

Transformative Reactions of Terpenoids and Studies on their Biological Activity

*Thesis submitted for the degree of Doctor of Philosophy in science
(Chemistry) under the
University of North Bengal*

By
AMITAVA MANDAL
(M.Phil. in Chemistry)



Under the Supervision of
Dr. Pranab Ghosh
Associate Professor in Chemistry

Department of Chemistry
University of North Bengal
Darjeeling 734 013
India

JULY 2012

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Dedicated to my Parents

Declaration

The research work embodied in this thesis entitled **“Transformative Reactions of Terpenoids and Studies on their Biological Activity”** has been carried out in the Department of Chemistry, University of North Bengal, Raja-Rammohunpur, Darjeeling, India-734 013, under the guidance and supervision of Dr. Pranab Ghosh, Associate Professor in Chemistry, Department of Chemistry, University of North Bengal. To my belief this thesis or any part of it has not been submitted before at any University or Institution for Ph.D. or any other Degree or Diploma.

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Place: N.B.U

Amitava Mandal

(AMITAVA MANDAL)

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Finally, I would like to express my thanks to UGC, New Delhi, India, for awarding me Junior and Senior Research Fellowship (Meritorious) and University of North Bengal for providing the infrastructural facilities.

Abbreviations

Entry	Expanded form	Abbreviations
1	Dimethyl sulfoxide	DMSO
2	Dimethyl formamide	DMF
3	Tertiary butanol	t-BuOH
4	Potassium tertiary butoxide	t-BuOK
5	Lead tetra acetate	LTA
6	Ethyl acetate	EtOAc
7	n-Butanol	n-BuOH
8	Methanol	MeOH
9	N-Bromosuccinimide	NBS
10	Nuclear magnetic resonance	NMR
11	Infrared	IR
12	Ultra violet	UV
13	Melting point	mp
14	Distortion less enhancement by polarization transfer	DEPT
15	Correlation spectroscopy	COSY
16	Nuclear overhauser effect spectroscopy	NOESY
17	Heteronuclear single-quantum spectrum	HSQC
18	Heteronuclear multiple-bond correlation spectroscopy	HMBC
19	Microwave	MW
20	Minimum inhibitory concentration	MIC
21	Specific rotation at sodium-D light	$[\alpha]_D$
22	Dynamic light scattering	DLS
23	Sodium dodecylsulfate	SDS
24	Critical micelle concentration	CMC
25	Electron spray ionization mass spectrometry	ESIMS
26	Time-of-flight mass spectrometry	TOFMS
27	Proton chemical shift	δ_H
28	Carbon chemical shift	δ_C
29	Two dimensional nuclear magnetic resonance spectroscopy	2D NMR
30	Tetra methyl silane	TMS
31	Fast atom bombardment mass spectrometry	FABMS
32	Fourier transform	FT
33	Thin layer chromatography	tlc
34	Nicotinamide adenine dinucleotide phosphate	NADP
35	Nicotinamide adenine dinucleotide phosphate hydride	NADPH
36	Microbial type culture collection	MTCC
37	petroleum ether	PE
38	Dichloromethane	DCM
39	Ethylenediamine	EDA

Chapter II describes the detail experimental procedures and a collection of important references used during the study.

Part II is divided into four chapters. Chapter I comprise a short review on pyrazine derivatives. Chapter II is divided in two sections. Section A is related to the development of a clean protocol for the synthesis of pyrazine derivatives of triterpenoids, the total optimization of the reaction condition and the details about the structure elucidation of the synthesized pyrazine derivatives.

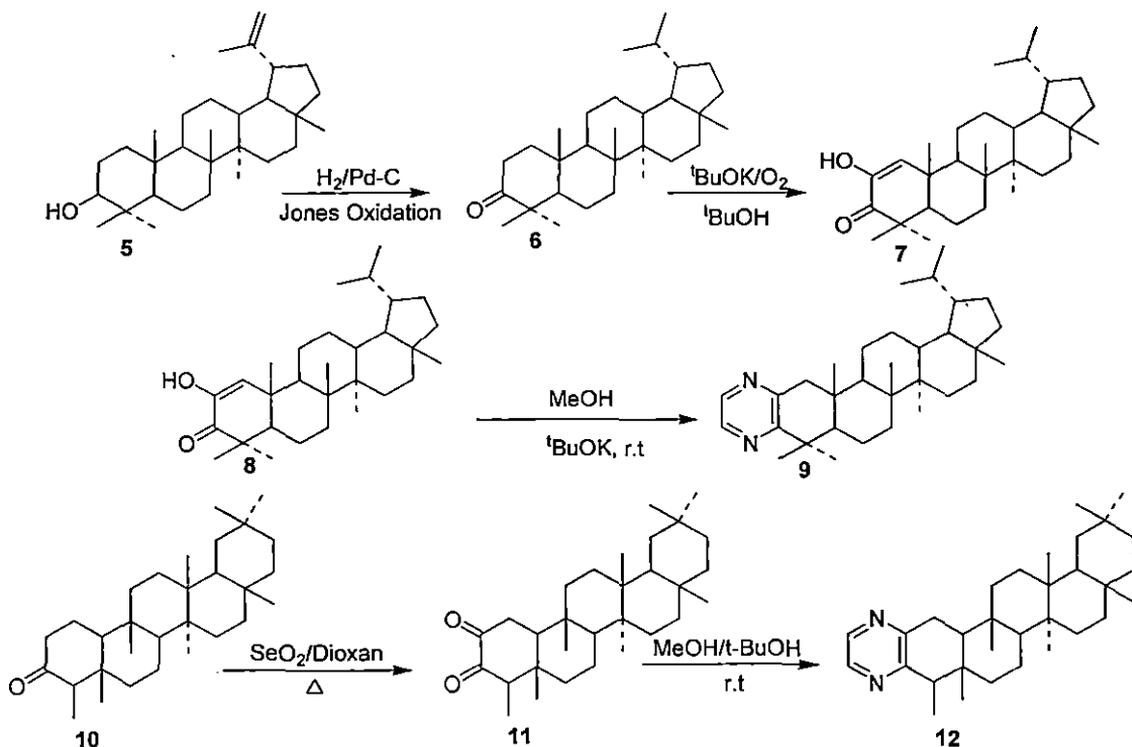


Figure 2 Synthesis of pyrazine derivatives of triterpenoids

Section B of this chapter describes the biological activity of the synthesized compounds. It represents the results of the antifungal activity and the antitopoisomerase activity with a through prediction of binding efficiency to the enzyme molecule by 3D docking studies. Chapter III is further classified into two different sections. Section A relates to the synthesis of benzopyrazine or quinoxaline derivative of pentacyclic triterenoid, friedelin by a novel protocol, standardization of the reaction condition, scope and application of the present protocol and a proposed mechanism of the developed method. Section B is related to the biological work. But because of the insolubility of the synthesized

Entry	Expanded form	Abbreviations
40	Tetranitro methane	TNM
41	Deoxyrib nucleic acid	DNA
42	Protein data bank	PDB
43	Topoisomerase II α	TOPO-II α
44	Trimethyl silyl chloride	TMSCI
45	Tetra-n-butylammonium bromide	TBAB
46	Cetyl trimethyl ammoniumbromide	CTAB
47	Cetyl pyridiniumchloride	CPC
48	Sodium dodecylbenzenesulfonate	SDBS
49	Tetra-n-butylammoniumiodide	TBAI
50	Polydispersity index	PDI
51	Ethyl acetate	EA
52	Ethylenediamine tetraacetic acid	EDTA
53	Natural product	NP
54	Human immunodeficiency virus	HIV
55	Optical rotatory dispersion	ORD
56	Lithium aluminium hydride	LAH
57	2,3-Dichloro-5,6-dicyano quinone	DDQ
58	N-methyl morpholine-N-oxide	NMMO
59	Metachloro perbenzoic acid	MCPBA
60	Central nervous system	CNS
61	Tetrahydro furan	THF

SUMMARY

The research work embodied in this thesis entitled “**Transformative Reactions of Terpenoids and Studies on their Biological Activity**” was carried out in the Department of Chemistry, University of North Bengal, Darjeeling – 734 013, under the guidance and supervision of Dr. P. Ghosh, Department of Chemistry, University of North Bengal. The studies described in this thesis comprises the phytochemical investigation of *Croton bonplandianum*, development of new methodology using greener and cleaner technology to synthesize some suitable derivatives of pentacyclic triterpenoids and investigation of their manifold applications in biology. The thesis has been divided into four parts.

Part I is divided into two chapters, chapter I and chapter II. Chapter I is divided into three sections. Section A comprises the main morphological feature and classification of the genera croton. Section B describes the detail phytochemical study of methanolic extract of root of *C. bonplandianum* Bail, isolation of various active components *etc.* The study reports the isolation and structure elucidation (IR, ^1H NMR, ^{13}C NMR, 2D NMR, MS *etc.*) of a new ursane type triterpenoid *viz.* 3 α -hydroxy-urs-12,15-dien (1), along with two known triterpenoids, oleanolic acid (2) and ursolic acid (3) and most abundant β -sitosterol (4).

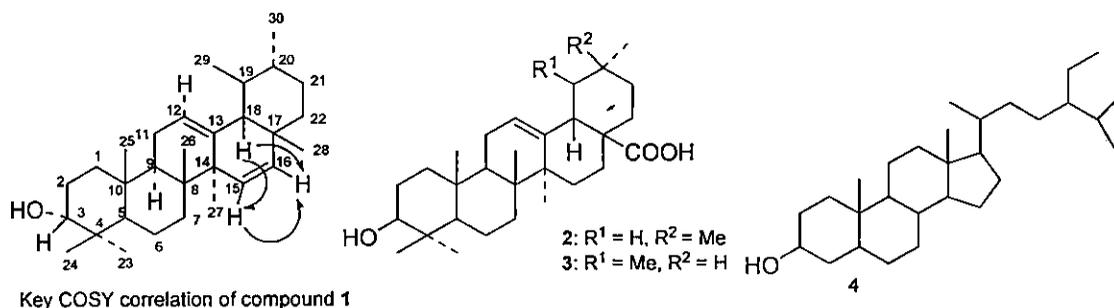


Figure 1 Isolated compounds from *C. bonplandianum* Bail.

Section C describes the results of the present investigation towards the antimicrobial activity of the isolated compounds against a series of fungal and bacterial pathogens. All the isolated compounds showed good antimicrobial activity and the determined MIC values are tabulated in table 3 and 4 respectively.

Chapter II describes the detail experimental procedures and a collection of important references used during the study.

Part II is divided into four chapters. Chapter I comprise a short review on pyrazine derivatives. Chapter II is divided in two sections. Section A is related to the development of a clean protocol for the synthesis of pyrazine derivatives of triterpenoids, the total optimization of the reaction condition and the details about the structure elucidation of the synthesized pyrazine derivatives.

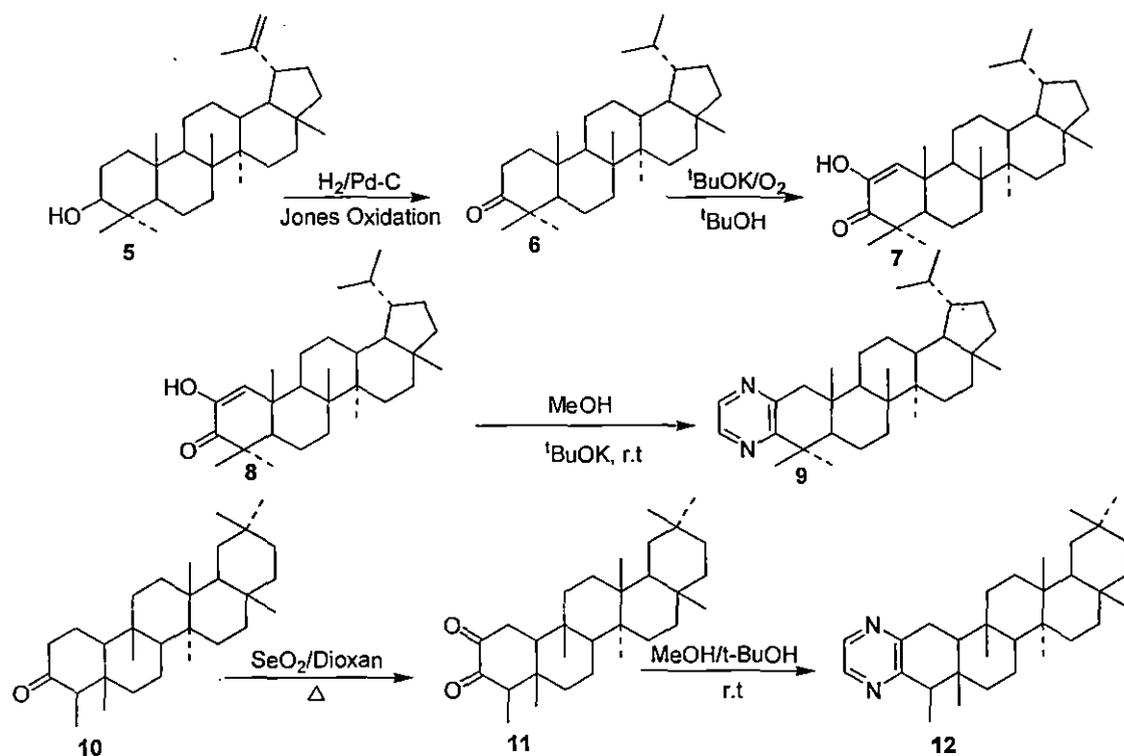


Figure 2 Synthesis of pyrazine derivatives of triterpenoids

Section B of this chapter describes the biological activity of the synthesized compounds. It represents the results of the antifungal activity and the antitopoisomerase activity with a through prediction of binding efficiency to the enzyme molecule by 3D docking studies. Chapter III is further classified into two different sections. Section A relates to the synthesis of benzopyrazine or quinoxaline derivative of pentacyclic triterenoid, friedelin by a novel protocol, standardization of the reaction condition, scope and application of the present protocol and a proposed mechanism of the developed method. Section B is related to the biological work. But because of the insolubility of the synthesized

quinoxaline derivative of friedelin in DMSO, the author was unable to carry out any biological work.

Chapter IV describes the detail experimental procedures and a list of references used during the study.

Part III of the present thesis is divided into three chapters. Chapter I comprises a short review of isolation and biological activity of triterpenoids having lupane skeleton.

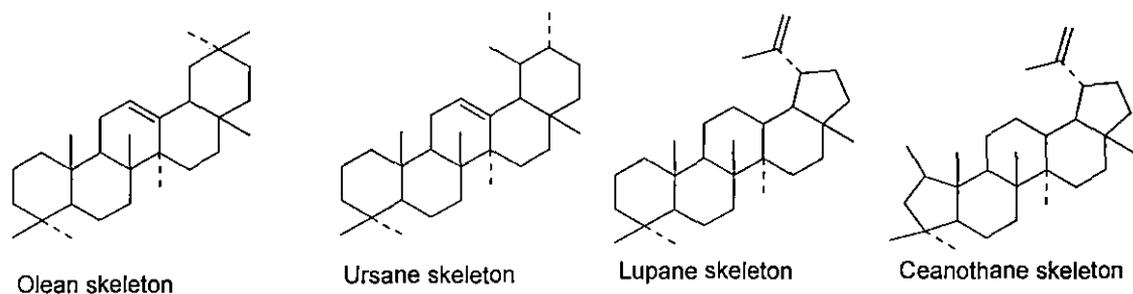
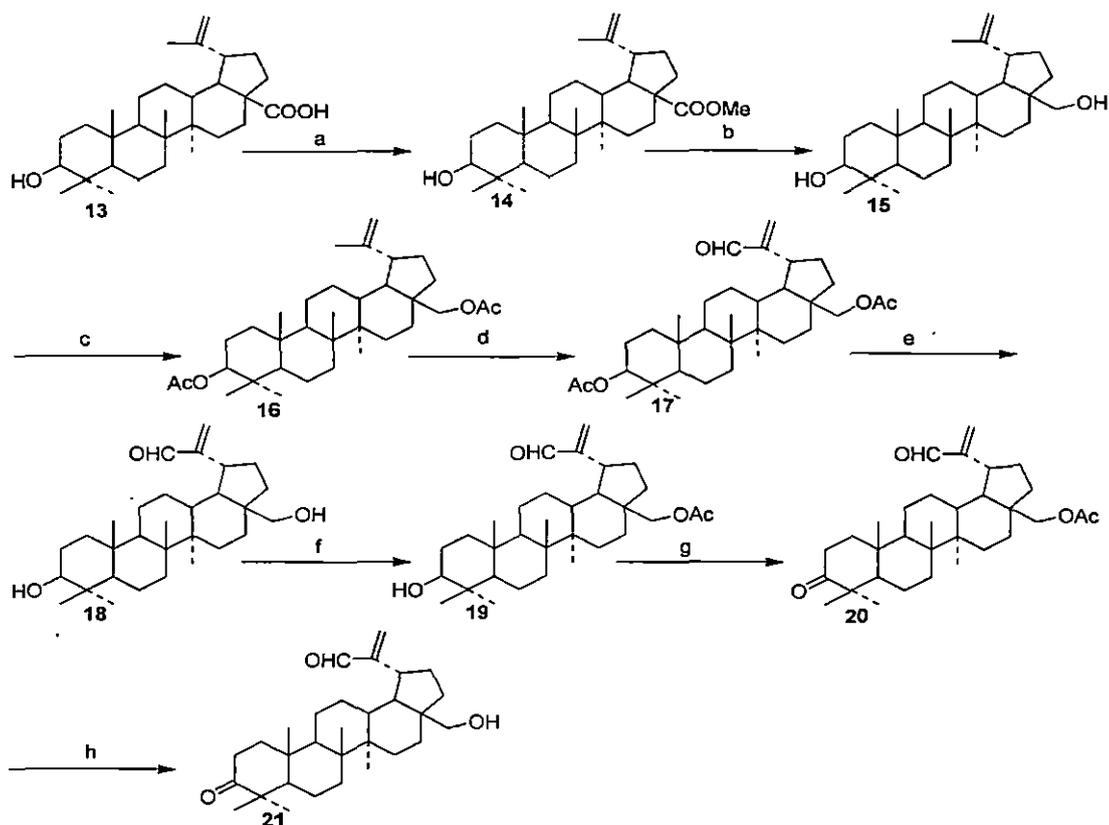


Figure 3 Structures of some important triterpene skeleton

Chapter II, Section A describes an efficient partial synthesis of 28-hydroxy-3-oxolup-20(29)-en-30-al from Betulinic acid. The structures of all the intermediates were elucidated by both chemical and spectral means.



Scheme 1. Partial synthesis of compound 21 from betulinic acid (13): Reagents and conditions: a, CH_2N_2 , ether, over night, AcOH (gal.), Na_2SO_4 ; b, LiAlH_4 , dry THF, 2 hrs., saturated Na_2SO_4 solution, ether, Na_2SO_4 ; c, $\text{C}_5\text{H}_5\text{N}$, Ac_2O , 6 hrs., (100°C) ice cold H_2O , ether, Na_2SO_4 ; d, SeO_2 , aq. dioxan, 2 hrs., ice cold H_2O , ether, Na_2SO_4 ; e, 10% alcoholic KOH, THF, 4 hrs., ice cold H_2O , ether, Na_2SO_4 ; f, $\text{C}_5\text{H}_5\text{N}$, Ac_2O , (5-10°C), 8 hrs., ice cold H_2O , Na_2SO_4 ; g, $\text{C}_5\text{H}_5\text{N}$, dry CrO_3 , overnight, ice cold H_2O , CH_2Cl_2 , MgSO_4 ; h, 10% alcoholic KOH, THF, 4 hrs., ice cold H_2O , ether, Na_2SO_4 .

Section B of this chapter describes the antileukemic activity of the compounds against three different cell lines *viz.* human K562 leukemia, murine WEHI3 leukemia and murine MEL erythroid progenitor.

Chapter III describes the detail experimental procedures and a collection of references used during the study.

Part IV is divided in three chapters. Chapter I deals with a short review of the transformative reactions of friedelan triterpenoids. Chapter II is further divided into two different sections, section A and section B. Section A describes the detail oxidative transformative reactions on friedelin and cerin, isolation and structure elucidation of the products.

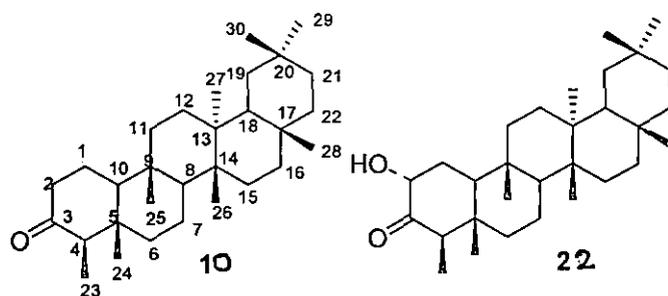


Figure 4 Chemical structures of triterpenoids from *Q. suber*

Section B of this chapter is related to the 3D molecular docking and the antitopoisomerase activity of all the compounds including the parent compounds isolated from natural sources. Both the theoretical docking results and the experimentally determined topoisomerase II α inhibitory activities are represented.

Chapter III describes the detail experimental procedures and a collection of references used during the study.

List of Publications and Poster Presentations

List of Publications:

1. **Amitava Mandal**, Shilpi Ghosh, Ashim Kumar Bothra, Ashis Kumar Nanda, Pranab Ghosh, Synthesis of friedelan triterpenoid analogs with DNA topoisomerase II α inhibitory activity and their molecular docking studies., *Eur. J. Med. Chem.*, 2012, 1-7, doi:10.1016/j.ejmech.2012.04.037.
2. Pranab Ghosh, **Amitava Mandal**, Joydip Ghosh, Chiranjib Pal, Ashis Kumar Nanda, Synthesis of bioactive 28-hydroxy-3-oxolup-20(29)-en-30-al with antileukemic activity, *J. Asian Nat. Prod. Res.*, 2012, 14, 141-153.
3. Pranab Ghosh and **Amitava Mandal**, Greener approach towards pyrazine synthesis, *Green Chemistry Letters and Reviews*, 2012, 5, 127-134.
4. Pranab Ghosh and **Amitava Mandal**, Sodium dodecyl sulfate in Water: Greener Approach for the Synthesis of Quinoxaline Derivatives, Communicated to *Green Chemistry Letters and Reviews*, MS ID-GCL-2011-133 (Accepted, Article in press).
5. Pranab Ghosh, **Amitava Mandal** and Md. Golam Rasul, A new bioactive ursane type triterpenoid from *Croton bonplandianum* Bail, Communicated to *Journal of Chemical Sciences*, MS ID-JCSC-D-2012-00067.

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1. Sixth CRSI (Kolkata) Chapter Symposium-University of North Bengal, 2008.
2. National Symposium on Diversity and Functionality of Plants and Microbes- University of North Bengal, 2008.
3. 3rd Mid Year Symposium of the CRSI-NIPER-2008.

4. Trends in Surface Science and Related Areas, University of North Bengal, 2008.
5. 11th CRSI National Symposium in Chemistry-National Chemical Laboratory, Pune, 2009.
6. 12th CRSI National Symposium in Chemistry & 4th CRSI-RSC Symposium in Chemistry, ICT, Hyderabad, 2010.
7. 13th CRSI National Symposium in Chemistry & 5th CRSI-RSC Symposium in Chemistry, NISER, Bhubaneswar, 2011.
8. National Seminar on Frontiers in Chemistry, 2011 & Celebration of the International Year of Chemistry 2011, University of North Bengal, 2011.
9. Regional Chemistry Seminar 2011, Biratnagar, Tribhuban University, Nepal (**First place in poster presentation**).
10. Microtrends 2012, University of North Bengal, Darjeeling, India, 2012.

Workshop or professional courses participated:

11. UGC Sponsored Research Scholars' Training programme 30.06.2011 to 01.07.2011 at University of North Bengal.
12. Workshop on Bioinformatics, September 2011 at University of North Bengal.

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Part I

**Phytochemical Investigation of the root of *Croton*
bonplandianum Bail**

Chapter I

Section A

1. Morphological feature of croton

Croton belongs to the family “**Euphorbiaceae**”. Euphorbiaceae, the spurge family consisting of about 322 genera and about 8910 species,¹ are predominantly cosmopolitan with the representation in the humid tropical and subtropical regions of both hemispheres. It is the sixth largest family in the world and occupies the seventh position in the Indian flora. Although most of the members of the family are readily discernible in the field, by the unisexual and mostly apetalous flowers, floral glands, the tricerpillary syncarpous pistil, and schizocarpic capsular fruits with three cocci and persistent columella or rarely with drupaceous fruits, the interrelationships among the genera are not sufficiently understood. The family is largely complex and forms a heterogenous assemblage of diverse growth forms and morphological features. It also includes many economically importance species. In spite of its many medicinal and economical importance very little attention about the detail phytochemistry of these species has been paid.

Croton bonplandianum is an evergreen or deciduous trees, shrubs, climbers or subshrubs, indumentum satellite. Leaves are alternate, opposite or whorled near branch ends, pinnately veinea or palmately veined, margins often serrate with two stalked or sessile glands at the top of the petiole and sometimes marginal glands, stipules minute or absent. There are two different types of flowers. Monoecious, flowers are solitary or clustered in terminal or auxiliary, simple or branched racemes. Male flowers have calyx 5-lobed, petals 5-6, stameas 10-12, free, receptacle hairy and disc-glands and free. Female flowers are sepals as in males, petals minute or absent, ovary 3-celied, styles long, bifid almost to the base. Fruits are 3-lobed and 3-seeded capsules. Seeds oblong, ellipsoid or often ovoid or squarish, usually 3-angled with a broad convex back, smooth, rarely sparsely stellate-pubescent; testa dry, more or less thin, endosperm coious, embryo straight, cotyledons broad²

It occurs in tropics and subtropics of Old and New World. The majority of the species are from South America and West Indies. Approximately 800 different species of are in the world. About 150 species are in Asia occurring in South China, Indo-China, South Asia and Malesia. In India about 16 different species are found.

2. Classification

The genera croton is further classified into several species:

1. *Croton roxburghii* Balakrishnan
2. *Croton joufra* Roxb
3. *Croton himalaicus* Long
4. *Croton tiglium* Linn.
5. *Croton caudatus* Geiseler and
6. *Croton bonplandianum* Baillon



Figure 1 Different species of croton

Croton bonplandianum Baillon is native to South America and has been introduced to India in late 1890s.

Species of croton contain a great diversity of chemical compounds, viz. several kinds of alkaloids, saponins, tannins and flavonoids. Various species exhibit antimicrobial, antomalarial, antiatherogenic, androgenic, insecticidal and antitumor activities. There is a great scope for further studies on the phytochemistry in this genus.



Figure 2 Identified species of *Croton bonplandianum* Bail

The present author has submitted two specimens of *Croton bonplandianum* with the tag numbers of R-1706, R-1707 to Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal, Darjeeling, India. Accession numbers of the submitted specimens were 9629 and 9630 and were stored in NBU Herbarium, Department of Botany, University of North Bengal, Darjeeling, India.

Section B

Phytochemical study of the methanolic extract of root of *Croton bonplandianum* Bail.

1. Introduction

Croton bonplandianum Bail belongs to the family of Euphorbiaceae, is native to southern Bolivia, Paraguay, southwestern Brazil and northern Argentina and was reported from India during late 1890 by Kaul.³ It now occurs widely along roadsides, railway abandoned field in wide open ravines, and paddy or sugarcane fields and on sandy or sandy clay soils. Due to the resemblance of the leaves and flower cymes to that of Tulsi, this plant is often called Ban Tulsi locally. *C. bonplandianum* was reported to have many medicinal uses including the repellent property against the insects,³⁻⁷ mosquito, *A. aegypti*.⁸ Local people in the remote area of West Bengal, India are using its root against snake bite and the leaf extract against high fever. In addition the plant is used both as a fuel and as detergent. The methanol extract of *C. bonplandianum* has been found to exhibit antitumor properties using *Agrobacterium tumefaciens* and has larvicidal activities.

Phytochemically Croton is rich in secondary metabolites including alkaloids and terpenoids.^{9,10} Diterpene resins found in many species of croton have been used experimentally in the studies of tumor initiation and conceivably prove to be useful in cancer therapy.¹¹ Apart from the above limited reports, no systematic study has yet been initiated for the phytochemical investigation of *C. bonplandianum* Bail specially that with the root of this plant species.

2. Results and Discussion

During our search towards bioactive natural products in tropical plants,¹² the author have investigated the chemical constituents of the MeOH extract of roots of *C. bonplandianum*, which showed potent antifungal activity against a series of fungal and bacterial pathogens. The investigation yielded the isolation of a new triterpene 3 α -hydroxy-urs-12,15-dien (1), along with two known triterpenoids, oleanolic acid (2) and ursolic acid (3) and most abundant β -sitosterol (4). All the compounds (1, 2 and 3) have been screened for their antimicrobial activity.

The MeOH extract of the roots of *C. bonplandianum* was suspended in water, partitioned successively between hexane, EtOAc and n-BuOH to get four fractions (hexane phase, ethylacetate phase, n-butanol phase and water phase). Since results of preliminary screening with the EtOAc soluble fraction was found to be most active against the fungal strain used, it was purified (through repeated column chromatography over silica gel) to isolate the active ingredients *viz.* 3 α -hydroxy-urs-12,15-dien (**1**), along with two known triterpenoids, oleanolic acid (**2**) and ursolic acid (**3**) and most abundant β -sitosterol (**4**). The structures of all the compounds were elucidated on the basis of chemical and spectroscopic analysis and by comparison (for compounds **2**, **3** and **4**) with the spectral data of already reported compounds.

The elucidation of the structure of the new compound was accomplished by extensive analysis of its spectral data. Compound **1** was obtained as a white powder of melting point (mp) 244-246 °C, $[\alpha]_D + 4.0$, UV inactive, showed strong absorption band at 3422 (-OH), 2850, 1461, 1376 (gem dimethyl), 1053, 970, 959, 838 cm^{-1} in the IR spectrum (Figure 3) indicative of the presence of hydroxyl, olefinic and gem dimethyl groups.

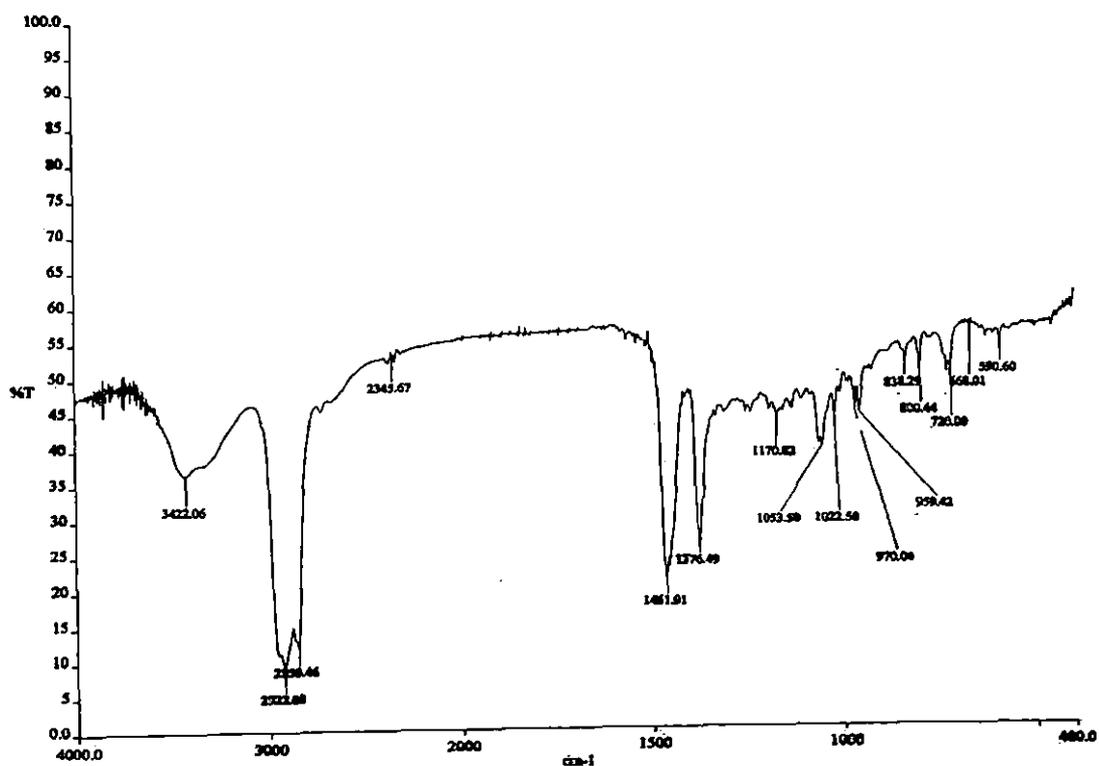


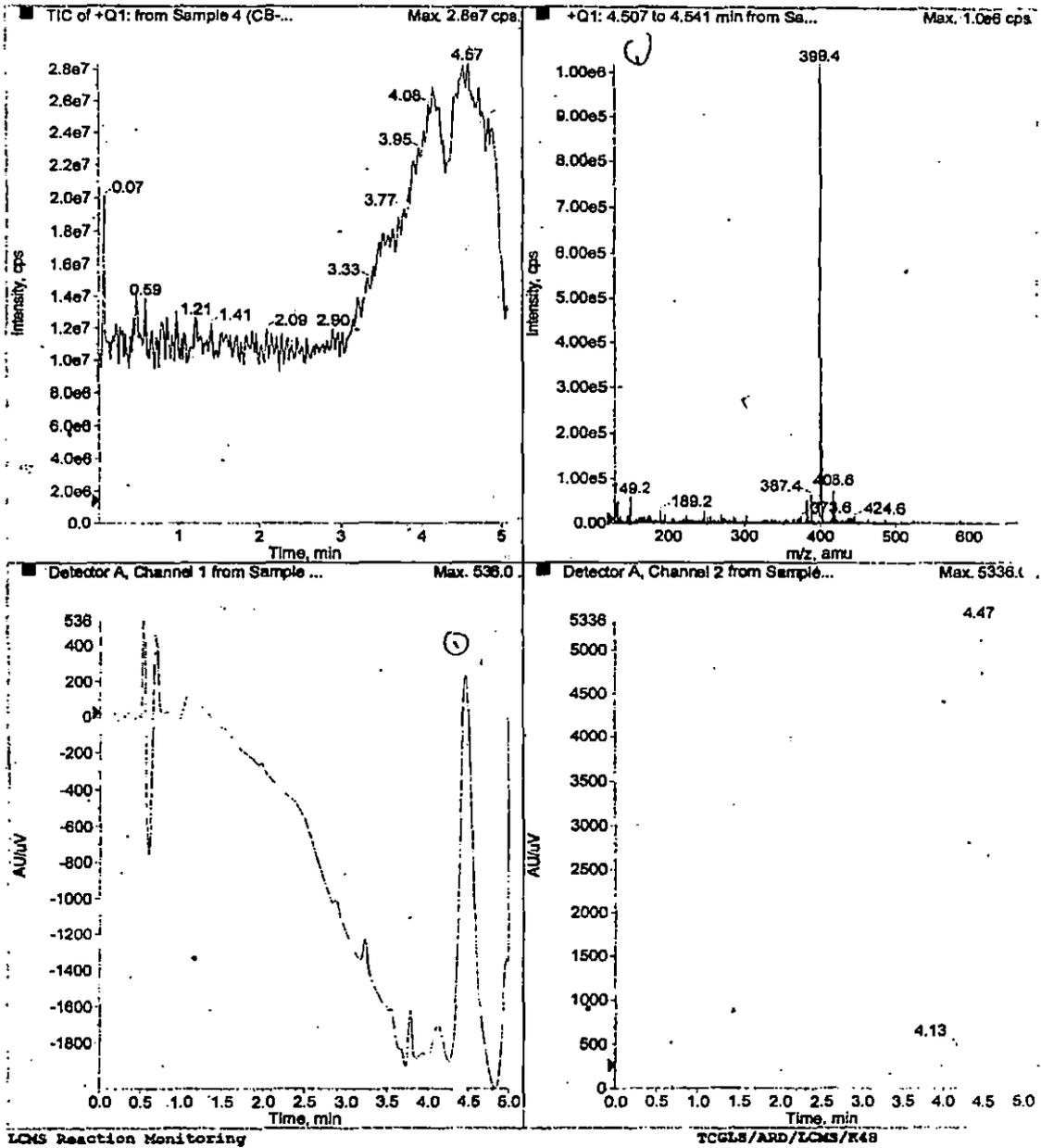
Figure 3 IR spectrum of the new compound, **1** isolated from *C. bonplandianum* Bail.

In the mass spectrum (Figure 4) it showed a molecular ion peak at m/z 425 ($m+1$). The ^1H NMR spectra of compound **1** (Figure 5, 6, 9, 10 and 11) showed characteristic proton signals due to six tertiary methyl groups at δ_{H} (proton chemical shift) 0.68 (3H, s), 0.69 (3H, s), 0.76 (3H, s), 0.84 (3H, s), 1.01 (3H, s), 1.25 (3H, s), and two secondary methyl signals as doublet centered at 0.79 (3H, d, $J = 6.3$ Hz) and 0.92 (3H, d, $J = 6.3$ Hz). ^{13}C NMR spectrum (Figure 7) at ambient temperature displayed signals for thirty carbons which were distinguished as eight methyls, seven methylenes, nine methines (five aliphatic, one oxygenated and three olefinic) and six quaternary with the aid of DEPT experiment (Figure 8). The assignment of the signals of the methyl groups and the remaining ^1H and ^{13}C signals were performed through analysis of the HSQC, HMBC and COSY experiments and the results were found consistent with pentacyclic triterpene skeleton and its molecular formula was suggested to be $\text{C}_{30}\text{H}_{48}\text{O}$ on the basis of ESIMS data [$m/z = 424$ (M^+)]. On acetylation it gave crystalline acetate of m.p. 223-224 °C. The molecular formula of this acetate was suggested to be $\text{C}_{32}\text{H}_{50}\text{O}_2$ [ESIMS data $m/z = 467$ ($M+1$)].

Sample Name: CB-R

Acq. Time: 17:08

Acq. Date: Friday, June 05, 2009



LCMS Reaction Monitoring

TCGLS/ARD/LCMS/R48

Channel 1 at wavelength 220 nm, *TFA:ACN
Channel 2 at wavelength 260 nm

Analysed by

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Figure 4a ESIMS spectrum of compound 1 isolated from *C. bonplandianum* Bail

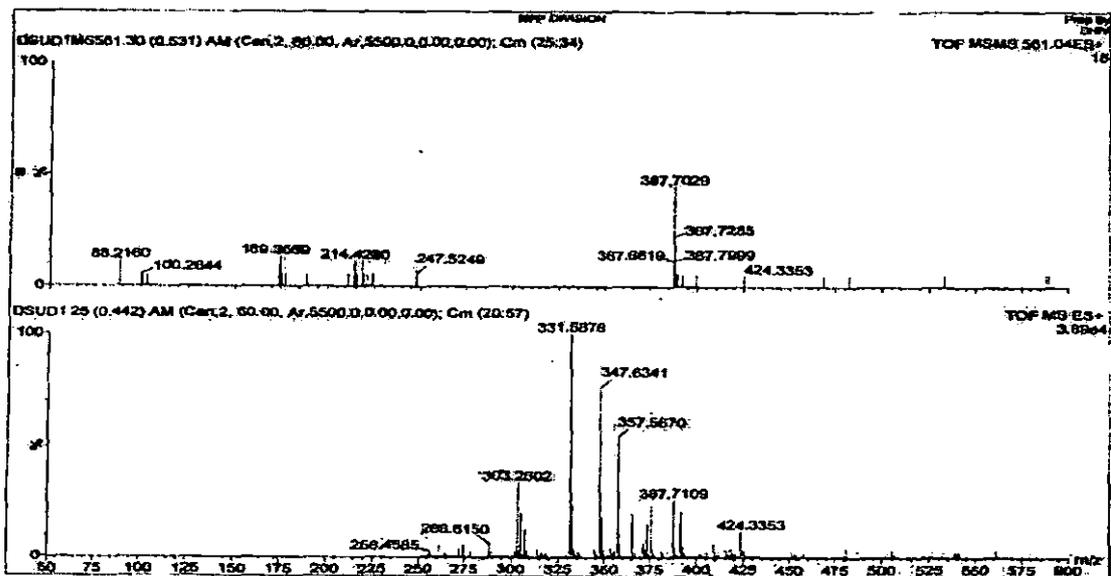


Figure 4b TOFMS spectrum of compound 1 isolated from *C. bonplandianum* Bail

The difference in mass spectral data of 1 and its acetate indicated the formation of a monoacetate and hence indicated the presence of only one hydroxyl group in compound 1. IR spectrum of the monoacetate showed peaks at 2850, 1461, 1376 (gem dimethyl), 1248 (-OCOMe), 1053, 959, 970, 838 cm^{-1} . The appearance of a sharp peak at 1376 cm^{-1} in the IR spectra of compound 1 and its monoacetate revealed the presence of a gem dimethyl group in ring A of the pentacyclic triterpenoid skeleton and the concordance of δ_{H} values in the ^1H NMR spectrum for the ring A with that of olean or ursane skeleton clearly rejected the possibility of the presence of friedelan skeleton. Mass fragmentation of the compound also followed the same pattern as was observed for typical pentacyclic triterenoids.¹³ A possible mass fragmentation pattern is depicted in scheme 1.

The comparison of the chemical shift positions (δ_{H}) of the A, B and C rings of 1, especially the resonances of the methyl groups and two secondary methyl signals on ring E provides the most useful indicator for the presence of ursane type triterpenoid skeleton.¹⁴ A hydroxy methine signal at δ_{H} 3.5 (1H, m, H-3) and a trisubstituted olefinic signal at δ_{H} 5.33 (1H, d, $J = 2.7$ Hz, H-12). However the splitting nature and small J value may be considered by the consideration of long range coupling involving H-12 and H-19,

both are closer in space. The through space distance is obviously more important than the number of intervening bonds, suggesting that **1** is a 3 α -hydroxy-urs-1,2-en type triterpenoid without possessing angular carboxyl group at C-28.¹² Additionally **1** showed an AB quartet (for >CH=CH<) centered at δ_H 5.11 (2H, $J = 15.1$ Hz and 8.5 Hz) signifying the presence of another olefinic double bond having two olefinic protons that must be attributed to the vinylic protons of a disubstituted double bond in a six membered ring.^{15, 16}

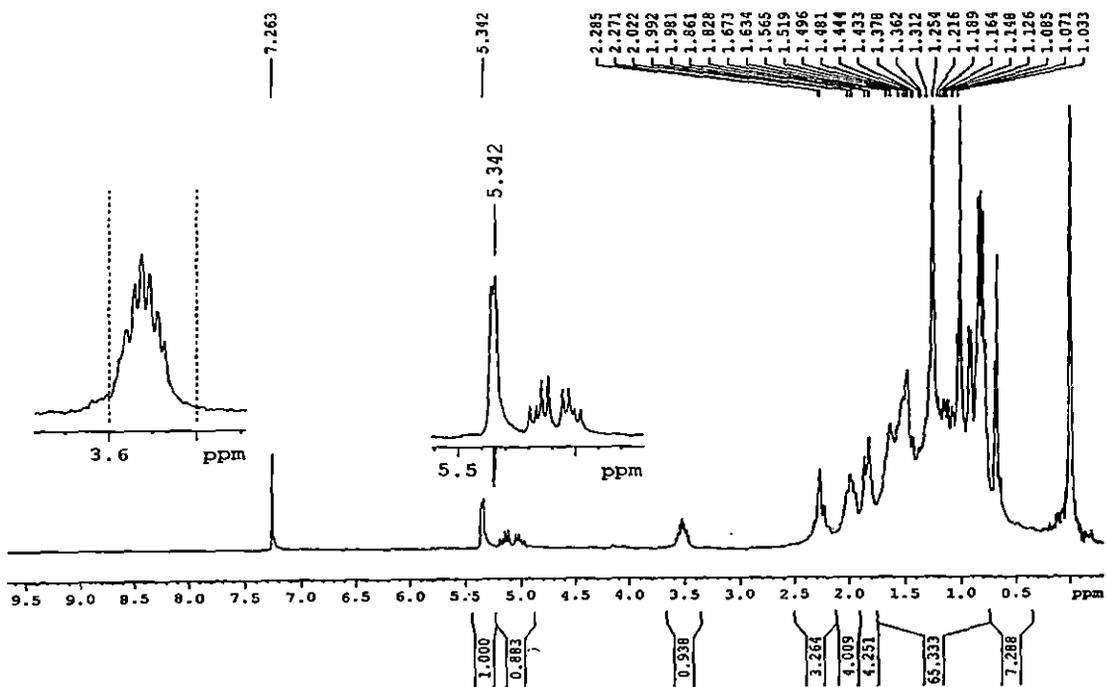


Figure 5 ¹H NMR spectrum of the new compound, **1** isolated from *C. bonplandianum* Bail.

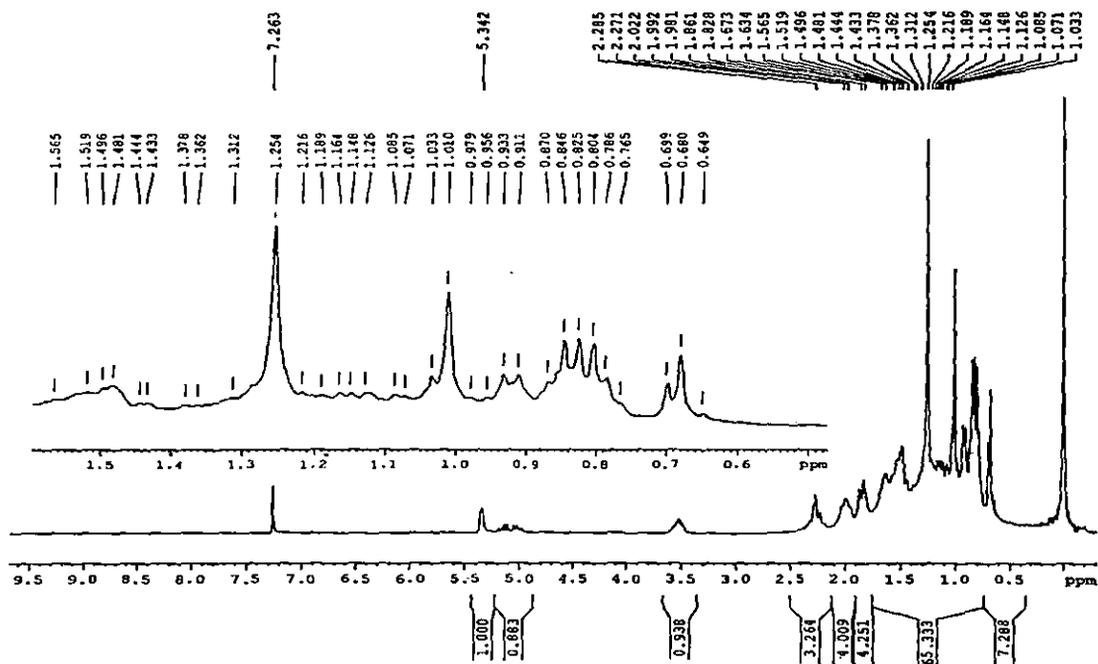


Figure 6 Expanded ^1H NMR spectrum of the new compound, **1** isolated from *C. bonplandianum* Bail.

^{13}C NMR spectrum showed all the 30 carbons and thereby confirmed the presence of a triterpene skeleton (Table 1). Four downfield signals at δ_{C} 121.7, 140.7, 129.2 and 138.3 indicated the presence of four sp^2 hybridized carbons. The signals at δ_{C} 121.7 and 140.7 are characteristic for a C-12/C-13' double bond in the ursane type structure.¹⁵ The signal at δ_{C} 140.7 is due to the presence of a trisubstituted olefinic carbon at C-13 and peaks at δ_{C} 138.3 and 129.2 were indicative of the presence of another double bond having two disubstituted olefinic carbon. The ^{13}C values for these two carbons (δ_{C} 138.3 and 129.2) clearly removed the possibility of any trisubstituted double bond. C-3 carbon appeared at δ_{C} 71.8 whereas for compounds **2** and **3** it appeared at δ_{C} 78.2, i.e. a shielding of seven units that signifying a stereochemical change at C-3. Therefore, for compound **1** the hydroxyl group is alpha instead of beta, i.e. it is an 3-epi-urs-1,2-en type triterpenoid. On the other hand, the chemical shift of C-18 (δ_{C} 55.9) in compound **1** was very close to that in compound **3** (δ_{C} 53.6), which indicated that these two compounds have the same configuration at C-18. Eight methyl signals appeared at δ_{C} 21.1 (C-23), 29.1 (C-24), 18.7 (C-25), 19.8 (C-26), 24.3 (C-27), 19.4 (C-28), 19.0 (C-29) and 23.0 (C-30). Assignments

of all the carbons were made by comparison with that reported in literature for ursane skeleton¹⁷ and are tabulated in table 1. Further confirmation about the structure of compound **1** was done by the proposed mass fragmentation pattern (Figure 13) of the compound.

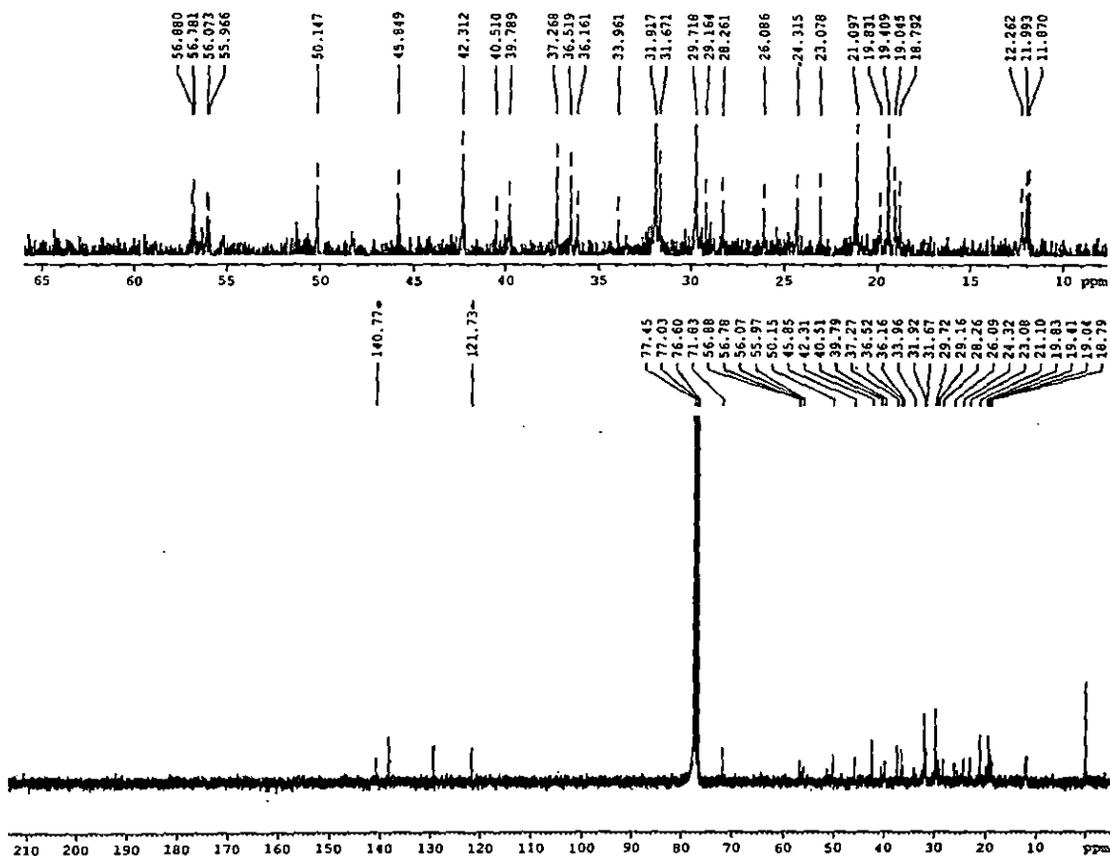


Figure 7 ¹³C NMR spectrum of the new compound, **1** isolated from *C. bonplandianum* Bail.

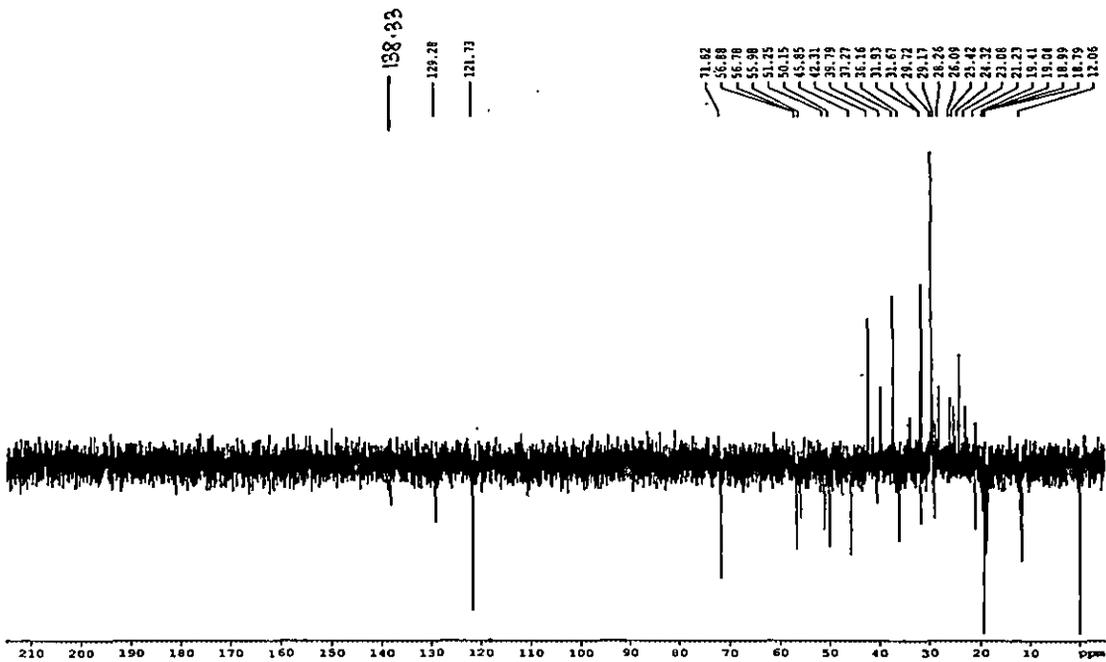


Figure 8 DEPT spectrum of the new compound, 1 isolated from *C. bonplandianum* Bail.

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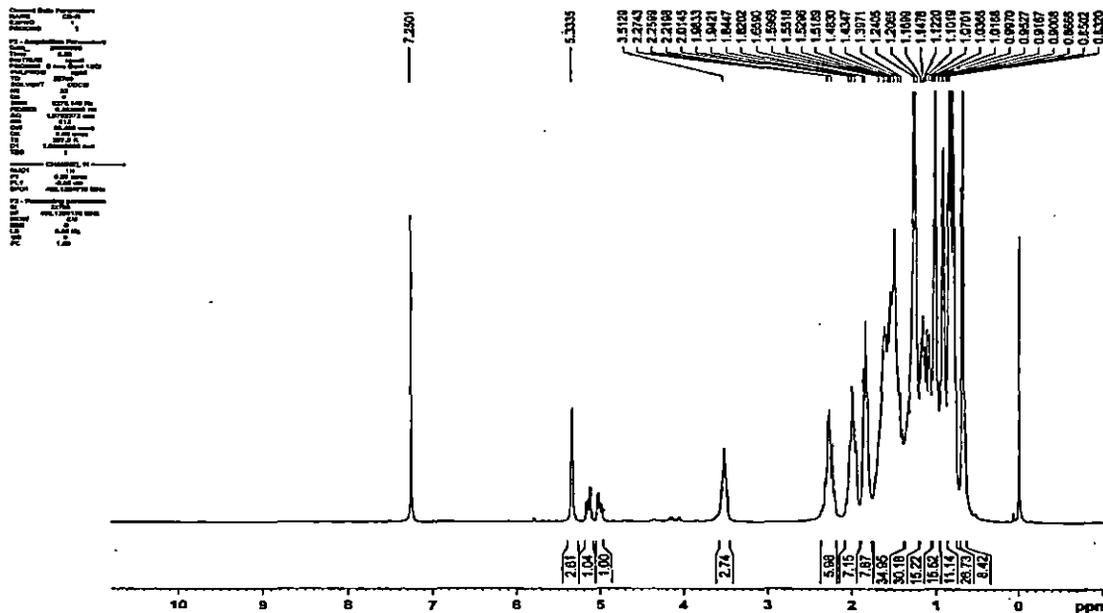


Figure 9 ¹H NMR spectrum of the new compound, 1 isolated from *C. bonplandianum* Bail at 400 MHz.

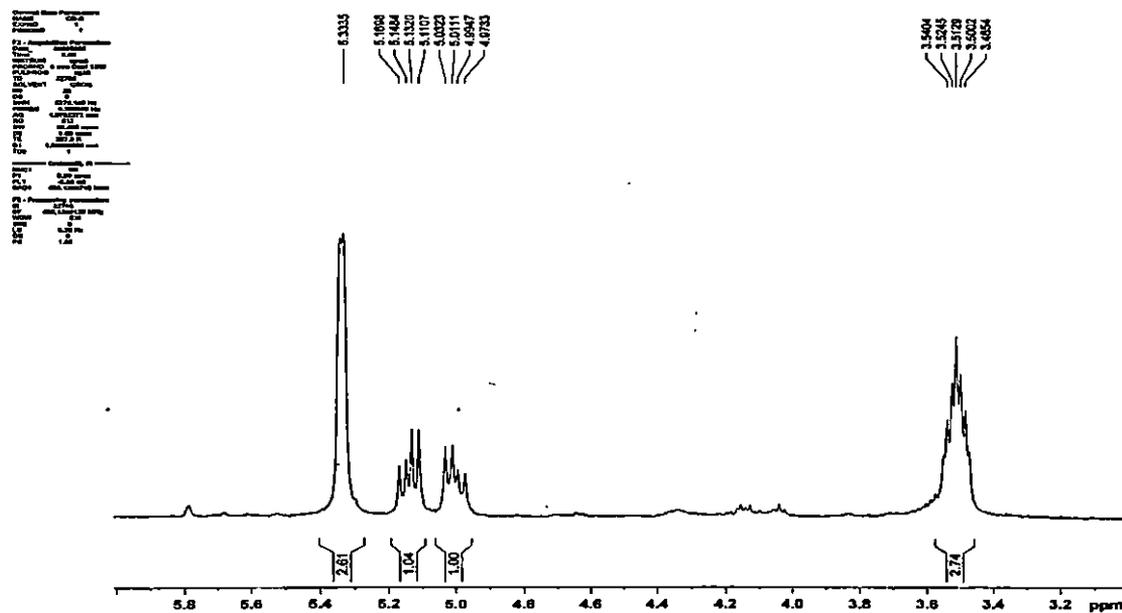


Figure 10 Expanded ^1H NMR spectrum of the new compound, **1** isolated from *C. bonplandianum* Bail at 400 MHz.

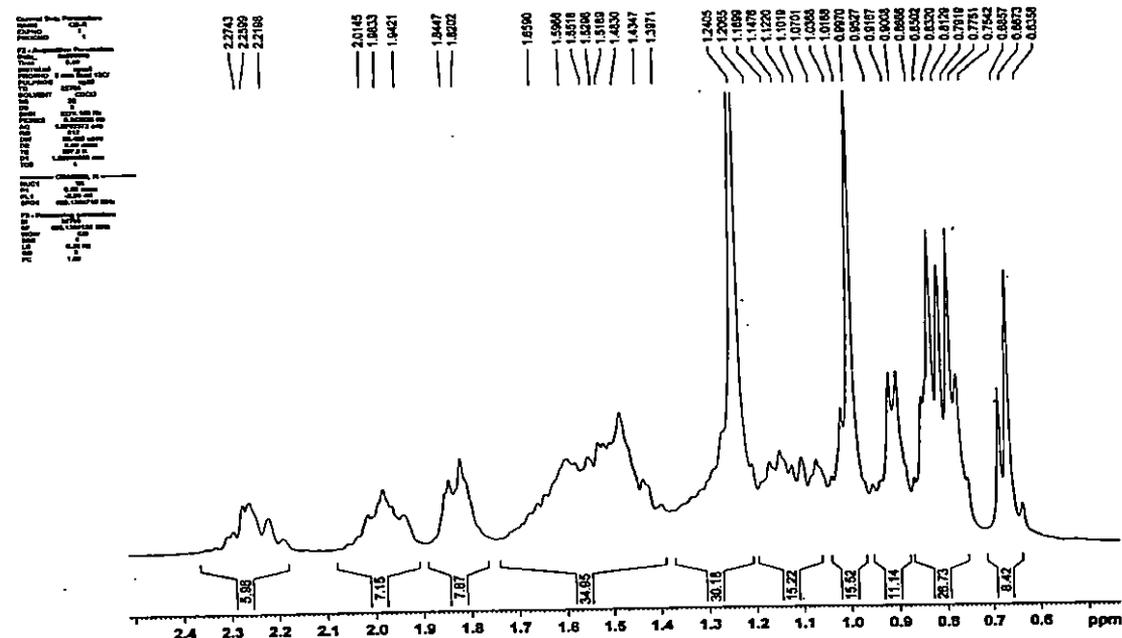


Figure 11 Expanded ^1H NMR spectrum of the new compound, **1** isolated from *C. bonplandianum* Bail at 400 MHz.

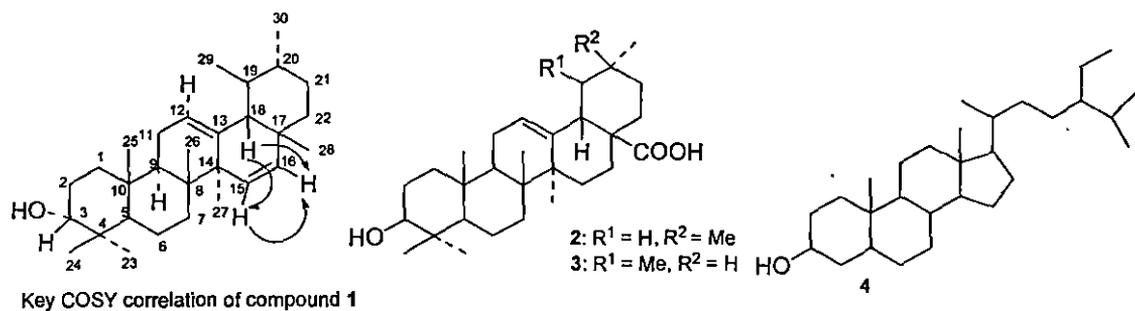
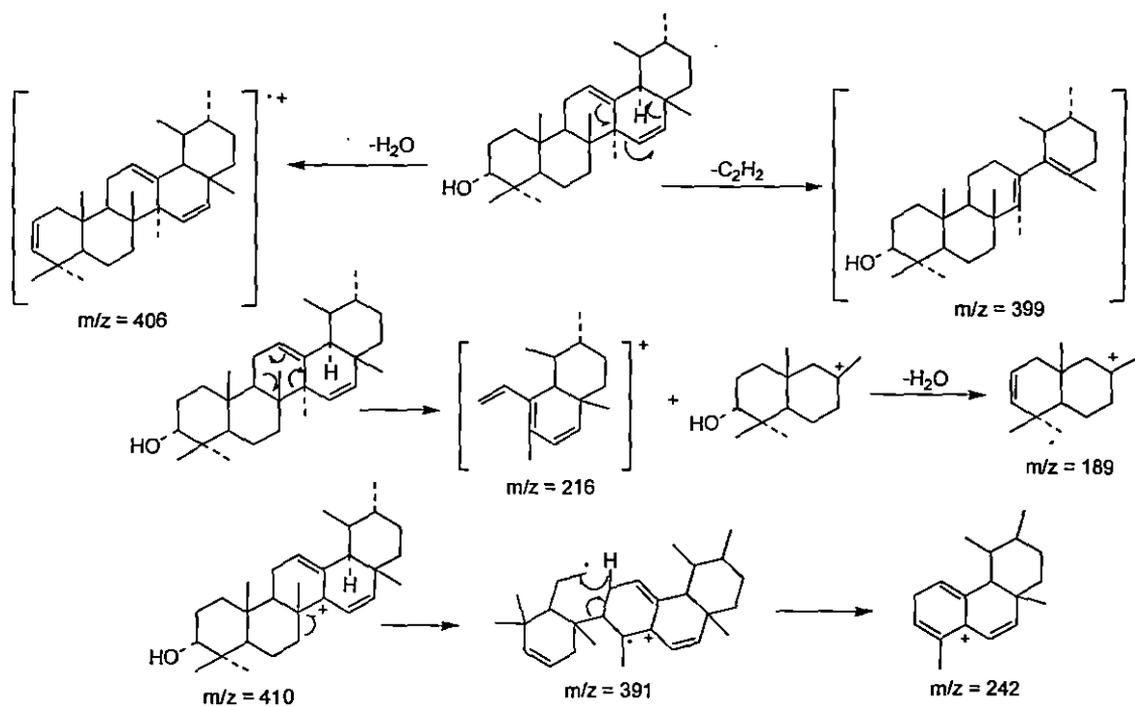


Figure 12 (A) Key COSY correlation of the isolated new compound 1, (B) Structures of other compounds isolated from *C. bonplandianum* Bail.



Scheme 1 Proposed mass fragmentation pattern of compound 1 isolated from *C. bonplandianum* Bail.

Table 1 ¹H, ¹³C chemical shift of compound 1 and ¹³C chemical shifts of 1^a, 2 and 3

C	1		1 ^a	2 ^b	3 ^b
	δC	δH	δC	δC	δC
1	40.5	1.03 (m), 1.59 (br. s)	40.5	39.0	39.2
2	29.7	1.65 (m), 1.94 (m)	29.7	28.1	28.2
3	71.8	3.50 (m)	74.0	78.2	78.2
4	33.9		33.9	39.4	39.6
5	56.8	0.86 (br. s)	56.7	55.9	55.9
6	18.7	1.39 (m), 1.48 (m)	18.7	18.8	18.8
7	31.9	1.51 (m), 1.82 (m)	31.8	33.4	33.7
8	39.7		39.7	39.8	40.1
9	50.2	1.55 (m)	50.0	48.2	48.1
10	36.5		36.5	37.4	37.5
11	24.3	1.84 (m), 1.98 (m)	24.3	23.8	23.7
12	121.7	5.33 (s, t-like)	122.6	122.6	125.7
13	140.7		139.6	144.8	139.3
14	42.3		42.3	42.2	42.6
15*	129.2		129.2	28.4	28.8
16*	138.3		138.3	23.8	25.0
17	45.8		45.8	46.7	48.1
18	55.9	2.26 (d, J = 4.3 Hz)	55.9	42.1	53.6
19	56.0	2.21 (m)	56.0	46.6	39.5
20	56.7	1.43 (br. s)	56.6	31.0	39.4
21	31.6	1.52 (d J = 5.6 Hz), 2.26 (d, J = 5.6 Hz)	31.8	34.3	31.1
22	37.2	2.01 (br. s), 2.21 (br. s)	36.9	33.2	37.4
23	21.1	0.68 (s)	21.1	28.8	28.8
24	29.1	0.69 (s)	29.1	16.5	16.5
25	28.2	0.76 (s)	28.2	15.6	15.7
26	19.8	0.84 (s)	19.8	17.5	17.5
27	26.0	1.01 (s)	26.0	26.2	24.0
28	19.4	1.25 (s)	19.3	180.0	179.7
29	19.0	0.79 (d, J = 6.3 Hz)	19.0	33.4	17.5
30	23.0	0.92 (d, J = 6.3 Hz)	23.0	23.8	21.4

^a Denotes acetyl derivative of compound 1; ^b measured in pyridine *d*₅ * Olefinic hydrogens attached to these carbons appeared as an AB q centered at 5.11 ppm with J = 15.1 and 8.5 Hz.

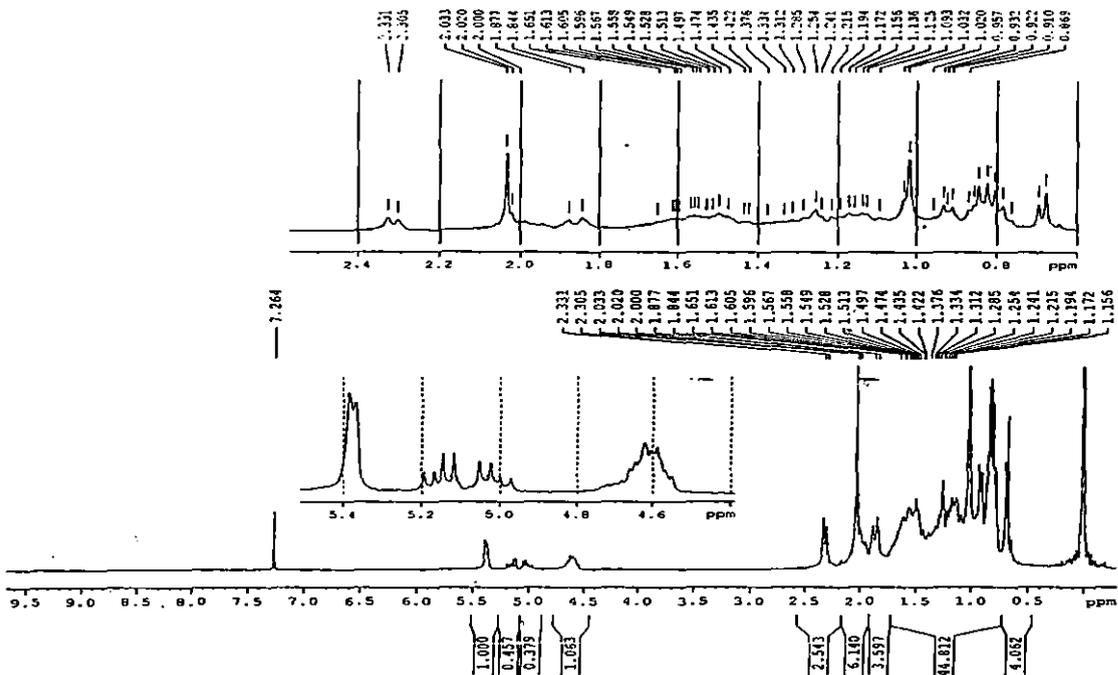


Figure 13 ^1H NMR spectrum of the acetyl derivative of the new compound isolated from *C. bonplandianum* Bail.

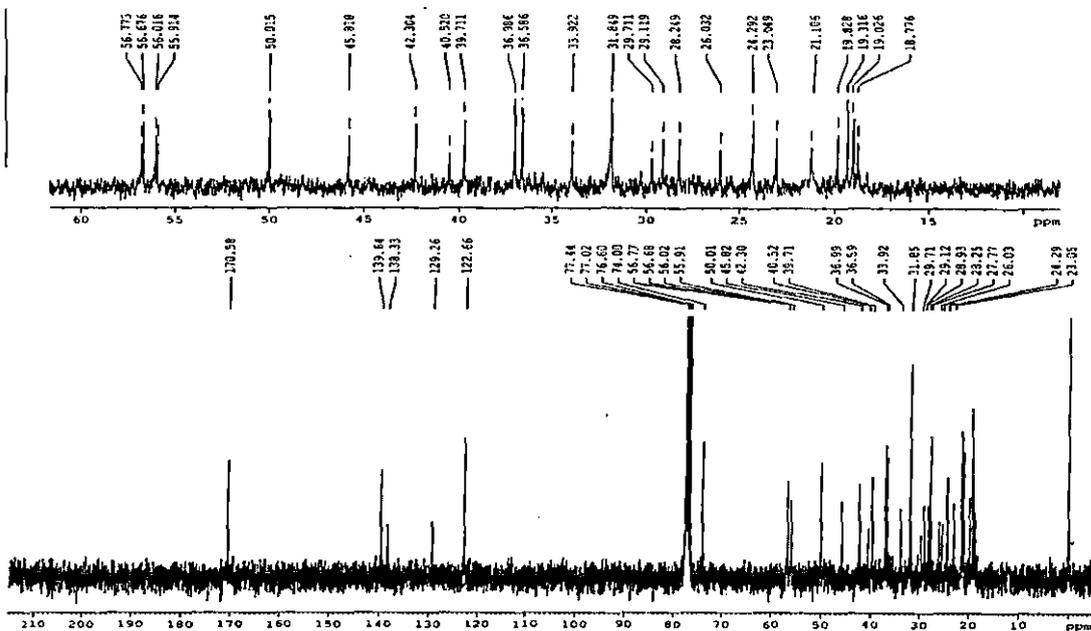


Figure 14 ^{13}C NMR spectrum of the acetyl derivative of the new compound isolated from *C. bonplandianum* Bail.

The structure of compound **1** was finally established by 2D NMR experiments. The methyl singlet at δ_{H} 1.25 was correlated with a carbon signal at δ_{C} 19.4 in the HMQC spectrum were assigned to H₃-28 from HMBC correlation observed from H-28 (δ_{H} 1.25) to C-17 (δ_{C} 45.8). Another Methyl singlet at δ_{H} 0.84, was correlated with carbon singlet at δ_{C} 19.8 in the HMQC spectrum, were assigned to H₃-26 from the HMBC correlation observed from H₃-26 (δ_{H} 0.84) to C-8 (δ_{C} 39.7). The presence of a trisubstituted olefin between C-12 and C-13 was revealed by the HMBC correlations observed for H₂-11/C-12, H₂-11/C-13, H-18/C-13 and H₃-27/C-13. Finally 2D NMR techniques were applied to locate the exact position of another double bond. Both the COSY and HMBC spectra gave significant information regarding the position of the double bond between C-15 and C-16. The COSY spectrum revealed two significant correlations of H-18 at δ_{H} 2.26 (1H, d, J = 4.3 Hz) with a proton at δ_{H} 5.13 (1H, ABq, J = 15.1 and 8.5 Hz) and a proton at δ_{H} 5.01 (1H, ABq, J = 15.1 and 8.5 Hz). In the HMBC spectrum cross peaks were observed due to H₃-27 at δ_{H} 1.01 (s, 3H)/ δ_{C} 129.2 and H₃-28 at δ_{H} 1.25 (s, 3H)/ δ_{C} 138.3. Two olefinic protons at δ_{H} 5.13 (1H, ABq, J = 15.1, 8.5 Hz) and δ_{H} 5.01 (1H, q, J = 15.1, 8.5 Hz) in effect showed correlations in COSY spectrum. From the above data it was confirmed that compound **1** has a second double bond between C₁₅ and C₁₆. The appearance of such an AB quartet for such type of double bond in ring D of pentacyclic triterpenoid, between C₁₅ and C₁₆, was reported in literature.^{16,18} Thus on the basis of the above, the structure of compound **1** was established as 3 α -hydroxy-urs-12,15-diene.

Spectral analysis (IR, NMR, Mass) of the prepared acetate derivative provide further evidence in favor of the proposed structure of compound **1** (3 α -hydroxy-urs-12,15-diene). Mass spectrum showed the incorporation of only one acetyl group (at m/z 467) and thus confirming the presence of only one hydroxyl group. IR spectrum of the monoacetate showed peak at 1248 cm⁻¹ in addition to other peaks for the acetate group. In the ¹H NMR spectrum acetate methyl appeared as a singlet centered at δ_{H} 2.33 (3H, s) and the C₃-H shifted downfield (δ_{H} 4.61, m, 1H) in comparison to the same (δ_{H} 3.52, m, 1H) for the hydroxyl methine part in compound **1**. The ¹³C spectrum showed all the carbons for the monoacetate derivative. C-3 appeared at δ_{C} 74.0 (shifted downfield with respect to that of the original compound, **1**) and the acetate carbonyl signal appeared at δ_{C}



170.5. The DEPT and COSY spectra of the acetylated derivative were in good agreement to that proposed for the acetylated derivative (5) and thus established the structure of compound 1 as 3 β -hydroxy-urs-12,15-dien.

Table 2 ^1H - ^{13}C long range correlation detected in the HMBC spectra of compound 1 (δ relative to TMS in CDCl_3)

Proton	Correlated C
1.01 (H-27)	39.7 (C-8), 140.7 (C-13), 42.3 (C-14), 129.2 (C-15), 50.1 (C-9), 128.3 (C-16)
0.84 (H-26)	39.7 (C-8), 42.3 (C-14)
1.25 (H-28)	45.8 (C-17)
0.68 (H-23)	33.9 (C-4), 56.8 (C-5), 71.8 (C-3)
1.55 (H-9)	36.5 (C-10), 39.7 (C-8), 19.8 (C-26), 28.2 (C-25)
5.33 (H-12)	50.1 (C-9), 42.3 (C-14), 55.9 (C-18)
2.26 (H-18)	121.7 (C-12), 140.7 (C-13), 42.3 (C-14), 128.3 (C-16), 45.8 (C-17), 56.0 (C-19), 56.7 (C-20), 19.4 (C-28)

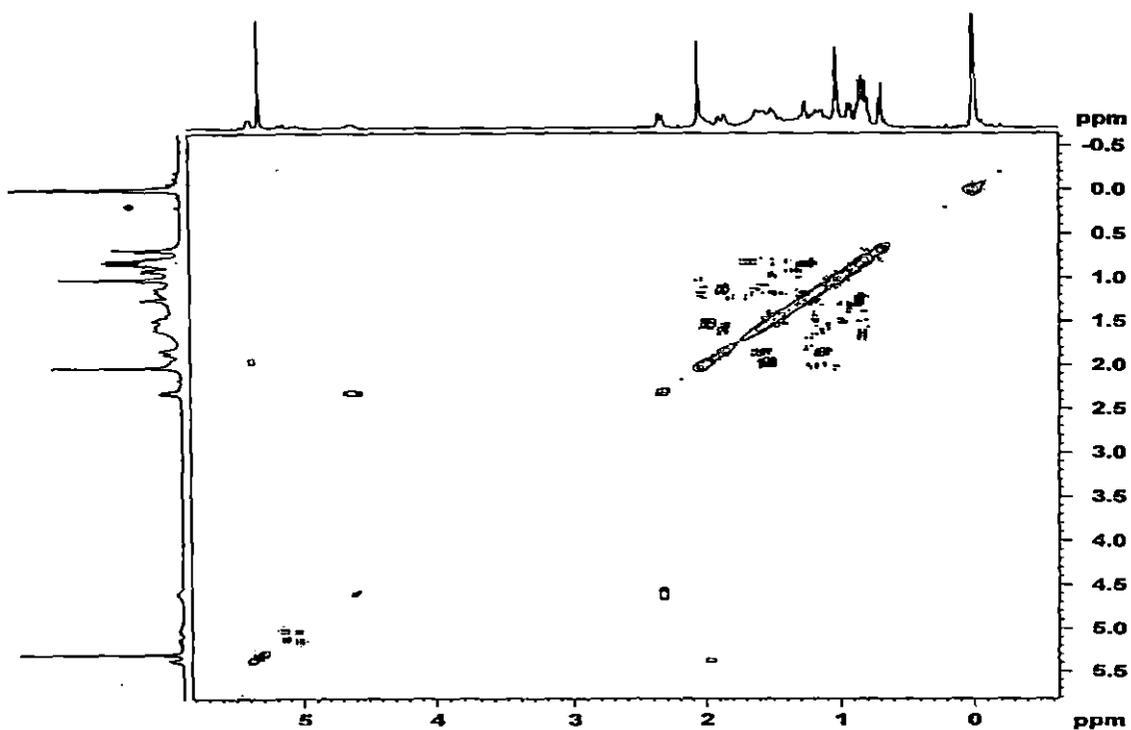


Figure 15 COSY spectrum of the acetyl derivative of the new compound isolated from *C. bonplandianum* Bail.

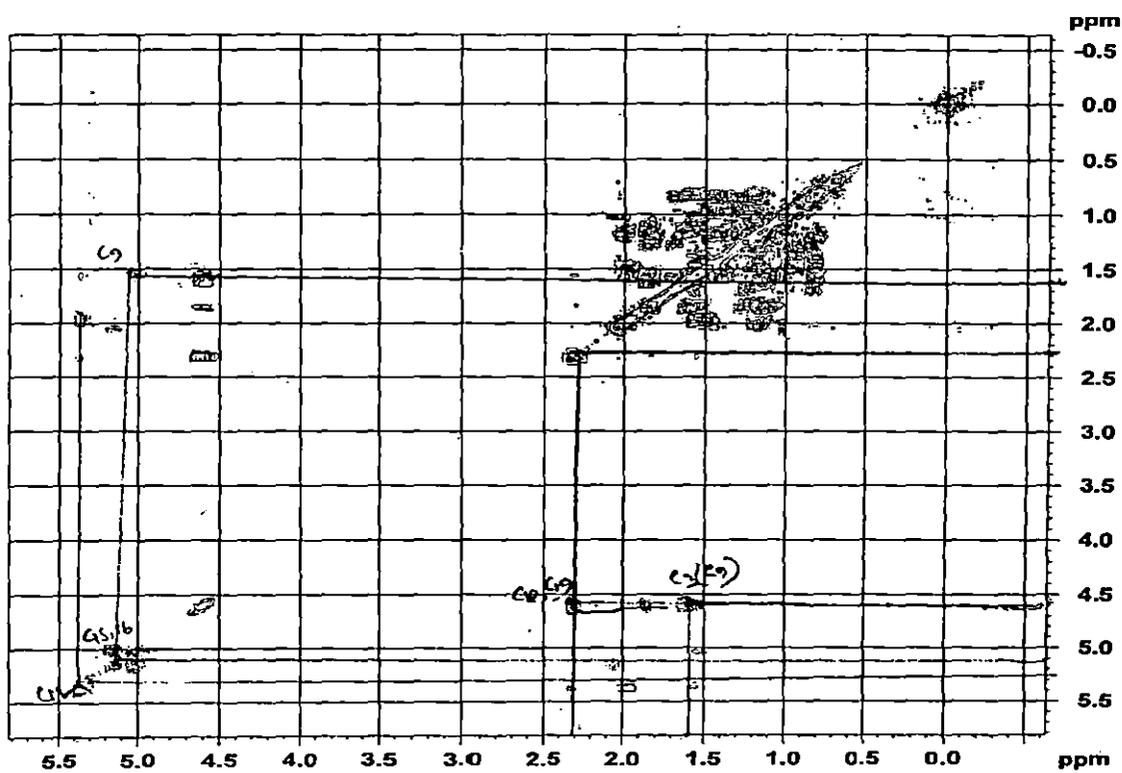


Figure 16 Expanded COSY spectrum of the acetyl derivative.

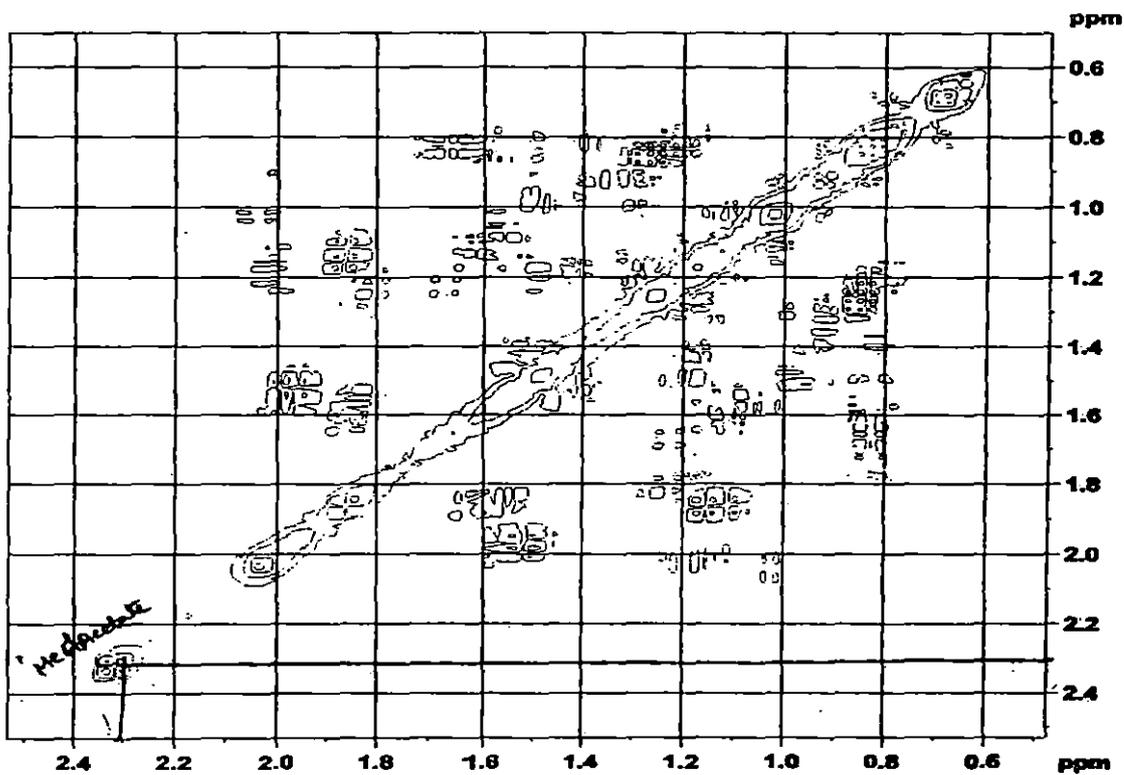


Figure 17 Expanded COSY spectrum of the acetyl derivative.

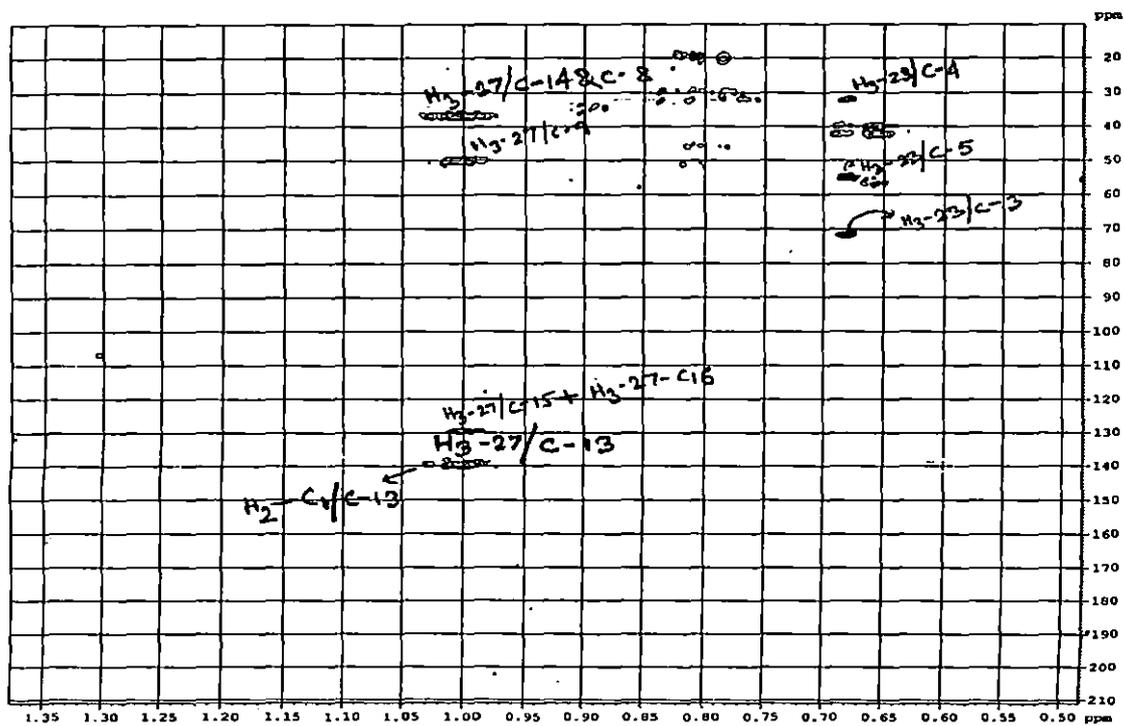


Figure 18 Expanded HMBC spectrum of the acetyl derivative.

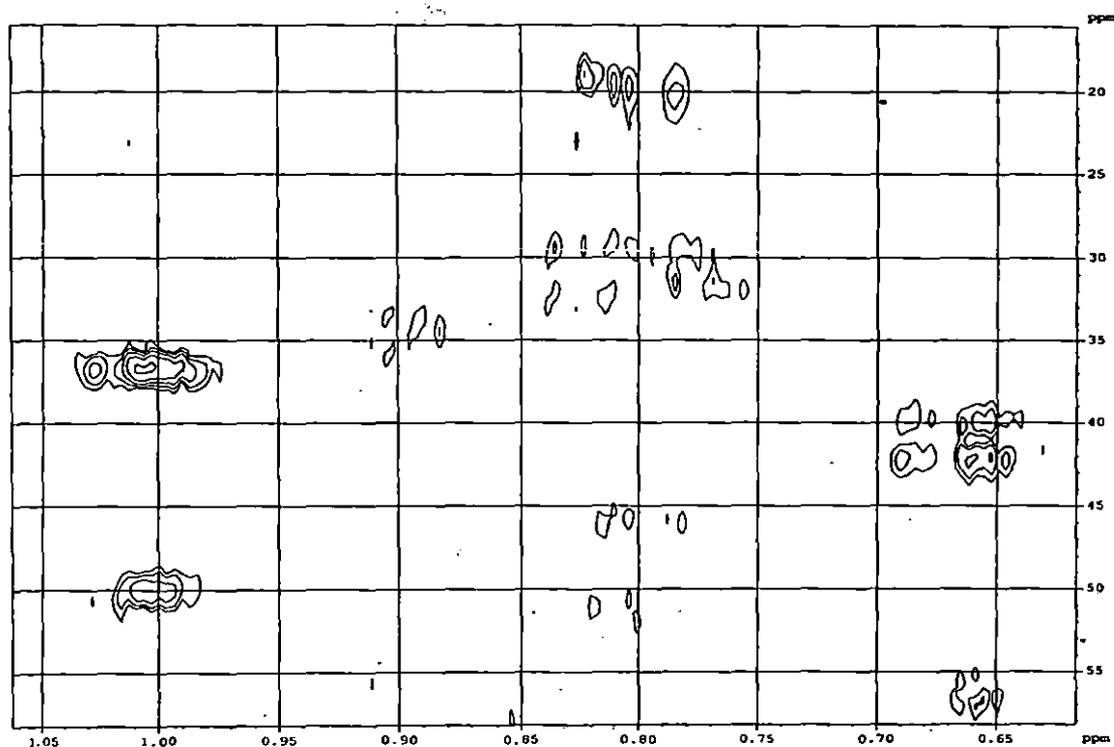


Figure 19 Expanded HMBC spectrum of the acetyl derivative.

Compound **2** after complete purification was obtained as a white powder of m.p. 306-308 °C and its molecular formula was suggested to be $C_{30}H_{50}O_3$ on the basis of FAB MS data [$m/z = 458.2 (M)^+$]. The 1H NMR spectra (Table 1) showed characteristic proton signals due to seven tertiary methyl groups at δ_H 1.24 (3H, s, C-23), 1.00 (3H, s, C-24), 0.98 (3H, s, C-25), 1.04 (3H, s, C-26), 1.30 (3H, s, C-27), 0.97 (3H, s, C-29), and 1.02 (3H, s, C-30). An oxygenated methine signal appeared at δ_H 3.44 (1H, m) and a trisubstituted olefinic signal at δ_H 5.49 (1H, s). The ^{13}C NMR spectra showed signals due to seven tertiary methyl groups at δ_C 28.8, 16.5, 15.6, 17.5, 26.2, 33.4 and 23.8. C-3 appeared at δ_C 78.2 and the angular carboxyl carbon came at δ_C 180.0. Two olefinic carbons C-12 and C-13 appeared at δ_C 122.6 and 144.8 respectively. All the above data confirmed compound **2** as oleanolic acid and these data were in good agreement to that reported in literature.¹⁹ Further verification was done by co-tlc, mixed melting point, co-IR with an authentic sample of oleanolic acid.

Compound **3** was also obtained as a white powder of m.p 280-282 °C and its molecular formula was suggested to be C₃₀H₅₀O₃ on the basis of FAB MS data [m/z = 458.5 (M)⁺]. The ¹H NMR spectra of **3** (Table 1) showed characteristic proton signals due to five tertiary methyl signals at δ_H 0.92 (3H, s), 1.02 (3H, s), 1.06 (3H, s), 1.24 (3H, s), 1.24 (3H, s), and two secondary methyl signals as doublet centered at 0.97 (3H, d, J = 6.5 Hz) and 1.02 (3H, d, J = 6.5 Hz) of the triterpenoid moiety. An oxygen methine signal at δ_H 3.44 (1H, m, H-3) and a trisubstituted olefinic signal at δ_H 5.49 (1H, t-like, H-12), suggesting that **3** is a 3β-hydroxy-urs-12-en type triterpenoid. ¹³C NMR spectra although showed 30 signals confirming the presence of triterpene skeleton (Table 1), along with two signals at δ_C 125.7 and 139.3 due to the presence of two sp² hybridised carbon. The signal at δ_C 139.3 is due to the presence of a trisubstituted olefinic carbon at C-13. C-3 carbon appeared at δ_C 78.2. Eight methyl signals appeared at δ_C 28.8 (C-23), 16.5 (C-24), 15.7 (C-25), 17.5 (C-26), 24.0 (C-27), 179.7 (C-28), 17.5 (C-29) and 21.4 (C-30). From the above data it was confirmed compound **3** as ursolic acid and also these data were in good agreement to that reported in literature.¹⁹ Further verification was done by co-tlc, mixed melting point, co-IR with an authentic sample of oleanolic acid.

Purification of the most polar fraction (8% ethyl acetate in petroleum ether) afforded white crystals of mp 136-137 °C, M⁺ 414, and was identified as β-sitosterol **4** by spectral analysis and by comparison with an authentic sample of β-sitosterol (mixed mp, co-IR, and co-tlc etc.). The IR, NMR and Mass spectra of β-sitosterol was given below (Figure 20, 21).

A probable biosynthetic pathway for the formation of the new triterpenoid **1** has depicted in figure 23 on the basis of the formation of 3-epi-α-amyrin.²⁰ This is followed by the formation of a transient carbanion intermediate (S3) and its subsequent rearrangement to S5. Catalytic role of NADP⁺ present in cells/tissue was then suggested for the formation of **1**.

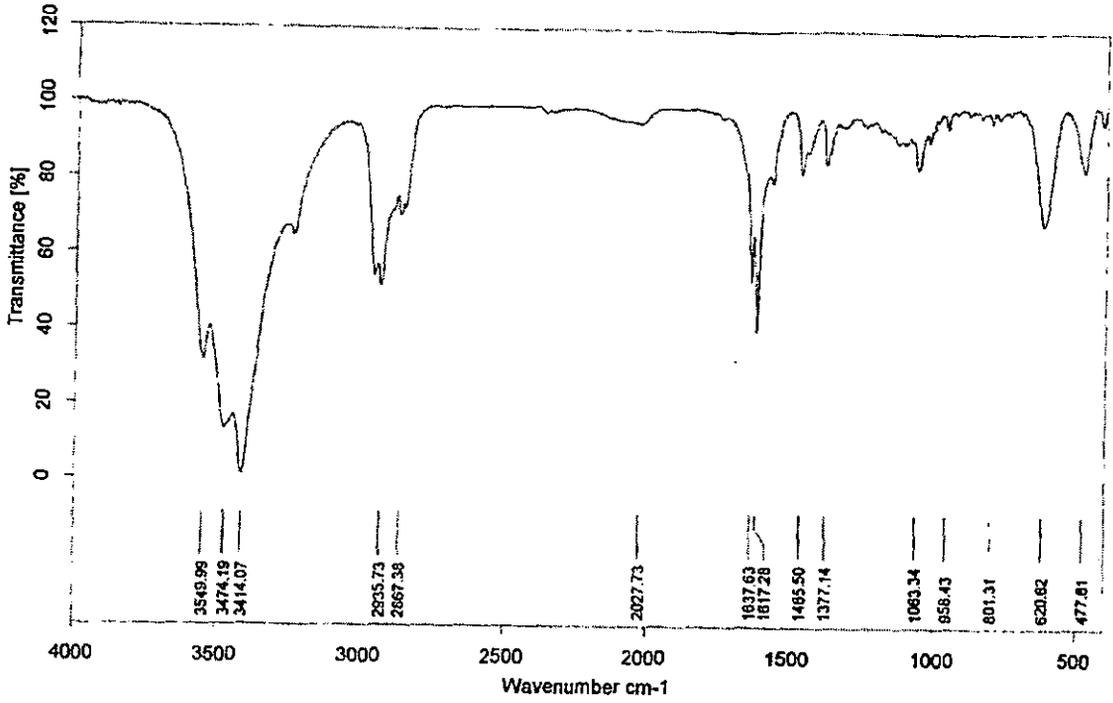


Figure 20 IR spectrum of β -sitosterol

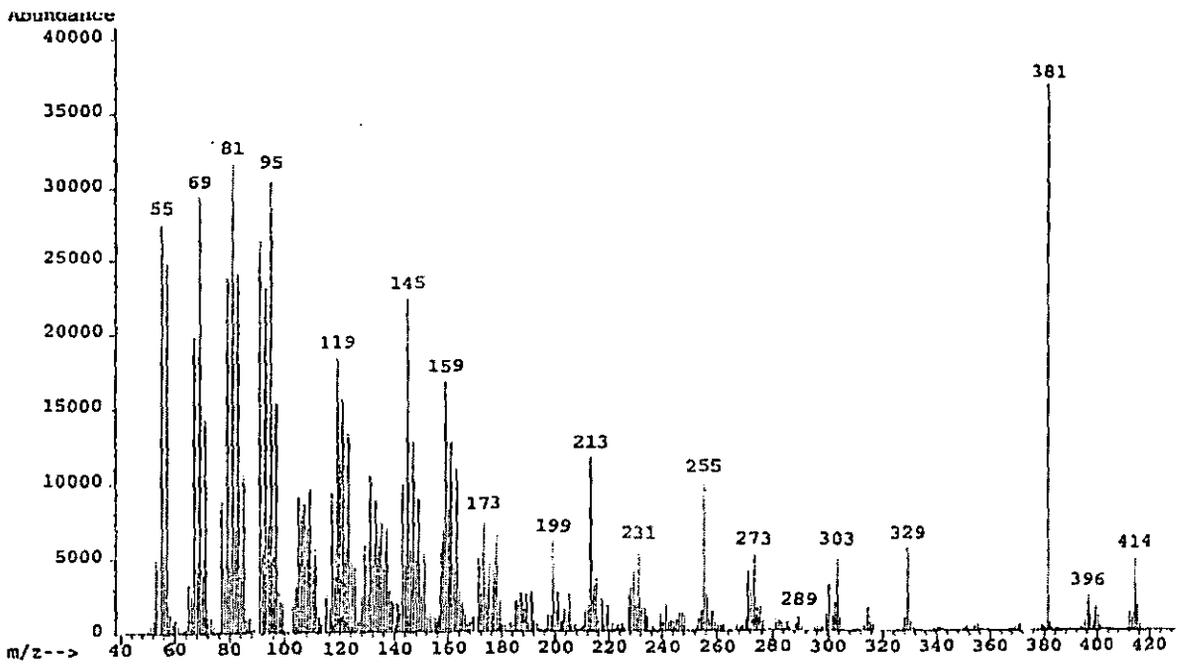


Figure 21 Mass spectrum of β -sitosterol

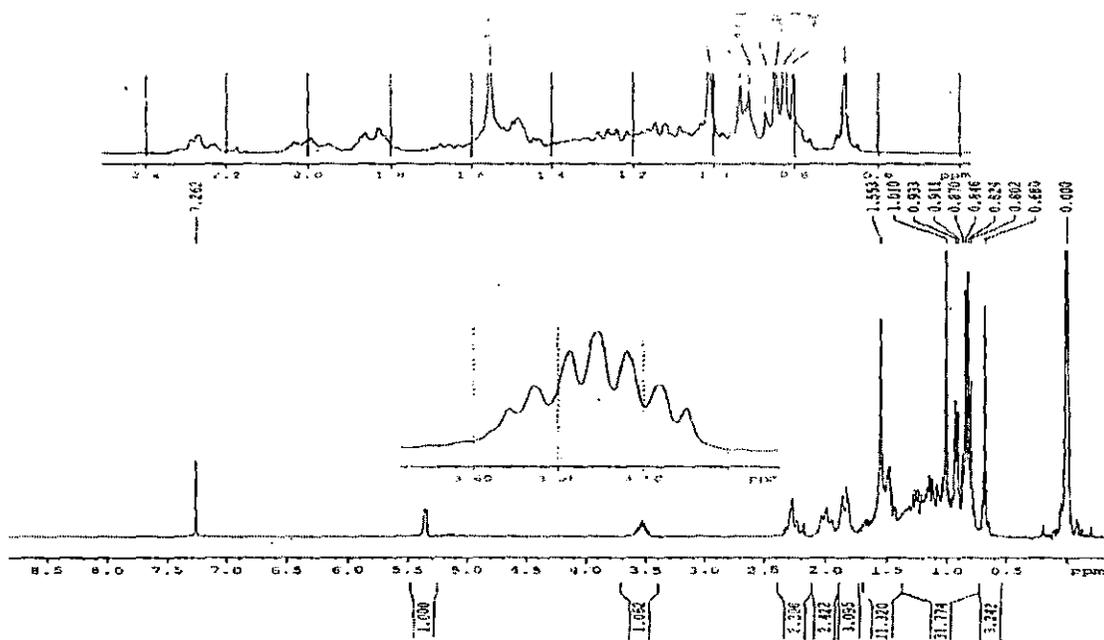


Figure 22 ^1H NMR spectrum of compound 4, β -sitosterol

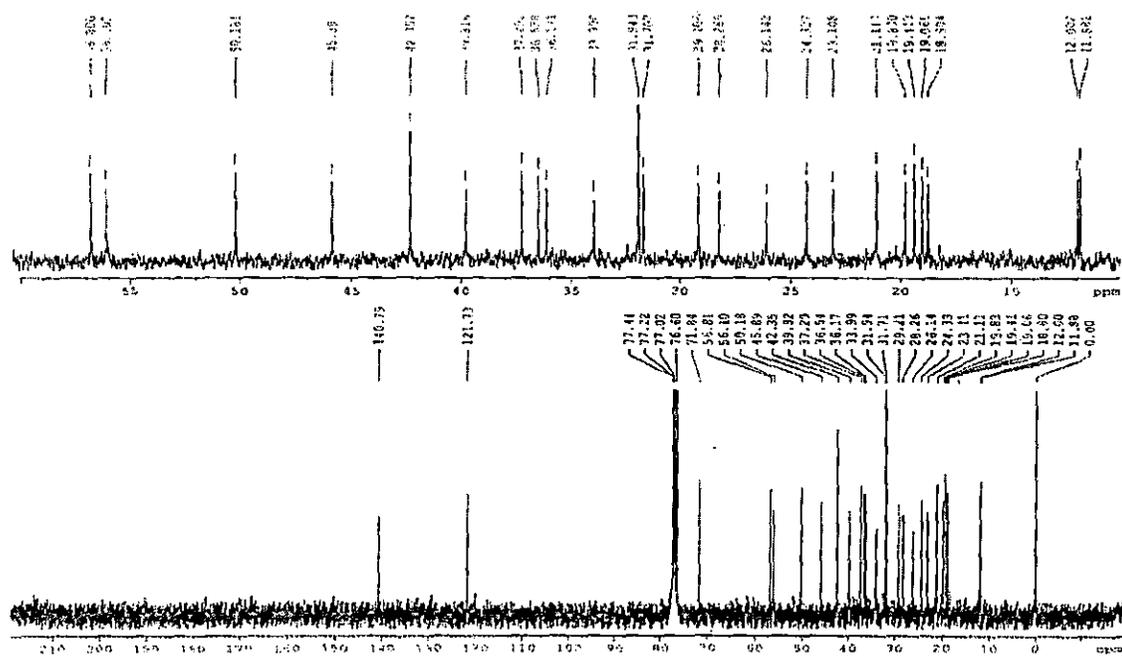
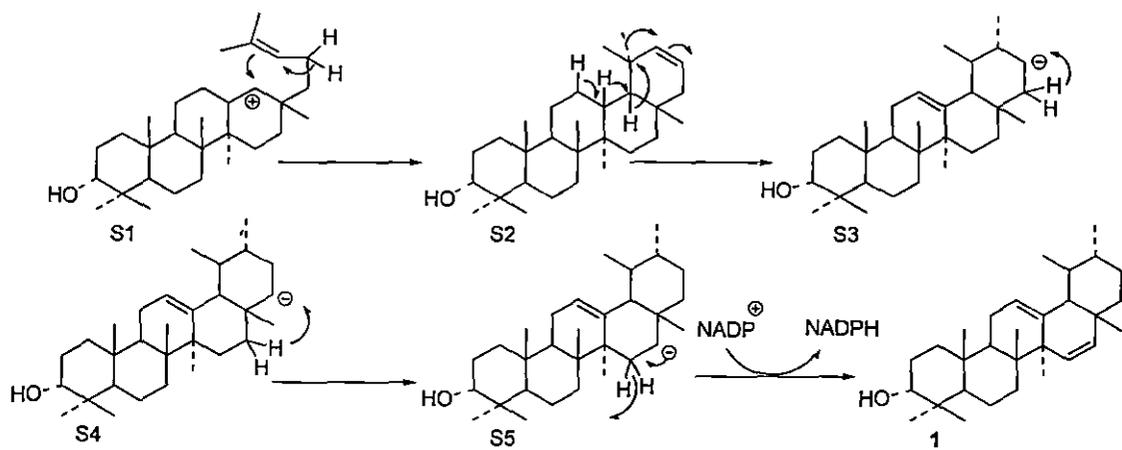


Figure 23 ^{13}C NMR spectrum of compound 4, β -sitosterol



Scheme 2 Probable biosynthetic pathway of compound 1

Section C

Antimicrobial activities of the isolated compounds from *C. bonplandianum* Bail

1. Antifungal activity of the isolated compounds

All the compounds (1, 2 and 3) isolated from the ethyl acetate soluble fraction were tested for their antifungal activity by the disc diffusion method (Table 3) against the microorganisms which are very much native to North Bengal,²¹ India; viz. *Calletotricheme camellie* (CC), *Fussarium equisetae* (FE), *Alterneria alternate* (AA), *Curvularia eragrostidies* (CE) and *Colletrichum Gleosproides* (CG). These fungal pathogens are responsible to cause wilt disease to tomatoes, pine apple *etc.* cultivated traditionally in this region. The MIC values including that of reference sample Bavistin are tabulated in table 1. The MIC values of Bavistin against these fungal pathogens were also determined. A comparison of antifungal activities of compound 1, 2 and 3 to that of Bavistin showed that although they are less active compare to Bavistin, but all of them exhibited moderately good activity against all the fungal pathogens tested.

Suitable fungal strains were procured from the microbiology laboratory of our institute. DMSO (dimethyl sulfoxide) was used as solvent to prepare different concentrations of the triterpenoid. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in petridishes and were incubated at 37 °C for 48 hour. Culture media for fungal pathogens were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber.

Table 3 MIC of Compound 1 against different fungi

Compound	MIC of 1 in µg/mL against different fungi				
	CC	CG	AA	FE	CE
1	10	<15	10	<10	15
2	<10	20	10	<10	10
3	15	<10	10	<15	<15
Bavistin	2.5	1.25	2.5	2.5	<2.5

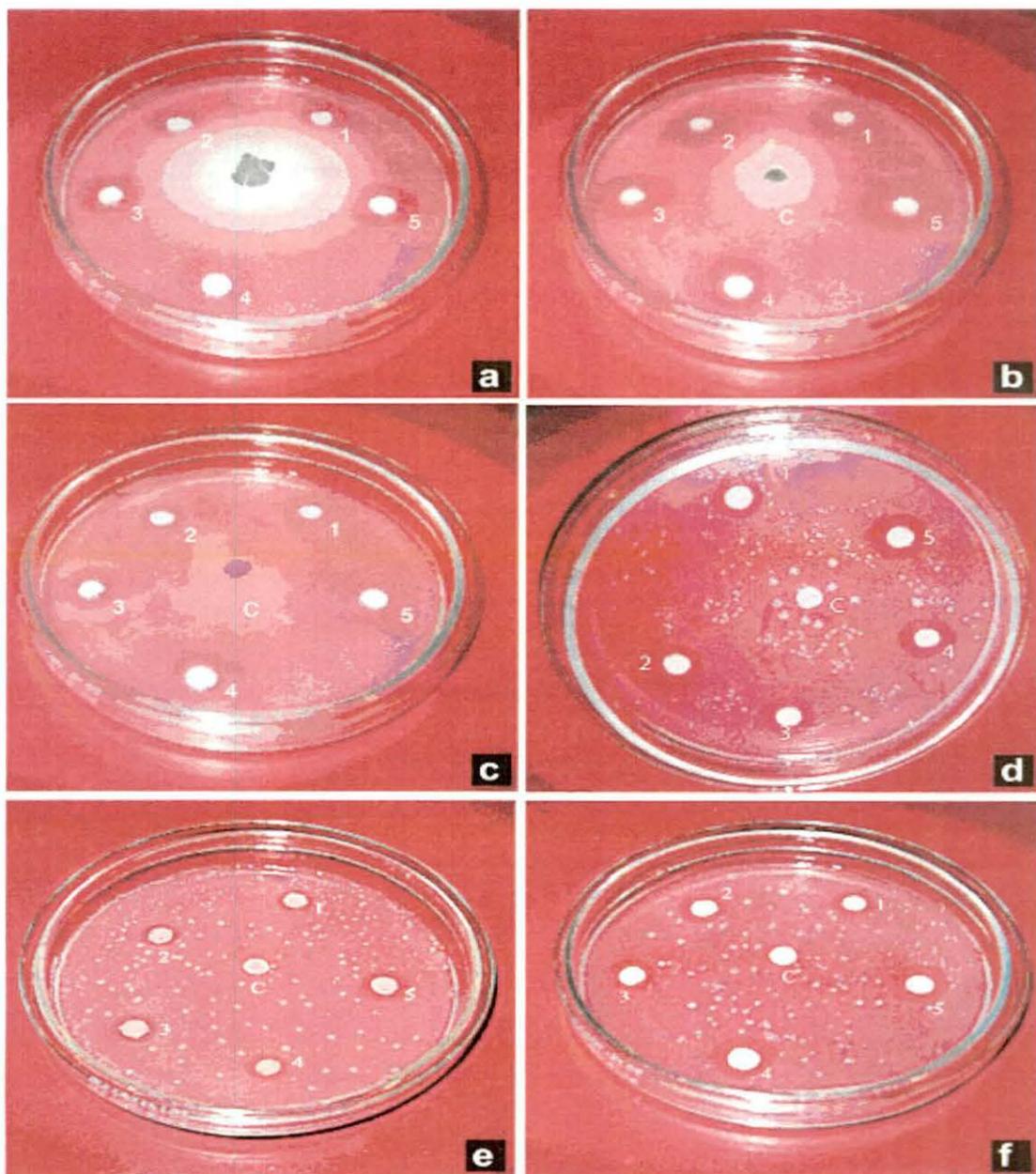


Figure 24 Representative Petri dishes of the antifungal activities, (c) for compound **1** against *Calletotricheme camellia*, (d) for compound **1** against *Fussarium equisetae*, (e) for compound **1** against *Alterneria alternate* and (f) for compound **1** against *Curvularia eragrostidies*.

To determine the antifungal efficiency of the isolated compounds the MIC values were compared to that of bavistin, an important antifungal drug molecule. Although the determined MIC values of bavistin against all the tested organisms were better than the isolated compounds, still the isolated compounds were promising as all of them showed good MIC values against all the tested organisms.

2. Antibacterial activity of the isolated compounds

All the isolated compounds were tested for their antifungal activity against a series of bacterial pathogens namely *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobactor sp.*, *S.dysenteriae*, *Aspergillus niger*, *Penicillium chrysogenum*. MIC values of different compounds are tabulated in table 4. The isolated compounds showed good antibacterial activities. The determined MIC values were compared with that for streptomycin.

Table 4 MICs in $\mu\text{g/mL}$ against different strains of bacteria

Compounds	EC	BS	SA	PA	CA	SD	EN	PC	AN
1	100>	200	100	75	75	100	75	100	75
2	100>	50	75	50	100	100	100	75	50
3	100	75	50	75	50	75	75	100	50
Streptomycin	8	5	8	8	8	8	5	8	8

BS-*Bacillus subtilis*, EC-*Escherichia coli*, SA-*Staphylococcus aureus*, PA-*Pseudomonas aeruginosa*, EN-*Enterobactor sp.*, SD-*S.dysenteriae*, AN-*Aspergillus niger*, PC-*Penicillium chrysogenum*, MIC - Minimum inhibitory concentratio

3. Conclusion

A new triterpenoid of ursane skeleton has been isolated from the root of *C. bonplandianum* and characterized as 3 β -hydroxy-urs-12,15-dien (1) along with two known triterpenic acids, oleanolic acid (2) and ursolic acid (3). β -sitosterol (4) was also obtained as the most polar fraction of the ethylacetate phase. Antifungal and antibacterial

potentiality of compound **1** was also detected against five different fungal pathogens. A plausible biosynthetic pathway was also suggested.

CHAPTER II

EXPERIMENTAL AND REFERENCES

1. Experimental

1.1 General experimental procedure

Melting points were determined by open capillary method and were uncorrected. IR spectra were measured on Shimadzu 8300 FT-IR spectrophotometer and Perkin Elmer FT-IR spectrophotometer. NMR spectra were recorded on Bruker-Avance 300 and 400 MHz FT-NMR spectrometers respectively. ESIMS was obtained on Applied Biosystem API 2000 and FAB MS were obtained on Jeol SX 102 mass spectrometer from CDRI Lucknow. TOF MS was recorded in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer.

1.2 Collection of Plant Material

Plants of *C. bonplandianum* used in this experiment were collected from North Bengal, India in May, 2008. After collection all the plants were washed thoroughly by plenty of water and the roots were separated by simple cutting through a knife in wet condition and separated those from the rest parts.

1.3 Drying and grinding

The plant's materials were shade dried and were cut into small pieces. It was then grinded in small lots to powdered form in a mechanical grinder and used for the extraction process.

1.4 Extraction and Isolation *C. bonplandianum* Bail

The air dried powdered roots of *C. bonplandianum* (2 Kg) was extracted with MeOH (2 L) in a soxhlet extractor for 7 days and MeOH was recovered in *vacuo*. The deep brown extracted mass (350 g) was suspended in distilled water (1L) and was partitioned successively between hexane (300 x 3), EtOAc (300 x 3) and n-BuOH (300 x 3) to obtain four fractions (hexane phase, ethyl acetate phase, n-butanol phase and water phase). The ethyl acetate phase (1.5 g) was purified by repeated column chromatography (silica gel) using petroleum ether (PE) and PE:ethyl acetate (EA) of varying concentrations as eluent.

