

**CHEMICAL FINGER-PRINTING, METABOLIC
PROFILING, AND BIOTRANSFORMATION OF NEEM
(*AZADIRACHTA INDICA*) LIMONOIDS**

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CERTIFICATE

This is to certify that the research work presented in thesis entitled “**Chemical Fingerprinting, Metabolic Profiling, and Biotransformation of Neem (*Azadirachta indica*) Limonoids**” has been carried out under my supervision at CSIR-National Chemical Laboratory, Pune and is a bonafide work of **Mr. Saikat Haldar**. This work is original and has not been submitted for any other degree or diploma to this or any other university.

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DECLARATION

I hereby declare that the research work presented in this thesis was carried out by me at CSIR-National Chemical Laboratory, Pune under the supervision of **Dr. H. V. Thulasiram**, Organic Chemistry Division, CSIR-National Chemical Laboratory, Pune. This work is original and has not been submitted in part or full for any degree or diploma to this or any other university.

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Dedicated to

My Parents & Teachers

who shaped me up to travel through the path

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--- Richelle E. Goodrich.

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Research Publications

1. Expedient preparative isolation and tandem mass spectrometric characterization of C-seco triterpenoids from Neem oil
Haldar, S.; Mulani, F. A.; Aarthy, T.; Dandekar, D. S.; Thulasiram, H. V. **2014** (*Communicated*)
2. Biocatalyst mediated functionalization of salannin, an insecticidal limonoid
Haldar, S.; Kolet, S. P.; Kale, B. S.; Dandekar, D. S.; Gonnade, R.; Thulasiram, H. V. *RSC Adv.* **2014**, 4, 27661-27664.
3. Lipase mediated separation of triterpene structural isomers, α - and β -amyrin
Haldar, S.; Kale, B. S.; Jadhav, D. D.; Thulasiram, H. V. *Tetrahedron Lett.* **2014**, 55, 3122-3125.
4. *Mucor hiemalis* mediated 14 α -hydroxylation on steroids: *In vivo* and *in vitro* investigations of 14 α -hydroxylase activity
Kolet, S. P.; **Haldar, S.**;[†] Niloferjahan, S.;[†] Thulasiram, H. V. *Steroids* **2014**, 85, 6-12. ([†]*Authors contributed equally*)
5. One-pot fluorescent labeling protocol for complex hydroxylated bioactive natural products
Haldar, S.; Kumar, S.; Kolet, S. P.; Patil, H. S.; Kumar, D.; Kundu, G. C.; Thulasiram, H. V. *J. Org. Chem.* **2013**, 78, 10192-10202.
6. Expedient preparative isolation, quantification and characterization of limonoids from Neem fruits
Haldar, S.; Phapale, P. B.; Kolet, S. P.; Thulasiram, H. V. *Anal. Methods* **2013**, 5, 5386-5391.
7. Biocatalyst mediated production of 6 β ,11 α -dihydroxy derivatives of 4-ene-3-one steroids
Kolet, S. P.; Niloferjahan, S.;[†] **Haldar, S.**;[†] Gonnade, R.; Thulasiram, H. V. *Steroids* **2013**, 78, 1152-1158. ([†]*Authors contributed equally*)
8. Biocatalysis: fungi mediated novel and selective 12 β - or 17 β -hydroxylation on the basic limonoid skeleton
Haldar, S.; Kolet, S. P.; Thulasiram, H. V. *Green Chem.* **2013**, 15, 1311-1317.
9. Novel anti-inflammatory activity of epoxyzadiradione against macrophage migration inhibitory factor: Inhibition of tautomerase and proinflammatory activities of macrophage migration inhibitory factor

Alam, A.; **Haldar, S.**; Thulasiram, H. V.; Kumar, R.; Goyal, M.; Iqbal, M. S.; Pal, C.; Dey, S.; Bindu, S.; Sarkar, S.; Pal, U.; Maiti, N. C.; Bandyopadhyay, U. *J. Biol. Chem.* **2012**, 287, 24844-24861.

10. Triterpenoid finger-printing, transcriptome analysis to identify and functionally characterize the initial genes involved in triterpenoid biosynthesis in Neem (*Azadirachta indica*)
Dandekar, D. S.; Pandreka, A.; **Haldar, S.**; Shinde, V. G.; Viragkar, U.; Mulani, F. A.; Thulasiram, H. V. (*Manuscript under preparation*)

Symposia and Presentations

1. Best poster award in 'INSA Platinum Jubilee International symposium' on 'Research in Molecular Medicine Based on Natural Resources and Traditional Knowledge' (November 21-23, 2009) held in CSIR-NCL, Pune, India.
2. Oral presentation (20 minutes) in 'RSC West India Ph. D. Students Symposium' held in University of Goa, Goa, India (September 03-04, 2010).
3. 'National Science Day Celebrations 2013' (February 26-17, 2013) held in CSIR-NCL Pune, India.
4. Best poster award in 'National Science Day Celebrations 2014' (February 25-26, 2014) held in CSIR-NCL Pune, India.

Abbreviations

UPLC	Ultra Pressure Liquid Chromatography	EtOAc	Ethyl Acetate
ESI	Electrospray Ionization	FBS	Fetal Bovine Serum
MS	Mass Spectrometry	AcOH	Acetic Acid
HRMS	High Resolution Mass Spectrometry	TMS	Tetramethylsilane
MPLC	Medium Pressure Liquid Chromatography	DCM	Dichloromethane
HPLC	High Pressure Liquid Chromatography	δ	Chemical Shift
APCI	Atmospheric Pressure Chemical Ionization	g	Gram
UV	Ultraviolet	TEA	Triethylamine
NMR	Nuclear Magnetic Resonance	nM	Nanomolar
PDA	Photo Diode Array	THF	Tetrahydrofuran
TLC	Thin Layer Chromatography	ppm	Parts per million
PTLC	Preparative Thin Layer Chromatography	$^{\circ}\text{C}$	Degree Celsius
NCE	Normalized Collision Energy	m/z	Mass to charge ratio
LC	Liquid Chromatography	R_t	Retention Time
SFC	Supercritical Fluid Chromatography	R_f	Retention Factor
HMBC	Heteronuclear Multiple Bond Coherence	MHz	Megahertz
DAPI	4',6-Diamidino-2-Phenylindole	L	Litre
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide	Kg	Kilogram
rpm	Revolutions per minute	h	Hour
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	min	Minute
DCC	N,N'-Dicyclohexylcarbodiimide	μL	Microlitre
TBAF	Tetra-n-butylammonium fluoride	mg	Milligram
DEPT	Distortionless Enhancement by Polarization Transfer	nm	Nanometre
COSY	Correlation Spectroscopy	mbar	Millibar
NOESY	Nuclear Overhauser Effect Spectroscopy	mmol	Millimole
HSQC	Heteronuclear Single Quantum Coherence	fmol	Femtomole
MIC	Minimum Inhibitory Concentration	mm	Millimetre
IC ₅₀	50% Inhibitory concentration	μg	Microgram
TBDMS	Tert-butyldimethylsilyl	cm	Centimetre
DMF	N,N-Dimethylformamide	μm	Micron
NBD	7-Nitrobenzo-2-oxa-1,3-diazole	ng	Nanogram
DMAP	4-(Dimethylamino)pyridine	mL	Millilitre
CDCl ₃	Deuterated chloroform	μM	Micromolar
PBS	Phosphate buffered saline	M	Molar
TBAI	Tetra-n-butylammonium iodide	μA	Microampere
DIC	N,N'-Diisopropylcarbodiimide	KV	Kilovolt
		mA	Milliampere

Abstract of the Thesis

The thesis entitled '**Chemical Finger-printing, Metabolic Profiling, and Biotransformation of Neem (*Azadirachta indica*) Limonoids**' deals with analytical and preparative scale isolation, spectroscopic characterization [NMR, ESI(+)-MS and ESI(+)-MS/MS] and UPLC-ESI(+)-HRMS based tissue specific metabolic profiling of limonoids from Neem plant. Whole-cell biocatalyst mediated functionalization of Neem limonoids has been studied to generate novel metabolites. Further, NBD based fluorescent labeling protocol was developed for the tagging and cell-imaging studies of Neem limonoids within cancer cell lines. The thesis comprises of four chapters.

Chapter 1

Introduction and Literature Review

Azadirachta indica (Neem), a fast-growing evergreen tree is native to Indian sub-continent and well-known as 'wonder tree' due to its diverse pharmaceutical and agrochemical uses. It is one the richest sources of secondary metabolites especially tetranortriterpenoids (limonoids) which have been reported to be responsible for the wide biological activities of Neem. They are highly complex, oxygenated and rearranged in structure and skeletally diverse. About 150 limonoids have been isolated and characterized from various parts of Neem plant. This chapter delineates the structural diversity of Neem limonoids in detail with individual structure of isolated triterpenoids. It also reviews the insecticidal (anti-feedant and growth regulatory) and pharmacological activities (anti-carcinogenic, anti-inflammatory, anti-plasmodial, anti-ulcerogenic and anti-microbial) of Neem limonoids. Further, the plausible biosynthetic pathway, separation techniques and chemical synthetic procedures for Neem limonoids have been discussed.

Chapter 2

Isolation, Characterization and Metabolic Profiling of Limonoids from Neem

Section A: Isolation and Metabolic Profiling of Limonoids from Neem

Metabolic profiling is about structural investigation of metabolites, their distribution and quantification for a specific class of metabolites. It is helpful for the quantification

(among different tissues, stages and conditions) of metabolites, identification of novel metabolite or better natural source and investigation of biosynthetic pathway. Except very few reports, no systematic efforts have been made for the profiling of Neem limonoids. In this chapter, isolation and characterization of fifteen triterpenoids from Neem seed kernel and fruit-coat have been described. Further, using these isolated triterpenoids as the reference standards the metabolic profiling has been carried out for triterpenoids in different tissues of Neem including flowers, leaves, stem, bark and five developmental stages of fruit-coat and seed kernel through UPLC-ESI(+)-HRMS.

Four major limonoids (Azadirachtin A, azadirachtin B, salannin, nimbin) and six minor limonoids (3-deacetylsalannin, 6-deacetylnimbin, nimbinene, 6-deacetylnimbinene, nimbanal, salannol acetate) have been isolated from seed kernel through solvent-partition followed by column chromatographic technique with high purity (Figure 1). Similarly, fruit-coat was extracted for the isolation of two major (azadiradione and epoxyazadiradione) and three minor limonoids (azadirone, gedunin and nimocinol) (Figure 1).

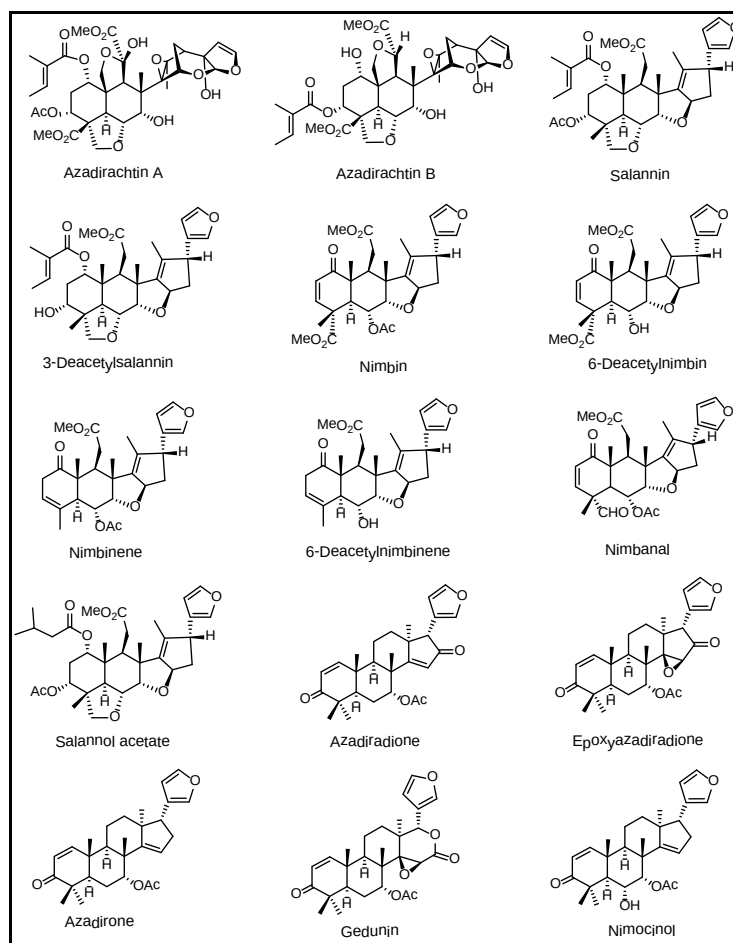


Figure 1 Isolated triterpenoids from Neem seed kernel and fruit-coat

Standard graphs were drawn for fifteen isolated triterpenoids in UPLC-ESI(+)-HRMS which were further utilized for the quantification of individual metabolites in crude extracts. Various Neem tissues (Figure 2A) were extracted by solvent-partition technique to obtain crude triterpenoid mixture and they were analyzed by UPLC-ESI(+)-HRMS. The amount of extracts obtained was directly correlated with the triterpenoid content of the tissues. Results indicated that seed kernel of stages 4 and 5 contained highest amount of triterpenoid extract (~80 mg/g) followed by fruit-coats of stages 1, 2 and 3 (~48-66 mg/g). The triterpenoid extract of fruit-coat of stages 4, 5 and kernel of stage 3 was in the range of ~25-35 mg/g of the tissue. Flowers, leaves, stem and bark samples contained ~22, 45 (including chlorophyll and other pigments), 15 and 10 mg extract respectively (Figure 2B).

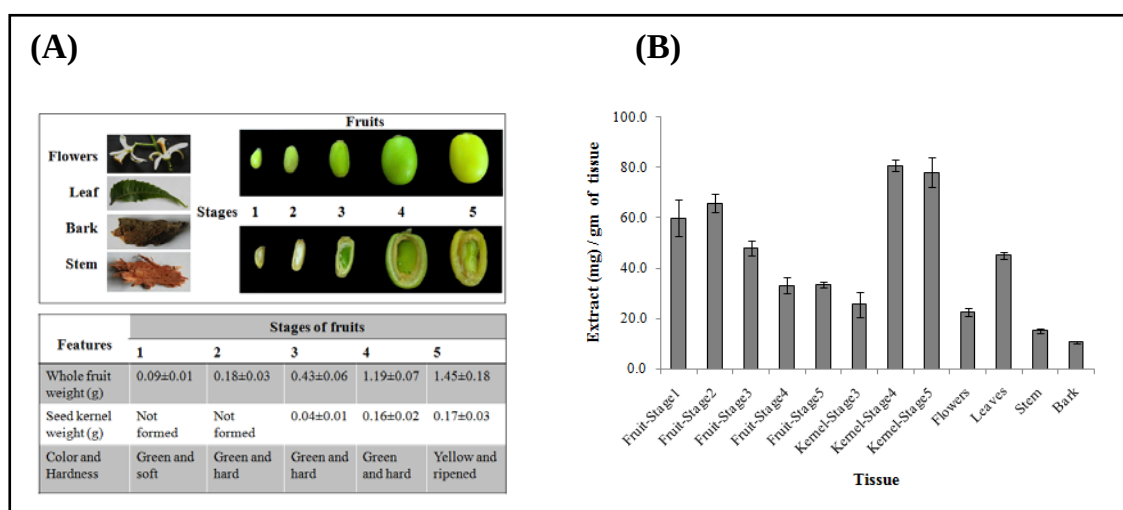


Figure 2 (A) Photographs of different tissues of Neem and physical characteristics of Neem fruits from various stages; **(B)** Amount of extracts obtained from various tissues and their stages from Neem.

The abundance of individual triterpenoids in the studied tissues has been represented in Figure 3. Metabolic profiling data revealed that seed kernel of stages 4 and 5 contained majorly C-seco triterpenoids (Azadirachtin A, azadirachtin B, salannin, nimbin, 6-deacetylnimbin, nimbinene, 6-deacetylnimbinene, nimbanal and salannol acetate) whereas all the developmental stages of fruit-coats were highly rich in triterpenoids of ring-intact limonoids (azadiradione, epoxyazadiradione, azadirone and gedunin). The limonoid profile of flowers and leaves also indicated the presence of basic/ring-intact limonoids. Stem and bark samples showed the abundance of mainly C-seco triterpenoids with lower level in comparison to seed kernel.

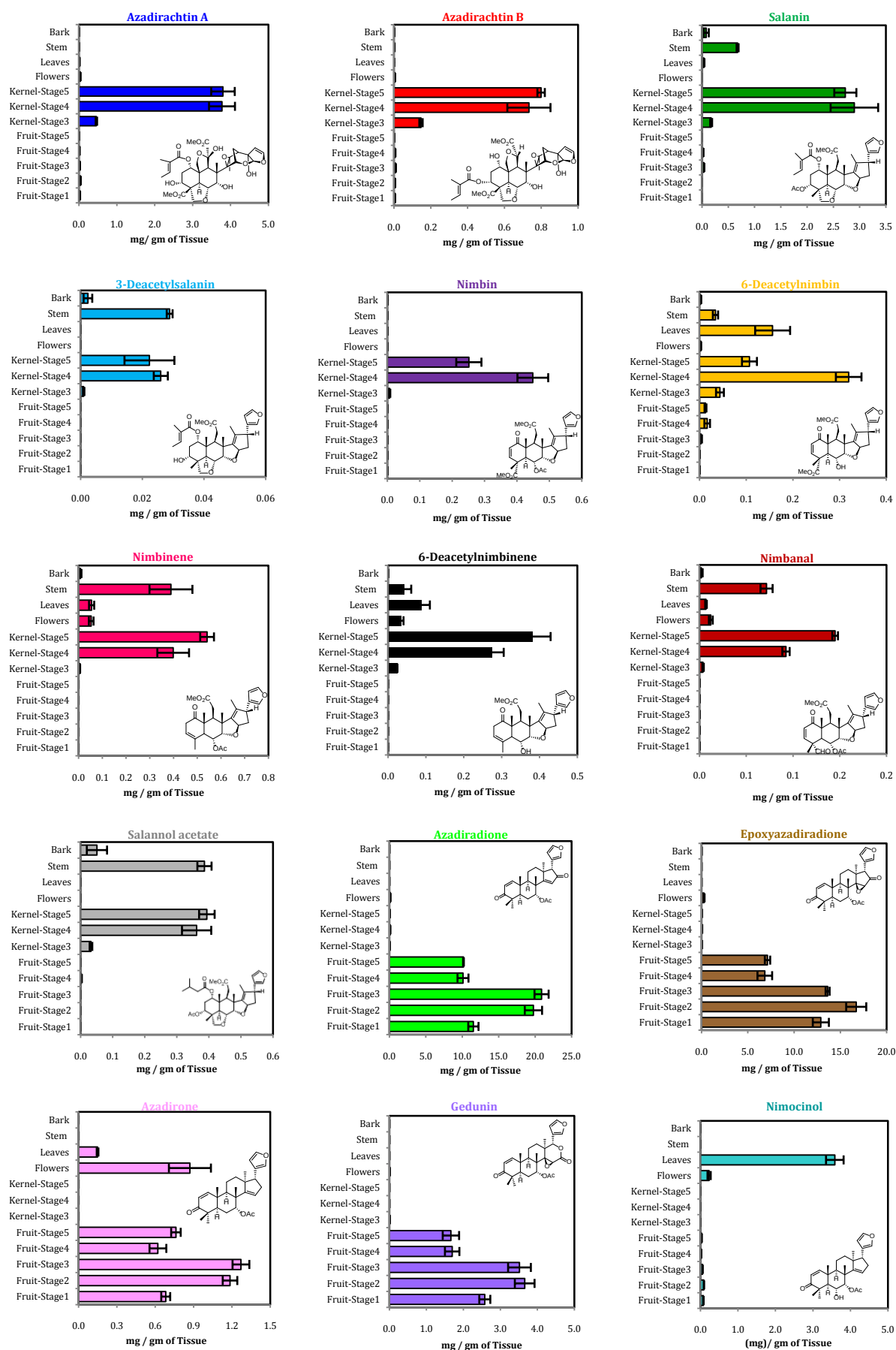


Figure 3 Quantitative abundance of individual triterpenoids in different tissues of Neem

Section B: Expedient MPLC Based Preparative Isolation, HPLC Based Quantification and MS/MS Characterization of Limonoids from Neem Fruits

Basic limonoids containing 4,4,8-trimethyl-17-furanylsteroidal skeleton are well-known as widely biologically active class of triterpenoids. The preparative scale availability and ‘in-mixture’ sensitive and accurate identification of these limonoids are highly demanding for the detailed biological investigation. In this section, we have optimized gram-scale isolation procedure for major basic limonoids such as azadirone (**I**), epoxyazadiradione (**II**) and azadiradione (**III**) from Neem fruits using automated medium pressure liquid chromatography (MPLC). Crude triterpenoid extract obtained from Neem fruit-coat by solvent-partition technique was subjected to purification over normal-phase silica gel Redisep cartridge with monitoring at 200-400 nm. A gradient solvent program of 54 min with increasing percentage of ethyl acetate in hexane was used as the eluent to furnish limonoids with high purity (>95%). Further, the separation process was scaled up to 2 g, 5 g and 10 g of the extract by varying the sizes of pre-packed columns and flow rate. Isolated basic limonoids were further quantified in three developmental stages of Neem fruits through HPLC utilizing the standard graphs drawn for individual compounds.

Crude extract (g)	Redisep Rf cartridge	Flow rate (mL/min)	Yield of limonoids (g)		
			I	II	III
1	24	20	0.04	0.21	0.26
2	40	25	0.10	0.40	0.54
5	120	50	0.24	1.03	1.36
10	220	90	0.51	2.05	2.67

* Solvent program: 0-3 min 5 % EtOAc, 6 min 10 % EtOAc, 3 min hold, 12 min 15 % EtOAc, 3 min hold, 18 min 20 % EtOAc, 3 min hold, 24 min 25 % EtOAc, 3 min hold, 30 min 30 % EtOAc, 6 min hold, 39 min 35 % EtOAc, 6 min hold, 48 min 40 % EtOAc, 6 min hold.

Table 1 Tunable parameters and yield obtained in MPLC scale up process

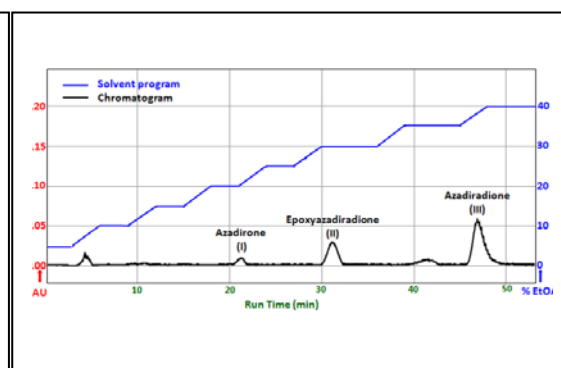


Figure 4 MPLC profile obtained with 10 g crude extract.

For rapid characterization in the complex phytochemical extract a LC-ESI(+)-quadrupole/orbitrap-MS/MS based technique was developed. MS/MS daughter ions were generated with high accuracy by the collisional activation of $[M+H]^+$ precursor ion in positive ion mode with varying stepped normalized collision energy (NCE) (15, 20, 25, 30, 35 and 40%). Structure-fragment relationships were established on the basis of chemical formulae obtained from m/z values (with error < 2.5 ppm) of the daughter ions. The MS/MS ions obtained were categorized into two groups. Ions with m/z 80-150

were generated due to the cleavage of C-C bonds and rearrangements. They were found to be ubiquitously present in the MS/MS spectra of all three limonoids as they were produced from cyclohexenone A ring, a common skeletal region of basic limonoids. Ions with m/z greater than 150 were produced by the removal of sensitive functional groups as the neutral molecules and they were found to be unique for studied limonoids. Applicability of the developed MS/MS identification technique was shown by the investigation of studied limonoids in Neem flower extract.

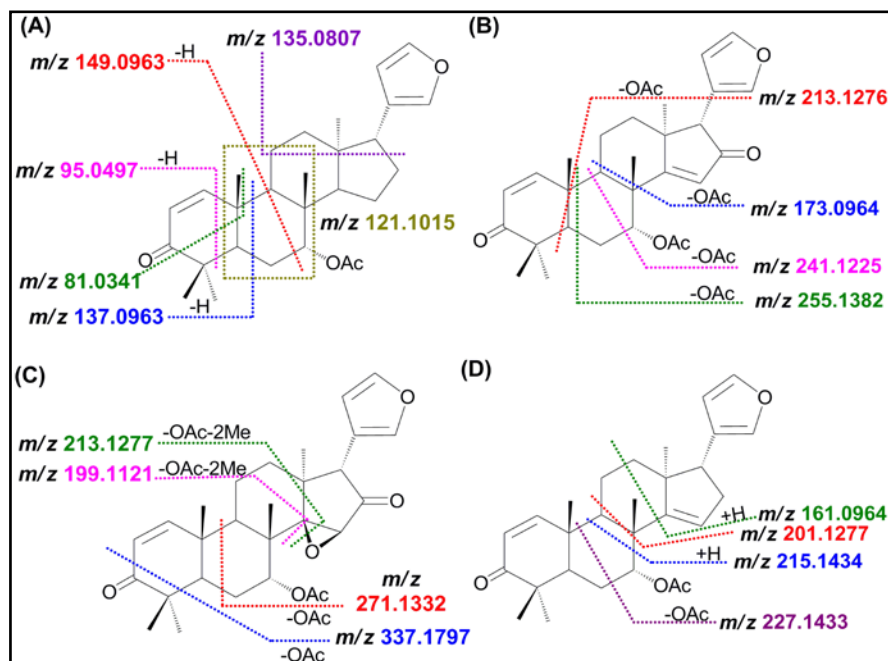


Figure 5 (A) Structure-fragment relationships of common m/z obtained for basic limonoids (I, II and III); (B) Structure-fragment correlations of diagnostic peaks for (B) III, (C) II and (D) I.

Section C: Expedient MPLC Based Preparative Isolation and MS/MS Characterization of C-Seco Triterpenoids from Neem Oil

Limonoids have been investigated to be active ingredients responsible for the diverse biological activities of Neem oil. However, there is no systematic preparative scale isolation protocol for the purification of C-seco limonoids from Neem oil. In this study, we have developed an MPLC based protocol for the isolation of eight major C-seco triterpenoids of salannin skeleton [6-deacetylnimbin (**1**), 6-deacetylnimbinene (**2**), nimbanal (**3**), nimbin (**4**), 3-deacetylsalannin (**5**), nimbinene (**6**), salannin (**7**) and salannol acetate (**8**)] from Neem oil. For rapid and sensitive identification, eight isolated triterpenoids along with five semi-synthesized derivatives were characterized by ESI(+)-quadrupole/orbitrap-MS/MS spectrometry for the first time.

An automated MPLC based protocol developed by successive application of normal phase and reverse phase silica gel pre-packed cartridges was utilized for the expedient and preparative purification of eight salannin type C-seco limonoids. Crude triterpenoid mixture obtained from Neem oil by solvent partition technique was fractionated into six unique fractions (**F1-F6**) through Redisep normal phase silica-gel cartridge (40 g, flow rate: 25 mL/min and solvent program: 105 min with increasing percentage of ethyl acetate in hexane). Each individual fraction (**F1-F6**) was further subjected to fine-purification over reverse phase silica gel cartridge (43 g, flow rate: 30 mL/min and solvent program: 80 min with increasing percentage of methanol in water) to furnish highly pure limonoids. The efficiency of developed protocol and purity of isolated triterpenoids was evaluated through HPLC-UV technique. The developed protocol has been schematically represented in Figure 6.

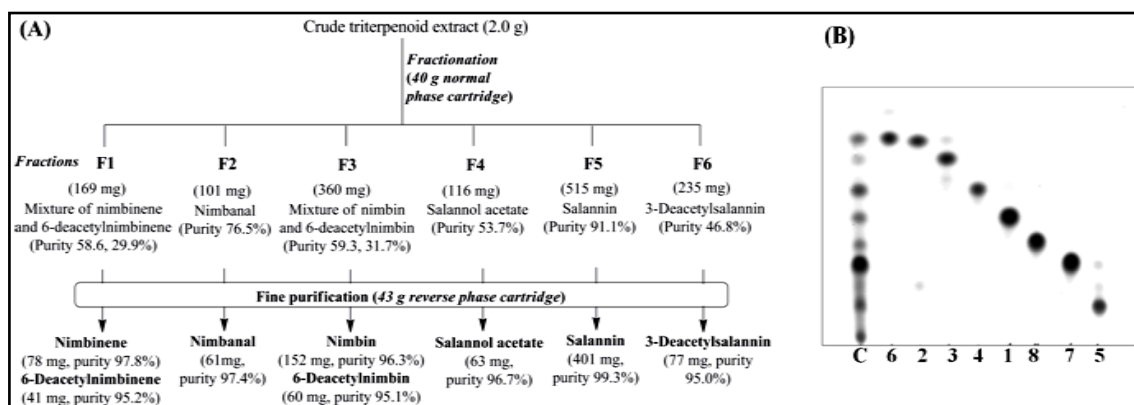
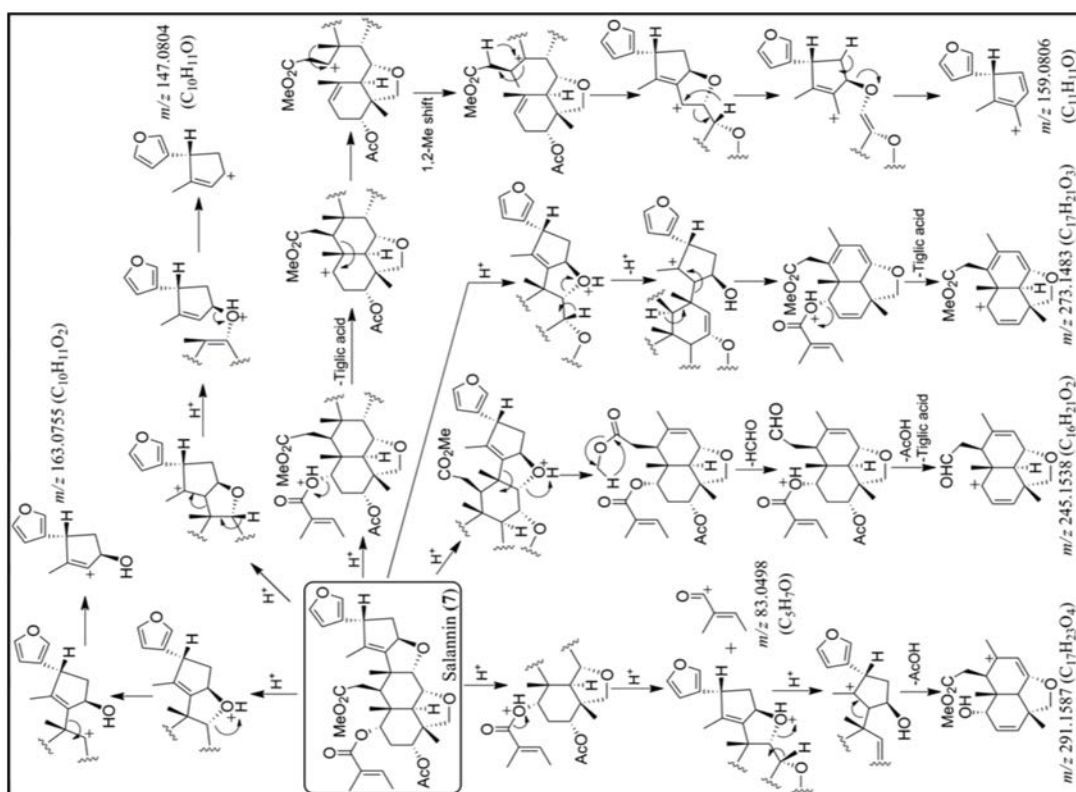
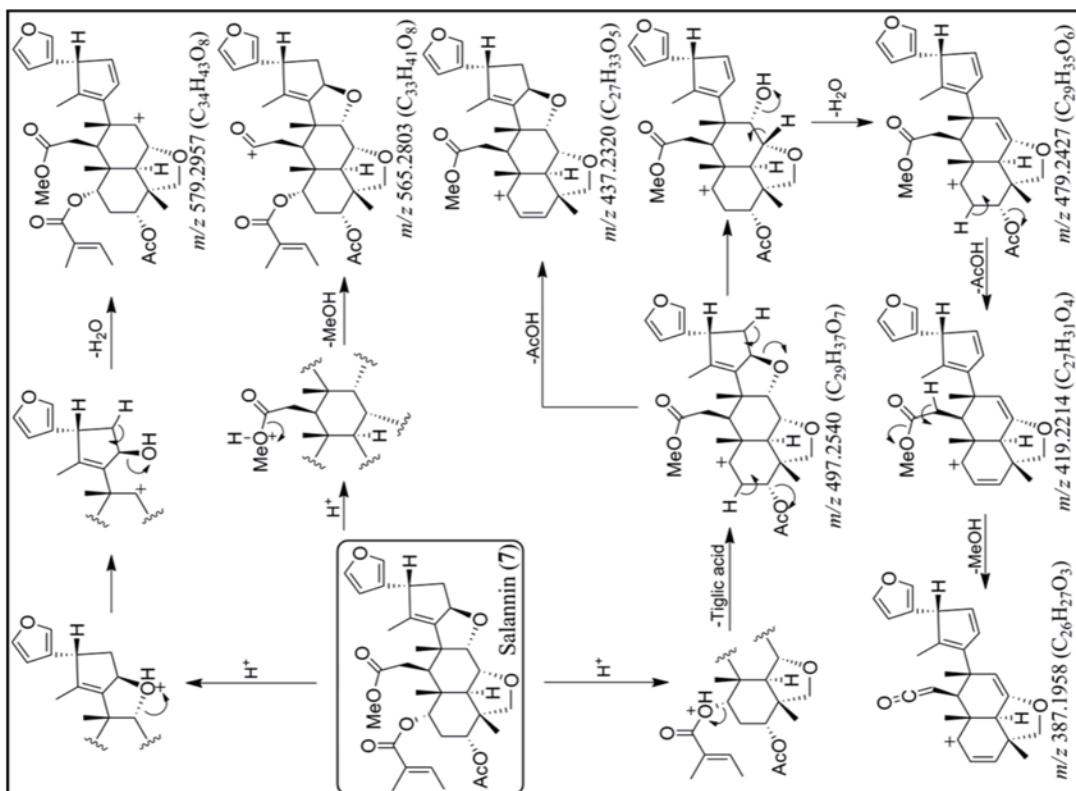


Figure 6 (A) Automated MPLC based protocol for the isolation of eight C-seco triterpenoids from Neem oil, **(B)** TLC profile of crude triterpenoid extract (lane 1, C) and purified triterpenoids (lane 2-9, triterpenoid 1-8).

Further, eight isolated C-seco triterpenoids of salannin type and five semi-synthetic derivatives were structurally characterized by the structure-fragment correlations of MS/MS daughter ions (generated from $[M+H]^+$ precursor ions) obtained through ESI(+)-quadrupole/orbitrap-MS/MS spectrometry with an optimized stepped normalized collision energy (NCE) of 20%. Structure-fragment relationships were predicted on the basis of chemical formulae obtained for each m/z values with high accuracy (error < 5.0 ppm) and their fragmentation pathway. MS/MS daughter ions were divided into two categories on the basis of fragmentation viewpoint; (i) high-mass fragments ($m/z > 300$) and (ii) low-mass fragments ($m/z < 300$). High-mass fragments were generated due to the loss of sensitive functional groups as neutral molecules. Low-



Scheme 1. Probable fragmentation pathway for low-mass MS/MS daughter ions ($m/z < 300$) of salannin (7).



Scheme 2. Probable fragmentation pathway for high-mass MS/MS daughter ions ($m/z > 300$) of salannin (7).

mass fragments were produced due to the C-C bond cleavages and rearrangements. High-mass fragments were important to recognize the functional groups arranged around the skeleton, whereas low-mass fragments can be utilized for the identification of skeletal framework of salannin type C-seco triterpenoids. To exemplify, Plausible mechanistic pathway leading to the formation of daughter ions ($[M+H]^+$ precursor ion) of salannin (7) has been schematically represented in scheme 1 and 2. To demonstrate the utility of developed MS/MS based characterization technique, reference spectra were utilized for the identification of studied triterpenoids in the extract of stem and bark tissues from Neem.

Chapter 3

Biocatalyst Mediated Functionalization of Neem Limonoids

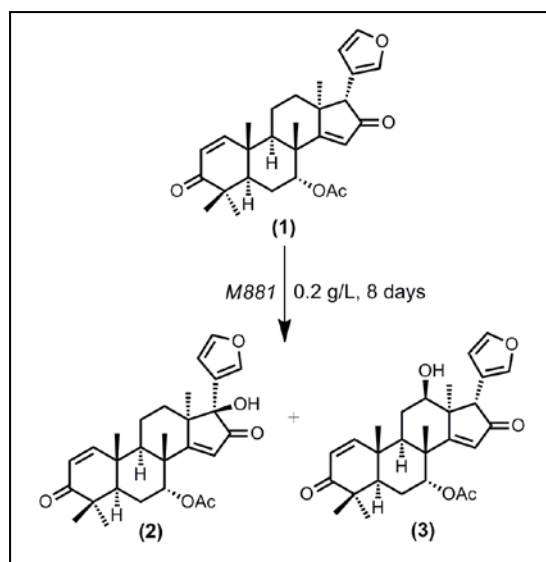
Section A: Biotransformation and Biocatalysis: an Introduction

Enzyme (isolated enzyme/whole cell) catalyzed transformation of chemical substances are collectively called as biotransformation and the phenomenon of catalysis is called biocatalysis. This introductory section describes the unrivalled power of ‘green’ biocatalysts over conventional chemical synthesis to achieve high stereo-, regio- and chemo-selectivity. It also provides a comparative discussion between the choice of isolated enzyme or whole cell as the biocatalyst, their scopes and obstacles. The application of biocatalysis in the field of organic chemistry for the production of bulk or fine chemicals has been demonstrated with real examples. Also, the biocatalyst mediated transformation of triterpenoids and Neem limonoids to generate structurally novel metabolites has been reviewed briefly.

Section A: Fungi Mediated Transformation of Basic Limonoids

Potent insecticidal activity and diverse pharmacological properties of basic limonoids have prompted us to study the biocatalyst mediated functionalization in search of structurally novel metabolites. Among various fungal systems screened, one of the *Mucor* sp. (NCIM catalogue no. 881 and abbreviated as *M881*) converted azadiradione efficiently to 17 β - and 12 β -hydroxy (~3:2) metabolites (Scheme 3). Time course study has indicated that *M881* transformed azadiradione quantitatively to its metabolites after 8 days of incubation period with 0.2 g/L substrate concentration (Figure 7). Preparative

scale fermentation was carried out for the isolation and characterization of the metabolites. Position and stereochemistry of the hydroxylation was elucidated by 1D-, 2D-NMR (COSY, NOESY, HSQC and HMBC) and HRMS studies.



Scheme 3 Biotransformation pathway for azadiradione (1) by *Mucor* sp. M881

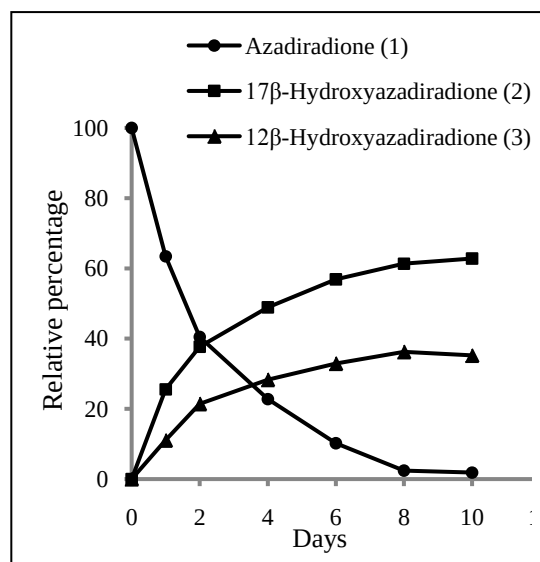


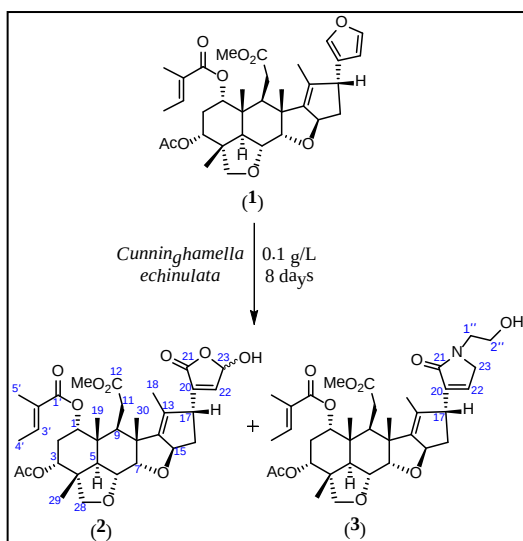
Figure 7 Time-course experiment with substrate concentration 0.2 g/L

To investigate the substrate specificity and effect of skeletal functional groups on the bioconversion product profile, another seven isolated/semi-synthesized derivatives of azadiradione was used as the substrate and their 12β- and/or 17β-hydroxylated metabolites were isolated and characterized. It was observed that cyclohexenone A ring was not critical for the bioconversion process. However, modifications in ring B and D could able to bring selectivity in product profile. For example, epoxyazadiradione and its derivatives furnished 12β-hydroxy metabolite selectivity and no trace of 17β-hydroxylation was observed. While screening with various substrates within basic limonoid family, rare and synthetically challenging 12β- and 17β-hydroxylation was achieved on the basic limonoid skeleton to produce a series of nine novel hydroxylated limonoids. Again, variation in whole cell biocatalyst was also found to bring selectivity in the ratio of metabolites. Some of the fungal systems belonging to the genera of *Mucor* and *Rhizopus* converted azadiradione selectively to 17β-hydroxy metabolite. Thus, modifications of specific functional groups and variation in biocatalyst were shown to bring selectivity among 12β- or 17β-hydroxylation. The fungal system mimicked the plant monooxygenase system for the production of 17β-hydroxyazadiradione which is one of the minor metabolites present in Neem fruit.

Moreover, whole cell mediated stereo- and regio-selective hydroxylation process was highly efficient and mild enough to sustain chemically sensitive functional groups around basic limonoid skeleton.

Section B: Fungi Mediated Functionalization of Salannin

Salannin (**1**), an insecticidal C-seco limonoid possesses a highly complex and multi-functional skeleton. Pronounced insecticidal activity and inefficiency of synthetic methods for selective modification have indulged us to study biocatalyst assisted functionalization of salannin. Among various fungal system screened, *Cunninghamella echinulata* (NCIM catalogue no. 691) was able to efficiently convert salannin to furan modified metabolites (Scheme 4). The major metabolite salanninolide (**2**) was γ -hydroxybutenolide analogue of C-17 furan and isolated previously from Neem seed extract. The minor metabolite was structurally novel and characterized as N-(2-hydroxyethyl)- α,β -unsaturated- γ -lactam analogue of furan moiety and named as salanninactam (**3**). Metabolites were characterized unambiguously by rigorous analyses of 1D-, 2D-NMR (COSY, NOESY, HSQC and HMBC), ESI(+)-HRMS and X-ray diffraction studies. Time course experiment revealed that *Cunninghamella echinulata* transformed salannin completely to its metabolites after 8 days of incubation period with 0.1 g/L substrate concentration (Figure 8). In previous studies salanninolide has been reported to be one of the photo-oxidized products of salannin. Herein, the fungal system selectively converted salannin to salanninolide as a major metabolite, thus indicating it as a metabolic product of limonoid biosynthetic pathway in Neem plant.



Scheme 4 Biotransformation of salannin (**1**) by *Cunninghamella echinulata*

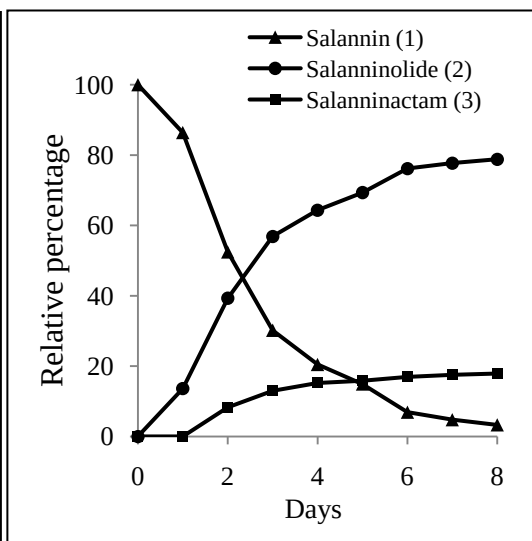


Figure 8 Time course experiment with substrate concentration: 0.1 g/L

Chapter 4

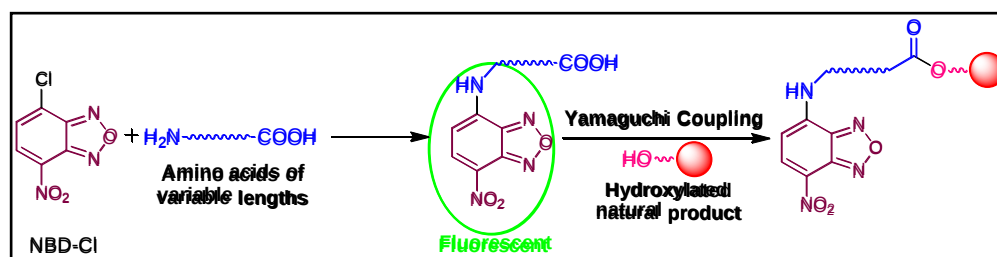
Fluorescent Labeling Protocol for Neem Limonoids and Cell-imaging Studies

Section A: Fluorescent Labeling and Cellular Imaging: an Introduction

Fluorescent labeling is the process of covalently attaching a fluorophore to the studied molecule to make it fluoresce. Qualitative or quantitative optical information can be obtained from fluorescently labeled molecules (fluorescent probes) through fluorescence detecting instruments. This technique is tremendously useful in natural product biology due to its high sensitivity in low concentration. Herein, the application of fluorescent labeling for the investigation of cellular phenomena (such as localization or interactions) associated with bioactive molecules has been demonstrated with real examples. Furthermore, commercially available tags for alcohols and reported protocols for the fluorescent labeling of complex organic molecules have been reviewed briefly.

Section B: One Pot Fluorescent Labeling Protocol for Complex Hydroxylated Bioactive Molecules and Cell-imaging Studies of Neem Limonoids

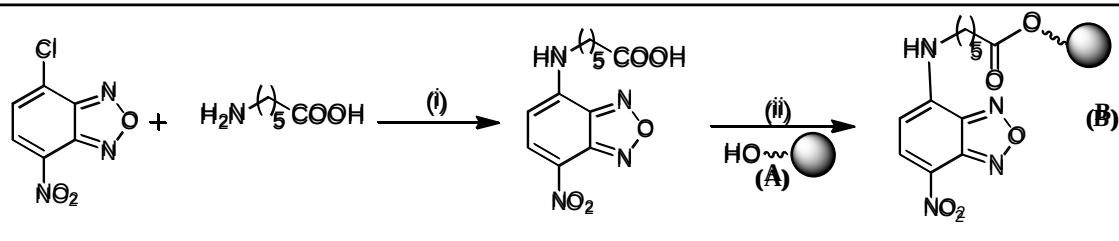
Reported protocols for the fluorescent labeling of small molecules are associated with several reaction steps along with protection-deprotection procedures. On the other hand, available commercial tags are expensive and require extreme conditions for labeling. These obstacles restrict the use of fluorescent labeling for the complex multi-functional and chemically sensitive molecules which are available in limited quantity (in milligram scale). In this study, 7-nitrobenzo-2-oxa-1,3-diazole (NBD) based one pot protocol for the fluorescent labeling of hydroxylated molecules have been developed with comprehensive demonstration of optimization and applicability of the protocol with complex bioactive molecules. Further, cellular imaging studies were performed using two NBD labeled qualified fluorescent probes of azadiradione within various cancer cell lines.



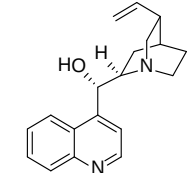
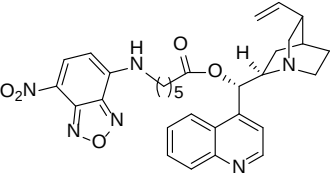
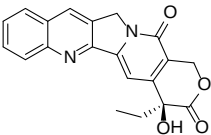
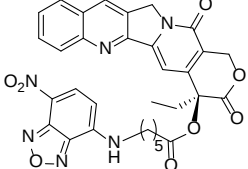
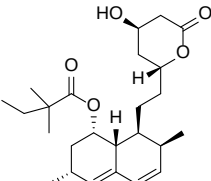
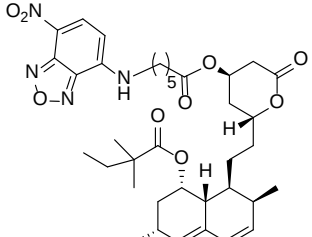
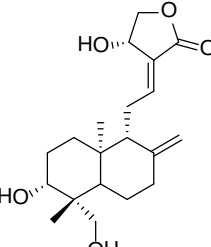
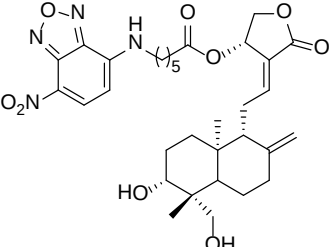
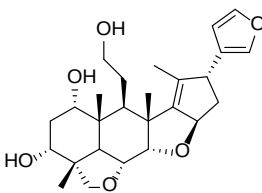
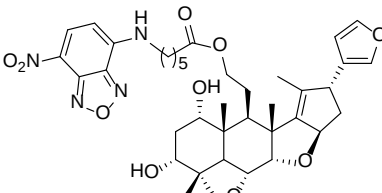
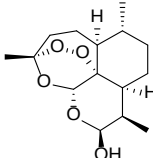
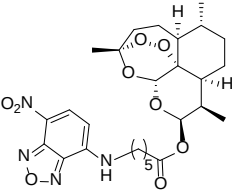
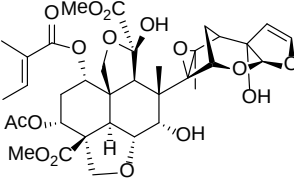
Scheme 5 One-pot protocol for the fluorescent labeling of hydroxylated bioactive molecules

Our protocol involves the aromatic nucleophilic substitution on NBD-Cl by amine functionality of amino acids in the initial step to generate fluorescent reagent followed by Yamaguchi coupling between free carboxylic end and hydroxylated bioactive molecule in the same vessel (Scheme 5). Among several common protocols available (DCC, EDC, DIC, Yamaguchi) for the coupling between carboxylic acid and alcohol, Yamaguchi was preferred on the basis of comparative kinetic results. In case of coupling between reduced azadiradione derivative (**1**) and NBD-6-aminocaproic acid by Yamaguchi, quantitative conversion was achieved after 30 min of reaction; whereas other coupling reactions such as DCC, EDC and DIC showed only 50-60% conversion even after 3 h. Prolonging the reaction time or increase in the mole percentage of coupling reagent didn't alter the progress of the reaction. The developed one pot protocol was flexible towards length of the linker joining the tag and studied molecule. Only by changing the amino acid in the initial step, length of the spacer can be manipulated with an ease. To demonstrate, **1** was labeled with three different fluorescent reagents obtained from three unlike amino acids. To investigate the efficiency of Yamaguchi coupling with respect to the nature of hydroxyl group, several structurally simple molecules bearing different kind of hydroxyl functionalities (including phenolic, primary, secondary, tertiary and allylic) were subjected to one pot protocol in presence of NBD-6-aminocaproic acid. It was observed that Yamaguchi coupling was highly efficient to furnish the coupled product in all instances with good to excellent yield except for tertiary. Further, applicability of the developed one pot protocol was studied with diverse complex and multi-functional hydroxylated bioactive molecules possessing chemically sensitive functional groups [nimbocinol (**2**), cholesterol (**3**), 11 α -hydroxyprogesterone (**4**), diosgenin (**5**), isoindolin skeleton (**6**), ezetimibe (**7**), cinchonine (**8**), camptothecin (**9**), simvastatin (**10**), andrographolide (**11**), salannin skeleton (**12**), β -dihydroartemisinin (**13**) and azadirachtin A (**14**)] (Table 2). Good to excellent yields were obtained in most cases except for the tertiary alcohols and molecules bearing multi-hydroxyl functionalities. In case of molecules with multi-hydroxyl groups the position of esterification was confirmed on the basis of 2D-NMR (HSQC and HMBC) studies. Although possessing three hydroxyl functionalities, Azadirachtin A (**14**) was not able to furnish any coupled product. An alternative approach was adopted by adding a small spacer (containing a hydroxyl group at the end) with azadirachtin skeleton followed by Yamaguchi coupling.

Table 2 Entries showing applicability of the developed one-pot protocol for fluorescent labeling of complex bioactive hydroxylated compounds. **Reagents and conditions:** (i) NaHCO₃, acetonitrile, water, 55 °C; (ii) 2,4,6- trichlorobenzoylchloride, TEA, DMAP, anhydrous THF.



Entry	(A)	(B)	Yield
1			87%
2			71%
3			96%
4			88%
5			94%
6			90%
7			52%

8		(8)		(8a)	82%
9		(9)		(9a)	09%
10		(10)		(10a)	81%
11		(11)		(11a)	33%
12		(12)		(12a)	93%
13		(13)		(13a)	71%
14		(14)	No reaction		—

To assess the applicability of NBD tagged bioactive molecules, two azadiradione derivatives (**1** and **2**) and their corresponding labeled analogues (**1a** and **2a**) were evaluated for cytotoxic activity against several cancer cell lines. Potency of their cytotoxic activity was monitored by cancer cell viability (MTT assay) and motility

assay (Wound migration assay). Both the analogues (**1a** and **2a**) were found to possess competitive cytotoxicity in comparison to parent molecules and considered as qualified fluorescent probes. Further, they were imaged successfully within cancer cell-lines to investigate cellular uptake and localization (Figure 9). Sub-cellular (mitochondrial and endoplasmic reticulum) localization of the fluorescent probes was investigated by colocalization studies.

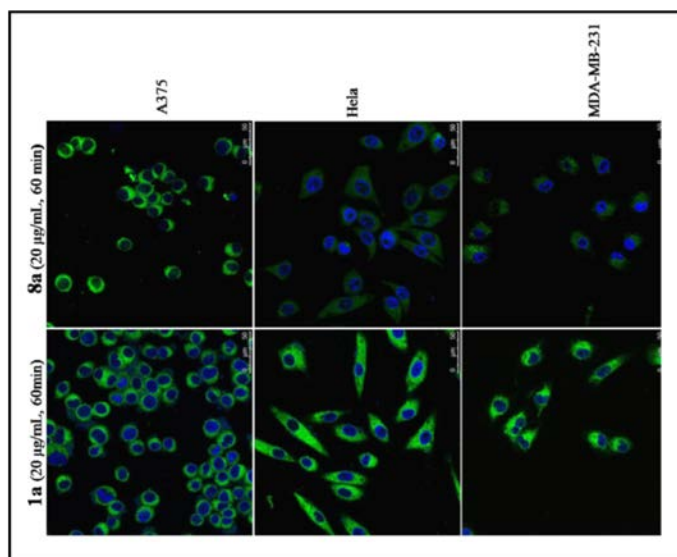
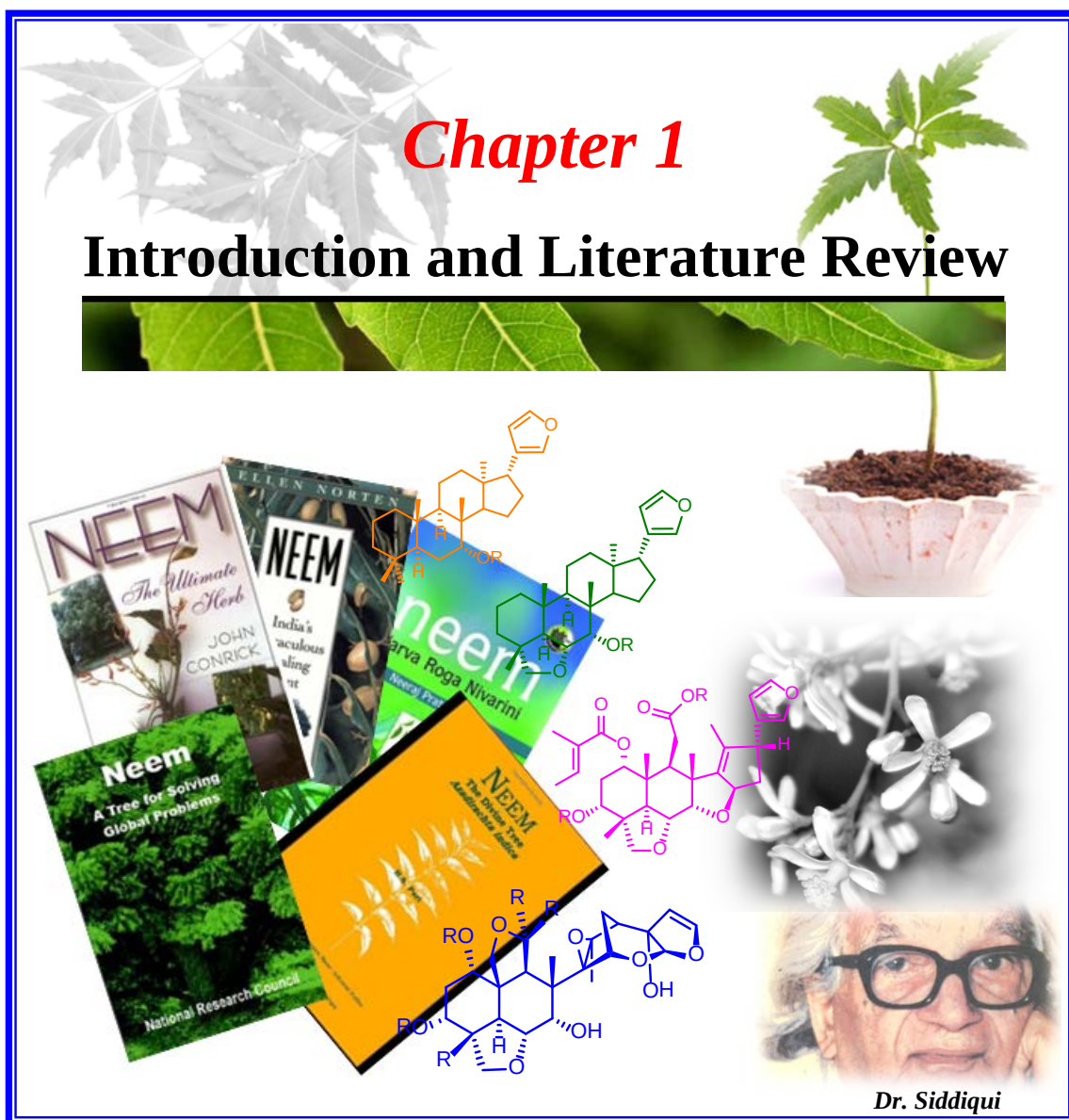


Figure 9 Cancer cells (A375, Hela and MDA-MB-231) were treated (20 µg/mL) with NBD labeled limonoids (**1a** and **8a**) for 1 h and analyzed under confocal microscope. Green: compounds labeled with NBD, Blue: Nucleus staining with DAPI. Micron bar: 50 µm.



Abstract

This chapter describes the blessings of Neem plant on mankind and structural diversity in its secondary metabolites especially limonoids. Wide spectrum of biological activities of Neem limonoids has been reviewed briefly. Further, plausible biosynthetic pathway, isolation techniques and synthetic routes developed till date have been discussed.

1.1 Neem: Nature's Gift to Mankind

Azadirachta indica (Neem, Margosa or Indian lilac), native to Indian subcontinent is well-known for its diverse medicinal uses since more than 2000 years.¹⁻³ 'Arishtha' (i.e. reliever of sickness), the 'Sanskrit' name of Neem itself reflects its usefulness and popularity in ancient times. A number of benefits have been documented in ancient Indian scripts such as 'Charak-Samhita' and 'Sushruta-Samhita'. Over 700 herbal preparations based on neem have been recognized in traditional health guides such as Ayurveda, Siddha, Unani and over 160 local practices are known in different parts of the country in which neem contributes as a key or the sole ingredient for curing sickness and disorders.⁴ Even in the modern times, the saying: 'Nimbati ivasthyamdadati' (i.e. Neem for good health), plays like a cradle to grave healthcare 'mantra' in the villages of India and neighbouring countries. All the parts of this evergreen tree have been used as traditional medicines in day-to-day household remedies and in ayurveda. In addition to its therapeutic potential, Neem is being widely used as eco-friendly commercial pesticides and agrochemicals.^{3,5} The past, present and future prospect of Neem tree has been recognized in a report 'Neem-A Tree for Solving Global Problems' published by USA National Academy of Sciences in 1992.⁶ Several review articles describing its therapeutic applications have been published in last decade reflecting its importance as a medicinal plant.^{5,7-12}

1.2 Neem: Origin, Distribution and Taxonomy

Neem is a fast-growing tree in tropical and semi-tropical region with a usual height of 15-25 meters. Bitterness is known characteristic of Neem and almost all the parts usually taste pungently bitter. The 'wonder tree' Neem is believed to be originated from Asian continent especially from the South and South-Eastern countries including India, Pakistan, Bangladesh, Myanmar, Thailand and Indonesia. Neem tree is considered to be so valuable that it has become an essential part of Indian ecosystem. Later, it was migrated to African countries such as Ghana, Nigeria, Cameroon, Sudan, Ethiopia, Somalia, Kenya by a person named 'Dogon Yaro' in 1928 (since then it is familiar as 'Dogonyaro tree' in northern Nigeria).⁹ Neem tree has been planted extensively to control the curse of desertification on the dry landscape of Nigeria around 1980.⁹ Neem plant has been scientifically named as *Azadirachta indica* in 1830 by De

Jussieu.¹³ The taxonomic position of Neem is as follows:

Order	Rutales
Suborder	Rutinae
Family	Meliaceae (Mahogany)
Subfamily	Melioideae
Tribe	Melieae
Genus	<i>Azadirachta</i>
Species	<i>indica</i>

Azadirachta indica and *Melia azedarach* (Chinaberry or Persian lilac) are two members of Meliaceae family which are very similar in appearance. Two species of *Azadirachta* are well known in Asian continent; *Azadirachta indica* (Indian subcontinent) and *Azadirachta excelsa* (South-East Asian subcontinent).⁷ India stands first in the production of Neem seeds worldwide with approximate annual estimation of 4,42,300 tons among which ~80% grows in Uttar Pradesh, Tamilnadu and Karnataka.¹²

1.3 Neem: Richest Source of Triterpenoids

Neem tree is one of the richest sources of secondary metabolites in nature. After the isolation of nimbin in 1942 by Siddiqui,¹⁴ more than 250 natural products (Diterpenoids, Triterpenoids, Steroids, Flavonoids, Coumarins, Hydrocarbons, Fatty acids etc.) have been characterized from various parts of Neem. Among them triterpenoid constitutes the major group and counts around 150 (Table 1.1). Triterpenoids present in Neem are highly oxygenated, rearranged, complex and skeletally diverse (Figure 1.1). They have been found to be majorly responsible for the diverse biological activities exhibited by the extracts of different Neem parts. Considering the skeletal diversity, Neem triterpenoids can be classified into various groups:

(A) Proto and apo-protolimonoids: They contain intact eight carbon side chain at C-17 of either butyrospermol (proto) or 4,4,8-trimethylsteroidal (apo-proto) skeleton. They are believed to be the biogenetic precursors of limonoids.

(B) Tetranortriterpenoids or Limonoids: Limonoids are structurally characterized as 4,4,8-trimethyl-17-furanyl steroid skeleton (Figure 1.1), derived by the loss of four

carbon unit from the eight carbon side chain ('tetranor-') at C-17 or further rearranged and functionalised triterpenoids. They are the major class of terpenoids abundant in Neem. Limonoids can be sub-divided into two categories:

(1) Ring Intact / Basic Limonoids: Limonoids, carrying intact A, B, C, D rings belong to this group. They can further be classified according to the skeletal modifications as follows:

- (i) Azadirone Skeleton
- (ii) Gedunin Skeleton
- (iii) Vilasinin Skeleton
- (iv) Ring-intact Butenolide Skeleton
- (v) Miscellaneous Ring-intact skeleton

(2) C-Seco Limonoids: C-ring of ring intact limonoids rearranges (cut and open *i.e.* 'seco') further to form C-seco limonoids. According to the skeletal diversity they can be classified as:

- (i) Nimbin Skeleton
- (ii) Salannin Skeleton
- (iii) Nimbolide Skeleton
- (iv) Azadirachtin Skeleton
- (v) Azadirachtinin Skeleton
- (vi) Nimbilin Skeleton
- (vii) C-Seco Butenolide Skeleton

(C) Pentanortriterpenoids: Triterpenoids, derived by the collective degradation of five carbon units ('pentanor') from proto or apo-proto skeleton (either from eight carbon side chain at C-17 or additionally from other skeletal region) are called as pentanortriterpenoids.

(D) Octanortriterpenoids: This class of triterpenoid is formed by the degradation of complete eight carbon side chain ('octanor') from protolimonoid structure resulting in un-substituted C-17.

(E) Nonanortriterpenoids: They are the nine carbon degraded ('nonanor') structure of triterpenoid. Eight carbon side chain along with one additional carbon from ring D removes to form this specific class of terpenoids.

(F) Mono, Di, Tri and Hexanortriterpenoids: They are structurally characterized by the presence of seven ('mononor'), six ('dinor'), five ('trinor') and two ('hexanor') carbon side chain respectively. Triterpenoids of these categories are found to be least abundant from Neem sources.

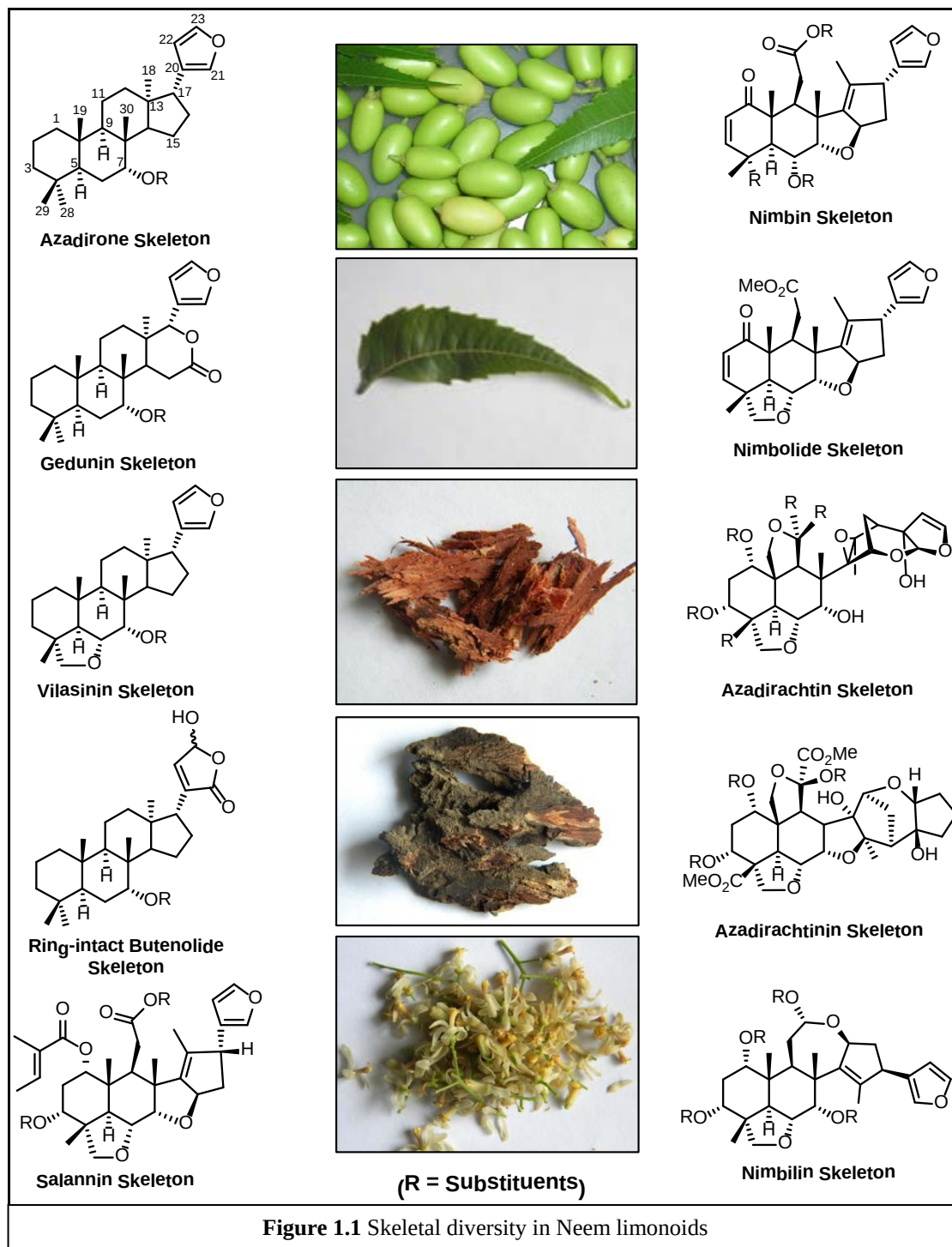
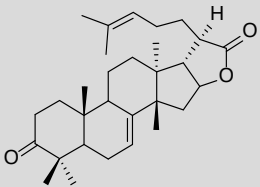
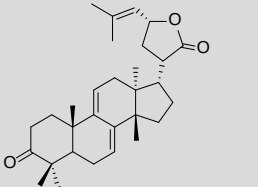
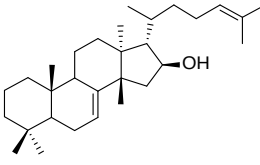
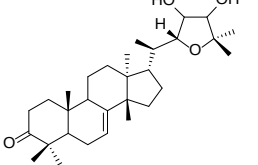
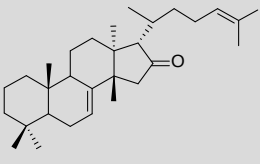
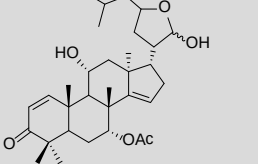
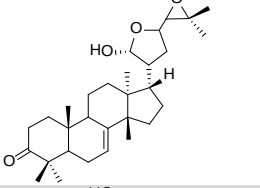
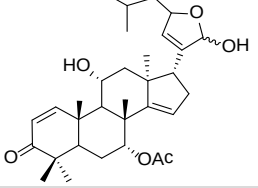
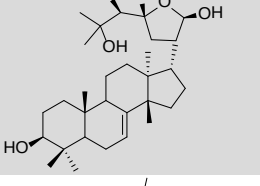
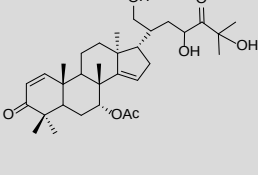
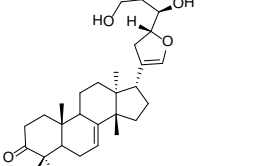
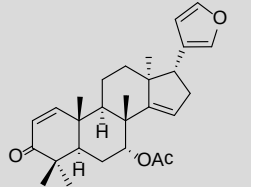
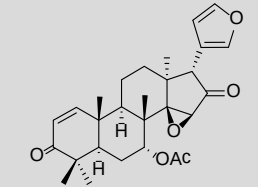
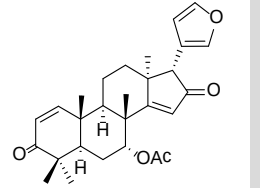
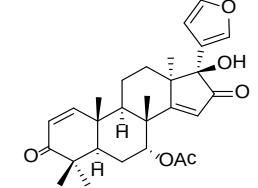
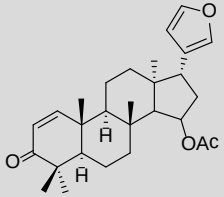
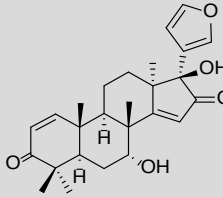
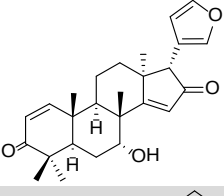
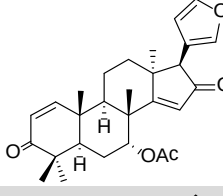
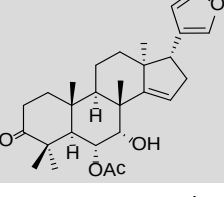
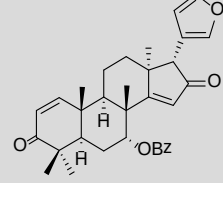
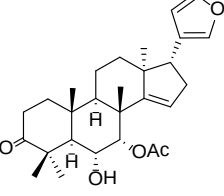
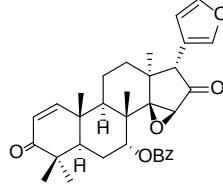
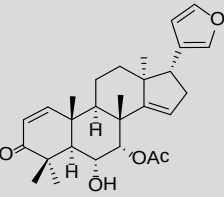
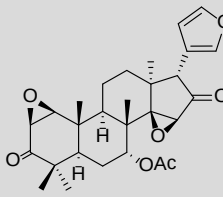
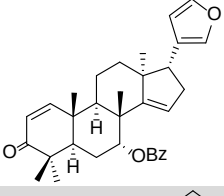
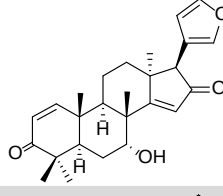
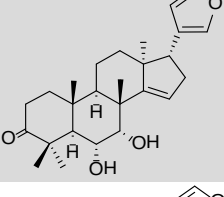
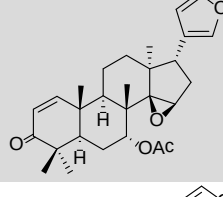
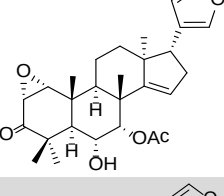
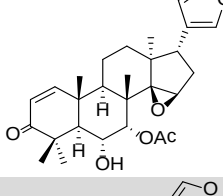
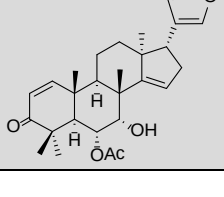
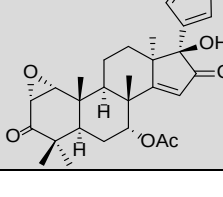


Table 1.1 Structure of triterpenoids isolated from *Azadirachta indica* (Neem)

No	Name (Molecular formula)	Structure	No	Name (Molecular formula)	Structure
(A) PROTO & APO-PROTOLIMONOIDS					
1.	Kulactone (C ₃₀ H ₄₄ O ₃) ¹⁵		2.	Nimolinone (C ₃₀ H ₄₂ O ₃) ¹⁶	
3.	Limocinol (C ₃₀ H ₅₀ O) ¹⁷		4.	Odoratone (C ₃₀ H ₄₈ O ₄) ¹⁸	
5.	Limocinone (C ₃₀ H ₄₈ O) ¹⁷		6.	Naheedin/ Azadirachnol (C ₃₂ H ₄₈ O ₆) ¹⁹	
7.	Melianone (C ₃₀ H ₄₆ O ₄) ²⁰		8.	Azadirachtol (C ₃₂ H ₄₆ O ₆) ²¹	
9.	Meliantriol (C ₃₀ H ₅₀ O ₅) ²²		10.	Azadirol (C ₃₂ H ₄₈ O ₇) ¹⁵	
11.	Nimbocinone (C ₃₀ H ₄₆ O ₄) ²³				

(B) TETRANORTRITERPENOID/LIMONOID: (1) Ring-intact Limonoids
(i) Azadirone Skeleton

12.	Azadirone (C ₂₈ H ₃₆ O ₄) ²⁴		13.	Epoxyazadiradione/ Nimbinin/ Nimolin (C ₂₈ H ₃₄ O ₆) ²⁴	
14.	Azadiradione/ Nimolicin (C ₂₈ H ₃₄ O ₅) ²⁴		15.	17β-Hydroxy azadiradione (C ₂₈ H ₃₄ O ₆) ²⁵	

16. Neeflone (C ₂₈ H ₃₈ O ₄) ²⁶		17. 17β-Hydroxy nimocinol (C ₂₆ H ₃₂ O ₅) ²⁷	
18. Nimocinol (C ₂₆ H ₃₂ O ₄) ²⁸		19. 17-Epiazadiradione (C ₂₈ H ₃₄ O ₅) ²⁹	
20. Meldenin (C ₂₈ H ₃₈ O ₅) ³⁰		21. 7α-Benzoyl Azadiradione (C ₃₃ H ₃₆ O ₅) ³¹	
22. Isomeldenin (C ₂₈ H ₃₈ O ₅) ³²		23. 7α-Benzoyl Epoxyazadiradione (C ₃₃ H ₃₆ O ₆) ³¹	
24. Nimocinol/ Nimanol (C ₂₈ H ₃₆ O ₅) ³²		25. 1β,2β-14β,15β-Di epoxyazadiradione (C ₂₈ H ₃₄ O ₇) ³¹	
26. Nimocin (C ₃₃ H ₃₈ O ₄) ³³		27. 17-Epi-nimocinol (C ₂₆ H ₃₂ O ₄) ³⁴	
28. Meldenindiol (C ₂₆ H ₃₆ O ₄) ³⁵		29. Trichilinone acetate (C ₂₈ H ₃₆ O ₅) ³⁶	
30. 1α,2α-Epoxy nimocinol (C ₂₈ H ₃₆ O ₆) ³⁷		31. 14,15-Epoxy Nimocinol (C ₂₈ H ₃₆ O ₆) ³⁸	
32. 6α-O-Acetyl- 7-deacetyl- nimocinol (C ₂₈ H ₃₆ O ₅) ³⁹		33. 1α,2α-Epoxy- 17β-hydroxy azadiradione (C ₂₈ H ₃₄ O ₇) ³⁷	

(ii) Gedunin Skeleton

34. Gedunin (C ₂₈ H ₃₄ O ₇) ⁴⁰		35. 6β-Hydroxygedunin (C ₂₈ H ₃₄ O ₈) ⁴¹	
36. Nimolicinol (C ₂₈ H ₃₄ O ₇) ³⁷		37. 7-Deacetyl-7α-benzoylgedunin (C ₃₃ H ₃₆ O ₇) ³¹	
38. Mahmoodin (C ₃₀ H ₃₈ O ₈) ⁴²		39. 7α-Deacetylgedunin (C ₂₆ H ₃₂ O ₆) ⁴⁰	
40. Azadirinin (C ₃₉ H ₄₆ O ₁₀) ⁴³		41. 1α,2α-Epoxy Nimolicinol (C ₂₈ H ₃₄ O ₈) ³⁷	
42. 7α-Deacetylnimolicinol (C ₂₆ H ₃₂ O ₆) ³⁷			

(iii) Vilasinin Skeleton

43. Vilasinin (C ₂₆ H ₃₆ O ₅) ⁴⁴		44. Vilasinin triacetate (C ₃₂ H ₄₂ O ₈) ⁴⁴	
45. Nimbidinin (C ₂₆ H ₃₄ O ₆) ⁴⁵		46. Meliacinol (C ₃₂ H ₄₂ O ₈) ³⁹	
47. Nimbolin A (C ₃₉ H ₄₆ O ₈) ⁴⁶		48. 1,3-Diacetyl vilasinin (C ₃₀ H ₄₀ O ₇) ⁴⁷	
49. 1-Acetyl-7-tigloyl vilasinin (C ₃₃ H ₄₄ O ₇) ⁴⁸		50. 1,3-Diacetyl-7-tigloyl-12α-hydroxyvilasinin (C ₃₅ H ₄₆ O ₉) ⁴⁹	

(iv) Ring-Intact Butenolide Skeleton

51. Azadironolide (C ₂₈ H ₃₆ O ₆) ⁵⁰		52. Azadiradionolide (C ₂₈ H ₃₄ O ₆) ⁵⁰	
53. Nimocinolide (C ₂₈ H ₃₆ O ₇) ³³		54. Isonimbocinolide (C ₃₂ H ₄₂ O ₁₀) ⁵¹	
55. Isonimbolide (C ₃₀ H ₃₈ O ₉) ⁵²		56. Isonimolicinolide (C ₃₀ H ₃₆ O ₉) ⁵³	
57. Isonimolide (C ₂₉ H ₃₈ O ₇) ⁵²		58. Nimbocinolide (C ₃₂ H ₄₂ O ₁₀) ⁵⁴	
59. 23-O-Methyl nimocinolide (C ₂₉ H ₃₈ O ₇) ⁵⁵		60. Isoazadironolide (C ₂₈ H ₃₆ O ₆) ⁵⁰	
61. 7α-Senecioid-7-deacetyl-23-O-methyl nimocinolide (C ₃₂ H ₄₂ O ₇) ⁵⁵		62. 23-O-Methyl azadironolide (C ₂₉ H ₃₈ O ₆) ³⁶	

(v) Miscellaneous Ring-Intact Skeleton

63. Limocin A (C ₂₉ H ₄₂ O ₅) ¹⁷		64. Azadirachtolide (C ₃₃ H ₄₆ O ₈) ⁵⁶	
65. Limocin B (C ₂₉ H ₄₂ O ₅) ¹⁷		66. Deoxy azadirachtolide (C ₃₃ H ₄₈ O ₇) ⁵⁶	

67.	Limocin C (C ₃₀ H ₄₄ O ₅) ⁵⁷	
68.	22,23-Dihydronimocinol (C ₂₈ H ₃₈ O ₅) ⁵⁸	
69.	Limocin D (C ₃₀ H ₄₄ O ₅) ⁵⁷	
70.	Meliacinanhydride (C ₃₁ H ₃₈ O ₁₀) ⁵⁹	
71.	Limocinin (C ₃₄ H ₄₄ O ₆) ¹⁷	
72.	Azadirachtanin (C ₃₂ H ₄₀ O ₁₁) ⁶⁰	
73.	Vepinin (C ₂₈ H ₃₆ O ₅) ⁶¹	
74.	Meliatetraolenone (C ₃₂ H ₄₂ O ₁₀) ¹⁸	
75.	Salimuzzalin (C ₃₀ H ₄₀ O ₇) ⁵⁹	
76.	Azadironic acid (C ₂₈ H ₃₈ O ₅) ⁶²	

(2) C-Seco limonoids

(i) Nimbin Skeleton

77.	Nimbin (C ₃₀ H ₃₆ O ₉) ⁶³	
78.	6-Deacetylnimbin (C ₂₈ H ₃₄ O ₈) ⁶³	
79.	Nimbanal (C ₂₉ H ₃₄ O ₈) ⁶³	
80.	6-Deacetylnimbanal (C ₂₇ H ₃₂ O ₇) ⁶³	
81.	Nimbinol (C ₂₉ H ₃₆ O ₈) ⁶³	
82.	4-Epi-nimbin (C ₃₀ H ₃₆ O ₉) ⁶⁴	

(ii) Salannin Skeleton

83.	Salannin (C ₃₄ H ₄₄ O ₉) ⁶⁵		84.	6-Deacetylsalannin (C ₃₂ H ₄₂ O ₈) ⁴⁷	
85.	Salannol (C ₃₂ H ₄₄ O ₈) ⁶⁶		86.	2',3'-Dehydrosalannol (C ₃₂ H ₄₂ O ₈) ⁶⁷	
87.	Salannol acetate (C ₃₄ H ₄₆ O ₉) ⁶⁶		88.	Nimbic acid (C ₂₆ H ₃₄ O ₇) ⁴⁵	

(iii) Nimbolide Skeleton

89.	Nimbolide (C ₂₇ H ₃₀ O ₇) ⁶³		90.	28-Deoxonimbolide (C ₂₇ H ₃₂ O ₆) ⁶³	
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(iv) Azadirachtin Skeleton

91.	Azadirachtin A (C ₃₅ H ₄₄ O ₁₆) ⁶⁸⁻⁷⁰		92.	Azadirachtin B/3-Tigloyl azadirachtol (C ₃₃ H ₄₂ O ₁₄) ⁷¹	
93.	Azadirachtin D (C ₃₄ H ₄₄ O ₁₄) ⁷²		94.	Azadirachtin E (C ₃₀ H ₃₈ O ₁₅) ⁷³	
95.	Azadirachtin I (C ₃₂ H ₄₂ O ₁₂) ⁷⁴		96.	Azadirachtin F (C ₃₂ H ₄₄ O ₁₂) ⁷⁴	
97.	Azadirachtin H (C ₃₃ H ₄₂ O ₁₄) ⁷⁴		98.	Azadirachtin G (C ₃₃ H ₄₂ O ₁₄) ⁷⁵	
99.	Azadirachtin K (C ₃₄ H ₄₀ O ₁₅) ⁷⁶		100.	Azadirachtin M (C ₃₂ H ₄₂ O ₁₃) ⁷⁷	

101.	11-Epi-azadirachtin H (C ₃₃ H ₄₂ O ₁₄) ⁷⁸		102.	13,14-desepoxy azadirachtin A (C ₃₅ H ₄₄ O ₁₅) ⁷⁹	
103.	Azadirachtol (C ₂₈ H ₃₆ O ₁₃) ⁷⁰		104.	3-Decetyl-11-desoxy Azadirachtin (C ₃₃ H ₄₂ O ₁₄) ⁸⁰	
105.	Vepaol (C ₃₆ H ₄₈ O ₁₇) ⁴⁰		106.	3-Deacetyl-3-cinnamoyl azadirachtin (C ₄₂ H ₄₈ O ₁₆) ⁶⁹	
107.	Isovepaol (C ₃₆ H ₄₈ O ₁₇) ⁴⁰		108.	3α-Acetoxy-1α-hydroxy azadirachtol (C ₃₀ H ₃₈ O ₁₄) ⁷⁵	

(v) Azadirachtinin Skeleton

109.	1-Tigloyl-3-acetyl Azadirachtinin (C ₃₅ H ₄₄ O ₁₅) ⁸¹		110.	Azadirachtin N (C ₃₃ H ₄₄ O ₁₅) ⁷⁷	
111.	1-Tigloyl-3-acetyl methoxy Azadirachtinin (C ₃₆ H ₄₆ O ₁₅) ⁶⁹		112.	3-Tigloyl azadirachtinin (C ₃₃ H ₄₂ O ₁₄) ⁸¹	

(vi) Nimbilin Skeleton

113.	Nimbinin (C ₄₂ H ₅₀ O ₁₀) ⁸²		114.	Nimbolicin (C ₄₁ H ₄₈ O ₁₀) ⁸³	
115.	Nimbolin B (C ₃₉ H ₄₆ O ₁₀) ⁸³		116.	Ochinolide B (C ₃₅ H ₄₄ O ₁₀) ⁷⁶	

(vii) C-Seco Butenolide Skeleton

117.	Salanninolide (C ₃₄ H ₄₄ O ₁₁) ⁸⁴		118.	Isosalanninolide (C ₃₄ H ₄₄ O ₁₁) ⁸⁴	
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119.	Margosinolide (C ₂₇ H ₃₂ O ₈) ⁸⁵		120.	Isoazadirolide (C ₃₂ H ₄₂ O ₁₀) ⁸⁶	
121.	6-Deacetyl nimbinolide (C ₂₈ H ₃₄ O ₁₀) ⁸⁷		122.	Isomargosinolide (C ₂₇ H ₃₂ O ₈) ⁸⁵	
123.	6-Deacetyl isonimbinolide (C ₂₈ H ₃₄ O ₁₀) ⁸⁷		124.	Isonimbinolide (C ₃₀ H ₃₆ O ₁₁) ⁸⁸	
125.	Salannolactam- 21 (C ₃₄ H ₄₅ NO ₉) ⁸⁹		126.	Salannolactam-23 (C ₃₄ H ₄₅ NO ₉) ⁸⁹	

(C) PENTANORTRITERPENOIDS

127.	Nimbandiol (C ₂₆ H ₃₂ O ₇) ⁹⁰		128.	6-Acetyl nimbandiol (C ₂₈ H ₃₄ O ₈) ⁹⁰	
129.	6-Deacetyl nimbinene (C ₂₆ H ₃₂ O ₆) ⁹⁰		130.	Nimbinene (C ₂₈ H ₃₄ O ₇) ⁹⁰	

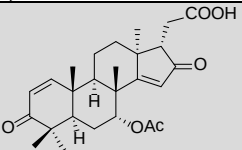
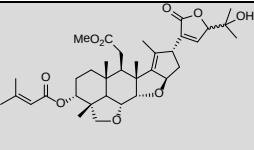
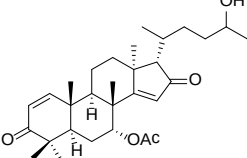
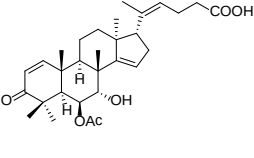
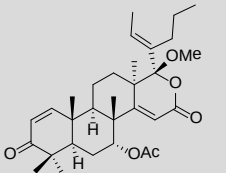
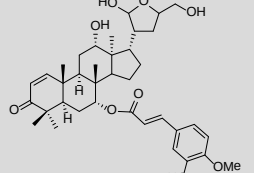
(D) OCTANORTRITERPENOIDS

131.	Azadironol (C ₃₂ H ₄₂ O ₆) ⁹¹		132.	Desfurano-6 α - hydroxy Azadiradione (C ₂₄ H ₃₂ O ₅) ⁵⁸	
133.	Desfurano azadiradione (C ₂₄ H ₃₂ O ₄) ¹⁹		134.	Desfurano- deacetyl-nimbin- 17-one (C ₂₄ H ₃₀ O ₈) ⁹²	

(C) NONANORTRITERPENOIDS

135.	β -Nimolactone (C ₂₃ H ₃₀ O ₅) ¹⁹		136.	α -Nimolactone (C ₂₃ H ₃₀ O ₅) ¹⁹	
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(C) MONO-, DI-, TRI-, HEXA-NORTRITERPENOIDS

137.	Nimolicinoic acid (C ₂₆ H ₃₄ O ₆) ⁵³		138.	Melianol (C ₃₅ H ₄₈ O ₉) ⁹²	
139.	Azadiradionol (C ₃₀ H ₄₄ O ₅) ⁹¹		140.	Azadirolic acid (C ₃₀ H ₄₂ O ₆) ⁹¹	
141.	Meliacinin (C ₃₁ H ₄₄ O ₆) ⁶²		142.	Meliacinolactol (C ₃₇ H ₅₀ O ₉) ⁵⁷	

1.4 Neem Limonoids: Insecticidal and Medicinal Activities

Investigation on limonoids started in the decade of 1950, when researchers were looking for the chemicals responsible for the bitterness of citrus juices. The first limonoid isolated was limonin from which the term 'limonoid' was derived and it was found to be responsible for the bitterness of navel orange juice in 1949.⁹³ But, limonoids came into real picture when they were found to possess potent anti-feedant and growth regulating activity against pests, in specific azadirachtin A (isolated by Morgan in 1968)⁹⁴, its derivatives and other C-seco limonoids.⁵ Later, they were demonstrated to exhibit a wide range of medicinal and pharmacological activities such as anti-inflammatory, anti-carcinogenic, anti-malarial, anti-ulcer, anti-microbial and hypoglycaemic.^{9,35,95,96}

1.4.1 Agricultural Application: Insecticidal Activity

Neem limonoids have been globally accepted as the natural pesticides and of great importance due to their ability to act against a broad range of pests without ecological disturbance.⁵ The mode of action of Neem insecticides is in contrast with traditional chemical pesticides which kills the insects as an immediate effect. Instead, it attacks systematically on each stages of the life cycle through oral feeding which results in anti-feedancy, growth retardation, abnormal bodily development, sterility and toxicity towards insects. Surprisingly, it does not harm those beneficial insects and

microorganisms which are not dependent on plants for their food. Selective toxicity, activity at low concentration, inactivity against mammals and beneficial organisms, biodegradability or non-persistence in the environment and economic compatibility has made these Neem based pesticides highly suitable for integrated pest management.^{70,95} There are a large number of commercial Neem based pesticides marketed in different parts of the world namely Margosan-O, Neemix, Azatin, NeemAzal and so on.¹¹ The term 'azadirachtin' has been coined for the insecticidal Neem extracts carrying azadirachtin A as the principal component along with other natural limonoids.¹¹

● Insect Antifeedant Activity

A feeding deterrent or an antifeedant molecule is defined as a chemical which inhibits feeding, though not able to kill the insect directly. The insect often dies through starvation as an indirect effect of anti-feedancy.⁹⁷ The most fascinating biological activity of limonoids was observed to be anti-feedancy on insects and their potency as anti-feedant molecules have been investigated widely on numerous insects.⁵

The most potent anti-feedant limonoid azadirachtin A is known to affect more than 600 species of insects and it was found to be active at 1-10 ppm.¹¹ A number of detailed reviews have been published describing its potency as antifeedant molecule against miscellaneous insects.^{5,96,98,99} Its anti-feedant efficacy on some of the insects has been listed in Table 1.2. For example, it showed dose dependent neural and anti-feedant behavioural response towards insects such as *S. littoralis*, *S. gregaria* and *L. migratoria*.⁹⁹ The pathological effects observed on these cases were due to the dose dependent reduction in intake of food. Azadirachtin B was found to be more or less comparable in anti-feedant potency compared to azadirachtin A in several cases.^{100,101} Azadirachtin A possesses higher deterrent activity in comparison to other non-azadirachtin limonoids such as salannin, 17 β -hydroxyazadiradione, 6-deacetylnimbin, gedunin and 7-deacetylgedunin from Neem against *H. armigera* and resulted in ~100% larval mortality at 1 ppm concentration.^{102,103} However, these non-azadirachtin limonoids affected feeding, development and reproduction in *H. armigera*. According to Govindachari *et al.* C-seco limonoids are more effective as anti-feedant agent towards pest in comparison to ring-intact limonoids.¹⁰⁴ The probable cause of anti-feedancy was shown to be interaction of limonoids with deterrent receptors and chemoreceptors.^{105,106}

Table 1.2 Anti-feedant potency of some Neem limonoids on various insects³⁵

Limonoid	Insect	Anti-feedant potency
Azadirachtin A	<i>Epilachna varivesti</i>	EC ₅₀ = 13 ppm; EC ₁₀₀ = 120 ppm
	<i>Spodoptera littoralis</i>	AI = 98.8 ± 1.11 (1 ppm), 100.0 ± 0.00 (10 ppm)
	<i>Helicoverpa armigera</i>	EC ₅₀ = 0.26 ppm (for neonates), 0.4
	<i>L. migratoria</i>	MIC = 25 ppm
	<i>L. migratoria</i>	ED ₅₀ = 3 ppm
	<i>Ostrinia nubilalis</i>	PC ₅₀ = 3.5 ppm (for neonate larvae), 24 ppm (for 3 rd instar larvae)
	<i>Peridroma saucia</i>	EC ₅₀ = 0.26 ppm
	<i>Pieris rapae</i>	AR = 100 (1000 ppm)
	<i>Phyllotreta striolata</i>	MIC = 10 ppm
	<i>Reticulitermes speratus</i>	PC ₉₅ = 65.293
	<i>Rhodnius prolixus</i>	ED ₅₀ = 25.0 µg/mL
	<i>Schistocerca gregaria</i>	ED ₅₀ = 0.001 ppm
Azadirachtin B	<i>Epilachna varivesti</i>	EC ₅₀ = 13 ppm; EC ₁₀₀ = 120 ppm
	<i>Epilachna varivesti</i>	EC ₅₀ = 30 ppm; EC ₁₀₀ = 150 ppm
	<i>Schistocerca gregaria</i>	ED ₅₀ = 80 µg/l
	<i>Locusta migratoria</i>	ED ₅₀ = 12 mg/L
Salannin	<i>Spodoptera frugiperda</i>	ED ₅₀ = 13 µg/cm ²
	<i>Reticulitermes speratus</i>	PC ₉₅ = 203.3 µg/disk
	<i>S. exigua</i> ; <i>S. eridania</i>	MAC = 1000 ppm
Azadirone	<i>Leptinotarsa decemlineata</i>	AI = 11.6 ± 6.3 (100 ppm), 22.4 ± 7.4 (300 ppm), 26.9 ± 5.1 (500 ppm)
Azadiradione	<i>Reticulitermes speratus</i>	PC ₉₅ = 827.5 µg/disk
	<i>Heliothis virescens</i>	EC ₅₀ = 560 ppm
Gedunin	<i>R. speratus</i>	PC ₉₅ = 218.4 µg/disk

†EC₅₀ (50% Effective concentration), EC₁₀₀ (100% Effective concentration), AI (Anti-feedant index), MIC (Minimum inhibitory concentration), ED₅₀ (50% Effective dose), PC₅₀ (50% protective concentration), AR (Anti-feedant rate), PC₉₅ (95% protective concentration against feeding), MAC (Minimum anti-feedancy concentration).

● Insect Growth Regulatory Activity

Retardation in growth and development of the insect may occur either due to the indirect effect of anti-feedancy or the direct toxic effect of the limonoids. In fact, growth disruption and anti-feedancy are two independent effects on the insects. Azadirachtin A has been proved to exert potent growth retardation activity against a large spectrum of insects along with its well-known anti-feedant activity. For example, it showed growth inhibitory activity on *Pieris brassicae* pupae which was not owing to the less intake of diet; rather it was a direct effect of azadirachtin A.¹⁰⁷ Feeding of foliage treated with azadirachtin A (5-10 ppm for 48 h) resulted in growth retardation of *S. litura* at initial stages of larvae. Higher concentration of azadirachtin A resulted in severe reduction in weight and metamorphosis inhibition (on last larval stage of *E. varivestis*)¹⁰⁸ along with several physical deformities such as wing deformities, unelasticization of wing lobes, development of wingless adults and larval mortality (on various stages of *D. Koenigii*)¹⁰⁹. Mortality and molting disorder were observed with early stages larvae of *A. ipsilon* when they were fed with azadirachtin sprayed diet. The ED₅₀ values for

Table 1.3 Growth regulatory potency of some Neem limonoids on various insects³⁵

Limonoid	Insect	Growth Regulating potency
Azadirachtin A	<i>Heliothis zea</i> , <i>H. virescens</i>	ED ₅₀ = 0.7 ppm
	<i>Spodoptera frugiperda</i> , <i>Pectinophora gossypiella</i>	ED ₅₀ = 0.4 ppm
	<i>Rhodnius prolixus</i>	ED ₅₀ = 0.04 µg/mL
	<i>Helicoverpa armigera</i>	EC ₅₀ = 0.26 ppm
	<i>Spodoptera litura</i>	EC ₅₀ = 0.21 ppm
Azadirachtin B	<i>Rhodnius prolixus</i>	ED ₅₀ = 0.015 µg/mL
Salannin	<i>Helicoverpa armigera</i>	EC ₅₀ = 74.5 ppm, EC ₉₅ = 187.4 ppm
	<i>Spodoptera litura</i>	EC ₅₀ = 72.0 ppm
Gedunin	<i>Spodoptera litura</i>	EC ₅₀ = 40.4 ppm
	<i>S. frugiperda</i>	LC ₅₀ = 39.0 ppm
	<i>Helicoverpa armigera</i>	EC ₅₀ = 50.8 ppm

† ED₅₀ (50% Effective dose), EC₅₀ (50% Effective concentration), EC₉₅ (95% Effective concentration).

molting inhibition by azadirachtin A were in the range of 10-25 ng/larvae for fourth-instar larvae of ten insect species of *Triatoma*, *Rhodnius* and *Panstrongylus*.¹¹⁰ Similarly, azadirachtin A exhibited growth retardation and various molting disorders on variety of pests in different stages of their life cycle.³⁵ The effects of molting disorders showed by azadirachtin A was investigated probably due to the disturbed ecdysteroids and juvenile hormone regulation and interference with the molting hormone pools.^{111,112} The growth inhibition activity was exerted probably through the disruption of gut motility and inhibition of gut enzymes (such as trypsin, phosphatase, dehydrogenase).^{113,114} Azadirachtin A and its derivatives were found to be most potent among Neem limonoids for the growth retardation and ecdysis inhibition of pests in comparison to intact and other C-seco limonoids (17 β -hydroxyazadiradione, 3-deacetylnimbin, gedunin, 7-deacetylgedunin, azadiradione, nimboicinol).¹¹⁵⁻¹¹⁸ Representative examples of growth regulatory potency of Neem limonoids are documented in Table 1.3.

1.4.2 Anti-carcinogenic Activity

Neem limonoids such as epoxyazadiradione, azadirone, gedunin, nimbolide, 28-deoxonimbolide, azadirachtin A and their derivatives have been reported to possess promising cytotoxicity against several cancer cell lines and their molecular mechanisms have been investigated in some cases.^{9,35,95,119} Cytotoxic potency of Neem limonoids against cancer cell lines has been listed in Table 1.4.

Kikuchi *et al.*¹²⁰ have screened several Neem limonoids of different skeletons (15 of azadirone type, 5 of gedunin type, 4 of azadirachtin type, 9 of nimbin type and 2 degraded limonoids) for the evaluation of cytotoxic activity against five different human cancer cell lines such as HL60 (leukemia), A549 (lung), AZ521 (stomach), SK-BR-3 (breast) and CRL1579 (melanoma). Seven limonoids including 7-benzoylnimbocinol, epoxyazadiradione, 7-deacetyl-7-benzoylpoxyazadiradione, gedunin, 7-deacetyl-7-benzoylgedunin, 28-deoxonimbolide and ohchinin acetate showed cytotoxicity ($IC_{50} < 10 \mu M$) against one or more cancer cell lines. Among them, 7-deacetyl-7-benzoylpoxyazadiradione, 7-deacetyl-7-benzoylgedunin and 28-deoxonimbolide exerted potent cytotoxicity ($IC_{50} = 2.7-3.1 \mu M$) against HL60 leukemia cancer cell line. Out of these three limonoids, 7-deacetyl-7-benzoylpoxyazadiradione was concluded to be the most useful anti-carcinogenic agent due to its weak cytotoxicity against normal

lymphocyte cells. The mode of action behind cytotoxicity was investigated to be the apoptotic cell death via both the mitochondrial and death receptor-mediated pathways. In other report by Nanduri *et al.*¹²¹ azadirone and its several semi-synthetic analogues were investigated for the cytotoxicity against a panel of human cancer cell lines (Breast/MCF-7, CNS/U251, colon/SW620, lung/H522, melanoma/M14, ovarian/SKOV3, prostate/DU145 and renal/A498) *in vitro*. Parent limonoid and seven analogues showed the potency against several cancer cell lines with GI₅₀ value less than 10 μ M. Azadirone and two of its analogues were found to possess good *in vivo* antitumor activity in modified hollow fiber animal models. Gedunin also showed cytotoxic potency (IC₅₀ < 5 μ M) against MCF-7 and SKBr3 breast cancer cell lines through inhibition of Hsp90 folding machinery and induced inhibition of Hsp90-dependent client proteins. Nineteen semi-synthetic analogues were tested for the evaluation of cytotoxicity against same cancer cell lines, though none of them was found to be more potent than the parent compound.^{122,123} Gedunin was also observed to be cytotoxic against CaCo-2 colon cancer cell line with IC₅₀ 16.83 μ M.¹²⁴ Among all the Neem limonoids, nimbolide has been studied in detail as a potent cytotoxic agent against several cancer cell lines. In 1996, Cohen *et al.*¹²⁵ reported nimbolide to be cytotoxic against neuroblastoma N1E-115 (mouse) cells with IC₅₀ less than 10 μ M. Other limonoids including epoxyazadiradione (IC₅₀ ~ 27 μ M), salannin (IC₅₀ ~ 112 μ M) were observed to be less potent than nimbolide whereas nimbin, 6-deacetylnimbin and azadirachtin were found to be practically non-toxic (IC₅₀ > 200 μ M). Further Roy *et al.*¹²⁶ had reported the ability of nimbolide to arrest G₂/M and G₀/G₁ phases of the cell cycle in HT-29 human colon carcinoma cells and moderate to very strong growth inhibitory activity in the concentration range 2.5-10 μ M. Effect on the cell cycle was accompanied by the up-regulation of p21, cyclin D2, Chk2 and down-regulation of cyclin A, cyclin E, cdk2, Rad17. Extension of the study on U937, HL-60, THP1 and B16 cell lines showed the similar anti-proliferative and apoptosis inducing activity through the interference on cell-cycle regulation.¹²⁷ Kumar *et al.*¹²⁸ had observed concentration and time dependent inhibition of growth of BeWo cells with IC₅₀ 2.01 and 1.91 μ M for 7 and 24 h respectively. Apoptosis was confirmed by the observation of nuclear fragmentation and condensation. Suppression of Bcl-2/Bax ratio along with higher expression level of Apaf-1, caspase-3 and cleavage of poly-(ADP-ribose) polymerase indicated a mitochondrial apoptotic pathway. Also, the increase in the level

Table 1.4 Cytotoxic activity of Neem limonoids against cancer cell lines^{35,95}

Limonoid	Cancer cell	Cytotoxic potency
Azadiradione	KB	IC ₅₀ = 9.38 µg/mL
	NCI-H187	IC ₅₀ = 6.44 µg/mL
	MCF7	IC ₅₀ = 7.13 µg/mL
	GPK	ED ₅₀ = 7.13 µg/mL
Epoxyazadiradione	KB	IC ₅₀ = 12.87 µg/mL
	NCI-H187	IC ₅₀ = 7.54 µg/mL
	143B.TK	IC ₅₀ = 24 µM
	MCF-7	IC ₅₀ = 4.68 µg/mL
Nimbolide	GPK	ED ₅₀ = 10.14 µg/mL
	143B.TK	IC ₅₀ = 4.3 µM
	BC-1	ED ₅₀ = 0.39 µg/mL, 3.1 µg/mL
	COL-2	ED ₅₀ = 0.41 µg/mL, 4.2 µg/mL
	HT-1080	ED ₅₀ = 0.31 µg/mL
	LU-1	ED ₅₀ = 0.42 µg/mL, 3.3 µg/mL
	MEL-2	ED ₅₀ = 0.53 µg/mL
	KB	ED ₅₀ = 0.25 µg/mL, 1.7 µg/mL
	P388	ED ₅₀ = 0.065 µg/mL
	LNCaP	ED ₅₀ = 0.9 µg/mL
	BC-1	ED ₅₀ = 1.34 µg/mL, 3.2 µg/mL
	COL-2	ED ₅₀ = 1.81 µg/mL, 9.0 µg/mL
	HT-1080	ED ₅₀ = 1.04 µg/mL
	LU-1	ED ₅₀ = 0.84 µg/mL, 8.5 µg/mL
	MEL-2	ED ₅₀ = 2.05 µg/mL
	KB	ED ₅₀ = 1.30 µg/mL, 4.1 µg/mL
28-Deoxonimbolide	P388	ED ₅₀ = 0.66 µg/mL
	LNCaP	ED ₅₀ = 1.9 µg/mL
	CaCo-2	IC ₅₀ = 16.83 µM
	GPK	ED ₅₀ = 275.10 µg/mL
Gedunin	P388	IC ₅₀ = 3.3 µg/mL
	CHAGO	IC ₅₀ = 16.00 µM
7-Deacetylgedunin	Hep-G2	IC ₅₀ = 10.26 µM
	P388	IC ₅₀ = 4.5 µg/mL

† IC₅₀ (50% Inhibitory concentration), ED₅₀ (50% Effective dose).

of ROS was accounted along with the apoptotic effects. Azadirachtin A was also observed to be active cytotoxic agent in some cases although possessing less potency in comparison to nimbolide. By monitoring the regulation of various key proteins (such as PCNA, p21, cyclin D1, GST-P, NF- κ B, p53, Fas, Bcl-2, Bax, Bid, Apaf-1, cytochrome C, survivin, caspases-3, -6, -8, -9 and PARP) in breast cancer cells (MCF-7 and MDA-MB-231),¹²⁹ hepatocarcinoma cells (HepG2),¹³⁰ cervical cancer cells (HeLa)¹³¹ and Hamster Buccal Pouch carcinogenesis model,¹³² induction of anti-proliferative effect by nimbolide and azadirachtin A was revealed through down-regulation of proteins involved in the cell cycle progression and apoptosis by both the intrinsic and extrinsic pathways. Thus, nimbolide was found to be a potent anti-proliferative and apoptosis inducing agent and thus offers to be a promising candidate in multi-targeted prevention and treatment of cancer.

1.4.3 Anti-plasmodial Activity

Ring intact Neem limonoids such as gedunin, azadiradione, epoxyazadiradione and azadirone have been reported to possess significant anti-plasmodial activity. Chianese *et al.*¹³³ have shown that these limonoids possess anti-plasmodial activity with IC₅₀ ranging from 1.0 to 6.0 μ M *in vitro* against D10 (Chloroquine sensitive) and W2 (Chloroquine resistant) strains of *P. falciparum*. Gedunin and azadirone were found to be most potent (IC₅₀ = 1.2-1.7 μ M) among the studied limonoids. In another study by MacKinnon *et al.*¹³⁴ gedunin and nine semi-synthetic derivatives were assayed *in vitro* against D6 (Chloroquine sensitive) and W2 (Chloroquine resistant) strains of *P. falciparum*. Among all the molecules screened, the parent compound gedunin showed most potent activity (IC₅₀ = 39 and 20 ng/mL for D6 and W2 respectively) and was found to be only limonoid possessing higher potency than chloroquine against chloroquine resistant W2 strain. Omar *et al.*¹³⁵ have also shown *in vivo* antimalarial efficacy of gedunin and 7-methoxygedunin in CD-1 mice infected with *Plasmodium berghei* and its synergistic effect with dillapiol (cytochrome P450 inhibitor). Several other studies also showed the *in vitro* anti-malarial potency of gedunin and its derivatives.¹³⁶⁻¹³⁹ Maneerat *et al.*¹⁴⁰ had also reported *in vitro* anti-plasmodial activity of azadiradione and epoxyazadiradione with IC₅₀ in the range of 2.1-6.3 μ g/mL. In a report by Jones *et al.*¹⁴¹ azadirachtin A has been shown to block the development of the motile malarial male gamete *in vitro*, though not able to inhibit growth and replication of

asexual blood stages. Mechanistically, it interfered with the formation of mitotic spindles and the assembly of microtubules into typical axonemes in *Plasmodium berghei*. However, either the replication of genome or the ability of tubulin monomers to assemble upon gametocyte activation was not inhibited, thereby suggesting interference of azadirachtin A with the patterning of microtubules into more complex structures.¹³⁶

1.4.4 Anti-inflammatory Activity

Azadiradione, epoxyazadiradione, 7-deacetylgedunin and nimolicinol inhibited 12-O-tetradecanoylphorbol-13-acetate induced inflammation (1 mg/ear) in mice with ID₅₀ 0.09-0.26 mg/ear and found to be more potent than standard anti-inflammatory drug indomethacin with ID₅₀ 0.30 mg/ear.¹⁴² According to the other report by Ravangpai *et al.*,¹⁴³ 7-deacetylgedunin exhibited significant inhibitory activity against nitric oxide production from activated macrophages with IC₅₀ value less than 10 µM, showing its ability as anti-inflammatory molecule. Penido *et al.*¹⁴⁴ also had demonstrated the anti-allergic activity of gedunin through the inhibition of allergic eosinophilia by disrupting the generation of eosinophilotactic chemokines through NFκB signaling pathway impairment in mice. Further studies indicated gedunin to interfere with the allergic responses primarily by modulating T lymphocyte and eosinophil functions.¹⁴⁵ The anti-inflammatory potency of azadiradione has been investigated by Ilango *et al.*¹⁴⁶ against acute paw oedema induced by carrageenan with doses of 50 and 100 mg/kg body weight of mice and it showed significant activity in time and dose dependent manner. Kaur *et al.*¹⁴⁷ had reported that nimbidin attenuated the functions of macrophages and neutrophils associated with inflammation *in vitro* and *in vivo*. Exposure of rat peritoneal macrophages to nimbidin inhibited phagocytosis and phorbol-12-myristate-13-acetate (PMA) stimulated respiratory burst *in vitro*. Oral administration of nimbidin to rats for 3 consecutive days with dose of 5-25 mg/kg significantly inhibited the migration of macrophages in response to inflammatory stimulations and inhibited phagocytosis and PMA stimulated respiratory burst in these cells. *In vitro* experiment also showed that nimbidin inhibited nitric oxide and prostaglandin E₂ production in lipopolysaccharide stimulated macrophages.

1.4.5 Anti-ulcerogenic Activity

In several occasions, the extracts from different parts of Neem tree especially leaves and

bark have been shown to possess anti-secretory and anti-ulcerogenic activities.¹⁴⁸ But very less effort have been made to find out the active principles responsible for its gastroprotective activities. In a report by Pillai *et al.*¹⁴⁹ nimbidin showed significant anti-ulcerogenic activity in various *in vivo* models. Results indicated significant gastroprotection by nimbidin in doses of 20 to 40 mg/kg in acetylsalicylic acid, stress, serotonin and indomethacin induced gastric lesions in rats. Lakshmi *et al.*¹⁵⁰ have shown the potency of gedunin as gastroprotective agent. Gedunin inhibited H⁺ K⁺-ATPase activity *in vitro* (IC₅₀ = 56.86 µg/mL) in comparison to standard anti-ulcer drug omeprazole with IC₅₀ value 30.24 µg/mL; thus indicating its significant anti-secretory activity.

1.4.6 Anti-bacterial and Anti-fungal Activity

Limonoids have been reported to possess anti-bacterial and anti-fungal properties in several studies. For example, azadiradione exerted inhibitory activity against *Mycobacterium tuberculosis* with MIC 6.25 µg/mL.¹⁴⁰ In other study, extracted Neem oil fraction showed the potent anti-fungal activity against *Drechslera oryzae*, *Fusarium oxysporum* and *Alternaria tenuis*. But, purified azadiradione, nimbin, salannin and epoxyazadiradione didn't show significant activity. However, when these limonoids were mixed and assayed, they showed antifungal activity in the same range as that of oil extract, indicating possible additive or synergistic effects.¹⁵¹ Rajanapo *et al.*¹⁵² reported antibacterial activity of nimbolide against three strains *Staphylococcus aureus*, *S. coagulase* (+) and *S. coagulsa* (-) with a concentration of 0.875 mg/disk. Kraus *et al.*¹⁵³ also showed that nimbolide inhibited *Pseudomonas stutzeri* even at a concentration of 0.5 µg/spot.

1.5 Biosynthesis of Neem Limonoids

Neem limonoids are highly complex in structure, multi-functionalized and skeletally diverse. Biosynthetic pathway leading to the formation of limonoids is tentative⁹⁶ and not based on any experimental evidences, except for a very few studies.^{154,155} Very less effort has been exerted for the elucidation of Neem limonoid biosynthetic pathway supported by proper experimental investigation. Practically, nothing is known about the cellular location of limonoid biosynthesis, biosynthetic pathway genes and enzymology.

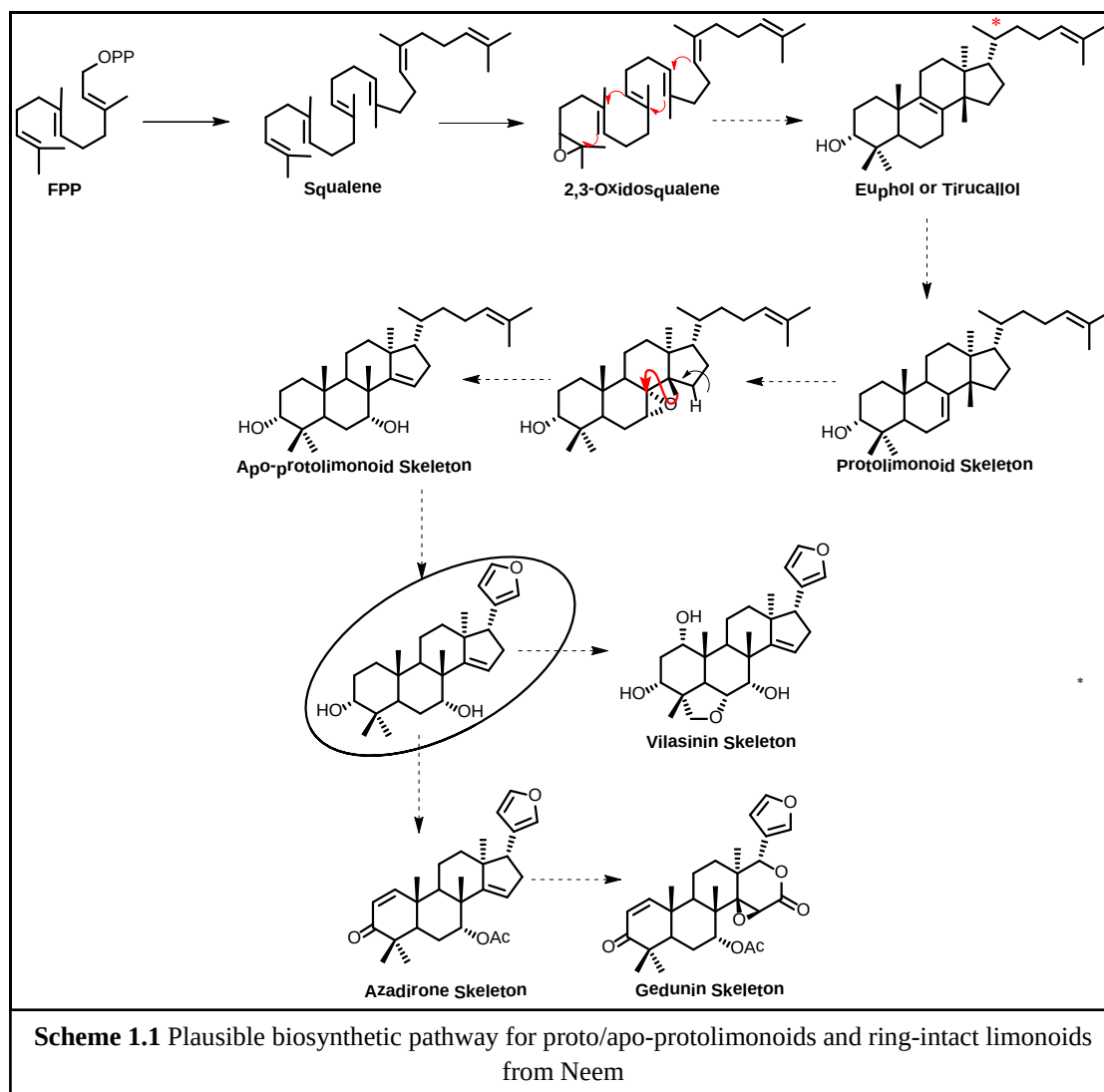
● Biosynthesis of protolimonoids

Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), synthesized from primary metabolites either through MVA or MEP pathway, condense in head-tail fashion to produce linear geranyl pyrophosphate (GPP). GPP further joins with one molecule of IPP to produce linear farnesyl pyrophosphate (FPP), a C-15 linear pyrophosphate. Two molecules of FPP further condense together to form squalene, the first committed precursor of triterpenoid biosynthetic pathway. Squalene is epoxidized to produce 2,3-oxidosqualene which undergoes tandem cyclization to form euphol (H-20 β , 20R) or tirucallol (H-20 α , 20S), one of which is believed to be the probable biogenetic precursor of ring intact limonoids (Scheme 1.1).¹⁵⁶ In the next step, $\Delta^{8,9}$ bond is shifted to $\Delta^{7,8}$ forming butyrospermol skeleton which is evident from the presence of several protolimonoids (Scheme 1.1, Table 1.1).¹⁵⁷

● Biosynthesis of ring-intact limonoids^{96,157}

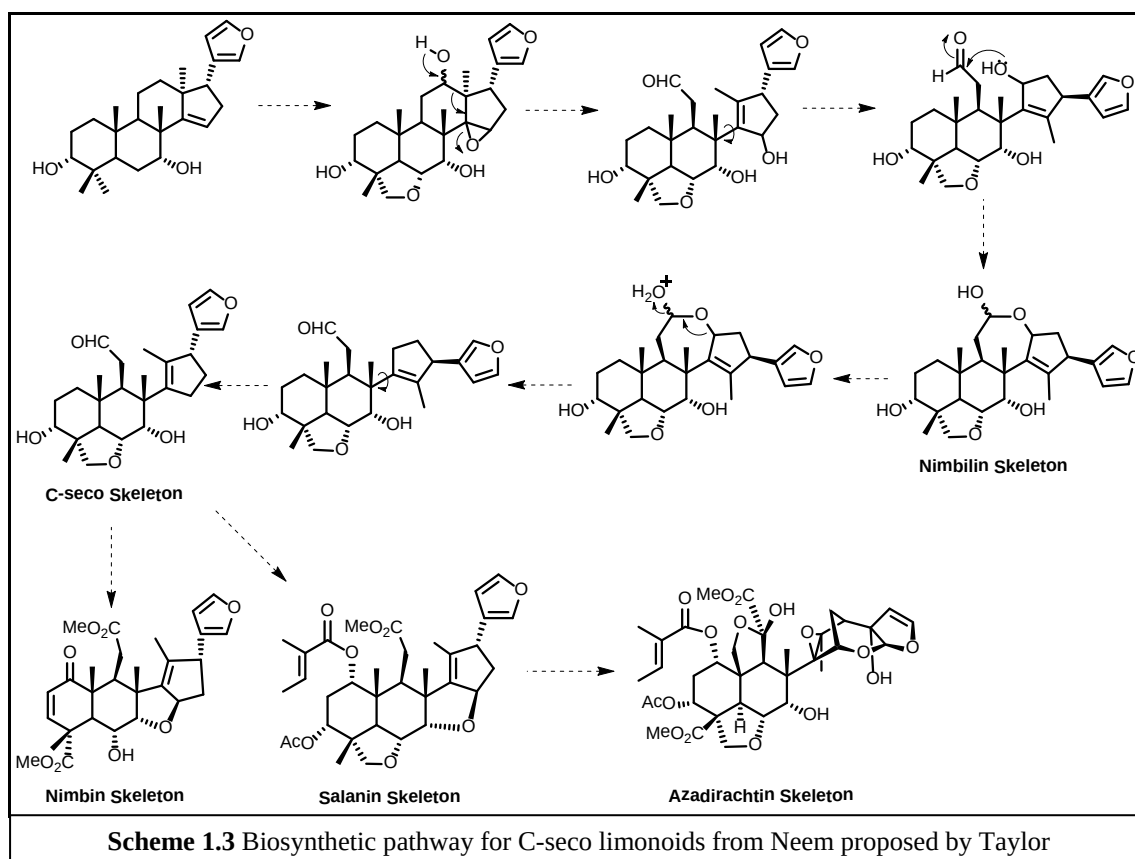
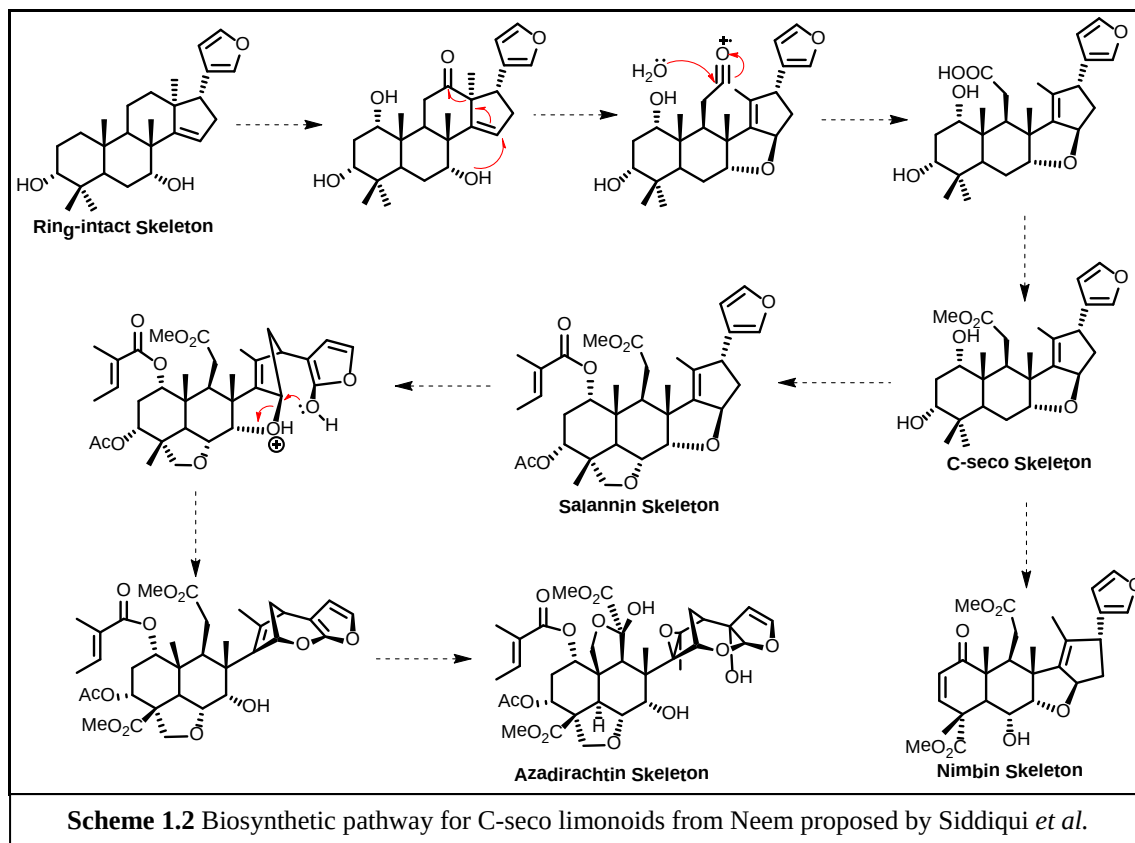
The $\Delta^{7,8}$ bond is epoxidized in α -orientation which is further opened up by the Wagner-Meerwein migration of Me-14 to C-8 generating hydroxyl group at 7 α -position and unsaturation at C-14/15, thus generating the skeleton of apo-protolimonoids (Scheme 1.1, Table 1.1). This pathway leading to the formation of 4,4,8-trimethylsteroidal structure supports the ubiquitous presence of 7 α -hydroxy and β -stereochemistry of C-30 methyl group. In the subsequent steps, the eight carbon side chain cyclises and undergoes oxidative degradation to lose four carbons to form 17 α -furan ring on 4,4,8-trimethylsteroidal skeleton (Ring-intact limonoids; Scheme 1.1, Table 1.1). The process of side chain degradation probably occurs after the formation of 4,4,8-trimethylsteroidal skeleton which is evident from the abundance of several apo-protolimonoids in Neem, carrying intact eight carbon side chain with 4,4,8-trimethylsteroidal structure. After the formation of basic structure, several oxidations and functionalization (such as acetylation of 7-hydroxy, introduction of carbonyl at C-16 or C-3, epoxidation at $\Delta^{14,15}$, dehydrogenation to generate $\Delta^{1,2}$) may occur to generate azadirone skeleton. Subsequent D-ring-expansion to lactone through Bayer-Villiger oxidation may synthesize the limonoids of gedunin skeleton. Formation of vilasinin skeleton may proceed through the hydroxylation at both C-6 and Me-28, followed by subsequent etherification (Scheme 1.1). Some of the ring-intact limonoids may undergo further oxidative modifications on

furan ring such as acetal formation (*e.g.* Limocin A, B, C, D, salimuzzalin), lactonization (*e.g.* azadirachtolide), hydroxylations (*e.g.* meliacinanhdyride) or dehydrogenation (*e.g.* 22,23-dihydronimocinol). In few cases it may undergo additional ring formation probably through hydroxylation and subsequent cyclization (*e.g.* azadirachtinin, vepinin) (Table 1.1).



● Biosynthesis of C-seco limonoids

Further skeletal rearrangement and extensive oxidations in one or more of the four rings (A, B, C and D) may give rise to different classes of limonoids. For example, C-seco limonoid, one of the major classes of Neem limonoids is formed through the ‘cut and open’ (*i.e.* seco) and further rearrangement of the C-ring. The biosynthetic origin of C-seco limonoids especially the mechanism behind the cleavage of C-12/C-13 bond is not



clear and there are debates regarding this unique rearrangement leading to the C-seco limonoid structure. According to Mitra *et al.*¹⁵⁸ and Siddiqui *et al.*¹⁵⁷ the C-12/C-13 bond on nimbidinin skeleton is cleaved to form 12-acylium ion with simultaneous attack of the OH-7 on C-15. Quenching of the acylium ion by water molecule to carboxylic acid and further oxidations may lead to salannin or nimbin skeleton (Scheme 1.2). Salannin may undergo extended rearrangement such as opening of C-ring and fusion of ring-D with furan to azadirachtin skeleton. Alternatively, Taylor¹⁵⁹ had proposed that the formation of C-seco limonoids initiates through the hydroxylation at C-12 and epoxidation at $\Delta^{14,15}$. Cleavage of the C-12/C-13 bond is accompanied by the simultaneous opening of 14,15-epoxide to generate aldehyde group at C-12 and hydroxyl at C-15. Rotation about the single bond may allow the OH-15 to cyclise with aldehyde at C-12 to form lactol containing nimbin skeleton (Table 1.1). Lactol C-ring may further undergo ring opening with the removal of water molecule followed by rotation around the same bond C-8/C-14 and recyclisation (Scheme 1.3). Proposed pathway was supported by Rajab *et al.*^{65,160} who showed biomimetic and thermodynamically favoured transformation of volkensin (a nimbin skeleton limonoid) to salannin. Further, nucleophilic attack of hydroxyl group on C-15 and simultaneous cleavage of C(15)-O bond may lead to the generation of azadirachtin skeleton. The ring-intact or C-seco butenolides (Table 1.1) were proposed either to be photo-degraded products of the parent limonoids or intermediates in the formation of C-17 furan ring.^{96,157}

1.6 Separation and Quantification Techniques for Neem Limonoids

Purification of individual components from the complex blend of triterpenoids from different parts of Neem plant is practically a challenging task due to narrow chromatographic resolution. In the last three decades several techniques have been developed describing the purification of major limonoids from seed kernel, especially azadirachtin A, B, salannin, nimbin and their derivatives. In gist, crude extract of triterpenoids was prepared by solvent extraction and partition technique. Initially ground Neem seeds were de-oiled by extracting with non-polar solvents (hexane or petroleum ether) to produce 'Neem cake' which was extracted with relatively polar solvents (methanol or dichloromethane) to obtain triterpenoids along with

macromolecules such as proteins or sugars. It was further partitioned between water and ethyl acetate to get the triterpenoids in organic layer which was concentrated to furnish crude mixture of triterpenoids. Subsequently the mixture was subjected to chromatographic purification (single or sequential) and/or analysis of individual triterpenoids.

In 1986 Yamasaki *et al.*¹⁶¹ had reported the systematic purification of azadirachtin A with low yield (56 mg from 1.0 kg seed) and high purity (>99%) by the combination of two step flash chromatography (silica gel and ODS) followed by two step preparative HPLC (silica gel and phenyl) technique from the crude limonoid mixture obtained by solvent partition extraction from Neem seeds. Schroeder *et al.*¹⁶² in 1987 had optimized the isolation protocol of azadirachtin A by the successive combination of vacuum liquid chromatography (Silica gel), CCl₄ crystallization technique, silica-gel flash column chromatography and CCl₄ re-crystallization with good yield (>5.0 g from 2.0 kg seeds) from the mixture of seed limonoids. In the isolation procedure reported by Govindachari *et al.*¹⁶³ in 1990 the steps after vacuum liquid chromatography were replaced by silica-gel MPLC and preparative reverse-phase HPLC (ODS column; solvent system methanol:water 60:40) techniques. Hull *et al.*¹⁶⁴ in 1993 had reported the reverse-phase HPLC (Supelcosil C₈ column) based sensitive (lower limit of detection 0.001% w/w) quantification technique of azadirachtin A within the concentration range 0.005-0.75% w/w in the insecticidal formulations based on Neem seed kernel extract. In another report by Govindachari *et al.*¹⁶⁵ in 1996 the major limonoid constituents of Neem oil (Azadirachtin A, B, D, H, I, salannin, nimbin, 6-deacetylnimbin, azadiradione and epoxyazadiradione) were purified by Shimpack reverse phase C₁₈ column. The same author in 1997 developed preparative HPLC based (Shimpack C₁₈ reverse phase column) purification of azadirachtin B using recycling mode.⁷¹ Johnson *et al.*¹⁶⁶ in 1997 had isolated eleven limonoids (including azadirachtin A and its six derivatives, salannin, nimbin, their deacetylated analogues and salanninolide) from the extract of Neem seed kernel using Florex RVM column. Further, comparative elution order of the purified limonoids was determined through supercritical fluid chromatography (SFC) and reverse-phase HPLC. Jarvis *et al.*⁸⁴ (1999) had shown that Biotage flash chromatography system could be used for the purification of eleven essential limonoids (Azadirachtin A, B, I, D, salannin, nimbin, 3-deacetylsalannin, 6-deacetylnimbin, azadirachtinin, 3-acetyl-1-tigloylazadirachtinin, isosalanninolide) from the

dichloromethane extract of Neem seeds. They found that Biotage columns were better than Florex RVM columns for the isolation of Neem limonoids with respect to time and solvent consumption. For rapid and sensitive qualitative and quantitative determination of limonoids (azadirachtin A, salannin, nimbin and 6-deacetylnimbin) from Neem seeds and tissue callus culture samples, Schaaf *et al.*¹⁶⁷ developed a reverse phase HPLC-APCI-MS (atmospheric pressure chemical ionization mass spectrometry) based analytical technique. The limit of detection for azadirachtin in extract samples was 10 pg in single ion monitoring (SIM) mode, approximately 1000-times higher sensitive than HPLC methods (10 ng) reported by Sundaram *et al.*¹⁶⁸ In addition to high sensitivity, the HPLC-MS method was facilitated with minimal sample preparation, purification and dramatically reduced analysis time. In a study by Jarvis *et al.*¹⁶⁹ the limonoid mixture was fractionated using solid-phase extraction technique to reduce the complexity of the crude extract prior to analyses by SFC or HPLC. Sharma *et al.*¹⁷⁰ demonstrated a reverse-phase MPLC (50:50 methanol:water as eluent and C₁₈ material with particle size 40-63 µm) based protocol for the preparative scale purification of azadirachtin A, B and H from azadirachtin enriched concentrate (azadirachtin A ~ 60%) prepared from methanolic extract of defatted neem seed kernels. Azadirachtins were further characterized through NMR and signature adducts in ESI(+)-MS spectra. In 2007 Silva *et al.*¹⁷¹ had exploited counter-current chromatography (CCC) with biphasic solvent system (hexane:butanol:methanol:water 1:0.9:1:0.9 v/v) followed by preparative reversed-phase HPLC (Supelcosil LC-8 column) to isolate seven limonoids (azadirachtin A, B, H, 6-deacetylnimbin, 3-deacetylsalannin, nimbin and salannin) from the Neem seed extract. Reproducibility, narrow polarity range and high purity were the advantages of this developed technique. In 2008 Sarais *et al.*¹⁷² developed a HPLC-ESI-MS based analytical technique for the rapid and accurate quantification of azadirachtin A, B, salannin, nimbin and their deacetylated derivatives in fruits and vegetables. The limit of detection and limit of quantification for azadirachtin A were determined to be 0.4 and 0.8 µg/kg respectively.

1.7 Chemical Synthesis of Neem Limonoids

Complexity in structural framework due to the presence of several tertiary centres, multi-functional groups and un-stability towards pH and light has extensively restricted the chemical synthesis of limonoids. Azadirachtin A is a classic example of natural

product possessing highly complex structural framework, carrying sixteen contiguous stereogenic centres among which seven are tetrasubstituted. In addition, it possesses a very rigid structural conformation due to the presence of several intramolecular hydrogen bonding and presence of diverse oxygen functionalities.¹⁷³ Again, it was observed to be highly sensitive towards extremes of pH and photolytic condition.¹⁷⁴ Despite enormous efforts by different groups of synthetic chemists, all the attempts were unsuccessful till twenty two years after the correct structural assignment of this insecticidal molecule in 1985.¹⁷⁴ In 2007 Ley *et al.* had reported the first total synthesis of azadirachtin A through convergent route joining two fragments at C-8/C-14.¹⁷⁵ In 2009 the same group had developed a second generation strategy.¹⁷⁶ In another report by Ley *et al.*¹⁷⁷ azadirachtin A and four of its analogues (vepaol, isovepaol, 3-deacetylazadirachtin A, 1-tigloyl-3-acetyl-11-methoxyazadirachtinin) were synthesized from a common intermediate.

Although possessing a wide spectrum of biological activities very less effort have been invested for the total synthesis of relatively less complex limonoids of azadirone skeleton. In fact very few groups had developed the synthetic strategies to access the ring-intact limonoids.¹⁷⁸ In 1989 Corey *et al.*¹⁷⁹ had reported the biomimetic total synthesis of azadiradione utilizing π -carbocationic cyclization for the construction of A, B and C ring. In another report by Corey *et al.*¹⁸⁰ in 2008 the synthesis of an azadiradione analogue was achieved by the cationic triple annulation process in an asymmetric fashion by installation of an initiating stereocenter to the polyolefin carbocyclization substrate. Fernández-Mateos and co-workers had demonstrated a semi-synthetic approach for azadiradione and analogues wherein the A-B decalin fragment is derived from the natural product starting material agathic acid.¹⁷⁸ Further they have used Nazarov reaction¹⁸¹ for the D-ring closing or alternatively an intramolecular dipolar [3+2] cycloaddition¹⁸² of a nitrile oxide with tethered olefin. Subsequently it was modified with a concise and stereo-controlled way to introduce the key structural functionalities leading to the close structural framework of azadiradione.

1.8 References

- (1) Chopra, R. N.; Nayer, S. L.; Chopra, I. C. *Glossary of Indian Medicinal Plants, New Delhi, CSIR* **1956**.
- (2) Thakur, R. S.; Singh, S. B.; Goswami, A. *Curr. Res. Med. Aromat. Plants* **1981**, 3, 135-140.
- (3) Schmutterer, H. *The Neem Tree: Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes*, VCH, Weinheim, Germany **1995**, 1-696.
- (4) Kumar, A. V. *Curr. Sci.* **2003**, 84, 265-267.
- (5) Schmutterer, H. *Annu. Rev. Entomol.* **1990**, 35, 271-297.
- (6) *Neem: A Tree for Solving Global Problems*; National Research Council, National Academy: Washington DC, **1992**.
- (7) Brahmachari, G. *Chembiochem* **2004**, 5, 409-421.
- (8) Biswas, K.; Chattopadhyay, I.; Banerjee, R. K.; Bandyopadhyay, U. *Curr. Sci.* **2002**, 82, 1336-1345.
- (9) Atawodi, S. E.; Atawodi, J. C. *Phytochem. Rev.* **2009**, 8, 601-620.
- (10) Govindachari, T. R. *Curr. Sci.* **1992**, 63, 117-122.
- (11) Morgan, E. D. *Bioorg. Med. Chem.* **2009**, 17, 4096-4105.
- (12) Sharma, P.; Tomar, L.; Bachwani, M.; Bansal, V. *Int. Res. J. Pharm.* **2011**, 2, 97-102.
- (13) De Jussieu, A. *Mem. Mus. Hist. Nat., Paris.* **1830**, 19, 220.
- (14) Siddiqui, S. *Curr. Sci.* **1942**, 11, 278-279.
- (15) Siddiqui, S.; Siddiqui, B. S.; Ghiasuddin; Faizi, S. *J. Nat. Prod.* **1991**, 54, 408-415.
- (16) Siddiqui, S.; Siddiqui, B. S.; Faizi, S.; Mahmood, T. *J. Chem. Soc. Pakistan* **1986**, 8, 341-347.
- (17) Siddiqui, S.; Siddiqui, B. S.; Ghiasuddin; Faizi, S. *Phytochemistry* **1991**, 30, 1615-1619.
- (18) Siddiqui, B. S.; Afshan, F.; Gulzar, T.; Sultana, R.; Naqvi, S. N. H.; Tariq, R. M. *Chem. Pharm. Bull.* **2003**, 51, 415-417.
- (19) Siddiqui, B. S.; Ghiasuddin; Faizi, S.; Siddiqui, S. *Phytochemistry* **1992**, 31, 4275-4278.
- (20) Lavie, D.; Jain, M. K.; Kirson, I. *J. Chem. Soc. C* **1967**, 1347-1351.
- (21) Siddiqui, S.; Siddiqui, B. S.; Faizi, S. *Planta Med.* **1985**, 51, 478-480.
- (22) Lavie, D.; Jain, M. K.; Shpangab, S. *J. Chem. Soc. Chem. Comm.* **1967**, 910-911.
- (23) Siddiqui, S.; Mahmood, T.; Siddiqui, B. S.; Faizi, S. *Phytochemistry* **1986**, 25, 2183-2185.
- (24) Lavie, D.; Levy, E. C.; Jain, M. K. *Tetrahedron* **1971**, 27, 3927-&.
- (25) Siddiqui, S.; Fuchs, S.; Lucke, J.; Voelter, W. *Tetrahedron Lett.* **1978**, 611-612.
- (26) Nanduri, S.; Banstola, K. P. *Indian J. Chem. B* **1995**, 34, 1019-1020.
- (27) Lee, S. M.; Olsen, J. I.; Schweizer, M. P.; Klocke, J. A. *Phytochemistry* **1988**, 27, 2773-2775.
- (28) Siddiqui, S.; Faizi, S.; Siddiqui, B. S. *Z. Naturforsch. B* **1986**, 41, 922-924.
- (29) Kraus, W.; Cramer, R. *Tetrahedron Lett.* **1978**, 2395-2398.
- (30) Connolly, J. D.; Handa, K. L.; McCrindle, R. *Tetrahedron Lett.* **1968**, 437-440.
- (31) Kraus, W.; Cramer, R.; Sawitzki, G. *Phytochemistry* **1981**, 20, 117-120.
- (32) Suresh, G.; Narasimhan, N. S.; Palani, N. *Phytochemistry* **1997**, 45, 807-810.
- (33) Siddiqui, S.; Faizi, S.; Mahmood, T.; Siddiqui, B. S. *J. Chem. Soc., Perkin Trans. 1* **1986**, 1021-1025.

-
- (34) Gaikwad, B. R.; Mayelvaganan, T.; Vyas, B. A.; Bhat, S. V. *Phytochemistry* **1990**, 29, 3963-3965.
- (35) Tan, Q. G.; Luo, X. D. *Chem. Rev.* **2011**, 111, 7437-7522.
- (36) Siddiqui, B. S.; Ali, S. T.; Rasheed, M.; Kardar, M. N. *Helv. Chim. Acta* **2003**, 86, 2787-2796.
- (37) Hallur, G.; Sivramakrishnan, A.; Bhat, S. V. *J. Nat. Prod.* **2002**, 65, 1177-1179.
- (38) Govindachari, T. R.; Malathi, R.; Gopalakrishnan, G.; Suresh, G.; Rajan, S. S. *Phytochemistry* **1999**, 52, 1117-1119.
- (39) Siddiqui, B. S.; Afshan, F.; Ghiasuddin; Faizi, S.; Naqvi, S. N. H.; Tariq, R. M. *Phytochemistry* **2000**, 53, 371-376.
- (40) Akihisa, T.; Noto, T.; Takahashi, A.; Fujita, Y.; Banno, N.; Tokuda, H.; Koike, K.; Suzuki, T.; Yasukawa, K.; Kimura, Y. *J. Oleo Sci.* **2009**, 58, 581-594.
- (41) Koul, O.; Multani, J. S.; Singh, G.; Daniewski, W. M.; Berlozecki, S. *J. Agric. Food Chem.* **2003**, 51, 2937-2942.
- (42) Siddiqui, S.; Faizi, S.; Siddiqui, B. S.; Ghiasuddin *J. Nat. Prod.* **1992**, 55, 303-310.
- (43) Ara, I.; Siddiqui, B. S.; Faizi, S.; Siddiqui, S. *Fitoterapia* **1992**, 63, 118-121.
- (44) Pachapurkar, R. V.; Kornule, P. M.; Narayanan, C. R. *Chem. Lett.* **1974**, 4, 357-358.
- (45) Mitra, C. R.; Garg, H. S.; Pandey, G. N. *Phytochemistry* **1971**, 10, 857-864.
- (46) Ekong, D. E. U.; Fakunle, C. O.; Fasina, A. K.; Okogun, J. I. *J. Chem. Soc. Chem. Comm.* **1969**, 1166-1167.
- (47) Kraus, W.; Cramer, R. *Liebigs. Ann. Chem.* **1981**, 181-189.
- (48) Kraus, W. *Stud. Organ. Chem.* **1984**, 17, 331.
- (49) Kumar, C.; Srinivas, M.; Yakkundi, S. *Phytochemistry* **1996**, 43, 451-455.
- (50) Siddiqui, B. S.; Ghiasuddin; Faizi, S.; Rasheed, M. *J. Nat. Prod.* **1999**, 62, 1006-1009.
- (51) Siddiqui, S.; Faizi, S.; Mahmood, T.; Siddiqui, B. S. *Heterocycles* **1986**, 24, 1319-1324.
- (52) Siddiqui, S.; Mahmood, T.; Siddiqui, B. S.; Faizi, S. *Heterocycles* **1987**, 26, 1827-1833.
- (53) Siddiqui, S.; Mahmood, T.; Faizi, S.; Siddiqui, B. S. *J. Chem. Soc. Perkin Trans. 1* **1987**, 1429-1432.
- (54) Siddiqui, S.; Siddiqui, B. S.; Mahmood, T.; Faizi, S. *Heterocycles* **1989**, 29, 87-96.
- (55) Siddiqui, B. S.; Afshan, F.; Ghiasuddin; Faizi, S.; Naqvi, S. N.; Tariq, R. M. *J. Chem. Soc. Perkin Trans. 1* **1999**, 2367-2370.
- (56) Ragasa, C. Y.; Nacpil, Z. D.; Natividad, G. M.; Tada, M.; Coll, J. C.; Rideout, J. A. *Phytochemistry* **1997**, 46, 555-558.
- (57) Siddiqui, B. S.; Rasheed, M. *Helv. Chim. Acta* **2001**, 84, 1962-1968.
- (58) Siddiqui, B. S.; Afshan, F.; Faizi, S.; Naqvi, S. N. H.; Tariq, R. M. *J. Nat. Prod.* **2002**, 65, 1216-1218.
- (59) Siddiqui, B. S.; Afshan, F.; Gulzar, T.; Hanif, M. *Phytochemistry* **2004**, 65, 2363-2367.
- (60) Podder, G.; Mahato, S. B. *Heterocycles* **1985**, 23, 2321-2325.
- (61) Narayanan, C. R.; Pachapurkar, R. V.; Sawant, B. M.; Wadia, M. S. *Indian J. Chem.* **1969**, 7, 187.
- (62) Siddiqui, B. S.; Rasheed, M.; Ghiasuddin; Faizi, S.; Naqvi, S. N. H.; Tariq, R. M. *Tetrahedron* **2000**, 56, 3547-3551.
- (63) Bokel, M.; Cramer, R.; Gutzeit, H.; Reeb, S.; Kraus, W. *Tetrahedron* **1990**, 46, 775-782.
- (64) Devakumar, C.; Mukerjee, S. K. *Indian J. Chem. B* **1985**, 24, 1105-1106.
- (65) Rajab, M. S.; Bentley, M. D.; Fort, R. C. *J. Nat. Prod.* **1988**, 51, 1291-1293.
-

-
- (66) Rojatkar, S. R.; Bhat, V. S.; Kulkarni, M. M.; Joshi, V. S.; Nagasampagi, B. A. *Phytochemistry* **1989**, *28*, 203-205.
- (67) Garg, H. S.; Bhakuni, D. S. *Phytochemistry* **1985**, *24*, 866-867.
- (68) Morgan, E. D.; Thornton, M. D. *Phytochemistry* **1973**, *12*, 391-392.
- (69) Kraus, W.; Bokel, M.; Bruhn, A.; Cramer, R.; Klaiber, I.; Klenk, A.; Nagl, G.; Pohnl, H.; Sadlo, H.; Vogler, B. *Tetrahedron* **1987**, *43*, 2817-2830.
- (70) Ley, S. V.; Denholm, A. A.; Wood, A. *Nat. Prod. Rep.* **1993**, *10*, 109-157.
- (71) Govindachari, T. R.; Gopalakrishnan, G.; Suresh, G. *J. Liq. Chromatogr. R. T.* **1997**, *20*, 1633-1636.
- (72) Rojatkar, S. R.; Nagasampagi, B. A. *Phytochemistry* **1993**, *32*, 213-214.
- (73) Rembold, H. *ACS Symp. Ser.* **1989**, *387*, 150.
- (74) Govindachari, T. R.; Sandhya, G.; Raj, S. P. G. *J. Nat. Prod.* **1992**, *55*, 596-601.
- (75) Rojatkar, S. R.; Nagasampagi, B. A. *Indian J. Chem. B* **1995**, *34*, 1016-1018.
- (76) Govindachari, T. R.; Sandhya, G.; Raj, S. P. G. *Indian J. Chem. B* **1992**, *31*, 295-298.
- (77) Luo, X. D.; Ma, Y. B.; Wu, S. H.; Wu, D. G. *J. Nat. Prod.* **1999**, *62*, 1022-1024.
- (78) Ramji, N.; Venkatakrishnan, K.; Madyastha, K. M. *Phytochemistry* **1996**, *42*, 561-562.
- (79) Govindachari, T. R.; Gopalakrishnan, G. *Phytochemistry* **1997**, *45*, 397-399.
- (80) Bilton, J. N.; Broughton, H. B.; Jones, P. S.; Ley, S. V.; Lidert, Z.; Morgan, E. D.; Rzepa, H. S.; Sheppard, R. N.; Slawin, A. M. Z.; Williams, D. J. *Tetrahedron* **1987**, *43*, 2805-2815.
- (81) Luo, X. D.; Wu, S. H.; Ma, Y. B.; Wu, D. G. *Nat. Prod. Res. Dev.* **2001**, *13*, 9.
- (82) Ara, I.; Siddiqui, B. S.; Faizi, S.; Siddiqui, S. *J. Nat. Prod.* **1989**, *52*, 1209-1213.
- (83) Ara, I.; Siddiqui, B. S.; Faizi, S.; Siddiqui, S. *Heterocycles* **1989**, *29*, 729-735.
- (84) Jarvis, A. P.; Morgan, E. D.; Edwards, C. *Phytochem. Anal.* **1999**, *10*, 39-43.
- (85) Siddiqui, S.; Faizi, S.; Mahmood, T.; Siddiqui, B. S. *Tetrahedron* **1986**, *42*, 4849-4856.
- (86) Siddiqui, S.; Siddiqui, B. S.; Faizi, S.; Mahmood, T. *Heterocycles* **1986**, *24*, 3163-3167.
- (87) Siddiqui, S.; Mahmood, T.; Siddiqui, B. S.; Faizi, S. *J. Nat. Prod.* **1986**, *49*, 1068-1073.
- (88) Ara, I.; Siddiqui, B. S.; Faizi, S.; Siddiqui, S. *Phytochemistry* **1988**, *27*, 1801-1804.
- (89) Kraus, W.; Klenk, A.; Bokel, M.; Vogler, B. *Liebigs Ann. Chem.* **1987**, *1987*, 337-340.
- (90) Kraus, W.; Cramer, R. *Chem. Ber.* **1981**, *114*, 2375-2381.
- (91) Siddiqui, B. S.; Ghiasuddin; Faizi, S. *Phytochemistry* **1998**, *47*, 1631-1636.
- (92) Siddiqui, B. S.; Afshan, F.; Faizi, S. *Tetrahedron* **2001**, *57*, 10281-10286.
- (93) Emerson, O. H. *Food Technol.* **1949**, *3*, 248-250.
- (94) Butterworth, J. H.; Morgan, E. D. *Chem. Commun.* **1968**, 23-24.
- (95) Roy, A.; Saraf, S. *Biol. Pharm. Bull.* **2006**, *29*, 191-201.
- (96) Champagne, D. E.; Koul, O.; Isman, M. B.; Scudder, G. G. E.; Towers, G. H. N. *Phytochemistry* **1992**, *31*, 377-394.
- (97) Munakata, K. *Pure Appl. Chem.* **1975**, *42*, 57-66.
- (98) Mordue, A. J.; Blackwell, A. *J. Insect Physiol.* **1993**, *39*, 903-924.
- (99) Simmonds, M. S. J.; Blaney, W. M. *Entomol. Exp. Appl.* **1996**, *80*, 23-26.
- (100) Klocke, J. A.; Lee, M. S.; Yamasaki, R. B.; EP Patent 0308044: **1989**.
- (101) Nasiruddin, M.; Mordue, A. J. *Entomol. Exp. Appl.* **1994**, *70*, 247-252.
- (102) Murugan, K.; Jeyabalan, D.; Kumar, N. S.; Babu, R.; Sivaramakrishnan, S.; Nathan, S. *S. Insect Sci. Appl.* **1998**, *18*, 157-162.
- (103) Nathan, S. S.; Kalaivani, K.; Murugan, K. *Acta trop.* **2005**, *96*, 47-55.
- (104) Govindachari, T. R.; Narasimhan, N. S.; Suresh, G.; Partho, P. D.; Gopalakrishnan, G.; Kumari, G. N. K. *J. Chem. Ecol.* **1995**, *21*, 1585-1600.
-

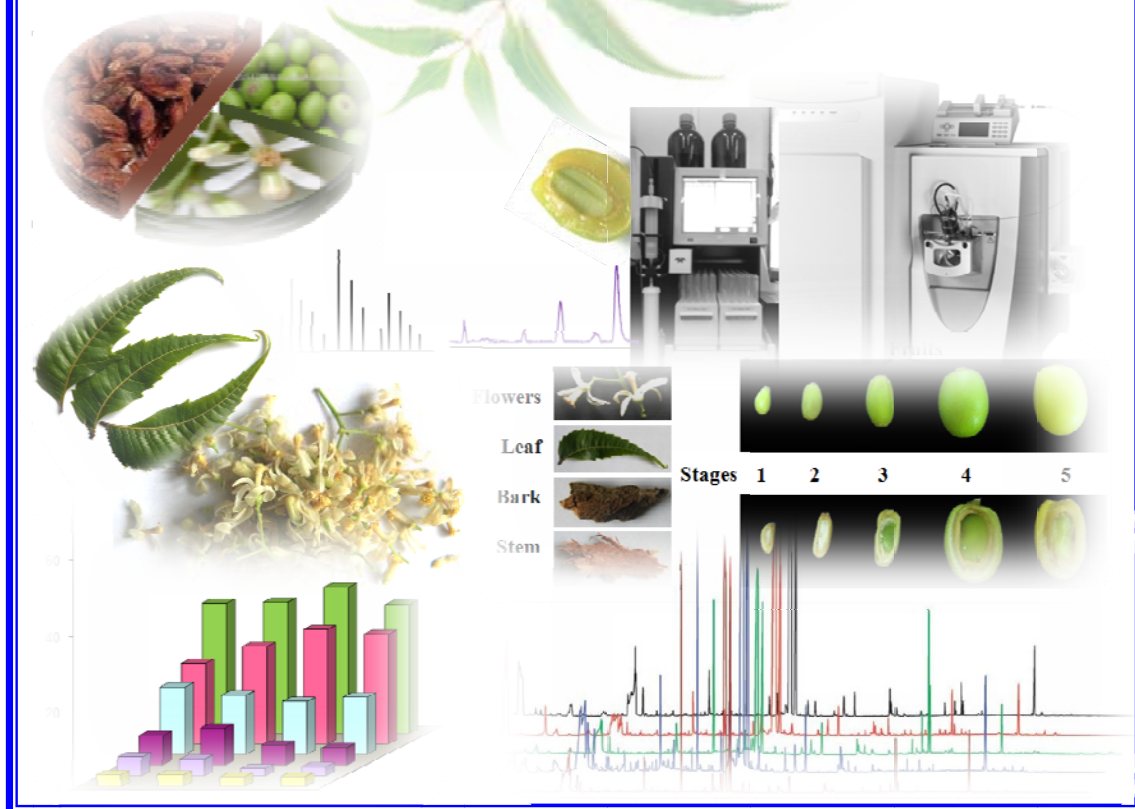
-
- (105) Lin ER, L. U. O.; Loon, J. J. A. v.; Schoonhoven, L. M. *Physiol. Entomol.* **1995**, *20*, 134-140.
- (106) Koul, O.; Shankar, J. S.; Kapil, R. S. *Entomol. Exp. Appl.* **1996**, *79*, 43-50.
- (107) Ruscoe, C. N. E. *Nat. New Biol.* **1972**, *236*, 159-160.
- (108) Schlueter, U. *Entomol. Exp. Appl.* **1985**, *39*, 191-195.
- (109) Koul, O. *Entomol. Exp. Appl.* **1984**, *36*, 85-88.
- (110) Garcia, E. S.; Gonzalez, M. S.; Azambuja, P.; Rembold, H. *Naturforsch. C* **1989**, *44*, 317-322.
- (111) Josephraj Kumar, A.; Subrahmanyam, B.; Srinivasan, S. *Eur. J. Entomol.* **1999**, *96*, 347-353.
- (112) Schlueter, U.; Bidmon, H. J.; Grewe, S. *J. Insect Physiol.* **1985**, *31*, 773-777.
- (113) Cottee, P. K.; Evans, K. A. *Physiol. Entomol.* **1985**, *10*, 431-437.
- (114) Timmins, W. A.; Reynolds, S. E. *Entomol. Exp. Appl.* **1992**, *63*, 47-54.
- (115) Nathan, S. S.; Kalaivani, K.; Chung, P. G.; Murugan, K. *Chemosphere* **2006**, *62*, 1388-1393.
- (116) Nathan, S. S.; Kalaivani, K.; Sehoon, K.; Murugan, K. *Chemosphere* **2006**, *62*, 1381-1387.
- (117) Govindachari, T. R.; Narasimhan, N. S.; Suresh, G.; Partho, P. D.; Gopalakrishnan, G. *J. Chem. Ecol.* **1996**, *22*, 1453-1461.
- (118) Kubo, I.; Klocke, J. *ACS Symp. Ser.* **1986**, *296*, 206-219.
- (119) Paul, R.; Prasad, M.; Sah, N. K. *Cancer Biol. Ther.* **2011**, *12*, 467-476.
- (120) Kikuchi, T.; Ishii, K.; Noto, T.; Takahashi, A.; Tabata, K.; Suzuki, T.; Akihisa, T. *J. Nat. Prod.* **2011**, *74*, 866-870.
- (121) Nanduri, S.; Thunuguntla, S. S. R.; Nyavanandi, V. K.; Kasu, S.; Kumar, P. M.; Ram, P. S.; Rajagopal, S.; Kumar, R. A.; Deevi, D. S.; Rajagopalan, R.; Venkateswarlu, A. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4111-4115.
- (122) Brandt, G. E. L.; Schmidt, M. D.; Prisinzano, T. E.; Blagg, B. S. J. *J. Med. Chem.* **2008**, *51*, 6495-6502.
- (123) Brandt, G. E. L.; Blagg, B. S. J. *Curr. Top. Med. Chem.* **2009**, *9*, 1447-1461.
- (124) Uddin, S. J.; Nahar, L.; Shilpi, J. A.; Shoeb, M.; Borkowski, T.; Gibbons, S.; Middleton, M.; Byres, M.; Sarker, S. D. *Phytother. Res.* **2007**, *21*, 757-761.
- (125) Cohen, E.; Quistad, G. B.; Casida, J. E. *Life Sci.* **1996**, *58*, 1075-1081.
- (126) Roy, M. K.; Kobori, M.; Takenaka, M.; Nakahara, K.; Shinmoto, H.; Tsushida, T. *Planta Med.* **2006**, *72*, 917-923.
- (127) Roy, M. K.; Kobori, M.; Takenaka, M.; Nakahara, K.; Shinmoto, H.; Isobe, S.; Tsushida, T. *Phytother. Res.* **2007**, *21*, 245-250.
- (128) Kumar, G. H.; Mohan, K.; Rao, A. J.; Nagini, S. *Invest. New Drug* **2009**, *27*, 246-252.
- (129) Elumalai, P.; Gunadharini, D. N.; Senthilkumar, K.; Banudevi, S.; Arunkumar, R.; Benson, C. S.; Sharmila, G.; Arunakaran, J. *Toxicol. Lett.* **2012**, *215*, 131-142.
- (130) Kavitha, K.; Priyadarsini, R. V.; Anitha, P.; Ramalingam, K.; Sakthivel, R.; Purushothaman, G.; Singh, A. K.; Karunakaran, D.; Nagini, S. *Euro. J. Pharmacol.* **2012**, *681*, 6-14.
- (131) Priyadarsini, R. V.; Murugan, R. S.; Sripriya, P.; Karunakaran, D.; Nagini, S. *Free Radical Res.* **2010**, *44*, 624-634.
- (132) Kumar, G. H.; Priyadarsini, R. V.; Vinothini, G.; Letchoumy, P. V.; Nagini, S. *Invest. New Drug* **2010**, *28*, 392-401.
-

-
- (133) Chianese, G.; Yerbanga, S. R.; Lucantoni, L.; Habluetzel, A.; Basilica, N.; Taramelli, D.; Fattorusso, E.; Taglialatela-Scafati, O. *J. Nat. Prod.* **2010**, *73*, 1448-1452.
- (134) MacKinnon, S.; Durst, T.; Arnason, J. T.; Angerhofer, C.; Pezzuto, J.; SanchezVindas, P. E.; Poveda, L. J.; Gbeassor, M. *J. Nat. Prod.* **1997**, *60*, 336-341.
- (135) Omar, S.; Godard, K.; Ingham, A.; Hussain, H.; Wongpanich, V.; Pezzuto, J.; Durst, T.; Eklun, C.; Gbeassor, M.; Sanchez-Vindas, P.; Poveda, L.; Philogene, B. J. R.; Arnason, J. T. *Ann. Appl. Biol.* **2003**, *143*, 135-141.
- (136) Billker, O.; Shaw, M. K.; Jones, I. W.; Ley, S. V.; Mordue, A. J.; Sinden, R. E. *J. Eukaryotic Microbiol.* **2002**, *49*, 489-497.
- (137) Lee, S. E.; Kim, M. R.; Kim, J. H.; Takeoka, G. R.; Kim, T. W.; Park, B. S. *Phytomedicine* **2008**, *15*, 533-535.
- (138) Lakshmi, V.; Srivastava, S.; Mishra, S. K.; Srivastava, M. N.; Srivastava, K.; Puri, S. K. *Nat. Prod. Res.* **2012**, *26*, 1012-1015.
- (139) Hay, A. E.; Ioset, J. R.; Ahua, K. M.; Diallo, D.; Brun, R.; Hostettmann, K. *J. Nat. Prod.* **2007**, *70*, 9-13.
- (140) Maneerata, W.; Laphookhieo, S.; Koysoomboon, S.; Chantrapromma, K. *Phytomedicine* **2008**, *15*, 1130-1134.
- (141) Jones, I. W.; Denholm, A. A.; Ley, S. V.; Lovell, H.; Wood, A.; Sinden, R. E. *FEMS Microbiol. Lett.* **1994**, *120*, 267-273.
- (142) Akihisa, T.; Takahashi, A.; Kikuchi, T.; Takagi, M.; Watanabe, K.; Fukatsu, M.; Fujita, Y.; Banno, N.; Tokuda, H.; Yasukawa, K. *J. Oleo Sci.* **2009**, *60*, 53-59.
- (143) Ravangpai, W.; Sommit, D.; Teerawatananond, T.; Sinpranee, N.; Palaga, T.; Pengprecha, S.; Muangsin, N.; Pudhom, K. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4485-4489.
- (144) Penido, C.; Costa, K. A.; Costa, M. F. D.; Pereira, J. D.; Siani, A. C.; Henriques, M. *Int. Immunopharmacol.* **2006**, *6*, 109-121.
- (145) Ferraris, F. K.; Moret, K. H.; Figueiredo, A. B. C.; Penido, C.; Henriques, M. *Int. Immunopharmacol.* **2012**, *14*, 82-93.
- (146) Ilango, K.; Maharajan, G.; Narasimhan, S. *Nat. Prod. Res.* **2013**, *27*, 1463-1467.
- (147) Kaur, G.; Alam, M. S.; Athar, M. *Phytother. Res.* **2004**, *18*, 419-424.
- (148) Maity, P.; Biswas, K.; Chattopadhyay, I.; Banerjee, R. K.; Bandyopadhyay, U. *Phytother. Res.* **2009**, *23*, 747-755.
- (149) Pillai, N. R.; Santhakumari, G. *Planta Med.* **1984**, *50*, 143-146.
- (150) Lakshmi, V.; Singh, N.; Shrivastva, S.; Mishra, S. K.; Dharmani, P.; Mishra, V.; Palit, G. *Phytomedicine* **2010**, *17*, 569-574.
- (151) Govindachari, T. R.; Suresh, G.; Gopalakrishnan, G.; Banumathy, B.; Masilamani, S. *Phytoparasitica* **1998**, *26*, 109-116.
- (152) Rojanapo, W.; Suwanno, S.; Somjaree, R.; Glinsukon, T.; Thebtaranont, Y. *J. Sci. Soc. Thailand* **1985**, *11*, 177-181.
- (153) Kraus, W.; Maile, R.; Vogler, B.; Wundrak, B. *J. Indian Chem. Soc.* **1997**, *74*, 870.
- (154) Ekong, D. E. U.; Ibiyemi, S. A. *Phytochemistry* **1985**, *24*, 2259-2261.
- (155) Ekong, D. E. U.; Ibiyemi, S. A.; Olagbemi, E. O. *J. Chem. Soc. Chem. Commun.* **1971**, 1117.
- (156) Xu, R.; Fazio, G. C.; Matsuda, S. P. T. *Phytochemistry* **2004**, *65*, 261-291.
- (157) Siddiqui, S.; Siddiqui, B. S.; Faizi, S.; Mahmood, T. *J. Nat. Prod.* **1988**, *51*, 30-43.
- (158) Mitra, C. R.; Garg, H. S.; Pandey, G. N. *Tetrahedron Lett.* **1970**, 2761-&.
-

-
- (159) Taylor, D. A. H. *Progress in the Chemistry of Organic Natural Products*; Springer, New York, **1984**.
- (160) Rajab, M. S.; Bentley, M. D.; Alford, A. R.; Mendel, M. J. *J. Nat. Prod.* **1988**, *51*, 168-171.
- (161) Yamasaki, R. B.; Klocke, J. A.; Lee, S. M.; Stone, G. A.; Darlington, M. V. *J. Chromatogr.* **1986**, *356*, 220-226.
- (162) Schroeder, D. R.; Nakanishi, K. *J. Nat. Prod.* **1987**, *50*, 241-244.
- (163) Govindachari, T. R.; Sandhya, G.; Ganeshraj, S. P. *J. Chromatogr.* **1990**, *513*, 389-391.
- (164) Hull, C. J.; Dutton, W. R.; Switzer, B. S. *J. Chromatogr.* **1993**, *633*, 300-304.
- (165) Govindachari, T. R.; Gopalakrishnan, G.; Suresh, G. *J. Liq. Chromatogr. R. T.* **1996**, *19*, 1729-1733.
- (166) Johnson, S.; Morgan, E. D. *J. Chromatogr. A* **1997**, *761*, 53-63.
- (167) Schaaf, O.; Jarvis, A. P.; van der Esch, S. A.; Giagnacovo, G.; Oldham, N. J. *J. Chromatogr. A* **2000**, *886*, 89-97.
- (168) Sundaram, K. M. S.; Sloane, L.; Curry, J. *J. Liq. Chromatogr.* **1995**, *18*, 363-376.
- (169) Jarvis, A. P.; Morgan, E. D. *Phytochem. Anal.* **2000**, *11*, 184-189.
- (170) Sharma, V.; Walia, S.; Kumar, J.; Nair, M. G.; Parmar, B. S. *J. Agric. Food Chem.* **2003**, *51*, 3966-3972.
- (171) Silva, J. C. T.; Jham, G. N.; Oliveira, R. D. L.; Brown, L. *J. Chromatogr. A* **2007**, *1151*, 203-210.
- (172) Sarais, G.; Caboni, P.; Sarritzu, E.; Russo, M.; Cabras, P. *J. Agric. Food Chem.* **2008**, *56*, 2939-2943.
- (173) Ley, S. V.; Abad-Somovilla, A.; Anderson, J. C.; Ayats, C.; Banteli, R.; Beckmann, E.; Boyer, A.; Brasca, M. G.; Brice, A.; Broughton, H. B.; Burke, B. J.; Cleator, E.; Craig, D.; Denholm, A. A.; Denton, R. M.; Durand-Reville, T.; Gobbi, L. B.; Gobel, M.; Gray, B. L.; Grossmann, R. B.; Gutteridge, C. E.; Hahn, N.; Harding, S. L.; Jennens, D. C.; Jennens, L.; Lovell, P. J.; Lovell, H. J.; de La Puente, M. L.; Kolb, H. C.; Koot, W. J.; Maslen, S. L.; McCusker, C. F.; Mattes, A.; Pape, A. R.; Pinto, A.; Santafianos, D.; Scott, J. S.; Smith, S. C.; Somers, A. Q.; Spilling, C. D.; Stelzer, F.; Toogood, P. L.; Turner, R. M.; Veitch, G. E.; Wood, A.; Zumbunn, C. *Chem. Eur. J.* **2008**, *14*, 10683-10704.
- (174) Veitch, G. E.; Boyer, A.; Ley, S. V. *Angew. Chem. Int. Ed.* **2008**, *47*, 9402-9429.
- (175) Veitch, G. E.; Beckmann, E.; Burke, B. J.; Boyer, A.; Maslen, S. L.; Ley, S. V. *Angew. Chem. Int. Ed.* **2007**, *46*, 7629-7632.
- (176) Boyer, A.; Veitch, G. E.; Beckmann, E.; Ley, S. V. *Angew. Chem. Int. Ed.* **2009**, *48*, 1317-1320.
- (177) Veitch, G. E.; Pinto, A.; Boyer, A.; Beckmann, E.; Anderson, J. C.; Ley, S. V. *Org. Lett.* **2008**, *10*, 569-572.
- (178) Heasley, B. *Eur. J. Org. Chem.* **2011**, 19-46.
- (179) Corey, E. J.; Hahl, R. W. *Tetrahedron Lett.* **1989**, *30*, 3023-3026.
- (180) Behenna, D. C.; Corey, E. J. *J. Am. Chem. Soc.* **2008**, *130*, 6720-6721.
- (181) Mateos, A. F.; Barba, A. M. L.; delaNava, E. M.; Coca, G. P.; Alonso, J. P.; Silvo, A. I. R.; Gonzalez, R. R. *Tetrahedron* **1997**, *53*, 14131-14140.
- (182) Mateos, A. F.; Coca, G. P.; Gonzalez, R. R. *Tetrahedron* **2005**, *61*, 8699-8704.

