Development of Small Molecules to Inhibit COX-2 by Perturbing Mitochondria of Cancer Cells

A THESIS

Submitted in partial fulfilment of the requirement for the award of the dual degree

Bachelor of Science – Master of Science

in

CHEMISTRY

by

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CERTIFICATE

This is to certify that this dissertation entitled "**Development of Small Molecules to Inhibit COX-2 by Perturbing Mitochondria of Cancer Cells**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the work and studies carried out by "**Deepshikha**" at "**Indian Institute of Technology, Gandhinagar**" under the supervision of "**Dr. Sudipta Basu**, Associate Professor, Department of Chemistry" during the academic year 2019-2020.

Signature of Supervisor

Dr. Sudipta Basu

DECLARATION

I hereby declare that the matter embodied in the report entitled "**Development of Small Molecules to Inhibit COX-2 by Perturbing Mitochondria of Cancer Cells**" are the results of the work carried out by me at Indian Institute of Technology (IIT-GN), Gandhinagar under the supervision of **Dr. Sudipta Basu** and the same as not been submitted elsewhere for any other degree.

Dshikha:

Signature of Student
Deepshikha

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor Dr. Sudipta Basu for guiding me throughout this project. I feel very honoured to be the student of brilliant teacher here who taught me the academic subjects and many more things about life. I appreciate his attitude for constantly encouraging me in developing my research and presentation skills. I am thankful to all professors at IISER-Pune for teaching me several different topics and subjects, some of which were extremely useful and needed for my project to be a complete piece. I would like to thank my TAC member Dr. S.G.Srivatsan who helped me in understanding and developing fundamentals of organic chemistry. I would like to thank Dr. Sopan Shinde, Shalini Pandey, Aman, Jaypal and Rahul, and all other labmates for being there for supporting me and helping me in every possible ways throughout my project also for the discussion to motivate me and encouraging me for my life goals. I would like to thank my friends who regularly keep me humoured and happy even in my worse times. Finally I would like to thank my family for their motivation, guidance and right direction they have provided me throughout my academic journey. I would like to convey my genuine appreciation and heart felt gratitude to all of them.

CONTENT

ABSTRACT

Over a long exposure of drugs through chemotherapy cancer cells develops drug resistance due to which drug is no more effective on cancer cells. To overcome this problem scientists are trying to target a specific organelle in cells such as nucleus, endoplasmic reticulum (ER), mitochondria etc. In this context, we have hypothesised an approach to target a specific organelle of cancer cells, to inhibit a particular enzyme that reduces the chance of cancer cells proliferation. We are mainly interested here to target mitochondria, as mitochondria are being found to be a key factors in biomedical science, and so inhibit the inducible COX-2 enzyme by using selective COX-2 inhibitors like Indomethacin and Ibuprofen, present inside the mitochondria as reported in many literatures. To target mitochondria specifically we have synthesised small molecules attached with a positively charged moiety that helps the molecules to drag and internalise towards mitochondrial negatively charged membrane. For the positive charge different derivatives of Triphenylphosphine (TPP) have been used, simultaneously it is also demonstrated that which of the derivatives is more effective in cellular internalizing the small molecules inside mitochondria, cell viability has been reported by MTT assay using MCF-7 cells which has reported good amount of killing in comparison to free drug. In order to further check the cellular internalization fluorescence compound has been synthesized of the same COX-2 inhibitors. The results suggest that these small molecules can be used to inhibit COX-2 by specifically targeting the mitochondria. Hence, by doing so we can demonstrate which among a particular inhibitor attached to one of the TPP derivatives is more efficient as cancer treatment approach.

1. INTRODUCTION

1.1 Cancer: Cancer one of the leading causes of morbidity and mortality worldwide. One of the reasons for thousands of deaths worldwide. The biological causes of cancer are uncontrolled signal transduction that causes uncontrolled cell proliferation, loss of apoptosis, tissue invasion and metastasis and angiogenesis. To understand the characteristics of cancer cells the hallmarks of cancer cells outlined by Hannah and Weinberg. Resistance to cell death, sustaining proliferation signalling[9] and deregulation of cellular energetic are the imperative hallmarks of cancer which are lightly governed by important sub cellular organelles such as mitochondrion, nucleus and endoplasmic reticulum (ER)[4]. Thus, specific targeting of the organelles in cancer cells is interesting strategy for future cancer therapy. By all the studies and experiments done so far we come to a point other than nucleus, ER and cytoplasm etc. mitochondria being the powerhouse of the cells is equally important for cell survival, also it leads to cell death via apoptosis. Howevercancer cells develop drug resistance through repairing pathways hence we have chosen mitochondria as our targeting organelle because of its apoptotic properties.

Cancer cells also develop apoptotic resistance and ratio of cells division accelerates over the cell death. Mitochondria apoptosis is resist by many proteins and enzymes like COX-2, 5-lipooxygenase, fatty acid CoA ligase-4 etc. our main target is to inhibit COX-2 protein and suppress its function. COX-2 is an inducible isoform of Cyclooxygenase (COX) induced by cytokines, mitogenic factors and environmental stress signals. By literature it is known that the CoX-2 is found to localize in mitochondria with Type IV calcium-independent PLA_2 ($iPLA_2$)[15] and heat shock (hsp60) proteins which was confirmed by confocal imaging microscopy. COX-2 converts arachidonic acid into prostaglandin H_2 which is a precursor of diverse prostanoids[31], also it upregulates antiapoptotic BcL_2 protein hence reduce apoptosis. To specifically target mitochondria and COX-2 in mitochondria we are synthesising small molecules having a positively charged head which will drag the molecules towards mitochondria as it has negatively charged membrane attached with a COX-2 inhibitor. Positive charge we are getting from Triphenylphosphine, also we are using different derivatives to check which derivative is more efficient to drag small molecules towards mitochondria.

1.2 Mitochondira as specific target:

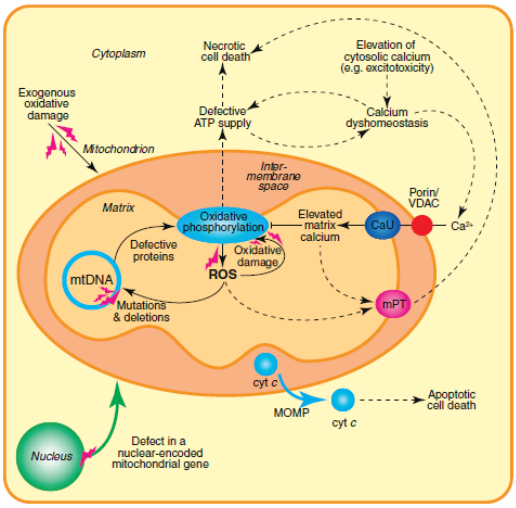


Figure 1: Mytochondrial dysfunction

Mitochondria are vitally important for energy production in cells therefore for a healthy survival, including apoptosis mitochondria also regulates various signaling pathways[6] as many of them converges to mitochondria hence plays a role of suicidal weapons heap in the cells.

So far for its crucial roles in cells survival and death it has allured and tempted many research scientists to work on mitochondria targeting. Any kind of maladjustment and debilitation in mitochondria results in various diseases which are linked to cancer neurodegenerative diseases Parkinson's or Alzheimer etc[23]. Mitochondria are serviceable in utmost all cancer cells. It has been bring that existence and metabolism of mitochondria is essential for cancer evolution or progression. Mitochondria bioenergetics, biosynthesis manufacture and redox signaling and stability are being targeted for the treatment of cancer. In few studies it is found that targeting mitochondril DNA (mtDNA) is also an amazing therapy. (*ACS Appl. Mater. Interfaces*, 2016, *8*, 13218-13231.)

1.3 COX-2 member of Cyclooxgenase enzyme family:

It is acknowledged that the connection between cancer and chronic inflammation intails cytokines and various inflammation pathways which takes place during variants steps of tumour growth. The rate limiting step of biosynthesis of prostaglandin is catalysed by a family of enzymes that is cyclooxygenase (COXs)[17]. This involves three main members COX-1 expressed ubiquitously, entails homeostasis; COX-2 inducible isoform, that upregulates at the time of inflammation and cancer; COX-3 found to be in brain and spinal cord. COX-2 harmonize cell proliferation and apoptosis mostly in solid tumours[16].

1.4 COX-2: Tumor promoter and better target for cancer therapy:

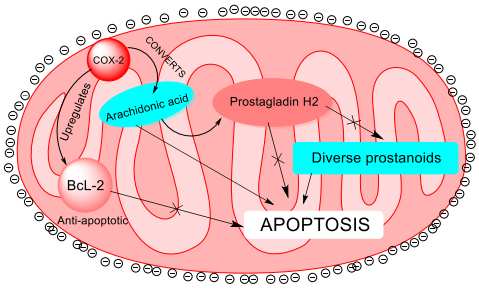


Figure 2: COX-2 inhibits apoptosis

Cyclooxygenase-2 (COX-2) abbreviate isoform of COX enzyme family has a very crucial impact on inflammation and tumorigenesis, factors such as environmental stress, cytokines and mitogenic component are involved in inducible form of COX-2. The produces forerunner of diverse prostanoids that is prostaglandin-H_2 by arachidonic acid through prostaglandin-G_2[20,21] intermediate of very less half-life.

COX-2 is revealed in many human cancer cells such as lung colon and gastric cancer cells. COX-2 has been found over expressed in many cancer cells, it has been reported that COX-2 plays a very crucial role at various steps in cancer promotion, via increment in mutated cancer cell proliferation, hence affect the efficacy of anticancer therapeutics and promotes tumour by affecting programmed cell death, overall affects the apoptosis of mitochondria as a result of loss in cell anchorage, that leads to metastasis formation[24]. Studies shows that the correlation of PGE2 and COX-2 plays a very vital role in tumour promotion via enhancement in tumour invasion cell proliferation also cell survival rate. This link of PGE2 with tumour progression can give a site to us that why using COX-2 inhibitors[8,9] (i.e ibuprofen and indomethacin) could be a better strategy in cancer treatments, therefore COXs are found to be great targets for several compounds that inhibit their function. Henceforth several NSAIDs are found to be better targeting compounds of COX-2. NASIDs bind in reversible competitive inhibition to active site of COX-2. Hence, COXIBs was developed as selective Cox-2 inhibitors as these are less gastrotoxic.

Apoptosis: In Multicellular organisms[16,17] tissue homeostasis is developed and maintained through the process of apoptosis

There are two important pathways for regulating apoptosis in intracellular regions. The intrinsic cascade[13], also known by stress-induced or mitochondrial apoptosis pathway which is an ultimate and quick response for the DNA damage. Mitochondrial outer membrane permealization (MOMP)[18] is a suitable hallmark for this cascade that escort along with disintegration of mitochondrial membrane potential that delivers requisite apoptosome

component cytochrome C and leads to apoptosis. On the other hand, extrinsic apoptosis cascade also termed as physiological apoptotic pathway that activates when a death receptor get binds with a certain ligand in the existence of death effector domain (DED) [20].

Our main focus is on intrinsic pathway of apoptosis. However, both the cascades are linked together as cross-talks between them takes place in which intrinsic pathway gets activate by extrinsic pathway via abbreviation of BH3 only protein through caspase-8[22].

1.5 Role of COX-2 in intrinsic apoptosis:

Cells upon activation through intrinsic apoptotic cascade encounter apoptosis because of DNA damage occurred due to various chemicals or surrounding and physical stress which is related to redox disequilibrium, associated with glutathione depletion (GSH) [23]. This is an ultimate requirement of translocation and activation the mitochondrial Bax[24] a pro-apoptotic Bcl-2 protein membrane. Bax forms oligomers that activate the release of cytochrome-c and MOMP. Similarly, Bak does the same process[25]. The upregulation of antiapoptotic proteins with addition of amended function of proapoptotic proteins or on the other hand downrange of one of the tumour suppressor genes[29] (i.e. p53), are rigorously in relation with disparity in between apoptotic and cell proliferation found in cancer cells. And it is also found in various studies that COX-2 plays a crucial role in all these apoptotic inhibition process because it was reported that inhibition of COX-2 mediate the apoptosis at an elevated rate in several cancer cells model.

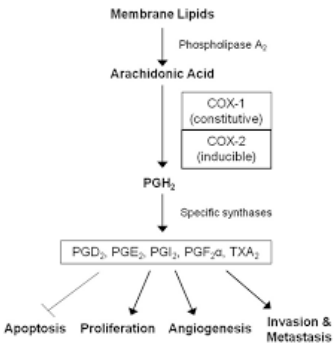


Figure 3: Role of COX-2

How apoptosis is stimulate by COX-2 inhibitors is explained through various mechanism in which one is increment of intercellular arachidonic acid is supposed to linked directly with COX-2 inhibition. Where intercellular Bcl-2 level[29] is altered by arachidonic acid, as Bcl-2 transfection inhibits apoptosis engendered by arachidonic acid. Sphingomyelinase activates when there is an increment of intercellular proapoptotic ceramides affects apoptosis by aggregation of arachidonic acid. Also, prostaglandin [19] production shows downstream as the Nitric Oxide (NO) level reduces in cancer cells because of

COX-2[18]. On the other hand prosurvival pathways (PI3K/ AKT) which is a survival cascade is activates by COX-2 inhibitors and this leads phosphorylation of Bad, member of BH-3 only proteins[18,19].

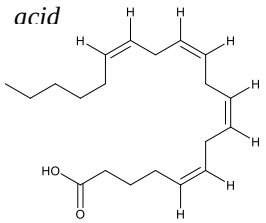
1.6 COX-2 inhibitors in Chemotherapy:

On the account of development of cancer cells which are multidrug resistance is a major reasons of failure of chemotherapeutic drug. Various mechanism comprise of over exposed apoptosis inhibiting proteins cause resistance to cancer treatment. The best interpreted mechanism is P-gp (P-glycoprotein)[5,6] that assist multidrug resistance (MDR). To target COX-2 we are using selective COX-2 inhibitors such as Indomethacin, Ibuprofen and Aspirin. To specifically target mitochondria and COX-2 in mitochondria we are synthesising small molecules having a positively charged head which will drag the molecules towards mitochondria as it has negatively charged membrane attached with a COX-2 inhibitor. Positive charge we are getting from Triphenylphosphine, also we are using different derivatives as mentioned in Fig1 to check which derivative is more efficient to drag small molecules towards mitochondria.

1.7 Localization of COX-2 in mitochondria of cancer cells:

Localization of Type-IV calcium independent PLA2 (iPLA2)[32] found to be mitochondria inner membrane and inhibition of (iPLA2) lead to enervated of apoptosis and loss of phospholipids in mitochondria. However, production of Arachidonic Acid (AA)[34] is catalysed by iPLA2 in mitochondria and leads to apoptosis through mitochondria and leads to apoptosis through mitochondria transition disruption, whereas COX-2 found to be aid of AA sink resisting against apoptosis. Hence, studies reported that mitochondrial localization of iPLA2 and COX-2 by confocal microscopy in cancer cells, also reported in many studies that COX-2 colocalised in mitochondria with heat shock protein (hsp60)[1,2] many experiments reported the localization of COX-2 in mitochondria of cancer cells but did not spotted in primary cultured cells.

Figure 4:Arachidonic acid



Also COX-2 was found largely in cytoplasm and small amount in nucleus this was confirmed by confocal microscopy experiments of COX-2 in HT-29 cells. Several results shows that COX-2 is localized with hsp60, the hsp60 primarily found to be colocalized into mitochondria and cytosolic COX-2 also found to be localized with hsp60.

All the results given in literature all together shows that mitochondria of cancer cells contains COX-2 whereas normal human healthy cells mitochondria do not contain COX-2. To further confirm these results of mitochondria localization of COX-2 different fractions of cytosolic and mitochondria were examined and COX-2, calregulin protein and hsp60 were taken and determined by western blotting (fig) exploration and found the abundance of COX-2 in mitochondrial fraction was detected and experimental confocal microscopy confirms the result that localization of iPLA2 and COX-2 are abundant in mitochondria of cancer cells. However, mitochondria of human fibroblast cells (HFb)[33] also show the localization of iPLA2 but not incorporate with COX-2.

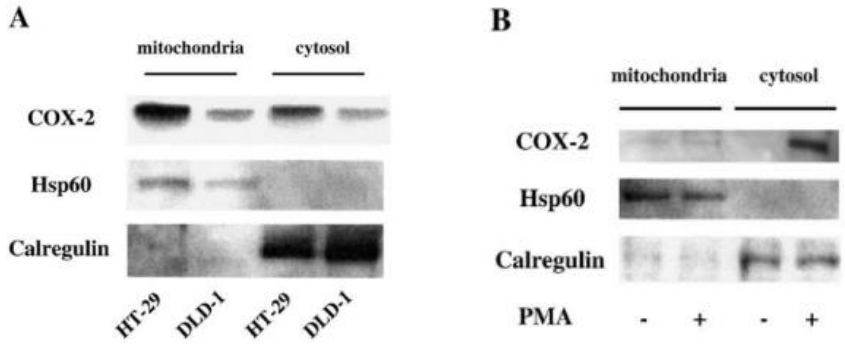


Figure 5: Analysis by western blotting of Sub cellular localization of COX-2 in (A) HT-29 and DLD-1 and (b) HFb (*Exp. Cell. Res.,* **2005,** *306,* 75-84)

2. RESULTS AND DISCUSSION

In order to improve the efficiency of cancer drugs and to improvise the cancer treatment therapy, targeting a specific organelle is challenging but possible. For which we had hypothesis a pathway to target one specific organelle that is mitochondria of cancer cells and to selectively inhibit the COX-2 enzyme present inside it. For this approach we have synthesised ten small molecules which are consist of selective COX-2 inhibitors (Indomethacin and Ibuprofen), six different derivatives of Triphenylphosphine both linked with Bromopentanol. The compunds are synthasised by using different coupling reagents and solvents in two step process involving nitrogen condition and reflux condition respectively. Similarly, two more compounds were synthesised in order to check the internalization of the molecules inside the mitochondria by using the fluorescence property by using 1,2,3,3-tertamethyl-1,H-indolium and COX-2 inhibitors attached via Parahydroxybenzaldehyde.

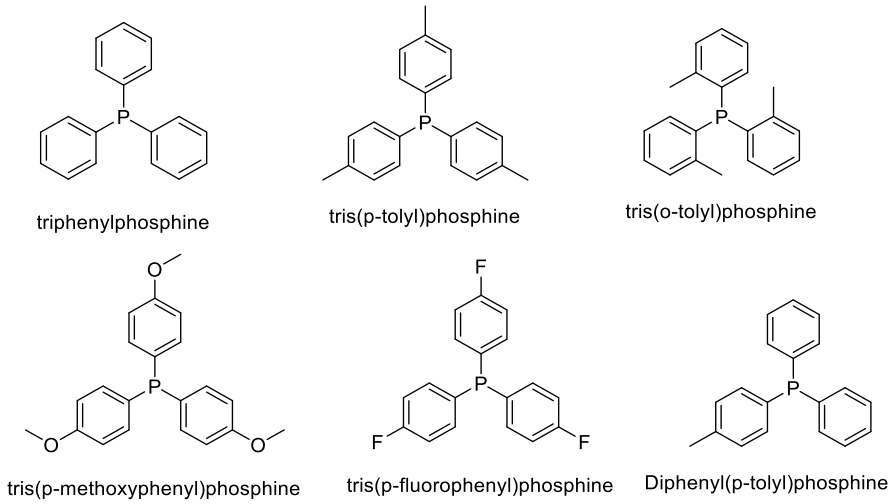


Figure 8: Derivatives of Triphenylphosphine

The purpose of this work is to develop a targeting approach where we can specifically target the mitochondria of cancer cells and inhibit the COX-2 enzyme inside mitochondria of cancer cells. To achieve this, covalent linking of cation such as triphosphosphonium moiety to some suitable pharmacophore[29] of our need, would be effective way to deliver our drug inside mitochondrial membrane. Because of the negatively charged membrane of mitochondria, accumulation of positively charged compound against their concentration gradient takes place in the mitochondrial matrix. Hence, we can add various lipophilic cations like TPP^+ can be linked to a bioactive compound of our need

to improve its mitochondria intake. The TPP^+ based targeting to mitochondria specifically to deliver small molecules have advantages over other approaches such as TPP^+ moieties are stable in biological system, have combination of hydrophilic and lipophilic properties, have very low chemical reactivity on exposure to cellular components and have very low chances of light absorption, hence found to be safe in humans. In this scenario the mitochondrial membrane potential and charge on the molecules are responsible forces for driving the accumulation of cations in mitochondria. However, lipophilicity of cation is a factor on which the dynamic of equilibration processes depends. The energy barrier of compound some time so higher that conjugation of TPP^+ moiety is not able to drag them into mitochondria. Hence, by trying different derivatives of TPP we can see the cellular internalization. Assuming that by increasing the lipophilic cation we can reduce or lower the energy barrier to make the transfer more compounds through phospholipid bilayer of mitochondria. Therefore we have chosen some derivatives of TPP with electron donating and electron withdrawing functional groups attached to them.

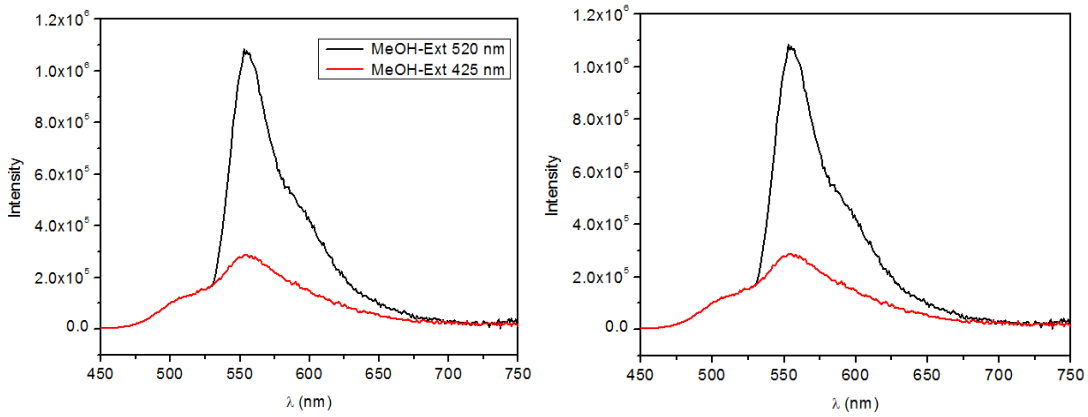
Simultaneously, we wanted to check that cancer cells are killing by the inhibition of COX-2 as per by proposed hypothesis, for this some biological experiments are in need to be performed such as western blotting and confocal imaging, in which by confocal we can able to check the cellular internalization of our COX-2 inhibitor small molecules inside mitochondria followed by which we can study the inhibition of COX-2 is done or not. However the small molecules containing COX-2 inhibitors linked to the derivatives of TPP are not fluorescence to be recorded in confocal imaging. Hence in order to see the cellular internalization of COX-2 inhibitors linked to cationic moiety inside the mitochondria of cancer cells we have synthesised a different molecules using 1,2,3,3-tetramethylindolium as positively charged moiety and parahydroxy benzaldehyde as a linker because of extended conjugation between indolium and parahydroxy benzaldehyde we can get intensified amount of fluorescence which we can use to do confocal imaging, and after getting the cellular internalization further we can use the same for western blotting to see the COX-2 inhibition.

The studies of biology suggest that the molecules are comparatively effective in killing the cancer cells with free drug that is only Indomethacin and Ibuprofen also we can see that the IC_{50} values are around 3-10 (mg/ml). The killing is more effective for the Tris-parafluoro-phosphine and Tris-paramethoxy-

phosphine showing that these electron withdrawing groups are more effective to drag the molecules inside the mitochondria as per values of IC_{50} given in the table 1.

2.1 Fluorescence data:

The fluorescence data was taken for the compound iii.**2** at the exciting wavelengths λ_{max}=520nm and λ_{max}=425nm in methanol.



