ISOLATION, IDENTIFICATION AND ENRICHMENT OF BIOLOGICALLY ACTIVE MOLECULES FROM PLANT SPECIES AND CHIRAL SEPARATION OF SOME BIOLOGICALLY IMPORTANT MOLECULES

A THESIS
SUBMITTED TO THE
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FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN
CHEMISTRY

BY
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UNDER THE GUIDANCE OF
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[FEBRUARY, 2010]

DEDICATED

TO

MY FAMILY

DECLARATION

I hereby declare that the work incorporated in the thesis entitled "**Isolation, identification and enrichment of biologically active molecules from plant species and chiral separation of some biologically important molecules**" submitted for the Ph. D. degree to the University of Pune has been carried out by me under the guidance of Dr. S. R. Rojatkar at National Chemistry Laboratory, Pune - 411 008.

This work is original and has not been submitted for any degree or diploma to this or any other university.

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CERTIFICATE

Certified that the work incorporated in the thesis "**Isolation, identification and enrichment of biologically active molecules from plant species and chiral separation of some biologically important molecules"** submitted by Shri Kiran. B. Sonawane was carried out by the candidate under my guidance. Such material as obtained from other sources has been duly acknowledged in the thesis.

Pune-411 008
February 2010

Dr. S. R. Rojatkar
(Research Guide)

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Kiran B. Sonawane

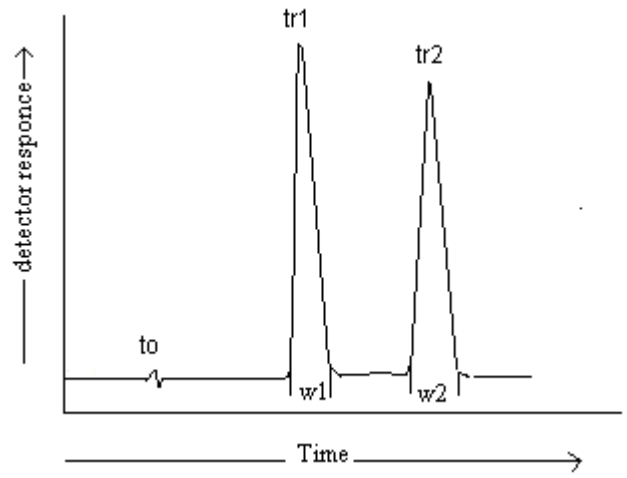
Abbreviations

Ac	Acetyl or acetate
Ac_2O	Acetic anhydride
AIBN	2, 2'-Azobisisobutyronitrile
Boc	*tert*-Butyloxycarbonyl
$CDCl_3$	Deuterated chloroform
CSP	Chiral stationary phase
COSY	Correlated spectroscopy
d	doublet
dd	doublet of doublet
DCM	Dichloromethane
DEPT	Distortion-less enhancement by polarization transfer
DIBAL	Diisobutylaluminium hydride
DMAP	*N, N'*-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO-d_6	Deuterated dimethyl sulfoxide
DNBPG	3,5-Dinitrobenzoylphenylglycine
g	gram
GC	Gas chromatography
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IR	Infra red
J	Coupling constant
Kg	Kilogram
m	multiplet
mg	miligram
MS	Mass spectrometry

M^+	Molecular ion
Me	Methyl
min	Minutes
ml	Millilitre
mm	Millimeter
mmol	Millimole
µm	Micrometer
µl	Microlitre
n-BuLi	*n*-Butyllithium
NBS	*N*-Bromosuccinimide
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
Ph	Phenyl
PDC	Pyridinium dichromate
PPA	Polyphosphoric acid
q	Quartet
p-TSA	*p*-Toluenesulfonic acid
rt	Room temperature
RT	Retention time
ROESY	Rotating-frame overhauser effect spectroscopy
s	Singlet
SMB	Simulated moving bed
SMBC	Simulated moving bed chromatography
t	Triplet
TBDMS	*tert*-Butyldimethylsilyl
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMB	True moving bed
TMBC	True moving bed chromatography
UV	ultraviolet

General remarks

1. All organic layers obtained after extractions were dried over anhydrous Na_2SO_4. All evaporations were carried out under reduced pressure on Buchi rotary evaporator. Silica gel for column chromatography was 60-120 mesh and 100-200 mesh. Pet ether used was the fraction of petroleum ether boiling in the range 60-80 °C.
2. All the temperatures are in °C. All the melting points and boiling points are in °C and are uncorrected. Melting points were recorded on Buchi B-540 melting point apparatus.
3. IR spectra were recorded on a Perkin-Elmer infra-red spectrometer model 599-B and model 1620 FT-IR (ν-max in cm^{-1}).
4. Unless otherwise stated, ^1H NMR spectra were recorded using TMS as internal reference on Bruker AC-200 or AC-400 instruments using $CDCl_3$ as solvent. All chemical shifts are reported in parts per million downfield from TMS. The coupling constants (*J* values) are reported in Hertz.
5. ^{13}C NMR spectra were recorded on Bruker AC-200 and AC-400 instruments operating at 50 MHz and 100 MHz.
6. Mass spectra were recorded on Finnigan-Mat 1020C mass spectrometer and were obtained at an ionization potential of 70 eV.
7. The compound numbers, scheme numbers and references given in each chapter refer to that particular chapter only.
8. Chromatographic parameters in chapter III are calculated as follows.



to: Nonsorbed time

tr1: retention time of peak 1

tr2: retention time of peak 2

w1: peak width of peak 1

w2: peak width of peak 2

Selectivity or separation factor = k2/k1

k1 (capacity factor of peak 1) = $(tr1 - t_0)/t_0$

k2 (capacity factor of peak 2) = $(tr2 - t_0)/t_0$

Resolution (R) = 2(tr2 – tr1)/w1+w2

Number of theoretical plates (N) = $5.55\ (tr)^2/(w_{1/2})^2$

where $w_{1/2}$ is peak width at half-height

ABSTRACT

Thesis Title

Isolation, identification and enrichment of biologically active molecules from plant species and chiral separation of some biologically important molecules

The thesis is divided into three chapters.

Chapter 1: Chemical Investigation of S*phaeranthus Indicus* and Enrichment of 7-Hydroxy Eudesmanoloid, a Biologically Important Compound

Chapter 2: Chemical Investigation of *Taxus Baccata*

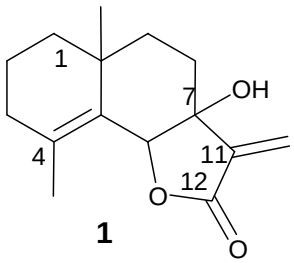
Chapter 3: Chiral Separation of Biologically Active Molecules by HPLC and SMBC

Chapter 1: Chemical Investigation of S*phaeranthus Indicus* and Enrichment of 7-Hydroxy Eudesmanoloid, a Biologically Important Compound

Sphaeranthus Indicus (Family: Asteraceae) is a medicinally important plant and all the parts of the plant have medicinal uses.[1] It is used to treat jaundice, hepatopathy, diabetes, leprosy, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia, skin diseases, insanity, tuberculosis, indigestion, bronchitis, spleen diseases, elephantiasis, anaemia, pain in uterus and vagina, piles, asthma, leucoderma, dysentery, vomiting, etc. The juice of the plant is styptic and diuretic and is useful against liver and gastric disorders.[1,2] It is also used in treating epileptic convulsions, mental illnesses and hemicranias. The flowers are highly alterative, depurative, cooling and tonic. They are also used as blood purifiers in skin diseases.[3] Roots and seeds are used as stomachic and anthelmintic.[4] Dried and powdered leaves are useful in the treatment of chronic skin diseases, urethral discharges and jaundice.[5] The oil from the roots is useful in treating scrofula and as an aphrodisiac. The external application of a paste of this herb is beneficial in treating pruritus and edema, arthritis, filariasis, gout and cervical adenopathy. It is also used in treatment of piles and hepatitis.[6]

Extracts of different parts of *S. indicus* have been found to have various biological activities, such as; alcoholic extract of flowers has hypotensive, peripheral vasodilatory, cathartic activity,[7,8] essential oil from leaves possesses antifungal properties[9] and an antimicrobial sesquiterpene has been isolated from its petroleum ether extract.[10]

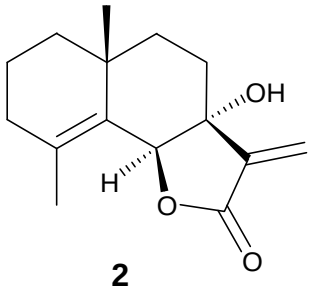
Some 7-hydroxysesquiterpene lactones (**1**) isolated from *S. indicus* exhibit biological activities such as immune stimulating activity,[11] antifungal, antibacterial, antiprotozoal activities.[12] A 7-hydroxysesquiterpene lactone is screened for its antitumor activity *in vitro* in various human and mouse tumor cell line and it inhibited telomerase and MMP-9 activity in tumor cells and induced apoptosis. It is formulated in injection, tablet and capsule formulations.[13] A herbal composition of the extract of *S. indicus* containing a compound, 7-hydroxy-4,11(13)-eudesmadien-12,6-olide, as an active ingredient and as a bioactive marker showed activity against inflammatory disorders.[14]



1

From *S. indicus* all eudesmanolides isolated have 7-hydroxy group. Other than eudesmanolides, the plant is reported to contain steroids, flavonoids and a novel 7-hydroxy eudesmanolide type of alkaloid.

In this chapter, the isolation of the major 7-hydroxyeudesmanolide (**2)** possessing biological activity by enriching the dichloromethane extracts of *S. indicus* by solvent-solvent extraction and broad column chromatography and finally by precipitation avoiding successive chromatography is described. Being a medicinally important plant possessing many biological activities and rich in 7-hydroxyeudesmanolides, further chemical investigation of its acetone extract was carried out by column chromatography and finally by preparative HPLC to isolate two more new eudesmanolides (**3** and **4**). Their structures are elucidated by extensive spectral analysis. The structure of **3** was established as 6β,7α,10β-4,11(13)-eudesmandiene-7β-hydroxy-3-one-12,6-olide and compound **4** was characterized as eudesman-4-en-6β,7α-olide.



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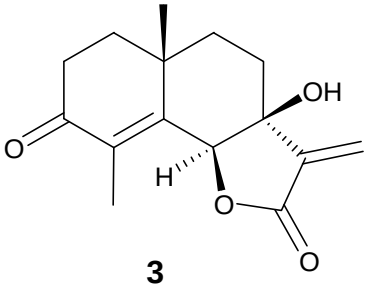

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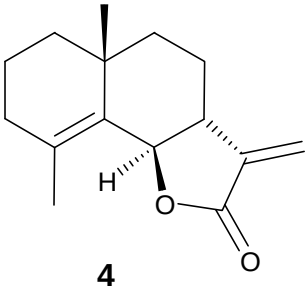

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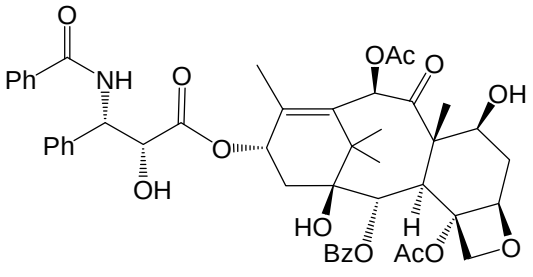
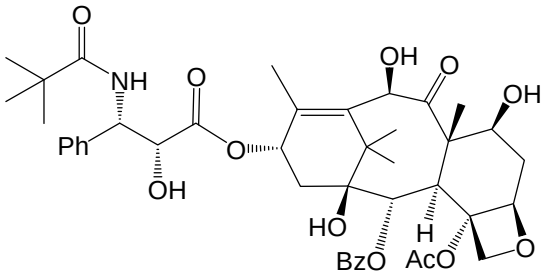
Chapter 2: Chemical Investigation of *Taxus Baccata*

Paclitaxel (Taxol, **5**), a highly potent anticancer natural product[15] was isolated from the bark of *Taxus brevifolia* in 1971.[16] Taxol was approved for treatment of drug-resistant ovarian cancer in 1992 and for breast cancer in 1994. Today it is used routinely to treat lung, head and neck, prostate, cervical cancers and AIDS-related Kaposi's sarcoma.[17] Semi-synthetic paclitaxel analogue docetaxel (Taxotere, **6**) was approved to treat breast cancer in 1996.



Taxol (Paclitaxel, 5)

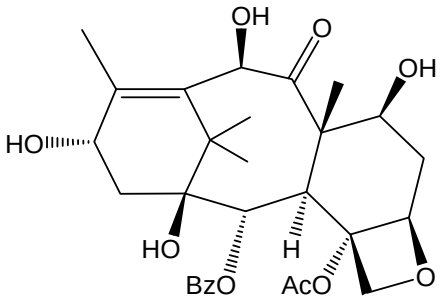


Taxotere (Docetaxel, 6)

More than 400 taxane-type diterpenoids have been isolated from various *Taxus* species. Before 1999, several comprehensive reviews have been published1[18] to list the diterpenes isolated from *Taxus* species. Recently a review has been published which includes new taxanes reported from 1999 to 2004.[19] Few taxanes including taxol precursor 10-deacetylbacctin III (**7**) have been isolated from *T. baccata* in our group.[20]

A short review of 46 new and known taxanes isolated from various *Taxus* species and reported after 2004 onward is described in this chapter. Further, enrichment of methanol extract of needles of *T. baccata* by solvent-solvent extraction for 10-deacetyl baccatin III (**7**) has been described. The residue after enrichment was subjected for

preparative HPLC to isolate two taxanes (**8** and **9**). The structure elucidation of both the compounds by ^{1}H NMR, ^{13}C NMR, ^{1}H - ^{1}H COSY, HMBC, HSQC, ROESY and NOESY is discussed in this chapter.



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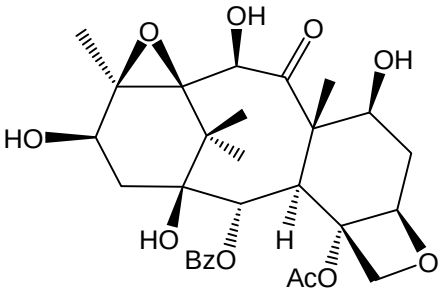
The compound **8** was characterized as 13-epi-11,12-epoxy-10-deacetylbaccatin III and was a new compound whereas compound **9** was characterized as 19-hydroxy-10-deacetyl baccatin III. Compound **9** found to be known compound from *T. baccata*, however its detailed characterization and ^{13}C NMR data was not reported, which is discussed in this chapter.



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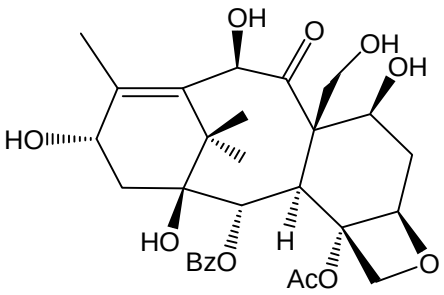

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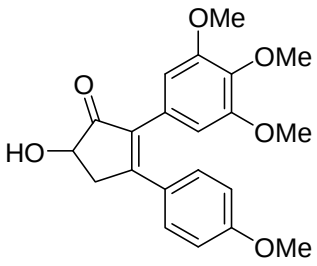
Chapter 3: Chiral Separation of Biologically Active Molecules by HPLC and SMBC

The enantiomers of a biologically active compound exhibit different activities and toxicities[21,22] has attracted worldwide attention and there is a move towards increasing single enantiomer use, wherever possible. As a result, of the top 100 drugs world wide, 50 are single enantiomers. Thus, the resolution of biologically active compound has become an inevitable step in the drug development process. Crystallization, use of resolving agents or enzymes, GC, HPLC[23] or SMBC[24] etc. are some of the methods used for resolution of various compounds and proper use of suitable methods to obtain both the

enantiomers in pure form holds the key position in the manufacture of various drugs. The application of simulated moving bed (SMB) technology for purification of intermediates and products in the pharmaceutical industry is today the attractive technique for economic reasons. SMB technology is a continuous countercurrent technology which is extensively used in the chiral separation of synthetic pharmaceuticals.[25-28]

In this chapter the separation of enantiomers of an anticancer agent[29] (**10**) which is an analogue of combretastatin A-4 (**11**) by chiral preparative HPLC is discussed. Similarly, enantiomers of a precursor (**12**) of a calcium sensitizer, levosimendan (**13**), an antiarrhythmic drug, cibenzoline (**14**) and two cibenzoline analogues (**15** and **16**) have been resolved and the process is discussed in this chapter. It is also shown that by SMBC process separation of enantiomers is faster and economical than preparative HPLC separation.



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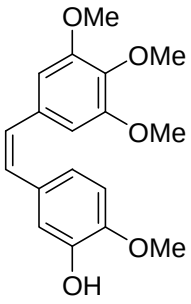

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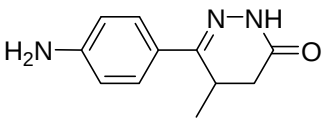

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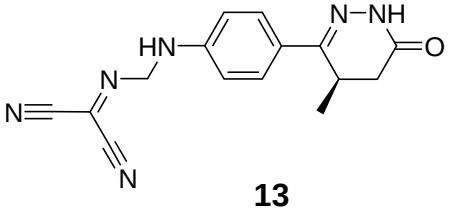

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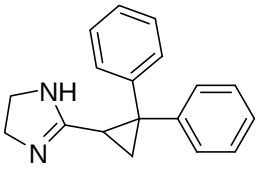

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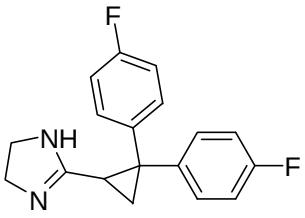

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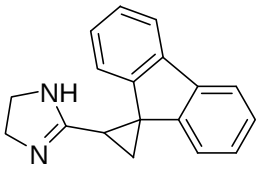

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Chapter I

Chemical investigation of *Sphaeranthus indicus* and enrichment of 7-hydroxy eudesmanoloid, a biologically important compound

Part of this work has been published in *Ind. J. Chem. Sect. B* **2007**, *46B*, 379

1.1 INTRODUCTION

The genus *Sphaeranthus* (Family: Asteraceae, tribe: Plucheeae) contains about forty species. Some of them are *Sphaeranthus indicus, S. africanus, S. senegalensis, S. hirtus, S. steetzii, S. suaveolens, S. kirkii, S. bullatus, S. cyathuloides* and *S. zeylanicus. Sphaeranthus indicus* is a medicinally important plant found abundantly growing as a common rabi weed in the rice fields all over India, Sri Lanka, Africa and Australia. It is a much branched annual spreading herb, which grows approximately 15-30 cm in height. It is known in Sanskrit as mahamundi, mundi or hapus and in Hindi language as gorkhmundi.[1]

According to Ayurveda, this herb is hot, laxative, digestible, tonic, fattening, alterative, anthelmintic and alexipharmic. All the parts of the plant have medicinal uses. It is used to treat jaundice, hepatopathy, diabetes, leprosy, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia, skin diseases, insanity, tuberculosis, indigestion, bronchitis, spleen diseases, elephantiasis, anaemia, pain in uterus and vagina, piles, asthma, leucoderma, dysentery, vomiting, etc. The whole herb is used in ayurvedic preparations to treat epilepsy and mental disorders. The juice of the plant is styptic and diuretic and it is said to be useful against liver and gastric disorders[1,2]. In folk medicine, the plant is reportedly used in treating epileptic convulsions, mental illnesses and hemicranias.[3a] It is reported that flowers are highly alterative, depurative, cooling and tonic. They are also used as blood purifiers in skin diseases.[3b] Roots and seeds are used as stomachic and anthelmintic.[4] Dried and powdered leaves of *S. indicus* are useful in the treatment of chronic skin diseases, urethral discharges and jaundice.[5] It is also used as a nervine tonic. The oil prepared using the plant root is reportedly useful in treating scrofula and as an aphrodisiac. The external application of a paste of this herb is beneficial in treating pruritus and edema, arthritis, filariasis, gout and cervical adenopathy. It is also used in treatment of piles and hepatitis.[6]

Extracts of different parts of *S. indicus* have been screened for their biological activities. In 1971, Srivastav *et al.*[7] observed that alcoholic extract of *S. indicus* flowers has hypotensive, peripheral vasodilatory and cathartic activity. Garg in 1982,[8] found that essential oil obtained from leaves possesses antifungal properties. Shaikh *et*

Sphaeranthus indicus

al.[9] isolated antimicrobial active alkaloidal and nonalkaloidal fractions from alcoholic extract. In 1988 Singh *et al*.[10] isolated an antimicrobial sesquiterpene from petroleum ether extract of *S. indicus*.

From 1995 onwards there are number of reports on different activities and uses of different extracts or isolated compounds from this plant. The extract is used in skin-lightening cosmetics,[11-15] antiaging cosmetics, skin moisturizer and anti-wrinkle cosmetics.[16-21] An Ayurvedic medicine has been prepared from the aqueous extracts of different herbs including extract of *S. indicus* for the treatment of AIDS.[22,23] Extract of *S. indicus* has also been reported to exhibit excellent antibacterial activity against Gram positive as well as Gram negative bacteria.[24] The extracts and constituents of *S. indicus* which are soluble in polar solvents have been found to show selective cytotoxicity against several different cancer cell lines.[25] A cream containing ethanolic extract of aerial parts significantly enhanced the rate of wound contraction and the period of epithelialization comparable to neomycin.[26] A bioactive fraction of *S. indicus* acts as potentiator of delayed type hypersensitivity (DTH). The fraction influences both humoral and cell-mediated immunity and offers protection against immunosuppression induced by the cytotoxic agent cyclophosphamide.[27] Free radical scavenging potential of the ethanolic extract of *S. indicus* was studied. The results justify the therapeutic applications of the plant in the indigenous system of medicine, augmenting its therapeutic value.[28] Recently petroleum ether extract of *S. indicus* flowers was found to produce prominent anxiolytic activity in mice.[29] The extract was further found to be effective in increasing phagocytic activity, hemagglutination antibody titer and delayed type hypersensitivity when tested in mice, thus shows good promise as an immunomodulatory agent, which acts by stimulating both humoral and cellular immunity.[30] Methanolic extract of plant showed macrofilaricidal activity against adult *Setaria digitata*, the cattle filarial worm.[31]

Though Gogte *et al*.[32] isolated first time three new sesquiterpene lactones, all of them having hydroxy group at C-7, from flower tops of the plant, Atta-ur-Rahman *et al*.[33] showed that 7-hydroxyfrullanolide, another new sesquiterpene lactone, isolated from ethanolic extract of flowers of *S. indicus* was the main antimicrobial component. They further isolated a new 7-hydroxysesquiterpene glycoside, sphaeranthanolide, from flowers exhibiting immune stimulating activity.[34] Chandra *et*

al.[35] isolated another new frullanolide, a sesquiterpene lactone, which showed to possess antifungal, antibacterial and antiprotozoal activities. A herbal anticancer agent comprising the extract or group of compounds obtained from the *S. indicus*, i.e. alkaloids, monoterpenes, sesquiterpenes, sesquiterpene lactones, sesquiterpene lactone glycosides, diterpenes, triterpenoids, fatty acid esters, hydrocarbons and amino acids was prepared. Further, 7-hydroxyeudesm-4-en-6,12-olide (HAC-1) isolated from *S. indicus* was screened for its antitumor activity *in vitro* in various human and mouse tumor cell lines. HAC-1 inhibited telomerase and MMP-9 activity in tumor cells and induced apoptosis. HAC-1 was formulated in injection, tablet and capsule formulations.[36] A herbal composition of the extract of *S. indicus* containing a compound, 7-hydroxy-4,11(13)-eudesmadien-12,6-olide, as an active ingredient and as a bioactive marker showed activity against inflammatory disorders.[37] A sesquiterpene lactone caused some developmental defects in the sensitive stages of mosquito *Anopheles stephensi*.[38]

1.2 PREVIOUS WORK

1.2.1 Identification of compounds from essential oils of *S. indicus*

A large number of constituents have been isolated from the extracts of the whole herb, flowers and leaves. Various types of steam volatile compounds such as methyl chavicol, α-ionone, d-cadinene, p-methoxy cinnamaldehyde as major constituents, and α-terpinene, α-citral, geraniol, geranyl acetate, β-ionone, ocimene, sphaerene as minor constituents of essential oil have been identified from oil of fresh flowering herb.[1,39,40] An alkaloid, sphaerenthrine, has been isolated from the essential oil of whole herb.[41,42] Some aromatic compounds have been reported from this plant.[43] Besides these compounds, three sesquiterpenes, sphaeranthol, sphaeranthene, indicusene and a phenolic ketone have been isolated from the oil,[1] however, the structures of these sesquiterpenes were not elucidated.

Recently the essential oil of flowers, roots and herb (stems with leaves) were investigated by gas chromatography spectroscopic (GC-FID and GC-MS) and olfactoric method to identify the compounds responsible for the characteristic odor as well as partly for the folk medicinal use of this plant. More than 95 volatiles were

found to be constituents of the three essential *S. indicus* oils with following composition of main components flower oil: β-eudesmol (21.4%), 2,5-dimethoxy-p-cymene (16.2%), β-caryophyllene (7.8%), τ-cadinol (7.2%), caryophyllene oxide (6.9%) and α-eudesmol (4.5%); root oil: 2,5-dimethoxy-p-cymene (28.3%), τ-cadinol (25.3%), (Z)-arteannuic alcohol (10.1%), β-maaliene (3.9%) and caryophyllene oxide (3.1%); herb oil: 2,5-dimethoxy-p-cymene (27.0%), τ-cadinol (12.5%), β-eudesmol (9.1%), α-eudesmol (7.0%) and caryophyllene oxide (4.7%). In addition, the odor impressions of the samples are described and the possible use of the essential *S. indicus* oils in medicinal, cosmetic and food flavoring discussed.[44] The Capitula of *S. indicus* gave 0.06-0.08 percent of essential oil having characteristic sweet aromatic odor. GC/MS examination of the oil has shown 15 constituents. The main constituents were cadinene, ocimene, citral, p-methoxycinnamaldehyde, geraniol, eugenol and geranyl acetate.[45] The hydrodistilled essential oil was analyzed by GC and GC/MS. Total 38 compounds making up 84.0% of the oil were identified. The composition of the oil is different from the earlier studies. The main constituents of the oil were: myrcene, borneol, β-cubebene, 2,5-dimethoxy-p-cymene, β-caryophyllene, 2,5-dimethoxy-1-isopropenyl 4-isopropylbenzene, α-agarofuran, caryophyllene oxide globulol, 10-epi-γ-eudesmol, α-muurolol, selin-ll-en-4α-ol and α-eudesmol valerianol constituting 75.2% of the oil.[46]

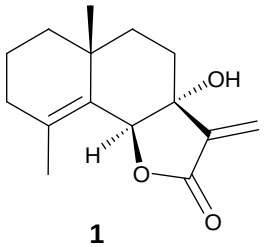
1.2.2 Compounds isolated from solvent extracts of different parts of *S. indicus*

Gupta *et al*.[47] isolated stigmasterol and β-sitosterol from the alcoholic extract of powdered caputula. However, Gogate *et al*.[32] isolated first time three new sesquiterpene lactones, i) 7α-hydroxy-4,11(13)-eudesmadien-12,6-olide (**1**), ii) 7β-hydroxy-4,11(13)-eudesmadien-12,6-olide (**2**) and iii) 7β-hydroxy-11β-methyleudesm-4-en-12,6-olide (**3**) (**Chart I**). Simultaneously Sohoni *et al*.[48] also isolated **1** and a new sesquiterpene acid, 2-hydroxycostic acid (**4**), along with the known compounds, β-eudesmol and ilicic acid from the acetone extract of *S. indicus*. The structure and relative stereochemistry of **1** was established by x-ray crystallographic studies of 4,5-epoxy derivative of **1**. At the same period Atta-ur-Rahman[33] isolated **1** which was the main component having antimicrobial activity in ethanolic extract of flowers of *S. indicus*. They further isolated a new 7-hydroxyeudesmanolide glycoside (**5**) from flowers exhibiting immunostimulant

activity.[34] In 1989 Singh *et al*.[49] isolated β-D-glucoside of (24S)-24-ethylcholesta-5,22-dien-3β-ol (**6**) from this plant. In 1991 Shekhani *et al*.[50] isolated three new 7α-hydroxy eudesmanolides (**7-9**) from flowers, whereas in 1992 Rojatkar *et al*.[51] isolated two more new 7α-hydroxy eudesmanolides (**10** and **11**) along with a known 7α-hydroxy eudesmanolide (**12**) earlier reported[52] from *Grangea maderaspatana* and two known sesquiterpenes, cryptomeridiol (**13**), possessing an antispasmodic activity earlier isolated from *Cymbopogon proximus*[53,54] and some other plant species and 4-epicryptomeridiol (**14**) which was earlier isolated from *Amanoa oblongifolia*.[55] Chughtai *et al*.[56] isolated and tentatively characterized two alkaloids (**15** and **16**) from flowers of *S. indicus*. Yadava *et al*.[57,58,59] isolated a new flavone diglycoside (**17**) from the stems and a new isoflavone glycoside (**18**) from leaves of *S. indicus*. Pujar *et al*.[60] isolated another new 7α-hydroxy eudesmanolide (**19**) and a new 7α-acetoxy eusdesmanolide (**20**) along with a new sesquiterpene, 3-keto-β-eudesmol (**21**). In 2004, we[61] isolated a 7α-hydroxy eudesmanolide (**22**) from aerial parts which was earlier reported from *Sphaeranthus suaveolens*.[62] Recently Mishra *et al*.[63] isolated a novel flavonoid *C*-glycoside (**23**) from the aerial part of *S. indicus*. In 2005, Selvanayagam *et al*.[64] isolated a novel 7-hydroxy eudesmanolide type of alkaloid (**24**) in which C-11 and lactone oxygen are replaced by nitrogen atoms.

Besides isolating above sesquiterpenes and other compounds, photooxidation reaction was carried out on major compound **1** to yield two known eudesmanolides (**10, 11**) and an epoxyeudesmanolide (**25**).[65]

CHART I



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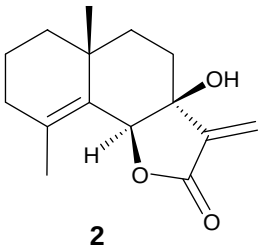

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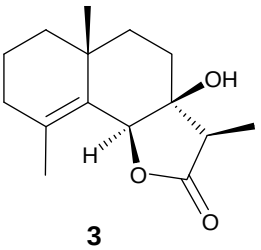

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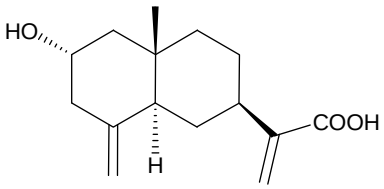

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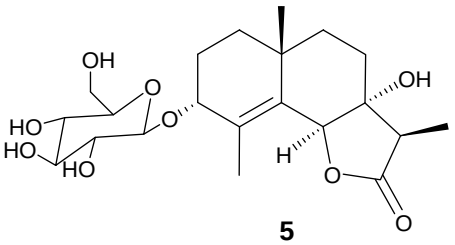

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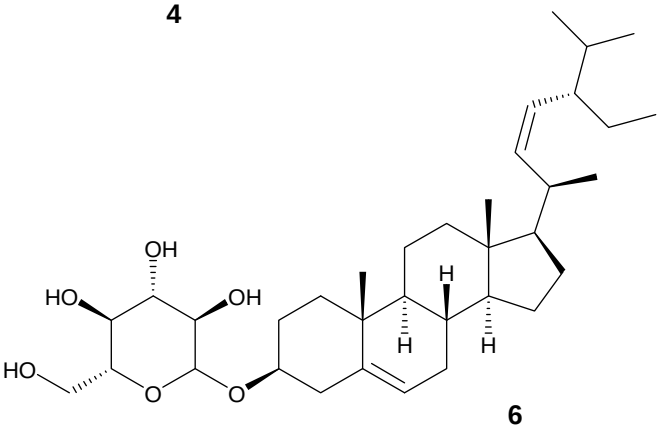

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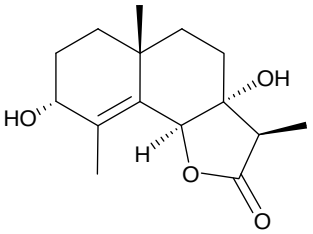

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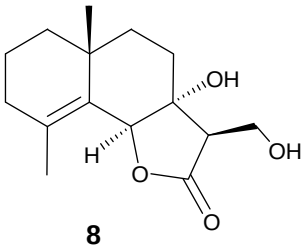

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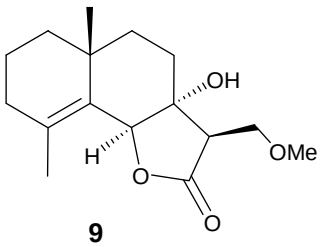

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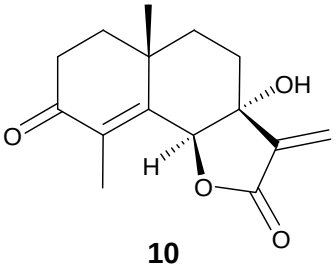

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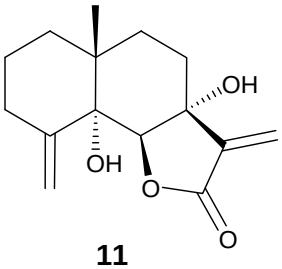

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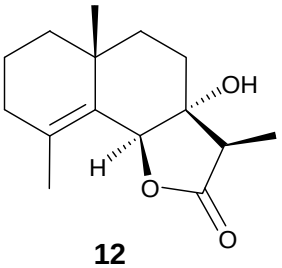

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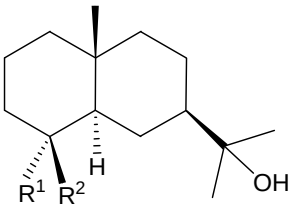
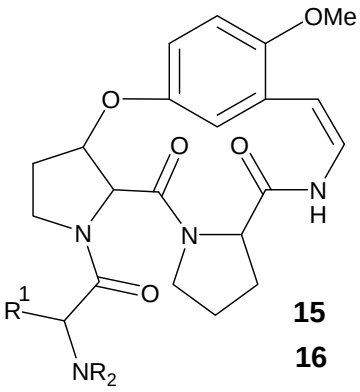


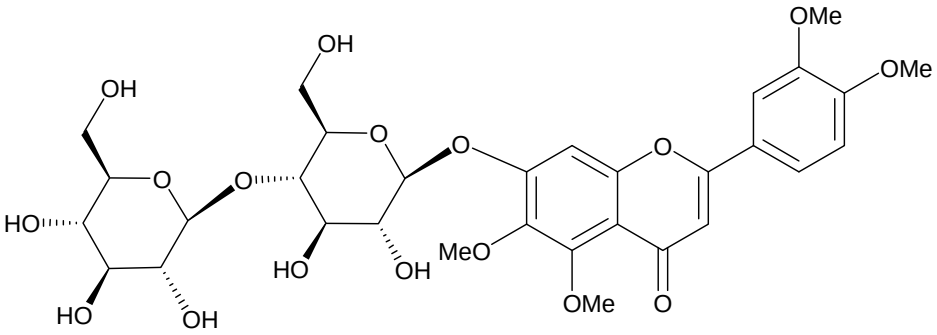
13 R^1 = OH R^2 = Me

14 R^1 = Me R^2 = OH



15 R = Me, R^1 = CH_2Ph

16 R = H, R^1 = Me



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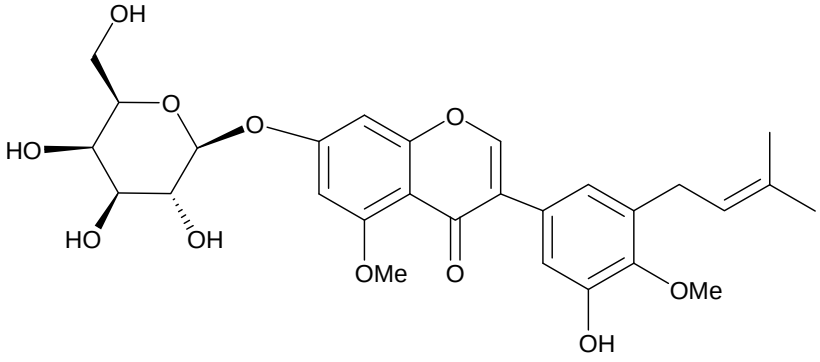

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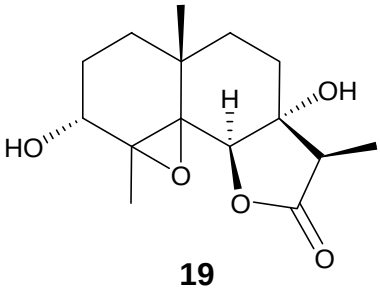

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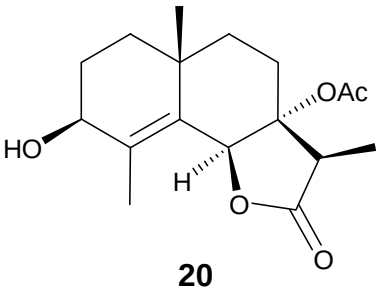

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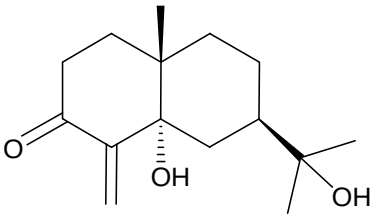

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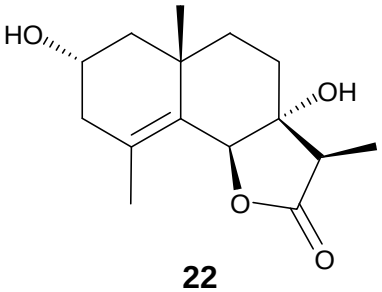

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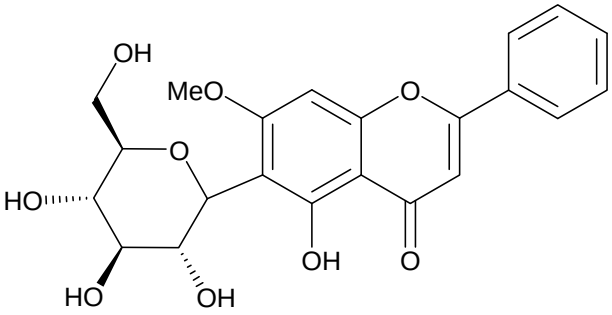

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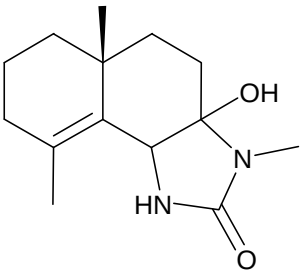

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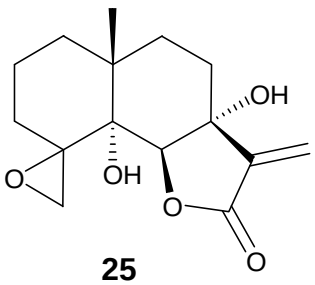

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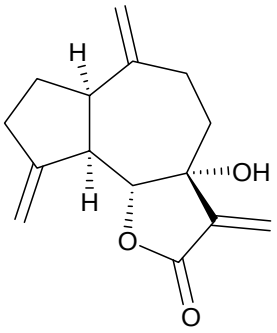
1.2.3 Compounds isolated from other *Sphaeranthus* species

Presence of sesquiterpenes being the characteristic of asteraceae (compositae) family, from *Sphaeranthus indicus*, belonging to this family, many sesquiterpenes have been isolated as mentioned above. Speciality of this plant is the presence of biologically active 7-hydroxyeudesmanolides. Such 7-hydroxysesquiterpenes are found rarely from plant kingdom. F. Bohlmann *et al*.[66] first time isolated two 7α-hydroxyguaianolides (**26, 27**) (**Chart II**) from *Podachaenium eminens*. Luengo *et al*.[67] isolated four new 7α-hydroxysesquiterpenes, two of them being the 7α-hydroxyeudesmanolides (**28**, **29**), one 7α-hydroxyguaianolide (**30**) and one 7α-hydroxycostunolide (**31**) from *Decachaeta ovatifolia*. A 7α-hydroxy eudesmanolide (**12**) has been isolated from *Grangea maderaspatana*[52] as mentioned above. Jakupovic *et al*.[62] isolated along with eudesmanolide **22** two more 7α-hydroxyeudesmanolides (**32, 33**) from *Sphaeranthus suaveolens*. However, no other *Sphaeranthus* species was found to contain 7-hydroxysesquiterpene.

CHART II



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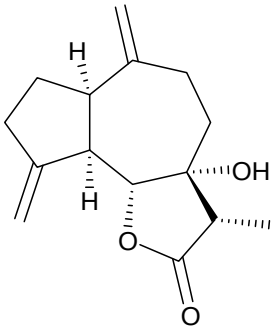

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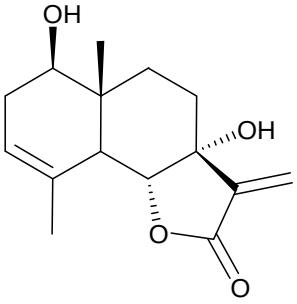

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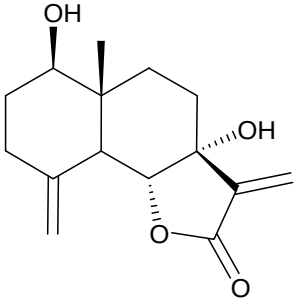

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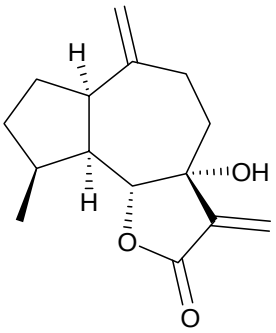

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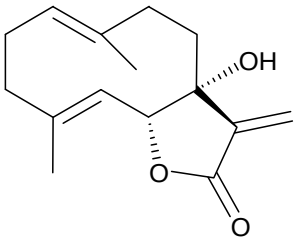

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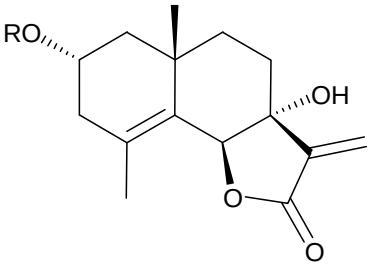


32 R = H

33 R = Ac

1.2.4 Characterization of sesquiterpene lactones

The advancement of modern spectral techniques has made it possible to find out the stereochemistry of asymmetric centers. In ^{1}H NMR, coupling constants can provide important information to assign the spatial arrangement of protons. Allylic coupling shows the stereochemistry of the α-methylene (C-13 exomethylene) γ-lactone and allylic H-7 in case of sesquiterpene lactones. The *trans*-lactones always have larger coupling constants than the *cis* lactones. The allylic couplings in the *trans*-lactones observed are normally equal to or greater than 3 Hz whereas allylic couplings in the *cis*-lactones are equal to or less than 3 Hz (Samek rule).[68] Furthermore the compounds having *trans*-lactone ring generally show larger coupling (J = 7 Hz) between the lactonic proton and the H-7, whereas in the compounds having *cis*-lactone the coupling between the lactonic proton and the H-7 is always small (J = 3 Hz).

Herz *et al*.[69] have pointed out that a small coupling constant (J = 1-1.5 Hz) between H-13 and H-7 protons is the characteristic of C-8 *cis* lactonised eudesmanolides and that somewhat larger coupling constant (J = 2-3 Hz) between H-13 and H-7 is the characteristic of guaianolides, pseudoguaianolides and C-6 *trans* lactonized eudesmanolides.

The important features in the ^{1}H NMR of sesquiterpene lactones are the appearance of the signals due to exomethylene protons (H-13a and H-13b), two doublets between δ 5.5 and δ 6.5 with small coupling constants (J =3 Hz and J =3.5 Hz) and a multiplet at δ 2.7 to δ 3.0 due to H-7 proton respectively.

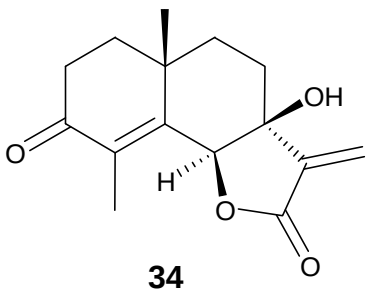
The α-methylene-γ-lactone moiety is further supported by the IR spectrum; the lactonic carbonyl normally appears at 1760-1770 cm^{-1} while the saturated sesquiterpene lactone carbonyl appears at 1780 cm^{-1}.

1.3 PRESENT WORK

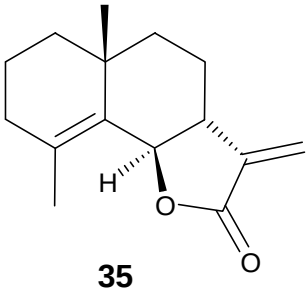
Literature survey revealed that *Sphaeranthus indicus*, a medicinally important plant possessing many biological activities, is rich in 7-hydroxyeudesmanolides and compound **1** is the major constituent of this plant possessing biological activity. It was thought that further chemical investigation of *S. indicus* could be undertaken to explore the possibilities of isolating novel compounds from this plant. It was also thought to enrich the total extract of *Sphaeranthus indicus* for compound **1**. With these aims in mind, the air-dried aerial part of *S. indicus* (850 g) was exhaustively extracted with acetone. The crude extract was successively chromatographed over silica gel and various fractions were collected as **A** to **G**. Purification of fraction **D** by column chromatography over silica gel (100-200 mesh) afforded major compound **1**. The residual fraction **D-6** after isolating compound **1** was subjected for preparative HPLC to afford a new eudesmanolide **34**. Purification of fraction **B** by successive column chromatography and preparative TLC afforded another new eudesmanolide **35** whereas purification of fraction **F** by successive column chromatography and preparative TLC afforded one more eudesmanolide **36** (**Chart III**).[70] All the compounds were fully characterized by extensive spectroscopic studies. The characterization of compound **36**, having the 7α-hydroxy group, has been discussed in Ph. D. thesis of Jadhav.[71] Characterization of compound **34** and **35** is being discussed in this chapter.

For the enrichment of compound **1** the aerial part of *S. indicus* (1 kg) was extracted with dichloromethane. Enrichment of compound **1** was monitored by TLC and HPLC.

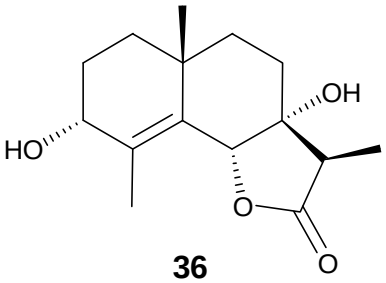
CHART III



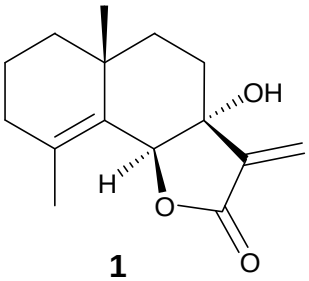
34



35



36



1

1.3.1 Enrichment of compound 1

For the enrichment study, first extraction time of compound **1** with different solvents was studied. The air-dried plant material (10 g each) was extracted with acetone, dichloromethane, methanol and acetonitrile at room temperature. All the extracts were analyzed quantitatively for percentage content of compound **1** after 4, 8 and 12 hours. This analysis revealed that compound **1** was maximum extracted in dichloromethane in 8 hours (**Table 1**). Hence extraction of plant material was carried out using dichloromethane as extraction solvent.

Thus the air-dried plant material (1 kg) was extracted with soaking in dichloromethane for 8 hours. The filtered extract was concentrated under vacuum (yield 50.72 g). This extract (50 g) was dissolved in n-hexane with sonication. The solution was kept at -5 °C for four hours. The precipitate obtained was filtered and washed with cold n-hexane. TLC of filtrate did not show presence of compound **1**.

Table 1: Quantitative HPLC analysis of different solvent extracts for the content of compound **1**

Solvent	Extraction Time (h)	Weight (mg)	Wt/wt % of compound 1	Calculated weight of compound 1 (mg)
Methanol	4	931	0.3233	3.01
	8	1634	0.2815	4.60
	12	1757	0.2788	4.99
Acetone	4	622	0.3376	2.10
	8	837	0.3835	3.21
	12	1501	0.3284	4.93
Dichloromethane	4	370	1.0594	3.92
	8	481	1.0374	4.99
	12	499	1.004	5.01
Ethyl acetate	4	125	0.554	0.69
	8	241	0.872	1.98
	12	411	0.953	3.92

The residue (15.5 g, HPLC purity of compound **1**, 3.06%) on filter paper was dissolved in acetone and adsorbed on silica gel (30 g, 60-120 mesh). The adsorbed silica gel was washed with 100 ml each of 10%, 20%, 30%, 40% and 50% acetone in n-hexane. These fractions were analyzed for presence of compound **1** by TLC and HPLC. Compound **1** was obtained in 30% and 40% acetone in n-hexane. Both fractions were mixed and concentrated (1.81 g, HPLC purity of compound **1**, 24.31%). The obtained mass was dissolved in ethyl acetate: pentane (40:30) by little warming and then chilled for 4 hrs at -5 °C. The precipitate obtained was filtered and dried to afford white powdered compound **1** (0.395 g, HPLC purity 88. 6 %).

1.3.2 Characterization of compounds 34 and 35

With the help of reported spectral data for various sesquiterpene lactones isolated from *S. indicus* the structure elucidation of compound **34** and compound **35** is discussed below.

1.3.2.1 Characterization of compound 34

Compound **34**, obtained as viscous liquid, showed in its mass spectrum M+1 peak at m/z 263 suggesting the possible molecular formula $C_{15}H_{18}O_4$, along with a

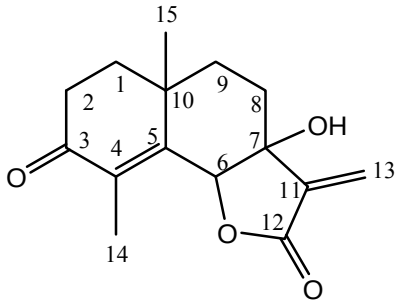
peak at m/z 244 $(M-H_2O)^+$. Its ^{13}C NMR spectrum (**Fig. 3, Table 3**) showed fifteen signals indicating the presence of fifteen carbon atoms. Its IR spectrum (**Fig. 1**) showed characteristic absorption bands at 1769 cm^{-1} for α-methylene-γ-lactone, 3401 cm^{-1} for hydroxy group, 1682 and 1598 cm^{-1} for enone and 1216 cm^{-1} (C-O stretching), thus satisfying the presence of four oxygen atoms in compound.

The ^1H NMR spectrum (**Fig. 2, Table 2**) of compound **34** showed two methyl signals, the first one at δ 1.287 which can be assigned to an angular methyl and second signal at δ 1.921 for a methyl on double bond. The spectrum further showed a singlet at δ 5.113 which could be assigned for the lactonic proton H-6. The multiplicity (singlet) of signal revealed that the lactonic proton was not coupling with either H-7 or with H-5. It also further indicated that there is a double bond at C4-C5. The spectrum further showed two singlets for one proton each at δ 5.950 and 6.415 which could be assigned to H-13a and H-13b. These clearly indicated that C-7 is a quaternary carbon and a hydroxy group is attached to C-7, which is the characteristic of this plant as described above in previous work. Thus from these spectral data, it was evident that the compound **34** should be a eudesmanolide with a double bond at C4-C5 and an α,β-unsaturated-γ-lactone at C6-C7 having a hydroxy group at C-7. The absorption at 1682 and 1598 cm^{-1} for enone in IR spectrum indicated that ketone group should be at C-3 which will be in conjugation to C4-C5 double bond. This was confirmed by appearance of signal at δ 198.51 due to enone carbonyl carbon and at δ 168.15 due to lactone carbonyl carbon in its ^{13}C NMR spectrum (**Fig**. **3**).

The ^{13}C NMR spectrum (**Fig. 3**) of compound **34** showed 15 signals revealing the presence of 15 carbon atoms which included signals at δ 198.51 and 168.15 for enone carbonyl carbon and lactone carbonyl carbon respectively, four signals at δ 150.51, 143.14, 137.92 and 123.55 due to four methylene carbons. The DEPT-135 pulse sequence (**Fig. 4**) showed that the only signal at δ 123.55 being a triplet could be due to C-13 and remaining three signals due to quaternary carbon atoms confirmed the double bond at C4-C5. The ^{13}C NMR spectrum further revealed the presence of two oxygenated carbon atoms at δ 81.70 and 76.41. The signal at δ 76.41 disappears in the DEPT experiment revealing presence of hydroxy group at C-7, and signal at δ 81.70 must be due to C-6. The DEPT experiment further revealed two quartets at δ

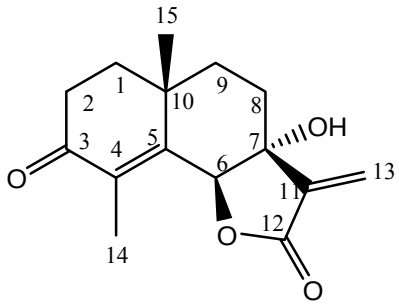
11.06 and 24.87 assignable for C-14 and C-15 respectively. The spectra further showed four triplets at δ 37.01, 34.07, 33.99, and 32.24 assigned for four methylene carbons as C-1, C-2, C-8 and C-9 and a singlet at δ 34.35 due to C-10.