Chapter 2

Isolation, Characterization and Metabolic Profiling of Limonoids from Neem



Abstract

Chapter 2 comprises of three sections. The chapter describes UPLC-ESI(+)-HRMS based tissue and stage specific metabolic profiling of limonoids in Neem using fifteen isolated triterpenoids as the reference standards. Further, the major limonoids abundant in fruit-coat and Neem oil have been isolated in preparative scale by expedient MPLC based protocol and they were structurally characterized through ESI(+)-quadrupole/orbitrap-MS/MS technique.

Chapter 2: Section A

Isolation and Metabolic Profiling of Limonoids from Neem

Highlights:

- *Fifteen triterpenoids were isolated and characterized from Neem seed kernel and fruit-coat.*
- *Triterpenoid content of various Neem tissues and their developmental stages was extracted.*
- *Crude triterpenoid mixture was resolved through UPLC-ESI(+)-HRMS and the level of metabolites was quantified utilizing standard graphs drawn for individual triterpenoids.*

2A.1 Introduction

Secondary metabolites and their distribution pattern is a typical characteristic of every life in nature and it can be considered as an intrinsic signature of that species. Metabolic finger-printing is all about the structural investigation of metabolites, their distribution and quantification among different tissues, stages and conditions.¹⁻¹² Quantitative finger-printing of secondary metabolites across different tissues of a species can be useful to predict the comparative expression levels of genes involved in the biosynthetic pathway of those metabolites. This information may be helpful to delineate the plausible biosynthetic pathway and identify the genes of interest from the targeted tissues. Secondly, quantitative finger-printing provides a clear idea about the abundance of different metabolites across various tissues and developmental stages. Fruitful time to harvest the plant material for isolation and commercialization of the products can be decided from the finger-printing information with an ease. Again, the quantification of secondary metabolites can lead to the discovery of a better plant source with richer abundance of specific metabolites.

Limonoids in Neem are diverse in skeletal architecture, huge in count and their abundance is highly tissue-specific. It will be of high importance to quantify the limonoids across various tissues and stages of Neem plant which may enlighten the probable biosynthetic pathway of complex limonoids, genes associated and their expression levels. As described earlier, azadirachtin A, B, salannin, nimbin and their deacetylated analogues have been quantified using HPLC-UV, HPLC-APCI-MS and HPLC-ESI-MS with high sensitivity from different sources including Neem tissues, various fruits and vegetables. Jhonson *et al.* in 1996 had investigated the variability of five major limonoids (namely azadirachtin A, salannin, nimbin, 3-deacetylsalannin, 6-deacethnimbin) and oil content in different growing stages of Neem seeds.¹³ Sidhu *et al.* in 2003 had examined oil content and HPLC based quantification of azadirachtin A and B in the Neem seeds from 43 different provinces of India.¹⁴ Except few discrete studies, there are no systematic investigations on the tissue- and stage-specific quantitative variation of Neem limonoids.

In the present study, fifteen triterpenoids from Neem fruits have been isolated using chromatographic techniques including HPLC and flash-chromatography. They were structurally characterized by various spectroscopic (NMR and HRMS) data. Six

major limonoids were isolated in gram-scale applying a common isolation protocol, which were further useful to investigate anti-inflammatory activities *in vitro* and *in vivo*. Further, isolated fifteen limonoids were quantified in various tissues of Neem such as flowers, bark, stem, leaves and five developing stages of fruit-coats and seed kernels using the standard graphs prepared for purified metabolites on UPLC-ESI(+)-HRMS.

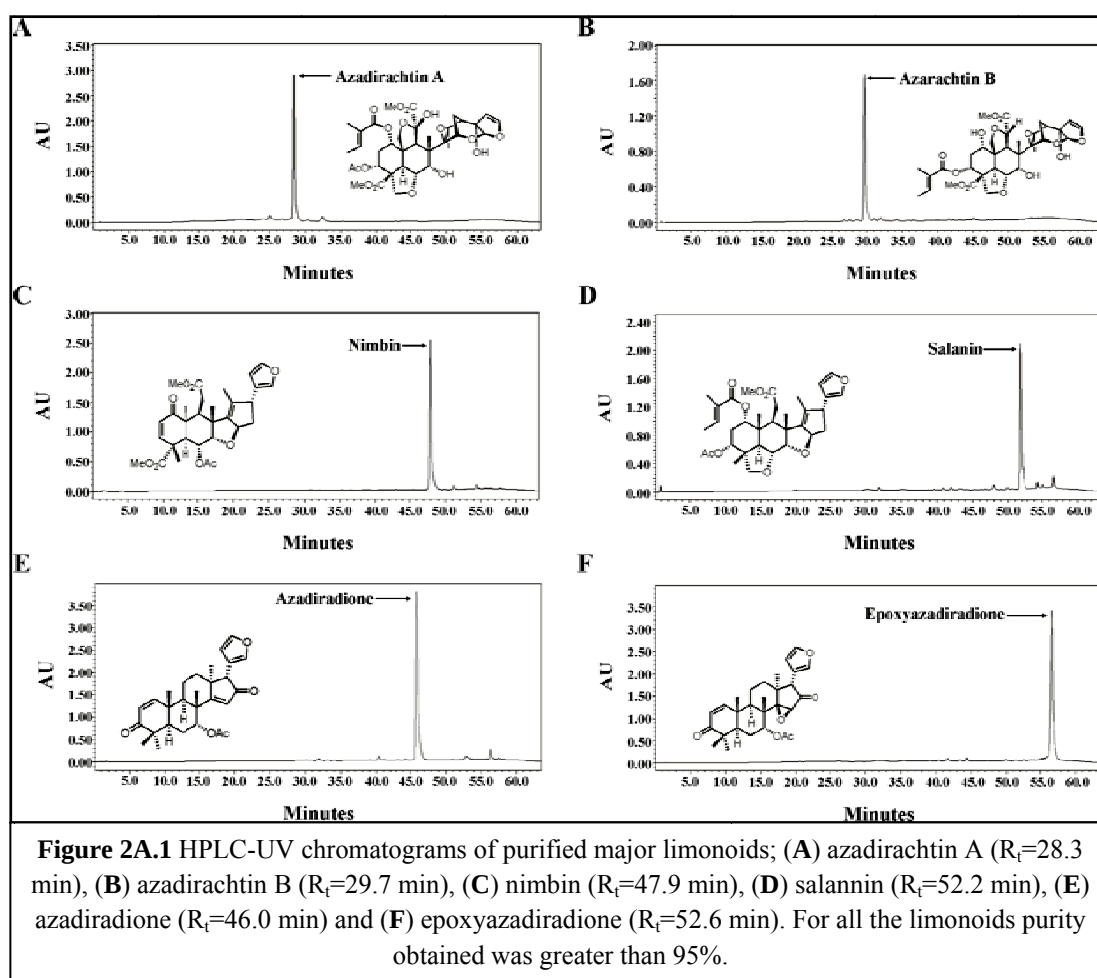
2A.2 Results and Discussion

2A.2.1 Isolation of Limonoids from Neem Fruit-coats and Seed Kernels

Extraction of dried Neem seed kernel resulted in the isolation of four major limonoids (Azadirachtin A, azadirachtin B, salannin, nimbin) and six minor triterpenoids (3-deacetylsalannin, 6-deacetylnimbin, nimbinene, 6-deacetylnimbinene, nimbanal, salannol acetate). Crude triterpenoid mixture was obtained by stepwise solvent partition technique. The sequential procedures of de-oiling, extraction of methanol-soluble secondary and primary metabolites followed by separation of triterpenoids by water-EtOAc partition were performed to yield the complex mixture of triterpenoids from seed kernels. Major constituents were purified by flash chromatographic technique from the crude triterpenoid mixture whereas purification of minor triterpenoids was achieved by repeated chromatographic techniques including semi-preparative reverse-phase HPLC-UV, flash chromatography and preparative TLC. Optimized separation protocol can be utilized for the gram-scale isolation of major limonoids from Neem seed-kernels with high purity. The purity of azadirachtin A, azadirachtin B, nimbin, salannin was 98.8%, 99.1%, 99.1% and 98.2% respectively, as determined by HPLC (Figure 2A.1).

Dried fruit-coats were extracted to isolate two major limonoids (azadiradione and epoxyazadiradione) and three minor limonoids (azadirone, gedunin and nimocinol). Separation protocol was similar to the seed-kernel except slight alternation. Since the oil content in fruit-coat is far less than the kernel, the process of de-fatting was not essential in this case. Significant difference in polarity and less complexity of the limonoid-mixture present in the fruit-coats led to the excellent separation of the components through flash chromatographic technique. The HPLC-purity of major isolated limonoids namely azadiradione and epoxyazadiradione was determined to be 98.9% and 99.6% respectively (Figure 2A.1).

The isolation protocol reported for the Neem limonoids till date are mainly focused on the isolation of azadirachtin A and its derivatives (Chapter 1). But, very less effort have been made for the preparative-scale isolation of other major limonoids from Neem fruits. Here in, the developed scheme can be exploited for the large-scale isolation of salannin, nimbin, azadiradione and epoxyazadiradione along with azadirachtin A and B with high purity. The gram-scale access to these molecules may facilitate the detailed biological investigations both *in vitro* and *in vivo*.



2A.2.2 Characterization of Neem Triterpenoids

Purified triterpenoids were characterized by the analyses of NMR spectrometric and HR-ESI(+)-MS data, which were in full agreement with the previously reported. Ring-intact limonoids were characterized by the presence of C-17 furan moiety and five quaternary methyl groups at C-18, 19, 28, 29 and 30. Characterized signals in ^1H (δ_{H} : 7.35-7.55 for H-21, 7.10-7.45 for H-23 and 6.25-6.35 for H-22) and ^{13}C NMR (δ_{C} :

~142, 140, 115-125 and 110 for 23, 21, 20 and 22 respectively) spectra were utilized to identify the presence of furan ring at C-17. Presence of five quaternary methyl groups at C-18, 19, 28, 29 and 30 were evident from ^1H and ^{13}C NMR signals within the chemical shift values ranging δ_{H} 0.80-1.40 and δ_{C} 15-25 respectively. The ubiquitous presence of an oxygenated functional group (hydroxyl or acetate in most cases) at C-7 (δ_{H} : 4.50-5.50 and δ_{C} : 65-75) was also observed in the ring-intact limonoid skeleton. Basic limonoids from azadirone group showed the presence of α,β -unsaturated carbonyl in the ring-A and a five membered ring-D. The presence of α,β -unsaturated carbonyl functionality was identified by the signals of two doublets (δ_{H} : 7.10-7.20 and 5.85-5.90) in ^1H NMR and the presence of unsaturated carbons (δ_{C} : 155-160 and 125-126) and carbonyl (δ_{C} : 204-205) in ^{13}C NMR. The presence of six-membered lactone D-ring as observed in gedunin skeleton was confirmed by the higher chemical shift values of C-17, both in ^1H (δ_{H} : ~5.50) and ^{13}C (δ_{C} : ~80) NMR spectra.

C-seco limonoids of salannin and nimbin skeletons were identified by the characteristic framework of ring C/D, the presence of furan moiety at C-17 and oxygen substituted C-6 and C-7. The unsaturation (C₁₃₋₁₄) in the ring C, characterized by ^{13}C NMR (δ_{C} : ~146 and 135 for C-14 and 13 respectively) spectra was a unique identity in these type of C-seco limonoid skeletons. Further, the C-D ring junction can be confirmed on the basis of signals for 15-C in ^1H (δ_{H} : 5.40-5.60) and ^{13}C (δ_{C} : ~87) NMR. Presence of C-7 and C-8 is indicated by the signals in ^1H (δ_{H} : 4.00-4.20 for H-7 and 4.00-5.20 for H-6) and ^{13}C (δ_{C} : 80-85 for C-7 and 65-75 for C-6) NMR spectra. The presence of carbonyl group at C-12 adjacent to methylene carbon (C-11) is also a common characteristic of C-seco limonoids. Nimbin type C-seco limonoid skeleton can be elucidated by the presence of α,β -unsaturated (C₂₋₃) ketone functionality in ring A (δ_{H} of doublets: 5.85-6.05 for H-2 and 6.10-6.50 for H-3; δ_{C} : ~201 for C-1, 125-130 for C-2, 140-150 for C-3) and a carbonyl group at C-28 (δ_{C} 170-205). Salannin type skeleton can be categorized by the presence of tigloyl moiety or further modified tigloyl derivative at C-1, cyclic ether ring (C-28-4-5-6) and an oxygenated substitution (acetate/hydroxyl) at C-3. In ^1H NMR, signals for the tigloyl group appear at δ_{H} 6.90-7.00 (H-3'), 1.80-1.85 (Me-4') and 1.90-1.95 (Me-5'). In ^{13}C NMR, corresponding signals can be found in the range δ_{C} ~166 (C-1'), 128 (C-2'), 137-139 (C-3'), 10-15 (C-4', 5'). Cyclic ether ring can be identified by the presence of C-28 through ^1H (δ_{H} of doublet: 3.60-3.70) and ^{13}C (δ_{C} : ~77) NMR. Oxygen substituted C-3 appears at δ_{H} 3.75-

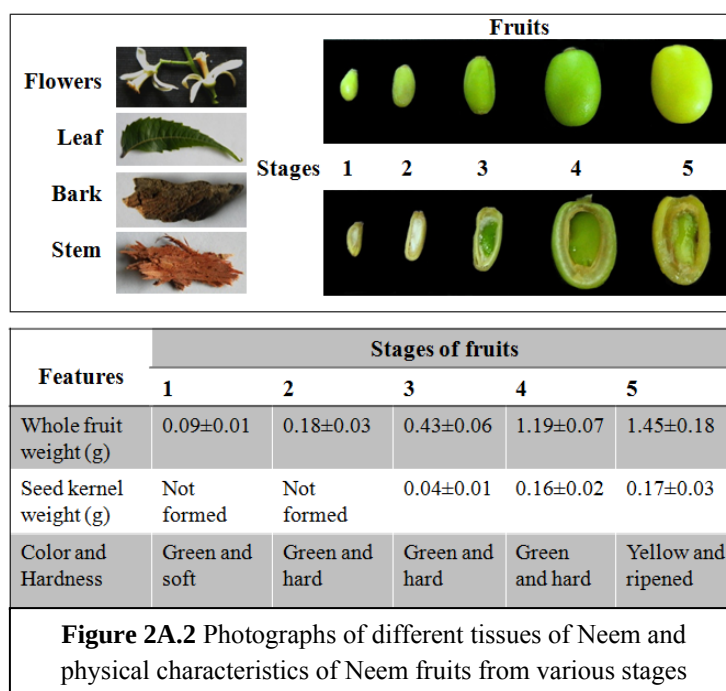
5.00 and $\delta_C \sim 70$. Pentanortriterpenoids of nimbinene skeleton can be characterized by the removal of Me-28 and the repositioning of unsaturation (C_{3-4}) (δ_H 5.40-5.50 for H-3; $\delta_C \sim 120$ for C-3 and 135-140 for C-4) in ring A.

Azadirachtin skeleton is characterized by the typical construction of rearranged C/D and furan ring. The unsaturation at C_{22-23} is identified by the signals at $\delta_H \sim 6.45$ (d, H-23) and 5.03 (d, H-22) in ^1H NMR. ^{13}C NMR chemical shift values ($\delta_C \sim 147$ for C-23 and 107 for C-22) also support the presence of C_{22-23} double bond. The existence of characteristic ring junction at C-20-21 can be identified by the signal at $\delta_H \sim 5.65$ (H-21) and $\delta_C \sim 108$ (C-21), 83 (C-20). The presence of cyclic five-membered ether ring (C-11-9-10-19) can be identified by the higher chemical shift values for C-11 (δ_C 75-105) and C-19 (δ_H 4.00-4.20; $\delta_C \sim 70$). Ring A and B contain various characteristic functional groups (similar to salannin skeleton) such as tigloyl, esters and hydroxyls with variability in their positions creating a large number of azadirachtin derivatives. The presence of cyclic ether joining the rings A/B (as in salannin type limonoid) and 7-hydroxyl (as in azadirone type limonoid) is a common structural feature of azadirachtin skeleton.

2A.2.3 Limonoid Profiling of Neem Tissues

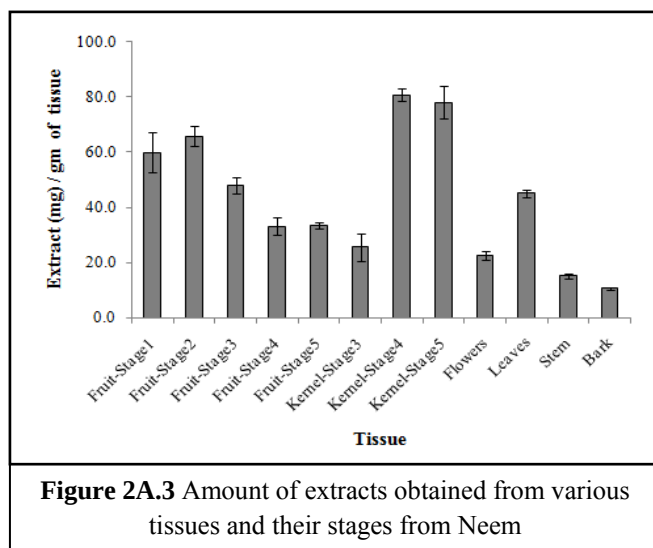
The levels of individual fifteen triterpenoids were determined in different tissues of Neem including flowers, leaves, stem, bark, 5 developmental stages of fruit-coats (fruit without kernel) and 3 stages of seed kernels. The division of fruits in various developmental stages was performed by considering the colour, weight and hardness of the fruits (Figure 2A.2). The crude mixture of triterpenoids were extracted from various fresh tissues of Neem using solvent partition technique and they were analyzed by UPLC-ESI(+)-HRMS (dissolving in methanol) in a gradient solvent program of methanol-water. Amount of crude extract obtained was directly correlated with the triterpenoid content of the corresponding tissue (Figure 2A.3). Quantification of the crude extract revealed that seed kernel of stages 4 and 5 contained the highest amount of triterpenoids (~ 80 mg/g of the tissue) followed by fruit-coats of stages 1, 2 and 3 (~ 48 -66 mg/g). Fruit-coats of stages 4, 5 and kernels of stage 3 were found to possess lesser amount of triterpenoids within the range of ~ 25 -35 mg/g. Flowers, leaves, stem and bark furnished ~ 22 , 45 (including chlorophylls and other pigments), 15 and 10 mg of

crude extract per gram of the tissue respectively. In fact, the chromatograms of tissues from stem and bark showed high abundance of secondary metabolites other than triterpenoids. Further, standard graphs were drawn for each of the fifteen isolated triterpenoids with regression coefficient greater than 0.99 (within the concentration range 0.04 to 0.003 mg/mL with injection volume 5 μ L) in UPLC-ESI(+)-HRMS (Figure 2A.4) and they were further utilized for the quantification of individual molecules in the extracts of different tissues of Neem by correlating with the area under respective peaks of extracted ion chromatograms.



Here in, we have studied the level of individual triterpenoids across different tissues of Neem plant (Figure 2A.5 and 2A.6). Azadirachtin A, the most documented Neem limonoid till date was found to be highly abundant in the seed kernels especially in the stages 4 and 5 (within the range of 3.7-3.8 mg/g of the tissue). Fruit-coats, flowers and leaves showed 100-500 fold lesser level ($\sim$ 0.005-0.04 mg/g) of azadirachtin A in comparison to seed kernel, whereas bark and stem contained very negligible quantity ($<$ 0.005 mg/g, 1000 fold lesser than kernel) of azadirachtin A. Similar distribution was observed with the levels of azadirachtin B. Highest level of azadirachtin B was observed in seed kernels of stages 4 and 5 (0.7-0.8 mg/g), whereas fruit-coats and flowers showed 100-150 fold lesser abundance in comparison. The level of azadirachtin B in stem and bark was highly negligible ($<$ 0.005 mg/g, 1000 fold lesser than kernel). Salannin

showed highest level of abundance in seed kernels of stages 4 and 5 (2.7-2.9 mg/g). Next higher level of salannin was observed in stem with ~0.7 mg/g of the tissue (4 fold lesser compared to kernel). Bark contained salannin with a level of ~0.08 mg/g which was 35 fold lesser compared to kernel. Flowers, leaves and fruit-coats showed highly negligible level of salannin (< 0.04 mg/g). Highest abundance of 3-deacetylsalannin was observed in seed kernel of stages 4, 5 and stem within the range of 0.02-0.03 mg/g of



the tissue. Other tissues did not show considerable quantity of 3-deacetylsalannin. Nimbin was observed mainly in seed kernels ranging from 0.3 to 0.4 mg/g and other parts with none or negligible quantity. The presence of 6-deacetylnimbin was observed in seed kernel of stages 4 and 5 along with leaves (0.14-0.30 mg/g). Very minor level was observed in fruit-

coats, flowers, stem and bark (< 0.04 mg/g). Nimbinene and 6-deacetylnimbinene, two pentanortriterpenoids exhibited similar pattern of distribution throughout different tissues. Highest abundance was observed in seed kernels of stages 4, 5 and stem within the range of 0.2-0.6 mg/g. Flowers and leaves showed minor quantity (0.05-0.09 mg/g), whereas bark and fruit-coats exhibited negligible level. Nimbanal showed higher level in seed kernel of stages 4, 5 and stem (0.07-0.15 mg/g) and the other parts showed very negligible levels. Salannol acetate was found to be most abundant in seed kernels and stem within the range of 0.3-0.4 mg/g and other tissues showed very minor abundances. Ring-intact limonoids azadirone, azadiradione, epoxyazadiradione and gedunin exhibited similar distribution profile across various tissues. Azadiradione showed highest level of abundance (10.0-20.0 mg/g) in all five developmental stages of fruit-coats especially in the stages 2 and 3 (19.0-20.0 mg/g) which was 100-200 fold higher than seed kernels and flowers (0.04-0.10 mg/g). Other tissues contained very negligible amount (< 0.002 mg/g). Similarly, epoxyazadiradione showed 200-300 times higher level in fruit-coats (6.0-17.0 mg/g, stage 2 and 3 with 13.0-17.0 mg/g) in comparison to seed kernels (0.02-0.06 mg/g) and 30-60 times than flowers (~0.30 mg/g). Azadirone

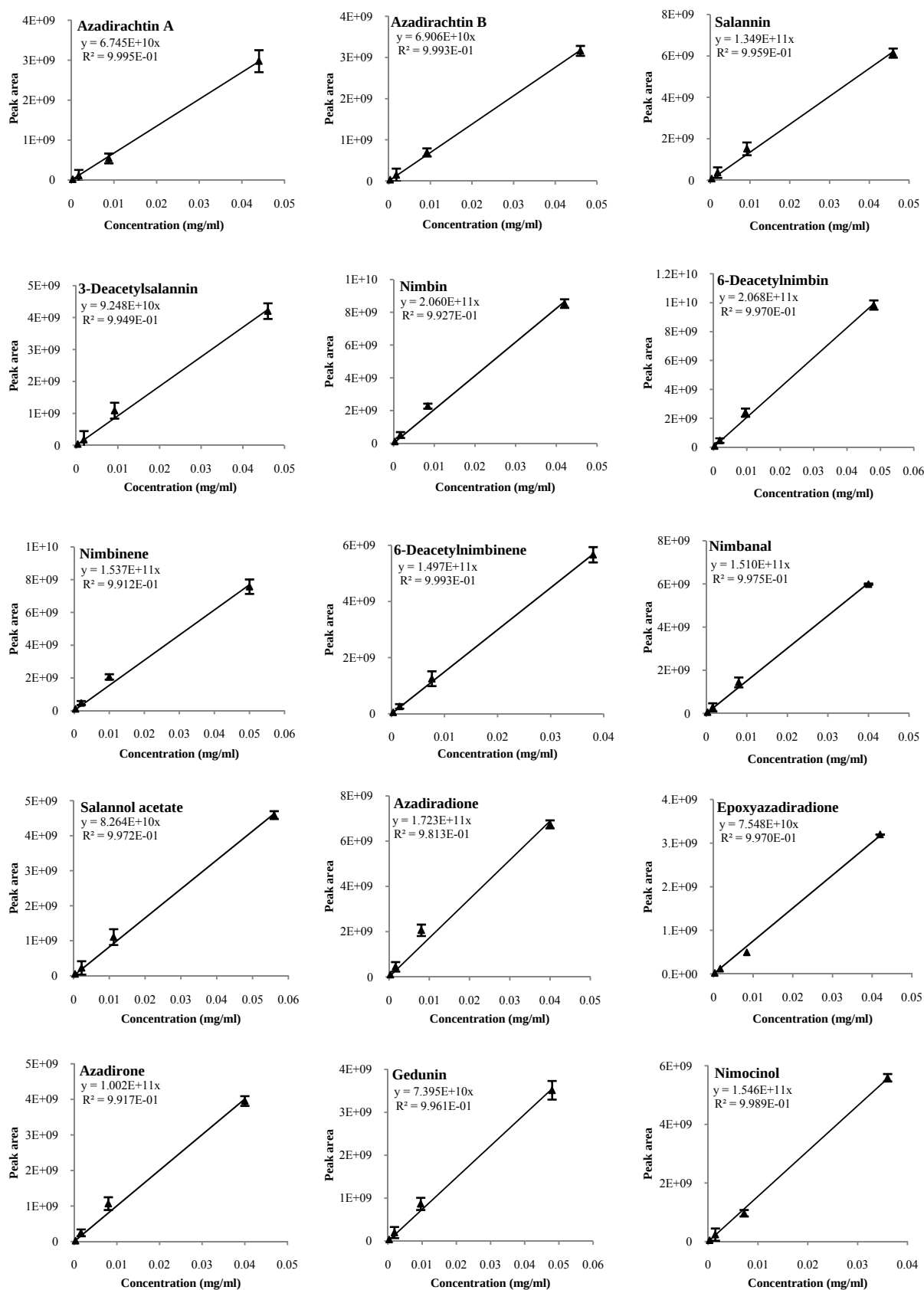
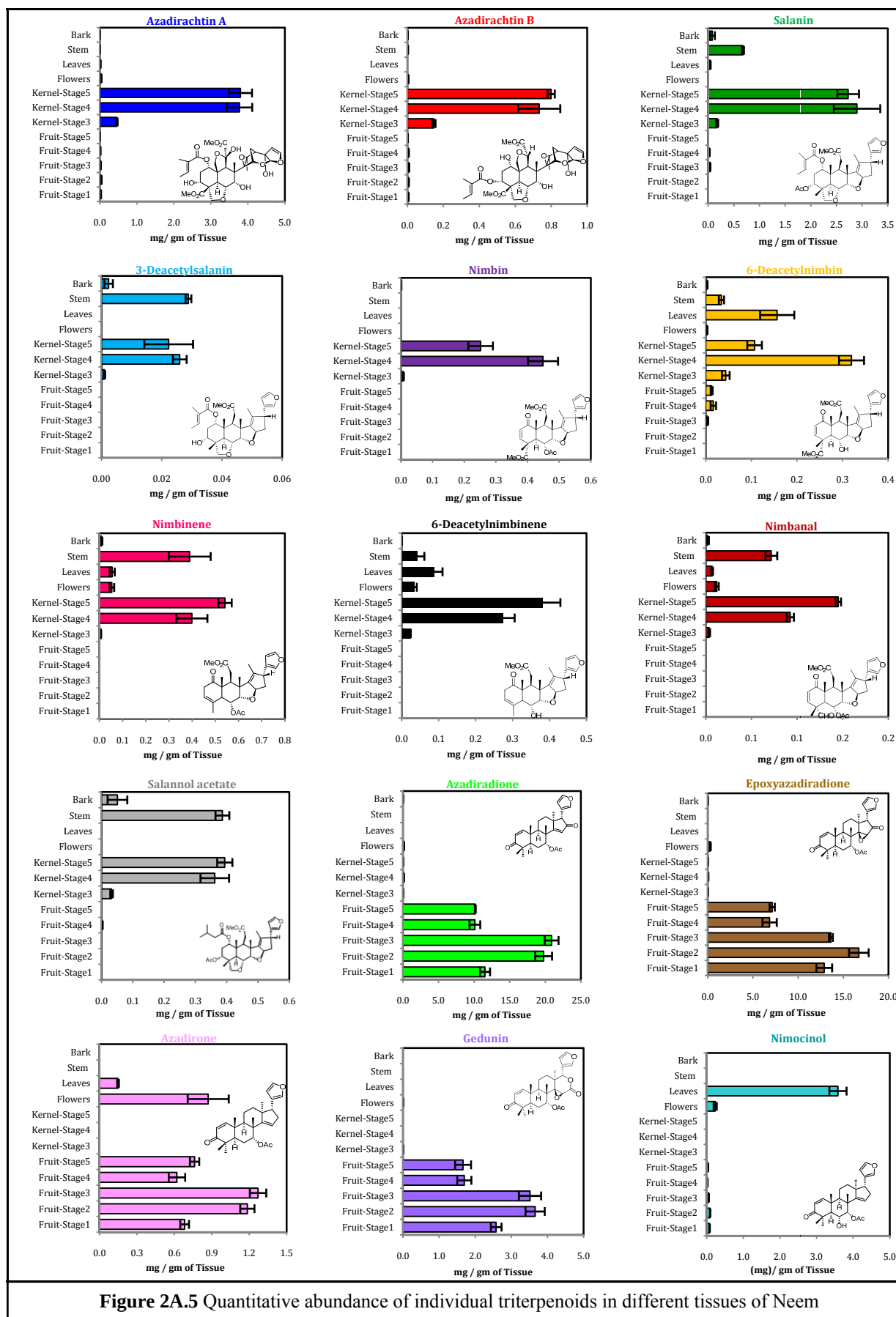


Figure 2A.4 Standard graphs for the purified triterpenoids prepared in UPLC-ESI(+)-HRMS; concentration range 0.04-0.003 mg/mL, injection volume 5 μ L



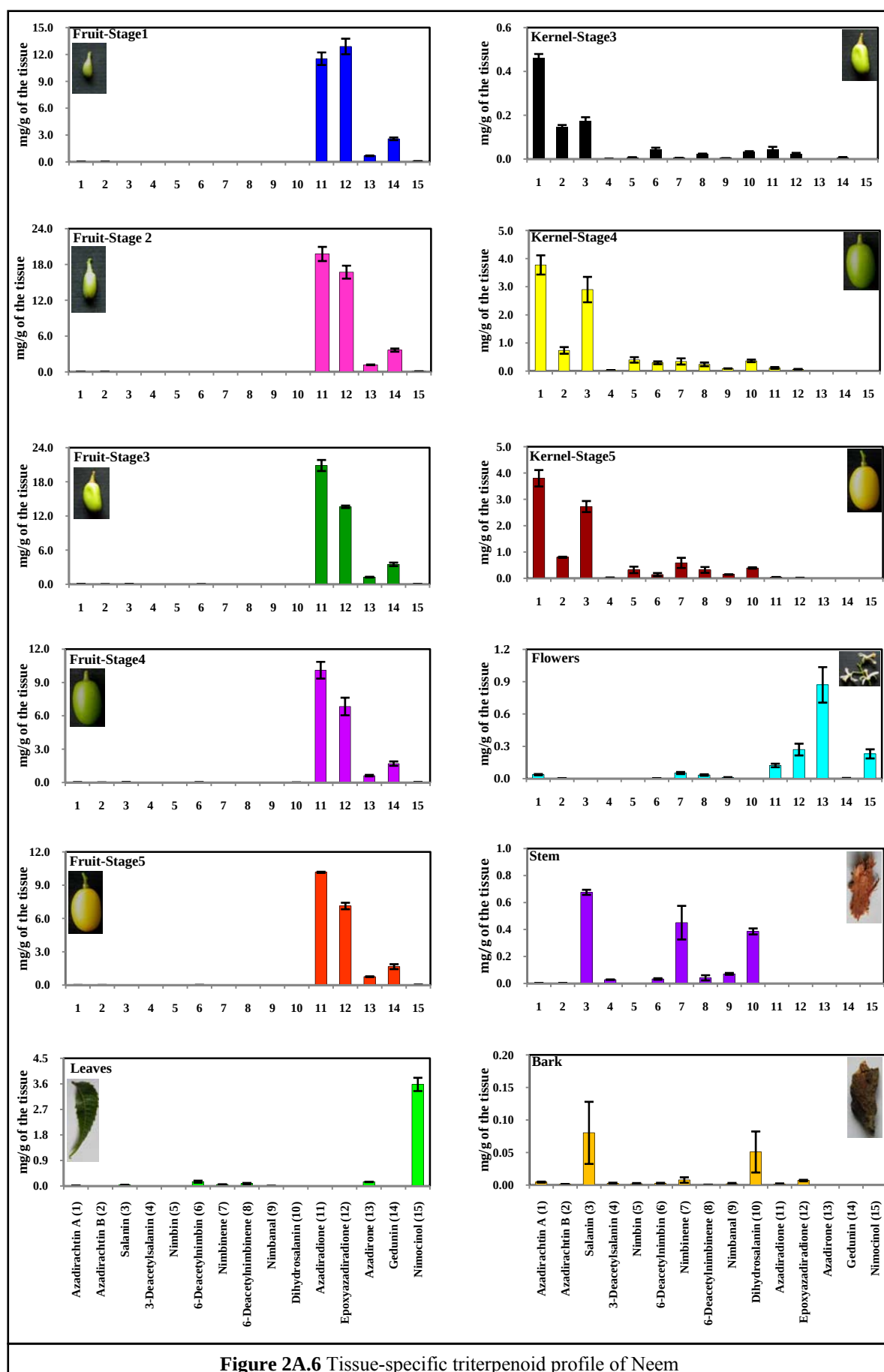


Figure 2A.6 Tissue-specific triterpenoid profile of Neem

was also found to be most abundant in all the developmental stages of fruit-coats (0.60-1.20 mg/g) specifically in the stages 2 and 3 (1.2-1.3 mg/g) and flowers (0.9 mg/g). Leaves showed very minor quantity (~0.15 mg/g) whereas other tissues exhibited very negligible level (< 0.001 mg/g). Gedunin, a potent anti-carcinogenic limonoid showed the highest level of abundance in fruit-coats, specifically in the stages 2 and 3 (3.5-3.7 mg/g). Other tissues were observed to be negligibly abundant with gedunin (< 0.01 mg/g). Nimocinol, 6 α -hydroxy derivative of azadirone was observed to be most abundant in leaves (3.6 mg/g) which was 15 fold higher than flowers (0.23 mg/g) and 50-180 times higher than fruit-coats (0.02-0.09 mg/g). Other tissues such as seed kernels, bark and stem showed very negligible abundance (< 0.001 mg/g).

2A.3 Conclusion

In brief, six major Neem limonoids namely azadirachtin A, azadirachtin B, salannin, nimbin, azadiradione and epoxyazadiradione were isolated in gram-scale with high purity using the combination of solvent-partition and flash-column chromatographic technique from the seed-kernels and fruit-coats. Another nine minor triterpenoids were also purified and characterized. Total fifteen isolated triterpenoids were further quantified by UPLC-ESI(+)-HRMS technique in various tissues (3 stages of kernel, 5 stages of fruit-coat, flowers, leaves, bark and stem) of Neem plant using the purified triterpenoids as the reference standards. Highest amount of triterpenoid content was observed in matured seed kernels followed by fruit-coats of initial stages. Tissues of flowers, leaves, stem and bark were relatively low in triterpenoid content. Fingerprinting data depicted C-seco limonoids to be the major constituents of triterpenoid pool from seed-kernel, stem and bark. Ring-intact limonoids were observed to be major metabolites of the triterpenoid mixture obtained from fruit-coats, flowers and leaves.

2A.4 Experimental Section

2A.4.1 General Experimental Procedures

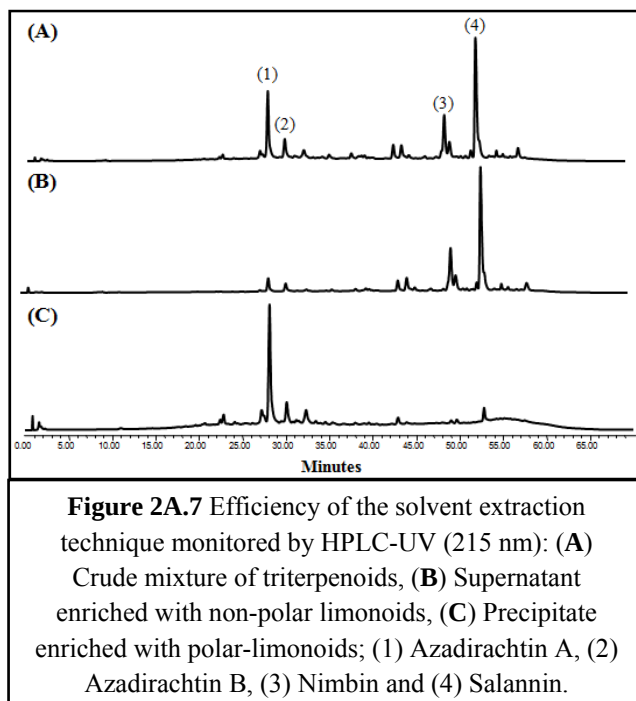
For the extraction and purification, required solvents were distilled prior to use. Solvents and chemicals were procured from Merck. For UPLC-ESI-HRMS experiments, LC-MS grade solvents were purchased from JT Baker. Thin layer

chromatography (TLC) was performed on pre-coated Merck silica gel plates (TLC Silica gel 60 F₂₅₄) using the solvent system dichloromethane: methanol (97:3) and the spots were visualized under UV lamp and developed using anisaldehyde charring reagent (92% ethanol, 3.2% anisaldehyde, 2.8% H₂SO₄ and 2% AcOH). For the preparative chromatography, 230-400 mesh silica gel was procured from Merck. Neem seeds for the isolation of compounds were purchased locally. NMR spectra were recorded in Bruker AV-400 (400MHz) spectrometer and the residual solvent signal was used as the reference. HPLC runs were performed on Waters HPLC system fitted with Merck, Chromolith RP-18C column (100-4.6 mm). For detection, Waters 2489 UV/Visible detector was used at 215 nm. A gradient program of water and methanol (0 min, 1% methanol/water; 15 min, 40% methanol/water; 30 min, 50% methanol/water, 45 min, 60% methanol/water; 50 min, 70% methanol/water; 55 min, 60% methanol/water; 60 min, 1% methanol/water; 65 min, 1% methanol/water) was employed as the mobile phase with a flow rate of 1 mL/min throughout the run for analytical injections. Neem fruits for limonoid finger-printing were collected from Pune region in Maharashtra during March to May. UPLC-ESI(+)-HRMS runs were performed on Q Exactive Orbitrap associated with Accela 1250 pump made by Thermo Scientific. Samples were resolved through Acquity BEH C18 UPLC column (2.1×100 mm) of particle size 1.7 μ M with a flow rate of 0.3 mL/min and gradient solvent program of 35 min (0.0 min, 40% methanol/water; 5.0 min, 50.0% methanol/water; 10.0 min, 60% methanol/water; 25.0 min, 65% methanol/water; 30.0 min, 90% methanol/water; 32.0 min, 90% methanol/water; 34.0 min, 40% methanol/water; 35.0 min, 40% methanol/water containing 0.1% formic acid). Profiling experiments were performed in ESI-positive ion mode using the tune method as follows: sheath gas flow rate 45 unit, auxiliary gas flow rate 10 unit, sweep gas flow rate 2 unit, spray voltage (|KV|) 3.60, spray current (μ A) 3.70, capillary temperature (°C) 320, s-lens RF level 50, heater temperature (°C) 350. ESI(+)-HRMS data were recorded in full scan mode within the mass range m/z 100 to 1000 and data were analyzed by Xcalibur software.

2A.4.2 Preparative-scale Extraction and Purification of Limonoids from Neem Seed Kernels and Fruit-coats

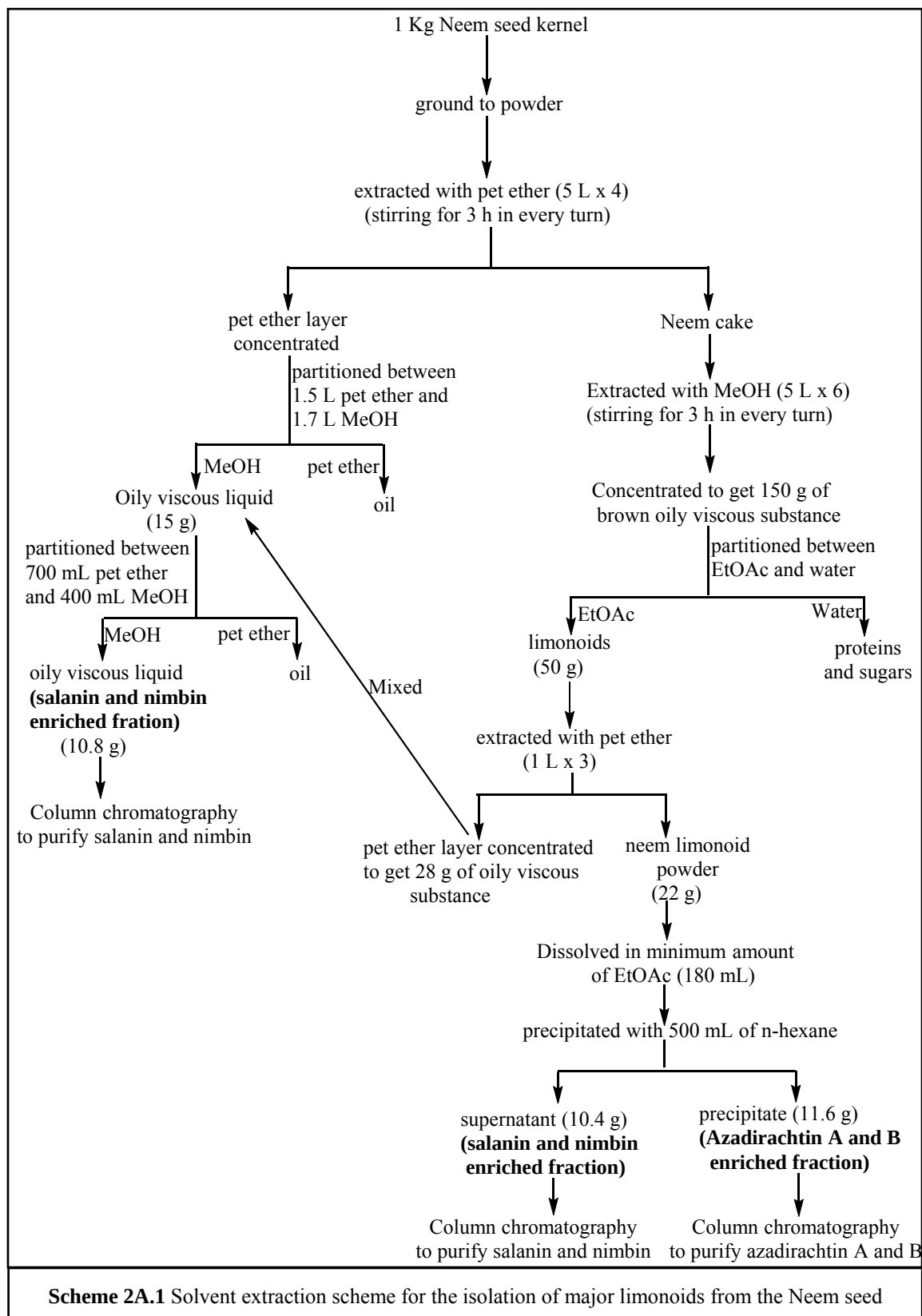
- *Extraction of seed kernels:* One kilogram dried and ground Neem seed kernel was extracted with petroleum ether (4 × 5 L) with stirring for at least 3 hours in every turn.

Combined petroleum ether layer was concentrated to obtain Neem oil, which was further partitioned between the layers of methanol (1.7 L) and petroleum ether (1.5 L). Petroleum ether layer containing residual oil was discarded and limonoids rich methanol layer was concentrated under vacuum at 52 °C which produced 15 g of oily viscous



liquid. The defatted powder (Neem cake) left after the removal of Neem oil from seed kernel was further extracted with methanol (6 × 5 L) in a similar manner and 153 g of oily viscous substance was obtained, which was further partitioned between ethyl acetate (2 L) and water (2 L) to remove water-soluble components. This concentrated ethyl acetate layer under reduced pressure at 50 °C (51 g) was washed with petroleum ether

(3 × 1 L) to remove the residual oil (28 g) and the removed residual oil fraction was combined with previously extracted 15 g of oily viscous liquid for further process. The residual powder (22 g) left after the Neem oil extraction from the Neem cake contained mainly limonoids (Figure 2A.7) which was dissolved in minimum volume of ethyl acetate (180 mL) and precipitated by adding 500 mL of n-hexane. Precipitate (11.6 g) contained mainly azadirachtin A and B whereas the supernatant (10.4 g) was enriched with salannin and nimbin (Figure 2A.7). Oily viscous liquid previously collected (15 + 28 = 43 g) was further partitioned between petroleum ether (700 mL) and methanol (400 mL). The petroleum ether layer was discarded and the methanol layer was concentrated under reduced pressure at 52 °C to get 10.8 g of relatively non-polar limonoids salannin and nimbin enriched fraction. The whole solvent partition protocol has been schematically outlined in scheme 2A.1. The solvent precipitation technique was highly efficient to separate the polar constituents such as azadirachtin derivatives (as precipitate) from the non-polar components (as supernatant) including salannin, nimbin and corresponding analogues. In fact, this step was critical to reduce the complexity of triterpenoid mixture obtained from seed kernel.



- Purification of limonoid mixture from seed-kernel:* The limonoid-enriched fractions obtained by solvent partition were further subjected to flash column chromatography to obtain individual pure limonoids. Twenty gram of the extract of non-polar limonoid

mixture (salannin and nimbin enriched fraction) was loaded on to a silica gel flash chromatography column (14 cm × 6 cm) equilibrated with petroleum ether. Initially, the column was eluted with petroleum ether (1 L) to remove residual oil. Further the column was eluted with gradient mixture of dichloromethane and methanol (700 mL of 0.1%, 1 L of 0.2% and 4.3 L of 0.4% methanol in dichloromethane) in sequential manner. The fractions collected were analyzed by TLC and LC-MS. The fractions containing pure nimbin (0.92 g, R_f : 0.65) and salannin (4.42 g, R_f : 0.51) were pooled and concentrated under reduced pressure. While purifying the major limonoids several minor components were also purified such as nimbinene, nimbanal, 6-decaetylnimbinene, 6-deacetylnimbin, 3-deacetylsalannin and salannol acetate using the combination of semi-preparative HPLC and preparative TLC (PTLC) techniques.

For the purification of azadirachtin A and B, the polar limonoid mixture (11.6 g) obtained as precipitate was subjected to column chromatography (13 cm × 6 cm) over silica gel (230-400 mesh) and eluted with gradient mixture of methanol and dichloromethane. The fractions obtained by eluting with 1.25% of methanol in dichloromethane contained single compound (0.60 g, R_f : 0.34). Based on the spectral characteristics, the compound was identified as azadirachtin B. Further elution of the column with 1.5% methanol in dichloromethane yielded a fraction containing a compound which was identified as azadirachtin A (2.04 g, R_f : 0.46) based on the spectral characteristics. Purification of the intermediate fractions through HPLC and PTLC techniques yielded one minor limonoid namely 3-deacetylsalannin.

- *Extraction of fruit-coats:* Dried Neem fruit powder (250 g) was extracted with (5 + 2 L) methanol and the combined extract was concentrated under reduced pressure at 52 °C to 1 L which was washed with petroleum ether to remove the oil. After concentration to dryness under reduced pressure, 53 g of brown viscous solid was obtained. This solid was partitioned between 1:1 mixture of ethyl acetate and water. The organic phase was concentrated under reduced pressure to dryness (24 g). The HPLC analyses indicated that, the crude mixture contained two major limonoids.

- *Purification of limonoid mixture from fruit-coats:* The above dried crude extract (24 g) from Neem fruit was then subjected to column chromatography (14 cm × 6 cm) over silica gel (230-400 mesh) and the compounds were eluted using gradient mixture of methanol in dichloromethane. Elution of the column with dichloromethane furnished a

compound which was identified as epoxyazadiradione (4.31 g, R_f : 0.82) based on various spectral studies. Further elution of the column with 0.1% methanol in dichloromethane yielded a fraction containing a compound (5.88 g, R_f : 0.66) which was identified as azadiradione by analyzing the spectral data. Azadirone, gedunin and nimocinol were purified as the minor metabolites while purifying the fruit-coat extract.

2A.4.3 Limonoid Profiling of Neem Tissues

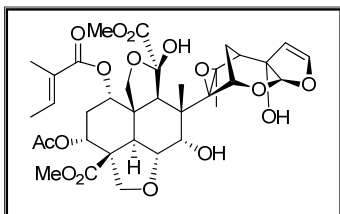
Fresh Neem tissues (0.5 g) were extracted with methanol (10 mL $\times$ 3) with continuous stirring for 3 h in every turn. The pooled methanol layer after concentration under reduced pressure at 50 °C was partitioned between EtOAc (20 mL) and water (20 mL). Organic layer was separated and concentrated under similar conditions to obtain the mixture of triterpenoids. Crude triterpenoid extracts were further dissolved in known volume of methanol (concentration $\sim$ 0.2 mg/mL), centrifuged to remove the suspended particles and injected (10 μ L) in LC-ESI(+)-HRMS. The retention times (R_t) and extracted ions for the individual studied triterpenoids have been listed in Table 2A.1.

Table 2A.1 The retention times (R_t) and extracted ions for the studied triterpenoids

Triterpenoid	R_t (min)	Extracted ion (m/z)	Fragmentation
Azadirachtin A	7.4	703	$[M+H-H_2O]^+$
Azadirachtin B	8.6	645	$[M+H-H_2O]^+$
Salannin	21.3	597	$[M+H]^+$
3-Deacetylsalannin	18.5	555	$[M+H]^+$
Nimbin	18.0	541	$[M+H]^+$
6-Deacetylnimbin	14.7	499	$[M+H]^+$
Nimbinene	20.7	483	$[M+H]^+$
6-Deacetylnimbinene	16.5	441	$[M+H]^+$
Nimbanal	17.8	511	$[M+H]^+$
Salannol acetate	28.4	599	$[M+H]^+$
Azadiradione	16.3	451	$[M+H]^+$
Epoxyazadiradione	27.4	467	$[M+H]^+$
Azadirone	31.7	437	$[M+H]^+$
Gedunin	20.4	483	$[M+H]^+$
Nimocinol	29.9	453	$[M+H]^+$

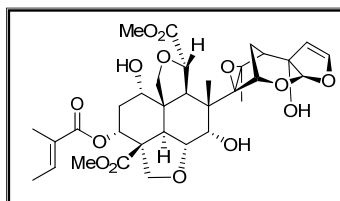
2A.4.4 Characterization of Neem Triterpenoids

Azadirachtin A: White solid. ^1H NMR (CDCl_3 , 400 MHz) δ : 6.92 (1H, m, H-3'), 6.45 (1H, d, $J=2.91$ Hz, H-23), 5.65 (1H, s, H-21), 5.50 (1H, t, $J=2.90$ Hz, H-3), 5.05 (1H, s, H-11-OH), 5.04 (1H, d, $J=2.78$ Hz, H-22), 4.76 (2H, m, H-1,7), 4.67 (1H, d, $J=3.28$ Hz, H-15), 4.60 (1H, dd, $J=12.63, 2.65$ Hz, H-6), 4.15 (1H, d, $J=9.47$ Hz, H-19), 4.07 (1H, d, $J=8.97$ Hz, H-28), 3.80 (3H, s, H-29-CO₂Me), 3.75 (1H, d, $J=9.08$ Hz, H-28), 3.69



(3H, s, H-12-CO₂Me), 3.63 (1H, d, $J=9.73$ Hz, H-19), 3.35 (1H, d, $J=12.51$ Hz, H-5), 3.33 (1H, s, H-9), 3.01, 2.93 (1H each, br s each, H-7-OH, 20-OH), 2.37 (1H, d, $J=5.18$ Hz, H-17), 2.25-2.32 (2H, m, H-2), 2.01 (3H, s, H-18), 1.95 (3H, s, C-COCH₃), 1.86 (3H, m, H-5'), 1.77 (3H, dd, $J=7.20, 1.01$ Hz, H-4'), 1.75 (3H, s, H-30), 1.32 (1H, d, $J=13.14$, H-16). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.33 (C-29), 171.77 (C-12), 169.68 (C-COCH₃), 166.21 (C-1'), 147.09 (C-23), 137.65 (C-3'), 128.58 (C-2'), 108.73 (C-21), 107.33 (C-22), 104.15 (C-11), 83.58 (C-20), 76.42 (C-15), 74.34 (C-7), 73.75 (C-6), 72.96 (C-28), 70.43 (C-1), 69.87 (C-14), 69.05 (C-19), 68.53 (C-13), 66.92 (C-3), 53.29 (C-29-CO₂Me), 52.77 (C-12-CO₂Me), 52.43 (C-4), 50.09 (C-10), 48.64 (C-17), 45.33 (C-8), 44.60 (C-9), 37.01 (C-5), 29.74 (C-2), 24.96 (C-16), 21.28 (C-30), 20.89 (C-COCH₃), 18.37 (C-18), 14.32 (C-4'), 11.95 (C-5'). **HRMS (ESI) m/z :** $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{35}\text{H}_{44}\text{O}_{16}\text{Na}$, 743.2522; found, 743.2518.

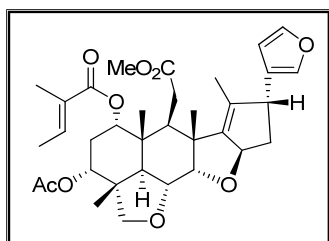
Azadirachtin B: White solid. ^1H NMR (CDCl_3 , 400 MHz) δ : 6.95 (1H, m, H-3'), 6.43 (1H, d, $J=2.91$ Hz, H-23), 5.66 (1H, s, H-21), 5.53 (1H, m, H-3), 5.03 (1H, d, $J=2.91$ Hz, H-22), 4.72 (1H, d, $J=2.65$ Hz, H-7), 4.51-4.58 (2H, m, H-6,15), 4.48 (1H, s, H-11), 3.79-4.06 (3H, m, H-19,28), 3.77 (6H, s, H-12-CO₂Me, 29-CO₂Me), 3.31-3.52 (4H, m, H-2,5,19,1), 3.20 (1H, m, H-9), 2.92 (1H, br s, H-20-OH), 2.36 (1H, d, $J=4.93$ Hz, H-17), 2.04 (3H, s, H-18), 1.84 (1H, m, H-5'), 1.79 (3H, dd, $J=7.07, 1.01$ Hz, H-4'), 1.45



(3H, s, H-30). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.96 (C-29), 173.35 (C-12), 166.92 (C-1'), 146.60 (C-23), 138.70 (C-3'), 128.30 (C-2'), 108.95 (C-21), 107.39 (C-22), 83.52 (C-20), 79.30 (C-11), 75.97 (C-15), 74.20 (C-6), 73.45 (C-7), 73.12 (C-28), 71.22 (C-19), 69.26 (C-1), 69.18 (C-14), 67.52 (C-3), 66.44 (C-13), 53.13 (C-4), 52.84 (C-29-CO₂Me), 52.47 (C-12-CO₂Me), 51.05 (C-10), 48.79 (C-17),

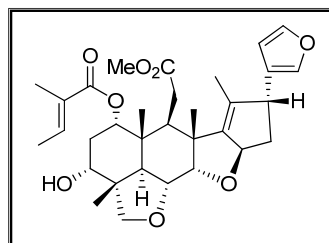
43.88 (C-8), 43.62 (C-9), 34.97 (C-5), 31.90 (C-2), 24.91 (C-16), 21.18 (C-30), 18.09 (C-18), 14.52 (C-4'), 11.91 (C-5'). **HRMS (ESI) m/z :** $[M+Na]^+$ Calcd for $C_{33}H_{42}O_{14}Na$, 685.2467; found, 685.2461.

Salannin: White solid. **^1H NMR ($CDCl_3$, 400 MHz) δ :** 7.33 (1H, m, H-21), 7.26 (1H, m, H-23), 6.96 (1H, q, $J=6.88$ Hz, H-3'), 6.30 (1H, m, H-22), 5.44 (1H, m, H-15), 4.96 (1H, m, H-3), 4.79 (1H, m, H-1), 4.18 (1H, d, $J=3.03$ Hz, H-7), 4.00 (1H, dd, $J=12.65$, 3.30 Hz, H-6), 3.58-3.70 (3H, m, H-17,28), 3.24 (3H, s, H-CO₂Me), 2.81 (1H, d, $J=12.65$ Hz, H-5), 2.75 (1H, m, H-9), 2.12-2.34 (6H, m, H-2,11,6), 1.95 (6H, s, H-5', COCH₃), 1.82 (3H, d, $J=7.15$ Hz, H-4'), 1.67 (3H, s, H-18), 1.30, 1.22, 0.99 (3H each, s each, H-29, 30, 19).



^{13}C NMR ($CDCl_3$, 100 MHz) δ : 172.77 (C-12), 170.39 (C-COCH₃), 166.75 (C-1'), 146.50 (C-14), 142.91 (C-21), 138.75 (C-23), 137.24 (C-3'), 134.88 (C-13), 128.99 (C-2'), 127.07 (C-20), 110.57 (C-22), 87.85 (C-15), 85.62 (C-7), 77.63 (C-28), 72.58 (C-6), 71.29 (C-3), 71.27 (C-1), 51.47 (C-CO₂Me), 49.36 (C-17), 49.03 (C-8), 42.67 (C-4), 41.35 (C-16), 40.56 (C-10), 39.91 (C-5), 39.39 (C-9), 30.65 (C-11), 27.55 (C-2), 20.84 (C-COCH₃), 19.56 (C-29), 16.88 (C-30), 15.09 (C-19), 14.34 (C-4'), 13.00 (C-18), 11.88 (C-5'). **HRMS (ESI) m/z :** $[M+H]^+$ Calcd for $C_{34}H_{45}O_9$, 597.3058; found, 597.3049.

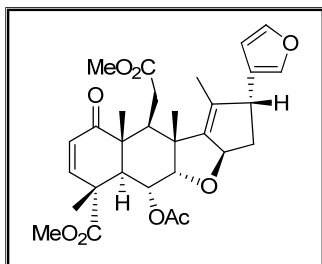
3-Deacetylsalannin: White solid. **^1H NMR ($CDCl_3$, 400 MHz) δ :** 7.32 (1H, m, H-21), 7.25 (1H, m, H-23), 6.91 (1H, m, H-3'), 6.28 (1H, m, H-22), 5.39 (1H, m, H-15), 5.02 (1H, t, $J=2.76$ Hz, H-1), 4.18 (1H, d, $J=3.26$ Hz, H-7), 4.14 (1H, d, $J=7.53$ Hz, H-28), 4.01 (1H, dd, $J=12.30$, 3.26 Hz, H-6), 3.87 (1H, dt, $J=9.03$, 2.76 Hz, H-3), 3.63 (1H, d, $J=7.28$ Hz, H-28), 3.62 (1H, d, $J=8.78$ Hz, H-17), 3.19 (3H, s, H-12-CO₂Me), 2.72 (1H, d, $J=12.30$ Hz, H-5), 2.64 (1H, dd, $J=9.29$, 3.01 Hz, H-9), 2.42 (1H, d, $J=9.29$ Hz, H-11), 1.92 (3H, s, H-5'), 1.85 (3H, dd, $J=7.03$, 1.00 Hz, H-4'), 1.65 (3H, d, $J=1.25$ Hz, H-18), 1.31 (3H, s, H-30), 1.16 (3H, s, H-29), 0.96 (3H, s, H-19).



^{13}C NMR ($CDCl_3$, 100 MHz) δ : 172.64 (C-12), 166.30 (C-1'), 146.49 (C-14), 142.86 (C-23), 138.72 (C-21), 138.09 (C-3'), 134.95 (C-13), 128.57 (C-2'), 127.13 (C-20), 110.66 (C-22), 87.83 (C-15), 85.79 (C-7), 77.83 (C-28), 72.81 (C-1), 72.43 (C-6), 70.85 (C-3), 51.41 (C-12), 49.33 (C-17), 48.93 (C-8), 44.20 (C-4), 41.25 (C-16), 40.91 (C-10), 39.46 (C-9), 38.80 (C-5), 30.50 (C-2), 30.39 (C-11), 19.85,

16.86, 15.19 (C-19, 29, 30), 14.57 (C-5'), 13.06 (C-18), 12.19 (C-4'). **HRMS (ESI) m/z :** $[M+H]^+$ Calcd for $C_{32}H_{43}O_8$, 555.2952; found, 555.2954.

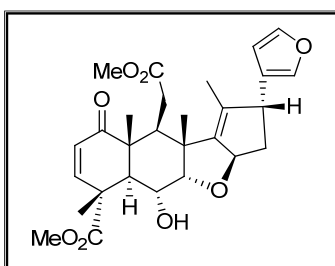
Nimbin: White solid. ^1H NMR ($CDCl_3$, 400 MHz) δ : 7.33 (1H, m, H-21), 7.23 (1H, m, H-23), 6.34 (2H, m, H-3,22), 5.88 (1H, d, $J=10.18$ Hz, H-2), 5.56 (1H, m, H-15), 5.22 (1H, dd, $J=12.38, 2.75$ Hz, H-6), 4.05 (1H, d, $J=2.48$ Hz, H-7), 3.74 (3H, s, H-28-CO₂Me), 3.66 (3H, s, H-12-CO₂Me), 3.62-3.69 (2H, m, H-17,5), 2.90 (1H, dd, $J=16.23,$



5.23 Hz, H-11), 2.84 (1H, t, $J=4.13$ Hz, H-9), 2.18-2.25 (2H, m, H-16,11), 2.04 (3H, s, H-COCH₃), 2.01 (1H, m, H-16), 1.67 (3H, s, H-18), 1.36, 1.35, 1.28 (3H each, s each, H-29,30,19). ^{13}C NMR ($CDCl_3$, 100 MHz) δ : 201.66 (C-1), 174.63 (C-28), 173.70 (C-12), 170.68 (C-COCH₃), 147.58

(C-3), 146.05 (C-14), 143.02 (C-23), 138.99 (C-21), 135.14 (C-13), 126.78 (C-20), 125.95 (C-2), 110.48 (C-22), 87.07 (C-15), 84.51 (C-7), 68.65 (C-6), 53.12 (C-28-CO₂Me), 51.73 (C-12-CO₂Me), 49.42 (C-17), 47.99 (C-10), 47.89 (C-4), 47.04 (C-8), 41.57 (C-16), 41.45 (C-5), 38.54 (C-9), 34.25 (C-11), 21.00 (C-COCH₃), 17.19 (C-29), 16.71 (C-30), 16.64 (C-19), 12.85 (C-18). **HRMS (ESI) m/z :** $[M+H]^+$ Calcd for $C_{30}H_{37}O_9$, 541.2432; found, 541.2435.

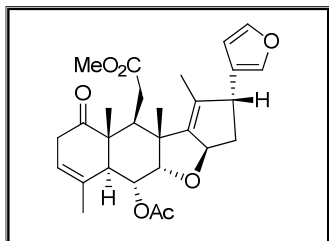
6-Deacetylnimbin: White solid. ^1H NMR ($CDCl_3$, 400 MHz) δ : 7.33 (1H, m, H-21), 7.24 (1H, m, H-23), 6.42 (1H, d, $J=10.09$ Hz, H-3), 6.34 (1H, m, H-22), 5.86 (1H, d, $J=10.27$ Hz, H-2), 5.56 (1H, t, $J=6.97$ Hz, H-15), 4.03 (1H, d, $J=3.12$ Hz, H-7), 3.93 (1H, td, $J=11.37, 3.12$ Hz, H-6), 3.70 (3H, s, H-28-CO₂Me), 3.66 (3H, s, H-12-CO₂Me),



3.40 (1H, d, $J=11.74$ Hz, H-5), 2.90 (1H, dd, $J=16.32, 5.69$ Hz, H-11), 2.77 (1H, m, H-9), 1.69 (3H, s, H-18), 1.59 (3H, s, H-29), 1.29 (3H, s, H-30), 1.22 (3H, s, H-19). ^{13}C NMR ($CDCl_3$, 100 MHz) δ : 202.18 (C-1), 175.54 (C-28), 173.66 (C-12), 148.07 (C-3), 146.80 (C-14), 143.05 (C-23), 138.99

(C-21), 134.91 (C-13), 126.80 (C-20), 126.41 (C-2), 110.42 (C-22), 87.42 (C-15), 86.93 (C-7), 66.18 (C-6), 52.99 (C-28-CO₂Me), 51.65 (C-12-CO₂Me), 49.62 (C-17), 47.75, 47.47, 47.32 (C-4, 8, 10), 43.64 (C-5), 41.42 (C-16), 39.05 (C-9), 34.35 (C-11), 17.51, 17.13, 16.38 (C-29, 30, 19), 12.83 (C-18). **HRMS (ESI) m/z :** $[M+H]^+$ Calcd for $C_{28}H_{35}O_8$, 499.2326; found, 499.2324.

Nimbinene: White solid. ^1H NMR (CDCl_3 , 400 MHz) δ : 7.30 (1H, t, $J=1.64$ Hz, H-21), 7.20 (1H, m, H-23), 6.27 (1H, m, H-22), 5.58 (1H, m, H-15), 5.48 (1H, m, H-3), 5.34 (1H, dd, $J=12.25, 3.16$ Hz, H-6), 4.06 (1H, d, $J=3.16$ Hz, H-7), 3.62 (1H, d, $J=8.08$

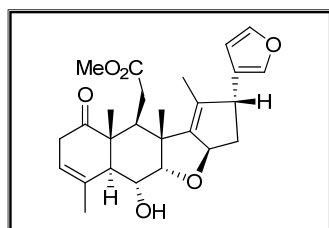


Hz, H-17), 3.55 (3H, s, H-12- CO_2Me), 3.28 (1H, d, $J=12.00$ Hz, H-5), 2.14 (3H, s, H- COCH_3), 1.76 (3H, d, $J=1.00$ Hz, H-29), 1.66 (3H, d, $J=1.52$ Hz, H-18), 1.37 (3H, s, H-30), 1.10 (3H, s, H-19). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 212.21 (C-1), 173.44 (C-12), 170.61 (C- COCH_3), 146.28 (C-14),

142.98 (C-23), 138.80 (C-21), 136.62 (C-4), 135.10 (C-13), 126.72 (C-20), 120.01 (C-3), 110.32 (C-22), 86.77 (C-15), 85.07 (C-7), 69.05 (C-6), 51.57 (C-12- CO_2Me), 49.34 (C-17), 49.31 (C-10), 47.71 (C-8), 42.94 (C-5), 41.27 (C-16), 39.79 (C-2), 36.84 (C-9), 33.43 (C-11), 21.59, 21.06 (C-29, COCH_3), 17.06, 14.13 (C-19, 30), 12.78 (C-18).

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{28}\text{H}_{35}\text{O}_7$, 483.2377; found, 483.2375.

6-Deacetylnimbinene: White solid. ^1H NMR (CDCl_3 , 400 MHz) δ : 7.32 (1H, m, H-21), 7.22 (1H, m, H-23), 6.29 (1H, m, H-22), 5.58 (1H, m, H-15), 5.46 (1H, m, H-3), 4.02 (2H, m, H-6, 7), 3.67 (1H, d, $J=8.62$, H-17), 3.59 (3H, s, H-12- CO_2Me), 2.06 (3H,

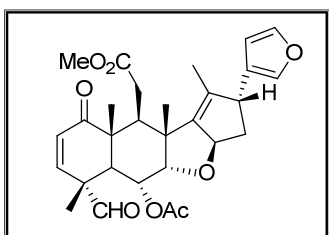


s, H- COCH_3), 1.70 (3H, s, H-29), 1.33 (3H, s, H-30), 1.06 (3H, s, H-19). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 213.20 (C-1), 173.65 (C-12), 147.26 (C-14), 143.08 (C-23), 138.91 (C-21), 138.64 (C-4), 134.85 (C-13), 126.79 (C-20), 119.13 (C-3), 110.38 (C-22), 87.98 (C-15), 86.38 (C-7), 66.38 (C-6),

51.63 (C-12- CO_2Me), 49.52 (C-17), 49.39 (C-10), 47.26 (C-5, 8), 41.32 (C-16), 39.95 (C-2), 37.14 (C-9), 33.62 (C-11), 21.73 (C-29), 17.43, 14.01 (C-19, 30), 12.87 (C-18).

HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{26}\text{H}_{33}\text{O}_6$, 441.2272; found, 441.2269.

Nimbanal: White solid. ^1H NMR (CDCl_3 , 400 MHz) δ : 9.25 (1H, s, H-28), 7.33 (1H, m, H-21), 7.24 (1H, m, H-23), 6.34 (1H, m, H-22), 6.10 (1H, d, $J=10.09$ Hz, H-3), 6.03

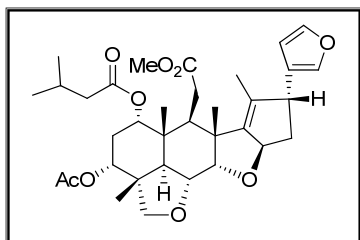


(1H, d, $J=10.09$ Hz, H-2), 5.57 (1H, m, H-15), 5.20 (1H, dd, $J=12.29, 2.93$ Hz, H-6), 4.09 (1H, d, $J=2.75$ Hz, H-7), 3.65 (4H, m, H-17, 12- CO_2Me), 3.27 (1H, d, $J=12.29$ Hz, H-5), 2.94 (1H, dd, $J=16.14, 5.14$ Hz, H-11), 2.86 (1H, t, $J=4.40$ Hz, H-9), 2.28 (1H, dd, $J=16.12, 4.40$ Hz, H-11), 1.99 (3H,

s, H- COCH_3), 1.69 (3H, s, H-18), 1.38 (3H, s, H-29), 1.32 (3H, s, H-30), 1.28 (3H, s, H-

19). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 201.02 (C-1), 199.40 (C-28), 173.42 (C-12), 170.19 (C-COCH₃), 145.62 (C-14), 144.66 (C-3), 143.04 (C-23), 138.97 (C-21), 135.61 (C-13), 129.04 (C-2), 126.65 (C-20), 110.38 (C-22), 87.18 (C-15), 84.30 (C-7), 68.49 (C-6), 51.77 (C-4), 51.69 (C-12-CO₂Me), 49.45 (C-17), 47.94, 47.27 (C-8, 10), 41.28 (C-16), 40.57 (C-5), 38.33 (C-9), 34.14 (C-11), 20.77 (C-COCH₃), 17.00, 16.82, 14.68 (C-19, 29, 30), 12.81 (C-18). HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{29}\text{H}_{35}\text{O}_8$, 511.2326; found, 511.2329.

Salannol acetate: White solid. ^1H NMR (CDCl_3 , 400 MHz) δ : 7.34 (1H, m, H-21), 7.29 (1H, m, H-23), 6.34 (1H, m, H-22), 5.48 (1H, m, H-15), 4.95 (1H, m, H-3), 4.85 (1H, m, H-1), 4.16 (1H, d, $J=3.05$ Hz, H-7), 3.97 (1H, dd, $J=12.51$, 3.05 Hz, H-6), 3.58-3.65 (3H, m, H-17, 28), 3.28 (3H, s, H-12-CO₂Me), 2.71 (2H, m, H-5, 9), 2.08 (3H, s,

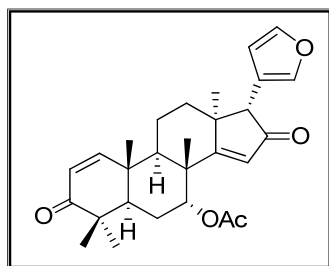


H-COCH₃), 1.65 (3H, s, H-18), 1.30 (3H, s, H-30), 1.21 (3H, s, H-29), 1.01 (3H, d, $J=1.21$ Hz, H-4'), 1.00 (3H, d, $J=1.53$ Hz, H-5'), 0.94 (3H, s, H-19). ^{13}C NMR (CDCl_3 ,

100 MHz) δ : 172.89, 172.65 (C-12, 1'), 170.61 (C-COCH₃), 146.44 (C-14), 142.86 (C-21), 138.90 (C-23),

134.74 (C-13), 127.16 (C-20), 110.78 (C-22), 87.89 (C-15), 85.61 (C-7), 77.79 (C-28), 72.68 (C-6), 71.33 (C-3), 70.61 (C-1), 51.50 (C-12-CO₂Me), 49.46 (C-17), 48.85 (C-8), 43.47 (C-2'), 42.69 (C-4), 41.15 (C-16), 40.22 (C-10), 39.87 (C-5), 39.19 (C-9), 30.16 (C-11), 28.59 (C-2), 25.11 (C-3'), 22.82, 22.66 (C-4', 5'), 21.18 (C-COCH₃), 19.55 (C-29), 16.92 (C-30), 15.54 (C-19), 12.90 (C-18). HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{34}\text{H}_{47}\text{O}_9$, 599.3215; found, 599.3210.

Azadiradione: White solid. ^1H NMR (CDCl_3 , 400 MHz) δ : 7.48 (1H, m, H-21), 7.44 (1H, m, H-23), 7.14 (1H, d, $J=10.18$ Hz, H-1), 6.28 (1H, m, H-22), 5.90 (1H, d, $J=10.18$ Hz, H-2), 5.89 (1H, s, H-15), 5.33 (1H, m, H-7), 3.43 (1H, s, H-17), 2.50 (1H, m, H-9),

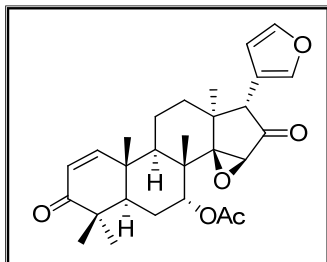


2.21 (1H, m, H-5), 1.96 (3H, s, -COCH₃), 1.35 (3H, s, H-30), 1.26 (3H, s, H-19), 1.11 (3H, s, H-29), 1.10 (3H, s, H-28), 1.04 (3H, s, H-18). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 204.96 (C-16), 203.96 (C-3), 192.24 (C-14), 169.58 (C-COCH₃), 156.67 (C-1), 142.80 (C-23), 141.67 (C-21), 125.96 (C-2),

123.33 (C-15), 118.44 (C-20), 111.13 (C-22), 73.92 (C-7), 60.76 (C-17), 47.97 (C-13), 46.15 (C-5), 44.56 (C-8), 44.09 (C-4), 40.01 (C-10), 38.23 (C-9), 30.36 (C-12), 27.00

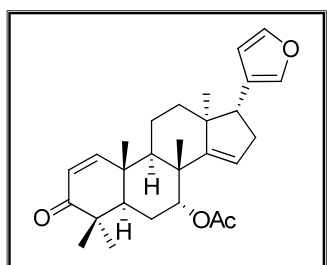
(C-28), 26.45 (C-18), 26.30 (C-30), 23.46 (C-6), 21.28 (C-29), 20.95 (C-COCH₃), 19.02 (C-19), 15.83 (C-11). **HRMS (ESI) *m/z***: [M+H]⁺ Calcd for C₂₈H₃₅O₅, 451.2479; found, 451.2478.

Epoxyazadiradione: White solid. **¹H NMR (CDCl₃, 400 MHz) δ** : 7.56 (1H, m, H-21), 7.40 (1H, m, H-23), 7.17 (1H, d, *J*=10.04 Hz, H-1), 6.24 (1H, m, H-22), 5.88 (1H, d, *J*=10.04 Hz, H-2), 4.73 (1H, m, H-7), 3.90 (1H, s, H-17), 3.41 (1H, s, H-15), 2.63 (1H,



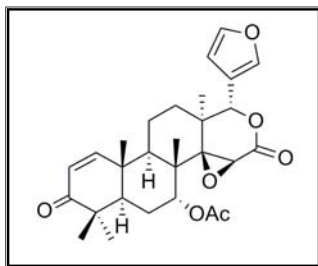
m, H-9), 2.20-2.15 (2H, m, H-5,12), 2.03 (3H, s, -COCH₃), 1.22 (3H, s, H-19), 1.21 (3H, s, H-30), 1.08 (3H, s, H-29), 1.07 (3H, s, H-28), 1.04 (3H, s, H-18). **¹³C NMR (CDCl₃, 100 MHz) δ** : 208.40 (C-16), 204.21 (C-3), 169.74 (C-COCH₃), 157.47 (C-1), 142.43 (C-23), 141.53 (C-21), 125.76 (C-2), 116.51 (C-20), 110.87 (C-22), 73.63 (C-7), 72.54 (C-14), 57.18 (C-15), 50.94 (C-17), 46.67 (C-5), 44.22 (C-4), 43.12 (C-8), 42.53 (C-13), 39.69 (C-9), 39.65 (C-10), 29.09 (C-12), 27.02 (C-28), 24.82 (C-18), 24.20 (C-6), 21.26 (C-COCH₃), 20.99 (C-29), 19.82 (C-19), 19.39 (C-30), 16.06 (C-11). **HRMS (ESI) *m/z***: [M+H]⁺ Calcd for C₂₈H₃₅O₆, 467.2428; found, 467.2426.

Azadirone: White solid. **¹H NMR (CDCl₃, 400 MHz) δ** : 7.38 (1H, m, H-21), 7.25 (1H, m, H-23), 7.16 (1H, d, *J*=10.04 Hz, H-1), 6.28 (1H, m, H-22), 5.85 (1H, d, *J*=10.04 Hz, H-2), 5.38 (1H, m, H-15), 5.28 (1H, m, H-7), 2.81 (1H, m, H-17), 1.96 (3H, s, -



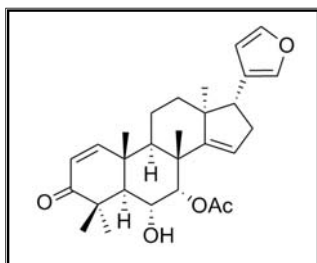
COCH₃), 1.23 (3H, s, H-30), 1.20 (3H, s, H-19), 1.08 (6H, s, H-29,28), 0.80 (3H, s, H-18). **¹³C NMR (CDCl₃, 100 MHz) δ** : 204.63 (C-3), 170.15 (C-COCH₃), 158.82 (C-14), 158.20 (C-1), 142.55 (C-23), 139.68 (C-21), 125.46 (C-2), 124.56 (C-20), 119.07 (C-15), 111.02 (C-22), 74.49 (C-7), 51.59 (C-17), 47.14 (C-13), 46.11 (C-5), 44.12 (C-4), 42.78 (C-8), 39.93 (C-10), 38.62 (C-9), 34.35 (C-16), 32.96 (C-12), 27.31 (C-28), 27.03 (C-30), 23.77 (C-6), 21.28, 21.15, 20.61 (C-COCH₃, 29, 18), 19.04 (C-19), 16.47 (C-11). **HRMS (ESI) *m/z***: [M+H]⁺ Calcd for C₂₈H₃₇O₄, 437.2686; found, 437.2685.

Gedunin: White solid. **¹H NMR (CDCl₃, 400 MHz) δ** : 7.42 (2H, m, H-21,23), 7.10 (1H, d, *J*=10.07 Hz, H-1), 6.34 (1H, m, H-22), 5.87 (1H, d, *J*=10.38 Hz, H-2), 5.62 (1H, s, H-17), 4.56 (1H, m, H-7), 3.53 (1H, s, H-15), 2.50 (1H, m, H-9), 2.17 (1H, m, H-5), 2.11 (3H, s, -COCH₃), 1.25 (3H, s, H-18), 1.23 (3H, s, H-19), 1.16 (3H, s, H-30), 1.08



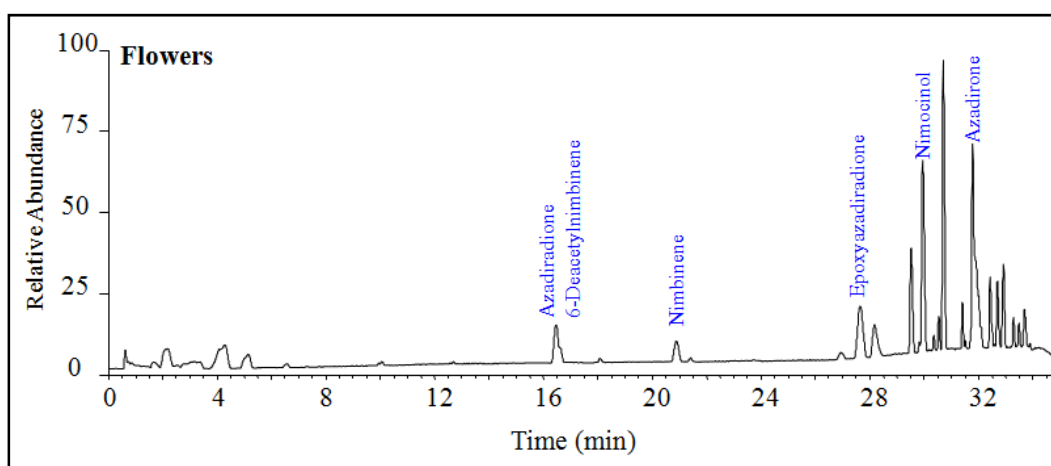
(3H, s, H-29), 1.07 (3H, s, H-28). **^{13}C NMR (CDCl_3 , 100 MHz) δ :** 203.93 (C-3), 169.87 (C-COCH₃), 167.42 (C-16), 156.90 (C-1), 143.08 (C-23), 141.20 (C-21), 126.01 (C-2), 120.44 (C-20), 109.86 (C-22), 78.26 (C-17), 73.24 (C-7), 69.76 (C-14), 56.89 (C-15), 46.05 (C-5), 44.05 (C-4), 42.64 (C-8), 40.04 (C-10), 39.54 (C-9), 38.74 (C-13), 27.16 (C-28), 26.02 (C-12), 23.26 (C-6), 21.18 (C-29), 21.05 (C-COCH₃), 19.72 (C-19), 18.30 (C-30), 17.74 (C-18), 14.99 (C-11). **HRMS (ESI) m/z :** $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{28}\text{H}_{35}\text{O}_7$, 483.2377; found, 483.2376.

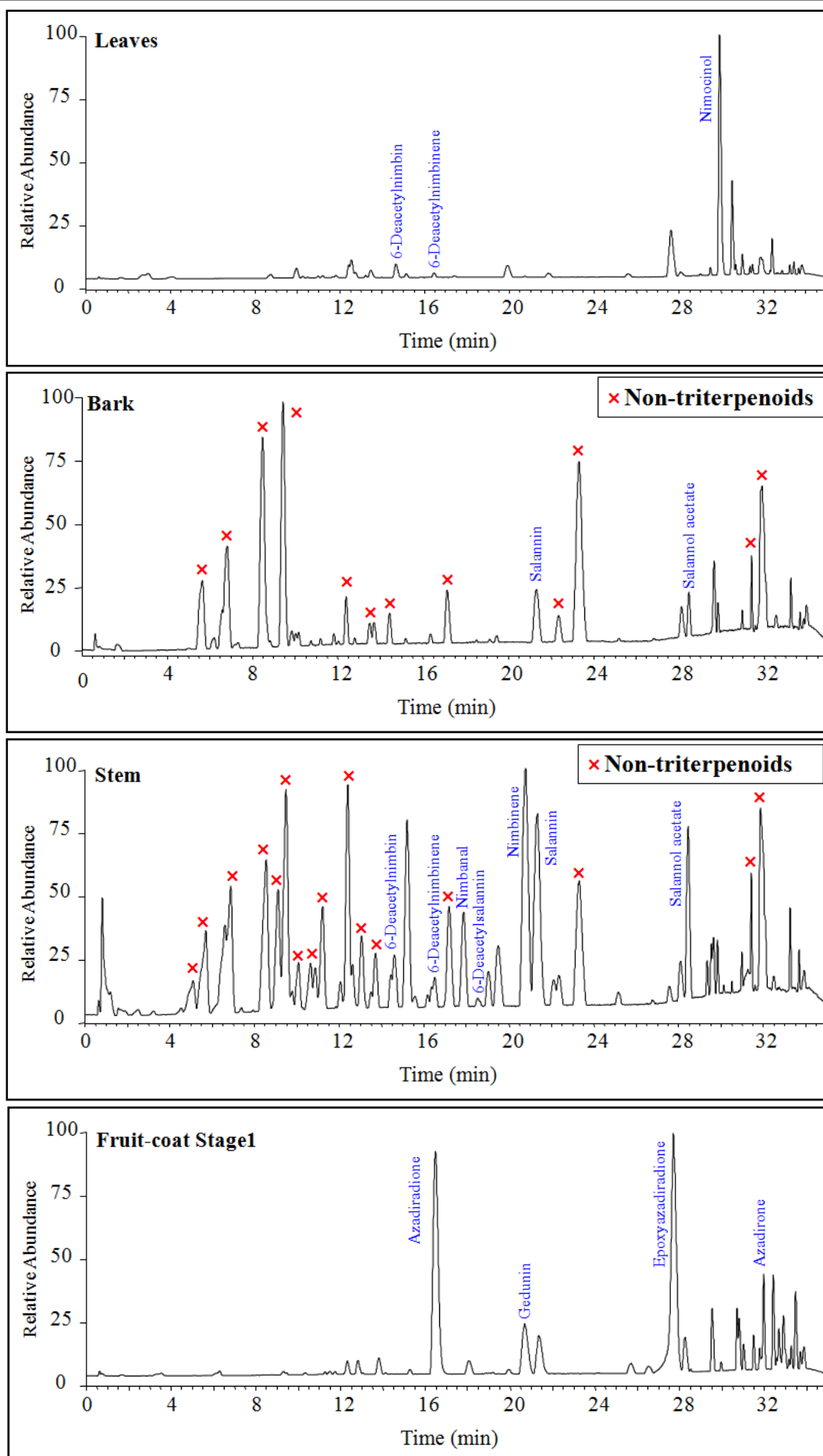
Nimocinol: White solid. **^1H NMR (CDCl_3 , 400 MHz) δ :** 7.39 (1H, m, H-21), 7.26 (1H, m, H-23), 7.11 (1H, d, $J=10.04$ Hz, H-1), 6.28 (1H, m, H-22), 5.90 (1H, d, $J=9.80$ Hz, H-2), 5.41 (1H, m, H-15), 5.36 (1H, d, $J=1.51$ Hz, H-7), 4.38 (1H, m, H-6), 2.82 (1H, dd, $J=10.54$, 7.78 Hz, H-17), 2.05 (3H, s, H-COCH₃), 1.41 (3H, s, H-30), 1.31 (3H, s, H-19), 1.27 (3H, s, H-28), 1.14 (3H, s, H-29), 0.82 (3H, s, H-18).

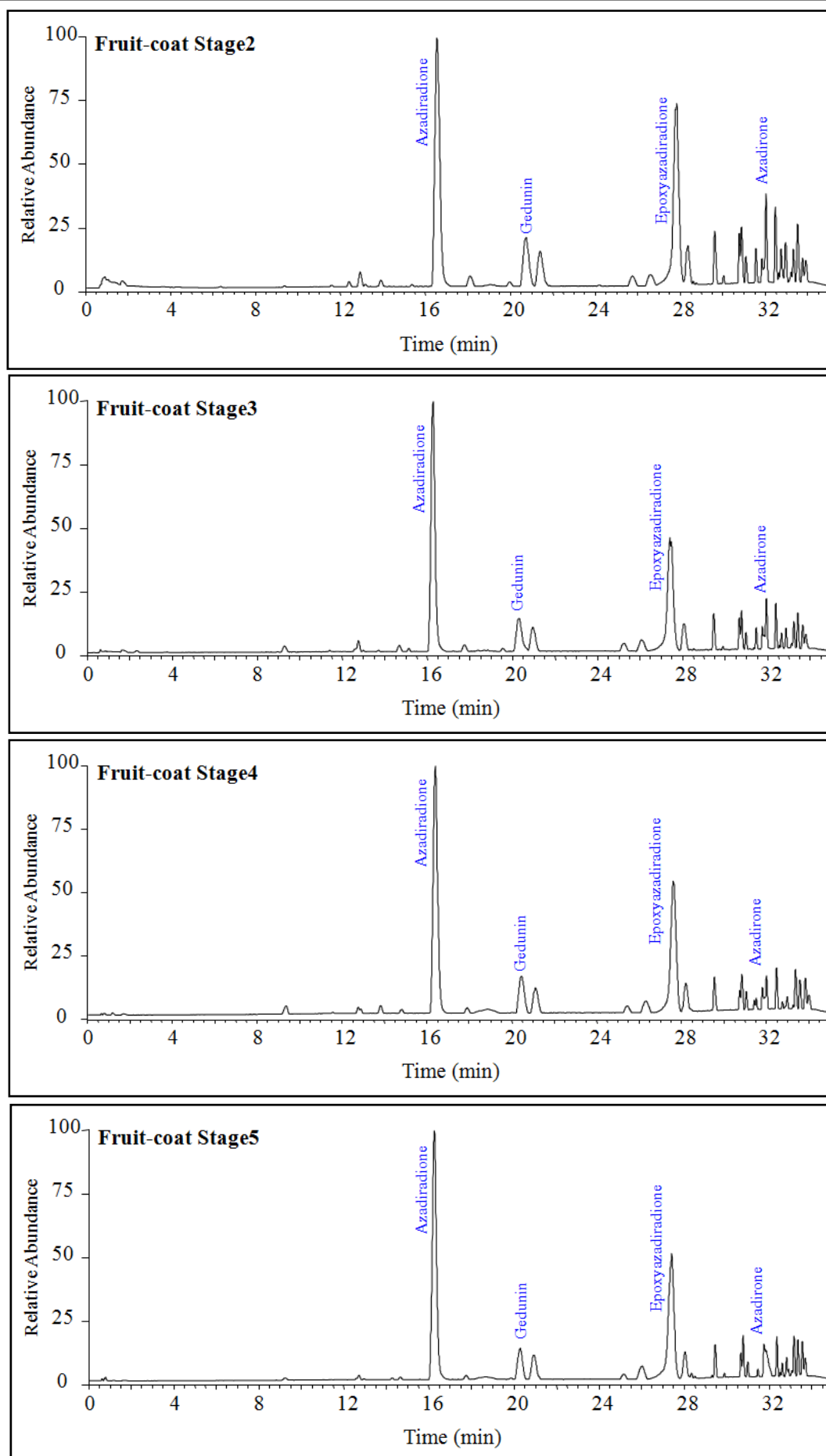


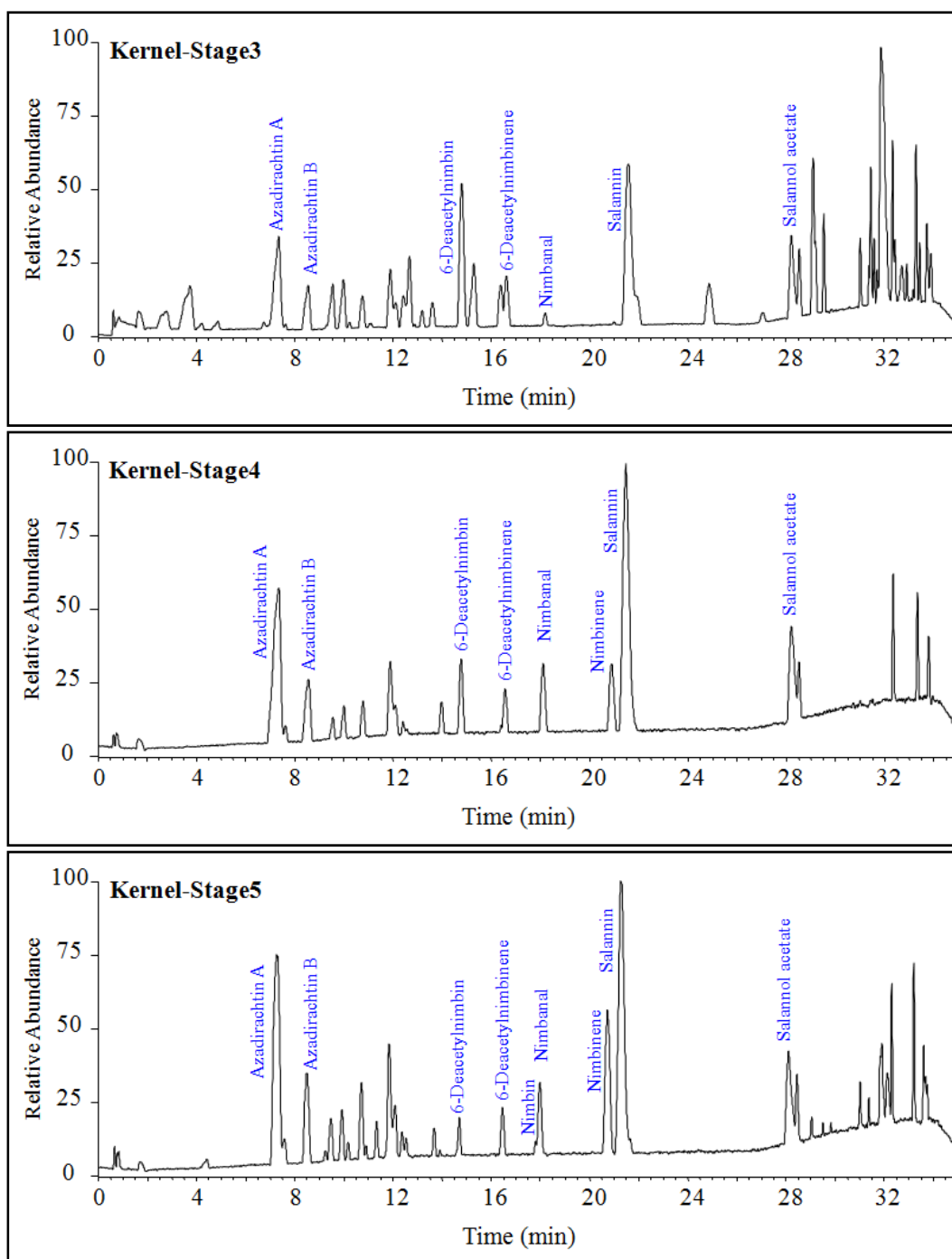
^{13}C NMR (CDCl_3 , 100 MHz) δ : 205.88 (C-3), 172.10 (C-COCH₃), 158.59 (C-14), 157.32 (C-1), 142.64 (C-23), 139.71 (C-21), 126.22 (C-2), 124.41 (C-20), 119.57 (C-15), 110.99 (C-22), 79.16 (C-7), 68.25 (C-6), 51.68 (C-17), 50.00 (C-5), 47.13 (C-13), 45.47, 43.15 (C-4,8), 40.55 (C-10), 37.21 (C-9), 34.37 (C-12), 32.77 (C-16), 31.93 (C-30), 27.13 (C-28), 21.14, 20.87, 20.83 (C-18, 29, COCH₃), 20.28 (C-19), 16.43 (C-11). **HRMS (ESI) m/z :** $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{28}\text{H}_{37}\text{O}_5$, 453.2636; found, 453.2631.

2A.4.5 Representative UPLC-ESI(+)-MS chromatograms of various Neem tissues

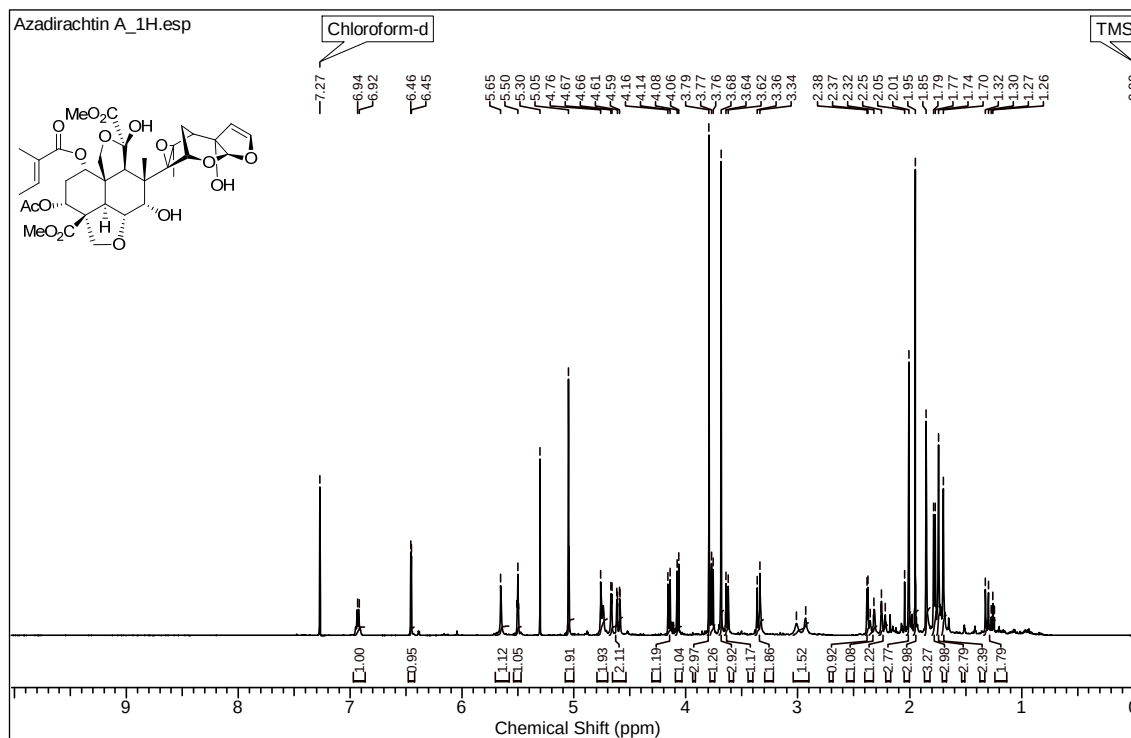
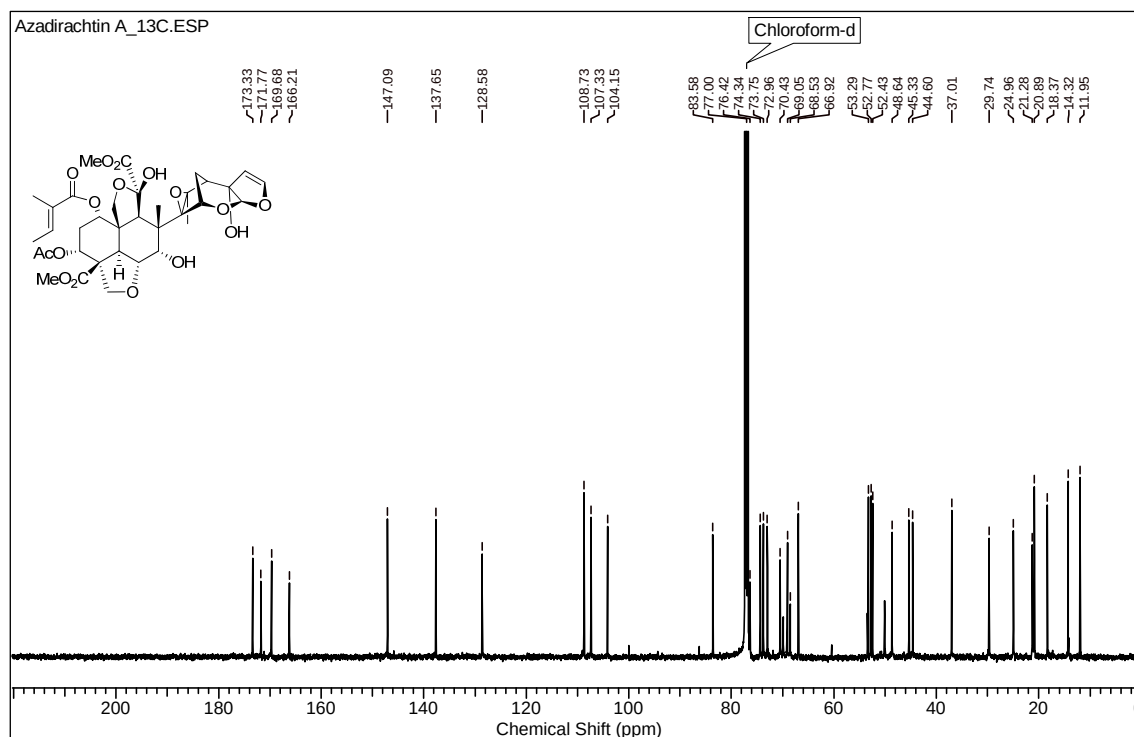


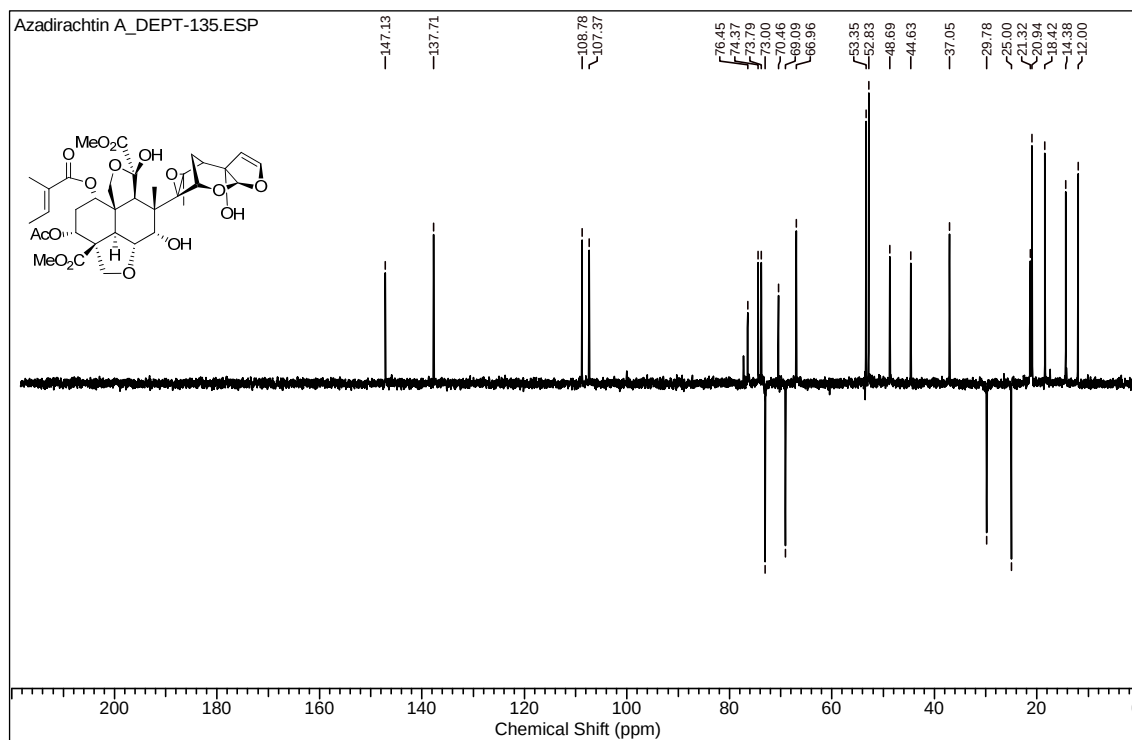
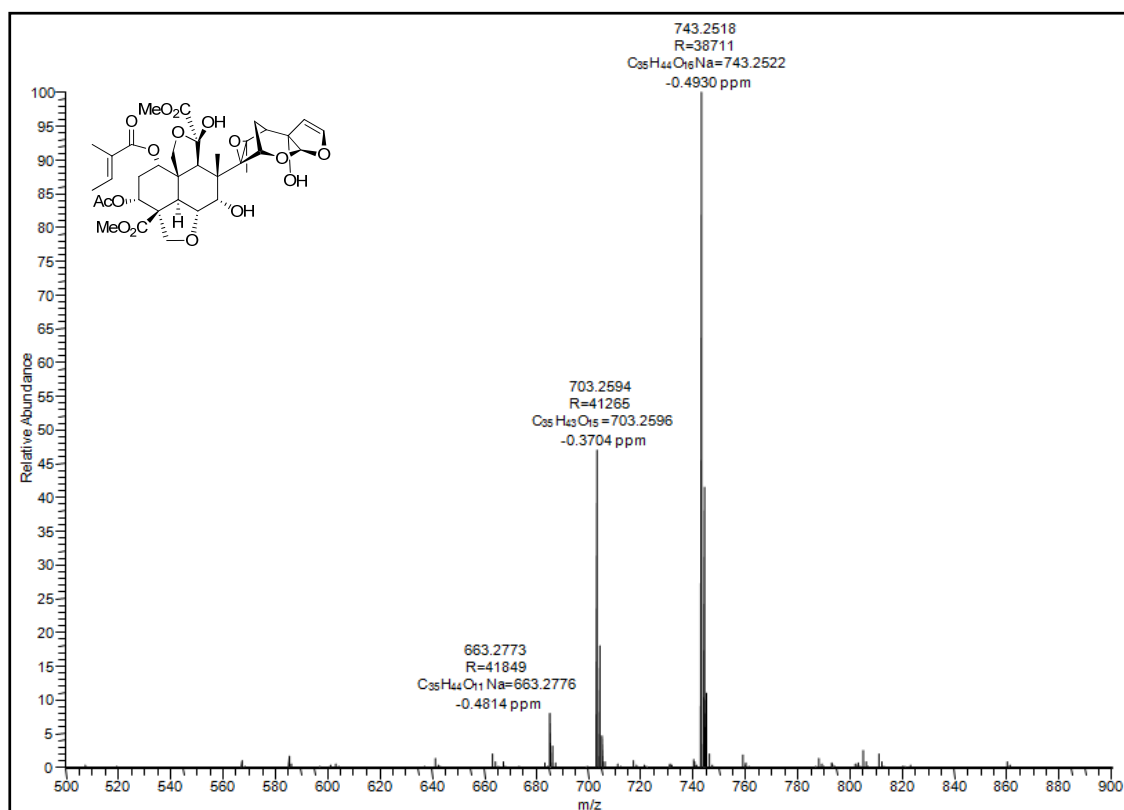




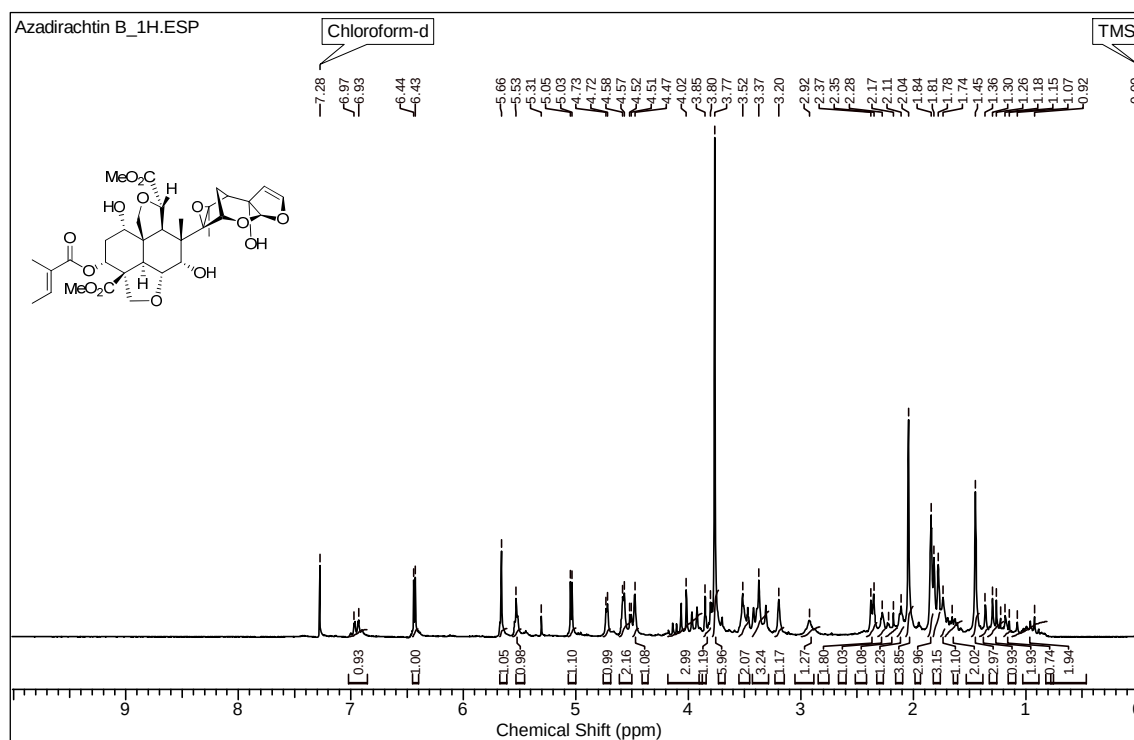


2A.5 Spectral Copies

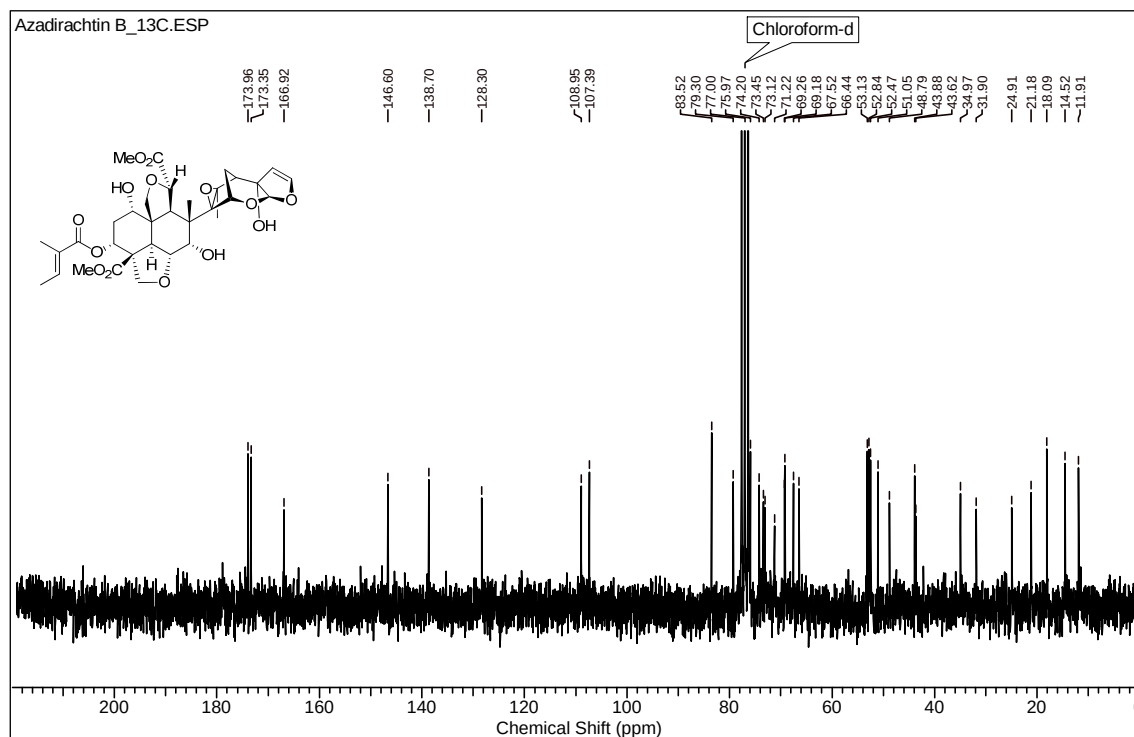
 ^1H NMR spectrum (CDCl_3 , 400 MHz) of azadirachtin A ^{13}C NMR spectrum (CDCl_3 , 100 MHz) of azadirachtin A

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of azadirachtin A

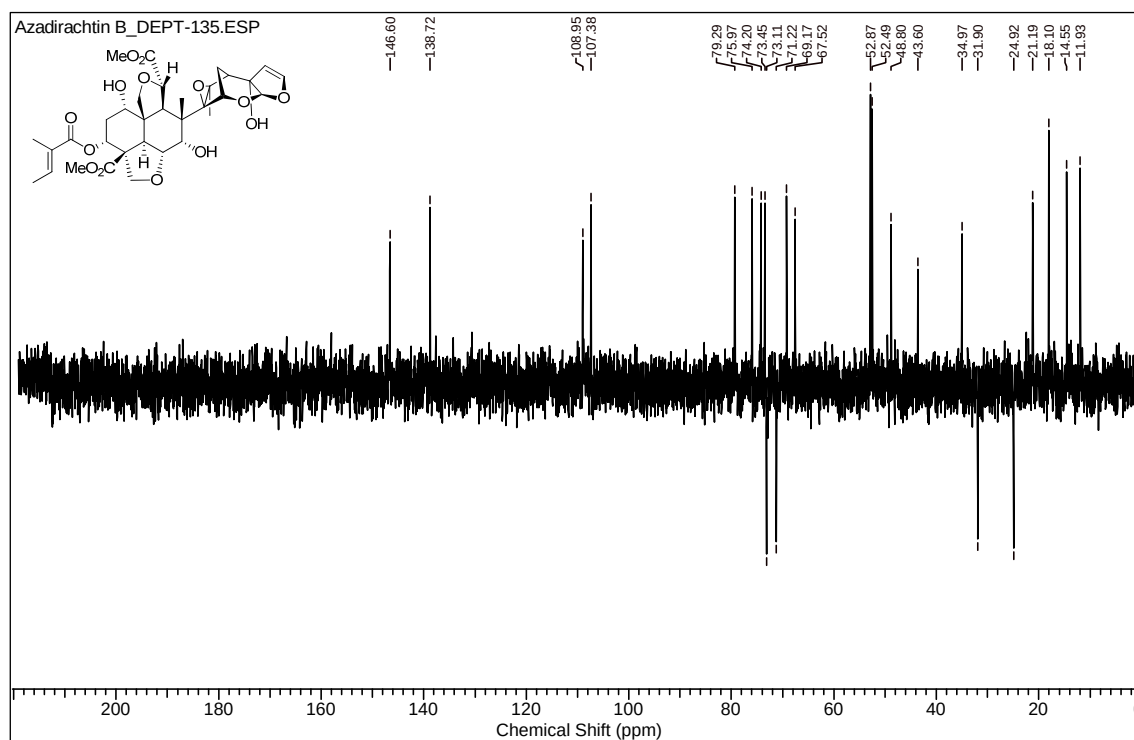
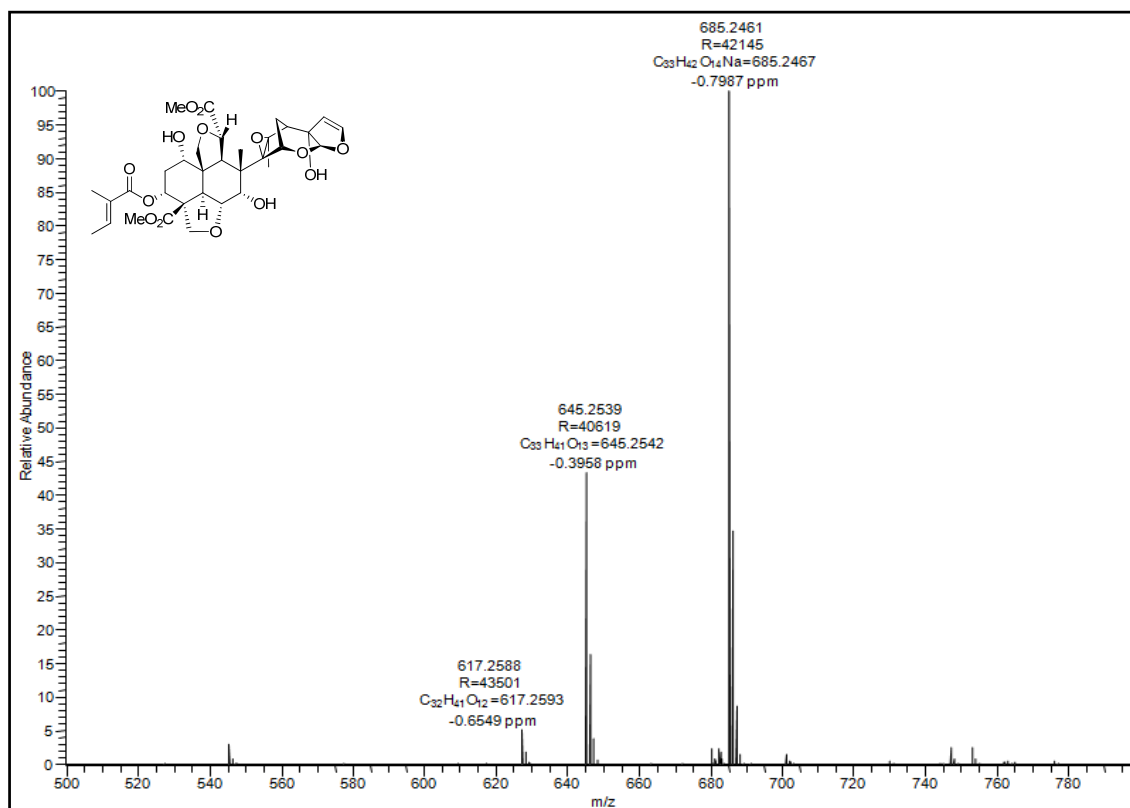
LC-ESI(+)-HRMS of azadirachtin A



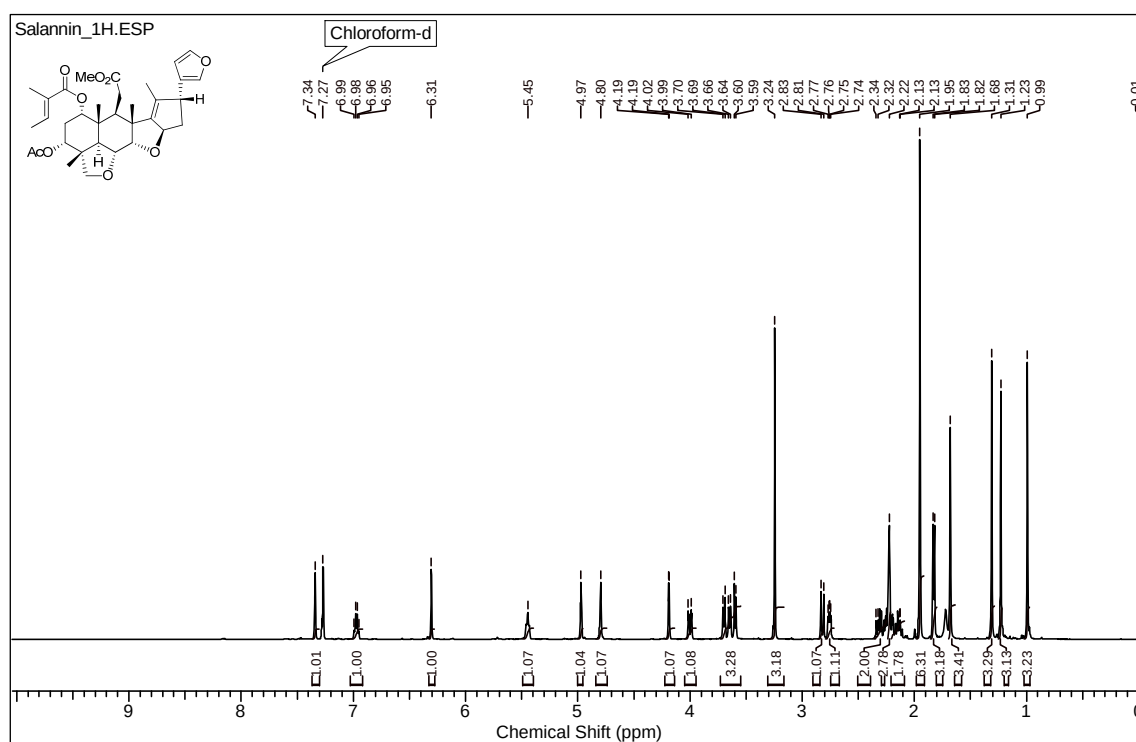
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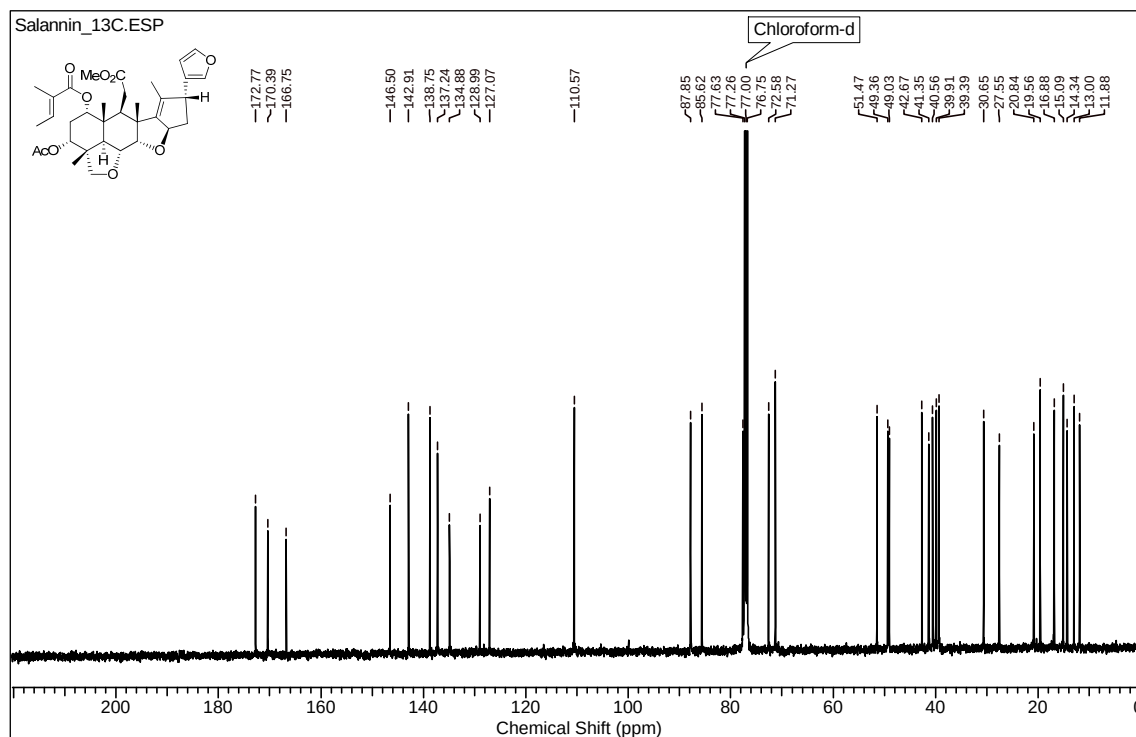
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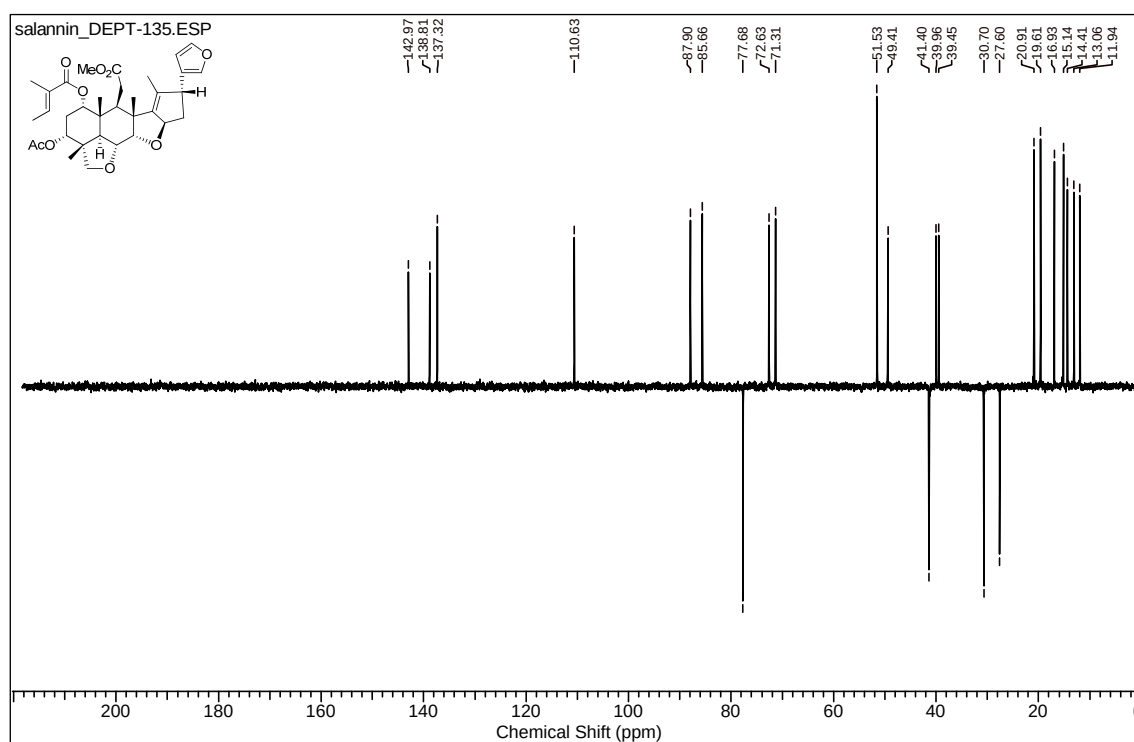
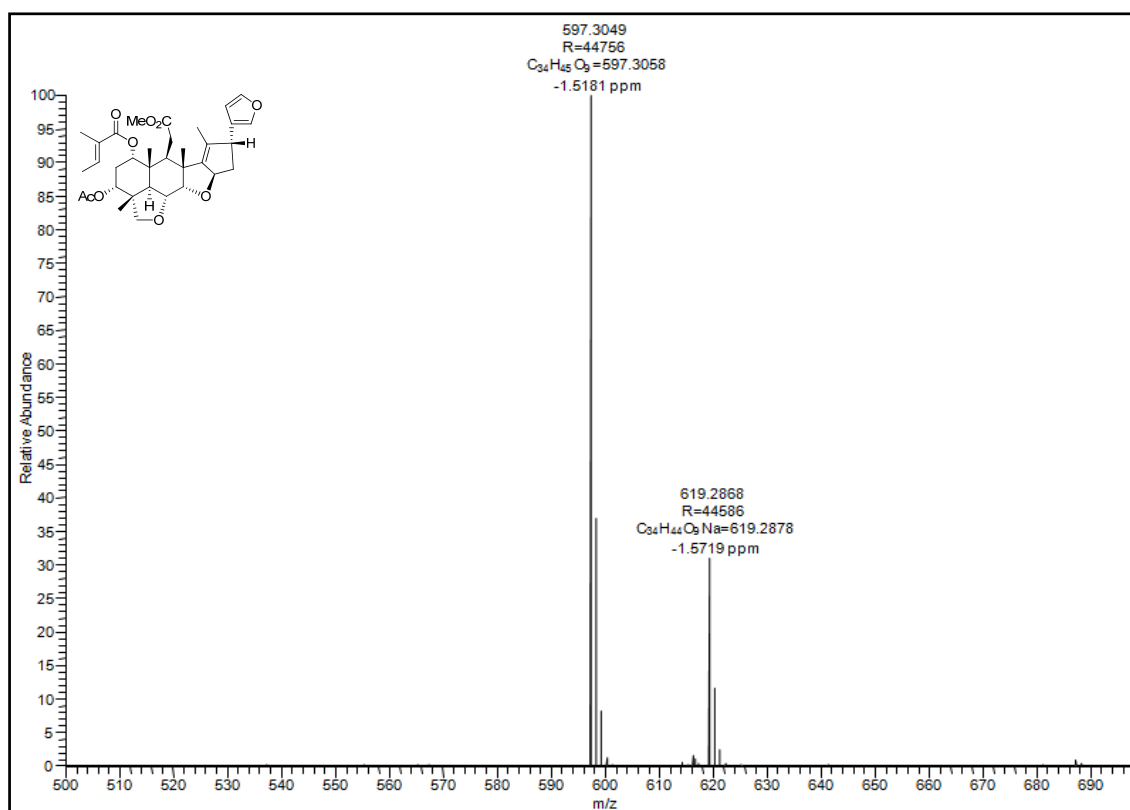
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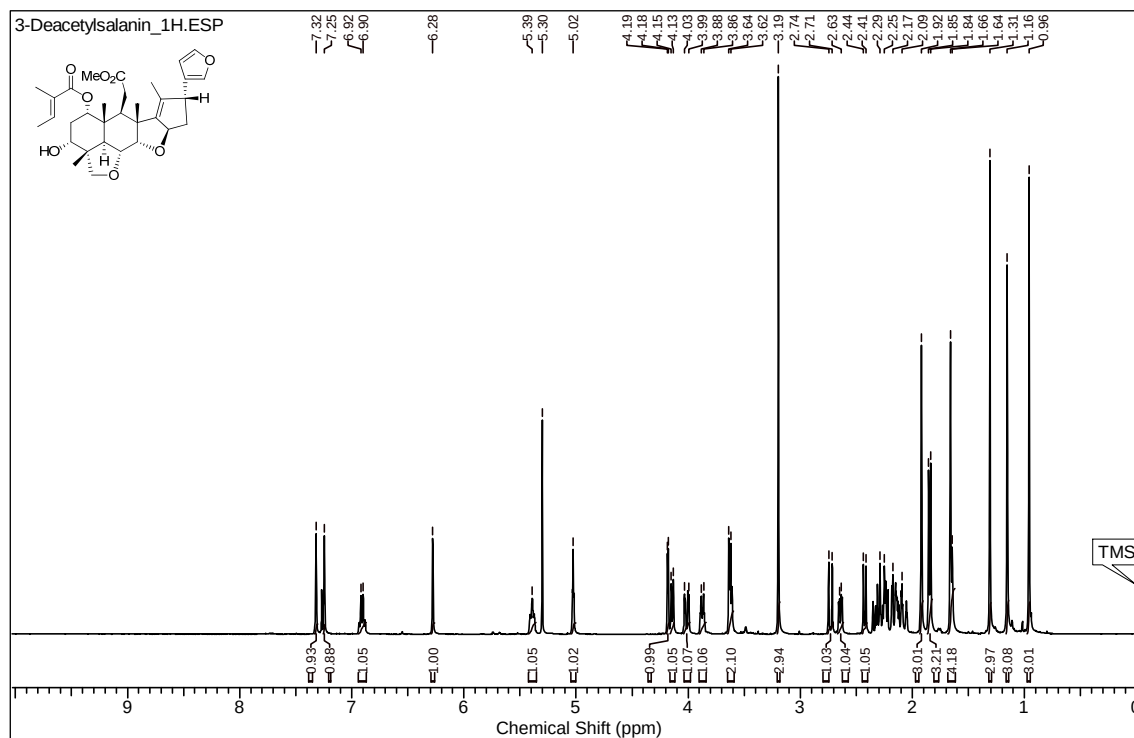
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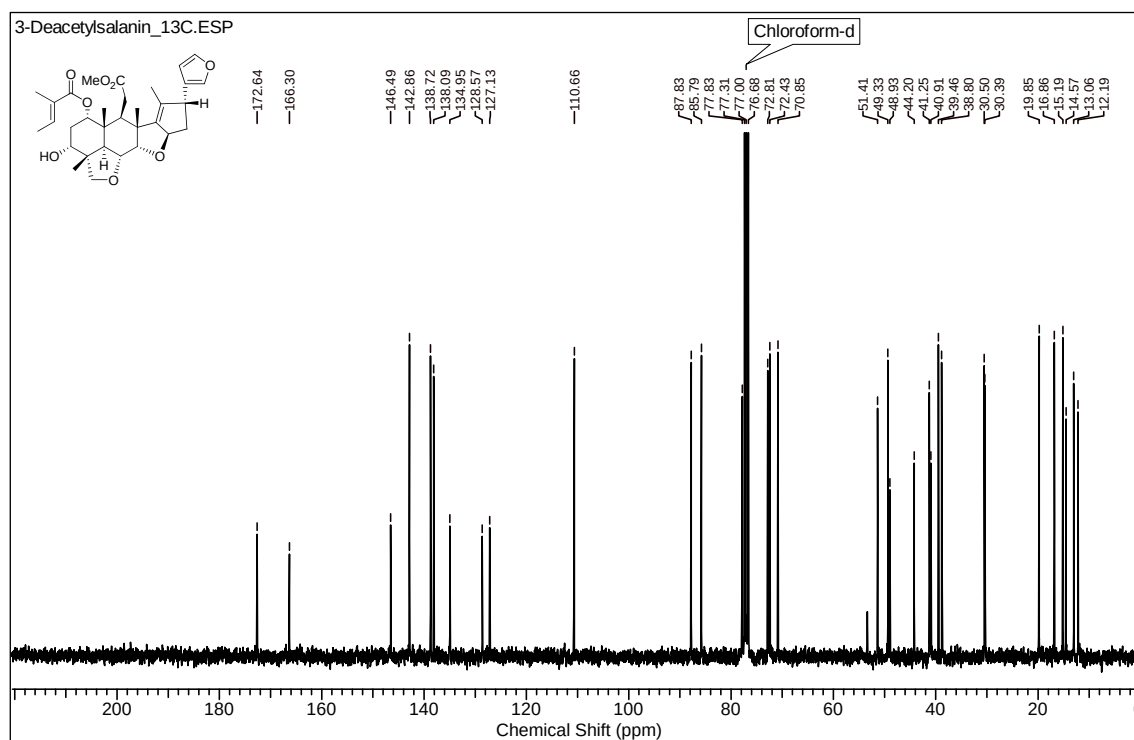
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DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of salannin

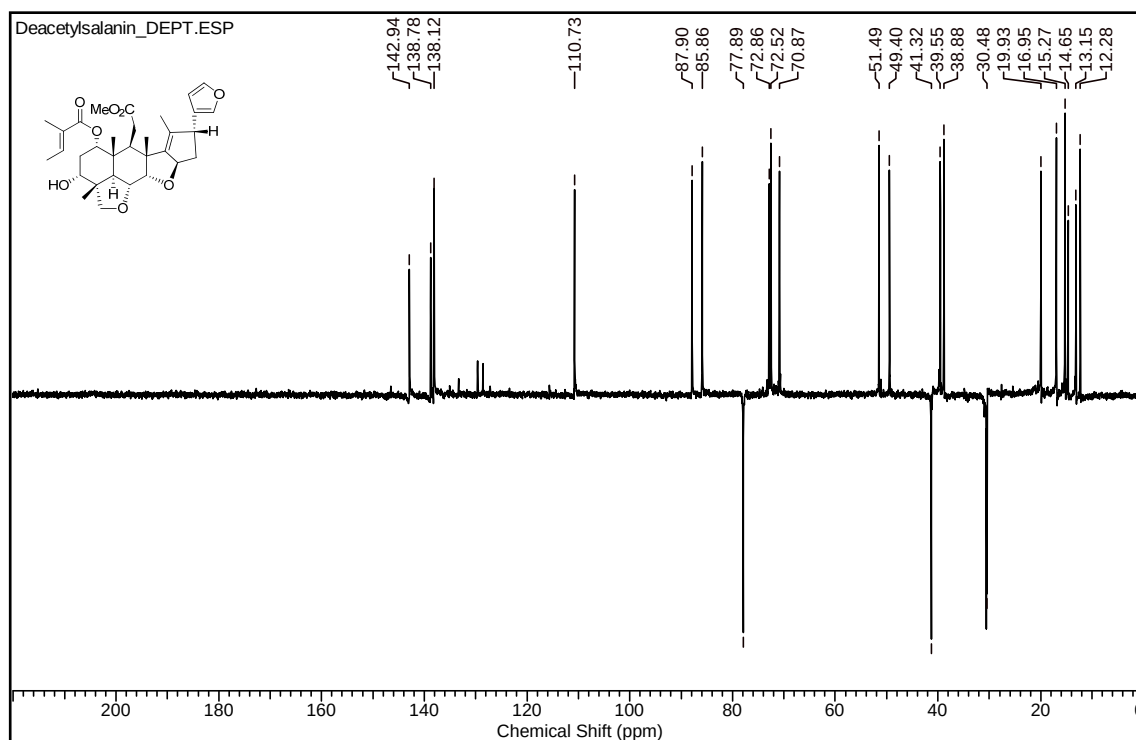
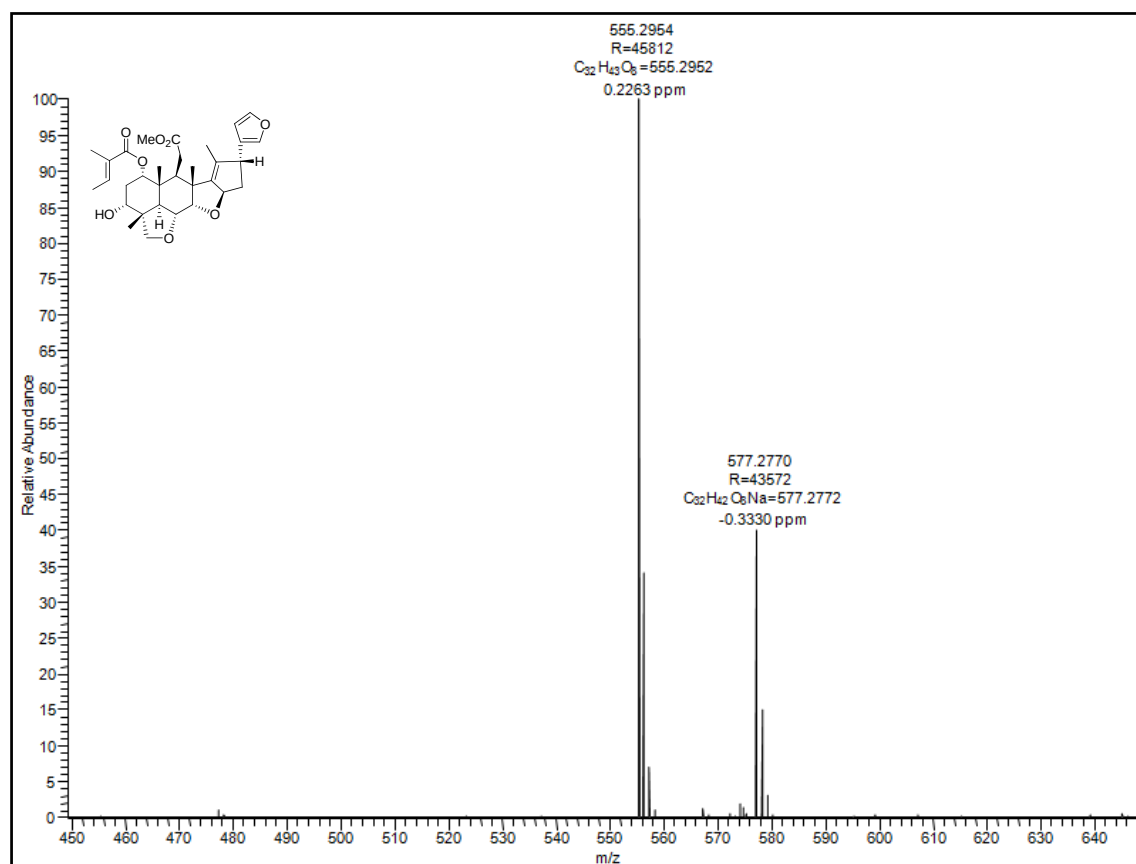
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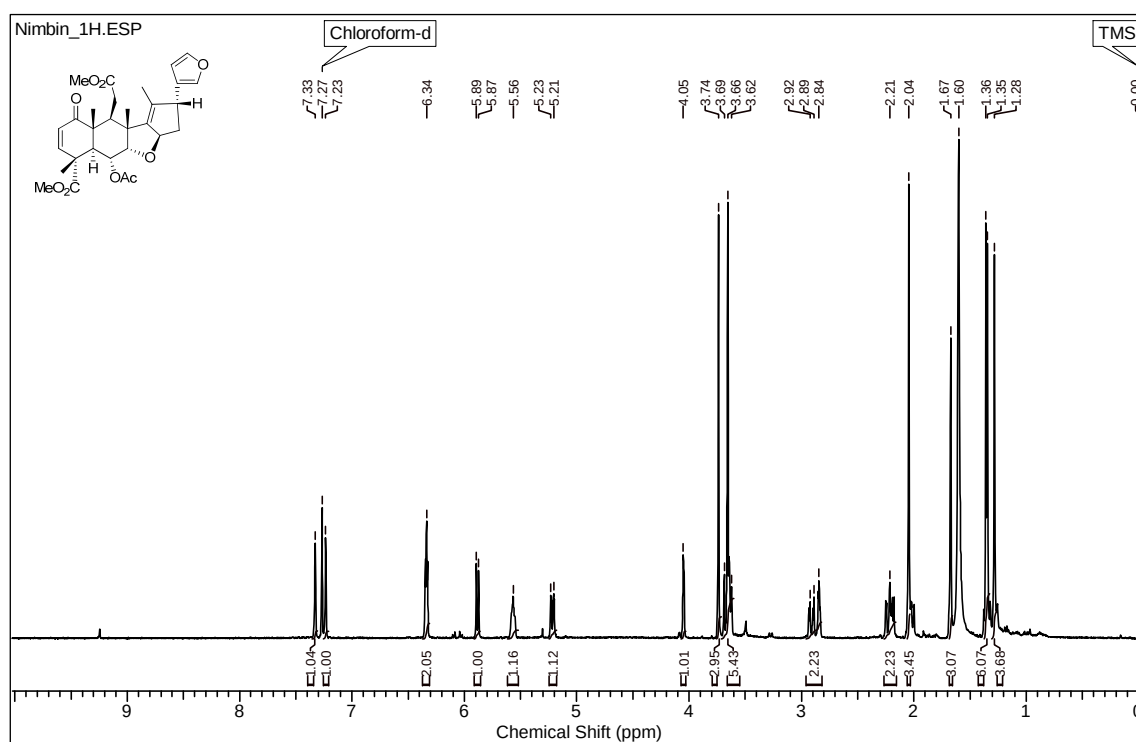
^1H NMR spectrum (CDCl_3 , 400 MHz) of 3-deacetylsalannin



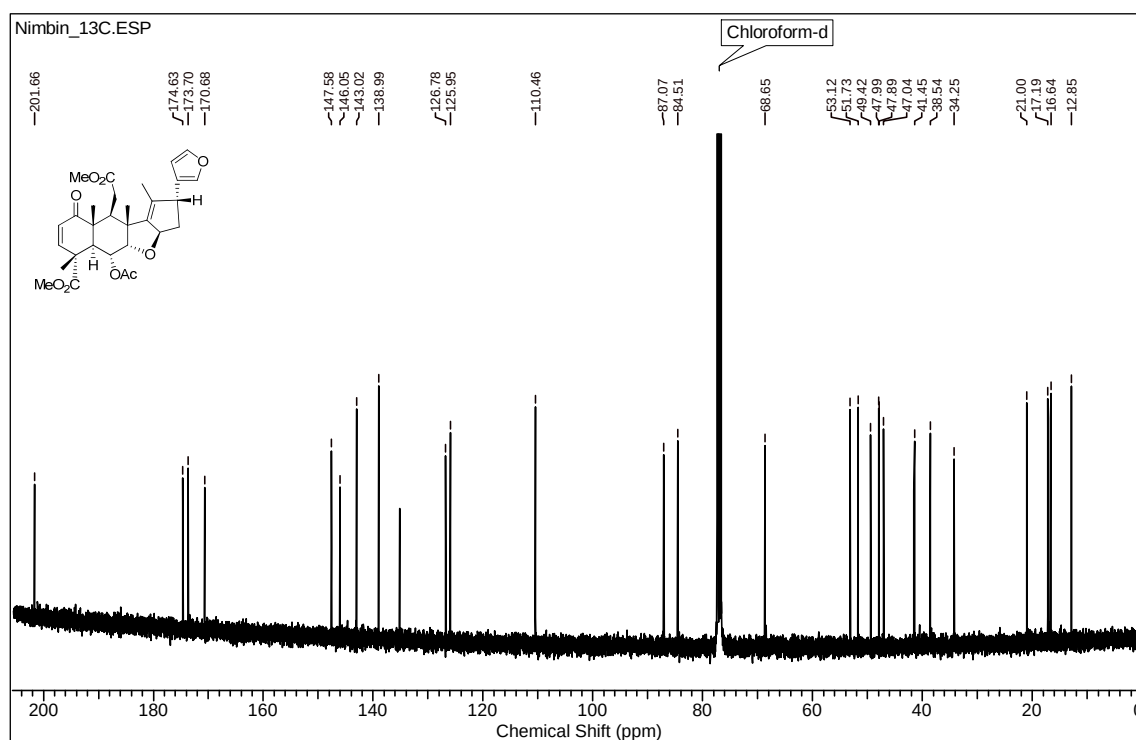
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DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of 3-deacetylsalannin

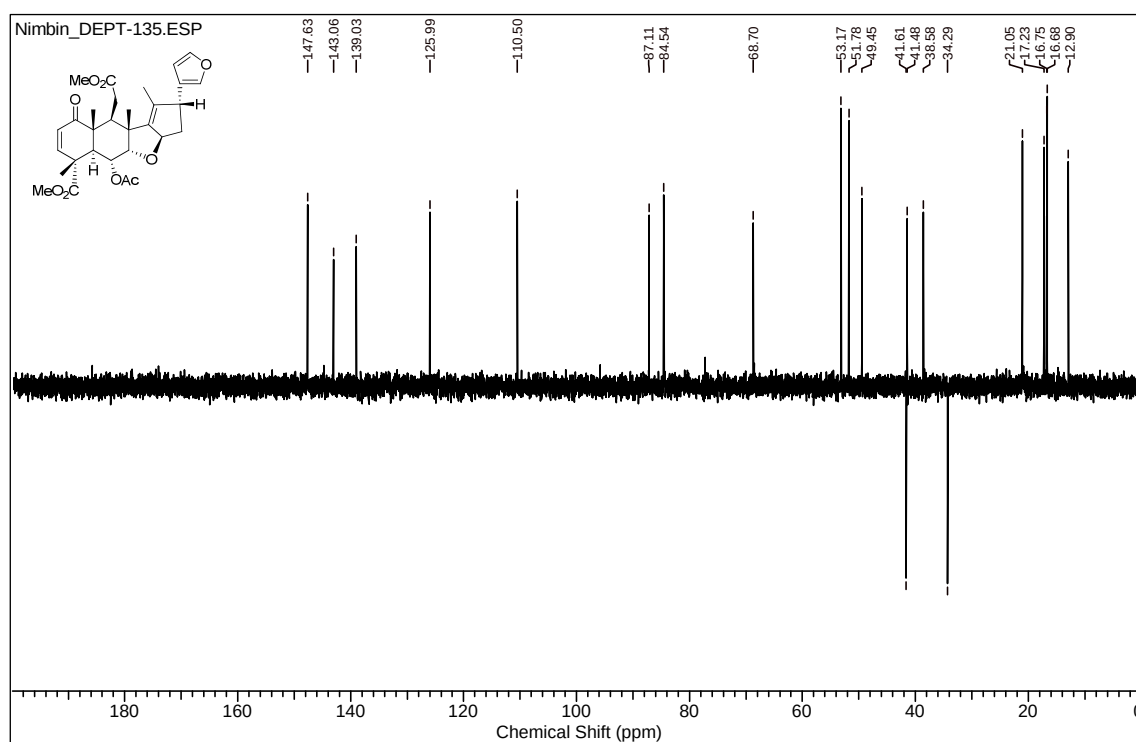
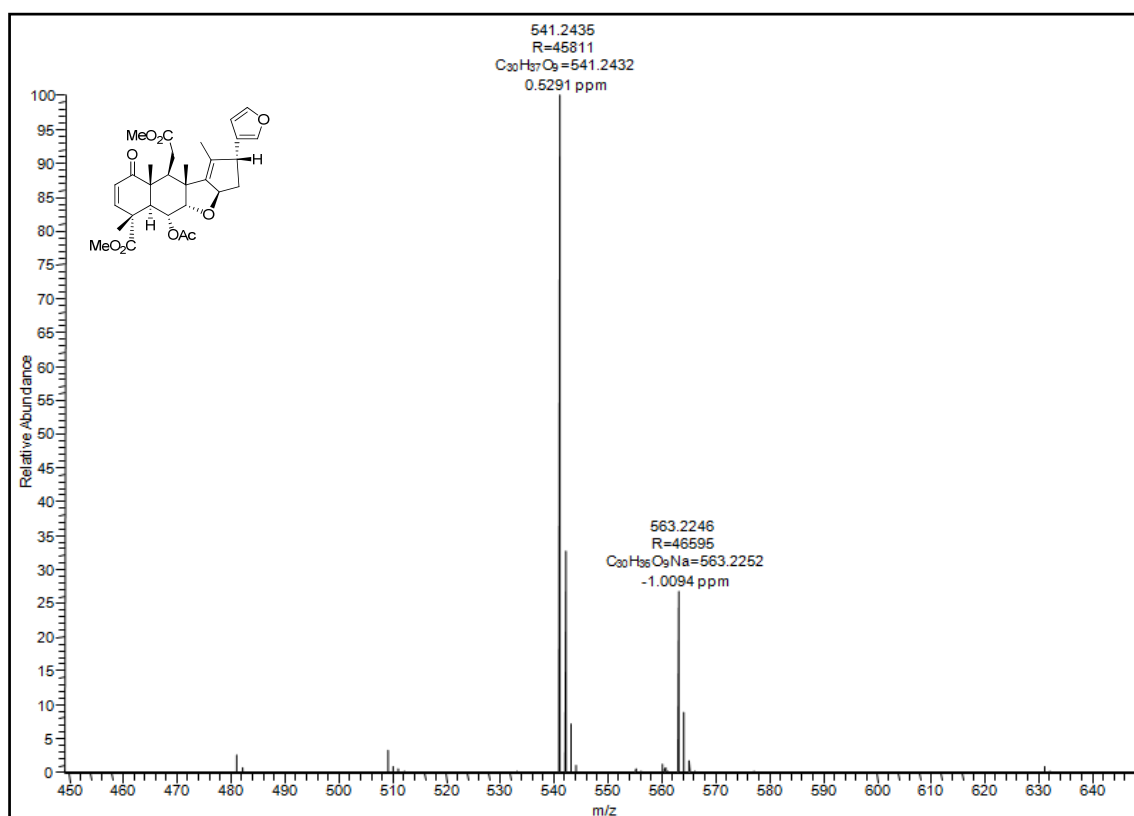
LC-ESI(+)-HRMS of 3-deacetylsalannin



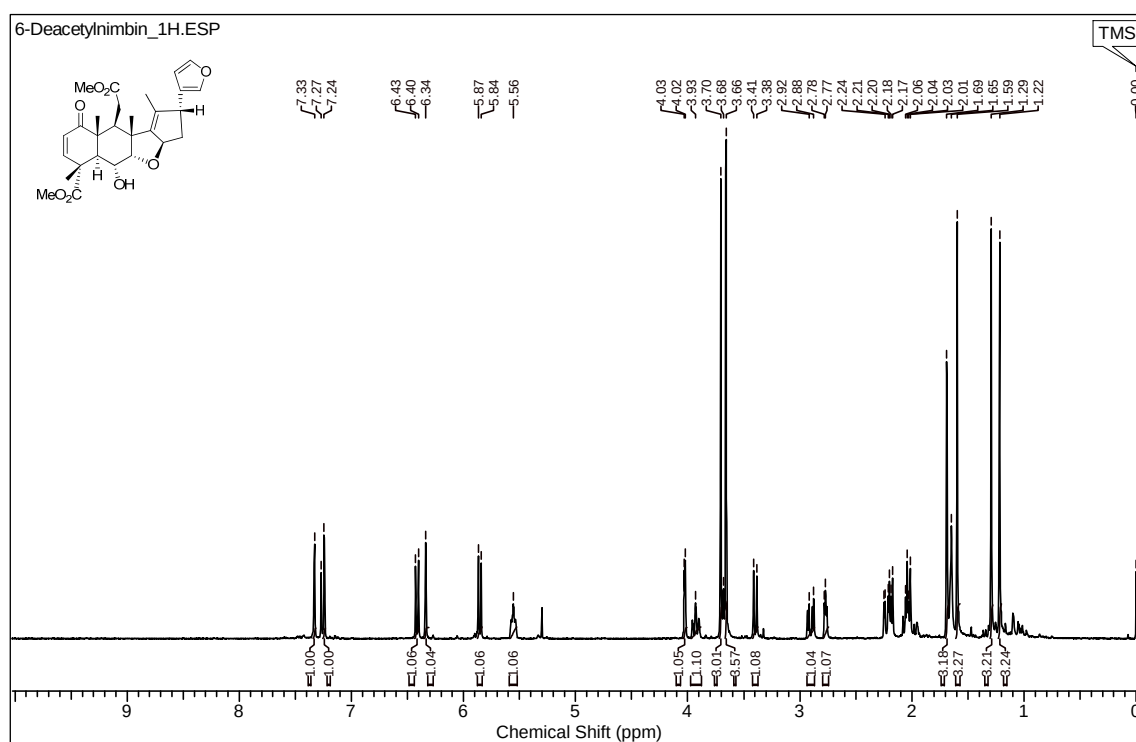
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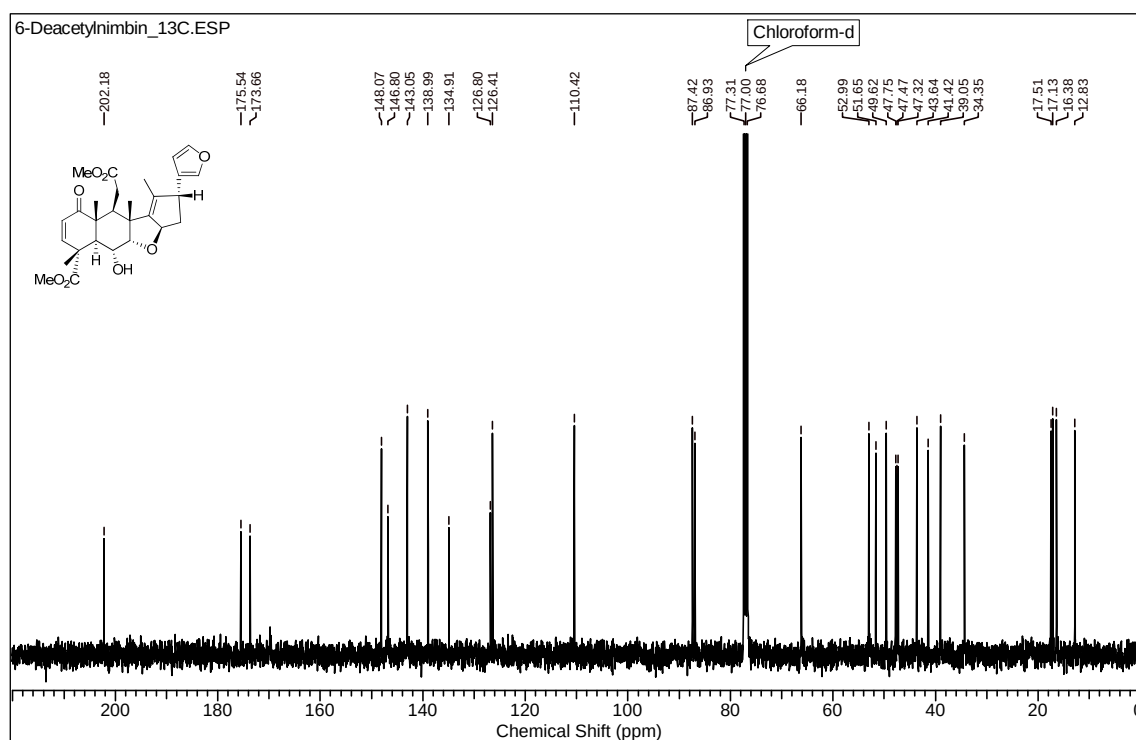
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DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of nimbin

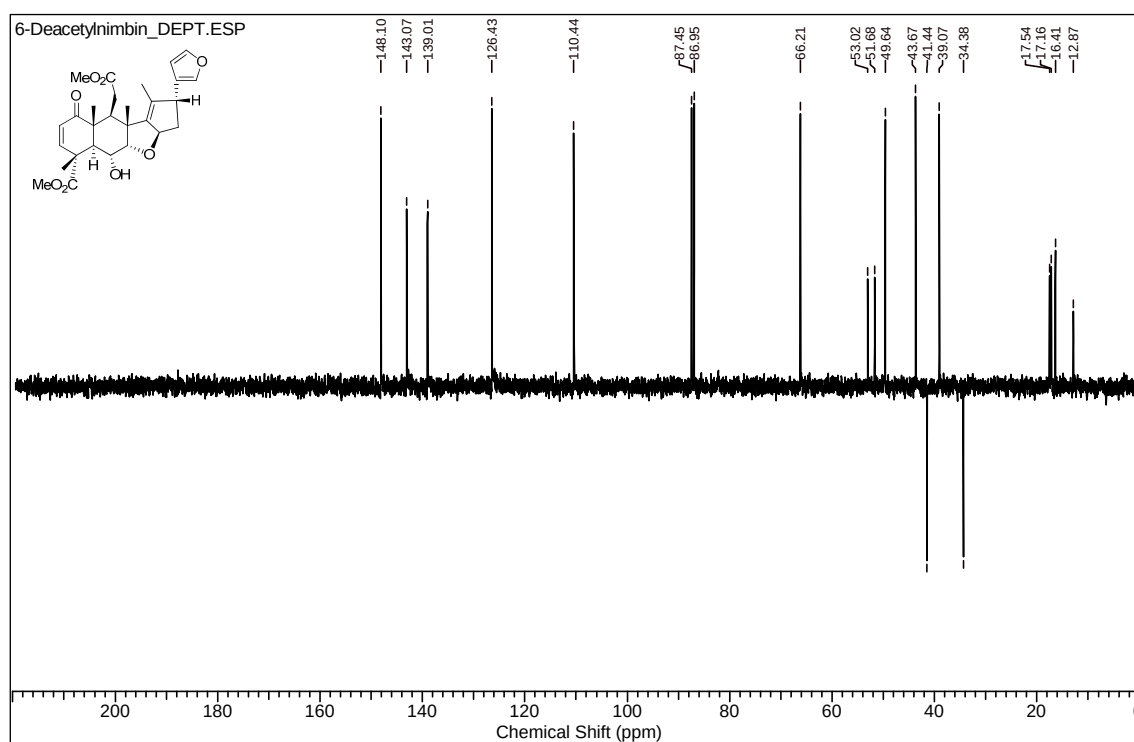
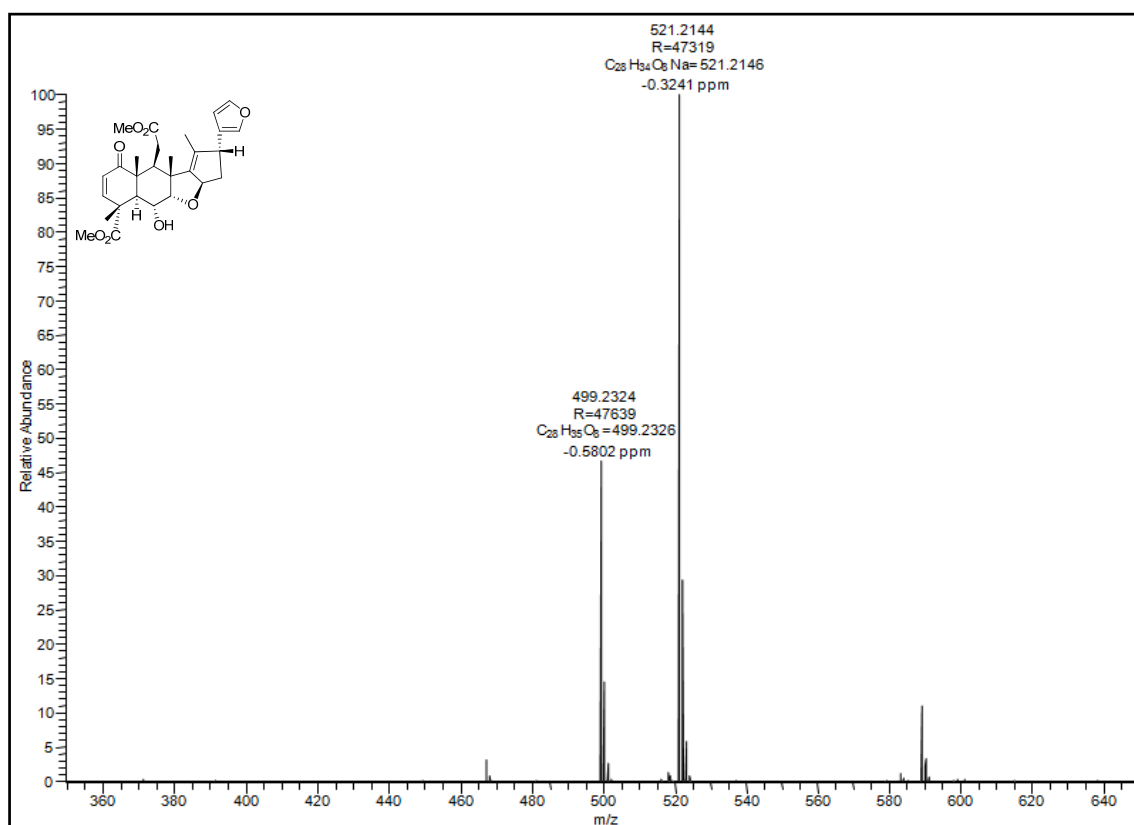
LC-ESI(+)-HRMS of nimbin