Graph 2.1.1: Fluorescence Emission wavelength graphs (a) Methanol as solvent and (b) Water as solvent

From the graph we can confirm that at λ_{max}=520nm and λ_{max}=425nm particular excitation wavelength the emission is at λ_{max}=590nm and λ_{max}=530nm respectively for both excitations in Methanol and water as solvent and blank, we have taken methanol and water as solvents according to further biology experiments, to check in which environment we will get the fluorescence intensively. From this we can infer that the compound will give fluorescence in green and yellowish green band region. Since, the small molecules or the COX-2 inhibitors do not have fluorescence property therefore the compound can be used to track the internalization of molecules inside the mitochondria using mito-trackers.

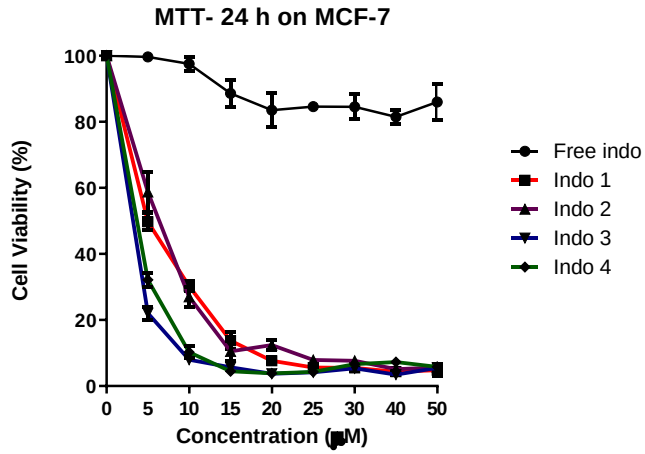
2.2 MTT procedure:

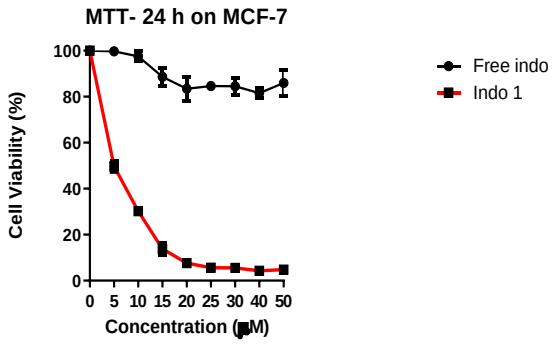
The cell viability and the anticancer ability of the compound or small molecules samples on cell line MCF-7 were resolute by MTT assay used for cytotoxicity assessment. In a 96 MTT well plate cell line MCF-7 (7000 per well) were plated in 200µl medium after incubation medium was removed from each wells for the MTT assay. Addition of 200µl of MTT (mg/ml) was done after washing each well by MEM (w/o) FCS 3 times carefully. After incubation was done in 5%

CO_2 incubator for 6-7hrs for cytotoxicity, 1ml of DMSO (solubilizing agent) added in each well and kept for 45sec after mixing it well using micropipette. After addition of DMSO, formation of formazon crystals develops the purple color which is used to visualise the viable cells. By using DMSO in blank the values of optical density (OD) was then taken in account at 595nm of the suspension taken in spectrometer cuvette.

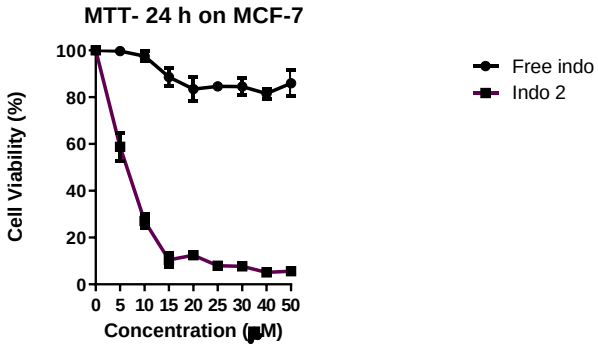
Putting the concentration of the drug on X-axis and relative cell viability on Y-axis, the standard graph was plotted and measured to determine the IC_{50} value were performed.

Cell viability (%) = (Mean OD / Control OD) x 100%

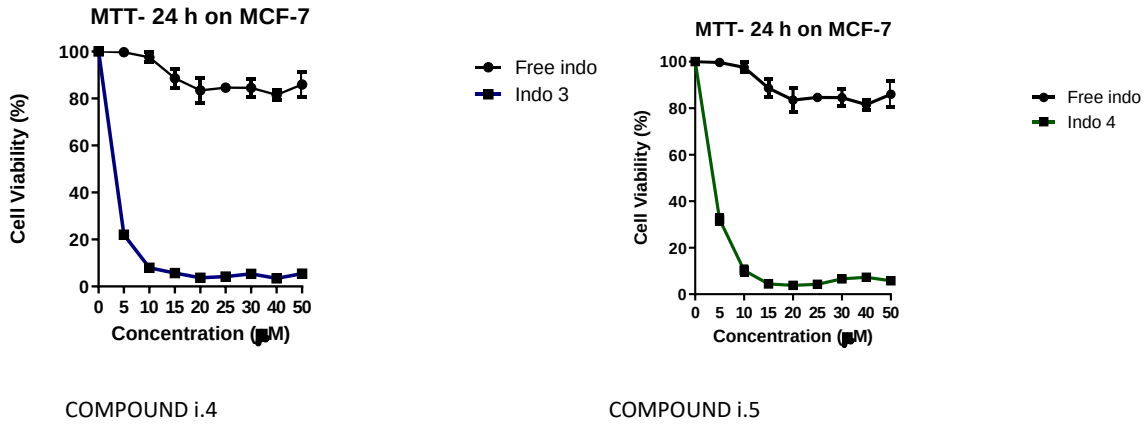




COMPOUND i.2



COMPOUND i.3



COMPOUND i.4 COMPOUND i.5

Graph 2.2.1. Cell viability assay graph (MTT assay)

COMPUNDS	IC_{50} µM
Compound i.2	4.5
Compound i.3	6.25
Compound i.4	3.5
Compound i.5	3.5

Table 2.2.2. IC_{50} values for the respective compounds.

From the graphs of cell viability of cell viability assay we can see the results of cytotoxicity assay. Cell viability was examined by MTT assay after incubation for 24hrs. It was found that all the respective molecules are able to inhibit the COX-2 and so the cell proliferation of cancer cells (MCF-7), from the results we are getting the (IC50) values around 3-10 (mg/ml) after 24 hrs exposure time. Here we get to know that the free drug indomethacin shows high IC50 values which means very less amount of cell killing, on the other hand the IC50 value of compound i.4 and compound i.5 are very low in compare to the IC50 values of other compounds. Therefore the COX-2 inhibitor with indomethacin attached to para-methoxy TPP and para-fluoro TPP are more efficient in killing of MCF-7 which is positive result and we can pursue for other experiments of biology. The compound synthesis is confirmed by NMR and HRMS Data.

The strategy is to deliver the positively charged moiety inside the mitochondria and inhibit the COX-2 present inside mitochondria of cancer cells. To target COX-2 we are using selective COX-2 inhibitors such as Indomethacin, Ibuprofen and Aspirin. To specifically target mitochondria and COX-2 in mitochondria we are synthesising small molecules having a positively charged

head which will drag the molecules towards mitochondria as it has negatively charged membrane (Fig.), attached with a COX-2 inhibitor. Positive charge we are getting from Triphenylphosphine, also we are using different derivatives as mentioned in Fig1 to check which derivative is more efficient to drag small molecules towards mitochondria.

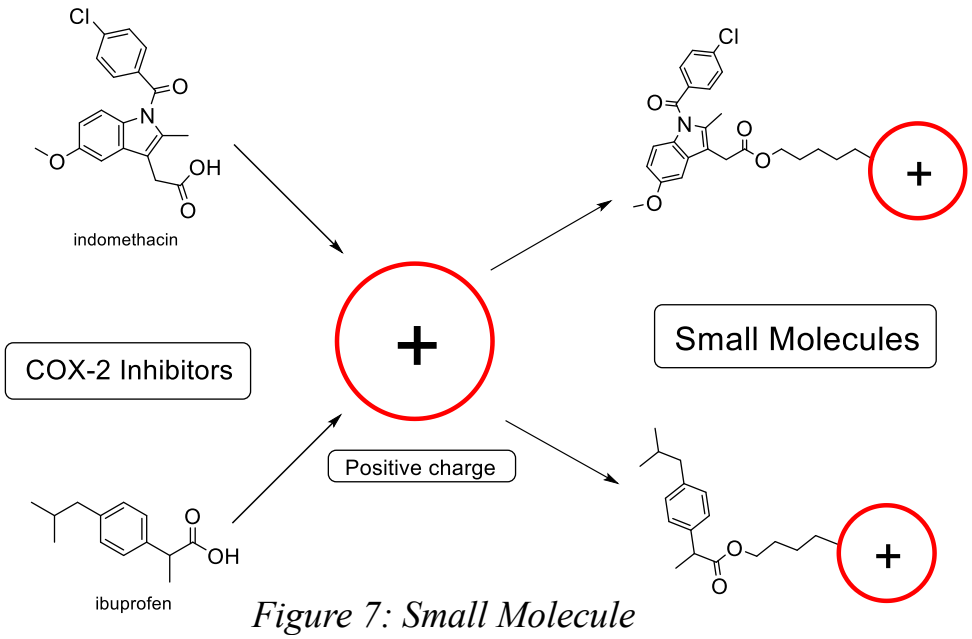
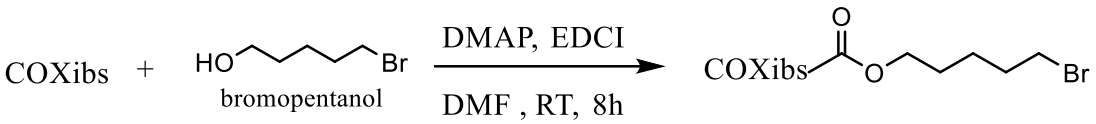


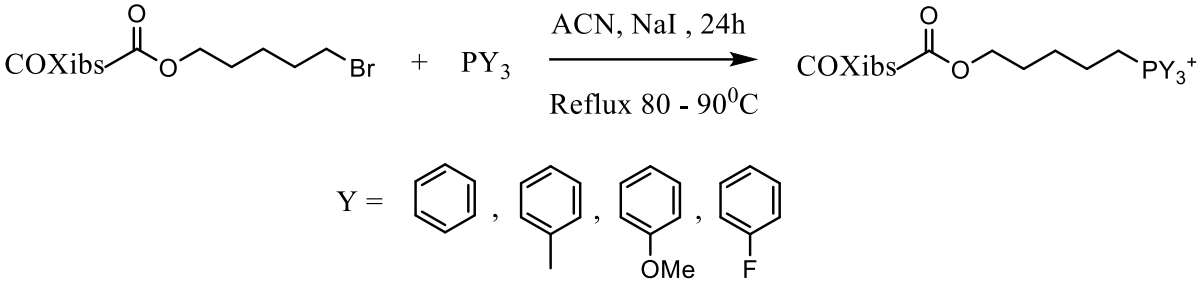
Figure 7: Small Molecule

2.3 Synthesis Approach for Small molecules

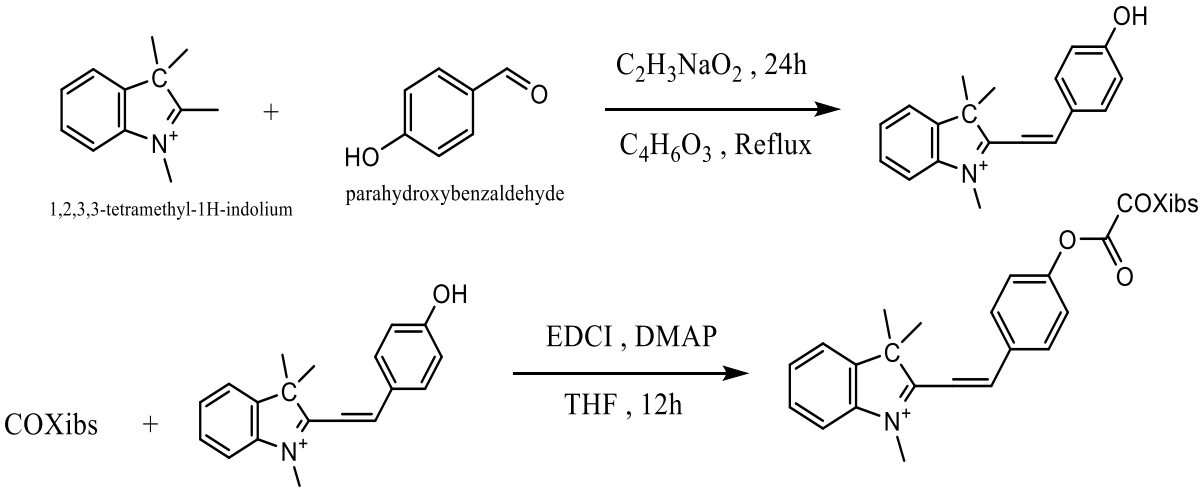


COXibs = Indomethcin and Ibuprofen

Scheme 1: The coupling reaction of Coxibs (COX-inhibitors) with Bromopentanol was performed under inert condition for 12h using suitable reagents. The coupling takes place between carboxyl group and hydroxyl group using EDCI: *1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide* and DMAP: *4-Dimethylaminopyridine* as coupling reagent. The yield obtained with this method was satisfactory, however using DCC as coupling reagent the yield obtained was too low.



Scheme 2: Triphenylphophonium salts are mostly synthesised by nucleophilic substitution of a leaving group such as halide, where substitution takes place at primary carbon atom giving high yield, initially the yield obtained was very low but after using Sodium Iodide as reagent there was a significant increase in the yield. The reaction was performed under reflux condition for 24h in solvents like Acetonitrile or Toluene. Using this method we have synthesised 10 different small molecules with different derivatives of TPP, however choosing the linker and reagents to obtained better yield and results was challenging.



Scheme 3: The Coxibs are attached with 1,2,3,3- tetramethyl indolium using Parahydroxy benzaldehyde, where Indolium and Benzaldehyde were linked through dehydration reaction in reflux for 24h using Sodium acetate and Acetic anhydride as reagents further using it for coupling with Coxibs (Indomethcin and Ibuprofen) in inert condition for 12h by using DMAP and EDCI as reagents. However the yield was satisfactory but the purification is difficult using column chromatography.

3. CONCLUSION

To specifically target mitochondria and selectively inhibit COX-2 present inside the mitochondria inside the mitochondria and to increase the efficacy of drug, small molecules has been synthesised attached with positively charged moiety, which was found to be efficient in killing the cancer cells. The small molecules consist of selective COX-2 inhibitors and positively charged moiety that is different derivatives of Triphenylphosphine and linker Bromopentanol. Similarly, other small molecules which contains COX-2 inhibitors attached to 1,2,3,3-tertramethyl-1H-indolium via Parahydroxybenzaldehyde in order to enhance the fluorescence, and to use further in tracking the cellular internalisation of molecules by confocal imaging. The efficacies of small molecules were demonstrated on MCF-7 cancer cell line and showed the IC_{50} value around 3-10 (mg/ml) which suggest the effective killing in compare to free drug. Further, its mechanism of action will be studied. The set of these molecules will be used to further study the cellular internalization into the mitochondria.

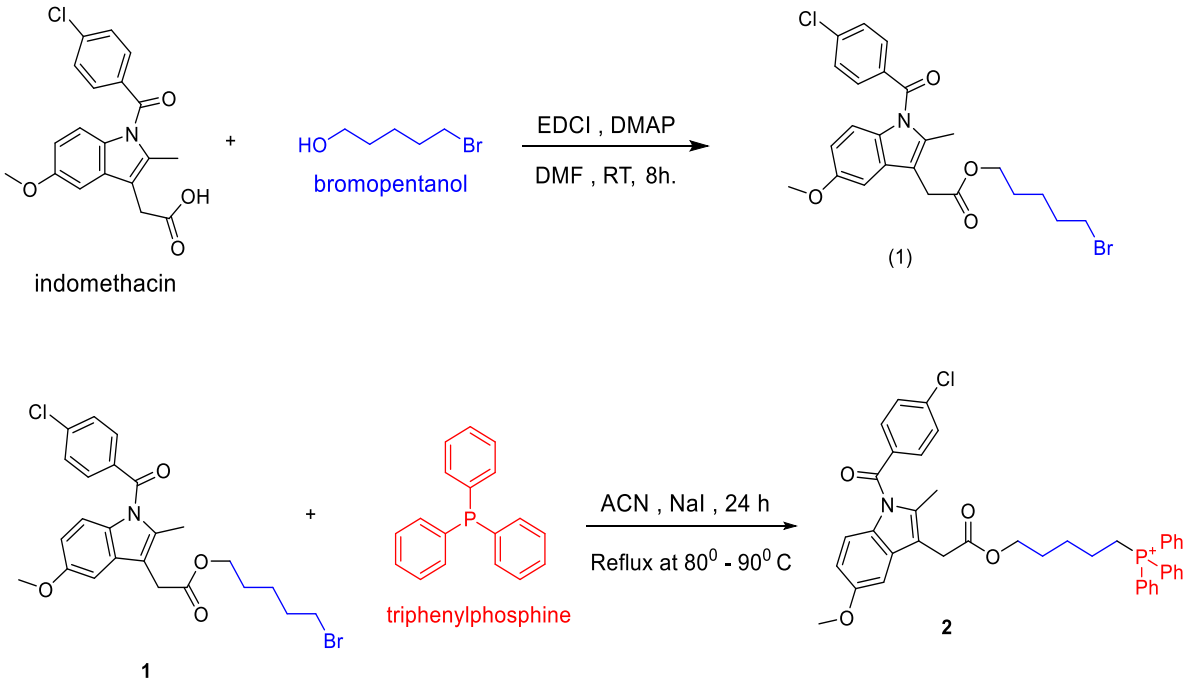
4. METHODS AND MATERIALS

Synthesis of small molecules, Indomethacin (i) and Ibuprofen (ii) attached with Triphenylphosphine. (**1)** Indomethacin/ Ibuprofen with Bromopentanol, (**2**) corresponding compound attached with PPh_3.

(i)

Synthesis of (i) Indomethacin attached with Bromopentanol and corresponding compound with Triphenylphosphine and its derivatives.

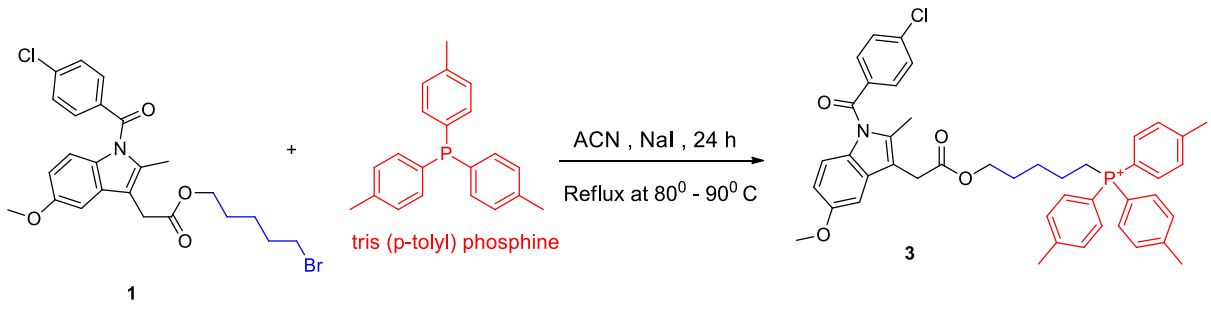
Chemicals Used: Indomethacin (1eq), Bromopentanol (2eq), Triphynelphosphine (2eq), EDCI: *1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide* (1.5eq), DMAP: *4-Dimethylaminopyridine* (0.5eq), NaI: *Sodium Iodide*(2eq), *Dimethylformamide*(DMF) solvent, *Acetonitrile* (ACN) solvent.



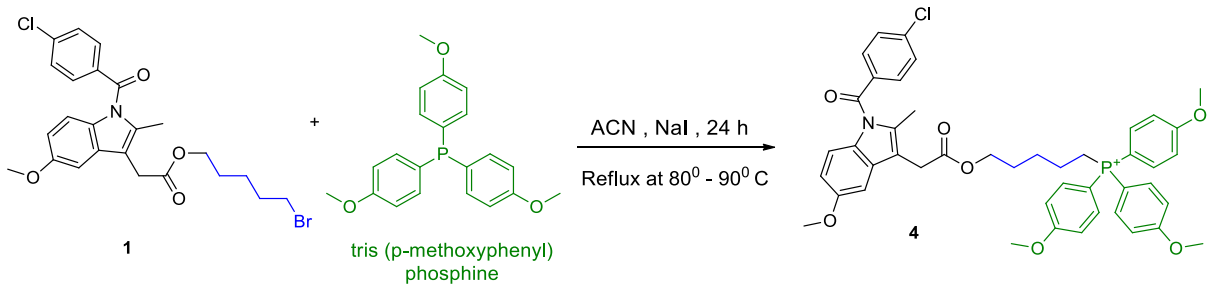
Procedure for synthesis of compound (1): In a 25 mL two neck round bottom flak, Indomethacin 200 mg (0.56 mmol, 1equiv) and EDCI 161.02 mg (0.84 mmol, 1.5equiv) were dissolved in DMF (3ml). The resulting reaction mixture was stirred under nitrogen condition for 15 minutes. After 15 min, DMAP34.16 mg (0.28 mmol, 0.5equiv) added and again kept for additional 15min. Finally, Bromopentanol 135µl (1.12 mmol, 2equiv) was added. Reaction was kept for stirring for additional 8 hours under nitrogen condition. After completion of reaction,(monitored by TLC in 20% Ethyl acetate in Pet

ether) DMF was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with PE:EA (90:10) solvent system. Yield= 87%

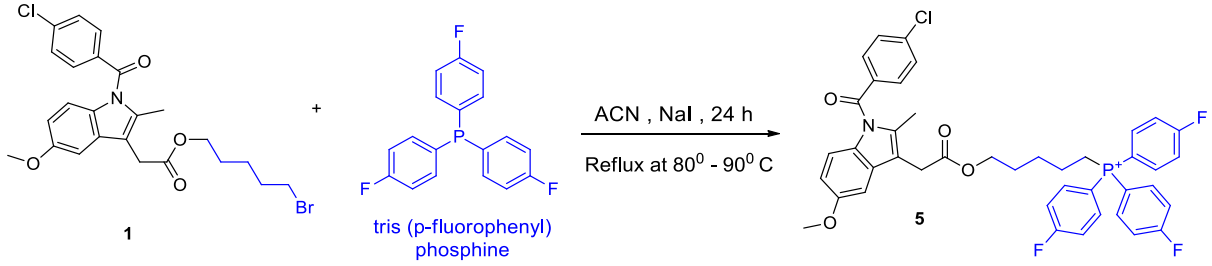
Procedure for synthesis of compound (2): In a 25 mL round bottom flak, Compound **1** 50 mg (0.099 mmol, 1.0 equiv) and NaI 60mg (0.297 mmol, 2equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Triphenylphosphine 91.4 mg (0.297 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (97:2.5:0.5) solvent system. Yield= 81.2%



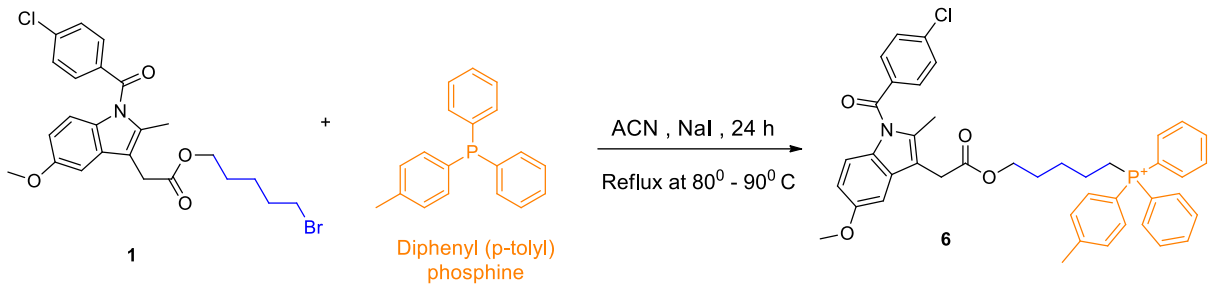
Procedure for compound (3): In a 25 mL round bottom flak, Compound **1** 50 mg (0.099 mmol, 1.0 equiv) and NaI 60mg (0.297 mmol, 2equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Tris(p-tolyl)phosphine 90.4 mg (0.297 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (97:2.5:0.5) solvent system. Yield= 78%



Procedure for compound (4): In a 25 mL round bottom flak, Compound **1** 50 mg (0.099 mmol, 1.0 equiv) and NaI 60mg (0.297 mmol, 2equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Tris(p-methoxy)phosphine 105.3mg (0.297 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (97:2.5:0.5) solvent system. Yield= 86%



Procedure for compound (5): In a 25 mL round bottom flak, Compound **1** 50 mg (0.099 mmol, 1.0 equiv) and NaI 60mg (0.297 mmol, 2equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Tris(p-fluoro)phosphine 95.6 mg (0.297 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (97:2.5:0.5) solvent system. Yield= 74%

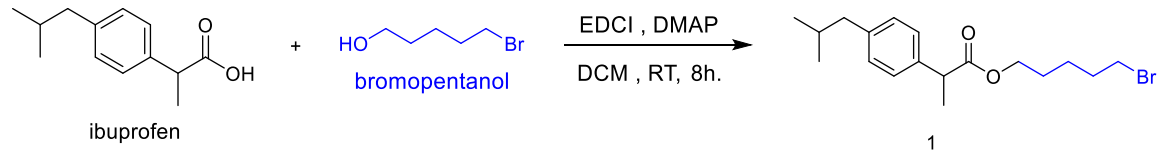


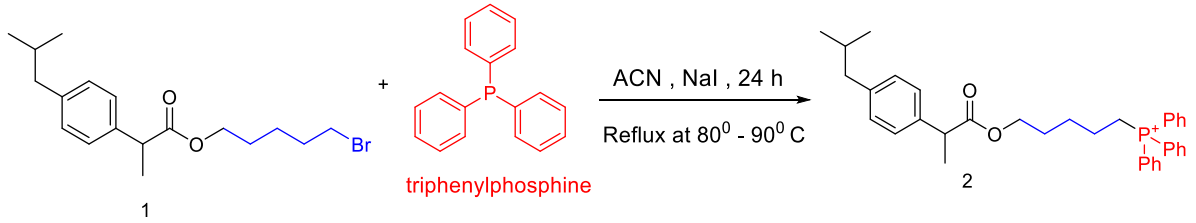
Procedure for compound (6): In a 25 mL round bottom flak, Compound **1** 50 mg (0.099 mmol, 1.0 equiv) and NaI 60mg (0.297 mmol, 2equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Diphenyl(p-tolyl)phosphine 92.4 mg (0.297 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (97:2.5:0.5) solvent system. Yield= 80%

(ii)

Synthesis of (ii) Ibuprofen attached with Bromopentanol and corresponding compound with Triphenylphosphine and its derivatives.

