400 MHz, Chloroform-*d*) δ 13.22 (s, 2H), 8.55 (s, 2H), 7.37 – 7.26 (m, 4H), 7.26 – 7.11 (m, 10H), 6.96 (d, J = 8.0 Hz, 2H), 6.87 (t, J = 7.5, 1.1 Hz, 2H), 3.97 (s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.18, 161.14, 146.62, 139.82, 133.12, 132.22, 129.90, 121.35, 119.21, 119.06, 117.26, 99.99, 41.03.

HRMS (ESI) m/z: [M+H]⁺ Calculated for C₂₇H₂₃N₂O₂ 407.1754; found 407.1758.

6.18 Synthesis of 2,2'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis-(methaneylylidene))dibenzonitrile from 4,4'-methylenedianiline and 2-formylbenzonitrile

- The scale of the reaction was halved. 0.5 mmol of MDA (0.099 g) was dissolved in 10 mL acetonitrile, and 1.5 mmol of Benzaldehyde (0.197 g) was dissolved in 5 mL acetonitrile.
- The product did not crash out during the reaction. The solvent was evaporated to gain an oily substance.
- The oil was dissolved in some toluene. Toluene and any toluene-water azeotrope was evaporated. The substance was still oily. The oil was mixed with 99.9 % methanol and mixed thoroughly. The methanol and any methanol-toluene azeotrope was evaporated to gain an orange powder.
- The powder was recrystallized from acetonitrile to gain 0.078 g (36.8%) product.

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 8.81 (s, 2H), 8.27 (d, *J* = 8.1, 1.2 Hz, 2H), 7.68 (t, 2H), 7.63 (d, *J* = 8.0, 1.2 Hz, 2H), 7.49 (t, *J* = 7.6, 1.3 Hz, 2H), 7.30 – 7.11 (m, 9H), 3.99 (s, 2H).

¹³C NMR (101 MHz, Chloroform-*d*) δ 155.04, 148.91, 140.14, 138.38, 133.16, 132.99, 131.05, 129.85, 127.67, 121.43, 116.97, 113.43, 41.09.

HRMS (ESI) m/z: [M+H]⁺ Calculated for C₂₉H₂₁N₄ 425.1761; found 425.1768.

6.19 Synthesis of (1*E*,1'*E*)-*N,N'*-((methylenebis(4,1-phenylene))bis(1-(2-chlorophenyl)-methanimine) from 4,4'-methylenedianiline and 2-chlorobenzaldehyde

- The product was weighed to be 0.365 g (82.4% yield).

Specter data:

^1H NMR (400 MHz, Chloroform-*d*) δ 8.85 (s, 2H), 8.17 (d, $J = 7.3, 2.2$ Hz, 2H), 7.37 – 7.25 (m, 6H), 7.22 – 7.10 (m, 9H), 3.97 (s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 156.50, 149.95, 139.43, 136.03, 133.31, 132.06, 129.94, 129.72, 128.54, 127.12, 121.32, 41.05.

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{27}\text{H}_{21}\text{N}_2\text{Cl}_2$ 443.1076; found 443.1077.

6.20 Synthesis of 4,4'-((1*E*,1'*E*)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis-(methaneylylidene))dibenzonitrile from 4,4'-methylenedianiline and 4-formylbenzonitrile

- The scale of the reaction was halved. 0.5 mmol of MDA (0.099 g) was dissolved in 5 mL acetonitrile, and 1.5 mmol of Benzaldehyde (0.197 g) was dissolved in 5 mL acetonitrile.
- The crude was recrystallized from acetonitrile.
- The final product weighed 0.156 g (73.5% yield).

Specter data:

^1H NMR (400 MHz, Chloroform-*d*) δ 8.44 (s, 2H), 7.93 (d, $J = 8.1$ Hz, 4H), 7.69 (d, $J = 8.0$ Hz, 4H), 7.19 (d, $J = 8.4$ Hz, 6H), 7.13 (d, $J = 8.3$ Hz, 4H), 3.98 (s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 157.33, 149.14, 140.01, 139.91, 132.54, 129.82, 129.05, 121.21.

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{29}\text{H}_{21}\text{N}_4$ 425.1761; found 425.1760.

6.21 Synthesis of (*E*)-4-(4-((3-nitrobenzylidene)amino)benzyl)aniline from 4,4'-methylenedianiline and 3-nitrobenzaldehyde

- 1 eq. MDA (3.7 mmol, 0.72 g) was dissolved in 25 mL of methanol in a round-bottom flask. 1 eq. of 3-Nitrobenzaldehyde (3.7 mmol, 0.55 g) was dissolved in 25 mL methanol in an Erlenmeyer flask.
- The diamine solution was heated to 50-60 °C and stirred. The benzaldehyde solution was added dropwise. The solution turned immediately yellow, and during the addition of benzaldehyde, a yellow solid crashed out.

- The reaction was run for 30 minutes before the reaction mixture was cooled down to room temperature.
- The precipitate was filtrated and washed with small amounts of methanol to yield a yellow solid.
- The solid was recrystallized from acetonitrile, filtrated and dried to yield 0.61 g of a yellow compound.

Specter data:

¹H NMR (400 MHz, Chloroform-*d*) δ 8.67 (s, 2H), 8.49 (s, 2H), 8.25 (d, *J* = 8.2, 2.3, 1.1 Hz, 2H), 8.18 (d, *J* = 7.8, 1.4 Hz, 2H), 7.59 (t, *J* = 7.9 Hz, 2H), 7.20 (t, *J* = 8.0 Hz, 7H), 7.16 (d, 4H), 3.99 (s, 2H).

¹³C NMR (101 MHz, Chloroform-*d*) δ 156.66, 148.99, 139.86, 137.96, 134.01, 129.84, 129.79, 125.51, 123.47, 121.21, 41.07.

HRMS (ESI) *m/z*: [M+H]⁺ Calculated for C₂₀H₁₈N₃O₂ 332.1394; found 332.1398.

6.22 Reduction of (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine) to form 4,4'-methylenebis(*N*-(3-nitrobenzyl)aniline)

- 0.200 g (0.43 mmol) of product from section 6.13 was dissolved in 50 mL 99.9% methanol in a round-bottom flask.
- Sodium borohydride was added to the solution under stirring. The reaction was screened with TLC. Due to the hydride likely being of poor quality, large amounts were used.
- Water was added to the solution. A yellow compound crashed out and was filtered.
- The solution remained yellow. It was extracted with ethyl acetate, leaving the water solution blank. Ethyl acetate was evaporated, but the amount of compound that had been extracted was insignificantly small.
- The yellow precipitate from earlier was dried and weighed 0.064 g.

Specter data:

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.84 (s, 2H), 8.77 – 8.69 (m, 2H), 8.43 – 8.32 (m, 4H), 7.83 (t, *J* = 8.0 Hz, 2H), 7.33 (dd, 8H), 4.03 (s, 2H).

^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ 158.66, 148.98, 140.48, 138.13, 134.99, 130.99, 130.07, 123.13, 121.92.

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{27}\text{H}_{24}\text{N}_4\text{O}_4$ 469.1870; found 469.1871.

6.23 Synthesis of 4-(4-aminobenzyl)-*N*-(3-nitrobenzyl)aniline from 4,4'-methylenedianiline and 1-(bromomethyl)-3-nitrobenzene

- 1 eq. of MDA (3 mmol, 0.595 g) was dissolved in 11 mL acetonitrile and 4 mL 1.0 M Na_2CO_3 solution in a round-bottom flask. 1 eq. of 1-(bromomethyl)-3-nitrobenzene (3 mmol, 0.648 g) was dissolved in 35 mL acetonitrile in an Erlenmeyer flask.
- The diamine solution was heated to 90 °C with reflux and stirred while the nitrobenzene solution was added dropwise. The reaction was run for 18 hours.
- The reaction mixture was cooled down to room temperature. Water was added until the total volume was 60 mL (a total of 20 mL of this mix was used for testing of different workup procedures).
- For the remaining 40 mL of the mixture, the solvents were evaporated, and the resulting oily mixture was dissolved in ethyl acetate and mixed with silica. The ethyl acetate was evaporated to get the mixture in the silica.
- The different compounds in the mix were separated using a silica column with a 1:1 ratio of ethyl acetate and heptane as the starting mobile phase. When most of the first compound had eluted, the ratio was increased to 3:2 to get the last two compounds (according to MS analysis the first compound is the disubstituted compound, the second compound the monosubstituted, and the third diamine starting material).
- Fractions containing the disubstituted compound were combined and the solvents evaporated. The same was done for the fractions containing the monosubstituted compound. In both cases an oil was obtained. Both oils were dissolved in benzene, and the benzene and any benzene-water azeotrope was evaporated. After this 99.9 % methanol was added to both oils, and after rigorous stirring, the methanol and any methanol-benzene azeotrope was evaporated. The monosubstituted compound was obtained as a yellow solid weighing 0.155 g (15.7% total yield (23.6% yield considering 2/3 of total solution was used in chromatography)).
- The disubstituted compound remained a viscous red oil. The oil would freeze solid using an acetone dry ice bath, but would melt when taken off the bath. After being exposed to air for

some days, the oil became solid and weighed 0.130 g (18.7% yield if the aim was to make disubstituted compound (28.1% yield considering 2/3 of total solution was used for chromatography)).

Specter data:

Monosubstituted product:

^1H NMR (400 MHz, Chloroform-*d*) δ 8.16 (s, 1H), 8.04 (d, $J = 8.2, 2.4, 1.1$ Hz, 1H), 7.63 (d, 1H), 7.42 (t, $J = 7.9$ Hz, 1H), 6.88 (dd, 4H), 6.54 (d, 2H), 6.45 (d, 2H), 4.36 (s, 2H), 3.69 (s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 145.47, 144.30, 142.20, 133.24, 131.91, 131.73, 129.67, 129.63, 129.53, 122.23, 122.09, 115.28, 113.09, 47.81, 40.13.

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{20}\text{H}_{20}\text{N}_3\text{O}_2$; found 334.1546.

Disubstituted compound:

^1H NMR (400 MHz, Chloroform-*d*) δ 8.15 (s, 2H), 8.04 (d, 2H), 7.62 (d, 2H), 7.42 (t, $J = 7.9$ Hz, 2H), 6.89 (d, 4H), 6.45 (d, 4H), 4.35 (s, 4H), 3.68 (s, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 148.58, 145.44, 142.15, 133.25, 131.64, 129.68, 129.53, 122.24, 122.09, 113.13, 99.99, 47.82, 40.07.

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calculated for $\text{C}_{27}\text{H}_{24}\text{N}_4\text{O}_4$ 469.1870; found 469.1856.

6.24 Synthesis of 4,4'-methylenebis(*N*-(3-nitrobenzyl)aniline) from 4,4'-methylene-dianiline and 1-(bromomethyl)-3-nitrobenzene

- 2 eq. of 1-(bromomethyl)-3-nitrobenzene (6 mmol, 1.296 g) was dissolved in 55 mL acetonitrile in a round-bottom flask. 1 eq. of MDA (3 mmol, 0.595 g) was dissolved in 19 mL acetonitrile and 6 mL 1.0 M Na_2CO_3 solution in an Erlenmeyer flask.
- The nitrobenzene solution was heated to 90 °C with reflux. The solution was stirred as the diamine solution was added dropwise. The reaction was run for 18 hours.
- MS analysis showed presence of both monosubstituted and disubstituted compound. TLC showed two spots corresponding to the mentioned compounds, with R_f values from the previous reaction as comparison. No additional spots were observed.

- Due to the disubstituted compound being isolated from the previous reaction, and due to time restraints, column chromatography was not performed to purify the compound.

Specter data:

HRMS (ESI) m/z: [M+H]⁺ Calculated for C₂₇H₂₄N₄O₄ 469.1870; found 469.1854.

7. References

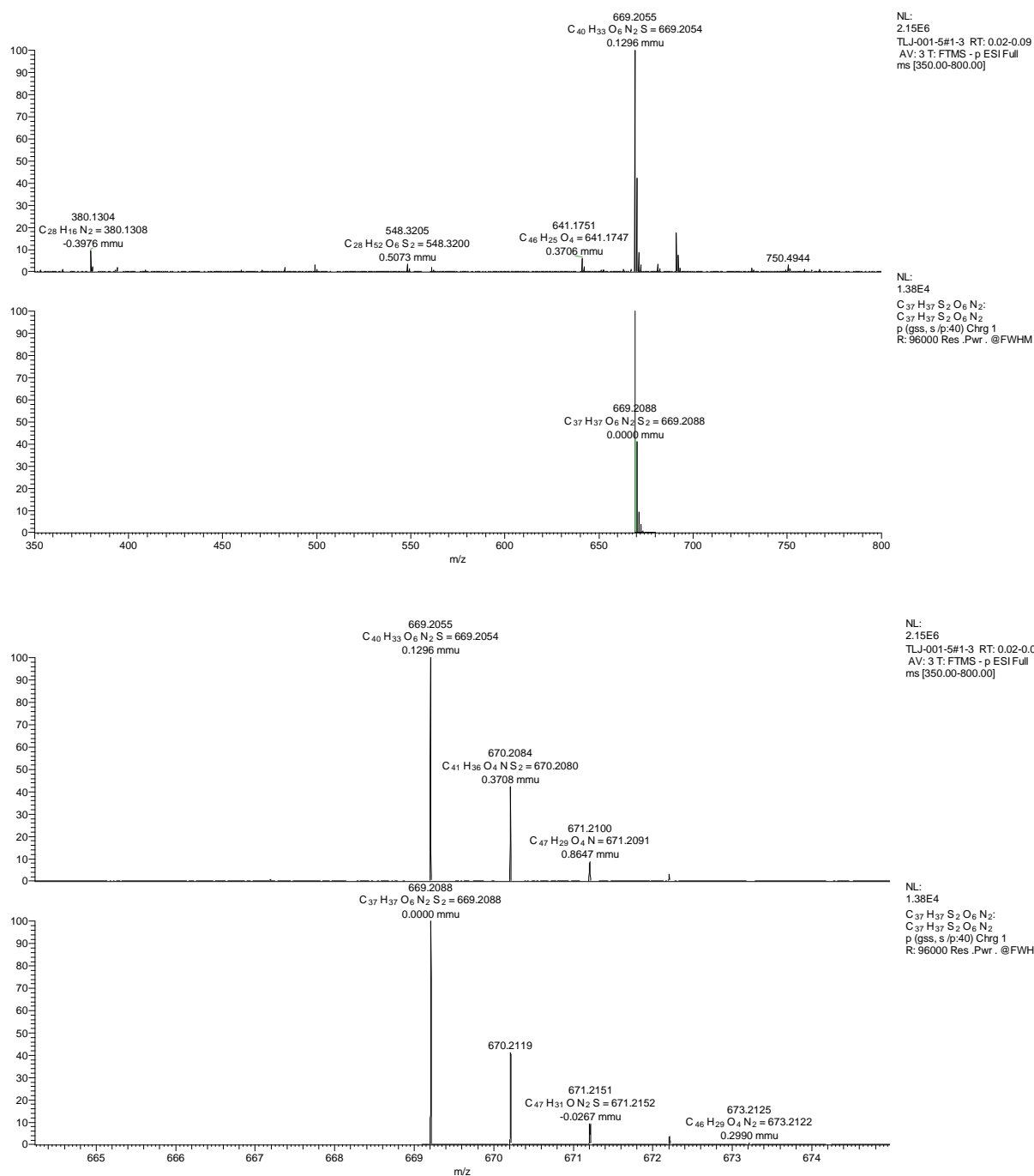
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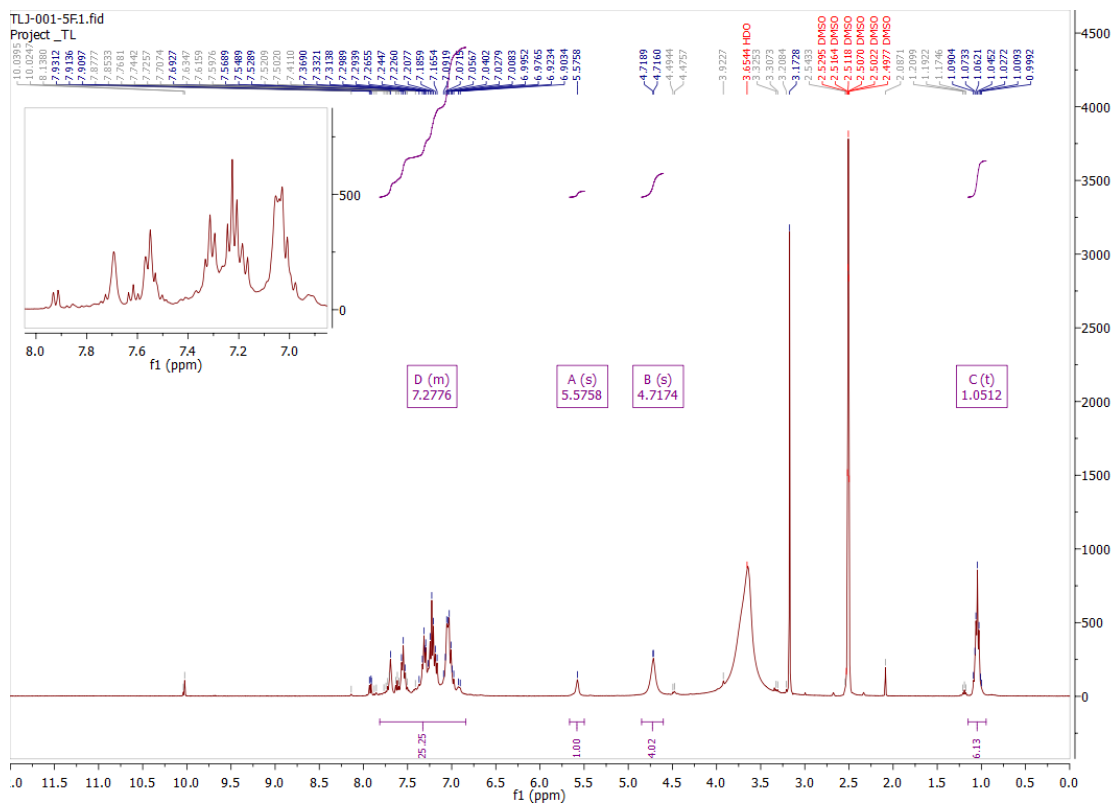
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8. Appendices

8.1 Spectra

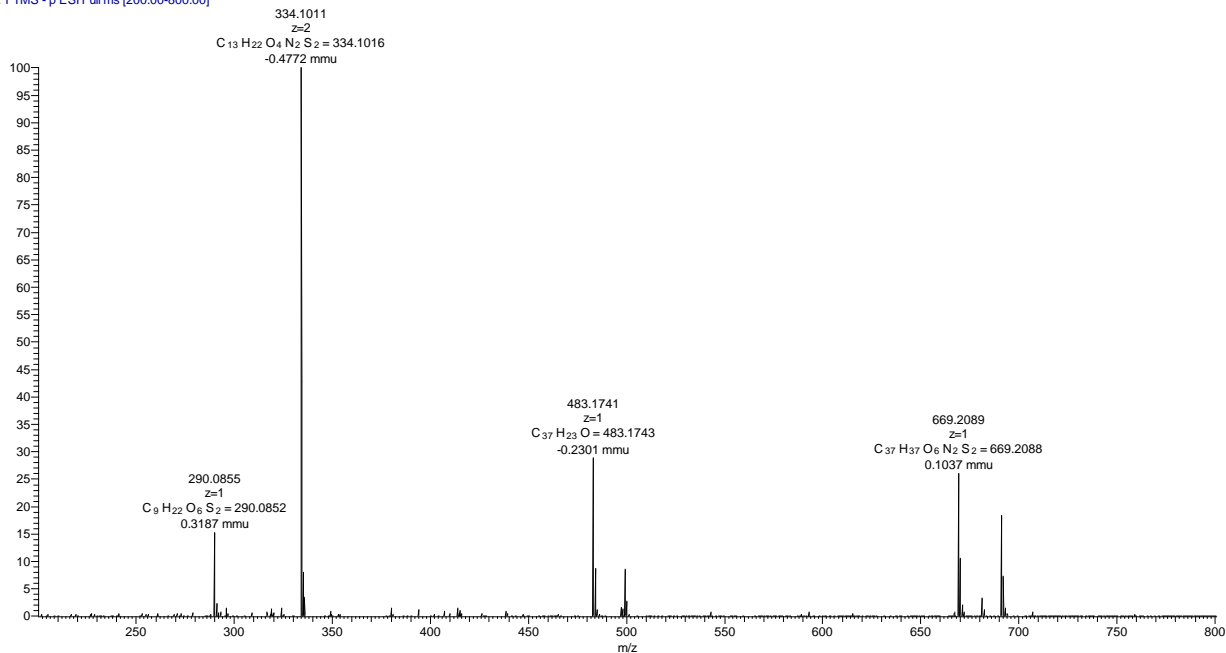
8.1.1 3,3'-((((phenylmethylene)bis(4,1-phenylene))bis(ethylazanediy))bis(methylene))-dibzenesulfonic acid

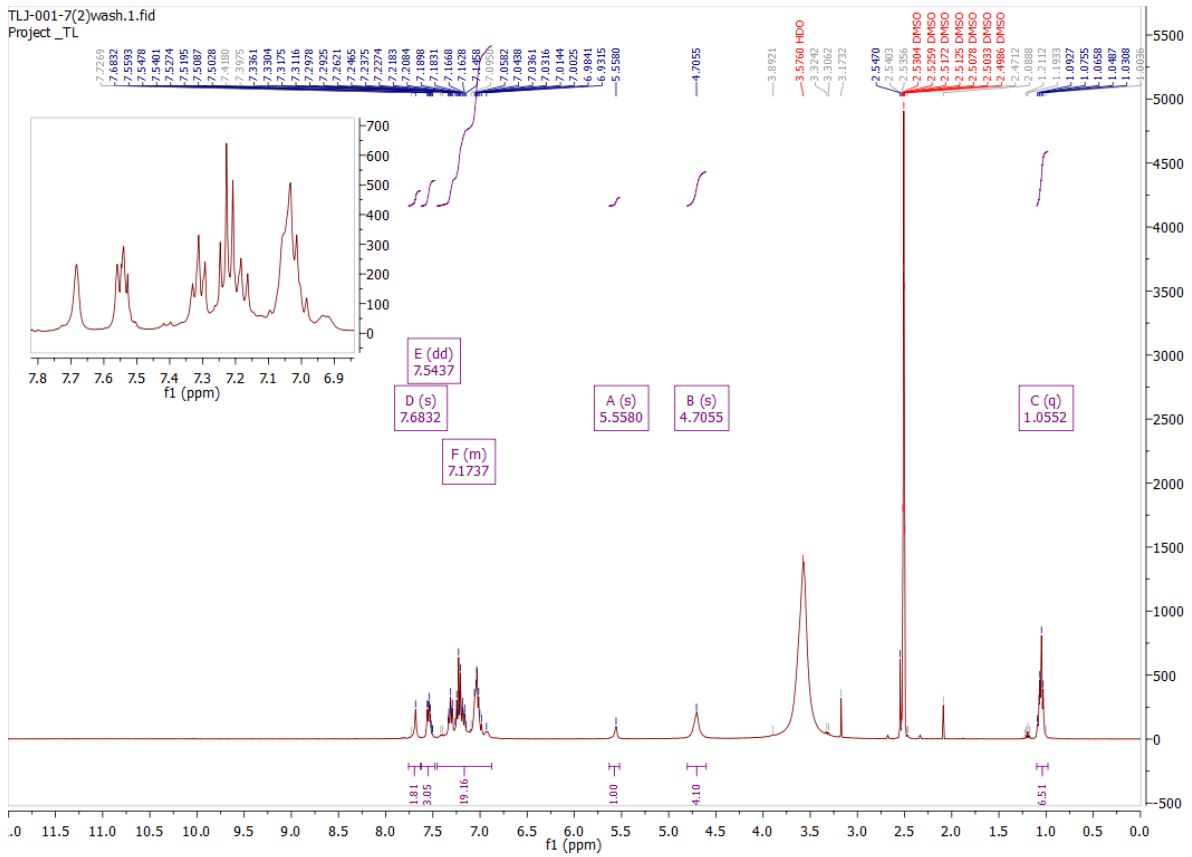
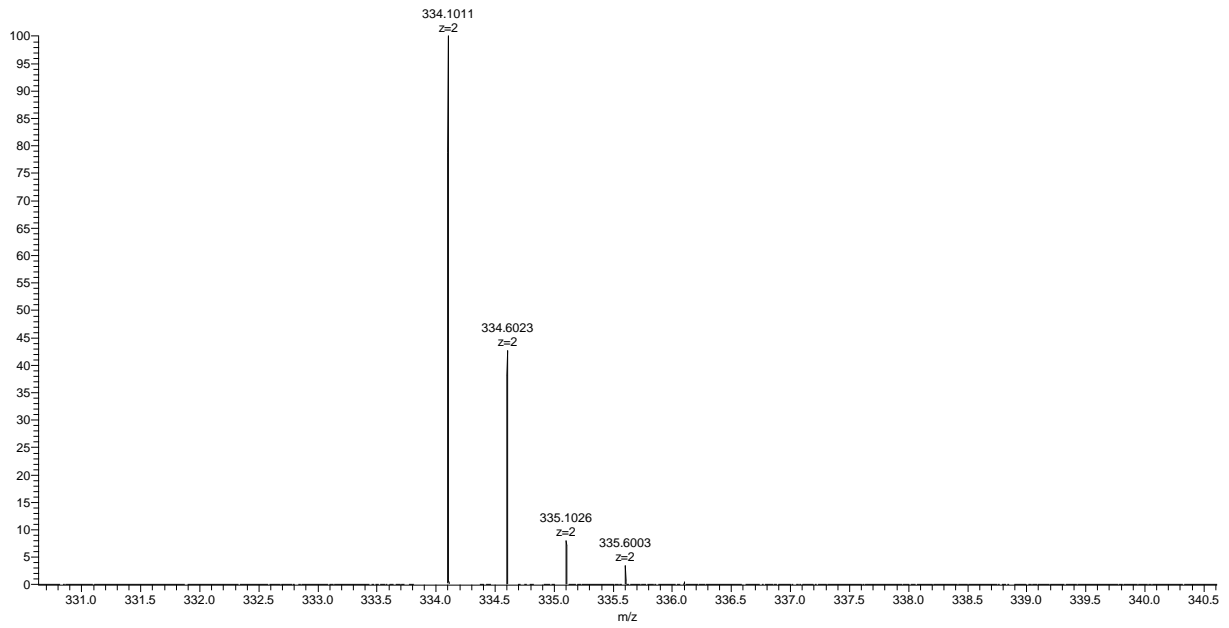


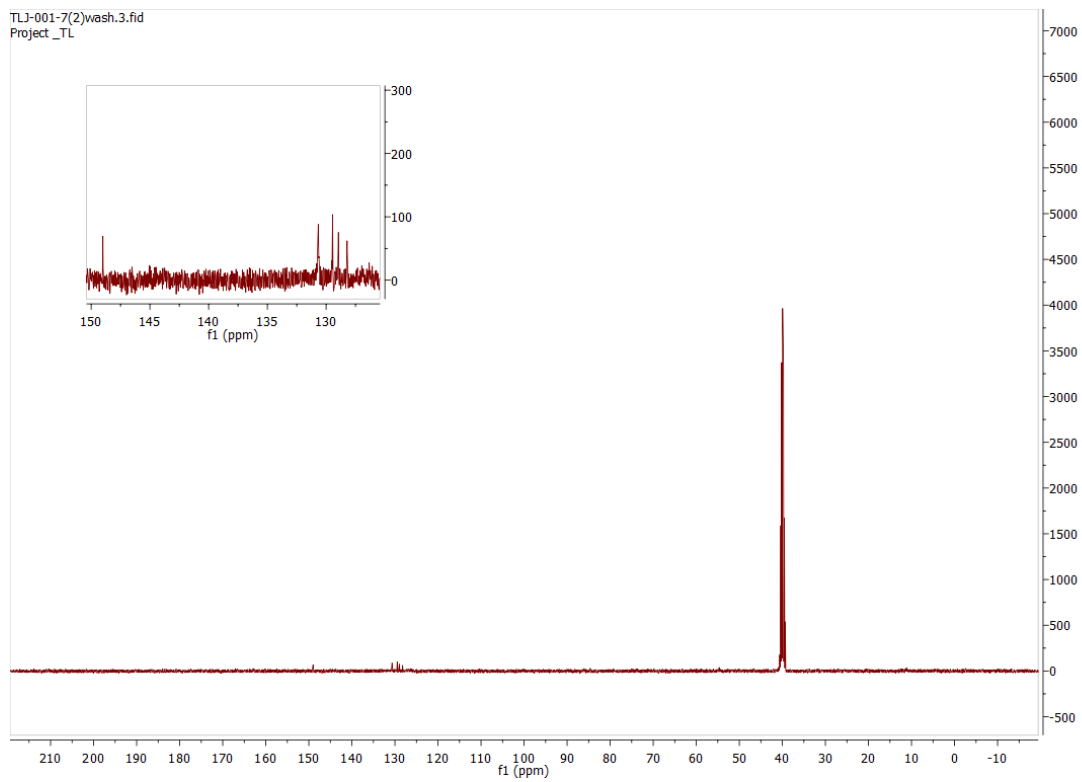
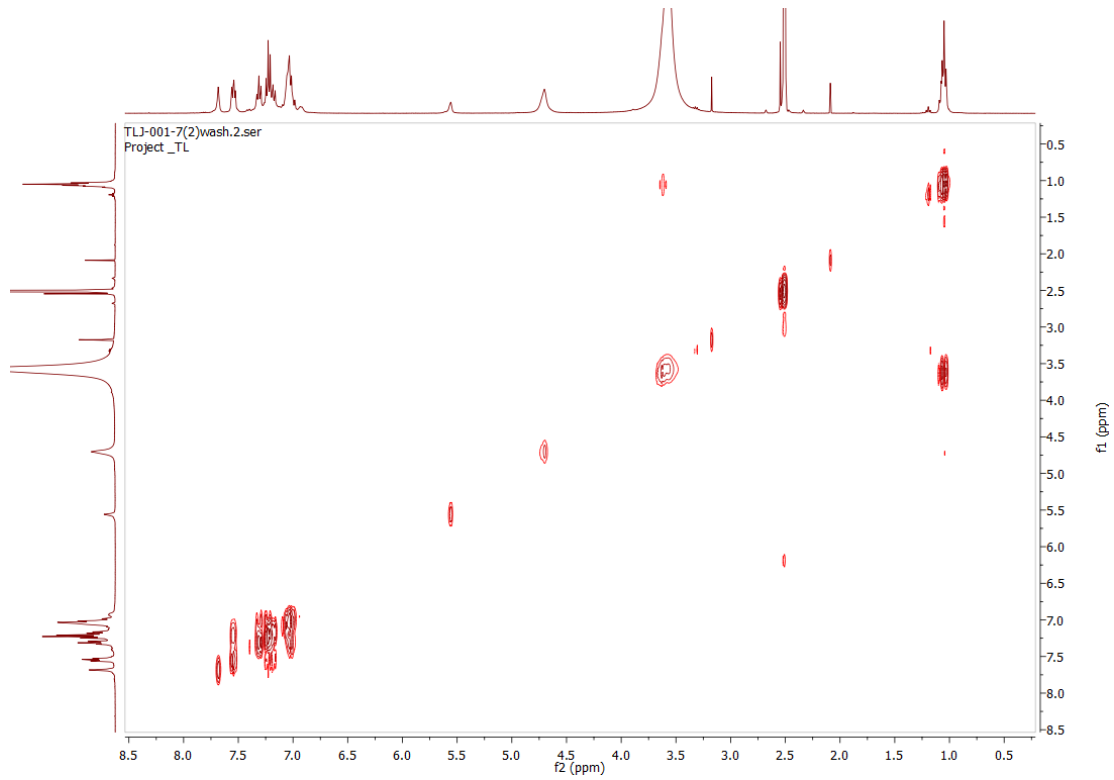


8.1.2 3,3'-(((phenylmethylene)bis(4,1-phenylene))bis(ethylazanediyl))bis(methylene)-dibenzene sulfonic acid

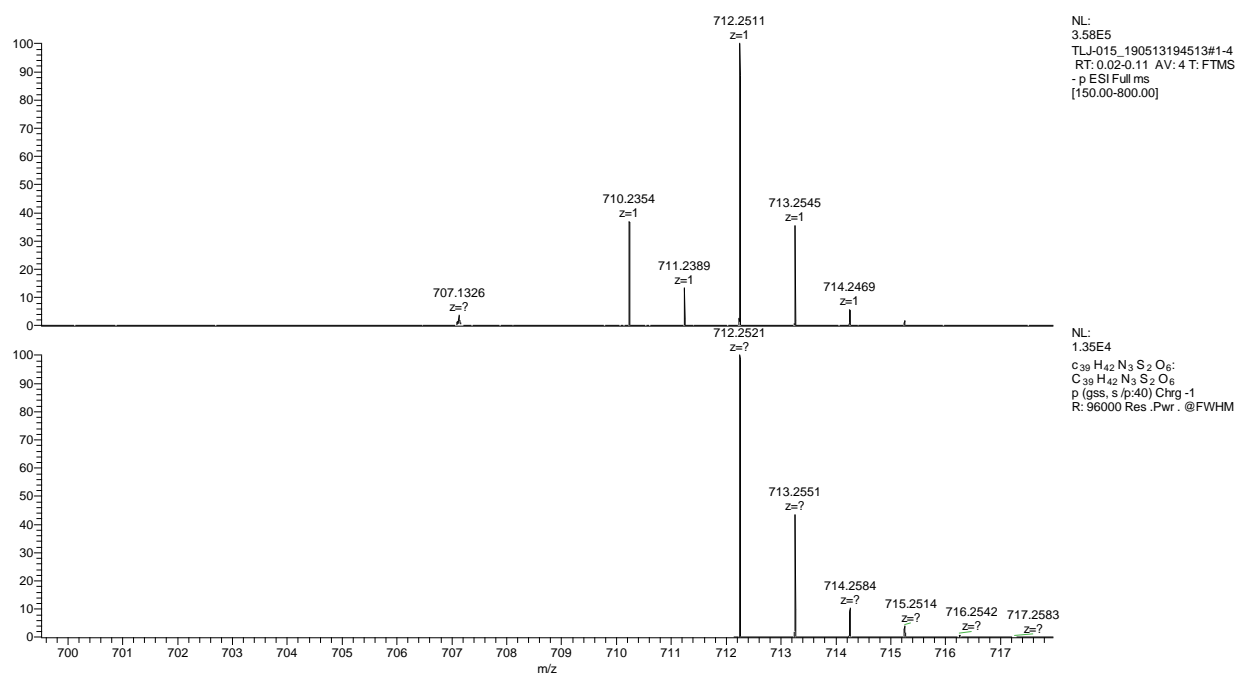
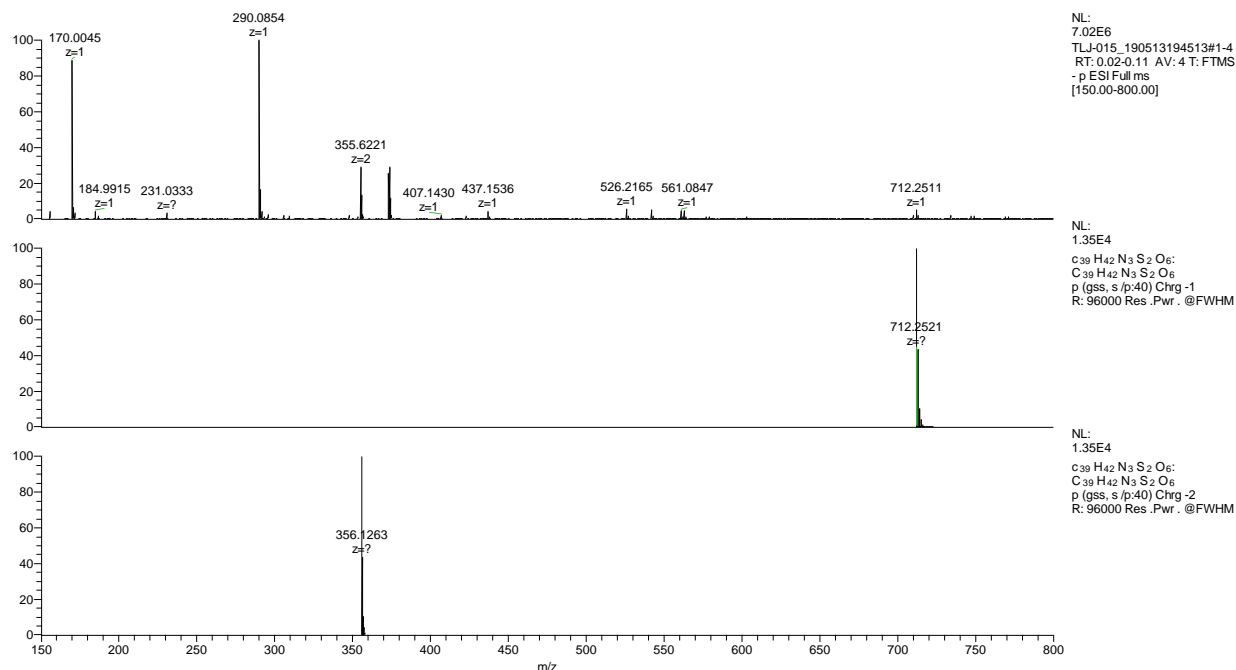
III-001-7 #1-5 RT: 0.00-0.12 AV: 5 NL: 3.58E6
T: FTMS - p ESI Full ms [200.00-800.00]

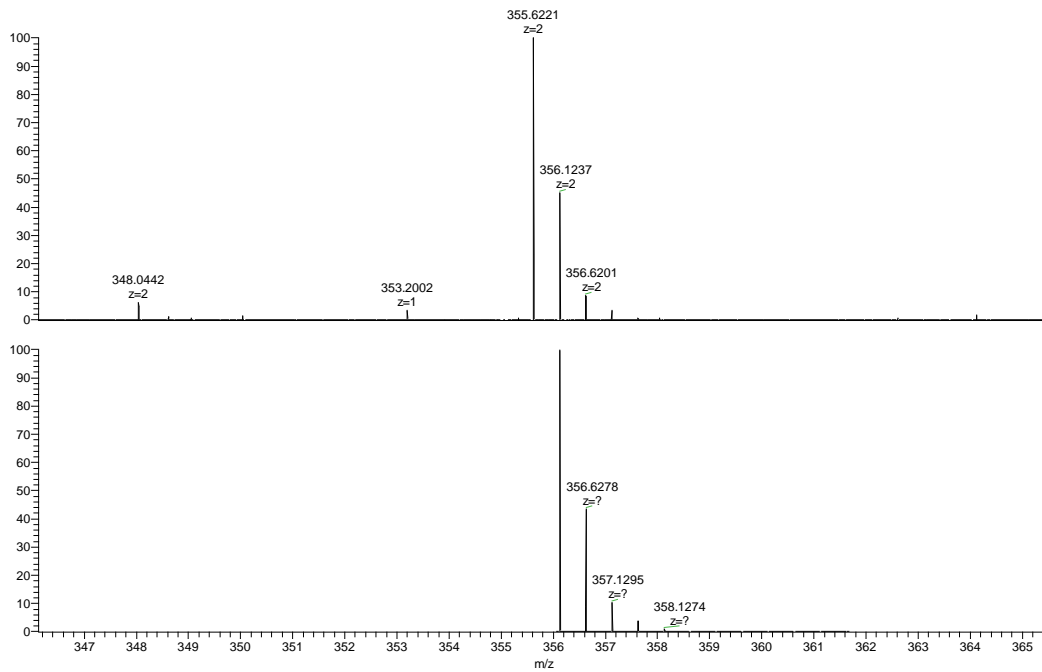






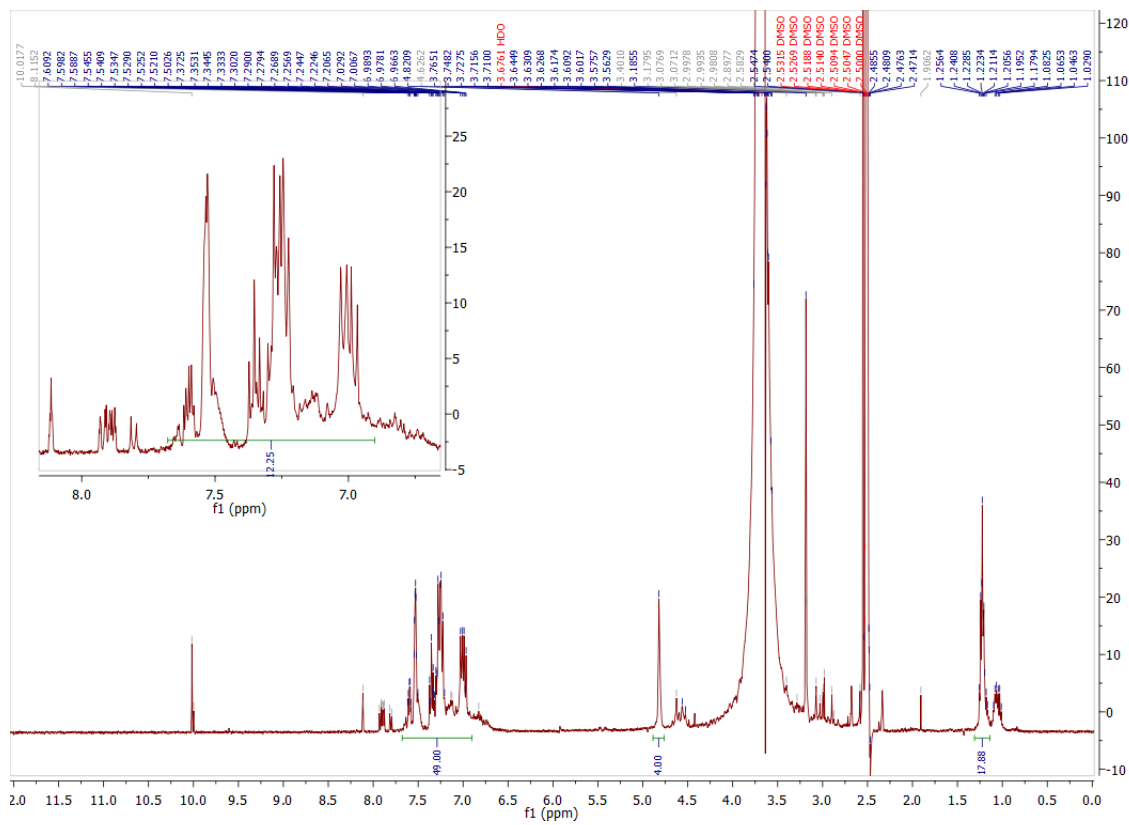
8.1.3 3,3'-((((4-(dimethylamino)phenyl)methylene)bis(4,1-phenylene))bis(ethylazanediy))bis-(methylene)dibenzenesulfonic acid