Thus from above discussed spectral data compound **34** was identified as a eudesmanolide having following structure.



In ^{1}H-^{1}H COSY spectrum (**Fig. 5**) of compound **34** signals at δ 5.113, 6.415 and 5.950 due to H-6, H-13a and H-13b respectively did not show relation with any other proton, thus revealing the presence of hydroxy group at C-7. From ^{1}H NMR spectrum it could be further revealed that protons at δ 2.724 and 2.524 must be C-2 protons next to carbonyl carbon (C-3). From the multiplicity and splitting of these signals it was further revealed that proton at δ 2.724 should be β-axially oriented whereas proton at δ 2.524 should be α-equatorially oriented. The ^{1}H-^{1}H COSY spectrum further revealed that these C-2 protons coupled with protons appearing at δ 2.056 and 1.730 which could be due to C-1 protons. From the multiplicity and splitting of these protons it could be concluded that the signal at δ 2.056 is due to β-equatorially oriented proton and the signal at δ 1.730 is for α-axially oriented proton. From ^{1}H-^{13}C heteroCOSY experiment (HMQC) (**Table 4, Fig. 6**) chemical shifts of C-1 and C-2 were assigned as δ 37.01 and 33.99 respectively. Further, one of the two C-9 protons appearing most upfield at δ 1.485 coupled with proton appearing at δ 1.652 as seen from ^{1}H-^{1}H COSY spectrum. Both these C-9 protons further coupled with C-8 protons appearing at δ 1.974 and 1.768 as multiplets. From ^{1}H-^{13}C heteroCOSY experiment, signals at δ 32.24 and 34.07 could be assigned to C-8 and C-9 respectively (**Table 4**).

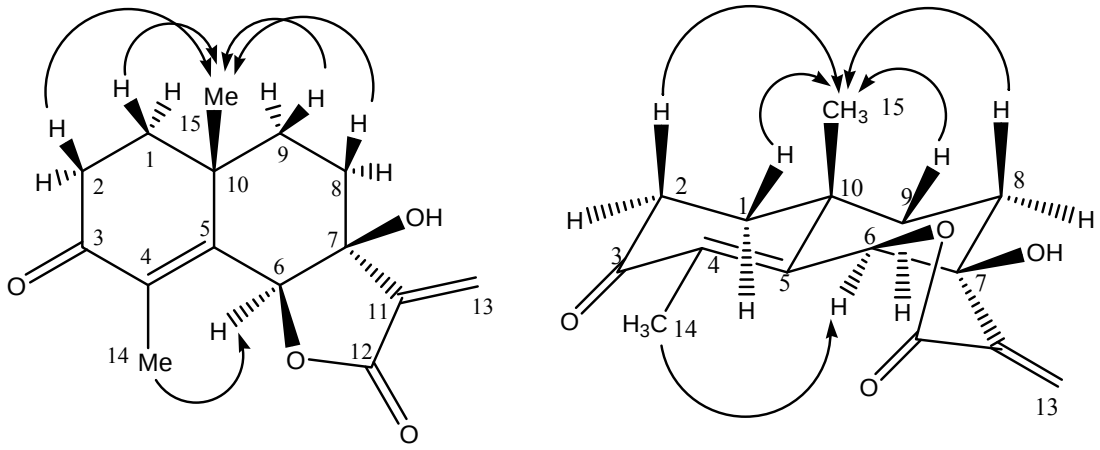
In 1992, in our group[51] compound **10** having *cis*-lactone was isolated from this plant.



10

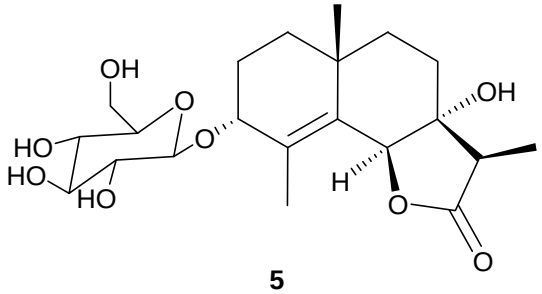
When spectral data of both compounds were compared, though there was not much difference in chemical shifts of protons in respective ^{1}H NMR spectra, there was considerable difference in chemical shifts of carbon atoms of both compounds in their ^{13}C NMR spectra, especially chemical shifts of C-3, C-4, C-6, C-7, C-12 and C-14. These facts clearly indicated that both compounds **34** and **10** are different and the difference may be either at all three C-6, C-7 and C-10 chiral centers or at any one or any two chiral centers.

Hence further NOESY experiment (**Fig. 7**) was performed to determine the spatial relationship between various protons of **34**. In this experiment axially oriented H-2β (δ 2.724) showed cross peaks with the C-15 methyl group (δ 1.287) confirming the stereochemistry of this methyl as β-axial. Further cross coupling of this methyl protons with protons at δ 2.056 (H-1, equatorial), 1.974 (H-8, axial) and 1.485 (H-9, equatorial) confirmed the β-orientation. In this NOESY experiment major cross coupling observed was between olefin methyl group (H-14) and H-6, which confirmed that H-6 was α-oriented and hence C-6 lactone oxygen bond must be β-oriented. But the NOESY experiment of compound **34** did not show correlation of hydroxy proton with other proton. All the above facts clearly indicated that hydroxy group in compound **34** must be β-oriented and hence lactone ring must be *trans*.



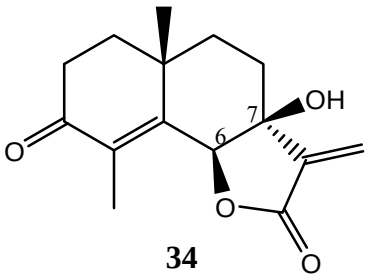
NOSEY correlation of compound **34**

The NOSEY experiment coupling result was similar to that observed by Shekhani *et al.*[34] between C-4 methyl group and H-6 and between C-10 β-methyl group and β-oriented H-1, H-2, H-8 and H-9 of compound **5**.



5

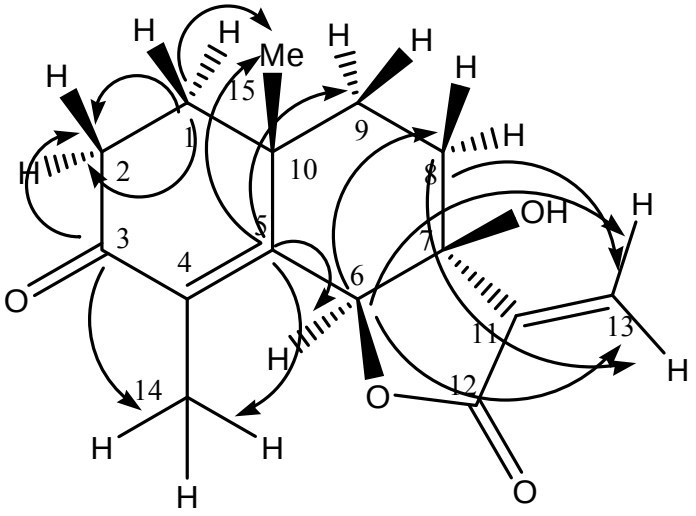
Thus from the above discussed spectral data, the structure of compound **34** was elucidated as 6β,7α,10β-4,11(13)-eudesmandiene-7β-hydroxy-3-one-12,6-olide as shown below, which is in fact 7-epimer of compound **10** and a **new 7β-hydroxy eudesmanolide**.



34

The optical rotation of compound **34** obtained was -57.57° ($CHCl_3$, *c* 0.07) whereas optical rotation of compound **10** obtained was -6.67° ($CHCl_3$).

The long range heteroCOSY experiment (HMBC) (**Table 5, Fig. 8**) was carried out to determine the couplings between carbon atoms with more distant coupled protons. In this experiment some of the long range couplings observed were as follows. C-3 showed coupling with H-2β and H-14 whereas C-12 showed coupling with H-13a and H-13b. C-13 showed coupling with only H-13a and H-13b. C-11 showed coupling with H-13a, H-13b, H-8α and H-8β. C-7 showed coupling with protons H-13a, H-13b, H-6α, H-9β and H-8β.



HeteroCOSY correlation of some carbon atoms with distinct protons of compound 34

Table 2: ^{1}H NMR spectral data of compounds **34** and **10**

Proton	**Chemical shift of Compound 34**	**Chemical shift of Compound 10**
H-1a	1.730 m	---
H-1e	2.056 m	---
H-2a	2.724 m	---
H-2e	2.524 m	---
H-6	5.113 s	5.11 s
H-8a	1.974 m	---
H-8b	1.768 m	---
H-9a	1.652 m	---
H-9b	1.485 m	---
H-13a	6.415 s	6.37 s
H-13b	5.950 s	5.94 s
H-14	1.921 s	1.90 s
H-15	1.287 s	1.27 s
OH	2.233 brs	---

Table 3: ^{13}C NMR spectral data of compounds **34** and **10**

Carbon	**Chemical shift of Compound 34**	**Chemical shift of Compound 10**
C-1	37.01 t	32.2 t
C-2	33.99 t	33.9 t
C-3	198.51 s	206.8 s
C-4	137.92 s	153.3 s
C-5	150.51 s	150.1 s
C-6	81.70 d	90.6 d
C-7	76.41 s	81.3 s
C-8	32.24 t	37.0 t
C-9	34.07 t	29.6 t
C-10	34.35 s	31.3 s
C-11	143.14 s	132.7 s
C-12	168.15 s	172.4 s
C-13	123.55 t	123.3 t
C-14	11.06 q	20.7 q
C-15	24.87 q	24.8 q

Table 4: ^{13}C NMR and heteroCOSY spectra of compound **34**

Carbon	**Chemical shift**	**Multiplicity (DEPT)**	**Connected to H (δ)**
C-1	37.01	CH_2	Hα (1.730), Hβ (2.056)
C-2	33.99	CH_2	Hα (2.524), Hβ (2.724)
C-3	198.51	quaternary	
C-4	137.92	quaternary	
C-5	150.51	quaternary	
C-6	81.70	CH	5.113
C-7	76.41	quaternary	
C-8	32.24	CH_2	Hα (1.768), Hβ (1.974)
C-9	34.07	CH_2	Hα (1.652), Hβ (1.485)
C-10	34.35	quaternary	
C-11	143.14	quaternary	
C-12	168.15	quaternary	
C-13	123.55	CH_2	Ha (6.415), Hb (5.950)
C-14	11.06	CH_3	1.921
C-15	24.87	CH_3	1.287

Table 5: Long range connectivity (hetero-COSY) of compound **34**

Carbon atom	Chemical shift	Connected to H
C-1	37.01 t	H-15, H-2α, H-2β
C-2	33.99 t	
C-3	198.51 s	H-2β, H-14
C-4	137.92 s	H-6α, H-2α, H-14
C-5	150.51 s	H-6α, H-14, H-15, H-9α, H-9β
C-6	81.70 d	H-13a, H-13b, H-8β
C-7	76.41 s	H-13a, H-13b, H-6α, H-9β, H-8β
C-8	32.24 t	H-13a, H-13b
C-9	34.07 t	
C-10	34.35 s	
C-11	143.14 s	H-13a, H-13b, H-8α, H-8β
C-12	168.15 s	H-13a, H-13b
C-13	123.55 t	H-13a, H-13b
C-14	11.06 q	H-1α, H-1β
C-15	24.87 q	H-9α, H-9β, H-15β

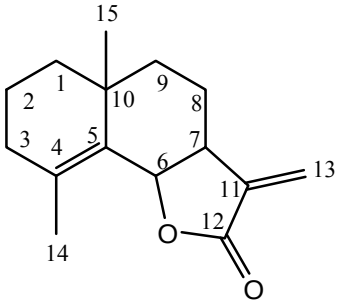
1.3.2.2 Characterization of compound 35

Compound **35,** obtained as viscous oil, showed in its mass spectrum the molecular ion peak at m/z 232 suggesting the possible molecular formula $C_{15}H_{20}O_2$, along with a peak at m/z 217 $(M\text{-}CH_3)^+$. Its ^{13}C NMR spectrum showed fifteen signals indicating the presence of fifteen carbon atoms. Its IR spectrum (**Fig. 9**) showed characteristic absorption bands at 1754 cm^{-1} for α-methylene-γ-lactone and 1216 cm^{-1} (C-O stretching), however it did not show absorption for hydroxy group, thus satisfying the two oxygen atoms in lactone ring. The ^1H NMR spectrum (**Fig. 10, Table 6**) of this compound showed two methyl signals, the first one at δ 1.05 which can be assigned to an angular methyl and second signal at δ 1.75 for a methyl on double bond. The spectrum further showed a doublet at δ 5.25 which could be

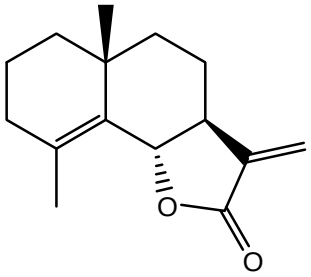
assigned for the lactonic proton H-6. The coupling constant (J = 7.0 Hz) and multiplicity of signal revealed that the lactonic proton was coupling with only H-7 and both these protons are *trans* to each other. It also further indicated that there is a double bond at C4-C5. The spectrum further showed two singlets for one proton each at δ 5.56 and 6.14 which could be assigned to H-13a and H-13b. Besides these signals, a multiplet for one proton at δ 2.95 was observed which could be due to H-7. Thus from these spectral data, it was evident that the compound **35** should be a eudesmanolide with a double bond at C4-C5 and a *trans* C6-C7 α,β-unsaturated-γ-lactone.

The ^{13}C NMR spectrum (**Fig. 11, Table 7**) of compound **35** showed 15 signals revealing the presence of 15 carbon atoms which included signal at δ 171.2 for lactone carbonyl carbon and four signals at δ 142.9, 138.8, 129.1 and 120.4 due to four methylene carbons. The DEPT-135 pulse sequence (**Fig. 12**) showed that the only signal at δ 120.4 being a triplet could be due to C-13 and remaining three signals due to quaternary carbon atoms confirmed the double bond at C4-C5. The DEPT experiment further revealed the presence of only one oxygenated carbon atom at δ 76.3 which must be due to C-6 methine. The DEPT-135 experiment further revealed two quartets at δ 16.6 and 26.1 assignable for C-15 and C-14 respectively. The spectrum further showed five triplets at δ 39.4, 38.2, 33.4, 25.3 and 18.4 assigned for five methylene carbons as C-1, C-2, C-3, C-8 and C-9 and a doublet at δ 41.5 due to C-7.

Thus from above discussed spectral data compound **35** was identified as a eudesmanolide having following structure.

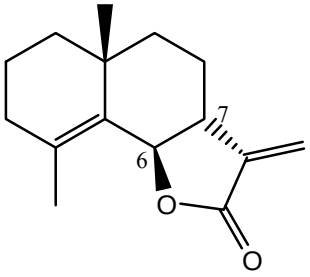


The ^{1}H NMR and ^{13}C NMR spectral data of compound **35** was further critically compared with ^{1}H NMR spectral data[72] (**Table 6**) and ^{13}C NMR spectral data[73] (**Table 7**) of (+)-arbusculine-B (**37**).



37

From comparison of spectral data and specific rotation of compound **35** ($[\alpha]_D^{26}$ = -24.32°, $CHCl_3$, *c* 0.7) and specific rotation of compound **37** ($[\alpha]_D^{26}$ = +22.2°, CH_3OH, *c* 0.135),[74] compound **35** was characterized[70] as eudesman-4-en-6β,7α-olide as shown below.



35

Table 6: ^{1}H NMR spectral data of compounds **35** and **37**

Proton	Compound **35**	Compound **37**
H-6α	5.25 d (*J* = 7.0 Hz)	---
H-6β	---	4.54 diffused doublet
H-7α	---	2.53 m
H-7β	2.95 m	---
H-13a	5.56 s	5.52 d (*J* = 3.0 Hz)
H-13b	6.14 s	6.09 d (*J* = 3.2 Hz)
H-14	1.79 s	1.86 d (*J* = 1.0 Hz)
H-15	1.05 s	1.11 s

Table 7: ^{13}C NMR spectral data of compounds **35** and **37**

Carbon	Compound **35**	Compound **37**
C-1	39.6	23.2
C-2	25.6	26.2
C-3	38.3	34.4
C-4	142.9	127.0
C-5	138.8	130.1
C-6	76.3	83.4
C-7	41.7	50.5
C-8	33.6	37.3
C-9	18.7	40.9
C-10	33.1	41.4
C-11	129.1	139.8
C-12	171.2	170.3
C-13	120.4	117.7
C-14	26.3	18.9
C-15	19.8	19.9

1.3.3 Conclusion

Thus a major 7-hydroxyeudesmanolide (**1)** possessing biological activity was isolated by enriching the dichloromethane extract of *Sphaeranthus indicus* by solvent-solvent extraction and broad column chromatography and finally by precipitation avoiding succesive chromatography. Being a medicinally important plant possessing many biological activities and rich in 7-hydroxyeudesmanolides, further chemical investigation of its acetone extract was carried out. By successive column chromatography and finally by preparative HPLC two more new eudesmanolides (**34** and **35**) have been isolated and their structures are elucidated by extensive spectral analysis. The structure of **34** was established as 6β,7α,10β-4,11(13)-eudesmandiene-7β-hydroxy-3-one-12,6-olide and compound **35** was characterized as eudesman-4-en-6β,7α-olide.

1.4 EXPERIMENTAL

1.4.1 Plant material

The plant *Sphaeranthus indicus* was collected from Mulshi village near Pune, Maharashtra, India, during January 2004. The plant was identified by Botanical Survey of India, Pune, India. Roots were separated from whole plant. The aerial part of the plant was shade dried and powdered.

1.4.2 Extraction

The powdered plant material (850 g) was extracted continuously with acetone in a soxhlet extractor on a water bath. The progress of the extraction was monitored by checking the yield of extract in 50 ml aliquot of fresh extract. The extract was concentrated on water-bath and the last traces of the solvent were removed under vacuum to obtain a dark colored residue (27.1 g).

1.4.3 Chromatographic separation

The extract (25 g) was chromatographed over silica gel (60-120 mesh, 500 g). The elution was carried out first using pet ether and then with pet ether-acetone with increasing percentage of acetone. Fractions of each 100 ml volume were collected. The progress of the column chromatographic separation was monitored by thin layer chromatography of the fractions. Fractions showing similar composition were combined together to obtain seven major fractions (**A** - **G**). The details of the chromatographic separation are summarized in **table 8**.

Table 8: Column chromatography of total extract

Fr. No.	Eluent	Total Volume collected	Weight	Approximate composition
A	Pet ether: Acetone (90:10)	3 x 100 ml	3.25 g	Straight chain hydro carbons
B	Pet ether: Acetone (80:20)	4 x 100 ml	2.74 g	Mixture of compound **35** and unidentified compounds
C	Pet ether: Acetone (80:20)	3 x 100 ml	2.52 g	Mixture of compound **1** + unidentified compounds
D	Pet ether: Acetone (70:30)	5 x 100 ml	4.27 g	Mixture of compound **1** + compound **34** and unidentified compounds
E	Pet ether: Acetone (70:30)	3 x 100 ml	1.78 g	Mixture of unidentified compounds
F	Pet ether: Acetone (60:40)	6 x 100 ml	1.96 g	Mixture of compound **36** + unidentified compounds
G	Pet ether: Acetone (50:50)	8 x 100 ml	2.98 g	Mixture of unidentified compounds

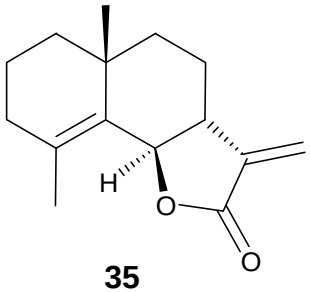
1.4.3.1 Chromatography of fraction B

A portion of fraction **B** (1.5 g) was rechromatographed over silica gel (100 – 200 mesh, 60 g) using petroleum ether-acetone mixture as eluent with successive increase in percentage of acetone. Fraction collected in pet ether-acetone (75:25) was concentrated (214 mg). This fraction (**B-5**) was further purified by successive preparative thin layer chromatography using pet ether-acetone (70:30) as mobile phase to afford compound **35** (12 mg). The details of the chromatography are summarized in **table 9**.

Table 9: Column chromatography of fraction **B**

Fraction No.	Eluent	Volume (ml)	Weight (mg)	Approximate Composition
B -1	Pet ether:acetone (95:05)	2 x 100	90	Mixture of unidentified compounds
B -2	Pet ether:acetone (90:10)	3 x 100	120	Mixture of unidentified compounds
B -3	Pet ether:acetone (85:15)	3 x 100	90	Mixture of unidentified compounds
B -4	Pet ether:acetone (80:20)	3 x 100	70	Mixture of unidentified compounds
B -5	Pet ether:acetone (75:25)	2 x 100	214	Mixture of unidentified compounds + compound **35**
B -6	Pet ether:acetone (70:30)	3 x 100	210	Mixture of unidentified compounds
B -7	Pet ether:acetone (65:35)	3 x 100	130	Mixture of unidentified compounds

Eudesm-4-en-6β,7α-olide (35)



35

Nature: Colorless gum; Yield: 12 mg; $[\alpha]_D^{26}$: - 24.32 ° ($CHCl_3$, *c* 0.7).

IR ($CHCl_3$): ν_{max} 1753, 1640, 1216 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ 1.05 (s, H-15), 1.79 (s, H-14), 2.95 (m, H-7β), 5.25 (d, *J* = 7.0 Hz, H-6α), 5.56 (s, H-13a), 6.14 (s, H-13b).

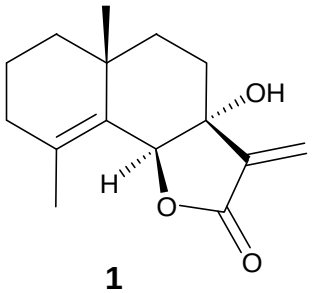
^{13}C NMR (100 MHz, $CDCl_3$): δ 18.7 (C-9), 19.8 (C-15), 25.6 (C-2), 26.3 (C-14), 33.1 (C-10), 33.6 (C-8), 38.3 (C-3), 39.6 (C-1), 41.7 (C-7), 76.3 (C-6), 120.4 (C-13), 129.1 (C-11), 138.8 (C-5), 142.9 (C-4), 171.2 (C-12).

Mass: 232 (M^+).

1.4.3.2 Chromatography of fraction D

Fraction **D** (4.2 g) was rechromatographed over silica gel (100 – 200 mesh, 120 g) using petroleum ether: ethyl acetate (80:20) as eluent with successive increase in percentage of ethyl acetate. Fractions showing similar TLC were mixed. Such ten fractions were collected (**D-1** to **D-10**). The details of the chromatographic separation are summarized in **table 10**. Fraction **D-6** eluted in pet ether: ethyl acetate (60:40) showed a major spot on TLC. The fraction was concentrated and crystallized from ethyl acetate-pentane. Crystals obtained (120 mg) were filtered and dried. Crystals were characterized as the major compound **1** comparing its spectral data with that of authentic compound earlier isolated in our group. The mother liquor was concentrated to give 150 mg residue.

7α-Hydroxyeudesm-4-en-6β,7β-olide (1)



1

Nature: White crystals; mp: 62-63 °C; $[\alpha]_D^{26}$: - 56.81° ($CHCl_3$, *c* 0.43).

IR ($CHCl_3$): ν_{max} 3400, 1760, 1650 cm^{-1}.

^{1}H NMR (90 MHz, $CDCl_3$): δ 1.04 (s, H-15), 1.73, (s, H-14), 5.04 (s, H-6α), 5.75, (s, H-13a), 6.31 (s, H-13b).

^{13}C NMR (50 MHz, $CDCl_3$): δ 18.31 (C-15), 19.49 (C-14), 26.32 (C-9), 31.78 (C-2), 32.88 (C-1), 33.27 (C-10), 35.09 (C-8), 38.99 (C-3), 76.12 (C-7), 81.75 (C-6), 121.27 (C-13), 127.31 (C-11), 140.5 (C-5), 145.25 (C-4), 169.71 (C-12).

Mass: 248 (M^+).

Table 10: Chromatography of fraction **D**

Fraction No.	Eluent	Volume (ml)	Weight (mg)	Approximate Composition
D-1	Pet ether: EtOAc (80:20)	2 x 100	310	Mixture of unidentified compounds
D-2	Pet ether: EtOAc (70:30)	3 x 100	430	Mixture of unidentified compounds
D-3	Pet ether: EtOAc (35:45)	3 x 100	290	Mixture of unidentified compounds
D-4	Pet ether: EtOAc (50:50)	3 x 100	270	Mixture of unidentified compounds
D-5	Pet ether: EtOAc (45:55)	2 x 100	330	Mixture of compound **1** and other unidentified compounds
D-6	Pet ether: EtOAc (40:60)	3 x 100	280	Mixture of compound **1** and **34** and other unidentified compounds
D-7	Pet ether: EtOAc (35:65)	3 x 100	410	Mixture of compound **1** and other unidentified compounds
D-8	Pet ether: EtOAc (35:65)	4 x 100	330	Mixture of unidentified compounds
D-9	Pet ether: EtOAc (30:70)	3 x 100	510	Mixture of unidentified compounds
D-10	Pet ether: EtOAc (25:75)	3 x 100	205	Mixture of unidentified compounds

TLC of the residual fraction **D-6** (150 mg) showed that it was a complex mixture of overlapping spots including spot of compound **1**. Hence it was analyzed by HPLC and further purified by preparative HPLC to afford compound **34** (3 mg).

1.4.3.3 Separation of compound 34 by preparative HPLC

Initially, compound **1** isolated by column chromatography (NMR pure) was analyzed. Its retention time was 10.6 min and purity was 95.4% (area).

The residual fraction (**D-6**) was analyzed using same parameters as those applied for analysis of compound **1**. It showed main two compounds, first compound **1** as major component [55.7% (area)] and another major component at RT 15.7 min having 14.3% area along with several minute components (**Fig. 13**).

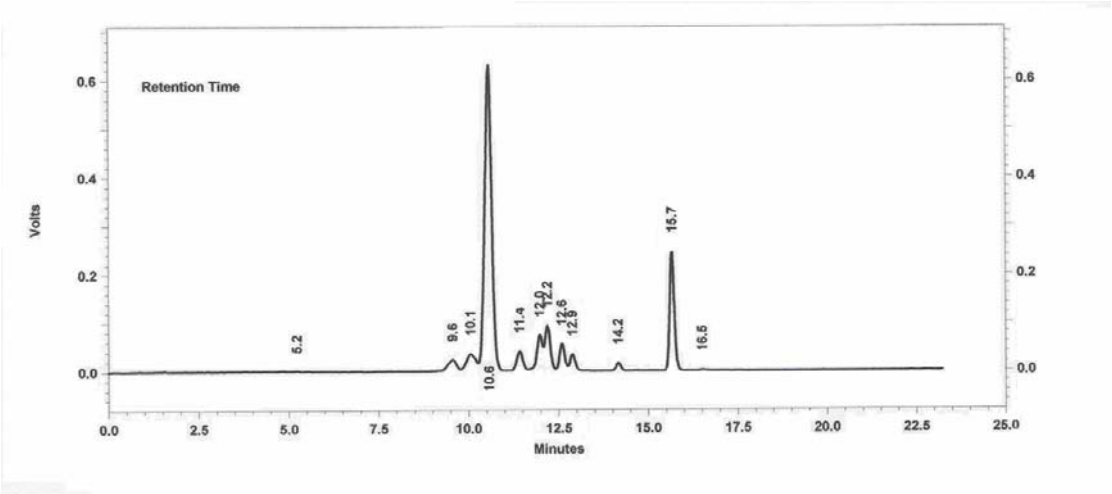


Fig. 13: Chromatogram of residual fraction **D-6**

To separate the major components from residual fraction **D-6** preparative HPLC was performed. The preparative HPLC was carried out on a Shimadzu SCL-8A system controller, SPD-10A*VP* multi wavelength detector, LC-8A HPLC pumps, CR-7A data acquisition and processing system and 7725i Rheodyne manual injector with 1 ml sample loop. Mobile phases for both A and B pumps were same as those used in analytical HPLC. Column used was C18 Waters XBridge, 5 µm (10 mm x 250 mm) and gradient elution was carried out at flow rate 4.7 ml/min.

Residual fraction **D-6** was dissolved in mobile phase (30 ml) used in pump B with sonication and was filtered through 0.45 µm nylon filter paper. Each time 0.8 ml of dissolved sample was loaded. Two chromatographic fractions first of compound **1** (RT 10.6 min) and second component at RT 15.7 min were collected manually. Both the fractions were concentrated at 40 °C under vacuum. The yield of compound **34**

(RT 15.7 min) was 3 mg. Purity both the compounds (**1** and **34**) were more than 99% (HPLC, **Fig. 14** and **Fig. 15**).

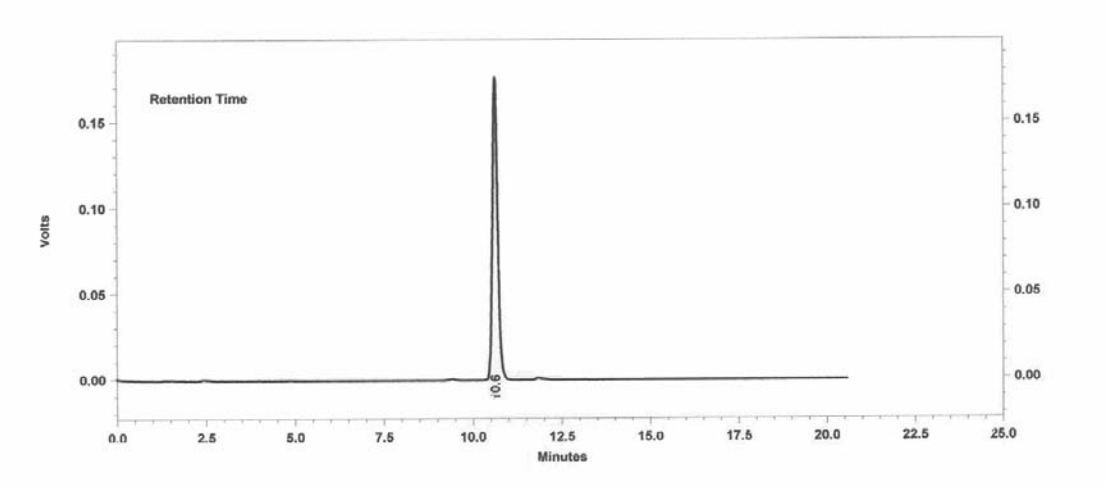


Fig. 14: Chromatogram of compound **1**

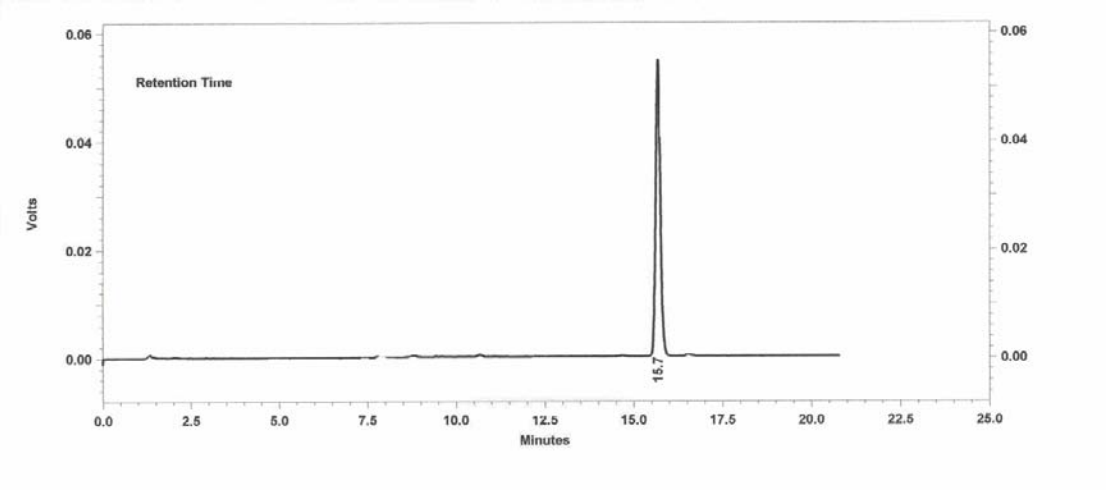
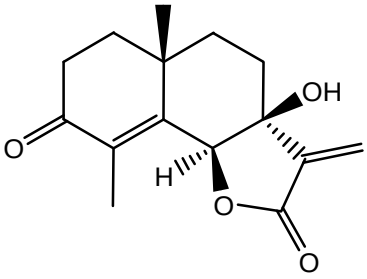


Fig. 15: Chromatogram of compound **34**

7β-Hydroxyeudesm-4-en-3-on-6β,7α-olide (34)



34

Nature: Colorless gum; Yield: 3 mg; $[\alpha]_D^{25}$: + 23° ($CHCl_3$, c 0.5)

IR ($CHCl_3$): ν_{max} 2900, 1760, 1650 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ1.287 (s, H-15), 1.485 (m, H-9b), 1.652 (m, H-9a), 1.730 (m, H-1a), 1.768 (m, H-8b), 1.921 (s, H-14), 1.974 (m, H-8a), 2.056 (m, H-1e), 2.524 (m, H-2e), 2.724 (m, H-2a), 5.113 (s, H-6), 5.950 (s, H-13b), 6.415 (s, H-13a).

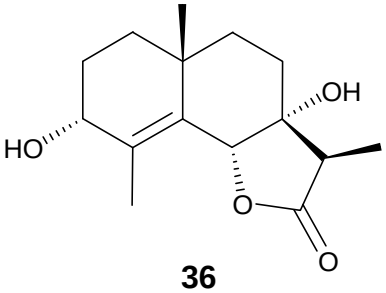
^{13}C NMR (100 MHz, $CDCl_3$): δ 11.06 (C-14), 24.87 (C-15), 32.24 (C-8), 33.99 (C-2), 34.07 (C-9), 34.35 (C-10), 37.01 (C-1), 76.41 (C-7), 81.70 (C-6), 123.55 (C-13), 137.92 (C-4), 143.14 (C-11), 150.51 (C-5), 168.15 (C-12), 198.51 (C-3).

Mass: 262 (M^+).

1.4.3.4 Rechromatography of fraction F

Fraction **F** (1.90 g) was rechromatographed over silica gel (100 – 200 mesh, 60 g) using petroleum ether:acetone mixture as eluent with successive increase in percentage of acetone. Fraction collected in pet ether-acetone (65:35) was concentrated (187 mg). This fraction was further purified by preparative thin layer chromatography using pet ether-acetone (65:35) to afford compound **36** (22 mg).

3α,7α-Dihydroxy-11 α,13-dihydroeudesm-4-en-6 α,7β,-olide (36)



36

Nature: Off white crystals.

mp: 123-124 °C; Yield: 17 mg; $[\alpha]_D^{25}$: +11.6° ($CHCl_3$, *c* 0.3).
IR ($CHCl_3$): ν_{max} 3608, 3397, 1771, 1655, 1215 cm^{-1}.
^1H NMR (400 MHz, $CDCl_3$): δ 1.03 (s, H-15), 1.22 (d, *J* = 7.5 Hz, H-13), 1.94 (s, H-14), 2.76 (q, *J* = 7.5 Hz, H-11), 4.00 (d, *J* = 2 Hz, H-3), 4.88 (s, H-6).
^{13}C NMR (100 MHz, $CDCl_3$): δ 7.1 (C-13), 17.7 (C-15), 24.0 (C-14), 25.0 (C-2), 27.4 (C-8), 33.8 (C-10), 33.9 (C-9), 34.8 (C-1), 47.8 (C-11), 69.6 (C-3), 77.2 (C-7), 79.9 (C-6), 131.3 (C-5), 139.7 (C-4), 176.3 (C-12).

1.4.4 Enrichment of compound 1

The air-dried plant material (10 g each) was soaked in each 50 ml of methanol, acetone, dichloromethane and ethyl acetate for 4 h, 8 h and 12 hours separately at room temperature. All the extracts were filtered and concentrated under reduced pressure at 40 °C and were analyzed quantitatively for percentage content of compound **1**.

The air-dried plant material (1 kg) was soaked in dichloromethane (5 lit) for 8 hours. The extract was filtered and the residual powder was washed with 1 lit of dichloromethane. Total filtrate was concentrated under reduced pressure (yield 50.72 g). The concentrated mass (50 g) was dissolved in n-hexane (200 ml) with sonication. The solution was kept at -5 °C for four hours. The turbid solution was centrifuged and the precipitate settled was filtered and washed with cold n-hexane. The sticky residue (15.5 g, HPLC purity of compound **1**, 3.06%) on filter paper was dissolved in acetone (20 ml) and adsorbed on silica gel (30 g, 60-120 mesh). The adsorbed silica gel was washed with each 100 ml of 10%, 20%, 30%, 40% and 50% acetone in n-hexane respectively. Fractions containing compound **1** (30% and 40% acetone in n-hexane) were mixed and concentrated to get a brown mass (1.81 g, HPLC purity of compound **1**, 24.31%). The brown mass was dissolved in ethyl acetate: pentane (40:30) and chilled for 4 h at -5 °C. The precipitate obtained was filtered and dried to afford white powdered compound **1** (0.395 g, HPLC purity 88. 6 %).

Linearity was studied for detector and compound **1** loading. The linear range of analysis was found from 1 µg to 30 µg. Relative standard deviation for results was 0.2%. Freshly prepared samples and standards in mobile phase were used for injection every day.

Analysis was done on binary gradient HPLC system (Shimadzu Japan) consisting of SPD-10Avp multi wavelength detector, SCL-10A*VP* system controller, LC-10AT*VP* HPLC pumps and Class *VP* software. Column used was C18 Waters XBridge, 5 μm, (4.6 mm x 250 mm) and the UV- detector was set at 254 nm. Mobile phase used in pump A was methanol:acetonitrile:water (20:10:70) and in pump B was methanol:acetonitrile:water (70:20:10). Column was equilibrated with mobile phase in pump A. Gradient elution was carried out at flow rate 1.0 ml/min. The linear gradient of mobile phase was changed within 20 min from 100% pump A to 100% pump B after injection of sample. Retention time of compound **1** was 10.6 min and its purity was 95.4% (area %).

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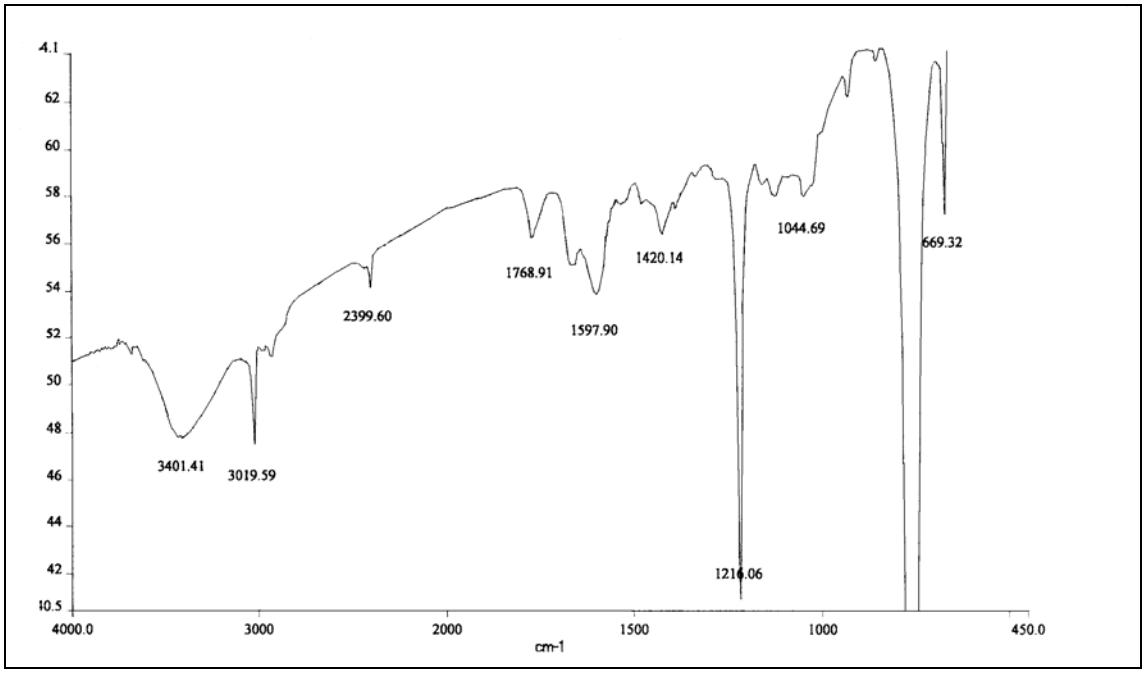
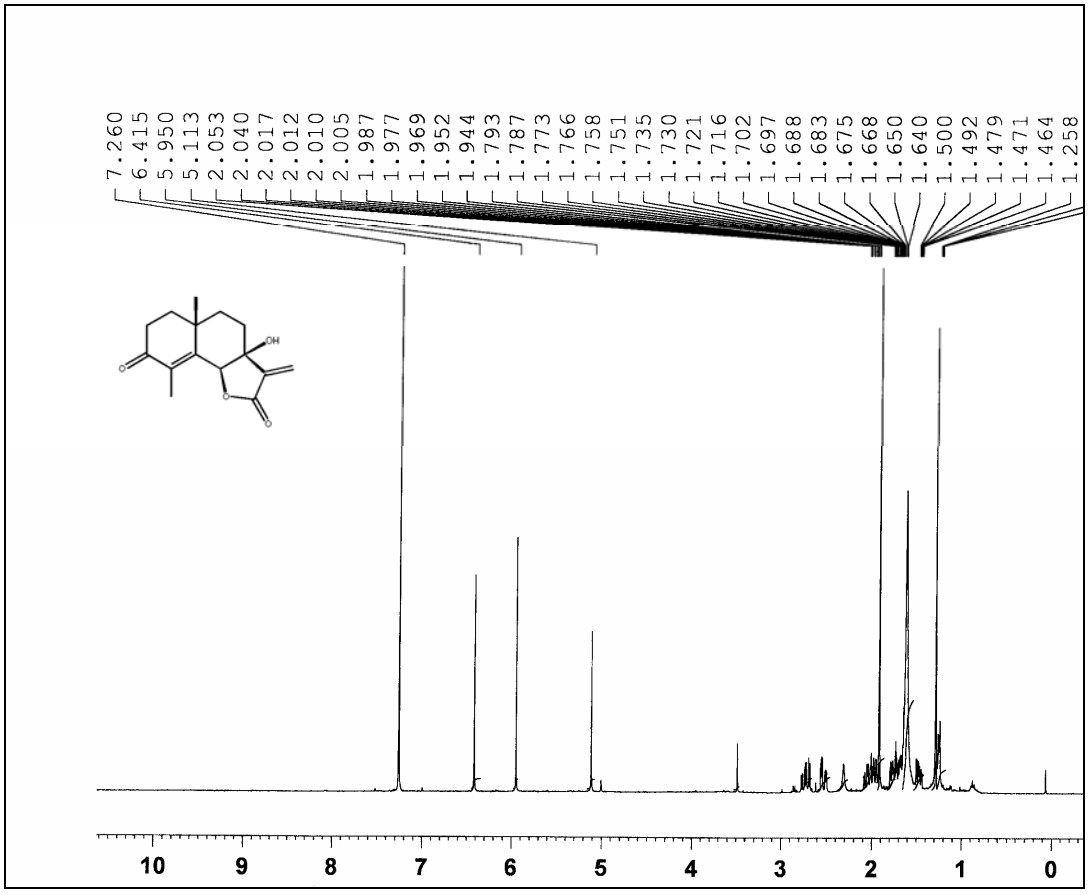


Fig. 1: IR spectrum of compound **34**



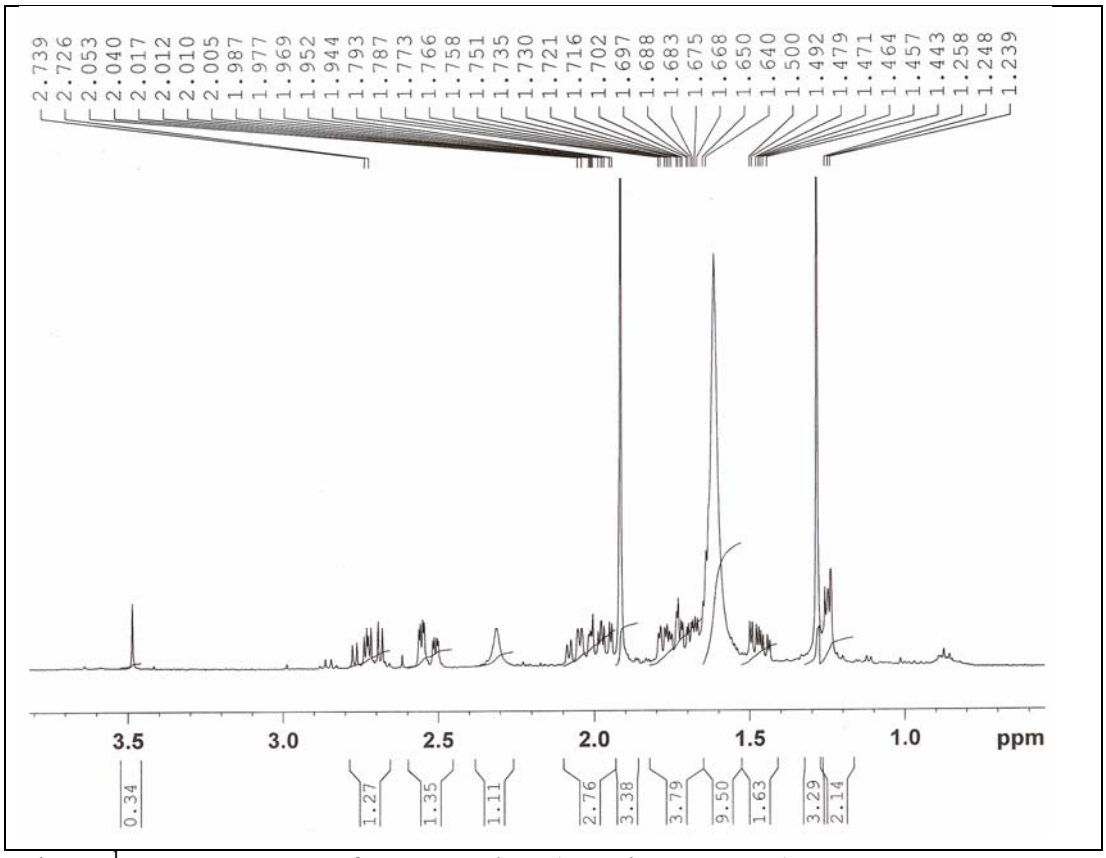


Fig. 2: ^{1}H NMR spectra of compound **34** ($CDCl_3$, 400 MHz)

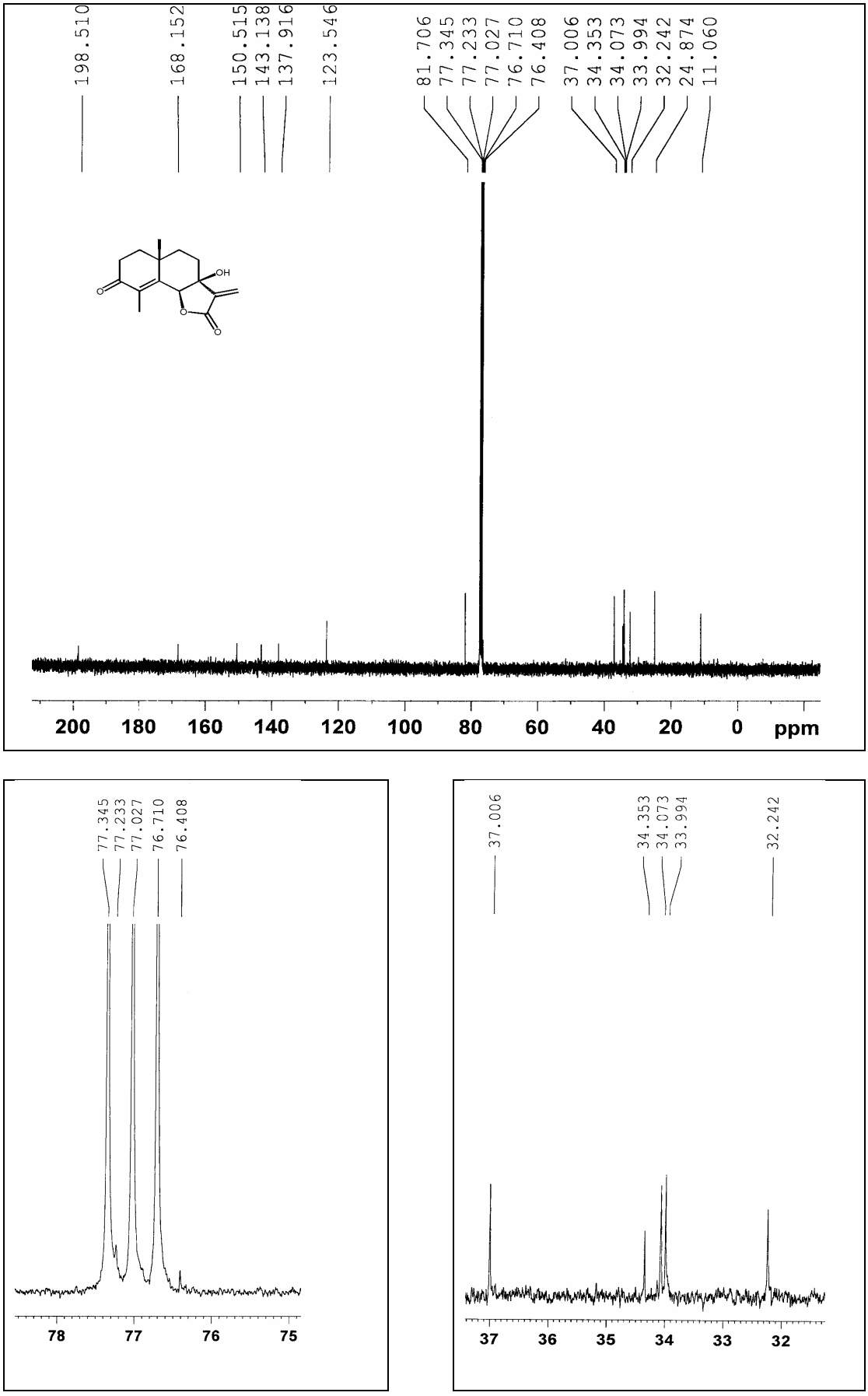


Fig. 3: ^{13}C NMR spectra of compound **34** ($CDCl_3$, 100 MHz)

