SECONDARY METABOLITES FROM A LOCAL MEDICINAL PLANT - *Gardenia erubescens*. Stapf. & Hutch.

by

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ABSTRACT.

A detailed chemical analysis of the stem of *Gardenia erubescens* Stapf. & Hutch. was carried out. The crushed stem was sequentially extracted with petroleum ether, ethyl acetate and methanol. Subsequently the petroleum ether, ethyl acetate and methanol extracts when subjected to phytochemical tests revealed the presence of steroids, flavonoids and saponins, while anthraquinones and alkaloids were not detected.

The petroleum ether extract and the n-butanol soluble fraction of the methanol extract were further subjected to chromatographic analyses using a combination of column, TLC and preparative TLC. The TLC analysis of petroleum ether extract showed the presence of steroids and flavonoids as the major components. TLC analysis of the n-butanol soluble fraction of methanol extract however revealed only the presence of saponins.

Purification of the petroleum ether extract on column of silica gel afforded, after further purification by preparative TLC, five known compounds, one fraction containing isomeric mixture of two compounds and one new bisnortriterpenoid compound. From the saponin fraction of the n-butanol soluble fraction of methanol extract, five compounds were isolated and identified. Four of these compounds were known while one was a new oleanene-type compound.

A total of fourteen compounds were isolated and identified from both the petroleum ether extract and n-butanol soluble fraction of the methanol

extract of the stems of *Gardenia erubescens*. Characterization of the known compounds was achieved through spectral correlation with authentic samples and/or derivatisation and comparison of melting points with the known compounds. Full characterization of the new compounds was accomplished by use of 2D nmr experiments - COSY and ^{1}H-^{13}C nmr correlation spectra, assisted by NOE difference spectroscopy.

The known compounds isolated and identified from *Gardenia erubescens* in this work were:

- D-Mannitol
- Stigmasterol
- Stigmasterol-3β-D-glucopyranoside
- 5-Hydroxy-7,4'-dimethoxyflavanone
- 5-Hydroxy-7,4'-dimethoxyflavone
- 5-Hydroxy-7,3',4'-trimethoxyflavanone
- 3β-Acetoxyolean-12en-30-oic acid
- Isomeric mixture of oleanolic and ursolic acids
- 3β,23-Dihydroxyolean-12-en-28-oic acid
- 2β,3β-Dihydroxyolean-12-en-28-oic acid
- 2β,3β,23-Trihydroxyolean-12-en-28-oic acid

and the two novel compounds were:

- Erubescenone and
- 3β,23,24-Trihydroxyolean-12-en-28-oic acid.

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I will always give thanks to the Lord for He is great and His greatness is beyond human understanding.

Esther Adebola Adelakun.

March 1995.

CERTIFICATION.

I certify that this work was carried out by Mrs. E. A. Adelakun in the department of Chemistry, University of Ibadan, Ibadan. Nigeria.

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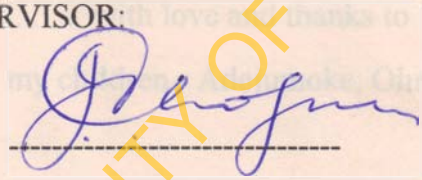

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DEDICATED

..... with love and thanks to

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GLOSSARY

General Abbreviations.

TLC =.thin layer chromatography

Prep. TLC = preparative thin layer chromatography

UV = ultraviolet

IR = infrared

NMR = nuclear magnetic resonance

DEPT = distortionless enhancement by polarization transfer

NOE = nuclear overhauser effect

COSY = correlated spectroscopy

HETEROCOSY = heteronuclear correlated spectroscopy

MS = mass spectroscopy

EIMS = electron impact mass spectroscopy

CIMS = chemical ionization mass spectroscopy

FABMS = fast atomic bombardment mass spectroscopy

R_f = mobility relative to front

nm = nanometers

FT = fourier transform

TMS = tetramethyl silane

Solvents and Reagents

DMSO = dimethyl sulphoxide

MeOH = methanol

$CHCl_3$ = chloroform

EtOAc = ethyl acetate

Ac_2O = acetic anhydride

H_2SO_4 = sulphuric acid

$AgNO_3$ = silver nitrate

NaOH = sodium hydroxide

HBr = hydrogen bromide

AcOH = acetic acid

$LiAlH_4$ = lithium aluminium hydride

CHAPTER ONE

INTRODUCTION.

During the long history of mankind, man's views on the cures of diseases have undergone radical changes. The early medicines of man were obtained from natural sources especially, the vegetable kingdom. There is an abundance of plant species in the world, and many are still being discovered. The knowledge of the use of these plants have accumulated over centuries of practice and were handed down by oral tradition from generation to generation. In the course of civilization the changes imposed by modern science on man's sociocultural practices now seem to cause a change in the disposition to such traditional values. However, some of the practices and methods of the use of these medicinal plants have survived to a greater extent in the developing countries of the world.

The use of plants either singly or with other natural products in the treatment of diseases within an organized indigenous system constitutes part of what is known as traditional medicine and is defined "as the sum total of all knowledge and practices whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation whether orally or in writing"[1]. It naturally

follows that the people engaged in the practice of traditional medicine are traditional medical doctors. The enomous varieties of plants used in this sense are known as medicinal plants, and "a medicinal plant is defined as any plant which in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs"[2].

Knowledge of the uses of medicinal plants though / jealously guarded by the traditional medical practitioners, has now become an area of great interest and exploitation in developing countries as Government in these countries now mobilize their people to be self-reliant in an attempt to save money through reduction in the / importation / of foreign products. Although the importance of naturally occuring drugs has long been recognised, it appears the developed countries - Europe and North America, are increasingly shifting emphasis to synthetic drugs. However an analysis shown in table 1, as reported by Prof. Okogun [3] in his lecture on "Drug production efforts in Nigeria: Medicinal Chemistry Research and a missing Link", delivered to the N.gerian Academy of science, suggested a significant contribution from natural products chemistry to drug production even in the so called technologically advanced countries.

But the situation is different in developing countries as 60-85% of the population in every developing country rely on traditional medicine because it is cheaper and more accessible than modern medicine. Research in African traditional medicine had been sponsored by OAU since 1968. The

OAU/STRC has continued to finance research in this area with regional co-ordinating centres scattered throughout Africa. The co-ordinating centre for West Africa is Ile-Ife[4]. The results of scientific investigations on medicinal plants continue to provide scientific proof of the efficacy of traditonal medicine.

Table 1 Value of Higher Plant Medicinals in the USA in 1973.
= $ 3,000,000.00

Source of prescription	Percentage	
	1973	1959
Higher Plant	25.2	25.2
Microbes	13.3	21.4
Animal	2.7	2.3
Total	41.2	48.9

A stage has therefore been reached whereby certain local claims to specific medicinal activities of some of these plants cannot be regarded as superstition or simply ignored. It is important to continue to explore this valuable cultural legacy in Nigeria so as to pave the way for integration of traditional and modern medicine as it is now in China. In essence, it has

become increasingly encouraging and necessary to subject many of these plants to scientific research so as to provide justification for their medicinal uses. This aspect of organic chemistry research is identified as natural products chemistry.

Natural products chemistry in its different aspects is an ancient science. For example, preparation of foodstuff, colouring matter, medicinals or stimulant are activities as old as mankind. When chemists took the final jump in the late 18th century from the world of myths into modern science, the true properties of extracts obtained from nature became the focus. They began to separate, purify and then analyse the compounds produced in living cells. Separation methods were developed and without doubt natural products chemistry has brought great stimuli to the development of the refined separation techniques available today, that is, column chromatography(CC), gas chromatography(GC), thin layer chromatography(TLC), high performance liquid chromatography(HPLC), paper chromatography(PC), electrophoresis, ion-exchange chromatography etc. These methods have made it possible to isolate compounds present in extremely small quantities. Structural elucidation was typically carried out by degradation to smaller fragments of known structure, but with computerised spectroscopy in its service, natural products chemistry has attained a highly refined status today. A large number of spectroscopic data correlating spectral properties with structure are available. These data give very valuable information about structure so much so that pure chemical transformation can be reduced to a minimum or

completely avoided. When the amount of sample available is very limited and structure too complicated, X-ray crystallography is the ultimate resource.

While natural products chemists are interested in secondary metabolites, the biochemists are concerned mostly with products of primary metabolism. Primary metabolism refers essentially to the photosynthetic and related processes producing the carbohydrates, fats and proteins which are widely distributed.

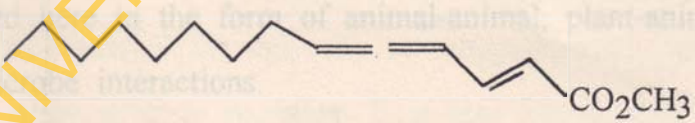
From the compounds identified as components of primary metabolism, only a handful serve as source material for the elaboration of the thousands of known secondary metabolites. The most important is acetic acid, the others are aromatic amino acids - tryptophan, phenylalanine and tyrosine, and the aliphatic amino acids - ornithine and lysine. When methionine is added as the natural methylating agent, the list is virtually complete.

Secondary metabolites are characteristic for the particular biological group -family or genus or species. Apparently, the synthetic process involved in the production of secondary metabolites is related to the mechanism of evolution of species. The specific pattern of constituents in species has been used for systematic determination.

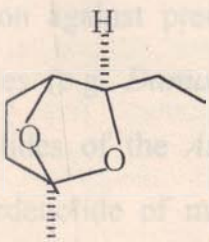
A characteristic feature of secondary metabolites is that their function is to a significant extent not always obvious. It is hard to believe that the organism should allocate so large a proportion of metabolic resources for purposes void of sense. The production of secondary metabolites is found to be connected with several external factors such as growth, flowering, season,

temperature, habitat etc. On this ground secondary metabolites are defined as substances which play a prominent role in the co-existence and co-evolution of species. The isolation of the sex-attractant, bombycol, 1 from the female silk moth was the first evidence in support of the idea that secondary metabolites produced in plants and animals have strong behaviour control on other species. This also signalled the beginning of research on pheromones. The structures of several specific sex attractants are known today[5]. Examples are bombycol, 1 from *Bombyx mori*, methyl (E)-2,4,5-tetradecatrienoate, 2 from *Acanthoscelides obtectus*, exo-brevicomin, 3 from *Dendroctonus brevicomio*, (E,E)-3,7-dimethyl-2,6-decadien-1,10-diol, 4 from *Danaus gilippus* and cis-verbenol, 5 from *Ips typographus*.

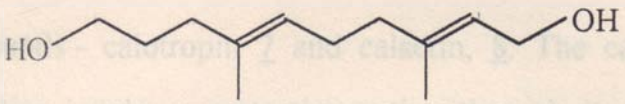
1, Bombycol.



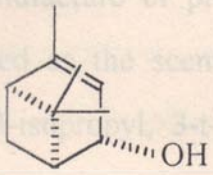
2, Methyl-(E)-2,4,5-tetradecatrienoate



3, Exo-brevicomin.



4, (E,E)-3,7-Dimethyl-2,6-decadien-1,10-diol.



5, cis-Verbenol.

1.2. SECONDARY METABOLITES AND THE ECOSYSTEM.

Today the role of natural products with respect to co-existence and co-evolution of species has been an area of intense research activity. The established role of some natural products in the ecosystem are briefly discussed here in the form of animal-animal, plant-animal, plant-plant and plant-microbe interactions.

1.2.1. Animal-Animal Interactions.

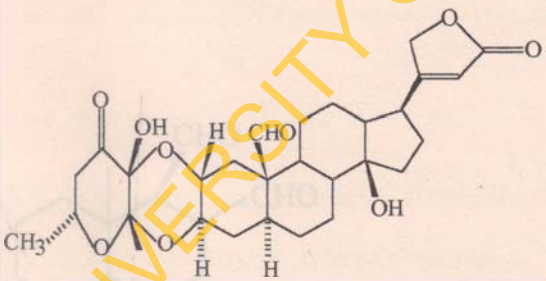
There have been reported cases where insects collect plant toxins for protection against predators. One such interaction is the ability of Monarch butterflies (e.g. *Danus plexippus*) to sequester, store and metabolize cardenolides of the *Asclepias species*[6]. The cardenolide, uscharidin 6, which is a cardenolide of milkweed, has been shown to be metabolized by tissue

homogenates of the larvae of Monarch butterflies to the more polar compounds - calotropin 7 and calactin, 8. The cardenolide content of these butterflies invoke a protective action through its emetic effect on predators. Similarly, the adult butterflies of the order - *Danainae*, sequester and store pyrrolizidine alkaloids from plant sources, using them both for defence and for manufacture of pheromones. The compound hydroxydanaidal, 9 has been identified as the scent produced in the coremata of *Creatonotos gangis*[7], while 3-isopropyl, 3-t-butyl and 3-isobutyl-2-methoxypyrazine 10, 11 & 12 respectively have been identified in the warning odour secretions of *Danaus plexippus* and *Zygaena ionicerae*. The brown aphid - *Aphis cytisorum* also accumulates cytisine as a defence chemical when fed on *Petteria ramentacea*[8].

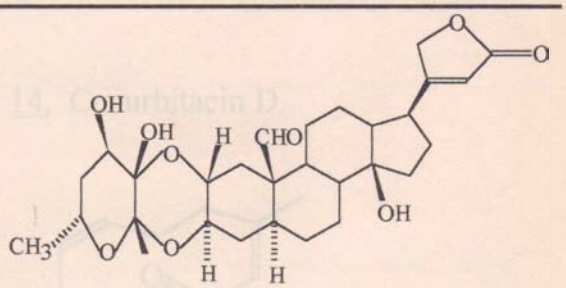
Other examples of sequestration of plant toxins include the use of the phenol, salicin 13, by the Chrysomelid beetle - *Chrysomela aenicollis*. The larvae sequester it from the leaves of *Salix species*, hydrolyse the glucosidic linkage and oxidize the aglycone to salicylaldehyde, which is the principal toxin in their defence secretion. It is also known that some species of the chrysomelidae have the ability to sequester a metabolite of cucurbitacin D, 14 which is then used to protect themselves against predation by mantids[5].

The marine world is not left out in this ecological chemistry, although it is less well developed. A few examples will be mentioned to illustrate the complex ecological situation that exists below the surface of the seas and oceans. The dorid nudibranch, *Dendrodoris limbata* produces polygodial, 15 -

a hot tasting sequiterpenoid - as an antifeedant against marine and fresh water fish. Other species like *Glossodoris quadricolor* sequester their defensive chemicals from sponges they feed on. For example, the ichthyotoxin of the sponge, latrunculin B 16, was identified in the mucus secretion of the mollusc, indicating that it is used as defensive agent[9]. Sponges may use their toxic secretion for their own purposes. Thus species of sighonodictyon burrow into living coral heads killing coral growth within a zone which extends for 1-2 cm. The toxin that is exuded is a guanidino-compound, 17.

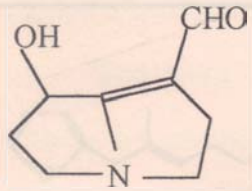


6, Uscharidin.

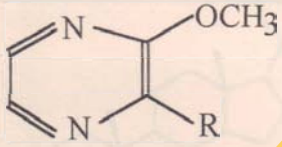


7, Calotropin, 3' α-OH.

8, Calactin, 3' β-OH.

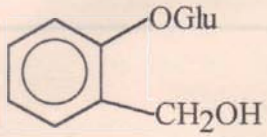


9, Hydroxydanaidal.

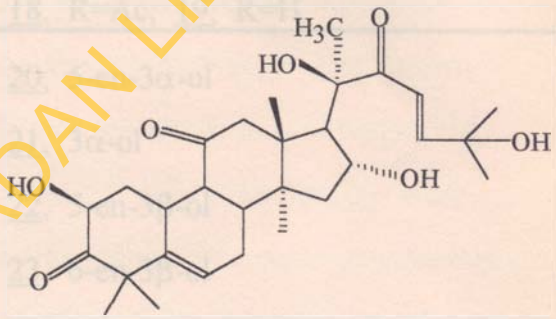


10, R=$CHMe_2$; 11, R=CMe_3;

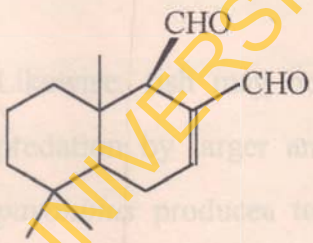
12, R=CH(Me)Et.



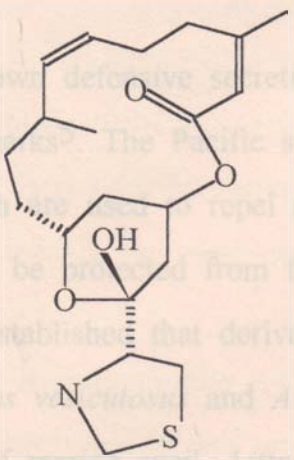
13, Salicin.



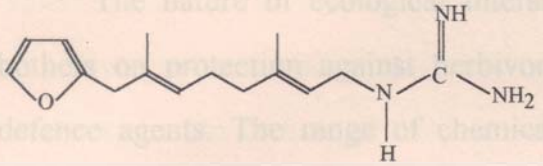
14, Cucurbitacin D.



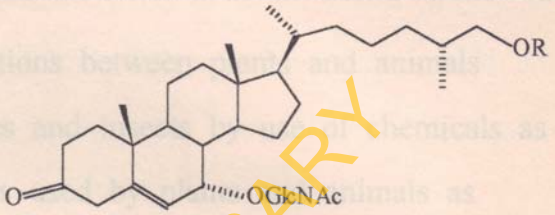
15, Polygodial.



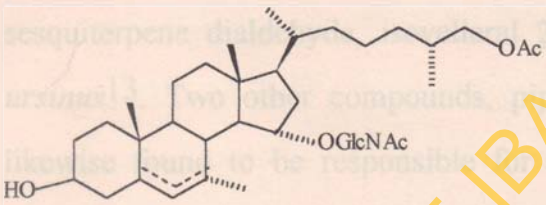
16, Latrunculin B.



17, Guanidino-compound.



18, R=Ac; 19, R=H.



20, 5-en-3α-ol

21, 3α-ol

22, 5-en-3β-ol

23, 6-en-3β-ol

(GlcNAc = 2-acetylamino-2-deoxy-D-glucosyl).

Likewise, fish may benefit from their own defensive secretions and avoid predation by larger animals, such as sharks[5]. The Pacific sole - *Paradachirus pavoninus* produces toxins 18-23, which are used to repel attack by sharks[10].On the other hand, algae may be protected from fish by their secondary constituents[11]. It has been established that derivatives of phloroglucinol in the brown algae *Fucus vesiculosus* and *Ascophyllum nodosum* effectively limit the feeding of marine snail, *Littorina littorea*[12].

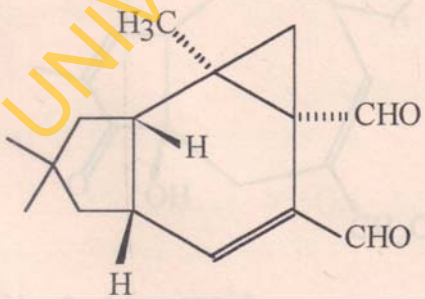
1.2.2. Plant - Animal Interactions.

The nature of ecological interactions between plants and animals bothers on protection against herbivores and insects by use of chemicals as defence agents. The range of chemicals used by plants and animals as toxins in response to predation or insect attack cuts across the major classes of secondary metabolites. The toxins that occur in the fruiting bodies of fungi are known to have protective role against herbivory. The known sesquiterpene dialdehyde, isovelleral 24, was obtained from *Lentinellus ursinus*[13]. Two other compounds, piperdial 25 and piperalol 26, were likewise found to be responsible for the pungency of *Lactarius piperatus*, *L. torminosus* and *Russula aqueletii*. These three aldehydes bear an obvious structural resemblance to the pungent principle, warburganol 27, which had been isolated from higher plants and shown to be antifeedant to Man and to insects[14]. Sesquiterpene lactones, which occur mainly in members of the family Compositae, are another group of sesquiterpenoids with defensive role against herbivory[15]. The compounds deoxylactucin 28, lactucin 29 and lactupicrin 30, from *Cichorium intybus* have been implicated as defensive agents as they occur in high concentrations in the most actively growing regions of the plant[16].

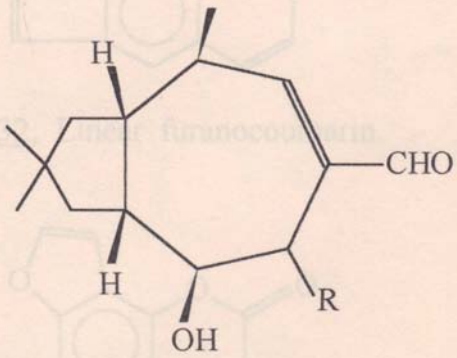
Some components of the croton oil from *Croton tiglium* known to be skin irritants and co-carcinogens in animals were tested on the larvae of the mosquito, *Culex pipiens*. One of the components, a diterpenoid 31, caused 100% mortality in the larvae at a concentration of 0.6ppm[17].

Furanocoumarins constitute another important class of plant toxins which are commonly found in plants of the family Umbelliferae. These compounds are photomutagenic and photocytotoxic and it has been demonstrated that most of the plants in this family are protected from herbivory by furanocoumarins[18]. It was also observed that plants which contain angularly fused furanocoumarins 33, are more toxic than those with linear furanocoumarins[19], 32.

A number of phenolic toxins have been identified. o- pentadecenyl-salicylic acid 34 and o- heptadecenylsalicylic acid 35, from the exudate of *Pelargonium hortorum* were found to be moderately toxic to the two-spotted spider mite, *Tetranyclus urticae*[20]. A series of prenylated hydroquinones 36, have been detected in species of *Phacelia* and are found to be responsible for the allergenic effects of these plants in Man[21].

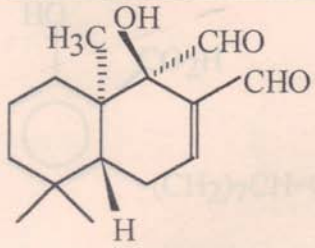


24, Isovelleral.

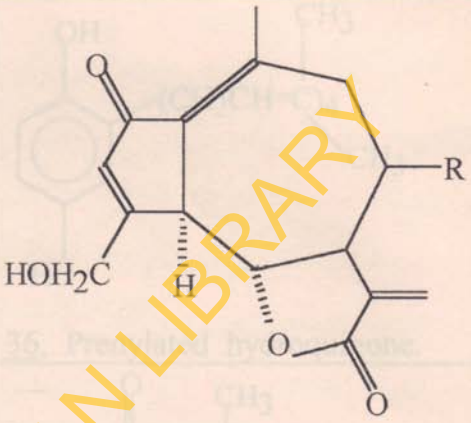


25, Piperdial, R= CHO

26, Piperalol, R= CH_2OH



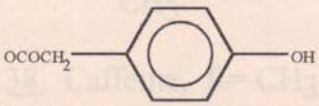
27, Warburganol.

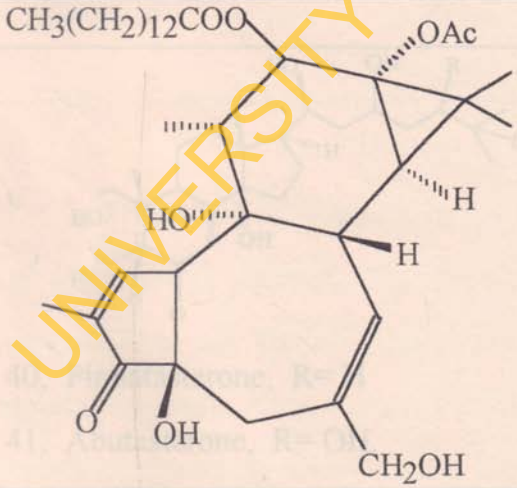


28, Deoxylactucin, R= H

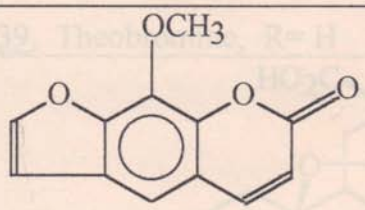
29, Lactucin, R= OH

30, Lactupicrin, R=

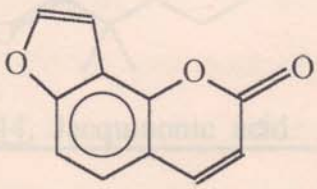




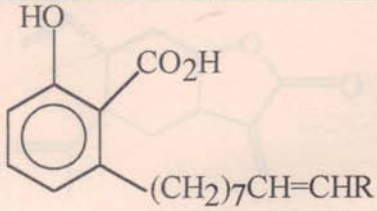
31, A diterpenoid.



32, Linear furanocoumarin.

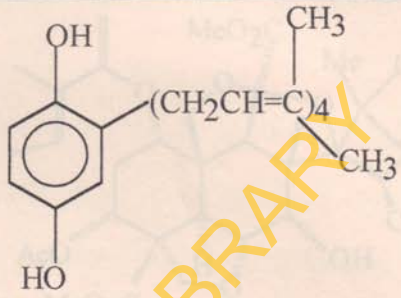


33, Angular furanocoumarin.

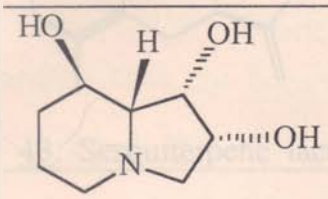


34, R= $(CH_2)_5CH_3$

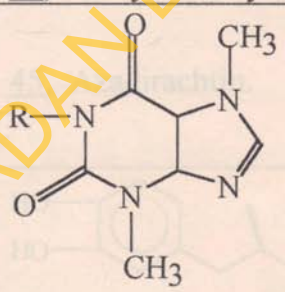
35, R= $(CH_2)_7CH_3$



36, Prenylated hydroquinone.

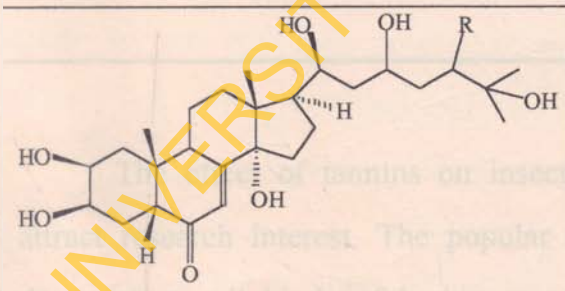


37, Swainsonine.



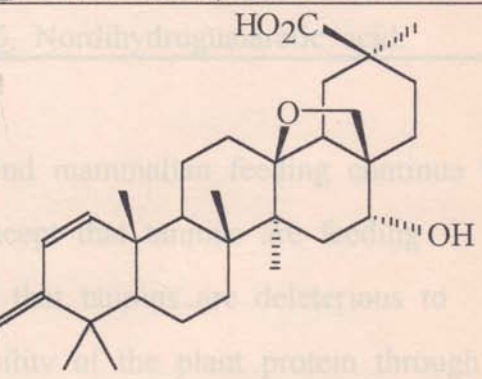
38, Caffeine, R= CH_3.

39, Theobromine, R= H

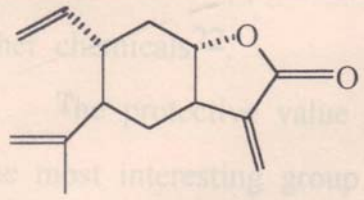


40, Pinnatasterone, R= H

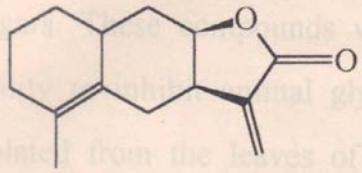
41, Abutasterone, R= OH.



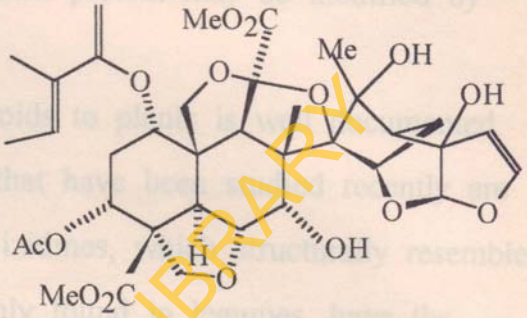
44, Jacquinonic acid.



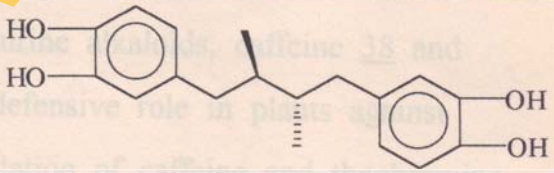
42



43. Sesquiterpene lactones.



45. Azadirachtin.



46. Nordihydroguaiaretic acid.

The effect of tannins on insects and mammalian feeding continue to attract research interest. The popular concept that tannins are feeding deterrents to all kinds of herbivores and that tannins are deleterious to animals because they reduce the digestibility of the plant protein through complex formation needs to be modified. It has now been realized that tannins are no different from other classes of toxins in that herbivores can adapt to their presence in the diet. Also the deleterious dietary effects

resulting from complex formation with plant protein may be modified by other chemicals[22].

The protective value of some alkaloids to plants is well documented. The most interesting group of alkaloids that have been studied recently are simple pyrrolidines, piperidines and indolizidines, which structurally resemble sugars. These compounds which are mainly found in legumes, have the ability to inhibit animal glycosidases. Swainsonine 37, has recently been isolated from the leaves of *Astragalus lentiginosus*[23] and is the active principle of this plant which causes neurological disturbances in cattle and eventually death. Even the well-known purine alkaloids, caffeine 38 and theobromine 39, now appear to have a defensive role in plants against attack by insects. The pattern of accumulation of caffeine and theobromine in plants of *Coffea arabica* is closely correlated with a proposed defence strategy, having very high concentrations of these compounds in young seedlings and during leaf development[24].

Phytoecdysones which are mimics of insect moulting hormones also provide a defence strategy by inhibiting the process of moulting in the larvae of insect invaders[25]. The search for phytoecdysones in plants continues to yield new compounds. New reports include pinnatasterone 40, from *Vitex pinnata*(Verbenaceae)[26], while abutasterone 41, from *Abuta velutina*[5] is also one of the recent addition. In most cases, plants which contain ecdysones often contain other terpenoids that possess antifeedant or insecticidal properties. Within a given plant species, the phytoecdysone

defence may thus be only one component of a complex defensive strategy to repel insect invaders.

Leaf-cutting ants constitute a serious menace to plants. These ants appear to be impervious to many secondary compounds which would in similar circumstances repel caterpillars or leaf miners. It is now obvious that plants provide immunity from attack by leaf-cutting ants by production of terpenoids which specifically stop invasion by ants[27-32]. The ant repellants range from simple monoterpenes such as β-ocimene[30] through sesquiterpenoids such as caryophyllene epoxide[27], sesquiterpene lactones 42, 43 and diterpenoids[31], to triterpenoids such as jacquinonic acid 44 and azadirachtin 45.

1.2.3. Plant- Plant Interactions.

Allelopathic effects seem to be an important factor which determines the pattern of vegetation in natural ecosystems. Allelopathic property is the production of secondary metabolites by a plant which is used to control the growth of other plants in its vicinity. A number of new classes of chemicals have been identified as allelopathic agents in particular instances. The lignan, nordihydroguaiaretic acid 46, which occurs in the leaves of *Larrea tridentata* has been implicated for the well-marked allelopathic effect of this shrub on surrounding vegetation[33]. Another known allelopathic substance is juglone, which is the toxic principle of walnut tree, *Juglans regia*. The well-known phenolic compounds such as p-coumaric and ferulic acids, have

also been implicated as allelopathic agents that are produced by the bamboo *Phyllostachys edulis*, the conifer *Cryptomeria japonica* and the grass *Setaria faberii*[34].

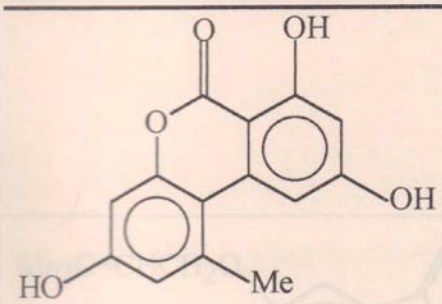
1.2.4. Plant- Microbe Interactions.

The interaction between plants and microbes usually takes the form of production of toxins by the microbes which renders the host plant toxic with symptoms of disease condition or production of toxins by plants as chemical defence agents against microbial infection. Depending on the form of interaction, mycotoxins, phytotoxins or anti microbial agents are produced.

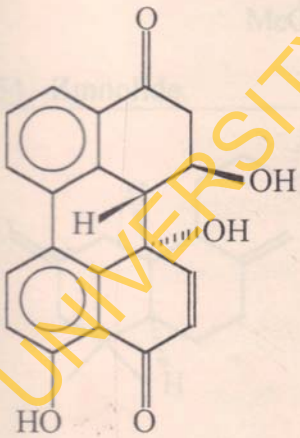
Mycotoxins are secondary metabolites of fungi origin which contaminate certain plant tissues, e.g. groundnuts and cereals, as a result of infection. Mycotoxins pose a threat to animals eating such plant tissues because they tend to be highly poisonous. The best known group of mycotoxins are the aflatoxins, which are formed by *Aspergillus flavus*. The aflatoxins are known to be phototoxic in their activity against animals while alternariol 47, which is a dibenzopyrone mycotoxin of *Alternaria* species, has also been shown to be a photosensitizing agent[35].

Phytotoxins are also microbial metabolites but they are responsible for the symptoms of disease in infected plants. Helminthosporoside, which has been shown as a mixture of three isomeric sesquiterpene glycosides 48-50, is the toxin of the sugar-cane pathogen, *Helminthosporium sacchari*[36]. The fungal pathogen (*Alternaria eichoniae*) of the water hyacinth, causes necrotic

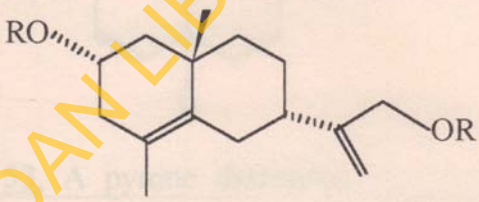
spots on its leaves by producing the quinonoid toxin, alteichin 51, while the citrus disease fungus *Alternaria citri*, wreaks its effects on lemon and lime trees by producing a complex of toxins[37]. The most active being the lactone, 52.



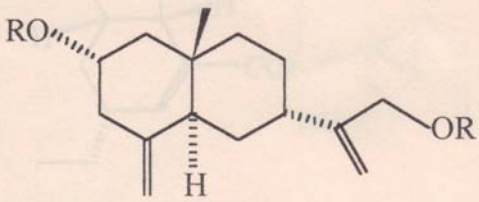
47, Alternariol.



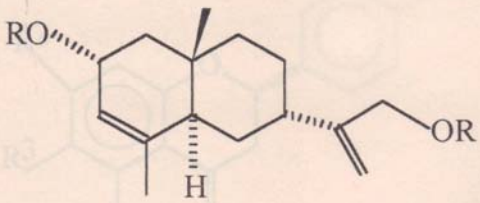
51, Alteichin.



48.

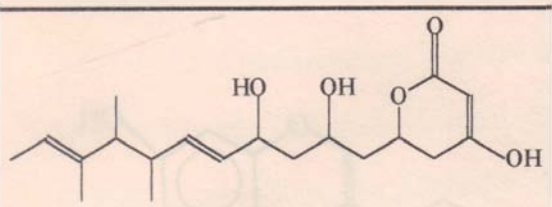


49.

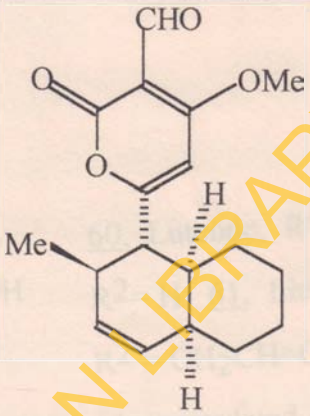


50.

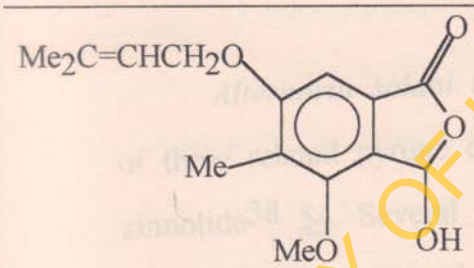
Sesquiterpene glycosides, R= Gal.



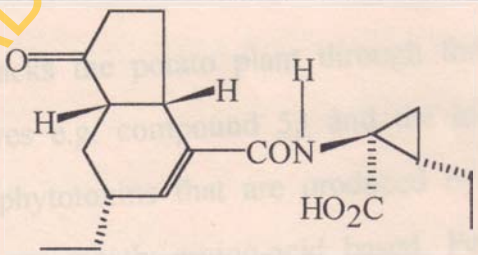
52, A lactone.



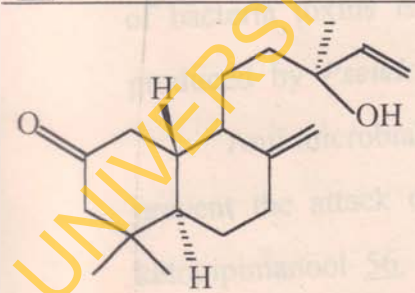
53, A pyrone derivative.



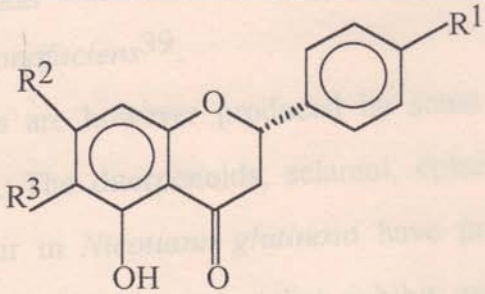
54, Zinnolide.



55, Cronatine.

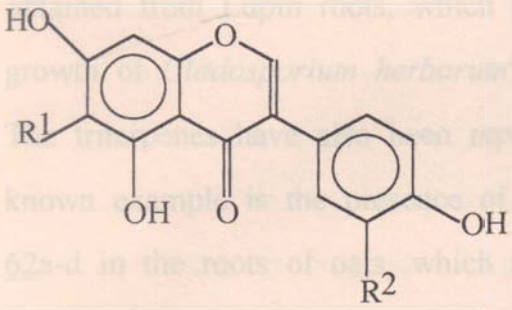


56, Ketoepimanool.



57, Pinocembrin, $R^1 = R^2 = R^3 = H$.

58, Sakuranetin, $R^1 = OH$, $R^2 = Me$, $R^3 = H$; 59, Naringenin, $R^1 = OH$, $R^2 = H$, $R^3 = CH_2CH{=}CMe_2$.



60, Luteone, $R^1 = CH_2CH=CMe_2$, R^2= H; 61, Licoisoflavone A, R^1 =H, $R^2 = CH_2CH=CMe_2$.

(isoprenylated isoflavones).

Alternaria solani also attacks the potato plant through the production of three related pyrone derivatives e.g. compound 53 and the lactone, zinnolide[38] 54. Several of the phytotoxins that are produced by bacteria have been characterized. These are mostly amino-acid based. Perhaps typical of bacteria toxins is cronatine 55, which is a chlorosis-inducing toxin that is produced by *Pseudomonas coronafaciens*[39].

Anti microbial substances are however produced by some plants to prevent the attack of microbes. The diterpenoids, sclareol, episclareol and ketopipimanool 56, which occur in *Nicotiana glutinosa* have proved to be effective antifungal agents[40]. Several flavanones that exhibit antifungal activity have also been reported. Pinocembrin 57, sakuranetin 58 and 6-isopentenyl-naringenin 59, have been shown to be very potent antifungal agents from plants[41-43].

Some isoflavonoids have also been found to afford significant antifungal protection. Examples are luteone 60 and licoisoflavone 61,

obtained from Lupin roots, which proved to be very potent by inhibiting the growth of *Cladosporium herbarum*[44-46].

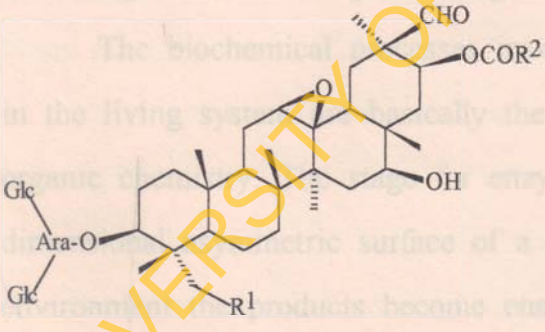
The triterpenes have also been reported as antimicrobial agents. A well known example is the presence of pentacyclic triterpene glycosides, avenacin 62a-d in the roots of oats, which affords protection against the fungus *Ophiobolus graminis* var. *tritici*. Avenacin has been identified as a mixture of four closely related glycosides[47].

Microbial toxins of two tropical African plants deserve mention because of their further utilization by mammals. The leaves of *Aspilia mossambicensis* contain the thiophene thiarubrine A 63, which is intensely antimicrobial. Its identification in the plant explains why this species is widely used in native medicines to cure sores and other infections. Remarkably, it is also an anthelmintic and wild chimpanzees have learnt to swallow the leaves whole, in order to rid themselves of nematodes and protozoan worms[48]. The second plant is *Maesa lanceolata*, the fruit of which has been shown to contain a particularly active antimicrobial agent, maesanin 64. The hot-water extract of the fruit is used to prevent cholera[49].

The knowledge acquired from various studies on secondary metabolites has been beneficial to Man in view of the exploitation of the therapeutic properties of these compounds for a wide variety of application.

1.3. EARLY DEVELOPMENT IN NATURAL PRODUCTS CHEMISTRY.

Initially the interest was simply in solving structural problems of natural products and grouping them according to origin, pharmacological activity or structure but soon the mass of information accumulated prompted chemists to see the need for a more coherent view of biosynthesis. A few chemists took the lead in the painstaking search for the hidden biosynthetic pathways and the peep-hole into the fascinating synthetic workshop of the living cell was gradually opened. The recognition of biosynthetic principles is the most significant development in natural products chemistry.

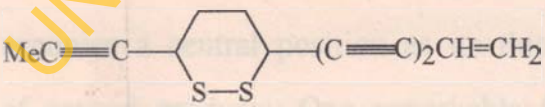


62 (a) R^1=OH, R^2= o-$MeHNC_6H_4$.