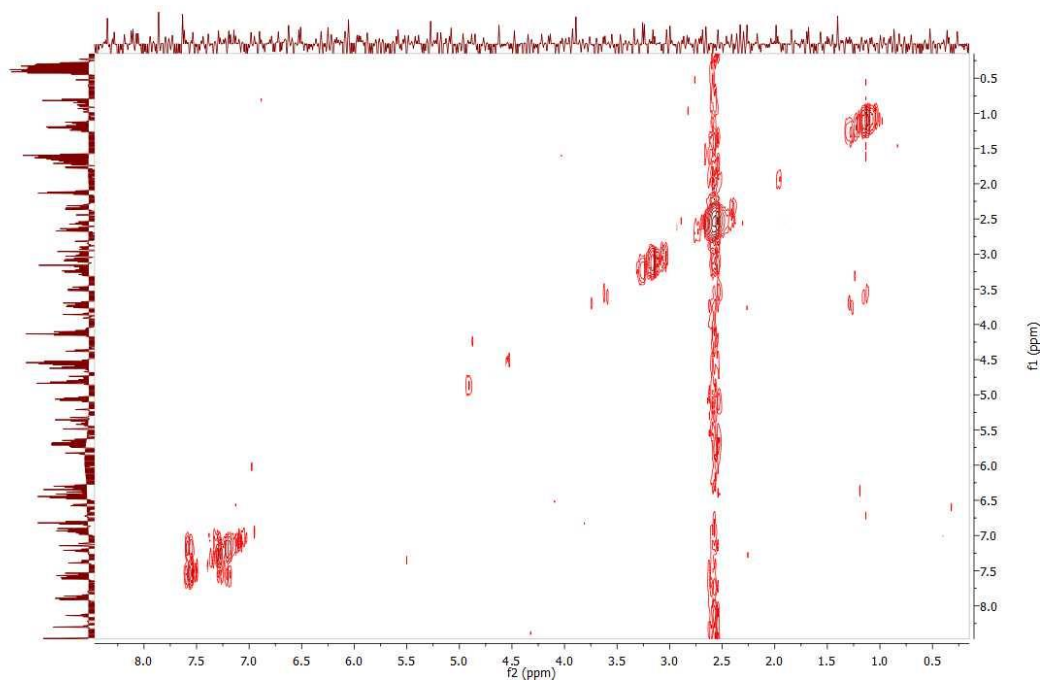




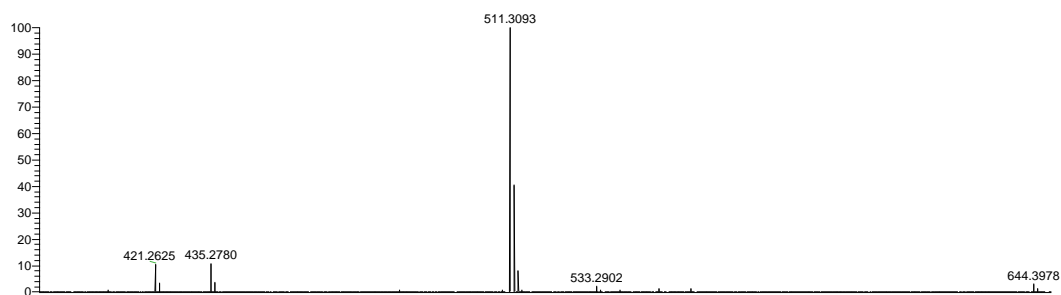
NL:
2.05E6
TLJ-015_190513194513#1-4
RT: 0.02-0.11 AV: 4 T: FTMS
- p ESI Full ms
[150.00-800.00]

NL:
1.35E4
C₃₉H₄₂N₃S₂O₆:
C₃₉H₄₂N₃S₂O₆
p (gss, s/p:40) Chrg -2
R: 96000 Res. Pwr. @FWHM

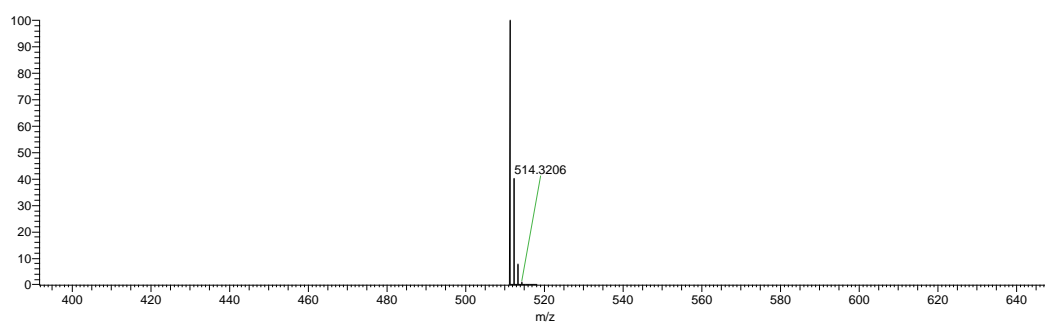




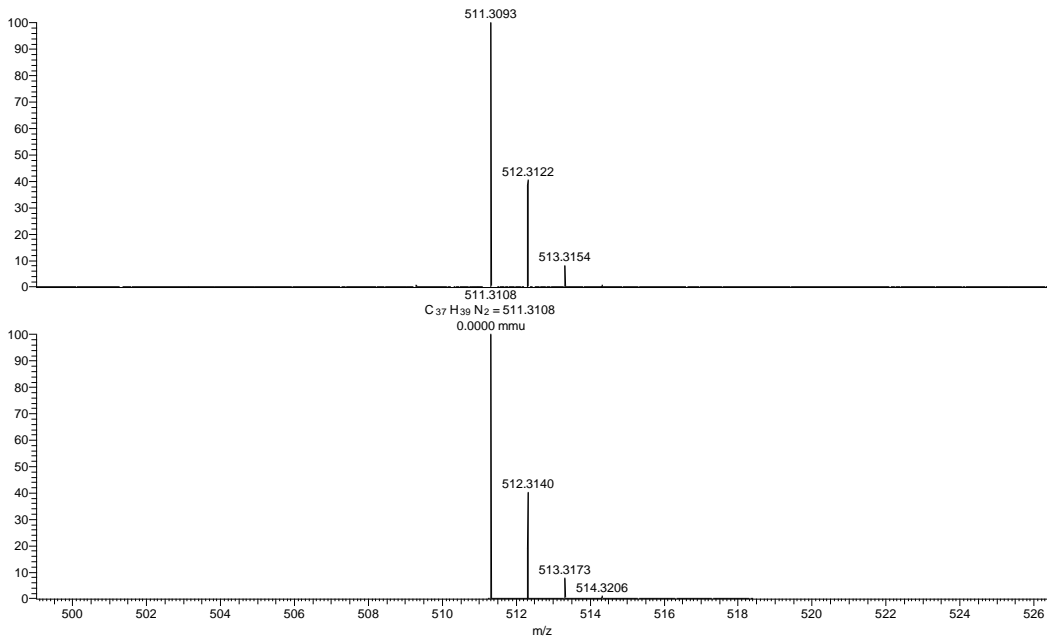
8.1.4 4,4'-(phenylmethylene)bis(*N*-benzyl-*N*-ethylaniline)



NL:
1.22E8
TLJ-005-4(2)#1-4 RT:
0.02-0.10 AV: 4 T: FTMS + p
ESI Full ms [150.00-800.00]

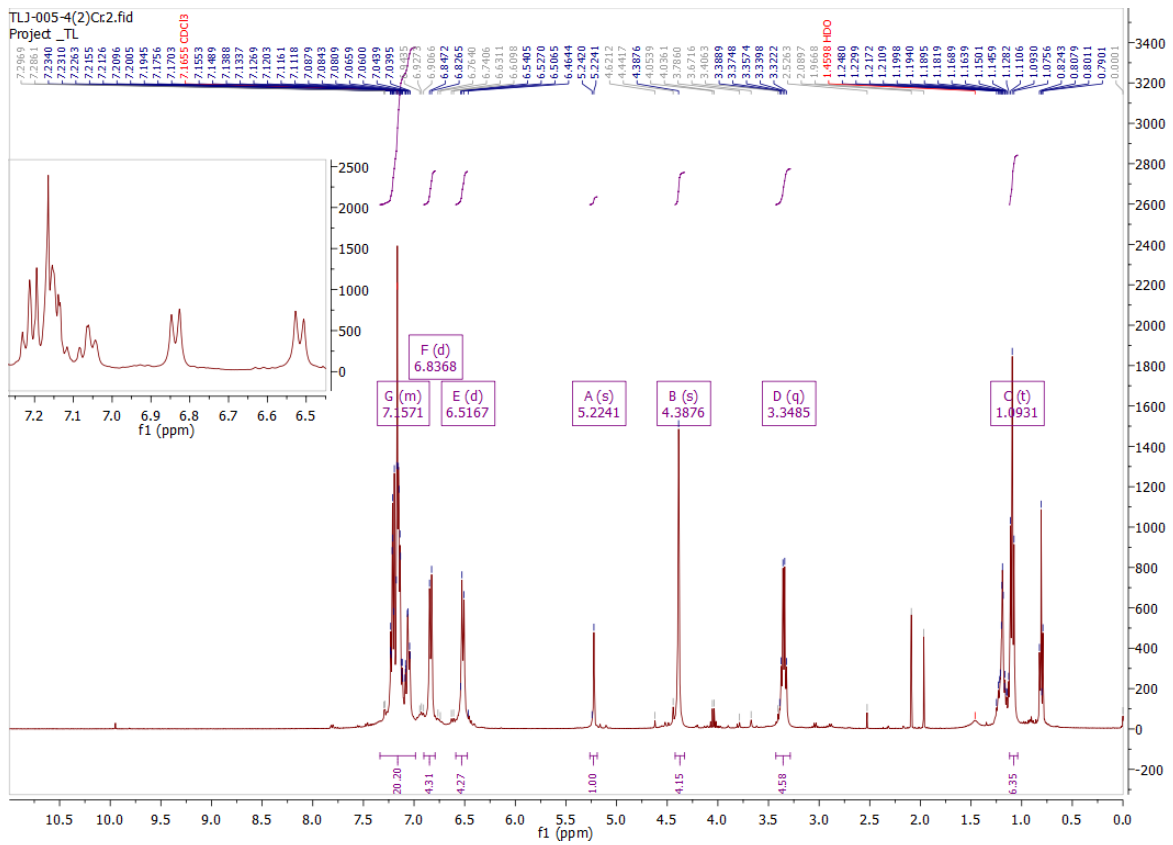


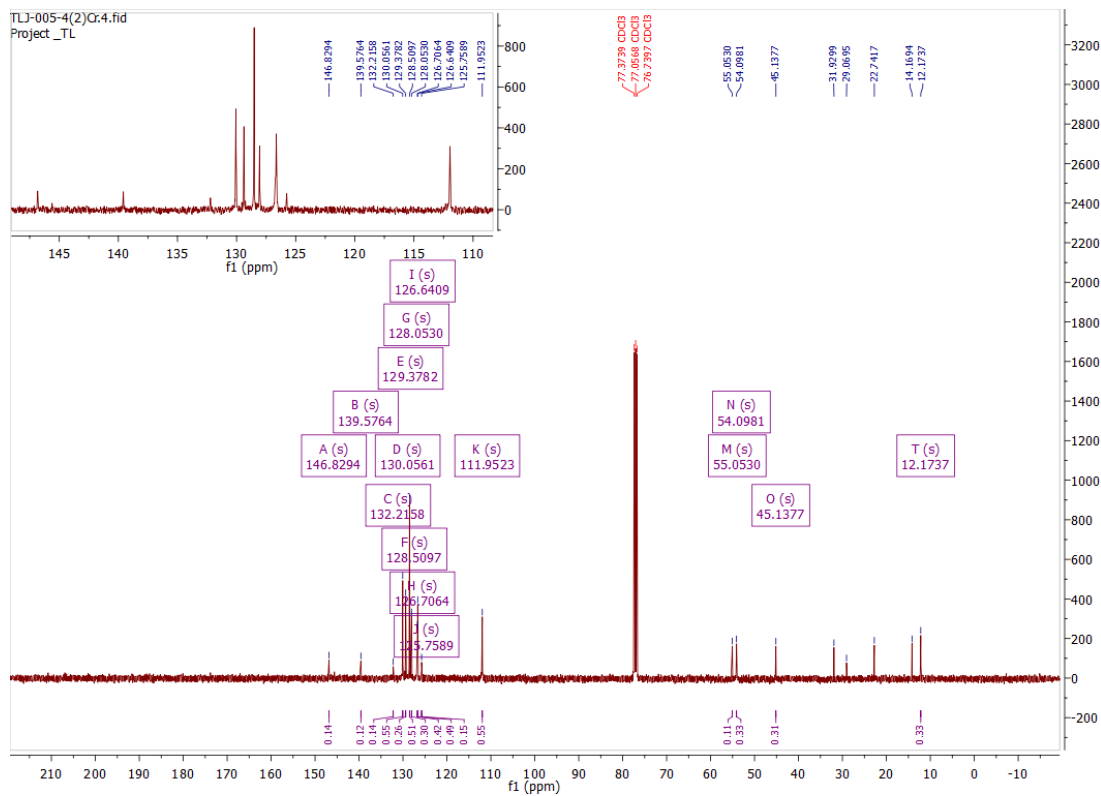
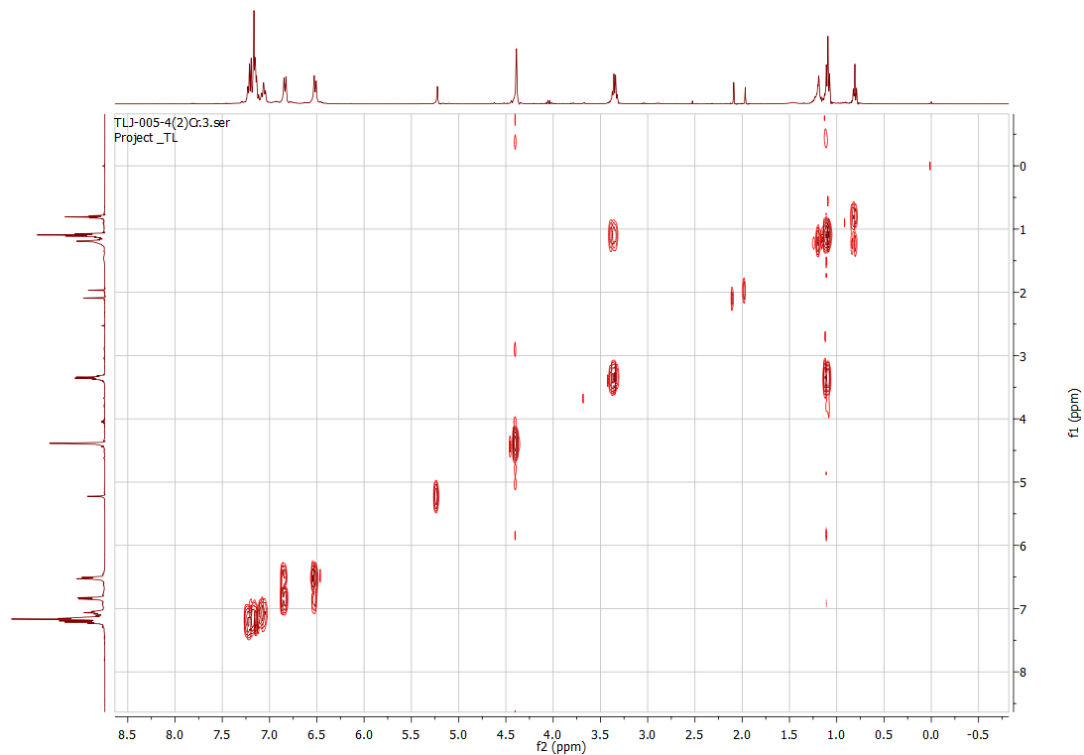
NL:
1.56E4
C₃₇H₃₉N₂:
C₃₇H₃₉N₂:
p (gss, s/p:40) Chrg 1
R: 96000 Res. Pwr. @FWHM



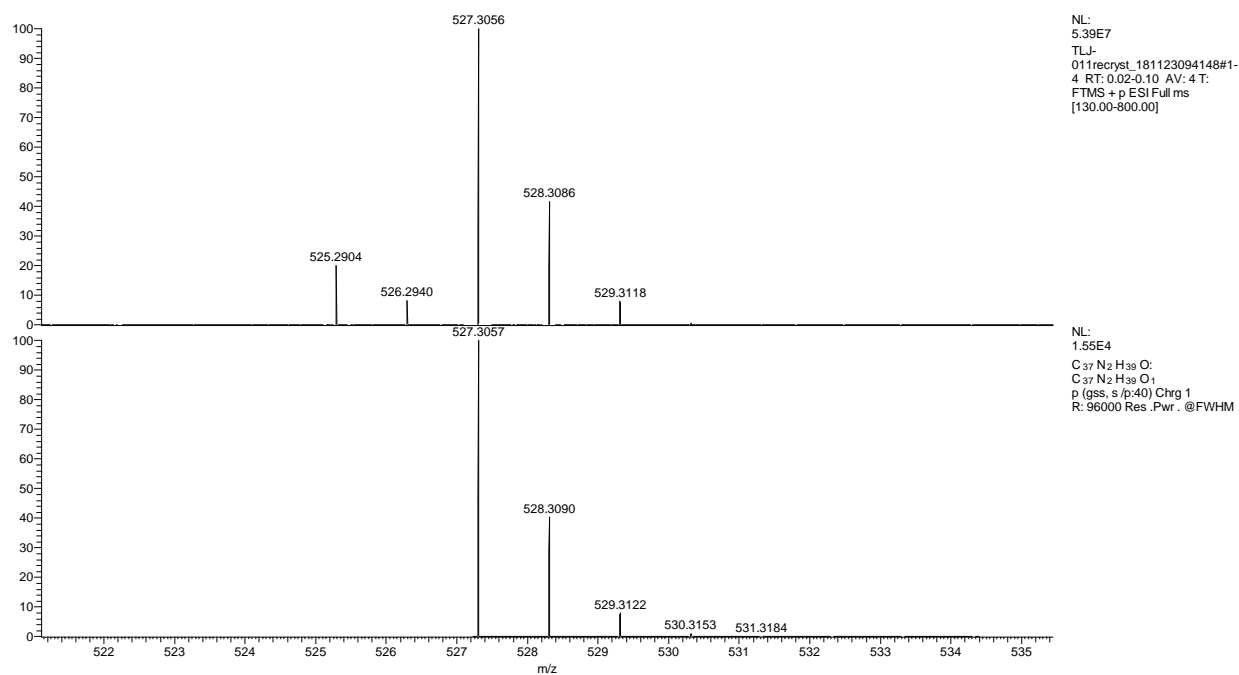
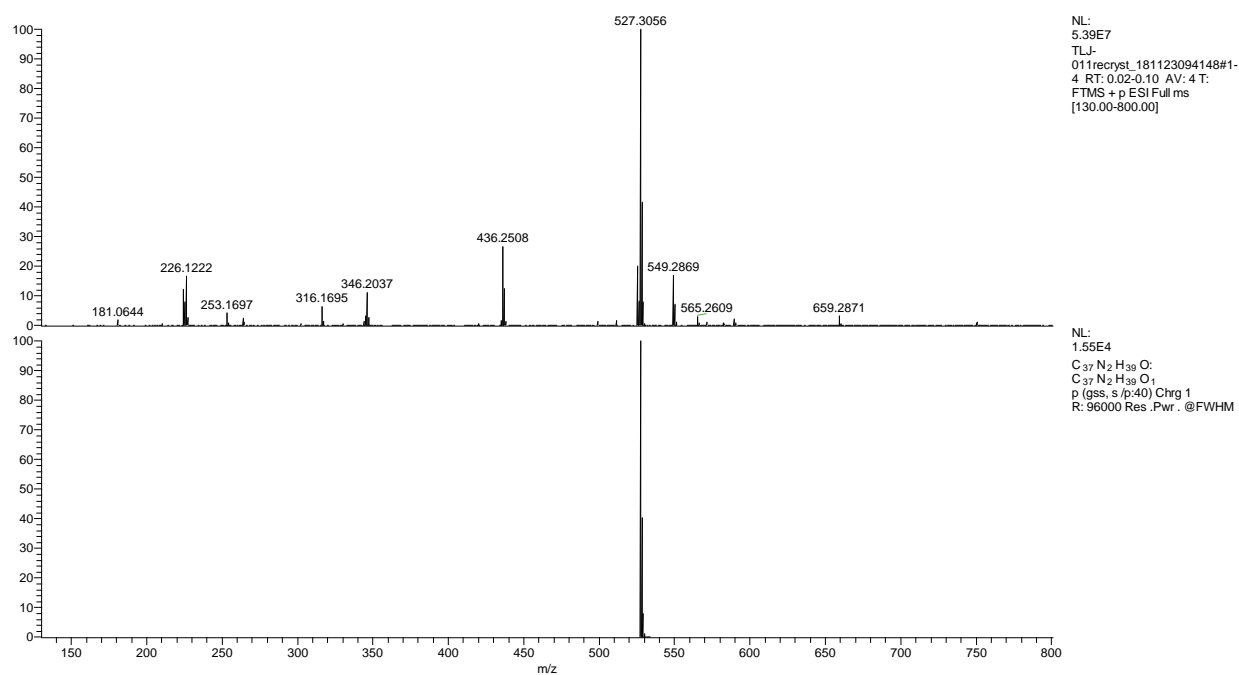
NL:
1.22E8
TLJ-005-4(2)#1-4 RT:
0.02-0.10 AV: 4 T; FTMS + p
ESI Full ms [150.00-800.00]

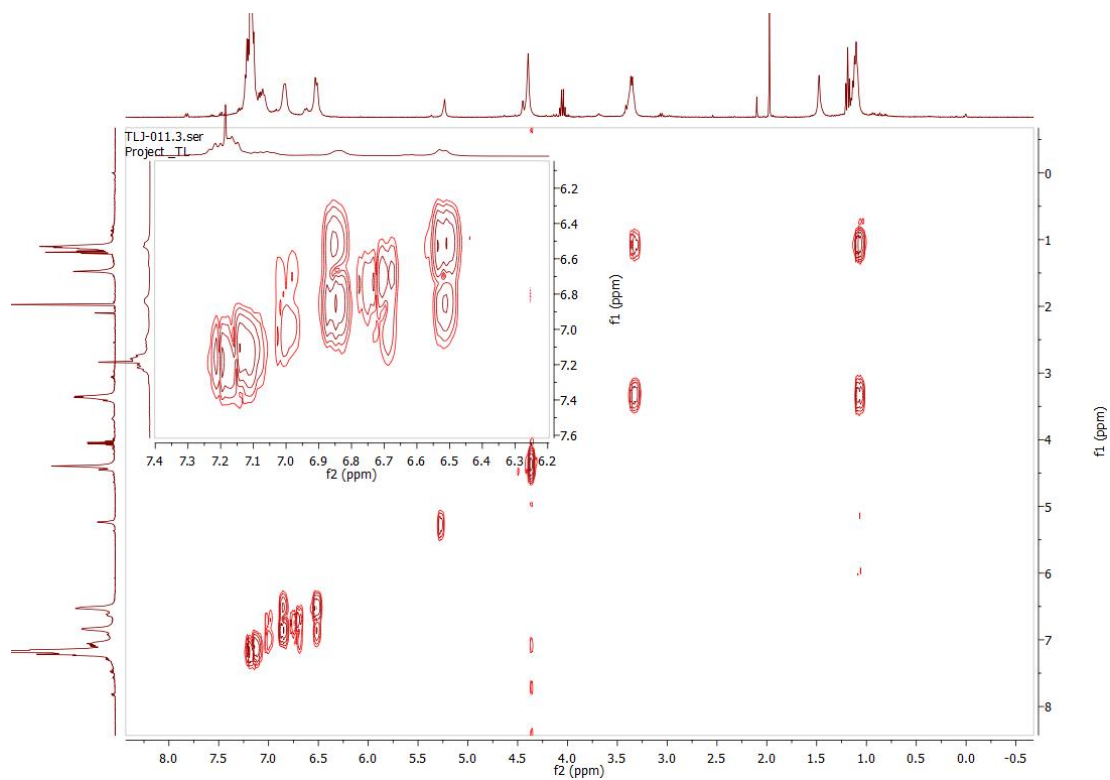
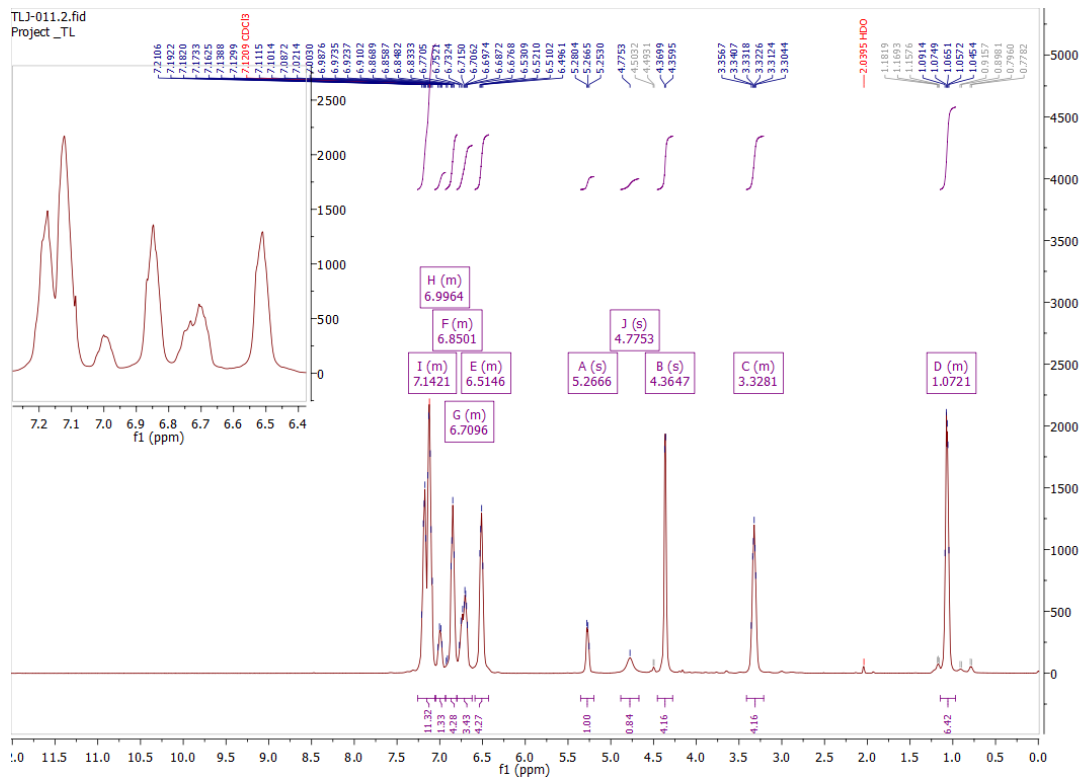
NL:
1.56E4
C₃₇H₃₉N₂:
C₃₇H₃₉N₂:
p (gss, s/p:40) Chrg 1
R: 96000 Res. Pwr. @FWHM

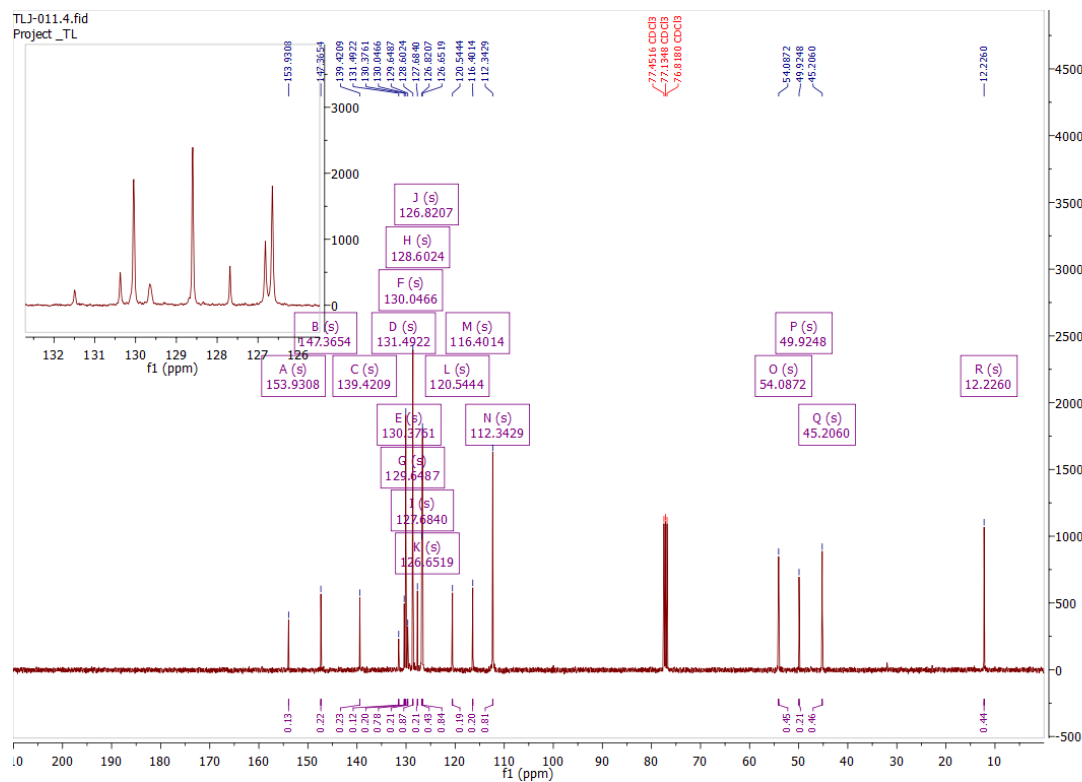




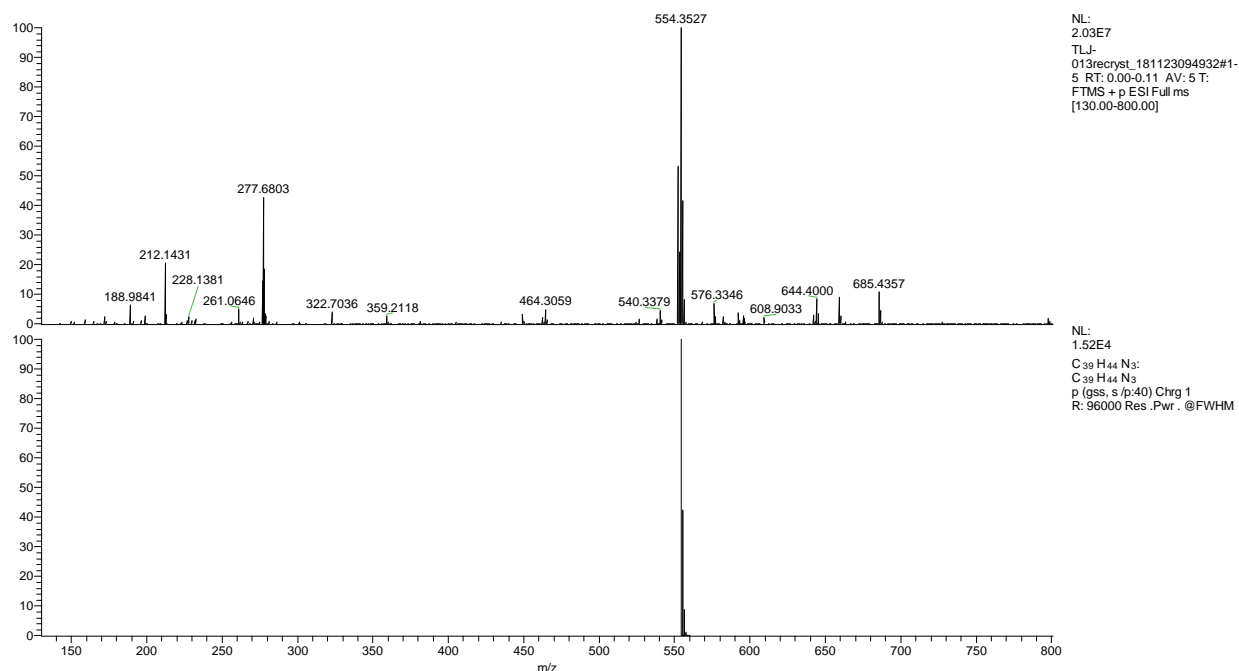
8.1.5 2-(bis(4-(benzyl(ethyl)amino)phenyl)methyl)phenol

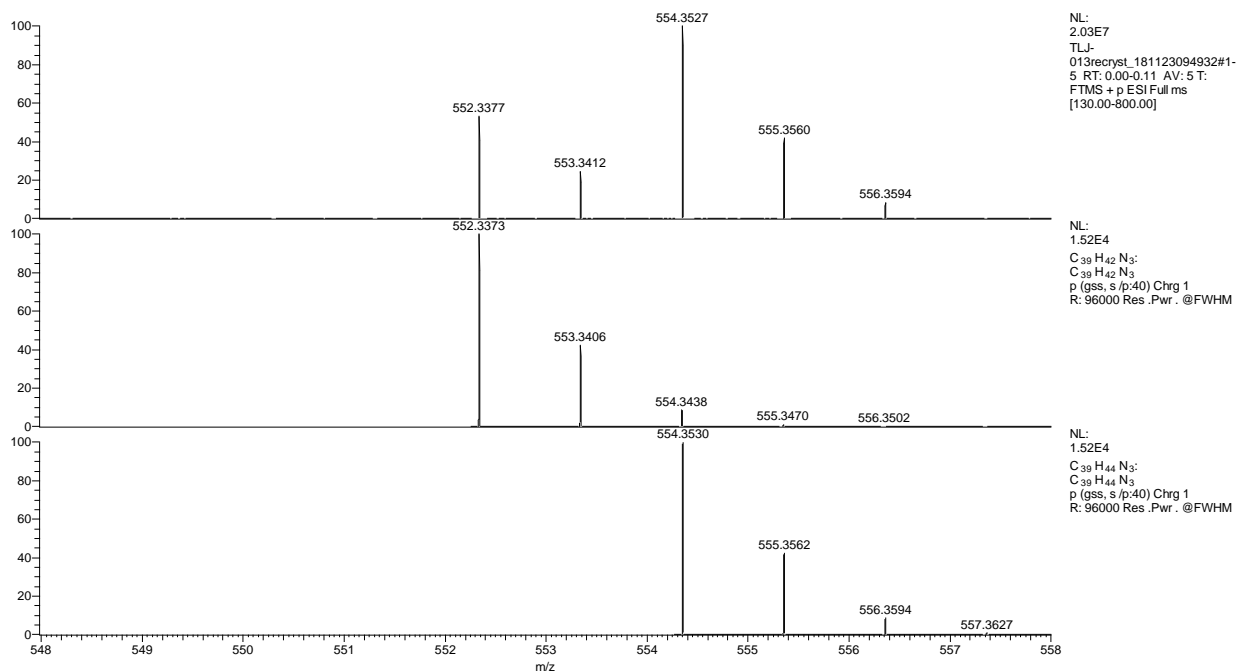




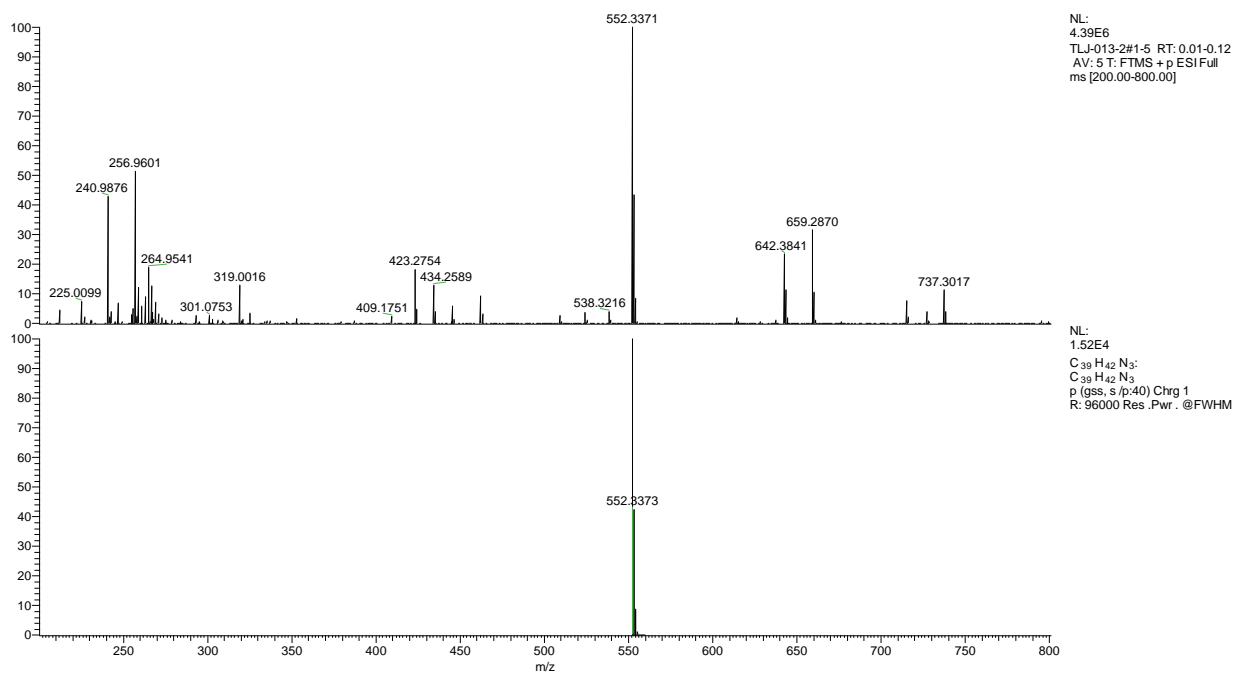


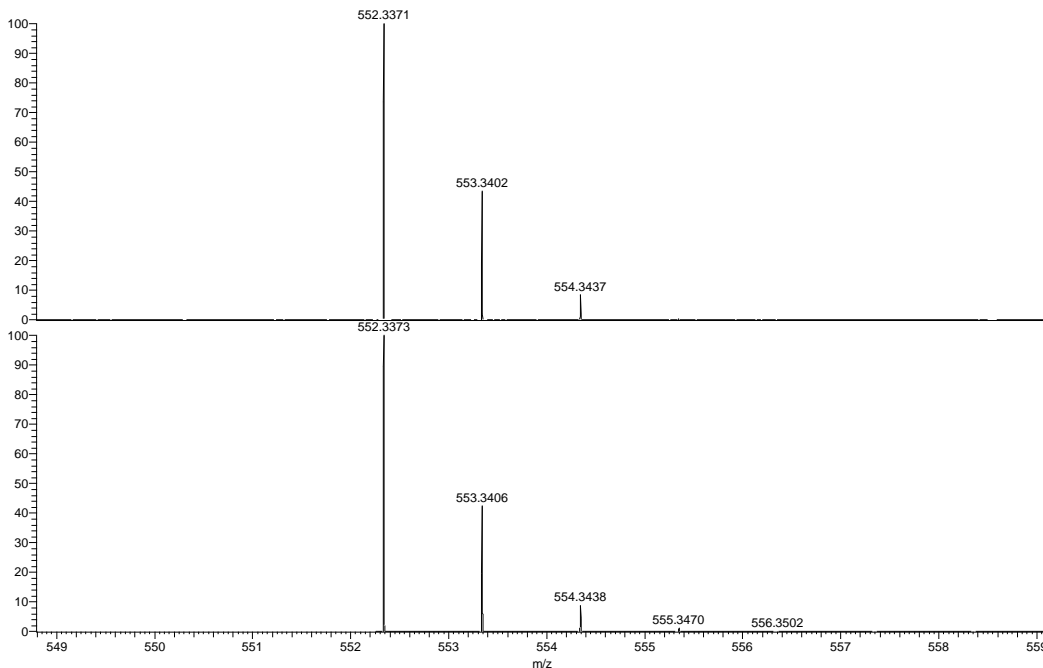
8.1.6 4,4'-((4-(dimethylamino)phenyl)methylene)bis(*N*-benzyl-*N*-ethylaniline)