Table 5 Purification of the crude extract of *C. bonplandianum* Bail

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-3	Nil	--
2	PE-EA (9.9:0.1)	4-10	Oil	--
3	PE-EA (9.8:0.2)	10-12	Nil	--
4	PE-EA (9.6:0.4)	13-18	White solid (\approx 0.2 g)	244-246 °C
5	PE-EA (9.5:0.5)	19-23	White solid (\approx 0.05 g)	306-308 °C
6	PE-EA (9.4:0.6)	24-31	White solid (\approx 0.05 g)	280-282 °C
7	PE-EA (9.3:0.7)	32-41	Nil	--
8	PE-EA (9.2:0.8)	42-66	White solid (\approx 0.3 g)	136-137 °C ^a
9	PE-EA (9.1:0.9)	66-82	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

^aDescribes melting point of the simple isolated compound as the it was hard to crystallize.

1.4.1 Characterization of compound 1

Compound 1 was obtained as a white powder of melting point (mp) 244-246 °C, $[\alpha]_D + 4.0$, UV inactive, showed strong absorption band at 3422 (-OH), 2850, 1461, 1376 (gem dimethyl), 1053, 970, 959, 838 cm^{-1} , ESIMS data [$m/z = 425$ (M+1)]. ¹H NMR spectra of compound 1 (Table 1) showed characteristic proton signals due to six tertiary methyl groups at δ_H (proton chemical shift) 0.68 (3H, s), 0.69 (3H, s), 0.76 (3H, s), 0.84 (3H, s), 1.01 (3H, s), 1.25 (3H, s), and two secondary methyl signals as doublet centered at 0.79 (3H, d, J = 6.3 Hz) and 0.92 (3H, d, J = 6.3 Hz). Elemental analysis: C, 84.82% (cald.

84.84), O, 3.68% (cald. 3.77) and H 11.50 (cald. 11.39). Thus compound **1** is 3 α -hydroxy-urs-12,15-dien.

1.4.2 Characterization of compound **2**

Compound **2** after complete purification was obtained as a white powder of mp 306-308 °C and its molecular formula was suggested to be C₃₀H₅₀O₃ on the basis of FAB MS data [$m/z = 458.2$ (M)⁺]. The ¹H NMR spectra showed characteristic proton signals due to seven tertiary methyl groups at δ_H 1.24 (3H, s, C-23), 1.00 (3H, s, C-24), 0.98 (3H, s, C-25), 1.04 (3H, s, C-26), 1.30 (3H, s, C-27), 0.97 (3H, s, C-29), and 1.02 (3H, s, C-30). An oxygenated methine signal appeared at δ_H 3.44 (1H, m) and a trisubstituted olefinic signal at δ_H 5.49 (1H, s). Therefore compound **2** is oleanolic acid. Finally it was confirmed by comparison with an authentic sample of oleanolic acid (mixed melting point, co-IR, co-tlc).

1.4.3 Characterization of compound **3**

Compound **3** was also obtained as a white powder of mp 280-282 °C and its molecular formula was suggested to be C₃₀H₅₀O₃ on the basis of FAB MS data [$m/z = 458.5$ (M)⁺]. The ¹H NMR spectra of **3** showed characteristic proton signals due to five tertiary methyl signals at δ_H 0.92 (3H, s), 1.02 (3H, s), 1.06 (3H, s), 1.24 (3H, s), 1.24 (3H, s), and two secondary methyl signals as doublet centered at 0.97 (3H, d, $J = 6.5$ Hz) and 1.02 (3H, d, $J = 6.5$ Hz) of the triterpenoid moiety. An oxygen methine signal at δ_H 3.44 (1H, m, H-3) and a trisubstituted olefinic signal at δ_H 5.49 (1H, t-like, H-12), suggesting that **3** is a 3 β -hydroxy-urs-12-en type triterpenoid. Finally it was confirmed by comparison with an authentic sample of ursolic acid (mixed melting point, co-IR, co-tlc).

1.4.4 Characterization of compound **4**

Purification of the most polar fraction afforded white crystals of m.p. 136-137°C, M⁺ 414, and was identified as β -sitosterol. Finally it was confirmed by comparison with an authentic sample of β -sitosterol (mixed melting point, co-IR, co-tlc).

1.5 Preparation of acetyl derivative of compound 1

Compound 1 (100 mg, 0.23 mmol) in a 50 mL round bottom flask was warmed over a water bath with 10mL of dry pyridine and 2 mL of acetic anhydride for 6 hours. The reaction mixture was then poured into 50 mL of ice cold water; a milky white precipitate appeared that was worked up with ether, dried over anhydrous sodium sulfate. In the tlc chromatogram, developed by vaporized iodine showed a single compound. Solvent ether was recovered to make a minimum volume and to that silica gel (60-120 mesh) was added. The dried silica adsorbed compound was added over a column of silica gel (60-120 mesh) and the column was run using petroleum ether (PE) and PE:ethyl acetate (EAA) of varying concentrations as eluent.

Table 6 Purification of the acetylated derivative of compound 1

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-6	Nil	--
2	PE-EA (9.95:0.05)	7-11	Oli	--
3	PE-EA (9.9:0.1)	12-18	White solid (\approx 0.085 g)	223-224 °C
4	PE-EA (9.8:0.2)	19-22	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.5.1 Characterization of acetyl derivative of compound 1

IR spectrum showed peaks at 2850, 1461, 1376, 1248, 1053, 959, 970, 838 cm^{-1} . In the mass spectrum it showed a molecular ion peak at 467. The ^1H NMR spectrum of the acylated derivative showed characteristic signals due to five tertiary methyl groups at δ_{H} 0.68 (3H, s, C-23), 0.69 (3H, s, C-24), 0.76 (3H, s, C-25), 0.84 (3H, s, C-26), 1.01 (3H, s, C-27), 1.25 (3H, s, C-28), and two secondary methyl signals as doublet centered at 0.79 (3H, d, $J = 6.3$ Hz) and 0.92 (3H, d, $J = 6.3$ Hz). The acetate methyl appeared as a

singlet centered at 2.33 (3H, s). Elemental analysis: C, 82.28% (cald. 82.35), O, 6.78% (cald. 6.86) and H 10.94 (cald. 10.80). Thus compound 1 is 3 α -hydroxy-urs-12,15-dien.

1.6 Bioassay

DMSO (dimethyl sulfoxide) was used as solvent to prepare different concentrations of the triterpenoids. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in petridishes and were incubated at 37 °C for 48 h. The bacterial growth was confirmed by a change of yellow to purple colour. Bacterial nutrient media was prepared by using agar, beef extract and bacto peptone in distilled water and the pH of the solution (6.8-7.0) was adjusted. Culture media for fungal strains were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber. Additionally slide germination method was also used for determination of antifungal activity (Table 3). The antifungal activities between these compounds and streptomycin and antibacterial activity with ampicillin, a β -lactam antibiotic were compared.

For studying the inhibitory effect of the two triterpenoids against test fungal pathogens following slide germination method, the spores of the pathogens were allowed to germinate in presence of the prepared and the 50% ethanol extracts. Compound solution was placed on the centre of the grease free microscope slide. In control the corresponding solvent, either sterile distilled water or 50% ethanol was placed. Thirty micro litre spore suspension prepared from ten days culture of the fungal pathogens were added to the spots in both experimental and control slides. In case of 50% ethanol extract, spore suspension was added after ethanol was evaporated. Three experimental slides were taken for each compound. The slides were then incubated at 28 °C in a humid chamber. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and a slide was placed on the rods in a uniformly balanced position. Then the petridish was filled with sterile distilled water so that the bottom of the slide remained just above the water surface. The petridish was then covered and incubated at 28 °C. Following 48 h of incubation, the slides were stained with lacto phenol-cotton blue mixture and observed in each slide for germination. Numbers of aspersoria formed were also observed and lengths of 50 germ tubes were measured. The entire experiment was repeated thrice.

1.6.1 Source of microbial cultures

Fungal and bacterial cultures used during the work were either isolated from the field or procured from Microbial type culture collection (MTCC), Chandigarh, India. The details of the source of the fungal cultures are given in Table 7.

Table 7 List of cultures used during the present study

Name of the microorganisms	Source	Number
<i>Staphylococcus aureus</i>	MTCC, Chandigarh, India	MTCC-26
<i>Escherichia coli</i>	MTCC, Chandigarh, India	MTCC-739
<i>Pseudomonas aeruginosa</i>	MTCC, Chandigarh, India	MTCC-2453
<i>Escherichia coli</i>	MTCC, Chandigarh, India	MTCC-2939
<i>Candida albicans</i>	MTCC, Chandigarh, India	MTCC-227
<i>Penicillium chrysogenum</i>	MTCC, Chandigarh, India	MTCC-160
<i>Colletotrichum gloeosporioides</i>	Plant pathology lab. NBU	Identification No. 5446.02
<i>Fusarium equiseti</i>	Plant pathology lab. NBU	Identification No. 6566.07
<i>Curvularia eragrostidis</i>	Plant pathology lab. NBU	Identified by Dr. A. Saha ²²
<i>Alternaria alternata</i>	Plant pathology lab. NBU	Identification No. 7065.08
<i>Colletotrichum camelliae</i>	Plant pathology lab. NBU	Identified by Dr. A. Saha ²²
<i>Bacillus subtilis</i>	Plant pathology lab. NBU	Identified by NBU ²²
<i>Enterobactor sp.</i>	Plant pathology lab. NBU	Identified by NBU ²²
<i>S. dysenteriae</i>	Plant pathology lab. NBU	Identified by NBU ²²
<i>Aspergillus niger</i>	Plant pathology lab. NBU	Identified by NBU ²²

1.6.2 Maintenance of stock cultures

Freshly prepared sterile slants of PDA medium were used for maintenance and preservation of fungal cultures. After four to eight weeks of sub culturing, the fungal pathogens grown on sterile PDA slants were stored in two different conditions, viz. at room temperature (25 °C) and in refrigerator (4-8 °C). Apart from weekly transfer for experimental purpose, the cultures were also examined at regular intervals under microscope.

1.6.3 Major Chemicals used

Table 8 In addition to the common laboratory reagents, following chemicals were used during the work

Chemicals	Company
$C_6H_{12}O_6$	Glaxo Laboratories (India) Ltd
$NaNO_3$	S d fine-CHEM limited
KCl	S d fine-CHEM limited
$MgSO_4, 7H_2O$	Merck, India
$FeSO_4, 7H_2O$	Merck, India
K_2HPO_4	Merck, India
$C_{12}H_{22}O_{11}$	Glaxo Laboratories (India) Ltd

1.6.4 Composition of media and solutions used

Some of the media/solutions used during the work are listed below along with their standard compositions.

Table 9 Nutrient Agar (NA)

Components	Amount
Beef extract	1.0 g
Yeast	2.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15 g
Distilled water	1 L

Table 10 Potato Dextrose Agar (PDA)

Component	Amount
Potato	40 g
Dextrose	2 g
Agar agar	2 g
Distilled water	100 mL

Table 11 Malt yeast Agar

Components	Amount
Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	20.0 g
Distilled water	1.0 g
pH	7.0

Table 12 Czapek concentrate

Components	Amount
NaNO ₃	30.0 g
KCl	5.0 g
MgSO ₄ , 7 H ₂ O	5.0 g
FeSO ₄ , 7 H ₂ O	0.1 g
Distilled water	1.0 L
K ₂ HPO ₄	1.0 g
Yeast extracts	5.0 g
Sucrose	30.0 g
Agar	15.0 g

1.6.4.1 *Staphylococcus aureus* were grown in nutrient agar media at 37±1 °C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.2 *Escherichia coli* were grown in nutrient agar media at 37±1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.3 *Pseudomonas aeruginosa* were grown in nutrient agar media at 37±1 °C in an incubator for 24 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals

1.6.4.4 *Candida albicans* were grown in nutrient agar media at 25±1 °C in an incubator for 48 h. The growth condition was malt yeast agar. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 60 days intervals.

1.6.4.5 *Penicillium chrysogenum* were grown in Czapek concentrate media at 25 ± 1 °C in an incubator for 7 days .The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 7 days old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.6 *Colletotrichum gloeosporioides* were grown in nutrient agar media at 37 ± 1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.7 *Fusarium equiseti* were grown in nutrient agar media at 37 ± 1 °C in an incubator for 48 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.8 *Curvularia eragrostidis* were grown in nutrient agar media at 37 ± 1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 45 days intervals

1.6.4.9 *Alternaria alternata* were grown in nutrient agar media at 37 ± 1 °C in an incubator for 24 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 60 days intervals

1.6.4.10 *Colletotrichum camelliae* was grown in nutrient agar media at 37 ± 1 °C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.11 *Bacillus subtilis* were grown in Czapek concentrate media at 25 ± 1 °C in an incubator for 7 days .The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 7

days old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.12 *Enterobacter sp.* was grown in nutrient agar media at 37 ± 1 °C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.13 *S. dysenteriae* was grown in nutrient agar media at 25 ± 1 °C in an incubator for 48 h. The growth condition was malt yeast agar. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 60 days intervals.

1.6.4.14 *Aspergillus niger* were grown in nutrient agar media at 37 ± 1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 45 days intervals

1.6.5 Assay of Antifungal activity

Fungi were grown on potato dextrose agar (PDA) medium at 28 ± 1 °C for mycelial growth. The fungicidal activities were determined using agar cup bioassay and spore germination bioassay.

1.6.6 Spore germination bioassay²³

The purified eluents (10 µL) were placed on two spots 3 cm apart on a clean grease free slide and the solvent was allowed to keep for some time to evaporate. One drop (0.02 mL) of spore suspension (10spores/mL) prepared from 15 days old culture of the test fungi was added on the same place where the purified eluents were placed and subsequently evaporated. Various compounds of five different concentrations were prepared (500 ppm, 400 ppm, 300 ppm, 200 ppm, 100 ppm). The studies were performed at 28 ± 1 °C for 24 h under humid conditions in Petri plates. Finally after proper incubation period, one drop of a cotton blue-lacto phenol mixture was added to each spot to fix the germinated spores. The number of spores germinated was compared with that of germinated spores of control (where no chemicals were used). Moist chamber was used for germination of spores. The number of germinated spores was calculated on the basis

of an average of 300 spores per treatment. The minimum inhibitory concentration required for complete inhibition was recorded in units of microgram per milliliter ($\mu\text{g/mL}$).

1.6.7 Antibacterial sensitivity test by the disc diffusion method²³

One ml of 48 h old culture of the test bacterium was taken in a petridish of 90 mm diameter. Then 20 mL of sterile NA medium was poured in the petridish and shaken carefully to mix the bacterial suspension with the medium. The petridishes were allowed to cool. Filter paper discs of 5 mm diameter containing desired concentration of the test samples were placed on the surface of the solidified media and incubated at 37 °C in an incubator for 48 h. Diameter of the inhibition zones was noted. In control sets no chemicals were used in the filter paper discs but sterile distilled water was used to soak the filter papers.

1.6.8 Antibacterial Assay

One ml of bacterial suspension was taken in a sterilized petridish of 90 cm diameter. Then Nutrient agar was poured in it. Sterilized filter paper discs (5 mm in diameter) were soaked in the desired test sample and finally placed on the surface of nutrient agar medium. The petridishes were incubated at 30 ± 2 °C in an incubator. Results were observed after 48 h of incubation

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Part II

**Synthesis of Pyrazine and Benzopyrazine
Derivatives of Pentacyclic Triterpenoids with
Antimicrobial and Antitopoisomerase Activity**

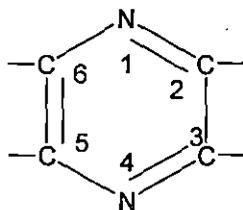
Chapter 1

1. A short review on pyrazine derivatives

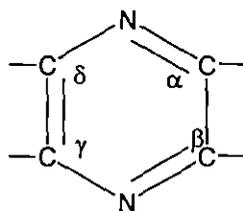
The flavor of foods has always been an interesting and complicated area worthy of investigation. Through the years many classes of compounds have been implicated as the key to the flavor properties of certain foods. During the past two decades, evidence has been accumulating that a class of heterocyclic nitrogen-containing compounds, namely pyrazines, directly contributes to the roasted or cooked flavor of foods.¹ A specific pyrazine compound that has been associated with potato flavor is 2,5-dimethylpyrazine.² At that time question arose how these pyrazine molecules were synthesized in foodstuffs. Many theories then came to explain the formation of pyrazines in foods. The role of carbohydrate degradation on pyrazine formation is well documented^{3,4} and early reports exist of the isolation of substituted pyrazines from reaction mixtures of ammonia and hexose sugars.⁵

Theories exist for the formation of various types of pyrazines. In the case of simple alkylated pyrazines, Dawes and Edwards in 1966, using model systems containing fructose and amino acids, identified 2,5-dimethyl- and trimethyl pyrazine and concluded that **pyrazines in heated foods resulted from the condensation reactions between sugars and amino acids.**

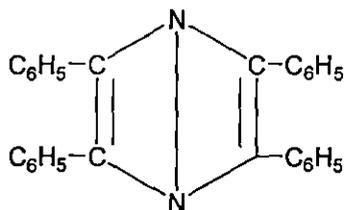
As will be indicated in the following historical section, the development of the nomenclature was beset with difficulties, owing to the confusion of the early workers with regard to the structure of pyrazine. In addition to understandable trivial names given to compounds discovered before their structure was elucidated, there arose such terms for pyrazine as aldine, paradiazine, and piazine. At one time, 2,5-dimethylpyrazine was thought to be the parent of the class, with the result that it was given the generic name "ketine." Following the Ring Index⁶ the name pyrazine was established and the numbering system is as follows-



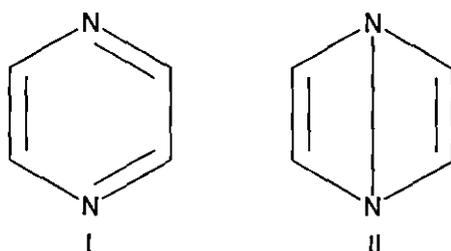
An alternative scheme is that used by T. Tictor and V. Richter in his *Lehrbuch der organischen Chemie*:



Historically the first structure elucidation of pyrazine derivative was made by Japp and Wilson and later by Pauling as well. Japp and Wilson⁷ undertook the task of determining the true nature of Erdman's benzoimidide, which they renamed ditolanazotide, by repeating the original experiments. In the following year Japp and Burton⁸ concluded that the compound was an azine, and assigned to it the structure, together with the name "tetraphenylazine."

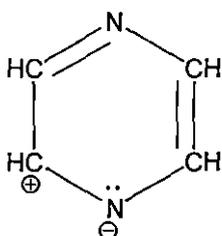


Snape and Brooke there upon repeated Laurent's original work and in 1897 published a paper^{9,10} in which they revealed that amarone, benzoimidide, ditolanazotide and tetraphenylazine were all one and the same substance: tetraphenylpyrazine. There yet remained doubt as to the exact location of the double bonds in the pyrazine molecule. The Kekule type with its conjugated double bond system (I) and the Dewar type (II) with the long para bond, each had its adherents.¹¹



Bruhl finally established the validity of I after a study of the molecular refractions of a number of pyrazine derivatives.

Pauling and his collaborators¹² have made electron-diffraction studies on some cyclic systems, including pyrazine. Their results showed that pyrazine and benzene have almost identical structures. Further, although the C-C distances for the two molecules were almost identical, 1.39 Å, the value for the C-N distance in pyrazine is 1.36 Å, was greater than expected for the Kekule resonance, which would give this bond 50 per cent double-bond character. This is interpreted as being due to the large electronegativity of the nitrogen atom, which results in the introduction of additional resonating ionic structures, as:



Simple pyrazines is a heterocyclic aromatic compound with the chemical formula $C_4H_4N_2$. It is a symmetrical molecule with point group D_{2h} .

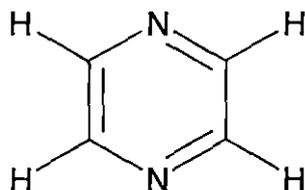


Figure 1 Finally accepted structure of pyrazine

Compounds containing *N*-heterocyclic moieties are a class of privileged compounds that have found numerous applications as pharmaceuticals as well as in medicines. Pyrazines are important component of aroma fragrances,¹ potential pharmacophore of a large

number of biologically active substances,¹³⁻¹⁶ and widely used as agrochemicals.¹⁷⁻¹⁹ For examples, methoxy pyrazines are relevant components of aromas of many fruits, vegetables and wines, methyl phenyl derivatives of dihydropyrazines inhibit the growth of *Echerichia coli* by generating hydroxyl and carbene-centered radicals that cause DNA strand breakage and alkylpyrazines have been recognized as flavor components in foods, as pheromones in various insect species¹³⁻¹⁶ and as versatile synthetic intermediates. Pyrazine derivatives are known for use as relaxing cardiovascular and uterine smooth muscle, anti-thrombotic, anti-aggregation, COX-2 inhibiting and analgesic effects.²⁰ Tetra methyl pyrazine also known as ligustrazine is reported to scavenge superoxide anion and decrease nitric oxide production in human polymorphonuclear leukocytes.²¹ It is also a component of some herbs in traditional Chinese medicine. Derivatives of pyrazine possess varieties of activities like antimicrobial, anti filarial, anti leukemia in mice against i.p. P388 and several pyrazines were more active than the corresponding oxazines or thiazines.²²⁻²⁴ Also a series of pyrazine-carboxymides has been described as eukalemic agents possessing diuretic and natriuretic properties. Hence pyrazine is a lead compound for designing potential bioactive agents.

The importance of the pyrazine nucleus in life processes is indicated in its condensed derivative, riboflavin or vitamin B2.



Figure 2 Structure of vitamin B2

The cephalostatins along with the structurally related ritterazines form a unique class of trisdecacyclic compounds that consist of two steroidal units linked through a pyrazine ring.²⁵ The first cephalostatins were isolated by Pettit *et al.* from the Indian Ocean tube worm *Cephalodiscus gilchristi*.²⁶

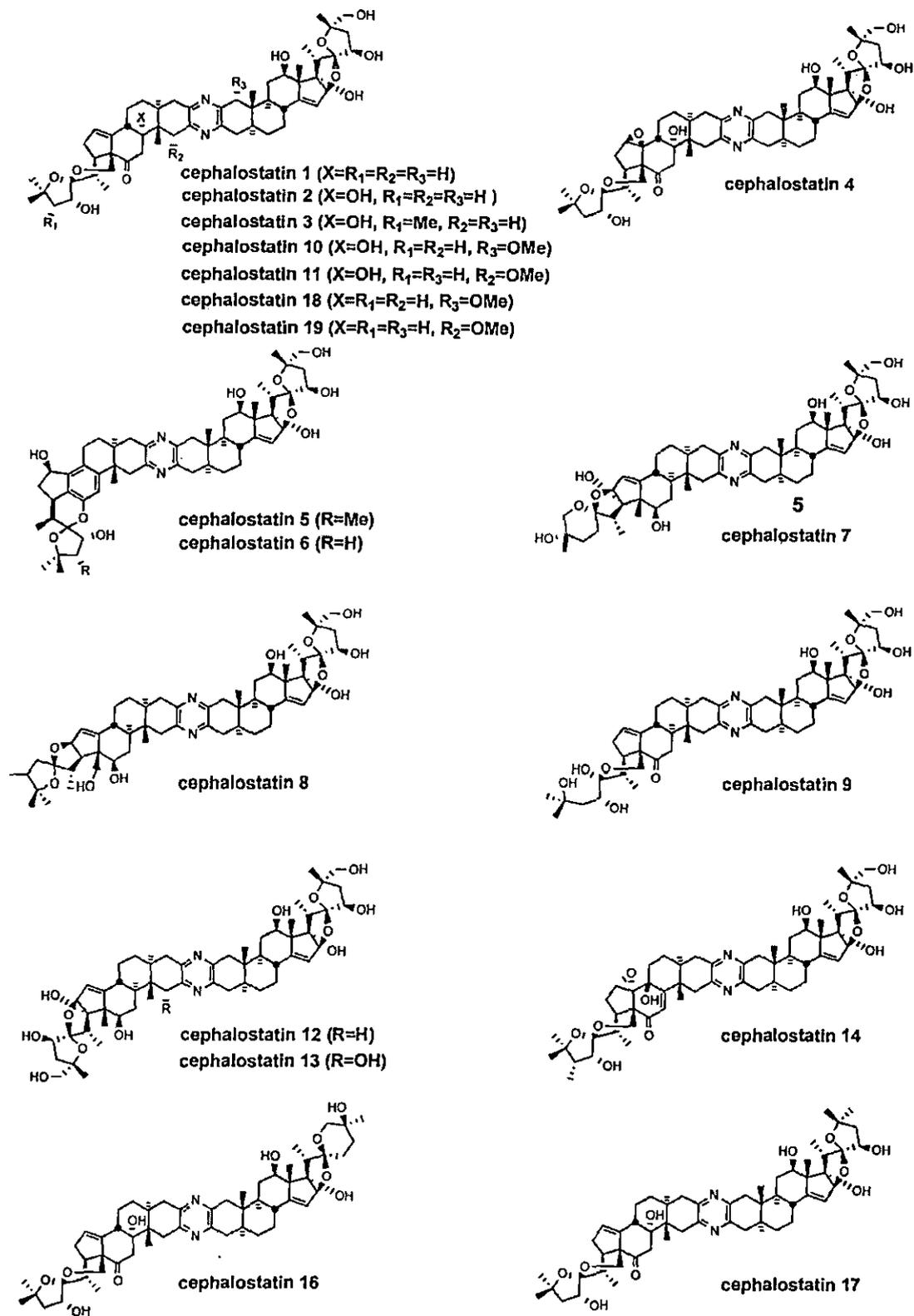


Figure 3 Structures of various naturally occurring cephalostatins

Cephalostatin 1 exhibits extraordinarily high cytostatic activity against a broad spectrum of cancer cell lines and proved to be one of the most powerful cell growth inhibitors ever tested in the NCI. It is considerably more active in vitro than paclitaxel and has an unprecedented mechanism of action.²⁷ More than 18 other cephalostatins were characterized and they showed the same unique cytotoxicity profile in the NCI-60 cell line panel.²⁸ Closely related ritterazines were isolated by Fusetani and co-workers from the Japanese marine tunicate *Ritterella tokioka* and they showed a similar pattern of cytotoxic activity.²⁹⁻³⁰

A great surprise was the isolation of ritterazines, compounds with structure analogous to cephalostatins, from the Japanese tunicates *Ritterella tokioka*.³¹⁻³² They are not related to the hemichordate family, to which *Cephalodiscus* belongs. The presence of the latter was not observed in the samples collected. Both species *C. gilchristi* and *R. tokioka* live in environments inhabited by marine predators and perhaps the production of the alkaloids under discussion is a form of chemical defense. To date, 24 ritterazines were isolated (ritterazines A-Z).³³ Their chemical relationship to the cephalostatins is obvious (some have the identical structure as one of the steroid units), although the same alkaloid has not yet been isolated from both species.

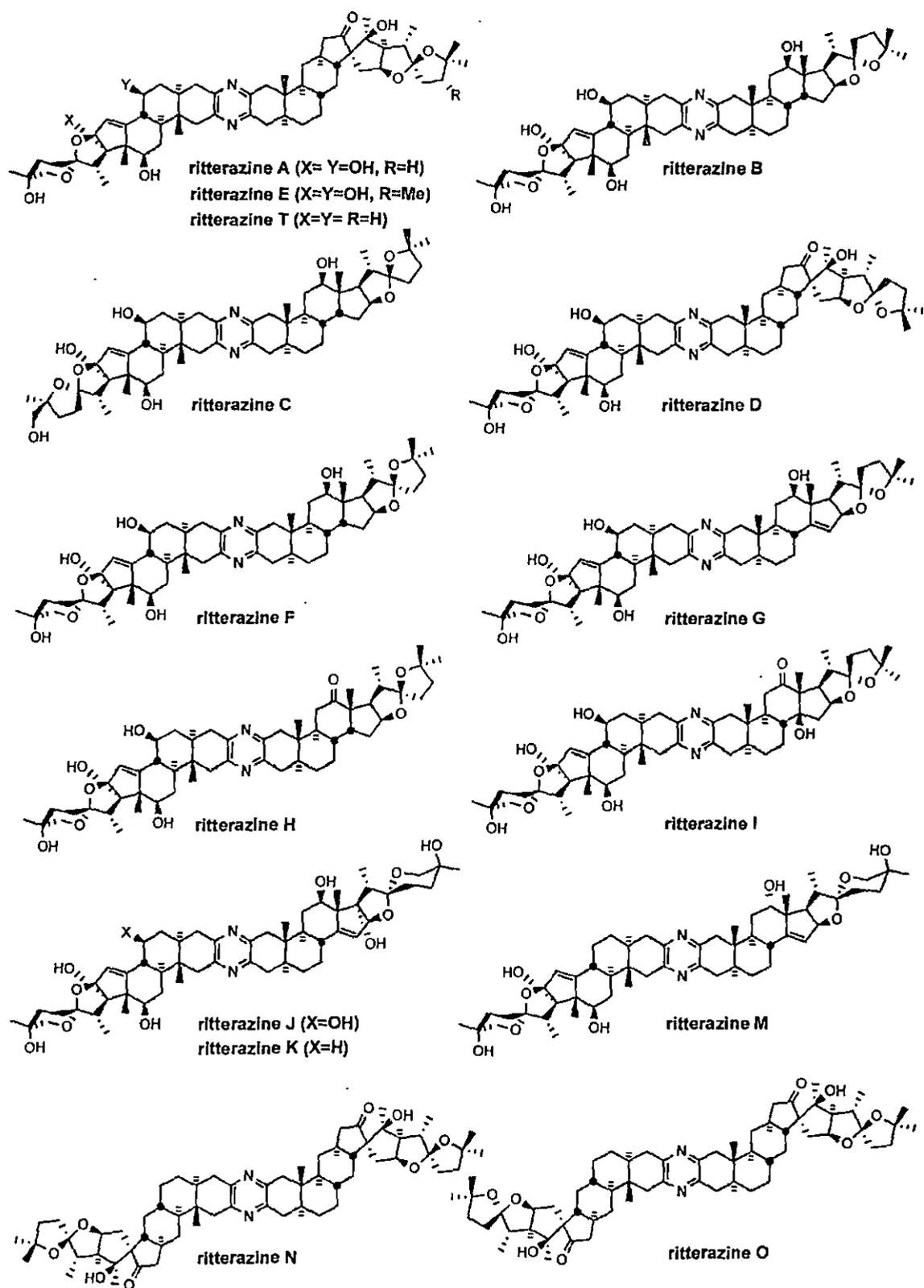
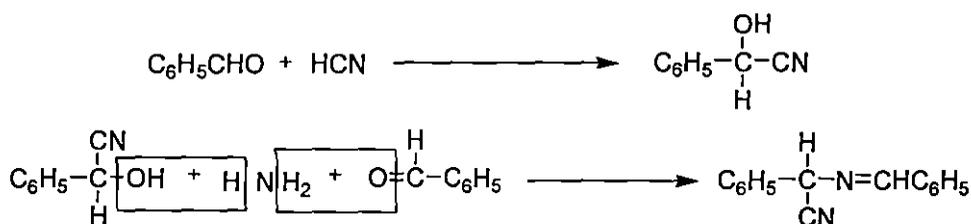


Figure 4 Structures of various naturally occurring ritterazines

Because of these associated wide variety of applications with the pyrazine moieties as well as their natural occurrence fused with steroidal skeleton, their synthesis has remained the goal of many research groups over the years.³⁴⁻⁴⁰

In recording the development of pyrazine chemistry, the author has attempted to preserve the original historical plan, presenting the facts in almost the same manner as they were exposed. This treatment may initially appear somewhat awkward, but it will be seen that the early and apparently unrelated observations form an important part of the entire pattern.

The first procedure for the synthesis of a pyrazine derivative was published by Laurent in 1844.³⁴ Starting with crude benzaldehyde, i.e., benzaldehyde containing some hydrogen cyanide, he treated it with ammonia to obtain what was then known as "benzoylazotid" actually α -benzalamino-phenylacetonitrile:



Scheme I Synthesis of α -benzalamino-phenylacetonitrile

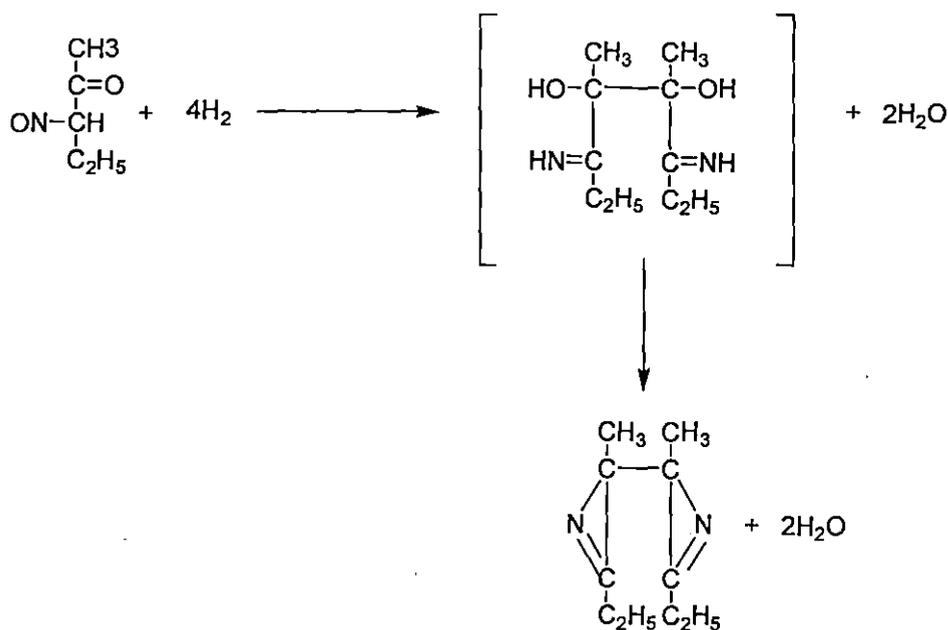
Then, in a fashion typical of that period, he more or less destructively distilled "benzoylazotid," and was able to isolate amongst the products a substance which he called amarone.

Twenty one years later Erdmann reported³⁵ an apparently new substance, benzoinimide, which he obtained, along with some others, by the action of ammonia on benzoin. No further progress was made until 1876, when Stadel and Rugheimer³⁶ published a paper describing the formation of a new compound, isoindol, by the action of ammonia on ω -chloroacetophenone. They postulated that isoindol was the inner anhydride of an amino ketone and was formed according to the following sequence:

advanced the possibility that the compound might contain one less hydrogen atom than was indicated by the formula.

This point was finally clarified by F. P. Treadwell³⁹ (of analytical fame) in the same laboratory in 1881. He had carried out a reduction of (iso) ‘nitrosoethylacetone’ and had isolated a crystalline hydrate. After placing these crystals in a desiccator over calcium chloride he noted that anhydrous oil had formed. Analysis of this oil proved that it possessed one hydrogen atom less than would be anticipated from the formula of the simple ‘inner anhydride’ of an amino ketone. Vapor-density determinations further indicated that the molecular weight was about twice that which would be expected for such a compound.

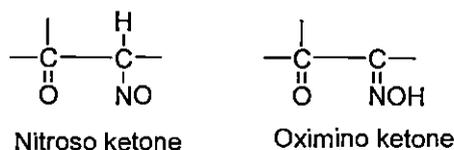
Using the reduction of acetone to pinacol as a model, Treadwell assumed that the reaction for (iso) ‘nitrosoethylacetone’ proceeded as follows:



Scheme 3 Synthesis of nitrosoethylacetone

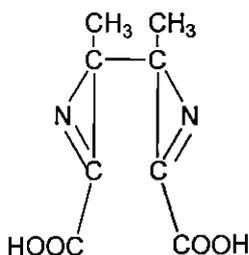
The name ‘ketine’ was applied to this new series of nitrogenous compounds to indicate their derivation from ketones. The simplest member, according to the Treadwell theory, would be that obtained from acetone; and this was specifically termed ketine. The other members were named as derivatives of ketine, so that Gutknecht’s compound was called dimethylketine, and Treadwell’s diethylketine.

The next year V. Meyer submitted a paper⁴⁰ in which he contributed two important suggestions. He first pointed out that although the products of the action of nitrous acid on the ketones were presumably nitroso compounds, they failed to respond to the Liebermann nitroso test. This led to the hypothesis that they were not true nitroso compounds but rather the isomeric oximes:

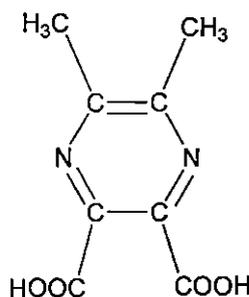


He then considered the reduction of these compounds. After making careful comparisons with the corresponding processes for nitro-amine and ketone-pinacol reductions, he concluded that the former was more analogous. This led to the abandonment of the Treadwell theory.

The first conception of the ketines as ring compounds was put forth, almost immediately, by Wleugel in an article⁴¹ concerned with the reduction of (iso) "nitrosoacetoacetic" ester. Thus, instead of writing the structure for the "ketine" this was obtained as



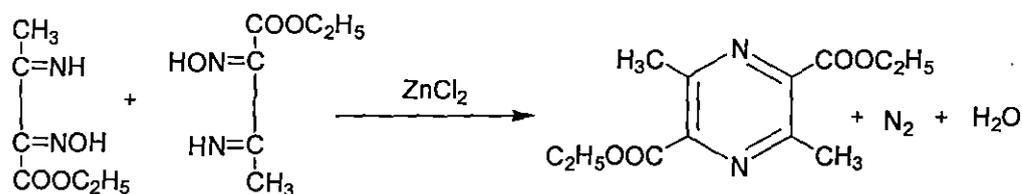
The six-membered ring was made closed by utilizing the C-C bonds involved in the two inner anhydride rings:



This resulted in the formulation of a heterocycle which, as Wleugel stated, could be conceived to be a pyridine in which the CH group para to the nitrogen atom was replaced

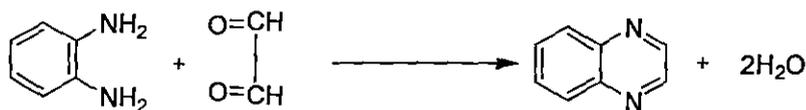
by another nitrogen atom. This is the modern concept of the pyrazine nucleus; but there remained a flaw in its derivation, since Wleugel had still utilized the ketine (pinacol) mechanism.

It remained for L. Oeconomides⁴² in 1886 to demonstrate experimentally that this mechanism was untenable. He attempted to dehydrate Wleugel's diacid to the acid anhydride, a reaction which should have been clearly possible if the two carboxyls were ortho to one another. This was unsuccessful, and the natural conclusion was that these functional groups had been assigned to incorrect positions on the ring. Verification came, together with a proof that the carboxyls were actually para, from the following experiment. Iminoisonitrosobutyric ester was heated with fused zinc chloride. An examination of the only plausible mechanism which could yield a "ketine" indicated that the carboxyl groups in such a compound would unambiguously be situated at the para positions:



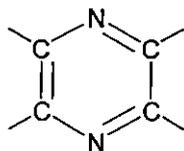
Scheme 4 Oeconomides's synthesis of pyrazine derivative

A small amount of free acid was isolated and compared with Wleugel's; the two were found to be identical. Oeconomides further called attention to the fact that Hinsberg⁴³ had synthesized quinoxaline, a condensed pyrazine, from *o*-phenylenediamine and glyoxal:

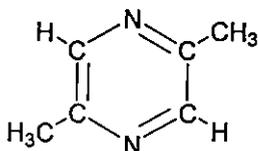


Scheme 5 Hinsberg's method of benzopyrazine synthesis

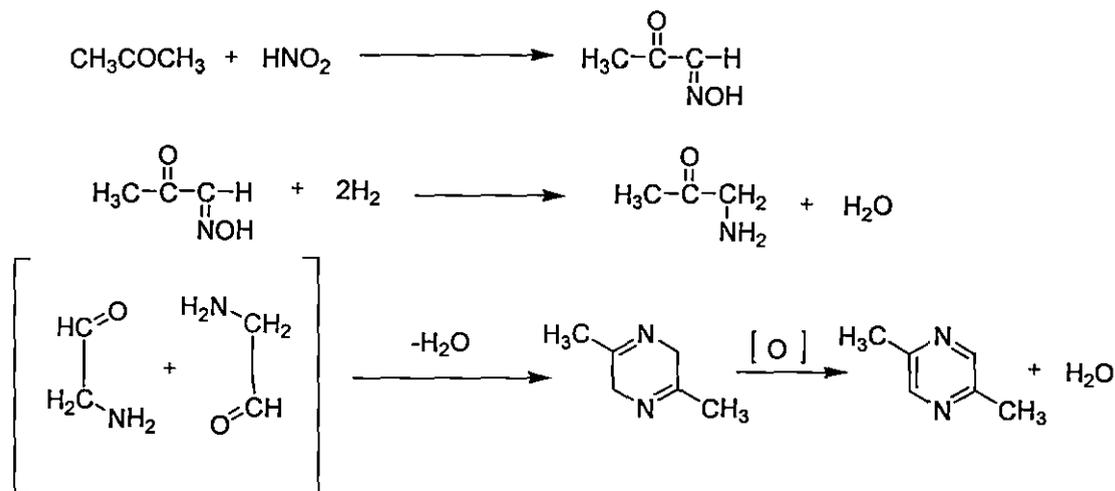
Thus, the ketine nucleus was firmly established as



and what was previously thought to be the simplest member, ketine, in fact was the dimethyl derivative.



The name "pyrazine" was independently suggested for the nucleus in the following year by Mason⁴⁴ and Wolff⁴⁵ in order to point up the correlation with pyridine. It is interesting to note that, in the same paper, Wolff acknowledged that the mechanism, first inferred by Meyer, for the preparation of a pyrazine by the reduction of an isonitroso ketone involved an intermediate amino ketone which immediately condensed with it to yield a dihydropyrazine that was oxidized to the desired pyrazine. Thus, starting with acetone:

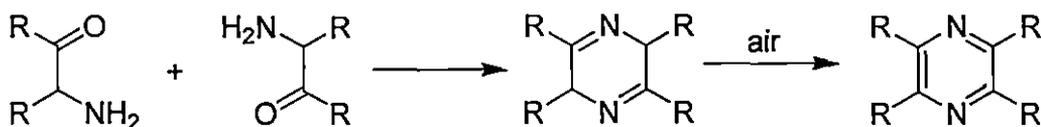


Scheme 6 Mason's synthesis of pyrazine synthesis

V. Meyer⁴⁶ objected to the term "pyrazine" on the grounds that Knorr⁴⁷ had already used it for pyrazole tetrahydride, and, in its stead, proposed the generic name "aldine," since the simplest member would result from the self-condensation of the hypothetical aminoacetaldehyde.

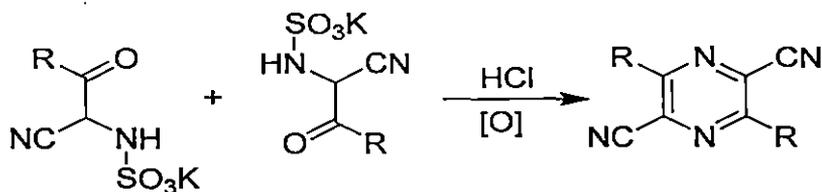
Widman⁴⁸ finally resolved the issues with a systematic nomenclature. He classified as azines those compounds which contained a six-membered ring consisting of nitrogen and carbon atoms. Hence, substances containing two nitrogen atoms in the ring were called diazines. These were further classified, according as to whether the nitrogens were ortho, meta or para, as o-diazines, m-diazines, or p-diazines. Mason condensed these names, respectively, into oiazines, miazines, and piazines; but it seems that he and his associates were the only ones to use this terminology consistently. In the light of the newly elucidated structures of the pyrazines, the results of the early workers were finally clarified.

Historically simple pyrazine was prepared as early as in 1876 by Stadel-Rugheimer.⁴⁹ In the Stadel-Rugheimer synthesis from 2-chloroacetophenone was reacted with ammonia to the amino ketone, then condensed and oxidized to pyrazine. In 1879 Gutknecht modified the process a little. The method was based on this self condensation but differing in the way the alpha-ketoamine is synthesised (the chlorine compound in the above method is a lachrymatory agent).⁵⁰



Scheme 7 Gutknecht's method of pyrazine synthesis

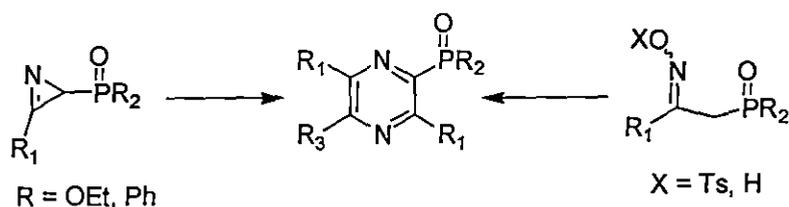
Fifty years later at the beginning of the twentieth century, Gastaldi synthesized pyrazine in a very convenient way.⁵¹⁻⁵²



Scheme 8 Gastaldi's method of pyrazine synthesis

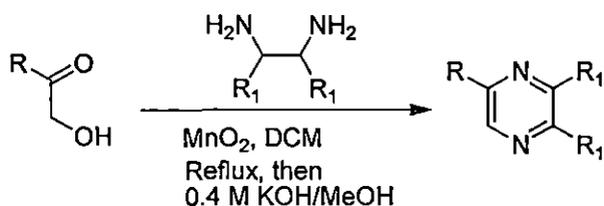
The above protocol served years to the synthetic organic chemistry. Moreover, it was a very good way for the incorporation of nitrile group into the heterocyclic moiety. Among the various methods developed, pyrazine compounds are synthesized by the reaction of diamines with diols in a vapour phase reaction in presence of granular alumina.¹⁰ Catalytic systems such as copper-chromium,¹¹ copper-zinc-chromium,¹² zinc-phosphoric acid-manganese,¹³ and silver¹⁴ are also patented as catalysts for the preparation of 2-methylpyrazine from ethylenediamine and propylene glycol. Pyrazines are also obtained from condensation reaction of diamines and epoxides using copper-chromium catalyst,¹⁵ condensation reaction between alkanolamines,¹⁶ or cyclodehydrogenation of N-(hydroxyalkyl) alkyldiamine¹⁷ using the same catalysts. In the presence of palladium catalyst, dehydrogenation of piperazines yields corresponding pyrazines in high yield.¹⁸

Palacios *et. al.* synthesized tetrasubstituted pyrazines containing two phosphonate groups in positions 2 and 5 and trisubstituted pyrazines containing a phosphonate or a phosphine oxide group in position 2 by thermal treatment of 2*H*-azirine-2-phosphonates and -phosphine oxides.⁵³ This method involved simply heating the substrates at 80 °C without any added solvent, catalyst or any solid inorganic support.



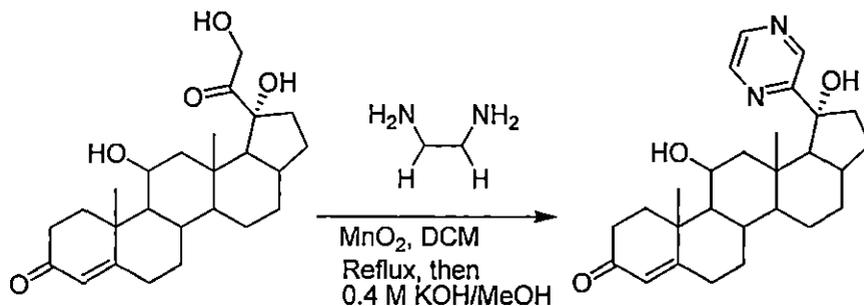
Scheme 9 Palacio's method of pyrazine synthesis

Recently, synthesis of pyrazines from α -hydroxy ketones and 1,2-diamines via MnO_2 catalyzed tandem oxidation process under refluxing conditions has been reported by Raw *et. al.* In this process α -hydroxy ketones underwent MnO_2 mediated oxidation followed by in situ trapping with aromatic and aliphatic 1,2-diamines to give quinoxalines or dihydropyrazines respectively in a one pot manner. Pyrazines were prepared in refluxing DCM with ten equivalents of MnO_2 and 0.4 M KOH solution.



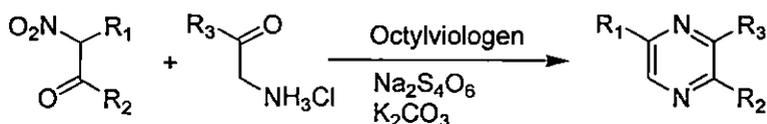
Scheme 10 Raw's method of pyrazine synthesis

In a dramatic move, they also reported the preparation of pyrazine derivatives of hydroxycortisone (a steroid) following their developed method in refluxing DCM with ten equivalents of MnO_2 and 0.4 M KOH solution. But the yield was very much poor, only 10 % isolated yield.⁵⁴



Scheme 11 Raw's method of synthesis of pyrazine derivative of hydroxycortisone

In the communication the authors did not reported the synthesis of pyrazine derivatives of terpenoids and also the developed process required high loading of the corrosive catalyst. Elmaaty *et al.* described a new regiochemically controlled synthetic method and the optimal reaction conditions of pyrazine synthesis from α -nitro ketones. According to their report α -nitro ketones can be transformed selectively into trialkyl-substituted pyrazines via reaction with α -amino ketones using octylviologen as an electron-transfer reagent in basic condition.⁵⁵

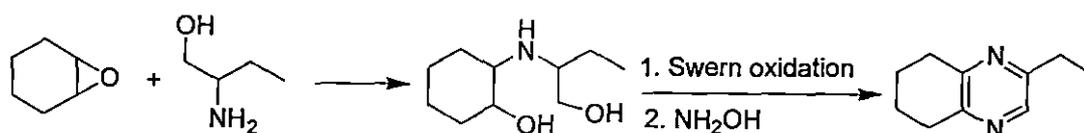


Scheme 12 Elmaaty's synthesis of pyrazine catalyzed by octylviologen

Although the process was very much utilizable for the synthesis of substituted pyrazines in good to excellent yields, but the syntheses of unsymmetrically substituted pyrazines

were a challenge to this developed method. Notably, it did not report the synthesis of pyrazine derivatives of naturally occurring steroids and terpenoids.

Taber *et al.*⁵⁶ in an interesting mode reported the preparation of pyrazine from epoxides under very mild way. Opening of the representative epoxide with 1,2-amino alcohols delivered the amino diols. The product diols were then oxidized under Swern conditions. The aminodiketones so prepared were not isolated, but were condensed directly with hydroxylamine to give the substituted pyrazines.



Scheme 13 Douglass's method of pyrazine synthesis from epoxides

In the experimental condition they⁵⁶ heated cyclohexene oxide and 2-amino-3-phenyl-1-propanol under solvent-free conditions, but after 7 days only starting materials were visible by tlc. While LiClO_4 and $\text{BF}_3 \cdot \text{OEt}_2$ failed to activate the epoxide, the addition of a catalytic amount of $\text{Yb}(\text{OTf})_3$ to the reaction facilitated an easy transformation to the amino diol. This is thought to be due to the oxophilicity of the early lanthanides. Further investigations later showed that identical loading of LiBr under solvent-free conditions effected an even faster transformation to the amino diol. When an activated epoxide was used, additions were carried out without catalyst. Indeed, if catalysts were added, an increased amount of the undesired regioisomer was observed.

The method of bubbling oxygen under refluxing condition⁵⁷ suffers from scientific drawbacks.

Synthetic transformations of natural compounds for the purpose of developing biologically active agents have become the basis of the actively advancing scientific direction of perfect organic synthesis and medical chemistry. The greatest attention of researchers is attracted by native compounds with reliably established biological activity. An attractive factor is the availability of natural metabolites due to frequent occurrence of the sources and technological reasonableness of the methods of isolation of natural substances. Widely known examples of medically successful transformations of steroids, antibiotics of penicillane and cephalosporane groups, alkaloids of morphinane series

have recently been supplemented with the modificants of cacinostatic taxol, anti-glaucoma terpenoid forskoline, antiplasmodium medicine artemisinin and other preparations. Compounds combining availability with valuable biological activity are frequent in the class of triterpenoids. Speaking of purposeful synthetic transformations of triterpenoids for medical chemistry, perhaps the most advanced object is the glycoside of licorice, glycyrrhizinic acid and its aglycones.⁵⁸ The recent two decades gave us grounds to expect that the preparations based on triterpenoids of lupane series could be involved in therapy of a number of diseases. The expectations are undoubtedly connected with betulinic acid (3 β -hydroxy-20(29)-lupene-28-oic acid) **1**, a triterpenoid surprisingly widespread in nature and easily available in almost any amount. The number of publications dealing with valuable biological activity of **1** and its natural and synthetic derivatives is increasingly growing. Numerous experimental and epidemiological studies have shown that several plant derived natural products may serve as effective anticancer drugs.⁵⁹ It was also documented in most of the literatures that suitable derivatives of naturally occurring triterpenoids often showed greater biological activities in comparison to that of the naturally occurring triterpenoids. In this regard researchers around the globe had so far reported the preparation of pyrazine derivatives of naturally occurring triterpenoids.

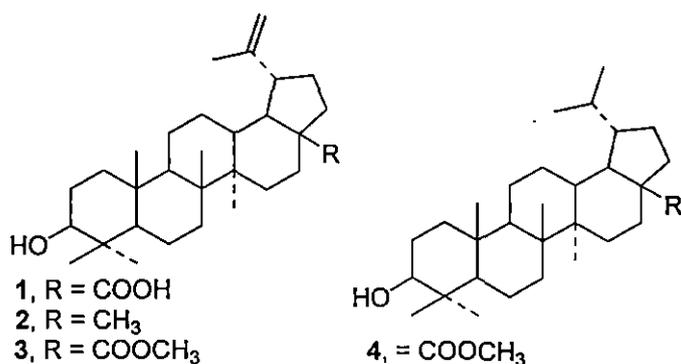
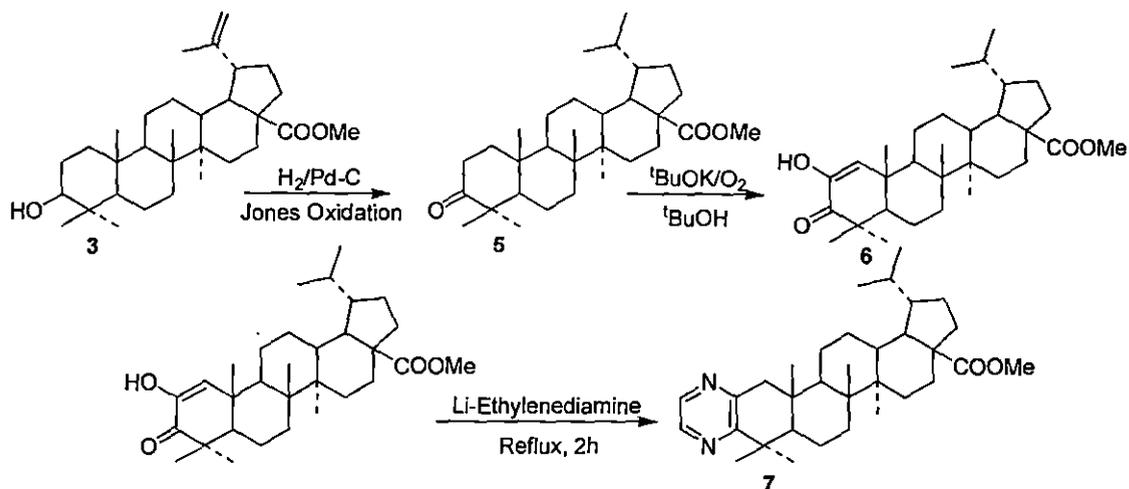


Figure 4 Lupane derived different triterpenoids

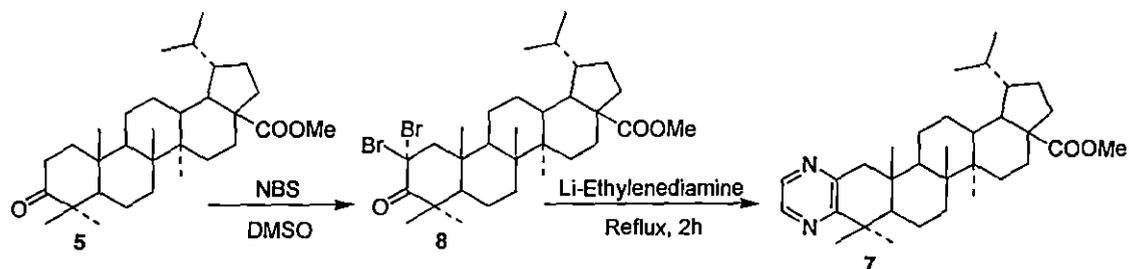
In an early attempt Pradhan *et al.* had reported the synthesis of pyrazine derivatives (scheme 7) of lupeol, **2** and methyl dihydrobetulinic acid, **4** in refluxing lithium-ethylenediamine in an expeditious manner.⁶⁰ Autooxidation of the respective triterpenoidal ketones (**5**) afforded the corresponding diosphenols, **6** that were then refluxed with excess ethylenediamine in presence of small pieces of lithium metals for 2

hours. After cooling to room temperature and usual work up followed by chromatographic purification afforded the corresponding pyrazine (7) derivatives with 52% yield. In this case, metallic lithium not only induced the required condensation of the diosphenol and ethylenediamine but also brought about the aromatization of the condensed compound in the same reaction media as well as in one pot.



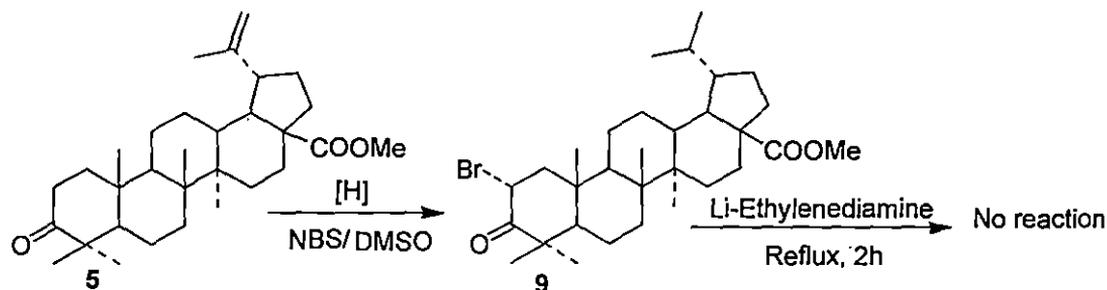
Scheme 14 Pradhan's synthesis of pyrazine derivatives of lupane triterpenoids using metallic lithium as the condensation agent

Later on the same authors also reported⁶¹ another method of synthesis of 7 derivatives from 2,2-dibromo-28-carbomethoxyupane-3-one (8) in the same way. 2,2-dibromolupanes were prepared from the corresponding ketones in a reaction with NBS (*N*-bromosuccinimide) in DMSO (dimethyl sulfoxide) under dark condition.



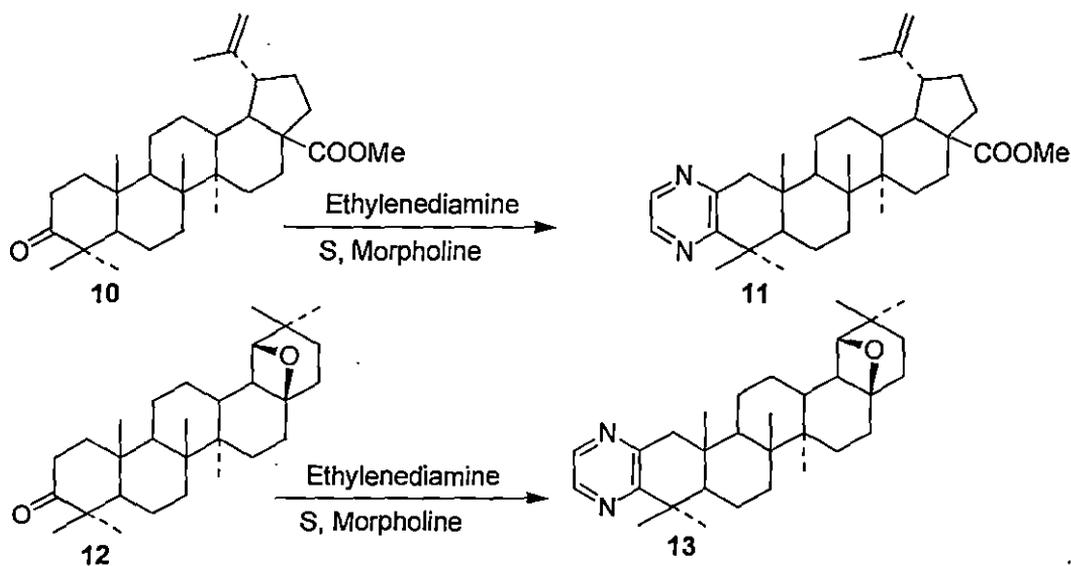
Scheme 15 Pradhan's synthesis of pyrazine derivatives of lupane triterpenoids from 2,2-dibromo-triterpenoid derivatives

Similar transformations to the corresponding pyrazine derivatives starting from monobromo triterpene derivatives *viz* 2 α -bromo-methylidihydrobetulonate (9) derivative of lupanone were also attempted but were not successful.



Scheme 16 Failure of pyrazine synthesis from monobromo triterpenoid derivatives

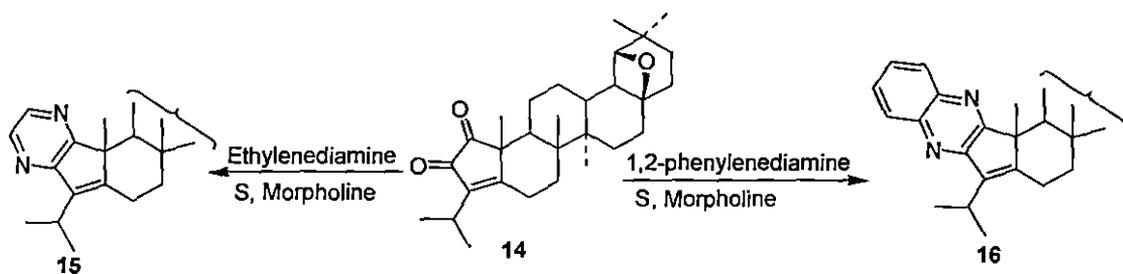
Very recently Urban *et al.*⁶² has reported the synthesis of pyrazine derivatives of various triterpenoids from the respective ketones in one pot. In the experimental procedure they refluxed the ketones with amorphous sulfur using morpholine as the solvent efficiently for 4 hours. After the usual workup and purification over silica gel column they yielded the corresponding pyrazine derivatives.



Scheme 17 Urban's method of pyrazine synthesis at ring A of pentacyclic triterpenoids

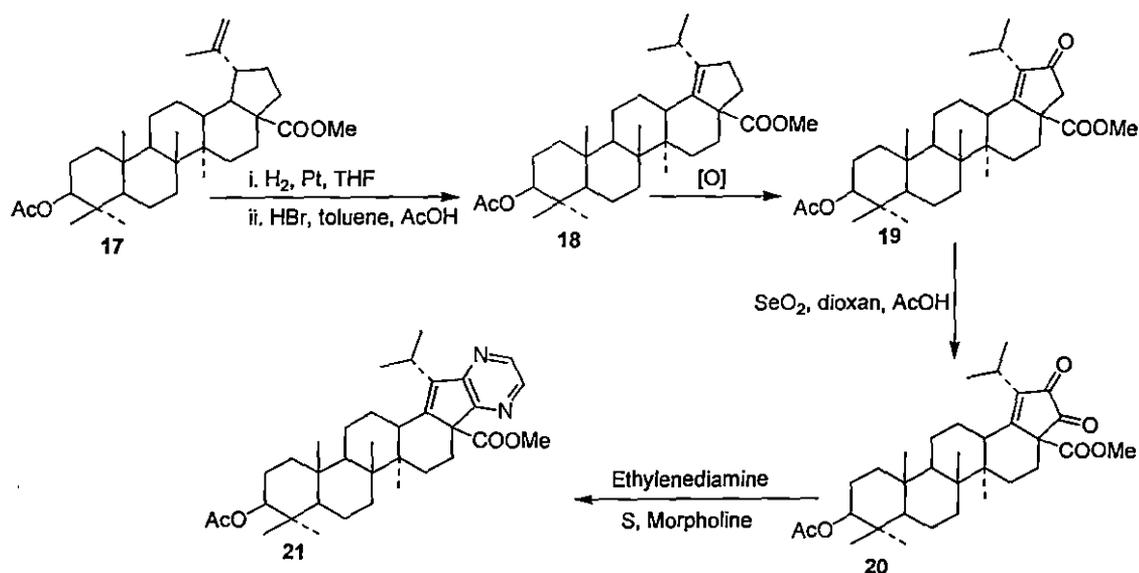
In an attempt in the same communication,⁶² they suitably modified the ring A of allobetulene into a cyclopentane ring containing an isopropyl group by a series of transformative reactions. Bromination and subsequent debromination introduced a double bond in the cyclopentane ring. Afterward, a series of reactions were performed to

introduce a 1,2-diketone that then transformed chemically to the corresponding pyrazine derivative. Here also they used amorphous sulfur as the oxidant and morpholine as solvent under refluxing condition for the oxidative condensation followed by in situ aromatization. Although the yields were not good enough, the developed process was pretty good to introduce the pyrazine nucleus into the ring A of the pentacyclic triterpene skeleton.



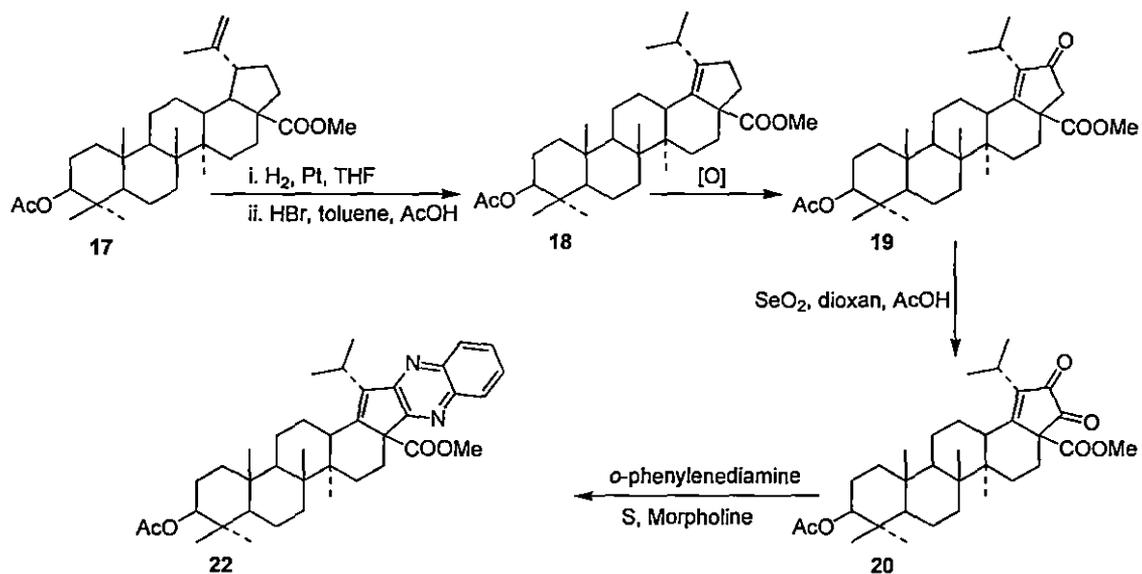
Scheme 18 Unban's method of pyrazine synthesis into transformed allobetulin derivatives

Following the procedure they⁶² not only incorporated the pyrazine ring into the ring A of pentacyclic triterpenoids, but also able to incorporate the same into the ring E of the pentacyclic triterpenoids in the following series of transformative reactions.

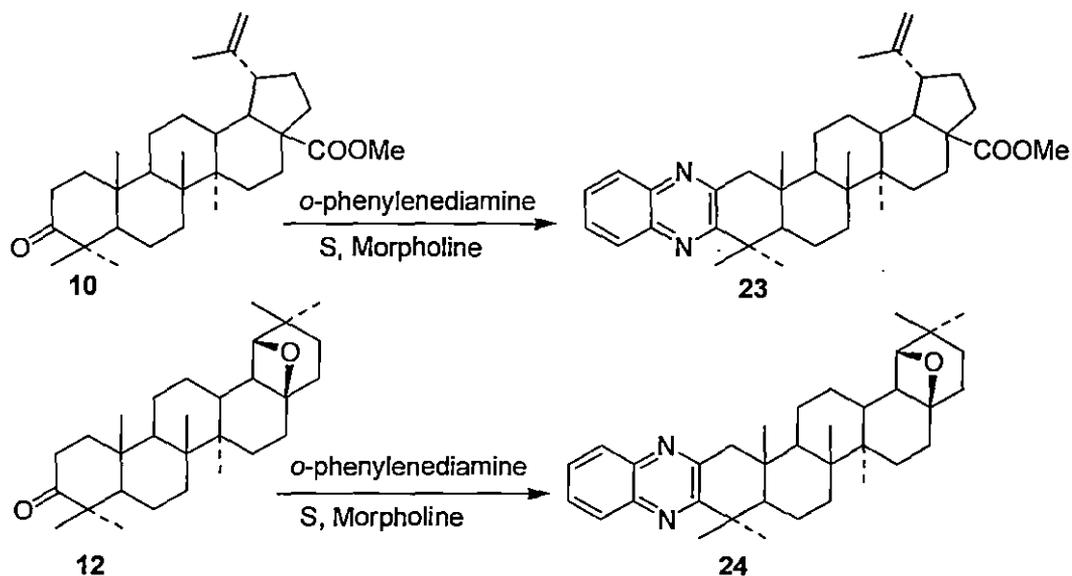


Scheme 19 Urban's method of pyrazine synthesis at ring E of pentacyclic teriterpenoids

In this same communication⁶² they have reported also reported the formation of benzopyrazines or quinoxaline derivatives of triterpenoids following the same method developed by them.



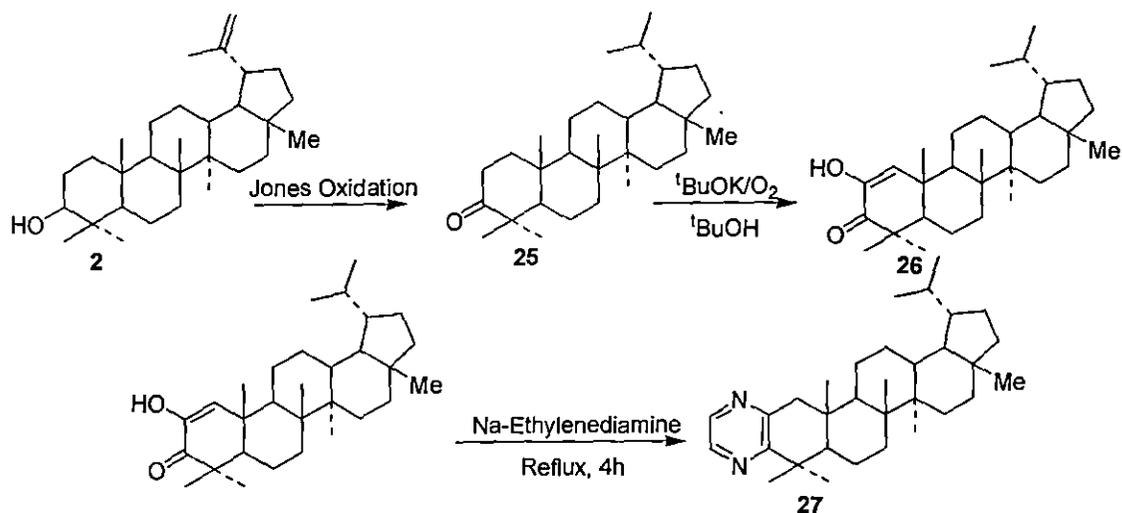
Scheme 20 Urban's method of benzopyrazine synthesis at ring E of pentacyclic teriterpenoids



Scheme 21 Urban's method of benzopyrazine synthesis at ring A of pentacyclic teriterpenoids

They method developed by Urban *et al.* is a slight modification to that developed by Sejbal *et al.*⁶³ as early as in 1986. They claimed that the purpose of their synthesis was to synthesize a series of pyrazine derivatives of pentacyclic triterpenes in a modified methodology. It although seemed that the method developed by Urban *et al.* was very efficient, the method used toxic amorphous sulfur and highly corrosive morpholine as the solvent which were against the principles of green chemistry as well as limits its widespread application as a modern green synthetic tool for the incorporation of pyrazine ring into ring A of triterpene skeleton.

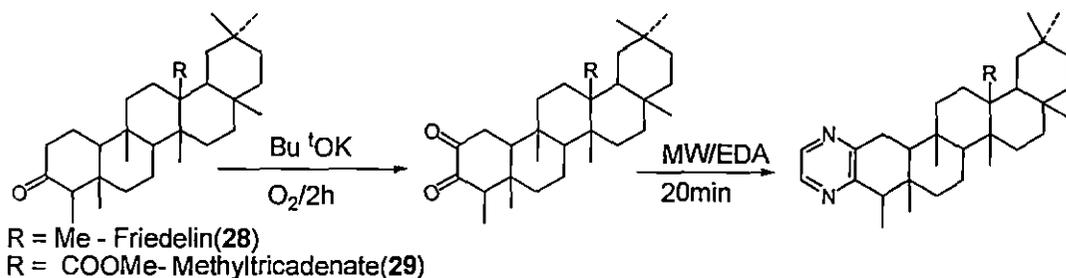
Ghosh *et al.*⁶⁴ in a good attempt to modify the work of Pradhan *et al.* carried out the same series of reactions (Scheme 8) to prepare the pyrazine derivatives of methyl dihydrobetulinate (4) and lupeol (2). In their modification they used metallic sodium instead of metallic lithium for the purpose of tandem condensation-aromatization. In their developed process they used a slight excess of ethylenediamine with the corresponding diketones or diosphenols in presence of metallic sodium without any other organic solvent and isolated the respective pyrazine derivatives in moderate to good yield. In that communication they used cheaper metallic sodium instead of lithium and therefore, the ease of toxicity associated with metallic lithium was eliminated and that their report is a forward footstep to the direction of “Green Chemistry”.



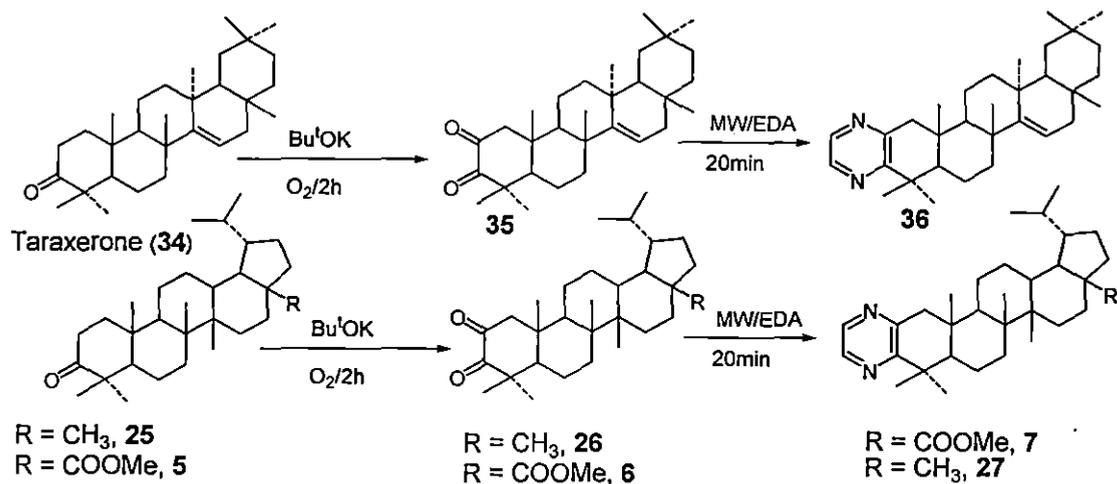
Scheme 22 Synthesis of pyrazine derivatives of lupanol by Ghosh *et al.*

Although their developed protocol is a footstep towards green chemistry, the method suffers from certain drawbacks from the present perspective of green chemistry. It is not atom economic, not energetically safer technique and the time required for the process was high too. Too much ethylenediamine was used to carry out the transformation.

To overcome the above limitations Ghosh *et al.*⁶⁵ attempted another procedure (Scheme 13 and 14) in which they used microwave irradiation as the energy source remaining all other things as same as that reported by Pradhan *et al.*⁶² The newly developed process is energetically efficient one and the duration of the reaction is very short too, only twenty minutes is sufficient to complete the reactions. In this method they were able to synthesize the pyrazine derivatives of five different pentacyclic triterpenoid molecules which include two examples from lupane skeleton, two from friedelan skeleton and one from taraxar skeleton. The potential application of microwave technology (MW) in organic synthesis is increasing⁶⁵ rapidly because of the reaction simplicity, less polluting and minimum reaction time providing rapid access to large libraries of diverse molecules. This technology has been implemented since the middle of 1980s in the field of organic chemistry.



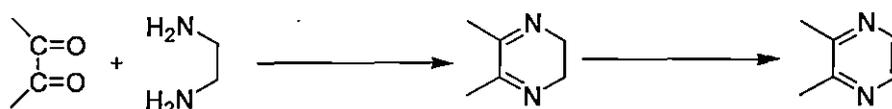
- 30 = Diketo friedelin
- 31 = Diketo derivative of the methyl ester of tricadenic acid
- 32 = Pyrazine derivative of friedelin
- 33 = Pyrazine derivative of methyltricadenate



Scheme 23 Synthesis of pyrazine derivatives of friedelin, lupanol and dihydro methylbetulonate by Ghosh *et al.* under microwave irradiation

Although the methods are useful for the incorporation of the pyrazine nucleus in the triterpenoid skeleton, but these methods do not satisfy completely the modern trends in “Green Chemistry”. The use of excess corrosive ethylenediamine limits its versatile application as a general tool for pyrazine synthesis. Although in modern days microwave irradiation has emerged as a safe source of energy to perform organic reactions, reactions performed at room temperature are always preferable. Additionally all the methods described above are not atom economic and in most of the cases the prepared side products are not environmentally safer.

Strategically, direct condensation reaction of 1,2-diketones with 1,2-diamine is the most straightforward as well as the classical route for the preparation of pyrazines via dihydropyrazine intermediates.



Scheme 24 Direct strategic approach to pyrazine

Although, a number of methods are reported in literature for the synthesis of pyrazine, none of them was found to be effective because of poor yield, harsh reaction condition and tedious work-up procedures.²¹ Attempts to carry out dehydrogenation under a variety of milder and more convenient laboratory procedures were not successful. Although, some of them are apparently useful, most of them are limited by long reaction time, low yields, and use of toxic solvents or heavy metals as the catalyst.

“*Green chemistry*”, also called sustainable chemistry, is a philosophy of chemical research and engineering that encourages the design of products and processes that minimize the use and generation of hazardous substances. Whereas environmental chemistry is the chemistry of the natural environment, and of pollutant chemicals in nature, green chemistry seeks to reduce and prevent pollution at its source. In 1990 the Pollution Prevention Act was passed in the United States. This act helped create a *modus operandi* for dealing with pollution in an original and innovative way. It aims to avoid problems before they happen. The main focus is on minimizing the hazard and maximizing the efficiency of any chemical choice. Paul Anastas, then of the United States Environmental Protection Agency, and John C. Warner developed 12 principles of green chemistry, which help to explain what the definition means in practice. The principles cover such concepts as:

- ❖ the design of processes to maximize the amount of raw material that ends up in the product;
- ❖ the use of safe, environment-benign substances, including solvents, whenever possible;
- ❖ the design of energy efficient processes;
- ❖ the best form of waste disposal: not to create it in the first place.

From green chemistry point of view a synthetic protocol should have the above criteria. All the existing methods for the synthesis of pyrazine derivatives although synthetically useful, but all of them suffer a number of limitations if we keep on looking from green chemistry point of view. Therefore, development of mild, efficient and environmentally

benign method for synthesizing pyrazine derivatives keeping the 12 points of green chemistry principles has been a major challenge in contemporary organic synthesis

Chapter II

Section A

Synthesis of pyrazine derivatives of triterpenoids

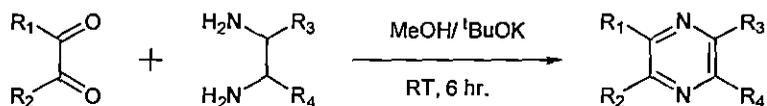
1. Introduction

Heterocyclic compounds occur widely in living organisms and many possess a broad range of biological activities. Researchers have also demonstrated the interesting biological activities of many natural terpenoids.⁶⁶ Triterpenoids are widely distributed in nature, and recent reports have demonstrated the interesting biological activities of this class of natural products.⁶⁷ However, triterpenoids possessing a nitrogen containing heterocycle condensed to an isoprenoid skeleton are rare. One example of less common isoprenoid is cephalostatin, isolated⁶⁶ from the sea worm *Cephalodiscus gilchristie*. This bis-steroidal pyrazine is highly cytotoxic,⁶⁸ which sparked interest in the synthesis of similar structures.⁶⁹⁻⁷¹ Since compounds containing *N*-heterocyclic moieties have found numerous applications as pharmaceuticals as well as in medicines, it is anticipated that incorporation of a pyrazine ring into the molecule of a pentacyclic triterpenoid may induce or enhance its biological activity. With this view in mind and in continuation of our studies on the transformative reactions of triterpenoids, we report herein the incorporation of pyrazine ring into ring A of the pentacyclic triterpenoids of lupane and friedelan skeleton (Schemes 1 and 2). Additionally, to date no parallel study have been reported that utilizes a green protocol for the synthesis of pyrazine derivatives of triterpenoids, a fact that motivated us to develop a green protocol for the synthesis of pyrazine derivatives from betulinic acid and friedelin. In order to make a series of pyrazine derivatives of triterpenoids and to evaluate their biological activities the present author has developed a novel green protocol for the same. The results of these investigations have been reported in the following sections.

2. Present Investigation

The present protocol comprises a direct condensation between the respective 1,2-diketo compounds with 1,2-diamines in aqueous methanol catalyzed by potassium tertbutoxide (t-BuOK) at room temperature. This high yielding process did not require any added expensive catalyst or bubbling of oxygen⁵⁷ at higher temperature (Scheme 3). Detection of dihydropyrazine along with pyrazines as well as the starting material at an early stage of the reaction indicated that the developed method involved aromatization following a very simple one pot route via the formation of non aromatic dihydropyrazine intermediate, removing any additional steps as reported in literature.

In order to develop the above convenient protocol we first chose the reaction of benzil, a common 1,2-diketone, with ethylenediamine (1:1) as the model case followed by optimisation of the reaction condition (Table 1).



Scheme 24 t-BuOK catalyzed synthesis of pyrazine derivatives in wet methanol

For the one pot synthesis of pyrazine derivatives we studied a good number of bases in various solvents including mixture. On this basis the combination of aqueous methanol-t-BuOK was found to be the best (Table 1) for the said transformation.

Table 1 Optimization of pyrazine synthesis reaction condition

Entry	Solvent	Base	Temperature	Time(hr.)	% Yield	
					%Dihydropyrazine	%Pyrazine
1	Water		RT	18	30	-
2	Water	Et ₃ N	"	18	30	-
3	Methanol	BuO ^t K	"	8	45	76
4	Acetonitrile	"	"	18	65	-
5	DCM	K ₂ CO ₃	"	18	20	-
6	DMSO	"	"	18	45	-
7	DMF	"	"	48	65	-
8	Methanol	"	"	8	24	52
9	Methanol	NaOAc	"	18	78	-
10	Isopropanol	Et ₃ N	"	18	56	-

% Yield refers to the isolated yield of all the compounds after chromatographic separation

But in order to establish a greener and cost effective protocol, we have optimized further both the volume of methanol as well as the molar proportion of base (in case of pyrazine preparation) in a reaction of 1:1 feed ratio of benzil and ethylene diamine. After a number of experiments we could able to minimise the proportion of base as well as the volume of solvent in the feed. From figure-1 it is clear that although there is a gradual increase in the % yield of the product but, after a certain voloume (only 3 mL methanol with respect to 1:1 millimolar ratio of diketone and diamine) there is no significant increase in % yield of the product. This observation clearly indicated that the reaction sequence depends upon the amount of the solvent in the reaction mixture taken during the course of the reaction at a given time for a single set of reaction.

Study towards the optimization of the amount of added base did reveal that only a catalytic amount of the t-BuOK in aqueous methanol was sufficient to carry out the reaction efficiently at room temperature within 6 hours in good yield. The present protocol utilized stoichimetric amount (1:1) of reactants, the reagent and catalytic amount of t-BuOK. It is also energetically efficient and did not produce any byproduct and hence it is indeed a green protocol. The generality of the developed process was justified by its successful application on a number of 1,2-diketones effectively⁷²

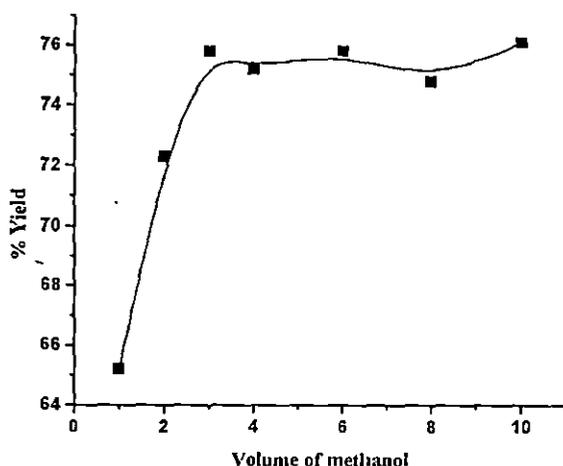
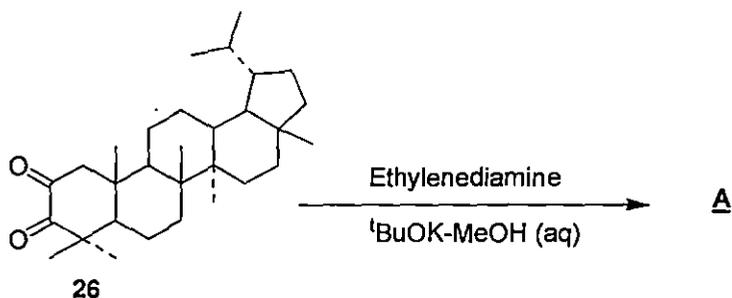


Figure 5 Variation of molar proportion of base in a 1:1 feed ratio of benzil and ethylenediamine

This general optimized protocol was then utilized to prepare some pyrazine derivatives of naturally occurring triterpenoids in the following way.

2.1 Preparation of pyrazine derivative of 2,3-diketo lupane (27)

2,3-diketo lupane (26) prepared by the autoxidation⁷³ of lupanone (see experimental), on treatment with ethylenediamine, EDA in aqueous methanol catalysed by t-BuOK afforded a single compound. Purification of the compound over a column of silica gel followed by crystallization from chloroform-petroleum ether furnished fine needle shaped crystals of compound **A**, analysed for $C_{32}H_{50}N_2$, mp 220-21°C, $[\alpha]_D + 24^\circ$.



Scheme 25 Treatment of 2,3-diketolupane with EDA in presence of t-BuOK in methanol

IR spectrum of the compound (Figure 6) showed peaks at 1650, 1120 cm^{-1} . It did not respond to the TNM (tetranitro methane) test for unsaturation. Its UV spectrum showed

peaks at 272 nm ($\epsilon = 5700$) and 278 nm ($\epsilon = 5600$). In the mass spectrum (Figure 9) of compound A it showed a molecular ion peak at m/z 462 ($M+1$). In elemental analysis compound A showed 83.10% C, 10.81% H. Thus from elemental analysis and mass spectrum, the molecular formula of compound A was established to, $C_{32}H_{50}N_2$. In 1H NMR spectrum (Figure 7) out of six tertiary methyl groups, each appeared as a sharp singlet at δ_H 0.78 (s, 3H), 0.83 (s, 3H), 0.98 (s, 3H), 1.11 (s, 3H), 1.29 (s, 3H) and 1.31 (s, 3H). Two secondary methyl groups each appeared as a doublet at δ_H 0.77 (d, 3H, $J = 7$ Hz) and 0.86 (d, 3H, $J = 7$ Hz). Two doublets at δ_H 2.45 and 3.04 with geminal coupling of 16 Hz could be assigned to the methylene protons at C-1 that has no neighboring protons. Two olefinic protons that appeared at δ_H 8.41 and 8.27 as doublet with $J = 3$ Hz. The former being splitted by long range 1,4 coupling with C-1 proton appearing at δ_H 2.45. The large downfield shift of these protons indicates that these are the aromatic protons deshielded by the hetero atom nitrogen.⁷⁴

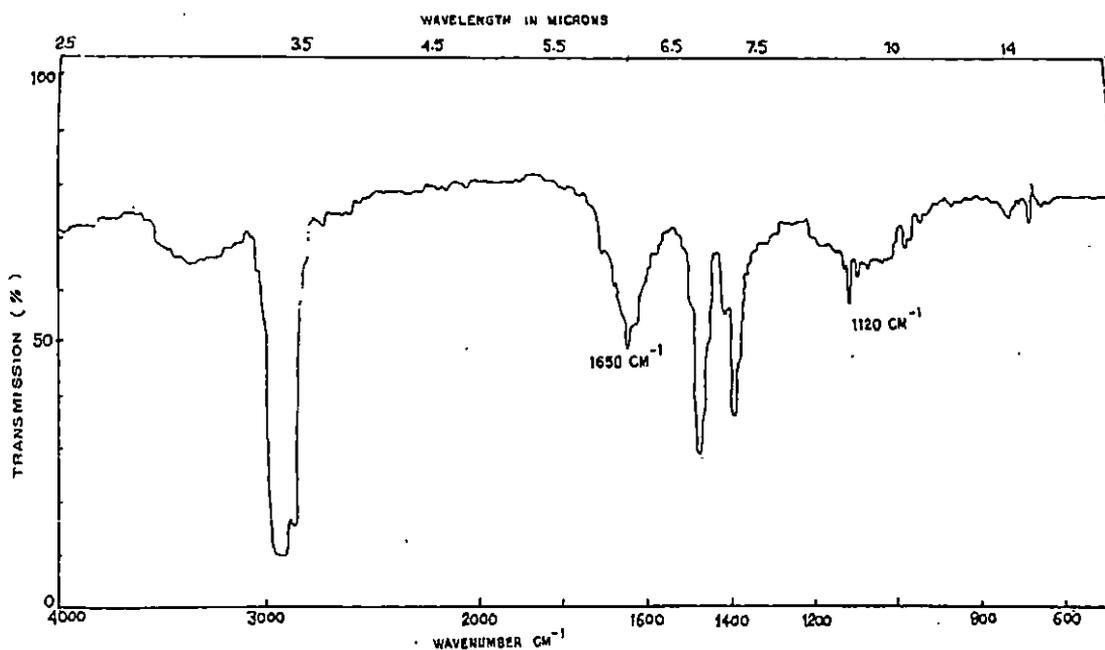


Figure 6 IR spectrum of pyrazine derivative of lupane

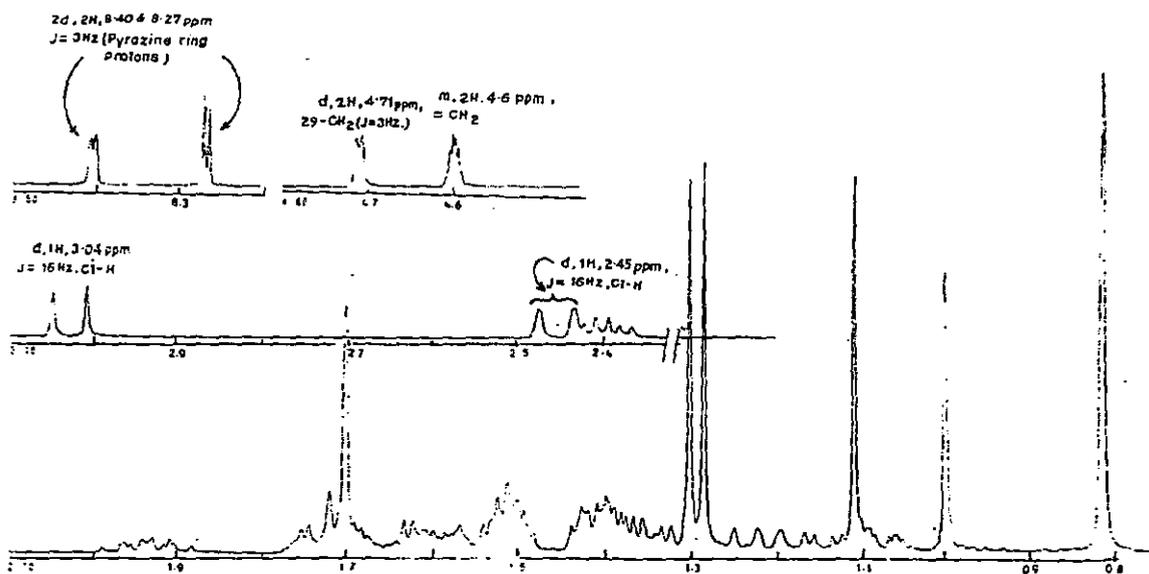


Figure 7 ¹H NMR spectrum of pyrazine derivative of lupane (27)

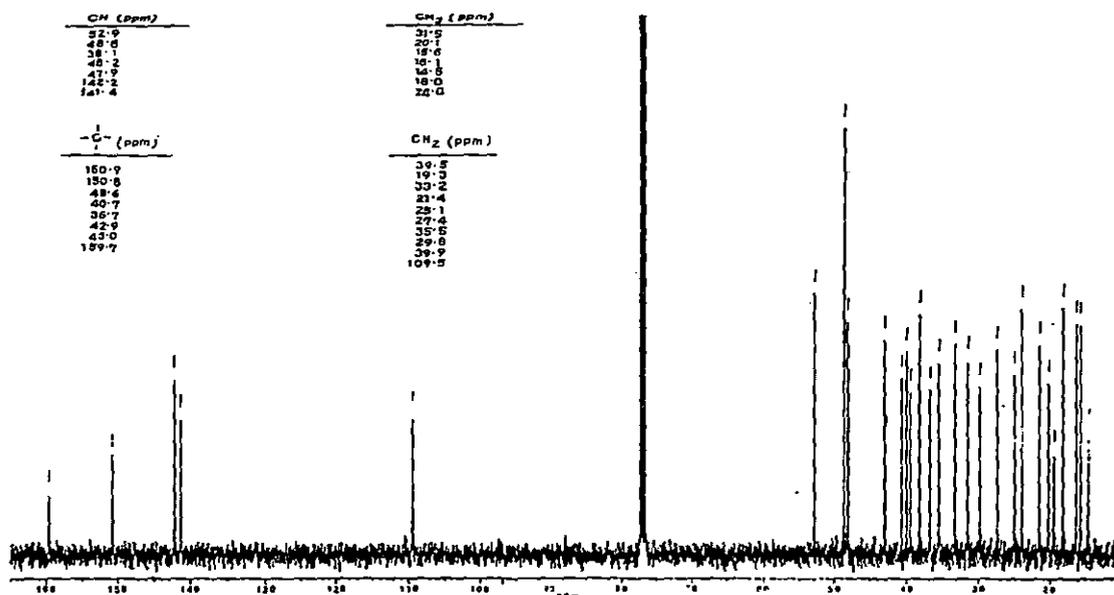


Figure 8 ¹³C NMR spectrum of pyrazine derivative of lupane (27)

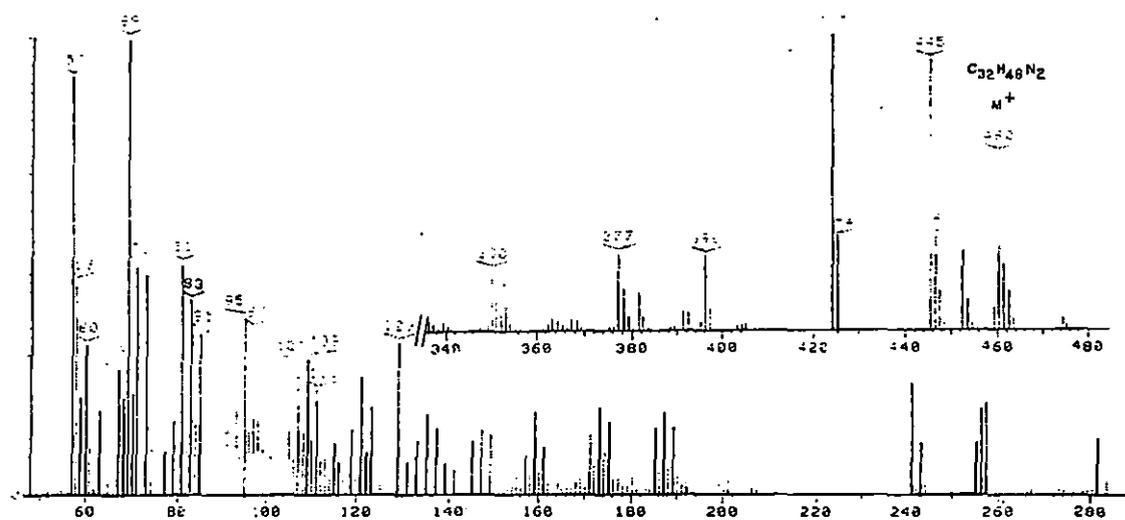
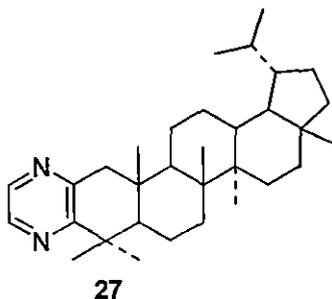
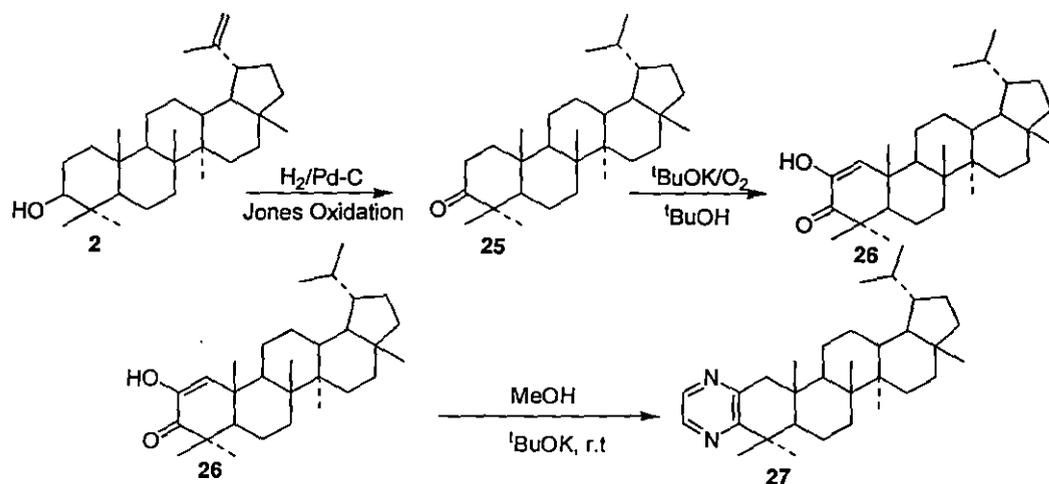


Figure 9 Mass spectrum of pyrazine derivative of lupane (27)

^{13}C NMR spectrum of compound **A** (Figure 8) accounted for all the 32 carbon atoms. The ^{13}C NMR data of compound **A** is represented along with those of lupanone in table 1. Thus from spectral analysis the exact structure of compound **A** has been assigned as structure **27**.

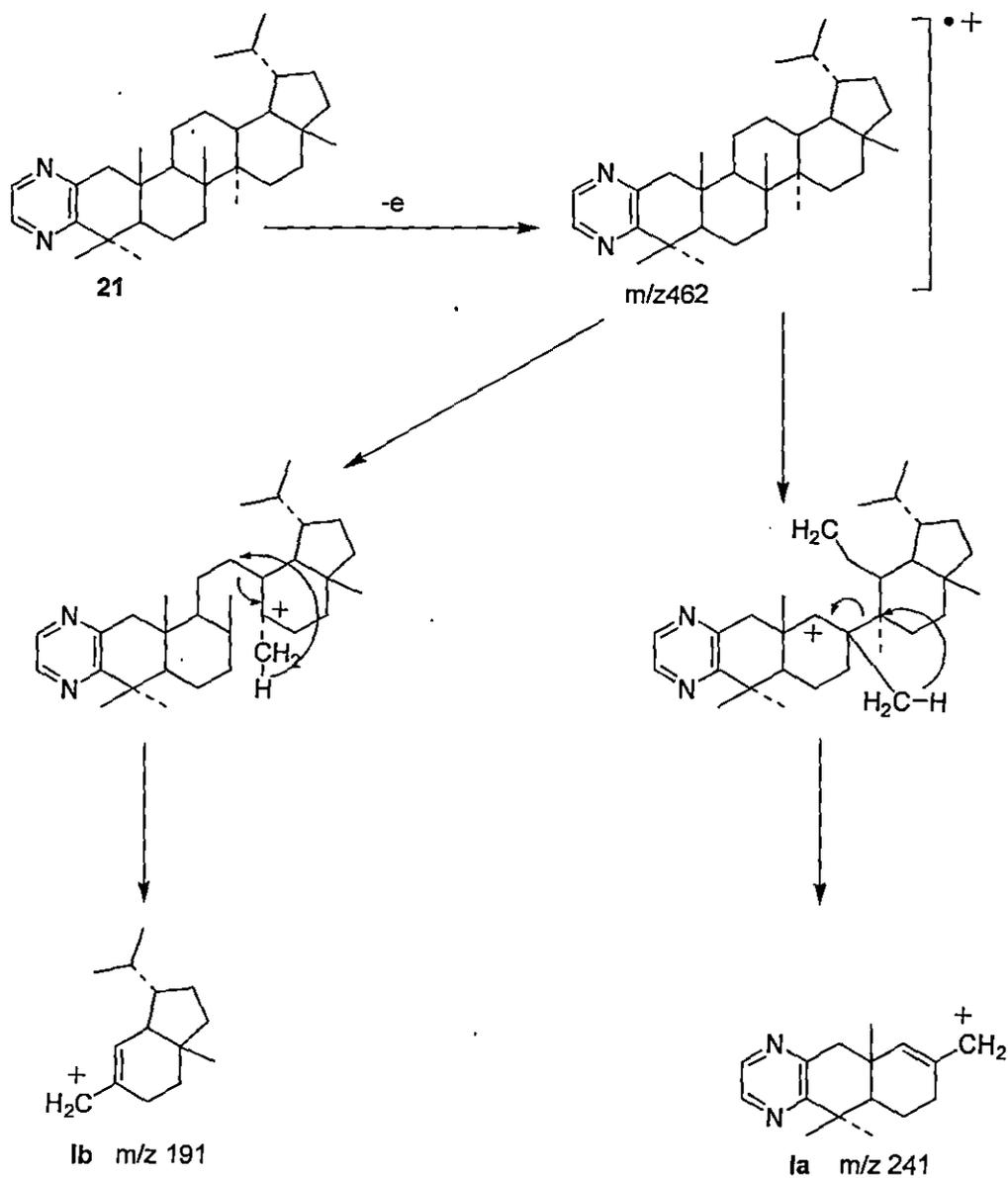


The total schematic representation of the synthetic strategy for the preparation of compound **27** is shown below. For a total description of experimental procedures please see the experimental section of this part. The parent compound for the synthesis of compound **27** was isolated from the outer bark of *Xanthoxylum budrunga* through soxhlet apparatus using toluene as the solvent.



Scheme 26 Synthesis of pyrazine derivative of lupane (27)

The structure of **27** for compound **A** was further established by a through mass spectrum analysis. Besides molecular ion peak (m/z 462), the mass spectrum (Figure 27) of compound **A** showed other peaks at m/z 445 (base peak), 377, 256, 241, 191 etc. The peak at m/z 445 was due to the elimination of methyl group from the molecular ion. The peak at m/z 241 was probably due to the fragment⁷⁵ **Ia** and that at m/z 191 was due to the existence of the fragment⁷⁵ **Ib** (Scheme 27).

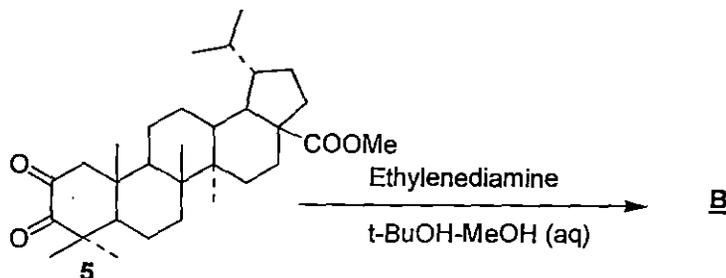


Scheme 27 Probable mass fragmentation pattern of compound 27

2.2 Preparation of pyrazine derivative of 2,3-diketo methyl dihydrobetulonate (6):

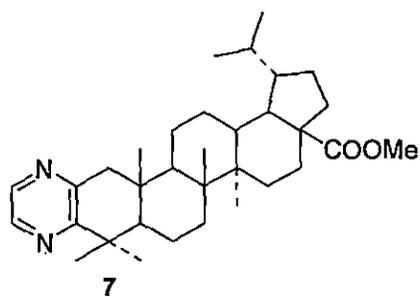
2,3-diketo methyl dihydrobetulonate (6) prepared by the autoxidation⁷³ of methyl dihydrobetulonate, 5 (see experimental), on treatment with ethylenediamine in aqueous methanol catalyzed by *t*-BuOK for 6 hours afforded a single compound. Purification of the compound over a column of silica gel followed by crystallization from

chloroform-petroleum ether furnished fine needle shaped crystals of compound **B**, analyzed for $C_{33}H_{50}O_2N_2$, mp 220-21 °C.

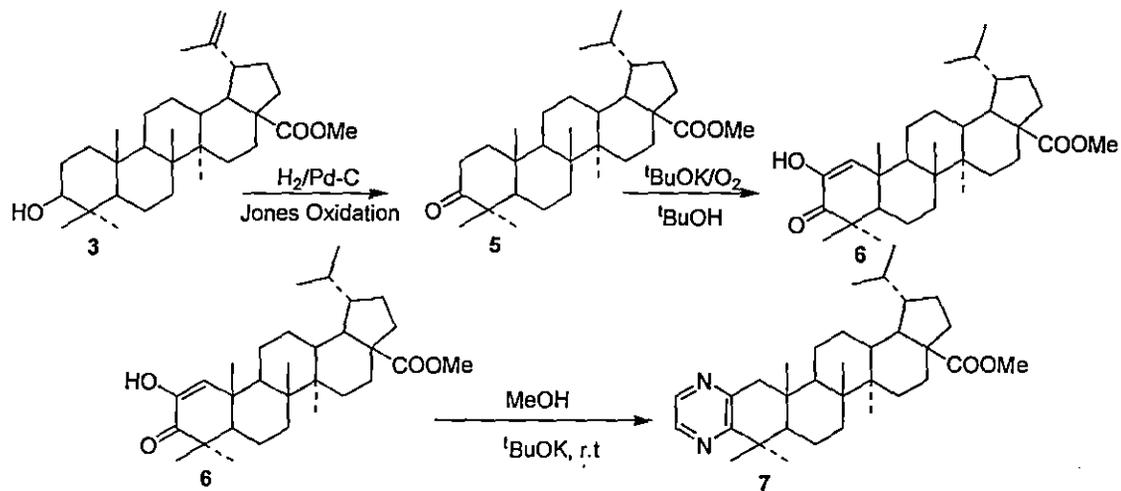


Scheme 28 Treatment of 2,3-diketomethyl dihydrobetulanate with EDA in $t\text{-BuOH-MeOH (aq.)}$

IR spectrum (Figure 12) of compound **B** showed peaks at 1710 cm^{-1} (CO_2Me), 1665 , 1430 and 1120 cm^{-1} . UV-visible spectrum (Figure 10) of compound **B** showed peaks at 272 ($\epsilon = 5712$) and 278 ($\epsilon = 5603$). It did not respond to the TNM test for active unsaturation. The mass spectrum (Figure 11) showed the molecular ion peak at m/z 506 as base peak. The UV-visible spectrum together with the mass spectrum indicated the incorporation of pyrazine nucleus into the ring A of pentacyclic triterpenoid. In the ^1H NMR spectrum (Figure 13) (CDCl_3 , δ ppm $^{-1}$ relative to TMS) it showed the presence of six tertiary methyl groups at δ_{H} 0.82 (s, 3H), 0.98 (s, 3H), 0.99 (s, 3H), 1.28 (s, 3H) and 1.305 (s, 3H). Two secondary isopropyl methyl groups appeared at δ_{H} 0.76 (d, 3H, $J = 7$ Hz) and at δ_{H} 0.88 (d, 3H, $J = 7$ Hz). Ester methyl at C-28 appeared as a sharp singlet at δ_{H} 3.66 (s, 3H, ester methyl). Two hydrogen atoms at C-1 were deshielded due to the magnetic anisotropy induced by the attached aromatic heterocyclic ring and each appeared as a distinct doublet at δ_{H} 2.48 (1H, d, $J = 16$ Hz) and at δ_{H} 3.04 (1H, d, $J = 16$ Hz). Two aromatic hydrogen atoms appeared at δ_{H} 8.27 (1H, d, $J = 3$ Hz) and at δ_{H} 8.41 (1H, d, $J = 3$ Hz). Analytical calculations were 78.26% C, 9.88% H, 5.53% N (calculated) and those found were 78.25% C, 9.73% H, 5.50% N. All the above facts lead to assign structure **7** to compound **C**.



Schematic representation for the whole steps of reaction was shown below-



Scheme 29 ^tBuOH-MeOH mediated synthesis of pyrazine derivative of methyl dihydrobetulanate

Finally the structure **7** was corroborated further by the mass fragmentation pattern of compound (Scheme 30).