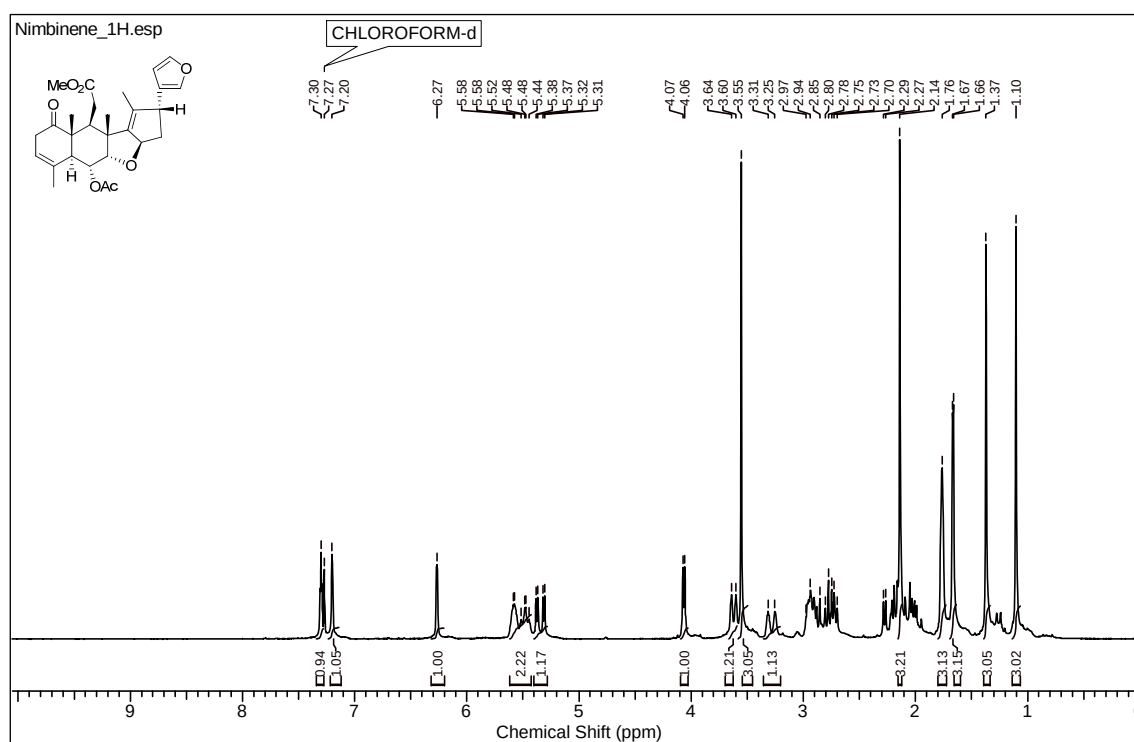
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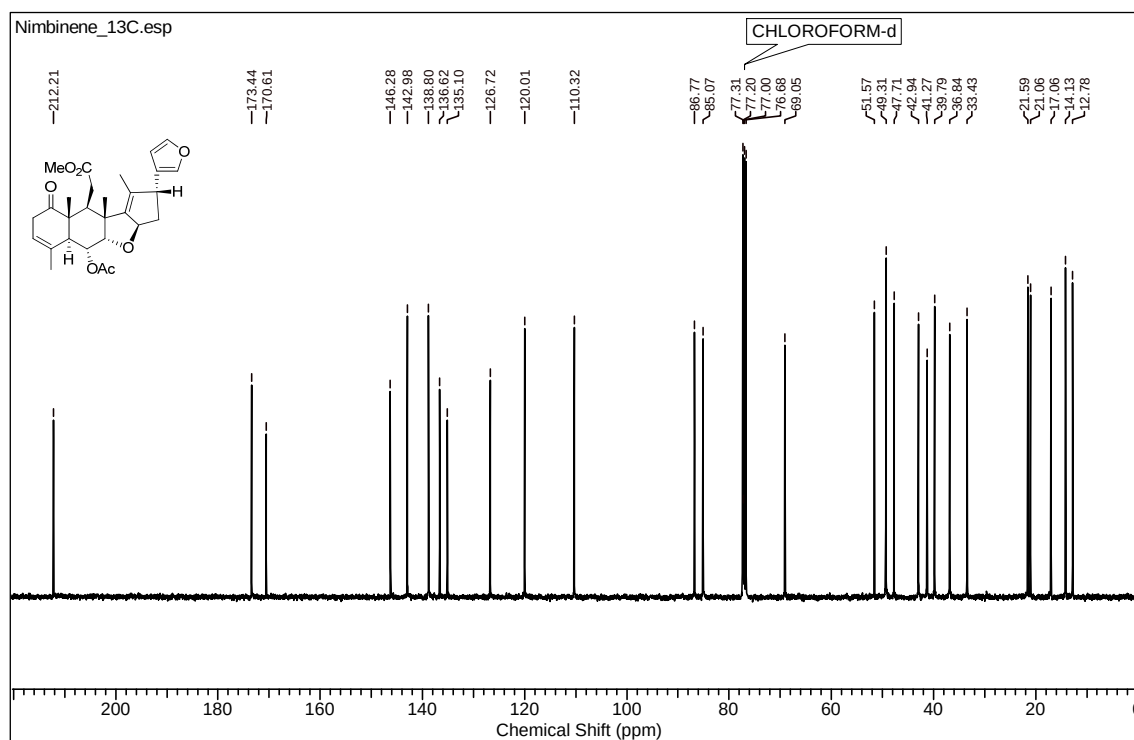
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 6-deacetylnimbin

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of 6-deacetylnimbin

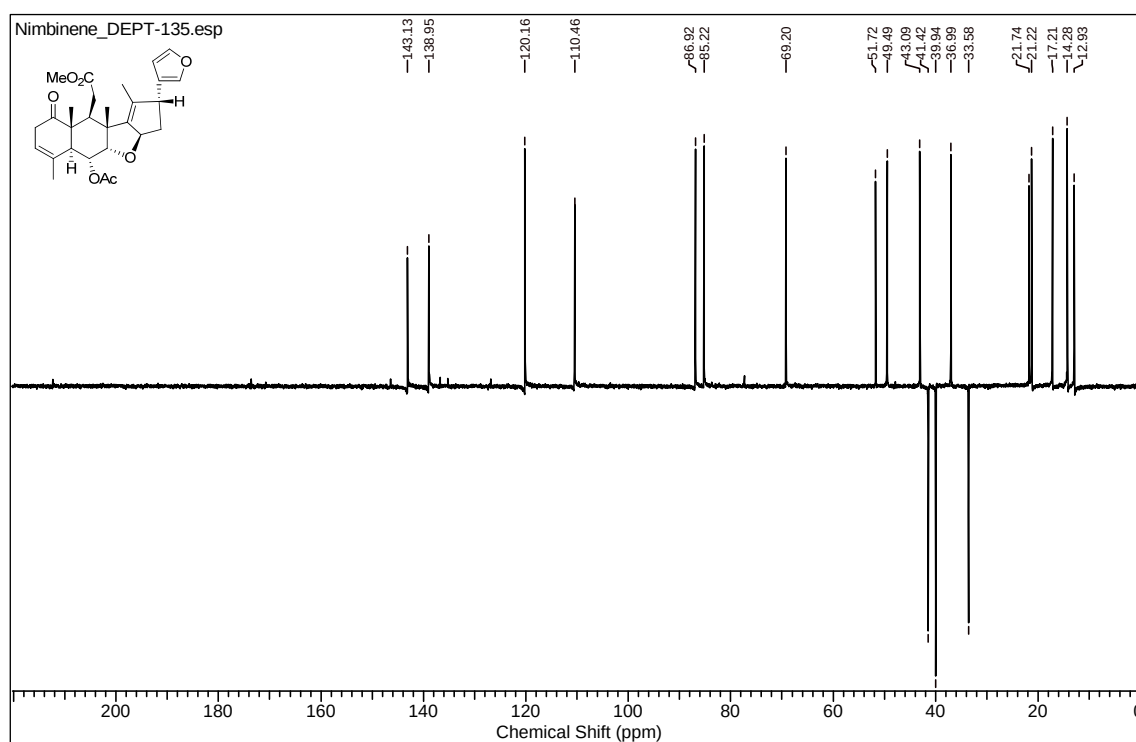
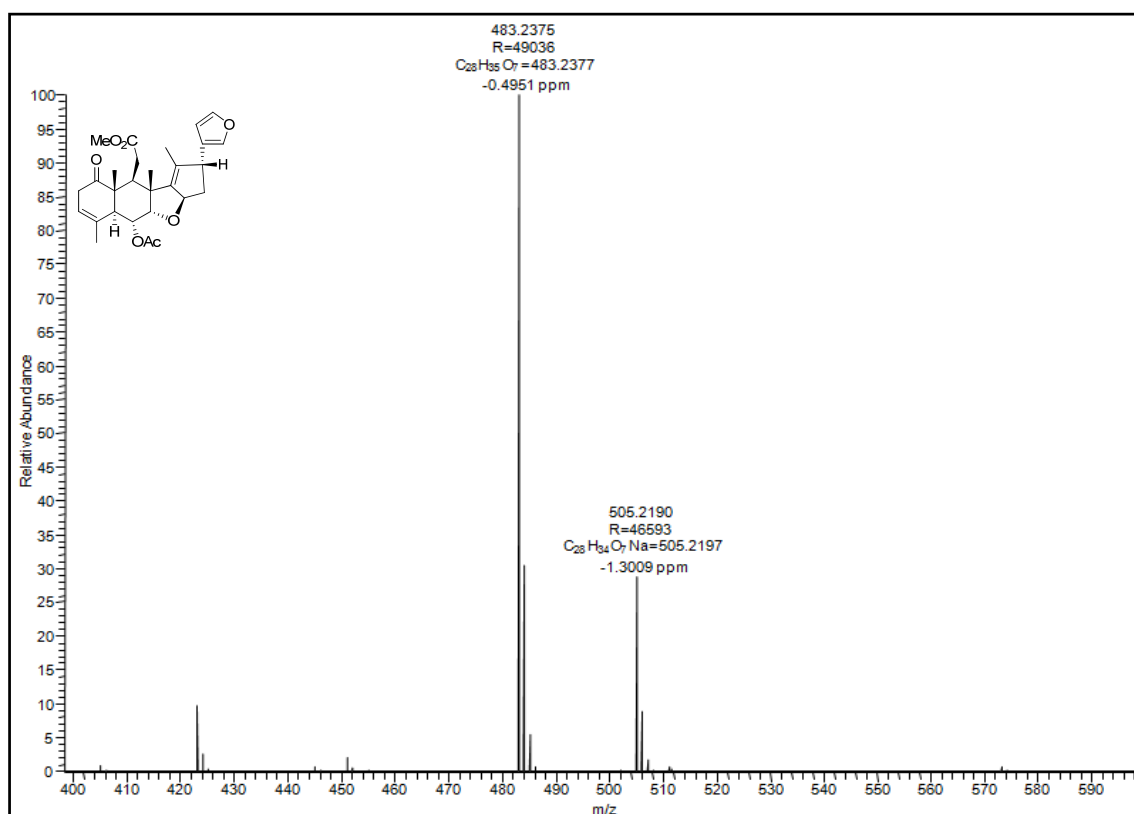
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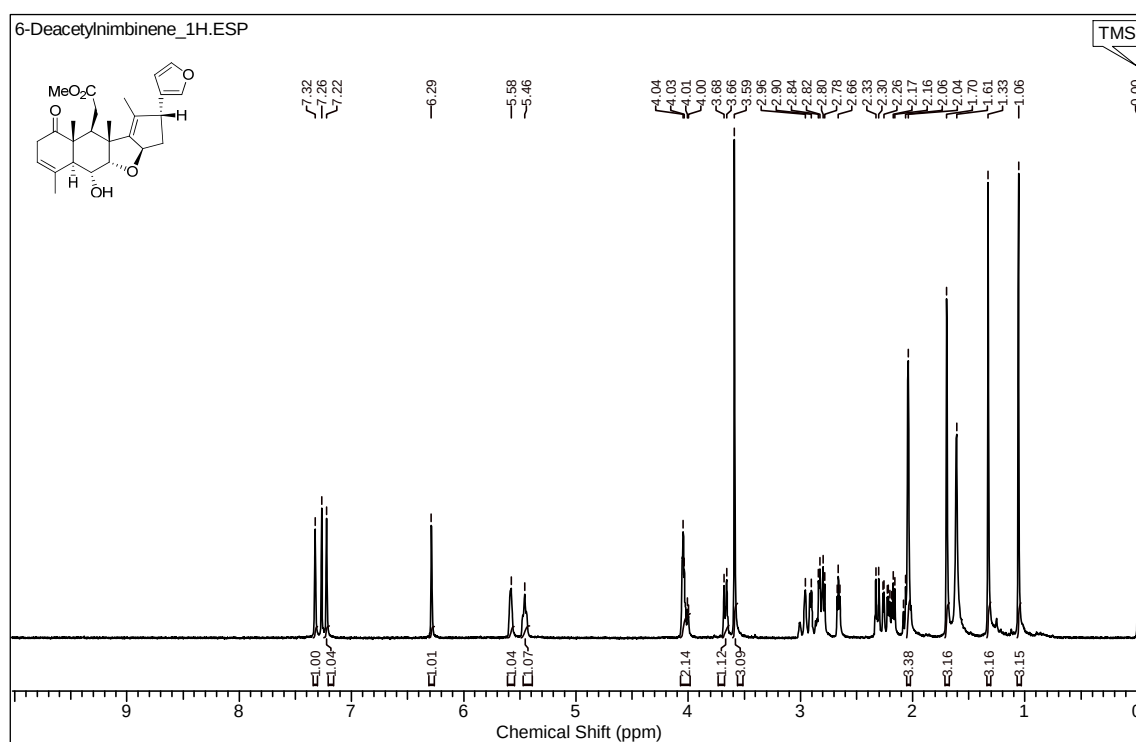
^1H NMR spectrum (CDCl_3 , 400 MHz) of nimbinene



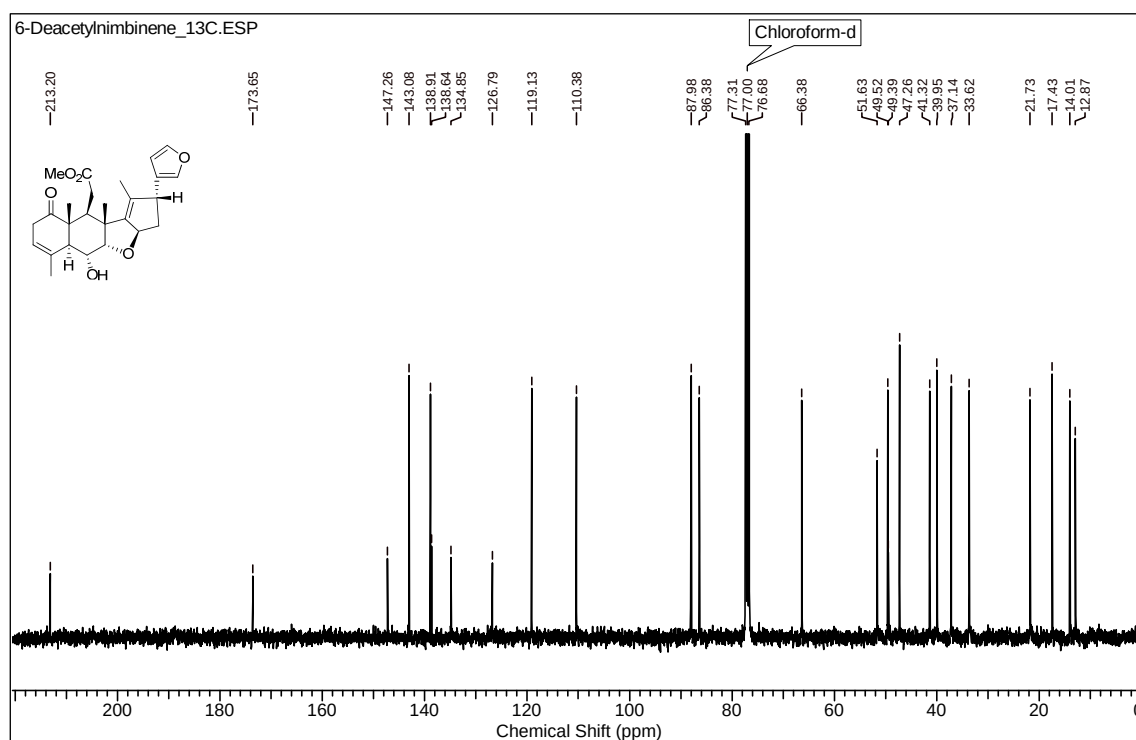
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DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of nimbinene

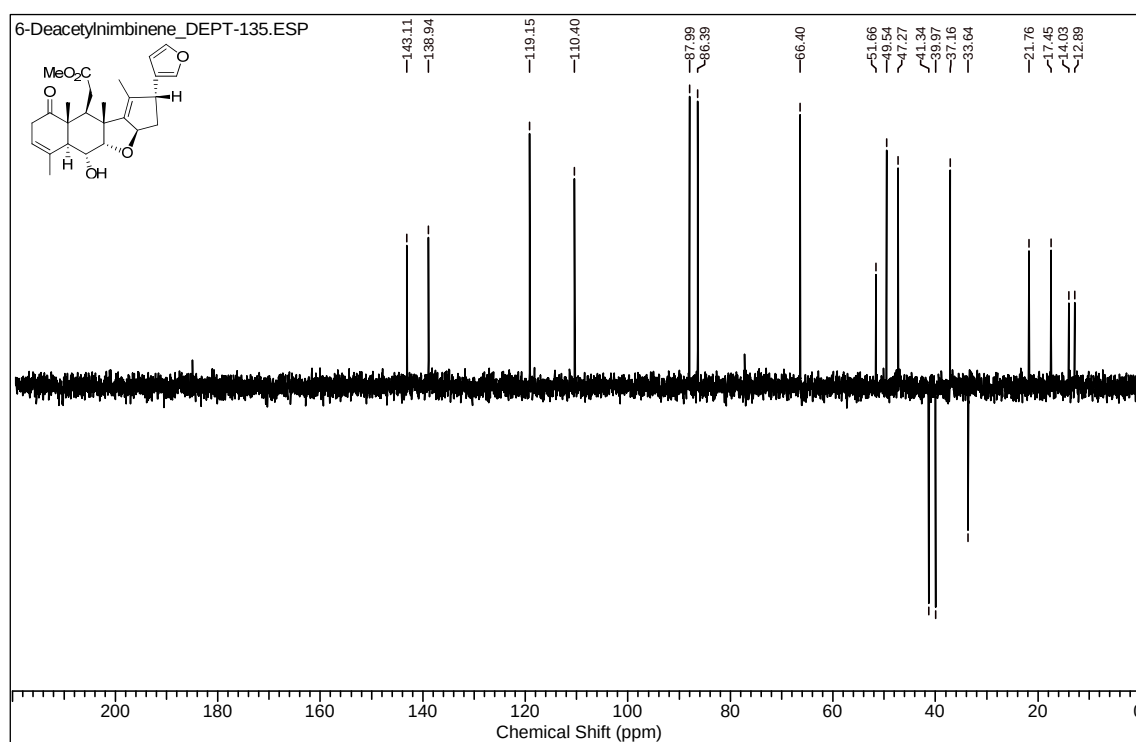
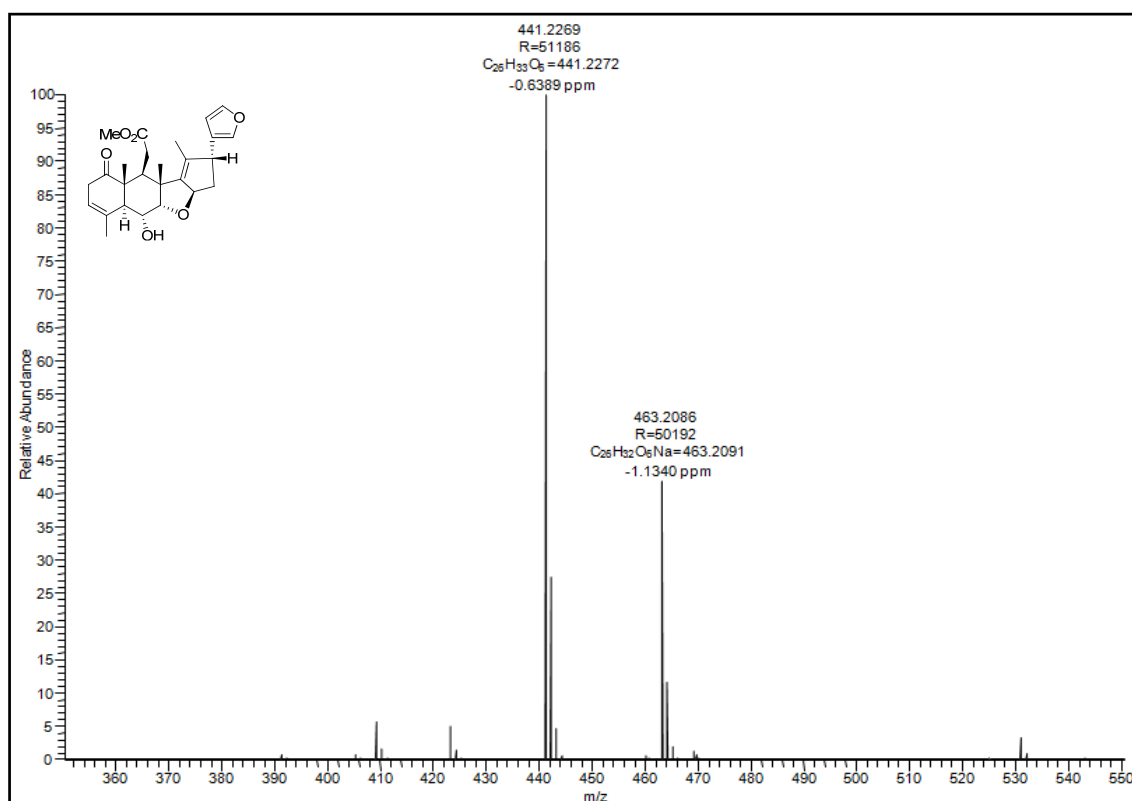
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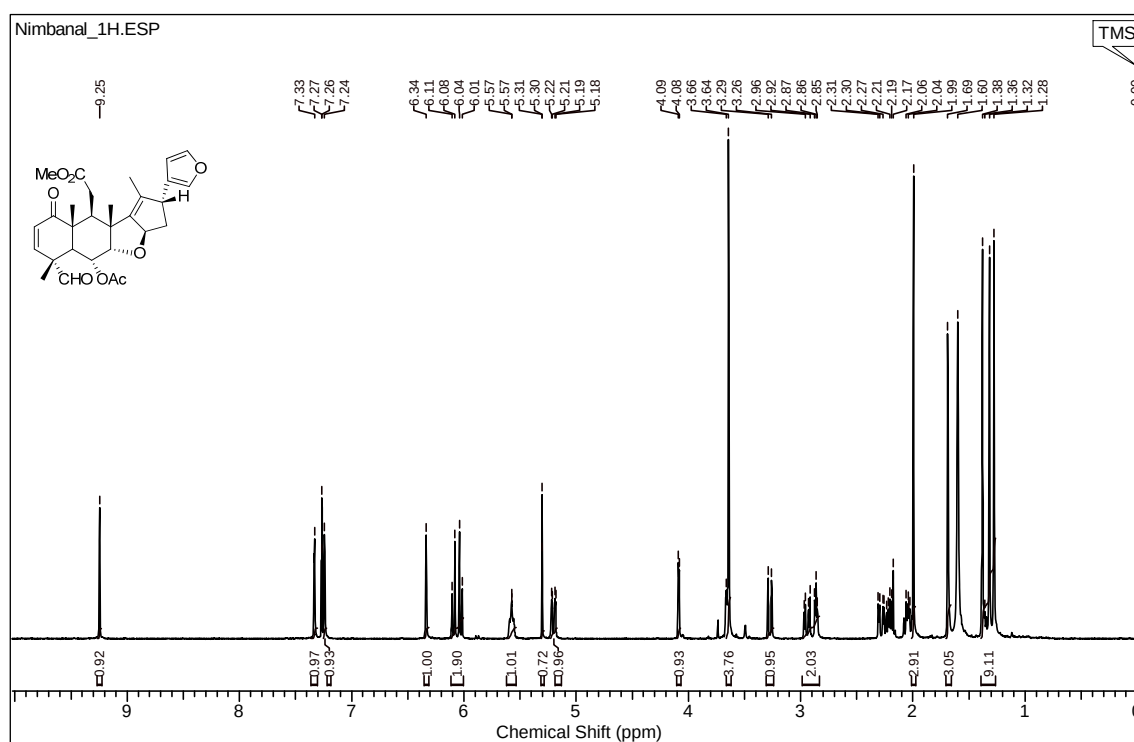
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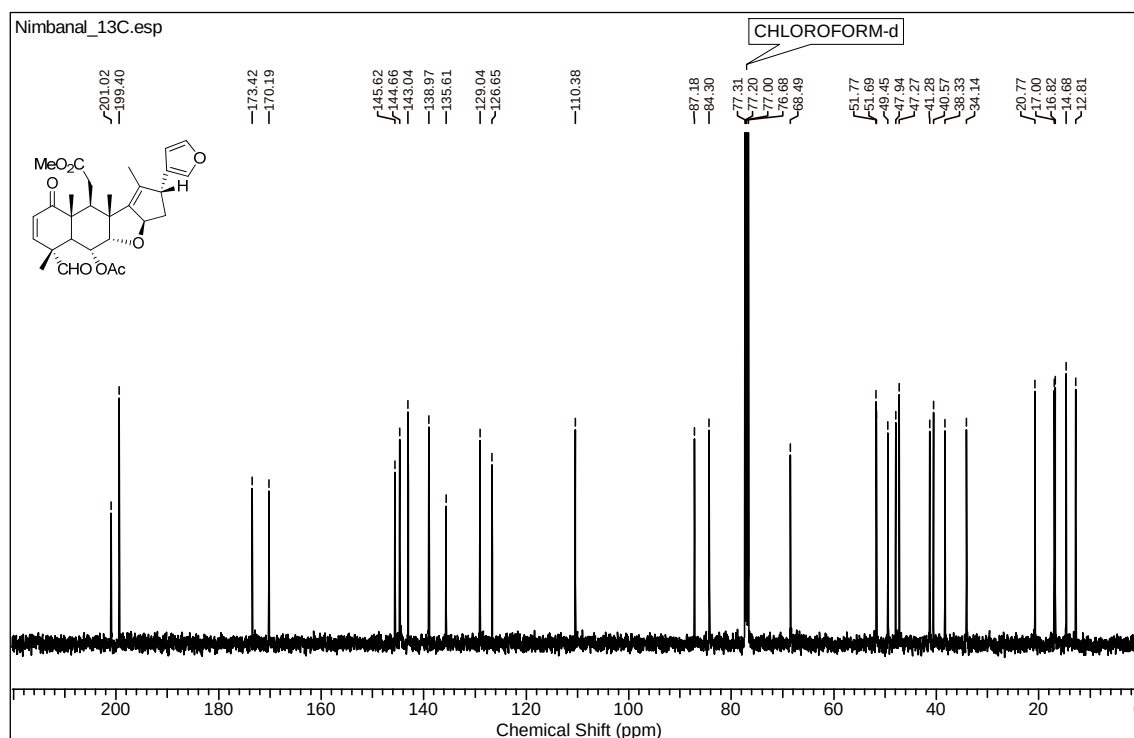
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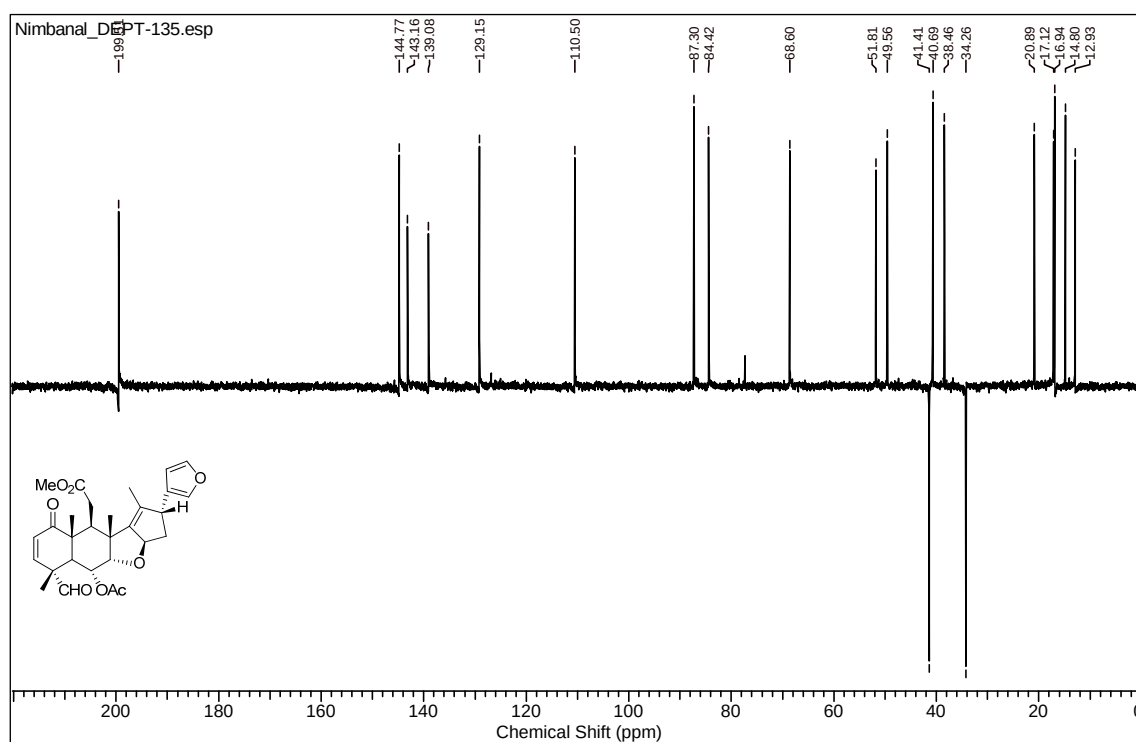
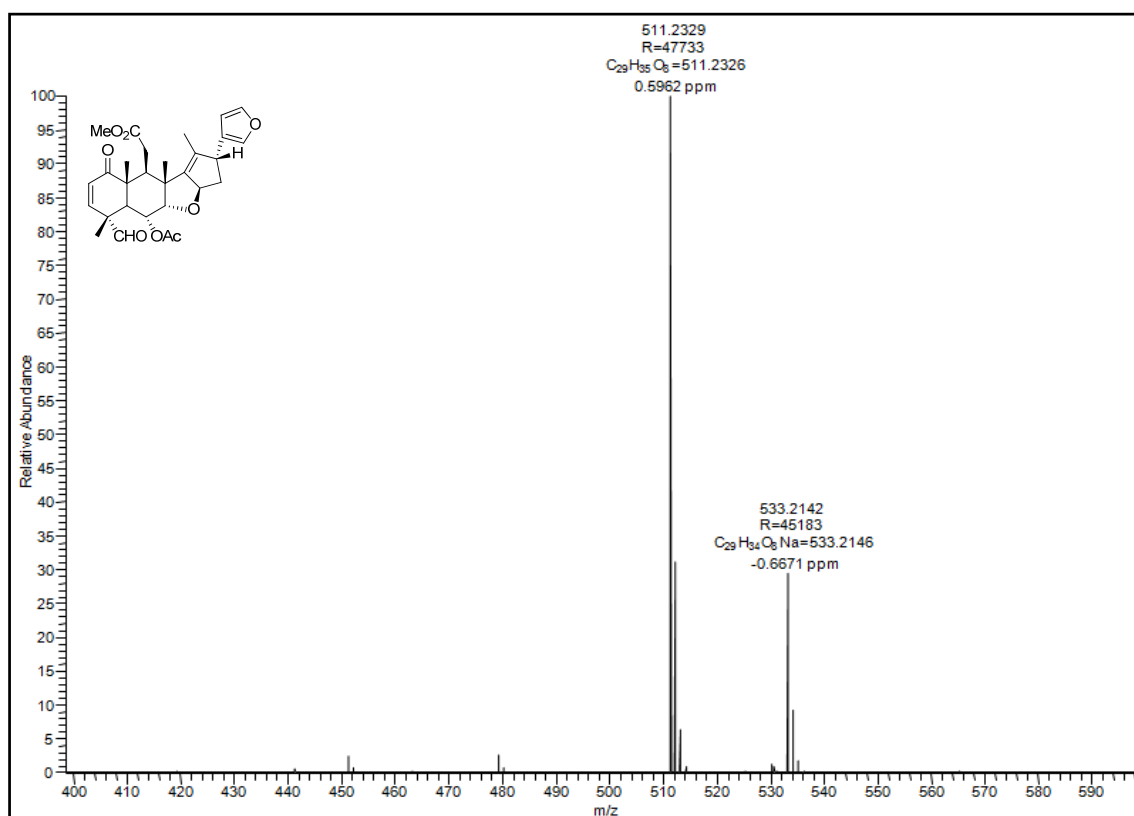
LC-ESI(+)-HRMS of 6-deacetylnimbinene



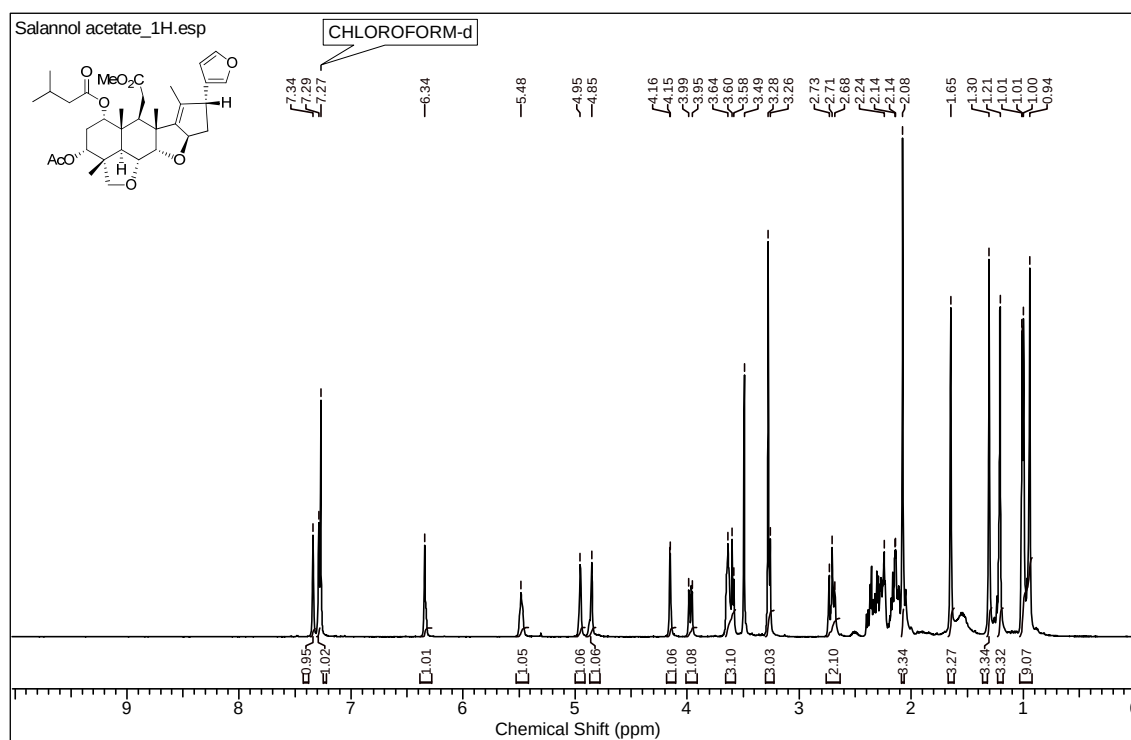
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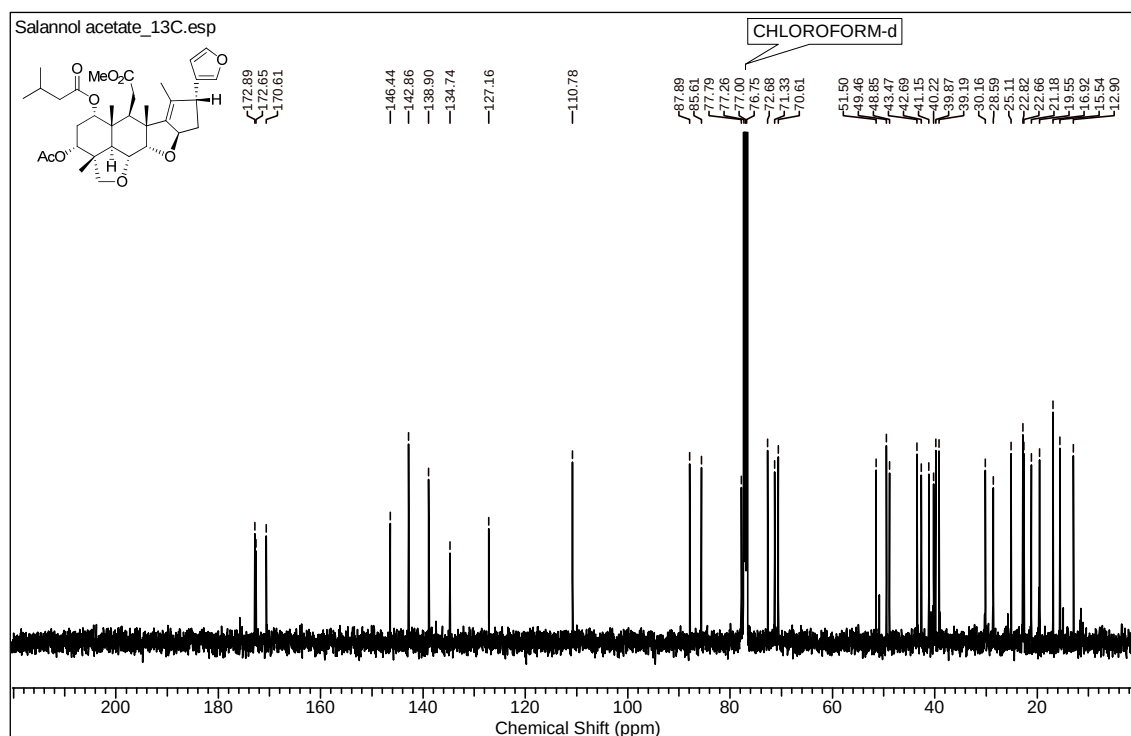
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DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of nimbanal

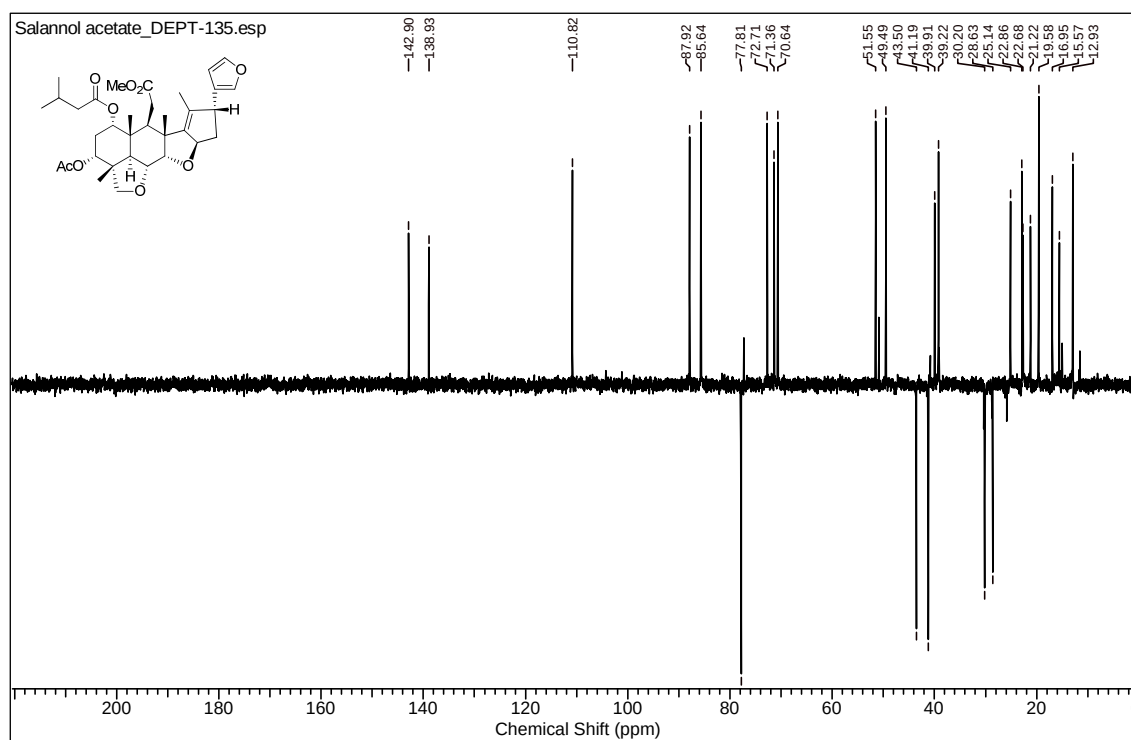
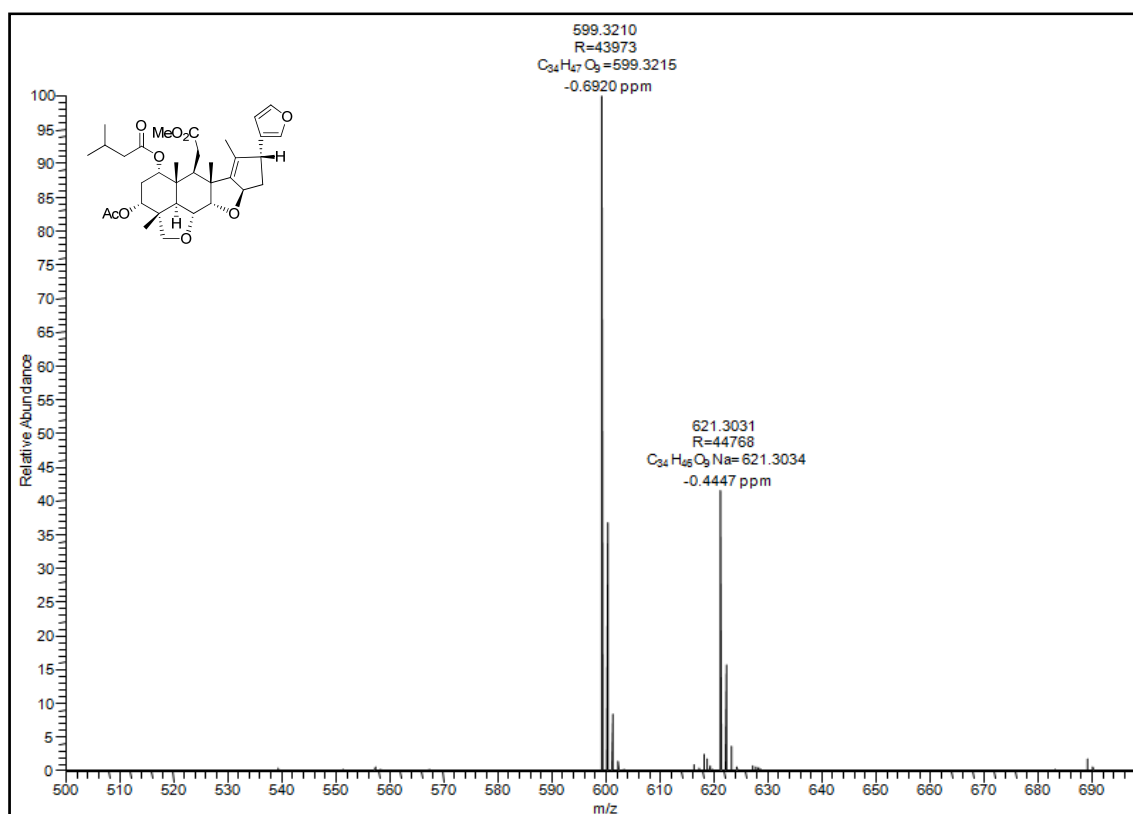
LC-ESI(+)-HRMS of nimbanal



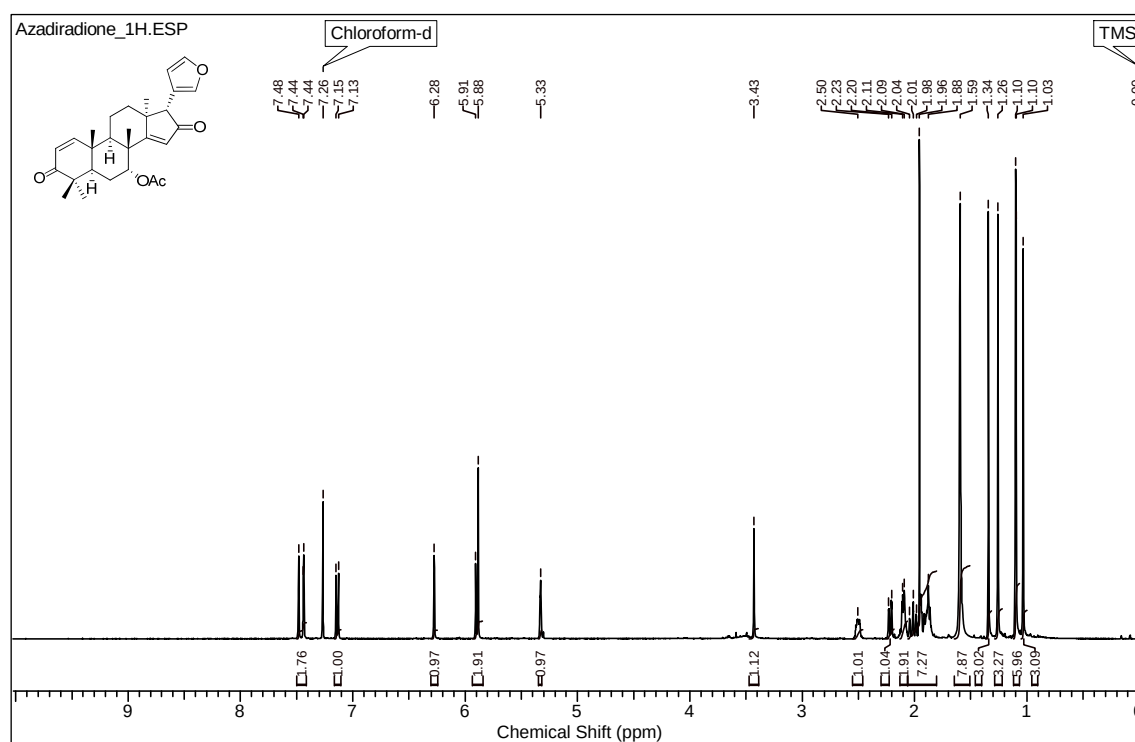
^1H NMR spectrum (CDCl_3 , 400 MHz) of salannol acetate



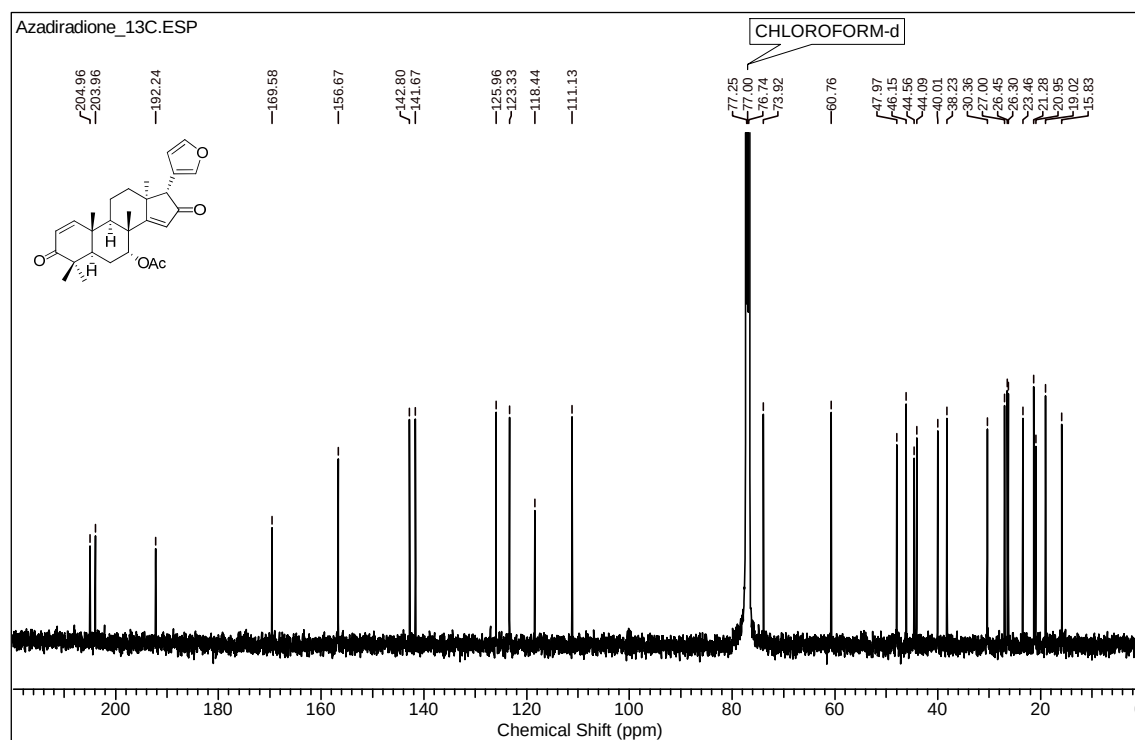
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of salannol acetate

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of salannol acetate

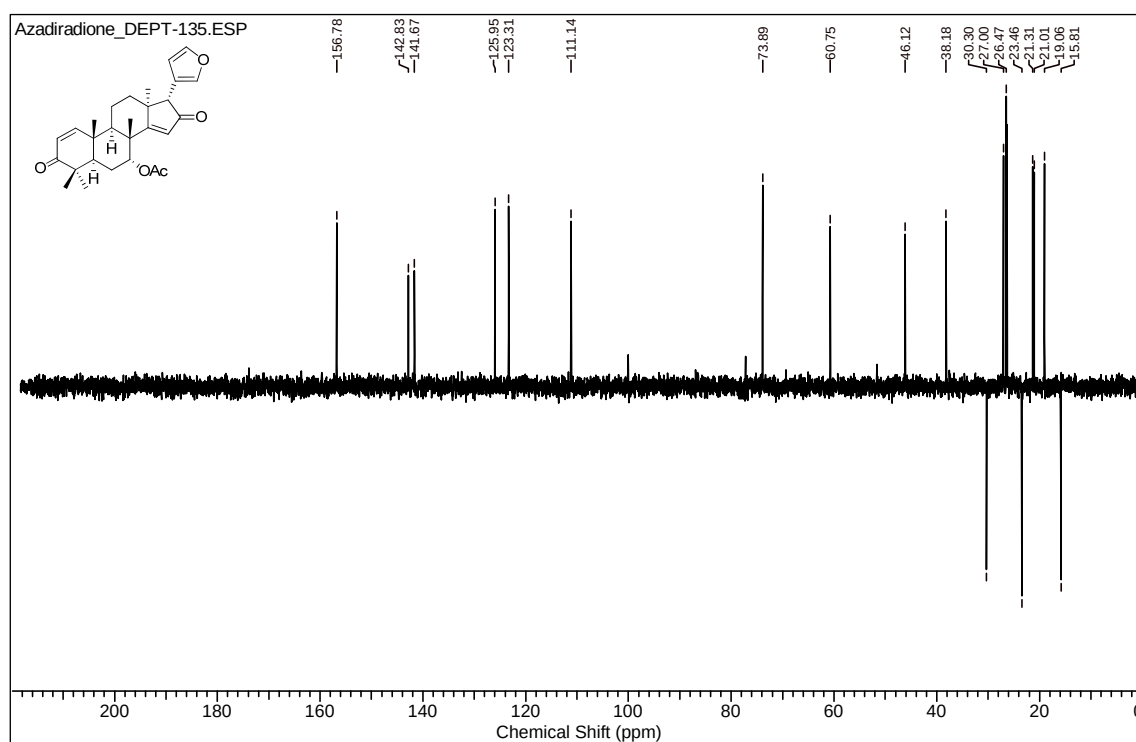
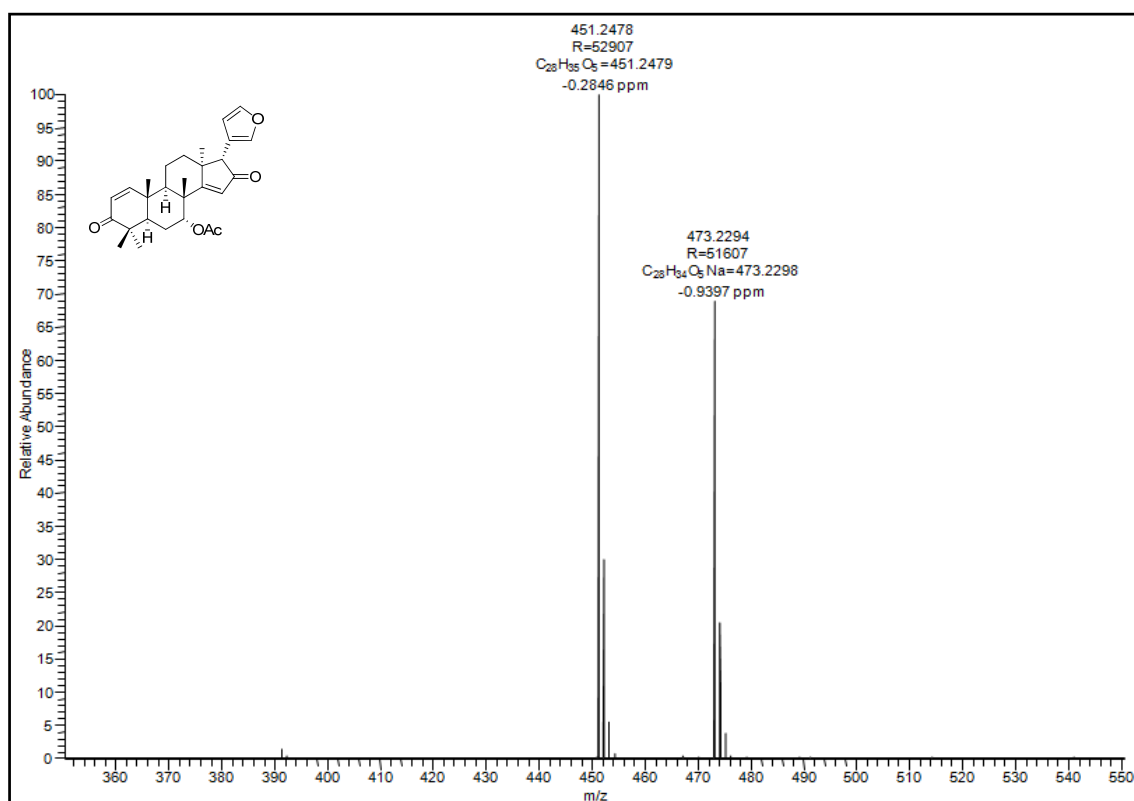
LC-ESI(+)-HRMS of salannol acetate



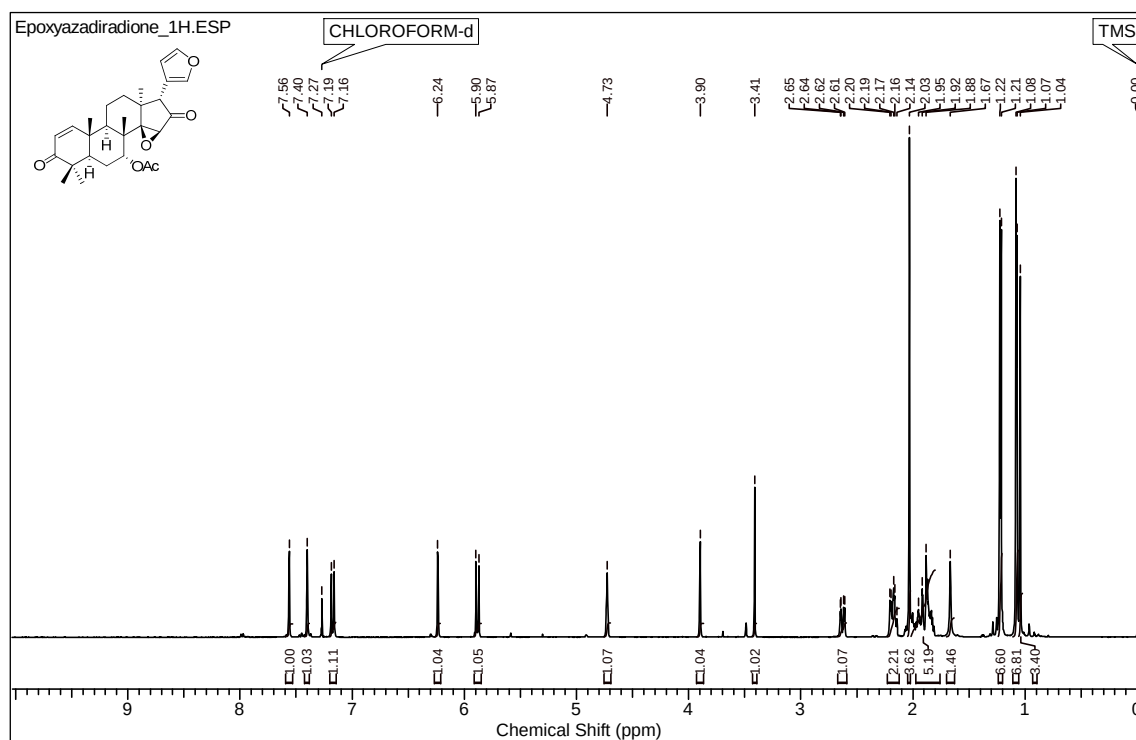
^1H NMR spectrum (CDCl_3 , 400 MHz) of azadiradione



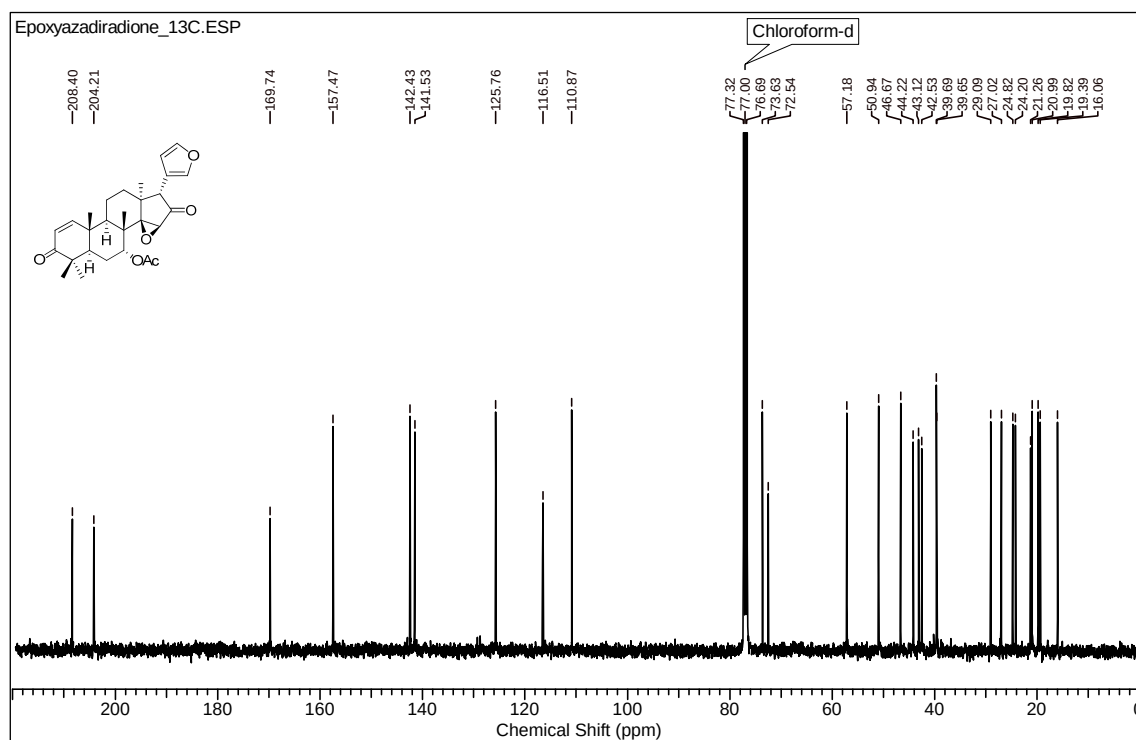
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of azadiradione

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of azadiradione

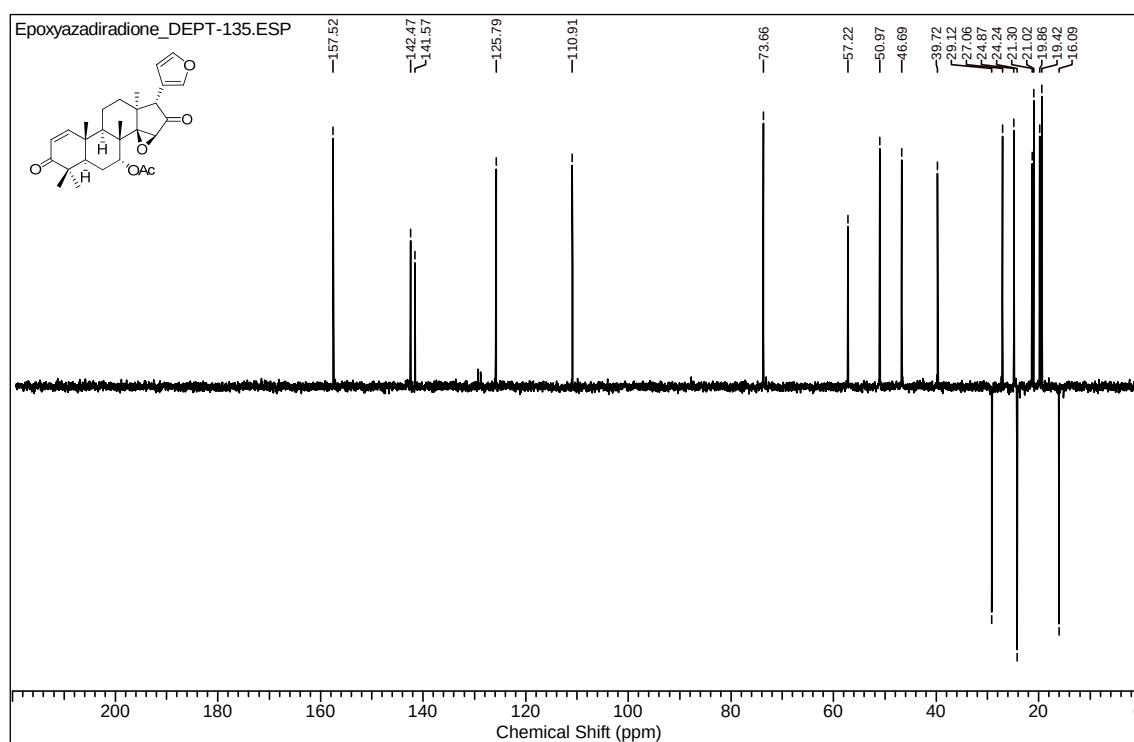
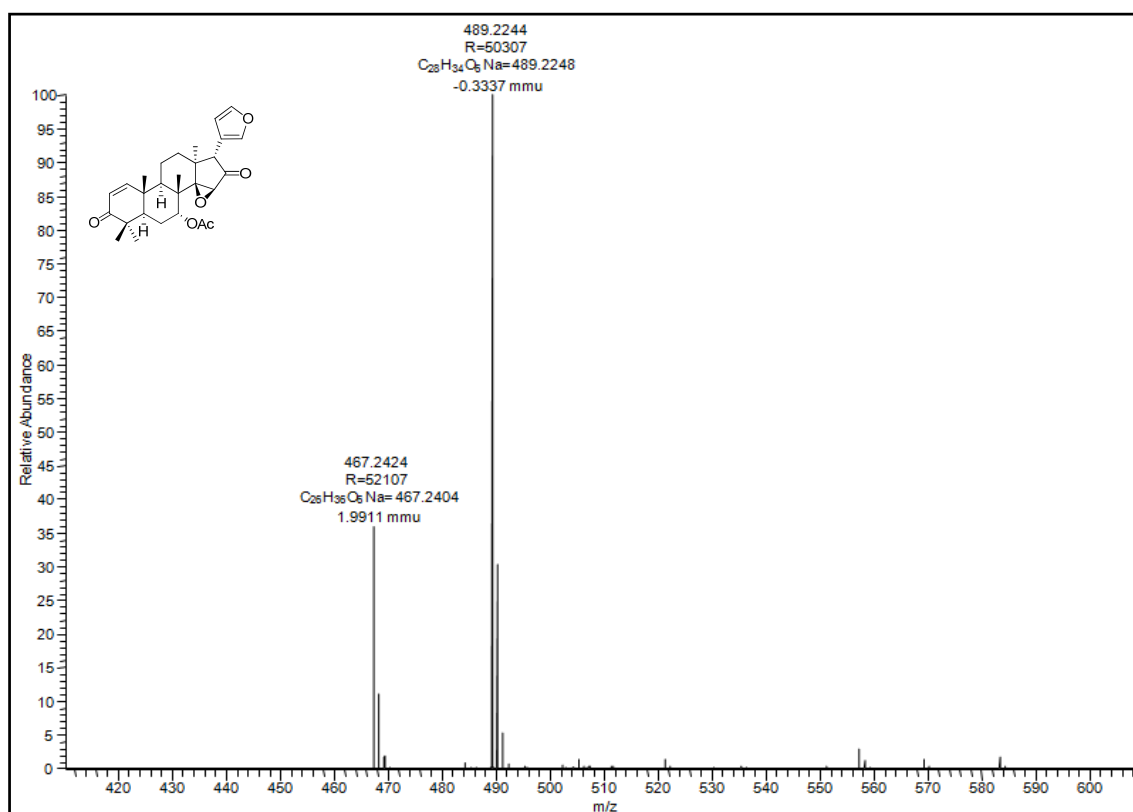
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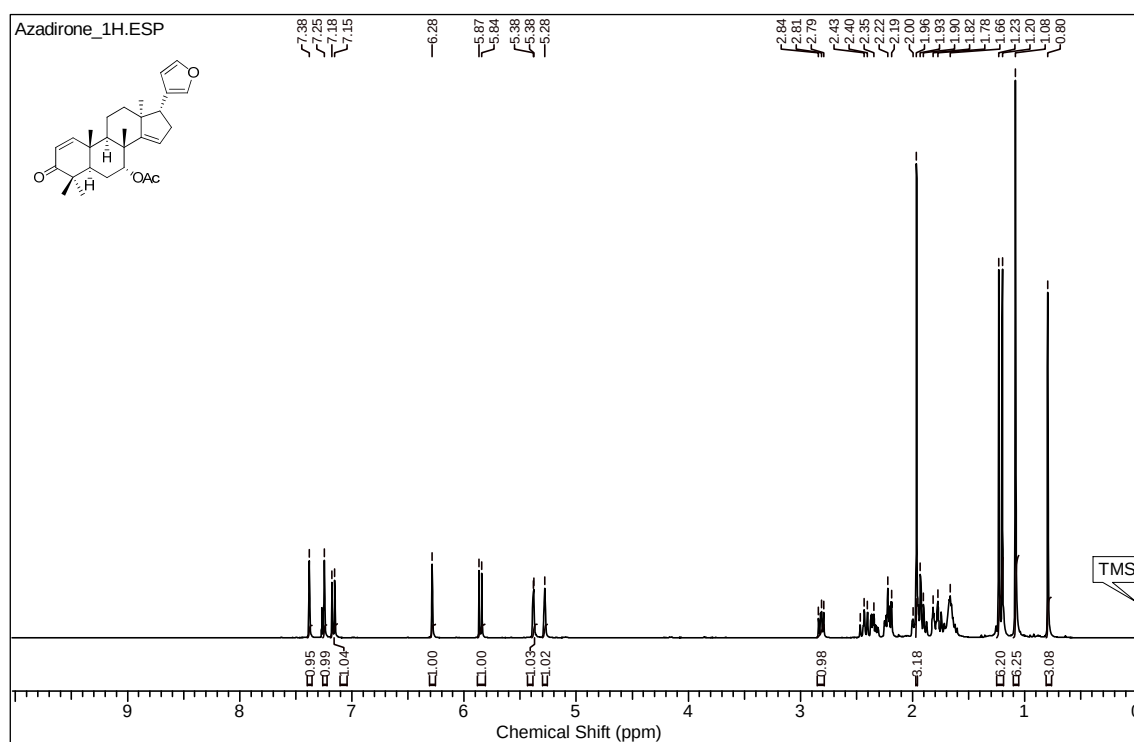
^1H NMR spectrum (CDCl_3 , 400 MHz) of epoxyzadiradione



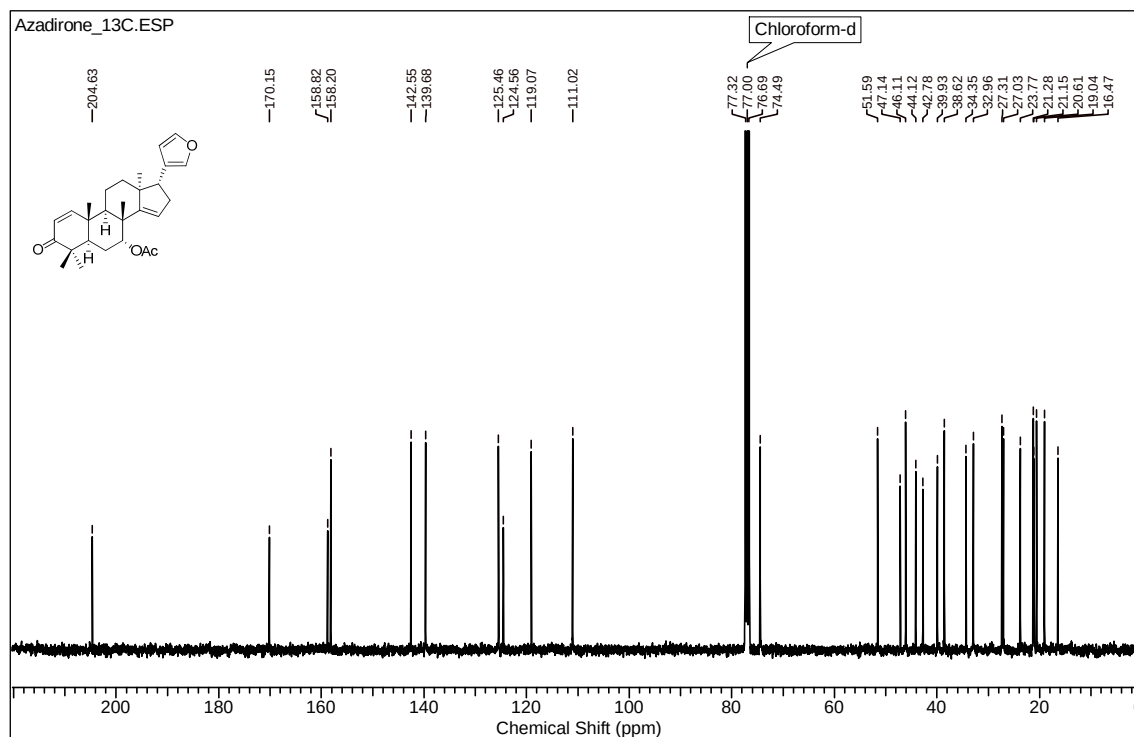
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DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of epoxyazadiradione

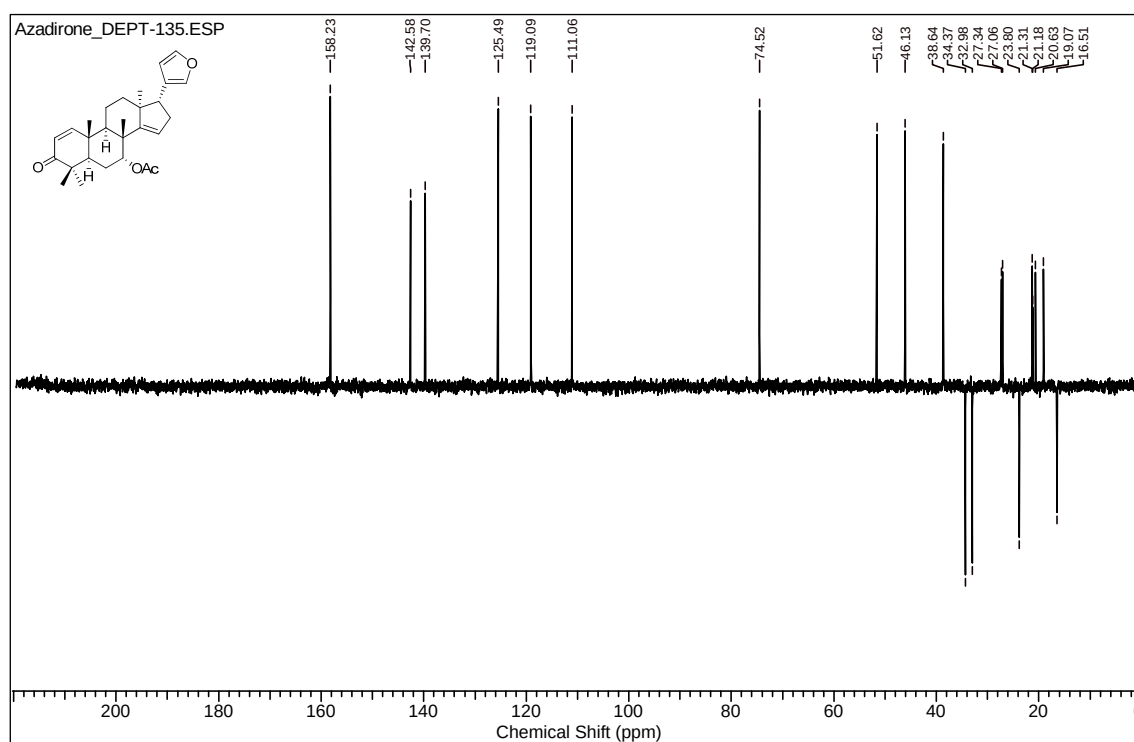
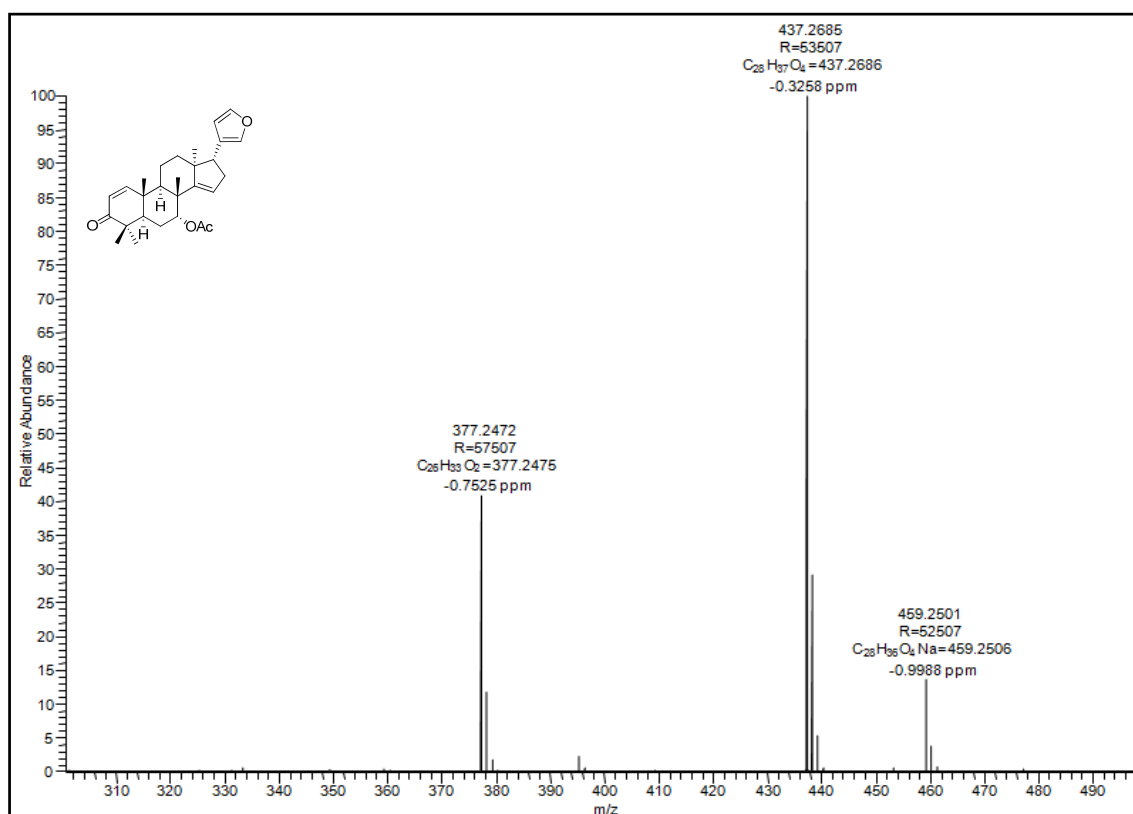
LC-ESI(+)-HRMS of epoxyazadiradione



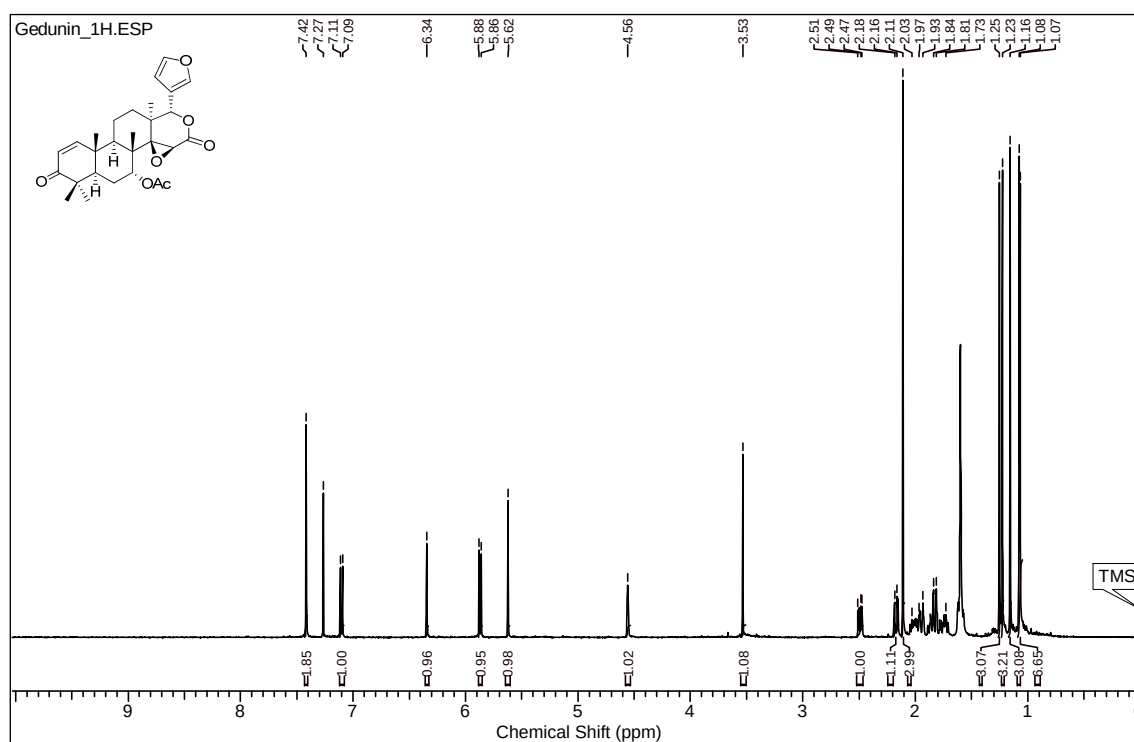
^1H NMR spectrum (CDCl_3 , 400 MHz) of azadirone



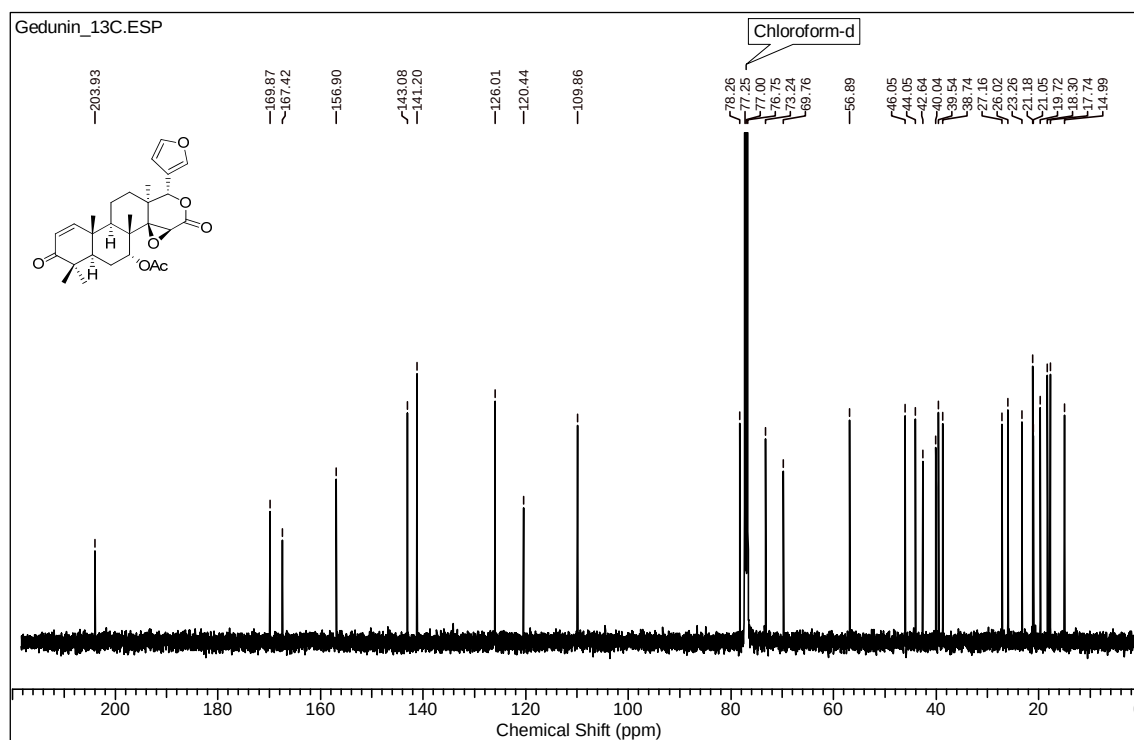
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of azadirone

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of azadirone

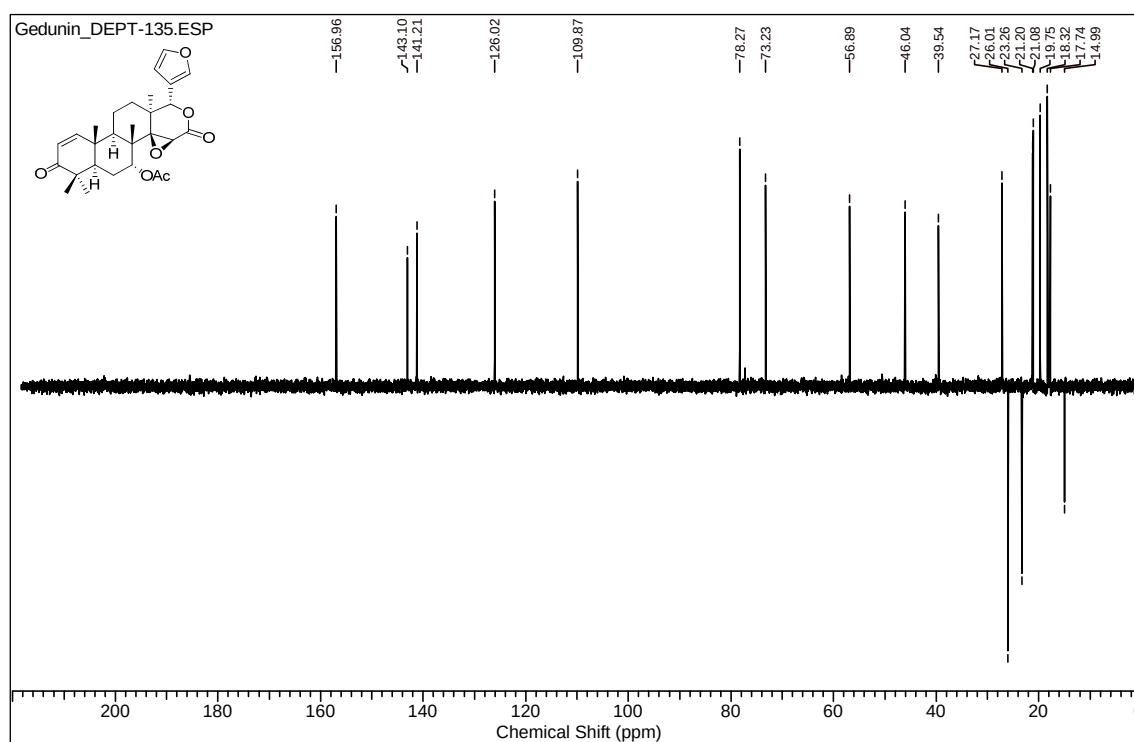
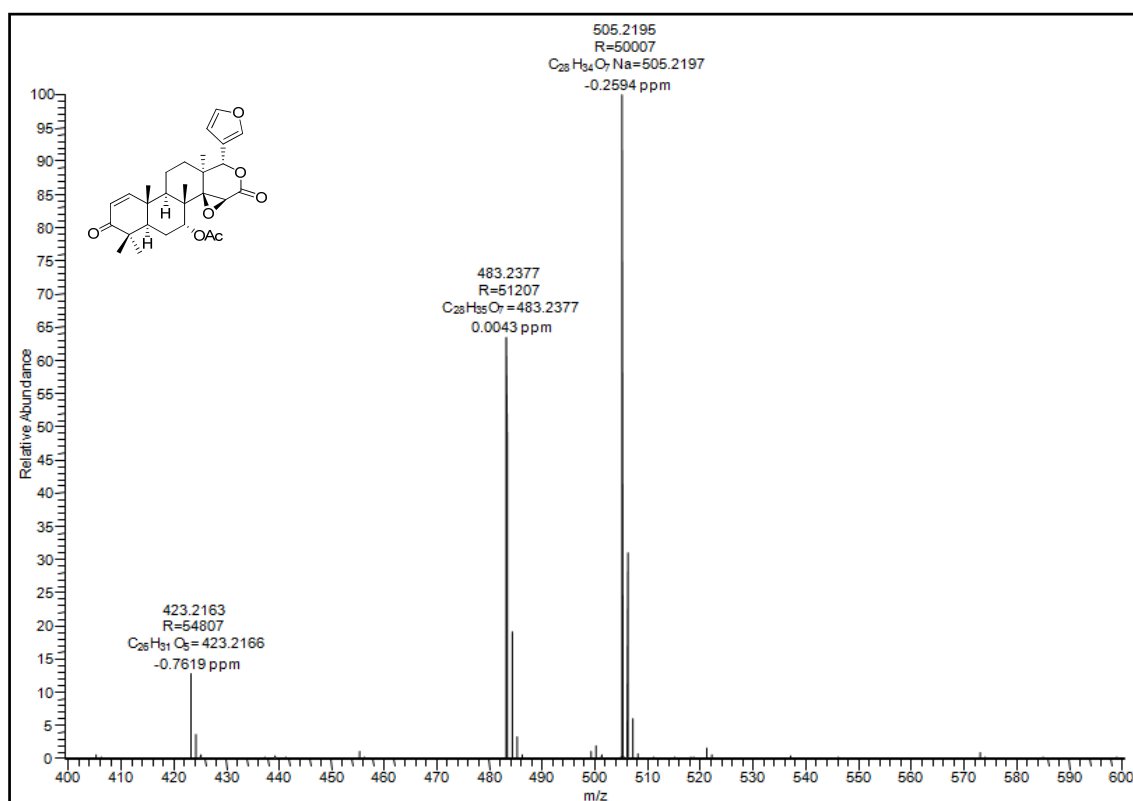
LC-ESI(+)-HRMS of azadirone



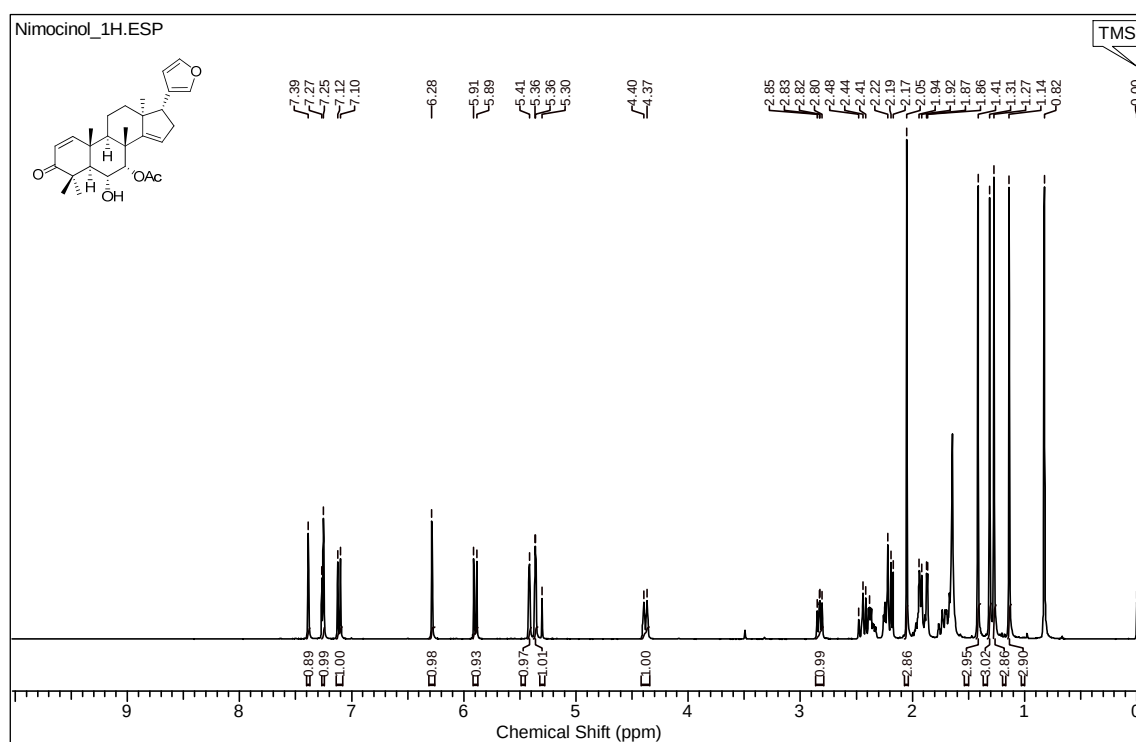
^1H NMR spectrum (CDCl_3 , 400 MHz) of gedunin



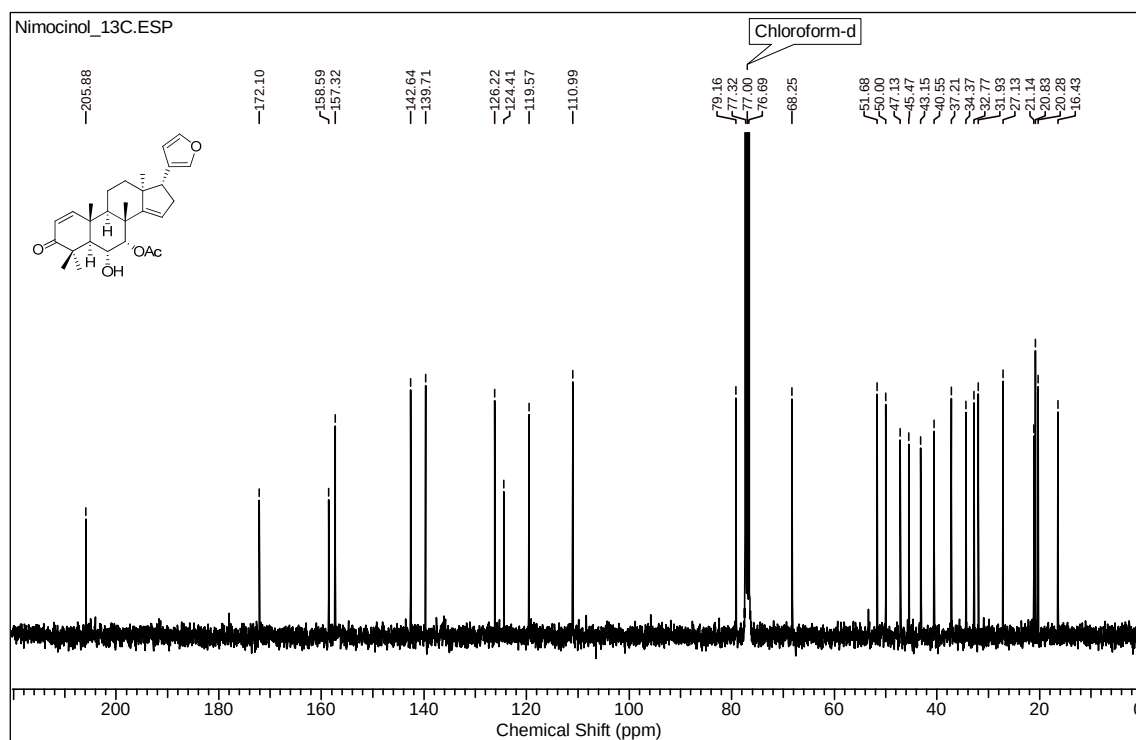
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of gedunin

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of gedunin

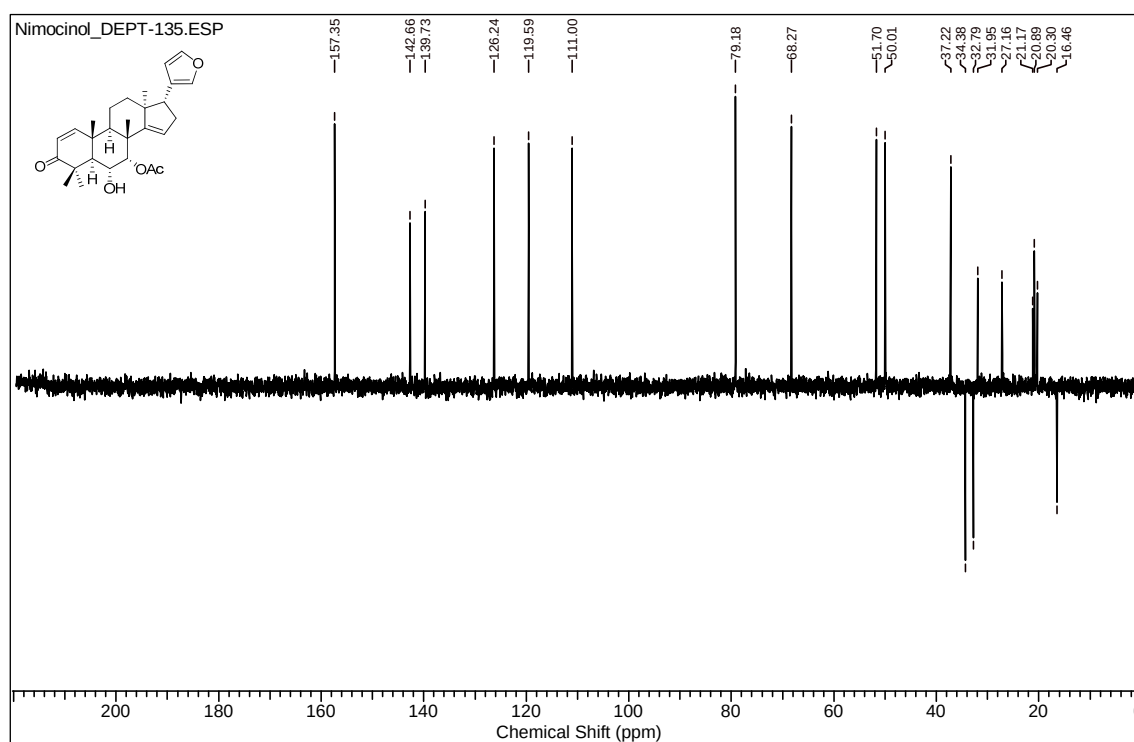
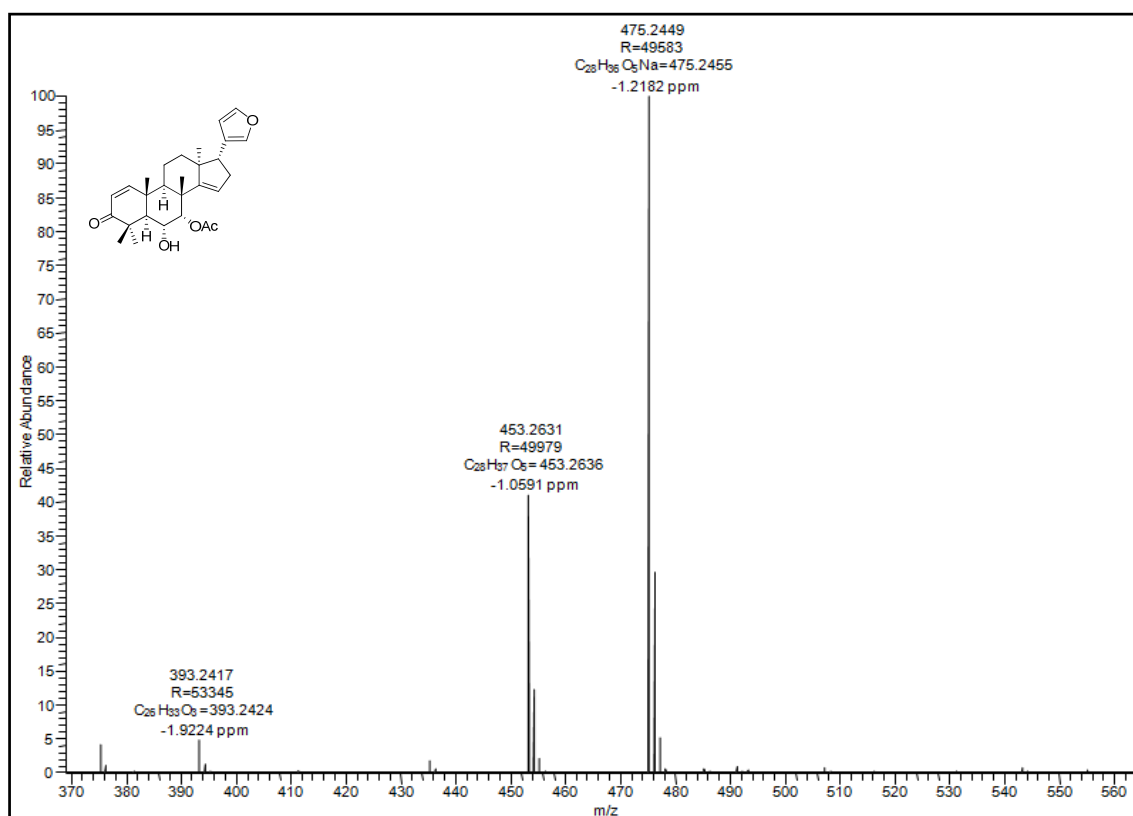
LC-ESI(+)-HRMS of gedunin



^1H NMR spectrum (CDCl_3 , 400 MHz) of nimocinol



^{13}C NMR spectrum (CDCl_3 , 100 MHz) of nimocinol

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of nimocinol

LC-ESI(+)-HRMS of nimocinol

Chapter 2: Section B

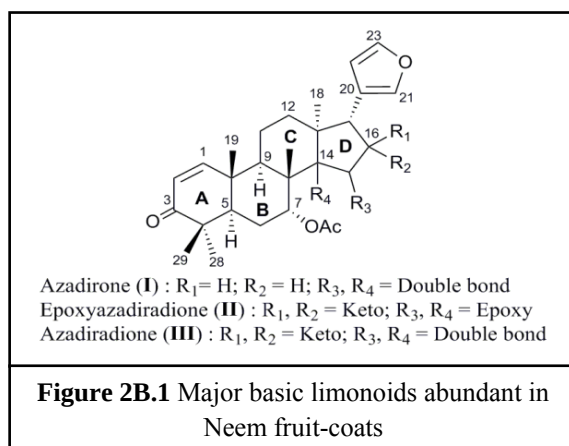
Expedient MPLC Based Preparative Isolation, HPLC Based Quantification and MS/MS Characterization of Limonoids from Neem Fruits

Highlights:

- *Three major basic limonoids were isolated in gram-scale from Neem fruit-coats using an automated and rapid MPLC based technique.*
- *Separation process was scaled up by fine-tuning chromatographic parameters.*
- *Isolated limonoids were quantified in developmental stages of fruit-coats using HPLC.*
- *Basic limonoids were characterized by ESI(+)-quadrupole/orbitrap-MS/MS technique.*

2B.1 Introduction

As discussed in chapter 1, basic limonoids are extremely promising pharmacologically active class of triterpenoids containing 4,4,8-trimethyl-17-furanylsteroidal signature skeleton (Figure 2B.1). Wide spectrum of biological activities such as insecticidal, anti-carcinogenic, anti-inflammatory, anti-malarial and anti-HIV properties (Chapter 1) of these limonoids is craving their gram scale availability for detailed investigations and applications. But complex structural architecture carrying several stereogenic and tertiary centres has restrained their synthetic access in spite of several trials in last two decades. On the other hand, isolation of limonoids from natural sources (mainly the plants belonging to the families Meliaceae and Rutaceae) in significant quantity is



tedious and time-consuming due to the complexity in separating the targeted one from cocktail of triterpenoids. Consequently, the development of a robust isolation protocol for rapid and preparative purification of these basic limonoids from plant extract will create an easy access to these valuable and biologically potent molecules. However,

there is no report on the rapid isolation of these limonoids in gram scale. Herein, we have developed for the first time, an optimized preparative scale isolation procedure for major basic limonoids such as azadirone (I), epoxyazadiradione (II) and azadiradione (III) from Neem fruit-coats (Figure 2B.1) using automated medium pressure liquid chromatography (MPLC) monitored by Photodiode Array (PDA) detector. Automated MPLC system with pre-packed cartridges of smaller particle size is a powerful high resolution technique for the time-saving large scale separation. But its applicability was highly restricted in case of complex mixtures from natural sources till date and a very few reports are available describing its utility in natural product research.¹⁵⁻¹⁸ In this section, we have demonstrated the ability of automated MPLC technique for the separation of major basic limonoids from Neem fruits. More importantly, the separation process was scaled up to 10 g of extract with fine-tuning of chromatographic parameters. Further, these limonoids were quantified in the plant extract using HPLC

standard curves drawn for individual compounds.

Identification of a specific metabolite in the limited quantity of a natural crude extract requires reference standard, which either may not be commercially available or too tedious to isolate or synthesize. Again, ESI-MS spectrum is also not faultlessly reliable for the identification of a compound as it provides only the molecular mass and corresponding chemical formula. On the other hand, LC system coupled with high resolution tandem mass spectrometry (LC-MSⁿ) is a widely applied sensitive and high-throughput technique for the rapid analyses of plant extract with greater confidence limit since structural information can be extracted through tandem fragmentations of the desired ion.¹⁹⁻²² For rapid characterization of these limonoids in the complex crude plant extract an LC-ESI-MS/MS based technique was developed to boost the screening procedure in search of richer plant source.

2B.2 Results and Discussion

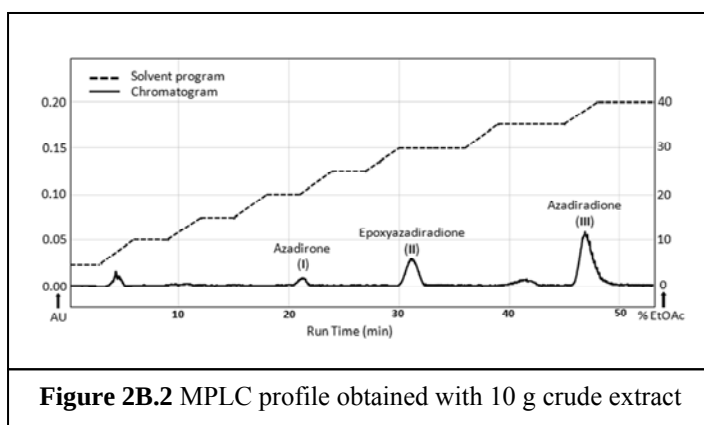
2B.2.1 Preparative Isolation by MPLC: Scale-up Approach

One kilogram fresh and mature Neem fruit-coats (*i.e.* fruits after the removal of seed kernels) were crushed using grinder and extracted with methanol (3 L × 2). The green mass obtained after concentrating methanol layer was partitioned between ethyl acetate

(800 mL) and water (800 mL).

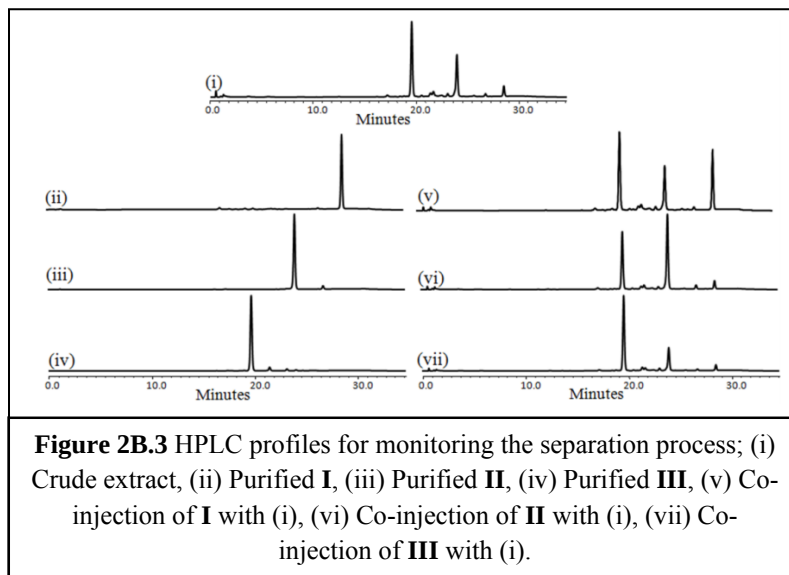
Further, ethyl acetate layer was concentrated to obtain 24.4 g green crude extract containing triterpenoids.

MPLC system fitted with in-built PDA detector was used for the automation in the separation process and



RediSep Rf cartridges of varying sizes, prefilled with normal phase silica gel with particle size 40-60 μm were used as the stationary phase for the purification and scaling up process. One gram of crude triterpenoid mixture was subjected to MPLC for the purification of individual limonoids using 24 g RediSep cartridge with a flow rate of 20 mL/min and monitoring at 200-400 nm. A gradient solvent program of 54 min with

increasing percentage of ethyl acetate in hexane was used as the eluent (Table 2A.1). Elution resulted in three major pure limonoids; azadirone (**I**) (0.04 g, R_t : 16.2 min), epoxyazadiradione (**II**) (0.21 g, R_t : 25.6 min), and azadiradione (**III**) (0.26 g, R_t : 40.0 min).

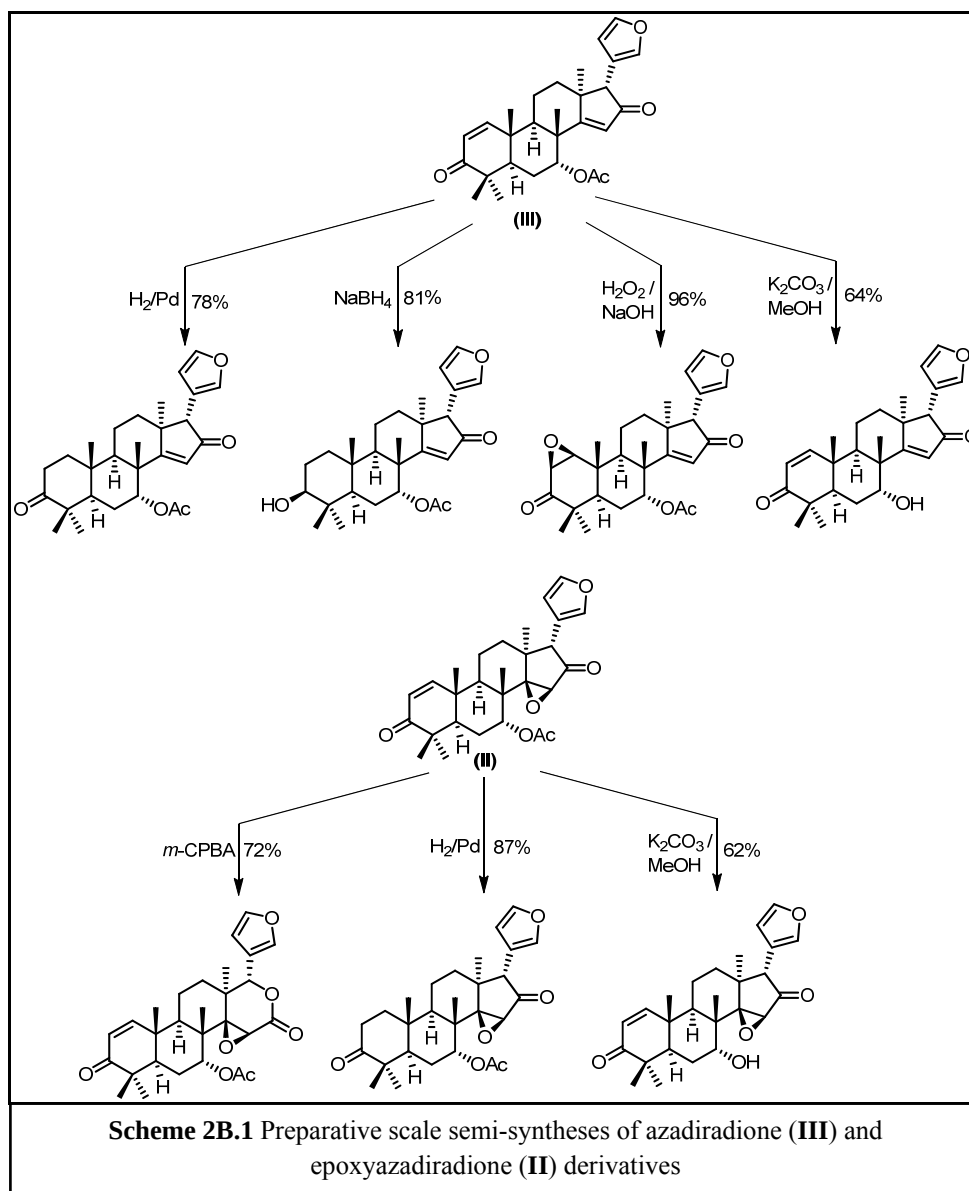


Further the separation process was scaled up to 2 g, 5 g and 10 g (Figure 2B.2, Table 2B.1) by varying the sizes of pre-packed columns and flow rate (keeping the solvent program identical). The purity of isolated limonoids obtained were more than 95% as indicated by HPLC analyses (Purity: **I**=98.1%, **II**=96.3%, **III**=95.2%) (Figure 2B.3). With gradual scaling up experiments, each limonoid was purified in large scale with high reproducibility in purity and retention time [elution ranges: $R_t(\text{I})$ =16-22 min, $R_t(\text{II})$ =25-33 min, $R_t(\text{III})$ =39-48 min throughout the scale up studies]. Ease in scaling

Crude extract (g)	RediSep Rf cartridge (g)	Flow rate (mL/min)	Yield of limonoids (g)		
			I	II	III
1	24	20	0.04	0.21	0.26
2	40	25	0.10	0.40	0.54
5	120	50	0.24	1.03	1.36
10	220	90	0.51	2.05	2.67
*Solvent program: 0-3 min 5% EtOAc, 6 min 10% EtOAc, 3 min hold, 12 min 15% EtOAc, 3 min hold, 18 min 20% EtOAc, 3 min hold, 24 min 25% EtOAc, 3 min hold, 30 min 30% EtOAc, 6 min hold, 39 min 35% EtOAc, 6 min hold, 48 min 40% EtOAc, 6 min hold.					

Table 2B.1 Tunable parameters and yield obtained in the MPLC scale up process

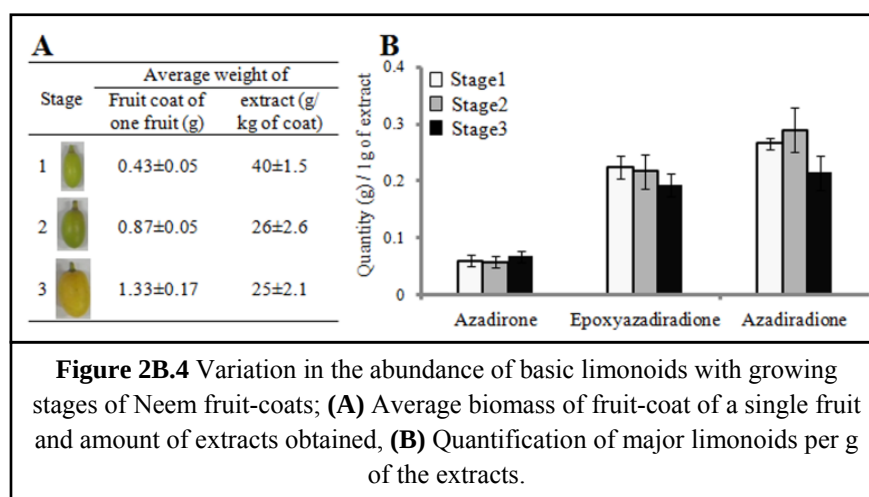
up, reproducibility and rapidity of the present protocol will make it more useful than usually adopted techniques such as HPLC or Counter Current Chromatography for the preparative purification of complex blend of limonoids. Further, these abundant limonoids were used for the preparative scale synthesis of a series of natural and semi-synthetic derivatives (Scheme 2B.1). For example, epoxyazadiradione was used for the gram scale semi-synthesis of gedunin, a well-known anti-carcinogenic limonoid.



2B.2.2 Quantification by HPLC: Developing Stages

Variation in expression levels of biosynthetic pathway genes in different growing stages of a specific tissue may vary the abundance of metabolites with progressive growth. The level of metabolites present in the Neem fruit-coats at different developmental stages

were determined with the help of standard curves for each purified triterpenoids drawn using HPLC (within the concentration range 0.03 to 1.0 mg/mL in methanol) with regression coefficient greater than 0.999. The peak position of individual limonoids in the crude extract was confirmed by co-injection studies of purified limonoids with the crude extract (Figure 2B.3). Specificity of the HPLC method for the analysis of crude extract was validated by LC-MS analyses. The levels of **I**, **II** and **III** in fruit-coats at different developing stages were determined individually. These results indicated that the quantity of individual limonoids per g of the extracts were in the same range throughout all developing stages (Figure 2B.4B). As the biomass of fruit-coats obtained in matured stages 2 and 3 are higher, Neem fruits of stages 2 and 3 are better suited for the large scale isolation of compounds **I**, **II** and **III** (Figure 2B.4A). These observations were in well-correlation with the finger-printing results discussed in the section A of Chapter 1.



2B.2.3 LC-ESI(+)-MS/MS: Rapid Identification

Q Exactive Orbitrap, an LC-ESI-Quadrupole/Orbitrap system was used for the full scan and MS/MS analyses in positive ion mode. Individual limonoids (**I**, **II** and **III**) in methanol (5μL) with a concentration of 0.1 mg/mL was subjected to chromatographic separation on LC system followed by full scan MS analysis. Further, a series of MS/MS experiments were performed by the collisional activation of $[M+H]^+$ ions with varying stepped normalised collision energy (NCE) in data dependent inclusion mode (specifying the R_t and m/z to be processed for MS/MS). In specific, R_t values for azadirone, epoxyazadiradione and azadiradione were observed to be 24.0, 20.4 and 16.7 min respectively in full scan ESI-MS run. Further $[M+H]^+$ ions, as observed in

individual ESI(+)-MS spectra (corresponding to m/z 437.2, 467.2 and 451.2 respectively) were subjected to MS/MS experiments by restricting the range of R_t in the inclusion mode as 23.5-25.5 min, 19.0-21.5 min and 15.5-17.7 min respectively. Each MS/MS experiment was performed in six different stepped normalized collision energies (NCE); 15, 20, 25, 30, 35 and 40% with stepped value 20%.

Variation in collision energy significantly influenced the relative intensity of daughter ions in a systematic way. It has been observed that the higher NCE increased the intensity of low mass fragments corresponding to the C-C bond cleavages and

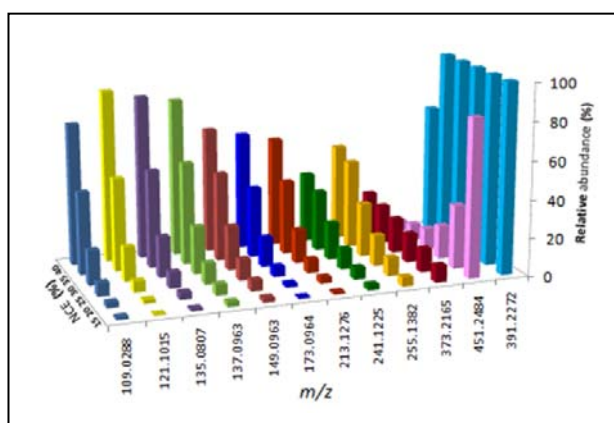
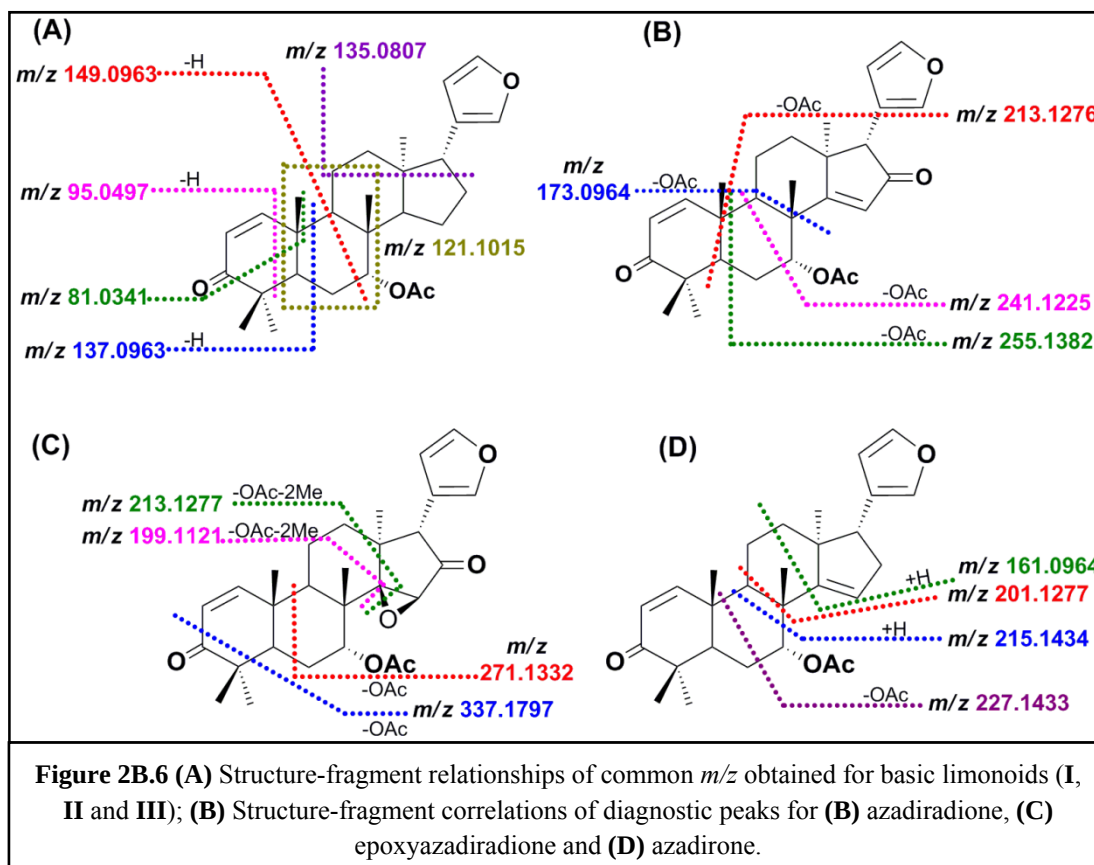


Figure 2B.5 Variation in relative abundance (Z-axis) of key MS/MS daughter fragments (X-axis) of $[M+H]^+$ ion with sweeping NCE (Y-axis) for azadiradione (**III**)

rearrangements, whereas lowering in NCE resulted in almost exclusive abundance of $[M+H]^+$ and higher mass fragments produced by easy removal of sensitive functional groups (e.g. acetyl) as neutral molecules. For example, in case of azadiradione, the relative intensity of m/z 451.2484 ($[M+H]^+$) diminished from 83.2% to 5.4% with increase in NCE from 15 to 40%, whereas the abundance of peak

at m/z 121.1015 ($[C_9H_{13}]^+$) enhanced from 0.5% to 89.9% with same enhancement in NCE (Figure 2B.5). Consequently, the mass spectra in weaker NCE will be devoid of lower fragments, but higher NCE will make them highly abundant. In essence, unique structural information can be extracted by sweeping collision energy in MS/MS spectrometry for these basic limonoids.

Structure-fragment correlations were established on the basis of chemical formula generated from corresponding m/z values obtained with high accuracy (errors < 2.5 ppm) (Table 2B.2). Systematic analyses of tandem mass spectral data revealed that secondary product ions obtained from $[M+H]^+$ precursor ions can be categorized into two groups. Fragments in the mass range m/z 80-150 are the characteristic peaks for basic limonoids having cyclohexenone A ring and commonly observed for all three, whereas fragments of mass higher than m/z 150 distinguish among **I**, **II** and **III**. To demonstrate, azadiradione (**III**) generated the diagnostic peaks at m/z 451.2484 ($[M+H]^+$), 391.2272 ($[M+H-AcOH]^+$), 373.2165 ($[M+H-AcOH-H_2O]^+$), 349.2163



($[M+H-AcOH-C_2H_2O]^+$), 255.1382, 241.1225, 213.1276, 173.0964, 149.0963, 137.0963, 135.0807, 121.1015, 95.0497 and 81.0341. Ions at m/z 81.0341, 95.0497, 121.1015, 135.0807, 137.0963 and 149.0963 are omnipresent among all three limonoids since they were generated from the common skeletal region (Figure 2B.6A). Peaks at m/z 255 and 241 were possibly generated from the C-C cleavages in ring B. The probable fragmentation for the generation of ion at m/z 213 may be due to the cleavage in ring A and loss of furan moiety. Similarly, fragment of mass 173 might be generated by the cleavage in ring C at the junction with ring B (Figure 2B.6B). Apart from common fragments in the lower mass range, epoxyazadiradione (**II**) produced characteristic peaks at m/z 467.2430 ($[M+H]^+$), 407.2223 ($[M+H-AcOH]^+$), 389.2113 ($[M+H-AcOH-H_2O]^+$), 379.2277 ($[M+H-AcOH-CO]^+$), 361.2166 ($[M+H-AcOH-CO-H_2O]^+$), 337.1797, 271.1332, 213.1277 and 199.1121. Fragmentations at m/z 337 and 271 probably generated by the cleavage in ring A and B respectively. Cleavage in ring C along with the removal of methyl groups produced the fragments at m/z 213 and 199 as shown in Figure 2B.6C. Similarly, azadirone (**I**) also generated its signature fragments at m/z 437.2694 ($[M+H]^+$), 419.2582 ($[M+H-H_2O]^+$), 377.2483 ($[M+H-AcOH]^+$), 359.2376 ($[M+H-AcOH-H_2O]^+$), 349.2534 ($[M+H-AcOH-CO]^+$), 227.1433,

Table 2B.2 MS/MS fragmentations of $[M+H]^+$ precursor ions of limonoids (**I**, **II** and **III**), abundance in two limiting NCE (15 and 40 %), corresponding chemical formula and errors

Limonoid	Precursor ion (m/z)	Daughter ions (m/z)	% Intensity (NCE 15/40)	Formula	Error (ppm)
III	451.2484 ($[M+H]^+$)	451.2484	83.2/5.4	$C_{28}H_{35}O_5$	0.00
		391.2272	100.0/67.2	$C_{26}H_{31}O_3$	0.25
		373.2165	8.8/22.7	$C_{26}H_{29}O_2$	0.80
		349.2163	2.7/12.4	$C_{24}H_{29}O_2$	1.43
		255.1382	4.7/50.2	$C_{17}H_{19}O_2$	1.17
		241.1225	2.9/36.3	$C_{16}H_{17}O_2$	1.66
		213.1276	1.1/57.0	$C_{15}H_{17}O$	1.40
		173.0964	0.6/61.3	$C_{12}H_{13}O$	1.15
		149.0963	2.4/65.2	$C_{10}H_{13}O$	2.01
		137.0963	2.5/82.4	$C_9H_{13}O$	2.19
		135.0807	1.2/85.4	$C_9H_{11}O$	2.22
		121.1015	0.5/89.9	C_9H_{13}	1.65
		95.0497	-/47.0	C_6H_7O	0.00
		81.0341	-/69.7	C_5H_5O	-1.23
II	467.2430 ($[M+H]^+$)	467.2430	31.1/-	$C_{28}H_{35}O_6$	0.85
		407.2223	100.0/17.8	$C_{26}H_{31}O_4$	-0.24
		389.2113	9.4/5.5	$C_{26}H_{29}O_3$	1.02
		379.2277	5.9/2.7	$C_{25}H_{31}O_3$	-1.05
		361.2166	2.8/3.4	$C_{25}H_{29}O_2$	0.55
		337.1797	3.1/3.4	$C_{22}H_{25}O_3$	2.07
		271.1332	9.8/8.9	$C_{17}H_{19}O_3$	0.73
		213.1277	-/15.7	$C_{15}H_{17}O$	0.94
		199.1121	-/13.0	$C_{14}H_{15}O$	1.00
		149.0964	15.3/100.0	$C_{10}H_{13}O$	1.34
		137.0964	2.2/31.2	$C_9H_{13}O$	1.46
		135.0807	2.9/54.4	$C_9H_{11}O$	2.22
		121.1016	-/33.6	C_9H_{13}	0.82
		95.0497	-/16.4	C_6H_7O	0.00
		81.0342	-/43.1	C_5H_5O	-2.47
I	437.2694 ($[M+H]^+$)	437.2694	82.6/-	$C_{28}H_{37}O_4$	-0.46
		419.2582	6.8/2.0	$C_{28}H_{35}O_3$	0.95
		377.2483	100.0/17.8	$C_{26}H_{33}O_2$	-0.53
		359.2376	10.7/7.6	$C_{26}H_{31}O$	-0.28
		349.2534	12.7/8.5	$C_{25}H_{33}O$	-0.86
		227.1433	3.6/28.1	$C_{16}H_{19}O$	1.32
		215.1434	7.5/41.4	$C_{15}H_{19}O$	1.39
		201.1277	9.2/37.9	$C_{14}H_{17}O$	0.99
		161.0964	5.4/51.0	$C_{11}H_{13}O$	1.24
		149.0964	6.0/100.0	$C_{10}H_{13}O$	1.34
		137.0964	2.0/46.7	$C_9H_{13}O$	1.46
		135.0807	-/34.9	$C_9H_{11}O$	2.22
		121.1016	-/34.6	C_9H_{13}	0.82
		95.0497	4.4/48.6	C_6H_7O	0.00
		81.0342	1.0/44.0	C_5H_5O	-2.47

*In the 4th column ‘-’ signifies % of intensity is less than 1.0.

215.1434, 201.1277, 161.0964 along with common fragments under m/z 150. C-C cleavage in ring B and C probably produced the fragment ions at m/z 227, 215, 201, and

161 as depicted in Figure 2B.6D. To validate the applicability of presently developed

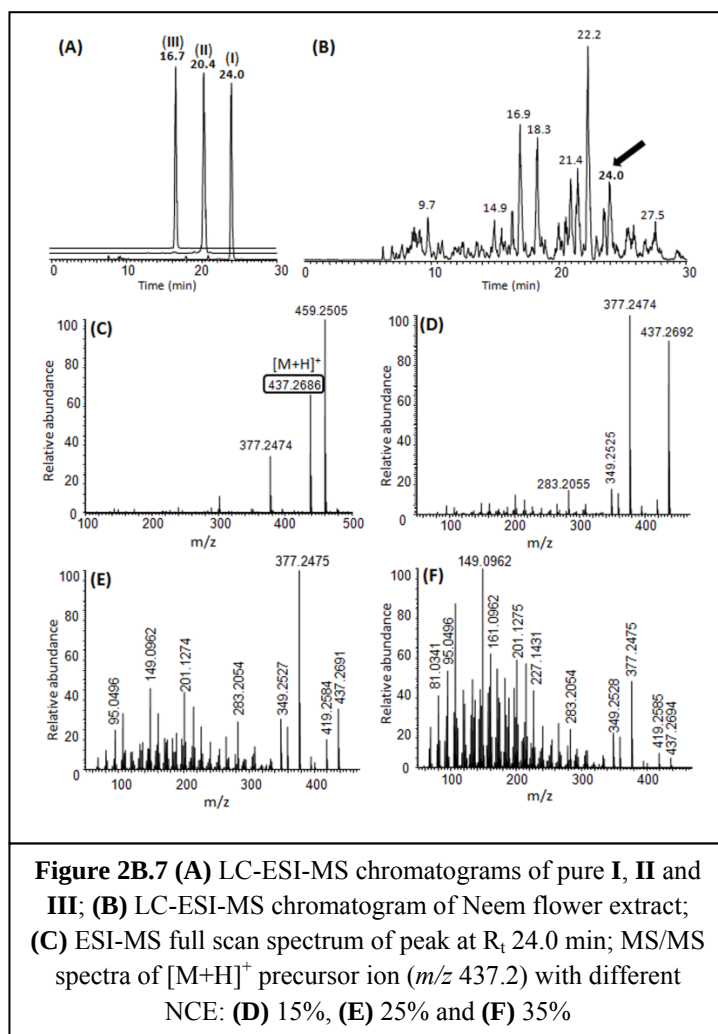


Figure 2B.7 (A) LC-ESI-MS chromatograms of pure I, II and III; (B) LC-ESI-MS chromatogram of Neem flower extract; (C) ESI-MS full scan spectrum of peak at R_t 24.0 min; MS/MS spectra of $[M+H]^+$ precursor ion (m/z 437.2) with different NCE: (D) 15%, (E) 25% and (F) 35%

LC-ESI(+)-MS/MS protocol for tracing these basic limonoids in unknown botanical sample. Neem flower extract was analysed. The LC chromatogram (showing R_t values) for the flower extract and full scan ESI(+)-MS spectra of resolved peaks (showing exact mass) showed the presence of azadirone (I), whereas azadiradione (III) and epoxyazadiradione (II) were found to be absent. Further the abundance of azadirone was confirmed on the basis of MS² experiments with varying NCE (15, 25 and

35%) which showed full agreement with the MS/MS data for purified azadirone (Figure 2B.7).

2B.3 Conclusion

In conclusion, an MPLC based scale-up approach was developed for the gram-scale isolation of major basic limonoids from Neem fruit-coats. This protocol can be used for the rapid and large-scale isolation of limonoids such as azadirone, epoxyazadiradione and azadiradione with high purity. The level of these limonoids were further determined at different developmental stages of Neem fruits and found to be in the same range throughout the growth. Further, an LC-ESI-MS/MS based technique was developed for the rapid characterization and screening of these limonoids in complex phytochemical mixtures. Henceforth, the scope of gram-scale availability and rapid characterization of

these ‘gold-mines’ will encourage the semi-syntheses of structurally diverse derivatives of limonoids and further investigations of known and exploration of unknown bioactivities.

2B. 4 Experimental section

2B.4.1 Chemicals and Materials

Neem fruits were collected from Aurangabad region in Maharashtra during March to May. Chemicals were procured from Sigma. For the extraction purpose technical grade solvents were purchased locally and distilled prior to use. For MPLC and HPLC purpose HPLC grade solvents (from Sigma) were used. For LC-ESI(+)-HRMS experiments, LC-MS grade solvents were purchased from JT Baker.

2B.4.2 Instrumentation

Automated MPLC system from Teledyne, Isco (Combiflash Rf 200) having integrated PDA detector was used for the preparative purification of the extracts. HPLC was performed on Waters HPLC system (Delta 600 pump and controller) coupled with Waters 2489 UV/Visible detector. LC-ESI(+)-MS/MS runs were performed on Q Exactive Orbitrap attached with Accela 1250 pump made by Thermo Scientific.

2B.4.3 HPLC Conditions

HPLC runs were carried out on analytical XBridge C₁₈ column (4.6 × 100 mm, 5 µm) with a gradient solvent program of 35 min (0 min, 40% methanol/water; 10 min, 60% methanol/water; 25 min, 90% methanol/water; 28 min, 60% methanol/water; 30 min, 40% methanol/water; 35 min, 40% methanol/water) and detection at 230 nm with a flow rate of 1 mL/min. Samples were dissolved in HPLC grade methanol and 20 µL of it was injected after filtration.

2B.4.4 LC-ESI(+)-MS and MS/MS Conditions

Samples were resolved through Thermo Scientific Hypersil Gold column of particle size 5 µm with a flow rate of 0.5 mL/min and gradient solvent program of 30 min (0.0 min, 10% methanol/water; 0.5 min, 10.0% methanol/water; 3.0 min, 45% methanol/water;

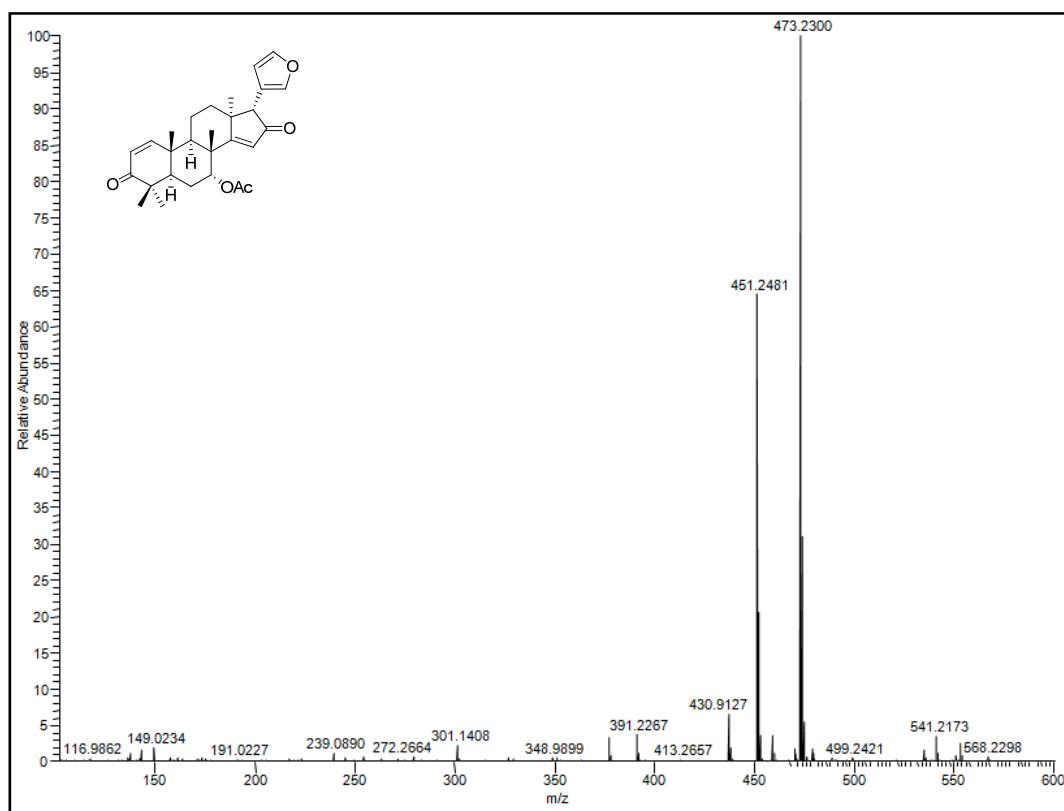
6.0 min, 60% methanol/water; 20.0 min, 90% methanol/water; 27.0 min, 90% methanol/water; 27.5 min, 10% methanol/water; 30.0 min, 10% methanol/water). Solvents (methanol and water) were added with 0.1% LC-MS grade formic acid. MS and MS/MS experiments were performed in ESI-positive ion mode using the tune method as followed: sheath gas flow rate 45, auxiliary gas flow rate 10, sweep gas flow rate 2, spray voltage 3.60 [KV], spray current 3.70 μ A, capillary temperature 320 °C, s-lens RF level 50, heater temperature 350 °C. ESI(+)-MS data were recorded in full scan positive ion mode within the mass range m/z 100 to 1000 whereas the ESI(+)-MS/MS experiments were performed in data dependent t- m/z^2 mode using inclusion input of desired R_t and m/z to be processed for MS/MS.

2B.4.5 Semi-synthesis of Derivatives

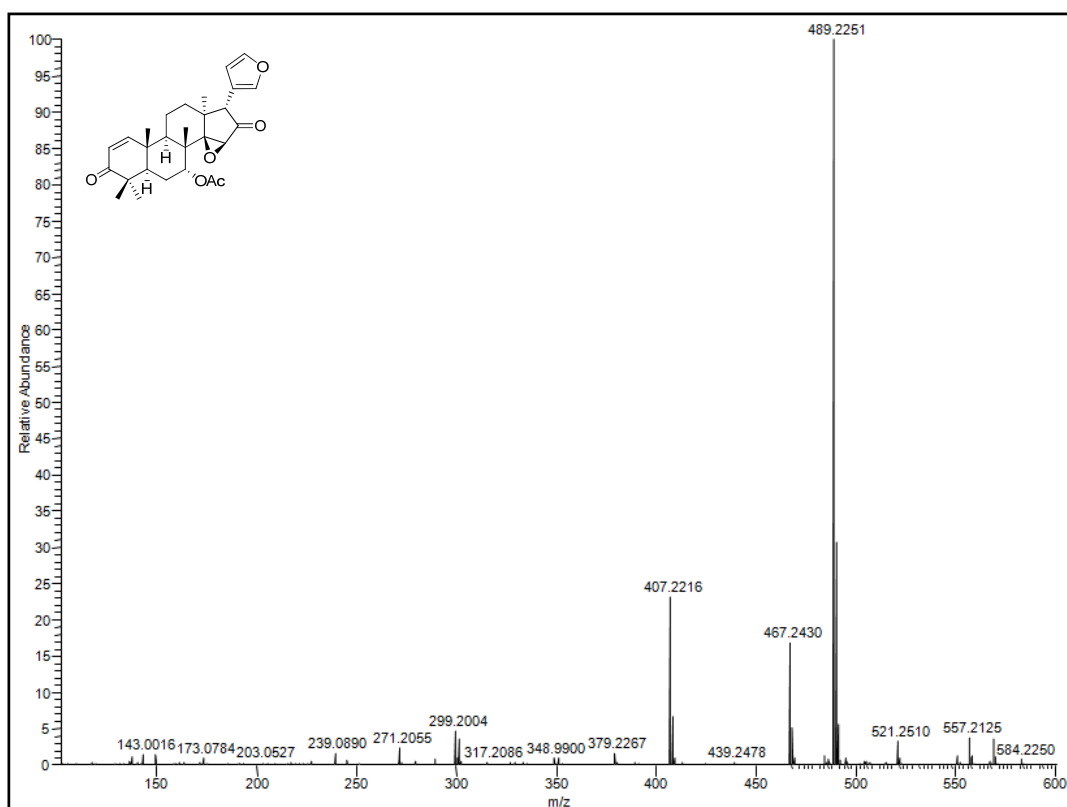
- **Synthesis of 1,2-dihydroazadiradione and 1,2-dihydroepoxyazadiradione:** 500 mg azadiradione (**III**) (1.1 mmol) in 60 mL HPLC grade methanol was stirred with 45 mg 10% Pd on activated carbon under 2 atmosphere pressure of hydrogen. After 10 h, the reaction mixture was filtered through celite bed and chromatographed on silica gel (230-400 mesh) column using a gradient mixture of methanol/dichloromethane as eluent to get 392 mg of desired 1,2-dihydroazadiradione (0.87 mmol, yield ~ 78%) (eluted at 0.6% methanol in dichloromethane). Similarly, 1,2-dihydroepoxyazadiradione (436 mg, 0.93 mmol) was prepared from the hydrogenation of epoxyazadiradione (**II**) (500 mg, 1.07 mmol) with a yield of ~ 87%.
- **Synthesis of nimboicinol and 7-Deacetylepoxiazadiradione:** 500 mg azadiradione (**III**) (1.1 mmol), dissolved in minimum amount of methanol was added by 200 mL saturated solution of K_2CO_3 and stirred for 18 h at room temperature. Since it was showing a complete consumption of azadiradione and formation of a more polar spot on TLC, the reaction mixture was neutralised to pH ~7.0 using 1(N) HCl and then methanol was concentrated in aspirator at 40 °C. Further, the mass was partitioned between brine solution and EtOAc. The EtOAc layer was concentrated, dried over Na_2SO_4 and 290 mg of pure nimboicinol (0.71 mmol, yield ~ 64%) was obtained after purification over silica gel (230-400 mesh) column at 0.8% methanol in dichloromethane. Similarly 7-deacetylepoxiazadiradione (280 mg, 0.66 mmol) was synthesised from epoxyazadiradione (**II**) (500 mg, 1.07 mmol) with a yield of 62%.

- **Synthesis of 1,2-epoxyazadiradione:** 500 mg azadiradione (**III**) (1.1. mmol) in 65 mL methanol was stirred for 5 h after addition of 20 drops of 5% aqueous NaOH followed by 20 mL of 30% H₂O₂. The reaction mixture was concentrated in vacuum at 50 °C and then extracted with dichloromethane (3 × 50 mL). The dichloromethane layer was dried over Na₂SO₄ and concentrated to get 498 mg 1,2-epoxyazadiradione (1.06 mmol, yield ~ 96%).
- **Synthesis of gedunin:** 1.50 g (3.21 mmol) epoxyazadiradione (**II**) taken in 30 mL of anhydrous dichloromethane in an inert atmosphere was cooled to 0 °C. Then 1.84 g (6.42 mmol) of 60% *m*-chloroperbenzoic acid was added in stirring condition followed by 0.54 g sodium bicarbonate. After 5 min the reaction vessel was shifted to room temperature and stirred for another 1 h. Further, the reaction mixture was concentrated under reduced pressure and taken in 30 mL of EtOAc. The organic layer was washed thrice with saturated aqueous solution of sodium bicarbonate (3 × 100 mL) and concentrated. The white crude obtained was purified over silica gel (100-220 mesh) column using 0.3% methanol in dichloromethane as the eluent to furnish 1.21 g (2.32 mmol, yield ~ 72%) pure gedunin.
- **Reduction of azadiradione:** 100 mg azadiradione (**III**) (0.23 mmol) taken in 2 mL of HPLC grade methanol in inert atmosphere was added by 04 mg (0.1 mmol) sodium borohydride at 0 °C. After addition, reaction vessel was shifted to room temperature and stirred for another 2 h. Concentrated reaction mixture was purified over silica gel column chromatography using 0.5% methanol in dichloromethane as the eluent to produce 82 mg (0.18 mmol, yield ~ 78%) reduced product of azadiradione. Spectroscopic characterization of the product revealed reduction of both 1,2-double bond and 3-keto functionality of azadiradione.