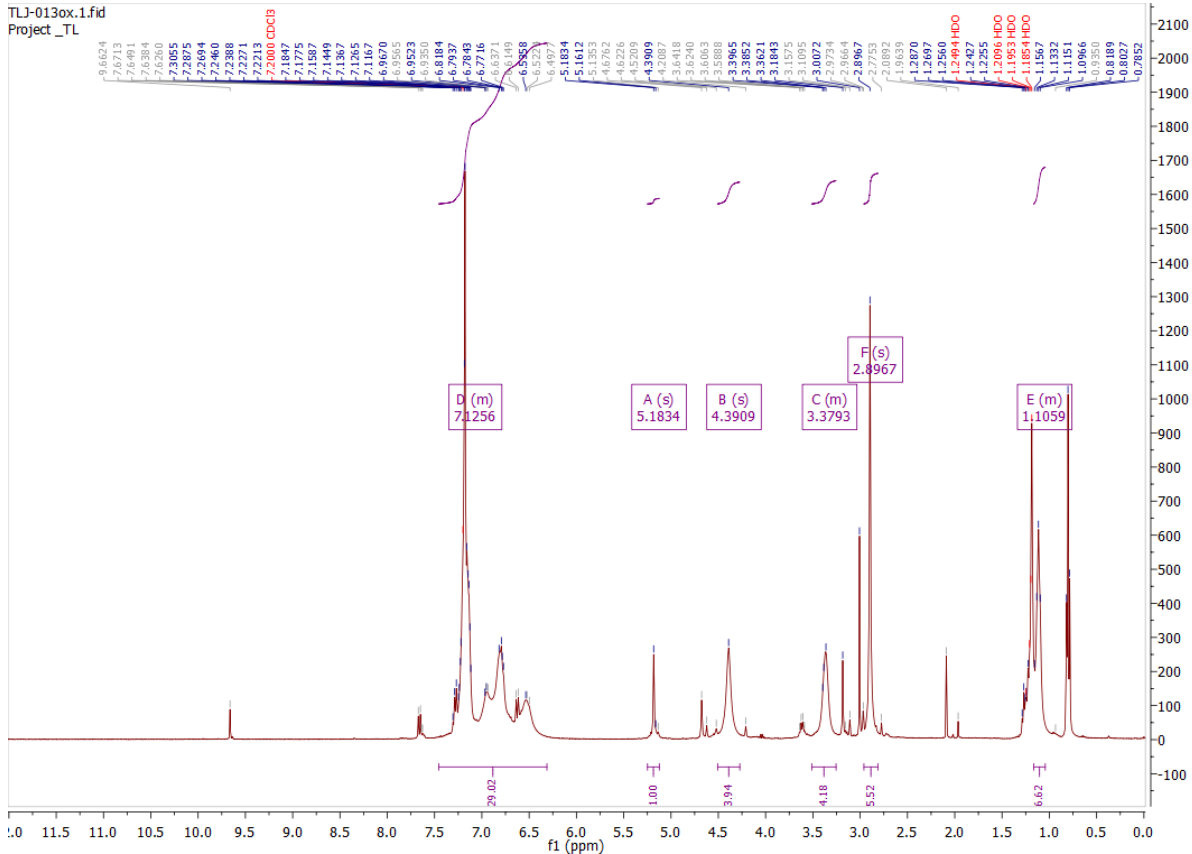
8.1.7 *N*-benzyl-*N*-((1*E*,4*E*)-4-((4-(benzyl(ethyl)amino)phenyl)(4-(dimethylamino)phenyl)-methylene)cyclohexa-2,5-dien-1-ylidene)ethanaminium



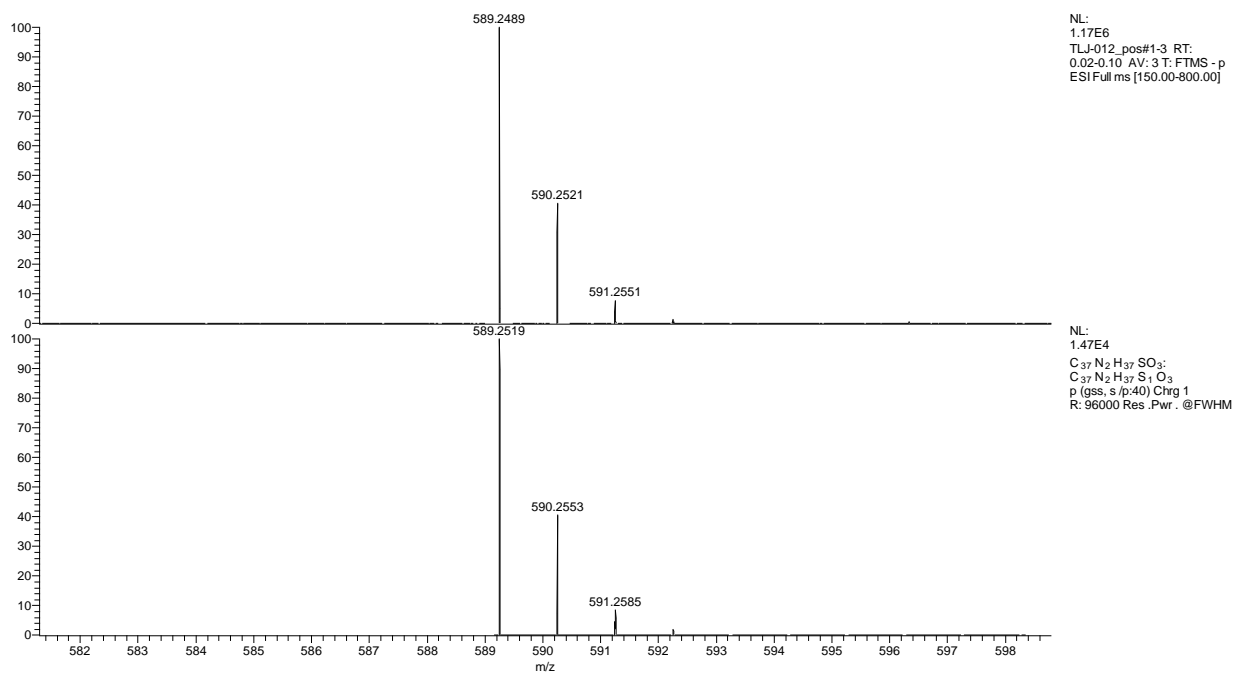
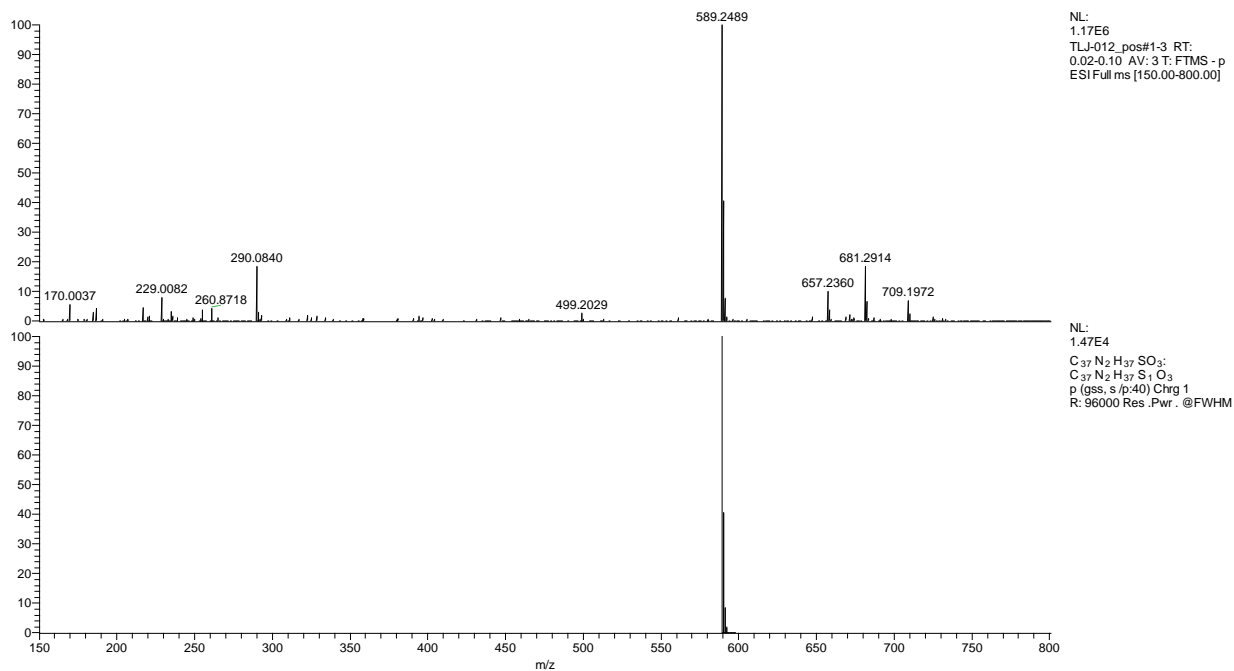


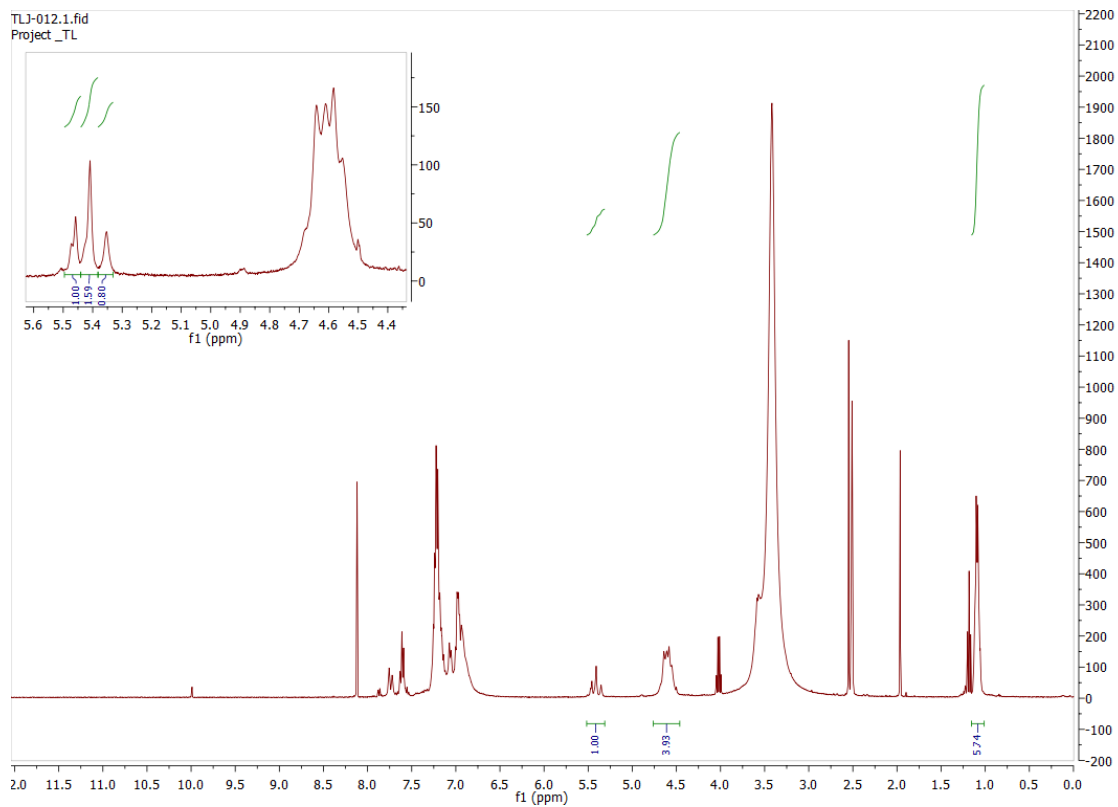
NL:
4.39E6
TLJ-013-2#1-S RT: 0.01-0.12
AV: 5 T: FTMS + p ESI Full
ms [200.00-800.00]

NL:
1.52E4
C₃₉ H₄₂ N₄:
C₃₉ H₄₂ N₄
p (gss, s/p:40) Chrg 1
R: 96000 Res. Pwr. @FWHM



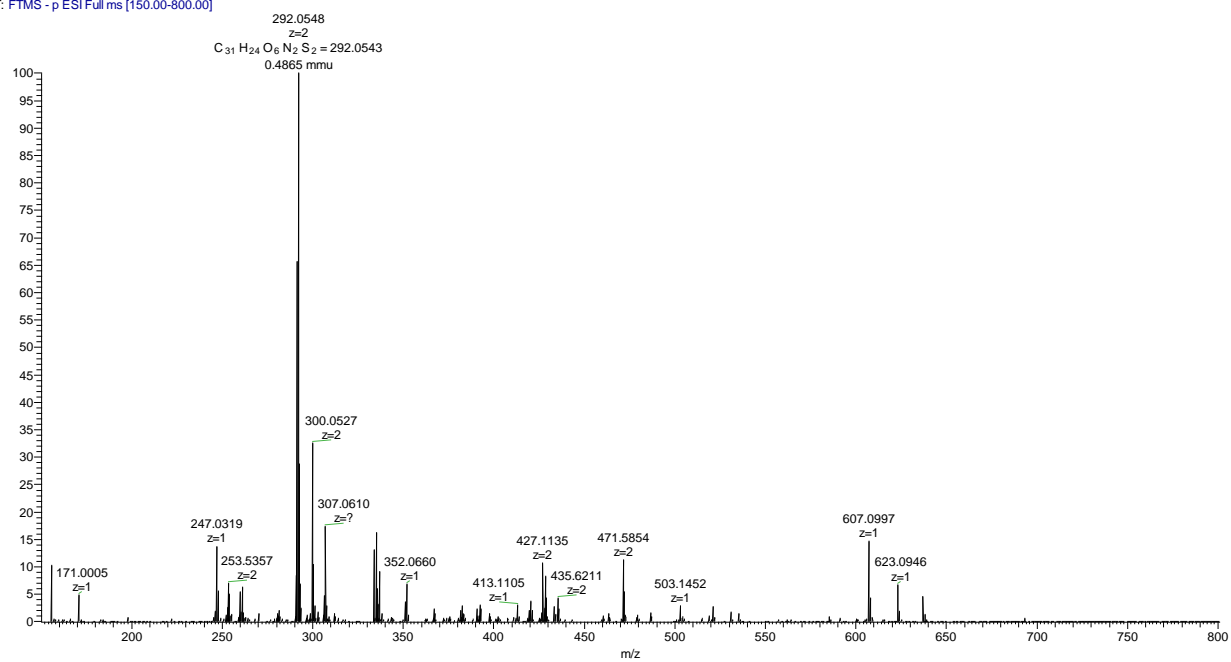
8.1.8 3-(((4-((4-(benzyl(ethyl)amino)phenyl)(phenyl)-methyl)phenyl)(ethyl)amino)methyl)-benzenesulfonic acid

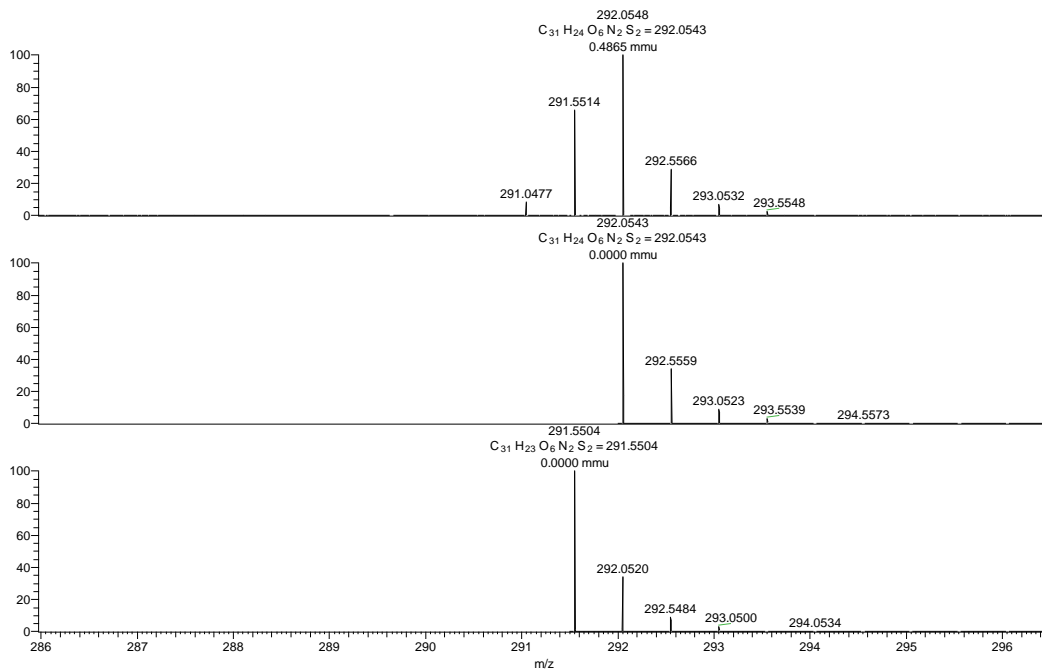




8.1.9 3,3'-(((phenylmethylene)bis(4,1-phenylene))bis-(azanediyl))dibzenesulfonic acid

TLJ-014_190430130101 #1-4 RT: 0.02-0.11 AV: 4 NL: 7.30E6
T: FTMS - p ESI Full.ms [150.00-800.00]

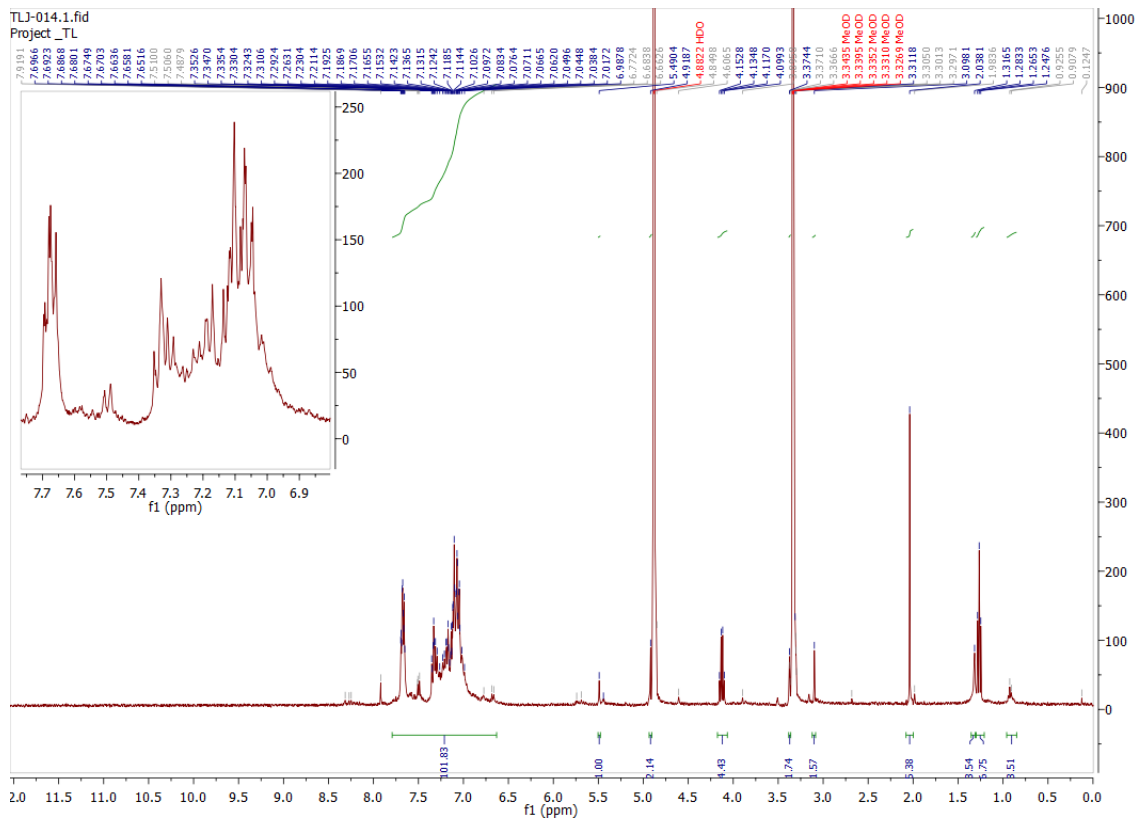




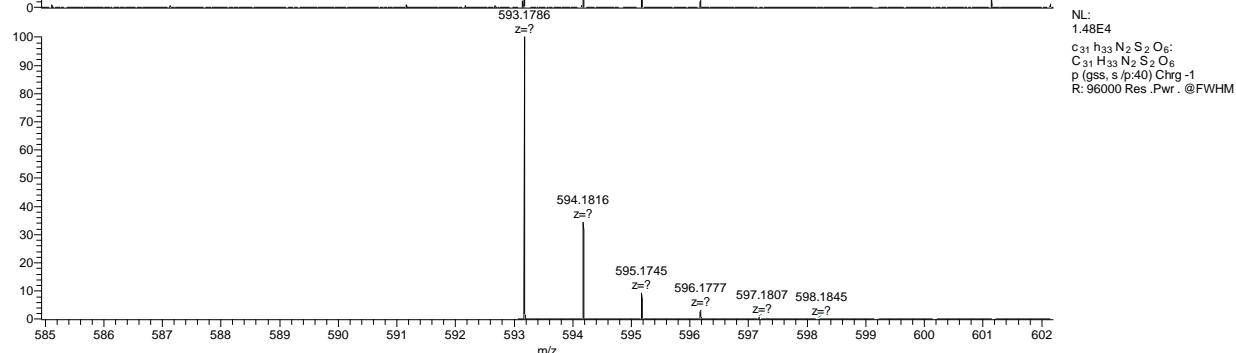
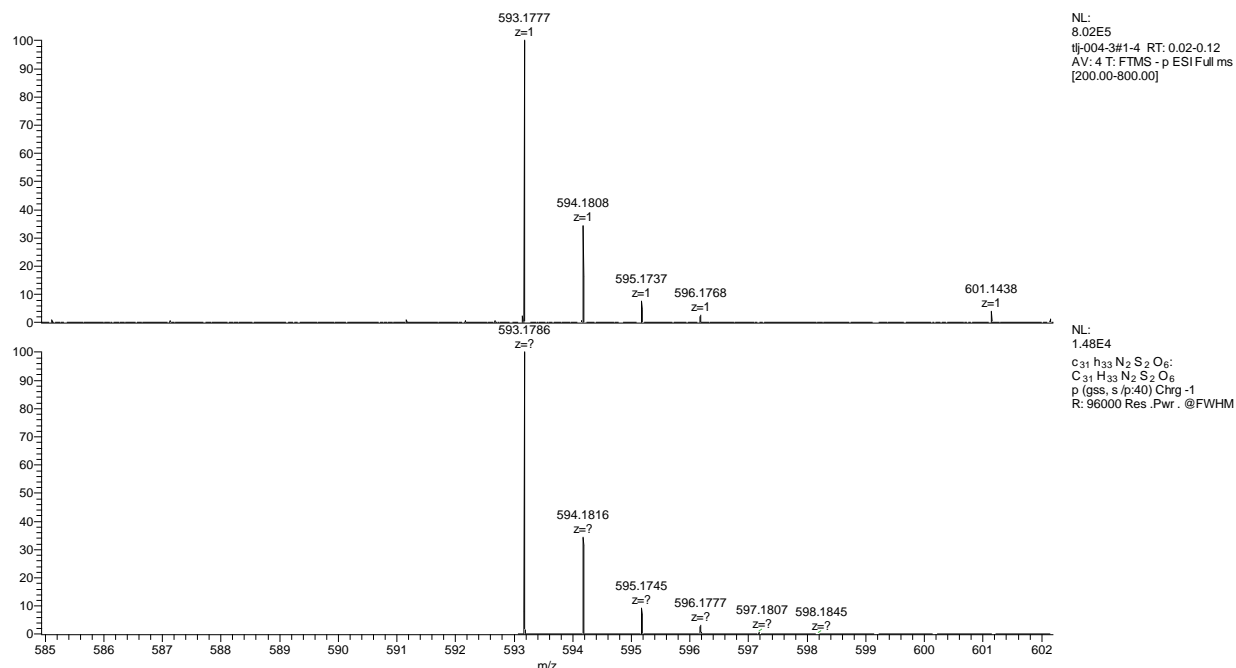
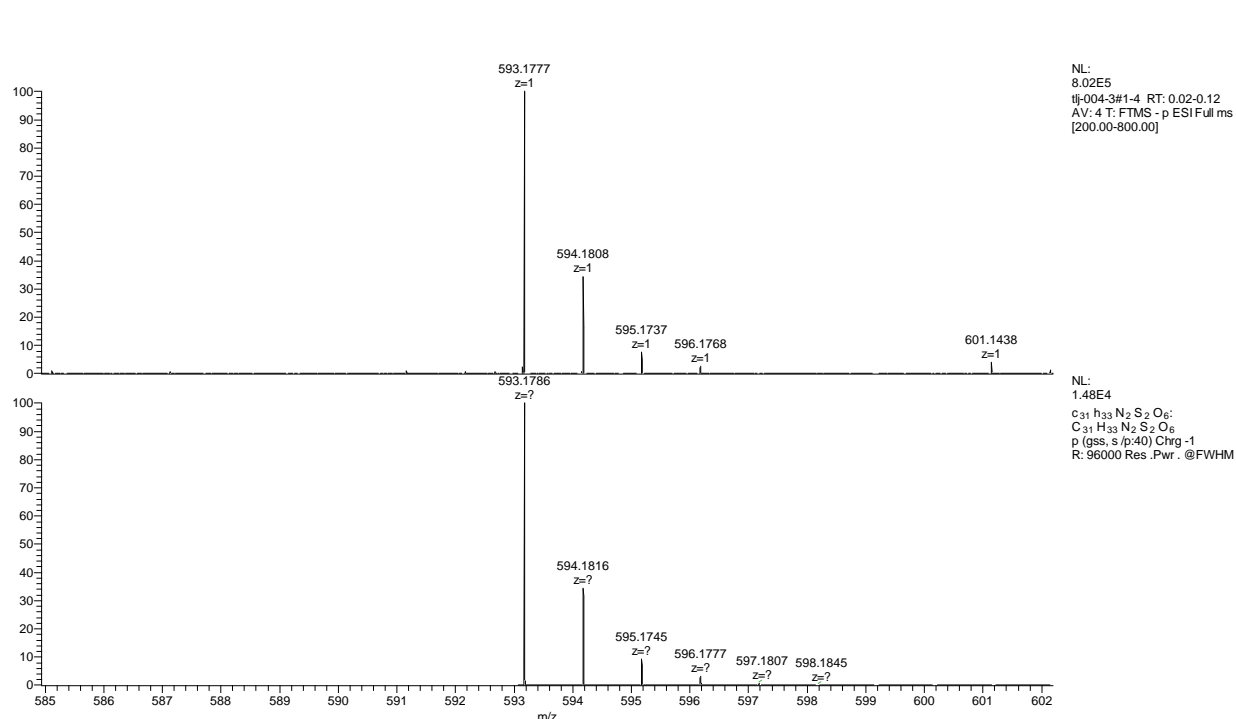
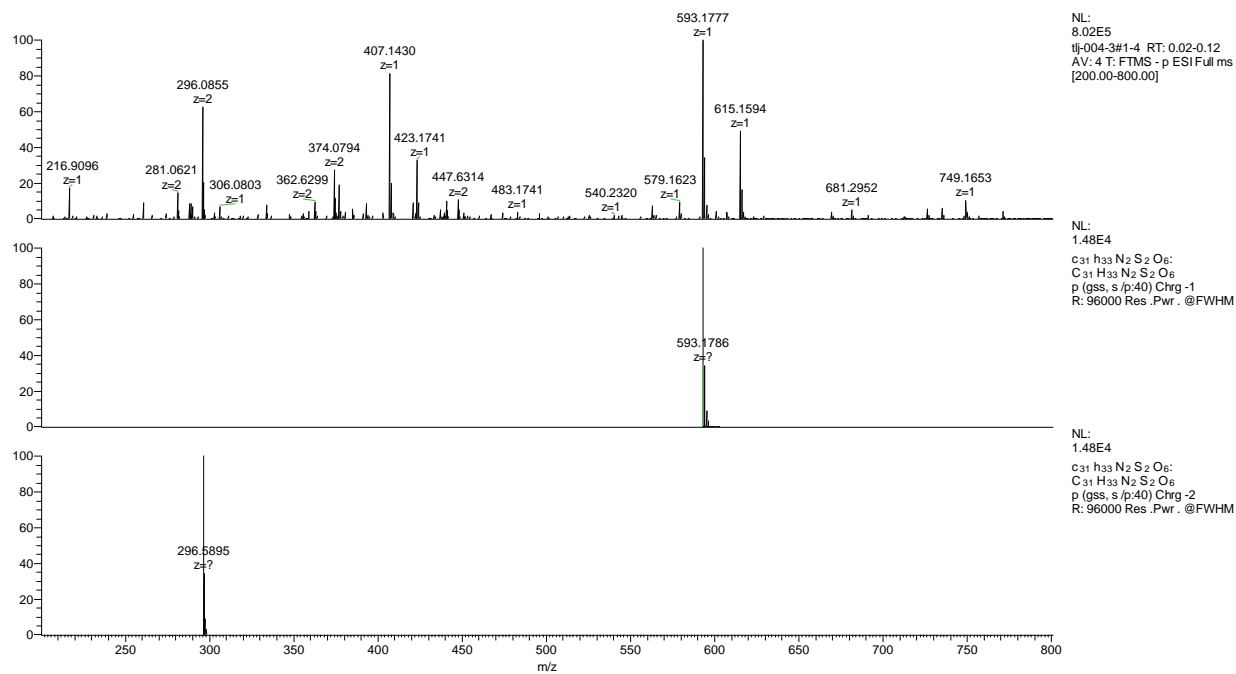
NL:
7.30E6
TLJ-014_190430130101#1-4
RT: 0.02-0.11 AV: 4 T: FTMS
- p ESI Full ms
[150.00-800.00]

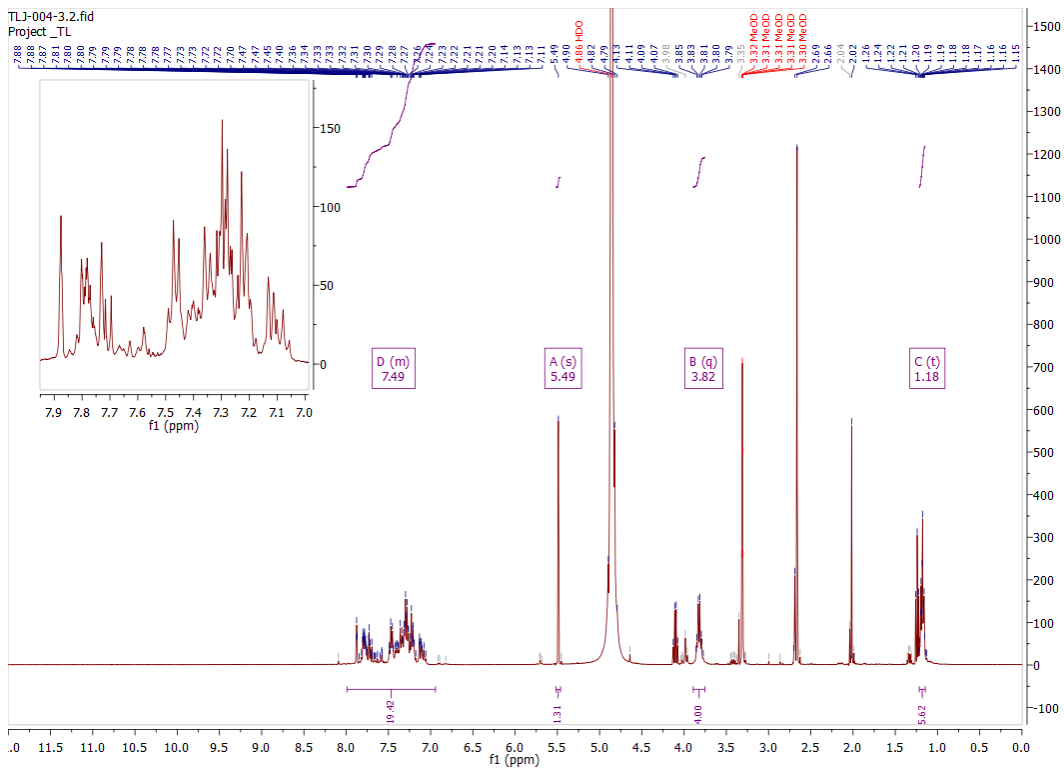
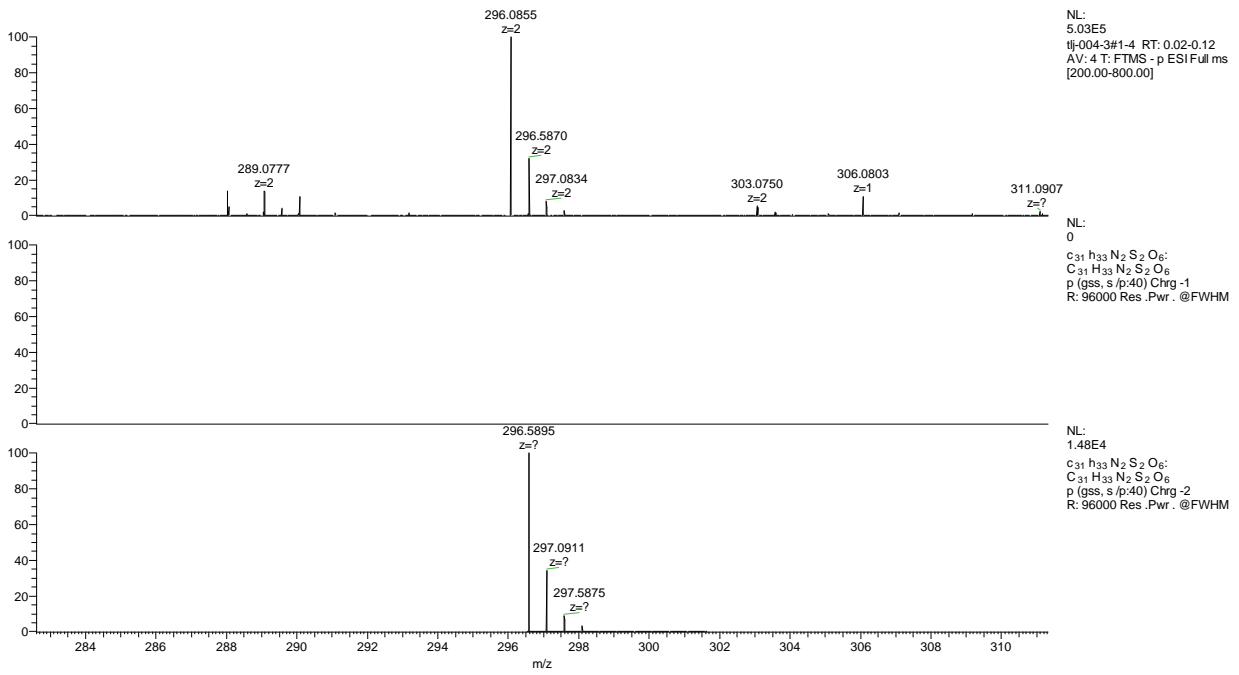
NL:
1.48E4
 $C_{31}H_{24}O_6N_2S_2$
 $C_{31}H_{24}O_6N_2S_2$
p (gss, s (p/40) Chrg -2
R: 96000 Res. Pwr. @FWHM

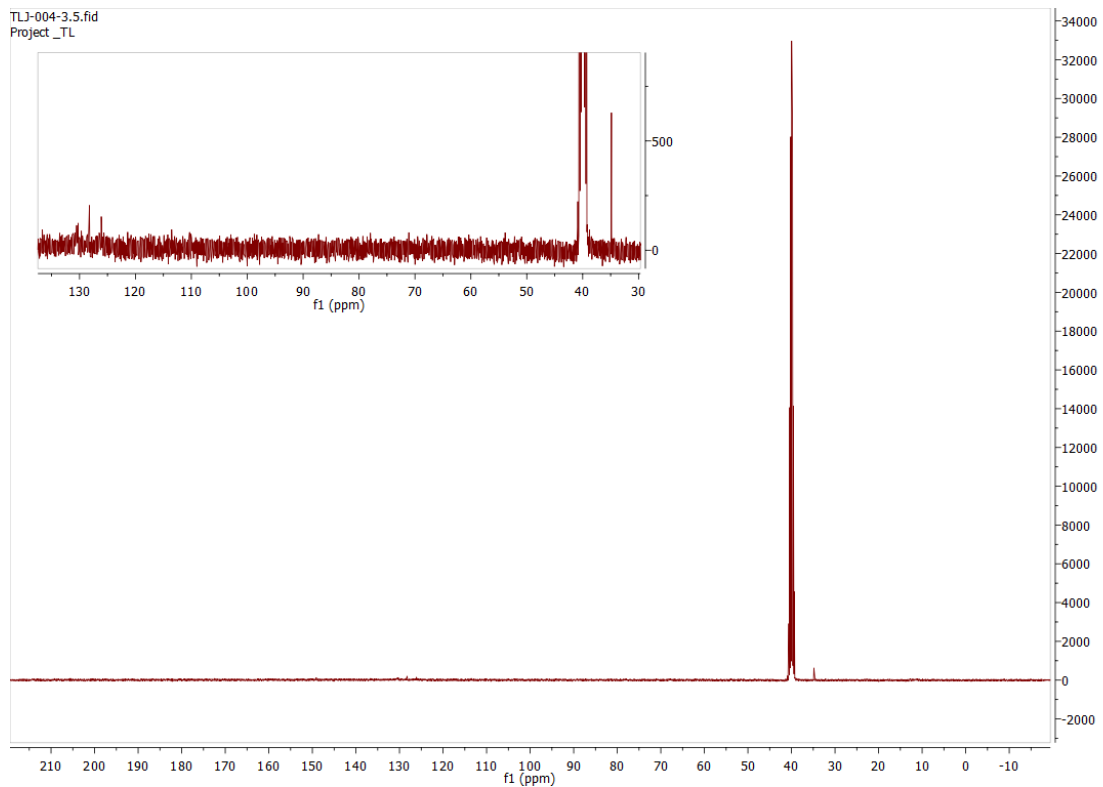
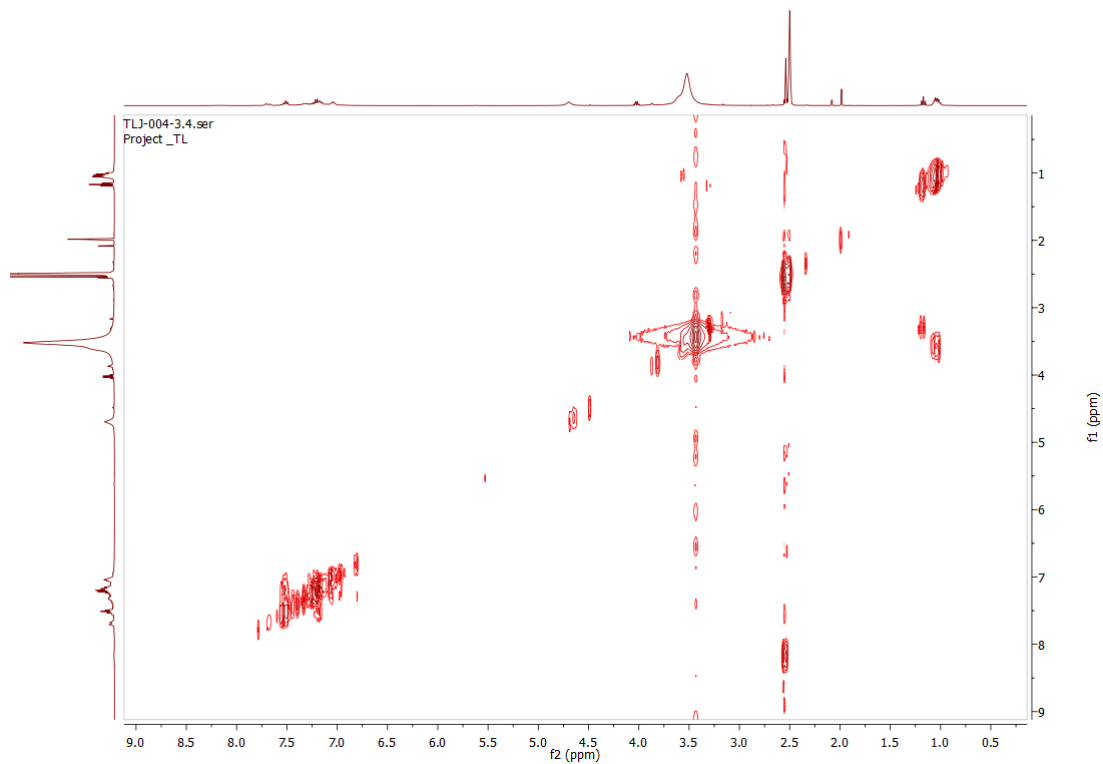
NL:
1.48E4
 $C_{31}H_{23}O_6N_2S_2$
 $C_{31}H_{23}O_6N_2S_2$
p (gss, s (p/40) Chrg -2
R: 96000 Res. Pwr. @FWHM