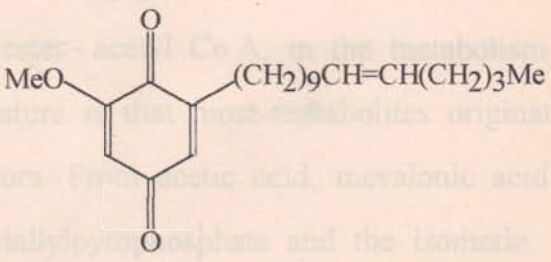
(b) R^1=OH, R^2=Ph.

(c) R^1=H, R^2= o-$MeHNC_6H_4$.

(d) R^1=H, R^2= Ph.



63, Thiarubrine A.



64, Maesanin.

Wallach and Ruzicka first recognized that the terpenes had a common building block, the isoprene unit and Winterstan and Trier suggested on good grounds that alkaloids were formed from α-amino acids. The biosynthetic routes of the various classes of compounds have now been well mapped out and these routes have been subjected to various biomimetic studies with identification of precursors and intermediates. Biomimetic studies have also led to novel and elegant synthetic procedures. Although it has been the tradition to confirm the structure of a compound isolated from natural source by synthesis, the motivation nowadays for total synthesis is not so much the question of confirmation but for the challenge and new knowledge inherent in synthesising intriguing structures.

The biochemical processes involved in the manufacture of metabolites in the living system are basically the same as the organic reactions used in organic chemistry. The stage for enzyme catalysed reaction is the three-dimensional asymmetric surface of a protein. As a result of the chiral environment the products become enantiomeric.

The link between primary and secondary metabolites is acetic acid. It occupies a central position as its thio ester - acetyl Co A, in the metabolism of natural products. One remarkable feature is that most metabolites originate from a very limited number of precursors. From acetic acid, mevalonic acid is derived, from which via 3,3-dimethylallylpyrophosphate and the isomeric isopentenylpyrophosphate, the isoprene unit and hence the terpenoids are formed. From carbohydrates, shikimic acid is derived which is the key to a

wealth of aromatic compounds, while the amino acids are precursors of the large variety of nitrogen containing compounds. Several groups of metabolites have mixed pathways in which one principal pathway acts as a substrate for another metabolite from a different pathway. Thus flavonoids are derived from the polyketide and acetate pathways. The indole alkaloids come from shikimate and acetate pathways.

Natural products were classified in the past according to structure or biological origin as fatty acids, carbohydrates, terpenes, mould metabolites, etc. The biosynthetic scheme now groups the compounds according to the synthetic routes employed by the cell as alkaloids, terpenoids, flavonoids, phenolics etc. There is of course overlap between the two but this has not posed any serious problem.

For many years, chemists were faced with the task of tracing out the pathways and the mechanism of each step involved in the biosynthesis of natural products. In the beginning accidental results contributed to the elucidation of intermediate steps. Knoop postulated in 1904 that degradation of fatty acids occur via β-oxidation. Collic also hypothesized in 1907 that the reverse reaction, the acetate condensation of Claisen type, is the origin of naturally occurring phenolics. The breakthrough came with the work on mutants and with isotopically labelled compounds. The fundamental investigations were run with the radioactive isotopes- ^{3}H, ^{14}C and ^{32}P. But in recent years with the advent of pulse Fourier-transform ^{13}C NMR

spectroscopy, biosynthetic studies have witnessed a new explosive development.

Radio tracing and mass spectrometry are the most sensitive methods now. Labelled precursors are usually introduced into the appropriate medium and the product analysed. In order to locate exactly the labelled atoms in a metabolite, it has to be degraded in an unambiguous way. The activity of each fragment isolated as CO_2, acetic acid or other well defined small organic molecule is then measured. Controlled degradation of a big molecule is a very difficult and time consuming exercise. This method was skilfully demonstrated by Bloch et al in the biosynthesis of cholesterol as being ultimately derived from acetate[50].

Biosynthetic studies is a significant aspect of natural products research. It has contributed in no small measure to the increased synthetic ingenuity of chemists.

1.4 PHYTOCHEMICAL AND PHARMACEUTICAL REVIEW OF THE PLANT FAMILY RUBIACEAE.

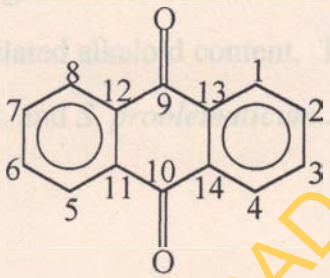
1.4.1. Natural Products from Rubiaceae Plants other than *Gardenia* species.

Gardenia erubescens Stapf. & Hutch. belongs to the family Rubiaceae. The plants in this family and specifically in the genera *Rubia*, *Galium* and *Morinda*, have long been known to contain substantial amounts of anthraquinones[51], with roots being especially rich sources of anthraquinones. These compounds are more often present as aglycones and sometimes in the form of glycosides. As a result of their anthraquinone content many plants of the Rubiaceae have been used for the preparation of natural dyes all over the world. The best known for this purpose is madder, which is the ground root of *Rubia tinctorum* L. from which alizarin is produced. Madder is used through the complexing of alizarin with metal oxides to produce various coloured dyes[52]. The synthesis and synthetic production of alizarin by Graebe and Liebermann in Germany killed the earlier high revenue yielding French trade on naturally sourced alizarin[53].

The anthraquinones found in the Rubiaceae do not have any laxative property[54]. Some of the rubiaceous anthraquinones however exhibit very interesting biological in vitro activities such as antimicrobial[55], hypotensive[56] and antileukemic[57-58] properties. In West and East Germany, extracts of the roots of *Rubia tinctorum* L. have been used for treatment of kidney stones[54,59]..

The anthraquinones found in the Rubiaceae constitute a homogeneous group of compounds, all being derivatives of the tricyclic structure, 65. Table 2 shows few examples of anthraquinones 66-72 isolated from the Rubiaceae.

The compounds,oruwal, 66 and oruwalol, 67 were isolated by



65.

Adesogan et al[60-61] from *Morinda lucida* Benth. ,a species found in the Western part of Nigeria.

Another important genus of the Rubiaceae is *Cinchona* species. These plants are cultivated for their bark which contains quinine, a potent antimalaria and quinidine, which is useful for cardiac arrhythmia in central South America. High yielding varieties of these trees have, for a long time, earned useful foreign exchange for the countries cultivating them. In malaria endemic countries of Africa, quinine was for a long time, the drug of choice for treating this disease. Although synthetic antimalaria drugs like chloroquine have long replaced quinine in the treatment of malaria because of its toxic side effects such as impaired hearing on prolonged use, quinine is still available as quinine hydrochloride

solution or tablets and it is still preferred particularly in the treatment of chloroquine-resistant strains of malaria.

The noreugenin-related alkaloids which is a class of chromone alkaloid have been reported in a genus of Rubiaceae family, that is *Schumanniophyton*. Only two species in this genus were mentioned in the review by Peter J. Houghton for their noreugenin-related alkaloid content. These are *Schumanniophyton magnificum* Harms. and *S. problematicum*.

Table 2 - Some Rubiaceous Anthraquinones.

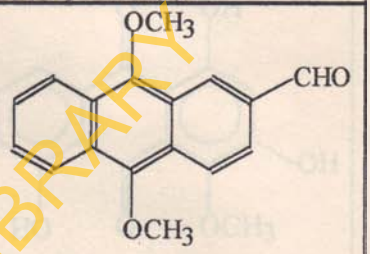
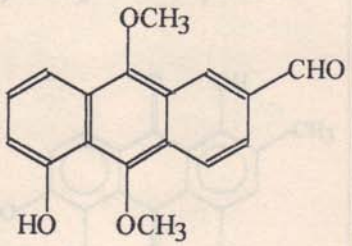
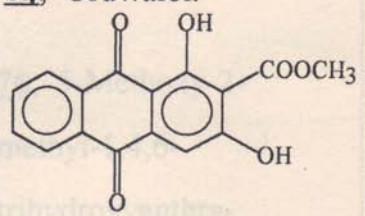
S/No.	Plant	Compound(s).
1.	*Morinda lucida* Benth. ref. 60, 61, 62.	 66 Oruwal.  67 Oruwalol.  68 Munjistin methyl ester.

Table 2 Contd.

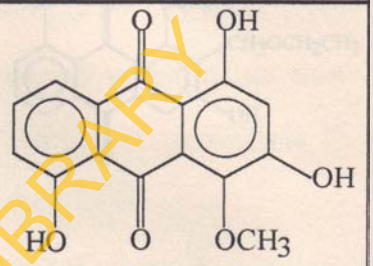
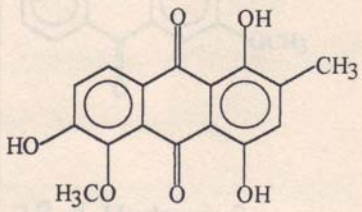
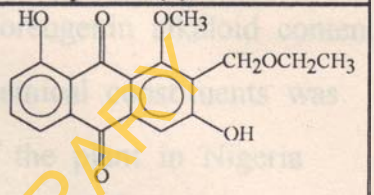
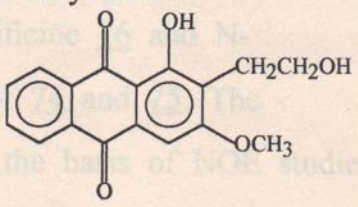
S/No.	Plant.	Compound(s).
2.	*Cinchona ledgetiana.* Moens. ref. 63.	 69, 4-Methyl-1,3,5-trihydroxylanthraquinone.  70, 5-Methoxy-2-methyl-1,4,6-trihydroxyanthraquinone.

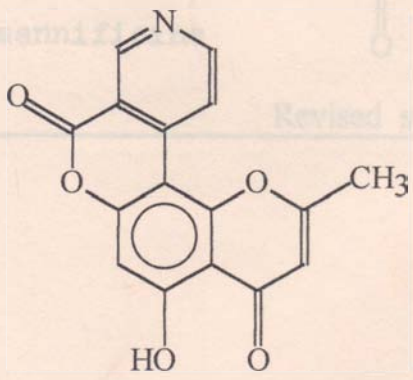
Table 2 Contd.

S/No.	Plant.	Compound(s).
3.	*Damnacanthus sub-spinosus*. Hand-Mazz. ref. 64.	 71, 8-Hydroxydamnacan-thol-2-ethyl ether.
4.	*Galium album* Mill. ref. 65	 72, 1-Hydroxy-2-hydroxyethyl-3-methoxy-anthraquinone.

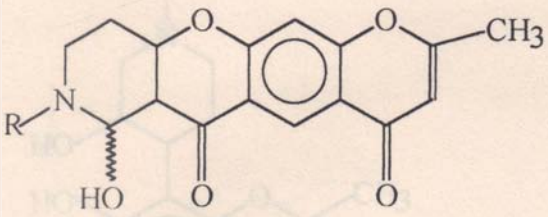
All the chromone alkaloids reported from the two plants are of noreugenin-pyridine or piperidine type. Three of these alkaloids were isolated from the root bark of *S. problematicum*[66]. These are schumanniophytine 73, a nicotinic acid noreugenin congener and two other unnamed noreugenin-piperidine-2-one derivatives.

Subsequent phytochemical interest was shifted on *S. magnificum* Harms., a species that was first reported for its noreugenin alkaloid content by Okogun et. al[67]. Their investigation on the chemical constituents was perhaps motivated by the medicinal importance of the plant in Nigeria where the stem juice serves as an antidote for snakebite[68].

The two alkaloids first isolated by Okogun et. al. from *S. magnificum* were schumannificine 74 and N-methylschumannificine *75*. Houghton et. al[69]., later isolated ten other alkaloids, 76-84, in addition to the first two mentioned above. Two of these, anhydroschumannificine 76 and N-methylanhydro-schumannificine 77, are analogues of 74 and 75. The structures of 74 and 75 were however revised on the basis of NOE studies on the N-methyl analogues of schumannificine. The structures 74-84 represent the chromone alkaloids of *Schumanniophyton magnificum* Harms.



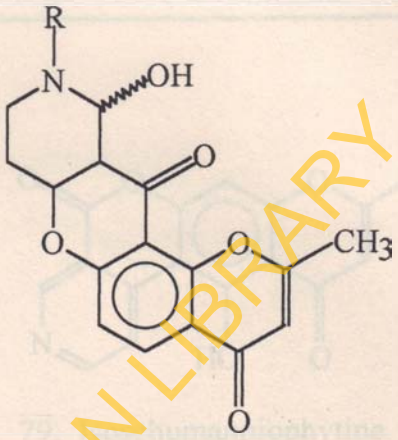
73, Schumanniophytine.



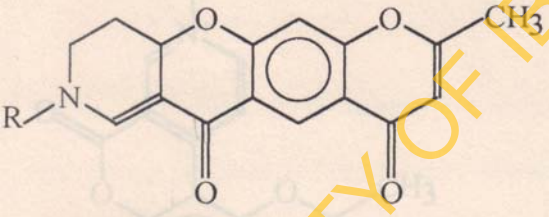
74, Schumannificine, R=H.

75, N-Methylschumannificine,

R=CH_3.



Revised structure.

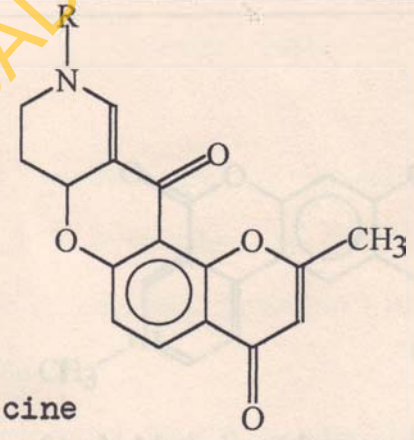


anhydro

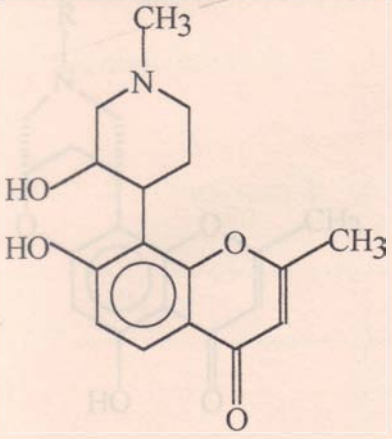
76, schumannificine, R=H.

77, N-Methylanhydro schumannificine

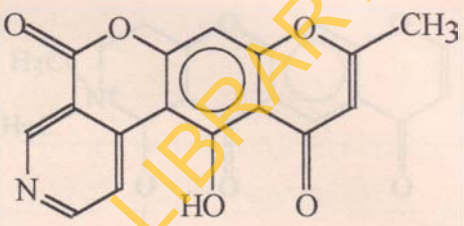
R=CH_3.



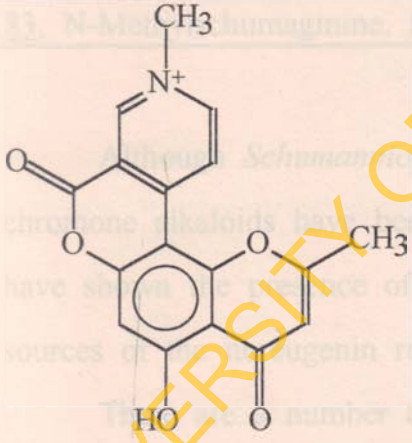
Revised structure.



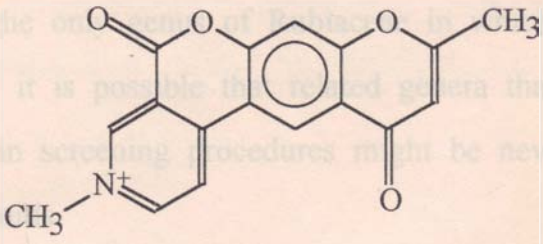
78, Rohitukine.



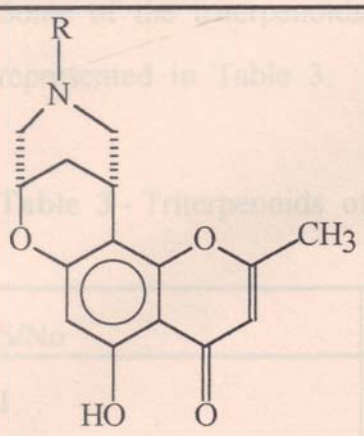
79, Isoschumanniophytine.

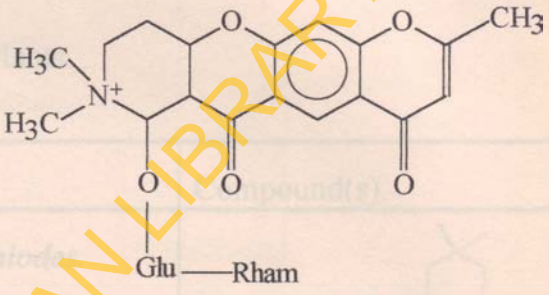


80, N-Methylschumanniophytine.



81, N-Methylisoschumanniophytine.





82, Schumaginine, R=H.

83, N-Methylschumaginine, R=CH_3. 84, Schumanniofoside.

Although *Schumanniophyton* is the only genus of Rubiaceae in which chromone alkaloids have been isolated, it is possible that related genera that have shown the presence of alkaloids in screening procedures might be new sources of the noreugenin related alkaloids.

There are a number of triterpenes that have been reported in Rubiaceae plants, some of which are used locally for medicinal purposes[70]. A systematic investigation of the Rubiaceae of Hong Kong revealed the presence of the common phytosterols, β-sitosterol and stigmasterol, together with triterpene saponins and sapogenins[71-74]. From the information available in the literature, it seems the quinovic acid derivatives are the most common triterpenoids in Rubiaceae plants[71].

Some of the triterpenoids, 85-95, reported in Rubiaceous plants are represented in Table 3.

Table 3 - Triterpenoids of Rubiaceae Plants.

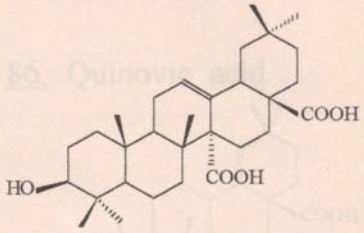
S/No	Plant(s).	Compound(s).
1.	*Randia canthiodes.* *Adinia pilulifera.* *Antirrhoea chinensis.* ref. 70,73.	 85, Cincholic acid.

Table 3 Contd.

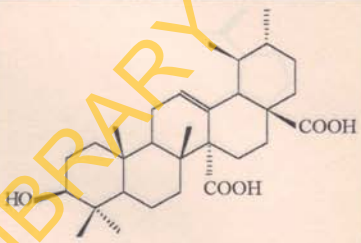
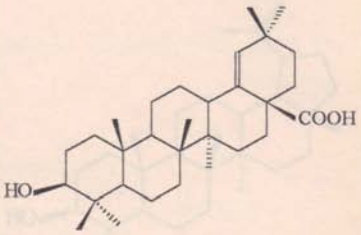
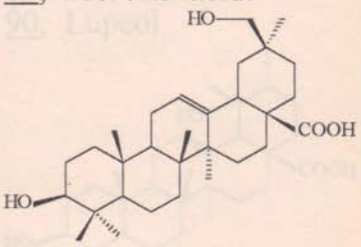
S/No.	Plant(s).	Compound(s).
2.	*Adinia pilulifera.* *Randia spinosa.* *Antirrhoea chinensis.* ref. 73,74.	 86, Quinovic acid.
3.	*Adinia pilulifera.* ref. 73.	 87, Morolic acid.
4.	*Randia sinensis.* ref. 70.	 88, Mesembryanthmoi-degenic acid.

Table 3 Contd.

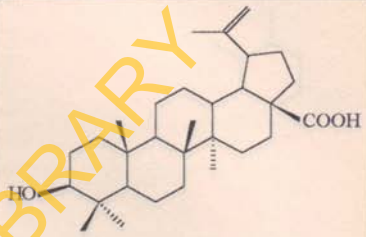
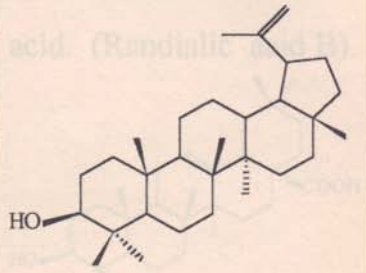
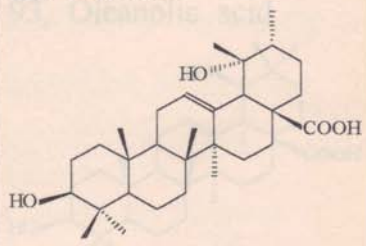
S/No.	Plant(s).	Compound(s).
5.	*Adinia pilulifera.* ref. 73.	 89, Betulinic acid.
6.	*Ixora chinensis.* ref. 70	 90, Lupeol.
7.	*Randia dumetorum.* ref. 74.	 91, 19α-Hydoxyursolic acid. (Randialic acid A).

Table 3 Contd.

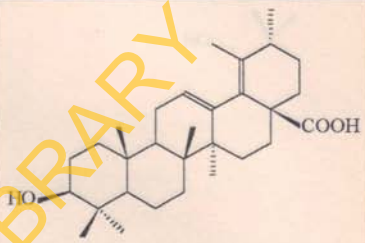
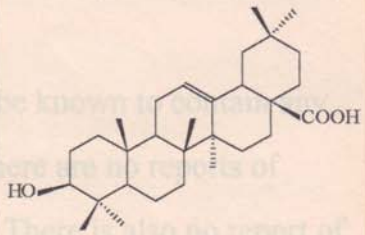
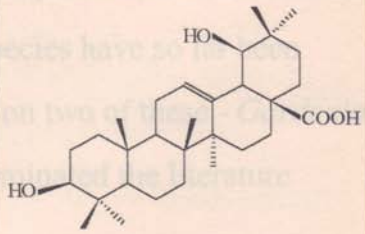
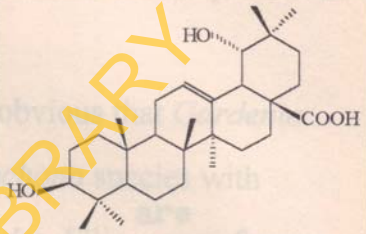
S/No.	Plant(s).	Compound(s).
7.	*Randia dumetorum.* ref. 74.	 92, 19-Dehydroursolic acid. (Randialic acid B).
8.	*R. dumetorum.* *R. uliginosa.* ref. 74.	 93, Oleanolic acid.
9.	Randia spinosa. ref. 74.	 94, Spinosic acid.

Table 3 Contd.

S/No.	Plant(s).	Compound(s).
9.	*Randia spinosa*. ref. 74.	 95, Siaresinolic acid.

1.4.2. Natural Products from *Gardenia* Species.

The plants of the genus *Gardenia* appear not to be known to contain any anthraquinone for which the family is well known, as there are no reports of isolation of anthraquinone from any *Gardenia* Species. There is also no report of the presence of alkaloid in any of these species. Nevertheless, literature survey on chemical constituents of Gardenia Species revealed these plants as rich sources of flavonoids, iridoids and triterpenes. A total of fifteen species have so far been subjected to phytochemical analysis and research work on two of these - *Gardenia jasminoides* Ellis and *G. lucida* (Roxb) significantly dominated the literature available on *Gardenia* plants.

The breakdown of compounds isolated from Gardenia plants together with their sources are presented in tables 4-6 below. The tables give a vivid picture of the various classes of natural products in these plants. It would be difficult to use the presence of any of the three major classes of compounds as a taxonomic maker

as the tables visibly show that the occurrence of these compounds do not follow any regular pattern in the species. The only compound which has been reported in almost all the species is D-mannitol[75].

Given the information available in the tables, it is obvious that *Gardenia* plants may never approach the significance of say the *Cinchona* species with respect to their therapeutic uses. The fruits of *G. jasminoides* Ellis are very famous in Japan for its laxative property which has been attributed to the iridoid constituents[76-79]. Out of about eight iridoid glycosides 96-103, identified so far (table 4) in the fruit of this plant, geniposide, 96 and genipin, 97 constitute the major components[78]. In addition to the iridoid glycosides, the leaf extract of *G. jasminoides* also contains a pseudoazulene iridoid, carbinal, 98 which has been identified as a potent anti-fungal compound[80-81]. This plant was also reported in China for its antifertility principles which was obtained from the ethyl acetate extract of its flowers. Gardenoic acid, 104 (Table 5), the active principle has been shown to be an early pregnancy terminating component[82].

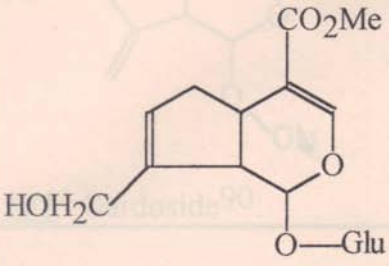
A significant proportion of the constituents from these plants are triterpenoids, 104-119 (Table 5). Most of the triterpenes reported have the Oleanane type skeleton and this is indicative of the preference for the biosynthetic pathway leading to the formation of Oleanane-type skeleton. As the processes involved are enzyme controlled, it is expected that the same type of enzymes should be present in the species since their genetic makeup is similar, consequently similar compounds are produced with same carbon skeleton which differ only in

the degree of substitution and the extent of oxidative transformation of the various substituents.

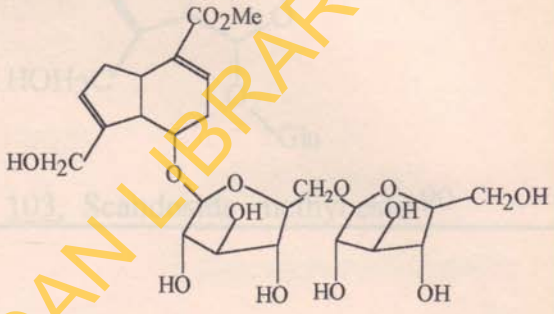
The report on isolation of triterpenes of *Gardenia* plants has followed the usual style of research in triterpene chemistry, as it had been the practice to simply identify the triterpenes from their natural sources as a matter of curiosity without any link to their therapeutic properties. However, the emphasis is gradually shifting in the direction of the therapeutic importance of triterpenoids in recent times with the discovery of anti-tumour and anti-leukemic properties within this group of compounds, particularly among the quassinoids and limonoids. There is certainly a renewed interest in the biological properties of triterpenes especially in Japan and China as more published data are now available on saponin constituents of medicinal plants[83-85].

The flavonoid constituents of *Gardenia* plants also present a unique feature as most of the flavonoids reported in these plants, 120-135 (table 6), are rare flavonoids with unusual A- and B-ring oxidation pattern.

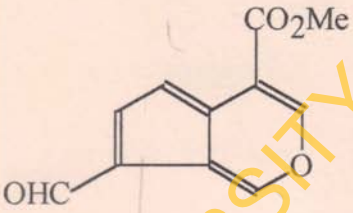
Table 4- Iridoid Glycosides from *Gardenia* species, *G. jasminoides*.



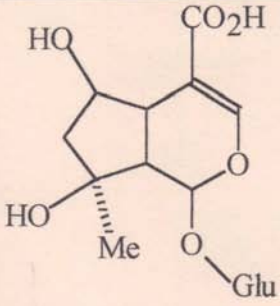
96, Geniposide[78].



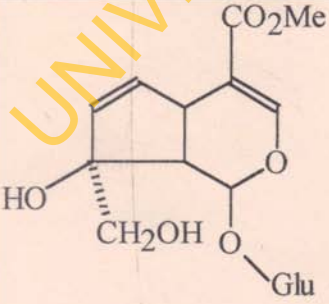
97, Genipin-1-β-gentiobioside[86].



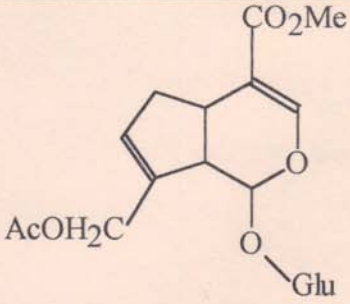
98, Cerbinal[80].



99, Shanzhiside[87].



100, Gardenoside[88].



101, 10-Acetylgeniposide[89].

Table 4 Contd.

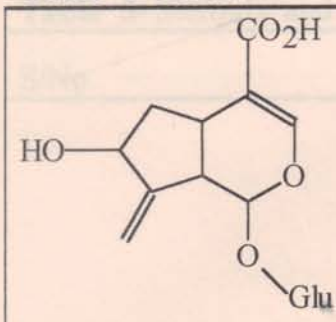
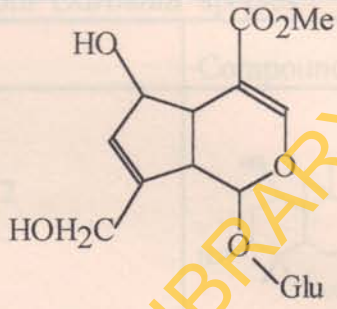
 102, Gardoside[90].	 103, Scandoside methyl ester[90].

Table 5- Steroids and Triterpenoids from *Gardenia* species.

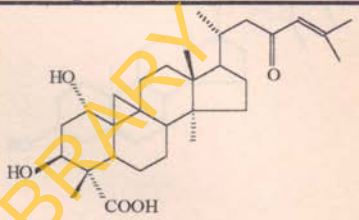
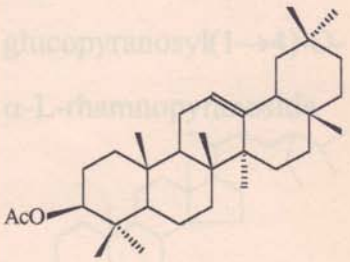
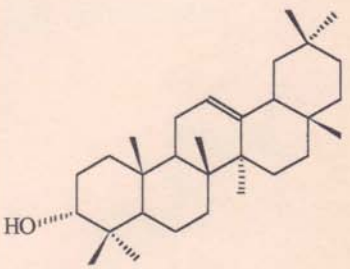
S/No.	Plant(s).	Compound(s).
1.	*Gardenia jasminoides*[82].	 104, Gardenoic acid B.
2.	*G. imperialis*.	 105, β- Amyrin acetate.
		 106, 3-Epi-β-amyrin-(pulcherol).

Table 5 Contd.

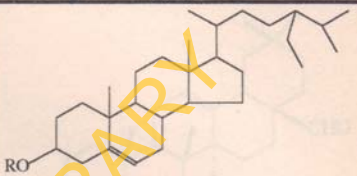
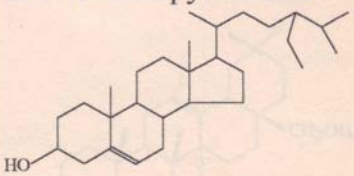
S/No.	Plant(s).	Compound(s).
3.	*G. lucida*[91].	 107, β-Sitosterol-3-O-β-glycoside., R= β-D-glucopyranosyl(1→4)-O-α-L-rhamnopyranoside.
4.	*G. jasminoides*[92]. *G. gummifera*[93]. G. latifolia[94]. G. turgida[95].	 108, β-Sitosterol.

Table 5 Contd.

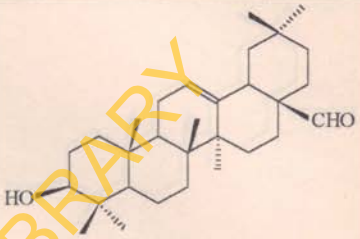
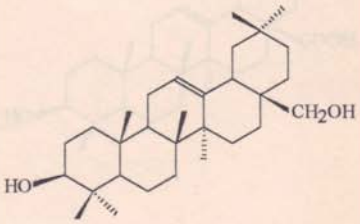
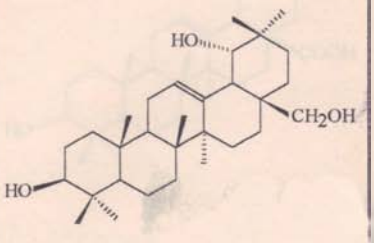
S/No.	Plant(s).	Compound(s).
5.	*G. gummifera*[93].	 109, Oleanonic aldehyde  110, Erythrodiol.  111, 19α-Hydroxy-erythrodiol.

Table 5 Contd.

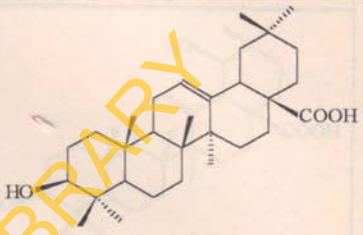
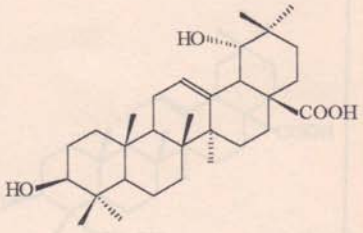
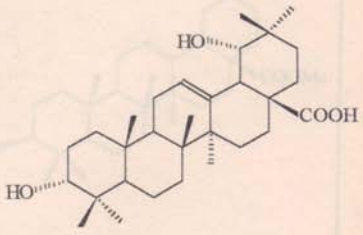
S/No.	Plant(s).	Compound(s).
6.	*G. latifolia*[94,96]. *G. turgida*[95,97].	 112, Oleanolic acid.  113, Siaresinolic acid.  114, 3-Episiaresinolic acid.

Table 5 Contd.

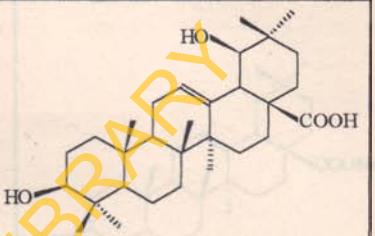
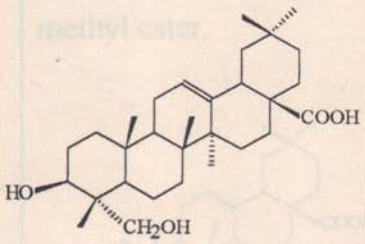
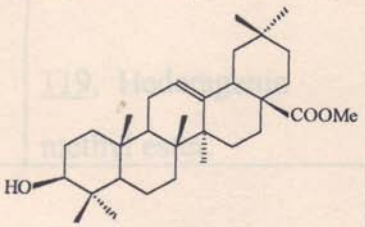
S/No.	Plant(s).	Compound(s).
6. Continued	*G. latifolia*[94,96].	
	G. turgida[95,97].	115, Spinosic acid.
		
		116, Hederagenin.
7.	*G. turgida*[97].	
		117, Oleanolic acid methyl ester.

Table 5 Contd.

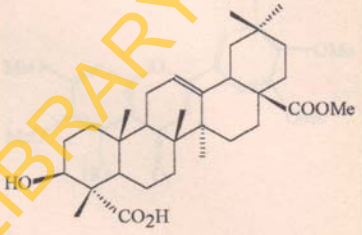
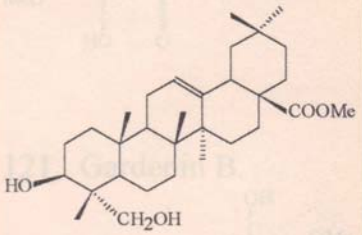
S/No.	Plant(s).	Compound(s).
7.	*G. turgida*[97].	 118, Gypsogenic acid methyl ester.  119, Hederagenin methyl ester.

Table 6- Flavonoid Constituents of *Gardenia* species.

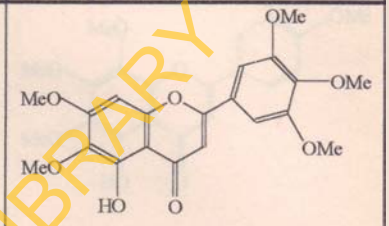
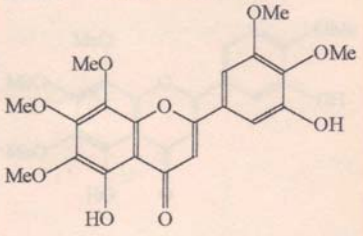
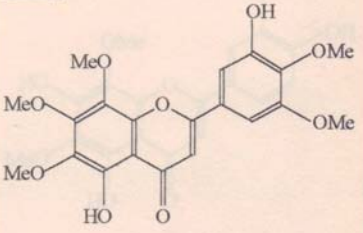
S/No.	Plant(s).	Compound(s).
1.	*G. lucida*[98,99]. *G. turgida*.	 120, Gardenin A.  121, Gardenin B.  122, Gardenin C.

Table 6 Contd.

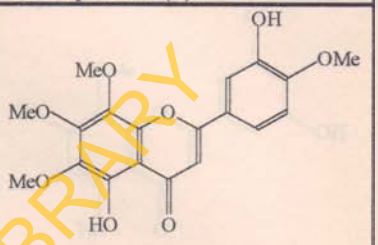
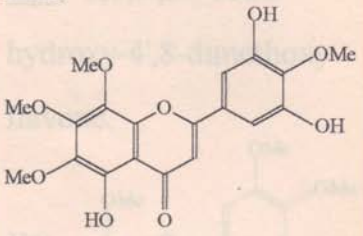
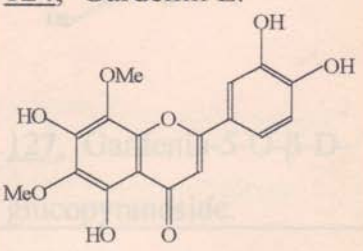
S/No.	Plant(s).	Compound(s).
1.	*G. lucida*[98,99]. *G. turgida*.	 123, Gardenin D.  124, Gardenin E.
2.	*G. lucida*[100]. G. gummifera[101].	 125, 5,7,3',4'- Tetra-hydroxy-6,8-dimethoxy-flavone.

Table 6 Contd.

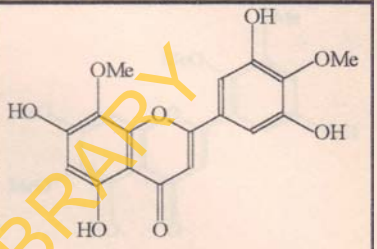
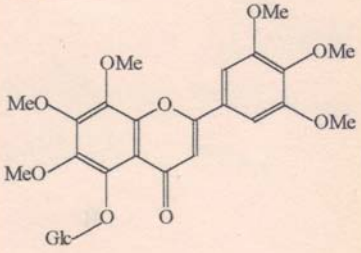
S/No.	Plant(s).	Compound(s).
2.	*G. lucida*[100]. *G. gummifera*[101].	 126, 5,7,3',5'- Tetra-hydroxy-4',8-dimethoxy-flavone.
3.	*G. florida*[102].	 127, Gardenin-5-O-β-D-glucopyranoside.

Table 6 Contd.

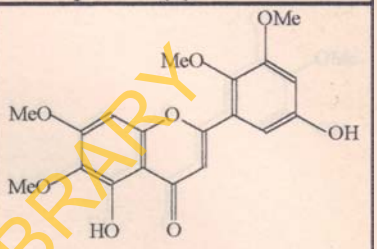
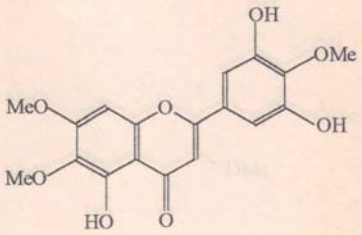
S/No.	Plant(s).	Compound(s).
4.	*G. Cramerii*[103]. *G. fosbergii*[103].	 128, 5,5'-Dihydroxy-6,7,2',3'-tetramethoxy-flavone.  129, 5,3',5'-Trihydroxy-3,6,7,4'-tetramethoxy-flavone.

Table 6 Contd.

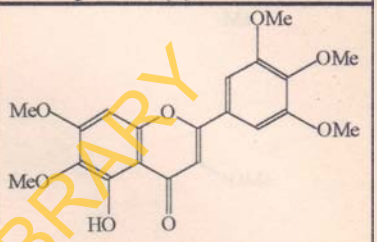
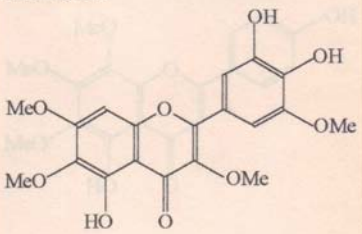
S/No.	Plant(s).	Compound(s).
4.	*G. Cramerii*[103]. *G. fosbergii*[103].	 130, 5-Hydroxy-6,7,3',4',5'-pentamethoxy-flavone.
5.	*G. fosbergii*[104].	 131, 5,3',4'-Trihydroxy-3,6,7,5'-tetramethoxy-flavone.

Table 6 Contd.

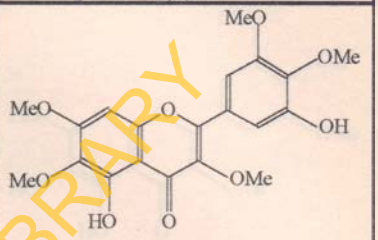
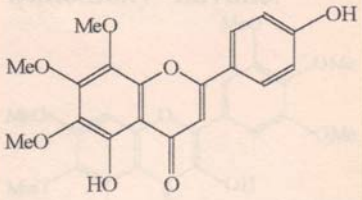
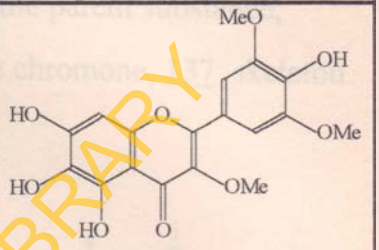
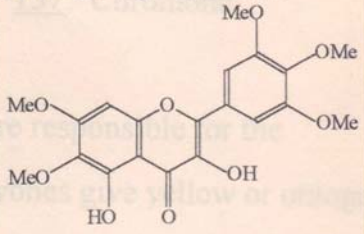
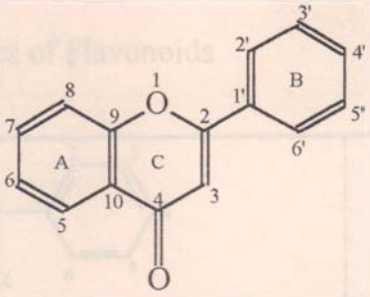
S/No.	Plant(s).	Compound(s).
5.	*G. fosbergii*[104]	 132, 5,5'-Dihydroxy-3,6,7,3',4'-pentamethoxy-flavone.  133, 5,4'-Dihydroxy-6,7,8-trimethoxyflavone.

Table 6 Contd.