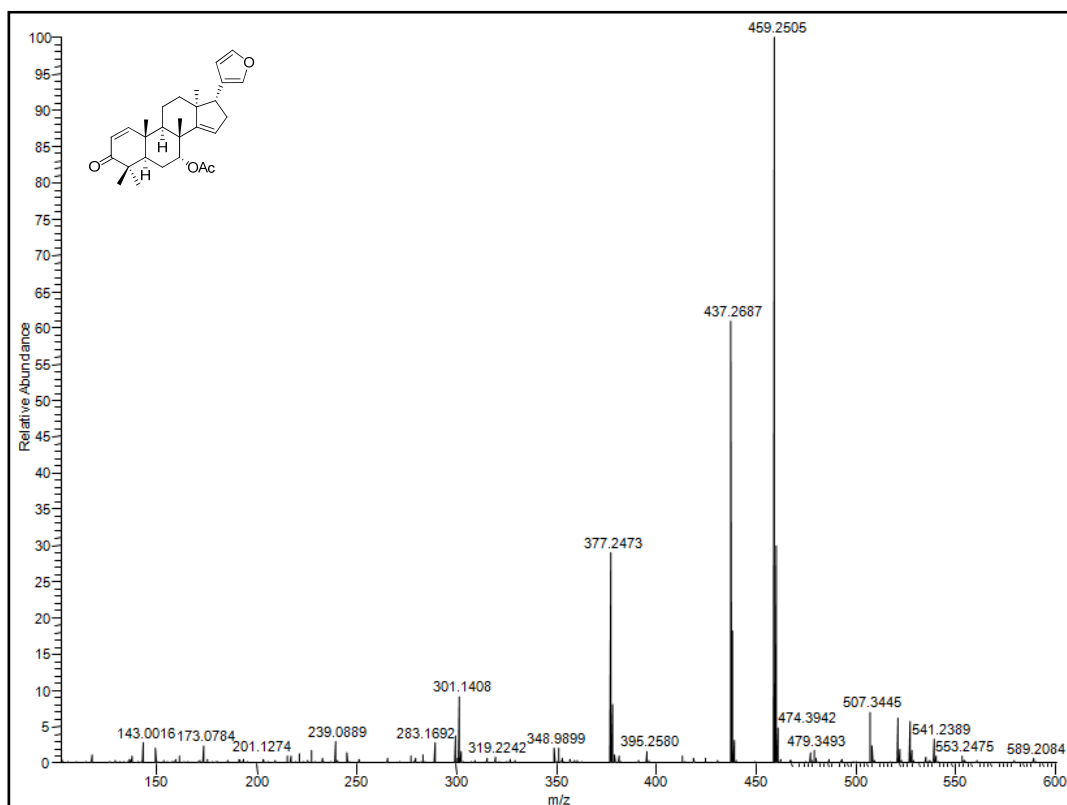
2B.5 Spectral Copies



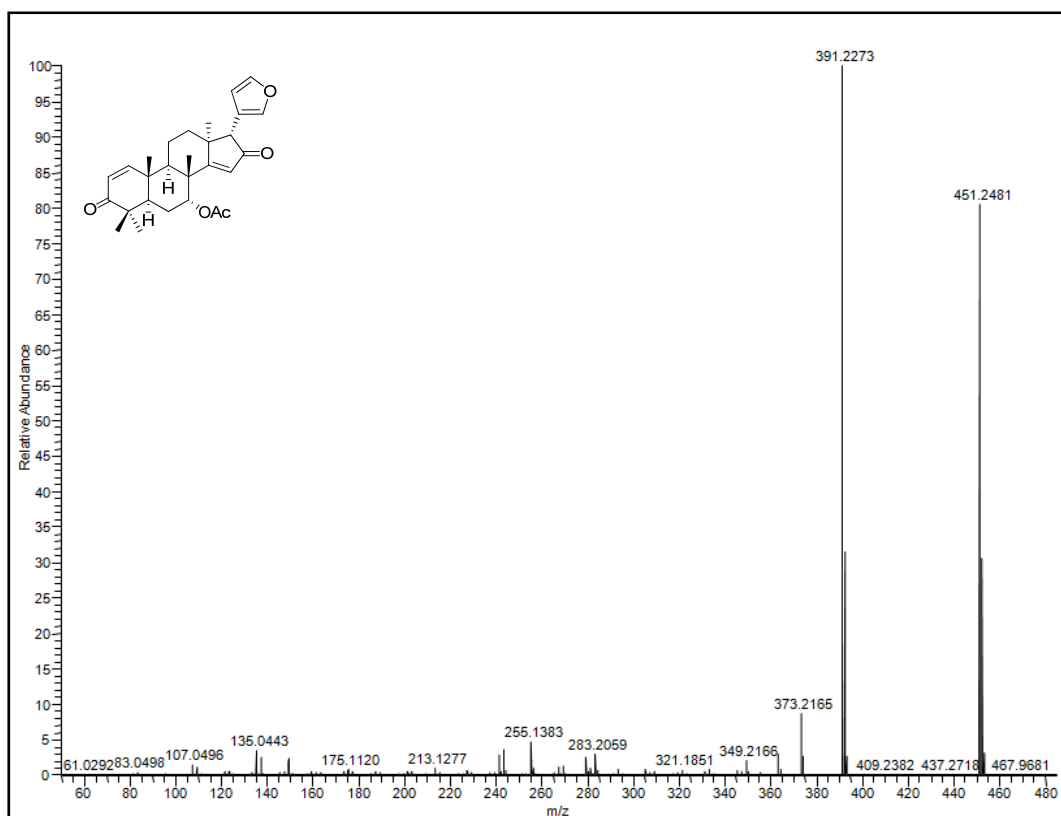
ESI(+)-MS spectrum of azadiradione (III)

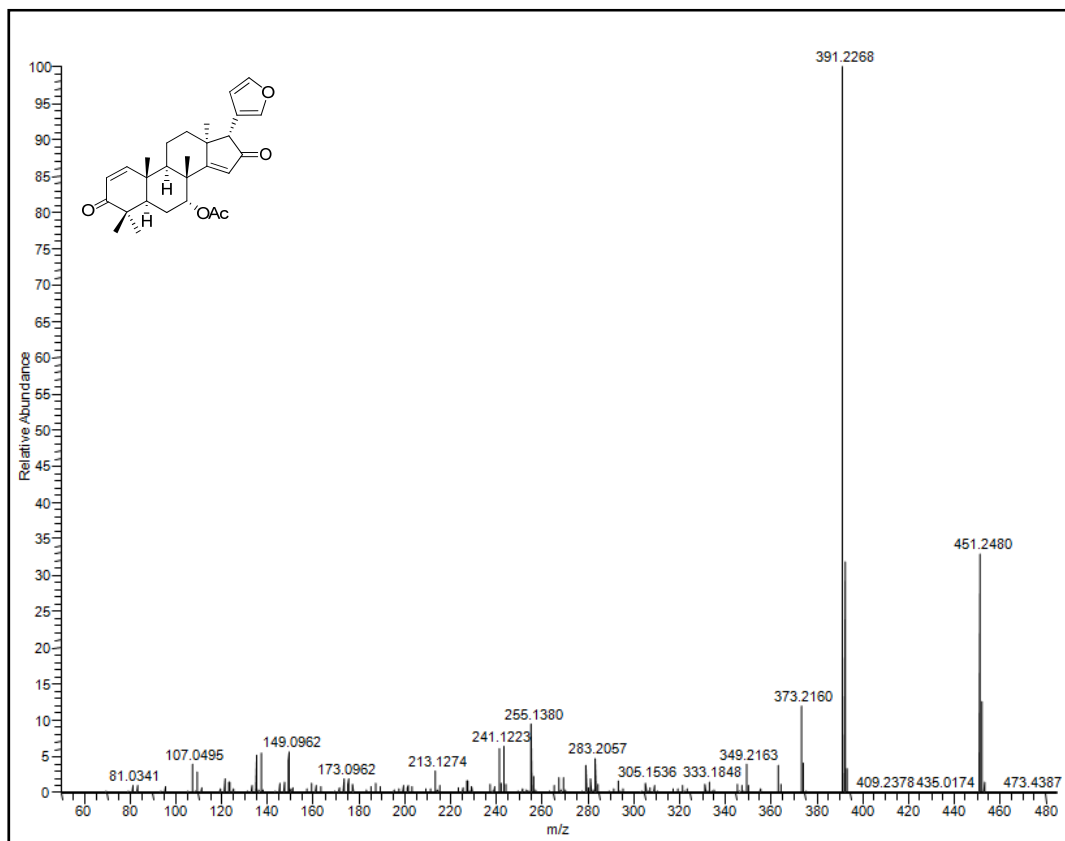
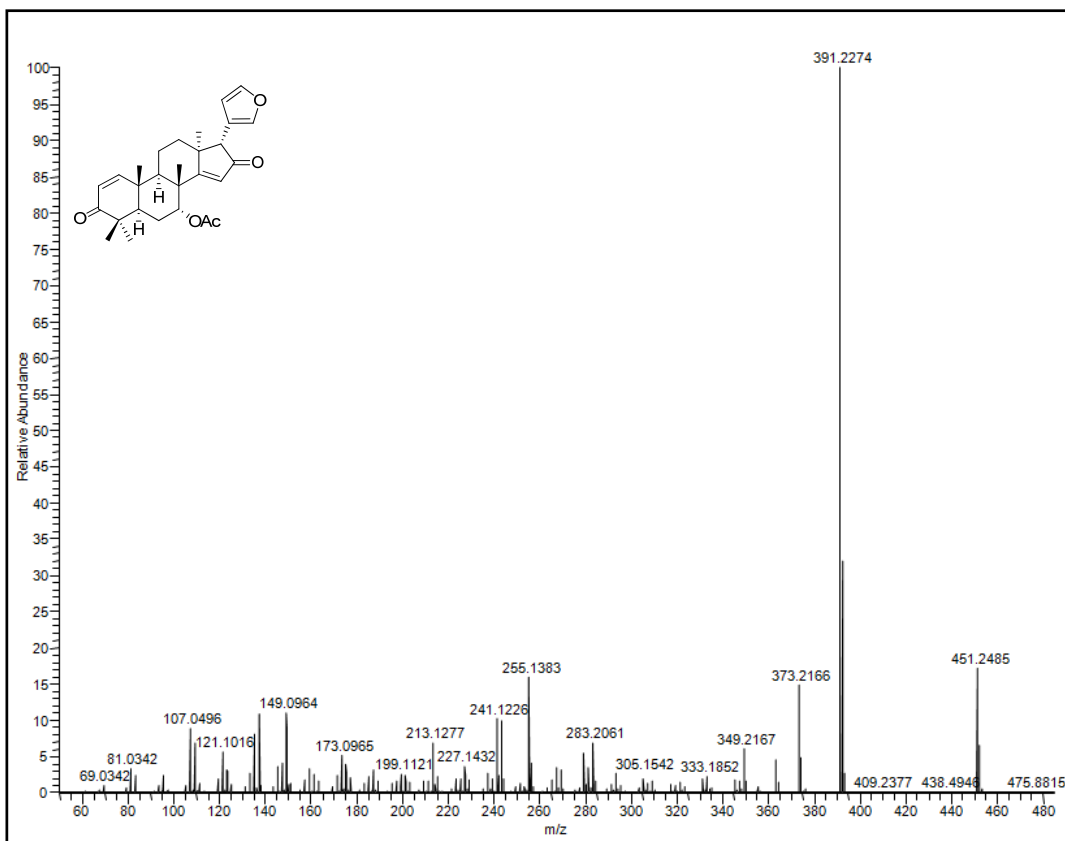


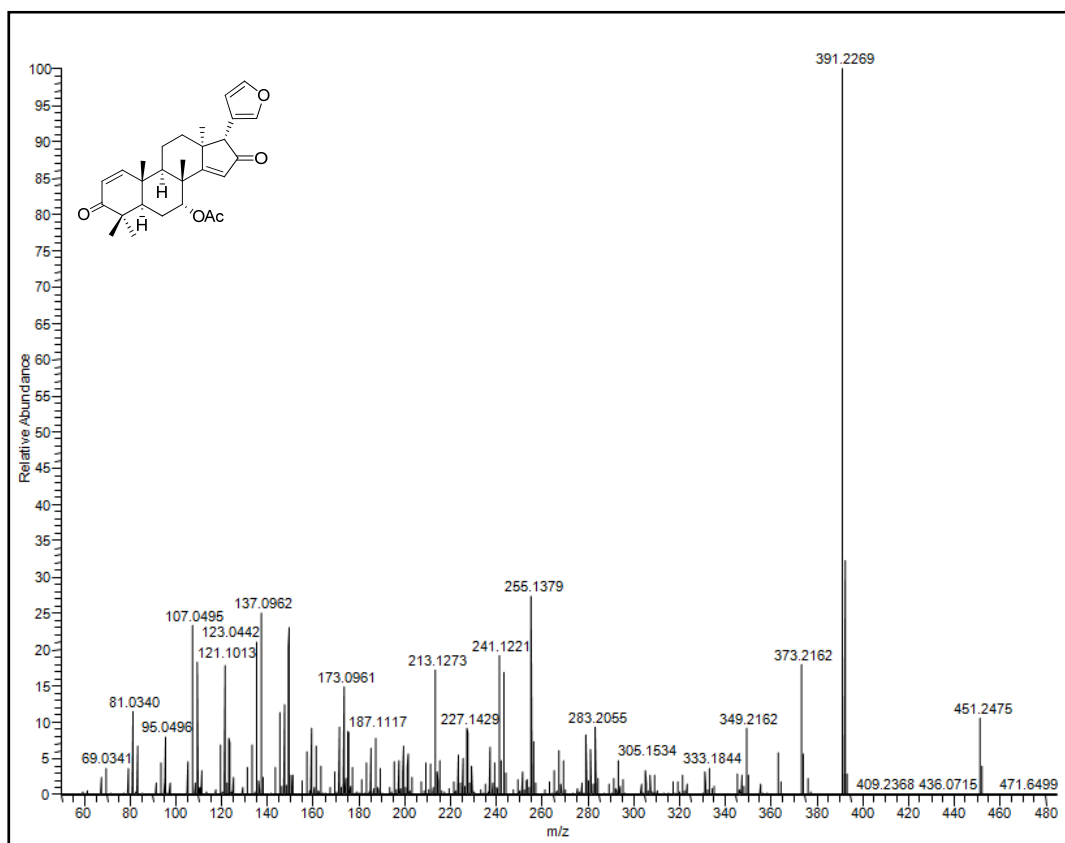
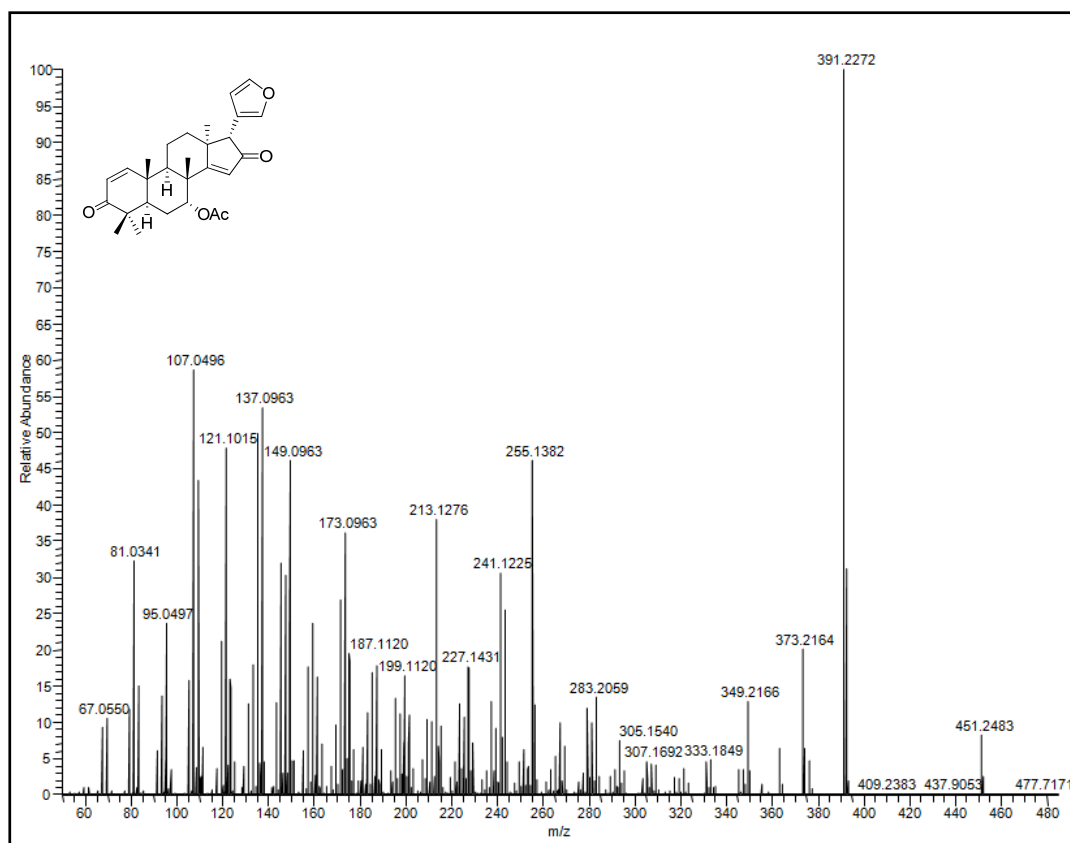
ESI-MS spectrum of epoxyazadiradione (II)

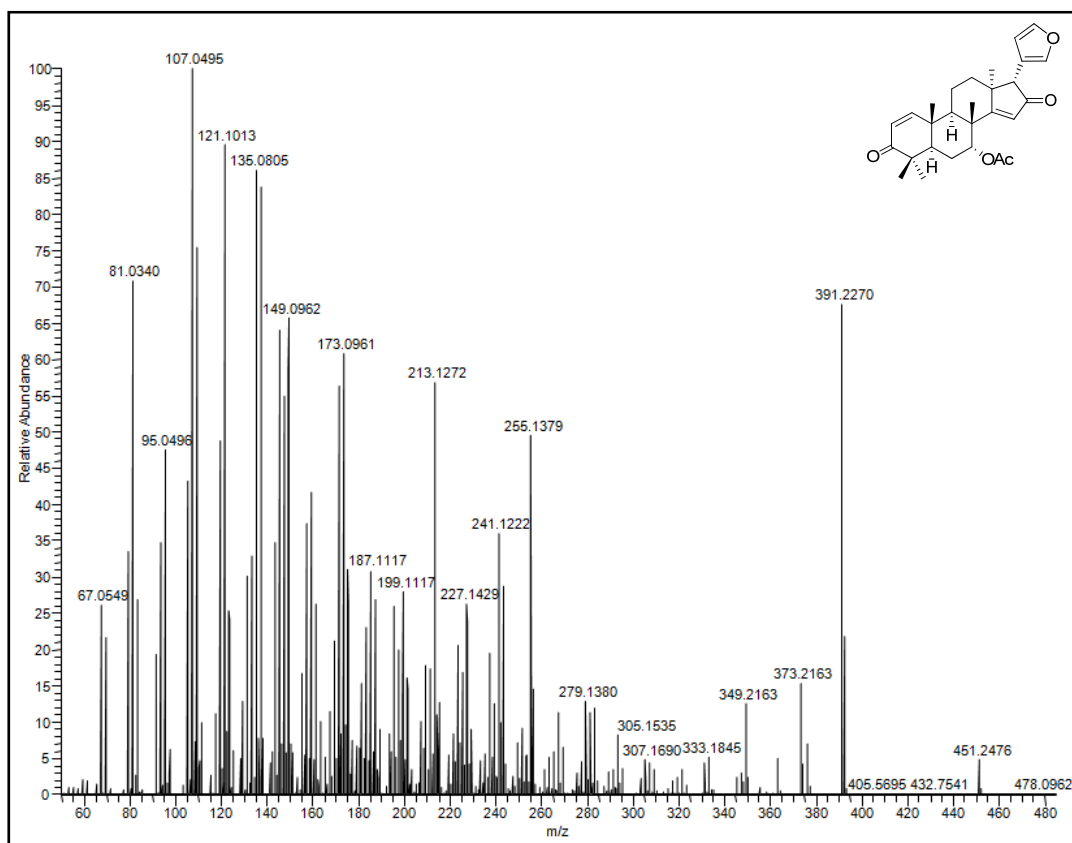


ESI-MS spectrum of azadirone (I)

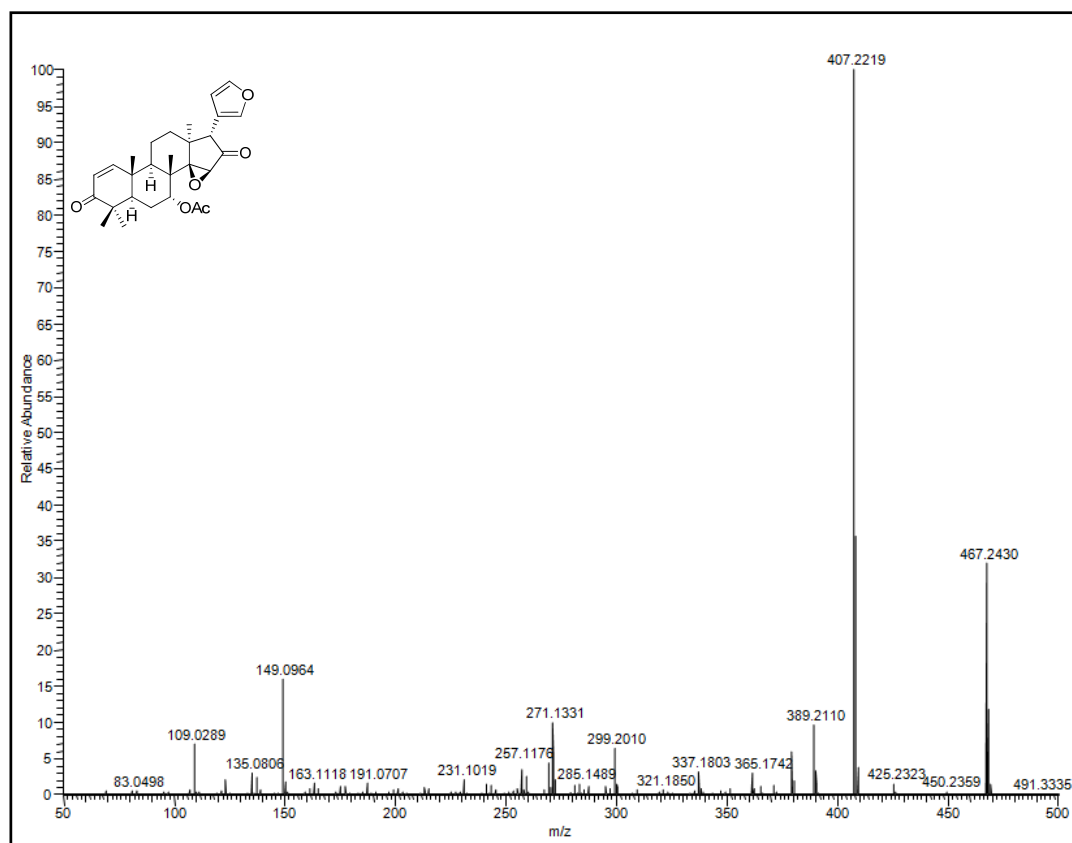
ESI-MS/MS spectrum of azadiradione (III) of m/z 451.2 in NCE = 15%

ESI-MS/MS spectrum of azadiradione (**III**) of m/z 451.2 in NCE = 20%ESI-MS/MS spectrum of azadiradione (**III**) of m/z 451.2 in NCE = 25%

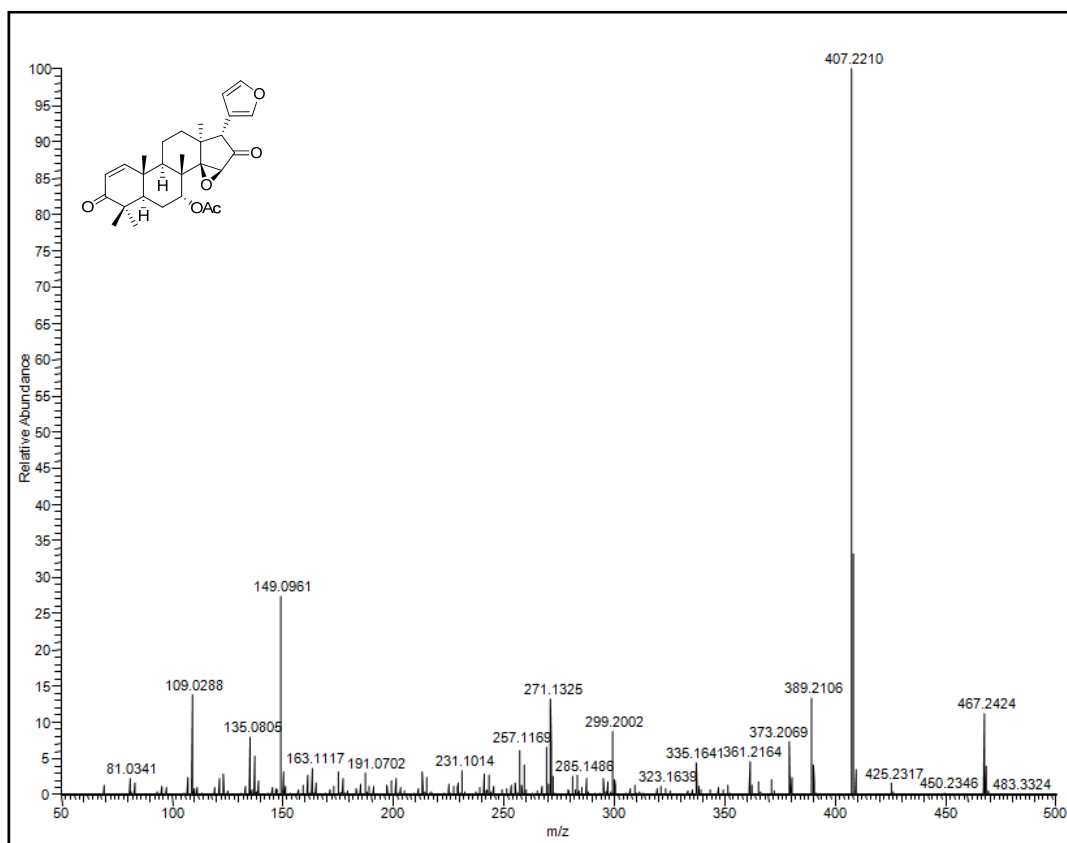
ESI-MS/MS spectrum of azadiradione (**III**) of m/z 451.2 in NCE = 30%ESI-MS/MS spectrum of azadiradione (**III**) of m/z 451.2 in NCE = 35%



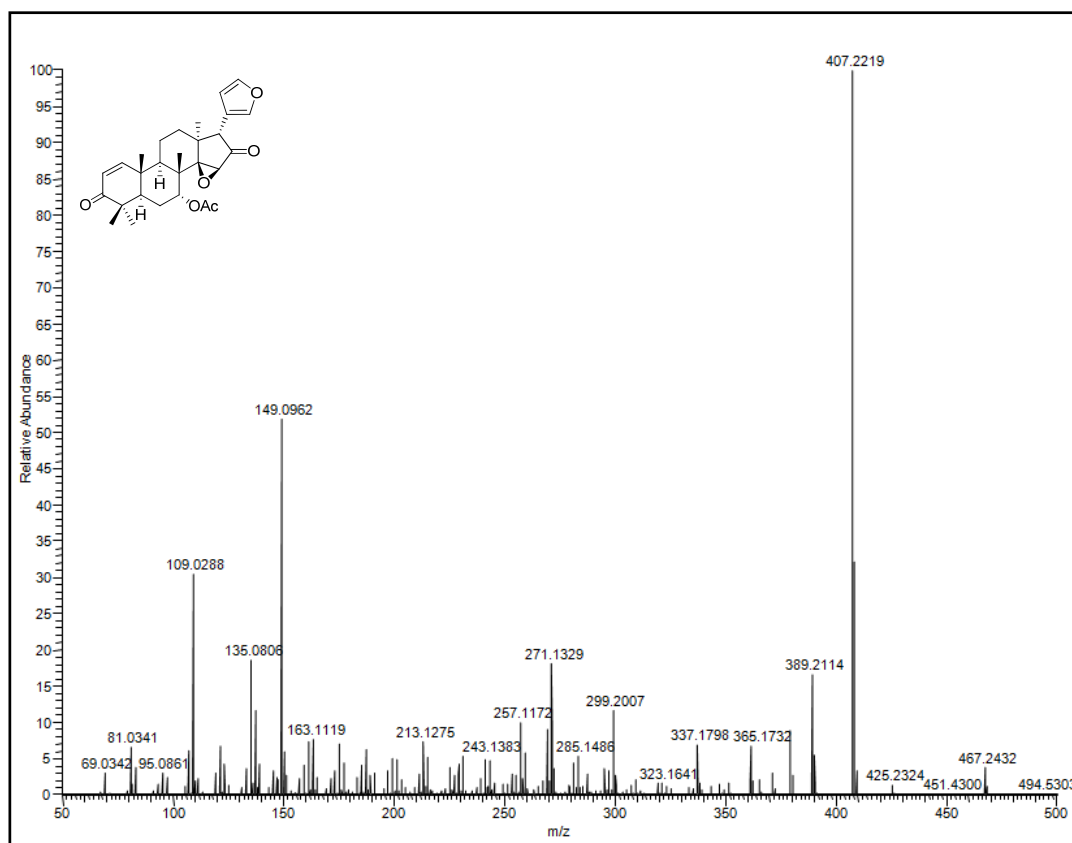
ESI-MS/MS spectrum of azadiradione (III) of m/z 451.2 in NCE = 40%



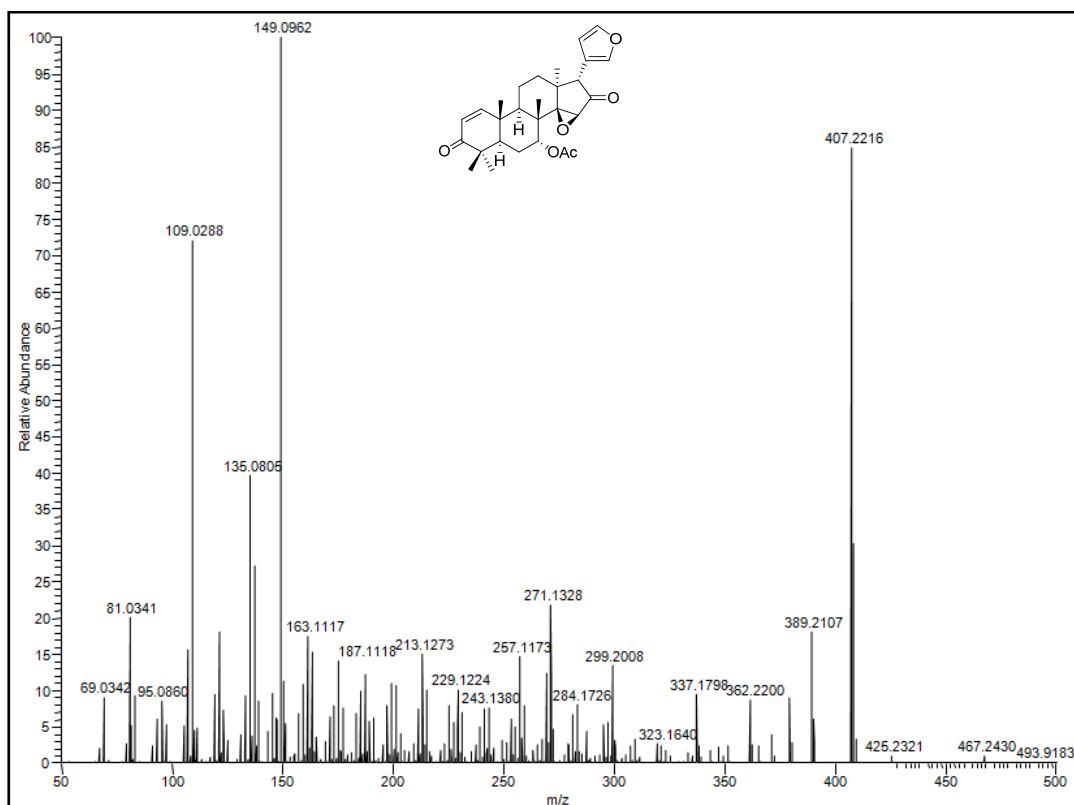
ESI-MS/MS spectrum of epoxyazadiradione (II) of m/z 467.2 in NCE = 15%



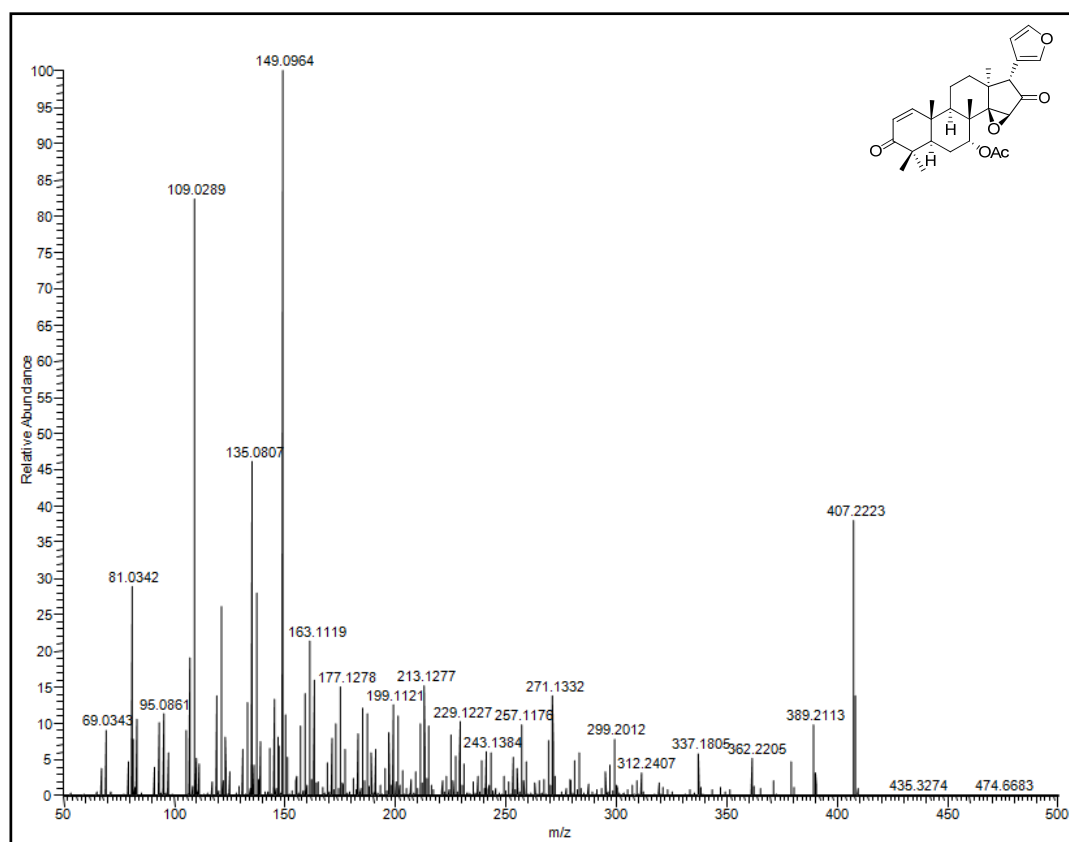
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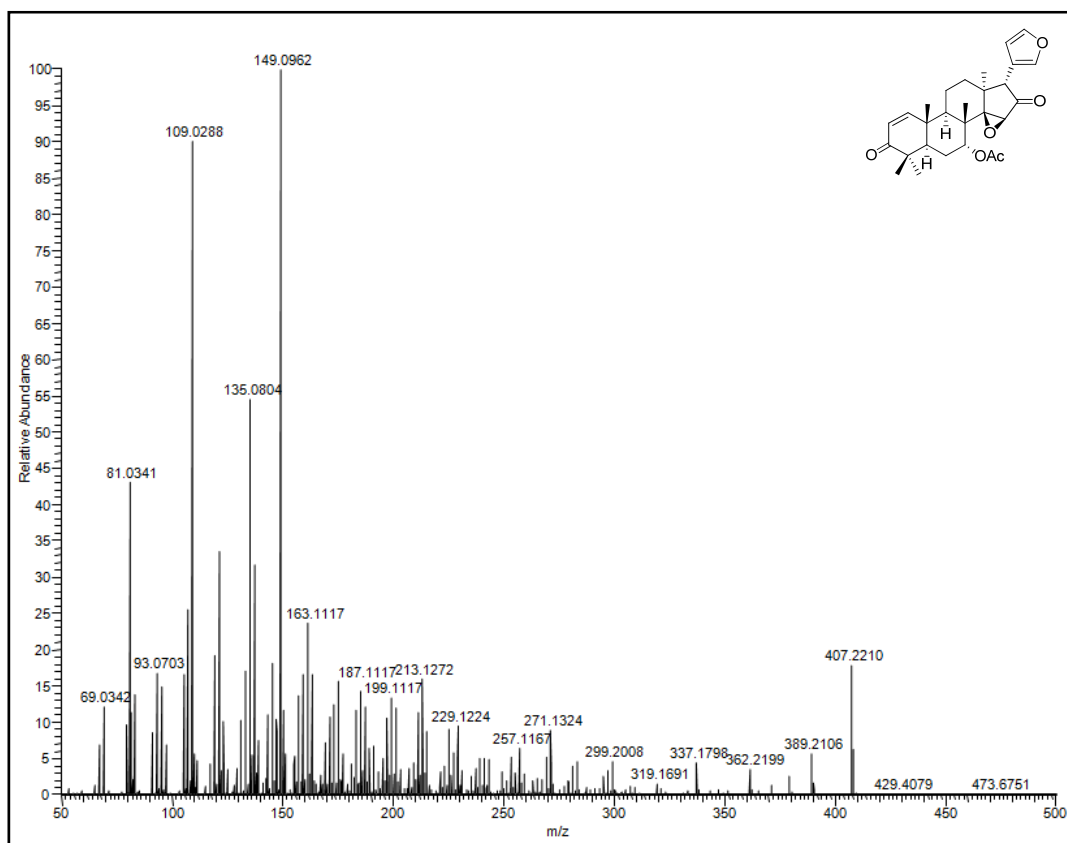
ESI-MS/MS spectrum of epoxyazadiradione (**II**) of m/z 467.2 in NCE = 25%



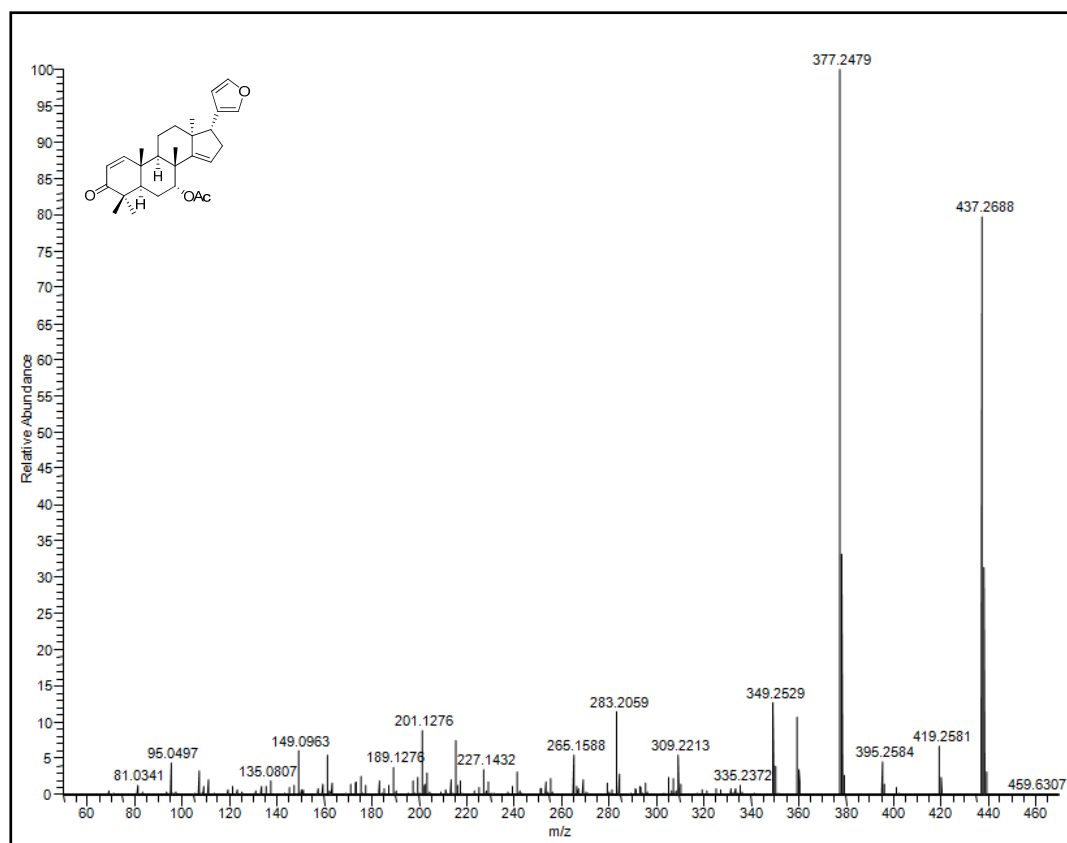
ESI-MS/MS spectrum of epoxyazadiradione (**II**) of m/z 467.2 in NCE = 30%



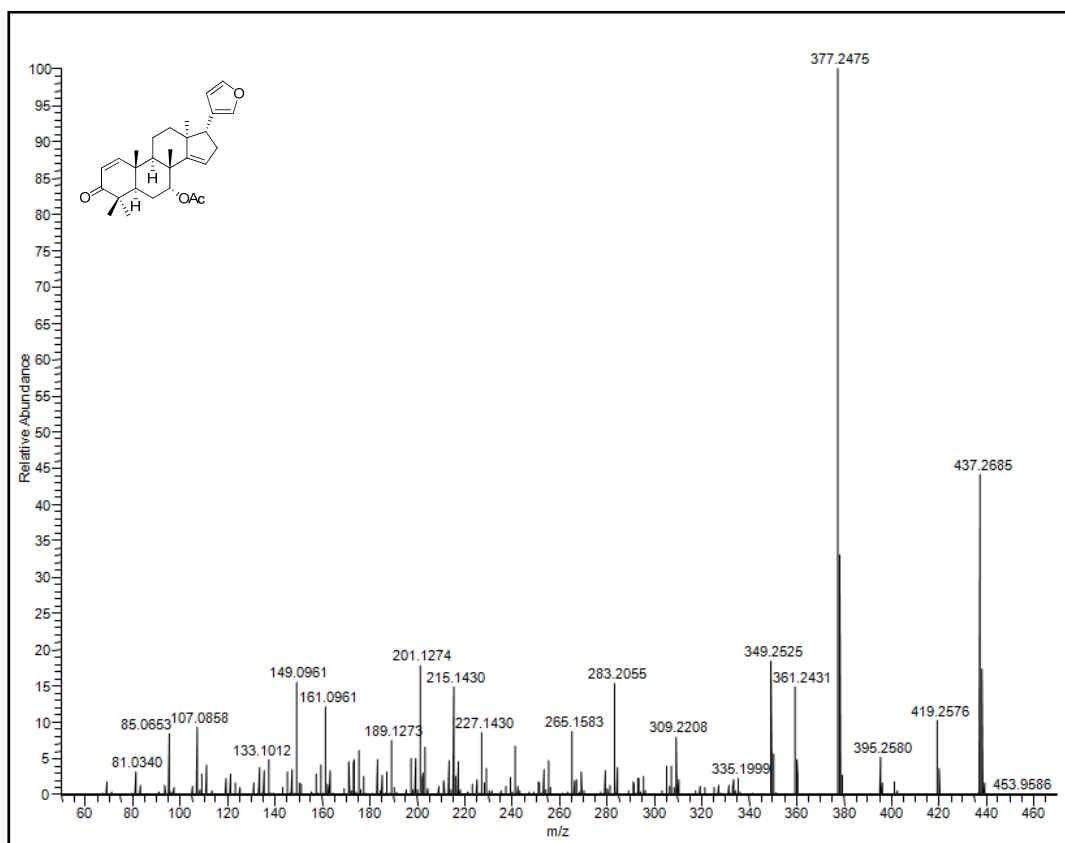
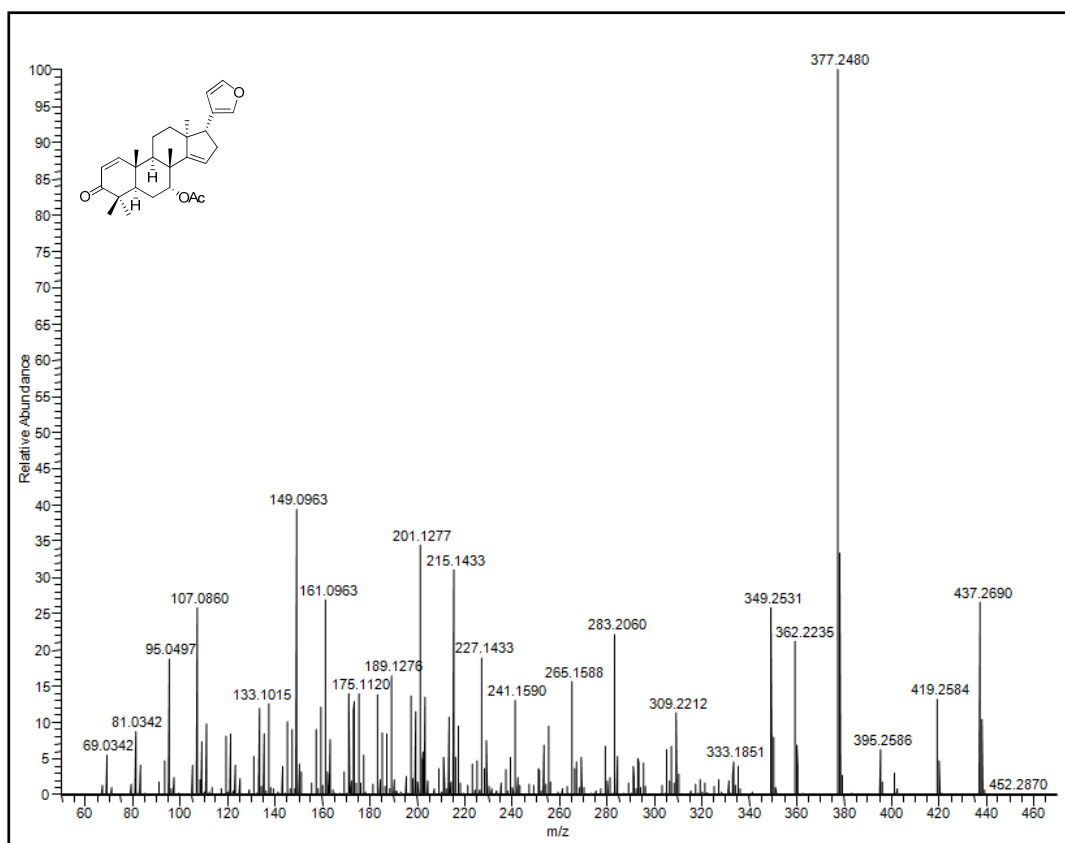
ESI-MS/MS spectrum of epoxyazadiradione (**II**) of m/z 467.2 in NCE = 35%

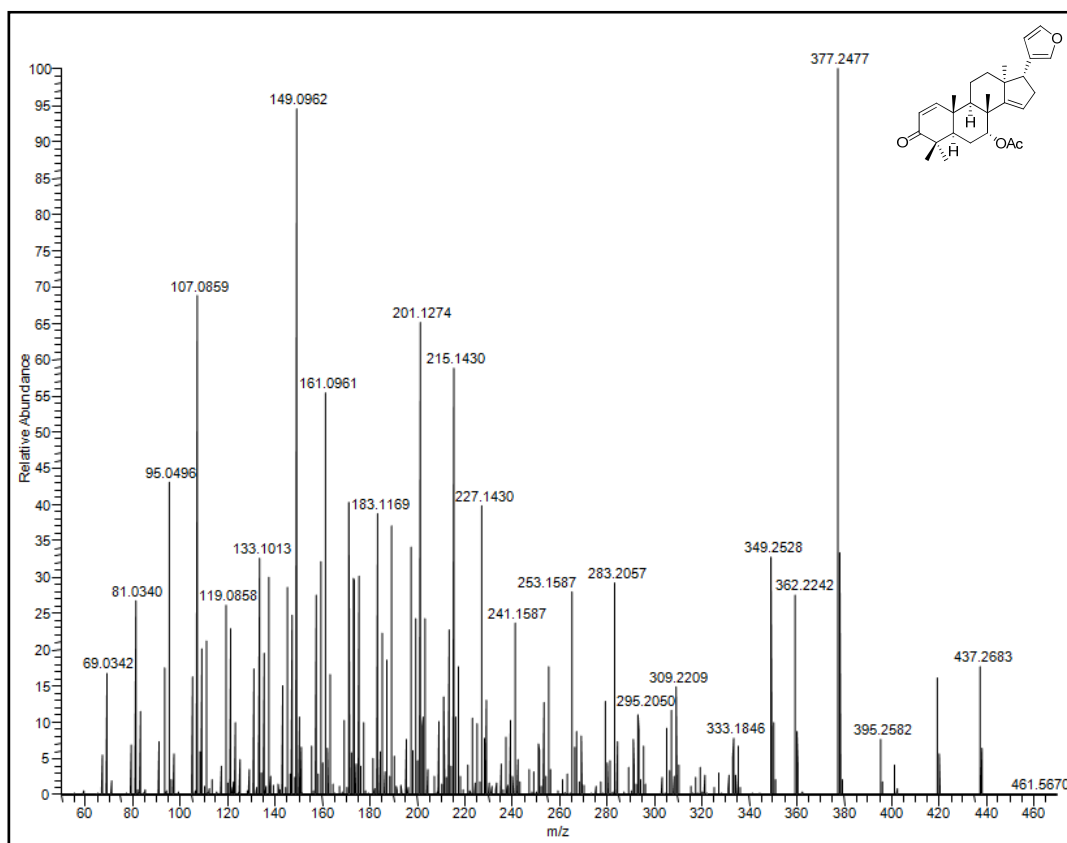
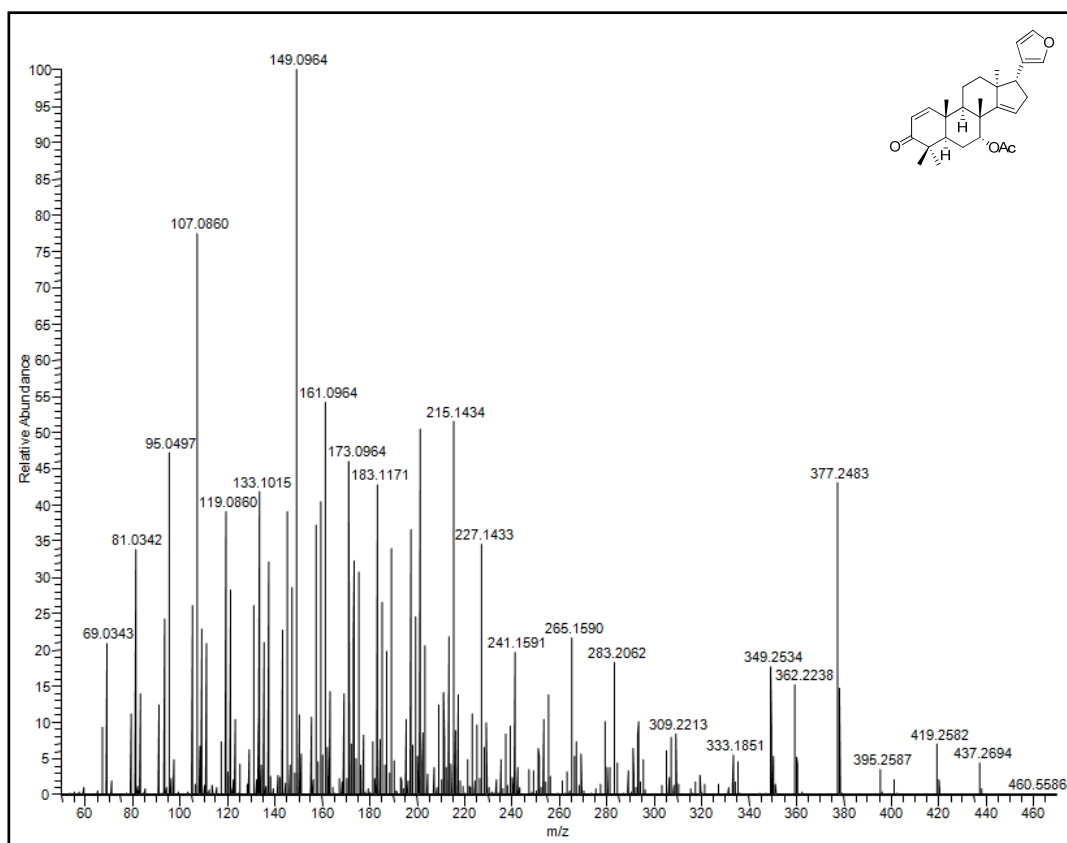


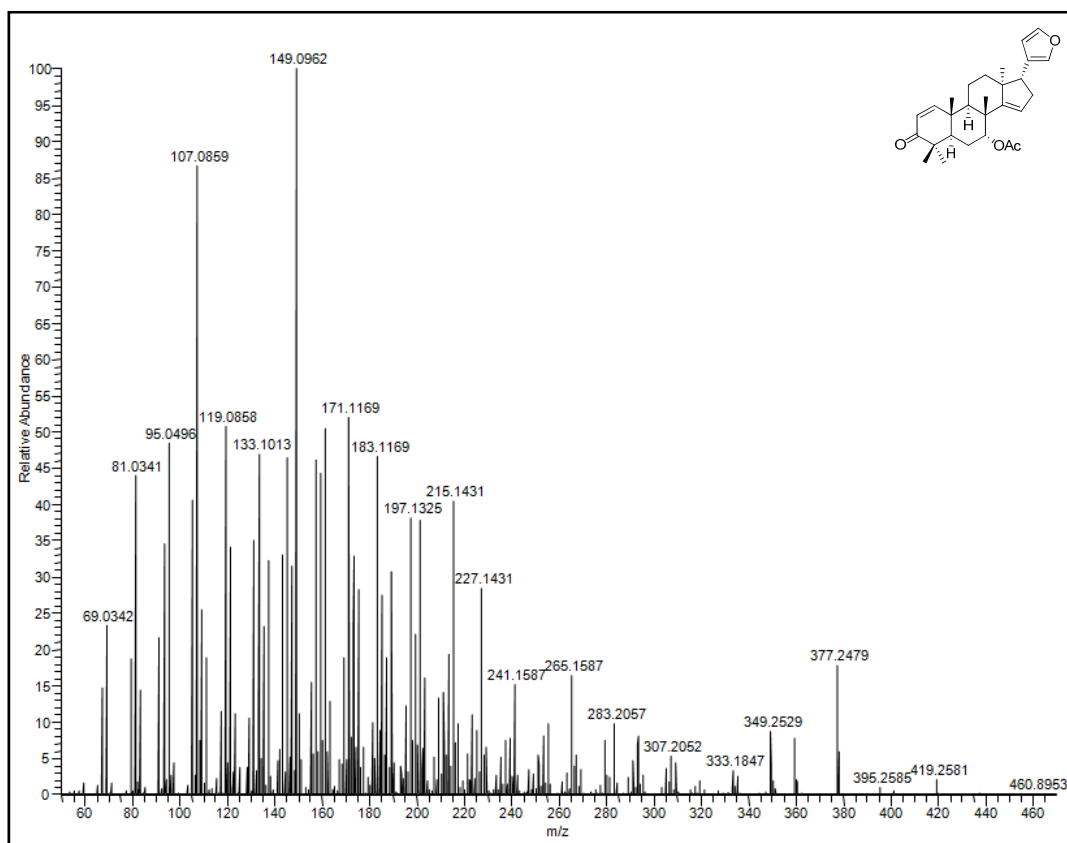
ESI-MS/MS spectrum of epoxyazadiradione (II) of m/z 467.2 in NCE = 40%



ESI-MS/MS spectrum of azadirone (I) of m/z 437.2 in NCE = 15%

ESI-MS/MS spectrum of azadirone (**I**) of m/z 437.2 in NCE = 20%ESI-MS/MS spectrum of azadirone (**I**) of m/z 437.2 in NCE = 25%

ESI-MS/MS spectrum of azadirone (I) of m/z 437.2 in NCE = 30%ESI-MS/MS spectrum of azadirone (I) of m/z 437.2 in NCE = 35%



ESI-MS/MS spectrum of azadirone (I) of m/z 437.2 in NCE = 40%

Chapter 2: Section C

Expedient MPLC Based Preparative Isolation and MS/MS Characterization of C-Seco Triterpenoids from Neem Oil

Highlights:

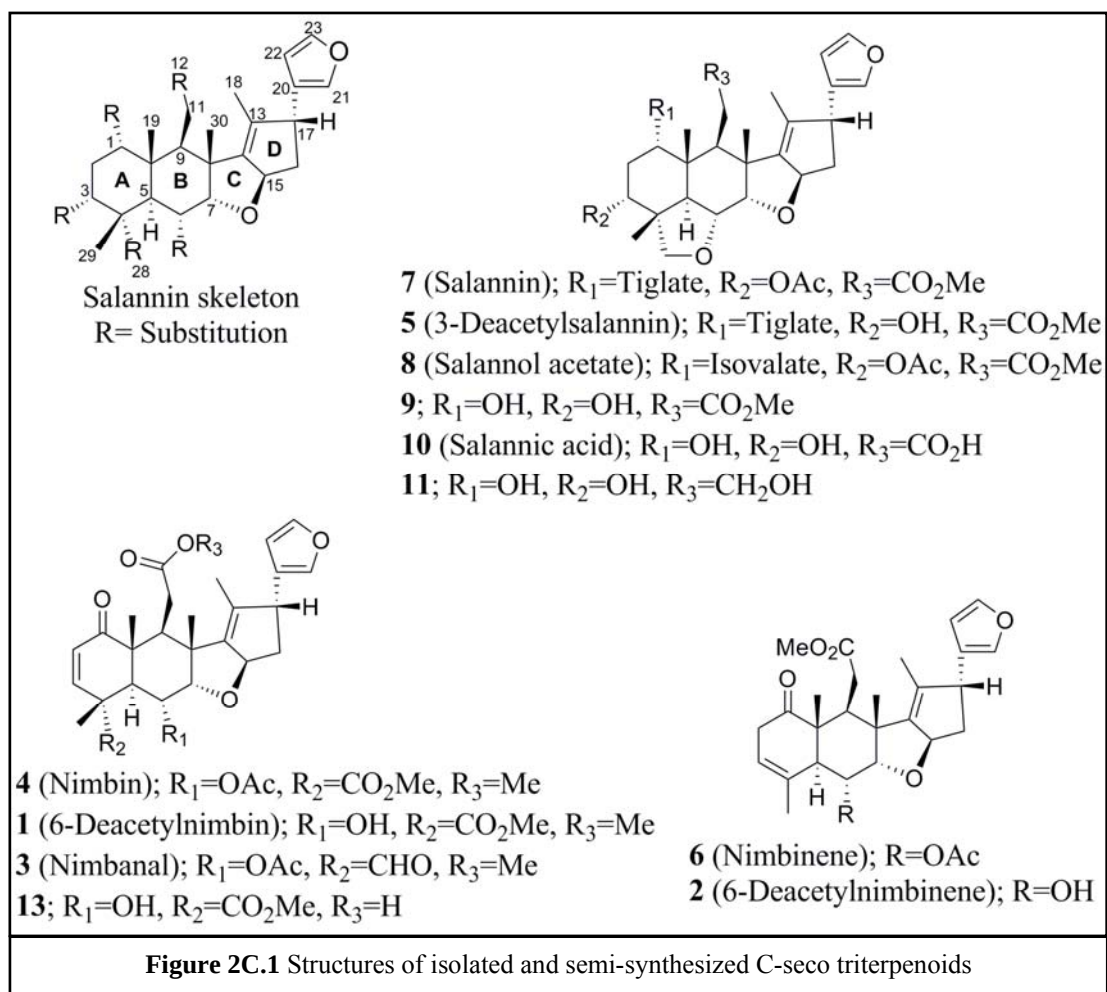
- *Eight major C-seco limonoids of salannin skeleton were isolated in preparative scale with high purity using a rapid MPLC based isolation technique from Neem oil extract.*
- *Eight isolated and five semi-synthesized derivatives were structurally characterized by ESI(+)-quadrupole/orbitrap-MS/MS technique.*
- *Plausible fragmentation pathway of MS/MS daughter ions was predicted and MS/MS identification technique was further applied for the investigation of studied C-seco triterpenoids in Neem bark and stem extract.*

2C.1 Introduction

C-seco (*i.e.* opened and modified C-ring) triterpenoids of salannin skeleton are well-known due to their potent anti-feedant and growth-regulating properties against a wide variety of insects.²³⁻²⁹ Abundance of C-seco triterpenoids is restrained to the family of *Maliaceae* and especially to the genera of *Azadirachta* and *Melia*.²³ Neem tree is one of the richest sources of C-seco triterpenoids in nature (Chapter 1).²⁵ Over fifty C-seco triterpenoids have been characterized from various parts of Neem plant, one third of which possess salannin or nimbin skeleton.²⁵ Potent insecticidal activity of this valuable class of triterpenoids has prompted the need of purified compounds for detailed investigation of known activity and exploration of unknown bioactivities. Due to highly complex and sensitive skeletal framework and presence of numerous stereogenic centres, synthetic approach is practically ineffective to make an easy access to these promising natural products. On the other hand, high structural resemblance among complex blend of C-seco triterpenoids results in narrow chromatographic resolution. Therefore, development of a rapid, effective and reproducible isolation protocol will facilitate the easy availability of these molecules. Reported separation protocols are mainly focused on the preparative isolation of azadirachtin A and its derivatives from seed extract and very less effort has been exerted aiming at other C-seco triterpenoids.³⁰⁻³⁶ Tetranortriterpenoids are known to be responsible for anti-carcinogenic,³⁷ anti-fertility,³⁸ anti-fungal,³⁹ acaricidal,⁴⁰ antifeedant and growth-regulatory^{26,41} activities of Neem oil. However, rapid and systematic preparative isolation procedure for individual triterpenoids from Neem oil is not well-established. As discussed in previous section, automated medium pressure liquid chromatography (MPLC) is a robust technique for developing expedient and high-resolution preparative-isolation procedure for natural products and to scale-up the developed protocol.^{17,18,42} Here in, we have demonstrated an automated MPLC based rapid and preparative purification procedure for eight major C-seco triterpenoids (Figure 2C.1) from Neem oil by successive application of normal phase and reverse phase silica gel pre-packed cartridges. The efficiency of the developed method and purity of the isolated triterpenoids were further evaluated by HPLC analyses.

Tandem mass spectrometry coupled with ultra performance liquid chromatography is a physicochemical technique to identify the individual natural

products in the phytochemical extracts even at a very low concentration (nm-pm range) with high rapidity, sensitivity, accuracy and low sample requirement.⁴³⁻⁴⁷ Signature MS/MS spectra and structure-fragment relationships can further be utilized for the high-throughput screening (in search of novel metabolites with similar skeleton or plant sources with higher abundance) and investigating the biological processes (such as metabolism, biosynthesis and degradation) associated with bioactive molecules qualitatively and quantitatively.^{19,20,48-50} In this study, UPLC-ESI(+)-quadrupole/orbitrap-MS/MS characterization of C-seco triterpenoids of salannin skeleton has been delineated for the first time. Plausible structure-fragment relationships of MS/MS daughter ions generated from thirteen isolated/semi-synthesized derivatives of salannin (Figure 2C.1) were deduced. Applicability of the technique was validated by the identification of same metabolites in the extract of Neem stem and bark by UPLC-ESI(+)-MS/MS using data-dependent inclusion mode.



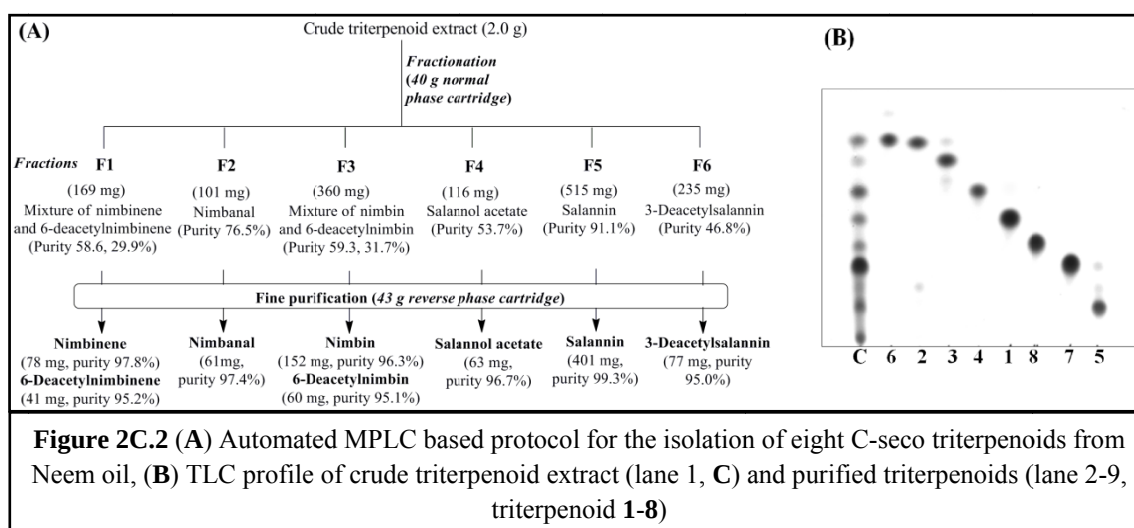
2C.2 Results and Discussion

2C.2.1 Automated MPLC Based Preparative Isolation

Application of normal phase silica gel cartridge for the fractionation of crude triterpenoid mixture (into six fractions, **F1-F6**) followed by fine purification of individual fractions using reverse phase silica gel cartridge was exploited for the isolation of eight major triterpenoids from Neem oil extract with high purity. The developed protocol has been schematically represented in Figure 2C.2. Purified triterpenoids were structurally characterized on the basis of NMR spectrometric and ESI(+)-HRMS data, which were in well correlation with the previous reports.^{28,33,51,52}

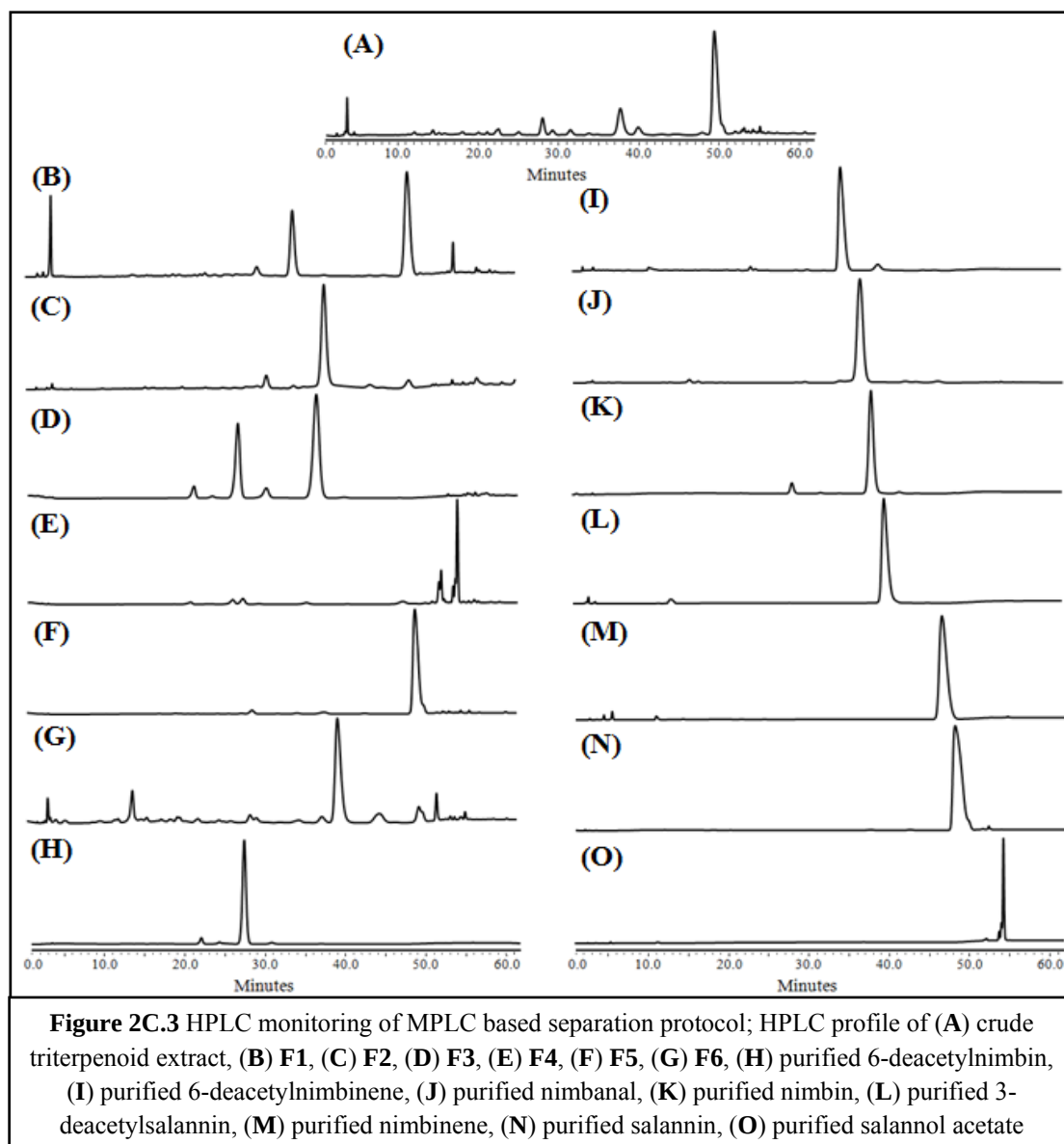
All six fractions (**F1-F6**) obtained through the fractionation over normal-phase silica-gel pre-packed cartridge were analyzed by HPLC (Figure 2C.3) and TLC. Further each of the fractions was purified individually by reverse phase cartridge to get purified metabolites. Fraction 1 (**F1**, 169 mg) contained two major triterpenoids as evident from HPLC chromatogram (purity 29.9% and 58.6%), although both appeared as the inseparable spot on TLC (normal phase). Purification of the fraction over reverse phase cartridge yielded two pure metabolites within the elution range 28-37 min (55-60% methanol in water) and 40-49 min (60% methanol in water) respectively. They were characterized as C-seco pentanortriterpenoids, namely 6-deacetylnimbinene (41 mg, R_t 33.5 min, R_f 0.82, purity 95.2%) and nimbinene (78 mg, R_t 45.6 min, R_f 0.82, purity 97.8%) respectively. HPLC analysis revealed that Fraction 2 (**F2**, 101 mg) contained one major metabolite with purity 76.5%. Upon purification over C18 stationary phase single metabolite was eluted within the elution range 35-43 min (55-60% methanol in water). NMR and ESI(+)-HRMS data elucidated the structure as nimbanal (61 mg, R_t 35.8 min, R_f 0.75, purity 97.4%), a C-seco tetranortriterpenoid. Fraction 3 (**F3**, 360 mg) was found to be a mixture of two metabolites as analyzed by HPLC and TLC with individual purity of 31.7% and 59.3% respectively. It was further subjected to reverse phase silica gel cartridge resulting in purification of two components with elution range 28-33 min (55-60% methanol in water) and 39-49 min (60% methanol in water). Metabolites were structurally characterized as C-seco tetranortriterpenoids, namely 6-decaetylnimbin (60 mg, R_t 26.6 min, R_f 0.54, purity 95.1%) and nimbin (152 mg, R_t 37.3 min, R_f 0.64, purity 96.3%) respectively. Fraction 4 (**F4**, 116 mg) contained majorly one metabolite (HPLC purity 53.7%) which upon purification through C18

silica gel cartridge furnished a C-seco tetranortriterpenoid (elution range 61-73 min corresponding to 60-70% methanol in water). The metabolite was characterized as salannol acetate (63 mg, R_t 53.4 min, R_f 0.45, purity 96.7%). Fraction 5 (**F5**, 515 mg) was consisted of major metabolite of the crude extract with HPLC purity 91.1%. Fine purification through reverse phase column eluted (elution range 38-58 min corresponding to 60% methanol in water) highly pure salannin (401 mg, R_t 48.5 min, R_f 0.36, purity 99.3%). Most polar fraction 6 (**F6**, 235 mg) of the crude extract contained one major metabolite with HPLC purity 46.8% along with numerous minor components. Purification of the same fraction over C18 silica gel cartridge yielded the major component within the elution range 38-49 min (60% methanol in water). The metabolite was characterized as deacetyl analogue of salannin, namely 3-deacetylsalannin (77 mg, R_t 38.8 min, R_f 0.16, purity 95.0%). TLC profile of the purified triterpenoids with reference to the crude extract has been represented in Figure 2C.2.



The developed procedure was highly efficient with respect to rapidity and purity of eight triterpenoids isolated. The automation utilized throughout the protocol expedited the whole process with very low risk of error. Chromatographic conditions (*i.e.* solvent program, flow rate and column parameters) used for the fine purification over reverse phase silica gel cartridge were common for six different fractions; thus reducing the required time and complexity of the process. Purity obtained for the isolated triterpenoids was $\geq 95\%$. The developed isolation protocol was performed thrice with high reproducibility in purity, yield and retention time. Simplicity in scaling-up, cost-

effectivity, rapidity and efficiency associated with the preparative isolation protocol developed on automated MPLC system will make it as promising technique over other commonly used advanced purification tools including HPLC or Counter Current Chromatography.



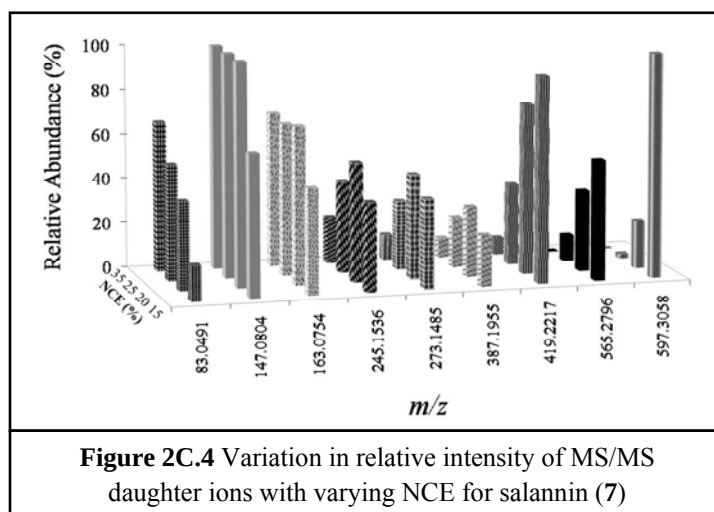
2C.2.2 UPLC-ESI(+)-MS and MS/MS Characterization

MS/MS signature spectra and structure-fragment correlations of the daughter ions (generated from $[M+H]^+$ precursor ions) corresponding to eight isolated C-seco triterpenoids of salannin type and five semi-synthetic derivatives (Figure 2C.1) were studied using ESI(+)-quadrupole/orbitrap-MS/MS spectrometry with an optimized stepped normalized collision energy (NCE) of 20%. Structure-fragment relationships

were established on the basis of chemical formulae obtained for each m/z values with high accuracy (error < 5.0 ppm) and the prediction of their pathway of genesis. The key fragments and their intensity of thirteen investigated C-seco triterpenoids have been documented in Table 2C.1. Observed fragments can be divided into two categories on the basis of mechanistic viewpoint; (i) high-mass fragments ($m/z > 300$) and (ii) low-mass fragments ($m/z < 300$). It was observed that fragments at higher than m/z 300 generated due to the loss of functional groups as neutral molecules such as H_2O , MeOH, CO, AcOH, HCHO, tiglic acid and isovaleric acid from intact C-seco triterpenoid skeleton. Lower-mass fragments were produced due to the C-C bond cleavages, rearrangements leading to skeletal fragmentation. High-mass fragments are essential to recognize the functional groups arranged around the skeleton, whereas low-mass fragments bear signature for the skeletal framework of salannin type C-seco triterpenoids. Therefore, fragments of both the types are highly essential for extracting valuable skeletal information.

2C.2.3 Optimization of Normalized Collision Energy (NCE)

The collision energy was optimized such a manner to produce both higher and lower mass fragments with comparable distribution of intensity. Salannin (7) was used as the



representative molecule for the optimization study with varying NCE (15, 20, 25, 35 and 50%). The variation in intensities of the fragments with sweeping collision energy has been depicted in Figure 2C.4. Higher NCE resulted in enhanced abundance of the low-mass

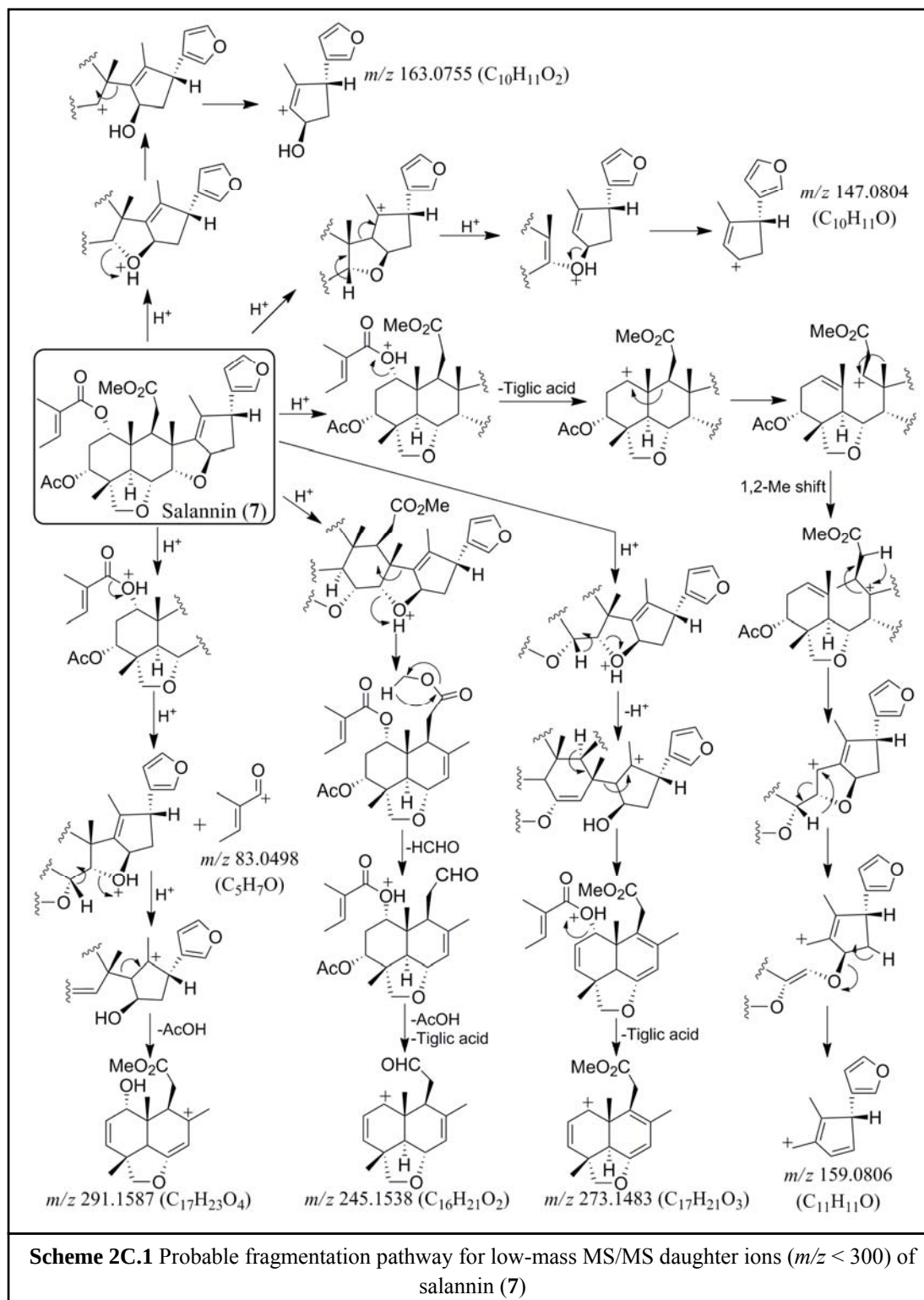
fragments, whereas lower NCE increased the intensity of high-mass fragments. 20% NCE was found to be most effective to generate the daughter ions whole throughout the high ($m/z > 300$) and low mass-range ($m/z < 300$) with significant intensity and used as the preferred NCE for other C-seco triterpenoids.

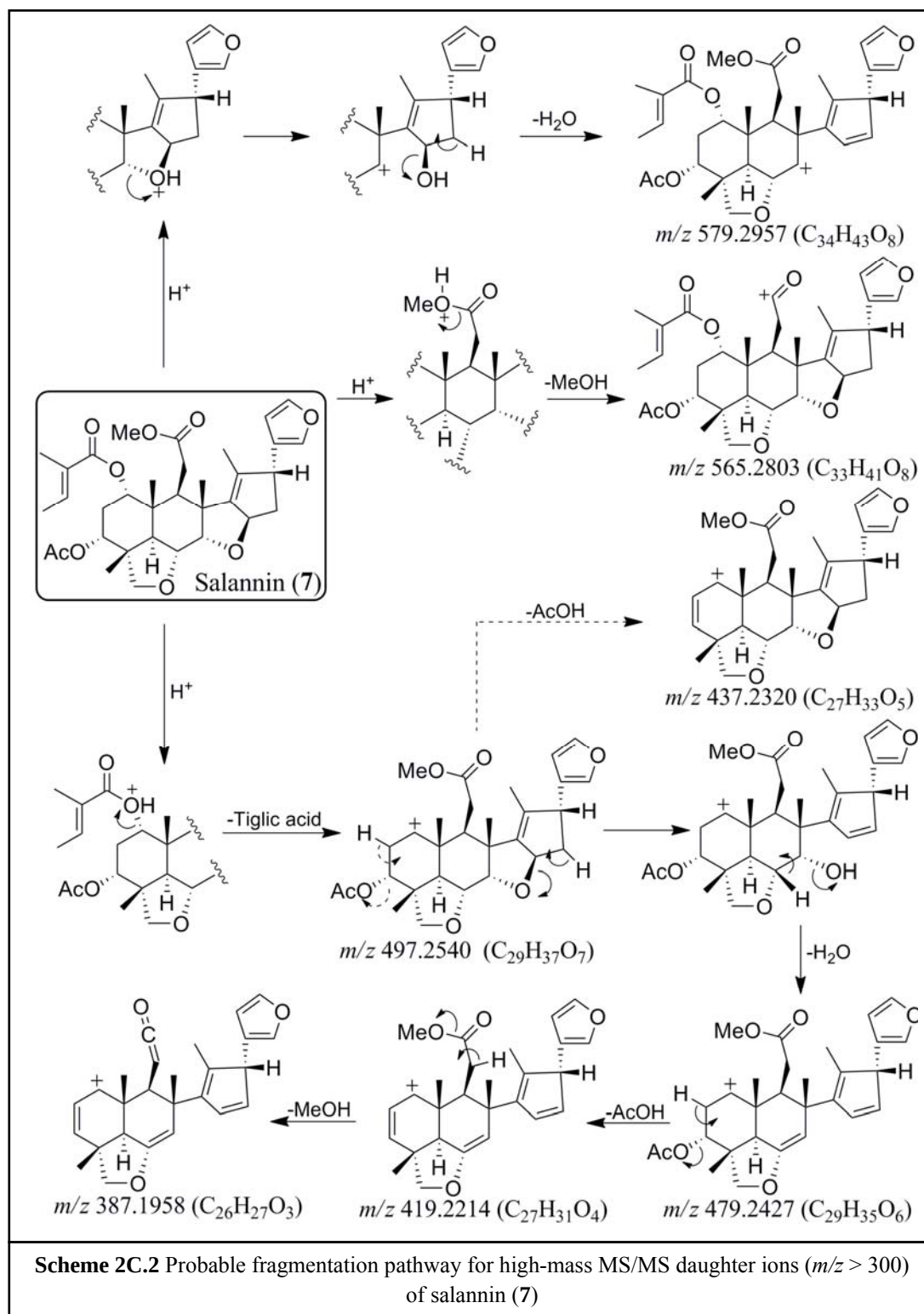
Table 2C.1 Structure, precursor ion, MS/MS daughter ions, their corresponding chemical formula and intensity in 20% NCE for triterpenoids **1-13**

No	Structure	Precursor ion (m/z)	Daughter ions m/z (Formula, % Intensity at NCE 20%)
7		[M+H] ⁺ 597.30	83.0498 (C ₅ H ₇ O, 39.7), 147.0804 (C ₁₀ H ₁₁ O, 100.0), 159.0806 (C ₁₁ H ₁₁ O, 31.1), 161.0962 (C ₁₁ H ₁₃ O, 31.7), 163.0755 (C ₁₀ H ₁₁ O ₂ , 31.7), 245.1538 (C ₁₆ H ₂₁ O ₂ , 53.1), 273.1483 (C ₁₇ H ₂₁ O ₃ , 46.7), 291.1587 (C ₁₇ H ₂₃ O ₄ , 13.4), 387.1958 (C ₂₆ H ₂₇ O ₃ , 31.1), 419.2214 (C ₂₇ H ₃₁ O ₄ , 75.6), 437.2320 (C ₂₇ H ₃₃ O ₅ , 25.8), 479.2427 (C ₂₉ H ₃₅ O ₆ , 16.7), 497.2540 (C ₂₉ H ₃₇ O ₇ , 9.0), 565.2803 (C ₃₃ H ₄₁ O ₈ , 35.7), 579.2957 (C ₃₄ H ₄₃ O ₈ , 14.1).
5		[M+H] ⁺ 555.20	147.0805 (C ₁₀ H ₁₁ O, 100.0), 159.0806 (C ₁₁ H ₁₁ O, 64.5), 161.0962 (C ₁₁ H ₁₃ O, 34.5), 163.0753 (C ₁₀ H ₁₁ O ₂ , 95.3), 245.1538 (C ₁₆ H ₂₁ O ₂ , 45.9), 273.1482 (C ₁₇ H ₂₁ O ₃ , 30.5), 291.1589 (C ₁₇ H ₂₃ O ₄ , 22.7), 387.1954 (C ₂₆ H ₂₇ O ₃ , 21.7), 405.2055 (C ₂₆ H ₂₉ O ₄ , 22.4), 419.2216 (C ₂₇ H ₃₁ O ₄ , 58.1), 437.2316 (C ₂₇ H ₃₃ O ₅ , 70.1), 455.2423 (C ₂₇ H ₃₅ O ₆ , 24.2), 523.2684 (C ₃₁ H ₃₉ O ₇ , 43.0), 537.2844 (C ₃₂ H ₄₁ O ₇ , 13.3).
8		[M+H] ⁺ 599.30	147.0804 (C ₁₀ H ₁₁ O, 100.0), 159.0804 (C ₁₁ H ₁₁ O, 30.9), 161.0960 (C ₁₁ H ₁₃ O, 31.1), 163.0752 (C ₁₀ H ₁₁ O ₂ , 64.8), 245.1535 (C ₁₆ H ₂₁ O ₂ , 52.4), 273.1481 (C ₁₇ H ₂₁ O ₃ , 40.6), 291.1578 (C ₁₇ H ₂₃ O ₄ , 10.1), 359.2005 (C ₂₅ H ₂₇ O ₂ , 17.2), 387.1947 (C ₂₆ H ₂₇ O ₃ , 26.7), 401.2104 (C ₂₇ H ₂₉ O ₃ , 29.0), 419.2211 (C ₂₇ H ₃₁ O ₄ , 68.7), 437.2323 (C ₂₇ H ₃₃ O ₅ , 21.9), 479.2430 (C ₂₉ H ₃₅ O ₆ , 14.7), 497.2523 (C ₂₉ H ₃₇ O ₇ , 8.3), 521.2899 (C ₃₂ H ₄₁ O ₆ , 4.2), 567.2958 (C ₃₃ H ₄₃ O ₈ , 28.2), 581.3101 (C ₃₄ H ₄₅ O ₈ , 16.5).
9		[M+H] ⁺ 473.20	147.0802 (C ₁₀ H ₁₁ O, 100.0), 159.0802 (C ₁₁ H ₁₁ O, 51.8), 161.0959 (C ₁₁ H ₁₃ O, 24.6), 163.0751 (C ₁₀ H ₁₁ O ₂ , 26.8), 245.1530 (C ₁₆ H ₂₁ O ₂ , 29.5), 273.1479 (C ₁₇ H ₂₁ O ₃ , 25.7), 291.1578 (C ₁₇ H ₂₃ O ₄ , 27.0), 405.2043 (C ₂₆ H ₂₉ O ₄ , 15.1), 411.2159 (C ₂₆ H ₃₁ O ₅ , 32.4), 419.2201 (C ₂₇ H ₃₁ O ₄ , 45.3), 437.2316 (C ₂₇ H ₃₃ O ₅ , 53.0), 441.2257 (C ₂₆ H ₃₃ O ₆ , 61.1), 455.2414 (C ₂₇ H ₃₅ O ₆ , 64.2).
10		[M+H] ⁺ 459.20	147.0803 (C ₁₀ H ₁₁ O, 62.9), 159.0802 (C ₁₁ H ₁₁ O, 25.9), 161.0959 (C ₁₁ H ₁₃ O, 12.6), 163.0752 (C ₁₀ H ₁₁ O ₂ , 14.8), 231.1374 (C ₁₅ H ₁₉ O ₂ , 23.6), 249.1479 (C ₁₅ H ₂₁ O ₃ , 18.6), 259.1325 (C ₁₆ H ₁₉ O ₃ , 16.2), 277.1422 (C ₁₆ H ₂₁ O ₄ , 15.6), 377.2100 (C ₂₅ H ₂₉ O ₃ , 13.3), 387.1946 (C ₂₆ H ₂₇ O ₃ , 11.6), 393.2048 (C ₂₅ H ₂₉ O ₄ , 10.7), 397.1994 (C ₂₄ H ₂₉ O ₅ , 17.9), 405.2051 (C ₂₆ H ₂₉ O ₄ , 38.6), 423.2158 (C ₂₆ H ₃₁ O ₅ , 45.9), 441.2258 (C ₂₆ H ₃₃ O ₆ , 83.8).
11		[M+H] ⁺ 445.20	147.0804 (C ₁₀ H ₁₁ O, 45.5), 159.0804 (C ₁₁ H ₁₁ O, 34.3), 161.0961 (C ₁₁ H ₁₃ O, 15.8), 163.0753 (C ₁₀ H ₁₁ O ₂ , 10.5), 217.1224 (C ₁₄ H ₁₇ O ₂ , 16.2), 245.1535 (C ₁₆ H ₂₁ O ₂ , 11.0), 337.2160 (C ₂₃ H ₂₉ O ₂ , 10.1), 347.2000 (C ₂₄ H ₂₇ O ₂ , 10.9), 363.2318 (C ₂₅ H ₃₁ O ₂ , 15.9), 373.2160 (C ₂₆ H ₂₉ O ₂ , 48.6), 391.2263 (C ₂₆ H ₃₁ O ₃ , 76.0), 409.2372 (C ₂₆ H ₃₃ O ₄ , 67.3), 427.2479 (C ₂₆ H ₃₅ O ₅ , 49.7).
12		[M+H] ⁺ 447.20	147.0804 (C ₁₀ H ₁₁ O, 31.4), 159.0802 (C ₁₁ H ₁₁ O, 26.8), 161.0958 (C ₁₁ H ₁₃ O, 9.9), 163.0750 (C ₁₀ H ₁₁ O ₂ , 8.7), 217.1224 (C ₁₄ H ₁₇ O ₂ , 9.1), 339.2278 (C ₂₃ H ₂₇ ² H ₂ O ₂ , 8.2), 349.2119 (C ₂₄ H ₂₅ ² H ₂ O ₂ , 6.9), 365.2434 (C ₂₅ H ₂₉ ² H ₂ O ₂ , 9.9), 375.2276 (C ₂₆ H ₂₇ ² H ₂ O ₂ , 30.6), 393.2383 (C ₂₆ H ₂₉ ² H ₂ O ₃ , 51.7), 411.2488 (C ₂₆ H ₃₁ ² H ₂ O ₄ , 50.0), 429.2596 (C ₂₆ H ₃₃ ² H ₂ O ₅ , 37.7).
4		[M+H] ⁺ 541.20	147.0805 (C ₁₀ H ₁₁ O, 70.2), 175.0754 (C ₁₁ H ₁₁ O ₂ , 24.6), 215.1069 (C ₁₄ H ₁₅ O ₂ , 24.1), 225.0912 (C ₁₅ H ₁₃ O ₂ , 27.0), 247.1328 (C ₁₅ H ₁₉ O ₃ , 31.9), 275.1278 (C ₁₆ H ₁₉ O ₄ , 29.0), 389.1750 (C ₂₅ H ₂₅ O ₄ , 15.5), 421.2003 (C ₂₆ H ₂₉ O ₅ , 19.6), 431.1857 (C ₂₇ H ₂₇ O ₅ , 37.9), 449.1952 (C ₂₇ H ₂₉ O ₆ , 46.8), 463.2119 (C ₂₈ H ₃₁ O ₆ , 14.2), 481.2225 (C ₂₈ H ₃₃ O ₇ , 7.1), 509.2166 (C ₂₉ H ₃₅ O ₈ , 100.0).
1		[M+H] ⁺ 499.20	147.0805 (C ₁₀ H ₁₁ O, 46.5), 175.0753 (C ₁₁ H ₁₁ O ₂ , 7.0), 215.1068 (C ₁₄ H ₁₅ O ₂ , 8.1), 225.0909 (C ₁₅ H ₁₃ O ₂ , 10.0), 247.1328 (C ₁₅ H ₁₉ O ₃ , 7.8), 275.1276 (C ₁₆ H ₁₉ O ₄ , 7.8), 371.1639 (C ₂₅ H ₂₅ O ₃ , 4.9), 389.1749 (C ₂₅ H ₂₅ O ₄ , 6.2), 421.2004 (C ₂₆ H ₂₉ O ₅ , 6.1), 431.1857 (C ₂₇ H ₂₇ O ₅ , 8.9), 435.1805 (C ₂₆ H ₂₇ O ₆ , 25.6), 449.1952 (C ₂₇ H ₂₉ O ₆ , 34.0), 467.2066 (C ₂₇ H ₃₁ O ₇ , 100.0).
3		[M+H] ⁺ 511.20	147.0805 (C ₁₀ H ₁₁ O, 37.5), 175.0753 (C ₁₁ H ₁₁ O ₂ , 22.6), 215.1068 (C ₁₄ H ₁₅ O ₂ , 6.8), 225.0909 (C ₁₅ H ₁₃ O ₂ , 16.5), 245.1173 (C ₁₅ H ₁₉ O ₃ , 13.4), 373.1797 (C ₂₅ H ₂₅ O ₃ , 12.2), 391.1897 (C ₂₅ H ₂₇ O ₄ , 20.6), 401.1741 (C ₂₆ H ₂₅ O ₄ , 21.7), 419.1854 (C ₂₆ H ₂₇ O ₅ , 31.0), 433.2003 (C ₂₇ H ₂₉ O ₅ , 2.8), 451.2118 (C ₂₇ H ₃₁ O ₆ , 14.6), 479.2070 (C ₂₈ H ₃₁ O ₇ , 100.0).
13		[M+H] ⁺ 485.20	147.0804 (C ₁₀ H ₁₁ O, 100.0), 175.0754 (C ₁₁ H ₁₁ O ₂ , 26.2), 187.1118 (C ₁₃ H ₁₅ O, 16.0), 215.1068 (C ₁₄ H ₁₅ O ₂ , 17.4), 225.0910 (C ₁₅ H ₁₃ O ₂ , 13.3), 233.1173 (C ₁₄ H ₁₇ O ₃ , 29.9), 275.1276 (C ₁₆ H ₁₉ O ₄ , 16.6), 361.1802 (C ₂₄ H ₂₅ O ₃ , 6.4), 371.1637 (C ₂₅ H ₂₅ O ₃ , 9.9), 389.1748 (C ₂₅ H ₂₅ O ₄ , 17.8), 407.1849 (C ₂₅ H ₂₇ O ₅ , 23.4), 431.1857 (C ₂₇ H ₂₇ O ₅ , 15.0), 435.1805 (C ₂₆ H ₂₇ O ₆ , 61.2), 449.1951 (C ₂₇ H ₂₉ O ₆ , 61.2), 453.1911 (C ₂₆ H ₂₉ O ₇ , 32.0), 467.2068 (C ₂₇ H ₃₁ O ₇ , 99.7).
6		[M+H] ⁺ 482.20	147.0802 (C ₁₀ H ₁₁ O, 81.2), 171.1165 (C ₁₃ H ₁₅ , 69.8), 187.1114 (C ₁₃ H ₁₅ O, 56.3), 217.1221 (C ₁₄ H ₁₇ O ₂ , 28.6), 227.1064 (C ₁₅ H ₁₅ O ₂ , 37.0), 245.1169 (C ₁₅ H ₁₇ O ₃ , 23.7), 247.1323 (C ₁₅ H ₁₉ O ₃ , 22.2), 259.1324 (C ₁₆ H ₁₉ O ₃ , 24.7), 277.1425 (C ₁₆ H ₂₁ O ₄ , 12.0), 313.1589 (C ₂₃ H ₂₁ O, 9.1), 327.1743 (C ₂₄ H ₂₃ O, 9.7), 331.1685 (C ₂₃ H ₂₅ O ₂ , 13.5), 345.1841 (C ₂₄ H ₂₅ O ₂ , 18.6), 363.1945 (C ₂₄ H ₂₇ O ₃ , 13.7), 373.1790 (C ₂₅ H ₂₅ O ₃ , 32.6), 391.1895 (C ₂₅ H ₂₇ O ₄ , 48.2), 405.2052 (C ₂₆ H ₂₉ O ₄ , 29.3), 423.2156 (C ₂₆ H ₃₁ O ₅ , 34.9), 451.2106 (C ₂₇ H ₃₁ O ₆ , 100.0).
2		[M+H] ⁺ 441.20	147.0804 (C ₁₀ H ₁₁ O, 37.1), 171.1165 (C ₁₃ H ₁₅ , 18.5), 187.1117 (C ₁₃ H ₁₅ O, 14.9), 217.1222 (C ₁₄ H ₁₇ O ₂ , 9.1), 227.1065 (C ₁₅ H ₁₅ O ₂ , 9.2), 245.1170 (C ₁₅ H ₁₇ O ₃ , 6.5), 247.1327 (C ₁₅ H ₁₉ O ₃ , 7.8), 259.1325 (C ₁₆ H ₁₉ O ₃ , 5.5), 277.1431 (C ₁₆ H ₂₁ O ₄ , 3.5), 331.1689 (C ₂₃ H ₂₅ O ₂ , 4.4), 345.1847 (C ₂₄ H ₂₅ O ₂ , 5.6), 363.1953 (C ₂₄ H ₂₇ O ₃ , 4.8), 373.1796 (C ₂₅ H ₂₅ O ₃ , 8.4), 391.1901 (C ₂₅ H ₂₇ O ₄ , 19.1), 405.2058 (C ₂₆ H ₂₉ O ₄ , 7.6), 409.2009 (C ₂₅ H ₂₉ O ₅ , 100.0), 423.2161 (C ₂₆ H ₃₁ O ₅ , 9.6).

2C.2.4 MS/MS Characterization of Salannin (7)

Plausible mechanistic pathway leading to the formation of daughter ions ($[M+H]^+$ precursor ion) of salannin (7) in 20% NCE has been schematically represented in scheme 2C.1 and 2C.2. Analysis of MS/MS fragments of salannin (7) revealed the





presence of low-mass key-fragments at m/z 83.0498, 147.0804, 159.0806, 163.0755, 245.1538 and 273.1483. The daughter ion at m/z 83.0498 (39.7%) was generated through the protonation mediated cleavage of tigloyl ester bond. Highly stabilized allylic daughter carbocation fragment at m/z 147.0804 was appeared with highest intensity (100%) and probably formed through the cleavage of C-ring at C(8)-C(14) and

C(15)-O bonds. This fragment was found to be ubiquitously present in all studied C-seco triterpenoids with very high intensity since it arises from a common skeletal region. Therefore, the specific fragment at m/z 147.0804 can be used as a diagnostic marker for the C-seco triterpenoid possessing characteristic C- and D-ring with 17-furan moiety. Similarly, the ion at m/z 163.0755 (31.7%) was an outcome of C-ring cleavage at C(8)-C(14) and C(7)-O. Protonation initiated removal of the tigloyl group followed by carbocation rearrangement through hydride and methyl shift produced the MS/MS peak at m/z 159.0806, thus bearing a typical marker for salannin skeleton. Fragment at m/z 245.1538, 273.1483 and 291.1587 were probably produced by the cleavage of ring-C from decalin system (consisting of ring-A and B) and simultaneous removal of acetate and tigloyl groups. These three fragments bear the signature for decalin system carrying methylene methyl ester moiety at C-9. Elucidation of fragmentation pathway of salannin (**7**) unveiled the presence of high-intensity (75.6%) key fragment at m/z 419.2214 $[M-\text{AcOH}-\text{tiglic acid}-\text{H}_2\text{O}+\text{H}]^+$, generating due to the combined loss of AcOH, tiglic acid and water from the precursor ion $[M+\text{H}]^+$ at m/z 597.3. Several daughter ions of lower intensity (< 40%) were also obtained as a result of neutral losses in the high mass-range at m/z 387.1958 $[M-\text{AcOH}-\text{tiglic acid}-\text{MeOH}-\text{H}_2\text{O}+\text{H}]^+$, 437.2320 $[M-\text{AcOH}-\text{tiglic acid}+\text{H}]^+$, 479.2427 $[M-\text{tiglic acid}-\text{H}_2\text{O}+\text{H}]^+$, 497.2540 $[M-\text{tiglic acid}+\text{H}]^+$, 565.2803 $[M-\text{MeOH}+\text{H}]^+$, 579.2957 $[M-\text{H}_2\text{O}+\text{H}]^+$. All these secondary ions arising out of neutral losses in various combinations correlate the presence of tigloyl, acetate, methyl ester and cyclic ether moieties on the structure of salannin (**7**). They can be considered as the diagnostic fragments for the identification of salannin among the mixture of numerous C-seco triterpenoids of similar skeleton.

2C.2.5 MS/MS Analyses of **5** (3-deacetylsalannin), **8** (salannol acetate) and **9**

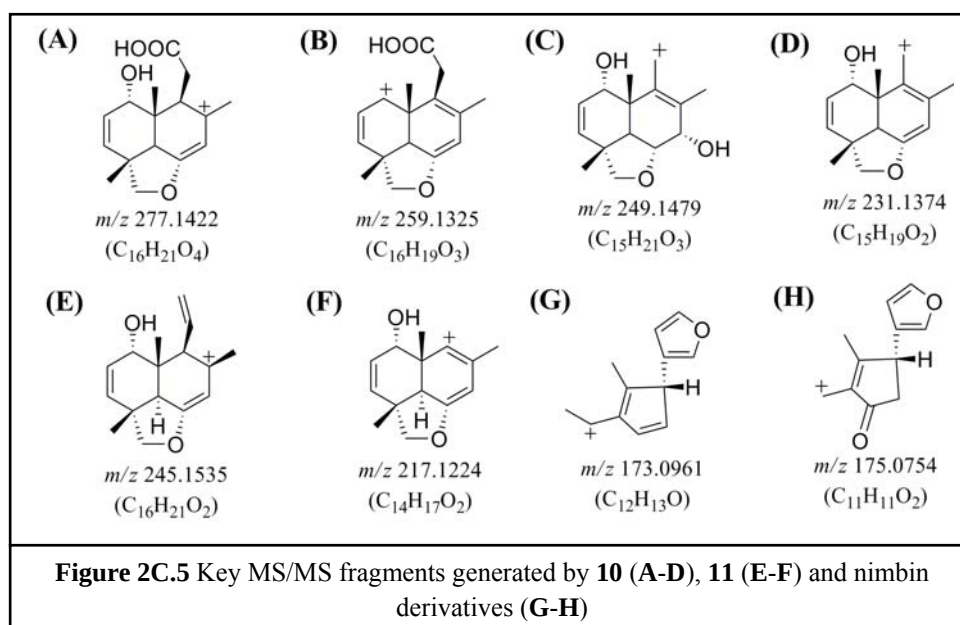
Three functionally modified derivatives **5** (3-deacetylsalannin), **8** (salannol acetate) and **9** possess variable substitutions at C-1 and C-3 though bearing 9-methylene methyl ester moiety in common. All of them produced same low-mass fragments at m/z 147 and 163, thus indicating the applicability of these two fragments to characterize C-seco triterpenoid skeleton carrying salannin-type C- and D-ring in unknown samples. Similarly, daughter ions at m/z 159, 245, 273 and 291 were observed as they are produced from the common skeletal region of salannin and these three derivatives. In MS/MS spectra of all three derivatives peak at m/z 147 was obtained with highest

relative abundance (100%). Similar to salannin (**7**) they also produced the higher mass fragments corresponding to the loss of several oxygenated functional groups. MS/MS spectrum of 3-deacetylsalannin (**5**) showed the presence of high-mass fragments at m/z 387.1954 [M-tiglic acid-MeOH-2H₂O+H]⁺, 405.2055 [M-tiglic acid-MeOH-H₂O+H]⁺, 419.2216 [M-tiglic acid-2H₂O+H]⁺, 437.2316 [M-tiglic acid-H₂O+H]⁺, 455.2423 [M-tiglic acid+H]⁺, 523.2684 [M-MeOH+H]⁺ and 537.2844 [M-H₂O+H]⁺. Salannol acetate (**8**) gave rise to MS/MS peaks at m/z 359.2005 [M-isovaleric acid-AcOH-MeOH-H₂O-CO+H]⁺, 387.1947 [M-isovaleric acid-AcOH-MeOH-H₂O+H]⁺, 401.2104 [M-isovaleric acid-AcOH-2H₂O+H]⁺, 419.2211 [M-isovaleric acid-AcOH-H₂O+H]⁺, 437.2323 [M-isovaleric acid-AcOH+H]⁺, 479.2430 [M-isovaleric acid-H₂O+H]⁺, 497.2523 [M-isovaleric acid+H]⁺, 521.2899 [M-AcOH-H₂O+H]⁺, 567.2958 [M-MeOH+H]⁺ and 581.3101 [M-H₂O+H]⁺. Daughter ions in high-mass range for compound **9** were observed at m/z 405.2043 [M-MeOH-2H₂O+H]⁺, 411.2159 [M-MeOH-HCHO+H]⁺, 419.2201 [M-3H₂O+H]⁺, 437.2316 [M-2H₂O+H]⁺, 441.2257 [M-MeOH+H]⁺ and 455.2414 [M-H₂O+H]⁺. In all three derivatives (**5**, **8** and **9**) common ions at m/z 419 and 437 were obtained with significant intensity.

2C.2.6 MS/MS Characterization of **10**, **11** and **12**

Another three semi-synthesized derivatives of salannin (**10**, **11** and **12**) structurally differ with respect to the presence of functional groups at C-1, C-3 and C-11. The expected characteristic skeletal fragments in low-mass range at m/z 147, 159 and 163 were observed in MS/MS spectra of all three molecules. Among them peak at m/z 147 was obtained with 45-65% relative abundance. Salannic acid (**10**), ester-hydrolyzed (at C-1, 3 and 11) derivative of salannin showed the daughter ions at m/z 259.1325 and 277.1422 which were mechanistically same fragments in comparison to m/z 273 and 291 respectively, observed with salannin derivatives bearing 9-methylene methyl ester moiety (Figure 2C.5A, 2C.5B). Another two fragments at m/z 231.1374 and 249.1479 showed by **10** was an outcome of further removal of carboxylic acid group as water and CO (Figure 2C.5C, 2C.5D). Ester-reduced (at C-1, 3 and 11) derivative of salannin (**11**) generated key skeletal fragment at m/z 245.1535 corresponding to the elimination of water from C-9 side-chain along with cleavage of C-ring, tigloyl and acetate moieties (Figure 2C.5E). The ion at m/z 217.1224 arose in similar manner though underwent complete removal of C-9 substitution as ethylene and water molecule (Figure 2C.5F).

Presence of MS/MS peak at the same position (m/z 217) in doubly deuterium-substituted (at C-12) derivative of ester-reduced salannin (**12**) further supported the removal of C-9 substituent. Higher-mass fragments of these derivatives were the outcome of neutral losses of multiple water molecules along with CO, HCHO and CH₄ in variable combinations. Salannic acid (**10**) showed the high-mass MS/MS peaks at m/z 377.2100 [M-CO-3H₂O+H]⁺, 387.1946 [M-4H₂O+H]⁺, 393.2048 [M-HCHO-2H₂O+H]⁺, 397.1994 [M-CH₄-CO-H₂O+H]⁺, 405.2051 [M-3H₂O+H]⁺, 423.2158 [M-2H₂O+H]⁺ and 441.2258 [M-H₂O+H]⁺. High-mass fragments at m/z 337.2160 [M-CH₄-2CO-2H₂O+H]⁺, 347.2000 [M-CH₄-CO-3H₂O+H]⁺, 363.2318 [M-CO-3H₂O+H]⁺, 373.2160 [M-4H₂O+H]⁺, 391.2263 [M-3H₂O+H]⁺, 409.2372 [M-2H₂O+H]⁺ and 427.2479 [M-H₂O+H]⁺ were generated by **11**. Deuterium labeled (at C-12) ester-reduced analogue of salannin (**12**) resulted in genetically identical high-mass fragments compared to non-substituted with a hike of 2 Da in exact mass as analyzed by fragmentation pathway. This observation indicates that higher mass MS/MS ions carry intact ethylene moiety at C-9.

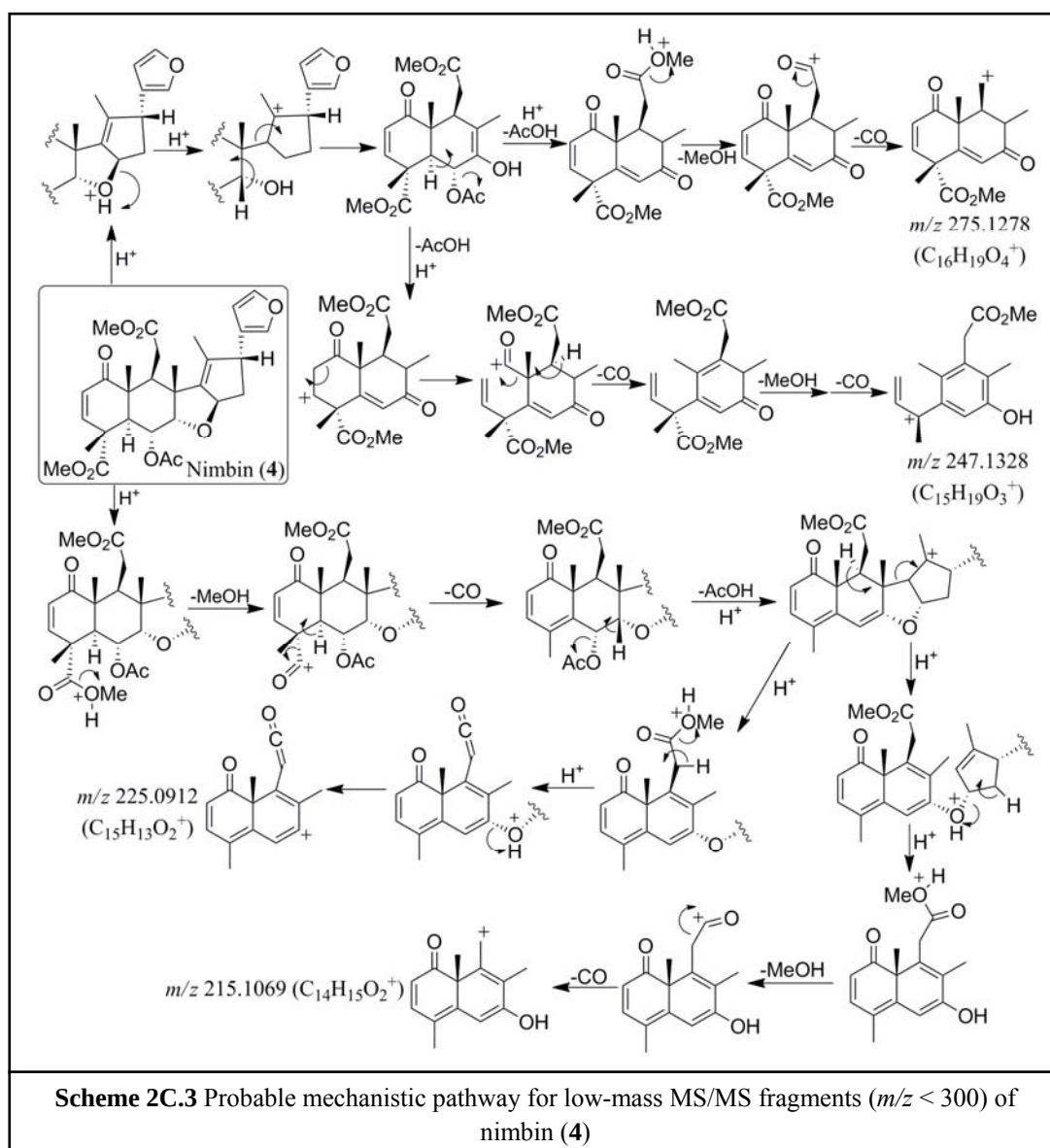


2C.2.7 MS/MS Characterization of **4** (nimbin), **1** (6-deacetylnimbin), **3** (nimbanal) and **13**

Nimbin (**4**) and its analogues are functionally modified derivatives of salannin on A- and B-ring. The cyclic ether ring joining C-4 and C-6 in salannin skeleton is replaced by acyclic oxygenated functional groups such as acetate, methyl ester, aldehyde and hydroxyl in nimbin skeleton. Diagnostic α,β -unsaturated carbonyl moiety at A-ring is an

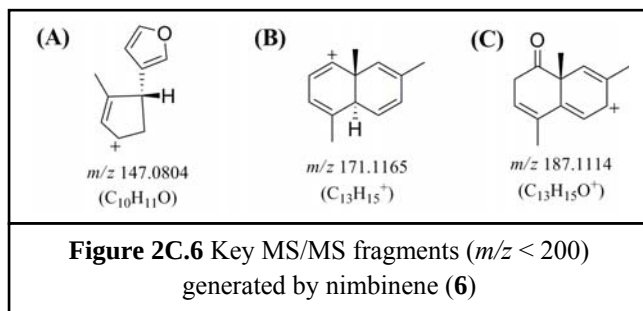
essential characteristic of their framework. Nimbin (**4**) and three of its isolated/semi-synthesized derivatives (**1**, **3** and **13**) were investigated and characterized structurally on the basis of MS/MS fragmentations obtained through collisional activation of $[M+H]^+$ parent ions. Skeletal fragments characteristic to ring C and D were observed at m/z 147, 173 and 175 for nimbin and its analogues (**1**, **3**, **4** and **13**) (Figure 2C.5G, 2C.5H). Nimbin (**4**) and 6-deacetylnimbin (**1**) generated the diagnostic A/B-ring fragments at m/z 215, 225, 247 and 275. All these fragments were generated by the cleavage of C-ring and simultaneous removal of oxygenated functional groups from decalin ring system. Probable structures of the daughter ions and pathway of their formation have been represented in Scheme 2C.3. The peak at m/z 275 showed by **4** (nimbin), **1** (6-deacetylnimbin) and **13** was produced through the removal of ring-C (at C8-C14 and C15-O) and C-11 substituent (methyl ester/carboxylic acid). The fragment at m/z 245 showed by nimbanal (**3**) was a daughter ion eventually produced by same mechanistic pathway. Nimbin (**4**) and 6-deacetylnimbin (**1**) showed a MS/MS peak at m/z 247 which was plausibly a highly stabilized benzylic carbocation formed through the removal of C-ring (at C8-C14 and C15-O), C-4 methyl ester and A-ring carbonyl. Ion produced by **13** at m/z 233 was originally the same ion by genesis in comparison to fragment at m/z 247. Fragments at m/z 215 and 225 were found to be present in MS/MS spectra of all nimbin analogues studied (**1**, **3**, **4** and **13**), indicating their generation from a common skeletal region. Probable fragmentation pathway revealed their structure bearing decalin framework with highly conjugated enone system. The MS/MS ions in low-mass range for nimbin and its derivatives were obtained with low to moderate intensity ranging from 5%-35% except for m/z 147 with high intensity. Higher mass fragments were also produced by nimbin derivatives with loss of neutral molecules and their pathway of genesis was predicted. Nimbin (**4**) showed high-mass MS/MS peaks at m/z 509.2166 $[M-MeOH+H]^+$, 481.2225 $[M-AcOH+H]^+$, 463.2119 $[M-AcOH-H_2O+H]^+$, 449.1952 $[M-MeOH-AcOH+H]^+$, 431.1857 $[M-MeOH-AcOH-H_2O+H]^+$, 421.2003 $[M-MeOH-AcOH-CO+H]^+$, 389.1750 $[M-2MeOH-AcOH-CO+H]^+$ corresponding to the removal of methyl esters, acetate and cyclic ether moieties. Similarly, 6-deacetylnimbin (**1**) produced high mass fragments at m/z 467.2066 $[M-MeOH+H]^+$, 449.1952 $[M-MeOH-H_2O+H]^+$, 435.1805 $[M-2MeOH+H]^+$, 431.1857 $[M-MeOH-2H_2O+H]^+$, 421.2004 $[M-MeOH-CO-H_2O+H]^+$, 389.1749 $[M-2MeOH-CO-H_2O+H]^+$, 371.1639 $[M-2MeOH-CO-2H_2O+H]^+$. C-4 aldehyde analogue of nimbin (**3**, nimbanal) was also found to produce

high-mass MS/MS fragments at m/z 479.2070 $[M-\text{MeOH}+\text{H}]^+$, 451.2118 $[M-\text{MeOH}-\text{CO}+\text{H}]^+$, 433.2003 $[M-\text{MeOH}-\text{H}_2\text{O}-\text{CO}+\text{H}]^+$, 419.1854 $[M-2\text{MeOH}-\text{CO}+\text{H}]^+$, 401.1741 $[M-2\text{MeOH}-\text{H}_2\text{O}-\text{CO}+\text{H}]^+$, 391.1897 $[M-2\text{MeOH}-2\text{CO}+\text{H}]^+$, 373.1797 $[M-2\text{MeOH}-\text{H}_2\text{O}-2\text{CO}+\text{H}]^+$. Ester hydrolyzed (at C-6 and C-11) derivative of nimbin (**13**) also produced diagnostic high-mass fragments at m/z 467.2068 $[M-\text{H}_2\text{O}+\text{H}]^+$, 453.1911 $[M-\text{MeOH}+\text{H}]^+$, 449.1951 $[M-2\text{H}_2\text{O}+\text{H}]^+$, 435.1805 $[M-\text{MeOH}-\text{H}_2\text{O}+\text{H}]^+$, 431.1857 $[M-3\text{H}_2\text{O}+\text{H}]^+$, 407.1849 $[M-\text{MeOH}-\text{H}_2\text{O}-\text{CO}+\text{H}]^+$, 389.1748 $[M-\text{MeOH}-2\text{H}_2\text{O}-\text{CO}+\text{H}]^+$, 371.1637 $[M-\text{MeOH}-3\text{H}_2\text{O}-\text{CO}+\text{H}]^+$, 361.1802 $[M-\text{MeOH}-2\text{H}_2\text{O}-2\text{CO}+\text{H}]^+$. The ions arising due to the removal of methanol (in case of **4**, **1** and **3**) or water (in case of **13**) from C-12 substituent were obtained with very high intensity ($> 99\%$) for nimbin and its derivatives.



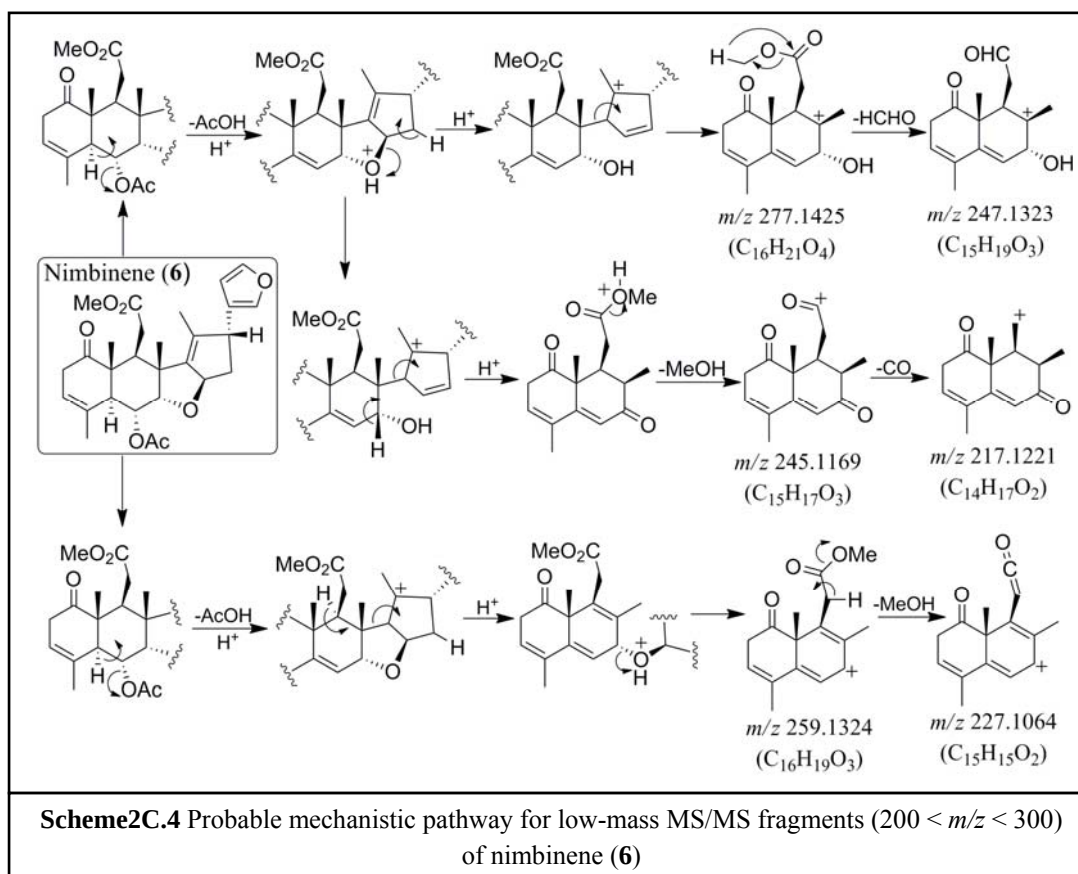
2C.2.8 MS/MS Characterization of 6 (nimbinene) and 2 (6-deacetylnimbinene)

Nimbinene (**6**) and 6-deacetylnimbinene (**2**), two isolated pentanortriterpenoids from Neem oil are A-ring modified analogues of nimbin. Unsaturation at C2-C3 in nimbin (**4**) is shifted to C3-C4 in nimbinene (**6**) skeleton with the removal of 4 α oxygenated functional group. For both the molecules (**6** and **2**) key low-mass fragment was observed at m/z 147 with very high intensity corresponding to the presence of typical C-



and D-ring substituted with 17-furan moiety. Another two fragments at m/z 171 and 187 were also observed with high abundance. Fragment at m/z 171 was probably the decalin ring fragment devoid of

oxygenated functional groups and bearing conjugated polyene system (Figure 2C.6B). Fragment at m/z 187 was probably generated through the cleavage of C-ring, C-9 and C-6 substituent from decalin ring system with an intact A-ring (Figure 2C.6C). The low-mass fragments in the range m/z 200-300 probably consisted of decalin system with intact A-ring and produced by the cleavage of C-ring with further removal of B-ring functional groups as depicted in Scheme 2C.4. These MS/MS fragments were observed at m/z 277, 259, 247, 245, 227 and 217 obtained with low to moderate intensity ranging from 5-45% for both the triterpenoids. Nimbinene (**6**) showed highest intensity (100%) fragment at m/z 451.2106 $[M-MeOH+H]^+$ generated by the removal of methanol from 11-methyl ester group. Other high-mass fragments included 423.2156 $[M-AcOH+H]^+$, 405.2052 $[M-AcOH-H_2O+H]^+$, 391.1895 $[M-MeOH-AcOH+H]^+$, 373.1790 $[M-MeOH-AcOH-H_2O+H]^+$, 363.1945 $[M-MeOH-AcOH-CO+H]^+$, 345.1841 $[M-MeOH-AcOH-CO-H_2O+H]^+$, 331.1685 $[M-MeOH-AcOH-C_2H_2O-H_2O+H]^+$, 327.1743 $[M-MeOH-AcOH-CO-2H_2O+H]^+$, 313.1589 $[M-MeOH-AcOH-C_2H_2O-2H_2O+H]^+$. These fragments were observed with low to moderate intensity (9-48%) with 20% NCE. 6-Deacetylnimbinene (**2**) also showed highest abundant (100%) high-mass MS/MS ion at m/z 409.2009 $[M-MeOH+H]^+$ due to the loss of methanol from 11-methyl ester. Other high-mass fragments generated by the same molecule was obtained at m/z 423.2161 $[M-H_2O+H]^+$, 405.2058 $[M-2H_2O+H]^+$, 391.1901 $[M-MeOH-H_2O+H]^+$, 373.1796 $[M-MeOH-2H_2O+H]^+$, 363.1953 $[M-MeOH-H_2O-CO+H]^+$, 345.1847 $[M-MeOH-2H_2O-CO+H]^+$, 331.1689 $[M-MeOH-C_2H_2O-2H_2O+H]^+$ with low intensity (< 20%).



As demonstrated, the MS/MS ions lower than m/z 300 were observed as fragmented skeleton along with loss of one or more functional groups. Therefore, these ions are highly important to characterize the skeleton of C-seco triterpenoids in unknown, complex phytochemical extracts and to identify the presence of individual characteristic rings. On the other hand, the significance of high-mass fragments lies in the characterization of flanked functionalities around the skeleton. In essence, ions in both the ranges of MS/MS spectrum provide important and complementary information to structurally elucidate these complex triterpenoids.

2C.2.9 MS/MS Based Identification of C-seco Triterpenoids in Neem Stem and Bark Extract

To showcase the applicability of developed MS/MS based identification technique, signature spectra were utilized for the identification of studied triterpenoids in the extract of stem and bark tissues from Neem. Comparing the retention time and ESI(+)-MS spectra of pure triterpenoids, presence of individual molecules were investigated in UPLC-ESI(+)-MS TIC (total ion chromatogram) of the extracts. Further, their

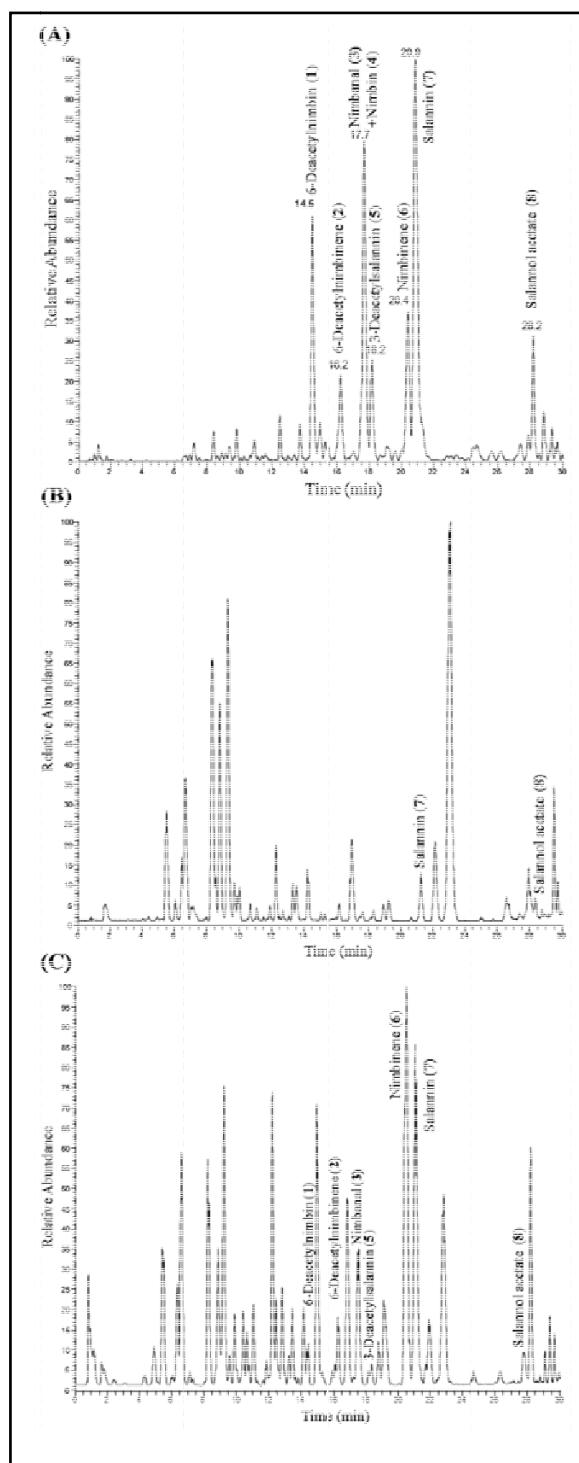


Figure 2C.7 UPLC-ESI(+)-MS TIC of (A) Neem oil extract, (B) Neem bark extract and (C) Neem stem extract. Peak identification and labeling was performed by comparing the retention time, ESI(+)-MS spectrum and further by ESI(+)-MS/MS spectrum of the purified triterpenoids

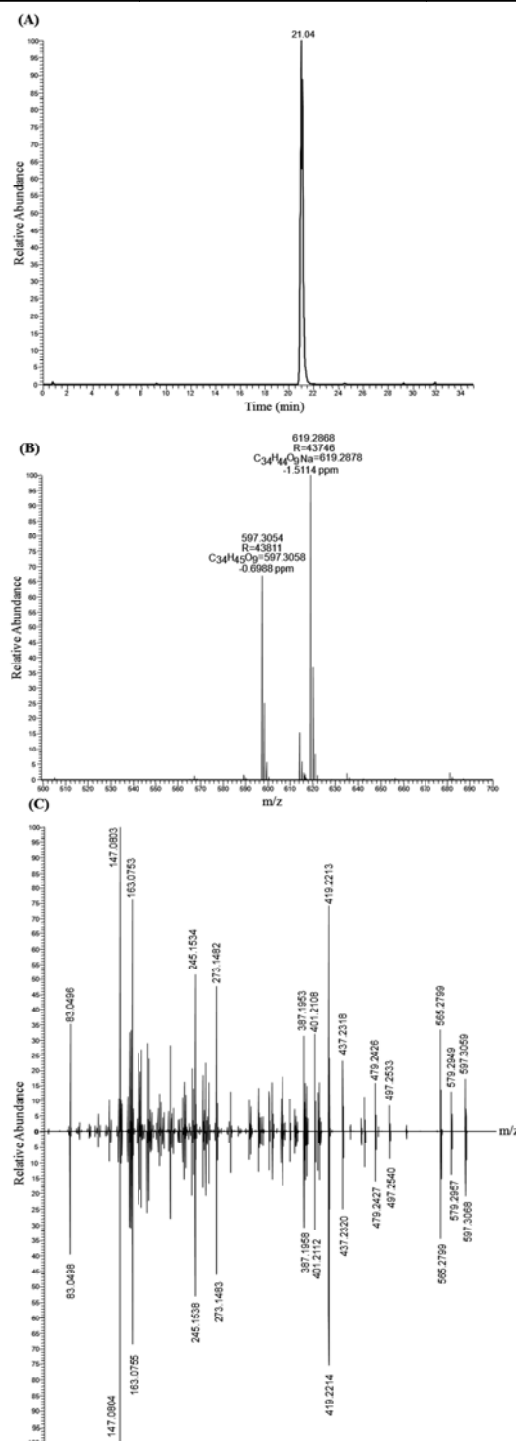


Figure 2C.8 (A) Extracted ion (m/z 597.2; $[M+H]^+$ ion of salannin) chromatogram from TIC of stem extract, (B) ESI(+)-MS spectrum of the extracted peak, (C) Comparative ESI(+)-MS/MS spectra of the identified peak (R_t 21.04 min) as salannin and purified salannin (inverted)

abundance was confirmed by comparing data dependent MS/MS spectra of the identified peaks (in the chromatograms of extracts) with purified triterpenoids. For

example, the peak at R_t 21.0 min in TIC of stem extract (Figure 2C.7C) showed protonated molecular ion peak ($[M+H]^+$) at m/z 597.3054 ($C_{34}H_{45}O_9$) (Figure 2C.8B) corresponding to the retention time and ($[M+H]^+$) ion of purified salannin (7). The data dependent MS/MS spectrum of $[M+H]^+$ (m/z 597.3) ion for the identified peak within retention time range 20.5-22.1 min (Figure 2C.8A) revealed the presence of key fragments identical to purified salannin (7) (Figure 2C.8C). Therefore, the peak at R_t 21.0 in TIC of stem extract was confirmed to be salannin (7). Other C-seco triterpenoids in Neem stem extract included salannol acetate (8, R_t 28.2 min), nimbinene (6, R_t 20.4 min), nimbanal (3, R_t 17.7 min), 3-deacetylsalannin (5, R_t 18.2 min) and 6-deacetylnimbinene (2, R_t 16.2 min) (Figure 2C.7C). Salannin and salannol acetate were observed to be only C-seco triterpenoids present in bark extract among all the molecules studied (Figure 2C.7B). The retention time, ESI(+)-MS spectrum and ESI(+)-MS/MS fragmentations of the identified triterpenoids were in well agreement with that of purified molecules, thus confirming their existence in the extract of Neem stem and bark.

2C.3 Conclusion

In conclusion, we have developed an MPLC based preparative isolation protocol for major C-seco triterpenoids from Neem oil. The developed procedure was efficient to furnish highly pure triterpenoids with reproducibility and rapidity. Further, the isolated C-seco triterpenoids and five semi-synthesized analogues were characterized by ESI(+)-quadrupole/orbitrap-MS/MS data obtained with high accuracy. Structure and fragmentation pathway of daughter ions were predicted. Low-mass key fragments characteristic to C-seco skeleton of salannin type and also high-mass key fragments specific to the skeletal functional groups were identified. The tandem mass spectrometric information was further applied for the identification of studied triterpenoids within the extracts of Neem stem and bark. Expedient preparative isolation technique of C-seco triterpenoids from Neem oil might be useful for large-scale availability and in depth investigation of bioactivities. Rationally depicted MS/MS characterization data of this class of triterpenoids might be highly beneficial for 'in-mixture' rapid, sensitive and accurate identification of individual molecules.

2C.4 Experimental Section

2C.4.1 Materials and Solvents

Neem seeds were collected from Aurangabad region of Maharashtra, India. For the extraction purpose technical grade solvents were purchased from Spectrochem, India and distilled prior to use. For MPLC and HPLC purpose HPLC grade solvents were purchased from Sigma. For LC-ESI(+)-MS experiments, LC-MS grade solvents were procured from JT Baker. The derivatives of triterpenoids were synthesized as reported earlier.²⁸

2C.4.2 Instrumentation

MPLC system from Teledyne, Isco (Combiflash Rf 200) with integrated PDA detector was used for the preparative purification. HPLC was performed on a Waters HPLC system (Delta 600 pump and controller) coupled with Waters 2489 UV/Visible detector. LC-ESI(+)-MS/MS runs were performed on Q Exactive Orbitrap attached with Accela 1250 pump (Thermo Scientific). NMR data (¹H, ¹³C, DEPT-135) were recorded on Bruker spectrophotometer (400 MHz for ¹H and 100 MHz for ¹³C) in CDCl₃ and the residual solvent or TMS signal was designated as the reference.

2C.4.3 Extraction of Neem Oil

One kilogram ground Neem seed kernel was extracted with petroleum ether (5 L × 4) with continuous stirring for 3 h in every turn. Combined petroleum ether layer was concentrated under reduced pressure at 50 °C to furnish 600 mL of oil. Neem oil was further extracted with methanol (1.8 L × 4) and combined methanol layer upon concentration under similar conditions yielded crude brown oily mixture of triterpenoids. Oily crude was loaded on silica gel bed (15 × 7 cm) and eluted with 25% ethyl acetate in petroleum ether to remove the residual oil. Subsequent elution of the column with 07% methanol in dichloromethane produced 10.1 g brown crude mixture of triterpenoids.

2C.4.4 Preparative Isolation by MPLC

Two gram of crude triterpenoid extract (adsorbed on normal phase silica gel, w/w 1:3) was fractionated over 40 g normal phase silica gel (particle size 35-70 μm) RediSep pre-

packed cartridge with 25 mL/min flow rate and monitoring at 240 nm by in-built UV detector. A gradient solvent program (105 min) of increasing percentage of ethyl acetate in hexane (0-10 min 20% EtOAc, 15 min 25% EtOAc, 10 min hold, 30 min 30% EtOAc, 10 min hold, 45 min 35% EtOAc, 10 min hold, 60 min 40% EtOAc, 10 min hold, 75 min 45% EtOAc, 10 min hold, 90 min 70% EtOAc, 15 min hold) was used for the fractionation process. Integrated fraction collector was used for the collection of elutes (25 mL each). Total 105 fractions after monitoring the triterpenoid content using TLC were pooled into six unique fractions. Six fractions (adsorbed on celite, *w/w* 1:3) were further purified individually over 43 g C18 reverse phase silica gel (particle size 40-63 μ m) RediSep pre-packed cartridge with 30 mL/min flow rate under similar condition. A common gradient solvent program (80 min) of increasing percentage of methanol in water (0-10 min 50% MeOH, 15 min 55% MeOH, 15 min hold, 35 min 60% MeOH, 25 min hold, 65 min 70% MeOH, 15 min hold) was applied for the purification of all six fractions. Fractions were collected in similar conditions and the pure fractions (as analyzed by TLC and HPLC) were pooled together to furnish eight pure triterpenoids.

2C.4.5 HPLC and TLC Conditions

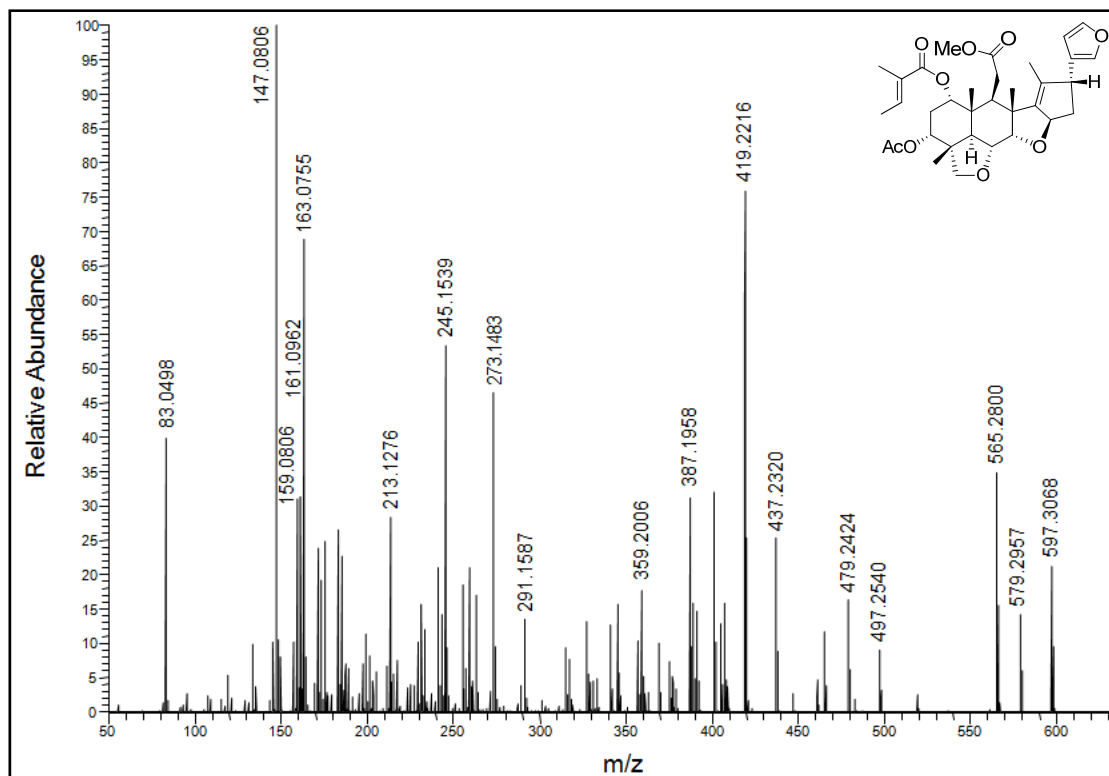
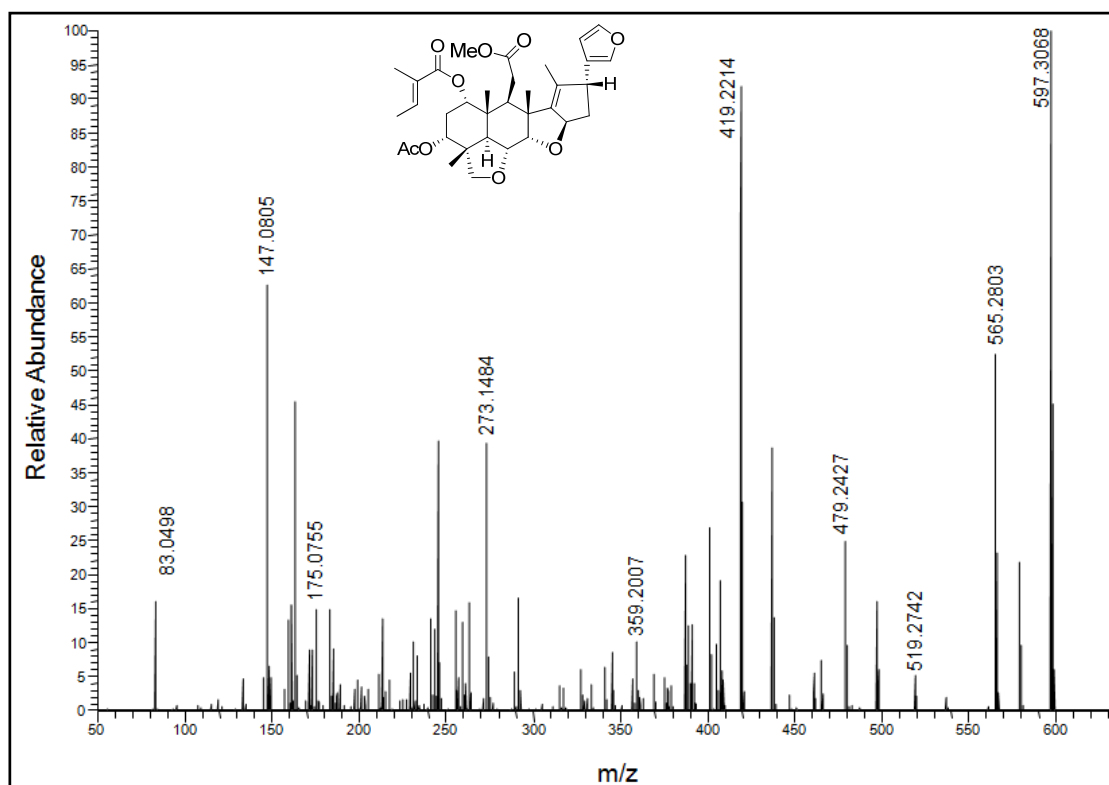
HPLC runs were performed on analytical XBridge C₁₈ column (4.6 $\times$ 250 mm, 5 μ m) and monitored by UV detection at 215 nm. Gradient solvent program of 60 min (0 min, 50% methanol/water; 10 min, 60% methanol/water; 40 min, 60% methanol/water; 45 min, 80% methanol/water; 55 min, 80% methanol/water; 58 min, 50% methanol/water; 60 min, 50% methanol/water) with a flow rate of 1 mL/min was used for the resolution of the components. Samples were dissolved in known volume of HPLC grade methanol and injected (15 μ L) manually after filtration. Thin layer chromatogram (TLC) was developed twice on silica gel G pre-coated plates (Merck, 0.25 mm) with 40% ethyl acetate in hexane as the mobile phase. Spots were visualized by dipping in a solution of 3.0% anisaldehyde, 2.8% H₂SO₄, 2% acetic acid in ethanol and subsequent heating.

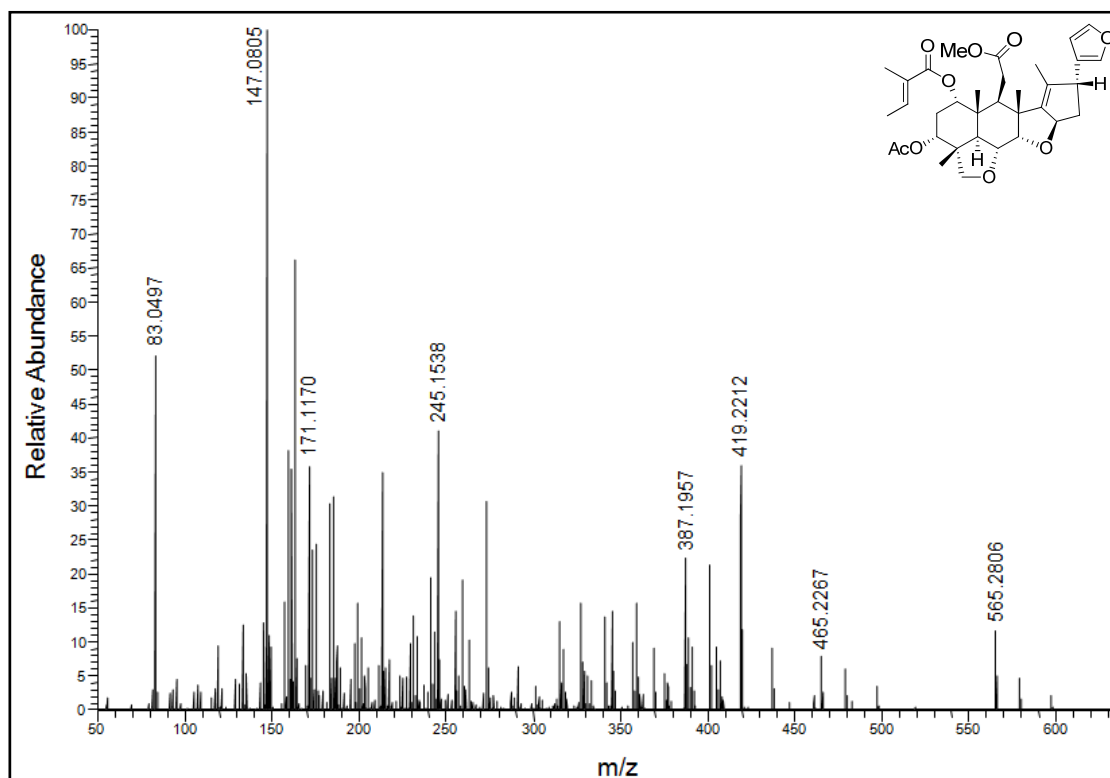
2C.4.6 UPLC-ESI(+)-MS and MS/MS Conditions

Samples were dissolved in methanol (concentration 0.1 mg/mL), filtered and 5 μ L of it was injected. Mixture of triterpenoids was resolved through Waters Acquity BEH C18 UPLC column (2.1 $\times$ 100 mm, particle size 1.7 μ M) using methanol-water gradient

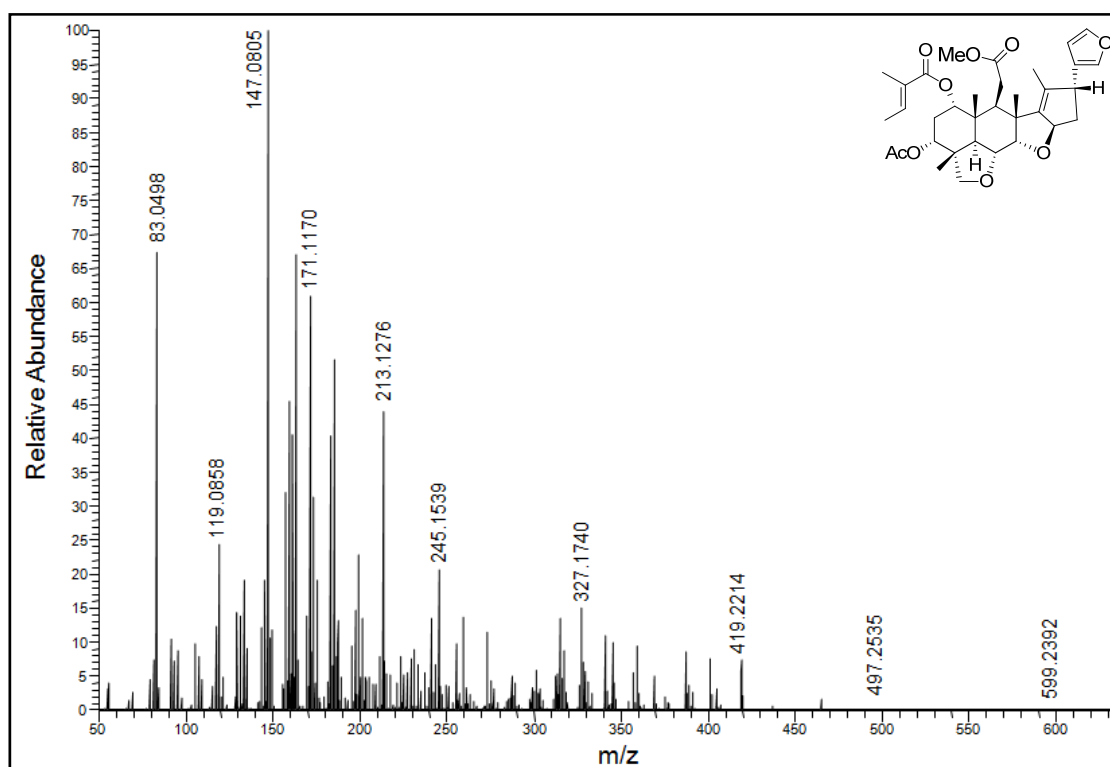
solvent program of 35 min (0.0 min, 40% methanol/water; 5.0 min, 50.0% methanol/water; 10.0 min, 60% methanol/water; 25.0 min, 65% methanol/water; 30.0 min, 90% methanol/water; 32.0 min, 90% methanol/water; 34.0 min, 40% methanol/water; 35.0 min, 40% methanol/water) with a flow rate of 0.3 mL/min. 0.1% LC-MS grade formic acid was added to water. MS and MS/MS runs were carried out using the tune method as follows: sheath gas (nitrogen) flow rate 45 units, auxiliary gas (nitrogen) flow rate 10 units, sweep gas (nitrogen) flow rate 2 units, spray voltage 3.60 [KV], spray current 3.70 μ A, capillary temperature 320 °C, s-lens RF level 50, heater temperature 350 °C. ESI-MS and MS/MS data were recorded within the mass range m/z 100 to 1000 in positive ion mode. ESI(+)-MS/MS experiments were performed on data dependent inclusion mode (t-ms²) with an external input of desired range of retention time and precursor ion $[(M+H)^+]$ to be processed for tandem fragmentation. Data were analyzed through Thermo Xcalibur software.

2C.5 Spectral Copies

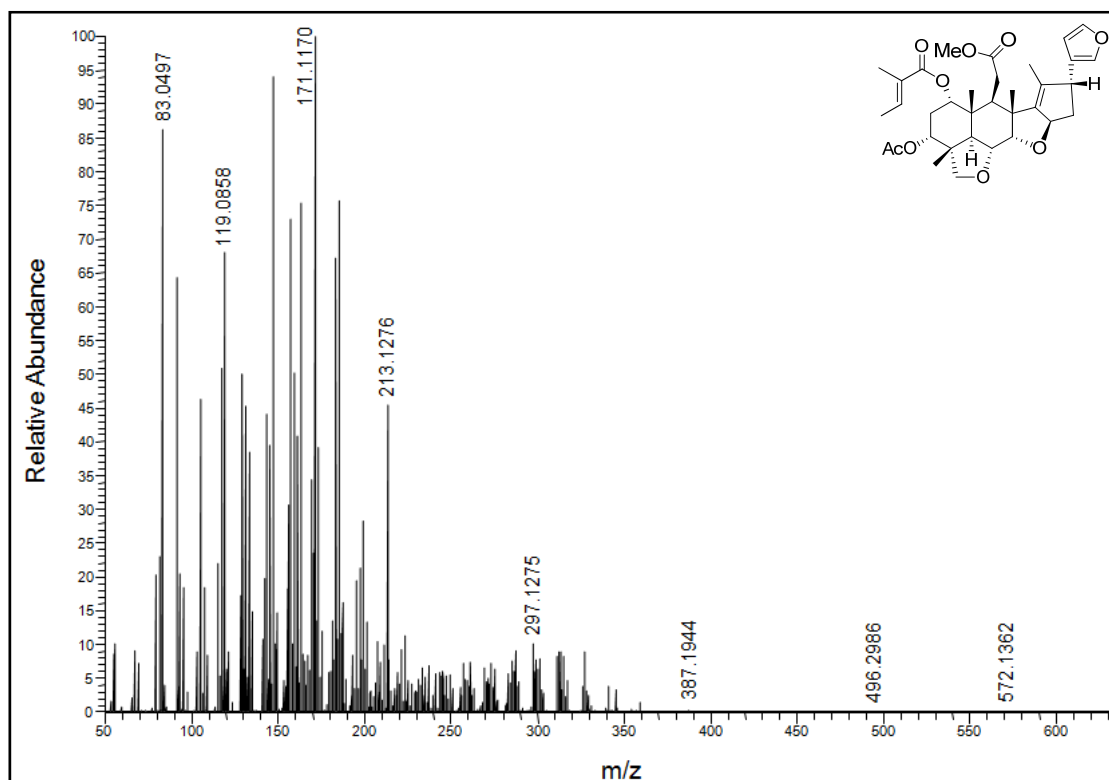
ESI(+)-MS/MS spectrum of 7 (Salannin) in NCE 20% (precursor ion $[M+H]^+$, m/z 597.3)ESI(+)-MS/MS spectrum of 7 (Salannin) in NCE 15% (precursor ion $[M+H]^+$, m/z 597.3)



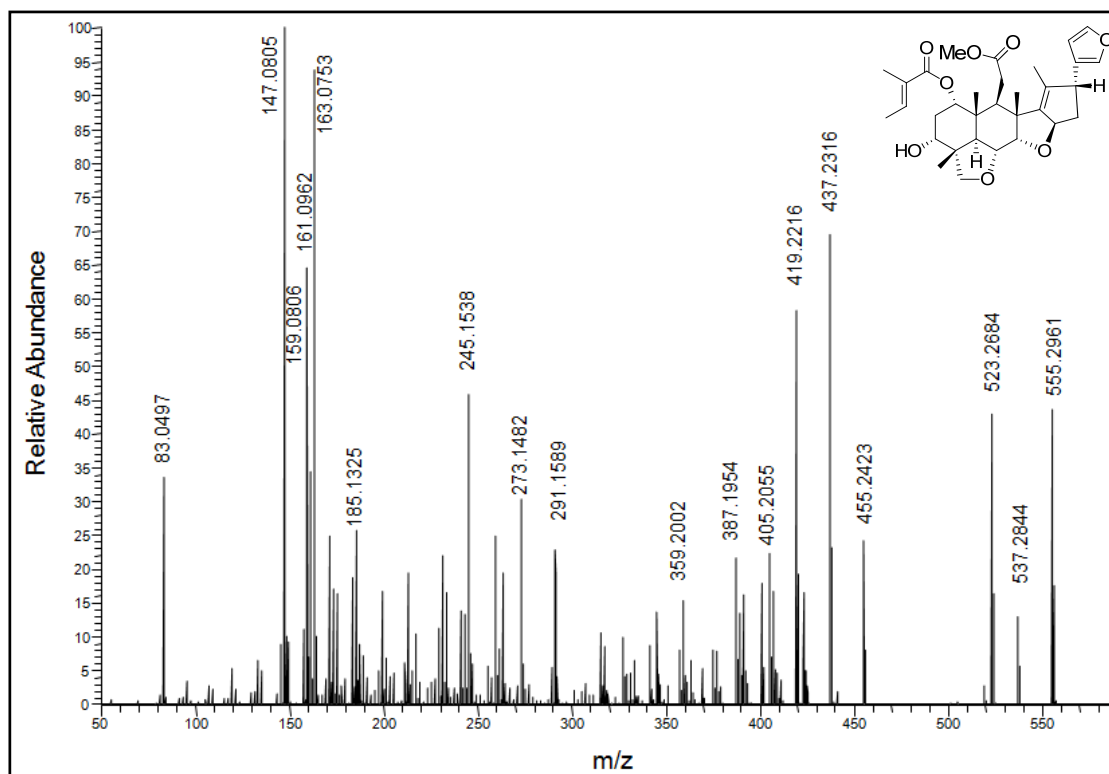
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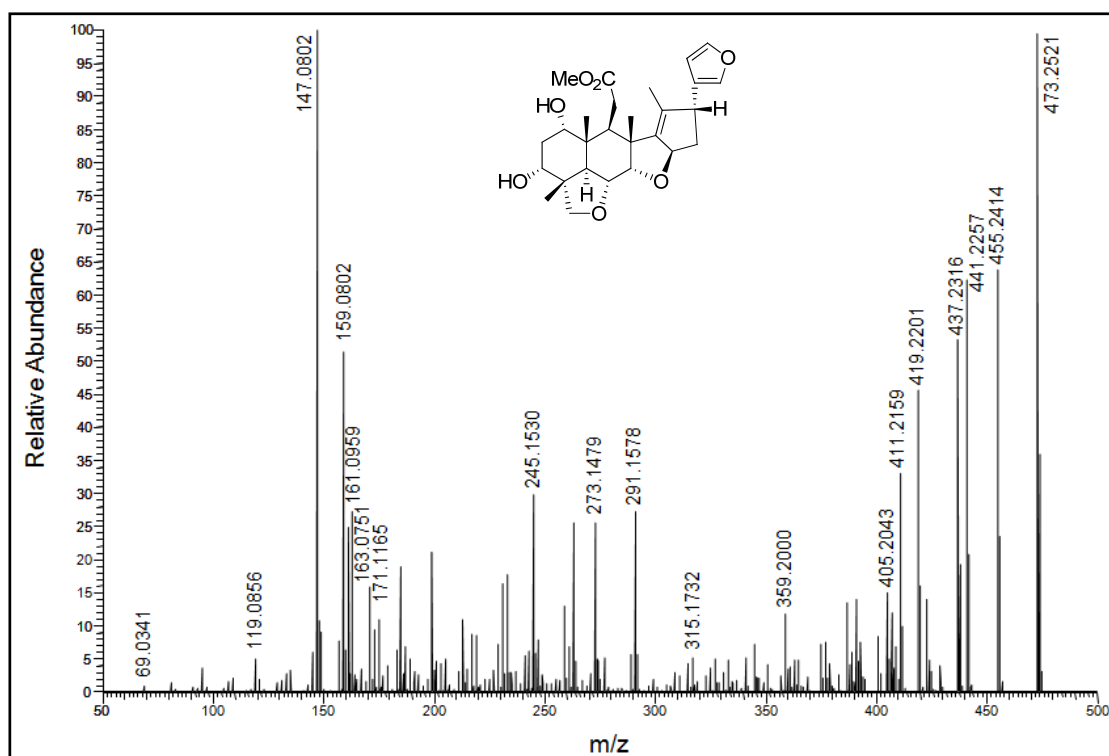
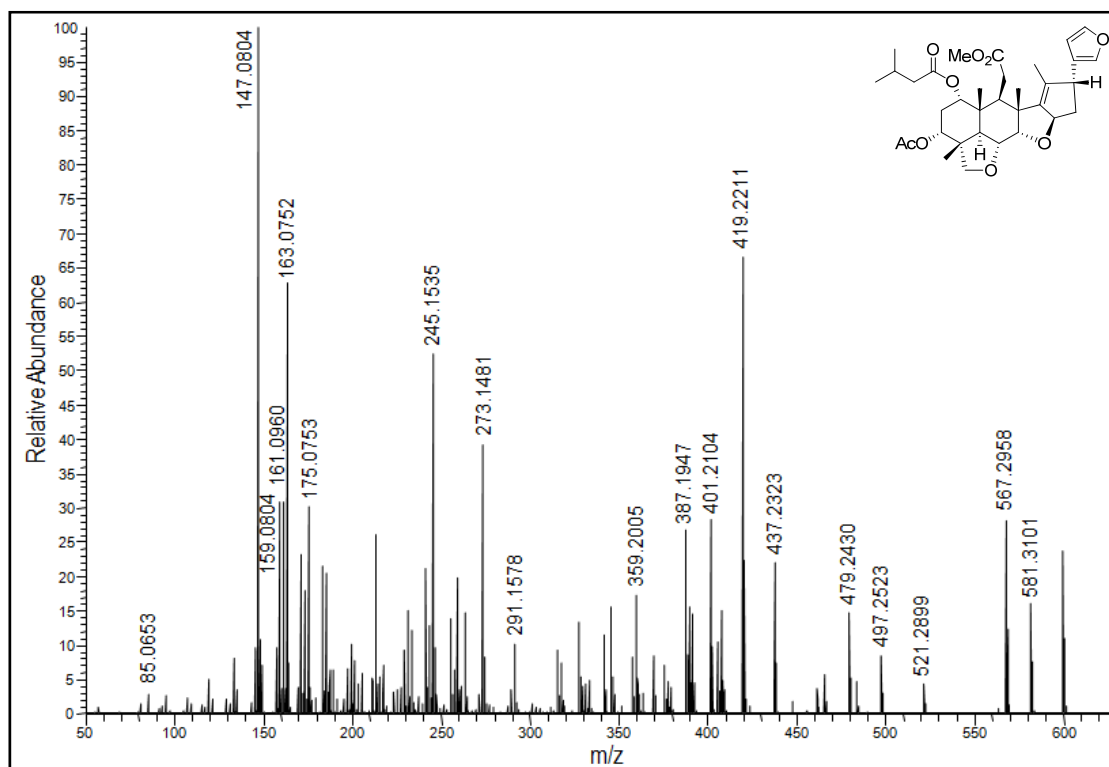
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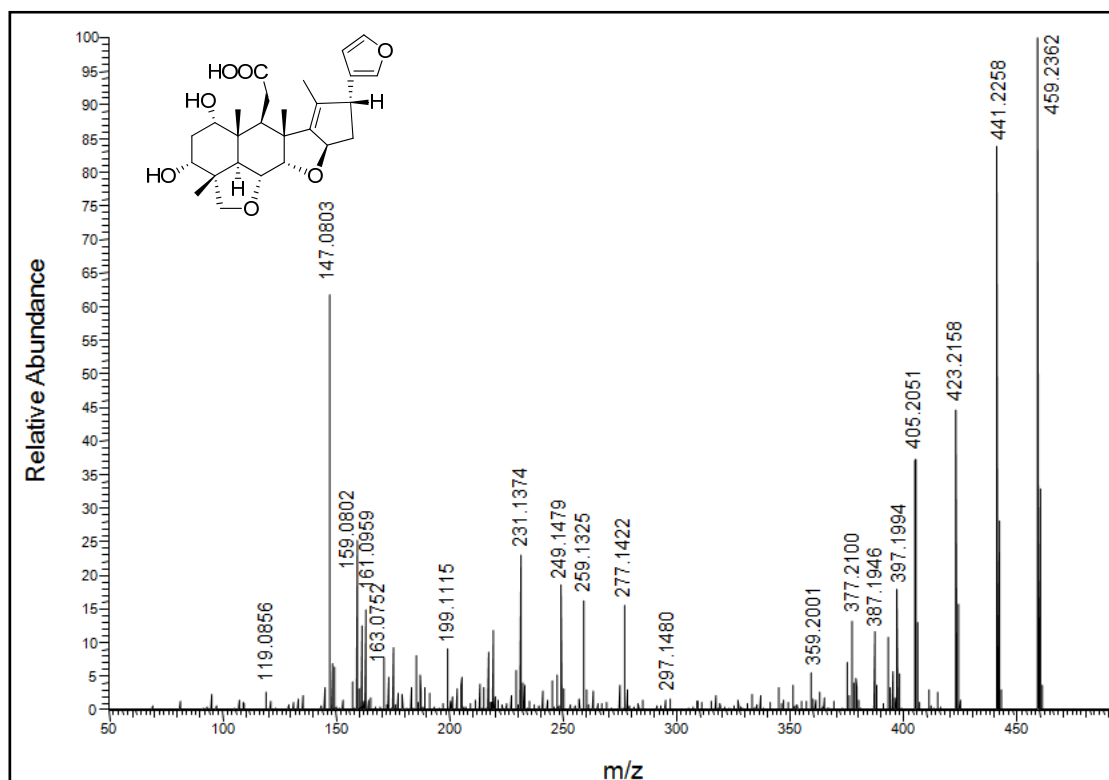


ESI(+)-MS/MS spectrum of 7 (Salannin) in NCE 50% (precursor ion $[M+H]^+$, m/z 597.3)

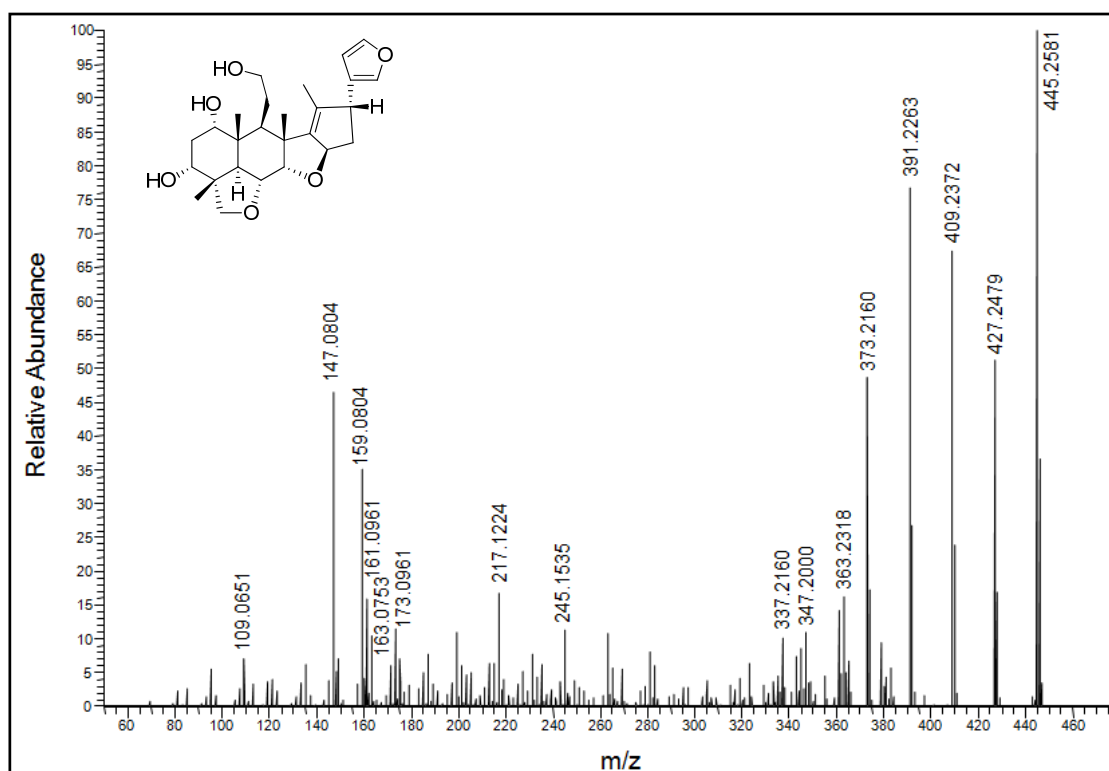


ESI(+)-MS/MS spectrum of 5 (3-Deacetylsalannin) in NCE 20% (precursor ion $[M+H]^+$, m/z 555.2)

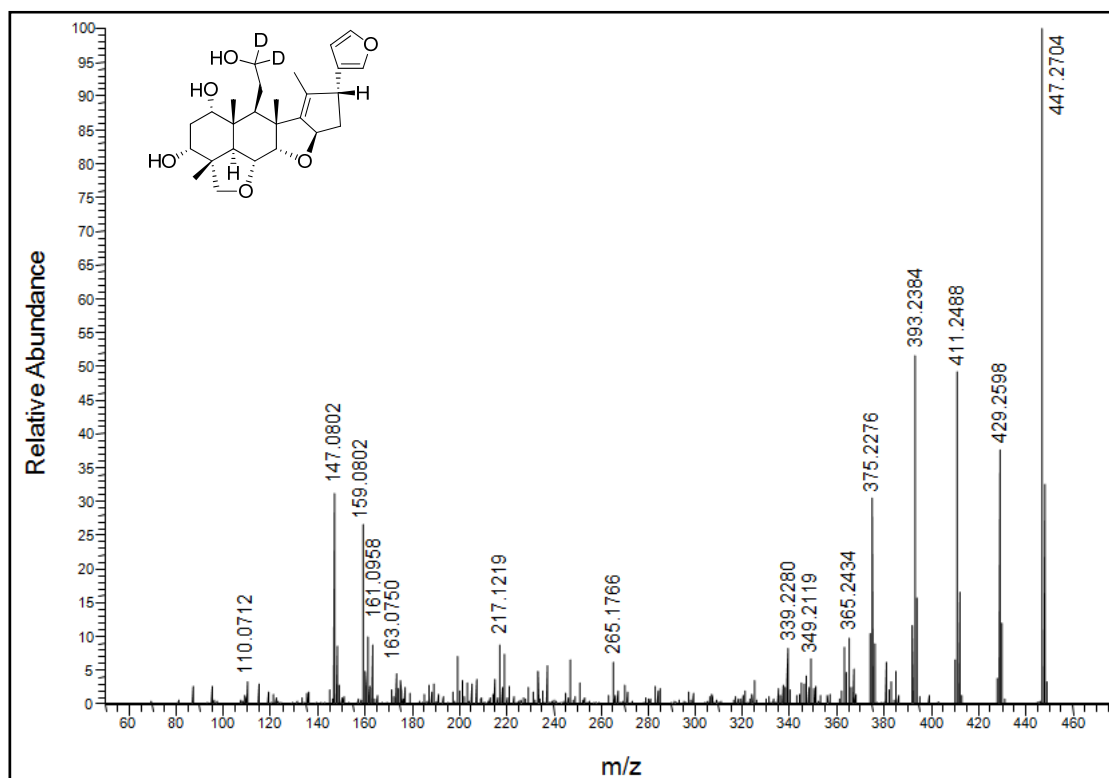
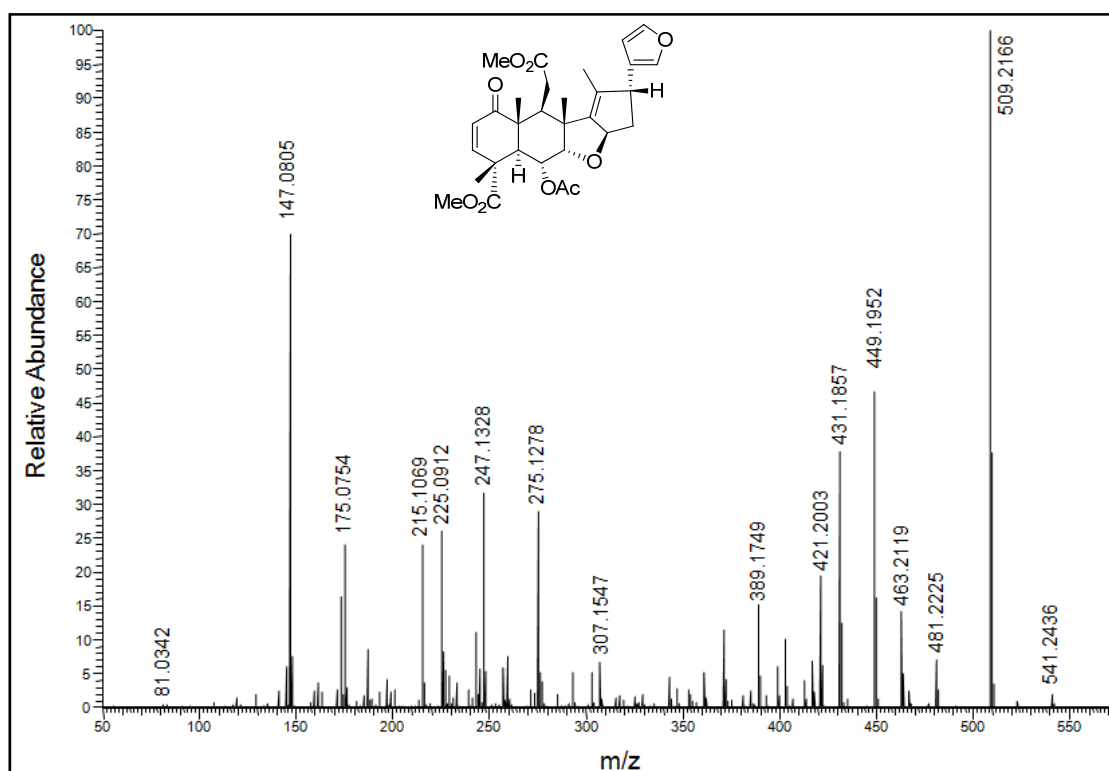


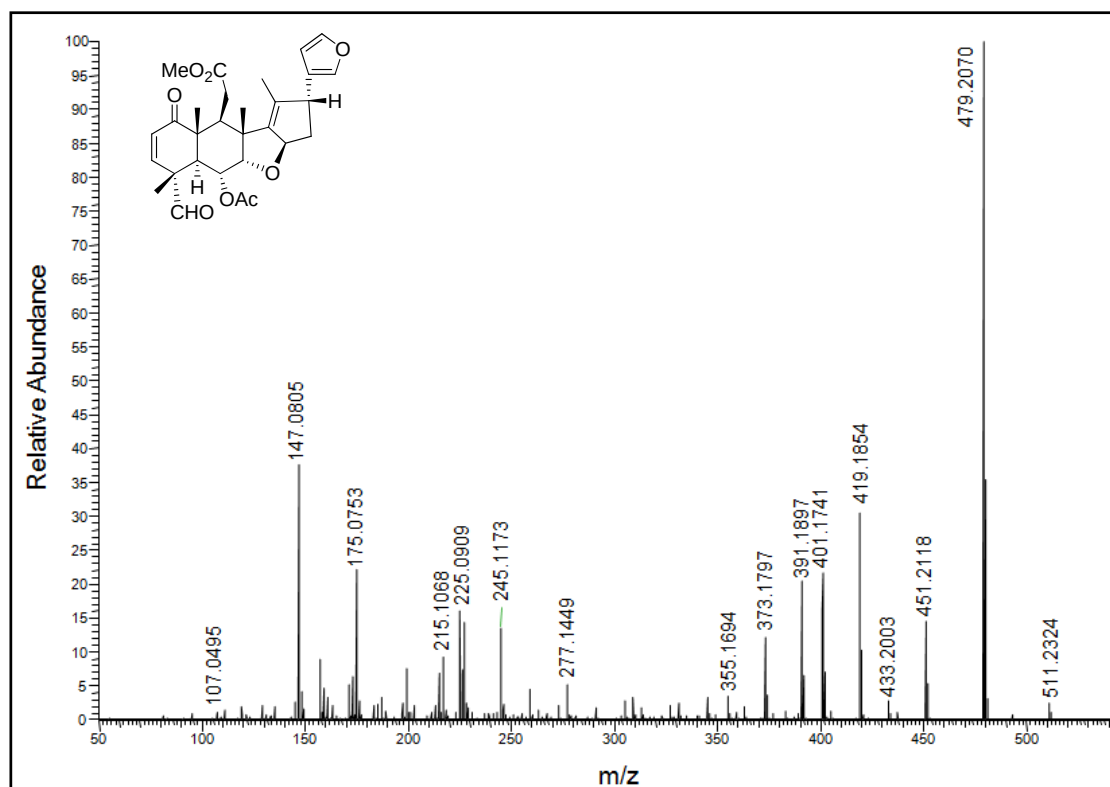
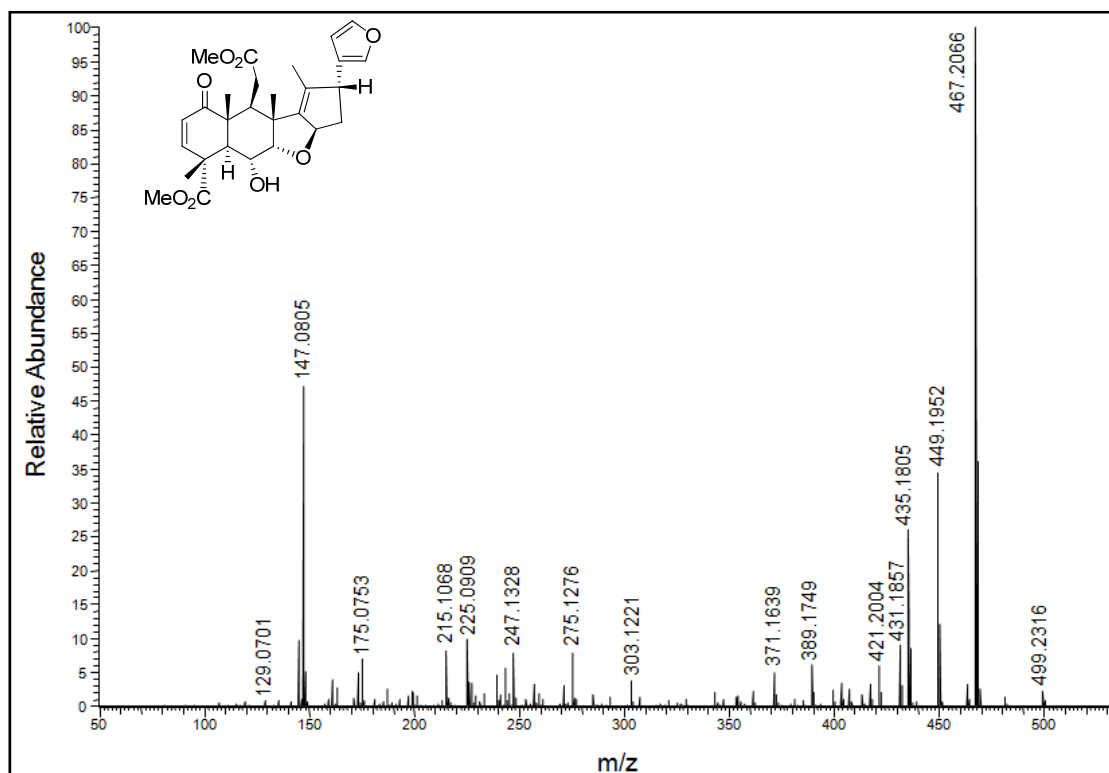


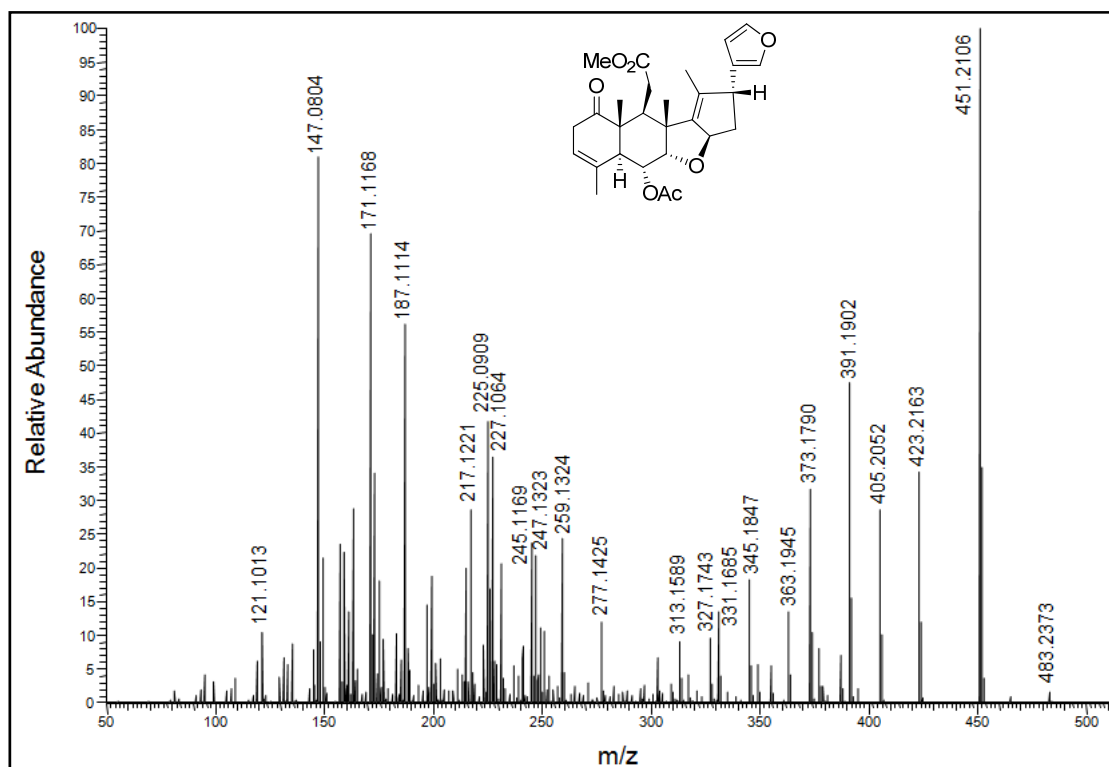
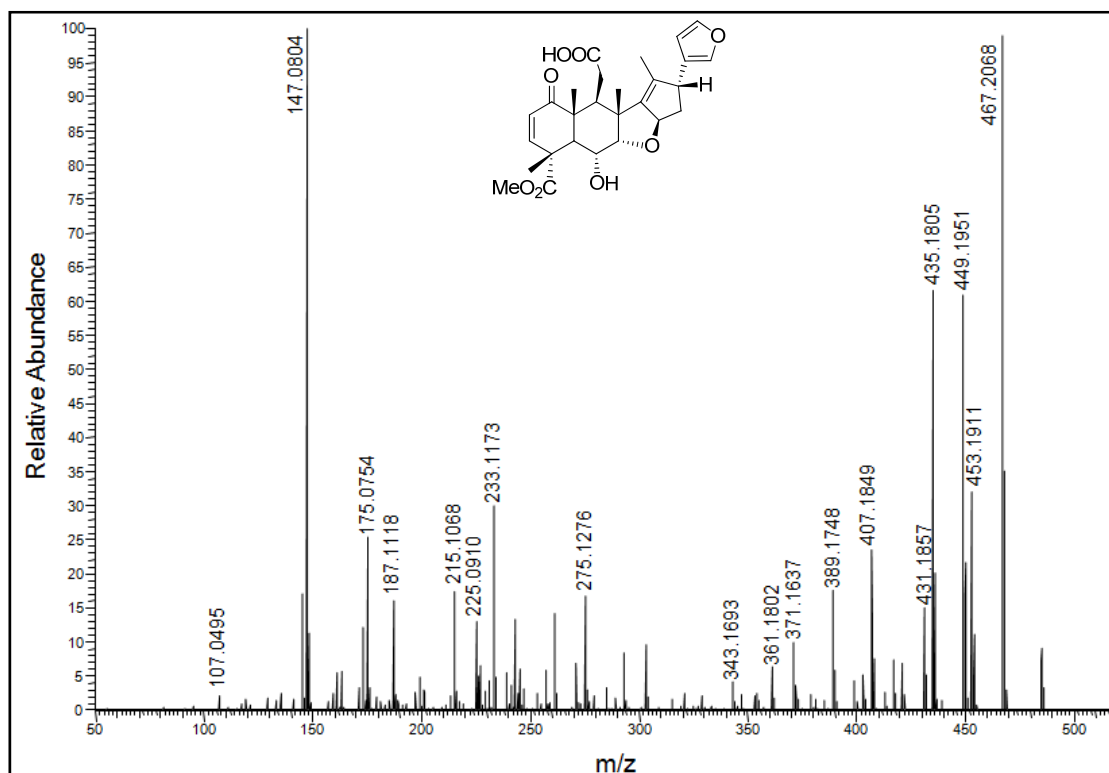
ESI(+)-MS/MS spectrum of **10** (Salannic acid) in NCE 20% (precursor ion $[M+H]^+$, m/z 459.2)

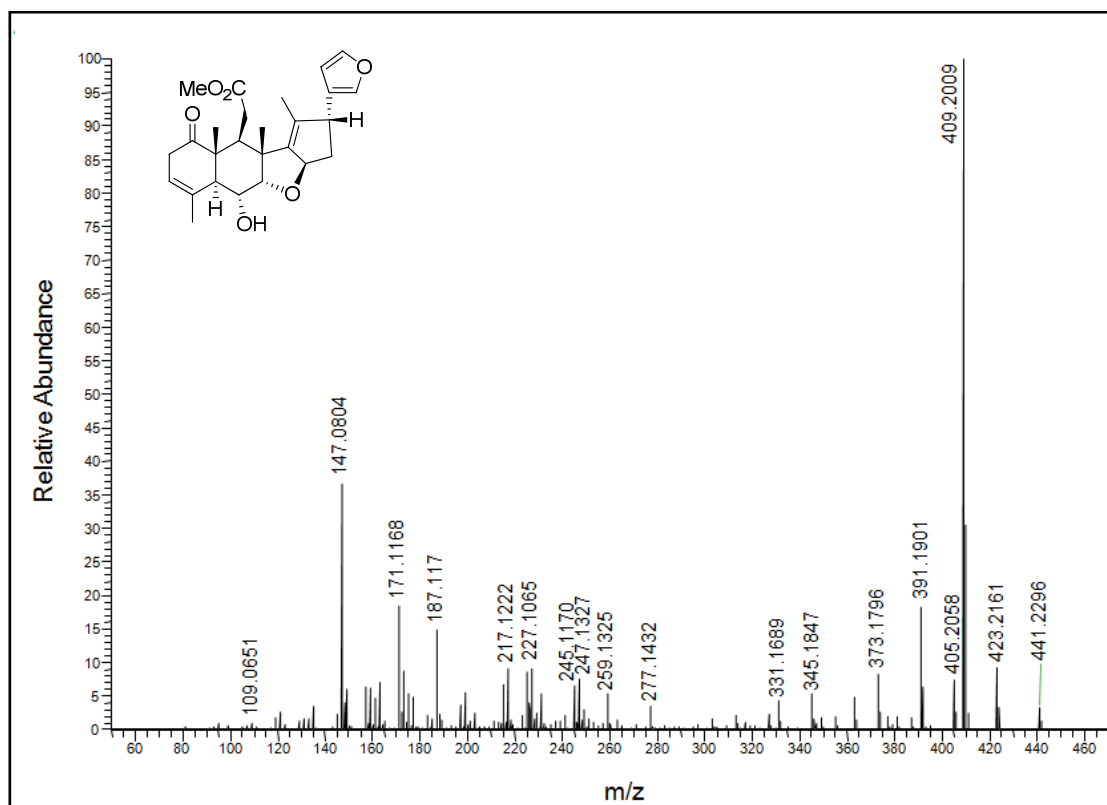


ESI(+)-MS/MS spectrum of **11** in NCE 20% (precursor ion $[M+H]^+$, m/z 445.2)

ESI(+)-MS/MS spectrum of **12** in NCE 20% (precursor ion $[M+H]^+$, m/z 447.2)ESI(+)-MS/MS spectrum of **4** (Nimbin) in NCE 20% (precursor ion $[M+H]^+$, m/z 541.2)







ESI(+)-MS/MS spectrum of **2** (6-Deacetylnimbinene) in NCE 20% (precursor ion $[M+H]^+$, m/z 441.2)

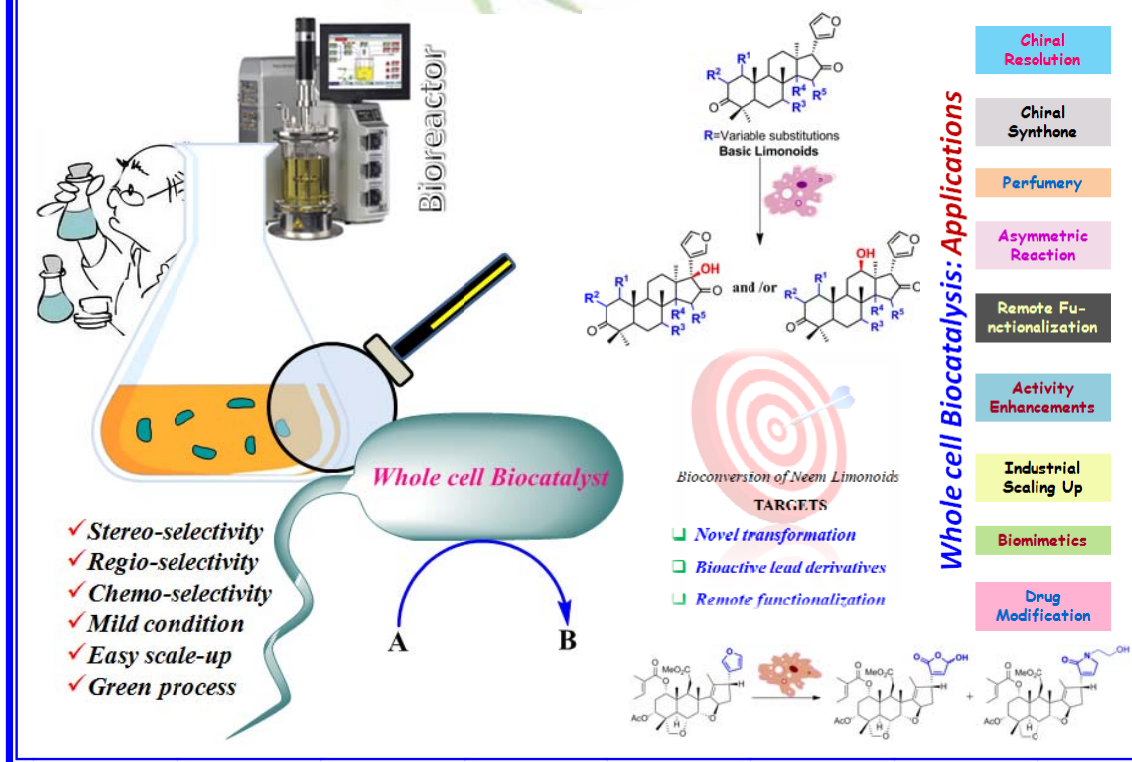
2D References

- (1) Ellis, D. I.; Goodacre, R. *Curr. Opin. Biotechnol.* **2011**, *23*, 22-28.
- (2) Fraser, P. D.; Pinto, M. E. S.; Holloway, D. E.; Bramley, P. M. *Plant J.* **2000**, *24*, 551-558.
- (3) Goodacre, R.; Vaidyanathan, S.; Dunn, W. B.; Harrigan, G. G.; Kell, D. B. *Trends Biotechnol.* **2004**, *22*, 245-252.
- (4) Hall, R. D. *New Phytol.* **2006**, *169*, 453-468.
- (5) Hollywood, K.; Brison, D. R.; Goodacre, R. *Proteomics* **2006**, *6*, 4716-4723.
- (6) Kell, D. B. *Curr. Opin. Microbiol.* **2004**, *7*, 296-307.
- (7) Lee, Y. J.; Perdian, D. C.; Song, Z.; Yeung, E. S.; Nikolau, B. J. *Plant J.* **2012**, *70*, 81-95.
- (8) Nguyen, Q.-T.; Merlo, M. E.; Medema, M. H.; Jankevics, A.; Breitling, R.; Takano, E. *FEBS Lett.* **2012**, *586*, 2177-2183.
- (9) Okazaki, Y.; Saito, K. *Plant Biotechnol. Rep.* **2012**, *6*, 1-15.
- (10) Roessner, U.; Luedemann, A.; Brust, D.; Fiehn, O.; Linke, T.; Willmitzer, L.; Fernie, A. R. *Plant Cell* **2001**, *13*, 11-29.
- (11) Schauer, N.; Fernie, A. R. *Trends Plant Sci.* **2006**, *11*, 508-516.
- (12) Zenobi, R. *Science* **2013**, *342*, 1243259.
- (13) Johnson, S.; Morgan, E. D.; Peiris, C. N. *Ann. Bot.* **1996**, *78*, 383-388.
- (14) Sidhu, O. P.; Kumar, V.; Behl, H. M. *Ind. Crop. Prod.* **2004**, *19*, 69-75.
- (15) Dalsgaard, P. W.; Potterat, O.; Dieterle, F.; Paululat, T.; Kuhn, T.; Hamburger, M. *Planta Med.* **2006**, *72*, 1322-1327.
- (16) Jarvis, A. P.; Morgan, E. D.; Edwards, C. *Phytochem. Anal.* **1999**, *10*, 39-43.
- (17) Weber, P.; Hamburger, M.; Schafroth, N.; Potterat, O. *Fitoterapia* **2011**, *82*, 155-161.
- (18) Xue, J.; Cao, C. Y.; Chen, J. M.; Bu, H. S.; Wu, H. M. *Chinese J. Chem.* **2001**, *19*, 82-90.
- (19) Cheng, C. R.; Yang, M.; Wu, Z. Y.; Wang, Y.; Zeng, F.; Wu, W. Y.; Guan, S. H.; Guo, D. A. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1323-1335.
- (20) Liu, X.; Li, S. L.; Zhou, Y.; Song, J. Z.; Zheng, Y. F.; Peng, G. P.; Xu, H. X. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1514-1522.
- (21) Ong, E. S.; Ong, C. N. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 589-598.
- (22) Tian, Q. G.; Schwartz, S. J. *Anal. Chem.* **2003**, *75*, 5451-5460.
- (23) Champagne, D. E.; Koul, O.; Isman, M. B.; Scudder, G. G. E.; Towers, G. H. N. *Phytochemistry* **1992**, *31*, 377-394.
- (24) Roy, A.; Saraf, S. *Biol. Pharm. Bull.* **2006**, *29*, 191-201.
- (25) Tan, Q. G.; Luo, X. D. *Chem. Rev.* **2011**, *111*, 7437-7522.
- (26) Govindachari, T. R.; Narasimhan, N. S.; Suresh, G.; Partho, P. D.; Gopalakrishnan, G. *J. Chem. Ecol.* **1996**, *22*, 1453-1461.
- (27) Simmonds, M. S.; Jarvis, A. P.; Johnson, S.; Jones, G. R.; Morgan, E. D. *Pest Manag. Sci.* **2004**, *60*, 459-464.
- (28) Yamasaki, R. B.; Klocke, J. A. *J. Agric. Food Chem.* **1989**, *37*, 1118-1124.
- (29) Koul, O.; Singh, G.; Singh, R.; Singh, J.; Daniewski, W. M.; Berlozecki, S. J. *Biosciences* **2004**, *29*, 409-416.
- (30) Govindachari, T. R.; Gopalakrishnan, G.; Suresh, G. *J. Liq. Chromatogr. R. T.* **1996**, *19*, 1729-1733.
- (31) Govindachari, T. R.; Sandhya, G.; Ganeshraj, S. P. *J. Chromatogr.* **1990**, *513*, 389-391.