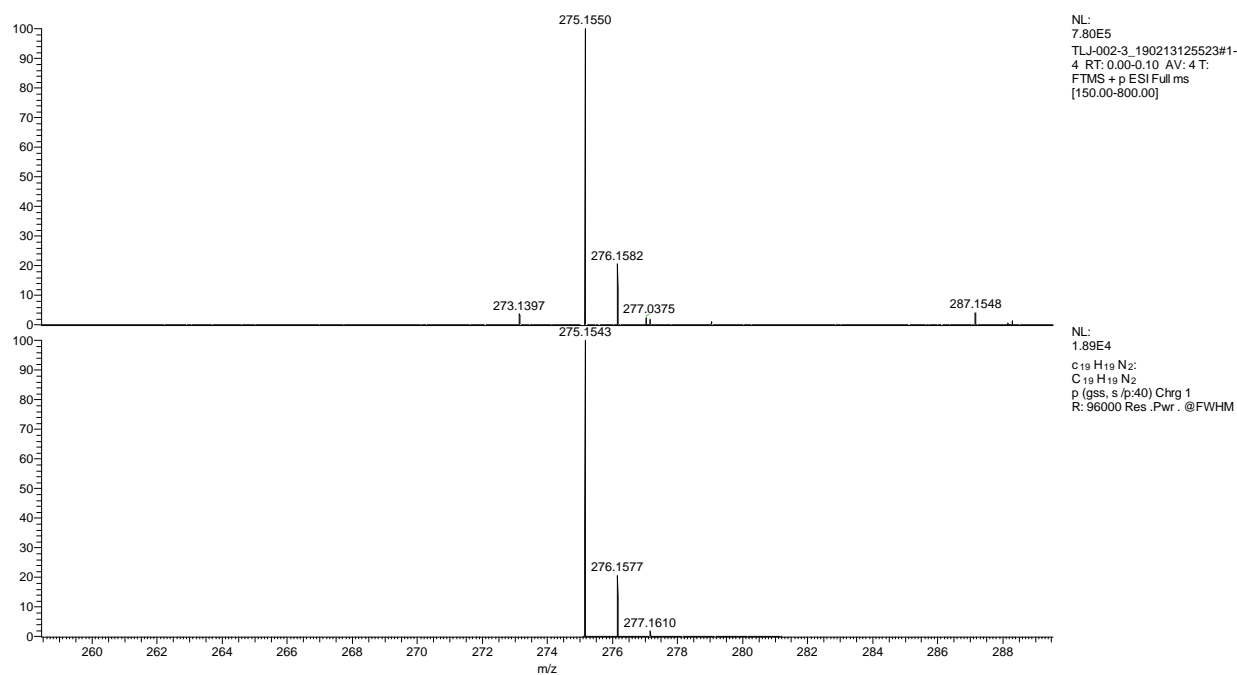
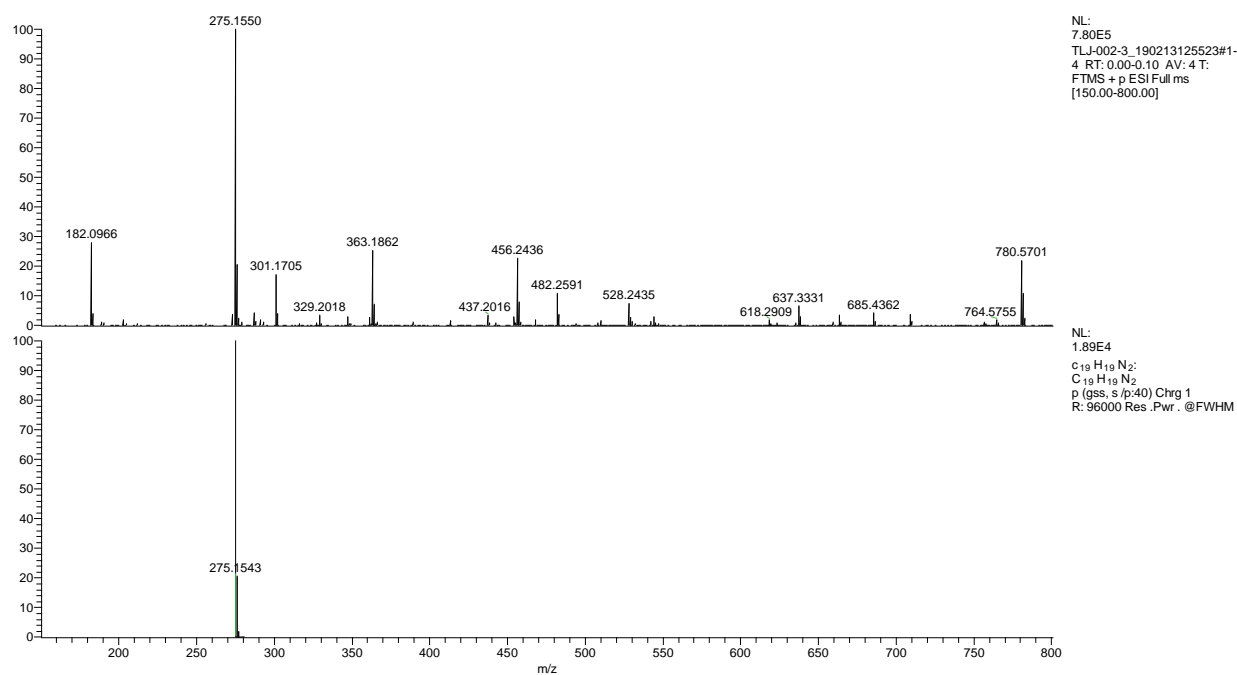
8.1.10 3,3'-(((methylenebis(4,1-phenylene))bis(ethylazanediyl))bis(methylene))-
dibzenesulfonic acid from

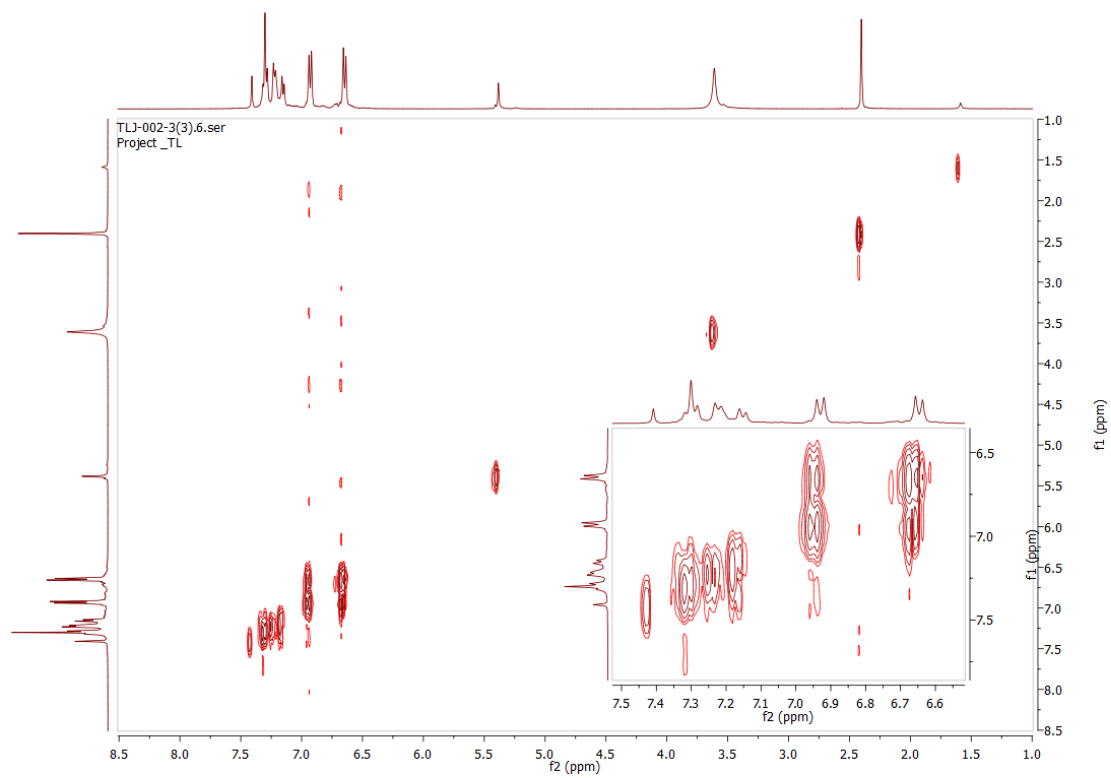
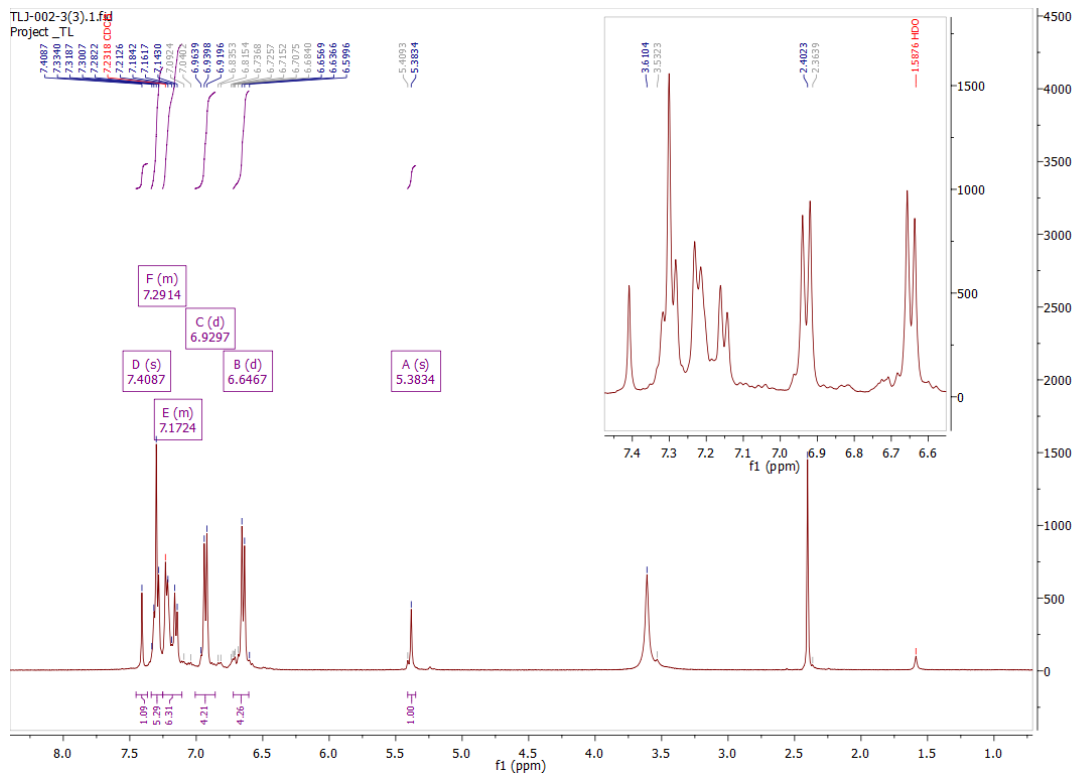


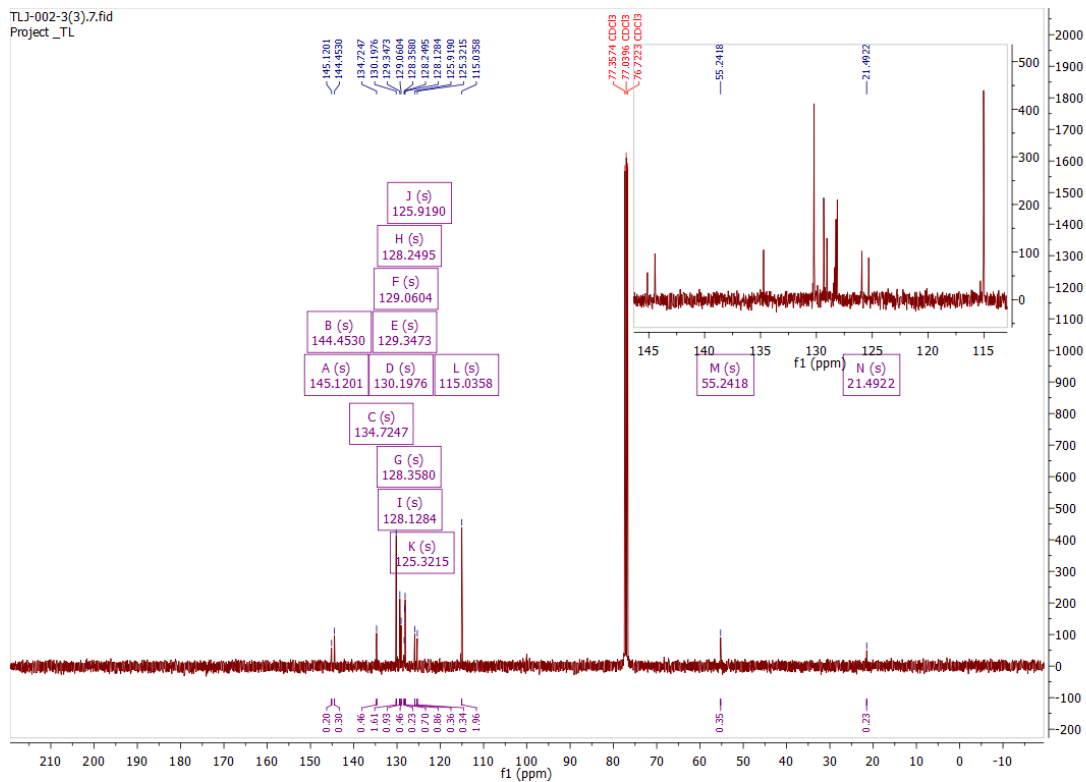




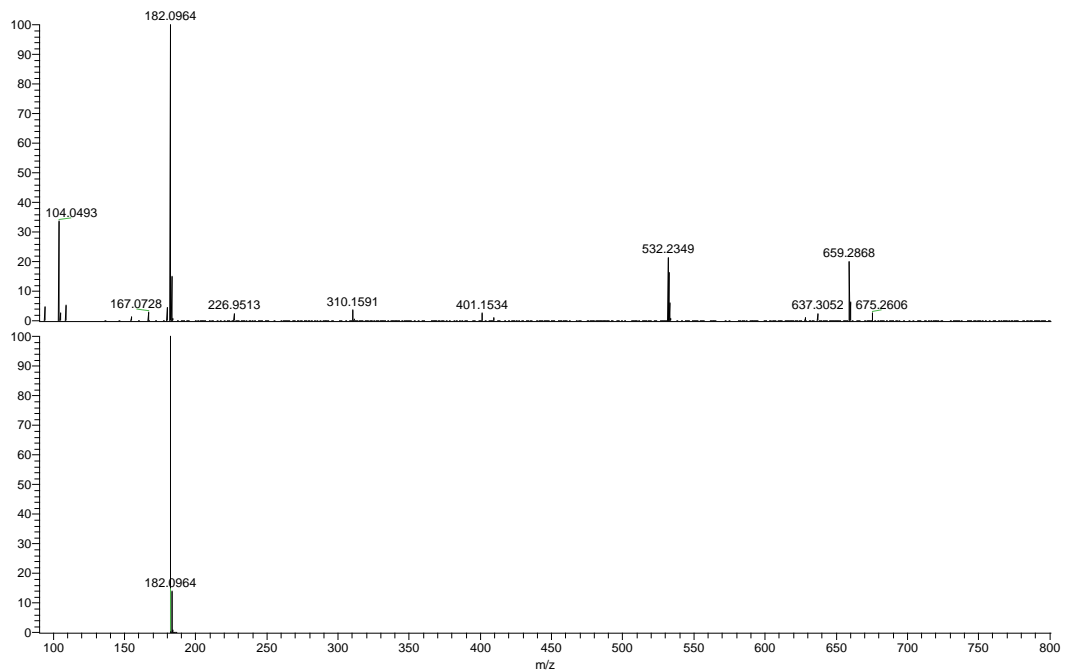
8.1.11 4,4'-(phenylmethylene)dianiline

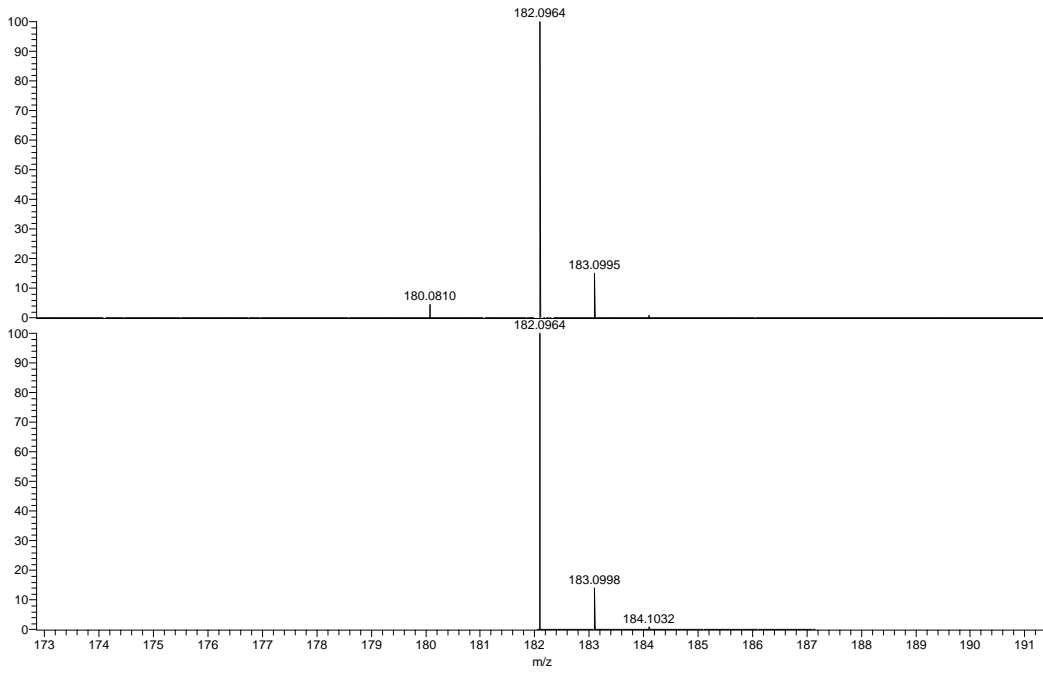






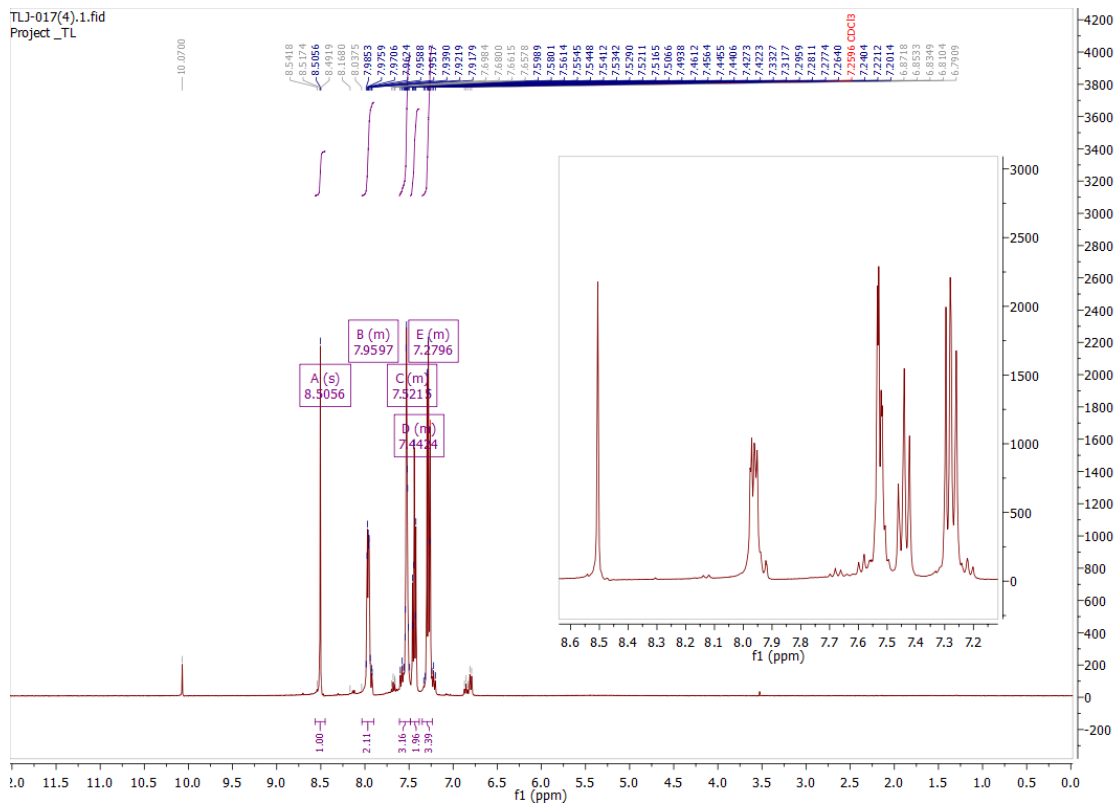
8.1.12 Synthesis of (*E*)-*N*,1-diphenylmethanimine



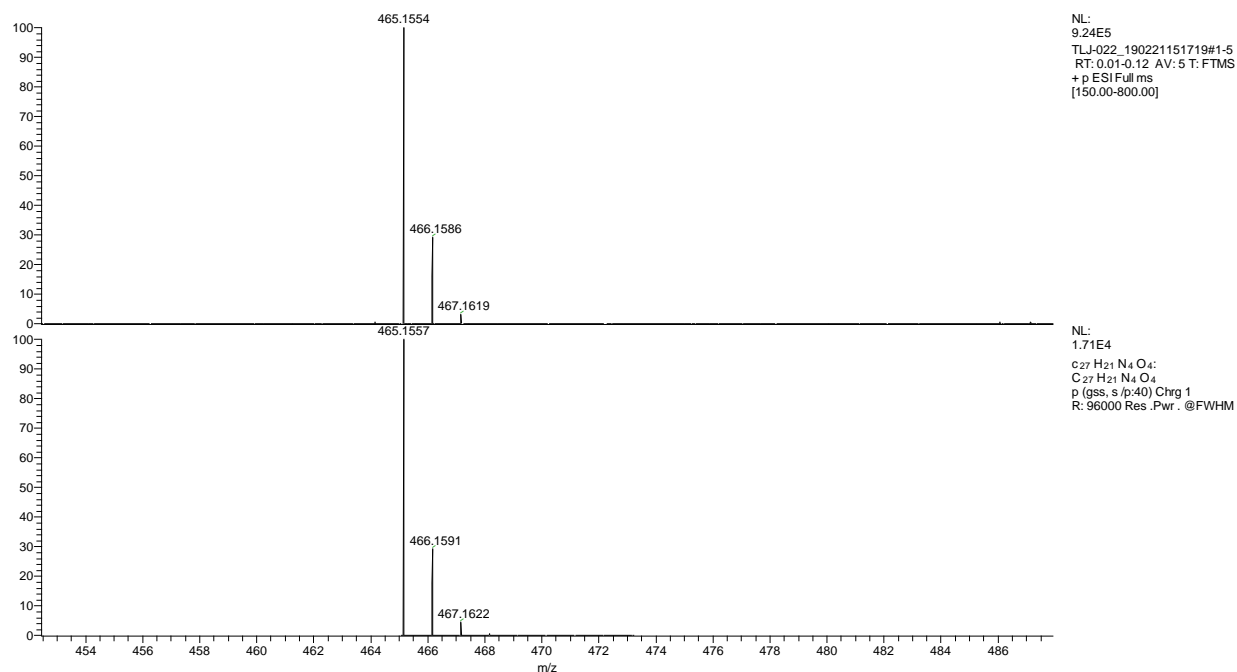
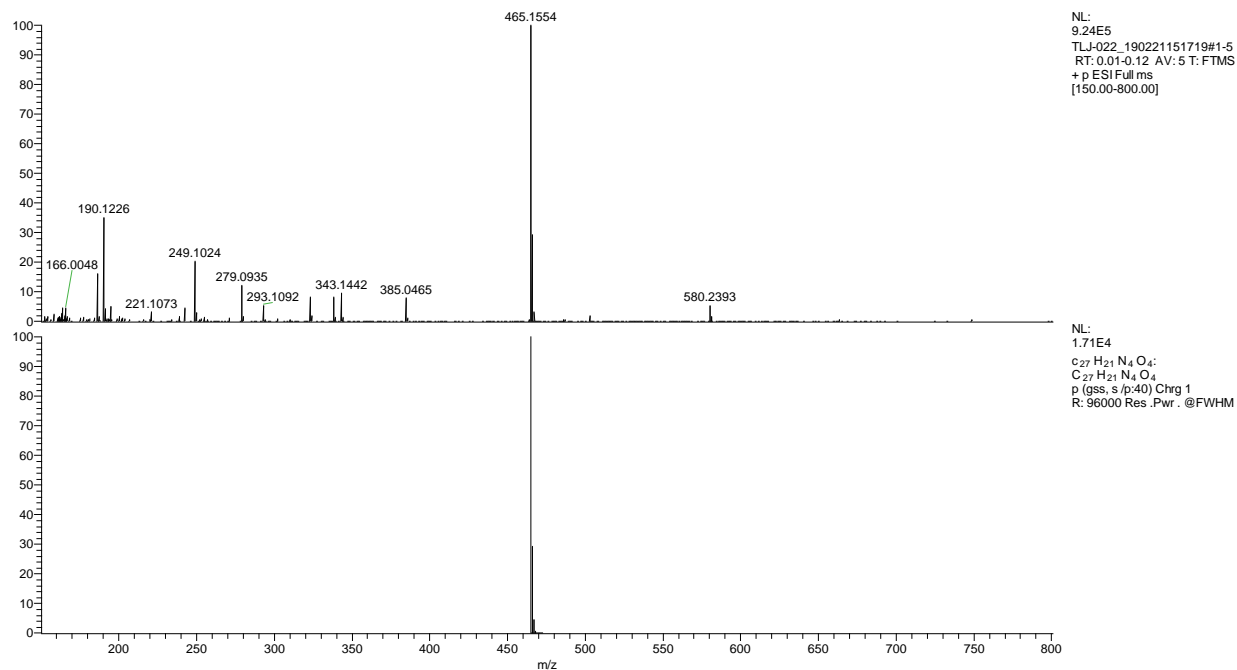


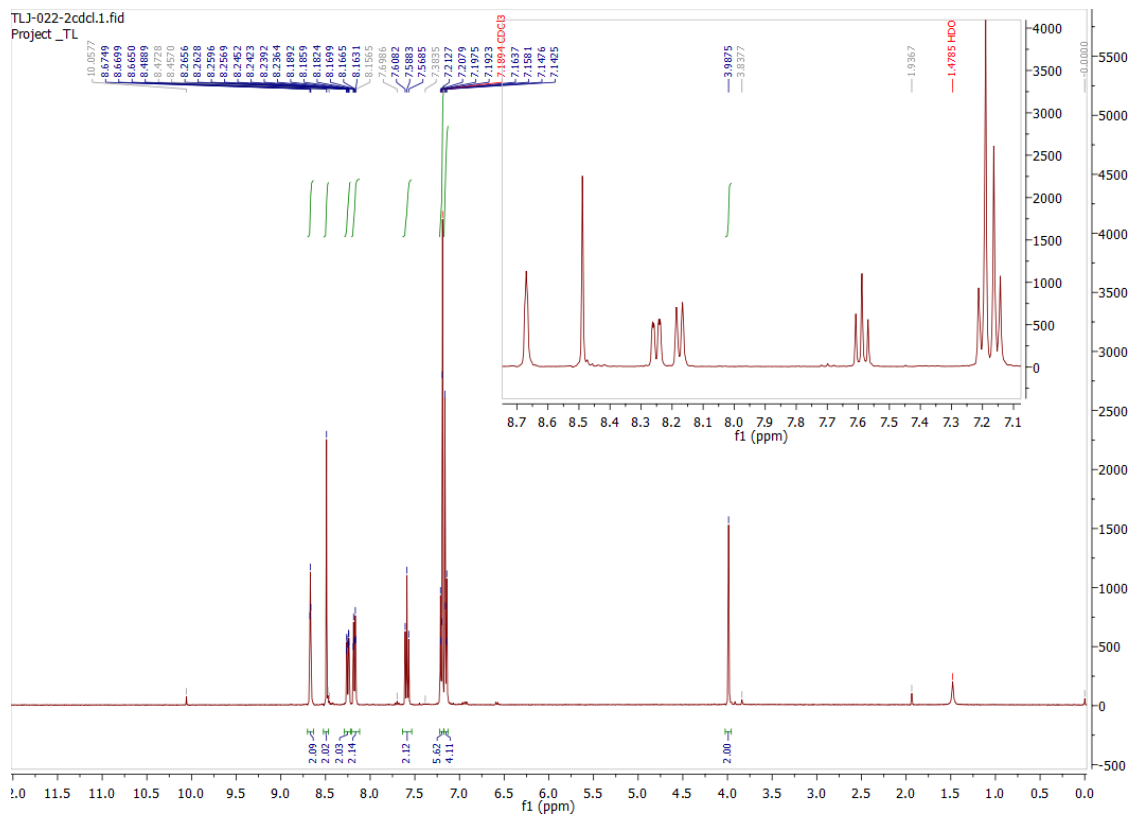
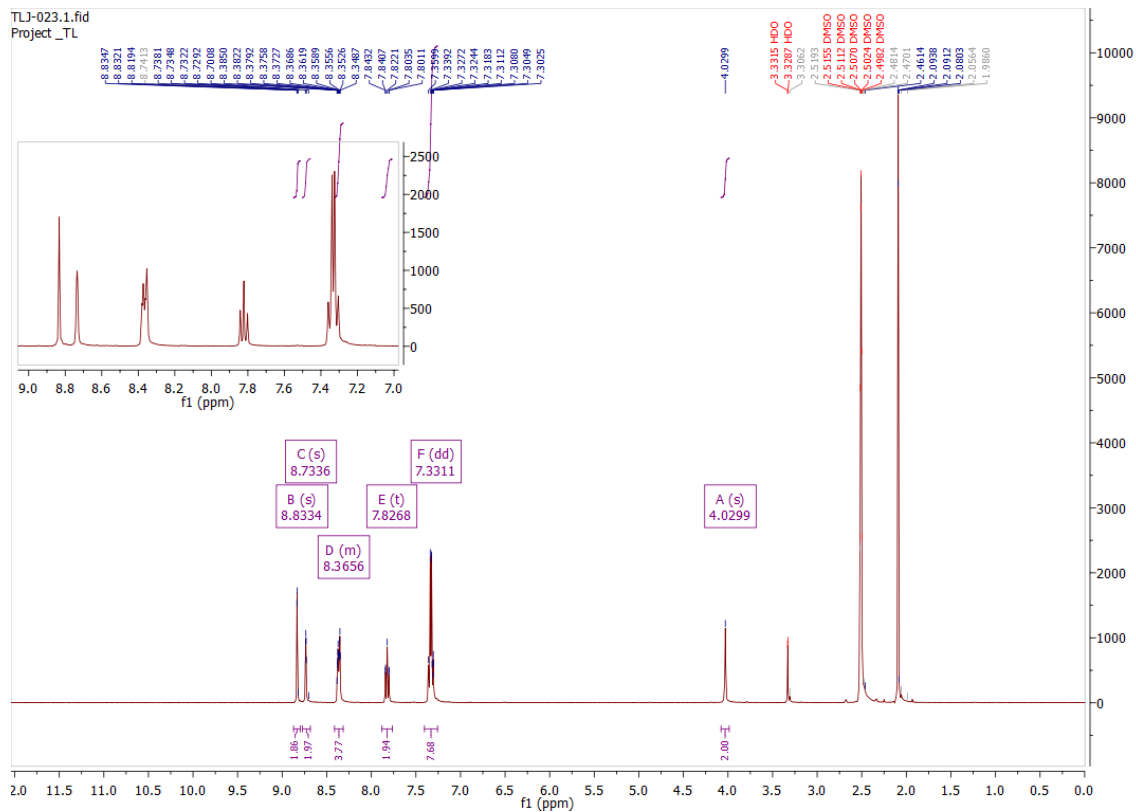
NL:
1.28E7
TLJ-017_pos#1-4 RT:
0.02-0.11 AV: 4 T: FTMS + p
ESI Full ms [90.00-800.00]

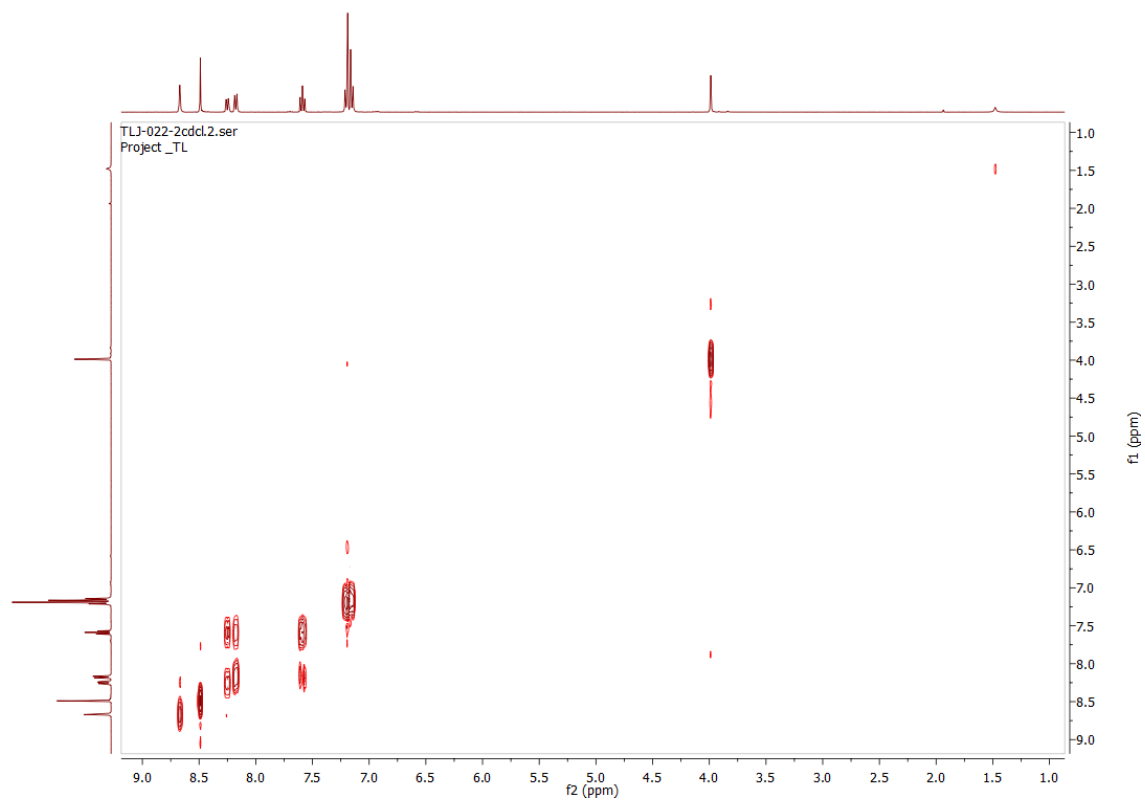
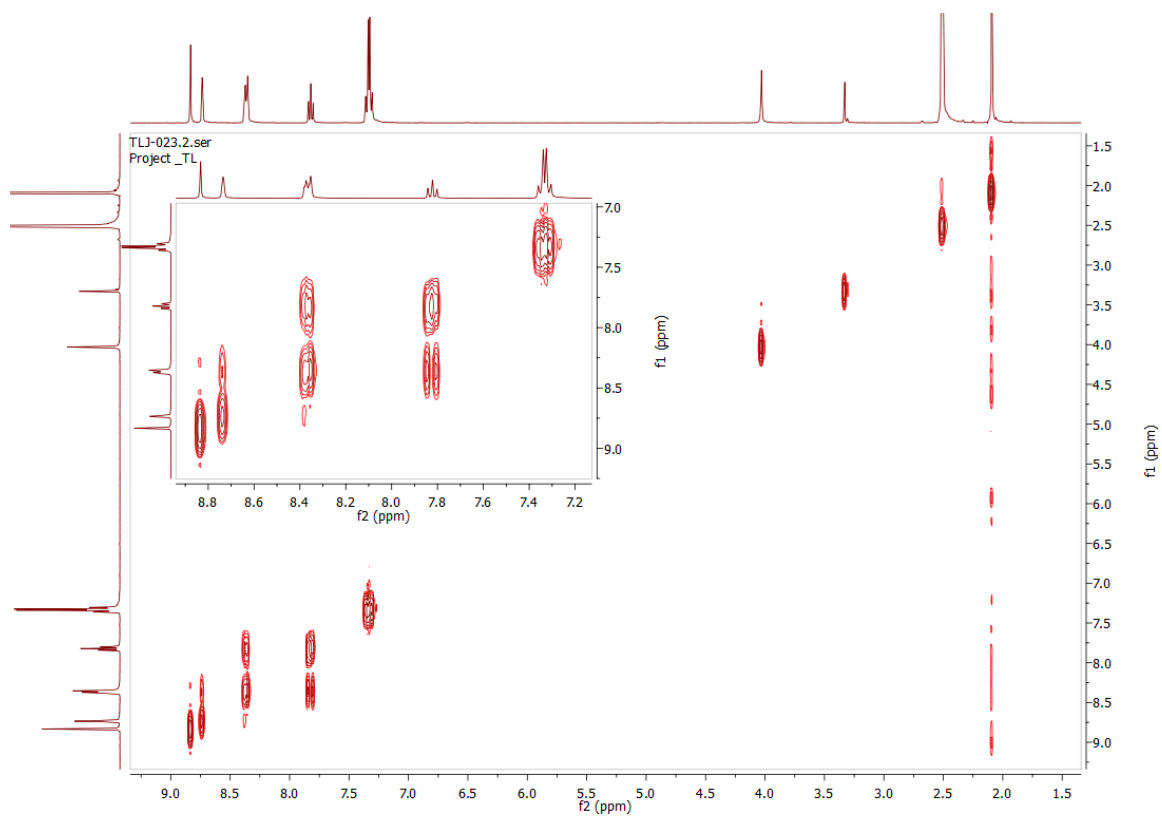
NL:
2.03E4
C₁₃H₁₂N:
C₁₃H₁₂N:
p (gss, s/p:40) Chrg 1
R: 96000 Res. Pwr. @FWHM

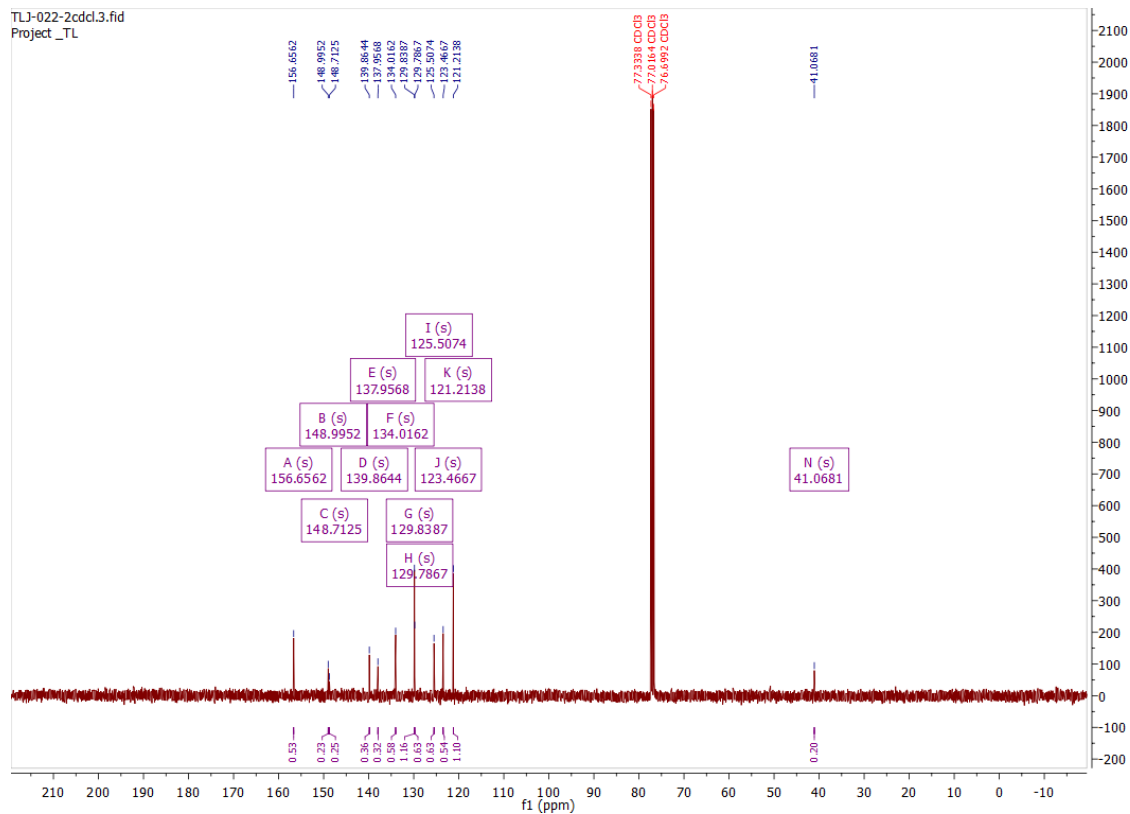
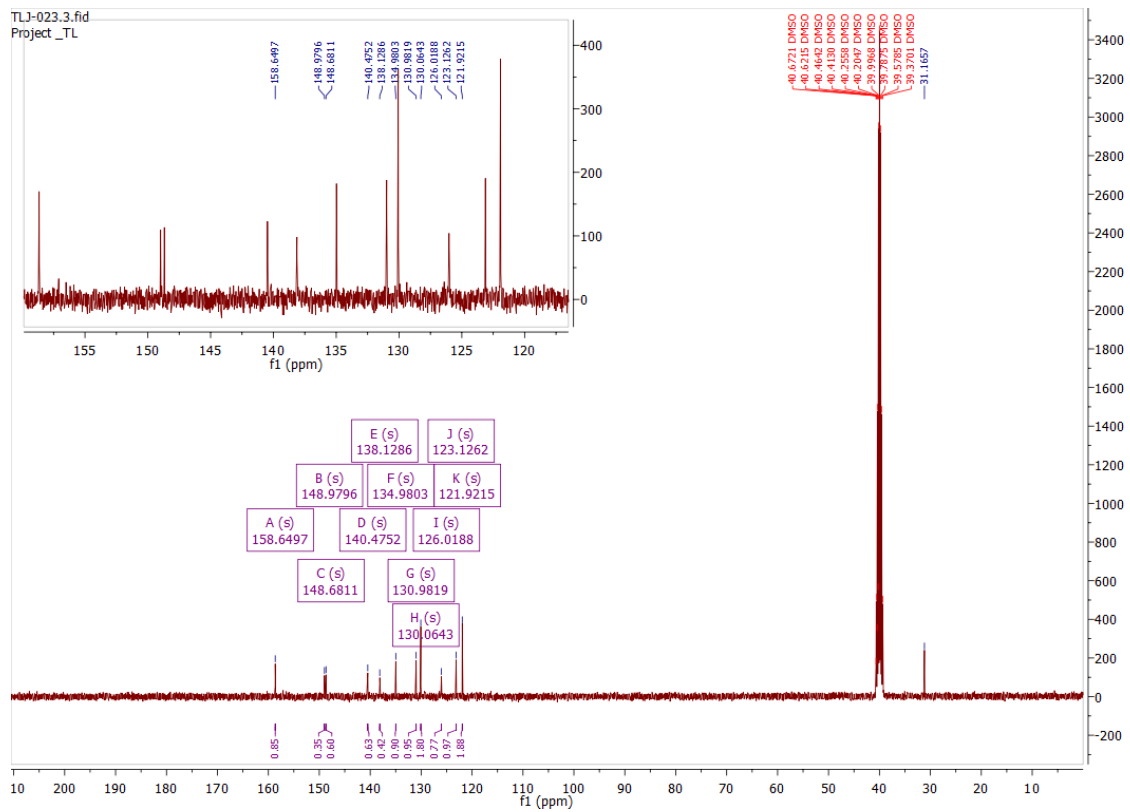