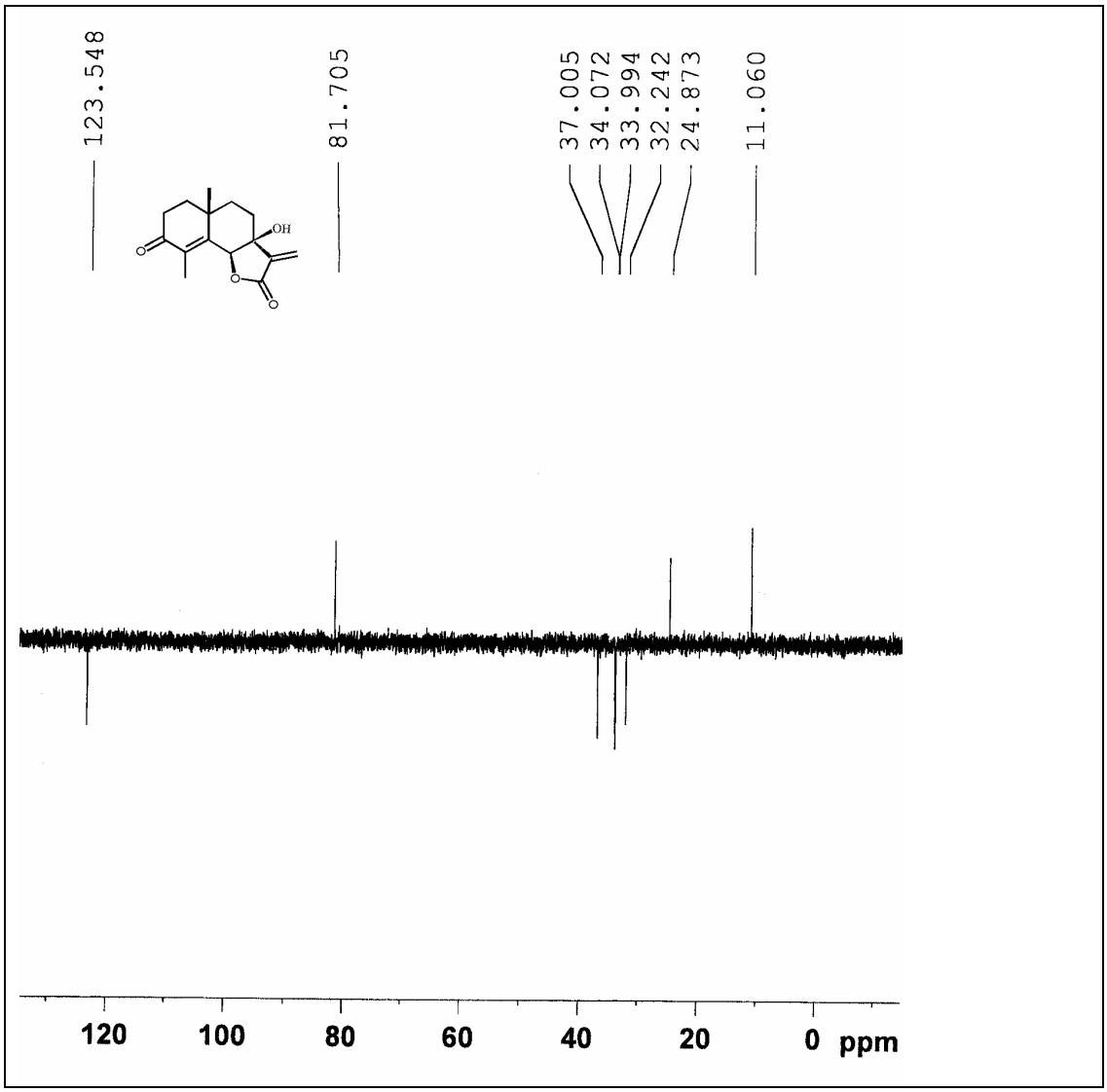


Fig. 4: DEPT spectrum of compound **34** ($CDCl_3$)

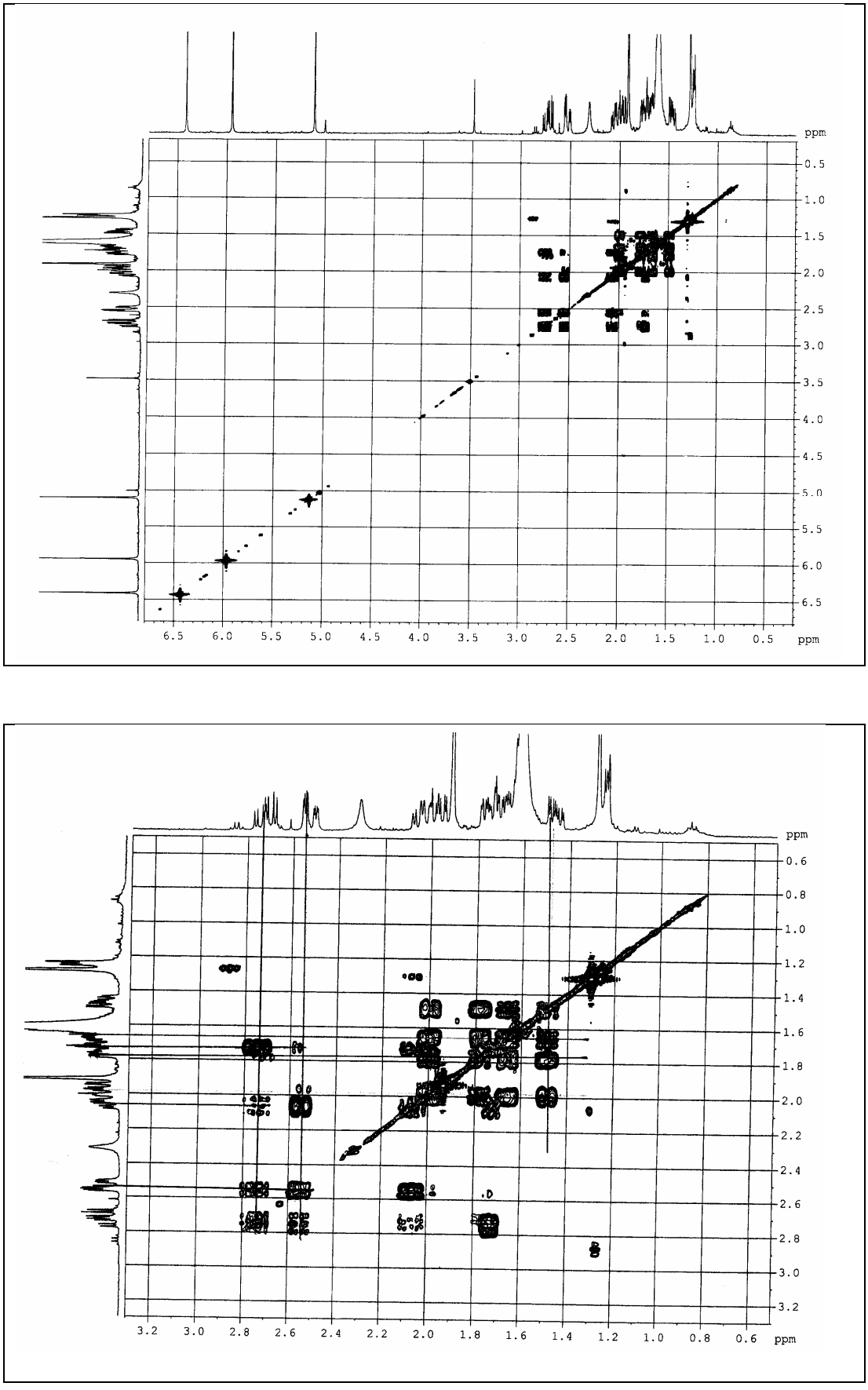
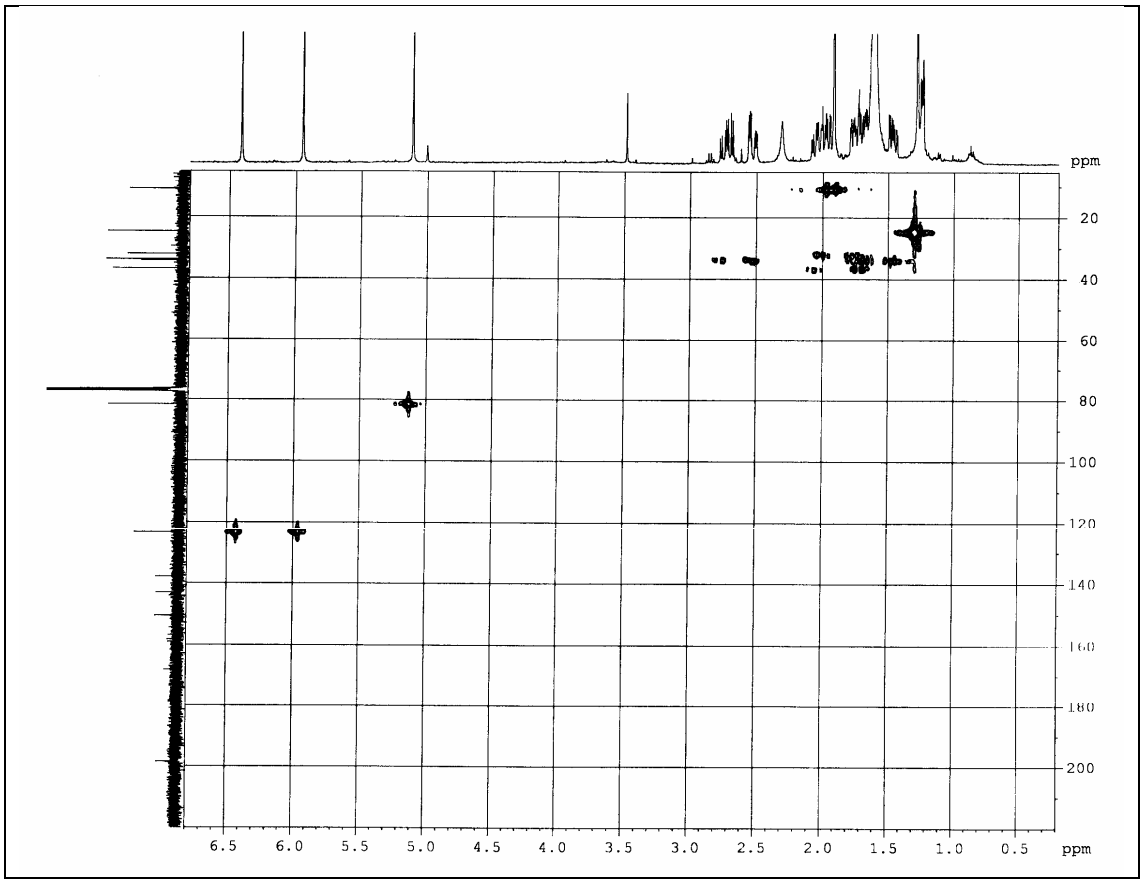


Fig. 5: ^{1}H-^{1}H 2D COSY experiment of compound **34**



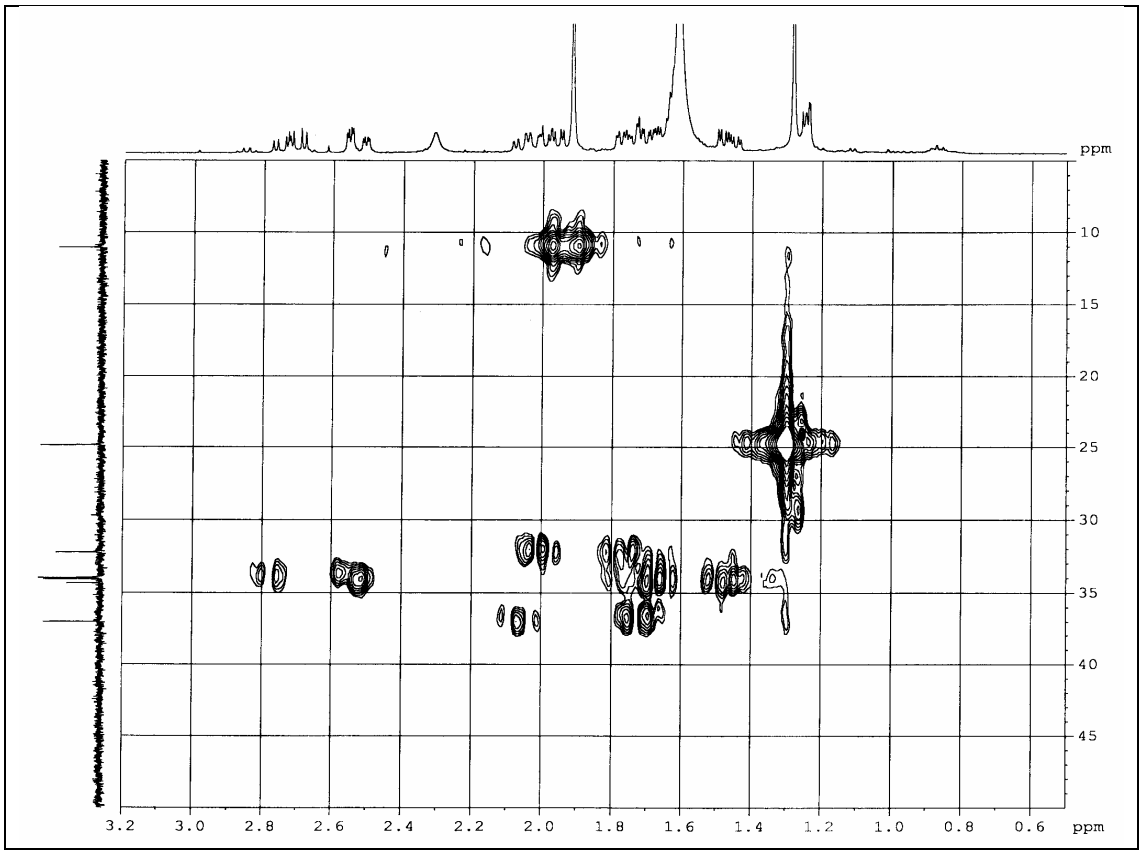


Fig. 6: HMQC experiment of compound **34**

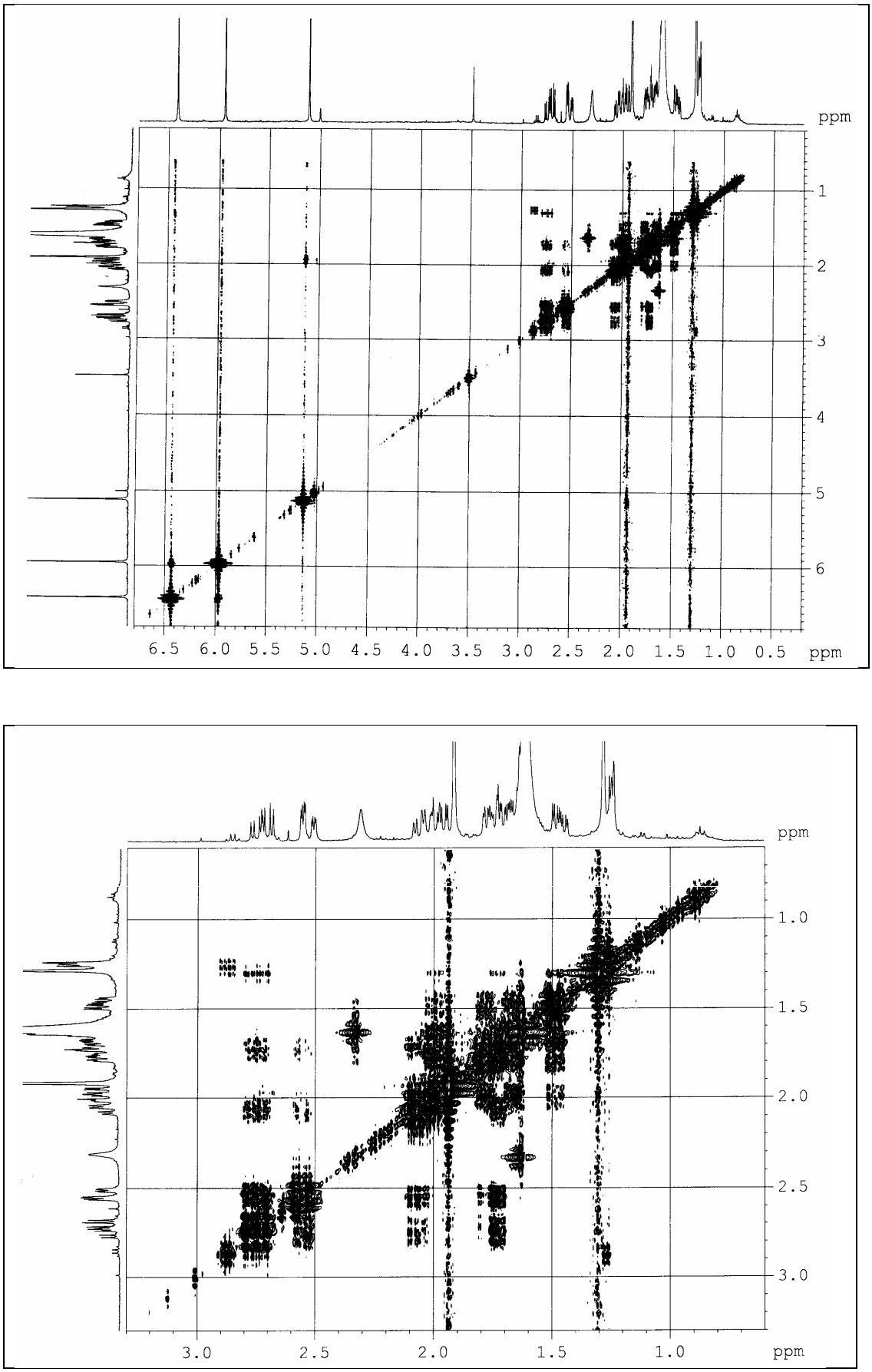
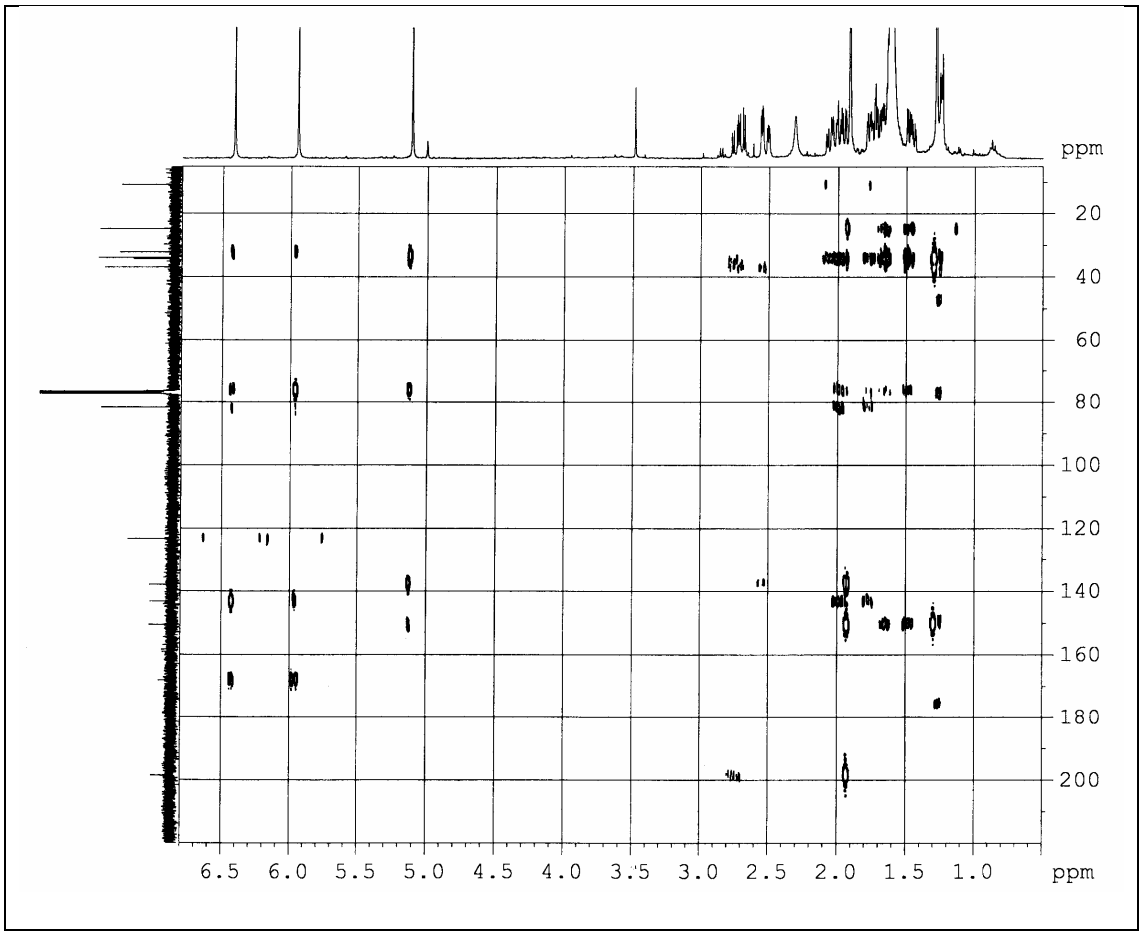


Fig. 7: NOESY experiment of compound **34**



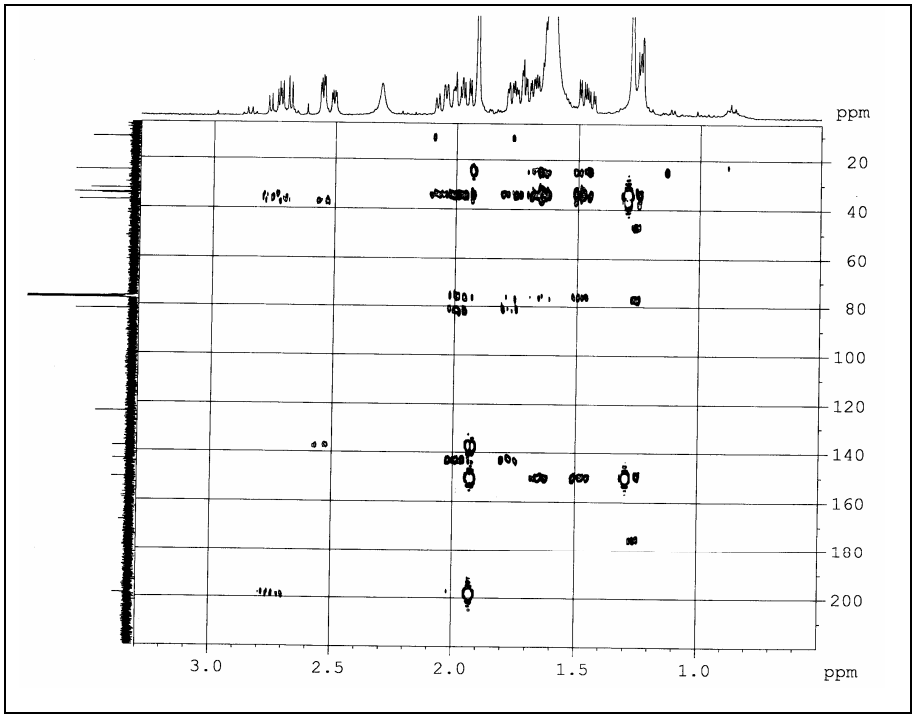


Fig. 8: HMBC experiment of compound **34**

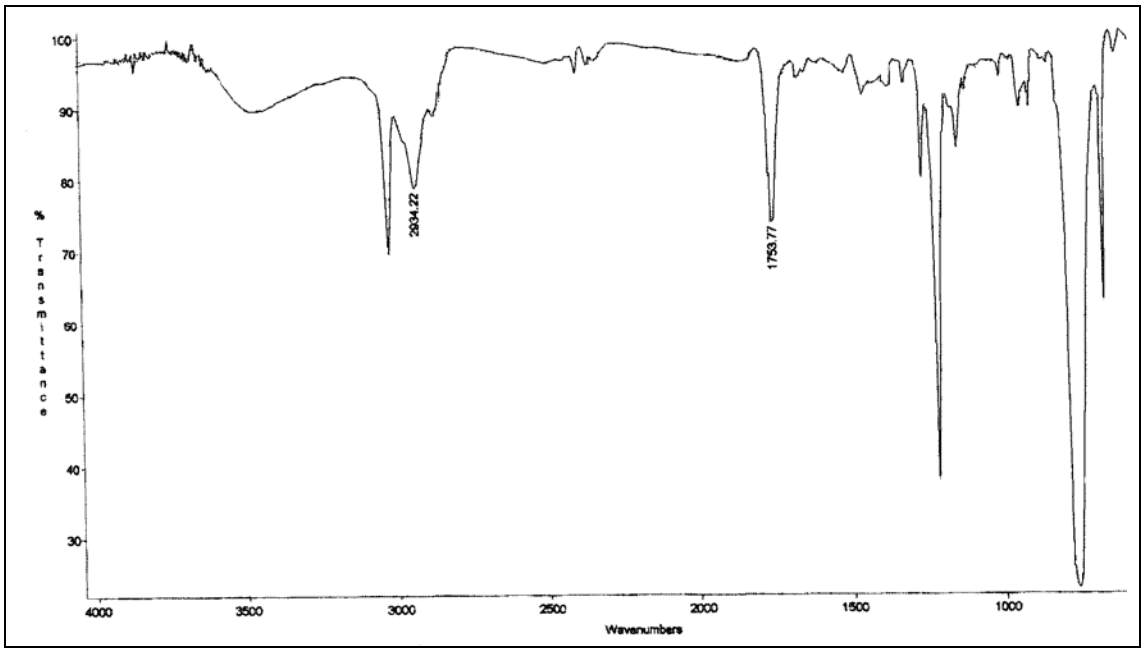
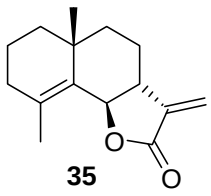


Fig. 9: IR spectrum of compound **35**



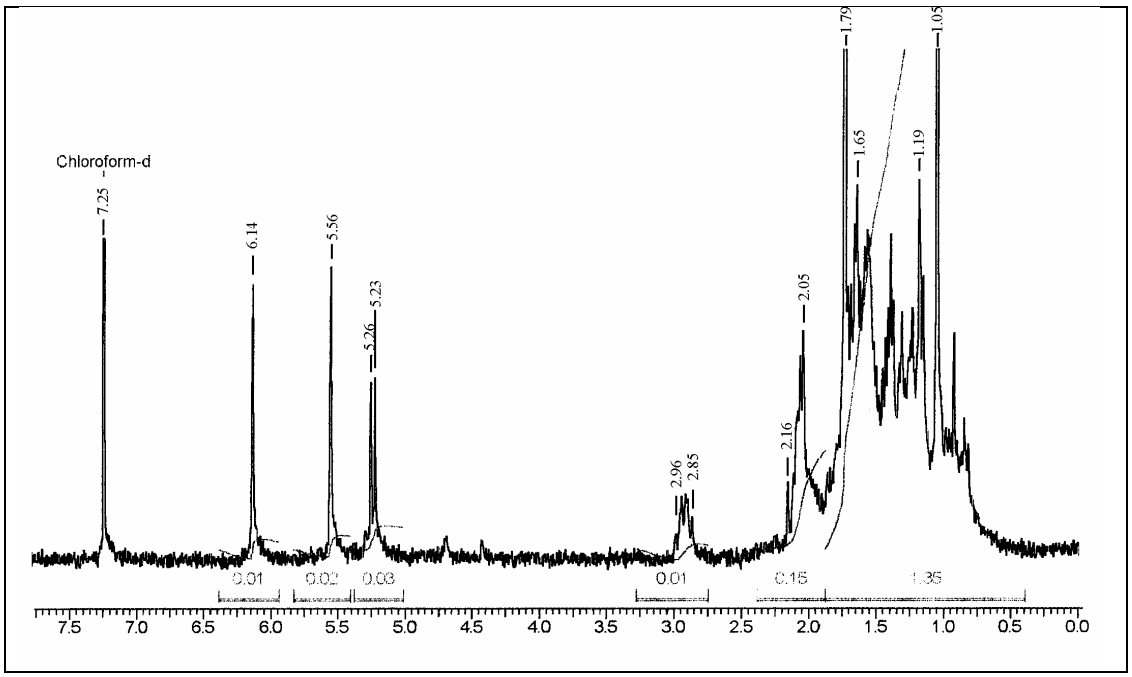


Fig. 10: ^{1}H NMR spectrum of compound **35** (200 MHz, $CDCl_3$)

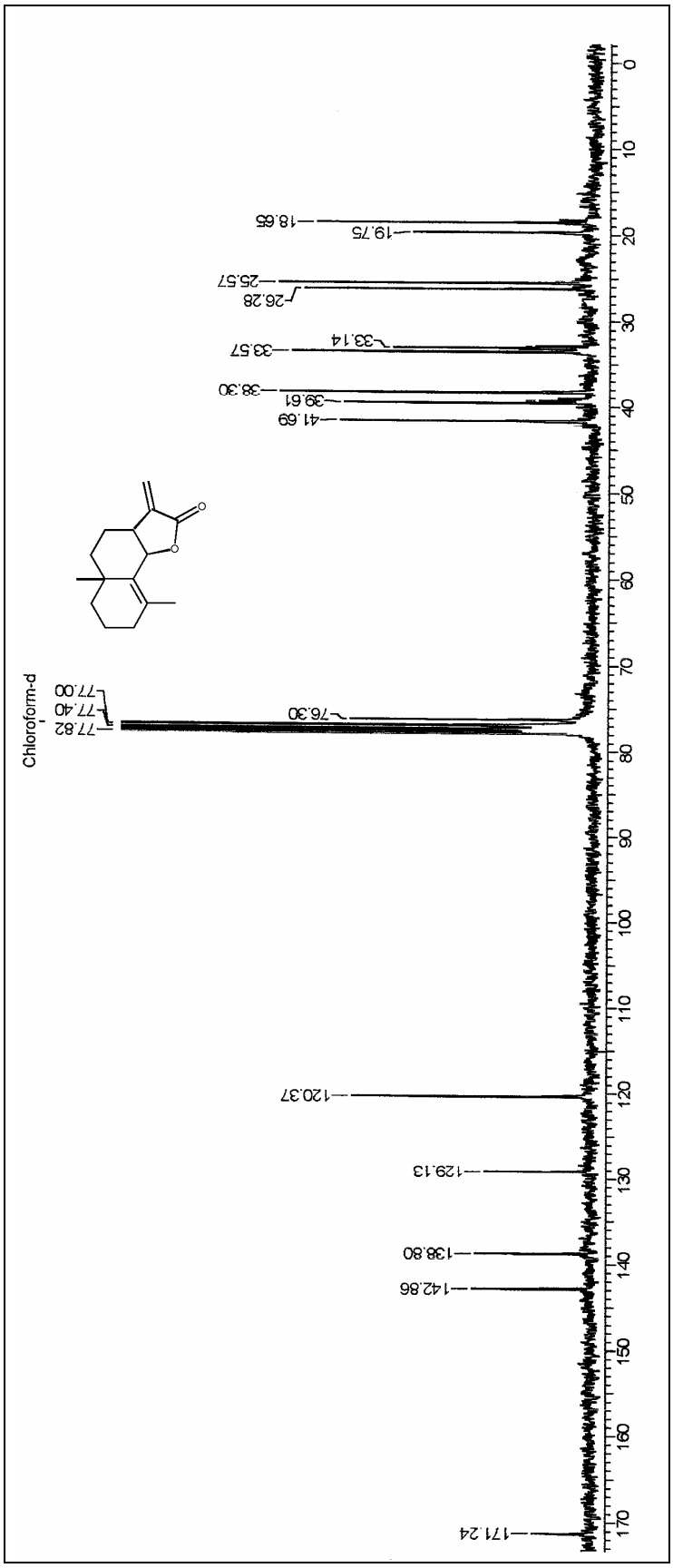


Fig. 11: ^{13}C NMR spectrum of compound **35** (50 MHz, $CDCl_3$)

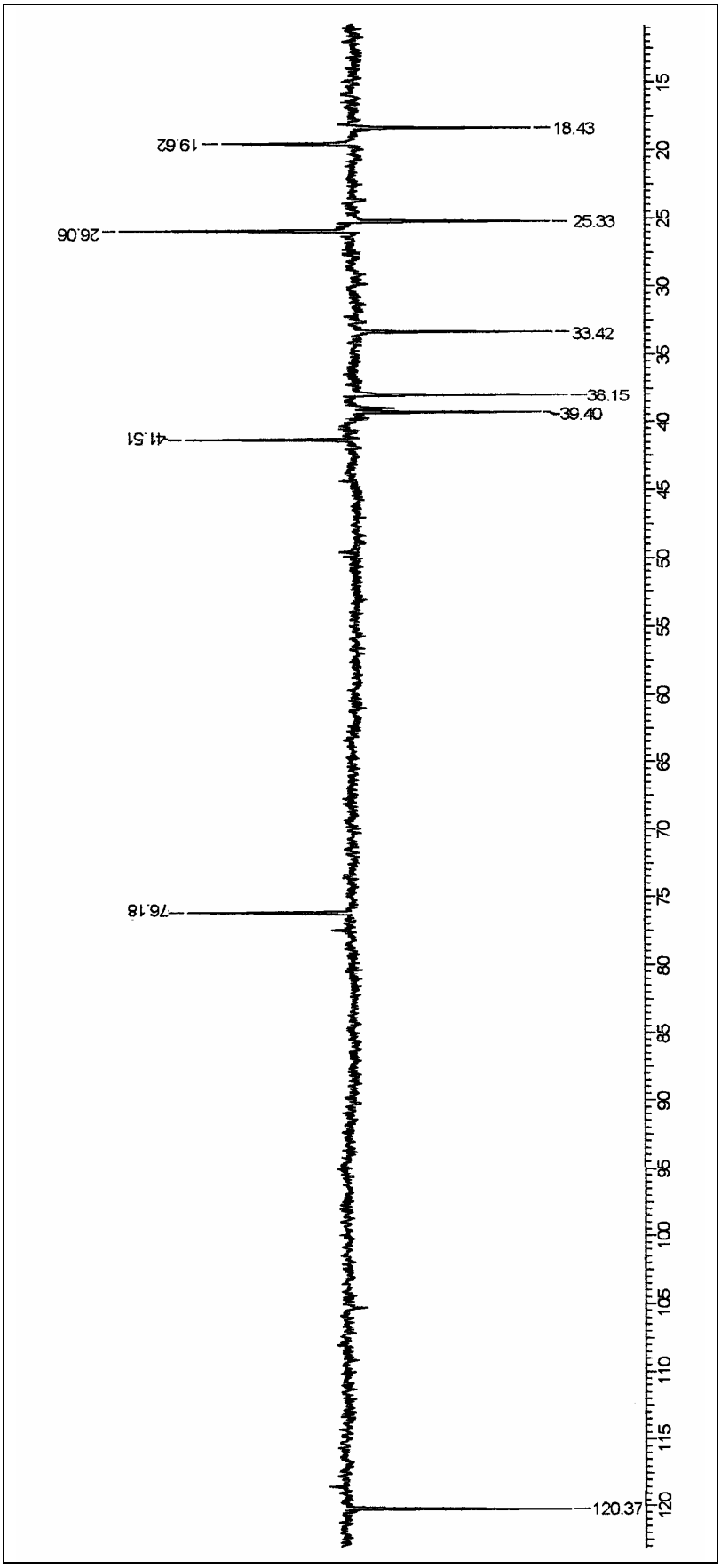


Fig. 12: DEPTH spectrum of compound **35** (50 MHz, $CDCl_3$)

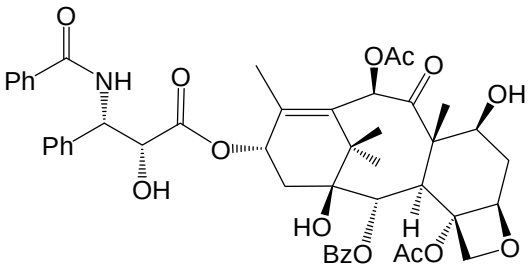
Chapter II

Chemical investigation of *Taxus baccata*

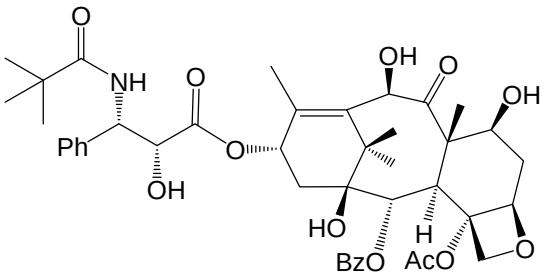
2.1 INTRODUCTION

Paclitaxel (Taxol, **1**), a chemically diverse diterpene and highly potent anticancer natural product[1] was isolated from the bark of the pacific yew (*Taxus brevifolia* Nutt) in 1971.[2] An important milestone in paclitaxel (**1**) development was the discovery of its unique mechanism of action. In 1979, Horwitz and co-workers[3] reported that **1** acted to promote the irreversible assembly of tubulin into microtubules, which was the first example as a promoter of microtubule assembly. It differs in anticancer activity from other anticancer drugs by inhibiting the cell division by a unique mechanism. Cell division takes place by duplication of chromosomes, which line up on spindles formed by microtubules during mitosis. Taxol gums up the tubules stopping the formation of spindles thus avoiding the cell division due to which the cancerous cells eventually die.[3]

Taxol (**1**) was approved for treatment of drug-resistant ovarian cancer by the Food and Drug Administration (FDA) in 1992 and for breast cancer in 1994. Clinical use of **1** has increased steadily since then, and today it is used routinely to treat lung, head and neck, prostate, and cervical cancers, and AIDS-related Kaposi's sarcoma[4] and semi-synthetic paclitaxel analogue docetaxel (Taxotere, **2**) was approved to treat breast cancer by FDA in 1996.



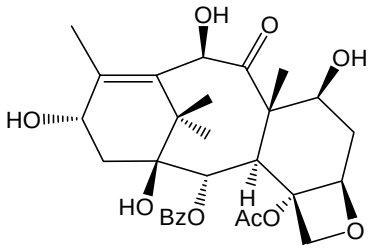
Taxol (Paclitaxel, 1)



Taxotere (Docetaxel, 2)

Taxol (**1**) and taxotere (**2**) both can be synthesized[5,6] from their precursor 10-deacetylbaccatin III (**3**) which can be isolated from the renewable resources such as leaves (needles) and stems of *Taxus* species.

Taxus baccata



10-Deacetylbaccatin III (3)

The discovery of taxol (**1**), as one of the most exciting leads in the cancer chemical therapy in the last three decades, has spurred a flurry of investigation on every part of all *Taxus* species in order to isolate **1,** 10-deacetylbaccatin III (**3**) and potentially more effective taxol derivatives for the treatment of various cancers, or as abundant starting material for the semi-synthesis of **1** and related compounds. As a result, more than 400 taxane-type diterpenoids have been isolated from various *Taxus* plants such as *T. baccata, T. wallichiana, T. cuspidata, T. mairei, T. chinensis, T. yunnanensis, T. canadensis, T. martrel, T. sumatrana* and some of them possess interesting anticancer activities.[7]

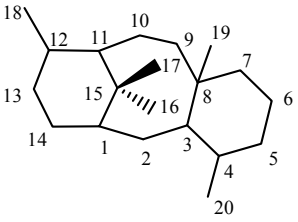
Before 1999, several comprehensive reviews have been published[8] to list the diterpenes isolated from *Taxus* species. Recently a review has been published which includes new taxanes reported since 1999.[9] These discoveries of new types of taxanes will encourage phytochemists to explore other interesting components of yew trees, and thus it will shed light on the understanding of the biogenesis of taxanes.

Thus in past few years, the yew trees have become an important commodity for the pharmaceutical industry. Taxol produced from one whole tree is enough for the treatment of one patient. The concentration of this compound is very low [0.0004 to 0.014%] in the yew tree. Highest concentration of taxol is in the bark of yew tree. Nine tons bark has to be worked-up in order to produce one kg of taxol. Such low concentrations are difficult for extraction and purification of compound to a pharmaceutical grade from raw materials. Further, yew tree is slow growing. It takes many years to mature from young plant. Deforestation and destruction of the particular yew tree are the obvious concerns.[10]

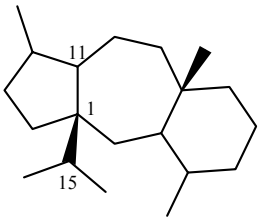
Since the demand for paclitaxel increases every year, the scientific community has been forced to look for the alternative ways of producing paclitaxel. Alternative methods for production of paclitaxel include use of renewable plant parts like branches and needles, nursery production of yew tree, plant cell culture,[11] synthesis from other natural products and total synthesis from easily available starting compounds.[12]

As a result of extensive research, several types of taxane diterpenoids are isolated from leaves (needles), seeds, bark, roots, and woods (stems and twigs) of the *Taxus* species; such as normal 6/8/6-ring taxanes **A**; rearranged taxanes: 11(15→1)abeotaxanes **B**; 11(15→1),11(10→9)diabeotaxanes **C**; 2(3→20)abeotaxanes **D**; transannular taxanes: 3,11-cyclotaxanes **E**; 3,8-secotaxanes (bicyclic taxanes) **F**. Besides these taxanes, some new types of compounds i.e. C(21)-homotaxane **G**, 3,11:12,20-dicyclotaxane **H** and 14,20-cyclotaxane **I** (**Chart I**) have been isolated from various parts of *taxus* species.

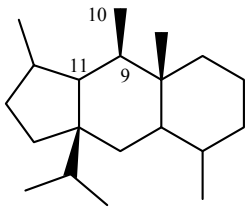
Chart I



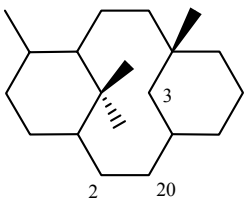
6/8/6 Taxane (**A**)



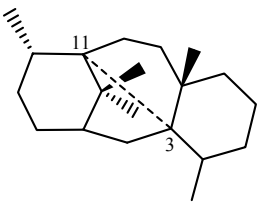
11(15→1)Abeotaxane (**B**)



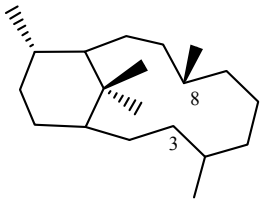
11(15→1),11(10→9)Diabeotaxane (**C**)



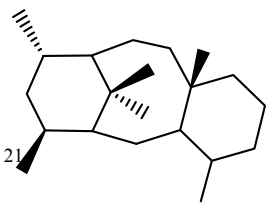
2(3→20)Abeotaxane (**D**)



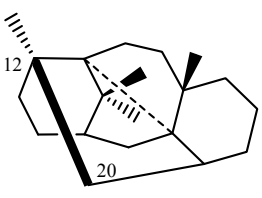
3,11-Cyclotaxane (**E**)



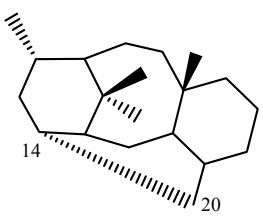
3,8-Secotaxane (**F**)



C(21)-Homotaxane (**G**)



3,11:12,20-Dicyclotaxane (**H**)



14,20-Cyclotaxane (**I**)

The trees of genus *Taxus* (Taxaceae) are dioecious and evergreen plants mainly distributed in the northern hemisphere. They usually grow in the mountainous areas at an altitude of 1500-3300 meter as tall trees or low trailing shrubs with distichous leaves that are linear with recurved margins. Shrubs are very popular as garden domination trees.

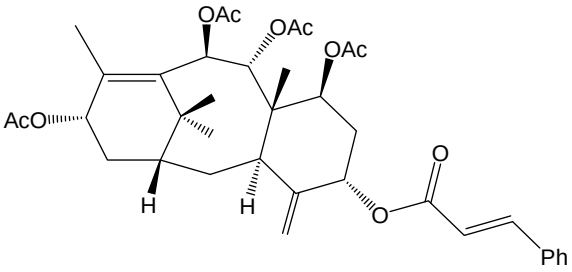
From various *Taxus* species, *T. baccata* and *T. wallichiana* are the species found in Himalaya Mountains. *Taxus baccata* is an evergreen tree usually 6 meters in height and 1.5-1.8 meter in girth found in the temperate Himalayas, at altitudes between 1800 to 3300 meters.[13] It is known as Himalayan yew, whereas *Taxus baccata* growing in the temperate region of Europe is known as European yew. A medicinal tincture made from the young shoots of *T. baccata* has long been used for the treatment of headache, giddiness, feeble and falling pulse, coldness of the extremities, diarrhea and severe biliousness. The leaves are credited with emmenagogue and antispasmodic properties. They are employed for the treatment of hysteria, epilepsy and nervousness.[13]

2.2 PREVIOUS WORK

With an aim to isolate 10-deacetylbaccatin III (**3**), chemical investigation of needles of *Taxus baccata* was carried out in our group affording first time isolation of four taxanes (**4-7**)[14, 15] and one aromatic compound (**8**)[16] (**Chart II**).

As mentioned above along with many *Taxus* species, *T. baccata* has been chemically investigated affording more than 100 different types of taxoids. From 2004 onwards many new taxoids have been isolated from different *Taxus* species. Ther is only one report[17] of isolation of a known taxane (**9**) from *taxus baccata*. Some lignanes and volatile compounds are reported from same species. The taxoids isolated from *Taxus* spceies from 2004 onward and which are not included in recent review[9] (2005) are listed in **tables 1-5, charts III – VII**.

CHART II



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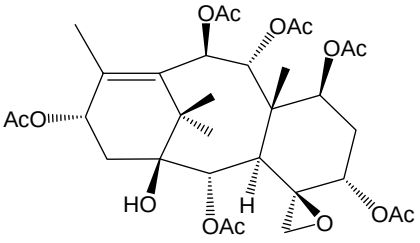

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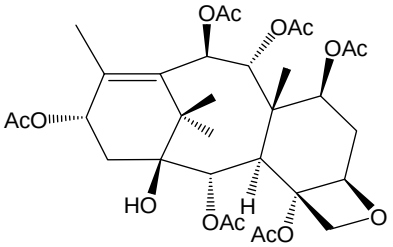

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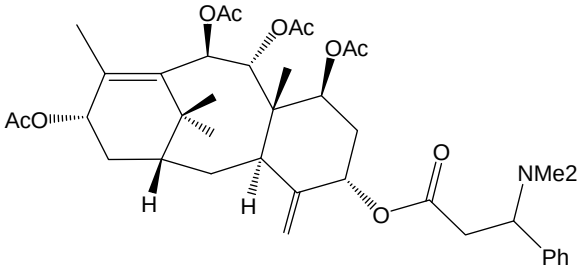

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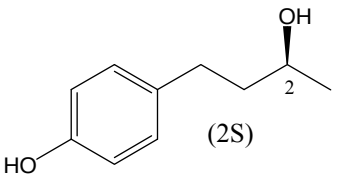

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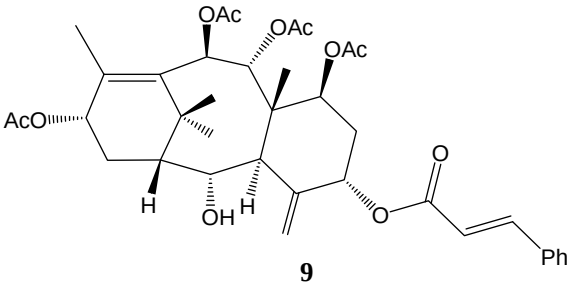


9

Table 1: Taxoids from needles and stems of *Taxus chinensis* (Chart III)

Comp. No.	Name	Ref. No.
10	2-Deacetyl-14β-hydroxybaccatin IV	**18**
11	14β-Hydroxybaccatin VI	**19**
12	1β-Hydroxy-2α,7β-deacetylbaccatin I	**20**
13	10β-Benzoyl-15-acetyltaxumairol X	**21**
14	5α-Acetyl-20-deacetyl-4,20-*p*-hydroxylbenzylidenedioxytaxuyunnanine L	**21**
15	2,20-Diacetyltaxumairol O	**22**
16	14β-Hydroxy-10-deacetyl-2-debenzoylbaccatin III	**22**
17	10-(β-Hydroxybutyryl)-10-deacetylbaccatin III	**23**
18	13,15-Epoxy-13-epi-taxayunnasin A	**24**
19	Taxchinin N	**24**

Table 2: Taxoids from leaves and twigs of *Taxus sumatrana* (Chart IV)

Comp. No.	Name	Ref. No.
20	Tasumatrol P	**25**
21	Tasumatrol Q	**25**
22	Tasumatrol R	**25**
23	Tasumatrol S	**25**
24	Tasumatrol T	**25**
25	Tasumatrol M	**26**
26	Tasumatrol N	**26**
27	Tasumatrol O	**26**

Table 3: Taxoids from *Taxus wallichiana* and *T. canadensis* (Chart V)

Comp. No.	Name	Species/Part	Ref. No.
28	2-Debenzoyl-2α-acetoxy-7,9,10,13-tetradeacetylabeobaccatin VI	***T. wallichiana* Bark**	**27**
29	2α,5α,10β-Triacetoxy-14β(2'-methylbutyraloxy)taxa-4(20),11-diene	***T. wallichiana* Heartwood**	**28**
30	1-Hydroxy-2-deacetoxy-5-decinnamoyltaxinine J	***T. wallichiana* Needles**	**29**
31	7β,10β,13α-Triacetoxy-5α-(3'-dimethylamino-3'-phenylpropanoyloxy)-2α-hydroxy-2(3→20)abeotaxa-4(20),11-dien-9-one	***T. canadensis* Needles**	**30**
32	2α,10β-Diacetoxy-9α-hydroxy-5α-(3'-dimethylamino-3'-phenylpropanoyloxy)-3,11-cyclotax-4(20)-en-13-one	***T. canadensis* Needles**	**30**
33	9α,10β-Diacetoxy-5α-cinnamoyloxy-2α,13α-dihydroxy-13(17)-epoxy-4(20),11-taxadiene	***T. canadensis* Needles**	**31**
34	2α,10β-Diacetoxy-5α-cinnamoyloxy-9α,13α-dihydroxy-13(17)-epoxy-4(20),11-taxadiene	***T. Canadensis* Needles**	**31**
35	13-Acetyl-9-dihydrobaccatin III	***T. Canadensis***	**32**

Table 4: Taxoids from different parts of *Taxus cuspidata* (Chart VI)

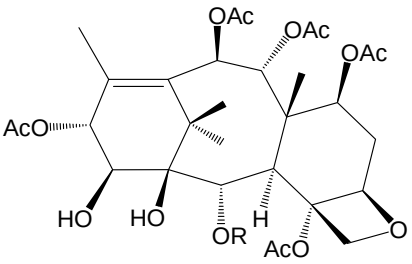
Comp. No.	Name	Part	Ref. No.
36	5α,10β,13α,20-Tetraacetoxytax-11-ene-2α,7β,9α -triol	Needles & branches	**33**
37	2α,9α,10β-Triacetoxy-5α-[(β-D-glucopyranosyl)oxy]-3,11-cyclotax-4(20)-en-13-one	Needles & branches	**33**
38	13α-Acetoxy-5α-cinnamoyloxy-2α,7β,10β-trihydroxy-2(3→20)abeotaxa-4(20),11-dien-9-one (Taxezopidine P)	Seeds	**34**
39	9α,10β-Diacetoxy-2α,13α-dihydroxy-5α-(3'-dimethylamino-3'-phenylpropanoyloxy)taxa-11,4(20)-diene (Taxezopidine O)	Seeds	**34**
40	2α,14β-Diacetoxy-10β-ethoxytaxa-11,4(20)-dien-5α-ol	Heartwood	**35**
41	2α,14β-Diacetoxy-10β-methoxytaxa-11,4(20)-dien-5α-ol	Heartwood	**35**
42	2α-Acetoxy-10β-ethoxytaxa-11,4(20)-diene-5α,14β-diol	Heartwood	**35**
43	7-*O*- β -Xylosyl-10-deacetyltaxuspinanane A	Needles	**36**
44	(12αH)-2α,10β-Diacetoxy-5α-cinnamoyloxy-9α,13α-epoxytax-4(20)-ene-11β,13β-diol	Needles	**37**

Table 5: Taxoids from seeds of *Taxus mairei* (Chart VII)

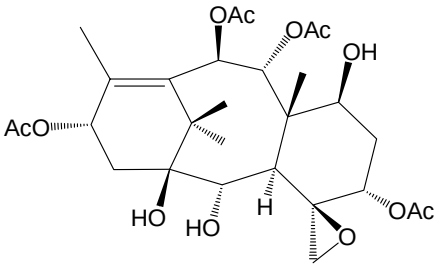
Comp. No.	Name	Ref. No.
45	1-Deoxypaclitaxel	**38**
46	2'-Acetyl taxol	**39**
47	5α,15-Dihydroxy-7β,9α-diacetoxy-11(15→1)abeotaxa-4(20),11-dien-13-one	**40**
48	2α-Hydroxy-9α,10β,13α-triacetoxy-5α-cinnamoyloxytaxa-11-en-4β,20-epoxide	**39**
49	7β,10β-Diacetoxy-2α,5α,13α-trihydroxy-2(3→20)abeotaxa-4(20),11-dien-9-one	**38 & 41**
50	2α,13α-Diacetoxy-10β-hydroxy-2(3→20)abeotaxa-4(20),6,11-triene-5,9-dione	**38**

From needles and stems of *T. chinensis*, taxoids **10** to **19** have been isolated (**Chart III, Table 1**) while from *T. sumatrana*, taxanes **20** to **27** have been isolated (**Chart IV, Table 2**). From *T. wallichiana*, taxanes **28** to **30** have been isolated and from *T. Canadensis*, taxanes **31** to **35** have been isolated (**Chart V, Table 3**). From *T. cuspidata* taxanes **36** to **44** have been isolated (**Chart VI, Table 4**) whereas from *T. mairei* taxanes **45** to **50** have been isolated (**Chart VII, Table 5**).