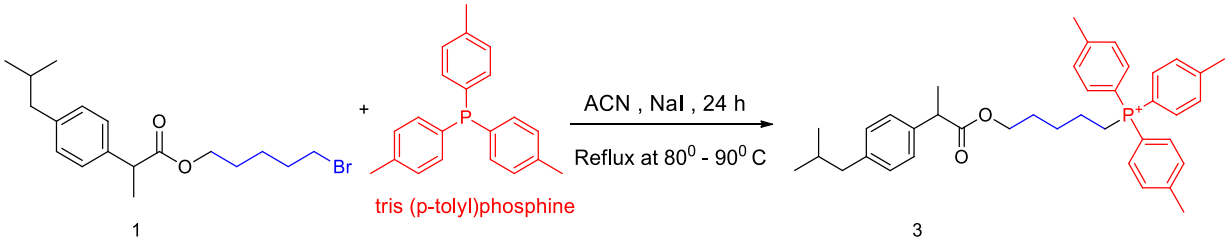
Chemicals Used: Ibuprofen (1eq), Bromopentanol (2eq), Triphynelphosphine (2eq), EDCI: *1-Ethyl- 3-(3-dimethylaminopropyl) carbodiimide* (1.5eq) , DMAP: *4-Dimethylaminopyridine* (0.5eq), NaI: *Sodium Iodide*(2eq), *Dichloromethane* (DCM) solvent, *Acetonitrile* (ACN)solvent.





Procedure for compound (1): In a 25 mL two neck round bottom flak, Ibuprofen 200mg (0.96 mmol, 1equiv) and EDCI 277.9 mg (1.45 mmol, 1.5equiv) were dissolved in DCM (3ml).The resulting reaction mixture was stirred under nitrogen condition for 15minutes. After 15min, DMAP 59.14mg (0.48mmol,0.5equiv) added and again kept for additional 15min. Finally,Bromopentanol 233.65µl (1.93 mmol, 2equiv) was added. Reaction was kept for stirring for additional 8 hours under nitrogen condition. After completion of reaction,(monitored by TLC in 20% Ethyl acetate in Pet ether) DMF was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with PE:EA (92:8) solventsystem. Yield= 84%

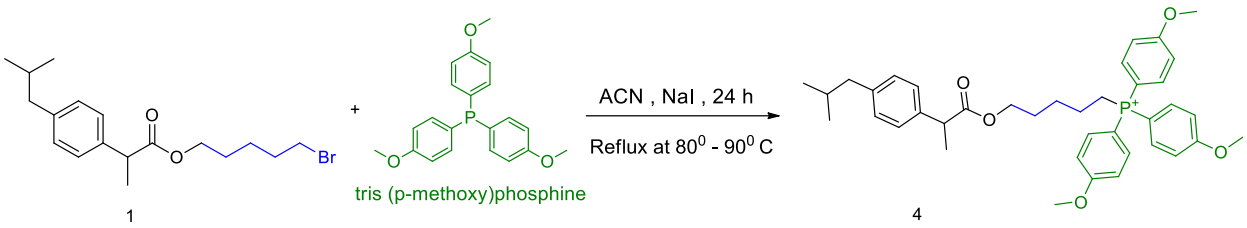
Procedure for compound (2): In a 25 mL round bottom flak, Compound **1** 50 mg (0.141 mmol, 1 equiv) and NaI 84.54 mg (0.564 mmol, 2.00 equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Triphenylphosphine 174.7 mg (0.564 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (98:2:0.5) solvent system. Yield= 93.1%



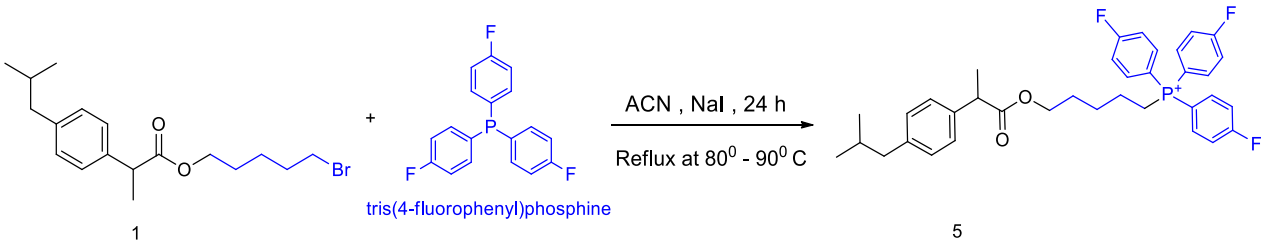
1 tris (p-tolyl)phosphine 3

Procedure for compound (3): In a 25 mL round bottom flak, Compound **1** 50 mg (0.141 mmol, 1 equiv) and NaI 84.54 mg (0.564 mmol, 2.00 equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15min, Tris(p-tolyl)phosphine 171.4mg (0.564 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and

resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (98:2:0.5) solvent system. Yield= 88%

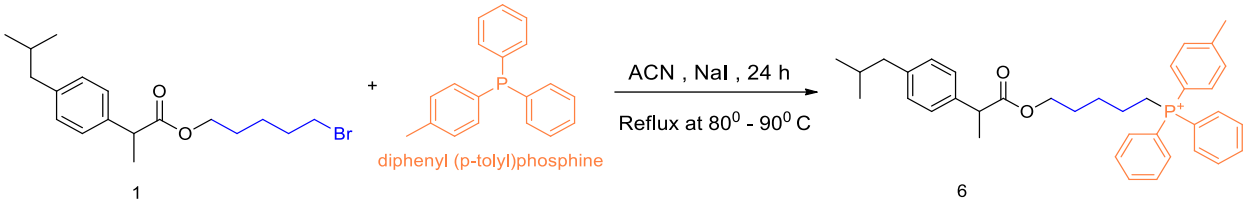


Procedure for compound (4): In a 25 mL round bottom flak, Compound **1** 50 mg (0.141 mmol, 1 equiv) and NaI 84.54 mg (0.564 mmol, 2.00 equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Tris(p-methoxy)phosphine 198.73mg (0.564 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (98:2:0.5) solvent system. Yield= 83%



Procedure for compound (5): In a 25 mL round bottom flak, Compound **1** 50 mg (0.141 mmol, 1 equiv) and NaI 84.54 mg (0.564 mmol, 2.00 equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Tris(p-fluoro)phosphine 178.3 mg (0.564 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-

200 mesh) eluting with DCM:MeOH:AcOH (98:2:0.5) solvent system. Yield= 74%

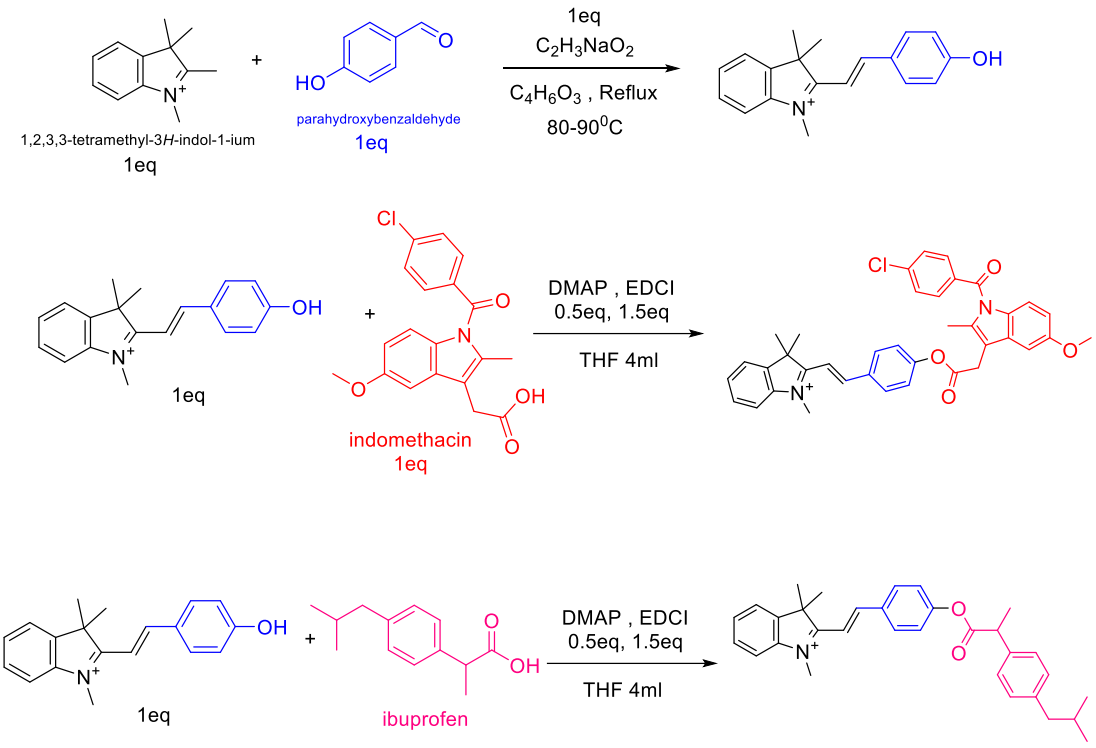


Procedure for compound (6): In a 25 mL round bottom flak, Compound **1** 50 mg (0.141 mmol, 1 equiv) and NaI 84.54 mg (0.564 mmol, 2.00 equiv) were dissolved in ACN (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, Diphenyl(p-tolyl)phosphine 102.8mg (0.564 mmol, 2equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 5% DCM in methanol) acetonitrile was removed under reduced pressure and resulted residue was purified by column chromatography on silica gel (100-200 mesh) eluting with DCM:MeOH:AcOH (98:2:0.5) solvent system. Yield= 92%

(iii)

Attempted synthesis of (i) Indomethacin and (ii) Ibuprofen attached to 1,2,3,3-tetramethly-3H-indol-1-ium by using parahydroxy Benzaldehyde as linker to enhance the fluorescence of small moleculesto check the internalization inside the mitochondria

Chemicals Used: (i) Indomethacin (1eq), (ii) Ibuprofen (1eq), Parahydroxybenzaldehyde (1eq), 1,2,3,3-tetramethly-3H-indol-1-ium (1eq), $C_2H_3NaO_2$: Sodium Acetate(1eq), EDCI: *1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide* (1.5eq) , DMAP: *4-Dimethylaminopyridine*(0.5eq), *Tetrahydrofuran* (THF) solvent, *Aceticanhydride* solvent.



Procedure for compound (iii.1): In a 25 mL round bottom flak, 1,2,3,3-tetramethly-3H-indol-1-ium 50 mg (0.141 mmol, 1 equiv) and $C_2H_3NaO_2$ 84.54 mg (0.564 mmol, 2.00 equiv) were dissolved in *Aceticanhydride* (3mL). The resulting reaction mixture was stirred under reflux for 15 minutes. After 15 min, parahydroxy benzaldehyde 84.54 mg (0.564 mmol, 2.00 equiv) was added. Reaction was kept for stirring for additional 24 hours under reflux condition. After completion of reaction,(monitored by TLC in 10% DCM in methanol) *Aceticanhydride* was removed under reduced pressure and resulted residue was purified by column chromatography on aluminium oxide eluting with DCM:MeOH (98:2) solvent system. Yield = 83.1%.

Procedure for compound (iii.2): In a 25 mL two neck round bottom flak, Ibuprofen 200mg (0.96 mmol, 1equiv) and EDCI 277.9 mg (1.45 mmol, 1.5equiv) were dissolved in THF (3ml). The resulting reaction mixture was stirred under nitrogen condition for 15minutes. After15min, DMAP59.14mg (0.48mmol,0.5equiv) added and again kept for additional 15min. Finally, Compound (iii.1) 233.65µl (1.93 mmol, 2equiv) was added. Reaction was kept for stirring for additional 12 hours under nitrogen condition. After completion of reaction,(monitored by TLC in 15% DCM in methanol) *THF* was removed under reduced pressure and resulted residue was purified by column

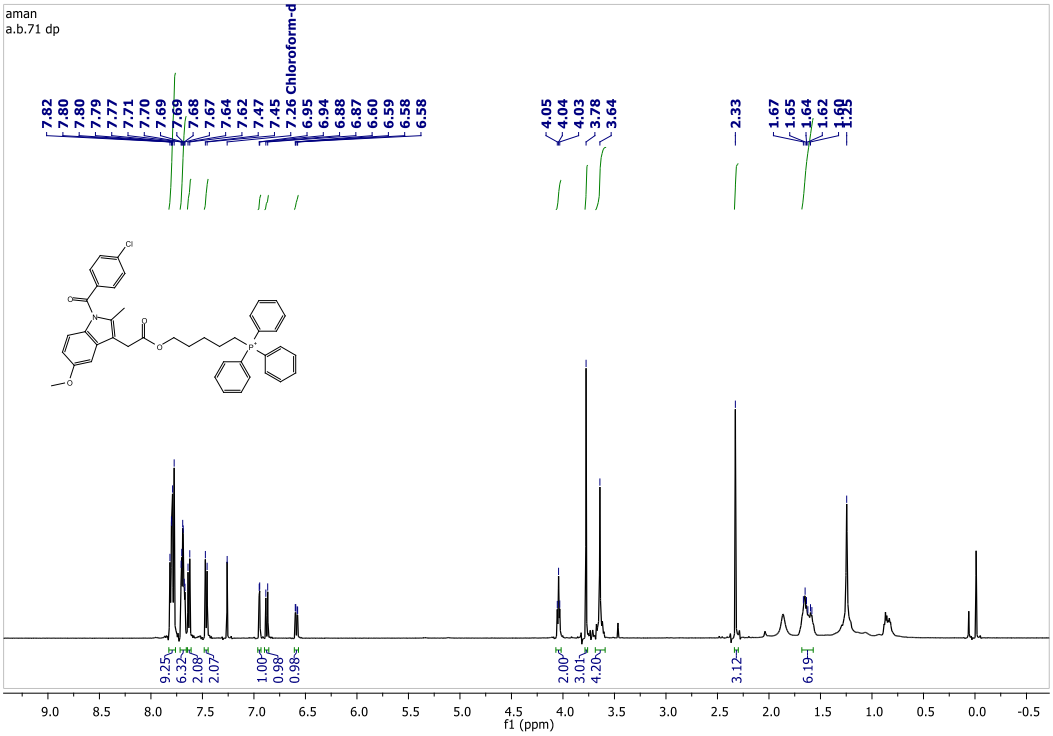
chromatography on aluminium oxide eluting with DCM:MeOH (98:4) solvent system. Yield = 72.3%.

Procedure for compound (iii.3): In a 25 mL two neck round bottom flak, Indomethacin 200mg (0.96 mmol, 1equiv) and EDCI 277.9 mg (1.45 mmol, 1.5equiv) were dissolved in THF (3ml). The resulting reaction mixture was stirred under nitrogen condition for 15minutes. After15min, DMAP59.14mg (0.48mmol,0.5equiv) added and again kept for additional 15min. Finally, Compound (iii.1) 233.65µl (1.93 mmol, 2equiv) was added. Reaction was kept for stirring for additional 12 hours under nitrogen condition. After completion of reaction,(monitored by TLC in 15% DCM in methanol) *THF* was removed under reduced pressure and resulted residue was purified by column chromatography on aluminium oxide eluting with DCM:MeOH (98:4) solvent system. Yield = 70.4%.

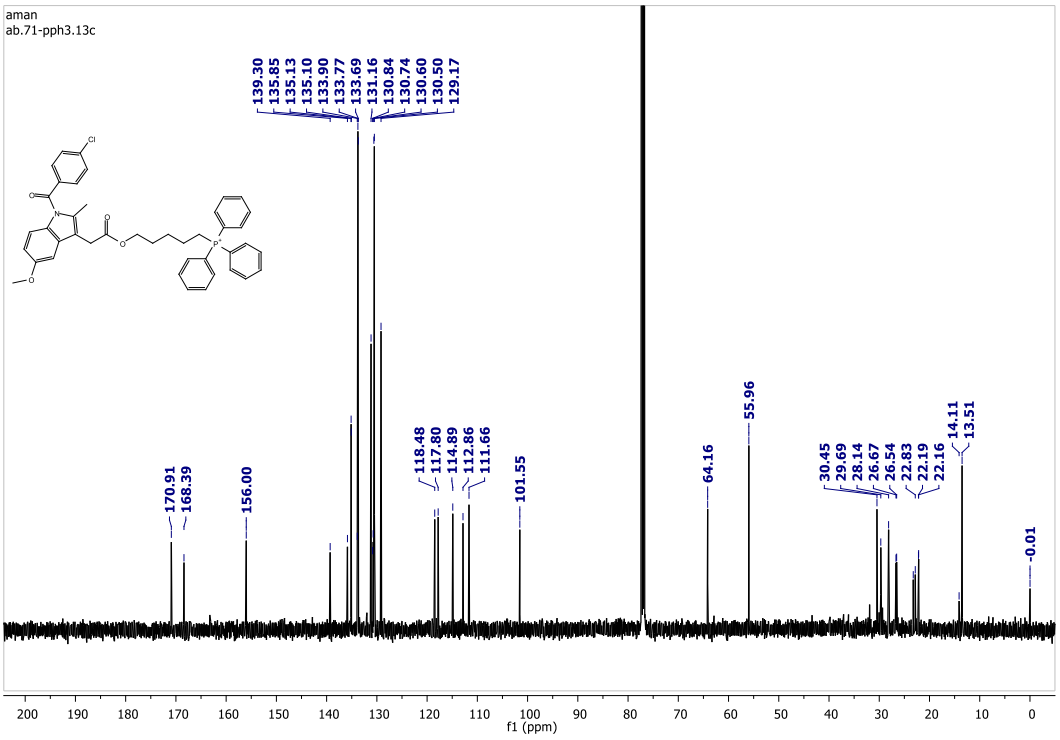
5. NMR DATA

4.1 NMR DATA CHARACTERIZATION OF COMPUND (i):

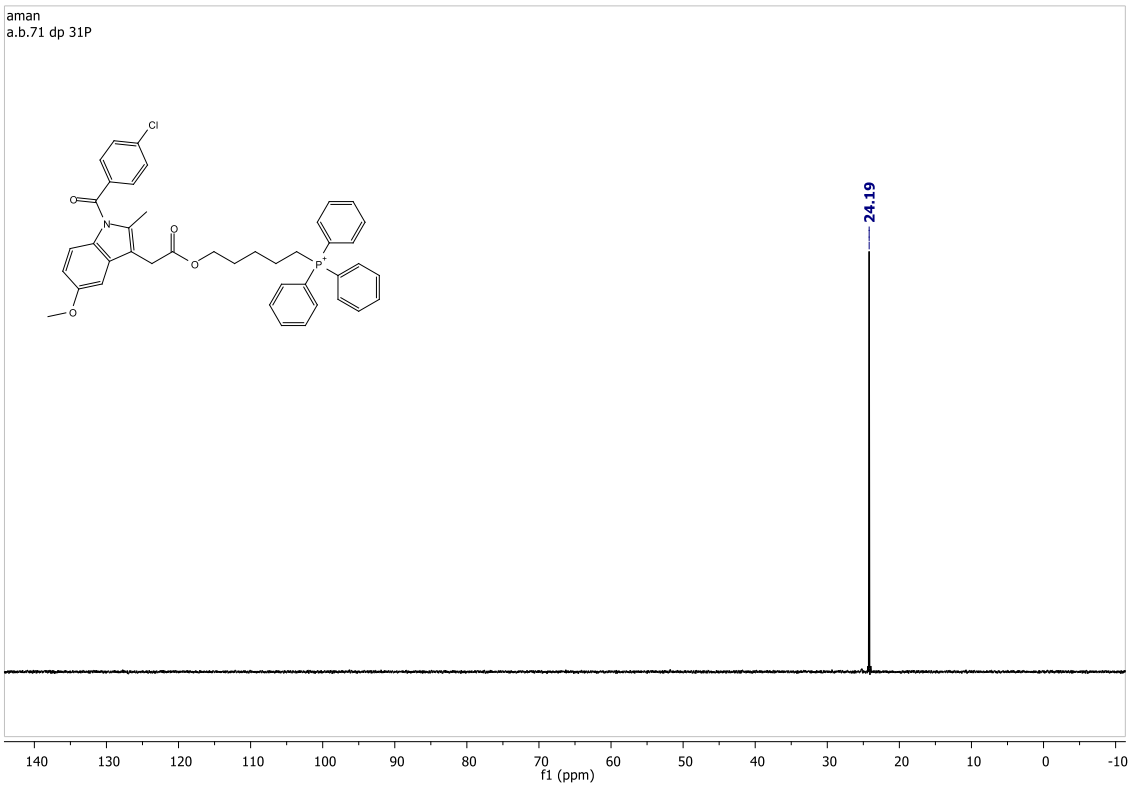
1H-NMR of (i.2):