8.1.13 (1E,1'E)-N,N'-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine)

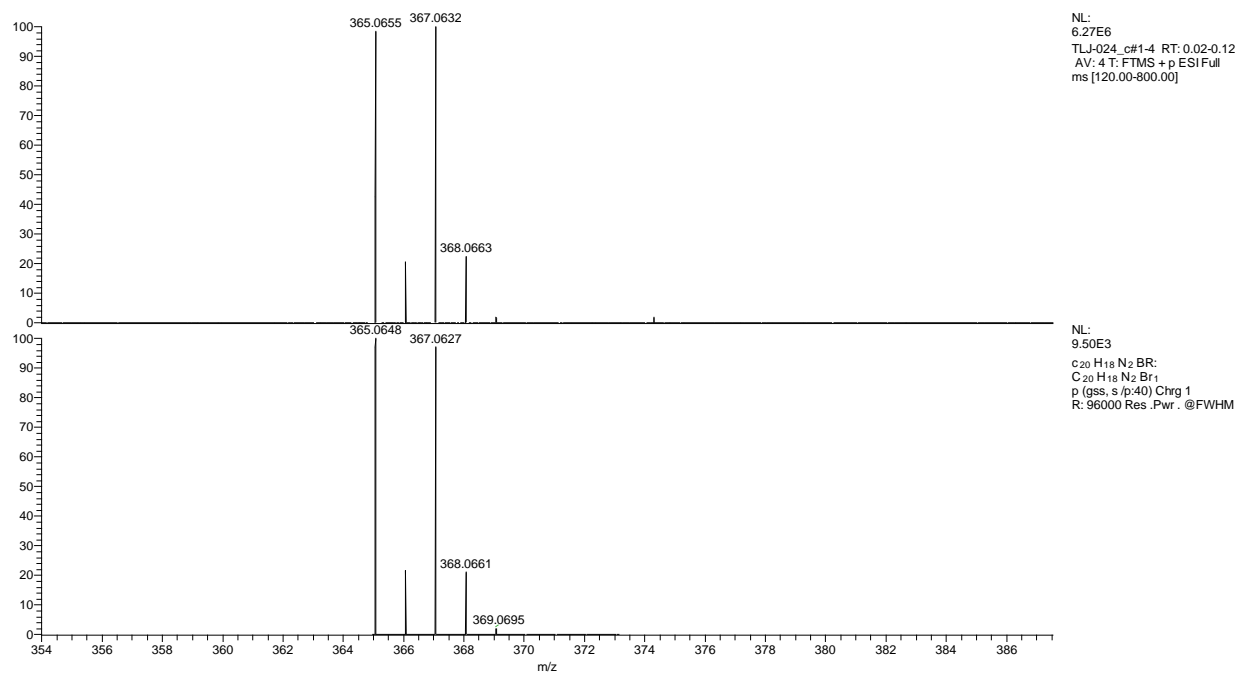
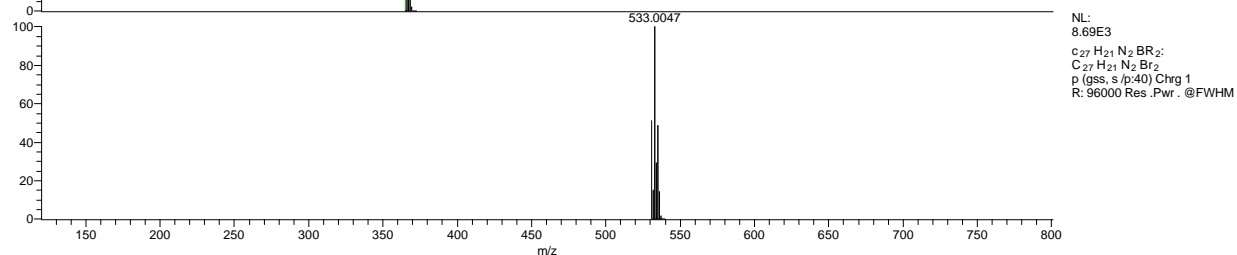
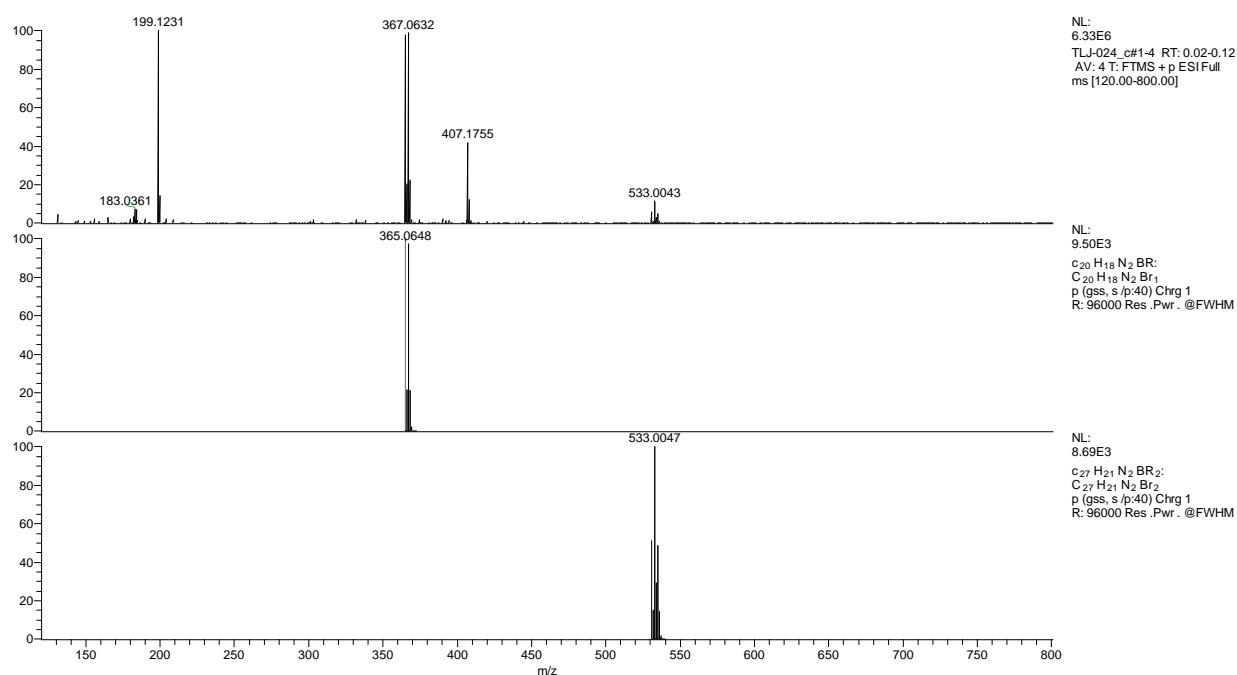


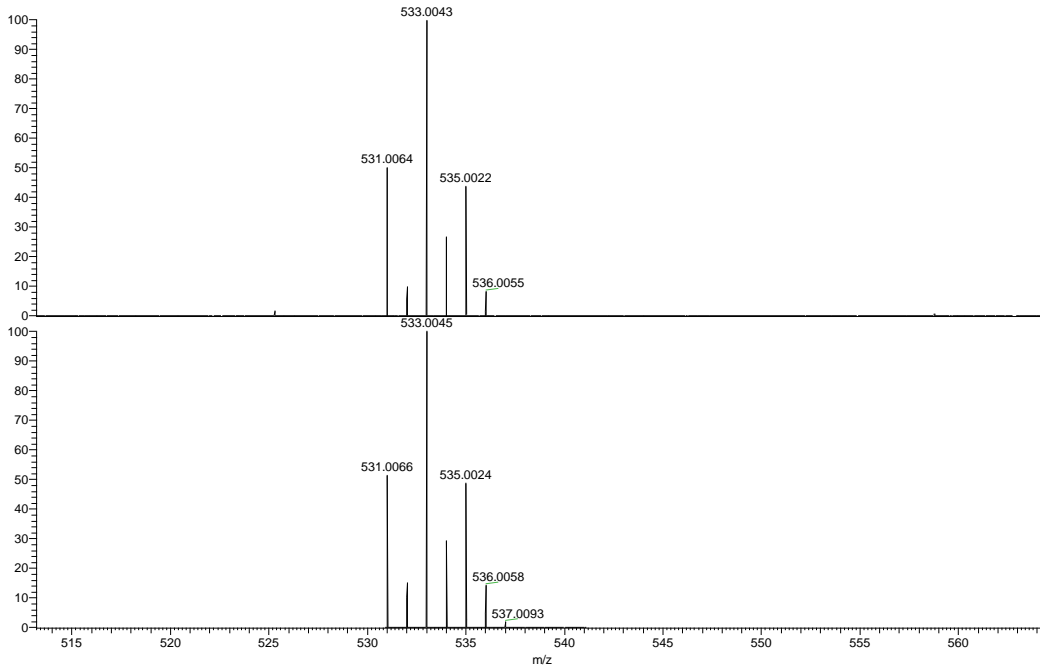






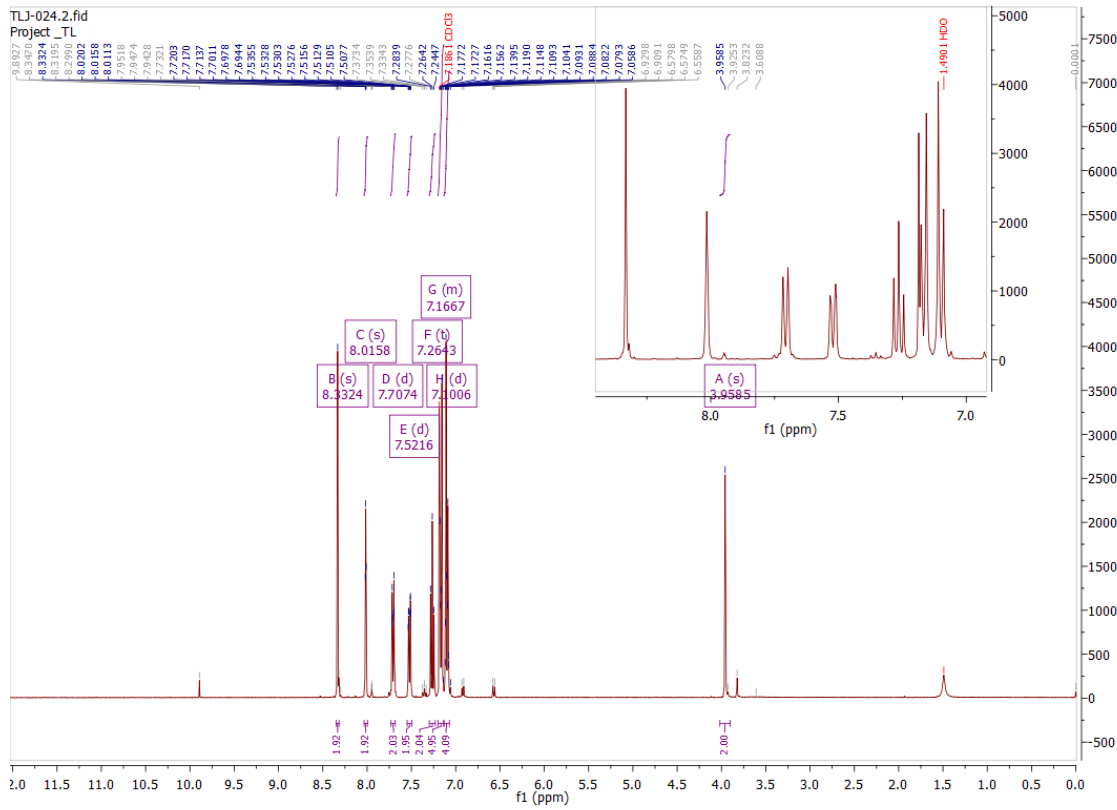
8.1.14 (1*E*,1'*E*)-*N,N'*-(methylenebis(4,1-phenylene))bis(1-(3-bromophenyl)-methanimine)

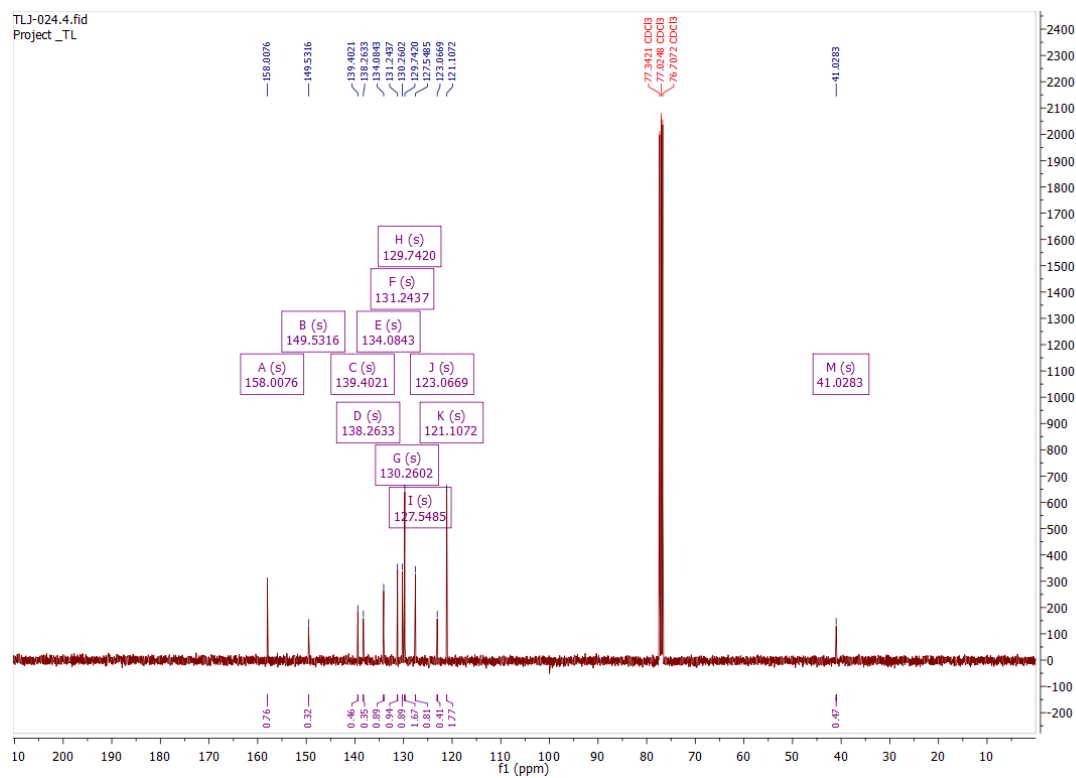
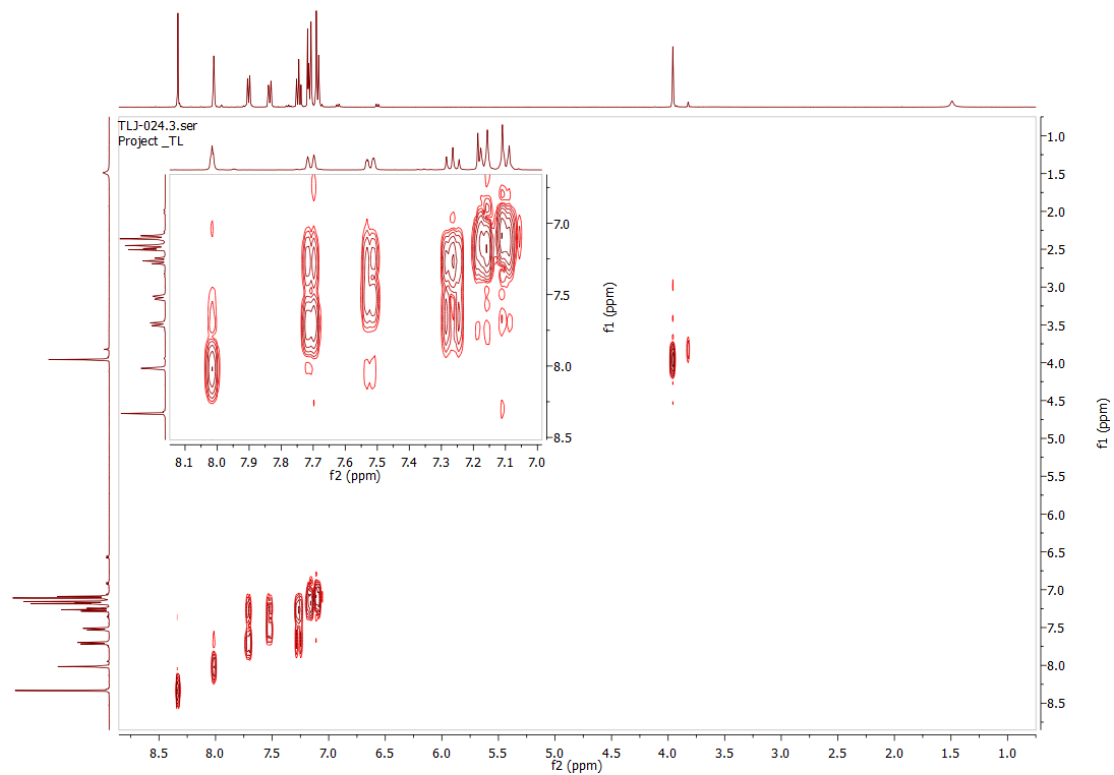




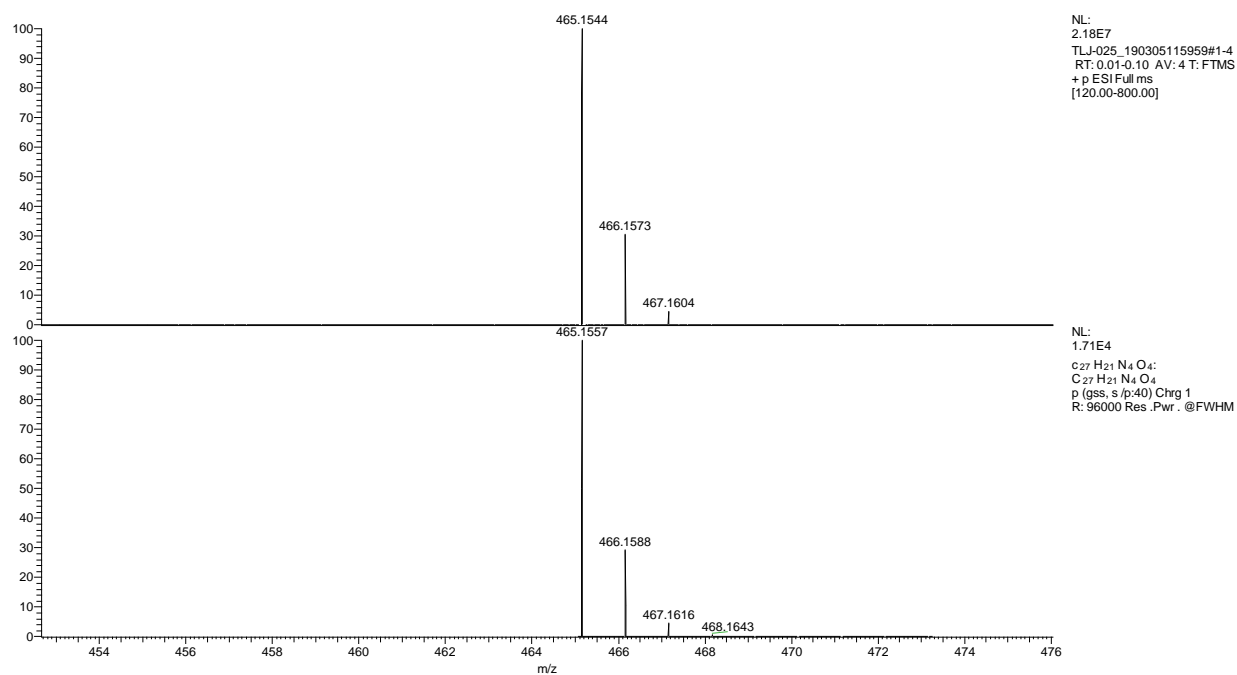
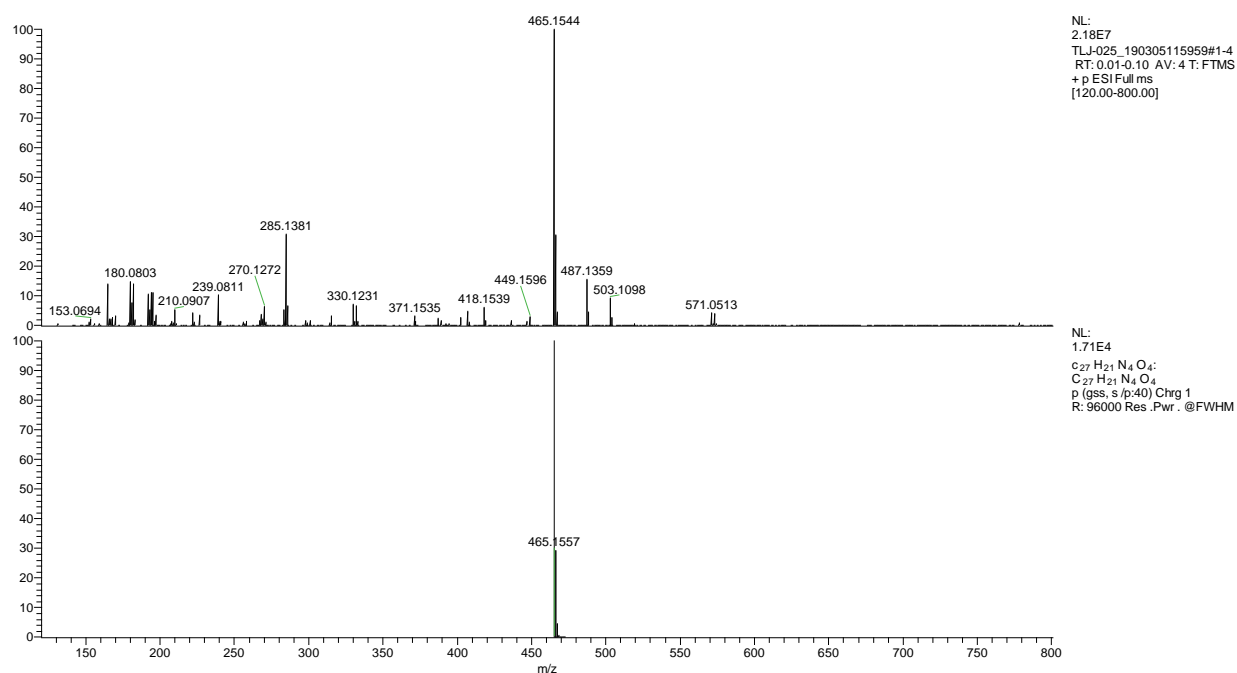
NL:
7.29E5
TLJ-024_c#1-4 RT: 0.02-0.12
AV: 4 T: FTMS + p ESI Full
ms [120.00-800.00]

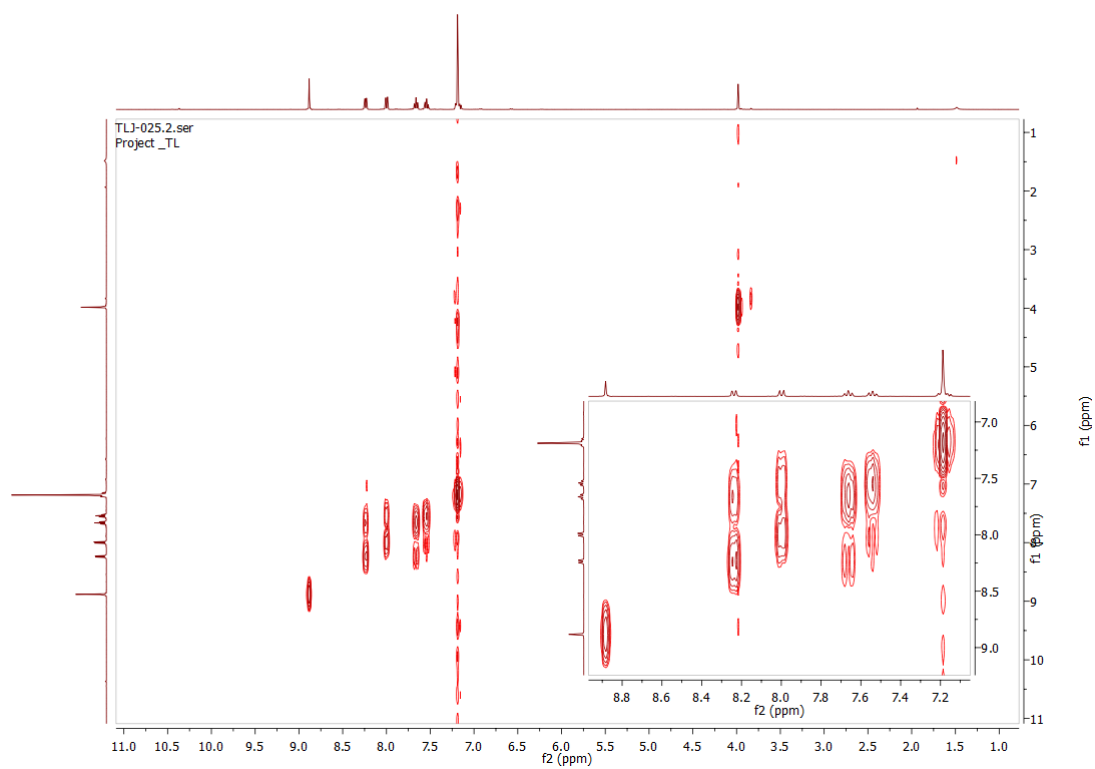
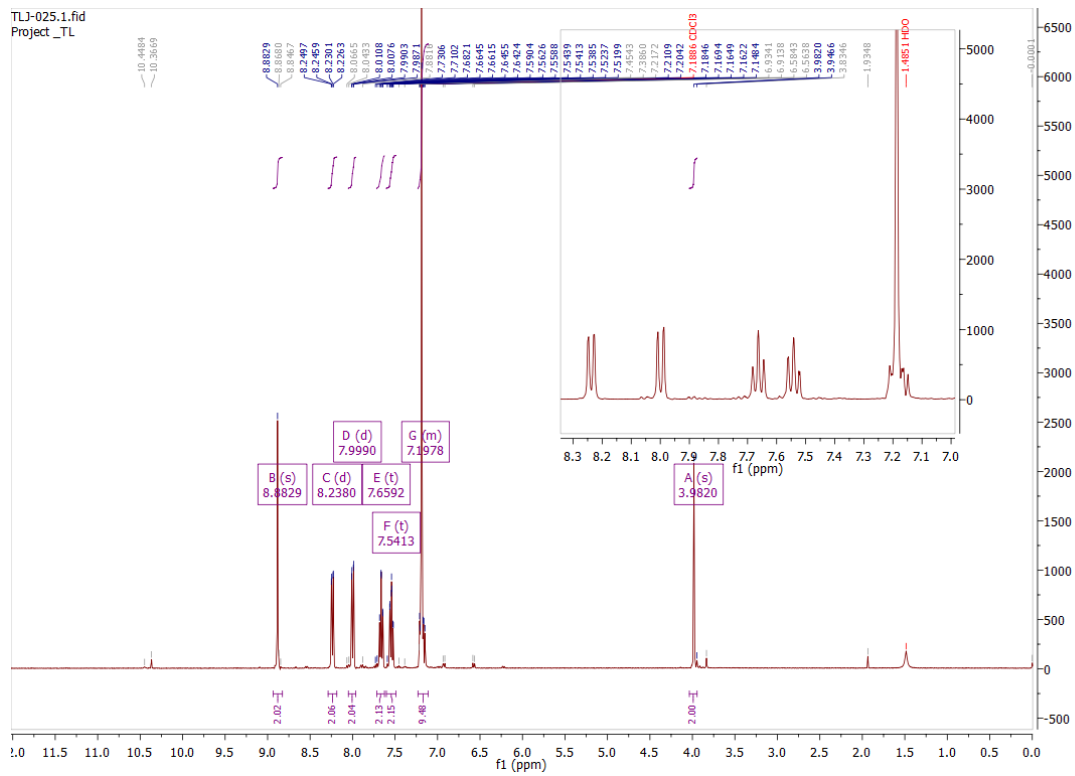
NL:
8.69E3
c27 H21 N2 BR2:
C27 H21 N2 BR2
p (gss, s/p:40) Chrg 1
R: 96000 Res. Pwr. @FWHM

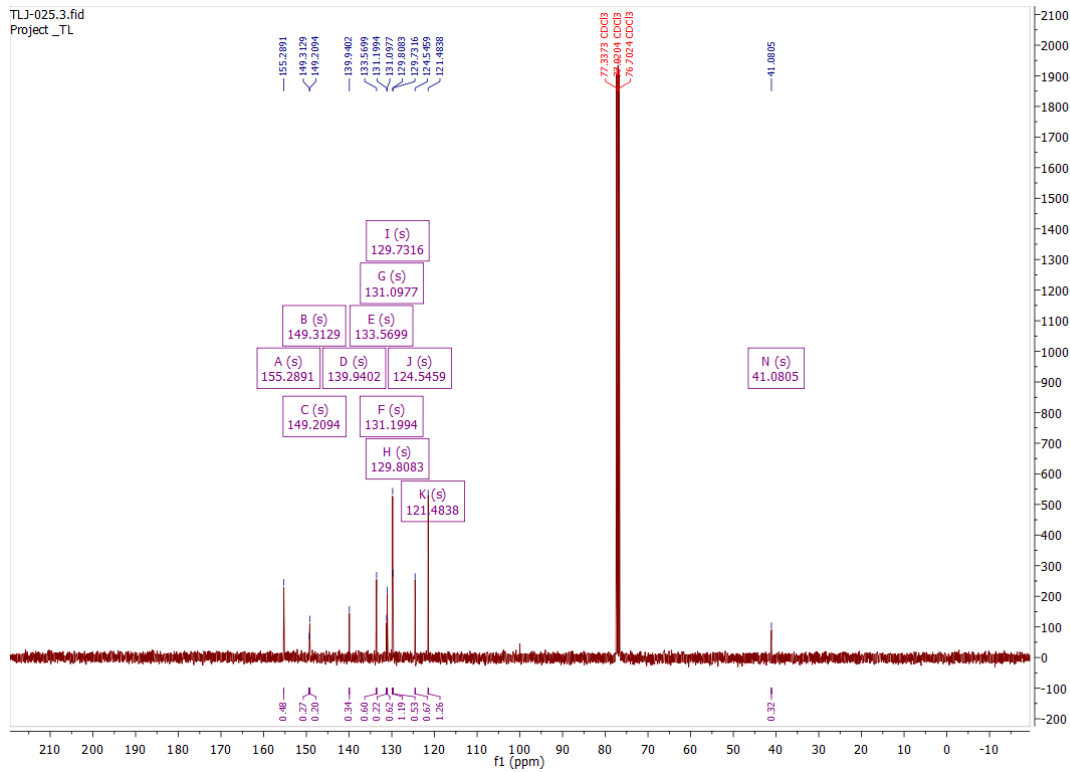




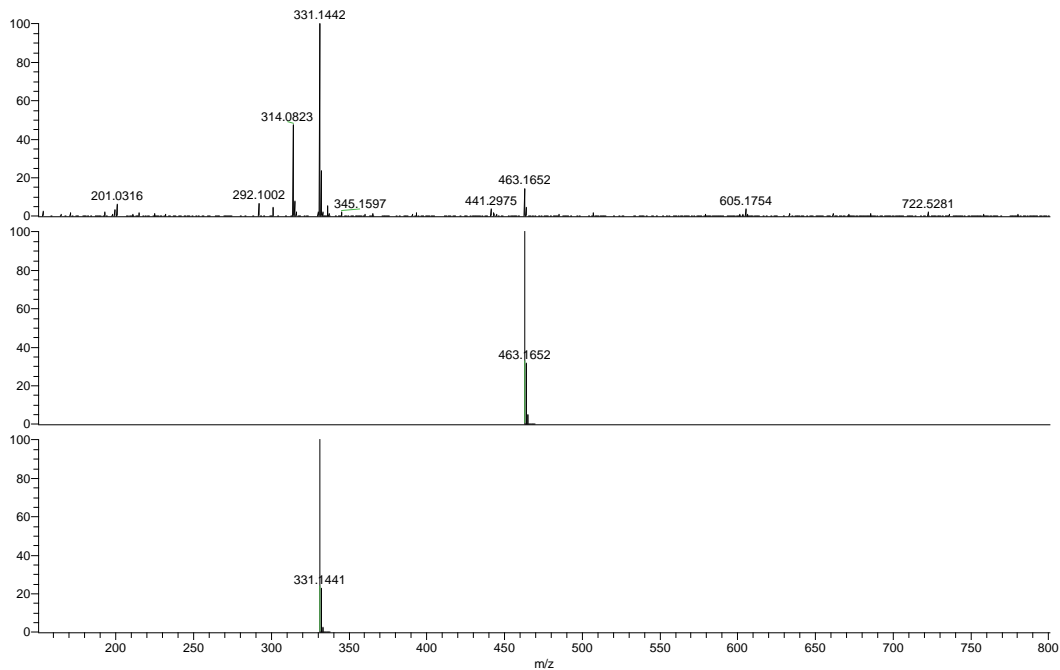
8.1.15 (1E,1'E)-N,N'-(methylenebis(4,1-phenylene))bis(1-(2-nitrophenyl)-methanimine)

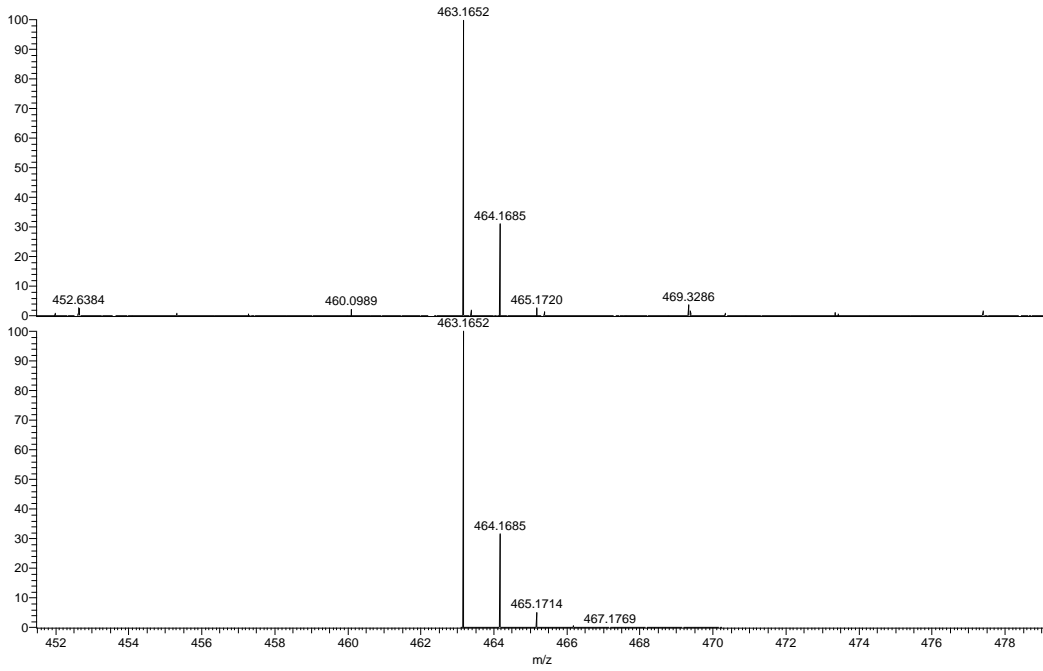






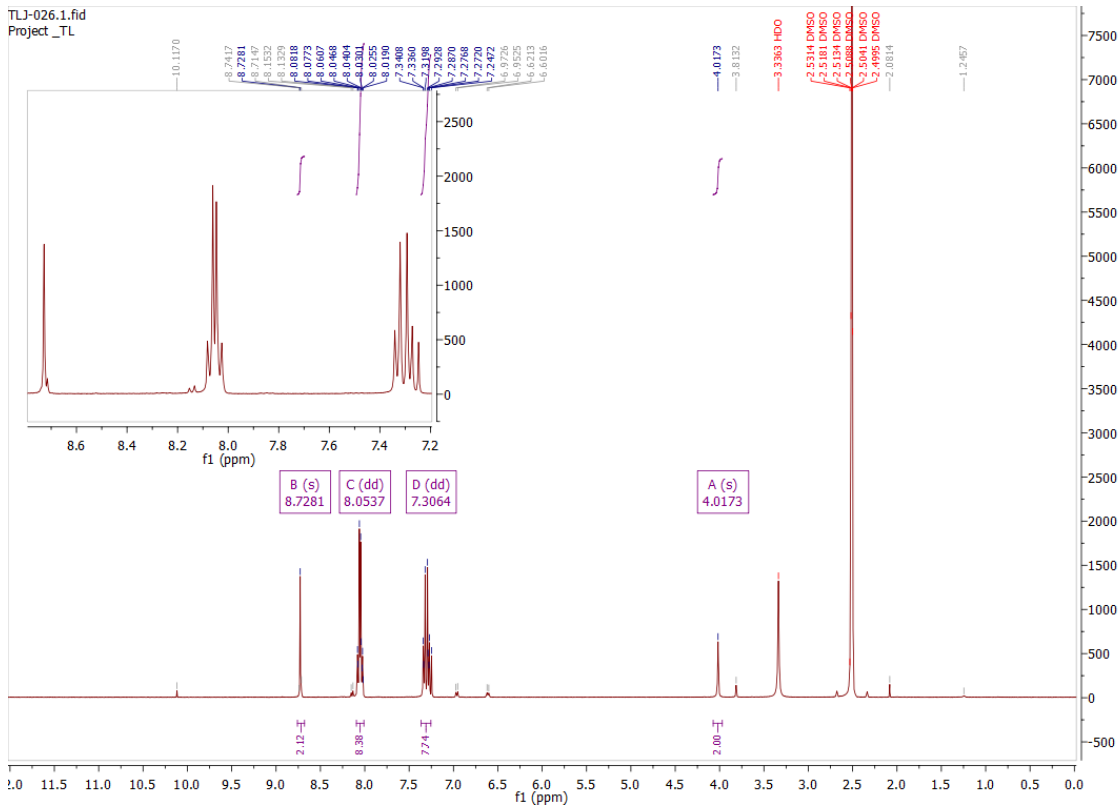
8.1.16 4,4'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))dibenzoic acid

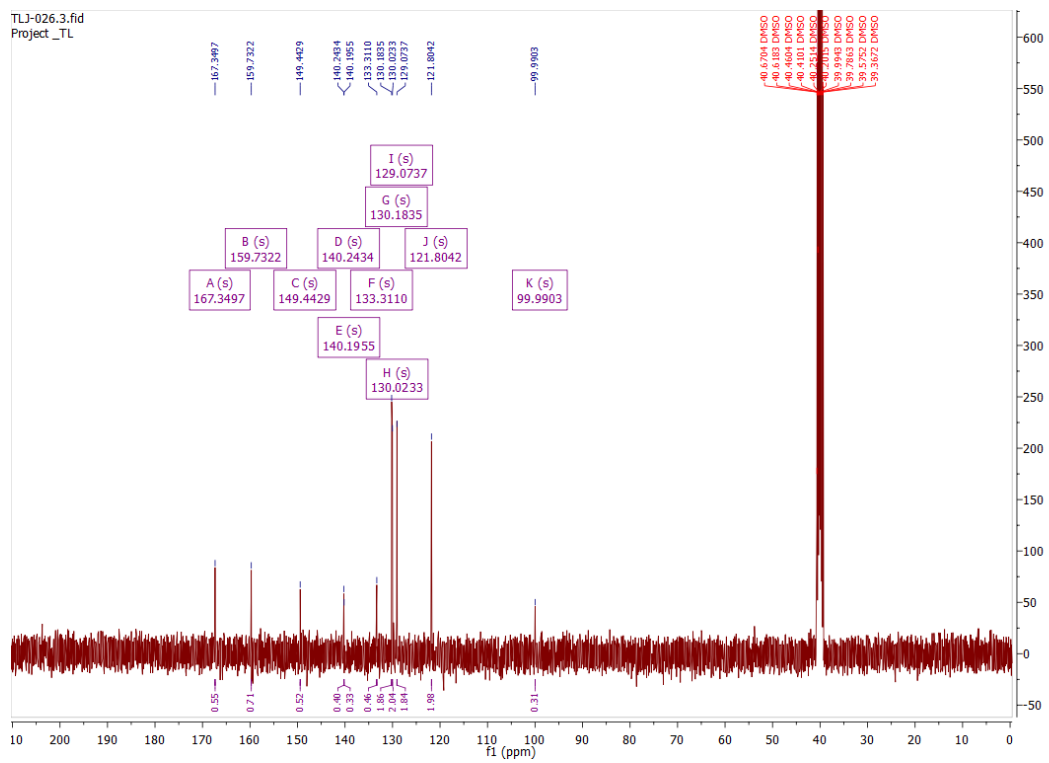
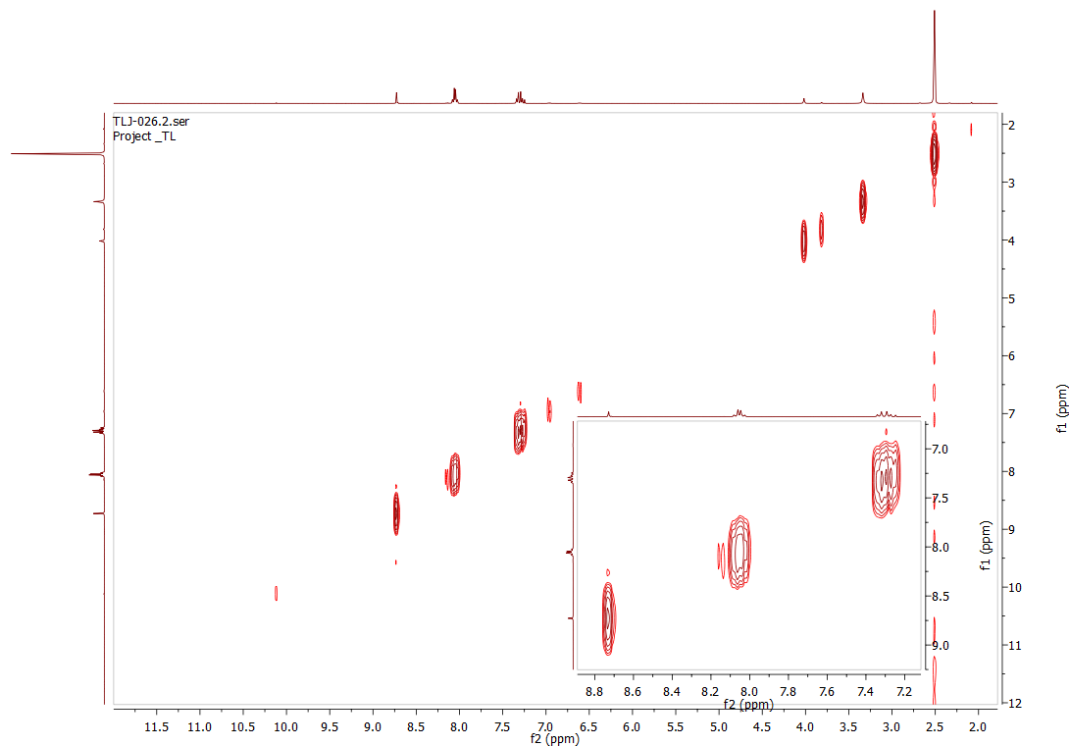