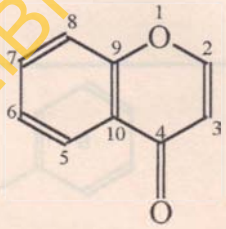
S/No.	Plant(s).	Compound(s).
5.	*G. fosbergii*[104]	 134, 5,6,7,4'-Tetra-hydroxy-3,3'5'-trimethoxy -flavone.  135, 3,5-Dihydroxy-6,7,3',4',5'pentamethoxy -flavone.

1.5 THE FLAVONOIDS.

The flavonoids are all structurally derived from the parent substance, flavone, 136 which in turn has a structure based on the chromone, 137 skeleton.



136, Flavone.



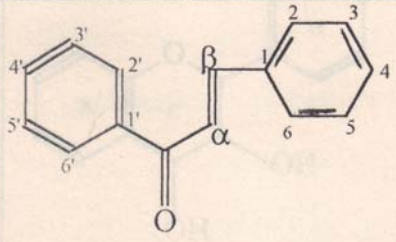
137, Chromone.

Most members of this class of natural products are responsible for the beautiful colours of flowers and fruits in nature. The flavones give yellow or orange colours and the anthocyanins give red, violet or blue colours. The occurrence of this numerous class of oxygen heterocycles is restricted to higher plants and ferns. Mosses contain a few flavonoid types but they are absent in algae, fungi and bacteria. The hydroxylation and methylation patterns appear to be genetically controlled, i.e. the distribution of flavonoids is a useful tool for classification purposes[105]. Biologically the flavonoids play a major role in relation to insect pollinating and feeding on plants.

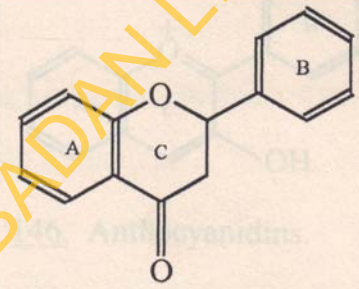
The flavonoids are structurally characterized by having two hydroxylated aromatic rings A and B, 136 joined by a three carbon fragment giving a C_{15} skeleton considered to be composed of two parts, C_6 and C_9 units.

Some eleven major classes of flavonoids are recognised and within each group there are members at various oxidation levels. The major classes of flavonoids are presented in table 7.

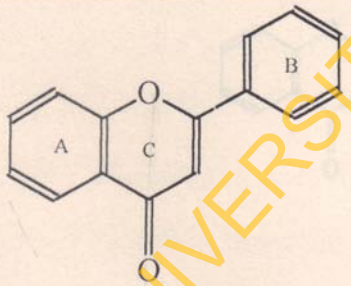
Table 7 - classes of Flavonoids



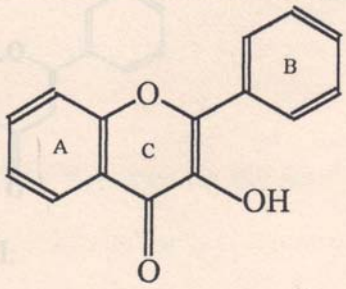
138, Chalcone.



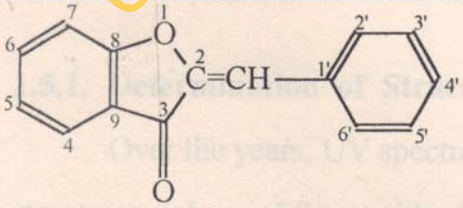
139, Flavanone.



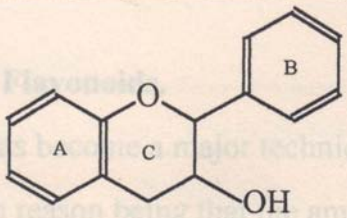
136, Flavone.



140, Flavonol.

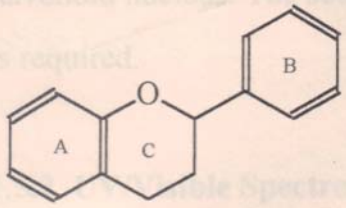


141, Aurone.

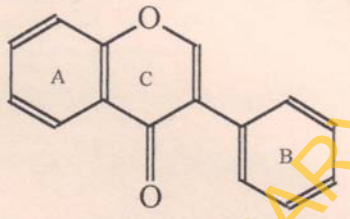


142, Cathechins.

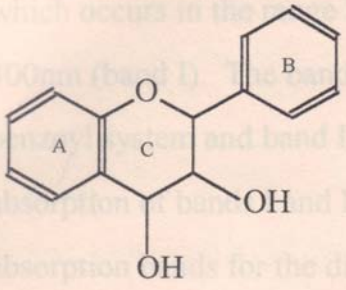
Table 7 Contd.



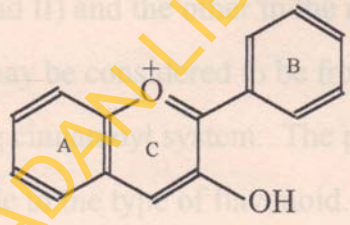
143, Flavanes.



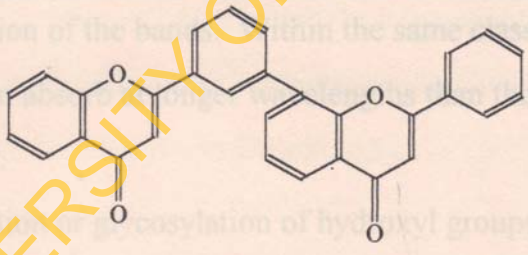
144, Isoflavone.



145, Leucoanthocyanidin.



146, Anthocyanidins.



147, Biflavonyl.

1.5.1. Determination of Structure of Flavonoids.

Over the years, UV spectroscopy has become a major technique for the structure analysis of flavonoids. The main reason being that the amount of structural information gained from a UV Spectrum is considerably enhanced by use of specific reagents which react with one or more functional groups on the

flavonoid nucleus. The second reason is that only a small amount of pure material is required.

1.5.2 UV/Visible Spectroscopy

The UV spectra of most flavonoids consists of two major absorption maxima, one of which occurs in the range 240-285nm (band II) and the other in the range 300-400nm (band I). The band II absorption may be considered to be from the A-ring benzoyl system and band I from the B-ring cinnamoyl system. The position of absorption of bands I and II provide a guide to the type of flavonoid. The major absorption bands for the different classes of flavonoids is shown in table 8.

The degree of hydroxylation of the flavone skeleton determines to a large extent the position of the bands. Within the same class, highly oxygenated members tend to absorb at longer wavelengths than those with fewer oxygen substituents[106].

Methylation or glycosylation of hydroxyl groups on the flavonoid nucleus usually result in hypsochromic shifts, particularly of band I, while acetylation tend to nullify the effect of the phenolic hydroxyl groups on the spectra.

Table 8 - Major Absorption Bands of Flavonoids.

Band I (λ, nm)	Band II (λ, nm)	Class of Flavonoid.
304-350	250-280	Flavones
352-385	250-280	Flavonols
300-340	245-270	Isoflavones

Table 8 Contd.

Band I (λ, nm)	Band II (λ, nm)	Class of Flavonoids.
300-340	270-295	Flavanones & Dihydro-flavonols
340-390	220-270	Chalcones
370-430	220-270	Aurones
465-550	270-280	Anthocyanidins & Antho-cyanins

The position of hydroxyl groups and the pattern of hydroxylation can be determined by the use of specific reagents which induce structurally significant shifts in the UV spectrum[107]. The reagents commonly used are sodium methoxide (NaOMe), sodium acetate (AcONa), sodium acetate/boric acid ($NaOAc/H_3BO_3$), aluminium chloride ($AlCl_3$) and aluminium chloride/hydrochloric acid ($AlCl_3/HCl$).

All hydroxyl groups on the flavonoid nucleus are ionized to some extent by sodium methoxide. Hence for most hydroxylated flavonoids, shifts to longer wavelength are observed in both bands. The most significant and informative of the shifts are viz. the bathochromic shift of 40-65nm in band I without a decrease in intensity which indicates the presence of a 4'-hydroxyl group in flavones and flavonols. Flavonols lacking 4'-hydroxylation also produce a 50-60nm red shift, but with a decrease in intensity.

For flavanones, Isoflavones and dihydroflavonols, a consistent 35-40nm bathchromic shift of band II suggests the presence of 5,7-dihydroxyl groups.

Sodium acetate being a much weaker base than sodium methoxide tend to ionize only the more acidic phenolic hydroxyl groups. Flavones and flavonols possessing a 7-hydroxyl group exhibit a 5-20nm bathochromic shift of band II while the presence of 7-hydroxyl group in isoflavones also produces a band II bathochromic shift of 6-20nm but 5,7-dihydroxy flavanones and dihydroflavonols give a shift of 60nm.

A mixture of sodium acetate and boric acid is used for the detection of o-dihydroxyl groups in flavonoids. Flavones and flavonols containing o-dihydroxyl groups in ring B give a 12-30nm bathochromic shift of band I while ring A o-dihydroxyl groups give rise to lesser shifts. But the isoflavones, flavanones and dihydroflavonols show a 10-15nm bathochromic shift only in band II when o-dihydroxyl groups are present on ring A.

Aluminium chloride chelates with functional groups such as the 5-hydroxy-4-keto, 3-hydroxy-4-keto and o-dihydroxyl groups. The reactions produce bathochromic shifts of one or both bands. The complex formed with 5-hydroxy-4-keto groups is stable in acid while the complexes formed with o-dihydroxyl groups and the 3-hydroxyl-4-keto groups decompose in acid medium. Hence treatment of a flavonoid solution with Aluminium chloride and then followed by addition of aqueous hydrochloric acid produces bathochromic shifts which may or may not disappear by addition of the aqueous acid depending on the type of groups involved in the complex formation.

The presence of a 5-hydroxyl group in a flavone gives a band I bathochromic shift of 35-55nm on addition of $AlCl_3$/HCl. Flavones with 3-or 3 and 5-hydroxyl groups however show a bathochromic shift in band I of 50-60nm. When a 5-hydroxyl group is present in isoflavones, a 10-14nm band II red shift is observed while flavanones and dihydroflavonols give a 20-26nm band II bathochromic shift on addition of $AlCl_3$/HCl reagent.

1.5.3 Proton NMR Spectroscopy

Initially the use of ^1H NMR for structural studies on flavonoids was confined to the relatively non-polar flavonoids such as the isoflavones and highly acetylated or methylated flavones which are soluble in solvents like $CDCl_3$ and CCl_4. However, the introduction of DMSO-d_6 and the use of TMS-ether derivatives now make it possible for the bulk of naturally occurring flavonoids to be studied by this method.

A lot of information which has accumulated over the period of studies on NMR spectroscopy of flavonoids have made it possible for NMR signals to be assigned tentatively to structural features. The distinguishing feature for such class of flavonoids seem to be endowed in the signals originating from ring C. Hence flavones and isoflavones are readily differentiated by using the position of C-2 and C-3 proton signals.Likewise, flavanones and dihydroflavonols are also differentiated on the basis of C-2 and C-3 proton signals (Table 9). The pattern of the signals of the aromatic rings A and B gives an indication of the substitution pattern on these aromatic rings[106].

The presence of hydroxyl protons are shown by signals in the range 9.70-12.40 ppm and the disappearance of these signals on addition of D_20 confirms their identity as hydroxyl proton resonance.

Table 9 - Chemical shift data for C-2 and C-3 protons in flavones, isoflavones, flavanones and dihydroflavonols.

Class of Compound	H-2 (ppm)	H-3 (ppm)
Flavone		6.3 (s)
Isoflavone	7.6-7.8 (s)	
Flavanone	5.0-5.5 (q)	2.8 (qq)
Dihydroflavonol	4.8-5.0 (d)	4.1-4.3 (d)

1.5.4 ^{13}C NMR Spectroscopy

The application of ^{13}C-NMR spectroscopy in structural studies of flavonoids has assumed an interesting dimension, although it is essentially complementary to ^{1}H NMR. By spectral correlation, it is possible to determine the structural features of an unknown flavonoid using a combination of ^{1}H and ^{13}C NMR data.

Flavones, flavanones and dihydroflavonoids are readily distinguished by the position of the C4 signal[108]. The value of the C4 signal is also indicative of the substitution on C5 (Table 10).

Generally the carbon atoms of rings A and B fall into the region for aromatic carbons with shifts corresponding to the oxygenation pattern on the rings.

Table 10 - ^{13}C Chemical shift data for C_4 signal in some flavonoid compounds.

Compound.	C_4 (ppm)
5-Hydroxyflavone	181-182
5-Methoxyflavone or 5-Glycosides	176-177
5-Hydroxyflavanone/Dihydroflavonol	195-197
5-Methoxyflavanone or 5-Glycosides/ Dihydroflavonol	189-192

The chemical shifts for the different types of carbon present in flavonoids are presented in Table 11.

Table 11 - ^{13}C Chemical shifts characteristic of flavonoid functional groups.

Type of Carbon	Chemical Shift (ppm)
Carbonyl	200-170
Aromatic	140-125 (without oxygen substituent)
	167-150 (C_9,C_7,C_5)
	110-90 (C_6,C_8,C_{10})
Ethylenic	160-165 (with oxygen substituent)
	100-110 (without oxygen substituent)
Carbinolic	77-79 (C2 of flavanones)

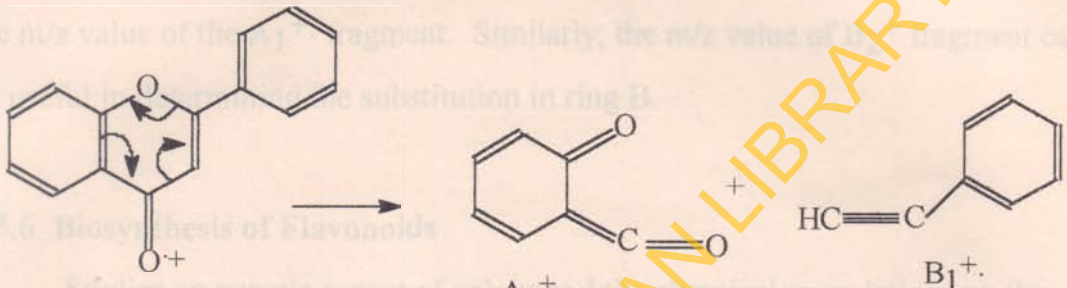
1.5.5 Mass Spectrometry

The use of mass spectrometry has been a valuable tool in determining the structures of flavonoids especially when small quantities of compounds are available. Electron impact (EI) mass spectrometry has been applied successfully to all classes of flavonoid aglycones and more recently to a number of different types of glycosides. Chemical ionization (CI) is less frequently utilized. It has only been applied to a few aglycones and gives few diagnostic fragments except for flavanones and dihydroflavonols. Fast atomic bombardment (FAB) on the other hand is becoming popular as regards the determination of the structures of flavonoid glycosides.

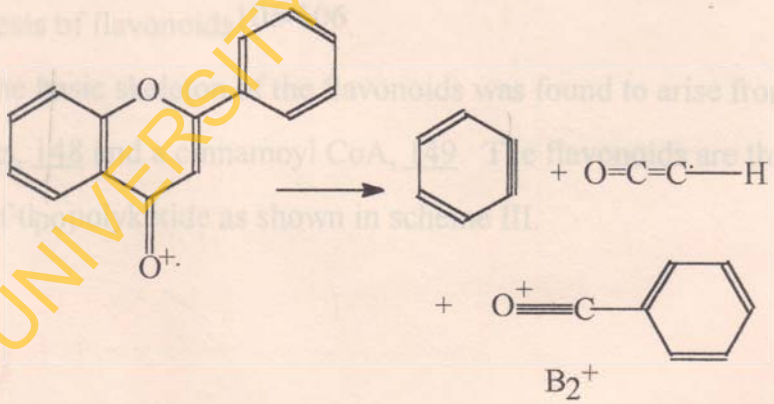
Most flavonoid aglycones give intense peaks for the molecular ion $[M^+]$ which is often the base peak. In addition to the molecular ion, flavonoid aglycones also give major peaks for $[M-H]^+$ and when methoxylated, $[M - CH_3]^+$. With respect to flavonoid identification, the most useful fragmentations have been found to be those which involve cleavage to give rings A and B fragments[106]. The common fragmentions of flavonoids are labelled as pathway I and II as in scheme I. Pathway I corresponds to a retro-Diels-Alder cleavage and it produces two ions A_1^+, and B_1^+ while pathway II gives a single charged species, B_2^+.

Scheme I: Major fragmentation pattern of flavonoids

Pathway I



Pathway II



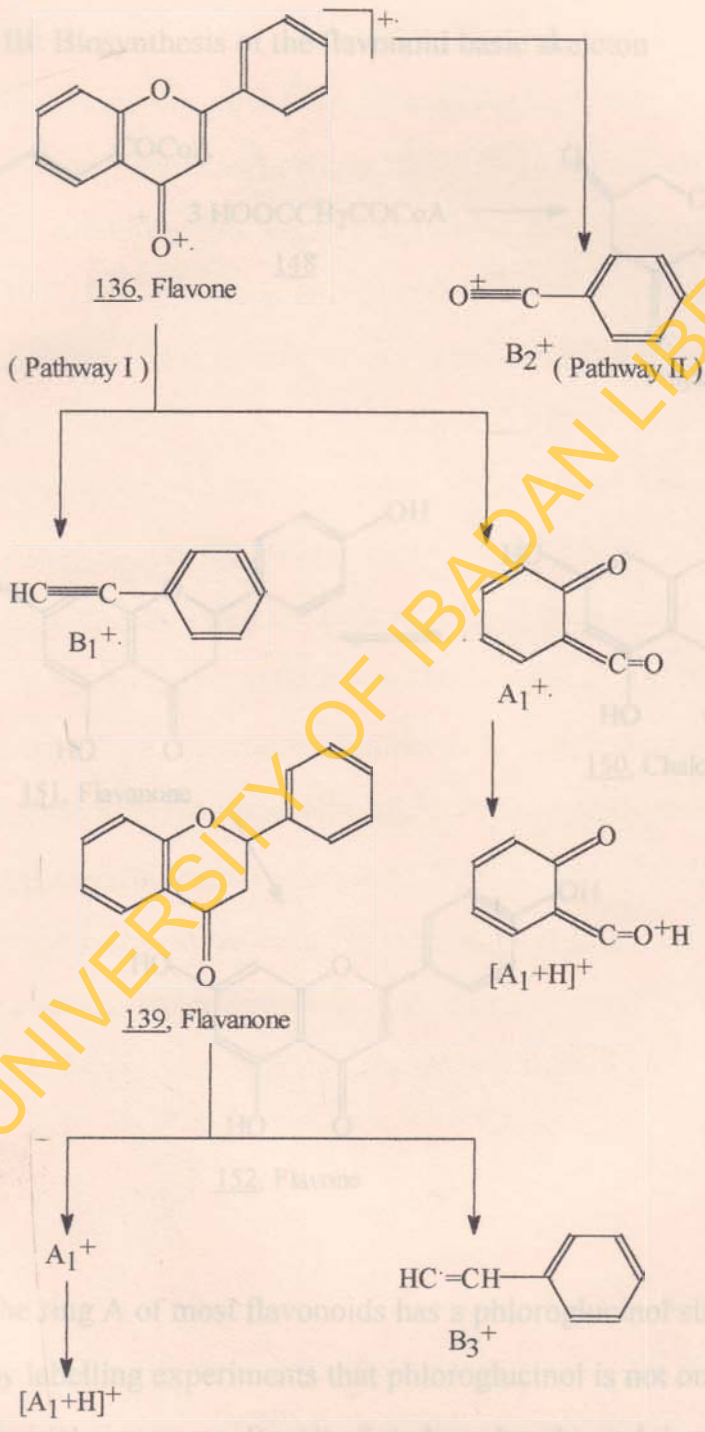
The diagnostic fragmentation patterns for flavones and flavanones are illustrated in Scheme II.

Although the base peak for most flavonoids is the molecular ion, $[M^+]$, peaks are usually prominent in the spectra for $[M - CO]^+$ and $[A_1 - CO]^+$ in addition to the fragments A_1^+ and B_1^+. Substitution in the A-ring can be detected by examining the m/z value of the A_1^+ fragment. Similarly, the m/z value of B_2^+ fragment can be useful in determining the substitution in ring B.

1.5.6 Biosynthesis of Flavonoids

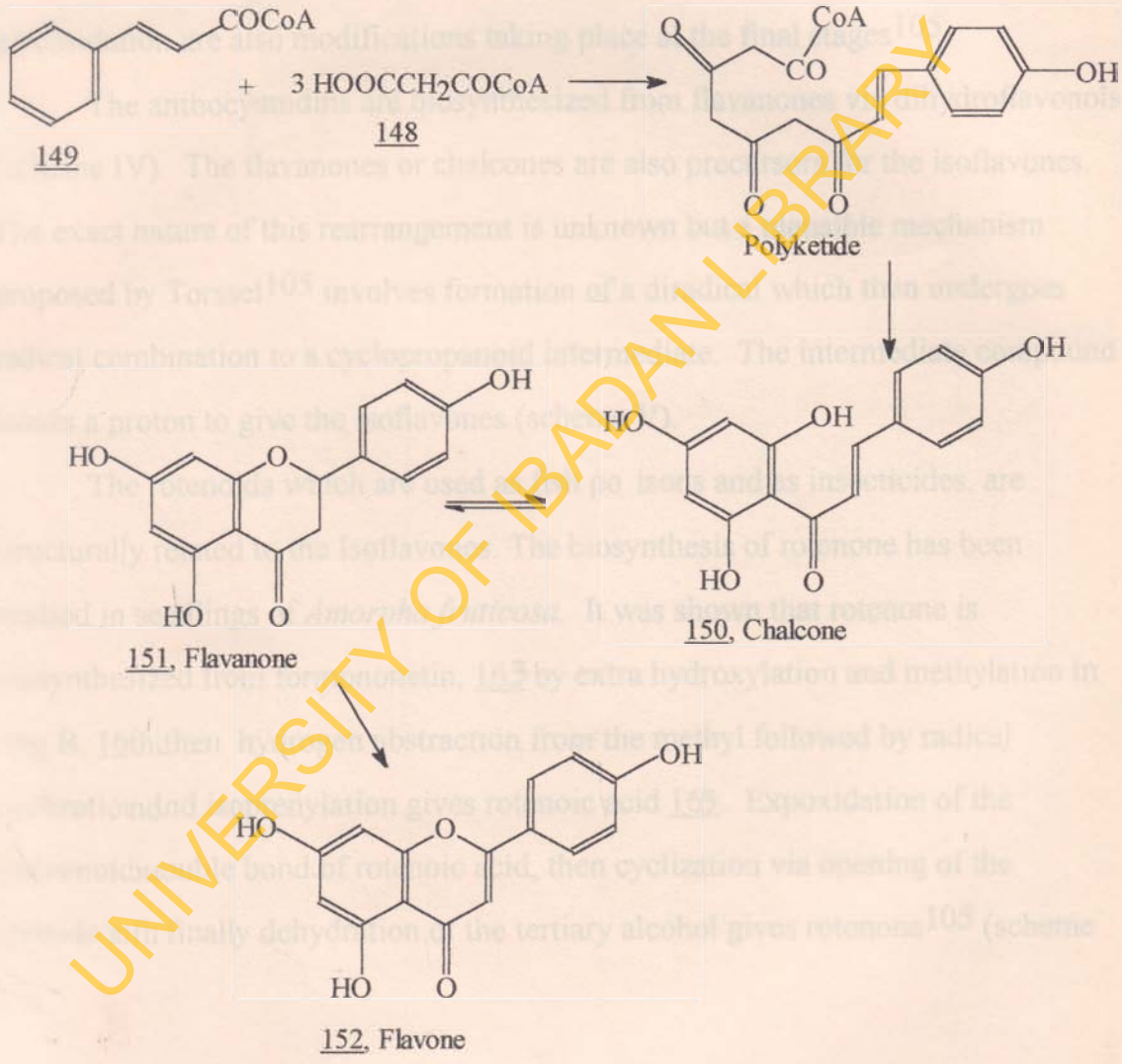
Studies on genetic aspect of colour and the chemical speculations on the mode of formation of the carbon skeleton of this class of compounds stimulated interest in the biosynthesis of flavonoids. Tracer Studies were first applied to the problem using intact plants or plant tissues. From the results of the studies, the precursors were identified and this led to an understanding of some details of the biosynthesis of flavonoids[105-106].

The basic skeleton of the flavonoids was found to arise from three malonyl CoA units, 148 and a cinnamoyl CoA, 149. The flavonoids are thus produced by folding of the polyketide as shown in scheme III.



Scheme II: Fragmentation pathways for flavones and flavanones

Scheme III: Biosynthesis of the flavonoid basic skeleton

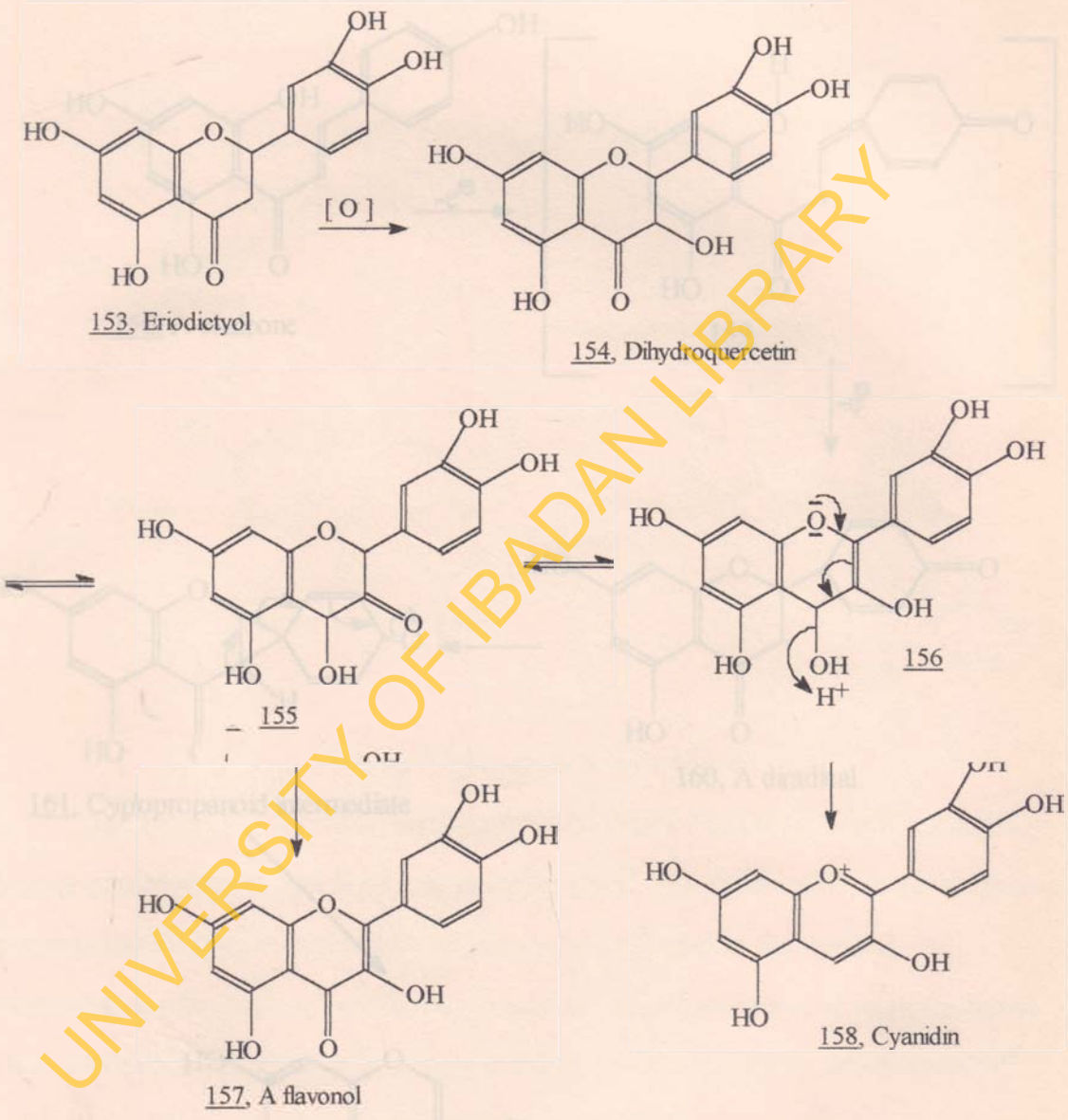


The ring A of most flavonoids has a phloroglucinol structure but it has been proved by labelling experiments that phloroglucinol is not on the pathway nor is phloroglucinol cinnamate. Result of studies also showed that the flavanone

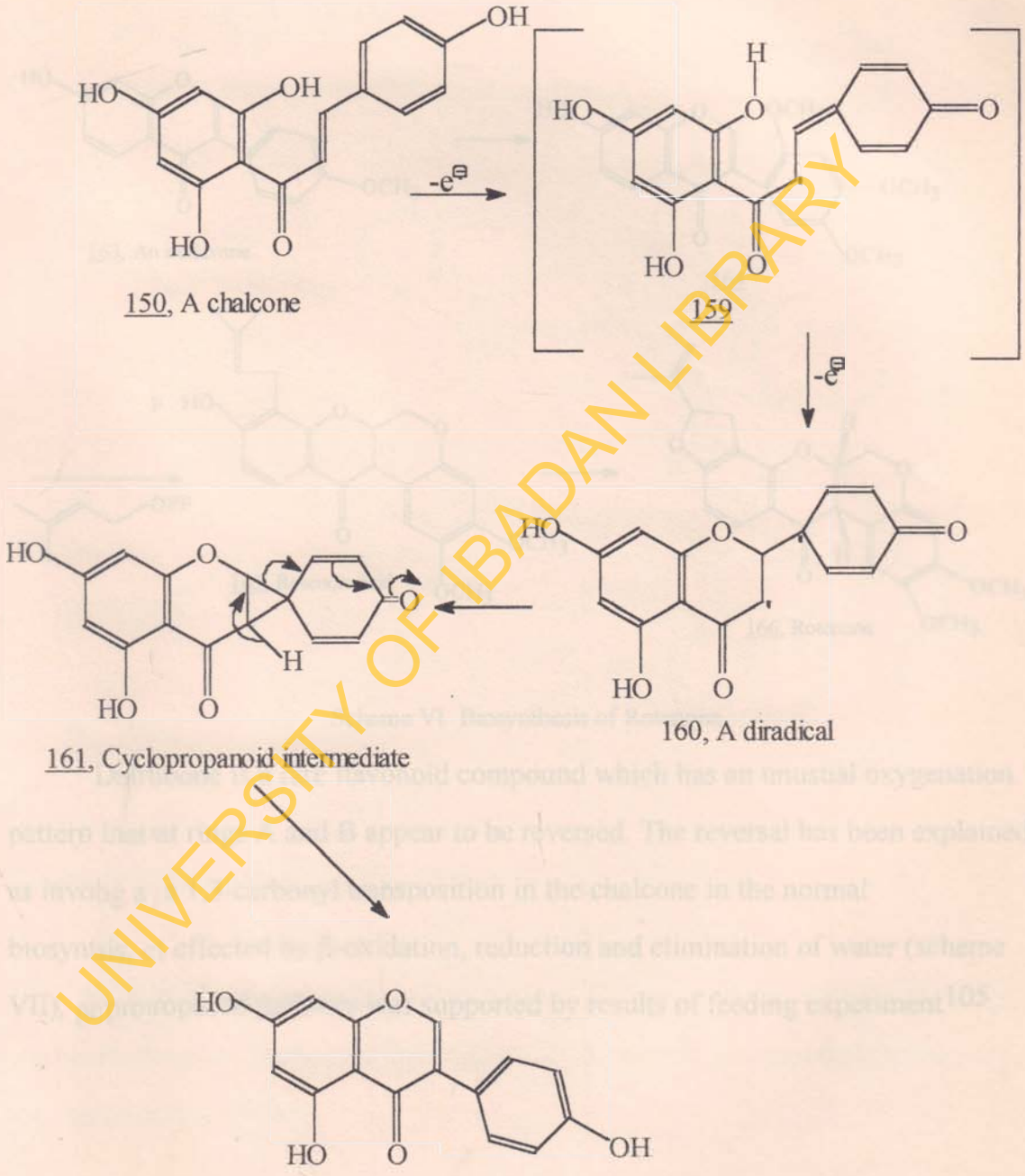
synthase complex is rather specific for p-OH-cinnamic acid which suggest that final hydroxylation of ring B takes place at the C_{15} stage. O-methylation and glycosidation are also modifications taking place at the final stages[105].

The anthocyanidins are biosynthesized from flavanones via dihydroflavonols (scheme IV). The flavanones or chalcones are also precursors for the isoflavones. The exact nature of this rearrangement is unknown but a plausible mechanism proposed by Torssel[105] involves formation of a diradical which then undergoes radical combination to a cyclopropanoid intermediate. The intermediate compound looses a proton to give the isoflavones (scheme V).

The rotenoids which are used as fish po isons and as insecticides, are structurally related to the Isoflavones. The biosynthesis of rotenone has been studied in seedlings of *Amorpha fruticosa*. It was shown that rotenone is biosynthesized from formononetin, 163 by extra hydroxylation and methylation in ring B, 164 ,then hydrogen abstraction from the methyl followed by radical cyclization and isoprenylation gives rotenoic acid 165. Expoxidation of the isoprenoid double bond of rotenoic acid, then cyclization via opening of the epoxide and finally dehydration of the tertiary alcohol gives rotenone[105] (scheme VI).



Scheme IV: Biosynthetic route for flavonols and cyanins



150, A chalcone

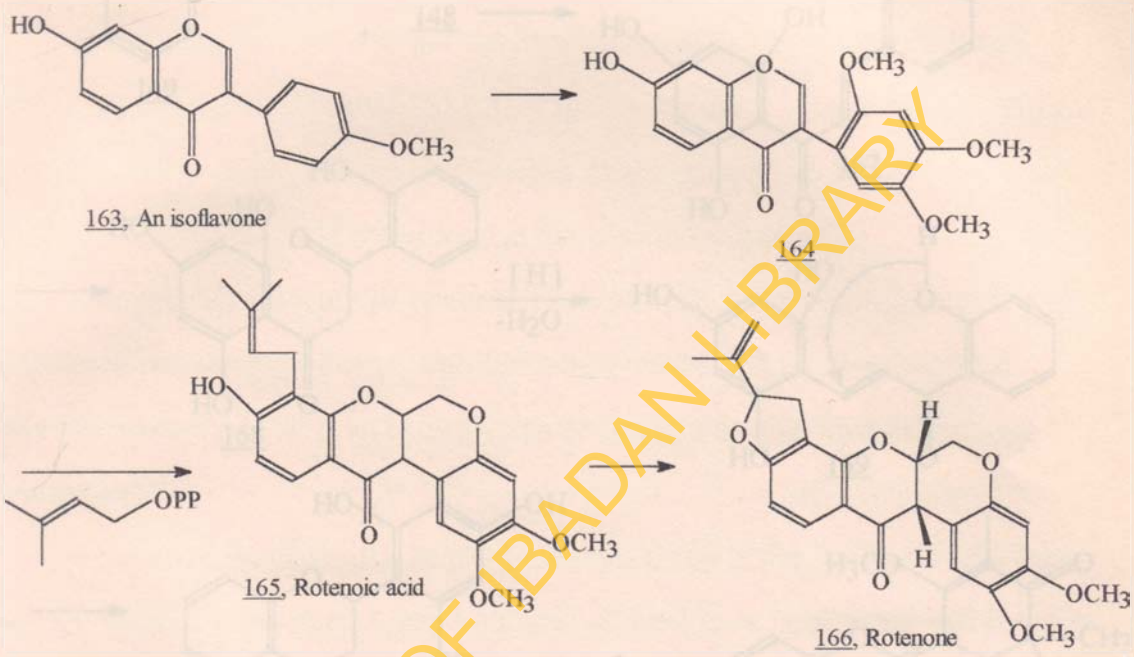
159

160, A diradical

161, Cyclopropanoid intermediate

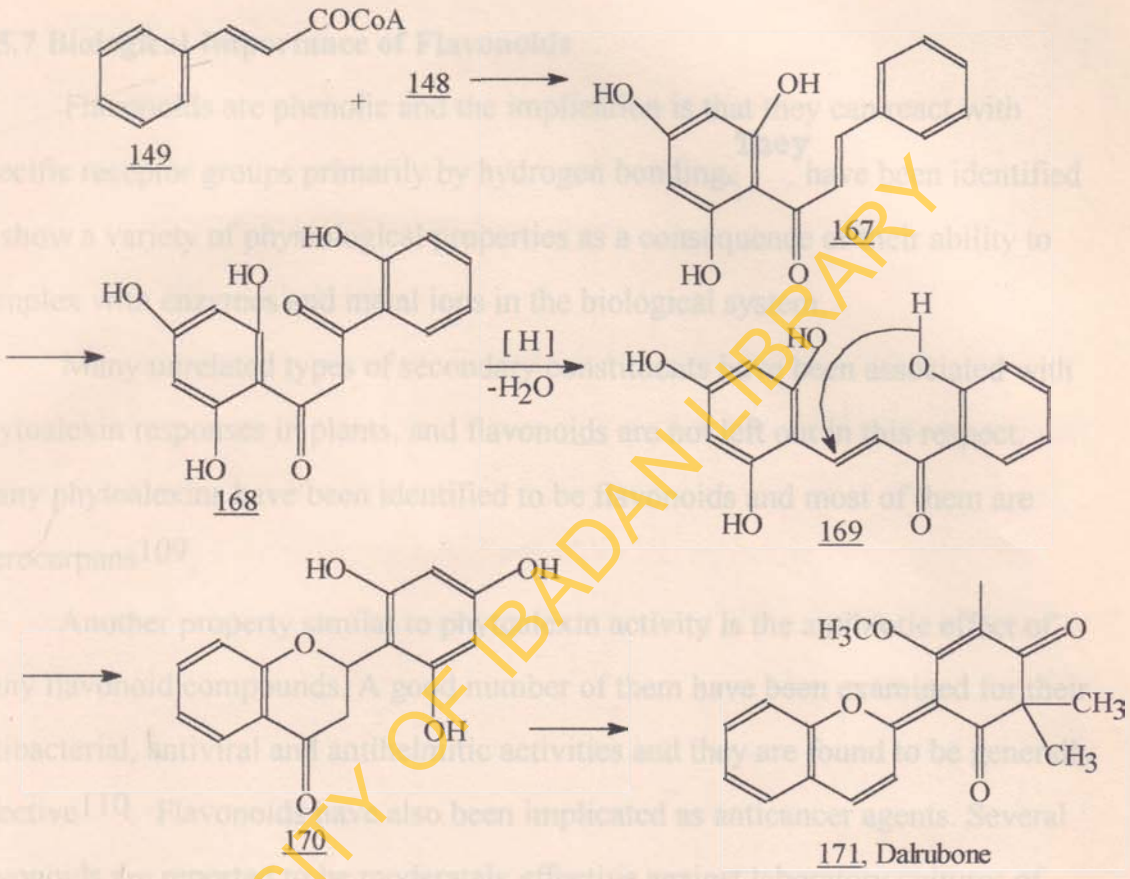
162, Genistain (an isoflavone)

Scheme V. Biosynthesis of Isoflavones



Scheme VI. Biosynthesis of Rotenone

Dalrubone is a rare flavonoid compound which has an unusual oxygenation pattern in that rings A and B appear to be reversed. The reversal has been explained as involving a 1,3-carbonyl transposition in the chalcone in the normal biosynthesis, effected by β-oxidation, reduction and elimination of water (scheme VII). The proposed pathway was supported by results of feeding experiment[105].



Scheme VII: Biosynthesis of Dalrubone

The brief account of the biosynthesis of flavonoids given above is inevitably selective. However an attempt has been made to mention the important features of the flavonoid biosynthesis.

1.5.7 Biological Importance of Flavonoids

Flavonoids are phenolic and the implication is that they can react with specific receptor groups primarily by hydrogen bonding. They have been identified to show a variety of physiological properties as a consequence of their ability to complex with enzymes and metal ions in the biological system.

Many unrelated types of secondary constituents have been associated with phytoalexin responses in plants, and flavonoids are not left out in this respect. Many phytoalexins have been identified to be flavonoids and most of them are pterocarpans[109].

Another property similar to phytoalexin activity is the antibiotic effect of many flavonoid compounds. A good number of them have been examined for their antibacterial, antiviral and antihelmitic activities and they are found to be generally effective[110]. Flavonoids have also been implicated as anticancer agents. Several flavonoids are reported to be moderately effective against laboratory cultures of malignant cells[105].

Some flavonoids have also been reported to show oestrogenic activity. Isoflavones are the only class of flavonoids so far recognised to possess this activity. The oestrogenic activity was attributed to the stilbene-like structure of the compounds which make them structurally similar to potent synthetic oestro gens like diethylstilboestrol.

Flavonoids are also known to have beneficial effects on vascular damage. This discovery was accidental. Recent research has revealed that flavonoids act on blood cell aggregation, a phenomenon that generally accompanies illness and

injury[106]. The action of flavonoids on cell aggregation is therefore consistent with the beneficial effects on capillaries and in disease condition since aggregation enhances symptoms of disease and induces pathology.

1.6 THE TERPENOIDS.

The name terpene was initially given to the group of compounds, mostly composed of the fragrant principles of plants, recognised by Wallach to be built up of branched C_5 units called isopentenyl or isoprene unit. Although isoprene unit was later perceived not to be the functional unit used by nature, it nevertheless provided a useful device for rationalizing the structures of many more complex compounds of higher molecular weight.

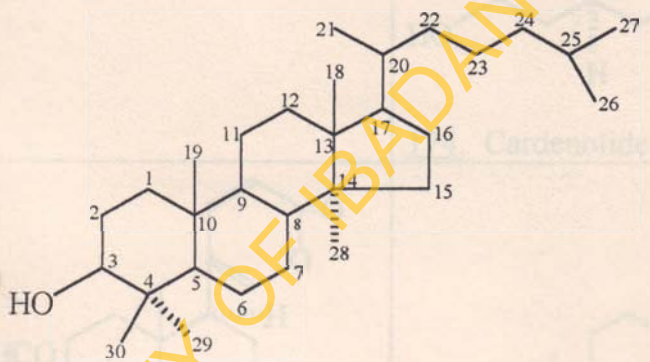
The isoprene rule was subsequently formulated to accommodate all compounds found to be built up of C_5 units. It states that terpenes are multiples of C_5 units linked together head to tail. Based on the number of C_5 units, the terpenes are classified as monoterpenes (C_{10}) sesquiterpenes (C_{15}), diterpenes (C_{20}), Sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}). However, the steroids and several other related compounds do not obey this rule apparently because degradation had occured, the head to tail principle was violated or there had been rearrangements of the original skeleton.