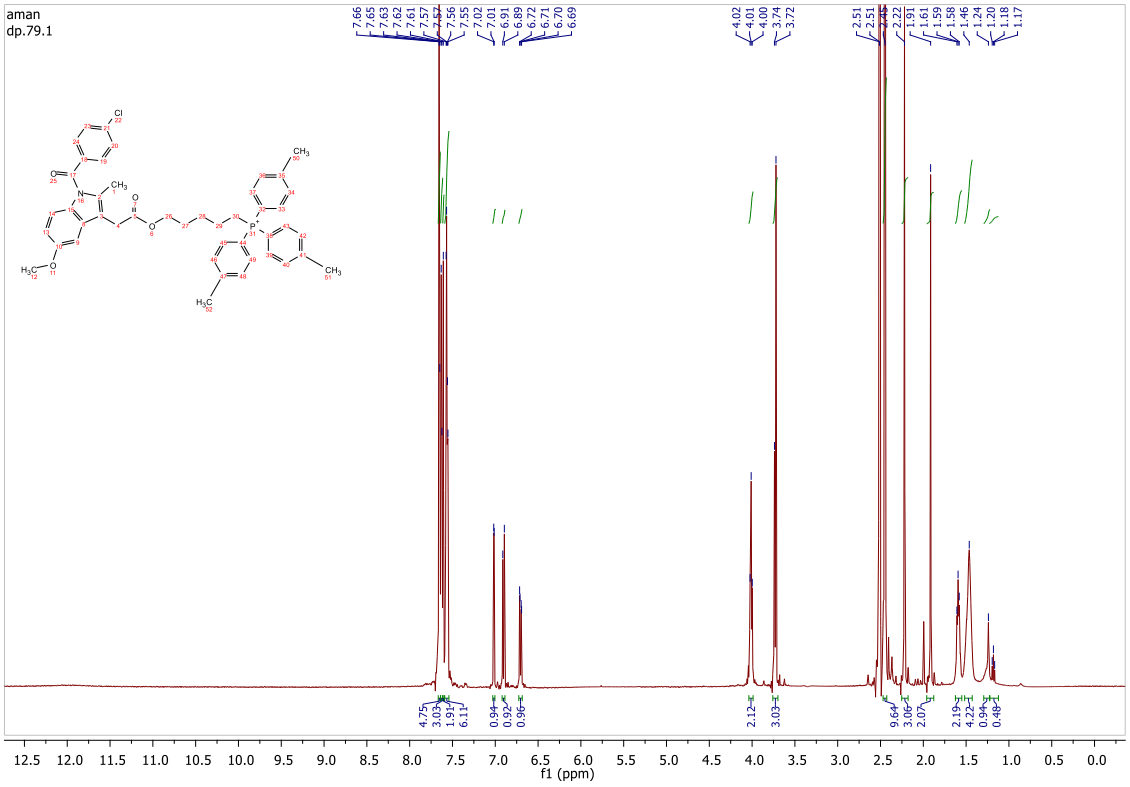
13C-NMR of (i.2)



31P-NMR of (i.2):



1H-NMR of (i.3):



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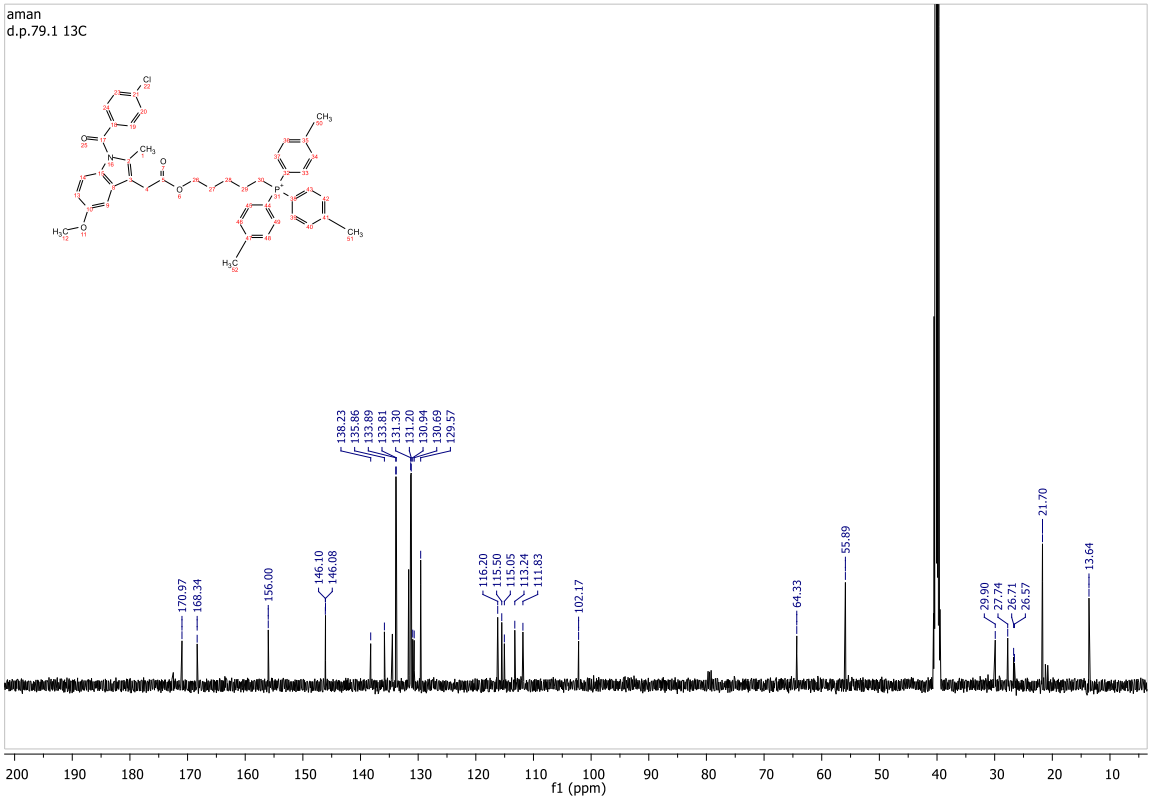

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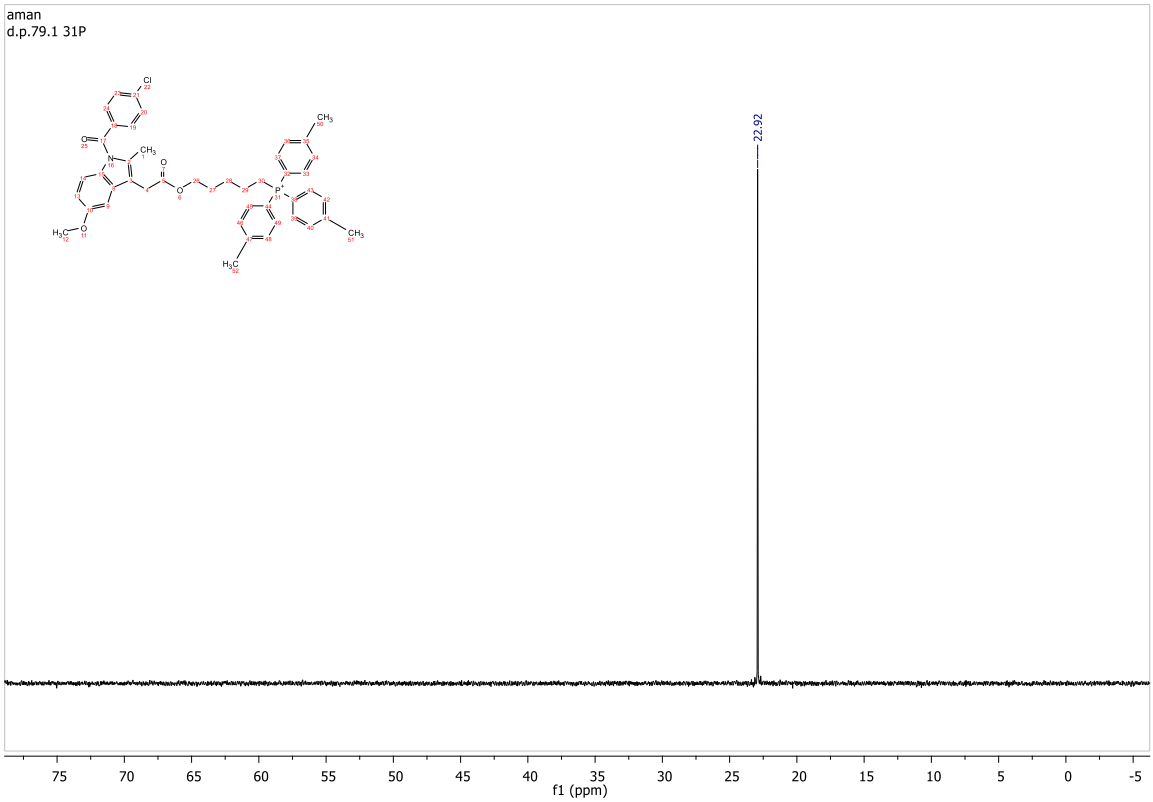
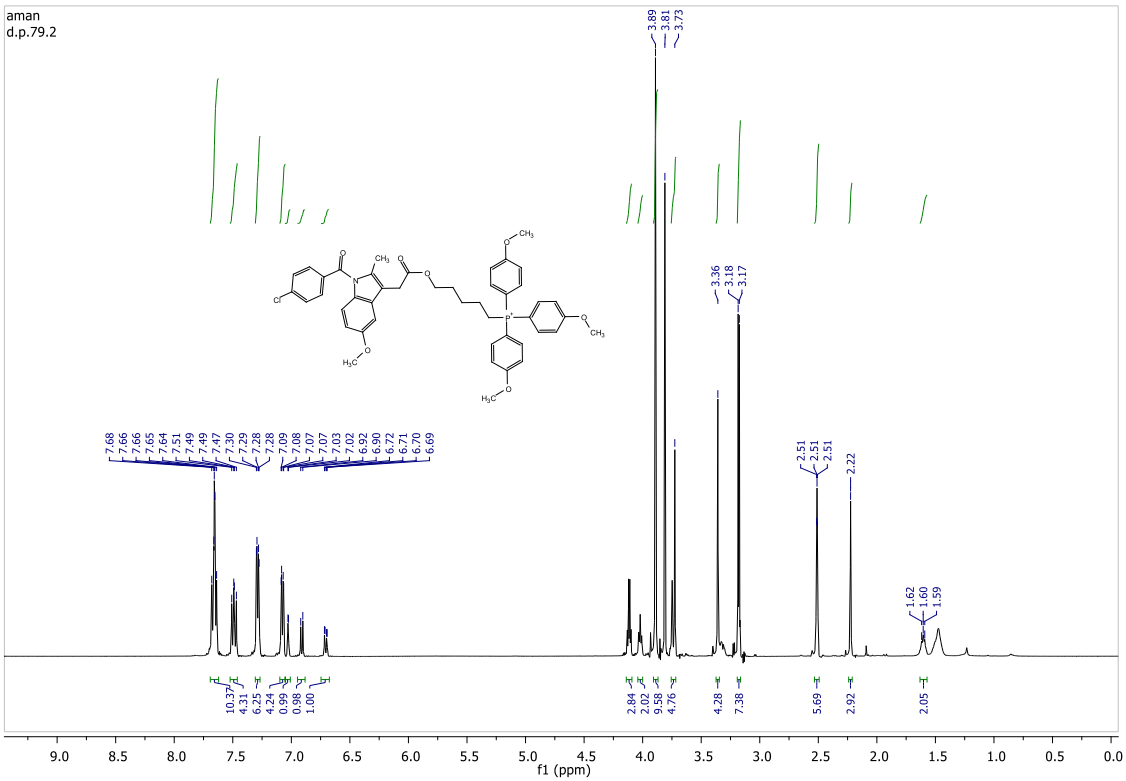


1H-NMR of (i.4):



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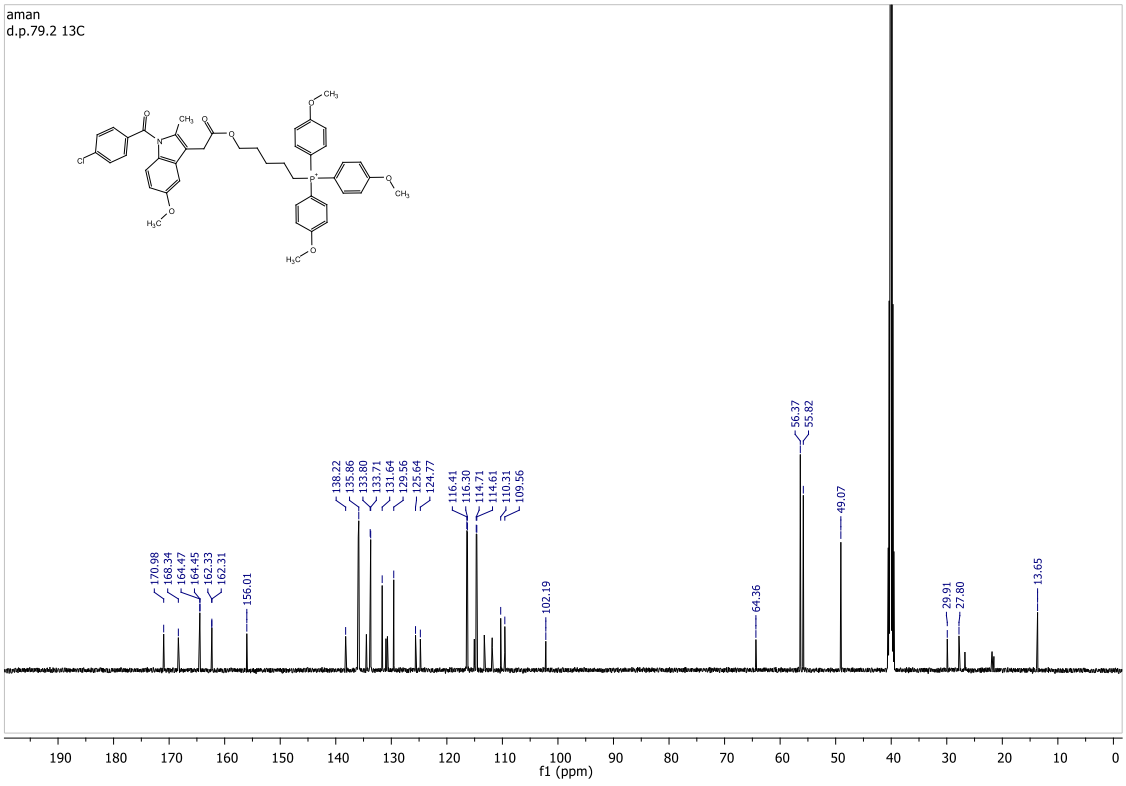

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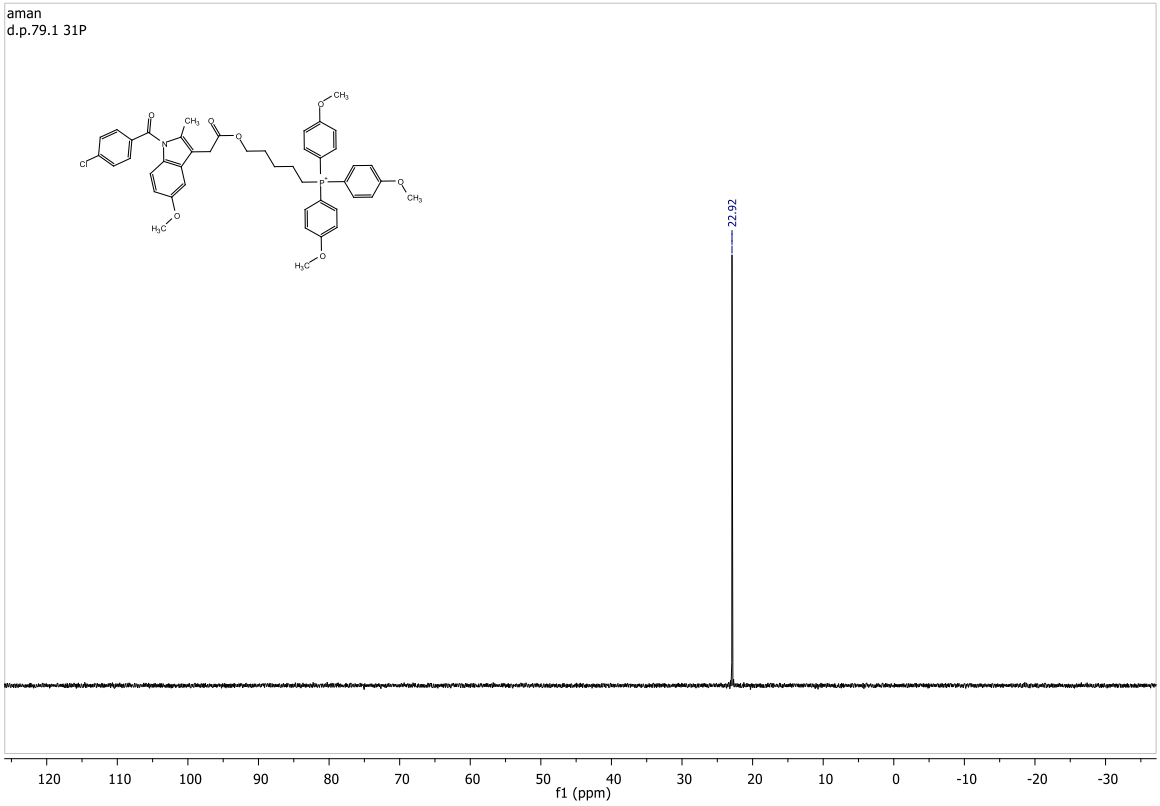
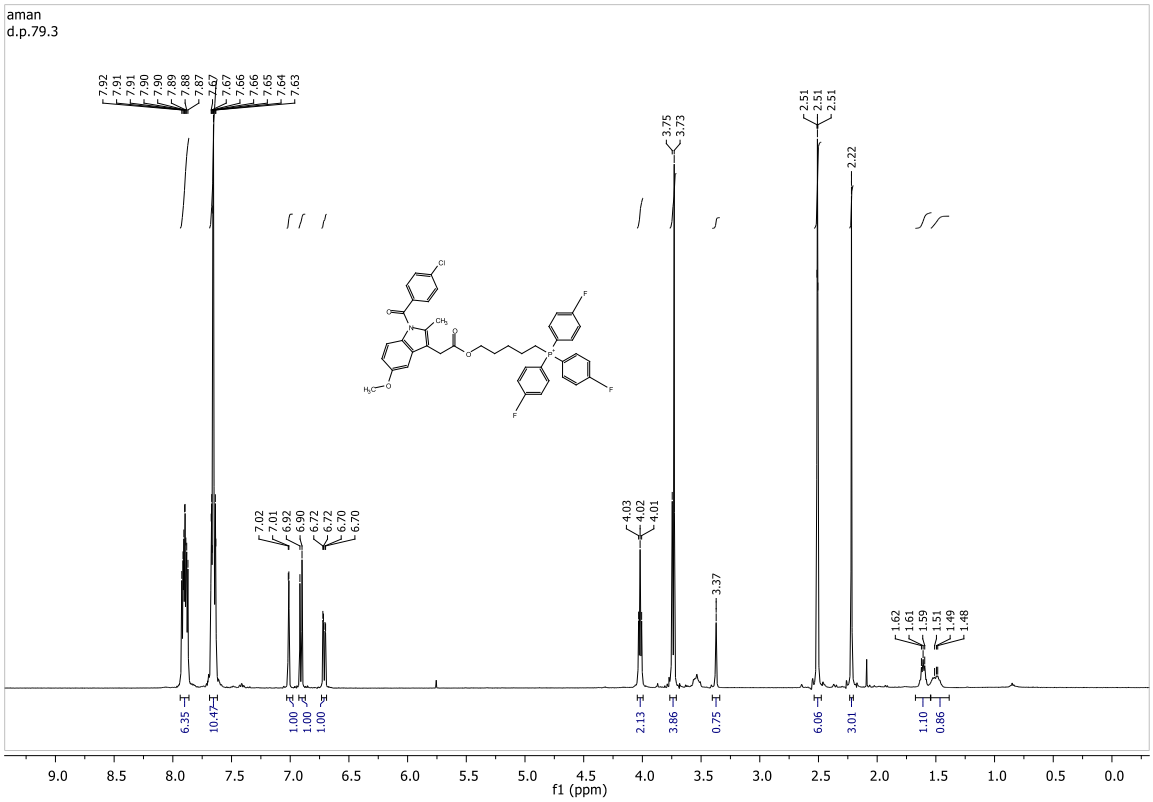


1H-NMR of (i.5):



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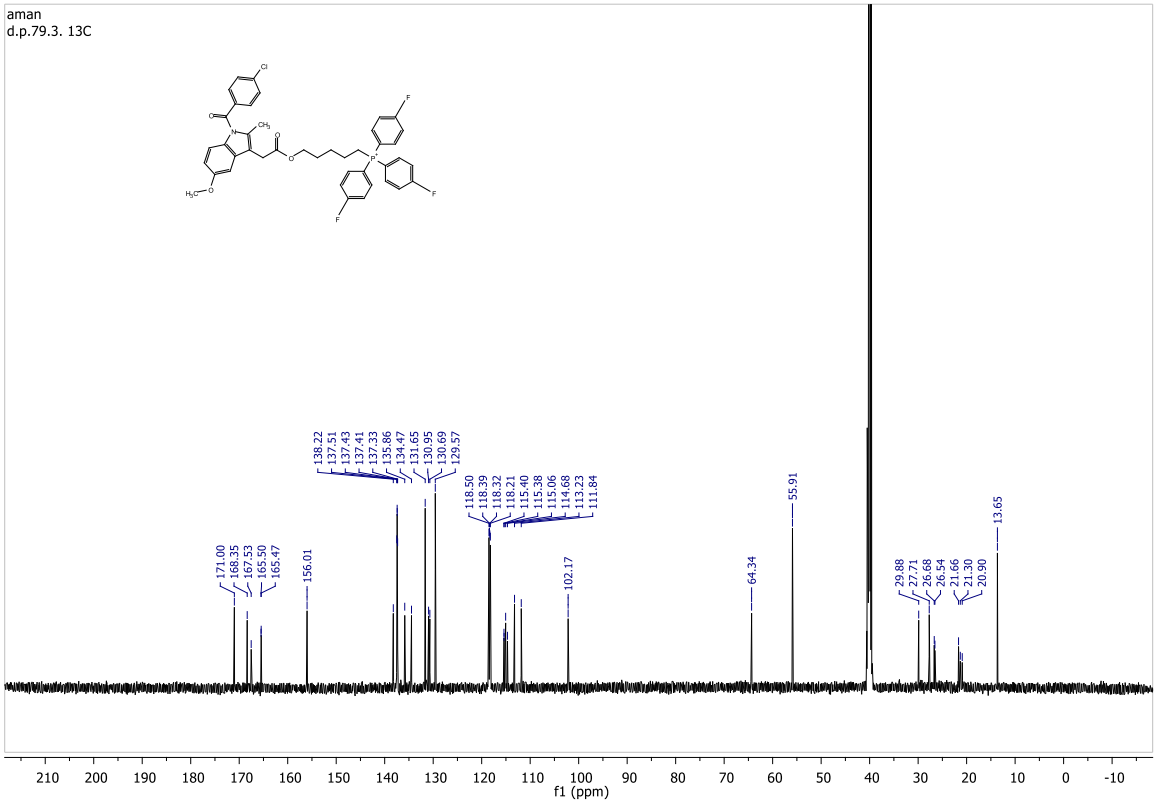