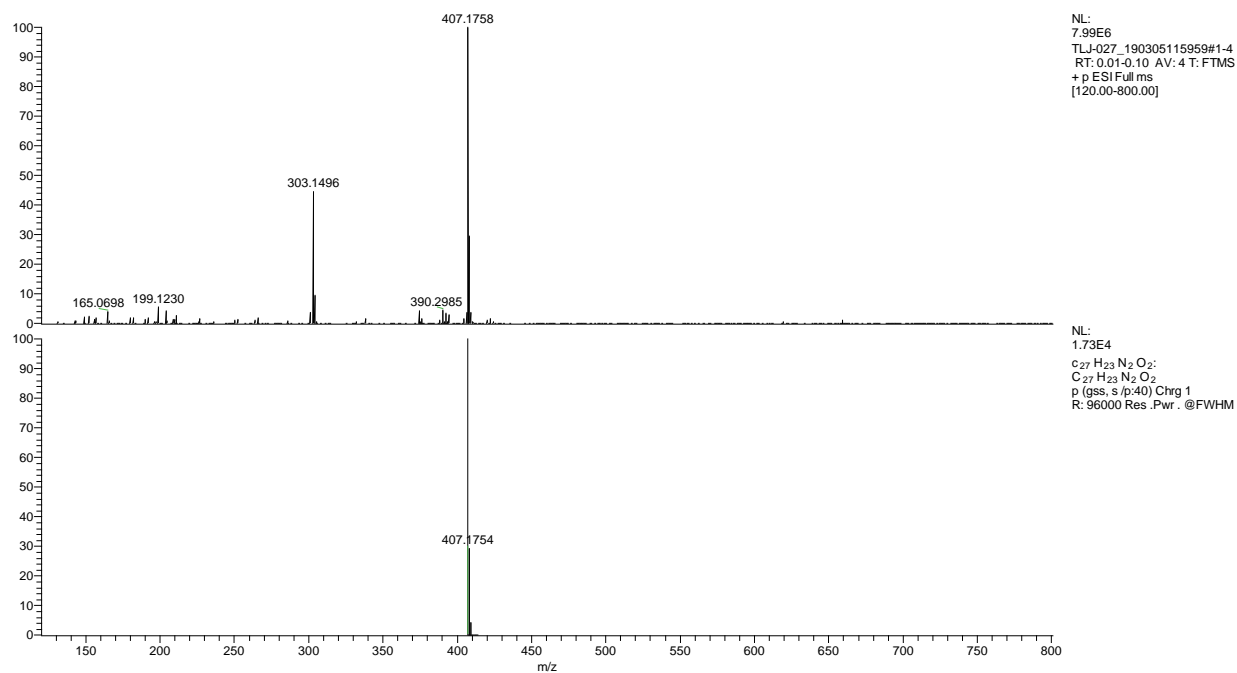
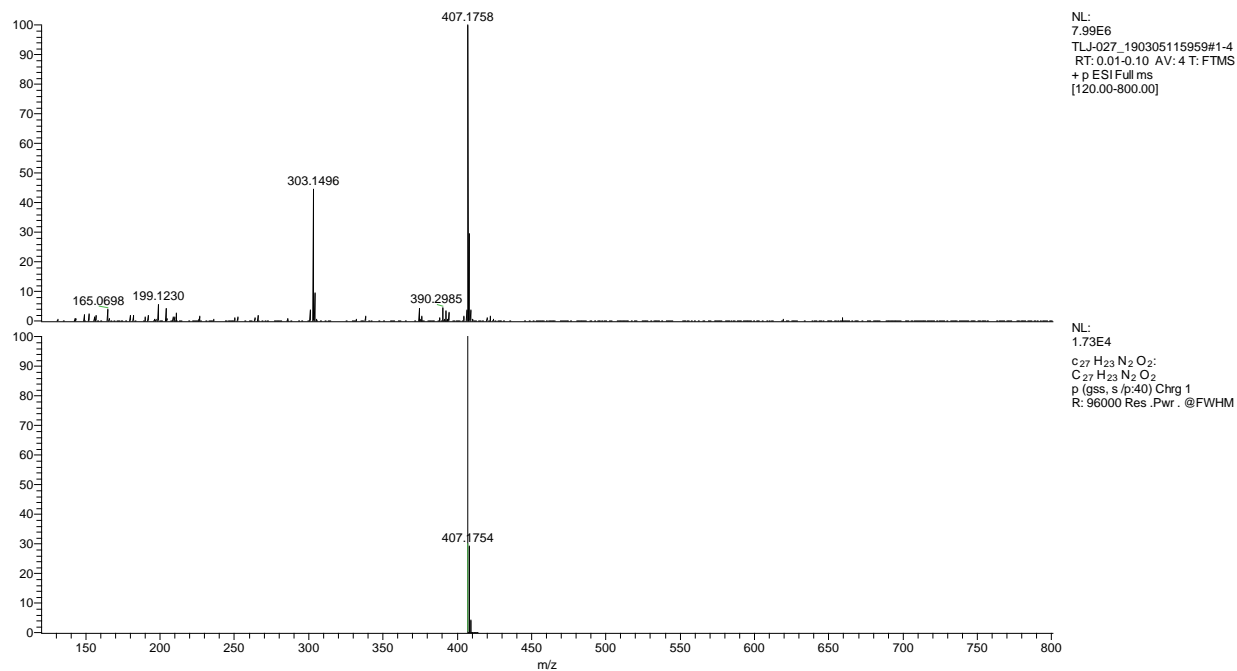
NL:
6.52E5
TLJ-026_190513194513#1-4
RT: 0.02-0.11 AV: 4 T: FTMS
+ p ESI Full ms
[150.00-800.00]

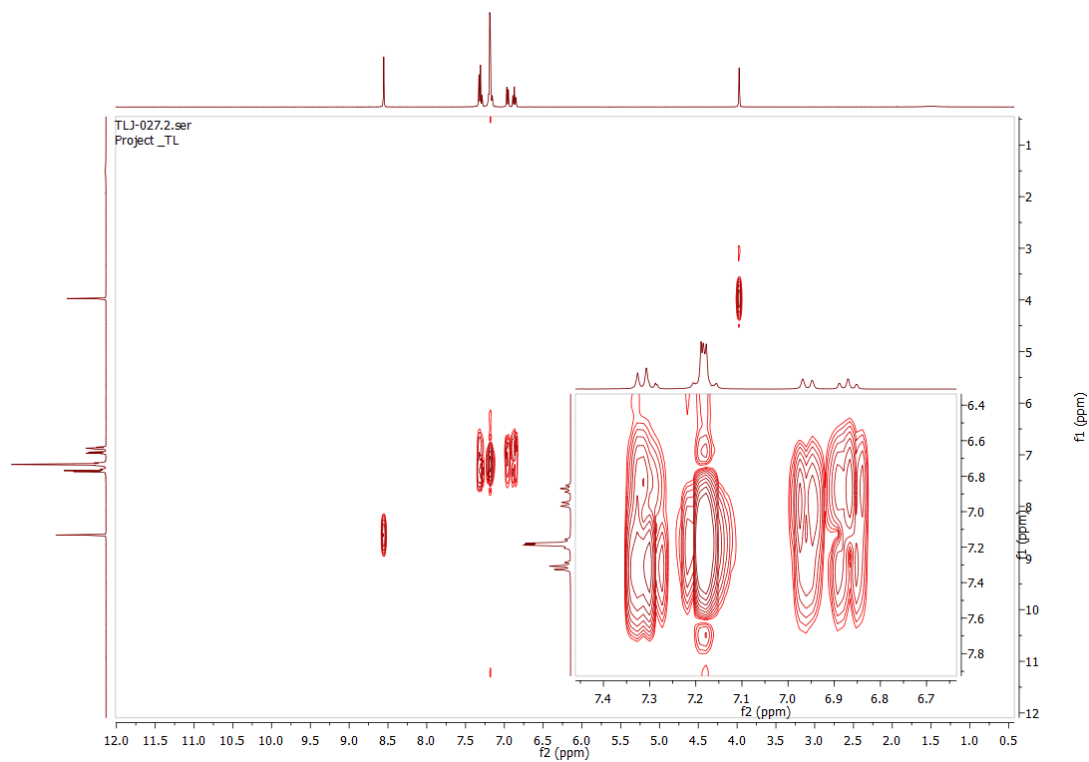
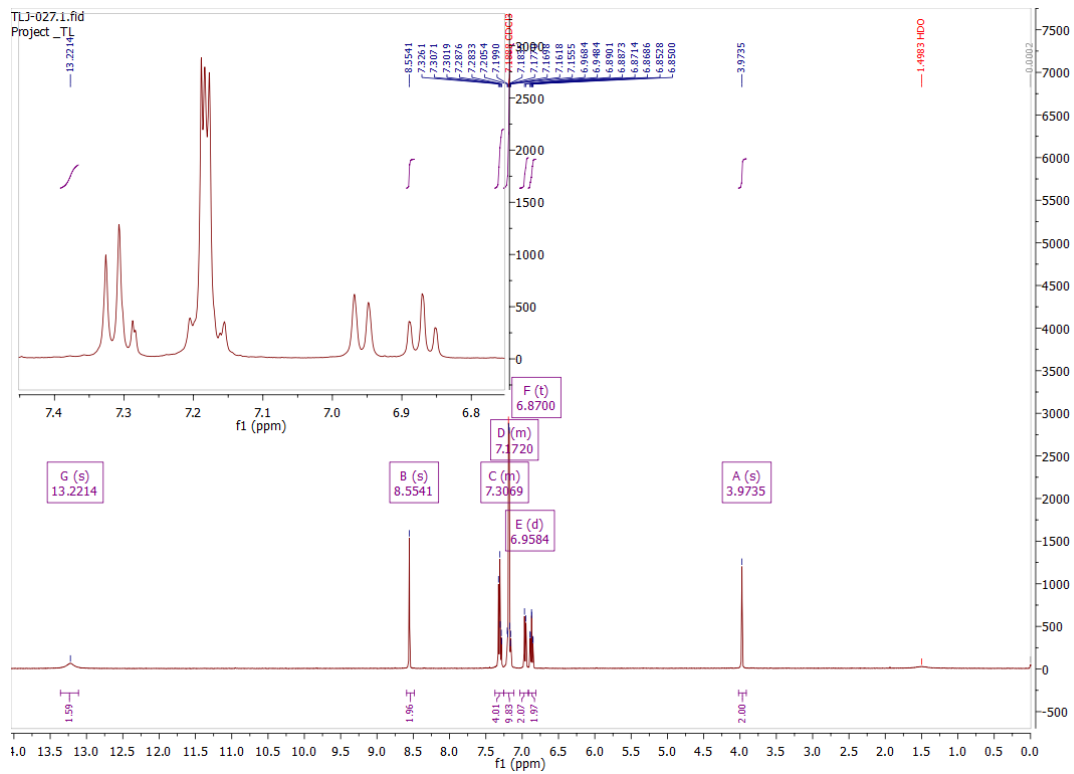
NL:
1.68E4
c29 H23 N2 O4:
p (gss, s/p:40) Chrg 1
R: 96000 Res. Pwr. @FWHM

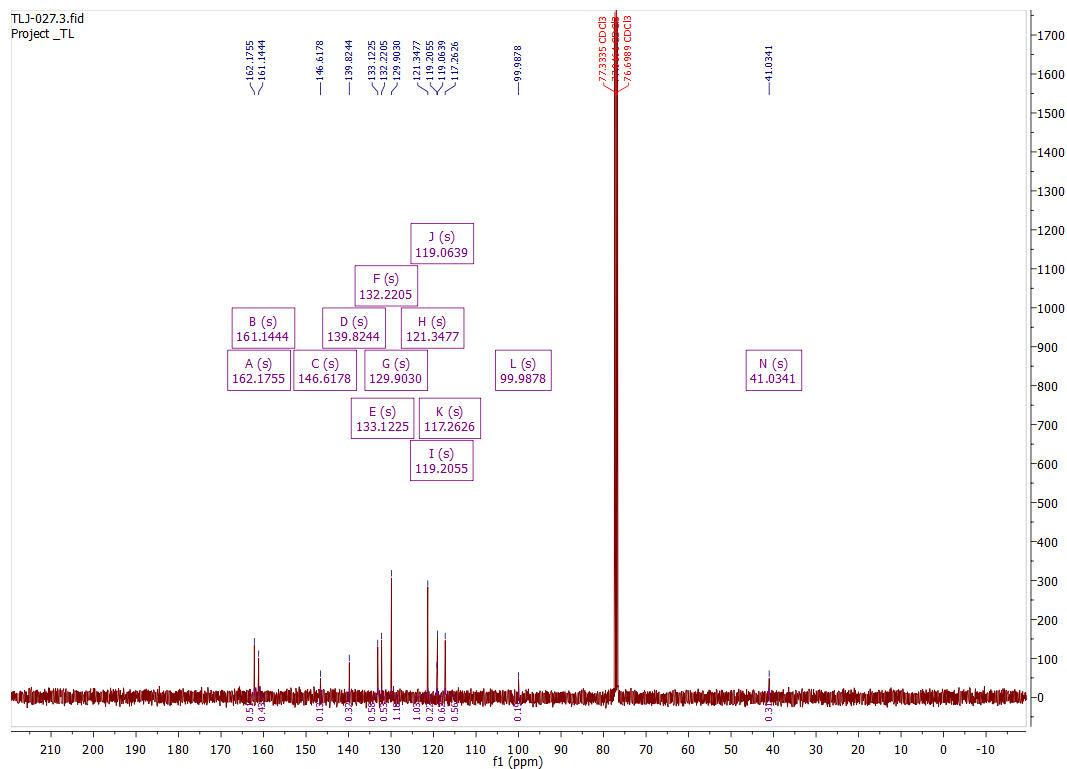




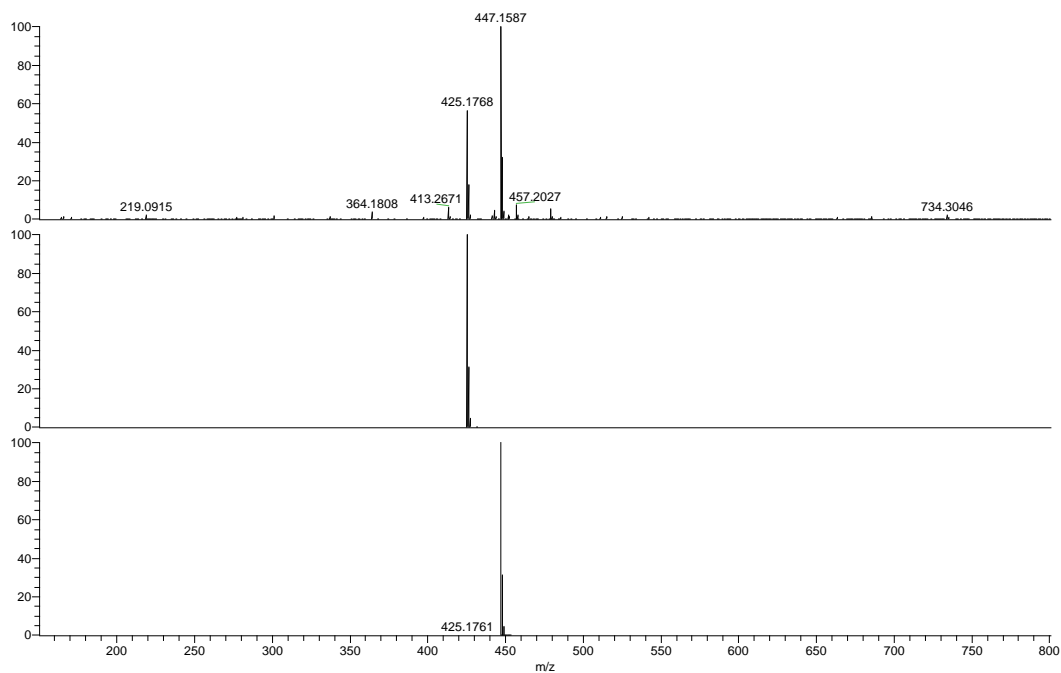
8.1.17 2,2'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis-(methaneylylidene))diphenol

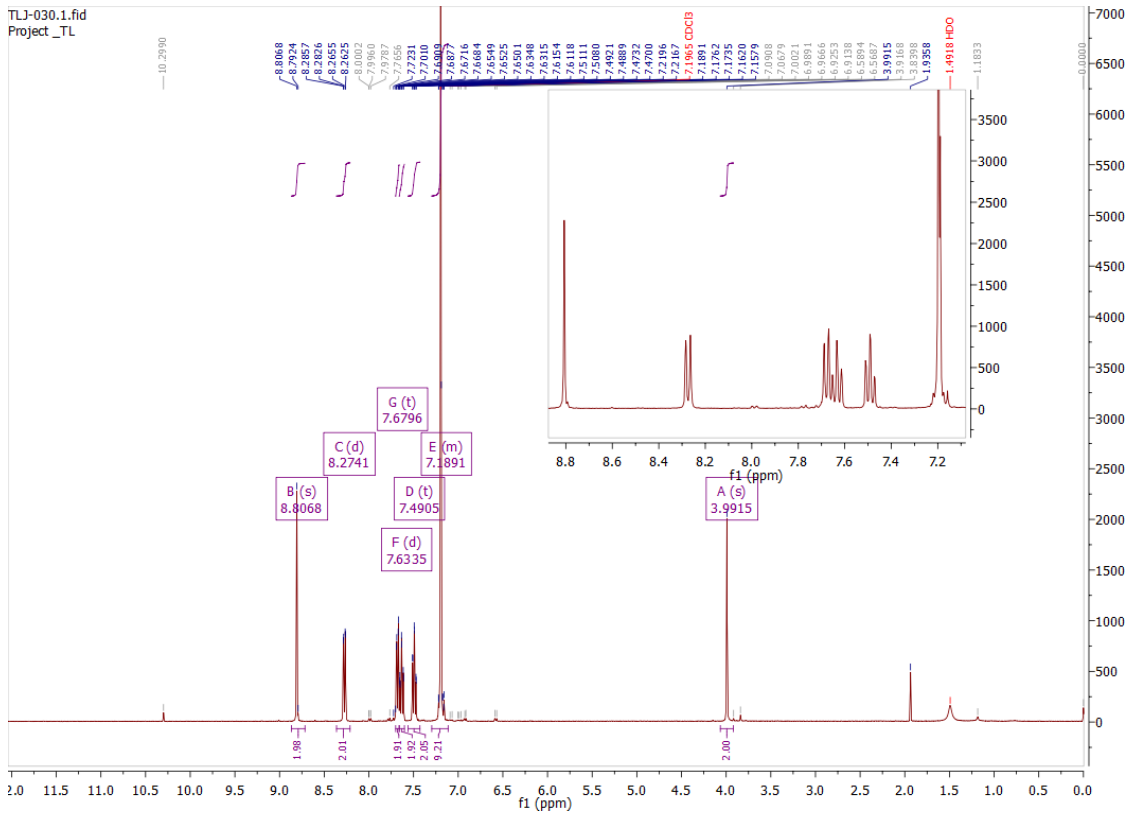
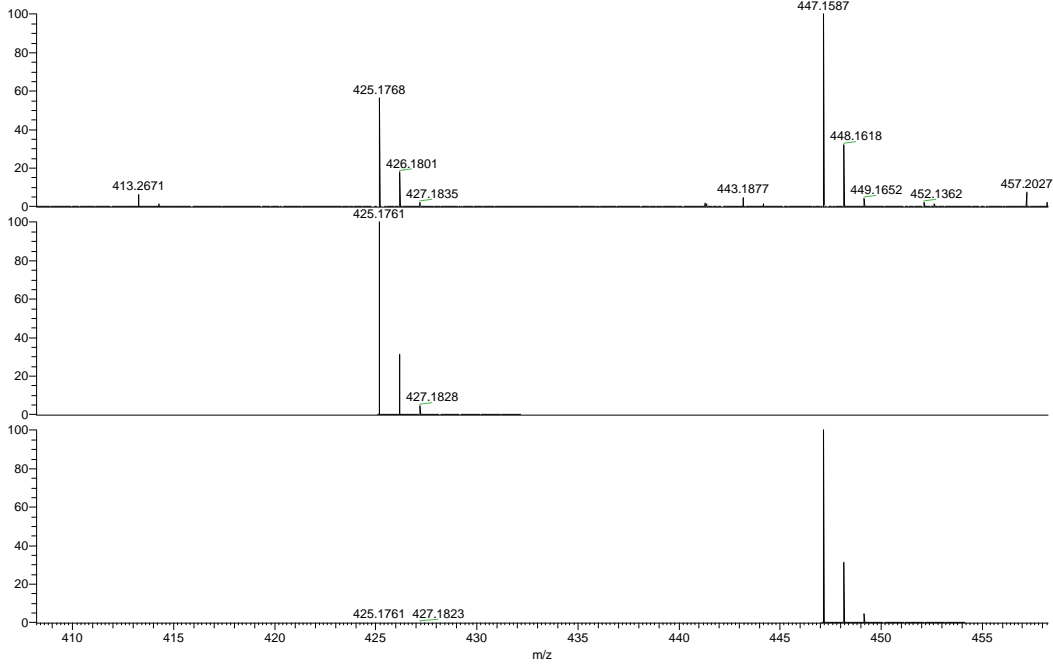


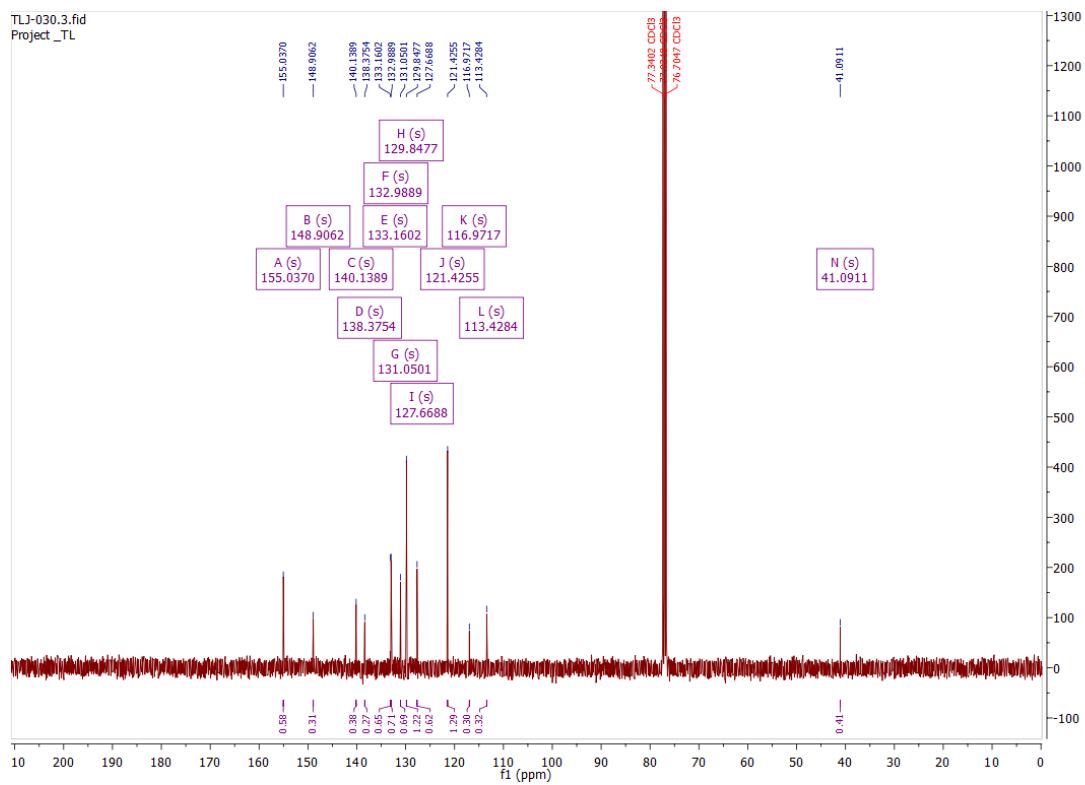
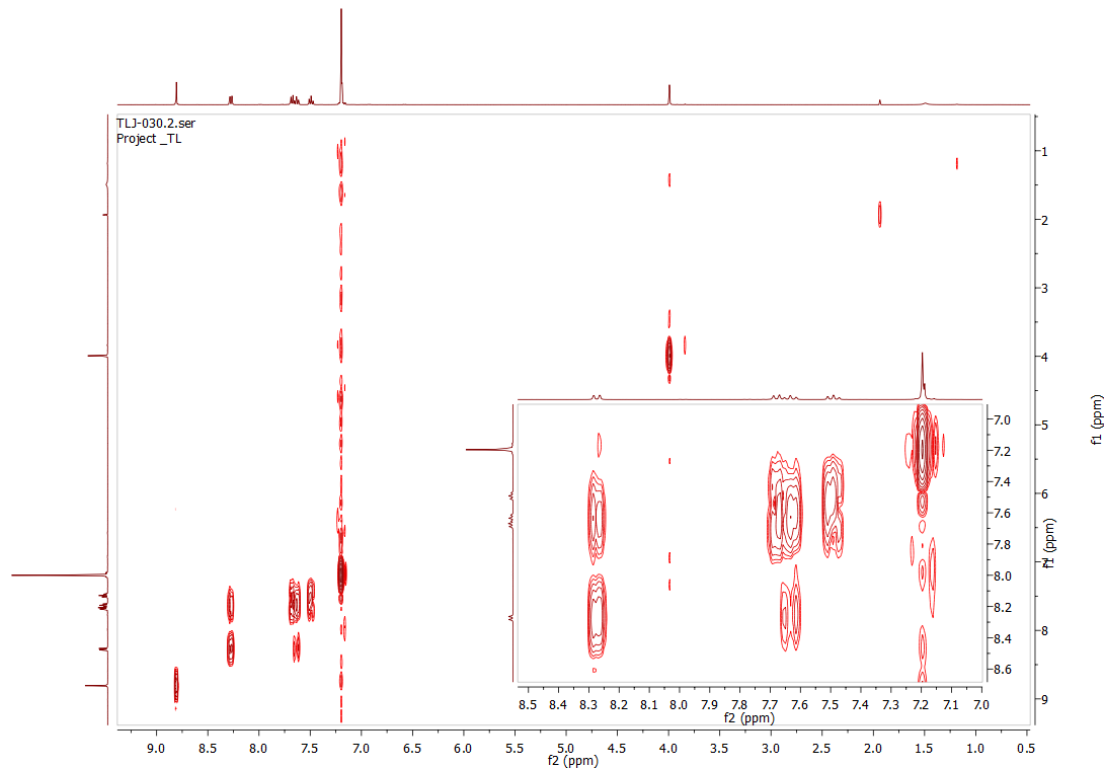




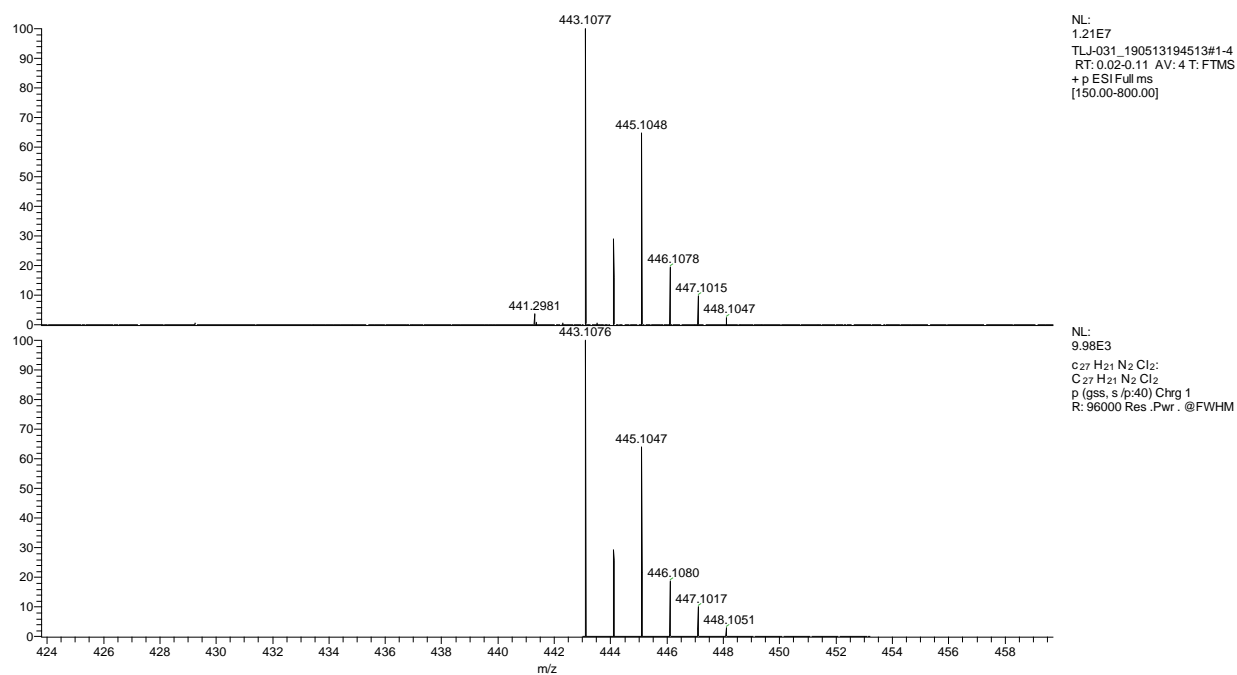
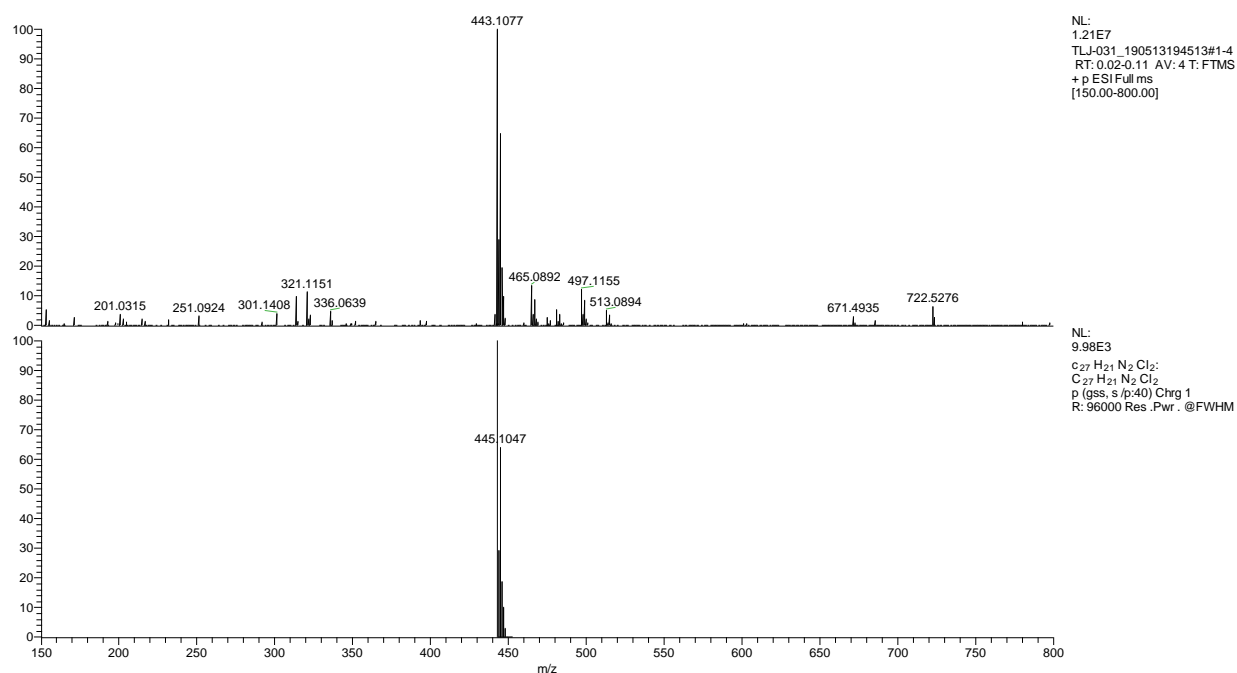
8.1.18 2,2'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))dibenzonitrile

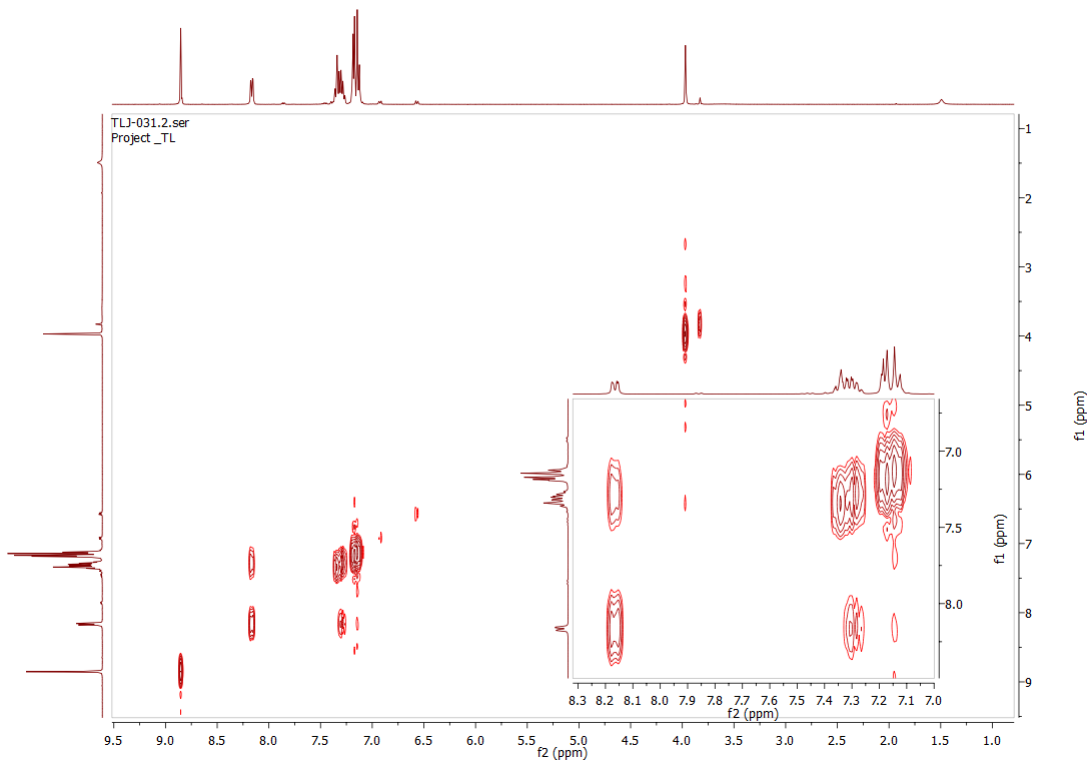
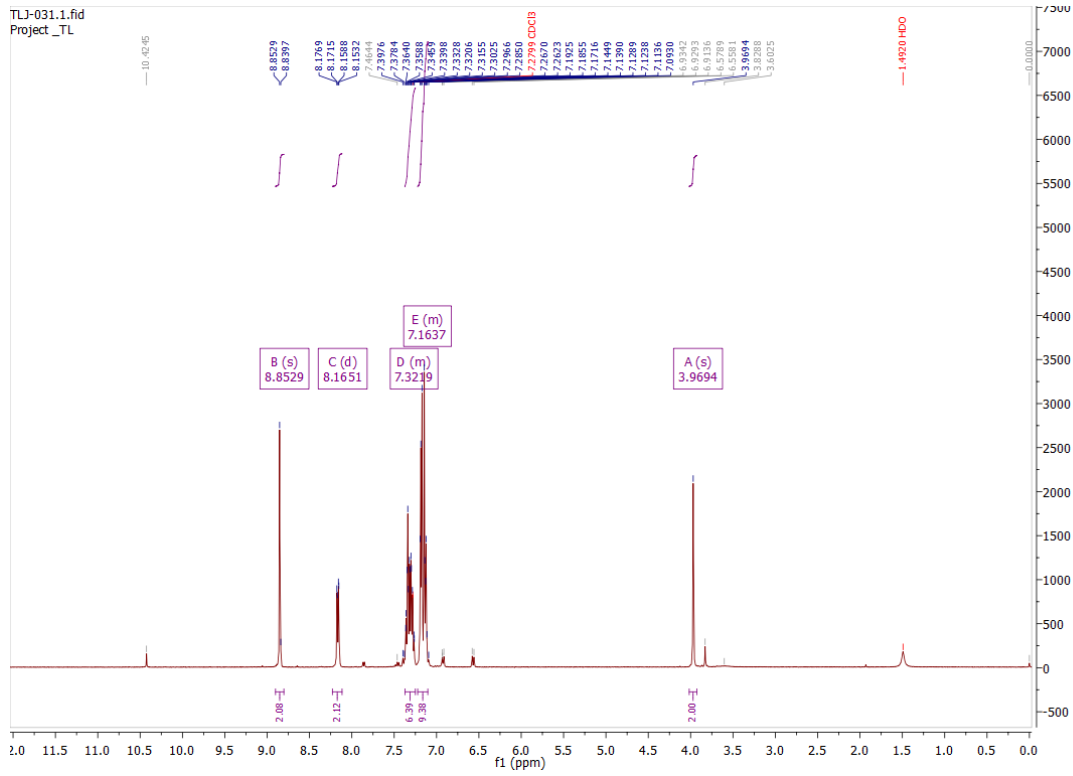


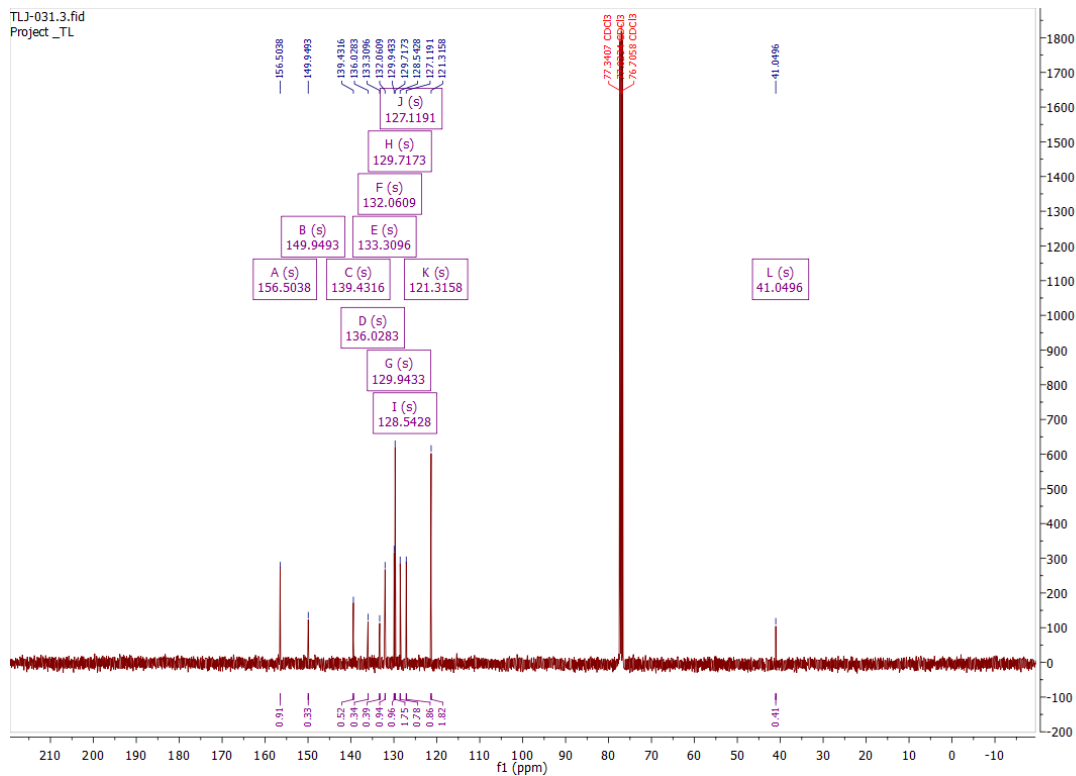




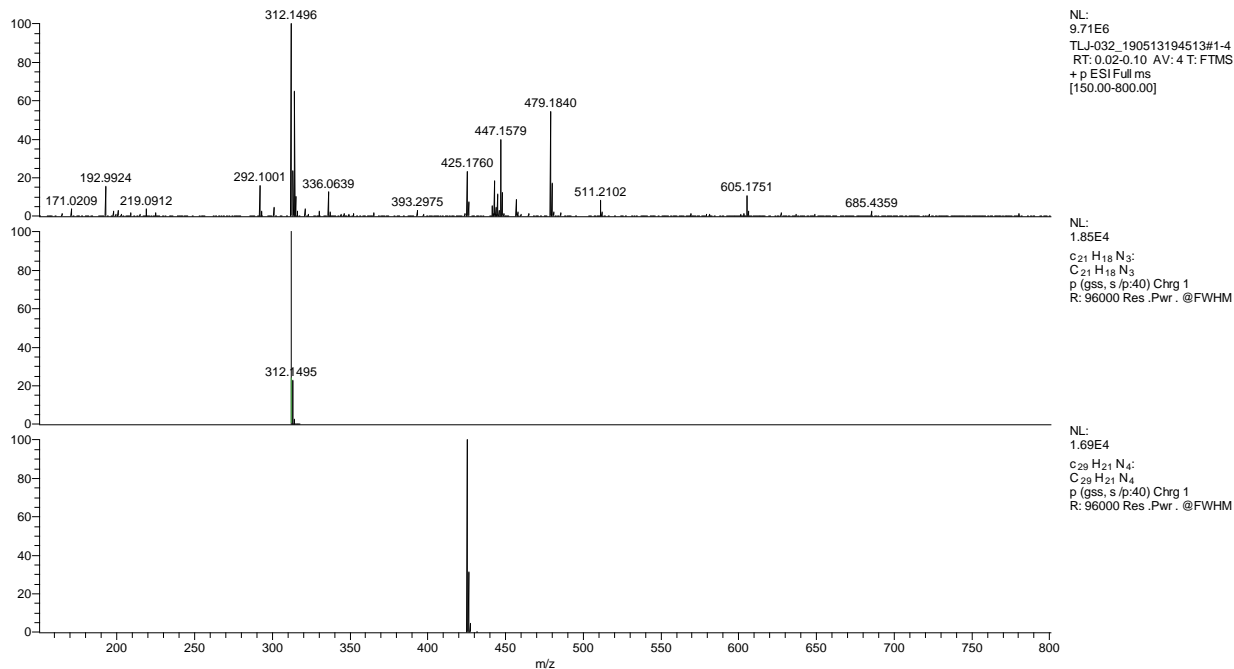
8.1.19 (1E,1'E)-N,N'-(methylenebis(4,1-phenylene))bis(1-(2-chlorophenyl)-methanimine)