The terpenes house a wealth of significant compounds. Most importantly, there are enormous number of terpenes which are physiologically very active compounds.

The triterpenes which are built up from six isoprene units are formed by cyclization of squalene. Squalene is in turn formed from tail to tail condensation of

two C_{15} units. Generally all triterpenes are recognised as being formed by cyclization of all-trans squalene in various chair-boat conformational sequences.

Structurally, the triterpenes show limited variations in skeletal modifications when compared with the lower terpenes like the sesquiterpenes. A salient feature of nearly all triterpenes is the equatorial hydroxyl group at C-3 as represented by the basic tetracycic triterpene skeleton, 172.



172

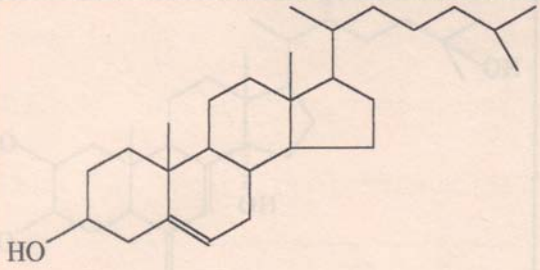
1.6.1. Classification of Triterpenes.

Triterpenes can be classified into two main groups, the tetracyclic triterpenes that include the sterols and the pentacyclic triterpenes.

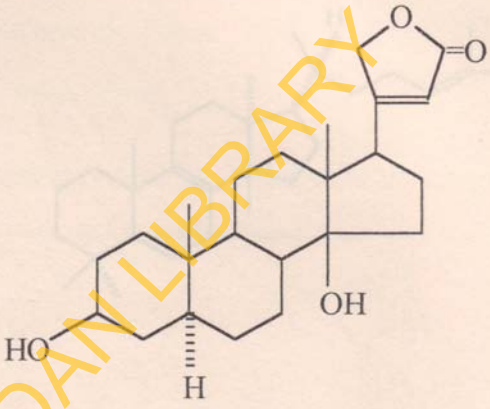
1.6.2. Tetracyclic Triterpenes.

This group of triterpenes possesses the cyclopentenophenanthrene nucleus. The group consists of the sterols, lanostane, euphane and dammarane compounds. The various subgroups of the tetracyclic triterpenes are illustrated in Table 12.

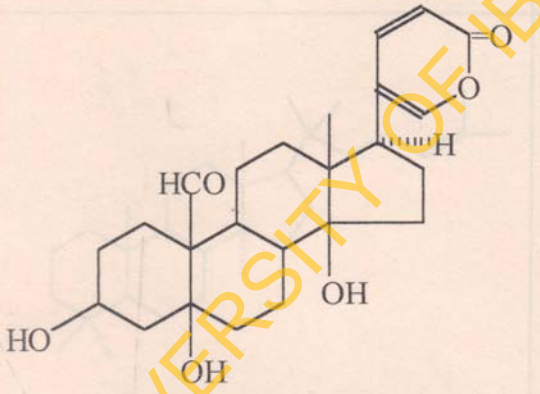
Table 12- Representative Structures of Tetracyclic Triterpenes.



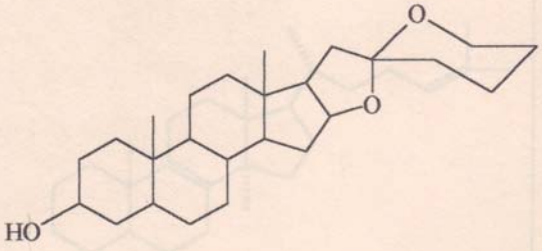
173, Sterol.



174, Cardenolide.

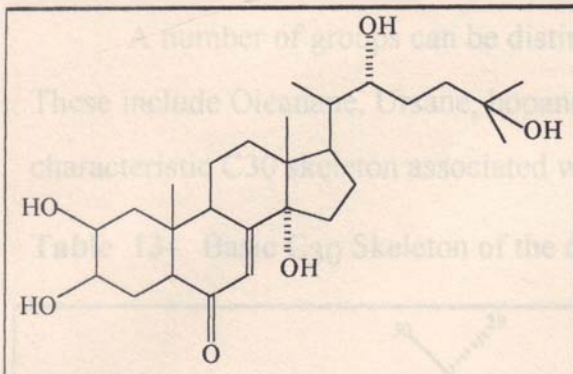


175, Bufadienolide.

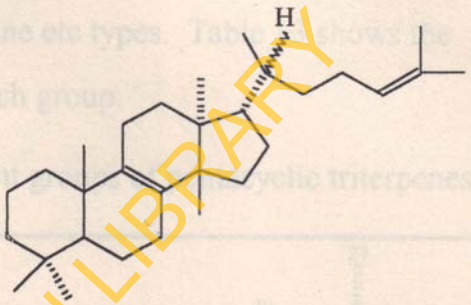


176, Spirostanol.

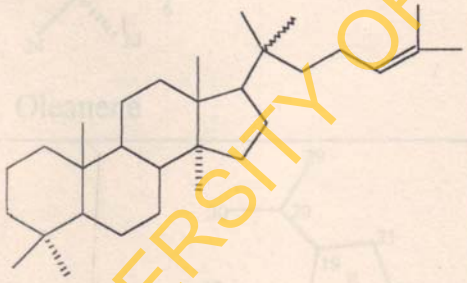
Table 12 Contd.



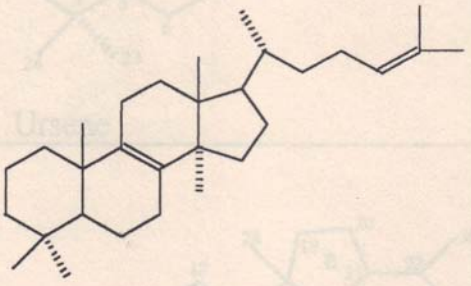
177, Ecdysone.



178, Euphane.



179, Dammarane.

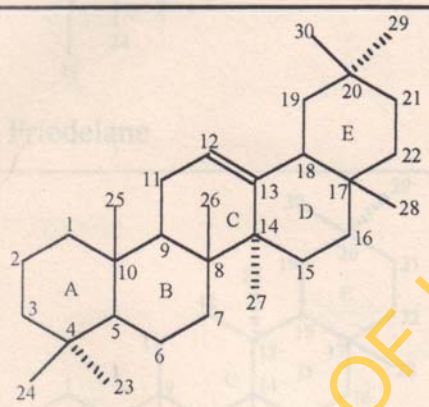


180, Lanostane.

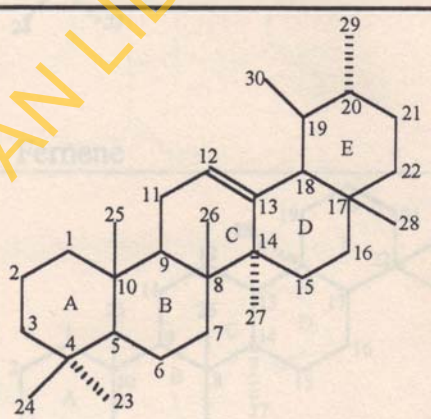
1.6.3 Pentacyclic triterpenes.

A number of groups can be distinguished within the pentacyclic triterpenes. These include Oleanane, Ursane, hopane, lupane etc types. Table 13 shows the characteristic C30 skeleton associated with each group.

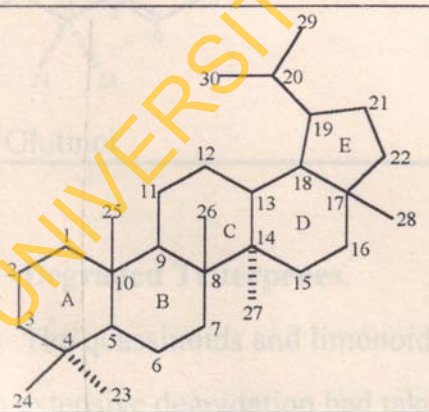
Table 13-. Basic C_{30} Skeleton of the different groups of pentacyclic triterpenes.



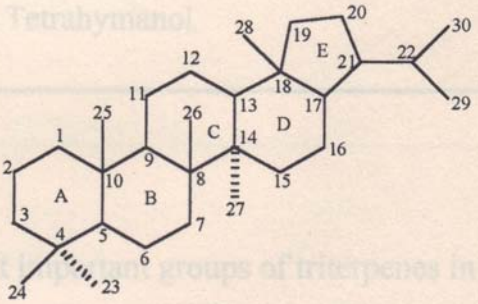
181, Oleanene



182, Ursene

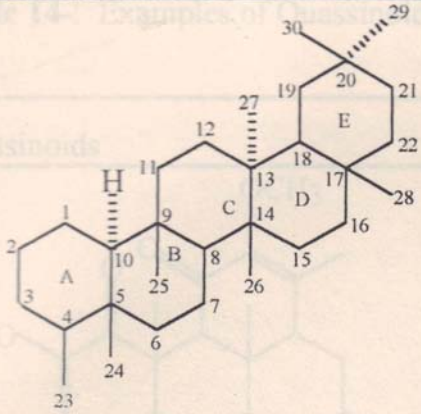


183, Lupane

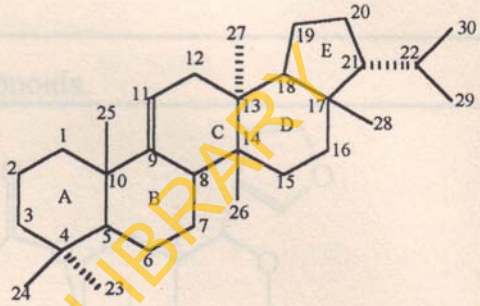


184, Hopane

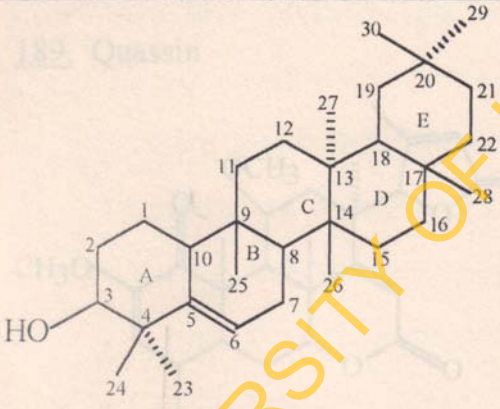
Table 13 Contd.



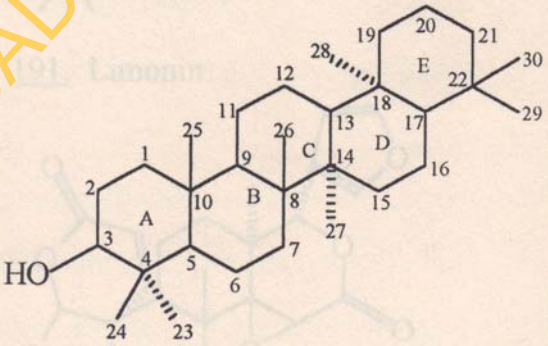
185, Friedelane



186, Fernene



187, Glutinol

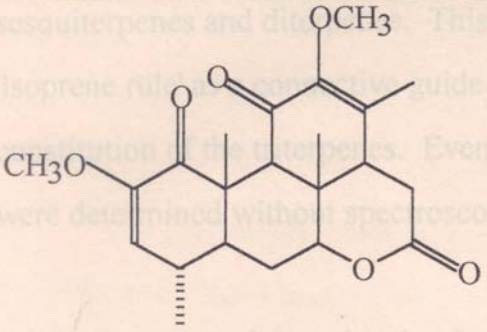
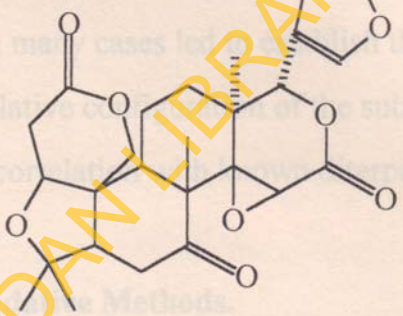
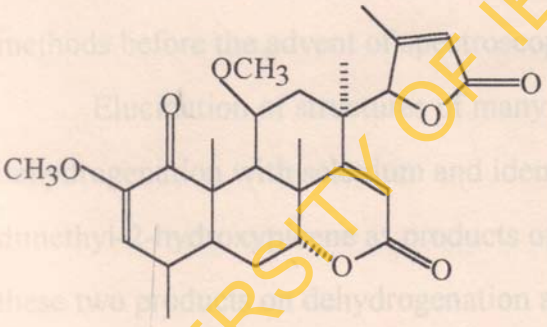
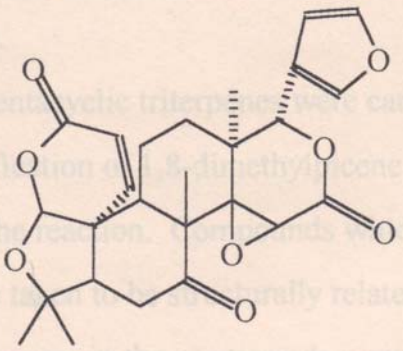


188, Tetrahymanol

1.6.4 Degraded Triterpenes.

The quassinoids and limonoids represent important groups of triterpenes in which extensive degradation had taken place. Consequently, they do not show any structural similarily with any of the major groups already mentioned above. Hence it becomes imperative to treat them separately as unique groups of triterpenes. Few examples of these two groups of compounds, 189-192, are shown in table 14.

Table 14-: Examples of Quassinoids and Limonoids.

Quassinoids.	Limonoids.
 189, Quassin	 191, Limonin
 190, Odyendane	 192, Jangomolide

1.6.5 Determination of structure of Triterpenes.

Reports on studies of the chemistry of triterpenes started to appear in the early thirties. At that time the laboratories were devoid of IR, NMR and Mass spectrometers. Therefore the characterization of compounds rested mainly on measuring m..p, b.p, δ, n_D and using combustion analysis.

Structure eludidation was largely accomplished by oxidative degradations and the most important of all was the pyrolysis in the presence of sulphur or selenium. These reactions had been used to determine the structures of cyclic sesquiterpenes and diterpenes. This experience together with employing the 'Isoprene rule' as a connective guide-line in many cases led to establish the correct constitution of the triterpenes. Even the relative configuration of the substituents were determined without spectroscopy by correlation with known diterpenes[111].

1.6.6 Structure determination by degradative Methods.

The structures of triterpenes were characterized mainly by degradative methods before the advent of spectroscopy.

Elucidation of structures of many pentacyclic triterpenes were carried out by dehydrogenation with selenium and identification of 1,8-dimethylpicene and 1,8-dimethyl-2-hydroxypicene as products of the reaction. Compounds which gave these two products on dehydrogenation are taken to be structurally related to β-amyrin. The position of C=C and methyl groups in the compounds were also determined through a series of chemical transformations usually involving oxidative cleavage of the C=C with subsequent pyrolysis which eventually produced fragments containing fewer rings. The products of these transformations were then correlated with known compounds[111].

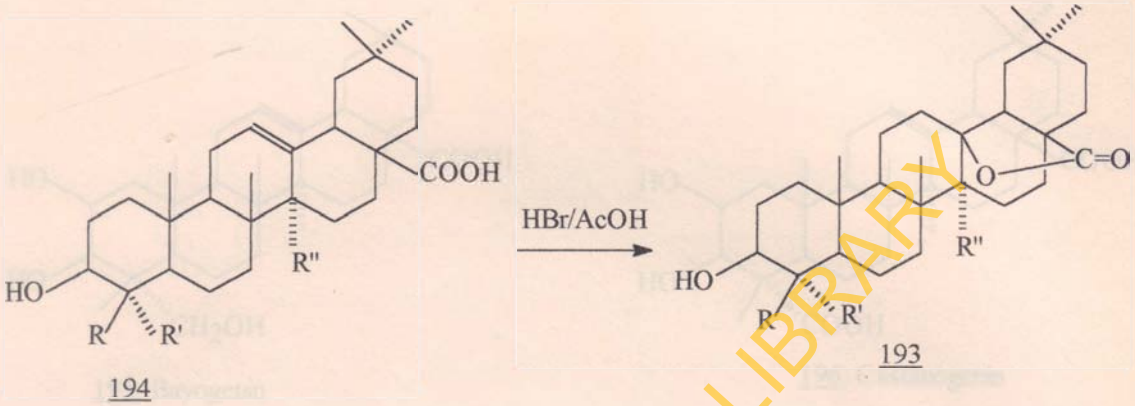
The structure of isomeric compounds generally presented challenging experience. However, through a sequence of chemical transformations many of the problems posed by such compounds were solved. For instance, the structure

elucidation of glycyrrhetic acid and the identification of the four soyasapogenols, A, C, B and D were particularly challenging problems[111].

In recent times, due to the availability of spectroscopic tools, structure elucidation by degradative methods has taken a new dimension. Only few chemical transformations are now necessary because derivatives of unknown compounds are now frequently identified by spectral correlation with known compounds.

The derivatives commonly employed in the establishment of structures of triterpenes are acetate and methyl ester derivatives. While the acetate derivatives help to confirm the presence and number of hydroxyl groups, their spectral data together with the melting point provide the basis for comparison with the spectra and melting point of authentic or known compounds[112]. The methyl ester derivatives similarly confirm the presence of carboxylic acid group and are also useful for comparison with available data on known compounds[113]. Other derivatives such as oxo-compounds are often used in solving structural problems of unknown triterpenes[114].

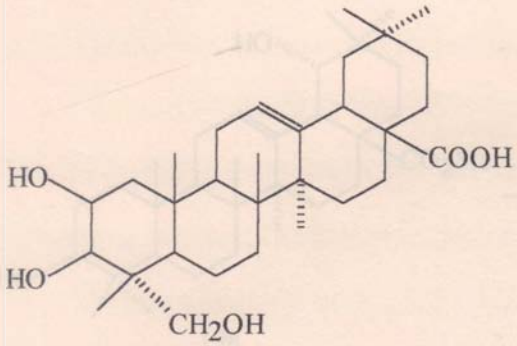
The location of carboxylic acid group on C_{17} of an Oleanene or Ursene skeleton is easily established by the formation of a γ-lactone[115], 193, from a Δ^{12}-Oleanene or Ursene compound, 194.



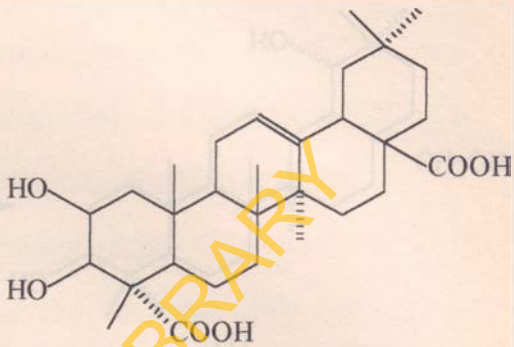
194 193

Some other important transformations used in structural elucidation include conversion of unknown compounds into other known compounds for easy comparison. The reactions commonly used are simple organic reactions such as reduction, oxidation, dehydration, etc.

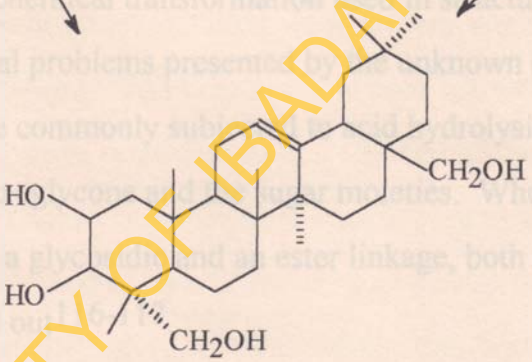
For example bayogenin, 195 and castanogenin, 196 were both reduced to the same tetrahydroxyl compound, 197. This result provided a basis for comparison between the two compounds[115].



195, Bayogenin

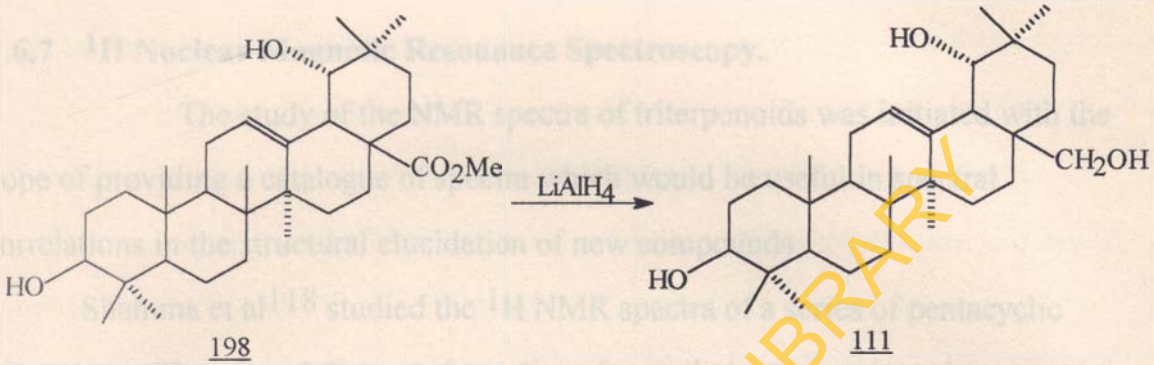


196, Castanogenin



197

Another example is the conversion of siaresinolic acid methyl ester, 198 into 19α -hydroxyerythrodiol, 111 by reduction with $LiAlH_4$[116]. The position of the hydroxyl groups in the compound 198 was thus identified by comparison with the structure of 111.



198 111

The nature of chemical transformation used in structure elucidation tend to vary with the structural problems presented by the unknown compound.

Sapogenins are commonly subjected to acid hydrolysis for full characterization of the aglycone and the sugar moieties. Where it is necessary to differenciate between a glycosidic and an ester linkage, both acid and alkaline hydrolyses are carried out[116-117].

Chemical degradative method contributed significantly to structure elucidation of triterpenoids before spectroscopy became available. However, it was very difficult at that time, to characterize compounds isolated in small quantities because large quantities of substances are usually needed to go through the host of transformation sequences.

Nevertheless, this method has contributed immensely to the development of conformational chemistry, understanding of mechanisms and development of elegant synthetic methods.

1.6.7 ^{1}H Nuclear Magnetic Resonance Spectroscopy.

The study of the NMR spectra of triterpenoids was initiated with the hope of providing a catalogue of spectra which would be useful in spectral correlations in the structural elucidation of new compounds.

Shamma et al[118] studied the ^{1}H NMR spectra of a series of pentacyclic triterpenes. They found distinct absorptions for methyl esters, acetoxyl groups and angular methyl groups. Certain other functional groups such as vinylic protons, protons α-to hydroxyl or acetoxyl and methylene protons were also found to be important because they gave clues to certain structural features of the triterpenes.

A number of empirical rules emanated from the results of their studies with respect to the chemical shift of common functional groups as a consequence of the position of such groups and or its interaction with neighbouring groups.

In the case of C-methyl groups, it was noticed that the chemical shift of the higest C-methyl group was particularly indicative of the position of the carbomethoxyl function if present in the molecule. The conclusion was that if a C-28 carbomethoxyl function is present in a triterpene of the ursane or oleanane series, the highest C-methyl absorption peak appears upfield from δ 0.775. Alternatively, when C-28 position was represented either by a hydroxymethylene, a methyl group or a lactone, the highest C-methyl absorption peak appears downfield from δ 0.775. The lupanes were also found to conform to this rule (Table 15).

The position of the absorption of the methoxyl moiety of a methyl ester is also partially indicative of the relative position of the carbomethoxyl group in the triterpene molecule. The observation was that absorption of a C-28 methyl ester

belonging to the Oleanane or Ursane group is usualy upfield from δ 3.595 while the carbomethoxyls located in other positions such as C-24 or C-30 absorb further downfield in the region from δ 3.595-3.650 (Table 16). The reason for the differences in the positions of the methyl ester absorptions is not known. It may be related to the fact that the C-28 carbomethoxyl function is extremely hindered or that this functional group is influenced by the magnetic anisotropy of the 12(13) double bond.

Vinyl proton absorption of the normal trisubstituted double bond, usually of 12|(13) position in the ursane and oleanane series was found to be in the region between δ4.93 and δ5.50. If a terminal double bond is present, such as in lupane series, the vinyl protons absorb at higher field around 4.30 to 5.07.

Another useful and characterisitc absorption is the vinylic methyl function, CH_3-C=C. Normal methyl groups absorb from δ0.63 to δ1.50 but vinylic methyl peaks are found to appear between δ1.63 and δ1.8 and are usually sharp and well defined. Many triterpenes of the lupane class have vinylic methyl groups and these are easily recognised. Thus betulin diacetate, melaleucic acid methyl ester and thurberogenin acetate exhibited vinylic methyl peaks at δ1.67, 1.64 and 1.80 respectively.

Acetoxyl protons are noted to give the sharpest absorption of any function in the triterpene serires, with majority of these protons absorbing between δ1.92 and δ1.97. There is however no clear differentiation between axial and equatorial acetoxyl functions in the triterpenes as was the case in cyclic polyol acetates[118]. The acetoxyl groups of 1,2-glycols were observed to appear at higher fields (δ1.85-

1.92) than the acetoxyl groups of analogous monoacetates. Thus vicinal glycols may be easily recognised by this method.

The absorptions of protons α-to acetoxyl goups usually appear between δ 3.65 to 5.60. This type of absorption fall into two categories depending on whether the acetoxyl group is primary or secondary. For secondary acetoxy groups, axial -protons are found between δ4.00 to 4.75 while the equatorial α-protons absorb at between δ5.00 to 5.48. The-protons α- to primary acetoxyl groups generally show absorptions at higher fields, beween δ3.65-5.20. Protons -α- to vicinal acetoxyl groups are found to appear at much lower field between δ4.9-5.4, however, these values should be used with discretion as it is not easy to clearly differentiate between these protons if only the one dimentional ^{1}H NMR is considered (Table 17).

Although the methyl groups in triterpenes usually appear as sharp peaks at the right end of the spectrum, their absorptions are frequently found to overlap. The assignments to most of the methyl groups are therefore not easy to make. From the investigation of the NMR spectra of some deuterated derivatives of the -Δ^{12}-Oleanene and Δ^{12}-Ursene by Karliner and Djerassi[119], assignments were made for the methyl groups in the NMR spectrum of Δ^{12}-Oleanene (table 18).

The values shown in table 18 can only serve as a guide because the positions of absorptions of angular methyl groups depend to a large extent on the substitution pattern on the carbon frame-work.

Table 15 - Chemical Shift of Highest C-methyl groups.

S/No.	Triterpene	Chemical Shift (ppm).
1.	Arjunolic acid methylester triacetate.	0.683.
2.	Echinocystic acid methyl ester diacetate.	0.713.
3	Oleanolic acid methyl ester.	0.730
4.	Ursolic acid methyl ester acetate.	0.735
5.	α-Amyrin benzoate.	0.865
6.	Erythrodiol diacetate.	0.875
7.	Lupanol.	0.803
8.	Betulin diacetate.	0.840

1.6.8 ^{13}C NMR Spectroscopy.

^{13}C NMR has always played a complementary role to ^{1}H NMR in structural elucidation of natural products. In the determination of structures of triterpenes it is significantly useful in providing a confirmatory evidence for the presence of certain functional groups. The presence of the functional groups listed in table 19 are readily recognised by mere inspection of ^{13}C NMR Spectrum of the

compounds. The table shows the chemical shifts of the functional groups commonly encountered in triterpenoid compounds.

Table 16 - Absorptions of Methyl Esters.

S/No.	Triterpene.	Methoxyl absorption.	Position of Carbomethoxyl function.
1.	Ursolic acid methyl ester.	3.578	C-28
2.	Cochalic acid methyl ester.	3.570	C-28
3.	Echinocystic acid methyl ester.	3.595	C-28
4.	11-Keto-α-boswellic acid methyl ester.	3.597	C-24
5.	Glycyrrhetic acid methyl ester.	3.645	C-30

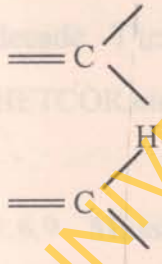
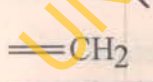
Table 17 - Absorptions of Protons Alpha to acetoxyl group.

S/No.	Triterpene.	Absorptions of α-protons.	Type of Acetoxyl group.
1.	Erythrodiol diacetate.	3.85	1°
2.	Betulin diacetate.	4.05	1°
3.	Soyasapogenol B triacetate.	4.22	1°
4.	Chichipegenin tetra-acetate	5.81	2°
5.	Longispinogenin triacetate.	5.62	2°
6.	Arjunolic acid methyl ester triacetate.	4.98	vicinal(2,3)
7.	Asiatic acid methyl ester triacetate.	5.00	vicinal(2,3)
8.	A1-Barrigenol penta-acetate.	5.54	vicinal(15,16)

Table 18 - Absorption of Methyl groups of Δ^{12}-oleanene.

δ-Value	1.13	o.97	0.93	0.87	0.83
Methyl group.	27	25	24	23,29,30	26,28

Table 19 - ^{13}C Chemical shifts of common functional groups of triterpenoids.

Functional Group.	Chemical Shift (ppm).
C-OH(1^o)	60-63
C-OH(2^o)	71-74
C-OH(3^o)	75-
-COOH	180-182
	138-145
	121-125
	105-110

The position of the chemical shift of olefinic carbons in triterpenoids usually gives a strong indication of whether it is an Oleanene, a ursene or a lupene

compound as the absorptions are characteristic of these classes of triterpenoids[120-122] (Table 20).

Table 20 - Characteristic Absorptions of Olefinic carbon atoms of Triterpenoids.

Class of Compound.	Position of Olefinic Carbon			
	C_{12}	C_{13}	C_{20}	C_{29}
Oleanene	122-126	140-145	-	-
Ursene	125-129	138-139.5	-	-
Lupene	-	-	150-	106-109

There has been a remarkable development in NMR spectroscopy in the past decade. The use of 2D NMR spectra in form of COSY, DEPT, HET 2DJ, HETCOR etc. now provide additional powerfull tool for structure determination.

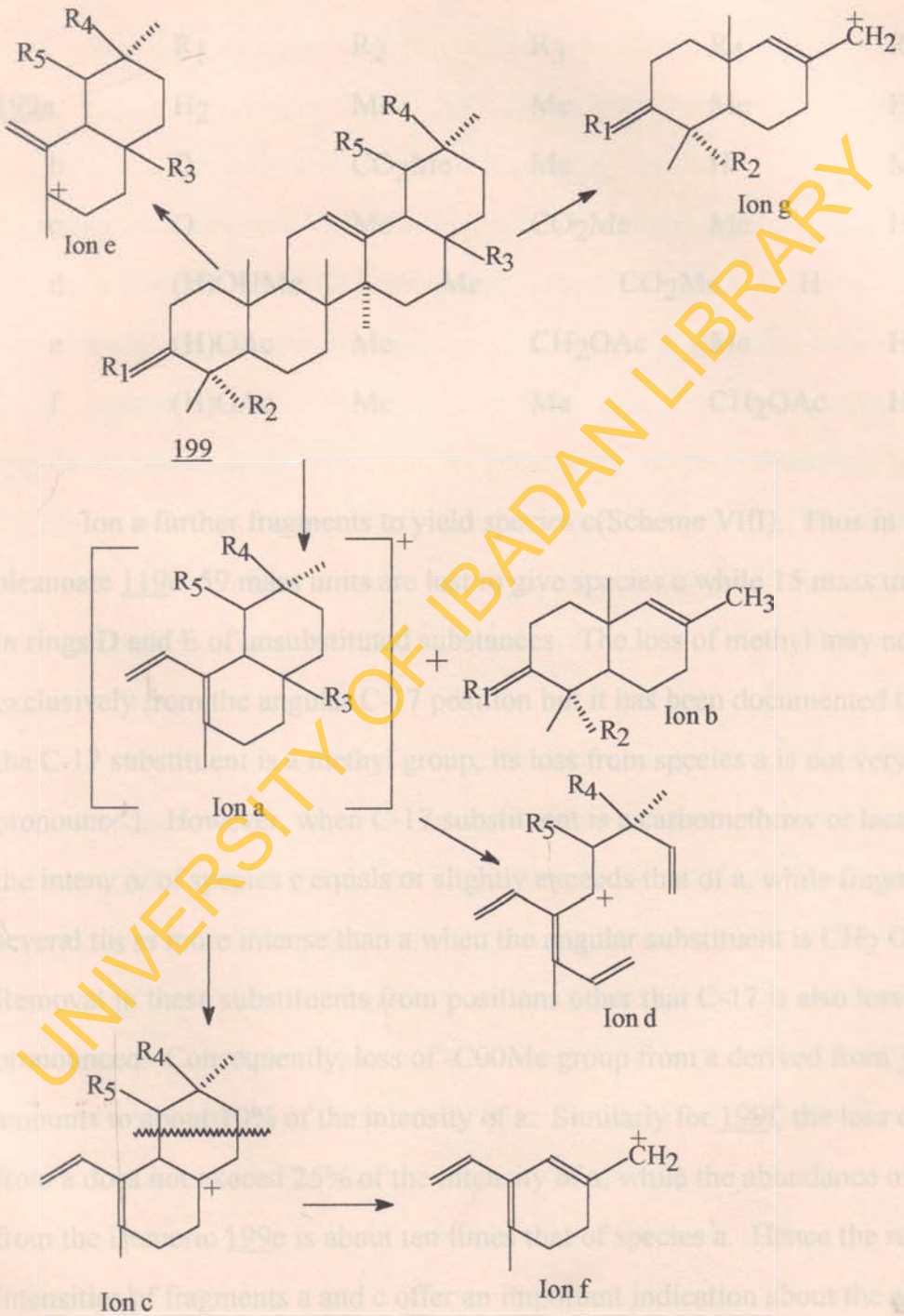
1.6.9 Mass Spectrometry.

Mass spectrometry has been used in recent times. to an increasing extent for the structure elucidation of triterpenoids. Before the early 60s very little was known about the mass spectra of this group of compounds apparently because of their low volatility. Budzikiewicz et al[123] however took the lead when they carried out investigation into the mass spectra of some pentacyclic triterpenes.

The possibilities and limitations of the use of the mass spectra as a diagnostic tool for unknown triterpenoids were also discussed. Since then, other reports[119] have been published on the mass spectra of trierpenes and they seem to confirm the observations made by Budzikiewicz et al.

1.6.10 Mass Spectrum of Oleanenes and Ursenes.

The most characteristic fragmentation of all compounds of this class is the retro-Diels-Alder cleavage of ring C when the 12-13 double bond is present. The retro-Diels-Alder fragmentation produces species a, which is subject to further fragmentation and a neutral fragment b(Scheme VIII). Charge retention with the fragment b can be observed to some extent but is usually of minor importance. Fragment a consists of rings D and E as shown by the fact that substitution in rings A and B does not change the mass, while alterations in rings D and E result in the appropriate mass shifts. Thus the unsubstituted parent hydrocarbon β-amyrin 199a yields fragment a of m/z 218. Methyl β-boswellonate 199b gives a with same mass (m/z 218) as 199a while the isomeric ether, methyl Oleanonate 199c with carbomethoxy group at C-17, exhibits species a at m/z 262 as does methyl 11-deoxyglycyrrhetate 199d. Erythrodiol diacetate 199e and the isomeric diacetate 199f both give fragment a at m/z 276. This typical retro-Diels-Alder fragmentation leading to species a has become a useful diagnostic tool for the presence of 12-13 double bond in the α- and β-amyrins[119].



Scheme VIII. Fragmentation pattern of Oleanenes and Ursenes.

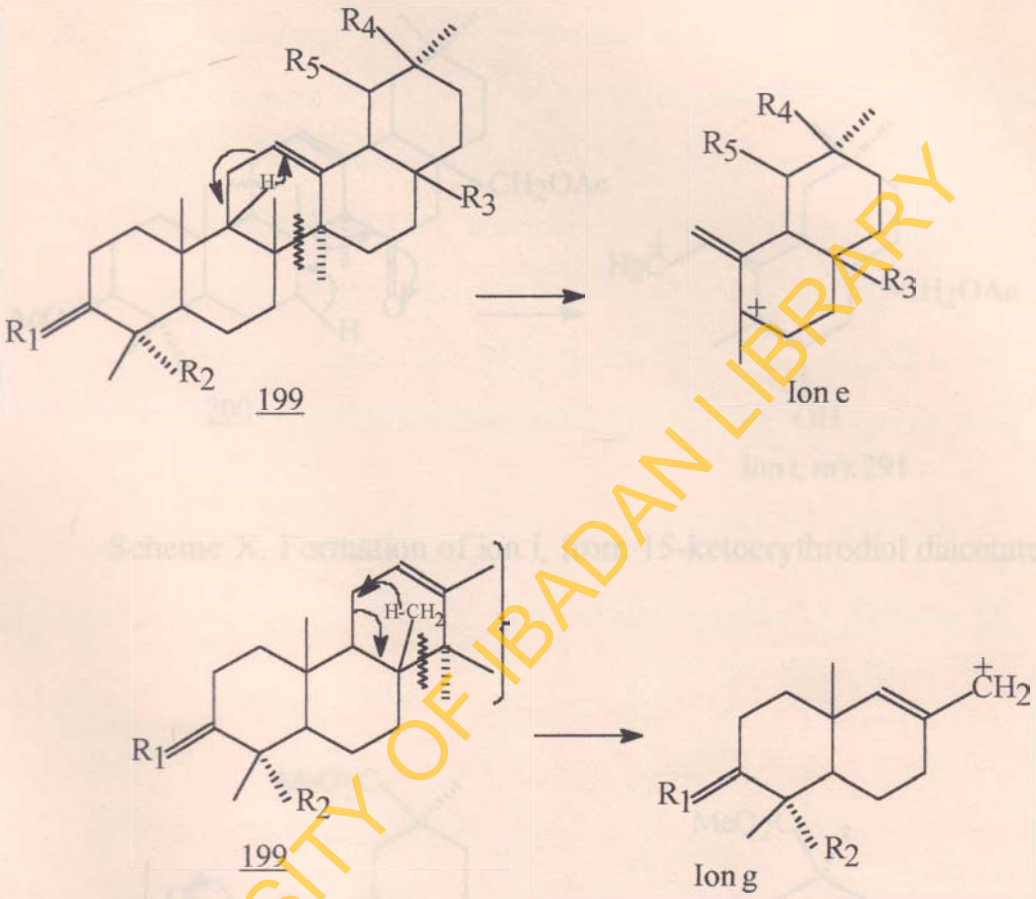
	R_1	R_2	R_3	R_4	R_5
199a.	H_2	Me	Me	Me	H
b	O	CO_2Me	Me	H	Me
c	O	Me	CO_2Me	Me	H
d	(H)OHMe	Me	CO_2Me	H	
e	(H)OAc	Me	CH_2OAc	Me	H
f	(H)OAc	Me	Me	CH_2OAc	H

Ion a further fragments to yield species c(Scheme VIII), Thus in methyl oleanoate 119c 59 mass units are lost to give species c while 15 mass units are lost in rings D and E of unsubstituted substances. The loss of methyl may not be exclusively from the angular C-17 position but it has been documented that when the C-17 substituent is a methyl group, its loss from species a is not very pronounced. However, when C-17 substituent is a carbomethoxy or lactone group the intensity of species c equals or slightly exceeds that of a, while fragment c is several times more intense than a when the angular substituent is CH_2 OAc. Removal of these substituents from positions other that C-17 is also less pronounced. Consequently, loss of -C00Me group from a derived from 199d amounts to about 10% of the intensity of a. Similarly for 199f, the loss of CH_2OAc from a does not exceed 25% of the intensity of a, while the abundance of species c from the isomeric 199e is about ten times that of species a. Hence the relative intensities of fragments a and c offer an important indication about the attachment of a substituent at C-17.

Species c decomposes to fragment f by loss of 70 mass unit. This cleavage is due to the partial loss of ring E. Species c is always accompanied by a less intense ion d, 15 mass units lower. It is probably formed by a one-step process from species a (Scheme VIII). Species a is also accompanied by fragment e of relatively low abundance and containing 13 mass units less. It is probably formed by one hydrogen transfer and cleavage of an allylically activated bond (Scheme IX).

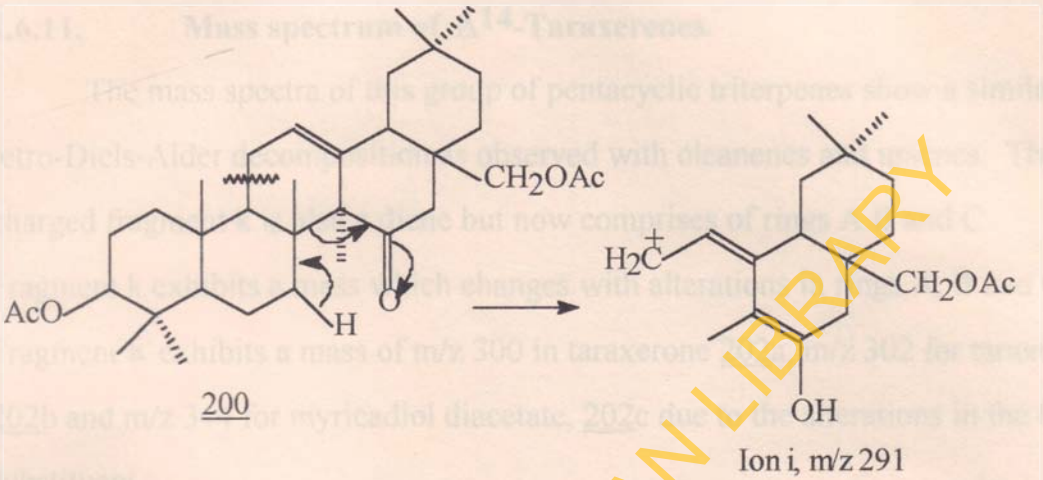
Another important fragment, g contains rings A and B as determined by the appropriate shifts upon substitution in these rings. In the hydrocarbon β-amyrin, it is found at m/z 191 while in the 3-ketone methyl oleanonate, it is shifted to m/z 205. A 3 - hydroxyl and 3-acetoxyl group causes shifts to m/z 207 and 249 respectively. The formation of g seem to involve transfer of one hydrogen atom (Scheme IX).