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- (32) Jarvis, A. P.; Morgan, E. D.; Edwards, C. *Phytochem. Anal.* **1999**, *10*, 39-43.
- (33) Johnson, S.; Morgan, E. D. *J. Chromatogr. A* **1997**, *761*, 53-63.
- (34) Schroeder, D. R.; Nakanishi, K. *J. Nat. Prod.* **1987**, *50*, 241-244.
- (35) Sharma, V.; Walia, S.; Kumar, J.; Nair, M. G.; Parmar, B. S. *J. Agric. Food Chem.* **2003**, *51*, 3966-3972.
- (36) Silva, J. C. T.; Jham, G. N.; Oliveira, R. D. L.; Brown, L. *J. Chromatogr. A* **2007**, *1151*, 203-210.
- (37) Srivastava, P.; Yadav, N.; Lella, R.; Schneider, A.; Jones, A.; Marlowe, T.; Lovett, G.; O'Loughlin, K.; Minderman, H.; Gogada, R.; Chandra, D. *Carcinogenesis* **2012**, *33*, 2199-2207.
- (38) Purohit, A.; Joshi, V. B.; Vyas, K. B. *Pharm. Biol.* **2008**, *46*, 660-664.
- (39) Govindachari, T. R.; Suresh, G.; Gopalakrishnan, G.; Banumathy, B.; Masilamani, S. *Phytoparasitica* **1998**, *26*, 109-116.
- (40) Deng, Y. X.; Shi, D. X.; Yin, Z. Q.; Guo, J. H.; Jia, R. Y.; Xu, J.; Song, X.; Lv, C.; Fan, Q. J.; Liang, X. X.; Shi, F.; Ye, G.; Zhang, W. *Exp. Parasitol.* **2012**, *130*, 475-477.
- (41) Govindachari, T. R.; Suresh, G.; Gopalakrishnan, G.; Wesley, S. D. *J. Appl. Ent.* **2000**, *124*, 287-291.
- (42) Haldar, S.; Phapale, P. B.; Kolet, S. P.; Thulasiram, H. V. *Anal. Methods* **2013**, *5*, 5386-5391.
- (43) Wu, H. F.; Guo, J.; Chen, S. L.; Liu, X.; Zhou, Y.; Zhang, X. P.; Xu, X. D. *J. Pharmaceut. Biomed.* **2013**, *72*, 267-291.
- (44) Allwood, J. W.; Goodacre, R. *Phytochem. Anal.* **2010**, *21*, 33-47.
- (45) Bumpus, S. B.; Kelleher, N. L. *Curr. Opin. Chem. Biol.* **2008**, *12*, 475-482.
- (46) Xing, J.; Xie, C. F.; Lou, H. X. *J. Pharmaceut. Biomed.* **2007**, *44*, 368-378.
- (47) Gika, H. G.; Theodoridis, G. A.; Plumb, R. S.; Wilson, I. D. *J. Pharmaceut. Biomed.* **2014**, *87*, 12-25.
- (48) Sartori, L. R.; Vessecchi, R.; Humpf, H. U.; Da Costa, F. B.; Lopes, N. P. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 723-730.
- (49) Musharraf, S. G.; Ali, A.; Ali, R. A.; Yousuf, S.; Atta ur, R.; Choudhary, M. I. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 104-114.
- (50) Yang, W.; Fang, D. M.; He, H. P.; Hao, X. J.; Wu, Z. J.; Zhang, G. L. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 1203-1212.
- (51) Rojatkari, S. R.; Bhat, V. S.; Kulkarni, M. M.; Joshi, V. S.; Nagasampagi, B. A. *Phytochemistry* **1989**, *28*, 203-205.
- (52) Kraus, W.; Cramer, R. *Chem. Ber.* **1981**, *114*, 2375-2381.

Chapter 3

Biocatalyst Mediated Functionalization of Neem Limonoids



Abstract

Chapter 3 comprises of three sections. The chapter starts with brief introduction on biocatalysis followed by discussion on fungi mediated transformation of basic limonoids and salannin. Fungal systems were utilized to achieve novel transformation on complex and sensitive skeleton of Neem limonoids. The metabolites were characterized through rigorous spectroscopic analyses.

Chapter 3: Section A

Biotransformation and Biocatalysis: an Introduction

Highlights:

This section deals with introduction, effectivity and scope of biocatalysis. Emphasis has been provided towards comparison between enzyme and whole cell mediated transformation and its application in the field of organic chemistry. Further, microbial conversion of triterpenoids and Neem limonoids available in the literature has been reviewed briefly.

3A.1 Biotransformation and Biocatalysis

Various enzyme catalyzed conversions of chemical substance(s) are collectively called as 'biotransformation' and associated phenomenon of catalysis is called 'biocatalysis'. Purified enzyme or alternatively whole-cell (*i.e.* source of enzymes) such as micro-organisms, plant/animal cells may be used as the biocatalyst. The utilization of biocatalysis started with early dawn of civilization under the form of fermentation for the preparation of ethanol, fermented dairy products and bread-making without perceiving the existence of micro-organisms. In comparison to the tremendous growth of classical chemical catalysts in last century, realm of biocatalysts remained highly restricted till 1960's from when structural and biochemical studies of enzymes started evolving rapidly. Till date, a number of biotransformation processes catalyzed by wild/engineered whole cell or purified enzymes have been industrialized in the sector of pharmaceuticals, foods, bulk/fine chemicals, polymer, biofuels etc. Still after stupendous progress in enzymology, structural biology, biochemical techniques and instrumentations, biologists struggle frequently to explain the extraordinarily high turn-over frequency and selectivity observed with efficient biocatalysts.

3A.2 Biocatalysis and its Unrivalled Power¹⁻¹⁰

In past few decades biocatalysts have attracted the attention of organic, medicinal and natural product chemists due to their high potential to introduce regio-, stereo- and chemo-selectivity in the reaction. Specific orientation of the substrate and large surface contact between substrate and enzyme within the active site render the scope of selectivity in product formation. During an enzyme-catalyzed reaction, only one area (*regio-selectivity*) and one face (*stereo-selectivity*) of the substrate is in proper position to react with the catalytic machinery of the enzyme resulting in an exquisite selectivity. Due to the same mechanism, specific group of the substrate undergoes reaction favourably over another with similar reactivity (*chemo-selectivity*). Such kind of selectivity attained through an enzyme catalyzed reaction, overcomes the disadvantages of protection-deprotection protocols and expensive chemical catalysts used in conventional chemical synthesis, thus reducing the number of steps, enhancing the overall yield and making the process less tedious and economic. While working with complex functionalized molecules or compounds with sensitive scaffolds, enzymatic

reactions are highly preferred as they are usually used in mild reaction conditions such as aqueous media and moderate temperature (<100 °C). Moreover, biocatalyzed reactions are devoid of externally added harsh chemical reagents. In case of large-scale industrial fermentation or enzymatic reaction, biocatalysts offer a ‘green’ process because usual components of the reaction are non-toxic, leaves no heavy metal or harmful chemical contamination in the product and generated wastes are usually biodegradable. However, most of the enzymes are highly selective to their substrates leading to a narrow synthetic substrate scope. In that case, enzymes those accept broader substrate range are advantageous and more applicable over others.

3A.3 Whole-cell Biocatalysis vs. Isolated Enzyme^{2,3,6,11,12}

Both isolated enzymes and whole-cell are used in industry and academia today. There is a trend to the choice of isolated enzyme or whole-cell biocatalyst. The reaction steps which require cofactors and their regeneration are usually carried out using whole-cell organisms. Although cofactors are available commercially and also their regeneration is possible *in vitro*, but it is easier and less expensive to use metabolically active cells as the source of cofactor and machinery for their regeneration *in situ*. In comparison to purified enzymes, whole-cell biocatalysts can easily be prepared and inexpensive. Micro-organisms are considered as ‘bags stuffed with catalysts’ and those enzymes are well-protected from the external environment. Enzymes are more stable in the cellular environment for long-term storage as compared to isolated one. Micro-organisms are known for their diversity, efficiency and ease of handling. There are several organizations for identifying, characterizing, preserving and distributing authentic microbial cultures worldwide (*e.g.* ATCC/American Type Culture Collection; WFCC/The World Federation for Culture Collections; JCM/Japan Collections of Micro-organisms; MCC/Microbial Culture Collection, India). Rapid advancement in molecular biological techniques also helped to engineer the micro-organisms to over-express the genes of interest (*i.e.* Recombinant DNA technology) and eventually over-production of corresponding enzymes in heterologous hosts. Isolated enzymes are usually used for the reactions which don’t require cofactors such as hydrolysis or isomerisation. However, micro-organisms can’t tolerate high concentration of substrate or product (usually > 1 g/L) and they are restricted to use with organic solvents. Moreover, they can generate

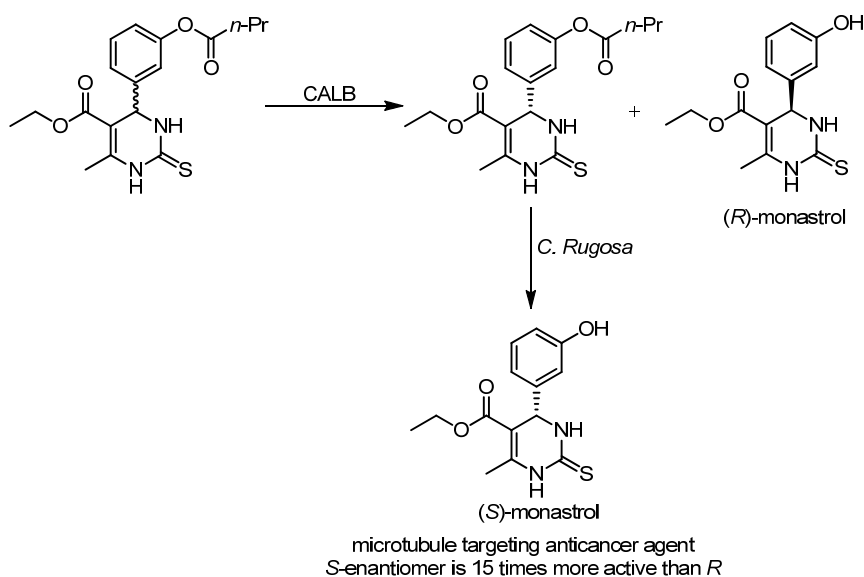
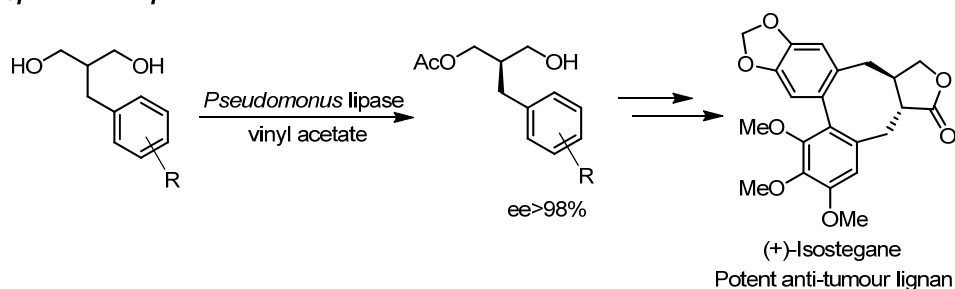
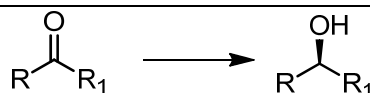
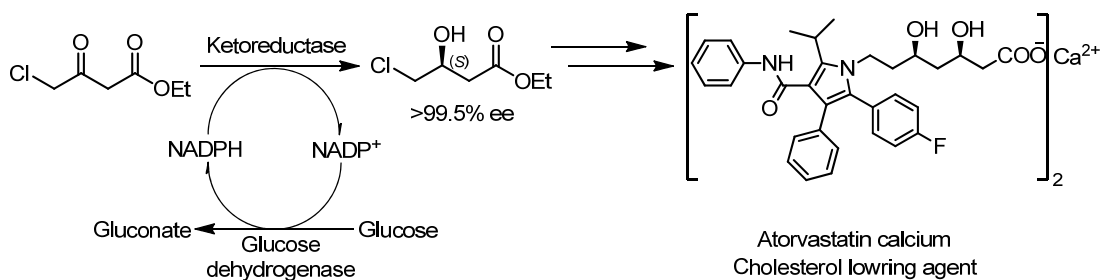
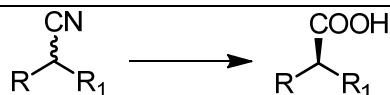
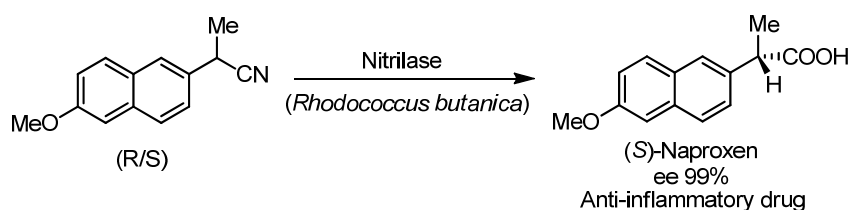
unwanted side products due to the presence of numerous enzymes. Therefore, the choice of biocatalyst (isolated enzyme/whole-cell) in case of industrial process development is sometimes tricky and depends on the comparable economic viability of both the processes.

3A.4 Biocatalysts: Contribution to the Field of Organic Chemistry^{3-5,7-10}

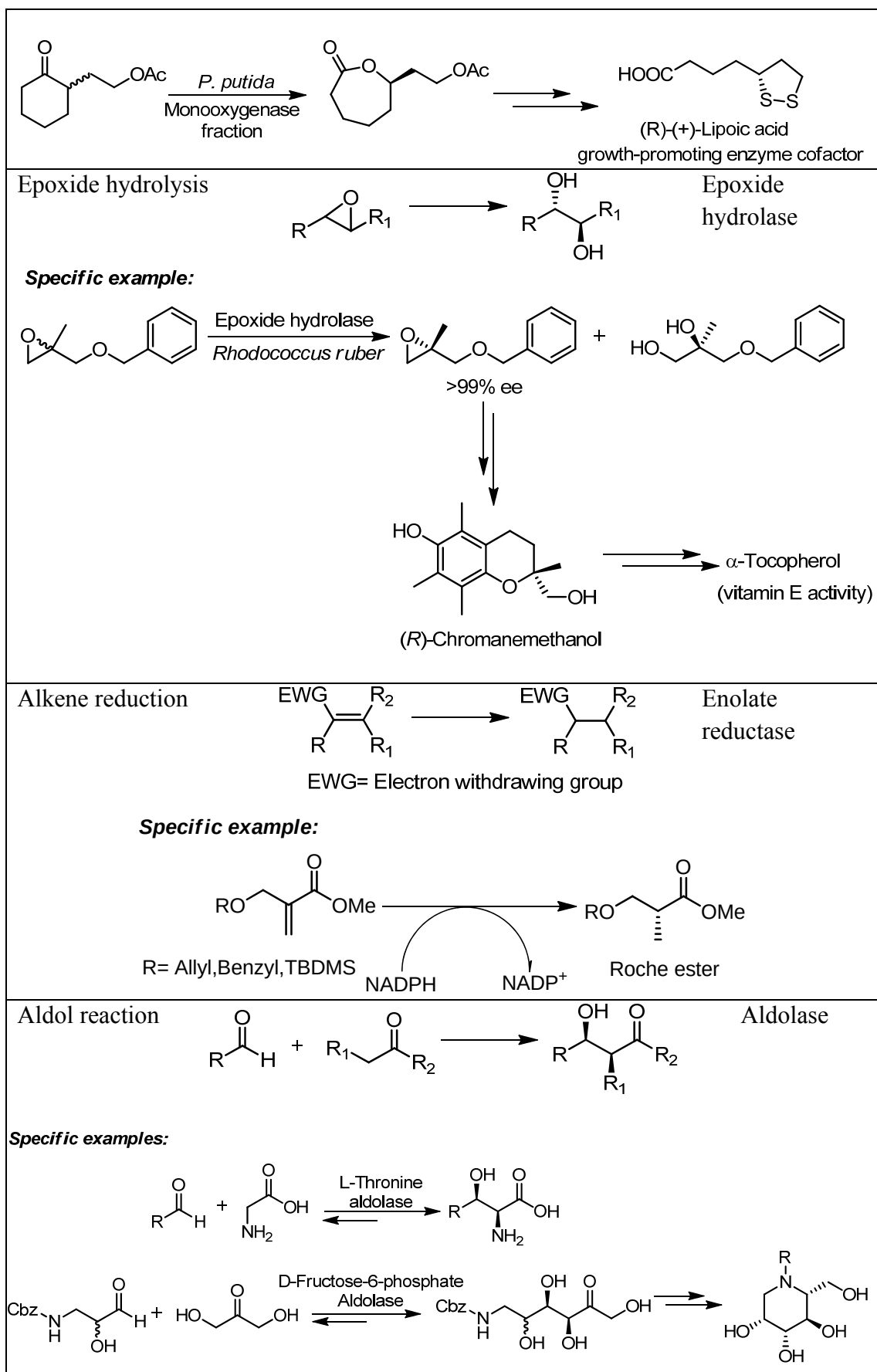
The documented history of whole-cell biotransformation is as old as 200 years. One of the oldest examples of industrial whole-cell biocatalysis was the production of acetic acid from ethanol (1815) using an immobilized strain of *Acetobacter*. Later on microbial production of vitamin C and steroid hydroxylations were developed in mid-twentieth century. Application of isolated enzymes became popular after the resolution of racemic amino acids by acylase method (Tanabe company, 1969). In last few decades, biocatalysts have taken momentum in the field of organic chemistry and associated industries. Biocatalytic reactions have contributed several useful tools (Table 3A.1) in organic chemistry toolbox and taking stride very rapidly. Several organic reactions such as transesterification, hydrolysis, oxidation, reduction, transamination, C-C and C-N bond forming reactions are carried out industrially using biocatalysts for the production of bulk or fine chemicals.

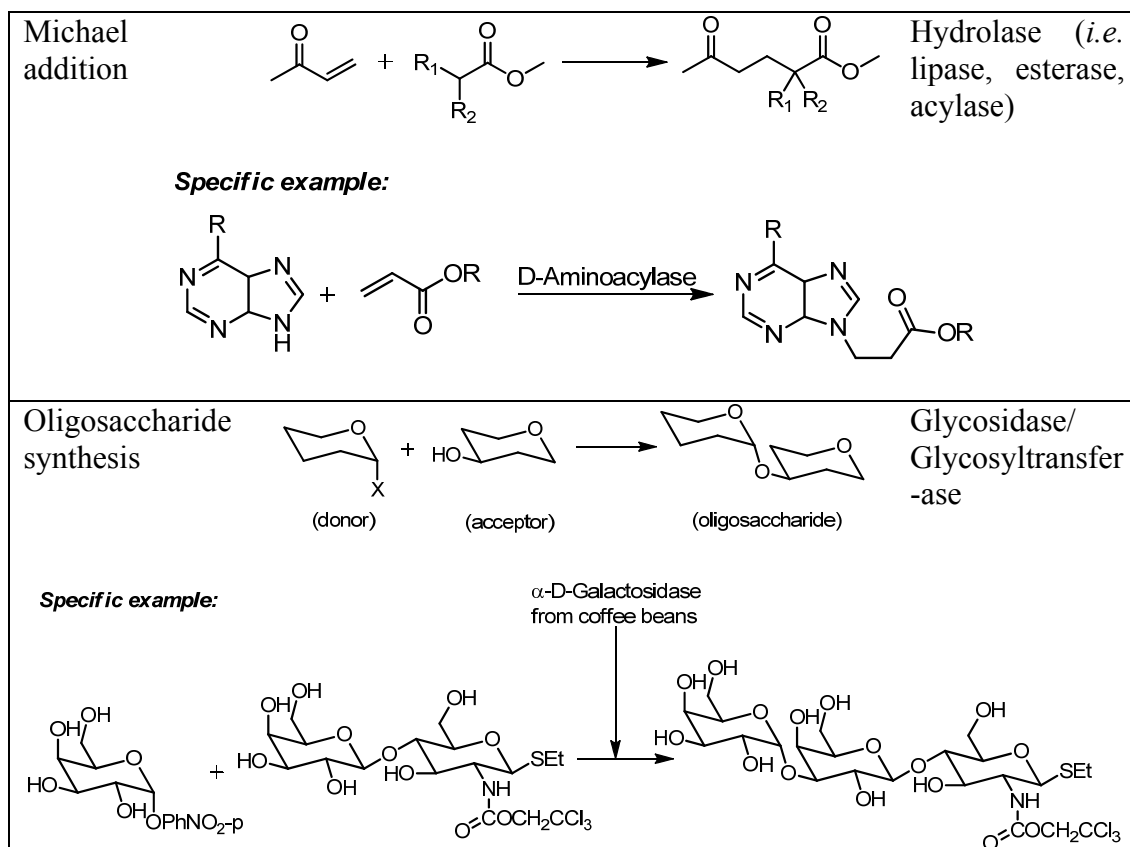
Table 3A.1 Various useful biocatalytic reactions used extensively in organic synthesis

Purpose	Reaction	Biocatalyst
Kinetic Resolution	Hydrolysis Esterification	Lipase/Esterase/ Acylase/ Peptidase/ Amidase

Specific example: hydrolysis**Specific example: esterification****Ketone
reduction****Ketoreductase****Specific example:****Nitrile
hydrolysis****Nitrilase****Specific example:**

Transamination	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{R}-\text{C}-\text{R}_1 \\ + \\ \begin{array}{c} \text{NH}_2 \\ \\ \text{CH} \\ \\ \text{COOH} \end{array} \end{array} \xrightarrow{\text{Transaminase}} \begin{array}{c} \text{NH}_2 \\ \\ \text{R}-\text{CH}-\text{R}_1 \\ + \\ \begin{array}{c} \text{O} \\ \parallel \\ \text{CH}_3-\text{COOH} \end{array} \end{array} $	Transaminase
Specific example:	ω-Transaminase <i>Vibrio fluvialis</i> L-alanine Pyruvate MOMO- <chem>COc1ccc(cc1)C(=O)O</chem> $\rightarrow$ <chem>COc1ccc(cc1)[C@H](N)C</chem> $\rightarrow$ <chem>CCN(C)C(=O)Oc1ccc(cc1)[C@H](N)C</chem> > 99% ee (S)-Rivastigmine Drug for Alzheimer disease	
Hydroxylation	$ \begin{array}{c} \text{R}-\text{CH}_2-\text{R}_1 \end{array} \longrightarrow \begin{array}{c} \text{OH} \\ \\ \text{R}-\text{CH}-\text{R}_1 \end{array} $	Hydroxylase
Specific example:	Rhizopus sp. Progesterone 11α-Hydroxyprogesterone Cortisone Steroidal hormone and anti-inflammatory drug	
Dihydroxylation	$ \begin{array}{c} \text{R}-\text{CH}=\text{CH}-\text{R}_1 \end{array} \longrightarrow \begin{array}{c} \text{HO}-\text{CH}-\text{CH}-\text{R}_1 \\ \quad \\ \text{R} \quad \text{R}_1 \end{array} $	Dioxygenase
Specific example:	Recombinant dioxygenase O₂ CO₂Et CO₂Et <chem>CCOC(=O)c1ccccc1</chem> $\rightarrow$ <chem>CCOC(=O)c1cc(O)c(O)cc1</chem> $\rightarrow$ <chem>CCOC(=O)c1cc(NC(=O)C)cc(O)c1</chem> Oseltamivir (Tamiflu) Anti-influenza drug	
Bayer-Villiger Oxidation	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{C}_6\text{H}_{10}-\text{R} \end{array} \longrightarrow \begin{array}{c} \text{O} \\ \parallel \\ \text{C}_7\text{H}_{11}-\text{R} \end{array} $	Monooxygenase
Specific examples:	Acinetobacter sp. <chem>C1CCCC1C(=O)CCCCCCCCCCCC</chem> $\rightarrow$ <chem>C1CCCC1C(=O)OCCCCCCCCCCCC</chem> $\rightarrow$ <chem>C1CCCC1C(=O)OCCCCCCCCCCCC</chem> (S) (R) mCPBA Pheromone from <i>Vespa orientalis</i>	

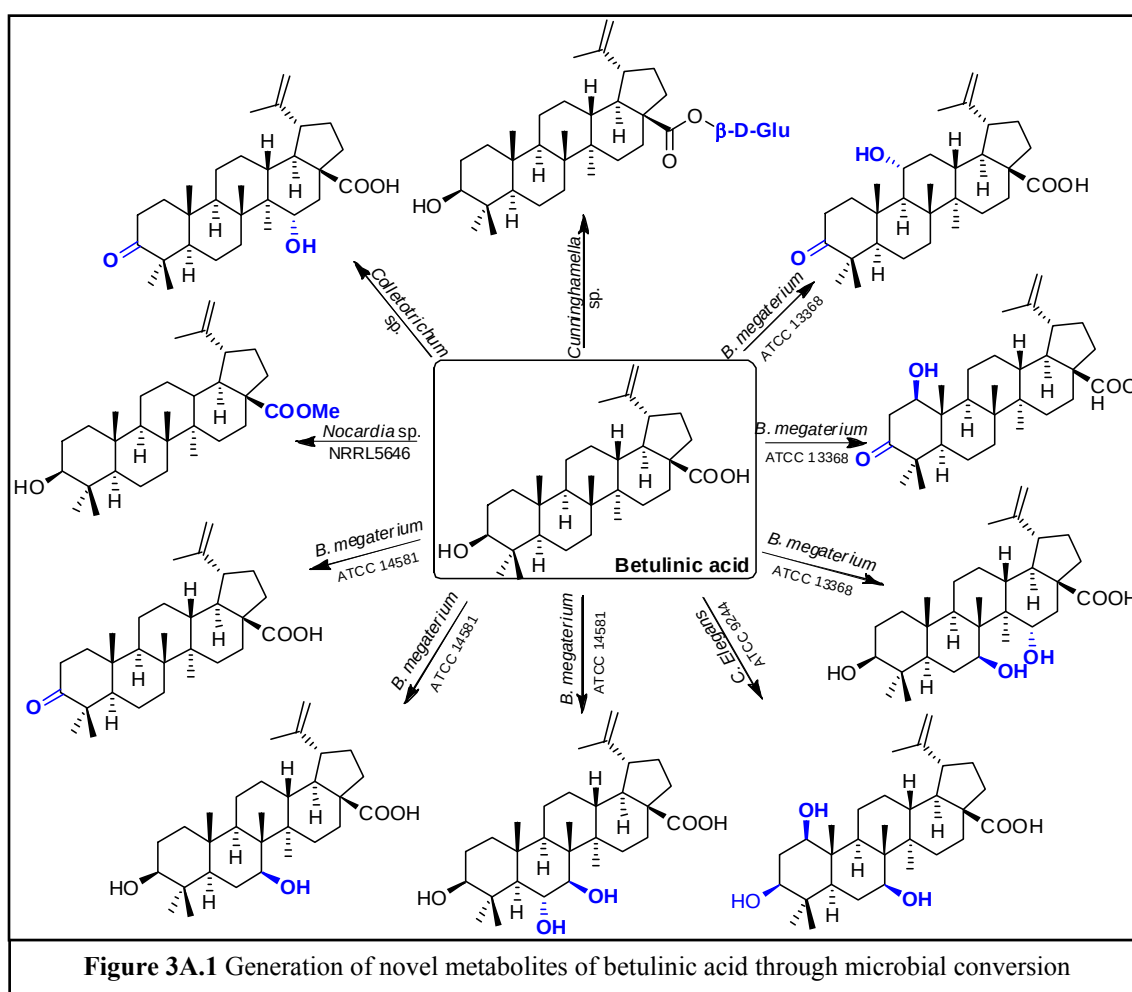




3A.5 Biotransformation of Triterpenoids

Triterpenoids are well-known for their structural complexity and diversity. Various oxygenated functional groups arranged around their skeleton play a key role to regulate the efficiency of biological activity. Presence of several sensitive functional groups of similar reactivity makes the choice of conventional chemical synthesis ineffective for selective structural modifications. Sometimes, functionalization at the remote skeletal carbon is either impossible or tedious to achieve through chemical methods. On the other hand biocatalysts offer a ‘green’ technique for regio-, chemo- and stereo-selective functionalization of these complex natural products in mild condition, which is unrivalled by any other techniques till date. Frequently they have been found to carry out synthetically challenging transformations to generate novel metabolites. Microbial transformations of triterpenoids especially pentacyclic triterpenes have been investigated in detail in last two decades.¹³⁻¹⁵ Several pentacyclic triterpenoids including betulinic acid, betulin, ursolic acid, glycyrrhetic acid, oleanolic acid, senegenin, liquiritic acid, lupeol have been biotransformed with various whole-cell microorganisms to produce numerous novel metabolites.¹³⁻¹⁵ To exemplify, Figure 3A.1 has been

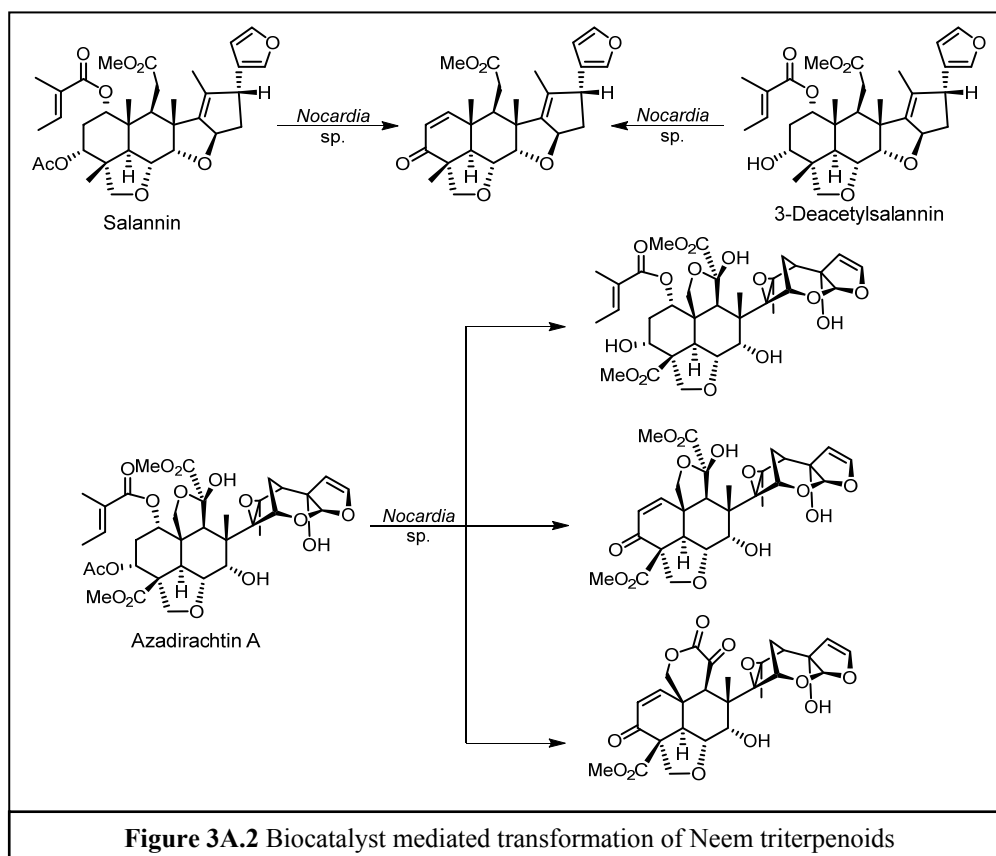
presented showing the generation of structurally diverse metabolites through microbial transformation of betulinic acid. Microbial conversion of betulinic acid by resting-cell suspension of *Cunninghamella* sp. (NRRL 5695) resulted in formation of a glucosidated metabolite, 28-*O*- β -D-glucopyranosyl-3 α -hydroxy-lup-20(29)-en-28-oate.¹⁶ In another study, incubation of betulinic acid by resting-cell suspension of phenobarbital-induced *Bacillus megaterium* (ATCC 14581) produced three oxidized metabolites, 3-oxo-lup-20(29)-en-28-oic acid (betulonic acid), 3 β ,7 β -dihydroxy-lup-20(29)-en-28-oic acid and 3 β ,6 α ,7 β -trihydroxy-lup-20(29)-en-28-oic acid.¹⁷ Transformation of the same substrate by *Cunninghamella elegans* (ATCC 9244) produced a novel trihydroxylated metabolite,



1 β ,3 β ,7 β -trihydroxy-lup-20(29)-en-28-oic acid.¹⁷ Another species of *Bacillus megaterium* (ATCC 13368) transformed betulinic acid to four metabolites, 3-oxo-lup-20(29)-en-28-oic acid, 3-oxo-11 α -hydroxy-lup-20(29)-en-28-oic acid, 1 β -hydroxy-3-oxo-lup-20(29)-en-28-oic acid and 3 β ,7 β ,15 α -trihydroxy-lup-20(29)-en-28-oic acid.¹⁸ In another report, *Colletotrichum* sp. converted betulinic acid into 3-oxo-15 α -hydroxy-lup-

20(29)-en-28-oic acid.¹⁹ Conversion of betulinic acid by *Nocardia* sp. (NRRL 5646) selectively yielded corresponding 28-methyl ester.²⁰

As discussed earlier, Neem triterpenoids are highly complex, multifunctional and sensitive which restrict the scope of selective structural modifications by conventional chemical methods. Very less effort has been made for the biocatalyst mediated transformation of Neem triterpenoids till date. However, the reported microbial transformations mainly led to the formation of metabolites by removal of sensitive functional groups such as tigloyl, acetate *etc.*, rather than synthetically challenging functionalization. For example, in a report by Madyastha *et al.* salannin and 3-deacetylsalannin was converted into 1-detigloyloxy-3-deacetylsalannin-1-en-3-one by *Nocardia* sp. leading to the formation of cyclohexenone A-ring (Figure 3A.2).²¹ Furthermore, to investigate the sequential events associated with this transformation and structural specificity of the bacterial system, several semi-synthesized derivatives of salannin were prepared and subjected to same biocatalyst.²² In a separate study by the same authors, azadirachtin A was converted into three metabolites by *Nocardia* sp., 3-deacetylazadirachtin, 1-detigloyl-3-deacetyl-azadirachtin-1-ene-3-one and 1-detigloyl-3-deacetyl-11,19-deoxa-12,19-oxa-11-oxo-azadirachtin-1-ene-3-one (Figure 3A.2).²³



Chapter 3: Section B

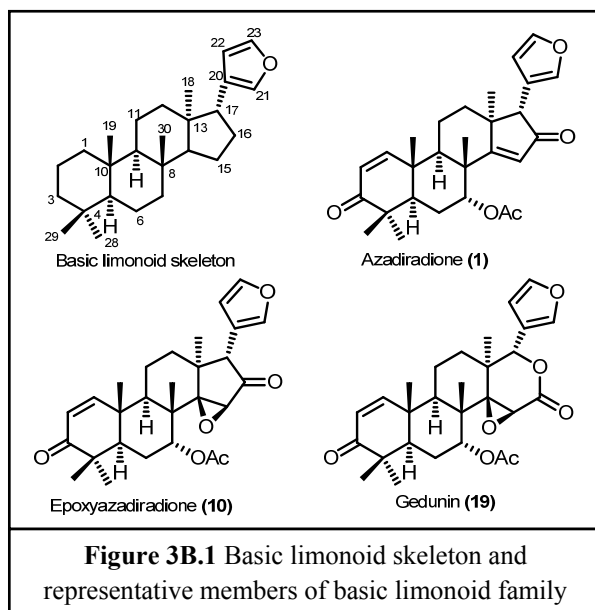
Fungi Mediated Transformation of Basic Limonoids

Highlights:

- *An efficient fungi mediated bioconversion technique was developed for rare and synthetically challenging 17 β - and 12 β -hydroxylation on the basic limonoid skeleton in mild condition by Mucor sp.*
- *Broader substrate scope within basic limonoid family generated a series of novel hydroxylated limonoids.*
- *Modifications of specific functional groups and variation in biocatalyst were shown to bring selectivity among 12 β - or 17 β -hydroxylation.*

3B.1 Introduction

As discussed in Chapter 1, strong antifeedant property²⁴ along with anti-plasmodial,²⁵ anti-HIV,²⁶ anti-inflammatory²⁷ activities of basic limonoids such as azadiradione and epoxyazadiradione (Figure 3B.1) have attracted the attention of synthetic chemists in last two decades.^{28,29} In particular, gedunin is a well-studied anti-malarial,³⁰ anti-



carcinogenic³¹ and anti-ulcerogenic agent.³² The potency of limonoids as a biologically active entity can be varied with the modification of functional groups ornamentally arranged around its skeleton. According to Champagne *et al.* limonoid biosynthetic pathway is featured by evolution towards increasing rearrangement and oxidation of basic skeleton to produce highly oxygenated terpenoids which are biosynthesized for self-defence against

insects.²⁴ So, increasing oxidation and rearrangement can lead to enhanced activity. But conventional synthetic tools are sometimes tedious and time consuming for desired functional group modifications on the 'active structural unit' due to the presence of inactive carbons, steric hindrance, sensitivity of functional groups towards external reagents and presence of same groups with similar reactivity. Frequently, synthesis involves several protection-deprotection steps which make the overall process low-yielding. On the other hand, biocatalysis is a well-established 'green' technique for carrying out stereo- and regio-selective functionalization of various sensitive and complex molecules in mild reaction conditions. Insecticidal property along with diverse pharmacological activities of basic limonoids directed us to screen various fungal systems for novel and useful biotransformation like C-H oxidation on synthetically inactive carbons.

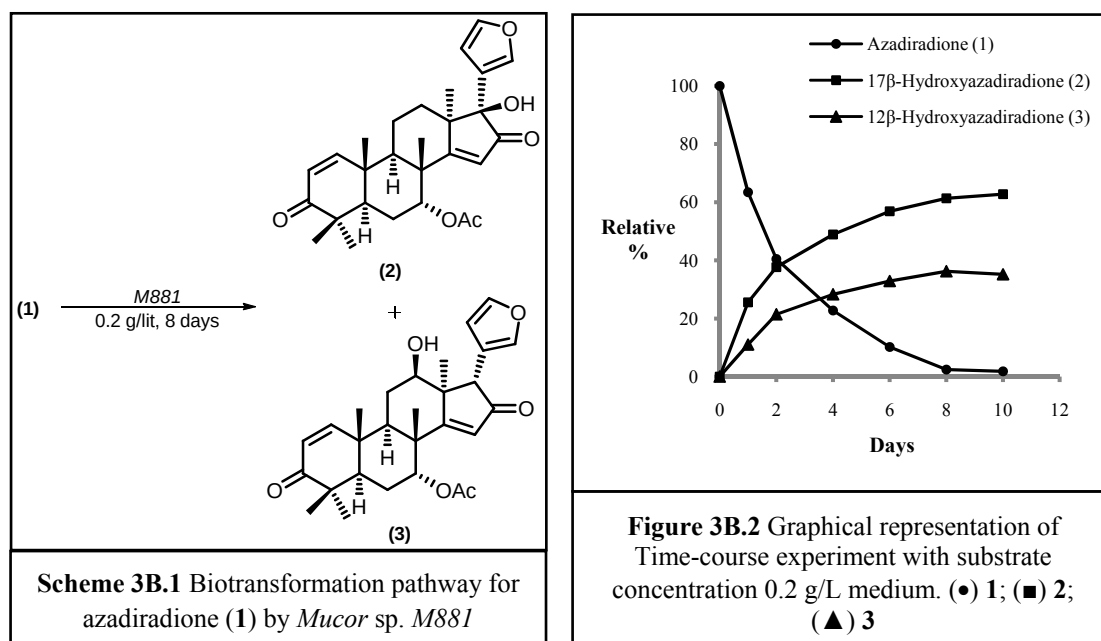
In this section, the scope of whole cell biocatalysis has been depicted for the production of 17 β - and 12 β -hydroxy metabolites of basic limonoids with excellent conversion. Indeed, it is the first investigation on the biotransformation of intact

limonoid skeleton furnishing novel stereo- and regio-selective hydroxylated metabolites which will be challenging to achieve by chemical synthetic methods. The ability of a specific biocatalyst to transform a variety of structurally modified basic limonoids in highly efficient manner was explored which broadened the substrate scope within the basic limonoid family. More interestingly, either of 17 β - or 12 β -hydroxylation was achieved selectively by varying the biocatalyst or skeletal functional groups.

3B.2 Results and Discussion

3B.2.1 Screening and Time-course Experiment

Azadiradione (**1**), an isolated limonoid from the fruit of *Azadirachta indica* (Neem) was used as the representative model substrate for the screening experiment in an analytical scale. Over twenty five different species of filamentous fungi belonging to genera *Aspergillus*, *Mucor*, *Cephalophora*, *Fusarium*, *Rhizopus* and *Gibberella* were screened



for their ability to carry out useful transformations of azadiradione. Screening experiments were carried out with 0.2 g/L of substrate concentration with 8 days of incubation period at 30 °C and 180 rpm. TLC, HPLC and LCMS analyses of the extracted biotransformed products indicated that one of the fungal system belonging to the genera *Mucor* (National Collection of Industrial Microorganisms or NCIM, Pune catalogue no. 881 and abbreviated as *M881*) could able to quantitatively carry out the

transformation and hence was selected for the fermentation in preparative scale. Bioconversion was validated by comparing with two control experiments; organism without substrate (organism control) and substrate without organism (substrate control). Resting cell experiment further confirmed that the hydroxylase system involved in the transformation is constitutive. Time-course experiment with the same substrate concentration clearly showed that *M881* quantitatively converted **1** into two metabolites after 8 days of incubation period (Scheme 3B.1, Figure 3B.2).

3B.2.2 Preparative Scale Fermentation and Purification

The experiment was further taken ahead in a preparative scale with an aim of

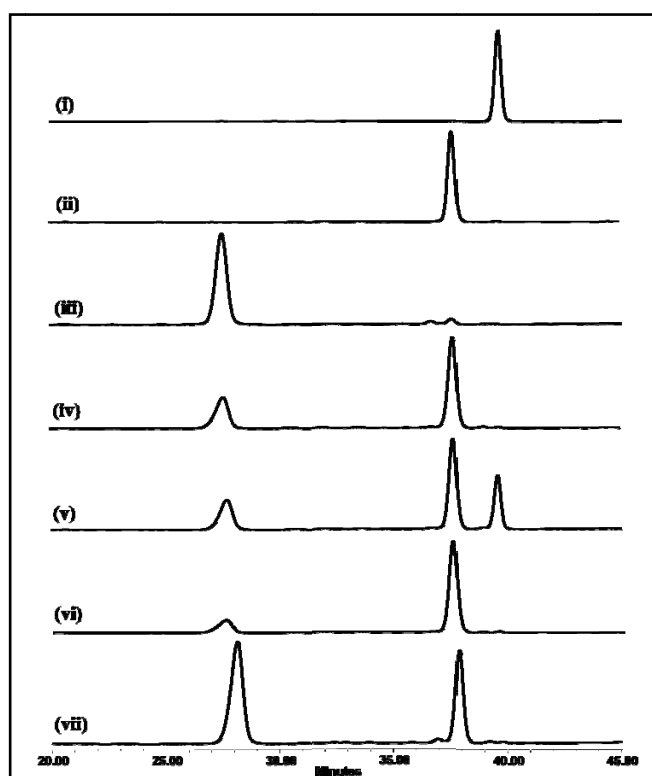


Figure 3B.3 (a) HPLC profiles of the biotransformation products obtained by incubating azadiradione (**1**) with *M881* for 8 days and co-injection studies: (i) Standard azadiradione, (ii) Purified **2**, (iii) Purified **3**, (iv) Fermented azadiradione (**1**) with *M881*, (v) Co-injection of **1** with (iv), (vi) Co-injection of **2** with (iv), (vii) Co-injection of **3** with (iv).

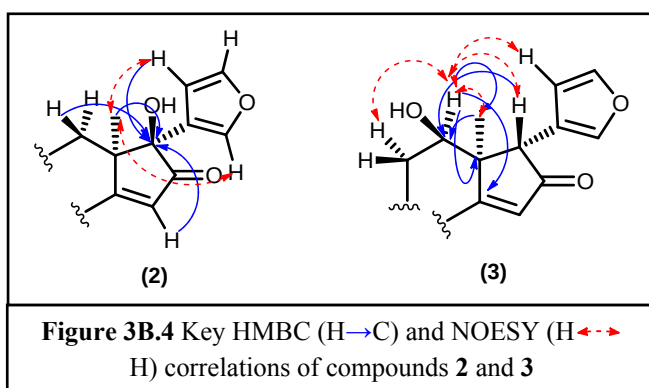
purification and characterization of the metabolites. Substrate concentration studies (0.1 g/L to 0.4 g/L) indicated that the fungal system *M881* quantitatively transformed **1** into **2** and **3** at the concentration of 0.2 g/L with 8 days of incubation period. Increase in the substrate concentration (>0.2 g/L) considerably decreased the rate of bioconversion. The optimized substrate concentration (0.2 g/L) and incubation period (8 days) as evidenced from time-course experiment were used for the preparative scale fermentation with 0.4 g of azadiradione. The purification over silica-gel column of the extracted crude biotransformation products

obtained by incubating azadiradione (**1**) with *M881* furnished two pure metabolites and characterized as 17 β -hydroxyazadiradione (**2**, 0.21 g, yield ~ 51%, R_f : 0.65, R_t : 38.0

min) and 12 β -hydroxyazadiradione (**3**, 0.14 g, yield ~ 33%, R_f : 0.56, R_i : 28.1 min) (Scheme 3B.1).

3B.2.3 Characterization of the Metabolites

Metabolites were structurally characterized on the basis of IR, LC-ESI-MS (positive ion mode), HRMS, ^1H , ^{13}C , DEPT and 2D (COSY, NOESY, HMBC, HSQC) NMR studies. The HRMS analysis of metabolite **2** showed an ion peak at m/z 489.2254



($\text{C}_{28}\text{H}_{34}\text{O}_6\text{Na}$), indicating the addition of an oxygen atom to azadiradione structure ($\text{C}_{28}\text{H}_{34}\text{O}_5$). Appearance of broad absorption band at 3448 cm^{-1} in IR spectrum and a highly abundant peak at m/z 449.2 ($[\text{M}-\text{H}_2\text{O}+\text{H}]^+$) in LC-ESI-MS of **2** indicated the presence of

a hydroxyl group. From the ^{13}C (DEPT) NMR studies, the number of quaternary carbons has increased by one with a loss of one tertiary carbon in comparison to the substrate which suggested that hydroxylation must occurred on a tertiary carbon. The ^1H NMR spectra of **2** was similar to that of **1** except the absence of peak at 3.43 ppm for 17-H. ^{13}C NMR showed downfield shift of C-17 from 60.76 to 80.56 ppm suggesting the presence of a hydroxyl group at C-17. HMBC experiment showed correlation of hydroxylated carbon (C-17) with H-15, 18, and 22 which further confirmed the 17-hydroxylation. The NOESY spectrum containing the cross peaks H-21/H-18, H-22/H12 α evidenced that the furan ring is in α -orientation (Figure 3B.4). Based on these spectral characteristics, the metabolite was identified as 17 β -hydroxyazadiradione (**2**). Also, the ^1H and ^{13}C NMR data matched well with the earlier report.³³

Similarly, from the HRMS data of metabolite **3**, the molecular formula was established as $\text{C}_{28}\text{H}_{34}\text{O}_6$ based on ion peak at m/z 489.2286 ($\text{C}_{28}\text{H}_{34}\text{O}_6\text{Na}$) and the presence of hydroxyl group was confirmed from the appearance of a broad absorption band at 3448 cm^{-1} in IR spectrum and a highly abundant peak at m/z 449.2 ($[\text{M}-\text{H}_2\text{O}+\text{H}]^+$) in LC-ESI-MS. Loss of a methylene carbon and increase in the number of tertiary carbon in the ^{13}C (DEPT) nmr of the metabolite, indicated that the

hydroxylation has occurred on a methylene carbon. Among three methylene carbons (C-6, 11 and 12) in the structure, the chemical shift of C-6 remained almost unchanged in the metabolite whereas C-11 and C-12 underwent downfield shift from 15.83 to 28.31

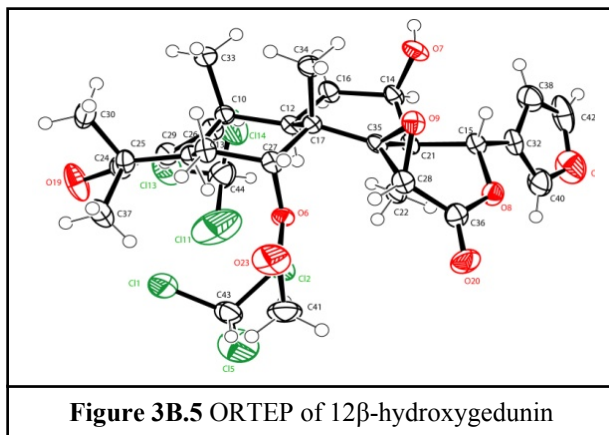


Figure 3B.5 ORTEP of 12 β -hydroxygedunin

and 30.36 to 68.37 ppm respectively accompanied by a downfield shift of quaternary C-13 carbon. These observations indicated the presence of hydroxyl group at C-12 position, which was again confirmed by HMBC correlation of hydroxylated carbon (C-12) with H-17, 18 and H-

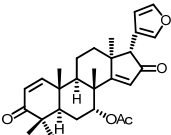
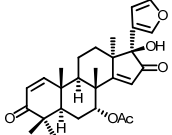
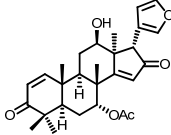
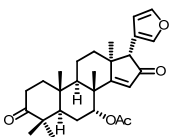
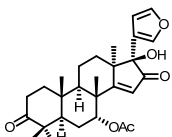
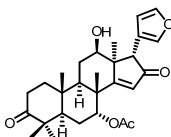
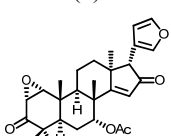
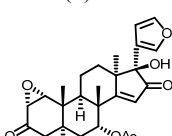
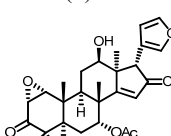
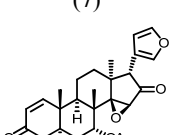
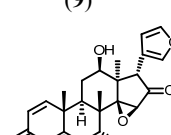
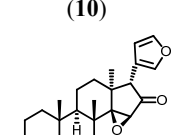
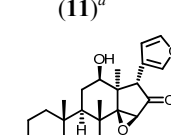
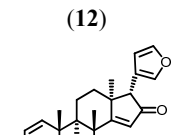
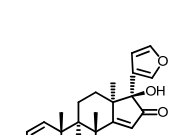
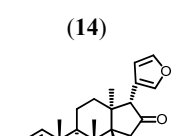
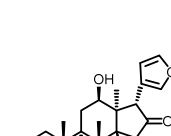
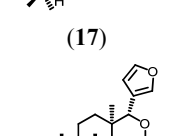
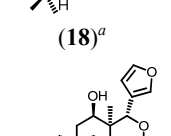
12 with C-18, 9, 13, 14. Hydroxylation was found to be equatorial (on the β -face) on the basis of strong NOESY cross peaks H-12/H-18, H-11 α , H-22 (Figure 3B.4). The presence of axial hydrogen on C-12 was also supported by the high coupling constant (7.32 Hz) of broad doublet assigned for H-12. Position and stereochemistry of 12 β -hydroxylation was further supported by the analysis of single crystal X-ray diffraction data obtained for the metabolite of gedunin (Figure 3B.5).

3B.2.4 Effects of Functional Groups on Bioconversion Profile

To further investigate the substrate specificity of the organism within basic limonoid family, biotransformation was studied with seven natural or semi-synthetic derivatives of azadiradione (Table 3B.1). Fermentation was carried out in preparative scale under similar conditions for each individual azadiradione derivative and the metabolites were characterized after purification.

Reduction and epoxidation of double bond in the A ring of azadiradione did not affect the ability of microorganism to carry out hydroxylation at 17 β - and 12 β -positions as observed with 1,2-dihydroazadiradione (**4**) and 1,2 α -epoxyazadiradione (**7**). Both the substrates were converted to 17 β - and 12 β -hydroxy metabolites almost in the same ratio (3:2) like azadiradione with excellent conversion rate. These results indicated that enone moiety in the ring A is not critical for carrying out the transformation. It is interesting to note that the organism specifically carried out hydroxylation at 12 β -position on epoxyazadiradione (**10**) and its double bond reduced derivative, 1,2-

Table 3B.1 Selectivity in biotransformation products with the variation in functional groups on the limonoid skeleton (substrate concentration 0.2 g/L and incubation period 8 days)

Entry No.	Substrate	Biocat -alyst	% Conversion	Metabolites			
				17 β -Hydroxy metabolite	%	12 β -Hydroxy metabolite	%
1.	 (1)	M881	99	 (2)	61	 (3) ^a	38
2.	 (4)	M881	99	 (5) ^a	59	 (6) ^a	40
3.	 (7)	M881	96	 (8) ^a	57	 (9) ^a	39
4.	 (10)	M881	99	Not produced	-	 (11) ^a	99
5.	 (12)	M881	93	Not produced	-	 (13) ^a	93
6.	 (14)	M881	92	 (15)	71	Not produced	-
7.	 (17)	M881	94	Not produced	-	 (18) ^a	94
8.	 (19)	M881	96	Not produced	-	 (20) ^a	96

^a Metabolites are novel *i.e.* synthesized or isolated for the first time. ^b Parentheses are showing the numbering of compounds

dihydroepoxyazadiradione (**12**). Indeed, the organism completely failed to carry out the 17 β -hydroxylation on **10** and **12** as indicated by HPLC and LC-MS analyses. The organism also accepted gedunin (**19**, expanded D ring as six membered lactone) as a substrate to produce 12 β -hydroxy gedunin as a sole biotransformed product. The absolute structure of 12 β -hydroxygedunin was elucidated by single crystal X-ray diffraction analysis³⁴ along with NMR studies. Similarly, organism was able to hydroxylate 7-deacetylepoxiazadiradione (**17**) at 12 β -position as a single metabolite. However, when 7-deacetylazadiradione (**14**, nimbocinol) was used as a substrate, the position of hydroxylation was changed to 17 β -position to produce 17 β -hydroxynimbocinol (**15**, 71%) along with oxidised product 7-oxo-17 β -hydroxynimbocinol (**16**, 21%) as metabolites. These results suggested that two functionalities (epoxy group in ring D and acetate in ring B) were critical in carrying out the hydroxylation and may bring about a sharp alternation in the product distribution ratio (17 β -hydroxy : 12 β -hydroxy). It is known that, chemically synthesized 17 β -hydroxynimbocinol (**15**) (~10% yield) from azadiradione (**1**) showed two fold potency against *Anopheles stephensi* Liston compared to nimbocinol.³⁵ Herein, the biocatalyst *M881* was able to oxidize nimbocinol (**14**) to a more potent insecticide 17 β -hydroxynimbocinol (**15**) under mild and more eco-friendly condition with much higher yields.

Modifications in skeletal functional groups didn't change the outcome (such as position and stereochemistry of hydroxylation) of bioconversion but it influenced the selectivity between two regio-isomers. As evidenced, 14,15 β -epoxidation on basic limonoid skeleton can eliminate 17 β -hydroxylation to produce 12 β -hydroxy derivative as the single metabolite. Importantly, biocatalyst *M881* can be used as the general tool for the production of 12 β - or 17 β -hydroxy limonoids of intact limonoid family.

3B.2.5 Biotransformation of Azadiradione by Various Fungi

From the screening experiments, we found that the fungal systems belonging to genera *Mucor* and *Rhizopus* have been able to efficiently and selectively hydroxylate at 12 β - and 17 β -positions of azadiradione. Analyses of HPLC profile of screening experiments revealed that some of the *Mucor* and *Rhizopus* species converted azadiradione to 17 β -hydroxy as the major metabolite in fairly good yields (Table 3B.2). For example, one of

the *Rhizopus sp.* (NCIM catalogue no. 1009) converted azadiradione to 17 β -hydroxy metabolite in 5:1 ratio with an overall 75.4% conversion (Table 3B.2). Therefore, these systems can be used for the selective 17 β -hydroxylation.

Table 3B.2 Product distribution table for the fungal systems showing considerable bioconversion of azadiradione (1)

Strain	NCIM catalogue no.	% Conversion	% 2	% 3
<i>Rhizopus oryzae</i>	1009	75.4	62.6	12.8
<i>Mucor heimalis</i>	873	69.3	51.6	17.7
<i>Rhizopus oryzae</i>	877	45.5	37.7	7.8
<i>Mucor piriformis</i>	- ^a	45.2	37.2	8.0
<i>Cephalophora irregularis</i>	1278	31.6	17.1	14.5
<i>Aspergillus foetidus</i>	505	20.4	13.2	7.2
<i>Fusarium proliferatum</i>	1105	7.3	7.3	-
<i>Aspergillus niger</i>	616	4.8	4.8	-
<i>Aspergillus giganteus</i>	568	4.7	4.8	-
^a soil isolated				

3B. 3 Conclusion

In conclusion, we have developed a highly efficient fungi mediated bioconversion technique for the 12 β - and 17 β -hydroxylation of the basic limonoid family. The synthetically challenging hydroxylation on inactive carbon (C-12) was achieved in a highly efficient and stereoselective manner. Chemically sensitive functional groups such as lactone, epoxide or ester were tolerable to the mild reaction condition. Using the biocatalyst *M88I*, variety of 12 β - and 17 β -hydroxylimonoids were generated among which many are hitherto unknown (**3**, **5**, **6**, **8**, **9**, **11**, **13**, **18** and **20**) and can prove to be potent bioactive molecules. Actually, 12 β -hydroxy natural products are rare in nature and therefore this report will create an opening to produce 12 β -hydroxy limonoids and evaluate their bioactivities. The fungal system mimicked the plant monooxygenase system for the production of 17 β -hydroxyazadiradione which is one of the minor metabolites present in Neem fruit.³³ Either of the regio-isomers (12 β - and 17 β - hydroxyl limonoids) was obtained selectively by specific modification of the skeletal functional groups or varying fungal strains within the genera of *Mucor* and *Rhizopus*.

3B. 4 Experimental Section

3B.4.1 General Information

Chemicals (GR grade) for preparing medium and performing the reactions were purchased from Merck and Sigma. All the HPLC Chromatograms were recorded on Waters HPLC system fitted with UV-Vis detector and analytical XBridge C₁₈ column (4.6 × 100 mm). Screening samples were injected in HPLC at a flow rate of 1 mL/min with a gradient solvent programme of: 0 min, 40% methanol/water; 25 min, 50% methanol/water; 40 min, 65% methanol/water; 42 min, 40% methanol/water; 45 min, 40% methanol/water; and monitored at 215 nm. LC-ESI-MS runs were performed in Waters Acquity Ultra Performance LC system attached with PDA and Single Quadrupole detectors. For extractions and preparative chromatography purpose technical grade solvents were distilled before use and for HPLC and LC-MS purpose LCMS grade solvents were procured from Sigma. Milli-Q water purification system (Millipore, USA) was used for purifying water required for HPLC and LC-MS experiments and making fermentation medium. Thin-layer chromatography (TLC) was performed on silica gel G-coated plates (0.25 mm for analytical) developed three times in 3 % methanol in dichloromethane. Compounds were visualized under UV light (254 nm) or by spraying with a solution of 3.0% anisaldehyde, 2.8% H₂SO₄, 2% acetic acid in ethanol followed by heating for 1-2 min. Purification of compounds was performed by flash chromatography using 230-400 mesh silica gel columns and dichloromethane/methanol gradient mixture as the eluent. Optical rotations were measured in chloroform at 589 nm on JASCO (P-2000) polarimeter using 10 mm cell (*c* in g/100 mL unit) and thin film IR spectra were recorded with Perkin Elmer FT-IR spectrophotometer in CHCl₃. Recording of NMR (¹H, ¹³C, DEPT-135) data were carried out on Varian INOVA spectrophotometer (200, 400 and 500 MHz) in CDCl₃ and the residual solvent or TMS signals were designated as the reference. Mass spectra (MS) were recorded on a MSI autoconcept UK with ionization energy 70eV and on Waters make QTOF (Synapt-HDMS). Single crystal X-ray data were collected on Super Nova Dual source X-ray Diffractometer system (Agilent Technologies) equipped with a CCD area detector and operated at 250 W power (50 kV, 0.8 mA) to generate Mo K α radiation (λ = 0.71073 Å) and Cu K α radiation (λ = 1.54178 Å). SHELX-9722 was used for the solution of structure.

3B.4.2 Limonoids and Microorganisms

Azadiradione and epoxyazadiradione were isolated from Neem fruits as discussed earlier. Synthetic procedure of the derivatives has been discussed previously (Chapter 2, Section 2B). The microorganisms used in the present study were obtained from National Collection of Industrial Microorganisms (NCIM), NCL, Pune. The *Mucor piriformis* was isolated from garden soil.³⁶

3B.4.3 General Biotransformation Procedure

(a) Fermentation condition

The cultures were stored at +4 °C and sub-cultured on PDA (potato-dextrose-agar) slant at 30 °C. The inoculum prepared from well (36-48 h) grown culture was used (3% v/v) for inoculation of 100 mL sterile modified Czapek Dox medium³⁷ of pH 5.8 in a 500 mL *Erlenmeyer* flask. Flasks were incubated at 30 °C on a rotary shaker (180 rpm) for growing the cultures.

(b) Screening experiment

To well-grown culture (pH of which was adjusted to 6.5-7.0 using sterile 1 M K₂HPO₄) 20 mg of azadiradione in 0.2 mL acetone was added and incubated at 30 °C and 180 rpm for 8 days. After this incubation period, mycelia and broth were extracted separately with ethyl acetate (three times) and analyzed by TLC and HPLC. All the screening experiments were analyzed by comparing with corresponding substrate control and organism control.

(c) Time-course experiment

In time course experiments, incubations were carried out for one to ten days using azadiradione (0.2 g/L) in acetone by *M881*. Aliquots were drawn at regular interval of every two days and extracted with ethyl acetate. The metabolites formed were monitored by HPLC analysis. The level of different metabolites formed was determined on the basis of the area under the respective peaks. All analyses were carried out under identical conditions.

(d) Resting cell experiment

Well-grown culture of *M881* (36 h) was filtered and the mycelia obtained were washed repeatedly with distilled water and phosphate buffer (pH 7.2). The mycelia were dried by pressing between the filter papers. 3 g of mycelia (wet weight) was weighed and added into 20 mL of phosphate buffer of pH 7.2 containing 150 mg dextrose. 3 mg of azadiradione in 0.2 mL acetone was added to the reaction mixture and incubated at 30 °C on a rotary shaker (180 rpm) for 36 hours. After this incubation period, filtered mycelia and broth were extracted with ethyl acetate (20 mL $\times$ 2) and analysed by TLC and HPLC.

(e) Preparative scale fermentation

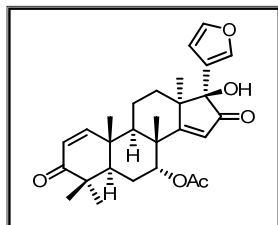
0.4 g limonoid (in 2 mL acetone) was distributed among 20 flasks each containing well-grown culture of *M881* after adjusting the pH of the broth to 6.5-7.0. Incubating at 30 °C and 180 rpm for 8 days, mycelia and broth were separated by filtration using muslin cloth and extracted separately with ethyl acetate (2 L $\times$ 3 for broth and 300 mL $\times$ 3 for mycelia). Both the ethyl acetate layers were combined and concentrated under reduced pressure at 50 °C. Extracted crude (~ 0.5 g) was purified over silica gel column using gradient mixture of dichloromethane and methanol. For example, elution of 230-400 mesh silica gel column (20 cm $\times$ 2.5 cm) loaded with 0.53 g crude biotransformed product of azadiradione furnished 17 β -hydroxyazadiradione (**2**) in 0.6% methanol in dichloromethane and 12 β -hydroxyazadiradione (**3**) in 0.8% methanol in dichloromethane as eluent.

(f) Characterization of the metabolites

The individual purified metabolites were characterised by NMR, IR and Mass spectra as discussed for the characterization of 12 β -hydroxy and 17 β -hydroxy azadiradione metabolites in the results and discussion section. 12 β -hydroxygedunin (**20**) was crystallized from chloroform (solvent of crystallization = 2) as colourless needles by slow evaporation of solvent at 4 °C and structure was determined by single crystal X-ray diffraction method.

3B.4.4 Characterization Data of the Metabolites

17 β -Hydroxyazadiradione: White solid. $[\alpha]_D^{28} = +64.7$ (*c* 3.25, CHCl₃). UV (Me₂CO)

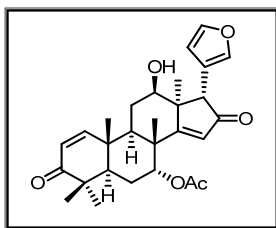


$\lambda_{\max}$: 210 nm. IR (CHCl₃) $\nu_{\max}$ (cm⁻¹): 3448 (-OH), 1736 (Acetate carbonyl), 1706 (D ring ketone), 1665 (A ring ketone), 1598 (D ring tri-substituted double bond), 1383 (A ring geminal methyls), 1243 (Acetate C-O), 1216, 1160, 1029, 874 (Furan ring). LC-MS (ESI) *m/z*: 489.3 ([M+Na]⁺), 467.3 ([M+H]⁺), 449.2 ([M-OH]⁺), 407.2 ([M-OAc]⁺).

HRMS (MALDI-TOF) *m/z*: [M+Na]⁺ Calcd for C₂₈H₃₄O₆Na, 489.2252; found, 489.2254. ¹H (CDCl₃, 400 MHz), ¹³C (CDCl₃, 100 MHz) and 2D NMR data:

Position	δ_H (ppm), <i>J</i> (in Hz)	δ_C (ppm)	HMBC (H→C)	NOESY (H→H)
1	7.14 (d,10.04)	157.21	3,5,9,10,19	19,2,9
2	5.86 (d,10.04)	125.71	4,10	1
3	-	204.19		
4	-	43.97		
5	2.17 (m)	45.89	4,7,10,19,28,29	28,9
6	1.79-2.06 (m)	23.39		
7	5.29 (m)	74.04	5,9,8,30, -COCH ₃	30,15
8	-	44.75		
9	2.3 (m)	38.62	1,8,10,11,14,19,30	18,5,1
10	-	39.85		
11	1.79-2.06 (m)	15.77		
12	β 2.56 (m), α 1.57(m)	22.20	β 11,13,17,18	(β) 30,12 α
13	-	50.24		
14	-	193.66		
15	5.74 (s)	122.40	8,13,14,16,17	30,7
16	-	206.02		
17	-	80.56		
18	0.94 (s)	30.76	12,13,14,17	12 α ,9,22,21
19	1.22 (s)	19.03	1,5,9,10	29,1,30
20	-	120.17		
21	7.52 (m)	141.31	20,22,23	18
22	6.37 (m)	109.52	17,20,21,23	18,12 α ,23
23	7.38 (m)	142.62	20,21	22
28	1.05 (s)	26.92	29	5
29	1.07 (s)	21.15	3,4,5,28	19
30	1.34 (s)	25.11	7,8,9,14	19,12 β ,7,15
-COCH ₃	1.91 (s)	20.9	-COCH ₃	
-COCH ₃	-	169.67		

12 β -Hydroxyazadiradione: White solid. $[\alpha]_D^{28} = -3.3$ (c 2.35, CHCl_3). UV (Me_2CO)

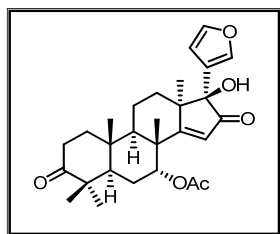


λ_{max} : 210 nm. IR (CHCl_3) ν_{max} (cm^{-1}): 3448 (-OH), 1735 (Acetate carbonyl), 1719 (A ring double bond), 1691 (D ring ketone), 1670 (A ring ketone), 1600 (D ring tri-substituted double bond), 1384 (A ring geminal methyls), 1243 (Acetate C-O), 1216, 1152, 1027, 874 (Furan ring). LC-MS (ESI) m/z :

489.3 ($[\text{M}+\text{Na}]^+$), 467.2 ($[\text{M}+\text{H}]^+$), 449.2 ($[\text{M}-\text{OH}]^+$), 407.3 ($[\text{M}-\text{OAc}]^+$). HRMS (MALDI-TOF) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{28}\text{H}_{34}\text{O}_6\text{Na}$, 489.2252; found, 489.2286. ^1H (CDCl_3 , 400 MHz), ^{13}C (CDCl_3 , 100 MHz) and 2D NMR data:

Position	δ_{H} (ppm), J (in Hz)	δ_{C} (ppm)	HMBC (H $\rightarrow$ C)	NOESY (H $\rightarrow$ H)
1	7.11 (d,10.38)	156.48	19,9,10,5,3	19,9,2
2	5.87 (d,10.07)	126.05	10,4	1
3	-	204.06		
4	-	44.13		
5	2.18 (m)	46.31	19,29,28,10,4,7	28,9
6	1.90 (m),2.03 (m)	23.43		
7	5.31 (m)	74.55	5,8,9,30, <u>C</u> OCH ₃	30,15
8	-	44.43		
9	2.49 (m)	37.92	19,11,10,14,30,8	18,5,1
10	-	39.76		
11	α 2.53 (m), β 1.90 (m)	28.31	(α) 9	(α) 12,11 β
12	4.05 (br. d, 7.32)	68.37	18,9,11,13,14	18,11 α ,17,22
13	-	52.73		
14	-	189.97		
15	5.90 (m)	125.02	17,14,16,8,13	30,7
16	-	205.76		
17	4.31 (s)	52.38	18,13,12,22,20,21,16	12,22,21
18	0.99 (s)	25.22	13,12,14	12,22,9,21
19	1.24 (s)	19.26	1,9,10,5	1,29,30
20	-	118.50		
21	7.46 (m)	141.98	20,22,23	18,17
22	6.27 (m)	111.28	20,21,17	18,12,17,23
23	7.40 (m)	142.87	20,21	22
28	1.07 (s)	26.94	29	5
29	1.08 (s)	21.21	28,4,5,3	19
30	1.48 (s)	25.49	9,8,7,14	7,15,19
-CO <u>C</u> H ₃	1.94 (s)	21.02	<u>C</u> OCH ₃	
- <u>C</u> OCH ₃	-	169.58		

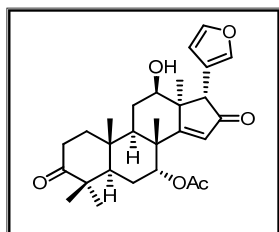
17 β -Hydroxy-1,2-dihydroazadiadione (5): White solid. $[\alpha]^{28}_D = +44.2$ (*c* 2.50,



CHCl_3). UV (Me_2CO) λ_{max} : 208 nm. IR (CHCl_3) ν_{max} (cm^{-1}): 3446 (-OH), 1733 (Acetate carbonyl), 1705 (D ring ketone and A ring ketone), 1598 (D ring tri-substituted double bond), 1381 (A ring geminal methyls), 1242 (Acetate C-O), 1158, 1030, 873 (Furan ring). ^1H NMR (CDCl_3 , 400 MHz) δ : 7.58 (1H, m, H-

21), 7.42 (1H, m, H-23), 6.40 (1H, m, H-22), 5.78 (1H, s, H-15), 5.31 (1H, m, H-7), 2.61 (1H, m, H-2 β), 2.44 (1H, m, H-2 α), 2.15 (1H, m, H-9), 1.95 (3H, s, COCH_3), 1.33 (3H, s, H-30), 1.12 (3H, s, H-19), 1.06 (3H, s, H-29), 1.03 (3H, s, H-28), 0.97 (3H, s, H-18). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 216.08 (C-3), 205.98 (C-16), 194.18 (C-14), 169.73 (C- COCH_3), 142.78 (C-23), 141.43 (C-21), 122.53 (C-20), 120.19 (C-15), 109.53 (C-22), 80.59 (C-17), 74.49 (C-7), 50.32 (C-13), 48.03 (C-5), 46.85 (C-4), 44.22 (C-8), 42.96 (C-9), 38.47 (C-1), 37.20 (C-10), 33.78 (C-2), 30.40 (C-18), 25.80 (C-28), 24.89 (C-30), 23.96 (C-6), 22.03 (C-12), 21.03, 21.02 (C-29, COCH_3), 15.67 (C-11), 15.14 (C-19). LC-MS (ESI) m/z : 491.2 ($[\text{M}+\text{Na}]^+$), 469.2 ($[\text{M}+\text{H}]^+$), 451.4 ($[\text{M}-\text{OH}]^+$), 409.2 ($[\text{M}-\text{OAc}]^+$). HRMS (MALDI-TOF) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{28}\text{H}_{36}\text{O}_6\text{Na}$, 491.2409; found, 491.2388.

12 β -Hydroxy-1,2-dihydroazadiadione (6): White solid. $[\alpha]^{28}_D = -42.6$ (*c* 3.50, CHCl_3).

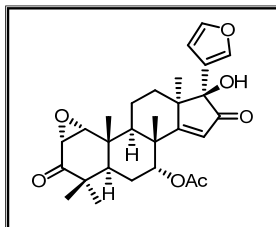


UV (Me_2CO) λ_{max} : 220, 201 nm. IR (CHCl_3) ν_{max} (cm^{-1}): 3462 (-OH), 1736 (Acetate carbonyl), 1700 (D ring ketone and A ring ketone), 1600 (D ring tri-substituted double bond), 1381 (A ring geminal methyls), 1242 (Acetate C-O), 1154, 1028, 874 (Furan ring). ^1H NMR (CDCl_3 , 400 MHz) δ : 7.46 (1H, m, H-21), 7.42

(1H, m, H-23), 6.27 (1H, m, H-22), 5.91 (1H, s, H-15), 5.30 (1H, m, H-7), 4.28 (1H, s, H-17), 4.03 (1H, br. d, $J = 6.7$ Hz, H-12), 2.61 (1H, m, H-2 β), 2.44 (1H, m, H-2 α), 2.29 (2H, m, H-9, 11 α), 1.95 (3H, s, COCH_3), 1.46 (3H, s, H-30), 1.11 (3H, s, H-19), 1.06 (3H, s, H-29), 1.02 (3H, s, H-28), 1.01 (3H, s, H-18). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 216.09 (C-3), 206.16 (C-16), 190.70 (C-14), 169.69 (C- COCH_3), 142.73 (C-23), 141.86 (C-21), 125.02 (C-15), 118.60 (C-20), 111.26 (C-22), 75.06 (C-7), 68.21 (C-12), 52.86 (C-13), 52.25 (C-17), 48.27 (C-5), 46.87 (C-4), 43.78 (C-8), 42.21 (C-9), 38.48 (C-1), 36.91 (C-10), 33.70 (C-2), 28.29 (C-11), 25.80 (C-28), 24.98 (C-30), 24.87 (C-18), 23.88 (C-6), 21.02 (C- COCH_3), 20.93 (C-29), 15.23 (C-19). LC-MS (ESI) m/z : 491.3

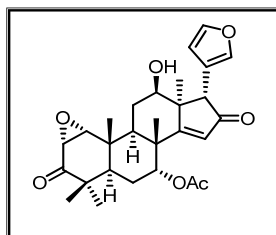
($[M+Na]^+$), 469.3 ($[M+H]^+$), 409.3 ($[M-OAc]^+$). **HRMS (MALDI-TOF) m/z :** $[M+Na]^+$ Calcd for $C_{28}H_{34}O_6Na$, 491.2409; found, 491.2388.

17 β -Hydroxy-1,2 α -Epoxyazadiradione (8): White solid. $[\alpha]_D^{28} = +132.4$ (c 1.50,



$CHCl_3$). **UV (Me_2CO) λ_{max} :** 220, 204 nm. **IR ($CHCl_3$) ν_{max} (cm^{-1}):** 3448 (-OH), 1738 (Acetate carbonyl), 1701 (D ring ketone and A ring ketone), 1598 (D ring tri-substituted double bond), 1381 (A ring geminal methyls), 1242 (Acetate C-O), 1159, 1033, 874 (Furan ring). **^1H NMR ($CDCl_3$, 400 MHz) δ :** 7.60 (1H, m, H-21), 7.43 (1H, m, H-23), 6.41 (1H, m, H-22), 5.78 (1H, s, H-15), 5.29 (1H, m, H-7), 3.61 (1H, d, $J=4.58$ Hz, H-1), 3.43 (1H, d, $J=4.58$ Hz, H-2), 2.62 (1H, m, H-9), 2.55 (1H, m, H-12 β), 2.46 (1H, m, H-5), 1.65 (1H, m, H-12 α), 1.99 (3H, s, $COCH_3$), 1.34 (3H, s, H-30), 1.07 (3H, s, H-19), 1.06 (3H, s, H-18), 1.03 (3H, s, H-28), 1.01 (3H, s, H-29). **^{13}C NMR ($CDCl_3$, 100 MHz) δ :** 211.24 (C-3), 205.80 (C-16), 193.31 (C-14), 169.84 (C- $COCH_3$), 142.88 (C-23), 141.50 (C-21), 122.44 (C-20), 120.09 (C-15), 109.50 (C-22), 80.63 (C-17), 73.83 (C-7), 62.90 (C-1), 56.55 (C-2), 50.28 (C-13), 44.38, 44.17 (C-4,8), 38.88 (C-10), 38.63 (C-5), 37.29 (C-9), 30.39 (C-18), 27.45 (C-28), 25.06 (C-30), 23.24 (C-6), 21.88 (C-12), 21.03 (C- $COCH_3$), 20.73 (C-29), 16.04 (C-11), 15.07 (C-19). **LC-MS (ESI) m/z :** 505.2 ($[M+Na]^+$), 483.2 ($[M+H]^+$), 465.4 ($[M-OH]^+$), 423.2 ($[M-OAc]^+$). **HRMS (MALDI-TOF) m/z :** $[M+Na]^+$ Calcd for $C_{28}H_{34}O_7Na$, 505.2202; found, 505.2164.

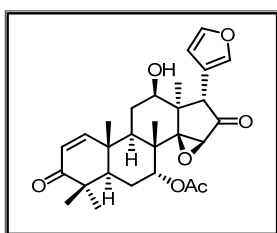
12 β -Hydroxy-1,2 α -Epoxyazadiradione (9): White solid. $[\alpha]_D^{28} = +21.7$ (c 2.50,



$CHCl_3$). **UV (Me_2CO) λ_{max} :** 217, 206 nm. **IR ($CHCl_3$) ν_{max} (cm^{-1}):** 3482 (-OH), 1735 (Acetate carbonyl), 1701 (D ring ketone and A ring ketone), 1600 (D ring tri-substituted double bond), 1381 (A ring geminal methyls), 1243 (Acetate C-O), 1154, 1035, 874 (Furan ring). **^1H NMR ($CDCl_3$, 400 MHz) δ :** 7.48 (1H, m, H-21), 7.44 (1H, m, H-23), 6.28 (1H, m, H-22), 5.93 (1H, s, H-15), 5.28 (1H, m, H-7), 4.30 (1H, s, H-17), 4.10 (1H, dd, $J=7.3, 2.4$ Hz, H-12), 3.55 (1H, d, $J=4.58$ Hz, H-1), 3.42 (1H, d, $J=4.58$ Hz, H-2), 2.78 (1H, m, H-9), 2.59 (1H, m, H-11 α), 2.45 (1H, m, H-5), 1.99 (3H, s, $COCH_3$), 1.47 (3H, s, H-30), 1.09 (3H, s, H-19), 1.07 (3H, s, H-18), 1.05 (3H, s, H-28), 1.01 (3H, s, H-29). **^{13}C NMR ($CDCl_3$, 100 MHz) δ :** 211.15 (C-3), 205.88 (C-16), 189.87 (C-14), 169.79 (C- $COCH_3$), 142.86 (C-23), 141.89 (C-21),

124.99 (C-15), 118.50 (C-20), 111.23 (C-22), 74.41 (C-7), 68.14 (C-12), 62.66 (C-1), 56.46 (C-2), 52.82 (C-13), 52.32 (C-17), 44.19, 44.01 (C-4, 8), 38.86 (C-5), 38.64 (C-10), 36.75 (C-9), 28.61 (C-11), 27.38 (C-28), 25.24 (C-30), 24.92 (C-18), 23.19 (C-6), 21.03 (C- COCH₃), 20.69 (C-29), 15.17 (C-19). **LC-MS (ESI) *m/z***: 505.4 ([M+Na]⁺), 483.2 ([M+H]⁺), 423.2 ([M-OAc]⁺). **HRMS (MALDI-TOF) *m/z***: [M+Na]⁺ Calcd for C₂₈H₃₄O₇Na, 505.2202; found, 505.2164.

12β-Hydroxyepoxyazadiradione (11): White solid. [α]_D²⁸ = +16.4 (*c* 2.60, CHCl₃).

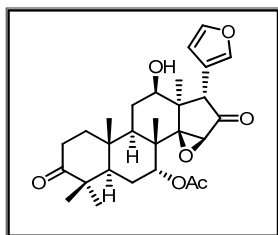


UV (Me₂CO) λ_{max}: 209 nm. **IR (CHCl₃) ν_{max} (cm⁻¹)**: 3481 (-OH), 1749 (Acetate carbonyl and D ring ketone), 1669 (A ring ketone), 1376 (A ring geminal methyls), 1239 (Acetate C-O), 1133, 1026, 875 (Furan ring). **LC-MS (ESI) *m/z***: 505.4 ([M+Na]⁺), 483.3 ([M+H]⁺), 423.4 ([M-OAc]⁺). **HRMS (MALDI-TOF) *m/z***: [M+Na]⁺ Calcd for C₂₈H₃₄O₇Na, 505.2202; found, 505.2195. **¹H (CDCl₃, 400 MHz), ¹³C (CDCl₃, 100 MHz) and 2D NMR data:**

Position	δ _H (ppm), <i>J</i> (in Hz)	δ _C (ppm)	HMBC (H→C)	NOESY (H→H)
1	7.13 (d,10.3)	157.08	3,5,10	19,11α,2
2	5.88 (d,10.0)	125.87	4,10	1
3	-	204.22		
4	-	44.18		
5	2.14 (m)	46.59	4,9,28	9
6	1.96-1.85 (m)	24.18		
7	4.72 (m)	73.49	5,9, -COCH ₃	15,6,30
8	-	43.00		
9	2.52 (m)	39.27	1,8,10,12,30	18,5
10	-	39.35		
11	α2.59 (m),β1.82 (m)	29.04		α 1,12
12	3.95 (dd,8.4,4.9)	70.66	13,14,18	18,11α,17,22
13	-	47.32		
14	-	71.24		
15	3.31 (s)	54.79	16,17	18,30,17,7, -COCH ₃
16	-	208.09		
17	4.48 (s)	44.89	12,13,16,18,20,21,23	21,22,12,15,18
18	1.02 (s)	23.26	13,14,17	21,22,17,12,15,9, COCH ₃
19	1.21 (s)	19.85	1,5,10	1
20	-	116.40		
21	7.61 (m)	141.98	20,22,23	17,18
22	6.35 (m)	111.18	20,21	23,17,12,18
23	7.38 (m)	142.37	20,21	22

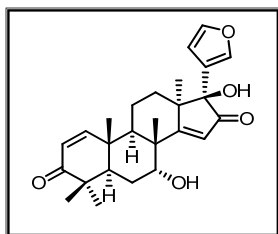
28	1.07 (s)	26.96	29	
29	1.08 (s)	20.88	28,3,4,5	
30	1.26 (s)	19.28	9,7,8,14	7,15
-COCH ₃	2.02 (s)	21.23	-COCH ₃	18,15
-COCH ₃	-	169.69		

12 β -Hydroxy-1,2-Dihydroepoxyazadiradione (13): White solid. $[\alpha]_D^{28} = -14.1$ (*c*



1.00, CHCl₃). **UV (Me₂CO) $\lambda_{\max}$:** 220, 204 nm. **IR (CHCl₃) $\nu_{\max}$ (cm⁻¹):** 3502 (-OH), 1748 (Acetate carbonyl), 1736 (D ring ketone), 1714 (A ring ketone), 1377 (A ring geminal methyls), 1240 (Acetate C-O), 1026, 875 (Furan ring). **¹H NMR (CDCl₃, 400 MHz) δ :** 7.63 (1H, m, H-21), 7.41 (1H, m, H-23), 6.35 (1H, m, H-22), 4.72 (1H, m, H-7), 4.49 (1H, s, H-17), 3.93 (1H, m, H-12), 3.32 (1H, s, H-15), 2.61 (1H, m, H-2 β), 2.48 (1H, m, H-2 α), 2.37-2.43 (2H, m, H-11 α and 9), 2.05 (3H, s, -COCH₃), 1.26 (3H, s, H-30), 1.10 (3H, s, H-19), 1.05 (3H, s, H-29), 1.04 (3H, s, H-28), 1.02 (3H, s, H-18). **¹³C NMR (CDCl₃, 100 MHz) δ :** 215.83 (C-3), 208.10 (C-16), 169.74 (C-COCH₃), 142.46 (C-23), 142.02 (C-21), 116.42 (C-20), 111.18 (C-22), 73.92 (C-7), 71.23 (C-14), 70.67 (C-12), 54.91 (C-15), 48.21 (C-5), 47.24 (C-13), 46.79 (C-4), 44.79 (C-17), 43.78 (C-9), 42.14 (C-8), 39.26 (C-1), 36.89 (C-10), 33.63 (C-2), 29.25 (C-11), 25.97 (C-28), 24.59 (C-6), 22.79 (C-18), 21.34 (C-COCH₃), 20.62 (C-29), 18.97 (C-30), 16.32 (C-19). **LC-MS (ESI) *m/z*:** 507.3 ([M+Na]⁺), 485.3 ([M+H]⁺), 425.4 ([M-OAc]⁺). **HRMS (MALDI-TOF) *m/z*:** [M+Na]⁺ Calcd for C₂₈H₃₆O₇Na, 507.2358; found, 507.2367.

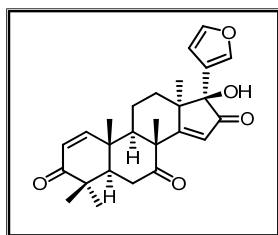
17 β -Hydroxynimbocinol (15): White solid. $[\alpha]_D^{28} = +54.1$ (*c* 3.50, CHCl₃). **UV**



(Me₂CO) $\lambda_{\max}$: 220, 201 nm. **IR (CHCl₃) $\nu_{\max}$ (cm⁻¹):** 3436 (-OH), 1698 (D ring ketone), 1664 (A ring ketone), 1595 (D ring tri-substituted double bond), 1383 (A ring geminal methyls), 1159, 1035, 873 (Furan ring). **¹H NMR (CDCl₃, 400 MHz) δ :** 7.59 (1H, m, H-21), 7.43 (1H, m, H-23), 7.15 (1H, d, *J*=10.23 Hz, H-1), 6.41 (1H, m, H-22), 5.91 (1H, s, H-15), 5.87 (1H, d, *J*=10.23 Hz, H-2), 4.19 (1H, m, H-7), 1.31 (3H, s, H-30), 1.23 (3H, s, H-19), 1.17 (3H, s, H-29), 1.12 (3H, s, H-28), 0.98 (3H, s, H-18). **¹³C NMR (CDCl₃, 100 MHz) δ :** 206.10 (C-16), 204.73 (C-3), 195.36 (C-14), 157.53 (C-1), 142.86 (C-23), 141.42 (C-21), 125.78 (C-2), 122.40 (C-20), 120.19 (C-15), 109.52 (C-22), 80.76 (C-17), 71.76 (C-7), 50.42 (C-13), 46.82 (C-

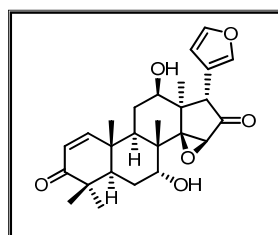
8), 44.44 (C-5), 44.19 (C-4), 40.18 (C-10), 36.89 (C-9), 30.15 (C-18), 27.11 (C-28), 25.57 (C-30), 25.44 (C-6), 22.14 (C-12), 21.42 (C-29), 19.19 (C-19), 15.63 (C-11). **LC-MS (ESI) m/z :** 447.4 ($[M+Na]^+$), 425.3 ($[M+H]^+$), 407.3 ($[M-OH]^+$). **HRMS (MALDI-TOF) m/z :** $[M+Na]^+$ Calcd for $C_{26}H_{32}O_5Na$, 447.2147; found, 447.2223.

17 β -Hydroxy-7-oxonimbocinol (16): White solid. $[\alpha]_D^{28} = +7.61$ (c 3.25, $CHCl_3$). **UV (Me_2CO) λ_{max} :** 219, 204 nm. **IR ($CHCl_3$) ν_{max} (cm^{-1}):** 3421 (-OH), 1708 (D ring ketone), 1673 (A ring ketone), 1595 (D ring



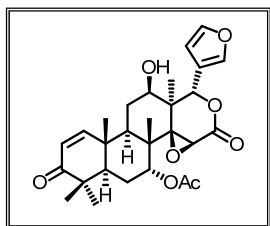
tri-substituted double bond), 1379 (A ring geminal methyls), 1160, 1030, 874 (Furan ring). **^1H NMR ($CDCl_3$, 400 MHz) δ :** 7.58 (1H, m, H-21), 7.42 (1H, m, H-23), 7.15 (1H, d, $J=10.04$ Hz, H-1), 6.38 (2H, m, H-22,15), 5.94 (1H, d, $J=10.29$ Hz, H-2), 2.98 (1H, t, $J=14.56$ Hz, H-6 β), 2.68 (2H, m, H-9,6 α), 2.48 (1H, dd, $J=14.05, 2.51$ Hz, H-5), 1.59 (3H, s, H-30), 1.42 (3H, s, H-19), 1.18 (3H, s, H-29), 1.15 (3H, s, H-28), 0.94 (3H, s, H-18). **^{13}C NMR ($CDCl_3$, 100 MHz) δ :** 206.80 (C-7), 206.07 (C-16), 203.07 (C-3), 185.34 (C-14), 155.57 (C-1), 142.84 (C-23), 141.53 (C-21), 126.76 (C-2), 126.32 (C-15), 122.40 (C-20), 109.40 (C-22), 80.27 (C-17), 54.23 (C-8), 52.79 (C-5), 50.62 (C-13), 45.33 (C-9), 44.80 (C-4), 39.72 (C-10), 36.01 (C-6), 32.03 (C-18), 26.73 (C-28), 25.86 (C-30), 23.47 (C-12), 20.94 (C-29), 18.80 (C-19), 16.99 (C-11). **LC-MS (ESI) m/z :** 445.3 ($[M+Na]^+$), 423.3 ($[M+H]^+$), 405.3 ($[M-OH]^+$). **HRMS (MALDI-TOF) m/z :** $[M+Na]^+$ Calcd for $C_{26}H_{30}O_5Na$, 445.1990; found, 445.1926.

12 β -Hydroxy-7-deacetylepoxызadiradione (18): White solid. $[\alpha]_D^{28} = +27.6$ (c 1.50, $CHCl_3$). **UV (Me_2CO) λ_{max} :** 208 nm. **IR ($CHCl_3$) ν_{max} (cm^{-1}):** 3525 (-OH), 1748 (D ring ketone), 1651 (A ring ketone), 1387 (A ring geminal methyls), 1215, 1024, 875 (Furan ring). **^1H NMR ($CDCl_3$, 400 MHz) δ :** 7.60 (1H, m, H-21), 7.40 (1H, m, H-23), 7.14 (1H, d, $J=10.04$ Hz, H-1), 6.36 (1H, m, H-22), 5.87 (1H, d, $J=10.29$ Hz, H-2), 4.50 (1H, s, H-17), 3.93 (1H, m, H-12), 3.67 (1H, m, H-7), 3.47 (1H, s, H-15), 2.57-2.60 (2H, m, H-9,11 α), 2.41 (1H, m, H-5), 1.20 (3H, s, H-19), 1.18 (3H, s, H-30), 1.14 (3H, s, H-29), 1.10 (3H, s, H-28), 1.06 (3H, s, H-18). **^{13}C NMR ($CDCl_3$, 100 MHz) δ :** 208.82 (C-16), 204.66 (C-3), 157.59 (C-1), 142.38, 142.05 (C-23,21), 125.82 (C-2), 116.75 (C-20), 111.38 (C-22), 72.00 (C-14), 71.57 (C-7), 71.14 (C-12), 55.19 (C-15), 47.53 (C-13), 45.29, 45.08 (C-17,5), 44.38, 44.33 (C-4,8), 39.54



(C-10), 37.84 (C-9), 29.39 (C-11), 27.99 (C-6), 27.13 (C-28), 23.60 (C-18), 21.19 (C-29), 20.02 (C-19), 19.51 (C-20). **LC-MS (ESI) m/z :** 463.0 ($[M+Na]^+$), 441.1 ($[M+H]^+$), 423.1 ($[M-OH]^+$). **HRMS (MALDI-TOF) m/z :** $[M+Na]^+$ Calcd for $C_{26}H_{32}O_6Na$, 463.2097; found, 463.2124.

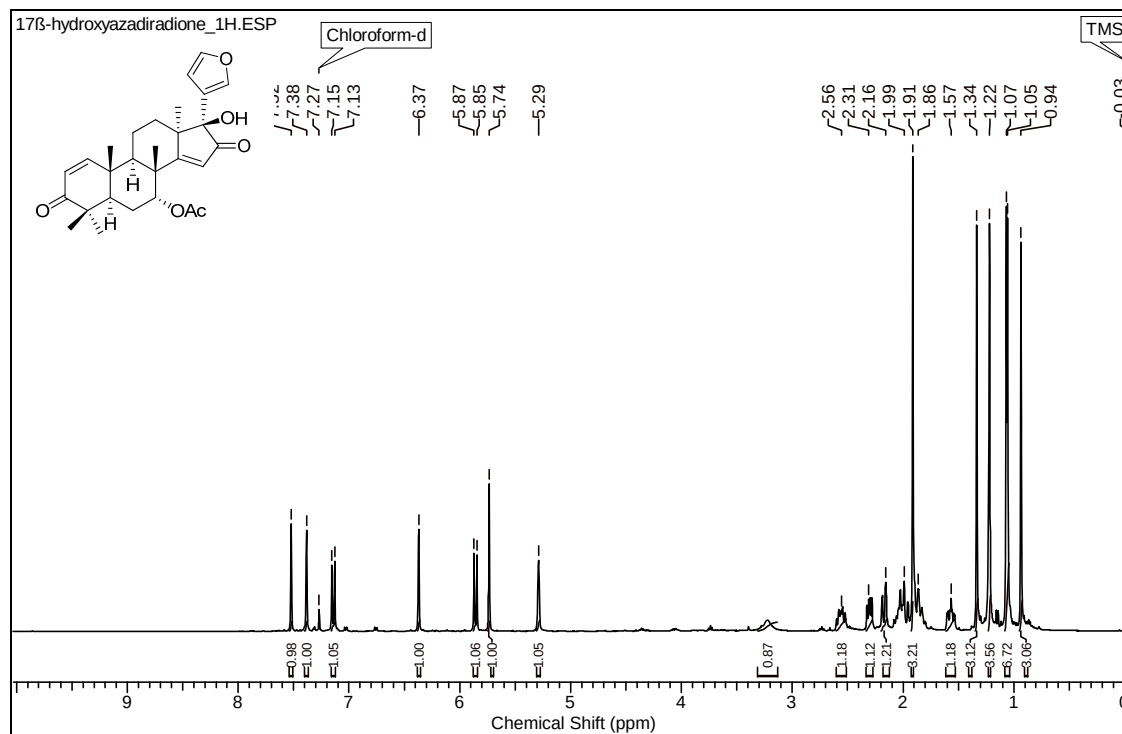
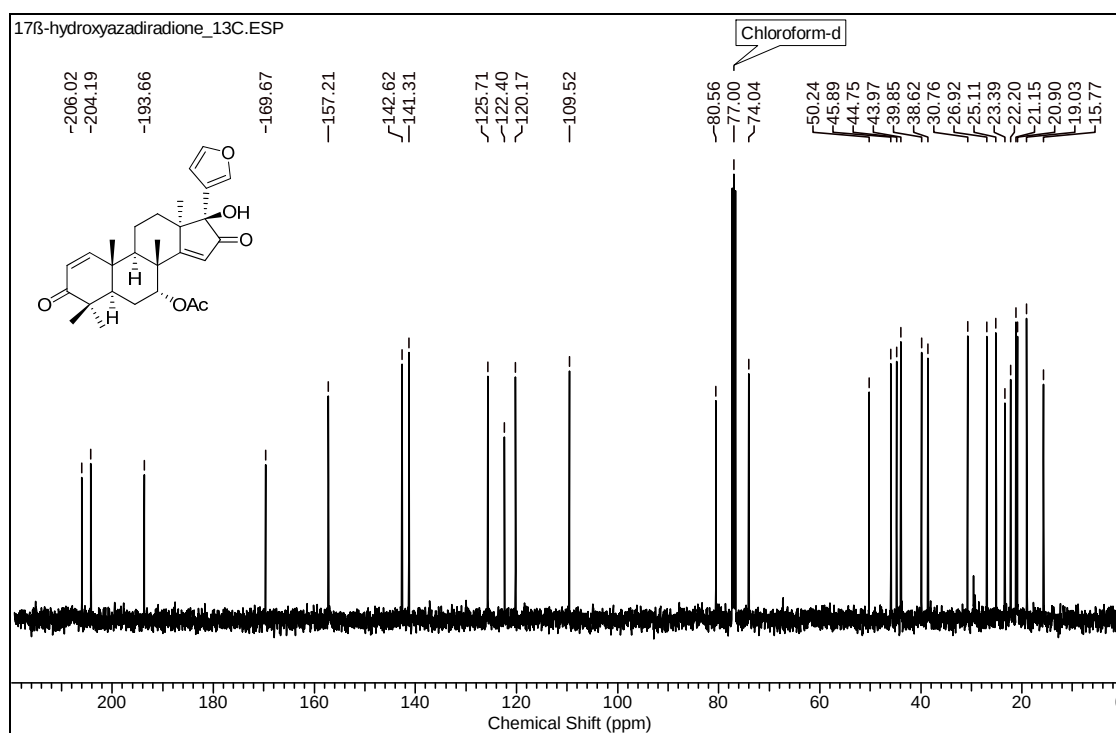
12 β -Hydroxygedunin (20): White solid. $[\alpha]_D^{28} = +11.6$ (c 1.00, $CHCl_3$). **UV** (Me_2CO)

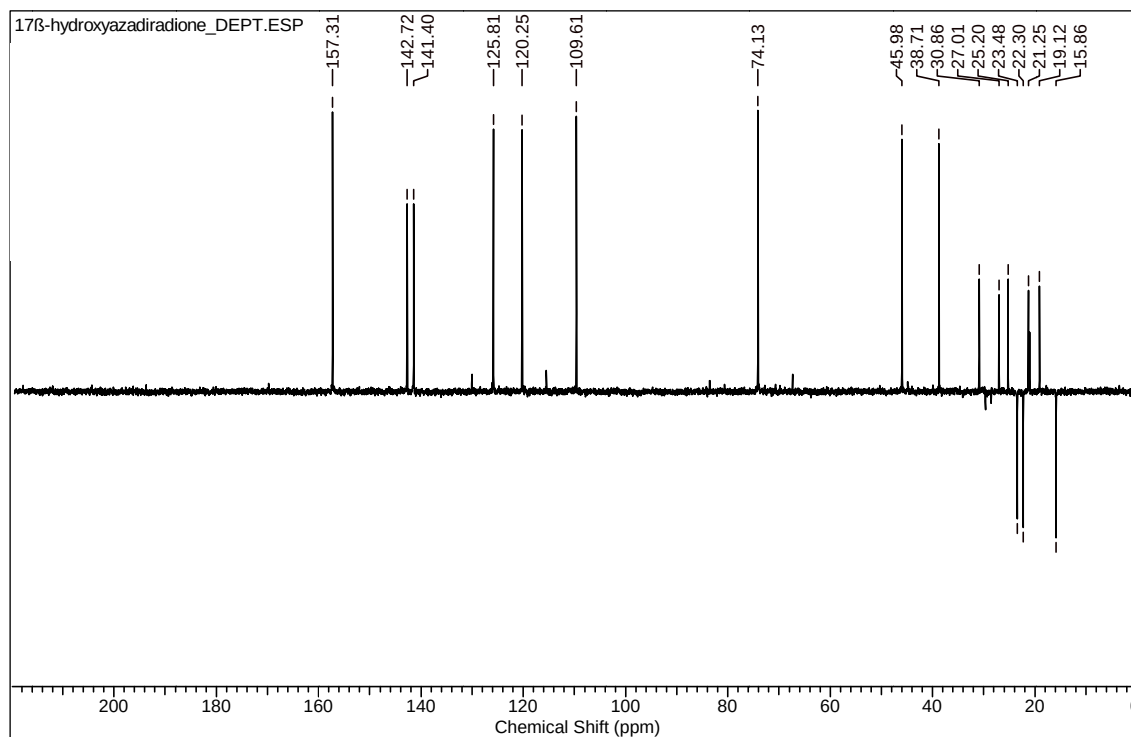
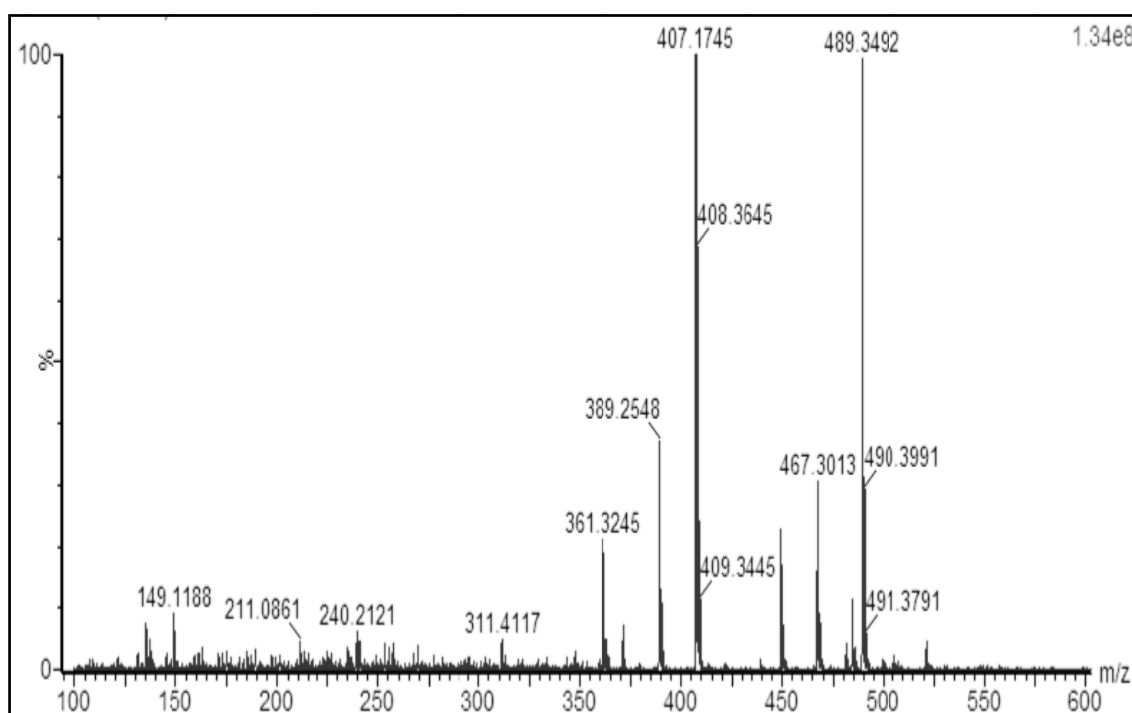


λ_{max} : 214 nm. **IR** ($CHCl_3$) ν_{max} (cm^{-1}): 3393 (-OH), 1734 (Acetate carbonyl and D ring ketone), 1657 (A ring ketone), 1370 (A ring geminal methyls), 1216 (Acetate C-O), 1026, 874 (Furan ring). **^1H NMR** ($CDCl_3$, 400 MHz) δ : 7.51 (1H, m, H-21), 7.43 (1H, m, H-23), 7.04 (1H, d, $J=10.29$ Hz, H-1), 6.47

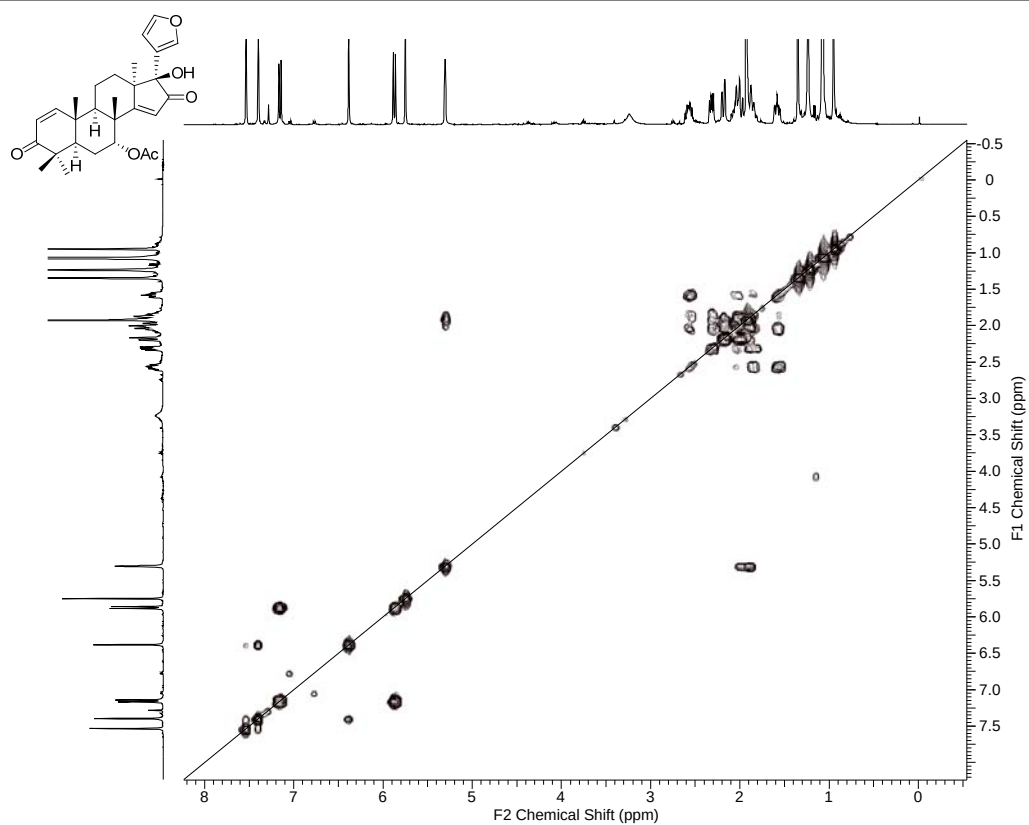
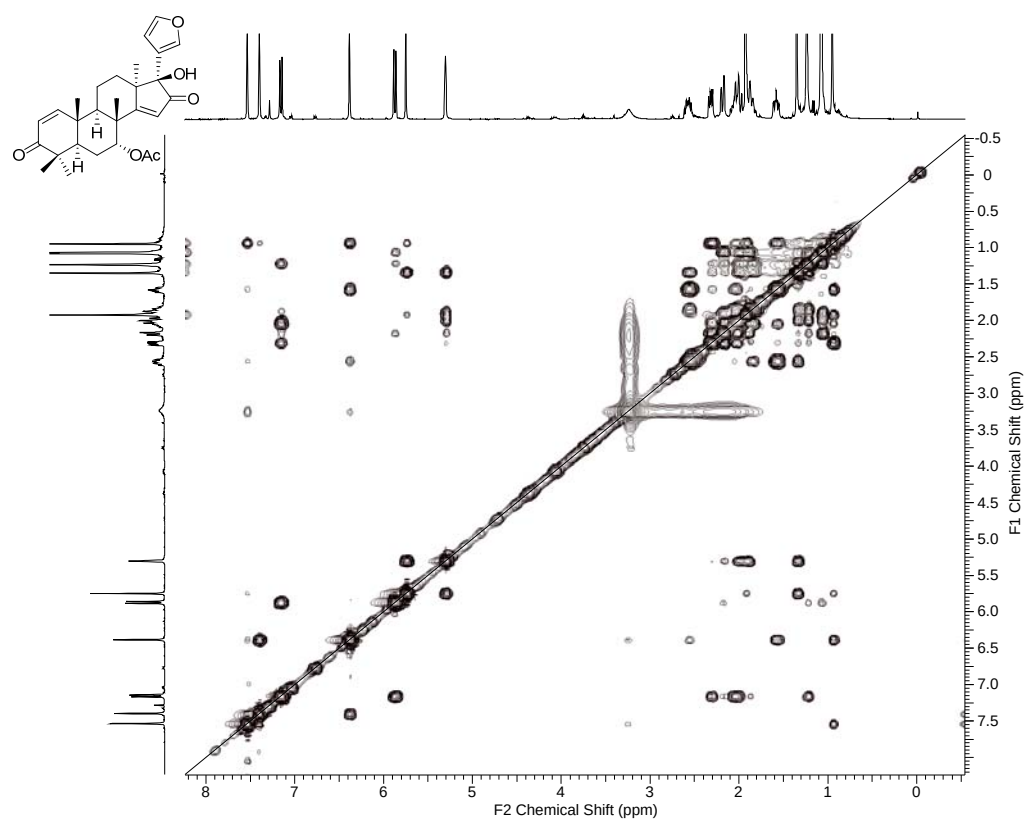
(1H, m, H-22), 6.18 (1H, s, H-17), 5.87 (1H, d, $J=10.04$ Hz, H-2), 4.57 (1H, m, H-7), 3.94 (1H, br. d, $J=7.6$ Hz, H-12), 3.45 (1H, s, H-15), 2.46-2.56 (2H, m, H-9,11 α), 2.17 (1H, m, H-5), 2.12 (3H, s, $-COCH_3$), 1.34 (3H, s, H-18), 1.25 (3H, s, H-19), 1.17 (3H, s, H-30), 1.08 (3H, s, H-29), 1.07 (3H, s, H-28). **^{13}C NMR** ($CDCl_3$, 100 MHz) δ : 203.90 (C-3), 169.89 ($C-COCH_3$), 167.41 (C-16), 156.42 (C-1), 143.21 (C-23), 141.45 (C-21), 126.19 (C-2), 120.02 (C-20), 109.86 (C-22), 75.57 (C-17), 73.38 (C-7), 69.22 (C-12), 68.28 (C-14), 55.02 (C-15), 46.09 (C-5), 44.06 (C-4), 42.82, 42.32 (C-8,13), 39.90 (C-10), 39.22 (C-9), 28.39 (C-11), 27.13 (C-28), 23.14 (C-6), 21.17 (C-29), 21.06 ($C-COCH_3$), 19.87 (C-19), 18.16 (C-30), 15.75 (C-18). **LC-MS (ESI) m/z :** 521.2 ($[M+Na]^+$), 499.2 ($[M+H]^+$), 439.1 ($[M-OAc]^+$). **HRMS (MALDI-TOF) m/z :** $[M+Na]^+$ Calcd for $C_{28}H_{34}O_8Na$, 521.2152; found, 521.2167.

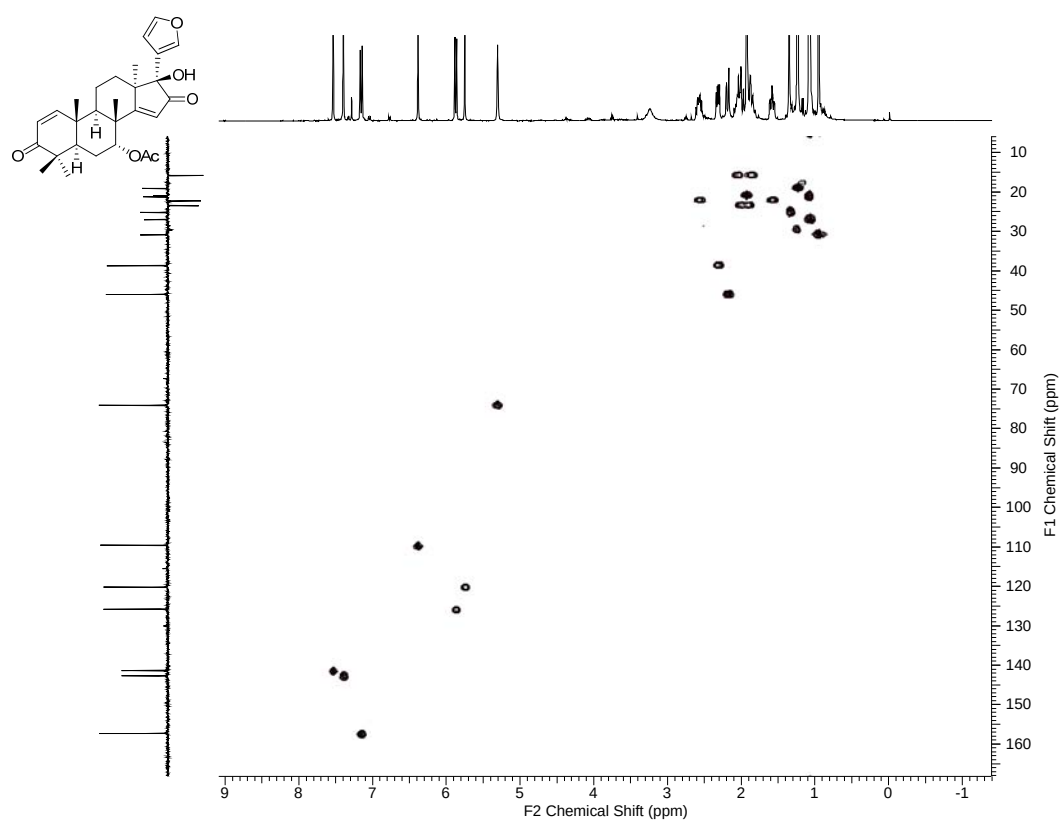
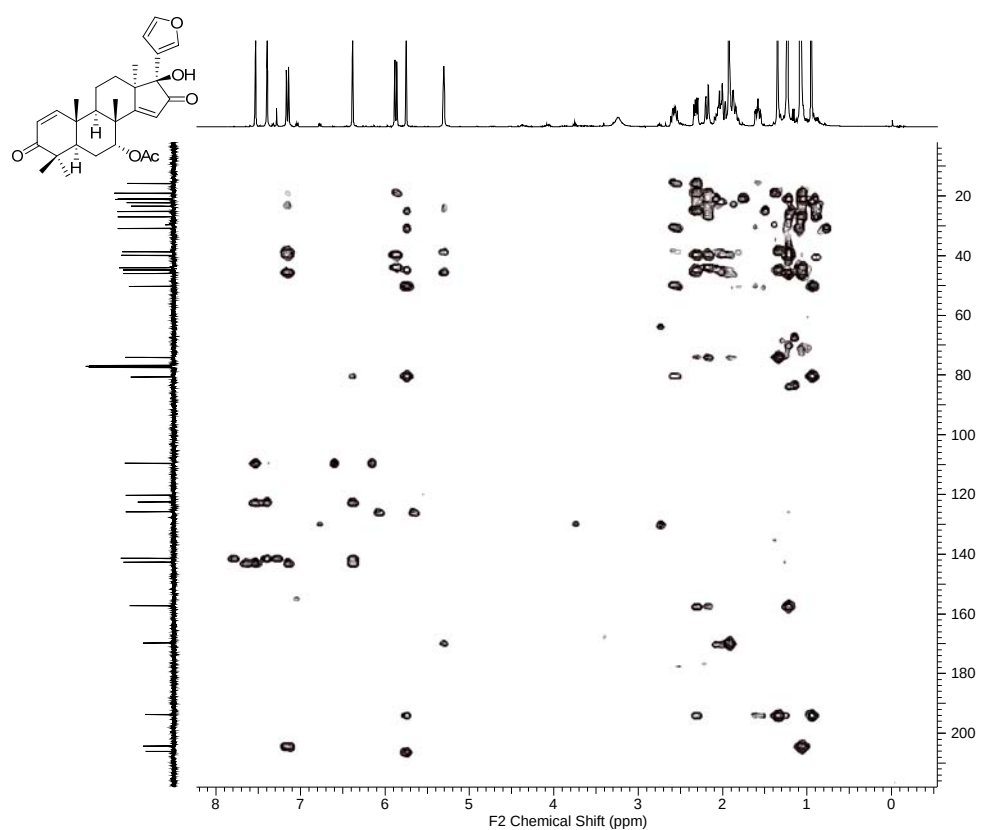
3B.5 Spectral Copies

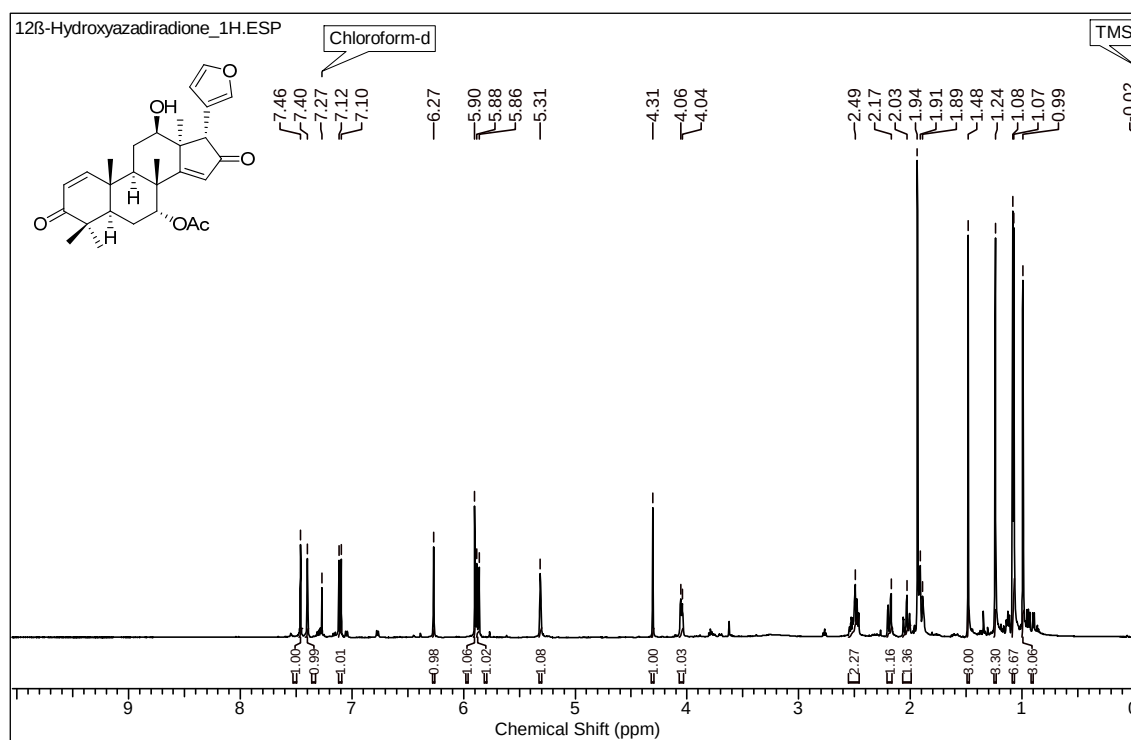
 ^1H NMR spectrum (CDCl_3 , 400 MHz) of 17 β -hydroxyazadiradione ^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 17 β -hydroxyazadiradione

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 17β-hydroxyazadiradione

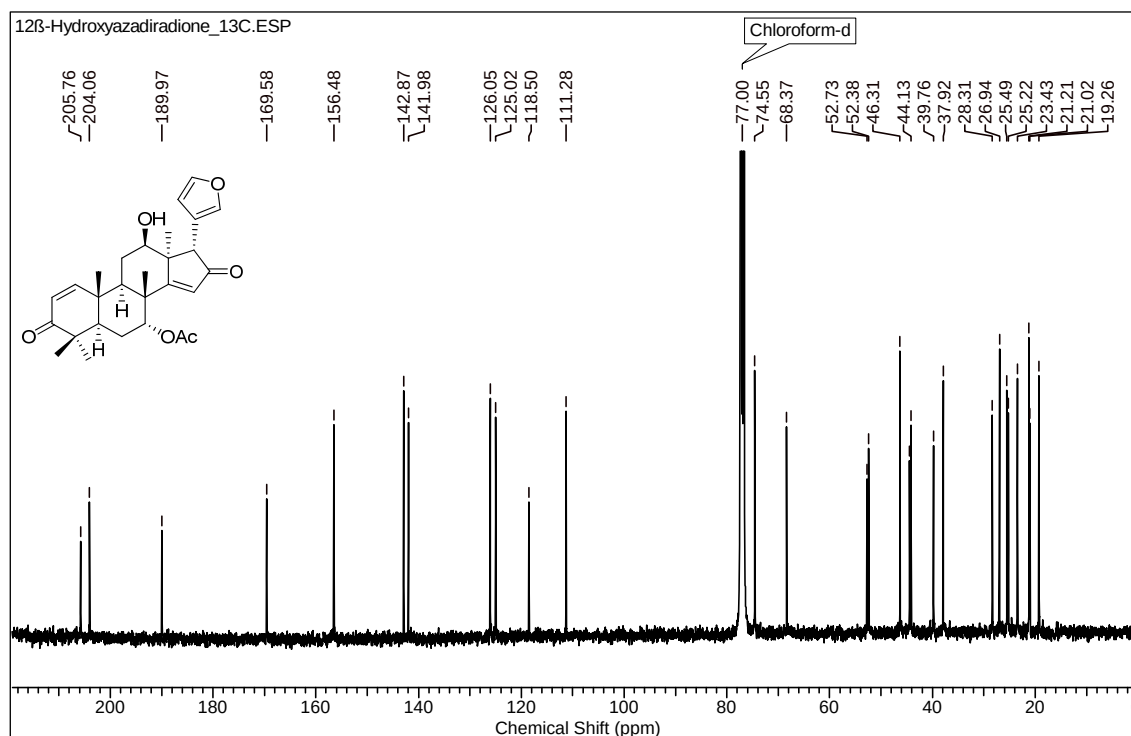
LC-ESI(+)-MS of 17β-hydroxyazadiradione

COSY spectrum of 17β-hydroxyazadiradione in CDCl₃NOESY spectrum of 17β-hydroxyazadiradione in CDCl₃

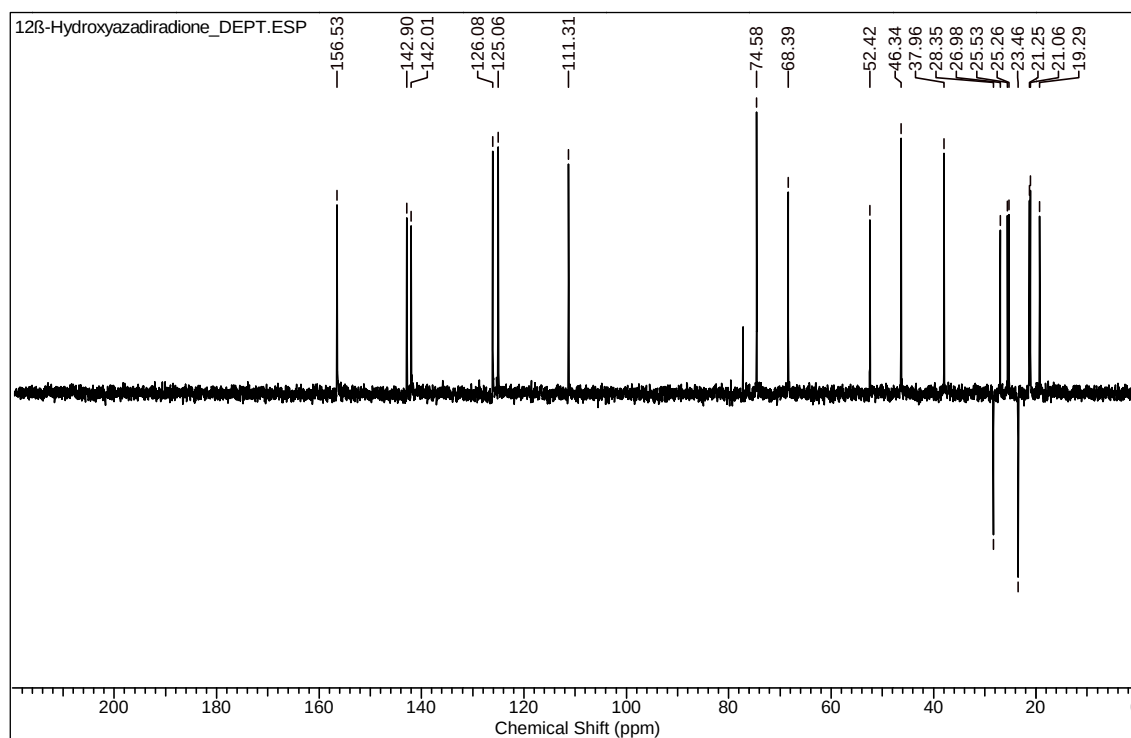
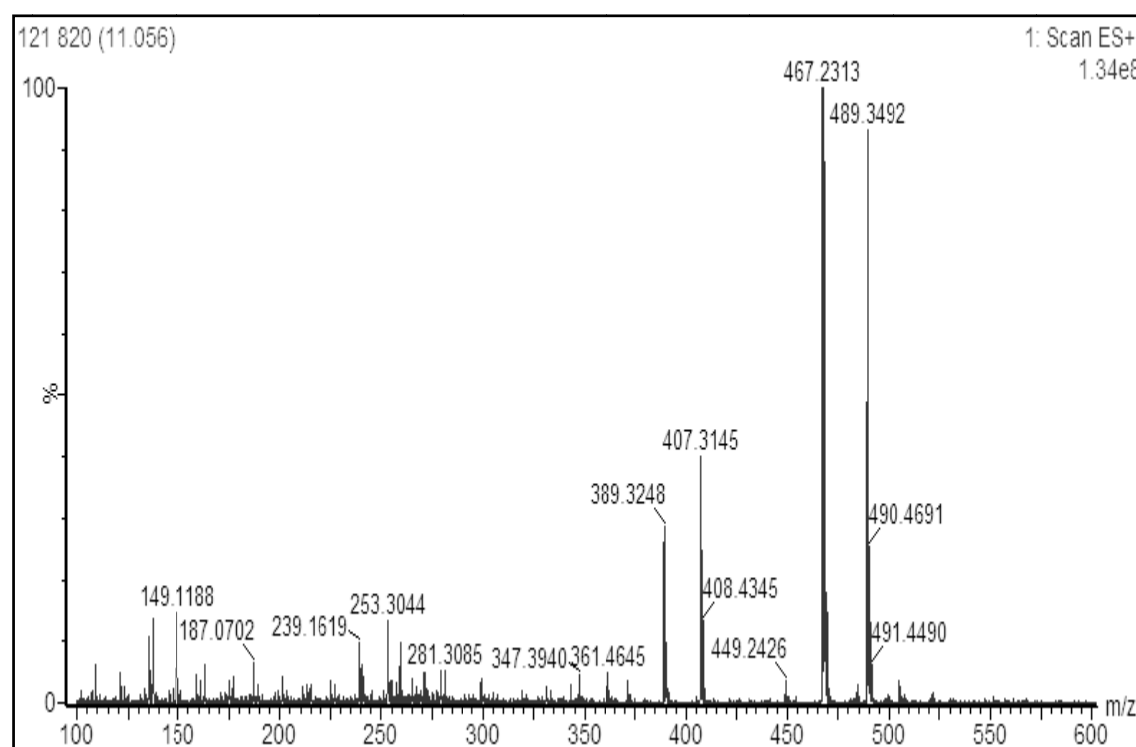
HSQC spectrum of 17β-hydroxyazadiradione in CDCl₃HMBC spectrum of 17β-hydroxyazadiradione in CDCl₃



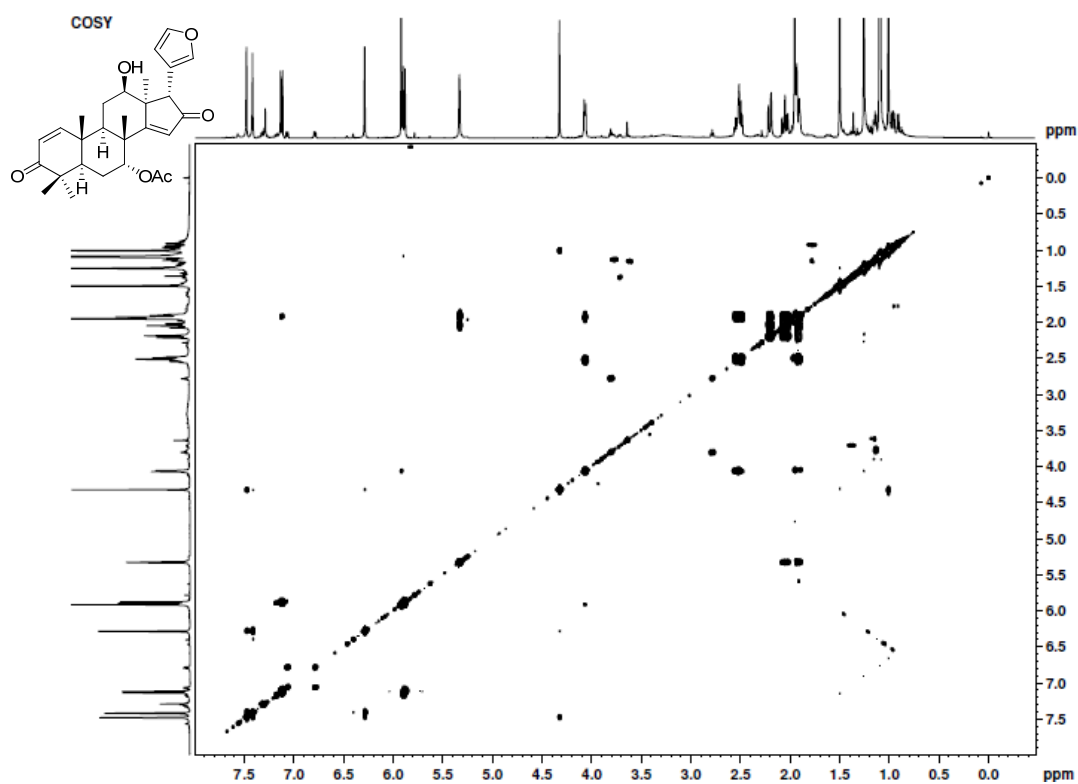
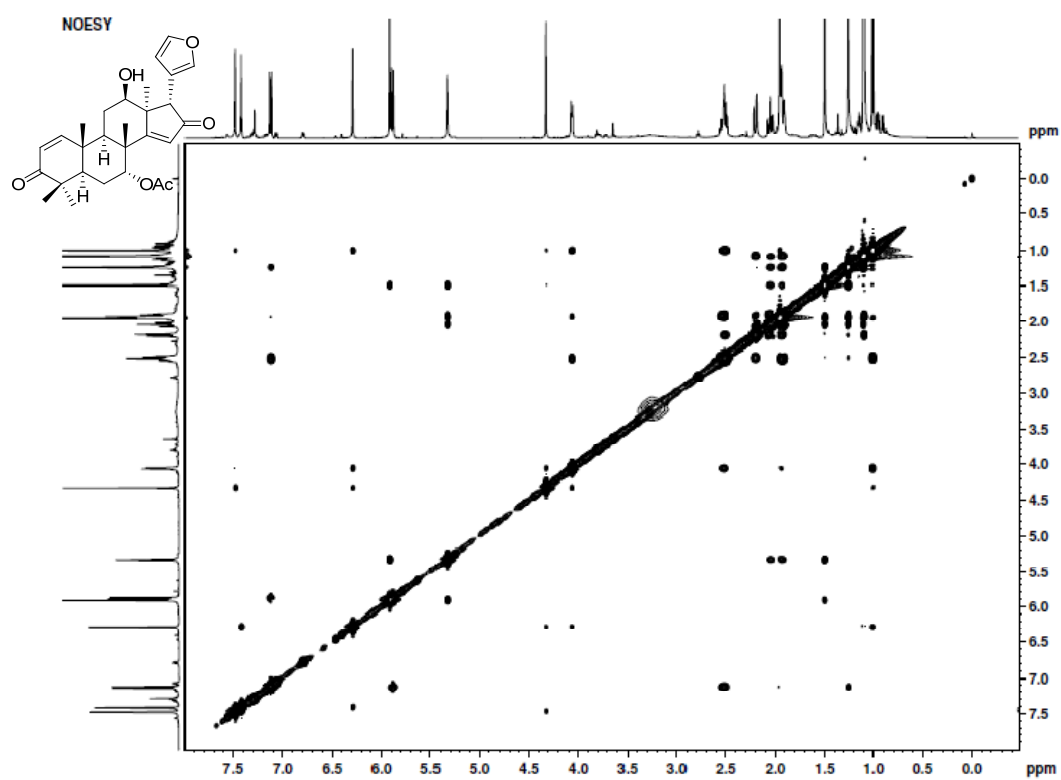
^1H NMR spectrum (CDCl_3 , 400 MHz) of 12 β -hydroxyazadiradione

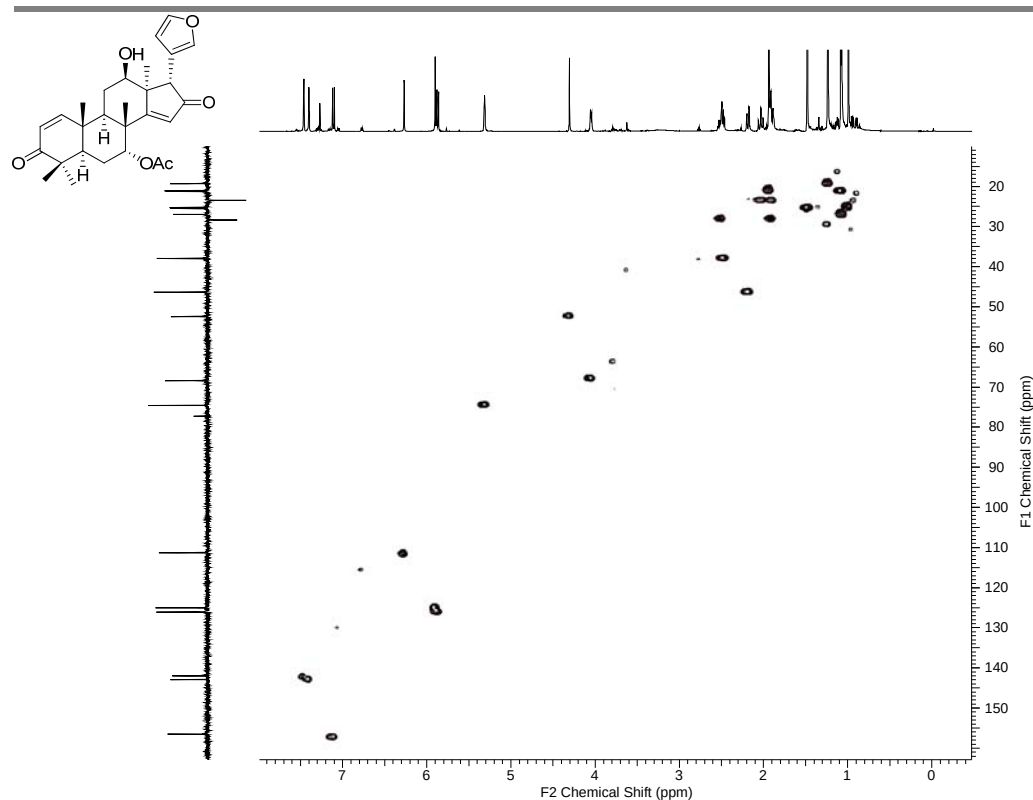
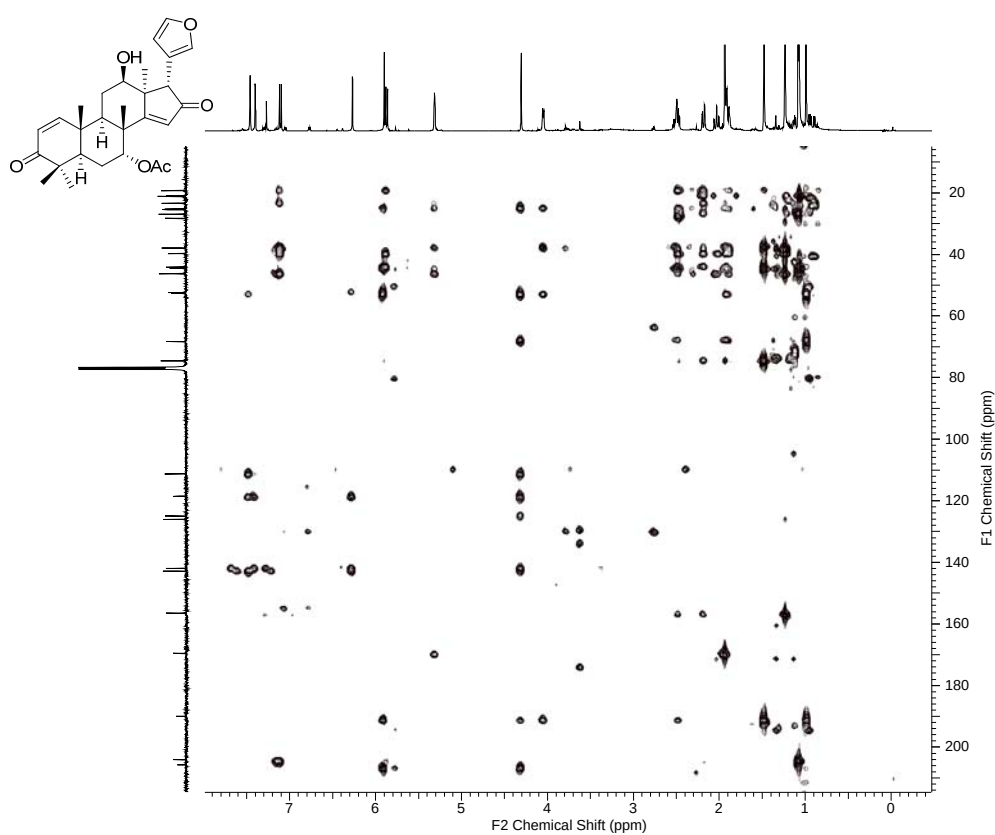


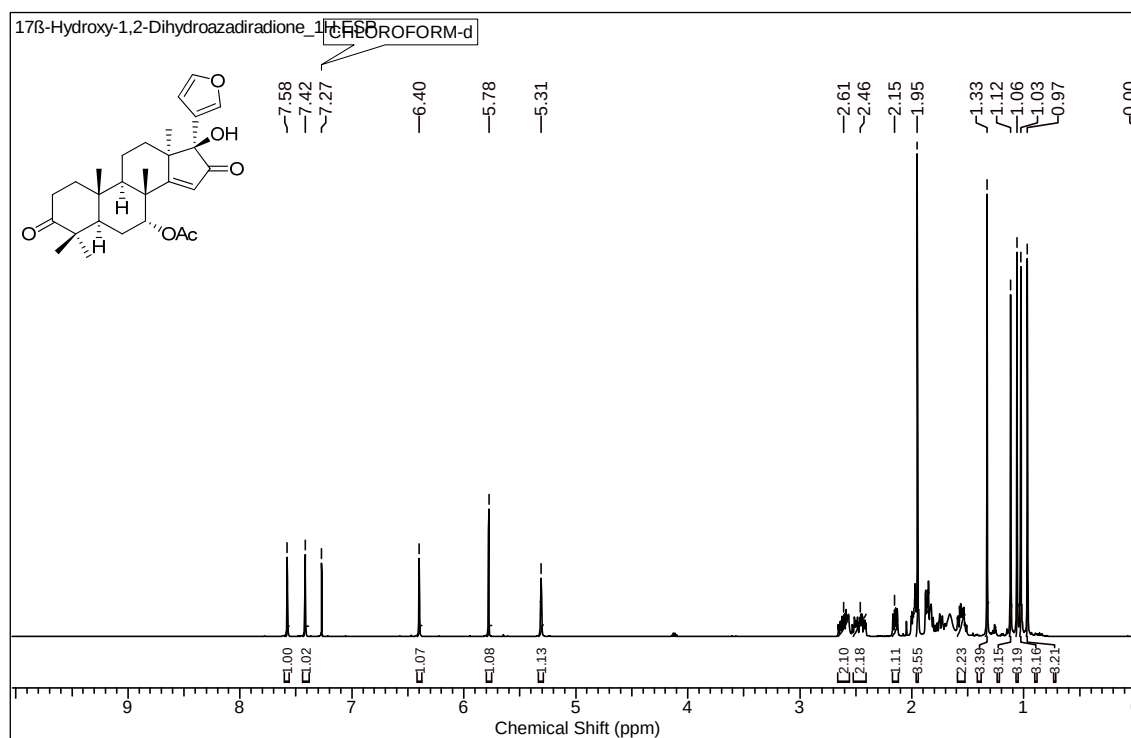
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 12 β -hydroxyazadiradione

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 12β-hydroxyazadiradione

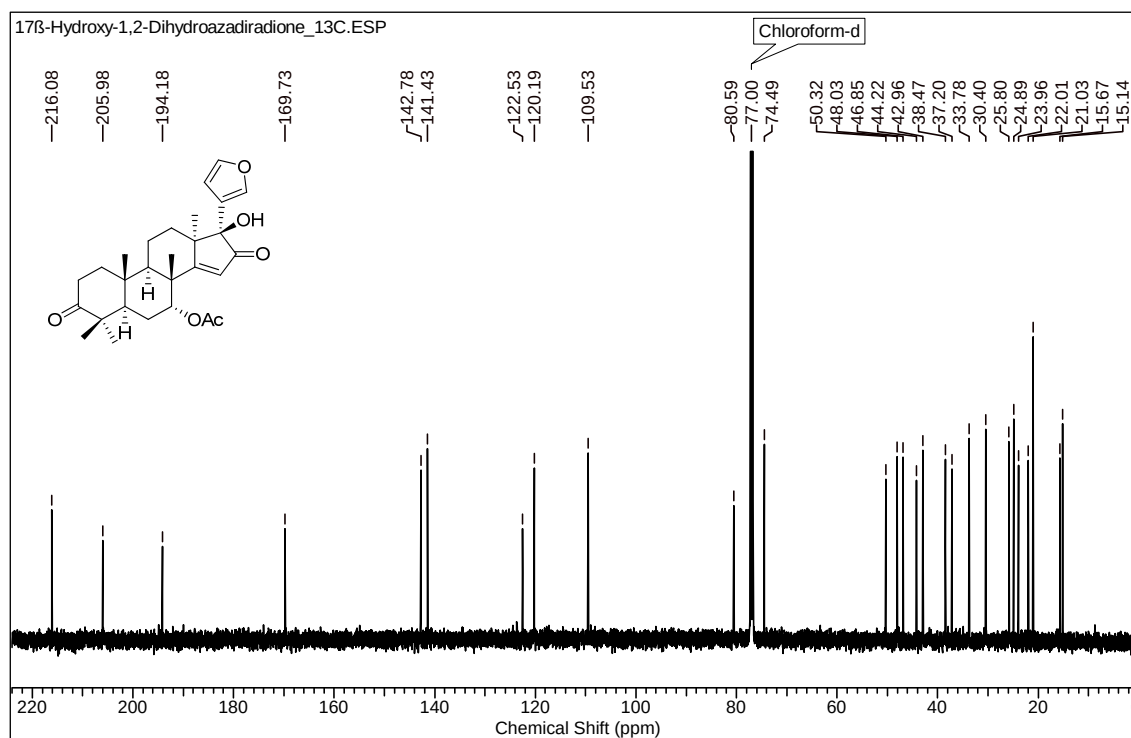
LC-ESI(+)-MS of 12β-hydroxyazadiradione

COSY spectrum of 12 β -hydroxyazadiradione in CDCl₃NOESY spectrum of 12 β -hydroxyazadiradione in CDCl₃

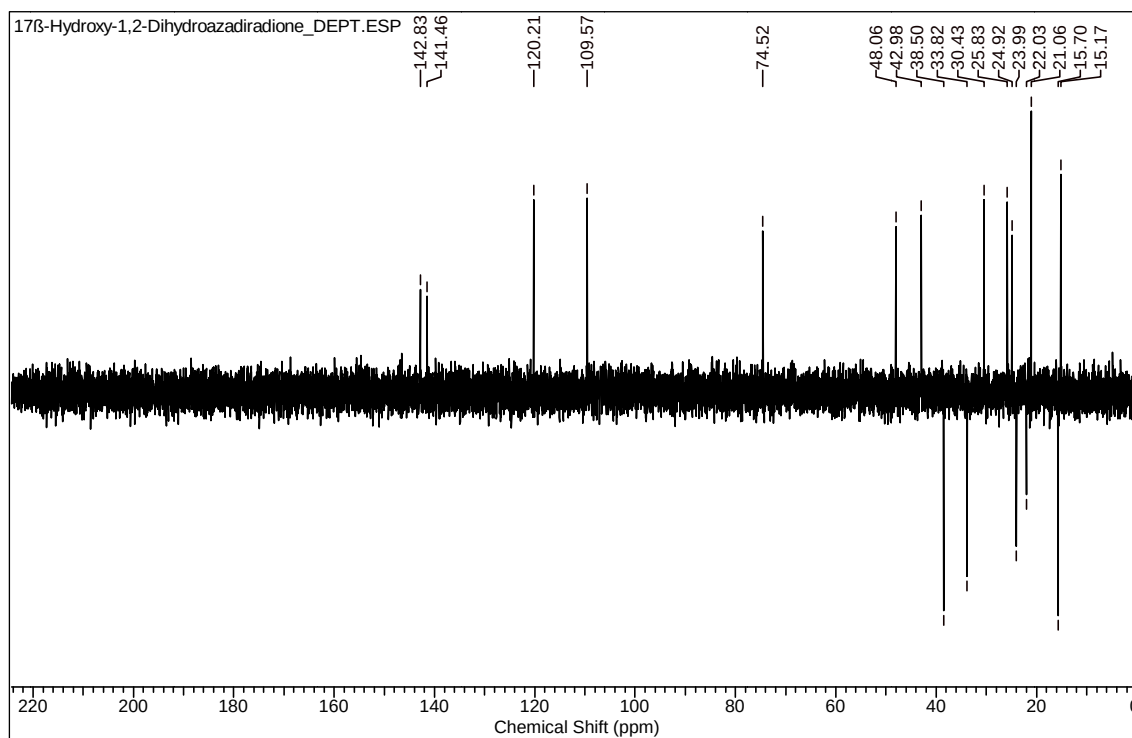
HSQC spectrum of 12β-hydroxyazadiradione in CDCl₃HMBC spectrum of 12β-hydroxyazadiradione in CDCl₃



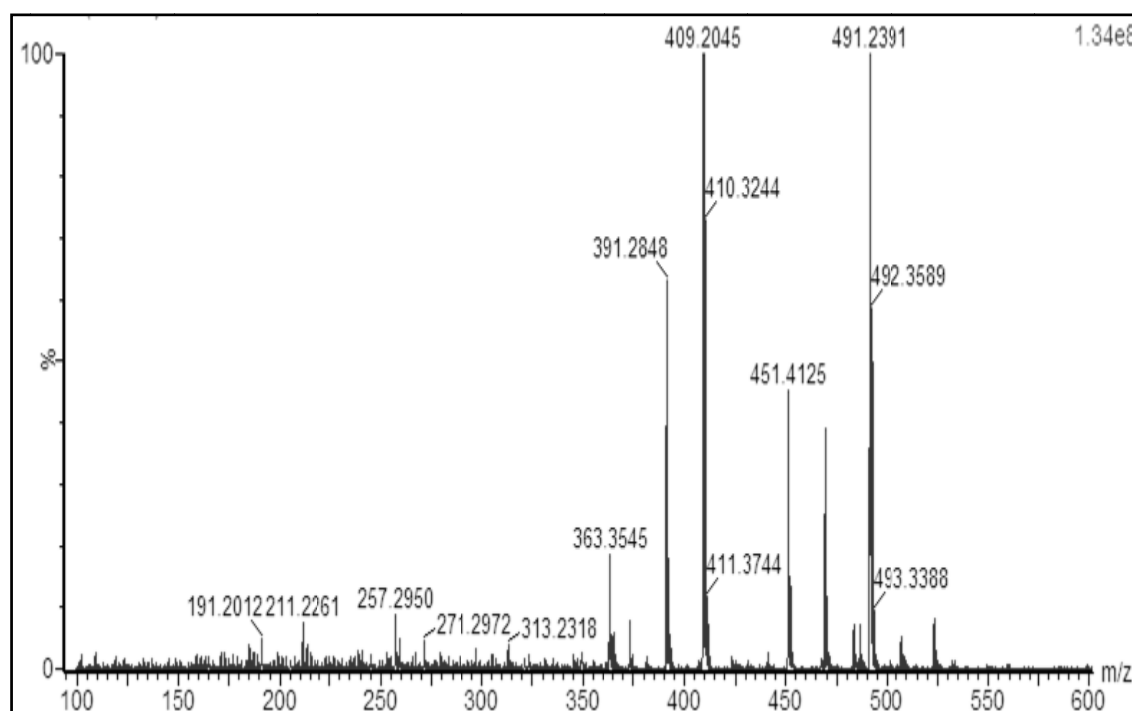
¹H NMR spectrum (CDCl₃, 400 MHz) of 17 β -hydroxy-1,2-dihydroazadiradione



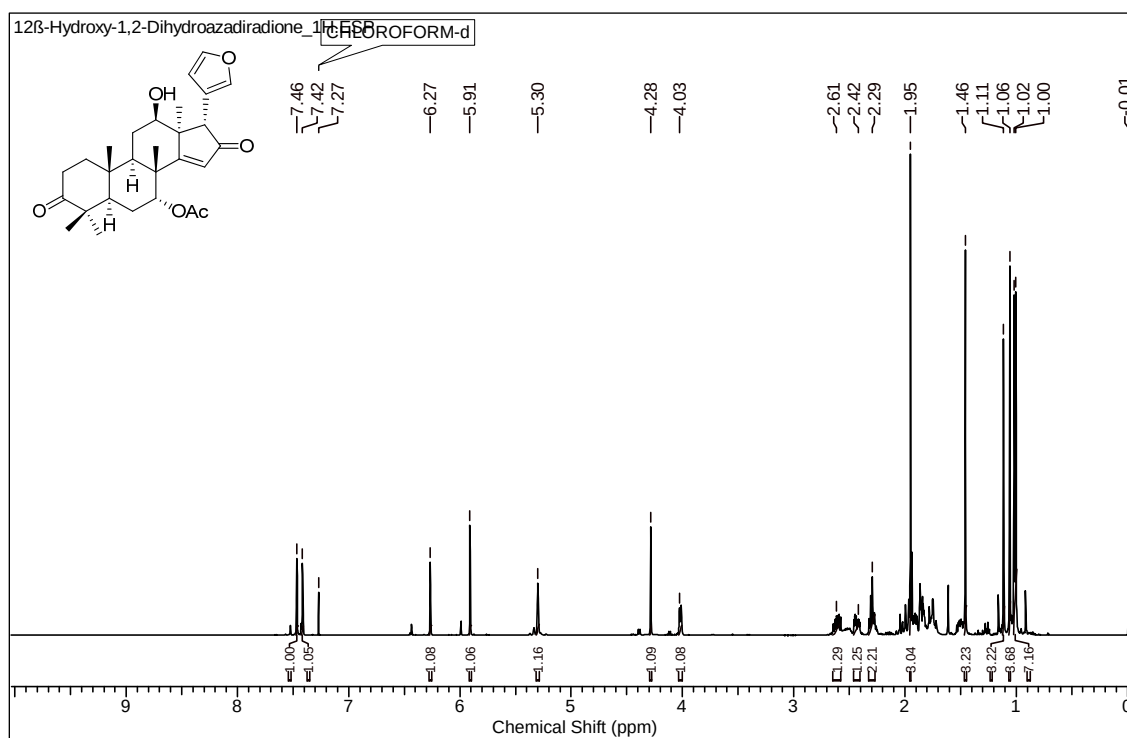
¹³C NMR spectrum (CDCl₃, 100 MHz) of 17 β -hydroxy-1,2-dihydroazadiradione



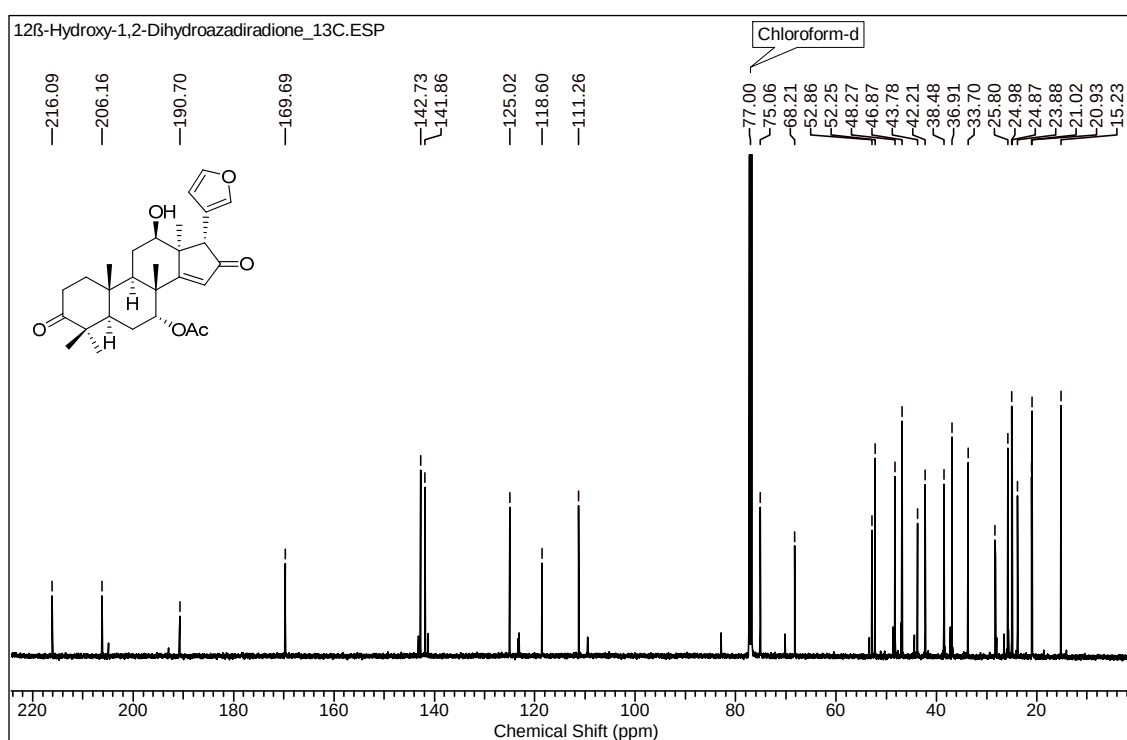
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 17β-hydroxy-1,2-dihydroazadiradione



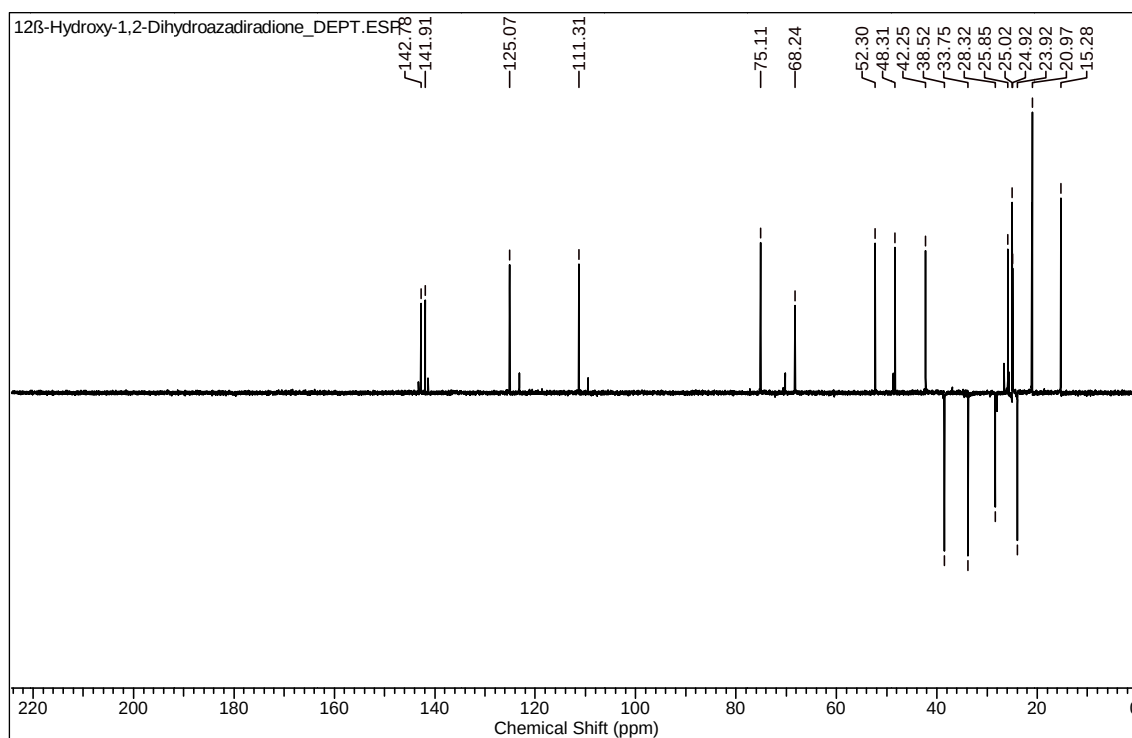
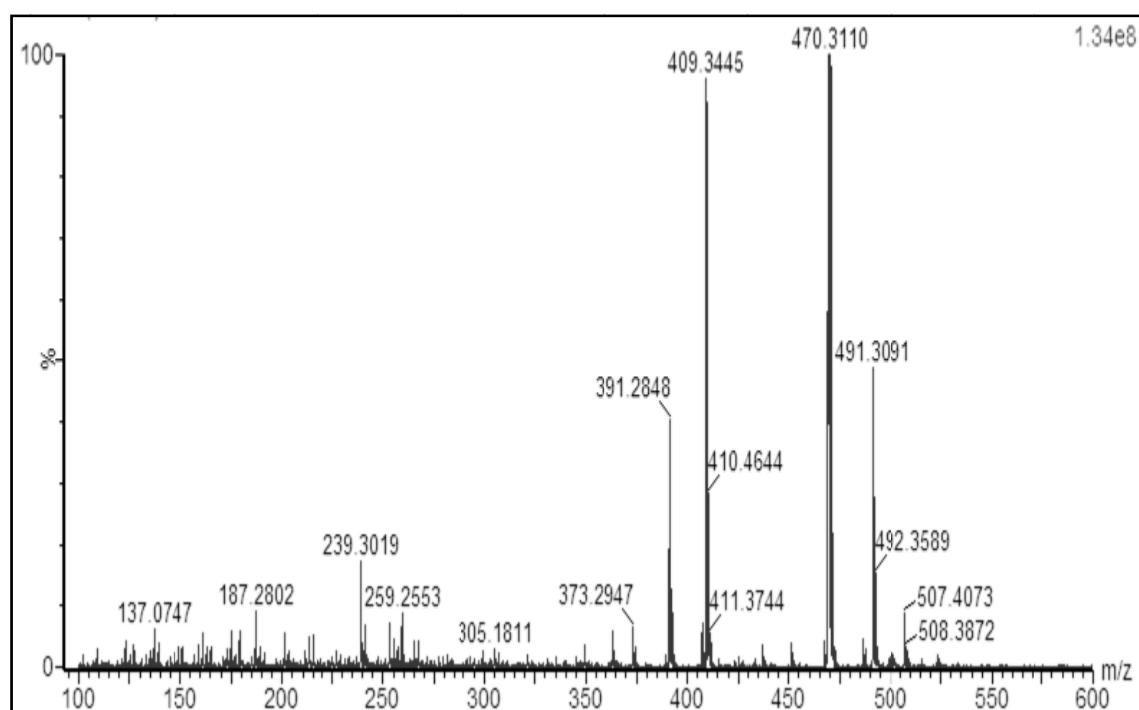
LC-ESI(+)-MS of 17β-hydroxy-1,2-dihydroazadiradione