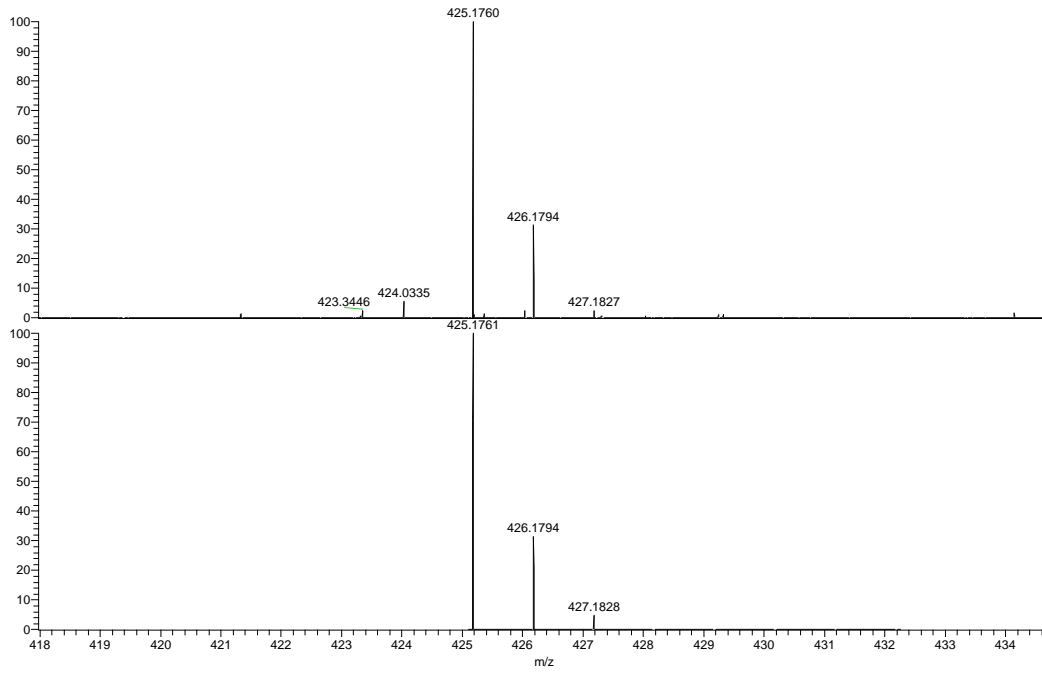






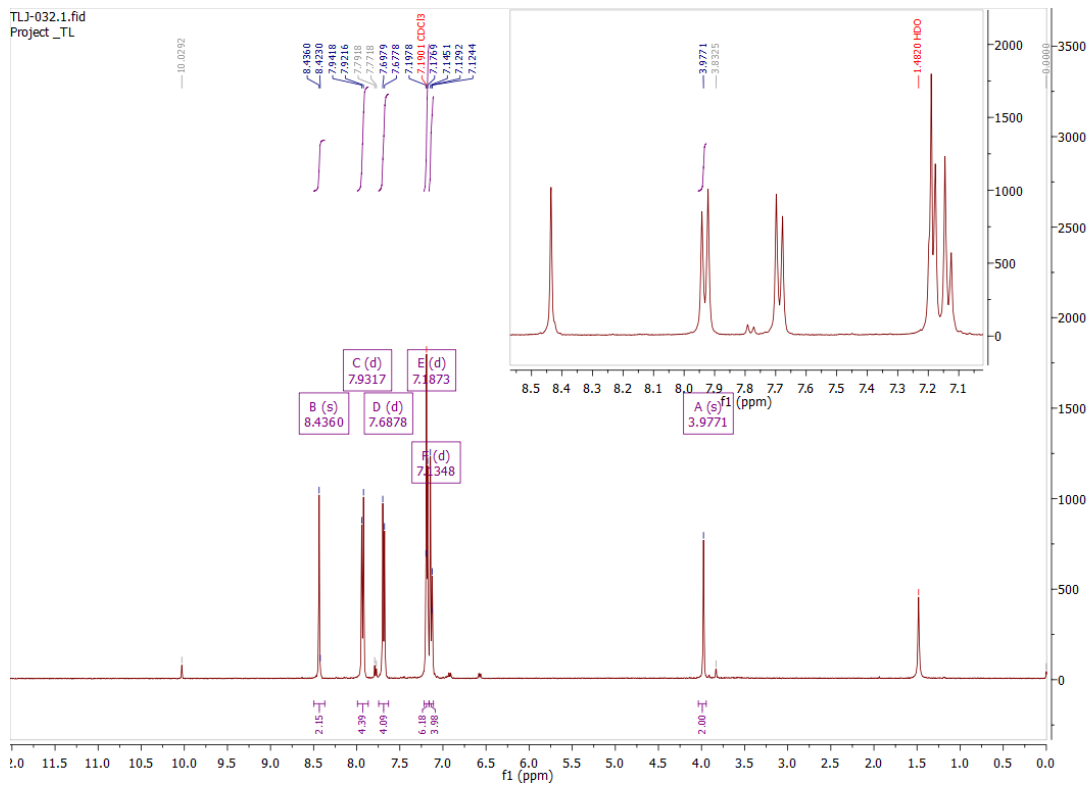
8.1.20 4,4'-((1E,1'E)-((methylenebis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))dibenzonitrile

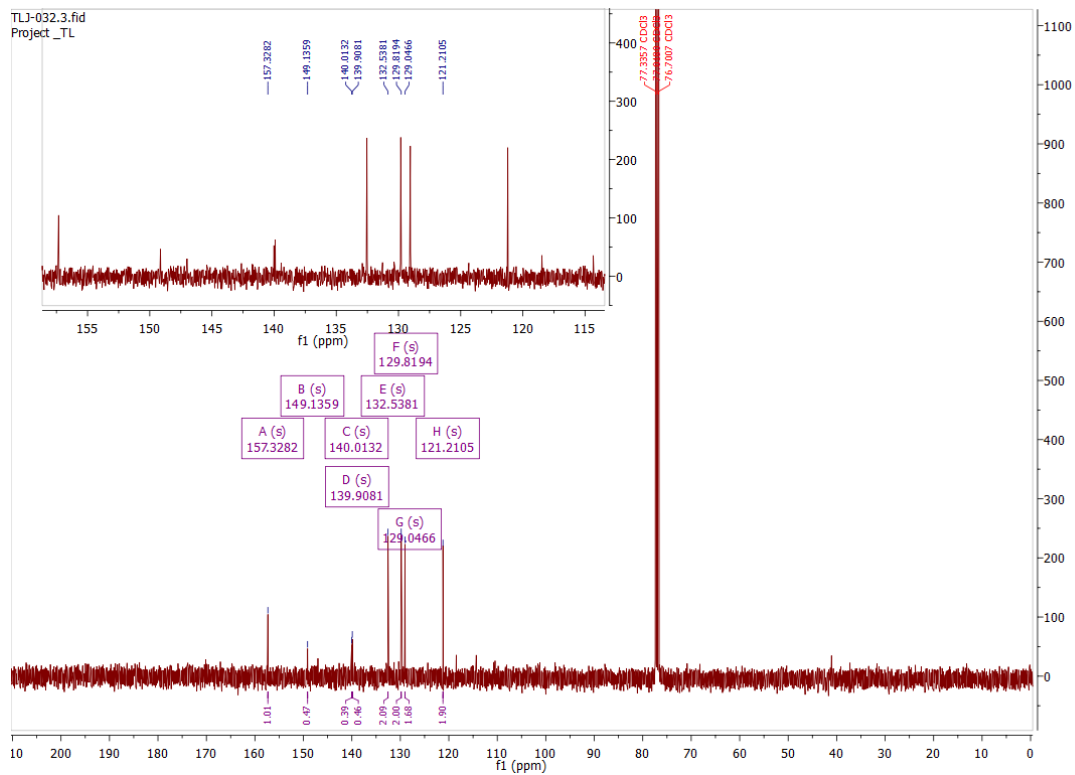
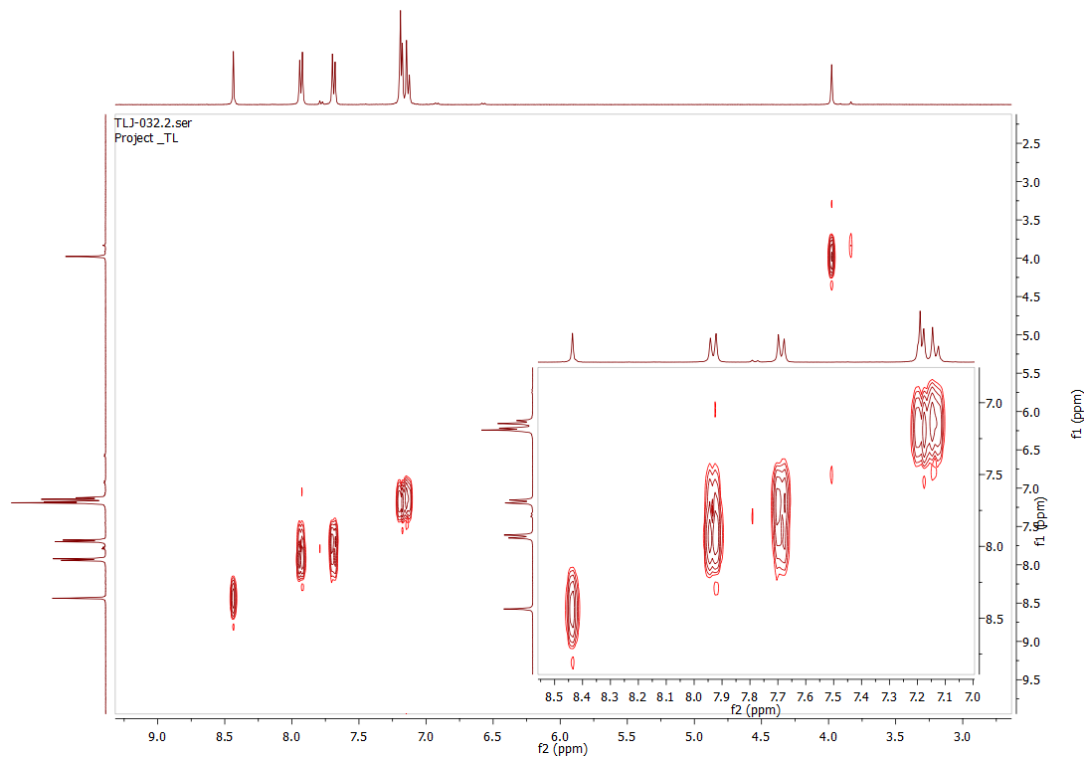




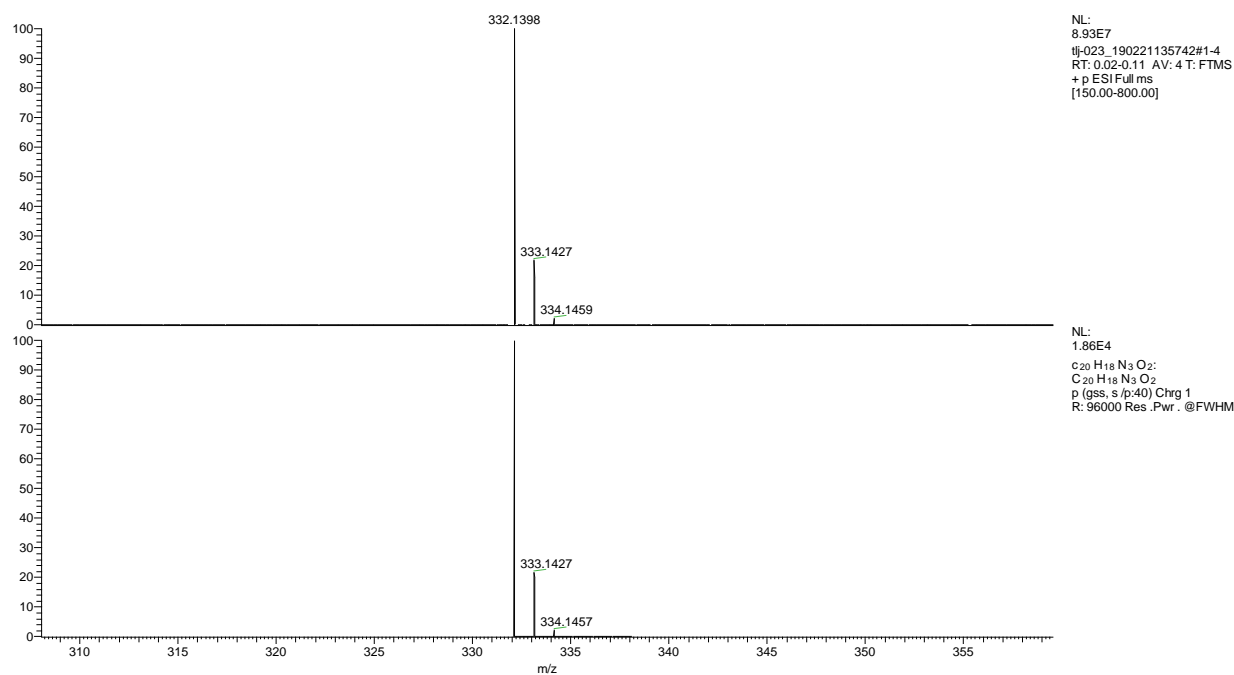
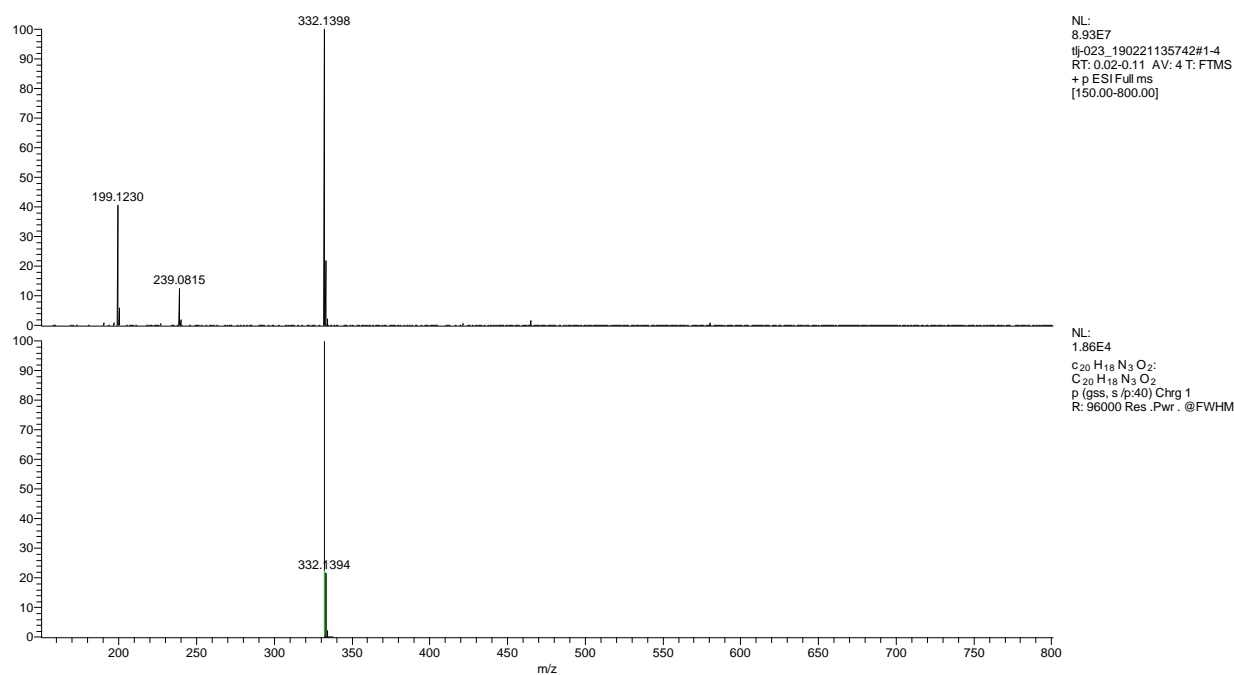
NL:
2.26E6
TLJ-032_190513194513#1-4
RT: 0.02-0.10 AV: 4 T: FTMS
+ p ESI Full ms
[150.00-800.00]

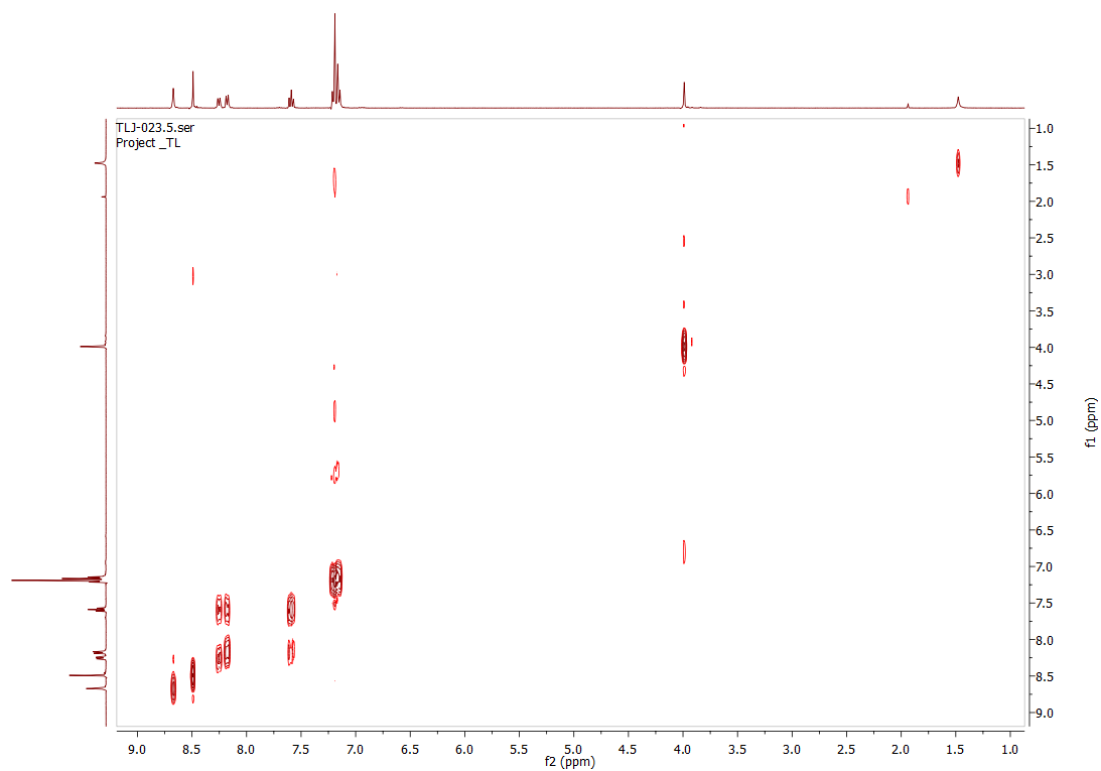
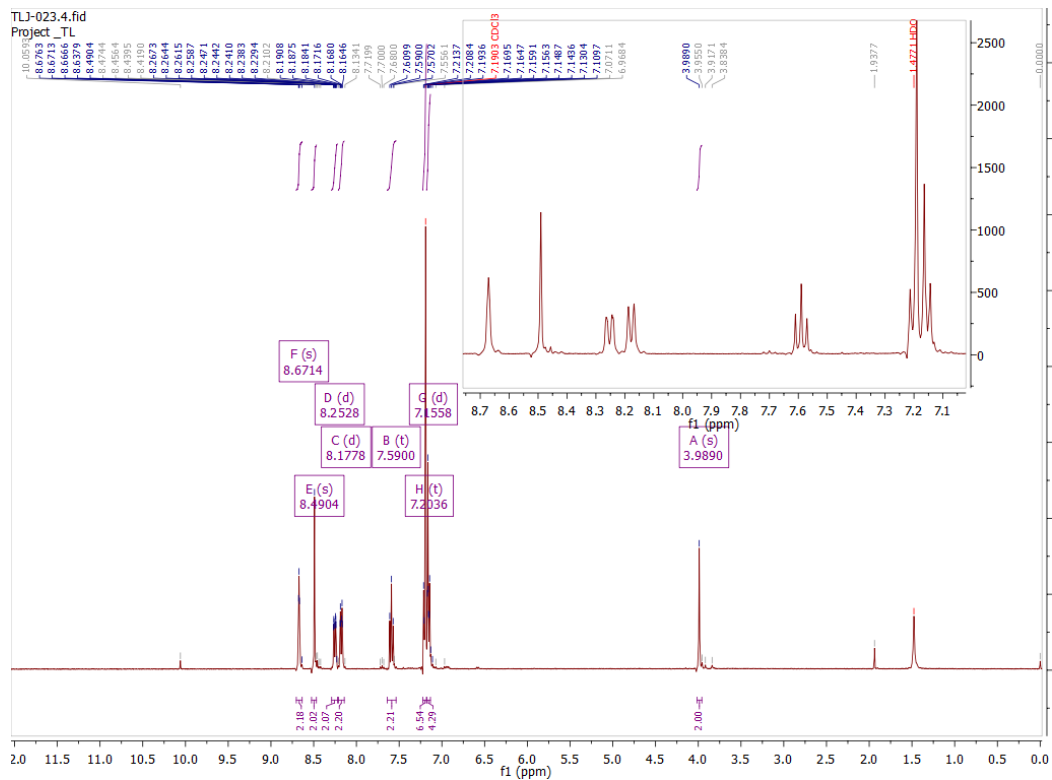
NL:
1.69E4
C₂₈H₂₁N₄:
C₂₉H₂₁N₄
p (gss, s/p:40) Chrg 1
R: 96000 Res. Pwr. @FWHM

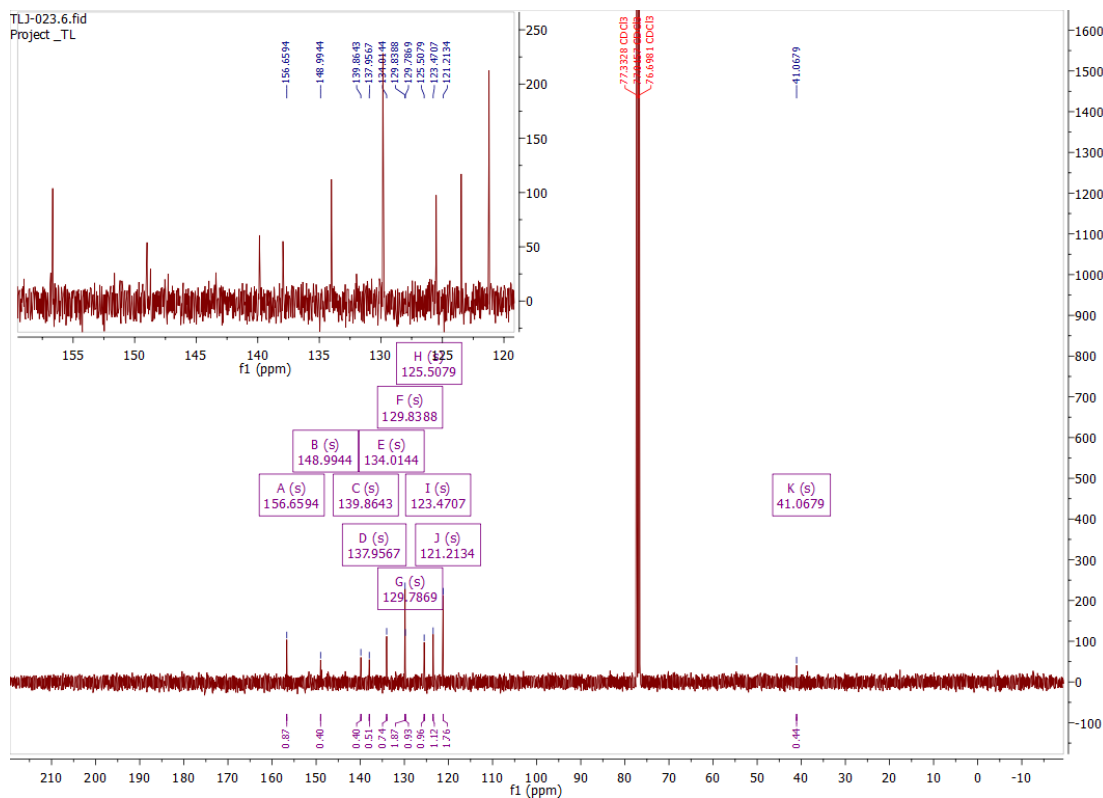




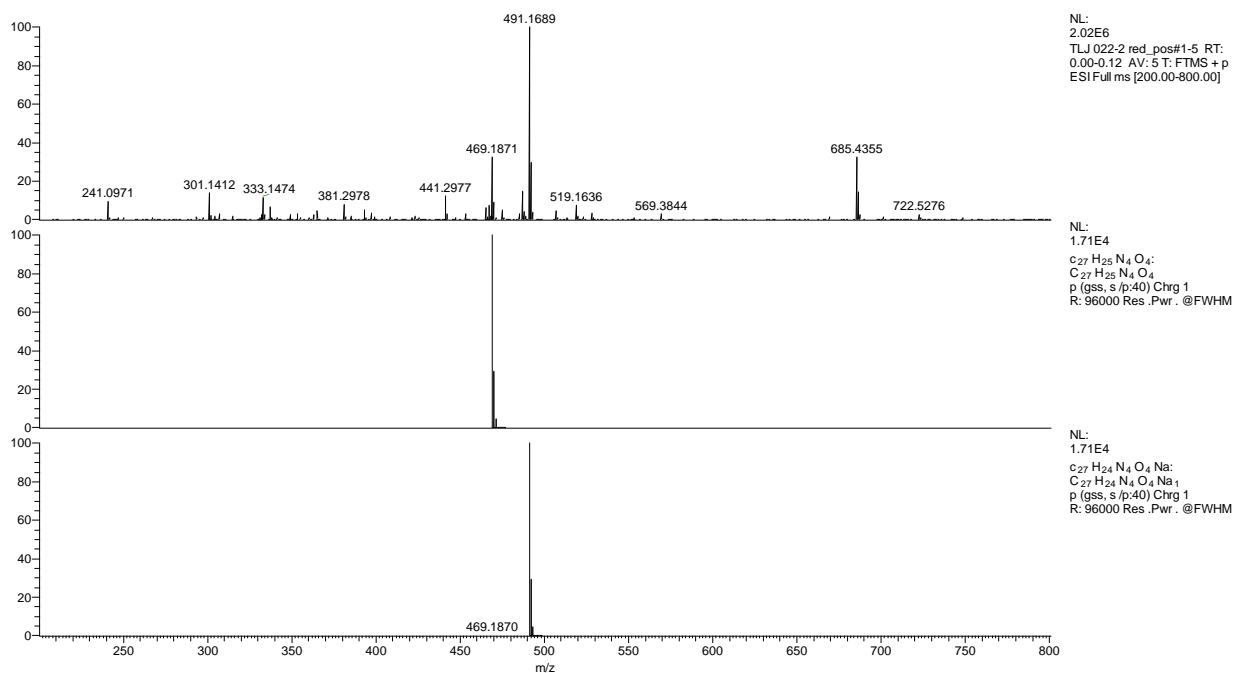
8.1.21 (E)-4-(4-((3-nitrobenzylidene)amino)benzyl)aniline

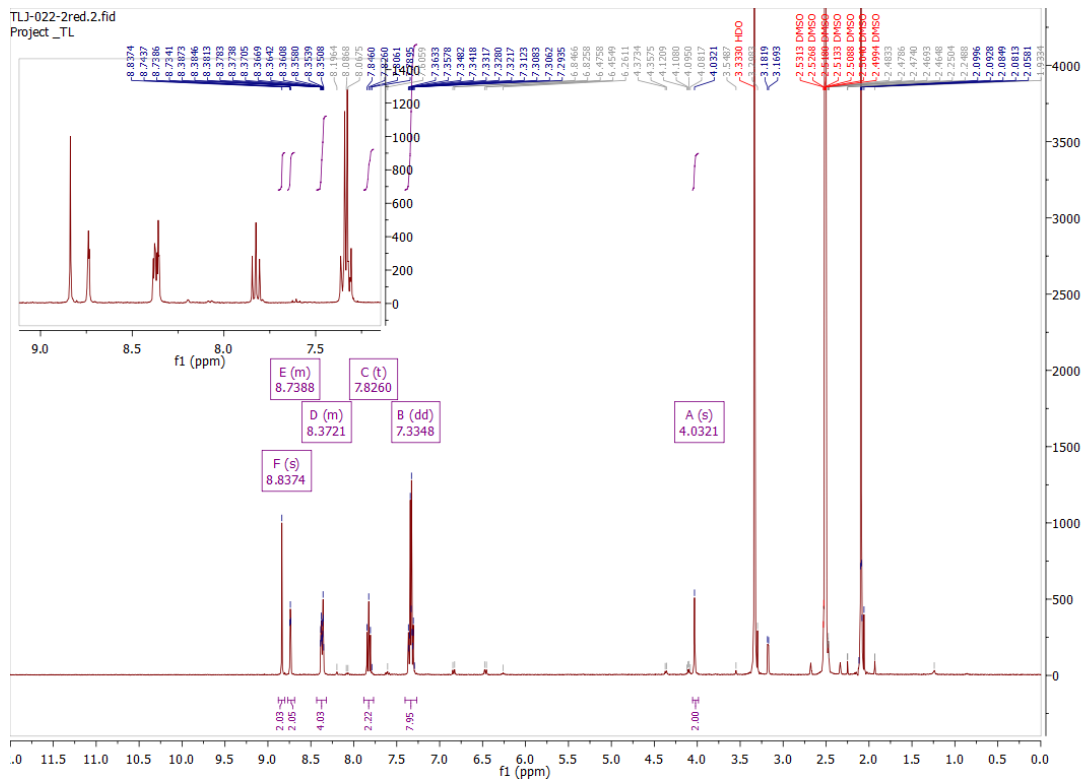
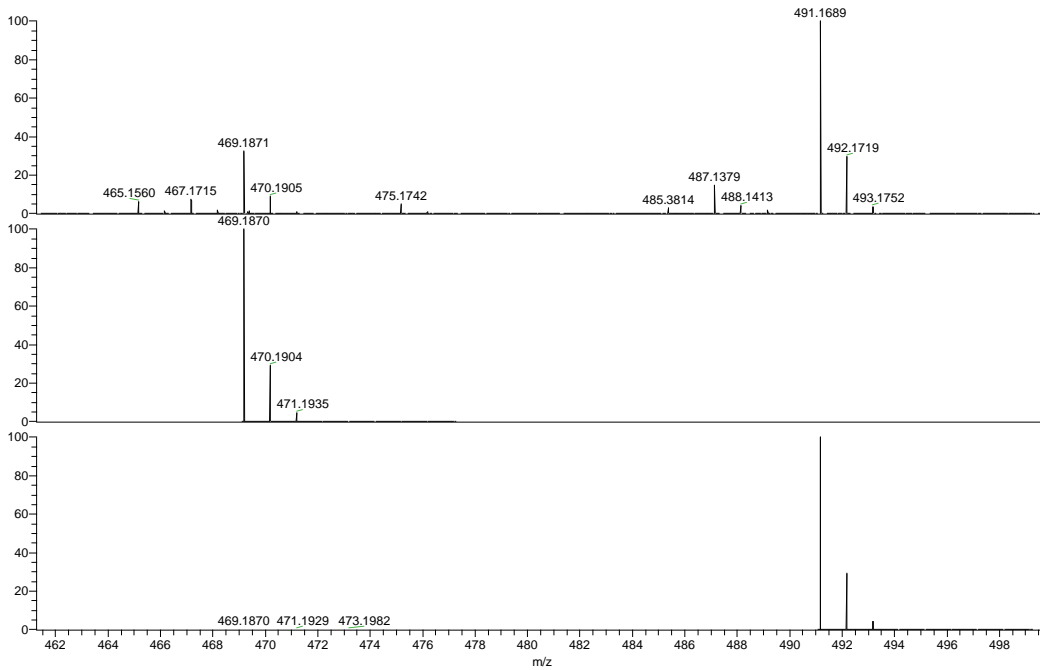


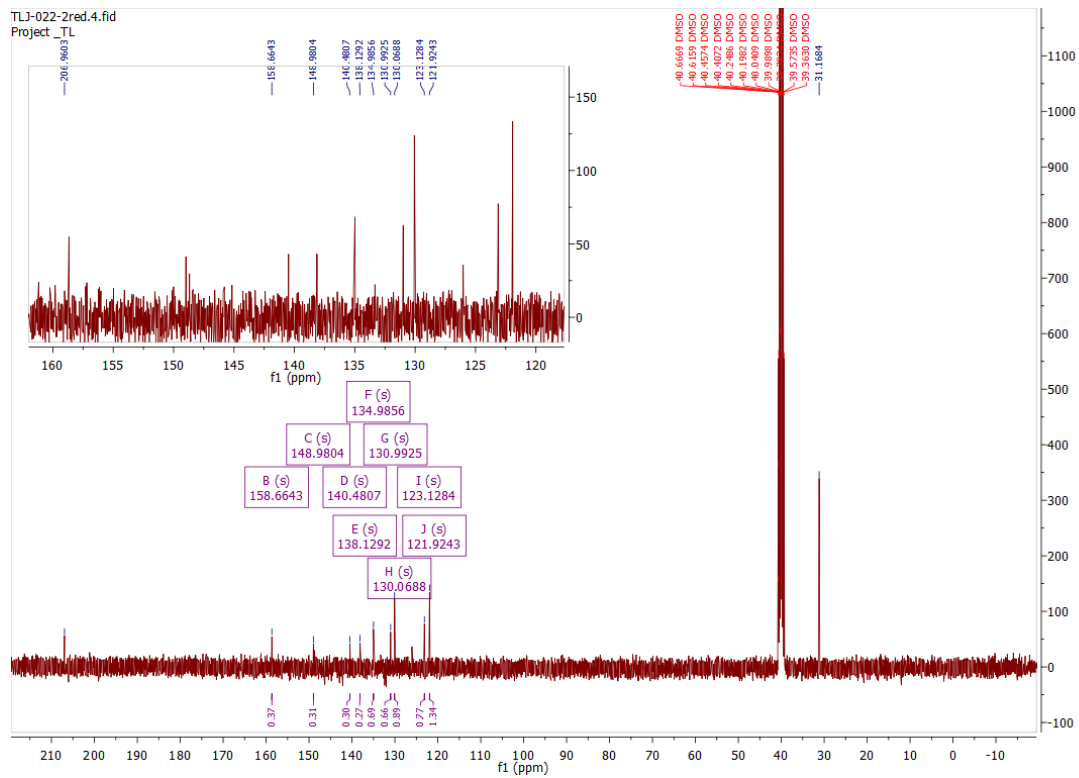
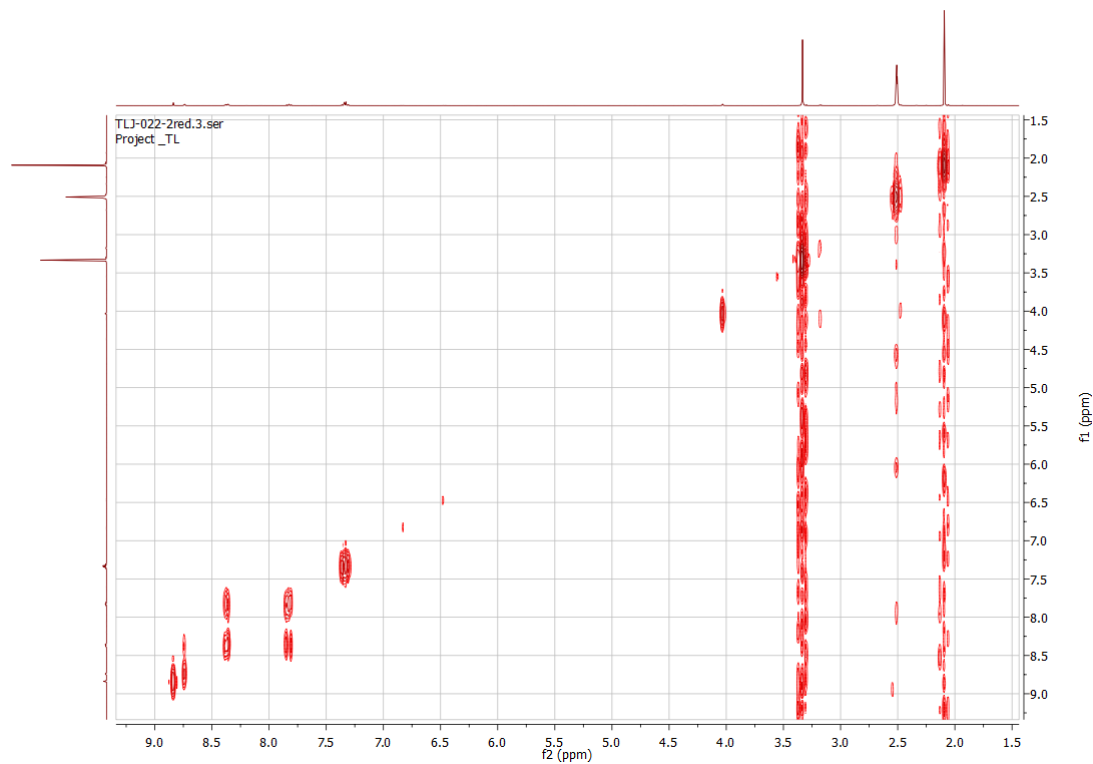




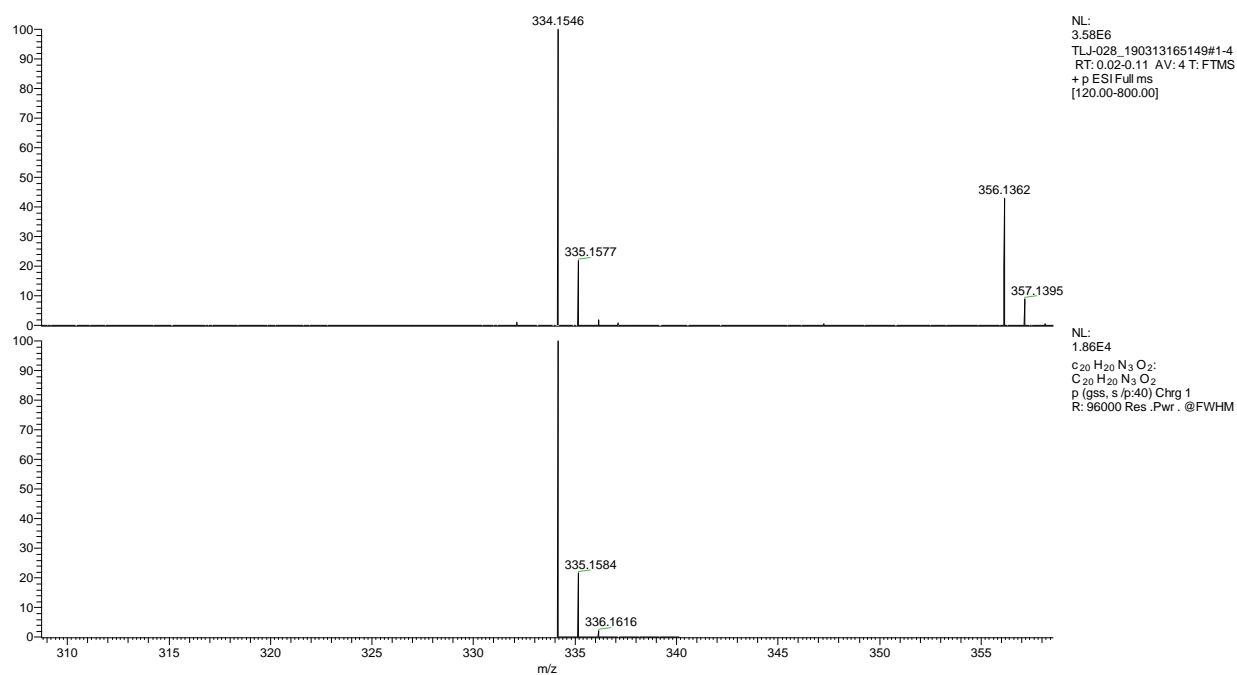
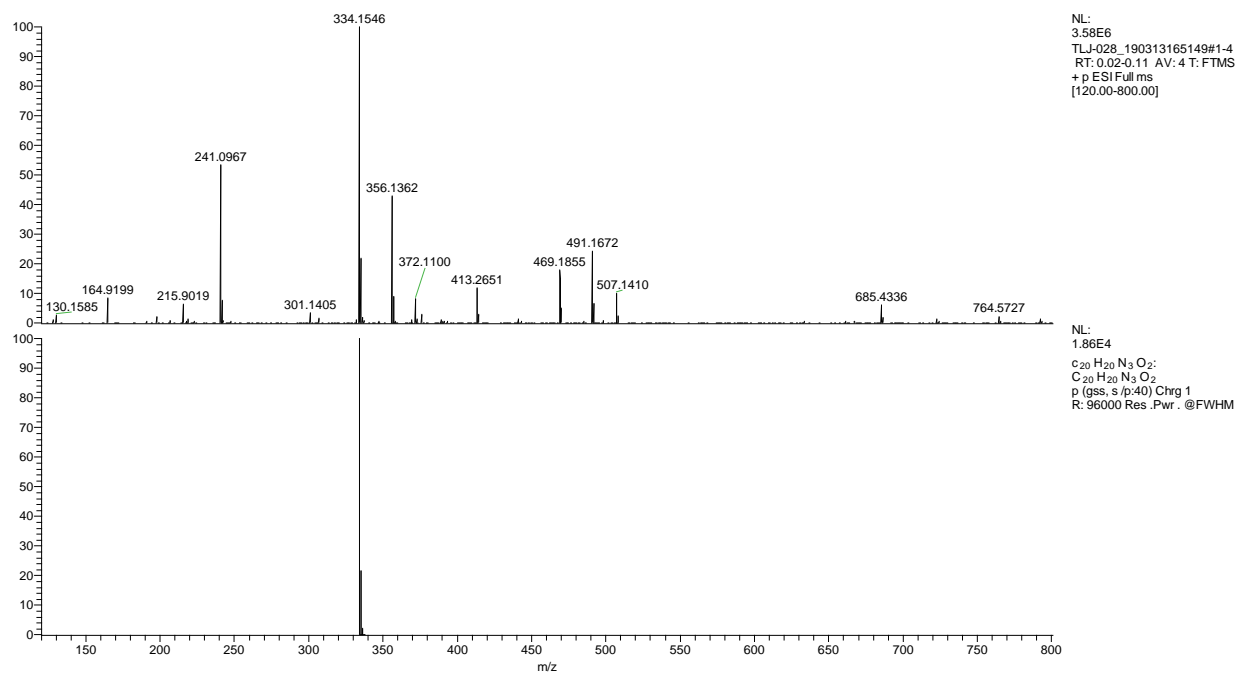
8.1.22 (1E,1'E)-N,N'-(methylenebis(4,1-phenylene))bis(1-(3-nitrophenyl)-methanimine)