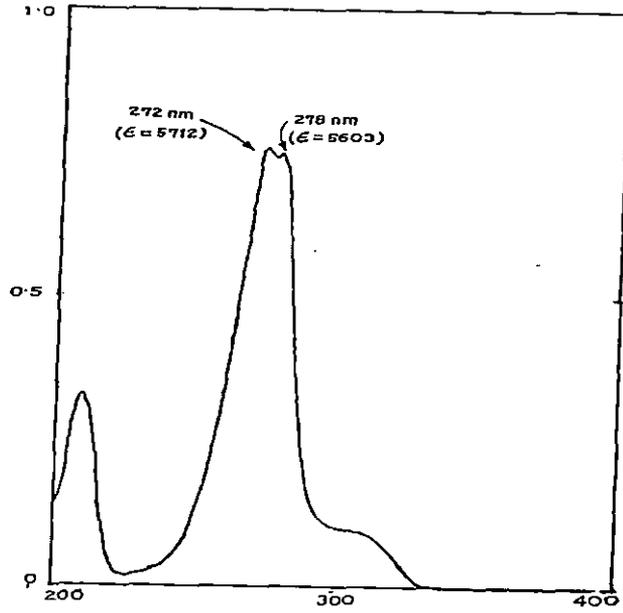


Figure 10 UV spectrum of pyrazine derivative of methyl dihydrobetulanate

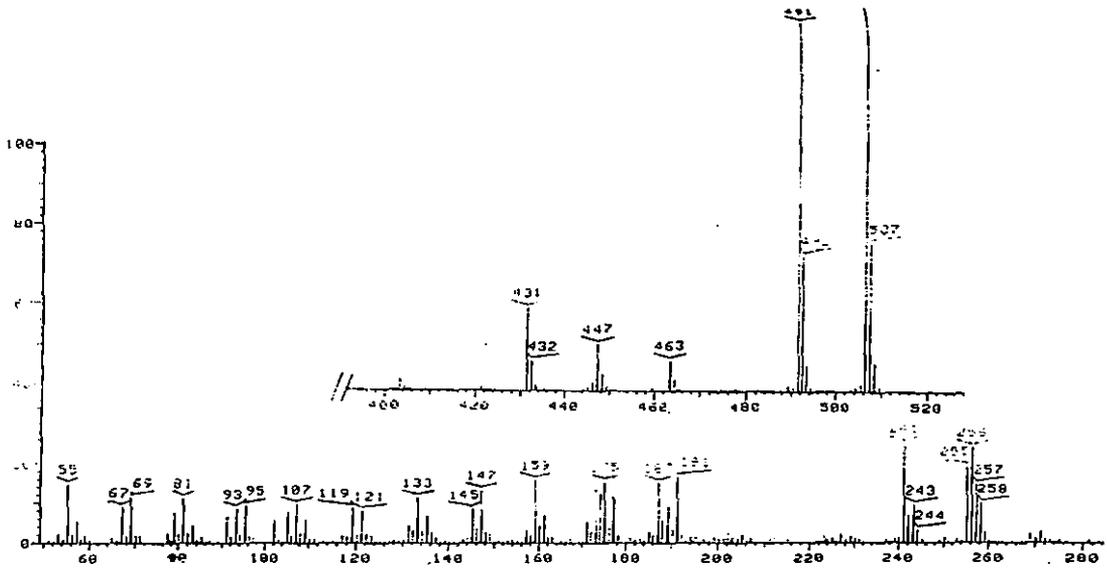


Figure 11 Mass spectrum of the pyrazine derivatives of methyl dihydrobetulanate

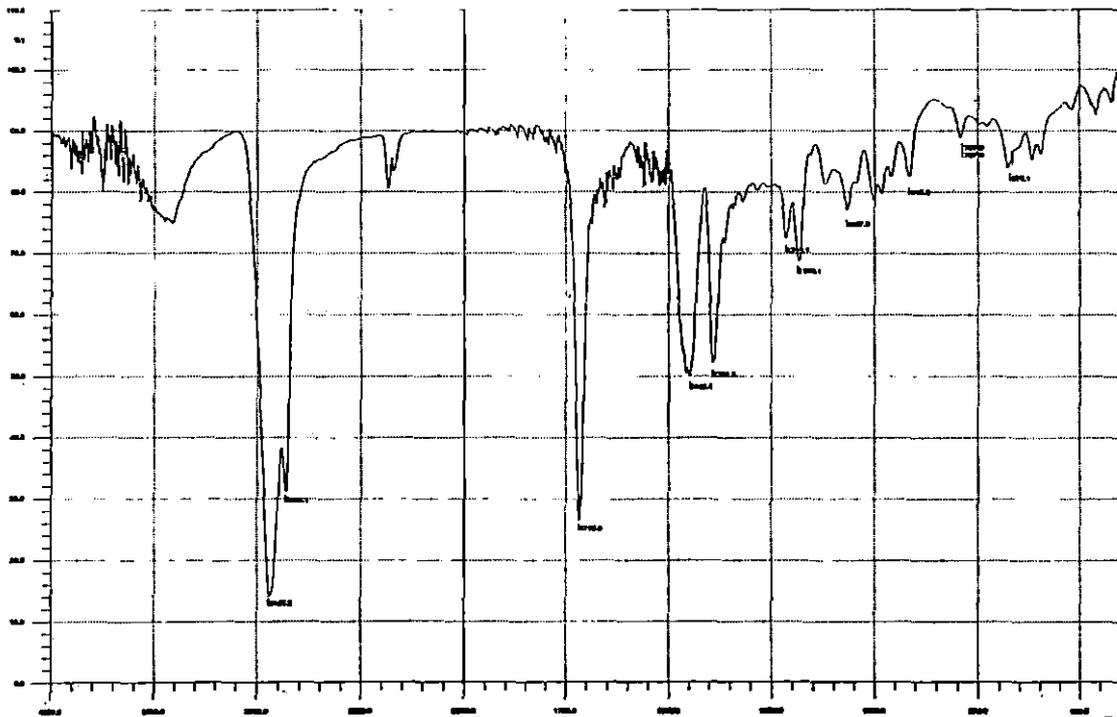


Figure 12 IR spectrum of pyrazine derivative of methyl dihydrobetulanate

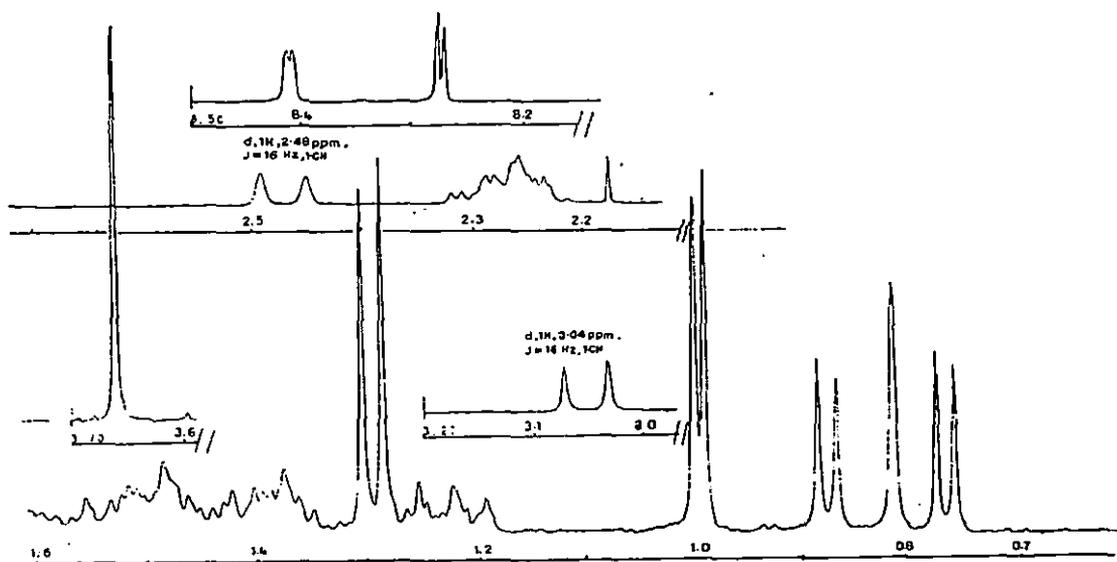
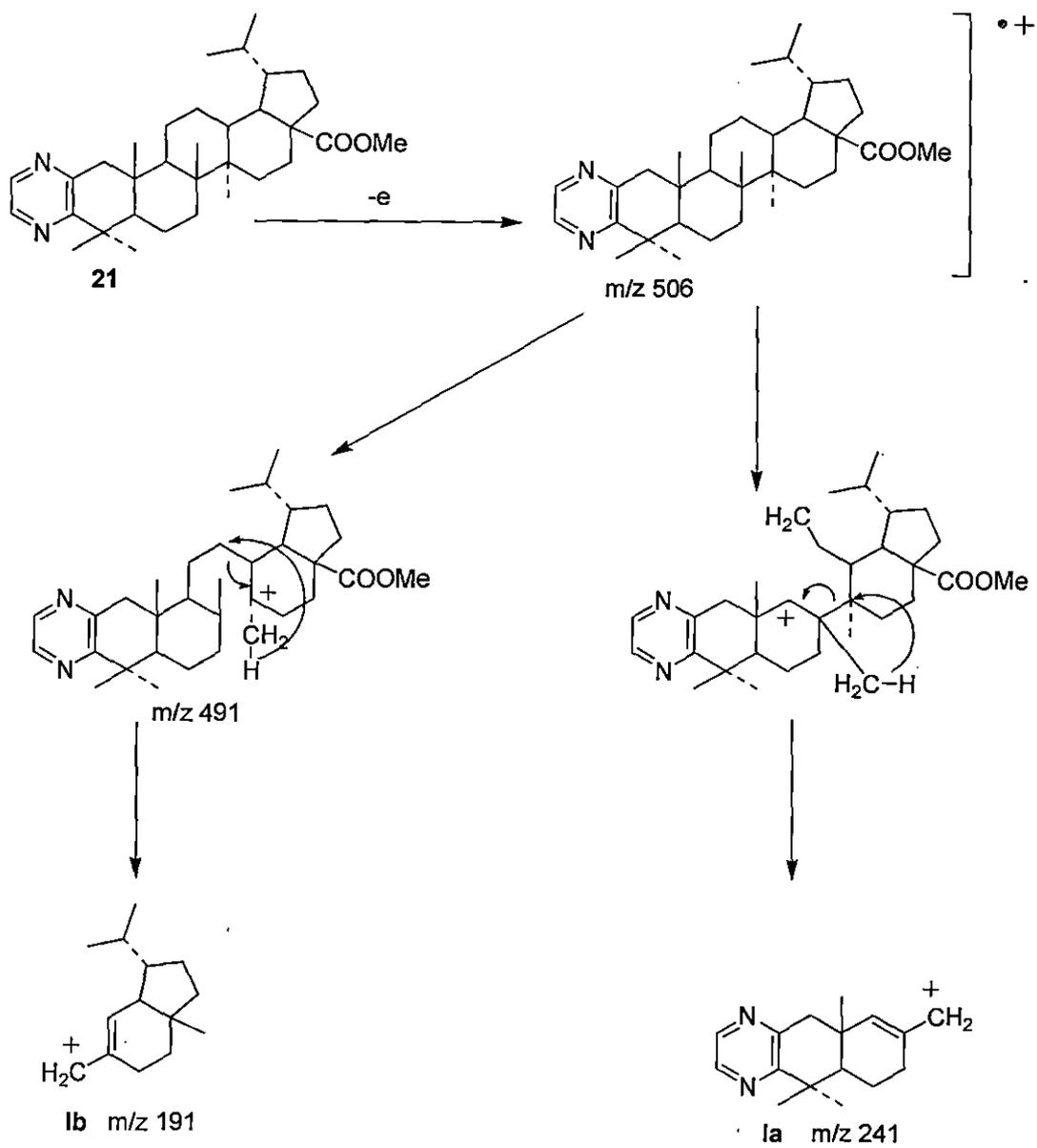
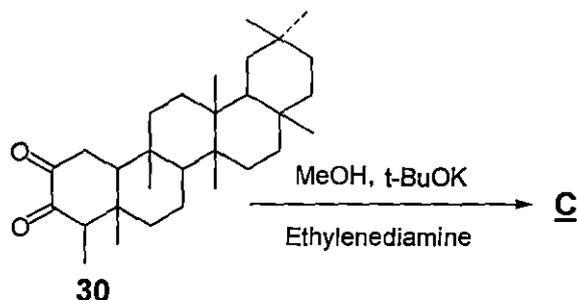


Figure 13 1H NMR spectrum of the pyrazine derivatives of methyl dihydrobetulanate



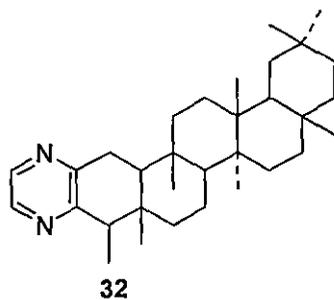
Scheme 30 Mass fragmentation pattern of compound 7

2.3 Preparation of pyrazine derivative of 2,3-diketo friedelin (6):

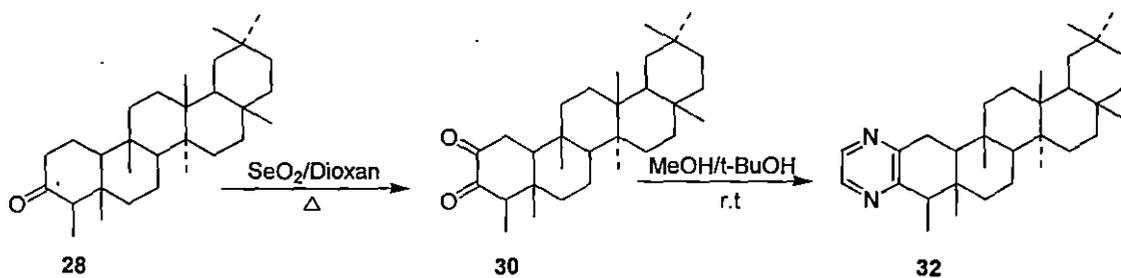


Scheme 31 Treatment of 2,3-diketofriedelin with ^tBuOH-MeOH (aq.)

2,3-Diketo friedelin **30** prepared by auto oxidation of friedelin **28** was treated with ethylenediamine, EDA in aqueous methanol catalysed by t-BuOK for 6 hours. The product obtained after usual work up showed a single spot in TLC and was purified over a column of silica gel (60-120 mesh). Crystallization of the compound from CHCl₃-MeOH mixture furnished compound **C**, analyzed for C₃₂H₅₀N₂, mp 228 °C. IR spectrum of the compound showed peaks at 1655, 1430, 1120 cm⁻¹ for pyrazine ring. UV spectrum showed peaks at 272 (ε = 5800) and 278 (ε = 5450) nm. Mass spectrum (Figure 17) of **C** showed molecular ion peak at *m/z* 462 as base peak, which is the characteristic feature of pyrazine compounds. The other peaks appeared at *m/z* 447, 420, 247, 107 and 71. The ¹H NMR spectrum (Figure 14 and 15) of **C** was indicative of the presence of seven tertiary methyls which appeared as sharp singlets (3H each) between δ 0.82–1.22 (7s, 21H, 7t CH₃), the doublet centered at δ 0.99 was due to the presence of secondary methyl protons (d, *J* = 6.5 Hz), two aromatic protons at δ 8.40 and 8.27 appeared as a doublet with *J* = 3 Hz. ¹³C NMR spectrum of the compound **C** showed the presence of 32 carbons, two singlets at δ 150.8 and 150.9 and two doublets at δ 141.4 and 142.3 were due to heterocyclic ring carbons typical to 2,3-disubstituted pyrazine skeleton. All the above facts led to assign structure **32** to compound **C**.



The formulation of structure **32** for compound **A** is further supported by the ^{13}C NMR (Figure 16) spectrum of the compound.



Scheme 32 $t\text{-BuOH}$ - MeOH mediated synthesis of pyrazine derivative of friedelin

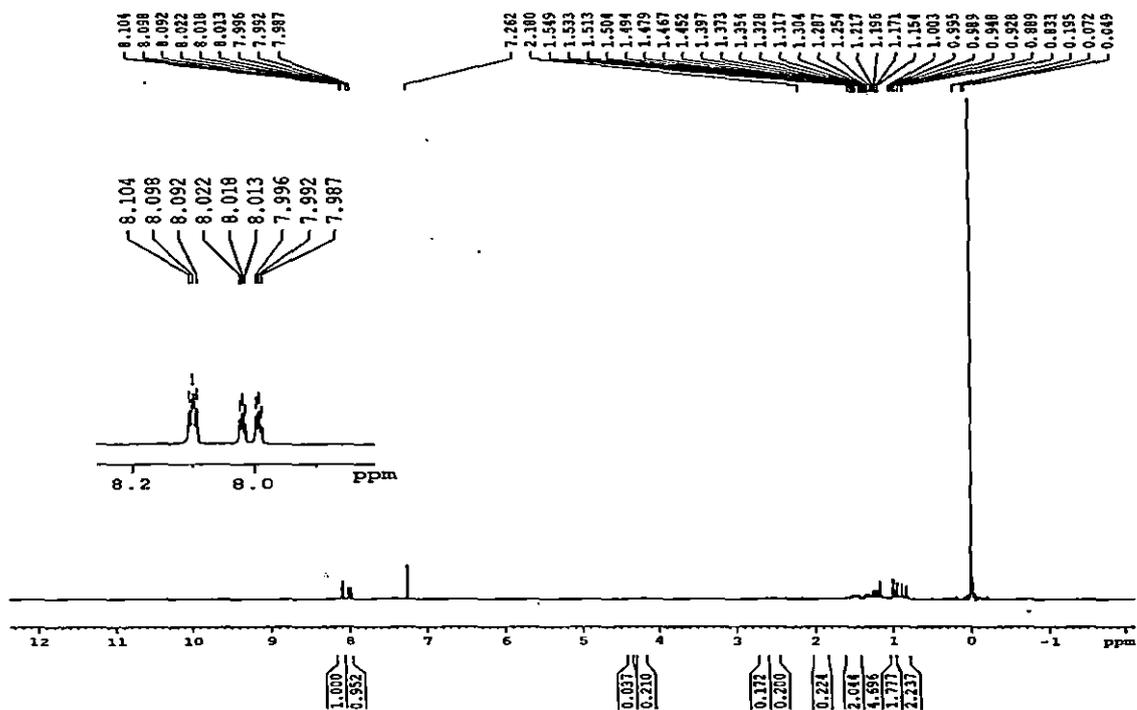


Figure 14 ^1H NMR spectrum of pyrazine derivative of friedelin

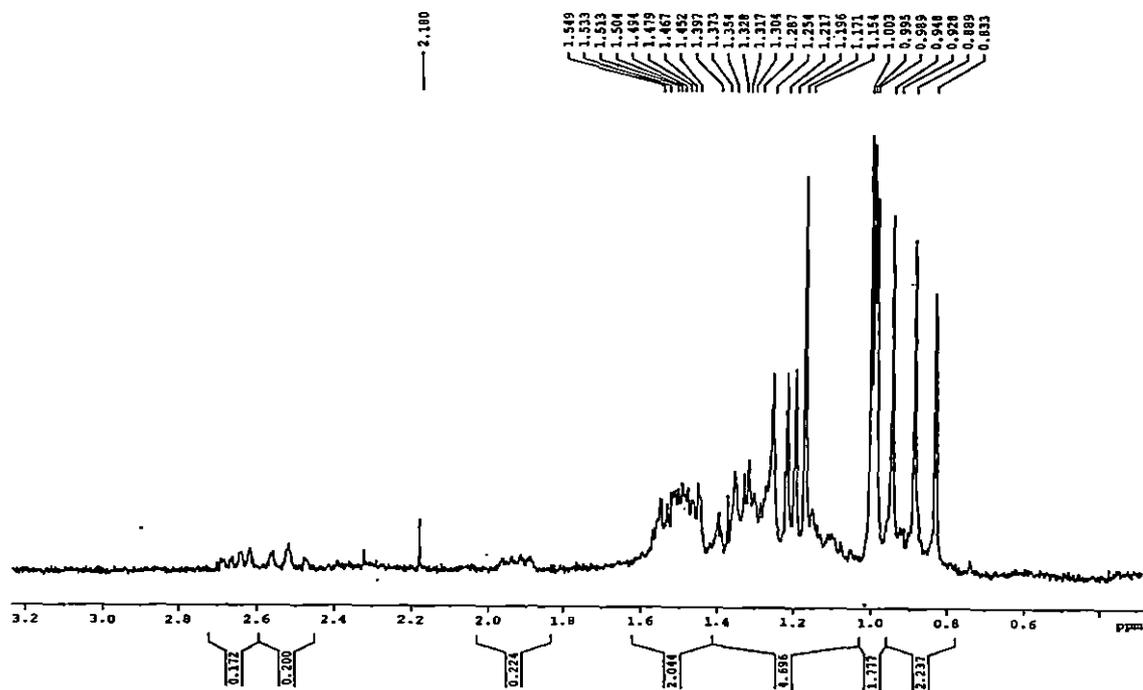


Figure 15 Expanded ^1H NMR spectrum of pyrazine derivative of friedelin

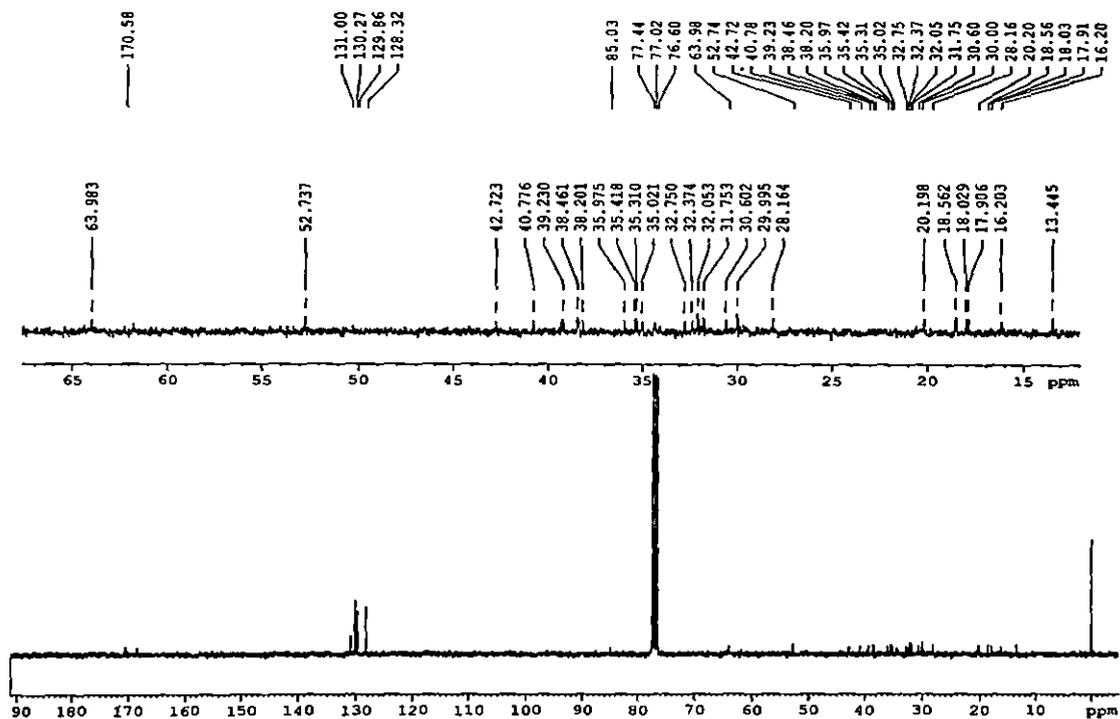


Figure 16 ^{13}C NMR spectrum of pyrazine derivative of friedelin

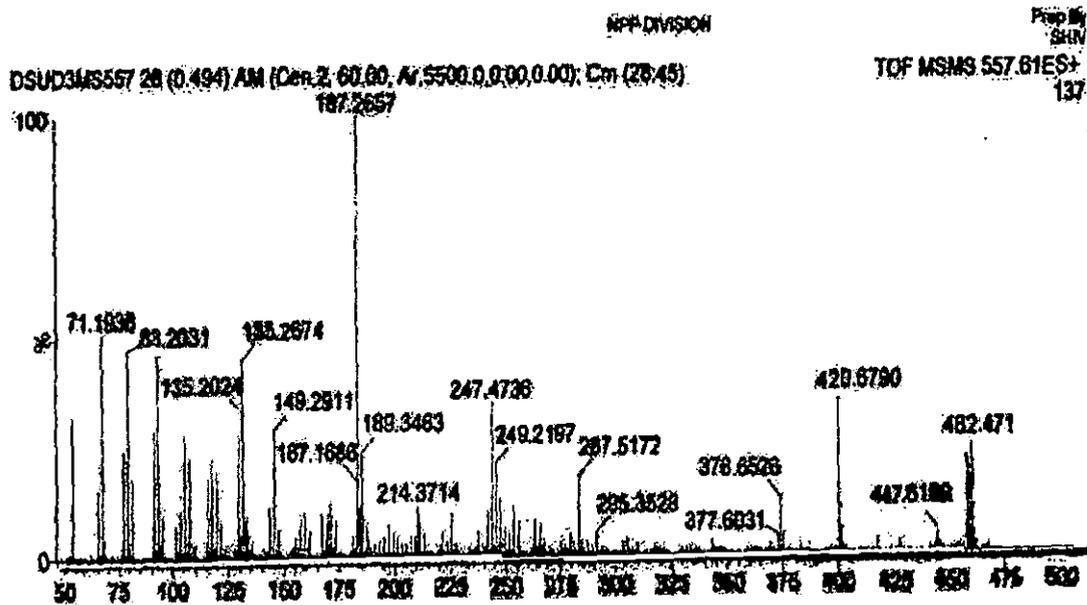


Figure 17 Mass spectrum of pyrazine derivative of friedelin

Section B

Biological activities of the prepared compounds

1. Introduction

Triterpenes represent a varied and important class of natural compounds. Among these, pentacyclic lupane-type triterpenes are one of the most significant subclass which has been shown to possess several medicinal properties. Triterpenoids have been used in traditional Asian medicine for centuries.⁷⁶ Naturally occurring triterpenoid such as betulinic acid, oleanolic acid and ursolic acid are known to have antibacterial, anti-inflammatory, antitumor and antileukemic activities.⁷⁶ But the reported activities of these naturally occurring triterpenoids are comparatively weak. To increase their usefulness, researchers around the globe are involved to synthesize various different novel derivatives that have exhibited superior activities against the tested organisms or the tested diseases. In a nut shell it is clear that suitable derivatives of these naturally occurring triterpenoids have superior activities against human ailments.

To this end the present author has determined the antifungal, antibacterial and anti topoisomerase activity of the prepared compounds. Because of the very poor solubility of the pyrazine derivative of friedelin in DMSO the present author was unable to determine its biological activities.

As the pyrazine derivative of betulinic acid showed better antifungal and antibacterial activity, the present author has determined the anti topoisomerase activity of this compound.

1. Antifungal and antibacterial activity

We studied both the *in vitro* antifungal and antibacterial activity of all isolated triterpenoids. Five different fungal pathogens namely, *Colletotrichum camelliae*, *Fusarium equiseti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides* were used for the antifungal study and for antibacterial study *E. Coli*, *B. Subtilis*, *S. aureus*, *Enterobactor* and some others were used as bacterial pathogen. Suitable strains of these organisms were procured from the microbiology laboratory of our institute. MICs (Minimum inhibitory concentration) of the triterpenoids against bacterial and fungal pathogen are reported in table 1& 2 respectively. DMSO (Dimethyl

sulfoxide) was used as solvent to prepare different concentrations of the triterpenoids. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in Petri dishes and were incubated at 37 °C for 48 h. The bacterial growth was confirmed by a change of yellow to purple colour. Bacterial nutrient media was prepared by using agar, beef extract and bacto peptone in distilled water and the pH of the solution (6.8 - 7.0) was adjusted. Culture media for fungal strains were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber. Additionally spore germination by wet chamber method was also used for determination of antifungal activity⁷⁷ (Table 3). We compared the antifungal activities of the compounds with streptomycin and antibacterial activity with ampicillin, a β -lactam antibiotic.

1.1. Results and Discussion

The antibacterial and antifungal activities of both the pyrazine derivatives were determined against four different bacterial and five different fungal pathogens. The results are tabulated in tables 1, 2 and 3. Both disc diffusion and spore germination techniques were used to evaluate the antifungal activity.

From the results as illustrated in various tables it is clear the all the compounds showed prominent antimicrobial activities against the tested fungal and bacterial pathogens as were observed from the experimental results (Table 2, 3 and 4). Compound 7, pyrazine derivative betulinic acid showed better MIC values against different bacterial strains (Table 1). Against *B. subtilis* it showed MIC values lower than 100 $\mu\text{g/mL}$. A clear comparison between compound 7 and ampicillin showed that the prepared pyrazine derivative had superior activity against *E. coli* and *Enterobactor* than the renowned ampicillin; it has lower MIC values than ampicillin (Table 1).

Table 2 MICs of compound **7** and **27** against different bacteria (Agar cup method)

Compounds	MIC in $\mu\text{g/mL}$ against different strains of bacteria			
	EC	BS	SA	EB
7	100	<100	100	100
27	150	100	200	100
Ampicillin	128	64	64	128

BS- *B. subtilis*, EC- *E. coli*, SA- *S. aureus*, EB- *Enterobactor*, MIC- Minimum inhibitory concentration.

Table 3 MICs of compound **7** and **27** against different fungi (Agar cup method)

Compounds	MIC in $\mu\text{g/mL}$ against different strains of fungi				
	CG	FE	CE	AA	CC
A	<5	20	40	10	<5
B	4.87	19.5	40	19.5	39
Streptomycin	1.25	2.5	<2.5	2.5	2.5

CG- *Colletrichum Gleosproides*, FE- *Fussarium equisetae*, CE- *Curvularia eragrostidies*, AA- *Alterneria alternata*, CC- *Calletotricheme camellie*.

Table 4 Evaluation of antifungal properties of reported triterpenoids against five virulent pathogens by spore germination bioassay (wet chamber method) after 48 h of incubation

Fungal pathogen	Compounds					
	7			27		
	PG ^a	PI	AL ^b	PG ^a	PI	AL ^b
	(μm)			(μm)		
CC	00	100	00	05	95	4.5
FE	00	100	00	00	100	00
AA	00	100	00	00	100	00
CG	00	100	00	00	100	00
CE	05	96	06	00	100	00

CG- *Colletrichum gleosporioides*, FE- *Fussarium equisetiae*, CE- *Curvularia eragrostidis*, AA- *Alternaria alternate*, CC- *Calletotricheme camellie*. PG-Percent germination, PI- Percent Inhibition, AL-Average germ tube length, ^aBased on 200 spores, ^bBased on 25 germ tubes.

The five different fungal strains used to determine the antifungal activities of the prepared pyrazine derivatives namely *Colletrichum gleosporioides*, *Fussarium equisetiae*, *Curvularia eragrostidis*, *Alternaria alternate*, *Calletotricheme camellie* cause mainly wilt disease to tomato or pineapples cultivated in this part of India.

2. Antitopoisomerase activity of the synthesized pyrazine derivatives

2.1 Introduction

Each of our cells contains about 2 meters of DNA, all folded into the tiny space inside the nucleus, which is a million times smaller.⁷⁸ As one might imagine, these long, thin strands can get tangled very easily in the busy environment of the nucleus. To make things even more complicated, DNA is a double helix, which must be unwound to access the genetic information. To help with these problems, our cells build several different topoisomerase enzymes that untangle and relax DNA strands.⁷⁸

Cellular DNA is extremely compacted, implying a high degree of structural organization. The folding mechanism not only must pack the DNA but also must permit access to the information in the DNA. The complicated structural folding pattern of DNA is commonly known as **supercoiling**. Supercoiling means the coiling of a coil. DNA is coiled in the form of a double helix, with both strands of DNA coiling around an axis. The further coiling of that axis upon itself produces DNA supercoiling. DNA supercoiling is generally a manifestation of structural strain. When there is no net bending of DNA axis upon itself, the DNA is said to be in relaxed state.

DNA supercoiling is a precisely regulated process that influences many aspects of DNA metabolism. Every cell has enzymes with the sole function of underwinding and/or relaxing DNA. The enzymes that increase or decrease the extent of DNA underwinding are topoisomerase. These enzymes play an especially important role in processes such as DNA packing.

Several measurable properties of supercoiling have been established. This work has drawn heavily on concepts derived from a branch of mathematics called **topology**, the study of the properties of an object that do not change under continuous deformations. For DNA continuous deformations include conformational changes due to thermal motions or interaction with proteins and other molecules.

For any type of DNA topological change, replication or transcription (Figure 18) supercoiled DNA molecules must unwind its supercoiling. This prerequisite unwinding of DNA supercoiling is catalyzed by the enzyme DNA topoisomerase through the catalytic TYR residue at the central DNA binding domain.

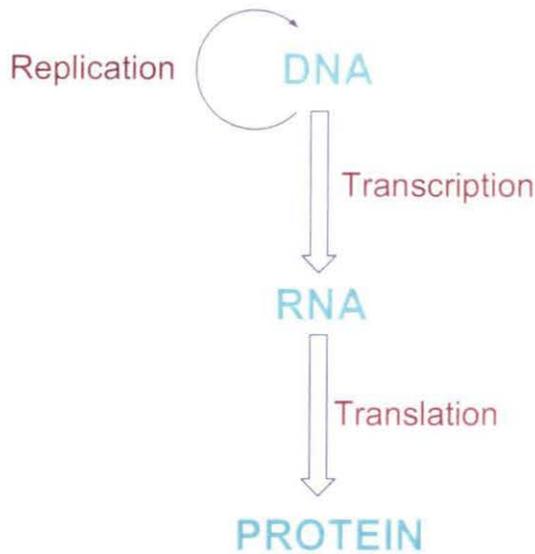


Figure 18 The central dogma of molecular biology showing the general pathway of information flow via replication, transcription and translation

Human topoisomerase I and II are ubiquitous nuclear enzymes involved in the control of DNA topology.⁷⁸⁻⁸⁰ During the catalytic cycle, the enzyme (topo II) transiently cleaves DNA, passes an intact double helix through the break and reseals it. Vertebrates contain two isoforms of the enzyme, topo II α and β .⁷⁸

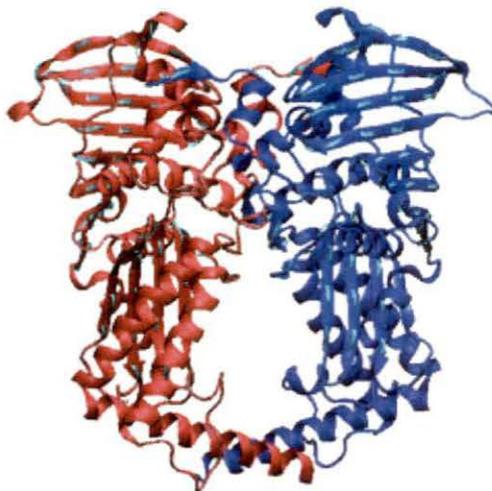


Figure 19 Complete secondary structure of topoisomerase II α

2.1.1 Relaxing DNA

Class I topoisomerases solve the problem of the tension caused during the winding and unwinding of DNA. It wraps around the DNA and makes a cut in one strand. Then, while holding onto the damaged spot, the enzyme allows the helix to spin, releasing any overwinding or underwinding. Once the DNA is relaxed, the topoisomerase reconnects the broken strand, restoring the DNA double helix.

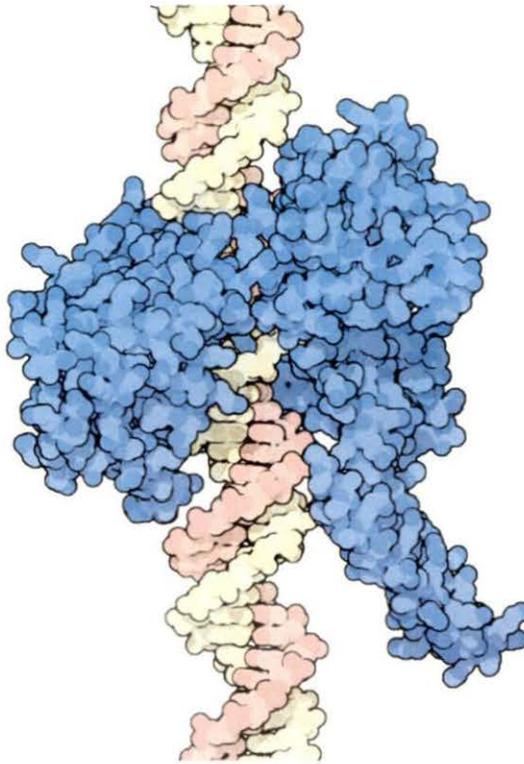


Figure 20 Schematic view of DNA bounded topoisomerase I

2.1.2 Class II Topoisomerase

Class II topoisomerase performs the amazing feat of breaking a DNA double helix, passing another helix through the gap, and resealing the double helix behind it. The picture shown here is built from two PDB entries: **1bgw**⁸² has the lower part of the topoisomerase, and **1eii**⁸³ is a domain from a gyrase, which is similar to the upper part of the topoisomerase. The topoisomerase is thought to be a highly dynamic structure, with several gates for entry of DNA into the two DNA-sized holes. Two tyrosine amino acids,

shown in red, cleave the DNA strands and form a covalent bond with them, holding them tightly until the DNA can be restored.

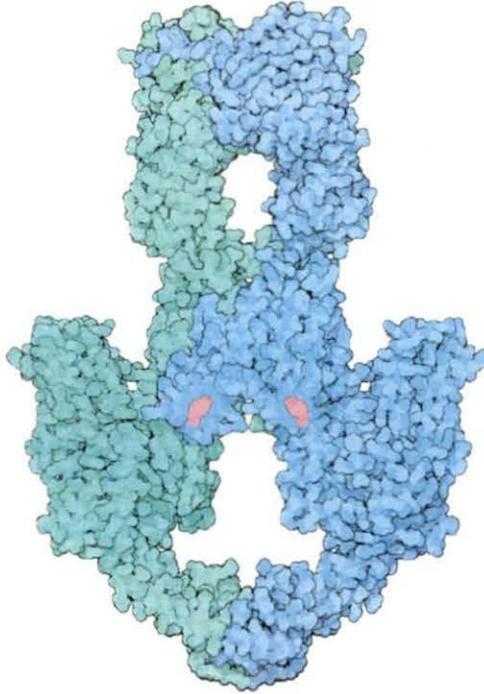


Figure 21 Complete structural model of topoisomerase II with several gates for entry of DNA

2.1.3 Untangling DNA

Class II topoisomerases are specialized in untangling DNA in the nucleus. For instance, when a cell is dividing, it needs to separate the two copies of each chromosome. During this process, portions of the two sister chromosomes may become looped around each other, getting hung up together as they are separated. Class II topoisomerase solves this problem by allowing one DNA helix to pass through the other one. It cuts both strands of one DNA double helix, keeping a firm grip on both halves. Then, it passes the other DNA through the gap, resolving the tangle. Finally, it reattaches the broken ends, restoring the DNA. The crystal structure of type II topoisomerase-DNA cleavable complex stabilized by the anticancer drug etoposide⁸⁴ is shown below. It clearly shows the number of amino acid residues for DNA binding site and cleavage core.

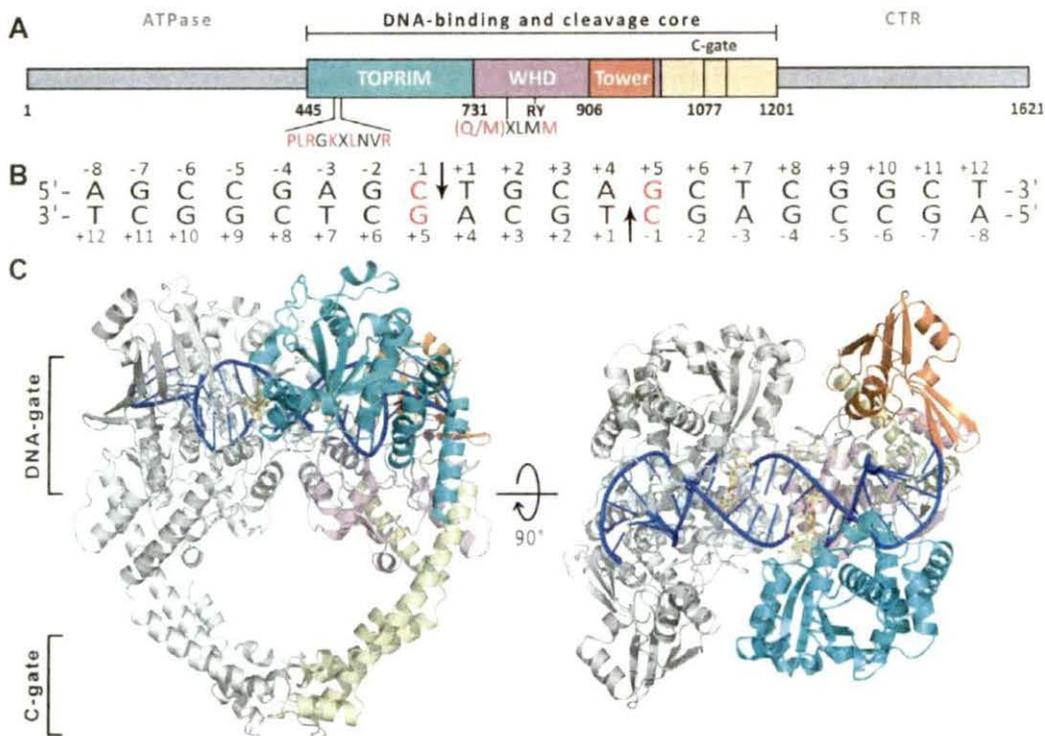


Figure 22 Structure of the hTOP2bcore-DNA cleavage complex stabilized by the anticancer drug etoposide. **(A)** Linear domain organization of hTOP2b. The middle fragment (residues 445 to 1201), corresponding to hTOP2bcore, was used in this study. The nomenclature of the TOP2 domains is adopted from the yeast enzyme.⁸⁵ Key drug-interacting residues whose mutations may confer drug resistance to antibiotics (for bacterial TOP2s) or anticancer agents (for eukaryotic TOP2s) are shown in red. **(B)** The palindromic DNA substrate used for crystallization. The cleavage sites are indicated by arrows. Positive and negative numbers (+1 to +12 and -1 to -8) designate nucleotides downstream and upstream of the scissile phosphate, respectively, with the +1 nucleotide forming a phosphotyrosyl linkage with Y821. The -1/+5 base pairs (in red) highlight the nucleotide preference for this position. **(C)** Orthogonal views of the ternary cleavage complex. DNA is in blue, one hTOP2bcore monomer is in gray, and the other follows the scheme shown in **(A)**.

These processes of relaxing and untangling are essential for the proper maintenance of our DNA, so topoisomerases are sensitive targets for poisons or inhibitors. If topoisomerases are blocked, the cell will encounter problems during transcription of the DNA and during cell division. Cancer chemotherapy takes advantage of this, using drugs that block topoisomerases to kill rapidly-dividing cancer cells. For instance, the widely-used anthracycline drugs, like doxorubicin and daunorubicin, attack class II topoisomerases, and the plant toxin camptothecin blocks the relaxing action of class I topoisomerases.

2.2 3D Molecular docking studies

To determine whether the pyrazine derivatives have potential as topoisomerase inhibitor, the synthesized pyrazine derivatives were initially screened for 3D molecular docking on the crystal structure of the enzyme (1bgw PDB for topoisomerase II α)⁸² using AutoDock 4 (vide infra).

From the present 3D molecular docking studies, it is clear that the pyrazine derivative of dihydrobetulinic acid binds to the central catalytic domain (please see figure 22 above) of topoisomerase II α . The conserved amino acid residues are TYR 806, TYR 813, VAL 814, LYS 1203, TYR 709, ASN 715, PHE 712, TYR 819, ARG 793, ASP 801 and PRO 798 (Figure 23). A closer look does reveal that the molecule binds to a condensed positively charged partially hydrophobic pocket of the enzyme. It forms two direct hydrogen bond interactions, one with the oxygen atom of the neighboring ASP 801 residue through the acidic hydrogen atom of the C-28 carboxylic acid group another with the oxygen atom of TYR 813 through the aromatic π -cloud of the pyrazine moiety.

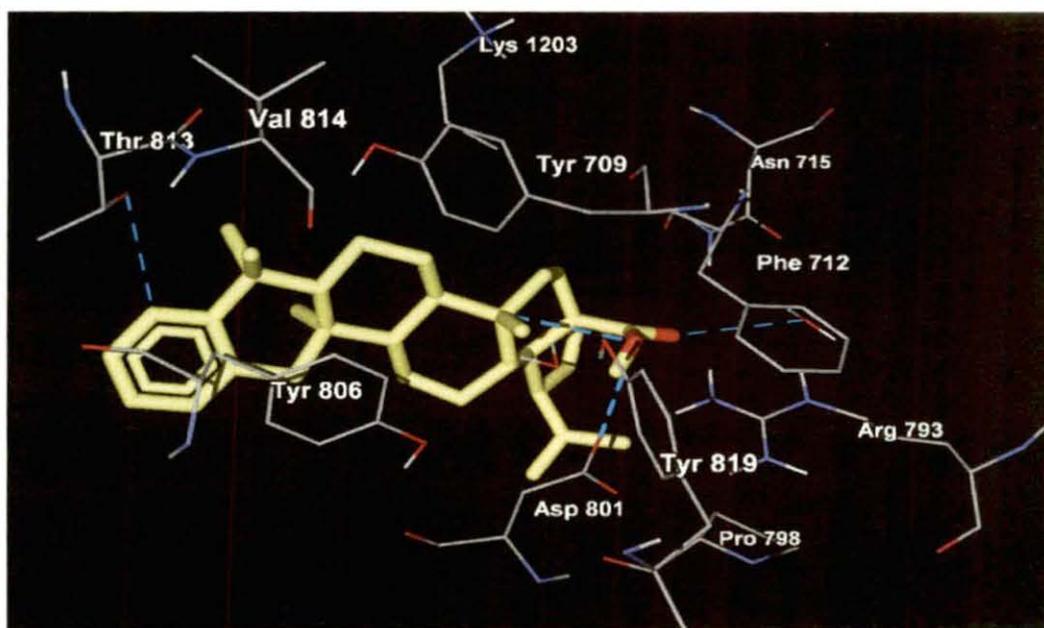


Figure 23 H-bond interaction view of the pyrazine derivative of dihydrobetulinic acid

In addition to this, it forms another one water mediated H-bond interaction to the π -cloud of PHE 712 involving the carbonyl oxygen of the C-28 carboxylic acid group.

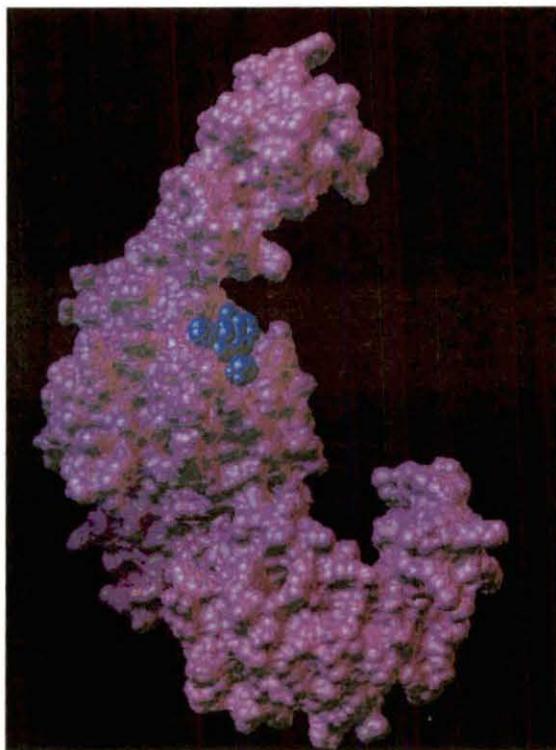
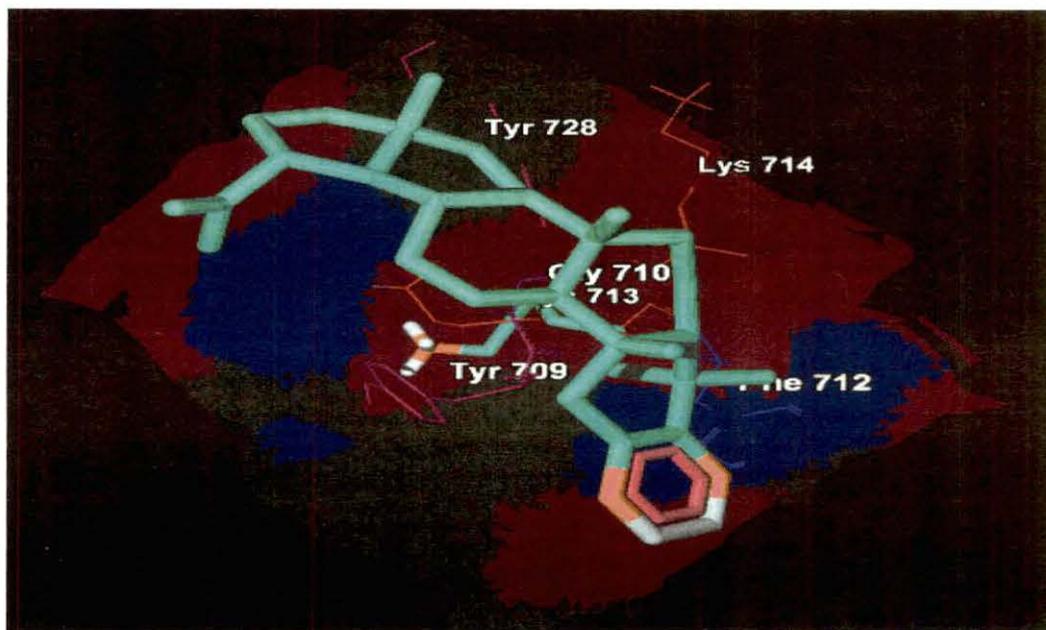


Figure 24 Detailed docked view of pyrazine derivative of dihydrobetulinic acid showing that the molecule has binded to one of the two possible gates for DNA binding

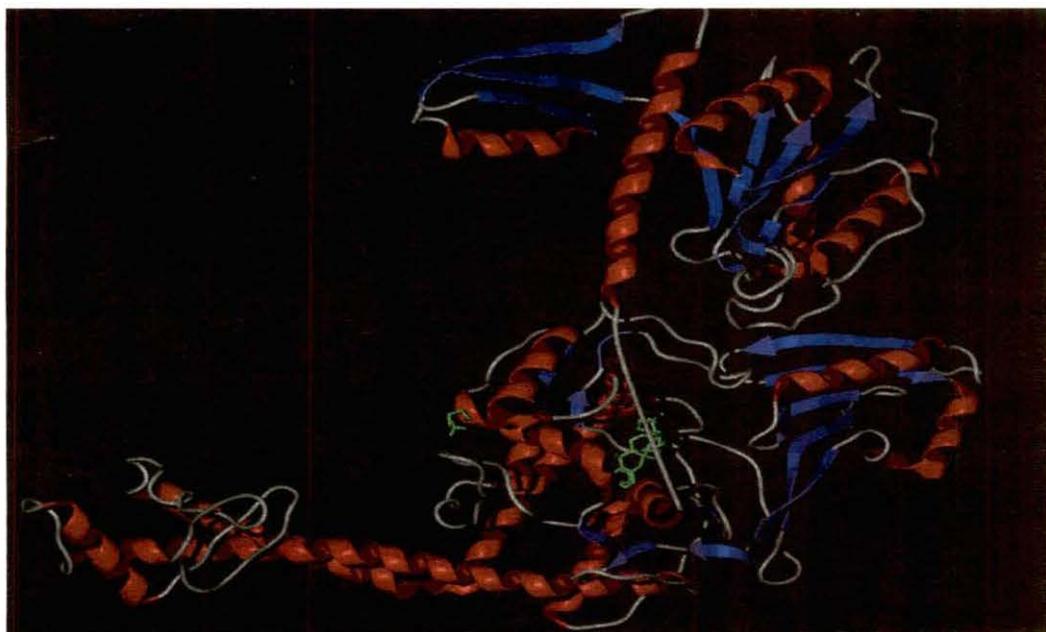
It was also interesting to note from the present docking studies that the synthesized pyrazine derivative has binded to the upper gate for DNA binding thus clearly inhibiting the further binding of DNA to the enzyme.

On the other hand the pyrazine derivative of lupeol binds to a condensed positively charged hydrophobic interior (Figure 25A and 25B) of the enzyme. It does not show any covalent bonding to a definite site of the binding pocket of the enzyme. It does not form any H-bond interaction with the any amino acid residues. The conserved amino acid residues that are in the vicinity are TYR 809, TYR 728, LYS 713, GLY 710, LYS 714 and PHE 712. Figure 25B shows the secondary interaction docked view; it is clear from

the view that there is no hydrogen bond between the synthesized pyrazine derivative and the neighboring amino acid residues.



(A)



(B)

Figure 25 Detailed docked view of the pyrazine derivative of lupeol, (A) Hydrophobic interaction view and (B) Secondary structure view.

Therefore the type of interaction between the ligand molecule and the host molecule must be either electrostatic interaction or dipole induced dipole interaction. It showed the binding energy of -9.76 kcal/mol.

Similar to these facts the prepared pyrazine derivative of friedelin does not show any H-bond interaction (Figure 26) with the enzyme 1bgw, pdb. It also binds to a hydrophobic pocket of the enzyme comprising of CYS 712, LYS 714, LYS 713, GLY 710, TYR 728 TYR 709 residues and the estimated binding energy value is -9.22 kcal/mol. Therefore the nature of interaction here also must be either electrostatic or dipole induced dipole interaction.

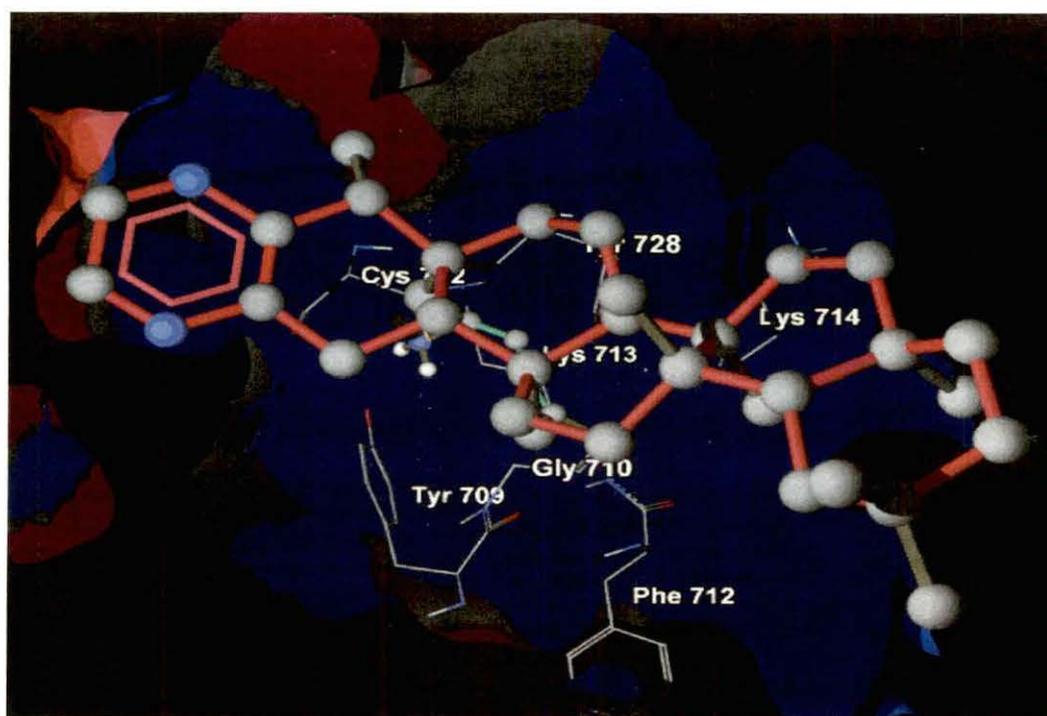


Figure 26 Detailed docked electrostatic interaction view of the pyrazine derivative of friedelin.

From the figure it can be said that the synthesized lead molecule can bind to the catalytic domain i.e. the DNA binding domain of the enzyme molecule. Except lysine all other neighboring amino acid residues are neutral in character. Another interesting fact is that the phenolic -OH group of TYR 709 turned inside towards the aromatic pyrazine moiety at the ring A of the pentacyclic skeleton. It might be possible that the expected nonionic

interaction is operating through that phenolic –OH group and with the aromatic ring current (Figure 26).

From the present docking studies on the crystal structure of topoisomerase II α (1bgw, pdb) it was clear that all the synthesized pyrazine derivatives can bind to the DNA binding domain of the enzyme. Thus it can be infer that all the three derivatives can inhibit topoisomerase II α to bind with DNA.

Of the three pyrazine derivatives, that of betulinic acid showed the best form of results as it binded to the upper DNA binding gate and it is the only compound that showed the important H-bond interaction with the enzyme. These attractive findings encouraged the present author to determine the experimental topoisomerase II α inhibitory activity of this compound.

2.3 Topoisomerase inhibitory activity of pyrazine derivative of dihydrobetulinic acid

Inhibition of catalytic activity of topoisomerase constitutes a useful strategy for the identification of potential antitumor agents. Topo II α creates transient breaks in supercoiled DNA resulting in DNA relaxation. The relaxed DNA can be distinguished from supercoiled DNA by gel electrophoresis analysis. The results of molecular docking studies on interaction of pyrazine derivative of dihydrobetulinic acid with DNA binding domain of human Topo II α , was further confirmed by examining DNA relaxation activity of Topo II α . The ATP dependent relaxation of supercoiled DNA by the enzyme was monitored in absence or presence of pyrazine derivative of dihydrobetulinic acid. From the results in figure 27, it is evident that the inhibitory effect of the compound was dose dependent. They showed complete inhibition of the catalytic activity of Topo II α at 100 and 50 μ M concentrations, whereas partial inhibition of the activity was observed at a concentration of 25 μ M.

The pyrazine derivative of methyl dihydrobetulanate was hydrolyzed successfully to the corresponding acid and to that the experimental work of DNA topoisomerase inhibition activity was carried out.

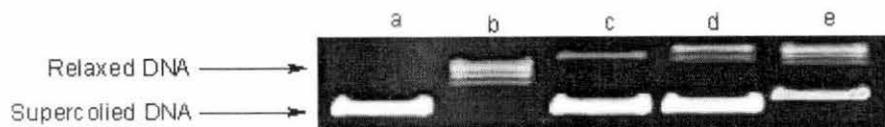


Figure 27 Effect of pyrazine derivative of dihydrobetulinic acid on DNA relaxation activity by DNA topoisomerase II α , Lane a: supercoiled DNA alone, Lane b: control no drug (with 2 units of topo II), Lane c, d and e: 100, 50 and 25 mM concentration of the synthesized pyrazine derivative respectively.

3. Conclusion

The present author has successfully synthesized the pyrazine derivatives of lupanol, methyl dihydrobetulanate and friedelin. All the molecules were characterized by spectral data and by comparison to that reported in literature. 3D molecular docking of these derivatives in the central catalytic domain of topoisomerase II α (1bgw PDB for topoisomerase II α) revealed the nature of the binding and the type of interactions between the synthesized compounds and the enzyme. The topoisomerase II α inhibitory activity was further confirmed by *in vitro* experiments. The present work will definitely enrich the modern drug designing towards the invention of newer plant based chemotherapeutics to fight against human ailments. In addition the findings may provide a better understanding of the structure activity relationship towards the topoisomerase inhibitory activity of the pentacyclic triterpenoids.

Chapter III

Section A

Synthesis of benzopyrazine or quinoxaline derivative of friedelin in water catalyzed by SDS

1. Introduction

Benzopyrazines or quinoxalines are ubiquitous heterocyclic units in pharmaceuticals and bioactive natural products.⁸⁹⁻⁹² They are used as pharmaceuticals and antibiotics such as echinomycin, levomycin and actinoleutin which are known to inhibit the growth of Gram-positive bacteria and are also active against various transplantable tumors.⁸⁹⁻⁹¹ Antitumoral properties of quinoxaline compounds have also been investigated. Beside these, they are well known for their application in dyes⁹³ as an efficient electroluminescent materials⁹⁴ in organic semiconductors⁹⁵ as building blocks for the synthesis of anion receptors⁹⁶ as cavitands^{97,98} dehydroannulenes⁹⁹ and DNA cleaving agents.^{100,101} Conventionally, quinoxalines are synthesized by a double condensation reaction involving a dicarbonyl precursor and *o*-phenylenediamine.^{102,103} Due to the highly reactive nature of the dicarbonyls, alternative routes have been proposed recently.¹⁰⁴ Antoniotti and Donach have reported one of these methods to synthesize quinoxalines from epoxides and ene-1,2-diamines.¹⁰⁴ Active manganese oxide and molecular sieves in combination or manganese oxides in combination with microwaves have also been used in producing quinoxalines.^{105,106} These processes, however, require excessive amounts of corrosive manganese oxide as stoichiometric oxidants and scaling them up for industrial processes can lead to the formation of large amounts of toxic waste leading to environmental issues. In additional studies, Robinson and Taylor reported a homogeneous catalytic process utilizing Pd(OAc)₂, RuCl₂ (PPh₃)₂ to synthesize quinoxalines from hydroxy ketones¹⁰⁷ and recently a copper catalyzed oxidative cyclization process has been reported.¹⁰⁸ An improved ruthenium catalyzed direct approach to synthesize quinoxalines from diols and *o*-diamines has also been reported.¹⁰⁹ These processes suffer from the major drawback that the catalysts are expensive, toxic and cannot be recovered and reused. In addition to the above catalytic methods, synthesis

of quinoxalines using zeolites¹¹⁰⁻¹¹³ microwave¹¹⁴⁻¹¹⁵ and solid supports¹¹⁶⁻¹¹⁸ has also been reported. Nevertheless, these methods suffer from unsatisfactory product yields, critical product isolation procedures, expensive and detrimental metal precursors and harsh reaction conditions, which limit their use as environmentally friendly protocol. In addition most of the reported methods are not recommended as a clean protocol.

Although very few of the recent reports have claimed α -bromoketones as an equivalent safe chemical precursor of α -hydroxyketones, epoxides or dicarbonyls as reaction partners of *o*-phenylenediamine to prepare quinoxalines¹¹⁹⁻¹²¹ they involved the use of either HClO₄-SiO₂ or TMSCl as catalyst. Although useful, HClO₄ has huge hazardous nature than its potential usefulness, whereas those catalyzed by TMSCl needs higher temperature, with lower yield of the desired products not satisfying the principles of green chemistry protocol in contemporary science as well as their acceptance for industrial applications.

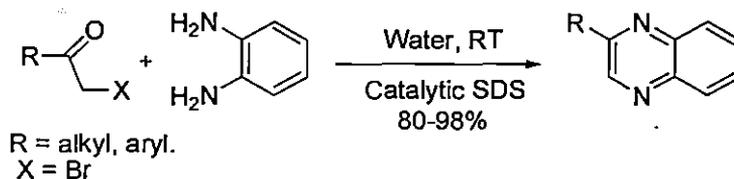
In this context the development of an alternative route to quinoxaline from less reactive α -bromoketones in aqueous medium was felt necessary not only due to the increased regulatory pressure focusing on organic solvents, but also because of the emphasis given towards the development of green protocol for organic synthesis nowadays.

The use of water as a medium for organic synthesis is one of the latest challenges in organic synthesis. Reactions in water emerged as a useful alternative route for several organic reactions owing to many of its potential advantages such as safety, economy and friendly towards catalytic and stereoselective processes and more importantly of environmental concern¹²¹⁻¹²² and the progress has been dramatic. Additionally, water facilitates ion separation through solvation which often results in altered behavior of reactants in an aqueous environment. Keeping these above facts in mind, the present author has recently tested water as a solvent in many of his ongoing studies towards organic syntheses and transformative reactions. Here the present author is reporting the results a successful attempt for an efficient synthesis of quinoxaline from α -bromoketones and *o*-phenylenediamine mediated by water and catalysed by SDS at room temperature in excellent yields. This is the first report of synthesizing quinoxaline derivatives in a very mild way in water catalyzed by nucleophilic SDS¹²¹ at ambient

temperature starting from the less reactive safer precursor α -bromoketones in an efficient manner.

2. The present work

Initially, efforts were directed towards the evaluation of catalytic ability of SDS for the synthesis of quinoxalines. Preliminary studies using phenacylbromide (1 mmol) and *o*-phenylenediamine (1 mmol) without SDS in water at room temperature did not afford the desired quinoxaline. Increase of the reaction time, temperature or by changing the molar proportion of the reactants did not make any influence on the course of the reaction. Addition of some common salts like NaCl, NH₄Cl, KBr *etc.* had no positive effect on the reaction. Similar molar ratios of substrates in tap water yielded the desired product only in presence of catalytic amounts of SDS (Scheme 33). The modified method gave excellent yield of the product within 6 hours at room temperature (Scheme 33). Thus, the catalytic role of SDS in the present transformation is well established.



Scheme 33 SDS catalyzed synthesis of quinoxaline

2.1 Results and Discussion

This excellent catalyzing ability of SDS inspired us to investigate the above transformation in details. In order to evaluate an optimized and general reaction protocol, a couple of experiments were carried out (Table 1) using varying amounts of SDS (0.34 mol%, 0.17 mol%, 0.06 mol%, 0.03 mol%, 0.02 mol% and 0.01 mol%) in combination with different types (both cationic and anionic) and proportions of surfactants *viz.* tetra-*n*-butylammonium bromide (TBAB), cetyl trimethyl ammoniumbromide (CTAB), cetyl pyridiniumchloride (CPC), sodium dodecylbenzenesulfonate (SDBS) and tetra-*n*-butylammoniumiodide (TBAI), in different reaction conditions for the above model study (Table 5). It is interesting to note that, although all the surfactants can afford quinoxaline as the major product but their combination with SDS showed excellent selectivity not only in forming the desired product but also in directing the reaction to proceed in a very

cleaner way (Entry 1-7, Table 1). Thus it was established that, α -bromoketone (1 mmol) and 1,2-diamine (1 mmol) in water (3 mL) gave the best result within 6 hrs in presence of SDS (10 mg, 0.03 mol%) at room temperature.

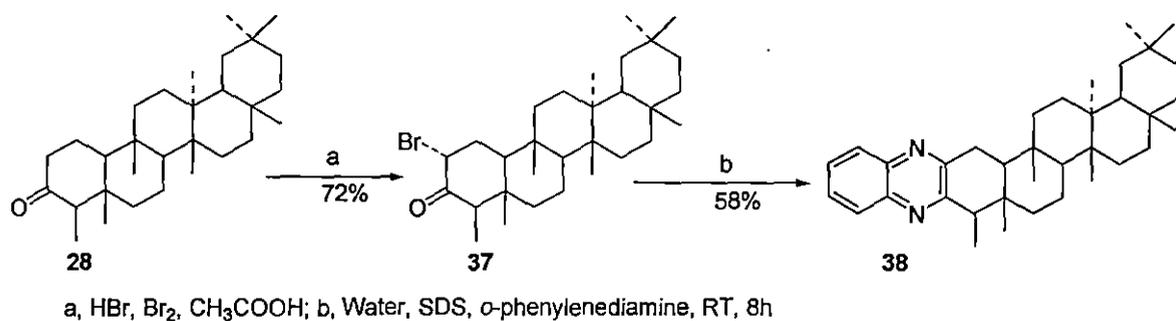
Table 5 Optimisation of quinoxaline synthesis using phenacyl bromide and *o*-phenylenediamine in presence of different surfactants and their amounts.

Entry	Ratio of aldehyde and diamine	Surfactant	Amount of surfactant (mg)	Temp (°C)	Time (hr)	% Yield ^a of 3
1	1:1	SDS	100	RT	6	96
2	1:1	SDS	50	RT	6	94
3	1:1	SDS	20	RT	6	96
4	1:1	SDS	15	RT	6	94
5	1:1	SDS	10	RT	6	95
6	1:1	SDS	7	RT	12	80
7	1:1	SDS	5	RT	15	64
8	1:1	SDS	5	50	8	68
9	1:1	TBAB	100	RT	10	78
10	1:1	TBAB	100	50	10	76
11	1:1	TBAB	200	100	10	80
12	1:1	CTAB	100	RT	10	66
13	1:1	CTAB	200	100	10	68
14	1:1	CPC	100	RT	10	78
15	1:1	CPC	200	100	10	80
16	1:1	TBAH	100	RT	10	76
17	1:1	TBAH	200	100	10	78
18	1:1	TBAI	100	RT	10	68
19	1:1	TBAI	200	100	10	74
20	1:1	SDBS	100	RT	8	82
21	1:1	SDBS	50	RT	8	80
22	1:1	SDBS	30	RT	8	64
23	1:1	SDBS	30	50	10	70

^a % Yield refers to the isolated yield of all the compounds after chromatographic separation.

It was also observed that during the reaction the substrates and reactants do not mix together in water; addition of SDS not only raised the solubility of the components in water but also catalyzed the process tremendously. Addition of catalytic amount of SDS (0.03 mol %) turned the reaction mixture into a clear yellowish colored solution that slowly transferred into reddish yellow as the reaction progressed. After completion of the reaction (checked by tlc), products were purified by simple filtration (and in some cases by column chromatography, silica 60-120 mesh) followed by crystallization to get the products in good to excellent yields.

Potential of pentacyclic triterenod as bioactive candidate is well described. In order to see the effect on their bioactivities by the introduction of quinoxaline ring on ring A, the present author applied the developed protocol on 2 α -bromofriedelin (**37**) (prepared from friedelin) (**28**) and isolated the corresponding quinoxaline derivative (**38**) in 58% yield within 8 hours under identical condition (0.03 mol% of SDS). This is also the very first report of preparing quinoxaline derivative of pentacyclic triterenoids in water at room temperature (Scheme 34).



Scheme 34 Synthesis of benzopyrazine or quinoxaline derivative of friedelin

For the synthesis of the benzopyrazine derivative of friedelin, first friedelin was isolated from cork through soxhlet apparatus (Vide infra). Friedelin was found as white powdered material of melting point 260-262 °C. In the IR spectrum it showed a characteristic peak for the presence of a six membered ketone moiety. In its ¹H NMR spectrum taken in CDCl₃, it showed the presence of eight methyl groups at δ_{H} 0.72 (s, 3H, -CH₃), 0.76 (s, 3H, -CH₃), 0.86 (s, 3H, -CH₃), 0.88 (s, 3H, -CH₃), 0.92 (s, 3H, -CH₃), 1.00 (s, 3H, -CH₃), 1.05(s, 3H, -CH₃), 1.18(s, 3H, -CH₃). All other ¹H NMR peaks are in good agreement with that reported for friedelin. In the ¹³C NMR spectrum C-3 appeared at δ_{C} 213.1 (C-3) and the carbon atoms of different methyl groups appeared at δ 7.2 (C-23), 14.6 (C-24), 18.5 (C-25), 15.7 (C-26), 18.7 (C-27), 32.1 (C-28), 31.8 (C-29), 32.8 (C-30). All other peaks are in good agreement with that reported for friedelin, **28**.

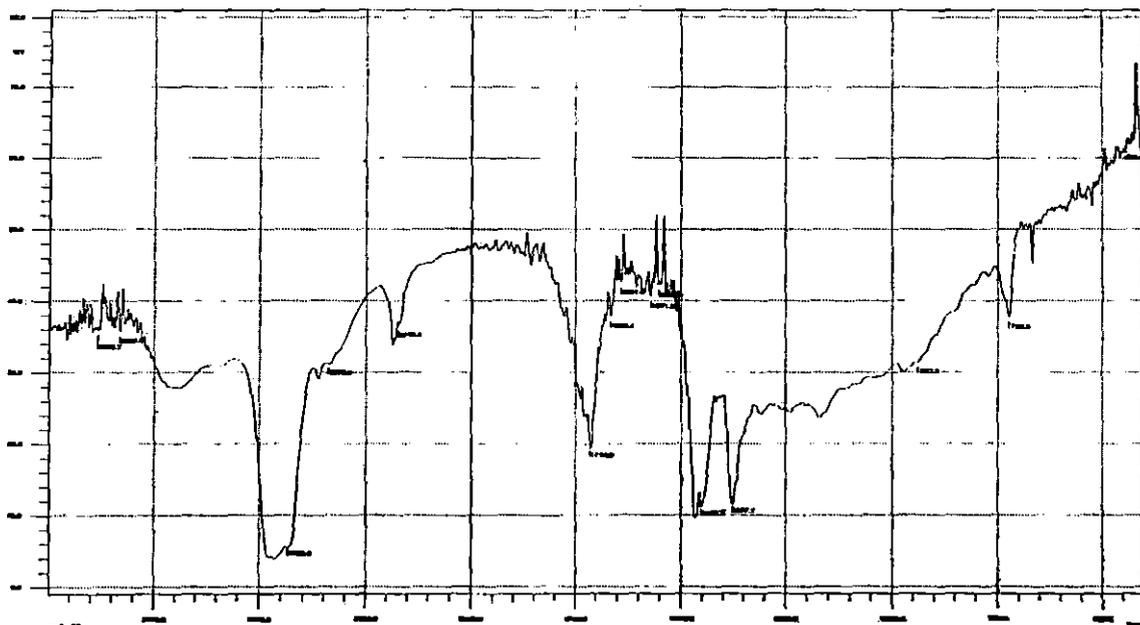


Figure 28 IR spectrum of friedelin, 28

2.365
2.322
2.301
2.276
2.137
2.131
1.937
1.771
1.718
1.689
1.557
1.474
1.453
1.418
1.390
1.340
1.311
1.251
1.180
1.095
1.059
1.004
0.754
0.727
0.683
0.787
0.725
0.663
0.615
0.597
0.465
0.401
0.227
0.151
0.062
0.000

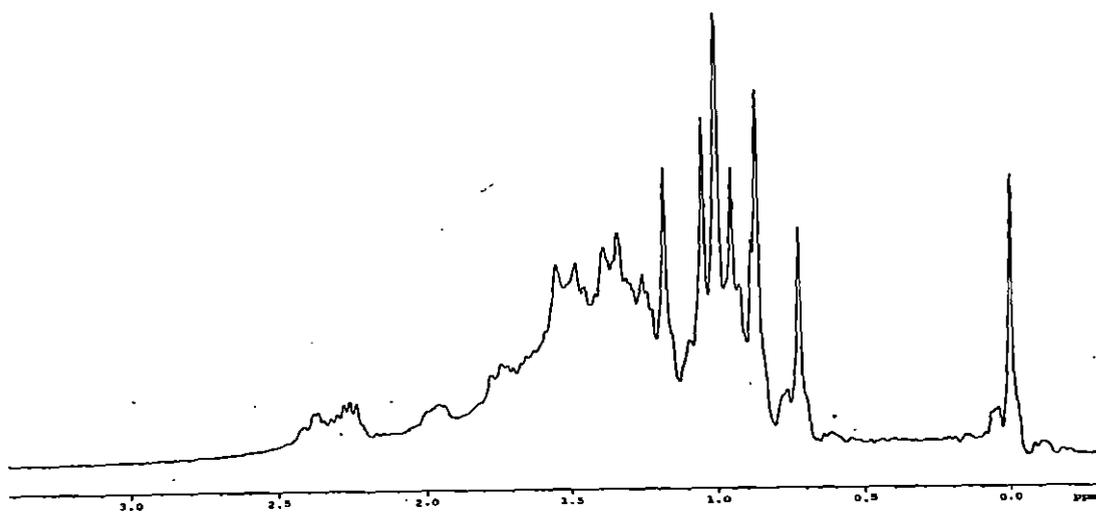
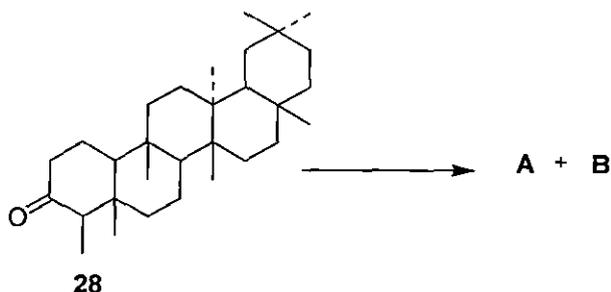


Figure 29 Expanded ^1H spectrum of friedelin, 28

2α -bromofriedelin was prepared from friedelin⁸⁷ following the process of E. J. Corey and J. J. Ursprung⁸⁷ in stirring chloroform solution by adding bromine (vide infra) at room temperature in 72% yield. The reaction mixture was worked up with chloroform and

purified over a column of silica gel (60-120 mesh). It showed two distinct spots in the tlc plate, thus signifying the presence of.



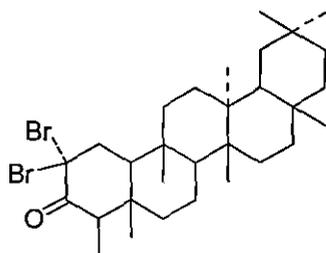
Scheme 34 Direct bromination of friedelin

2.1.1 Characterization of compound A

Compound A was purified as white powdered material of melting point 199-200 °C. In its IR spectrum it gave peaks at 1715, 1448, 1391, 916 and 787 cm^{-1} . The IR spectrum showed the presence of a six membered cyclic ketone moiety. In its mass spectrum (Figure 30) it showed molecular ion peak at m/z 584 and three distinct peaks at m/z 582, 584, 586 in a ration of 1:2:1. The appearance of such type of peaks in definite ratio indicated the presence of two bromine atoms in the molecule of A. From elemental analysis and mass spectral data the molecular formula of compound A was corroborated as $\text{C}_{30}\text{H}_{48}\text{Br}_2\text{O}$. The six degrees of unsaturation coupled with the molecular formula as obtained from the mass and elemental analysis data signified the presence of five rings and a double bond in the molecule.

In the ^1H NMR spectrum (Figure 31) taken in CDCl_3 taking TMS as an internal standard at ambient temperature it showed a triplet at δ_{H} 4.01 with an integration of one proton having coupling constant, $J_{1\alpha\text{H}, 2\alpha\text{H}} = 2.7$ Hz. This was the αH atom at C-1 of the friedelan moiety. Another triplet appeared at δ_{H} 3.13 with an integration of one proton having coupling constant, $J_{1\beta\text{H}, 2\alpha\text{H}} = 6.6$ Hz. This was attributed to the βH atom at C-1 of the friedelin moiety. The difference in the coupling constants for the two attached hydrogen atoms at C-1 might be due to the cis or trans orientation of the βH and αH respectively to that of the βH at C-2. Eight methyl groups each appeared as a sharp singlet at δ_{H} 0.72 (s, 3H, $-\text{CH}_3$), 0.76 (s, 3H, $-\text{CH}_3$), 0.86 (s, 3H, $-\text{CH}_3$), 0.88 (s, 3H, $-\text{CH}_3$), 0.92 (s, 3H, $-\text{CH}_3$),

1.00 (s, 3H, -CH₃), 1.05 (s, 3H, -CH₃) and 1.18 (s, 3H, -CH₃) satisfactory to that reported for friedelan skeleton. A multiplet centered at δ_H 1.97 was due to the presence of C-4 α hydrogen atom in the ring A of the pentacyclic friedelan triterpenoid skeleton. All other peaks were in agreement to that of friedelan skeleton. From these above all data it was clear that the structure of compound **A** is the following 2,2-dibromo friedel-3-one, **39**. In the ¹³C NMR spectrum (Figure 32) C-2 was deshielded significantly and appeared at δ_C 80.6. Carbonyl group at C-3 appeared at δ_C 206.5. All other peaks were in good agreement to that of friedelan skeeton.



39

2,2-Dibromo friedel-3-one

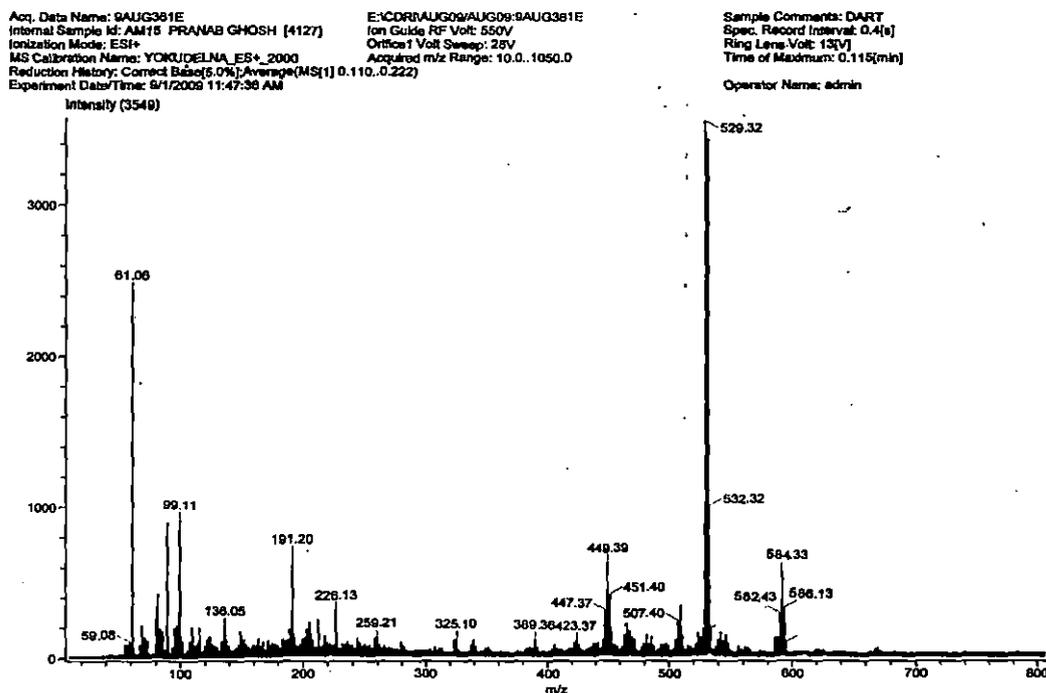


Figure 30 Mass spectrum of 2,2-dibromo friedelin, **39**

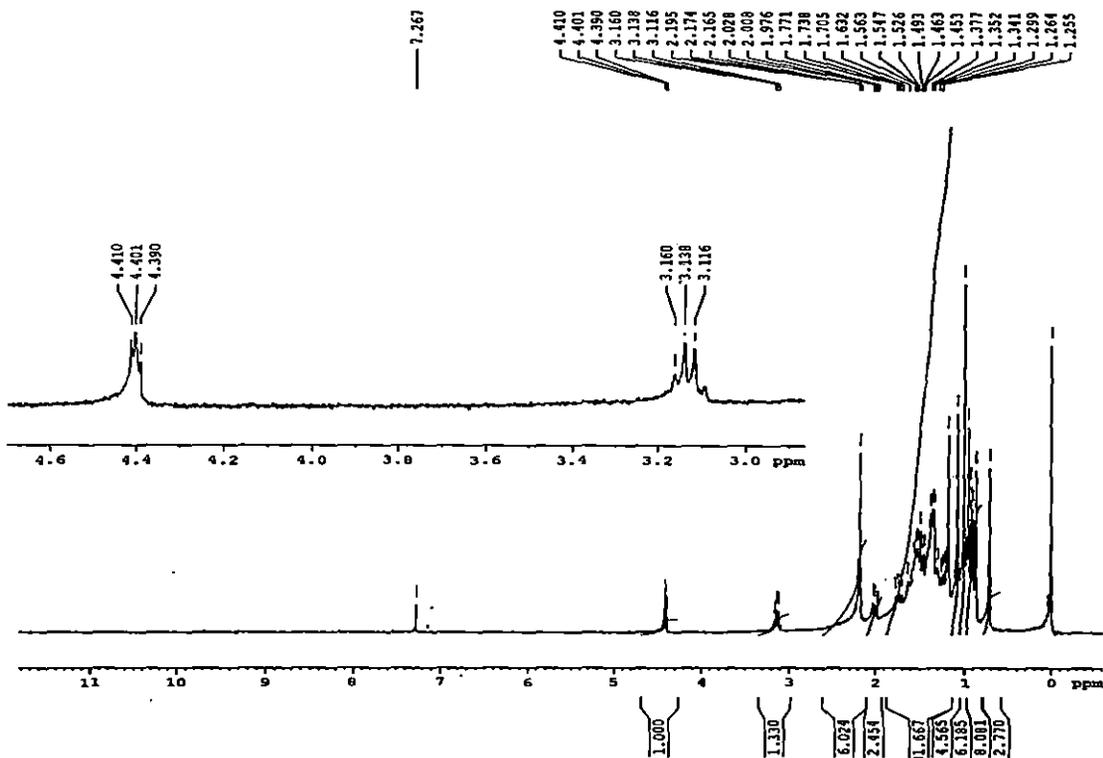


Figure 31 ^1H NMR spectrum of 2,2-dibromo friedelin, 39

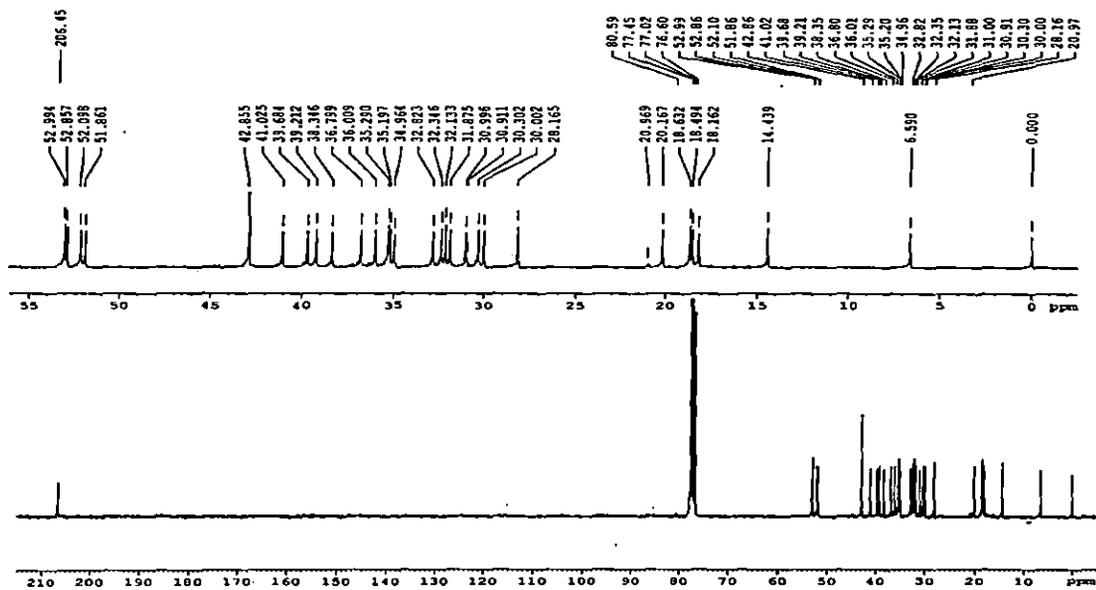


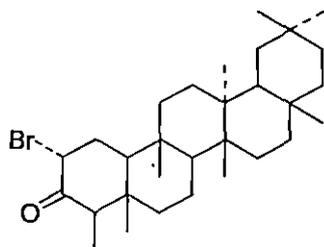
Figure 32 ^{13}C NMR spectrum of 2,2-dibromo friedelin, 39

2.1.2 Characterization of compound **B**

The isolated white powdered compound, the major one, showed a melting point of 208-210 °C (the reported⁸⁷ melting point of 2 α -bromofriedelin is 210 °C). Very good correlation of melting point of compound **B** to that of the reported melting point of 2 α -bromofriedelin indicated that compound **B** might be the target compound. In the IR spectrum (Figure 33) it showed sharp peaks at 1715 cm⁻¹ for the presence of a six membered ketone. Other peaks appeared at 2940, 2864, 1457, 1388, 1181 and 594 cm⁻¹. In the mass spectrum (Figure 34) it showed the molecular ion peak at m/z 504, another peak at m/z 506 appeared at a ratio of 1:1 to that of the molecular ion peak. The appearance of such type of peaks in the definite ratio indicated the incorporation of a single bromine atom into the triterpenoid skeleton. From the elemental analysis and the mass spectral data the molecular formula of compound **B** was assigned as C₃₀H₄₉BrO. Here also the six degrees of unsaturation coupled with the molecular formula as obtained from the mass and elemental analysis data signified the presence of five rings and a double bond in the molecule.

In the ¹H NMR spectrum (Figure 35, 36), it showed the presence of eight tertiary methyl groups at δ_H 0.72 (s, 3H, -CH₃), 0.89 (s, 3H, -CH₃), 0.96 (s, 3H, -CH₃), 1.00 (s, 3H, -CH₃), 1.03 (s, 3H, -CH₃), 1.07 (s, 3H, -CH₃), 1.18 (s, 3H, -CH₃) and 1.25 (s, 3H, -CH₃). A doublet of a doublet centered at δ_H 1.87 (dd, 1H, J = 2.3 and 15.2 Hz) was due to the presence of C-4 α hydrogen atom in the ring A of the pentacyclic friedelan triterpenoid skeleton. Another multiplet with an integration of only one proton appeared at δ_H 3.22 (m, 1H). A large deshielding nature of the appeared proton might be attributed by the fact that it is the β H at C-2 and the observed large deshielding obviously due to the magnetic anisotropy induced by the attached electronegative bromine atom. Two hydrogen atoms at C-1 of the friedelan skeleton were also deshielded by the magnetic anisotropy induced by the bromine atom at C-2 and each appeared as a distinct multiplet at δ_H 2.94 (m, 1H, α H at C-1) and 2.71 (m, 1H, β H at C-1). All other peaks are in close similarity to that of friedelan skeleton. In the ¹³C NMR spectrum (Figure 37) C-2 appeared at δ_C 70.4 and the carbonyl carbon at C-3 appeared at δ_C 198.2. Eight methyl groups each gave a sharp singlet at δ_C 7.6 (C-23), 14.5 (C-24), 18.0 (C-25), 20.2 (C-26), 18.6 (C-27), 32.1 (C-28),

35.0 (C-29) and 31.8 (C-30). All other skeletal carbon atoms gave distinct peaks and all are in very good agreement to the for friedelan skeleton. On the basis of all these data, the structure of compound **B** was suggested as 2 α -bromofriedel-3-one, **37**.



37
2 α -bromofriedel-3-one

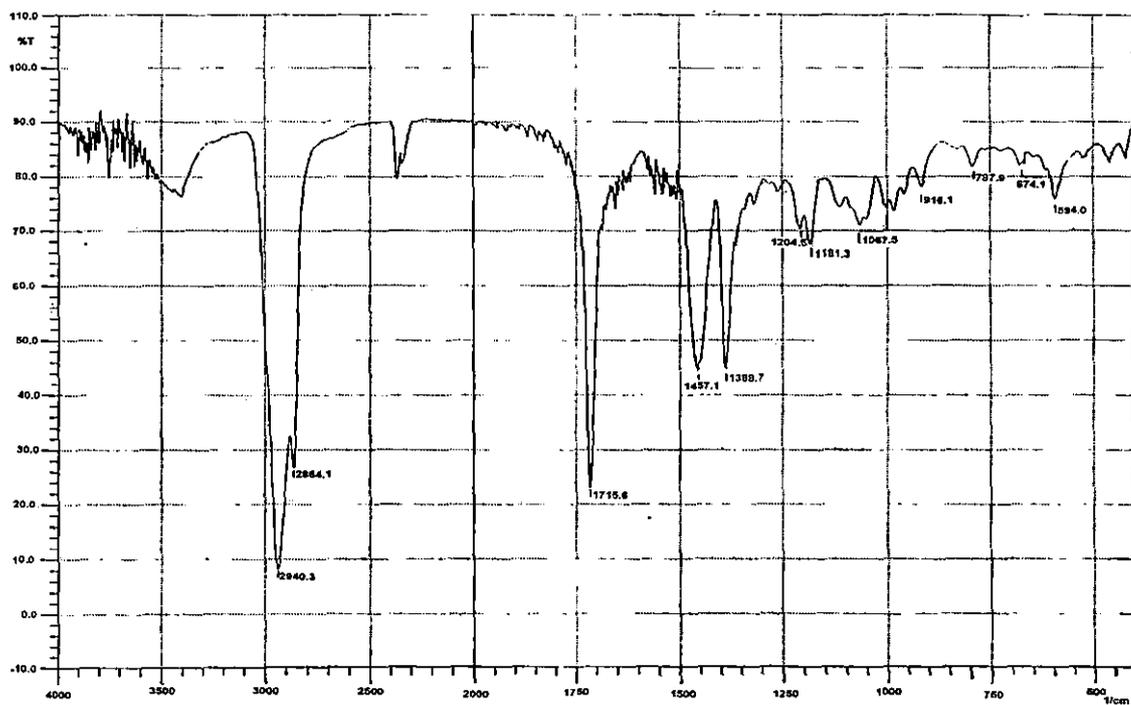


Figure 33 IR spectra of 2 α -bromofriedelin, **37**

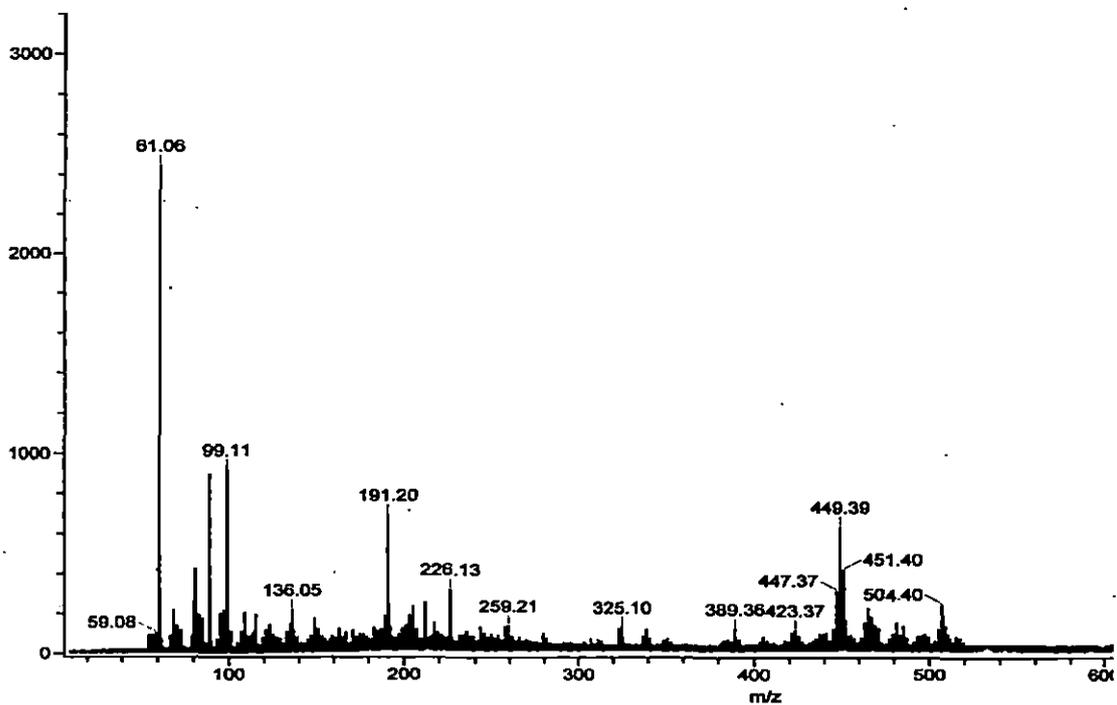


Figure 34 Mass spectrum of 2 α -bromo friedelin

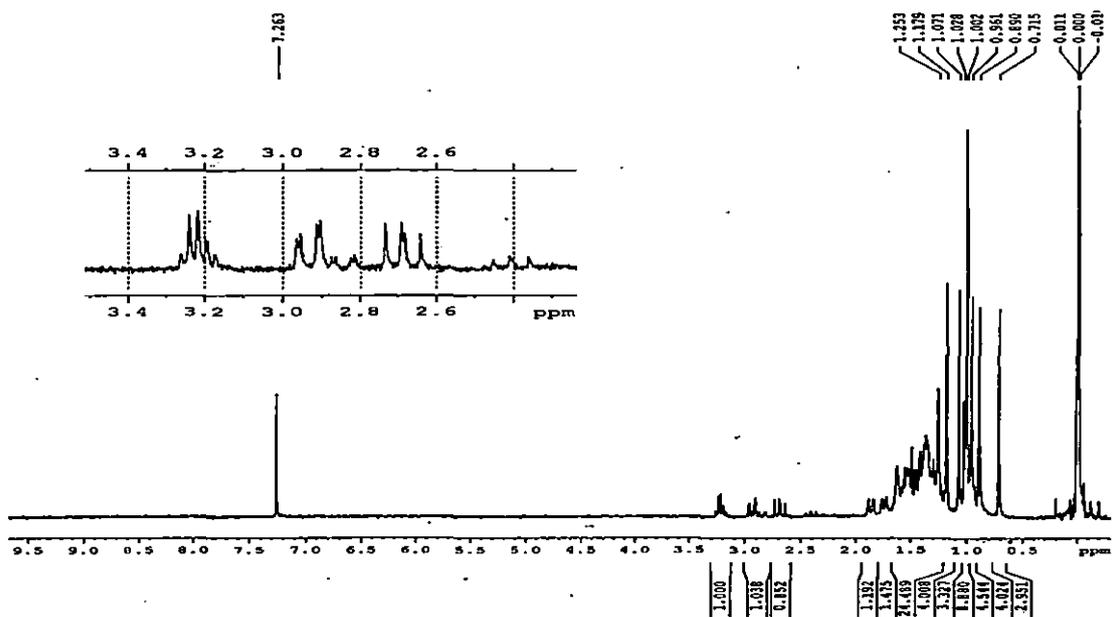


Figure 35 ^1H NMR spectrum of 2 α -bromofriedelin

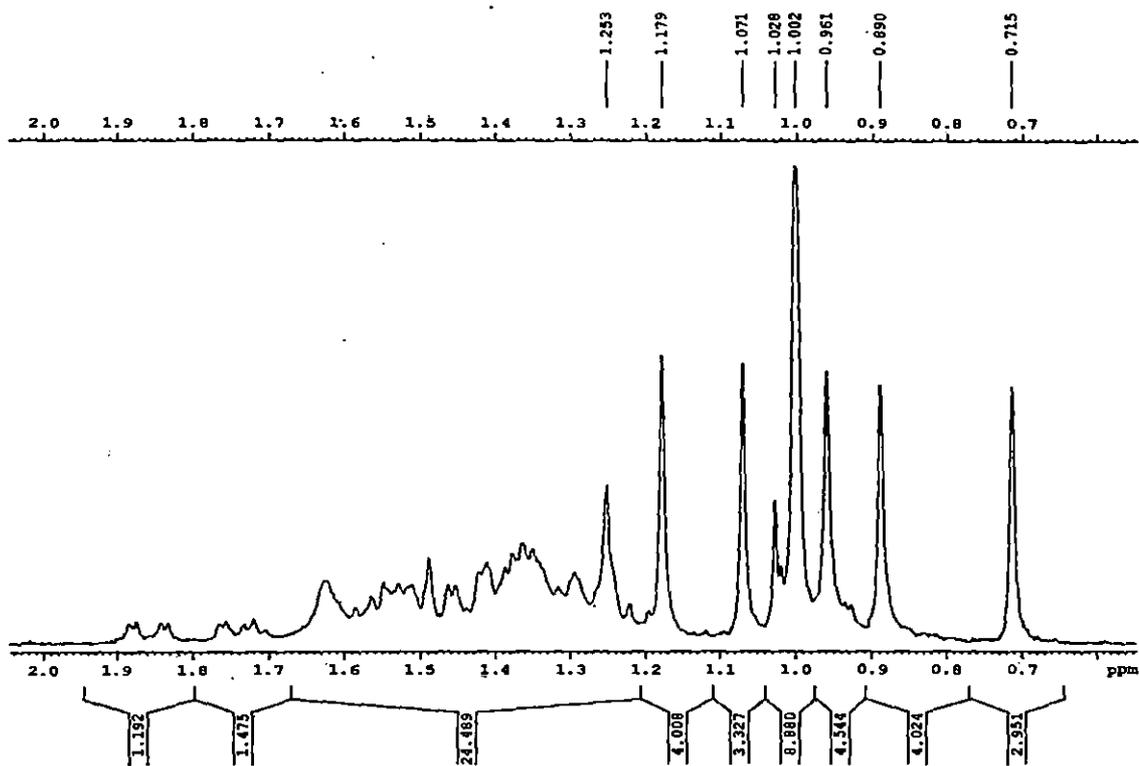


Figure 36 Expanded ¹H NMR spectrum of 2α-bromofriedelin

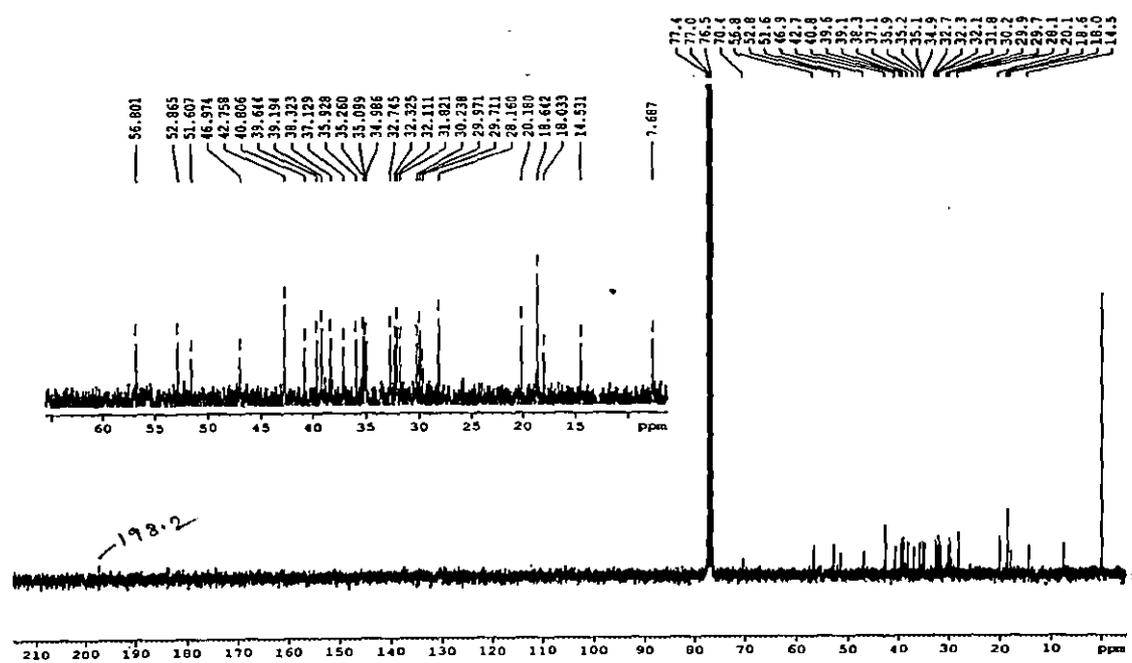


Figure 37 Expanded ¹³C NMR spectrum of 2α-bromofriedelin

2.2 Synthesis of benzopyrazine or quinoxaline derivative of friedelin

2 α -bromofriedel-3-one prepared so far was treated with *o*-phenylenediamine in tap water using SDS as the catalyst under stirring condition at room temperature. After 8 hours at the end of the reaction as revealed by tlc, the reaction mixture was worked up with ethyl acetate (Videinfra) and dried over anhydrous sodium sulfate. After drying the reaction mixture showed a single compound in tlc. It was then purified over a column of silica gel (60-120 mesh).

Purification of the reaction mixture gave a white powdered compound of melting point 248-250 °C (248-151 °C as reported).⁸⁸ In its mass spectrum it showed a molecular ion peak at m/z 512. From the elemental analysis and the mass spectral data the molecular formula of the synthesized compound should be C₃₆H₅₂N₂. In the IR spectrum (Figure 38) it gave peaks at 2941, 2863, 1715, 1448, 1391, 1214, 1186, 1067, 916, 787.9 and 674 cm⁻¹. It showed a molecular ion peak at m/z 512 in its mass spectrum (Figure 39), thus confirming the incorporation of quinoxaline moiety. In the ¹H NMR spectrum (Figure 40) eight methyl groups appeared as a sharp singlet at δ_H 0.72 (s, 3H, -CH₃), 0.76(s, 3H, -CH₃), 0.83(s, 3H, -CH₃), 0.88(s, 3H, -CH₃), 0.92(s, 3H, -CH₃), 1.00(s, 3H, -CH₃), 1.05(s, 3H, -CH₃), 1.15(s, 3H, -CH₃). Four aromatic hydrogen atoms appeared at δ_H 7.43 (t, 1H, $J = 7.8$ Hz); 7.58 (dq, 1H, $J = 2.4$ Hz and 8.1 Hz); 8.00 (dt, 1H, $J = 2.7$ Hz and 7.8 Hz); 8.09 (m, 1H). In the ¹³C NMR spectrum (Figure 41) eight methyl groups gave singlet at δ_C 13.5 (C-23), 16.2 (C-24), 18.0 (C-25), 20.2 (C-26), 18.6 (C-27), 32.1 (C-28), 35.0 (C-29) and 31.8 (C-30). The attached heterocyclic ring structure to the ring A of the pentacyclic triterpenoid induced a deshielding effect to the methyl groups at C-4 (C-23 methyl) and at C-5 (C-24 methyl) and that is why these two methyl groups appeared in a larger deshielded position with respect to that for simple friedelan skeleton. Moreover, the extent of deshielding influence for the methyl group at C-4 (δ_C 13.5, whereas the same appeared at δ_C 7.6 in 2 α -bromofriedel-3-one) is found to be larger than that for at C-5 (δ_C 16.2, whereas the same appeared at δ_C 14.5 in 2 α -bromofriedel-3-one). This observed greater deshielding of the methyl group at C-4 (C-23 methyl) might be due to the fact that it is nearer to the incorporated heterocyclic moiety at ring A of the pentacyclic structure than that at C-5 (C-24 methyl). Aromatic carbon atoms appeared at δ_C 128.3, 129.8, 130.3, 131.0, 133.8, 134.7, 168.3 and 170.6. All the above spectral and

physical data can be explained by considering the following structure to the synthesized compound.

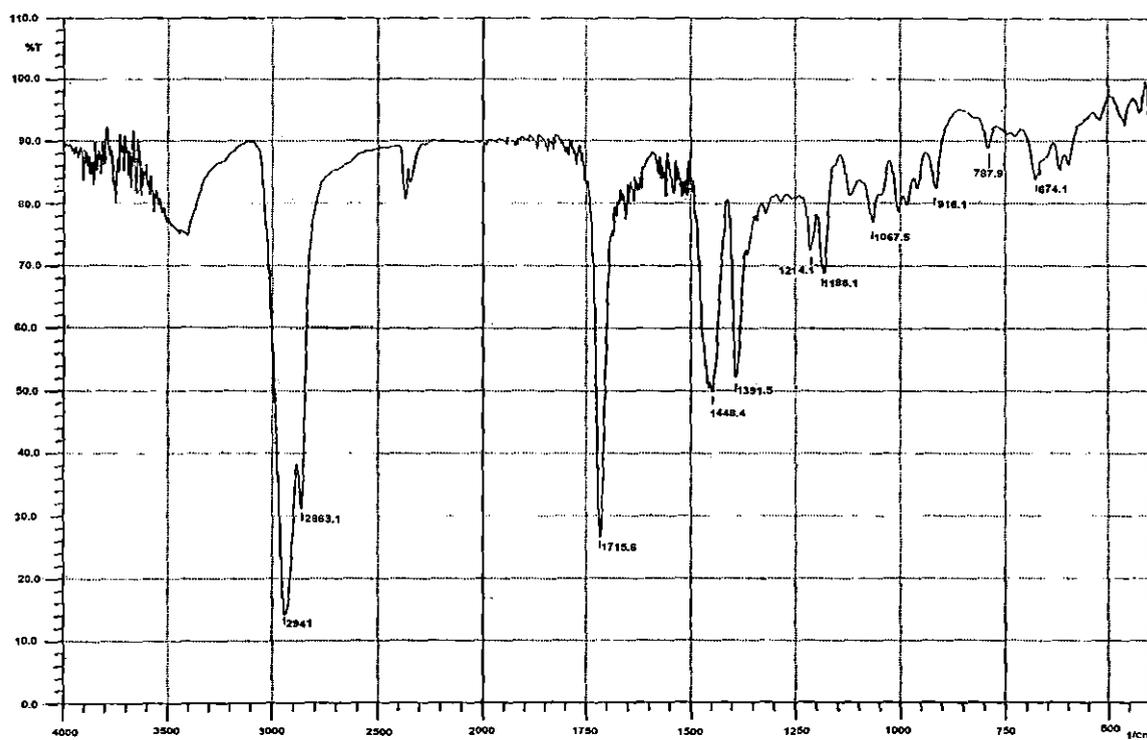
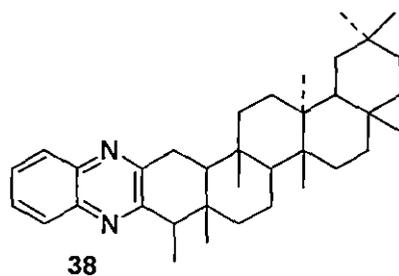


Figure 38 IR spectrum of friedelin quinoxaline derivative, 38

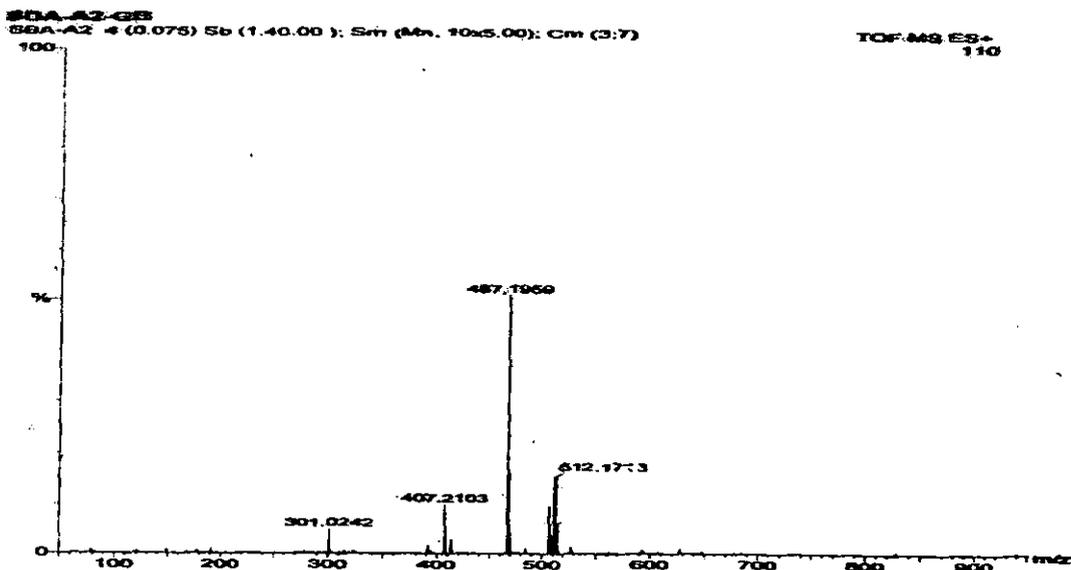


Figure 39 Mass spectrum of the quinoxaline derivative of friedelin, 38

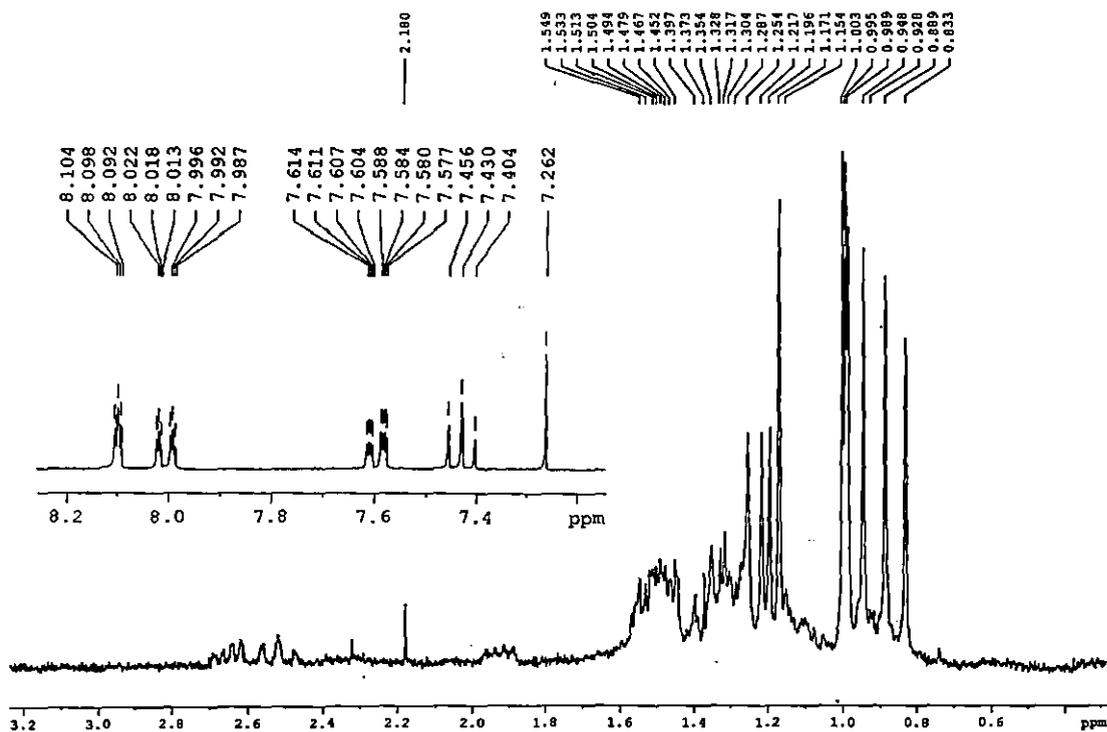


Figure 40 ^1H NMR spectrum of benzopyrazine derivative of quinoxaline, 38

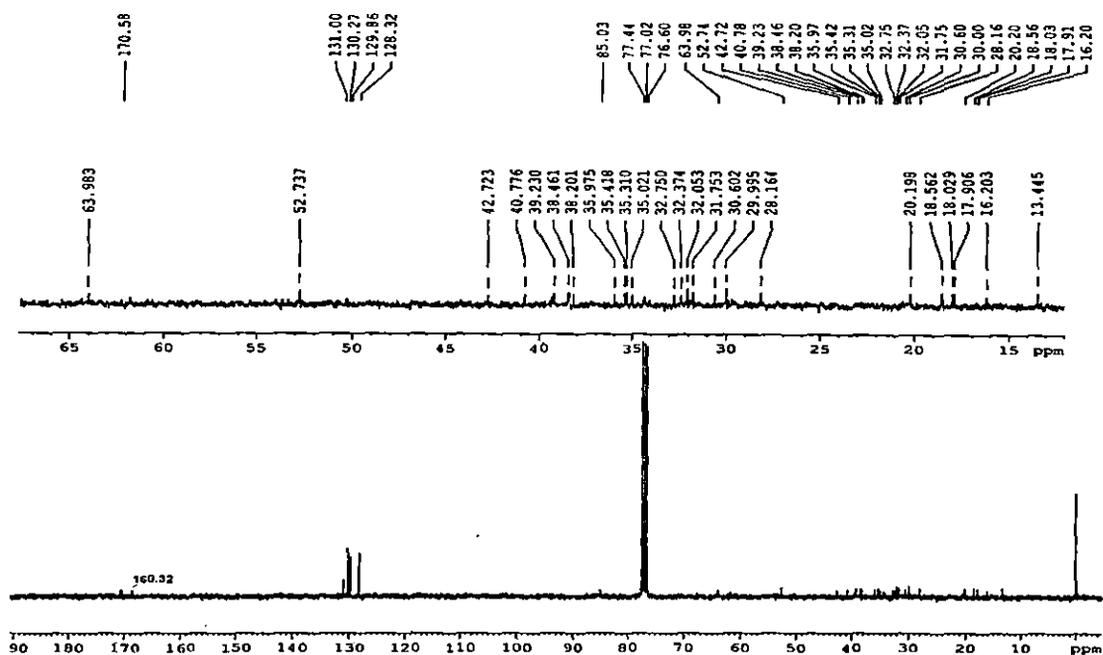


Figure 41 ^{13}C NMR spectrum of friedelin quinoxaline derivative, 38

Unfortunately similar transformation with the 2,2-dibromo friedelin did not yield the desired product under the same reaction condition.

2.3 Versatility of the developed protocol

In order to demonstrate the versatility of SDS as a catalyst for the synthesis of quinoxalines, a series of α -bromoketones and 1,2-diamines were subjected to undergo one pot condensation-aromatization in presence of SDS under the optimized reaction protocol (Table 6). All of the reactions tried showed good selectivity with excellent isolated yields. While investigating the influence of the substituents present either on ketone part or on 1,2-diamine on the course of the reaction, it was observed that compounds having electron donating or withdrawing groups on the ketone (Entry 2, 3, 5, 6, 7 and 8, 9, of Table 6) both underwent the reactions in almost similar fashion and gave good yields. Although, *p*-bromo phenacylbromide (Entry 5, of Table 6) gave better yield than its meta isomer (Entry 9, of Table 6), the corresponding *p*-nitro and *m*-nitro derivative underwent the reaction in identical fashion (Entry 10, of Table 6). Disubstituted α -bromoketones (Entry 11, 12, of Table 6) also gave excellent yields of the

expected quinoxalines. Sensitive molecules like 1,2-diaminomalonitrile (Entry 15, of Table 6) was also found compatible to the reaction condition and gave 84% yield of the corresponding quinoxaline. All the observed results were summarized in table 6.

Table 6 Preparation of quinoxaline derivatives.

Entry	α -Bromo carbonyl compound	Diamine	Time (h)	Product	%Yield
1			6		94
2			6		92
3			6		92
4			6		86
5			6		92
6			6		89
7			6		87
8			7		84
9			6		87
10			8		85
11			7		83
12			6.5		98
13			7		82
14			6		92
15			5		84

% Yield refers to the isolated yield of all the compounds.

Continuation of Table 6

Entry	α -Bromo carbonyl compound	Diamine	Time (h)	Product	%Yield
16			6.5		88
17			6		88
18			6		88
19			6		88

% Yield refers to the isolated yield of all the compounds.

2.3 Proposed mechanism for the present transformation

It is well known that under ambient condition surfactant molecules can aggregate in an aqueous phase to micelles with hydrophobic core and a hydrophilic corona.^{122,123}

To determine whether micellisation had occurred or not we first measured the CMC (critical micellisation concentration) of SDS (Figure 42) and the value was found to be 8.33 mM. In the present study, under the optimized reaction condition the concentration of SDS was 11.57 mM (10 mg of SDS in 3 ml water). Since the value was far beyond the CMC value of SDS (8.33 mM), micellization was anticipated.

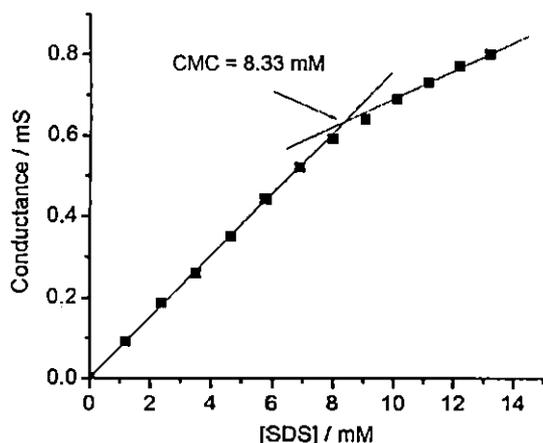


Figure 42 Plot of conductance vs. concentration of SDS for the calculation of CMC value of SDS

It was reported in the literature¹²⁴ that the dimensionless packing parameter P of the molecular geometry as an index to predict the size and shape of the micelles. P was defined as $V/(a_0l)$, where V is the hydrocarbon chain volume, a_0 is the optimum head group area per molecule, and l is the hydrocarbon chain length that is taken to be ca. 80-90% of the fully extended chain length.¹²⁴ The overall prediction was concluded as follows:

Spherical micelles $P < 1/3$

Cylindrical micelles $1/3 < P < 1/2$

Bilayers (or vesicles) $1/2 < P < 1$

Inverted structures $P > 1$

The value of packing parameter P , an index to predict the size and shape of the micelles,¹²⁴ of SDS was found to be 0.235 (taking l as 90 % of the fully extended chain length) indicating the spherical nature of the developed micelles.