Generally introduction of a keto group at C-6, C-16 or C-12 does not affect the fragmentation path of the Δ^{12}-unsaturated oleanenes and ursenes but only causes additional fragmentations. The existence of a carbonyl function close to the centers of principal fragmentation can change the mode of cleavage as shown in the mass spectra of 15-ketoerythrodiol diacetate 200 and methyl glycyrrhetate 201. 15-ketoerythrodiol diacetate does not show the expected fragment at m/z 290 but its most abundant ion occurs at m/z 291. This ion i results from the hydrogen transfer process similar to that observed in 15-keto steroids[124] (scheme X).

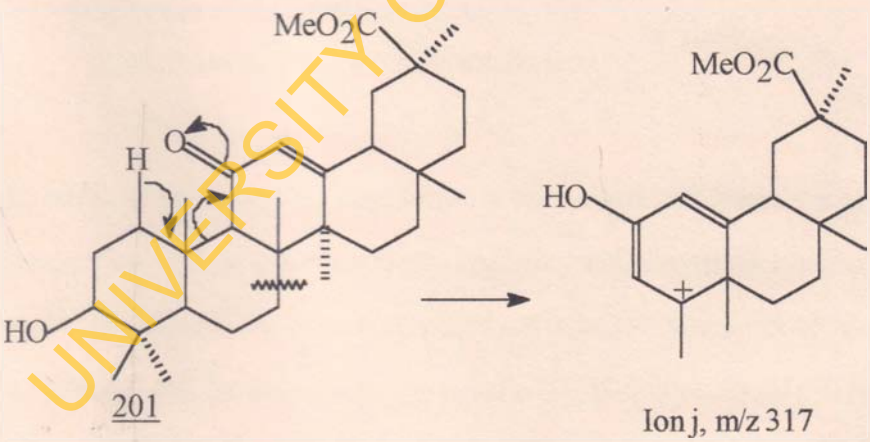


Scheme IX: Formation of ions e and g from the oleanenes and ursenes.

In methyl glycyrrhetate the expected fragment a (m/z 276) is present but in addition another fragment j at m/z 317 with higher intensity can be found. Its formation is also adduced to hydrogen transfer process (scheme XI).



Scheme X. Formation of ion i, from 15-ketoerythrodiol diacetate

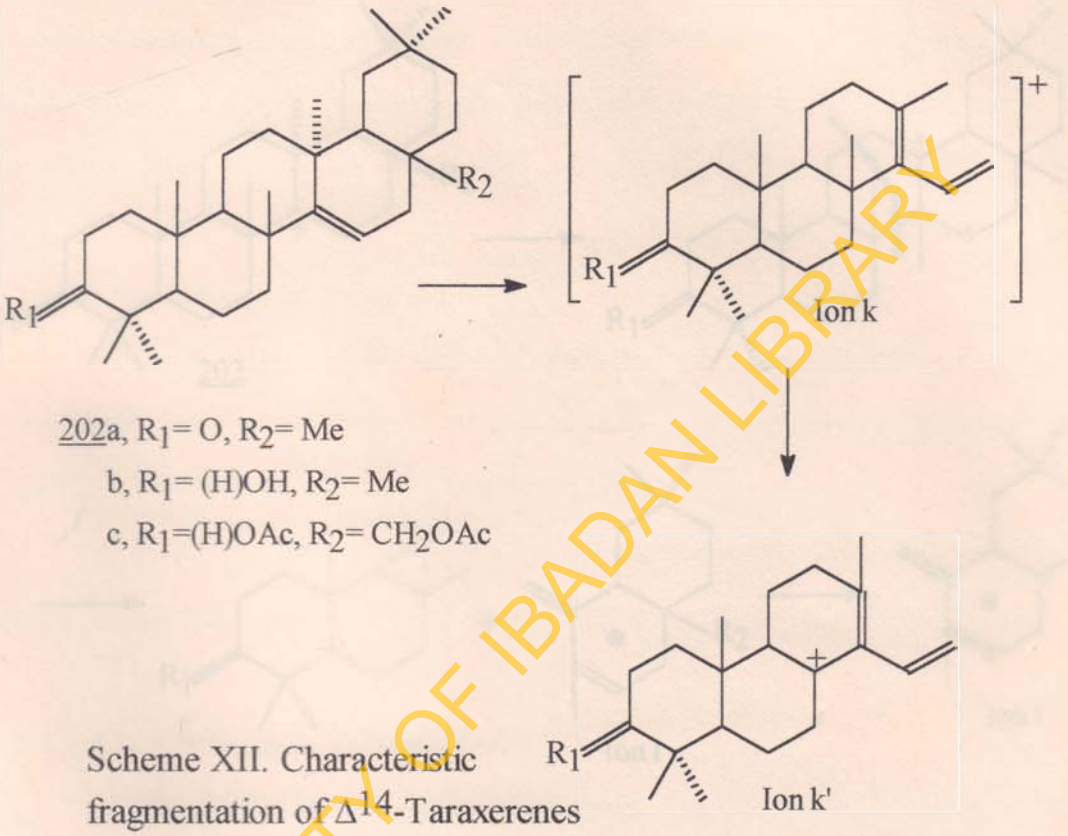


Scheme XI. Fragmentation of methyl glycyrrhetate to give ion j.

1.6.11. Mass spectrum of Δ^{14}-Taraxerenes.

The mass spectra of this group of pentacyclic triterpenes show a similar retro-Diels-Alder decomposition as observed with oleanenes and ursenes. The charged fragment k is also a diene but now comprises of rings A.B and C. Fragment k exhibits a mass which changes with alterations in rings A, B and C. Fragment k exhibits a mass of m/z 300 in taraxerone 202a, m/z 302 for taraxerol, 202b and m/z 344 for myricadiol diacetate, 202c due to the alterations in the C-3 substituent.

Ion k is accompanied by an ion k', 15 mass units lower, which is formed by the loss of a methyl group, the allylically activated one at C-8 (Scheme XII).



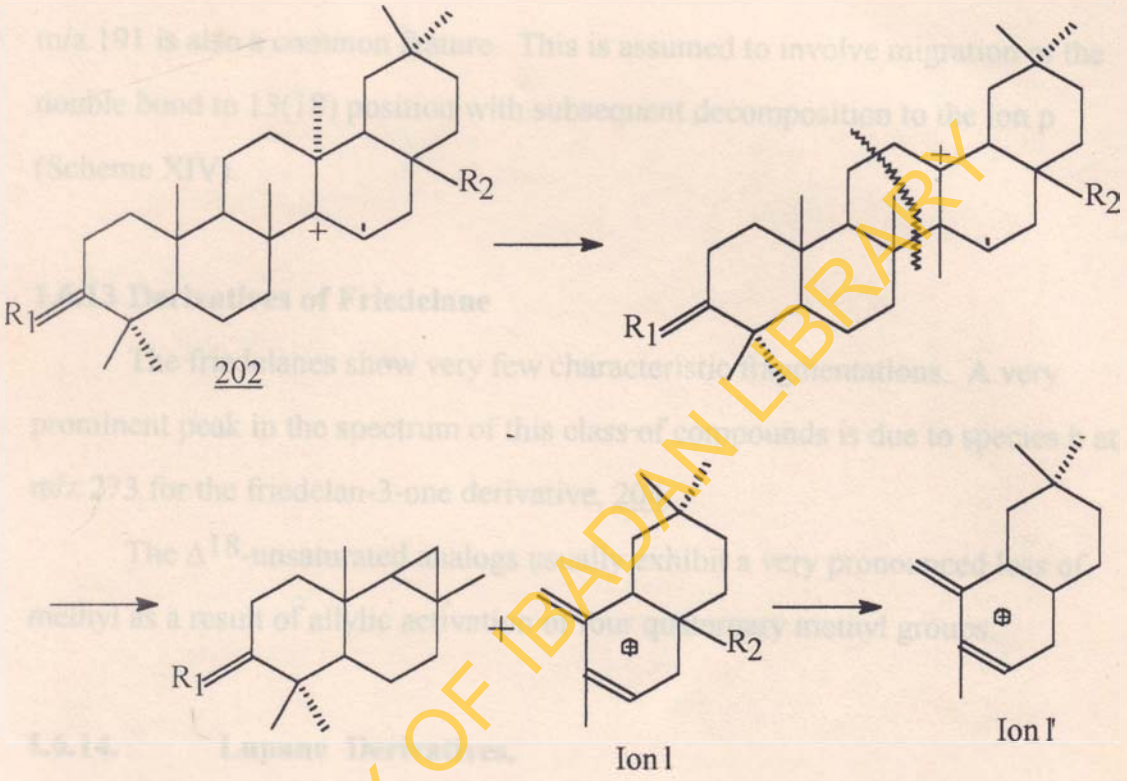
202a, R_1= O, R_2= Me

b, R_1= (H)OH, R_2= Me

c, R_1=(H)OAc, R_2= CH_2OAc

Scheme XII. Characteristic fragmentation of Δ^{14}-Taraxerenes

In addition to these two fragments, a very abundant fragment at m/z 204 for taraxerone and taraxerol is characteristic. The fragment l, is derived from rings D and E as verified by the spectrum of myricadiol which contains a rather small peak at m/z 262 but an abundant one at m/z 202 (l-CH_3 COOH). The formation of l is proposed to involve the fission of C-11-12 and C-8-14 bonds (Scheme XIII).



Scheme XIII. Formation of ions l and l' from Δ^{14}-Taraxerenes.

Further fragmentation of l produces fragment l-CH_3, or l-CH_2 OAc depending on the substituent on C-17 to give l', m/z 189.

1.6.12 28-Nor-$\Delta^{17(18)}$-Oleanenes.

The most abundant fragment ion is given by retro-Diels-Alder cleavage of ring D. 28-Nor-$\Delta^{17(18)}$-oleanen-3-one and the corresponding alcohol thus exhibit their most abundant fragment ion, 0 at m/z 163 (Scheme XIV). A strong peak at

m/z 191 is also a common feature. This is assumed to involve migration of the double bond to 13(18) position with subsequent decomposition to the ion p (Scheme XIV).

1.6.13 Derivatives of Friedelane

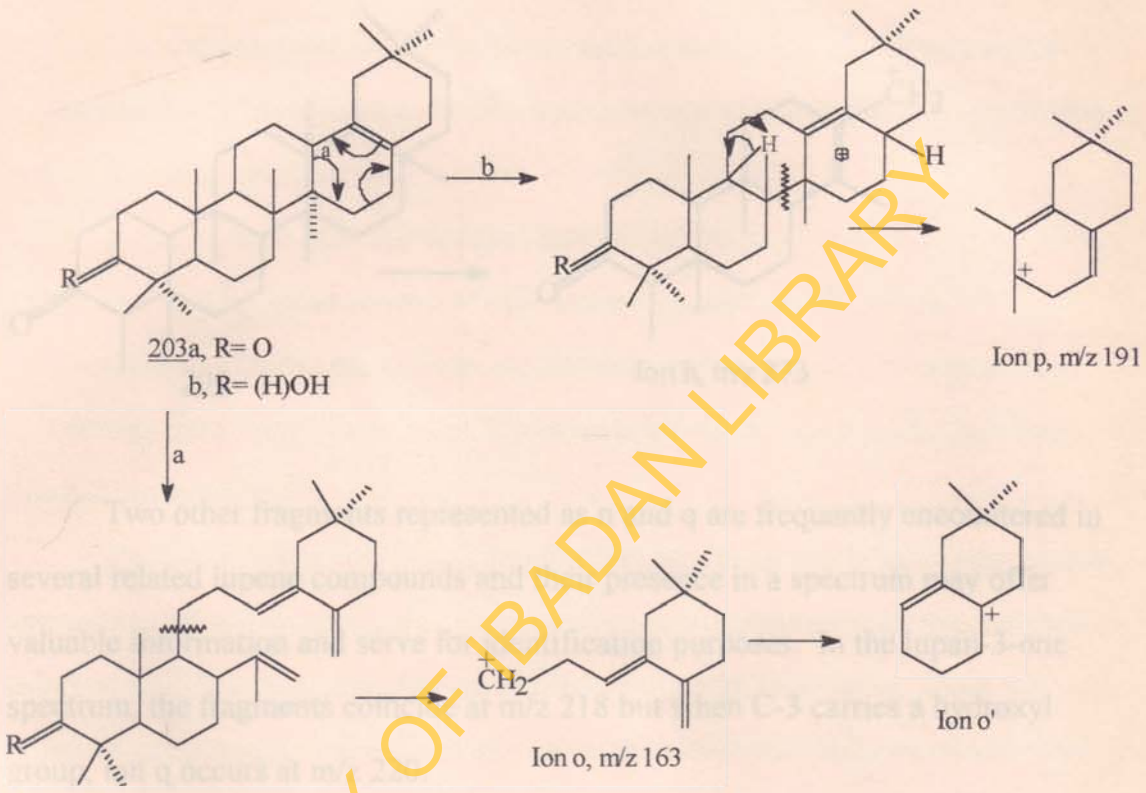
The friedelanes show very few characteristic fragmentations. A very prominent peak in the spectrum of this class of compounds is due to species h at m/z 273 for the friedelan-3-one derivative, 204.

The Δ^{18}-unsaturated analogs usually exhibit a very pronounced loss of methyl as a result of allylic activation of four quaternary methyl groups.

1.6.14. Lupane Derivatives.

This series which contains a 5-membered ring E to which an isopropyl or isopropenyl group is attached show very pronounced loss of 43 mass unit ($-C_3H_7$) in certain members but becomes minimal in highly substituted derivatives.

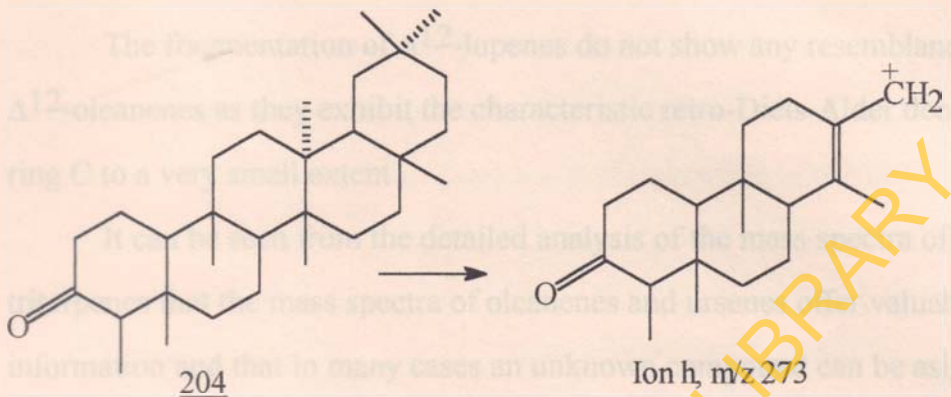
Saturated lupanes yield a species which corresponds to species g as the most abundant fragment in addition to M-15 and M-43 peaks. In lupan-3-one,



Scheme XIV

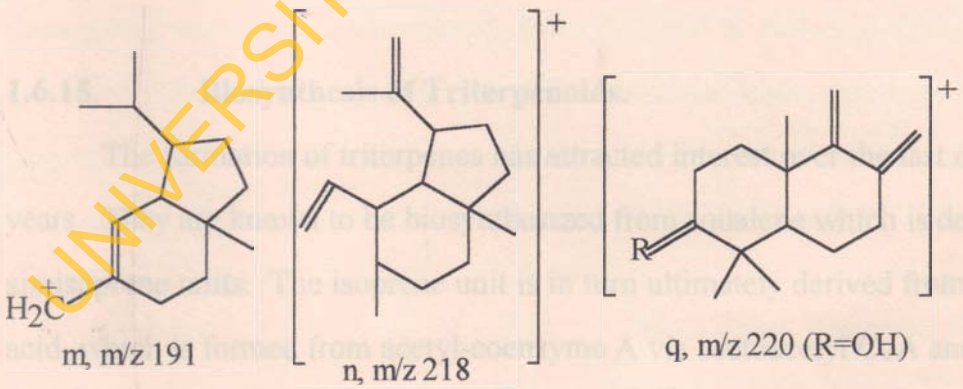
Fragmentation of $\Delta^{17(18)}$-Oleanenes to give ions p, o and o'

this fragment occurs at m/z 203. A fragment at m/z 191 is given structure m. A common characteristic feature is the presence of very pronounced molecular ion.



204 Ion h, m/z 273

Two other fragments represented as n and q are frequently encountered in several related lupene compounds and their presence in a spectrum may offer valuable information and serve for identification purposes. In the lupan-3-one spectrum, the fragments coincide at m/z 218 but when C-3 carries a hydroxyl group, ion q occurs at m/z 220.



m, m/z 191 n, m/z 218 q, m/z 220 (R=OH)

The fragmentation of Δ^{12}-lupenes do not show any resemblance to the Δ^{12}-oleanenes as they exhibit the characteristic retro-Diels-Alder decomposition of ring C to a very small extent.

It can be seen from the detailed analysis of the mass spectra of pentacyclic triterpenes that the mass spectra of oleanenes and ursenes offer valuable information and that in many cases an unknown compound can be asigned to a certain structural type. In addition the location of substituents can be narrowed down considerably[123].

The mass spectra of lupane derivatives seem to be much less characteristic and only in simplest cases are a few fragments outstanding enough to offer useful information. Therefore only the molecular weight and information about the presence of certain functional groups can be derived in the majority of members of the lupane series.

1.6.15. Biosynthesis of Triterpenoids.

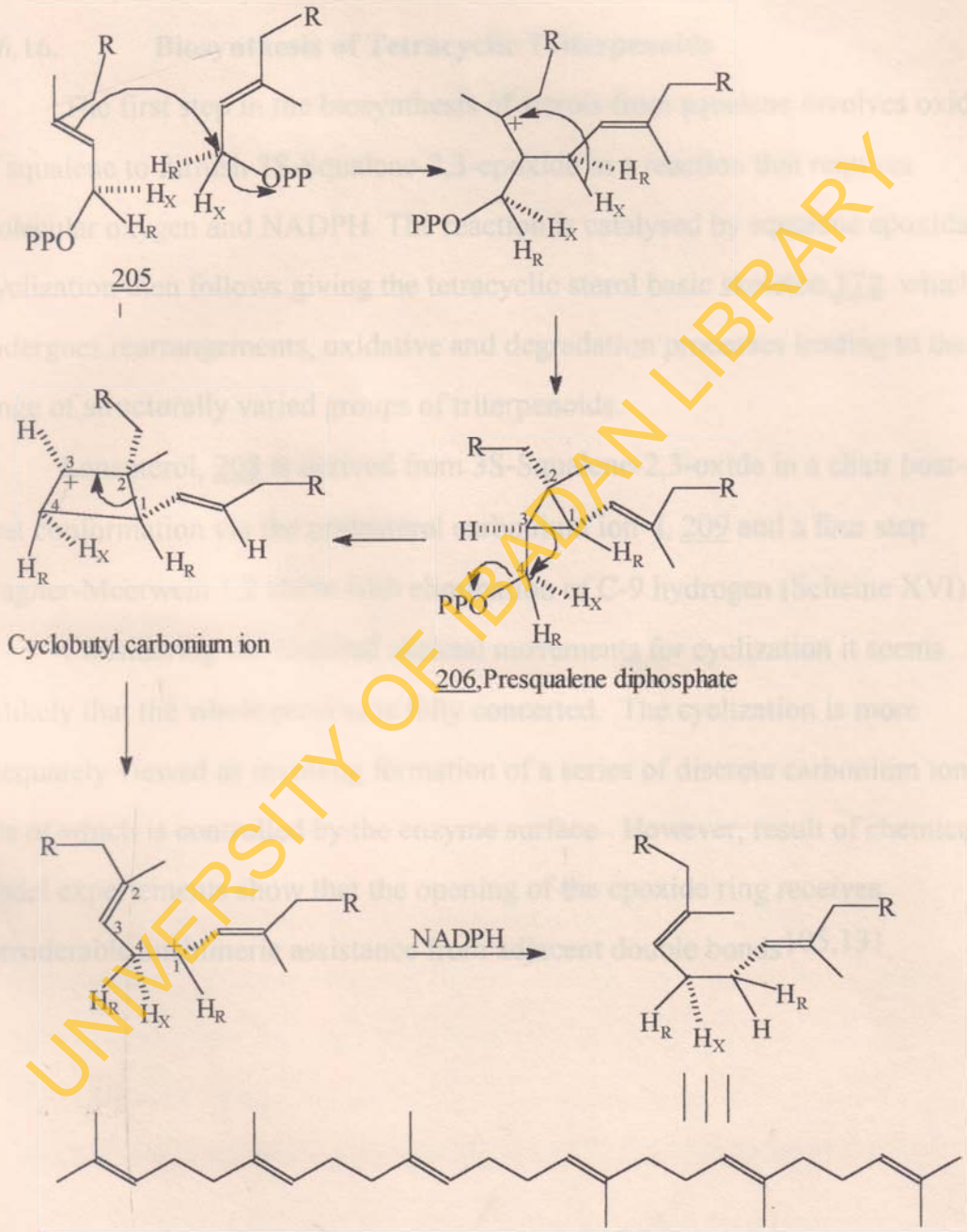
The formation of triterpenes has attracted interest over the last couple of years. They are known to be biosynthesized from squalene which is derived from six isoprene units. The isoprene unit is in turn ultimately derived from mevalonic acid, which is formed from acetyl-coenzyme A via acetoacetyl CoA and 3(S)-3-hydroxyl-3-methylglutaryl-Co A (HMG-CoA)[125].

Squalene is formed from two all-trans farnesyl diphosphate, 205 joind tail to tail. The 2,3-double bond of one farnesyl diphosphate molecule is alkylated by another farnesyl diphosphate molecule with inversion of configuration and the

stereospecific elimination of Hx gives rise to the cyclopropane moiety of the intermediate compound, presqualene, 206. In the absence of NADPH the intermediate compound accumulates but in the presence of NADPH, the cyclopropane rearranges by ring expansion and inversion of configuration at C-4, the discrete cyclobutyl carbonium ion then collapses to the linear all-trans squalene[105,126-128], 207, (Scheme XV).

The conversion of farnesyl diphosphate into squalene is catalysed by squalene synthase.

Parallel studies on the different aspects of terpene and steroid chemistry gradually focussed the interest around the C_{30} hydrocarbon, squalene, as a conceivable progenitor of the higher terpenoids. Squalene was first isolated from shark liver, *Squalus* spp, but was latter found to be ubiquitously distributed. By folding this compound in certain models, the basic triterpenoid skeleton can be constructed with the angular methyls and side chain in correct positions. There exist also the possibility that the naturally occuring all-trans squalene may isomerize at an olefinic centre at some stage of the cyclization, but surprisingly there are few skeletal variations in the triterpene series compared with the sesquiterpene and the diterpene series[129-130].



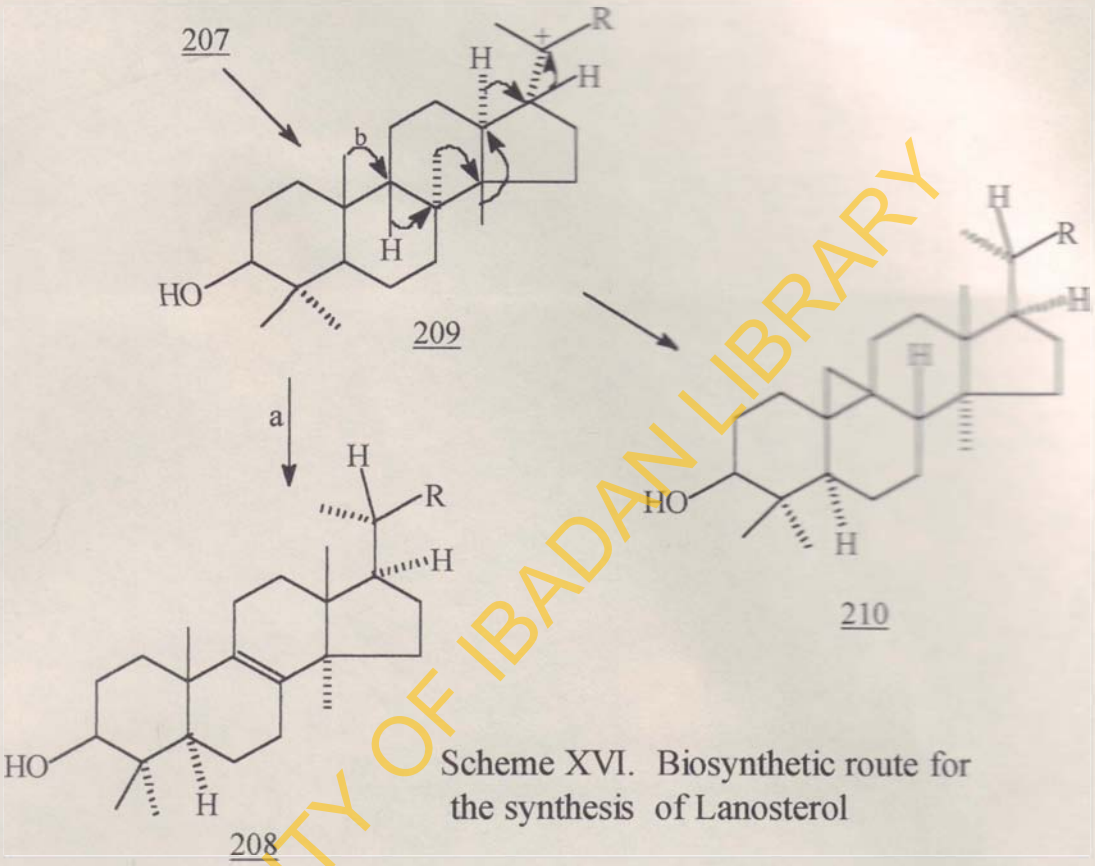
Scheme XV. Biosynthesis of squalene from all trans farnesyl diphosphate

1.6.16. Biosynthesis of Tetracyclic Triterpenoids

The first step in the biosynthesis of sterols from squalene involves oxidation of squalene to furnish 3S-Squalene-2,3-epoxide in a reaction that requires molecular oxygen and NADPH. The reaction is catalysed by squalene epoxidase. Cyclization then follows giving the tetracyclic sterol basic skeleton, 172 which then undergoes rearrangements, oxidative and degradation processes leading to the range of structurally varied groups of triterpenoids.

Lansoterol, 208 is derived from 3S-Squalene-2,3-oxide in a chair boat-chair-boat conformation via the protosterol carbonium ion I, 209 and a four step Wagner-Meerwein 1,2 shifts with elimination of C-9 hydrogen (Scheme XVI).

Considering the required skeletal movements for cyclization it seems unlikely that the whole process is fully concerted. The cyclization is more adequately viewed as involvng formation of a series of discrete carbonium ions, the fate of which is controlled by the enzyme surface. However, result of chemical model experiements show that the opening of the epoxide ring receives considerable anchimeric assistance from adjacent double bonds[105,131].

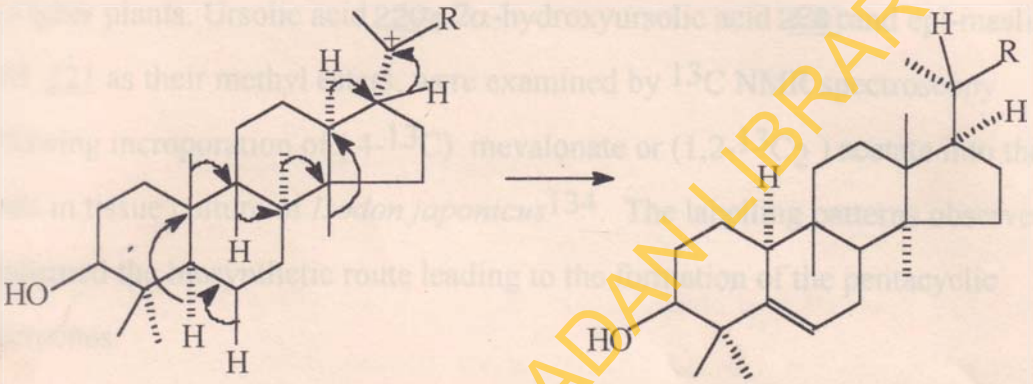


Scheme XVI. Biosynthetic route for the synthesis of Lanosterol

One further shift of C-9 Hβ→C8 Hβ and C-9 alkylation by C-19 leads to cycloartenol 210 containing a cyclopropane ring. It is generally accepted that cycloartenol is the starting triterpene for the biosynthesis of sterols in photosynthetic plants and algae while lanosterol performs the equivalent role in animals and in the few fungi that have been investigated[132-133].

Further deep-seated rearrangement of protosterol carbonium ion I leads to the cucurbitanes, 211 the toxic principles of cucurbitaceous plants, a highly

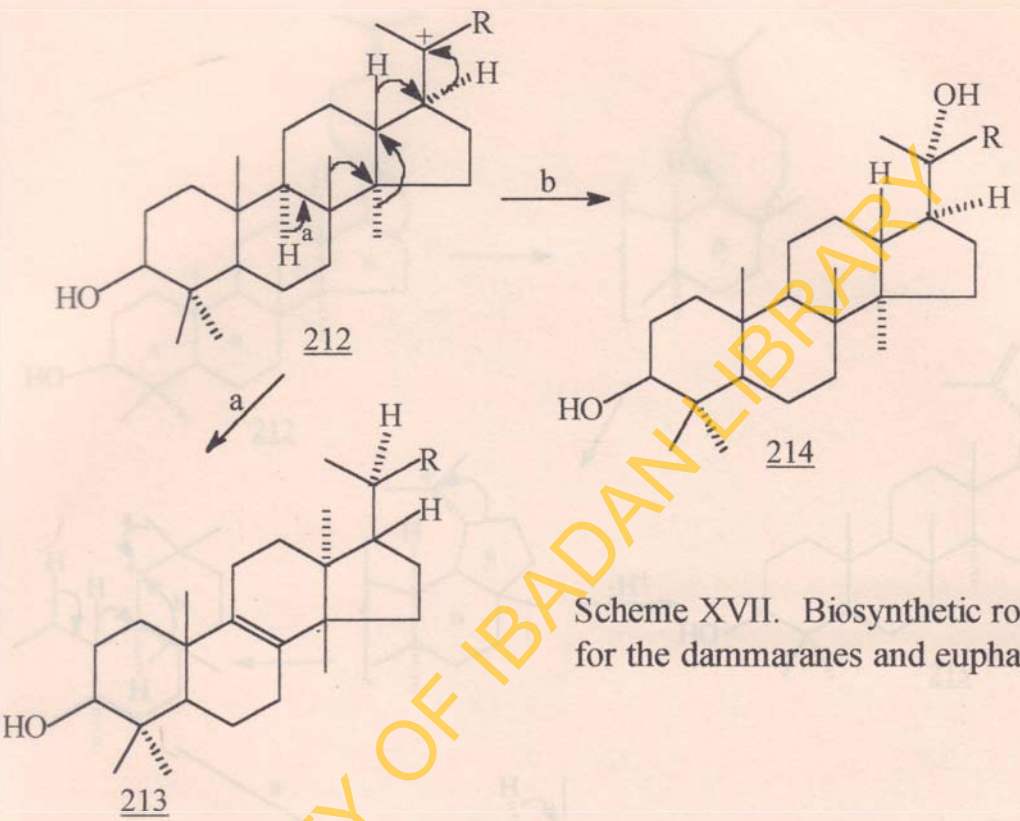
oxygenated group of tetracyclic triterpenes which have continued to attract interest because of their toxicity[134].



209 211, Cucurbitane skeleton

Studies have shown that the stereochemistry that is induced at C-20 in the lanosterol formed is determined not by the relative size of the substituents at C-19 in the analogues of squalene-2,3-epoxide but by the stereochemical disposition of these substituents about the Δ^{18}-double bond[135].

Two other tetracyclic triterpenoid groups are derived from the protosterol carbonium ion II, 212. These are the dammaranes and euphanes. The euphanes 213, differ from the lanostanes in the configuration around C-13,14 and 20, while the dammaranes 214, retain the protosterol carbonium ion II skeleton (Scheme XVII).

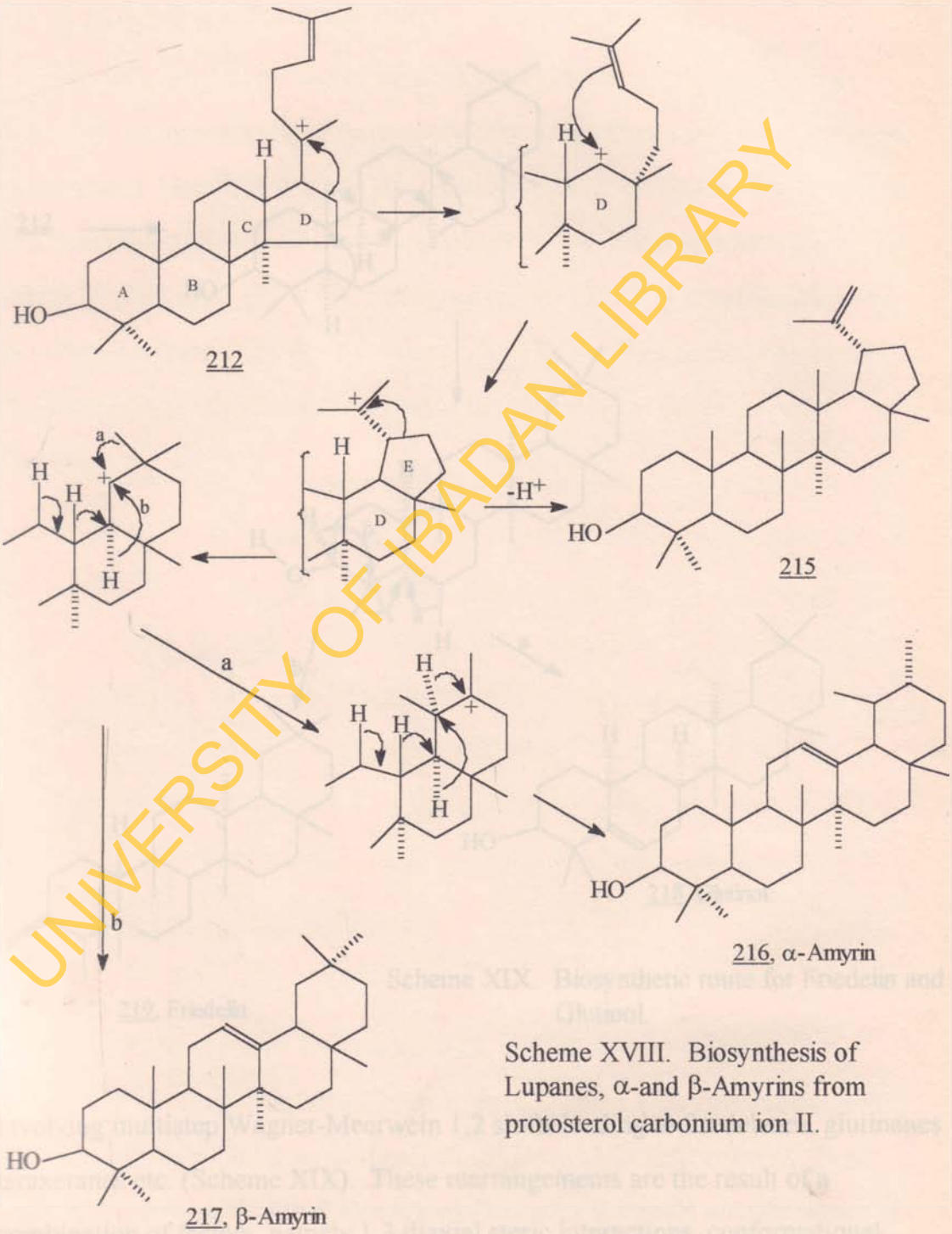


Scheme XVII. Biosynthetic route for the dammaranes and euphanes

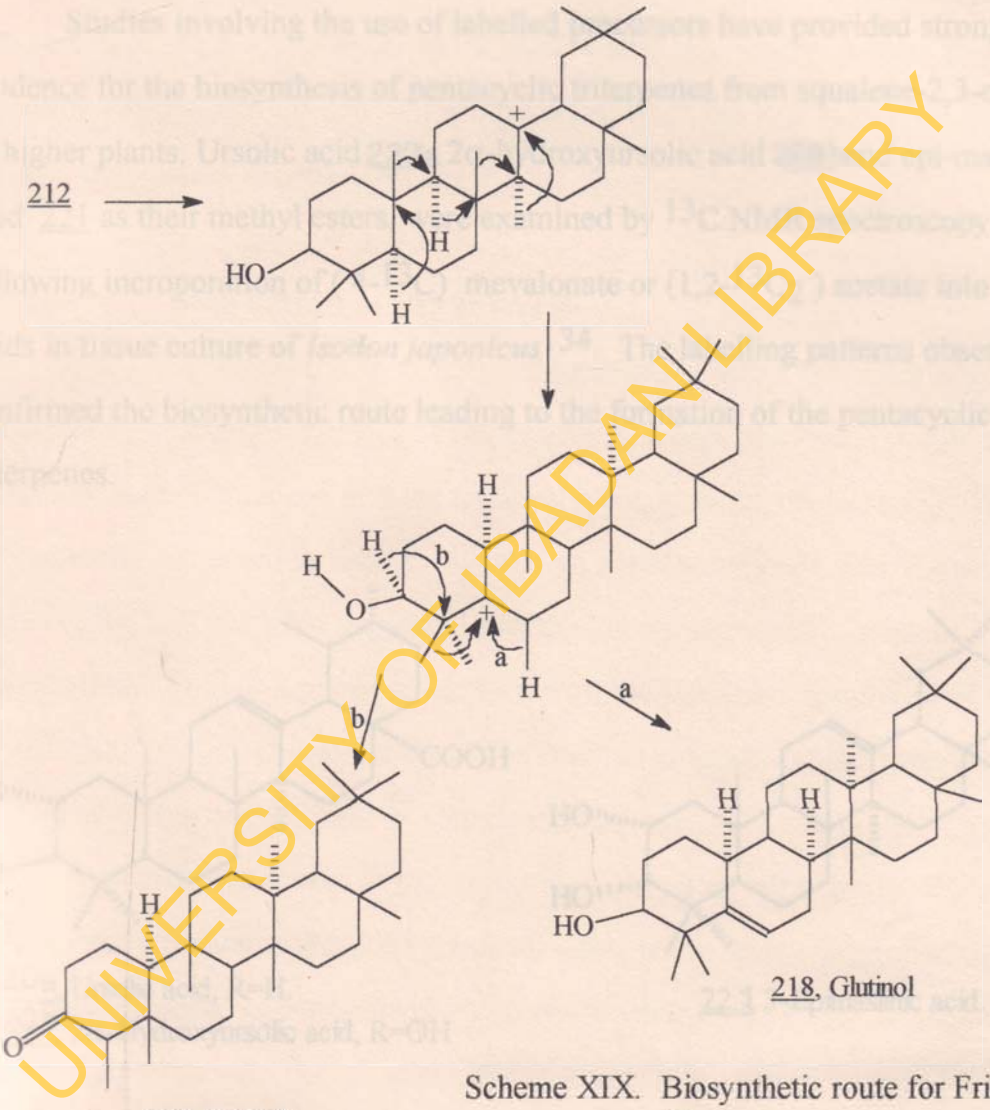
1.6.17. Biosynthesis of Pentacyclic Triterpenes.

The pentacyclic triterpenes are mostly derived from protosterol carbonium ion II by expansion of the ring D and cyclization with the side chain.

Lupeol 215, pseudo taraxasterol 216, β-amyrin 217 and their oxygenated derivatives have rearrangements within D and E rings only (Scheme XVIII). A large group of pentacyclic triterpenes are formed by backbone rearrangement



Scheme XVIII. Biosynthesis of Lupanes, α-and β-Amyrins from protosterol carbonium ion II.

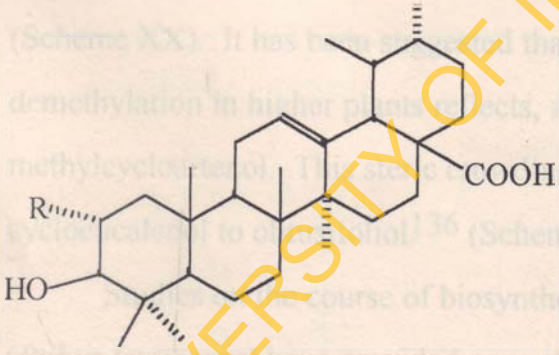


Scheme XIX. Biosynthetic route for Friedelin and Glutinol.

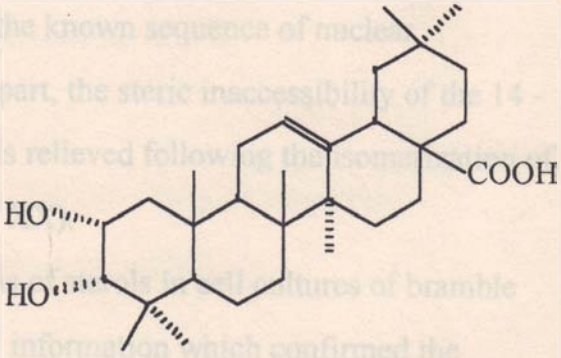
involving multistep Wagner-Meerwein 1,2 shifts leading to friedelanes, glutinanes taraxeranes etc. (Scheme XIX). These rearrangements are the result of a combination of factors, namely 1,3 diaxial steric interactions, conformational

factors and stereoelectronic effects[133].

Studies involving the use of labelled precursors have provided strong evidence for the biosynthesis of pentacyclic triterpenes from squalene-2,3-epoxide in higher plants. Ursolic acid 220a, 2α-hydroxyursolic acid 220b and epi-maslinic acid 221 as their methyl esters, were examined by ^{13}C NMR spectroscopy following incroporation of (4-^{13}C) mevalonate or (1,2-$^{13}C_2$) acetate into these acids in tissue culture of *Isodon japonicus*[134]. The labelling patterns observed confirmed the biosynthetic route leading to the formation of the pentacyclic triterpenes.



220a, Ursolic acid, R=H.
220b, 2α-Hydroxyursolic acid, R=OH



221, 3-Epimaslinic acid.