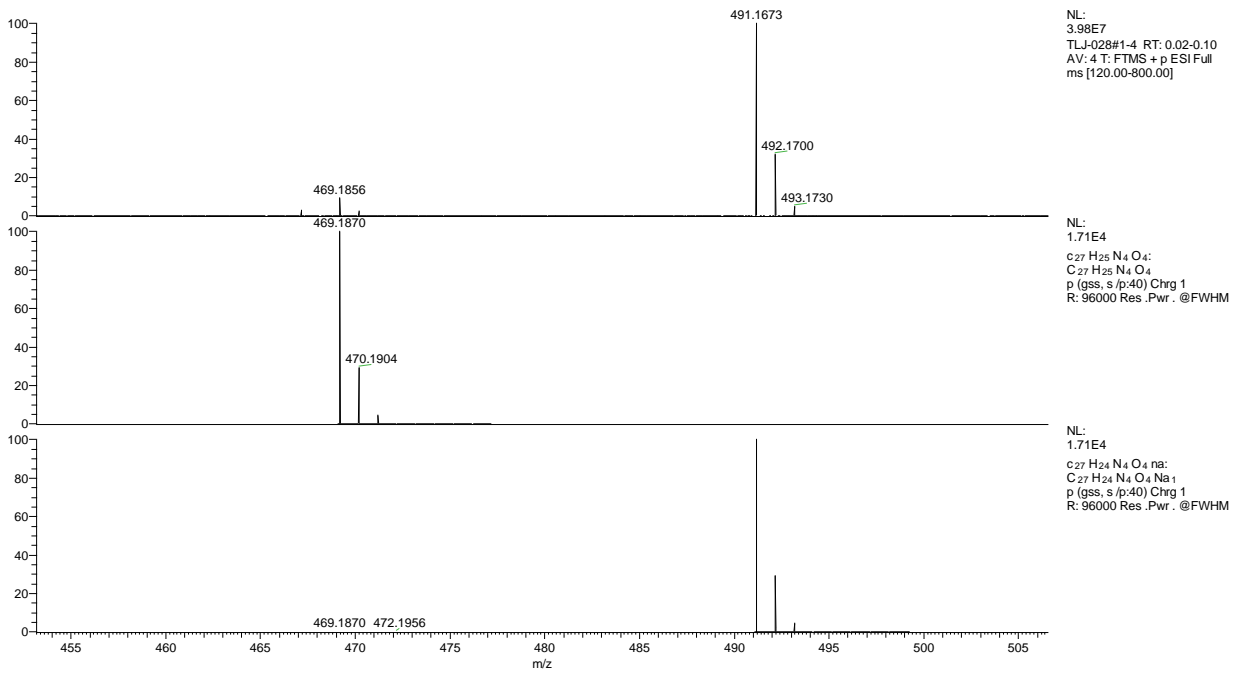
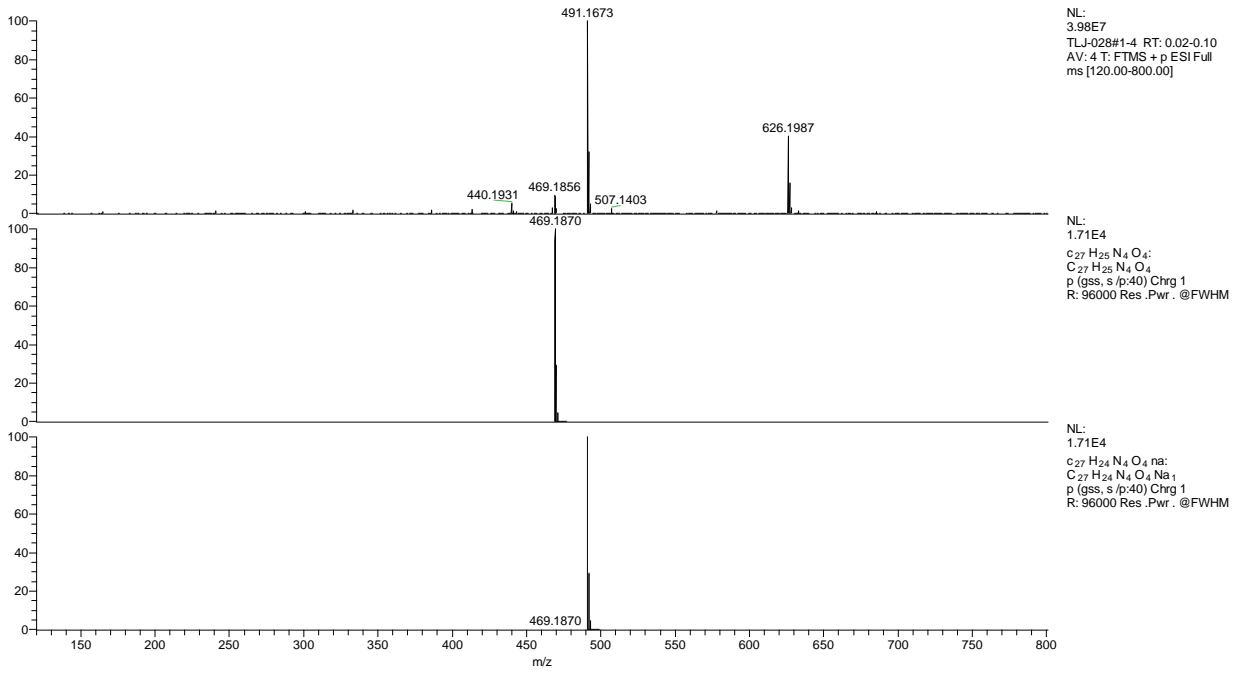


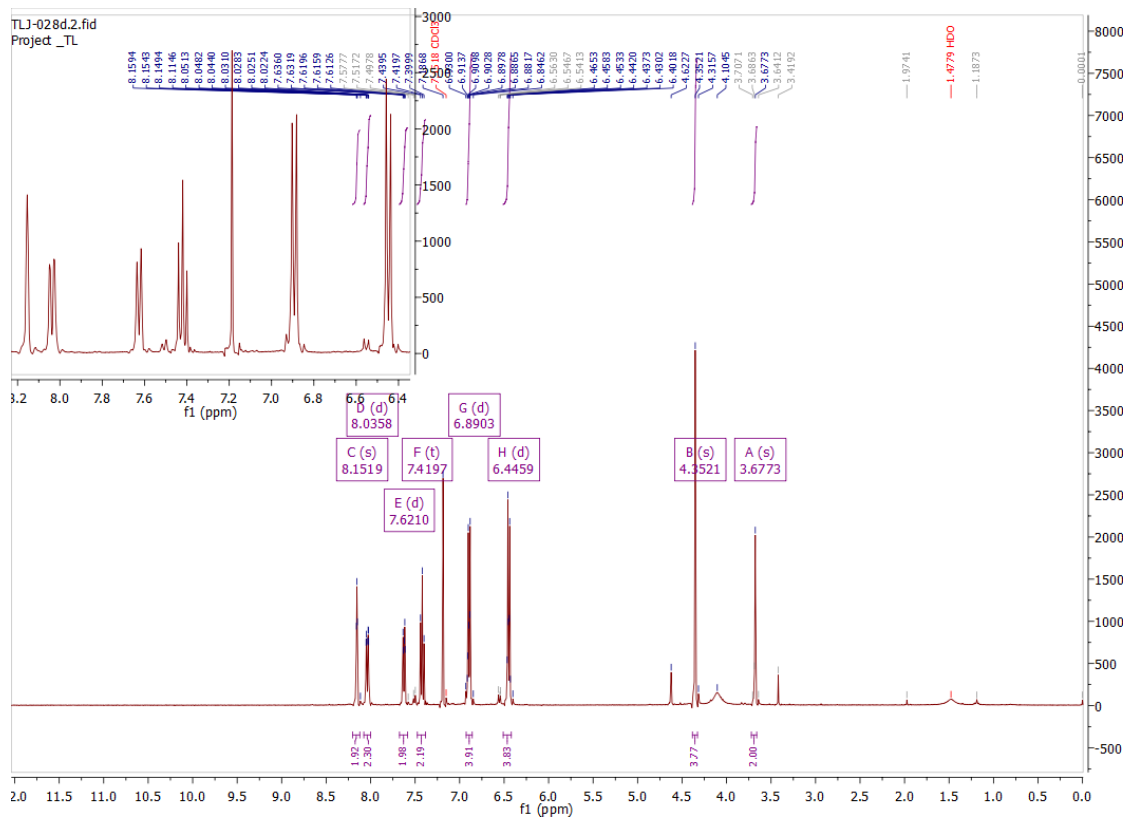
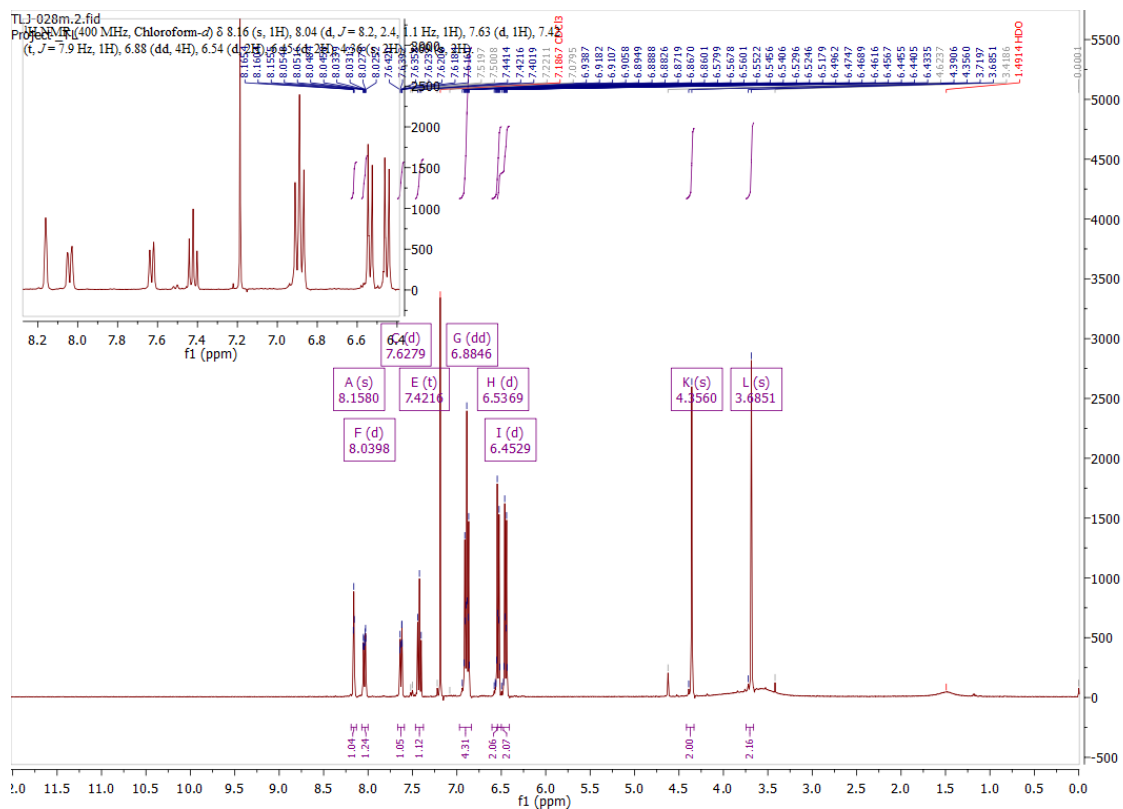


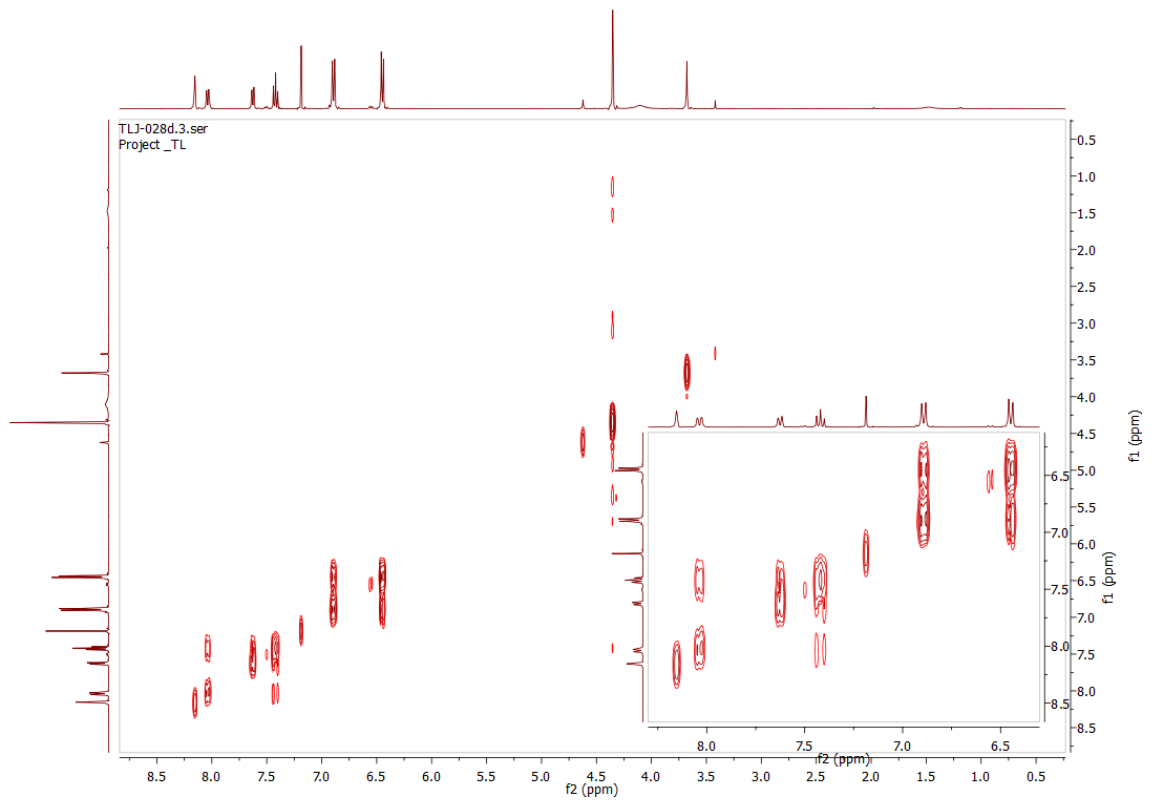
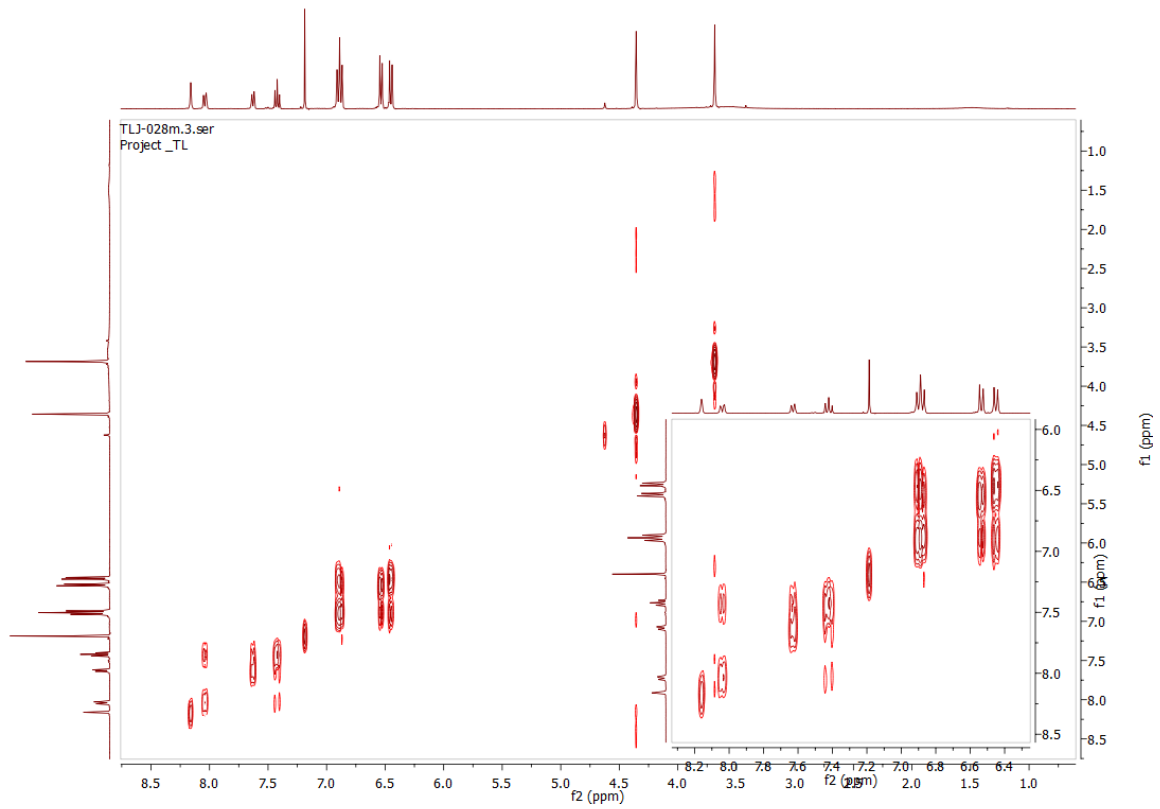


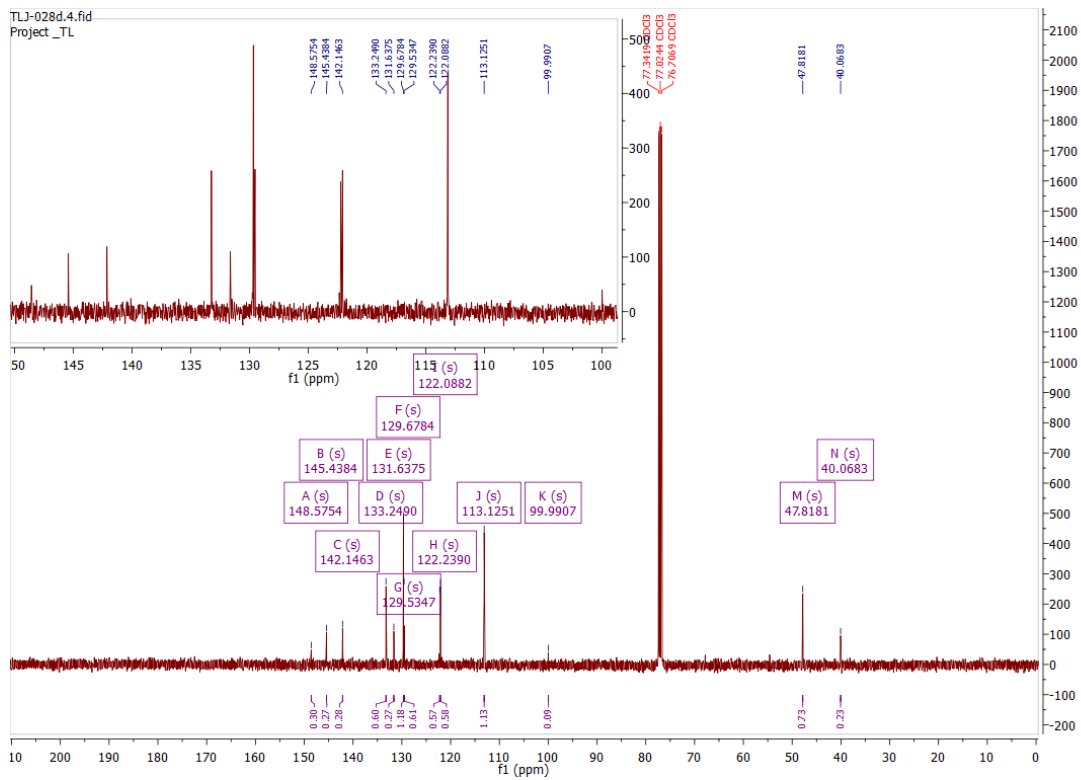
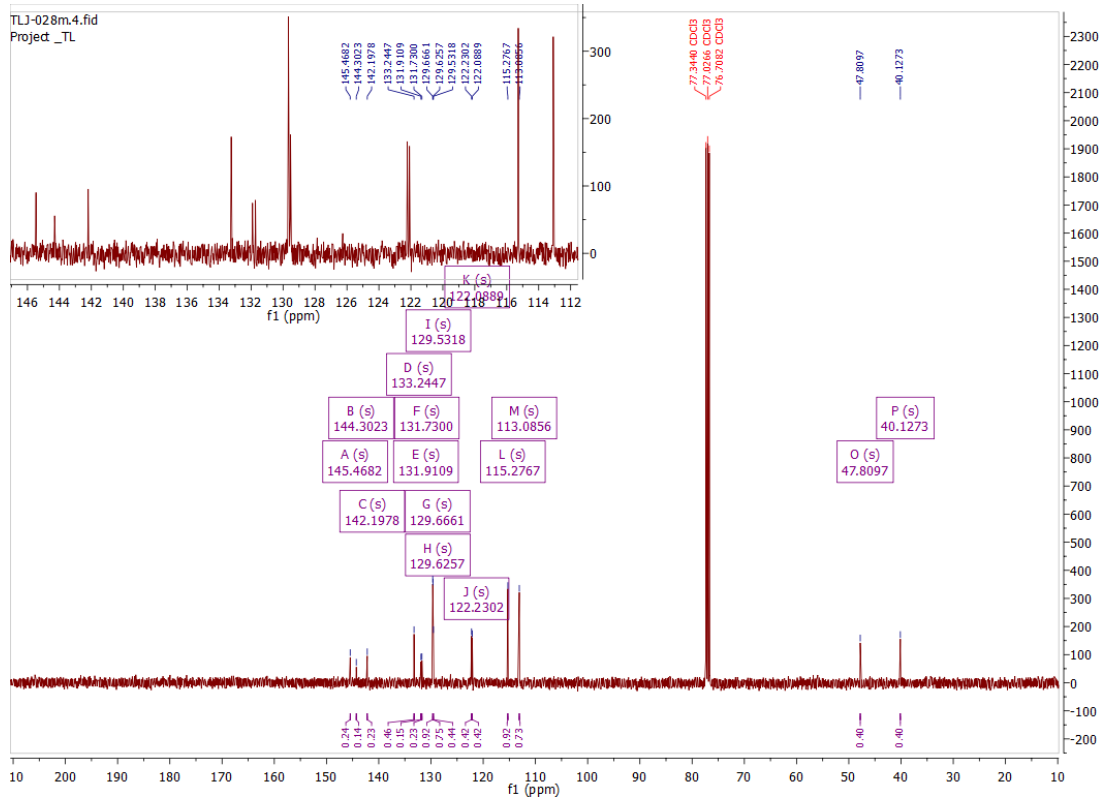
8.1.23 4-(4-aminobenzyl)-*N*-(3-nitrobenzyl)aniline and 4,4'-methylenebis(*N*-(3-nitrobenzyl)aniline)





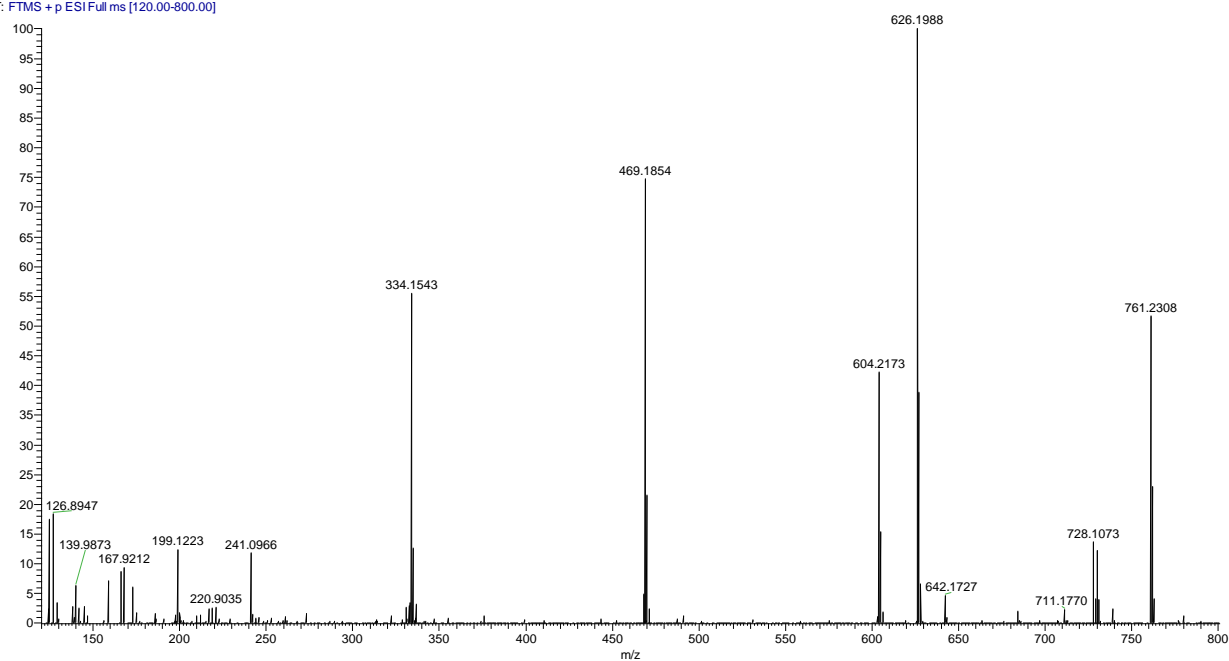






8.1.24 4,4'-methylenebis(*N*-(3-nitrobenzyl)aniline)

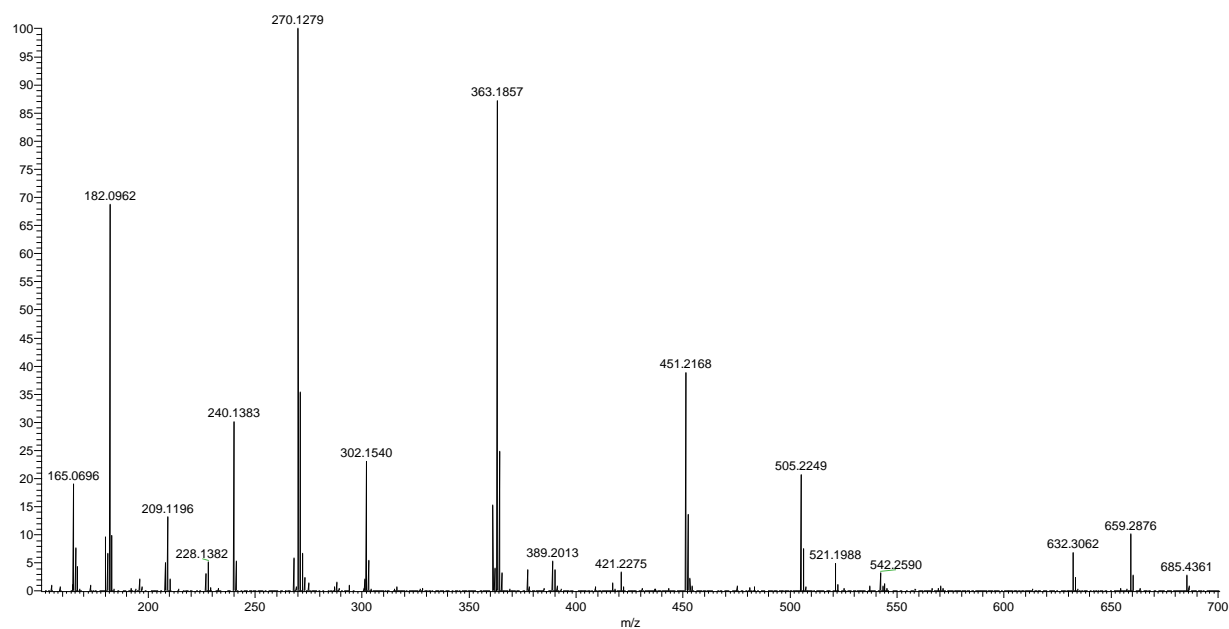
TLJ-029_posb #1-5 RT: 0.00-0.12 AV: 5 NL: 2.12E6
T: FTMS + p ESI Full ms [120.00-800.00]



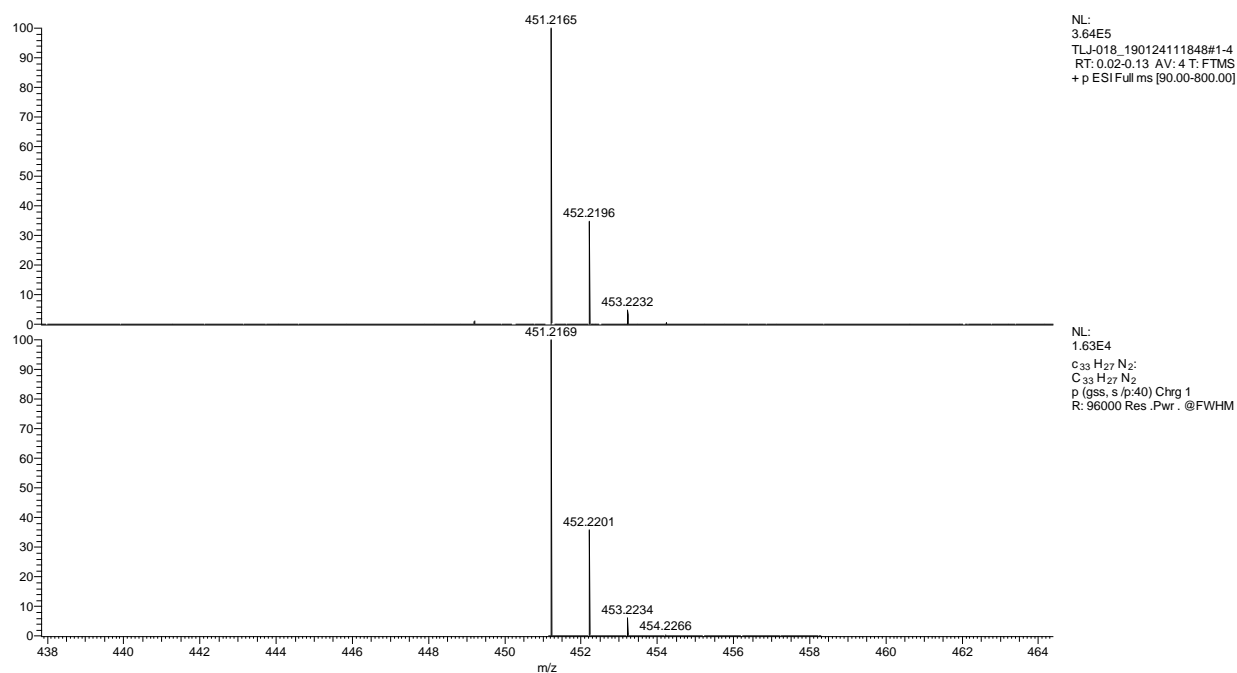
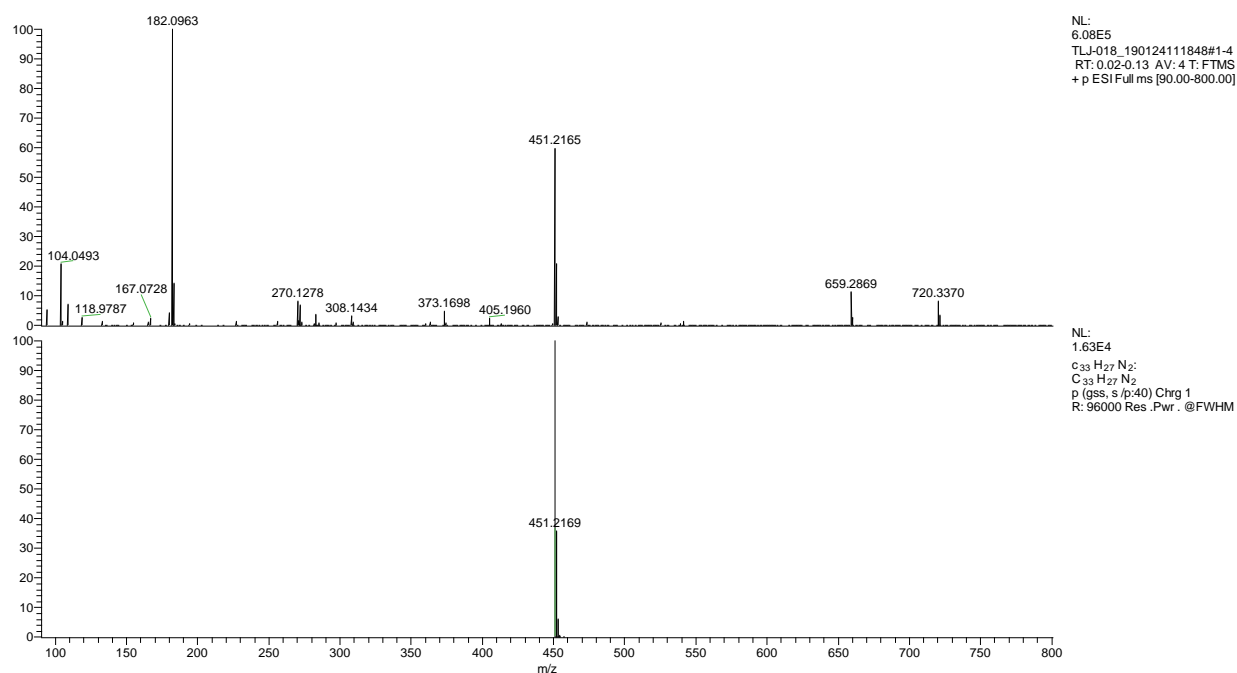
8.2 Additional HRMS spectra

8.2.1 4.1.11 high acid conc. MW synthesis

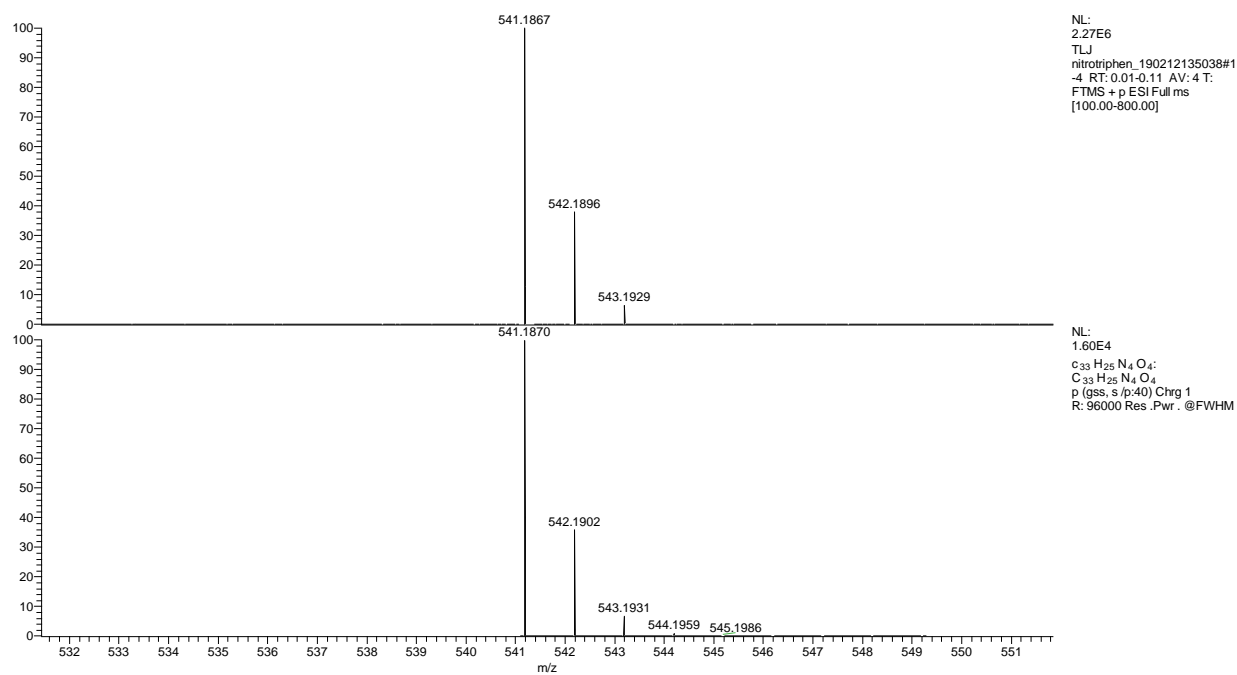
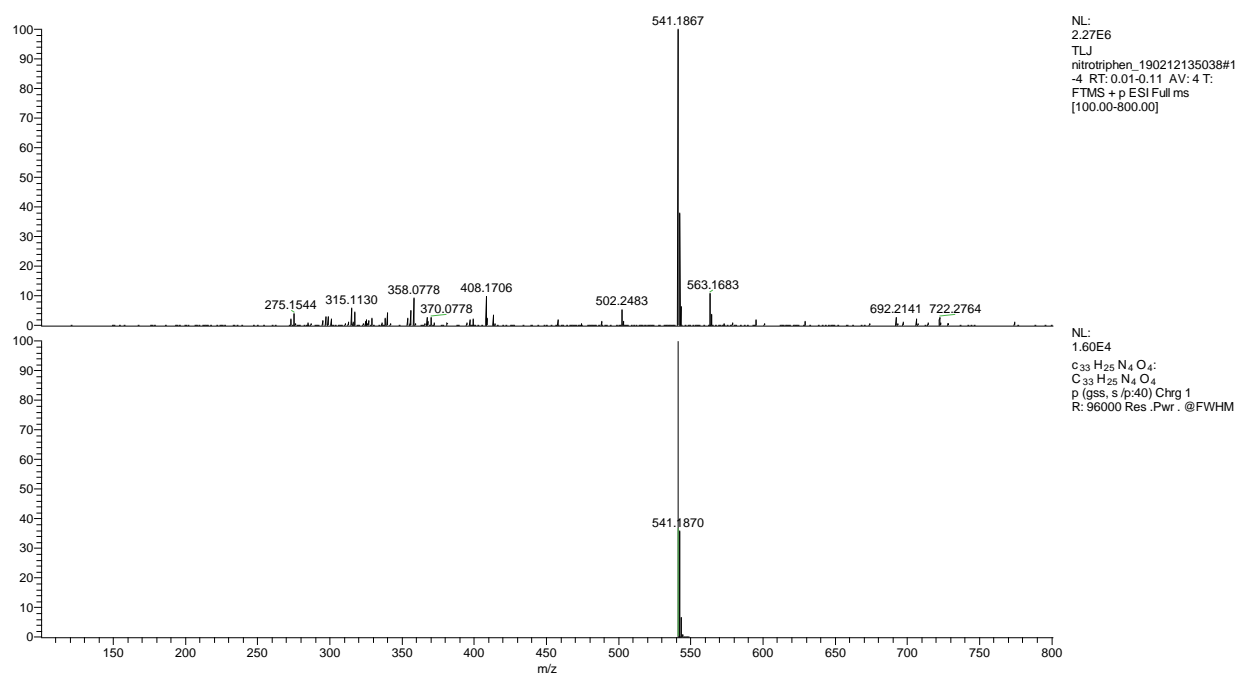
TLJ-002-2_181214120005 #1-5 RT: 0.00-0.11 AV: 5 NL: 1.61E7
T: FTMS + p ESI Full ms [150.00-700.00]



8.2.2 4.1.11 Attempt at one-pot MW synthesis



8.2.3 4.1.11 Attempt at reacting PDMA with 3-nitrobenzaldehyde



8.2.4 4.1.11 Attempt at synthesizing a different PMDA compound