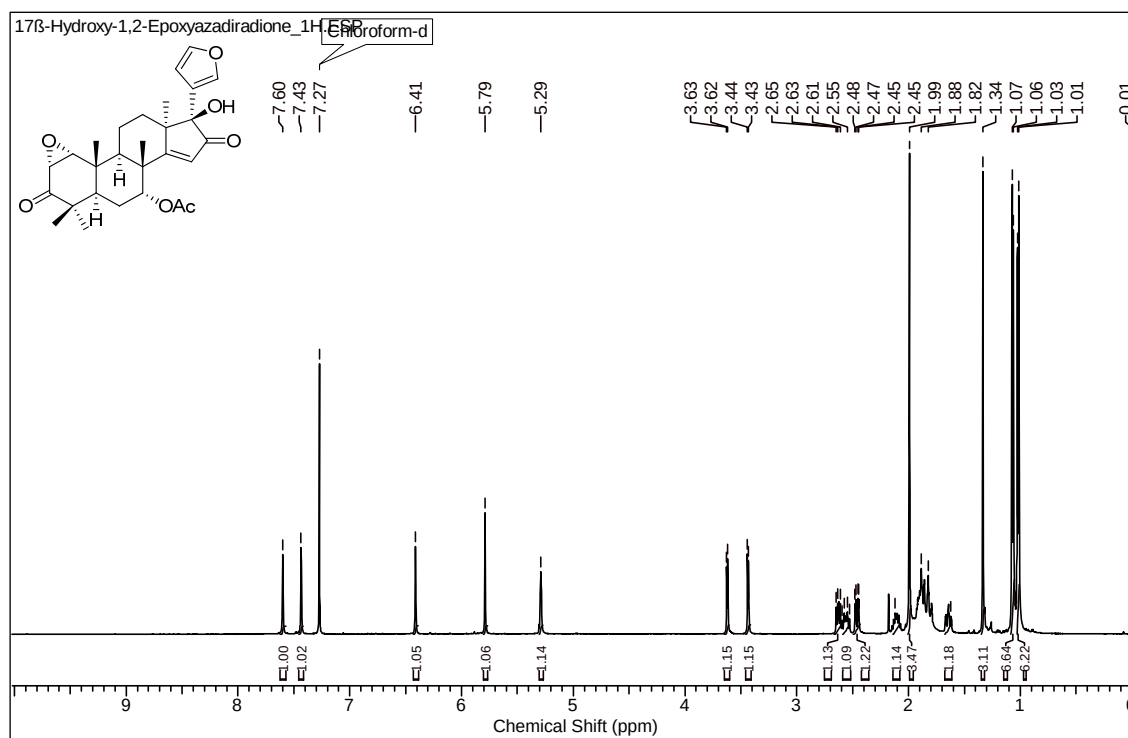
^1H NMR spectrum (CDCl_3 , 400 MHz) of 12 β -hydroxy-1,2-dihydroazadiradione



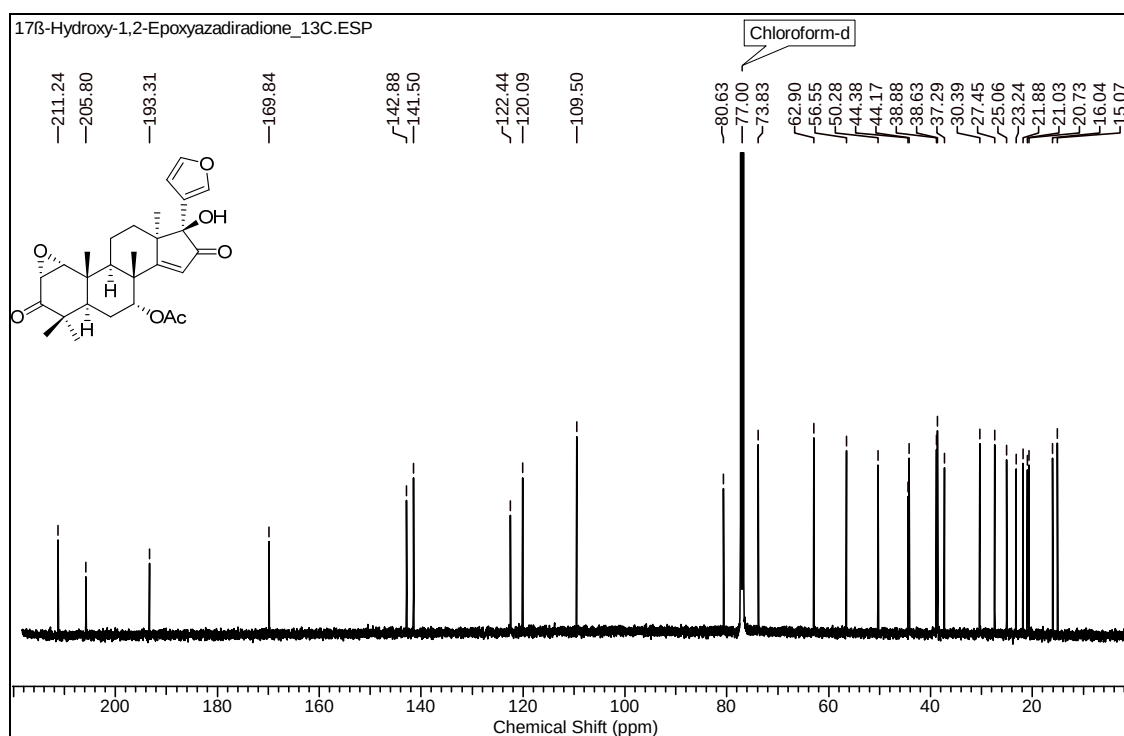
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 12 β -hydroxy-1,2-dihydroazadiradione

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 12β-hydroxy-1,2-dihydroazadiradione

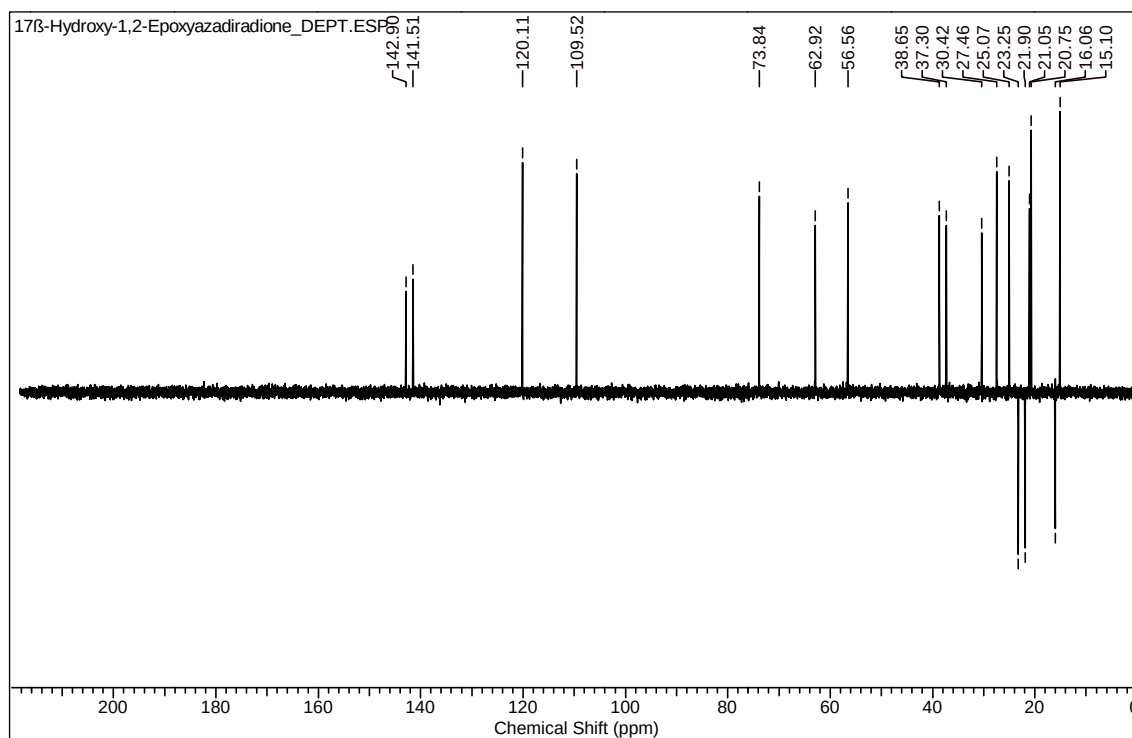
LC-ESI(+)-MS of 12β-hydroxy-1,2-dihydroazadiradione



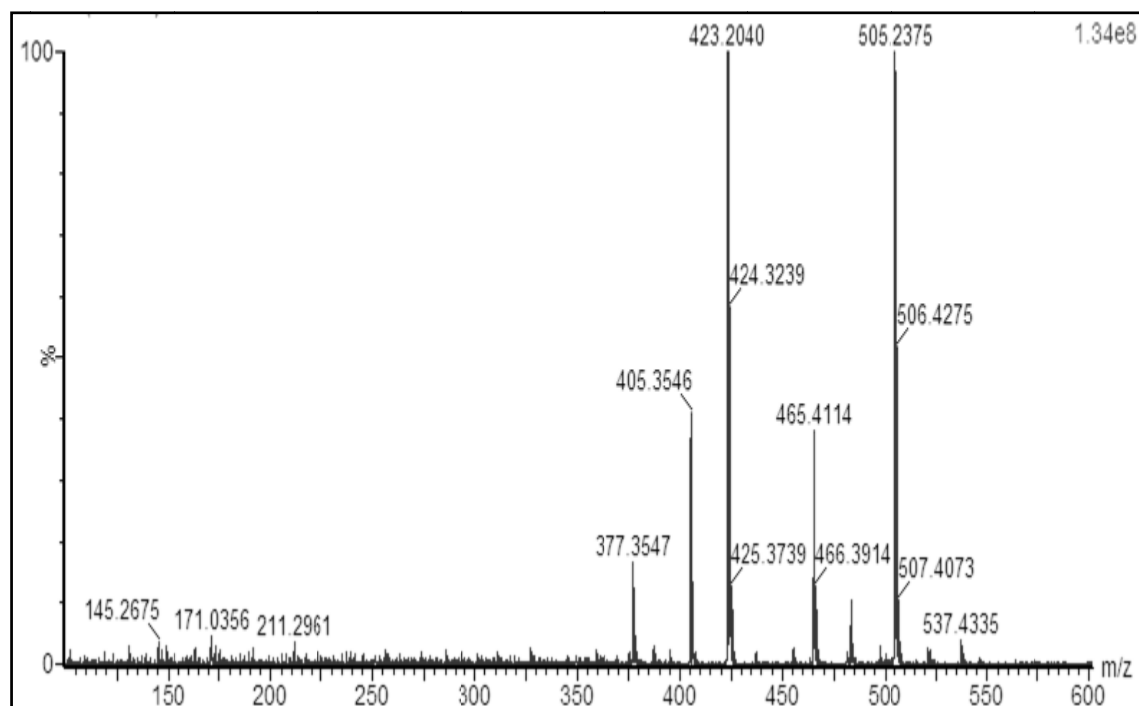
^1H NMR spectrum (CDCl_3 , 400 MHz) of 17 β -Hydroxy-1,2 α -Epoxyazadiradione



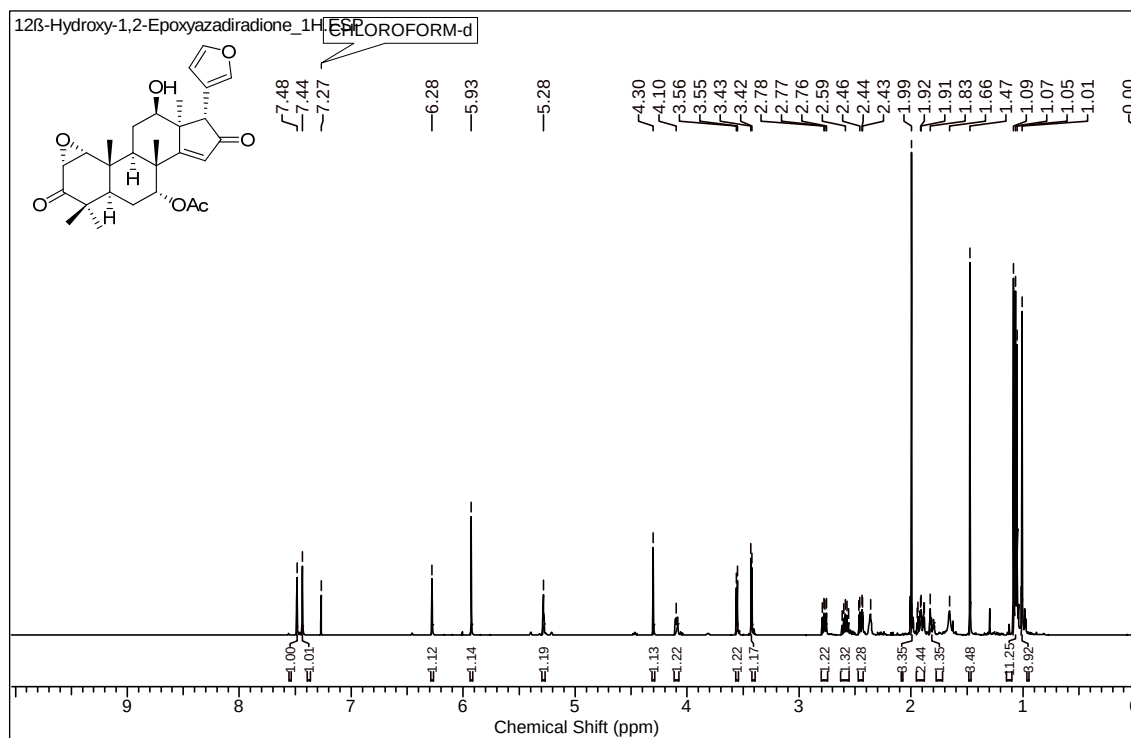
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 17 β -Hydroxy-1,2 α -Epoxyazadiradione



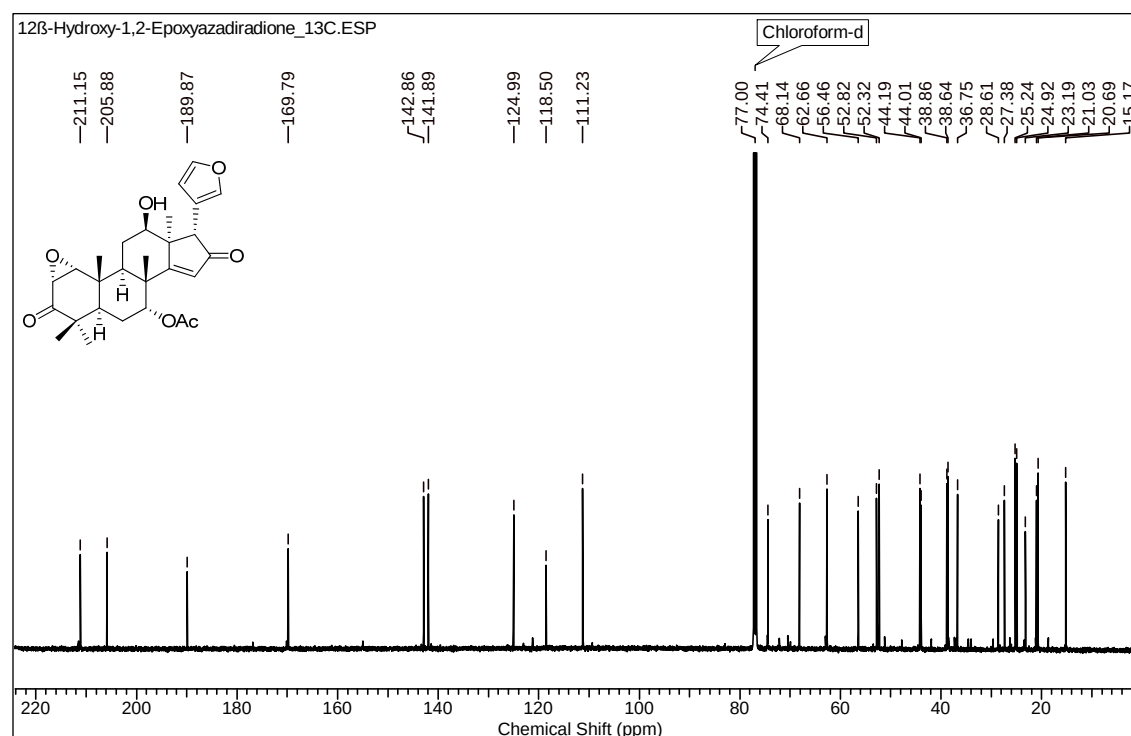
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 17β-Hydroxy-1,2α-Epoxyazadiradione



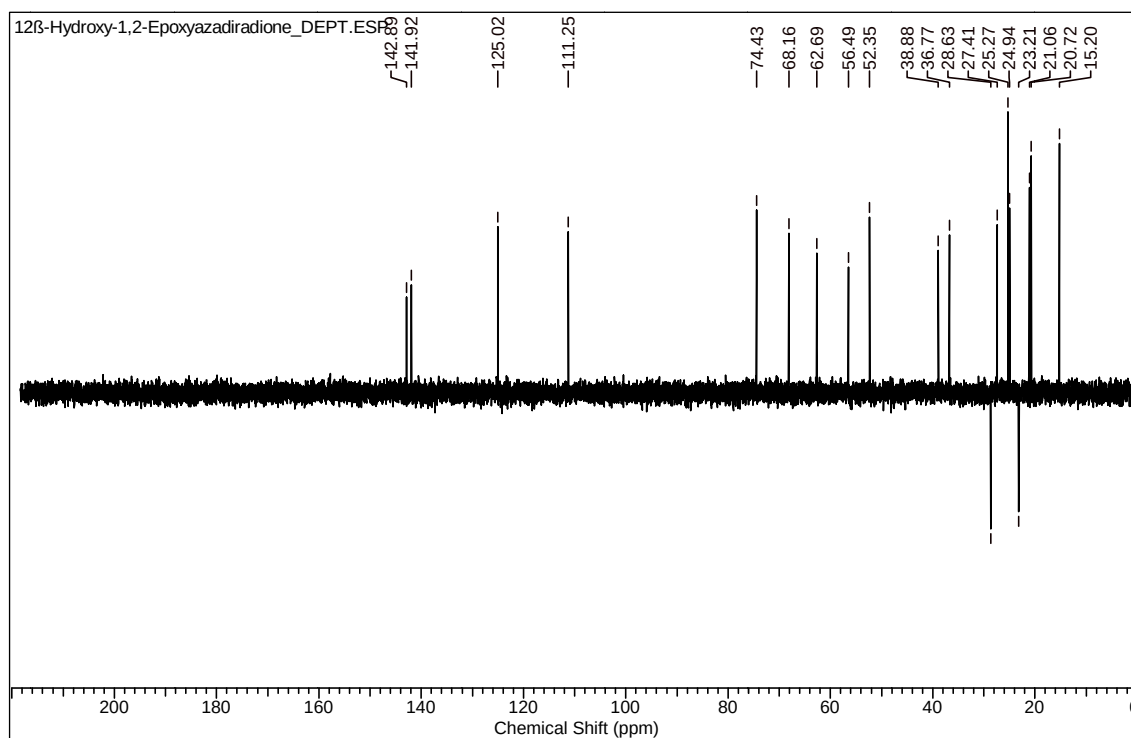
LC-ESI(+)-MS of 17β-hydroxy-1,2-dihydroazadiradione



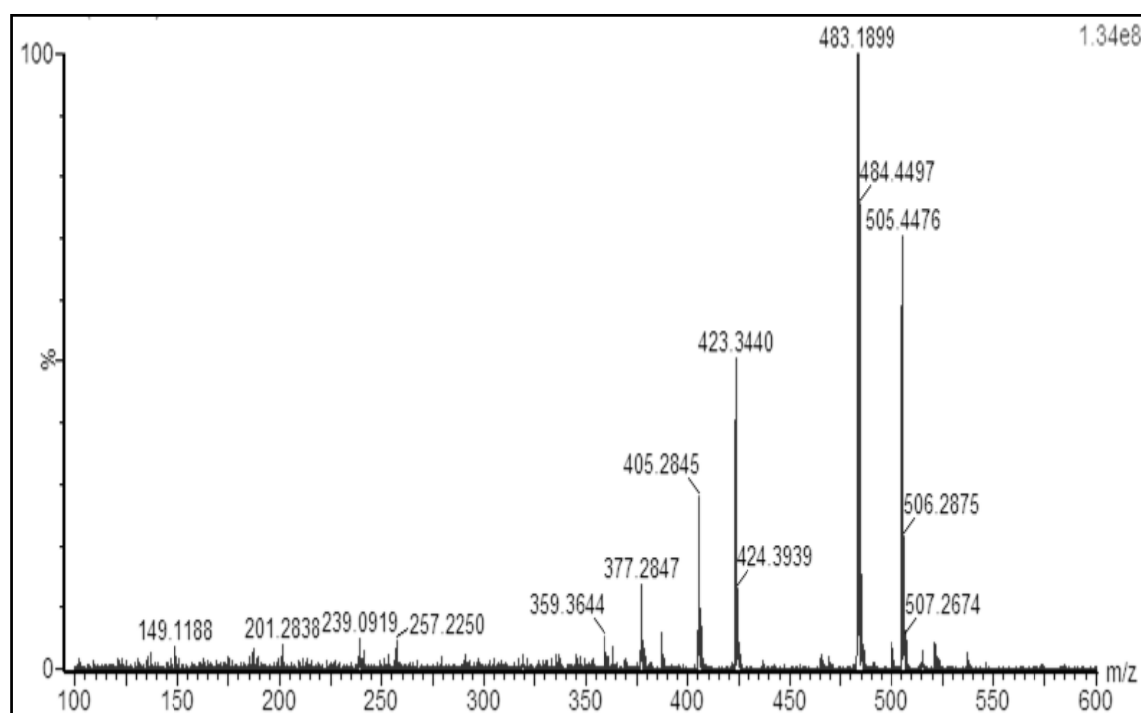
^1H NMR spectrum (CDCl_3 , 400 MHz) of 12 β -Hydroxy-1,2 α -Epoxyazadiradione



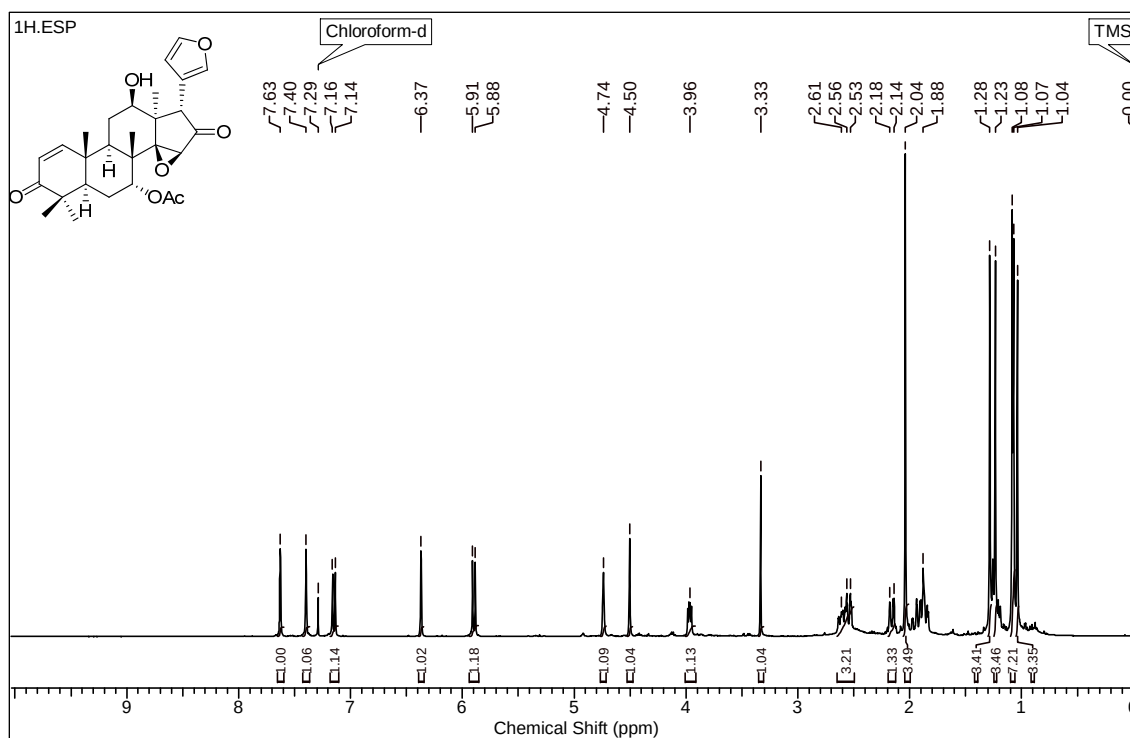
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 12 β -Hydroxy-1,2 α -Epoxyazadiradione



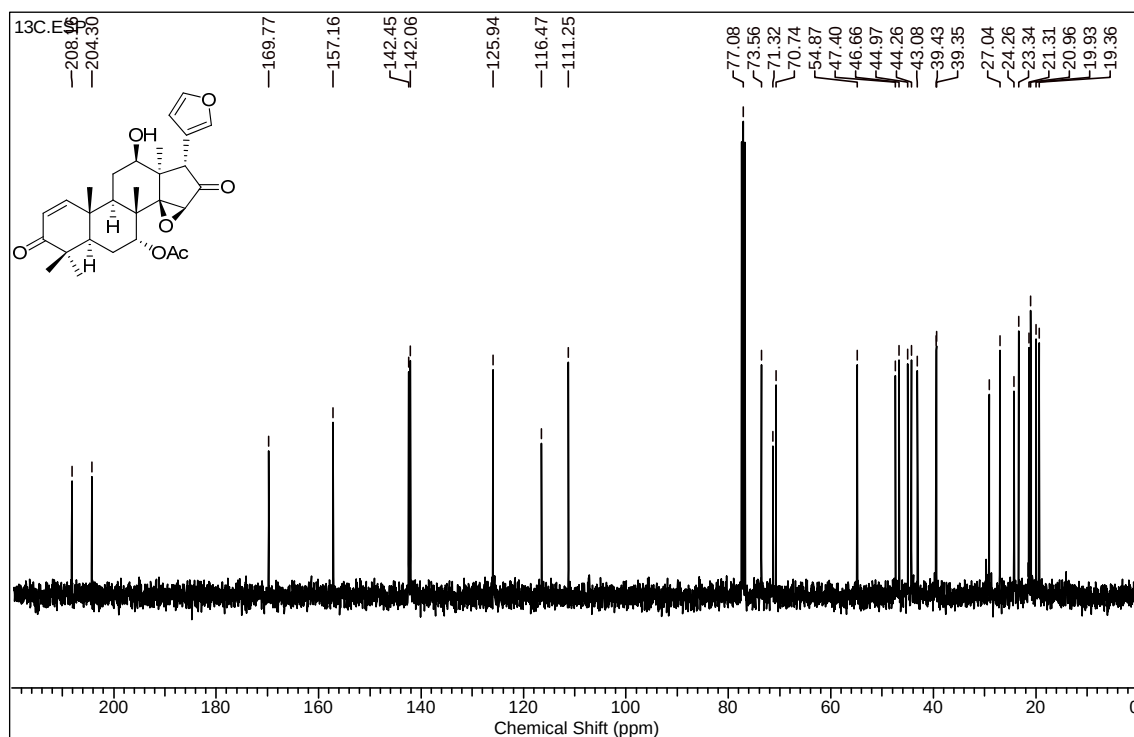
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 12β-Hydroxy-1,2α-Epoxyazadiradione



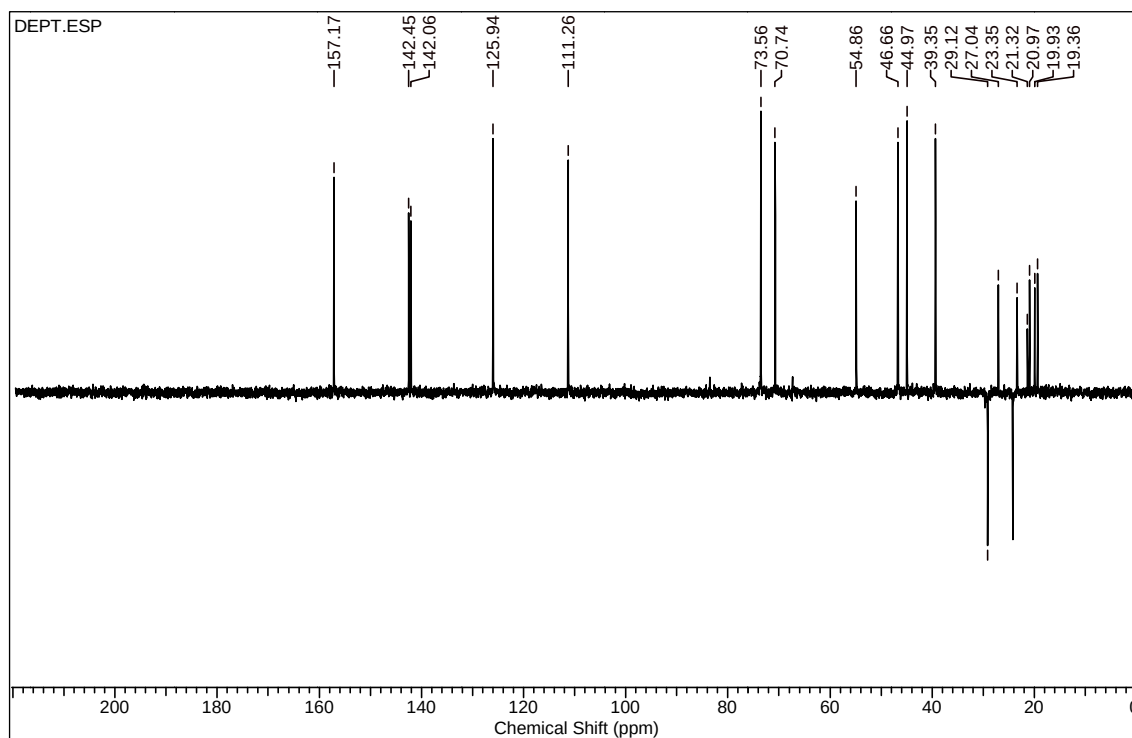
LC-ESI(+)-MS of 12β-hydroxy-1,2-dihydroazadiradione



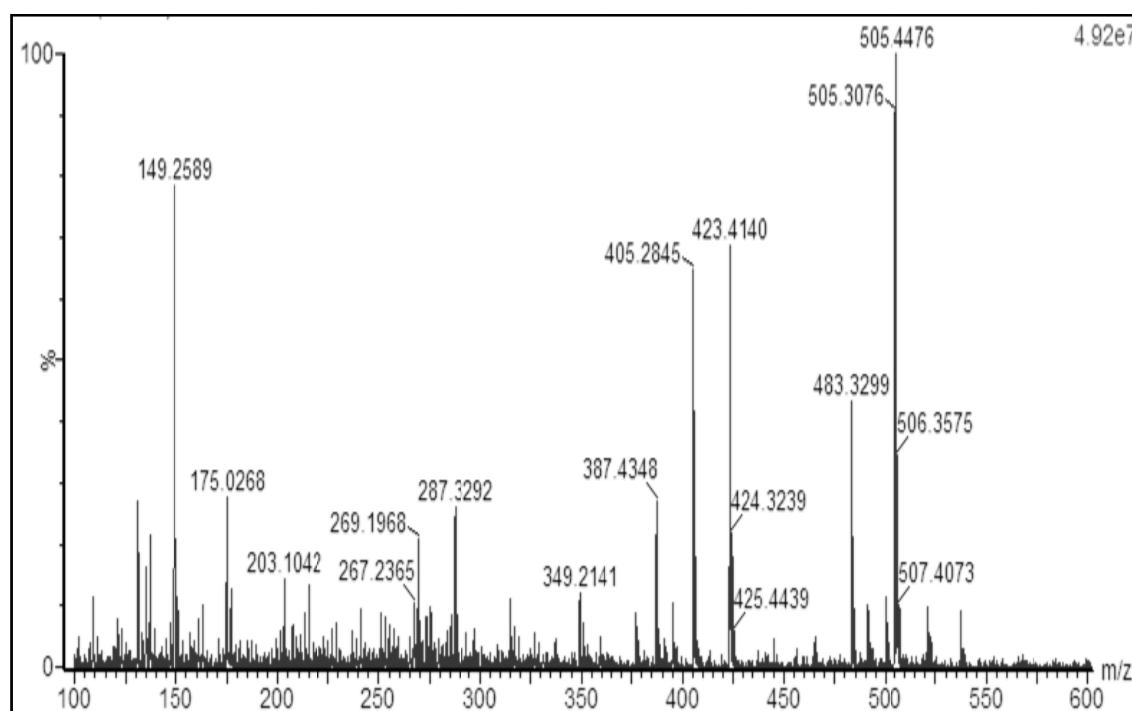
¹H NMR spectrum (CDCl₃, 400 MHz) of 12β-Hydroxyepoxyazadiradione



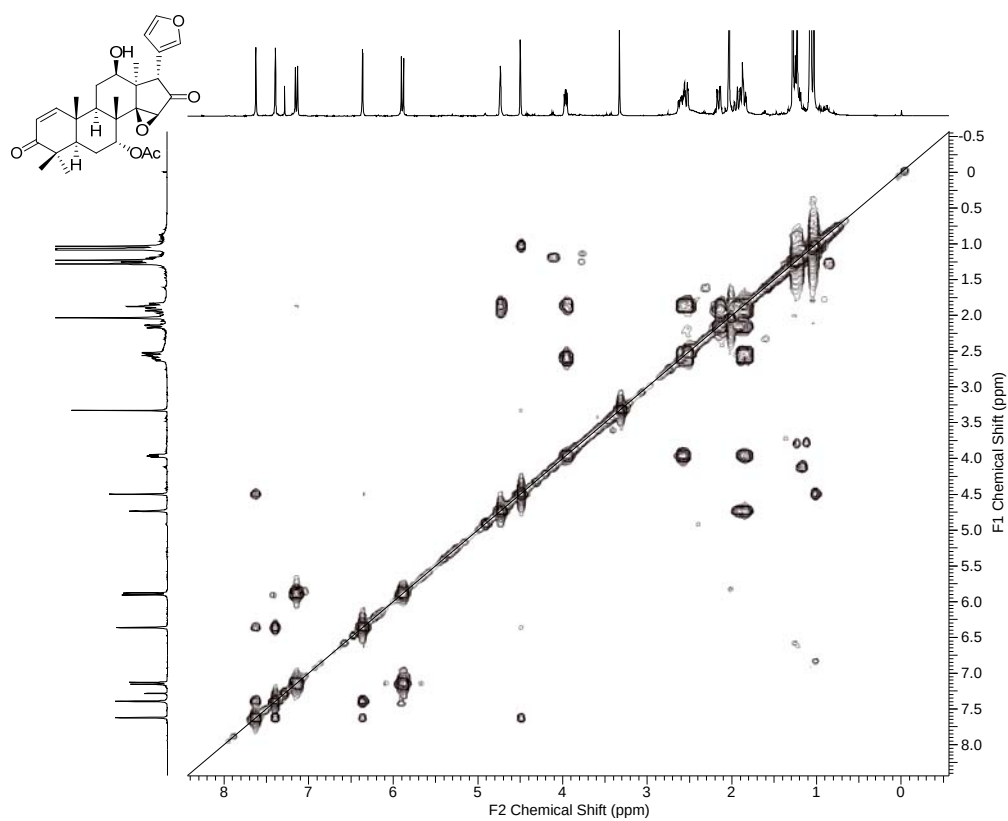
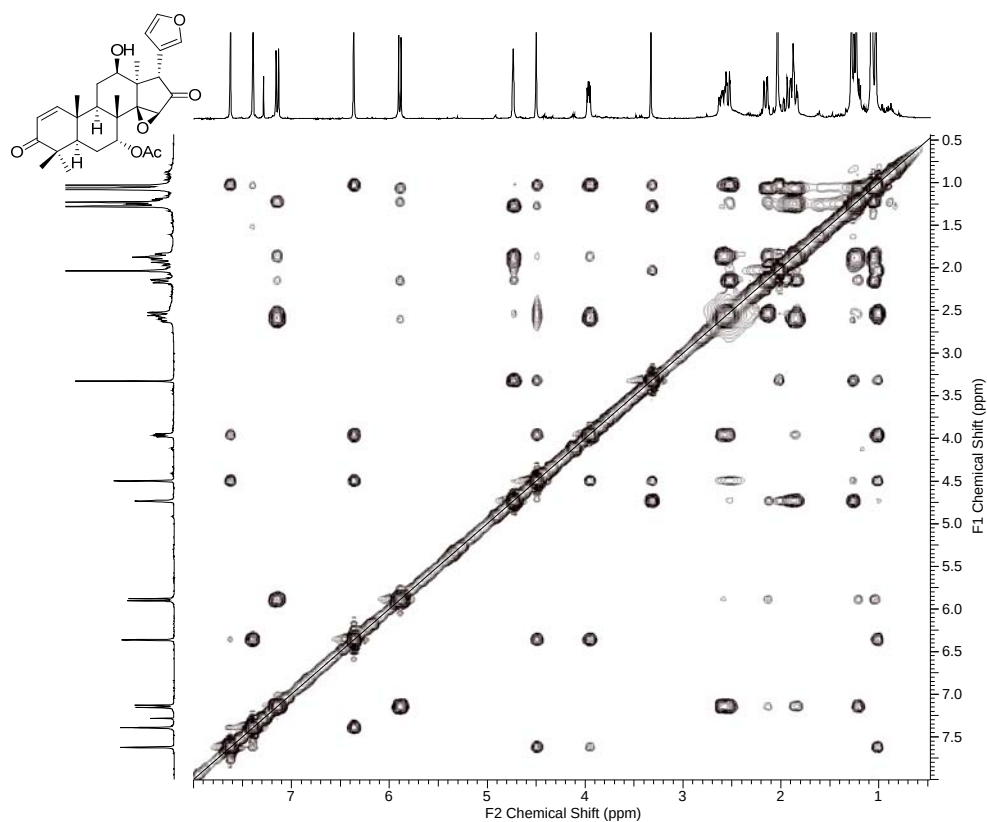
¹³C NMR spectrum (CDCl₃, 100 MHz) of 12β-Hydroxyepoxyazadiradione

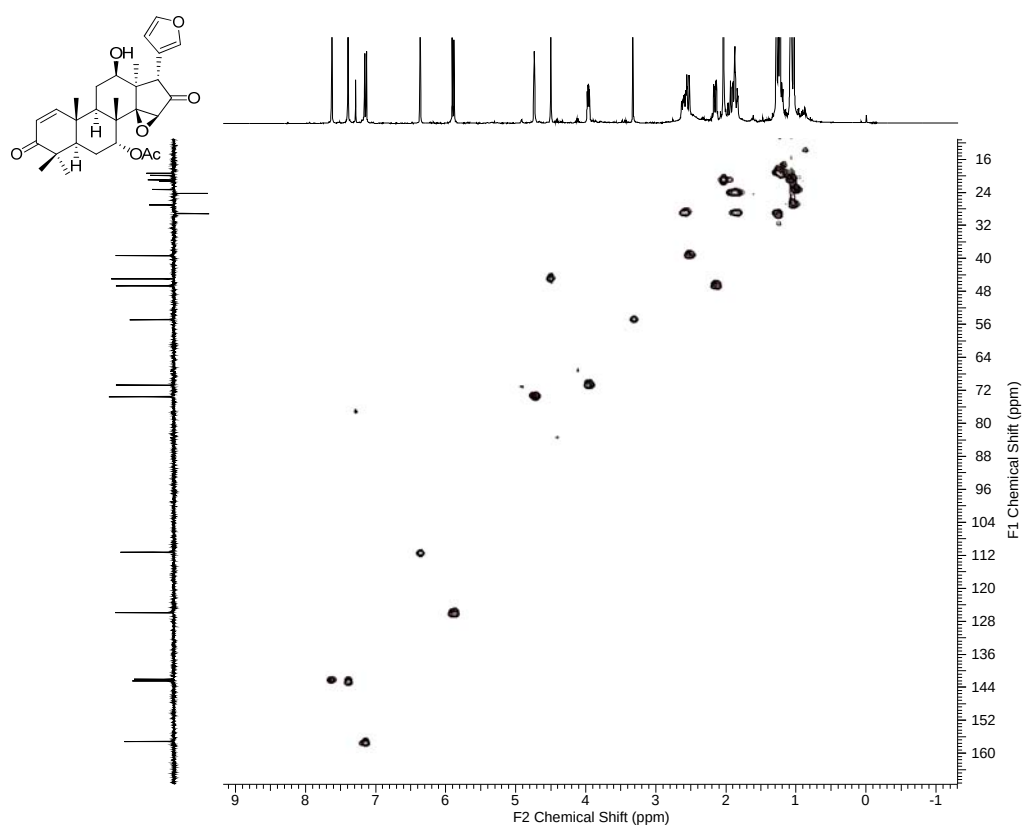
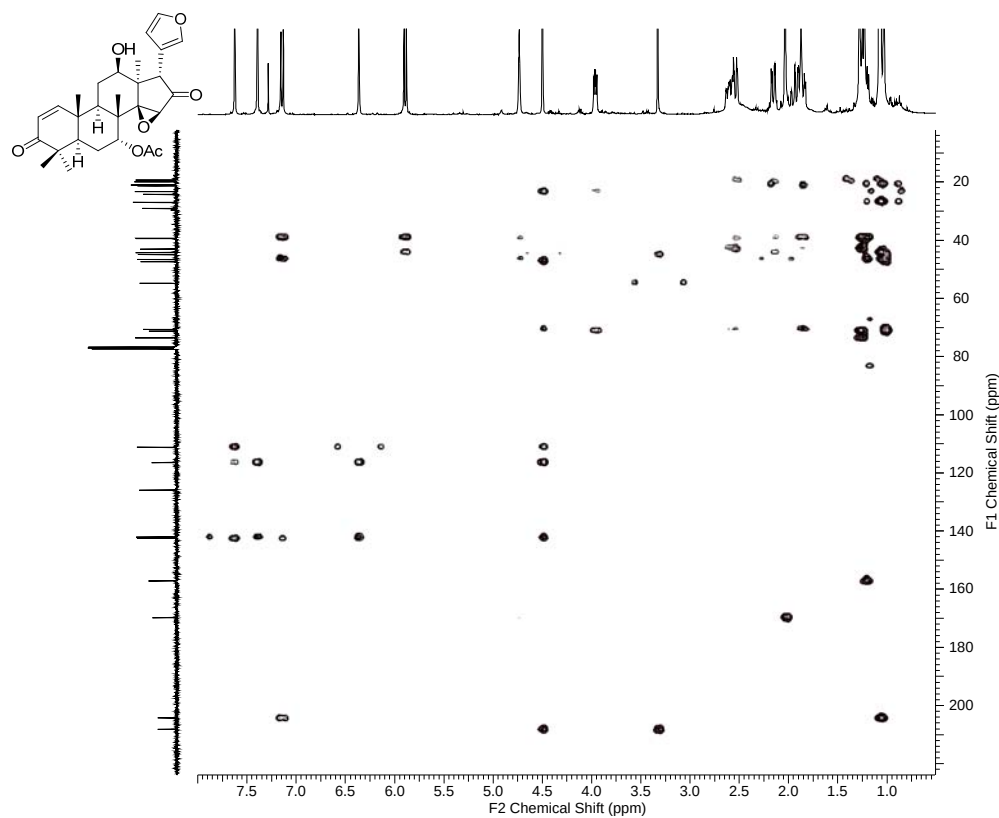


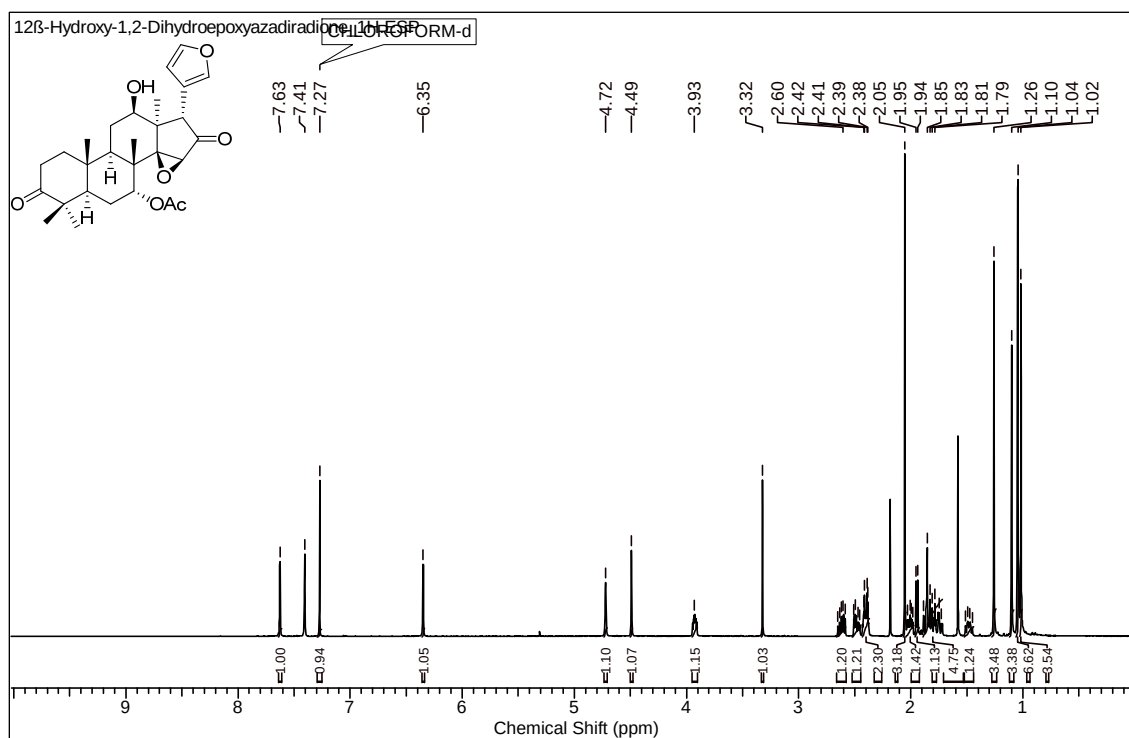
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 12β-Hydroxyepoxyazadiradione



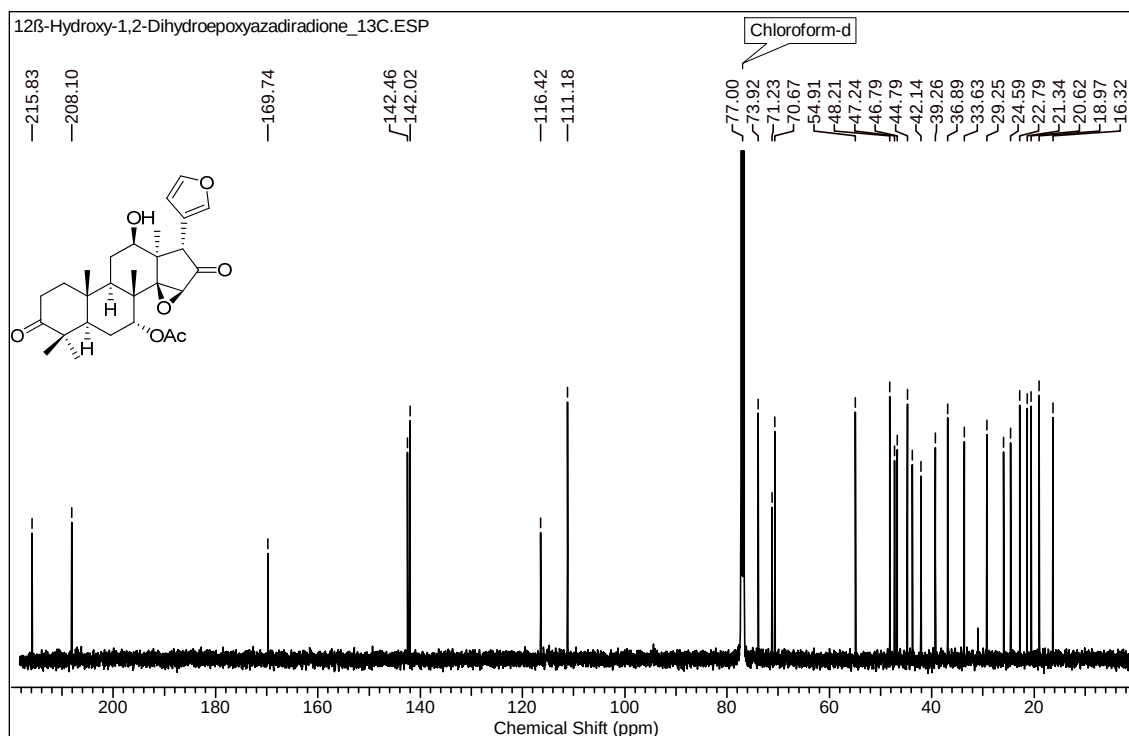
LC-ESI(+)-MS of 12β-Hydroxyepoxyazadiradione

COSY spectrum of 12β-hydroxyepoxyazadiradione in CDCl₃NOESY spectrum of 12β-hydroxyepoxyazadiradione in CDCl₃

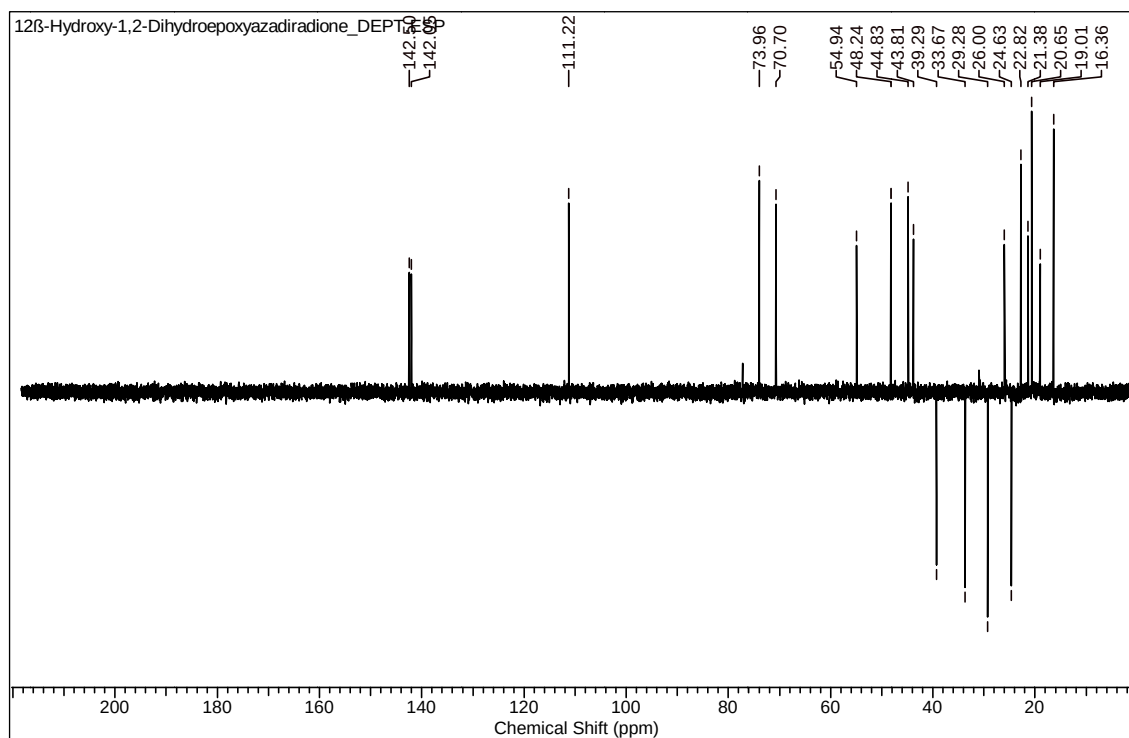
HSQC spectrum of 12β-hydroxyepoxyazadiradione in CDCl₃HMBC spectrum of 12β-hydroxyepoxyazadiradione in CDCl₃



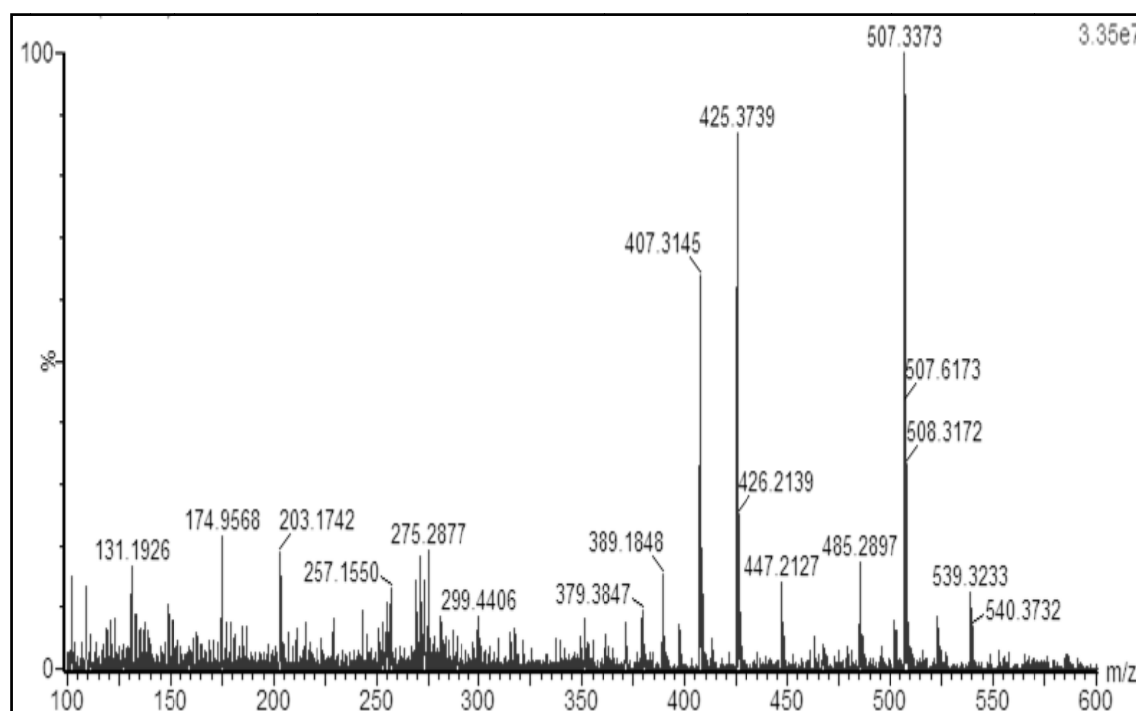
^1H NMR spectrum (CDCl_3 , 400 MHz) of 12 β -Hydroxy-1,2-dihydroepoxyazadiradione



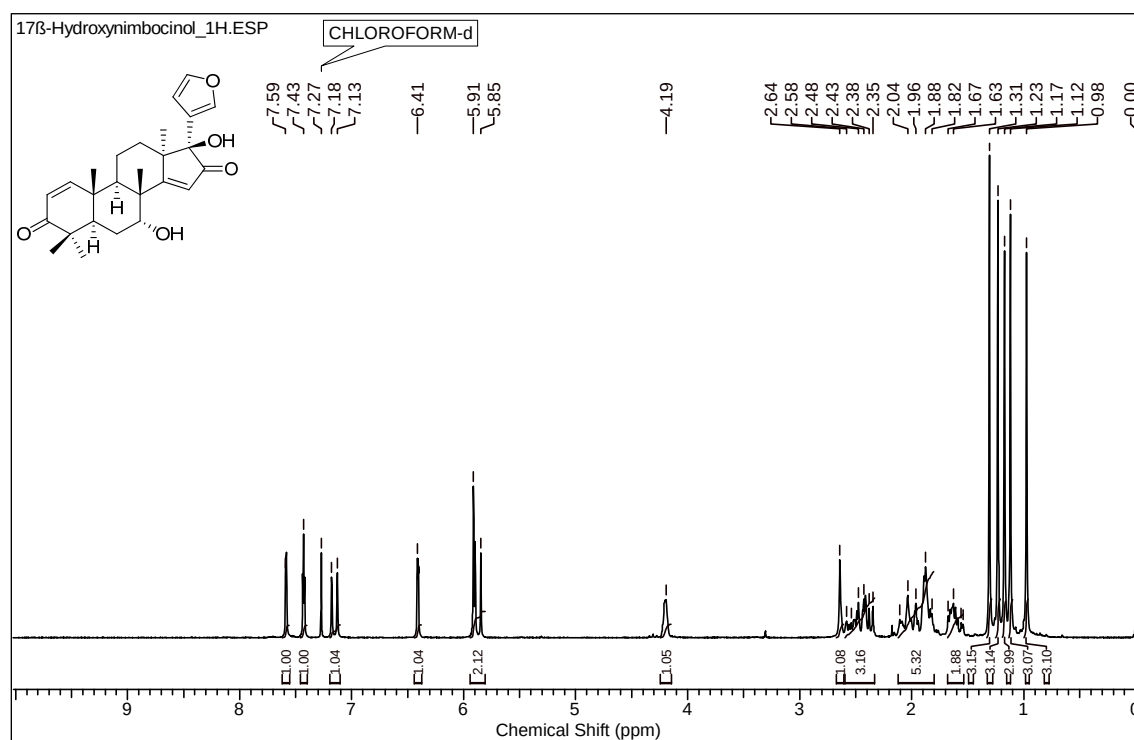
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 12 β -Hydroxy-1,2-dihydroepoxyazadiradione



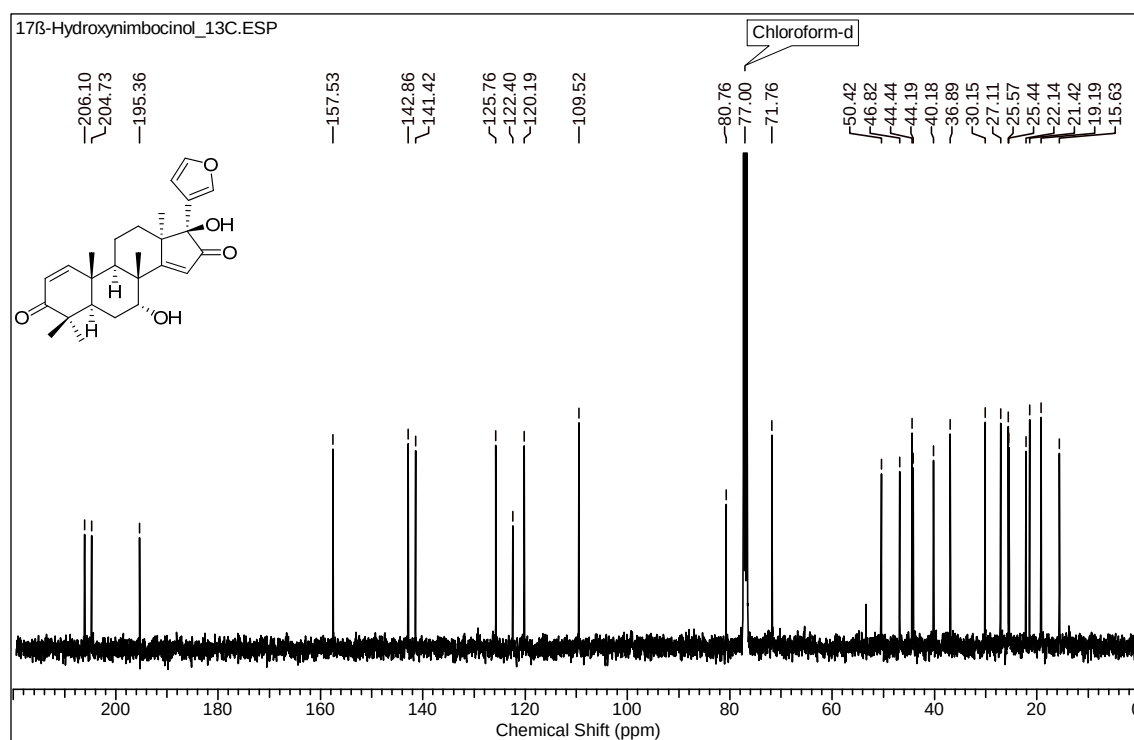
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 12β-Hydroxy-1,2-dihydroepoxyazadiradione



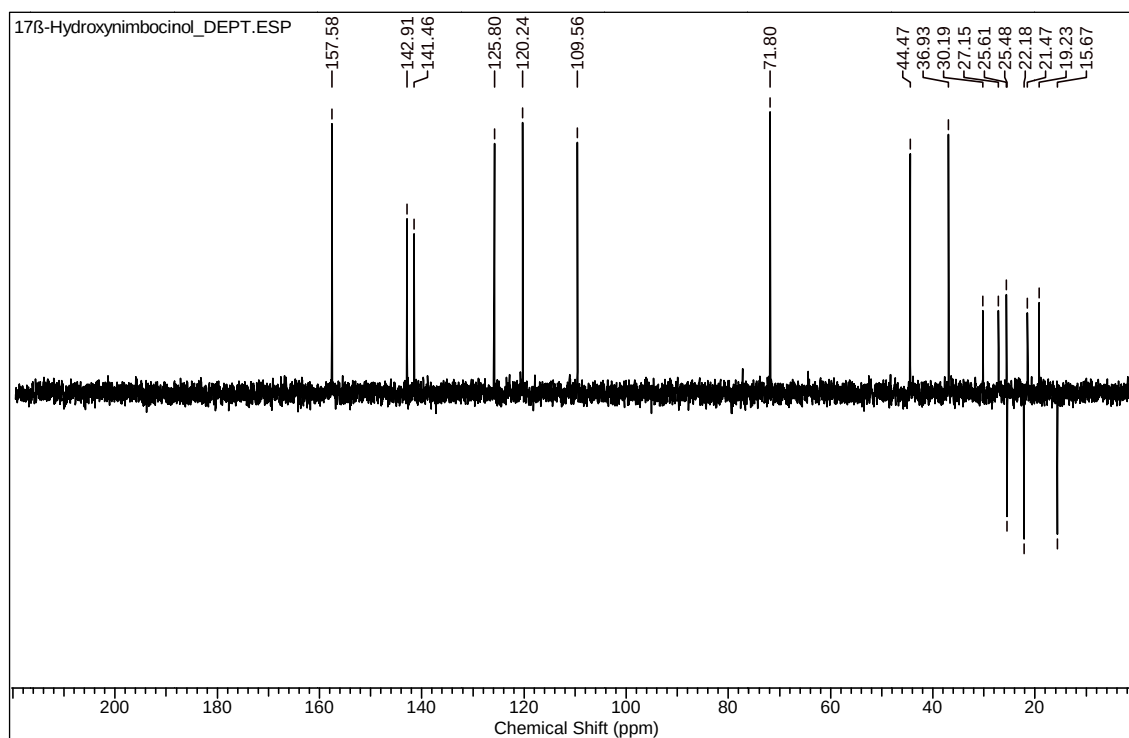
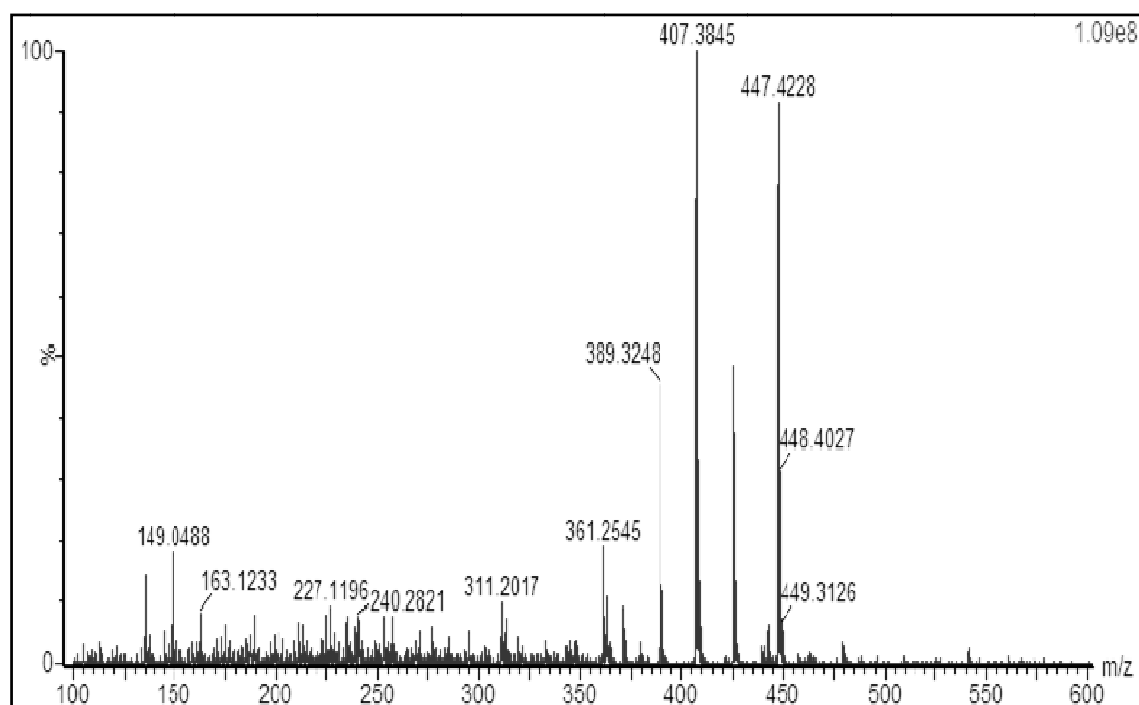
LC-ESI(+)-MS of 12β-Hydroxy-1,2-dihydroepoxyazadiradione



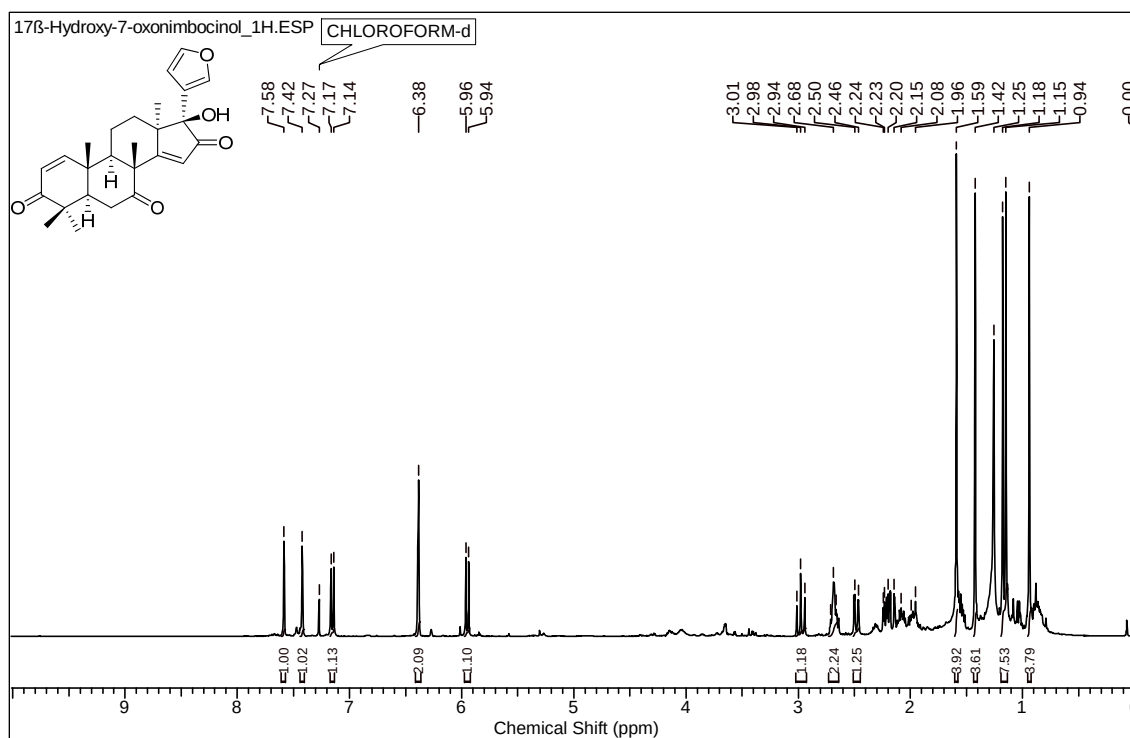
^1H NMR spectrum (CDCl_3 , 400 MHz) of 17 β -hydroxynimbocinol



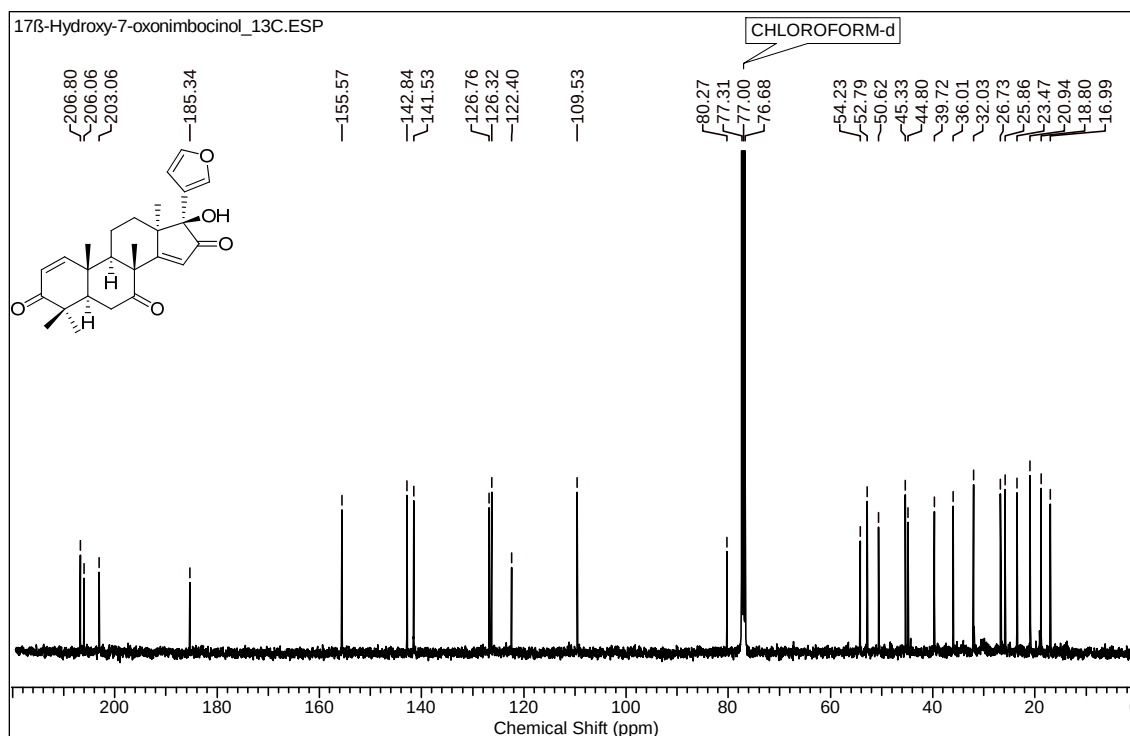
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 17 β -hydroxynimbocinol

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 17β-hydroxynimbocinol

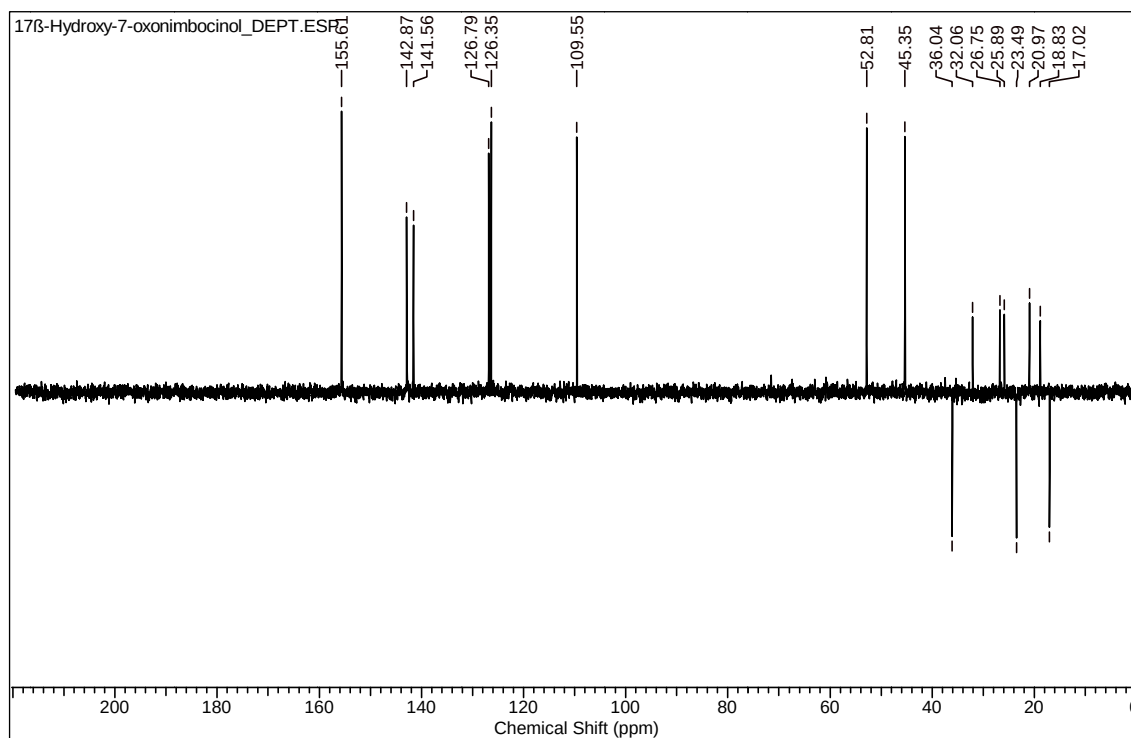
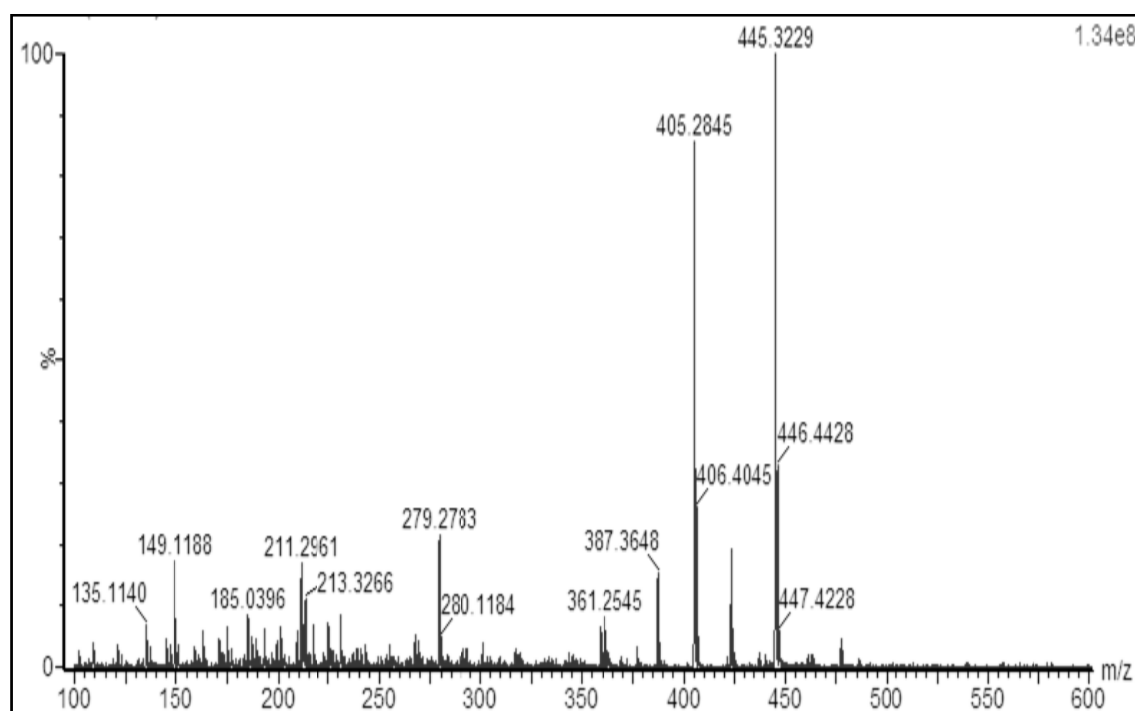
LC-ESI(+)-MS of 17β-hydroxynimbocinol



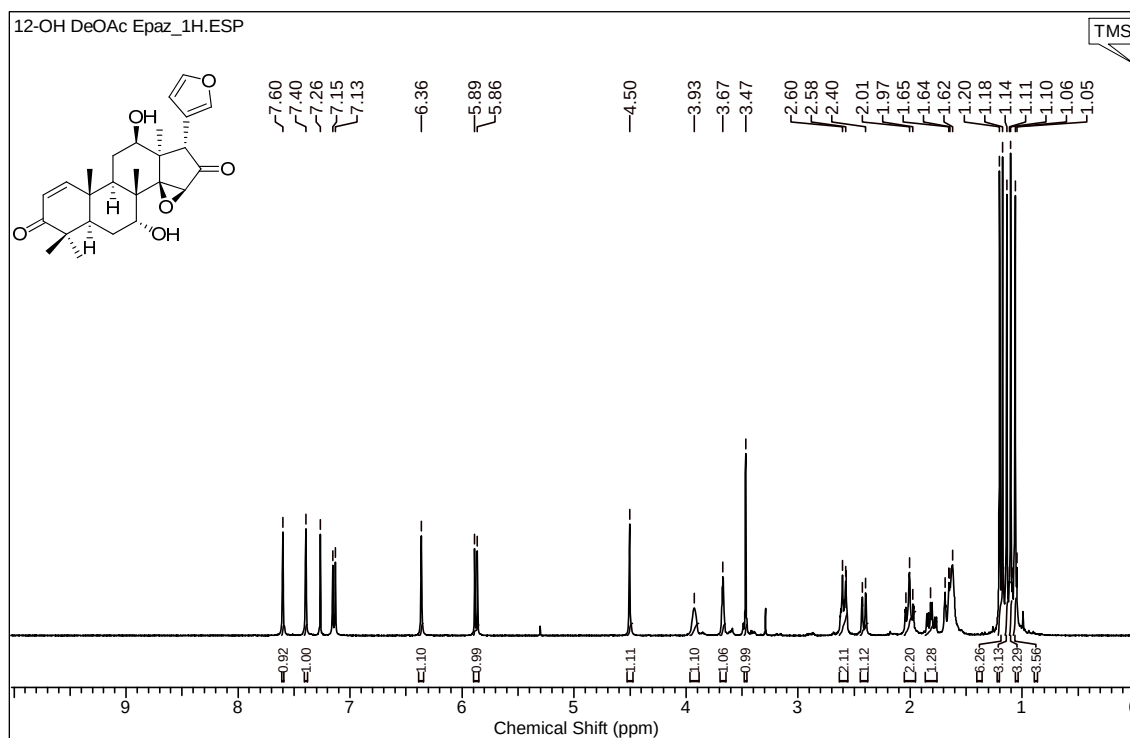
^1H NMR spectrum (CDCl_3 , 400 MHz) of 17 β -hydroxy-7-oxonimbocinol



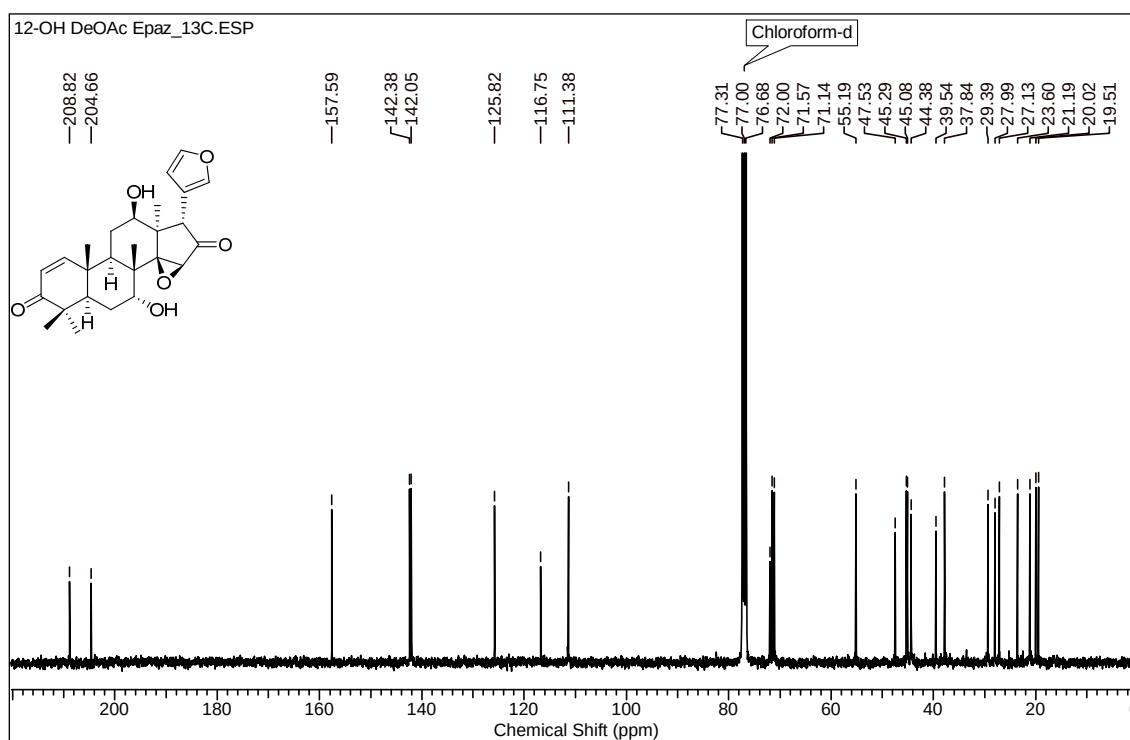
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 17 β -hydroxy-7-oxonimbocinol

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 17β-hydroxy-7-oxonimbocinol

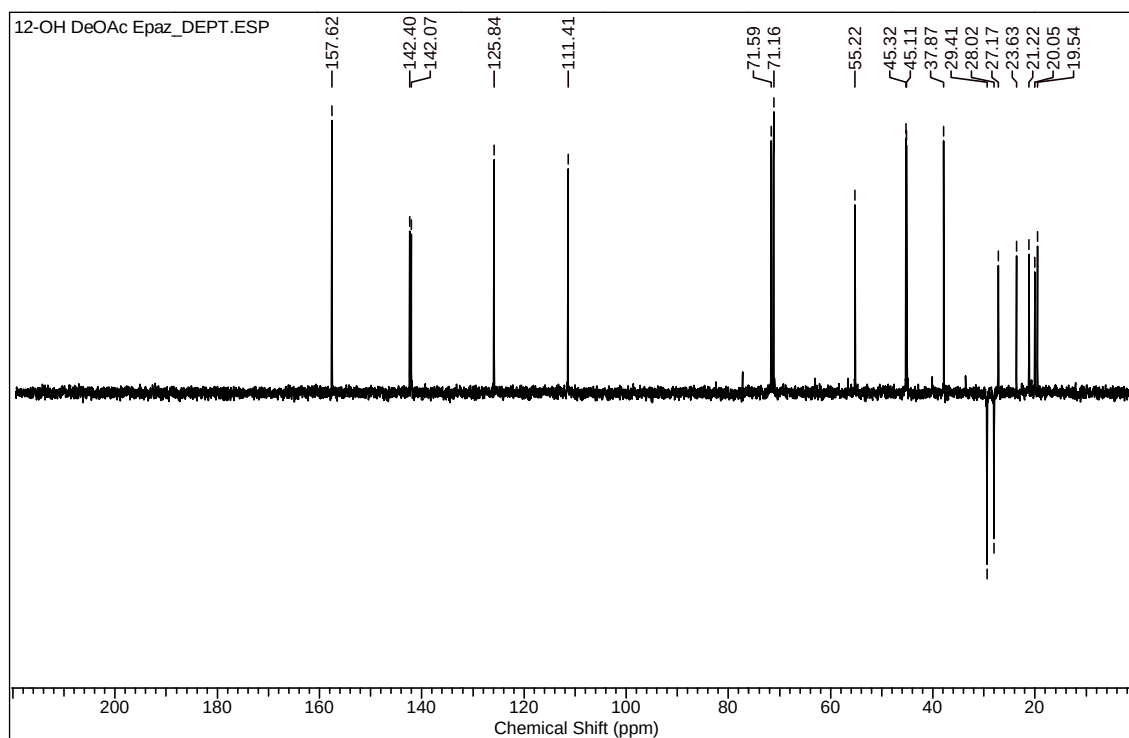
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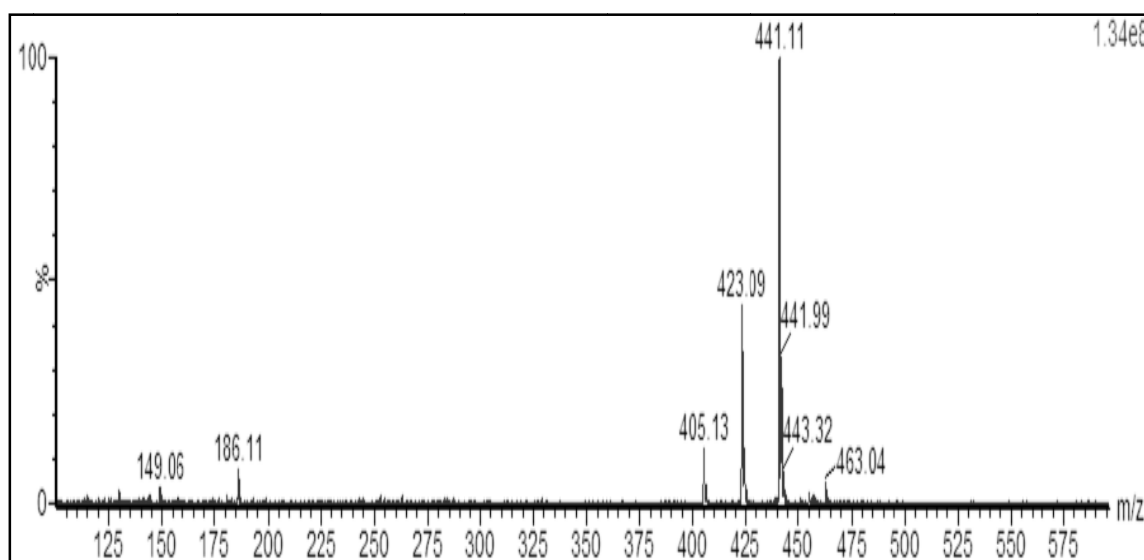
^1H NMR spectrum (CDCl_3 , 400 MHz) of 12β-hydroxy-7-deacetylepoxyzadiradione



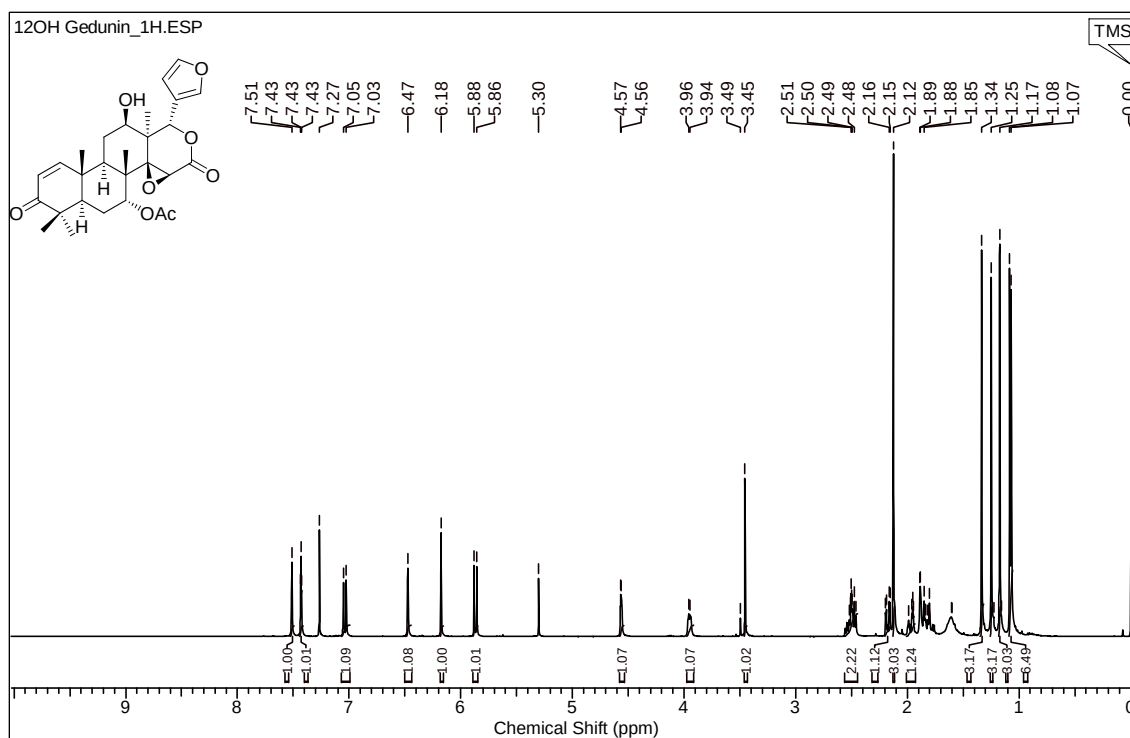
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 12β-hydroxy-7-deacetylepoxyzadiradione



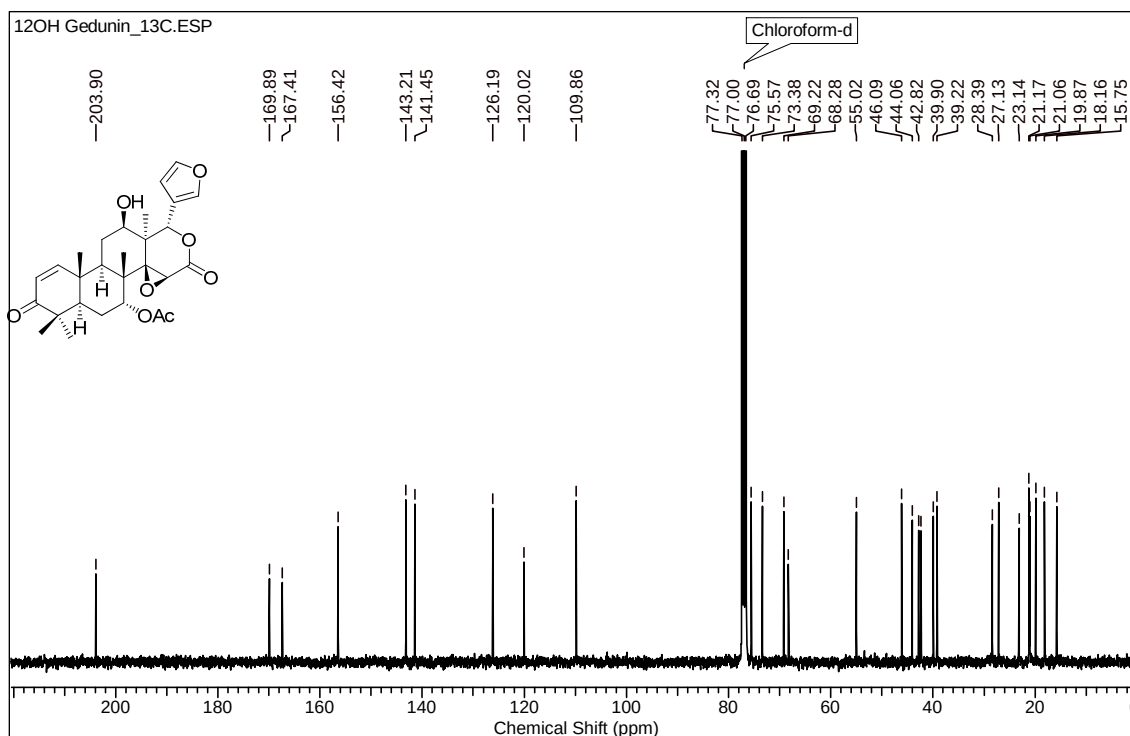
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of 12 β -hydroxy-7-deacetyloxyazadiradione



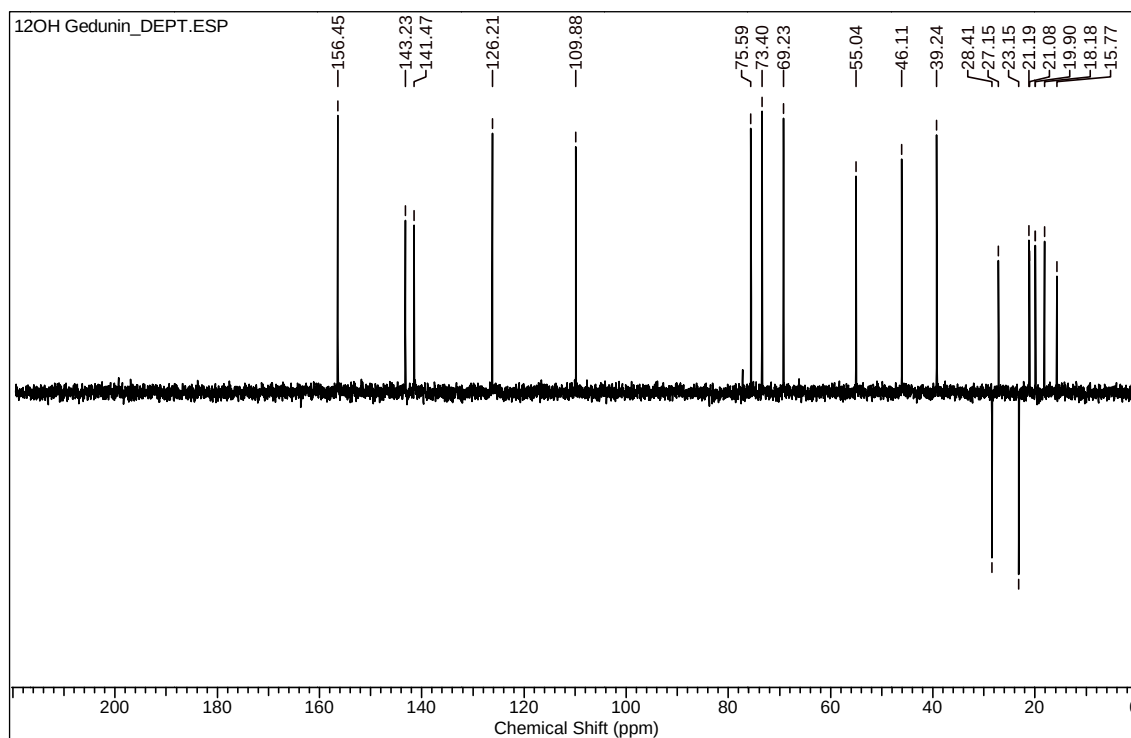
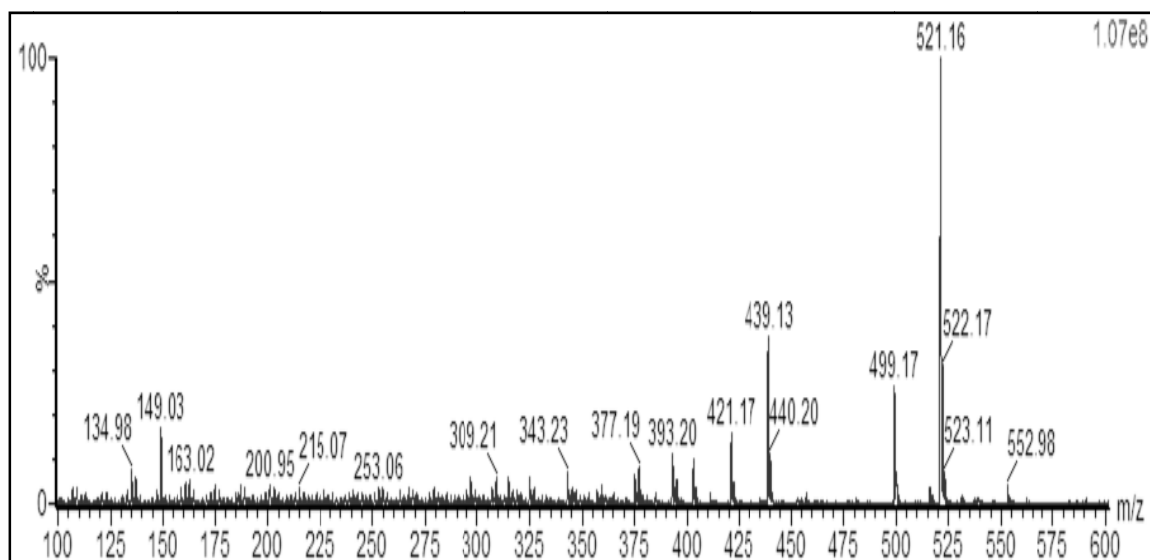
LC-ESI(+)-MS of 12 β -hydroxy-7-deacetyloxyazadiradione



^1H NMR spectrum (CDCl_3 , 400 MHz) of 12β-hydroxygedunin



^{13}C NMR spectrum (CDCl_3 , 100 MHz) of 12β-hydroxygedunin

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of 12β-hydroxygedunin

LC-ESI(+)-MS of 12β-hydroxygedunin

Chapter 3: Section C

Fungi Mediated Functionalization of Salannin

Highlights:

- *Insecticidal C-seco limonoid salannin was efficiently converted into two metabolites by Cunninghamella echinulata.*
- *Major metabolite (salanninolide) was γ -hydroxybutenolide analogue of furan moiety and minor metabolite (salanninactam) was N-(2-hydroxyethyl)- α,β -unsaturated- γ -lactam substituted analogue of furan at C-17.*
- *Present studies have indicated salanninolide to be an intermediate in the C-seco limonoid biosynthetic pathway in the Neem plant.*

3C.1 Introduction

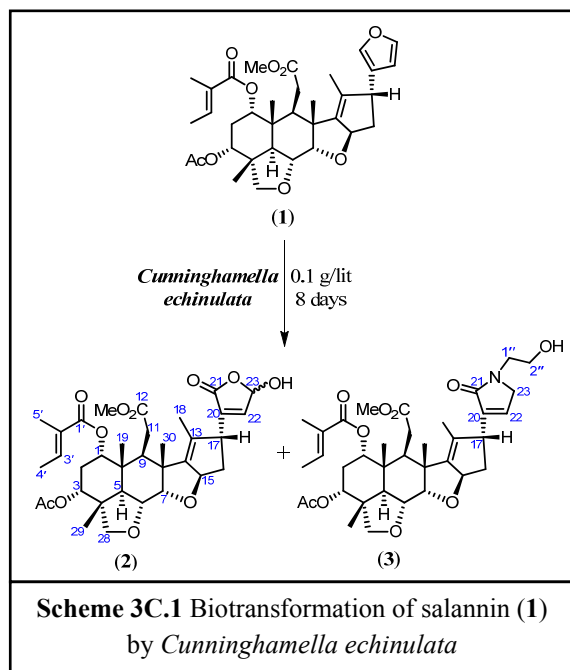
Salannin (**1**), a C-seco limonoid is well-known due to its strong anti-feedant and growth-inhibiting properties towards insects.^{24,38-40} Abundance of salannin among plant resources is restricted to the family of *Maliaceae* and specifically in the genera of *Azadirachta* and *Melia* (e.g. *Azadirachta indica*, *Melia dubia*, *Melia volkensii*).²⁴ Modifications in various functional groups flanked around its skeleton can lead to the variability in its potency as an insecticidal molecule. For example, hydrogenation of furan moiety at C-17 resulted in an enhanced activity against Colorado potato beetle.⁴⁰ However, the complex skeletal architecture and presence of sensitive functional groups make the use of conventional synthesis for structural and functional modifications tedious and time consuming. On the other hand, biocatalysts offer synthetically challenging highly regio- and stereo-selective structural and functional modifications on these complex natural products at mild conditions. Biocatalysts are known to modify natural products to generate bioactive lead derivatives.^{6,41-43} The pronounced insecticidal activity of salannin have prompted to study biocatalyst mediated transformation of salannin. In this section, a novel bioconversion of salannin (**1**) by using the fungal strain *Cunninghamella echinulata* to produce two metabolites salanninolide (**2**) and salanninactam (**3**) was investigated of which metabolite **3** is hitherto not known (Scheme 3C.1).

3C.2 Results and Discussion

3C.2.1 Screening, Time-course Experiment and Preparative Scale Fermentation

Various fungal systems belonging to the genera of *Mucor*, *Aspergillus*, *Rhizopus*, *Neurospora*, *Penicillium* and *Cunninghamella* were screened for the novel and efficient biotransformation of salannin. Of these, *Cunninghamella echinulata* (National Collection of Industrial Microorganisms, catalogue no. 691) was found to carry out efficient transformation of salannin into two metabolites, which were absent in substrate control (i.e. substrate without organism) as well as organism control (i.e. organism without substrate) experiments as monitored by TLC and LC-ESI-HRMS (Figure 3C.1). Stability of salannin in the substrate control experiment excluded the possibility of degradation in the experimental condition. The substrate concentration of 0.1 g/L with

an incubation period of 8 days was optimized from the substrate concentration and time course experiments (Figure 3C.1). Time course experiments with salannin (**1**) revealed that during early stages of incubation (48 h) nearly 48% of **1** was transformed into

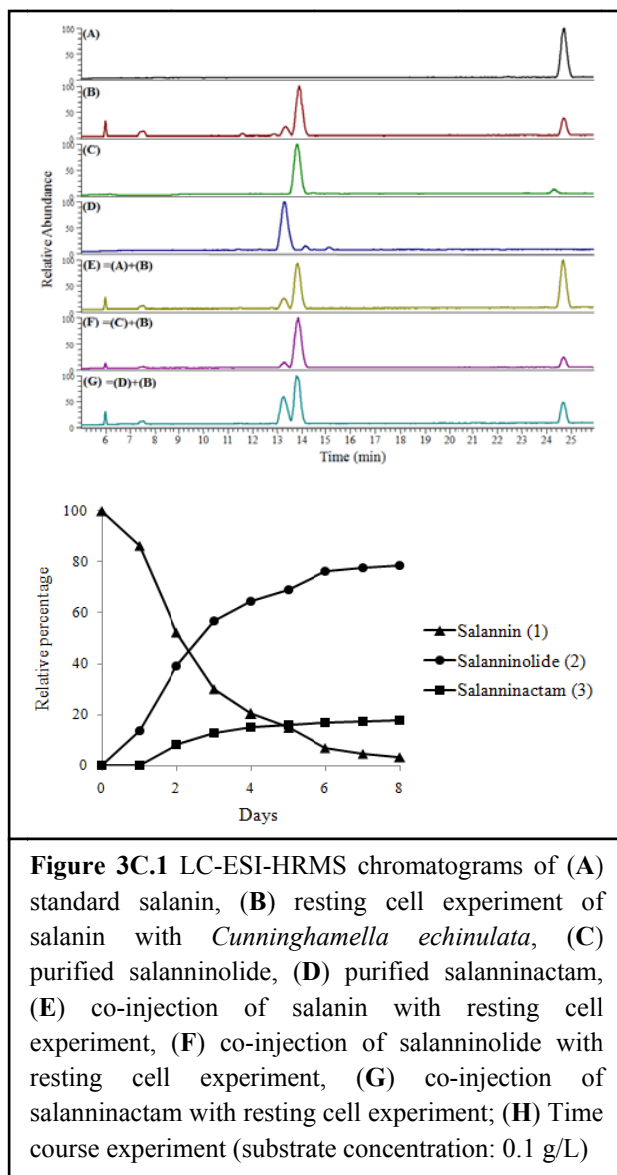


metabolites **2** and **3**. However, prolonging the incubation period to seven days, the transformation increased to 95% (Figure 3C.1). In resting cell experiment, about 80% of the substrate (**1**) was transformed into two metabolites (**2** and **3**) after 36 h of incubation period with substrate concentration 2mg/3g of mycelia (Figure 3C.1). Further, formation of same level of metabolites was observed when the resting cell experiments were carried out in dark indicating that these metabolites were formed through enzymatic

transformation and ruling out the possibility of photo-oxidation of the substrate. Large-scale fermentation of salannin (400 mg) with *Cunninghamella echinulata* yielded a crude extract (433 mg) which upon TLC and LC-ESI-HRMS analyses indicated the presence of three limonoids including un-reacted **1**. The extract was subjected to column chromatography and the metabolites were eluted with methanol-dichloromethane gradient mixture. The purified metabolites **2** (78 mg, R_f : 0.23, R_t : 13.8 min) and **3** (11 mg, R_f : 0.21, R_t : 13.3 min) were structurally characterized on the basis of HRMS, IR, one- and two-dimensional NMR spectrometric and crystallographic studies.

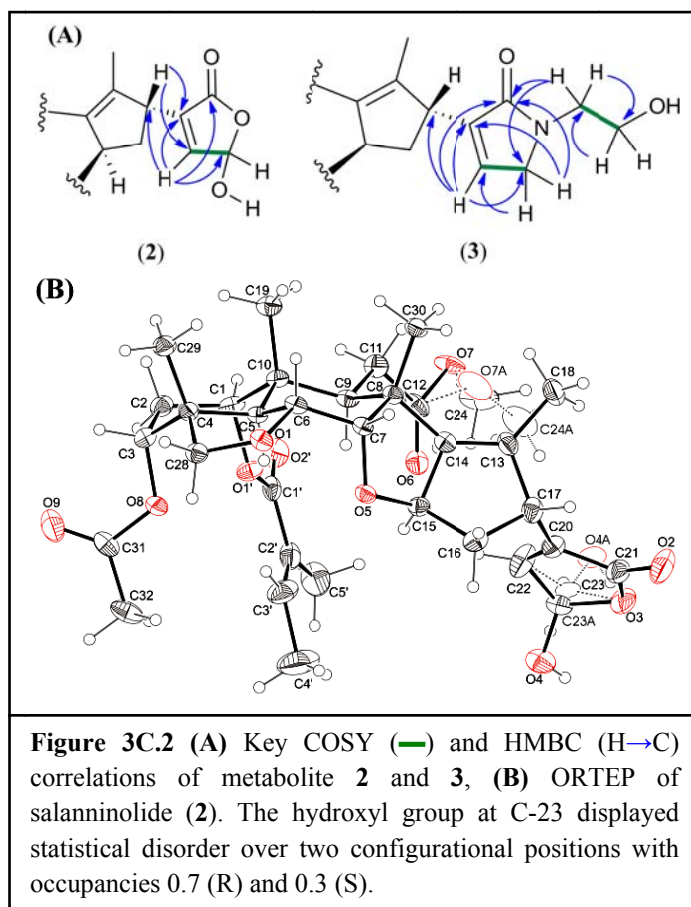
3C.2.2 Characterization of the Metabolites

LC-ESI-HRMS analysis of metabolite **2** showed an ion peak at m/z 651.2762 ($C_{34}H_{44}O_{11}Na$; $[M+Na]^+$) indicating insertion of two oxygen atoms to the salannin ($C_{34}H_{44}O_9$) skeleton maintaining same degree of unsaturation. Exhibition of a broad and strong absorption band at 3397 cm^{-1} (broad and strong) in IR spectrum and $[M+H-H_2O]^+$ ion peak at m/z 611.2848 ($C_{34}H_{43}O_{10}$) in LC-ESI-HRMS implied the presence of hydroxyl functionality in the metabolite **2**. In comparison to salannin (**1**), NMR spectra



(^1H and ^{13}C) of metabolite **2** revealed significant alternation only in the chemical shifts of furan moiety. Therefore, basic skeleton of salannin (**1**) consisting of A, B, C and D rings was concluded to be intact in the metabolite **2**. The conversion of furan moiety to γ -hydroxybutenolide attached to C-17 in metabolite **2** was confirmed on the basis of NMR signals observed for hemiacetal (δ_{C} 98.6, CH) and α,β -unsaturated lactone (δ_{C} 137.4, C and 141.6, CH for double bond; δ_{C} 171.3, C for lactone carbonyl). The presence of α,β -unsaturated lactone and hemiacetal was further substantiated by the peaks at δ_{H} 6.76 (1H, s) and 5.97 (1H, s) respectively in ^1H NMR. The structural connectivity and assignment of NMR chemical shift values were further evaluated through

homo- and hetero-nuclear 2D NMR studies (COSY, NOESY, HSQC and HMBC) (Figure 3C.2). Presence of a spin-system constituting H-17-22-23 in COSY spectrum and correlations (H-22 with C-20, 21, 23, 17 and H-17 with C-20, 22) in HMBC spectrum further affirmed the existence of γ -hydroxybutenolide ring (α,β -unsaturated- γ -hydroxy- γ -lactone) at C-17. Analyzing all the spectroscopic data, metabolite **2** was identified as salanninolide (γ -hydroxybutenolide analogue of salannin) which was previously isolated from the seeds of *Azadirachta indica* (Neem).^{44,45} However, metabolite **2** was isolated as an inseparable diastereomeric mixture (86:14 from ^1H NMR spectrum) at C-23 as γ -hydroxybutenolides are highly susceptible to isomerisation at the hydroxy-bearing centre as observed with scalarane sesterterpene skeletons.⁴⁶ The structure of metabolite **2** and its existence as diastereomeric mixture was further



unambiguously confirmed on the basis of single crystal X-ray diffraction data (Figure 3C.2).⁴⁷ LC-ESI-HRMS spectrum of metabolite **3** showed major ion peak at m/z 678.3251 ($C_{36}H_{49}O_{10}NNa$; $[M+Na]^+$) corresponding to the insertion of two carbons, one oxygen and one nitrogen atoms to the salannin ($C_{34}H_{44}O_9$) skeleton. Absorption bands at 3422 (broad) and 1671 (sharp) cm^{-1} in IR spectrum indicated the presence of hydroxyl and amide/lactam functionality. As observed with metabolite **2**,

chemical shift values for the rings A, B, C and D were similar to salannin for metabolite **3** except the furan moiety at C-17. Presence of α,β -unsaturated lactam was predicted on the basis of peaks at δ_C 141.2, 135.3 (C and CH respectively, double bond) and 172.7 (C, lactam carbonyl) in ^{13}C NMR spectrum. The peak at δ_H 6.80 (1H, m) in ^1H NMR spectrum further supported the presence of α,β -unsaturated lactam containing tri-substituted double bond. ^{13}C and DEPT-135 NMR data indicated the presence of two additional methylene carbons in metabolite **3** compared to salannin. Considering the degree of unsaturation (*i.e.* 13, same as that of salannin) and chemical shift values of methylene carbons (^{13}C NMR δ_C : 62.2, 52.6 and 46.6) for the side chain of metabolite **3**, a N-(2-hydroxyethyl) substituted α,β -unsaturated- γ -lactam structure was constructed. Existence of methylene protons at δ_H 3.97 (2H, s), 3.83 (2H, t) and 3.57-3.68 (2H, overlapped with H-17 and 28) in ^1H NMR and their correlation with δ_C 52.6, 62.2 and 46.6 respectively in HSQC spectrum further supported the structural framework. COSY spectrum showed the presence of only two spin systems (H-22-23 and H-1''-2'') in the side chain at C-17. Key correlations such as H-22 $\leftrightarrow$ C-20, C-21, C-23, C-17 and H-23 $\leftrightarrow$ C-20, C-21, C-22 in HMBC spectrum further upheld the presence of α,β -

Table 3C.1 ^1H (400 MHz) and ^{13}C NMR (100 MHz) assignments of metabolites **2** and **3** in CDCl_3

No.	Salanninolide (2)		Salanninactam (3)	
	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)
1	70.5 CH	4.89, t (2.4)	71.6 CH	4.73, t (2.4)
2	28.2 CH_2	^a	27.4 CH_2	^b
3	71.2 CH	4.98, t (2.4)	71.3 CH	4.95, t (2.4)
4	42.6 C	-	42.7 C	-
5	40.2 CH	2.72, d (12.8)	39.9 CH	2.79, d (12.5)
6	72.5 CH	3.98, dd (12.5, 3.4)	72.5 CH	3.99, dd (12.8, 3.4)
7	86.0 CH	4.25, d (3.0)	85.6 CH	4.20, d (3.0)
8	48.3 C	-	49.4 C	-
9	39.2 CH	2.53, m	39.4 CH	2.71, t (6.1)
10	40.5 C	-	40.6 C	-
11	29.9 CH_2	^a	30.8 CH_2	^b
12	174.6 C	-	173.2 C	-
13	132.7 C	-	133.4 C	-
14	147.8 C	-	148.4 C	-
15	87.4 CH	5.42, m	87.6 CH	5.30, m
16	40.2 CH_2	^a	40.0 CH_2	^b
17	48.7 CH	3.49, d (6.7)	49.1 CH	^c
18	13.3 CH_3	1.83, s	13.2 CH_3	1.72, s
19	15.3 CH_3	0.94, s	15.0 CH_3	1.01, s
20	137.4 C	-	141.2 C	-
21	171.3 C	-	172.7 C	-
22	141.6 CH	6.76, s	135.3 CH	6.80, m
23	96.8 CH	5.97, s	52.6 CH_2	3.97, s
23-OH	-	5.30, br s	-	-
28	77.6 CH_2	3.72, d (7.6) 3.61, d (7.6)	77.6 CH_2	^c
29	19.4 CH_3	1.21, s	19.7 CH_3	1.22, s
30	16.3 CH_3	1.30, s	16.9 CH_3	1.30, s
12-OMe	52.5 CH_3	3.44, s	51.5 CH_3	3.43, s
3-OAc	21.0 CH_3	2.00, s	20.8 CH_3	1.92, s
	170.3 C		170.3 C	
1'	166.5 C	-	166.7 C	-
2'	128.9 C	-	129.1 C	-
3'	137.3 CH	6.96, m	137.1 CH	6.92, m
4'	14.4 CH_3	1.85, d (7.0)	14.4 CH_3	1.79, d (7.0)
5'	11.9 CH_3	1.90, s	11.9 CH_3	1.90, s
1''	-	-	46.6 CH_2	^c
2''	-	-	62.0 CH_2	3.83, t (4.9)

Overlapped in the region ^a δ_{H} 2.10-2.40, ^b δ_{H} 2.04-2.32 and ^c δ_{H} 3.57-3.68.

unsaturated lactam moiety. The presence of N-(2-hydroxyethyl) was confirmed on the basis of correlations $H-2'' \leftrightarrow C-1''$ and $H-1'' \leftrightarrow C-2''$, C-23, C-21 as observed by HMBC analysis. The observations obtained from COSY and HMBC spectra (Figure 3C.2) were in full agreement with the construction and arrangement of various functionalities in the metabolite **3**. Thus, metabolite **3** was structurally characterized as novel N-substituted α,β -unsaturated- γ -lactam analogue of salannin on the basis of spectral data and named as salanninactam.

3C.3 Conclusion

Salanninolide (**2**), a minor constituent of the diverse limonoid pool from Neem was reported to be photo-oxidized product of salannin (**1**) in organic solvent.^{48,49} Consequently, salanninolide and other γ -hydroxybutenolides from Neem were proposed either to be photo-degraded products of the corresponding limonoids or alternatively an intermediate in the formation of C-17 furan ring.⁵⁰ In this study, fungi mediated transformation indicated an enzymatic pathway leading to the biosynthesis of salanninolide from salannin. Therefore, the abundance of salanninolide (**2**) in Neem might not be due to the photo-oxidation of salannin and indicated it as a metabolic product of limonoid biosynthetic pathway.

Photo-mediated oxidation of salannin (**1**) is reported to produce salanninolide (**2**) along with two other isomeric analogues, namely isosalanninolide and Δ^{17} -isosalanninolide.⁴⁸ In comparison, microbial system selectively transformed furan to γ -hydroxybutenolide as a major metabolite which is truly rare.⁵¹ Bioconversion reaction leading to the transformation of furan to α,β -unsaturated- γ -lactam ring (**3**) is one of the rare biotransformation reactions.⁵² In fact, natural existence of α,β -unsaturated five-membered lactam ring at the side chain of limonoids has been reported rarely.⁵³⁻⁵⁶ In the present study, rare and novel bioconversion of furan moiety of salannin to γ -hydroxybutenolide and N-(2-hydroxyethyl) substituted α,β -unsaturated- γ -lactam analogue demonstrated the potential of microbial system, especially *Cunninghamella echinulata* to transform complex natural products into novel metabolites which might be useful further for the large scale production of these metabolites for various applications.

3C. 4 Experimental Section

3C.4.1 General Information

Thin film IR spectra were recorded in CHCl_3 using Perkin Elmer FT-IR spectrophotometer and optical rotations were measured in JASCO (P-2000) polarimeter at 589 nm in CHCl_3 (c in g/100 mL). NMR (^1H , ^{13}C , DEPT-135, COSY, NOESY, HSQC and HMBC) data were recorded on Bruker 400 MHz spectrophotometer in CDCl_3 and signal for the residual solvent was marked as the reference. HRMS data and quantification of the individual limonoids were performed on Q Exactive Quadrupole/Orbitrap MS system (Thermo Scientific) attached with Accela 1250 pump. Thermo Scientific Hypersil Gold column of particle size 5 μm with a flow rate of 0.5 mL/min and gradient solvent program of 27.0 min (0.0 min, 10% methanol/water; 0.5 min, 10.0% methanol/water; 2.0 min, 45% methanol/water; 10.0 min, 50% methanol/water; 20.0 min, 65% methanol/water; 22.0 min, 90% methanol/water; 24.0 min, 90% methanol/water; 25.0 min, 10% methanol/water; 27.0 min, 10% methanol/water) was used for the resolution of individual components. HRMS experiments were performed in ESI-positive ion mode within the mass range m/z 100-1000 using the tune method as sheath gas flow rate 45, auxiliary gas flow rate 10, sweep gas flow rate 2, spray current (μA) 3.70, spray voltage (KV) 3.60, capillary temperature ($^\circ\text{C}$) 320, heater temperature ($^\circ\text{C}$) 350, s-lens RF level 50. For LC-ESI-HRMS analyses LC-MS grade solvents were purchased from JT Baker. Technical grade solvents were used for the extraction and preparative chromatography purpose and distilled prior to use. Chemicals for preparing medium were procured from Himedia. Thin layer chromatography (TLC) was carried out on Merck silica gel-G pre-coated plates of thickness 0.25 mm and developed twice using the mobile phase of 3.0% methanol in dichloromethane. Anisaldehyde charring reagent was sprayed on TLC and heated for visualizing the spots. Metabolites were purified over silica-gel (230-400 mesh) flash column chromatography using the gradient mixture of methanol in dichloromethane as the eluent. X-ray intensity data measurements of salanninolide (**2**) was carried out on a Bruker SMART APEX II CCD diffractometer with graphite-monochromatized ($\text{MoK}\alpha = 0.71073\text{\AA}$) radiation at 90 (2) K. The X-ray generator was operated at 50 kV and 30 mA. A preliminary set of cell constants and an orientation matrix were calculated from three sets of 12 frames. Data were collected with ω scan width of 0.5° at

eight different settings of ϕ and 2θ with a frame time of 10 sec keeping the sample-to-detector distance fixed at 5.00 cm. The X-ray data collection was monitored by APEX2 program.

3C.4.2 Substrate, Organisms and Screening

Salannin, used in this study was isolated from the seed kernel of *Azadirachta indica* (Neem) as discussed earlier (Chapter 2). Micro-organisms used for the screening and preparative scale fermentation purpose were obtained from National Collection of Industrial Microorganisms (NCIM), CSIR-NCL, Pune. Cultures were stored at 4 °C and propagated at 30 °C on PDA (Potato dextrose agar) slants. Inoculum (3.0% v/v) prepared from 2 days grown culture was used for the inoculation of 100 mL sterile modified Czapek-dox medium contained in a 500 mL conical flask maintaining aseptic condition. Flasks were further left for orbital shaking on rotary shaker at 30 °C and 180 rpm for 2 days. To the well grown culture of different fungi in 100 mL of medium, 10 mg salannin (**1**) was added dissolving in 0.2 mL of acetone. Before addition of the substrate pH of the cultured medium was adjusted to 6.0-7.0 using 1 M K_2HPO_4 . After incubation at 30 °C and 180 rpm for 5 days, both the broth and mycelia was extracted separately with ethyl acetate (100 mL $\times$ 2) and ethyl acetate layer after concentration was analyzed for bioconversion of salannin (**1**) by TLC and LC-ESI-HRMS.

3C.4.3 Time-course and Resting Cell Experiment

Time-course experiment with *Cunninghamella echinulata* was carried out by aseptically taking out 10 mL of medium (100 mL containing 10 mg of salannin) in every 24 h and extracting the fermented medium by ethyl acetate (10 mL $\times$ 2). Progress of the reaction was monitored by injecting concentrated organic layer in LC-ESI-HRMS and the level of metabolites or substrate was quantified using the standard graphs drawn for **1**, **2** and **3** using area under respective peaks. The peak positions of individual components were confirmed by co-injection studies. For the resting cell experiment, well grown culture of *Cunninghamella echinulata* was filtered through muslin cloth and mycelia was washed repeatedly by distilled water followed by phosphate buffer of pH 7.2. Squeezed mycelia (3 g) were added to 50 mL of phosphate buffer (pH 7.2) containing 250 mg dextrose and 2 mg salannin (in 0.2 mL acetone). Reaction mixture was incubated at 30 °C and

180 rpm for 36 h. After the incubation the reaction mixture was extracted with ethyl acetate (50 mL $\times$ 2) and analyzed by TLC and LC-ESI-HRMS. Similarly, dark-control resting cell experiments were carried out by incubating the reaction mixture in brown conical flask under dark condition.

3C.4.4 Preparative Scale Biotransformation

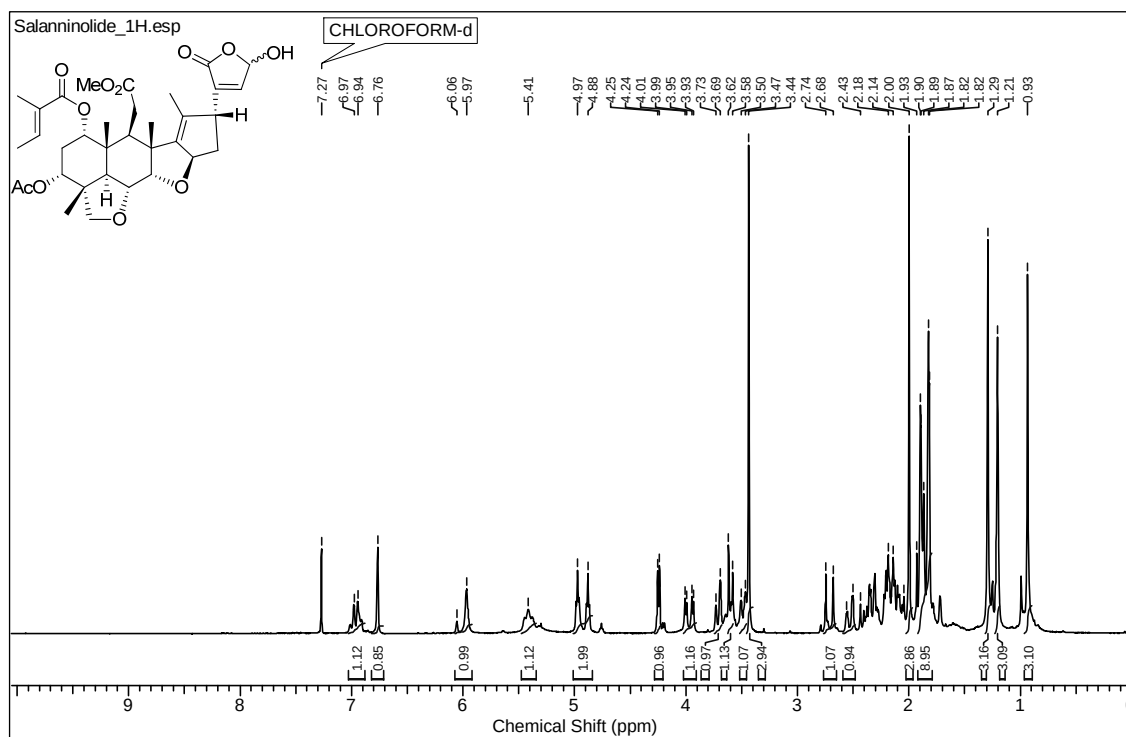
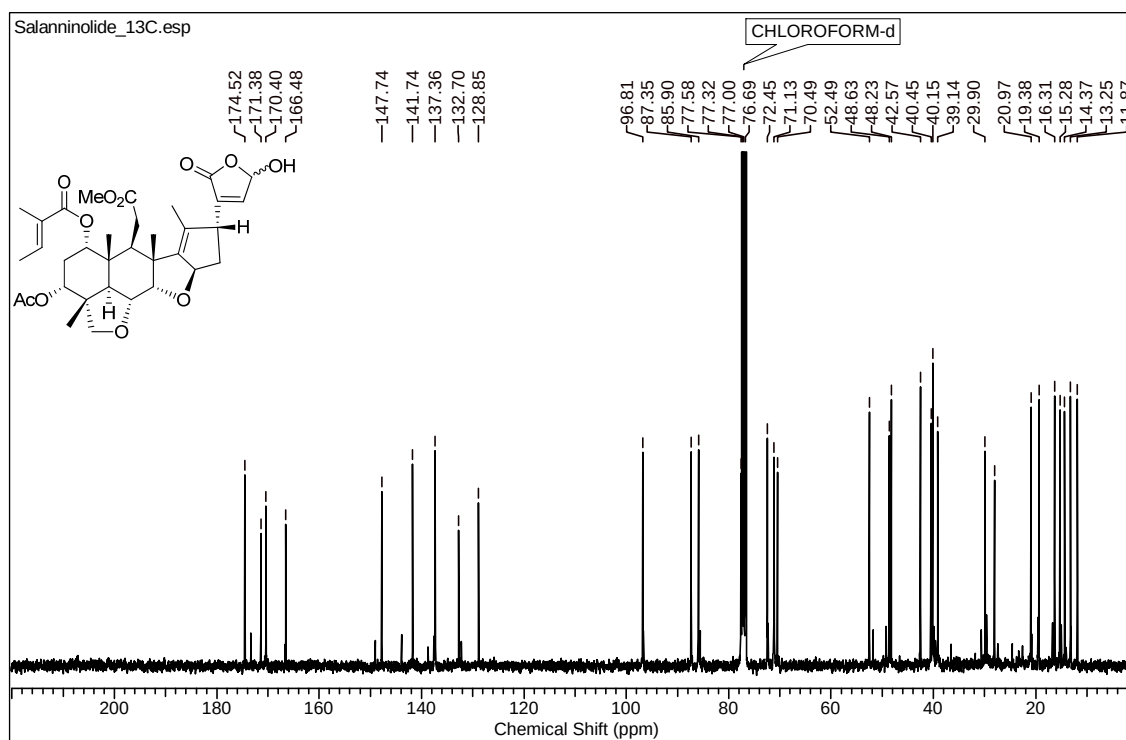
Large scale fermentation was carried out by distributing 0.40 g salannin (dissolved in 8.0 mL acetone) in 40 flasks each containing 100 mL well-grown culture of *Cunninghamella echinulata* and incubating under identical conditions. At higher substrate concentration, the level of metabolite formation was reduced. After 8 days of incubation, contents of all the flasks were pooled together and mycelia were separated by filtration. Broth and mycelia were extracted individually using ethyl acetate (4 L $\times$ 3 and 500 mL $\times$ 3 respectively). Both the ethyl acetate layers were concentrated separately and analyzed to check for the metabolites. According to the TLC and LC-ESI-HRMS, metabolites were observed only in the broth extract (0.56 g) and not in the mycelial extract. The brown crude broth extract was further purified by flash column chromatography by gradient elution of increasing percentage of methanol in dichloromethane. Unmetabolized salannin was eluted at 1.0% methanol in dichloromethane followed by salanninolide (**2**) at 1.4% and salanninactam (**3**) at 2.8%. Purified metabolites **2** and **3** were characterized using numerous spectroscopic and crystallographic studies.

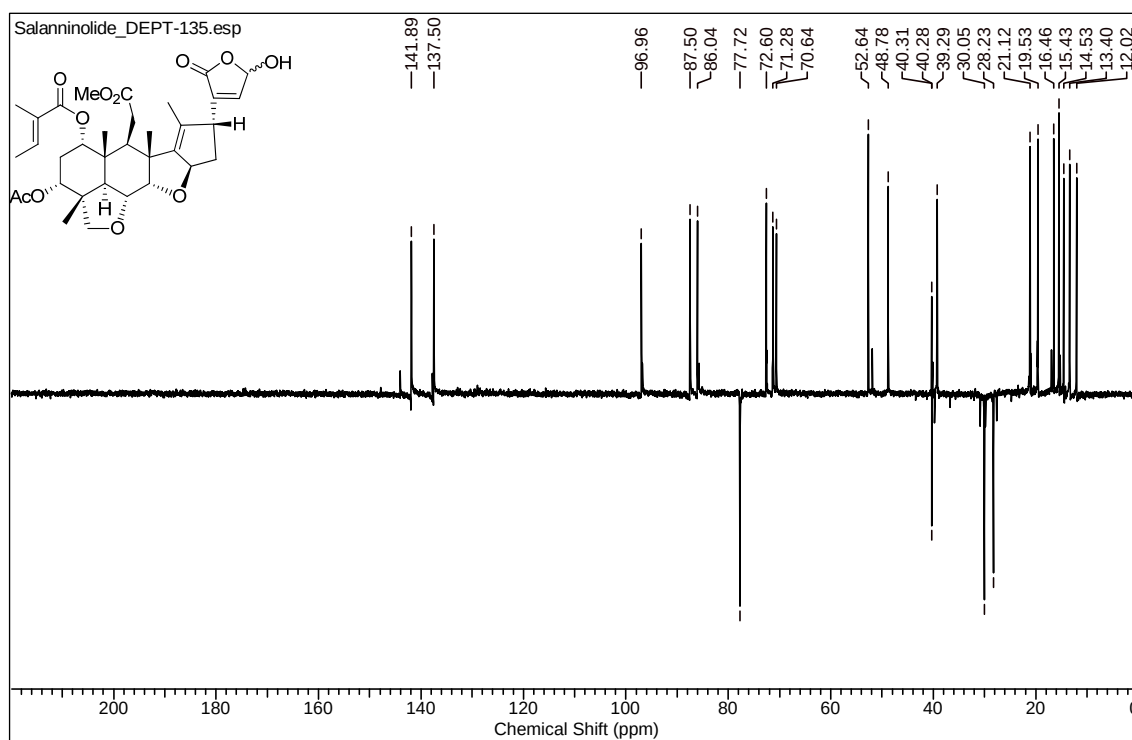
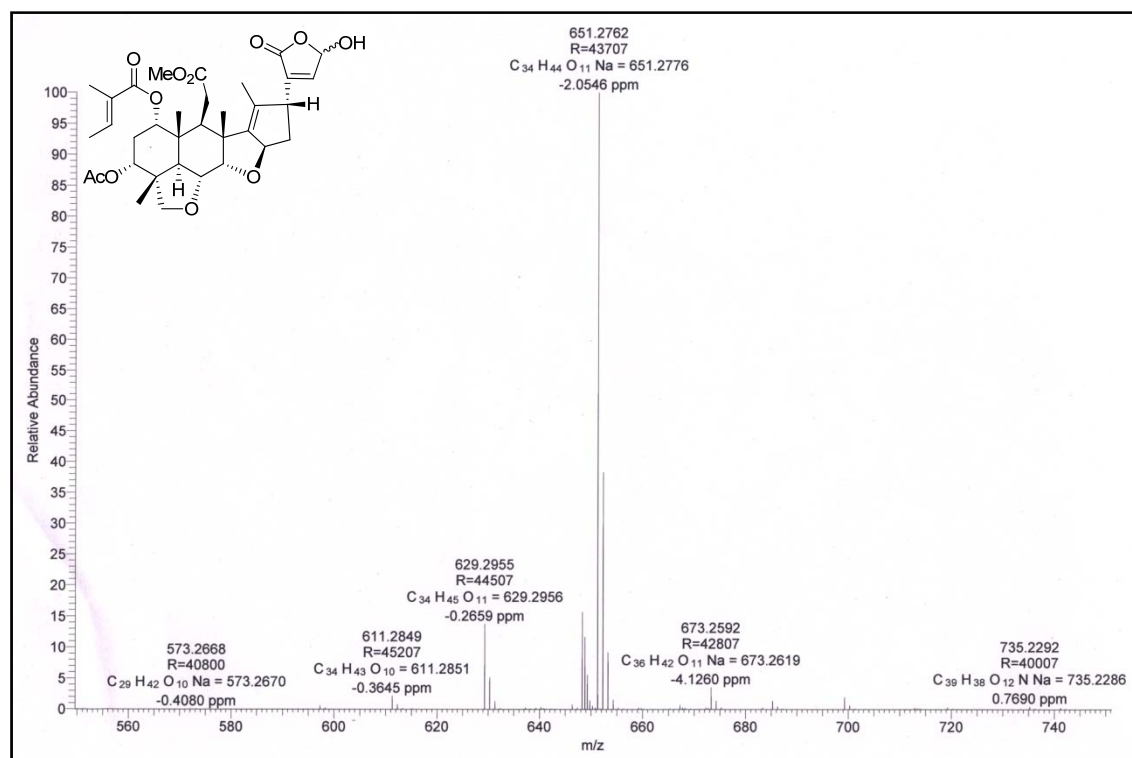
3C.4.5 Characterization Data of the Metabolites

Salanninolide (2): White crystalline solid. $[\alpha]_D^{25} = +148.8$ (*c* 2.2, CHCl₃). IR (CHCl₃) $\nu_{\max}$ (cm⁻¹): 3397, 1763, 1735, 1718, 1700. ¹H NMR (CDCl₃, 400 MHz) δ : Table 3C.1. ¹³C NMR (CDCl₃, 100 MHz) δ : Table 3C.1. HRMS (ESI) *m/z*: [M+Na]⁺ Calcd for C₃₄H₄₄O₁₁Na, 651.2776; found, 651.2762.

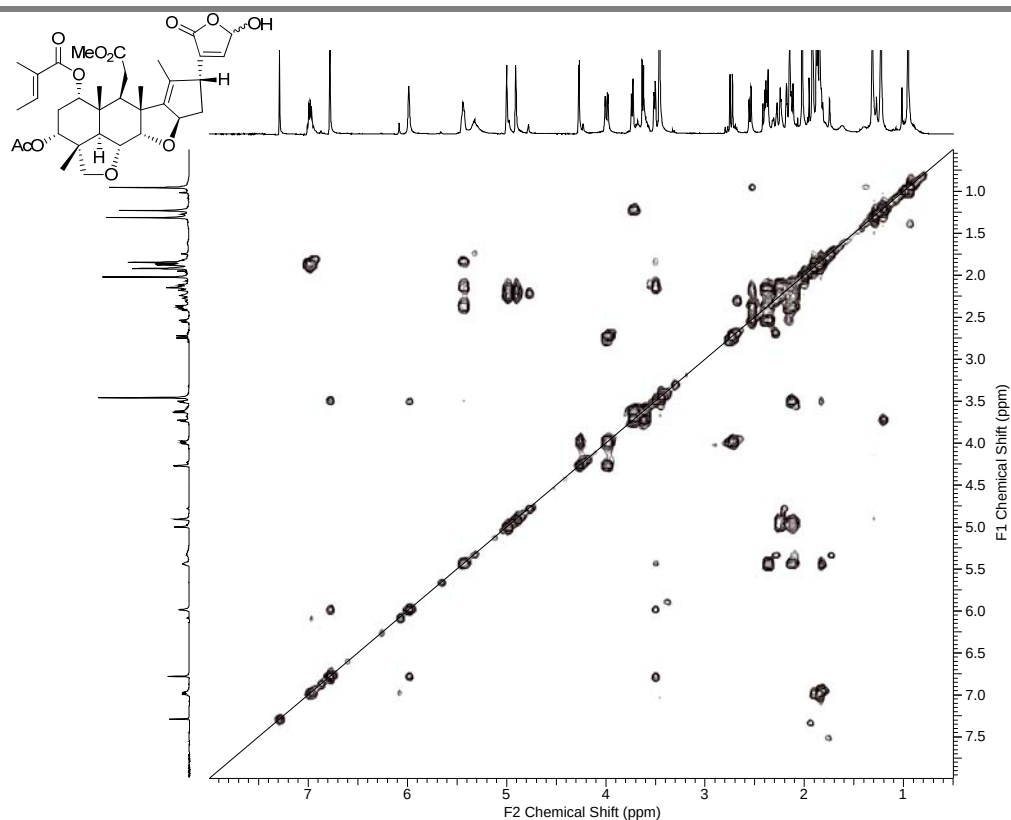
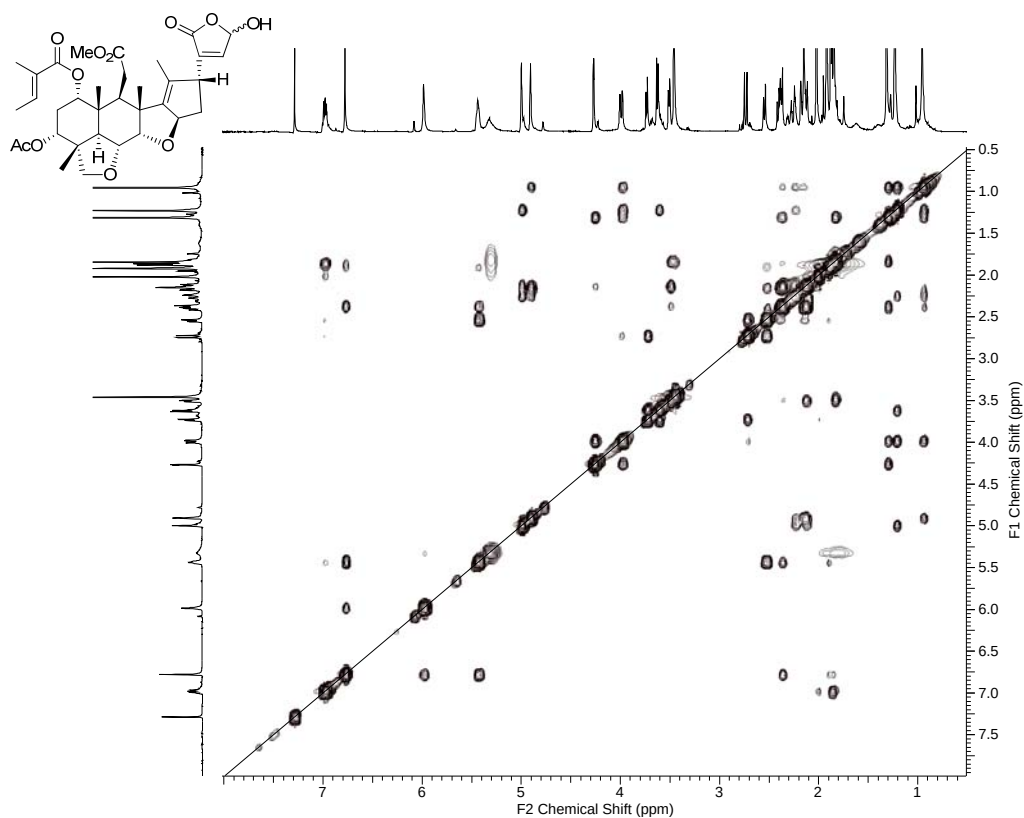
Salanninactam (3): White solid. $[\alpha]_D^{25} = +109.4$ (*c* 0.9, CHCl₃). IR (CHCl₃) $\nu_{\max}$ (cm⁻¹): 3422, 1733, 1700, 1671. ¹H NMR (CDCl₃, 400 MHz) δ : Table 3C.1. ¹³C NMR (CDCl₃, 100 MHz) δ : Table 3C.1. HRMS (ESI) *m/z*: [M+Na]⁺ Calcd for C₃₆H₄₉O₁₀NNa, 678.3249; found 678.3251.

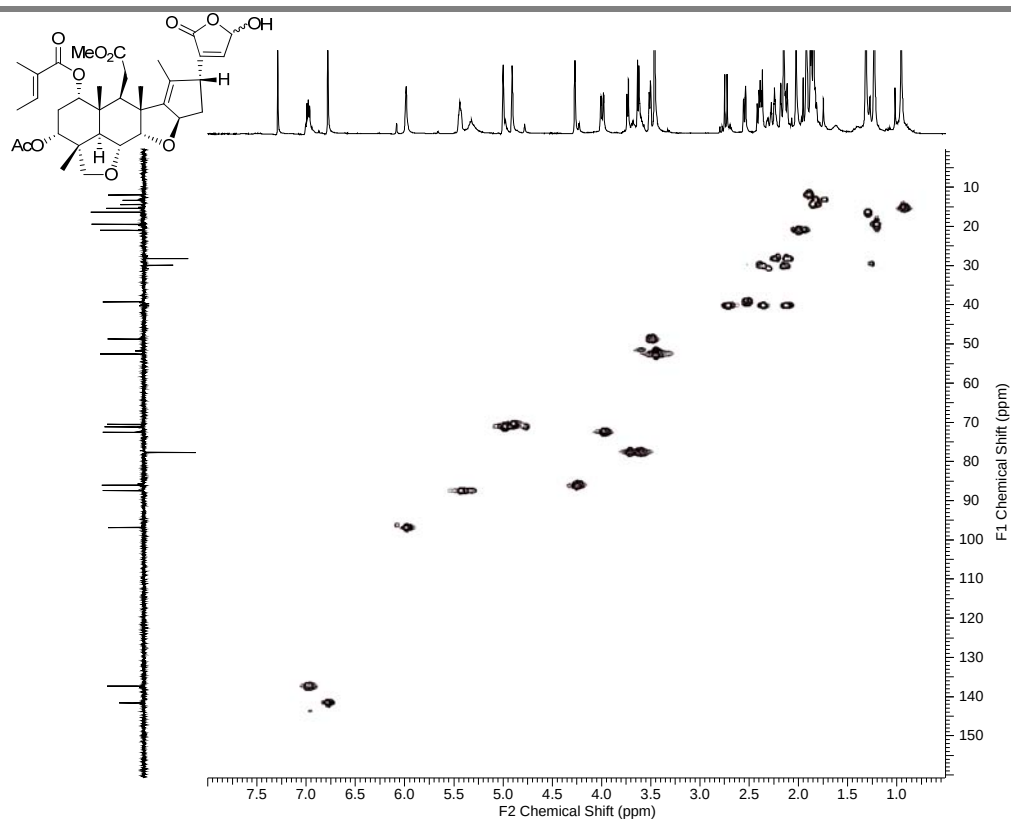
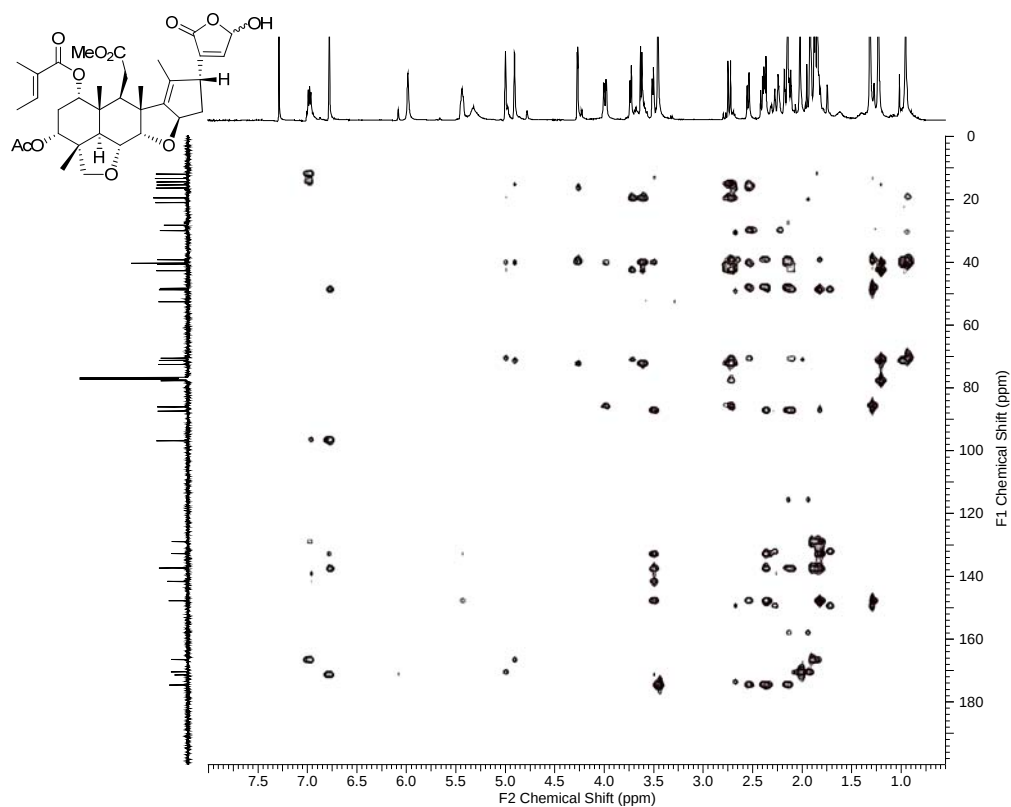
3C.5 Spectral Copies

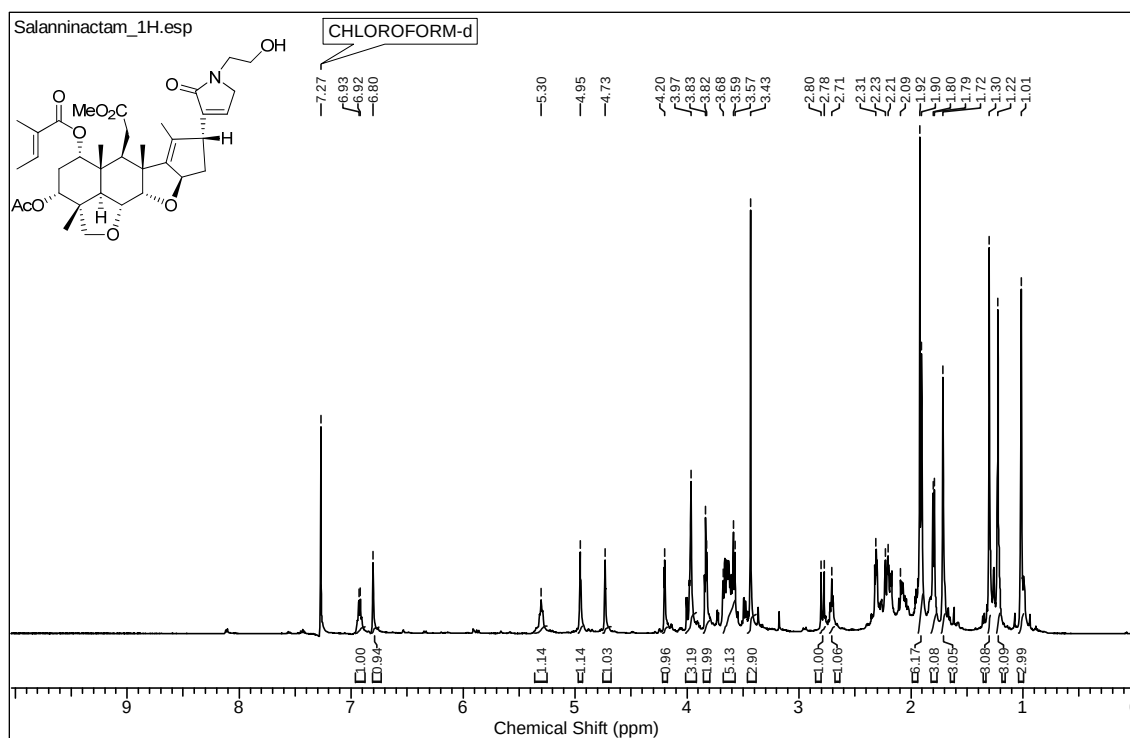
 ^1H NMR spectrum (CDCl_3 , 400 MHz) of salanninolide ^{13}C NMR spectrum (CDCl_3 , 100 MHz) of salanninolide

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of salanninolide

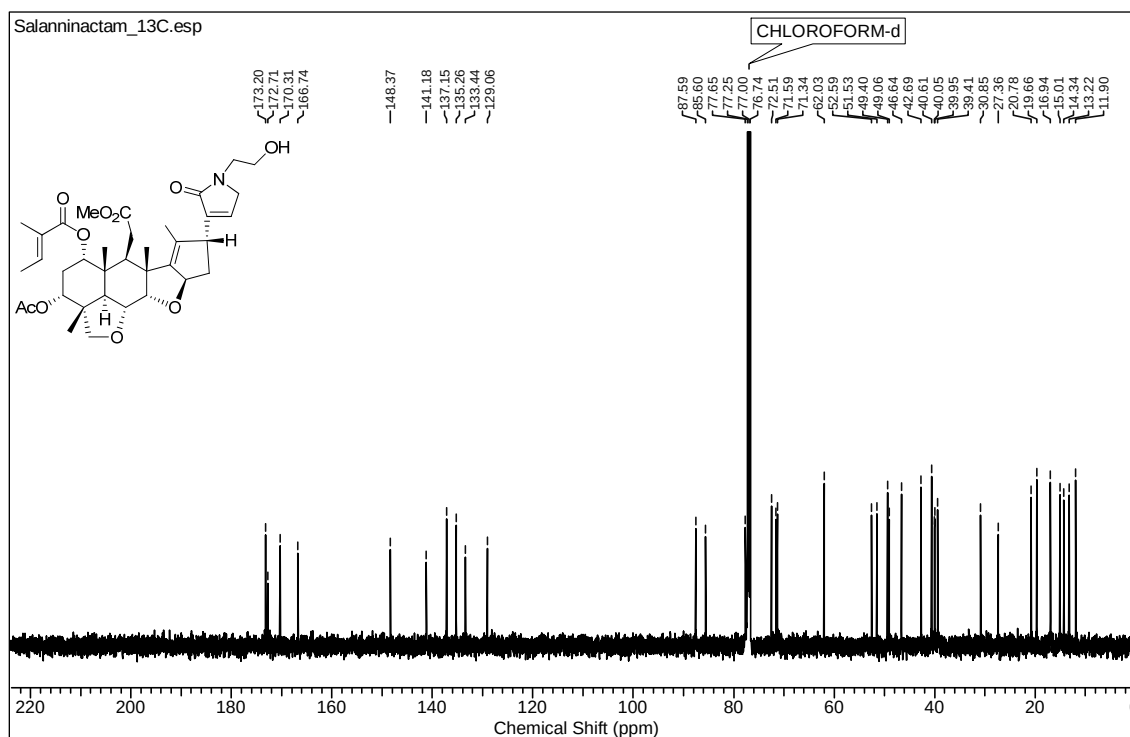
LC-ESI(+)-HRMS of salanninolide

COSY spectrum of salanninolide in CDCl₃NOESY spectrum of salanninolide in CDCl₃

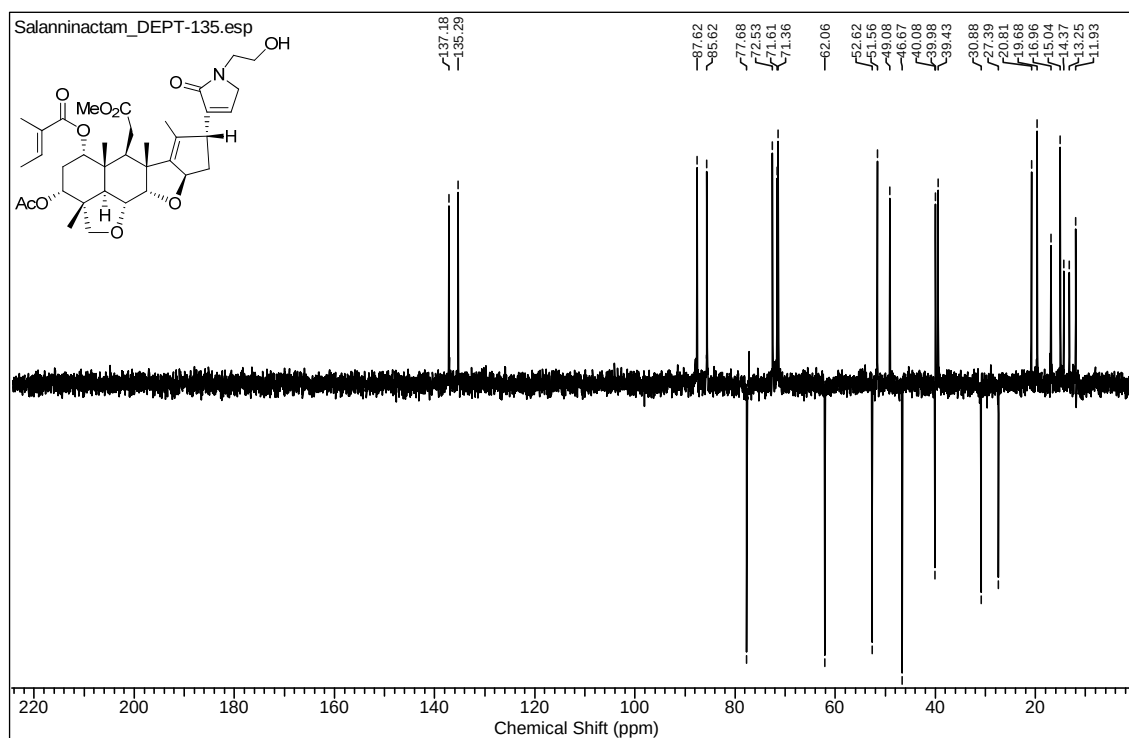
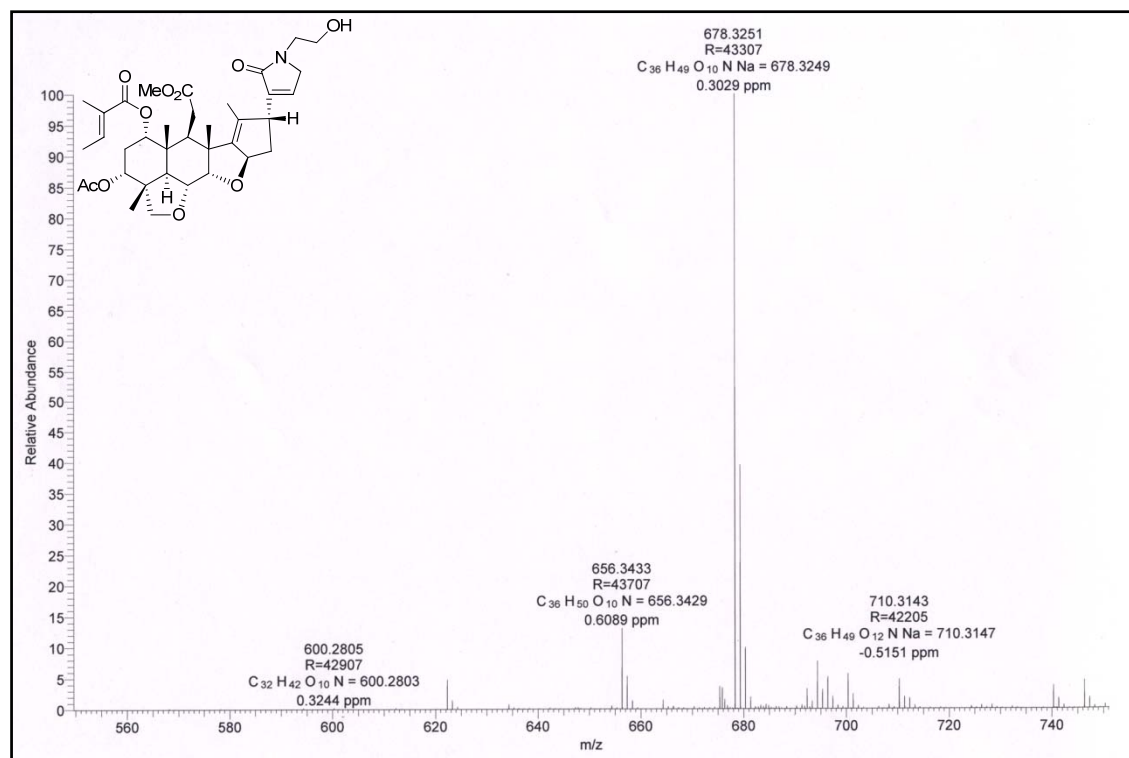
HSQC spectrum of salanninolide in CDCl₃HMBC spectrum of salanninolide in CDCl₃



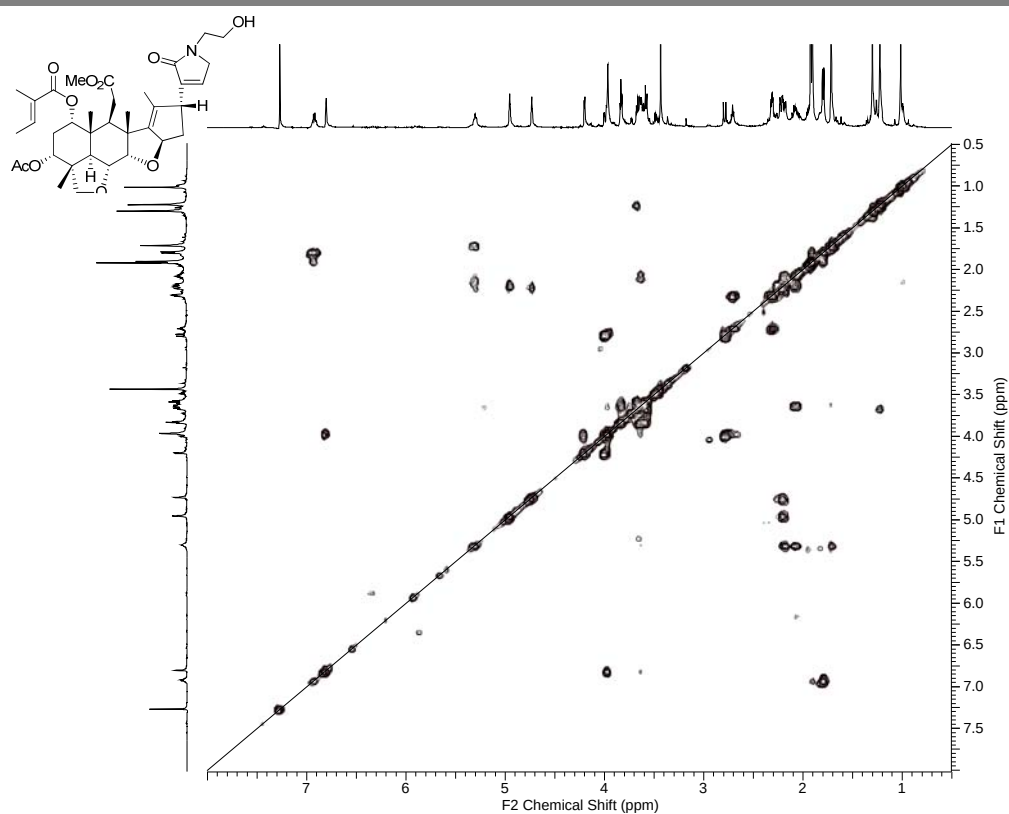
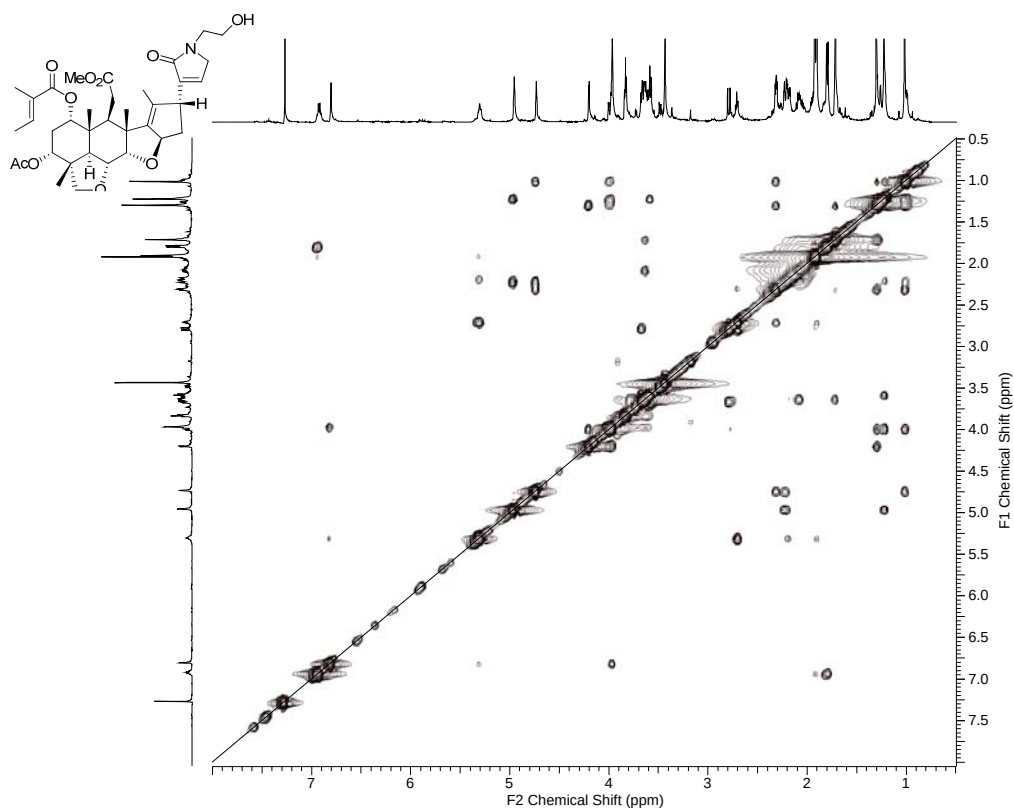
^1H NMR spectrum (CDCl_3 , 400 MHz) of salanninactam

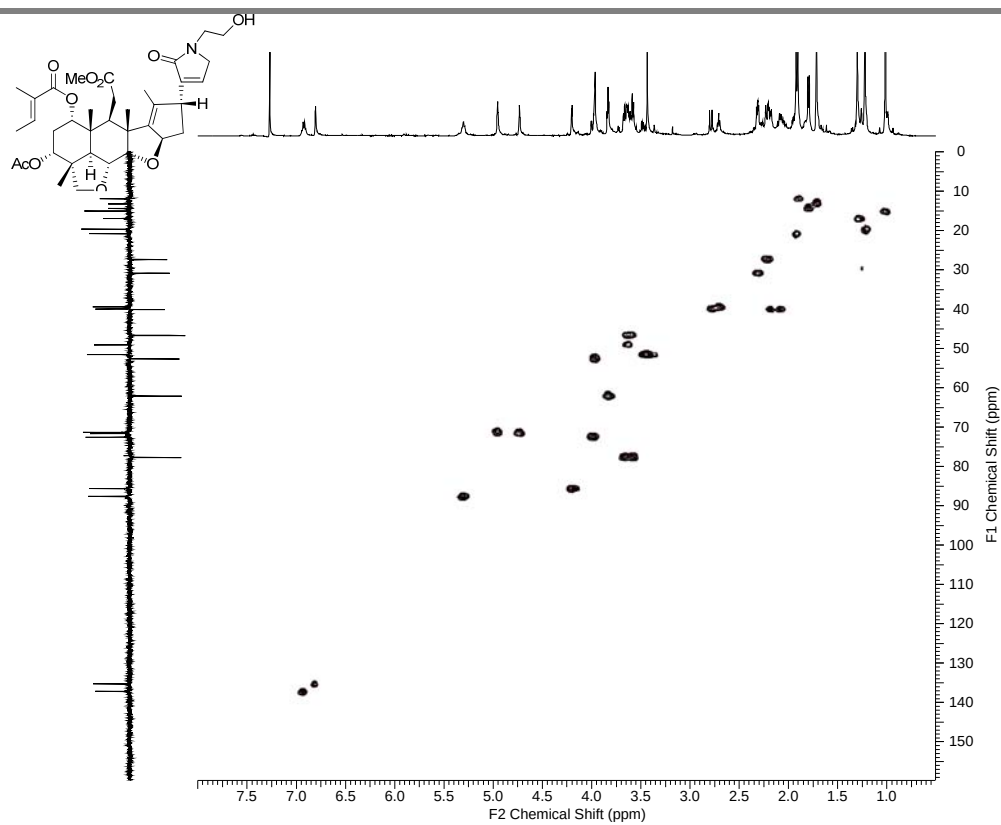
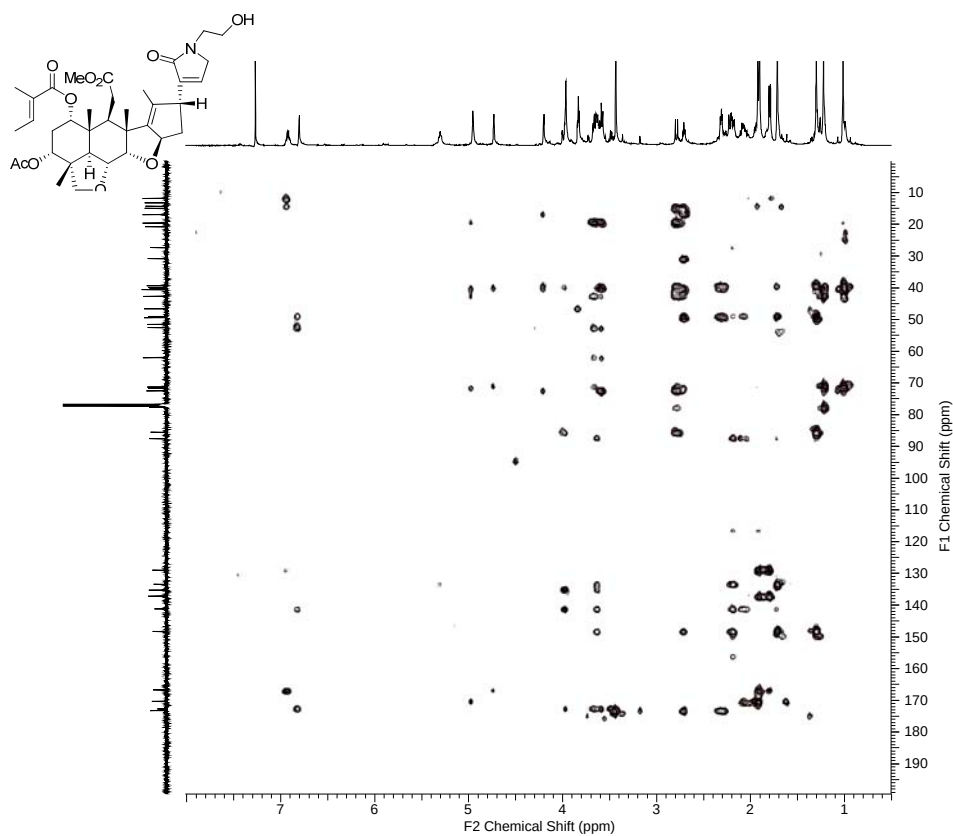


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of salanninactam

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of salanninactam

LC-ESI(+)-HRMS of salanninactam

COSY spectrum of salanninactam in CDCl₃NOESY spectrum of salanninactam in CDCl₃

HSQC spectrum of salanninactam in CDCl₃HMBC spectrum of salanninactam in CDCl₃

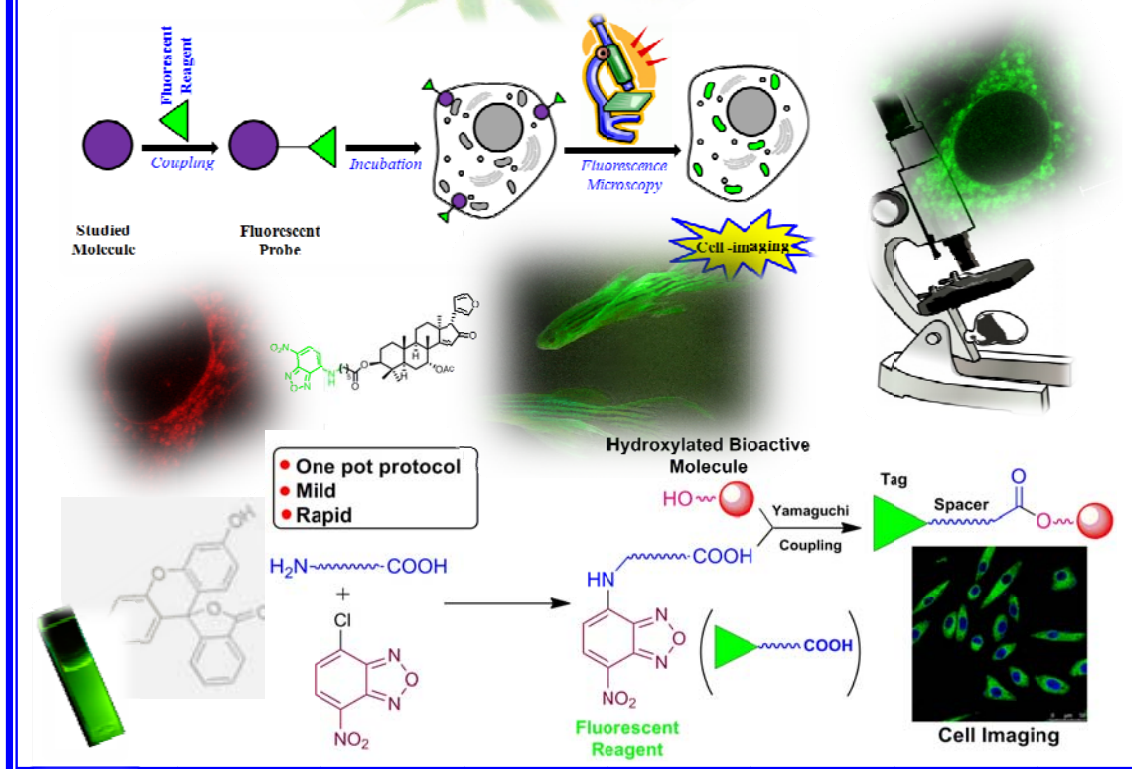
3D References

- (1) Schoemaker, H. E.; Mink, D.; Wubbolts, M. G. *Science* **2003**, *299*, 1694-1697.
- (2) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258-268.
- (3) Pollard, D. J.; Woodley, J. M. *Trends Biotechnol.* **2007**, *25*, 66-73.
- (4) Patel, R. N. *ACS Catal.* **2011**, *1*, 1056-1074.
- (5) Patel, R. N. *Coordin. Chem. Rev.* **2008**, *252*, 659-701.
- (6) Ishige, T.; Honda, K.; Shimizu, S. *Curr. Opin. Chem. Biol.* **2005**, *9*, 174-180.
- (7) Davis, B. G.; Borer, V. *Nat. Prod. Rep.* **2001**, *18*, 618-640.
- (8) Clouthier, C. M.; Pelletier, J. N. *Chem. Soc. Rev.* **2011**, *41*, 1585-1605.
- (9) Reetz, M. T. *Tetrahedron* **2012**, *68*, 7530-7548.
- (10) Ran, N. Q.; Zhao, L. S.; Chen, Z. M.; Tao, J. H. *Green Chem.* **2008**, *10*, 361-372.
- (11) Woodley, J. M. *Biochem. Soc. Trans.* **2006**, *34*, 301-303.
- (12) Burton, S. G.; Cowan, D. A.; Woodley, J. M. *Nature Biotech.* **2002**, *20*, 37-45.
- (13) Muffler, K.; Leipold, D.; Scheller, M.-C.; Haas, C.; Steingroewer, J.; Bley, T.; Neuhaus, H. E.; Mirata, M. A.; Schrader, J.; Ulber, R. *Process Biochem.* **2011**, *46*, 1-15.
- (14) Bhatti, H. N.; Khera, R. A. *J. Asian Nat. Prod. Res.* **2014**, *16*, 70-104.
- (15) Parra, A.; Rivas, F.; Garcia-Granados, A.; Martinez, A. *Mini-Rev. Org. Chem.* **2009**, *6*, 307-320.
- (16) Chatterjee, P.; Pezzuto, J. M.; Kouzi, S. A. *J. Nat. Prod.* **1999**, *62*, 761-763.
- (17) Kouzi, S. A.; Chatterjee, P.; Pezzuto, J. M.; Hamann, M. T. *J. Nat. Prod.* **2000**, *63*, 1653-1657.
- (18) Chatterjee, P.; Kouzi, S. A.; Pezzuto, J. M.; Hamann, M. T. *Appl. Environ. Microb.* **2000**, *66*, 3850-3855.
- (19) Bastos, D. Z. L.; Pimentel, I. C.; de Jesus, D. A.; de Oliveira, B. s. H. *Phytochemistry* **2007**, *68*, 834-839.
- (20) Zhang, J.; Cheng, Z.-H.; Yu, B.-Y.; Cordell, G. A.; Qiu, S. X. *Tetrahedron Lett.* **2005**, *46*, 2337-2340.
- (21) Madyastha, K. M.; Venkatakrishnan, K. *J. Chem. Soc. Perkin Trans. 1* **1998**, 1183-1184.
- (22) Madyastha, K. M.; Venkatakrishnan, K. *J. Chem. Soc. Perkin Trans. 1* **2000**, 3055-3062.
- (23) Madyastha, K. M.; Venkatakrishnan, K. *Tetrahedron Lett.* **1999**, *40*, 5243-5246.
- (24) Champagne, D. E.; Koul, O.; Isman, M. B.; Scudder, G. G. E.; Towers, G. H. N. *Phytochemistry* **1992**, *31*, 377-394.
- (25) Chianese, G.; Yerbanga, S. R.; Lucantoni, L.; Habluetzel, A.; Basilica, N.; Taramelli, D.; Fattorusso, E.; Taglialatela-Scafati, O. *J. Nat. Prod.* **2010**, *73*, 1448-1452.
- (26) Delafuente, J. A.; Marugan, J. J.; Cross, S. S.; Mateos, A. F.; Garcia, S.; Menendez, A. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1471-1474.
- (27) Alam, A.; Haldar, S.; Thulasiram, H. V.; Kumar, R.; Goyal, M.; Iqbal, M. S.; Pal, C.; Dey, S.; Bindu, S.; Sarkar, S.; Pal, U.; Maiti, N. C.; Bandyopadhyay, U. *J. Biol. Chem.* **2012**, *287*, 24844-24861.
- (28) Corey, E. J.; Hahl, R. W. *Tetrahedron Lett.* **1989**, *30*, 3023-3026.
- (29) Mateos, A. F.; Barba, A. M. L. *J. Org. Chem.* **1995**, *60*, 3580-3585.
- (30) MacKinnon, S.; Durst, T.; Arnason, J. T.; Angerhofer, C.; Pezzuto, J.; SanchezVindas, P. E.; Poveda, L. J.; Gbeassor, M. *J. Nat. Prod.* **1997**, *60*, 336-341.