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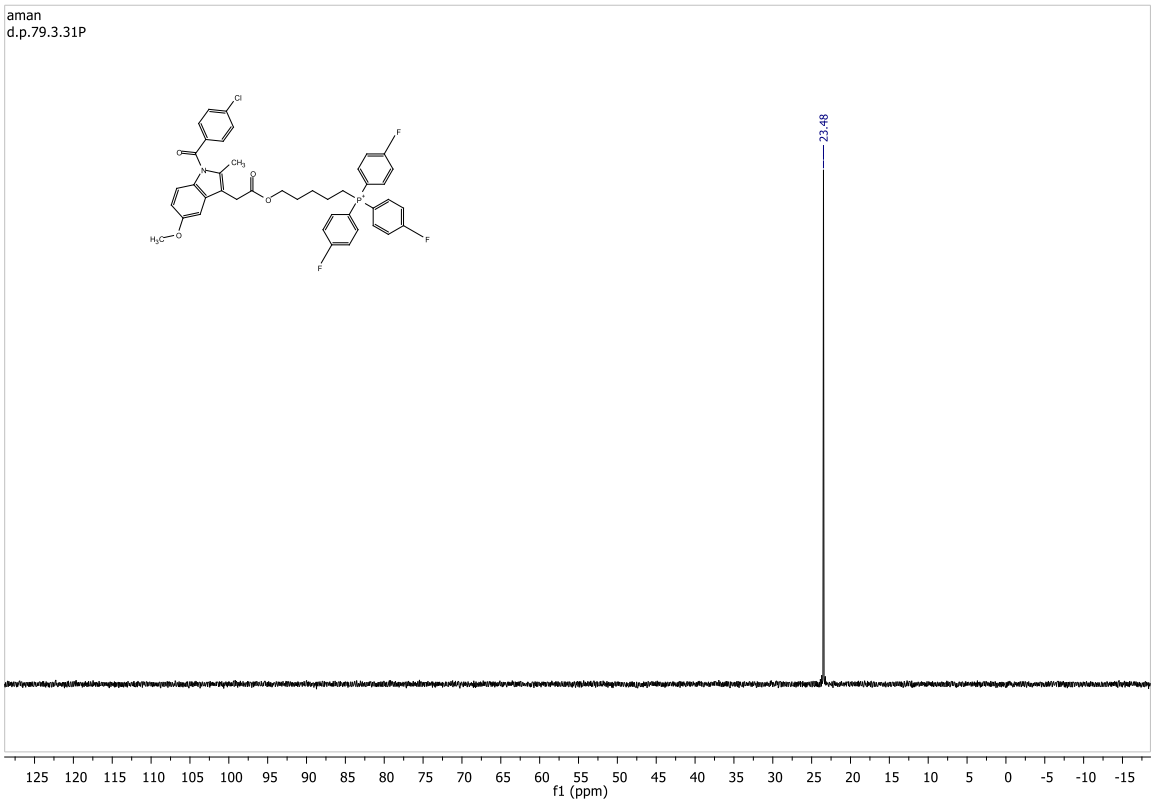
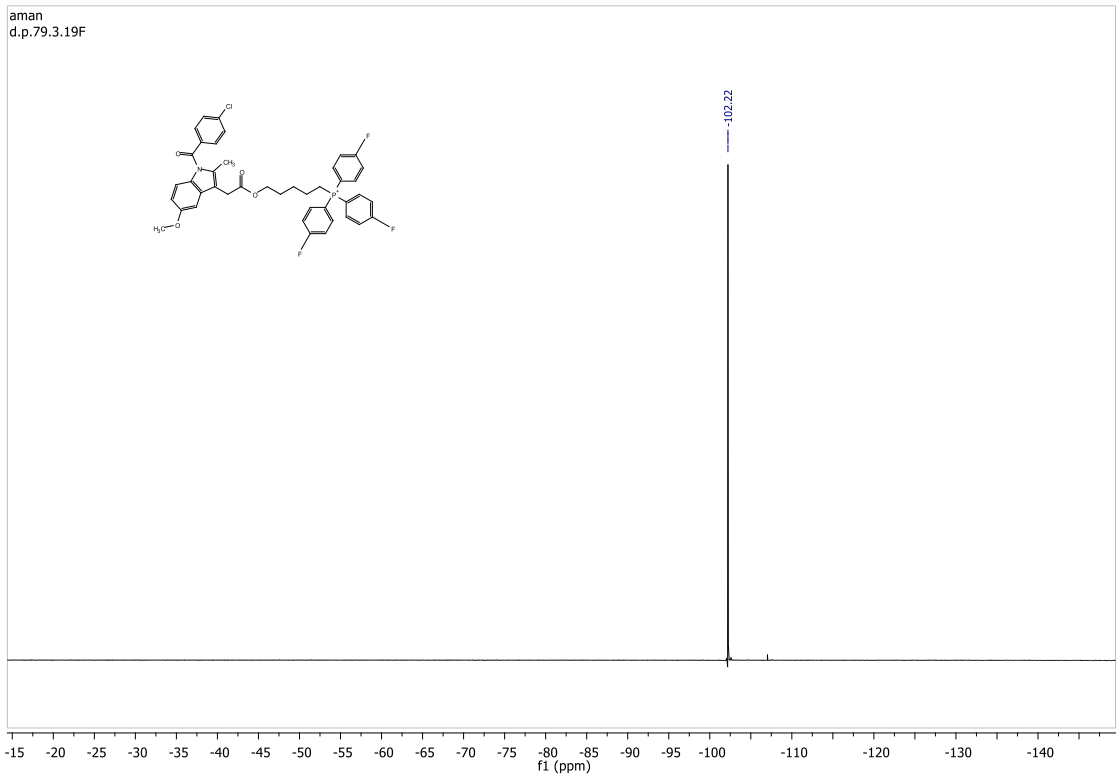
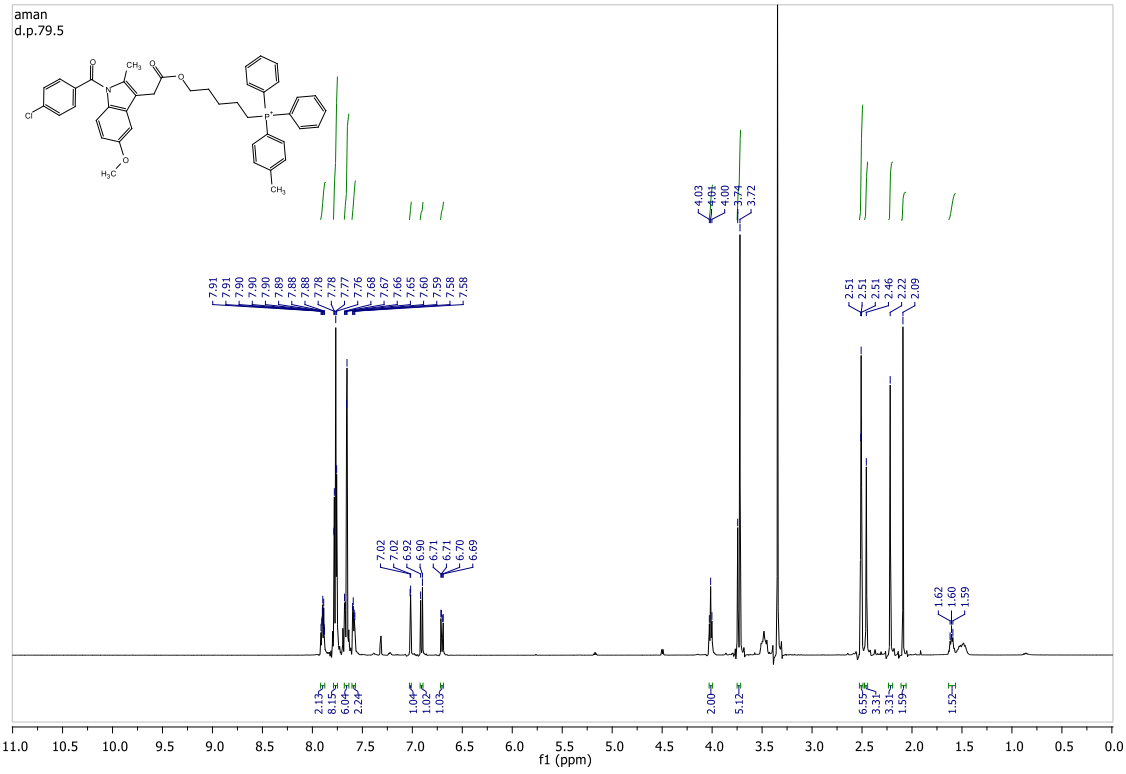


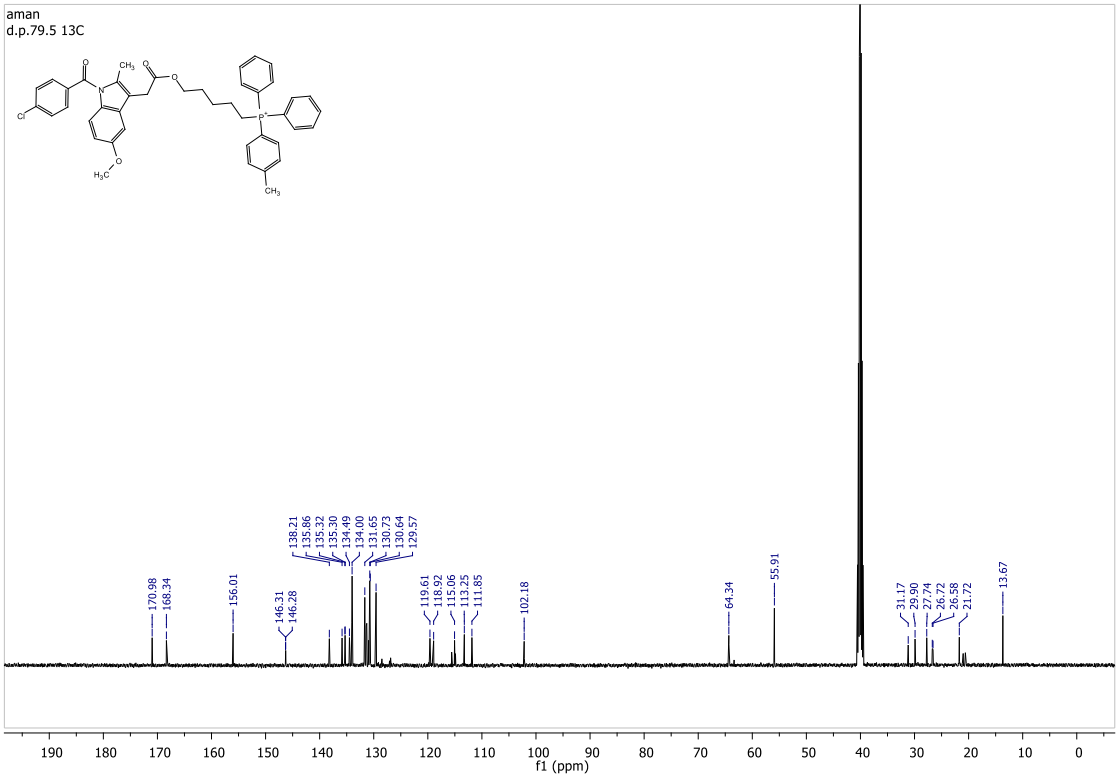
19F-NMR of (i.5):



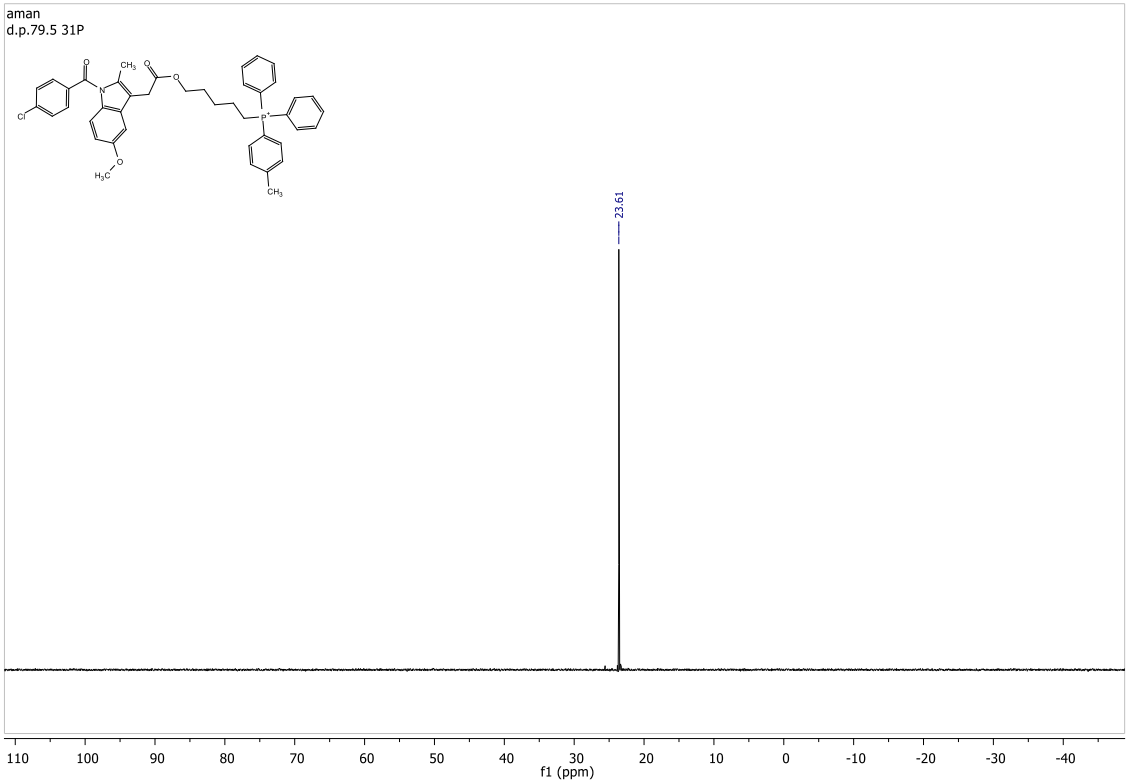
1H-NMR of (i.6):



13C-NMR of (i.6):



31P-NMR of (i.6):



4.2 NMR DATA CHARACTERIZATION OF COMPUND (ii):

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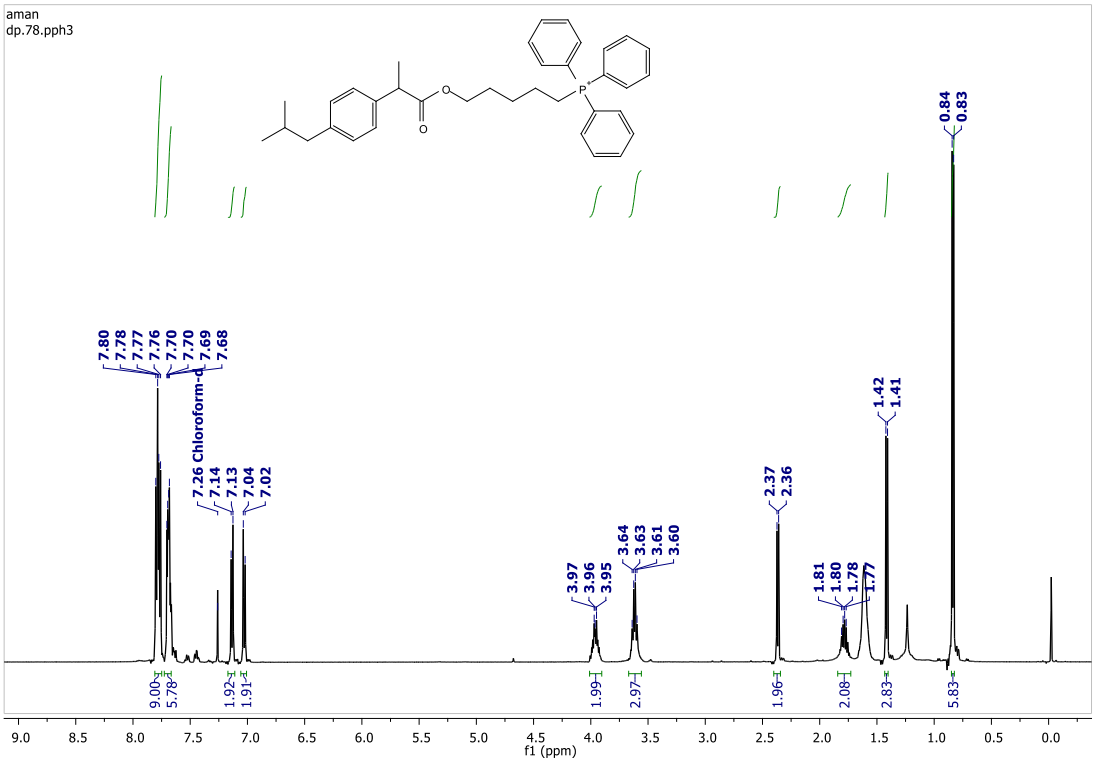
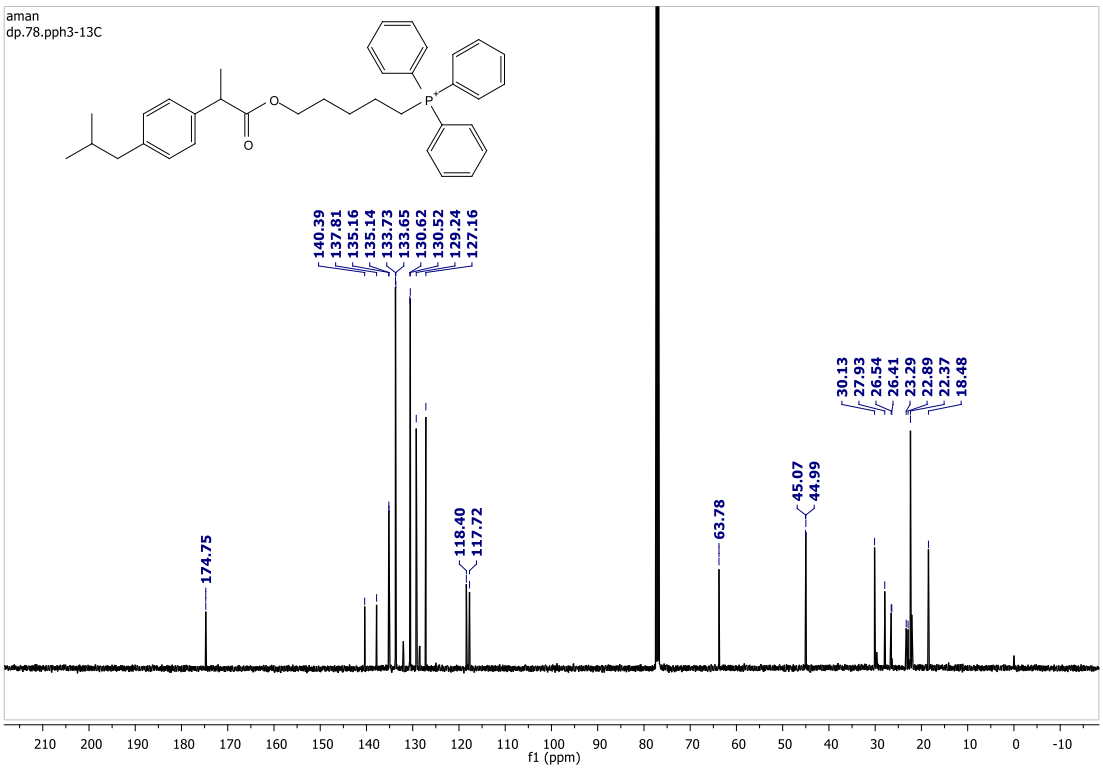
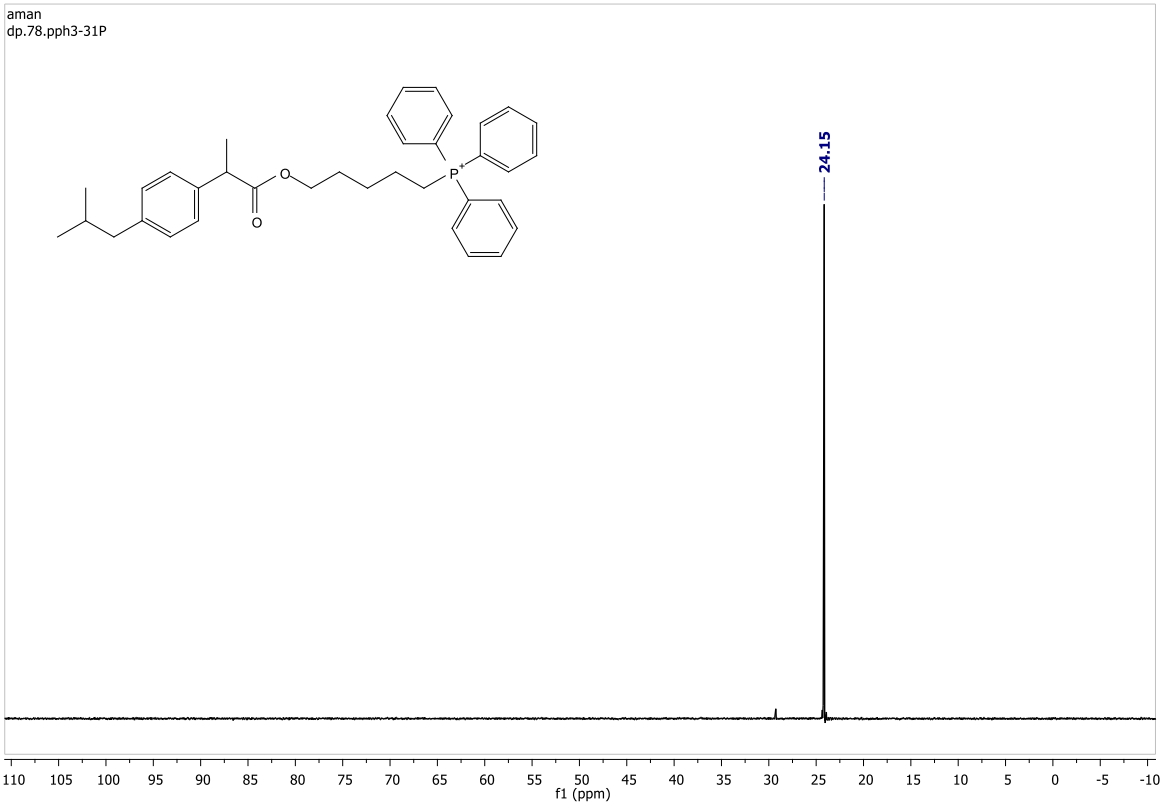


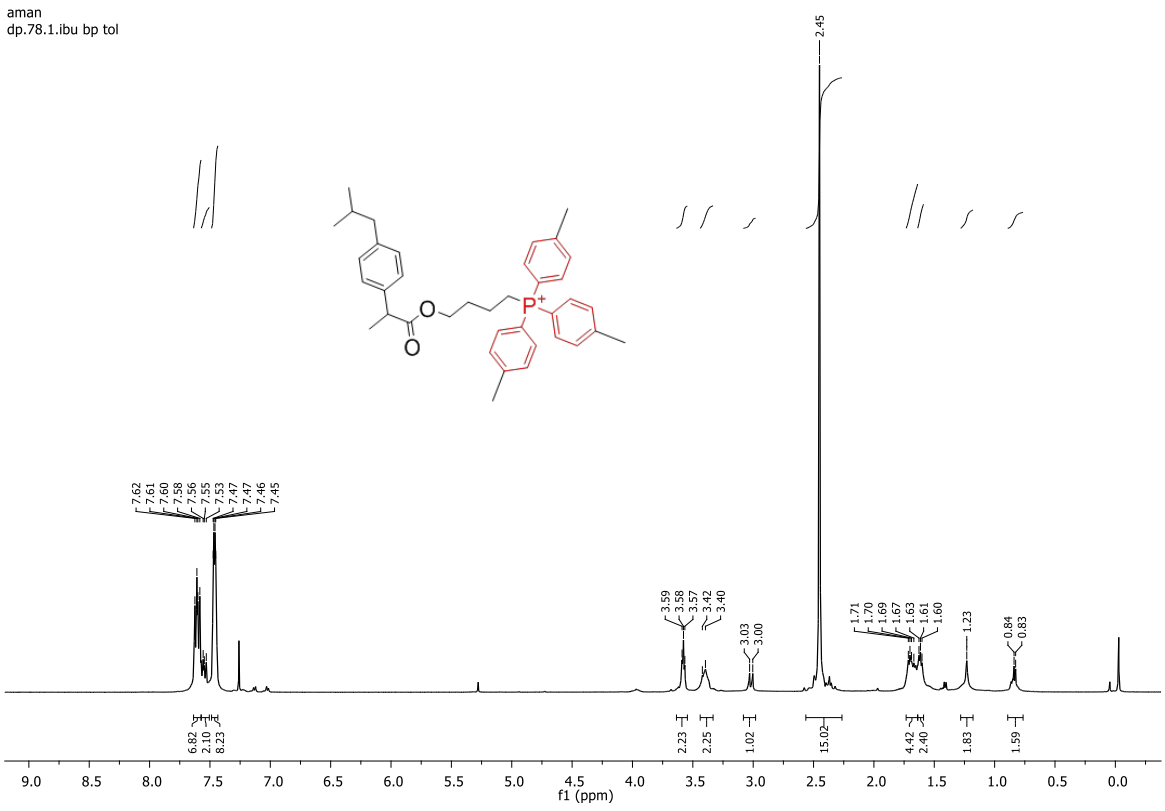
13C-NMR of (ii.2):