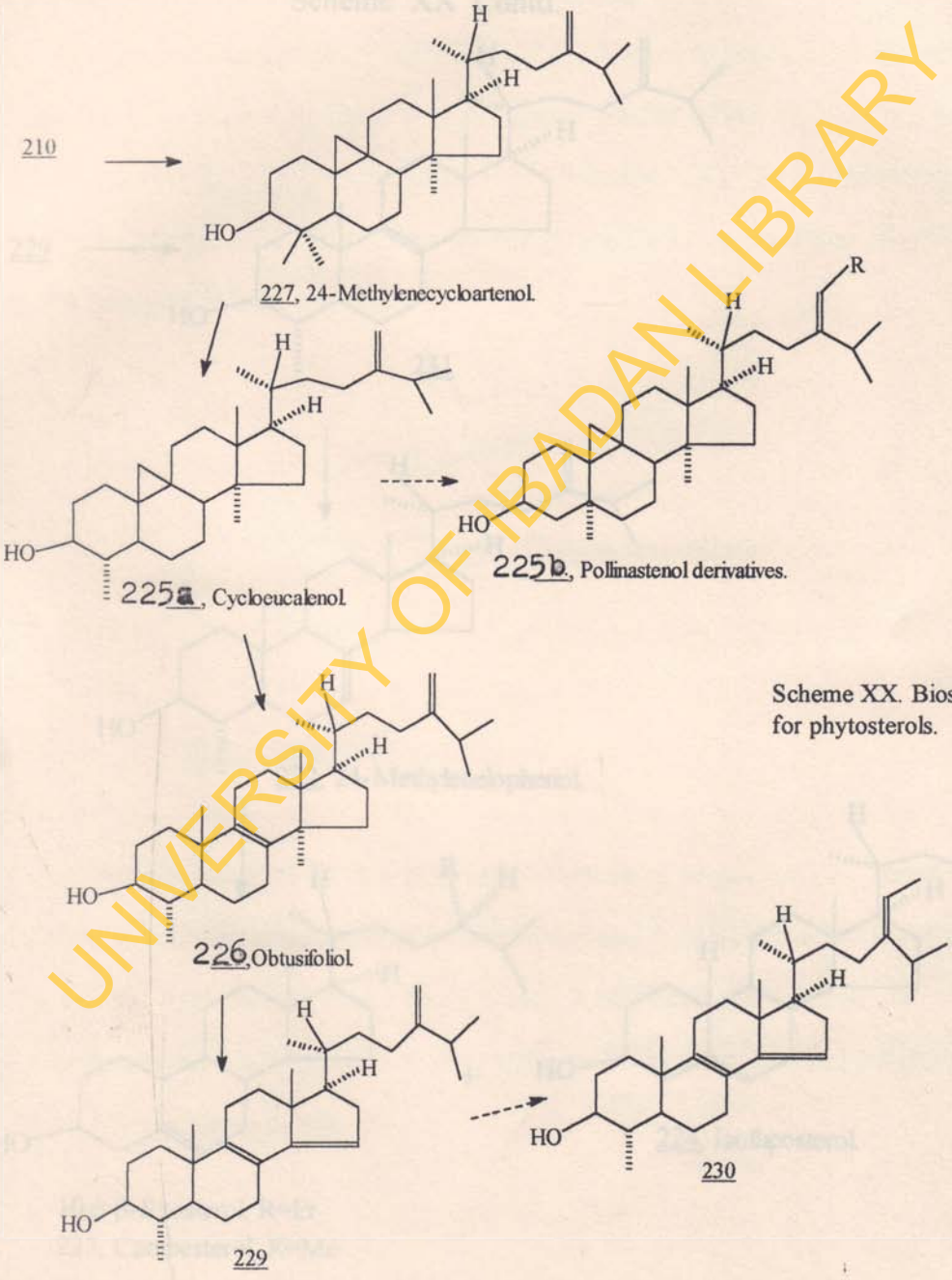
The labelling patterns for carbons 4,23, and 24 in epi-maslinic acid is consistent with its formation from 3S-Squalene-2,3-oxide, with epimerization of the 3β-hydroxyl function (at some stage) which must be formed initially. This evidence precludes the idea that 3α-hydroxyl function is formed via cyclization of

3R-Squalene-2,3-epoxide[133]. Labelling experiments have also given a clue as to the particular stage in the biosynthetic route when 12β proton is lost. The labelling pattern showed that 12β proton is lost from the intermediate ion for Urs-12-ene and Olean-12-enederivatives .

1.6.18. Biosynthesis of Phytosterols

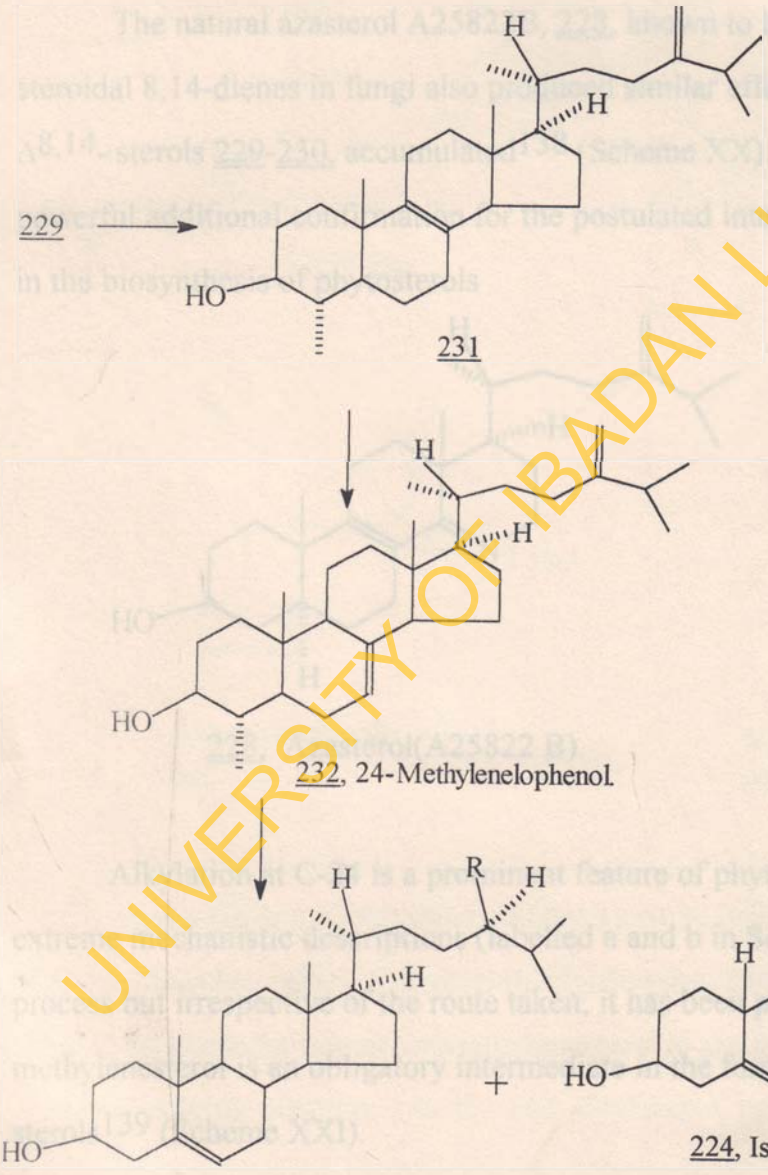
The biosynthetic transformation of squalene to the Δ^5-steroids such as β-sitosterol 109, stigmasterol 222, campesterol 223 and isofucosterol, 224, involve the conversion of squalene into the tetracyclic triterpenoid, cycloartenol, which is subsequently subjected to further degradative transformation to give the sterols (Scheme XX). It has been suggested that the known sequence of nuclear demethylation in higher plants reflects, in part, the steric inaccessibility of the 14 -methylcycloartenol. This steric crowding is relieved following the isomerization of cycloeucalenol to obtusifoliol[136] (Scheme XX).

Studies on the course of biosynthesis of sterols in cell cultures of bramble (*Rubus fruticosus*) have provided powerful information which confirmed the postulated sequence of biosynthetic route for Δ^5-sterols. The major steroids of untreated cultures were the Δ^5-sterol, β-sitosterol, 70%, campesterol, 15% and isofucosterol, 12% (Scheme XX). After four weeks of growth in the presence of the systemic fungicide, tridemorph, bramble cells no longer produced Δ^5-steroids, owing to specific blocking of the key enzyme that isomerizes cycloeucalenol, 225a to furnish the Δ^8-sterol, obtusifoliol, 226. As a result cyclo-sterols such as 24-



Scheme XX. Biosynthetic route for phytosterols.

Scheme XX Contd.

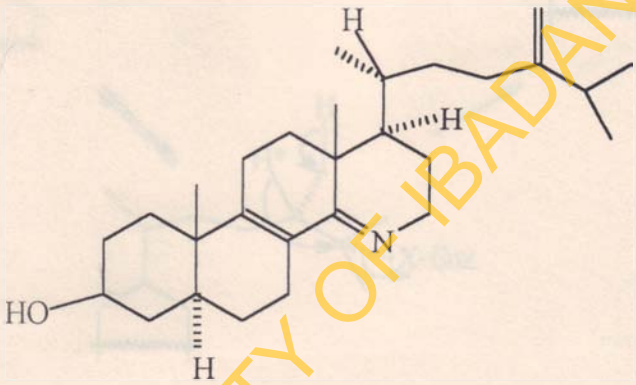


10 & β-Sitosterol, R=Et

223, Campesterol, R=Me

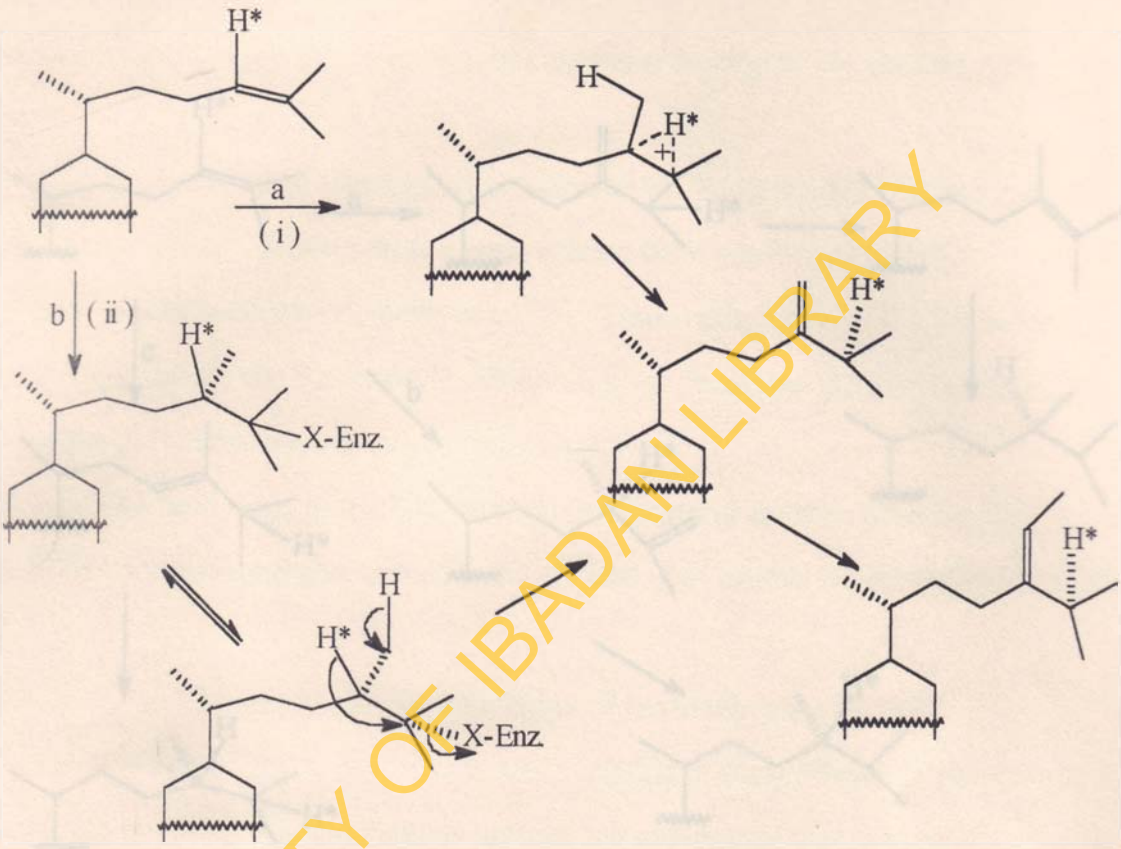
methylene-cycloartanol, 227, cycloeucalenol 225a and pollinastanol derivatives, 225b constituted 80% of the sterols that were present[137].

The natural azasterol A25822B, 228, known to block nuclear reduction of steroidal 8,14-dienes in fungi also produced similar effect on bramble cells as $\Delta^{8,14}$- sterols 229-230, accumulated[138] (Scheme XX). This observation provides powerful additional confirmation for the postulated intermediacy of $\Delta^{8,14}$-sterols in the biosynthesis of phytosterols



228, Azasterol(A25822 B).

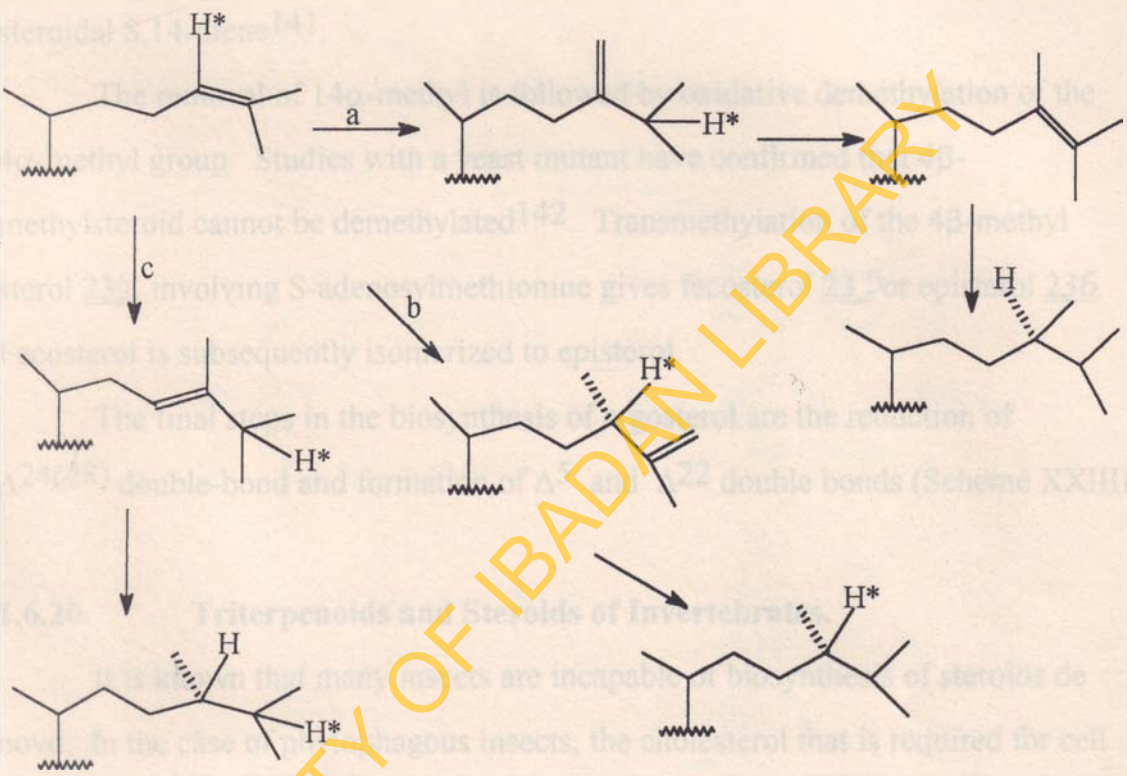
Alkylation at C-24 is a prominent feature of phytosterols. There are two extreme mechanistic descriptions (labelled a and b in Scheme XXI) for alkylation process but irrespective of the route taken, it has been proved that 24-methylenesterol is an obligatory intermediate in the formation of 24-ethyl-sterols[139] (Scheme XXI).



(i) S-adenosylmethionine(SAM).
(ii) SAM. Enz.-$X^{\ominus}$

Scheme XXI. Alkylation of phytosterol at C-24.

Phytosterols also fall into two broad groups with respect to the configuration at C-24. The 24α-series, β-sitosterol, stigmasterol, campesterol are suggested to be formed by isomerization of 24-methylenesterol to the $\Delta^{24(25)}$- isomer followed by stereospecific reduction to give the 24α-alkyl sterol, route a (Scheme XXII). Two possibilities have been discussed for the biosynthesis of 24 β-methyl sterols, namely reduction of a 24β-methyl-Δ^{25}-steroid (route b) or stereospecific reduction of a 24 -methyl-Δ^{23}-steroid[140](route C).



Scheme XXII. Biosynthesis of 24-α and β-alkyl sterols.

1.6.19. Biosynthesis of sterols in Fungi.

Demethylation of lanosterol is the first major step in the biosynthesis of ergosterol, 233 in yeast.

Several commercial fungicides owe their antifungal activity, at least in part, to interference with the biosynthesis of ergosterol by blocking the 14α-

demethylation of lanosterol or by blocking the reduction of the intermediate steroidal 8,14-diene[141].

The removal of 14α-methyl is followed by oxidative demethylation of the 4α-methyl group. Studies with a yeast mutant have confirmed that 4β-methylsteroid cannot be demethylated[142]. Transmethylation of the 4β-methyl sterol 234, involving S-adenosylmethionine gives fecosterol 235 or episterol 236. Fecosterol is subsequently isomerized to episterol.

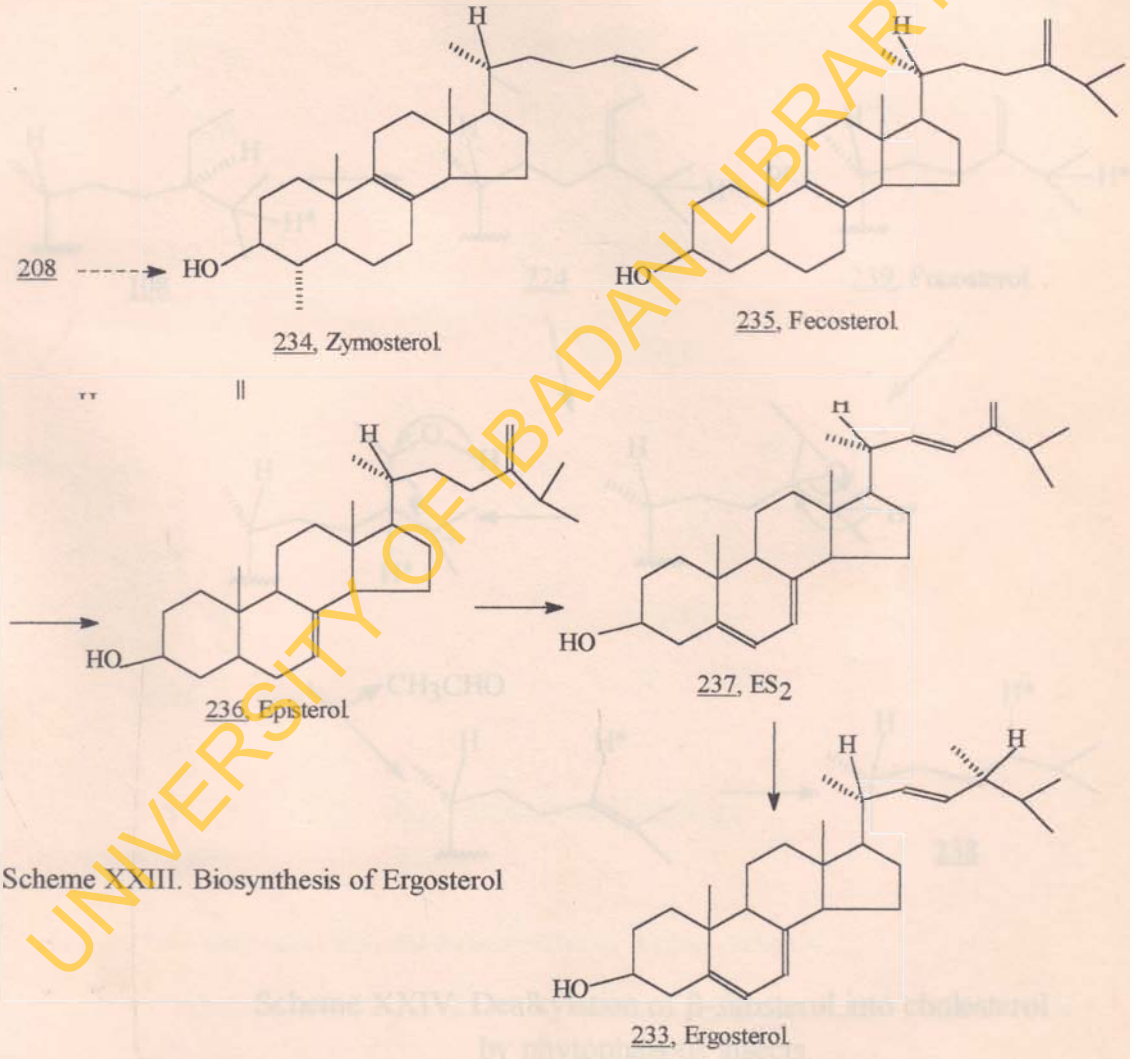
The final steps in the biosynthesis of ergosterol are the reduction of $\Delta^{24(28)}$ double-bond and formation of Δ^{5} and Δ^{22} double bonds (Scheme XXIII).

1.6.20. Triterpenoids and Steroids of Invertebrates.

It is known that many insects are incapable of biosynthesis of steroids de novo. In the case of phytophagous insects, the cholesterol that is required for cell membrane functions and for the synthesis of hormones is acquired by dealkylation (at C-24) of dietary phytosterols. The degradative route is illustrated in Scheme XXIV for the conversion of β-sitosterol into cholesterol, 238.

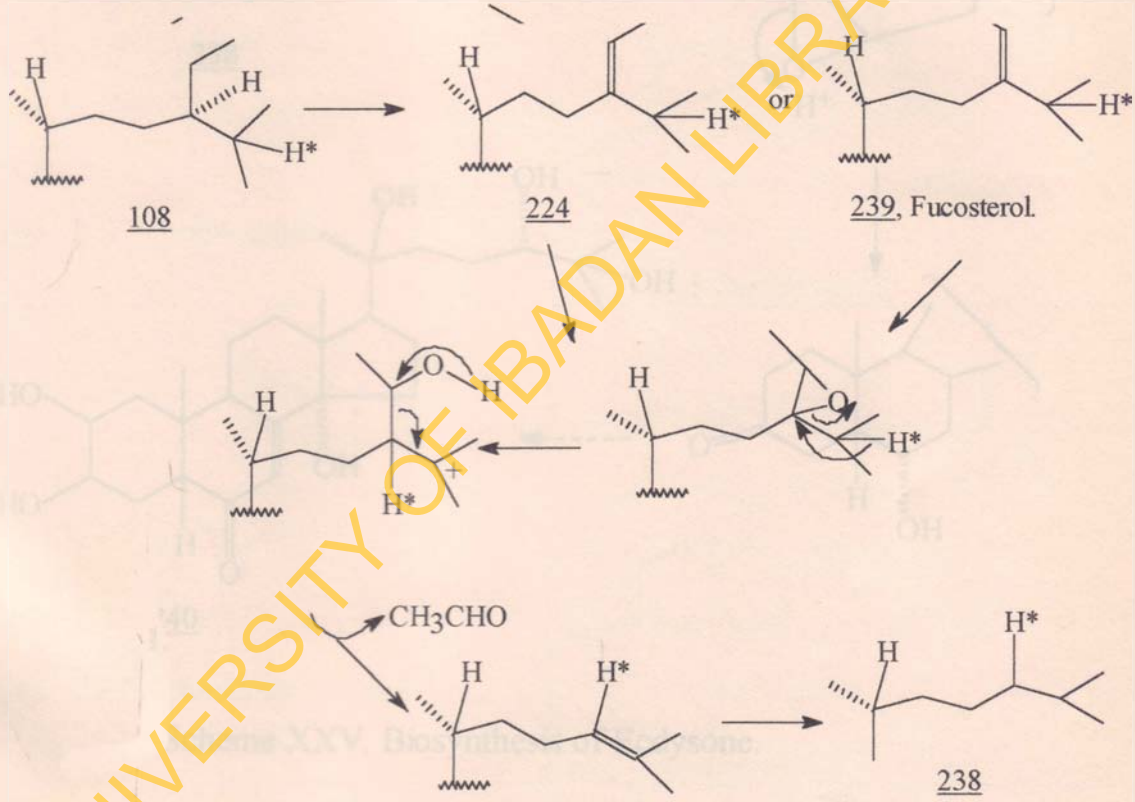
The intermediates in Scheme XXIV - isofucosterol, 224 and fucosterol 239 could be formed (in principle) by hydroxylation of β-sitosterol at C-24 or C-28 followed by dehydration. However, observations from experiments[144] suggested that the conversion of β-sitosterol into fucosterol and isofucosterol involves a dehydrogenation reaction rather than hydroxylation-dehydration sequence. The cholesterol is subsequently metabolised into the ecdysteroids by a series of

oxidative/reductive processes as illustrated by the biosynthesis of 20-hydroxyecdysone, 240 in Scheme XXV.



Scheme XXIII. Biosynthesis of Ergosterol

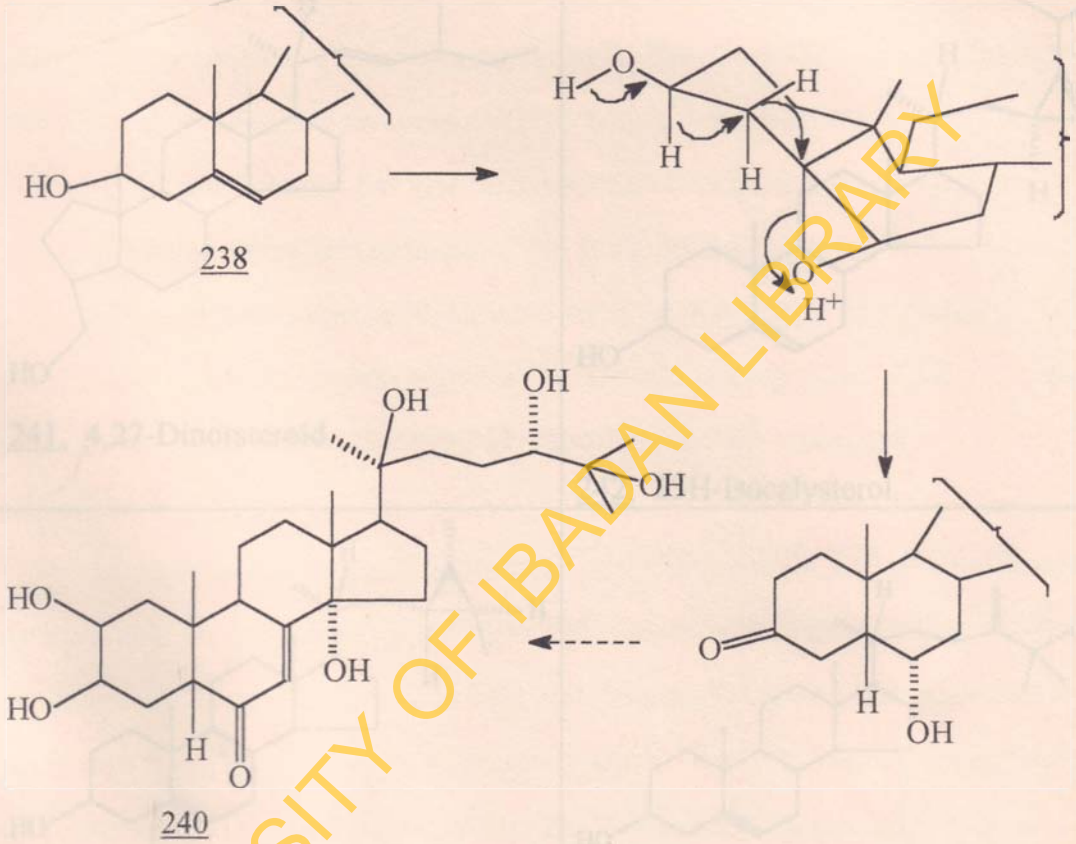
Marine invertebrates have made significant contribution to the steroidal compounds. They have continued to provided novel and biosynthetically intriguing steroids[145].



108 224 239, Fucosterol. 238

Scheme XXIV. Dealkylation of β-sitosterol into cholesterol by phytophagous insects.

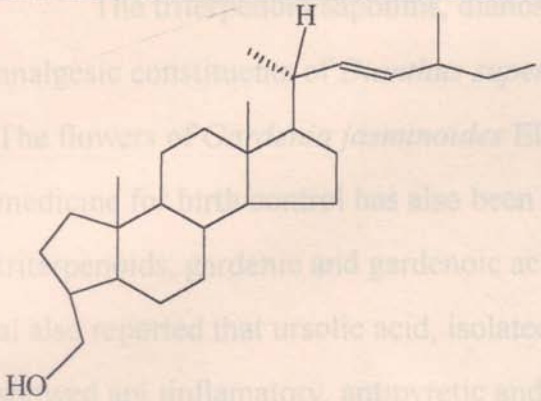
Some of the recent isolations include 4,27-dinor-steroid[146], 241, the cyclopropanes[147], 23H- isocalysterol 242 and 23, 24-dihydrocalysterol 243, mutasterol 244 and pulchrasterol[148] 245.



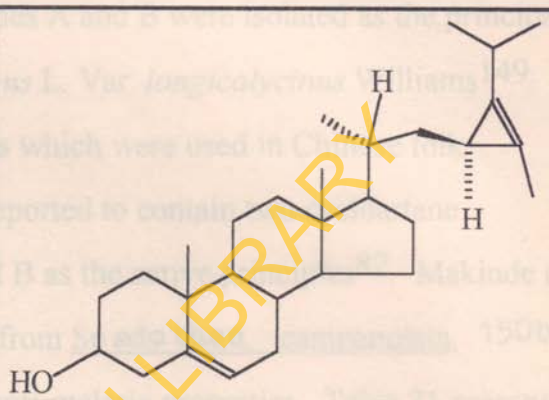
Scheme XXV. Biosynthesis of Ecdysone.

1.6.21. Pharmacological properties of Triterpenoids.

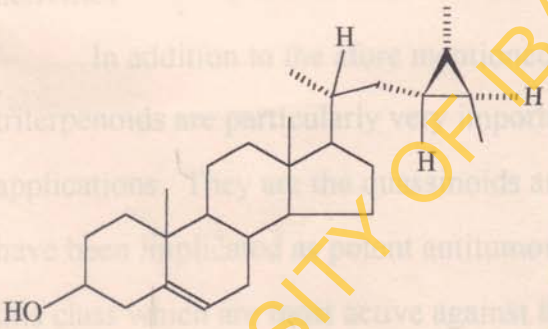
The medicinal importance of triterpenoids for the most part is obscure as they do not show any impressive therapeutic properties like the sesquiterpenes and the diterpenes. However, there are a number of reports in the literature on the medicinal uses of some plants whose active principles have been identified as triterpenoids.



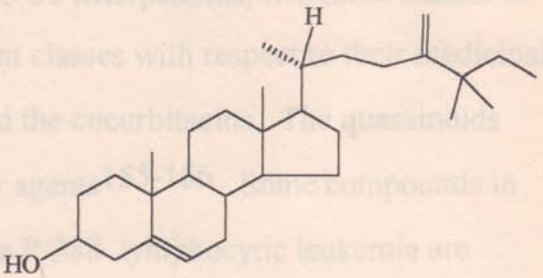
241, 4,27-Dinorsteroid.



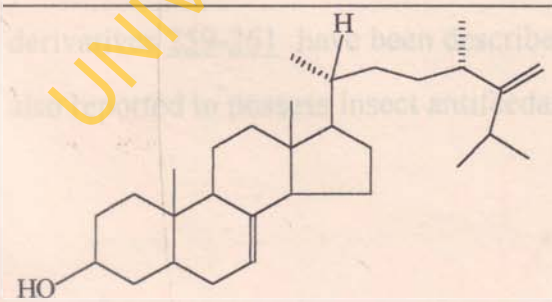
242, 23H-Isocalysterol.



243, 23,24-Dihydrocalysterol.



244, Mutasterol.



245, Pulchrasterol.

The triterpenoid saponins, dianosides A and B were isolated as the principal analgesic constituents of *Dianthus superbus* L. Var. *longicalycinus* Williams[149]. The flowers of *Gardenia jasminoides* Ellis which were used in Chinese folk medicine for birth control has also been reported to contain two cycloartane triterpenoids, gardenic and gardenoic acid B as the active principles[82]. Makinde et al also reported that ursolic acid, isolated from Spondo thea campanulata, [150b-c] showed anti-inflamatory, antipyretic and anti-malaria properties. Table 21 presents some C-30 triterpenoids, 104, 246-252, reported for their biological activities[150-154].

In addition to the afore mentioned C-30 triterpenoids, two other classes of triterpenoids are particularly very important classes with respect to their medicinal applications. They are the quassinoids and the cucurbitacins. The quassinoids have been implicated as potent antitumour agents[155-156]. Some compounds in this class which are most active against the P-388 lymphocytic leukemia are represented by compounds 253-258 (Table 22). The cucurbitacins have also been found to show antitumour properties. A number of new tumour inhibitory derivatives 259-261 have been described[157-158] (Table 23). Some derivatives are also reported to possess insect antifeedant activity.

Table 21 - Some biologically active triterpenoids

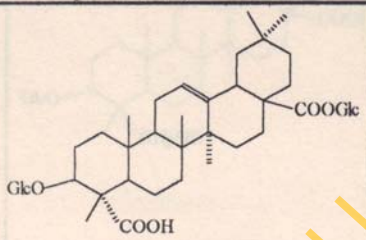
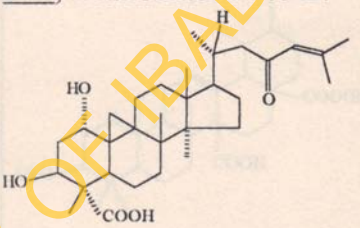
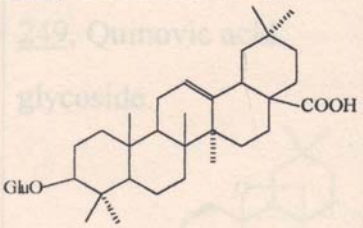
S/No.	Compound(s).	Medicinal properties.
1.	 246, Dianosides A & B.	Analgesic[149].
2.	 104, Gardenoic acid.	Antifertility[82].
3.	 247, Oleanolic acid-3-β-glycoside.	Molluscidal[150].

Table 21 Contd.

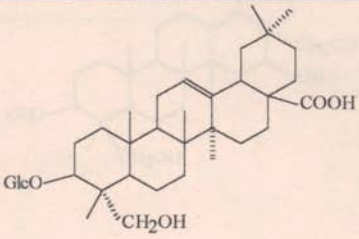
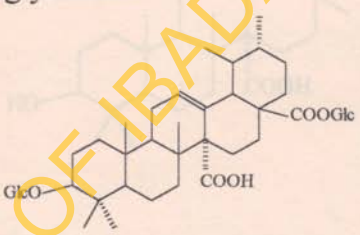
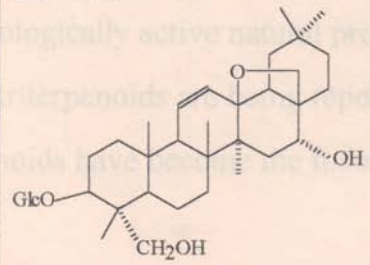
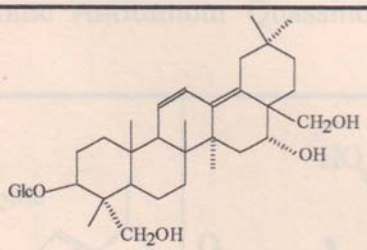
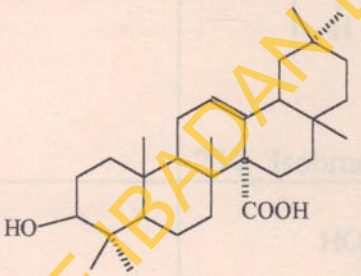
S/No.	Compound(s).	Medicinal properties.
4.	 248, Hederagenin-3-β-glycoside.	Hypotensive[151].
5.	 249, Quinovic acid glycoside.	Cytotoxic[152].
6.	 250, Prosaikogenin G.	Anti-inflammatory and Anti-hepatotoxic[153].

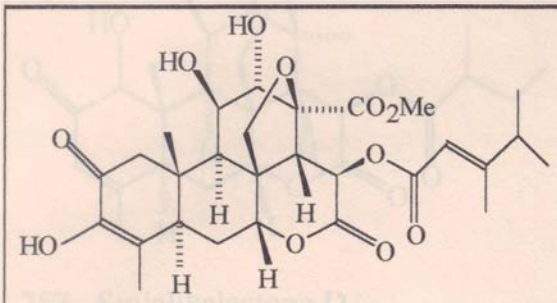
Table 21 Contd.

7.	 251, Saikosaponin b2.  252, - Peltoboykinolic acid.	Cytotoxic[154].

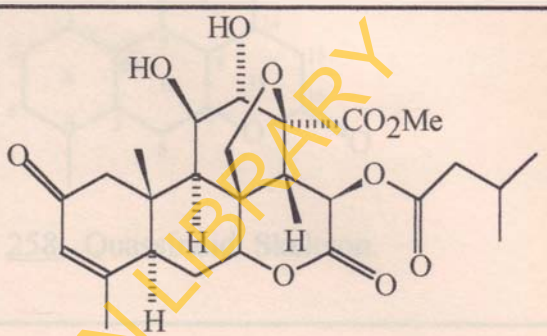
As the search for biologically active natural products continues, an increasing number of new triterpenoids are being reported. It is particularly noteworthy that the quassinoids have become the focus of research interest in recent years.

Although the triterpenoids are not as popular as the alkaloids or the sesquiterpenoids in terms of biological activities, they have certainly offered a new direction and hope in the search for biologically active natural products for the treatment of cancer in recent times.

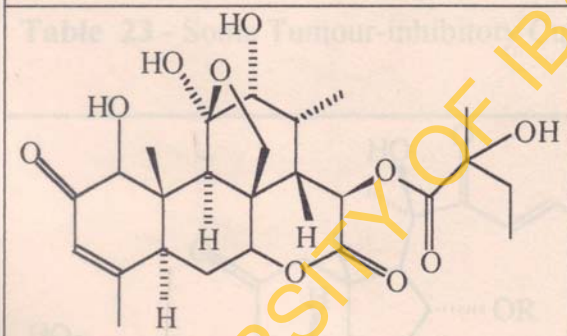
Table 22 - Structures of some Antitumour Quassinoids.



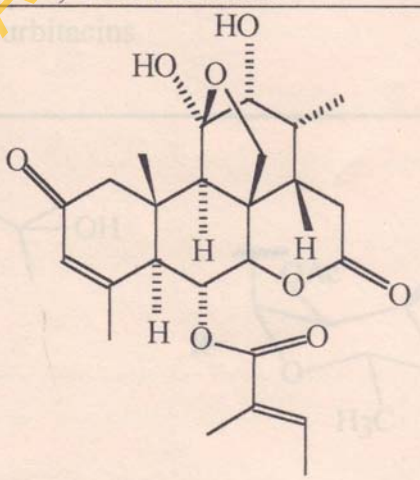
253, Bruceantin.



254, Isobruceine A.

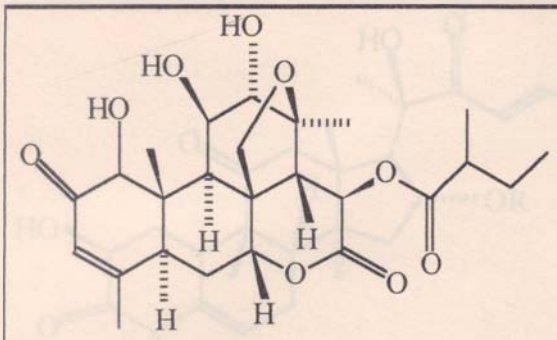


255, Glaucarubinone.

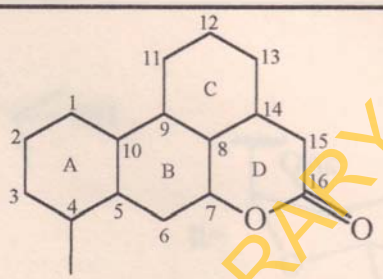


256, 6-α-Tigloyloxychaparcinone.

Table 22 Contd.

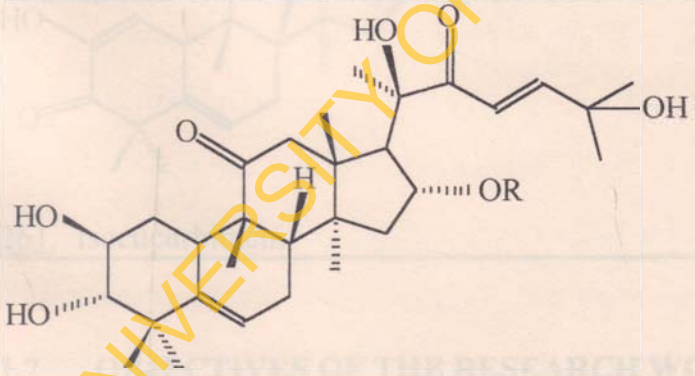


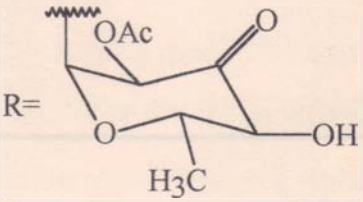
257, Sinialikalactone D.



258, Quassinoid Skeleton.