CHART III (Taxoids from *Taxus chinensis*)



9 R = H
10 R = Bz



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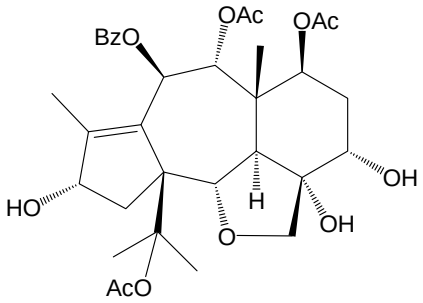

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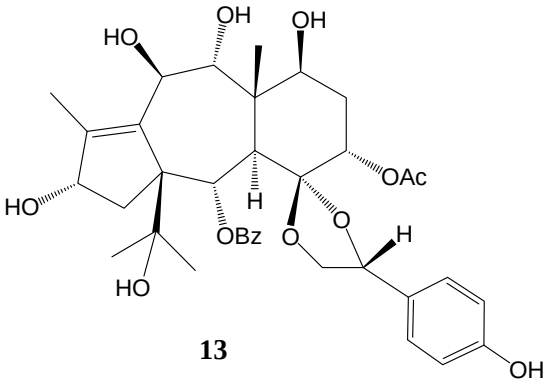

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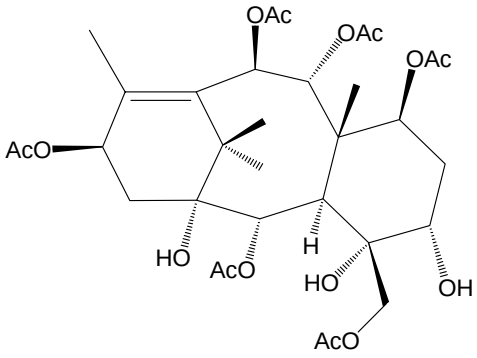

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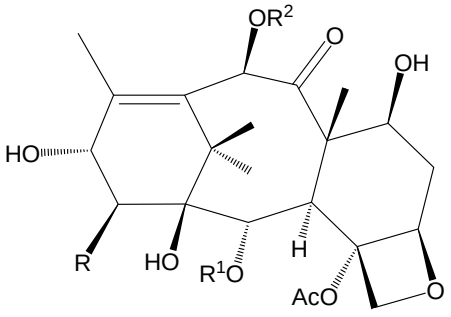
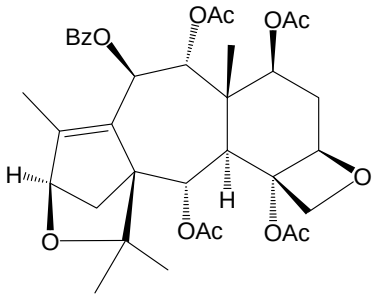


15 R = OH, R^1 = R^2 = H
16 R = H, R^1 = Bz, R^2 = $COCH_2CH(CH_3)OH$



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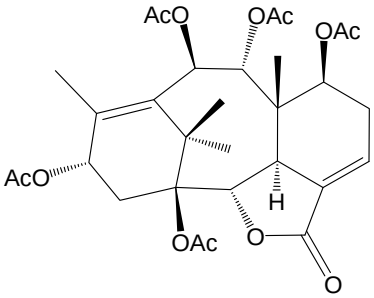
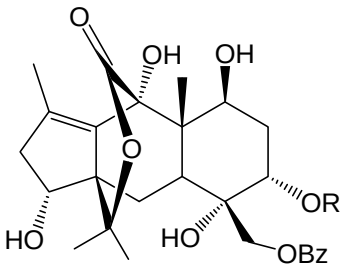
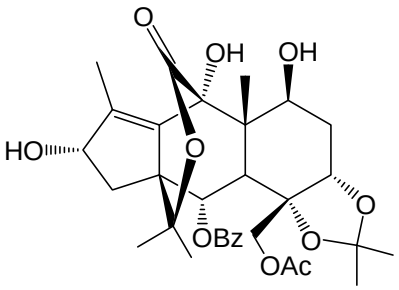


18

CHART IV (Taxoids from *Taxus sumatrana*)



19 R = Ac
20 R = H



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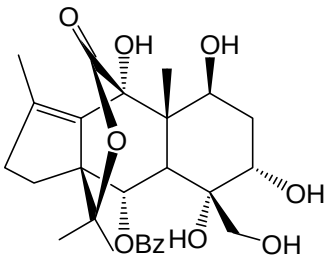

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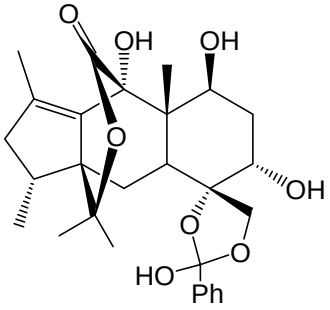

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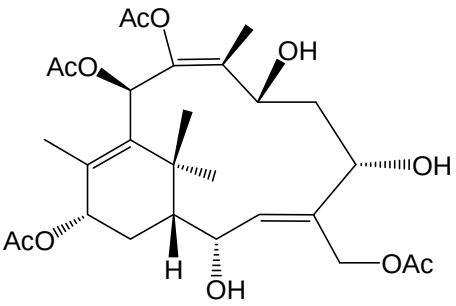

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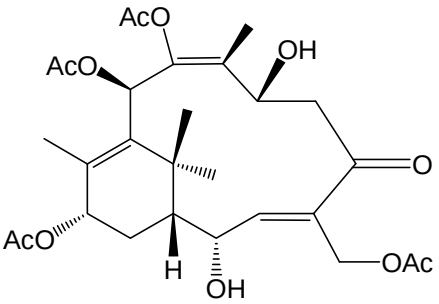

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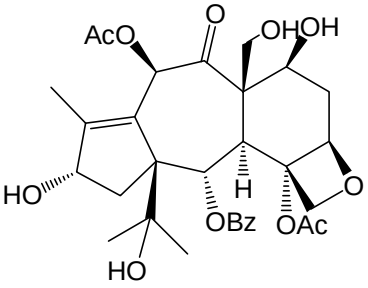
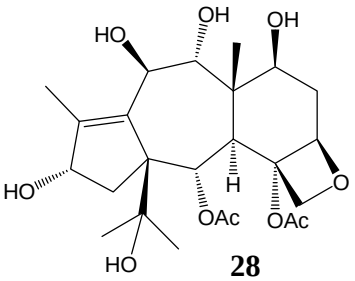


26

CHART V

(Taxoids from *Taxus wallichiana* and *T. canadensis*)



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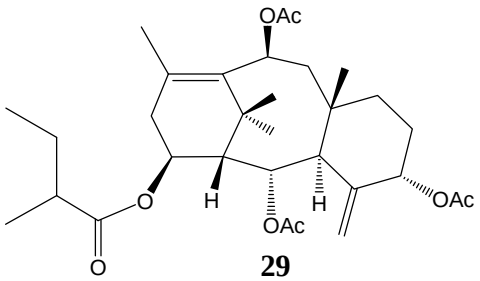

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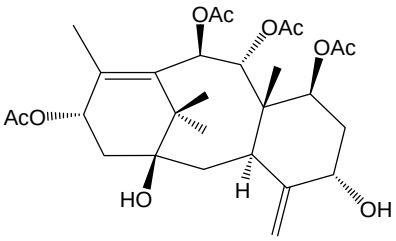

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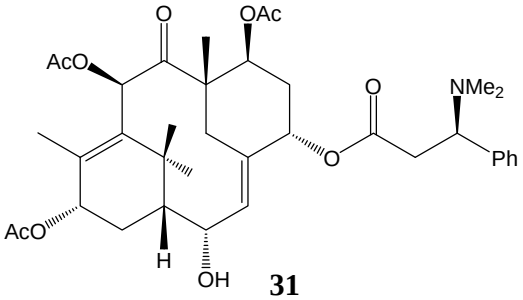

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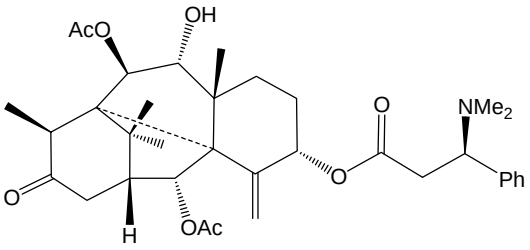

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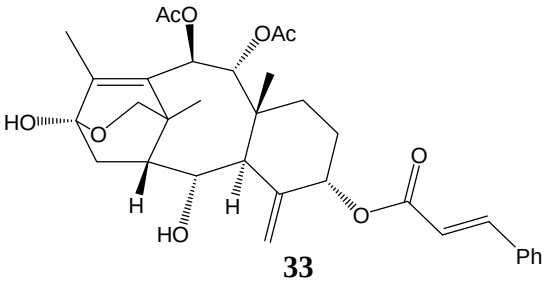

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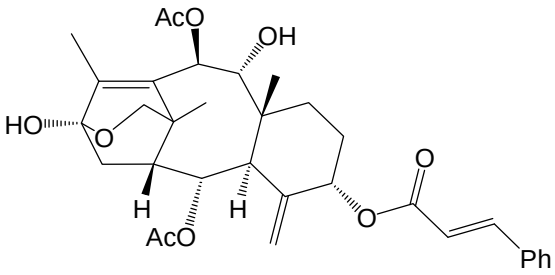

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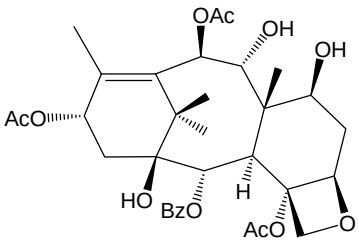
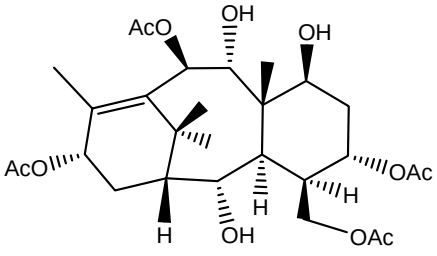


35

CHART VI (Taxoids from *Taxus cuspidata*)



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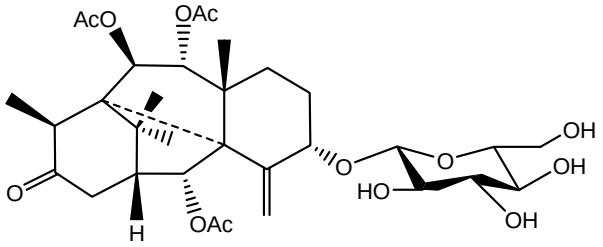
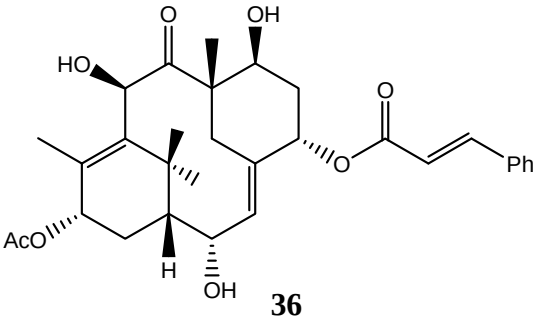


35



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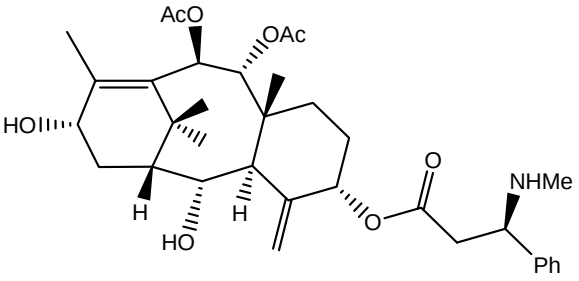

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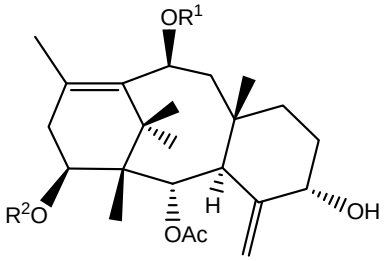
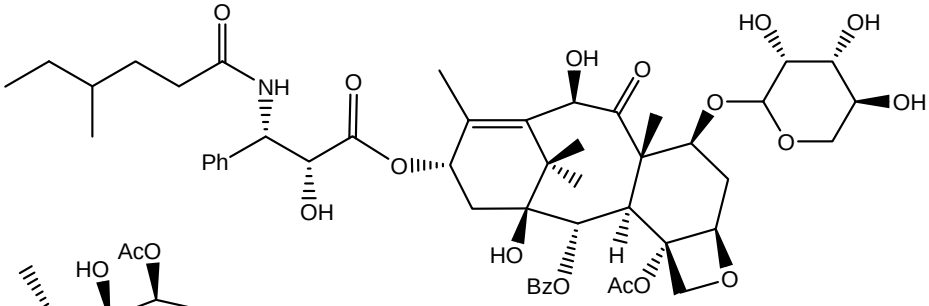


	R^1	R^2
38	**Et**	**Ac**
39	**Me**	**Ac**
40	**Et**	**H**



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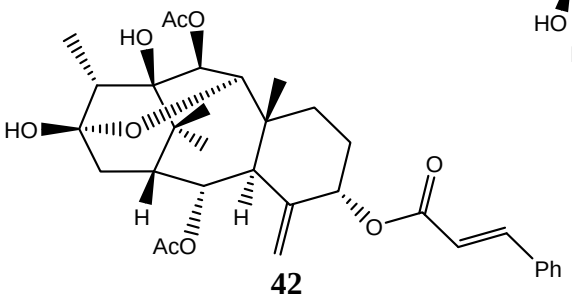
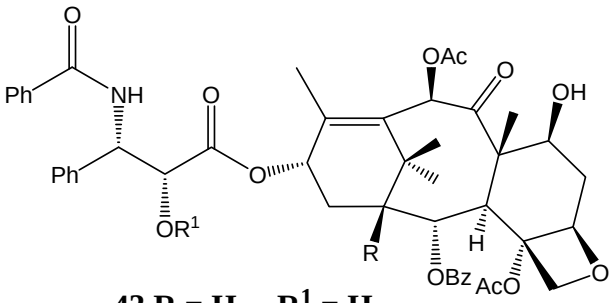


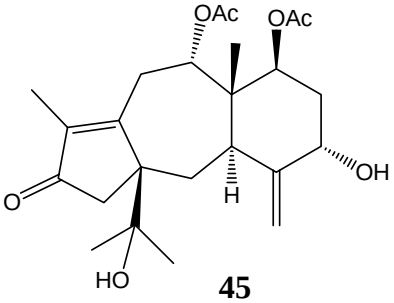
42

CHART VII (Taxoids from *Taxus mairei*)



43 R = H, R^1 = H

44 R = OH, R^1 = Ac



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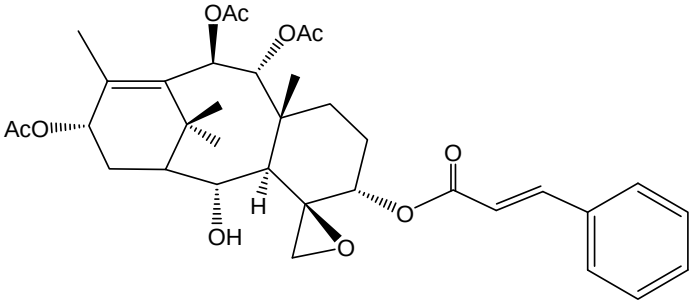
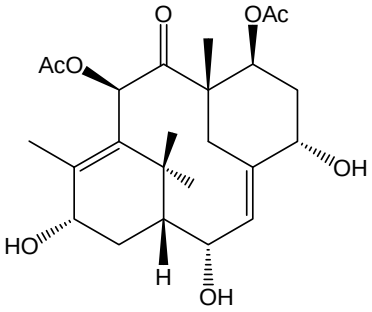
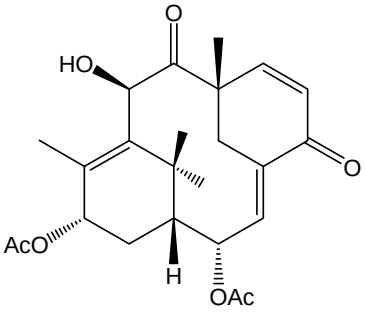


46



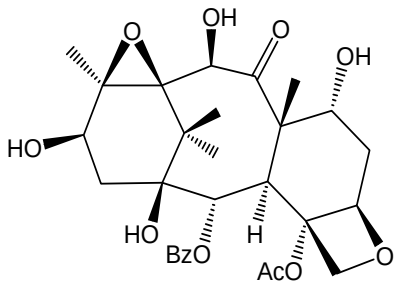
47



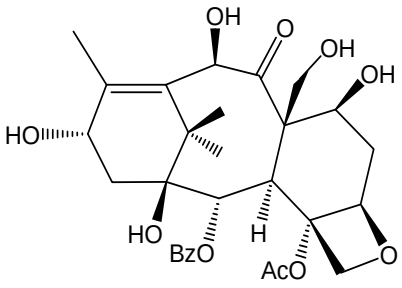
48

2.3 PRESENT WORK

Thus, being a medicinally important plant, it was thought to enrich methanol extract of needles of *T. baccata* for 10-deacetyl baccatin III (**3**), a precursor of taxol (**1**). The enrichment was carried out by solvent-solvent extraction. The residue after final enrichment was subjected for preparative HPLC to isolate two more taxoids (**51** and **52**). The structures of both the compounds are elucidated by intensive spectral studies.



51



52

2.3.1 Enrichment of 10-DAB III (3)

The air-dried needles of *Taxus baccata* (1 kg) were extracted with methanol. The crude methanol extract was dissolved in acetone. The insoluble portion was removed by filtration. The filtrate was concentrated to a solid mass, further solvent partition and crystallization at low temperature offered 10-DAB III up to 89% purity. Mother liquor obtained after separating 10-DAB III was analyzed by HPLC. It showed three major peaks including peak of 10-DAB III and other two compounds (**51** and **52**). These were separated by preparative HPLC.

2.3.2 Characterization of compound 51

Compound **51,** obtained as viscous oil, showed in its ESI-mass spectrum (**Fig. 1**) the molecular ion peak $[M+H]^+$ at m/z 561.5 and $[M+Na]^+$ at m/z 583.6 suggesting one of the possible molecular formula as $C_{29}H_{36}O_{11}$. Its IR spectrum (**Fig. 2**) showed characteristic broad absorption bands at 1718.3 cm^{-1} for carbonyl groups

and 3425.7 cm^{-1} for hydroxy groups. Its ^{13}C NMR spectrum (**Fig. 5, Table 7**) showed twenty seven signals out of which two intense signals at δ 129.19 and 130.03 might be due to two similar carbons each, thus indicating the presence of twenty nine carbon atoms in molecule.

The ^{1}H NMR spectrum (**Fig. 3, Table 6**) of compound **51** revealed the presence of an acetate methyl at δ 2.276 and four methyls at δ 0.771, 1.017, 1.532 and 1.636 on quaternary carbons. Two of the methyl singlets at δ 0.771 and 1.017 showed correlation in ^{1}H - ^{1}H COSY experiment (**Fig. 7**) with each other indicating that they are geminal methyls, Me-16 and Me-17, located at C-15 in taxoids. The other two methyl singlets could be due to Me-18 and Me-19.

The ^{1}H NMR spectrum of this compound further showed signals at δ 8.013 (d, *J* = 7.2 Hz, 2H), 7.569 (m, 2H) and 7.667 (m, 1H) revealing the presence of benzoyl group. The intense signals at δ 129.19 and 130.03 appearing as doublets, a doublet at δ 133.77 and a signal at δ 130.49 as singlet in ^{13}C NMR spectrum and in DEPT-135 experiment (**Fig. 6**) confirmed the presence of benzoyl group. Absence of any more signal in olefinic region clearly indicated that C11-C12 double bond is not present. Further appearance of two signals at δ 66.99 and 67.20 as singlet indicated that C11-C12 double bond is oxygenated to C11-C12 epoxide which could be β-oriented as observed in all the taxoids isolated from *Taxus* species.[42]

The signals at δ 165.66 and 170.34 in ^{13}C NMR spectrum revealed the presence of two ester carbonyl carbons, which should be an acetate carbonyl and another benzoyl carbonyl. The ^{13}C NMR spectrum also showed a signal at δ 209.73 for normal carbonyl which could be at C-9 as observed in most of taxoids.[43]

Besides methyl and benzoyl signals, the ^{1}H NMR spectrum of the compound showed four D_2O exchangeable signals (^{1}H NMR spectrum after D_2O exchange, **Fig. 4**) at δ 5.474 (d, *J* = 4.4 Hz), 5.121 (d, *J* = 7.2 Hz), 4.804 (d, *J* = 2.8 Hz) and 4.504 (s) indicating the presence of hydroxy groups. These three doublets indicated that these are due to protons of three secondary hydroxy groups which could be at any three carbons out of four carbons (C-2, C-7, C-10 and C-13) as observed in most of

taxoids,[43] whereas the singlet could be due to proton of tertiary hydroxy group either at C-1 in taxol type of compounds or at C-15 in 11(15→1)abeotaxanes.[8,9]

The ^{1}H NMR spectrum (**Fig. 3**) of compound further showed an AB quartet for two protons at δ 4.097 (J = 5.6 Hz) which are coupling with each other as observed in ^{1}H - ^{1}H COSY experiment (**Fig. 7**) of compound. The ^{1}H - ^{13}C heterocoupling experiment (HSQC) (**Fig. 8**) of compound showed that these both protons are on same carbon atom appearing at δ 76.21 as triplet and thus confirming the presence of oxetane ring in compound.

In ^{1}H - ^{1}H COSY experiment of compound a proton at δ 3.076 (d, J = 6.7 Hz), which is typical H-3α at ring junction in taxane diterpenes[43,44] showed coupling with a proton appearing at δ 5.396 (d, J = 6.7 Hz) which has to be H-2. The chemical shift of this proton clearly indicated that benzoyl group is at C-2 and the coupling constant of both protons, as observed in several C-2 derivated taxanes[43,45] also clearly indicated that they are *trans* to each other, i.e. benzoyl group is α-oriented. The ^{1}H - ^{13}C heterocoupling (HSQC) experiment revealed the correlation of H-2 signal in ^{1}H NMR spectrum with signal at δ 74.62 in ^{13}C NMR spectrum which must be due to C-2. It was, therefore, indicative that the three secondary hydroxy groups must be at C-7, C-10 and C-13. The fact was further confirmed by five signals in ^{13}C NMR spectrum at δ 66.08, 71.08, 74.62, 76.12 and 83.44 as doublets for five oxygenated carbon atoms which should be C-2, C-5, C-7, C-10 and C-13.

From ^{1}H - ^{13}C heterocoupling experiment (HSQC) of compound it was revealed that two single protons appearing at δ 1.935 (dd, J = 6.4, 10 Hz) and 2.187 (dd, J = 7.2, 9.2 Hz) are on same carbon atom appearing as triplet at δ 38.92 which could be either C-6 or C-14. The ^{1}H - ^{13}C heterocoupling experiment further showed that a proton at δ 1.684 (m) and a proton at δ 2.351 (m) are on same carbon atom which is appearing as triplet at δ 36.97 in ^{13}C NMR spectrum and this carbon again could be either C-6 or C-14. The ^{1}H - ^{1}H COSY experiment of compound showed that both the protons at δ 1.935 and 2.187 couple with only one proton at δ 3.978 (m) whereas protons at δ 1.684 and at δ 2.351 couple with two different protons, a proton at δ 4.044 (m) and one of two protons at δ 5.102 respectively. This clearly indicated

that signals at δ 1.935 and 2.187 are due to H-14a and H-14b and signal at δ 3.978 is due to H-13. The chemical shift of H-13 clearly indicated that this proton is α-oriented.[43b,43d,43h,45,46] The ^{1}H - ^{1}H COSY experiment showed correlation of H-13 with hydroxy proton signal at δ 5.474, thus indicating that it is due to C13-OH proton. Further, from HSQC experiment it was also confirmed that doublet at δ 66.08 was due to C-13 and a triplet at δ 38.92 was due to C-14.

It was further confirmed that signals at δ 1.684 and δ 2.351 were due to H-6a and H-6b and a triplet at δ 36.97 in ^{13}C NMR spectrum was due to C-6. Further, the protons at δ 4.044 and at δ 5.130 must be H-5 and H-7 or *vice versa*. From the literature[43] it could be concluded that signal at δ 5.102 must be due to H-5α and signal at δ 4.044 must be due to H-7. The chemical shift of H-7 further indicated that H-7 is α-oriented and C7-OH is β-oriented.[47] From ^{1}H - ^{13}C heterocoupling experiment chemical shifts of C-5 and C-7 in ^{13}C NMR spectrum were identified as δ 83.44 and δ 71.08 respectively.

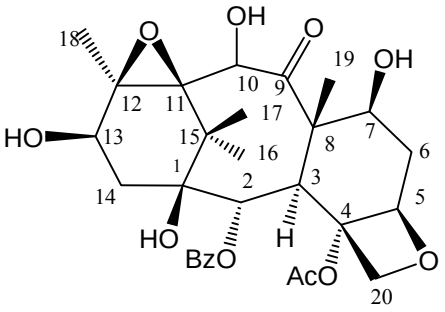
The ^{1}H - ^{1}H COSY experiment showed correlation of H-7 with hydroxy proton signal at δ 5.121, thus indicating that it was due to C7-hydroxyl proton. The ^{1}H - ^{1}H COSY experiment further showed coupling of H-5 at δ 5.102 with C-6 proton at δ 2.351. From the coupling constant (J = 9.2 Hz) of H-5, as observed in ^{1}H NMR spectrum after D_2O exchange (**Fig. 4**), it could be concluded that this proton at C-6 must be *trans* to H-5α, i.e. H-6 at δ 2.351 must be β-oriented and thus H-6 at δ 1.684 must be α-oriented.

^{1}H NMR spectrum further showed that the proton signal at δ 4.203 appearing as doublet (J = 2.8 Hz) appeared as singlet after D_2O exchange. This indicated that this signal must be due to H-10. The ^{1}H - ^{1}H COSY experiment showed correlation of H-10 with a hydroxy proton appearing as doublet at δ 4.804 (J = 2.8 Hz) which disappears after D_2O exchange indicating that this signal must be due to C10-hydroxyl proton. The HSQC experiment revealed that C-10 is appearing at δ 76.12 in ^{13}C NMR spectrum.

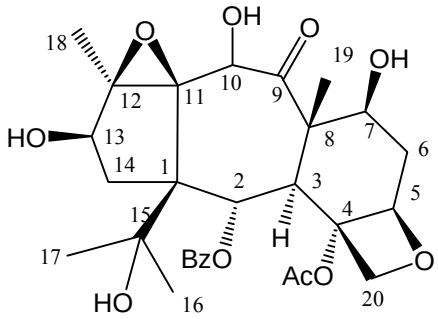
It can be further concluded that the fourth exchangeable signal which appeared as singlet at δ 4.504 must be due to either C1-OH proton in taxol type of compounds or due to C15-OH proton in 11(15→1)abeotaxanes.

The ^{13}C NMR spectrum of compound further showed four signals at δ 43.19, 57.07, 77.70 and 80.85 as singlets which could be attributed to oxygenated C-1, C-4 where α-oriented acetoxy group is found in all taxoids having oxetane ring, C-8 and C-15. Chemical shift of C-8 in both taxane series[43] and in 11(15→1)abeotaxane series[26,43d,43h] compounds having carbonyl group at C-9 is found to be δ 54 – 62. Thus it can be concluded that signal at δ 57.07 must be due to C-8.

Thus the compound **51** could be a taxane or 11(15→1)abeotaxane having one of the following structures.

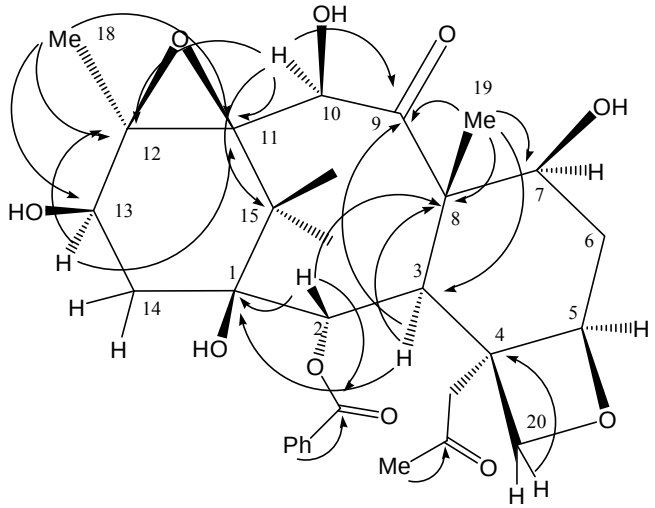


Taxane



11(15→1)Abeotaxane

The HMBC experiment (**Fig. 9**) of the compound showed correlation of C-9 signal with signals due to H-3 at δ 3.076, H-10 at δ 4.203 and methyl signal at δ 1.636 which has to be Me-19. This methyl signal at δ 1.636 showed correlation with quartet at δ 10.12 in ^{13}C NMR spectrum of compound thus indicating this chemical shift was due to C-19. It can be further concluded that the other methyl appearing at δ 1.532 must be due to Me-18 which showed correlation with another quartet at δ 15.74 in ^{13}C NMR spectrum. It was further confirmed by the correlation observed in HMBC experiment between Me-18 and C-11, C-12 and C-13. The HMBC experiment further showed the correlation of Me-19 with C-3, C-7 (δ 71.08) and a signal at δ 57.07 which has to be due to C-8. The experiment further showed correlation of C-8 with H-3 and H-2 and weak correlation with H-10.



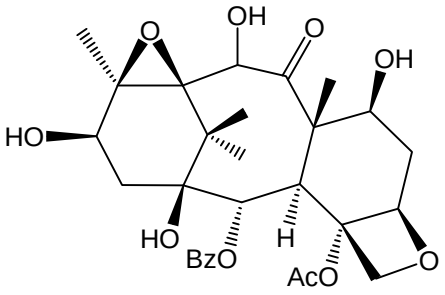
Some HMBC correlations of compound **51**

The HMBC experiment of the compound showed further correlation of acetyl carbonyl carbon at δ 170.34 with acetate methyl at δ 2.276 and benzoyl carbonyl carbon at δ 165.66 with benzoyl protons and H-2 as expected.

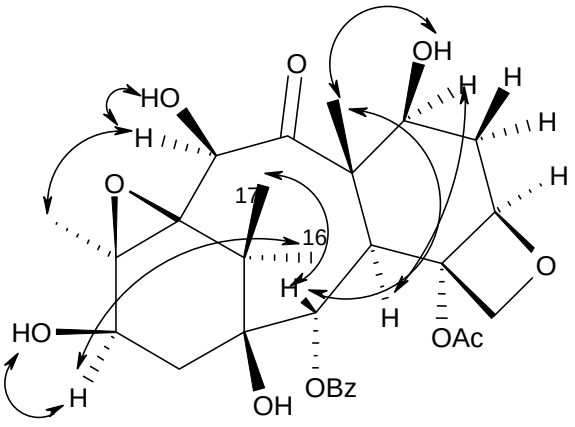
The HMBC experiment further showed correlation of singlet at δ 80.85 with signals due to H-20a and H-20b revealing that this singlet must be due to C-4. C-4 also showed weak correlation with H-7 and acetate methyl.

From above observations, it was clear that the remaining two singlets at δ 43.19 and 77.70 in ^{13}C NMR spectrum must be due to C-1 and C-15 or *vice versa*. In 11(15→1)abeotaxane series[43b,43d,43h,45,48] the chemical shifts of C-1 and C-15 are observed at around δ 61-70 and 74-90 respectively whereas in taxane series they are observed at around δ 42-45 and 75-78. This clearly indicated that compound **51** belongs to taxane series and singlet at δ 77.7 must be due to C-1 and singlet at δ 43.19 must be due to C-15. This inference was further confirmed by correlation of signal at δ 43.19 with H-10, Me-16 and Me-17; and correlation of signal at δ 77.17 with H-2, Me-16 and Me-17 and weak correlation with H-3.

From this discussion it could be concluded that compound **51** belongs to taxane series having following structure.



In ^{1}H - ^{1}H NOESY experiment (**Fig. 10**) H-3 at δ 3.076 showed correlation with H-7 at δ 4.044 thus confirming the stereochemistry of hydroxy group at C-7 as β-oriented. The experiment further showed correlation of H-10 with Me-18, thus confirming the stereochemistry of H-10 as α-oriented and hydroxy group as β-oriented. Me-18 also showed weak correlation with H-3. The experiment also showed correlation of H-2 with methyl signal at δ 1.017 which has to be β-oriented and was assigned as Me-17 and the other methyl at δ 0.771 was assigned as Me-16 which would be α-oriented. Me-16 showed correlation with H-13. Both Me-16 and Me-17 showed weak correlation with C1-hydroxyl proton. Me-17 also showed weak correlation with C10-hydroxyl proton. The experiment also showed correlation between H-13 and C13-hydroxyl proton, and H-10 and C10-hydroxyl proton. The experiment further showed correlation of H-14a at δ 1.935 with C1-hydroxyl proton. It also showed strong correlation of C7-hydroxyl proton with H-6α and weak correlation with H-6β which again confirmed the stereochemistry of C7-hydroxy group.



NOESY correlation of compound **51**

Thus from above discussed spectral data, the compound **51** was characterized as 13-epi-11,12-epoxy-10-deacetylbaccatin III and found to be a new compound.

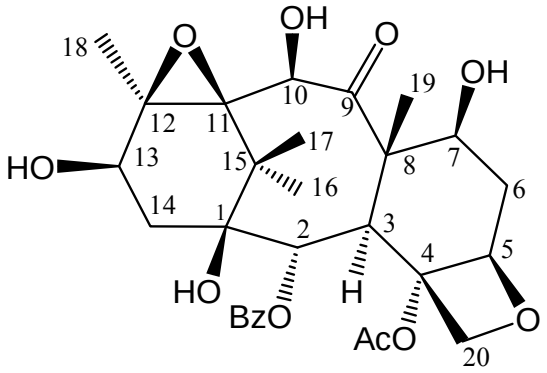


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Table 6: ^{1}H NMR spectral data of compound **51** (DMSO-d_6)

Proton	Chemical Shift in δ	Multiplicity
H-2β	5.396	d, *J* = 6.7 Hz
H-3α	3.076	d, *J* = 6.7 Hz
H-5α	5.102	d, *J* = 9.2 Hz
H-6β	2.351	m (merged with acetate methyl)
H-6α	1.684	m (merged with H-18)
H-7α	4.044	m (merged with H-13)
H-10α	4.203	d, *J* = 2.8 Hz
H-13α	3.978	m (merged with H-7)
H-14a	2.187	dd, *J* = 7.2, 9.2 Hz
H-14b	1.935	dd, *J* = 6.4, 10.0 Hz
H-16	0.771	s
H-17	1.017	s
H-18	1.532	s
H-19	1.636	s
H-20a & H-20b	4.097	AB quartet, *J* = 5.6 Hz
OCOC$\underline{H}_3$	2.276	s
OBz:H-2'& H-6'	8.013	d, *J* = 7.2 Hz
H-3' & H-5'	7.569	m
H-4'	7.667	m
C-1 O$\underline{H}$	4.504	s
C-7 O$\underline{H}$	5.121	d, *J* = 7.2 Hz
C-10 O$\underline{H}$	4.804	d, *J* = 2.8 Hz
C-13 O$\underline{H}$	5.474	d, *J* = 4.4 Hz

Table 7: ^{13}C NMR spectral data of compound **51** (DMSO-d_6)

Carbon	Chemical shift in δ
C-1	77.70, s
C-2	74.62, d
C-3	44.94, d
C-4	80.85, s
C-5	83.44, d
C-6	36.97, t
C-7	71.08, d
C-8	57.07, s
C-9	209.73, s
C-10	76.12, d
C-11	**66.99**, s
C-12	**67.20**, s
C-13	66.08, d
C-14	38.92, t
C-15	43.19, s
C-16	26.41, q
C-17	20.67, q
C-18	15.74, q
C-19	10.12, t
C-20	76.22, t
O<u>C</u>OCH$_3$	170.34, s
OCO<u>C</u>H$_3$	22.70, q
O<u>C</u>OBz	165.66, s
C-1’	130.49, s
C-2’	130.03, d
C-3’	129.19, d
C-4’	133.78, d
C-5’	129.19, d
C-6’	130.03, d

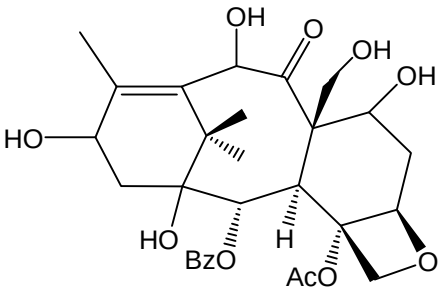
2.3.3 Characterization of compound 52

Compound **52,** obtained as white amorphous powder, showed in its ESI-mass spectrum (**Fig. 12**) the molecular ion peak at *m/z* 583.3 $[M+Na]^+$ suggesting one of the possible molecular formulas $C_{29}H_{36}O_{11}$. Its IR spectrum (**Fig. 11**) showed characteristic broad absorption bands at 1713 cm^{-1} for carbonyl groups and 3428 cm^{-1} for hydroxy groups. Its ^{13}C NMR spectrum (**Fig. 14, Table 9**) showed twenty seven signals out of which two signals at δ 129.49 and 130.42 might be due to two similar *ortho* and *meta* carbons of benzoyl group as discussed before, thus indicating the presence of twenty nine carbon atoms in molecule. The presence of benzoyl group was confirmed by signals at δ 8.09 (d, *J* = 7.2 Hz, 2H), 7.59 (m, 2H) and 7.68 (m, 1H) in ^1H NMR spectrum (**Fig. 13, Table 10**) of compound **52**.

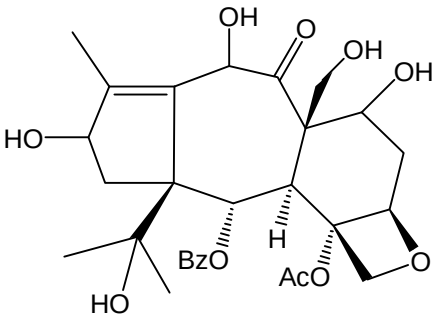
The ^{13}C NMR spectrum further showed signals at δ 165.44 and 170.48 for two ester carbonyl carbons and a signal at δ 208.96 for normal carbonyl. It showed six signals in olefinic region, two signals as mentioned above were for two carbons each, thus accounting for eight carbon atoms. The DEPT-135 experiment (**Fig. 15, Table 9**) of ^{13}C NMR spectrum showed that out of these eight carbon atoms three carbon atoms appearing at δ 142.29, 135.83 and 131.87 are tetravalent, whereas five carbon atoms which included above four at δ 129.49 and 130.42 and fifth one at δ 133.77 are appearing as doublets. This clearly indicated that compound has a benzoyl group and a tetra-substituted double bond.

The ^1H NMR spectrum of compound showed two methyl signals on quaternary carbon at δ 0.99 and 1.18 and a vinyl methyl signal at δ 1.93 along with an acetate methyl signal at δ 2.27. The presence of four methyl groups was confirmed by appearance of only four signals in ^{13}C NMR spectrum at higher field at δ 15.64, 21.23, 23.16 and 27.54. The ^1H NMR spectrum of compound further showed two doublets each for one proton at δ 4.11 (d, *J* = 8.1 Hz) and δ 4.37 (d, *J* = 7.7 Hz) which are coupling with each other as observed in ^1H – ^1H COSY experiment (**Fig. 16**) of compound. The ^1H - ^{13}C heterocoupling experiment (HSQC) (**Fig. 17**) of compound showed that both of these protons are on same carbon atom appearing at δ 76.21 as triplet and thus confirming the presence of oxetane ring in compound.

The ^{13}C NMR spectrum further showed five signals at δ 67.05, 71.58, 75.39, 77.57 and 84.81 as doublets for five oxygenated carbon atoms which could be C-2, C-5, C-7, C-10 and C-13. The signal appearing at δ 208.96 must be due to C-9 carbonyl carbon. The ^{13}C NMR spectrum also showed a triplet at δ 58.35 which should be due to CH_2OH as a methyl is missing in ^{1}H NMR spectrum and it is oxygenated to primary alcohol; this can be C-8 methyl oxygenated to C-8 CH_2OH. The ^{1}H - ^{13}C HSQC experiment showed correlation of C-19 with two overlapping AB quartets at δ 4.503 and 4.513 which are due to H-19a and H-19b. Besides these signals, ^{13}C NMR spectrum showed two triplets at δ 37.54 and 40.23 for C-6 and C-14 and a doublet at δ 47.69 for C-3 as generally observed for taxanes.



Taxane

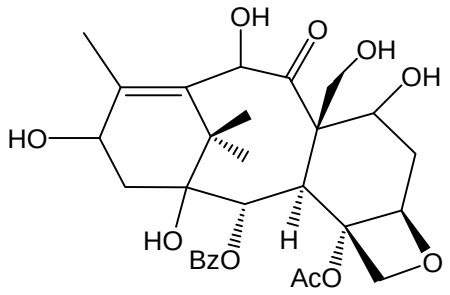


11(15→1)Abeotaxane

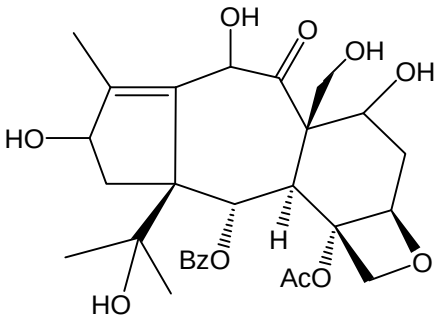
The ^{13}C NMR spectrum further showed four signals at δ 43.40, 60.96, 78.68 and 81.20 as singlets which could be attributed to C-1, C-4, C-8 and C-15 out of which the signal at δ 60.96 could be assigned to C-8. Further, one of the two signals at δ 78.69 and 81.2 must be due to oxygenated C-4 corresponding to α-oriented acetoxy group found in all taxoids having oxetane ring.

In ^{1}H - ^{1}H COSY experiment of compound **52**, a proton at δ 3.825 (d, J = 7 Hz) which is typical H-3α at ring junction in taxane diterpenes[43,44] showed coupling with a proton appearing at δ 6.525 (d, J = 7 Hz) which has to be H-2. The chemical shift of this proton clearly indicated that benzoyl group is at C-2 and the coupling constant (7 Hz) of both protons, as observed in several C-2 derivated taxanes,[43,45] also clearly indicated that they are *trans* to each other, i.e. benzoyl group is α-oriented.

Thus the compound **52** could be a taxane or 11(15→1)abeotaxane having following structures.



Taxane



11(15→1)Abeotaxane

^{1}H - ^{1}H COSY experiment further showed signals at δ 1.58 (t, *J* = 12.8 Hz) and at δ 2.31 (m) integrating for one proton each coupling with each other. In ^{1}H - ^{13}C heterocoupling experiment, both of these protons showed to be on same carbon atom at δ 37.54. Similarly a multiplet at δ 2.14 integrating for two protons showed coupling with carbon appearing at δ 40.23. This clearly suggested that multiplet at δ 2.14 was due to either H-14a & H-14b or H-6a & H-6b and multiplets at δ 1.524 and δ 2.252 were due to H-6a & H-6b or H-14a & H-14b. However, in ^{1}H - ^{1}H COSY experiment multiplet at δ 2.14 showed coupling with only one signal at δ 4.66, whereas multiplets at δ1.58 and δ 2.31 showed coupling with signals due to one proton each at δ 4.07 and δ 4.985 respectively. This clearly suggested that multiplet at δ 2.14 is due to H-14a and H-14b which will couple with only H-13 which is appearing at δ 4.66 and signals at δ1.58 and δ 2.31 are due to H-6a and H-6b which are coupling with two different oxygenated protons H-5 and H-7 appearing at δ 4.07 and δ 4.98 or vice versa.

^{1}H - ^{1}H COSY experiment also showed coupling of oxygenated proton at δ 4.985 with only one proton at δ 2.31 (H-6α or H-6β), whereas oxygenated proton at δ 4.07 showed coupling not only with proton at δ1.58 (H-6α or H-6β) but also with a proton at δ 5.04 (d, *J* = 7 Hz). In ^{1}H - ^{13}C heterocoupling experiment, this particular proton at δ 5.04 did not show relation with any carbon atom signal, which indicated that this signal must be due to hydroxyl proton. Thus signal at δ 4.07 must be due to H-7 and signal at δ 5.04 must be due to C7-OH and signal at 4.985 must be due to H-

5. The ^{1}H - ^{13}C heterocoupling experiment further showed the correlation between assigned protons and corresponding carbon atoms (**Fig. 17**). However the experiment did not show correlation of proton signals at δ 3.93, 4.66, 4.74, 5.04 and 5.175 with any carbon atom signal thus confirming the presence of five hydroxyl groups and these signals must be due to hydroxyl protons.

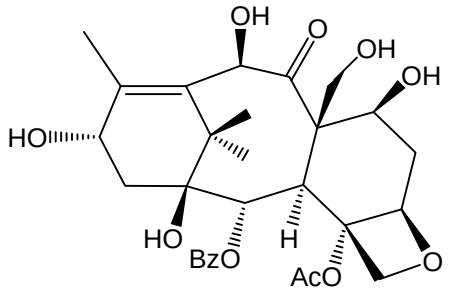
In ^{1}H - ^{1}H ROESY experiment (**Fig. 18**) H-5 showed ROE correlation with a C-6 proton at δ 2.31. Since biogenetically H-5 is α-oriented, this H-6 at δ 2.31 must also be α-oriented i.e. signal at δ 2.31 is due to H-6α and hence proton at δ1.58 must be H-6β.

Now though in ^{1}H - ^{1}H COSY experiment the H-6β showed coupling with H-7 at δ 4.07, in ^{1}H-^{1}H ROESY experiment the ROE correlation is not observed, which clearly indicated that these two protons are *trans* to each other i.e. H-7 appearing at δ 4.07 is α-oriented and C7-OH is β-oriented.

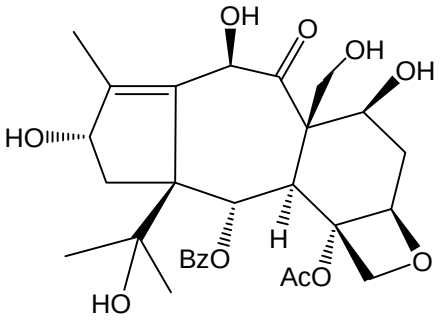
The ^{1}H-^{1}H ROESY experiment further showed correlation between H-7 and a proton appearing at δ 5.11 (brs) which has to be H-10. Since H-7 is α-oriented, H-10 must also be α-oriented and C10-OH must be β-oriented.

Further the chemical shift of H-13 at δ 4.66 clearly suggested that this proton is β-oriented and C13-OH is α-oriented as observed in all α-oriented C13-hydroxyl group of taxoids where β-oriented proton is appearing at δ 4.5 – 4.8.[43b,43d,43h,45] The only compound reported having β-oriented C13-hydroxyl group is 13-epi-10-deacetylbaccatin[46] in which the chemical shift of α-oriented H-13 is reported at much higher field at δ 3.71.

Thus the compound **52** could be a taxane or 11(15→1)abeotaxane having following structure with stereochemistry assigned at C-7, C-10 and C-13.



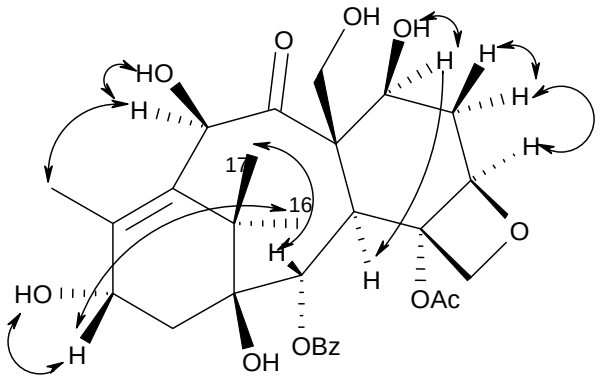
Taxane



11(15→1)Abeotaxane

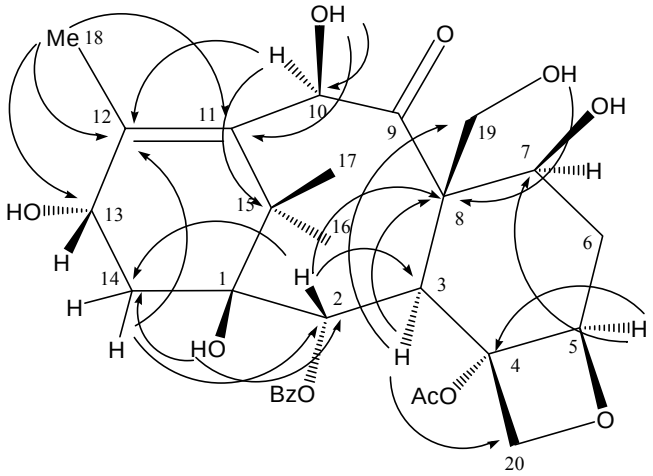
The ^{1}H-^{1}H ROESY experiment of compound **52** showed correlation between C10-H and Me-18. It further showed correlation between β-oriented H-2 and a methyl appearing at δ 1.18 which is designated as Me-17 and the Me-16 appearing at δ 0.99 showed correlation with H-13. The ROESY experiment also showed the correlation between H-6α and H-6β, H-7 and C7-hydroxy proton at δ 5.04 (d, *J* = 7 Hz), H-10 and C10-hydroxy proton at δ 4.74 (d, *J* = 2 Hz), and H-13 and C13-hydroxy proton at δ 5.175 (d, *J* = 3.8 Hz).

In ROESY experiments of most of the taxane compounds and 11(15→1)abeotaxane compounds, β-oriented H-2 and a C-15 methyl showed correlation, they also showed correlation between second C-15 methyl and H-13.[43b,43c,43d,45,49] Thus from these correlations it was not possible to identify the ring system of compound **52**. Hence HMBC correlation experiment was further carried out on compound **52**. In this experiment (**Fig. 19**) along with other correlations, the major correlation obtained was between H-14 and C-12. It did not show correlation between H-14 and C-11. In 11(15→1)abeotaxane derivatives H-14 showed correlation with both C-11 and C-12 as both carbons are equidistant from H-14.[43d,48] However in taxane series H-14 showed correlation with only C-12; it did not show correlation with C-11 as it is one bond away from H-14.[43d,48b] Thus from this observation it was concluded that compound **52** has taxane ring system and thus compound **52** was characterized as 19-hydroxy-10-deacetyl baccatin III.



ROESY correlation of compound **52**

HMBC experiment also revealed the correlation of hydroxyl proton at δ 4.66 with C-14 and C-2 thus confirming this hydroxyl group to be at C-1. It also revealed the correlation of hydroxyl proton at δ 3.93 with C-8 and thus confirming this hydroxyl group to be at C-19. The experiment further revealed correlation of Me-18 with C-11, C-12 and C-13 and thus confirming the olefinic methyl at C-12. The experiment also showed correlations between H-2 and C-2, C-3, C-8, C-14; H-3 and C-8, C-19, C-20; H-5 and C-4, C-7; C7-O<u>H</u> and C-7, C-8; H-10 and C-12, C-15; C10-O<u>H</u> and C-10, C-11; H-14 and C-2, C-12, O<u>C</u>OCH$_3$; H-17 and C-2, C-11, C-15, C-16; H-18 and C-11, C-12, C-13; H-20a and C-4; H-20b and C-5.



Some HMBC correlations of compound **52**

Literature survey revealed that this compound **52**, 19-hydroxy-10-deacetyl baccatin III is already isolated[50] from *Taxus baccata*. The ^{1}H NMR spectral data

matches with that of reported compound. However ^{13}C NMR data and detailed characterization of compound is not reported, which is described here.

2.3.4 Conclusion

Thus 10-deacetyl baccatin III (**3**), a precursor of taxol (**1**), a highly potent anticancer drug, has been isolated in almost 89% purity by enriching the methanol extract of needles of *T. baccata* by solvent-solvent extraction and final crystallization without time consuming column chromatography. During enrichment a new and a known taxoid have been isolated by preparative HPLC. Structures of both the compounds are elucidated by intensive spectral studies including ^{1}H - ^{1}H COSY, HSBC, HMBC, ROESY, NOESY experiments. The new compound **51** has been characterized as 13-epi-11,12-epoxy-10-deacetylbaccatin III and the known compound **52** has been characterized as 19-hydroxy-10-deacetyl baccatin III using various NMR techniques. The ^{13}C NMR data of compound **52** have been reported first time.