For further confirmation DLS (Dynamic Light Scattering) measurement was carried out of a 11.57 mM aqueous solution of SDS that indicated the presence of micelles (Figure 43) of radius 161 nm (diameter of 322 nm) with the PDI (Polydispersity index) of 0.348.

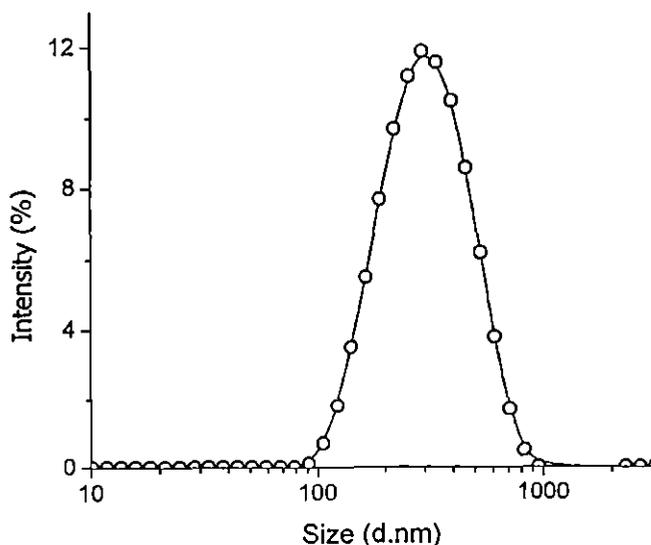


Figure 43 Graph of intensity vs. size (nm) of the micelles based on DLS measurement.

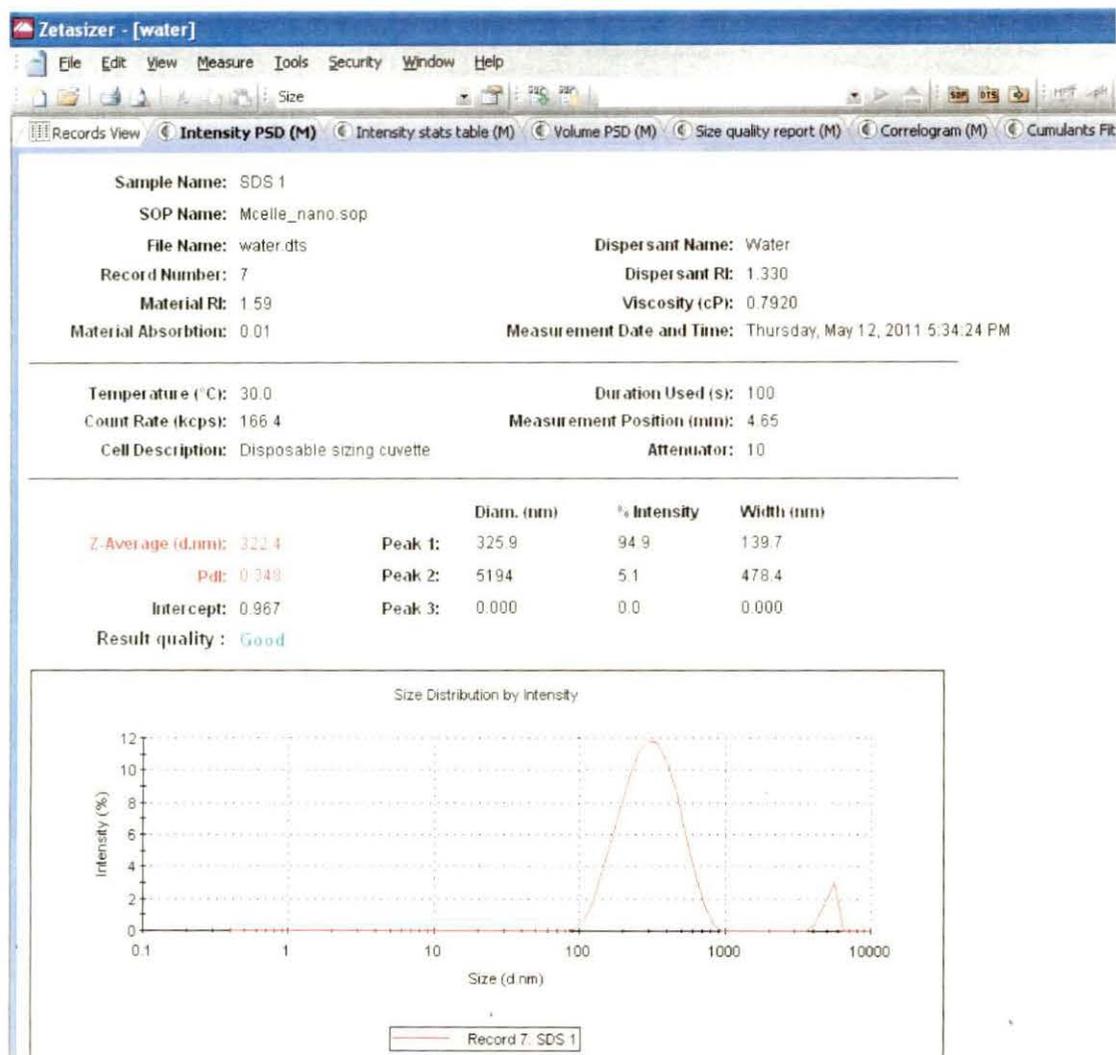
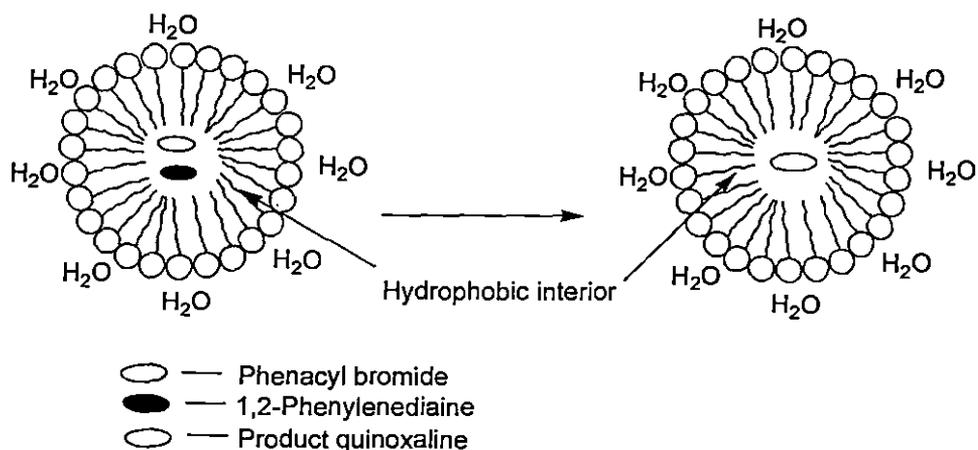
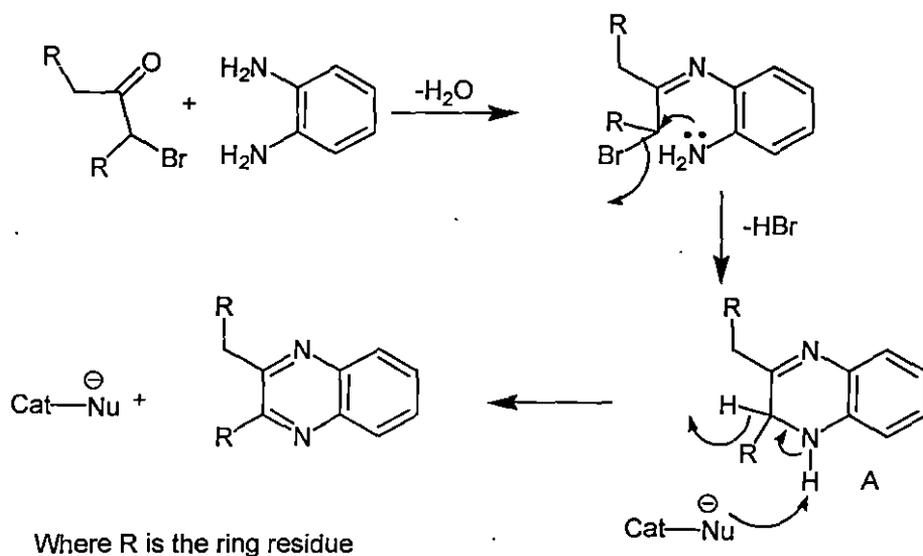


Figure 44 Machine characterization of the formation of the spherical micelle of radius 161 nm.

The role of SDS as a nucleophile is well investigated in literature.¹²¹ Considering the above characteristics of SDS, the most probable mechanism of the micellar SDS in effecting the present transformation may be depicted as shown in scheme 35. In the micellar solution, 1,2-phenylenediamine and phenacyl bromide, both of which are hydrophobic in nature, are entered into the hydrophobic core of the micelles and thus assist the condensation between the phenacyl bromide and *o*-phenylenediamine to form dihydroquinoxaline derivative, **A** (Scheme 35 and 36). Nucleophilic nature of SDS may have assisted the in situ aromatization of the dihydro derivative (**A**) to afford quinoxalines (Scheme 36).



Scheme 35 Proposed model for the synthesis of quinoxaline in water-SDS



Scheme 36 Plausible mechanism of the SDS catalysed quinoxaline formation.

2.4 Recyclability of the catalyst

As was mentioned earlier, a simple filtration or easy work up procedure of the reaction and reuse of the catalyst, SDS directly from the aqueous extract of the reaction mixture for a fresh run, are the great advantages of the developed process. Gratifyingly, it was tested that the recovered water layer can be reused for six consecutive runs (Table 7).

Table 7 Recycling experiment using SDS

Entry	No. of Cycle	% Yield
1	0	92
2	1	87
3	2	82
4	3	78
5	4	72
6	5	68

% Yield refers to the isolated yield of the compound after chromatography.

3. Conclusion

A simple, energy efficient, one step SDS catalyzed (0.03 mol%) greener method for the synthesis of benzopyrazines or quinoxaline derivatives under water mediating condition has been developed. Structurally diversified α -bromoketones, commonly regarded as safer chemicals, were used as reaction partners of 1,2-diamines in water at ambient temperature. Effect of the nature and position of the substituents on both the reactants in consideration to the reaction condition was also studied. Disubstituted α -bromoketones and 2 α -bromo friedelin (a representative of pentacyclic triterpenoids) also formed the corresponding quinoxalines that may serve as lead compound in near future. Except water, no other organic solvents were used. The ambient reaction conditions, comparatively lower reaction time, excellent product yields and simple work up procedure not only make this methodology an alternative platform to the conventional acid/base catalyzed thermal processes, but also found to be significant under the umbrella of environmentally greener and safer processes that may find its place in industry. Moreover, water as a solvent used with micellar SDS has both economic and environmental advantages. As micelles of diameter of 322 nm (radius 161 nm) were formed, it was anticipated that the entitled reactions were occurring inside the hydrophilic core of the micelles. Scaling up the reaction upto 5 moles scale gave good results. We believe our developed process not only satisfied the principles of green chemistry, can open a new way of synthesizing bioactive molecules by catalyzing SDS in water.

SECTION B

Biological activity of the synthesized benzopyrazine derivative of friedelin

Because of the poor solubility of the synthesized benzopyrazine derivative of friedelin, the present author was unable to carry out any practical biological work of this compound.

Chapter IV

Experimental and references

1. Experimental for chemical work

1.1 General

Melting points were recorded in open capillary method and are uncorrected. IR spectra were recorded in Shimadzu 800 FT-IR spectrophotometer as well as in Perkin Elmer FT-IR spectrophotometer using both KBr disc as well as nujol. NMR spectra were recorded in Bruker-Avance 300 MHz FT-NMR instrument at ambient temperature with a 5mm BBO probe. The NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of CDCl₃ solvent as the standards. ¹H spectra were recorded in 300 MHz frequencies and ¹³C NMR spectra were recorded in 75.4 MHz frequencies. Coupling constant 'J' was calculated in Hz. The mass spectra were taken in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer. Elemental Analyses were done in Vario EL-III. Hydrodynamic diameter measurements of aqueous SDS micelles were performed using Nano ZS-90 (Malvern, UK) dynamic light scattering spectrometer. Friedelin and cerin was isolated from cork through soxhlet apparatus using petroleum ether (60-80 °C) as the solvent. All the chemicals used were commercial grade and were purified prior to their use.

1.1.1 Isolation of lupeol from *Xanthoxylum budrunga*

Lupeol was isolated from the bark of *Xanthoxylum budrunga*, collected from Kalimpong, Darjeeling, India. After collection it was shade dried, cut into small pieces and grinded in powdered form through mechanical grinder.

Dried and powdered trunk bark of *Xanthoxylum budrunga* (2 kg) was extracted with toluene in Soxhlet apparatus for 20 hours. Toluene was distilled off and the gummy residue (1 kg) was taken up in ether (1.5 L). The ether solution was washed with 10% aqueous sodium hydroxide solution (3x300 ml). The aqueous alkaline layer was thoroughly shaken with ether to remove any neutral material that might be present. The ether portion was washed with water till neutral and then dried (Na₂SO₄). Ether was removed when a gummy residue (250 g) of lupeol was obtained. Purification was done

over a column of silica gel (60-120 mesh) developed with petroleum ether (PE) and varying concentrations of PE:ethyl acetate (EA) as eluent.

Table 8 Purification of the crude extract of *X. budrunga*

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-7	Nil	--
2	PE-EA (9.9:0.1)	8-13	Oil	--
3	PE-EA (9.8:0.2)	14-17	Oil	--
4	PE-EA (9.6:0.4)	18-25	Nil	--
5	PE-EA (9.5:0.5)	26-55	White solid (\approx 0.5 g)	211-213 °C
6	PE-EA (9.4:0.6)	56-65	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.1.A Characterization of lupeol

Fractions 26-55 (Table 1) were combined and crystallized from a mixture of chloroform and methanol. The compound was coded as 'A' and was characterized. Melting point (mp) was determined as 215 °C. On the basis of IR, TLC and melting point, the compound resembled as lupeol. Finally, the compound was confirmed as lupeol when compared with an authentic sample of lupeol (co-IR, co-tlc, mixed mp *etc.*).

1.1.2 Hydrogenation of lupeol: Preparation of lupanol

Lupeol (5 g) dissolved in a mixture of ethyl-acetate and acetic acid (80 mL each) was shaken in an atmosphere of hydrogen in presence of PtO₂ catalyst (0.2 g) for three hours until absorption of hydrogen ceased. Ethyl-acetate (EA) was removed by distillation and the solution was diluted with water whereby a white solid (4.5 g) separated out which was collected by filtration. Crystallisation from a mixture of chloroform and methanol furnished colourless plates of lupanol and characterized. Melting point was determined as 204 °C, $[\alpha]_D +15^\circ$. On the basis of IR, TLC and melting point the compound resembled

as lupanol. Finally, the compound was confirmed as lupanol when compared with an authentic sample of lupanol (co-IR, co-tlc, mixed mp *etc.*).

1.1.3 Jone's oxidation of lupanol: preparation of lupanone

To a solution of lupanol (4 g) in pure acetone (400 mL) was added Jone's reagent drop wise with shaking until a faint orange colour persisted. The mixture was kept at room temperature for 1 hour, dilute with water and extracted with ether. The ether layer was washed thoroughly with water, dried (anhydrous Na₂SO₄) and the ether evaporated. The residue (3.5 g) was chromatographed over a column of silica gel (60-120 mesh). The chromatogram was developed with petroleum-ether and then eluted with the following solvents (Table 2).

Table 9 Purification of the Jone's oxidation product of lupeol

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Oil	--
2	PE-EA (9.95:0.05)	9-14	Nil	--
3	PE-EA (9.9:0.1)	15-48	White solid (\approx 3.5 g)	211-213 °C
4	PE-EA (9.8:0.2)	49-55	Nil	--
5	PE-EA (9.6:0.4)	56-67	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.3.A Characterization of lupanone

Fractions 15-48 (Table 2) were combined and crystallized from a mixture of chloroform and methanol. The crystallization furnished colourless needles of lupanone and characterized. Melting point (mp) was determined as 208 °C, $[\alpha]_D^{+15}$. On the basis of IR, TLC and melting point, the compound resembled as lupanone. Finally, the compound was confirmed as lupanone when compared with an authentic sample of lupanone (co-IR, co-tlc, mixed mp *etc.*).

1.1.4 Autoxidation of lupanone: Preparation of diosphenol derivative⁷³

Lupanone (0.6 g) isolated from bark of *Xanthoxylum budrunga* was suspended in potassium tertiary butoxide (prepared from 6 g of potassium and 60 mL of tertiary butanol) was shaken in a stream of oxygen for two hours. The reaction mixture was then diluted with water and then 6*N* hydrochloric acid was added till the solution was acidic. It was then extracted with chloroform (100 mL) and the combined extract was dried (anhydrous Na₂SO₄) and the solvent was removed under reduced pressure to yield a colorless solid, **B**. The prepared solid was used as recovered without further purification for the synthesis of pyrazine derivatives into the ring A of lupane skeleton.

1.1.5 Preparation of the 1,4-pyrazine derivative

In a typical reaction procedure, in a 50 ml round bottom flask 200 mg of **B** was dissolved in 15 mL of wet methanol and the solution was made homogeneous by stirring with a magnetic spinning bar. To this 200 mg of ethylene diamine and small amount of ^tBuOK (traces) were added. Stirring was continued until the reaction was complete (checked by tlc). Methanol was evaporated under reduced pressure and the crude product was purified by chromatography using silica gel (60-120 mesh) column. Petroleum ether (PE) and varied proportion of PE:ethyl acetate (EA) was used as eluent.

Table 10 Purification of the Jone's oxidation product of lupeol

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-6	Oil	--
2	PE-EA (9.9:0.1)	7-12	Nil	--
3	PE-EA (9.8:0.2)	13-28	White solid (≈ 0.15 g)	219-220 °C

Further elution with more polar solvent mixture did not afford any solid material

1.1.5.A Characterization of the 1,4-pyrazine derivative lupanone

Crystallization from chloroform-methanol mixture (fractions 13-28) afforded white needle shaped crystals, $C_{32}H_{50}N_2$, mp 220 °C, IR at 1650, 1430 and 1120 cm^{-1} . UV absorption maxima at 272nm ($\epsilon = 5831$) and 278nm ($\epsilon = 5792$). Anal.Calc.: 83.12% C, 10.82% H; found 83.10% C, 10.81% H. Mass spectrum showed molecular ion peak at m/z 462. 1H NMR ($CDCl_3$, δ ppm $^{-1}$ relative to TMS): 0.78, 0.83, 0.98, 1.11, 1.29, 1.31 (6s, 18H, 6t- CH_3); 0.77, 0.86 (2d, 6H, CH (CH_3) $_2$, J = 7 Hz); 2.47, 3.04 ppm (2d, J = 16 Hz); 8.27(d, J = 3 Hz); 8.41(dd, J = 3 Hz).

1.1.6 Isolation of betulinic acid from *Bischofia javanica* Linn

Betulinic acid was isolated from the outer bark of *Bischofia javanica*, collected from the sukna belt of Darjeeling foot hills of West Bengal, India. After collection the bark materials were cut into small pieces and shade dried. The dried plant materials were then grinded to powdered form by a mechanical grinder.

Dried and powdered trunk bark of *Bischofia javanica* (2 kg) was extracted with toluene in a soxhlet apparatus for twenty hours. Benzene was distilled off and the gummy residue (9 g) was taken up in ether (1 L). The ether solution was washed with 10% aqueous sodium hydroxide solution (3x300 mL). The aqueous alkaline layer was thoroughly shaken with ether to remove any neutral material that might be present. The aqueous layer was acidified (1 L) when some insoluble solid separated out. The acidified portions was extracted with ether, washed with water till neutral and then dried (anhydrous Na_2SO_4). Ether was removed when a gummy residue (2 g) of betulinic acid was obtained.

Table 11 Purification of the crude extract of betulinic acid

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-4	Oil	--
2	PE-EA (9.9:0.1)	5-11	Nil	--
3	PE-EA (9.8:0.2)	12-18	Nil	--

Table 11 continued

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
4	PE-EA (9.7:0.3)	19-53	White solid (\approx 1.5 g)	295-297 °C
5	PE-EA (9.6:0.4)	54-67	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.6.A Characterization of betulinic acid

Fractions 19-53 (Table 4) were combined and crystallized from a mixture of chloroform and methanol. The compound was coded as 'A' and was characterized. Melting point (mp) was determined as 295 °C. On the basis of IR, TLC and melting point, the compound resembled as betulinic acid. Finally, the compound was confirmed as betulinic acid when compared with an authentic sample of lupeol (co-IR, co-tlc, mixed mp *etc.*).

1.1.7 Esterification of betulinic acid: Preparation of methyl betulinate

To the crude acid (1 g) dissolved in cold ether (150 ml) was added a solution of diazomethane in ether at cold prepared from *N*-nitrosomethyl urea (1.5 g) with constant hand stirring and was kept overnight. Next day excess of diazomethane was destroyed with glacial acetic acid (1 ml). The ether solution was washed with water, 10% sodium bicarbonate solution and again with water till neutral and then dried (anhydrous Na₂SO₄). Evaporation of the ether yielded a gummy residue (1.5 g).

Table 12 Purification of methyl betulinate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-3	Oil	--
2	PE-EA (9.9:0.1)	4-11	Nil	--
3	PE-EA (9.8:0.2)	12-32	White solid (\approx 1.0 g)	222-223 °C
4	PE-EA (9.7:0.3)	33-39	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.7.A Characterization of methyl betulinate

Solid obtained from the fractions 12-32 (Table 5) were combined (1.0 g) and crystallized from a mixture of chloroform and methanol to afford colorless needles of methyl betulinate⁸⁶ mp 223-24 °C, $[\alpha]_D + 5^\circ$ mp 224-25 °C, $[\alpha]_D + 5^\circ$, identical with an authentic sample of methyl betulinate (mixed mp, co-tlc and co-IR).

1.1.8 Hydrogenation of methyl betulinate: Preparation of methyl dihydrobetulinate

Methyl betulinate (1.0 g), dissolved in a mixture of ethyl acetate and acetic acid (50 ml each) was shaken in an atmosphere of hydrogen in presence of PtO₂ catalyst (0.1 g) for three hours until absorption of hydrogen ceased. Ethyl acetate was removed by distillation and the solution was diluted with water whereby a white solid (1.0 g) separated out which was collected by filtration. Crystallisation from a mixture of chloroform and methanol furnished colorless needles of mp 236-38 °C, identical (mixed mp, co-IR and co-tlc) with an authentic sample of dihydromethyl betulinate.

1.1.9 Jone's oxidation of methyl dihydrobetulinate: Preparation of methyl dihydrobetulonate

To a solution of methyl dihydrobetulinate (1.0 g) in pure acetone (250 mL) was added Jone's reagent dropwise with shaking until a faint orange color persisted. The mixture was kept at room temperature for 1 hour, diluted with water and extracted with ether. The ether layer was washed thoroughly with water, dried (anhydrous Na₂SO₄) and the ether evaporated. The residue (0.6 g) was chromatographed over a column of silica gel (60-120 mesh). The chromatogram was developed with petroleum-ether and then eluted with the following solvents (Table 6)

Table 13 Purification of methyl dihydrobetulonate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-7	Nil	--
2	PE-EA (9.9:0.1)	8-27	White solid (≈ 0.5 g)	196-199 °C

Further elution with more polar solvent mixture did not afford any solid material

1.1.9.A Characterization of methyl dihydrobetulonate

Fractions 8-27 (Table 6) were combined and on crystallization from chloroform-methanol mixture furnished needle shaped crystals of compound, mp 202-4 °C, $[\alpha]_D +8^\circ$, identical with an authentic samples of methyl dihydrobetulonate (mixed mp, co-IR and co-tlc). In IR it showed peaks at 1730 (-COOCH₃), 1708 cm⁻¹.

1.1.10 Autoxidation of methyl dihydrobetulonate: Preparation of diosphenol

Methyl dihydrobetulonate (0.5 g) suspended in potassium tertiary butoxide in tertiary butanol (prepared from 5 g potassium and 150 ml of tertiary butanol) was shaken in a stream of oxygen for 75 minutes. The residue (0.45 g) after usual work up was chromatographed over a column of silica gel (60-120 mesh) developed with petroleum ether (PE) and varying concentrations of PE:ethyl acetate (EA).

Table 14 Purification of the autoxidation product of methyl dihydrobetulonate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-9	Nil	--
2	PE-EA (9.9:0.1)	10-14	Nil	--
3	PE-EA (9.7:0.3)	15-18	Nil	--
4	PE-EA (9.6:0.4)	19-22	Nil	--
5	PE-EA (9.2:0.8)	23-26	Nil	--
6	PE-EA (9.0:1.0)	27-31	Nil	--
7	PE-EA (8.8:1.2)	32-57	White solid (≈ 0.4 g)	126-129 °C
8	PE-EA (8.7:1.3)	58-64	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.10.A Characterization of diosphenol derivative⁷³

Fractions 32-57 (Table 7) of same R_f values were combined and on recrystallization from a mixture of chloroform-methanol afforded colorless crystals of mp 131-32 °C, $[\alpha]_D -1.94^\circ$ mp 131-33 °C, $[\alpha]_D -1.96^\circ$]. It gave positive ferric chloride coloration for

diosphenol and was identified as 2,3-diketo methyl dihydrobetulanate. At UV showed absorption maxima at 271 nm ($\epsilon = 7829$) and in IR it showed peaks at 3460 (-OH), 1730 (-COOCH₃), 1670, 1650, 860 cm⁻¹.

1.1.11 Preparation of the 1,4-pyrazine derivative of 2,3-diketo dihydro betulanate

In a typical reaction procedure, in a 50 ml round bottom flask 200 mg of 2,3-diketo methyl dihydrobetulanate was dissolved in 15 mL of wet methanol and the solution was made homogeneous by stirring with a magnetic spinning bar. To this 200 mg of ethylene diamine and small amount of ^tBuOK (traces) were added. Stirring was continued until the reaction was complete (checked by tlc). Methanol was evaporated under reduced pressure and the crude product was purified by chromatography using silica gel (60-120 mesh) column. Petroleum ether (PE) and varied proportion of PE:ethyl acetate (EA) was used as eluent.

Table 15 Purification of the 1,4-pyrazine derivative of 2,3-diketo dihydro betulanate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-6	Oil	--
2	PE-EA (9.9:0.1)	7-12	Nil	--
3	PE-EA (9.8:0.2)	13-28	White solid (≈ 0.15 g)	218-220 °C
4	PE-EA (9.7:0.3)	29-34	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.11.A Characterization of pyrazine derivative of 2,3-diketo methyl dihydrobetulanate

Crystallization from CHCl₃-MeOH mixture, C₃₃H₅₀O₂N₂, mp 220 °C, IR spectrum showed peaks at 1710 cm⁻¹(CO₂Me); 1665, 1430 and 1120 cm⁻¹. UV spectrum showed absorption maximum at 272nm ($\epsilon = 5712$) and 278 nm ($\epsilon = 5603$). Anal. Calc.: 78.26%

C, 9.88% H, 5.53% N; found 78.25% C, 9.73% H, 5.50% N. Mass spectrum showed molecular ion peak at m/z 506 as base peak. ^1H NMR (CDCl_3 , δ ppm $^{-1}$ relative to TMS): 0.82, 0.985, 0.99, 1.28, 1.305, 0.76 and 0.88 ppm (2d, 6H, $j = 7$ Hz); 2.48, 3.04 ppm (2d $J = 16$ Hz); 8.27, 8.41 ppm (2d, $J = 3$ Hz); 3.66 (s, 3H, ester methyl).

1.1.12 Isolation of friedelin and cerin form cork

Friedelin was extracted from cork (*Quercus suber*). Cork was purchased from Kolkata, India in June 2009. The dried cork materials were then cut into very small pieces and used as such for the extraction of friedelin.

Very small pices of cork material (2 Kg) were extracted with petroleum ether, PE (60-120 °C) for a period of 5 days. After five days a second batch was run in the similar way. From the resulting yellowish colored solution solvent PE was recovered in reduced pressure. After the recovery of the solvent a brown gummy residue (500 g) was obtained that was then dissolved in hot chloroform. The solution was then filtered for any traces of impurities. Total volume of the solution was then decreased by a slow evaporation of the solvent chloroform. The resulting relatively concentrated solution was cooled to room temperature and kept in dark place for over night. Being very sparingly soluble in cold chloroform, cerin was crystallized out as slightly yellowish crystals at the bottom of the container. It was the filtered through a sintered Buckner funnel, washed twice with cold chloroform and dried. Repeated crystallization was performed to afford pure cerin in small needle shaped crystals of mp 248-250 °C.

All the washings and the filtrate were combined and solvent chloroform was evaporated out. The resulting brown gummy residue was then purified to afford pure friedelin over a column of silica gel (60-120 mesh). Pure PE and mixture of PE:ethyl acetate (EA) of varying concentrations were then used as eluent.

Table 16 Purification of the crude extract of cork, isolation of friedelin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-10	Nil	--
2	PE-EA (9.95:0.05)	11-14	Oil	--
3	PE-EA (9.95:0.05)	15-19	White solid (Traces)	--
4	PE-EA (9.95:0.05)	20-24	Nil	--
5	PE-EA (9.95:0.05)	24-72	White solid (\approx 0.8 g)	260-261 °C (Friedelin)

Further elution with more polar solvent mixture did not afford any solid material

1.1.12.A Characterization of friedelin

Friedelin was crystallized from chloroform-methanol solvent mixture as pure white crystals of mp 260-261 °C. In IR it gave peaks at 1715 cm^{-1} . In ^1H NMR eight methyl groups of friedelan skeleton appeared at δ_{H} 0.71 (s, 3H), 0.86 (s, 3H), 0.88 (s, 3H), 0.92 (s, 3H), 1.00 (s, 6H, two methyl groups), 1.05 (s, 3H) and 1.18 (s, 3H). Final confirmation of the isolated compound cerin was done by comparing (mixed mp, co-IR, co-tlc *etc.*) the compound with an authentic sample of friedelin.

1.1.13 Preparation of diketofriedelin

In a 500 mL three necked flask was equipped with a reflux condenser, a thermometer and a dropping funnel. To it 500 mg (1.22 mmol) of pure friedelin and 100 mL of ethanol was heated at 75-80 °C and a solution of 125 mg (1.36 mmol) of selenium dioxide in 100 mL of rectified spirit from a dropping funnel over a period of 2 hours, maintaining the temperature at 75-80 °C. The reaction mixture was further refluxed for another 2 hours after the addition of selenium dioxide. As much as ethanol was distilled off from the reaction mixture and the reaction mixture was cooled to room temperature. It was then poured into ice cold water whereby a yellowish white solid separated out at the walls of the container as well as at the bottom of water. It was then worked up with chloroform

and dried by anhydrous sodium sulfate. The chloroform layer showed a single prominent peak at the tlc plate, solvent was recovered and the final product was purified over a column of neutral alumina.

Table 17 Purification of the selenium dioxide oxidation product of friedelin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Oil	--
2	PE-EA (9.95:0.05)	9-14	Nil	--
3		15-18	Nil	--
4		19-24	Nil	--
5	PE-EA (9.8:0.2)	25-32	Nil	--
6	PE-EA (9.1:0.9)	33-46	Nil	--
7	PE-EA (9.0:1.0)	47-54	Nil	--
8	PE-EA (8.5:1.5)	55-72	White solid	--
	PE-EA (8.0:2.0)		(\approx 0.22 g)	131-32 °C
9	PE-EA (7.9:2.1)	73-77	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.13.A Characterization of 2,3-diketo friedelin

Fractions 55-72 (Table 7) of same R_f values were combined and on recrystallization from a mixture of chloroform-methanol afforded colorless crystals of mp 131-32 °C, It gave positive ferric chloride coloration for diosphenol and was identified as 2,3-diketo friedelin. At UV it showed absorption maxima at 271 nm ($\epsilon = 7829$) and in IR it showed peaks at 3460 (-OH), 1715 (-COOCH₃), 1670, 1650, 860 cm⁻¹. In ¹H NMR eight methyl groups of friedelin skeleton appeared at δ_H 0.71 (s, 3H), 0.86 (s, 3H), 0.88 (s, 3H), 0.92 (s, 3H), 1.00 (s, 6H, two methyl groups), 1.05 (s, 3H) and 1.18 (s, 3H).

1.1.14 Preparation of the 1,4-pyrazine derivative of 2,3-diketo friedelin

In a typical reaction procedure, in a 50 ml round bottom flask 200 mg of 2,3-diketo friedelin was dissolved in 15 mL of wet methanol and the solution was made homogeneous by stirring with a magnetic spinning bar. To this 200 mg of ethylene diamine and small amount of t-BuOK (traces) were added. Stirring was continued until the reaction was complete (checked by tlc). Methanol was evaporated under reduced pressure and the crude product was purified by chromatography using silica gel (60-120 mesh) column. Petroleum ether (PE) and varied proportion of PE:ethyl acetate (EA) was used as eluent.

Table 18 Purification of the 1,4-pyrazine derivative of 2,3-diketo friedelin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-5	Oil	--
2	PE-EA (9.9:0.1)	6-12	Nil	--
3	PE-EA (9.8:0.2)	13-25	White solid (\approx 0.15 g)	248-250 °C
4	PE-EA (9.7:0.3)	26-34	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.14.A Characterization of the 1,4-pyrazine derivative of 2,3-diketo friedelin

UV spectrum showed peaks at 272 ($\epsilon = 5800$) and 278 ($\epsilon = 5450$) nm. The ^1H NMR spectrum of was indicative of the presence of seven tertiary methyls which appeared as sharp singlets (3H each) between δ 0.82–1.22 (7s, 21H, 7t CH_3), the doublet centered at δ 0.99 was due to the presence of secondary methyl protons (d, $J = 6.5$ Hz), two aromatic protons at δ 8.40 and 8.27 appeared as a doublet with $J = 3$ Hz.

1.1.15 Preparation of 2 α -bromofriedelin from friedelin

A solution of 1.28 g of friedelin in 50 mL of chloroform was treated with 1 mL of saturated solution of HBr in chloroform followed by a solution of 0.55 g of bromine in 5 mL chloroform. The decolorization was instantaneous. Chloroform was evaporated in reduced pressure and the residue was purified by column chromatography Silica 60-120 mesh). Mixtures of petroleum ether and ethyl acetate were used to run the column.

Table 19 Purification of the bromonated products of friedelin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	m.p. after crystallization
1	PE (60-80 °C)	1-7	Nil	--
2	PE-EA (9.95:0.05)	8-12	White solid (\approx 0.1 g)	199-200 °C (A)
3	PE-EA (9.9:0.1)	13-17	White solid (\approx 0.5 g)	208-210 °C (B)
4	PE-EA (9.8:0.2)	18-34		--

Further elution with more polar solvent mixture did not afford any solid material

1.1.15.A Characterization of compound **A**

Fractions 8-12 of same R_f values were combined and recrystallized after evaporation of the solvent from chloroform-methanol. It showed melting point 199-200 °C. In its IR spectrum it gave peaks at 1715, 1448, 1391, 916 and 787 cm^{-1} . In its mass spectrum it showed molecular ion peak at m/z 584. In the ^1H NMR spectrum it showed a triplet at δ_{H} 4.01 (t, 1H, $J_{1\alpha\text{H}, 2\alpha\text{H}} = 2.7$ Hz), 3.13 (t, 1H, $J_{1\beta\text{H}, 2\alpha\text{H}} = 6.6$ Hz). Eight methyl groups each appeared as a sharp singlet at δ_{H} 0.72 (s, 3H, -CH₃), 0.76(s, 3H, -CH₃), 0.86(s, 3H, -CH₃), 0.88(s, 3H, -CH₃), 0.92(s, 3H, -CH₃), 1.00(s, 3H, -CH₃), 1.05(s, 3H, -CH₃), 1.18(s, 3H, -CH₃) satisfactory to that reported for friedelan skeleton. A multiplet centered at δ_{H} 1.97 was due to the presence of C-4 α hydrogen atom in the ring A of the pentacyclic friedelan

triterpenoid skeleton. From these data compound A was assigned as 2,2-dibromofriedelin.

1.1.15.B Characterization of compound B

The isolated white powdered compound, the major one, showed a melting point of 208-210 °C (the reported melting point of 2 α -bromofriedelin⁸⁷ is 210 °C. ¹H NMR (CDCl₃, 300 MHz) δ 0.72 (s, 3H, -CH₃), 0.76(s, 3H, -CH₃), 0.86(s, 3H, -CH₃), 0.88(s, 3H, -CH₃), 0.92(s, 3H, -CH₃), 1.00(s, 3H, -CH₃), 1.05(s, 3H, -CH₃), 1.18(s, 3H, -CH₃), 3.13 (dd, 2H, J = 6.6 and 13.2 Hz, 2H-C-1), 4.40 (m, 1H, 1H-C-2). All other peaks are in good agreement with that reported for friedelin. ¹³C NMR (CDCl₃, 75 MHz) δ 213.1 (C-3), 7.2 (C-23), 14.6 (C-24), 18.5 (C-25), 15.7 (C-26), 18.7 (C-27), 32.1 (C-28), 31.8 (C-29), 32.8 (C-30), 53.0 (C-2). Elemental analysis: %C 71.07 (calcd. 71.26), %H 9.65 (calcd. 9.65) and %Br15.38 (calcd. 15.81). from these data the structure of compound B was assigned as 2 α -bromofriedelin.

1.1.16 Synthesis of the benzopyrazine derivative of friedelin

In the experimental procedure, *o*-phenylenediamine (1 mmol) and 2 α -bromofriedelin (1 mmol) in 1:1 molar ratios was taken in a 50 ml round bottom flask. To this water (3 ml) and 10 mg (0.03 mol %) sodium dodecylsulfate (SDS) was admixed. The reaction mixture was then allowed to stir with magnetic spinning bar at room temperature. After the completion of the reaction (checked by tlc), the residue was filtered, washed with water, dried and finally recrystallized from chloroform-methanol.

1.1.16.A Characterization of the benzopyrazine derivative of friedelin

After crystallization it showed mp 248-250 °C (248-151 °C as reported.⁸⁸ ¹H NMR (CDCl₃, 300 MHz) δ 7.43 (t, 1H, J = 7.8 Hz); 7.58 (dq, 1H, J = 2.4 Hz and 8.1 Hz); 8.00 (dt, 1H, J = 2.7 Hz and 7.8 Hz); 8.09 (m, 1H). 0.72 (s, 3H, -CH₃), 0.76(s, 3H, -CH₃), 0.86(s, 3H, -CH₃), 0.88(s, 3H, -CH₃), 0.92(s, 3H, -CH₃), 1.00(s, 3H, -CH₃), 1.05(s, 3H, -CH₃), 1.15(s, 3H, -CH₃). Elemental analysis: %C 83.92 (calcd. 84.32), %H 10.27 (calcd. 10.22).

2. Antifungal activity of the synthesized pyrazine derivatives

DMSO (dimethyl sulfoxide) was used as solvent to prepare different concentrations of the triterpenoids. Solvent control (DMSO) was also maintained throughout the.

experiment. All experiments were performed in petridishes and were incubated at 37 °C for 48 h.

For studying the inhibitory effect of the two triterpenoids against test fungal pathogens following slide germination method, the spores of the pathogens were allowed to germinate in presence of the prepared and the 50% ethanol extracts. Compound solution was placed on the centre of the grease free microscope slide. In control the corresponding solvent, either sterile distilled water or 50% ethanol was placed. Thirty micro litre spore suspension prepared from ten days culture of the fungal pathogens were added to the spots in both experimental and control slides. In case of 50% ethanol extract, spore suspension was added after ethanol was evaporated. Three experimental slides were taken for each compound. The slides were then incubated at 28 °C in a humid chamber. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and a slide was placed on the rods in a uniformly balanced position. Then the petridish was filled with sterile distilled water so that the bottom of the slide remained just above the water surface. The petridish was then covered and incubated at 28 °C. Following 48 h of incubation, the slides were stained with lacto phenol-cotton blue mixture and observed in each slide for germination. Numbers of aspersoria formed were also observed and lengths of 50 germ tubes were measured. The entire experiment was repeated thrice.

3. Molecular Docking

Three dimensional molecular docking studies were carried out with AutoDock 4. Molecular drawing and 3D structure optimizations were carried out by Chem Sketch 12.0 software package. Initially we selected the ligand and by neighbour selection through Arguslab we located amino acid residues within 3.5Å surrounding the ligand. Molecular viewing was performed by Molegro molecular viewer as well as in AutoDock 4.

4. Topoisomerase II assay

Human TOPO-II α activity was measured by relaxation of supercoiled pBR322 plasmid DNA. Reaction mixture contained 10 mM Tris (pH-6.9), 50 mM KCl, 50 mM NaCl, 5mM MgCl₂, 1mM ATP, pBR 322 plasmid DNA (100 ng) and 2 Units of Topo II α , in a final volume of 20 μ l. For inhibition studies, the compounds were preincubated with human TOPO-II α and DNA for 15 min. Compounds were used at the appropriate concentrations by dissolving in 2% (v/v) DMSO. DMSO didn't show detrimental effect

on the enzyme activity at concentration up to 2 % v/v. Reaction mixture was incubated at 37⁰C for 30 min and stopped by addition of 2ml of 7mM EDTA. Reaction product was mixed with DNA loading dye and electrophoresed on 1% TAE-Agarose. The gel was stained with ethidium bromide (0.5 µg/ml) for 20 min, destained twice in TAE buffer and then visualized using a Gel Doc-Imaging system (Spectronics, USA).

5. Notes and References

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Part III

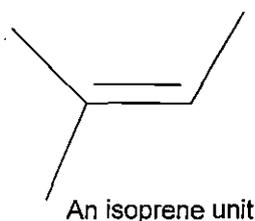
Partial Synthesis of 28-hydroxy-3-oxo-lup-20(29)-en-30-al with Antileukemic Activity

Chapter I

1. A short review about triterpenoids of lupane skeleton

Natural products (NPs) are an incredibly diverse group of small (usually molecular weight less than 1500 Da) organic compounds isolated from a variety of natural sources, principally from plants. The reason that NPs capture the imagination of organic chemists and pharmaceutical scientists is because of their well documented and wide ranging biological activities and their skeletal diversity and intriguing functional group characteristics, which render them as indispensable leads for probing biological systems status and for drug discovery with new bioassay systems.

Terpenes are a class of NPs possessing a carbon frame work comprising of units of five carbon arrangements. The monomeric unit is called isoprene unit indicated by the symbol C_5 .



Most terpenes possess a carbon in multiples of C_5 arrangement and the isoprene unit is considered as one of the nature's favorite building block of terpene biosynthesis. Terpenes are classified in the following groups-

- | | | |
|-------|-----------------------------|--------------------|
| i. | Hemiterpenes (C_5) | One C_5 unit |
| ii. | Monoterpenes (C_{10}) | Two C_5 unit |
| iii. | Sesquiterpenes (C_{15}) | Three C_5 unit |
| iv. | Diterpenes (C_{20}) | Four C_5 unit |
| v. | Sesterpene (C_{25}) | Five C_5 unit |
| vi. | Triterpene (C_{30}) | Six C_5 unit |
| vii. | Tetraterpenes (C_{40}) | Eight C_5 unit |
| viii. | Polyterpenes ($C_{>40}$) | > Eight C_5 unit |

These units of five carbons are easily recognized in terpenes. These units generally consist of head to tail bonded isoprenes along with supplementary bonds. These structural features are a consequence of the common biosynthetic origin of terpenes.¹ Terpene skeletons are produced from acyclic substrates by carbocation chemistry through successive electrophilic additions followed by rearrangements.

Triterpenoids are a large and structurally diverse group of natural secondary metabolites¹ that display nearly 200 distinct skeletons. Most triterpenoids are 6-6-6-5 tetracycles, 6-6-6-6-5 pentacycles, or 6-6-6-6-6 pentacycles, but acyclic, monocyclic, bicyclic, tricyclic, and hexacyclic triterpenoids have also been isolated from natural sources. Compounds combining availability with valuable biological activity are frequent in the class of triterpenoids. Several reviews have been published to document the occurrence and variable biological activities of these classes of terpenoids.² Of all the groups' pentacyclic triterpenoids form a distinct class that is now getting too much attention by the synthetic chemists as well as medicinal chemists in the field of lead research because of the fascinating biological activities associated with them. Pentacyclic triterpenoids have several skeletal arrangements. Most important arrangements are –

- ❖ Olean skeleton
- ❖ Ursane skeleton
- ❖ Lupane skeleton and
- ❖ Ceanothan skeleton

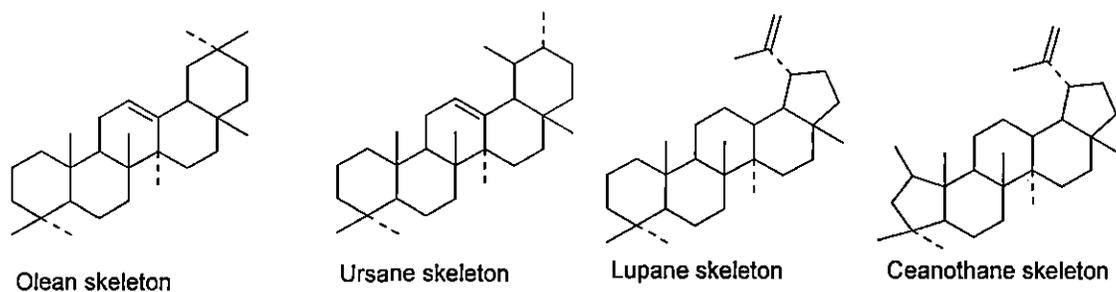
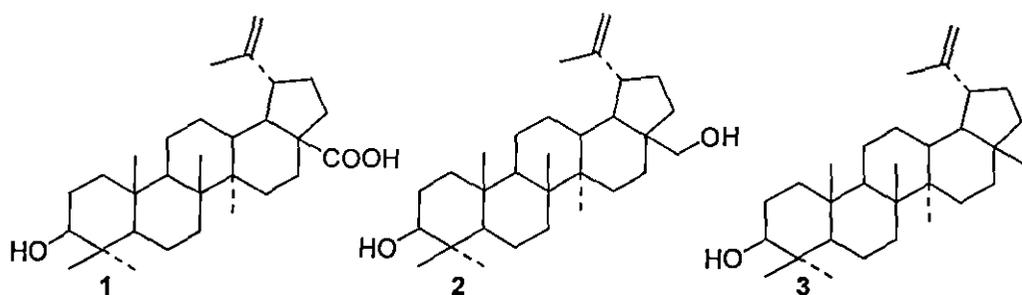


Figure 1 Important triterpene skeleton

As triterpene is wide in nature and it is not easy to discuss in detail about all the skeletons of this class of natural product, only the triterpenoids of lupane skeleton is described here.

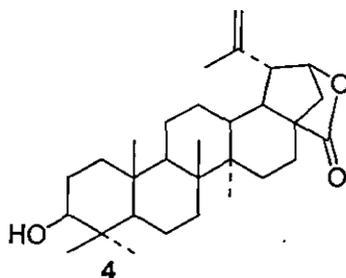
Lupane-type triterpenes, are widely distributed in the plant kingdom; the birch tree being one of the most widely reported sources. Betulinic acid (1), betulin (2) and lupeol (3) are the most important lupane triterpenoid that has been reported to have anti-melanoma,³ anti-neuroblastoma,⁴ anti-leukemia,⁵ anti-HIV,⁶ and anti-malarial properties.⁷ To date the literature is increasing by documenting the isolation of various lupane triterpenoids from diverse natural sources mainly from plants.



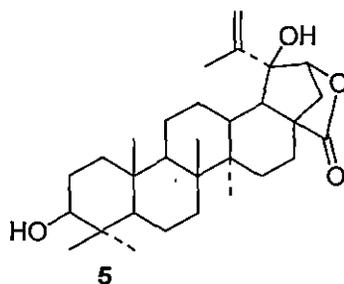
The main source of betulin is birch bark. When the bark is heated, sublimation occurs. Lovits was the first to observe this in 1788. The name betulin was given to this compound by Mason in 1831. Later betulin was obtained by extraction. The birch bark has two clearly distinguished parts: external and internal. The external part of birch bark is especially rich in the extractives: their content reaches 40 %. The main component of almost all the extracts is betulin which determines the white colour of bark.² The betulin content of the external part of bark varies within 10-35 % depending on birch species, site of ground and conditions, the age of a tree, and other factors. Betulin was detected in pendent birch (*Betula verrucosa* Ehrh. = *Betula pendula* Roth.) and in downy birch (*B. pubescens* Ehrh.), the species widespread in Russia.⁸ Betulin is also present in the bark of white birch (*Betula alba* L.) growing in Europe.⁹ Along with betulin, extracts of the bark of these species contain its oxidized derivatives: betulic acid, betulic aldehyde, methyl ester of betulic acid, betulonic aldehyde, betulonic acid.¹⁰ A permanent satellite of betulin in birch bark is lupeol (~10 % of betulin). Anomalously high content of lupeol close to the amount of betulin was described by Tolstikov *et al.*¹¹ Lupeol was isolated for the first time from sprouts of lupine (*Lupinus albus*), a fodder plant; later it was detected in other sources.¹¹ Along with lupane derivatives, birch bark contains triterpenes of oleanane and ursane series. In some birch species, for example in *B. dahurica* Pall., prevailing

compounds are oleananic acid and its derivatives.¹² Among other oleanane derivatives detected in the extracts of birch bark, noteworthy is a product of rearrangement of betulin, allobetulin which is easily obtained under the action of acidic reagents.¹³ The qualitative composition of triterpenoids of the bark of black birch species *B. pubescens* and *B. pendula* is the same as that of white-bark birch, but their amount is 2.3 times smaller. The content of betulin in white-bark birch and in black bark *B. pubescens* is substantially lower than that in the bark of *B. pendula*.¹⁴ Extracts of the internal part of bark contain small amounts of betulin; however, they can be used as sources of phenolic compounds.¹⁵ Betulin, lupeol and the products of their metabolic hydroxylation were detected in at least twenty different plant species belonging to different genera and families. For example, betulin, lupeol and betulic acid were extracted from the bark of alder tree (*Aldus subcordata* L.),¹⁶ zizyphus (*Ziziphus jujuba* M.)^{17,18} and from the top parts of thistle (*Atractylis carduus* L.).¹⁹ Betulic acid was detected in the leaves of plumeria (*Plumeria obtusa*),²⁰ triadenum (*Triadenum japonicum*),²¹ in orchids (Orchid *Lusia indivisa*)²² and other plants (*Dillenia papuana*, *Tryphyllum peltatum*, *Ancistrocladus heyneaus*, *Diospyros leucomelas*, *Tetracera boliviana*, *Sizyphus joazeiro*, *Syzigium claviflorum*, *Aerva javanica*) widespread in the tropics and of course from *Biscofia javanica*.

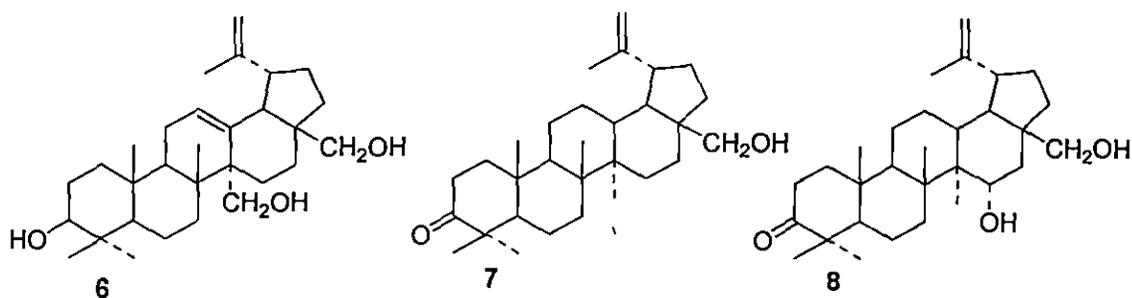
In an early attempt Carl Djerassi isolated²³ and characterized thurberogenin, a new triterpene from the cactus *Lemaireocereus thurberi*. Later on he revised the structure of thurberogenin (4) and gave its modified structure as a lupane triterpenoid.²⁴



In that same communication²³ the same authors also reported the isolation of stellatogenin (5) from *Lemaireocereus stellatus*, a new lupane type triterpenoid.

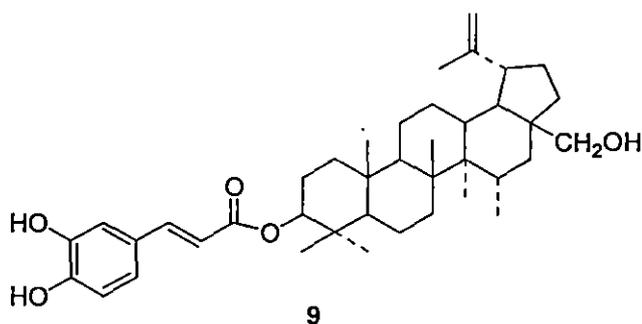


Siddiqui *et al.*²⁵ isolated a new lupane type triterpenoid oleanderol (6) from the leaves of *Nerium oleander* L. *Nerium oleander* L. (syn. *Nerium odorum*, Apocynaceae), distributed in the Mediterranean region and subtropical Asia, is indigenous to the Indo-Pakistan subcontinent. The plant is commonly known as “kaner”, and its various parts are reported as therapeutic agents in the treatment of swellings, leprosy, eye and skin diseases. The leaves possess cardiotoxic and antibacterial properties.²⁵

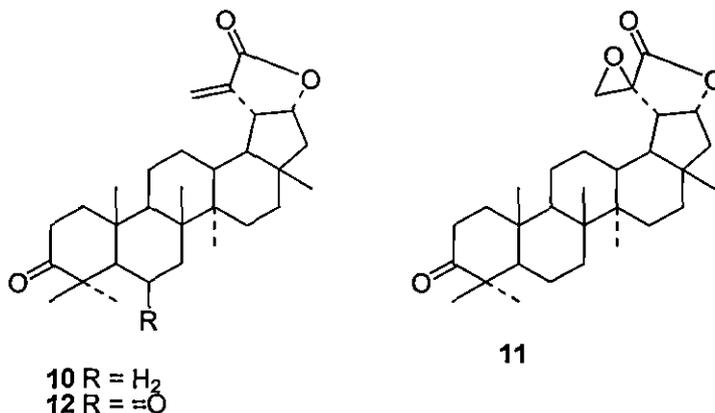


The family Celastraceae is well rich in triterpenoid as evident by the enormous number of publications in current literature. Tinto and his co-workers²⁶ in 1992 reported the isolation of some lupane type triterpenoids from the methanolic extract of the bark of *Salacia cordata*. The most abundant triterpenoid isolated were identified as 28-hydroxylup-20(29)-en-3-one or commonly betulone which has been reported previously as an oxidation product of betulin (2), and 30-hydroxylup-20(29)-en-3-one. The third compound, 15,28-dihydroxylup-20(29)-en-3-one (8), has not been described previously. Its molecular formula, $C_{30}H_{48}O_3$, was determined (HREIMS), and structural and functional features were recognized from IR and NMR spectra.

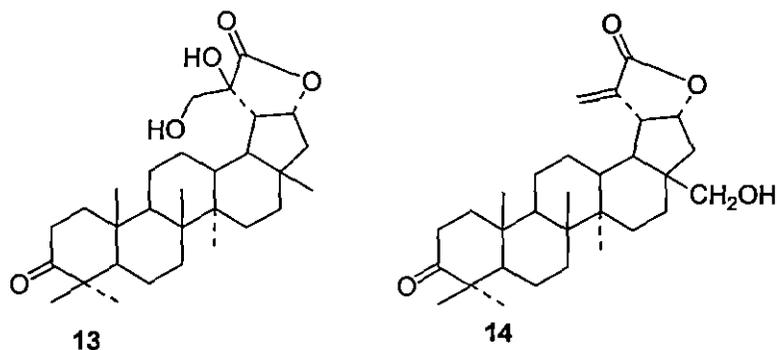
Patra *et al.*²⁷ for the first time ever isolated and reported the existence of new somewhat air sensitive α -lupene ester, lup-20(29)-en-28-ol-3P-yl caffeate (9).



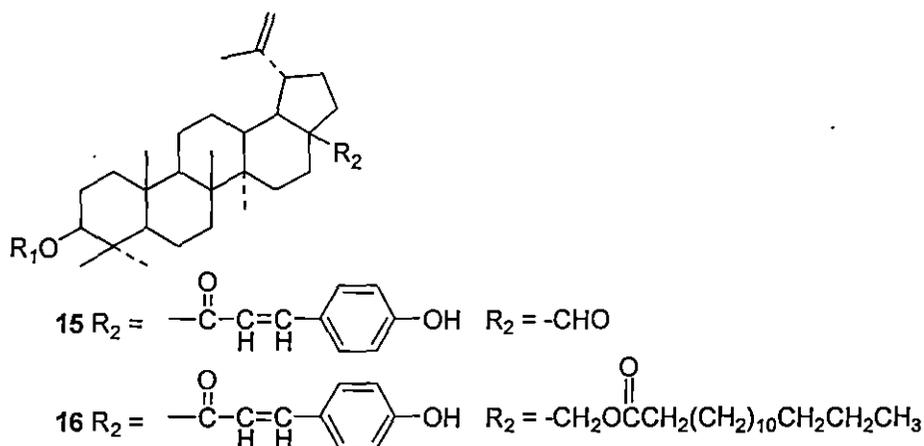
Ngassapa *et al.*²⁸ for the first time reported the isolation of three new lupane lactones from the nonpolar fraction of the stem bark of *Kokoona ochracea*. From extensive 1D and 2D NMR studies they established the structures as 3-oxolup-20(29)en-30,21 α -olide (10), 20,29-epoxy-3-oxolupan-30,21 α -olide (11) and 3,6-dioxolup-20(29)en-30, 21 α -olide (12).



Later on in another communication Ngassapa *et al.*²⁹ isolated another two novel cytotoxic lupane lactones Ochraceolide D and E from the same plant, *Kokoona ochracea* of family Celastraceae. The structure of compound D and E were established as 20,29-dihydroxy-3-oxolupan-30,21 α -olide (13) and 28-hydroxyl-3-oxolup-20(29)-en-30,21 α -olide (14) respectively. Both the compounds showed promising anticancer activity against a series of cancer cell lines *in vitro*. All the structures of the isolated compounds were elucidated by a detail study of the spectroscopic data as well as chemical analysis. In this case both the lactones were very much stable at room temperature.



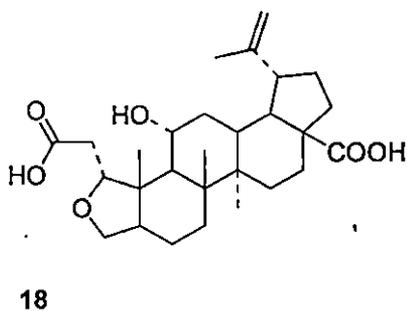
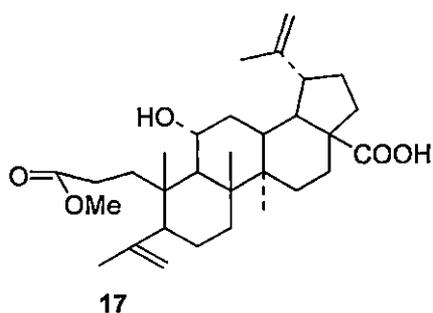
Of 13 species of *Diospyros* (Ebenacea) growing in Taiwan, several have been studied for their chemical constituents, resulting in the isolation and structure elucidation of various triterpenes, lignans, steroids, benzoquinones, and naphthoquinone. Chi-I Chang and Yueh-Hsiung Kuo³⁰ had isolated and characterized two new lupane derivatives, 3-(*E*)-coumaroylbetulinaldehyde (**15**) and 3-(*E*)-coumaroyl-28-palmitoylbetulin(**16**), have been isolated from the stems of *Diospyros maritima*. Their structures were determined by using spectral and chemical methods.



Sonja Sturm and coworkers³¹ had isolated compounds **15** and **16** from stem bark of *Lophopetalum wallichii* along with some others known ones all having lupane skeleton. They also examined the Farnesyl Protein Transferase Inhibitory Activity of all the isolates.

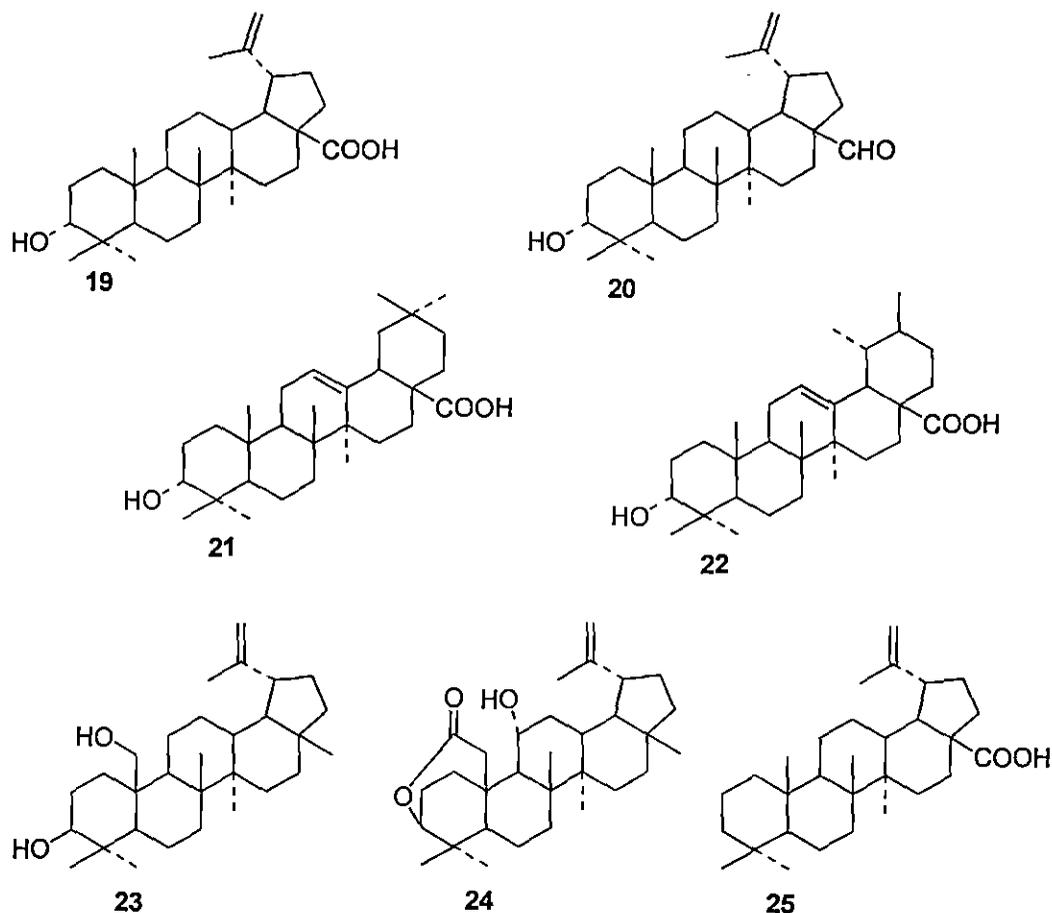
The root and stem barks of *Acanthopanax* species have been used as a tonic and prophylactic in oriental herbal medication from olden times. The leaves and roots of this species have been also taken as a health drink and drug in Korea. These observation

attracted Park³² and his coworkers to search the phytochemicals and they reported the isolation of some 3,4-*seco*-lupane triterpenes from this plant. The names of the compounds are inermoside, **17** and 11-deoxyisochiisnoside, **18**.

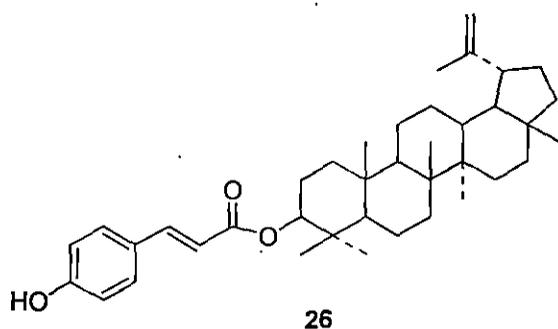


Takeoka *et al.*³³ in an interesting investigation had reported the isolation of betulinic acid (**1**) and its some derivatives from Almonds [*Prunus dulcis* (Mill.) D.A. Webb].

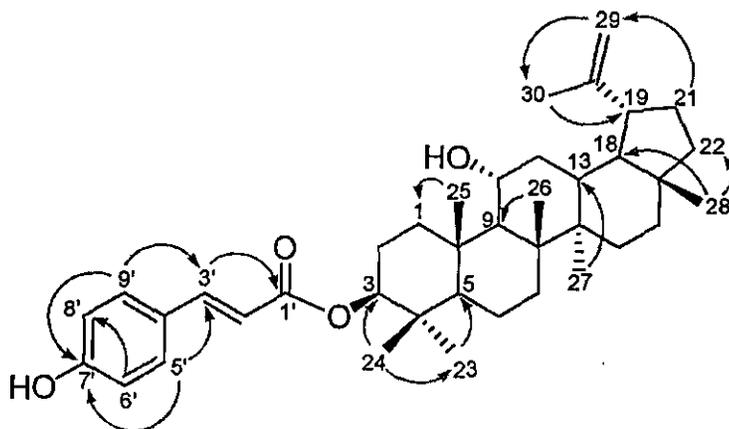
Chaturvedula *et al.*³⁴ in their ongoing study on natural products reported the isolation of three novel lupane triterpenoids from the twigs of *Coussarea paniculata* using a yeast-based assay for potential DNA-damaging agents in addition to eight known triterpenoids, lupeol (**3**), lupenyl acetate, betulin (**2**), betulinic acid (**1**), 3-*epi*-betulinic acid (**19**), 3-*epi*-betulinaldehyde (**20**), oleanolic acid (**21**), and ursolic acid (**22**). The structures of the new compounds were established as lup-20(29)-en-3 β ,25-diol (**23**), lup-20(29)-en-11 α -ol-25,3 β -lactone (**24**), and 3-deoxybetulonic acid (**25**), on the basis of extensive 1D and 2D NMR spectroscopic data interpretation and chemical conversions.



Oramas-Royo and his coworkers had isolated seven new triterpenoids and 36 known compounds from the root bark of *Maytenus retusa*. Their structures were determined by 1D and 2D spectroscopic studies. Several compounds were evaluated for their cytotoxicity against the human tumor cell lines HL-60 and MCF-7. Some of them were cytotoxic, with IC₅₀ values ranging between 0.2 and 4.7 μ M. Out of all the seven new compounds one compound had the lupane skeleton and the structure of the compound was elucidated as 3 β -(*E*)-*p*-coumaroylnepeticin (26).

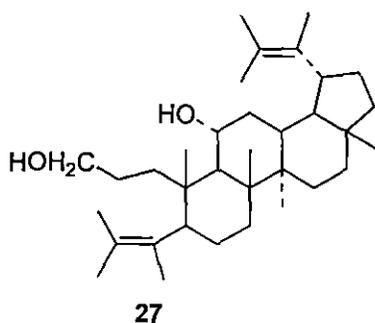


The structure of the compound was elucidated by extensive interpretation of the 1D and 2D NMR spectra of the compound. The HMBC correlation was so much indicative about the proposed structure of the compound (26).



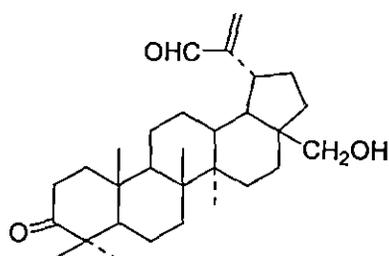
Selected HMBC correlation of compound (26)

Dallavalle *et al.*³⁵ isolated a new *seco*-ring A lupane triterpene derivative (27), along with lupenone, lupeol, α -sitosterol, ursolic acid, and stigmasterol 3-*O*- β -D-glucoside, were isolated from a methanol extract of mature stems of *Lasianthus gardneri*, a shrub from the family Rubiaceae growing in Sri Lanka. The structure and stereochemistry of the new compound were determined using a combination of ^{13}C and ^1H homo- and heteronuclear 2D NMR experiments and from mass spectral data. The structure of the compound was further confirmed by partial synthesis from lupeol.



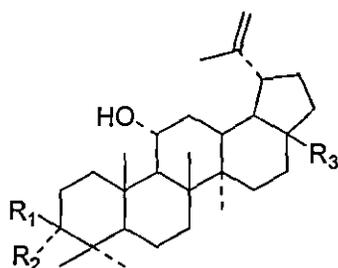
The stem bark of *Acacia mellifera* (Leguminosae) is used in the traditional African ethnomedicine for the treatment of pneumonia, malaria, primary infection of syphilis, sterility and stomach-ache.³⁶ These uses attracted Charles Mutai and his coworkers for a thorough photochemical study of the bark of *Acacia mellifera*. In that study they reported

the isolation of the new lupane triterpene 28-hydroxy-3-oxo-lup-20-(29)-en-30-al (**28**) along with eight known triterpenoids from the DCM extract of the outer bark.

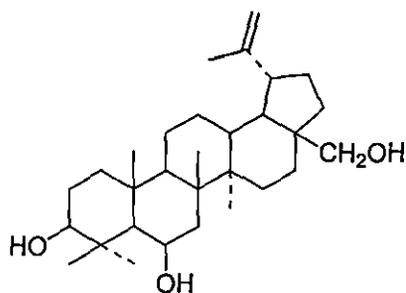


28

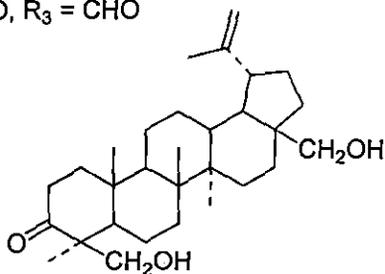
Five new lupane triterpenes, in addition to 24 known ones, were isolated from *Maytenus cuzcoina* and *M. chiapensis* by Nunez *et al.*³⁷ Their structures were elucidated on the basis of spectroscopic analysis, including homonuclear and heteronuclear correlation NMR (COSY, ROESY, HSQC, and HMBC) experiments. New triterpenoids were named as 11 α -hydroxy-*epi*-betulin (**29**), 6 β -hydroxybetulin (**30**), 24-hydroxybetulone (**31**), rigidinol-28-aldehyde (**32**) and 28-hydroxyglochidone (**33**).



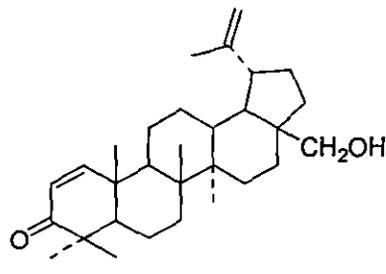
29 R₁ = H, R₂ = OH, R₃ = CH₂OH
32 R₁ = R₂ = O, R₃ = CHO



30

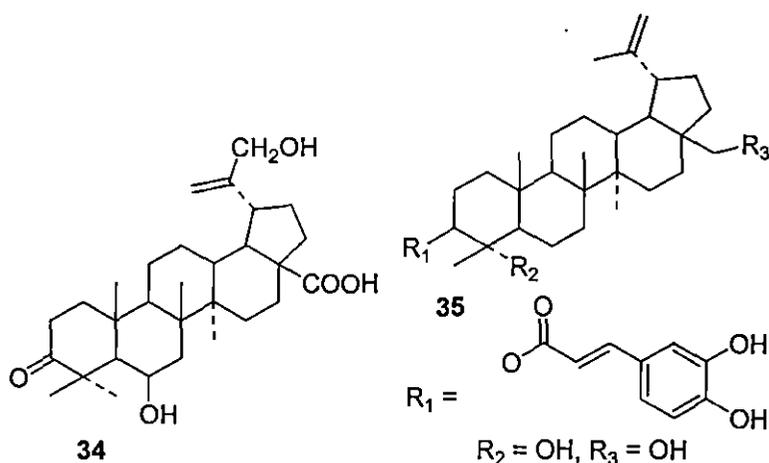


31



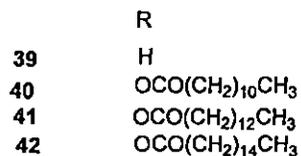
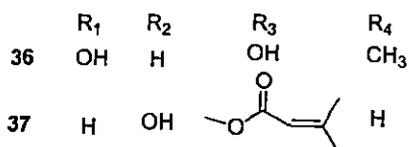
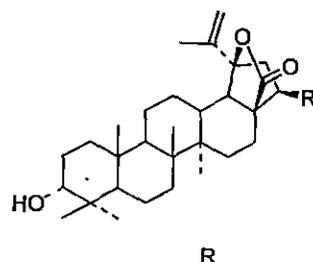
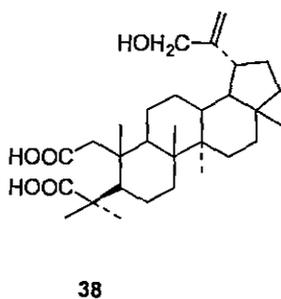
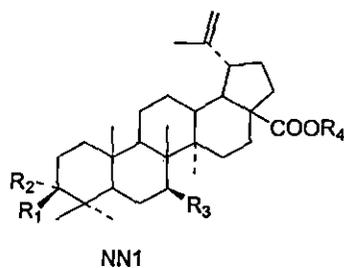
33

Fukuda *et al.*³⁸ isolated 6 β -hydroxy-3-oxolup-20(29)-en-30-ol-28-oic acid (**34**) and **35** from the cones of *Liquidamber styraciflua* L. (Hamamelidaceae) together with another novel triterpenoid having olean skeleton and six already known triterpenes. They also showed the anticancer potential of all the isolates against some cancer cell lines.



The family Celastraceae has almost 100 genera and about 1300 species³⁹ that are widespread in the tropical and subtropical regions of North Africa, South America, and East Asia.⁴⁰

Chen *et al.*⁴¹ in their on going study about the phytochemical investigations of natural products of plant origin had isolated seven new lupane triterpenoids from bioactive methanol extracts of *Microtropis fokienensis* (**36-38**) and *Perrottetia arisanensis* (**39-42**), along with 18 known compounds including compound **28**. The structures of the new compounds were elucidated on the basis of spectroscopic data analysis. All triterpenoids were evaluated for their in vitro cytotoxicity toward seven human cancer cell lines. Compound **28** (28-hydroxy-3-oxo-lup-20(29)-en-30-al) was among the most cytotoxic substances obtained and was found to induce apoptosis of human leukemia HL60 cells and mediate cleavage of PARP and upregulation of Bax proteins.



In 2010 Guerrero-Analco reported⁴² the bioassay guided isolation of 23,28-dihydroxylupan-20(29)-ene-3 β -caffeate from the crude extract (80% EtOH in H_2O) of *Sorbus decora* C.K. Schneid. (Rosaceae), commonly known as “Showy mountain ash”, is a widely distributed tree of the boreal forest of North America.

Oyo-Ita *et al.*⁴³ in a dramatic work isolated and established the presence of higher triterpenoid methyl ether and acetates in the sediments of the cross-river system, southwest Nigeria. They proved the presence of lupane derived compounds.

Chapter II

Section A

Partial synthesis of 28-hydroxy-3-oxolup-20(29)-en-30-al from Betulinic acid

1. Introduction

Triterpenes represent a varied and important class of natural compounds. Among these, pentacyclic lupane-type triterpenes are one of the most significant subclass which has been shown to possess several medicinal properties.^{44,45} The antitumor properties of plant extracts comprising lupane-derived triterpenoids have been demonstrated over the past 25 years for their cytostatic activity on various *in vitro* and *in vivo* cancer model systems.⁴⁶ Betulinic acid, one of the lupane-derived triterpenoid, exerts a selective anti-tumor activity on cultured human melanoma,⁴⁶ neuroblastoma,^{47,48} malignant brain tumor,⁴⁹ and leukemia cells.^{3,50} Other pharmacological activities of lupane type triterpenoids include anti-inflammatory activity,⁵¹ anti carcinogenic activity,⁴⁶ photosynthetic inhibitors,⁵² anti HIV,⁶ anti diabetic⁵³ *etc.* Therefore, it is important to supply those triterpenoids with novel structures in sufficient amounts for further biological testing. And, in this regard, synthesis rather than isolation from natural sources is more efficient and also economical. Medicinal plants and their phytoconstituents have always been a better choice for leukemia and nutraceuticals have been proven to have antileukemic activity in experimental studies.⁵⁴ Derivatives of betulin, basically triterpenoids of lupane skeleton, are reported to possess significant cytotoxicity against wide variety of cancer cell lines.⁵⁵ In a recent publication, one such naturally occurring compound, 28-hydroxy-3-oxolup-20(29)-en-30-al (**28**), which has reported³⁶ for the first time from the bark of *Acacia mellifera*, showed significant cytotoxicity against NSCLC-N6 cell line. Subsequently Chen *et al.* reported⁴¹ the presence of **28** in the methanol extract of *Microtropis fokienensis*, reported⁴¹ to induce apoptosis of human leukemia HL60 cells and mediate-cleavage of PARP and up regulation of Bax proteins. Compound **28** (28-hydroxy-3-oxolup-20(29)-en-30-al) was among the most cytotoxic substances⁵⁶ obtained among all the isolated compounds. They also investigated the potential effects of **28** on growth inhibition of HL60 cells.⁴¹ According to their results⁴¹ this compound induced apoptosis

in a dose-dependent manner. The molecular mechanism of compound **28** towards cancer cells is still a subject of continuous investigation and a specific target(s) has yet to be identified. Therefore, suitable derivatives of lupane may be considered as a group of compounds having promising bioactivity that can be used for further chemical as well as biological research. Henceforth, the present demands of this type of rare (scarce) naturally occurring triterpenoids are enormous in the contemporary medicinal research. Thus, it was felt necessary to supply this novel triterpenoid, **28** in sufficient amounts for further biological testing. And, in this regard, synthesis rather than isolation from natural sources is more efficient and economical.

2. Present investigation

In view of the above and in continuation of our studies towards the chemical transformations of pentacyclic triterpenoids⁵⁷ the present author is reporting herein a multistep protocol for the synthesis of 28-hydroxy-3-oxolup-20(29)-en-30-al (**28**) from betulinic acid (**1**) (Figure 2). *In vitro*, anti cancer activities of all the compounds having C-30 -CHO group is also tested against different cell lines. Derivatization of C-30 methyl group was accomplished in good yield by SeO₂ oxidation in refluxing aqueous dioxan. This is the first report of the partial synthesis of compound **28** from **1** and its potent anticancer activity against human K562 leukemia, murine WEHI3 leukemia and murine MEL erythroid progenitor cell lines.

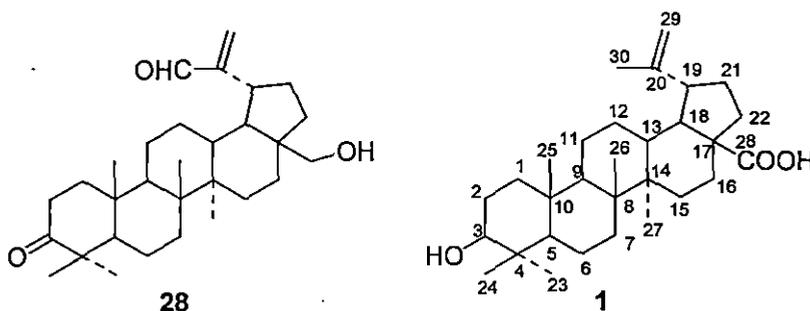


Figure 2 Structures of 28-hydroxy-3-oxolup-20(29)-en-30-al and betulinic acid

2.1 Results and Discussion

The sequential steps involved in the synthesis of compound **28** are illustrated in scheme 1. Betulinic acid (**1**), isolated from *Bischofia javanica*, was esterified (diazomethane) at C-28 to form 28-carbomethoxy-lup-20(29)-en-3β-ol (**43**) almost quantitatively.

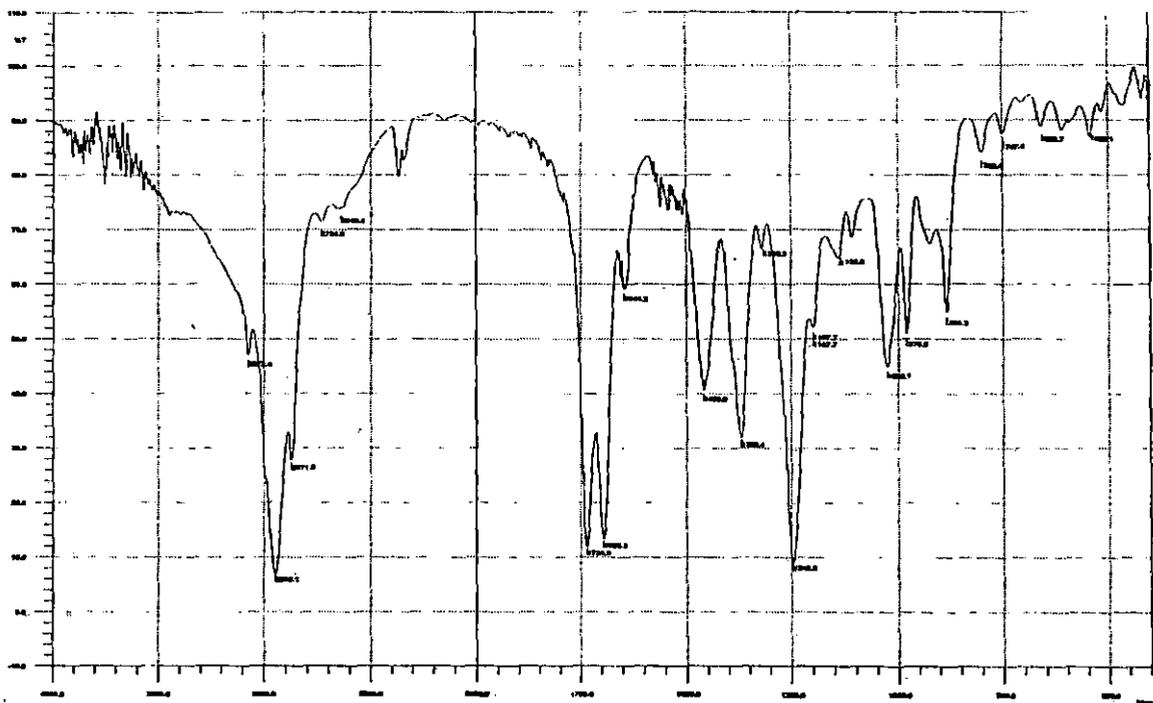


Figure 3 IR spectrum of betulinic acid, 1

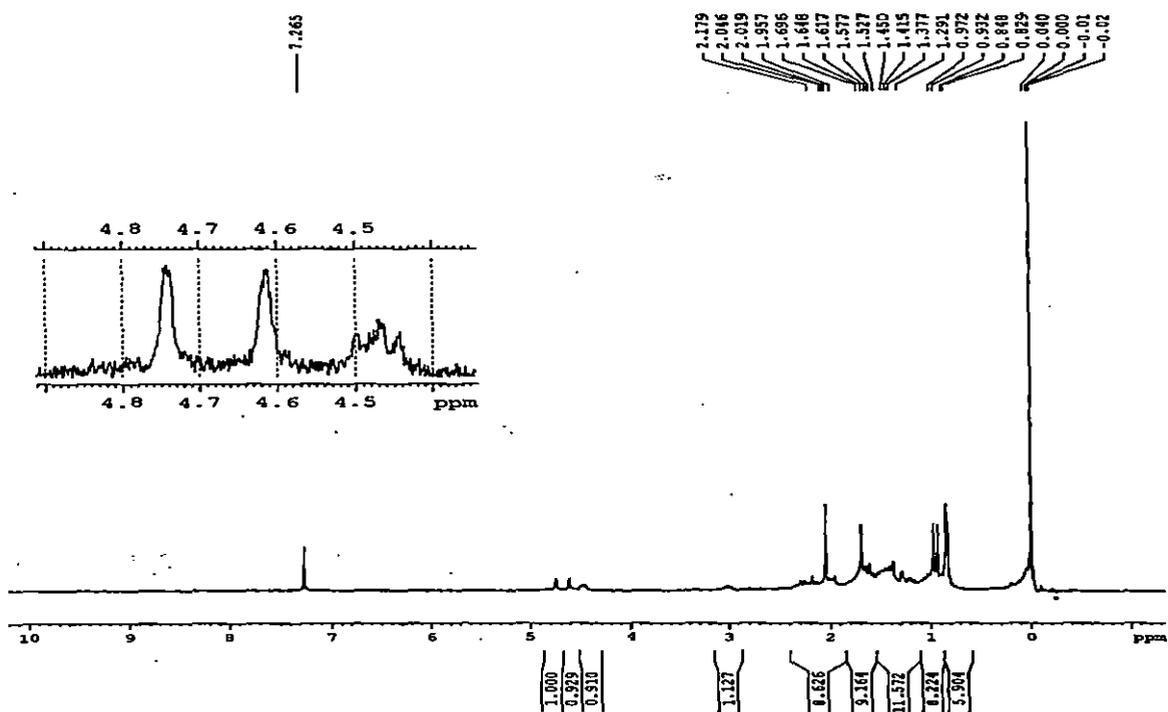


Figure 4 ^1H NMR spectrum of betulinic acid, 1

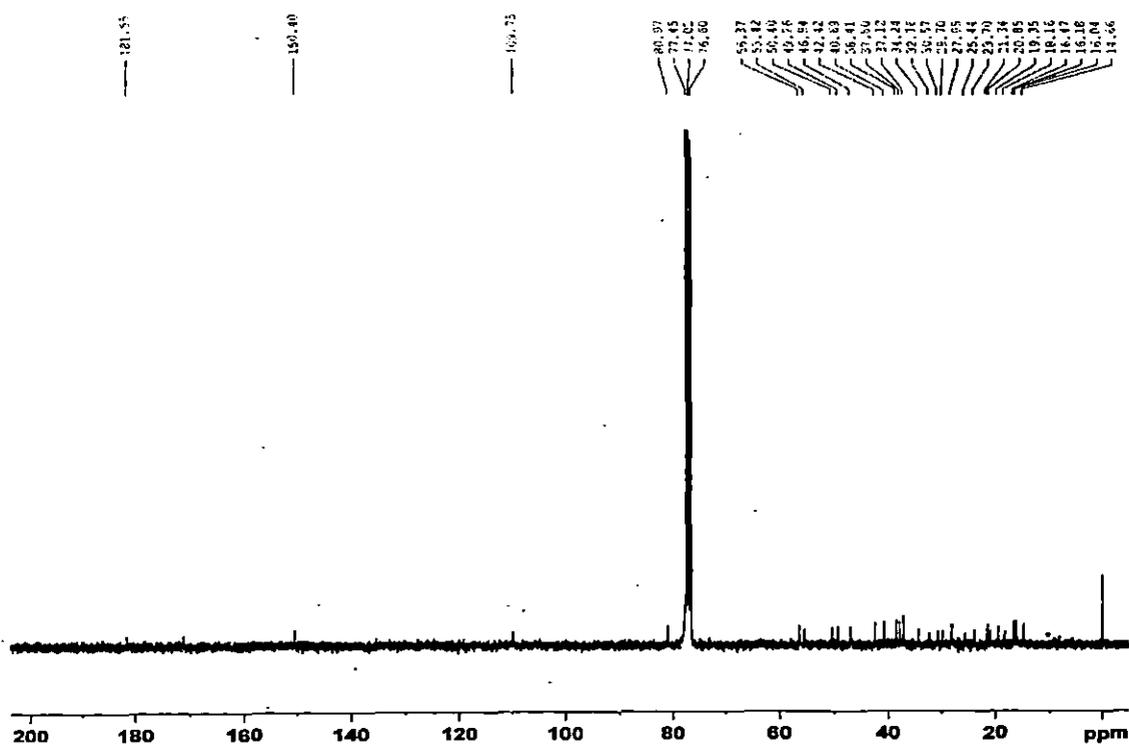


Figure 5 ^{13}C NMR spectrum of betulinic acid, 1

Compound **43** was obtained (2.9g, 98%) as a white solid with mp 222-224°C and $[\alpha]_{\text{D}}^{25} +5.0$ (CHCl_3). The IR spectrum gave peaks at 3537 (-OH), 1725 (-COOMe), 1704, 1641, 1449, 1166, 881 ($=\text{CH}_2$) cm^{-1} . Elemental analysis: Found: % C 78.79, % H 10.52 and calcd. for $\text{C}_{31}\text{H}_{50}\text{O}_3$: % C 79.10, % H 10.71. In the ^1H NMR spectrum it gave six singlets due to six methyl groups at δ_{H} 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.40 (s, 3H) and 1.68 (s, 3H). Peak at δ_{H} 3.18 are due to the proton at C-3 (dd, 1H, $J = 5.1$ and 10.8 Hz, proton at C-3). Ester methyl at C-28 appeared as a sharp singlet at δ_{H} 3.66 (s, 3H, -OMe). Two olefinic protons at C-29 each appeared as singlet at δ_{H} 4.59 (s, 1H, H-C-29) and 4.73 (s, 1H, H-C-29). The compound was also found identical with an authentic sample (co-TLC, mixed mp).

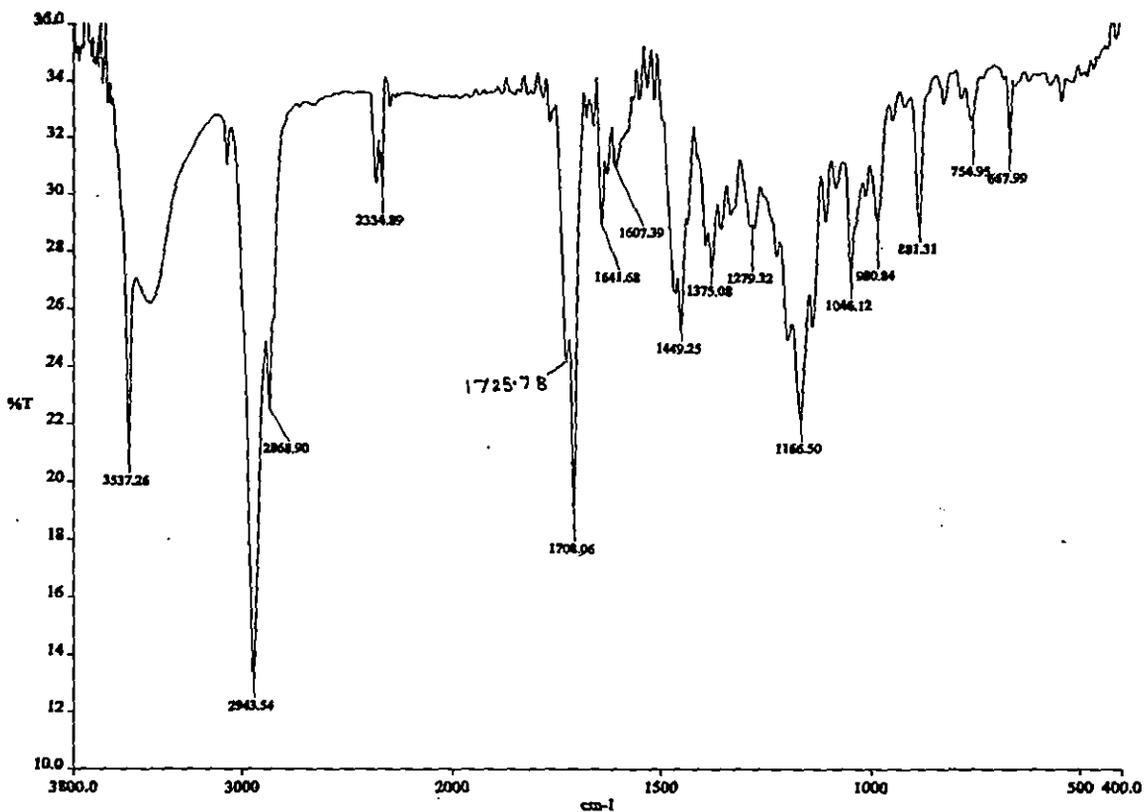


Figure 6 IR spectrum of methylbetulinate 43

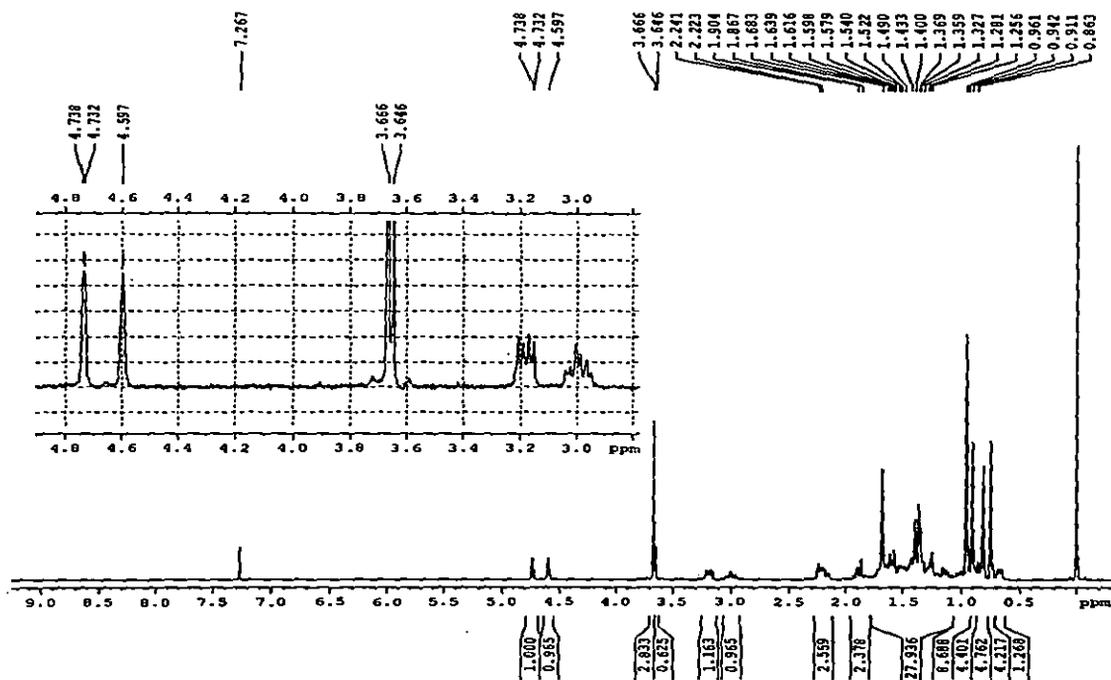


Figure 7 ¹H NMR spectrum of methylbetulinate, 43

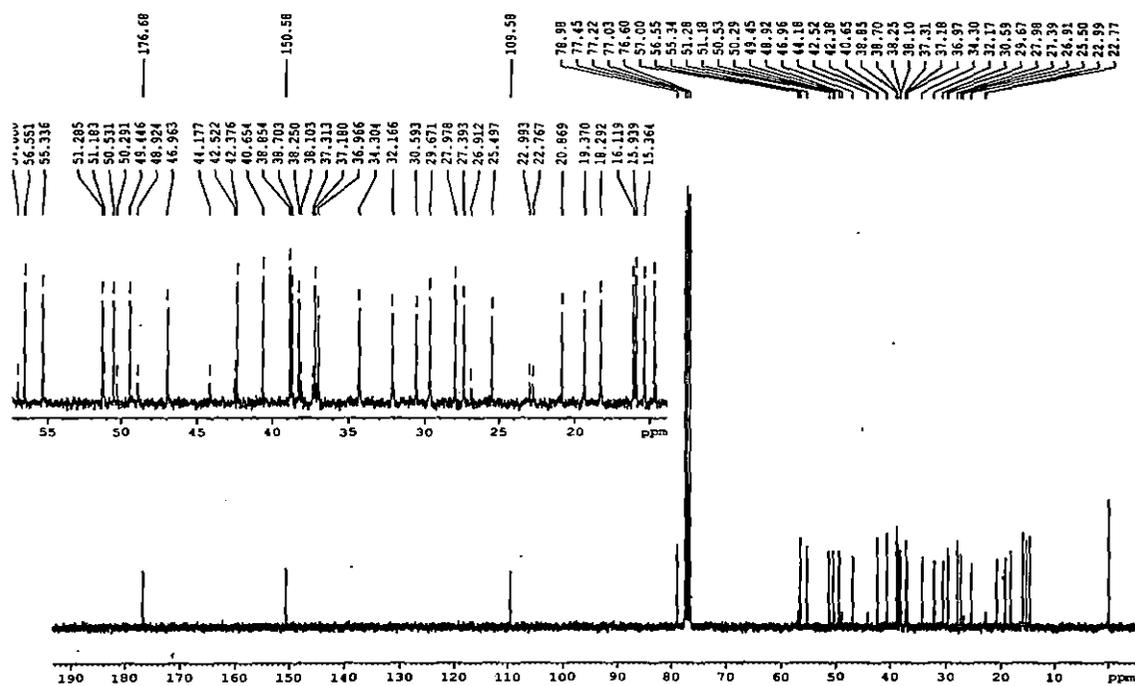


Figure 8 ^{13}C NMR spectrum of methylbetulinate, 43

Lithium aluminium hydride (LAH) reduction of **3** in anhydrous THF gave betulins (**2**) in 72% yield. Allylic oxidation of the C-30 methyl was then carried out with SeO_2 in aqueous dioxan under refluxing condition after protecting C-3 and C-28 hydroxyl groups as acetate of compound **44**. The incorporation of formyl group was assigned by IR and NMR (both ^1H and ^{13}C) spectra of compound **45**. The IR spectrum showed peaks at 1732 ($-\text{OCOCH}_3$), 1691 (conjugated aldehyde), 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 889 ($=\text{CH}_2$) cm^{-1} . The molecular formula of compound **45** was assigned to $\text{C}_{34}\text{H}_{52}\text{O}_5$ (M^+ 540.29, Analytical calculation % C 75.42, % H 9.56). ^1H NMR spectrum of compound **6**, taken in CDCl_3 , gave a singlet at δ_{H} 9.56 with integration of one proton in addition to five methyls at δ_{H} 0.93 (s, 3H), 0.94 (s, 3H), 1.02 (s, 3H), 1.06 (s, 3H), 1.39 (s, 3H) for lupane skeleton. Two olefinic protons appeared at δ_{H} (proton chemical shift position) 6.28 (s, 1H) and at δ_{H} 5.93 (s, 1H) and two geminal hydrogens of C-28 each gave a doublet at δ_{H} 4.24 (d, 1H, $J = 10.6$ Hz) and at δ_{H} 3.68 (d, 1H, $J = 10.6$ Hz). Two acetyl methyls appeared as two sharp singlets at δ_{H} 2.07 (s, 3H) and at δ_{H} 2.03 (s, 3H). In

the proton decoupled ^{13}C NMR spectrum of **6** a singlet at δ_{C} 194.6 (Table 1) clearly indicated the presence of a formyl group at C-30. Careful hydrolysis of **45** gave a yellowish gummy residue after evaporation of the solvent in *vacuo*. Purification of this gummy material over a column of silica gel gave almost quantitatively a powdered white solid of compound **46**, melting point (mp) 274-276 °C. The IR spectrum gave peaks at 3393 (-OH), 1688 (conjugated aldehyde), 1453, 1375 (gem dimethyl), 1029, 942, 890 ($=\text{CH}_2$) cm^{-1} . The molecular formula of compound **46** was assigned to $\text{C}_{30}\text{H}_{48}\text{O}_3$ (M^+ 456.32, Analytical calculation % C 78.66, % H 10.52). Disappearance of ^1H NMR signals at δ_{H} 2.03 (s, 3H) and 2.07 (s, 3H) and the presence of two singlets at δ_{C} 78.9 (for C-3) and 60.2 (for C-28) in the ^{13}C NMR spectrum of **46** clearly indicated the deprotection of both the hydroxyl groups at C-3 and at C-28 during the formation of **46** from the diacetate (**45**).