31P-NMR of (ii.2):



1H-NMR of (ii.3):



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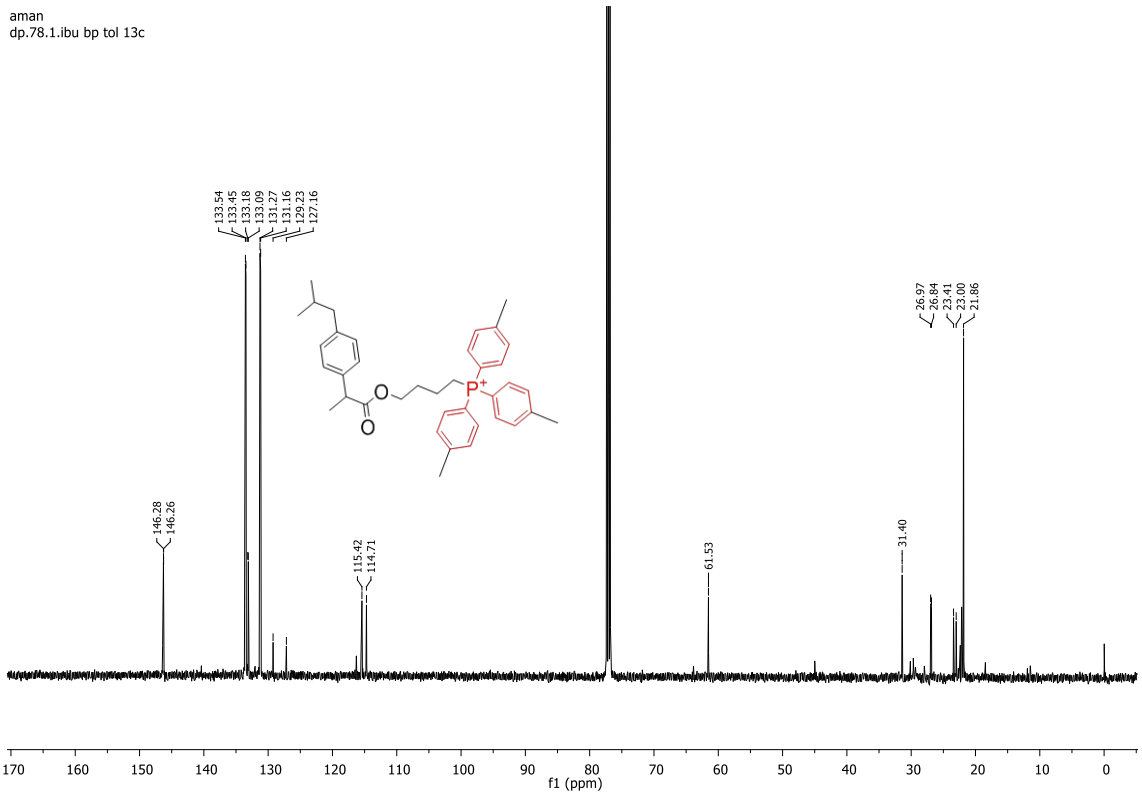

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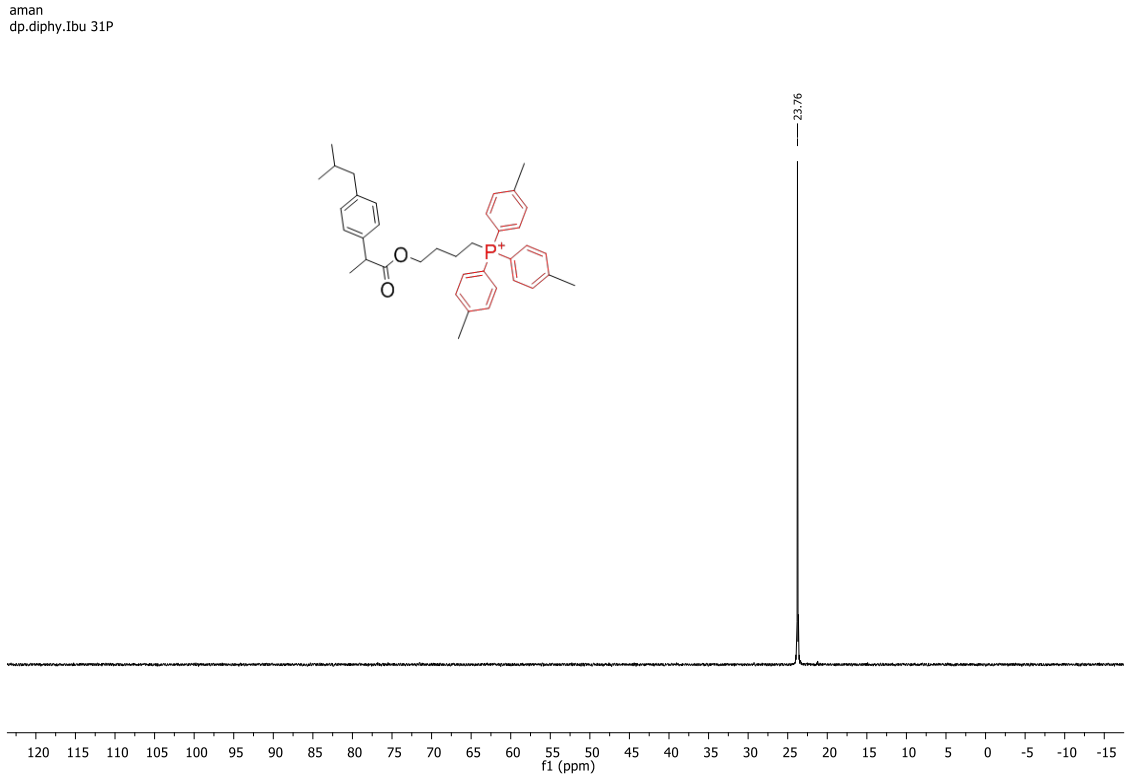

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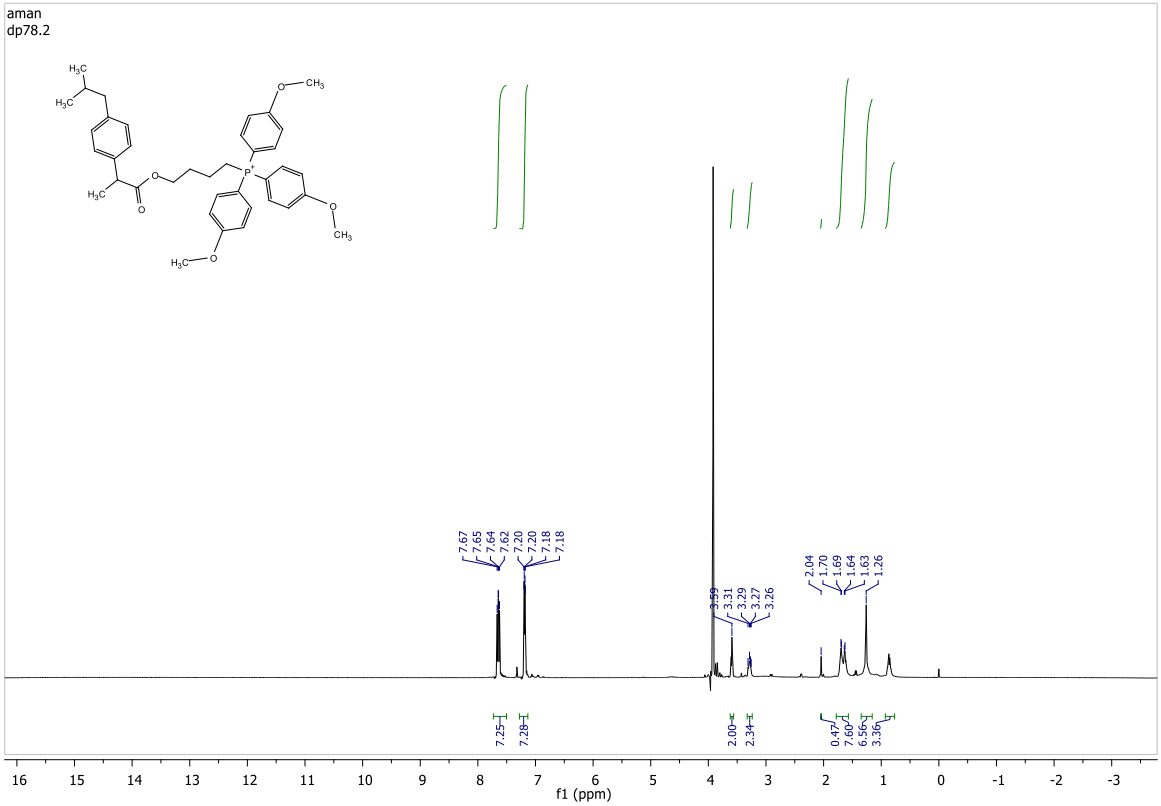

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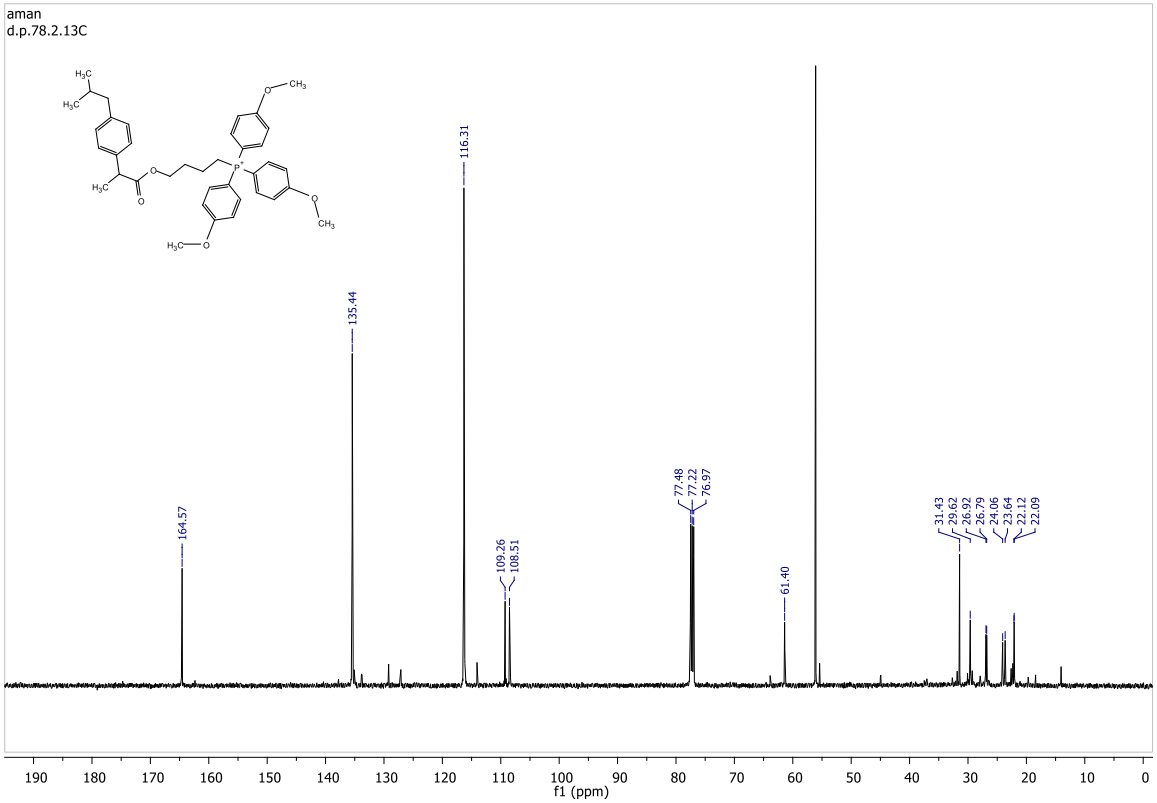

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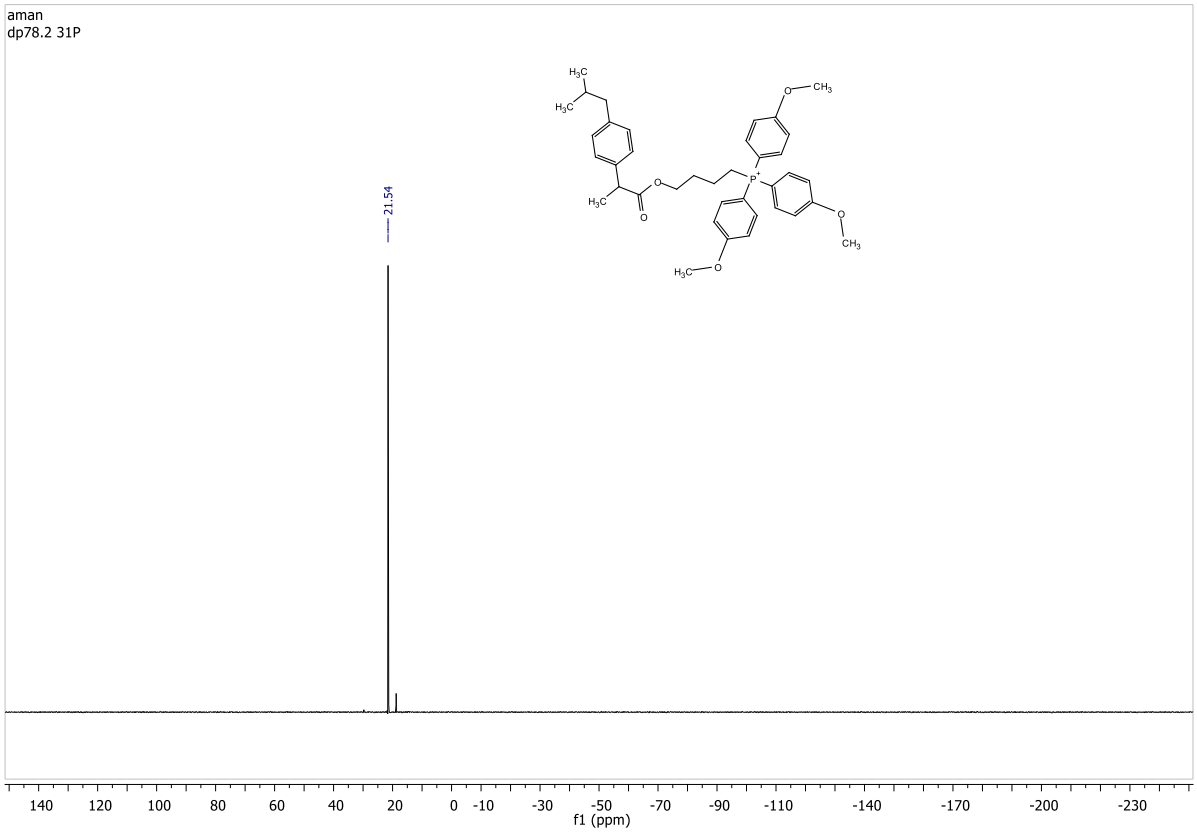

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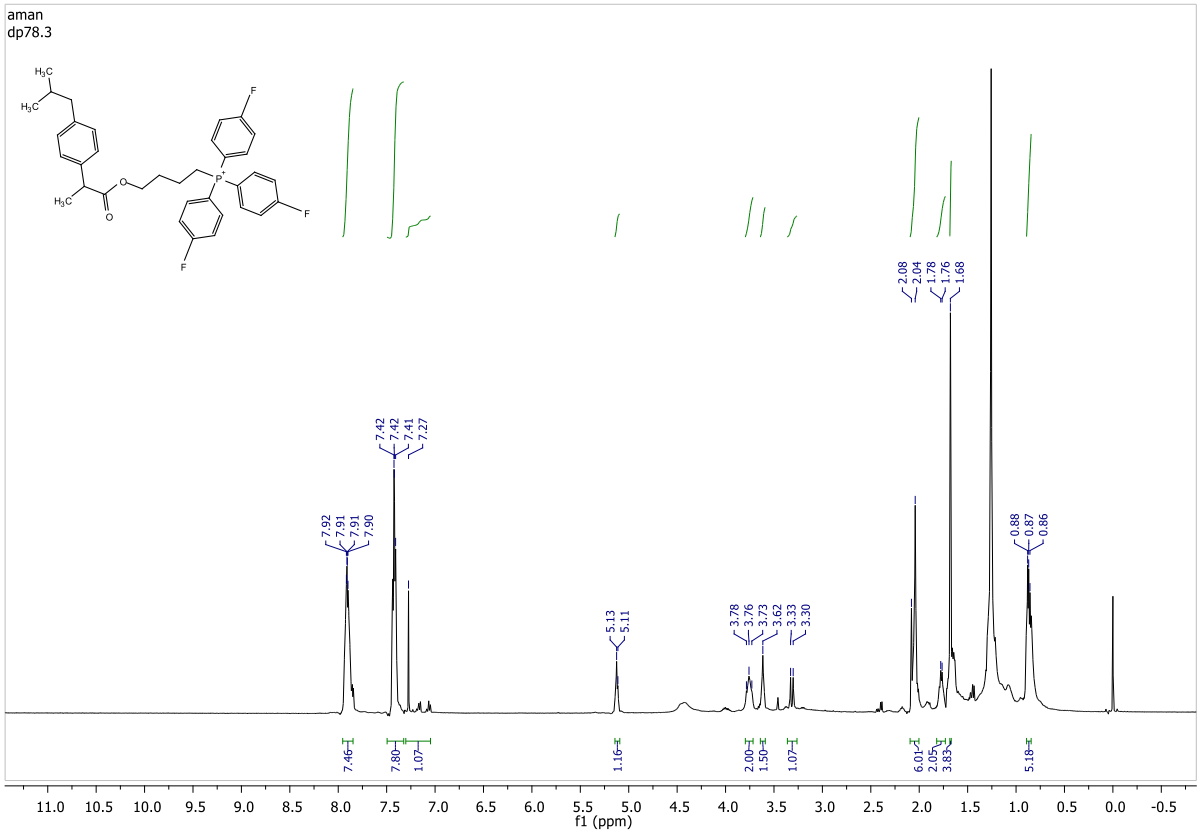

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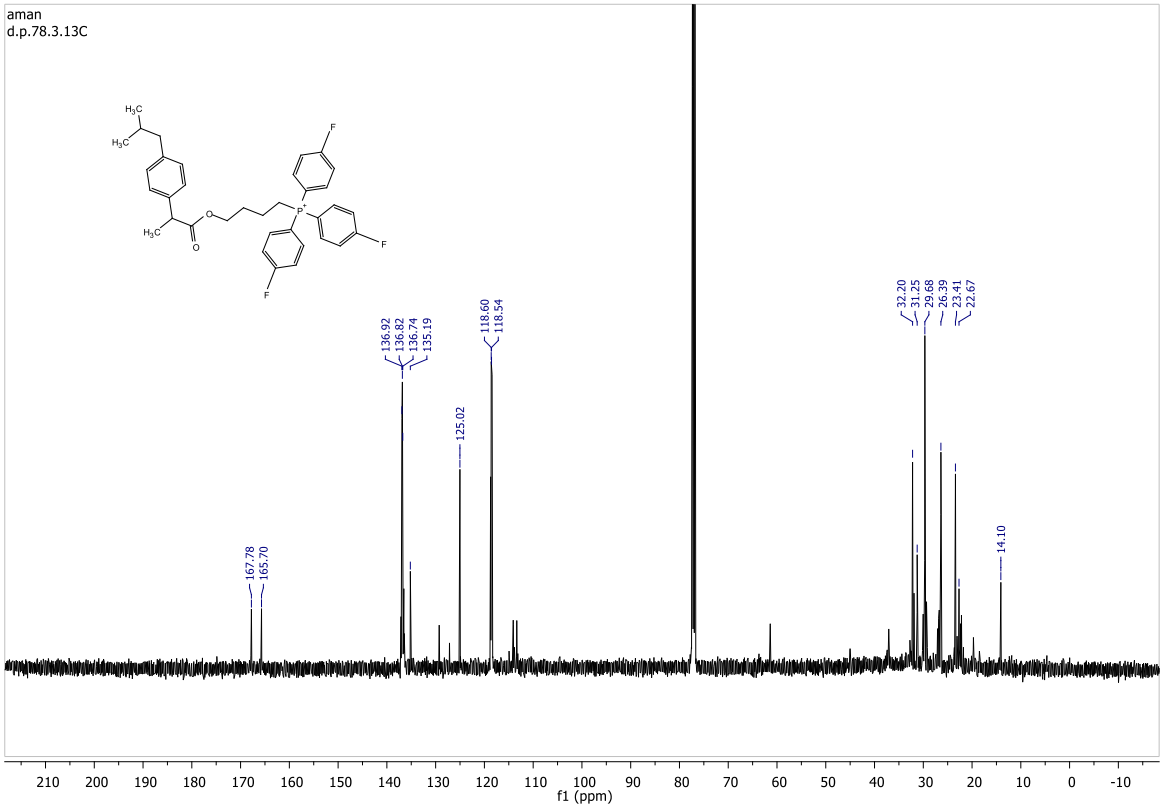