Table 8: ^{1}H NMR spectral data of compound **52** (DMSO-d_6)

Proton	Chemical Shift in δ	Multiplicity
H-2β	6.525	d, *J* = 7.0 Hz
H-3α	3.825	d, *J* = 6.8 Hz
H-5α	4.985	d, *J* = 9.2 Hz
H-6α	2.31	m (merged with acetate methyl)
H-6β	1.58	t, *J* = 12.8 Hz
H-7α	4.07	m (merged with H-20a)
H-10α	5.11	d, *J* = 2.0 Hz
H-13β	4.66	m
H-14a & H-14b	2.14	m
H-16	0.99	s
H-17	1.18	s
H-18	1.93	s
H-19a & H-19b	4.503 & 4.513	AB quartets, *J* = 12.2 Hz
H-20a	4.11	d, merged with H-7
H-20b	4.37	d, *J* = 7.7 Hz
OCOC$\underline{H}_3$	2.27	s
OBz:H-2'& H-6'	8.09	d, *J* = 7.3 Hz
H-3' & H-5'	7.59	m
H-4'	7.68	m
C1-O$\underline{H}$	3.93	s
C7-O$\underline{H}$	5.04	d, *J* = 7.0 Hz
C10-O$\underline{H}$	4.74	d, *J* = 2.0 Hz
C13-O$\underline{H}$	5.175	d, *J* = 3.8 Hz
C19-O$\underline{H}$	4.66	m

Table 9: ^{13}C NMR spectral data of compound **52** (DMSO-d_6)

Carbon	Chemical shift in δ
C-1	78.68, s
C-2	77.57, d
C-3	47.69, d
C-4	81.20, s
C-5	84.81, d
C-6	37.54, t
C-7	71.58, d
C-8	60.96, s
C-9	208.96, s
C-10	75.39, d
C-11	135.83, s
C-12	142.29, s
C-13	67.05, d
C-14	40.23, t
C-15	43.40, s
C-16	27.54, q
C-17	21.23, q
C-18	15.64, q
C-19	58.35, t
C-20	76.21, t
O<u>C</u>OCH$_3$	170.48, s
OCO<u>C</u>H$_3$	23.16, q
O<u>C</u>OBz	165.44, s
C-1’	131.87, s
C-2’	130.42, d
C-3’	129.49, d
C-4’	133.77, d
C-5’	129.49, d
C-6’	130.42, d

2.4 EXPERIMENTAL

2.4.1 Extraction and enrichment of 10-deacetylbaccatin III

Powdered air-dried leaves (1 kg) of *Taxus baccata* were extracted with methanol (5 lit X 2) with stirring for 12 hrs at ambient temperature. The extract was concentrated under reduced pressure at 50-55 °C to obtain dark mass (250 g). The extract was stirred with hexane (1.2 lit) at ambient temperature for two hrs. The mixture was filtered and the residue was washed with hexane (200 ml). The insoluble solid (180 g) was stirred with acetone (1.7 lit) for two hrs at room temperature. The mixture was centrifuged and the clear acetone solution part was separated. The acetone solution was concentrated to dryness at 50-55 °C under reduced pressure to give 113 g of residue. This residue was dissolved in chloroform (2 lit) and was extracted with water:methanol (95:5) (1 lit X 2). Aqueous layer was removed; chloroform layer was dried over anhydrous sodium sulphate and concentrated to dryness at 50-55 °C under reduced pressure to obtain brown colored mass (82 g). This brown colored mass was dissolved in methanol (400 ml) with gentle heating and the solution was kept at -5 °C for twelve hrs. Precipitate obtained was filtered and dried under reduced pressure to afford yellowish powder (3.82 g). It was dissolved in warm acetonitrile (150 ml) and cooled at -5 °C for 12 hrs. The precipitate formed was filtered and washed with cold acetonitrile to afford 0.935 g of 10-DAB III (**3**). The filtrate was concentrated to give 2.62 g of residue. 10-Deacetylbaccatin III (**3**) isolated was analyzed by analytical HPLC, the purity of 10-DAB III (**3**) was found to be 89% w/w by quantitative HPLC.

2.4.2 Analysis of 10-DAB III by HPLC

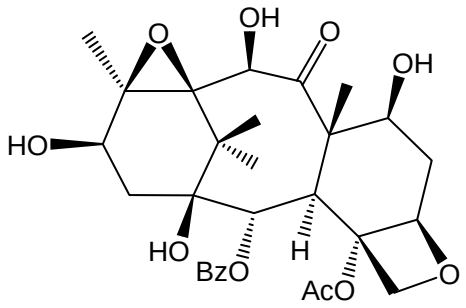
Analytical HPLC chromatographic binary gradient system (Shimadzu Japan) consisted of SPD-10A*VP* multi wavelength UV detector, SCL-10A*VP* system controller, LC-10AT*VP* HPLC pumps and Class *VP* software. Column used was C18 Waters XBridge 5 µm (4.6 mm x 250 mm) and the detector was set at wavelength 227 nm. Mobile phases used were acetonitrile in pump A and water in pump B. Column was equilibrated with 28 % A. Linear gradient elution was carried out at flow rate 1.0 ml/min 28% A for 16 min, then changed to 33% A at 17 min, held at 33% A until 29 min and then changed to 45% A until 60 min. Standard 10-DAB III was obtained

from Bristol-Mayer Ltd USA. Samples were dissolved in 45% acetonitrile in water. The standard solution was stored in freeze at 0 °C. Linearity was studied for detector and 10-DAB III loading. The linear range of analysis was found from 1 µg to 25 µg of standard 10-DAB III. Relative standard deviation was 0.2%.

2.4.3 Isolation of compounds 51 and 52 by preparative HPLC

HPLC analysis of the residue (2.62 g) from acetonitrile mother liquor obtained above showed that it was a mixture of compounds containing major peaks at RT 12.1 min (40% area) and RT 15.4 min (23.3 % area) including 10-DAB III (**3**) (13.8 % area). The residual mixture was separated by preparative HPLC using C18 Waters XBridge 5 µm (10 mm x 250 mm) column. Shimadzu LC8A pumps, Rheodyne injector with 2 ml loop and SPD10A UV detector were used. The detector was set at wavelength 227 nm. Mobile phases used were same as those of analytical HPLC. The flow rate used was 4.6 ml/min and the injection size was 1.2 ml for sample solution. The mother liquor after removing 10-DAB III (**3**) was concentrated to yield 2.6 g of residue. It was dissolved in 300 ml 45% acetonitrile in water with the help of ultrasonic bath. Separation was achieved with a gradient elution as per analytical method. All three fractions for three peaks were collected separately. Fraction collected at RT 12.0 min to 12.4 min was labeled as **F1**, fraction collected from RT 14.7 min. to 15.2 min was labeled as **F2** and fraction collected from RT 15.3 min. to 15.7 min was labeled as **F3**. Acetonitrile from all the fractions was removed under reduced pressure. The residual aqueous layers were extracted with ethyl acetate to yield 0.264 g of compound **52** from **F1** and 0.032 g of compound **51** from **F3**. From **F2**, 13 mg of 10-DAB III (**3**) was obtained. Spectral data of 10-DAB III (**3**) were identical to those reported in literature.[51]

13-Epi-11,12-epoxy-10-deacetylbaccatin III (51)



Nature: White solid; Yield: 32 mg; $[\alpha]_D^{25}$: + 41° ($CH_3CH_2OCOCH_3$, *c* 0.6)

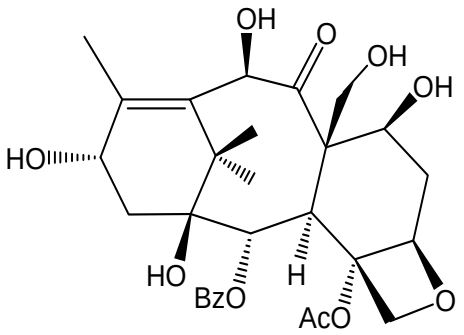
IR ($CHCl_3$): ν_{max} 3425.7, 1718.3, 1591.3, 1248.5 cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ 0.771 (s, H-16), 1.017 (s, H-17), 1.532 (s, H-18), 1.636 (s, H-19), 1.684 (m, merged with H-18, H-6α), 1.935 (dd, *J* = 6.4, 10.0 Hz, H-14b), 2.187 (dd, *J* = 7.2, 9.2 Hz, H-14a), 2.276 (s, OCOC<u>H</u>$_3$), 2.351 (m, merged with acetate methyl, H-6β), 3.076 (d, J = 6.7 Hz, H-3α), 3.978 (m, merged with H-7, H-13α), 4.044 (m, merged with H-13, H-7α), 4.097 (AB quartet, *J* = 5.6 Hz, H-20a & 20b), 4.203 (d, *J* = 2.8 Hz, H-10α), 4.504 (s, C1-O<u>H</u>), 4.804 (d, J = 2.8 Hz, C10-O<u>H</u>), 5.102 (d, *J* = 9.2 Hz, H-5α), 5.121 (d, *J* = 7.2 Hz, C7-O<u>H</u>), 5.396 (d, *J* = 6.7 Hz, H-2β), 5.474 (d, *J* = 4.4 Hz, C13-O<u>H</u>), 7.569 (m, H-3' & H-5'), 7.667 (m, H-4'), 8.013 (d, *J* = 7.2 Hz, H-2' & H-6').

^{13}C NMR (100 MHz, DMSO-d_6): δ 10.12 (C-19), 15.74 (C-18), 20.67 (C-17), 22.70 (C-OCO<u>C</u>H$_3$), 26.41 (C-16), 36.97 (C-6), 38.92 (C-14), 43.19 (C-15), 44.94 (C-3), 57.07 (C-8), 66.08 (C-13), 67.00 (C-11), 67.20 (C-12), 71.08 (C-7), 74.62 (C-2), 76.12 (C-10), 76.22 (C-20), 77.70 (C-1), 80.85 (C-4), 83.44 (C-5), 129.19 (C-3' & C-5'), 130.03 (C-2' & C-6'), 130.49 (C-1'), 133.78 (C-4'), 165.66 (O<u>C</u>OBz), 170.34 (O<u>C</u>OCH$_3$), 209.73 (C-9).

ESI-Mass: 583.6 $[M + Na]^+$, 561.5 $[M + H]^+$, 501.6, 361.3.

19-Hydroxy-10-deacetyl baccatin III (52)



Nature: White solid; Yield: 264 mg; $[\alpha]_D^{25}$: -56.3 ° ($CH_3CH_2OCOCH_3$, *c* 0.55)

IR ($CHCl_3$): ν_{max} 3428.13, 1713.06, 1246.4 cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ 0.99 (s, H-16), 1.18 (s, H-17), 1.58 (t, *J* = 12.8 Hz, H-6β), 1.93 (s, H-18), 2.14 (m, H-14), 2.27 (s, OCOC<u>H</u>$_3$), 2.31 (m, H-6α), 3.825 (d, *J* = 6.8 Hz), 3.93 (s, C1-O<u>H</u>), 4.07 (m, H-7α), 4.11 (d, merged with H-7, H-20a), 4.37 (d, *J* = 7.7 Hz, H-20b), 4.503 & 4.513 (two ABq, *J* = 12.2 Hz, H-19a & H-19b), 4.66 (m, H-13β), 4.66 (m, C19-O<u>H</u>), 4.74 (d, J = 2.0 Hz, C10-O<u>H</u>), 4.985 (d, *J* = 9.2 Hz, H-5α), 5.04 (d, *J* = 7.0 Hz, C7-O<u>H</u>), 5.11 (d, *J* = 2.0 Hz, H-10α), 5.175 (d, J = 3.8 Hz, C13-O<u>H</u>), 6.525 (d, *J* = 7.0 Hz, H-2β), 7.59 (m, H-3' & H-5'), 7.68 (m, H-4'), 8.09 (d, J = 7.3 Hz, H-2' & H-6').

^{13}C NMR (100 MHz, DMSO-d_6): δ 15.64 (C-18), 21.23 (C-17), 23.16 (OCO<u>C</u>H$_3$), 27.54 (C-16), 37.54 (C-6), 40.23 (C-14), 43.40 (C-15), 47.69 (C-3), 58.35 (C-19), 60.96 (C-8), 67.05 (C-13), 71.58 (C-7), 75.39 (C-10), 76.21 (C-20), 77.57 (C-2), 78.68 (C-1), 81.20 (C-4), 84.81 (C-5), 129.49 (C-3'), 129.49 (C-5'), 130.42 (C-2'), 130.42 (C-6'), 131.87 (C-1'), 133.77 (C-4'), 135.83 (C-11), 142.29 (C-12), 165.44 (OBz-<u>C</u>O), 170.48 (O<u>C</u>OCH$_3$), 208.96 (C-9).

ESI-Mass: 583.3 $[M + Na]^+$, 464.1, 360.4, 304.3.

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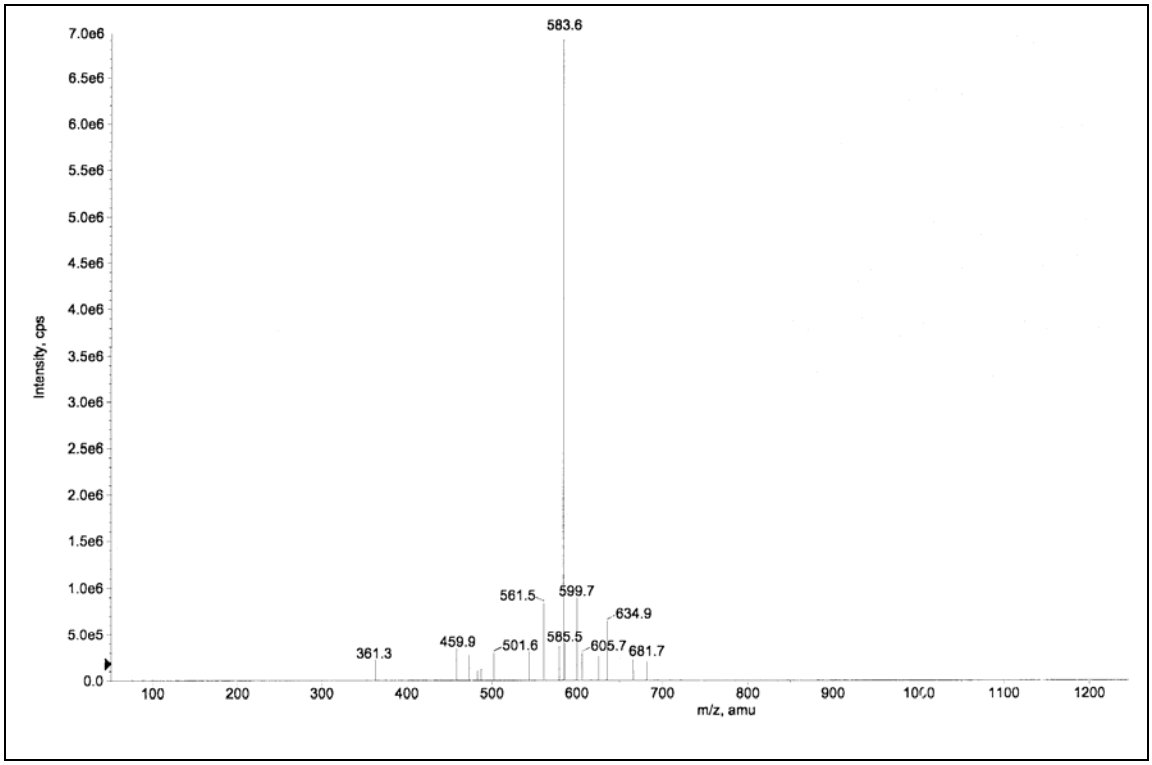


Fig. 1: ESI-mass spectrum of compound **51**

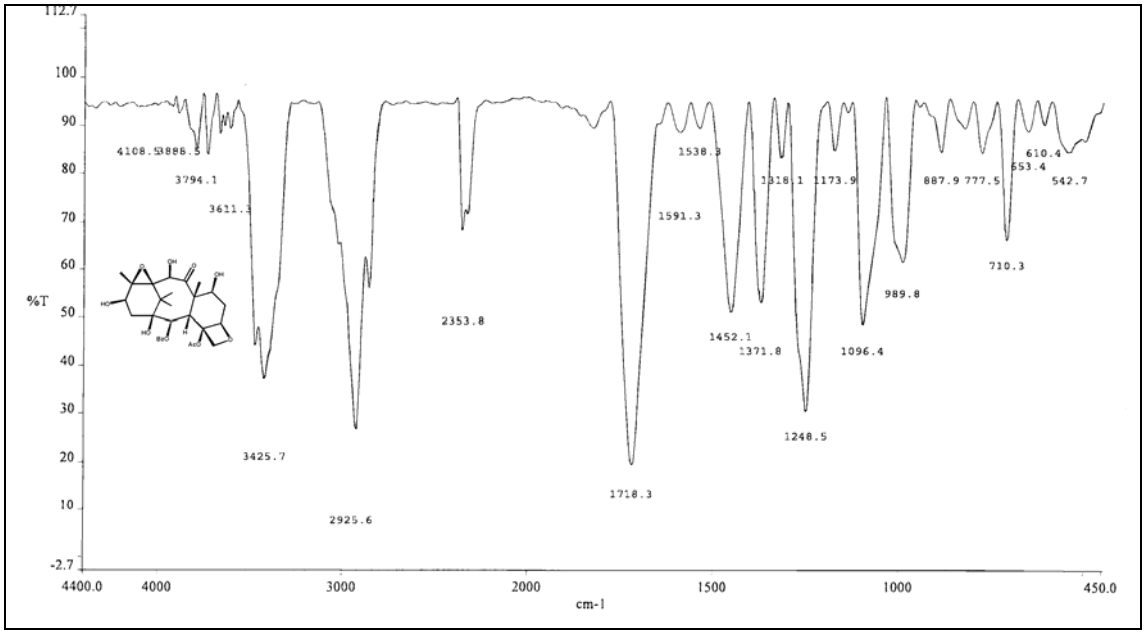
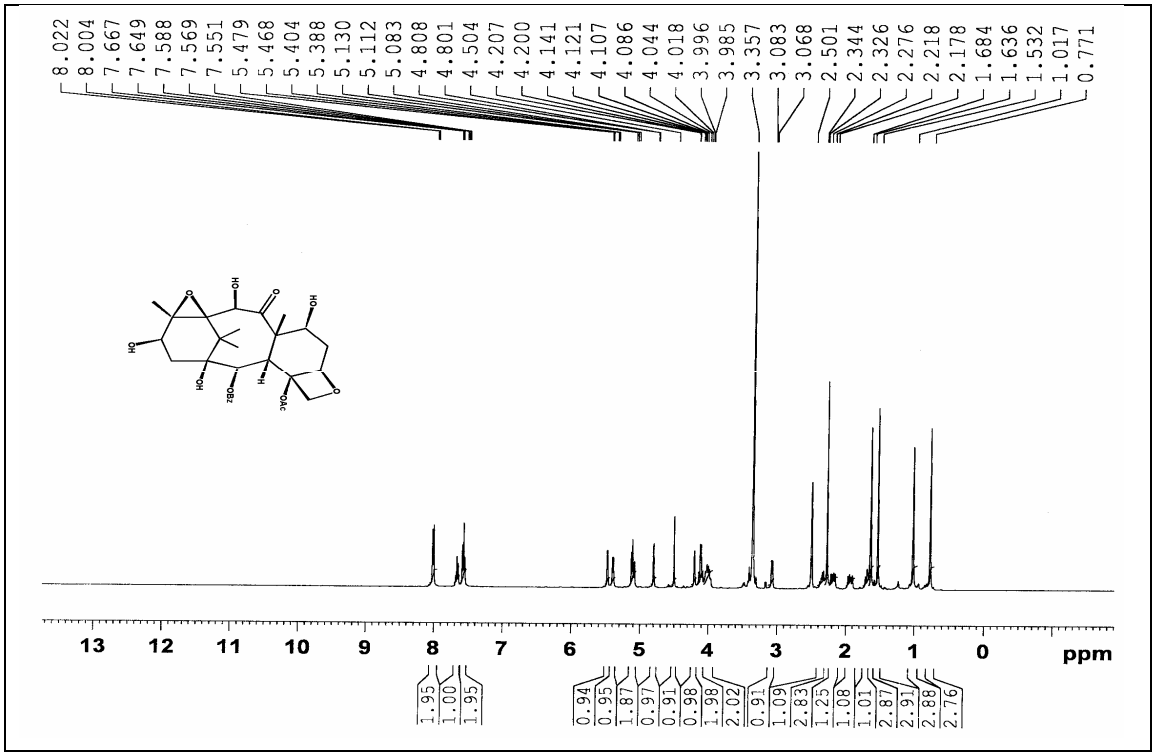


Fig. 2: IR spectrum of compound **51**



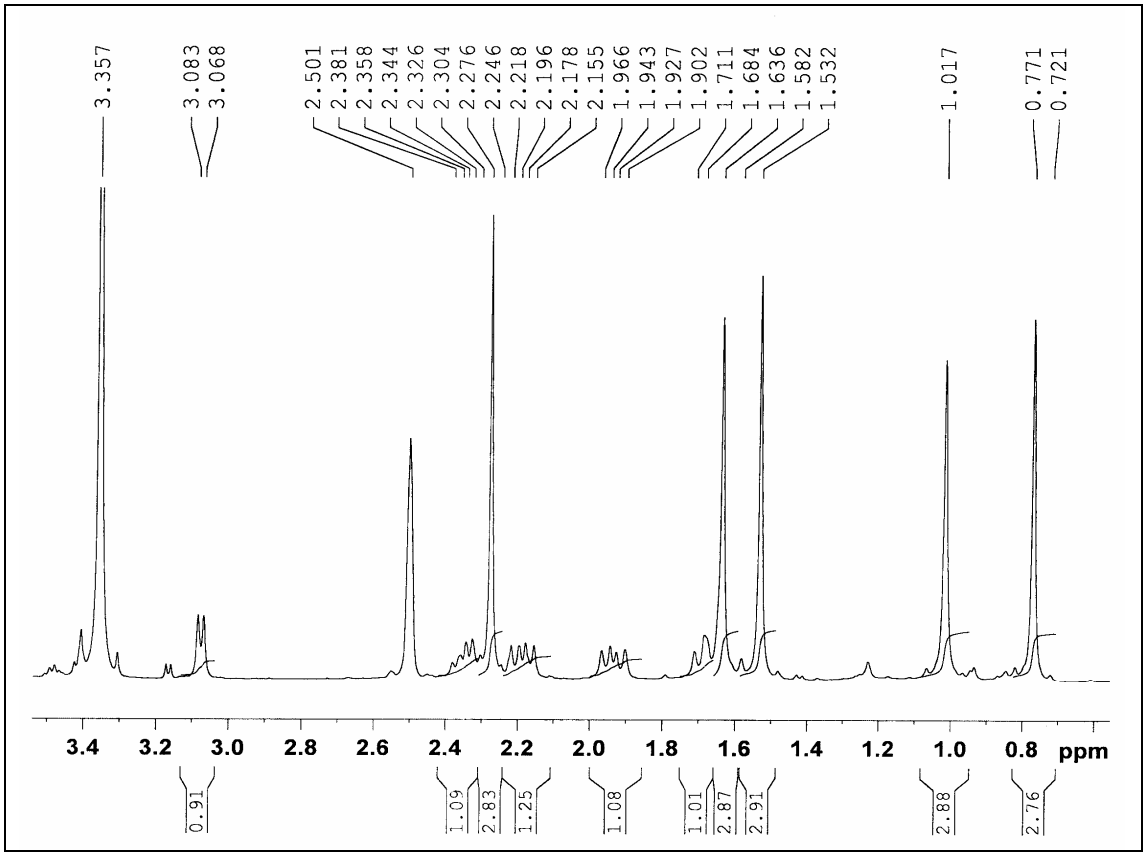


Fig. 3 : ^{1}H NMR spectra of compound **51** (DMSO-d_6, 400 MHz)

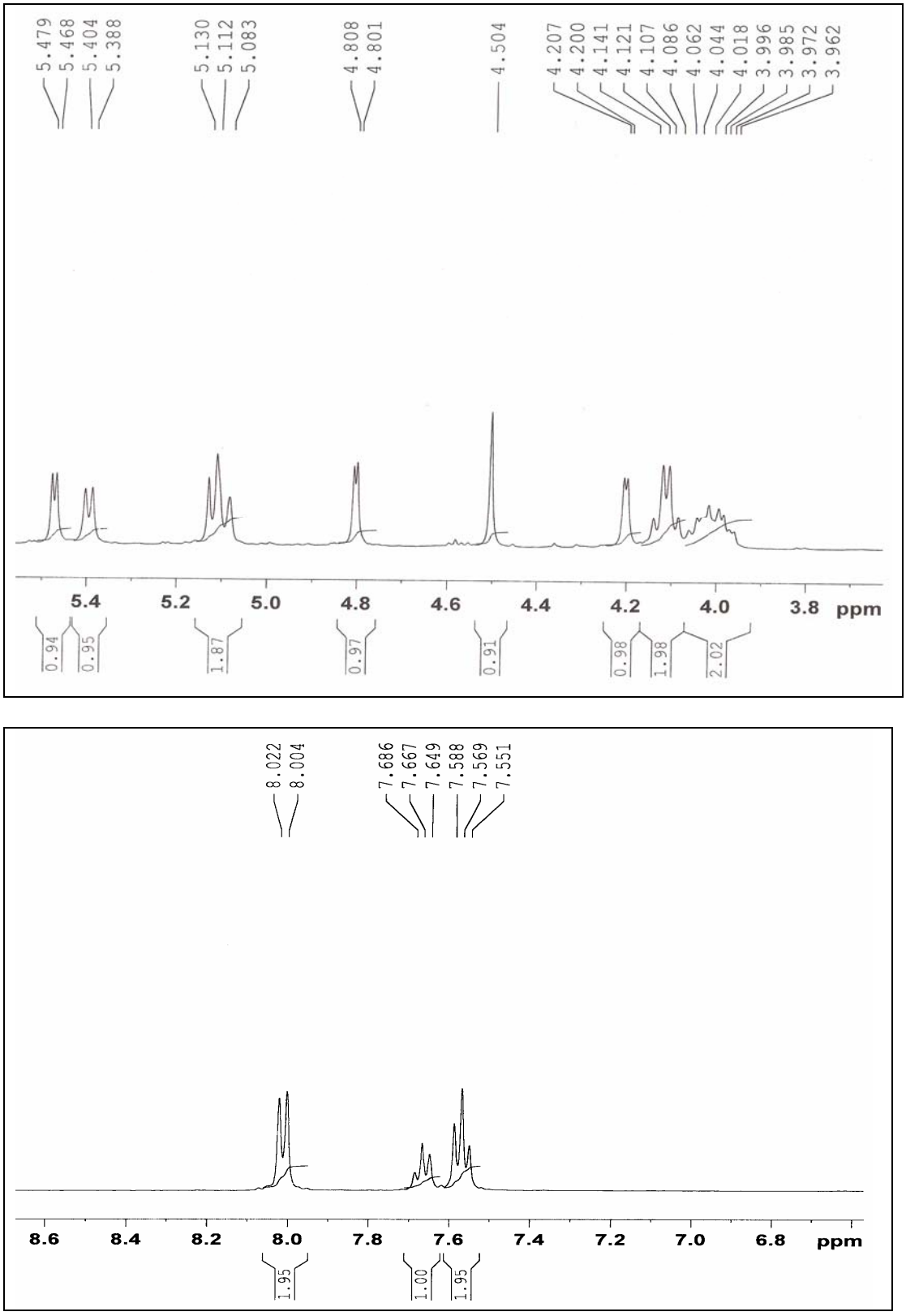


Fig. 3: ^{1}H NMR spectra of compound **51** (DMSO-d_6, 400 MHz)

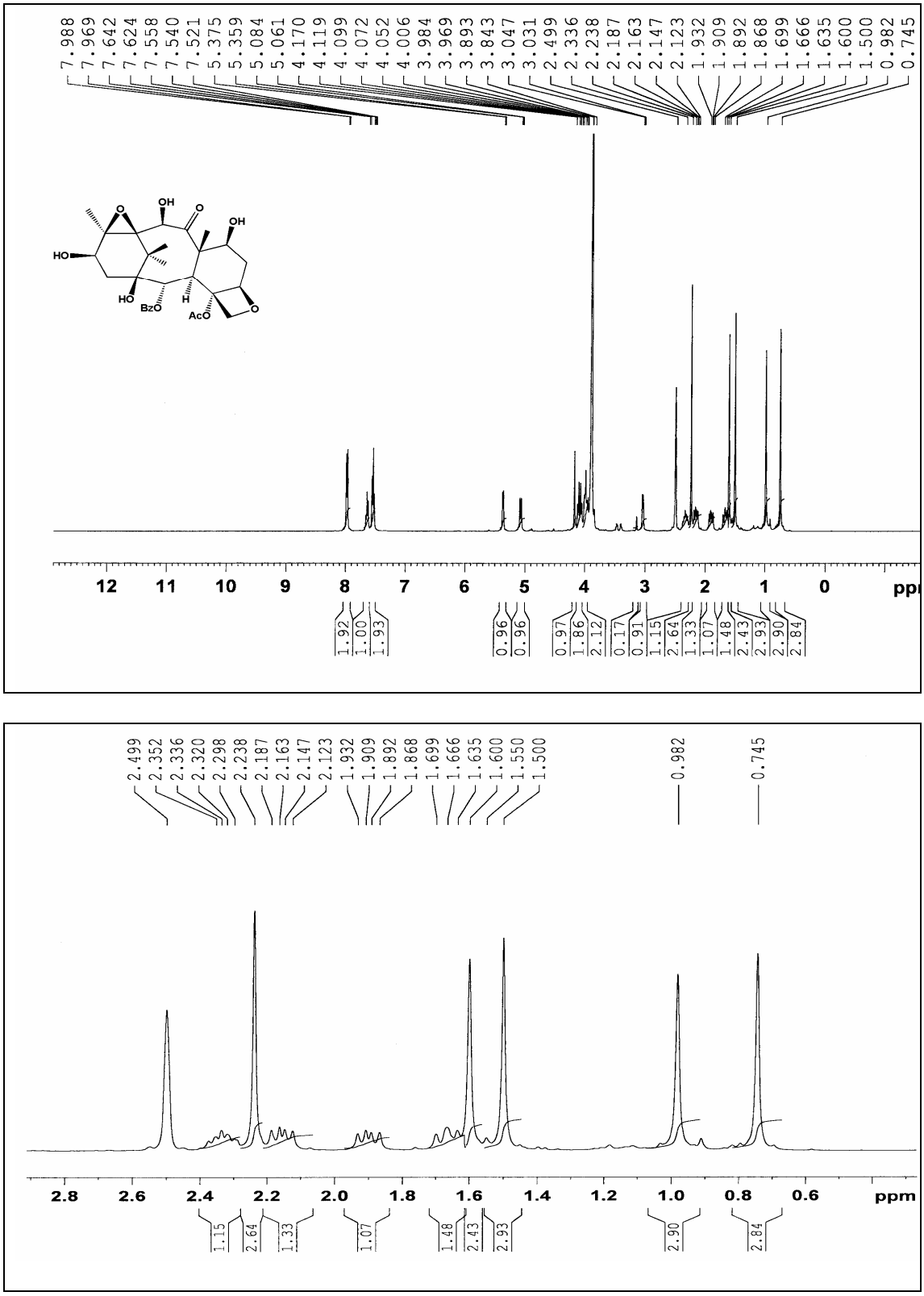


Fig. 4: ^{1}H NMR spectra after D_2O exchange of compound **51** (DMSO-d_6, 400 MHz)

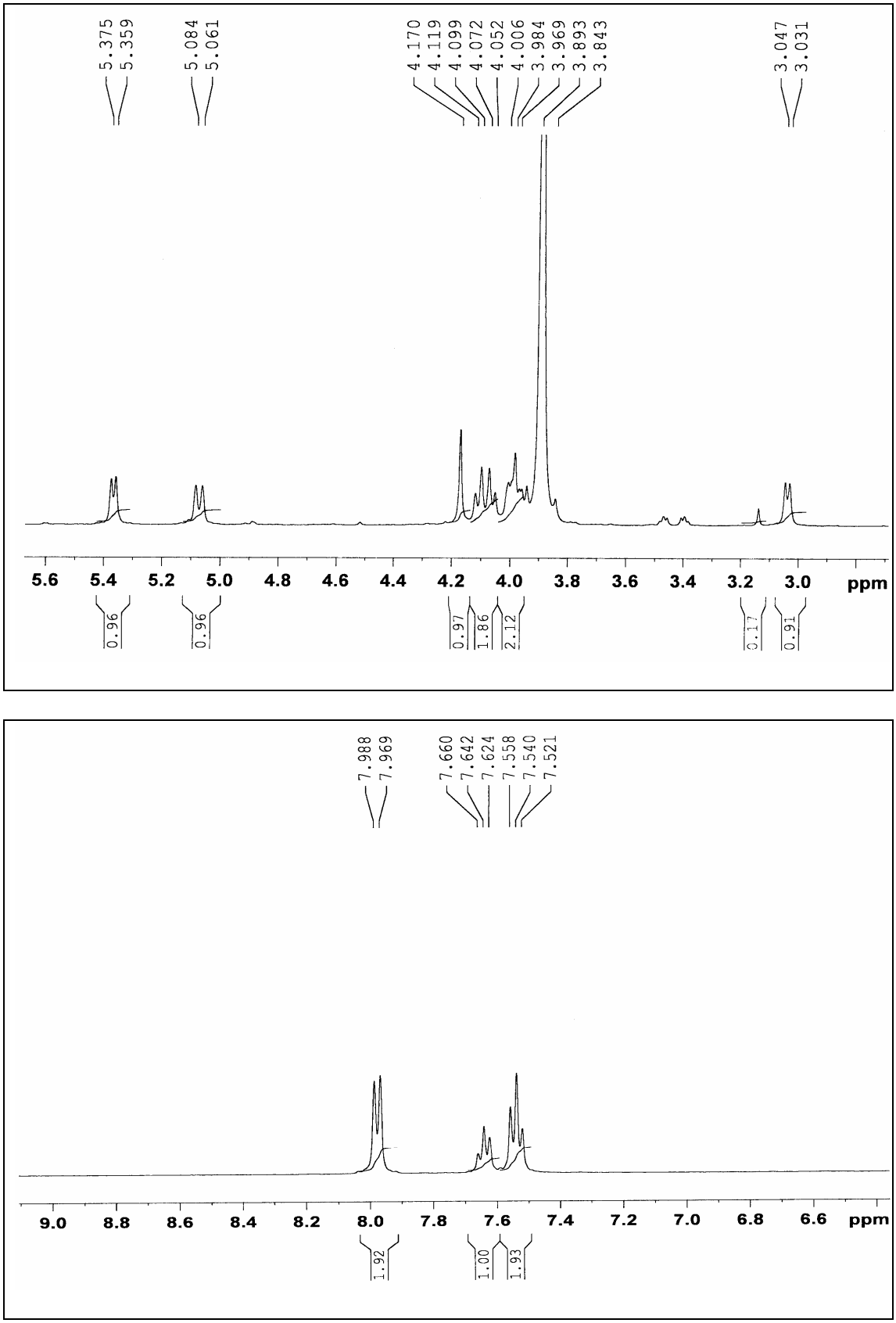


Fig. 4: ^{1}H NMR spectra after D_2O exchange of compound **51** (DMSO-d_6, 400 MHz)

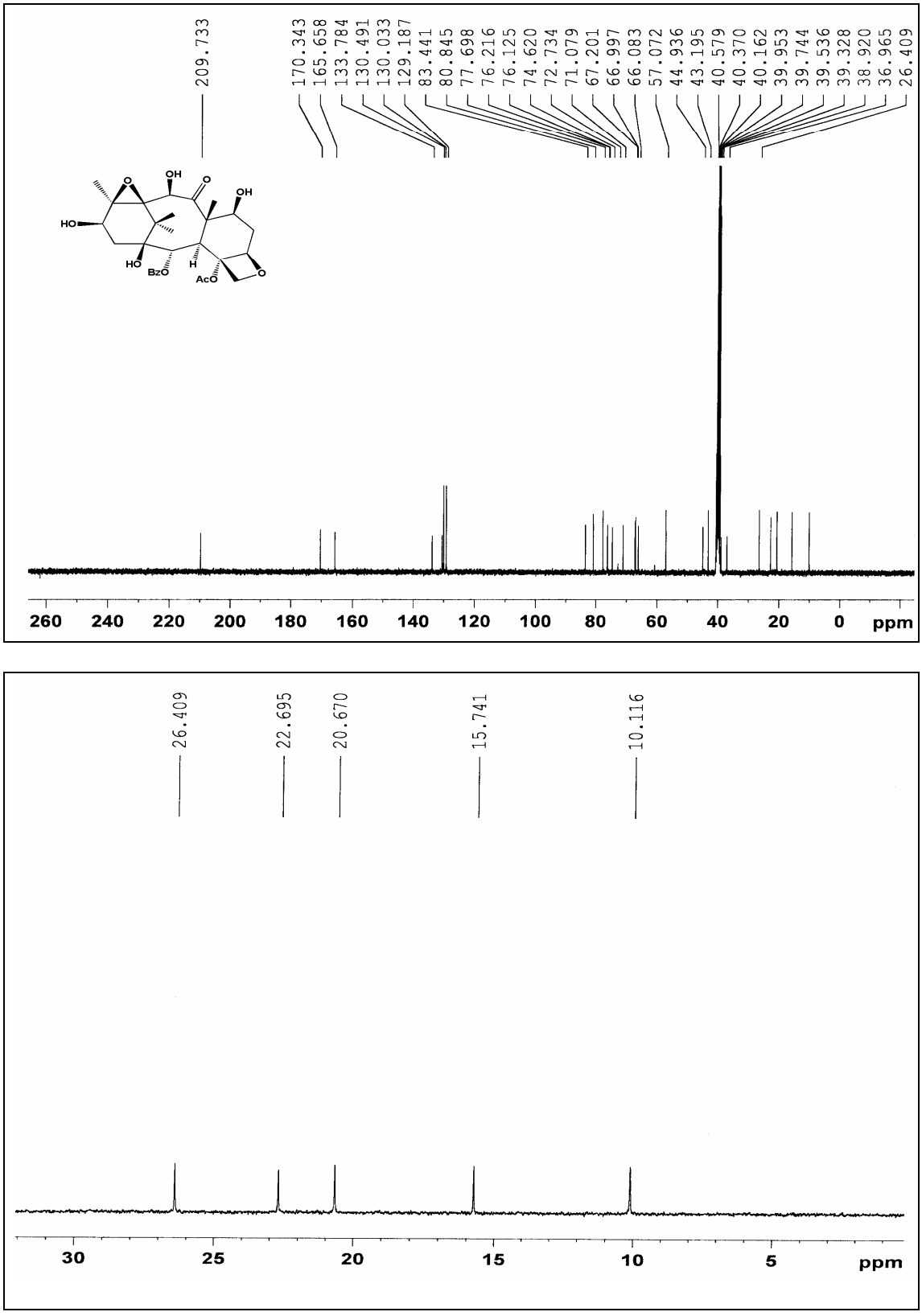


Fig. 5: ^{13}C NMR spectra of compound **51** (DMSO-d_6, 100 MHz)

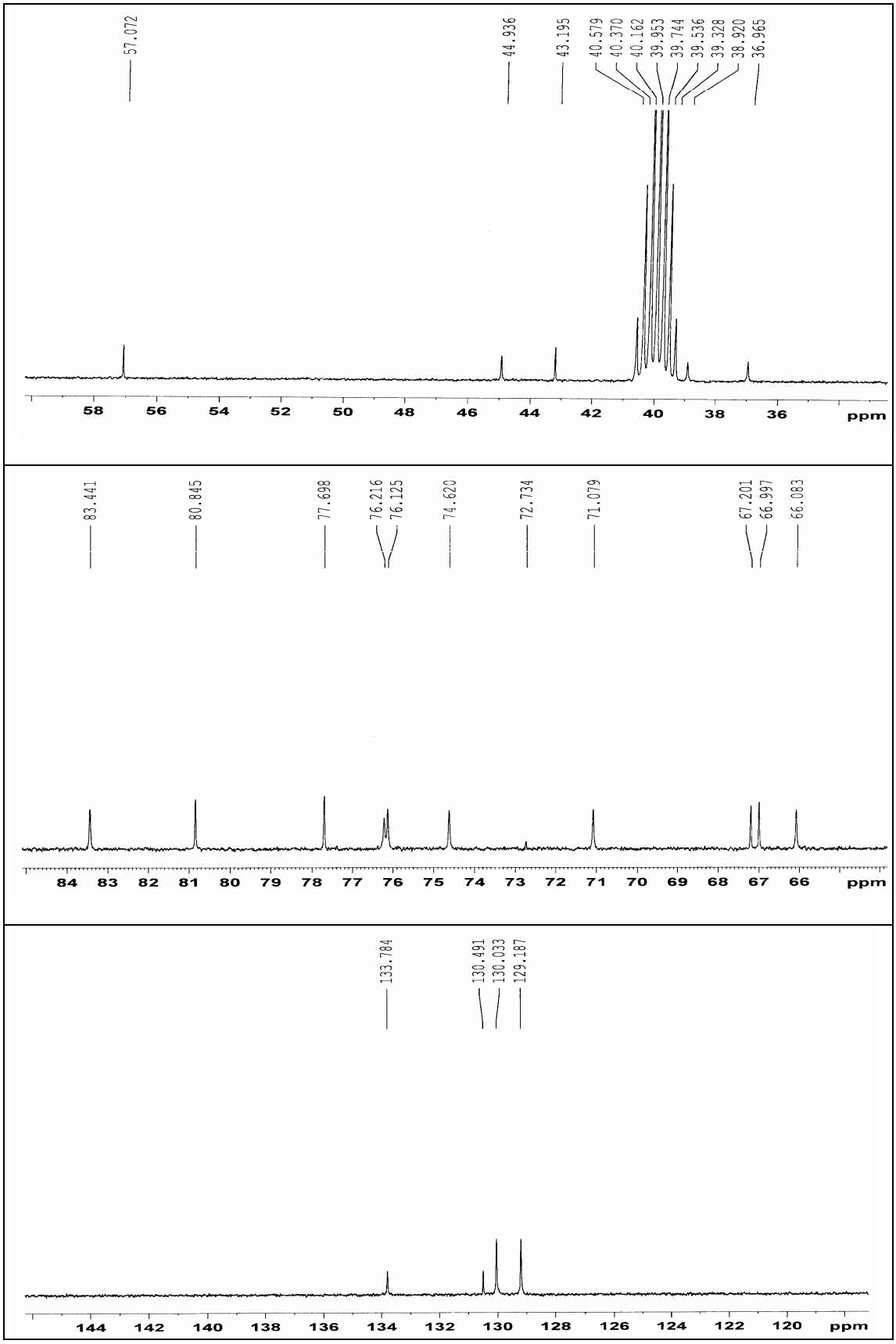
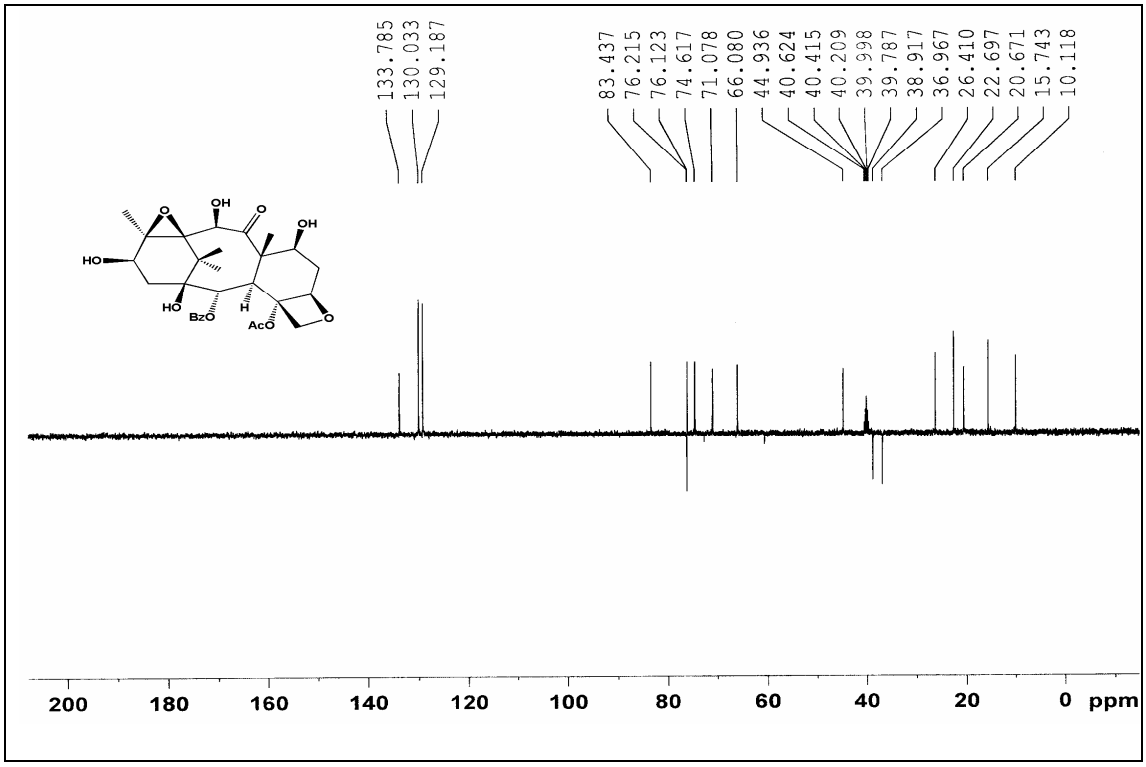


Fig. 5: ^{13}C NMR spectra of compound **51** (DMSO-d_6, 100 MHz)



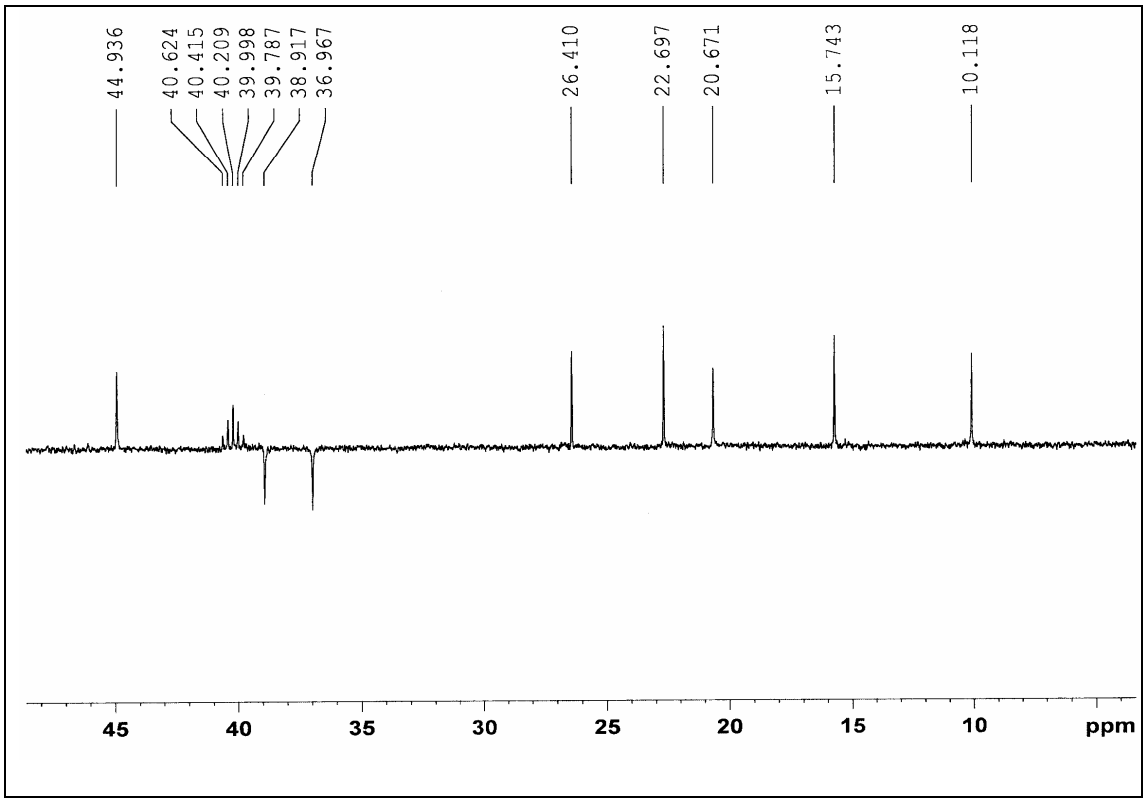


Fig. 6: DEPT-135 experiment spectra of compound **51** (DMSO-d_6, 100 MHz)

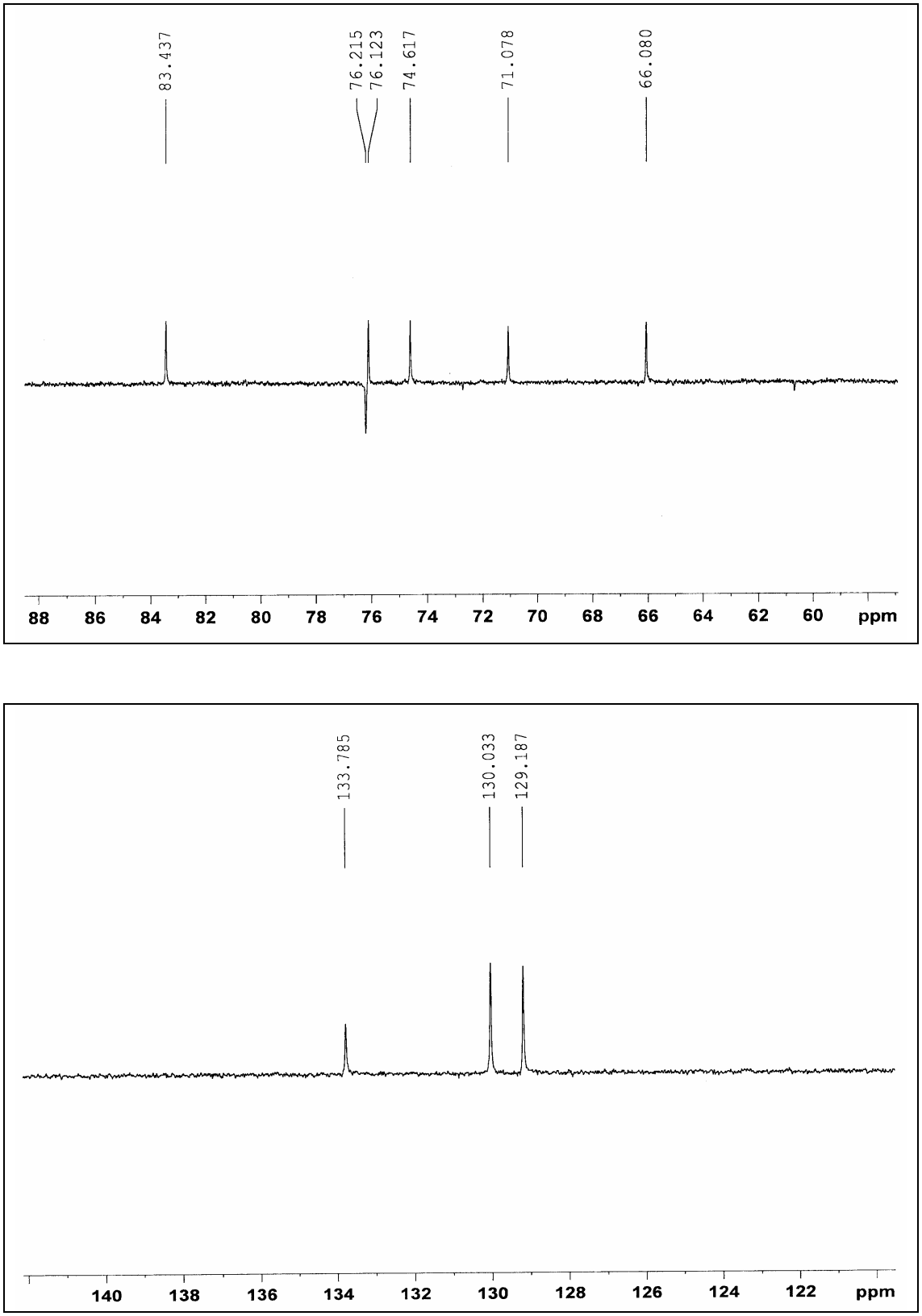


Fig. 6: DEPT-135 experiment spectra of compound **51** (DMSO-d_6, 100 MHz)