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- (31) Brandt, G. E. L.; Schmidt, M. D.; Prisinzano, T. E.; Blagg, B. S. J. *J. Med. Chem.* **2008**, *51*, 6495-6502.
- (32) Lakshmi, V.; Singh, N.; Shrivastva, S.; Mishra, S. K.; Dharmani, P.; Mishra, V.; Palit, G. *Phytomedicine* **2010**, *17*, 569-574.
- (33) Kraus, W.; Cramer, R. *Tetrahedron Lett.* **1978**, 2395-2398.
- (34) X-ray crystallographic data of **20**: CCDC reference number CCDC 918564, Formula weight: 737.29, Orthorhombic, Unit cell parameters: $a = 9.7594(4)$ $b = 18.5524(6)$ $c = 18.8335(5)$ Å, space group: P212121.
- (35) Siddiqui, B. S.; Rasheed, M.; Faizi, S.; Firdous; Ali, S. T.; Tariq, R. M.; Naqvi, S. *Helv. Chim. Acta.* **2003**, *86*, 3342-3353.
- (36) Madyastha, K. M.; Thulasiram, H. V. *J. Agric. Food Chem.* **1999**, *47*, 1203-1207.
- (37) Daramwar, P. P.; Rincy, R.; Niloferjahan, S.; Krithika, R.; Gulati, A.; Yadav, A.; Sharma, R.; Thulasiram, H. V. *Bioresource Technol.*, *115*, 70-74.
- (38) Govindachari, T. R.; Narasimhan, N. S.; Suresh, G.; Partho, P. D.; Gopalakrishnan, G. *J. Chem. Ecol.* **1996**, *22*, 1453-1461.
- (39) Simmonds, M. S.; Jarvis, A. P.; Johnson, S.; Jones, G. R.; Morgan, E. D. *Pest Manag. Sci.* **2004**, *60*, 459-464.
- (40) Yamasaki, R. B.; Klocke, J. A. *J. Agric. Food Chem.* **1989**, *37*, 1118-1124.
- (41) Hudlicky, T.; Reed, J. W. *Chem. Soc. Rev.* **2009**, *38*, 3117-3132.
- (42) Muffler, K.; Leipold, D.; Scheller, M. C.; Haas, C.; Steingroewer, J.; Bley, T.; Neuhaus, H. E.; Mirata, M. A.; Schrader, J.; Ulber, R. *Process Biochem.* **2006**, *46*, 1-15.
- (43) Wohlgemuth, R. *Curr. Opin. Microbiol.* **2010**, *13*, 283-292.
- (44) Garg, H. S.; Bhakuni, D. S. *Phytochemistry* **1984**, *23*, 2383-2385.
- (45) Johnson, S.; Morgan, E. D. *J. Chromatogr. A* **1997**, *761*, 53-63.
- (46) Jeon, J. E.; Bae, J.; Lee, K. J.; Oh, K. B.; Shin, J. *J. Nat. Prod.* **2011**, *74*, 847-851.
- (47) X-ray crystallographic data of **2**: CCDC no 964596, colorless plate, monoclinic, space group: $P2_1$, Unit cell parameters: $a = 10.6696(13)$, $b = 13.6752(17)$, $c = 11.3635(14)$ Å.
- (48) Gopalakrishnan, G.; Singh, N. D. P.; Kasinath, V. *Molecules* **2001**, *6*, 551-556.
- (49) Jarvis, A. P.; Johnson, S.; Morgan, E. D.; Simmonds, M. S. J.; Blaney, W. M. *J. Chem. Ecol.* **1997**, *23*, 2841-2860.
- (50) Siddiqui, S.; Siddiqui, B. S.; Faizi, S.; Mahmood, T. *J. Nat. Prod.* **1988**, *51*, 30-43.
- (51) Hashimoto, T.; Noma, Y.; Asakawa, Y. *Heterocycles* **2001**, *54*, 529-559.
- (52) El Sayed, K. A.; Mayer, A. M. S.; Kelly, M.; Hamann, M. T. *J. Org. Chem.* **1999**, *64*, 9258-9260.
- (53) Cheplogoi, P. K.; Mulholland, D. A. *Phytochemistry* **2003**, *62*, 1173-1178.
- (54) Kraus, W.; Klenk, A.; Bokel, M.; Vogler, B. *Liebigs. Ann. Chem.* **1987**, 337-340.
- (55) Qi, S. H.; Chen, L.; Wu, D. G.; Ma, Y. B.; Luo, X. D. *Tetrahedron* **2003**, *59*, 4193-4199.
- (56) G.-Y. Zhu, G. Chen, L. Liu, L.-P. Bai and Z.-H. Jiang, *J. Nat. Prod.*, **2014**, *77*, 983-989.

Chapter 4

Fluorescent Labeling and Cell-Imaging Studies of Neem Limonoids



Abstract

Chapter 4 comprises of two sections. Section A introduces the concept of fluorescent labeling and its application in natural product biology. Section B describes development of NBD based one-pot fluorescent labeling protocol for complex hydroxylated bioactive molecules. Further, fluorescently labeled Neem limonoids were subjected to cell-imaging studies to investigate the cellular localization within cancer cell lines.

Chapter 4: Section A

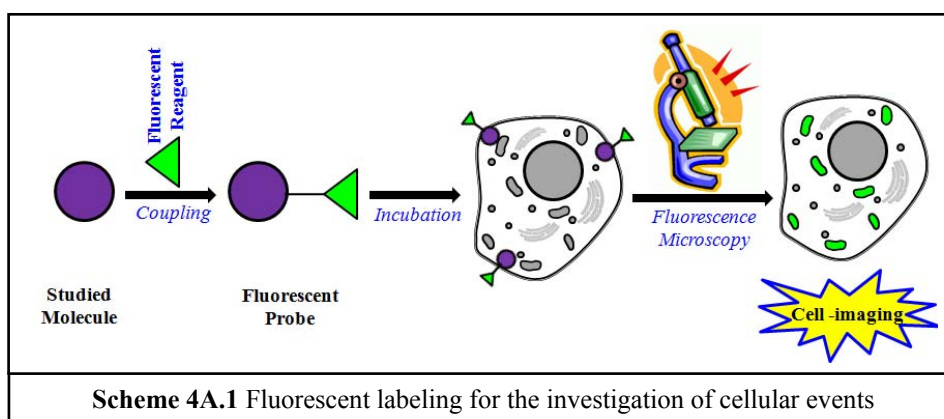
Fluorescent Labeling and Cellular Imaging: an Introduction

Highlights:

This section deals with introduction and applicability of labeling organic molecules with fluorescent dyes. Real examples were cited to show its utility in natural product biology. Further, limitations of commercially available tags and reported protocols have been discussed in brief which prompted us to develop efficient fluorescent labeling method.

4A.1 Fluorescent Labeling and Cellular Imaging

Fluorescent labeling is a process of covalently attaching a fluorophore to the studied molecule to get optical information about the parent molecule of interest through fluorescence detecting instruments. This technique is well-known due to its high sensitivity even at very low concentrations rendering it highly suitable for studying biological experiments. Moreover, fluorescent labeled bioactive molecules (*i.e.* fluorescent probe) are useful to obtain real-time visual information (qualitative/quantitative) inside the living cells through fluorescence microscope or fluorescence detector.¹⁻⁵



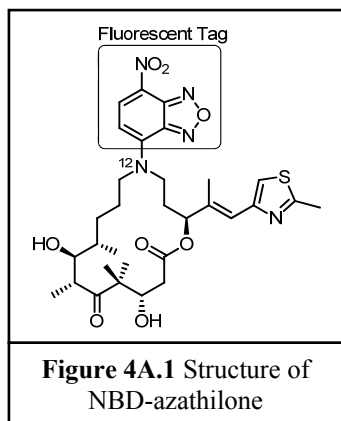
4A.2 Fluorescent Labeling in Natural Product Biology

Fluorescence imaging of the living cells to study the cellular events or visualize the associated phenotypes has become an attractive technique in recent years.⁶⁻¹³ After the investigation on tagging of phalloidin with fluorescein isothiocyanate in 1967,¹⁴ fluorescent labeling has been developed in a broader way as an important technique in the study of natural product biology. Bioactive molecules or biochemical probes covalently linked with the fluorescent organic dyes are used to unveil the cellular processes involved with their activity. Fluorescent probe based cell-imaging can be used to study several cellular phenomena associated with the bioactive natural products at the molecular level such as target discovery, cell-based assays, investigation of mode-of-action and *in vivo* studies to monitor cell fate.¹⁻⁵ In the last decade, rationally designed fluorescent probes of numerous bioactive natural products including paclitaxel,¹³ acetogenin,¹⁵ naringenin,⁸ azathilone,⁹ artemisinin,¹⁶ wortmanin,⁷ trioxolane¹⁰ have been prepared and studied through fluorescence imaging of live cells to find their target,

localization, mode of action, fate *etc.* For better understanding, examples describing the preparation of fluorescent probes and relevant cell-imaging studies of three bioactive molecules have been discussed below.

Example 1:

An azamacrolide anti-cancer agent azathilone (or 12-aza-epothilone) was labeled with

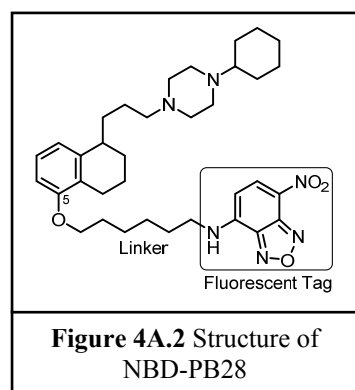


4-nitro-1,2,3-benzoxadiazole (NBD) fluorophore at N-12 (Figure 4A.1) and investigated for its mode of anti-cancer activity.⁹ Although the fluorescent probe (NBD-azathilone) was found to be less potent than the parent, NBD-azathilone promoted tubulin assembly, inhibited cancer cell proliferation *in vitro* and arrested the cell cycle at the G2/M transition as like the parent molecule. Binding of the probe to cellular microtubules was visualized by confocal

fluorescence microscopy; thus supporting the *in vitro* observations.

Example 2:

1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine is a high affinity ligand (PB28) of σ_2 receptors. Fluorescent probes linked to 5-

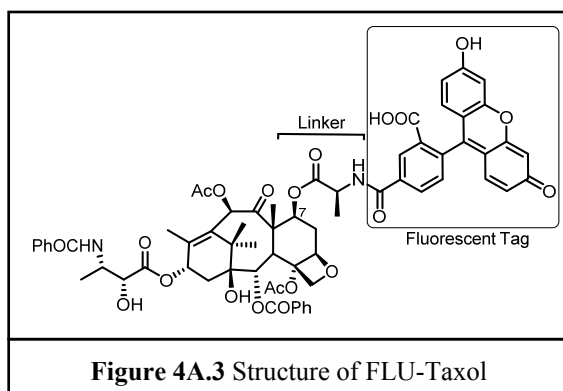


methoxytetralin nucleus of PB28 were synthesized using NBD or dansyl fluorescent tags joined with 5/6-atom linker. NBD-bearing probe (NBD-PB28, Figure 4A.2) showed high σ_2 affinity and optimal fluorescent properties.⁶ Fluorescence property of NBD-PB28 was used to further investigate the mechanism of σ_2 receptors. Its uptake in pancreatic tumor cells was evaluated by flow cytometry. The uptake was higher in proliferating cells

indicating σ_2 receptor to be potential biomarker of cell proliferation. Higher the proliferation, stronger was the anti-proliferative effect of σ_2 agonist. To investigate the localization of σ_2 receptors, colocalization studies of NBD-PB28 were performed with sub-cellular organelles by confocal microscopy which indicated highest localization in endoplasmic reticulum and lysosomes.

Example 3:

Paclitaxel (taxol) is a natural diterpenoid used in cancer therapy. Among numerous



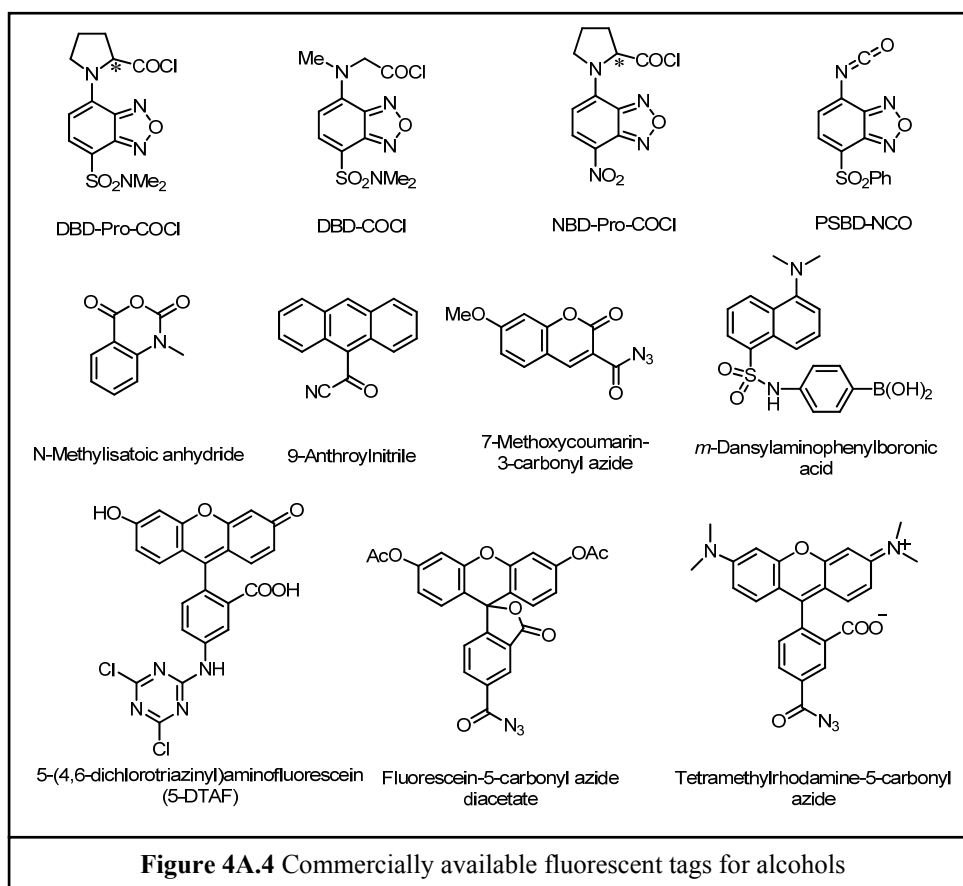
functional groups present around its complex structure, 7-hydroxyl group was chosen for the fluorescent tagging by analysing the structure-activity relationship. 4'-fluoresceincarboxylic acid was attached as a fluorescent tag with 7-hydroxyl group through L-alanine linker (FLU-Taxol, Figure 4A.3).¹³ Synthesized

fluorescent probe was more water soluble than the original natural product and it was utilized for the first direct visualization of taxol-microtubule interaction in epithelial PtK2 cultured cells; thus confirming its mode of action.

4A.3 Commercially Available Fluorescent Tags for Alcohols

Although hydroxyl functionality is highly abundant in natural products or biomacromolecules, its chemical reactivity is low especially in comparison to other nucleophiles such as amines or thiols. Therefore, it is a difficult task to selectively modify hydroxyl group in a complex multifunctional molecule. Fluorescent labeling reagents for alcohols based on 1,2,3-benzoxadiazole skeleton are well-explored and available commercially. DBD-Pro-COCl (chiral), DBD-COCl, NBD-Pro-COCl (chiral), PSBD-NCO are well-known examples of that category (Figure 4A.4).¹⁷ They react quantitatively with alcohols at 60-80 °C in 2-4 h in organic solvent (sometimes in presence of mild organic base). The usual detection limit of the labeled derivatives was found to be in the range of 5-50 fmol on the fluorescence detector. These tags have been used in several occasions for the fluorescent derivatization and quantification of alcohols through HPLC attached with fluorescence detector.¹⁷ In several other studies, the free hydroxyl group of serine/threonine residues in proteins, carbohydrates of glycoproteins, polysaccharides have been modified with various commercially available fluorescent reagents such as N-methylisatoic anhydride,¹⁸ *m*-dansylaminophenylboronic acid,¹⁹ 5-(4,6-dichlorotriazinyl) aminofluorescein (5-DTAF),²⁰ 9-anthrolynitrile²¹ (Figure 4A.4). Several commercially available fluorescent reagents carrying acyl azide

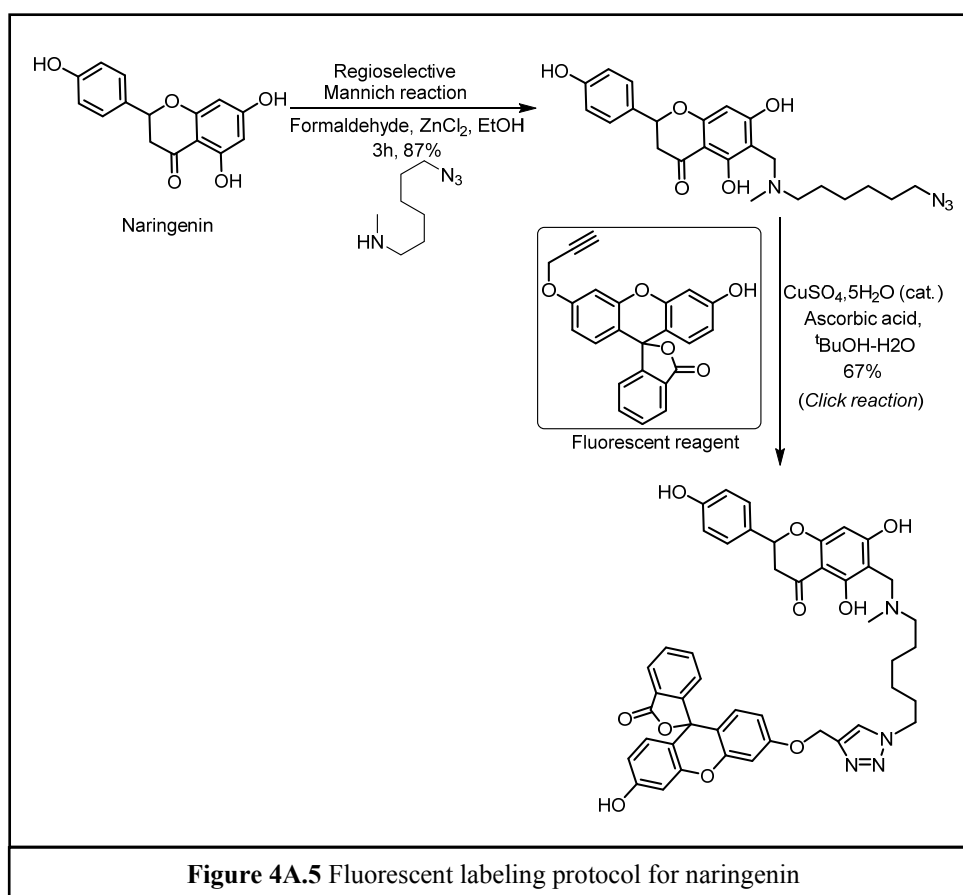
moiety (such as 7-methoxycoumarin-3-carbonyl azide,²² fluorescein-5-carbonyl azide diacetate or tetramethylrhodamine-5-carbonyl azide²³) are useful for sensitive quantitative analysis of alcohols (Figure 4A.4). When an acyl azide and alcohol are heated together in an organic solvent at 80 °C, acyl azide will rearrange to form an isocyanate that reacts with the alcohol to generate a stable urethane. The detection limit of these urethane fluorescent conjugates is less than 50 femtograms on the HPLC fluorescence detector. 9-Anthroynitrile possesses an acyl nitrile moiety which reacts with alcohols (such as steroids and acylglycerols) in organic solvents to yield carboxylate esters that can be monitored by HPLC.²⁴ Lipophilicity of this tag helps it to modify hydroxyl groups of proteins and hydroxylated fatty acids that are present within cell membranes. But most of the alcohol modifying tags reacts faster with amines due to higher nucleophilicity. Thus, it is a troublesome job to selectively label hydroxyl functionality in the presence of amine(s).



4A.4 Available Protocols for Fluorescent Labeling of Natural Products

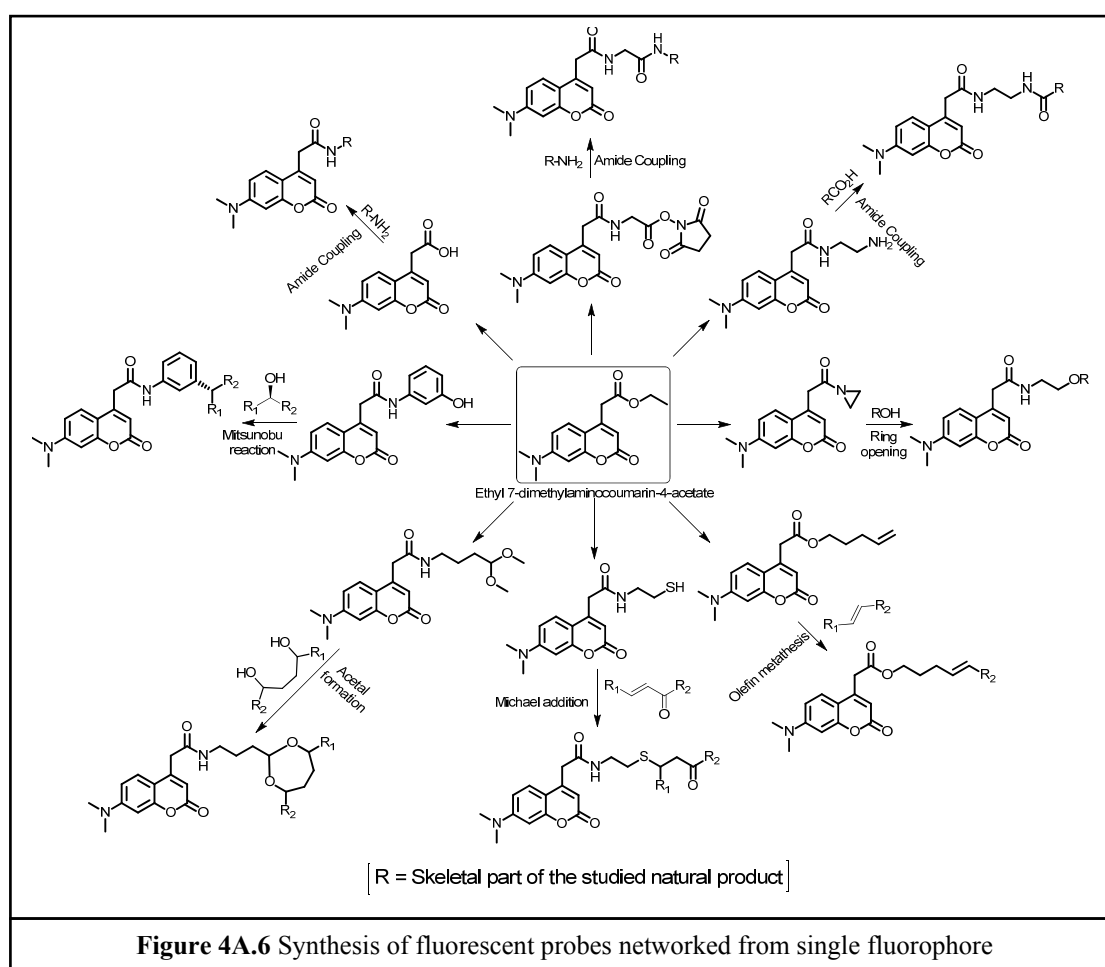
Systematic generalized protocol for the fluorescent labeling of complex natural products

is not well-established. Among very few methods reported, Cu(I)-catalyzed azide-alkyne [3+2] cycloaddition (*click reaction*) based protocol using inexpensive fluorescein alkyne derivative was well-demonstrated.^{8,25} Introduction of azido functionality to specific natural products by a selective and efficient manner followed by coupling with fluorescein derivative carrying terminal alkyne functionality was the essential of the developed protocol. The protocol was applied successfully on four bioactive small molecules of different skeletons such as naringenin, muramyl dipeptide, sinomenine and artemisinin.²⁵ Advantages of this method included the use of inexpensive reagents and applicability with wide range of bioactive molecules bearing reactive functionalities. To demonstrate a specific example, adopted protocol for the labeling of naringenin has been represented in Figure 4A.5.



In another study, diverse set of natural products have been labeled with a single dye possessing different linkers.²⁶ According to the nature of functional groups present in the natural products, central fluorophore carrying suitable terminal functionality on the linker was chosen and coupled together using various coupling reactions such as amide coupling, olefin metathesis, Michael addition, Mitsunobu reaction, diol to acetal

formation *etc.* Ethyl 7-dimethylaminocoumarin-4-acetate was preferred as the central dye which was further functionalized synthetically to prepare a set of eight fluorescent reagents carrying different terminal functional groups (Figure 4A.6). Among numerous fluorophores available, it was selected on the basis of its excellent photophysical properties, sufficient cellular uptake, lack of biological activity and sub-cellular localization, water solubility, small size, availability and photo-stability. Eight different fluorescent reagents were further utilized to label different complex bioactive natural products (such as amphotericin B, okadaic acid, borrelidin, microsclerodermin F, 22,23-dihydroavermectin B1, lissoclinolide, mucocin, nogalamycin) and their relevant bioactivity, quantitative cellular uptake and localization were evaluated.²⁶



Chapter 4: Section B

One Pot Fluorescent Labeling Protocol for Complex Hydroxylated Bioactive Molecules and Cell-imaging Studies of Neem Limonoids

Highlights:

- *Highly efficient one-pot methodology for the NBD based fluorescent labeling of hydroxylated small molecules was developed in mild reaction condition.*
- *Cellular internalization and localization of two qualified NBD tagged azadiradione derivatives were evaluated through fluorescence imaging studies of various cancer cell lines.*

4B.1 Introduction

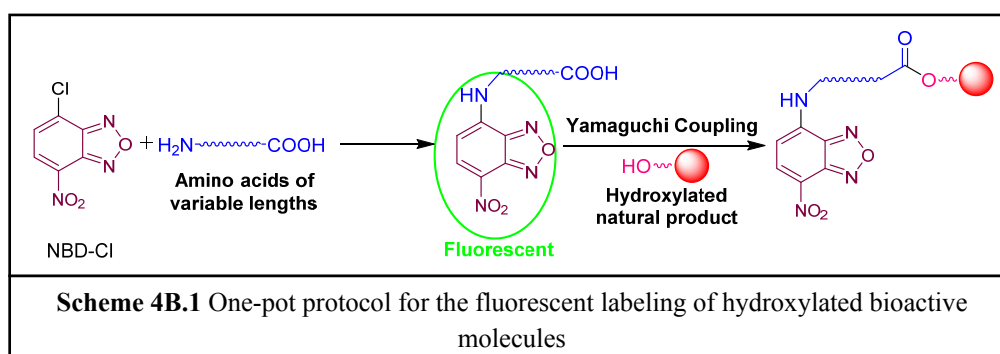
Fluorescent labeling of natural products involving several reaction steps along with purification and protection-deprotection procedures results in low overall yield and makes the process tedious.^{8,25,26} Indeed the availability of natural products is scanty in most of the cases due to their low abundance in the natural sources, making it difficult to carry out all the required steps. Secondly, the widely used tags (such as fluorescein, rhodamine) are bulkier in nature, which may lead to loss of biological activity.^{7,8} As discussed, ready to use fluorescent tags are available commercially but they are quite expensive and require extreme reaction conditions for labeling (e.g. NBD-F or DBD-COCl needs 60 °C at basic pH) which restrict their use for tagging the sensitive multifunctional natural products.^{17,27,28} Consequently, there is a need for the development of an inexpensive, mild and high-yielding protocol for the fluorescent labeling of biologically active complex natural products capable of maintaining the activity in the labeled derivative. Therefore, exploration of rapid, inexpensive and effective fluorescent tagging protocol for the bioactive natural products and successful implementation of the developed procedure will facilitate the integration of fluorescence imaging and investigation of cellular events. In this section, we have demonstrated a one-pot labeling protocol for the hydroxylated bioactive compounds by using 7-nitrobenzo-2-oxa-1,3-diazole (NBD) based tag which was successfully applied to label several complex multifunctional molecules. Further, two semi-synthetic derivatives of azadiradione (azadiradione and its derivatives are known for their cytotoxic activity against several human cancer cell lines as described in Chapter 1)²⁹⁻³³ and their fluorescently labeled analogues were subjected to comparative cytotoxicity studies. Also, the cellular uptake and intracellular localization of these labeled compounds in various cancer cell lines were monitored by fluorescence imaging.

4B.2 Results and Discussion

4B.2.1 One Pot Labeling Protocol

NBD-Cl is a non-fluorescent molecule, which undergoes nucleophilic substitution by amine functionality and becomes a highly sensitive fluorescent entity (depending on the polarity of the environment). This technique is being used over the years for the labeling

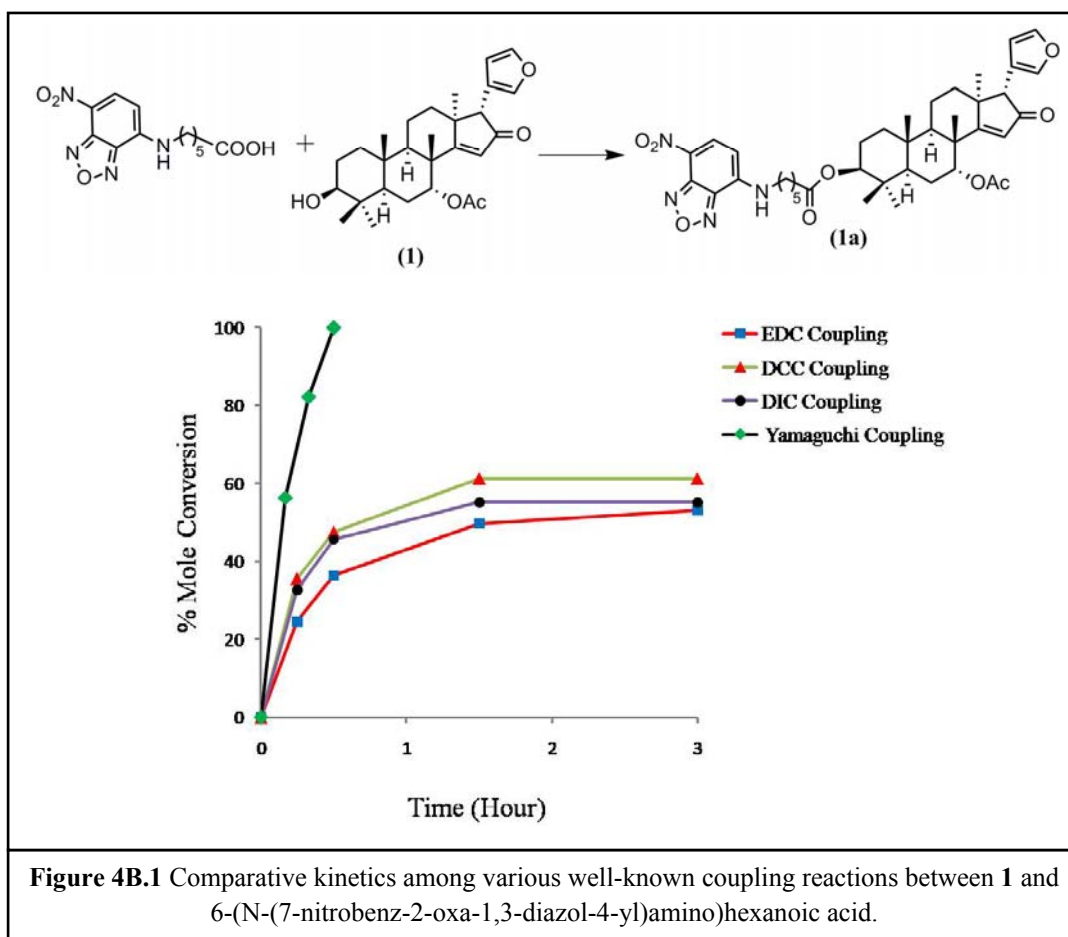
of proteins and amine containing small molecules.^{9,11,34-39} But, the reactivity of NBD-Cl towards hydroxyl group is very poor and this method cannot be used directly for the tagging of hydroxylated molecules.²⁷ Here, our protocol involves the aromatic nucleophilic substitution on NBD-Cl by the amine functionality of amino acids in the initial step to generate fluorescent reagent followed by coupling between the free carboxylic end and the hydroxylated bioactive molecule in the same vessel (Scheme 4B.1). It is always preferable to synthesize fluorescent reagent freshly in one-pot rather than using it as an individual tagging dye due to several reasons. Length of the amino acid used in the initial step determines the length of the spacer between the tag and natural product (as demonstrated later). Therefore, for achieving different spacer lengths in the labeled analogues individual fluorescent reagents (containing carboxylic acid at the end) of various lengths have to be prepared and purified separately. Instead it is more convenient to use one-pot protocol utilizing the commercially available amino acids of own choice corresponding to the desired spacer length. Secondly, fluorescent reagents are photosensitive and they require special care during preparation and storage such as dim light and low temperature. Preferably they are used immediately after preparation and purification.⁴⁰ Therefore, it is more convenient to generate fluorescent reagent freshly in one-pot from two non-fluorescent starting materials (NBD-Cl and amino acids). Again, after the preparation of fluorescent reagent it is essentially needed to be purified before storage. But, the reported one-pot protocol is devoid of one additional purification step making it less tedious.



4B.2.2 Comparative Kinetics among Coupling Reactions

Among several well-established coupling reactions available between carboxylic acid and alcohol, Yamaguchi was preferred on the basis of outcome of comparative kinetic experiments. **1** was used as the model substrate and allowed to couple with 6-(N-(7-

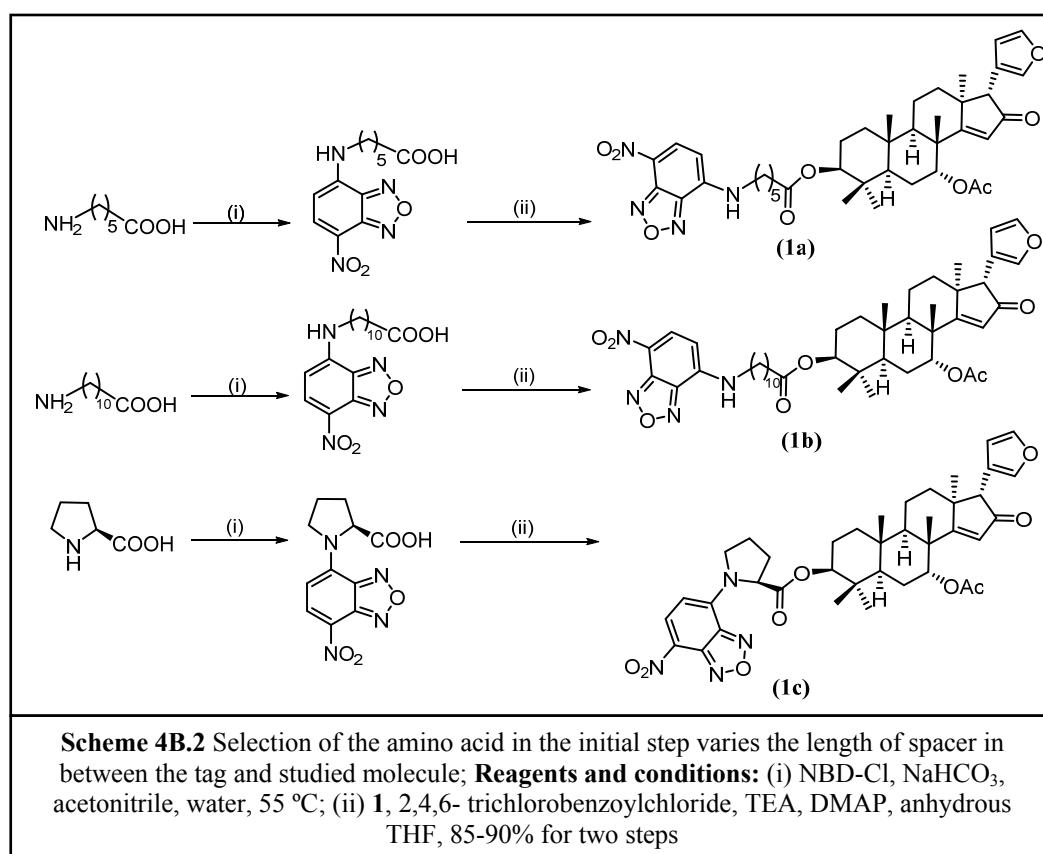
nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid in presence of various coupling reagents (DCC, EDC, DIC, Yamaguchi) (Figure 4B.1). Progress of the reactions was monitored by HPLC using the standard graphs prepared for **1** and **1a**. The coupling reagents, DCC, EDC and DIC showed similar reaction kinetic profile. About 50-60% of **1** (by mole) was converted to **1a** in 90 min of reaction time by all three coupling agents. However, no significant change in the product level was observed till 10 h (Figure 4B.1). The higher stoichiometric ratio of coupling reagents (DCC, EDC and DIC) also didn't alter the progress or rate of the reaction significantly even after the prolonged reaction time. On the other hand, Yamaguchi coupling showed quantitative conversion in 30 min; hence was chosen as the preferred coupling reaction for other molecules.



4B.2.3 Flexibility in Spacer Length

The choice of amino acid to be used in the initial step will determine the length of spacer joining the NBD skeleton and the bioactive molecule of interest. Indeed, the length of the spacer can vary the hydrophobicity of the labeled molecule which might be

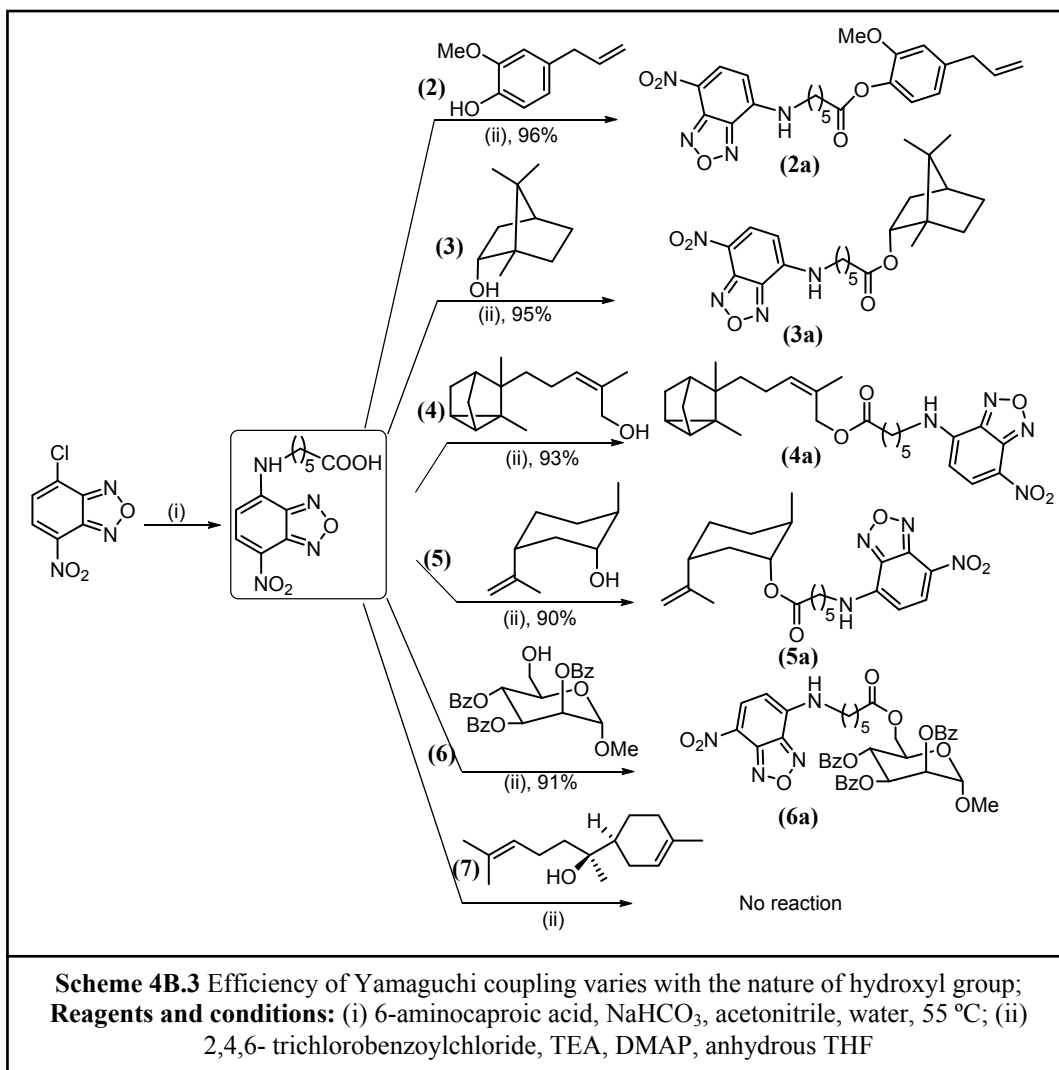
a guiding factor for the cellular uptake. Also, a spacer of optimum length is important for retaining the activity observed with the parental molecule.⁶ This flexibility in our methodology makes it unique and will be helpful for successfully labeling a complex molecule while preserving its activity. To demonstrate this, **1** was labeled with spacers of three different lengths using three unlike amino acids; 6-aminocaproic acid (six carbon spacer), 11-aminoundecanoic acid (eleven carbon spacer) and L-proline (creating turn in the structure) (Scheme 4B.2).



4B.2.4 Nature of the Hydroxyl Group

To investigate the efficiency of Yamaguchi coupling with varying nature of hydroxyl groups, several structurally simple compounds bearing different kind of hydroxyl functionalities (such as phenolic, primary, secondary and tertiary) were chosen and subjected to one-pot protocol using 6-aminocaproic acid (Scheme 4B.3). Eugenol (**2**, phenolic hydroxyl), (-)-borneol (**3**, secondary hydroxyl), α -santalol (**4**, allylic primary hydroxyl), (-)-dihydrocarveol (**5**, secondary hydroxyl) and a partially protected mannose (**6**), carrying primary hydroxyl furnished the labeled products with excellent yields. However, the coupling did not work out when α -(-)-bisabolol (**7**), a sesquiterpene

carrying tertiary hydroxyl group was used as the substrate. These results indicated that the developed protocol for NBD based fluorescent tagging of bioactive molecules possessing phenolic, primary and secondary hydroxyl groups using Yamaguchi coupling was highly efficient.

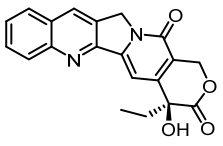
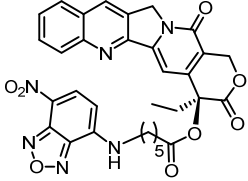
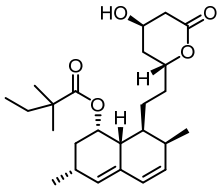
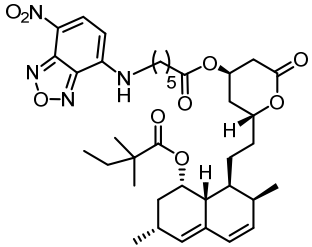
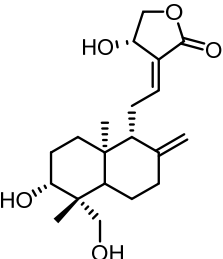
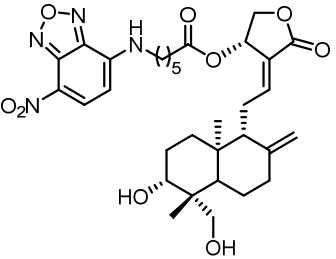
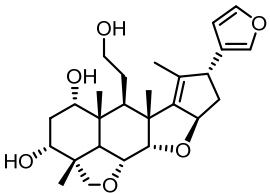
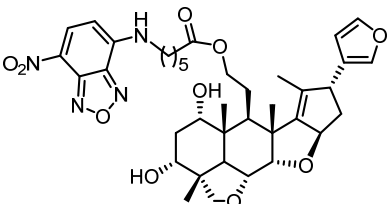
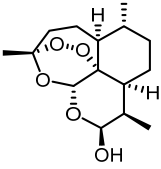
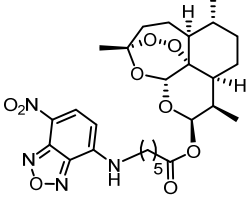
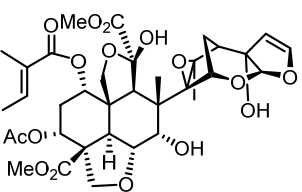


4B.2.5 Applicability with Complex Molecules

To showcase broad applicability of the present methodology several structurally diverse and multifunctional molecules were selected for fluorescent labeling (Table 4B.1) using afore-mentioned one-pot protocol. The fluorescent tagged compound (**8a**) was obtained in good yield (71%), when nimbocinol³¹ (**8**, deacetylated azadiradione) carrying a sterically hindered axial hydroxyl group at C-7 of basic limonoid skeleton was used as the substrate. Similarly, cholesterol (**9**), 11 α -hydroxyprogesterone (**10**) and diosgenin (**11**, a steroidal sapogenin containing a spiroketal moiety)⁴¹ furnished corresponding

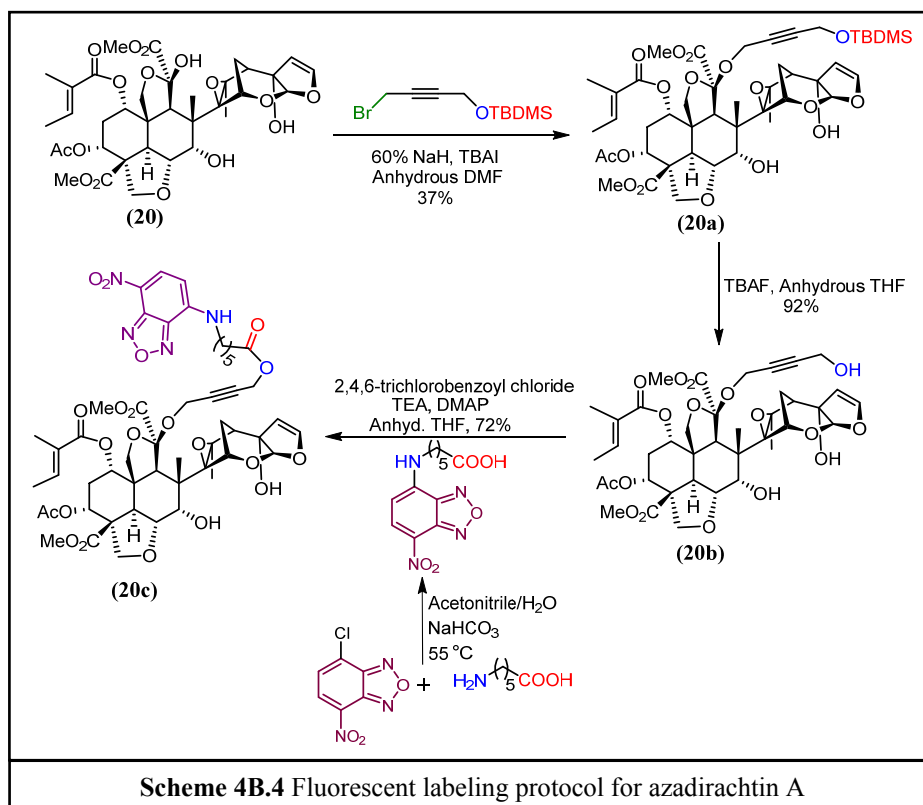
Table 4B.1 Entries showing applicability of the developed one-pot protocol for fluorescent labeling of complex bioactive hydroxylated compounds; **Reagents and conditions:** (i) NaHCO_3 , acetonitrile, water, 55 °C; (ii) 2,4,6- trichlorobenzoylchloride, TEA, DMAP, anhydrous THF

Entry	(A)	(B)	Yield	
1			(8a)	71%
2			(9a)	96%
3			(10a)	88%
4			(11a)	94%
5			(12a)	90%
6			(13a)	52%
7			(14a)	82%

8		(15)		(15a)	09%
9		(16)		(16a)	81%
10		(17)		(17a)	33%
11		(18)		(18a)	93%
12		(19)		(19a)	71%
13		(20)	No reaction		

tagged compounds **9a**, **10a** and **11a** in good to excellent yields. Phenolic hydroxyl on the isoindolin skeleton of compound **12** responded to the reaction with an excellent yield. Ezetimibe (**13**, a cholesterol lowering drug)⁴² possessing both alkylic and phenolic hydroxyl underwent a competitive reaction. However, at -15 °C with 5 min of reaction time, tagged phenolic hydroxyl was obtained as a major product (52%) along with doubly tagged product (27%). An antimalarial alkaloid cinchonine⁴³ (**14**) carrying a secondary hydroxyl group was labeled with a very good yield (82%), whereas another anticancer alkaloid (S)-(+)-camptothecin⁴⁴ (**15**) yielded the coupled product with a poor

yield (09%) due to the tertiary nature of its hydroxyl functionality. A hypolipidemic drug simvastatin⁴² (**16**) carrying a secondary hydroxyl group on a six membered lactone ring produced the tagged derivative with 81% yield. Andrographolide⁴⁵ (**17**), an anticancer natural product has three different hydroxyl groups around its skeleton; an allylic secondary hydroxyl on five membered lactone ring, one primary and another hindered secondary hydroxyl group. Although at room temperature the same reaction condition produced a mixture of products, at -15 °C with 5 min stirring the mono-labeled (on allylic secondary hydroxyl) product was obtained with a moderate yield (33%) and characterized by extensive 1D- and 2D-NMR studies. Interestingly, a reduced product of salannin (**18**) having two hindered secondary hydroxyl and another primary hydroxyl groups, furnished mono-labeled product on primary hydroxyl with an excellent yield (93%). β -dihydroartemisinin (**19**) a well-known antimalarial⁴⁶ agent having a sensitive peroxo bridge along with an adjacent ketal moiety in its skeleton was also able to produce the coupled product with 71% yield. A well-studied highly potent antifeedant neem limonoid azadirachtin A (**20**) possesses a unique molecular architecture carrying ester, epoxide, ether and ketal functionalities which makes it highly sensitive towards pH and light.⁴⁷ Surprisingly, it was not able to furnish any product and remained unreacted under the same reaction condition despite having three

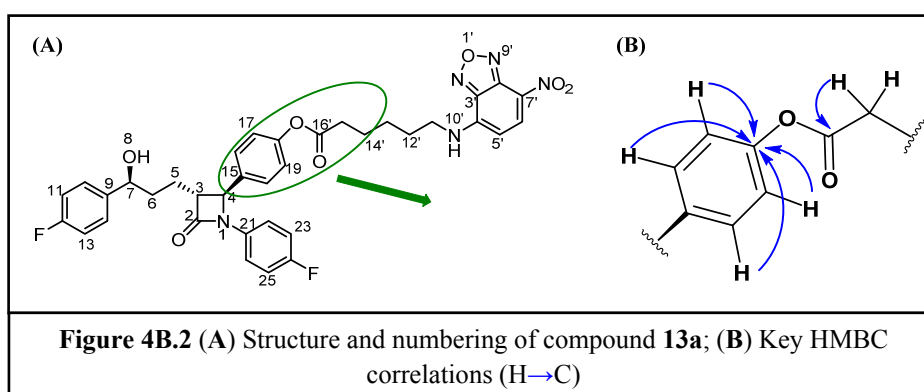


hydroxyl groups around its skeleton. Inability of azadirachtin A (**20**) to produce any product may be attributed to the extreme steric hindrance around hydroxyl groups and their involvement in the intramolecular hydrogen bonding network.⁴⁸ To avoid these obstacles, an alternative approach was adopted. Initially, a short spacer carrying a protected hydroxyl group at the tail was attached followed by deprotection and Yamaguchi coupling. Azadirachtin A (**20**) was reacted with a propargyl bromide derivative carrying a TBDMS protected hydroxyl group at the other end in the presence of sodium hydride. After nucleophilic substitution by 11-OH (as characterized on the basis of earlier reports),^{49,50} hydroxyl was deprotected by TBAF and further subjected to one-pot protocol to get labeled azadirachtin (**20c**) in 24% overall yield (Scheme 4B.4).

4B.2.6 Structural Characterization of the Labeled Analogues

Synthesized fluorescent probes were structurally characterized on the basis of NMR (¹H, ¹³C and DEPT-135) and ESI(+)-HRMS studies. In case of labeled compounds bearing two or more hydroxyl functionalities (**13a**, **17a** and **18a**), the position of esterification was confirmed on the basis of 2D-NMR (HMBC and HSQC) correlations. To demonstrate, details of the characterization for compounds **13a**, **17a** and **18a** have been discussed as follows.

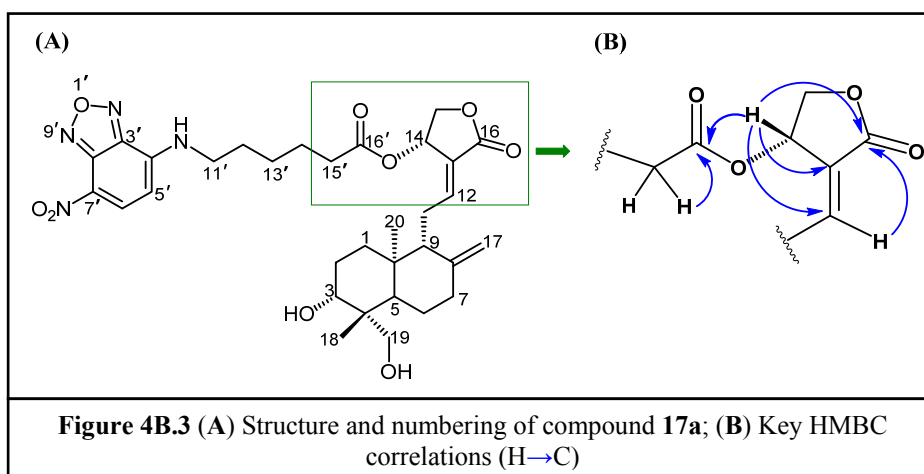
(a) Characterization of NBD labeled ezetimibe (**13a**):



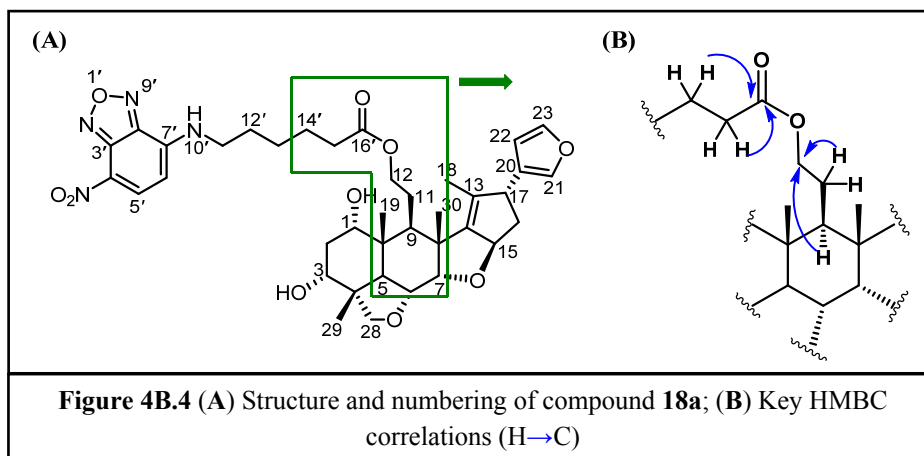
The regio-selective esterification on phenolic hydroxyl group was confirmed by the extensive analyses of ¹H, ¹³C, DEPT-135, HSQC and HMBC NMR spectra. Existence of an additional quaternary signal at δ_C 171.7 in ¹³C NMR of NBD labeled ezetimibe (**13a**) suggested the esterification of hydroxyl functionality. Further, the signal at δ_C 171.7 confirmably ascribed to the ester carbonyl on the basis of HMBC correlation of

C-16' with H-15' (Figure 4B.2). Except the phenolic ring (consisting of C-15, C-16, C-17, C-18, C-19 and C-20), the chemical shift values for other carbons of ezetimibe skeleton was similar to the parent (**13**). An upfield shift by 6 ppm was observed for C-18. The signals for C-15 and C-17, 19 shifted downfield by 7 ppm and 6 ppm respectively. These observations confirmed the esterification on phenolic OH-18. The correlation of C-18 (δ_C 150.6) with H-17, 19 (δ_H 7.10) and H-16, 20 (δ_H 7.34) additionally affirmed the assignment of δ_C 150.6 to C-18. The accurate allocation of the ^1H signals were achieved by the mutual correlations (C-16,20/H-16,20 and C-17,19/H-17,19) obtained from HSQC spectrum.

(b) Characterization of NBD labeled andrographolide (17a**):**



The presence of ester bond in NBD tagged andrographolide (**17a**) was confirmed due to the presence of a newly appeared quaternary signal at δ_C 172.9 compared to andrographolide (**17**) and was assigned to the ester carbonyl. The chemical shift values for andrographolide skeleton in ^{13}C NMR of **17a** remained similar in comparison to **17** except for C-14, C-13 and C-15. The downfield shift of C-14 (by 3 ppm) and upfield shift of C-13 (by 5 ppm) and C-15 (by 3 ppm) indicated the esterification at 14-OH. Further the position of attachment of NBD was confirmed on the basis of HSQC and HMBC spectra (Figure 4B.3). The signal at δ_H 5.94 (d, $J=5.52$ Hz) was assigned to H-14 according to the correlations of H-14 with C-12 (δ_C 150.6), C-13 (δ_C 123.8) and C-16 (δ_C 169.1) in the HMBC spectrum. The signal at δ_C 172.9 was ascribed to C-16' due to the presence of its correlation with H-15' (δ_H 2.43). The esterification at 14-OH was unambiguously affirmed on the basis of strong correlation between H-14 (δ_H 5.94) and C-16' (δ_C 172.9).

(c) Characterization of NBD labeled reduced salannin (**18a**):

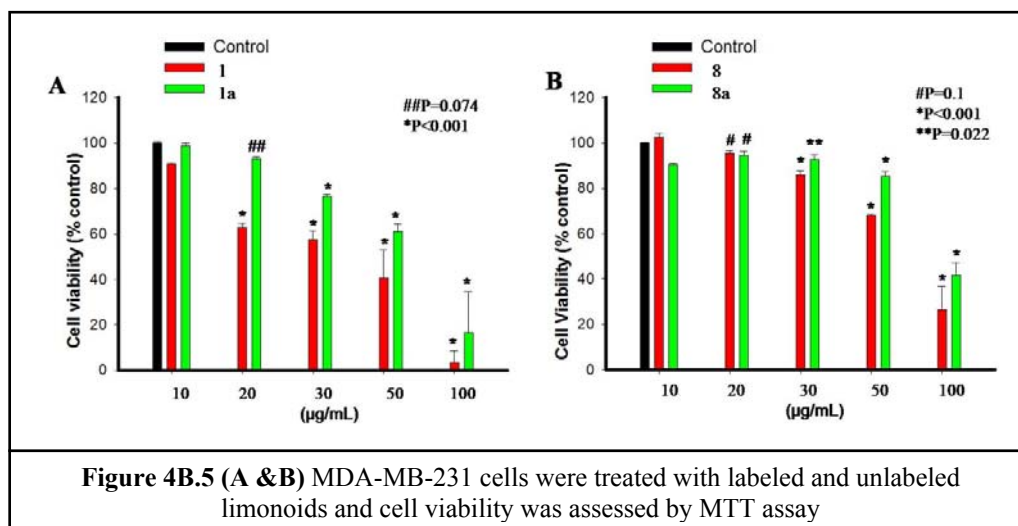
Appearance of an additional quaternary signal at δ_C 173.3 in ^{13}C NMR of **18a** with respect to **18** implied the esterification of one of the hydroxyl functionalities. The correlation of C-16' (δ_C 173.3) with H-15' (δ_H 2.33) and H-14' (δ_H 1.70) in HMBC spectrum further confirmed the assignment of δ_C 173.3 to the ester carbonyl. A downfield shift of 2 ppm for methylene C-12 (δ_C 64.8) was observed after esterification. The neighbouring C-11 (δ_C 64.8) underwent an upfield shift by 3 ppm. The other carbons of the esterified product (**18a**) showed similar chemical shift values with respect to the original natural product confirming the esterification on 12-OH. This was further supported by comparing DEPT-135 NMR data, since change in chemical shift values was observed only for the methylene carbons (C-12 and C-11) and not for any methine carbons. The assignment of chemical shift value for the esterified centre C-12 at δ_C 64.8 was further confirmed on the basis of HMBC correlations of C-12 (δ_C 64.8) with H-11 (δ_H 1.80) and H-9 (δ_H 2.22) (Figure 4B.4). All the assignments of ^1H and ^{13}C signals were supported well by the mutual correlations observed in HSQC spectrum.

4B.2.7 Evaluation of Anticancer Activity of Azadiradione Fluorescent Probes

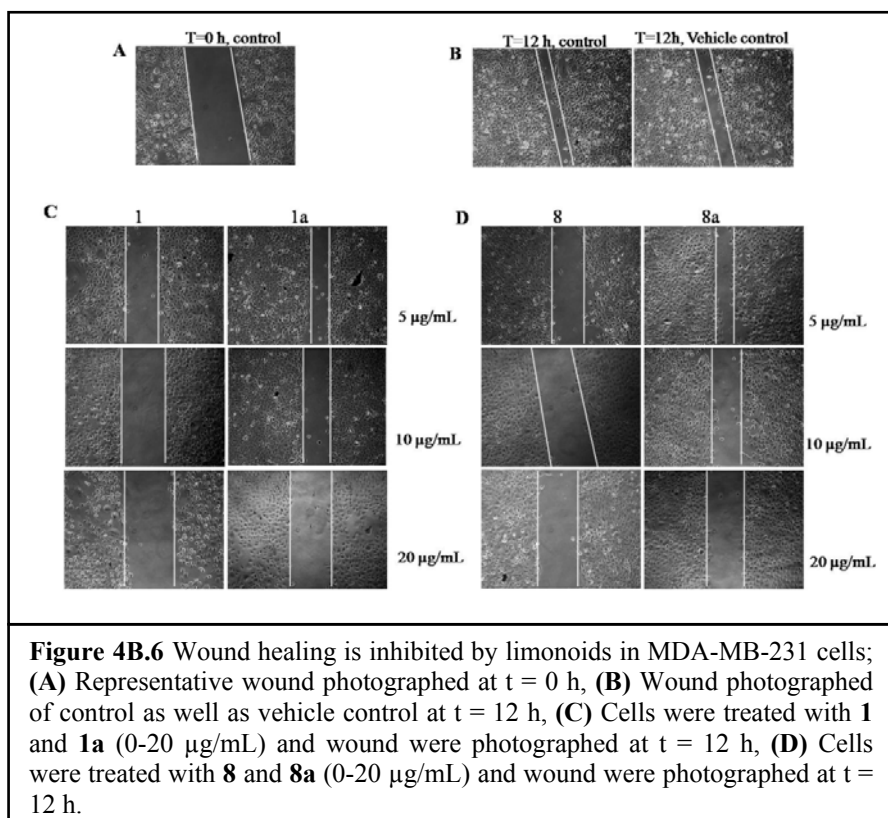
To assess the applicability of fluorescent tagged bioactive molecules synthesized in this study (Scheme 4B.2, Table 4B.1), two limonoids were chosen for their biological evaluation and cellular imaging. Two azadiradione derivatives (**1** and **8**) and their corresponding labeled analogues (**1a** and **8a**) were evaluated for cytotoxic activity against several cancer cell lines. Potency of their cytotoxic activity was monitored by cancer cell viability (MTT assay) and motility assay (Wound healing assay).

(a) Cancer cell viability studies

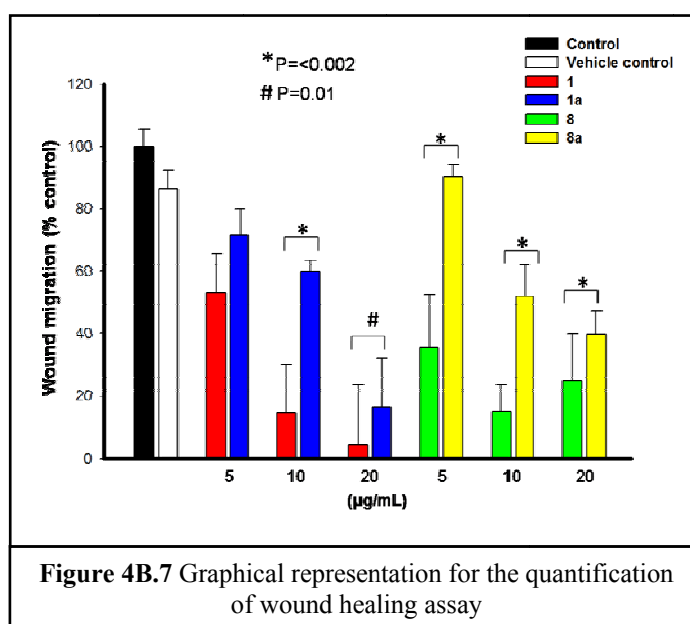
MDA-MB-231 cells were treated with either of labeled (**1a** and **8a**) and parent (**1** and **8**) azadiradione derivatives with varying concentrations (0-100 $\mu\text{g/mL}$) and MTT assay was performed. The results depicted comparable decrease in cell viability in labeled and unlabeled limonoids in a concentration dependent manner (Figure 4B.5).

**(b) Cancer cell motility studies**

Cancer metastasis is basically the migration of tumor cells from the primary tumors to

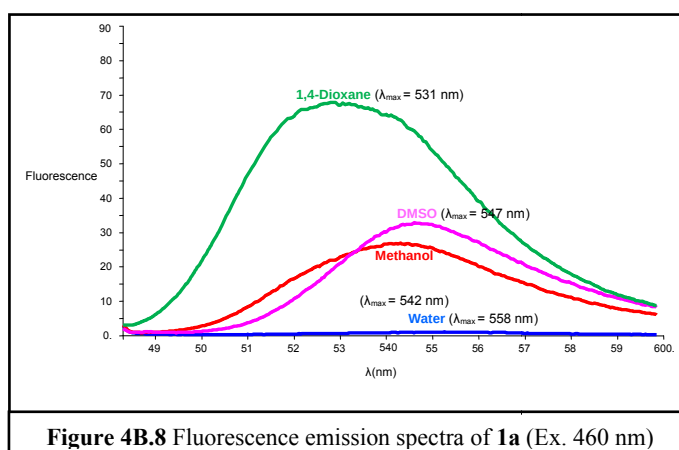


the distant body parts to develop secondary tumors. Any potent drug should target cancer cell viability as well as motility. Therefore to check the effect of labeled and unlabeled limonoids on breast cancer cell motility, wound healing assay was performed. Confluent monolayer of MDA-MB-231 cells were wounded uniformly, treated with labeled (**1a** and **8a**) as well as unlabeled (**1** and **8**) limonoids (0-20 $\mu\text{g/mL}$) for 12 h and motility was assessed (Figure 4B.6). The results demonstrated the inhibition in MDA-MB-231 cell motility with increasing concentration of limonoids (labeled as well as unlabeled) though considerable loss of potency in labeled limonoids was observed (Figure 4B.7).



4B.2.8 Fluorescence Emission Spectra

Sensitivity of NBD fluorophore is highly dependent on polarity of the surrounding environment. Polarity dependent fluorescence intensity of NBD skeleton can be



correlated with the polarity of the environment or binding site of the target inside the cell. Higher the polarity of the environment lower the fluorescence sensitivity was reported.⁹ Here we have recorded fluorescence emission spectra of **1a** (0.1 $\mu\text{g/mL}$) in solvents of different polarity (Excitation at 460 nm). Emission spectra clearly indicated that the fluorescence intensity is reduced with increase in the polarity of the environment (Figure 4B.8).

4B.2.9 Stability of the Ester Bond in Intracellular Condition

Standard NBD tagged azadiradione derivative (**1a**), alcohol (Azadiradione derivative, **1**)

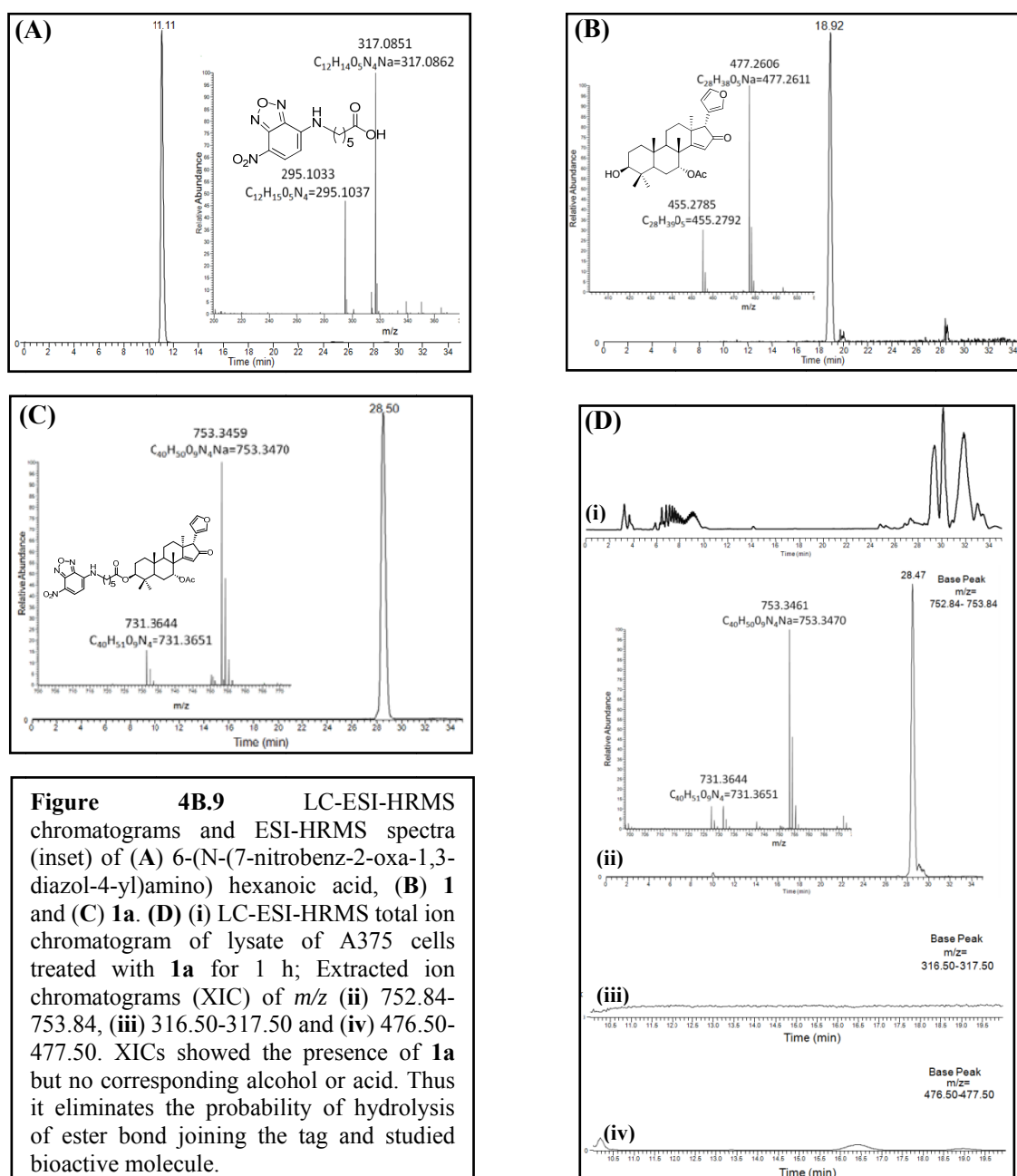


Figure 4B.9 LC-ESI-HRMS chromatograms and ESI-HRMS spectra (inset) of (A) 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoic acid, (B) **1** and (C) **1a**. (D) (i) LC-ESI-HRMS total ion chromatogram of lysate of A375 cells treated with **1a** for 1 h; Extracted ion chromatograms (XIC) of m/z (ii) 752.84-753.84, (iii) 316.50-317.50 and (iv) 476.50-477.50. XICs showed the presence of **1a** but no corresponding alcohol or acid. Thus it eliminates the probability of hydrolysis of ester bond joining the tag and studied bioactive molecule.

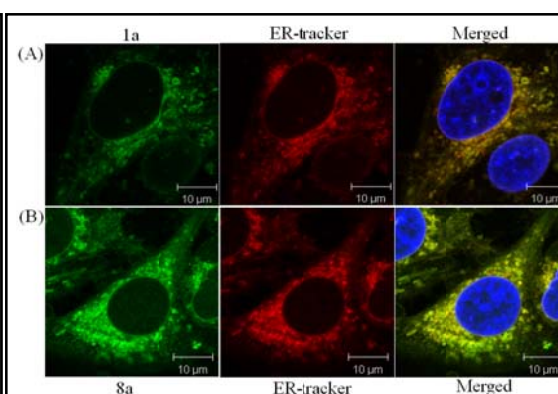
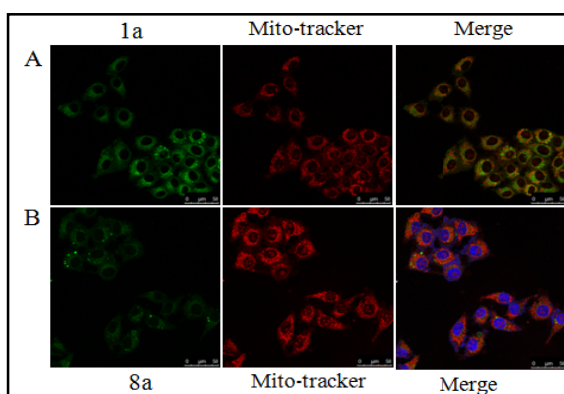
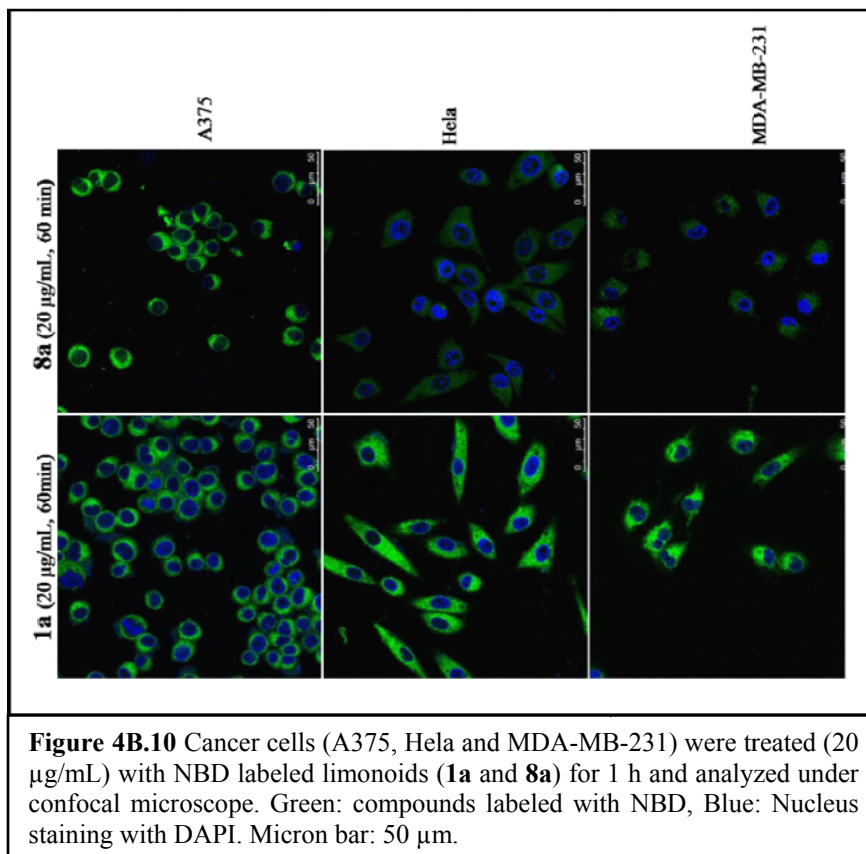
and acid (NBD tag) were analyzed by LC-ESI-HRMS (Figure 4B.9). Then, cell lysate of A375 cells treated with **1a** and two controls (lysate of untreated cells and buffer) were injected in identical conditions. By comparing R_t and HRMS with the standards, the abundance of **1a**, **1** and NBD tag was determined in the lysate of A375 cells (treated with **1a**) using extracted ion chromatograms (XIC) (Figure 4B.9). XICs of $[M+Na]^+$ adducts (m/z 752.84-753.84 for **1a**, m/z 316.50-317.50 for NBD tag and m/z 476.50-477.50 for **1**) showed the presence of only **1a** in the total ion chromatogram, whereas the corresponding alcohol (**1**) and acid (may be formed due to the hydrolysis of ester bond) were absent (Figure 4B.9D). These results clearly ascertained the stability of the ester bond joining the tag with the studied azadiradione derivative under intracellular conditions.

4B.2.10 Cell Imaging Studies

The comparative inhibition studies of cancer cell viability and motility have indicated the concentration dependent cytotoxic activity for both the labeled analogues (**1a** or **8a**) and comparable inhibitory potencies with respect to the parent azadiradione derivatives. The retention of activity in labeled analogues may be attributed to the smaller size of NBD and presence of highly flexible six-carbon spacer joining the tag and bioactive molecule. However, the slight alternation in potency of tagged derivatives with respect to the parent molecules may be due to the structural modification caused by the attachment of NBD tag and esterification of hydroxyl functionality. Therefore, considering the extent of cytotoxic activity preserved, **1a** and **8a** can be considered as the valid cancer cell imaging probe for azadiradione derivatives.

Cancer cells (A375, HeLa and MDA-MB-231) were treated either with **1a** or **8a** (20 $\mu\text{g/mL}$) for 1 h and analyzed for cellular internalization and localization under confocal microscope. Results depicted the cellular uptake of NBD labeled compounds irrespective of the cancer cell lines and no specific localization in the nucleus (Figure 4B.10). Further these imaging probes can be used for studying the localization in sub-cellular organelles. To demonstrate specific examples, HeLa cells treated either with **1a** or **8a** were stained by Mito-tracker Red and analyzed through confocal microscopy which did not show any colocalization with the mitochondria (Figure 4B.11). Similarly, the colocalization experiment was performed with ER-tracker red and labeled analogues

(**1a** and **8a**) were found to be localized in the endoplasmic reticulum (ER) on the basis of yellow fluorescence observed in the overlaid image (Figure 4B.12).



4B.3 Conclusion

Simple and highly efficient one-pot methodology for the NBD based fluorescent labeling of hydroxylated small molecules was developed, which can be applied to track their mode of action through fluorescence cell imaging. Among several common coupling reactions, Yamaguchi was found to be best on the basis of yield and kinetic studies. The protocol was executed with a mild condition so that it was able to sustain sensitive functionalities such as lactone, ketal, ether, peroxo or epoxide in the reaction environment. Both nucleophilic substitution and coupling steps were carried out in the same vessel and required a single purification step. Even the natural products isolated in trace amounts (a few milligrams) can be labeled in sufficient yield and more economically using the present protocol. Importantly, coupling reaction was very selective between acid and alcohol functionality, which allows tagging the complex molecules bearing multiple functional groups. Fluorescein, a widely used fluorescent tag, was replaced by NBD as it is less bulkier than the former. Again, length of the spacer can easily be modified by a suitable choice of the amino acid. Further, evaluation of the two NBD tagged azadiradione derivatives for cytotoxic activity indicated that the labeling did not alter the activity drastically. The internalization and localization of those probes were imaged successfully inside the cancer cells and found to be localized in the endoplasmic reticulum. Therefore, this methodology can be used to synthesize the fluorescent imaging probes of hydroxylated bioactive compounds to study the cellular processes especially cellular uptake, localization or specific interactions.

4B.4 Experimental Section

4B.4.1 General Information

Technical grade solvents were distilled prior to use for preparative chromatography purpose. Compounds were purified over silica gel (230-400 mesh) flash chromatography columns using dichloromethane/methanol gradient mixture as the eluent. HPLC runs were performed on HPLC system coupled with UV-Vis detector and analytical XBridge C₁₈ column (4.6 × 100 mm) at a flow rate of 1 mL/min with a gradient solvent programme of: 0 min, 40% methanol/water; 10 min, 60% methanol/water; 25 min, 90% methanol/water; 28 min, 60% methanol/water; 30 min, 40% methanol/water; and monitored at 230 nm. Reactions and purification processes

were monitored by thin layer chromatography, which was carried out on pre-coated silica gel plates using UV light (254 nm) as the visualization medium and anisaldehyde charring solution as the staining agent. Thin film IR spectra were recorded in CHCl_3 and optical rotations were determined in the same solvent at 589 nm using 10 mm cell (c in gm/100 mL unit). Chemical shift values in NMR (^1H , ^{13}C , DEPT-135) spectra were reported in ppm with respect to the residual solvent or TMS signals as the reference. HRMS data were collected on Q Exactive Quadrupole-Orbitrap Mass Spectrometer. The human cervical carcinoma (HeLa), breast adenocarcinoma (MDA-MB-231), and skin carcinoma (A375) cells were procured from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in L-15 (MDA-MB-231), MEM (HeLa) and DMEM (A375, NIH-3T3) media (Gibco BRL) supplemented with 10% FBS, 100 units penicillin and 100 gm/mL streptomycin. The cells were maintained under humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cell imaging was performed on confocal microscope. LC-ESI-HRMS runs were performed on Q Exactive Orbitrap attached with LC system. Samples were resolved through Hypersil Gold C_{18} column of particle size 5 μm with a flow rate of 0.5 mL/min and gradient solvent program of 35 min (0.0 min, 10% methanol/water; 0.5 min, 10.0% methanol/water; 3.0 min, 45% methanol/water; 6.0 min, 60% methanol/water; 30.0 min, 90% methanol/water; 32.0 min, 90% methanol/water; 32.5 min, 10% methanol/water; 35.0 min, 10% methanol/water). 0.1% LC-MS grade formic acid was also added to the solvents (methanol and water). HRMS data were recorded in ESI-positive ion mode within the mass range m/z 100 to 1000 using the tune method as followed: sheath gas flow rate 45, auxiliary gas flow rate 10, sweep gas flow rate 2, spray voltage ($|\text{KV}|$) 3.60, spray current (μA) 3.70, capillary temperature (°C) 320, s-lens RF level 50, heater temperature (°C) 350.

4B.4.2 One-pot Labeling Protocol

A solution of NBD-Cl (1.00 equiv) in acetonitrile (18 mL/mmol) was added dropwise to a solution of amino acid (1.00 equiv) and sodium bicarbonate (3.00 equiv) in water (6 mL/mmol) at 55 °C and incubated for 1 h. Then acetonitrile was concentrated under reduced pressure and pH of the aqueous reaction mixture was adjusted to ~2.0 using 1N HCl. Further it was concentrated to dryness under 20 mbar pressure and 62 °C temperature. The deep orange crude solid was again dissolved in minimum amount of

acetonitrile and dried in the same condition to make sure that there is no residual moisture remaining. Further Yamaguchi coupling was carried out in the same vessel following the reported procedure⁵¹ with slight modifications. The natural product/bioactive molecule (0.75 equiv) with hydroxyl functionality in anhydrous THF (20 mL/mmol) was added to the dried crude under inert atmosphere with stirring. 2,4,6-trichlorobenzoyl chloride (1.00 equiv) and anhydrous TEA (1.00 equiv) were successively added dropwise to the reaction mixture. After 5 min, DMAP (1.00 equiv) was added to the reaction vessel and the stirring was continued for another 30 min. The reaction was quenched by adding a few drops of water. Then it was concentrated to dryness and directly purified over silica gel column. All the steps were performed under dim light and labeled compounds were stored at -20 °C in dark.

4B.4.3 Coupling Reaction (DCC, EDC and DIC) Procedure

To a mixture of acid (1.00 equiv), alcohol (0.75 equiv) and DMAP (1.00 equiv) in an inert atmosphere, anhydrous DCM (30 mL/mmol) was added. The mixture was stirred to dissolve all the components followed by addition of coupling reagent (1.00 equiv) (DCC, EDC or DIC) in stirring condition. After addition, reaction mixture was left for stirring at room temperature.

4B.4.4 Preparation of Propargylated Derivative of Azadirachtin A (21a)

Azadirachtin A (100 mg, 0.14 mmol) taken in argon atmosphere was dissolved in 1.0 mL of anhydrous DMF and the mixture was cooled to 0 °C. 60% sodium hydride (10.2 mg, 0.42 mmol) was added to the mixture and stirred at 0 °C for 8 min. The colorless reaction mixture became light brown by this time period. Then propargyl bromide derivative was added (52.6 mg, 0.20 mmol) to it followed by a catalytic amount (pinch) of TBAI. Continuing the stirring at 0 °C for another 4 min, the reaction mixture was shifted to room temperature and allowed to stir for 20 min. After quenching the reaction with a few drops of chilled water, it was taken in ethyl acetate (20 mL) and washed thrice with brine solution (50 mL). Ethyl acetate layer was concentrated and purified by silica gel column chromatography using a gradient mixture of DCM and methanol as the eluent to get the desired product (**21a**) (46.72 mg, 0.052 mmol, yield 37%).

4B.4.5 Deprotection of Propargylated Derivative of Azadirachtin A (21b)

1M TBAF (62 μ L, 0.062 mmol) was added dropwise to **21a** (46.72 mg, 0.052 mmol) taken in 2 mL of anhydrous THF and the reaction mixture was stirred for 10 min in an inert atmosphere. Further, it was concentrated and purified over silica gel column using 2.0% methanol in DCM as the eluent to produce the deprotected product **21b** in 92% yield (37.7 mg, 0.048 mmol).

4B.4.6 Cell Viability Assay

MDA-MB-231 cells (2×10^4 cells per well) were seeded in a 96-well microplate. Cells were treated with either **8** or **8a** (0-100 μ g/mL) for 24 h. In separate experiments, cells were treated either with **1** or **1a** (0-100 μ g/mL) for 24 h. 200 μ L of MTT (0.5 mg/mL) was added into each well and incubated at 37 °C for 4 h. Formazan crystals were dissolved with isopropanol and optical density of formazan solution were measured at 570 nm, using a microplate reader. Experiments were performed in triplicates. Mean values of the reading were calculated, analyzed statistically and percentage change with respect to control was defined. A graph was plotted as cell viability (% control) versus doses using sigma plot software.

4B.4.7 Cell Motility Assay

Cells were grown, synchronized for 24 h in serum-free medium and wounded uniformly using sterile tip. The wound photograph were captured and the cells were treated with NBD-labeled or unlabeled azadiradione derivatives (0-20 μ g/mL). After 12 h, wound closures were photographed using phase contrast microscope and analyzed.

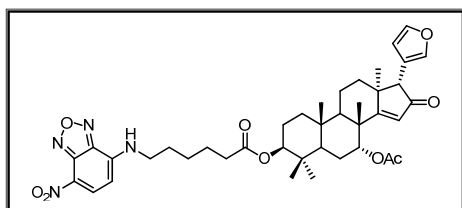
4B.4.8 Confocal Microscopy

MDA-MB-231, A375 and HeLa cells were grown on cover slips, treated with **1a** or **8a** (20 μ g/mL) for 1 h and fluorescence imaging analysis was performed. For colocalization studies, HeLa cells were treated with either **8a** or **1a** for 1 h, followed by treatment with mito-tracker (50 nM) for 20 min. Treated cells were fixed with 2% paraformaldehyde for 10 min, washed twice with PBS and mounted onto a glass slide with mounting media. Nuclei were stained with DAPI and visualized under a confocal microscope (excitation 460 nm, emission 530 nm) with 60x magnification. Similarly,

colocalization experiment was performed with fluorescently (green) tagged derivatives of azadiradione (**1a** and **8a**) and red ER tracker in MDA-MB-231 cells. Cells treated either with **1a** or **8a** (20 $\mu\text{g/mL}$ for 1 h) were stained with ER-tracker (1 μM for 20 min) and analyzed under a confocal microscope with 100x magnification after fixation and nuclear staining with DAPI (blue).

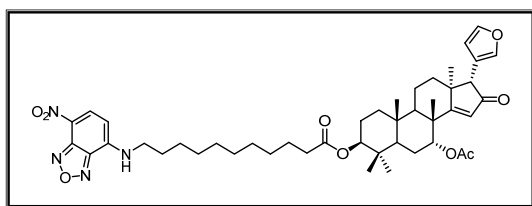
4B.8.9 Yield and Characterization Data of the Synthesized Compounds

Compound 1a: 41.9 mg (0.057 mmol) of **1a** was isolated from 30.0 mg (0.066 mmol)



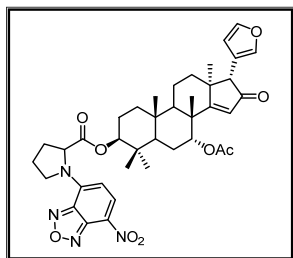
of **1** with 87% yield. Orange solid. $[\alpha]_D^{25} = -15.5$ (c 3.0, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3327, 1719, 1700. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.47 (d, $J=8.55$ Hz, 1H), 7.45 (m, 1H), 7.41 (m, 1H), 6.52 (m, 1H), 6.25 (m, 1H), 6.17 (d, $J=8.54$ Hz, 1H), 5.83 (s, 1H), 5.25 (m, 1H), 4.53 (m, 1H), 3.53 (dd, $J=12.51, 6.41$ Hz, 2H), 3.37 (s, 1H), 2.37 (m, 2H), 1.95 (s, 3H), 1.25 (s, 3H), 1.02 (s, 3H), 0.97 (s, 3H), 0.86 (s, 3H), 0.77 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 205.5, 193.5, 173.1, 169.7, 144.2, 143.9, 142.6, 141.5, 136.5, 123.8, 123.1, 118.6, 111.2, 98.5, 80.2, 74.7, 60.5, 48.1, 48.0, 44.0, 43.6, 43.1, 37.3, 37.2, 34.2, 30.3, 28.0, 27.6, 26.3, 26.2, 26.1, 24.3, 23.3, 22.8, 21.0, 16.4, 15.7, 15.5. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{40}\text{H}_{51}\text{N}_4\text{O}_9$, 731.3659; found, 731.3656.

Compound 1b: 47.3 mg (0.059 mmol) of **1b** was isolated from 30.0 mg (0.066 mmol)



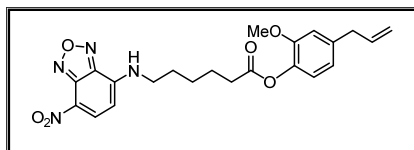
of **1** with 89% yield. Orange solid. $[\alpha]_D^{25} = -12.6$ (c 1.7, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3327, 1715, 1700. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.50 (d, $J=8.72$ Hz, 1H), 7.46 (m, 1H), 7.42 (m, 1H), 6.35 (m, 1H), 6.27 (m, 1H), 6.17 (d, $J=8.59$ Hz, 1H), 5.85 (s, 1H), 5.26 (m, 1H), 4.54 (m, 1H), 3.50 (m, 2H), 3.38 (s, 1H), 2.31 (m, 2H), 1.95 (s, 3H), 1.25 (s, 3H), 1.02 (s, 3H), 0.99 (s, 3H), 0.87 (s, 3H), 0.78 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 205.4, 193.4, 173.6, 169.7, 144.3, 143.9, 143.8, 142.7, 141.6, 136.4, 124.0, 123.2, 118.6, 111.2, 98.5, 79.8, 74.8, 60.6, 48.1, 48.1, 44.0, 44.0, 43.1, 37.5, 37.4, 37.2, 34.7, 30.4, 29.3, 29.2, 29.1, 29.1, 28.5, 27.6, 26.9, 26.2, 26.1, 25.1, 23.4, 22.8, 21.0, 16.4, 15.8, 15.6. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{45}\text{H}_{61}\text{N}_4\text{O}_9$, 801.4438; found, 801.4405.

Compound 1c: 40.0 mg (0.056 mmol) of **1c** was isolated from 30.0 mg (0.066 mmol)



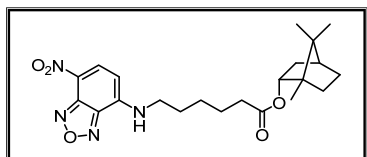
of **1** with 85% yield. Orange solid. $[\alpha]_D^{25} = +101.2$ (c 2.25, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 1736, 1701. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.46 (d, $J=8.84$ Hz, 1H), 7.45 (m, 1H), 7.41 (m, 1H), 6.26 (m, 1H), 6.14 (d, $J=8.72$ Hz, 1H), 5.83 (s, 1H), 5.52 (br. m, 1H), 5.26 (m, 1H), 4.52 (m, 1H), 3.78 (br. m, 2H), 3.37 (s, 1H), 1.93 (s, 3H), 1.23 (s, 3H), 1.00 (s, 3H), 0.91 (s, 3H), 0.86 (s, 3H), 0.78 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 205.4, 193.3, 170.5, 169.7, 144.7, 144.3, 143.3, 142.7, 141.6, 135.5, 123.2, 123.1, 118.5, 111.1, 101.7, 82.1, 74.5, 64.7, 60.5, 50.7, 48.1, 48.0, 44.0, 43.0, 37.4, 37.3, 37.2, 31.2, 30.2, 27.6, 26.2, 26.1, 22.9, 22.7, 21.0, 16.3, 15.7, 15.5. HRMS (ESI) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{39}\text{H}_{46}\text{N}_4\text{O}_9\text{Na}$, 737.3162; found, 737.3157.

Compound 2a: 77.4 mg (0.176 mmol) of **2a** was isolated from 30.0 mg (0.183 mmol)



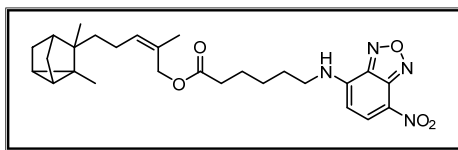
of **2** with 96% yield. Orange solid. $[\alpha]_D^{25} = +0.15$ (c 4.1, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3330, 1754. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.47 (d, $J=8.85$ Hz, 1H), 6.92 (d, $J=7.93$ Hz, 1H), 6.77 (m, 2H), 6.34 (m, 1H), 6.18 (d, $J=8.54$ Hz, 1H), 5.96 (m, 1H), 5.11 (m, 2H), 3.79 (s, 3H), 3.54 (dd, $J=13.12, 6.17$ Hz, 2H), 3.38 (m, 2H), 2.64 (t, $J=7.32$ Hz, 2H), 1.88 (m, 4H), 1.64 (m, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 171.5, 150.7, 144.2, 143.9, 143.8, 139.1, 137.8, 136.9, 136.4, 124.1, 122.4, 120.7, 116.2, 112.8, 98.5, 55.8, 43.7, 40.0, 33.6, 28.1, 26.2, 24.3. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{22}\text{H}_{25}\text{N}_4\text{O}_6$, 441.1774; found, 441.1753.

Compound 3a: 79.1 mg (0.184 mmol) of **3a** was isolated from 30.0 mg (0.195 mmol)



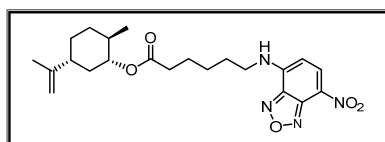
of **3** with 95% yield. Orange solid. $[\alpha]_D^{25} = -18.6$ (c 3.8, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3326, 1719. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.50 (d, $J=8.53$ Hz, 1H), 6.40 (m, 1H), 6.18 (d, $J=8.78$ Hz, 1H), 4.90 (m, 1H), 3.52 (m, 2H), 2.39 (m, 3H), 0.91 (s, 3H), 0.87 (s, 3H), 0.82 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.7, 144.2, 143.9, 143.8, 136.5, 123.9, 98.5, 79.9, 48.7, 47.8, 44.8, 43.7, 36.8, 34.2, 28.1, 28.0, 27.1, 26.3, 24.4, 19.6, 18.8, 13.5. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{22}\text{H}_{31}\text{N}_4\text{O}_5$, 431.2294; found, 431.2272.

Compound 4a: 62.5 mg (0.126 mmol) of **4a** was isolated from 30.0 mg (0.136 mmol)



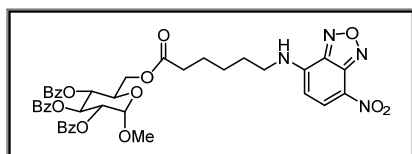
of **4** with 93% yield. Orange solid. $[\alpha]_D^{25} = +3.51$ (c 1.3, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3328, 1719. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.50 (d, $J=8.54$ Hz, 1H), 6.34 (m, 1H), 6.18 (d, $J=8.54$ Hz, 1H), 5.41 (t, $J=7.32$ Hz, 1H), 4.61 (s, 2H), 3.52 (m, 2H), 2.40 (t, $J=7.32$ Hz, 2H), 1.73 (s, 3H), 0.99 (s, 3H), 0.82 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.4, 144.2, 143.9, 143.8, 136.4, 132.0, 129.0, 124.1, 98.5, 63.2, 45.8, 43.7, 38.1, 34.6, 33.9, 31.5, 31.0, 28.1, 27.3, 26.3, 24.3, 23.1, 21.4, 19.5, 19.4, 17.5, 10.6. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{27}\text{H}_{37}\text{N}_4\text{O}_5$, 497.2764; found, 497.2740.

Compound 5a: 75.2 mg (0.175 mmol) of **5a** was isolated from 30.0 mg (0.195 mmol)



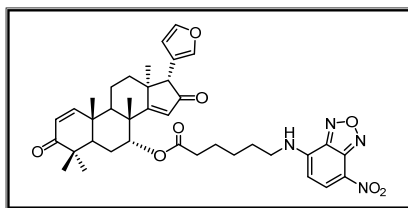
of **5** with 90% yield. Orange solid. $[\alpha]_D^{25} = -34.7$ (c 2.2, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3325, 1727. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.49 (d, $J=8.72$ Hz, 1H), 6.32 (m, 1H), 6.18 (d, $J=8.59$ Hz, 1H), 4.68 (m, 2H), 4.50 (m, 1H), 3.50 (m, 2H), 2.37 (m, 2H), 1.70 (s, 3H), 0.89 (d, $J=6.32$ Hz, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.1, 148.8, 144.2, 143.9, 136.5, 123.8, 108.8, 98.5, 78.3, 43.7, 43.6, 37.1, 36.9, 34.2, 33.0, 30.8, 28.1, 26.3, 24.4, 20.8, 18.2. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{22}\text{H}_{31}\text{N}_4\text{O}_5$, 431.2294; found, 431.2285.

Compound 6a: 42.2 mg (0.054 mmol) of **6a** was isolated from 30.0 mg (0.059 mmol)



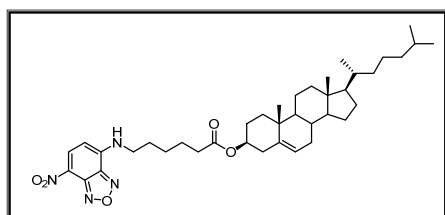
of **6** with 91% yield. Orange solid. $[\alpha]_D^{25} = -55.3$ (c 2.5, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3339, 1732. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.43 (d, $J=8.70$ Hz, 1H), 8.10-7.22 (m, 15H), 6.53 (m, 1H), 6.13 (d, $J=8.24$ Hz, 1H), 5.97 (t, $J=10.08$ Hz, 1H), 5.89 (dd, $J=10.07$, 3.21 Hz, 1H), 5.68 (m, 1H), 5.01 (d, $J=1.83$ Hz, 1H), 4.42 (dd, $J=12.36$, 4.58 Hz, 1H), 4.33 (m, 2H), 3.54 (s, 3H), 3.47 (m, 2H), 2.37-2.52 (m, 2H), 1.71-1.83 (m, 4H), 1.50 (m, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.0, 165.6, 165.4, 165.3, 144.2, 143.9, 143.8, 136.4, 133.5, 133.2, 129.8, 129.7, 129.6, 129.3, 129.0, 128.8, 128.5, 128.5, 128.2, 123.8, 98.6, 98.4, 70.4, 69.9, 68.5, 66.8, 62.6, 55.6, 43.6, 33.6, 28.0, 26.2, 24.0. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{40}\text{H}_{39}\text{N}_4\text{O}_{13}$, 783.2514; found, 783.2508.

Compound 8a: 35.7 mg (0.052 mmol) of **8a** was isolated from 30.0 mg (0.073 mmol)



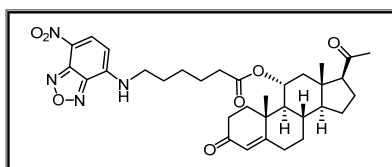
of **8** with 71% yield. Orange solid. $[\alpha]_D^{25} = -66.3$ (c 1.6, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3307, 1729, 1694, 1667. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.48 (d, $J=8.85$ Hz, 1H), 7.70 (m, 1H), 7.48 (m, 1H), 7.43 (m, 1H), 7.13 (d, $J=10.38$ Hz, 1H), 6.25 (m, 1H), 6.19 (d, $J=8.85$ Hz, 1H), 5.88 (m, 2H), 5.35 (m, 1H), 3.53 (s, 1H), 3.50 (m, 2H), 2.50 (m, 1H), 2.32 (m, 1H), 1.37 (s, 3H), 1.27 (s, 3H), 1.09 (s, 3H), 1.06 (s, 3H), 1.06 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 206.2, 203.9, 193.1, 172.1, 156.6, 144.4, 144.1, 142.9, 141.5, 136.7, 125.8, 123.2, 123.0, 118.1, 111.0, 98.3, 73.8, 60.9, 48.3, 46.1, 44.6, 44.0, 43.7, 40.0, 38.2, 34.1, 29.9, 28.1, 26.8, 26.8, 26.4, 26.2, 24.4, 23.4, 21.2, 18.9, 15.7. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{38}\text{H}_{45}\text{N}_4\text{O}_8$, 685.3237; found, 685.3245.

Compound 9a: 49.4 mg (0.075 mmol) of **9a** was isolated from 30.0 mg (0.078 mmol)



of **9** with 96% yield. Orange solid. $[\alpha]_D^{25} = -20.1$ (c 0.9, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3331, 1721. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.50 (d, $J=8.54$ Hz, 1H), 6.28 (m, 1H), 6.18 (d, $J=8.54$ Hz, 1H), 5.36 (m, 1H), 4.63 (m, 1H), 3.52 (dd, $J=13.12$, 6.17 Hz, 1H), 2.35 (m, 2H), 2.30 (m, 2H), 1.02 (s, 3H), 0.92 (d, $J=6.41$ Hz, 3H), 0.88 (d, $J=2.41$ Hz, 3H), 0.86 (d, $J=2.41$ Hz, 3H), 0.68 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 172.8, 144.3, 143.9, 143.8, 139.5, 136.4, 124.2, 122.8, 98.5, 74.1, 56.7, 56.1, 50.0, 43.6, 42.3, 39.7, 39.5, 38.2, 36.9, 36.6, 36.2, 35.8, 34.2, 31.9, 31.8, 28.2, 28.2, 28.0, 27.8, 26.3, 24.3, 24.3, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{39}\text{H}_{59}\text{N}_4\text{O}_5$, 663.4485; found, 663.4460.

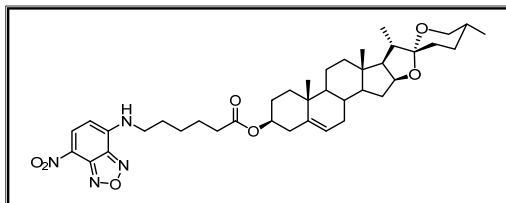
Compound 10a: 48.5 mg (0.080 mmol) of **10a** was isolated from 30.0 mg (0.091



mmol) of **10** with 88% yield. Orange solid. $[\alpha]_D^{25} = +93.1$ (c 1.21, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3309, 1727, 1701, 1665. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.49 (d, $J=8.54$ Hz, 1H), 6.66 (m, 1H), 6.19 (d, $J=8.54$ Hz, 1H), 5.76 (s, 1H), 5.28 (m, 1H), 3.54 (m, 2H), 2.52 (m, 1H), 2.11 (s, 3H), 1.26 (s, 3H), 0.76 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 208.7, 199.2, 172.3, 169.6, 144.2, 143.9, 143.9, 136.5, 124.7, 123.8, 98.5, 70.7, 62.8, 55.3, 54.7, 45.2, 43.7, 43.6, 39.7, 36.6, 35.0, 34.6, 34.0, 33.2,

31.7, 31.2, 28.1, 26.4, 24.2, 24.0, 23.1, 18.3, 14.0. HRMS (ESI) m/z : $[M+Na]^+$ Calcd for $C_{33}H_{42}N_4O_7Na$, 629.2951; found, 629.2945.

Compound 11a: 46.9 mg (0.068 mmol) of **11a** was isolated from 30.0 mg (0.072

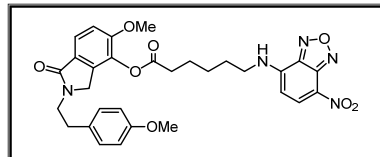


mmol) of **11** with 94% yield. Orange solid.

$[\alpha]_D^{25} = -64.2$ (c 3.2, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3324, 1728. ^1H NMR ($CDCl_3$, 400 MHz) δ : 8.49 (d, $J=8.72$ Hz, 1H), 6.29 (m,

1H), 6.18 (d, $J=8.59$ Hz, 1H), 5.36 (d, $J=4.42$ Hz, 1H), 4.62 (m, 1H), 4.42 (m, 1H), 3.32-3.57 (m, 4H), 2.35 (m, 4H), 1.03 (s, 3H), 0.98 (d, $J=6.69$ Hz, 3H), 0.80 (s, 3H), 0.79 (d, $J=5.49$ Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ : 172.8, 144.2, 143.8, 139.5, 136.5, 123.8, 122.4, 109.2, 98.5, 80.7, 73.9, 66.8, 62.0, 56.4, 49.9, 43.6, 41.5, 40.2, 39.6, 38.1, 36.9, 36.7, 34.2, 32.0, 31.8, 31.3, 30.2, 28.7, 28.1, 27.7, 26.2, 24.3, 20.8, 19.3, 17.1, 16.2, 14.5. HRMS (ESI) m/z : $[M+H]^+$ Calcd for $C_{39}H_{55}N_4O_7$, 691.4071; found, 691.4073.

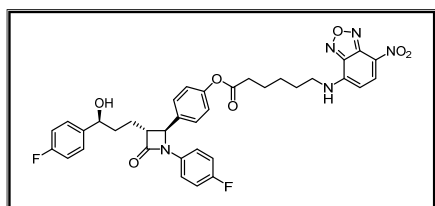
Compound 12a: 50.8 mg (0.086 mmol) of **12a** was isolated from 30.0 mg (0.096



mmol) of **12** with 90% yield. Orange solid. $[\alpha]_D^{25} = +0.29$ (c 1.48, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3333, 1759, 1683. ^1H NMR ($CDCl_3$, 400 MHz) δ : 8.48 (d,

$J=8.24$ Hz, 1H), 7.24 (d, $J=2.29$ Hz, 1H), 7.13 (d, $J=8.70$ Hz, 2H), 6.82 (d, $J=8.70$ Hz, 2H), 6.79 (d, $J=1.83$ Hz, 1H), 6.50 (m, 1H), 6.18 (d, $J=8.70$ Hz, 1H), 4.06 (s, 2H), 3.85 (s, 3H), 3.78 (s, 3H), 3.79 (t, $J=7.33$ Hz, 2H), 3.55 (m, 2H), 2.91 (t, $J=7.33$ Hz, 2H), 2.63 (t, $J=7.33$ Hz, 2H), 1.80-1.94 (m, 4H), 1.61 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ : 170.6, 167.6, 161.0, 158.2, 145.7, 144.2, 143.8, 143.8, 136.4, 135.7, 130.6, 129.6, 125.0, 124.0, 114.0, 112.4, 104.8, 98.6, 56.0, 55.3, 48.2, 44.5, 43.6, 33.9, 33.7, 28.1, 26.3, 24.3. HRMS (ESI) m/z : $[M+H]^+$ Calcd for $C_{30}H_{32}N_5O_8$, 590.2251; found, 590.2255.

Compound 13a: 26.1 mg (0.038 mmol) of **13a** was isolated from 30.0 mg (0.073

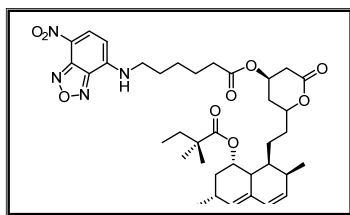


mmol) of **13** with 52% yield. Orange solid. $[\alpha]_D^{25} = -25.7$ (c 2.5, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3336, 1743. ^1H NMR ($CDCl_3$, 400 MHz) δ : 8.47 (d, $J=8.53$ Hz, 1H), 6.90-7.35 (m, 12H), 6.46 (m, 1H),

O=[N+]([O-])c1ccc(NC(=O)CCCCC(=O)O[C@H]2c3ccccc3c4ncncc4[C@@H]2C5=C(C)CCN5C)cc1C[C@H]1C(=O)OC(=O)C1c2c3c(c4c2cnc5ccccc45)C(=O)N(CCCCNc6cc([N+](=O)[O-])ccc6N=O)c3

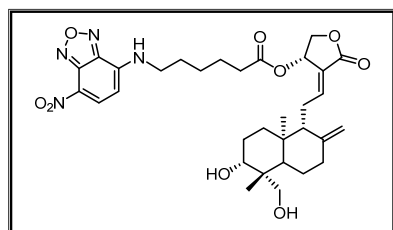
267

Compound 16a: 40.3 mg (0.058 mmol) of **16a** was isolated from 30.0 mg (0.072



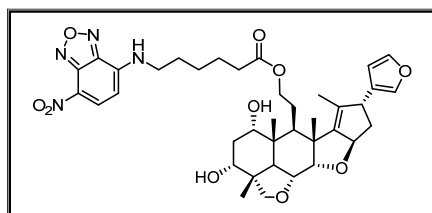
mmol) of **16** with 81% yield. Orange solid. $[\alpha]_D^{25} = +140.0$ (*c* 2.6, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3323, 1733. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.50 (d, $J=8.54$ Hz, 1H), 6.70 (m, 1H), 6.20 (d, $J=8.85$ Hz, 1H), 5.99 (d, $J=9.77$ Hz, 1H), 5.78 (dd, $J=9.77, 6.10$ Hz, 1H), 5.52 (t, $J=3.36$ Hz, 1H), 5.36 (q, $J=3.05$ Hz, 1H), 5.28 (m, 1H), 4.48 (m, 1H), 3.54 (m, 2H), 2.75 (m, 2H), 2.40 (t, $J=7.32$ Hz, 2H), 1.13 (s, 3H), 1.12 (s, 3H), 1.09 (d, $J=7.32, 7.02$ Hz, 3H), 0.89 (d, $J=7.32, 7.02$ Hz, 3H), 0.81 (t, $J=7.63$ Hz, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 177.9, 172.3, 168.8, 144.3, 144.0, 143.9, 136.5, 132.7, 131.3, 129.8, 128.4, 123.8, 98.5, 77.1, 68.0, 65.7, 43.7, 43.0, 37.4, 36.7, 35.5, 34.0, 33.3, 33.2, 33.0, 32.8, 30.6, 28.1, 27.2, 26.3, 24.8, 24.7, 24.6, 24.4, 23.0, 13.9, 9.3. HRMS (ESI) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{37}\text{H}_{50}\text{N}_4\text{O}_9\text{Na}$, 717.3475; found, 717.3434.

Compound 17a: 17.7 mg (0.028 mmol) of **17a** was isolated from 30.0 mg (0.086



mmol) of **17** with 33% yield. Orange solid. $[\alpha]_D^{25} = -80.7$ (*c* 0.95, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3409, 1738. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.50 (d, $J=8.53$ Hz, 1H), 7.01 (m, 1H), 6.51 (m, 1H), 6.18 (d, $J=8.53$ Hz, 1H), 5.94 (d, $J=5.52$ Hz, 1H), 4.85 (s, 1H), 4.56 (m, 1H), 4.47 (s, 1H), 4.23 (m, 1H), 4.15 (d, $J=11.04$ Hz, 1H), 3.50 (m, 3H), 3.32 (d, $J=11.04$ Hz, 1H), 2.35-2.45 (m, 5H), 1.25 (s, 3H), 0.62 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 172.9, 169.1, 150.6, 146.9, 144.2, 143.9, 143.8, 136.5, 123.9, 123.8, 108.7, 98.6, 80.3, 71.6, 67.8, 64.1, 55.7, 55.0, 43.6, 42.8, 38.7, 37.6, 36.9, 33.7, 29.7, 28.1, 26.3, 25.3, 24.2, 23.6, 22.7, 15.1. HRMS (ESI) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{32}\text{H}_{43}\text{N}_4\text{O}_9$, 627.3030; found, 627.3024.

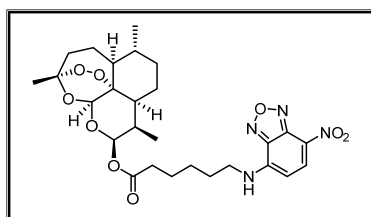
Compound 18a: 30.2 mg (0.042 mmol) of **18a** was isolated from 20.0 mg (0.045



mmol) of **18** with 93% yield. Orange solid. $[\alpha]_D^{25} = +73.0$ (*c* 0.9, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3140, 1719. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.49 (d, $J=8.85$ Hz, 1H), 7.32 (m, 1H), 7.21 (m, 1H), 6.67 (m, 1H), 6.19 (m, 1H), 6.17 (d, $J=8.85$ Hz, 1H), 5.36 (m, 1H), 4.22 (m, 1H), 4.18 (d, $J=3.36$ Hz, 1H), 3.98 (m, 4H), 3.67 (m, 3H), 3.57 (d, $J=7.02$ Hz, 1H), 3.50 (m, 2H),

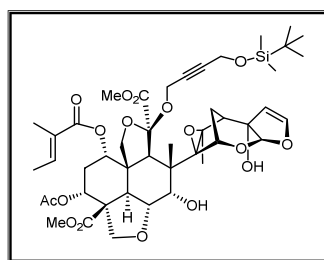
3.34 (d, $J=6.71$ Hz, 1H), 2.61 (d, $J=12.82$ Hz, 1H), 2.33 (t, $J=7.02$ Hz, 2H), 1.78 (s, 3H), 1.32 (s, 3H), 1.11 (s, 3H), 0.89 (s, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.3, 148.0, 144.2, 144.0, 143.9, 143.1, 138.4, 136.6, 133.4, 127.1, 123.7, 110.4, 98.5, 87.5, 85.9, 77.5, 72.9, 72.6, 71.8, 64.8, 49.2, 49.0, 44.3, 43.7, 41.5, 41.5, 38.5, 37.6, 33.8, 31.4, 28.0, 26.2, 24.9, 24.2, 19.3, 17.1, 15.0, 13.3. HRMS (ESI) m/z : $[\text{M}+\text{NH}_4]^+$ Calcd for $\text{C}_{38}\text{H}_{52}\text{N}_5\text{O}_{10}$, 738.3714; found, 738.3698.

Compound 19a: 42.0 mg (0.075 mmol) of **19a** was isolated from 30.0 mg (0.106



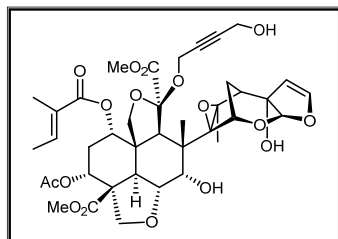
mmol) of **19** with 71% yield. Orange solid. $[\alpha]_D^{25} = +1.1$ (c 0.62, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3337, 1747. ^1H NMR (CDCl_3 , 400 MHz) δ : 8.50 (d, $J=8.53$ Hz, 1H), 6.56 (m, 1H), 6.19 (d, $J=8.78$ Hz, 1H), 5.81 (d, $J=9.79$ Hz, 1H), 5.45 (s, 1H), 3.53 (m, 2H), 1.41 (s, 3H), 0.98 (d, $J=6.02$ Hz, 3H), 0.84 (d, $J=7.28$ Hz, 3H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 172.2, 144.2, 143.9, 136.5, 123.8, 104.5, 98.5, 91.9, 91.5, 80.1, 51.5, 45.1, 43.6, 37.2, 36.1, 34.0, 33.8, 31.7, 27.9, 26.1, 25.9, 24.5, 23.8, 22.0, 20.2, 12.1. HRMS (ESI) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{27}\text{H}_{36}\text{N}_4\text{O}_9\text{Na}$, 583.2380; found, 583.2370.

Compound 20a: 46.7 mg (0.052 mmol) of **20a** was isolated from 100.0 mg (0.139



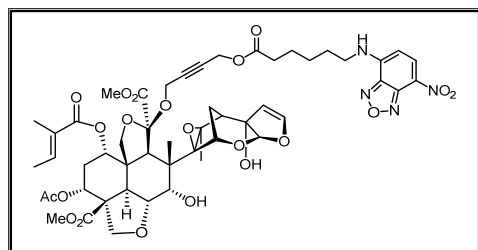
mmol) of **20** with 37% yield. White solid. $[\alpha]_D^{25} = -20.1$ (c 3.2, CHCl_3). IR (CHCl_3) ν_{max} (cm^{-1}): 3480, 1741. ^1H NMR (CDCl_3 , 400 MHz) δ : 6.93 (m, 1H), 6.42 (d, $J=2.75$ Hz, 1H), 5.63 (s, 1H), 5.48 (t like, 1H), 5.02 (d, $J=3.05$ Hz, 1H), 4.74 (m, 1H), 4.71 (t, $J=2.75$ Hz, 1H), 4.59 (dd, $J=2.44$, 12.51 Hz, 1H), 4.53 (m, 1H), 4.44 (m, 1H), 4.26 (m, 3H), 4.11 (d, $J=9.46$ Hz, 1H), 4.05 (d, $J=8.85$ Hz, 1H), 3.79 (s, 3H), 3.74 (d, $J=8.85$ Hz, 1H), 3.65 (m, 1H), 3.63 (s, 3H), 3.54 (s, 1H), 3.37 (d, $J=12.51$ Hz, 1H), 3.01 (1H, br s), 2.92 (1H, br s), 2.34 (m, 1H), 2.28 (d, $J=5.49$ Hz, 1H), 2.22 (m, 1H), 1.94 (s, 3H), 1.89 (s, 3H), 1.85 (m, 3H), 1.77 (m, 3H), 1.64 (s, 3H), 1.24 (m, 2H), 0.87 (s, 9H), 0.08 (s, 6H). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 173.4, 169.7, 169.1, 166.4, 146.9, 137.9, 128.5, 108.7, 107.5, 106.5, 84.6, 83.6, 80.6, 76.8, 75.2, 73.7, 72.9, 70.6, 69.4, 66.9, 53.1, 53.0, 52.7, 52.5, 51.7, 50.1, 47.8, 46.2, 45.4, 37.0, 29.7, 25.8, 25.1, 21.0, 20.8, 18.8, 18.2, 14.3, 11.9, (-)5.2. HRMS (ESI) m/z : $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{45}\text{H}_{62}\text{O}_{17}\text{SiNa}$, 925.3654; found, 925.3630.

Compound 20b: 37.7 mg (0.048 mmol) of **20b** was isolated from 46.7 mg (0.052



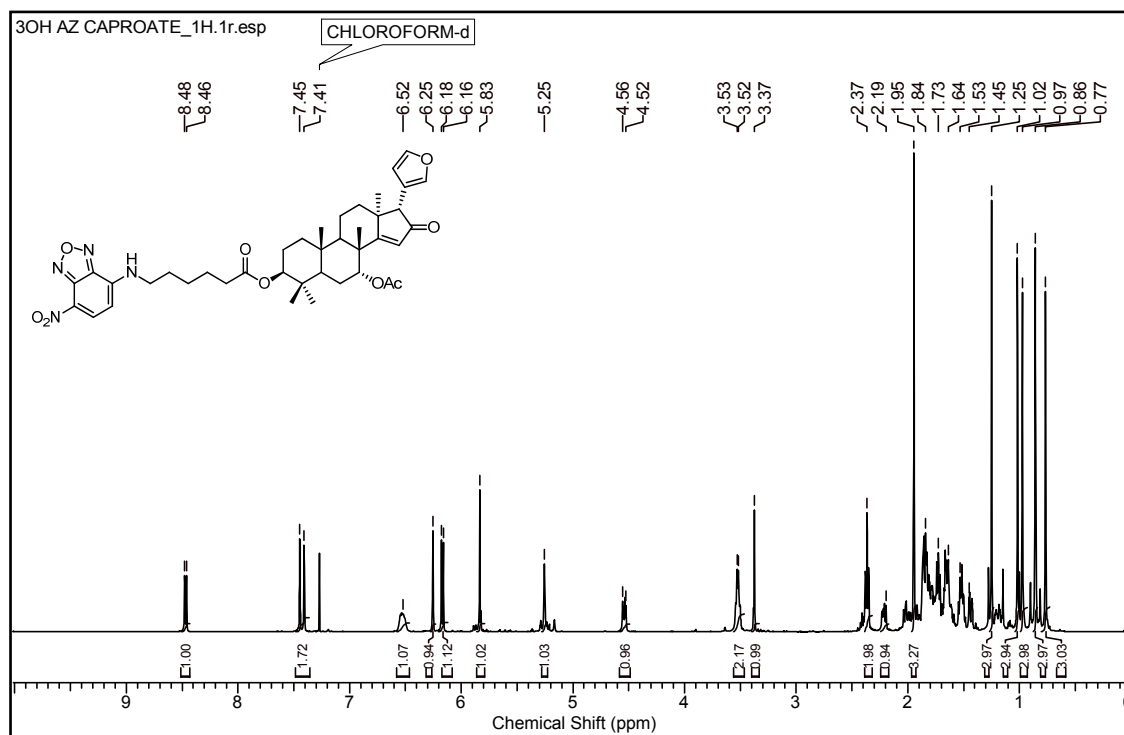
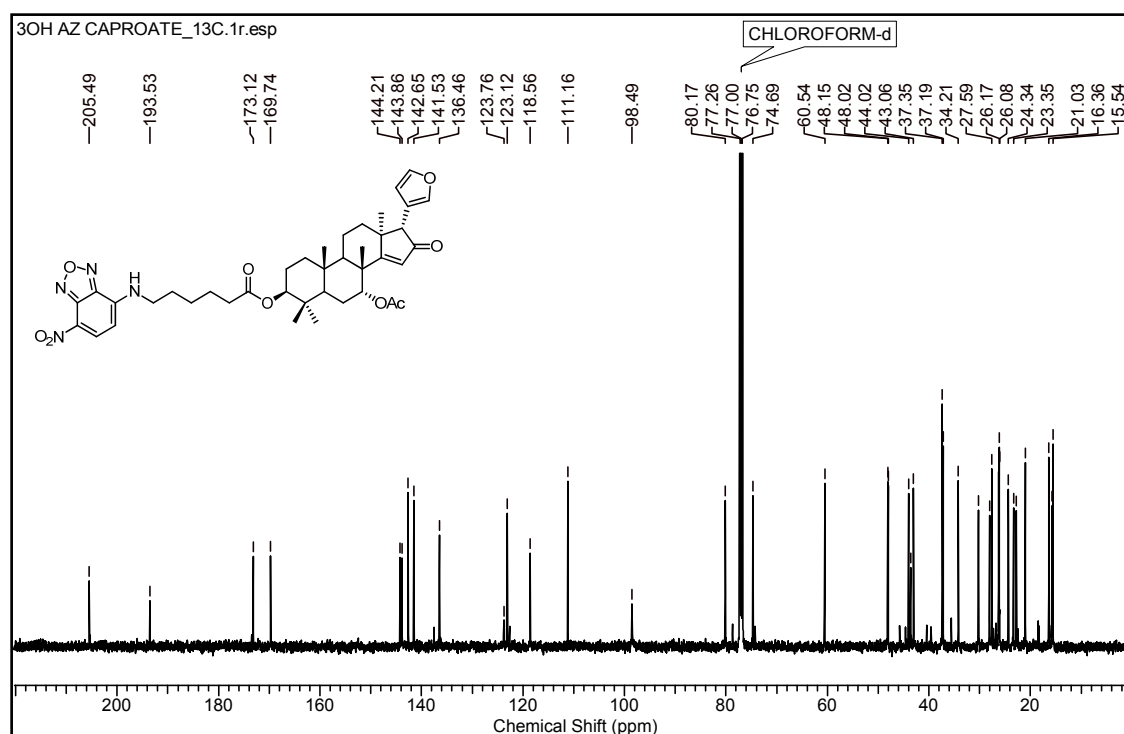
mmol) of **20a** with 92% yield. White solid. $[\alpha]_D^{25} = -20.2$ (c 0.9, CHCl₃). IR (CHCl₃) $\nu_{\max}$ (cm⁻¹): 3460, 1740. ¹H NMR (CDCl₃, 400 MHz) δ : 6.94 (m, 1H), 6.43 (d, *J*=2.75 Hz, 1H), 5.69 (s, 1H), 5.49 (t like, 1H), 5.04 (d, *J*=2.75 Hz, 1H), 4.82 (m, 1H), 4.72 (t, *J*=2.75 Hz, 1H), 4.58 (dd, *J*=12.51, 2.75 Hz, 1H), 4.47 (m, 1H), 4.43 (m, 1H), 4.33 (m, 1H), 4.23 (m, 2H), 4.13 (d, *J*=9.77 Hz, 1H), 4.06 (d, *J*=8.85 Hz, 1H), 3.80 (s, 3H), 3.74 (d, *J*=8.85 Hz, 1H), 3.66 (m, 5H), 3.39 (d, *J*=12.51 Hz, 1H), 2.90 (1H, br s), 2.74 (1H, br s), 2.40 (m, 1H), 2.30 (d, *J*=5.49 Hz, 1H), 2.22 (m, 1H), 1.94 (s, 3H), 1.91 (s, 3H), 1.87 (m, 3H), 1.78 (dd like, 3H), 1.58 (s, 3H), 1.26 (m, 2H). ¹³C NMR (CDCl₃, 100 MHz) δ : 173.4, 169.7, 169.2, 166.5, 147.0, 137.9, 128.5, 108.3, 107.8, 106.5, 84.6, 83.6, 81.8, 75.3, 73.8, 72.9, 70.8, 69.8, 69.5, 66.9, 53.0, 52.7, 52.6, 51.0, 50.2, 47.8, 46.3, 45.5, 37.1, 29.7, 25.4, 20.8, 20.7, 18.9, 14.3, 11.9. HRMS (ESI) *m/z*: [M+Na]⁺ Calcd for C₃₉H₄₈O₁₇Na, 811.2789; found, 811.2741.

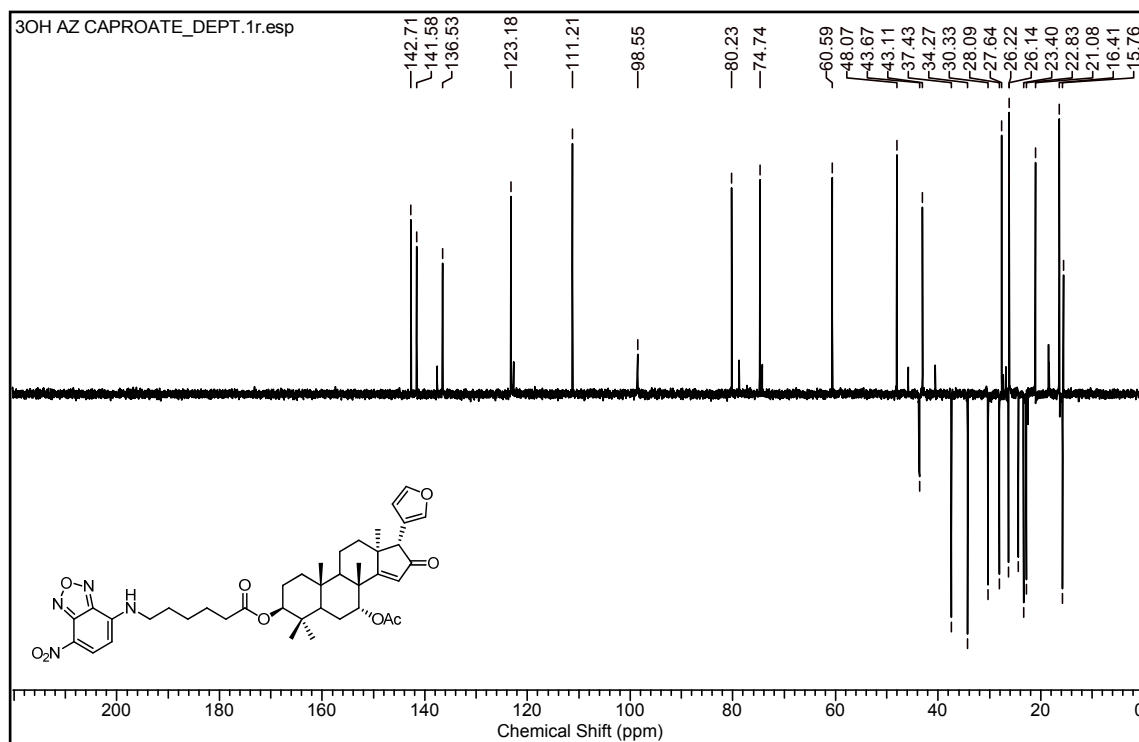
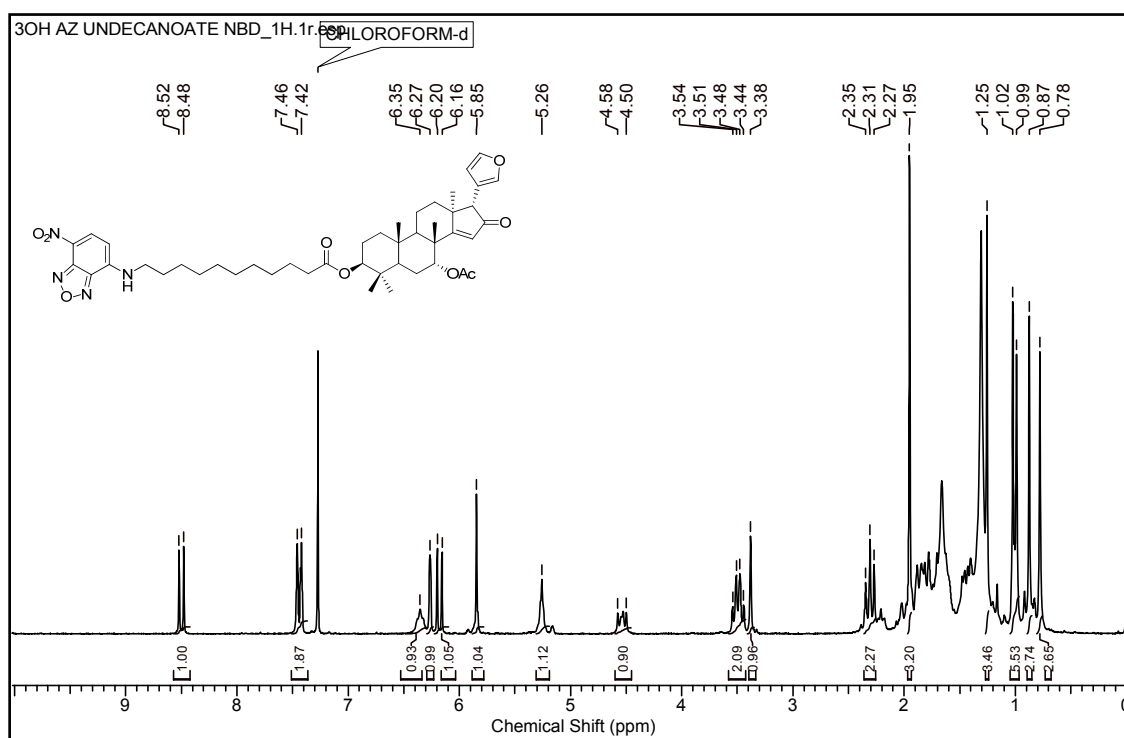
Compound 20c: 36.9 mg (0.035 mmol) of **20c** was isolated from 37.7 mg (0.048

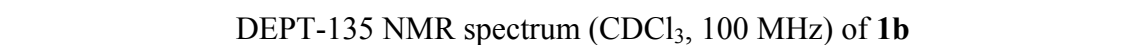


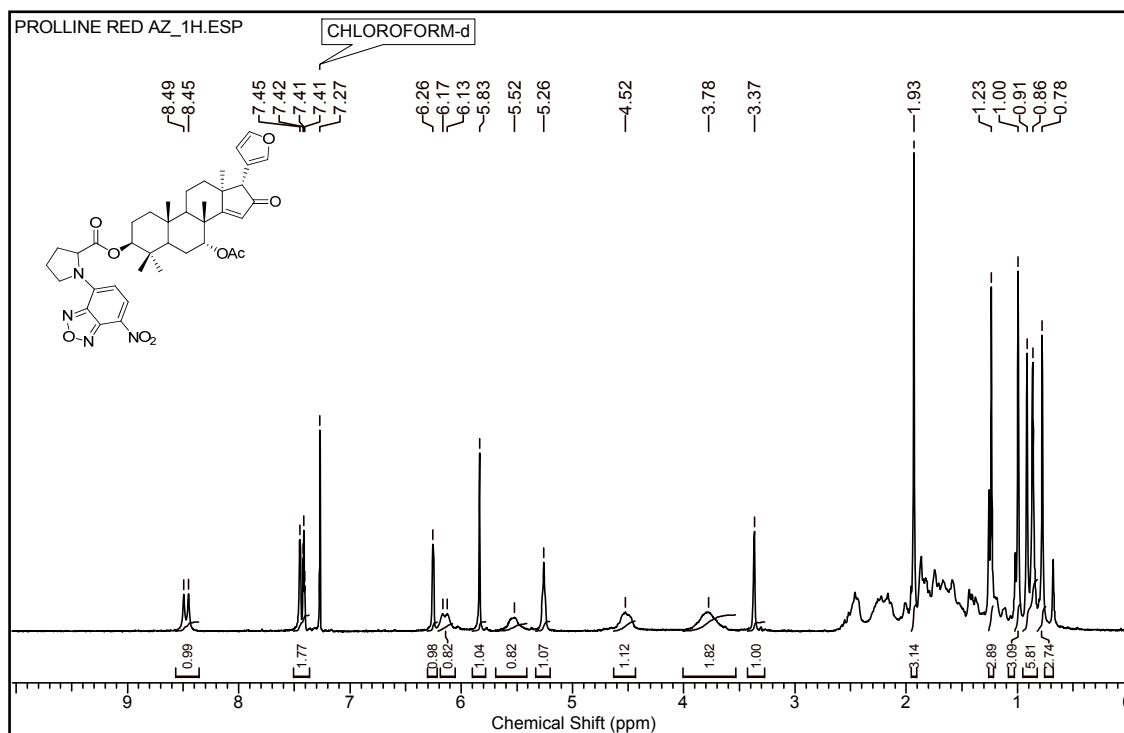
mmol) of **20b** with 72% yield. Orange solid. $[\alpha]_D^{25} = -12.3$ (c 1.8, CHCl₃). IR (CHCl₃) $\nu_{\max}$ (cm⁻¹): 3413, 1738. ¹H NMR (CDCl₃, 400 MHz) δ : 8.51 (d, *J*=8.54 Hz, 1H), 6.93 (m, H-33), 6.50 (m, 1H), 6.44 (d, *J*=2.75 Hz, 1H), 6.19 (d, *J*=8.85 Hz, 1H), 5.62 (s, 1H), 5.50 (t, *J*=2.75 Hz, 1H), 5.04 (d, *J*=2.75 Hz, 1H), 4.71 (m, 2H), 4.67 (t like, 2H), 4.59 (dd, *J*=12.51, 2.75 Hz, 1H), 4.53 (m, 1H), 4.47 (m, 1H), 4.28 (m, 1H), 4.14 (d, *J*=9.77 Hz, 1H), 4.06 (d, *J*=8.85 Hz, 1H), 3.80 (s, 3H), 3.76 (d, *J*=9.16 Hz, 1H), 3.65 (m, 4H), 3.52 (m, 3H), 3.37 (d, *J*=12.51 Hz, 1H), 2.92 (m, 2H), 2.38 (t, *J*=7.32 Hz, 2H), 2.34 (m, 1H), 2.29 (d, *J*=5.49 Hz, 1H), 2.21 (m, 1H), 1.95 (s, 3H), 1.90 (s, 3H), 1.87 (m, 3H), 1.77 (m, 3H), 1.66 (s, 3H), 1.15 (d, *J*=6.41 Hz, 1H). ¹³C NMR (CDCl₃, 100 MHz) δ : 173.4, 172.4, 169.7, 169.0, 166.4, 146.9, 144.3, 143.9, 143.8, 137.9, 136.4, 128.5, 124.0, 108.9, 107.6, 106.4, 98.5, 83.7, 82.7, 79.9, 76.9, 75.2, 73.7, 73.1, 70.6, 69.7, 69.3, 66.9, 53.0, 52.8, 52.8, 52.6, 52.2, 50.2, 47.9, 46.3, 45.4, 43.7, 37.0, 33.6, 29.8, 28.1, 26.3, 25.1, 24.2, 23.5, 20.9, 18.9, 14.3, 11.9. HRMS (ESI) *m/z*: [M+Na]⁺ Calcd for C₅₁H₆₀N₄O₂₁Na, 1087.3648; found, 1087.3611.