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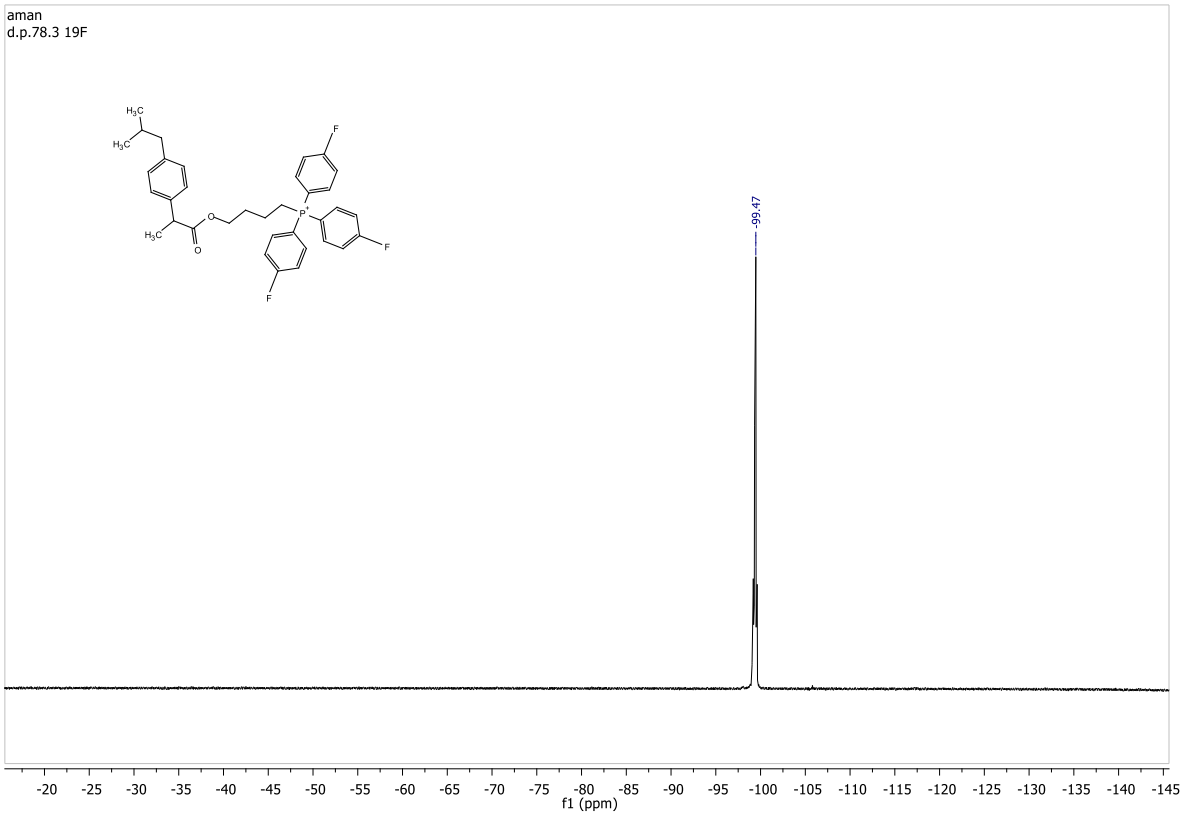
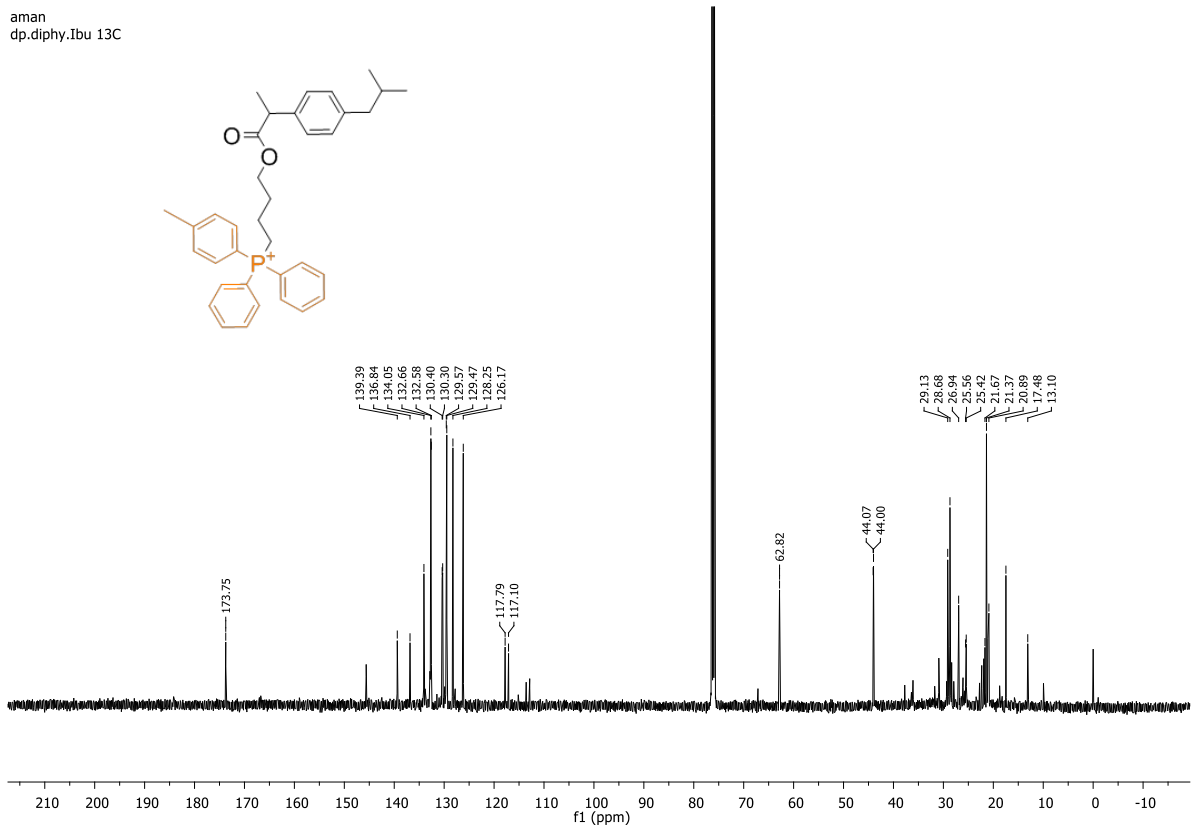


31P-NMR of (ii.5):

1H-NMR of (ii.6):



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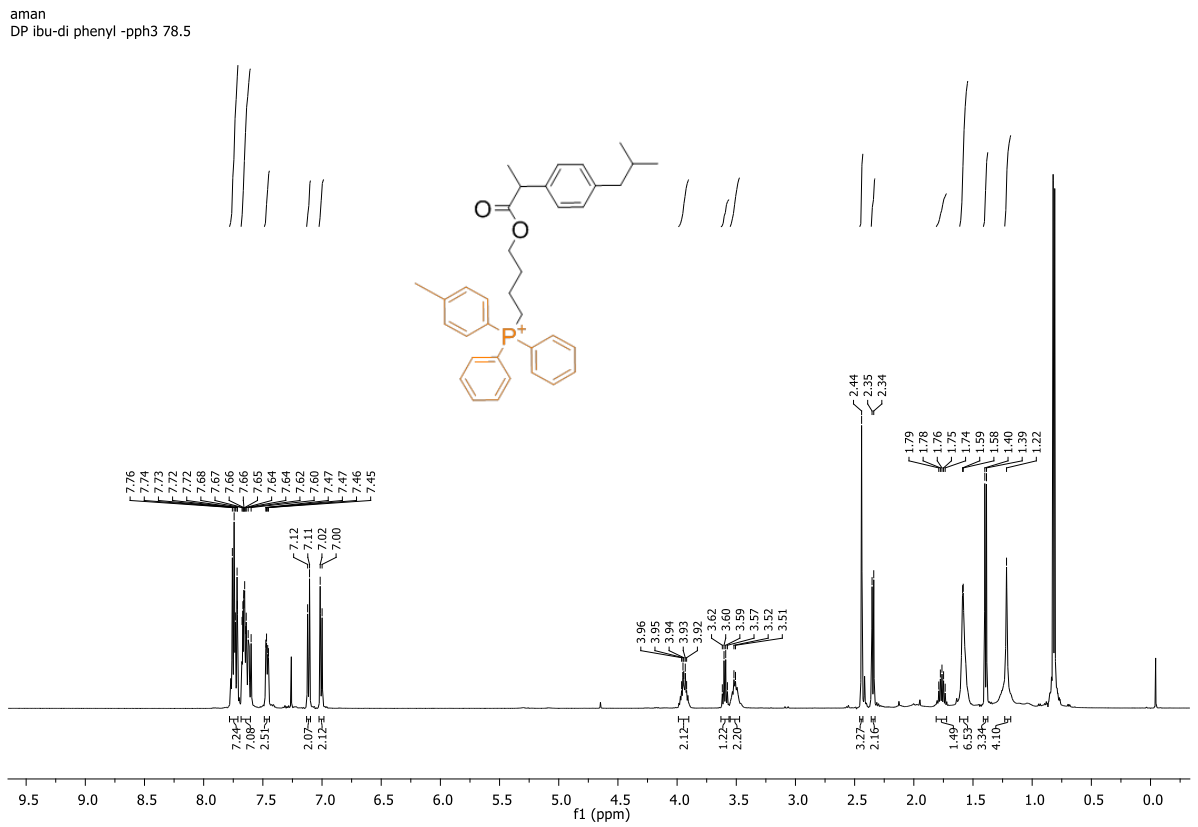

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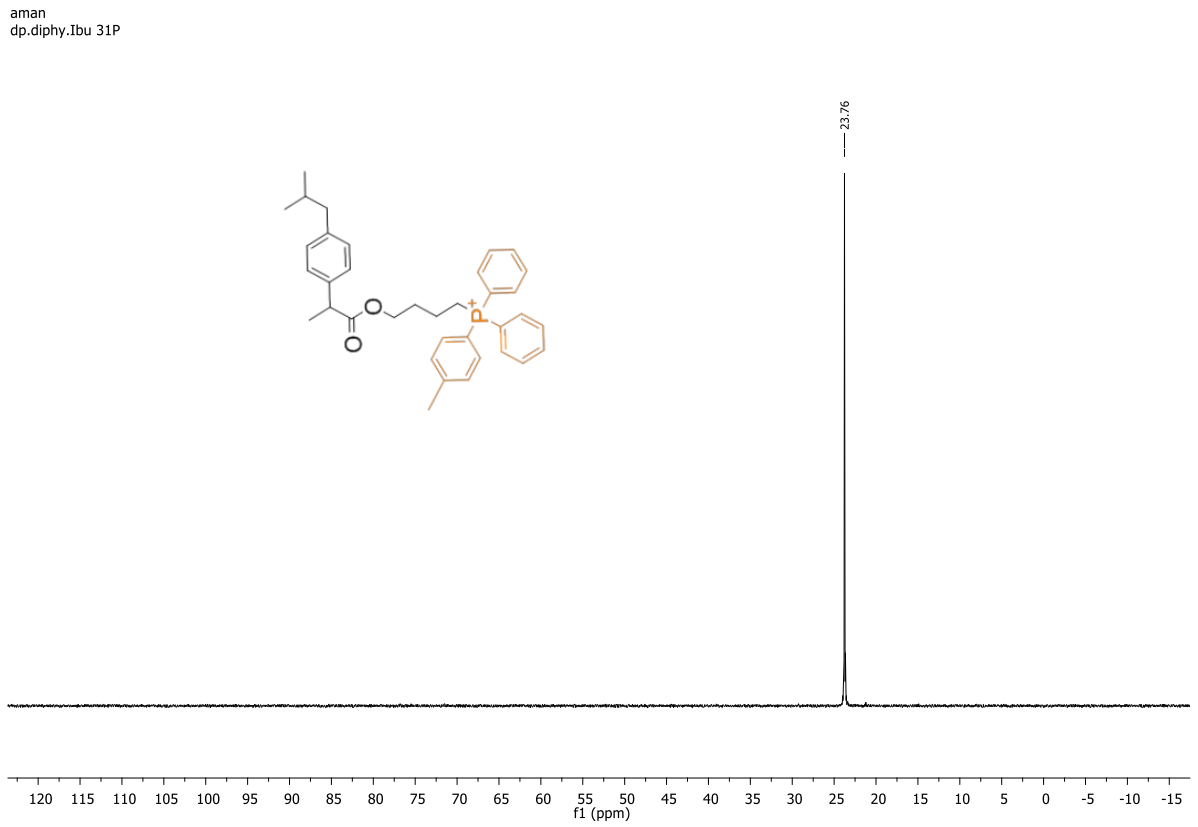
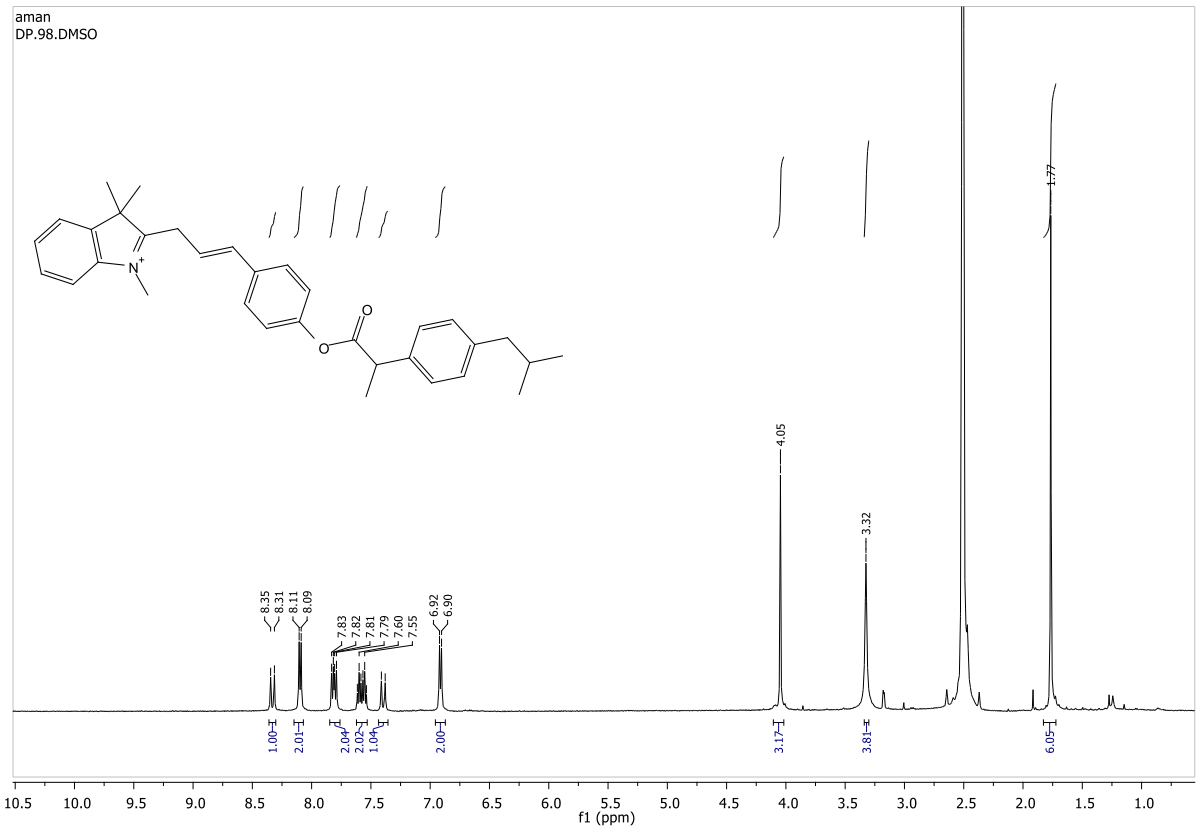
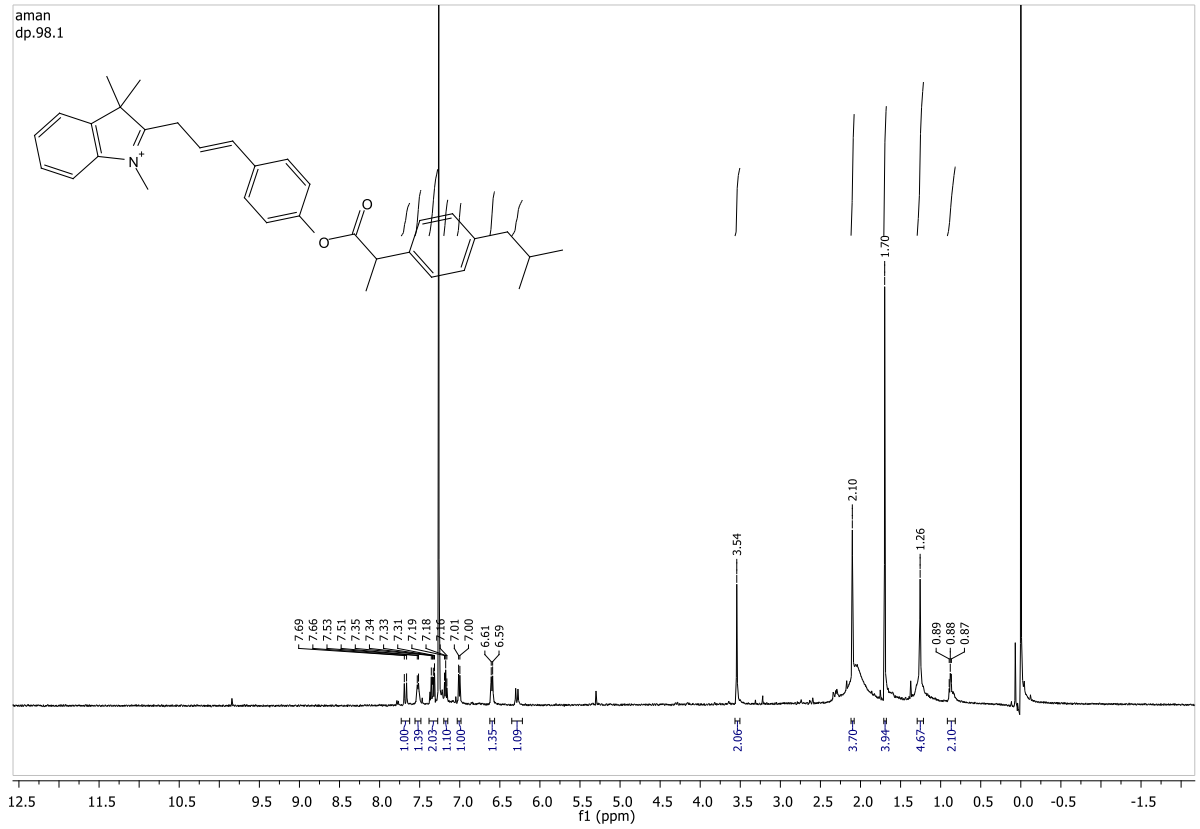


1H-NMR of scheme 3a :



1H-NMR of scheme 3b:



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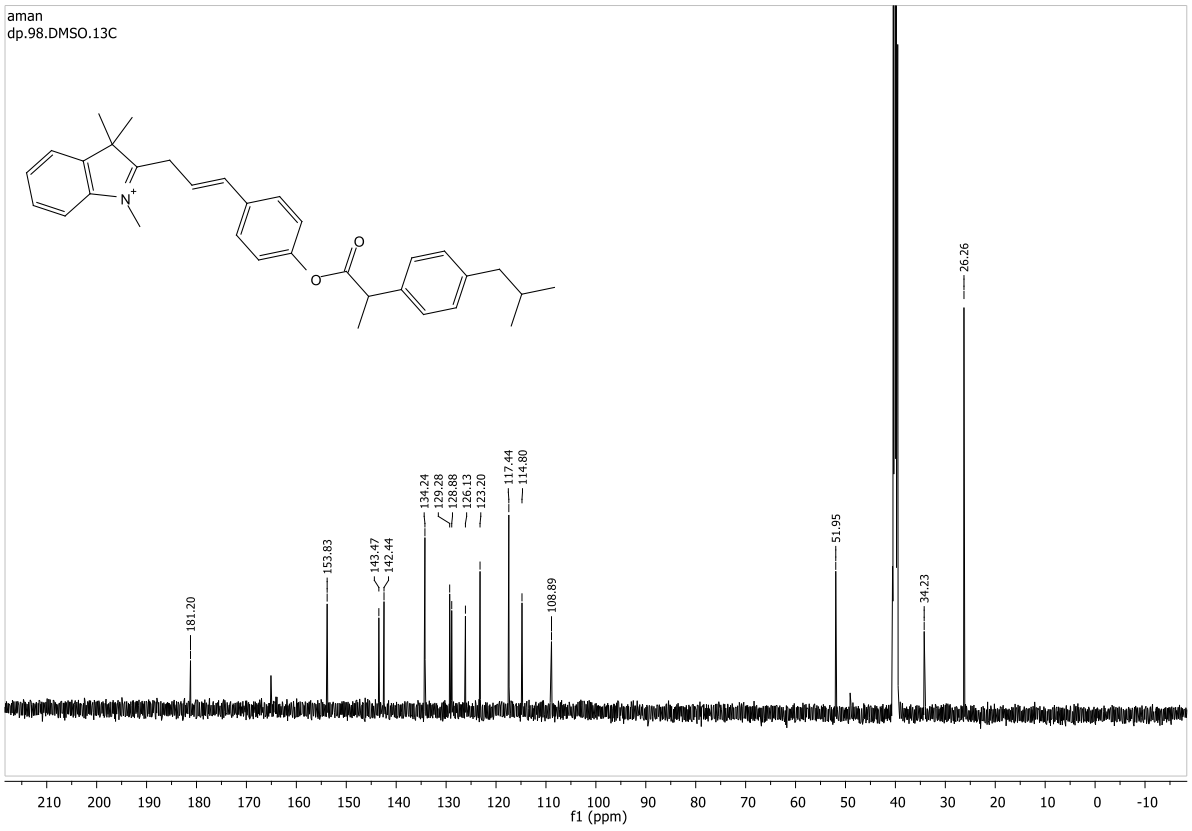

6. MASS DATA

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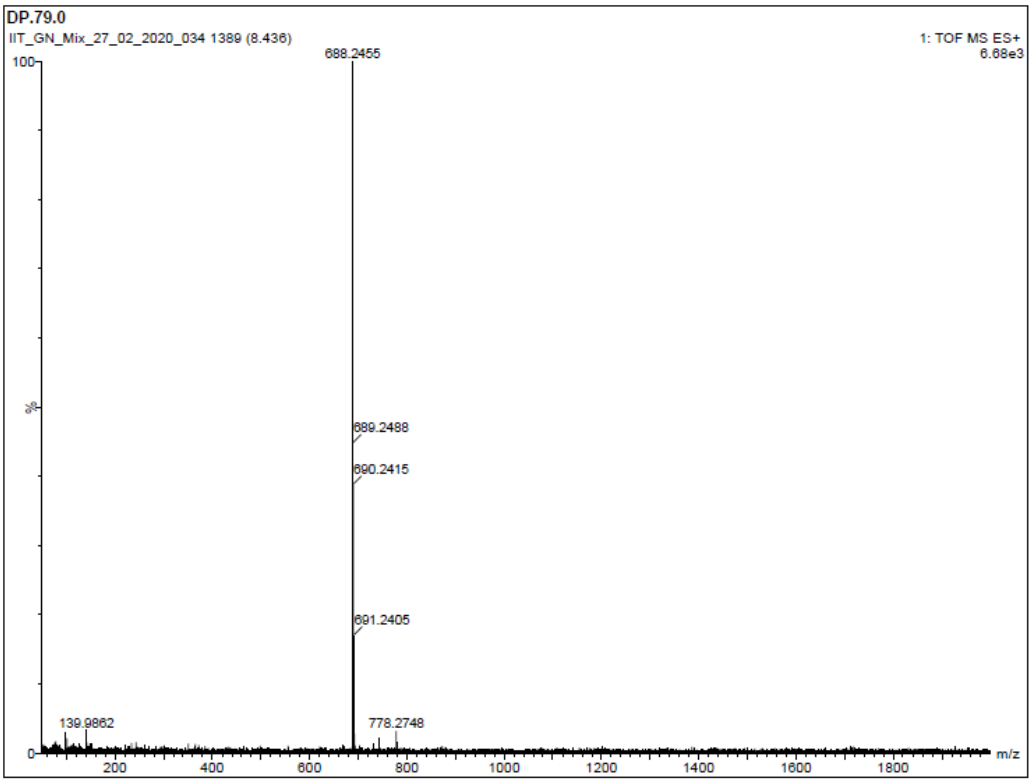

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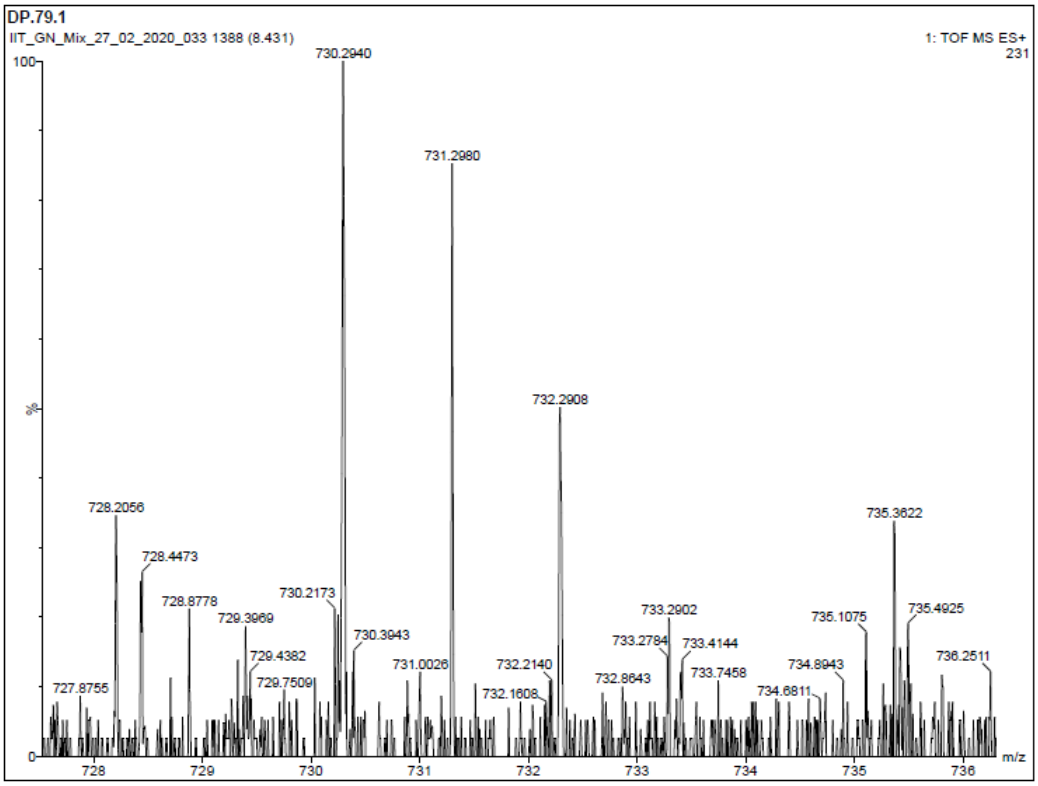