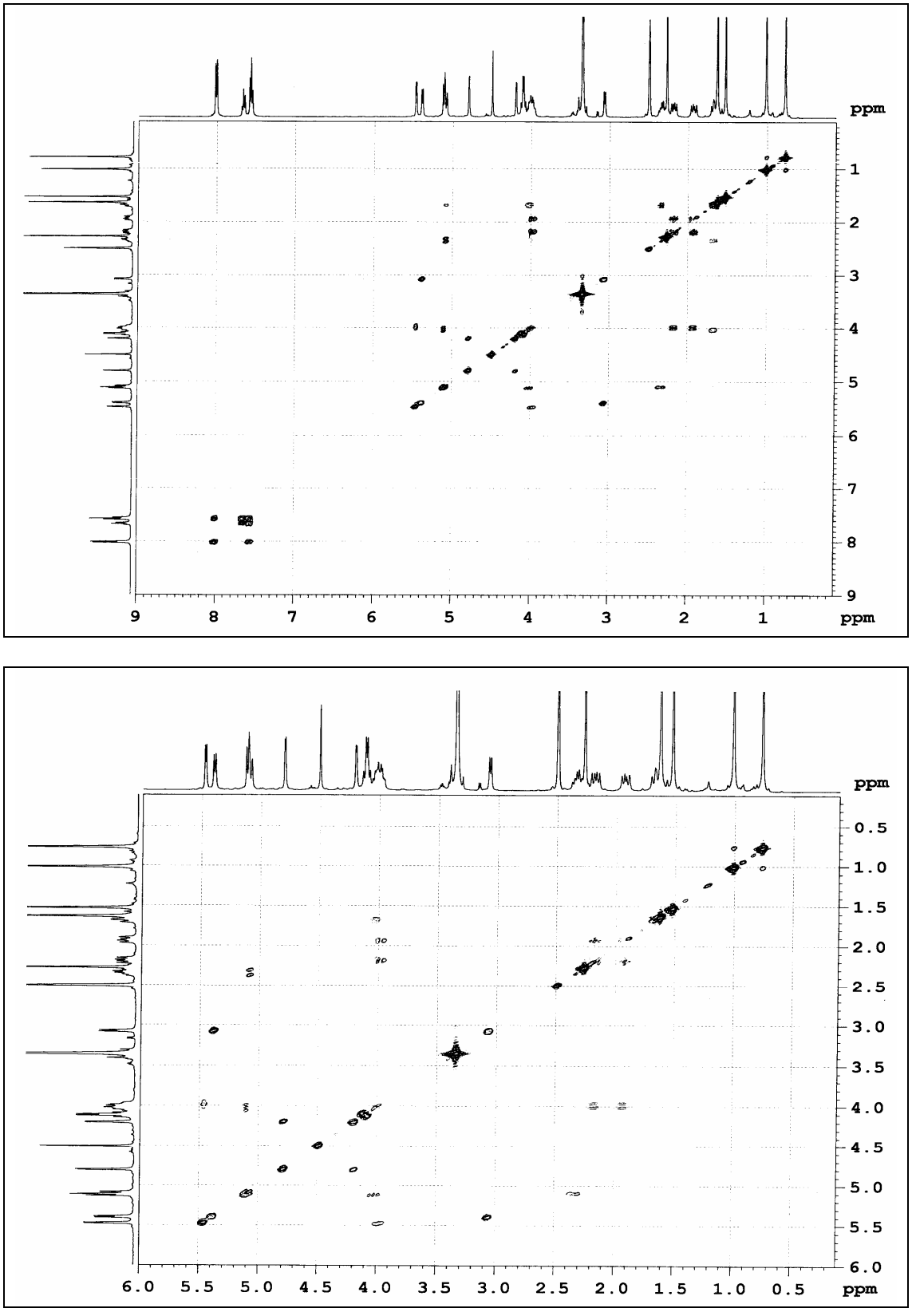


Fig. 7: ^{1}H-^{1}H COSY experiment of compound **51** (DMSO-d_6, 400 MHz)

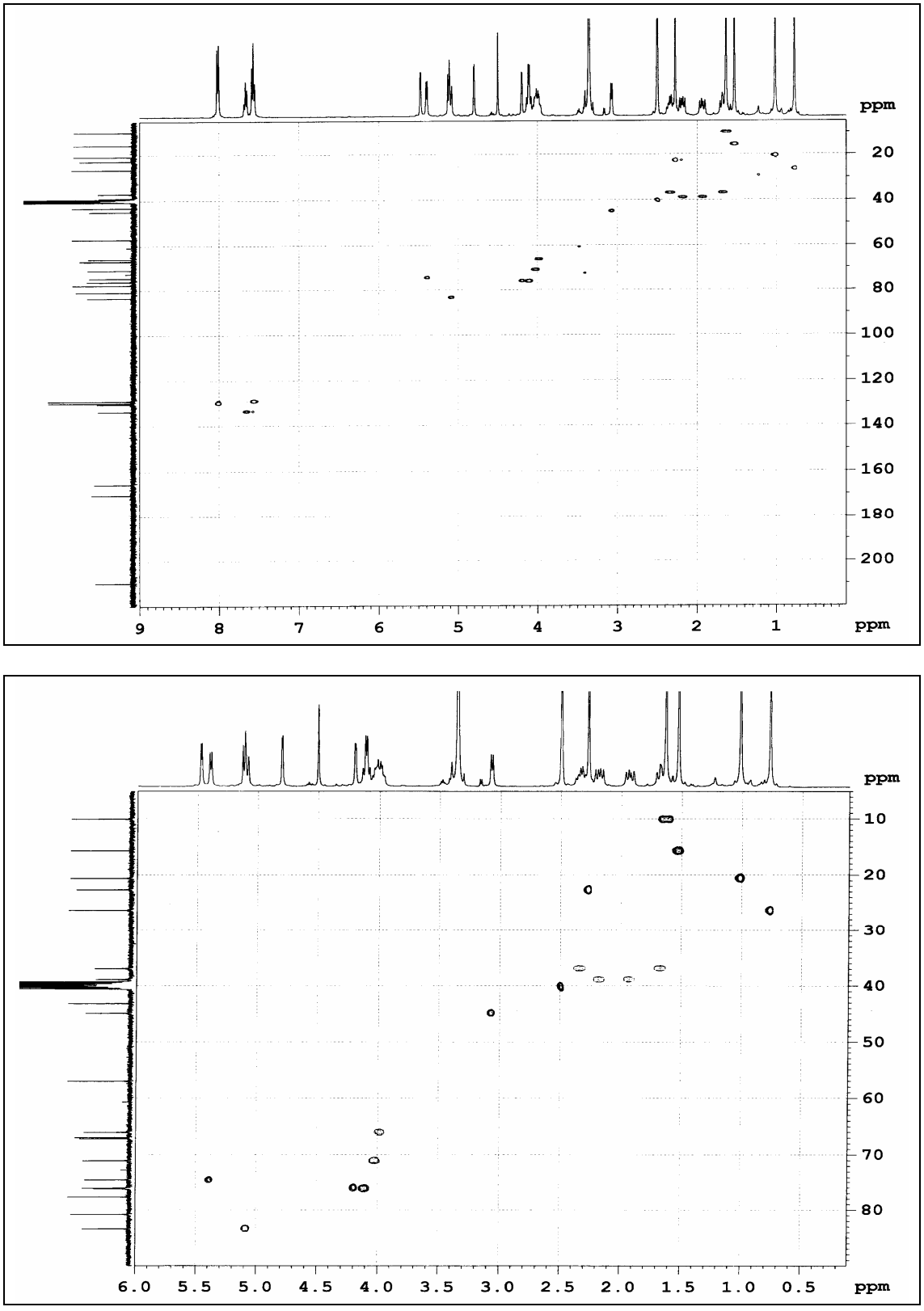


Fig. 8: ^{1}H-^{13}C HSQC experiment of compound **51** (DMSO-d_6)

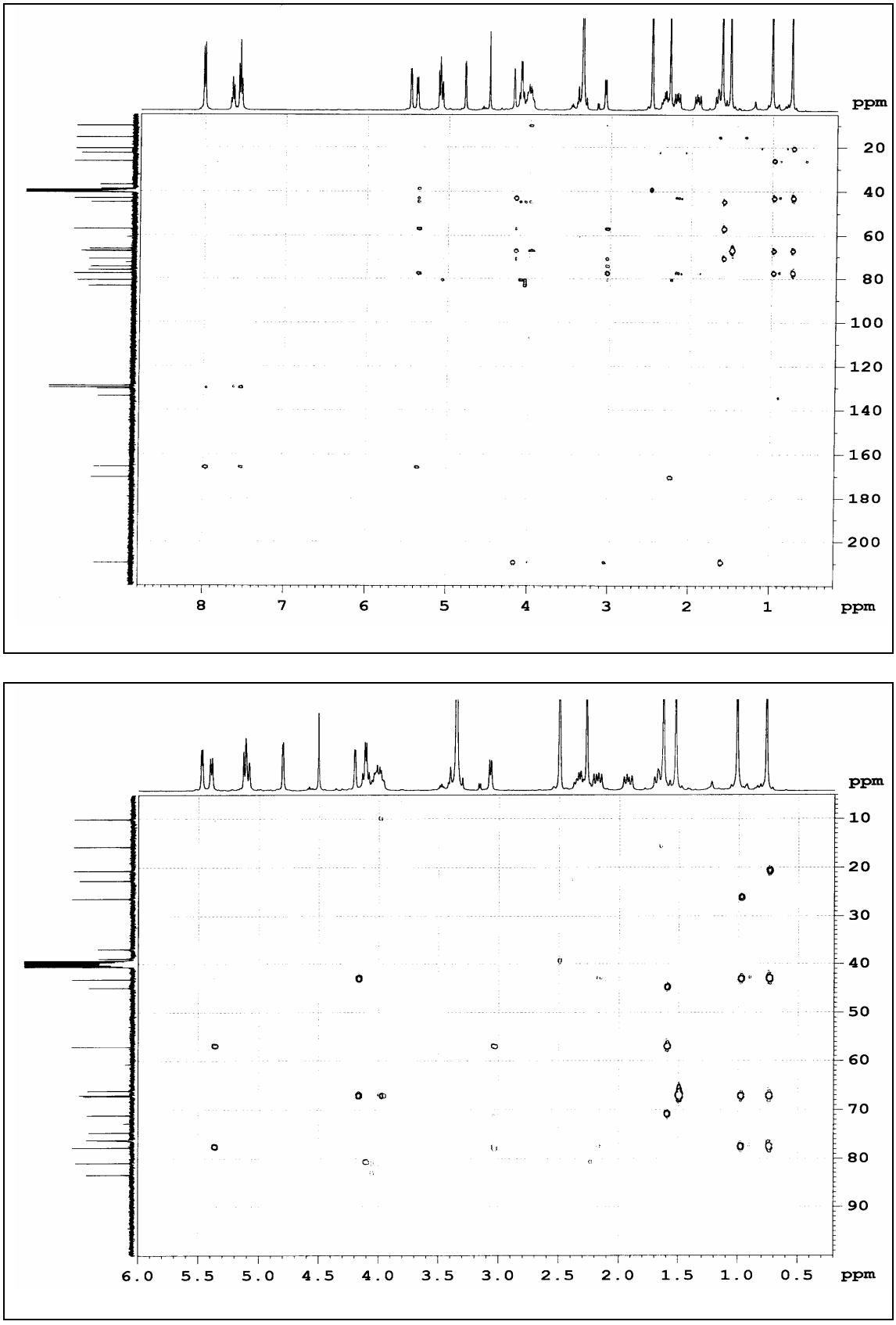


Fig. 9: ^{1}H-^{13}C HMBC experiment of compound **51** (DMSO-d_6)

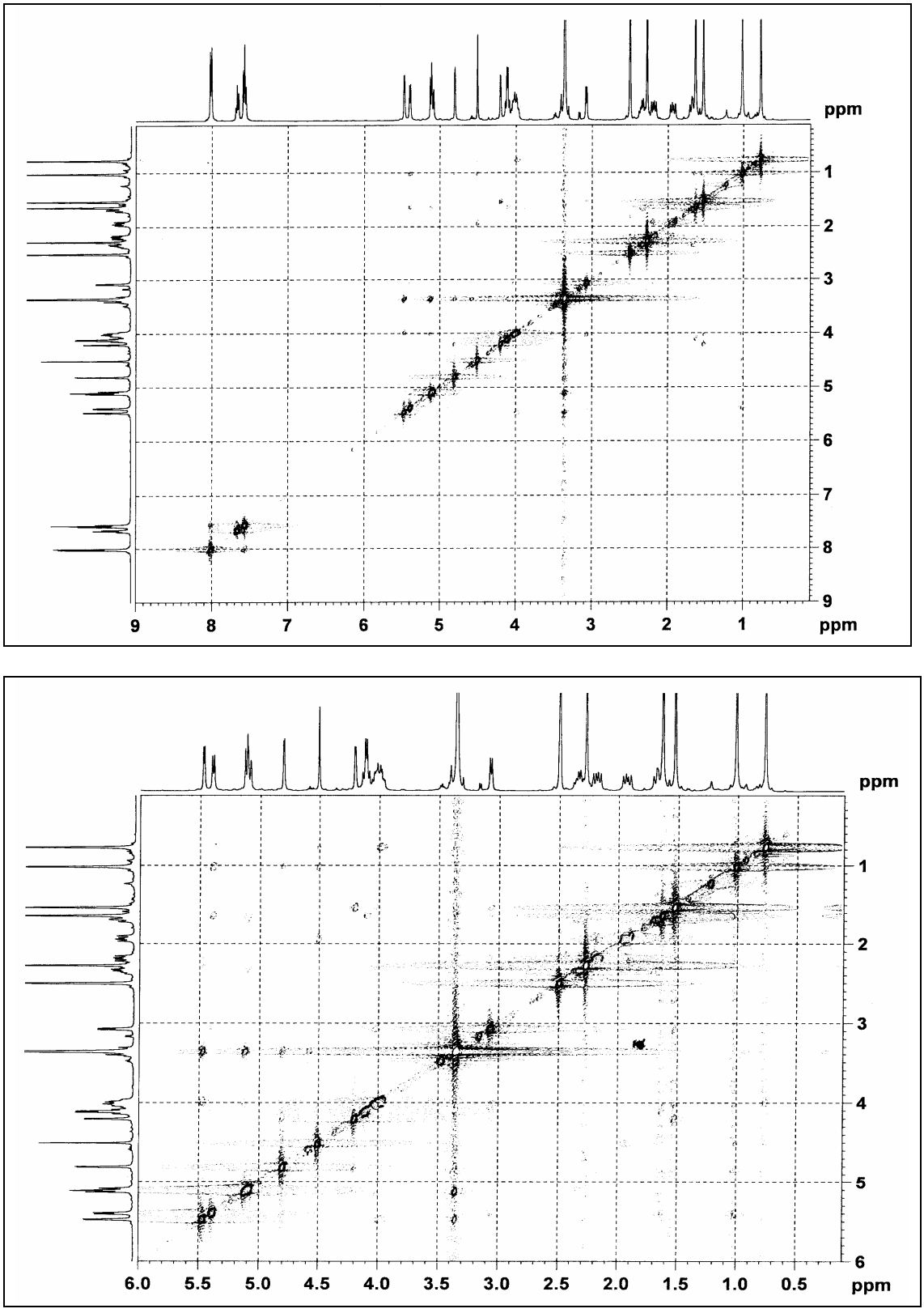


Fig. 10: ^{1}H- ^{1}H NOESY experiment of compound **51** (DMSO-d_6)

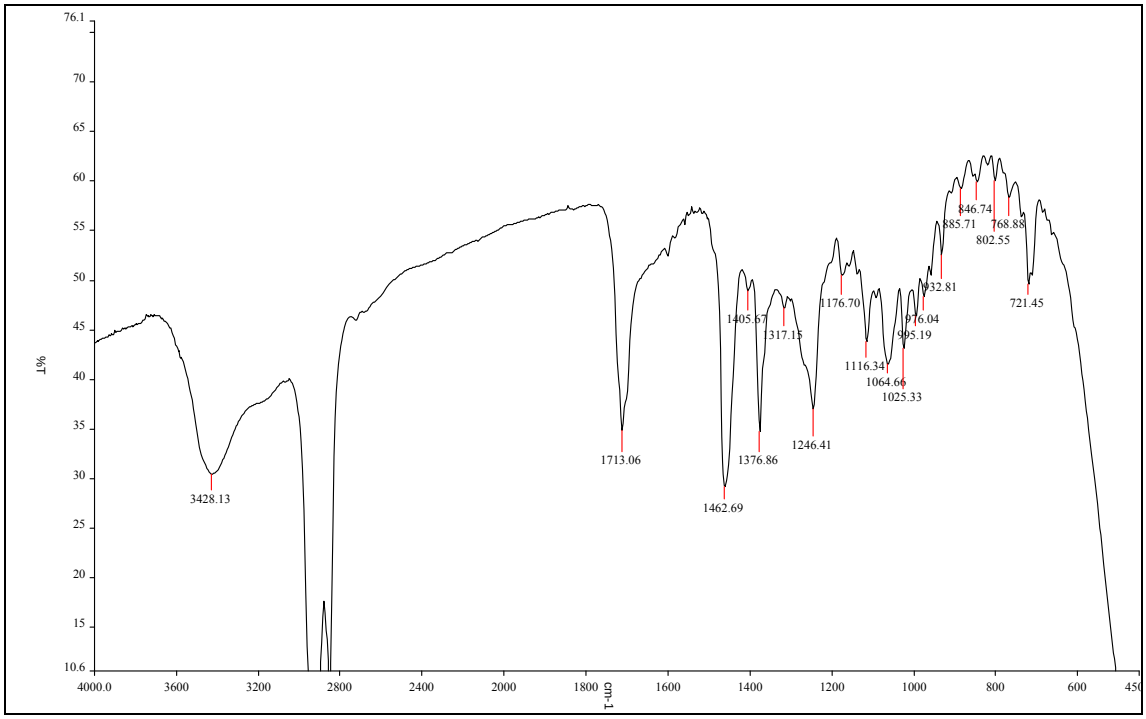


Fig. 11: IR spectrum of compound **52**

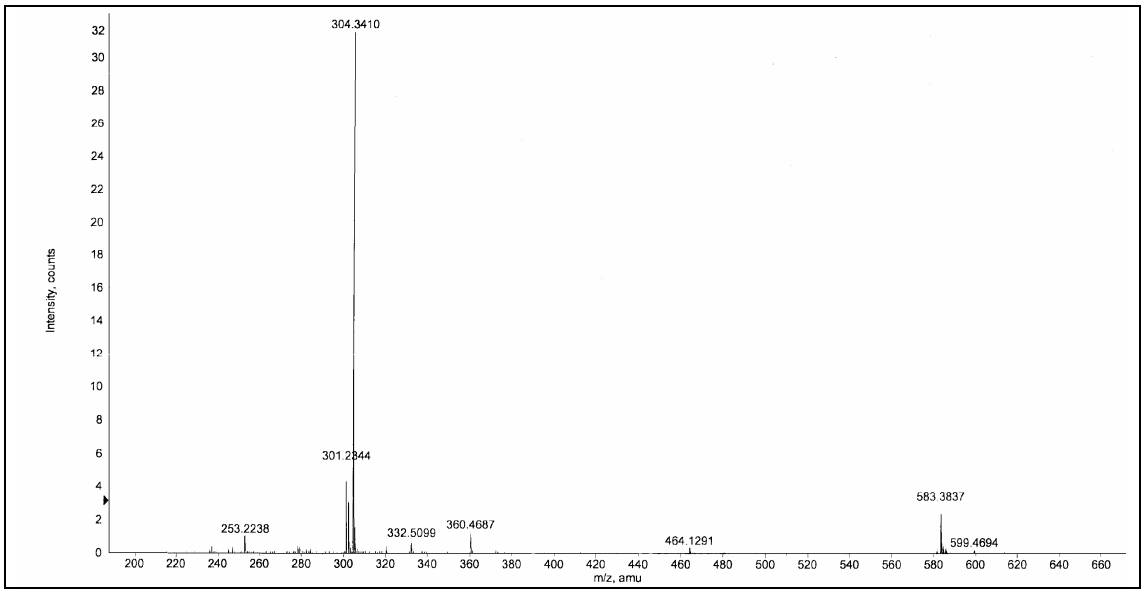


Fig. 12: ESI-mass spectrum of compound **52**

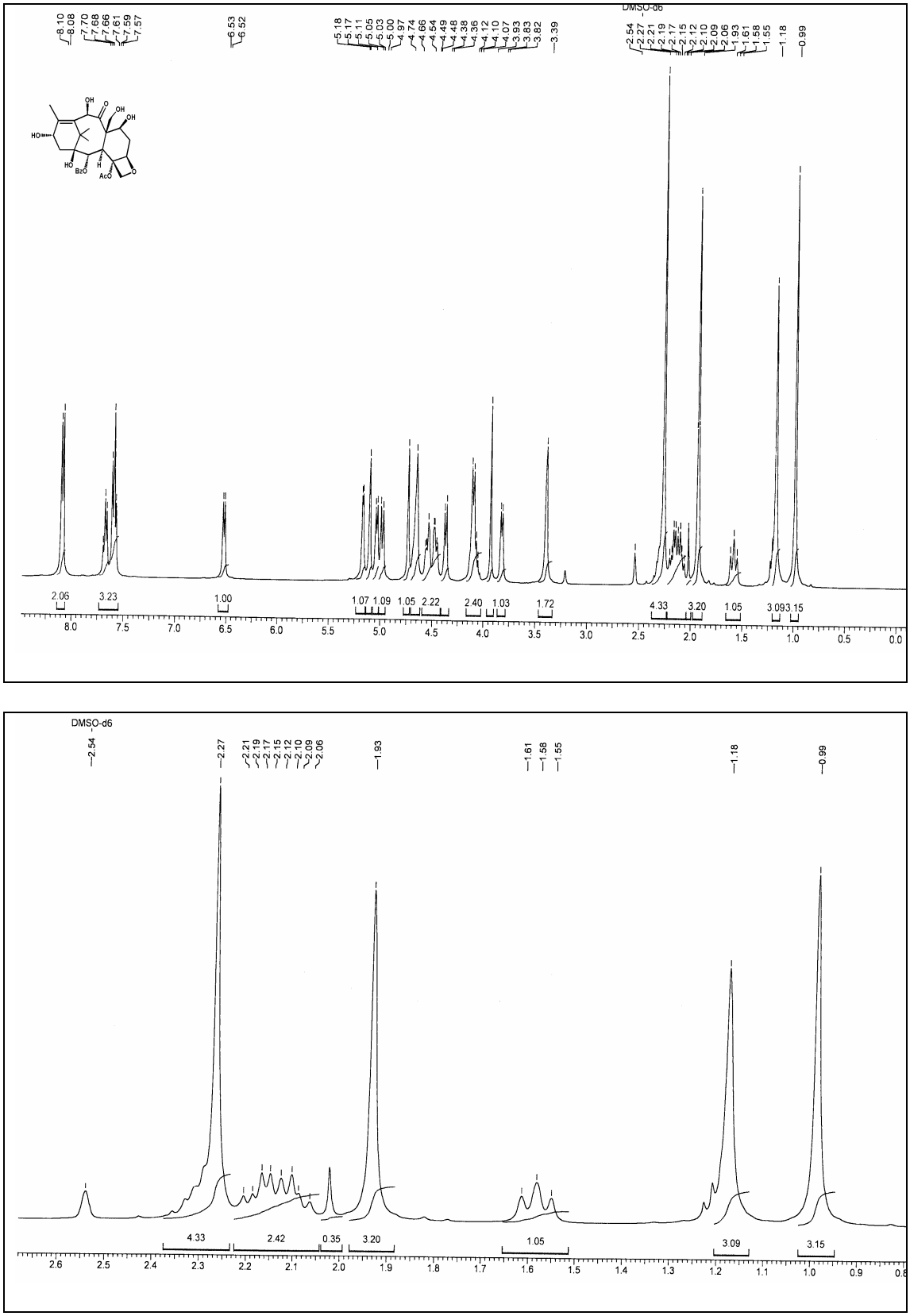


Fig. 13: ^{1}H NMR spectra of compound **52** (DMSO-d_6, 400 MHz)

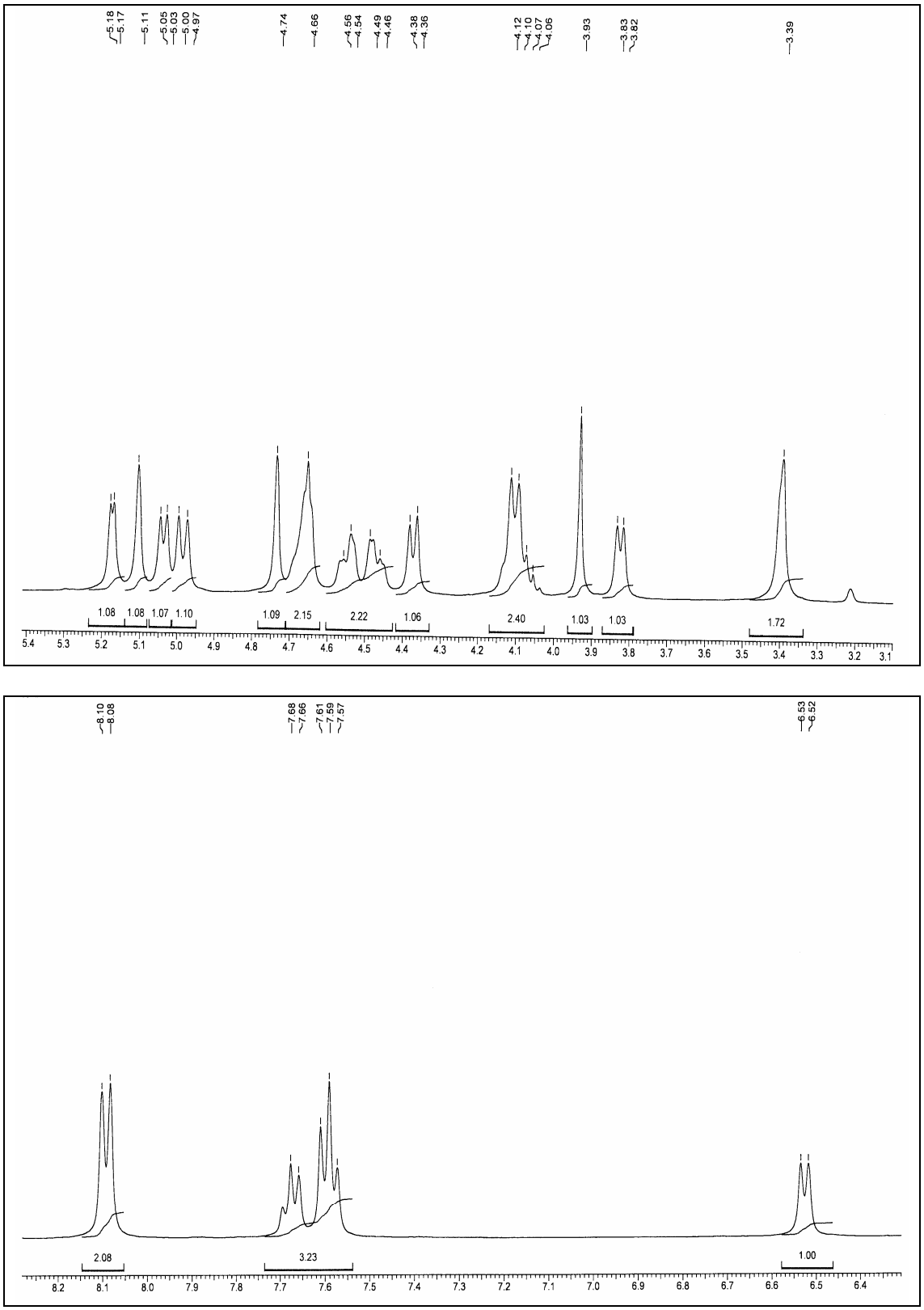
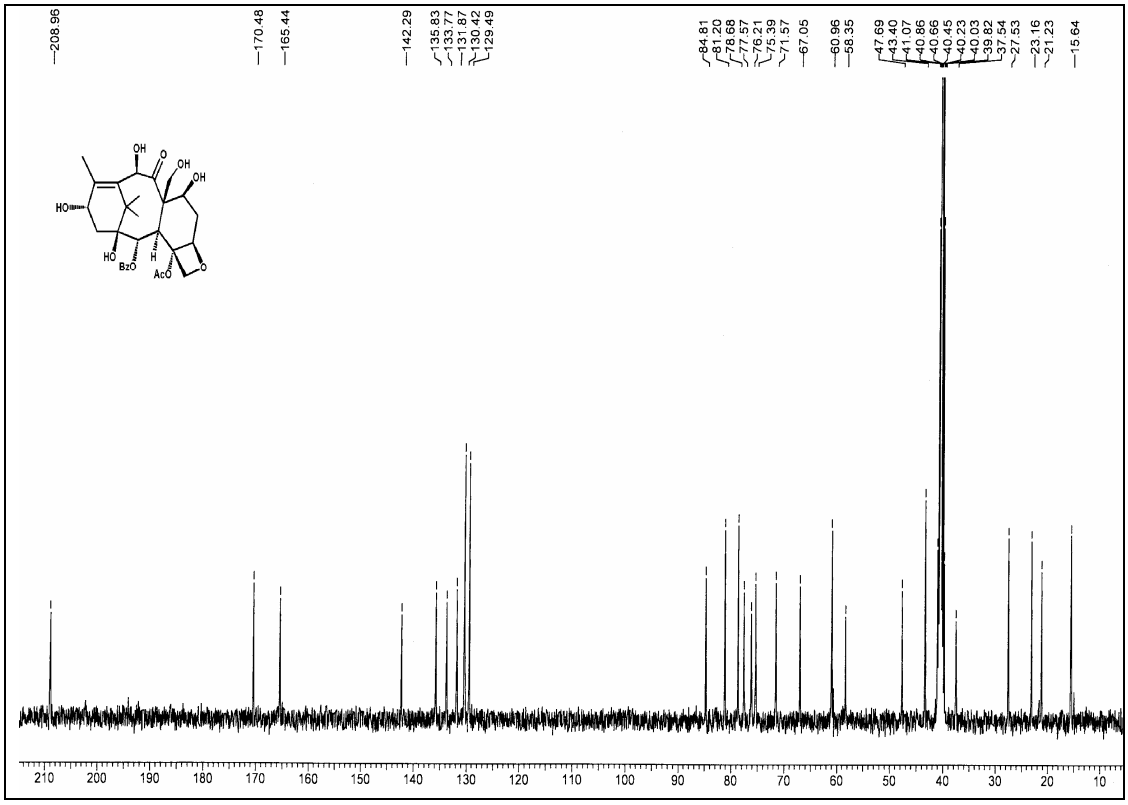


Fig. 13: ^{1}H NMR spectra of compound **52** (DMSO-d_6, 400 MHz)



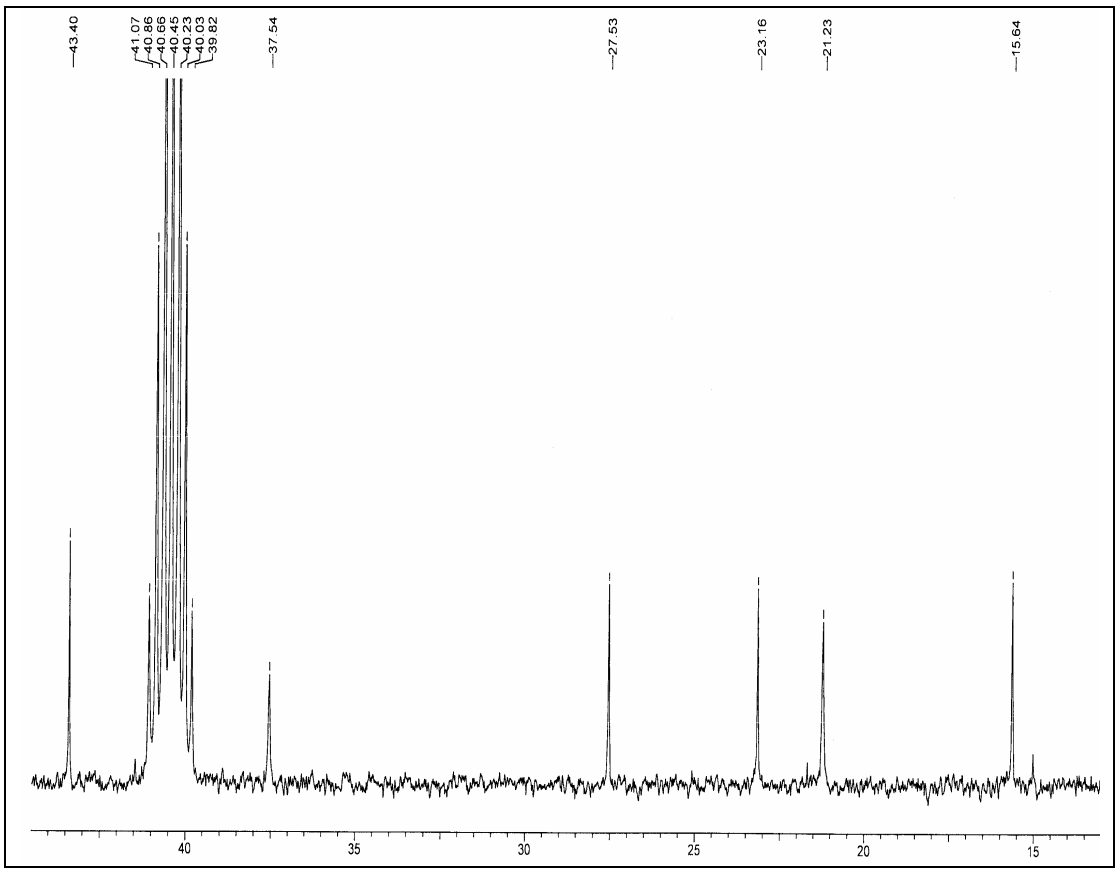


Fig. 14: ^{13}C NMR spectra of compound **52** (DMSO-d_6, 100 MHz)

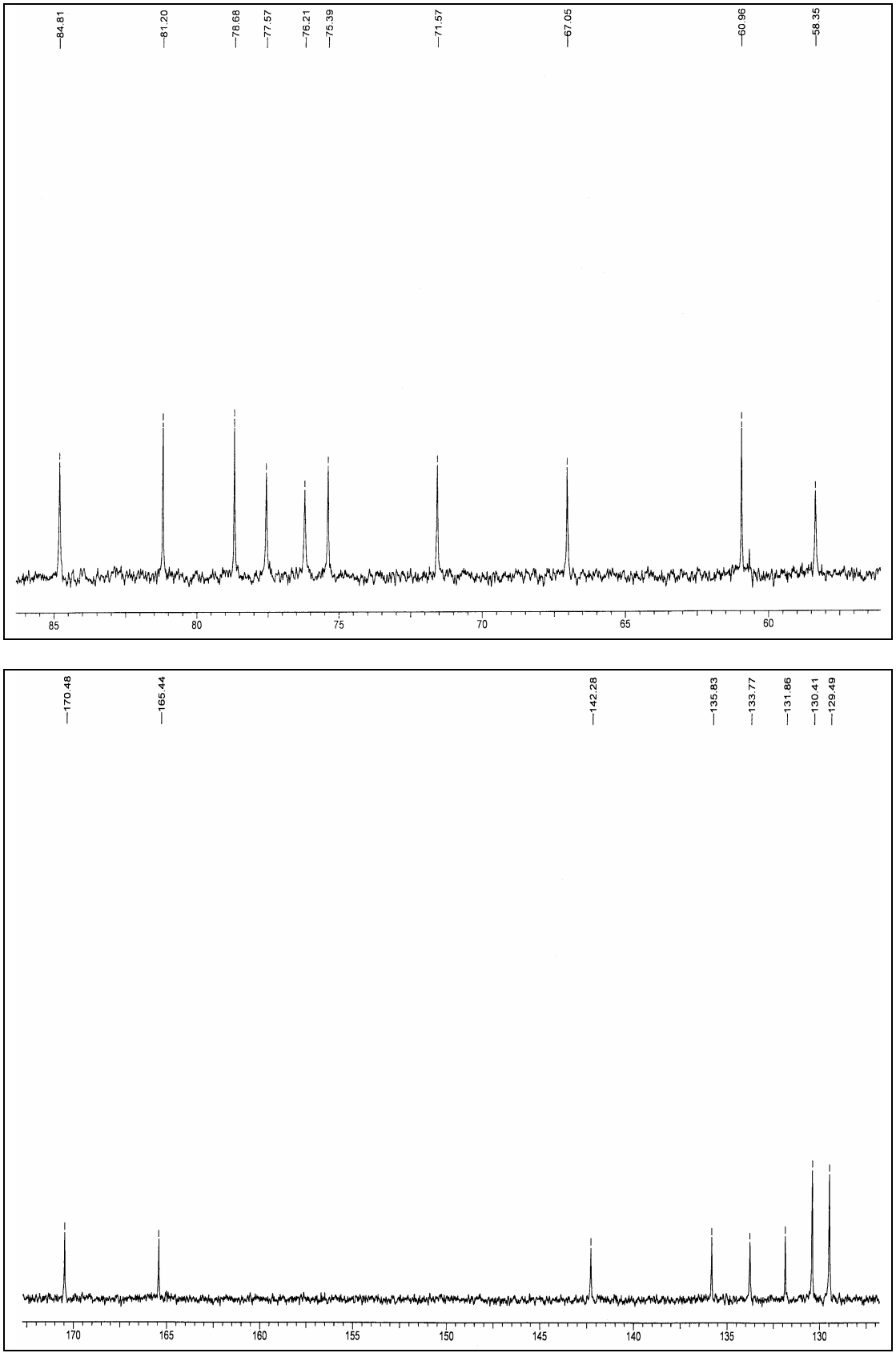


Fig. 14: ^{13}C NMR spectra of compound **52** (DMSO-d_6, 100 MHz)

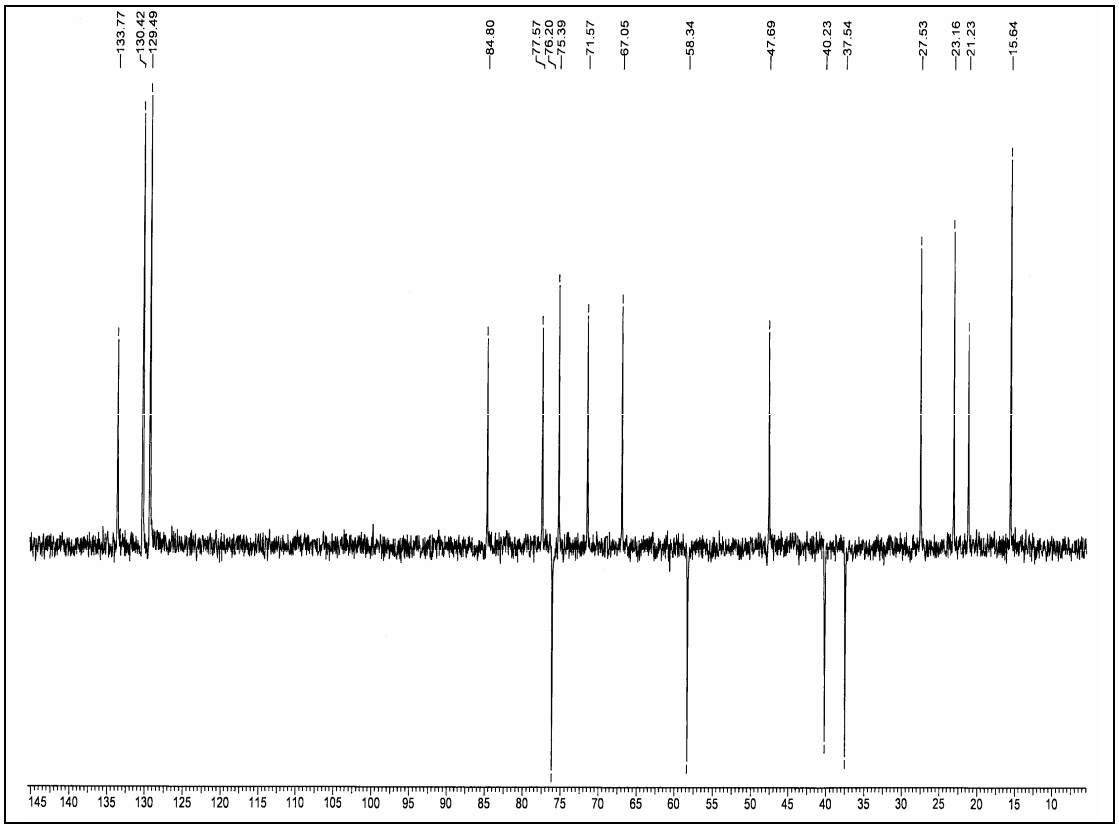
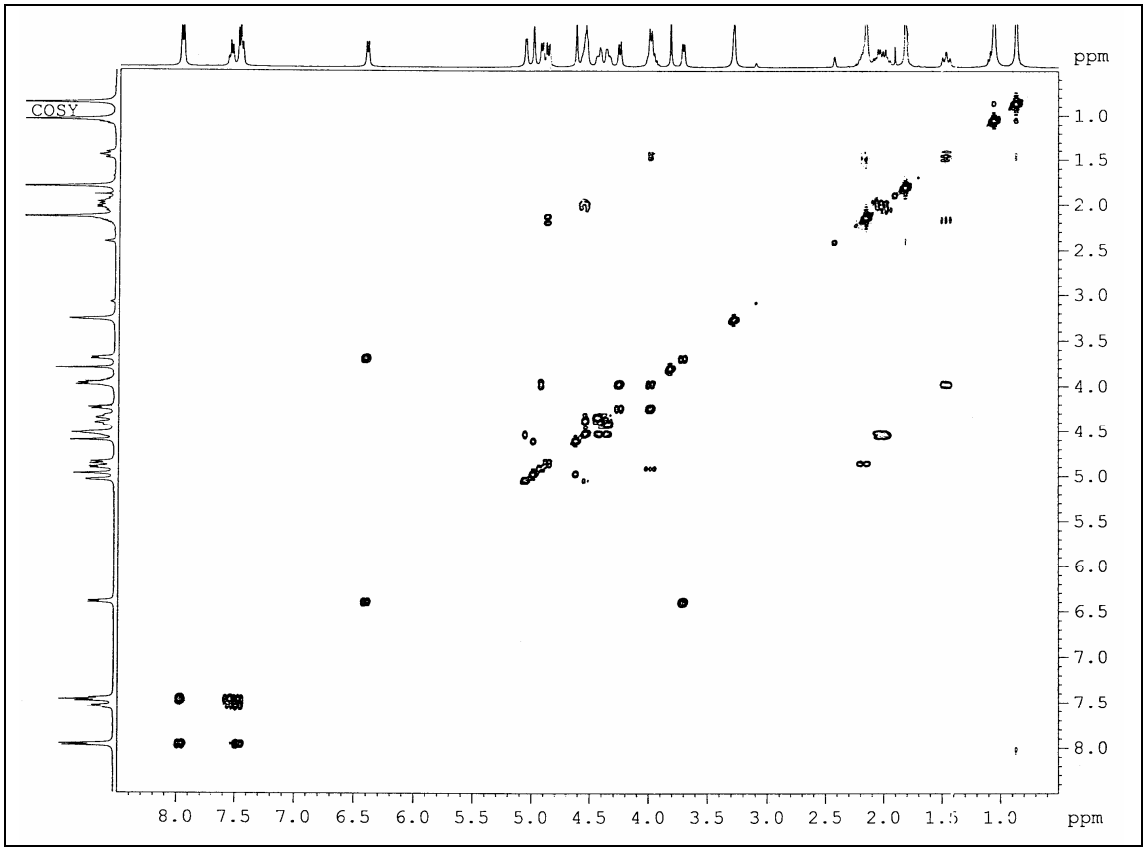


Fig. 15: DEPT-135 experiment spectrum of compound **52** (DMSO-d_6, 100 MHz)



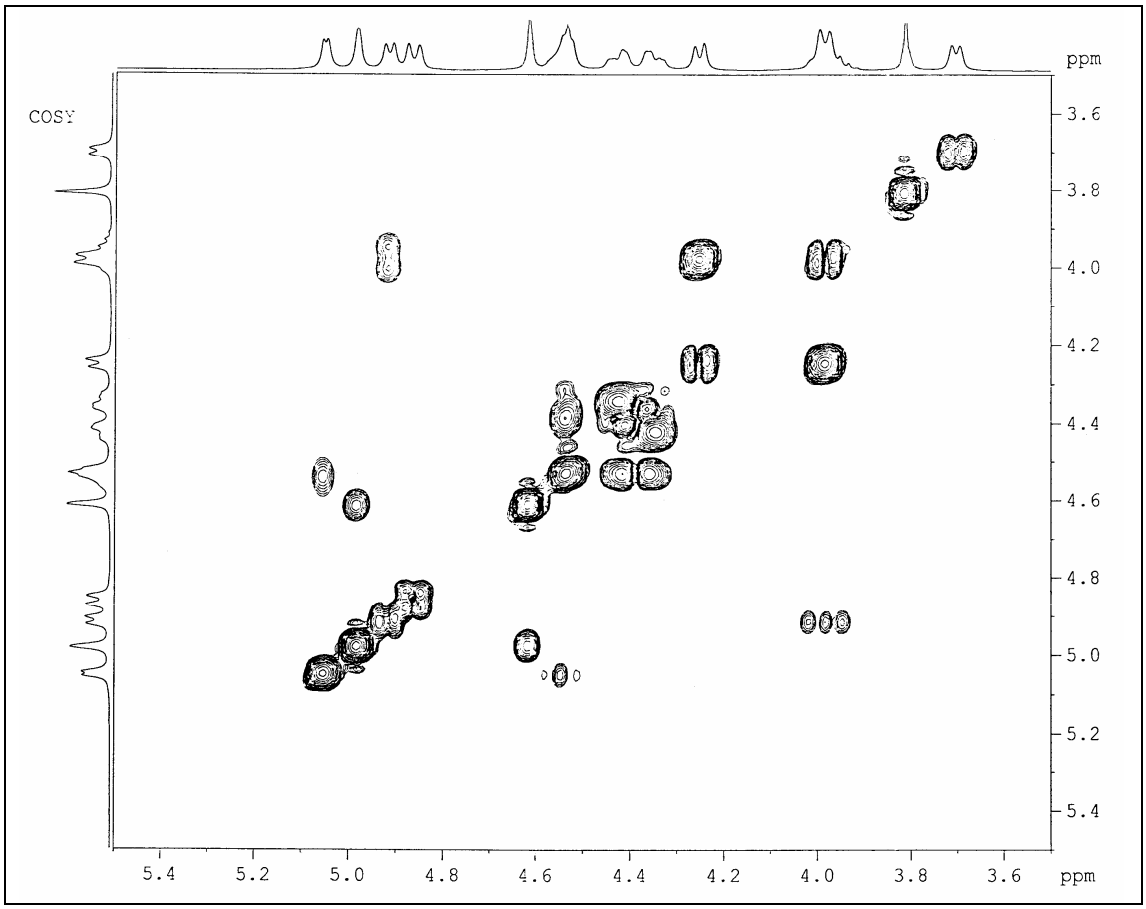


Fig. 16: ^{1}H-^{1}H-COSY spectrum of compound **52** (DMSO-d_6)

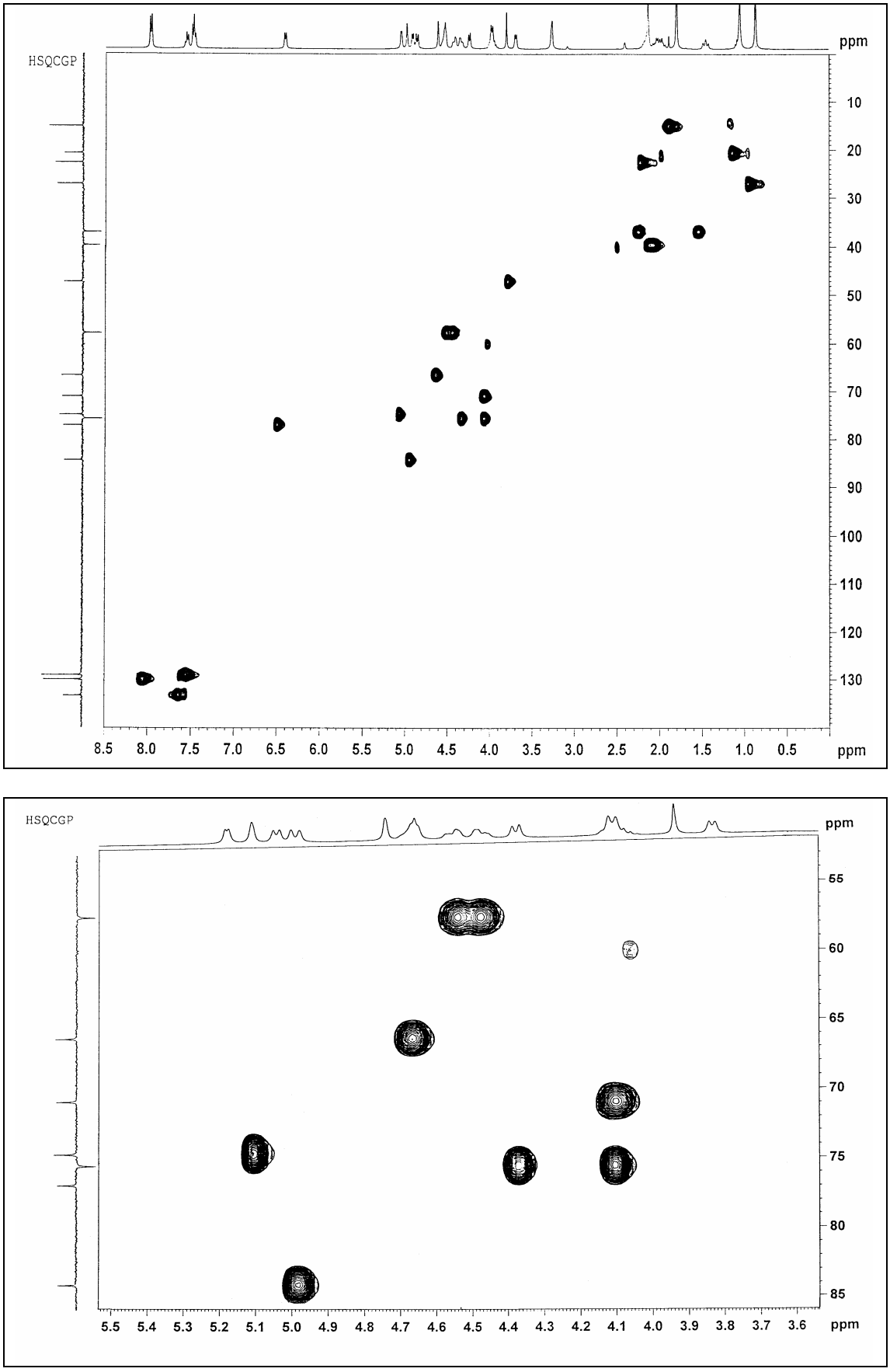


Fig. 17: HSQC experiment of compound **52** (DMSO-d_6)