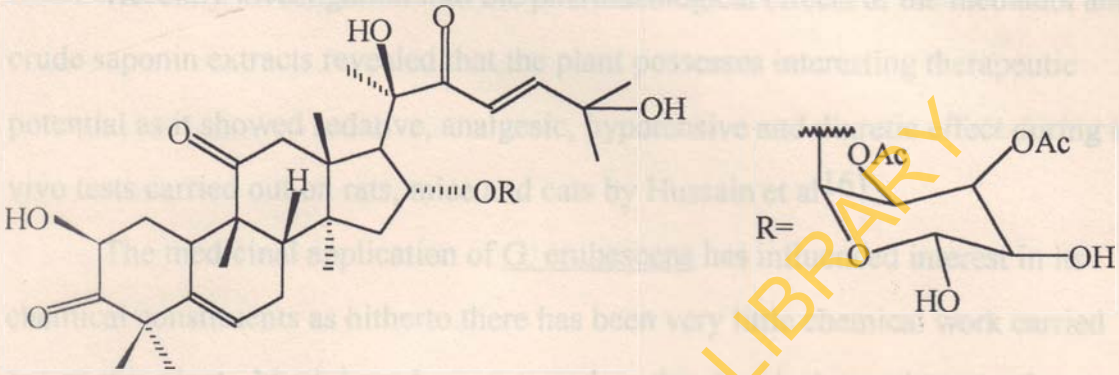
Table 23 - Some Tumour-inhibitory Cucurbitacins.



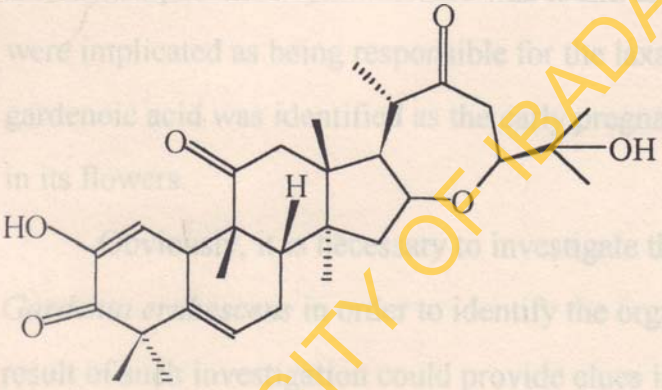


259, Datiscoside B.

Table 23 Contd.



260. Datiscoside C.



261. Isocucurbitacin.

1.7 OBJECTIVES OF THE RESEARCH WORK.

Gardenia erubescens Stapf. and Hutch. is a shrub found growing throughout the savannah region in Nigeria. It is also found in Uganda and Sudan. The plant is used by the natives in Northern Nigeria as aphrodisiacs and as a traditional remedy for a variety of other ailments particularly for the treatment of gonorrhea, abdominal disorders, loss of appetite and insomnia. Its fruits are edible and were reported to have been used to give strength on longer journeys[159-160].

Recently investigation into the pharmacological effects of the methanol and crude saponin extracts revealed that the plant possesses interesting therapeutic potential as it showed sedative, analgesic, hypotensive and diuretic effect during in vivo tests carried out on rats, mice and cats by Hussain et al[161].

The medicinal application of G. erubescens has influenced interest in its chemical constituents as hitherto there has been very little chemical work carried out on this plant. Much have been reported on the chemical constituents of some *Gardenia* species. *G. jasminoides* was found to be rich in iridoid glycosides which were implicated as being responsible for the laxative properties of its fruits while gardenoic acid was identified as the early pregnancy terminating principle present in its flowers.

Obviously, it is necessary to investigate the chemical constituents of *Gardenia erubescens* in order to identify the organic metabolites present and the result of such investigation could provide clues in rationalizing the medicinal uses.

It is along this perspective of providing scientific evidence for the use of *G.* erubescens as a local medicinal plant, that the main objective of this research work is based. The trust of the work is on isolation and characterization of secondary plant products from *G. erubescens* with particular interest on the saponin constituents as a means of providing scientific insight into its pharmacological properties.

The work is also aimed at identification of novel structures as this constitutes a significant contribution to the arsenal of natural products and also to scientific knowledge in general.

As this investigation was partly motivated by the absence of detailed documentation on the chemical constituents of *G. erubescens*, one of the objectives of this work is also to achieve a detailed chemical analysis of the extracts of stems of *G. erubescens* Stapf. and Hutch.

It was intended that hereafter the isolated constituents would be individually subjected to bioassay experiments.

CHAPTER TWO

RESULTS AND DISCUSSION.

2.1 Extraction and Preliminary Examination of Extracts.

The investigation into the chemical constituents of *Gardenia erubescens* was stimulated by the pharmacological activities reported on the crude extracts of the roots and stembark of the plant[161].

The whole stem of *G erubescens* was used in this work. The plant material was collected in Jos, in September 1992 and identified by the herbarium staff of the School of Forestry Jos. A voucher specimen has been deposited in the School of Forestry, Jos.

The plant material was air-dried and pulverized before extraction was carried out. Each extraction was sequentially performed with petroleum ether for 24h in a Soxhlet extractor and methanol for 24h or by soaking in 60% aqueous methanol for 72h after extraction with petroleum ether for 24h. A set of extraction was also carried out sequentially with petroleum ether for 24h, ethylacetate for 24h and finally with methanol for 24h.

Concentration of the petroleum ether extract gave a dark greenish-yellow gum. The yield of extracts ranged from 5-8g for every 2-3kg of plant material extracted. The methanol extract gave a dark brown gum on concentration and the yield was on the average 300g for 3kg of plant material. The ethylacetate extract gave a thick dark-green oil (~2.5g). The partitioning of the methanol soluble

fraction of methanol extract between water and n-butanol and then between water and ethylacetate gave n-butanol soluble and ethylacetate soluble extracts respectively. 100g of the methanol soluble fraction afforded 15g of n-butanol soluble fraction and about 1g of ethylacetate soluble fraction.

The various extracts, that is the petroleum ether, ethylacetate, methanol, n-butanol soluble and ethylacetate soluble extracts were subjected to phytochemical tests for the presence of flavonoids, steroids, alkaloids anthraquinones, saponins and tannins. The result of the phytochemical screening is presented in Table 24.

Table 24 - Result of Phytochemical Tests on Extracts of Stems of *G. erubescens*.

Class of Compound.	EXTRACTS.				
	Petroleum ether.	Ethyl-acetate.	Methanol.	n-Butanol soluble	EtOAc soluble.
Flavonoid.	+	-	-	-	-
Steriod.	++	+	+	+	-
Alkaloid.	-	-	-	-	-
Anthra-quinone.	-	-			
Saponins.		-	++	+	-
Tannins.		-	++	-	-

- Negative, + Positive, ++ Srongly positive.

Table 24 showed the presence of flavonoids, steroids, saponins and tannins in the extracts of the stems of *G. erubescens*. The presence of flavonoid compounds only in the petroleum ether extract suggested that relatively less polar flavonoid compounds are present and these are commonly the methyl ether derivatives of the hydroxyl group s on A and B rings of the flavonoids. Alkaloids and anthraquinones which are very popular among the members of Rubiaceae plants were also notably absent. This is however in consonance with other *Gardenia* plants as there has been no report of the presence of alkaloids and anthraquinones in any *Gardenia* plant. The result of the test for steroids and saponins showed a substancial amount of these compounds to be present. The petroleum ether extract was strongly positive for the presence of steroidal nucleus while the methanol and n-butanol soluble extracts gave positive reaction for saponins. Tannins were present in the polar extracts but much higher quantity was observed to be present in the crude methanol extract while as expected, the petroleum ether extract gave a negative response. The overall picture was that steroids and saponins constituted the major components of *Gardenia erubescens*.

In continuation of the search for the secondary plant products of *G. erubescens*, isolation, purification and characterization processes were performed on the petroleum ether and methanol extracts.

The petroleum ether extract was subjected to tlc analysis on silica gel plates in various solvent systems and the chromatoplate were sprayed with detection reagents for alkaloids, steroids and flavonoids. The results obtained by spraying the chromatoplates with detection reagents complement the earlier observations

from phytochemical tests carried out on dilute solutions of the crude extract. The presence of flavonoids and steroids were visibly corroborated. Flavonoid tlc components appeared at R_f values - 0.72, 0.49 and 0.36 in EtOAc/Hexane, 1:3.

The result of the phytochemical analysis carried out on both crude methanol and n-butanol soluble extracts informed the decision to limit the tlc analysis to n-butanol soluble fraction particularly as the crude methanol extract contained a lot of brown pigment. The sprayed chromatoplates confirmed the presence of triterpenoids while other classes of compounds were not implicated. It was observed that 5-6 components located at R_f values 0.4-0.2 in the solvent system, chloroform: methanol: water, 65:45:12, appeared as major triterpenoid components on the chromatoplates. The results of the tlc analyses on the petroleum and methanol extracts provided useful data which served as guide in the isolation and purification of the components of the extracts of *Gardenia erubescens*.

2.2 Isolation and Purification of Components from the extracts of *G. erubescens*.

Column chromatrography and preparative tlc served as a means of separation and purification of the components from the petroleum ether, ethylacetate and n-butanol soluble extracts of *G. erubescens*.

Column chromatographic separation of the petroleum ether extract was achieved on a column of silica gel G. (230-400 mesh) eluted with EtOAc/Hexane mixtures and further purification of the isolated compounds where necessary was accomplished by preparative tlc. Altogether, eight components were isolated and

identified from the petroleum ether extract. Six of the components were obtained as single pure compounds while the seventh fraction was composed of two isomeric compounds. The purity of each of the compounds was confirmed by tlc in one or two solvent systems. These compounds were labelled as GSH2, GSH 14, GSH 24, GSH 26, GSH 32 GSH 41 and GSH 49. The most abundant component was GSH 14 (~1g), while GSH 49 (~500mg) was also obtained in high quantity relative to other components which were obtained in amounts less than 100mg from 6-10g of crude extract.

GSH 2, GSH 26 and GSH 41 gave positive flavonoid reaction when the chromatoplates were sprayed with 5% $AlCl_3$/MeOH reagent. Each of them appeared as dark absorbing spots in UV light before treatment and as bright yellow spots after treatment. GSH 14 gave a dark grey colour while GSH 24 gave brown colour and GSH 32, GSH 49, both gave reddish brown colour with acetic anhydride/sulphuric acid reagent.

The compounds labelled as GSH 2, GSH 26 and GSH 41 were thus suspected to be flavonoid compounds and this had been confirmed by their spectral data. Also on the basis of the colour reaction with acetic anhydride/sulphuric acid reagent, GSH 14, GSH 24, GSH 32 and GSH 49 were identified as steroids and/or triterpenoid compounds. Their identities were unambiquously defined by their spectral data. The ^{1}H and ^{13}C nmr spectra of GSH 49 suggested it as a mixture of two isomeric compounds.

Column chromatographic analysis of ethylacetate extract performed with ethylacetate/hexane mixtures and then methanol/ethylacetate mixtures afforded one

pure component which was eluted with 5% Methanol/ethylacetate. This compound which appeared as a single component on tlc in 30% methanol/ethylacetate (R_f value 0.28) and methanol-chloroform-water, 65:45:12 (R_f value 0.72) gave a dark brown colour with Ac_20/H_2SO_4 reagent. The compound was later found to be identical to GS 13 (nmr spectra).

From the concentrated methanol extract, a white solid was obtained by tituration with methanol. The solid after washing thoroughly with methanol was recrystallized from methanol/water mixture and obtained as shining white crystals. This compound was labelled as GS 1 and was later identified by m.p. and spectral data as D-mannitol, m.p. 166-168^oC.

The mother liquor obtained from the trituration with methanol of the methanol extract was concentrated and partitioned between water and n-butanol in order to selectively extract the saponins into the n-butanol phase. The result of tlc analysis of the n-butanol soluble extract confirmed the effective concentration of the saponins in the n-butanol phase as it showed up to six components which reacted positively with AC_20/H_2SO_4 and chlorosulphonic acid reagents.

Chromatographic purification of the n-butanol soluble extract on a column of silica gel using methanol/chloroform mixtures as eluting solvent produced a steroidal glucoside labelled as GS 13 which was eluted with 15% methanol/chloroform. Several attemtps were made to isolate the remaining saponin components in the extract. First, the eluting mixture was changed to methanol-chloroform-water, 65:45:12 and then the column was repeated using methanol-chloroform-water, 65:35:10. Secondly, the n-butanol soluble extract was further

treated to remove unwanted pigments (see experimental), thus obtaining a more concentrated saponin fraction. The column chromatographic processes were then repeated on the treated n-butanol soluble extract but all efforts failed to yield any components.

Although it is a common knowledge that highly polar components do not separate well on silica gel, there are reports in the literature where saponin constituents of extracts were separated on a column of silica gel using methanol-chloroform-water (65:35:10)[162-163]. It is on the basis of this information that preparative tlc purification of the n-butanol soluble extract was attempted since the column chromatography failed. The preparative tlc carried out in solvent systems, methanol-chloroform-water, 65:45:12 and 65:35:10, was also not successful.

The implication of the failure to achieve any level of separation of the saponin components is that the glycosides most likely contain oligosaccharides as the sugar moiety, thus making the saponins highly polar. The only documentation of the presence and isolation of a saponin from a *Gardenia* plant was from the work reported by Shukla et al[91]. In their report, the saponin isolated was -β-sitosterol-3-0-β-Dglucopyranosyl-(1→4) - O-α-L-rhamnopyranoside from *Gardenia lucida* (Roxb.-), and it was obtained by solvent extraction.

Generally, the most popular and effective method of isolation of saponins from extracts involves the use of polyamide as column materials with methanol/water mixtures as eluent. This method is frequently reported by the Japanese working on saponins. Infact quite a substantial number of published work on saponins make use of chromatographic separation on a column of polyamide.

Apparently this method seems to be the only option left since the two methods tried have failed. Unfortunately, the polyamide material was not available and attempts to isolate the saponins from the n-butanol soluble extract was therefore abandoned.

In the light of the problems enumerated above, the saponin components were subsequently isolated and characterized as their aglycones. Mild acid hydrolysis (with 2% H_2SO_4/MeOH) of n-butanol soluble extract produced the aglycone mixture which showed three major triterpene spots (R_f values 0.52, 0.46, 0.40) on tlc with 10% MeOH/$CHCl_3$.

Chromatographic purification of the aglycone mixture on a column of silica gel eluting with MeOH/$CHCl_3$ mixtures afforded four triterpene components which on further purification by preparative tlc produced GSA 5, GSA 8, GSA 11 and GSA 16 as the saponin aglycone present in the n-butanol soluble extract of *Gardenia erubescens*.

The total number of components thus isolated from the methanol extract was six. These were GS 1, GS 13, GSA 5, GSA 8, GSA 11 and GSA 16. Apart from GS 1, the rest gave positive reaction for steroid or triterpenes.

2.3 Derivatives of Compounds Isolated from *G. erubescens*.

The identification of compounds from natural sources is deemed not to be complete until the functional groups present in such compounds are derivatized. The preparation of derivatives of a compound gives a strong evidence for the presence of the identified functional groups and this lends support for the confirmation of the structure of the compound. The exercise requires that the compound should be available in reasonable quantity, implying that for compounds available in very small quantities (1-10mg), it becomes difficult to prepare derivatives.

In the pioneering years of the development of isolation and characterization of natural products, a number of compounds isolated in trace amounts were not fully identified. But with the advent of FT nmr and the application of COSY, NOE etc to structural problems, the characterization of compounds available in few milligrams (up to 10mg) is effectively carried out now, in most cases, exclusively on the basis of nmr studies and other spectral data (UV, IR and MS) available.

Most of the compounds isolated from both the petroleum ether and n-butanol soluble extracts of *Gardenia erubescens* were in few milligrams (less than 50 mg). The most abundant being D-mannitol. The method of characterization of the various compounds was therefore infuenced by the quantity available for each compound.

The compounds labelled as GSH 2, GSH 26, GSH 32, GSH 41 and GSA 8 were characterized by comparison of their spectral data and melting point with the literature data. GSH 24 which was identified to be a new compound was subjected

to exhaustive nmr studies to arrive at the proposed structure. For GSH 49, identified as a mixture of ursolic and oleanolic acids, the composition was established by direct comparison of spectroscopic data and R_f values with those of authentic samples.

The other compounds, GSH 14, GS 1, GS 13, GSA 5, GSA 11 and GSA 16 were each subjected to one form of chemical reaction or another in addition to the spectral data available.

The acetates of GS 1, GSH 14, GSA 5 and GSA 11 were prepared by the reaction with 1:1 mixture of pyridine/acetic anhydride. The acetate derivatives for each compound lend additional support for the number of hydroxyl groups identified.

For GS 13, characterization was achieved through acid hydrolysis to the aglycone. The aglycone was subsequently identified by comparison of spectral data with that of GSH 14 and the authentic sample and this eventually gave the clue to the structure of GS 13 which was found to be a 3β-glucoside of GSH 14.

GSA 16 was identified also as a new compound. It was subjected to detailed nmr studies together with preparation of derivatives. The acetate derivative gave a triacetate compound, indicating the presence of three hydroxyl groups while the reaction with diazomethane produced a methylester confirming the presence of a carboxyl function.

2.4 NMR Studies on the Triterpenoid Components of *Gardenia erubescens.*

The standard nmr spectra and 2D nmr spectra in particular were of tremendous assistance in elucidation of structures of GSH 32, GSA 5, GSA 8, GSA 11 and specifically the structures of the new compounds - GSH 24 and GSA 16.

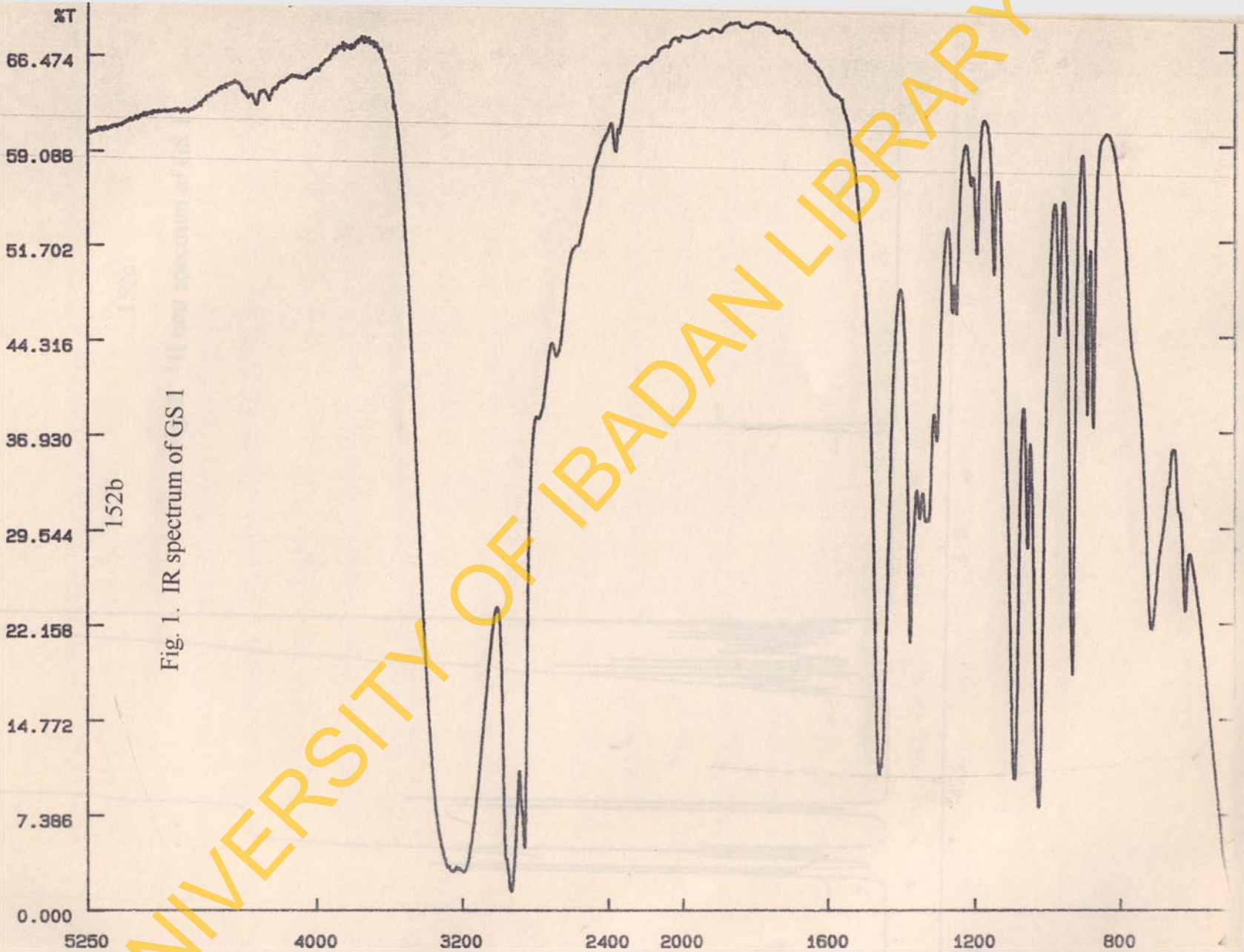
COSY and HETEROCOSY were the 2D nmr spectra employed while DEPT and NOE difference spectra supplied complimentary data[164-166].

The spectral data obtained from these nmr studies were used, in combination with melting points and derivatization, to arrive at the structures proposed for the new compounds - GSH 24 and GSA 16.

2.5 Characterization of Compounds Isolated from *Gardenia erubescens.*

2.5.1 The Structure of GS 1

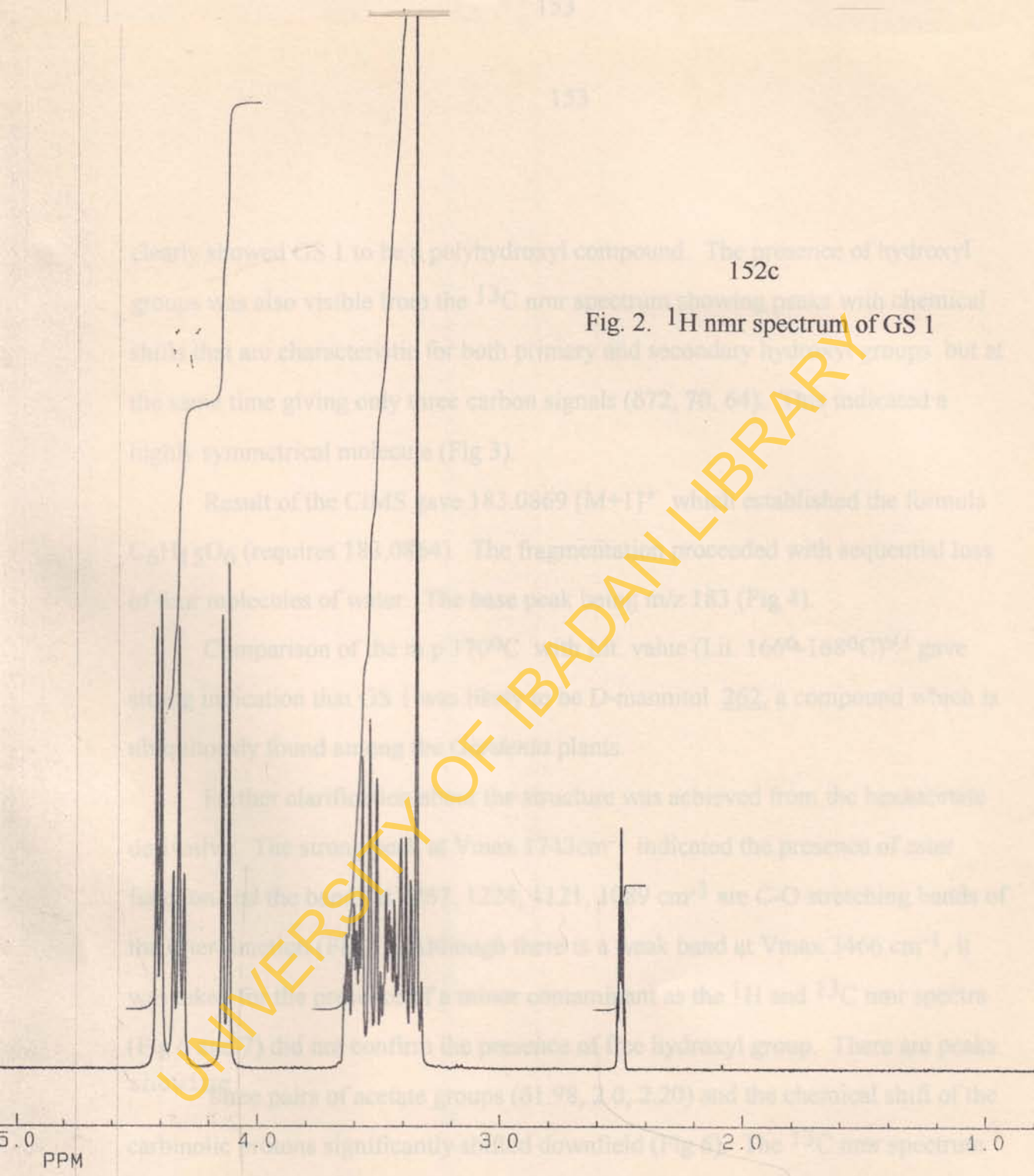
The IR spectrum of GS 1 showed the presence of -OH group with an intense broad band at max 3300-3150cm^{-1}. The presence of the hydroxyl group was further indicated by the bands at 1262, 1147, 1092 and 1062 cm^{-1} for C-O stretching vibrations (Fig 1). Information about the number of protons present in the compound was obtained from the ^{1}H nmr spectrum, a total of fourteen protons were shown to be present (Fig 2). Six protons appeared around δ4.42-4.13 as 2H,d (δ4.42), 2H,t (δ4.35) and 2H,d (δ4.13). These are considered to be the hydroxylic protons. The remaining eight protons appeared as a multiplet centered at δ3.50. This chemical shift is characteristic of carbinolic protons. The ^{1}H nmr spectrum



152b

Fig. 1. IR spectrum of GS 1

Fig. 2. ^{1}H nmr spectrum of GS 1



clearly showed GS 1 to be a polyhydroxyl compound. The presence of hydroxyl groups was also visible from the ^{13}C nmr spectrum showing peaks with chemical shifts that are characteristic for both primary and secondary hydroxyl groups but at the same time giving only three carbon signals (δ72, 70, 64). This indicated a highly symmetrical molecule (Fig 3).

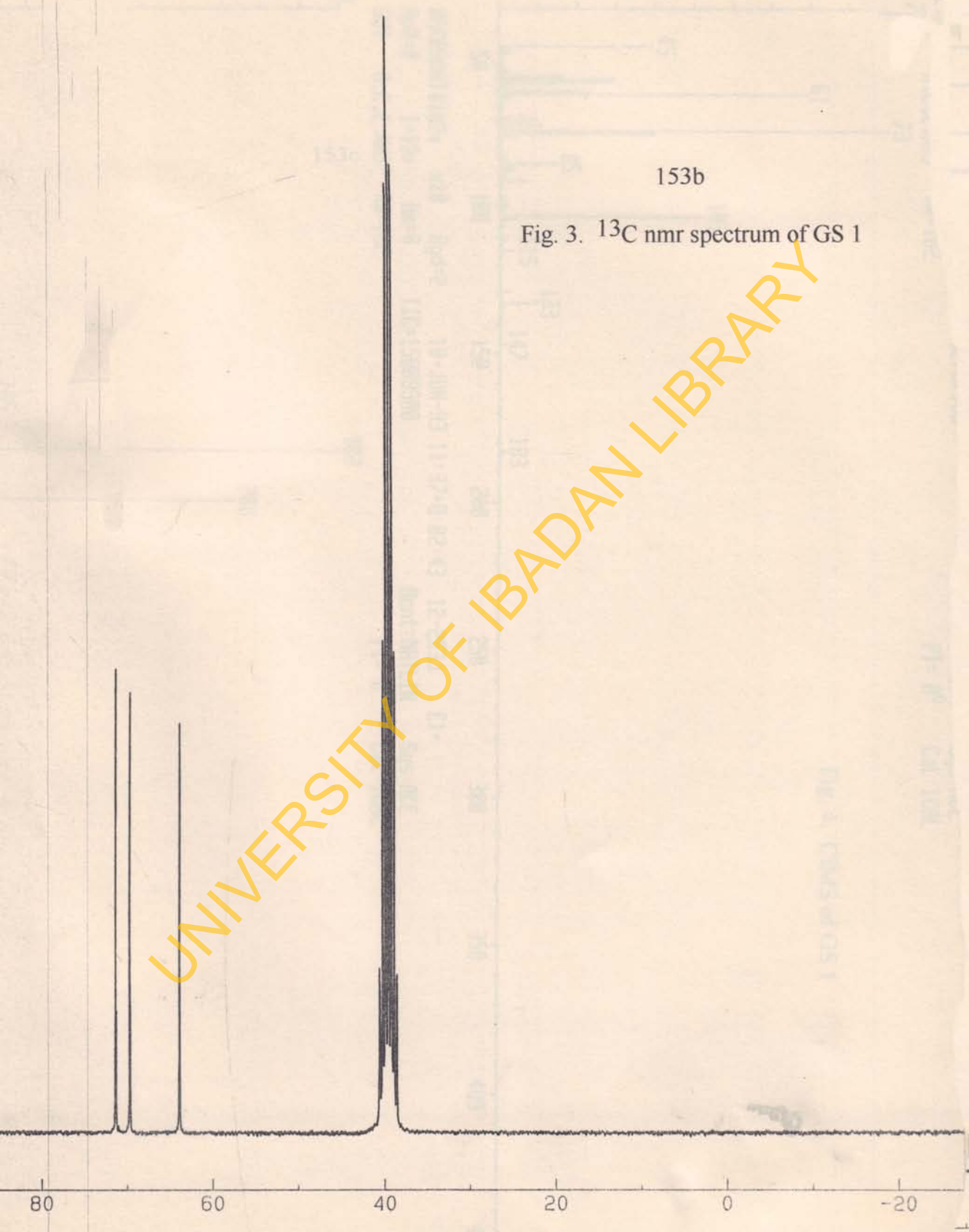
Result of the CIMS gave 183.0869 $[M+1]^+$ which established the formula $C_6H_{15}O_6$ (requires 183.0864). The fragmentation proceeded with sequential loss of four molecules of water. The base peak being m/z 183 (Fig 4).

Comparison of the m.p 170°C with Lit. value (Lit. 166°-168°C)[93] gave strong indication that GS 1 was likely to be D-mannitol 262, a compound which is ubiquitously found among the *Gardenia* plants.

Further clarification about the structure was achieved from the hexaacetate derivative. The strong peak at Vmax 1743cm^{-1} indicated the presence of ester function and the bands at 1267, 1224, 1121, 1089 cm^{-1} are C-O stretching bands of the ester function (Fig 5). Although there is a weak band at Vmax 3466 cm^{-1}, it was taken for the presence of a minor contaminant as the ^1H and ^{13}C nmr spectra (Fig 6 and 7) did not confirm the presence of free hydroxyl group. There are peaks showing three pairs of acetate groups (δ1.98, 2.0, 2.20) and the chemical shift of the carbinolic protons significantly shifted downfield (Fig 6). The ^{13}C nmr spectrum also confirmed the presence of three pairs of acetate groups and also three pairs of carbinolic carbons, further indicating that the molecule is highly symmetrical (Fig. 7).

153b

Fig. 3. ^{13}C nmr spectrum of GS 1



GS1 A.C.E.NH3 MW=182 PT= 0⁰ Cal:1CAL MAS

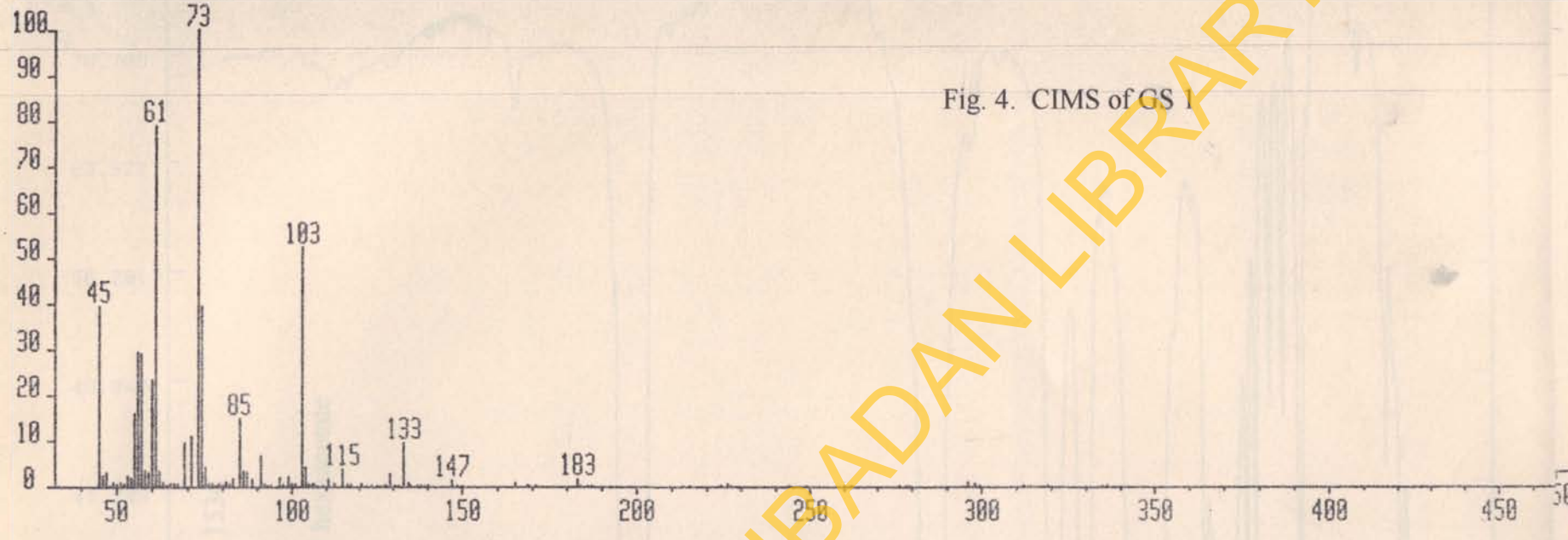


Fig. 4. CIMS of GS 1

WAR09MAT#102* x10 Bgd=2 10-JUN-93 11:37+0:02:43 12-253J C1+
BpM=0 I=10v Hm=0 TIC=15009900 Acnt:MATLIN Sys:ACE HM
GS1 A.C.E.NH3 MW=182 PT= 0⁰ Cal:1CAL MAS

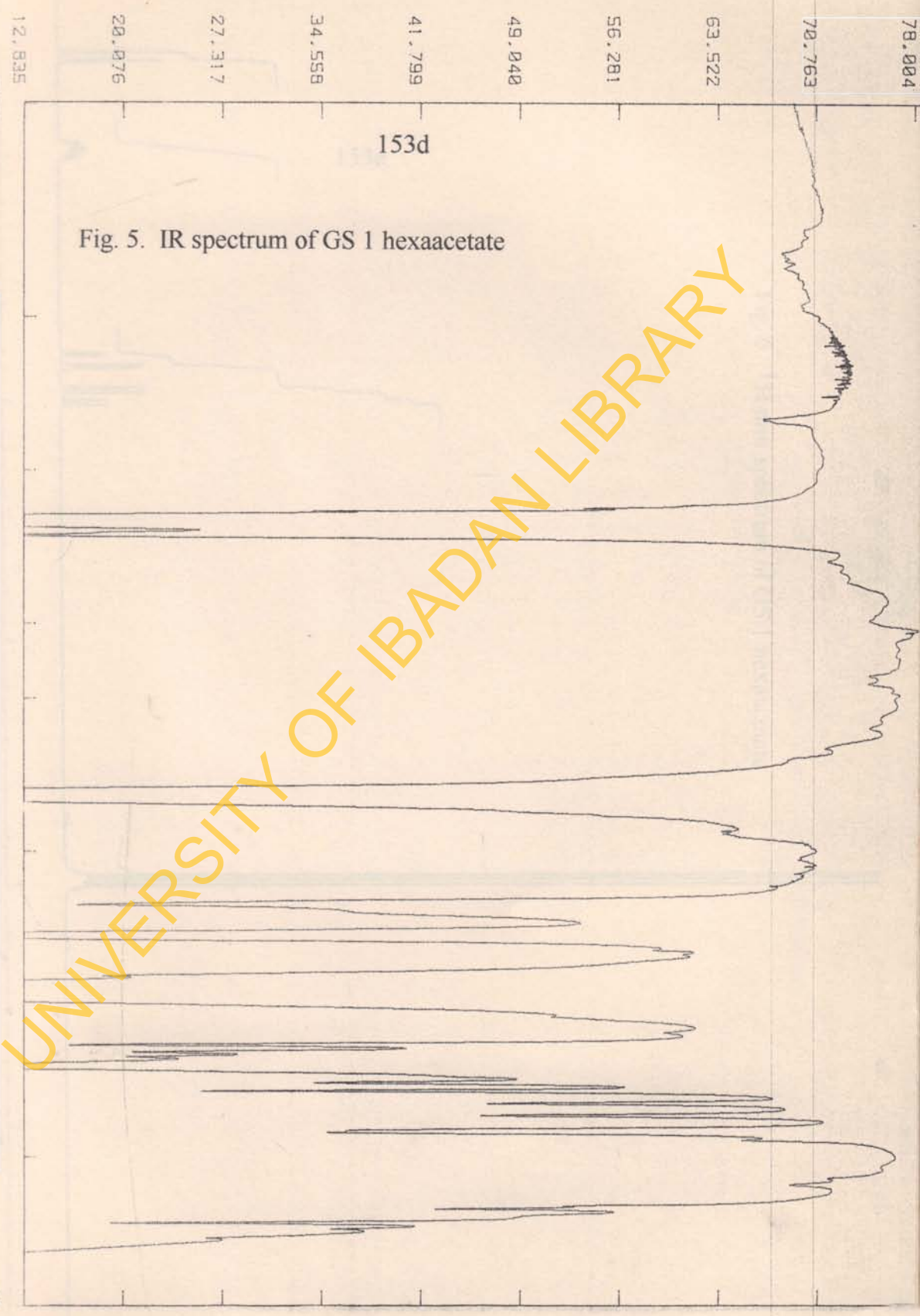


Fig. 5. IR spectrum of GS 1 hexaacetate

Fig. 6. ^{1}H nmr spectrum of GS 1 hexaacetate

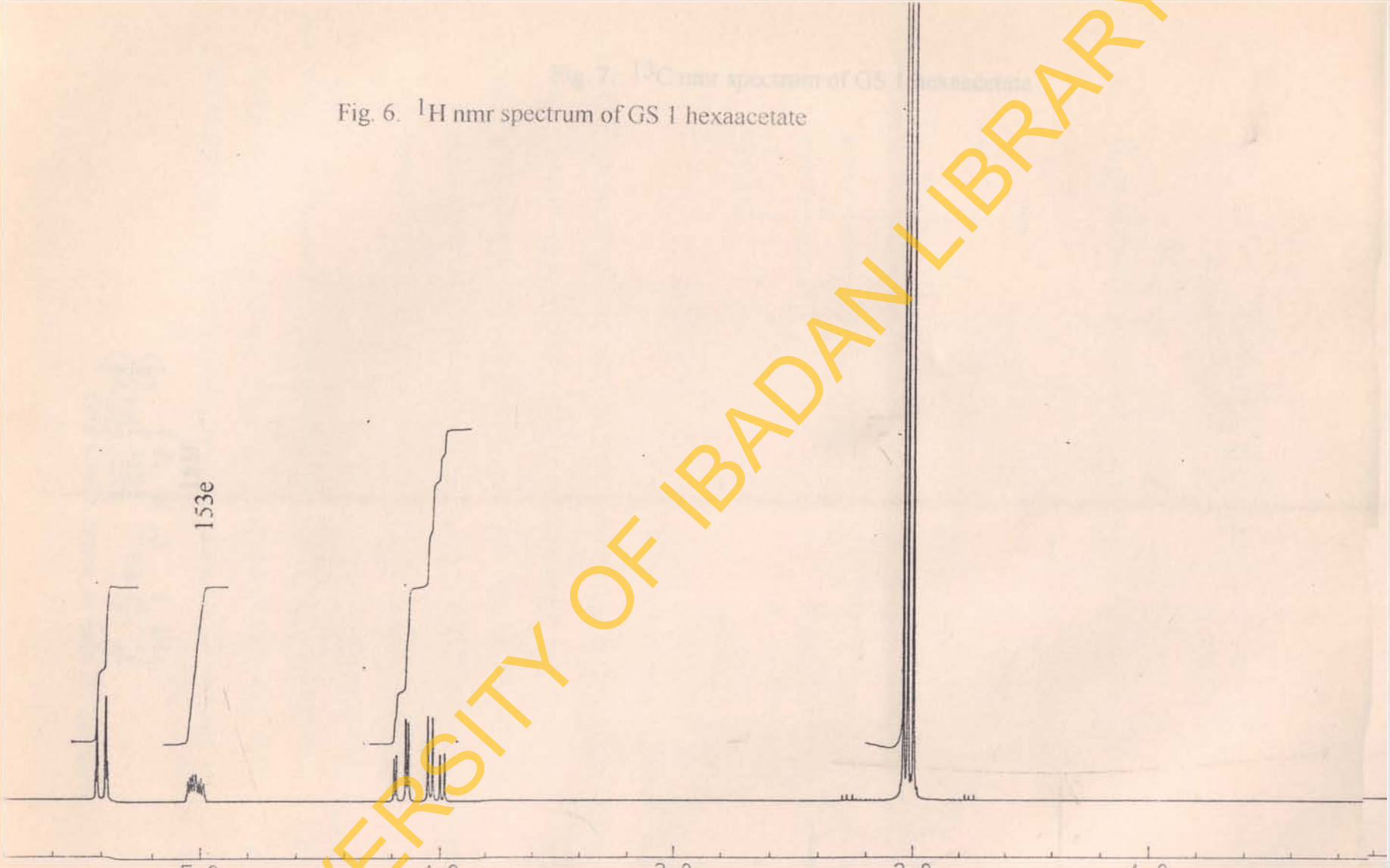


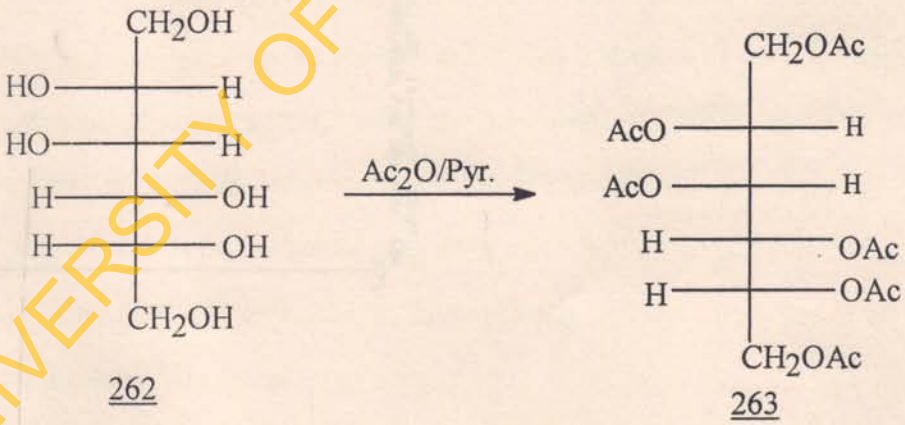
Fig. 7. ^{13}C nmr spectrum of GS 1 hexaacetate

153f

The CIMS also confirmed the derivative to be a hexaacetate with m/z 435 $[M+1]^+$ peak. This corresponds to $C_{18}H_{27}O_{12}$. The peaks at m/z (rel. int) 375 (100), 333(4), 289(3), 273(12) and 213(18) indicated progressive loss of acetic acid or acetyl unit from the molecule (fig 8).