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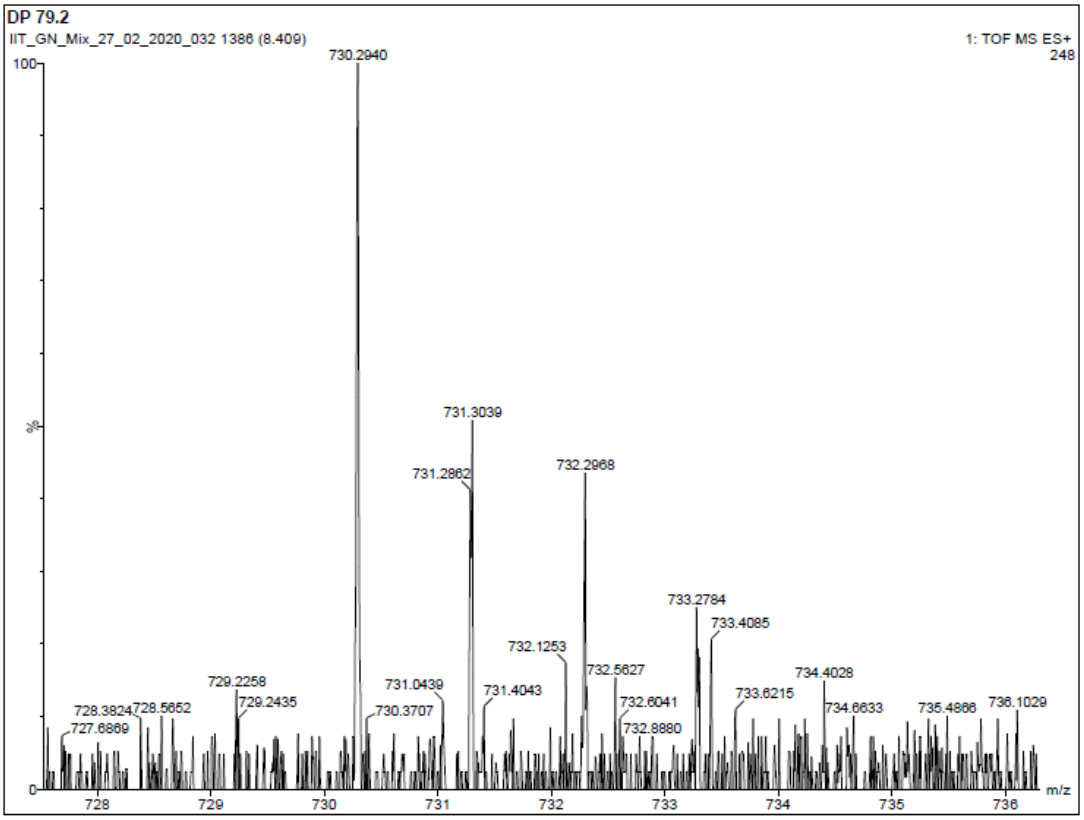

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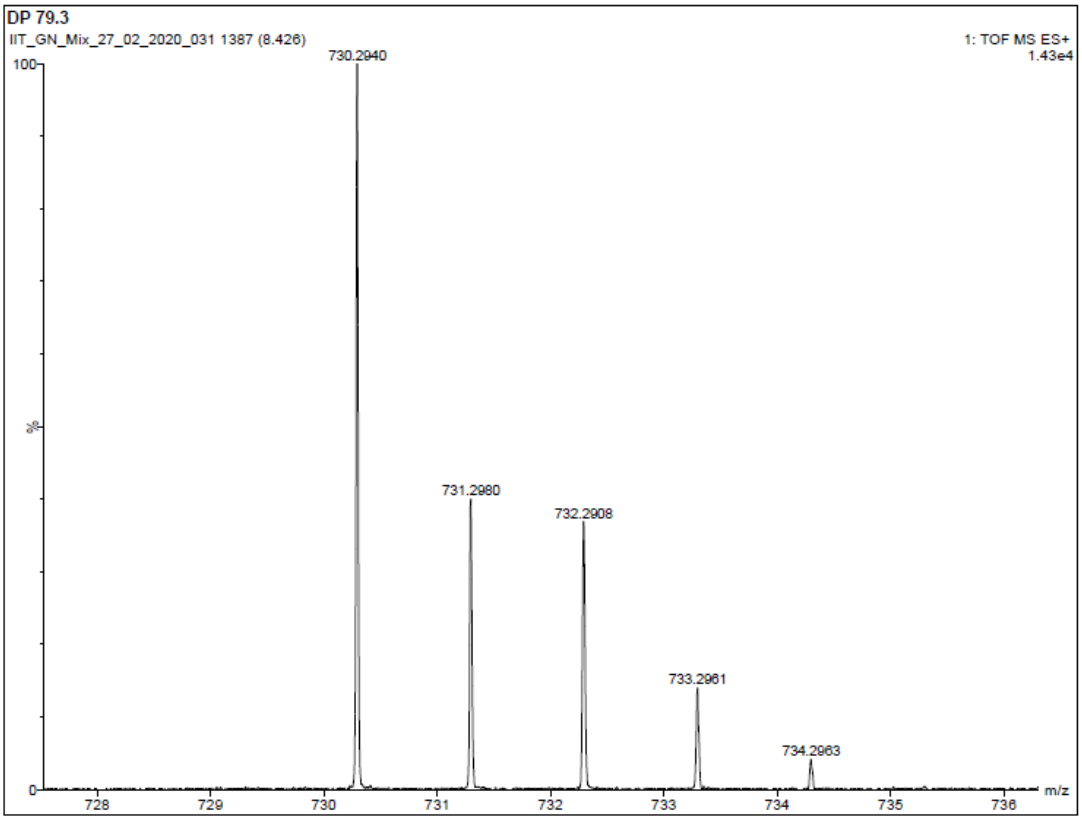

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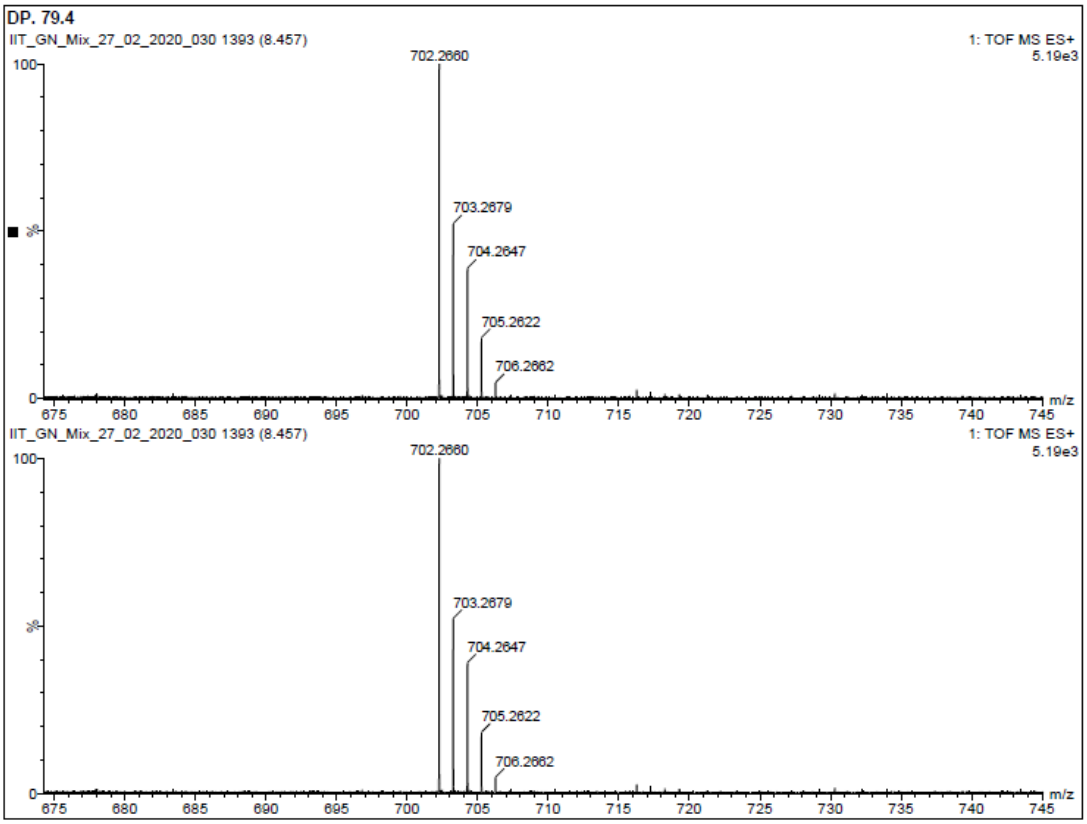

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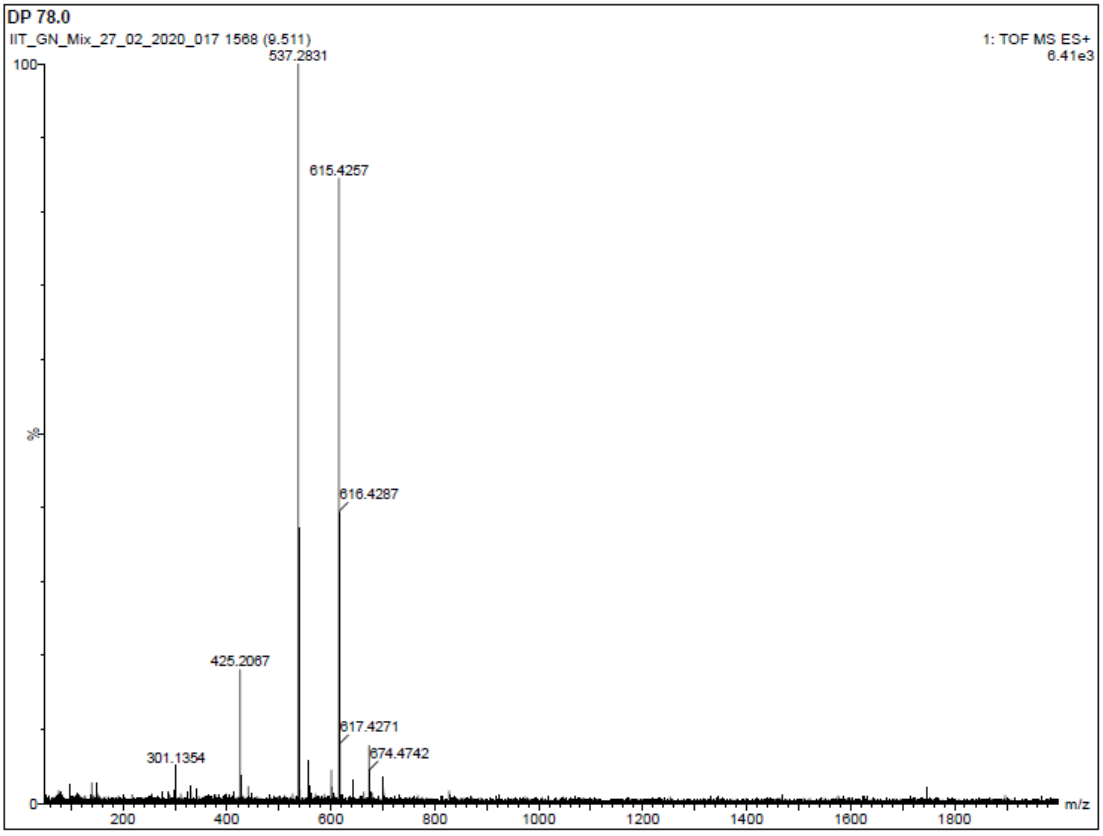

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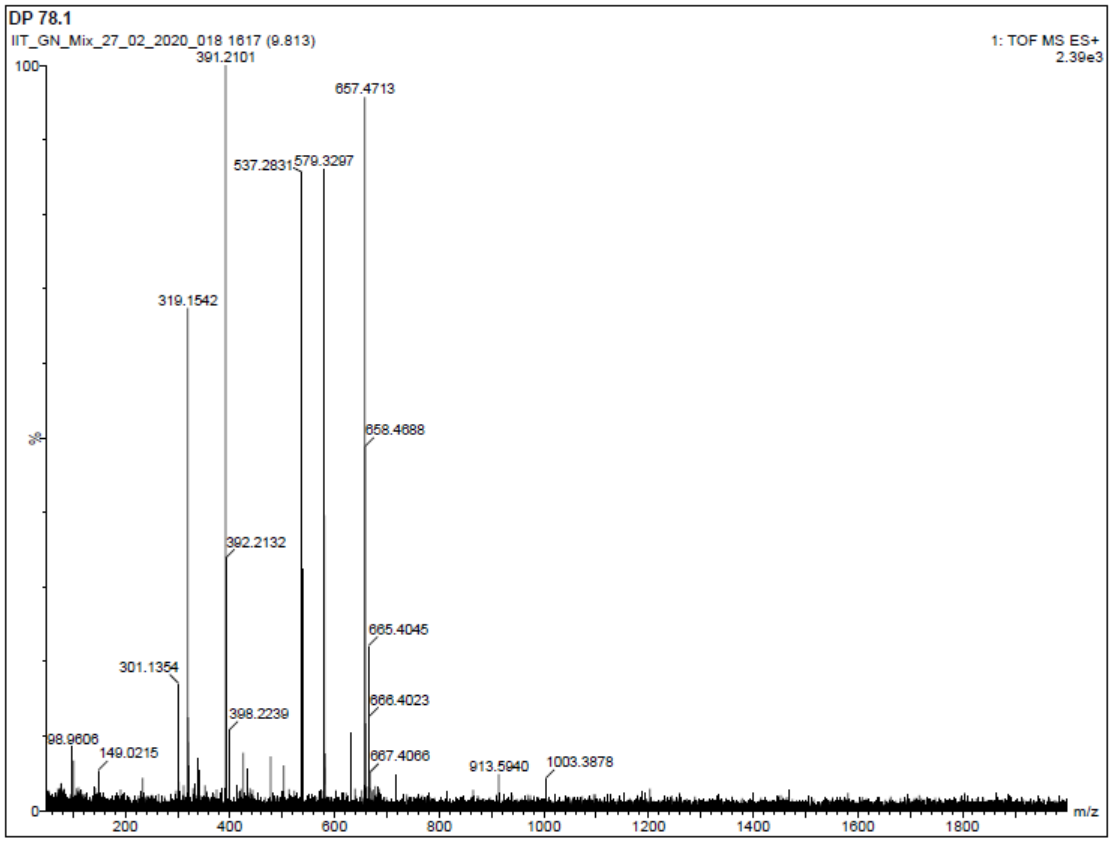

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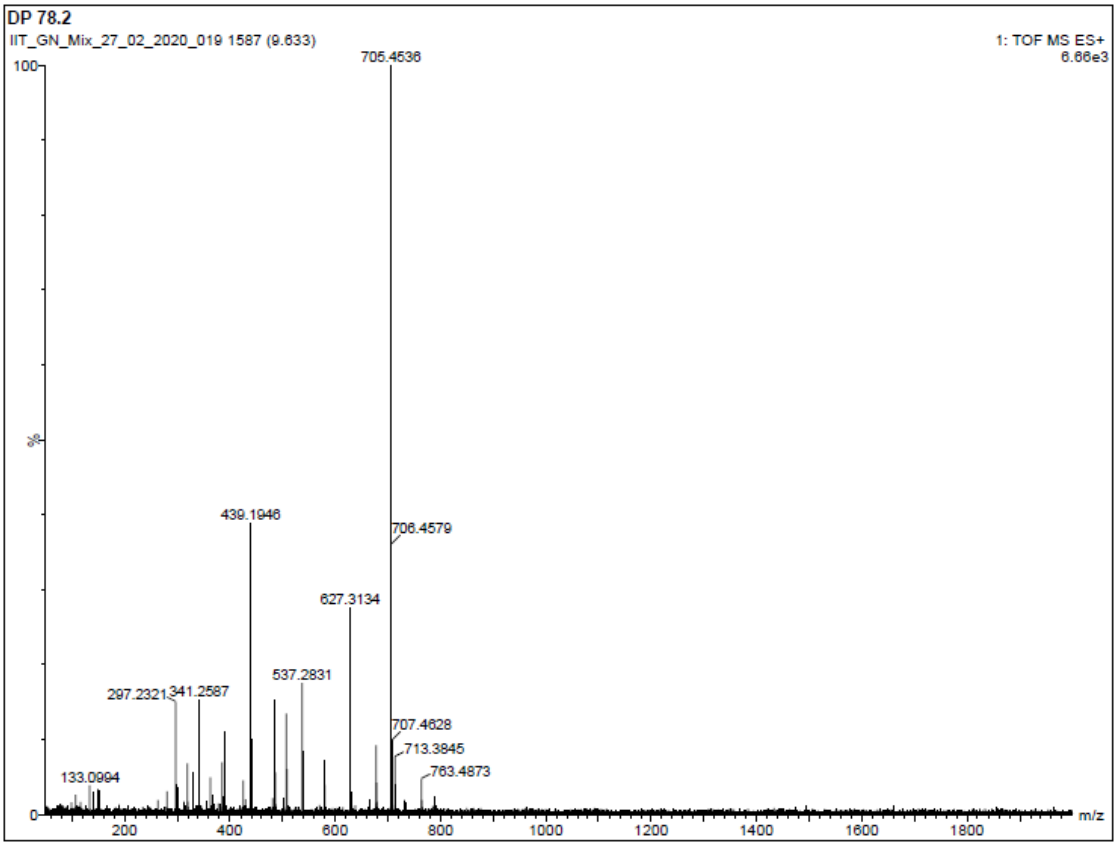

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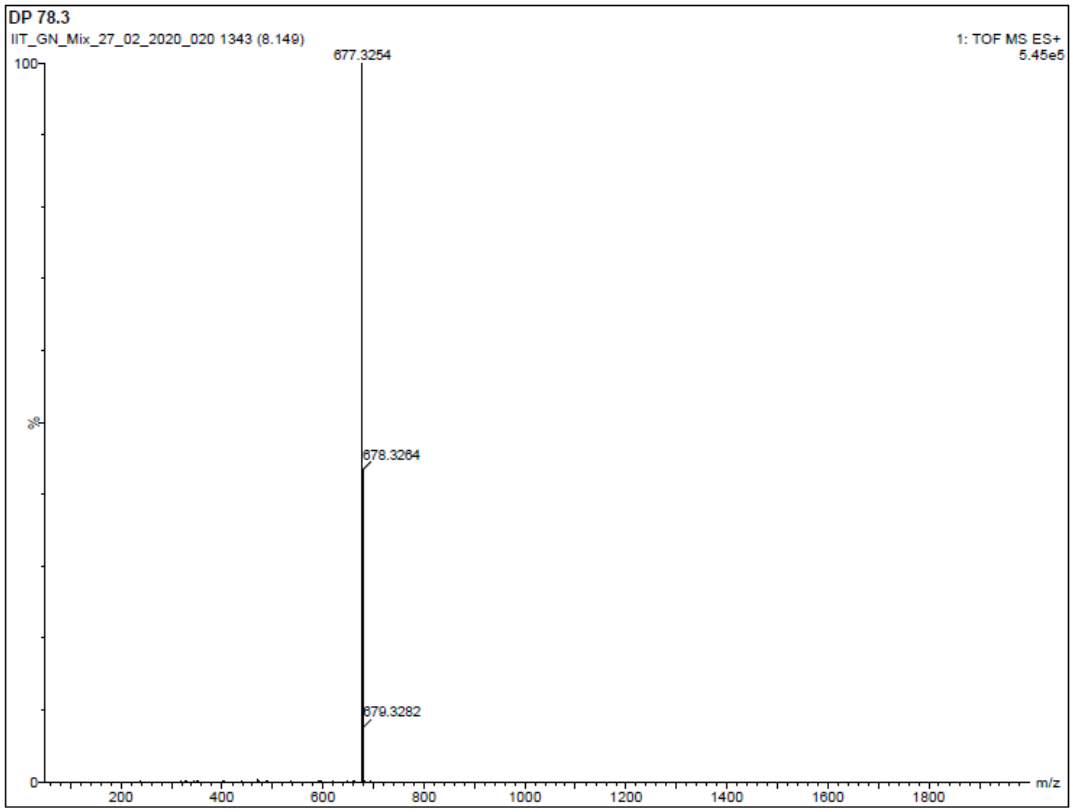

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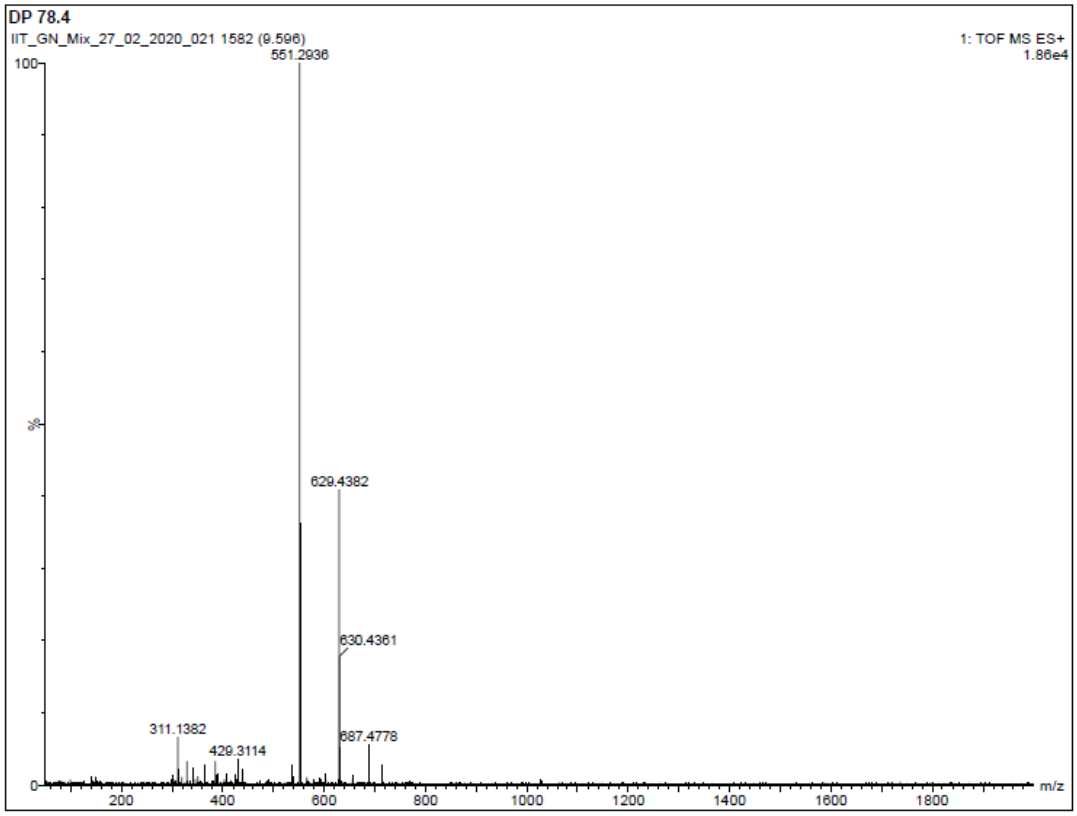

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