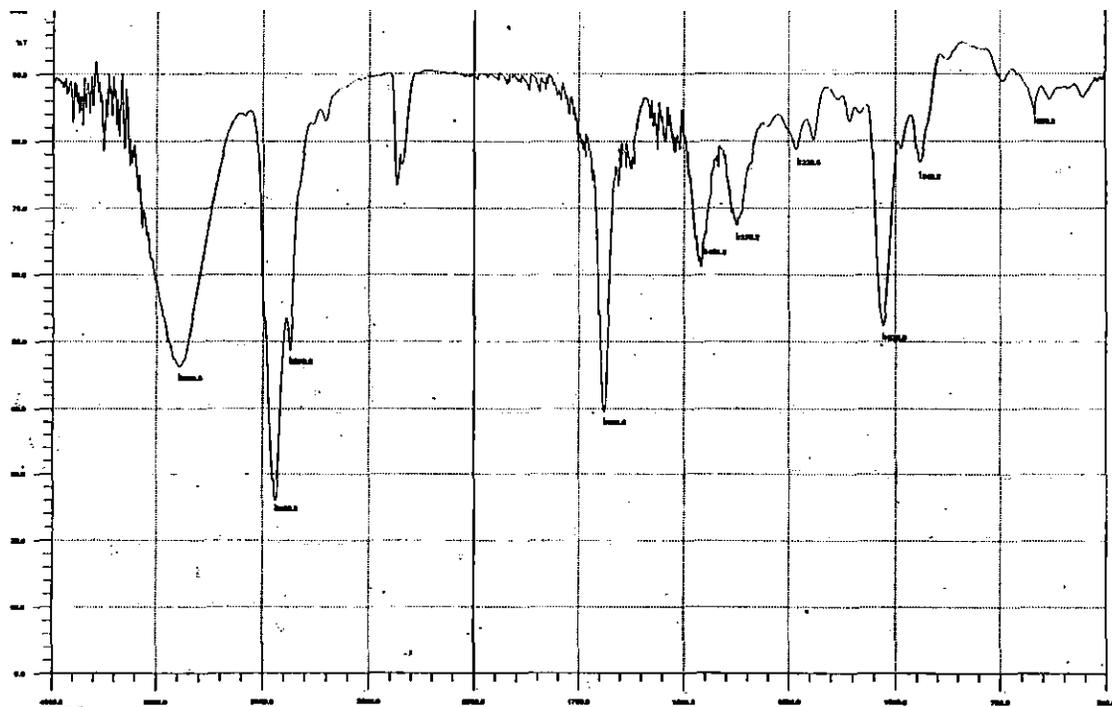


Figure 9 IR spectrum of betulin, 2

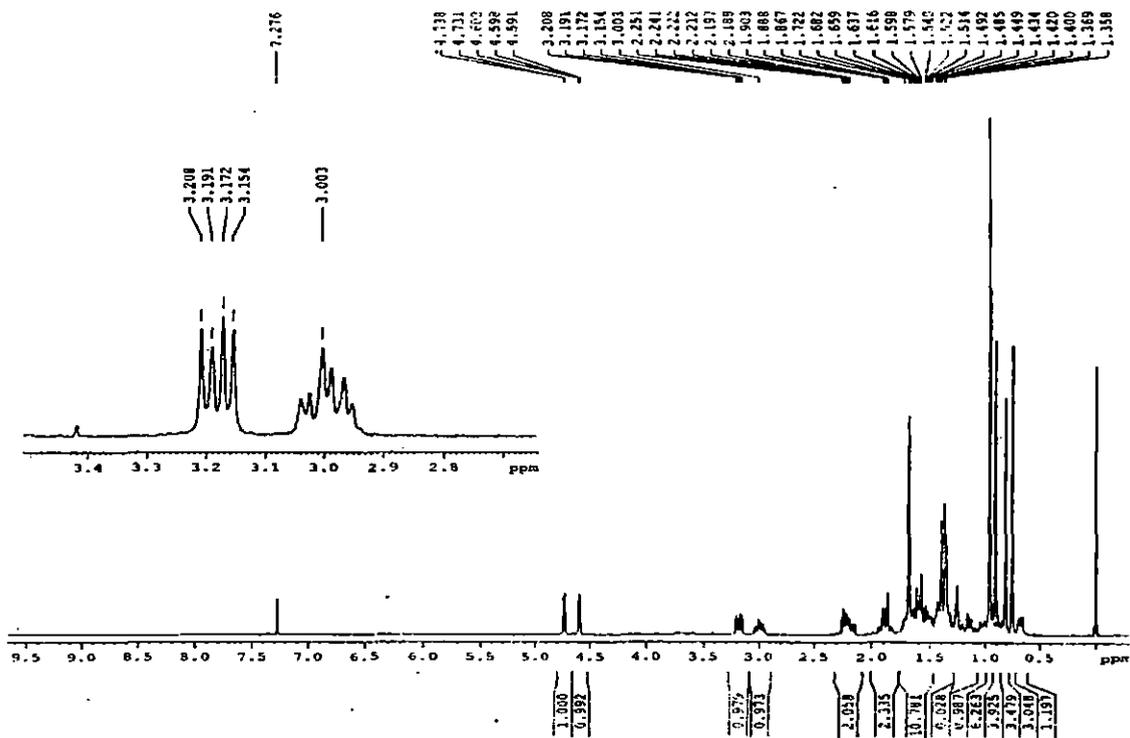


Figure 10 ^1H NMR spectrum of betulin, 2

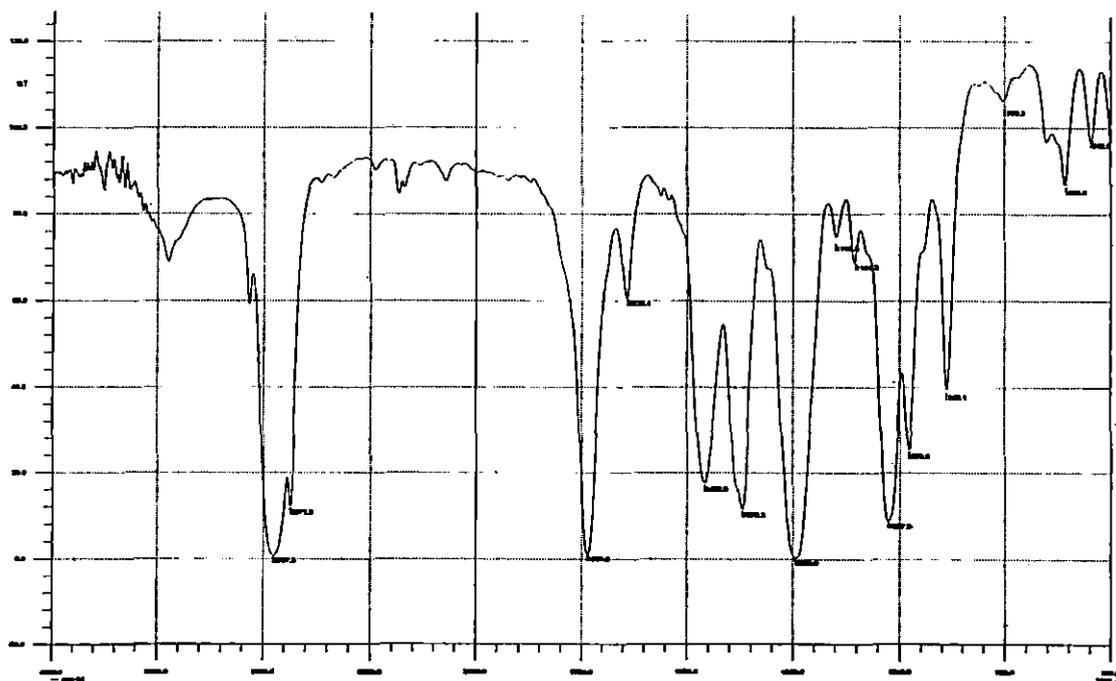


Figure 11 IR spectrum of betulin diacetate, 44

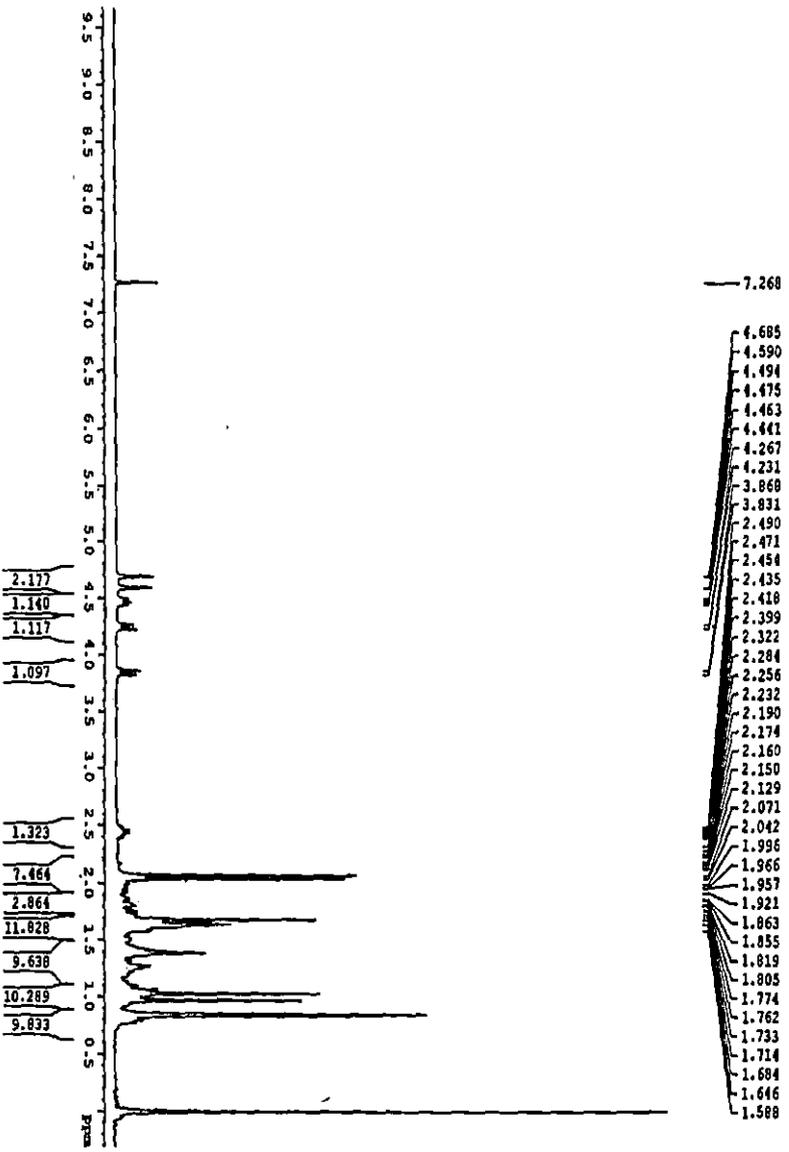


Figure 12 ¹H NMR spectrum of betulin diacetate, 44

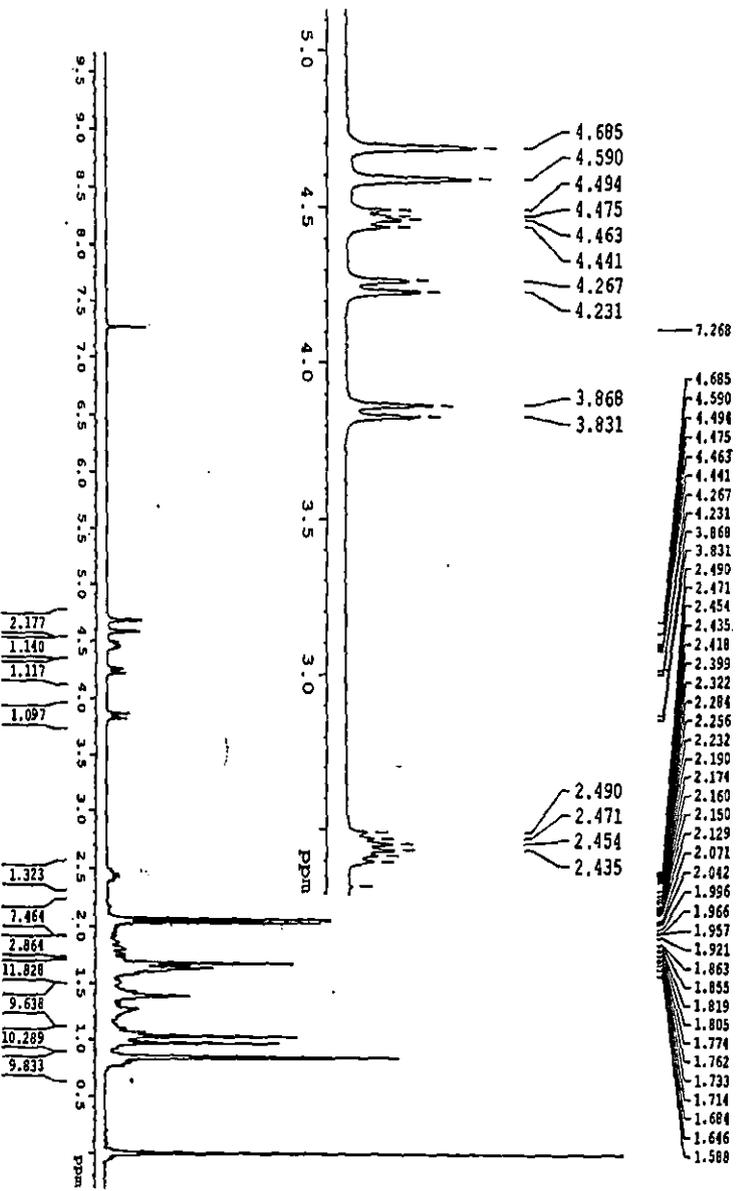


Figure 13 Expanded ¹H NMR spectrum of betulin diacetate, 44

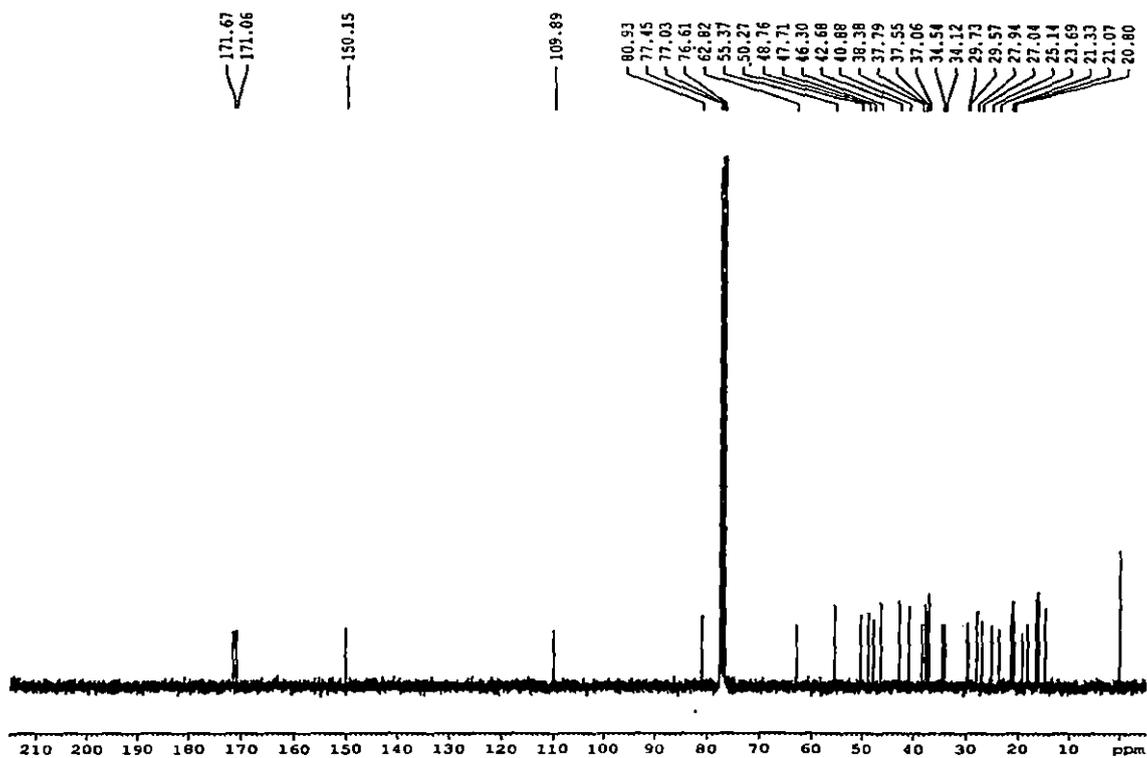


Figure 14 ^{13}C NMR spectrum of betulin diacetate, 44

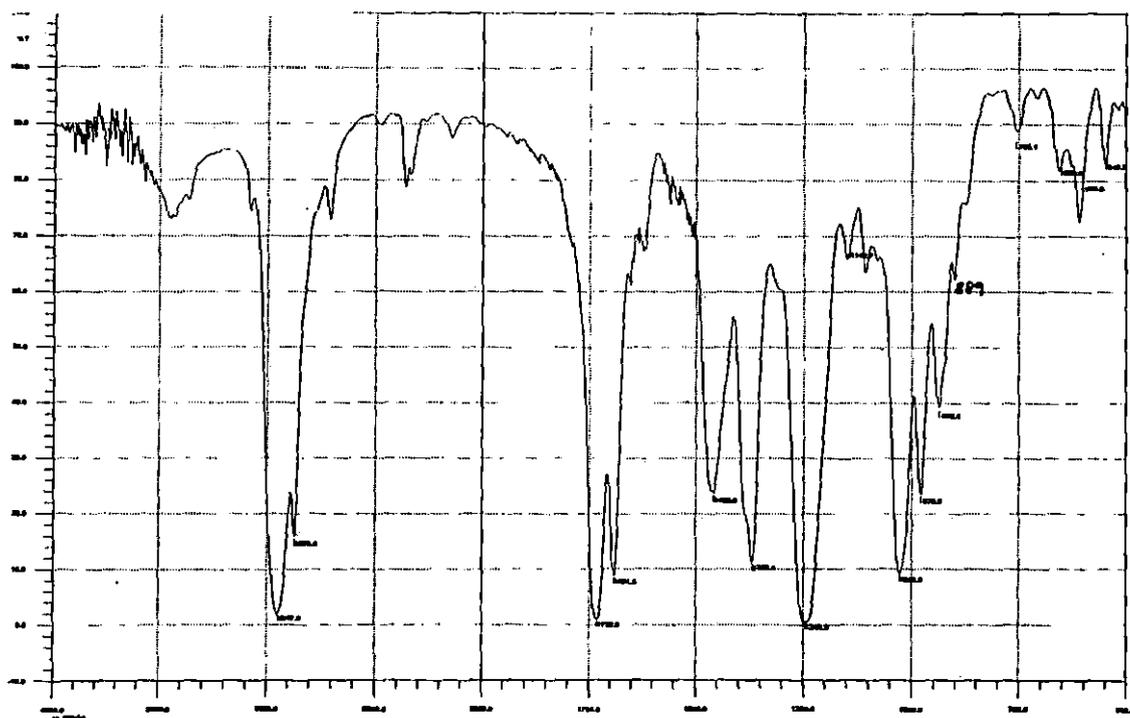


Figure 15 IR spectrum of diacetyl betulin-30-al, 45

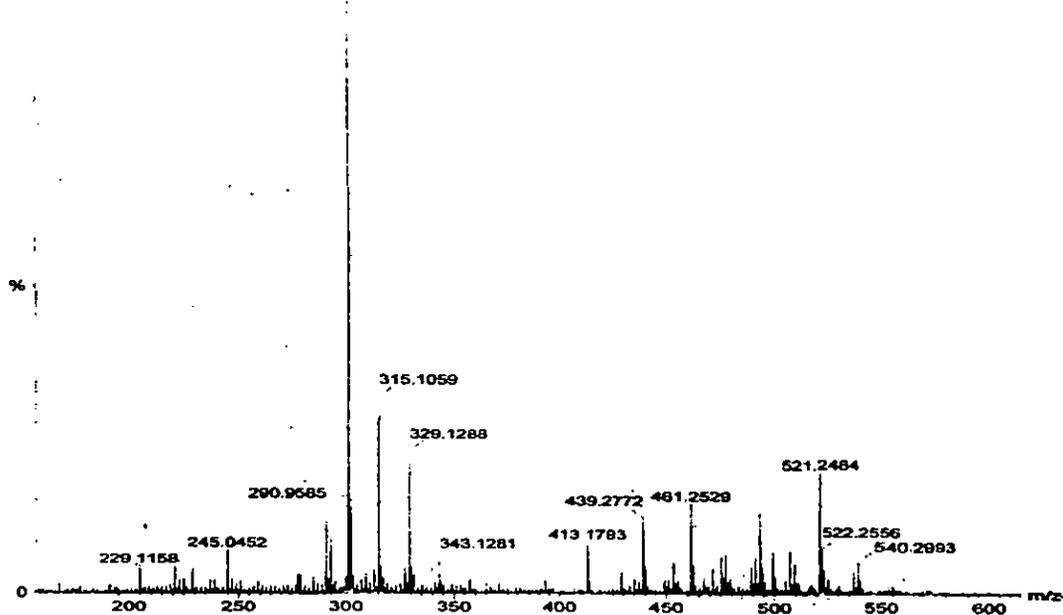


Figure 16 Mass spectrum of compound 45

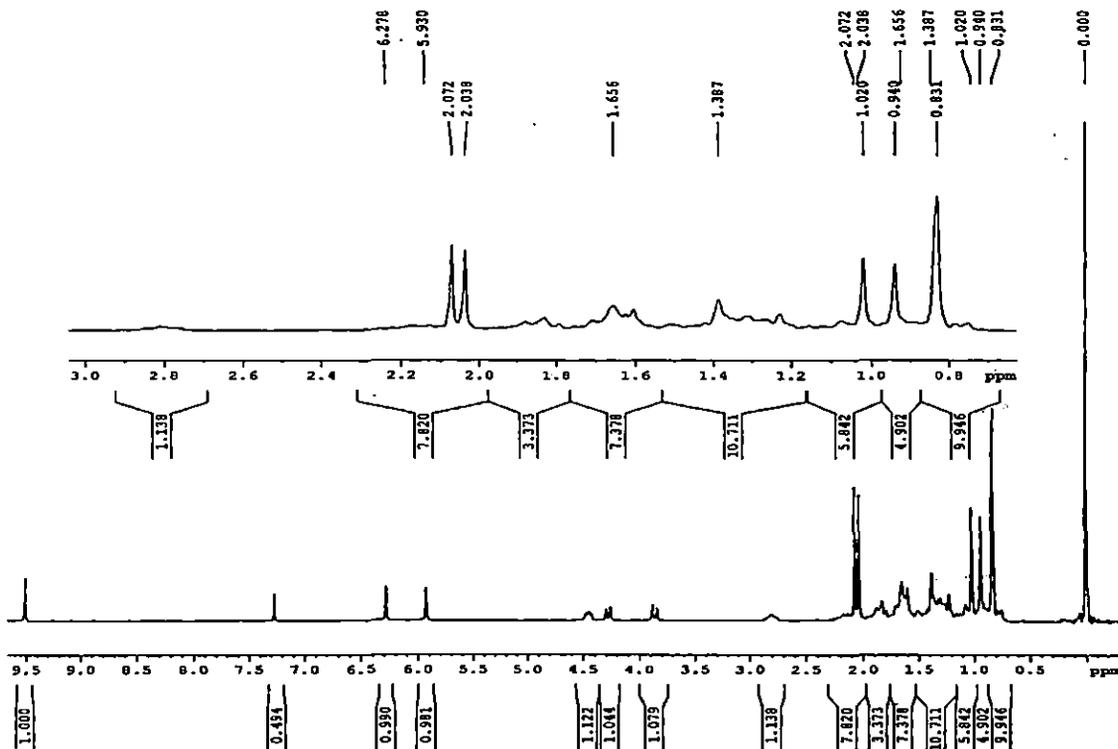


Figure 17 ^1H NMR spectrum of diacetyl betulin aldehyde, 45

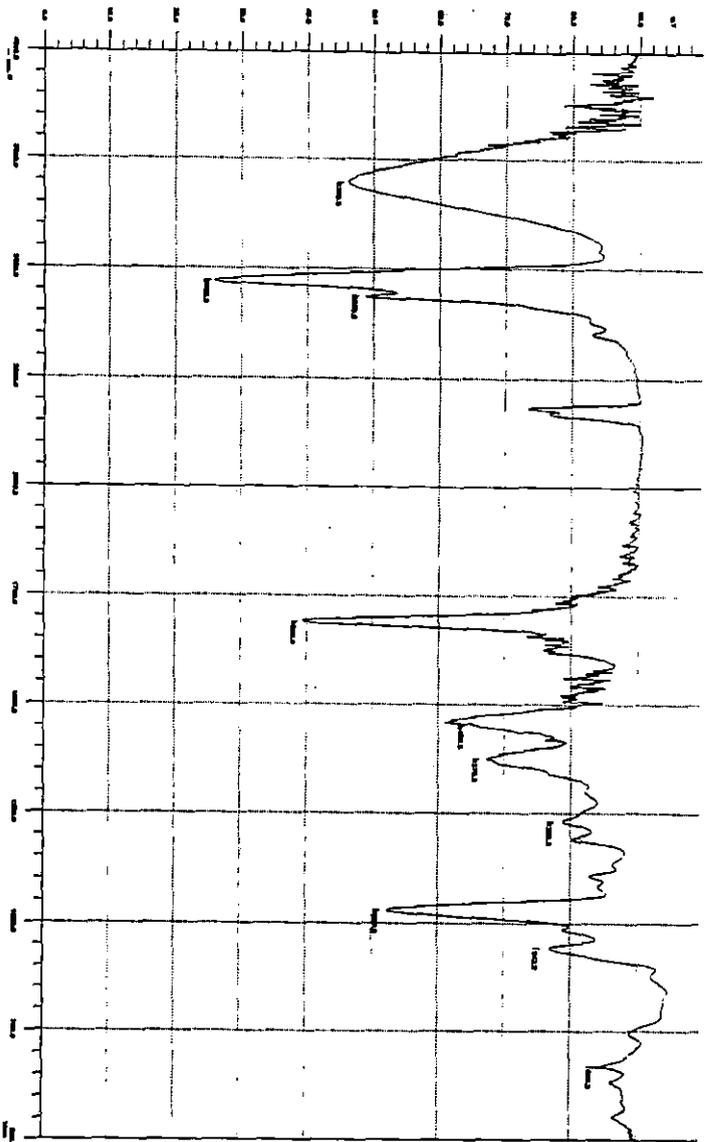


Figure 19 IR spectrum of betulin-30-aldehyde, 46

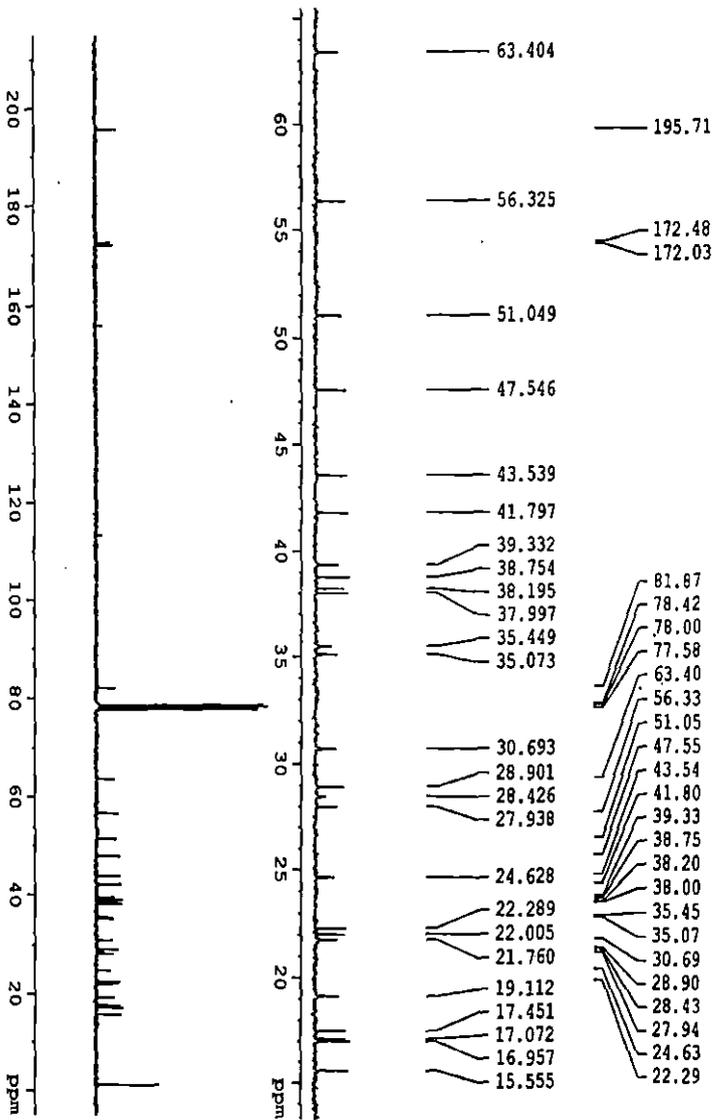


Figure 18 ¹³C NMR spectrum of diacetyl betulin-30-aldehyde, 45

ABA-AC-SKS
ABA-AC 7 (0.131) Sm (Mn, 10x5.00); Cm (1:10)
100- 301.0969

TOF MS ES+
83.9

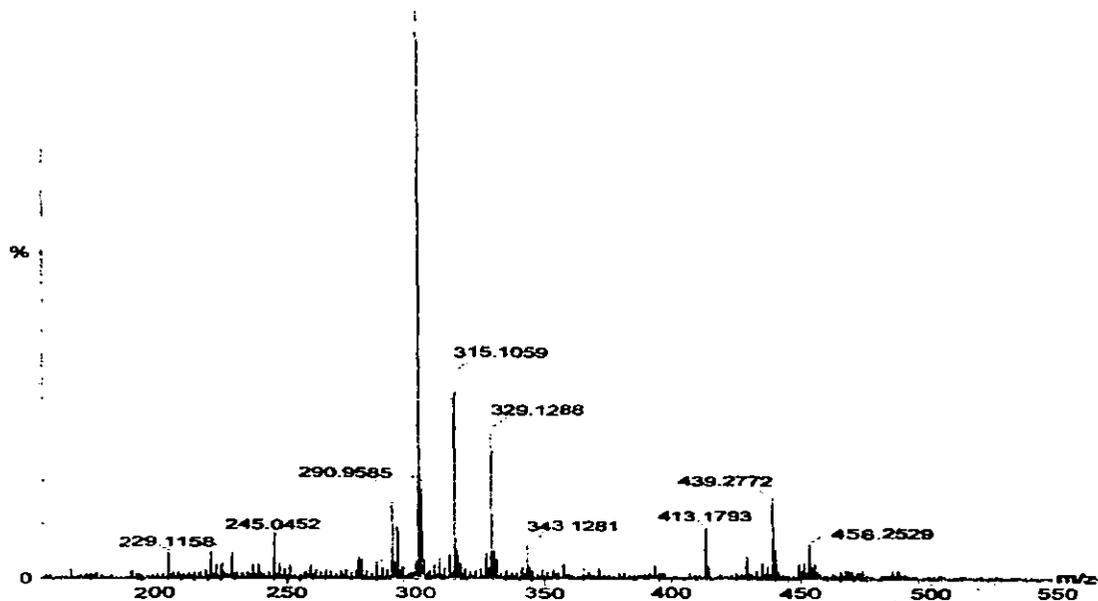


Figure 20 Mass spectrum of compound 46

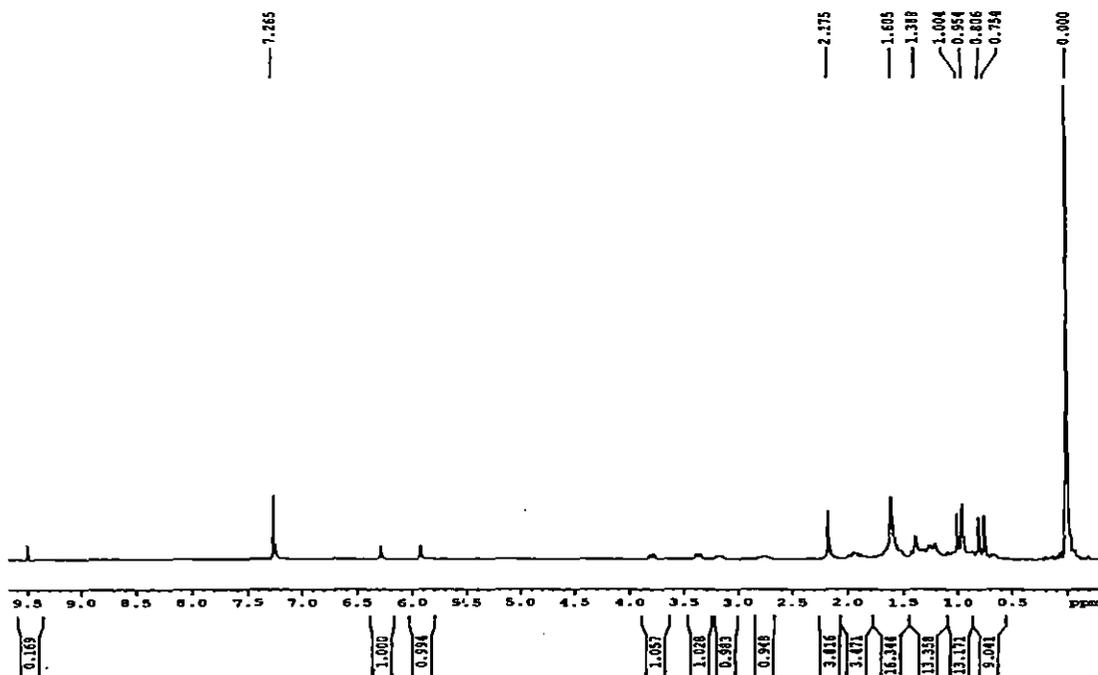


Figure 21 ¹H NMR spectrum of betulin-30-aldehyde, 46

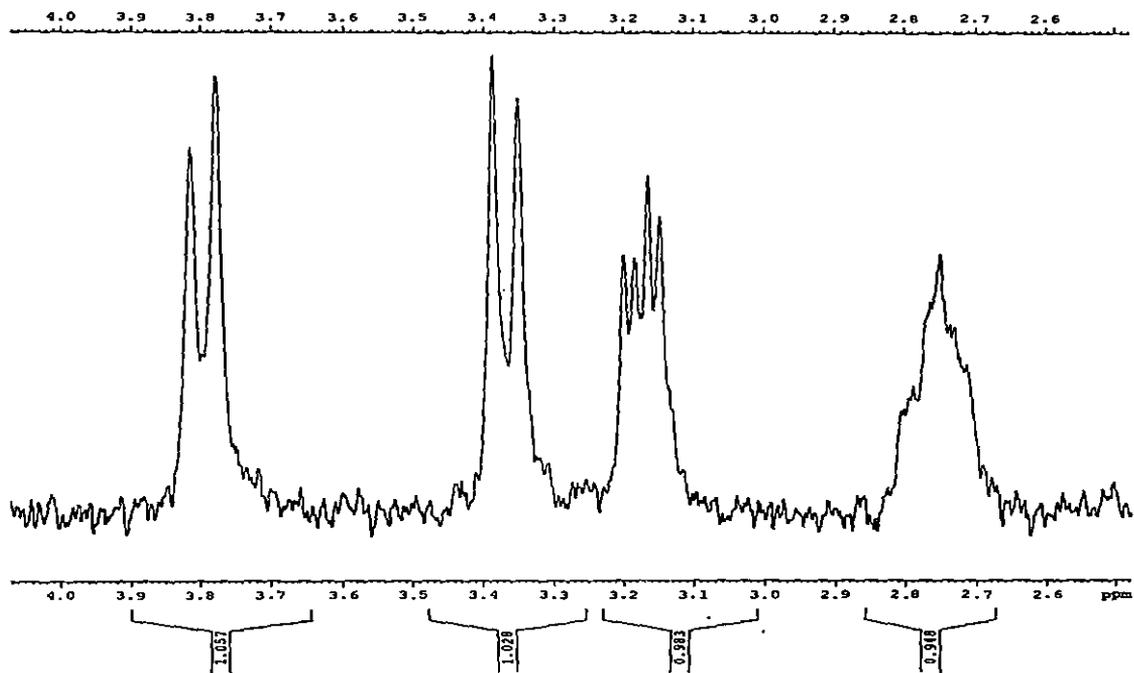


Figure 22 Expanded ^1H NMR spectrum of betulin-30-aldehyde, 46

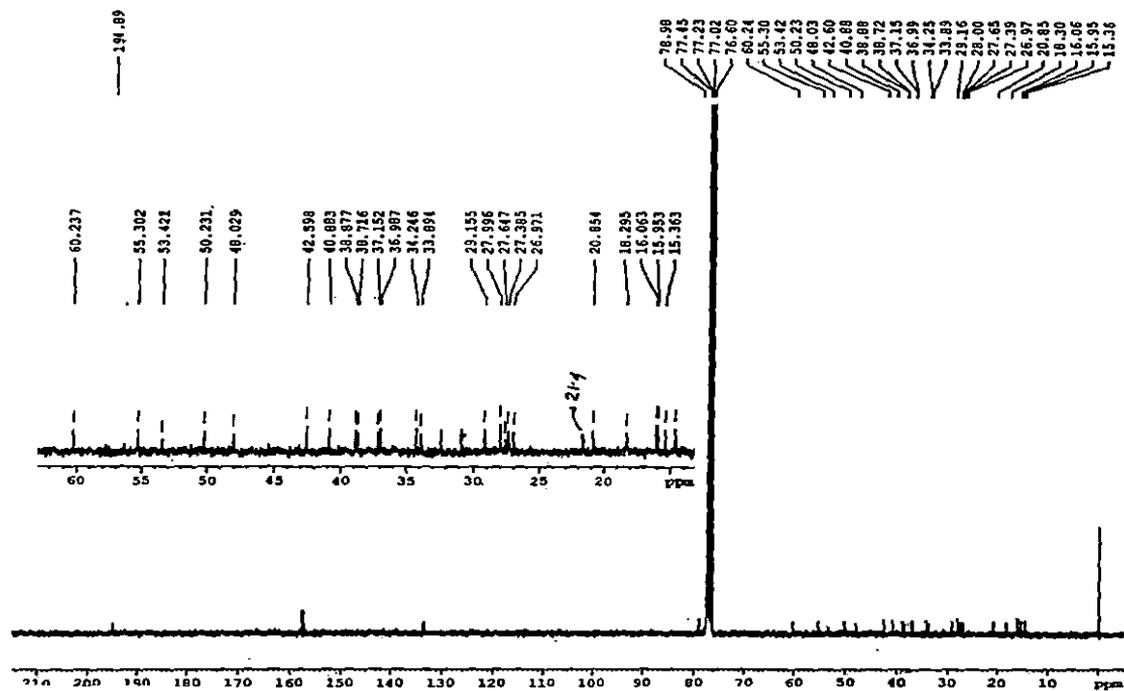
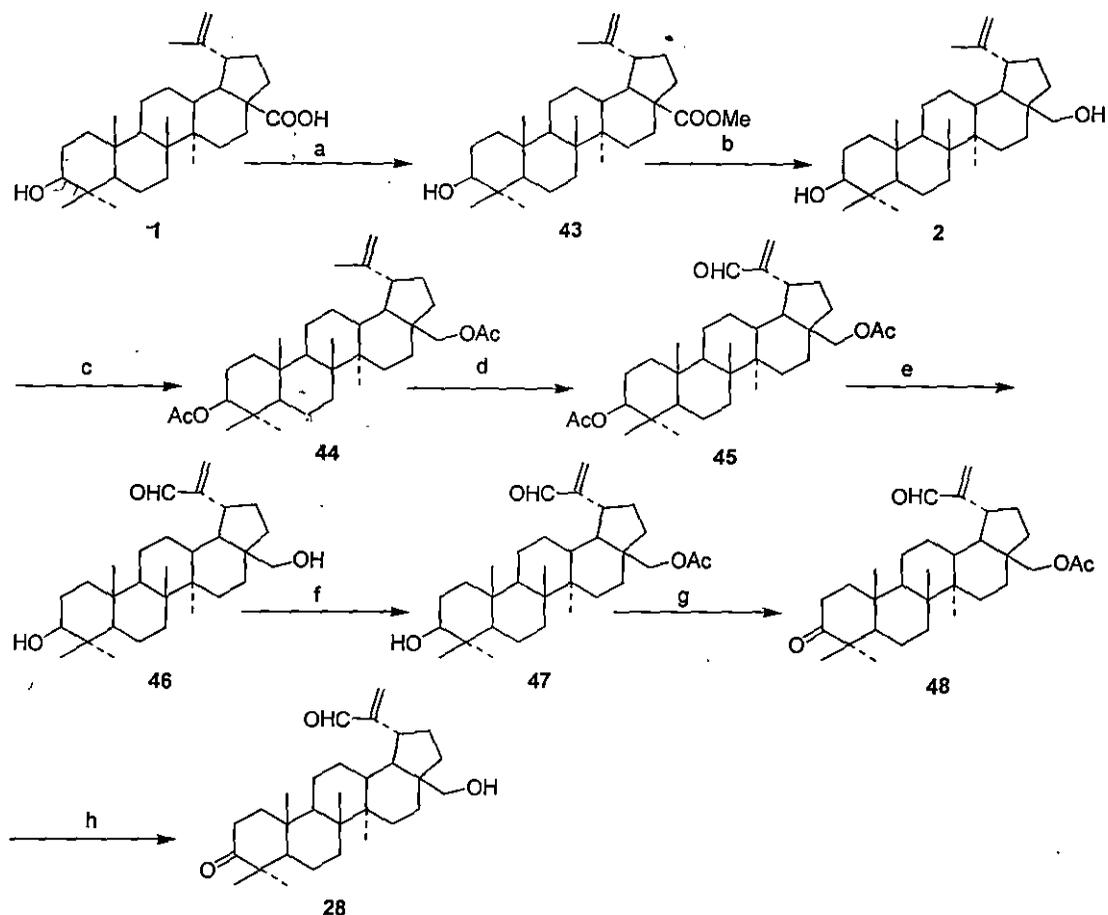


Figure 23 ^{13}C NMR spectrum of betulin-30-aldehyde, 46

A number of methods⁵⁸ were then applied for the selective oxidation of C-3 secondary hydroxyl group keeping C-28 primary hydroxyl group intact. None of the existing

methods⁵⁸ including the one developed by Mckillop *et al.* in 1979,⁵⁸ was found effective to produce the desired selectivity. Thus once again the protection-deprotection method was applied for the formation of desired aldehyde, **47**.

A solution of **46** (350 mg, 0.76 mmol) in CHCl₃ (10 mL) and pyridine (15 mL) was treated with Ac₂O (10 mL, 0.098 mmol) at very low temperature (5-10 °C). After the reaction is over (checked by tlc), the mixture was diluted with CHCl₃ (20 mL), then the organic layer was washed with saturated aq. NaCl (10 mL x 3), dried over anhydrous sodium sulfate, and concentrated in *vacuo*. Purification of the reaction mixture gave compound **47** mp 260-262 °C. The IR spectrum gave peaks at 3461 (-OH), 1732 (-OCOCH₃), 1691 (>C=C-CHO), 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 890 (=CH₂) cm⁻¹. The molecular formula of compound **47** was assigned to C₃₂H₅₀O₄ (M⁺ 498.3, Analytical calculation % C 77.02, % H 10.12). The ¹H NMR spectrum of **47** showed a singlet at δ_H 9.56 (s, 1H) for the aldehyde proton. ¹³C NMR of compound **47** (Table 1) accounted for all the carbons. Acetylation of only the C-28 -OH group was confirmed by comparison of ¹H and ¹³C NMR data of C-3 hydroxy compound and with the acetate as reported in literature.³⁶ Assigned ¹³C NMR data of all the compounds are given in table 1. Thus on the basis of the above spectral data, structure of compound **47** was assigned as 28-acetoxy-lup-20(29)-en-3β-ol-30-al.



Scheme 1. Partial synthesis of compound 28 from betulinic acid (1): Reagents and conditions: a, CH_2N_2 , ether, over night, AcOH (gal.) Na_2SO_4 ; b, LiAlH_4 , dry THF, 2 hrs., saturated Na_2SO_4 solution, ether, Na_2SO_4 ; c, $\text{C}_5\text{H}_5\text{N}$, Ac_2O , 6 hrs. (100°C) ice cold H_2O , ether, Na_2SO_4 ; d, SeO_2 , aq. dioxan, 2 hrs., ice cold H_2O , ether, Na_2SO_4 ; e, 10% alcoholic KOH , THF, 4 hrs., ice cold H_2O , ether, Na_2SO_4 ; f, $\text{C}_5\text{H}_5\text{N}$, Ac_2O , (5-10°C), 8 hrs., ice cold H_2O , Na_2SO_4 ; g, $\text{C}_5\text{H}_5\text{N}$, dry CrO_3 , overnight, ice cold H_2O , CH_2Cl_2 , MgSO_4 ; h, 10% alcoholic KOH , THF, 4 hrs., ice cold H_2O , ether, Na_2SO_4 .

In the subsequent step C-3 -OH group was converted to the ketone 48 (175 mg), using anhydrous CrO_3 in dry pyridine (see experimental) at ambient temperature. After purification it showed mp 276-278 °C. The exact structure of compound 48 was elucidated by spectroscopic studies. The IR spectrum gave peaks at 1730 ($>\text{C}=\text{O}$), 1706, 1696 ($\text{CH}_2=\text{C}-\text{CHO}$). The molecular formula of compound 48 was assigned to $\text{C}_{32}\text{H}_{48}\text{O}_4$ (M^+ 496.34, Analytical calculation % C 77.28, % H 9.62). In its ^1H NMR spectrum, compound 48 gave a singlet at δ_{H} 9.56 (s, 1H) for the aldehydic proton. Two olefinic protons of C-29 appeared at δ_{H} 6.23 (s, 1H) and 5.92 (s, 1H). Two geminal protons of C₂₈ - CH_2 group, each appeared as a doublet at δ_{H} 3.83 (d, 1H, $J = 10.6$ Hz) and at δ_{H} 3.38 (d,

1H, J = 10.6 Hz). All the five methyls appeared between δ_H 0.75 to 0.95 along with other peaks of lupane skeleton. ^{13}C NMR spectrum (Table 1) was also in good agreement with the proposed structure of compound **48** (Table 1). Deprotection of C-28 hydroxyl group finally afforded a white, amorphous solid, designated as **X** (44% overall yield), mp 288-290 °C.

The IR spectrum gave peaks at 3424 (-OH), 1725 (>C=O), 1706, 1697 (conjugated aldehyde), 1461, 1380 (gem dimethyl), 1163, 987 cm^{-1} . The molecular formula was established as $C_{30}H_{46}O_3$ by HREIMS (m/z 554.38 $[M]^+$, calcd. 554.34). In the 1H NMR spectrum five tertiary methyl groups appeared at δ_H 0.91 (s, 3H), 0.93 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H) and 1.15 (s, 3H). Two olefinic C-29 protons appeared at δ_H 6.91 (s, 1H) and 6.28 (s, 1H). The singlet at δ_H 9.52 (s, 1H) was due to the aldehydic proton of C-30. Two geminal protons of C₂₈-H₂ appeared at δ_H 3.78 (d, 1H, J = 10.6 Hz) and at δ_H 3.36 (d, 1H, J = 10.6 Hz). The ^{13}C spectrum revealed the presence of two olefinic carbons at δ_C 133.6 (C-29) and 156.2 (C-20). The aldehydic C-30 carbon appeared at δ_C 194.9. C-3 appeared at δ_C 218.1 and C-28 appeared at δ_C 60.2.

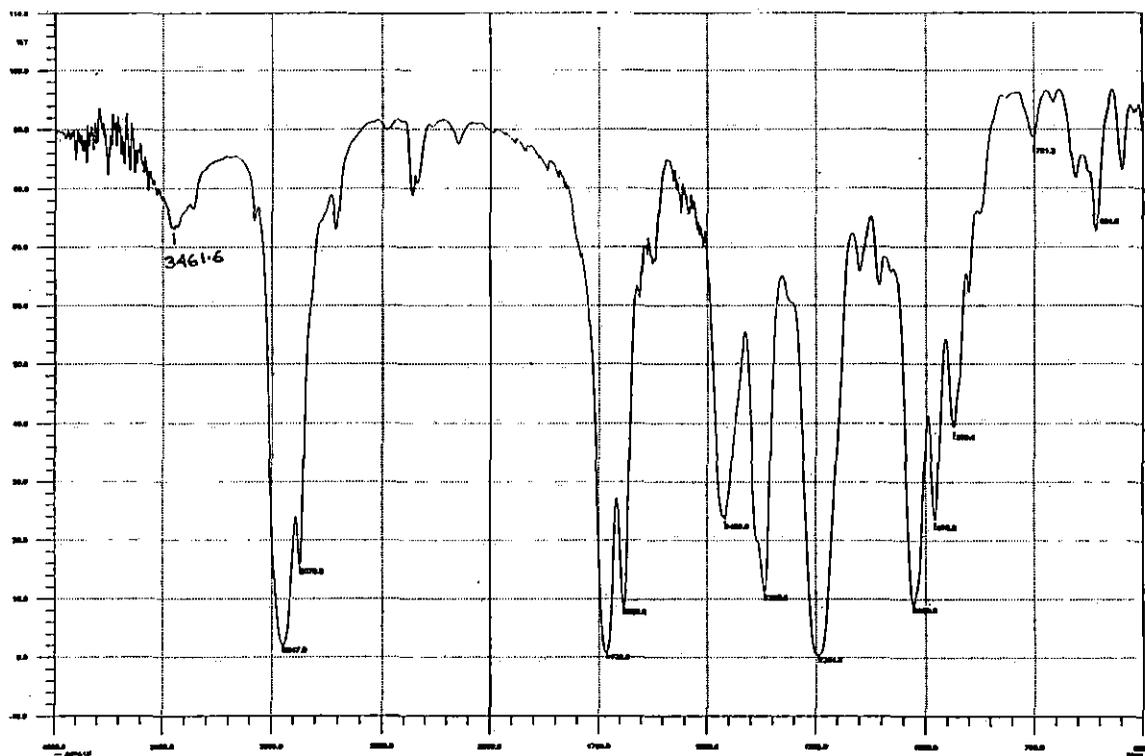


Figure 24 IR spectrum of 28-acetylbetulin-30-aldehyde, **47**

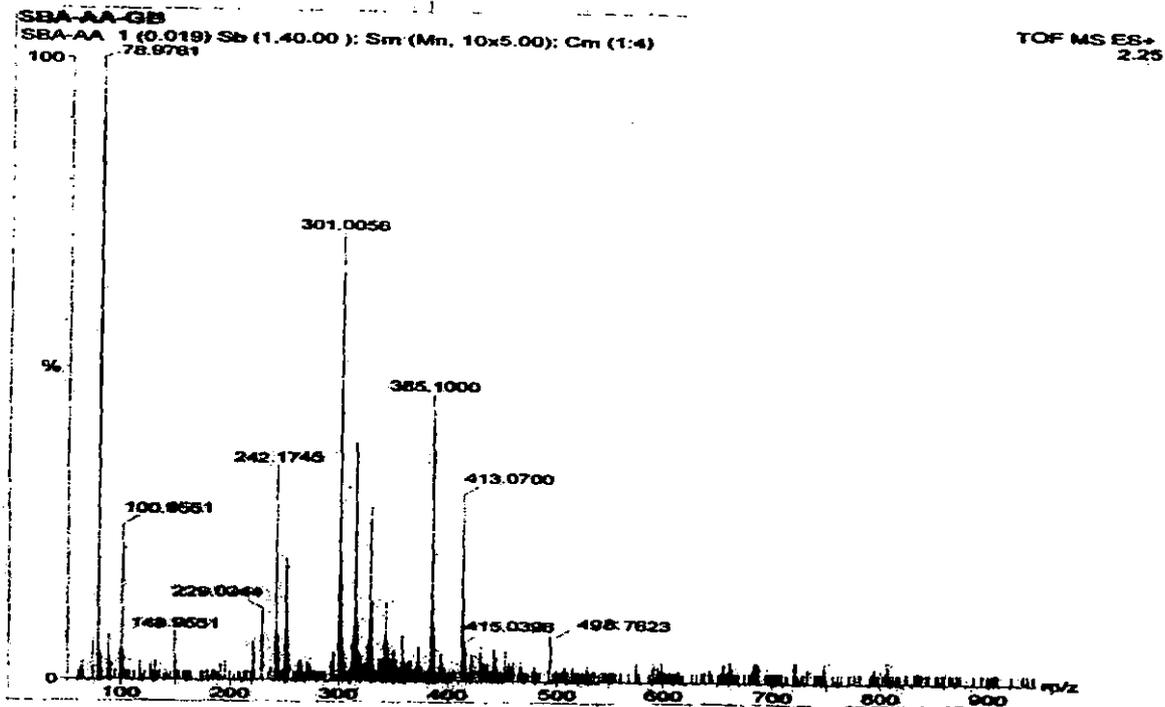


Figure 25 Mass spectrum of compound 47

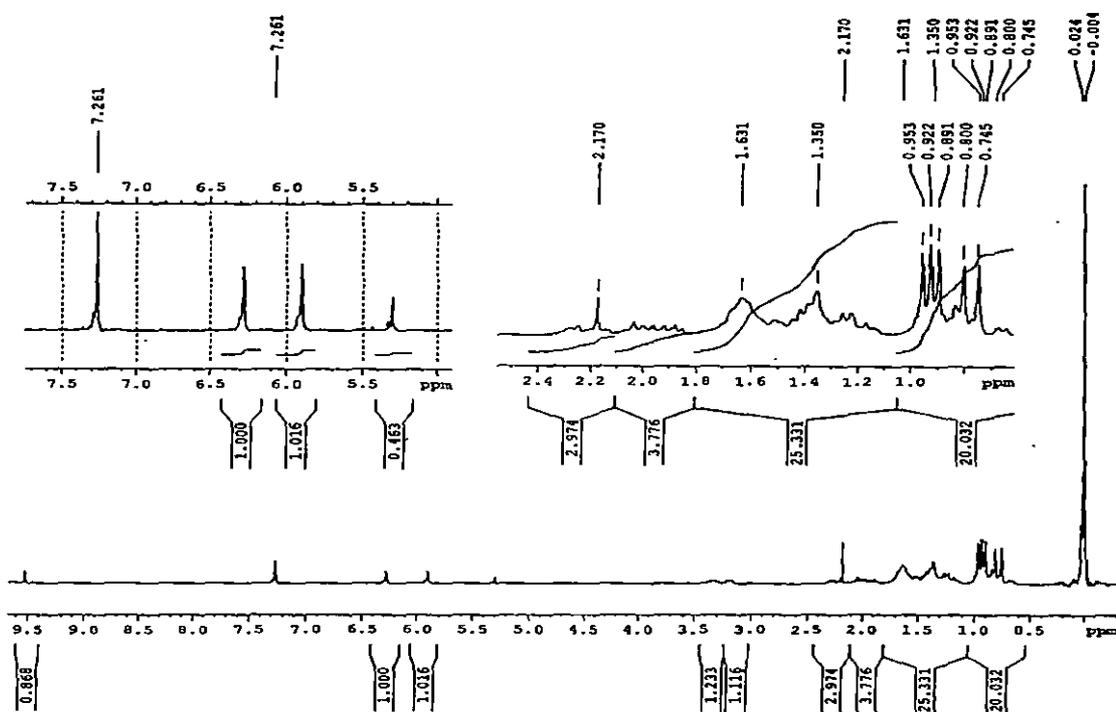


Figure 26 ^1H NMR spectrum of 28-acetylbetulin-30-aldehyde, 47

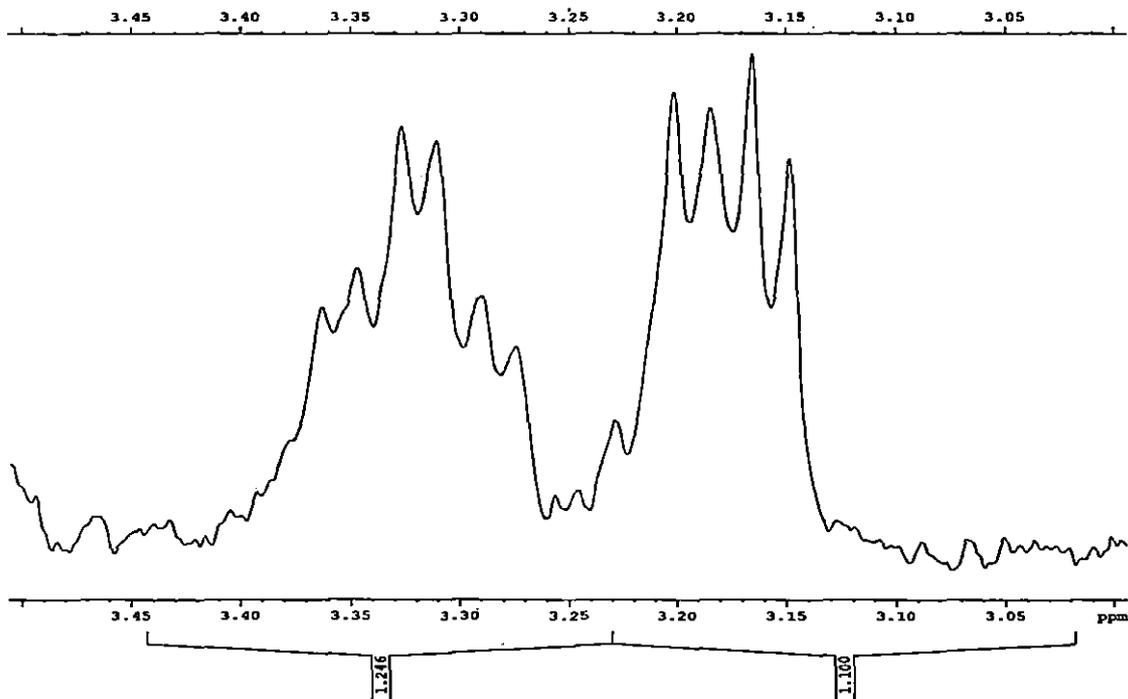


Figure 27 Expanded ^1H NMR spectrum of 28-acetylbetulin-30-aldehyde, 47

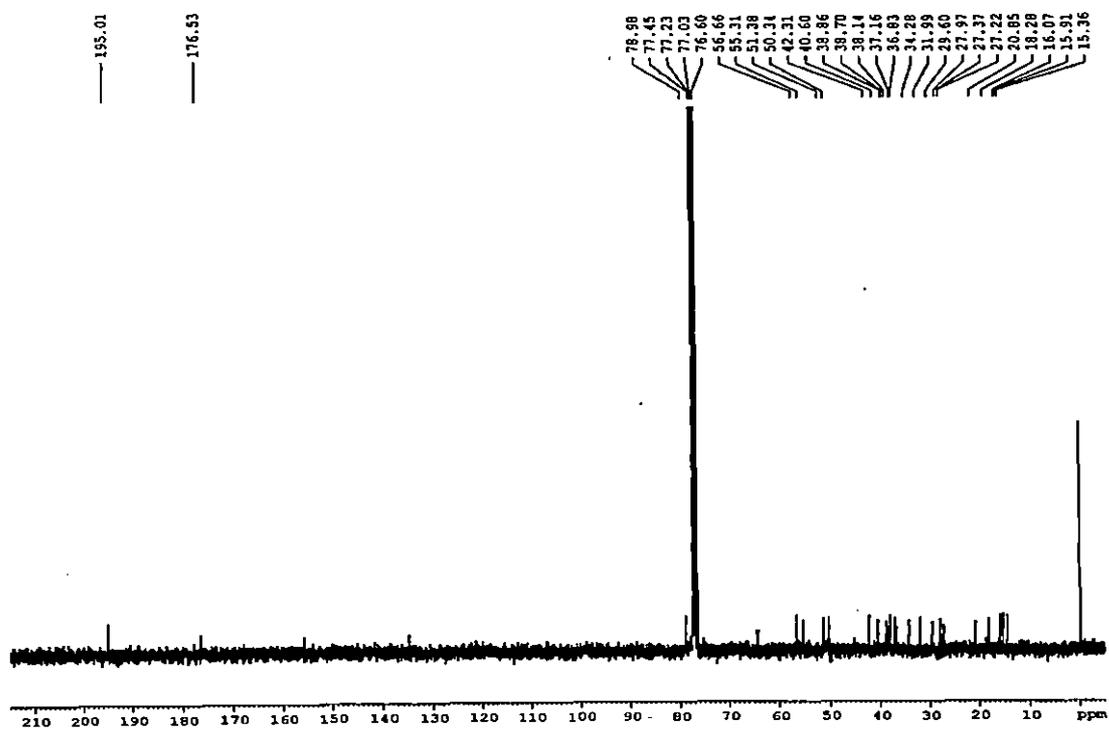


Figure 28 ^{13}C NMR spectrum of 28-acetylbetulin-30-aldehyde, 47

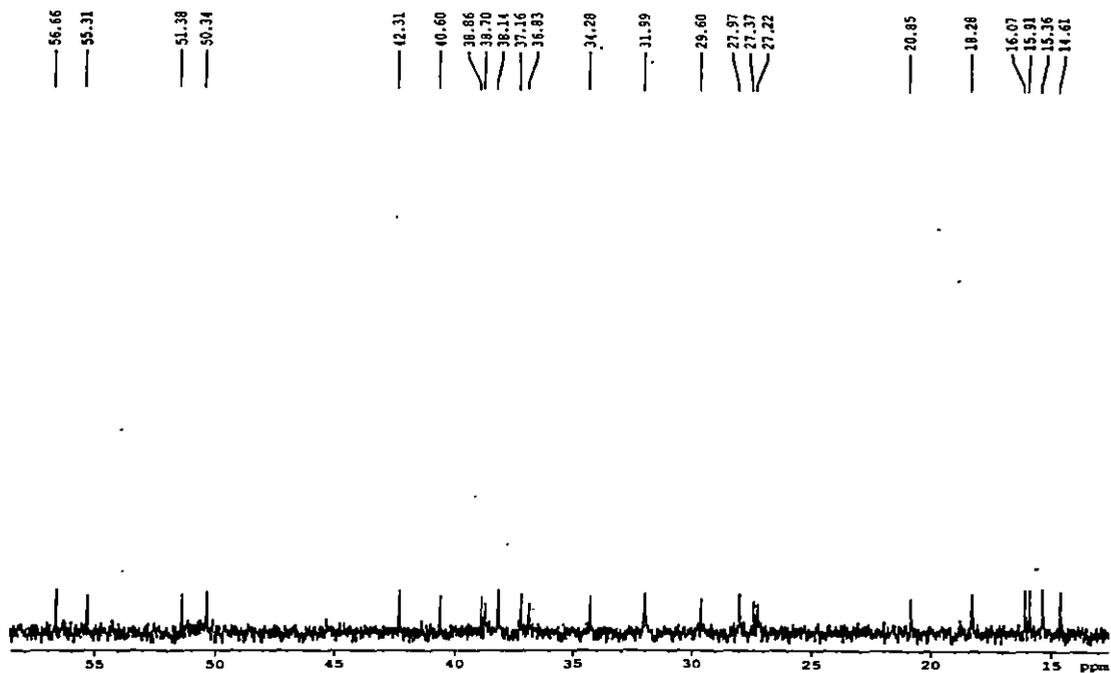


Figure 29 Expanded ^{13}C NMR spectrum of 28-acetylbetulin-30-aldehyde, 47

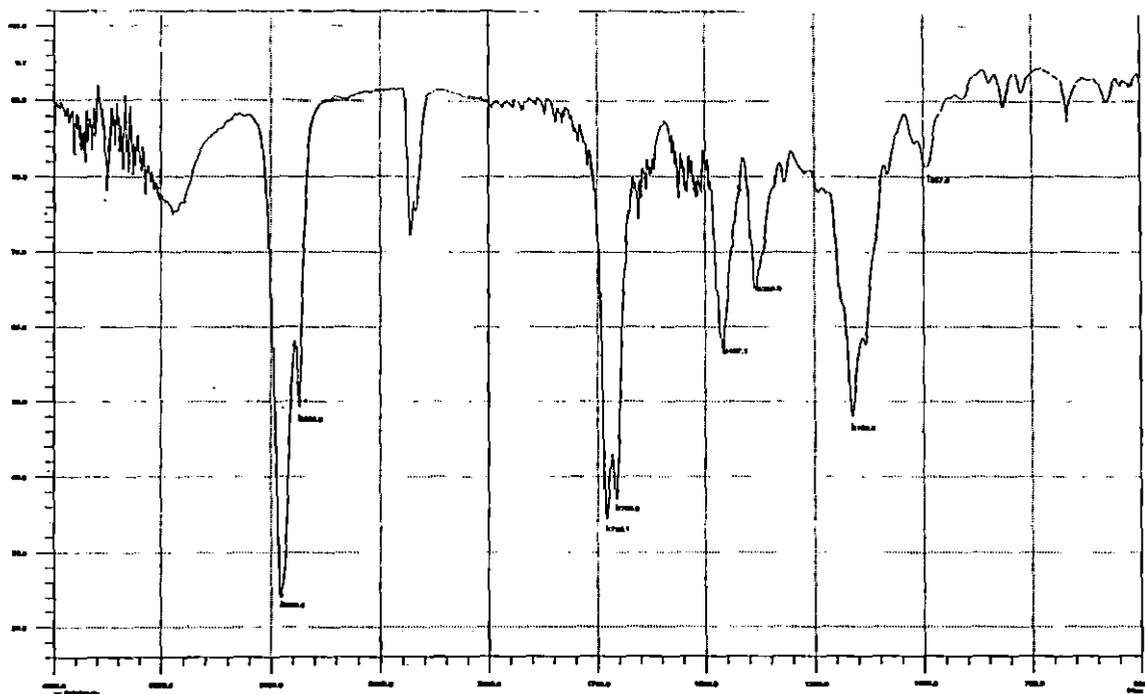


Figure 30 IR spectrum of 3-keto28-acetylbetulin-30-aldehyde, 48

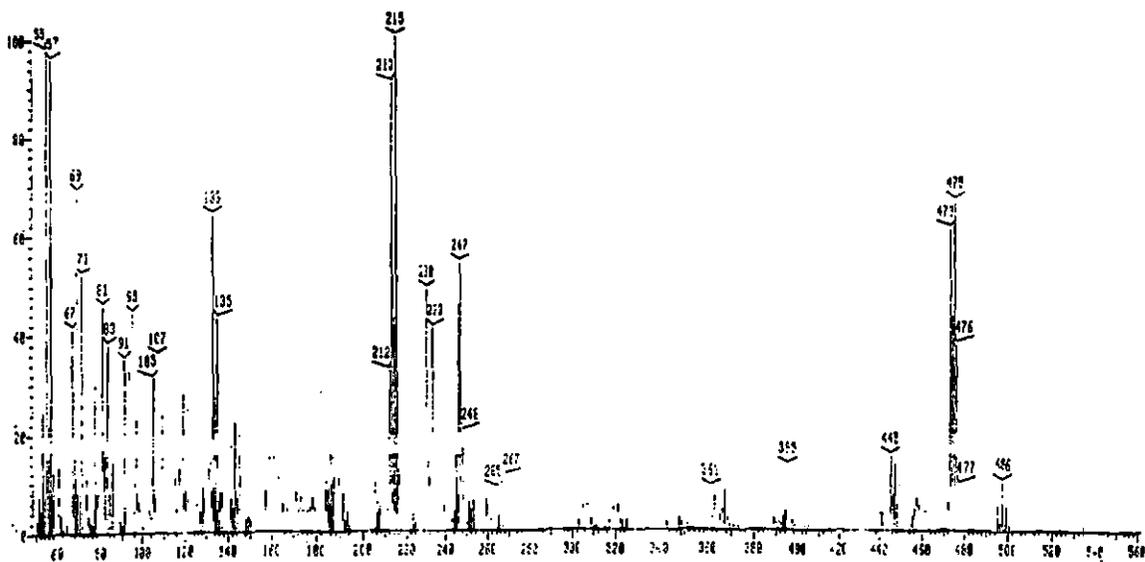


Figure 31 Mass spectrum of compound 48

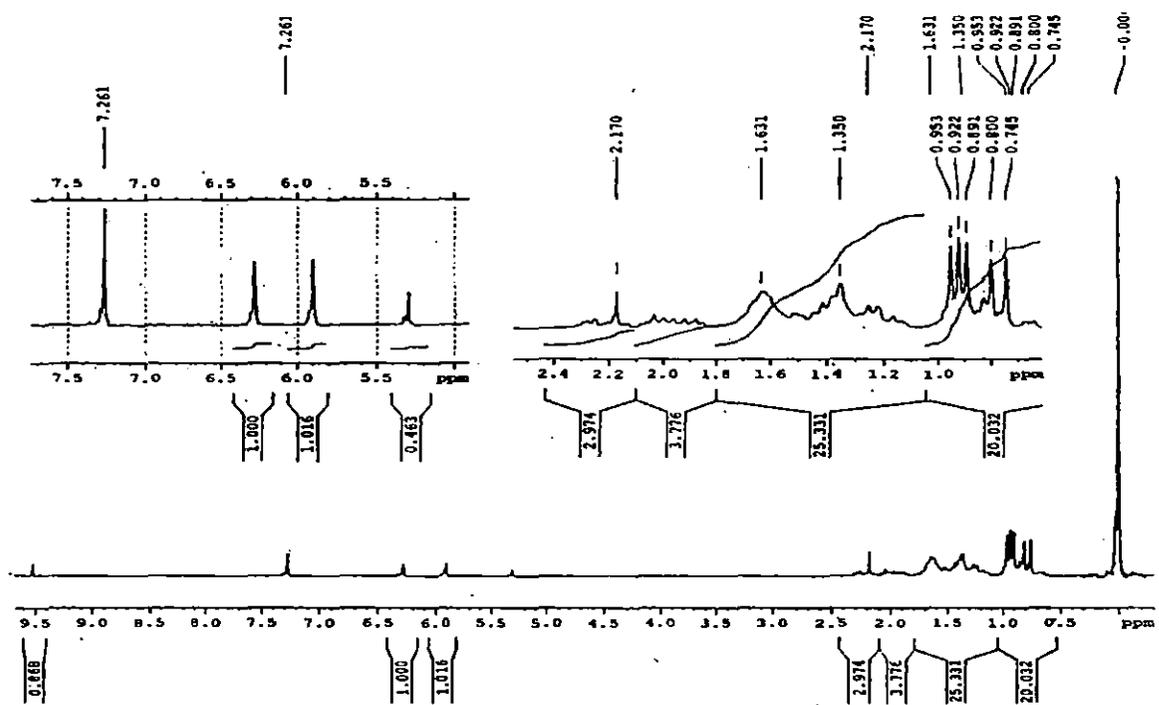


Figure 32 ¹H NMR spectrum of 3-keto28-acetylbetulin-30-aldehyde, 48

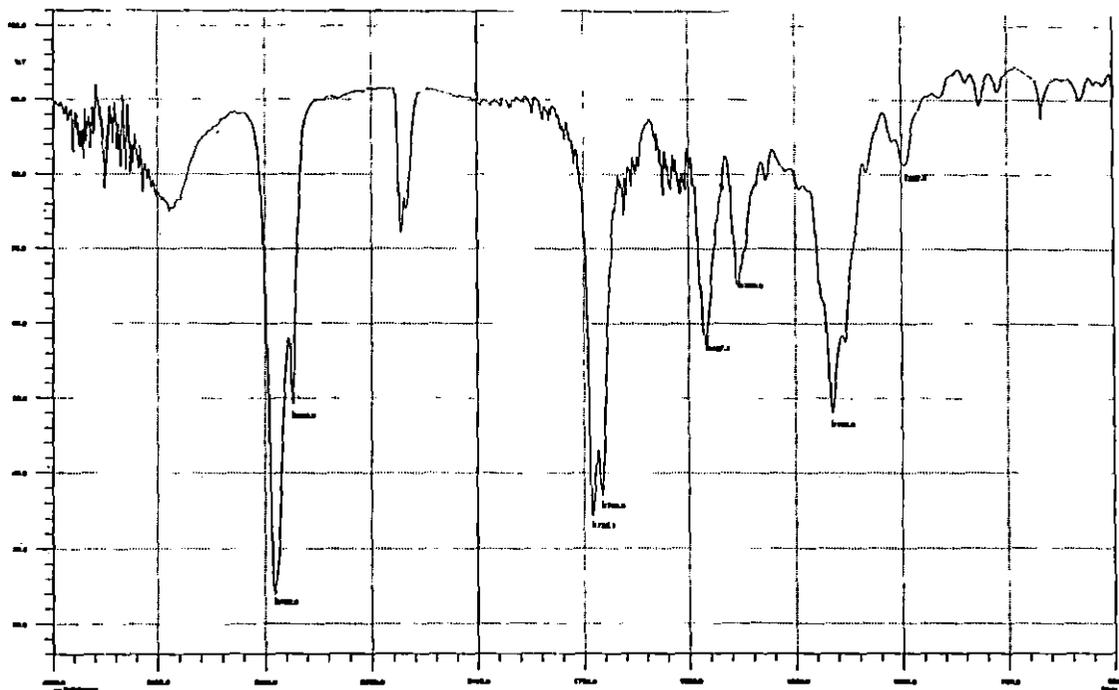


Figure 33 IR spectrum of compound 28

ABA-AC-SKS
 ABA-AC 7 (0.131) Sm (Mn, 10x5.00); Cm (1:10)
 100 301.0989

TOF MS ES+
 83.9

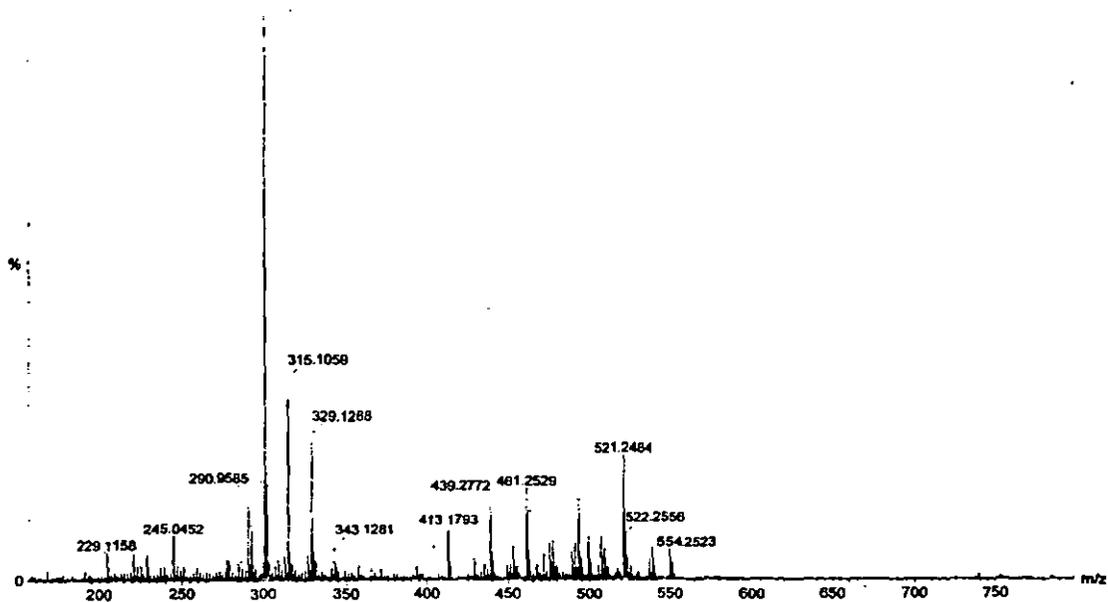


Figure 34 Mass spectrum of compound 28

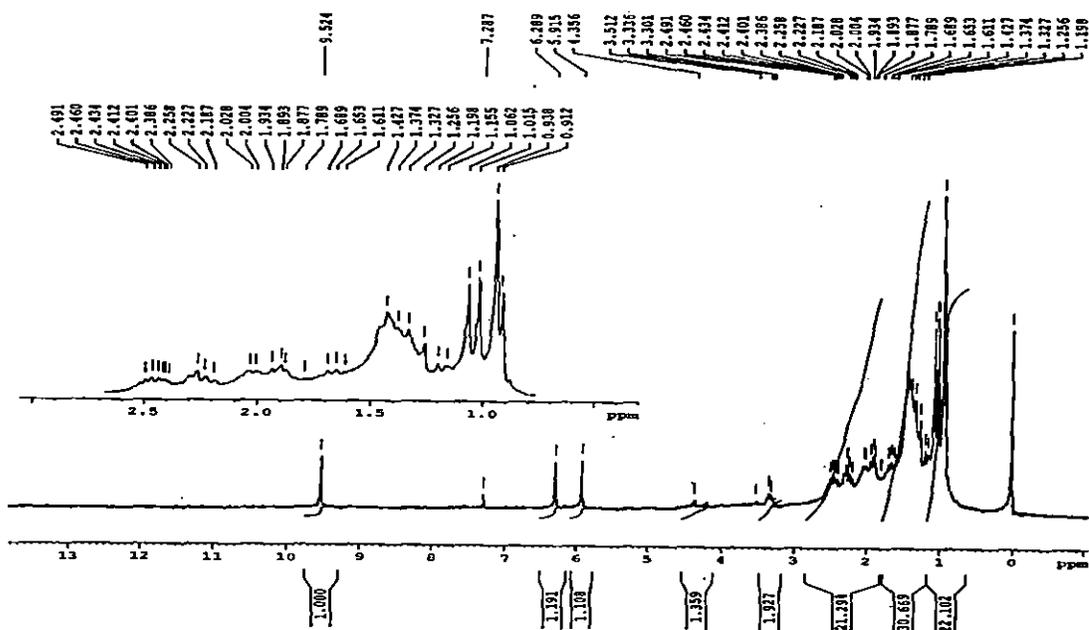


Figure 35 ^1H NMR spectrum of compound 28

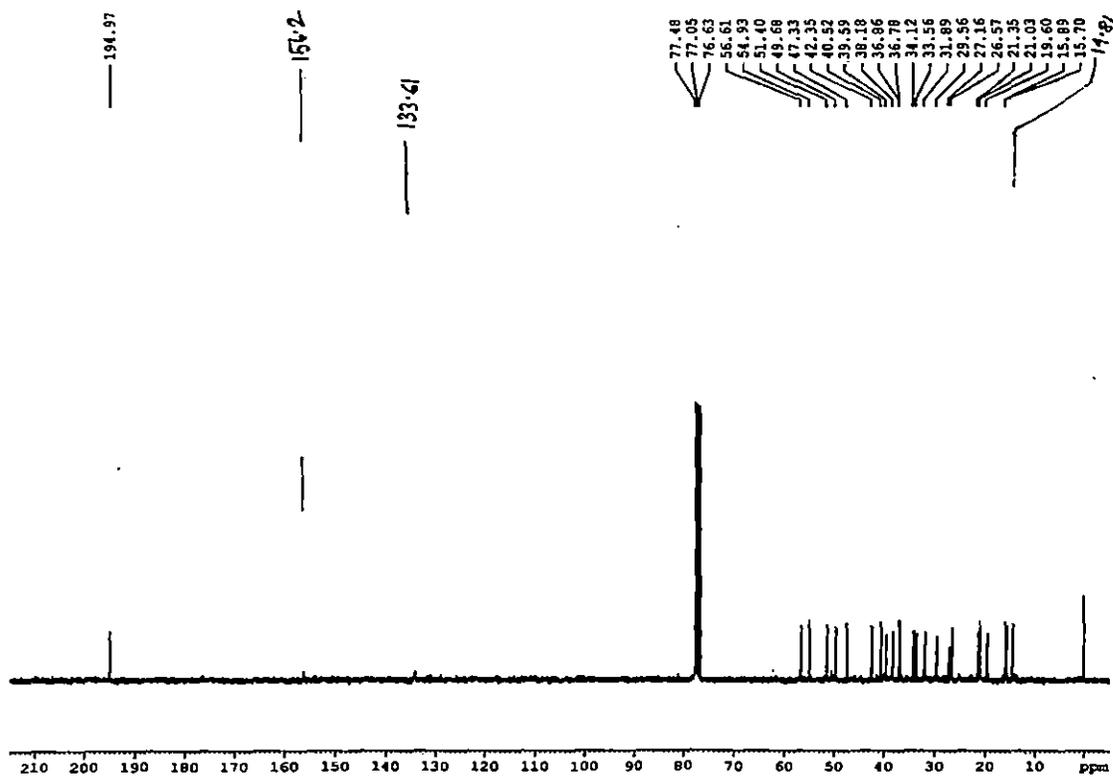


Figure 36 ^{13}C NMR spectrum of compound 28

Table 1 ^{13}C NMR data of compounds 1-9 and betulonic acid taken in CDCl_3

Position	1*	1	2	3	4	5	6	7	8	9	Betulonic acid
1	39.6	39.6	38.8	38.8	38.8	38.4	39.3	39.0	39.0	39.0	38.8
2	34.1	34.1	34.0	34.3	34.2	34.1	35.0	34.3	34.3	34.3	34.0
3	218.0	218.1	78.9	79.0	78.9	80.9	81.8	78.9	78.9	218.1	218.1
							(172.0 ^a)				
4	47.4	47.3	47.3	50.5	50.5	50.2	47.6	48.0	48.0	48.0	47.3
5	55.0	54.9	54.8	55.3	55.3	55.3	56.3	55.3	55.3	55.3	54.8
6	19.6	19.6	19.5	19.5	19.3	19.1	19.1	19.3	19.3	19.3	19.5
7	33.5	33.5	33.6	32.1	32.3	34.1	35.0	33.8	33.8	33.8	33.6
8	42.7	42.3	40.6	42.3	42.4	42.6	43.5	42.6	42.6	42.6	40.6
9	49.6	49.6	49.8	49.4	49.4	48.8	48.7	48.0	48.0	48.0	49.8
10	36.9	36.8	36.8	36.9	36.9	37.0	35.5	36.9	36.9	36.9	36.8
11	21.4	21.3	21.3	20.8	20.8	21.0	21.7	20.9	20.9	20.9	21.3
12	27.6	27.1	25.4	25.5	25.4	27.0	27.9	27.6	27.6	27.6	25.4
13	37.1	38.1	38.5	38.2	38.2	37.8	37.9	37.1	37.1	37.1	38.5
14	40.8	40.5	42.4	40.6	40.6	40.8	41.7	40.8	40.8	40.8	42.4
15	26.9	26.5	29.6	29.6	29.6	27.0	28.9	26.9	26.9	26.9	29.6
16	29.1	29.5	32.0	27.9	27.9	27.9	28.4	28.1	28.1	28.1	32.0
17	48.0	47.1	56.3	46.5	47.6	47.7	47.3	47.3	47.3	47.3	56.3
18	52.3	51.4	49.1	51.2	51.3	51.2	51.0	50.2	50.2	50.2	49.1
19	36.5	37.5	46.8	46.9	46.9	37.5	38.7	38.7	38.7	38.7	46.8
20	157.0	156.2	150.2	150.6	150.7	150.2	157.1	157.1	157.1	157.1	150.2
21	32.8	31.8	30.5	30.6	30.5	34.5	31.0	31.8	31.8	31.8	30.5
22	33.9	36.7	36.8	37.2	37.1	37.0	38.1	36.4	36.4	36.4	36.8
23	26.6	26.6	26.6	27.4	27.4	27.0	24.6	26.9	26.9	26.9	26.6
24	21.1	21.0	20.9	19.3	19.3	20.8	22.0	21.4	21.4	21.4	20.9
25	15.9	15.8	15.8	15.3	15.3	16.0	17.1	15.9	15.9	15.9	15.8
26	15.8	15.7	15.9	15.9	15.9	16.1	16.9	15.3	15.3	15.3	15.9
27	14.6	14.8	14.6	14.7	14.7	14.7	15.6	14.6	14.6	14.6	14.6
28	60.2	60.2	181.6	176.6	61.3	62.8	63.4	60.2	64.2	60.2	181.6
							(172.5 ^a)				
29	133.2	133.6	109.7	109.5	109.5	109.8	133.7	133.6	133.6	133.6	109.7
30	194.9	194.6	19.3	18.3	18.3	18.1	194.6	194.6	194.6	194.6	19.3

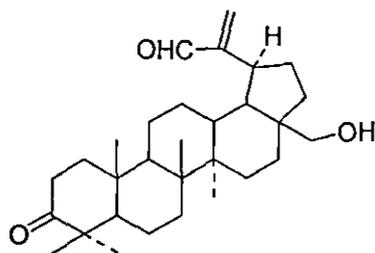
*Represents that reported in literature, ^a represents the chemical shift position of the diacetate

Thus the presence of C-30 aldehyde and C₂₀-C₂₉ double bond is obvious from the above ^{13}C and ^1H NMR spectra of the compound **X**. However, the shift in δ values for carbons C-12, C-13, C-17, C-18, C-19 and C-21 (Table 1) in compound **X** in comparison to that observed for betulonic acid⁴¹ and lupeol,⁵⁷ may be either because of the isomeric nature of the attached isopropenyl group of the cyclopentane ring at C-19 of lupane skeleton, as-observed earlier,⁵⁷ or because of the introduction of C-30 -CHO group during the SeO_2

oxidation of the C-30 methyl.⁵⁷ At this juncture it is relevant to mention that the ¹³C signals for the above mentioned carbons started appearing in all the compounds (*viz.* compound **45**, **46**, **47** and **48**) once the SeO₂ oxidation step was performed. The shifts in NMR spectrum and possible epimerization of the side chain during the SeO₂ oxidation are already reported in literature from our laboratory⁵⁷ and the existence of such a conformational isomer is also documented.⁵⁹ However, in the present case, we could not get the isomeric peaks for the relevant carbons in the ¹³C NMR of **28**, **45**, **46**, **47** and **48** observed by the previous workers.^{57,59} Therefore, on the basis of the above spectral analysis the structure of compound **X** has been assigned as 28-hydroxy-3-oxolup-20(29)-en-30-al (**28**) or **49** (Fig 2).

Further confirmation of the stereochemistry at C-19 was settled by carrying out the 2D NMR techniques on compound **45** (first compound after the SeO₂ oxidation) where the probable stereochemical change would occur. The NOSEY spectrum of compound **45** gave significant information about the stereochemistry at C-19. All NOE cross peaks have opposite phase to the diagonal, indicating these arose from positive NOE enhancements as anticipated for a molecule of this size (*M*⁺ 540.4) under ambient conditions. H-19 at δ_H 2.76 (m, 1H) has showed strong correlations (Figure 38) with β-H-12 at δ_H 1.03 (m, 1H), β-H-13 at δ_H 1.66 (td, 1H, *J* = 12.2, 3.6 Hz) and β-H-21 at δ_H 2.18 (m, 1H). The nuclear overhauser effect (NOE) observed in the NOESY spectrum of compound, **6** indicated the involvement of β-H-12 at δ_H 1.03 (m, 1H) and β-H-13 at δ_H 1.66 (td, 1H, *J* = 12.2, 3.6 Hz) with β-H-21 at δ_H 2.18 (m, 1H) respectively. These observations may be explained by considering the H-19 configuration as β as depicted in figure 38. Additionally, β-H-13 at δ_H 1.66 (td, 1H, *J* = 12.2, 3.6 Hz) showed positive NOE effect with β-H-12 at δ_H 1.03 (m, 1H) (Fig 2). From these data it can be concluded that the original stereochemistry at C-19 was retained during SeO₂ oxidation on **44** and the chemical shift values for C-12, C-13, C-17, C-18, C-19 and C-21 in ¹³C NMR spectrum of subsequent oxidized products with respect to that for lupane skeleton are due to the angularly dependent through space effects, such as the anisotropic magnetic susceptibility and /or electric field effect,³⁶ offered by the conjugated carbonyl group. The through space distance is obviously more important than the number of intervening bonds since the chemical shift changes are much smaller for C-13 than for C-12, which is one

bond further away but closer in space.⁵⁹ SeO₂ oxidation of compound **44** to **45** may only be considered as the step where the stereochemical change at C-19 would have occurred, since in all other subsequent steps no reaction was carried out that can alter the stereochemistry at C-19. Therefore, the stereochemistry at C-19 would be the same as **45** for all the subsequent molecules (**46**, **47**, **48** and **28**) derived from it.



49

Figure 37 Epimeric form of compound **28**

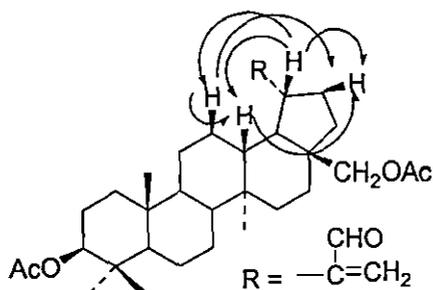


Figure 38 Key NOESY correlation of compound **45**

Therefore, from the above all spectral evidences, it can be concluded that the present author have synthesized the titled compound effectively from the naturally abundant betulinic acid.

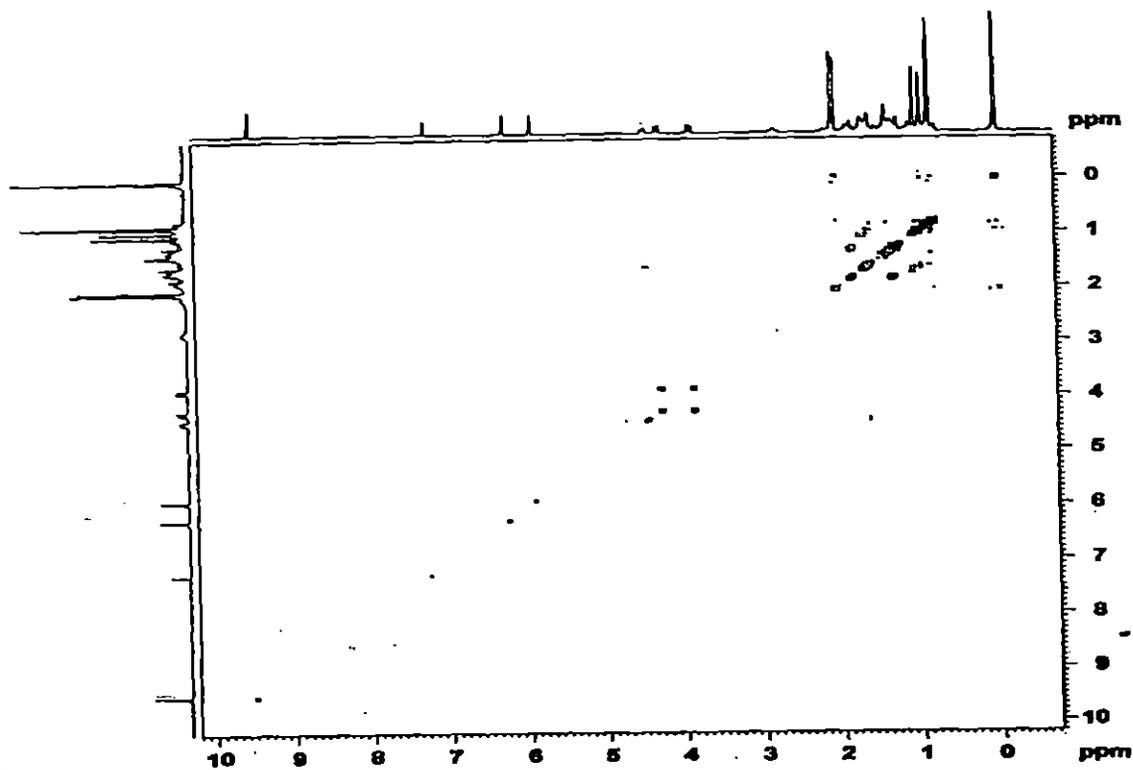


Figure 39 COSY spectrum of compound 45

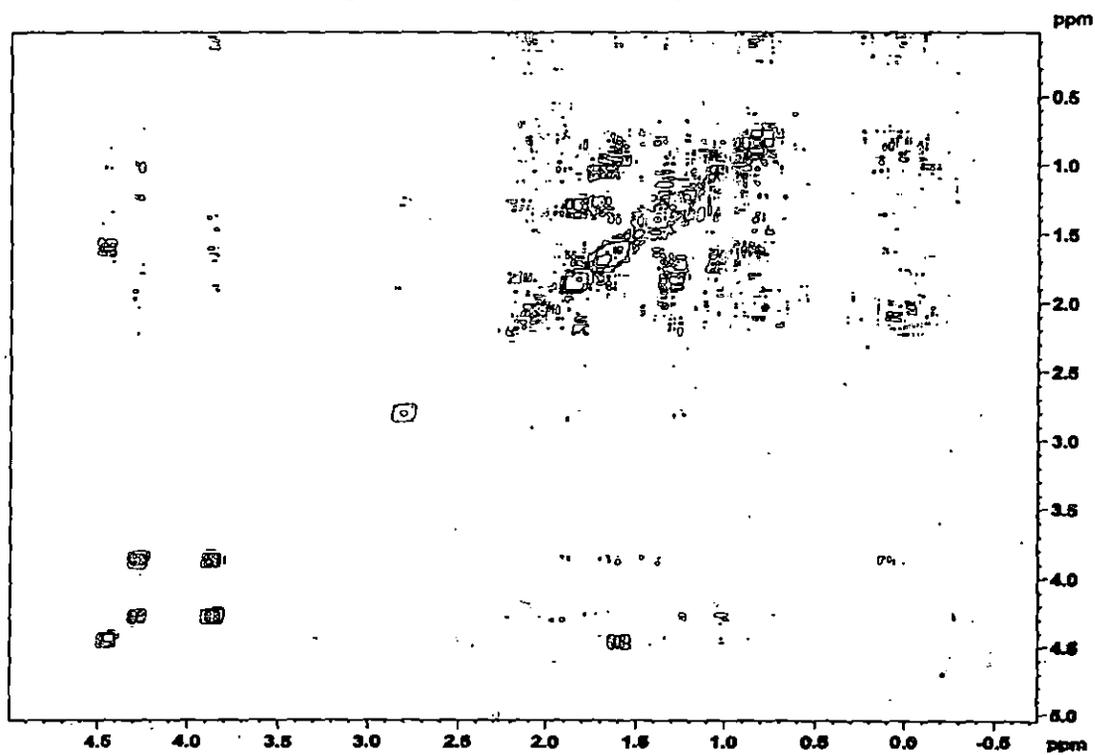


Figure 40 Expanded COSY spectrum of compound 45

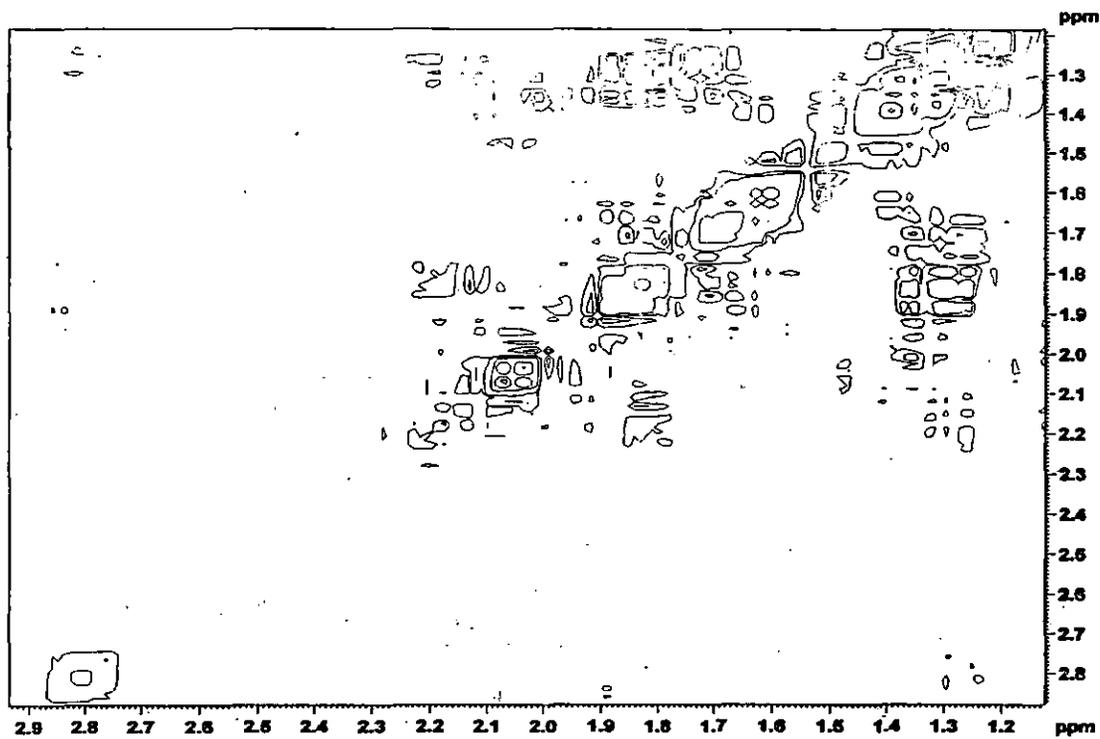


Figure 41 Expanded COSY spectrum of compound 45

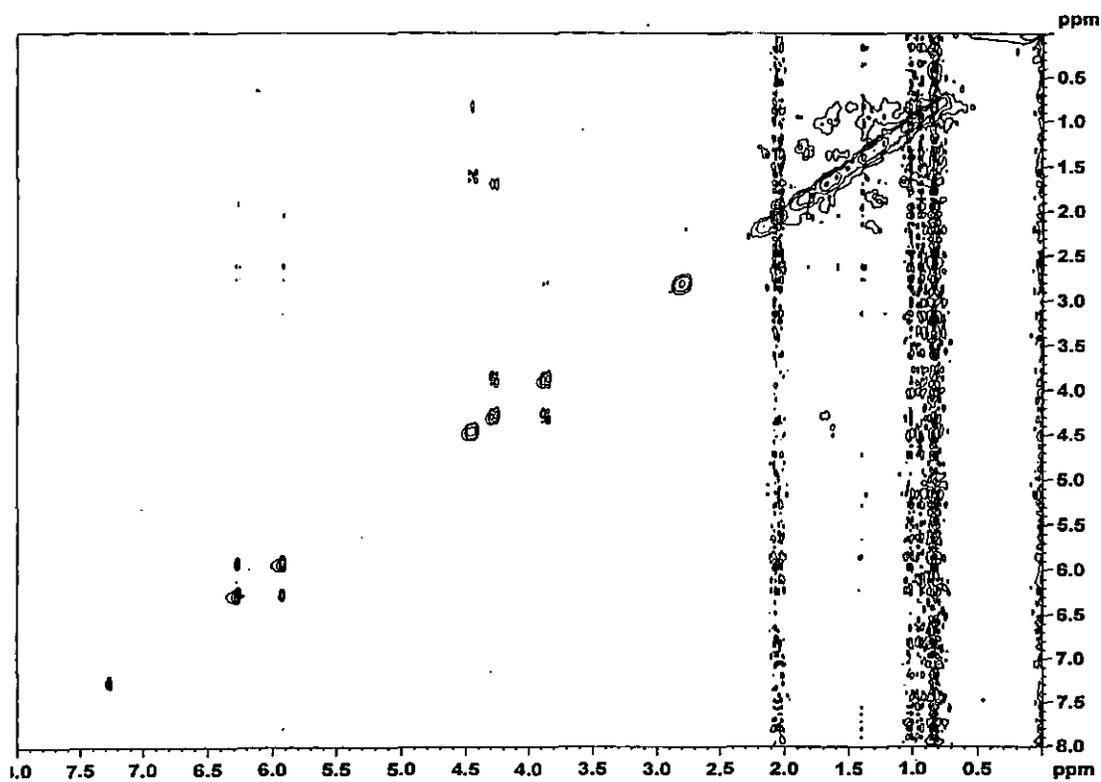


Figure 42 NOESY spectrum of compound 45

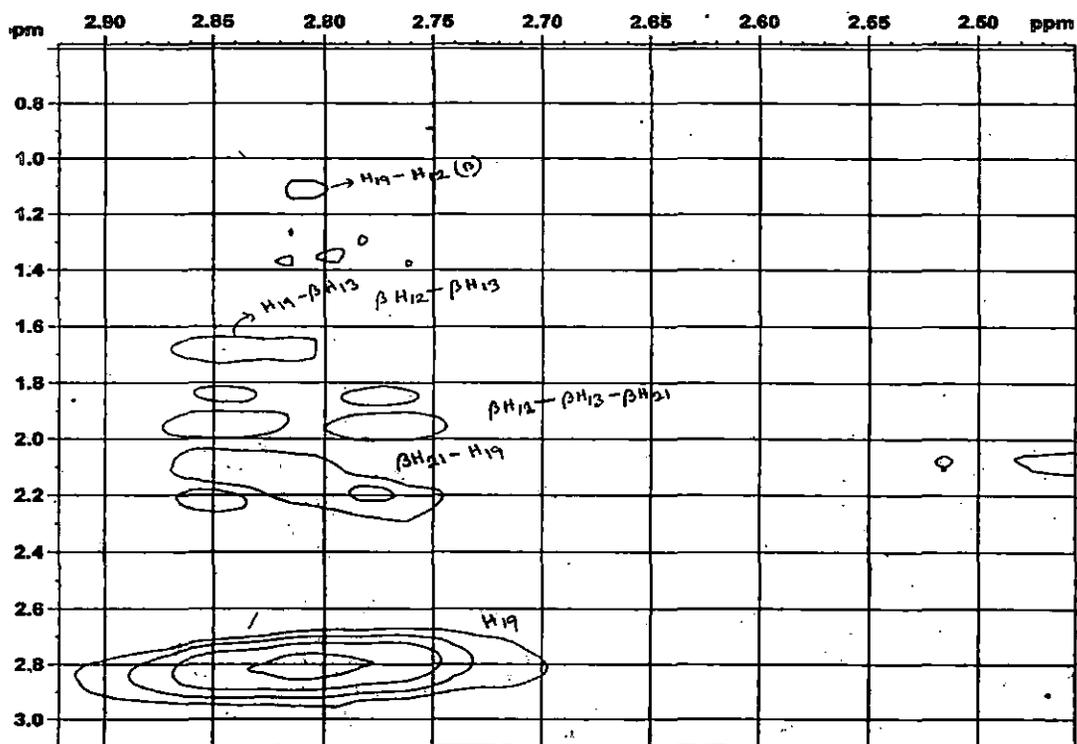


Figure 43 Expanded NOESY spectrum of compound 45

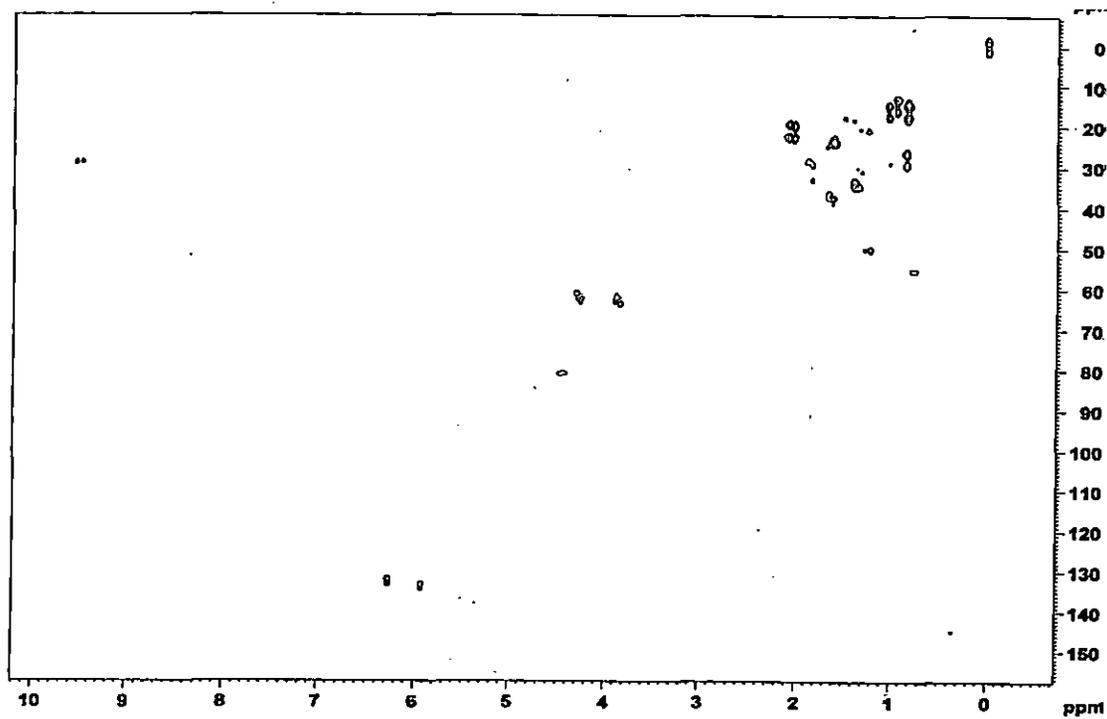


Figure 44 HMQC spectrum of compound 45

The synthesized compounds were then studied for their antileukemic activity against three different cell lines *viz.* human K562 leukemia, murine WEHI3 leukemia and murine MEL erythroid progenitor.

Section B

Antileukemic activities of different compounds

1. Introduction

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of white blood cells. Leukemia is a broad term covering a spectrum of diseases. In turn, it is part of the even broader group of diseases called hematological neoplasms. In 2000, approximately 256,000 children and adults around the world developed some form of leukemia, and 209,000 died from it.⁶⁰ About 90% of all leukemias are diagnosed in adults. Most forms of leukemia are treated with pharmaceutical medications, typically combined into a multi-drug chemotherapy regimen. Some are also treated with radiation therapy. In some cases, a bone marrow transplant is useful. All these treatments are useful but only to a very limited extent and the chemotherapeutics used to date are not specific to the affected cells, thus causing severe damage to the body. Despite recent improvements in the treatment of early-stage disease, leukemia blast crisis remains a therapeutic challenge because it is highly refractory to standard induction chemotherapy, with a response rate in myeloid blast crisis of less than 30%.⁶¹ Therefore, development of mild selective chemotherapeutics is a real demand in contemporary medical sciences.

2. Present investigation

In the present study, compounds were subjected to cytotoxic assay against human K562 leukemia, murine WEHI3 leukemia and murine MEL erythroid progenitor and the assays were performed in three independent experiments as per the guidelines of biosafety committee of West Bengal State University, West Bengal, India. (Figures 45, 46 and 47). To determine whether the compounds had any effect on cell lines, cell cultures were incubated with various concentrations of compounds (dissolved in 0.1% v/v DMSO). DMSO had no effect on the growth of cell lines at a final concentration of 0.1% (v/v). The compounds **28**, **1**, **43**, **44** and **46** had differential effects on the growth of the cell lines. The effect of **47** and **45** could not be checked due to its poor solubility in DMSO.

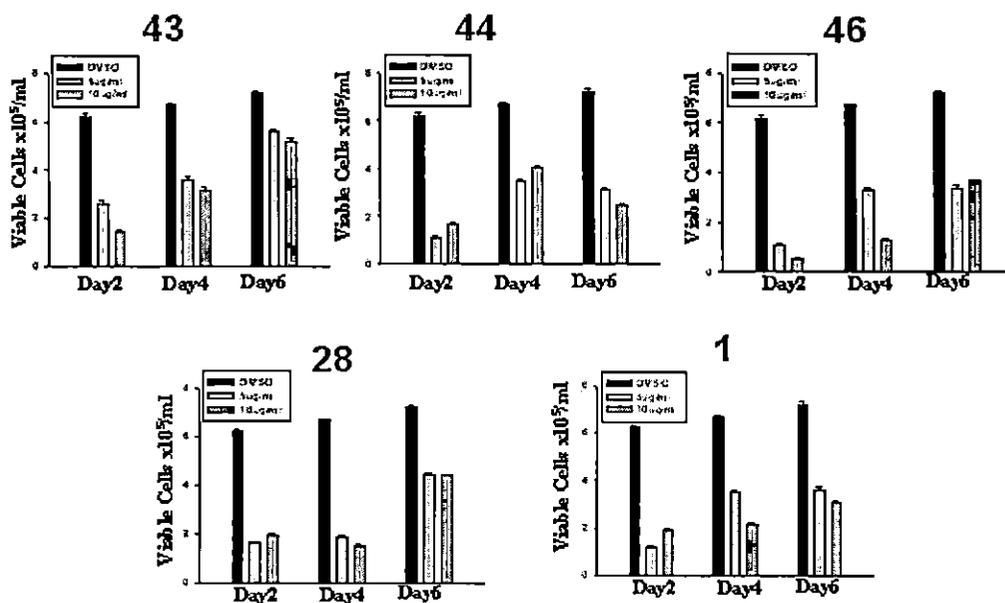


Figure 45 Anti-proliferative effect on WEHI3 Cells

All the compounds showed potent activities against the entire cell lines used although; the activity against murine MEL erythroid progenitor was not as good as for other two cell lines (Table 4, Fig 5). However, 1 was the most cytotoxic against murine MEL erythroid progenitor. The antiproliferative effect of all the compounds helped us to predict some the structure activity relationship. A comparison of bioassay data between 2 and 3 revealed that compound 2 was more active than 3 against human K562 leukemia, murine WEHI3 leukemia.

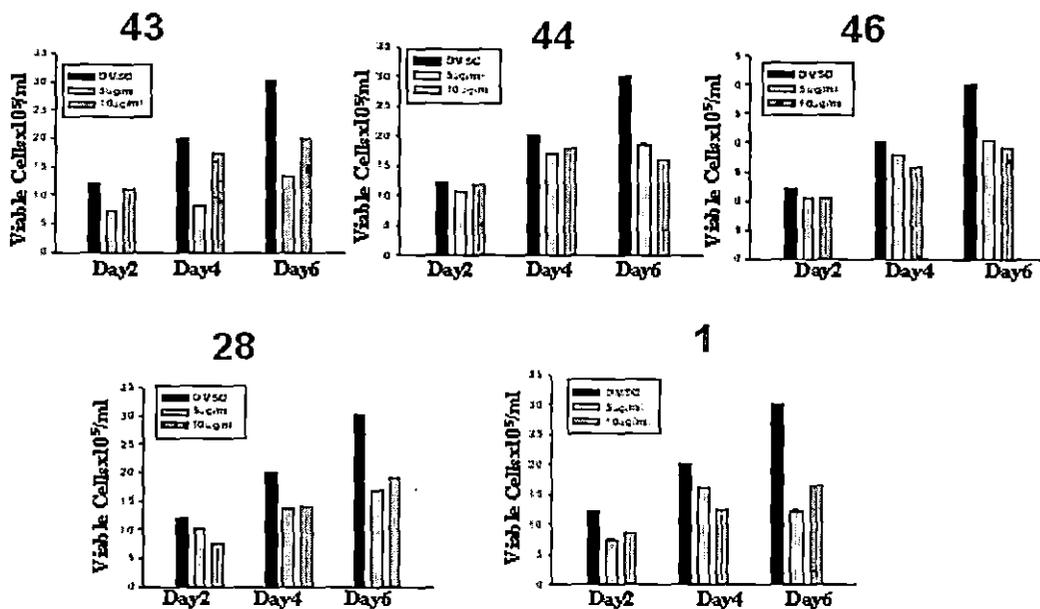


Figure 46 Anti-proliferative effect on MEL Cells

The only difference between their structures was at C-28. Compound 2 had a $-\text{COOH}$ group and 3 had $-\text{COOCH}_3$ group i.e. more polar grouping is necessary ($-\text{COOH}$) to have high activity. Incorporation of aldehyde group at C-30 had increased the activity further. The study also revealed that against human K562 leukemia and murine WEHI3 leukemia cell lines 7 showed better activities than that for 1. A closer look on the structures of these two compounds revealed that they had a difference in their structure only at C-3 and the data indicated that $-\text{OH}$ group at C-3 was the preferred one for having higher activity.

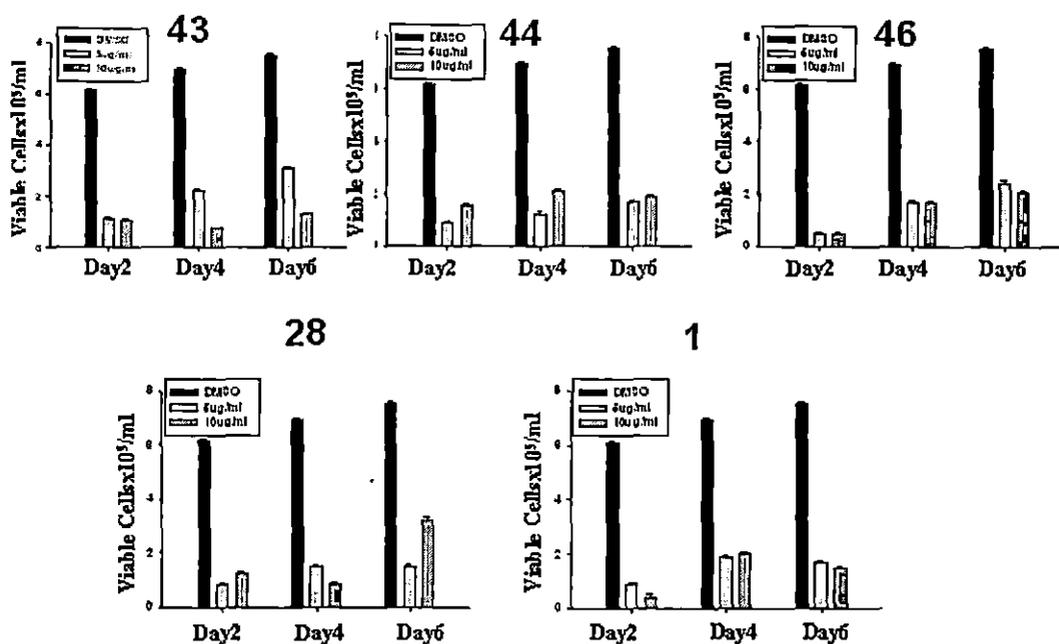


Figure 47 Anti-proliferative effect on K562 Cells

The in detail antiproliferative activities of different compounds were presented in tabular form also.

Table 2 Anti-proliferative effect on K562 cells

Experimental parameter	% of Inhibition P (students' <i>t</i> test)		
	Day 2	Day 4	Day 6
28 Control vs 5µg / mL	85.97% P < 0.0006	78.93% P < 0.0001	80.61% P < 0.0004
28 Control vs 10 µg / mL	79.44% P < 0.0007	87.59% P < 0.004	57.50% P < 0.0009
1 Control vs 5µg / mL	85.97% P < 0.0006	73.16% P < 0.0006	77.95% P < 0.0005
1 Control vs 10 µg / mL	93.47% P < 0.0005	70.70% P < 0.0004	80.07% P < 0.00003
43 Control vs 5µg / mL	82.05% P < 0.0003	68.25% P < 0.0007	58.83% P < 0.0007
43 Control vs 10 µg / mL	82.40% P < 0.00004	89.03% P < 0.00002	82.73% P < 0.0003
44 Control vs 5µg / mL	85.97% P < 0.0006	82.68% P < 0.0005	77.95% P < 0.0005
44 Control vs 10 µg / mL	75.04% P < 0.001	70.27% P < 0.0007	75.29% P < 0.0005
46 Control vs 5µg / mL	92.49% P < 0.0001	76.04% P < 0.0006	68.12% P < 0.0006
46 Control vs 10 µg / mL	92.49% P < 0.0005	75.46% P < 0.00004	72.64% P < 0.0005

Table 3 Anti-proliferative effect on WEHI 3cells

Experimental parameter	% of Inhibition P (students' <i>t</i> test)		
	Day 2	Day 4	Day 6
28 Control vs 5µg / mL	74.19% P < 0.0001	72.68% P < 0.00004	38.88% P < 0.001
28 Control vs 10 µg / mL	68.87% P < 0.0009	77.61% P < 0.001	38.88% P < 0.0004
1 Control vs 5µg / mL	80.64% P < 0.0001	47.76% P < 0.001	50.00% P < 0.001
1 Control vs 10 µg / mL	68.87% P < 0.0009	68.65% P < 0.0001	56.94% P < 0.0005
43 Control vs 5µg / mL	58.06% P < 0.001	46.26% P < 0.0003	22.22% P < 0.005
43 Control vs 10 µg / mL	77.41% P < 0.0005	52.23% P < 0.0008	27.77% P < 0.001
44 Control vs 5µg / mL	82.25% P < 0.0001	47.76% P < 0.0009	56.94% P < 0.0005
44 Control vs 10 µg / mL	72.58% P < 0.0001	38.80% P < 0.001	65.25% P < 0.0004
46 Control vs 5µg / mL	82.25% P < 0.0003	50.74% P < 0.001	52.77% P < 0.001
46 Control vs 10 µg / mL	91.93% P < 0.0003	81.19% P < 0.0002	48.61% P < 0.0008

Table 4 Anti-proliferative effect on MEL cells

Experimental parameter	% of Inhibition P (students' <i>t</i> test)		
	Day 2	Day 4	Day 6
28 Control vs 5µg / mL	16.85% P < 0.001	31.90% P < 0.001	44.31% P < 0.000006
28 Control vs 10 µg / mL	38.01% P < 0.0001	30.05% P < 0.00003	36.54% P < 0.00008
1 Control vs 5µg / mL	38.01% P < 0.0001	19.94% P < 0.001	59.03% P < 0.000003
1 Control vs 10 µg / mL	28.42% P < 0.00009	37.53% P < 0.0003	45.18% P < 0.00007
43 Control vs 5µg / mL	39.42% P < 0.0006	59.62% P < 0.0001	55.71% P < 0.000003
43 Control vs 10 µg / mL	8.26% P < 0.001	12.61% P < 0.002	33.22% P < 0.00003
44 Control vs 5µg / mL	10.49% P < 0.0006	14.95 P < 0.001	37.87 P < 0.00002
44 Control vs 10 µg / mL	1.40% P < 0.02	9.97% P < 0.001	46.17 P < 0.00001
46 Control vs 5µg / mL	14.62% P < 0.004	10.11% P < 0.0002	31.89 P < 0.0001
46 Control vs 10 µg / mL	13.22% P < 0.003	21.93% P < 0.001	36.54% P < 0.0001

3. Conclusion

In Conclusion it can be said that the present author has discovered a simple and effective method for the partial synthesis of 28-hydroxy-lup-20(29)-en-30-al, a rare triterpenoid from betulinic acid, an abundant triterpenoid of lupane skeleton. Besides these chemical works the present author has also done the antileukemic activity of the synthesized triterpenoid, 28-hydroxy-lup-20(29)-en-30-al and also the intermediate derivatives that are soluble in DMSO against three leukemic cell lines, viz. human K562 leukemia, murine WEHI3 leukemia and murine MEL erythroid progenitor. All the tested compounds showed good antiproliferative activity against all the cell lines tested. Expectedly 28-hydroxy-lup-20(29)-en-30-al showed the best results against all the tested cell lines. It is very unfortunate that the present author is unable to evaluate the mechanism of action of these compounds.

Chapter III

EXPERIMENTAL AND REFERENCES

1. Experimental for chemical work

1.1 General

Melting points were recorded in open capillary method and are uncorrected. IR spectra were recorded in Shimadzu 800 FT-IR spectrophotometer as well as in Perkin Elmer FT-IR spectrophotometer using both KBr disc as well as nujol. NMR spectra were recorded in Bruker-Avance 300 MHz FT-NMR instrument at ambient temperature with a 5mm BBO probe. The NMR chemical shift was reported in ppm relative to 7.26 and 77.0 ppm of CDCl₃ solvent as the standards. ¹H spectra were recorded in 300 MHz frequencies and ¹³C NMR spectra were recorded in 75.4 MHz frequencies. Coupling constant 'J' was calculated in Hz. The mass spectra were taken in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer. Elemental Analyses were done in Vario EL-III. Friedelin and cerin was isolated from cork through soxhlet apparatus using petroleum ether (60-80 °C) as the solvent. All the chemicals used were commercial grade and were purified prior to their use.

1.1.1 Extraction from outer bark of *Bischofia javanica*: Isolation of Betulinic acid

The isolation of betulinic acid from the powdered bark of *Bischofia javanica* Linn. is already reported in the experimental part of the previous chapter (Para 1.1.6 in page number 132).

1.1.2 Esterification of betulinic acid, isolation of methyl betulinate (43)

The process of esterification of betulinic acid and the total purification procedure was already stated in the previous chapter (Para 1.1.7 in page number 133).

1.1.3 LAH reduction of methyl betulinate: Isolation of lup-20(29)-en-3 β ,28-diol

2.8 g (5.95 mmol) of methyl betulinate was added to 50 ml of dry THF in a 150 mL round bottom flask. The solution was homogenized and to this LAH (75.28 g, 1.98 mmol) was added in small lots at cold (10-15 °C) and the solution was stirred for 15 minutes. Stirring was continued for another 3 hours at room temperature and saturated solution of sodium sulfate was added drop wise till the excess LAH is destroyed. The reaction mixture was poured in 200 mL cold water and extracted with diethylether and

dried over anhydrous sodium sulfate. Evaporation of solvent gave a residue that was then purified over a column of silica gel (60-120 mesh).

Table 1 Purification of the LAH reduction product of methyl betulinate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-9	Nil	--
2	PE-EA (9.9:0.1)	10-14	Nil	--
3	PE-EA (9.7:0.3)	15-18	Nil	--
4	PE-EA (9.6:0.4)	19-22	Nil	--
5	PE-EA (9.2:0.8)	23-26	Nil	--
6	PE-EA (9.0:1.0)	27-31	Nil	--
7	PE-EA (8.8:1.2)	32-57	White solid (\approx 2.0 g)	256-257 °C --
8	PE-EA (8.7 :1.3)	58-64	Nil	

Further elution with more polar solvent mixture did not afford any solid material

1.1.3.A Characterization of lup-20(29)-en-3 β ,28-diol

All the fractions between 32-57 having the same R_f values were combined and recrystallized as a white crystals of mp 256-257 °C and $[\alpha]_D +16$ (10% v/v MeOH in CHCl_3 ; 0.35 g/ml). The IR spectrum gave peaks at 3393 (-OH), 1453, 1375 (gem dimethyl), 1229, 1029, 942 and 889 ($=\text{CH}_2$) cm^{-1} . $^1\text{H NMR } \delta_{\text{H}}$ 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.37 (s, 3H), 1.68 (s, 3H), 2.43 (m, 1H, H-C-18), 3.84 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.24 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.46 (m, 1H, H-C-3), 4.59 (s, 1H), 4.68 (s, 1H). The compound was found identical with an authentic sample (co-tlc, mixed mp *etc.*).

1.1.4 Acetylation of lup-20(29)-en-3 β ,28-diol: Isolation of lup-20(29)-en-3 β ,28-diyl acetate

1.8 g (4.06 mmol) lup-20(29)-en-3 β ,28-diol was dissolved in 50 mL dry pyridine in a 100 mL round bottom flask and to this 10 mL acetic anhydride was added. The reaction mixture was warmed over water bath for 6 hours. After cooling, it was poured into 150 mL ice cold water and worked up with diethylether. The ether layer was washed with 6N HCl and again with water till neutral. After drying and evaporation of solvent at reduced pressure the residue obtained was purified over a column of silica gel (60-120 mesh).

Table 2 Purification of lup-20(29)-en-3 β ,28-diyl acetate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-7	Nil	--
2	PE-EA (9.9:0.1)	8-27	White solid (\approx 1.6 g)	222-223 °C

Further elution with more polar solvent mixture did not afford any solid material

1.1.4.A Characterization of lup-20(29)-en-3 β ,28-diyl acetate

All the fractions between 8-27 having the same R_f values were combined and recrystallized as a white crystals of mp 222-223 °C and $[\alpha]_D + 2$ (CHCl₃; 0.4 g/ml). The IR spectrum gave peaks at 1735 (-OCOCH₃), 1639, 1459, 1370 (gem dimethyl), 1243, 1027, 979 and 889 (=CH₂) cm⁻¹. Analytical calculation % C 77.46, % H 10.31 (found) and % C 77.52, % H 10.33 (calculated). ¹H NMR δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.59 (s, 3H), 1.65 (s, 3H), 2.43 (m, 1H, H-C-18), 3.84 (d, 1H, J = 11.1 Hz, H-C-28), 4.24 (d, 1H, J = 11.1 Hz, H-C-28), 4.46 (m, 1H, H-C-3), 4.59 (s, 1H), 4.68 (s, 1H). Acetate peaks appeared at δ_H 2.04 (s, 3H) and 2.04 (s, 3H). The compound was found identical with an authentic sample (co-tlc, mixed mp *etc.*).

1.1.5 Selenium dioxide oxidation of lup-20(29)-en-3 β ,28-diyl acetate: Isolation of lup-20(29)-en-3 β ,28-diyl acetate-30-al

lup-20(29)-en-3 β ,28-diyl acetate (1.5 g 2.85 mmol) was dissolved in 10 mL of aqueous dioxin and 20 mL of SeO₂ was added to it. The resultant reaction mixture was refluxed for 2 hours and after cooling poured into 100 mL ice cold water. A curdy white precipitate developed. After usual work up with diethylether, it was dried over anhydrous sulfate and the solvent was evaporated in reduced pressure. The yellow gummy residue obtained was purified over a column of silica gel (60-120 mesh).

Table 3 Purification of the oxidation product of lup-20(29)-en-3 β ,28-diyl acetate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Oil	--
2	PE-EA (9.95:0.05)	9-14	Nil	--
3	PE-EA (9.9:0.1)	15-18	Nil	--
4	PE-EA (9.8:0.2)	19-24	Nil	--
5	PE-EA (9.1:0.9)	25-32	Nil	--
6	PE-EA (9.0:1.0)	33-46	White solid (\approx 1.1 g)	246-248 °C --
7	PE-EA (8.8:1.2)	47-53	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.5.A Characterization of lup-20(29)-en-3 β ,28-diyl acetate-30-al

All the fractions between 33-46 having the same R_f values were combined and recrystallized as a white crystals of mp 246-248 °C. IR ν_{\max} : 1732 (-OCOCH₃), 1691 (>C=C-CHO) 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 889 (=CH₂) cm⁻¹. ¹H NMR: δ_H 0.93 (s, 3H), 0.94 (s, 3H), 1.02 (s, 3H), 1.06 (s, 3H), 1.39 (s, 3H), 6.28 (s, 1H, H-C-29), 5.93 (s, 1H, H-C-29), 4.24 (d, 1H, J = 10.6 Hz, H- C-28) and δ_H 3.68 (d, 1H, J =

10.6 Hz, H- C-28), 9.56 (s, 1H, aldehyde hydrogen at C-30). Two acetyl methyls appeared at δ_H 2.07 (s, 3H) and at 2.03 (s, 3H). C-3 hydrogen appeared as a broad multiplet centered at δ_H 4.44 (m, 1H). Analytical calculation % C 75.42, % H 9.56 (found) and % C 75.51, % H 9.69 (calculated).

1.1.6 Hydrolysis of lup-20(29)-en-3 β ,28-diyl acetate-30-al: Isolation of lup-20(29)-en-3 β ,28-diol-30-al

900 mg (1.66 mmol) of lup-20(29)-en-3 β ,28-diyl acetate-30-al was refluxed with 10% (w/v) alcoholic KOH solution for 4 hours. After completion of the reaction it was poured into 100 mL ice cold water. After usual work up with diethylether, the ether layer was washed several times with water, dried over anhydrous sodium sulfate and the recovered material was purified over silica gel (60-120 mesh) chromatography.

Table 4 Purification of the hydrolysis product of lup-20(29)-en-3 β ,28-diyl acetate-30-al

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Oil	--
2	PE-EA (9.95:0.05)	9-14	Nil	--
			Nil	--
3	PE-EA (9.9:0.1)	15-18	Nil	--
4	PE-EA (9.8:0.2)	19-24	Nil	--
5	PE-EA (9.1:0.9)	25-32	Nil	--
6	PE-EA (9.0:1.0)	33-46	Nil	--
7	PE-EA (8.5:1.5)	47-54	Nil	--
8	PE-EA (8.0:2.0)	55-72	White solid (\approx 0.72 g)	274-276 °C

Further elution with more polar solvent mixture did not afford any solid material

1.1.6A Characterization of lup-20(29)-en-3 β ,28-diol-30-al

All the fractions between 55-72 having the same R_f values were combined and recrystallized as a white crystals of mp 274-276 °C, IR ν_{max} : 3393 (-OH), 1688 (>C=C-

CHO), 1453, 1375 (gem dimethyl), 1029, 942, 890 (=CH₂) cm⁻¹. ¹H NMR: δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.95 (s, 3H), 1.00 (s, 3H), 1.38 (s, 3H), 2.62 (m, 1H, hydrogen at C-3), 3.15 (m, 1H, proton of-OH at C-3), 3.38 (d, 1H, J = 10.6 Hz, H-C-28), 3.83 (d, 1H, J = 10.6 Hz, H-C-28), 5.94 (s, 1H, H-C-29), 6.32 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30). Analytical calculation % C 78.66, % H 10.52 (found) and % C 78.90, % H 10.59 (calculated).

1.1.7 Selective acetylation of lup-20(29)-en-3β,28-diol-30-al: 3β-hydroxy-28-acetoxy lup-20(29)-en-30-al

A solution lup-20(29)-en-3β,28-diol-30-al (350 mg, 0.76 mmol) in CHCl₃ (10 mL) and pyridine (15 mL) was treated with Ac₂O (10 mL, 0.098 mmol) at very low temperature (0-5 °C) for 12 hours. The solution was diluted with CHCl₃ (20 mL), the organic layer was washed with saturated aq. NaCl (10 mL x 3), dried, concentrated, purified by silica gel (60-120 mesh) chromatography.