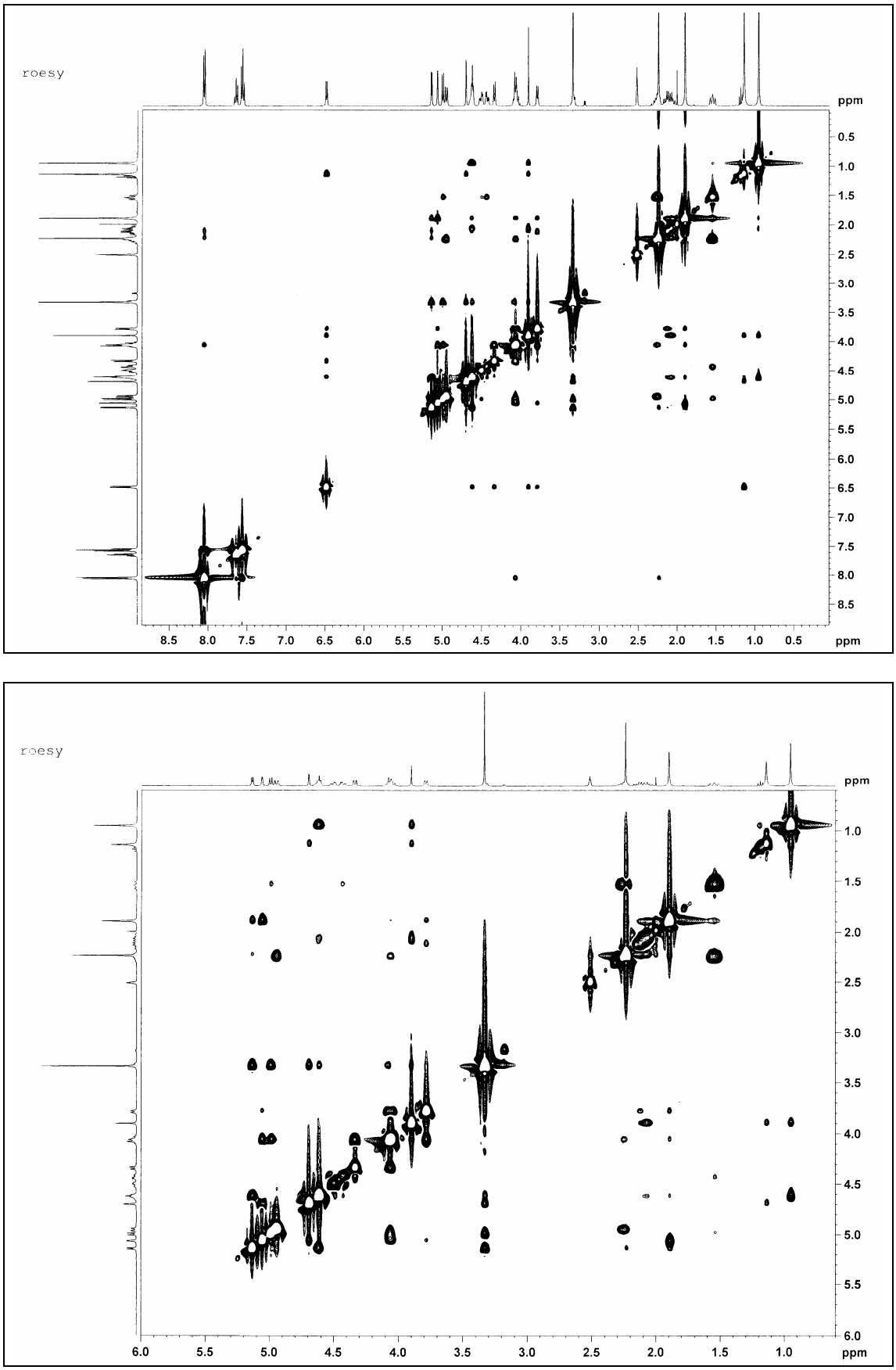
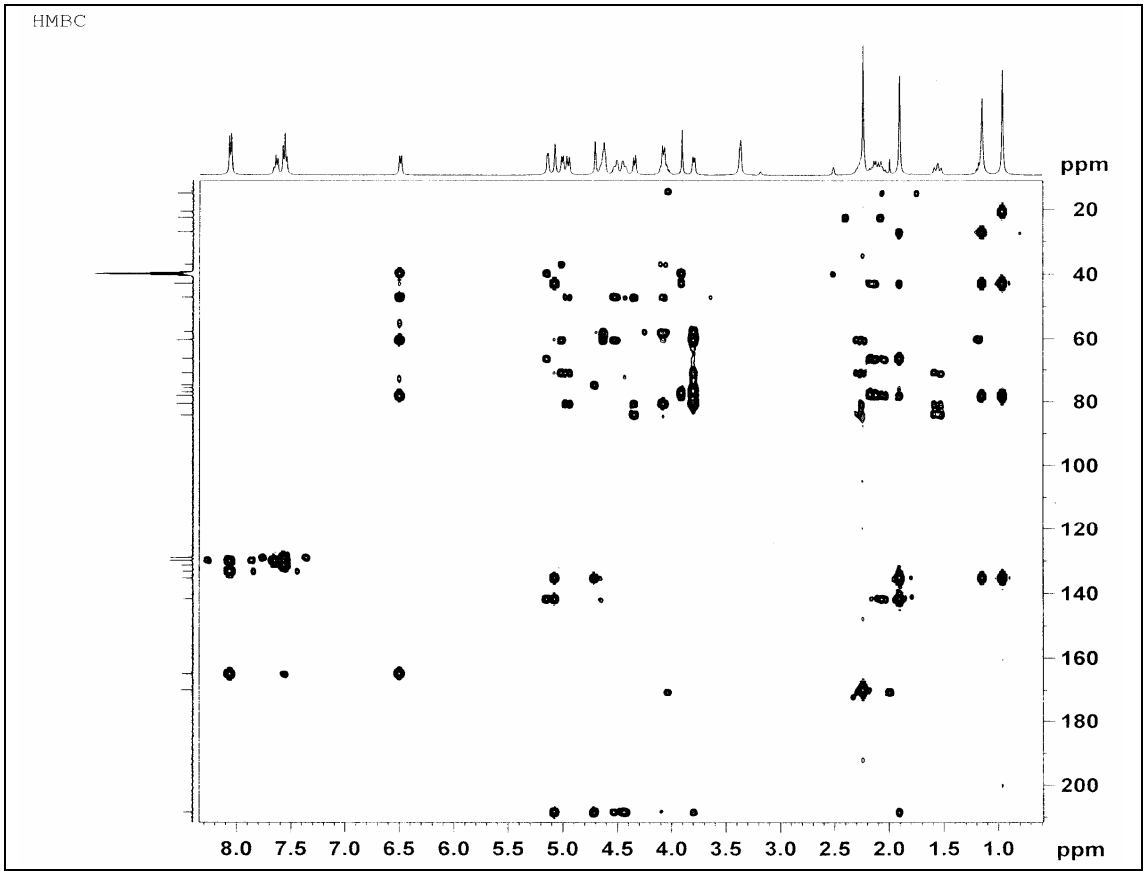


Fig. 18: ^{1}H-^{1}H ROESY spectrum of compound **52** (DMSO-d_6)



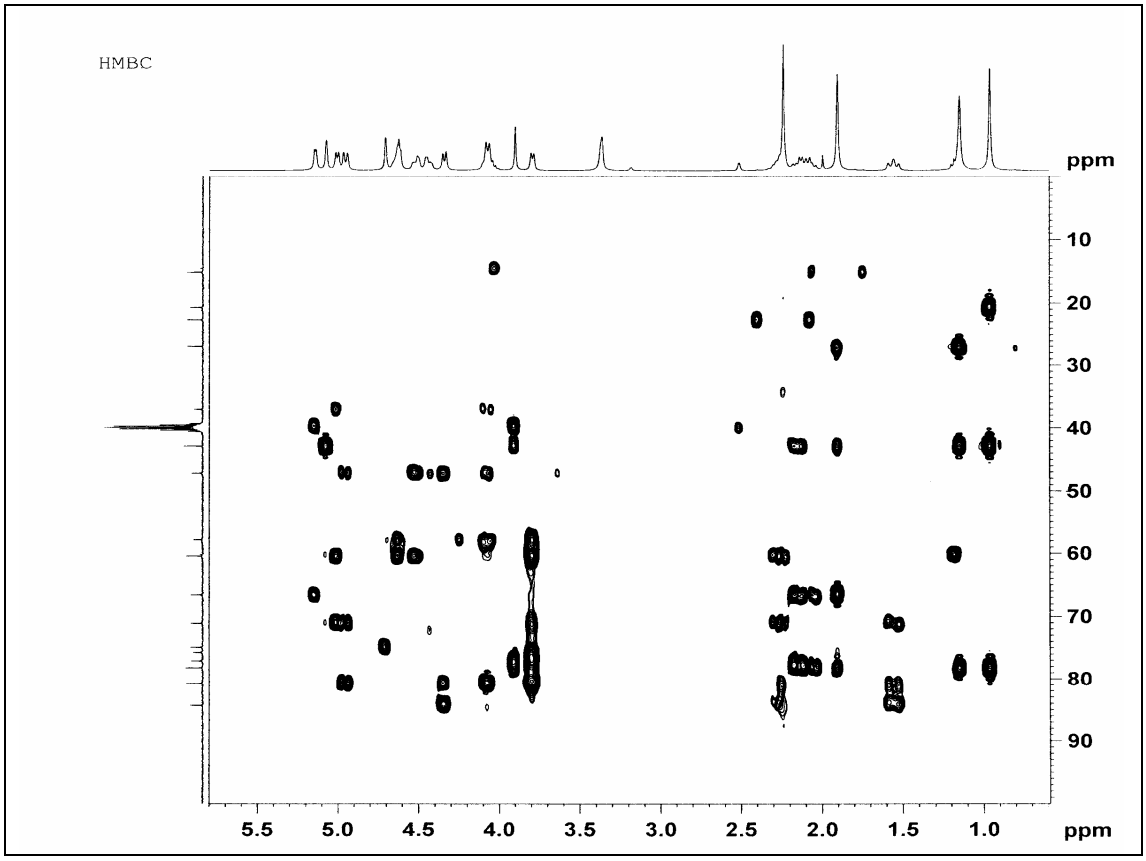


Fig. 19: HMBC experiment of compound **52** (DMSO-d_6)

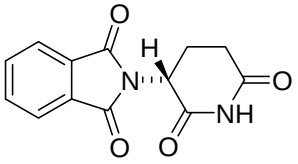
3.1 INTRODUCTION

3.1.1 Biologically active molecules

The advances in stereoselective bioanalysis led to a new awareness of the importance of stereoselective pharmacodynamics and pharmacokinetics, enabling the differentiation of the relative contributions of enantiomers to overall drug action. When one enantiomer is responsible for the activity of interest, its paired enantiomer could be inactive, be an antagonist of the active enantiomer or have a separate activity that could be desirable or undesirable.[1-3]

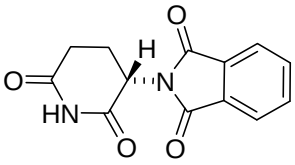
The reasons for producing optically pure molecules include the following: i) biological activity is often associated with only one enantiomer; ii) enantiomers may exhibit very different types of activity, both of which may be beneficial or one may be beneficial and other undesirable (**Fig. 1**). Racemic thalidomide consumed by expectant mothers as sedative in the early sixties created a generation of maltransformed babies because of teratogenicity of the (*S*)-enantiomer[4] which made the company to close down for ever and opened the eyes of scientists against the potential harm from the wrong enantiomer usage. Production of only one enantiomer allows the separation of the effects; iii) the unwanted enantiomer is at best enantiomeric ballast[5] gratuitously applied to the environment; iv) optically pure compound may be more than twice as active as the racemate because of antagonism; v) registration consideration;[6] vi) production of materials as the required enantiomer is now a question of law in certain countries, the unwanted enantiomer being considered as an impurity.

The fact that enantiomers of a biologically active compound exhibit different activities and toxicities[5,7] has attracted worldwide attention and there is a move towards increasing single enantiomer use, wherever possible as a matter of choice. Drug regulatory authorities are insisting that enantiomers should be studied separately for their biological activity and toxicological properties as well as by dictates of regulates in bioactive materials for different reasons including the biological ones. As a result, of the top 100 drugs world wide, 50 are single enantiomers.

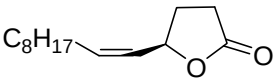


(R)-sedative, nontoxic

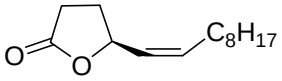
Thalidomide



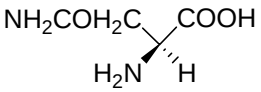
(S)-potent teratogen



(R, *Z*)-pheromone activity

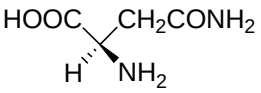


(S, *Z*)-potent inhibitor of pheromone activity

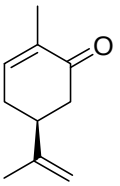


(S) bitter taste

Asparagine

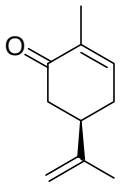


(R) sweet taste

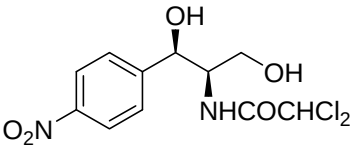


(S) caraway flavor

Carvone

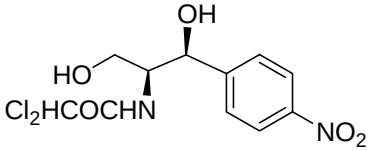


(R) spearmint flavor

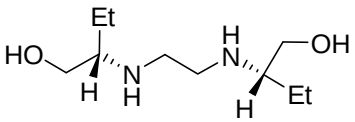


(R, R) antibacterial

Chloramphenicol

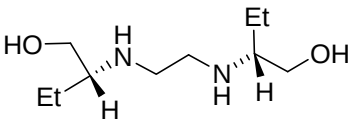


(S, S) inactive

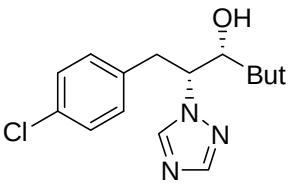


(S, S) tuberculostatic

Ethambutol

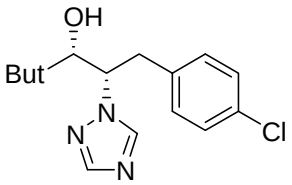


(R, R) causes blindness



(2R, 3R) fungicide

Paclobutrazol



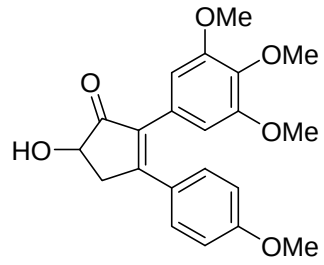
(2S, 3S) plant growth regulator

Fig. 1

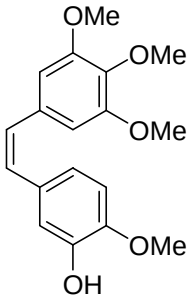
Thus, the resolution of biologically active compound has become an inevitable step in the drug development process. Crystallization,[8] use of resolving agents or enzymes,[9] GC,[10,11] HPLC[12] or simulated moving bed chromatography (SMBC) [13] etc. are some of the methods used for resolution of various compounds and proper use of suitable methods to obtain both the enantiomers in pure form holds the key position in the manufacture of various drugs. The application of simulated moving bed (SMB) technology for purification of intermediates and products in the pharmaceutical industry is today the attractive technique for economic reasons. SMB technology is a continuous countercurrent technology which is extensively used in the chiral separation of pharmaceuticals[14-18] and of various synthetic pharmaceuticals.[19,20]

In this chapter the resolution of some biologically active compounds by preparative HPLC and SMBC has been described.

5-Hydroxy-3-(4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-cyclopent-2-en-1-one (**1**), a novel anticancer agent[21] and an analogue of combretastatin A-4 (**2**) has been resolved by chiral preparative HPLC. Further, racemic (±)-(RS)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**3**), a precursor of a calcium sensitizer, levosimendan, (-)-(R)-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono] propanedinitrile, (**4**), an antiarrhythmic drug, cibenzoline (**5**) and two new cibenzoline analogues (**6** and **7**) have also been resolved by simulated moving bed chromatography (SMBC).



1



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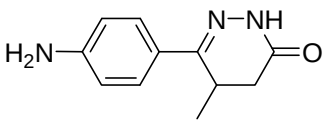

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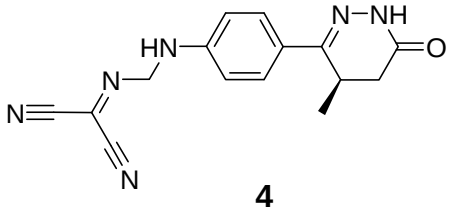

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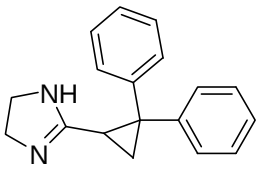

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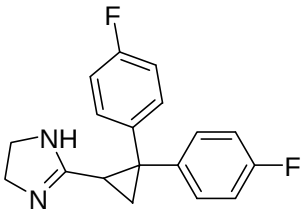

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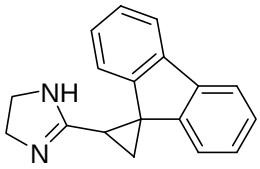

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3.1.2 High performance liquid chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a powerful analytical technique for the separation of samples especially biological, pharmaceutical, food, environmental, industrial, etc. In HPLC a solvent is forced through under high pressures of up to 400 atmospheres. Columns are packed with very much smaller size particles as stationary phase, which gives a much greater surface area for chemical interactions between the stationary phase and the molecules flowing through it which allows a much better separation of the components of the mixture.

There are two types of phases by which HPLC is carried out, i) normal phase HPLC and ii) reverse phase HPLC. It operates on the basis of hydrophilicity and lipophilicity.

Normal phase HPLC is essentially just the same as thin layer chromatography or column chromatography. Although it is described as "normal", it is not the most commonly used form of HPLC.

In reverse phase HPLC, the stationary phase consists of silica based packings with n-alkyl chains covalently bound. For example, C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendancy of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds. A polar solvent is used, e.g. a mixture of water and an alcohol such as methanol.

There are several types of mobile phases which include isocratic, gradient, and polytyptic. In isocratic elution, compounds are eluted using constant mobile phase composition. In gradient elution, different compounds are eluted by increasing the strength of the organic solvent. The sample is injected while a weaker mobile phase is being applied to the system. The strength of the mobile phase is later increased in increments by raising the organic solvent fraction, which subsequently results in elution of retained components. Polytyptic Mobile Phase, sometimes referred as mixed-mode chromatography, is a versatile method in which several types of chromatographic techniques, or modes, can be employed using the same column.[22]

The majority of the compounds which include biomedical substances are separated by reversed phase HPLC. In reverse phase HPLC, aqueous mixtures with methanol, acetonitrile and additives such as buffers or ion-pairing reagents are used.

There are many types of detectors that can be used with HPLC. The more common detectors include: Ultra-Violet and Refractive Index. Ultra-Violet detectors can be accomplished at one or several wavelengths.[23]

There are two approaches for chiral resolution by HPLC; i) on chiral stationary phases (CSPs) based on polysaccharide, cyclodextrin, macrocyclic glycopeptide antibiotics, protein, ligand exchange, crown ether etc and ii) using chiral mobile phase additives.

For analytical HPLC, columns with small diameter up to 5 mm are used and in preparative HPLC, columns usually have larger diameter which are designed to facilitate large volume injections into the HPLC system.

Before starting a preparative chiral separation, it is essential to identify chiral stationary phase (CSP) exhibiting good chiral recognition ability. This is usually done with an analytical column because it is less substance and time-consuming. If the phase exhibits useful properties for analytical purposes, it will be appropriate for preparative applications. Most chiral stationary phases have relatively low saturation capacity, so the enantiomer separations are usually done under strongly nonlinear conditions.[24]

Preparative chromatography is generally carried out under mass or volume overloaded conditions in order to increase the product throughput. In volume overloading, the sample concentrations are maintained in the linear region of the isotherm and the volume is increased until the throughput is optimized.[25] A fundamental problem with this technique is the underutilization of the column and the corresponding low throughputs. In mass overloading, the sample concentration is increased beyond the linear adsorption region resulting in asymmetric band profiles. A combination of volume and mass overloading is normally employed to maximize throughput in preparative chromatography.[26] Chromatography on large scale consumes large volumes of expensive solvents, which is one of several reasons why optimization of the process is essential.

3.1.3 Simulated Moving Bed Chromatography (SMBC)

3.1.3.1 Principle of SMBC

Unlike normal HPLC, SMB operates continuously for large amounts, without loss of the enantiomeric purity and is a more efficient alternative.[27] This process consists of simulating the countercurrent movement of the adsorbent bed by switching the positions of inlet and outlet streams to produce two outlet streams, one of which is rich in the more adsorbed component (extract stream), while the other is rich in the less adsorbed component (raffinate stream).

The principle behind SMBC[28] is the true moving bed chromatography (TMBC) which is only a theoretical concept where a counter-current between a solid (adsorbent) and a liquid (eluent) is achieved in a single column. A binary mixture (feed), to be separated, is injected at the middle of the column. Both the solid

(adsorbent) and the liquid (eluent) are such chosen, so that the compounds A and B present in the feed are not adsorbed equally on the solid.

The separation column can be divided into four zones or sections as shown in **fig. 2**. These zones are fulfilling different functions.

By injecting a particular concentrated binary mixture at a well defined flow rate into a column at time $t_{0,}$ all species are transported into zone III. Following the laws of adsorption, the components of the mixture will be distributed between the liquid and the solid phase. The more adsorbed component B will be enriched in the solid phase.

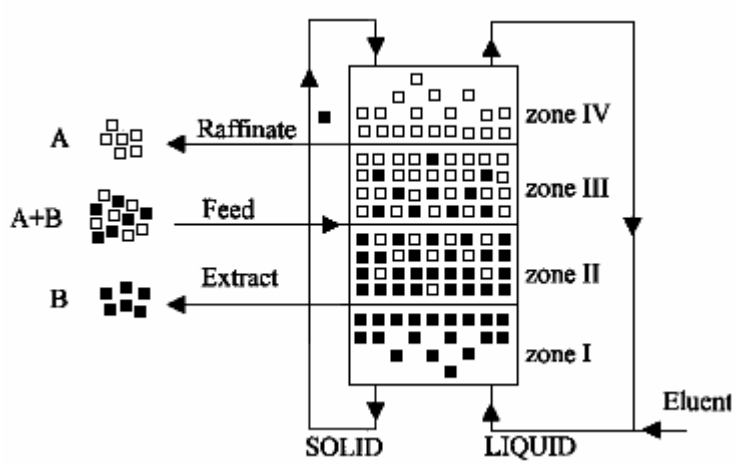


Fig. 2: Principle of TMB

Since a high purity of component A is required in the raffinate stream, component B will be adsorbed in zone III in the volume between feed inlet and raffinate outlet. Thus the function of zone III is to adsorb component B or to separate it from A. The inverse problem exists in zone II, where component A will be desorbed from the solid phase and will be pushed back into zone III. Zone II has the function to separate component A from B.

Zone I has to clean the solid (adsorbent). All components flowing into this zone have to be desorbed, so that only the unloaded solid reaches the lower end of the column and is recycled to the upper end. In zone IV, vice versa, the liquid has to be cleaned. Thus, there are two separation sections (zone II and III) and two cleaning zones (zone I and IV).

If a suitable ratio of the volumetric flow rates of the liquid and the solid phase are released in each zone, a concentration profile is created as shown in **fig. 3**. The raffinate stream contains nearly no B and the extract stream contains nearly no A.

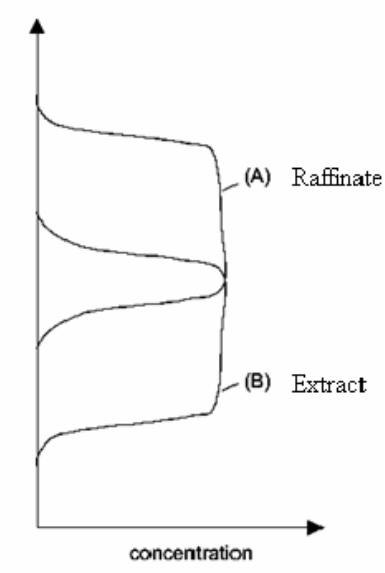


Fig. 3

As the circulation of a solid in a column is not possible practically, technically the TMB concept cannot be implemented. Consequently, the only way to implement the moving bed concept is to discretize the counter-current column in identical fixed bed columns connected in series, to achieve a liquid circulation in the columns, and periodically switch the injection and withdrawal points from one column to the following one**,** so that it simulates the movement of the solid (**Fig. 4**). In the SMB implementation, the solid is not moving anymore like in the TMB, the counter-current effect being associated to the shift of the inlet/outlet lines.

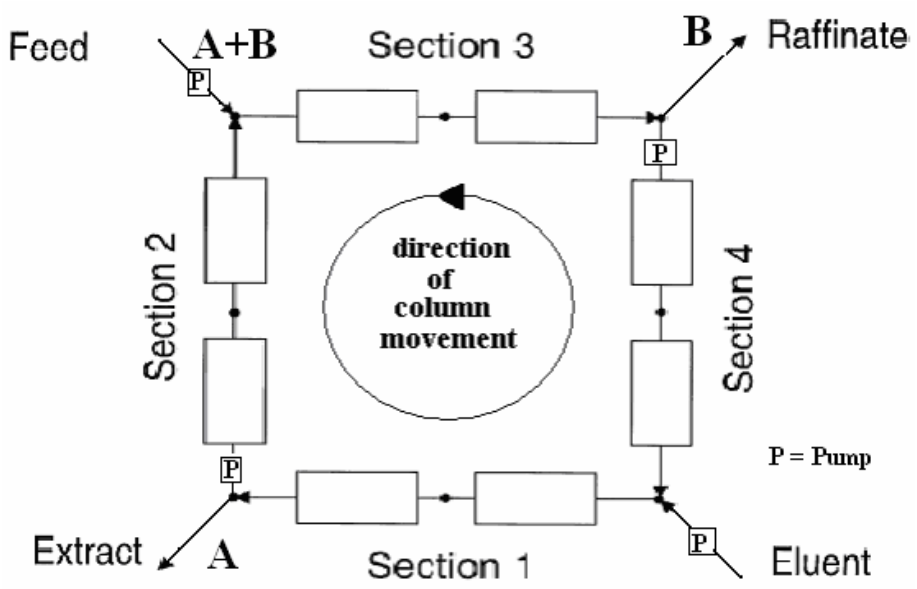


Fig. 4: Principle of the SMB

SMB requires a more complex equipment setup and some mathematical computer assisted modeling for the flow rate selection in each zone.

An SMB system consists of several identical columns (usually 8-12), which are placed in series and between each of them four valves are placed which can be individually opened and closed. Two recycling pumps inside the column circle deliver the mobile phase flow through all columns. Two additional pumps constantly inject the feed and fresh eluent, and two pumps withdraw the raffinate and extract flows.

SMB process optimization is done by computer simulations. It is much more difficult to optimize SMB during nonlinear conditions as compared to linear conditions. In fact, empirical approaches for optimization during overload and non-linear conditions are in most cases even impossible.[29,30] Computer-assisted optimization is therefore especially important for chiral separations since these CSPs have in general lower saturation capacities compared to non-chiral columns.

A number of factors must be considered for a successful SMB separation. The mixture to be separated must be i) soluble in the mobile phase, ii) stable both in the solvent and on the stationary phase, iii) stable upon re-isolation from the mobile phase, iv) have a high saturation capacity on adsorbent.

3.1.3.2 Procedure of determining the operating conditions for complete separation

Storti *et al.*[31] have described a method for determining the operation conditions for complete separation based on the Equilibrium theory in SMB technology. The diagram (**Fig. 5**), called the Morbidelli-triangle, is used to analyze the optimum conditions, the robustness and the efficiency of the novel SMB process. The methodology is briefly described below.

To achieve separation of the feed components, the net flow rates of the most retained component (Extract) must be set such that it will be transported to the extract port, while the least retained component (Raffinate) must be transported to the raffinate port. A set of parameters is set up on the basis of the separation factors of the components in each of the two sections, where the separation factor is defined as follows.[32]

$$S_{in} = \frac{F_s}{F_n} * K_i \quad \text{..... 1}$$

where S_{in} is separation factor of i (i = extract or raffinate) component in n^{th} zone. F_s is the sorbent (solid) flow rate, F_n is the liquid flow in section n and K_i is the distribution coefficient (Henri constant) of component i. The flow rate ratio (m) in n^{th} zone is defined as

$$m_n = \frac{F_n}{F_s} \quad \text{..... 2}$$

A separation factor larger than unity means that the component is moving with the sorbent. Separation factor smaller than one means the component is moving with solvent. The following set of constraints must be met to achieve complete separation of the two feed components:

$$K_R < m_2 < K_E \; ; K_R < m_3 < K_E \text{ and } m_4 < K_R \quad \text{..... 3}$$

where K_E is the equlibrium constant for extract and K_R is the equilibrium constant for the raffinate. The subscripts are representing the zone of the SMB. In order to have a positive feed flow m_3 must be larger than m_2

$$m_2 < m_3 \quad \cdots\cdots\cdots\cdots \quad 4$$

The region of complete separation was constructed by projecting the inequalities in the m_2-m_3 plane. This region is shown in **fig. 5**.

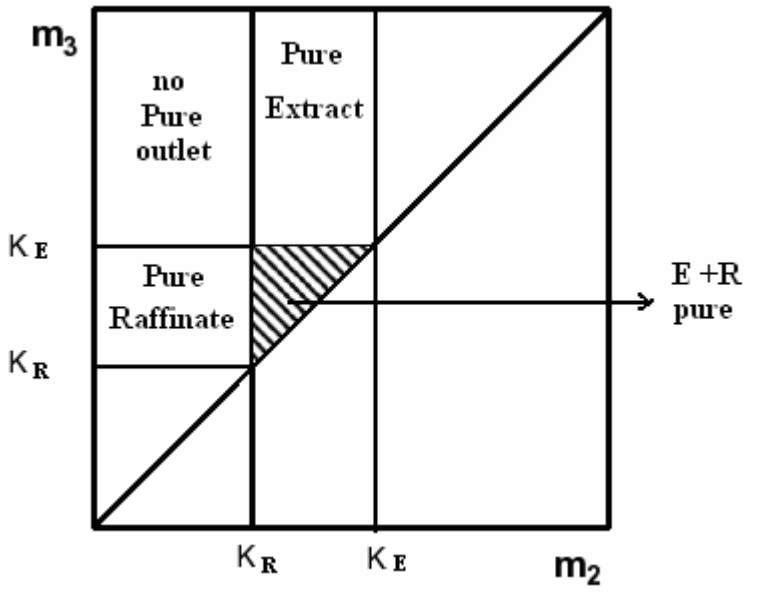


Fig. 5: Schematic diagram (Morbidelli triangle) for an SMB in isocratic mode

Flow ratio in section n is given by[33]

$$m_n = \frac{Q_n^{LF} t^* - V\varepsilon - V_n^D}{V(1-\varepsilon)} \quad \cdots\cdots\cdots\cdots \quad 5$$

Q_n^{LF} is the liquid flow rate in zone n of the SMB unit, t* is the switching time for the stream positions, V and ε are the volume and the overall porosity of the column, respectively while V_n^D is the dead volume of the system. The value of V_n^D of SMB system is obtained by dividing the overall extra column dead volume in section n by the number of columns in that section. The condition expressed in (3)

defines the projection of the complete separation region and yields points in the plane (m_2–m_3), as depicted in **fig. 5**. The point within the Morbidelli triangle is the geometric representation of one condition for the separation of the racemate.

Before running the SMB unit it is necessary to determine the interactions between the enantiomers of the racemic mixture and the chiral stationary phase. This information is obtained by injecting diluted solutions of racemate into each of the columns of the SMB unit. Retention times for each enantiomer are then measured allowing the determination of the linear isotherms. The isotherms are represented by the Henry constants given by:

$$Hi = \frac{t_i - t_o}{t_o} \times \frac{\varepsilon}{1 - \varepsilon} \quad \text{..... 6}$$

where t_i is the retention time of enantiomer i, t_o is retention time of nonretained component and ε is the bed porosity.

The total porosity of the bed of stationary phase packed in the columns is necessary to measure as described by Pedeferri *et al*.[34] The residence time of nonretained component through the bed is proportional to total bed porosity (ε). The relationship between porosity and residence time is given by the equation:

$$\varepsilon = t_o \times \frac{Q}{V} \quad \text{..... 7}$$

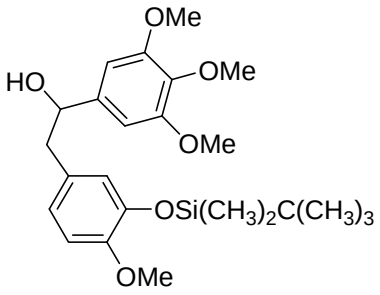
where t_o is the inert compound migration time, Q is the flow rate and V is the column volume. t_o is usually measured by injecting non-interacting solute with the adsorbent.

3.2 PREVIOUS WORK

3.2.1 Combretastatin A-4 analogue

Pettit *et al*.[35] have reported the resolution of protected racemic combretastatin (**8**) by a semipreparative HPLC column Pirkle type prep-10-DNBPG, (10 mm x 500 mm, l0 μm). However, they found that it showed almost base-line resolution on a similar analytical column (4.6 mm x 250 mm, 5 μm). A solution of 700 mg of **8** in

hexane-isopropyl alcohol (9:1) was applied to the semi-preparative column in 0.5 ml aliquots. The resolution was performed by using hexane-isopropyl alcohol (9:1) as mobile phase at a flow rate of 2.5 ml per min.



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Ramacciotti *et al*.[36] have reported the *ee* determination of (S)-enantiomer of **8** by HPLC on a CHIRALCEL OD-H column (4.6 mm x 250 mm) eluting with hexane/2-propanol (1:9).

3.2.2 Levosimendan precursor

Earlier in 1996, Wikberg *et al*.[37] have developed a HPLC method for determination of the ratio of simendan enantiomer concentrations in blood plasma samples. They achieved the direct resolution of the enantiomers by using a chiral β-cyclodextrin stationary phase in reversed phase mode. An adequate resolution was achieved with an eluent containing 24-33% of methanol in a 0.5% (v/v) triethylammonium acetate buffer, pH 6.0, and a flow rate of 1 ml/min.

In 2002, Liu *et al*.[38] developed a chiral separation method for the separation of enantiomers of simendan by using a commercially available chiral stationary phase (CSP) of Kromasil CHI - TBB, with hexane:iso-propanol (85-92.5:7.5-15) as mobile phase and the detection wavelength of 380 nm, and 0-02% acetic acid as mobile phase modifier. The direct enantiomeric separation of simendan was studied by HPLC in normal phase mode. It was found that composition of the mobile phase and mobile phase additives could tune the resolution and retention time of simendan. Larger resolution and longer retention time were generated by adding less amount of polar additive to the mobile phase.

Li *et al.*[39] studied the chiral separation of simendan enantiomers using capillary electrophoresis with β-cyclodextrin (β-CD) as chiral selector. They further investigated the influences of the concentration and pH of borate buffer solution, β-CD concentration and methanol content in the background electrolyte. A baseline separation of simendan enantiomers was achieved in the background electrolyte of 20 mmol/lit borate buffer (pH 11.0) containing 12 mmol/lit β-CD-methanol (50:50 V/V).

Simultaneously Zhang *et al.*[40] also studied the enantiomeric resolution of simendan by using reversed-phase HPLC with β-cyclodextrin as chiral mobile phase additive. The chromatography system with a dynamically-generated stationary phase with β-cyclodextrin was proved to be an effective method for simendan enantiomer separation. Zirchrom Kromasil ODS-1, 5 μm (4.6 mm x 150 mm) column was used. The optimized mobile phase was 20 mmol/lit phosphate buffer (pH 6.0) containing 12 mmol/lit β-cyclodextrin-methanol (70:30, V/V) with the flow rate set at 0.8 ml/min at 17 °C. The retention time of L-simendan and D-simendan were 22.5 min and 24.5 min respectively and resolution of 1.57 was achieved.

Ding *et al.*[41] used the antibiotic chiral chromatographic stationary phase prepared from teicoplanin or its derivatives as stereoselective agent for chiral resolution of simendan enantiomer. The chromatography carrier was amorphous or spheric silica gel.

Lu *et al.*[42] separated the simendan enantiomers by capillary zone electrophoresis using β-cyclodextrin as chiral selector. They also studied the influences of the concentration and pH of borate buffer solution, concentration of β-CD and methanol content. A baseline separation of simendan enantiomers was achieved with background electrolyte of 20 mmol/lit borate buffer (pH 11.0) containing 12 mmol/lit β-CD-methanol (50:50, V/V). The linear range for simendan enantiomer was 25 - 500 mg/lit (0.997). They claimed that this method can be applied in qualitative and quantitative analysis for simendan enantiomers.

Ding *et al.*[43] prepared teicoplanin-bonded chiral stationary phase and assessed in reversed phase mode by HPLC for the direct chiral separation of racemic simendan and α-amino acids (hydroxy acids), etc. They found that hydrophobic/hydrophilic, electrostatic effects are important for the retention behavior and resolution. Thus with

increase in hydrophobicity, better resolution can be obtained in mobile phase with lower organic modifier content.

In all the above reported methods either HPLC methods were developed for determination of the ratio of simendan enantiomer concentrations in blood plasma samples or the resolution of simendan was carried out on milligram scale. Hence precursor **3** was synthesized and attempts were made to resolve it chemically according to literature process.[44] However, the yields were poor. Hence the racemic precursor was resolved by an efficient and economical SMBC process.

3.2.3 Cibenzoline and its analogues

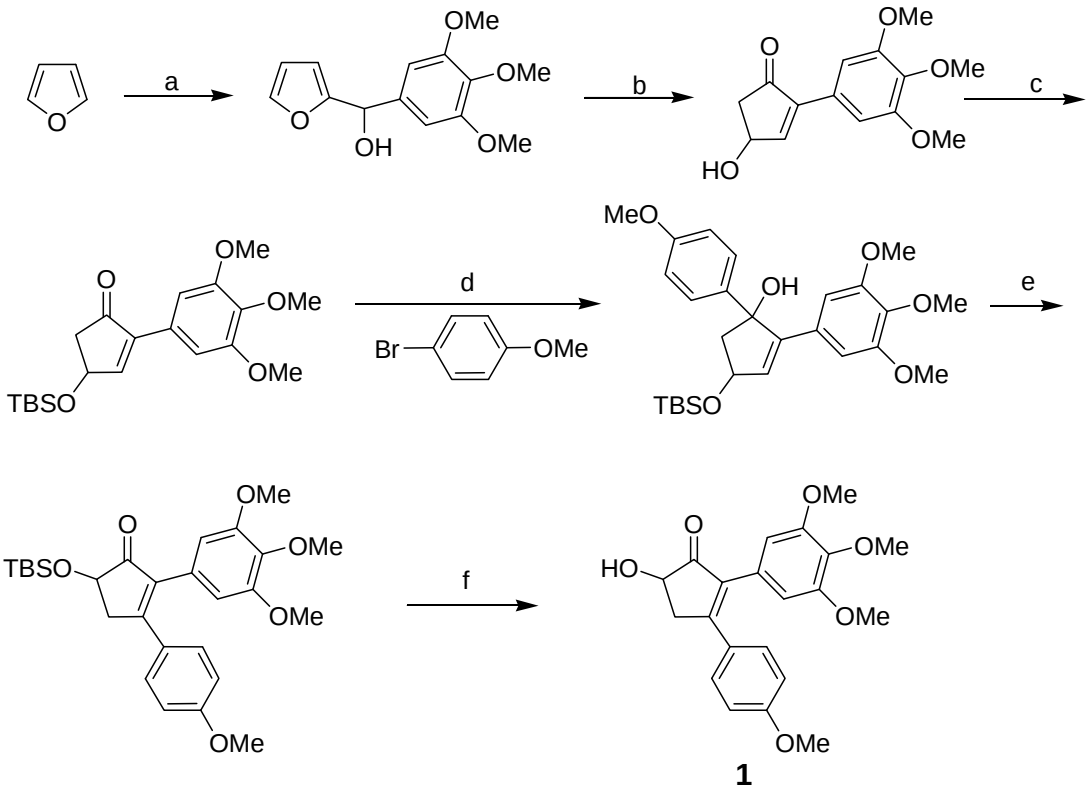
Till date, literature cites only few reports on HPLC assay for the determination of cibenzoline in human plasma and urine.[45-47] Analysis was carried out i) on ion-exchange (sulfonate) column using UV-detection at 214 nm and a mobile phase consisting of acetonitrile-phosphate buffer (0.015 mol/lit, pH 6.0) (80:20); ii) on a Nucleosil CN reversed-phase column using UV-detection at 214 nm and a mobile phase consisting of 65% of the solution A (971.5 ml water, 25 ml 1-octanesulfonic acid, 1 ml butylamine, 2.5 ml dibutylamine phosphate) and 35% acetonitrile and iii) on a Zorbax C8 column and a mobile phase of a mixture of acetonitrile and 0.06% phosphoric acid (40:60, V/V), at a flow rate of 1 ml/min, UV detection was carried out at 230 nm. In 1989, Martin *et al*.[48] has reported the determination of enantiomeric purities of the cibenzoline enantiomers by using CHIRALCEL OD column; mobile phase n-hexane:ethanol:iso-propanol:diethylamine (80:15:5:0.1) and flow rate 1 ml/min. Recently, the enantiomeric excess of cibenzoline and some of its derivatives were determined using same HPLC conditions.[49]

3.3 PRESENT WORK

3.3.1 Resolution of combretastatin A-4 analogue (1) by preparative HPLC

Combretastatin A-4 (**2**), a substituted *cis*-stilbene isolated from South African tree *Combretum caffrum*,[50] is an important anticancer agent. To study the structure–activity relationship a number of diaryl cyclopentenones[51] were synthesized in our group. It is known that 3,4,5-trimethoxy substituents in the A ring and *cis*-orientation

between A and B rings of combretastatin A-4 are the necessary structural features for its activity. On the basis of the activity data,[21,51] racemic compound **1**, synthesized as shown in **scheme 1** was selected for further study and both the enantiomers were separated by derivatisation and column chromatography as well as by preparative HPLC.[52] The resolution of **1** by preparative HPLC is described herein.



Reagents and conditions: a) i) n-BuLi, THF ii) $MgBr_2$, THF, -30 °C, 4 h iii) 3,4,5-trimethoxybenzaldehyde, 93% b) $ZnCl_2$, dioxane/water, reflux, 24 h, 90% c) TBDMS-Cl, DCM, Et_3N, 3 h, 74% d) Mg, THF, 0 °C-rt, 2 h, 72% e) PDC (2 eq), DCM, rt, 12 h, 46% f) CH_3COOH-THF-H_2O (3:1:1), 50 °C, 20 h, 84%.

Scheme 1

3.3.1.1 Selection of chiral column

Initially analytical chiral HPLC experiments for enantioseparation were carried out on (R,R) Whelk-O 1 [4.6 mm x 250 mm] column with n-hexane:iso-propanol and various concentrations as mobile phase. A very small resolution (R = 0.3, **Fig. 6A**) was obtained; whereas reverse phase combinations, methanol:water, acetoniltrile:water at different proportions did not show any resolution on same column. Similarly, no resolution was obtained using CHIRALCEL OJ-H [4.6 mm x

250 mm] and CHIRALCEL OD-H [4.6 mm x 250 mm] columns using hexane-ethanol or n-hexane:iso-propanol with different proportions as mobile phase.

Reverse phase trials on CHIRALCEL OD-RH column using water:methanol, water-ethanol or water-IPA as mobile phases with varying concentrations did not show any resolution for racemic compound **1**, whereas acetonitrile:water (40:60) showed resolution (R = 1.32) on CHIRALCEL OD-RH [4.6 mm x 150 mm] column (**Fig. 6B**). It was better than resolution obtained on (R,R) Whelk-O 1 column. From this study it was decided to carry out preparative resolution on CHIRALCEL OD-R (10 mm x 250 mm) column.

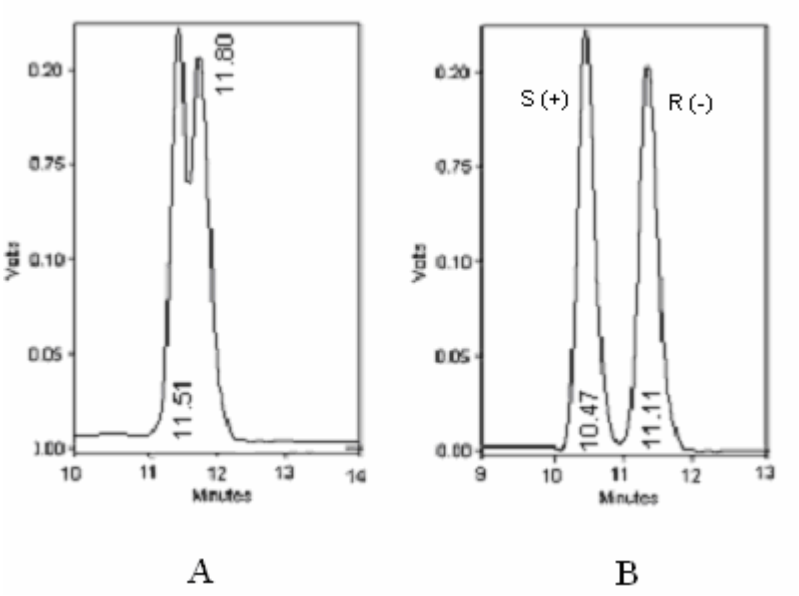


Fig 6: Chromatogram of compound **1**

(A) Separation of compound **1** on (R,R) Whelk-O 1 column (4 mm x 250 mm). Mobile phase n-hexne:iso-propanol (50:50), flow rate 0.5 ml/min, pressure 710 psi, detection wavelength 254 nm (R = 0.3, α = 1.02).

(B) Separation of compound **1** on CHIRALCEL OD-RH column (4.6 mm x 250 mm). Mobile phase acetonitrile:water (40:60, flow rate 0.5 ml/min, pressure 510 psi. Detection wavelength 254 nm (R=1.32, α = 1.03).

3.3.1.2 Optimization of mobile phase

Optimization of the composition of mobile phase (acetonitrile:water) was achieved by conducting the resolution with different compositions of acetonitrile: water such as (25:75), (30:70), (35:65), (40:60), (45:55) and (50:50) with constant flow rate 4 ml/min, detection at wavelength 254 nm and injection load 20 µl. Best resolution (1.61) was obtained using acetonitrile:water (40:60). The resolution, separation factor and solubility of compound **1** are shown in **table 1** and depicted in **fig. 7**.

Table 1: Solubility, selectivity (α) and resolution (R) for compound **1** with various compositions of acetonitrile:water using semi-preparative CHIRALCEL OD-R column, flow rate 4 ml/min

Composition ($CH_3CN:H_2O$)	**Selectivity (α)**	**Resolution (R)**	**Solubility in mobile phase (mg/ml)**
25:75	1.03	1.01	Less than 0.1
30:70	1.06	1.42	0.11
35:65	1.08	1.52	0.18
40:60	1.12	1.61	0.32
45:55	1.12	1.42	0.41
50:50	1.13	1.32	0.51

As indicated in **fig. 7**, resolution increases as percentage of acetonitrile increases and maximum resolution was gained at 40% acetonitrile. However at higher percentage, though solubility of compound was more, decrease in resolution was observed due to the peak broadening. Similarly selectivity (α) increases up to 40 % acetonitrile. However, at higher percentage it slightly decreases or remains constant. From these facts, acetonitrile:water (40:60), as mobile phase, was considered for further study.

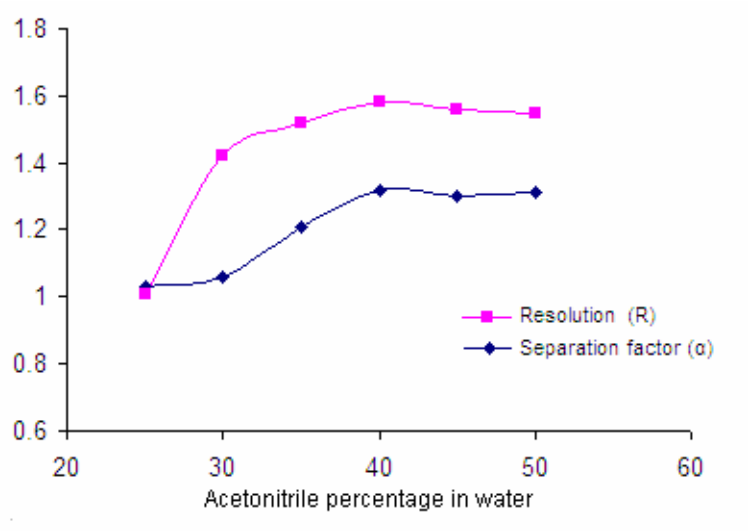


Fig. 7: Effects of the acetonitrile percentage on separation factor (α) and resolution factor (R) for compound **1** on preparative CHIRALCEL OD-R column (10 mm x 250 mm), mobile phase: acetonitrile:water, flow rate: 4.0 ml/min, detection 254 nm.

3.3.1.3 Optimum flow rate and sample load

The loading capacity of sample is a critical factor in preparative separation. It indicates the maximum amount of racemate that column can tolerate without compromising the resolution. The concentration of the sample was 0.32 mg/ml. The resolution was carried out at different sample loads, such as 0.1 ml, 0.25 ml, 0.5 ml and 1.0 ml at different flow rates of mobile phase (acetonitrile:water, 40:60) varying from 2 ml/min to 5 ml/min. The results obtained are summarized in **table 2** and depicted in **fig. 8**.

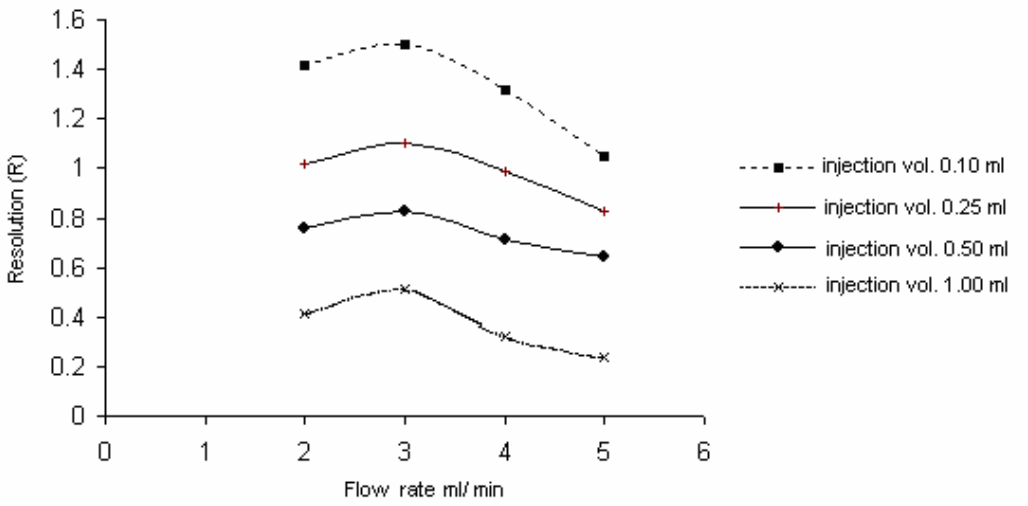


Fig. 8: Resolution at different sample load at different flow rates.

Table 2: Enantiomer separation properties with various sample loads at different flow rates

Sample load	Flow rate (ml/min)	Selectivity (α)	Resolution (R)	Pressure (psi)	Purity* Area%	
					Extr.	Raff.
0.1 ml	2	1.42	1.42	610-620	99.9	99.6
	3	1.32	1.51	810-820	99.8	99.7
	4	1.21	1.32	840-850	99.8	99.4
	5	1.12	1.05	1190-1200	99.1	99.3
0.25 ml	2	1.40	1.02	610-620	98.0	98.4
	3	1.28	1.12	810-820	98.5	98.2
	4	1.16	0.99	840-850	97.8	97.4
	5	1.08	0.83	1190-1200	95.2	95.8
0.5 ml	2	1.37	0.76	610-620	93.0	93.5
	3	1.25	0.83	810-820	94.2	93.9
	4	1.13	0.71	840-850	95.3	95.6
	5	1.05	0.64	1190-1200	92.4	92.1
1 ml	2	1.35	0.41	610-620	67.0	66.8
	3	1.22	0.51	810-820	82.0	81.4
	4	1.10	0.32	840-850	79.0	78.0
	5	1.01	0.24	1190-1200	62.0	60.9

*Purity of individual enantiomer

Fig. 8 illustrates the resolution (R) of the sample at different sample loads. As it can be seen, at sample load 0.1 ml the resolution was more than 1 at different flow rates with maximum at flow rate 3 ml/min. Thus this should be the best condition for good resolution. However, under these conditions the sample load was too low. At higher sample load, though selectivity was more than 1, the resolution was more than 1 only for sample load 0.25 ml for flow rate 2 ml/min and 3 ml/min and slightly lower than 1 (0.99 and 0.83) for flow rate 4 ml/min and 5 ml/min. For sample load 0.5 ml and 1 ml, the resolution was much lower than 1. The purity of each enantiomer

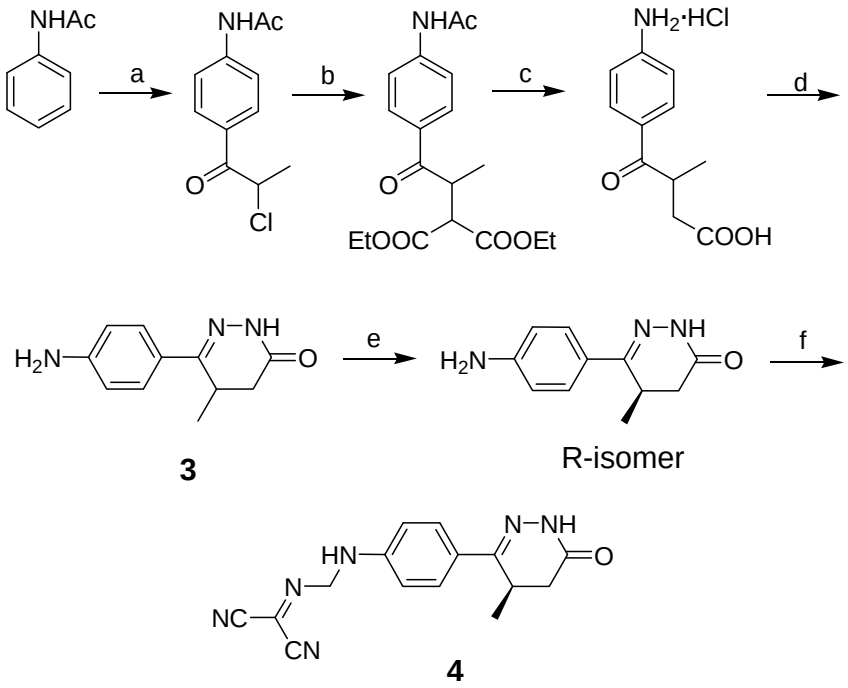
separated at sample load 0.1 ml and 0.25 ml at all four flow rates was checked by analytical HPLC. It was found that again at sample load 0.1 ml the purity was more than 99% (area) at all flow rates. At sample load 0.25 ml, the maximum purity (98.5% area) was for flow rate 3 ml/min. From above study it was concluded that sample load 0.25 ml at flow rate 3 ml/min was the best sample load for preparative separation of compound **1**.