4B.5 Spectral Copies

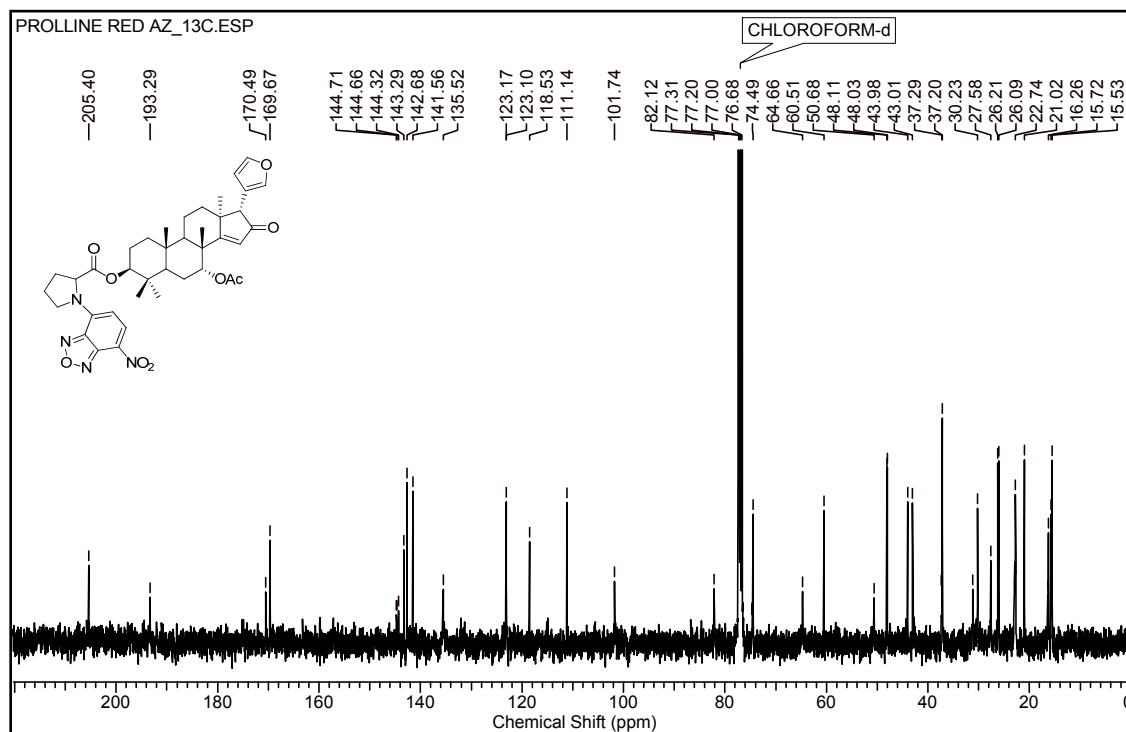
 ^1H NMR spectrum (CDCl_3 , 400 MHz) of **1a** ^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **1a**

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **1a** ^1H NMR spectrum (CDCl_3 , 400 MHz) of **1b**

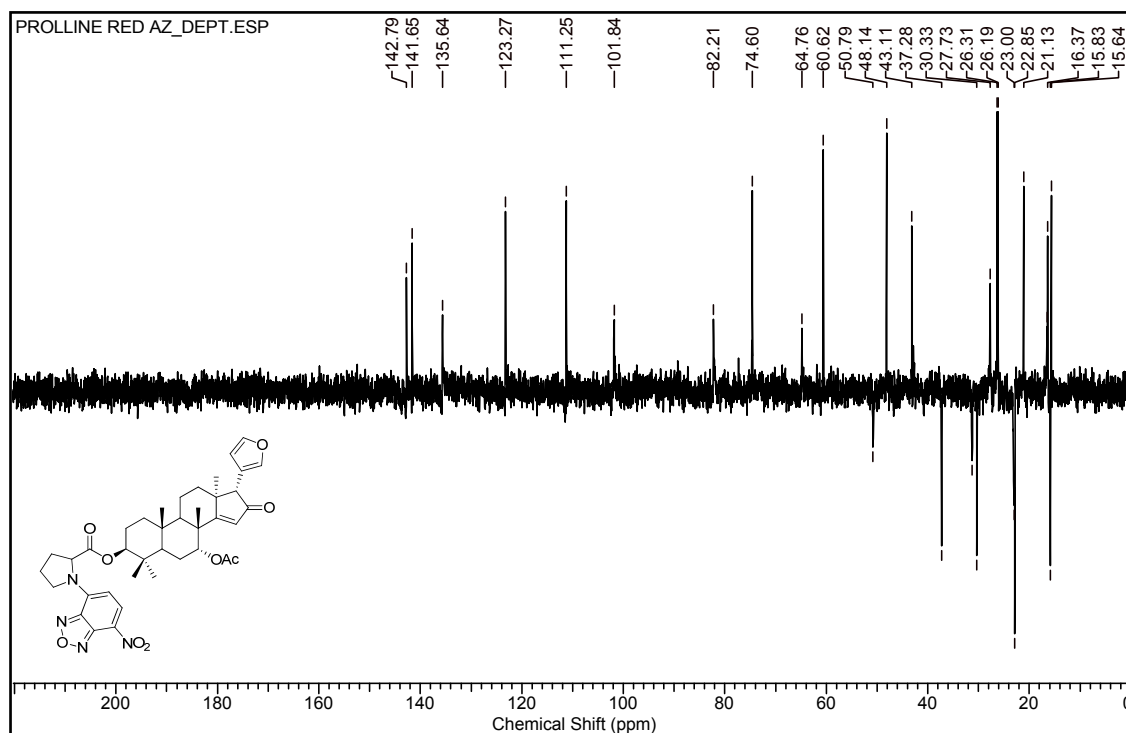
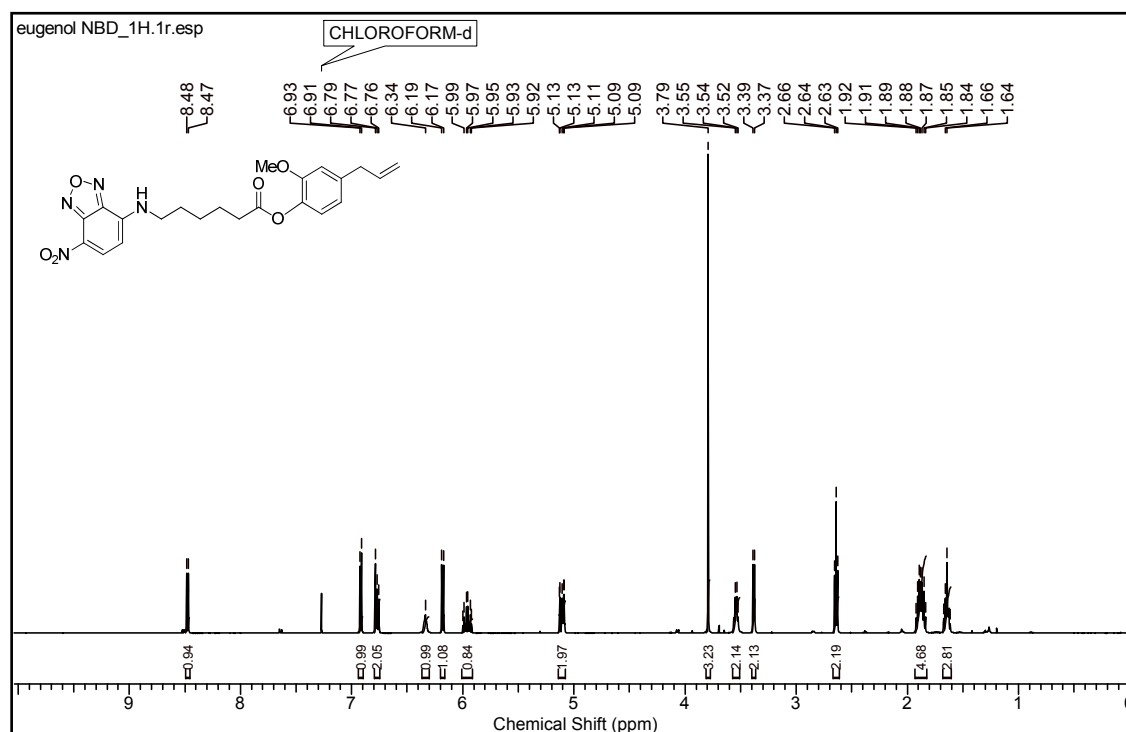


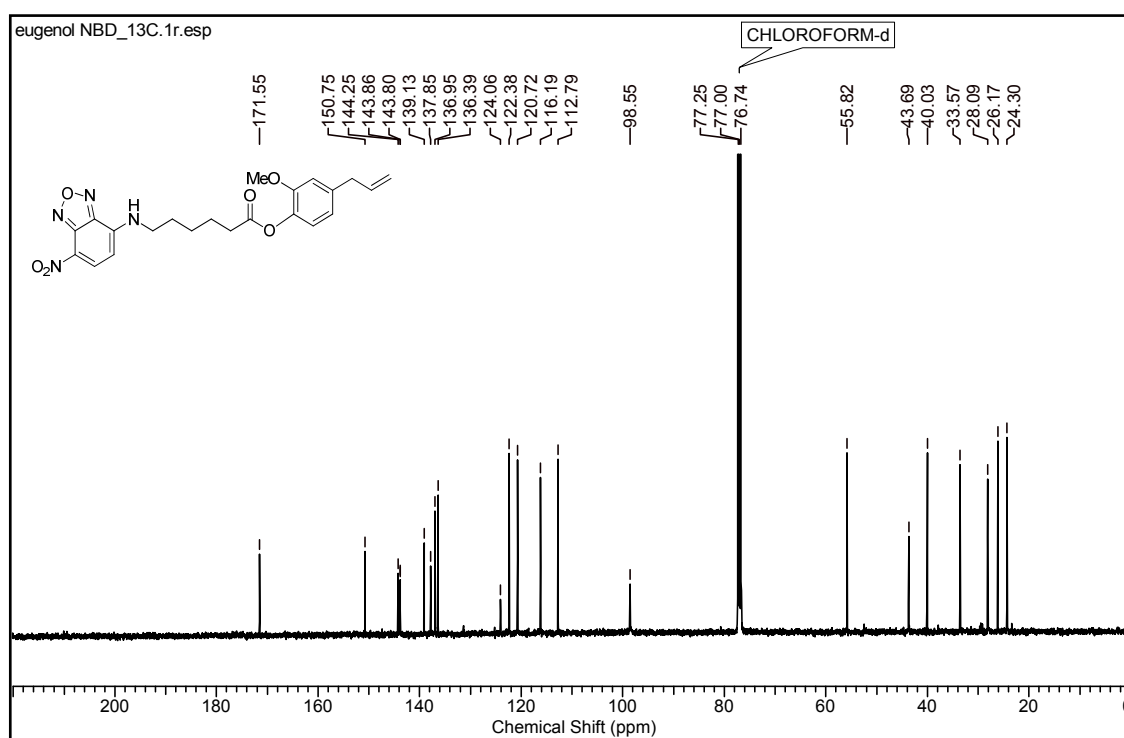


^1H NMR spectrum (CDCl_3 , 400 MHz) of **1c**

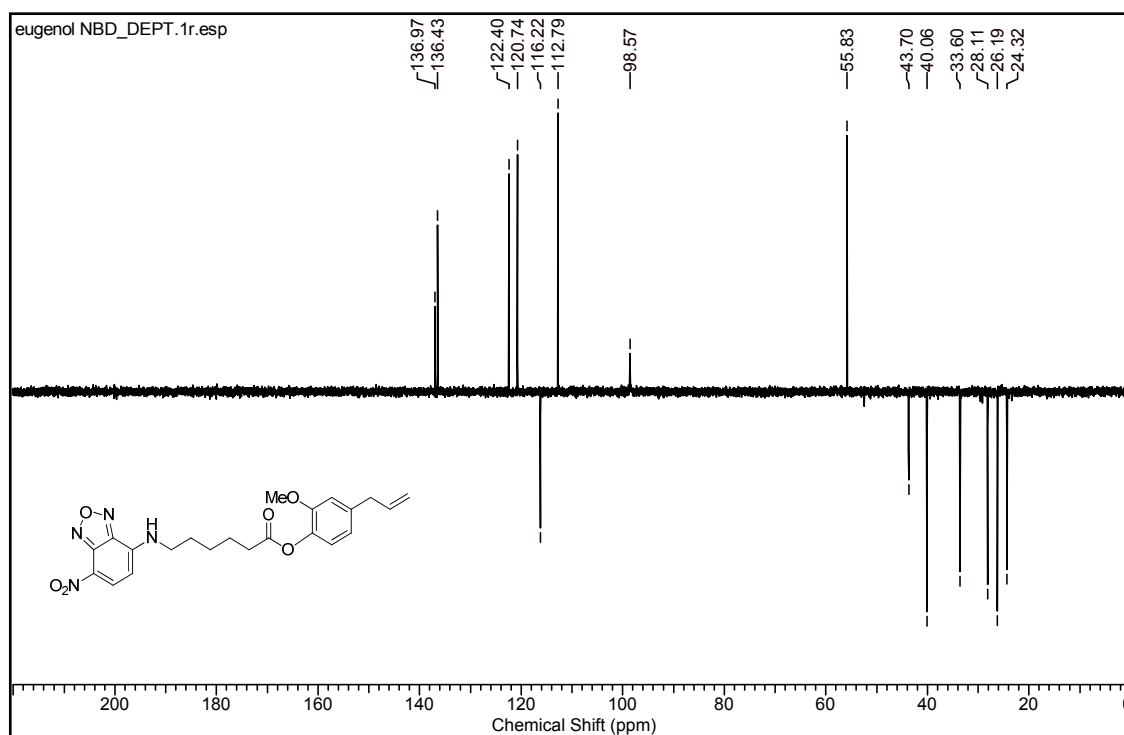


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **1c**

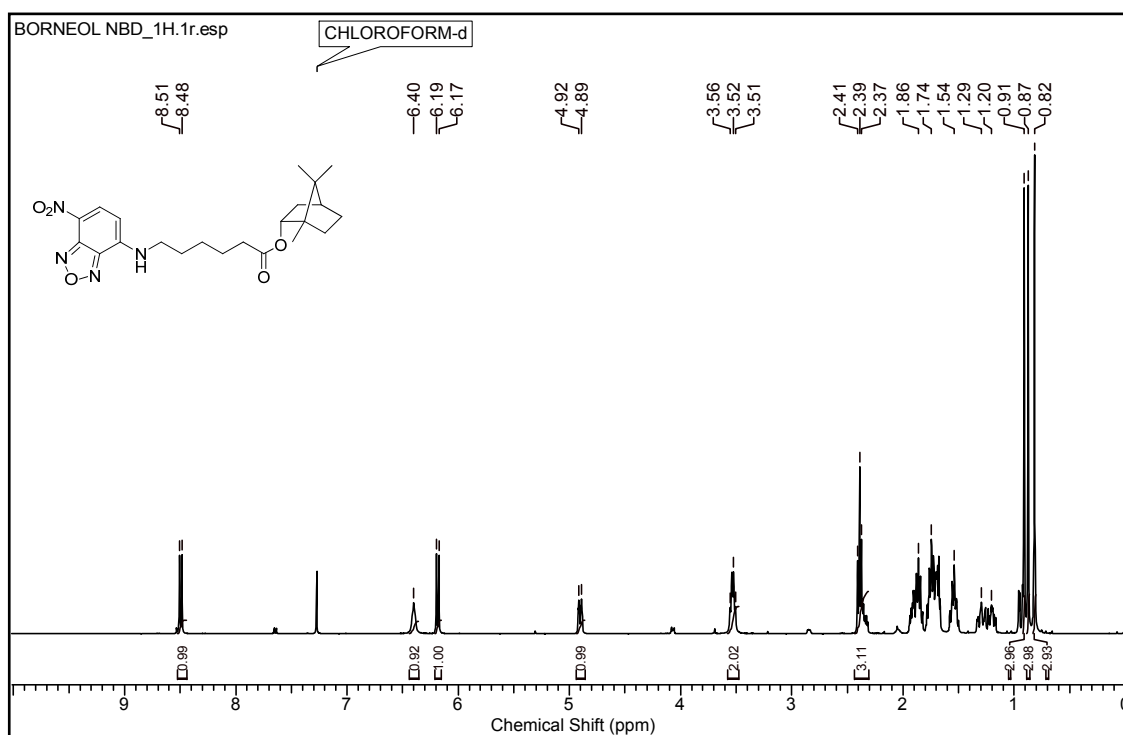
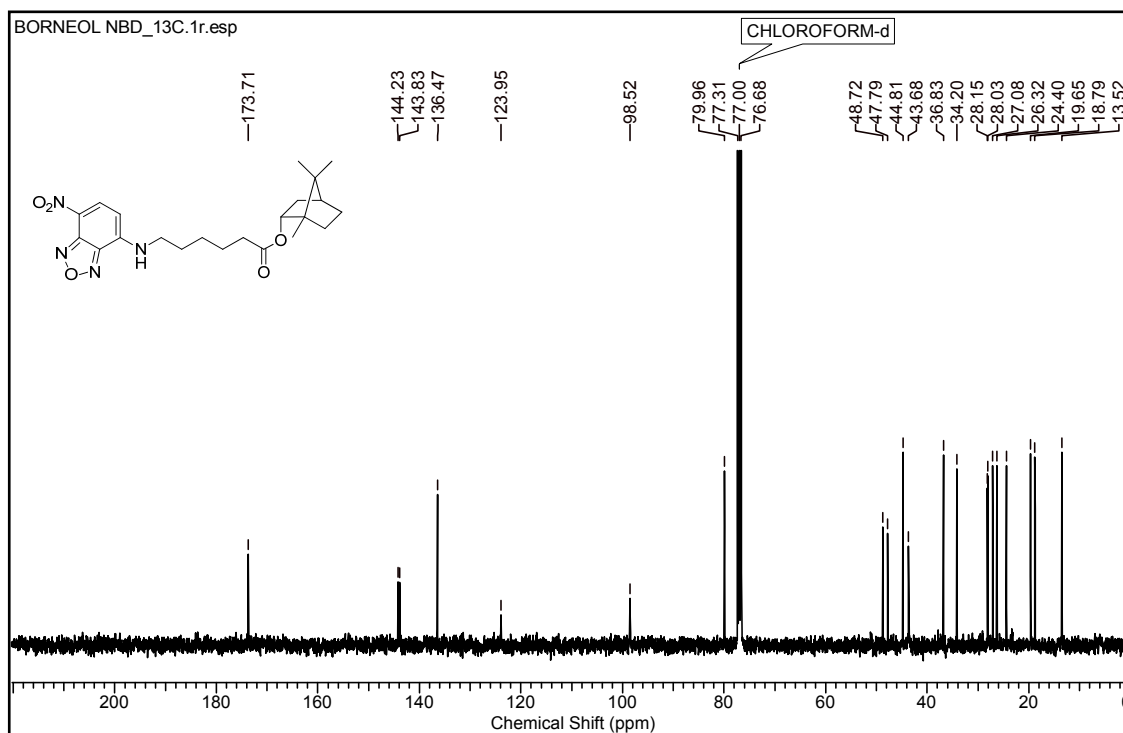
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **1c** ^1H NMR spectrum (CDCl_3 , 400 MHz) of **2a**

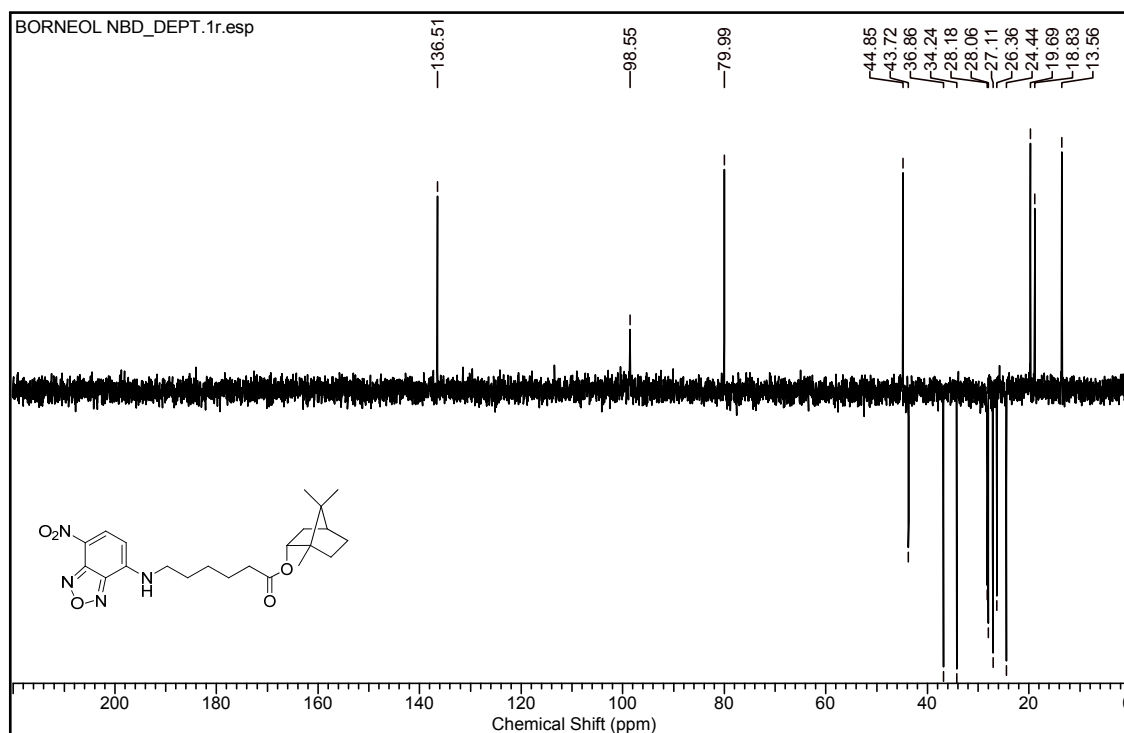
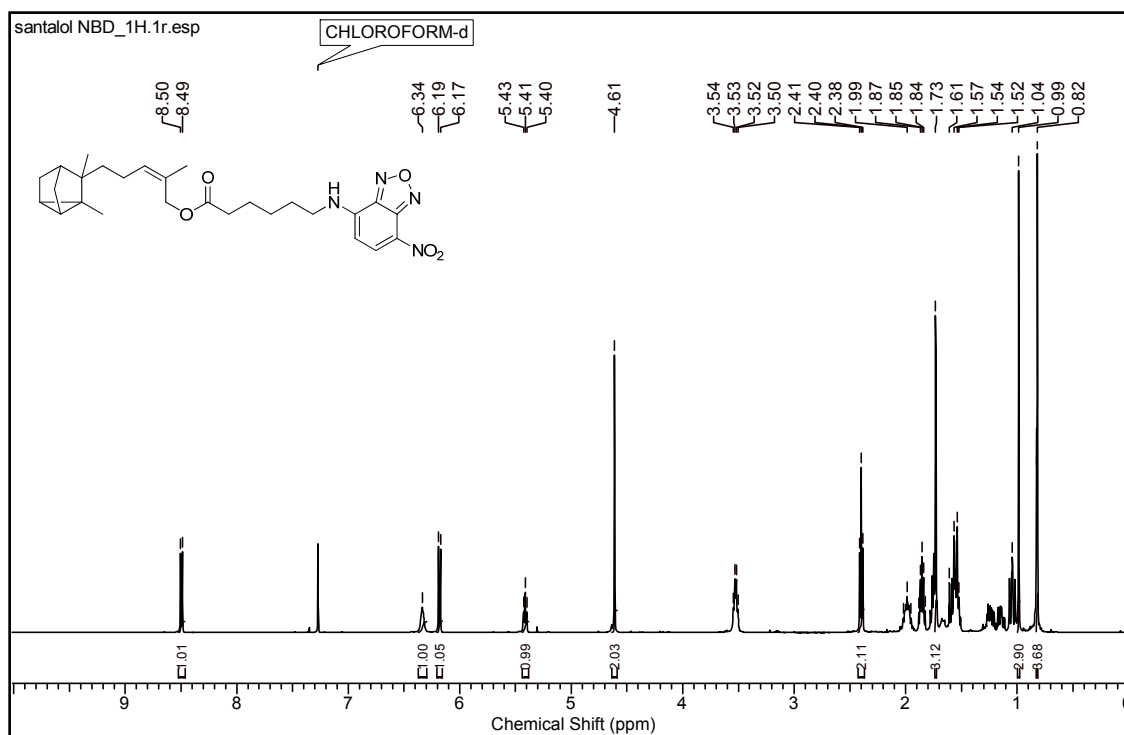


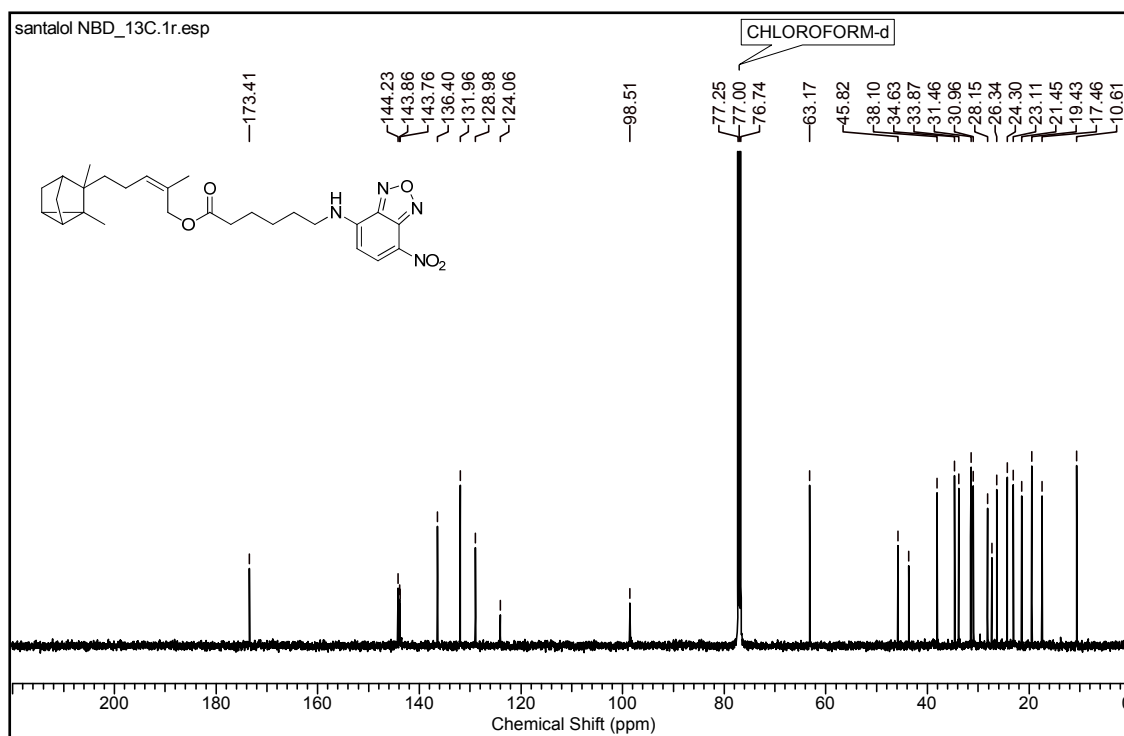
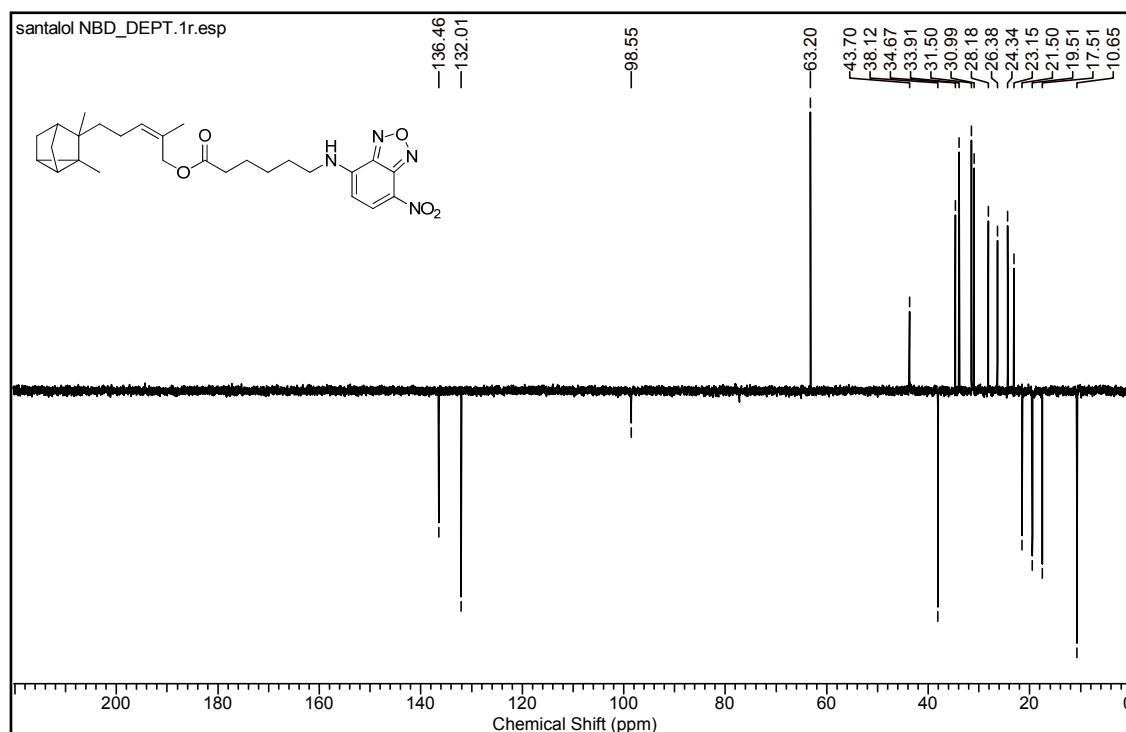
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **2a**

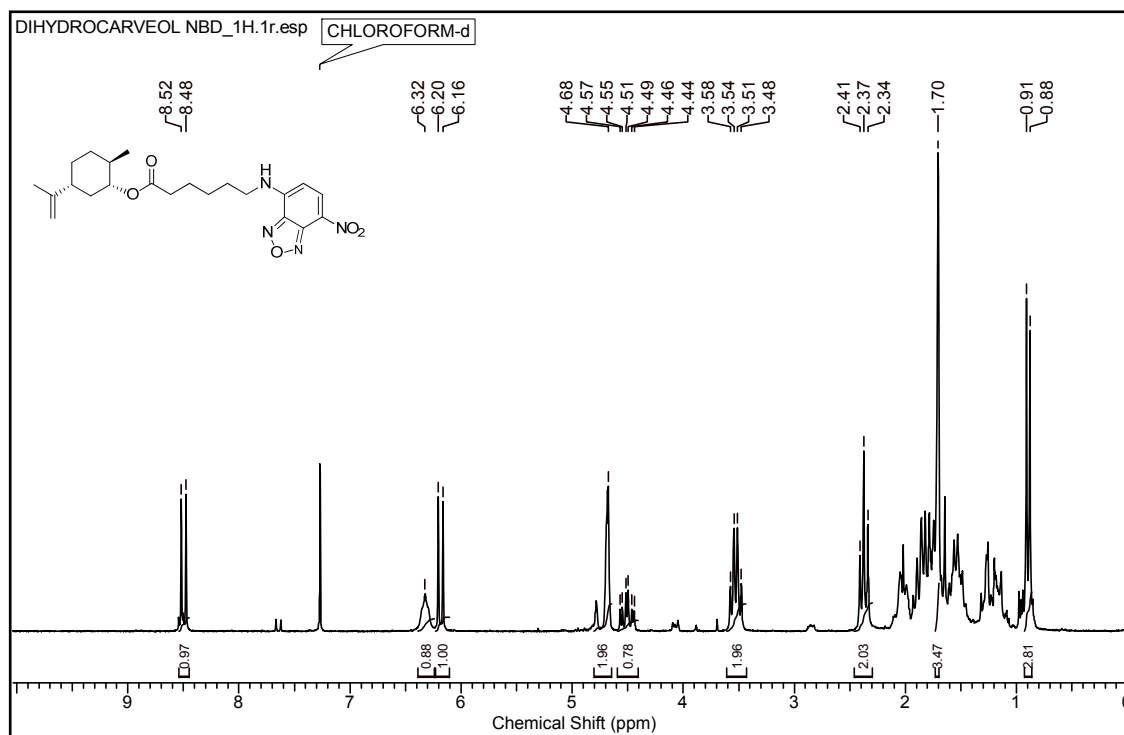


DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **2a**

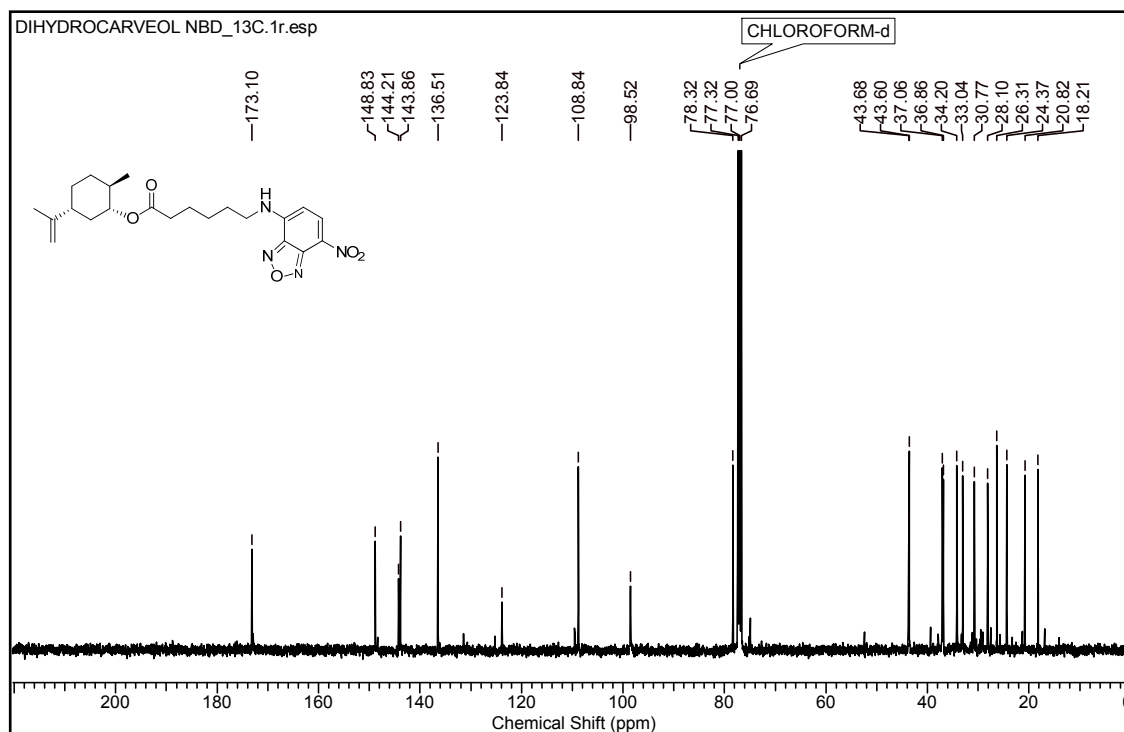
 ^1H NMR spectrum (CDCl_3 , 400 MHz) of **3a** ^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **3a**

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **3a** ^1H NMR spectrum (CDCl_3 , 400 MHz) of **4a**

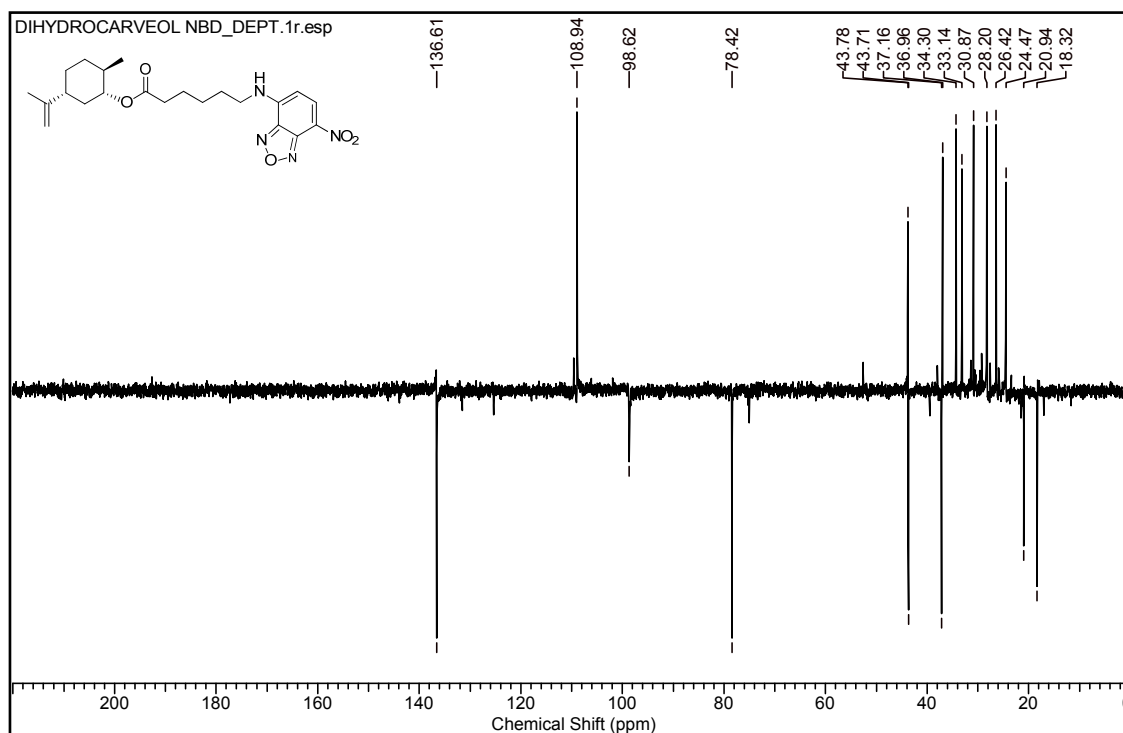
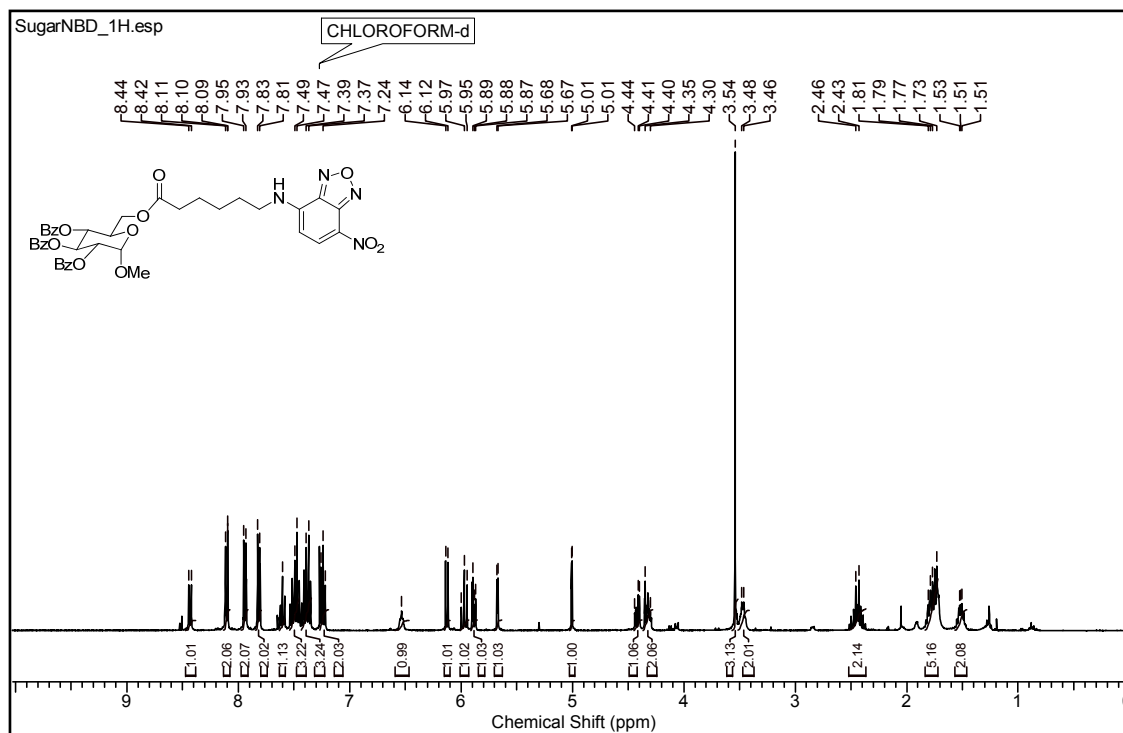
 ^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **4a**DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **4a**

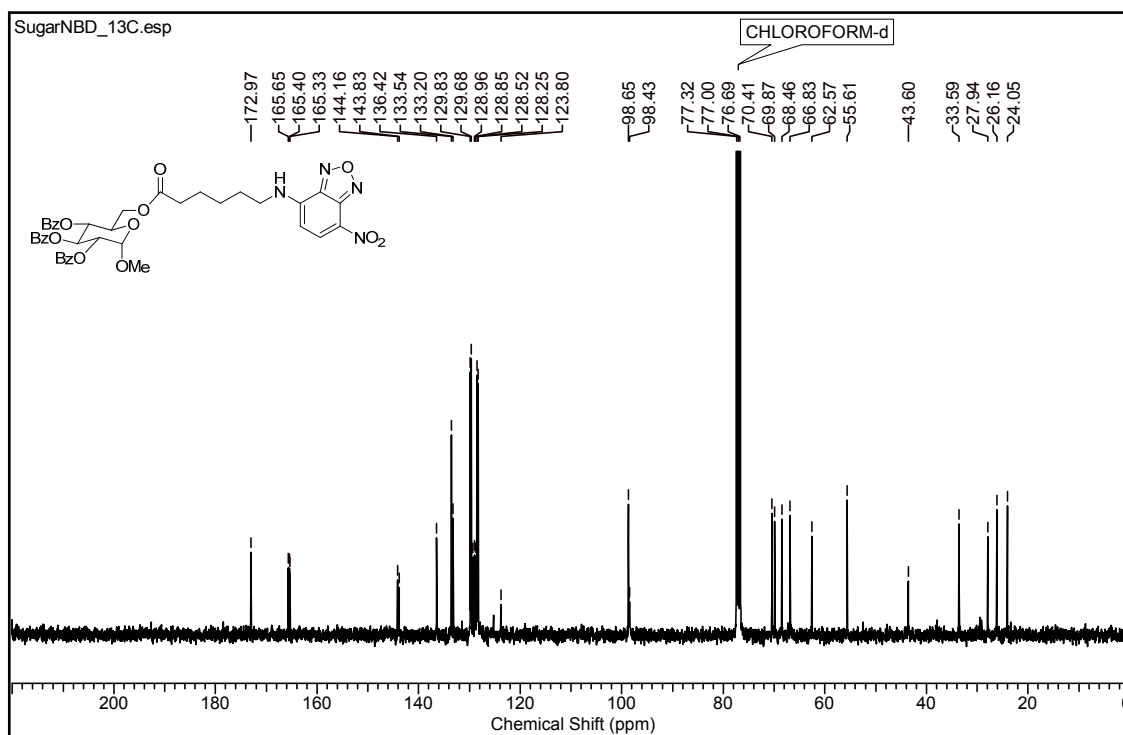


^1H NMR spectrum (CDCl_3 , 400 MHz) of **5a**

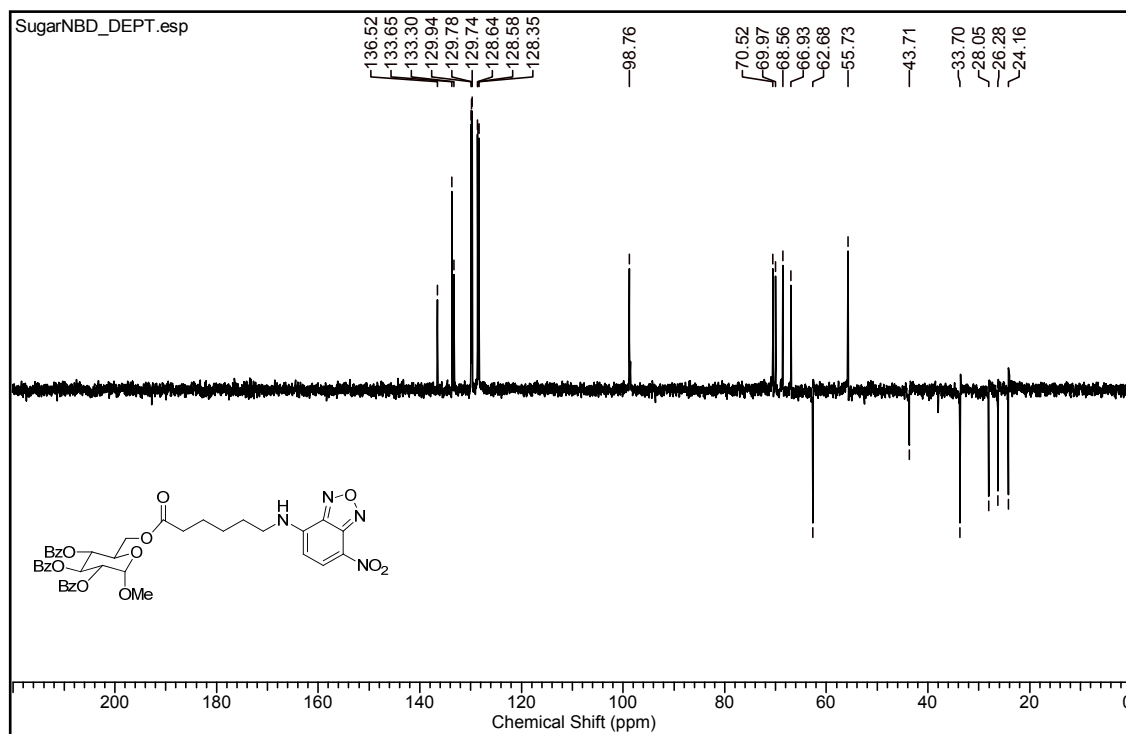


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **5a**

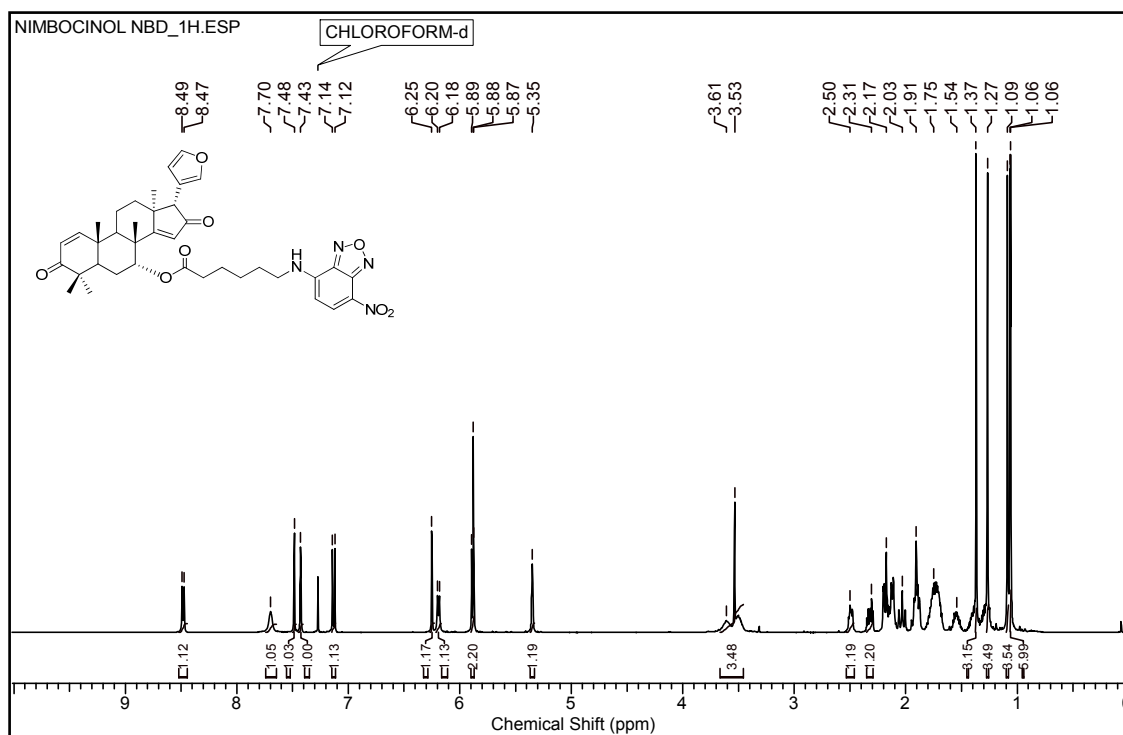
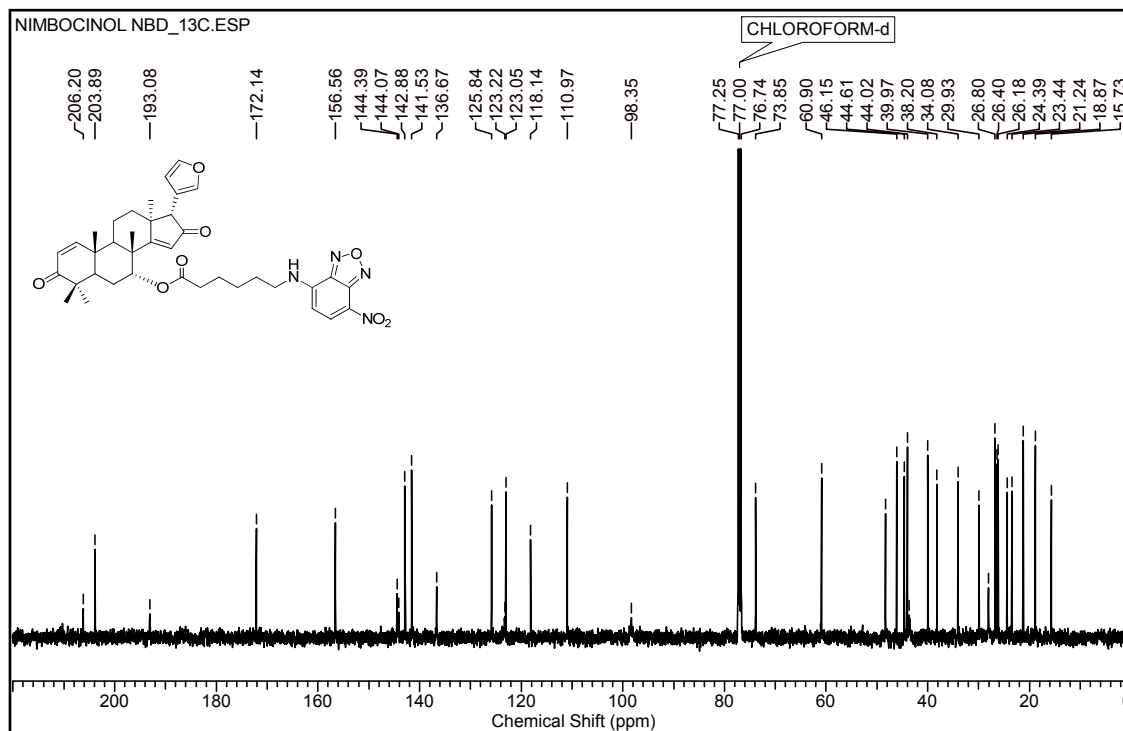
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of **5a**¹H NMR spectrum (CDCl₃, 400 MHz) of **6a**

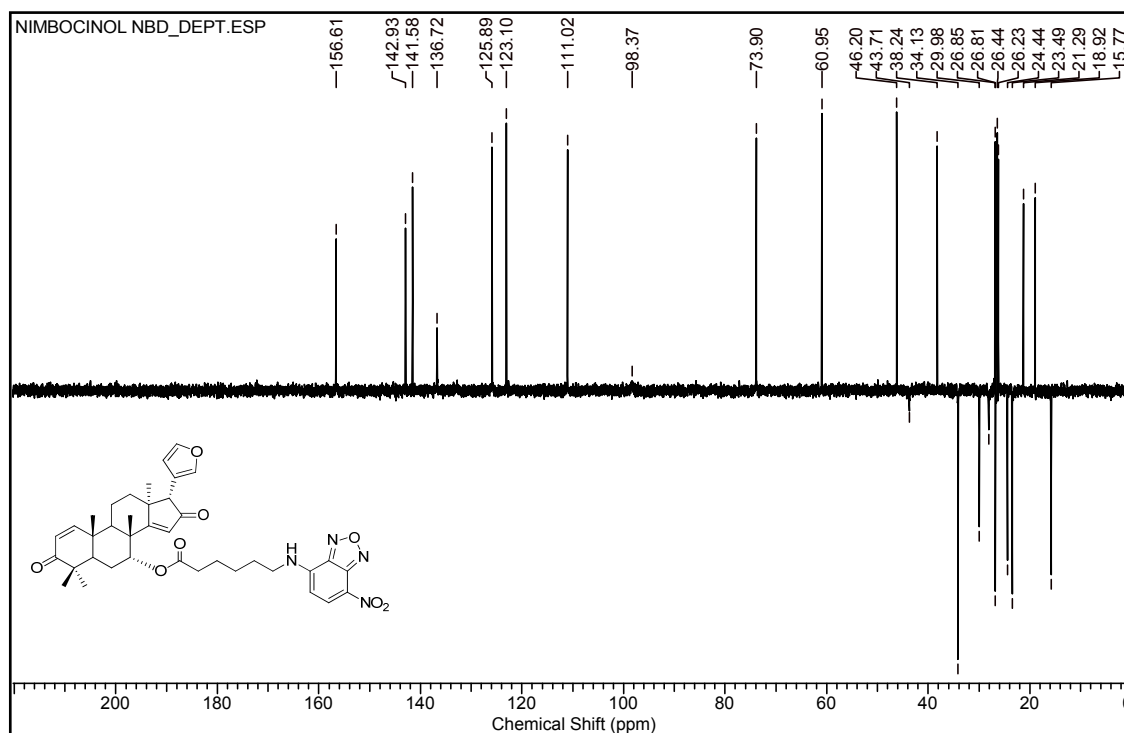
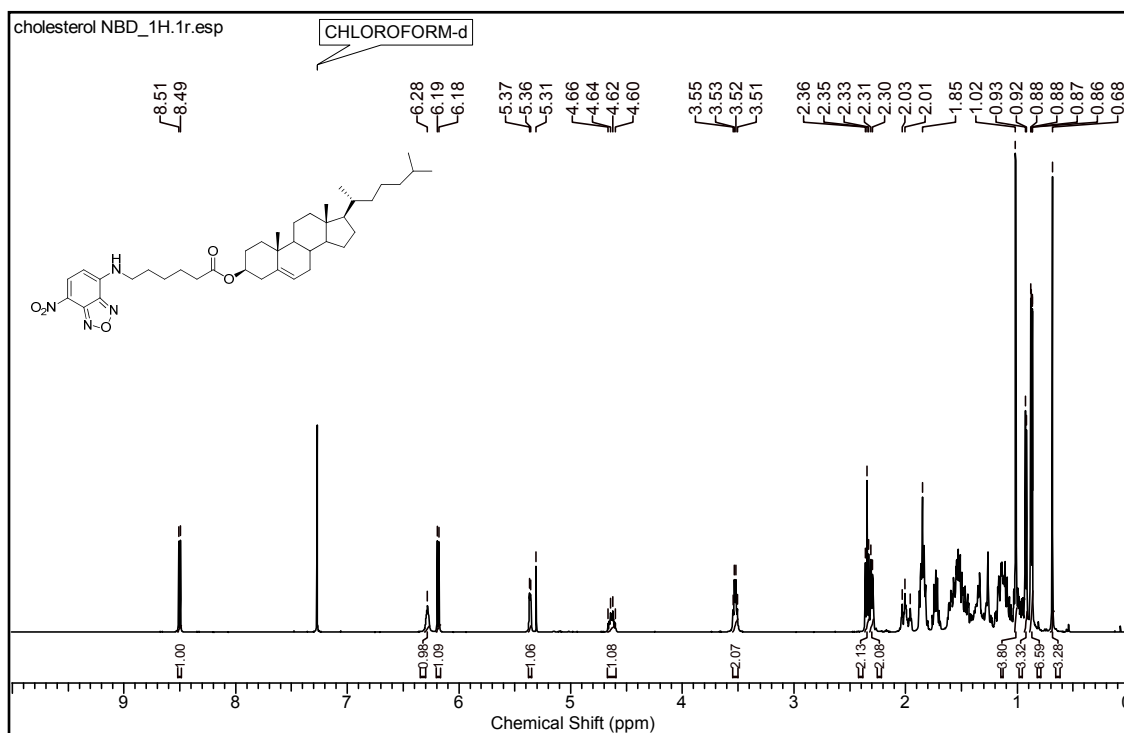


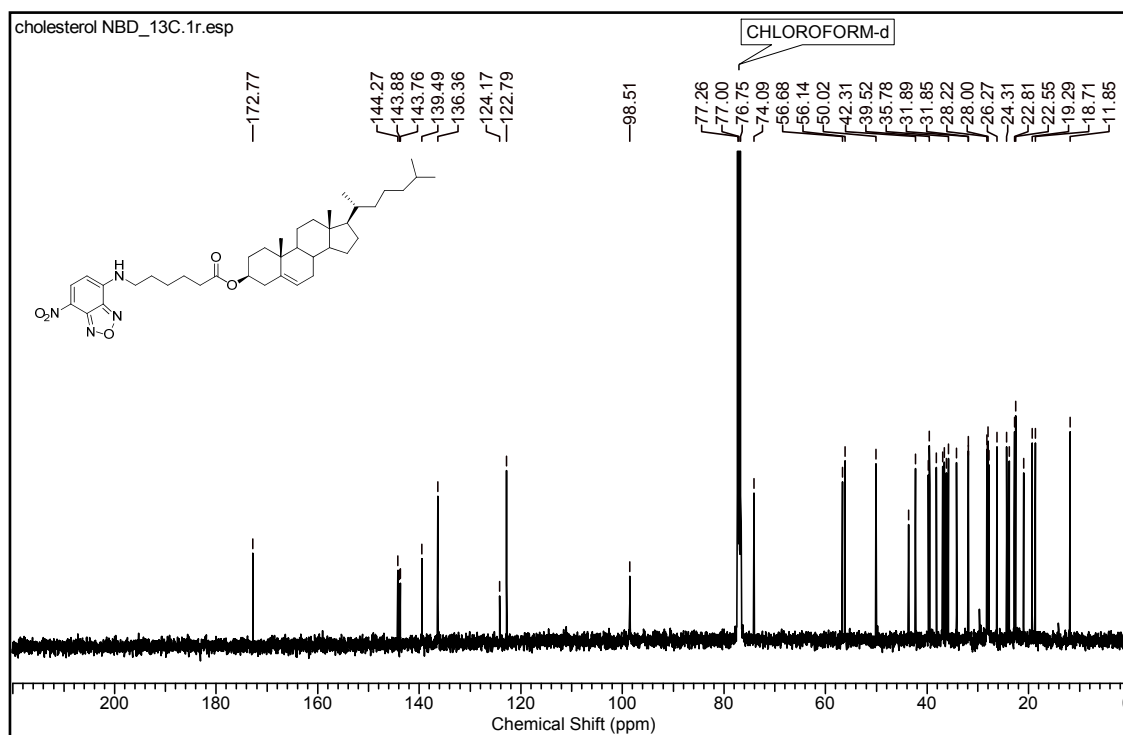
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **6a**



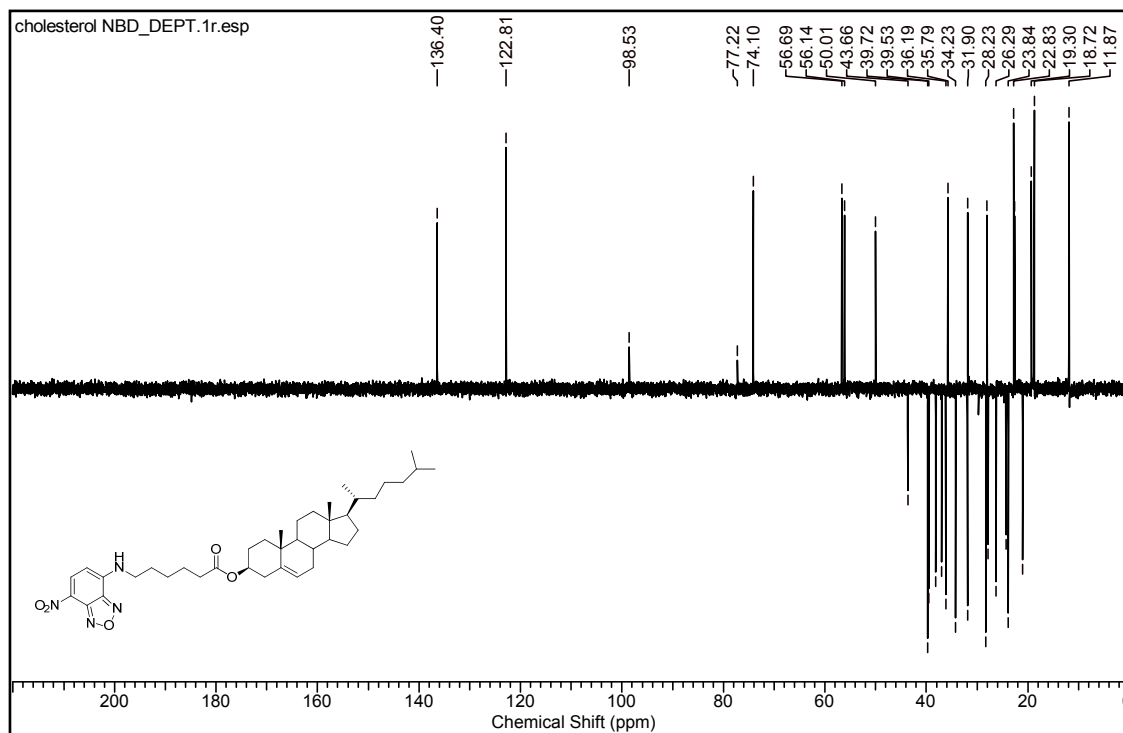
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **6a**

 ^1H NMR spectrum (CDCl_3 , 400 MHz) of **8a** ^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **8a**

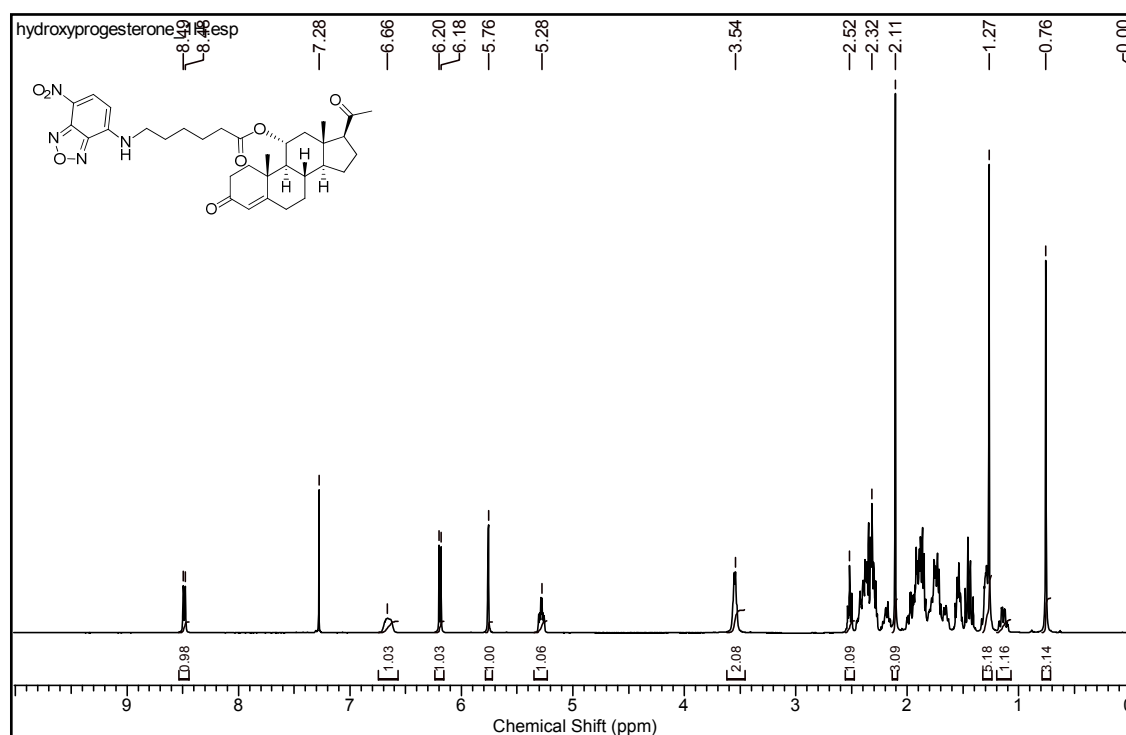
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **8a** ^1H NMR spectrum (CDCl_3 , 400 MHz) of **9a**



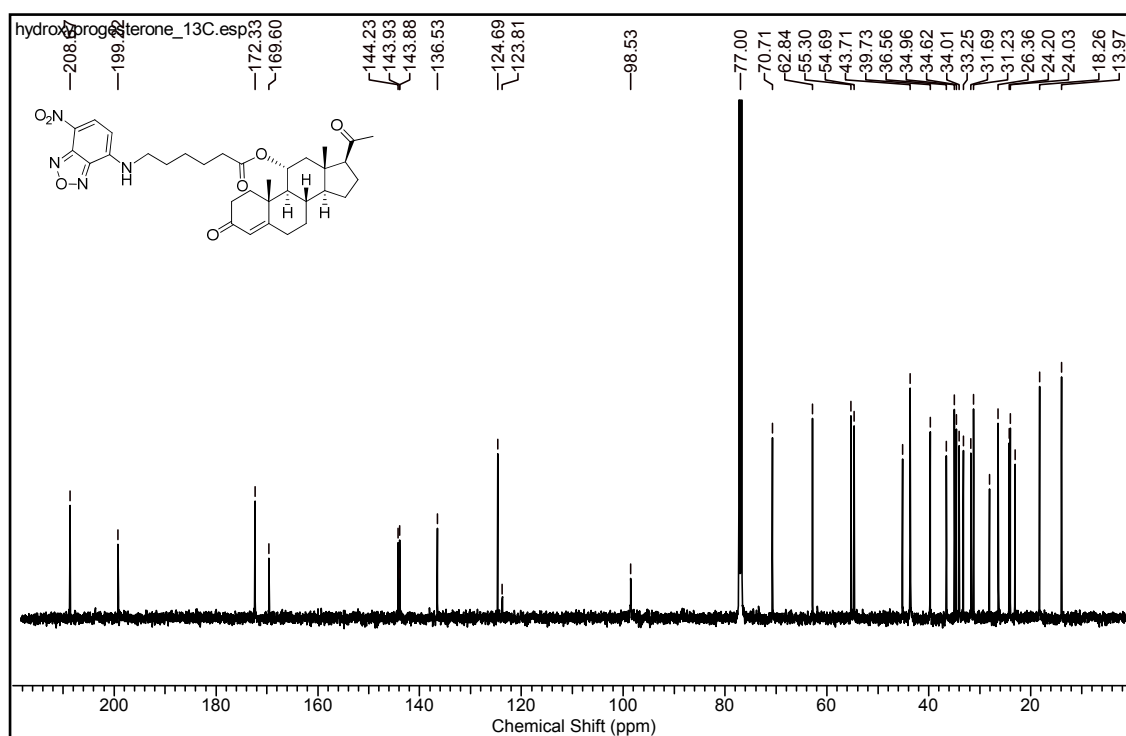
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **9a**



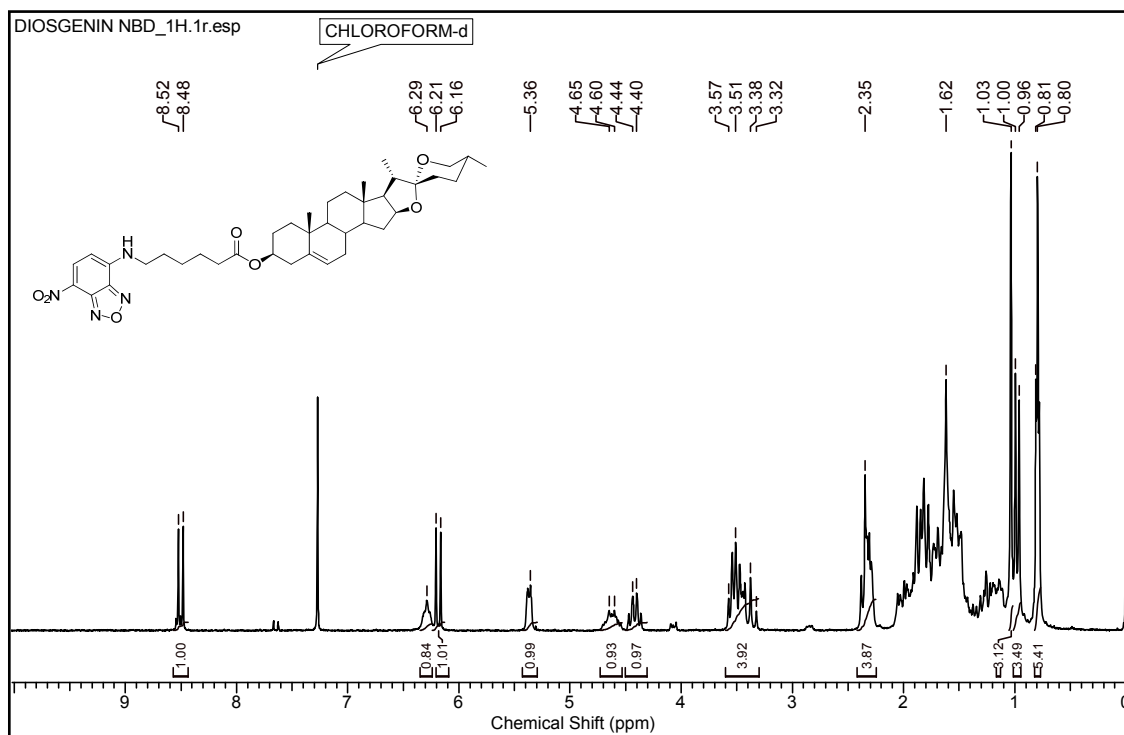
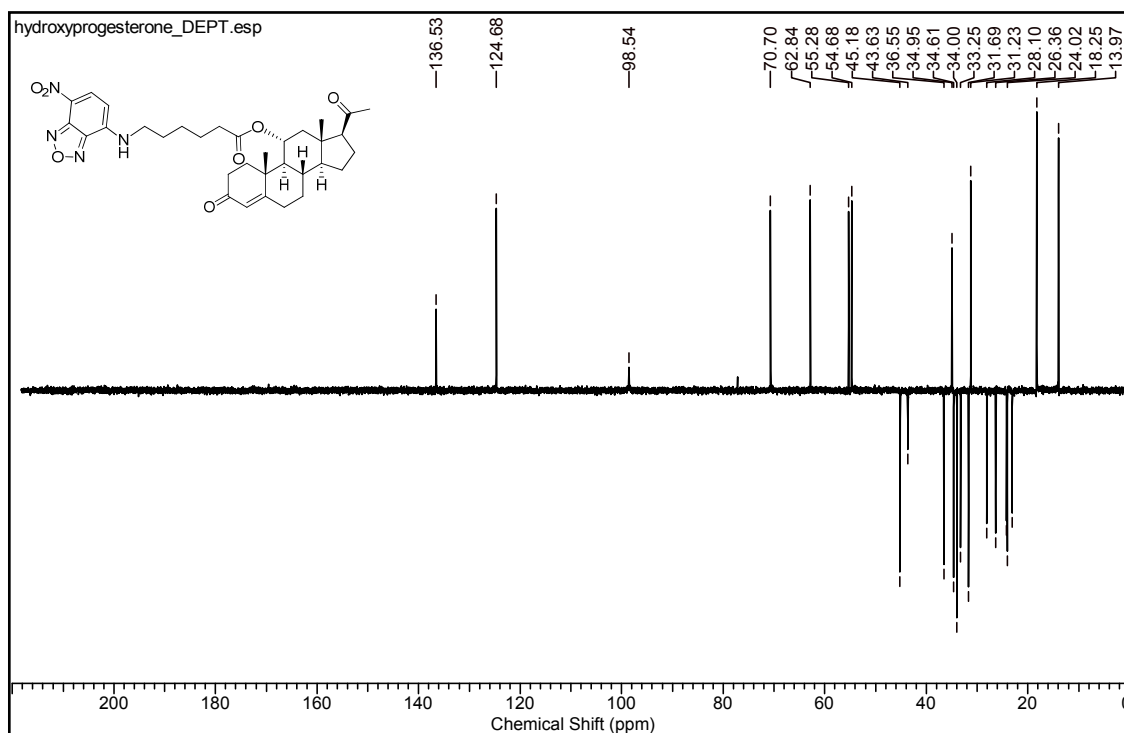
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **9a**

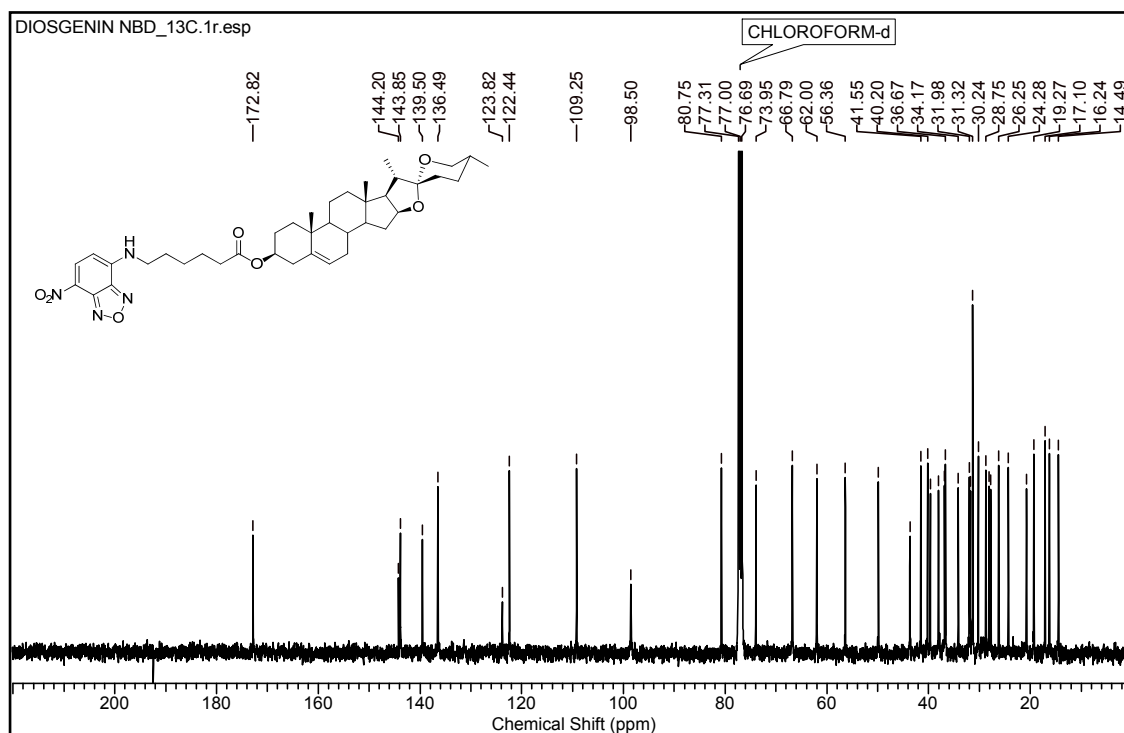


^1H NMR spectrum (CDCl_3 , 400 MHz) of **10a**

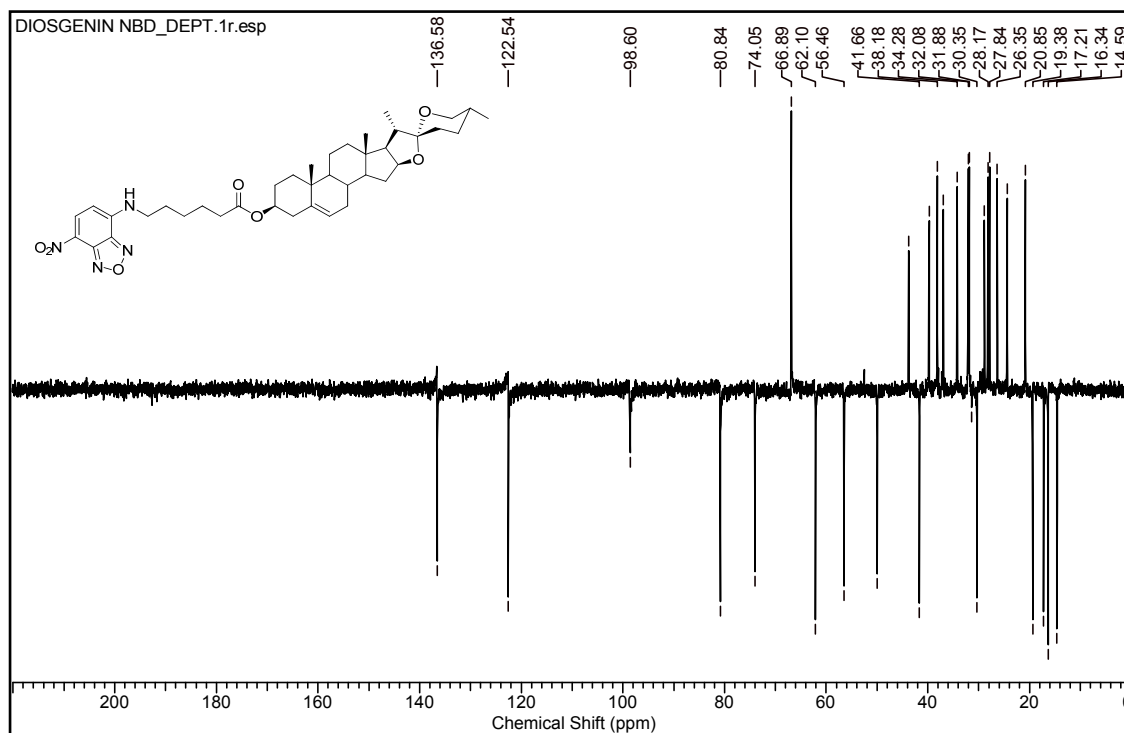


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **10a**

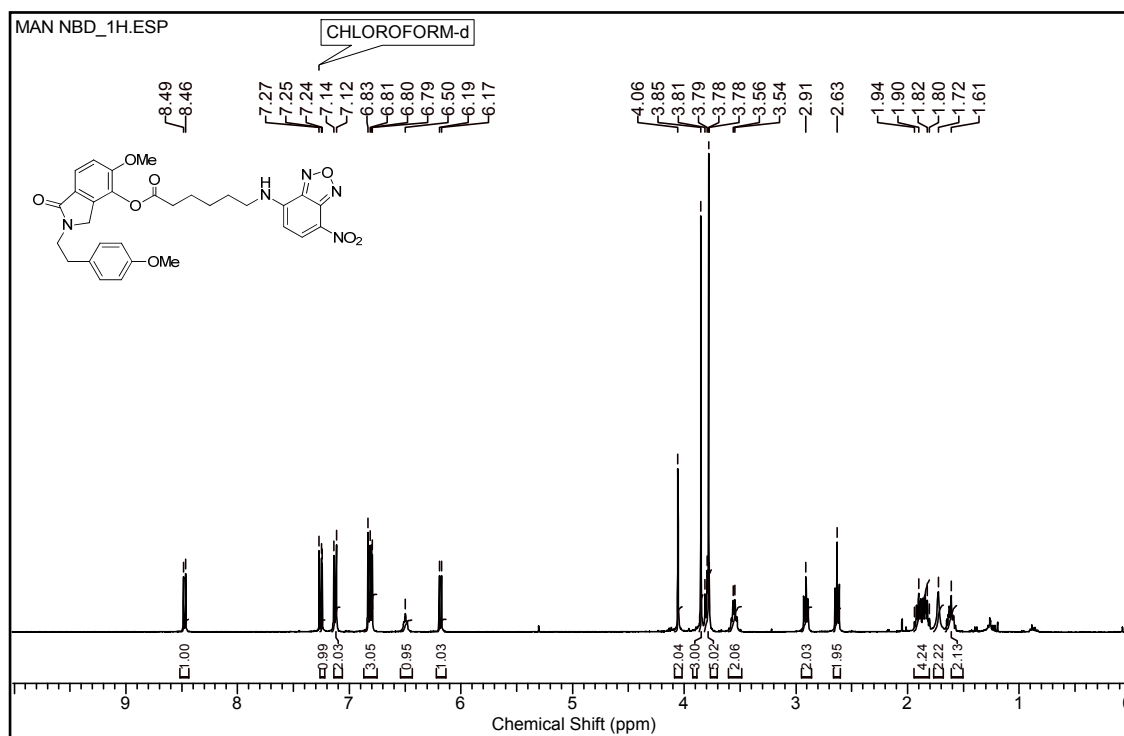




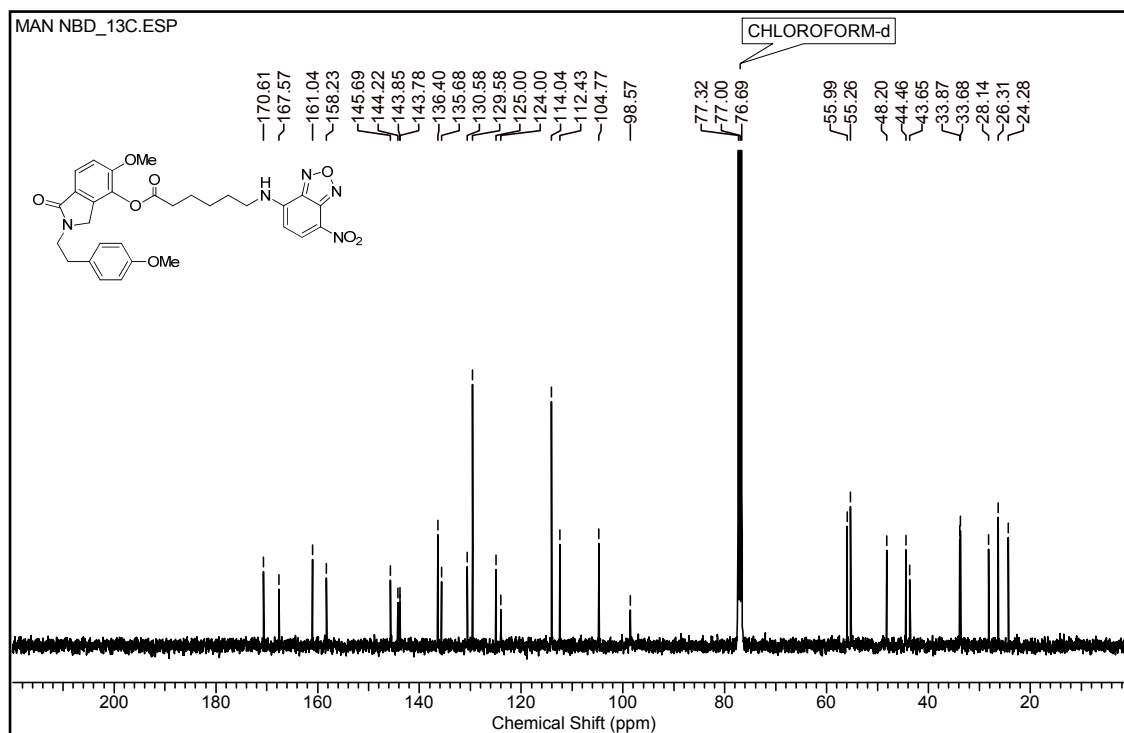
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **11a**



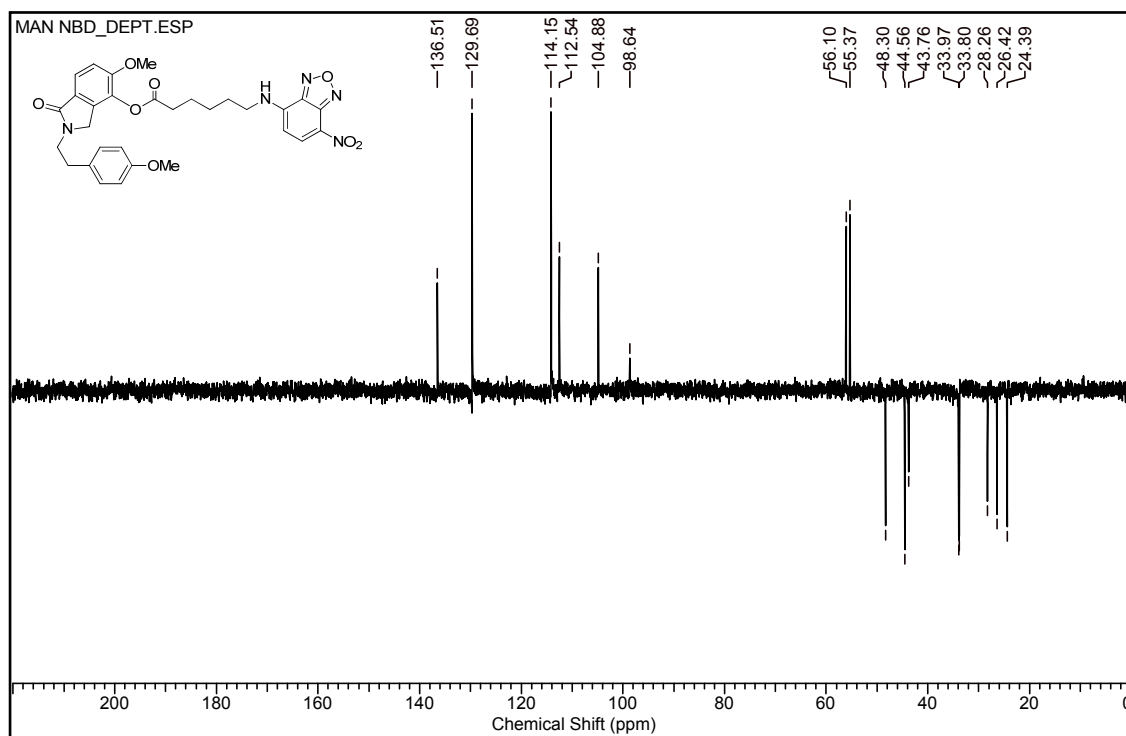
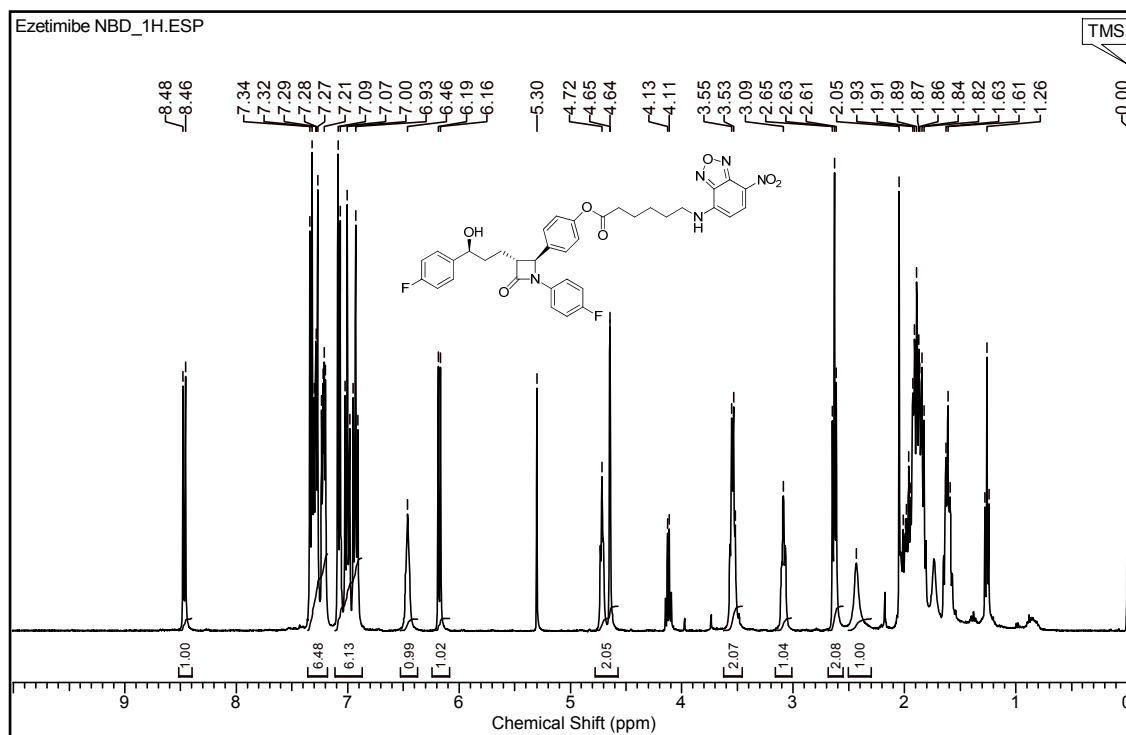
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **11a**

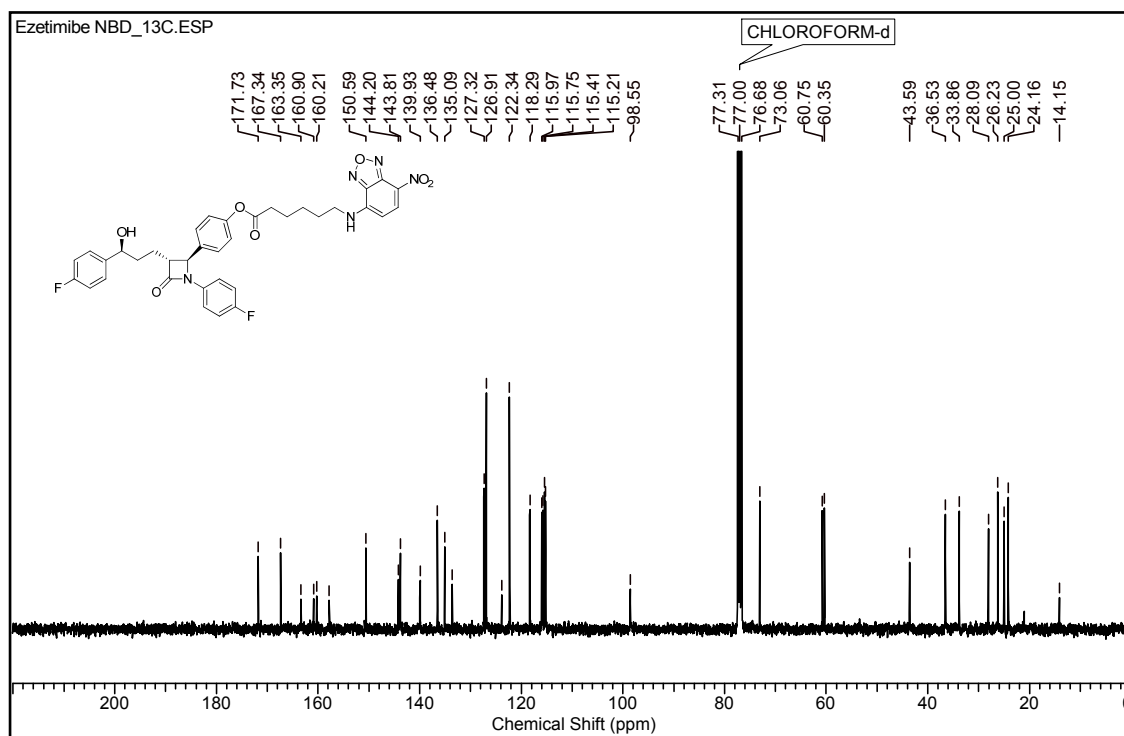


^1H NMR spectrum (CDCl_3 , 400 MHz) of **12a**

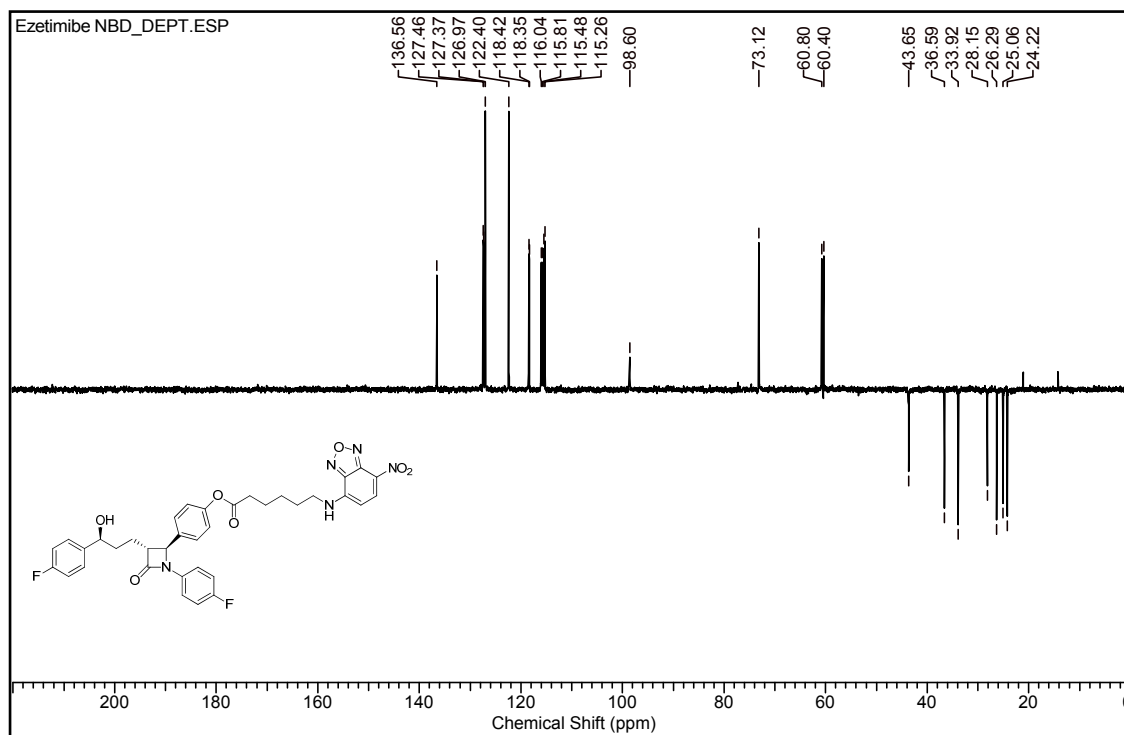


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **12a**

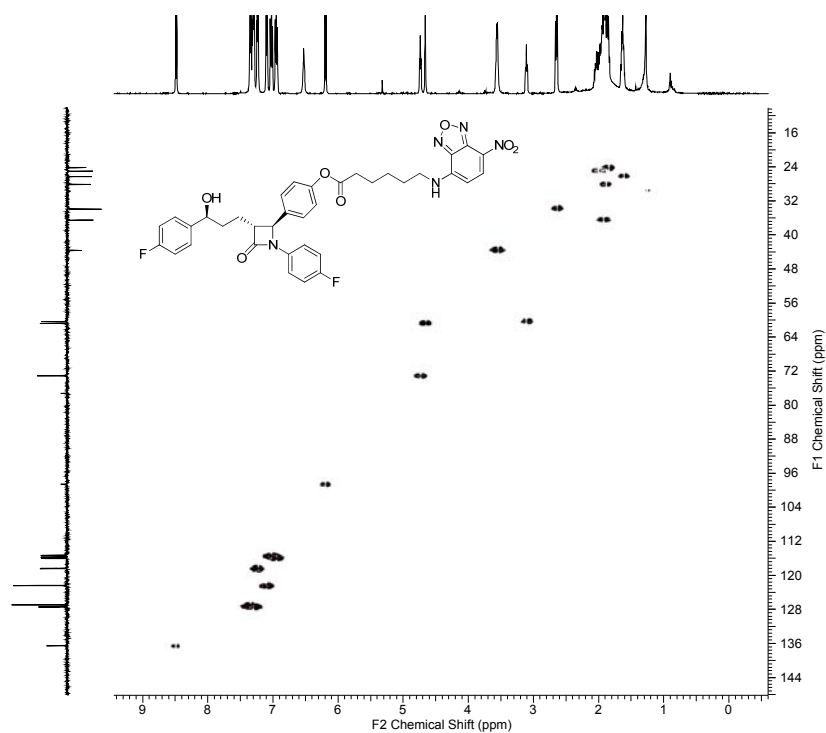
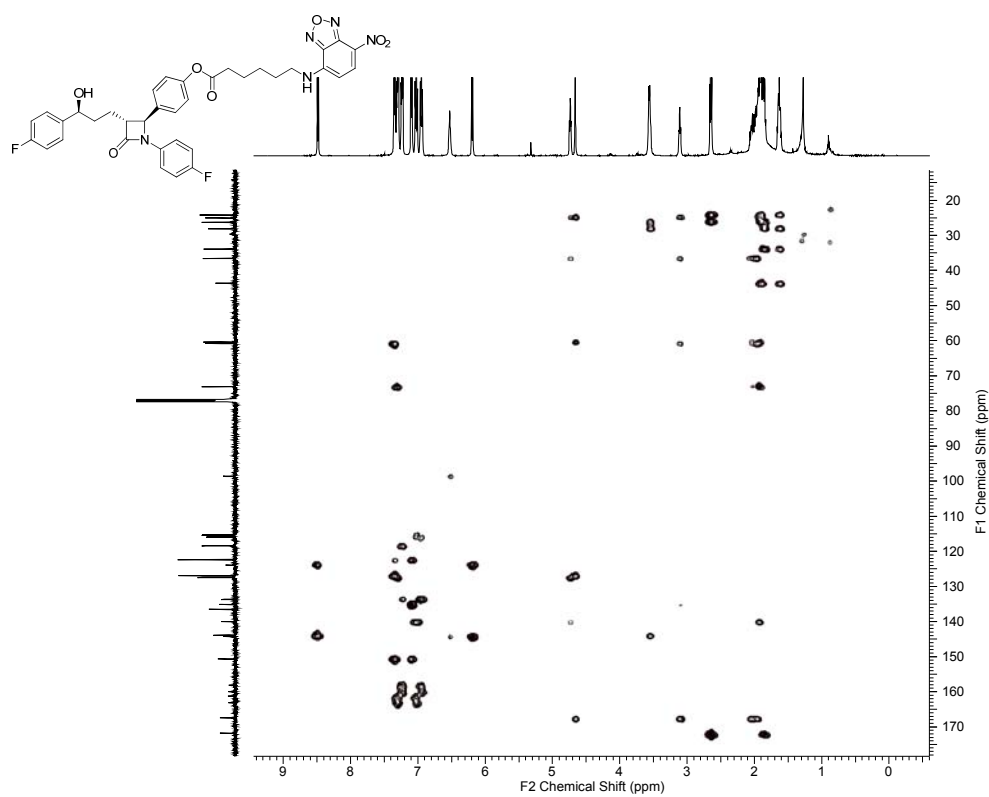
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of **12a**¹H NMR spectrum (CDCl₃, 400 MHz) of **13a**

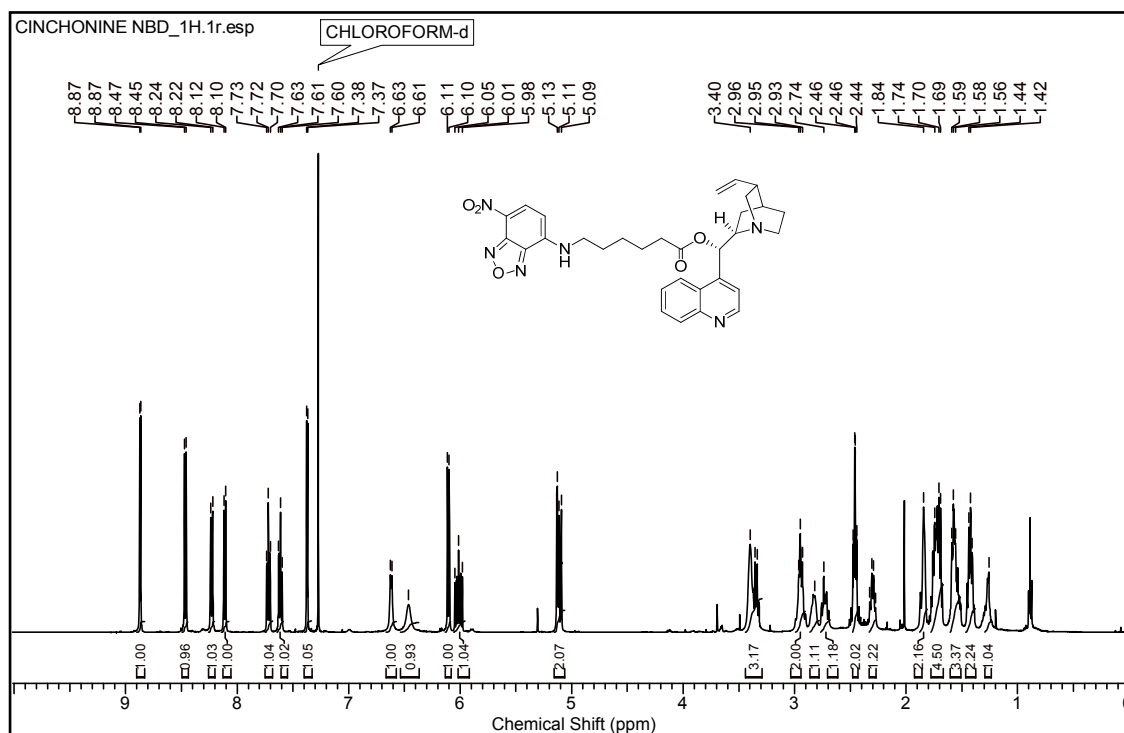


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **13a**

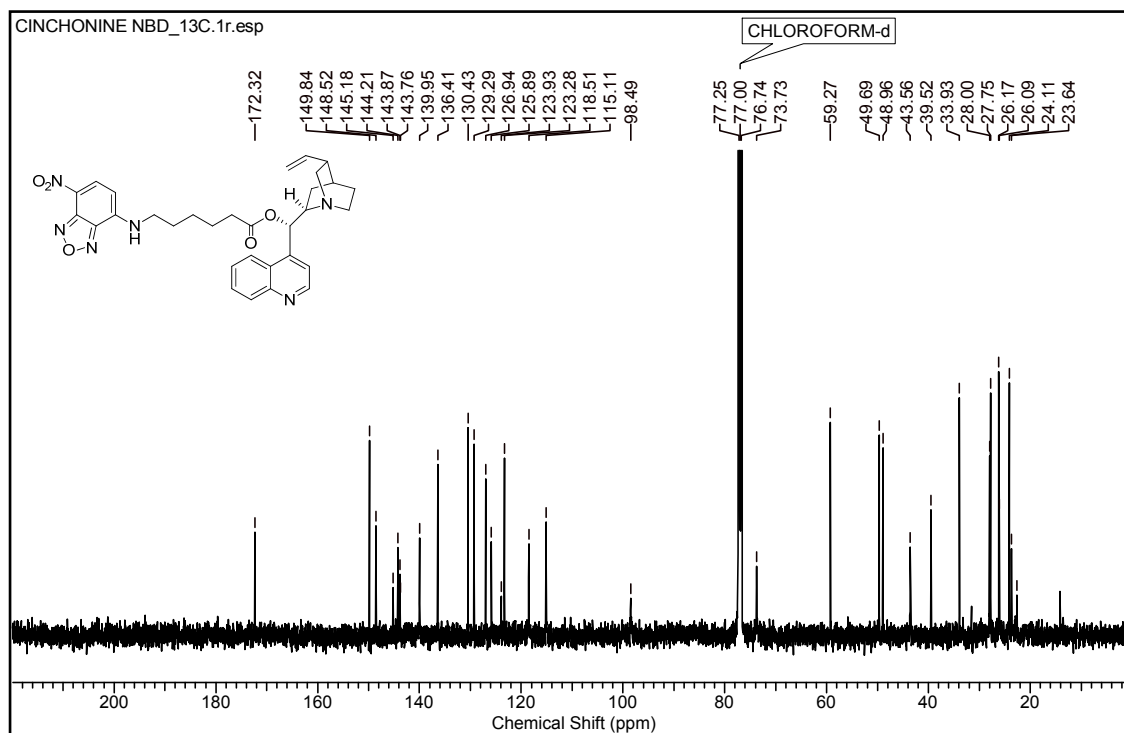


DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **13a**

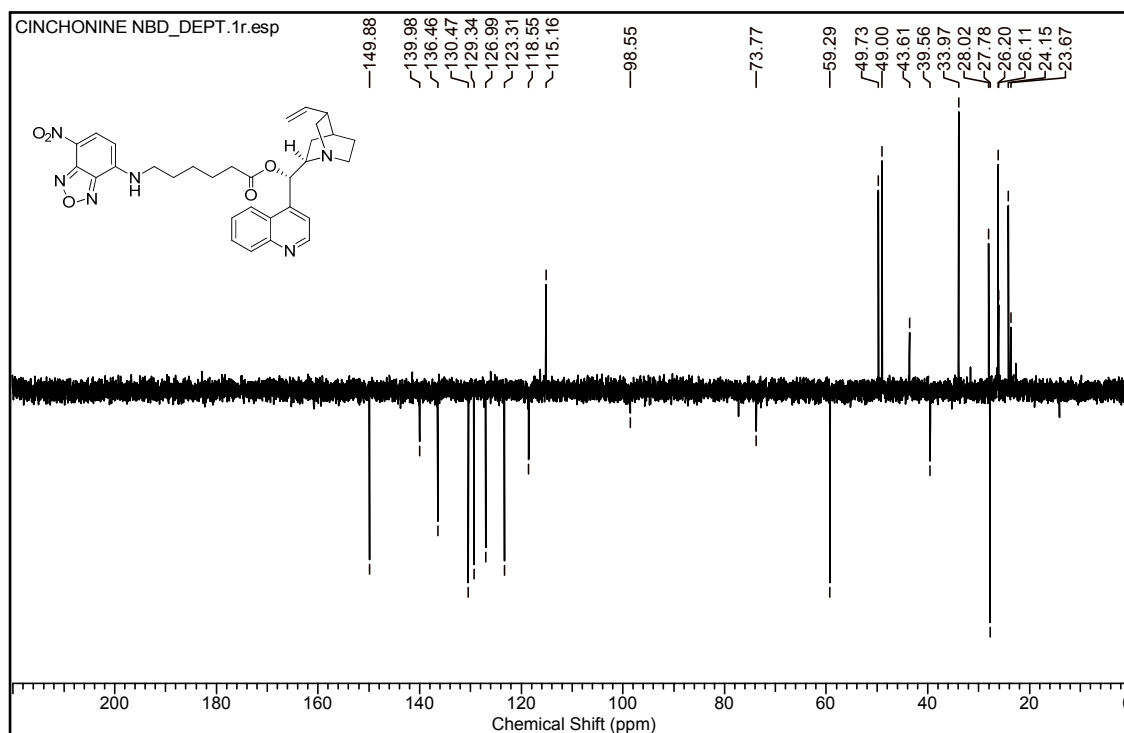
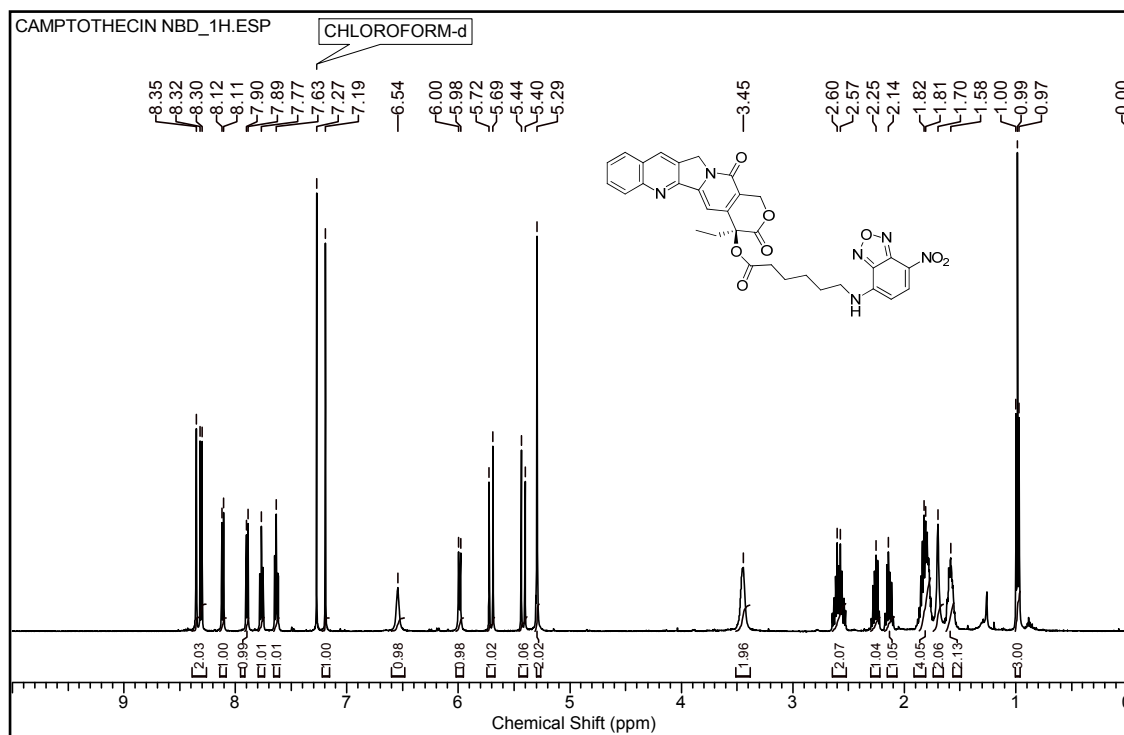
HSQC spectrum of **13a**HMBC spectrum of **13a**

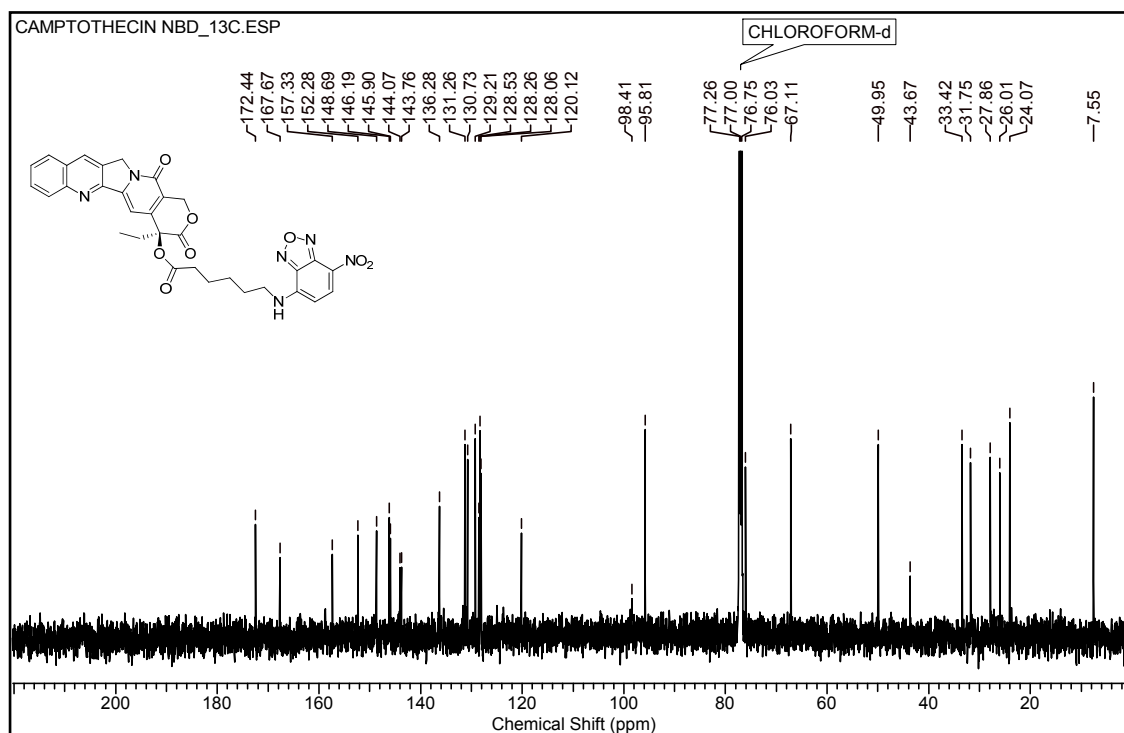


^1H NMR spectrum (CDCl_3 , 400 MHz) of **14a**

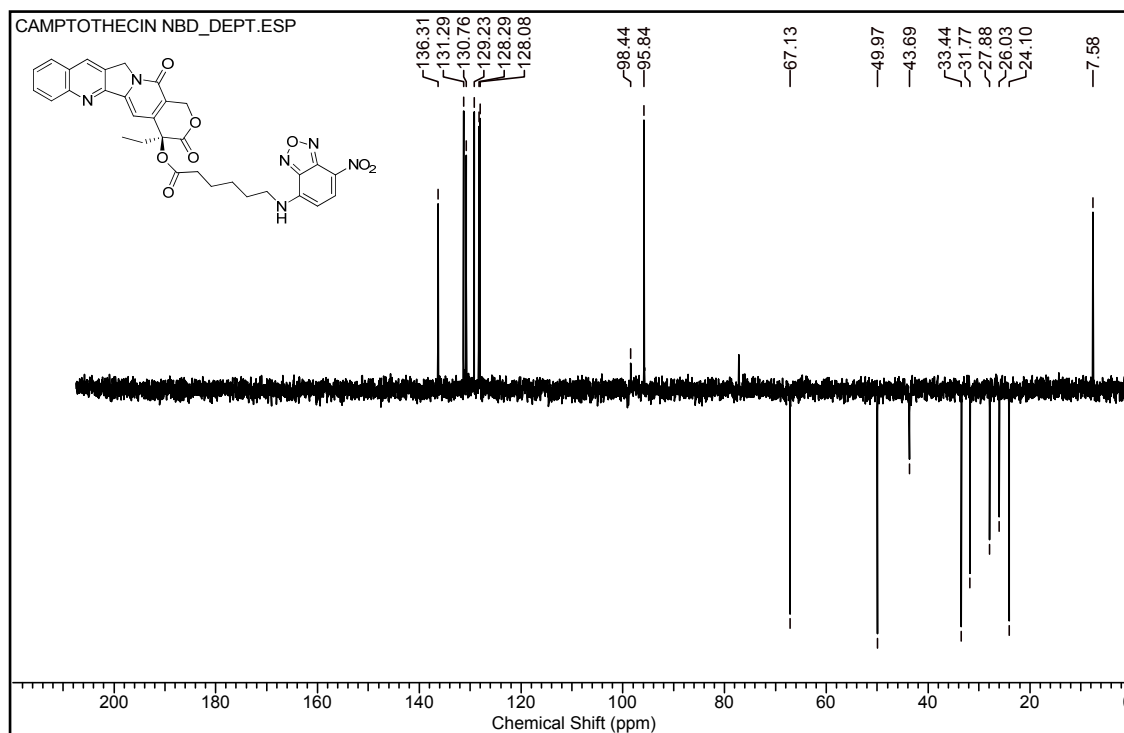


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **14a**

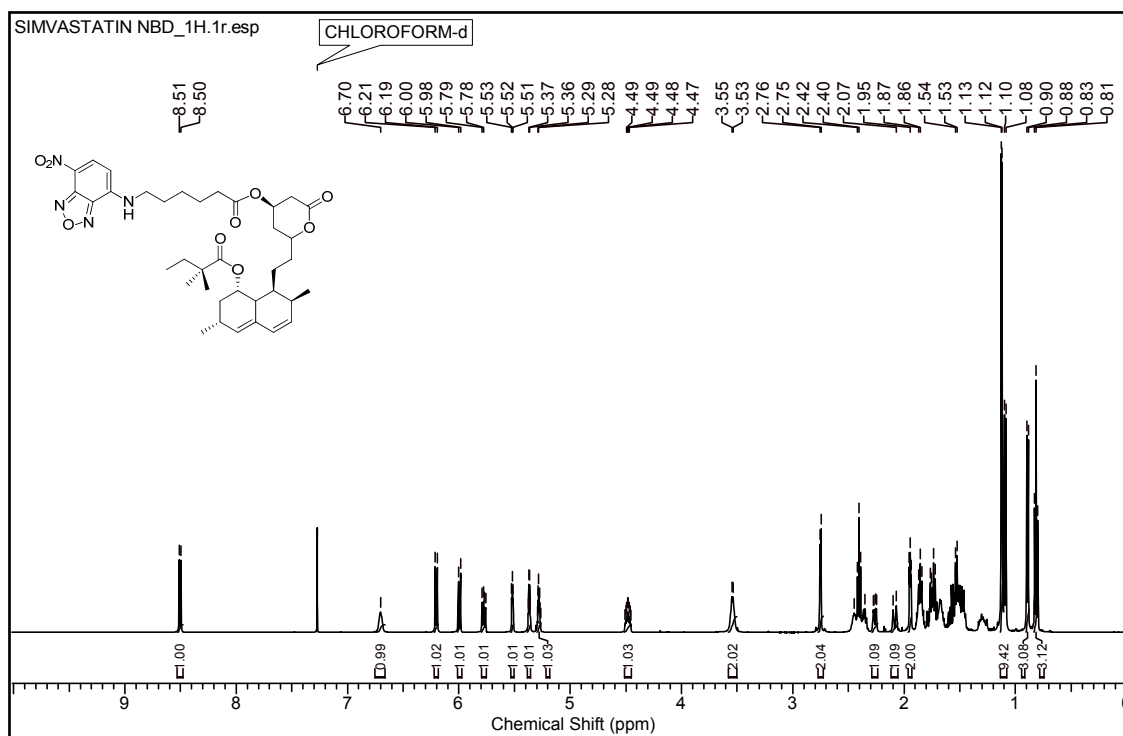
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of **14a**¹H NMR spectrum (CDCl₃, 400 MHz) of **15a**



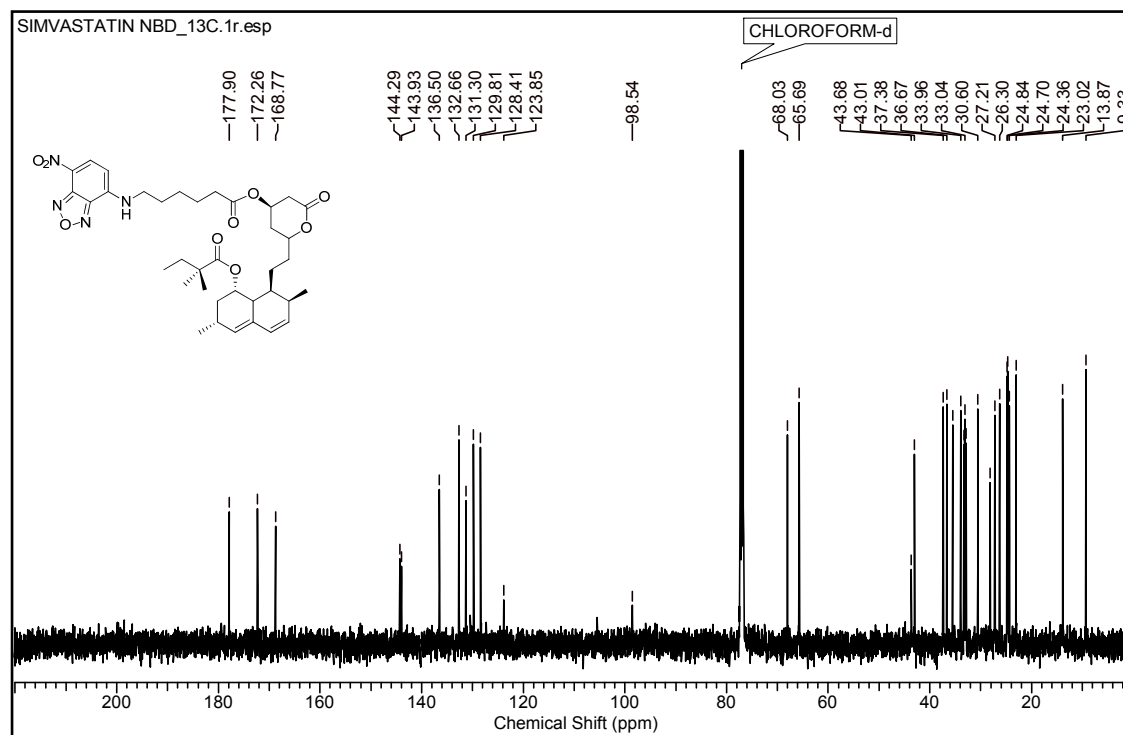
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **15a**



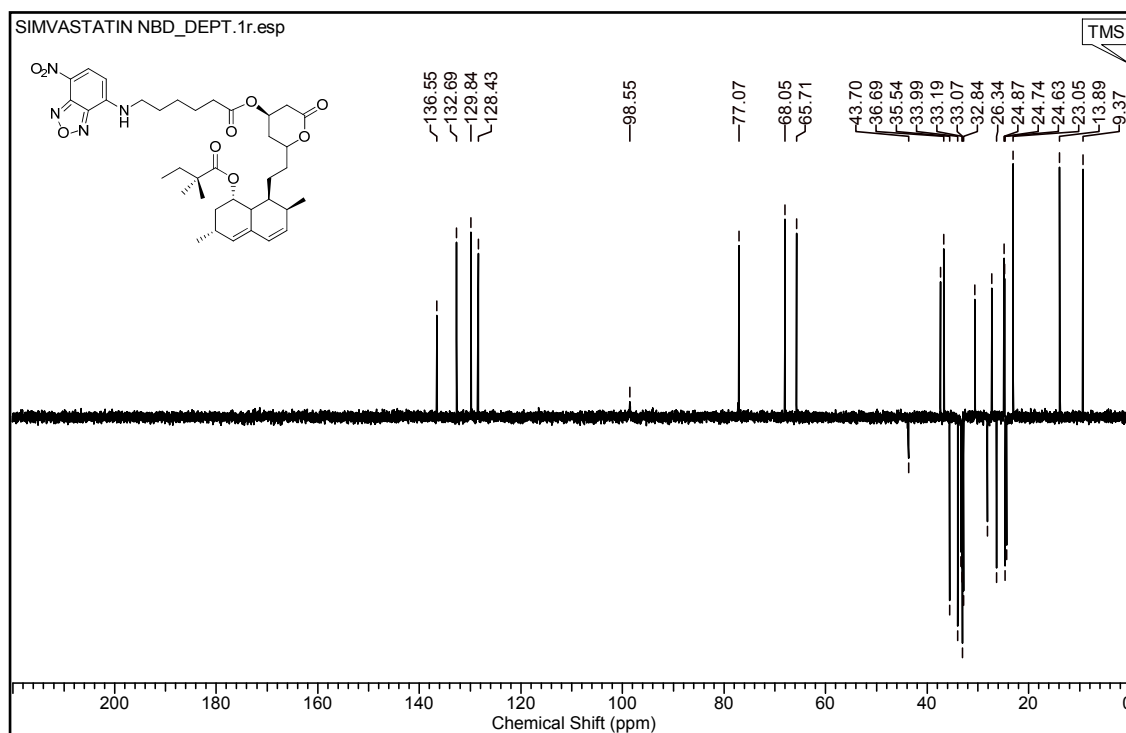
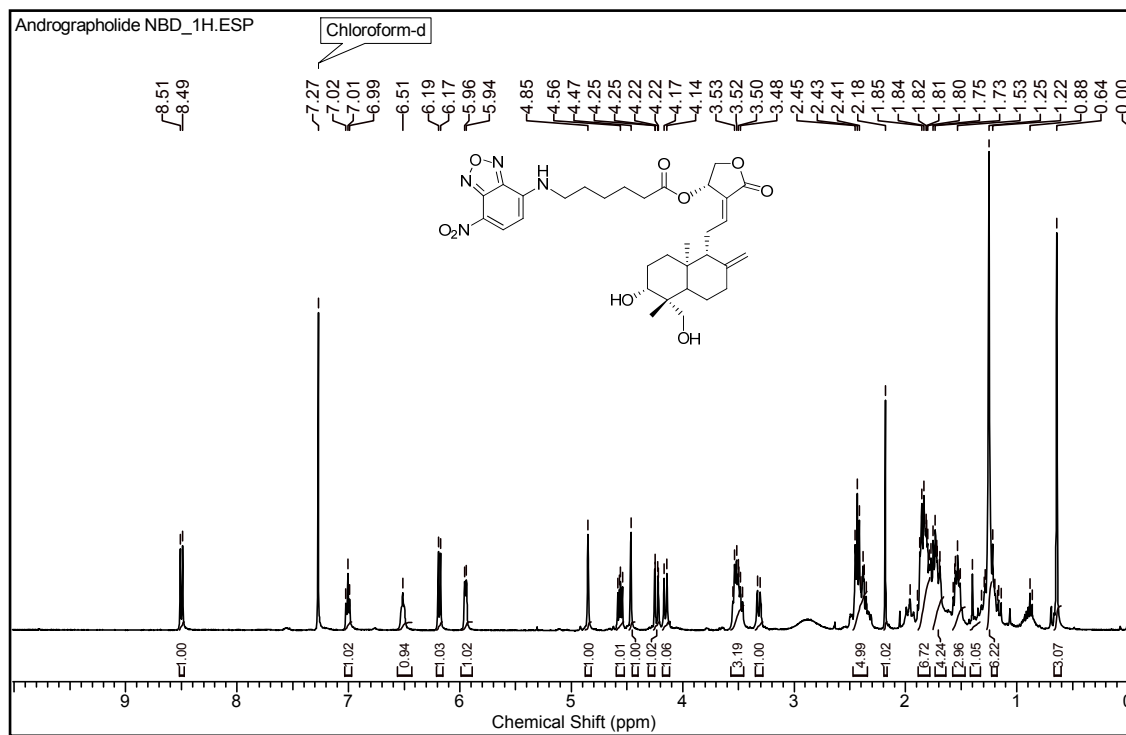
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **15a**

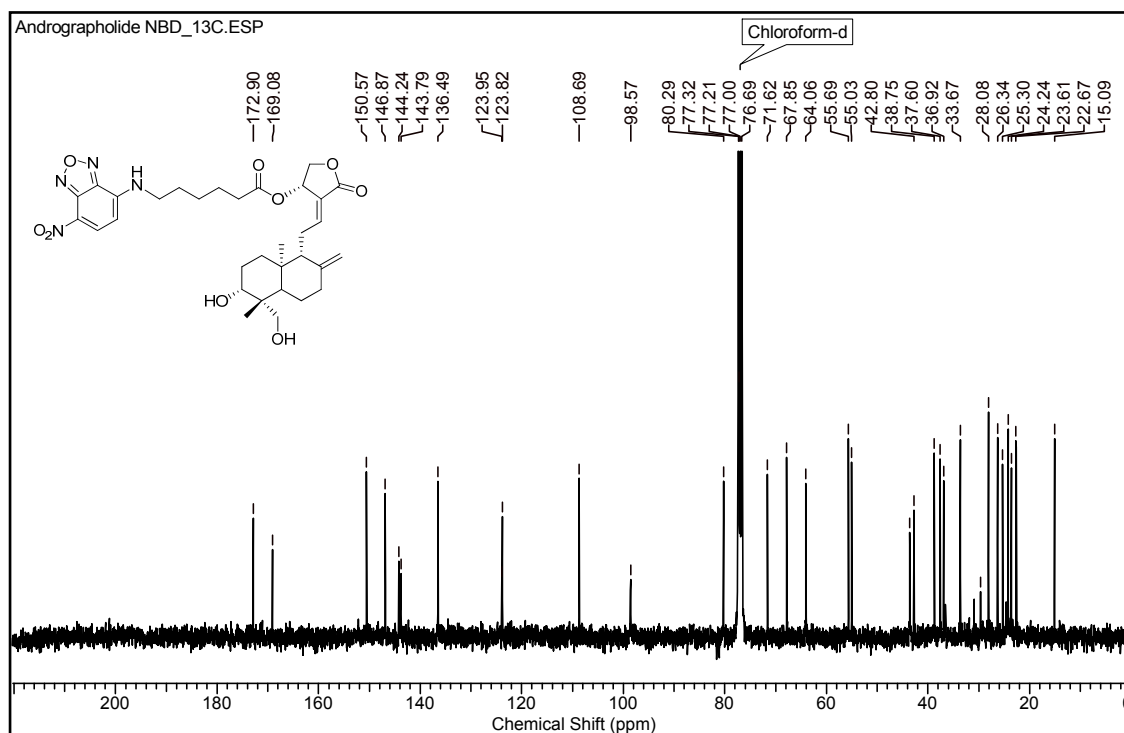


^1H NMR spectrum (CDCl_3 , 400 MHz) of **16a**

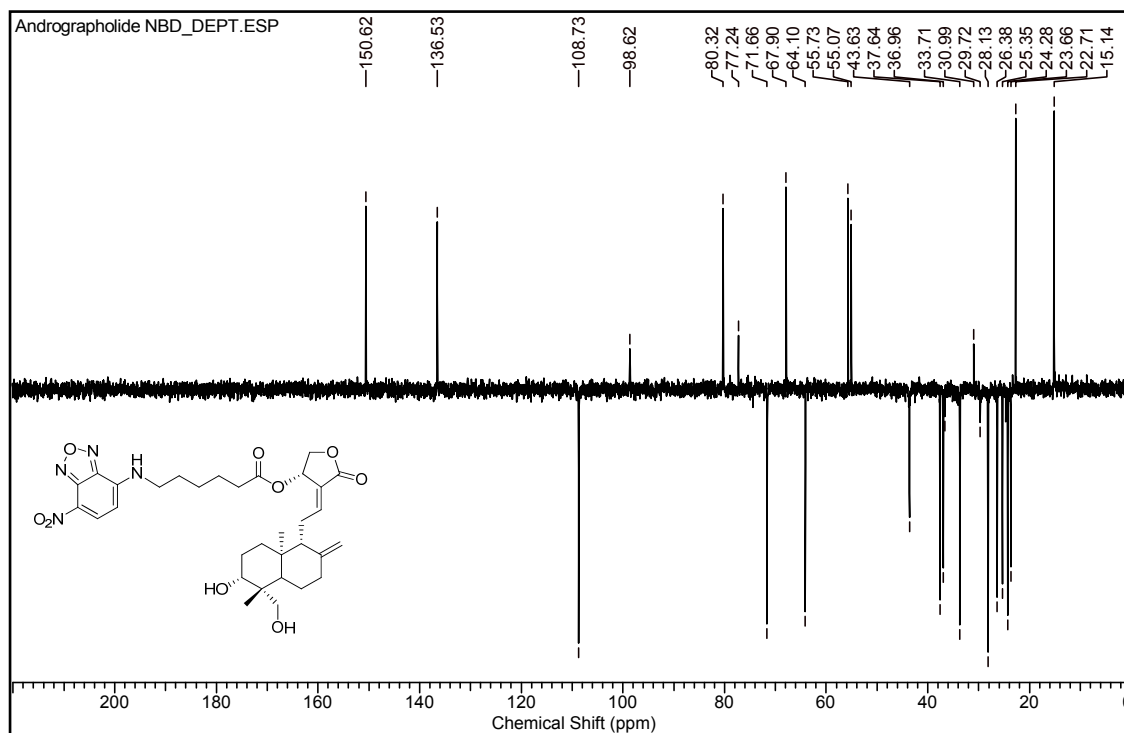


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **16a**

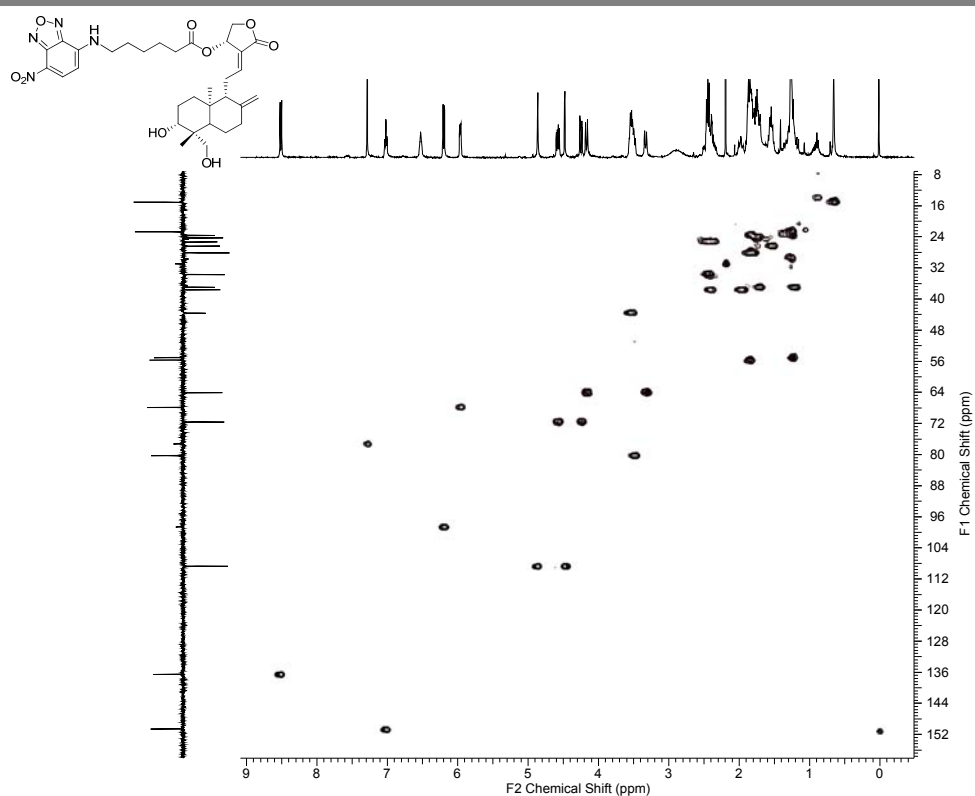
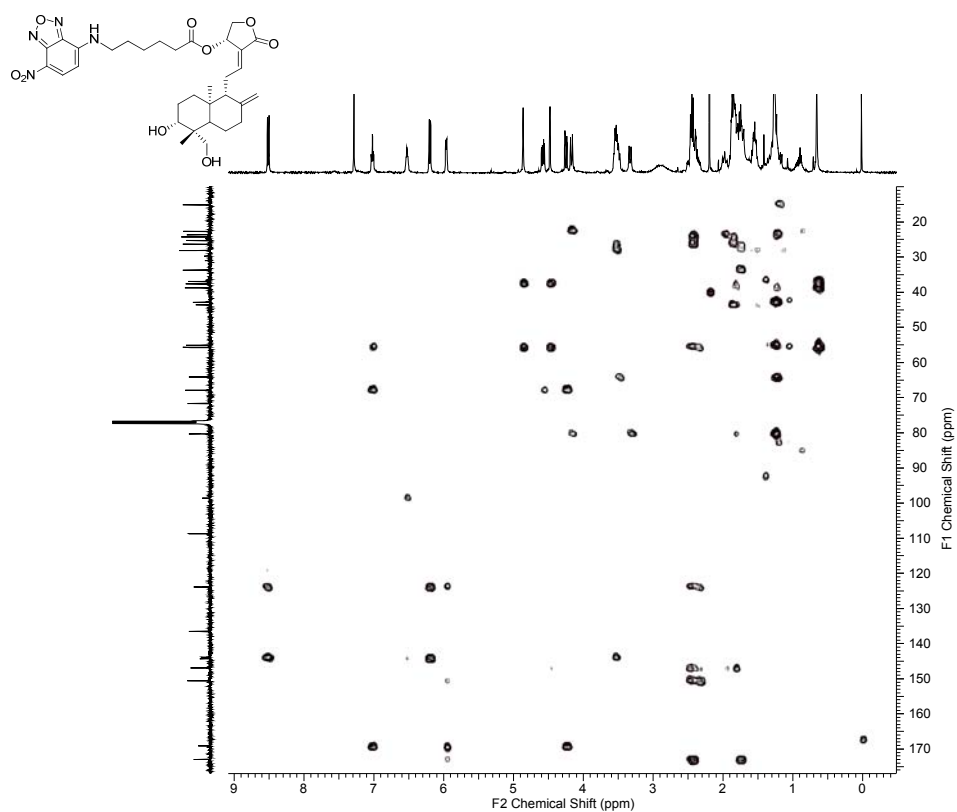
DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of **16a**¹H NMR spectrum (CDCl₃, 400 MHz) of **17a**

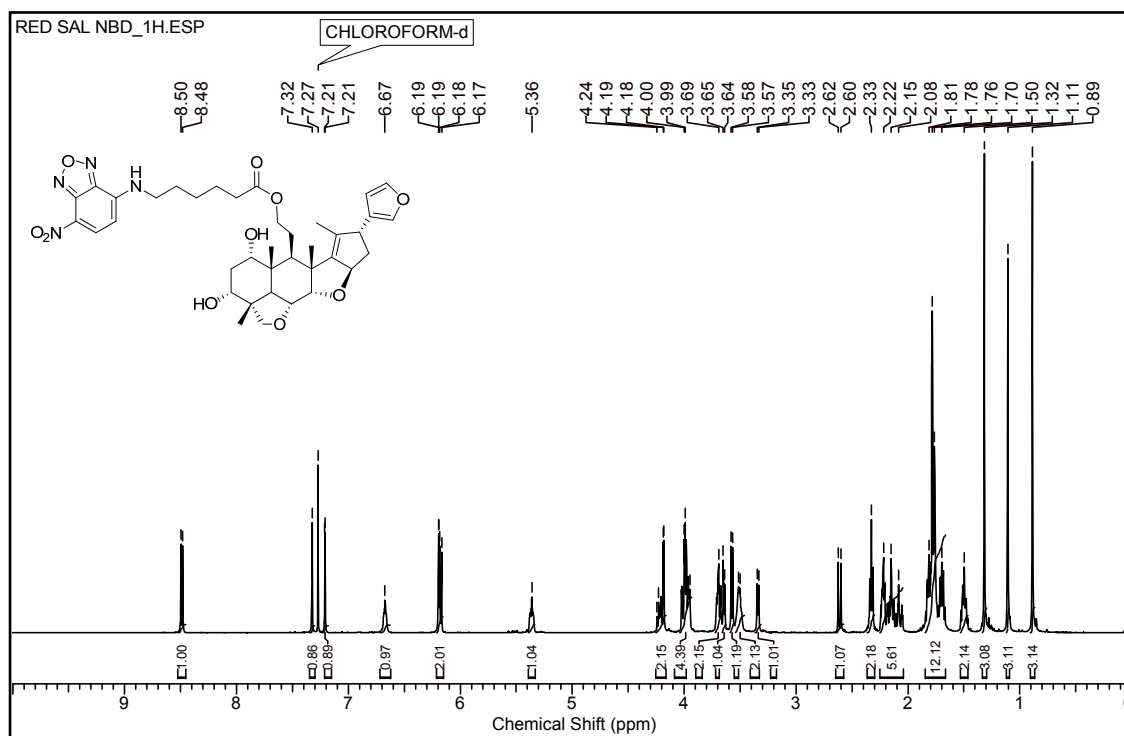


¹³C NMR spectrum (CDCl₃, 100 MHz) of **17a**

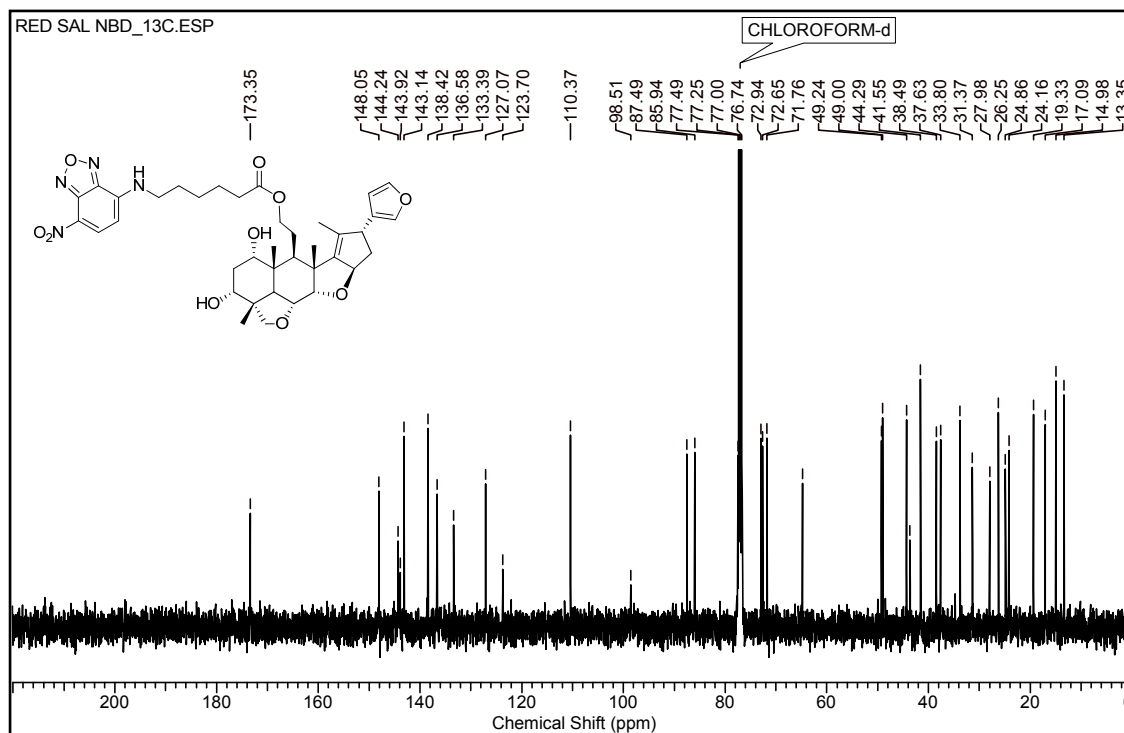


DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of **17a**

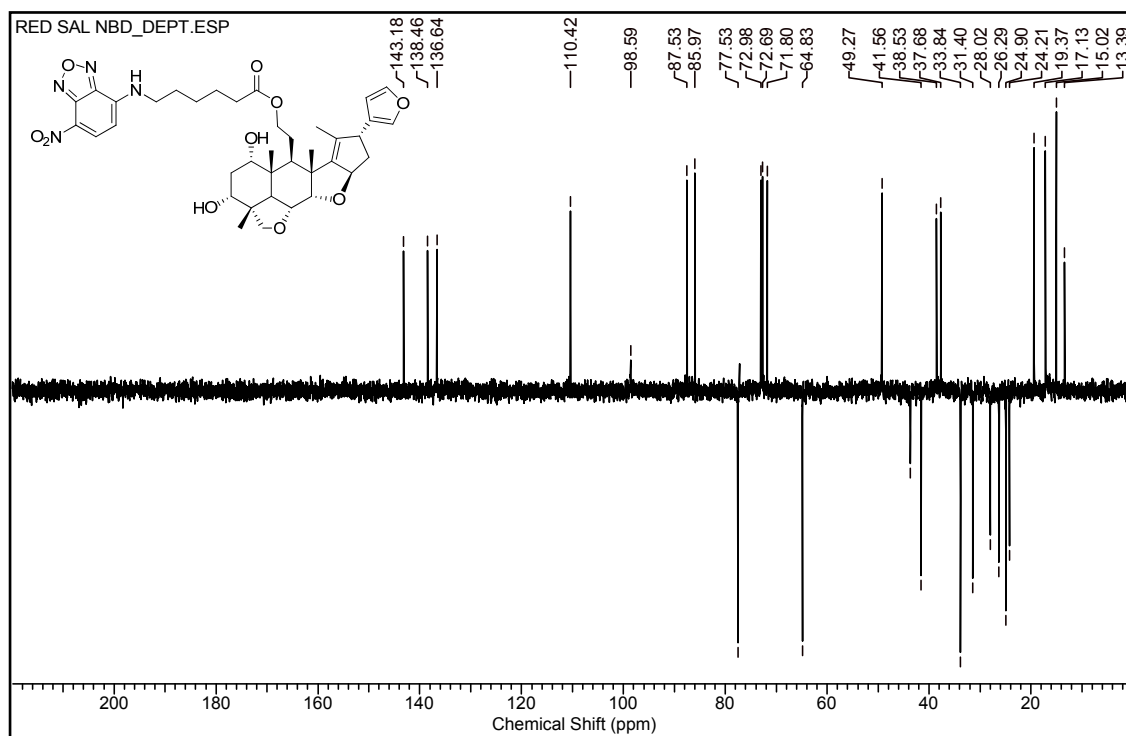
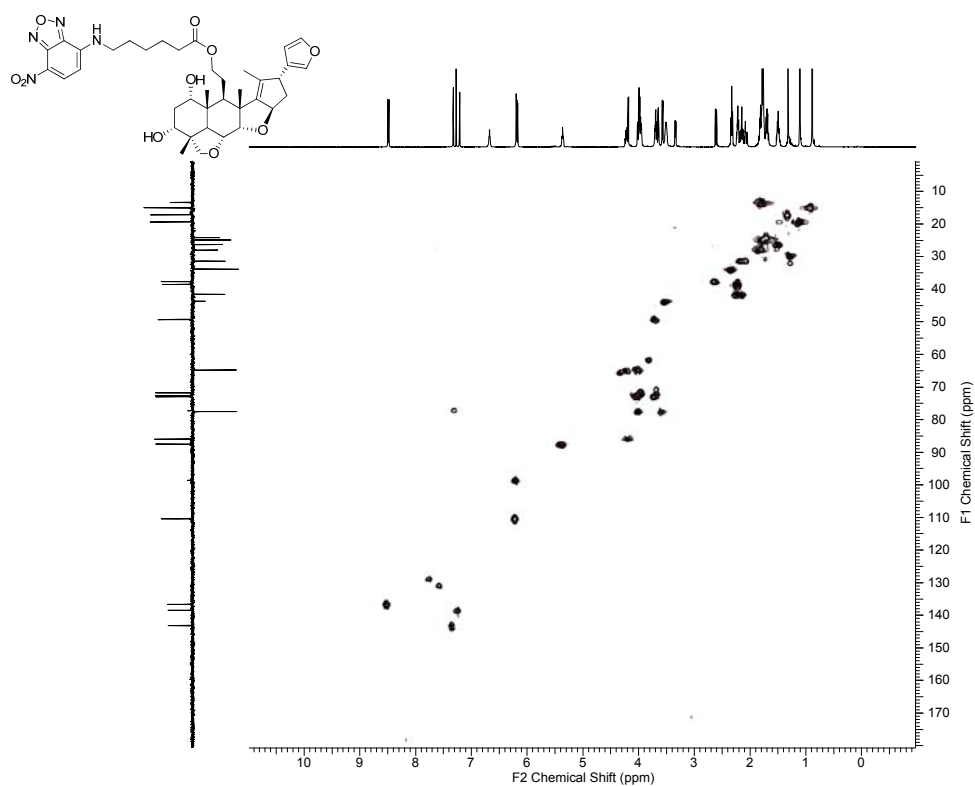
HSQC spectrum of **17a**HMBC spectrum of **17a**

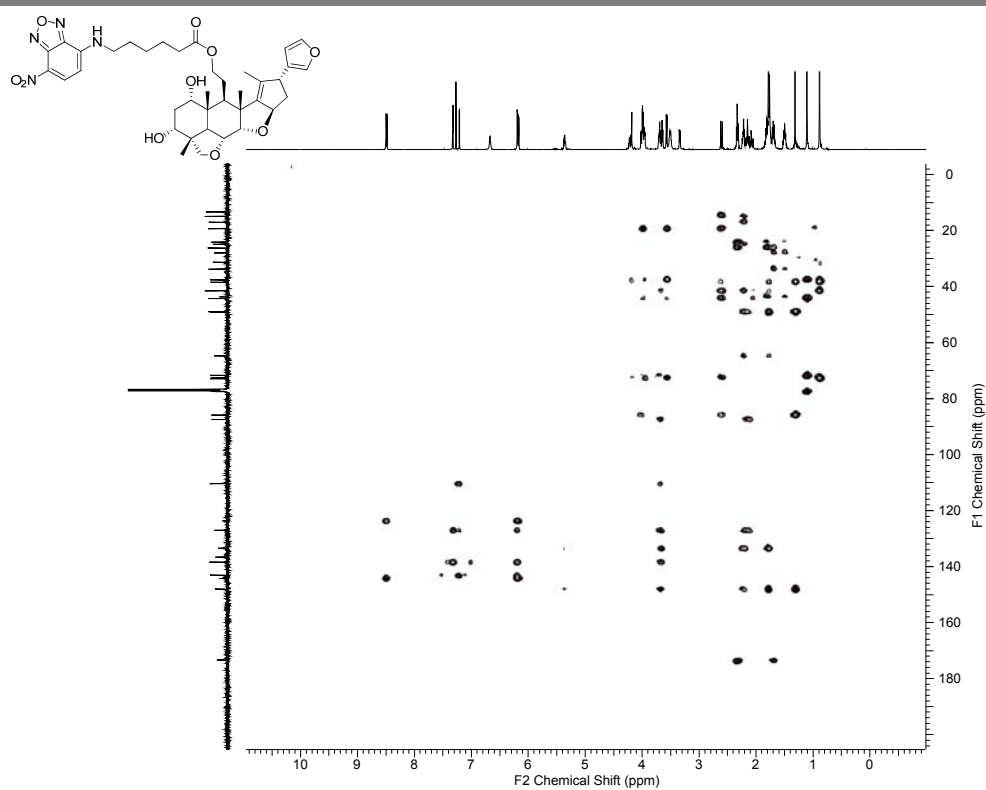
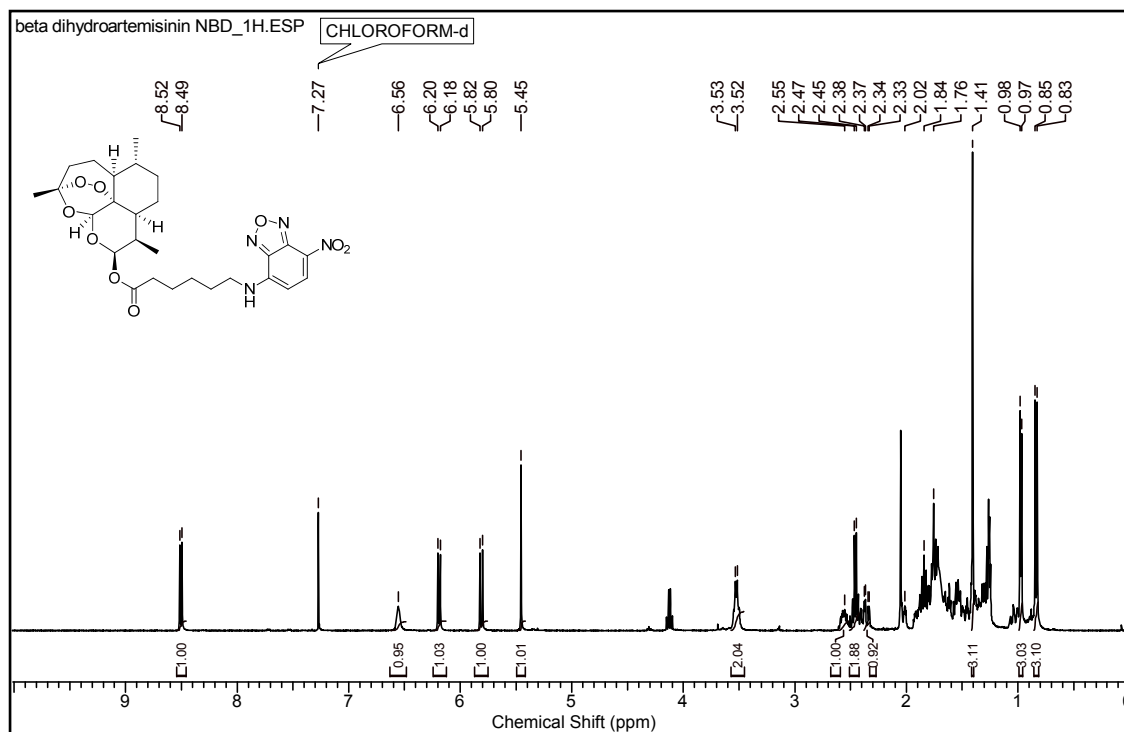


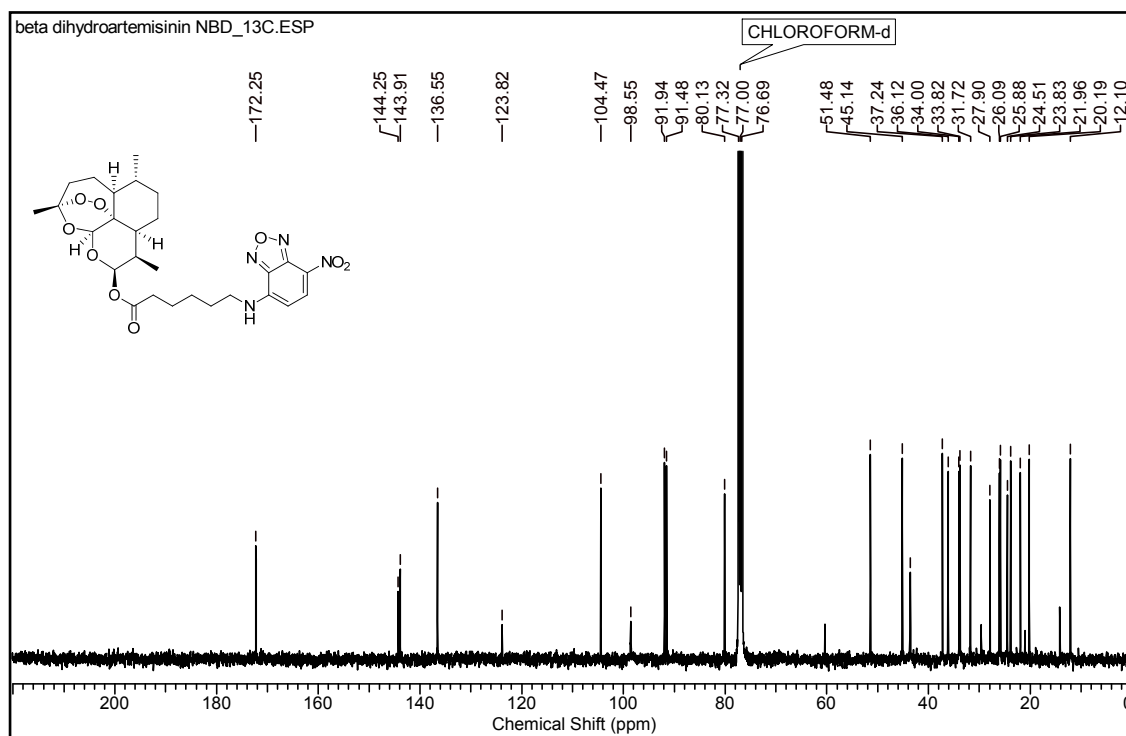
^1H NMR spectrum (CDCl_3 , 400 MHz) of **18a**



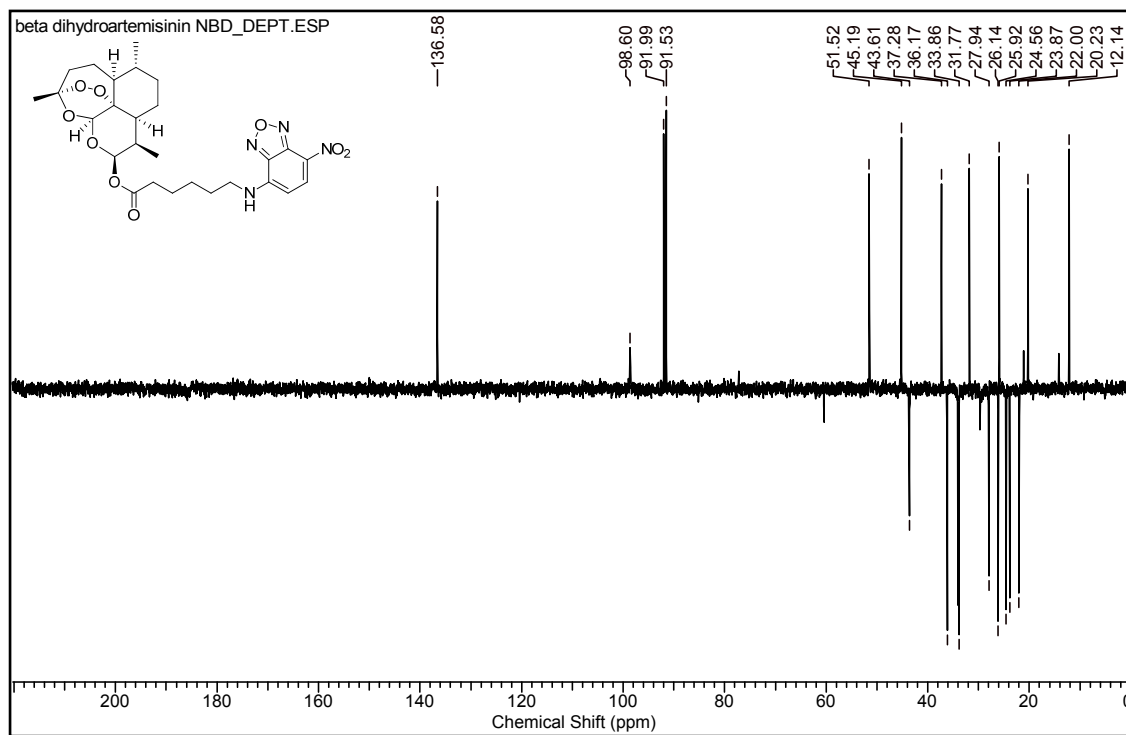
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **18a**

DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **18a**HSQC spectrum of **18a**

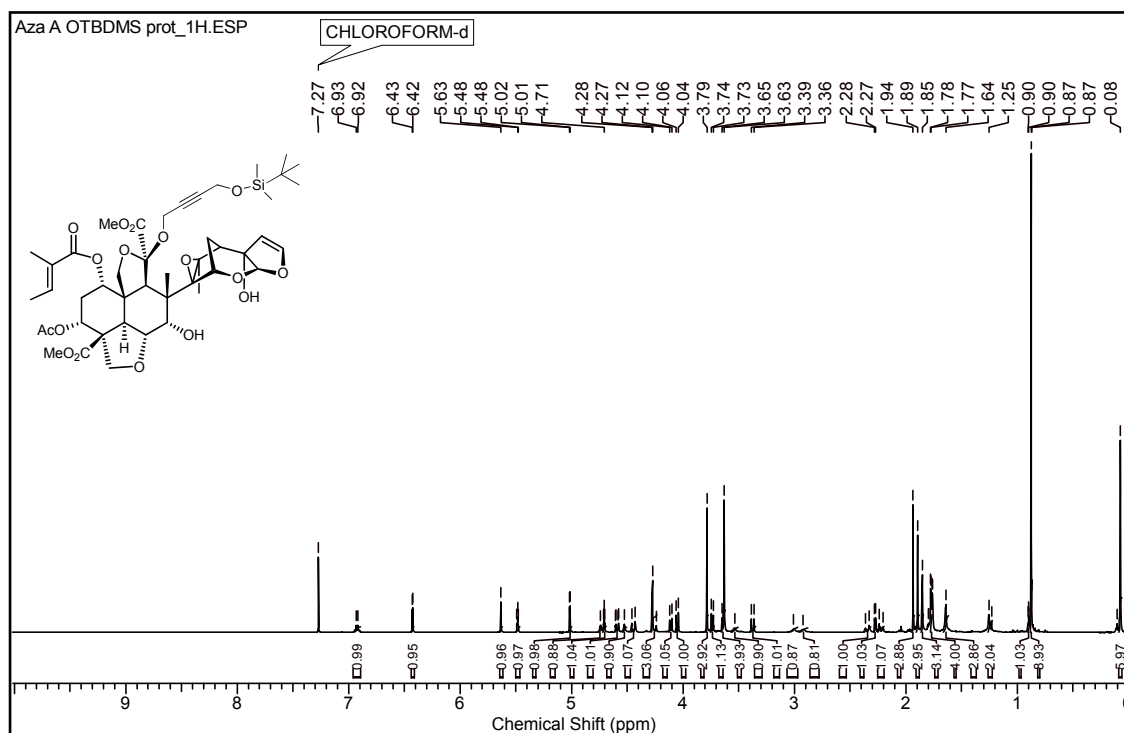
HMBC spectrum of **18a** ^1H NMR spectrum (CDCl_3 , 400 MHz) of **19a**



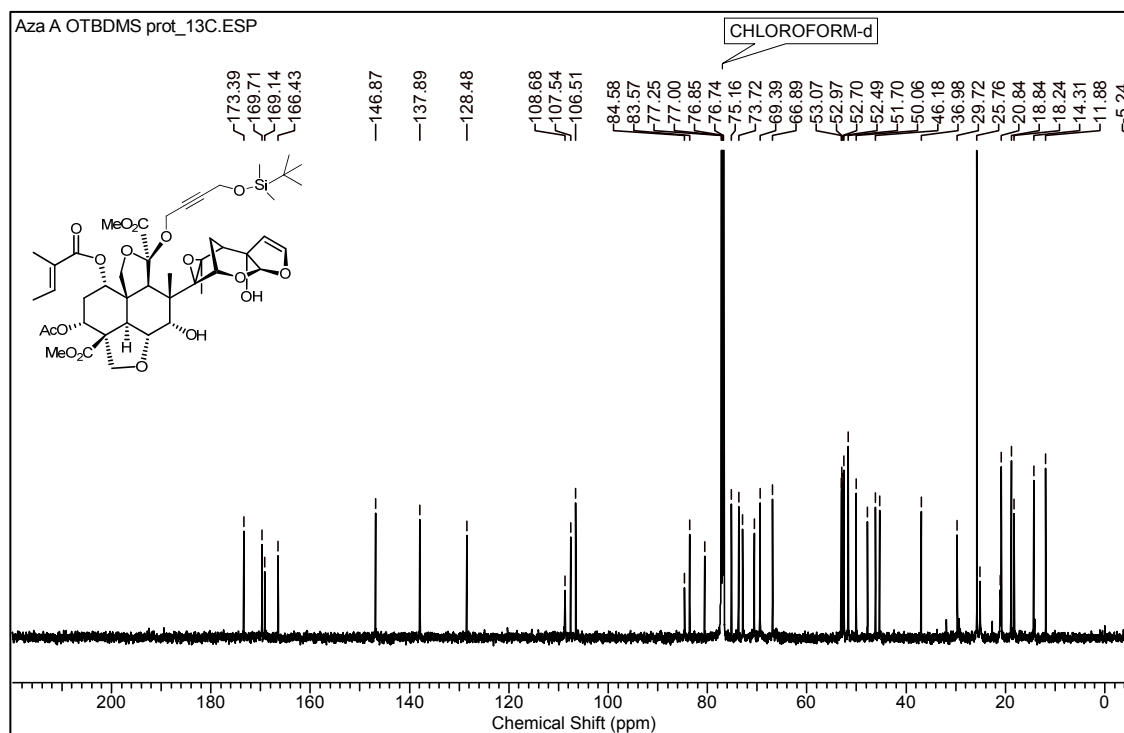
^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **19a**



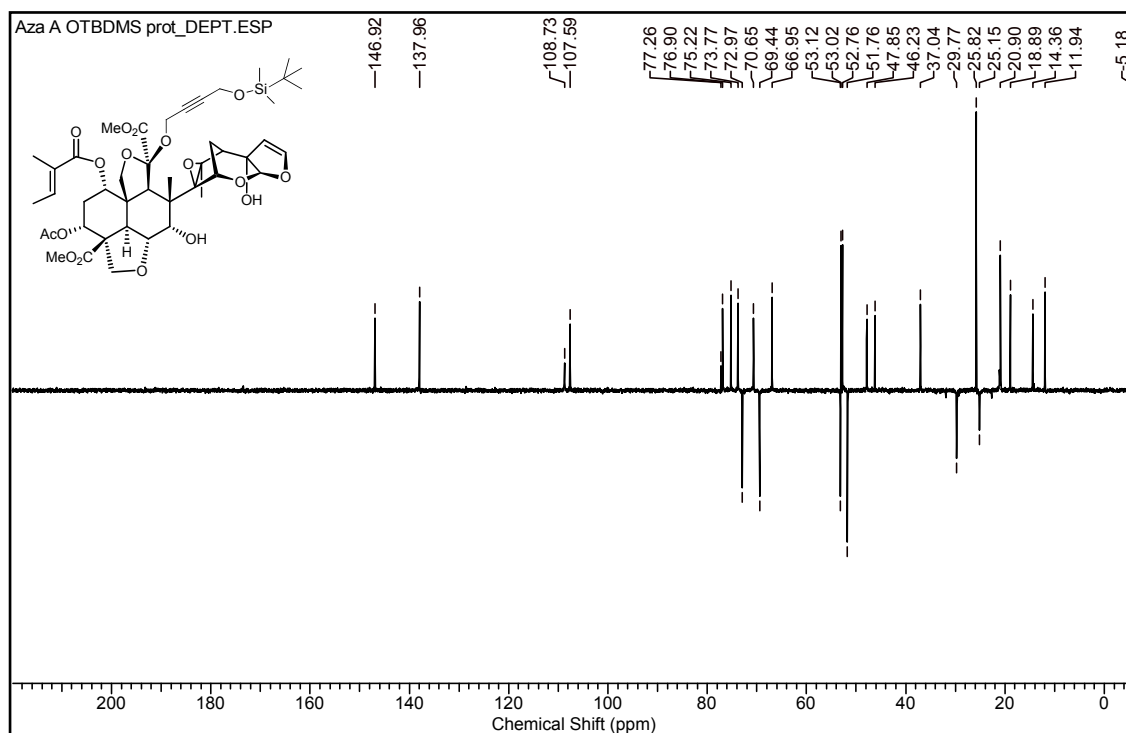
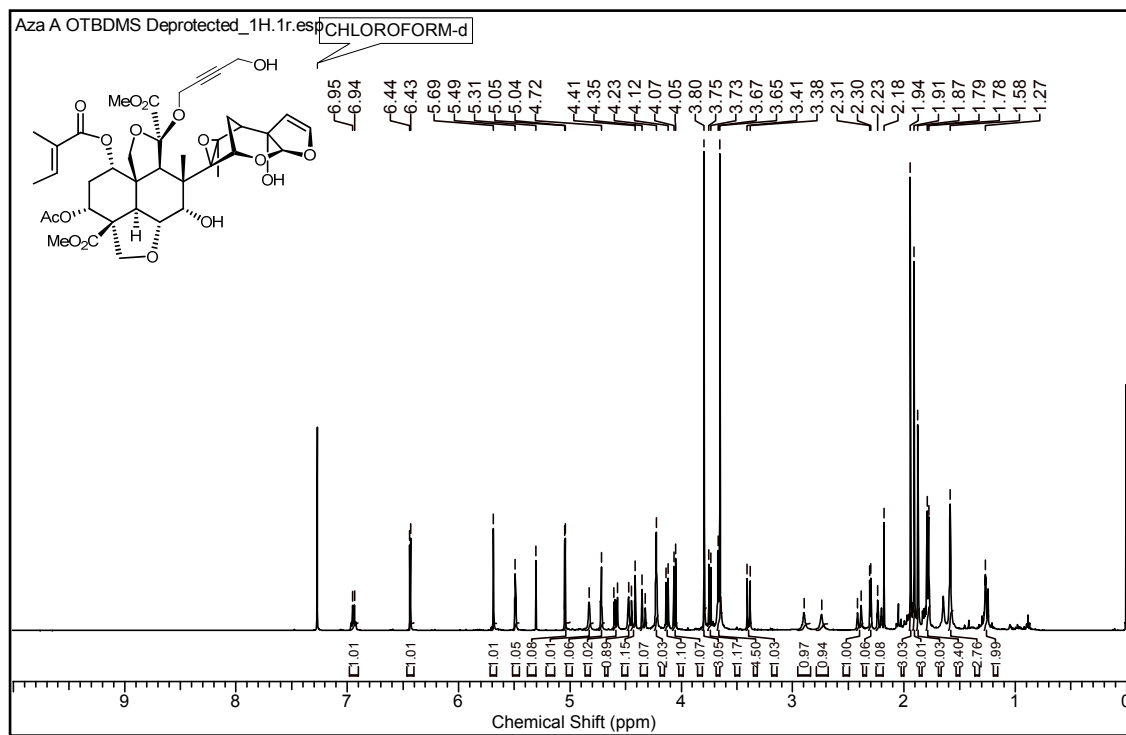
DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **19a**

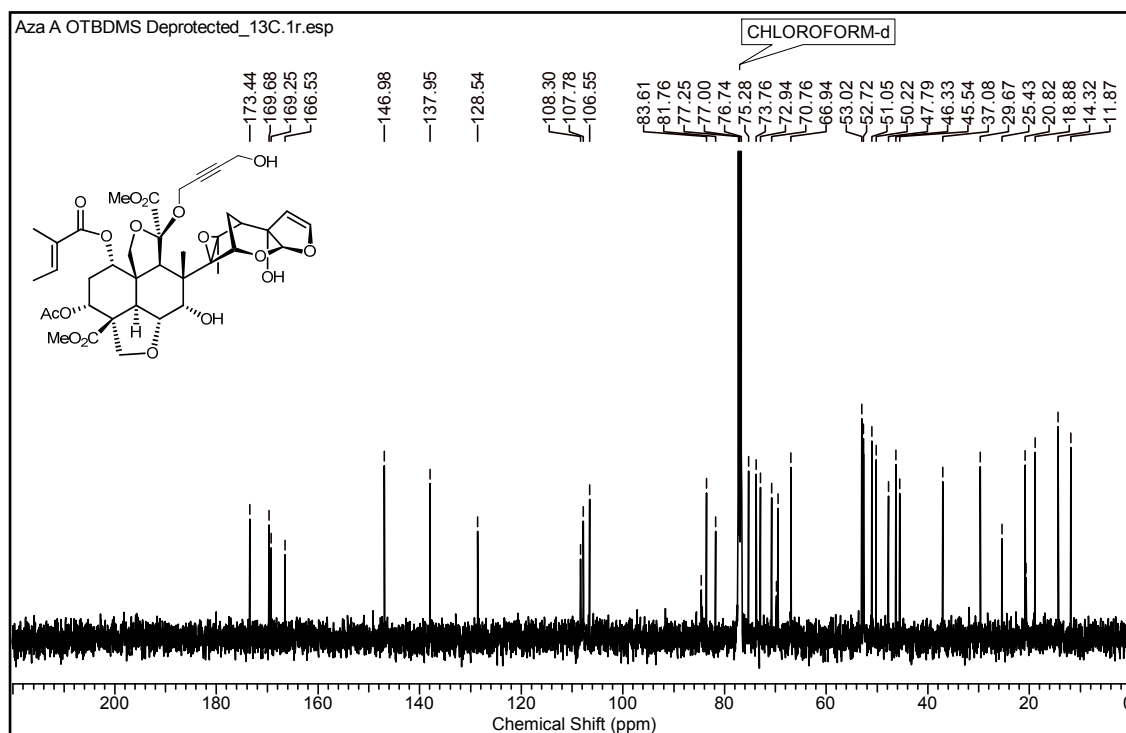


^1H NMR spectrum (CDCl_3 , 400 MHz) of **20a**

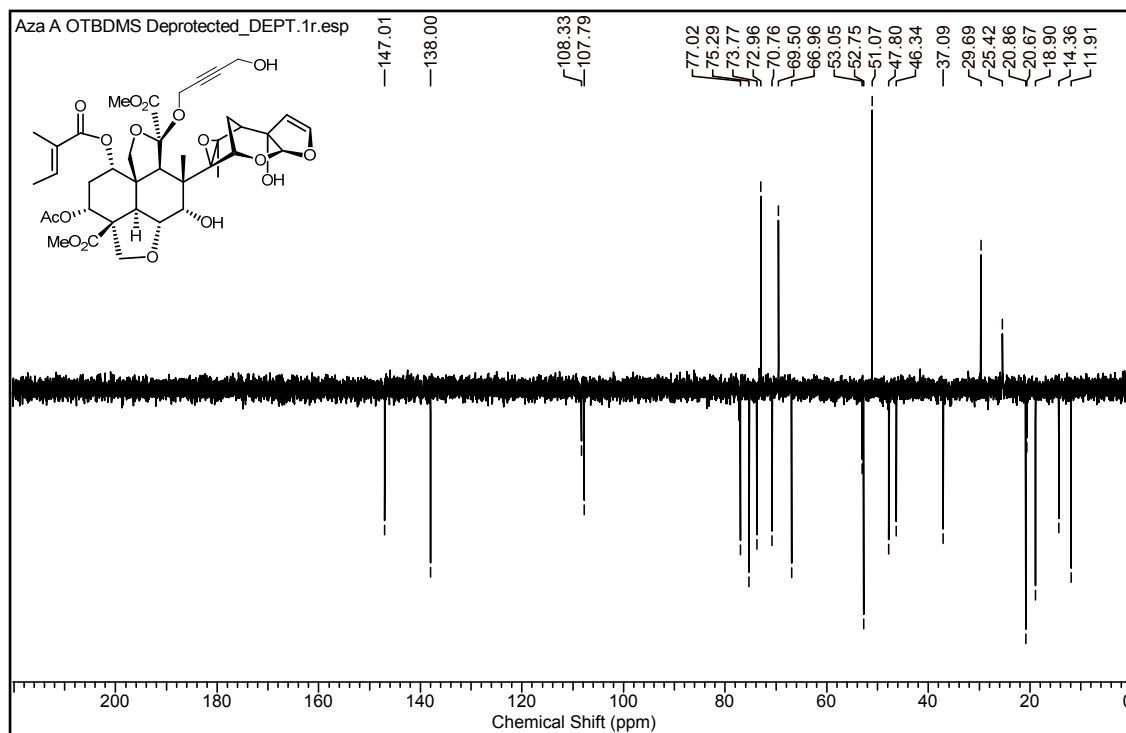


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **20a**

DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of **20a**¹H NMR spectrum (CDCl₃, 400 MHz) of **20b**

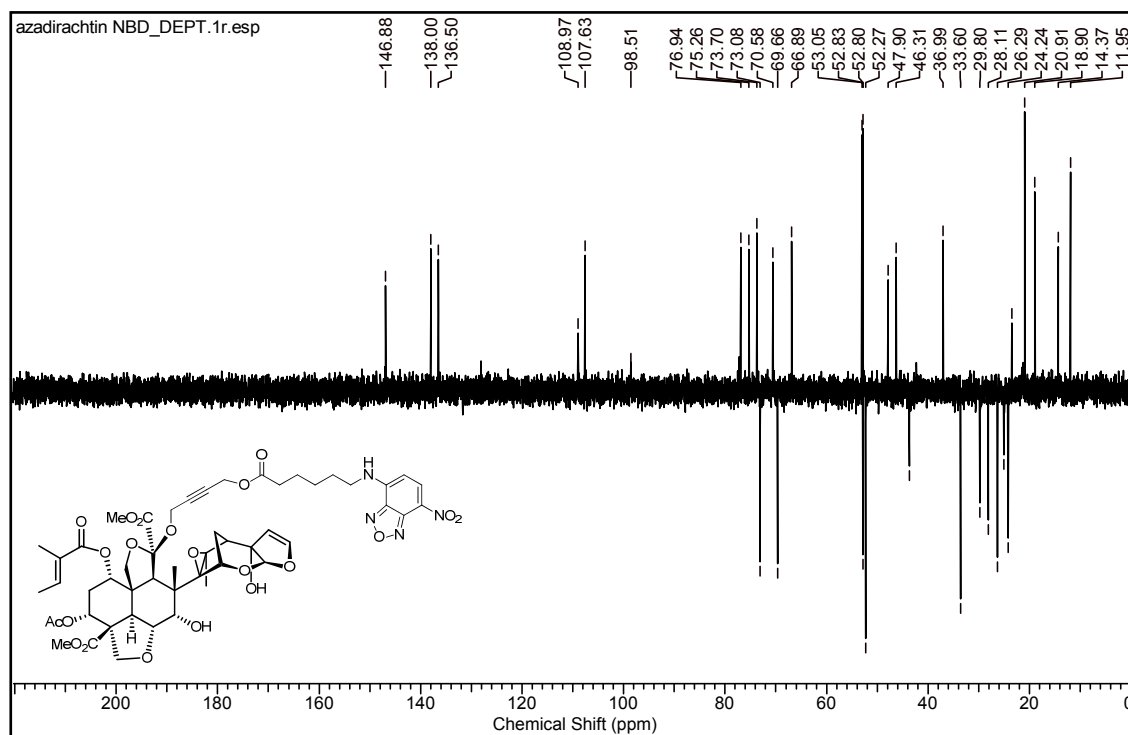


^{13}C NMR spectrum (CDCl_3 , 100 MHz) of **20b**



DEPT-135 NMR spectrum (CDCl_3 , 100 MHz) of **20b**



DEPT-135 NMR spectrum (CDCl₃, 100 MHz) of **20c**

4C **References**

- (1) Lang, P.; Yeow, K.; Nichols, A.; Scheer, A. *Nat. Rev. Drug Discov.* **2006**, *5*, 343-356.
- (2) Miyawaki, A.; Sawano, A.; Kogure, T. *Nat. Cell Biol.* **2003**, S1-S7.
- (3) Sekar, R. B.; Periasamy, A. *J. Cell Biol.* **2003**, *160*, 629-633.
- (4) Watson, P.; Jones, A. T.; Stephens, D. J. *Adv. Drug Deliv. Rev.* **2005**, *57*, 43-61.
- (5) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*.
- (6) Abate, C.; Hornick, J. R.; Spitzer, D.; Hawkins, W. G.; Niso, M.; Perrone, R.; Berardi, F. *J. Med. Chem.* **2011**, *54*, 5858-5867.
- (7) Barnes, K. R.; Blois, J.; Smith, A.; Yuan, H.; Reynolds, F.; Weissleder, R.; Cantley, L. C.; Josephson, L. *Bioconjugate Chem.* **2008**, *19*, 130-137.
- (8) Chen, L.; Li, F. Q.; Hou, B. H.; Hong, G. F.; Yao, Z. J. *J. Org. Chem.* **2008**, *73*, 8279-8285.
- (9) Gertsch, J.; Feyen, F.; Butzberger, A.; Gerber, B.; Pfeiffer, B.; Altmann, K. H. *Chembiochem* **2009**, *10*, 2513-2521.
- (10) Hartwig, C. L.; Lauterwasser, E. M. W.; Mahajan, S. S.; Hoke, J. M.; Cooper, R. A.; Renslo, A. R. *J. Med. Chem.* **2011**, *54*, 8207-8213.
- (11) Hiratsuka, T.; Kato, T. *J. Biol. Chem.* **1987**, *262*, 6318-6322.
- (12) Levi, J.; Cheng, Z.; Gheysens, O.; Patel, M.; Chan, C. T.; Wang, Y. B.; Namavari, M.; Gambhir, S. S. *Bioconjugate Chem.* **2007**, *18*, 628-634.
- (13) Souto, A. A.; Acuña, A. U.; Andreu, J. M.; Barasoain, I.; Abal, M.; Amat-Guerri, F. *Angew. Chem. Int. Edit.* **1996**, *34*, 2710-12.
- (14) Wieland, T. *Fortschr. Chem. Org. Naturst.* **1967**, *25*, 214.
- (15) Liu, H. X.; Huang, G. R.; Zhang, H. M.; Jiang, S.; Wu, J. R.; Yao, Z. J. *Chembiochem* **2007**, *8*, 172-177.
- (16) Pan, Q. Y.; Wang, S. Z.; Lu, J. J.; Meng, L. H.; Yao, Z. J. *Sci. China Chem.* **2010**, *53*, 119-124.
- (17) Uchiyama, S.; Santa, T.; Okiyama, N.; Fukushima, T.; Imai, K. *Biomed. Chromatogr.* **2001**, *15*, 295-318.
- (18) DeAngelis, P. L. *Anal. Biochem.* **2000**, *284*, 167-169.
- (19) Burnett, T. J.; Peebles, H. C.; Hageman, J. H. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 157-162.
- (20) Stearns, N. A.; PrigentRichard, S.; Letourneur, D.; Castellot, J. J. *Anal. Biochem.* **1997**, *247*, 348-356.
- (21) Arata, T. *Biochemistry* **1996**, *35*, 16061-16068.
- (22) Akira, T.; Mitsuru, I.; Tsutomu, S.; Hiroyuki, S.; Shujiro, G. *Chem. Pharm. Bull.* **1985**, *33*, 1164-1169.
- (23) Luo, L. B.; Tam, J.; Maysinger, D.; Eisenberg, A. *Bioconjugate Chem.* **2002**, *13*, 1259-1265.
- (24) Yan, B.; Liu, L.; Astor, C. A.; Tang, Q. *Anal. Chem.* **1999**, *71*, 4564-4571.
- (25) Zhang, Y. H.; Gao, Z. X.; Zhong, C. L.; Zhou, H. B.; Chen, L.; Wu, W. M.; Peng, X. J.; Yao, Z. J. *Tetrahedron* **2007**, *63*, 6813-6821.
- (26) Alexander, M. D.; Burkart, M. D.; Leonard, M. S.; Portonovo, P.; Liang, B.; Ding, X.; Joullie, M. M.; Gullledge, B. M.; Aggen, J. B.; Chamberlin, A. R. *Chembiochem* **2006**, *7*, 409-416.
- (27) Imai, K.; Fukushima, T.; Yokosu, H. *Biomed. Chromatogr.* **1994**, *8*, 107-113.

-
- (28) Toyooka, T.; Liu, Y. M.; Hanioka, N.; Jinno, H.; Ando, M.; Imai, K. *J. Chromatogr. A* **1994**, 675, 79-88.
- (29) Brandt, G. E. L.; Schmidt, M. D.; Prisinzano, T. E.; Blagg, B. S. J. *J. Med. Chem.* **2008**, 51, 6495-6502.
- (30) Nanduri, S.; Thunuguntla, S. S. R.; Nyavanandi, V. K.; Kasu, S.; Kumar, P. M.; Ram, P. S.; Rajagopal, S.; Kumar, R. A.; Deevi, D. S.; Rajagopalan, R.; Venkateswarlu, A. *Bioorg. Med. Chem. Lett.* **2003**, 13, 4111-4115.
- (31) Kikuchi, T.; Ishii, K.; Noto, T.; Takahashi, A.; Tabata, K.; Suzuki, T.; Akihisa, T. *J. Nat. Prod.* **2011**, 74, 866-870.
- (32) Cohen, E.; Quistad, G. B.; Casida, J. E. *Life Sci.* **1996**, 58, 1075-1081.
- (33) Paul, R.; Prasad, M.; Sah, N. K. *Cancer Biol. Ther.* **2011**, 12, 467-476.
- (34) Ma, L. H.; Takanishi, C. L.; Wood, M. J. *J. Biol. Chem.* **2007**, 282, 31429-31436.
- (35) Martensson, L. G.; Jonsson, B. H.; Freskgard, P. O.; Kihlgren, A.; Svensson, M.; Carlsson, U. *Biochemistry* **1993**, 32, 224-231.
- (36) Ricci, G.; Caccuri, A. M.; Lobello, M.; Pastore, A.; Piemonte, F.; Federici, G. *Anal. Biochem.* **1994**, 218, 463-465.
- (37) Lipsky, N. G.; Pagano, R. E. *Proc. Natl. Acad. Sci.* **1983**, 80, 2608-2612.
- (38) Elliott, J. T.; Prestwich, G. D. *Bioconjugate Chem.* **2000**, 11, 832-841.
- (39) Wise, E. T.; Singh, N.; Hogan, B. L. *J. Chromatogr. A* **1996**, 746, 109-121.
- (40) Xia, Z. P.; Smith, C. D. *J. Org. Chem.* **2001**, 66, 5241-5244.
- (41) Chen, S.; Shih, Y. W.; Huang, H. C.; Cheng, H. W. *Plos One* **2011**, 6.
- (42) Huang, X. H.; Chen, D. Y. K. *Chemmedchem* **2012**, 7, 1882-1894.
- (43) Earle, D. P.; Welch, W. J.; Shannon, J. A. *J. Clin. Invest.* **1948**, 27, 87-92.
- (44) Zhang, W.; Song, J. J.; Mu, L. Y.; Zhang, B. Z.; Liu, L. W.; Xing, Y. H.; Wang, K. R.; Li, Z. Y.; Wang, R. *Bioorg. Med. Chem. Lett.* **2011**, 21, 1452-1455.
- (45) Kumar, S.; Patil, H. S.; Sharma, P.; Kumar, D.; Dasari, S.; Puranik, V. G.; Thulasiram, H. V.; Kundu, G. C. *Curr. Mol. Med.* **2012**, 12, 952-966.
- (46) Singh, C.; Chaudhary, S.; Puri, S. K. *Bioorg. Med. Chem. Lett.* **2008**, 18, 1436-1441.
- (47) Veitch, G. E.; Beckmann, E.; Burke, B. J.; Boyer, A.; Maslen, S. L.; Ley, S. V. *Angew. Chem. Int. Edit.* **2007**, 46, 7629-7632.
- (48) Broughton, H. B.; Ley, S. V.; Slawin, A. M. Z.; Williams, D. J.; Morgan, E. D. *J. Chem. Soc. Chem. Commun.* **1986**, 46-47.
- (49) Yamasaki, R. B.; Klocke, J. A. *J. Agric. Food Chem.* **1987**, 35, 467-471.
- (50) Veitch, G. E.; Boyer, A.; Ley, S. V. *Angew. Chem. Int. Edit.* **2008**, 47, 9402-9429.
- (51) Dhimitruka, H.; SantaLucia, J. *Org. Lett.* **2006**, 8, 47-50.

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