Table 5 Purification of the crude extract of cork, isolation of friedelin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-10	Nil	--
2	PE-EA (9.95:0.05)	11-14	Oil	--
3	PE-EA (9.95:0.05)	15-19	White solid (Traces)	--
4	PE-EA (9.95:0.05)	20-24	Nil	--
5	PE-EA (9.95:0.05)	24-72	White solid (≈ 0.32 g)	260-262 °C

Further elution with more polar solvent mixture did not afford any solid material

1.1.7.A Characterization of 3β-hydroxy-28-acetoxy-lup-20(29)-en-30-al

All the fractions between 24-27 having the same R_f values were combined and recrystallized as a white crystals of mp 260-262 °C, IR ν_{max}: 3461 (-OH), 1732 (-

OCOCH₃), 1691 (>C=C-CHO), 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 890 (=CH₂) cm⁻¹. ¹H NMR δ_H 0.75 (s, 3H), 0.80 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.95 (s, 3H), 2.1 (s, 3H, acetate methyl), 3.18 (m, 1H, proton of -OH at C-3), 3.38 (d, 1H, J = 10.6 Hz, H-C-28), 3.83 (d, 1H, J = 10.6 Hz, H-C-28), 5.94 (s, 1H, H-C-29), 6.32 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30). Analytical calculation % C 77.02, % H 10.12 (found) and % C 77.06, % H 10.10 (calculated).

1.1.8 Selective oxidation of 3β-hydroxy-28-acetoxy-lup-20(29)-en-30-al: 28-Acetoxy-3-oxolup-20(29)-en-30-al

3β-hydroxy-28-acetoxy-lup-20(29)-en-30-al (150 mg, 0.30 mmol) was dissolved in dry pyridine (30 mL). 230 mg (2.91 mmol) of dry CrO₃ was added in small lots and the reaction mixture was kept overnight. It was then poured into ice cold water and the resultant yellowish solid was extracted with ether, washed with 6 N HCl and again with water till neutral and dried over anhydrous magnesium sulfate to get a gummy residue. Purification of the gum over a column of silica gel (60-120 mesh) yielded compound.

Table 6 Purification of methyl dihydrobetulonate

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-7	Nil	--
2	PE-EA (9.9:0.1)	8-27	White solid (≈ 0.084 g)	276-278 °C

Further elution with more polar solvent mixture did not afford any solid material

1.1.8.A Characterization of 28-Acetoxy-3-oxolup-20(29)-en-30-al

All the fractions between 24-27 having the same R_f values were combined and recrystallized as a white crystals of mp 276-278 °C. IR ν_{max}: 1730 (>C=O), 1706, 1697 (>C=C-CHO), 1461 1380 (gem dimethyl), 1244, 1163, 987, 890 (=CH₂) cm⁻¹. Analytical calculation % C 77.28, % H 9.62 (found) and % C 77.38, % H 9.74 (calculated). ¹H NMR δ_H 0.75 (s, 3H), 0.80 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.95 (s, 3H), 2.17 (s, 3H, acetate methyl at C-28) 3.38 (d, 1H, J = 10.6 Hz, H-C-28), 3.83 (d, 1H, J = 10.6 Hz, H-C-28),

5.92 (s, 1H, H-C-29), 6.23 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30). Analytical calculation % C 77.28, % H 9.62 (found) and % C 77.38, % H 9.74 (calculated).

1.1.9 Hydrolysis of 28-Acetoxy-3-oxolup-20(29)-en-30-al: Isolation of 28-Hydroxy-3-oxolup-20(29)-en-30-al

75 mg (0.15 mmol) of 28-Acetoxy-3-oxolup-20(29)-en-30-al was refluxed with 10 % alcoholic KOH solution for 4 hours. After completion of the reaction it was poured into 100 mL ice cold water. After usual work up with ether, the ether layer was washed several times with water, dried over anhydrous magnesium sulfate and the recovered material was purified by column chromatography (silica gel, 60-120 mesh).

Table 7 Purification of 28-Hydroxy-3-oxolup-20(29)-en-30-al

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-3	Oil	--
2	PE-EA (9.9:0.1)	4-11	Nil	--
3	PE-EA (9.8:0.2)	12-32	White solid (\approx 0.05 g)	288-290 °C
4	PE-EA (9.7:0.3)	33-39	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.1.9.A Characterization of 28-Hydroxy-3-oxolup-20(29)-en-30-al

All the fractions between 33-39 having the same R_f values were combined and recrystallized as a white crystals of m.p. 288-290 °C. IR ν_{\max} : 3424 (-OH), 1725 (>C=O), 1706, 1697 (>C=C-CHO), 1461 1380 (gem dimethyl), 1163, 987 cm^{-1} . $[\alpha]_D + 16.3$ (MeOH), $^1\text{H NMR}$: δ_{H} 0.91 (s, 3H), 0.93(s, 3H), 1.01 (s, 3H), 1.06 (s, 3H) ans 1.15 (s, 3H), 6.91 (s, 1H, H-C-29), 6.28 (s, 1H, H-C-29), 3.36 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.78 (d, 1H, $J = 10.6$ Hz, H-C-28), 9.52 (s, 1H, aldehyde hydrogen at C-30). $^{13}\text{C NMR}$ spectral data see Table 1. HREIMS: m/z 554.38 $[\text{M}]^+$ (calcd for $\text{C}_{30}\text{H}_{46}\text{O}_3$, 554.34). Elemental analysis: Found: C, 79.18 %, H, 10.21% (found); calcd for $\text{C}_{30}\text{H}_{46}\text{O}_3$: C, 79.25%, H, 10.20%.

1.2 Bioassay

1.2.1 Antileukemic activity of the synthesized compounds

The assays against human K562 leukemia, murine WEHI3 leukemia and murine MEL erythroid progenitor cells were performed in three independent experiments as per the guidelines of biosafety committee of West Bengal States University. K562 and WEHI 3 cells were obtained from cell repository of National Centre for Cell Science, Pune (agreement between two institutes). MEL cells were obtained as gift from Professor Michael H. Kershaw, Cancer Immunotherapy Research, Peter MacCallum Cancer Centre, Australia (through an agreement with Walter and Eliza Hall Institute, Australia). The cells were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mmol glutamine, supplemented with 1% penicillin-streptomycin, and were incubated at 37 °C under 5% CO₂ atmosphere. In these experiments, cells were seeded in quadruplicate in 24-wellplates (10⁵ cells/mL) with the compounds (**28**, **1**, **43**, **44** and **46**) dissolved in DMSO (0.1% v/v) at various concentrations. The effect of **47** and **45** could not be checked due to its poor solubility in DMSO. The cells were incubated for 2, 4 and 6 days. Growth of cells was monitored by counting the number of live cells microscopically using Neubauer haemocytometer by trypan blue exclusion method.⁶² Statistical analyses for all experiments were performed by Student's *t*-test with the program SigmaPlot.

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Part IV

**Oxidative Transformation of Cerin and Friedelin
with 3D Molecular Docking and Antitopoisomerase
Activity**

Chapter I

Transformative reactions on friedelan triterpenoids

1. Introduction

The constituents of cork have been the subject of many researches for the past 150 years, and over this period a considerable number of pure substances have been isolated and characterized. The scientific literature on friedelin, 3-keto derivative of the hydrocarbon friedelan (Figure 1) is widely scattered. Kuloshreshtha *et al.*¹ reviewed the occurrence of triterpenoids for the period 1963-70. However in that review there were few listing of friedelan related compounds.

Friedel² according to Karrer³ first isolated friedelin, however as much earlier reference is provided by Elsevier to Chevreul who obtained substances from cork which he called “Cerine”.³ In 1889 Istrati and Ostrogovich reported that cerine was actually composed of two different compounds, one they name friedelin in honour of their friend Friedel and the other as cerin.³

After the work of Karrer, almost 40 years later Drake and Jacobson⁴ had repeated the isolation of friedelin and cerin and determined their empirical molecular formula. For the first time they claimed that both friedelin and cerin have the same skeleton (Figure 1) and friedelin has a keto group and cerin a hydroxyl ketone.

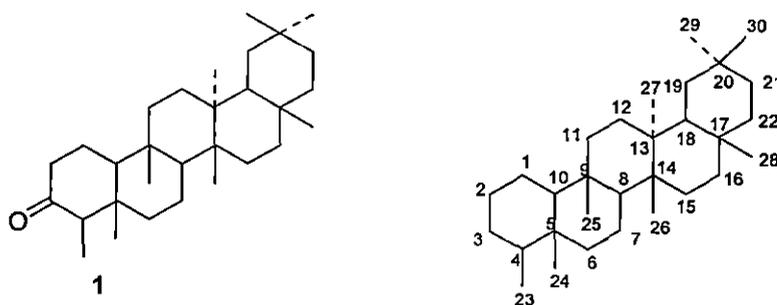
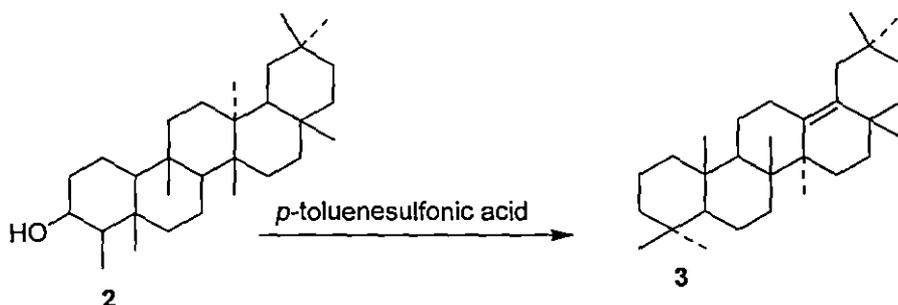


Figure 1 Structure of friedelan skeleton and friedelin, 1

Efforts to elucidate the structure and confirmation were based initially on the preparation of derivatives and their comparison with the known compounds. Most modern physical

method was yet to be developed, but Drake and Wolfe⁵ investigated the surface films formed by friedelin and cerin. Lander and his coworkers⁶ studied the structure of friedelin by using the dipole moment of cerin, friedelin and various other friedelan isomers. During the period of 1944-49 Ruzicka *et al.*^{7,8} published the results of their attempts to derive the structure of friedelin by some chemical means.^{7,8} However it was 1955, two groups (Ruzicka *et al.* and Corey *et al.*) working independently, arrived at the accepted structure as a result of dehydrogenation studies and the isomerization^{9,10} of friedel-3 β -ol, **2** to olean-13(18)-ene, **3** (Scheme 1).

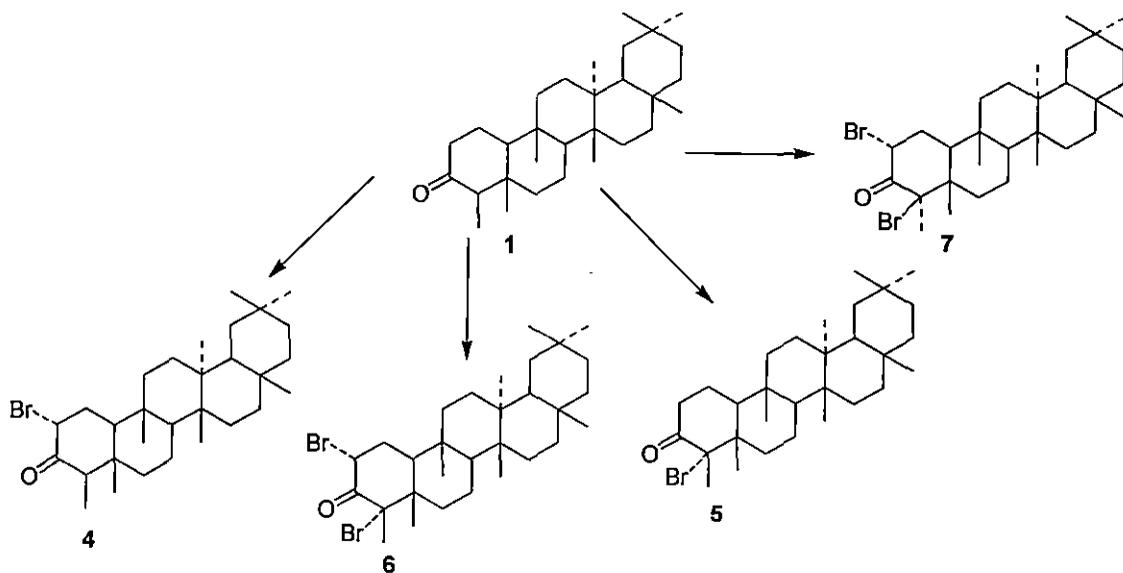


Scheme 1 Acid catalyzed conversion of friedelan to olean skeleton

The rearrangement also proceeds well with Δ^3 -friedelene and somewhat less readily with Δ^2 -friedelene, the optimum results (55% yields) so far having been obtained from friedelan-3 β -ol and hydrogen chloride in phenol at 110 °C. Further studies to friedelan-olean rearrangement continued with attempts to isolate the intermediated compounds.¹¹ Finally Carl Djerassi and his coworkers confirmed the configuration of friedelin by optical rotatory dispersion studies.¹²⁻¹³ After that several publications appeared dealing with the isolation and purification of friedelin and related triterpenoids. As the whole description of various methods of isolation and purification are beyond the scope of this review, the present author is more concerned to represent the various transformative reactions on friedelin and cerin.

Corey and Ursprung⁹ had shown (Scheme 2) that friedelin on direct bromination gave 2 α -bromo friedelin (**4**) and bromination of appropriate enol benzoate gave the isomeric 4 α -bromo friedelin (**5**). They have also prepared a dibromo friedelin (**6**) in presence of hydrobromic acid in chloroform. The dibromo friedelin has been assigned as 2 α ,4 α -dibromo friedelin from the UV absorption at 332 nm. Carl Djerassi and his coworkers

have synthesized another dibromo friedelin (7) by bromination of 2 α -bromofriedelin in glacial acetic acid. This prepared from dibromo friedelin was found different from that prepared by Corey. From UV absorption data (310.5 nm) they designated the compound as 2 α ,4 β -dibromo friedelin (7).



Scheme 2 Direct bromination of friedelin

Takahashi *et al.* and Ourrison¹⁴ also prepared a dibromo friedelin by the action of bromine in chloroform and acetic acid on friedelin. But they could not assign the structure of this compound although the compound showed UV absorption at 320 nm.

The reaction of friedelin and its derivatives with NBS was studied by Stevenson *et al.*¹⁵ They found that friedelin on treatment with molar equivalent of NBS in carbon tetrachloride gave 4 α -bromofriedelin in satisfactory yield. They also isolated 2 α -bromofriedelin from the 4 α -bromofriedelin by further treatment with bromine in glacial acetic acid. Hence in this reaction isomerization occurred rather than substitution. As was expected from the results, it was found that 4 α -bromofriedelin, $[\alpha]_D = +92^\circ$ was unstable in chloroform-hydrobromic acid, the presumed equilibrium mixture $[\alpha]_D = -75^\circ$ being formed after 24 h. 2 α -bromofriedelin, 4 also gave the same result on similar equilibration.

For obtaining dibromofriedelin, since this route was unsuccessful, an alternative route of treatment of 2 α -bromofriedelin, **4** with NBS was attempted by Stevenson *et al.*¹⁶ Treatment of 2 α -bromofriedelin, **4** with NBS gave an unsaturated monoketone C₃₀H₄₇OBr which showed positive TNM test, indicating thereby the presence of ethylinic linkage (Figure 2). UV and IR spectra of this compound showed that the double bond of this compound was not in conjugated to the carbonyl group and the α -bromine atom retained an axial orientation. Since it was known that acid isomerization of friedel-3-ene (**8**) afforded a mixture of olean-13(18)-ene (**3**) and 18 α -olean-12-ene (**9**),^{16,17} it was assumed that this nonconjugated bromoketone had probably arisen by molecular rearrangement of 2 α ,4 α -dibromoketone intermediate with elimination of hydrobromic acid.

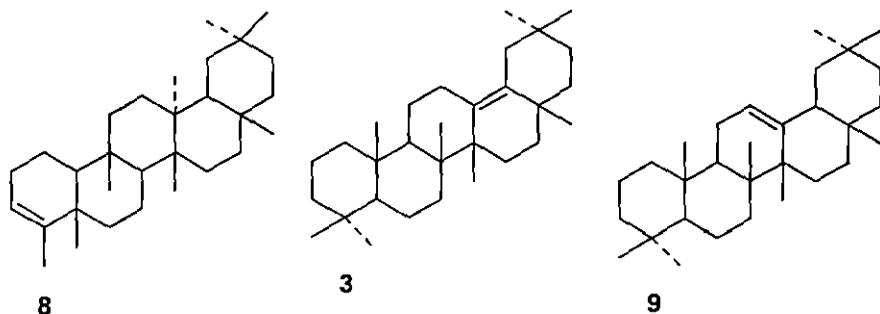


Figure 2 Structures of some different dehydrobrominated products from friedelin

A precedent for such a rearrangement was provided by the action of silver acetate on 4 α -bromofriedelin to yield a product **3** which was shown to be a mixture¹⁸ of alnus-5-ene (**10**) and alnus-5-enone (**11**). The probability that the unsaturated bromoketone derived from 4 α -bromofriedelin could be represented as 2-bromoalnus enone (**12**) was elucidated from the fact that the zinc debromination in neutral solution was different from either alnus enone **10** or **11** (Figure 3).

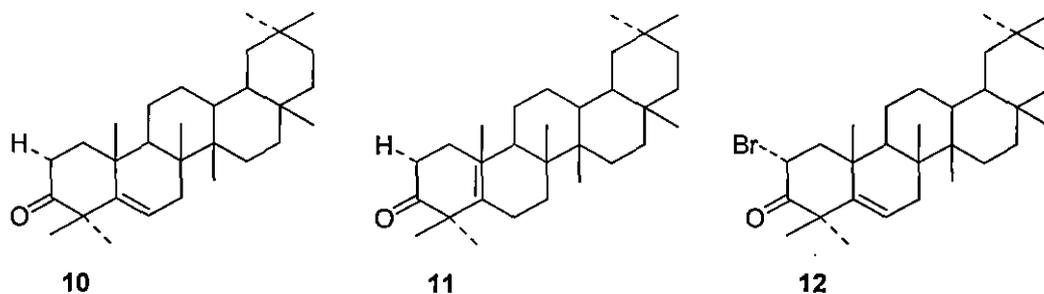
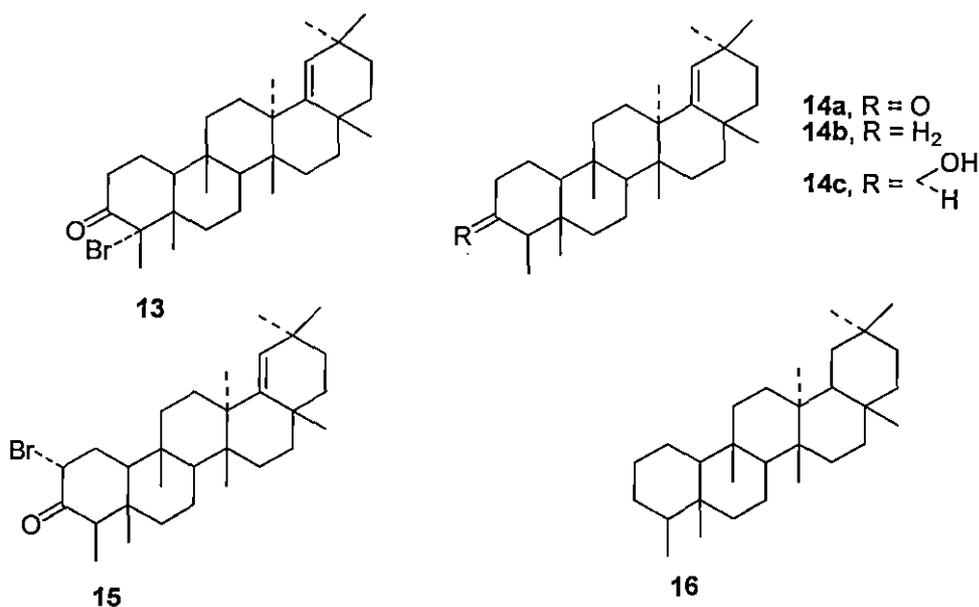


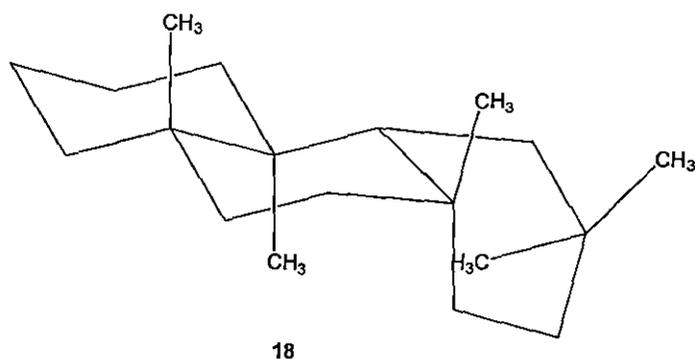
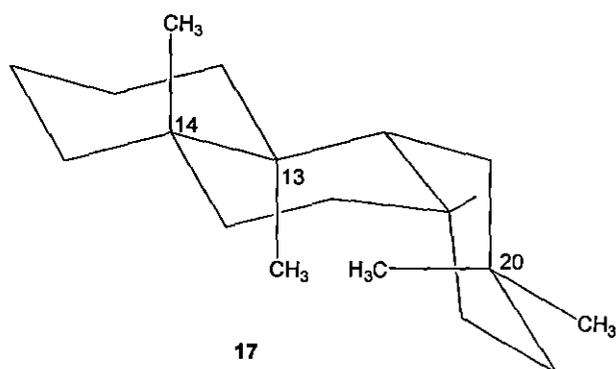
Figure 3 Structures of some alnus derivatives synthesized from friedelin

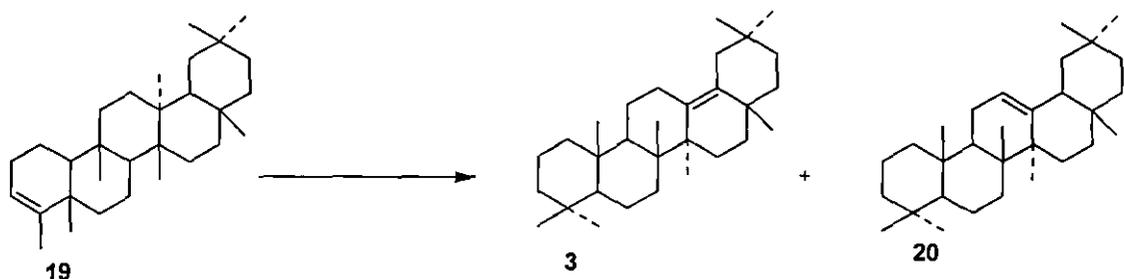
Treatment of 4 α -bromofriedelin, **5** with NBS gave an isomeric non-conjugated axial bromo substituted ketone (**13**) having the molecular formula of $C_{30}H_{47}OBr$ which on dehydrobromination gave the identical ketone $C_{30}H_{48}O$ (**14**). LAH reduction of **14a** gave an alcohol **14c** and on Hung-Minlon reduction gave the hydrocarbon **14b**. From these observations the isomeric monobromoketones obtained from 2 α -bromofriedelin and 4 α -bromofriedelin was assigned structures 2 α -bromofriedel-18-en-3-one, **15** and 2 α -bromofriedel-18-en-3-one, **13** respectively. These assignments were also supported by specific rotation and ORD studies.



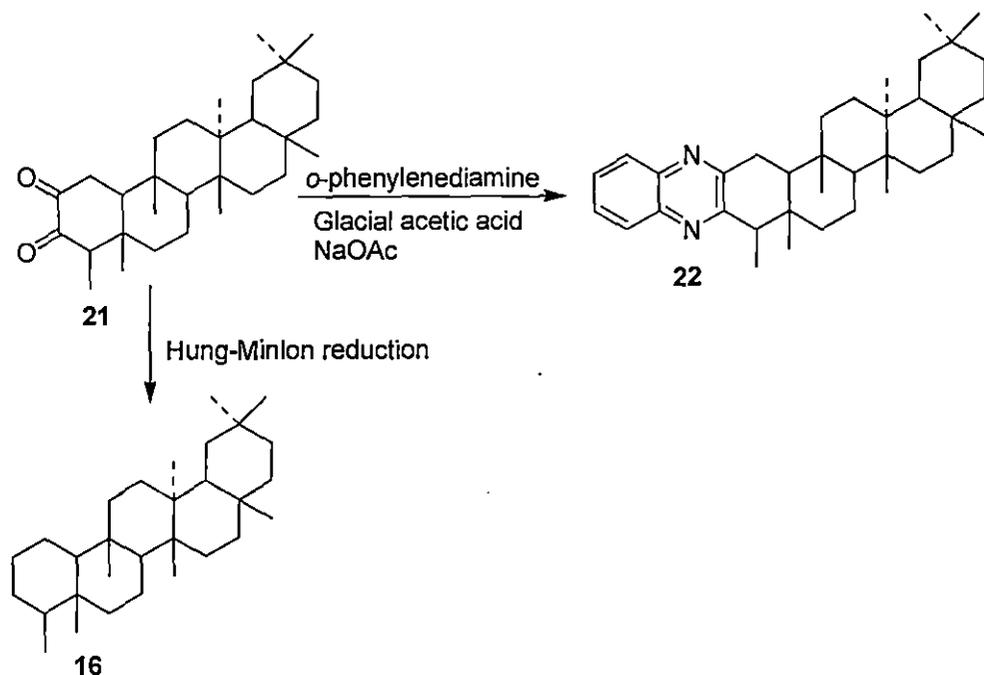
The experiments on friedelin and related triterpenoids as described by Kane and Stevenson established that in friedelin the tertiary axial α -hydrogen atom at position C-4 is more reactive towards NBS than the secondary hydrogen atoms at position C-2, but

that the presence of a 2α -bromine atom effectively prevents abstraction of the 4α -hydrogen atom by its 1:3-diaxial blocking effect to the approach of a succinimide radical. In absence of the activating C-3 carbonyl group or where there is deactivation due to the steric influence of the neighbouring axial halogen, the most reactive hydrogen is the tertiary C-18 atom. An examination of an all chair form (17) of friedelan shows that, as a consequence of the *cis*-junction of D and E rings, a severe steric interaction must exist between the 13α - and 20α -methyl groups. Whereas this interference is removed if the terminal ring E adopts a boat conformation (18), an unfavorable 1:4-diaxial "boat prow-and-stern" methyl interaction is a consequence. The steric strain inherent in both conformations of this *cis* D/E system is relieved by dissociation of the 18β -hydrogen atom and formation of the ethylenic trigonal system. The driving force for the backbone rearrangement of friedelene (19) to the oleanenes 3 and 20 can be attributed also to the same steric interactions.





V. V Kane and R. Stivenson¹⁹ in their continuous work on cork triterpenoid isolated friedelan-2,3-dione from cork smoker wash solid. The structure of this triterpenoid was established following the results of some chemical transformation. Hung-Minlon reduction of the isolated derivative gave exclusively the hydrocarbon friedelan readily identified by direct comparison with an authentic specimen and consequently restricted the possible formulations to friedelan-1,2-dione, friedelan-6,7-dione or friedelan-2,3-dione. A decision in favor of friedelan-2,3-dione, **21** was reached by conversion to the diketone to friedelan-3-one. For a final verification (Scheme 3) about their proposed structure the workers synthesized the quinoxaline derivative of friedelin under refluxing condition in glacial acetic acid in presence of sodium acetate. The ready formation of the quinoxaline derivative (**22**) (Scheme 3) firmly established the exact structure of the isolated triterpenoid that have two carbonyl groups at C-2 and C-3 positions of the pentacyclic ring structure.



Scheme 3 Transformation of 1,2-friedelin by Kane

Pradhan *et al.*²⁰ studied the reaction of NBS in DMSO on friedelin (Figure 4) and observed that it gave a mixture of five different products. From the spectral as well as chemical evidences they designated the compounds as 2 α ,23-dibromofriedelin (**23**), 2 β ,23-dibromofriedelin (**24**), 2 α -bromofriedelin, 2,2-dibromofriedelin, **25** and 2,3-diketo friedelin, **21**.

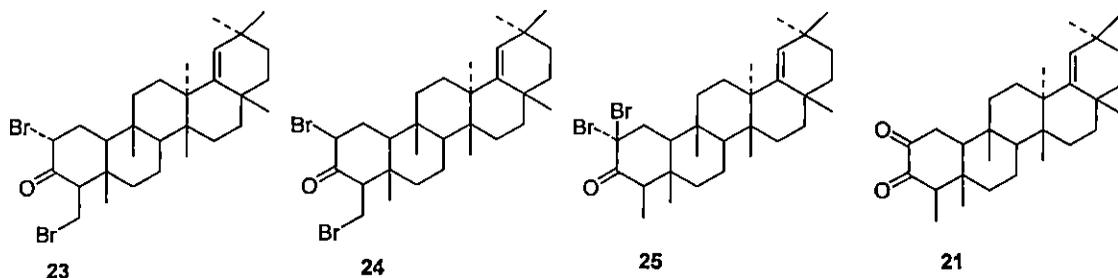
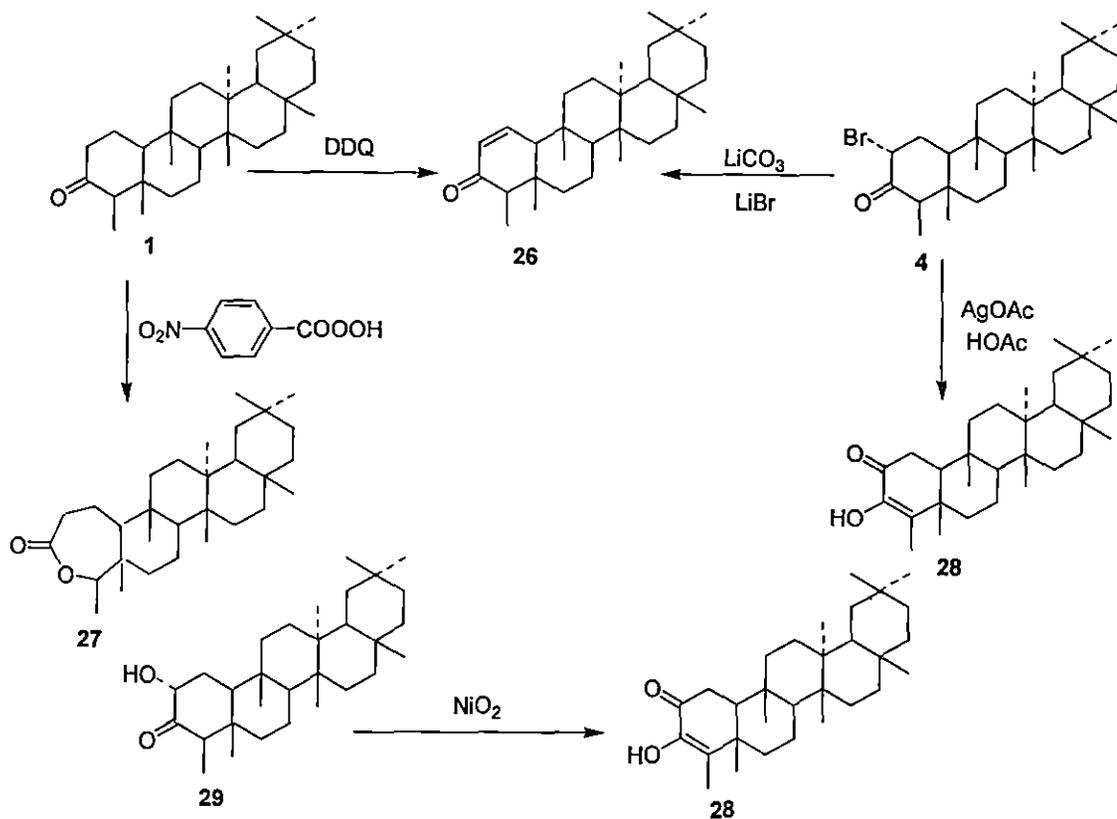
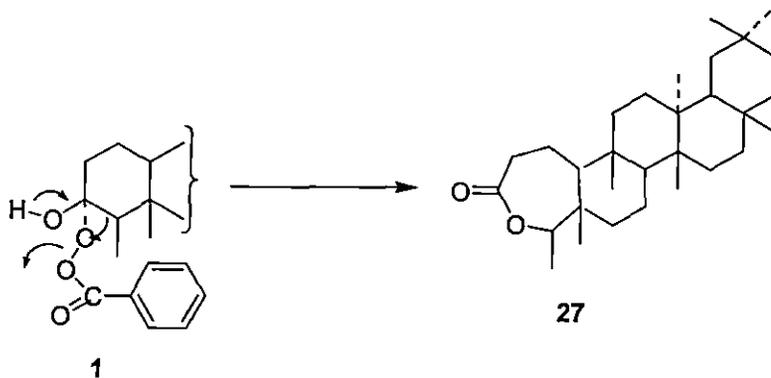


Figure 4 Products of NBS bromination of friedelin in DMSO by Pradhan *et al.*

Talapatra *et al.*²¹ studied some oxidative transformation on friedelin (Scheme 4). DDQ dehydrogenation of friedelin afforded friedel-1-ene-3-one in a very low yield (9%). Treatment of 2 α -bromofriedelin with LiBr and LiCO₃ furnished friedel-1-ene-3-one (20%) along with friedelin (15%). Reaction of 2 α -bromofriedelin with AgOAc and glacial acetic acid leads to the formation of 3-hydroxyfriedel-3-ene-2-one (66%). Baeyer Villiger oxidation of friedelin with *p*-nitroperbenzoic acid produced the ϵ -lactone with 41% yield while NiO₂ oxidation of cerin afforded 3-hydroxyfriedel-3-ene-2-one and 3 α -hydroxyfriedel-3-ene-2-one with 15%. They have also suggested a mechanism for their formation (Scheme 5).



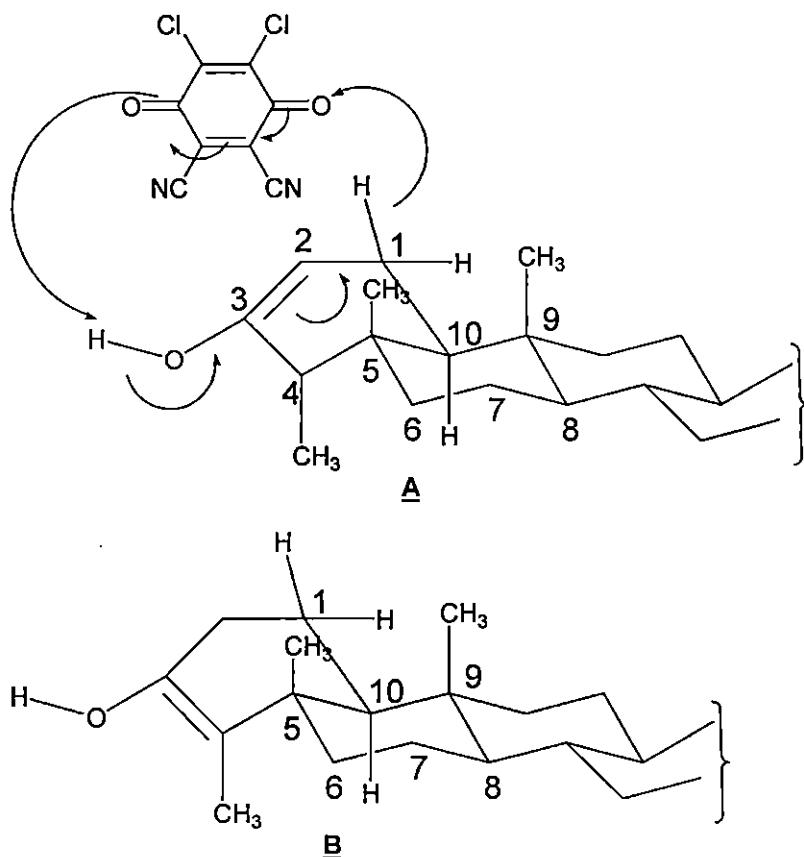
Scheme 4 Transformative reactions on friedelin and cerin by Talapatra *et al.*



Scheme 5 Proposed mechanism of formation of the ϵ -lactone by Talapatra *et al.*

Additionally, they have suggested an explanation (Scheme 6) to the observed low yield of friedelin-1-ene-3-one by DDQ dehydrogenation of friedelin. According to them of the two enols **A** and **B** that friedelin can form, the population of **A** is much lower, **B** being more stabilized by the inductive effect of 4-Me and the pseudo-axial orientation of 5-Me,

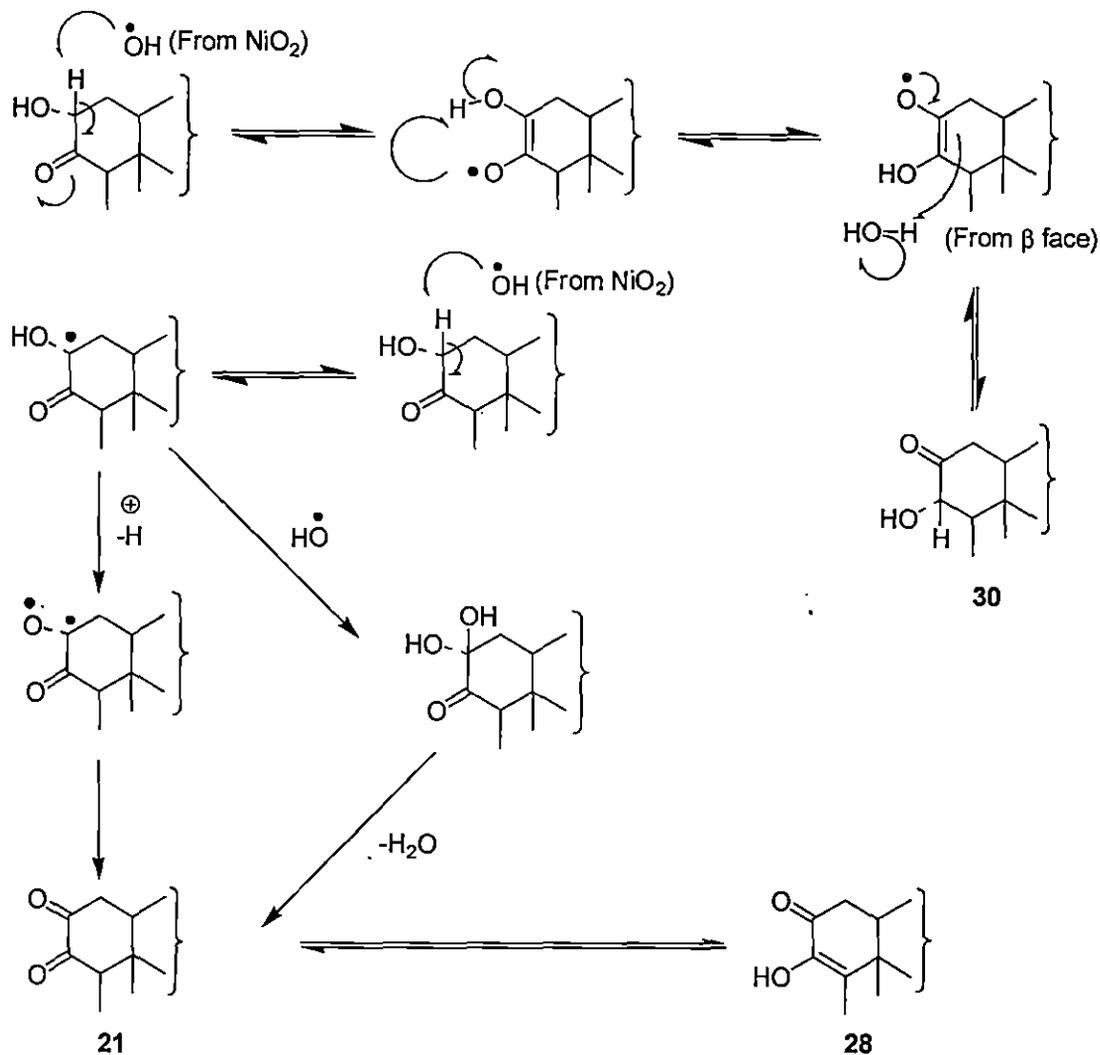
thereby alleviating the steric interaction with syn-axial 9-Me and 1 β -H to some extent. Enolization is the first step for this dehydrogenation and the product is formed from the enol by the transfer of the pseudo-axial γ -H to the quinone maintaining continuous overlap of the π -orbitals of the double bond being formed with the remainder of the conjugated system. Thus the stable enol **B** is inert toward DDQ as it does not possess any pseudo-axial γ -H at C-5. On the contrary the less stable enol **A** possesses a pseudo-axial γ -H at C-1 sufficiently hindered due to the syn-axial 5-Me and 9-Me and hence its transfer to the quinone involves high activation energy resulting in a low yield of friedel-1-ene-3-one.



Scheme 6 Proposed mechanism for the dehydrogenation reaction by DDQ by Talapatra *et al.*

The naturally occurring cerin on being refluxed with nickel peroxide in benzene (Scheme 7) afforded a solid residue which on subsequent chromatography over silica gel furnished 3-hydroxyfriedel-3-ene-2-one and 3 α -hydroxyfriedel-3-ene-2-one. The mechanism as

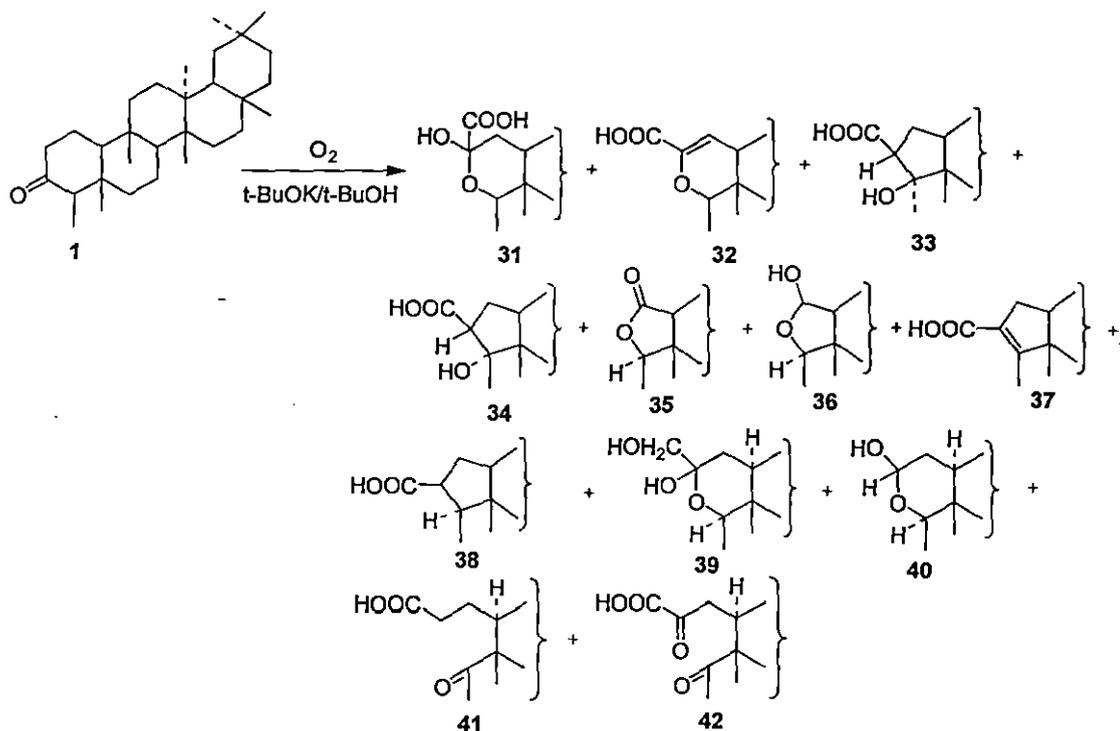
suggested for the formation of 3-hydroxyfriedel-3-ene-2-one from cerin was as follows.



Scheme 7 Proposed mechanism of formation of 3-hydroxyfriedel-3-ene-2-one by Talapatra *et al.*

Auto oxidation of ketones with gaseous oxygen in the presence of strong bases is known as the convenient method for the preparation of α -diketones from the corresponding α -methylene ketones. The autooxidation of triterpene with a 4,4-dimethyl-3-keto structure was examined by many workers and in most of the cases they reported the formation of α -diketones in moderate yield. Nishihama *et al.*²² for the first time reported the base catalyzed autooxidation of friedelin (Scheme 8) and also studied the mechanism of their

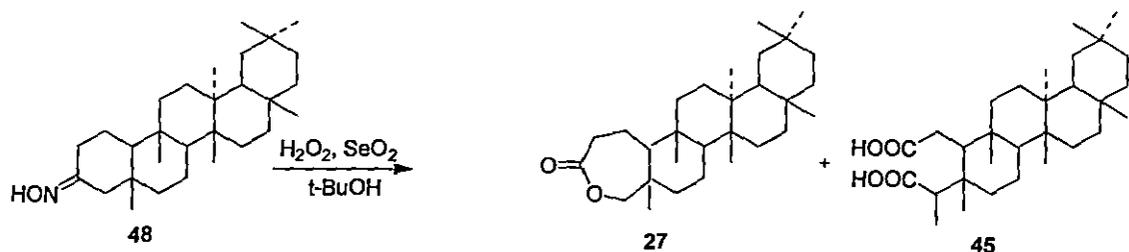
formation. They have synthesized a number of compounds, of which 2-hydroxy-3-oxafriedelane-2-carboxylic acid was the main product. 3-oxafriedel-1-ene-2-carboxylic acid was the dehydrated product of 2-hydroxy-3-oxafriedelane-2-carboxylic acid. The common intermediates to this compound was suggested to be 4-hydroperoxyfriedelan-3-one, not the friedelan-2,3-dione.



Scheme 8 Basic auto oxidation of friedelin by Nishihama *et al.*

In 1991 Pradhan *et al.*²³ once again studied the autooxidation of friedelin in $t\text{-BuOH}$ in presence of $t\text{-BuOK}$ as the catalyst (Scheme 9). Oxygenation of friedelin in presence of $t\text{-BuOK}$ in $t\text{-BuOH}$ furnished $t\text{-butyl A-nor-friedel-2(4)-en-2-carboxylate}$ (43), friedel-2,3-dione (21), friedelan-3 β -ol (2), 2 β -hydroxy-A-nor-friedelan-2 α -carboxylic acid (44) and friedelin dicarboxylic acid (45). Lithium-ethylenediamine reduction of $t\text{-butyl A-nor-friedel-2(4)-en-2-carboxylate}$ gave A-nor-friedelan-2 α -carboxylic acid (46) and A-nor-friedelene (47). Acetylation of 2 β -hydroxy-A-nor-friedelan-2 α -carboxylic acid gave friedel-2(4)-en-2-ol-acetate. They have characterized all the products with by spectral as well as chemical evidences.

Pradhan *et al.* in a different attempt tried the oxidation of friedelin oxim with hydrogen peroxide and selenium dioxide in *t*-BuOH (Scheme 11). The reaction furnished a ϵ -lactone (27) and 2,3-seco-friedelonic acid (45).

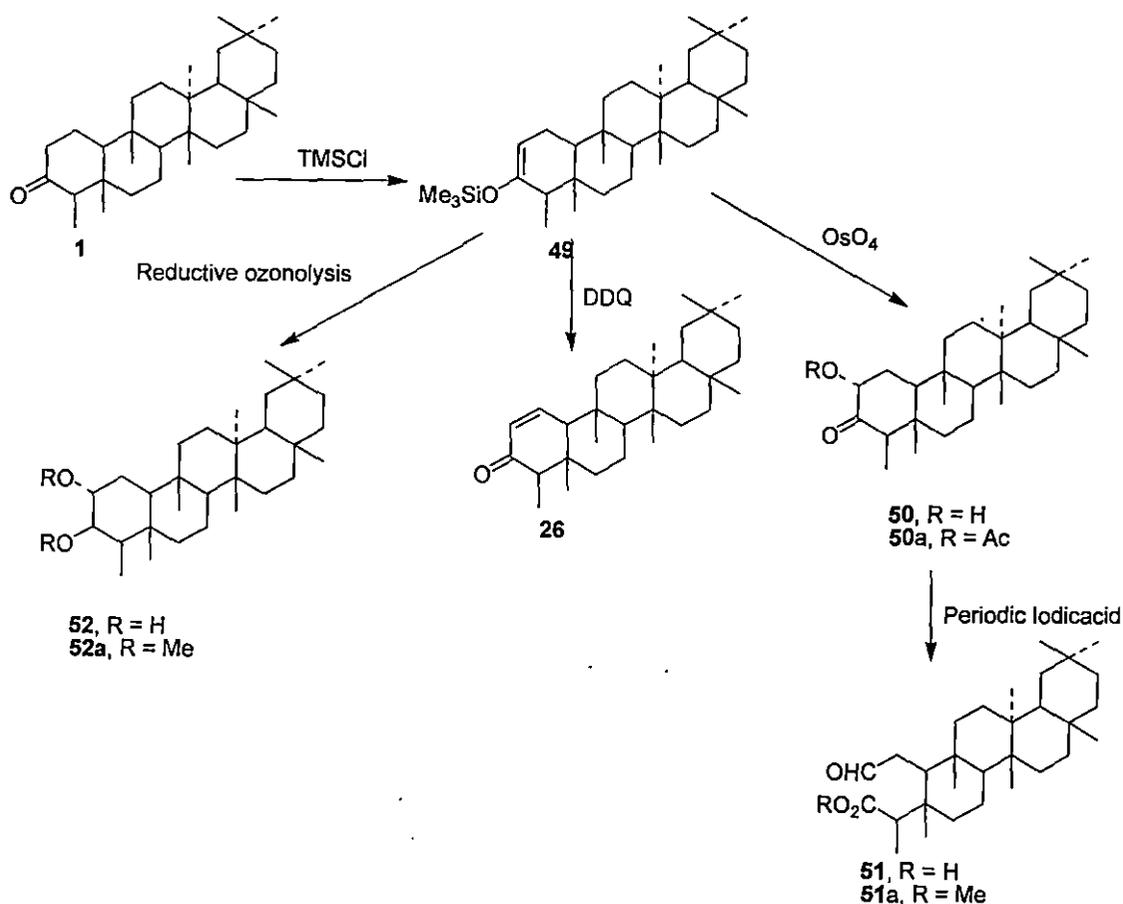


Scheme 11 SeO_2 - H_2O_2 oxidation of friedelin oxim by Pradhan *et al.*

V. Anjaneyulu and G. Sambasiva Rao²⁴ also carried out the oxidation of friedelin under SeO_2 - H_2O_2 in *t*-BuOH. They isolated the same compounds as found by Pradhan *et al.* except friedel-1-ene-3-one.

It is interesting to note that these authors had not done any biological work on friedelin itself as well the synthesized derivatives from the various routes. Some biological works on friedelin and its derivatives had started late nineties of last century. The following part of the review will demonstrate some chemical transformative reactions as well as some reported biological activities of friedelin and its derivatives.

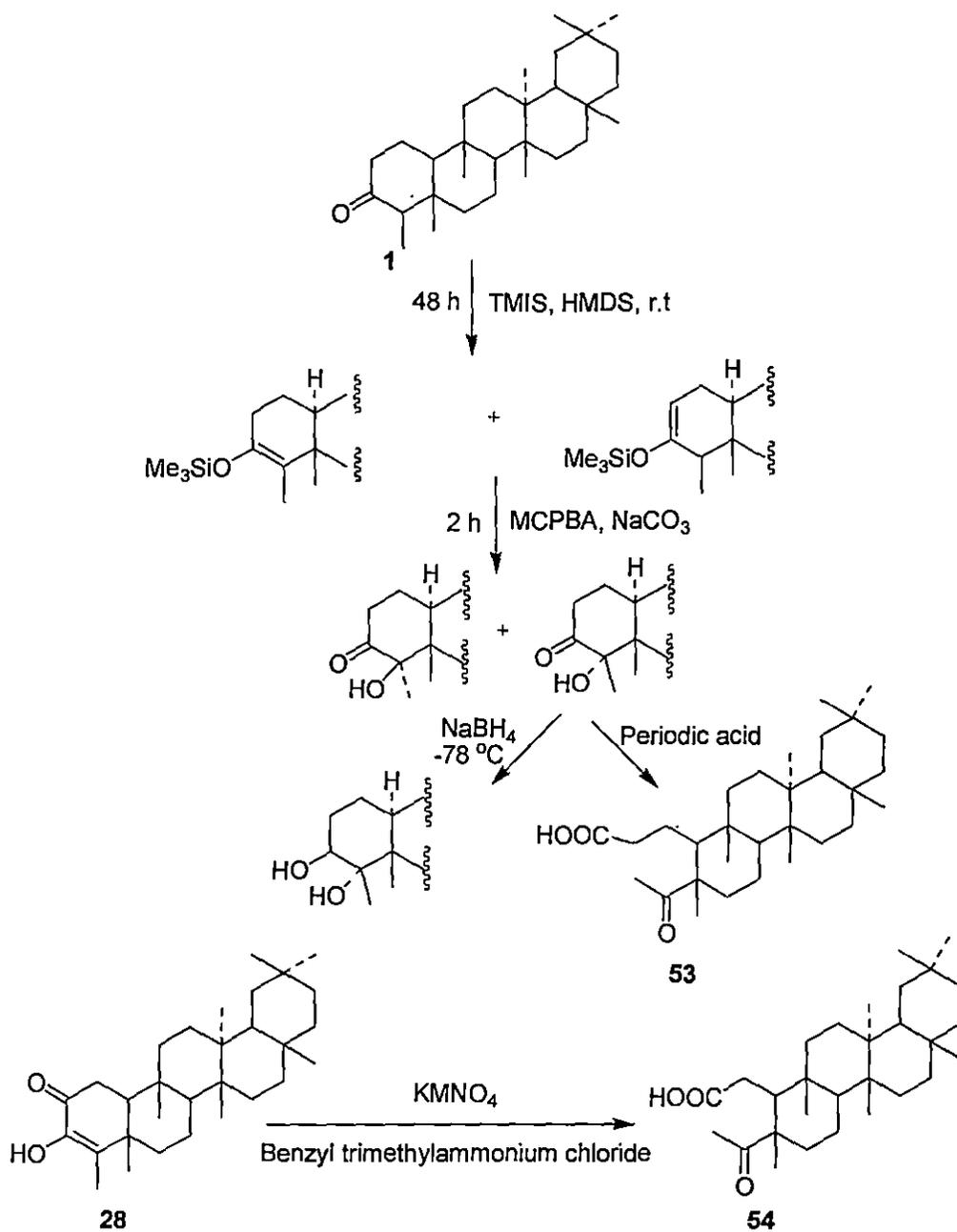
Moiteiro *et al.*²⁵ reported the formation of 3-trimethylsilyloxyfriedel-2-ene (49) in high yields under the controlled silylation of friedelin (1) (Scheme 12) from cork smoker wash solids, a byproduct generated during processing of corkboard by steam baking. Oxidation of 3-trimethylsilyloxyfriedel-2-ene with OsO_4 /NMMO produced 2 α -hydroxyfriedelan-3-one (cerin) (50) from which the new 2,3-secofriedelan-2-al-3-oic acid (51) as obtained quantitatively by periodic acid oxidation. Oxidation of 49 with DDQ afforded friedel-1-en-3-one (26). Reductive ozonolysis of 49 gave 2 α ,3 β -dihydroxyfriedelane, pachysandiol A (52). Compound 51 proved to be a potent inhibitor of human lymphocyte proliferation ($\text{IC}_{50} = 10.7 \mu\text{M}$) and of the growth of a human cancer cell line ($\text{GI}_{50} = 5.4-17.2 \mu\text{M}$).



Scheme 12 Transformative reactions on friedelin by Moiteiro *et al.*

Moiteiro *et al.*²⁶ in another attempt in 2004 reported the synthesis of two different seco-friedelinic acids **53** and **54** though silylation followed by oxidation (Scheme 13). **53** was isolated in a reaction between the trimethylsilyl ether of friedelin and periodic acid. On the other hand **54** was prepared by an effective reaction between friedelin-2-keto-3-hydroxy-3-ene and potassium permanganate in benzyltrimethyl ammonium chloride. They have also synthesized three hydroxyl compounds by oxidation with MCPBA and via sodium borohydride reduction of the intermediate keto-hydroxy derivative. The total reaction sequence is shown below (Scheme 13). These hydroxyl and seco derivatives were evaluated for their ability to inhibit *in vitro* the growth of three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and SF-268 (CNS cancer). Only compounds 3,4-Secofriedelin-4-oxo-3-oic acid (**53**) and 3-Nor-

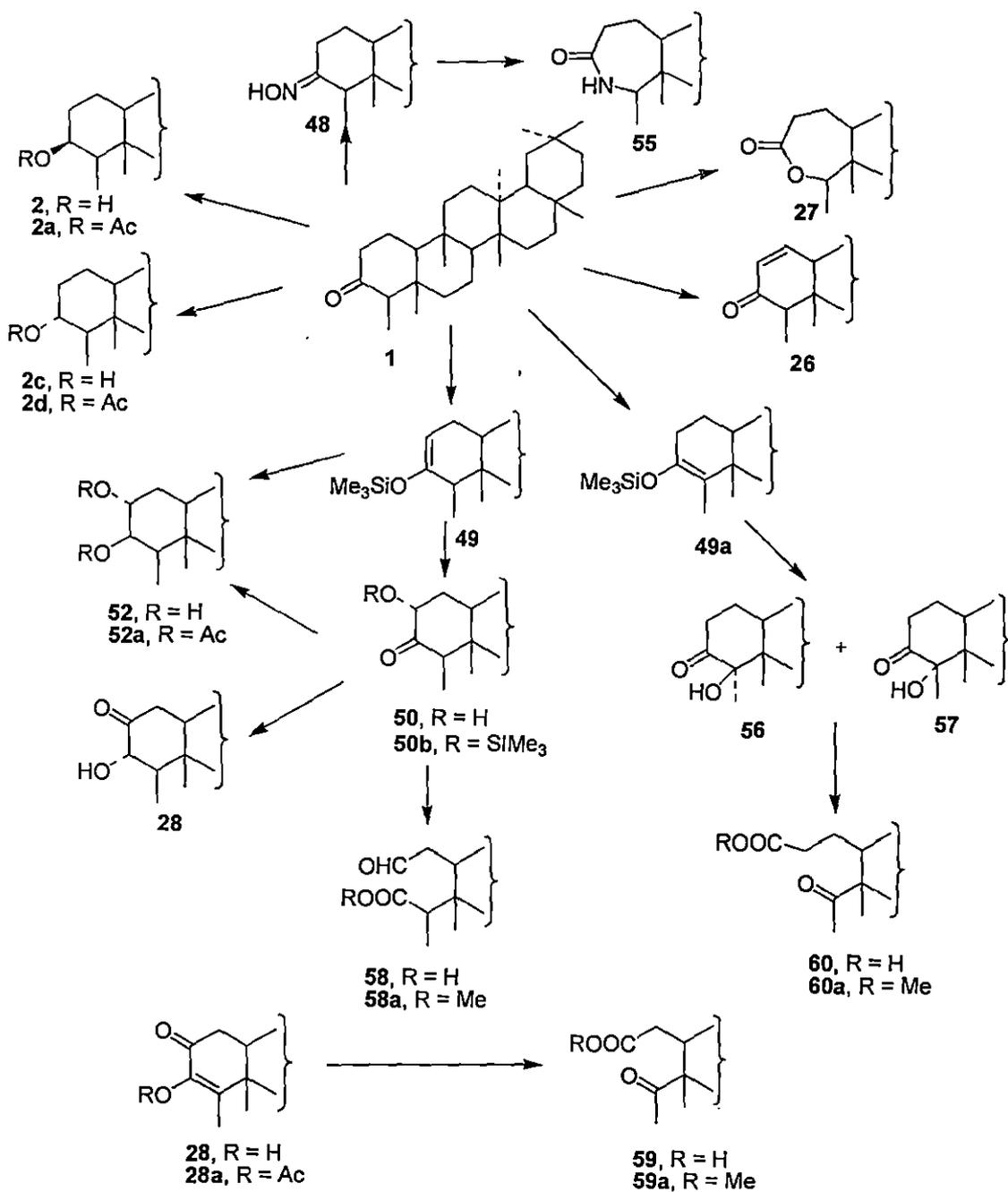
2,4-secofriedelan-4-oxo-2-oic acid (**54**) showed significant activities against all the tested cell lines exhibiting GI_{50} of 24.6 to 32.8 μM and 10.9 to 17.6 μM , respectively.



Scheme 13 Synthesis of some hydroxy and seco derivatives of friedelin by Moiteiro *et al.*

Gonzalez-Coloma and his co-workers²⁷ described the synthesis, bioactivity screening, and structure-activity relationships of various synthetic triterpenoids prepared from the

cork processing byproducts friedelin (**1**) and 3-hydroxyfriedel-3-en-2-one (**28**) via oxidative procedures (Scheme 14). The synthesis of compounds 2R-trimethylsiloxyfriedelan-3-one (**50b**), friedelin-2,3-lactone (**27**), friedelin-3-oxime (**48**), and friedelin-3,4-lactam (**55**) was also described. They have studied the insecticidal and phytotoxic potential of these compounds, their selective cytotoxic effects on insect and mammalian cells, and their antiparasitic effects. Structural modifications of the A-ring of friedelin (**1**) improved its insecticidal activity with derivatives **50**, 2,3-secofriedelan-2-al-3-oic acid (**58**), its acetylated derivative **58a**, 3 α - and 3R-hydroxyfriedelane (**2**, **2a**, **2c** and **2d**), 3R-hydroxyfriedel-2-one (**28**), 4 α -hydroxyfriedel-3-one (**57**), the acetylated **2d**, 3,4-secofriedelan-4-oxo-3-oic-acid (**59**), lactone **27**, and the oxime **48** being stronger insecticides than the parent compound. Methyl-3-nor-2,4-secofriedelan-4-oxo-2-oic acid (**59**) and its acetylated derivative **59a** also showed insecticidal activity in contrast to their inactive parent compound **28** and **28a**. The postingestive effects and cytotoxicity of these compounds suggested a multifaceted insecticidal mode of action. According to them these structural modifications did not result in better phytotoxic agents than the parent compounds except for lactam **55** and yielded several moderately active antiparasite derivatives (seco acids **58**, **59**, **60** and 4 α -hydroxyfriedel-3-one **57**) with cytotoxic effects on mammalian cells.



Scheme 14 Oxidative transformative reactions on friedelin by Gonzalez *et al.*

Sheppard *et al.*²⁸ investigated the selective palladium mediated C–H functionalisation of appropriately functionalised derivatives of lanosterol, cholesterol, and friedelin. The desired equatorial aldehyde functionality was successfully introduced into the lanosterol skeleton as expected. Cyclopalladation of a cholesterol derivative proceeded as expected,

but during oxidation of the organopalladium intermediate, participation of the adjacent alkene functionality led to stereoselective formation of a cyclopropane and introduction of an acetate group into the steroid backbone at C-6. But the friedelin oxim failed to undergo a successful cyclopalladation reaction, presumably due to the unfavorable orientation of the single methyl group and/or the potential for β -hydride elimination from any resultant σ -organopalladium complex.

Chapter II

Section A

Transformative reactions on friedelin

1. Introduction

The discovery of lead for pharmaceutical investigations requires identification of new molecules that are able to interact with and modify a biological target.^{29,30} Natural products represent one of the most relevant approaches to this goal. Natural products are produced in living organisms by the activity of biosynthetic enzymes. They are thus recognized by the enzymes at specific binding sites complementary in shape and physicochemical properties. Natural products may possess the imprint for binding to the therapeutic target proteins containing the ligand binding motif similar to the biosynthetic enzyme.²⁹ Therefore, it is important to identify novel compounds those are complementary to biological structure space.

Triterpenoids are a large, ubiquitous and structurally diverse group of natural products that exhibit nearly 200 diverse skeletons.³¹ Most significant triterpenoids are 6-6-6-5 tetracycles, 6-6-6-6-5 pentacycles, or 6-6-6-6-6 pentacycles³² with physiological functions allied with chemical protection of plants.³³ Triterpenes, highly oxidized at ring A have been reported to possess a wide spectrum of biological activities.³⁴

Pentacyclic triterpenes, such as betulinic, boswellic, ursolic, and oleanolic acids are highly abundant in many edible fruits and vegetables. They are reported to inhibit cultured human melanoma, neuroblastoma, malignant brain tumor and leukemic cells.^{35,36} They inhibit topoisomerases I and II α by contending with DNA for topoisomerase binding sites, thus preventing topoisomerase-DNA cleavable complex formation.^{32,35,36} The general pentacyclic ring structure of triterpenoids has been reported to be essential for topoisomerase inhibitory activity.^{37,38} However, the structure itself is inadequate for inhibition and the nature and arrangement of the side groups/functionality are the key factors.^{37,38}

Although, friedelan group of triterpenoids are 6-6-6-6-6 pentacycles and are wide spread in nature, surprisingly modern "lead research" on friedelan skeleton is not much

prevalent. In recent times only few works on the transformative reactions on friedelin (1) have been reported.^{33,39,40} Some recent studies have indicated the *in vitro* anti-tumor activity of some of hemisynthetic friedelin derivatives,^{33,39,40} but the mechanisms through which these compounds achieve this effect has not yet been elucidated. Moreover, the systematic studies on transformative reactions and biological activity of cerin (2) are limited.

2. The present investigation

In the present study structurally modified friedelan derivatives, highly oxidized on ring A were synthesised from friedelin (1) and rare cerin (29) and characterized by IR, 1D, 2D-NMR and MS.

2.1 Results and Discussion

We have synthesized some hemisynthetic friedelan compounds (59, 59a, 61, 45 and 45a) by simple chemical modifications of triterpenes 1 and 29 (Figure 1) isolated from *Quercus suber* (Cork).

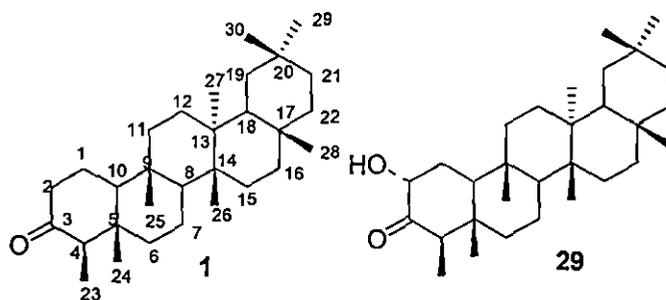


Figure 5 Chemical structures of triterpenoids from *Q. suber*

Two different schemes (Scheme 1 and Scheme 2) were used for their synthesis. Both these schemes are quite suitable for possible large-scale applications. The conversions mainly focused on the oxidative cleavage of the ring A of the natural triterpenoids. The oxidative cleavage of 1 and 29 produced 2,3-secofriedelan-2,3-dioic acid (45) and 4-oxa-3,4-secofriedelan-3-oic acid (59), respectively. A controlled lead tetraacetate (LTA) oxidation on both 59 and 45 at room temperature selectively produced a A-nor-lactone, 61 (Scheme 1 and 2) with 68% yield.

Friedelin, **1** and cerin, **29** were isolated from cork by using soxhlet apparatus. Cerin was obtained as slightly yellowish crystals of melting point (mp) 260-261 °C.

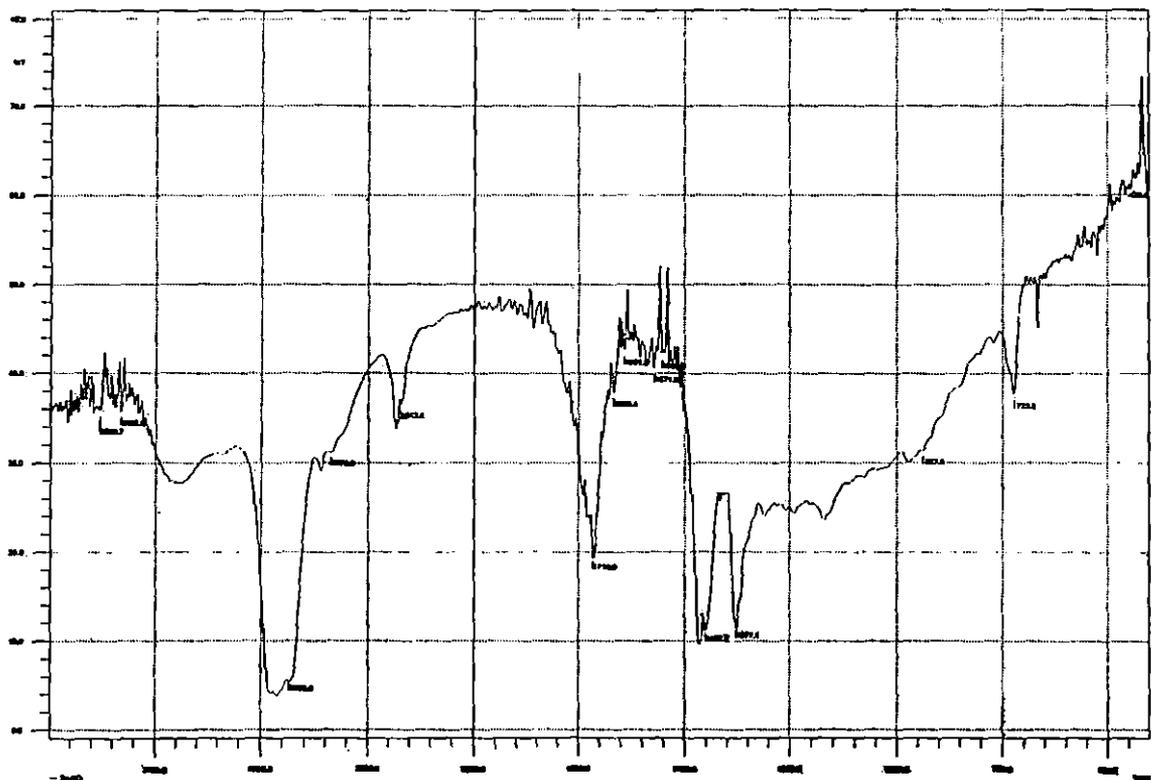


Figure 6 IR spectrum of friedelin, **1**

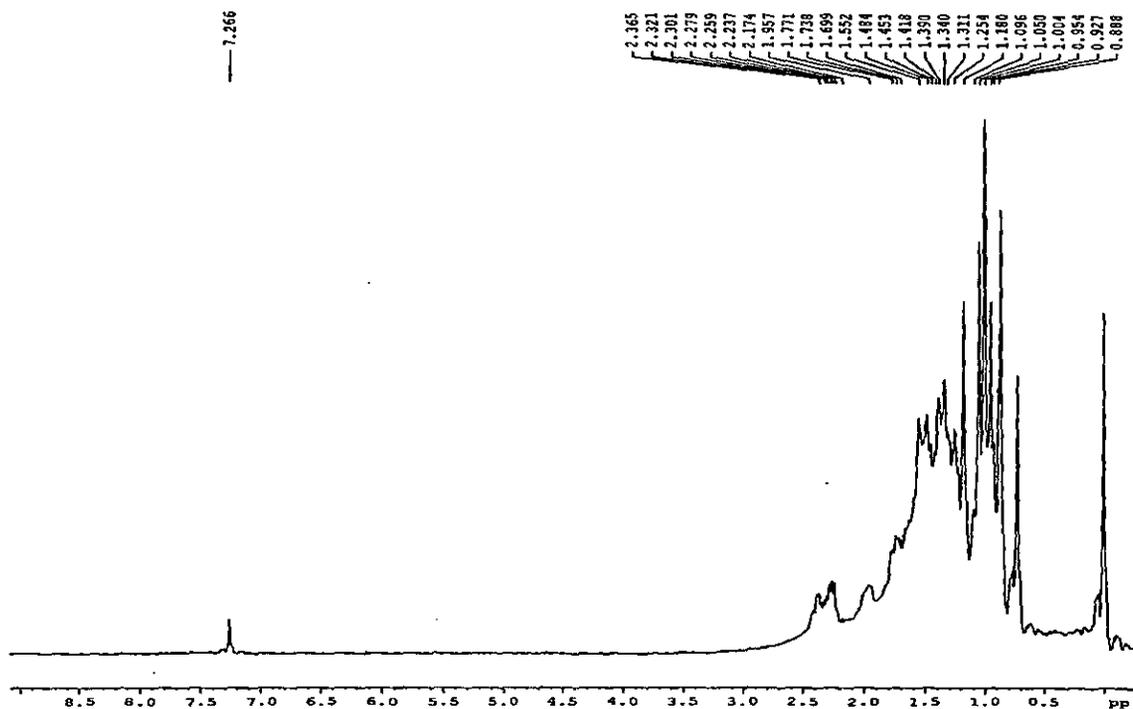


Figure 7 ^1H NMR spectrum of friedelin, 1

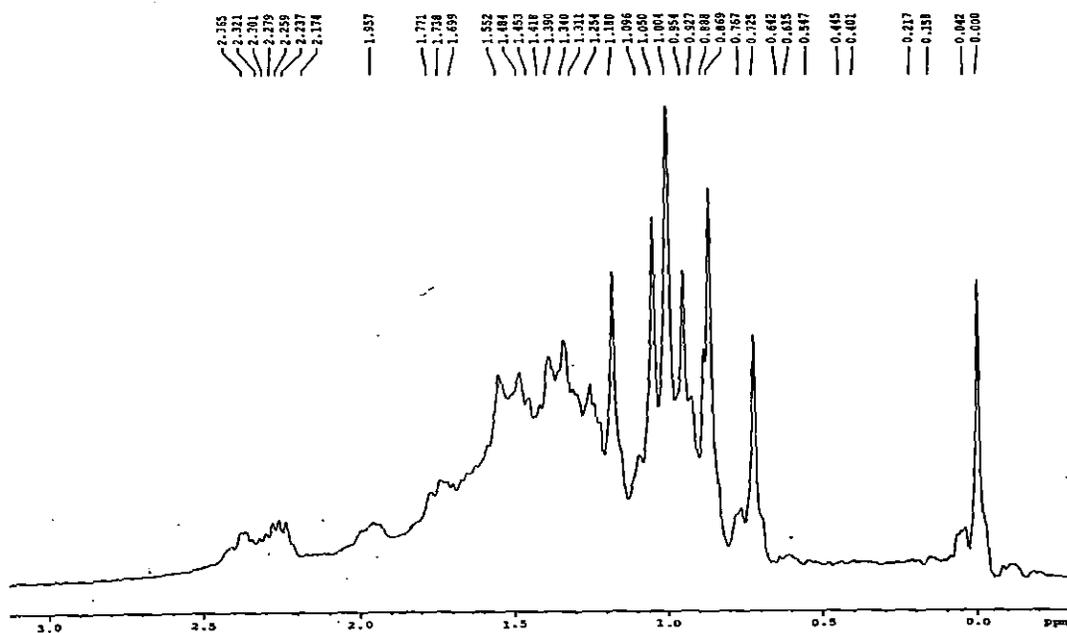


Figure 8 Expanded ^1H spectrum of friedelin, 1

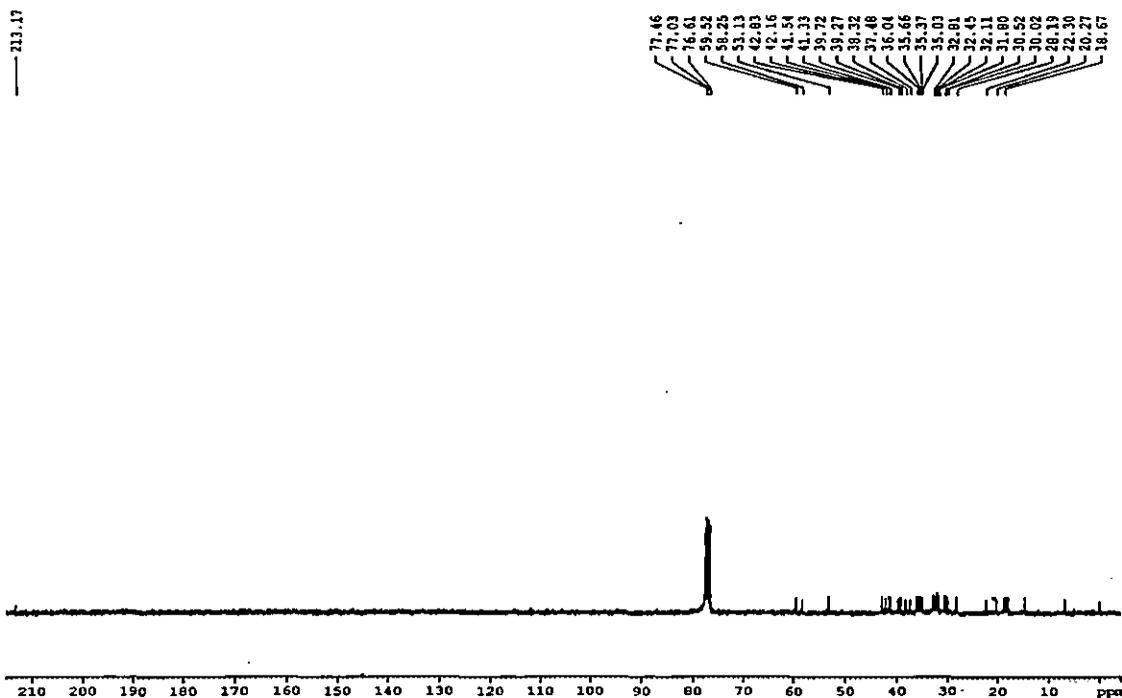
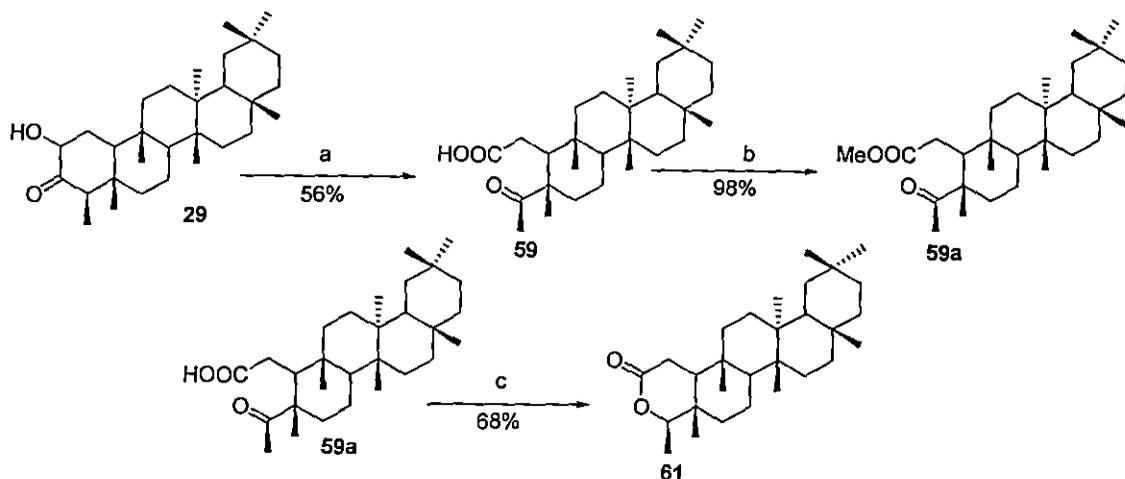


Figure 9 ^{13}C NMR spectrum of friedelin, 1

Oxidation of **29** in glacial acetic acid in presence of anhydrous CrO_3 , followed by evaporation of the solvent at reduced pressure gave a yellow gummy residue (Scheme 1). Purification of the residue over a column of silica gel gave white powdered compound, **59** of melting point (mp) 214-215 $^\circ\text{C}$, which upon methylation with diazomethane afforded the corresponding methyl ester **59a** of mp 167-168 $^\circ\text{C}$. In the IR spectrum compound **59** showed peaks at 3079, 1734 (H-bonded $>\text{C}=\text{O}$), 1696 (carbonyl of COOH group), 1465 (C-O), 1419, 1302, 1074 and 899 cm^{-1} . ^1H NMR spectra of compound **59** gave signals for the presence of eight tertiary methyls at δ_{H} 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23). Two methylene hydrogens at C_1 appeared at δ_{H} 1.90 (dd, 1H, $J_{1\text{eq}10\text{ax}} = 4.0$ Hz, $J_{\text{gem}} = 15.6$ Hz, H-1) and 2.35 (1H, dd, $J_{1\text{ax}10\text{ax}} = 6.6$ Hz, $J_{\text{gem}} = 15.6$ Hz, H-1). C_{10} axial hydrogen atom appeared at δ_{H} 2.30 (dd, 1H, $J_{10\text{ax}1\text{eq}} = 4.0$ Hz, $J_{10\text{ax}1\text{ax}} = 6.6$ Hz, H-10) and the carboxyl hydrogen appeared as a singlet at δ_{H} 9.96 (s, 1H, COOH). ^{13}C spectral data are tabulated in table 1. On the basis of the above data, structure of compound **59** was established as 4-oxa-3,4-secofriedelan-

3-*o*-ic acid. Compound **59** on esterification with diazomethane gave the corresponding ester, **59a** with 94% yield. In its ^1H NMR spectrum it gave a sharp singlet at δ_{H} 3.67 (s, 3H, $-\text{OCOCH}_3$) due to the ester methyl and all other signals were in good correlation to the proposed structure of **59a**. The IR, NMR data of **59** and **59a** were comparable to that reported in literature.^{25,26}



Scheme 15 Oxidative ring cleavage of compound **2**. Reagents and conditions: **a**, Dry CrO_3 , glacial AcOH, ice cold water, CHCl_3 , anhyd. $\text{Al}_2(\text{SO}_4)_3$; **b**, Diethyl ether, CH_2N_2 , glacial AcOH, ice cold water, CHCl_3 , anhyd. $\text{Al}_2(\text{SO}_4)_3$; **c**, Lead tetra acetate, glacial AcOH, ice cold water, CHCl_3 , anhyd. $\text{Al}_2(\text{SO}_4)_3$

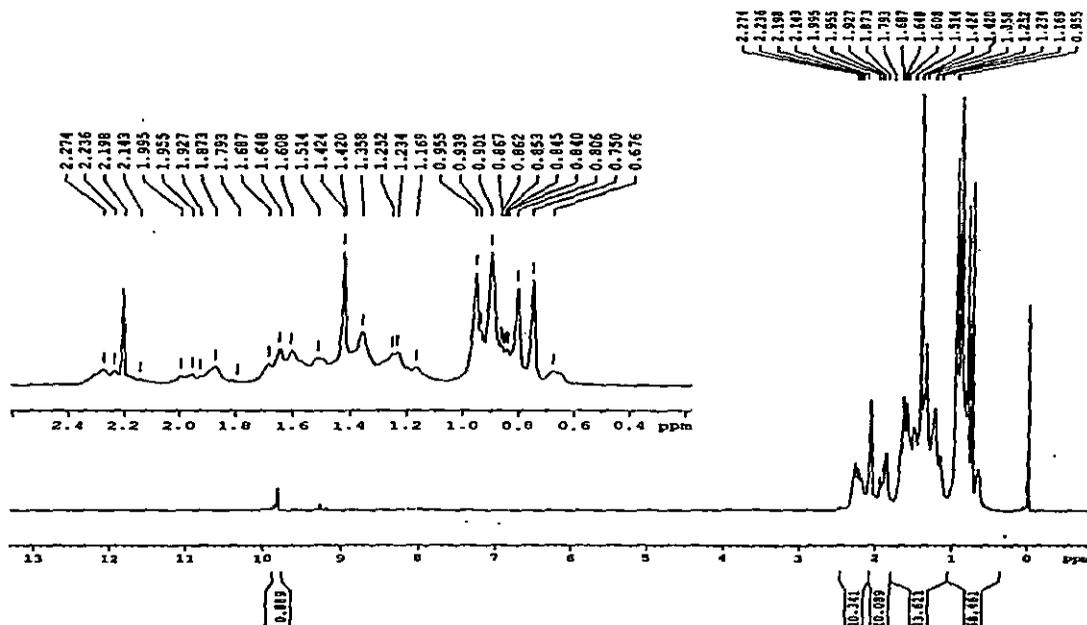


Figure 10 ^1H NMR spectrum of compound **59**

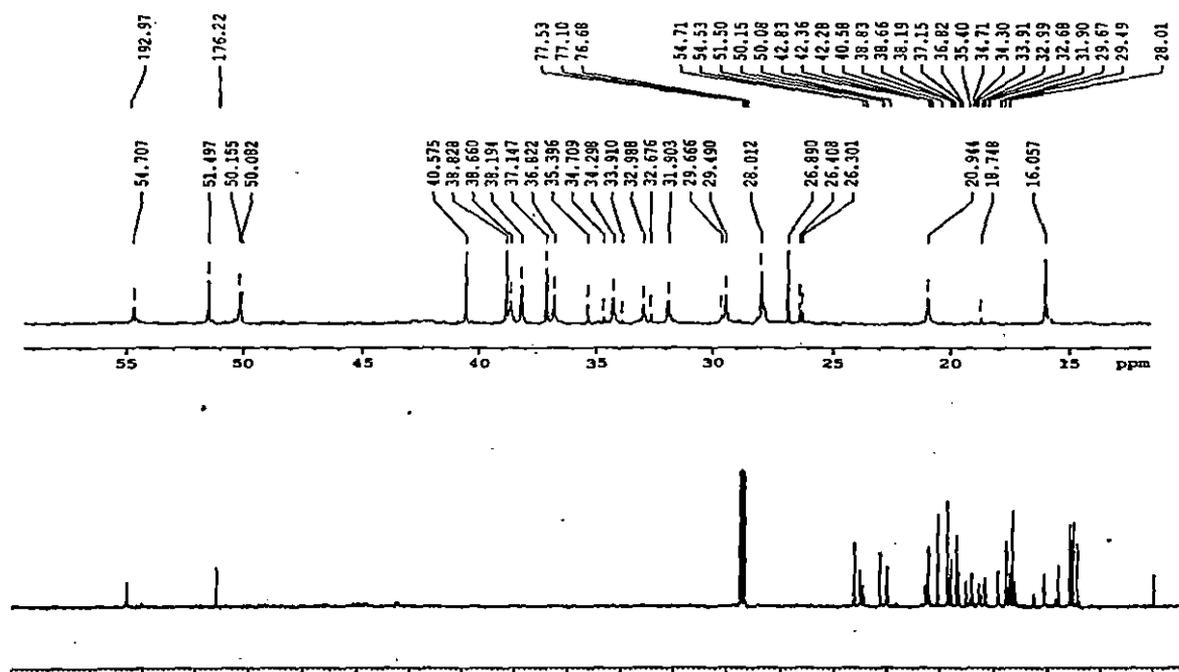


Figure 11 ^{13}C NMR spectrum of compound 59

In another attempt to synthesize friedlan derivatives highly oxidized at ring A, the oxidation of **1** was undertaken with ammonium vanadate in concentrated HNO_3 -glacial acetic acid at 0°C . Purification of the reaction mixture through column chromatography yielded a white powdered compound, **45** (Scheme 2). Compound **45a** on esterification by diazomethane yielded the corresponding ester **45a** exclusively of mp 167 - 169°C . The IR, MS and NMR (both ^1H and ^{13}C) data of **45** and **45a** were comparable with the data reported in the literature.^{23,25,26}

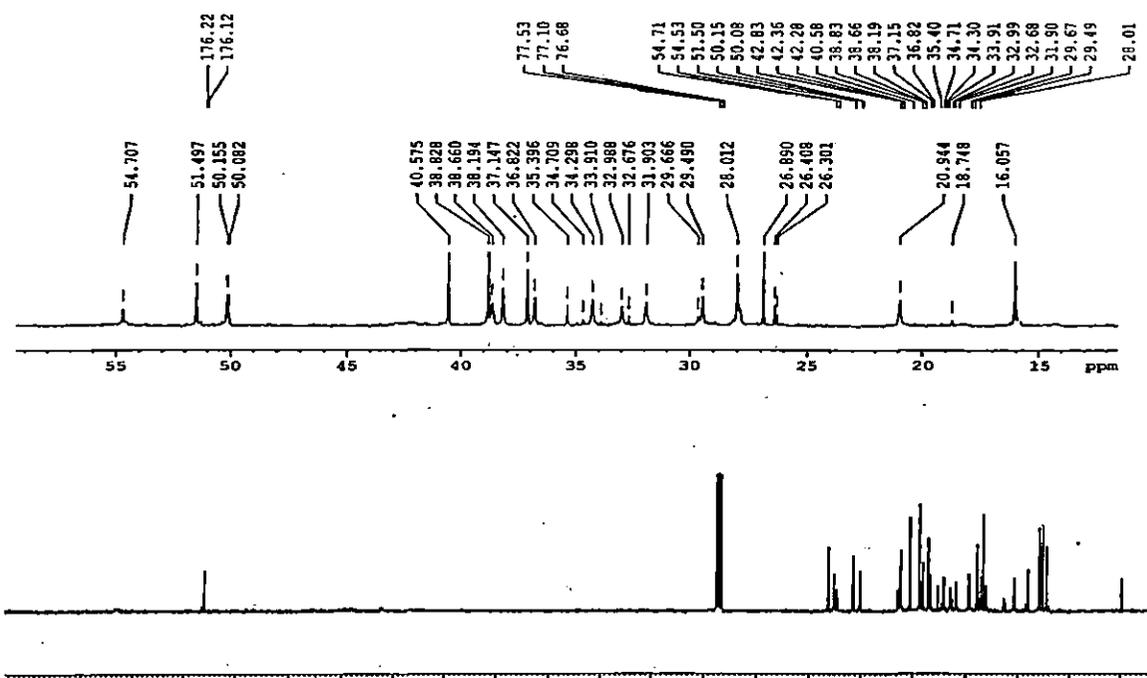


Figure 13 ^{13}C NMR spectrum of compound 45

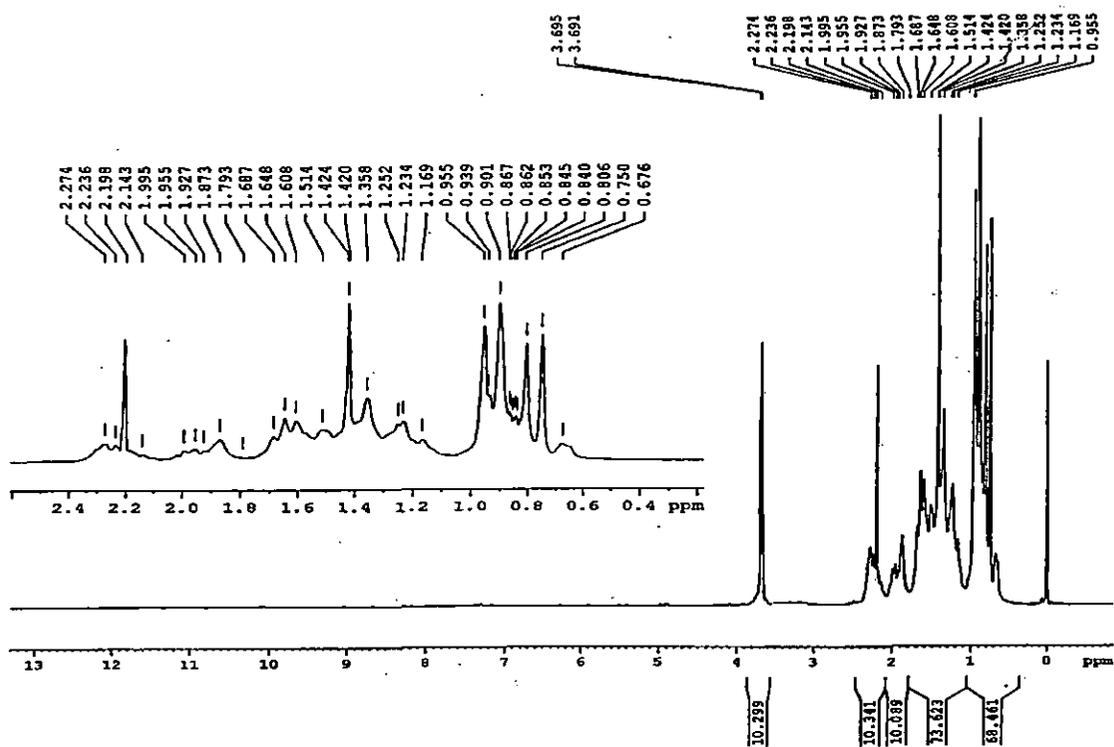


Figure 14 ^1H NMR spectrum of compound 45a

Further oxidation of **59** and **45** separately with LTA in glacial acetic acid furnished the same compound **61**, as white powder of mp 266-267 °C. The molecular formula of the compound, as determined by ESI (m/z 429 $M+1$) and TOF (m/z 428 M^+) MS was $C_{29}H_{48}O_2$. This molecular formula was also corroborated by 1H and ^{13}C NMR spectroscopic data (Table 1). The ^{13}C and DEPT spectroscopic data of **61** revealed the presence of 29 carbon signals including an ester carbonyl (δ_c 172.1), eight primary, ten secondary, four tertiary and seven quaternary carbon atoms.

The six degrees of unsaturation inherent in the molecular formula of **61**, coupled with the NMR data showed the presence of one carbonyl group and five rings in the molecule **61**. These results indicated that a probable cyclization of the nor-seco acid **59** and seco-diacid **45** had occurred during the LTA oxidation and the nor-lactone, **61** had been generated.

In the IR spectrum, compound **61** gave peaks at 2939, 2866, 1730 (six membered lactone), 1459 (C-O), 1388 (CH-CH₃), 1241 and 1082 cm^{-1} . In its 1H NMR spectrum (Figure 10-11) it showed seven tertiary methyl signals at δ_H 0.84 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H) and 1.17 (s, 3H, H₃-28) (Figure 1). Another secondary methyl group at C₄ appeared at δ_H 1.20 (d, 3H, $J = 9$ Hz, H₃-23). C₄-H appeared as a quartet at δ_H 4.05 (1H, q, $J = 6.3$ Hz). C₁-Hs are deshielded due to the magnetic anisotropy induced by the neighboring carbonyl group at C₂ and each appeared as a doublet of a doublet (dd) centered at δ_H 2.55 (1H, dd, $J = 6.6$ and 12.3 Hz, α H) and δ_H 2.40 (1H, dd, $J = 6.6$ and 12.9 Hz, β H). The slight difference in the observed 'J' value may be due to the unequal coupling of the axial and equatorial hydrogens on that carbon. H₁₀ (α) appeared as a singlet at δ_H 1.56 (1H, s). All this data is in good agreement with that for friedelan skeleton.

Table 1 ^{13}C NMR data of parent and different hemisynthetic friedelan derivatives

Position	δ_{C}				
	1	59	61	1*	59*
1	22.3	32.9	34.9	22.3	32.9
2	41.5	176.2	172.0	41.5	178.2
3	213.2			213.3	
4	58.3	193.3	87.6	58.2	233.9
5	42.2	54.7	53.9	42.1	53.4
6	41.3	37.2	39.2	41.3	37.5
7	18.2	18.2	18.6	18.2	17.5
8	53.1	51.5	52.8	53.1	52.5
9	37.5	38.2	37.2	37.4	38.2
10	59.5	50.0	76.6	59.4	49.8
11	35.7	34.3	35.3	35.6	34.4
12	30.5	29.5	30.0	30.5	29.9
13	39.7	40.5	39.7	39.7	39.6
14	38.3	38.6	36.1	38.3	38.3
15	32.5	32.6	32.2	32.4	32.3
16	36.0	35.4	35.8	36.0	35.9
17	30.0	29.7	29.9	30.0	29.9
18	42.8	50.1	42.7	42.7	48.8
19	35.4	36.8	35.0	35.3	35.8
20	28.2	28.0	28.1	28.1	28.1
21	32.8	31.9	32.7	32.7	32.8
22	39.3	38.9	38.1	39.2	39.2
23	6.8	25.3	16.6	6.8	25.3
24	14.7	26.8	12.4	14.6	17.6
25	18.0	18.7	17.5	17.9	17.8
26	20.2	16.1	20.7	20.2	20.2
27	18.7	20.9	16.6	18.6	18.7
28	32.1	33.9	32.1	32.1	32.2
29	35.0	34.7	34.1	35.0	34.9
30	31.8	31.7	31.8	31.7	31.8

* Reported data in literature

The COSY, NOESY and HMBC spectra (Figure 13-24) of **61** allowed assignment of all the proton and carbon signals. All the ^{13}C data is presented in table 1. All the above evidence supports the final compound as a nor-lactone. The probability of formation of the other possible six membered lactone (2-oxafriedelan-3-one) was ruled out because of the greater deshielding nature of H_4 (δ_{H} 4.05, 1H, q, $J = 6.3$ Hz). Nevertheless as compound **61** had been formed from a 2,3-seco compound (either **59** or **45**), there remains every possibility that the stereochemistry at C_4 had been changed, which may

give rise to structure II (Figure 2). Thus, the probable structures of the compound are either I or II (Figure 2).

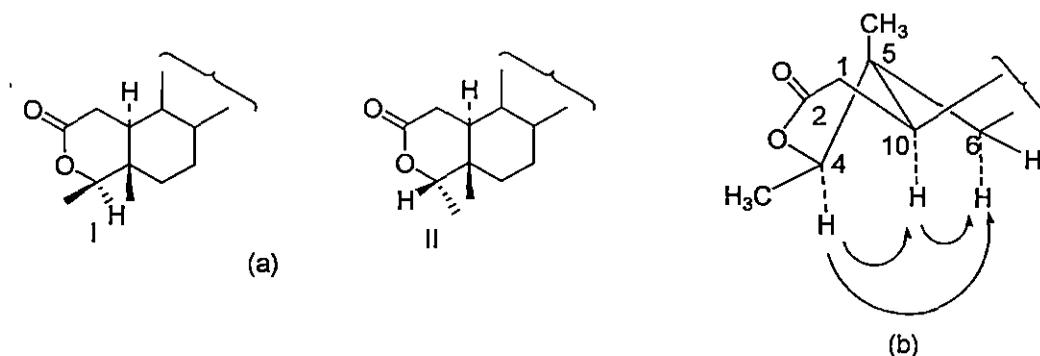


Figure 16 (a) Partial structures of the two possible lactones; (b) Key NOESY correlation of compound **61**

The exact stereochemistry at C₄ was confirmed by 2D NMR techniques. The NOESY spectrum (Figure 19 and 20) of compound **61** gave significant information to this end. All NOE cross peaks have opposite phase to the diagonal, indicating that these arose from positive NOE enhancement as anticipated for a molecule of the size (having M⁺ 428) under ambient conditions. In the NOESY spectrum, H₄ at δ_{H} 4.05 (1H, q, J = 6.3 Hz) showed two important correlations between H₁₀ at δ_{H} 1.56 (1H, s) and H₆ (α) δ_{H} 1.09 (1H, m). In addition, cross peaks were observed by the NOE effects due to H₁₀ at δ_{H} 1.56 (1H, s) with H₆ (α) δ_{H} 1.09 (1H, m). The above data established the stereochemistry of C₄-H as α and hence structure I (Figure 2) is the exact structure of **61**. Thus compound **61** is 3-oxafriedelan-2-one.