With above standardized conditions 325 mg of racemic compound **1** was resolved. Both the fractions were collected separately, extracted with methylene chloride, dried over anhydrous sodium sulphate and concentrated under reduced pressure. The yield of first enantiomer eluted at RT 10.4 min was 128 mg which showed optical rotation $[\alpha]_{25}{}^{D}$ + 21.33° ($CHCl_3$, *c* 0.55). The second enantiomer (wt. 135 mg) eluted at RT 11.1 min had optical rotation $[\alpha]_{25}{}^{D}$ - 21.26° ($CHCl_3$, *c* 0.55).

3.3.2 Resolution of simendan precursor (3), cibenzoline (5) and its analogues (6 and 7) by simulated moving bed chromatography (SMBC)

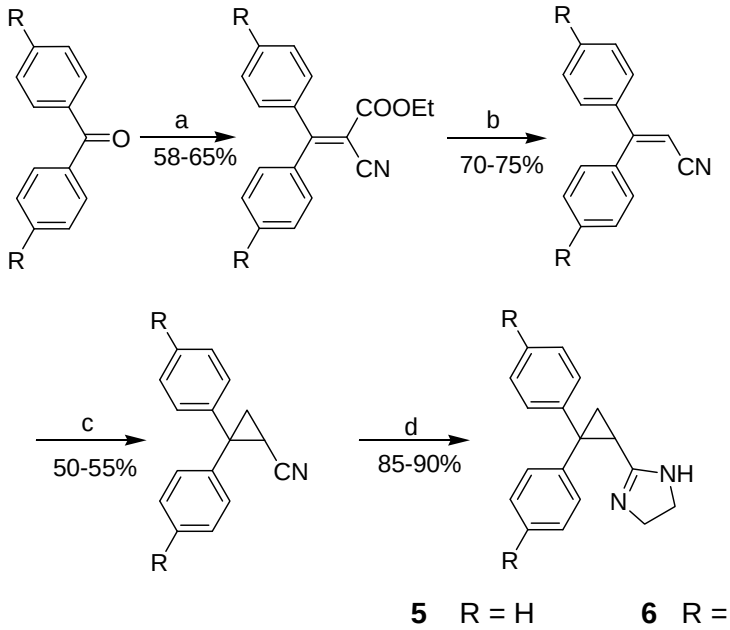
Simendan is a racemic mixture of levosimendan (LS) (**4**) and dextrosimendan (DS). Both the racemate and the pharmacologically active enantiomer, levosimendan, are calcium sensitizer and are novel positive inotropic drug candidates intended for the treatment of congestive heart failure.[37,53] In our group a precursor of simendan, 6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2*H*)-one (**3**), was synthesized by reported[54] procedure (**Scheme 2**) and its resolution was carried out by SMBC to isolate R-isomer, which is described in this chapter. The resolved R-precursor was further treated with malononitrile/sodium nitrite to afford levosimendan (**4**).

Cibenzoline has been clinically used as one of the class I antiarrhythmic agents.[55,56] The syntheses of cibenzoline (**5**) and its two analogues (**6** and **7**) were carried out (**Scheme 3** and **Scheme 4**) in order to create a more potent and well tolerated therapeutic agent which will not induce spordic hypoglycemia[57,58] and stimulate insulin secretion in the potential treatment of cardiac arrhythmias[59] avoiding toxic reagents like mercuric oxide and avoiding high temperature for the final condensation process from commercially available benzophenone as starting material.[60] All these compounds (**5**, **6** and **7**) have been resolved by SMBC process.



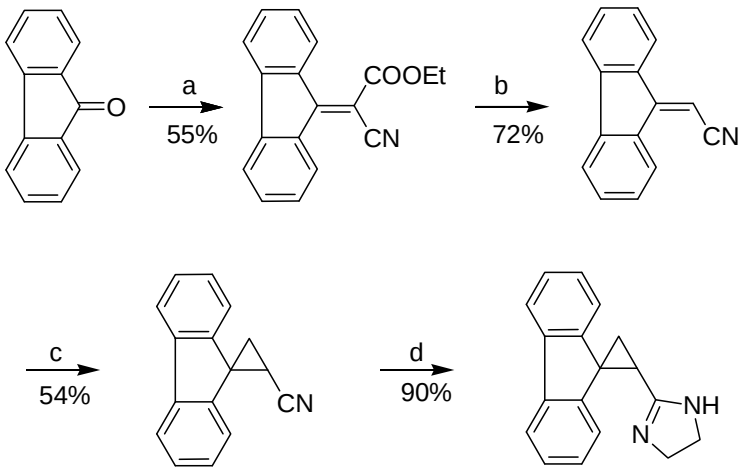
Reagents and conditions: a) i) 2-Chloropropionyl chloride, $AlCl_3$, 80% b) Diethyl malonate, base, 61% c) EtOH/HCl, reflux, 75% d) $N_2H_4.H_2O$, 65% e) Resolution f) Malononitrile/sodium nitrite, 60%.

Scheme 2



Reagents and conditions: a) $CNCH_2COOEt$, $HOAc/C_6H_6$, β-alanine at reflux, 90 h b) NaCl, H_2O, DMSO, 160-170 °C, 4 h c) $Me_3S(O)I$, NaH, DMSO, rt, 24 h d) Sulphur, ethylenediamine, reflux, 4h.

Scheme 3



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Reagents and conditions: a) $CNCH_2COOEt$, $HOAc/C_6H_6$, β-alanine at reflux, 90 h b) NaCl, H_2O, DMSO, 160-170 °C, 4 h c) $Me_3S(O)I$, NaH, DMSO, rt, 24 h d) Sulphur, ethylenediamine, reflux, 4 h.

Scheme 4

3.3.2.1 Resolution of simendan precursor (3)

3.3.2.1.1 Solubility, selectivity and resolution (R) of 3

As solubility of simendan precursor (**3**) was very less in iso-propanol (0.02 mg/ml) compared to ethanol (4.8 mg/ml), ethanol:n-hexane was considered for resolution. The solubility of **3** was determined in ethanol:n-hexane (mobile phase) at various proportions. It was observed that solubility of **3** was increased with the increasing percentage of ethanol (**Table 3**). Further, **3** was analyzed using saturated solution of **3** in various mobile phases having different proportions of ethanol:n-hexane as mentioned above. It was observed that the selectivity (α) and resolution (R) were minimum in 100% ethanol i. e. 1.29 and 1.14 respectively, but solubility was maximum (4.8 mg/ml); whereas in ethanol:n-hexane (50:50) both selectivity (α) and resolution (R) were maximum (1.92 and 2.02 respectively), but solubility of **3** was less (0.4 mg/ml) (**Table 3**). It could be observed from the table and the graph (**Fig. 9**) that even though selectivity (α) and resolution (R) are better at lower proportions of ethanol, solubility of **3** is less. The best selectivity (1.83) and resolution (1.54) were observed with 90:10 ethanol:n-hexane at 3.6 mg/ml concentration (**Fig. 10**) which was considered to be the best composition for SMB chromatography.

Table 3: Solubility of **3** and its selectivity (α) and resolution (R) on single SMB column, flow rate 6ml/min, wave length 254 nm

Mobile phase* Ethanol: n-hexane	**Solubility of compound 3 (mg/ml)**	**(α)**	**(R)**	**Pump pressure Psi units**
100:00	4.8	1.29	1.14	500
90:10	3.6	1.83	1.54	491
80:20	2.5	1.83	1.54	478
70:30	1.4	1.86	1.72	457
60:40	0.8	1.90	1.98	442
50:50	0.4	1.92	2.02	425

*Each 100 ml of mobile phase contains 1 ml of iso-propanol and 0.1 ml of diethyl amine.

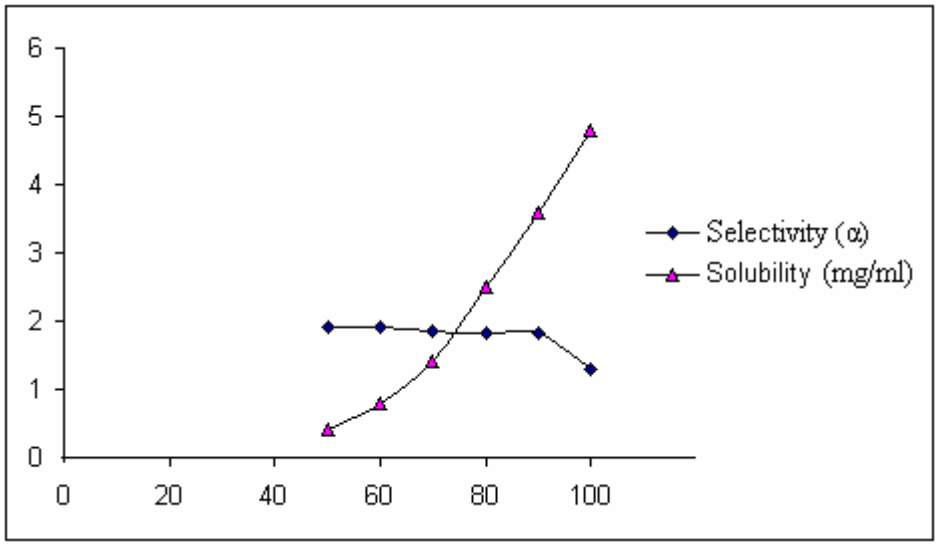


Fig. 9: Solubility of **3** in different mobile phases and its selectivity on single SMB column, flow rate 6ml/min, wave length 254 nm

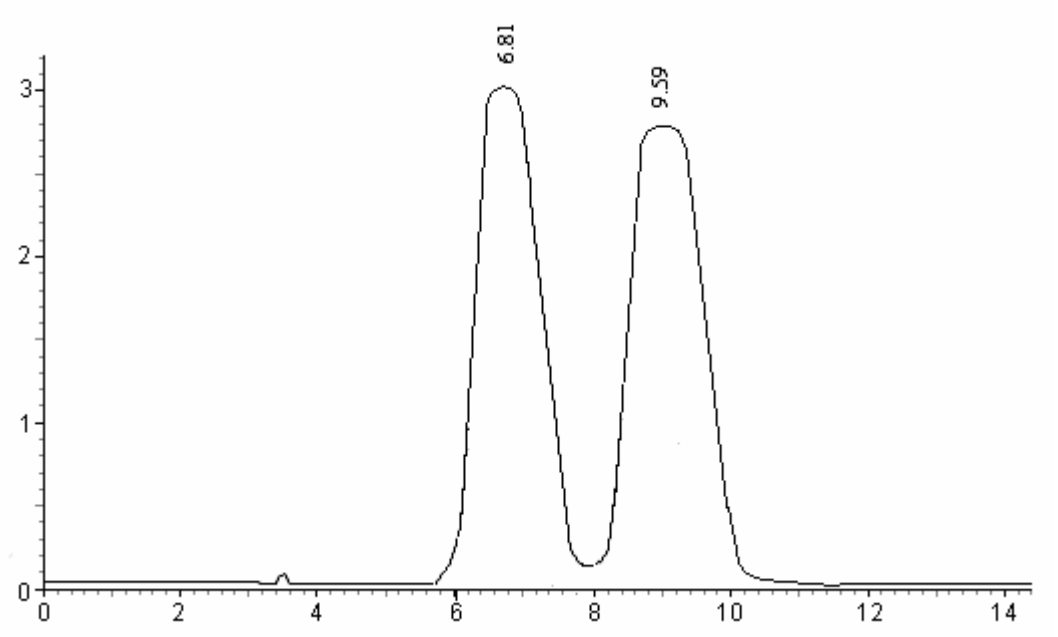


Fig. 10: Separation of **3** on one SMB column, mobile phase: 90:10:0.1:1 ethanol:n-hexane:diethylamine:isopropyl alcohol, flow rate 6 ml/min injection volume 100 µl, wavelength 254 nm, temperature 30 °C.

3.3.2.1.2 Calculation of SMB parameters and separation of enantiomers

After deciding the mobile phase composition, the analysis was carried out on all eight columns to be used in SMB unit. Average migration time (t_0) of non-retained component, retention time of both enantiomers (raffinate and extract, T_R and T_E), tube delay time, column length, column diameter and flow rate of mobile phase were fed in linear isotherm software. This software provided the isotherm parameters, K (Henry coefficient) for raffinate (K_R 5.259) and for extract (K_E 9.663) and average porosity (0.843) of the columns. These obtained values were fed to the Chromsim simulation software. This software provided the 'Morbidelli triangle' (**Fig. 11**) which gave the range of the flow rate ratios (m_n) of zone 1 to zone 4. The flow rate ratios of zone 2 and zone 3 were from 5.41 to 9.81. According to SMB theory the flow rate ratio of zone 1 (m_1) should be more than 9.81 whereas flow rate ratio of zone 4 (m_4) should be less than 5.41. Several variations in flow rate ratios of zone 1 to zone 4, feed flow rate, number of switches of port valve were fed in Chromsim simulation software so that the flow rates in each zone should not exceed the pressure limit, the flow rate of mobile phase should be minimum and the switch time of port valve should not be too low or too high to damage its seal. After satisfying all above parameters the computer

simulation was done. It was observed that less number of switches of valves did not give steady state of purity of both raffinate and extract. Hence number of switches was also fed in such a way that the steady state of 100% purity of both raffinate and extract was achieved while simulation.

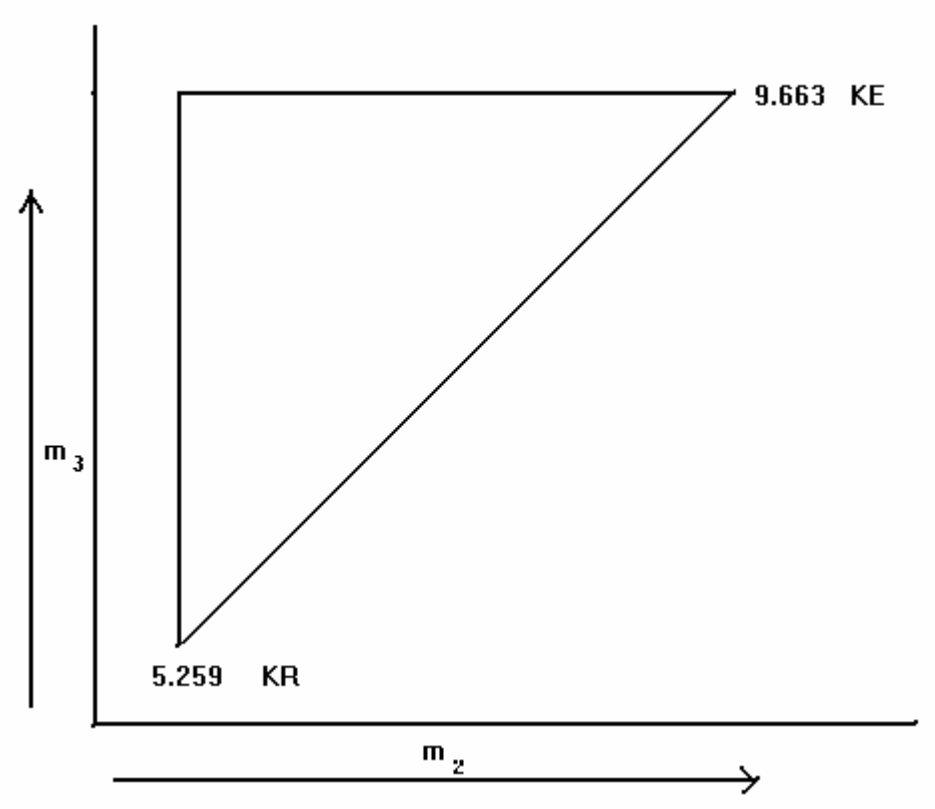


Fig. 11: Morbidelli triangle for compound **3**

After obtaining the good calculated values for steady separation of raffinate and extract, all eight columns were fitted to SMB unit in such a way that columns with matching retention time of any one isomer of racemate are diagonally symmetric to each other and then the SMB instrument was switched on. The mobile phase (ethanol:hexane:diethylamine:isopropanol, 90:10:0.1:1) was pumped through eluent and feed inlets by applying their flow rates calculated from Chromsim simulation software. The flow rates at raffinate and extract outlets were monitored by flow meters and were adjusted with help of regulator valves to calculated flow rates.

After achieving the steady flow rates of all four inlets/outlets for about 1 h, feed inlet was switched over to racemic mixture solution prepared in same mobile phase. After each cycle the analysis of extract and raffinate was carried out on

analytical HPLC to check individual purity. After obtaining the 100 % purity of both raffinate and extract the analysis of each cycle was further carried out till steady state of purity and concentration of outlets was achieved. Necessary variations of flow rate ratios of zone 1 to zone 4 (m_1 to m_4) were done occasionally on Chromsim simulation software and after satisfying all parameters same were reapplied to SMB instrument for the purpose of separation. This process was continued till unaffected purity of extract and raffinate was achieved i. e. it achieved the steady state.

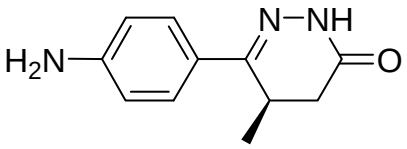
The optimum calculated and practically applied parameters for resolution of simendan precursor (**3**) are shown in **table 4**.

Table 4: Operating parameters of SMB for compound **3**

Mobile phase	**Ethanol:n-hexane:diethylamine:isopropanol 90:10:0.1:1**
Feed concentration	3.4 mg/ml
Flow rate ratios	
m_1	10.0647
m_2	6.26847
m_3	7.57645
m_4	4.56810
Feed flow rate	0.70 ml/min
Eluent flow rate	2.94 ml/min
Flow rate in zone 1	8.26 ml/min
Flow rate in zone 2	6.23 ml/min
Flow rate in zone 3	6.93 ml/min
Flow rate in zone 4	5.32 ml/min
Extract flow rate	2.03 ml/min
Raffinate flow rate	1.61 ml/min
Switching time	3.54 min
Pressure (bar)	8.6

The simulated purity of raffinate and extract were 100%, whereas obtained purity was 99.96% and 99.99% respectively (**Fig. 12**). Total 5.2 g of simendan

precursor (**3**) was resolved to obtain 2.15 g of raffinate and 2.08 g of extract. Optical rotation of both isomers were taken and found that the extract was the required R-isomer (**10**) of simendan precursor.



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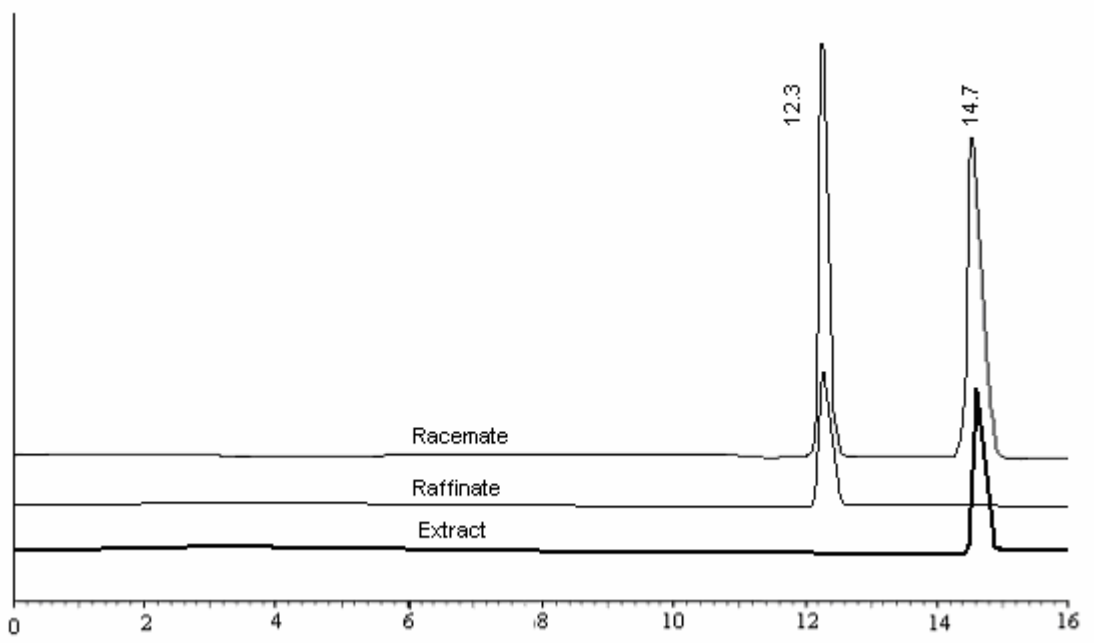


Fig. 12: Analysis of simendan precursor **3** on CHIRALCEL OD-H (250 mm x 4.6 mm), mobile phase 92:8:0.1:1 ethanol:n-hexane:diethyl amine:iso-propanol, flow rate 1 ml/min, wavelength 254 nm.

3.3.2.1.3 Comparison of separation under batch and SMB conditions

From the SMB parameters applied (**Table 4**) it could be calculated that on 84.4 g of CSP 1.166 lit of mobile phase will be necessary to resolve 1 g of simandan precursor **3** in 6.62 h. For the comparison between batch separation and SMB separation, simadan precursor **3** was resolved using single SMB column on preparative HPLC. The flow rate of mobile phase was 6 ml/min, sample injection volume 0.5 ml and one batch time was 12 min. By extrapolating these results, for

resolution of 1 g of **3**, the requirement of mobile phase would be 42.6 lit and 118 h would be necessary.

3.3.2.2 Resolution of cibenzoline (5) and its analogues 6 and 7

3.3.2.2.1 Determination of solubility

Solubility of cibenzoline (**5**) its analogues **6** and **7** was determined in ethanol:n-hexane (mobile phase) at various proportions. It was observed that solubility of all three compounds increased with the increasing percentage of ethanol (**Table 5**).

Table 5: Solubility of cibenzoline (**5**) and its analogues **6** and **7**

Mobile phase* Ethanol:n-hexane	**Cibenzoline(5) mg/ml**	**Cibenzoline analogue (6)**	**Cibenzoline analogue (7)**
100:0	1.42	1.6	3.12
90:10	1.21	1.4	2.28
80:20	1.03	1.1	1.50
70:30	0.81	0.8	0.8
60:40	0.60	0.4	0.4
50:50	0.30	0.07	0.05

*Each 100 ml of mobile phase contains 1 ml of iso-propanol and 0.1 ml of diethyl amine.

3.3.2.2.2 Selectivity (α) and resolution (R)

After finding out the solubility of all three compounds, each compound was analyzed on HPLC instrument using saturated solution in various mobile phases on single SMB column in all mobile phases mentioned above.

It was observed that in 100% ethanol the selectivity (α) was minimum (1.01) and it goes on increasing as proportion of n-hexane increases. Though in case of cibenzoline in 90:10 ethanol:n-hexane selectivity is again 1.01, it increases with the percentage of n-hexane. The maximum selectivity (α) was 1.36, 1.28 and 1.45 for

compounds **5, 6** and **7** respectively in three different mobile phases i.e. ethanol:n-hexane (60:40), ethanol:n-hexane (80:20) and ethanol:n-hexane (70:30) respectively. There was no resolution (R) in 100% ethanol in case of all three compounds. Resolution of **5** was less than one in all mobile phases with maximum of 0.93 in ethanol:n-hexane (70:30). Resolution and selectivity of **6** were maximum in ethanol:n-hexane (80:20) whereas resolution and selectivity of **7** were maximum in ethanol:n-hexane (70:30) (**Table 6**).

Table 6: Selectivity (α) and resolution (R) of compounds **5**, **6** and **7** in various mobile phases on single SMB column, flow rate 6ml/min, wave length 254 nm

Mobile phase* **Ethanol:n-hexane**	**Cibenzoline (5)**		**Compound 6**		**Compound 7**	
	(α)	**(R)**	**(α)**	**(R)**	**(α)**	**(R)**
100:0	1.01	-	1.01	-	1.01	-
90:10	1.01	-	1.16	0.8	1.21	1.03
80:20	1.11	0.82	1.28	1.37	1.36	1.10
70:30	1.30	0.93	1.26	1.30	1.45	1.63
60:40	1.36	0.88	1.17	0.93	1.39	1.51
50:50	1.33	0.85	1.14	0.88	1.39	1.51

*Each 100 ml of mobile phase contains 1 ml of iso-propanol and 0.1 ml of diethyl amine.

It could be observed from the **table 6** and the graph (**Fig. 13**) that even though selectivity (α) of **5** was high in 60% ethanol, its solubility was less (0.6 mg/ml). The suitable selectivity (1.30) and resolution (0.93) were observed with 70:30 ethanol:n-hexane at 0.81 mg/ml concentration (**Fig. 14**) which was considered as the best composition for SMB chromatography. In case of compound **6** in 80% ethanol selectivity (α) and resolution (R) were maximum i.e. 1.28 and 1.37 respectively; its solubility (1.1 mg/ml) was also suitable to be considered for SMB separation. While in case of compound **7** though in 80% ethanol selectivity (α) and resolution (R) were less than those of in 70%, its solubility (1.5 mg/ml) in 80% ethanol was much better

than it was in 70% ethanol (0.8 mg/ml) and hence 80% ethanol in n-hexane was considered as the best mobile phase for its SMB separation.

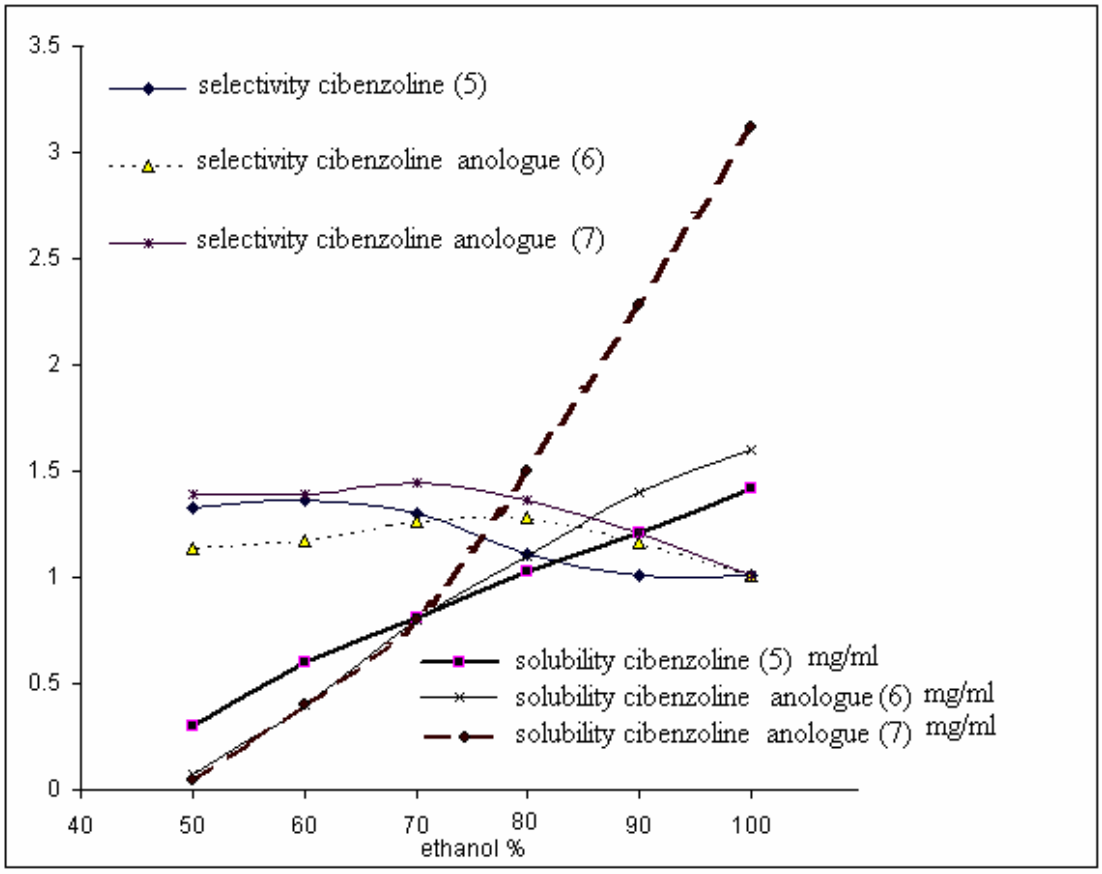


Fig. 13: Solubility of **5, 6** and **7** in different mobile phases and their selectivity on single SMB column

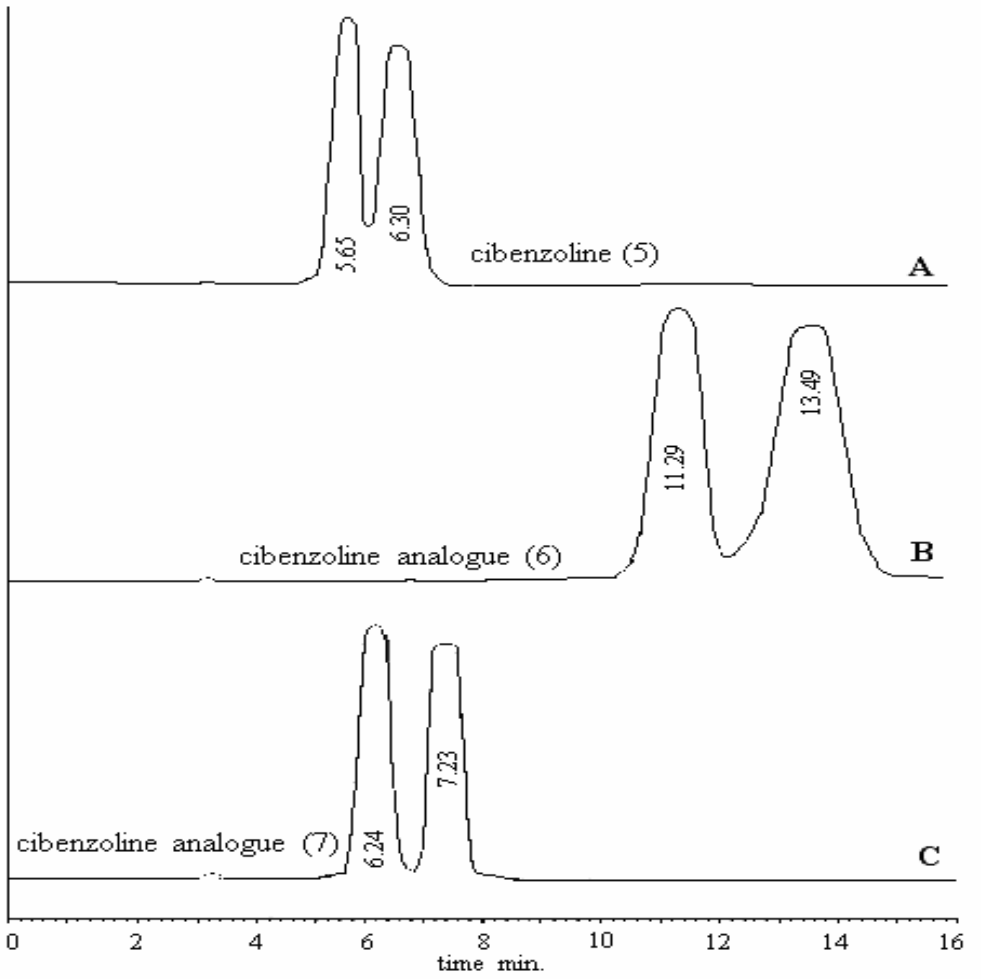


Fig. 14: Analysis of cibenzoline (**5**) and cibenzoline analogues **6** and **7**

Column CHIRALCEL OD (16 mm x 120 mm, 20 μm), flow rate 6 ml/min, wavelength 254 nm, injection vol. 100 μl, mobile phase: ethanol:n-hexane:diethyl amine:iso-propanol A) 70:30:0.1:1; B) 80:20:0.1:1; C) 80 :20 :0.1:1.

3.3.2.2.3 Calculation of SMB parameters and separation of enantiomers

After deciding the mobile phase compositions for SMB separation of all three compounds, their individual analysis was carried out on all eight columns used in SMB unit. Average migration time (t_0) of non-retained component, retention time of both enantiomers (raffinate and extract, T_R and T_E), tube delay time, column length, column diameter and flow rate of mobile phase were fed in linear isotherm software. This software provided the isotherm parameters, K (Henry coefficient) for raffinate (K_R) and extract (K_E) for each individual compound (**Table 7**) along with average porosity (0.843) of the columns which was same as that of obtained previously.

Table 7: Henry coefficients of compounds **5**, **6** and **7**

Compound	K_R	K_E
5	3.422	4.452
6	12.356	15.841
7	3.590	4.890

These obtained values were fed to the Chromsim simulation software. This software provided the 'Morbidelli triangle' for each compound which gave the individual range of the flow rate ratios (m_n) of zone 1 to zone 4 (**Table 8**) for all three compounds.

Table 8: Flow rate ratios of compounds **5**, **6** and **7** obtained from Morbidelli triangle

compounds	m_1	Limits of m_2 and m_3	m_4
5	> 4.75	3.69 to 4.75	< 3.69
6	> 16.0	12.5 to 16.0	< 12.5
7	> 5.02	3.72 to 5.02	< 3.72

The SMB parameters of all three compounds were then separately calculated by feeding their various flow rate ratios m_1 to m_4, feed flow rates, number of switches of port valve in Chromsim simulation software to satisfy all the parameters as described for the resolution of compound **3**. After satisfying all above parameters the computer simulation was done in such a way that the steady state of 100% purity of both raffinate and extract was achieved for all three compounds.

These compounds **5**, **6** and **7** were then resolved separately by pumping the individual mobile phases through eluent and feed inlets by applying their respective flow rates calculated from Chromsim simulation software. The flow rates at raffinate and extract outlets were monitored by flow meters and were adjusted with help of regulator valves to calculated flow rates. After achieving the steady flow rates of all four inlets/outlets for about one hour, feed inlet was switched over to racemic mixture solution prepared in same mobile phase. After each cycle the analysis of extract and raffinate was carried out on analytical HPLC to check individual purity. After obtaining the 100% purity of both raffinate and extract, the analysis of each cycle was

further carried out till steady state of purity and concentration of outlets was achieved. After few analysis necessary variations of flow rate ratios of zone 1 to zone 4 (m_1 to m_4) were done on Chromsim simulation software and after satisfying all parameters same were reapplied to SMB instrument for the purpose of separation. This process was continued till unaffected purity of extract and raffinate was achieved for all three compounds.

The optimum calculated and practically applied parameters for resolution of compounds **5**, **6** and **7** are shown in **table 9**. Purity of both enantiomers of all three compounds was analyzed by HPLC (**Fig. 15**). Quantity of compounds **5**, **6** and **7** resolved was 1.4 g, 1.36 g and 1.52 g respectively.

Table 9: Operating parameters of SMB for cibenzoline (**5**) and its analogues **6** and **7**

Operation parameters	**Cibenzoline (5)**	**Compound 6**	**Compound 7**
Mobile phase*	70:30:0.1:1	80:20:0.1:1	80:20:0.1:1
Feed concentration (mg/ml)	0.8	1.0	1.4
m_1	4.9999	16.7976	5.3000
m_2	4.0753	13.2042	4.0444
m_3	4.3252	15.0709	4.5644
m_4	3.1252	11.9442	3.0644
Feed flow rate (ml/min)	0.3	0.9	0.9
Eluent flow rate (ml/min)	2.25	2.43	3.87
Flow rate in zone 1 (ml/min)	12.7	10.68	16.84
Flow rate in zone 2 (ml/min)	11.6	8.95	14.68
Flow rate in zone 3 (ml/min)	11.89	9.85	15.58
Flow rate in zone 4 (ml/min)	10.45	8.35	12.98
Extract flow rate (ml/min)	1.1	1.73	2.17
Raffinate flow rate (ml/min)	1.44	1.50	2.59
Switching time (min)	1.5	3.93	1.28
Pressure (bar)	1580	1479	1720
Purity of raffinate (area %)	99.98	99.96	99.99
Purity of extract (area %)	99.99	99.98	99.99

*Ethanol:n-hexane:diethylamine:iso-propanol

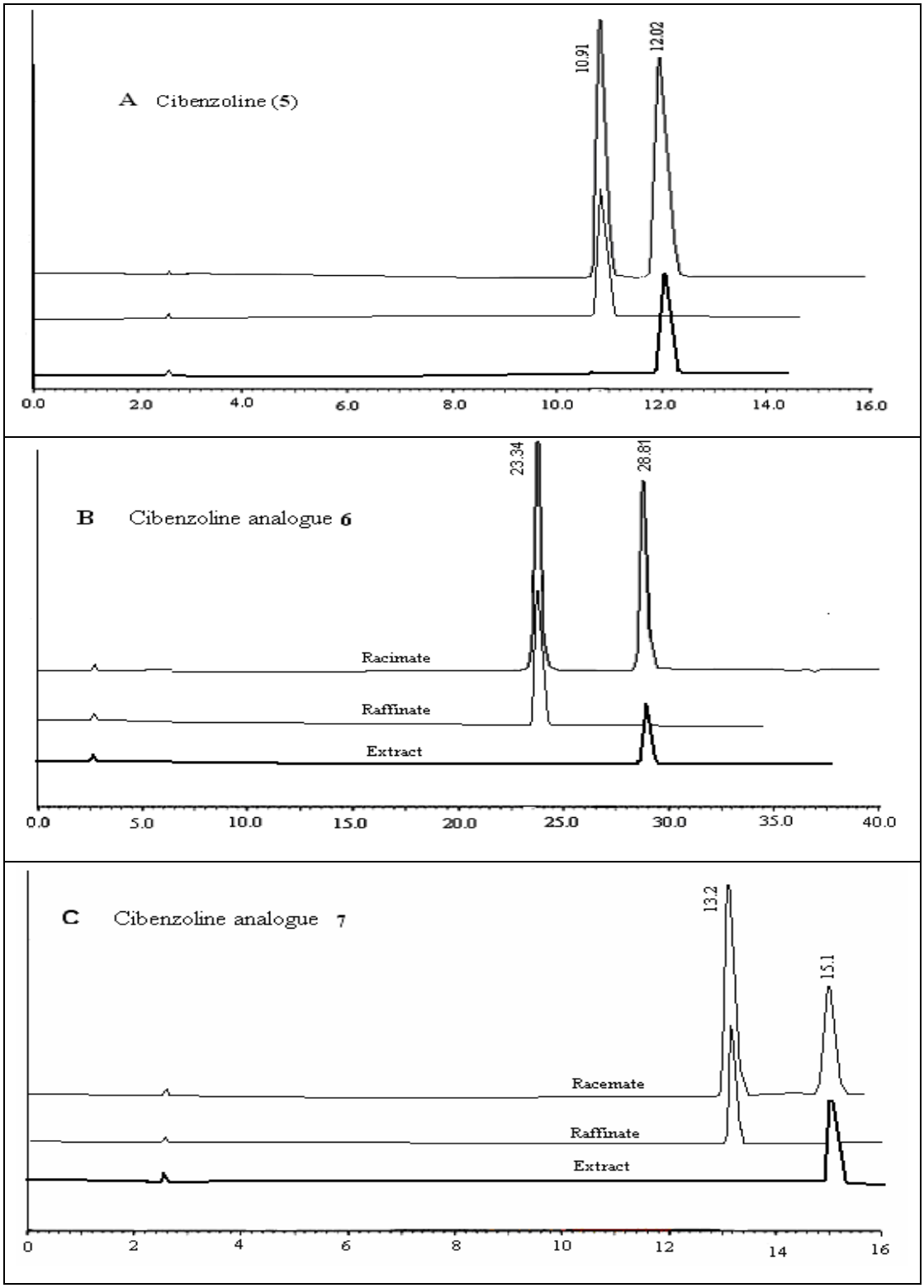


Fig. 15: Overlay of analysis of reffinate, extract and racemate on column CHIRALCEL OD (4.6 mm x 250 mm, 10 µm), wavelength 254 nm, flow rate 1 ml/min, mobile phase: ethanol:n-hexane:diethyl amine:isopropyl alcohol (**A**) 70:30:0.1:1), (**B**) 70:30:0.1:1, (**C**) 80:20:0.1:1.

3.3.2.2.4 Comparison of separation under batch and SMB conditions

From the SMB parameters applied for all three compounds (**Table 10**) it could be calculated that on 84.4 g of CSP 10.625 lit, 3.7 lit and 3.785 lit of mobile phase will be required to resolve 1 g of compounds **5**, **6** and **7** in 69.45, 18.52 and 13.22 h respectively. For the comparison between batch separation and SMB separation, all three compounds were resolved using single SMB column on preparative HPLC. Flow rate of mobile phase was 6 ml/min, sample injection volume: 0.2 ml, 0.4 ml and 0.5 ml for compounds **5**, **6** and **7** with batch period of 8 min, 15 min and 9 min respectively. By extrapolating these results, by batch separation method for 1 g of racemates **5**, **6** and **7** mobile phase requirement would be 300 lit, 225 lit and 77.14 lit in 833 h, 625 h and 214.3 h respectively.

Table 10: Comparison between preparative HPLC on single SMB column and on SMBC with eight columns for resolution of 1 g of compounds

Parameter	**Cibenzoline (5)**		**Cibenzoline analogue (6)**		**Cibenzoline analogue (7)**	
	Batch	**SMB**	**Batch**	**SMB**	**Batch**	**SMB**
Feed conc. (mg/ml)	0.8	0.8	1.1	1.1	1.5	1.5
Amount of CSP (g)	10.55	84.4	10.55	84.4	10.55	84.4
Eluent flow rate (ml/min)	6.0	2.25	6 .0	2.43	6.0	3.87
Feed rate (ml/min)	0.2	0.30	0.4	0.90	0.5	0.90
Mobile phase (lit)	300	10.62	225	3.7	77.14	3.785
Time required	833.3	69.45	625	18.52	214.3	13.22

3.3.3 Conclusion

Thus 5-hydroxy-3-(4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-cyclopent-2-en-1-one (**1**), a novel anticancer agent, has been resolved by chiral preparative HPLC. The racemic (±)-(RS)-6-(4-aminophenyl)-5-methyl-4,5-duhydropyridazin-3(2*H*)-one (**3**), a precursor of a calcium sensitizer, levosimendan, (-)-(R)-[4-(1,4,5,6-

tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono] propanedinitrile, (**4**), an antiarrhythmic drug, cibenzoline (**5**) and two new cibenzoline analogues (**6** and **7**) have also been resolved successfully by simulated moving bed chromatography. It is also shown that by SMBC resolution of these compounds is much faster and economical by using SMBC than by batch preparative HPLC.

3.4 EXPERIMENTAL

Instrument

High performance liquid chromatography (HPLC)

Analytical HPLC was carried out on a Merck–Hitachi HPLC system (Merck KGaA, Darmstadt, Germany) consisting of model L-7100 (quaternary) pump, a Rheodyne 7725i manual injector valve with 20 µl injection loop and model L-7400 UV detector operated at 254 nm. The chromatography data were processed by model D-7000 HPLC system manager.

Preparative separation was carried out on Shimadzu HPLC system (Japan) consisting of model LC-8A pump, SCL-8A system controller, CR-7A integrator with Rheodyne manual injector valve having 1 ml injection loop and SPD-10AVP UV detector operated at 254 nm.

Simulated moving bed (SMB) chromatography unit

Simulated moving bed chromatography (SMBC) was performed at 30 °C using CSEP® C916 SMB unit (Knauer Berlin, Germany) equipped with 8 columns (16 mm x 120 mm) containing 24.1 ml of the chiral stationary phase arranged in series and distributed in four pairs of two columns in each zone. The chiral stationary phase was CHIRALCEL OD (Daicel, Tokyo, Japan), 20 µm. Sixty four port valve changed the positions of the feed, desorbent inlet, raffinate and extract outlets at preset switch times. This valve was connected to two semi-preparative liquid chromatographic pumps (Knauer K-500) and two analytical liquid chromatographic pumps (Knauer K-501). The sixty four port valve was controlled by instrument software (ValveChrom 2000). The unit also contained two sampling valves connected in series with flow-meter that permitted the collection of extract and raffinate separately. A Knauer membrane degasser was used to prevent air bubbles in the system before feed was loaded. Software ValveChrom 2000 (ver. 1.6) was used to control SMBC. Isothermfit (ver. 1.0) was used to calculate porosity, selectivity and Henry constant. SMB_Guide (ver. 1.2) was used to calculate flow rates in different zones and switch time.

The solvents were HPLC grade from MERCK (India) and were filtered on a Millipore membrane of 0.45 μm.

Determination of solubility

The saturated solution of compound was prepared by dissolving maximum quantity with sonication at 30 ºC in respective mobile phase. The filtered and measured quantity of saturated solution was evaporated till dryness on a dish. Residual weights were considered as solubility of that compound in corresponding mobile phase.

Resolution of 5-hydroxy-3-(4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-cyclopent-2-en-1-one (1) by preparative HPLC

Compound **1** (325 mg) was dissolved in 1.02 lit of mobile phase (acetonitrile:water, 40:60) with sonication and was analyzed using CHIRALCEL OD-RH (4.6 mm x 150 mm) column at flow rate 0.5 ml/min.

The preparative HPLC was carried out on CHIRALCEL OD-R (10 mm x 250 mm) column using same mobile phase with flow rate 3 ml/min, sample load 0.25 ml. The fraction was cut at valley of two peaks. Both the fractions were collected separately, extracted with methylene chloride, dried over anhydrous sodium sulfate and concentrated under reduced pressure.

The yield of first enantiomer eluted at RT 10.4 min was 128 mg which showed optical rotation $[\alpha]_{25}^{D}$ +21.33° ($CHCl_3$, *c* 0.55). The second enantiomer (wt. 135 mg) eluted at RT 11.1 min had optical rotation $[\alpha]_{25}^{D}$ -21.26° ($CHCl_3$, *c* 0.55).

Resolution of simendan precursor (3), cibenzoline (5) and its analogues (6 and 7) by simulated moving bed chromatography (SMBC)

Typical resolution of simendan precursor (3)

Selectivity and resolution (R)

Selectivity and resolution (R) of simendan precursor (**3**) were determined in ethanol:n-hexane (mobile phase) at various proportions using 6ml/min on single SMB

column at wave length 254 nm. The best selectivity (1.83) and resolution (1.54) were observed with 90:10 ethanol:n-hexane at 3.6 mg/ml concentration at which SMB chromatography was performed.

Calculation of SMB parameters

Initially, the analysis was carried out on all eight columns of SMB unit. Average t_0 (3.49 min) of non-retained component, retention time of both enantiomers, raffinate (6.85 min) and extract (9.63 min), tube delay time (0.1 min), column length, column diameter and flow rate of mobile phase were fed in linear isotherm software. The software provided Henry coefficient for raffinate (K_R 5.259) and for extract (K_E 9.663) and average porosity (0.843) of the columns. These obtained values were fed to the Chromsim simulation software. This software provided the 'Morbidelli triangle' which gave the range of the flow rate ratios (m_n) of zone 1 to zone 4. The flow rate ratios of zone 2 and zone 3 were from 5.41 to 9.81. Several variations in flow rate ratios of zone 1 to zone 4, feed flow rate, number of switches of port valve were fed in Chromsim simulation software so that the flow rates in each zone should not exceed the pressure limit, the flow rate of mobile phase should be minimum and the switch time of port valve should not be too low or too high to damage its seal. After satisfying all above parameters the computer simulation was done. The number of switches was also fed in such a way that the steady state of 100% purity of both raffinate and extract was achieved while simulation.

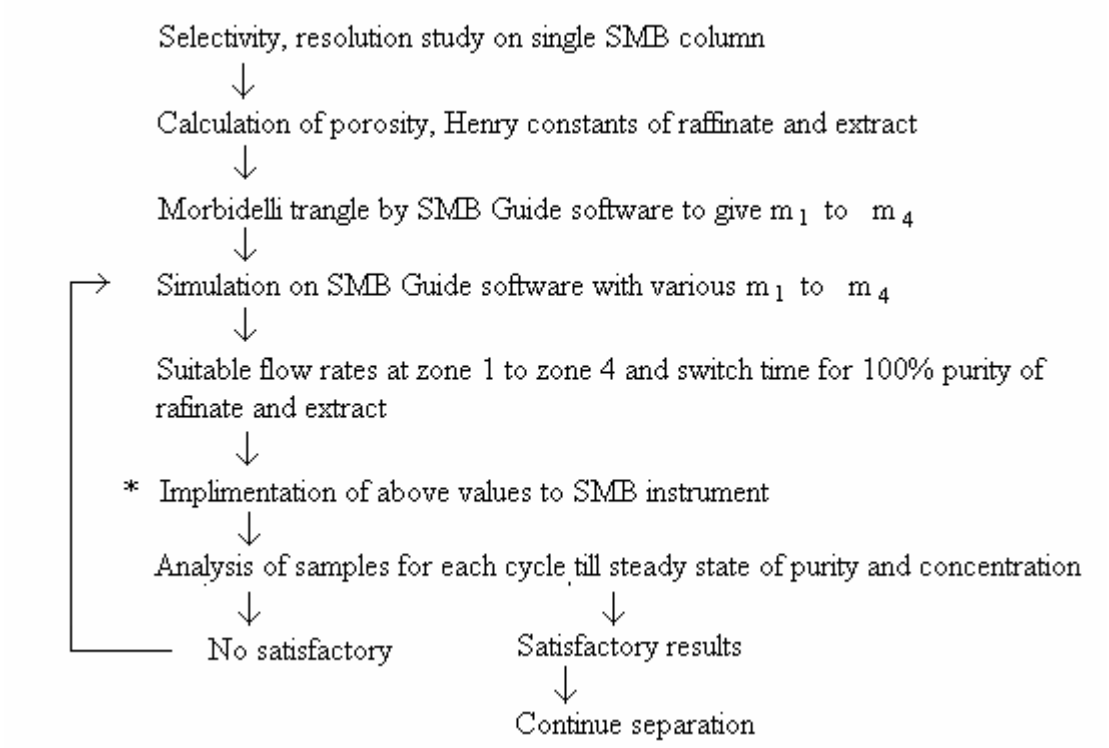
SMB operation

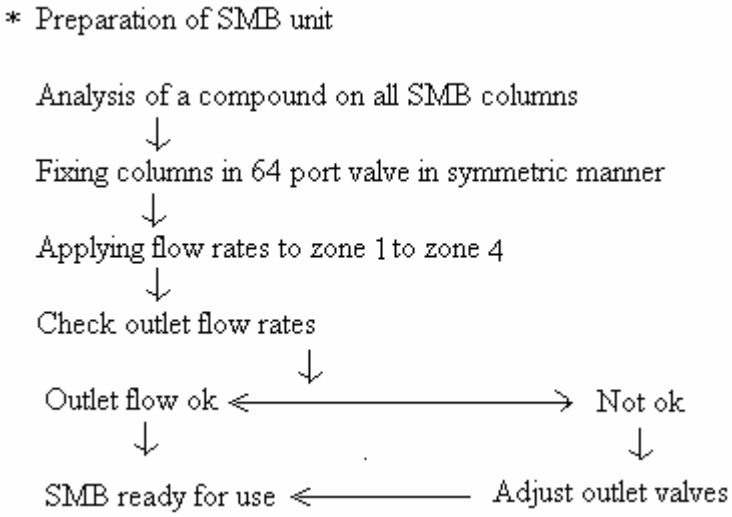
After obtaining the optimum values for steady separation of raffinate and extract the SMBC instrument was switched on. The mobile phase (ethanol:hexane:diethylamine:isopropanol, 90:10:0.1:1) was pumped through eluent and feed inlets by applying their flow rates calculated from Chromsim simulation software. The flow rates at raffinate and extract outlets were monitored by flow meters and were adjusted with help of regulator valves to calculated flow rates.

After achieving the steady flow rates of all four inlets/outlets for about 1 h, feed inlet was switched over to racemic mixture solution prepared in same mobile phase. After each cycle the analysis of extract and raffinate was carried out on

analytical HPLC to check individual purity. After obtaining the 100 % purity of both raffinate and extract the analysis of each cycle was further carried out till steady state of purity and concentration of outlets was achieved. Necessary variations of flow rate ratios of zone 1 to zone 4 (m_1 to m_4) were done occasionally on Chromsim simulation software and after satisfying all parameters same were reapplied to SMB instrument for the purpose of separation. This process was continued till unaffected purity of extract and raffinate was achieved.

Schematic diagram of SMBC





Total 2.9 g of simendan precursor (**3**) was resolved to obtain 0.95 g of raffinate and 0.96 g of extract (levosimendan). The purity of raffinate and extract were 99.96% and 99.99% respectively.

Further 2.4 g of cibenzoline (**5**) was resolved to obtain 0.55g of raffinate and 0.46 g of extract. The purity of raffinate and extract were 99.94% and 99.89%. 1.36 g of cibenzoline derivative **6** was resolved to obtain 0.35 g of raffinate and 0.36 g of extract. The purity of raffinate and extract were 99.86% and 99.88%, and 1.52 g cibenzoline derivative **7** was resolved similarly to obtain 0.25 g of raffinate and 0.24 g of extract. The purity of raffinate and extract were 99.66% and 99.98% respectively.

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