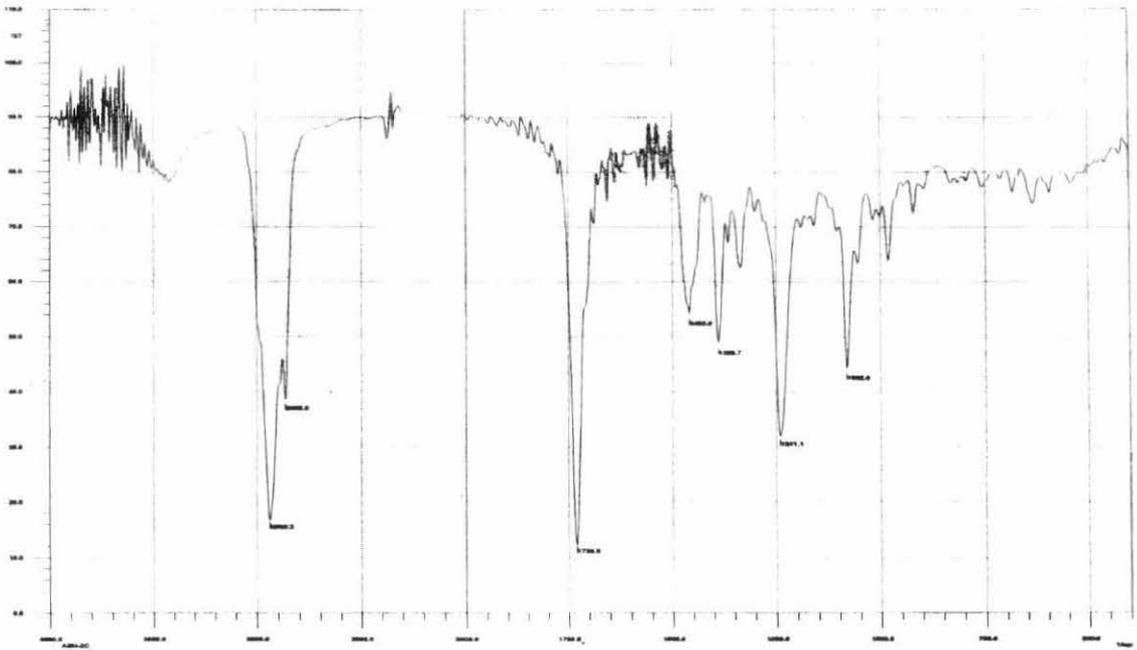


Figure 17 IR spectrum of compound 61

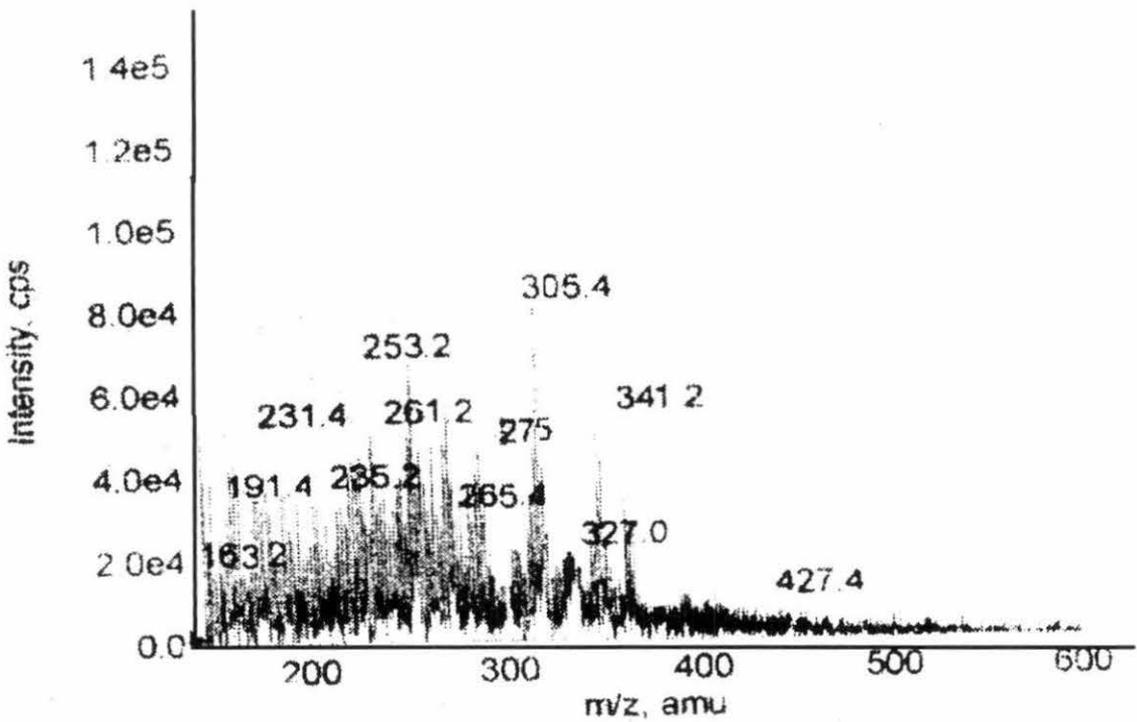


Figure 18 Mass spectrum (ESIMS) of compound 61

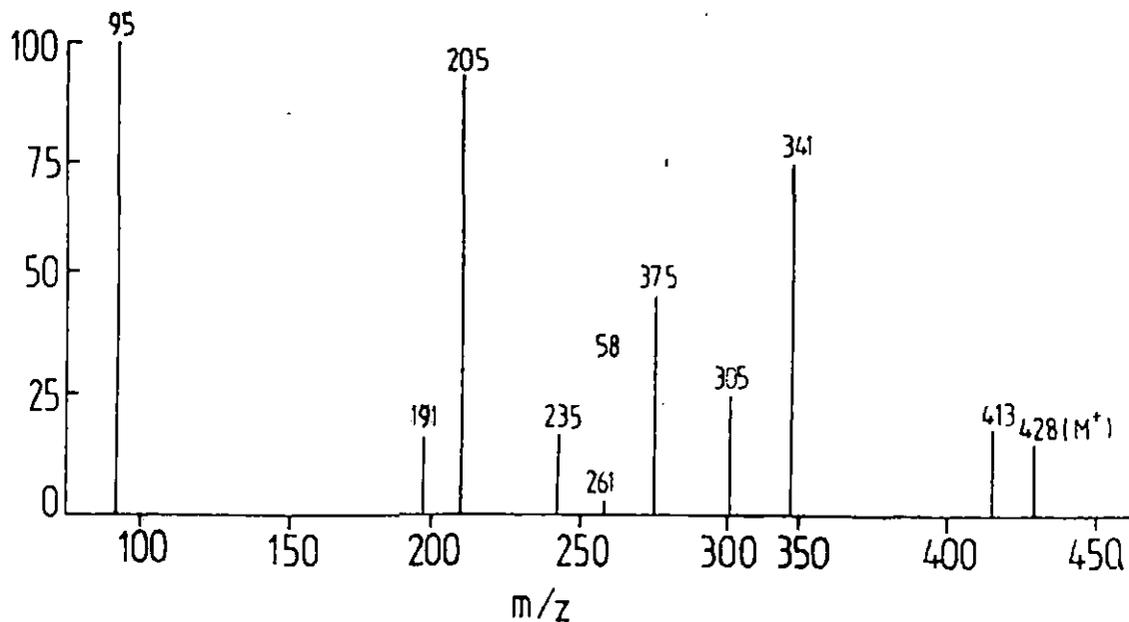
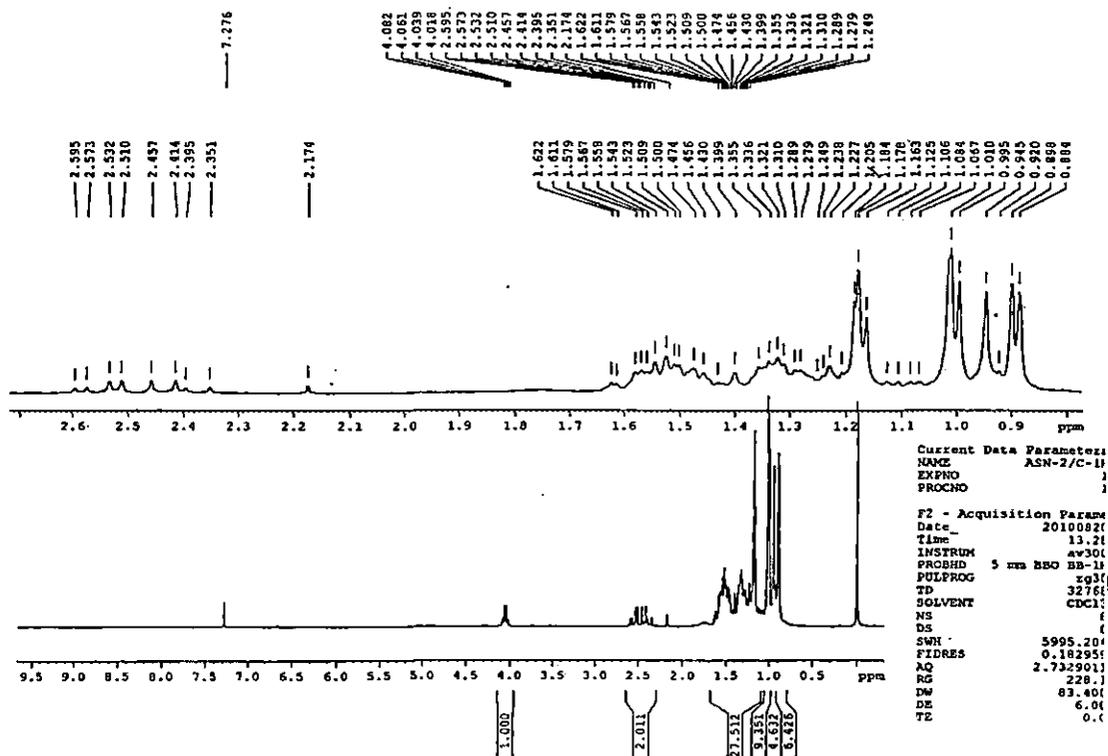


Figure 19 ToF MS spectrum of compound 61



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PROCNO   1

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Figure 20 ¹H NMR spectrum of compound 61

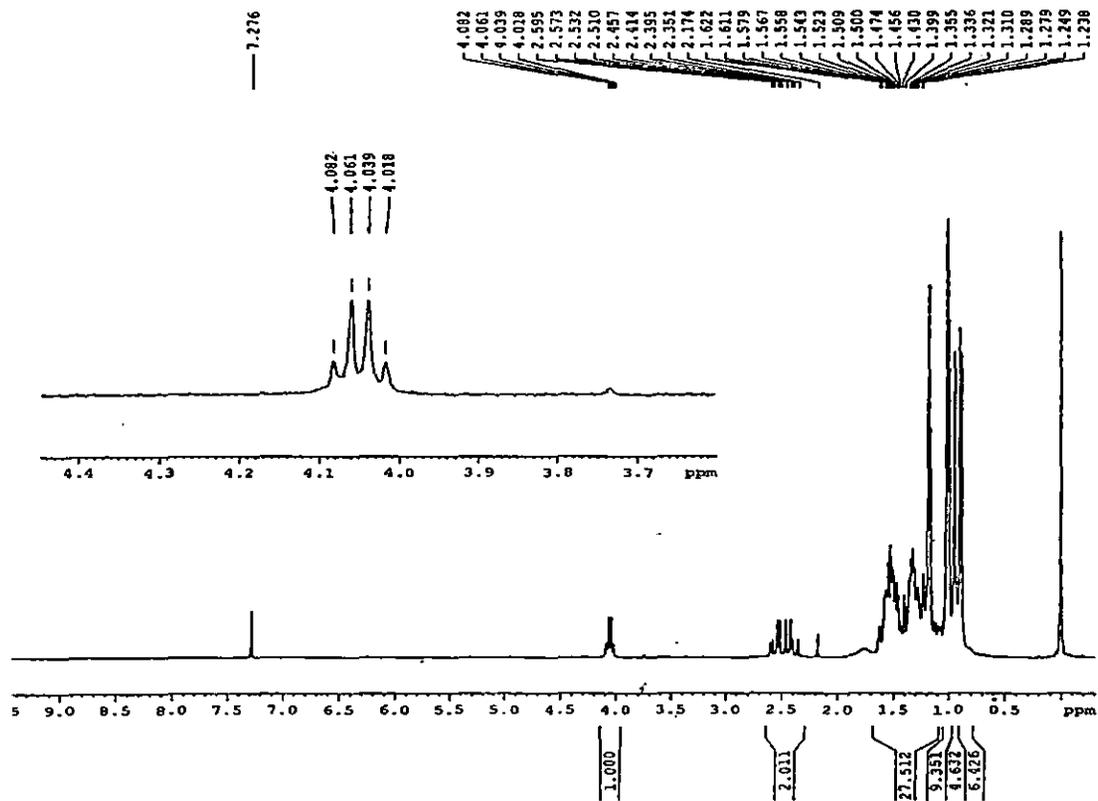


Figure 21 Expanded ^1H NMR spectrum of compound 61

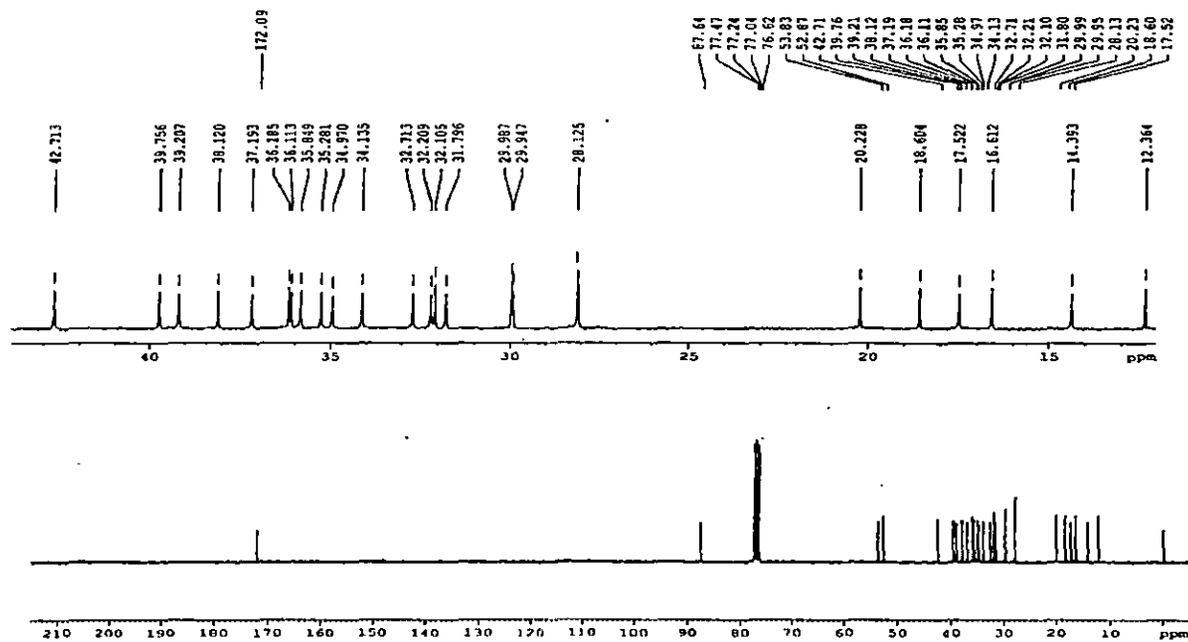


Figure 22 ^{13}C NMR spectrum of compound 61

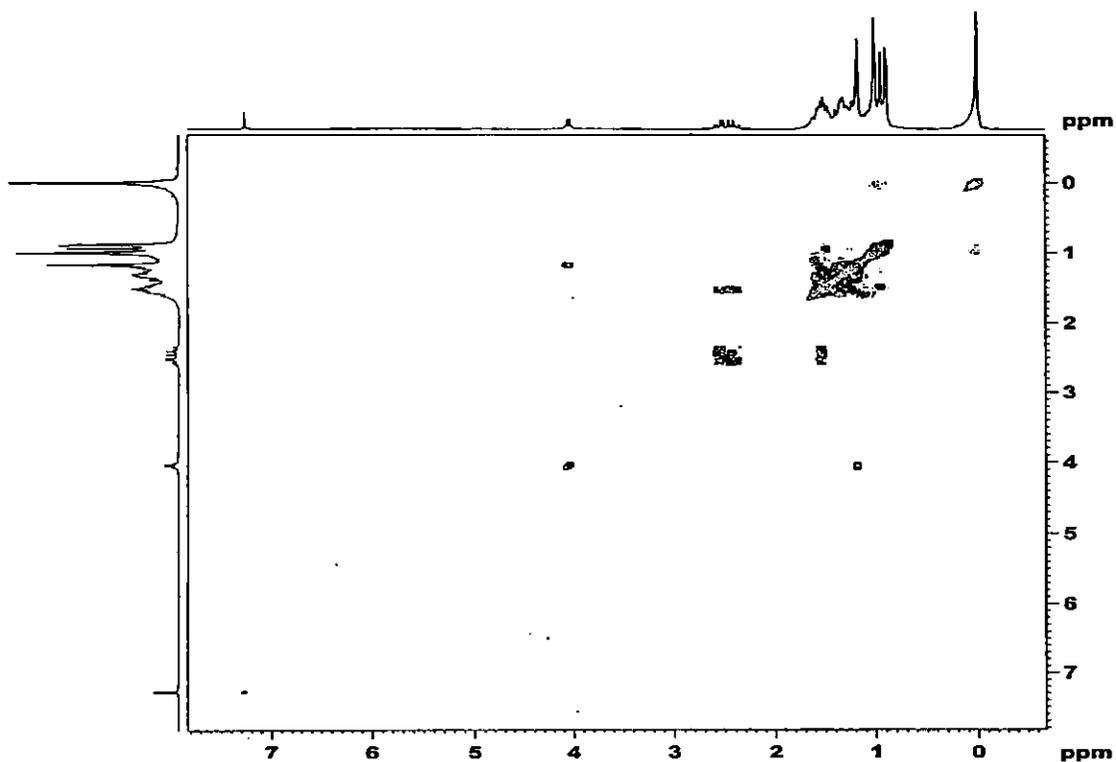


Figure 23 COSY spectrum of compound 61

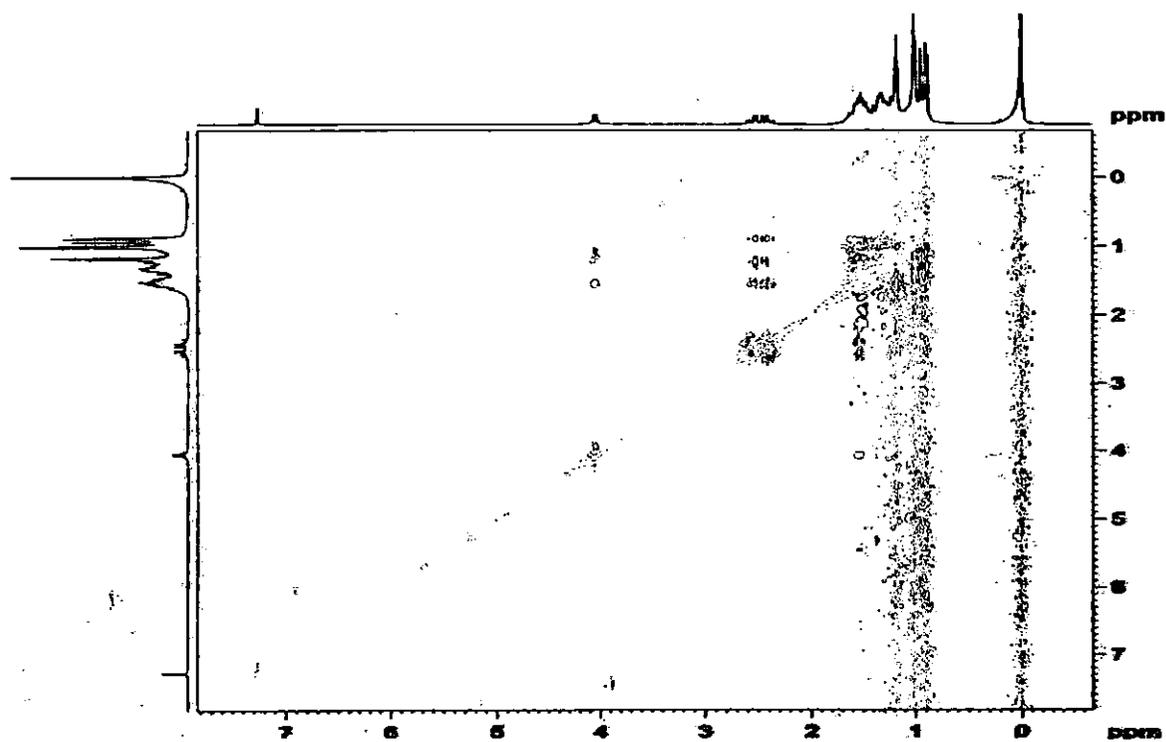


Figure 24 ROESY spectrum of compound 61

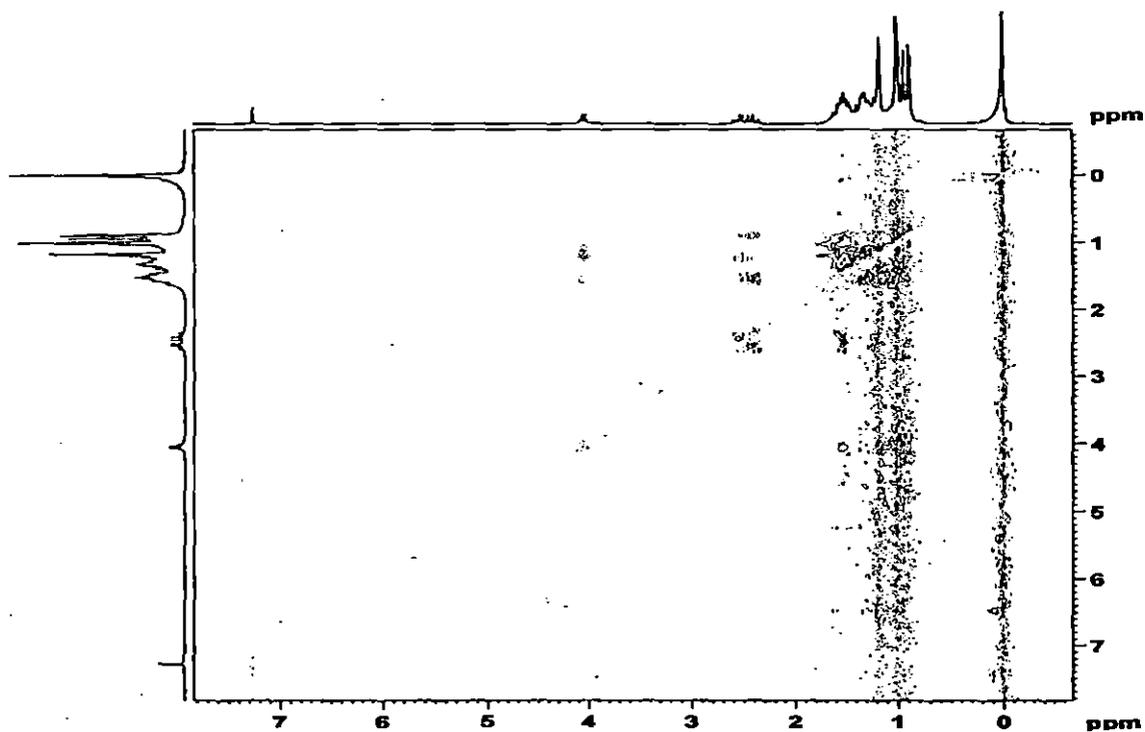


Figure 25 NOESY spectrum of compound 61

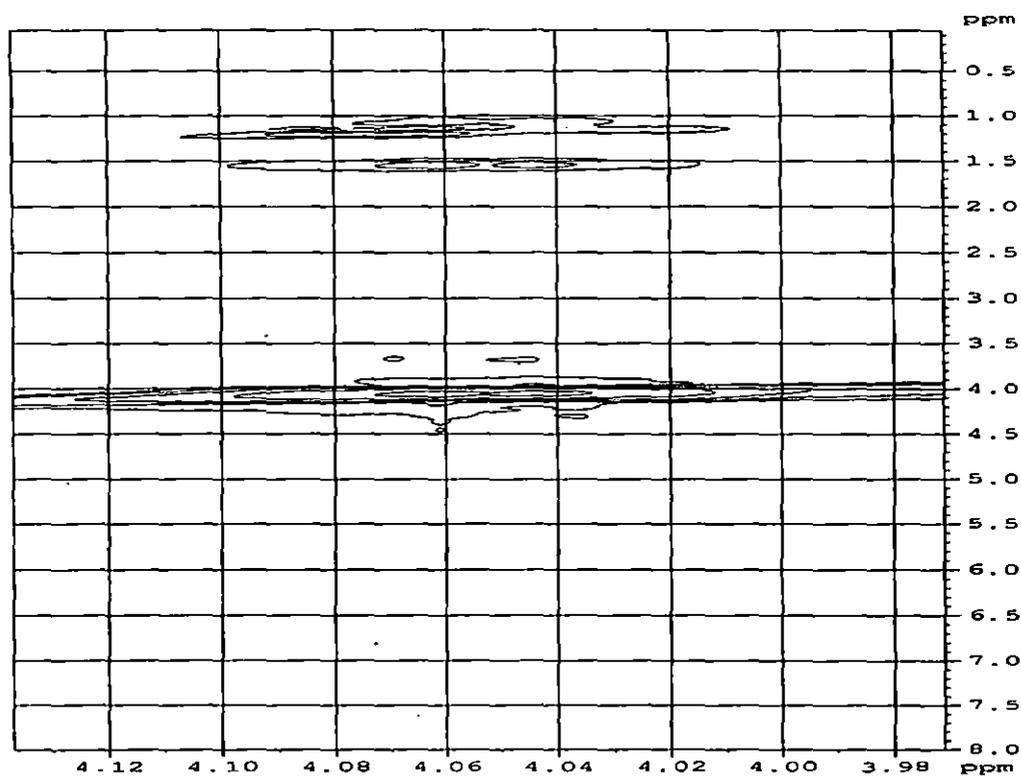


Figure 26 Expanded NOESY spectrum of compound 61

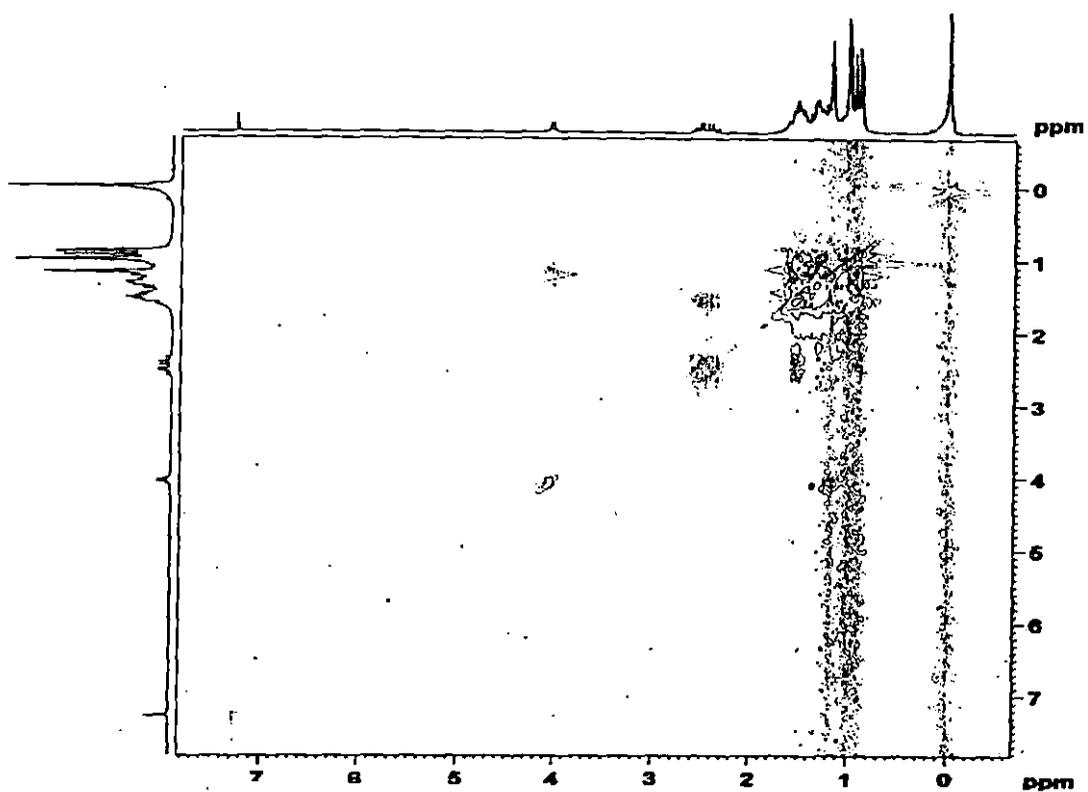


Figure 27 TCOSY spectrum of compound 61

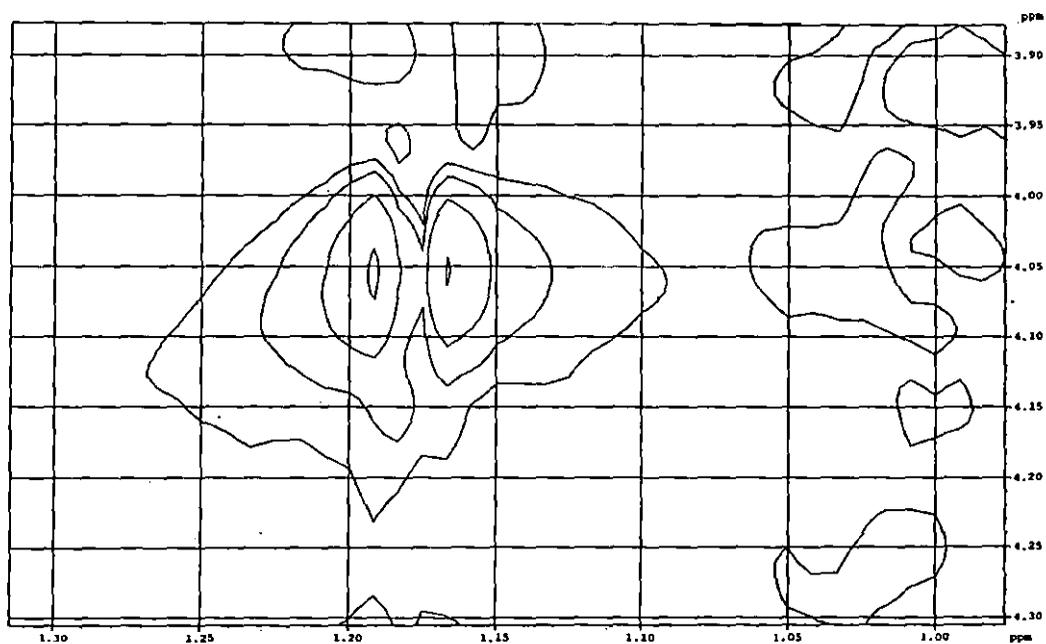


Figure 28 Expanded TCOSY spectrum of compound 61

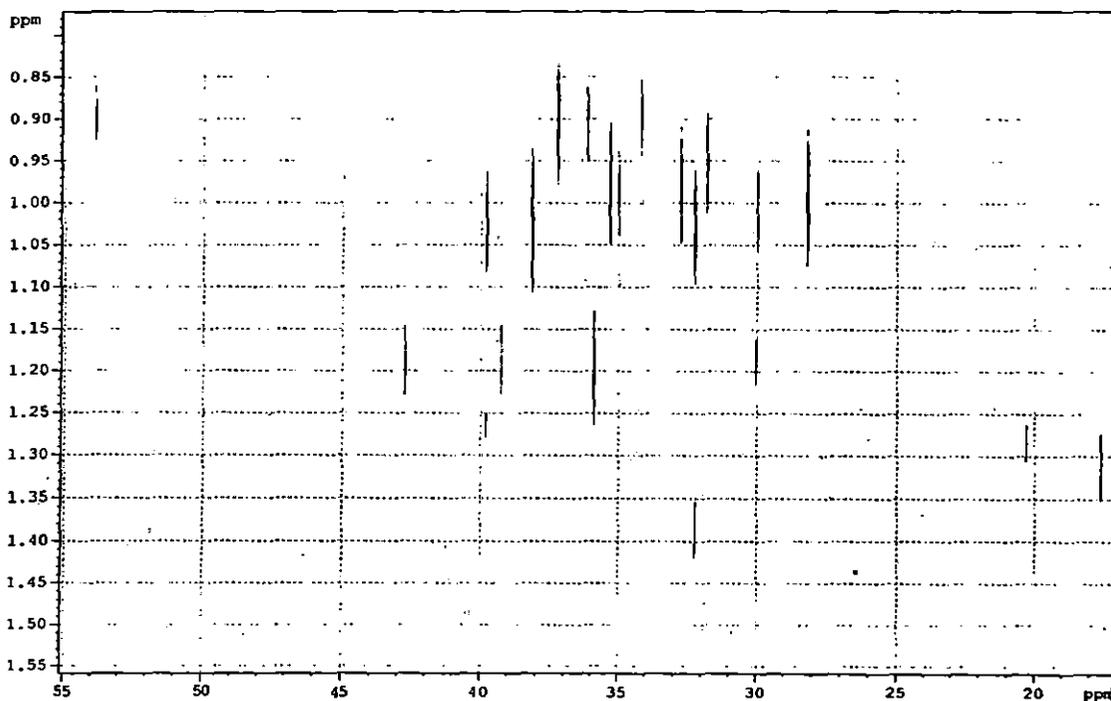
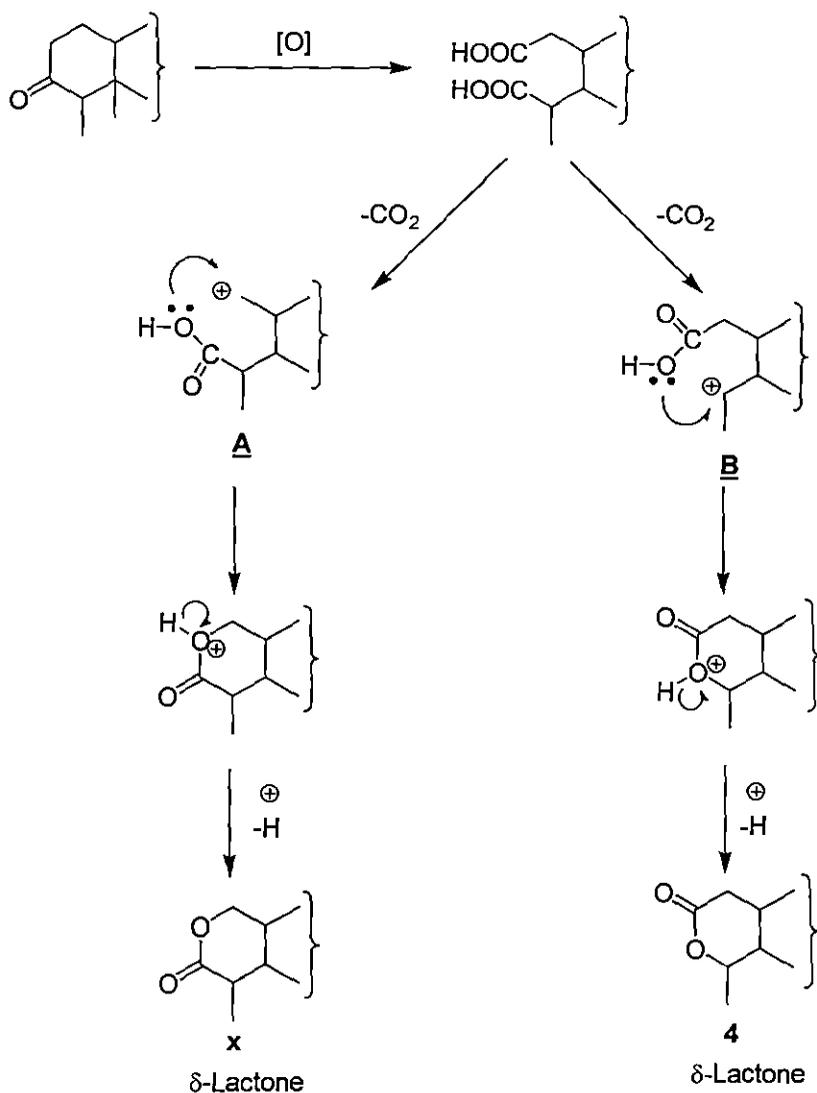


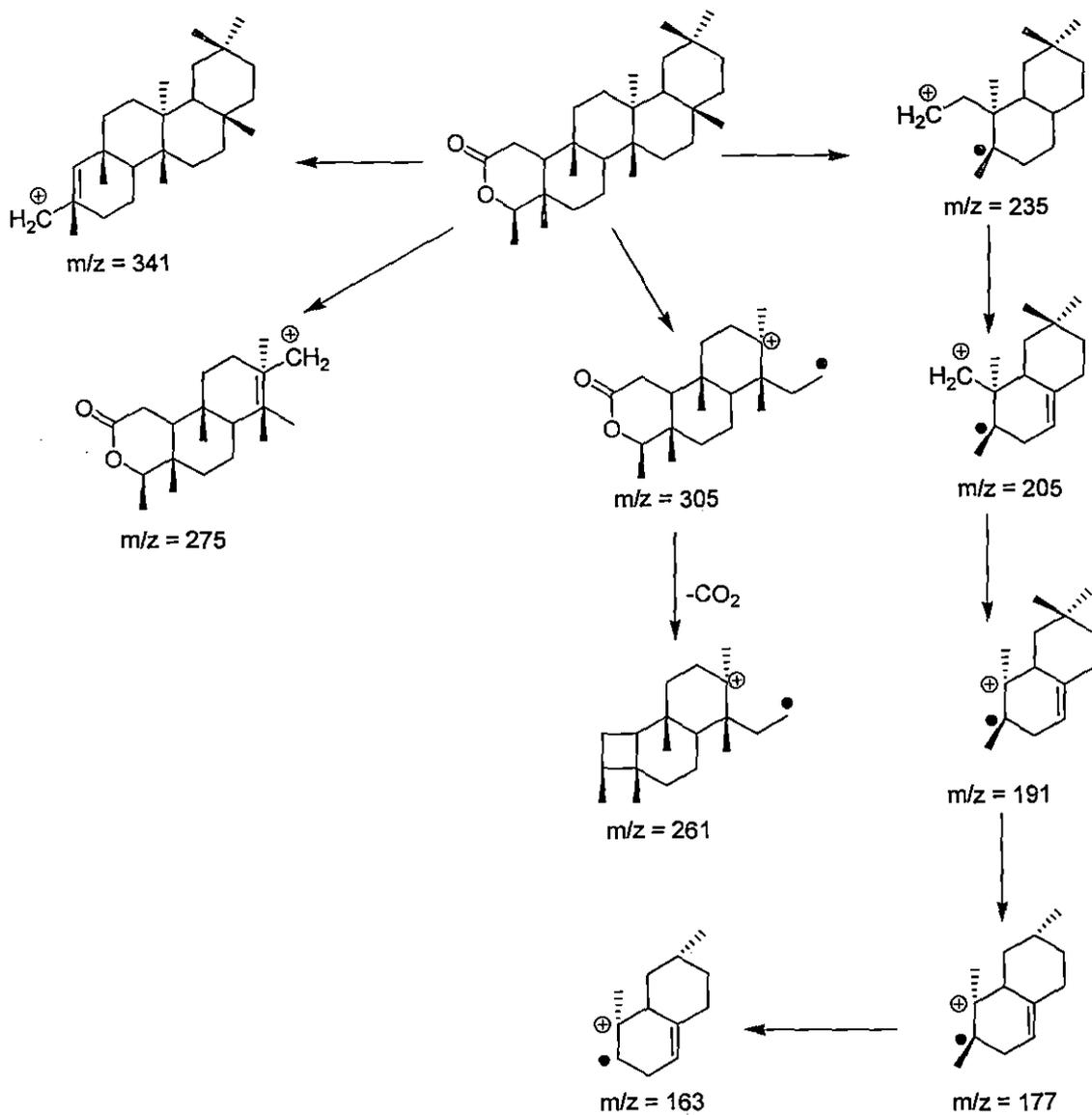
Figure 29 HETCOR spectrum of compound 61

The present author also predicted the mechanism of formation of the δ -lactone. It is depicted below. While LTA oxidation, decarboxylation will occur from either of the two positions to generate the carbocation intermediate A or B. Subsequent attack on the carbon site of the cation by the lone pair of electrons on the oxygen atom hydroxyl group of the retained carboxyl acid moiety followed by proton elimination resulted in the formation of two different isomeric δ -lactones. However, in practice compound **61** was solely generated. This fact can also be explained by considering the stability of the intermediated carbocations A and B. Being secondary in character, B was more stable than A (a primary carbocation) and therefore, in the reaction medium carbocation had generated exclusively thus forming **61** as the final product of the reaction.



Scheme 17 Probable mechanism of formation of the δ -lactone

Finally the structure of the synthesized δ -lactone, **61** was confirmed by the mass fragmentation pattern, schematically represented below (Scheme 18).



Scheme 18 Probable mass fragmentation pattern of the synthesized δ -lactone, 61

Section B

3D Molecular docking and Antitopoisomerase activities of the derived compounds

1. Introduction

Topoisomerases are ubiquitous enzymes that direct and modify the topological state of DNA.⁴¹⁻⁴⁶ They play crucial role in several aspects of DNA metabolism like replication, transcription, recombination and chromosomal segregation at mitosis.^{45,46} These enzymes act by sequential breakage and reunion of either one DNA strand (Topoisomerase I) or both DNA strands (Topoisomerase II).^{45,46} All vertebrates have two highly similar, though functionally discrete, Topo II isoforms, α and β .^{31,41-46} Multiple studies have shown the Topo II α levels increase during cell proliferation and this enzyme appears to be the isoform involved in mitosis.⁴¹⁻⁴⁶ The pharmacological inhibition of this enzyme has therefore been identified as target for anticancer drug development.⁴¹⁻⁴⁶ To date several topoisomerase inhibitors have been identified. Among them plant derived camptothecin and podophyllotoxins/etoposides have notable therapeutic efficacy as antitumor drugs. Despite their numerous applications, one cannot ignore the toxicity associated to these compounds.^{47,48} Therefore, the development of newer drugs with lesser side effects, more chemical stability and better efficacy is indispensable.

In the present study 3D molecular docking of structurally modified friedelan derivatives, highly oxidized on ring A were studied on crystal structure of topoisomerase II α (1bgw for topoisomerases II α , PDB)⁴⁹ was performed to evaluate the binding energies as well as their mode of interaction. Finally, the molecules were tested for their ability to inhibit the catalytic activity of topoisomerases II α .

2. 3D Molecular Docking studies of Friedelan analogs

To determine whether the friedelan analogs have potential as topoisomerase inhibitor, the parent compounds (**1** and **29**) and their hemisynthetic derivatives ((**59**, **61** and **45**) were docked into the central catalytic domain of the enzyme (1bgw PDB for topoisomerase II α)⁴⁹ by using AutoDock 4.

A previously modeled lupane bound structure was used as a starting point for calculating the lowest energy conformation of the bound ligands. The amino acids ARG 1016, HIS

1012, TYR 805, LYS 812 and LYS 713, have been shown to be present in the triterpenoids (lupane skeleton) binding domain of Topo II α .⁴⁹ An initial docking was therefore performed considering these amino acid residues as flexible for binding of the present triterpenoid derivatives. Although, the calculated binding energies are negative for all friedelan derivatives (ligands) against the reported residues, the lowest value is obtained for LYS 713 (feasible binding, -9.46 Kcal/mol for **1**, -8.54 Kcal/mol for **29**, -8.13 Kcal/mol for **59** and -8.81 Kcal/mol for **61**). However, in docked complexes LYS 713 is not within 3.5 Å from the ligand. Hence, despite good binding energy values, there remain scientific limitations to consider any type of noncovalent interactions (H-bonding or electrostatic interaction) between the ligands and the flexible residues. These observations preclude a similar/common binding for lupane and friedelan derivatives in Topoisomerase II α (1bgw, PDB).

Table 2 Estimated binding energy of different compounds against LYS 713

Entry	Compound	Estimated energies (Kcal/mol)				
		BIE	IME	INE	TE	UE
1	1	-9.46	-9.46	+0.00	+0.00	+0.00
2	29	-8.54	-8.84	+0.01	+0.30	-0.01
3	59	-8.13	-9.32	-0.26	+1.19	+0.26
4	61	-8.81	-8.81	+0.00	+0.00	+0.00

BIE, Binding energy; IME Intermolecular energy; INE, Internal energy; TE, Torsional energy; UE, Unbound system's energy

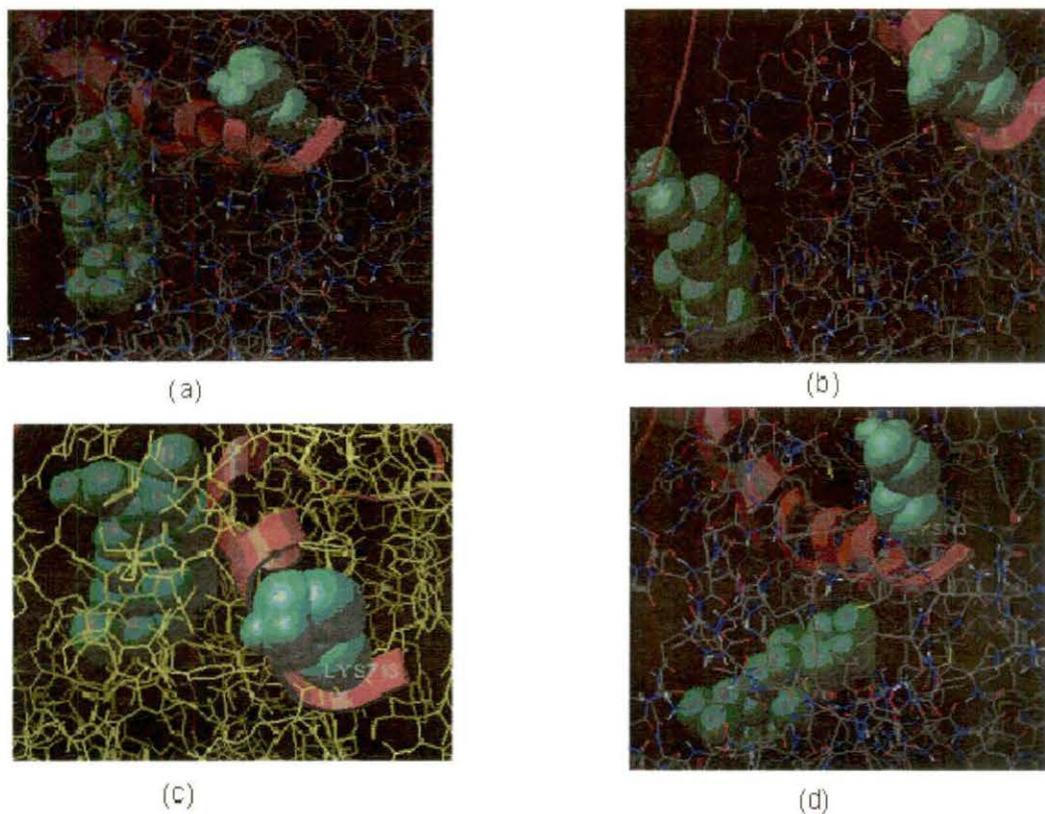


Figure 30 Detailed view of docked structures of compounds **1** (a), **29** (b), **59** (c) and **61** (d) against LYS713 of Topo II α .

To obtain a better understanding of the binding site, amino acids neighbors within 3.5 Å from the ligand were considered for final docking studies. Interestingly, the calculated binding energies of the ligands to the residues that are well within 3.5 Å are acceptable values in every case.

The results of molecular docking of hemisynthetic friedelan analogs on TopoII α are overlaid in figure 3. The compound **1** binds to the hydrophilic region of the enzyme involving ASN 765, ASN 752, GLY 766, TYR 760, ARG 1172, indicating the bulk of intermolecular interactions as non-ionic. In addition, the carbonyl group in ring A of the compound binds via a hydrogen bond to amino group of LEU 791. It also forms a water mediated hydrogen bond with ASN 769 (Figure 3a). Compound **29** differs from compound **1**, in having an additional beta- hydroxyl group at position C-2. The presence of the beta hydroxyl group modifies the binding properties of the molecule to the DNA-binding domain of the Topo II enzyme. Most of the interactions between compound **29** and TopoII are essentially hydrophobic and the molecule interacts with aliphatic side

chains of TopoII, including ALA 778, ALA 781, ALA 723, GLY 633 and ALA743 (Figure 3b). Presence of the carbonyl group and non bonding electron pair in oxygen atom of the lactone ring impart some polar properties to the compound **61**. From the results in Figure 3d, it is evident that lactone ring in the molecule interacts with a polar region of the enzyme containing ASP 513, GLU 512, TYR 511, ARG 508. Moreover, carbonyl group in lactone ring also forms hydrogen bond with hydroxyl group of TYR 511 and side chain amino group of ARG 508. On the other hand, rest of the nonpolar part of compound **61** binds to an alanine rich hydrophobic pocket of TopoII consisting of aliphatic side chains of ALA 778, ALA 779, ALA 780 and ALA 781, and also to ILE 553.

Compounds **59** and **45** both are seco-friedelan derivatives and they have certain structural similarities. Their skeletons contain carboxylic acids, which on deprotonation will generate the carboxylate ion. Hence, it can be predicted that both **59** and **45** will bind at similar sites in the structural space of the enzyme and the binding site should have a condensed positive charge for the best fitting of the ligands. As shown in figure 3c and 3e, compounds **59** and **45** bind to a condensed positively charged polar site of the enzyme that permits attractive electrostatic interactions with the neighboring residues. In addition, GLY 703, GLN 704 and LYS 701 residues are in common in their binding domain, thus confirming the prediction. The compound **59** also showed three H-bond interactions with the neighboring THR 745, SER 741 and TYR 735 residues.

From the present docking studies it can be inferred that some subtle change in molecular structure of the drug molecule alters the ligand binding domain in the drug target. These phenomena is very interesting as well as desirable for drug designing because repeated application of same drug/compound leads to the development of resistance to the action of the drug, due to unavoidable conformational modifications in the drug target. A suitable change in structure or more precisely specific modification in the structural arrangement/connectivity of the drug molecule may enable it to bind with some other nearby residues within the radius of binding domain. As a result, the newer molecule may again act as a good inhibitor to the host molecule and subsequently can show its drug efficacy.

Table 3 Names of the neighbouring residues

Entry	Ligand	Neighbouring residues		BIE (Kcal/mol)
		Within 3.5 A	Within 2.5 A	
1	1	ARG 691	ARG 691	-9.48
		GLN 744	GLY 703 ^a	
		GLN 751	TYR 735	-8.82
		GLY 703		
2	29	TYR 735		
		ARG 691	ARG 691	-8.51
		GLN 704	SER 741	-10.00
		GLN 744	TYR 735	-9.95
		SER 741		
		THR 745		
3	59	TYR 735		
		ALA 831	ASN 757	-8.82
		ARG 907	ASN 829	-8.88
		ASN 757	GLN 704	-8.39
		ASN 829		
		GLN 704		
		GLN 744		
		GLY 830		
4	61	LYS 701		
		SER 756		
		ALA 780	GLN 632	-8.82
		ARG 508	LYS 515	-8.82
		ARG 782		
		ASP 513		
		GLN 632		
		GLN 704		
		GLU 512		
		ILE 553		
		LYS 515		
5	45	TRY 511		
		ALA 780	GLN 632	-8.01
		ARG 508	ASP 513	-9.38
		ARG 782	GLY 633 ^a	
		GLN 632	LYS 515	-5.61
		ASP 513		
		GLY 633		
		LYS 515		
		LEU 631		
		GLU 512		
		ILE 553		
		TYR 511		
PHE 628				

BIE, Estimated binding energy of the most stable conformation

a, For this residue no active torsion is present

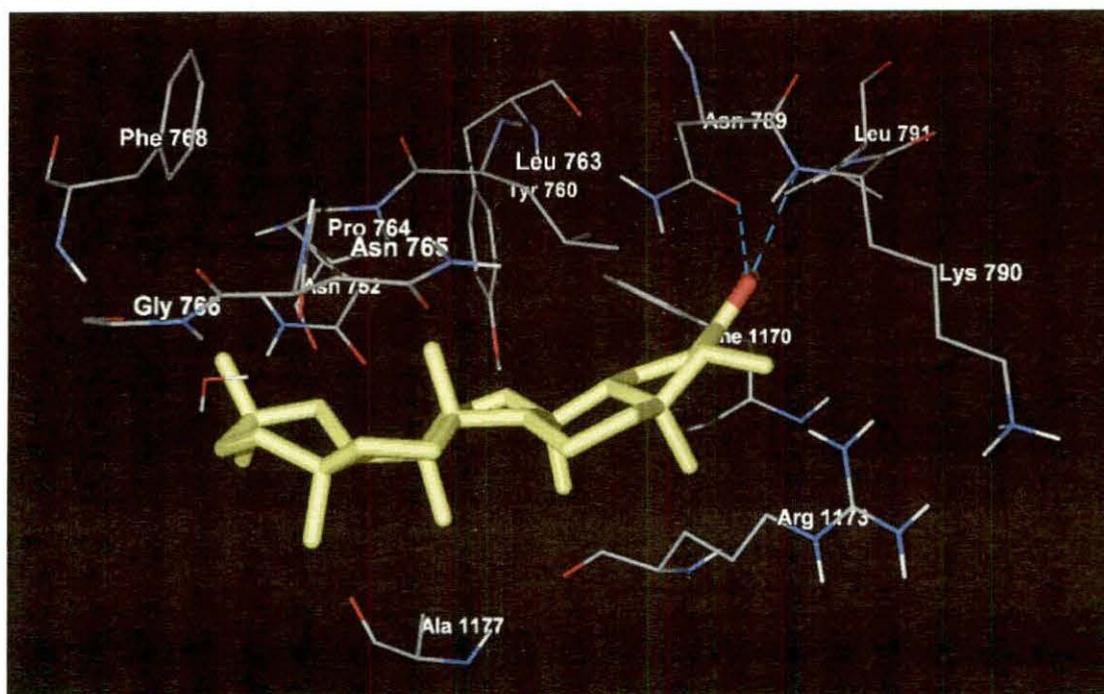


Figure 31 Detailed docked view of friedelin, 1 on the 1bgw.pdb

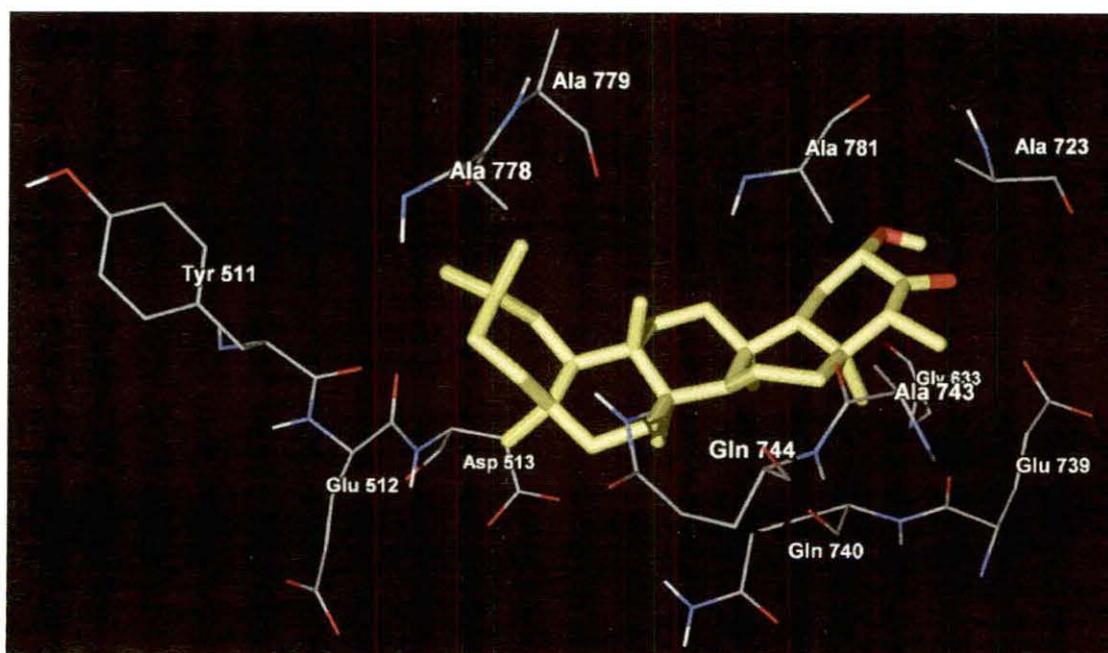


Figure 32 Detailed docked view of cerin, 29 on the 1bgw.pdb

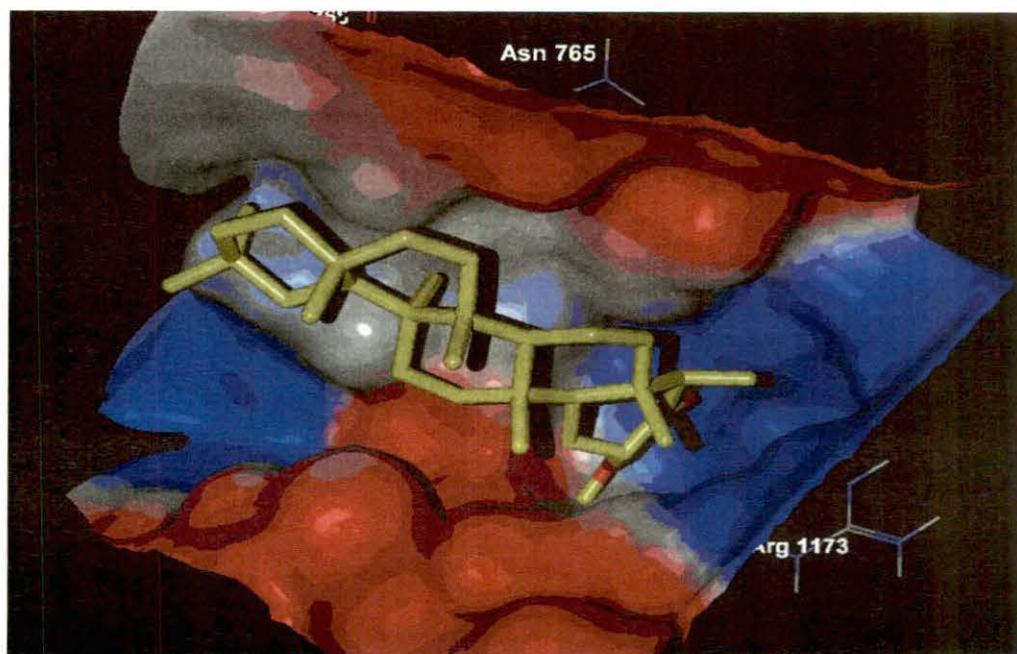


Figure 33 Electrostatic interaction view of cerin, 29 inside the hydrophobic pocket of the binding site in the protein

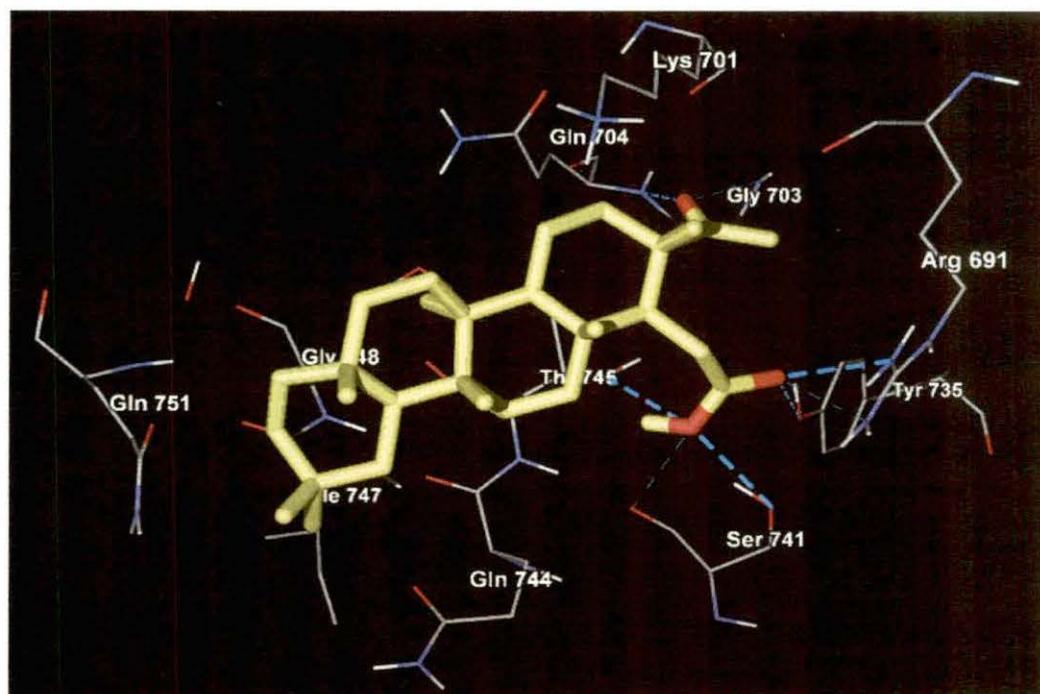


Figure 34 Detailed docked view of compound 59 on the 1bgw.pdb

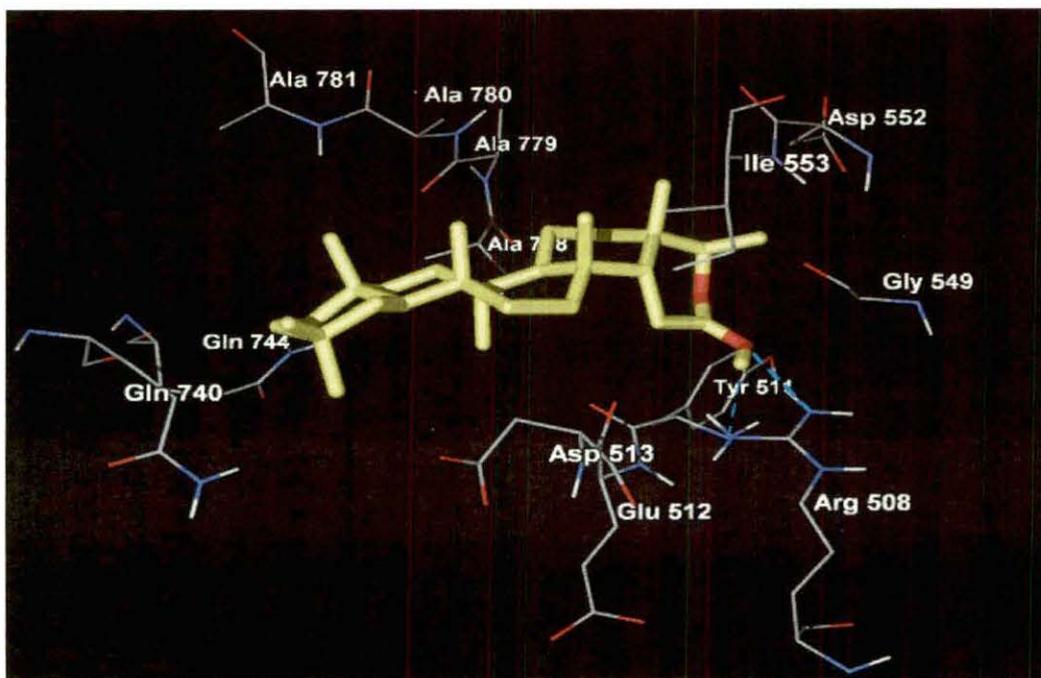


Figure 35 Detailed docked view of compound 61 on the 1bgw, pdb

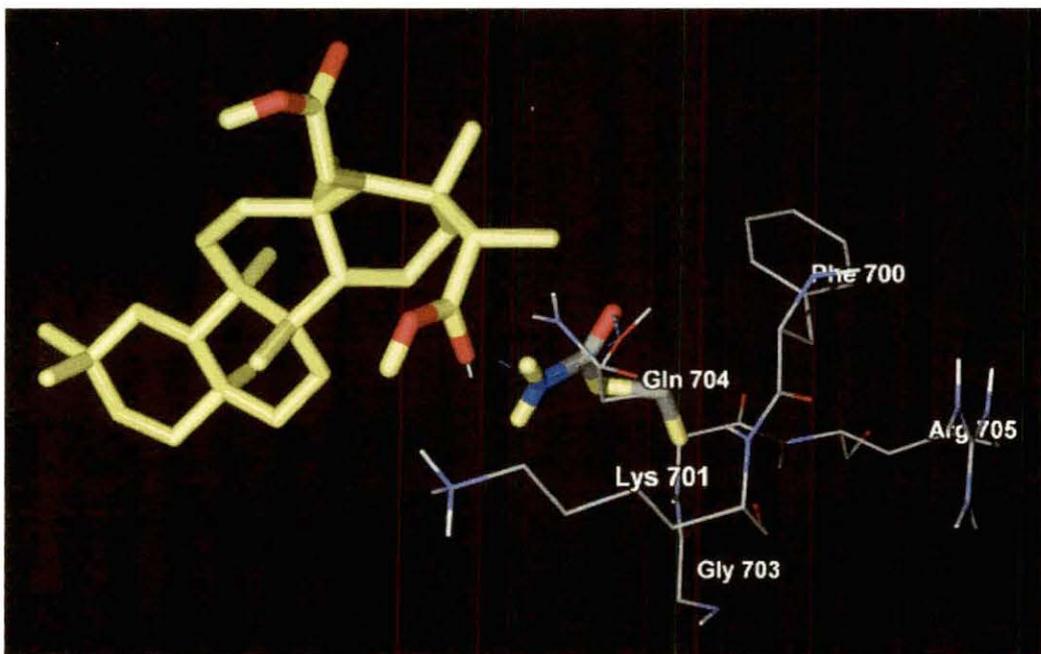


Figure 36 Detailed docked view of compound 45 on the 1bgw, pdb

Additionally, the estimated binding energies of ten different conformations of compounds **59** and **45** against the surrounding residues (only that within 2.5Å) are presented in figure 38.

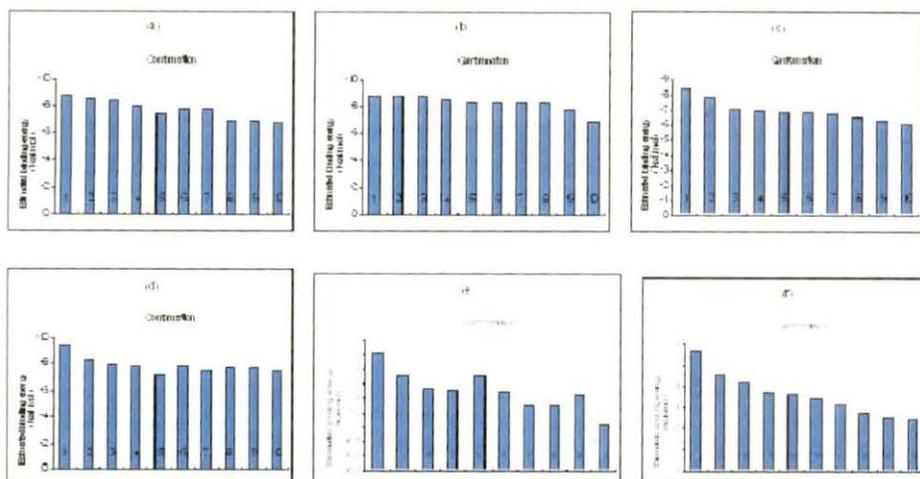


Figure 38 Plots of estimated binding energy (Kcal/mol) vs the ten possible conformations of the ligand; plots (a), (b) and (c) are for compounds **59** for ASN 757, ASN 829 and GLN 704 residues respectively, plots (d), (e) and (f) are for compound **45** for ASP 513, GLN 632 and LYS 515 residues respectively.

3. Topoisomerase inhibitory activity of Friedelan derivatives

Inhibition of catalytic activity of topoisomerase constitutes a useful strategy for the identification of potential antitumor agents. Topo II α creates transient breaks in supercoiled DNA resulting in DNA relaxation. The relaxed DNA can be distinguished from supercoiled DNA by gel electrophoresis analysis. The results of molecular docking studies on interaction of hemisynthetic friedelan derivatives with DNA binding domain of human Topo II α , was further confirmed by examining DNA relaxation activity of Topo II α . The ATP dependent relaxation of supercoiled DNA by the enzyme was monitored in absence or presence of hemisynthetic friedelan derivatives **59** and **45**. From the results in figure-39, it is evident that the inhibitory effects of compounds **59** and **45** were dose dependent. They showed complete inhibition of the catalytic activity of Topo II α at 100 and 50 μ M concentrations; whereas partial inhibition of the activity was observed at a concentration of 25 μ M. Compounds **59** and **45** couldn't be studied due to their partial solubility in DMSO.

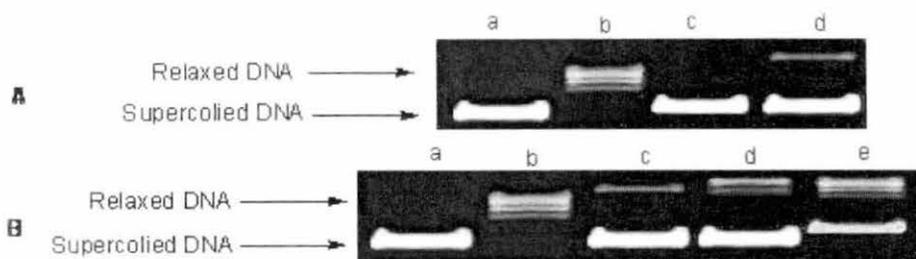


Figure 39 Effect of compound **3a** (A) and **5a** (B) on DNA relaxation activity by DNA topoisomerase II α (A), Lane a: supercoiled DNA alone; Lane b: control no drug (2 unit of topo II); Lanes c and d: 100 and 50 μ M of **3a** respectively. (B) Lane a; supercoiled DNA alone; Lane b: control no drug (2 unit of topo II); Lanes c, d and e: 100, 50 and 25 μ M of **5a** respectively.

4. Conclusion

The present author has successfully synthesized some oxygenated friedelan derivatives. All the molecules were characterized by spectral data and by comparison to that reported in literature. 3D molecular docking of these derivatives in the central catalytic domain of topoisomerase II α (1bgw PDB for topoisomerase II α) revealed the nature of the binding and the type of interactions between the synthesized compounds and the enzyme. The topoisomerase II α inhibitory activity was further confirmed by *in vitro* experiments. This is the first report of the antitopoisomerase activities of friedelan derivatives. The present author believes that the findings will definitely enrich the modern drug designing towards the invention of newer plant based chemotherapeutics to fight against human ailments. In addition the findings may provide a better understanding of the structure activity relationship towards the topoisomerase inhibitory activity of the pentacyclic triterpenoids, whether the carboxylation at ring A is truly the key factor of the defined activity or not.

Chapter III

EXPERIMENTAL AND REFERENCES

1. Experimental

1.1 General

Melting points (mp) were recorded in open capillary method and are uncorrected. IR spectra were recorded in Shimadzu 800 FT-IR spectrophotometer and Perkin-Erlmer FT-IR spectrophotometer using both KBr disc as well as nujol. NMR spectra were recorded in Bruker-Avanve 300 MHz FT-NMR instrument at ambient temperature with a 5mm BBO probe. The NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of CDCl₃ solvent as the standards. ¹H spectra were recorded in 300 MHz frequencies and ¹³C NMR spectra were recorded in 75.4 MHz frequencies. Coupling constant 'J' was calculated in Hz. The mass spectra were taken in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer. Elemental Analyses were done in Vario EL-III. Friedelin and cerin was isolated from cork through soxhlet apparatus using petroleum ether (60-80 °C) as the solvent. All the chemicals used were commercial grade and were purified prior to their use.

1.2 Collection of plant material

Friedelin was extracted from cork (*Quercus suber*). Cork was purchased from Kolkata, India in June 2009. The dried cork materials were then cut into very small pieces and used as such for the extraction of friedelin.

1.3 Isolation of friedelin and cerin

Very small pices of cork material (2 Kg) were extracted with petroleum ether, PE (60-120 °C) for a period of 5 days. After five days a second batch was run in the similar way. From the resulting yellowish colored solution solvent PE was recovered in reduced pressure. After the recovery of the solvent a brown gummy residue (500 g) was obtained that was then dissolved in hot chloroform. The solution was then filtered for any traces of impurities. Total volume of the solution was then decreased by a slow evaporation of the solvent chloroform. The resulting relatively concentrated solution was cooled to room temperature and kept in dark place for over night. Being very sparingly soluble in cold chloroform, cerin was crystallized out as slightly yellowish crystals at the bottom of the

container. It was filtered through a sintered Buckner funnel, washed twice with cold chloroform and dried. Repeated crystallization was performed to afford pure cerin in small needle shaped crystals of mp 248-250 °C.

All the washings and the filtrate were combined and solvent chloroform was evaporated out. The resulting brown gummy residue was then purified to afford pure friedelin over a column of silica gel (60-120 mesh). Pure PE and mixture of PE:ethyl acetate (EA) of varying concentrations were then used as eluent.

Table 4 Purification of the crude extract of cork, isolation of friedelin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-10	Nil	--
2	PE-EA (9.95:0.05)	11-14	Oil	--
3	PE-EA (9.95:0.05)	15-19	White solid (Traces)	--
4	PE-EA (9.95:0.05)	20-24	Nil	--
5	PE-EA (9.95:0.05)	24-72	White solid (≈ 0.8 g)	260-261 °C (Friedelin)

Further elution with more polar solvent mixture did not afford any solid material

1.3.1 Characterization of cerin

Cerin was recrystallized from hot chloroform and finally from ethyl acetate as slightly yellowish crystals of mp 248-250 °C. In its IR spectrum it gave peaks at 3394, 1713, 1462, 1377, 985, 696 cm⁻¹. Because of the poor solubility of cerin in most of the deuterated solvents the present author was unable to take the NMR spectra of cerin. Final confirmation of the isolated compound cerin was done by comparing (mixed mp, co-IR, co-tlc *etc.*) the compound with an authentic sample of cerin.

1.3.2 Characterization of friedelin

Friedelin was crystallized from chloroform-methanol solvent mixture as pure white crystals of mp 260-261 °C. In IR it gave peaks at 1715 cm⁻¹. In ¹H NMR eight methyl groups of friedelan skeleton appeared at δ_{H} 0.71 (s, 3H), 0.86 (s, 3H), 0.88 (s, 3H), 0.92 (s, 3H), 1.00 (s, 6H, two methyl groups), 1.05 (s, 3H) and 1.18 (s, 3H). Final confirmation of the isolated compound cerin was done by comparing (mixed mp, co-IR, co-tlc *etc.*) the compound with an authentic sample of friedelin.

1.4 Oxidation of 2 with CrO₃ in glacial acetic acid, 29

In a 50 mL round bottom flask 750 mg (1.7 mmol) of cerin, **29**, was dissolved in 5 mL of glacial acetic acid. In another 50 mL round bottom flask 168.3 mg (1.7 mmol) of CrO₃ was dissolved. Both the solutions were made homogeneous and the first solution was added slowly to the second solution with shaking. Shaking was continued for another two hours. The resulting solution was then poured into ice cold water and worked up with chloroform. It was then purified over a column of silica gel (60-120 mesh). Pure PE and mixture of PE:ethyl acetate (EA) of varying concentrations were then used as eluent.

Table 5 Purification of the oxidation product of cerin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Oil	--
2	PE-EA (9.95:0.05)	9-14	Nil Nil	-- --
3	PE-EA (9.9:0.1)	15-18	Nil	--
4	PE-EA (9.8:0.2)	19-24	Nil	--
5	PE-EA (9.1:0.9)	25-32	White solid	214-215 °C
6	PE-EA (9.0:1.0)	33-46	(\approx 0.52 g)	

Further elution with more polar solvent mixture did not afford any solid material

1.4.1 Characterization of the oxidized product, 59

It showed mp of 214-215 °C, IR at 3079, 1734 (H-bonded >C=O), 1696 (carbonyl of COOH group), 1465 (C-O), 1419, 1302, 1074 and 899 cm⁻¹, ¹H NMR eight tertiary methyls at δ_H 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23), 1.90 (dd, 1H, $J_{1eq10ax} = 4.0$ Hz, $J_{gem} = 15.6$ Hz, H-1), 2.35 (1H, dd, $J_{1ax10ax} = 6.6$ Hz, $J_{gem} = 15.6$ Hz, H-1), 2.30 (dd, 1H, $J_{10ax1eq} = 4.0$ Hz, $J_{10ax1ax} = 6.6$ Hz, H-10) and at δ_H 2.32 (s, 1H, COOH). Elemental analysis: C, 78.13% (cald. 78.33), O, 10.67% (cald. 10.79) and H 11.20 (cald. 10.88), signified the molecular formula C₂₉H₄₈O₃. Therefore the compound is 4-oxa-3,4-secofriedelan-3-oic acid (59).

1.4.2 Esterification of 59, preparation of 59a

500 mg (1.05 mmol) of 59 was dissolved in diethyl ether (50 mL) at cold (0-5 °C) in a 250 mL conical flask. To this an excess of diazomethane dissolved in ether at cold (0-5 °C) was added slowly with constant shaking. The whole reaction sequence was carried out in a fume cupboard. The resultant yellowish solution was kept overnight at dark. After that 2 mL of glacial acetic acid was added to this to neutralize the traces of diazomethane. The solution was then diluted with cold water and extracted with ether. Evaporation of solvent at vacuum gave a yellowish compound that was purified over a column of silica gel (60-120 mesh). Pure PE and mixture of PE:ethyl acetate (EA) of varying concentrations were then used as eluent.

Table 6 Purification of the acetylated product, 59a

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-7	Nil	--
2	PE-EA (9.95:0.05)	8-12	Nil	--
3	PE-EA (9.9:0.1)	13-17	Nil	--
4	PE-EA (9.8:0.2)	18-34	White solid (≈ 0.5 g)	167-168 °C

Further elution with more polar solvent mixture did not afford any solid material

1.4.2.1 Characterization of compound 59a

Compound **59a** was crystallized from chloroform-petroleum ether solvent mixture as white needle shaped crystals of mp 167-168 °C, IR at 3079, 1734, 1465 (C-O), 1419, 1302, 1248, 1074 and 899 cm^{-1} . All ^1H NMR peaks are similar to that of **3a**, except 3.66 (s, 3H, $-\text{OCOCH}_3$). Elemental analysis: C, 78.43% (cald. 78.55), O, 10.87% (cald. 10.99) and H 10.70 (cald. 10.46), signified the molecular formula $\text{C}_{30}\text{H}_{50}\text{O}_3$. Therefore, the compound is 4-oxa-3,4-secofriedelan-3-methanoate. 10.78

1.5 Oxidation of 1 with ammonium vanadate in glacial acetic acid

To a stirred mixture of ammonium vanadate (32 mg) and concentrated HNO_3 (10 mL) maintained at 0 °C was added slowly a solution of **1** (1 g) in glacial acetic acid (15 mL) during the course of 15 minutes. Then the reaction mixture was stirred for an additional one hour at 0 °C. The reaction mixture was then poured in ice cold water whereby a white solid separated out. The solid was extracted out with ether and the ether solution was washed with water and a solution of 10 % aqueous NaOH solution (3 x 100 mL) and with water till neutral and dried. The total aqueous layer was acidified with dilute HCl (6 N) and the white precipitated so formed was extracted with chloroform, washed with water till neutral and dried (anhydrous Na_2SO_4) and purified over a column of silica gel (60-120 mesh). Pure PE and mixture of PE:ethyl acetate (EA) of varying concentrations were then used as eluent.

Table 7 Purification of the oxidation product of friedelin

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Oil	--
2	PE-EA (9.95:0.05)	9-14	Nil	--
			Nil	--
3	PE-EA (9.9:0.1)	15-18	Nil	--
4	PE-EA (9.8:0.2)	19-24	Nil	--
5	PE-EA (9.1:0.9)	25-32	Nil	--
6	PE-EA (9.0:1.0)	33-46	Nil	--
7	PE-EA (8.5:1.5)	47-54	Nil	--
8	PE-EA (8.0:2.0)	55-72	White solid (≈ 0.52 g)	181-184 °C

Further elution with more polar solvent mixture did not afford any solid material

1.5.1 Characterization of compound 45

Compound **45** was hard to crystallize. In the ^1H NMR spectrum it showed eight tertiary methyls at δ_{H} 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23), the two acidic protons appeared at 9.89 (s, 2H). Elemental analysis: C, 75.83% (cald. 75.90), O, 13.67% (cald. 13.48) and H 10.50 (cald. 10.62), signified the molecular formula $\text{C}_{30}\text{H}_{50}\text{O}_4$.

1.5.2 Esterification of 45, preparation of 45

Esterification of compound **45** was carried out following the same procedure as that for compound **45** (Please follow paragraph 1.4.2 of this chapter). After the reaction was over, the reaction mixture was poured into ice cold water, a white precipitate appeared. It was the worked up with chloroform and checked for the tlc. In the tlc it showed the presence of a single compound that was very much lower in polarity than the starting compound. Solvent chloroform was recovered in reduced pressure and further purification was done

by column chromatography. Pure PE and mixture of PE:ethyl acetate (EA) of varying concentrations were then used as eluent.

Table 8 Purification of the acetylated product **45a**

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-5	Nil	--
2	PE-EA (9.95:0.05)	6-9	Nil	--
3	PE-EA (9.9:0.1)	10-14	Nil	--
4	PE-EA (9.8:0.2)	15-28	White solid (≈ 0.5 g)	167-169 °C

Further elution with more polar solvent mixture did not afford any solid material

1.5.3 Characterization of compound **45a**

Compound **45a** was crystallized chloroform-petroleum ether solvent mixture as white needle shaped crystals of mp 167-169 °C. All ^1H NMR peaks are similar to that of **45**, except 3.66 (s, 6H, 2-OCOCH₃), thus signifying that both the carboxylic acid group had been converted to ester groups. ^1H NMR spectrum showed eight tertiary methyls at δ_{H} 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23). Elemental analysis: C, 76.33% (cald. 76.45), O, 12.69% (cald. 12.73) and H 10.98 (cald. 10.83), signified the molecular formula C₃₂H₅₄O₄.

1.6 LTA Oxidation of **59**

A mixture of compound **59** (400 mg, 0.84 mmol), LTA (744.8 mg, 1.6 mmol) and glacial acetic acid (10 mL) was heated at 80 °C with stirring under nitrogen atmosphere for four hours. Ethane diol (15 mL) was added to neutralize the excess LTA and then the mixture was poured into ice cold water. A white precipitate was appeared. This aqueous portion was extracted with ether, washed with 10% sodium bicarbonate solution again washed with water till neutral and then dried over anhydrous sodium sulfate and purified by

column chromatography (60-120 mesh). Pure PE and mixture of PE:ethyl acetate (EA) of varying concentrations were then used as eluent.

Table 9 Purification of the LTA oxidation product of **59**, isolation of compound **61**

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Nil	--
2	PE-EA (9.95:0.05)	9-14	Nil	--
3	PE-EA (9.9:0.1)	15-19	Nil	--
4	PE-EA (9.8:0.2)	20-32	White solid (≈ 0.1 g)	266-267 °C

Further elution with more polar solvent mixture did not afford any solid material

1.6.1 Characterization of compound **4**

Compound **61** was recrystallized from chloroform-petroleum ether as white crystals of mp 266-267 °C, molecular formula $C_{29}H_{48}O_2$ from ESI (m/z 429 $M+1$), MALDI-TOF (m/z 428 M^+), IR at 2939, 2866, 1730 (six membered lactone), 1459 (C-O), 1388 (CH- CH_3), 1241 and 1082 cm^{-1} , 1H NMR at δ_H 0.84 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 0.16 (s, 3H) and 1.17 (s, 3H, H_3-28), 1.20 (d, 3H, $J = 9$ Hz, H_3-23), 4.05 (1H, q, $J = 6.3$ Hz). Elemental analysis: C, 81.18% (cald. 81.25), O, 7.36% (cald. 7.46) and H 11.46 (cald. 11.29), signified the molecular formula $C_{29}H_{48}O_2$. Therefore compound **61** is 3-oxafriedelan-2-one.

1.7 LTA Oxidation of **45**

A mixture of compound **45** (400 mg, 0.84 mmol), LTA (744.8 mg, 1.6 mmol) and glacial acetic acid (10 mL) was heated at 80 °C with stirring under nitrogen atmosphere for four hours. Ethane diol (15 mL) was added to neutralize the excess LTA and then the mixture was poured into ice cold water. A white precipitate was appeared. This aqueous portion was extracted with ether, washed with 10% sodium bicarbonate solution again washed with water till neutral and then dried over anhydrous sodium sulfate and purified by column chromatography (60-120 mesh).

Table 10 Purification of the LTA oxidation product of **45**, isolation of compound **61**

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-8	Nil	--
2	PE-EA (9.95:0.05)	9-14	Nil	--
3	PE-EA (9.9:0.1)	15-19	Nil	--
4	PE-EA (9.8:0.2)	20-32	White solid (\approx 0.1 g)	266-267 °C

Further elution with more polar solvent mixture did not afford any solid material

1.7.1 Characterization of the oxidized product, **61**

Compound **61** was recrystallized from chloroform-petroleum ether as white crystals of mp 266-267 °C, molecular formula $C_{29}H_{48}O_2$ from ESI (m/z 429 $M+1$), MALDI-TOF (m/z 428 M^+), IR at 2939, 2866, 1730 (six membered lactone), 1459 (C-O), 1388 (CH-CH₃), 1241 and 1082 cm^{-1} , ¹H NMR at δ_H 0.84 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 0.16 (s, 3H) and 1.17 (s, 3H, H₃-28), 1.20 (d, 3H, J = 9 Hz, H₃-23), 4.05 (1H, q, J = 6.3 Hz). Elemental analysis: C, 81.18% (cald. 81.25), O, 7.36% (cald. 7.46) and H 11.46 (cald. 11.29), signified the molecular formula $C_{29}H_{48}O_2$. Therefore compound **61** is 3-oxafriedelan-2-one.

1.8 3D Molecular Docking

Three dimensional molecular docking studies were carried out with AutoDock 4. Molecular drawing and 3D structure optimizations were carried out by Chem Sketch 12.0 software package. Initially we selected the ligand and by neighbour selection through Arguslab we located amino acid residues within 3.5Å surrounding the ligand. Molecular viewing was performed by Molegro molecular viewer as well as in AutoDock 4.

1.9 Topoisomerase II assay

Human TOPO-II α activity was measured by relaxation of supercoiled pBR322 plasmid DNA. Reaction mixture contained 10 mM Tris (pH-6.9), 50 mM KCl, 50 mM NaCl, 5mM MgCl₂, 1mM ATP, pBR 322 plasmid DNA (100 ng) and 2 Units of Topo II α , in a final volume of 20 μ l. For inhibition studies, the compounds were preincubated with

human TOPO-II α and DNA for 15 min. Compounds were used at the appropriate concentrations by dissolving in 2% (v/v) DMSO. DMSO didn't show detrimental effect on the enzyme activity at concentration up to 2 % v/v. Reaction mixture was incubated at 37⁰C for 30 min and stopped by addition of 2ml of 7mM EDTA. Reaction product was mixed with DNA loading dye and electrophoresed on 1% TAE-Agarose. The gel was stained with ethidium bromide (0.5 μ g/ml) for 20 min, destained twice in TAE buffer and then visualized using a Gel Doc-Imaging system (Spectronics, USA).

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Appendix



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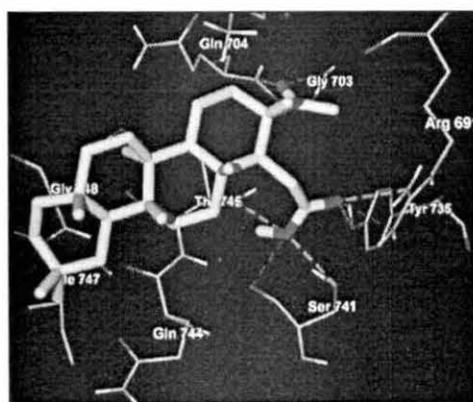
Original article

Synthesis of friedelan triterpenoid analogs with DNA topoisomerase II α inhibitory activity and their molecular docking studiesAmitava Mandal^a, Shilpi Ghosh^b, Ashim Kumar Bothra^c, Ashis Kumar Nanda^d, Pranab Ghosh^{a,*}^a Natural Products and Polymer Chemistry Laboratory, Department of Chemistry, University of North Bengal, Darjeeling, West Bengal 734 013, India^b Department of Biotechnology, University of North Bengal, Darjeeling, West Bengal 734 013, India^c Cheminformatics and Bioinformatics Laboratory, Department of Chemistry, Raiganj University College, Raiganj, North Dinajpur, West Bengal 733 134, India^d Department of Chemistry, University of North Bengal, Darjeeling, West Bengal 734 013, India

HIGHLIGHTS

- ▶ Very first report of topoisomerase inhibitory activity of the friedelan derivatives.
- ▶ 3D molecular docking on topoisomerase II α (1 bgw, PDB).
- ▶ Prediction of nature of interactions of the drug molecules with enzyme.
- ▶ Helpful for drug designing and mechanism of drug resistance phenomena.
- ▶ Prediction of dose dependent inhibition activity against topoisomerase II α .

GRAPHICAL ABSTRACT



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ABSTRACT

Five highly oxygenated friedelan derivatives (**3a**, **3b**, **4**, **5a** and **5b**) were synthesized. The structures of these compounds were established on the basis of spectral (IR, 1D and 2D NMR, MS etc.) and chemical data. The molecules, including the parent compounds were screened for three-dimensional (3D) molecular docking on the crystal structure of topoisomerase II α (1 bgw for topoisomerase II α , PDB). Compounds **3a** and **5a** showed a dose dependent inhibition of catalytic activity of human topoisomerase II α .

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1. Introduction

The discovery of lead for pharmaceutical investigations requires identification of new molecules that are able to interact with and modify a biological target [1,2]. Natural products represent one of the most relevant approaches to this goal. Natural products are

* Corresponding author. Tel.: +91 353 2776381; fax: +91 353 2699 001.
 E-mail address: pizy12@yahoo.com (P. Ghosh).

produced in living organisms by the activity of biosynthetic enzymes. They are thus recognized by the enzymes at specific binding sites complementary in shape and physicochemical properties. Natural products may possess the imprint for binding to the therapeutic target proteins containing the ligand binding motif similar to the biosynthetic enzyme [1]. Therefore, it is important to identify novel compounds those are complementary to biological structure space.

Triterpenoids are a large, ubiquitous and structurally diverse group of natural products that exhibit nearly 200 diverse skeletons [8]. Most significant triterpenoids are 6-6-6-5 tetracycles, 6-6-6-5 pentacycles, or 6-6-6-6-6 pentacycles [3] with physiological functions allied with chemical protection of plants [4]. Triterpenes, highly oxidized at ring A have been reported to possess a wide spectrum of biological activities [5].

Topoisomerases are ubiquitous enzymes that direct and modify the topological state of DNA [6,7]. They play crucial role in several aspects of DNA metabolism like replication, transcription, recombination and chromosomal segregation at mitosis [6,7]. These enzymes act by sequential breakage and reunion of either one DNA strand (Topoisomerase I) or both DNA strands (Topoisomerase II) [6,7]. All vertebrates have two highly similar, though functionally discrete, Topo II isoforms, α and β [8]. Multiple studies have shown the Topo II α levels increase during cell proliferation and this enzyme appears to be the isoform involved in mitosis [9,10]. The pharmacological inhibition of this enzyme has therefore been identified as target for anticancer drug development [9,10]. To date several topoisomerase inhibitors have been identified. Among them plant derived camptothecin and podophyllotoxins/etoposides have notable therapeutic efficacy as anti-tumor drugs. Despite their numerous applications, one cannot ignore the toxicity associated to these compounds [11,12]. Therefore, the development of newer drugs with lesser side effects, more chemical stability and better efficacy is indispensable.

Pentacyclic triterpenes, such as betulinic, boswellic, ursolic, and oleanolic acids are highly abundant in many edible fruits and vegetables. They are reported to inhibit cultured human melanoma, neuroblastoma, malignant brain tumor and leukemic cells [13,14]. They inhibit topoisomerases I and II α by contending with DNA for topoisomerase binding sites, thus preventing topoisomerase-DNA cleavable complex formation [3,13,14]. The general pentacyclic ring structure of triterpenoids has been reported to be essential for topoisomerase inhibitory activity [15]. However, the structure itself is inadequate for inhibition and the nature and arrangement of the side groups/functionality are the key factors [15].

Although, friedelan group of triterpenoids are 6-6-6-6-6 pentacycles and are wide spread in nature, surprisingly modern "lead research" on friedelan skeleton is not much prevalent. In recent times only few works on the transformative reactions on friedelin (1) have been reported [4,16,17]. Some recent studies have

indicated the *in vitro* anti-tumor activity of some of hemisynthetic friedelin derivatives [4,16,17], but the mechanisms through which these compounds achieve this effect has not yet been elucidated. Moreover, the systematic studies on transformative reactions and biological activity of cerin (2) are limited.

In the present study structurally modified friedelan derivatives, highly oxidized on ring A were synthesized from friedelin (1) and rare cerin (2) and characterized by IR, 1D, 2D NMR and MS. The 3D molecular docking of these molecules on crystal structure of topoisomerase II α (1 bgw for topoisomerases II α , PDB) [18] was performed to evaluate the binding energies as well as their mode of interaction. Finally, the molecules were tested for their ability to inhibit the catalytic activity of topoisomerases II α .

2. Results and discussion

2.1. Chemistry

We have synthesized some hemisynthetic friedelan compounds (3a, 3b, 4, 5a and 5b) by simple chemical modifications of triterpenes 1 and 2 (Fig. 1) isolated from *Quercus suber* (Cork).

Two different schemes (Schemes 1 and 2) were used for their synthesis. Both these schemes are quite suitable for possible large-scale applications. The conversions mainly focused on the oxidative cleavage of the ring A of the natural triterpenoids. The oxidative cleavage of 1 and 2 produced 2,3-secofriedelan-2,3-dioic acid (5a) and 4-oxa-3,4-secofriedelan-3-oic acid (3a), respectively. A controlled lead tetra acetate (LTA) oxidation on both 3a and 5a at room temperature selectively produced an A-nor-lactone, 4 (Schemes 1 and 2) with 68% yield.

Friedelin, 1 and cerin, 2 were isolated from cork by using soxhlet apparatus. Cerin was obtained as slightly yellowish crystals of melting point (mp) 260–261 °C. Oxidation of 2 in glacial acetic acid in presence of anhydrous CrO₃ followed by evaporation of the solvent at reduced pressure gave a yellow gummy residue (Scheme 1). Purification of the residue over a column of silica gel gave white powdered compound, 3a of melting point (mp) 214–215 °C, which upon methylation with diazomethane afforded the corresponding methyl ester 3b of mp 167–168 °C. In the IR spectrum compound 3a showed peaks at 3079, 1734 (H-bonded >C=O), 1696 (carbonyl of COOH group), 1465 (C–O), 1419, 1302, 1074 and 899 cm⁻¹. ¹H NMR spectra of compound 3a gave signals for the presence of eight tertiary methyls at δ_H 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23). Two methylene hydrogens at C₁ appeared at δ_H 1.90 (dd, 1H, $J_{1eq10ax} = 4.0$ Hz, $J_{gem} = 15.6$ Hz, H-1) and 2.35 (1H, dd, $J_{1ax10ax} = 6.6$ Hz, $J_{gem} = 15.6$ Hz, H-1). C₁₀ axial hydrogen atom appeared at δ_H 2.30 (dd, 1H, $J_{10ax1eq} = 4.0$ Hz, $J_{10ax1ax} = 6.6$ Hz, H-10) and the carboxyl hydrogen appeared as a singlet at δ_H 9.95 (s, 1H, COOH). ¹³C spectral data are tabulated in Table 1. On the basis of the above data, structure of compound 3a was established as 4-oxa-3,4-secofriedelan-3-oic acid. Compound 3a on esterification with diazomethane gave the corresponding ester, 3b with 94% yield. In its ¹H NMR spectrum it gave a sharp singlet at δ_H 3.67 (s, 3H, –OCOCH₃) due to the ester methyl and all other signals were in good correlation to the proposed structure of 3b. The IR, NMR data of 3a and 3b were comparable to that reported in literature [19–21].

In an another attempt to synthesize friedelan derivatives highly oxidized at ring A, the oxidation of 1 was undertaken with ammonium vanadate in concentrated HNO₃-glacial acetic acid at 0 °C. Purification of the reaction mixture through column chromatography yielded a white powdered compound, 5a (Scheme 2). Compound 5a on esterification by diazomethane yielded the

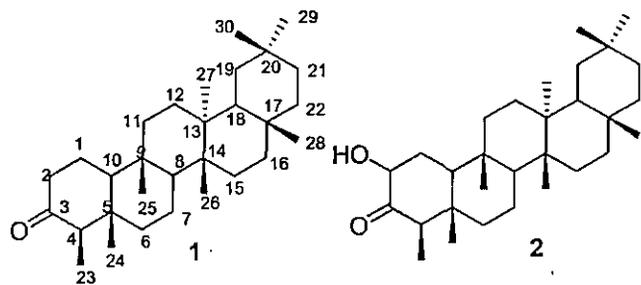
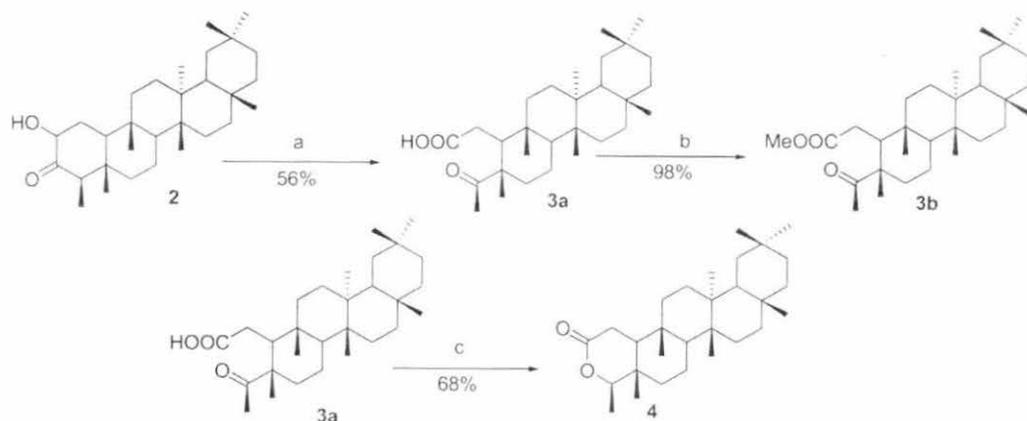


Fig. 1. Chemical structures of triterpenoids from *Quercus suber* (1) friedelin and (2) cerin.



Scheme 1. Oxidative ring cleavage of compound **2**. Reagents and conditions a, Dry CrO₃, glacial AcOH, ice cold water, CHCl₃, anhyd. Al₂(SO₄)₃; b, Diethyl ether, CH₂N₂, glacial AcOH, ice cold water, CHCl₃, anhyd. Al₂(SO₄)₃; c, Lead tetra acetate, glacial AcOH, ice cold water, CHCl₃, anhyd. Al₂(SO₄)₃.

corresponding ester **5b** exclusively of mp 167–169 °C. The IR, MS and NMR (both ¹H and ¹³C) data of **5a** and **5b** were comparable with the data reported in the literature [19–21].

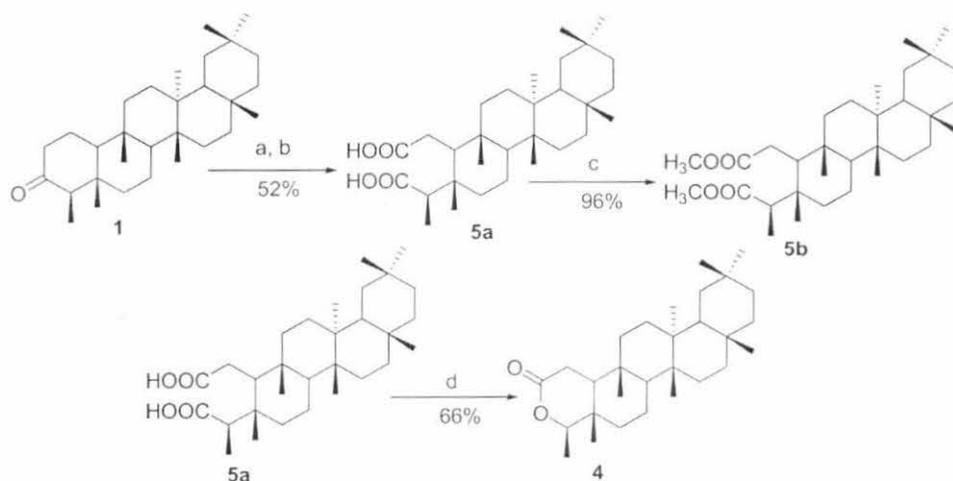
Further oxidation of **3a** and **5a** separately with LTA in glacial acetic acid furnished the same compound **4**, as white powder of mp 266–267 °C. The molecular formula of the compound, as determined by ESI (*m/z* 429 M+1) and TOF (*m/z* 428 M⁺) MS was C₂₉H₄₈O₂. This molecular formula was also corroborated by ¹H and ¹³C NMR spectroscopic data (Table 1). The ¹³C and DEPT spectroscopic data of **4** revealed the presence of 29 carbon signals including an ester carbonyl (δ_c 172.1), eight primary, ten secondary, four tertiary and seven quaternary carbon atoms. The six degrees of unsaturation inherent in the molecular formula of **4**, coupled with the NMR data showed the presence of one carbonyl group and five rings in the molecule **4**. These results indicated that a probable cyclization of the nor-seco acid **3a** and seco-diacid **5a** had occurred during the LTA oxidation and the nor-lactone, **4** had been generated.

In the IR spectrum, compound **4** gave peaks at 2939, 2866, 1730 (six membered lactone), 1459 (C-O), 1388 (CH-CH₃), 1241 and 1082 cm⁻¹. In its ¹H NMR spectrum it showed seven tertiary methyl signals at δ_H 0.84 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H) and 1.17 (s, 3H, H₃-28) (Fig. 1). Another

secondary methyl group at C₄ appeared at δ_H 1.20 (d, 3H, *J* = 9 Hz, H₃-23). C₄-H appeared as a quartet at δ_H 4.05 (1H, q, *J* = 6.3 Hz). C₁-Hs are deshielded due to the magnetic anisotropy induced by the neighboring carbonyl group at C₂ and each appeared as a doublet (dd) centered at δ_H 2.55 (1H, dd, *J* = 6.6 and 12.3 Hz, α H) and δ_H 2.40 (1H, dd, *J* = 6.6 and 12.9 Hz, β H). The slight difference in the observed '*J*' value may be due to the unequal coupling of the axial and equatorial hydrogens on that carbon. H₁₀ (α) appeared as a singlet at δ_H 1.56 (1H, s). All this data is in good agreement with that for friedelan skeleton.

The COSY, NOESY and HMBC spectra of **4** allowed assignment of all the proton and carbon signals. All the ¹³C data is presented in Table 1. All the above evidence supports the final compound as a nor-lactone. The probability of formation of the other possible six membered lactone (2-oxafriedelan-3-one) was ruled out because of the greater deshielding nature of H₄ (δ_H 4.05, 1H, q, *J* = 6.3 Hz). Nevertheless as compound **4** had been formed from a 2,3-seco compound (either **3a** or **5a**), there remains every possibility that the stereochemistry at C₄ had been changed, which may give rise to structure II (Fig. 2). Thus, the probable structures of the compound are either I or II (Fig. 2).

The exact stereochemistry at C₄ was confirmed by 2D NMR techniques. The NOESY spectrum of compound **4** gave significant



Scheme 2. Oxidative transformation of compound **1**. Reagents and conditions: a, Glacial CH₃COOH, Vanadium pentoxide, HNO₃ cold H₂O, Chloroform, Na₂SO₄ (Anhy.); b, H₂O₂; c, CH₂N₂, dry ether, glacial acetic acid, Na₂SO₄(Anhy.); d, LTA, glacial CH₃COOH, CHCl₃, Na₂SO₄ (Anhy.).

Table 1
 ^{13}C NMR data of parent and different hemisynthetic friedelan derivatives.

Position	δ_{c}				
	1	3a	4	1 ^a	3a ^a
1	22.3	32.9	34.9	22.3	32.9
2	41.5	176.2	172.0	41.5	178.2
3	213.2			213.3	
4	58.3	193.3	87.6	58.2	233.9
5	42.2	54.7	53.9	42.1	53.4
6	41.3	37.2	39.2	41.3	37.5
7	18.2	18.2	18.6	18.2	17.5
8	53.1	51.5	52.8	53.1	52.5
9	37.5	38.2	37.2	37.4	38.2
10	59.5	50.0	76.6	59.4	49.8
11	35.7	34.3	35.3	35.6	34.4
12	30.5	29.5	30.0	30.5	29.9
13	39.7	40.5	39.7	39.7	39.6
14	38.3	38.6	36.1	38.3	38.3
15	32.5	32.6	32.2	32.4	32.3
16	36.0	35.4	35.8	36.0	35.9
17	30.0	29.7	29.9	30.0	29.9
18	42.8	50.1	42.7	42.7	48.8
19	35.4	36.8	35.0	35.3	35.8
20	28.2	28.0	28.1	28.1	28.1
21	32.8	31.9	32.7	32.7	32.8
22	39.3	38.9	38.1	39.2	39.2
23	6.8	25.3	16.6	6.8	25.3
24	14.7	26.8	12.4	14.6	17.6
25	18.0	18.7	17.5	17.9	17.8
26	20.2	16.1	20.7	20.2	20.2
27	18.7	20.9	16.6	18.6	18.7
28	32.1	33.9	32.1	32.1	32.2
29	35.0	34.7	34.1	35.0	34.9
30	31.8	31.7	31.8	31.7	31.8

^a Reported data in literature.

information to this end. All NOE cross peaks have opposite phase to the diagonal, indicating that these arose from positive NOE enhancement as anticipated for a molecule of the size (having M^+ 428) under ambient conditions. In the NOESY spectrum, H_4 at δ_{H} 4.05 (1H, q, $J = 6.3$ Hz) showed two important correlations between H_{10} at δ_{H} 1.56 (1H, s) and H_6 (α) δ_{H} 1.09 (1H, m). In addition, cross peaks were observed by the NOE effects due to H_{10} at δ_{H} 1.56 (1H, s) with H_6 (α) δ_{H} 1.09 (1H, m). The above data established the stereochemistry of C_4 -H as alpha and hence structure I (Fig. 2) is the exact structure of 4. Thus compound 4 is 3-oxafriedelan-2-one.

2.2. 3D molecular docking studies of friedelan analogs

To determine whether the friedelan analogs have potential as topoisomerase inhibitor, the parent compounds (1 and 2) and their hemisynthetic derivatives (3a, 4 and 5a) were docked into the

central catalytic domain of the enzyme (1 bgw PDB for topoisomerase II α) by using AutoDock 4.

A previously modeled lupane bound structure was used as a starting point for calculating the lowest energy conformation of the bound ligands. The amino acids ARG 1016, HIS 1012, TYR 805, LYS 812 and LYS 713, have been shown to be present in the triterpenoids (lupane skeleton) binding domain of Topo II α [22]. An initial docking was therefore performed considering these amino acid residues as flexible for binding of the present triterpenoid derivatives. Although, the calculated binding energies are negative for all friedelan derivatives (ligands) against the reported residues, the lowest value is obtained for LYS 713 (feasible binding, -9.46 kcal/mol for 1, -8.54 kcal/mol for 2, -8.13 kcal/mol for 3a and -8.81 kcal/mol for 4). However, in docked complexes LYS 713 is not within 3.5 Å from the ligand. Hence, despite good binding energy values, there remain scientific limitations to consider any type of noncovalent interactions (H-bonding or electrostatic interaction) between the ligands and the flexible residues. These observations preclude a similar/common binding for lupane and friedelan derivatives in Topoisomerase II α (1 bgw, PDB).

To obtain a better understanding of the binding site, amino acids neighbors within 3.5 Å from the ligand were considered for final docking studies. Interestingly, the calculated binding energies of the ligands to the residues that are well within 3.5 Å are acceptable values in every case.

The results of molecular docking of hemisynthetic friedelan analogs on Topo II α are overlaid in Fig. 3. The compound 1 binds to the hydrophilic region of the enzyme involving ASN 765, ASN 752, GLY 766, TYR 760, ARG 1172, indicating the bulk of intermolecular interactions as non-ionic. In addition, the carbonyl group in ring A of the compound binds via a hydrogen bond to amino group of LEU 791. It also forms a water mediated hydrogen bond with ASN 769 (Fig. 3a). Compound 2 differs from compound 1, in having an additional beta hydroxyl group at position C-2. The presence of the beta hydroxyl group modifies the binding properties of the molecule to the DNA-binding domain of the Topo II enzyme. Most of the interactions between compound 2 and Topo II are essentially hydrophobic and the molecule interacts with aliphatic side chains of Topo II, including ALA 778, ALA 781, ALA 723, GLY 633 and ALA743 (Fig. 3b). Presence of the carbonyl group and non bonding electron pair in oxygen atom of the lactone ring impart some polar properties to the compound 4. From the results in Fig. 3d, it is evident that lactone ring in the molecule interacts with a polar region of the enzyme containing ASP 513, GLU 512, TYR 511, ARG 508. Moreover, carbonyl group in lactone ring also forms hydrogen bond with hydroxyl group of TYR 511 and side chain amino group of ARG 508. On the other hand, rest of the nonpolar part of compound 4 binds to an alanine rich hydrophobic pocket of Topo II consisting

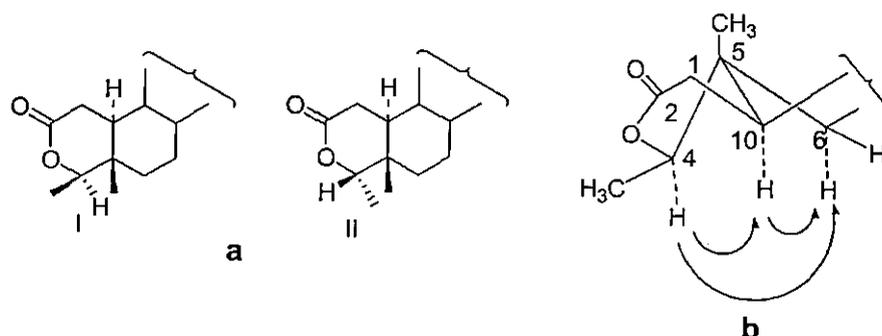


Fig. 2. (a) Partial structures of the two possible lactones; (b) Key NOESY correlation of compound 4.

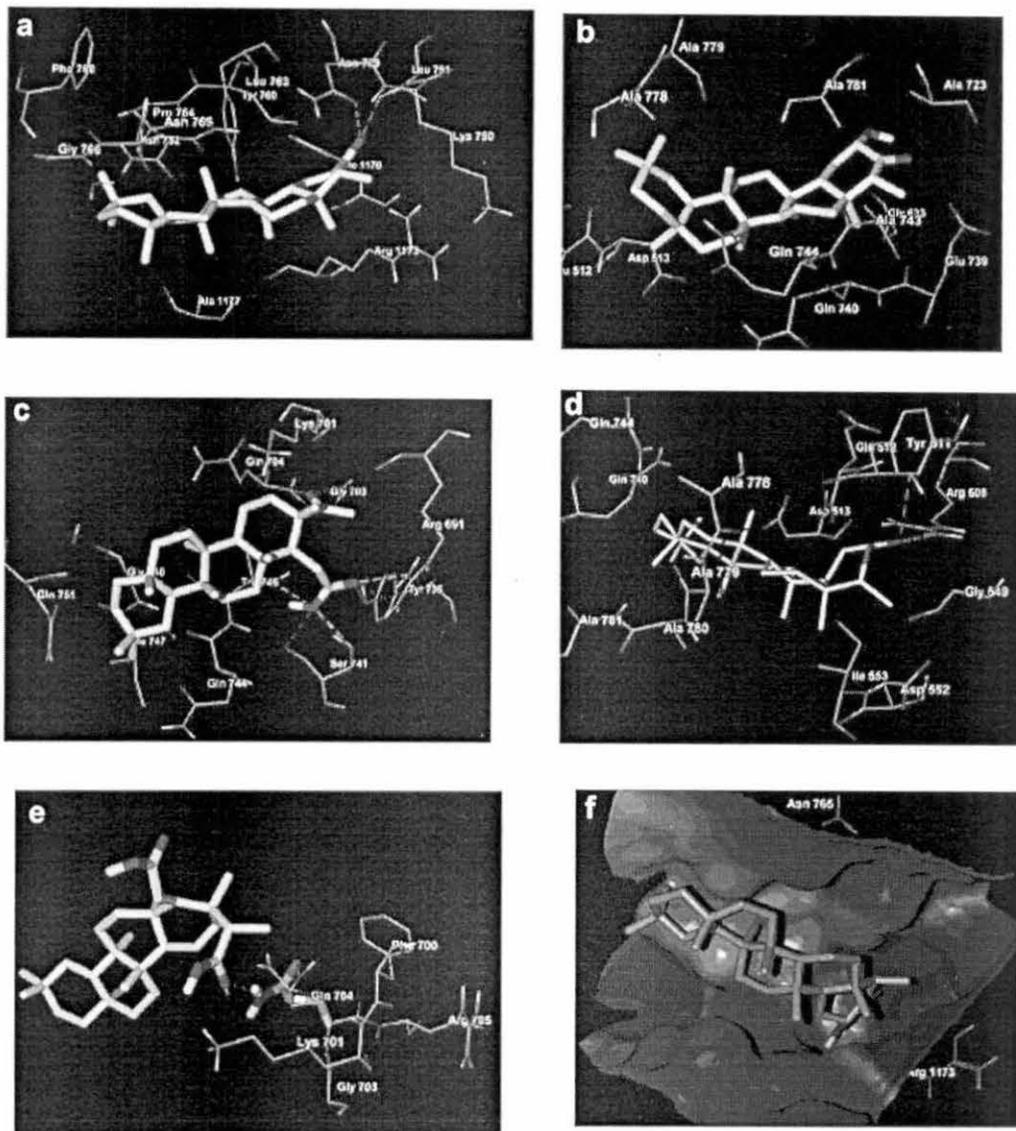


Fig. 3. Detailed docked view of different compounds; (a) for compound 1, (b) for compound 2, (c) for compound 3a, (d) for compound 4, (e) for compound 5a and (f) electrostatic interaction view for compound 2.

of aliphatic side chains of ALA 778, ALA 779, ALA 780 and ALA 781, and also to ILE 553.

Compounds **3a** and **5a** both are secofriedelan derivatives and they have certain structural similarities. Their skeletons contain carboxylic acids, which on deprotonation will generate the carboxylate ion. Hence, it can be predicted that both **3a** and **5a** will bind at similar sites in the structural space of the enzyme and the binding site should have a condensed positive charge for the best fitting of the ligands. As shown in Fig. 3c and e, compounds **3a** and **5a** bind to a condensed positively charged polar site of the enzyme that permits attractive electrostatic interactions with the neighboring residues. In addition, GLY 703, GLN 704 and LYS 701 residues are in common in their binding domain, thus confirming the prediction. The compound **3a** also showed three H-bond interactions with the neighboring THR 745, SER 741 and TYR 735 residues.

From the present docking studies it can be inferred that some subtle change in molecular structure of the drug molecule alters the

ligand binding domain in the drug target. These phenomena is very interesting as well as desirable for drug designing because repeated application of same drug/compound leads to the development of resistance to the action of the drug, due to unavoidable conformational modifications in the drug target. A suitable change in structure or more precisely specific modification in the structural arrangement/connectivity of the drug molecule may enable it to bind with some other nearby residues within the radius of binding domain. As a result, the newer molecule may again act as a good inhibitor to the host molecule and subsequently can show its drug efficacy.

2.3. Topoisomerase inhibitory activity of friedelan derivatives

Inhibition of catalytic activity of topoisomerase constitutes a useful strategy for the identification of potential anti-tumor agents. Topo II α creates transient breaks in supercoiled DNA resulting in DNA relaxation. The relaxed DNA can be distinguished

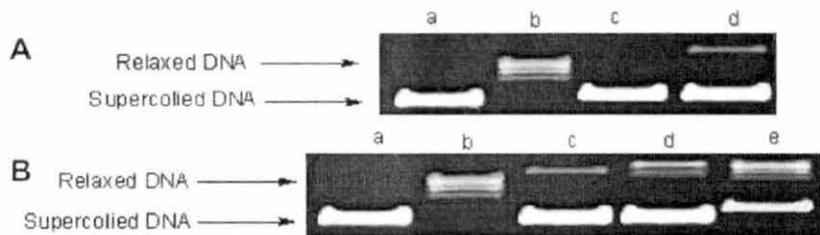


Fig. 4. Effect of compound **3a** (A) and **5a** (B) on DNA relaxation activity by DNA topoisomerase II α (A). Lane a: supercoiled DNA alone; Lane b: control no drug (2 unit of topo II); Lanes c and d: 100 and 50 μ M of **3a** respectively. (B) Lane a: supercoiled DNA alone; Lane b: control no drug (2 unit of topo II); Lanes c, d and e: 100, 50 and 25 μ M of **5a** respectively.

from supercoiled DNA by gel electrophoresis analysis. The results of molecular docking studies on interaction of hemisynthetic friedelin derivatives with DNA-binding domain of human Topo II α , was further confirmed by examining DNA relaxation activity of Topo II α . The ATP dependent relaxation of supercoiled DNA by the enzyme was monitored in absence or presence of hemisynthetic friedelin derivatives **3a** and **5a**. From the results in Fig. 4, it is evident that the inhibitory effects of compounds **3a** and **5a** were dose dependent. They showed complete inhibition of the catalytic activity of Topo II α at 100 and 50 μ M concentrations, whereas partial inhibition of the activity was observed at a concentration of 25 μ M. Compounds **3b** and **5b** couldn't be studied due to their partial solubility in DMSO.

3. Conclusion

We have successfully synthesized some oxygenated friedelin derivatives. All the molecules were characterized by spectral data and by comparison to that reported in literature. 3D molecular docking of these derivatives in the central catalytic domain of topoisomerase II α (1 bgw PDB for topoisomerase II α) revealed the nature of the binding and the type of interactions between the synthesized compounds and the enzyme. The topoisomerase II α inhibitory activity was further confirmed by *in vitro* experiments. This is the first report of the antitopoisomerase activities of friedelin derivatives. We do believe our findings will definitely enrich the modern drug designing toward the invention of newer plant based chemotherapeutics to fight against human ailments. In addition the findings may provide a better understanding of the structure activity relationship toward the topoisomerase inhibitory activity of the pentacyclic triterpenoids, whether the carboxylation at ring A is truly the key factor of the defined activity or not.

4. Experimentals

4.1. Material and method

The bark of *Q. suber* (Cork) was collected from commercial source. All the chemicals used were of commercial grade and were purified prior to their use.

Melting points were recorded in open capillary method and are uncorrected. IR spectra were recorded in Shimadzu 800 FT-IR spectrophotometer using both KBr disc as well as nujol. NMR spectra were recorded in Bruker-Avance 300 MHz FT-NMR instrument at ambient temperature with a 5 mm BBO probe. The NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of CDCl₃ solvent as the standards. ¹H spectra were recorded in 300 MHz frequencies and ¹³C NMR spectra were recorded in 75.4 MHz frequencies. Coupling constant 'J' was calculated in Hz. The mass spectra were taken in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer.

4.2. Extraction and isolation of friedelin (**1**) and cerin (**2**)

Mixture of friedelin and cerin was extracted by petroleum ether (60–80 °C) in a soxhlet apparatus. From the mixture cerin was isolated as a chloroform insoluble part that was then recrystallized from hot chloroform. Friedelin was collected from the chloroform solution, dried and purified by column chromatography.

4.3. Oxidation of **2** with CrO₃ in glacial acetic acid

Cerin, **2** (750 mg, 1.7 mmol) was dissolved in 5 mL of glacial acetic acid in a 50 mL round bottom flask. In another 50 mL round bottom flask 168.3 mg (1.7 mmol) of CrO₃ was dissolved. Both the solutions were made homogeneous and the first solution was added slowly to the second solution with shaking. Shaking was continued for another 2 h. The resulting solution was then poured into ice cold water and worked up with chloroform. It was then purified over a column of silica gel.

4-oxa-3,4-secofriedelan-3-oic acid (**3a**), mp 214–215 °C, IR at 3079, 1734 (H-bonded >C=O), 1696 (carbonyl of COOH group), 1465 (C-O), 1419, 1302, 1074 and 899 cm⁻¹, ¹H NMR eight tertiary methyls at δ_H 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23), 1.90 (dd, 1H, $J_{1eq10ax} = 4.0$ Hz, $J_{gem} = 15.6$ Hz, H-1), 2.35 (1H, dd, $J_{1ax10ax} = 6.6$ Hz, $J_{gem} = 15.6$ Hz, H-1), 2.30 (dd, 1H, $J_{10ax1eq} = 4.0$ Hz, $J_{10ax1ax} = 6.6$ Hz, H-10) and at δ_H 2.32 (s, 1H, COOH).

4.4. Esterification of **3a** to prepare **3b**

Compound **3a** (500 mg, 1.05 mmol) of was dissolved in diethyl ether (50 mL) at cold (0–5 °C) in a 250 mL conical flask. To this an excess of diazomethane dissolved in cold ether (0–5 °C) was added slowly with constant shaking. The whole reaction sequence was carried out in a fume cupboard. The resultant yellowish solution was kept overnight in dark. To this 2 mL of glacial acetic acid was added to neutralize the traces of diazomethane. The solution was then diluted with cold water and extracted with ether. Evaporation of solvent in vacuum gave a gummy residue that was purified over a column of silica gel (60–120 mesh). 4-oxa-3,4-secofriedelan-3-methanoate (**3b**), mp 167–168 °C, IR at 3079, 1734, 1465 (C-O), 1419, 1302, 1248, 1074 and 899 cm⁻¹. All ¹H NMR peaks are similar to those of **3a**, except 3.66 (s, 3H, –OCOCH₃).

4.5. Oxidation of friedelin (**1**) with ammonium vanadate in glacial acetic acid

To the stirred mixture of ammonium vanadate (32 mg, 0.27 mmol) and concentrated HNO₃ (10 mL) maintained at 0 °C was added slowly a solution of **1** (1 g, 2.34 mmol) in glacial acetic acid (15 mL) during the course of 15 min. The reaction mixture was stirred for an additional 1 h at 0 °C and then poured in ice cold

water whereby a white solid separated out. The solid was extracted out with ether and the ether solution was washed with water and a solution of 10% aqueous NaOH solution (3 × 100 mL) and with water till neutral and dried. The total aqueous layer was acidified with dilute HCl (6 N) and the white precipitated so formed was extracted with chloroform, washed with water till neutral and dried (anhydrous Na₂SO₄) and purified over a column of silica gel (60–120 mesh).

4.6. LTA oxidation of 3a/5a

A mixture of compound 3a (400 mg, 0.84 mmol), LTA (744.8 mg, 1.6 mmol) and glacial acetic acid (10 mL) was heated with stirring under nitrogen atmosphere for 4 h. Ethane diol (15 mL) was added to neutralize excess LTA and then the mixture was poured into ice cold water. The aqueous portion was extracted with ether, washed with 10% sodium bicarbonate solution again washed with water till neutral and then dried over anhydrous sodium sulfate and purified by column chromatography.

3-oxafriedelan-2-one (4), mp 266–267 °C, molecular formula C₂₉H₄₈O₂ from ESI (*m/z* 429 M+1), TOF (*m/z* 428 M⁺), IR at 2939, 2866, 1730 (six membered lactone), 1459 (C–O), 1388 (CH–CH₃), 1241 and 1082 cm⁻¹, ¹H NMR at δ_H 0.84 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H) and 1.17 (s, 3H, H₃-28), 1.20 (d, 3H, *J* = 9 Hz, H₃-23), 4.05 (1H, *q*, *J* = 6.3 Hz).

4.7. 3D molecular docking

Three-dimensional molecular docking studies were carried out with AutoDock 4. Structural drawing, 3D structure optimization and energy minimization were done by ACD Labs 12.0 and Arguslab respectively. Initially we selected the ligand and by neighbor selection through Arguslab we located amino acid residues within 3.5 Å surrounding the ligand. Molecular viewing was performed by Molegro molecular viewer as well as in AutoDock 4.

4.8. Topoisomerase II assay

Human TOPO-IIα activity was measured by measuring the relaxation of supercoiled pBR 322 plasmid DNA. Reaction mixture contained 10 mM Tris (pH-6.9), 50 mM KCl, 50 mM NaCl, 5 mM MgCl₂, 1 mM ATP, pBR 322 plasmid DNA (100 ng) and 2 Units of Topo IIα (USB, USA), in a final volume of 20 μL. For inhibition studies, the compounds were preincubated with human TOPO-IIα and DNA for 15 min. Compounds were used at the appropriate concentrations by dissolving in 2% (v/v) DMSO. DMSO didn't show detrimental effect on the enzyme activity at concentration up to 2% v/v. Reaction mixture was incubated at 37 °C for 30 min and

stopped by addition of 2 mL of 7 mM EDTA. Reaction product was mixed with DNA loading dye and electrophoresed on 1% TAE-Agarose. The gel was stained with ethidium bromide (0.5 μg/ml) for 20 min, destained twice in TAE buffer and then visualized using a Gel Doc-Imaging system (Spectronics, USA).

Acknowledgement

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.04.037.

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Synthesis of bioactive 28-hydroxy-3-oxolup-20(29)-en-30-al with antileukemic activity

Pranab Ghosh^{a*}, Amitava Mandal^a, Joydip Ghosh^b, Chiranjib Pal^b and Ashis Kumar Nanda^c

^aNatural Product and Polymer Chemistry Laboratory, Department of Chemistry, University of North Bengal, Darjeeling 734 013, West Bengal, India; ^bCellular Immunology and Experimental Therapeutics Laboratory, Department of Zoology, West Bengal States University, Barasat 700 126, 24 PGS (N), West Bengal, India; ^cDepartment of Chemistry, University of North Bengal, Darjeeling 734 013, West Bengal, India

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An easy and efficient route to partial synthesis of bioactive 28-hydroxy-3-oxolup-20(29)-en-30-al (1), starting from betulinic acid (2), has been developed (eight steps, 44% overall yield). Structures of all the compounds were determined by spectral studies (IR, ¹H, ¹³C NMR, MS, NOESY, COSY, etc.). Compound 1 and the precursors (2, 3, 5, and 7) showed antiproliferative activities against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor.

Keywords: triterpenoid; partial synthesis; betulinic acid; antileukemic activity

1. Introduction

Triterpenes represent a varied and important class of natural compounds. Among these, pentacyclic lupane-type triterpenes are one of the most significant subclasses which have shown to possess several medicinal properties [1,2]. The antitumor properties of plant extracts comprising lupane-derived triterpenoids have been demonstrated during the past 25 years for their cytostatic activity on various *in vitro* and *in vivo* cancer model systems [3]. Betulinic acid, one of the lupane-derived triterpenoids, exerts a selective antitumor activity on cultured human melanoma [3], neuroblastoma [4,5], malignant brain tumor [6], and leukemia cells [7]. Other pharmacological activities of lupane-type triterpenoids include anti-inflammatory activity [8], anti-carcinogenic activity [3], photosynthetic inhibitors [9], anti-HIV

[10], antidiabetic [11] and so on. Therefore, it is important to supply those triterpenoids with novel structures in sufficient amounts for further biological testing. And, in this regard, synthesis rather than isolation from natural sources is more efficient and also economical.

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of white blood cells. It is a broad term covering a spectrum of diseases. In turn, it is part of the even broader group of diseases called hematological neoplasms. In 2000, approximately 256,000 children and adults around the world developed some form of leukemia, and 209,000 died from it [12]. About 90% of all leukemias are diagnosed in adults. Most forms of leukemia are treated with pharmaceutical medications, typically combined into a multi-drug chemotherapy

*Corresponding author. Email: pizy12@yahoo.com

regimen. Some are also treated with radiation therapy. In some cases, a bone marrow transplant is useful. All these treatments are useful but only to a very limited extent, and the chemotherapeutics used to date are not specific to the affected cells, thus causing severe damage to the body. Despite recent improvements in the treatment of early-stage disease, leukemia blast crisis remains a therapeutic challenge because it is highly refractory to standard induction chemotherapy, with a response rate in myeloid blast crisis of less than 30% [13]. Therefore, development of mild selective chemotherapeutics is a real demand in contemporary medical sciences.

Medicinal plants and their phytoconstituents have always been a better choice for leukemia and nutraceuticals have been proven to have antileukemic activity in experimental studies [14]. Derivatives of betulin, basically triterpenoids of lupane skeleton, are reported to possess significant cytotoxicity against a wide variety of cancer cell lines [15]. In a recent publication, one such naturally occurring compound, 28-hydroxy-3-oxolup-20(29)-en-30-al (1), which has been reported [16] for the first time from the bark of *Acacia mellifera*, showed significant cytotoxicity against NSCLC-N6 cell line. Subsequently, Chen et al. [17] reported the presence of 1 in the methanol extract of *Microtropis fokienensis*, which has been reported [17] to induce apoptosis of human

leukemia HL60 cells and mediate cleavage of PARP and upregulation of Bax proteins. Compound 1 (28-hydroxy-3-oxolup-20(29)-en-30-al) was among the most cytotoxic substances obtained [22]. They also investigated the potential effects of 1 on growth inhibition of HL60 cells [17]. According to their results [17], this compound induced apoptosis in a dose-dependent manner. The molecular mechanism of compound 1 toward cancer cells is still a subject of continuous investigation and a specific target(s) has yet to be identified. Therefore, suitable derivatives of lupane may be considered as a group of compounds having promising bioactivity which can be used for further chemical as well as biological research. Henceforth, the present demands of this type of rare (scarce) naturally occurring triterpenoids are enormous in the contemporary medicinal research. Thus, it was felt necessary to supply this novel triterpenoid 1 in sufficient amounts for further biological testing. And, in this regard, synthesis rather than isolation from natural sources is more efficient and economical.

In view of the above and in continuation of our studies toward the chemical transformations of pentacyclic triterpenoids [18], we report herein a multistep protocol for the synthesis of 28-hydroxy-3-oxolup-20(29)-en-30-al (1) from betulinic acid (2; Figure 1). *In vitro*, anticancer activities of all the compounds having C-30 -CHO group (1, 2, 3, 5, and 7) are

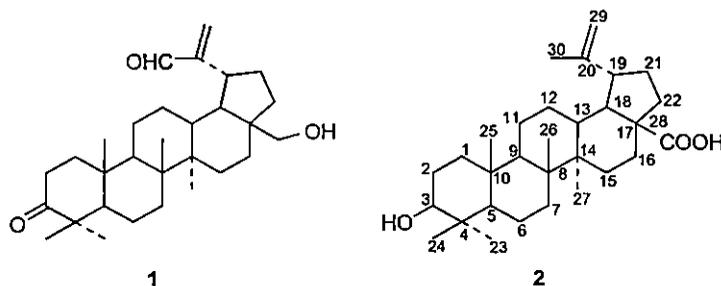


Figure 1. Structures of compounds 1 and 2.

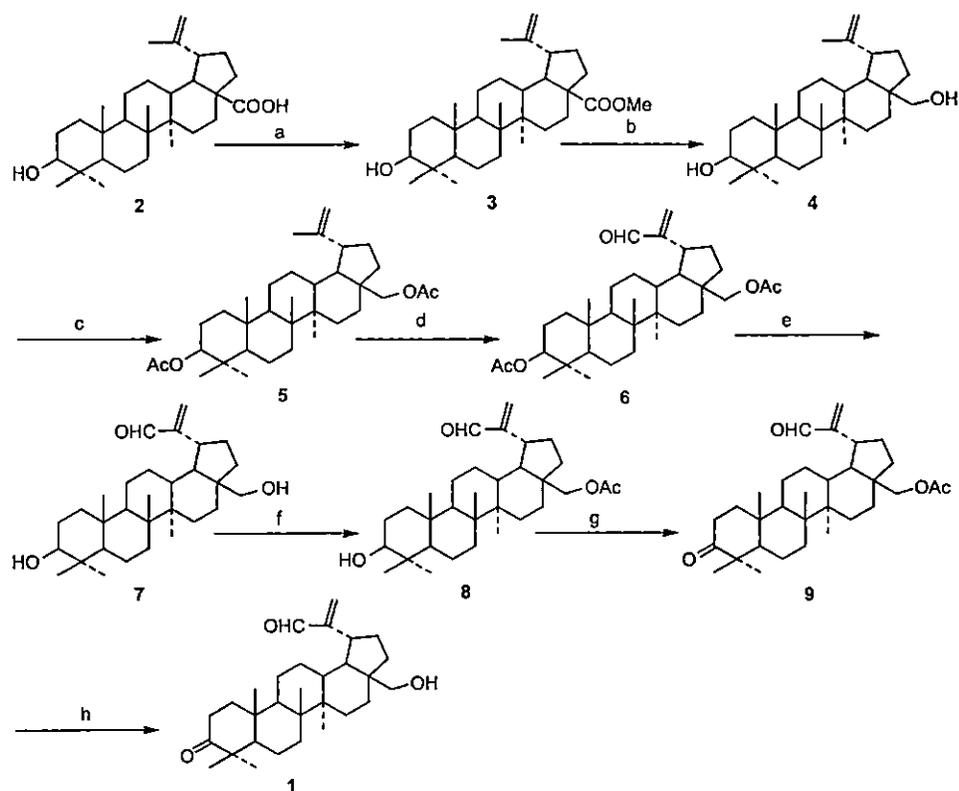
also tested against different cell lines. Derivatization of C-30 methyl group was accomplished in good yield by SeO_2 oxidation in refluxing aqueous dioxan. This is the first report of synthesis of compound **1** from **2** and its potent anticancer activity against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor cell lines.

2. Results and discussion

2.1 Chemical part

The sequential steps involved in the synthesis of compound **1** are illustrated in Scheme 1. Betulinic acid (**2**), isolated from *Bischofia javanica*, was esterified

(diazomethane) at C-28 to form 28-carbomethoxy-lup-20(29)-en-3 β -ol (**3**) almost quantitatively. Lithium aluminum hydride reduction of **3** in anhydrous THF gave betulin (**4**) in 72% yield. Allylic oxidation of the C-30 methyl was then carried out with SeO_2 in aqueous dioxan under refluxing condition after protecting C-3 and C-28 hydroxyl groups as acetate. The incorporation of formyl group was assigned by IR and NMR (both ^1H and ^{13}C) spectra of compound **6**. The IR spectrum showed peaks at 1732 ($-\text{OCOCH}_3$), 1691 (conjugated aldehyde), 1459 and 1369 (gem dimethyl), and 1244, 1028, 978, 936, and 889 ($=\text{CH}_2$) cm^{-1} . The molecular formula of



Scheme 1. Partial synthesis of compound **1** from betulinic acid (**2**). Reagents and conditions: (a) CH_2N_2 , ether, over night, AcOH (gal.), Na_2SO_4 ; (b) LiAlH_4 , dry THF, 2 h, saturated Na_2SO_4 solution, ether, Na_2SO_4 ; (c) $\text{C}_5\text{H}_5\text{N}$, Ac_2O , 6 h (100°C) ice-cold H_2O , ether, Na_2SO_4 ; (d) SeO_2 , aq. dioxan, 2 h, ice-cold H_2O , ether, Na_2SO_4 ; (e) 10% alcoholic KOH , THF, 4 h, ice-cold H_2O , ether, Na_2SO_4 ; (f) $\text{C}_5\text{H}_5\text{N}$, Ac_2O , ($5-10^\circ\text{C}$), 8 h, ice-cold H_2O , Na_2SO_4 ; (g) $\text{C}_5\text{H}_5\text{N}$, dry CrO_3 , overnight, ice-cold H_2O , CH_2Cl_2 , MgSO_4 ; (h) 10% alcoholic KOH , THF, 4 h, ice-cold H_2O , ether, Na_2SO_4 .

compound **6** was assigned as $C_{34}H_{52}O_5$ (M^+ 540.29, analytical calculation % C 75.42, % H 9.56). The 1H NMR spectrum of compound **6**, taken in $CDCl_3$, gave a singlet at δ_H 9.56 with an integration of one proton in addition to five methyls at δ_H 0.93 (s, 3H), 0.94 (s, 3H), 1.02 (s, 3H), 1.06 (s, 3H), and 1.39 (s, 3H) for lupane skeleton. Two olefinic protons appeared at δ_H (proton chemical shift position) 6.28 (s, 1H) and at δ_H 5.93 (s, 1H) and two geminal hydrogens of C-28 each gave a doublet at δ_H 4.24 (d, 1H, $J = 10.6$ Hz) and at δ_H 3.68 (d, 1H, $J = 10.6$ Hz). Two acetyl methyls appeared as two sharp singlets at δ_H 2.07 (s, 3H) and at δ_H 2.03 (s, 3H). In the proton-decoupled ^{13}C spectrum of **6**, a singlet at δ_c 194.6 (Table 1) clearly indicated the presence of a formyl group at C-30. Careful hydrolysis of **6** gave a yellowish gummy residue after evaporation of the solvent *in vacuo*. Purification of this gummy material over a column of silica gel gave almost quantitatively a powdered white solid of compound **7** with melting point (mp) 274–276°C. The IR spectrum gave peaks at 3393 (—OH), 1688 (conjugated aldehyde), 1453, and 1375 (gem dimethyl), and 1029, 942, and 890 ($=CH_2$) cm^{-1} . The molecular formula of compound **7** was assigned as $C_{30}H_{48}O_3$ (M^+ 456.32, analytical calculation % C 78.66, % H 10.52). Disappearance of 1H NMR signals at δ_H 2.03 (s, 3H) and 2.07 (s, 3H) and the presence of two singlets at δ_c 78.9 (for C-3) and 60.2 (for C-28) in the ^{13}C NMR spectrum of **7** clearly indicated the deprotection of both the hydroxyl groups at C-3 and at C-28 during the formation of **7** from diacetate (**6**).

A number of methods [19] were then applied for the selective oxidation of C-3 secondary hydroxyl group, keeping C-28 primary hydroxyl group intact. None of the existing methods [19], including the one developed by Mckillop et al. [20] in 1979, was found effective in producing the desired selectivity. Thus, once again the protection–deprotection method was

applied for the formation of the desired aldehyde **8**.

A solution of **7** (350 mg, 0.76 mmol) in $CHCl_3$ (10 ml) and pyridine (15 ml) was treated with Ac_2O (10 ml, 0.098 mmol) at very low temperature (5–10°C). After the reaction was over (checked by TLC), the mixture was diluted with $CHCl_3$ (20 ml), and then the organic layer was washed with saturated aq. NaCl (10 ml \times 3), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. Purification of the reaction mixture gave compound **8** with mp 260–262°C. The IR spectrum gave peaks at 3461 (—OH), 1732 (—OCOCH₃), 1691 ($>C=C-CHO$), 1459, 1369 (gem dimethyl), and 1244, 1028, 978, 936, and 890 ($=CH_2$) cm^{-1} . The molecular formula of compound **8** was assigned as $C_{32}H_{50}O_4$ (M^+ 498.35, analytical calculation % C 77.02, % H 10.12). The 1H NMR spectrum of **8** showed a singlet at δ_H 9.56 (s, 1H) for the aldehyde proton. The ^{13}C NMR spectrum of compound **8** (Table 1) accounted for all the carbons. Acetylation of only the C-28 —OH group was confirmed by comparison of 1H and ^{13}C NMR data of C-3 hydroxy compound and with the acetate as reported in the literature [21]. The assigned ^{13}C NMR data of all the compounds are given in Table 1. Thus, on the basis of the above spectral data, the structure of compound **8** was assigned as 28-acetoxy-lup-20(29)-en-3 β -ol-30-al.

In the subsequent step, C-3 —OH group was converted to ketone **9** (175 mg), using anhydrous CrO_3 in dry pyridine (see Experimental section) at ambient temperature. After purification it showed mp 276–278°C. The exact structure of compound **9** was elucidated by spectroscopic studies. The IR spectrum gave peaks at 1730 ($>C=O$) and at 1706, 1696 ($CH_2=C-CHO$) cm^{-1} . The molecular formula of compound **9** was assigned as $C_{32}H_{48}O_4$ (M^+ 496.34, analytical calculation % C 77.28, % H 9.62). In its 1H NMR spectrum, compound **9** gave a singlet at δ_H 9.56 (s, 1H) for the aldehydic proton. Two olefinic

Table 1. ^{13}C NMR data of compounds 1–9 and betulonic acid taken in CDCl_3 .

Position	1*	1	2	3	4	5	6	7	8	9	Betulonic acid
1	39.6	39.6	38.8	38.8	38.8	38.4	39.3	39.0	39.0	39.0	38.8
2	34.1	34.1	34.0	34.3	34.2	34.1	35.0	34.3	34.3	34.3	34.0
3	218.0	218.1	78.9	79.0	78.9	80.9	81.8 (172.0 ^b)	78.9	78.9	218.1	218.1
4	47.4	47.3	47.3	50.5	50.5	50.2	47.6	48.0	48.0	48.0	47.3
5	55.0	54.9	54.8	55.3	55.3	55.3	56.3	55.3	55.3	55.3	54.8
6	19.6	19.6	19.5	19.5	19.3	19.1	19.1	19.3	19.3	19.3	19.5
7	33.5	33.5	33.6	32.1	32.3	34.1	35.0	33.8	33.8	33.8	33.6
8	42.7	42.3	40.6	42.3	42.4	42.6	43.5	42.6	42.6	42.6	40.6
9	49.6	49.6	49.8	49.4	49.4	48.8	48.7	48.0	48.0	48.0	49.8
10	36.9	36.8	36.8	36.9	36.9	37.0	35.5	36.9	36.9	36.9	36.8
11	21.4	21.3	21.3	20.8	20.8	21.0	21.7	20.9	20.9	20.9	21.3
12	27.6	27.1	25.4	25.5	25.4	27.0	27.9	27.6	27.6	27.6	25.4
13	37.1	38.1	38.5	38.2	38.2	37.8	37.9	37.1	37.1	37.1	38.5
14	40.8	40.5	42.4	40.6	40.6	40.8	41.7	40.8	40.8	40.8	42.4
15	26.9	26.5	29.6	29.6	29.6	27.0	28.9	26.9	26.9	26.9	29.6
16	29.1	29.5	32.0	27.9	27.9	27.9	28.4	28.1	28.1	28.1	32.0
17	48.0	47.1	56.3	46.5	47.6	47.7	47.3	47.3	47.3	47.3	56.3
18	52.3	51.4	49.1	51.2	51.3	51.2	51.0	50.2	50.2	50.2	49.1
19	36.5	37.5	46.8	46.9	46.9	37.5	38.7	38.7	38.7	38.7	46.8
20	157.0	156.2	150.2	150.6	150.7	150.2	157.1	157.1	157.1	157.1	150.2
21	32.8	31.8	30.5	30.6	30.5	34.5	31.0	31.8	31.8	31.8	30.5
22	33.9	36.7	36.8	37.2	37.1	37.0	38.1	36.4	36.4	36.4	36.8
23	26.6	26.6	26.6	27.4	27.4	27.0	24.6	26.9	26.9	26.9	26.6
24	21.1	21.0	20.9	19.3	19.3	20.8	22.0	21.4	21.4	21.4	20.9
25	15.9	15.8	15.8	15.3	15.3	16.0	17.1	15.9	15.9	15.9	15.8
26	15.8	15.7	15.9	15.9	15.9	16.1	16.9	15.3	15.3	15.3	15.9
27	14.6	14.8	14.6	14.7	14.7	14.7	15.6	14.6	14.6	14.6	14.6
28	60.2	60.2	181.6	176.6	61.3	62.8	63.4 (172.5 ^b)	60.2	64.2	60.2	181.6
29	133.2	133.6	109.7	109.5	109.5	109.8	133.7	133.6	133.6	133.6	109.7
30	194.9	194.6	19.3	18.3	18.3	18.1	194.6	194.6	194.6	194.6	19.3

Notes: *Represents that reported in the literature; ^brepresents the chemical shift position of diacetate.

protons of C-29 appeared at δ_{H} 6.23 (s, 1H) and 5.92 (s, 1H). For two geminal protons of C₂₈-H₂ group, each appeared as a doublet at δ_{H} 3.83 (d, 1H, $J = 10.6$ Hz) and 3.38 (d, 1H, $J = 10.6$ Hz). All the five methyls appeared between δ_{H} 0.75 and 0.95 along with other peaks of lupane skeleton. The ¹³C NMR spectrum (Table 1) was also in good agreement with the proposed structure of compound **9** (Table 1). Deprotection of C-28 hydroxyl group finally afforded a white amorphous solid **X** (44% overall yield), mp 288–290°C.

The IR spectrum gave peaks at 3424 (—OH), 1725 ($>\text{C}=\text{O}$), 1706, 1697 (conjugated aldehyde), 1461, 1380 (gem dimethyl), and 1163, 987 cm^{-1} . The molecular formula was established as C₃₀H₄₆O₃ by HR-EI-MS (m/z 554.38 [M]⁺, calcd. 554.34). In the ¹H NMR spectrum, five tertiary methyl groups appeared at δ_{H} 0.91 (s, 3H), 0.93 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H), and 1.15 (s, 3H). Two olefinic C-29 protons appeared at δ_{H} 6.91 (s, 1H) and 6.28 (s, 1H). The singlet at δ_{H} 9.52 (s, 1H) was due to the aldehydic proton of C-30. Two geminal protons of C₂₈-H₂ appeared at δ_{H} 3.78 (d, 1H, $J = 10.6$ Hz) and 3.36 (d, 1H, $J = 10.6$ Hz). The ¹³C spectrum revealed the presence of two olefinic carbons at δ_{C} 133.6 (C-29) and 156.2 (C-20). The aldehydic C-30 carbon appeared at δ_{C} 194.6. C-3 appeared at δ_{C} 218.1 and C-28 appeared at δ_{C} 60.2.

Thus, the presence of C-30 aldehyde and C₂₀-C₂₉ double bond is obvious from the above ¹³C and ¹H NMR spectra of compound **X**. However, the shift in δ values for carbons C-12, C-13, C-17, C-18, C-19, and C-21 (Table 1) in compound **X** in comparison with that observed for betulinic acid [17] and lupeol [18] may be either because of the isomeric nature of the attached isopropenyl group of the cyclopentane ring at C-19 of lupane skeleton [18] or because of the introduction of C-30 —CHO group during the SeO₂ oxidation of the C-30 methyl [18]. At this

juncture, it is relevant to mention that the shift in ¹³C signals for the above-mentioned carbons started appearing in all the compounds (*viz.* compounds **6**, **7**, **8**, and **9**) once the SeO₂ oxidation step was carried out. The shifts in the NMR spectrum and possible epimerization of the side chain during the SeO₂ oxidation were already reported in the literature from our laboratory [18] and the existence of such a conformational isomer is also documented [23]. However, in the present case, we could not get the isomeric peaks for the relevant carbons in the ¹³C NMR spectra of **1**, **6**, **7**, **8**, and **9** as observed by the previous workers [18,23]. Therefore, on the basis of the above spectral analysis, the structure of compound **X** has been assigned as 28-hydroxy-3-oxolup-20(29)-en-30-al (**1**) or **10** (Figure 2).

Further confirmation of the stereochemistry at C-19 was settled by carrying out the 2D NMR techniques on compound **6** (first compound after the SeO₂ oxidation) in which the probable stereochemical change would occur. The NOSEY spectrum of compound **6** gave significant information about the stereochemistry at C-19. All nuclear overhauser effect (NOE) cross-peaks have opposite phase to the diagonal, indicating that these arose from positive NOE enhancements as anticipated for a molecule of this size (M^+ 540.4) under ambient conditions. H-19 at δ_{H} 2.76 (m, 1H) showed strong correlations (Figure 3) with β -H-12 at δ_{H} 1.03 (m, 1H), β -H-13 at δ_{H} 1.66 (td, 1H, $J = 12.2$,

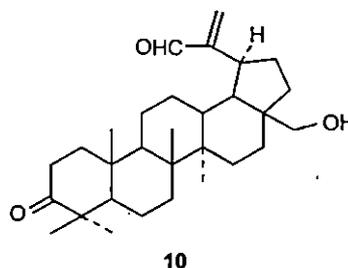


Figure 2. Epimeric form of compound **1**.

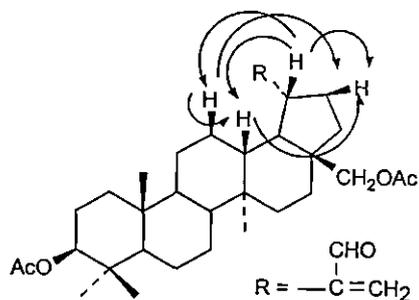


Figure 3. Key NOESY correlations of compound 6.

3.6 Hz), and β -H-21 at δ_H 2.18 (m, 1H). The NOE observed in the NOESY spectrum of compound 6 indicated the involvement of β -H-12 at δ_H 1.03 (m, 1H) and β -H-13 at δ_H 1.66 (td, 1H, $J = 12.2, 3.6$ Hz) with β -H-21 at δ_H 2.18 (m, 1H). These observations may be explained by considering the H-19 configuration as β as depicted in Figure 3. Additionally, β -H-13 at δ_H 1.66 (td, 1H, $J = 12.2, 3.6$ Hz) showed positive NOE with β -H-12 at δ_H 1.03 (m, 1H; Figure 2). From these data, it can be concluded that the original stereochemistry at C-19 was retained during SeO_2 oxidation on 5 and the shift in chemical shift values for C-12, C-13, C-17, C-18, C-19, and C-21 in the ^{13}C NMR spectrum of subsequent oxidized products with respect to that for lupane skeleton is due to the angular dependence through space effects, such as the anisotropic magnetic susceptibility and/or electric field effect [16], offered by the conjugated carbonyl group. The through space distance is obviously more important than the number of intervening bonds since the chemical shift changes are much smaller for C-13 than for C-12, which is one bond further away but closer in space [23]. SeO_2 oxidation of compound 5 to 6 may only be considered as the step where the stereochemical change at C-19 would have occurred, since in all other subsequent steps no reaction was carried out that can alter the stereochemistry at C-19.

Therefore, the stereochemistry at C-19 would be the same as 6 for all the subsequent molecules (7, 8, 9, and 1) derived from it.

2.2 Antileukemic activity

In this study, compounds were subjected to cytotoxic assay against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor, and the assays were carried out in three independent experiments as per the guidelines of biosafety committee of West Bengal State University (Figures 4–6). To determine whether the compounds had any effect on cell lines, cell cultures were incubated with various concentrations of compounds (dissolved in 0.1% v/v DMSO). DMSO had no effect on the growth of cell lines at a final concentration of 0.1% (v/v). Compounds 1, 2, 3, 5, and 7 had differential effects on the growth of the cell lines. The effect of 4 and 6 could not be checked due to its poor solubility in DMSO.

All the compounds showed potent activities against the entire cell lines used; although the activity against murine MEL erythroid progenitor was not as good as for other two cell lines (Figure 5). However, 1 was the most cytotoxic against murine MEL erythroid progenitor. The anti-proliferative effect of all the compounds helped us to predict some of the structure–activity relationships. A comparison of bioassay data between 2 and 3 revealed that compound 2 was more active than 3 against human K562 leukemia and murine WEHI3 leukemia. The only difference between their structures was at C-28. Compound 2 had a $-\text{COOH}$ group and 3 had a $-\text{COOCH}_3$ group, i.e. more polar grouping is necessary ($-\text{COOH}$) to have high activity. Incorporation of aldehyde group at C-30 had increased the activity further. The study also revealed that against human K562 leukemia and murine WEHI3 leukemia cell lines, 7 showed better

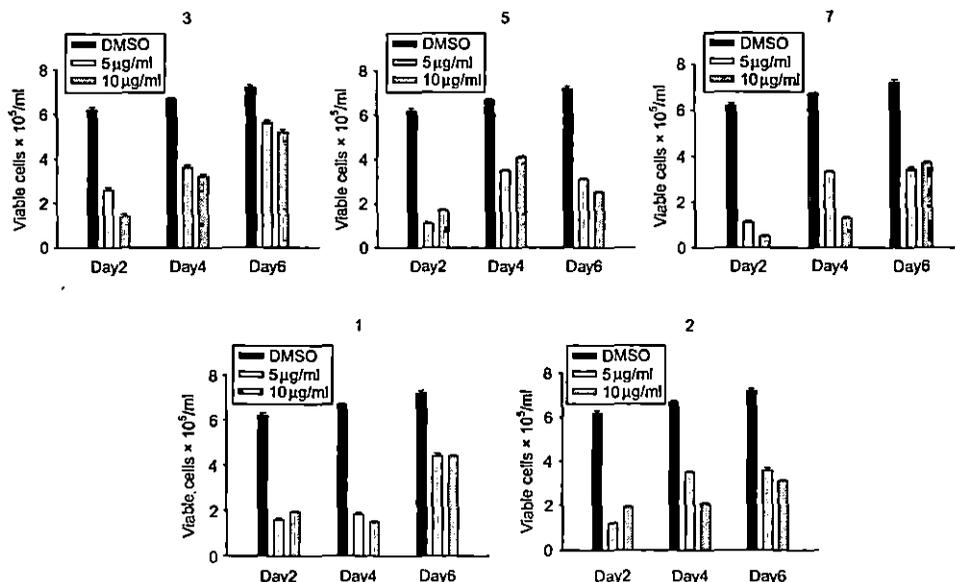


Figure 4. Anti-proliferative effect of 3, 5, 7, 1, and 2 on WEHI3 cells.

activities than 1. A closer look at the structures of these two compounds revealed that they had a difference in their structures only at C-3 and the data indicated that $-OH$ group at C-3 was the one which contributed to higher activity.

3. Experimental

3.1 General experimental procedures

$[\alpha]_D$ was measured in Autopol III Automatic Polarimeter. Melting points were recorded by open capillary method and are uncorrected. IR spectra were recorded in

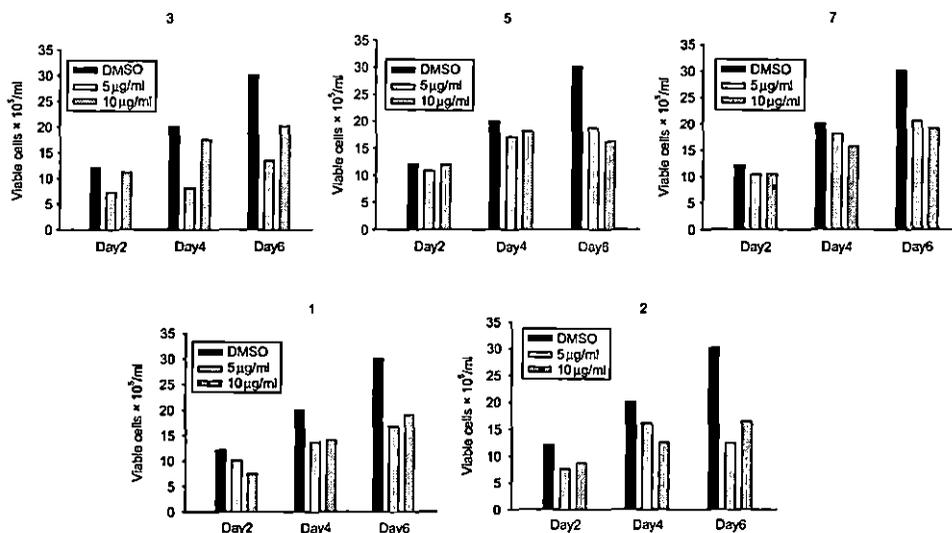


Figure 5. Anti-proliferative effect of 3, 5, 7, 1, and 2 on MEL cells.

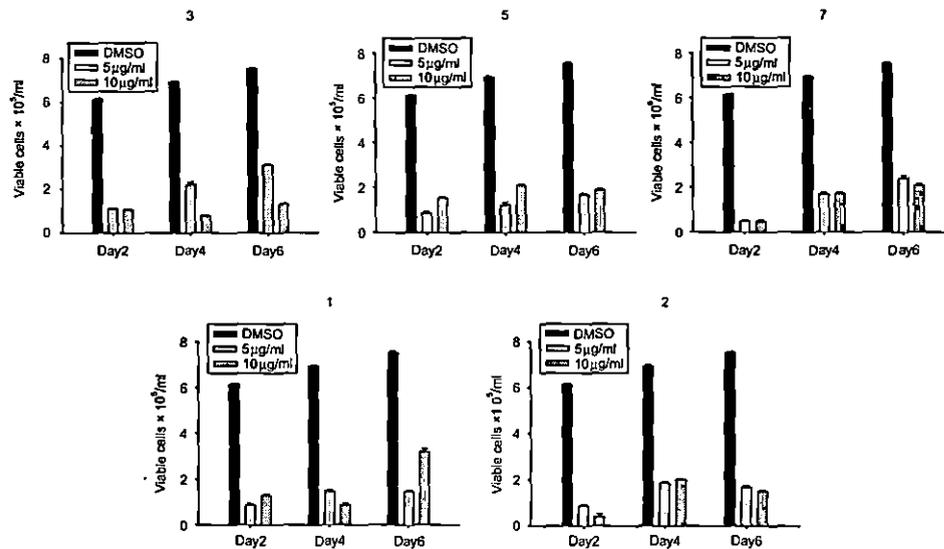


Figure 6. Anti-proliferative effect of 3, 5, 7, 1, and 2 on K562 cells.

Shimadzu 800 FT-IR spectrophotometer and NMR spectra were recorded in Bruker-Avance 300 MHz FT NMR spectrometer located in the Department of Chemistry, University of North Bengal, India – 734 013. IR spectra were recorded using both the KBr disk and nujol and the NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of CDCl_3 solvent as the standards. ^1H spectra were recorded at 300 MHz frequencies and ^{13}C NMR spectra were recorded at 75.4 MHz frequencies. Coupling constant ' J ' was calculated in Hz. Betulinic acid was isolated from the outer bark of *B. javanica* through soxhlet apparatus in toluene. All the chemicals used were of commercial grade and were purified prior to their use. The mass spectra were recorded in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer and elemental analyses were carried out in Vario EL-III from CDRI, Lucknow, India – 226 001.

3.2 Methyl betulinate (3)

Three grams (6.58 mmol) of betulinic acid were dissolved in diethyl ether (100 ml) at very low temperature (0–5°C) in a 250 ml

conical flask. To this an excess of diazomethane dissolved in ether at very low temperature (0–5°C) was added slowly with constant shaking. The whole reaction sequence was carried out in a fume cupboard. The resultant yellowish solution was kept overnight under darkness. After that 2 ml of glacial acetic acid was added to this to neutralize the traces of diazomethane. The solution was then diluted with cold water and extracted with ether. Evaporation of solvent at vacuum gave a gummy residue that was purified over a column of silica gel (60–120 mesh).

Compound 3 was obtained (2.9 g, 98%) as a white solid with mp 222–224°C and $[\alpha]_D + 5.0$ (CHCl_3). The IR spectrum gave peaks at 3540 (–OH), at 1733 (–COOMe), and at 1660 and 890 ($=\text{CH}_2$) cm^{-1} . Elemental analysis: found: C, 78.79%, H, 10.52%; calcd for $\text{C}_{31}\text{H}_{50}\text{O}_3$: C, 79.10%, H, 10.71%. ^1H NMR δ_{H} 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.40 (s, 3H), 1.68 (s, 3H), 2.99 (m, 1H, H-C-3), 3.18 (dd, 1H, $J = 5.1, 10.8$ Hz, H-3), 3.66 (s, 3H, –OMe), 4.59 (s, 1H, H-C-29), and 4.73 (s, 1H, H-C-29). The compound was found

identical to an authentic sample (co-TLC, mixed mp).

3.3 *Lup-20(29)-en-3 β ,28-diol (4)*

Compound 3 (2.8 g, 5.95 mmol) was added to 50 ml of dry THF in a 150 ml round-bottomed flask. The solution was homogenized and to this LAH (75.28 mg, 1.98 mmol) was added in small lots at low temperature (10–15°C), and the solution was stirred for 15 min. Stirring was continued for another 3 h at room temperature and saturated solution of sodium sulfate was added dropwise till the excess LAH was destroyed. The reaction mixture was poured into 200 ml cold water and extracted with diethyl ether and dried over anhydrous sodium sulfate. Evaporation of solvent gave a residue that was then purified over a column of silica gel (60–120 mesh).

Compound 4 was obtained (76%) as a white solid with mp 256–257°C and $[\alpha]_D + 16$ (10% v/v MeOH in CHCl₃; 0.35 g/ml). The IR spectrum gave peaks at 3393 (–OH), 1453 and 1375 (gem dimethyl), and 1229, 1029, 942, and 889 (=CH₂) cm⁻¹. ¹H NMR δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.37 (s, 3H), 1.68 (s, 3H), 2.43 (m, 1H, H-C-18), 3.84 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.24 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.46 (m, 1H, H-C-3), 4.59 (s, 1H), 4.68 (s, 1H). The compound was found identical to an authentic sample (co-TLC, mixed mp, etc.).

3.4 *Lup-20(29)-en-3 β ,28-diol acetate (5)*

Compound 4 (1.8 g, 4.06 mmol) was dissolved in 50 ml dry pyridine in a 100 ml round-bottomed flask and to this 10 ml acetic anhydride was added. The reaction mixture was warmed under water bath for 6 h. After cooling, it was poured into 150 ml ice-cold water and extracted with diethyl ether. The ether layer was

washed with 6 N HCl and again with water till neutral. The residue obtained after evaporation of the solvent at reduced pressure was dried and purified over a column of silica gel (60–120 mesh).

Compound 5 was obtained (1.6 g, 94%) as a white solid with mp 222–223°C and $[\alpha]_D + 22$ (CHCl₃; 0.4 g/ml). The IR spectrum gave peaks at 1735 (–OCOCH₃), 1639, 1459, and 1370 (gem dimethyl), and 1243, 1027, 979, and 889 (=CH₂) cm⁻¹. Elemental analysis: found: C, 77.46%, H, 10.31%; calcd for C₃₄H₅₄O₄: C, 77.52%, H, 10.33%. ¹H NMR δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.59 (s, 3H), 1.65 (s, 3H), 2.43 (m, 1H, H-C-18), 3.84 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.24 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.46 (m, 1H, H-C-3), and 4.59 (s, 1H), 4.68 (s, 1H). Acetate peaks appeared at δ_H 2.04 (s, 6H). The compound was found identical to an authentic sample (co-TLC, mixed mp, etc.).

3.5 *Lup-20(29)-en-3 β ,28-diol acetate-30-al (6)*

Compound 5 (1.5 g, 2.85 mmol) was dissolved in 10 ml of aqueous dioxan and 20 ml of SeO₂ was added to it. The resultant reaction mixture was refluxed for 2 h and after cooling poured into 100 ml ice-cold water. A curdy white precipitate developed. After usual work up with diethyl ether, it was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The yellow gummy residue obtained was purified over a column of silica gel (60–120 mesh).

Compound 6 was obtained (62%) as a white solid with mp 246–248°C. IR ν_{max} : 1732 (–OCOCH₃), 1691 (>C=C–CHO) 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 889 (=CH₂) cm⁻¹. ¹H NMR: δ_H 0.93 (s, 3H), 0.94 (s, 3H), 1.02 (s, 3H), 1.06 (s, 3H), 1.39 (s, 3H), 6.28 (s, 1H, H-C-29), 5.93 (s, 1H, H-C-29), 4.24 (d, 1H,

$J = 10.6$ Hz, H-C-28) and δ_{H} 3.68 (d, 1H, $J = 10.6$ Hz, H-C-28), 9.56 (s, 1H, aldehyde hydrogen at C-30). Two acetyl methyls appeared at δ_{H} 2.07 (s, 3H) and at 2.03 (s, 3H). C-3 hydrogen appeared as a broad multiplet centered at δ_{H} 4.44 (m, 1H). Elemental analysis: found: C, 75.42%, H, 9.56%; calcd for $\text{C}_{34}\text{H}_{52}\text{O}_5$: C, 75.51%, H, 9.69%.

3.6 Lup-20(29)-en-3 β ,28-diol-30-al (7)

Compound 6 (900 mg, 1.66 mmol) was refluxed with 10% (w/v) alcoholic KOH solution for 4 h. After completion of the reaction, it was poured into 100 ml ice-cold water. After usual work up with diethyl ether, the ether layer was washed several times with water, dried over anhydrous sodium sulfate, and the recovered material was purified by column chromatography over silica gel (60–120 mesh).

Compound 7 was obtained (756 mg, 84%) as a white solid, mp 274–276°C, IR ν_{max} : 3393 (–OH), 1688 (>C=C–CHO), 1453, 1375 (gem dimethyl), 1029, 942, 890 (=CH₂) cm^{-1} . ¹H NMR: δ_{H} 0.75 (s, 3H), 0.81 (s, 3H), 0.95 (s, 3H), 1.00 (s, 3H), 1.38 (s, 3H), 2.62 (m, 1H, hydrogen at C-3), 3.15 (m, 1H, proton of–OH at C-3), 3.38 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.83 (d, 1H, $J = 10.6$ Hz, H-C-28), 5.94 (s, 1H, H-C-29), 6.32 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30). Elemental analysis: found: C, 78.66%, H, 10.52%; calcd for $\text{C}_{30}\text{H}_{48}\text{O}_3$: C, 78.90%, H, 10.59%.

3.7 3 β -Hydroxy-28-acetoxy-lup-20(29)-en-30-al (8)

A solution of 7 (350 mg, 0.76 mmol) in CHCl_3 (10 ml) and pyridine (15 ml) was treated with Ac_2O (10 ml, 0.098 mmol) at very low temperature (0–5°C) for 12 h. The solution was diluted with CHCl_3 (20 ml), and then the organic layer was washed with saturated aq. NaCl (10 ml \times 3), dried, concentrated, and pur-

ified by column chromatography over silica gel (60–120 mesh).

Compound 8 was obtained (72%) as a white solid, mp 260–262°C, IR ν_{max} : 3461 (–OH), 1732 (–OCOCH₃), 1691 (>C=C–CHO), 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 890 (=CH₂) cm^{-1} . ¹H NMR δ_{H} 0.75 (s, 3H), 0.80 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.95 (s, 3H), 2.1 (s, 3H, acetate methyl), 3.18 (m, 1H, proton at C-3), 3.38 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.83 (d, 1H, $J = 10.6$ Hz, H-C-28), 5.94 (s, 1H, H-C-29), 6.32 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30). Elemental analysis: found: C, 77.02%, H, 10.12%; calcd for $\text{C}_{32}\text{H}_{50}\text{O}_4$: C, 77.06%, H, 10.10%.

3.8 28-Acetoxy-3-oxolup-20(29)-en-30-al (9)

Compound 8 (150 mg, 0.30 mmol) was dissolved in dry pyridine (30 ml). Two hundred and thirty milligrams (2.91 mmol) of dry CrO_3 were added in small lots and the reaction mixture was kept overnight. It was then poured into ice-cold water and the resultant yellowish solid was extracted with ether, washed with 6 N HCl and again with water till neutral, and dried over anhydrous magnesium sulfate to get a gummy residue. Purification of the gum over a column of silica gel (60–120 mesh) yielded compound 9.

Compound 9 was obtained (84 mg, 56%) as a white solid, mp 276–278°C. IR ν_{max} : 1730 (>C=O), 1706, 1697 (>C=C–CHO), 1461, 1380 (gem dimethyl), 1244, 1163, 987, 890 (=CH₂) cm^{-1} . Elemental analysis: found: C, 77.28%, H, 9.62%; calcd for $\text{C}_{32}\text{H}_{48}\text{O}_4$: C, 77.38%, H, 9.74%. ¹H NMR δ_{H} 0.75 (s, 3H), 0.80 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.95 (s, 3H), 2.17 (s, 3H, acetate methyl at C-28) 3.38 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.83 (d, 1H, $J = 10.6$ Hz, H-C-28), 5.92 (s, 1H, H-C-29), 6.23 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30).

3.9 28-Hydroxy-3-oxolup-20(29)-en-30-al (1)

Compound 9 (75 mg, 0.15 mmol) was refluxed with 10% alcoholic KOH solution for 4 h. After completion of the reaction, it was poured into 100 ml ice-cold water. After usual work up with ether, the ether layer was washed several times with water, dried over anhydrous magnesium sulfate, and the recovered material was purified by column chromatography (silica gel, 60–120 mesh).

Compound 1 was obtained (80.6 mg, 96%) as a white amorphous solid; $[\alpha]_D + 16.3$ (MeOH), mp 288–290°C. IR ν_{\max} : 3424 (–OH), 1725 (>C=O), 1706, 1697 (>C=C–CHO), 1461, 1380 (gem dimethyl), 1163, 987 cm^{-1} . ^1H NMR: δ_{H} 0.91 (s, 3H), 0.93 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H), and 1.15 (s, 3H), 6.91 (s, 1H, H-C-29), 6.28 (s, 1H, H-C-29), 3.36 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.78 (d, 1H, $J = 10.6$ Hz, H-C-28), 9.52 (s, 1H, aldehyde hydrogen at C-30). ^{13}C NMR spectral data are depicted in Table 1. HR-EI-MS: m/z 554.38 $[\text{M}]^+$ (calcd for $\text{C}_{30}\text{H}_{46}\text{O}_3$, 554.34). Elemental analysis: found: C, 79.18%, H, 10.21%; calcd for $\text{C}_{30}\text{H}_{46}\text{O}_3$: C, 79.25%, H, 10.20%.

4. Bioassay

The assays against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor cells were carried out in three independent experiments as per the guidelines of biosafety committee of West Bengal States University. K562 and WEHI3 cells were obtained from cell repository of National Centre for Cell Science, Pune (agreement between two institutes). MEL cells were obtained as gift from Prof. Michael H. Kershaw, Cancer Immunotherapy Research, Peter MacCallum Cancer Centre, Australia (through an agreement with Walter and Eliza Hall Institute, Australia). The cells were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mmol glutamine,

supplemented with 1% penicillin–streptomycin, and were incubated at 37°C under 5% CO_2 atmosphere. In these experiments, cells were seeded in quadruplicate in 24-well plates (105 cells/mL) with the compounds (1, 2, 3, 5, and 7) dissolved in DMSO (0.1% v/v) at various concentrations. The effect of 4 and 6 could not be checked due to its poor solubility in DMSO. The cells were incubated for 2, 4, and 6 days. Growth of cells was monitored by counting the number of live cells microscopically using Neubauer hemocytometer by trypan blue exclusion method. Statistical analyses for all experiments were carried out by Student's t -test using the program SigmaPlot.

Acknowledgement

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RESEARCH LETTER

Greener approach toward one pot route to pyrazine synthesis

Pranab Ghosh* and Amitava Mandal

Department of Chemistry, University of North Bengal, Siliguri, Darjeeling, West Bengal, India

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A very simple, cost effective, and environmentally benign method has been reported for the preparation of pyrazine derivatives of pentacyclic triterpenoids. The versatility of the method is determined by synthesizing a large number of pyrazine derivatives of smaller molecules.

Keywords: green synthesis; one pot; triterpenoid; pyrazine; cost effective

Introduction

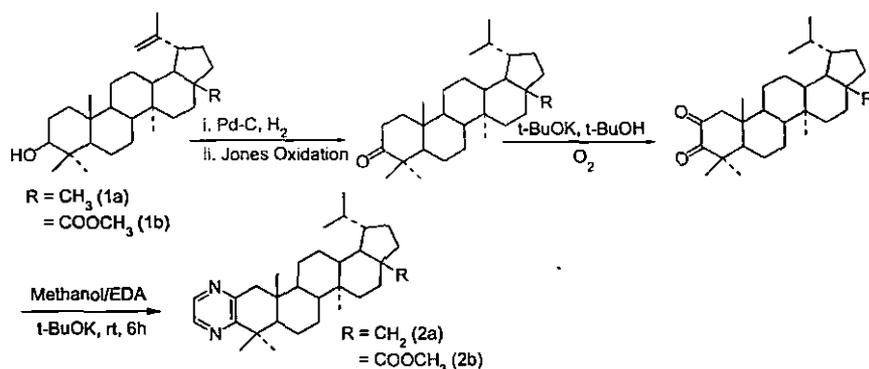
Compounds containing *N*-heterocyclic moieties are a class of privileged compounds that have found numerous applications as pharmaceuticals. Pyrazines are important components of aroma fragrances (1), potential pharmacophore of a large number of biologically active substances (2–6), and widely used as agrochemicals (7–9). For example, methoxy pyrazines are relevant components of aromas of many fruits, vegetables, and wines; methyl phenyl derivatives of dihydropyrazines inhibit the growth of *E. coli* by generating hydroxyl and carbene-centered radicals that cause DNA strand breakage; and alkylpyrazines have been recognized as flavor components in foods, as pheromones in various insect species (7, 8), and as versatile synthetic intermediates. Pyrazine derivatives are known for use as relaxing cardiovascular and uterine smooth muscle, anti-thrombotic, anti-aggregation, COX-2 inhibiting, and analgesic effects (10). Because of the wide variety of applications associated with the pyrazine moieties, their synthesis has remained the goal of many research groups over the years. Among the various methods developed, pyrazine compounds are synthesized by the reaction of diamines with diols in a vapor phase reaction in presence of granular alumina (11). Catalytic systems such as copper-chromium (12), copper-zinc-chromium (13), zinc-phosphoric acid-manganese (14), and silver (15) are also patented as catalysts for the preparation of 2-methylpyrazine from ethylenediamine and propylene glycol. Pyrazines are also obtained from condensation reaction of diamines and epoxides using copper-chromium catalyst (16), condensation reaction between alkanolamines (17), or

cyclodehydrogenation of *N*-(hydroxyalkyl) alkyldiamine (18) using the same catalysts. In the presence of a palladium catalyst, dehydrogenation of piperazines yields corresponding pyrazines in high yield (19). Recently, synthesis of pyrazines from α -hydroxy ketones and 1,2-diamines via MnO₂ catalyzed tandem oxidation process under refluxing conditions has been reported, but the yields are not encouraging and the loading of the catalyst was also high (20). The method of bubbling oxygen under refluxing condition (21) suffers from scientific drawbacks. Strategically, direct condensation reaction of 1,2-diketones with 1,2-diamine (22) is the most straightforward as well as the classical route for the preparation of pyrazines via dihydropyrazines (22). Although, a number of methods are reported in literature for the synthesis of pyrazine, none of them was found to be effective because of poor yield, harsh reaction condition, and tedious work-up procedures (23). Attempts to carry out dehydrogenation under a variety of milder and more convenient laboratory procedures were not successful (24). Although, some of them are apparently useful, most of them are limited by long reaction time, low yields, and use of toxic solvents or heavy metals as the catalyst (24). Therefore, development of mild, efficient, and environmentally benign method for synthesizing pyrazines has been a major challenge in contemporary organic synthesis.

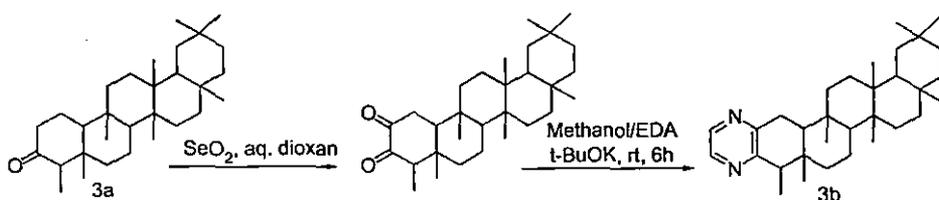
Results and discussion

Triterpenoids are widely distributed in nature, and recent reports have demonstrated the interesting biological activities of this class of natural products. However, triterpenoids possessing a nitrogen containing

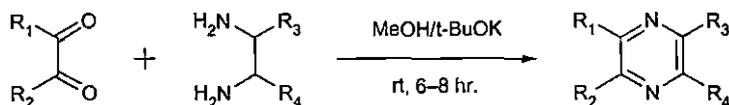
*Corresponding author. Email: pizy12@yahoo.com



Scheme 1. Preparation of diketo derivatives and synthesis of pyrazine derivative.



Scheme 2. Preparation of diketofriedelin and synthesis of pyrazine derivative.

Scheme 3. Preparation of pyrazines. R_1 may be alkyl, aryl or furyl group; R_2 may be alkyl, aryl or furyl group or hydrogen; R_3 may be hydrogen, alkyl or nitrile group; R_4 may be hydrogen or nitrile group.

heterocycle condensed to an isoprenoid skeleton are rare. Since compounds containing *N*-heterocyclic moieties have found numerous applications as pharmaceuticals as well as in medicines, it is also anticipated that incorporation of a pyrazine ring into the molecule of a pentacyclic triterpenoid may induce or enhance its biological activity. With this view in mind and in continuation of our studies on the transformative reactions of triterpenoids, we report herein the incorporation of pyrazine ring into ring A of the pentacyclic triterpenoids of lupane and friedelin skeleton (Schemes 1 and 2). The protocol comprises a direct condensation between the respective 1,2-diketo compounds with 1,2-diamines in aqueous methanol catalyzed by potassium tert-butoxide (*t*-BuOK) at room temperature. This high yielding process did not require any added expensive catalyst or bubbling of oxygen (2l) at higher temperature (Scheme 1). Detection of dihydropyrazine along with pyrazines as well as the starting material at an early stage of the reaction indicated that the developed method involved aromatization following

a very simple one pot route via the formation of dihydropyrazine, removing any additional steps as reported in literature.

In order to show the general applicability, we attempted our procedure using a number of both structurally and chemically diversified 1,2-dicarbonyls and 1,2-diamines to synthesize pyrazine derivatives (Table 1) and were able to get identical results in each case. Thus, this cost-effective process may also be considered as an excellent environmentally benign alternative for the preparation of pyrazine derivatives from a host of compounds (Scheme 3).

General experimental detail

All the melting points were determined in an open capillary method; UV spectra were recorded in JASCO V-530 UV/VIS spectrophotometer; IR was recorded in Perkin-Elmer FT-IR spectrophotometer; and NMR was recorded in Bruker-Avance 300 MHz FT-NMR instrument using TMS as the internal standard. NMR spectra were recorded in CDCl_3 .

Table 1. Selective synthesis of pyrazine.

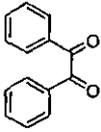
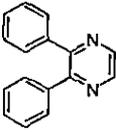
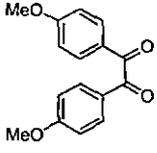
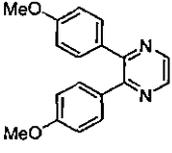
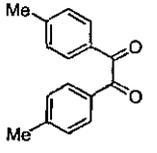
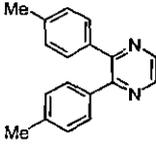
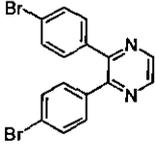
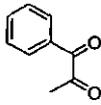
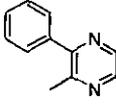
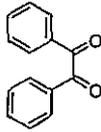
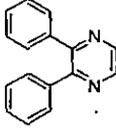
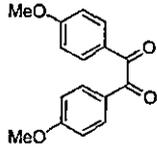
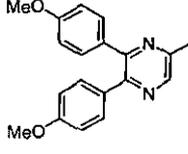
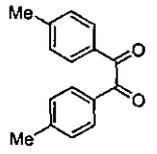
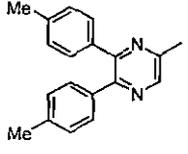
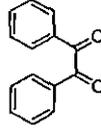
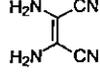
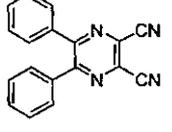
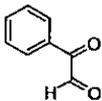
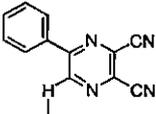
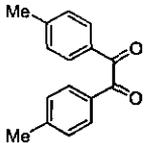
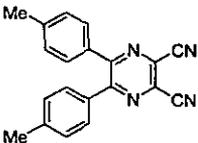
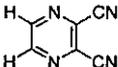
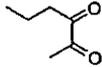
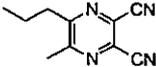
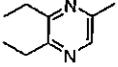
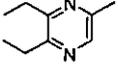
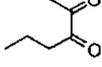
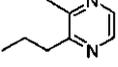
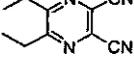
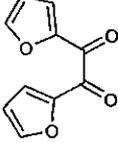
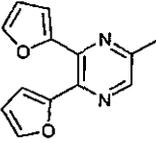
Entry	1,2-Diketone	1,2-Diamine	Time (h)	Pyrazine	% Yield
1			8		82
2			8		78
3			7.5		82
4			10		84
5			8		76
6			8		78
7			8.5		78
8			8		86
9			4		86

Table 1 (Continued)

Entry	1,2-Diketone	1,2-Diamine	Time (h)	Pyrazine	% Yield
10			3.5		88
11			5		78
12			3.5		82
13			4		76
14			4.5		74
15			5		76
16			4.5		78
17			6		76
18			7		82

Note: % Yield refers to the isolated yield of all the compounds.

The entire chemicals were purchased from Merck, Fluka, SRL, and S.D. fine chemicals companies. The reagents from Merck and Fluka were used as received and others were purified following the standard methodology prior to their use. Betulinic acid, lupeol, and friedelin were isolated from their natural sources (see supporting information).

Preparation of 1,4-pyrazine derivatives

In a typical reaction procedure, in a 50 ml round bottom flask, 2 mmol of recrystallized benzil was dissolved in 3 ml of aqueous methanol and was made homogeneous by stirring with a magnetic spinning bar. To this 2 mmol of ethylene diamine and catalytic amount of t-BuOK (10 mg or 0.08 mmol) were added. Stirring was continued until the reaction is completed (checked by TLC). Methanol was evaporated under reduced pressure, and the crude product was purified by chromatography using silica gel. Varied proportion of petroleum ether and ethyl acetate was used as eluent.

Conclusion

A mild, efficient, and environmentally benign method has been developed for the synthesis of pyrazines that is superior in every respect than the already reported methods. Introduction of the pyrazine nucleus is expected to induce potent biological activity into the triterpenoids that will be tested with the help of a sister institution having these facilities. The data obtained in the process may be helpful to study the SAR (structure–activity relationship) among this particular class of compounds, especially the triterpenoids of above skeletons.

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SUPPORTING INFORMATION

General experimental detail

All the melting points were determined in an open capillary method, UV spectra were recorded in JASCO V-530 UV/VIS spectrophotometer; IR spectra were recorded in Perkin-Elmer FT-IR spectrophotometer; NMR was recorded in Bruker-Avance 300 MHz FT-NMR instrument using TMS as the internal standard. NMR spectra were recorded in CDCl₃. The chemicals/reagents purchased from Merck, Fluka, SRL, S d fine chemicals companies and were used either as received (Merck, Fluka) or after purification prior to use. Triterpenoids, Betulinic acid, lupeol and friedelin were isolated from their natural sources (Please see the supporting information) and were used as starting materials in the present investigation.

Isolation of triterpenoids

Betulinic acid, lupeol and friedelin were isolated from *Bischofia javanica*, *Xanthozylum budrungs* and *quercus suber* respectively in a soxhlet extractor using petroleum ether (60-80°C) as the solvent. All the triterpenoids were purified by column chromatography followed by crystallization.

Preparation of diketo derivatives

Betulinic acid and lupeol were hydrogenated and oxidized to get the corresponding 3-keto compound. The corresponding diketo compounds were prepared by auto-oxidation of each of them following the method as reported elsewhere (Gangully, A. K.; Govindachari, T. R.; Mohamed, P. A. *Tetrahedron*, 1966, 22, 3597-3599.). Diketo derivative of friedelin was prepared by SeO₂ oxidation of friedelin in aq. Dioxin.

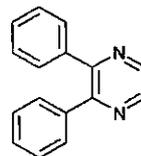
Preparation of 1, 4-pyrazine derivatives

In a typical reaction procedure, in a 50 ml round bottom flask 2 mmol of recrystallized benzil was dissolved in 3 ml of wet methanol and was made homogeneous by stirring with a magnetic spinning bar. To this 2 mmol of ethylene diamine and small amount of ^tBuOK were added. Stirring was continued until the reaction was complete (checked by tlc). Methanol was evaporated under reduced pressure and the crude product was purified by chromatogra-

phy using silica gel. Varied proportion of petroleum ether and ethyl acetate was used as eluent.

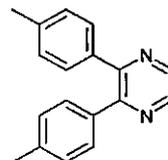
CHARACTERIZATION OF SOME REPRESENTATIVE COMPOUNDS:

2, 3-diphenyl pyrazine



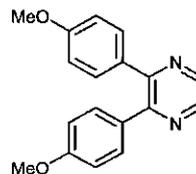
¹H NMR (CDCl₃, 300MHz): δ 7.14-7.25 (m, 5H, five aromatic hydrogen); 7.37-7.44 (m, 5H, five aromatic hydrogen); 8.52 (s, 2H, 2 aromatic hydrogen of the heterocyclic moiety). ¹³C NMR (CDCl₃, 75MHz): δ 128.1, 128.2, 128.5, 129.5, 138.5, 141.9 and 152.6.

2, 3-di *p*-tolyl pyrazine



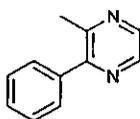
¹H NMR (CDCl₃, 300MHz): δ 2.31 (s, 6H, 2-CH₃); 7.04-7.12 (m, 3H, aromatic hydrogen); 7.27-7.46 (m, 5H, five aromatic hydrogen); 8.51 (s, 2H, two aromatic hydrogen of the heterocyclic moiety). ¹³C NMR (CDCl₃, 75MHz): δ 21.3, 129.0, 129.4, 135.8, 138.5, 141.7 and 152.6.

2, 3-bis (4-methoxy phenyl) pyrazine



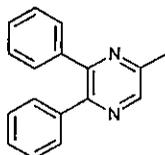
¹H NMR (CDCl₃, 300MHz): δ 3.77 (s, 6H, 2-OCH₃); 6.75-6.85 (m, 4H, four aromatic hydrogen); 7.33-7.43 (m, 4H, four aromatic hydrogen); 8.45 (s, 2H, two aromatic hydrogen of the heterocyclic moiety). ¹³C NMR (CDCl₃, 75MHz): δ 55.2, 113.7, 130.9, 131.2, 141.4, 152.1 and 159.9.

2-methyl-3-phenyl pyrazine



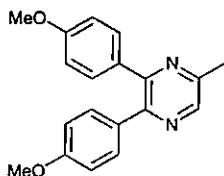
^1H NMR (CDCl_3 , 300MHz): δ 2.54 (s, 3H, $-\text{CH}_3$); 7.46-7.59 (m, 5H, five aromatic hydrogen); 8.44 (d, 2H, $J = 2.4\text{Hz}$). ^{13}C NMR (CDCl_3 , 75MHz): δ 23.1, 128.4, 128.7, 128.9, 138.5, 141.5, 142.1, 151.8 and 154.4.

2, 3-diphenyl-5-methyl pyrazine



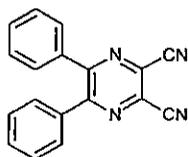
^1H NMR (CDCl_3 , 300MHz): δ 1.86 (s, 3H, $-\text{CH}_3$); 6.63 (d, 10H, $J = 5.1\text{Hz}$, ten aromatic hydrogen); 7.68 (s, 1H, one aromatic hydrogen of the heterocyclic moiety). ^{13}C NMR (CDCl_3 , 75MHz): δ 20.5, 127.4, 127.6, 128.7, 128.8, 137.8, 141.0, 148.8, 150.3, 150.7.

2, 3-bis (4-methoxy phenyl)-5-methyl pyrazine



^1H NMR (CDCl_3 , 300MHz): δ 2.62 (s, 3H, $-\text{CH}_3$); 3.80 (s, 6H, 2- OCH_3); 6.82 (dd, 4H, $J = 1.8\text{Hz}$, four aromatic hydrogen); 7.38 (dd, 4H, $J = 1.8\text{Hz}$, four aromatic hydrogen); 8.39 (s, 1H, one aromatic hydrogen of the heterocyclic moiety). ^{13}C NMR (CDCl_3 , 75MHz): δ 21.2, 53.4, 55.2, 130.8, 130.9, 131.2, 131.4, 141.1, 149.0, 150.5, 150.9, 159.8, 159.6.

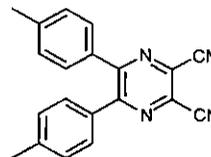
5, 6-diphenyl pyrazine-2, 3-dicarbonitrile



^1H NMR (CDCl_3 , 300MHz): δ 7.16-7.30 (m, 5H, five aromatic hydrogen); 7.45 (t, 2H, $J = 7.3\text{Hz}$, two aromatic hydrogen); 7.57 (t, 1H, $J = 7.3\text{Hz}$, aromatic

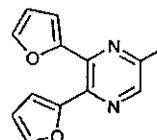
hydrogen); 7.78 (d, 2H, $J = 7.2\text{Hz}$, aromatic hydrogen). ^{13}C NMR (CDCl_3 , 75MHz): δ 126.5, 127.5, 128.2, 128.4, 130.0, 132.4, 137.5, 143.8, 196.8 (carbon of nitrile group).

5, 6-dip-tolylpyrazine-2, 3-dicarbonitrile



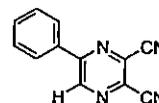
^1H NMR (CDCl_3 , 300MHz): δ 2.32 (m, 6H, 2- $-\text{CH}_3$); 6.99 (m, 4H, aromatic protons); 7.43 (m, 1H, aromatic proton); 7.94 (m, 3H, aromatic protons). ^{13}C NMR (CDCl_3 , 75MHz): δ 21.6, 126.8, 128.6, 129.3, 130.0, 139.2, 144.0, 144.3, 193.3.

2, 3 di-(furan-2-yl)-5-methyl pyrazine



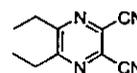
^1H NMR (CDCl_3 , 300MHz): δ 2.59 (s, 3H, $-\text{CH}_3$); 6.56 (m, 4H, aromatic protons); 7.52 (m, 2H, aromatic protons), 8.37 (s, 1H, aromatic proton). ^{13}C NMR (CDCl_3 , 75MHz): δ 21.3, 112.1, 112.7, 139.2, 140.8, 141.7, 143.4, 143.7, 150.5, 150.6, 151.2.

5-phenylpyrazine 2, 3-dicarbonitrile



^1H NMR (CDCl_3 , 300MHz): δ 7.61 (d, 3H, $J = 7.5\text{Hz}$); 8.13 (d, 2H, $J = 6.6\text{Hz}$); 8.51 (s, 1H). ^{13}C NMR (CDCl_3 , 75MHz): δ 128.0, 129.8, 130.8, 132.5, 133.0, 144.1, 154.8.

5, 6-diethylpyrazine-2, 3-dicarbonitrile



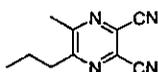
^1H NMR (CDCl_3 , 300MHz): δ 1.39 (m, 6H); 1.97 (m, 2H); 2.97 (m, 2H); ^{13}C NMR (CDCl_3 , 75MHz): δ 11.2, 19.8 (2- CH_3); 25.1, 27.8 (2- CH_2); 113.4, 130.2 (aromatic carbon); 161.3 ($-\text{CN}$).

Pyrazine-2, 3-dicarbonitrile



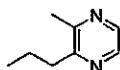
^1H NMR (CDCl_3 , 300MHz): δ 9.00 (s, 2H, aromatic protons). ^{13}C NMR (CDCl_3 , 75MHz): δ 113.1, 133.84 (aromatic carbons); 147.5 (-CN).

5-methyl-6-propiopyrazine-2, 3-dicarbonitrile



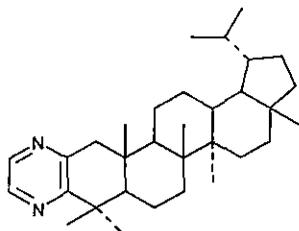
^1H NMR (CDCl_3 , 300MHz): δ 1.06 (t, 3H, $J = 7.2\text{Hz}$); 1.78-1.88 (m, 2H); 2.75 (s, 2H, $-\text{CH}_3$); 2.94 (t, 2H, $J = 7.5\text{Hz}$). ^{13}C NMR (CDCl_3 , 75MHz): δ 13.8, 20.4, 22.3, 36.9, 113.3, 113.4, 129.9, 130.4, 157.7, 161.2.

2-methyl-3-propylpyrazine



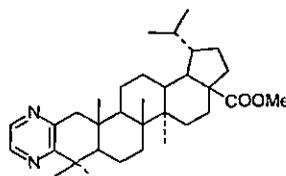
^1H NMR (CDCl_3 , 300MHz): δ 0.96-1.04 (m, 3H, $-\text{CH}_3$); 1.70-1.82 (m, 2H); 2.57 (s, 3H, $-\text{CH}_3$); 2.79 (t, 2H, $J = 7.5\text{Hz}$). ^{13}C NMR (CDCl_3 , 75MHz): δ 14.0, 21.5, 21.7, 36.9, 141.1, 141.4, 152.3, 156.0.

1, 4-pyrazine derivative of 1, 2-diketo lupane



Crystallization afforded white needle shaped crystals, $\text{C}_{32}\text{H}_{50}\text{N}_2$, m.p. 220°C , IR at 1650, 1430 and 1120cm^{-1} . UV absorption maxima at 272nm ($\epsilon = 5831$) and 278nm ($\epsilon = 5792$). Anal. Calc.: 83.12% C, 10.82% H; found 83.10% C, 10.81% H. Mass spectrum showed molecular ion peak at m/z 462. ^1H NMR (CDCl_3 , δ ppm $^{-1}$ relative to TMS): 0.78, 0.83, 0.98, 1.11, 1.29, 1.31 (6s, 18H, 6t- CH_3); 0.77, 0.86 (2d, 6H, CH (CH_3) $_2$, $J = 7\text{Hz}$); 2.47, 3.04 ppm (2d, $J = 16\text{Hz}$); 8.27(d, $J = 3\text{Hz}$); 8.41(dd, $J = 3\text{Hz}$).

1, 4-pyrazine derivative of 1, 2-diketone of methyl dihydrobetulonate



Crystallization from CHCl_3 -MeOH mixture, $\text{C}_{33}\text{H}_{50}\text{O}_2\text{N}_2$, m.p. 220°C . IR spectrum showed peaks at 1710cm^{-1} (CO_2Me); 1665, 1430 and 1120cm^{-1} . UV spectrum showed absorption maximum at 272nm ($\epsilon = 5712$) and 278 nm ($\epsilon = 5603$). Anal. Calc.: 78.26% C, 9.88% H, 5.53% N; found 78.25% C, 9.73% H, 5.50% N. Mass spectrum showed molecular ion peak at m/z 506 as base peak. ^1H NMR (CDCl_3 , δ ppm $^{-1}$ relative to TMS): 0.82, 0.985, 0.99, 1.28, 1.305, 0.76 and 0.88 ppm (2d, 6H, $j = 7\text{Hz}$); 2.48, 3.04 ppm (2d $J = 16\text{Hz}$); 8.27, 8.41 ppm (2d, $J = 3\text{Hz}$); 3.66 (s, 3H, ester methyl).

Sodium dodecyl sulfate in Water: Greener Approach for the Synthesis of Quinoxaline Derivatives

Pranab Ghosh, * and Amitava Mandal

Department of Chemistry, University of North Bengal, Darjeeling, West Bengal 734 013,

India, E-mail: pizy12@yahoo.com

Ph. No. (+ 91) 353 277 6381, Fax: (+ 91) 353 2699001

Abstract

A mild and efficient synthetic method has been developed for the preparation of biologically important quinoxalines in excellent yield from relatively safe precursor α -bromoketones and 1,2-diamines using catalytic amount of micellar sodium dodecylsulfate (SDS) in water at ambient temperature. The method is also found effective for the introduction of quinoxaline moiety into the ring A of pentacyclic triterpenoid, friedelin. Ambient reaction conditions, renewable catalytic condition, inherently safer chemistry, excellent product yields and water as a reaction medium display both economic and environmental advantages.

Key words: Quinoxaline, water, micellar SDS, room temperature, greener approach.

Introduction

Quinoxalines are ubiquitous heterocyclic units in pharmaceuticals and bioactive natural products (1-4). They are used as pharmaceuticals and antibiotics such as echinomycin, levomycin and actinoleutin which are known to inhibit the growth of Gram-positive bacteria and are also active against various transplantable tumors (1-3). Antitumoral properties of quinoxaline compounds have also been investigated (4) Beside these, they are well known for their application in dyes (5) as an efficient electroluminescent materials (6) in organic semiconductors (7) as building blocks for the synthesis of anion receptors (8) as cavitands (9-10) dehydroannulenes (11) and DNA cleaving agents (12-13). Conventionally, quinoxalines are synthesized by a double condensation reaction involving a dicarbonyl precursor and *o*-phenylenediamine (14-15). Due to the highly reactive nature of the dicarbonyls, alternative routes have been proposed recently (16). Antoniotti and Donach have reported one of these methods to synthesize quinoxalines

from epoxides and ene-1,2-diamines (16) Active manganese oxide and molecular sieves in combination or manganese oxides in combination with microwaves have also been used in producing quinoxalines (17-18). These processes, however, require excessive amounts of corrosive manganese oxide as stoichiometric oxidants and scaling them up for industrial processes can lead to the formation of large amounts of toxic waste leading to environmental issues. In additional studies, Robinson and Taylor reported a homogeneous catalytic process utilizing Pd(OAc)₂, RuCl₂ (PPh₃)₂ to synthesize quinoxalines from hydroxy ketones (19) and recently a copper catalyzed oxidative cyclization process has been reported (20). An improved ruthenium catalyzed direct approach to synthesize quinoxalines from diols and *o*-diamines has also been reported (21). These processes suffer from the major drawback that the catalysts are expensive, toxic and cannot be recovered and reused. In addition to the above catalytic methods, synthesis of quinoxalines using zeolites (22-25) microwave (26-27) and solid supports (28-30) has also been reported. Nevertheless, these methods suffer from unsatisfactory product yields, critical product isolation procedures, expensive and detrimental metal precursors and harsh reaction conditions, which limit their use as environmentally friendly protocol. In addition most of the reported methods are not recommended as a clean protocol.

Although very few of the recent reports have claimed α -bromoketones as an equivalent safe chemical precursor of α -hydroxyketones, epoxides or dicarbonyls as reaction partners of *o*-phenylenediamine to prepare quinoxalines (31-32) they involved the use of either HClO₄-SiO₂ or TMSCl as catalyst. Although useful, HClO₄ has huge hazardous nature than its potential usefulness, whereas those catalyzed by TMSCl needs higher

temperature, with lower yield of the desired products not satisfying the principles of green chemistry protocol in contemporary science as well as their acceptance for industrial applications.

In this context the development of an alternative route to quinoxaline from less reactive α -bromoketones in aqueous medium was felt necessary not only due to the increased regulatory pressure focusing on organic solvents, but also because of the emphasis given towards the development of green protocol for organic synthesis nowadays.

The use of water as a medium for organic synthesis is one of the latest challenges in organic synthesis. Reactions in water emerged as a useful alternative route for several organic reactions owing to many of its potential advantages such as safety, economy and friendly towards catalytic and stereoselective processes and more importantly of environmental concern (32-33) and the progress has been dramatic. Additionally, water facilitates ion separation through solvation which often results in altered behavior of reactants in an aqueous environment. Keeping these above facts in mind, we recently have tested water as a solvent in many of our ongoing studies towards organic syntheses and transformative reactions. Very recently we have reported³³ the selective synthesis of 1,2-disubstituted benzimidazole in water catalysed by SDS, and now reporting herein the result of another successful attempt for an efficient synthesis of quinoxaline from α -bromoketones and *o*-phenylenediamine mediated by water and catalysed by SDS at room temperature in excellent yields. This is the first report of synthesizing quinoxaline derivatives in a very mild way in water catalyzed by nucleophilic SDS (33) at ambient temperature starting from the less reactive safer precursor α -bromoketones in an efficient manner.

Results and Discussion

Initially, efforts were directed towards the evaluation of catalytic ability of SDS for the synthesis of quinoxalines. Preliminary studies using phenacylbromide (1 mmol) and *o*-phenylenediamine (1 mmol) without SDS in water at room temperature did not afford the desired quinoxaline. Increase of the reaction time, temperature or by changing the molar proportion of the reactants did not make any influence on the course of the reaction. Addition of some common salts like NaCl, NH₄Cl, KBr *etc.* had no positive effect on the reaction. Similar molar ratios of substrates in tap water yielded the desired product (**3**) only in presence of catalytic amounts of SDS. The modified method gave excellent yield of the product within 6 hours at room temperature (Scheme 1). Thus, the catalytic role of SDS in the present transformation is well established.

This excellent catalyzing ability of SDS inspired us to investigate the above transformation in details. In order to evaluate an optimized and general reaction protocol, a couple of experiments were carried out (Table 1) using varying amounts of SDS (0.34 mol%, 0.17 mol%, 0.06 mol%, 0.03 mol%, 0.02 mol% and 0.01 mol%) in combination with different types (both cationic and anionic) and proportions of surfactants *viz.* tetra-*n*-butylammonium bromide (TBAB), cetyl trimethyl ammoniumbromide (CTAB), cetyl pyridiniumchloride (CPC), sodium dodecylbenzenesulfonate (SDBS) and tetra-*n*-butylammoniumiodide (TBAI), in different reaction conditions for the above model study (Table 1). It is interesting to note that, although all the surfactants can afford (**3**) as the major product but their combination with SDS showed excellent selectivity not only in forming the desired product (**3**) but also in directing the reaction to proceed in a very cleaner way (Entry 1-7, Table 1). Thus it was established that, α -bromoketone (1 mmol)

and 1,2-diamine (1 mmol) in water (3 mL) gave the best result within 6 hrs in presence of SDS (10 mg, 0.03 mol%) at room temperature.

It was also observed that during the reaction the substrates and reactants do not mix together in water; addition of SDS not only raised the solubility of the components in water but also catalyzed the process tremendously. Addition of catalytic amount of SDS (0.03 mol %) turned the reaction mixture into a clear yellowish colored solution that slowly transferred into reddish yellow as the reaction progressed. After completion of the reaction (checked by tlc), products were purified by simple filtration (and in some cases by column chromatography, silica 60-120 mesh) followed by crystallization to get the products in good to excellent yields.

In order to demonstrate the versatility of SDS as a catalyst for the synthesis of quinoxalines, a series of α -bromoketones and 1,2-diamines were subjected to undergo one pot condensation-aromatization in presence of SDS under the optimized reaction protocol (Table 2). All of the reactions tried showed good selectivity with excellent isolated yields.

While investigating the influence of the substituents present either on ketone part or on 1,2-diamine on the course of the reaction, it was observed that compounds having electron donating or withdrawing groups on the ketone (Entry 2, 3, 5, 6, 7 and 8, 9, of Table 2) both underwent the reactions in almost similar fashion and gave good yields. Although, *p*-bromo phenacylbromide (Entry 5, of Table 2) gave better yield than its meta isomer (Entry 9, of Table 2), the corresponding *p*-nitro and *m*-nitro derivative underwent the reaction in identical fashion (Entry 10, of Table 2). Disubstituted α -bromoketones (Entry 11, 12, of Table 2) also gave excellent yields of the expected quinoxalines.

Sensitive molecules like 1,2-diaminomalonitrile (Entry 15, of Table 2) was also found compatible to the reaction condition and gave 84% yield of the corresponding quinoxaline. All the observed results were summarized in table 2.

Potential of pentacyclic triterenod as bioactive candidate is well described (34). In order to see the effect on their bioactivities by the introduction of quinoxaline ring on ring A, we applied our protocol on 2 α -bromofriedelin (5) (prepared from friedelin) (4) and to our delight we have isolated the corresponding quinoxaline derivative (6) in 58% yield within 8 hours under identical condition (0.03 mol% of SDS). This is also the very first report of preparing quinoxaline derivative of pentacyclic triterenoids in water at room temperature (Scheme 2).

As was mentioned earlier, a simple filtration or easy work up procedure of the reaction and reuse of the catalyst, SDS directly from the aqueous extract of the reaction mixture for a fresh run, are the great advantages of the developed process. Gratifyingly, it was tested that the recovered water layer can be reused for six consecutive runs (Table 3).

It is well known that under ambient condition surfactant molecules can aggregate in an aqueous phase to micelles with hydrophobic core and a hydrophilic corona (35-36). To determine whether micellisation had occurred or not we first measured the CMC (critical micellisation concentration) of SDS (Fig 1) and the value was found to be 8.33 mM. In the present study, under the optimized reaction condition the concentration of SDS was 11.57 mM (10 mg of SDS in 3 ml water). Since the value was far beyond the CMC value of SDS (8.33 mM), micellization was anticipated.

It was reported in the literature (37) that the dimensionless packing parameter P of the molecular geometry as an index to predict the size and shape of the micelles. P was defined as $V/(a_0l)$, where V is the hydrocarbon chain volume, a_0 is the optimum head group area per molecule, and l is the hydrocarbon chain length that is taken to be ca. 80-90% of the fully extended chain length (37). The overall prediction was concluded as follows:

Spherical micelles $P < 1/3$

Cylindrical micelles $1/3 < P < 1/2$

Bilayers (or vesicles) $1/2 < P < 1$

Inverted structures $P > 1$

The value of packing parameter P , an index to predict the size and shape of the micelles (37), of SDS was found to be 0.235 (taking l as 90 % of the fully extended chain length) indicating the spherical nature of the developed micelles.

For further confirmation DLS (Dynamic Light Scattering) measurement was carried out of a 11.57 mM aqueous solution of SDS that indicated the presence of micelles (Fig 2) of radius 161 nm (diameter of 322 nm) with the PDI (Polydispersity index) of 0.348.

The role of SDS as a nucleophile is well investigated in our previous communication (33). Considering the above characteristics of SDS, the most probable mechanism of the micellar SDS in effecting the present transformation may be depicted as shown in scheme 3. In the micellar solution, 1,2-phenylenediamine and phenacyl bromide, both of which are hydrophobic in nature, are entered into the hydrophobic core of the micelles and thus assist the condensation between the phenacyl bromide and *o*-

phenylenediamine to form dihydroquinoxaline derivative (A) (Scheme 3). Nucleophilic nature of SDS may have assisted the in situ aromatization of the dihydro derivative (A) to afford quinoxalines (Scheme 4).

Conclusion

A simple, energy efficient, one step SDS catalyzed (0.03 mol%) greener method for the synthesis of quinoxaline derivatives under water mediating condition has been developed. Structurally diversified α -bromoketones, commonly regarded as safer chemicals, were used as reaction partners of 1,2-diamines in water at ambient temperature. Effect of the nature and position of the substituents on both the reactants in consideration to the reaction condition was also studied. Disubstituted α -bromoketones and 2 α -bromo friedelin (a representative of pentacyclic triterpenoids) also formed the corresponding quinoxalines that may serve as lead compound in near future. Except water, no other organic solvents were used. The ambient reaction conditions, comparatively lower reaction time, excellent product yields and simple work up procedure not only make this methodology an alternative platform to the conventional acid/base catalyzed thermal processes, but also found to be significant under the umbrella of environmentally greener and safer processes that may find its place in industry. Moreover, water as a solvent used with micellar SDS has both economic and environmental advantages. As micelles of diameter of 322 nm were formed, it was anticipated that the entitled reactions were occurring inside the hydrophilic core of the micelles. Scaling up the reaction upto 5 moles scale gave good results. We believe our developed process not only satisfied the principles of green chemistry, can open a new way of synthesizing bioactive molecules

by catalyzing SDS in water. Further explorative studies of this efficient combination to various organic syntheses is undergoing in our laboratory.

Experimental

General

All the chemicals used were reagent grade and purified prior to their use. SDS was purchased from Sigma-Aldrich, India. The NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of CDCl₃ solvent as the standards. ¹H spectra were recorded in 300 MHz frequencies and ¹³C NMR spectra were recorded in 75.4 MHz frequencies. Coupling constant 'J' was calculated in Hz. Conductance was measured in Systronics conductivity meter 304 at 25°C (298 K). DLS measurement was performed in Nano ZS90 (Malvern, UK).

General procedure for quinoxalines

In a typical experimental procedure, *o*-phenylenediamine (1 mmol) and α -bromoketone (1 mmol) in 1:1 molar ratios was taken in a 50 ml round bottom flask. To this water (3 ml) and 10 mg (0.03 mol %) sodium dodecylsulfate was admixed. The reaction mixture was then allowed to stir with magnetic spinning bar at room temperature. After the completion of the reaction (checked by tlc), the residue was filtered, washed with water, dried and finally recrystallized from methanol. The desired pure product was characterized by spectral (IR, ¹H- and ¹³C-NMR) data and compared to those reported in literature.

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Table 1 Optimisation of quinoxaline synthesis using phenacyl bromide and *o*-phenylenediamine in presence of different surfactants and their amounts.

Entry	Ratio of aldehyde and diamine	Surfactant	Amount of surfactant (mg)	Temp (°C)	Time (hr)	% Yield ^a of 3
1	1:1	SDS	100	RT	6	96
2	1:1	SDS	50	RT	6	94
3	1:1	SDS	20	RT	6	96
4	1:1	SDS	15	RT	6	94
5	1:1	SDS	10	RT	6	95
6	1:1	SDS	7	RT	12	80
7	1:1	SDS	5	RT	15	64
8	1:1	SDS	5	50	8	68
9	1:1	TBAB	100	RT	10	78
10	1:1	TBAB	100	50	10	76
11	1:1	TBAB	200	100	10	80
12	1:1	CTAB	100	RT	10	66
13	1:1	CTAB	200	100	10	68
14	1:1	CPC	100	RT	10	78
15	1:1	CPC	200	100	10	80
16	1:1	TBAH	100	RT	10	76
17	1:1	TBAH	200	100	10	78
18	1:1	TBAI	100	RT	10	68
19	1:1	TBAI	200	100	10	74
20	1:1	SDBS	100	RT	8	82
21	1:1	SDBS	50	RT	8	80
22	1:1	SDBS	30	RT	8	64
23	1:1	SDBS	30	50	10	70

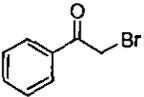
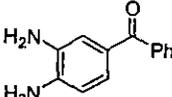
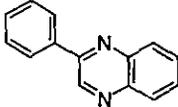
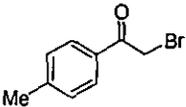
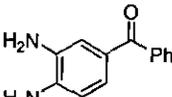
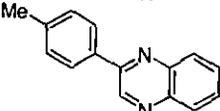
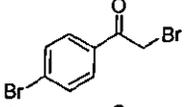
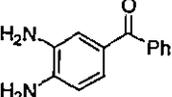
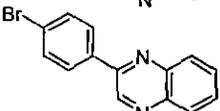
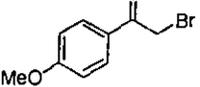
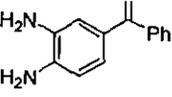
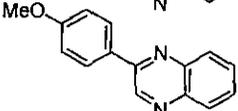
^a % Yield refers to the isolated yield of all the compounds after chromatographic separation.

Table2 Preparation of quinoxaline derivatives.

Entry	α -Bromo carbonyl compound	Diamine	Time (h)	Product	%Yield
1			6		94
2			8		92
3			6		92
4			6		88
5			6		92
6			6		89
7			6		87
8			7		84
9			6		87
10			8		85
11			7		83
12			6.5		98
13			7		82
14			6		92
15			5		84

% Yield refers to the isolated yield of all the compounds.

Continuation of Table2

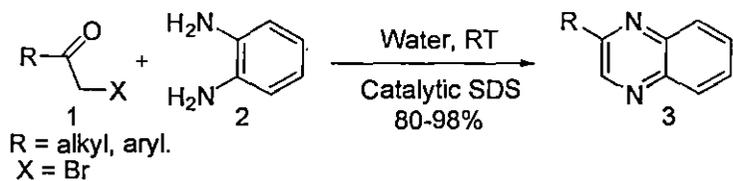
Entry	α -Bromo carbonyl compound	Diamine	Time (h)	Product	%Yield
16			6.5		88
17			6		86
18			6		86
19			6		86

% Yield refers to the isolated yield of all the compounds.

Table 3 Recycling experiment using SDS

Entry	No. of Cycle	% Yield
1	0	92
2	1	87
3	2	82
4	3	78
5	4	72
6	5	68

% Yield refers to the isolated yield of the compound after chromatography.



Scheme 1 SDS catalysed synthesis of quinoxalines in water

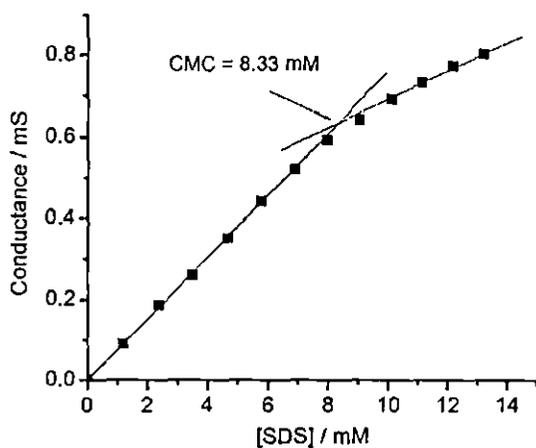
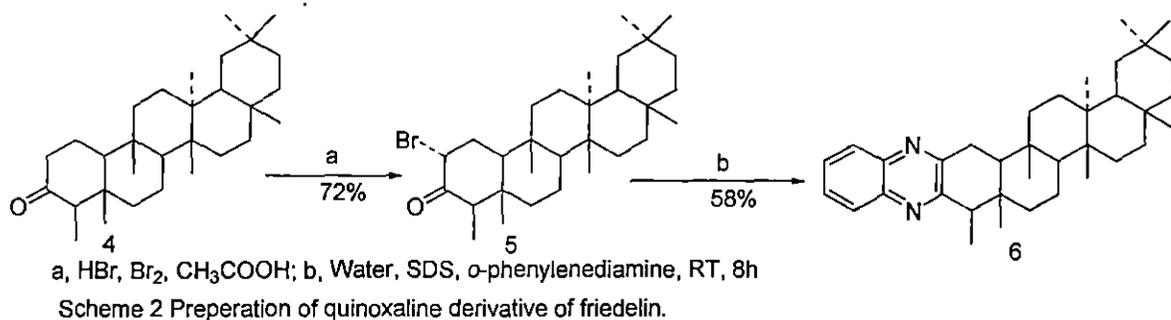


Fig 1 Plot of conductance vs. concentration of SDS for the calculation of CMC value of SDS.

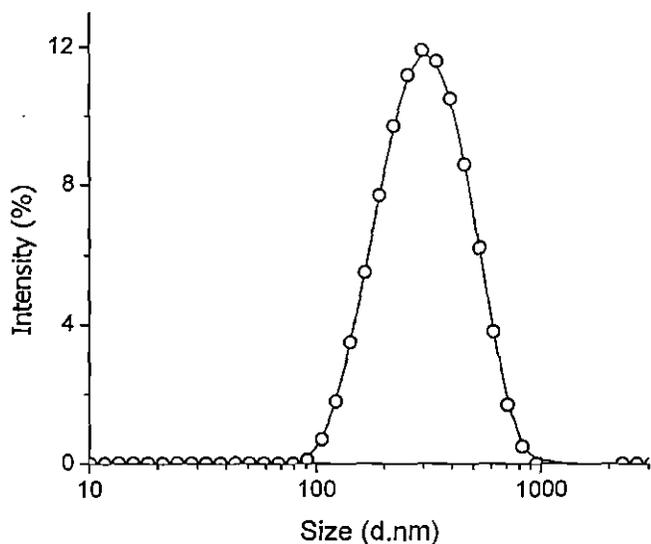
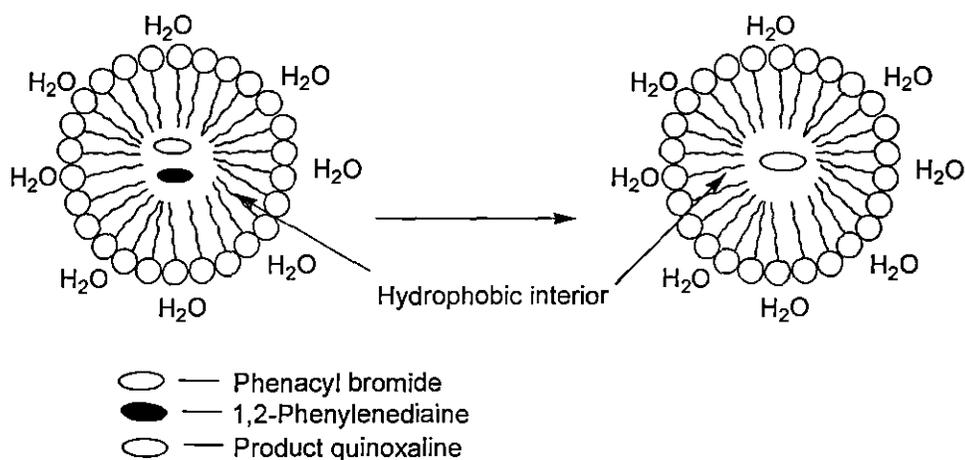
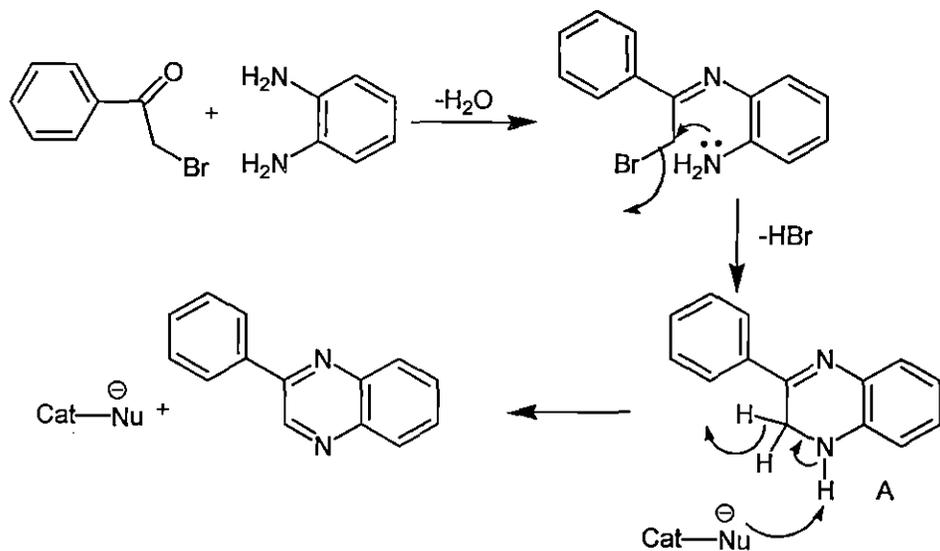


Fig 2 Graph of intensity vs. size (nm) of the micelles based on DLS measurement.



Scheme 3 Proposed model for the synthesis of quinoxaline in water-SDS



Scheme 4 Plausible mechanism of the SDS catalysed quinoxaline formation.

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**A New Bioactive Ursane Type Triterpenoid from *Croton
bonplandianum* Bail**

Pranab Ghosh* Amitava Mandal and Mohammad Golam Rasul

Department of Chemiatry, University of North Bengal, Darjeeling, West Bengal, India

Pin-734013, E-mail: pizy12@yahoo.com.

Ph. No.: (+91) 353 2776381; Fax: (+91) 353 2776381

Running title: Triterpenoids from croton

ABSTRACT

A new triterpenoid 3 α -hydroxy-urs-12,15-dien (**1**) of ursane skeleton along with three others, viz. oleanolic acid (**2**) and ursolic acid (**3**) and β -sitosterol (**4**) were isolated from the root of *Croton bonplandianum* Bail. Their chemical structures were established on the basis of spectroscopic analysis (IR, 1D NMR, 2D NMR, Mass *etc.*) and by chemical means. A probable biosynthetic pathway of compound **1** was also proposed. Preliminary investigation with the new compound showed potent activity against a series of fungal pathogens.

Keywords *Croton bonplandianum*, 3 α -hydroxy-urs-12,15-dien, antifungal.

1. Introduction

Croton bonplandianum Bail belongs to the family of Euphorbiaceae, is native to southern Bolivia, Paraguay, southwestern Brazil and northern Argentina and was reported from India during late 1890 by Kaul.¹ It now occurs widely along roadsides, railway abandoned field in wide open ravines, and paddy or sugarcane fields and on sandy or sandy clay soils. Due to the resemblance of the leaves and flower cymes to that of Tulsi, this plant is often called Ban Tulsi locally. *C. bonplandianum* was reported to have many medicinal uses including the repellent property against the insects,²⁻⁵ mosquito, *A. aegypti*.⁶ Local people in the remote area of West Bengal, India are using its root against snake bite and the leaf extract against high fever. In addition the plant is used both as a fuel and as detergent. The methanol extract of *C. bonplandianum* has been found to exhibit antitumor properties using *Agrobacterium tumefaciens* and has larvicidal activities.

Phytochemically Croton is rich in secondary metabolites including alkaloids and terpenoids.^{7,8} Diterpene resins found in many species of croton have been used experimentally in the studies of tumor initiation and conceivably prove to be useful in cancer therapy.⁹ Apart from the above limited reports, no systematic study has yet been initiated for the total phytochemical investigation of *C. bonplandianum* Bail.

2. Results and Discussion

During our search towards bioactive natural products in tropical plants,¹⁰ we have investigated the chemical constituents of the MeOH extract of roots of *C. bonplandianum*, which showed potent antifungal activity against a series of fungal and bacterial pathogens. The investigation yielded the isolation of a new triterpene 3 α -

hydroxy-urs-12,15-dien (**1**), along with two known triterpenoid oleanolic acid (**2**), ursolic acid (**3**) and most abundant β -sitosterol (**4**). All the compounds (**1**, **2** and **3**) have been screened for their antimicrobial activity.

The MeOH extract of the roots of *C. bonplandianum* was suspended in water, partitioned successively between hexane, EtOAc and n-BuOH to get four fractions (hexane phase, ethylacetate phase, n-butanol phase and water phase). Since results of preliminary screening with the EtOAc soluble fraction was found to be most active against the fungal strain used, it was purified (through repeated column chromatography over silica gel) to isolate the active ingredients *viz.* 3 α -hydroxy-urs-12,15-dien (**1**), along with two known triterpenoids, oleanolic acid (**2**) and ursolic acid (**3**) and most abundant β -sitosterol (**4**). The structures of all the compounds were elucidated on the basis of chemical and spectroscopic analysis and by comparison (for compounds **2**, **3** and **4**) with the spectral data of already reported compounds.

The elucidation of the structure of the new compound was accomplished by extensive analysis of its spectral data. Compound **1** was obtained as a white powder of melting point (mp) 244-246 °C, $[\alpha]_D +4.0$, UV inactive, showed strong absorption band at 3422 (-OH), 2850, 1461, 1376 (gem dimethyl), 1053, 970, 959, 838 cm^{-1} in the IR spectrum indicative of the presence of hydroxyl, olefinic and gem dimethyl groups. ^{13}C NMR spectrum at ambient temperature displayed signals for thirty carbons which were distinguished as eight methyls, seven methylenes, nine methines (five aliphatic, one oxygenated and three olefinic) and six quaternary with the aid of DEPT experiment. The ^1H NMR spectra of compound **1** (Table 1) showed characteristic proton signals due to six tertiary methyl groups at δ_{H} (proton chemical shift) 0.68 (3H, s, C₂₃-Me), 0.69 (3H, s,

C₂₄-Me), 0.76 (3H, s, C₂₅-Me), 0.84 (3H, s, C₂₆-Me), 1.01 (3H, s, C₂₇-Me), 1.25 (3H, s, C₂₈-Me), and two secondary methyl signals as doublet centered at 0.79 (3H, d, J = 6.3 Hz, C₂₉-Me) and 0.92 (3H, d, J = 6.3 Hz, C₃₀-Me). The assignment of the signals of the methyl groups and the remaining ¹H and ¹³C signals were performed through analysis of the HSQC, HMBC and COSY experiments and the results were found consistent with pentacyclic triterpene skeleton and its molecular formula was suggested to be C₃₀H₄₈O on the basis of ESIMS and TOF MS data [m/z = 424 (M⁺)]. On acetylation it gave crystalline acetate of mp 223-224 °C. The molecular formula of this acetate was suggested to be C₃₂H₅₀O₂ [ESIMS data m/z = 467 (M+1)]. The difference in mass spectral data of **1** and its acetate indicated the formation of a monoacetate and hence indicated the presence of only one hydroxyl group in compound **1**. IR spectrum of the monoacetate showed peaks at 2850, 1461, 1376 (gem dimethyl), 1248 (-OCOMe), 1053, 959, 970, 838 cm⁻¹. The appearance of a sharp peak at 1376 cm⁻¹ in the IR spectra of compound **1** and its monoacetate revealed the presence of a gem dimethyl group in ring A of the pentacyclic triterpenoid skeleton and the concordance of δ_H values in the ¹H NMR spectrum for the ring A with that of olean or ursane skeleton clearly rejected the possibility of the presence of friedelan skeleton. Mass fragmentation of the compound also followed the same pattern as was observed for typical pentacyclic triterenoids.¹¹ A possible mass fragmentation pattern is depicted in scheme 1.

The comparison of the chemical shift positions (δ_H) of the A, B and C rings of **1**, especially the resonances of the methyl groups and two secondary methyl signals on ring E provides the most useful indicator for the presence of ursane type triterpenoid skeleton.¹² A hydroxy methine signal at δ_H 3.5 (1H, m, H-3) and a trisubstituted olefinic

signal at δ_{H} 5.33 (1H, d, $J = 2.7$ Hz, H-12). However the splitting nature and small J value may be considered by the consideration of long range coupling involving H-12 and H-19, both are closer in space. The through space distance is obviously more important than the number of intervening bonds, suggesting that **1** is a 3-hydroxy-urs-1,2-en type triterpenoid without possessing angular carboxyl group at C-28.¹⁰ Additionally **1** showed an AB quartet ($>\text{CH}=\text{CH}<$) centered at δ_{H} 5.11 (2H, $J = 15.1$ Hz and 8.5 Hz, H-15 and H-16) signifying the presence of another olefinic double bond having two olefinic protons that must be attributed to the vinylic protons of a disubstituted double bond in a six membered ring.^{13,16}

¹³C NMR spectrum showed all the 30 carbons and thereby confirmed the presence of a triterpene skeleton (Table 1). Four downfield signals at δ_{C} 121.7 (C-12), 140.7 (C-13), 129.2 (C-15) and 138.3 (C-16) indicated the presence of four sp^2 hybridized carbons. The signals at δ_{C} 121.7 and 140.7 are characteristic for a C-12/C-13 double bond in the ursane type structure.¹³ The signal at δ_{C} 140.7 is due to the presence of a trisubstituted olefinic carbon at C-13 and peaks at δ_{C} 138.3 and 129.2 were indicative of the presence of another double bond having two disubstituted olefinic carbon. The ¹³C values for these two carbons (δ_{C} 138.3 and 129.2) clearly removed the possibility of any trisubstituted double bond. C-3 carbon appeared at δ_{C} 71.8 whereas for compounds **2** and **3** it appeared at δ_{C} 78.2, i.e. a shielding of seven units that signifying a stereochemical change at C-3. Therefore, for compound **1** the hydroxyl group is alpha instead of beta, i.e. it is an 3-epi-urs-1,2-en type triterpenoid. On the other hand, the chemical shift of C-18 (δ_{C} 55.9) in compound **1** was very close to that in compound **3** (δ_{C} 53.6), which indicated that these two compounds have the same configuration at C-18. Eight methyl signals appeared at δ_{C}

21.1 (C-23), 29.1 (C-24), 18.7 (C-25), 19.8 (C-26), 24.3 (C-27), 19.4 (C-28), 19.0 (C-29) and 23.0 (C-30). Assignments of all the carbons were made by comparison with that reported in literature for ursane skeleton¹⁴ and are tabulated in table 1.

The structure of compound **1** was finally established by 2D NMR experiments. The methyl singlet at δ_{H} 1.25 was correlated with a carbon signal at δ_{C} 19.4 in the HMQC spectrum were assigned to H₃-28 from HMBC correlation observed from H₃-28 (δ_{H} 1.25) to C-17 (δ_{C} 45.8). Another Methyl singlet at δ_{H} 0.84 (3H, s, C₂₆-Me), was correlated with carbon singlet at δ_{C} 19.8 in the HMQC spectrum, were assigned to H₃-26 from the HMBC correlation observed from H₃-26 (δ_{H} 0.84) to C-8 (δ_{C} 39.7). The presence of a trisubstituted olefin between C-12 and C-13 was revealed by the HMBC correlations observed for H₂-11/C-12, H₂-11/C-13, H-18/C-13 and H₃-27/C-13. Finally 2D NMR techniques were applied to locate the exact position of another double bond. Both the COSY and HMBC spectra gave significant information regarding the position of the double bond between C-15 and C-16. The COSY spectrum revealed two significant correlations of H-18 at δ_{H} 2.26 (1H, d, $J = 4.3$ Hz) with a proton at δ_{H} 5.13 (1H, AB q, $J = 15.1$ and 8.5 Hz) and a proton at δ_{H} 5.01 (1H, AB q, $J = 15.1$ and 8.5 Hz). In the HMBC spectrum cross peaks were observed due to H₃-27 at δ_{H} 1.01 (s, 3H)/ δ_{C} 129.2 and H₃-28 at δ_{H} 1.25 (s, 3H)/ δ_{C} 138.3. Two olefinic protons at δ_{H} 5.13 (1H, AB q, $J = 15.1, 8.5$ Hz) and δ_{H} 5.01 (1H, q, $J = 15.1, 8.5$ Hz) in effect showed correlations in COSY spectrum. From the above data it was confirmed that compound **1** has a second double bond between C₁₅ and C₁₆. The appearance of such an AB quartet for such type of double bond in ring D of pentacyclic triterpenoid, between C₁₅ and C₁₆, was reported in literature.^{15,16}

Thus on the basis of the above data the structure of compound **1** was established as 3 α -hydroxy-urs-12,15-diene.

Spectral analysis (IR, NMR, Mass *etc.*) of the prepared acetate derivative provide further evidence in favor of the proposed structure of compound **1** (3 α -hydroxy-urs-12,15-diene). Mass spectrum showed the incorporation of only one acetyl group (at m/z 467) and thus confirming the presence of only one hydroxyl group. IR spectrum of the monoacetate showed peak at 1248 cm^{-1} in addition to other peaks for the acetate group. In the ^1H NMR spectrum acetate methyl appeared as a singlet centered at δ_{H} 2.33 (3H, s) and the C₃-H shifted downfield (δ_{H} 4.61, m, 1H) in comparison to the same (δ_{H} 3.52, m, 1H) for the hydroxyl methine part in compound **1**. The ^{13}C spectrum showed all the carbons for the monoacetate derivative. C-3 appeared at δ_{C} 74.0 (shifted downfield with respect to that of the original compound, **1**) and the acetate carbonyl signal appeared at δ_{C} 170.5. The DEPT and COSY spectra of the acetylated derivative were in good agreement to that proposed for the acetylated derivative (**5**) and thus established the structure of compound **1** as 3 α -hydroxy-urs-12,15-dien.

Purification of compound **2** yielded a white powder of mp 306-308 °C and its molecular formula was suggested to be C₃₀H₅₀O₃ on the basis of FAB MS data [m/z = 458.2 (M)⁺] and its structure was elucidated as oleanolic acid by ^1H and ^{13}C NMR data and that already reported in literature.¹⁷

Compound **3** was also obtained as a white powder of mp 280-282 °C. Its molecular formula was suggested to be C₃₀H₅₀O₃ on the basis of FAB MS data [m/z = 458.5 (M)⁺] and was finally identified as ursolic acid by ^1H and ^{13}C NMR data and that already reported in literature.¹⁷

Purification of the most polar fraction (8% ethyl acetate in petroleum ether) afforded white crystals of mp 136-137°C, M^+ 414, and was identified as β -sitosterol **4** by spectral analysis and by comparison with an authentic sample of β -sitosterol (mixed m.p., co-IR, and co-tlc etc.).

A probable biosynthetic pathway for the formation of the new triterpenoid **1** has depicted in scheme 2 on the basis of the formation of α -amyrin.¹⁸ This is followed by the formation of a transient carbanion intermediate (S3) and its subsequent rearrangement to S5. Catalytic role of $NADP^+$ present in cells/tissue was then suggested for the formation of **1**.

All the compounds (**1**, **2** and **3**) isolated from the ethyl acetate soluble fraction were tested for their antifungal activity by the disc diffusion method (Table 3) against the microorganisms which are very much native to North Bengal,¹⁹ India; viz. *Calletotricheme camellie*, *Fussarium equisetae*, *Alternaria alternate*, *Curvularia eragrostidies* and *Colletrichum Gleosproides*. These fungal pathogens are responsible to cause wilt disease to tomatoes, pine apple *etc.* cultivated traditionally in this region. The MIC values including that of reference sample Bavistin are tabulated in table I. The MIC values of Bavistin against these fungal pathogens were also determined. A comparison of antifungal activities of compound **1**, **2** and **3** to that of Bavistin showed that although they are less active compare to Bavistin, but all of them exhibited moderately good activity against all the fungal pathogens tested.

3. Conclusion

A new triterpenoid of ursane skeleton has been isolated from the root of *C. bonplandianum* and characterized as 3α -hydroxy-urs-12,15-dien (**1**) along with two

known triterpenic acids, oleanolic acid (2) and ursolic acid (3). β -sitosterol (4) was also obtained as the most polar fraction of the ethylacetate phase. Antifungal potentiality of compound 1 was also detected against five different fungal pathogens. A plausible biosynthetic pathway was also suggested.

4. Experimental

4.1. General experimental procedure

Melting points were determined by open capillary method and were uncorrected. IR spectra were measured on Shimadzu 8300 FT-IR spectrophotometer. NMR spectra were recorded on Bruker-Avance 300 and 400 MHz FT-NMR spectrometer. ESIMS was obtained on Applied Biosystem API 2000 and FAB MS were obtained on Jeol SX 102 mass spectrometer.

4.2. Plant Material

Plants of *C. bonplandianum* used in this experiment were collected from North Bengal, India in May, 2008.

4.3. Extraction and Isolation

The air dried roots of *C. bonplandianum* (2 Kg) was chopped into small pieces and extracted with MeOH (2 L) in a soxhlet extractor for 7 days and MeOH was recovered in *vacuo*. The extracted mass (350 g) was suspended in distilled water (1L) and was partitioned successively between hexane (300 x 3), EtOAc (300 x 3) and n-BuOH (300 x 3) to obtain four fractions (hexane phase, ethyl acetate phase, n-butanol phase and water phase). The ethyl acetate phase (1.5 g) was purified by repeated column chromatography (silica gel) using petroleum ether (PE) and PE:ethyl acetate of varying concentrations as eluent.

4.4. Preparation of acetyl derivative of compound 1

Compound 1 (100 mg, 0.23 mmol) in a 50 mL round bottom flask was warmed over a water bath with 10mL of pyridine and 2 mL of acetic anhydride for 6 hours. The reaction mixture was then poured into 50 mL of ice cold water, worked up with ether, dried over anhydrous sodium sulfate. The crude product was then purified over a column of silica gel.

Purification of the gummy material, gave white crystalline monoacetate of mp 223-224 ° C. IR spectrum showed peaks at 2850, 1461, 1376, 1248, 1053, 959, 970, 838 cm^{-1} . In the mass spectrum it showed a molecular ion peak at 467. The ^1H NMR spectrum of the acylated derivative showed characteristic signals due to five tertiary methyl groups at δ_{H} 0.68 (3H, s, C-23), 0.69 (3H, s, C-24), 0.76 (3H, s, C-25), 0.84 (3H, s, C-26), 1.01 (3H, s, C-27), 1.25 (3H, s, C-28), and two secondary methyl signals as doublet centered at 0.79 (3H, d, $J = 6.3$ Hz) and 0.92 (3H, d, $J = 6.3$ Hz). The acetate methyl appeared as a singlet centered at 2.33 (3H, s).

4.5. Bioassay

Suitable fungal strains were procured from the microbiology laboratory of our institute. DMSO (dimethyl sulfoxide) was used as solvent to prepare different concentrations of the triterpenoid. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in petridishes and were incubated at 37 °C for 48 hour. Culture media for fungal pathogens were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber.

5. Acknowledgement

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6. References

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Table 1 ¹H, ¹³C chemical shift of compound 1 and ¹³C chemical shifts of 1^a, 2 and 3

C	1		1 ^a	2 ^b	3 ^b
	δC	δH	δC	δC	δC
1	40.5	1.03 (m), 1.59 (br. s)	40.5	39.0	39.2
2	29.7	1.65 (m), 1.94 (m)	29.7	28.1	28.2
3	71.8	3.50 (m)	74.0	78.2	78.2
4	33.9		33.9	39.4	39.6
5	56.8	0.86 (br. s)	56.7	55.9	55.9
6	18.7	1.39 (m), 1.48 (m)	18.7	18.8	18.8
7	31.9	1.51 (m), 1.82 (m)	31.8	33.4	33.7
8	39.7		39.7	39.8	40.1
9	50.2	1.55 (m)	50.0	48.2	48.1
10	36.5		36.5	37.4	37.5
11	24.3	1.84 (m), 1.98 (m)	24.3	23.8	23.7
12	121.7	5.33 (s, t-like)	122.6	122.6	125.7
13	140.7		139.6	144.8	139.3
14	42.3		42.3	42.2	42.6
15*	129.2		129.2	28.4	28.8
16*	138.3		138.3	23.8	25.0
17	45.8		45.8	46.7	48.1
18	55.9	2.26 (d, J = 4.3 Hz)	55.9	42.1	53.6
19	56.0	2.21 (m)	56.0	46.6	39.5
20	56.7	1.43 (br. s)	56.6	31.0	39.4
21	31.6	1.52 (d J = 5.6 Hz), 2.26 (d, J = 5.6 Hz)	31.8	34.3	31.1
22	37.2	2.01 (br. s), 2.21 (br. s)	36.9	33.2	37.4
23	21.1	0.68 (s)	21.1	28.8	28.8
24	29.1	0.69 (s)	29.1	16.5	16.5
25	28.2	0.76 (s)	28.2	15.6	15.7
26	19.8	0.84 (s)	19.8	17.5	17.5
27	26.0	1.01 (s)	26.0	26.2	24.0
28	19.4	1.25 (s)	19.3	180.0	179.7
29	19.0	0.79 (d, J = 6.3 Hz)	19.0	33.4	17.5
30	23.0	0.92 (d, J = 6.3 Hz)	23.0	23.8	21.4

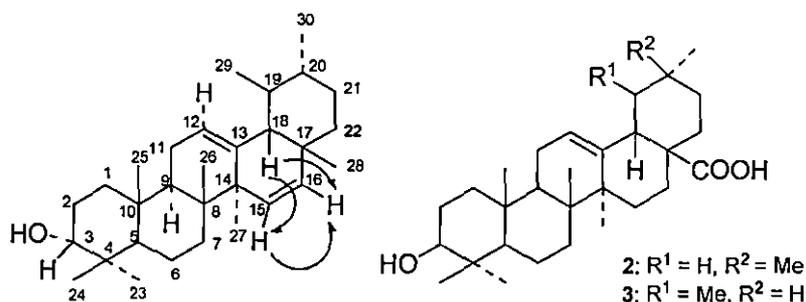
^a Denotes acetyl derivative of compound 1, ^b measured in pyridine d₅ * Olefinic hydrogens attached to these carbons appeared as an AB q centered at 5.11 ppm with J = 15.1 and 8.5 Hz.

Table 2 ¹H-¹³C long range correlation detected in the HMBC spectra of compound 1 (δ relative to TMS in CDCl₃)

Proton	Correlated C
1.01 (H-27)	39.7 (C-8), 140.7 (C-13), 42.3 (C-14), 129.2 (C-15), 50.1 (C-9), 128.3 (C-16)
0.84 (H-26)	39.7 (C-8), 42.3 (C-14)
1.25 (H-28)	45.8 (C-17)
0.68 (H-23)	33.9 (C-4), 56.8 (C-5), 71.8 (C-3)
1.55 (H-9)	36.5 (C-10), 39.7 (C-8), 19.8 (C-26), 28.2 (C-25)
5.33 (H-12)	50.1 (C-9), 42.3 (C-14), 55.9 (C-18)
2.26 (H-18)	121.7 (C-12), 140.7 (C-13), 42.3 (C-14), 128.3 (C-16), 45.8 (C-17), 56.0 (C-19), 56.7 (C-20), 19.4 (C-28)

Table 3 MIC of Compound 1 against different fungi

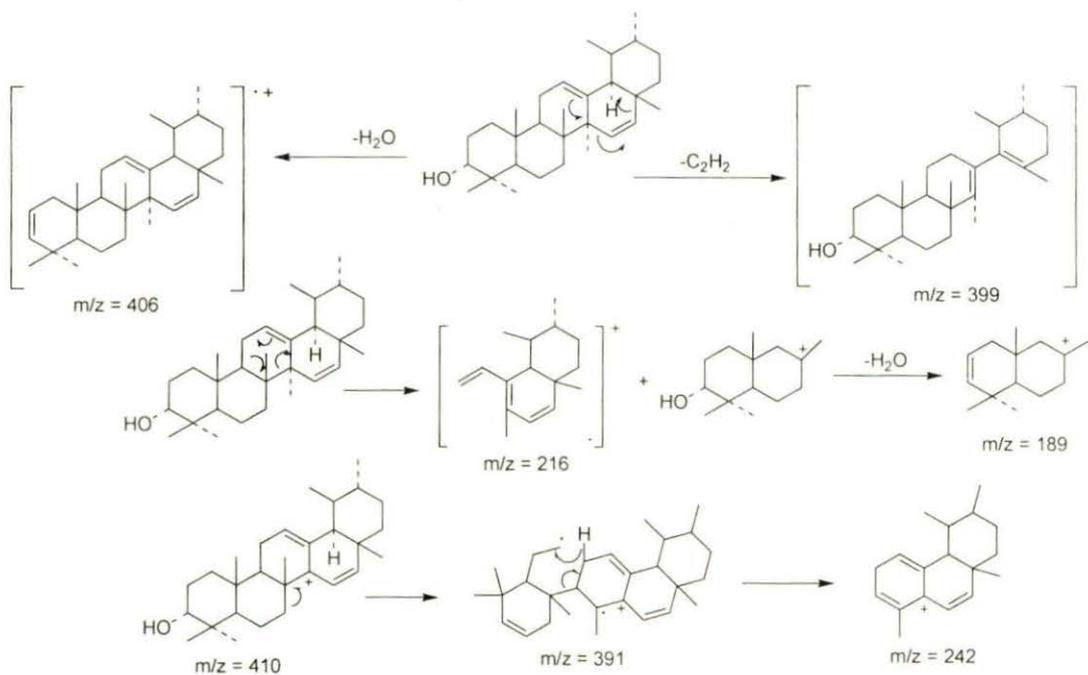
Compound	MIC of 1 in µg/mL against different fungi				
	CC	CG	AA	FE	CE
1	10	<15	10	<10	15
2	<10	20	10	<10	10
3	15	<10	10	<15	<15
Bavistin	2.5	1.25	2.5	2.5	<2.5



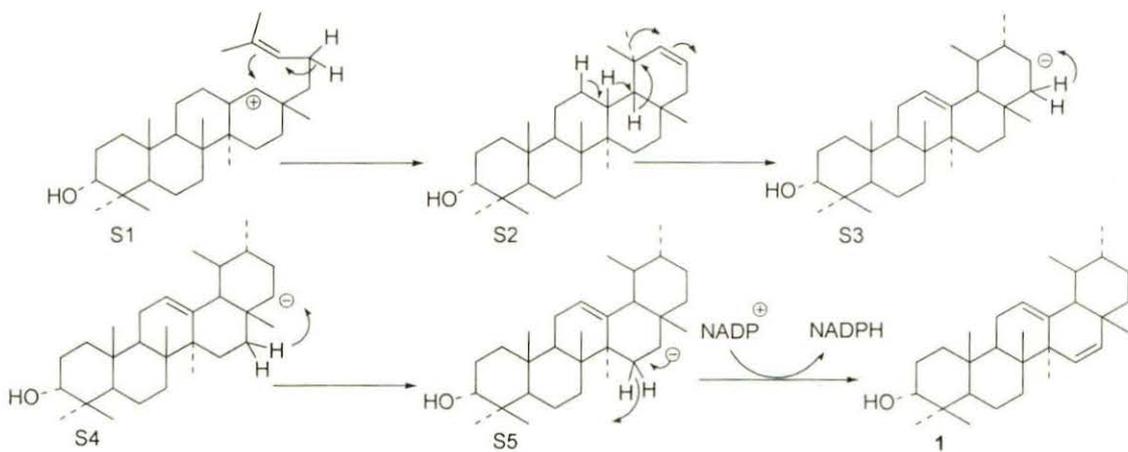
Key COSY correlation of compound 1

1.01 8.0

0.84 8.0
1.25 8.0



Scheme 1 Proposed mass fragmentation pattern of compound 1



Scheme 2 Probable biosynthetic pathway of compound 1