The melting point of GS 1 hexaacetate was 117-119°C and that of D-mannitol hexacetate 263 (Lit. 121°c)[93]. The two values are comparable.

Consequently, on the basis of the spectral data and melting point of GS 1 and its derivative, GS 1 hexaacetate, GS 1 was identified as D-mannitol. D-mannitol is a derivative of D-mannose in which the aldehyde group of the sugar has been reduced to a primary alcohol group.

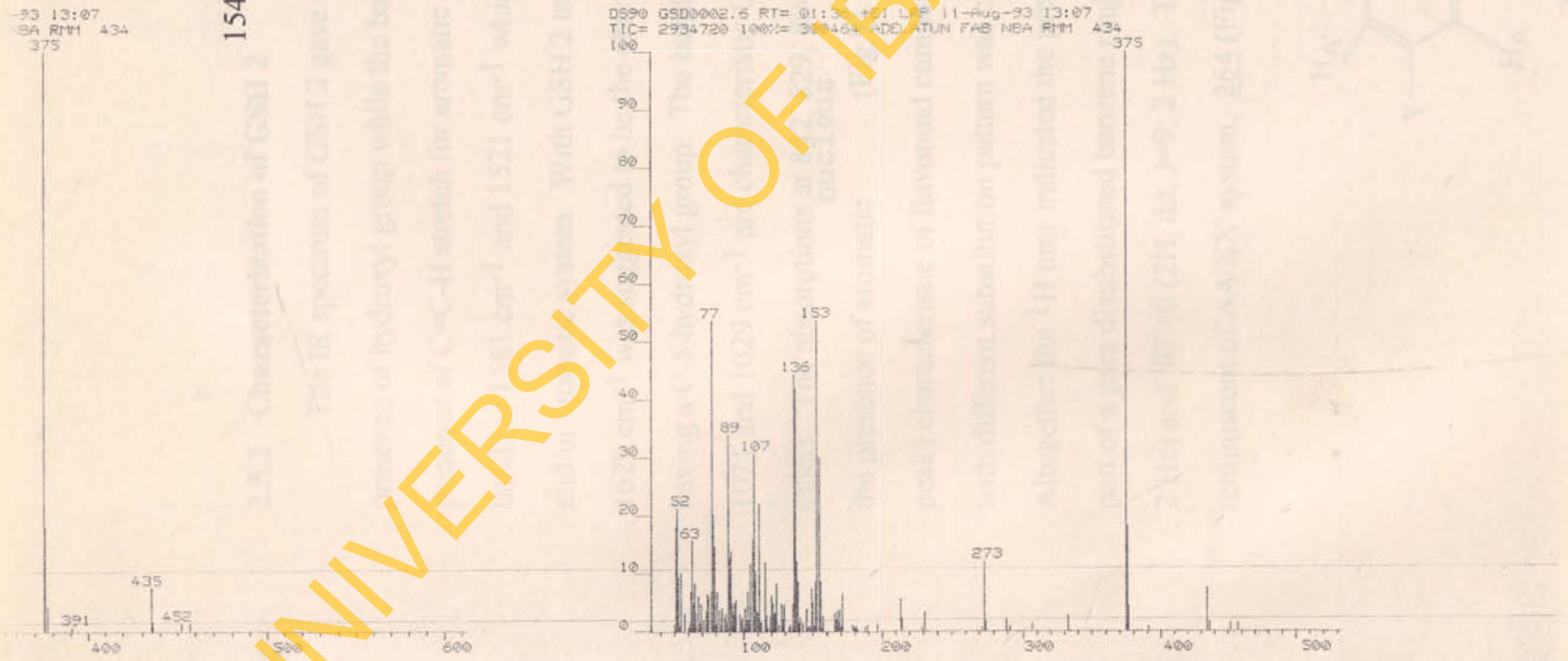


262 263

D-mannitol was reported for its low diuretic activity by Hussain et al[161].

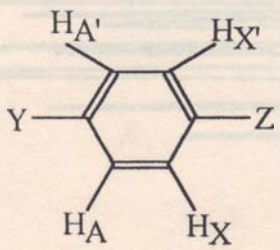
Fig. 8. EIMS of GS 1 hexaacetate

154b



2.5.2 Characterization of GSH 2

The IR spectrum of GSH 2 gave a weak band at Vmax 4331 cm^{-1} indicating the presence of hydroxyl group while the band at Vmax 3096 cm^{-1} indicated the presence of C=C-H stretch for aromatic system. This was further supported by the bands at 1581 cm^{-1} and 1521 cm^{-1} which are C=C stretch bands for conjugated and/or aromatic system. With GSH 2 testing positive for flavonoid, the band at 1629 cm^{-1} was suggested to be the characteristic C=O stretch band of flavonoids having a C-5 hydroxyl group. The bands at 1276, 1255, 1212, 1199, 1159, 1090, 1071 and 1029 cm^{-1} are characteristic C-O stretching absorptions of aryl alkyl ethers. The absorptions at 842, 829, 804, 746, and 733 cm^{-1} strongly suggested the presence of aromatic nucleus (Fig 9). Both ^{1}H and ^{13}C nmr of GSH 2 showed peaks characteristic of flavonoid compounds. The presence of two aromatic rings with different substitution pattern was clearly evident in the ^{1}H nmr (Fig 10). Altogether the ^{1}H nmr indicated the presence of six aromatic protons, four being part of a para disubstituted benzene ring with chemical shifts at δ6.97 (2H, dd, J=9, 2 Hz) and δ7.40 (2H, dd, J=9, 2 Hz). The splitting pattern (distorted doublet) is reminiscent of AA'XX' system, 264 (Fig. 11).



264

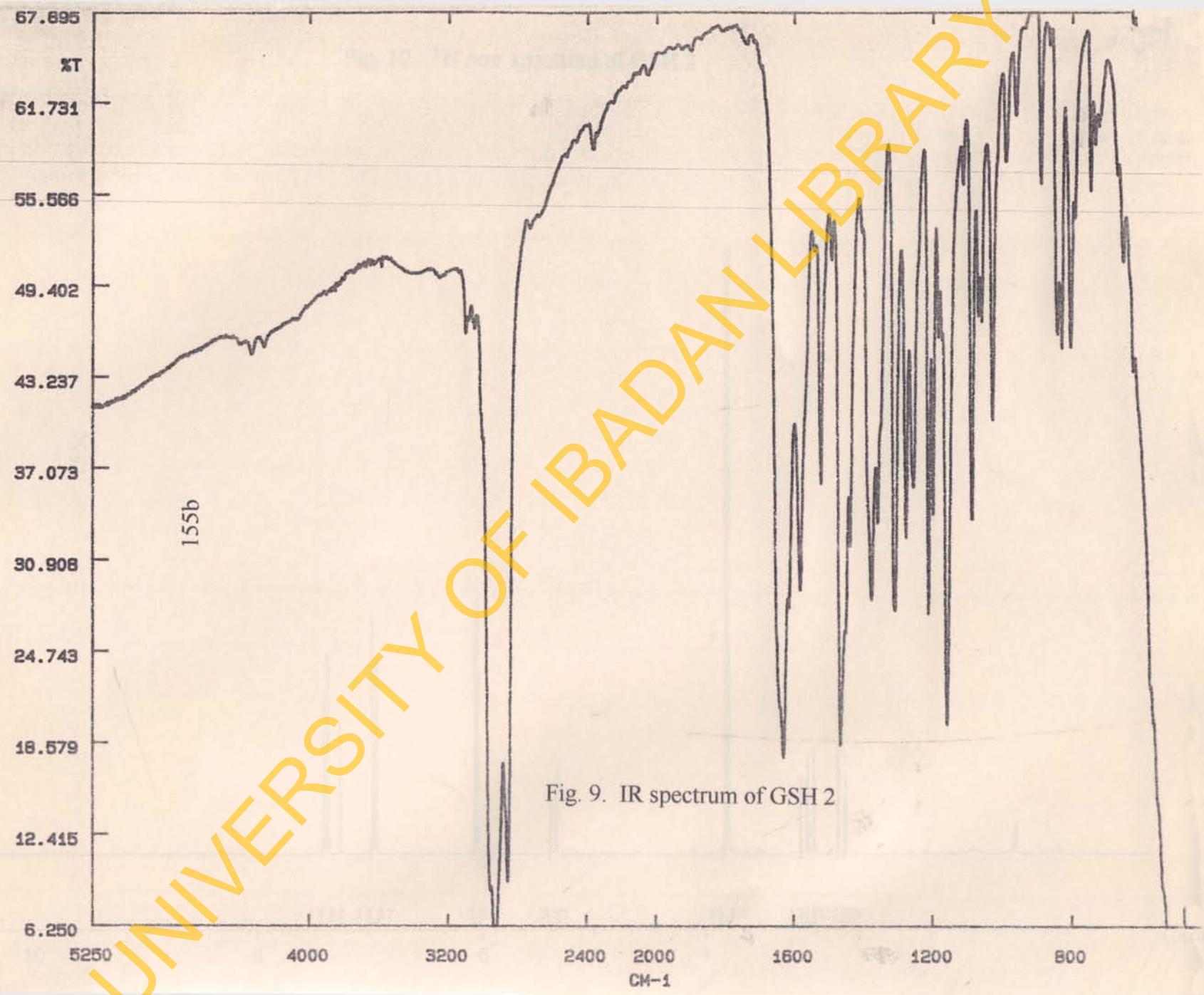


Fig. 9. IR spectrum of GSH 2

Fig. 10. ^{1}H nmr spectrum of GSH 2

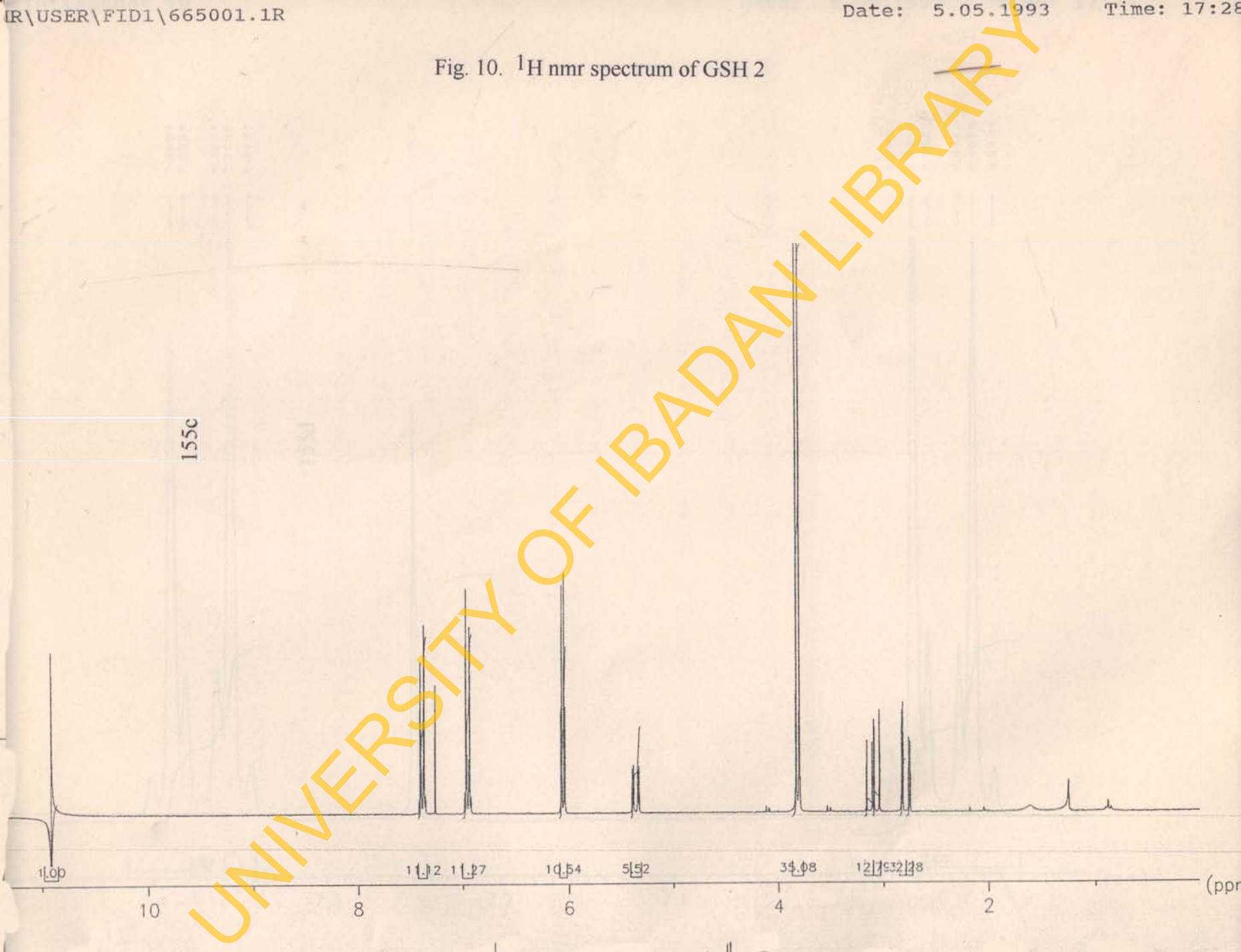
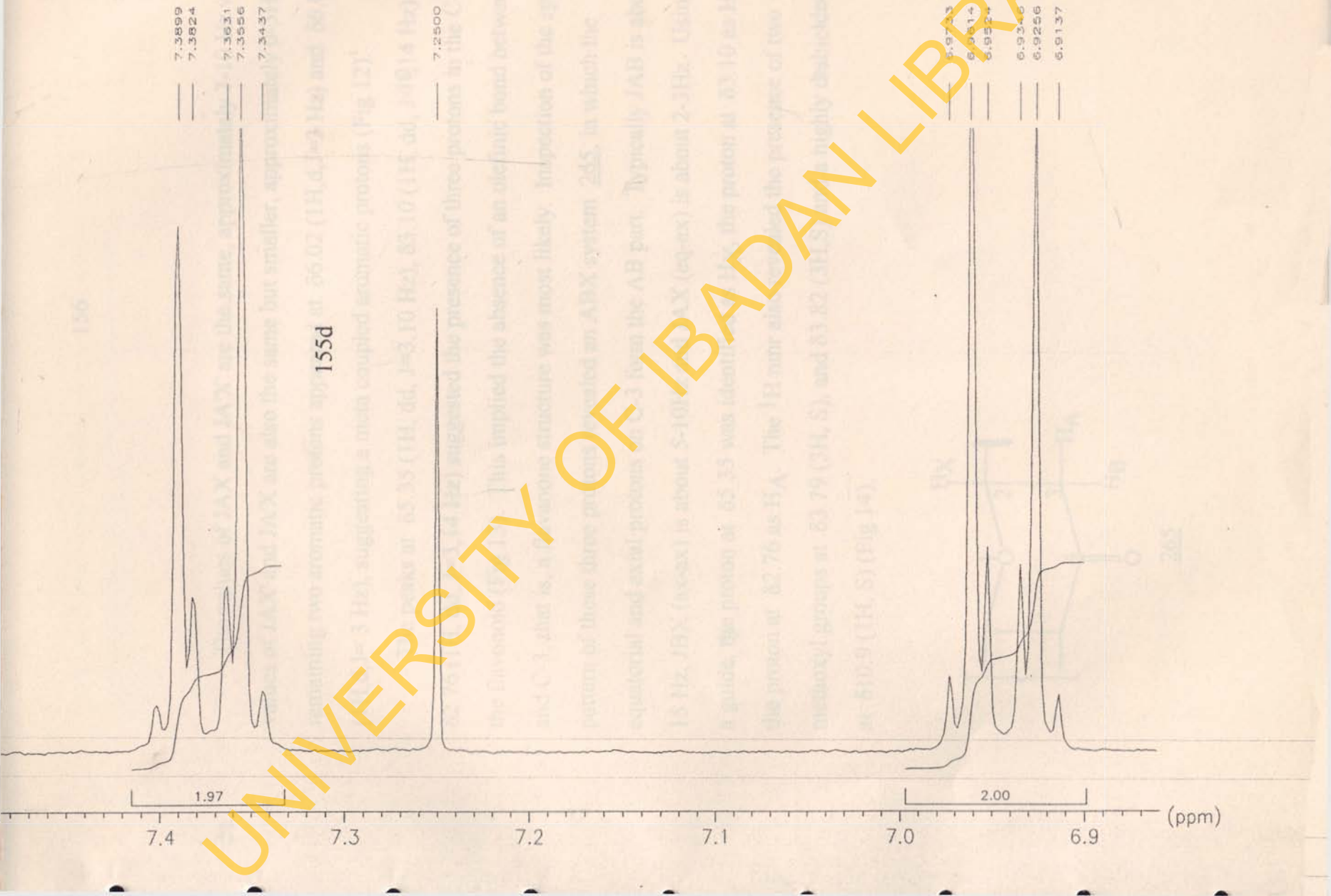
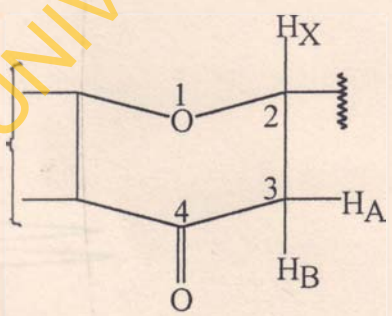


Fig. 11. ^{1}H nmr spectrum of GSH 2



The values of JAX and JA'X' are the same, approximately 7-10 Hz while the values of JAX' and JA'X are also the same but smaller, approximately 0-3Hz. The remaining two aromatic protons appeared at δ6.02 (1H,d,J=3 Hz) and δ6.05 (1H,d,J= 3 Hz), suggesting a meta coupled aromatic protons (Fig 12).

The peaks at δ5.35 (1H, dd, J=3,10 Hz), δ3.10 (1H, dd, J=10,14 Hz) and δ2.76 (1H, dd, J=3,14 Hz) suggested the presence of three protons in the C-ring of the flavonoid (Fig 13). This implied the absence of an olefinic bond between C-2 and C-3, that is, a flavanone structure was most likely. Inspection of the splitting pattern of these three protons revealed an ABX system 265, in which the equatorial and axial protons on C-3 form the AB part. Typically JAB is about 12-15 Hz, JBX (ax-ax) is about 5-10Hz and JAX (eq-ax) is about 2-3Hz. Using this as a guide, the proton at δ5.35 was identified as H_X, the proton at δ3.10 as H_B and the proton at δ2.76 as H_A. The ^1H nmr also revealed the presence of two methoxyl groups at δ3.79 (3H, S), and δ3.82 (3H,S) and a highly deshielded proton at δ10.9 (1H, S) (Fig 14).



265

Fig. 12. ^{1}H nmr spectrum of GSH 2

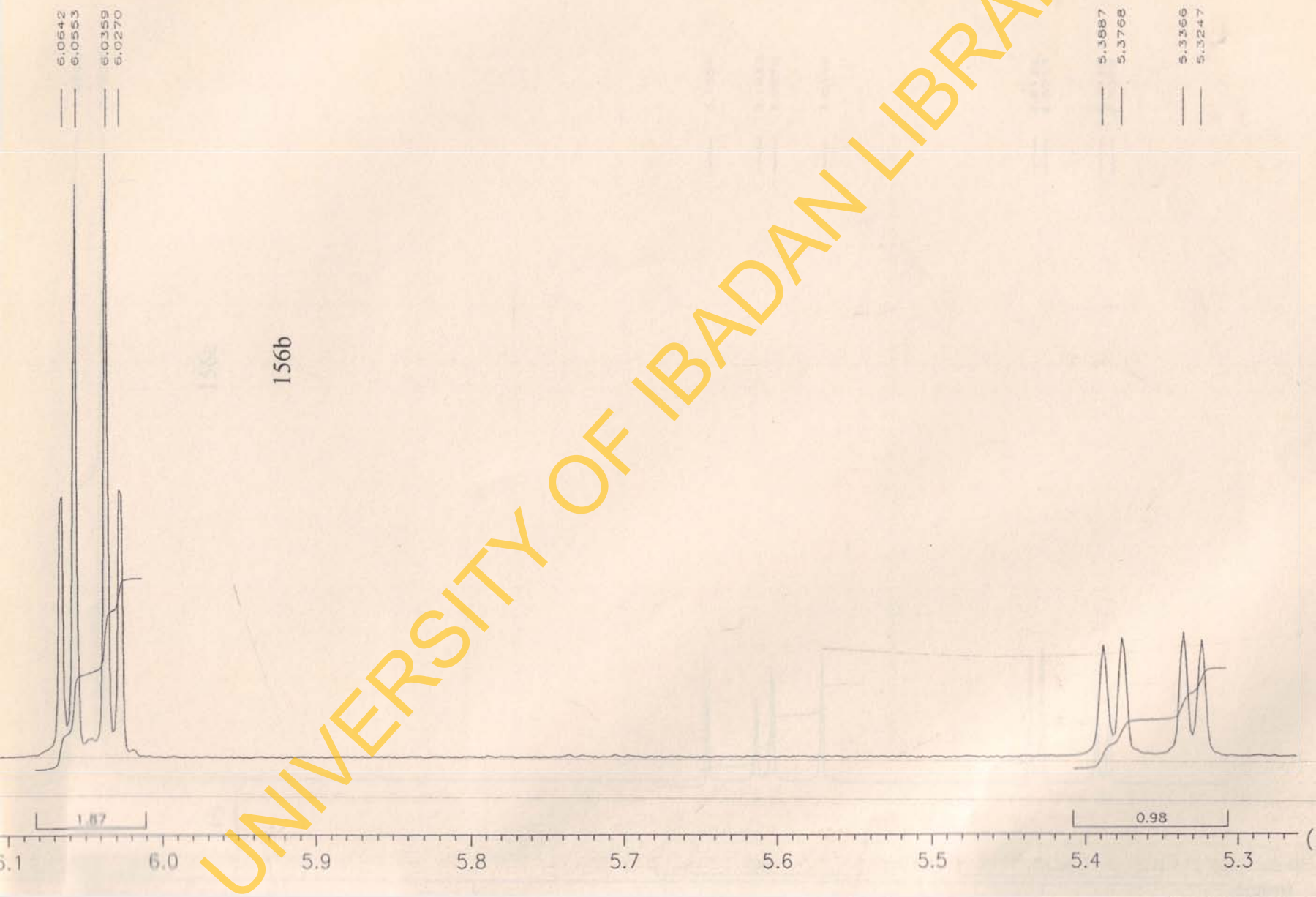


Fig. 13. ^{1}H nmr spectrum of GSH 2

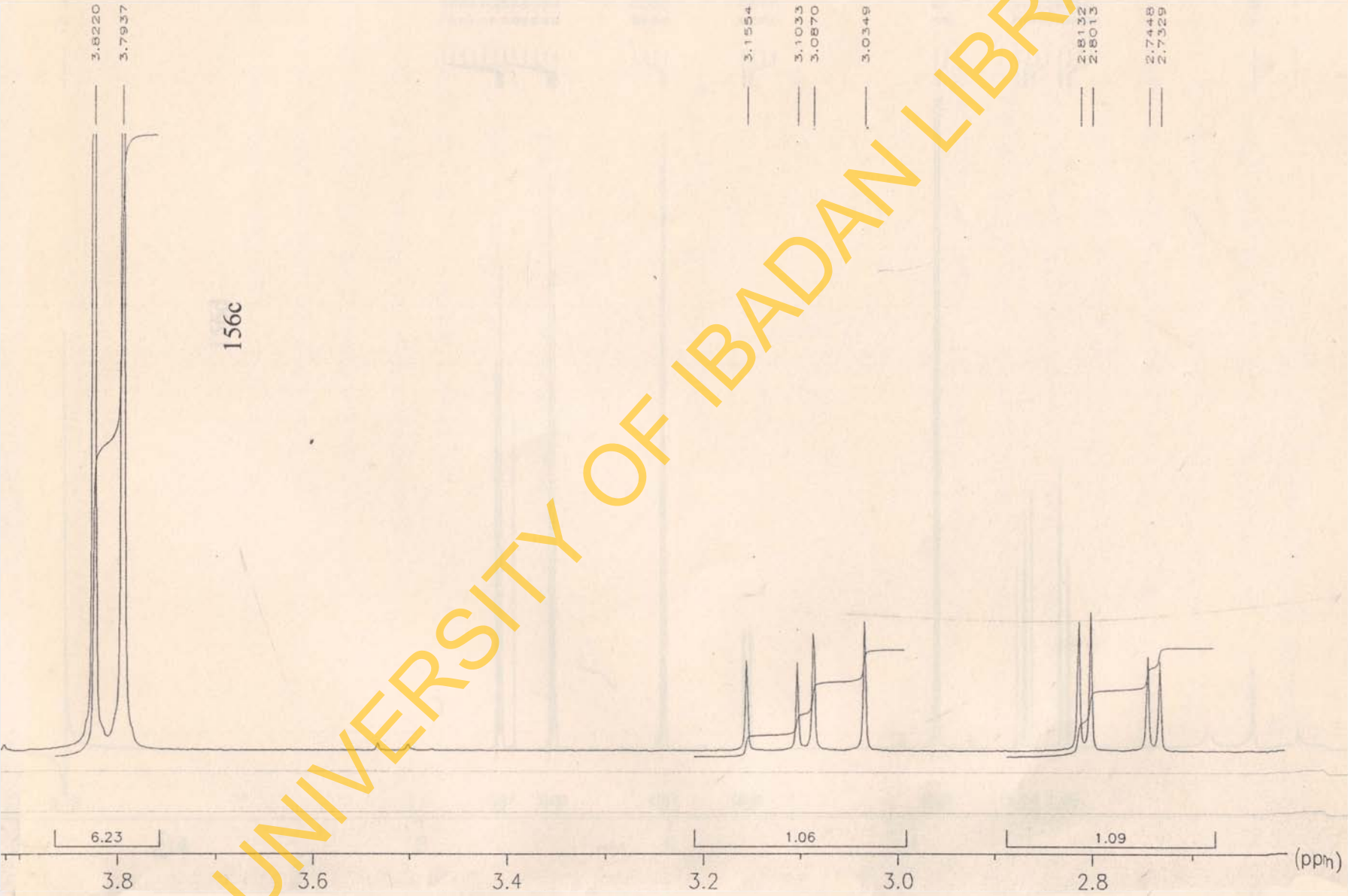
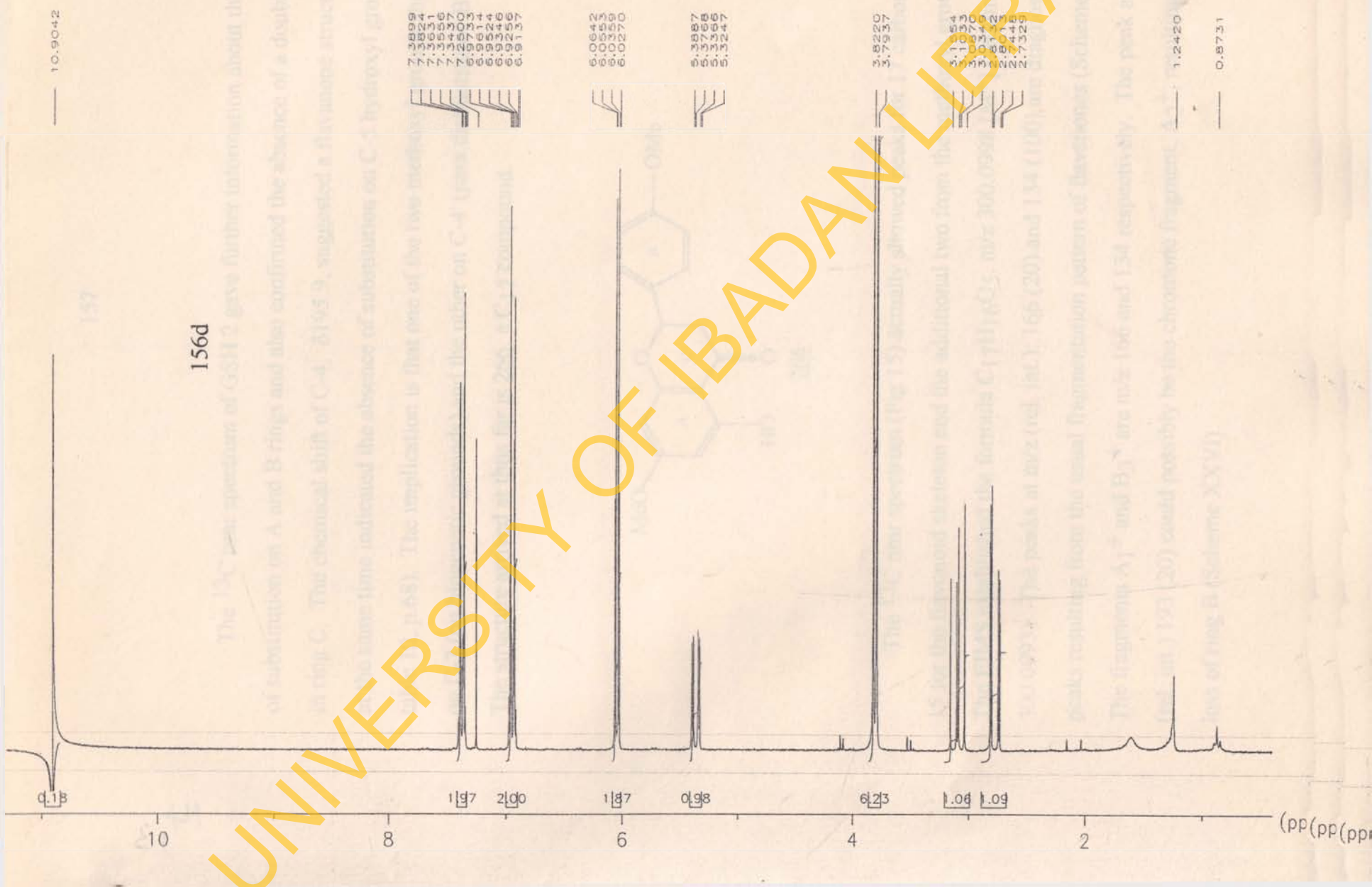
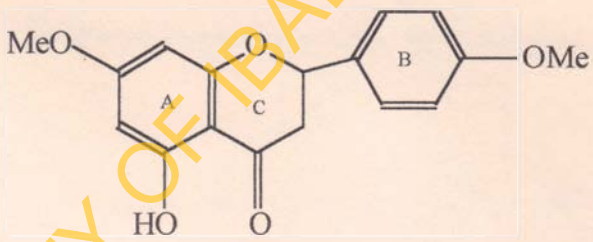


Fig. 14. ^{1}H nmr spectrum of GSH 2



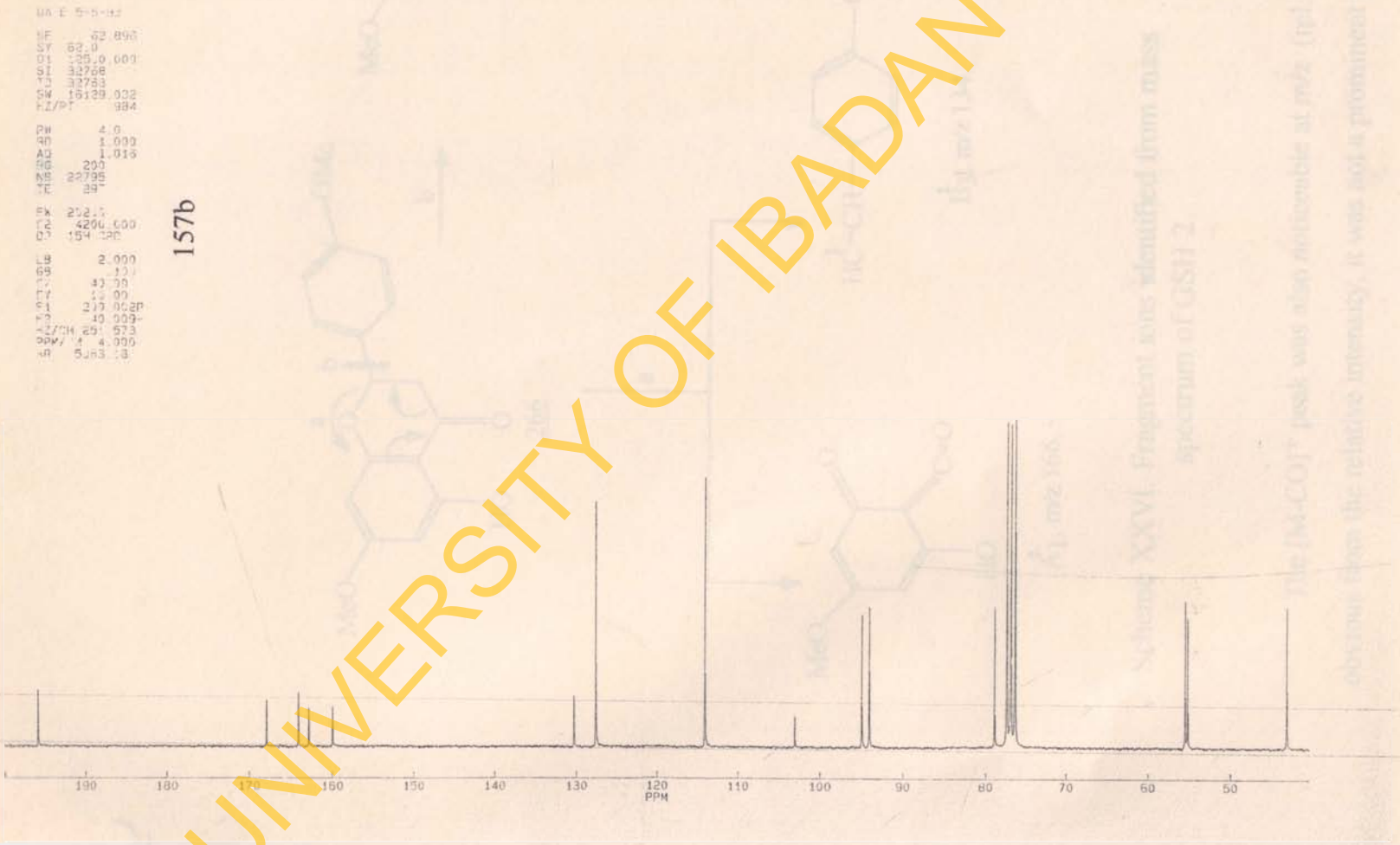
The ^{13}C nmr spectrum of GSH 2 gave further information about the nature of substitution on A and B rings and also confirmed the absence of a double bond in ring C. The chemical shift of C-4, δ195.9, suggested a flavanone structure and at the same time indicated the absence of substitution on C-5 hydroxyl group (see table 10, p.68). The implication is that one of the two methoxyl groups should be on C-7 (on biogenetic grounds) and the other on C-4' (para disbustituted B ring). The structure arrived at thus far is 266, a C_{17} compound.

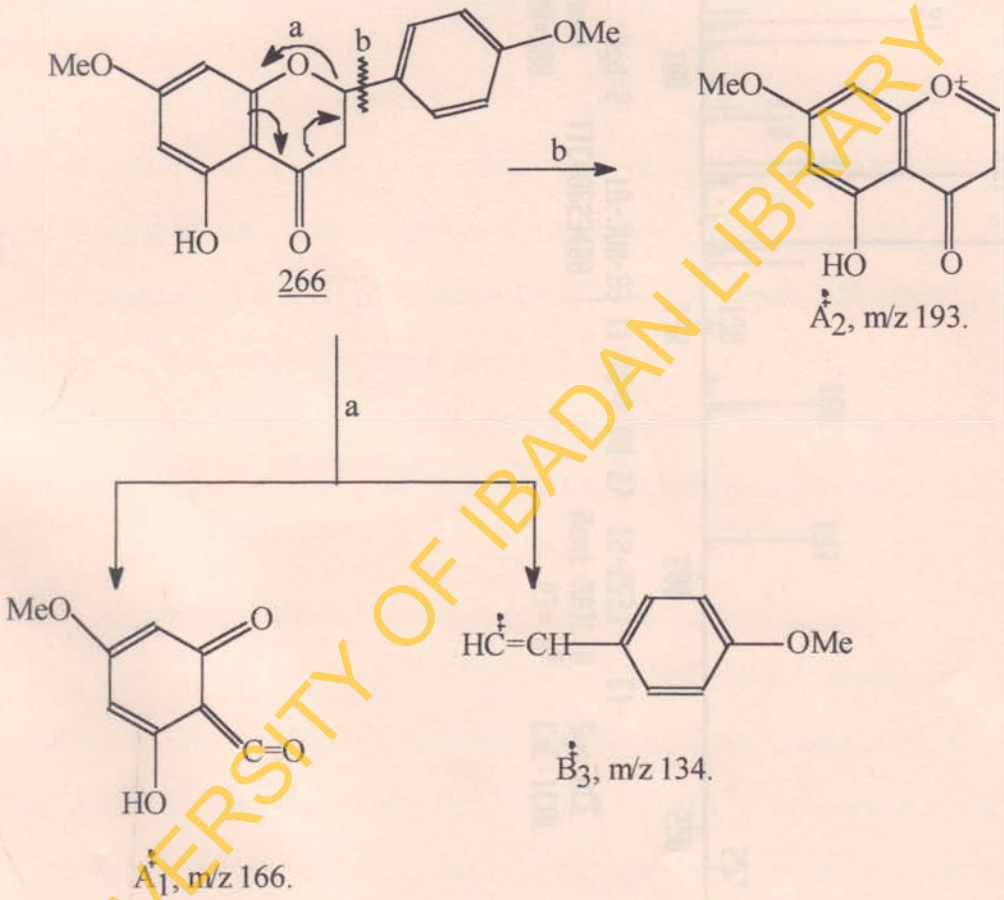


266

The ^{13}C nmr spectrum (Fig 15) actually showed peaks for 17 carbon atoms, 15 for the flavonoid skeleton and the additional two from the methoxyl groups. The EIMS established the formula $C_{17}H_{16}O_5$, m/z 300.0998 [M^+] (requires 300.0993). The peaks at m/z (rel. int.): 166 (20) and 134 (100) are diagnostic peaks resulting from the usual fragmentation pattern of flavanones (Scheme XXVI). The fragments A_1^+ and B_3^+ are m/z 166 and 134 respectively. The peak at m/z (rel. int.) 193 (20) could possibly be the chromone fragment, A_2^+, resulting from loss of ring B (Scheme XXVI).

Fig. 15. ^{13}C nmr spectrum of GSH 2





Scheme XXVI. Fragment ions identified from mass spectrum of GSH 2.

The $[M-CO]^+$ peak was also noticeable at m/z (rel. int.) 272 (2) but as it is obvious from the relative intensity, it was not a prominent peak (Fig 16).

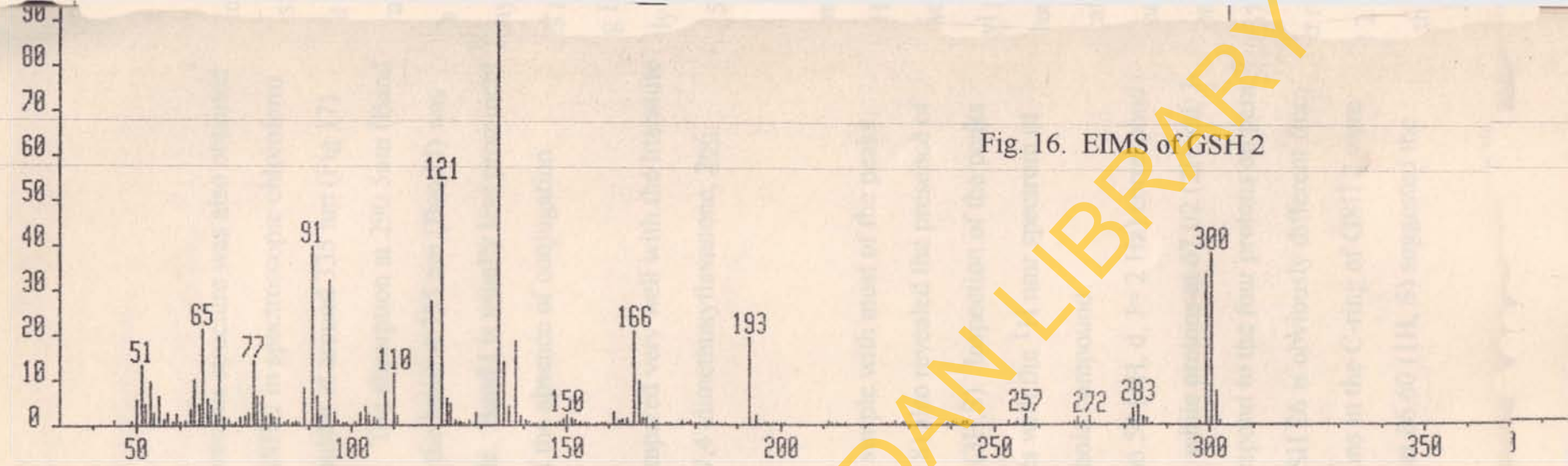
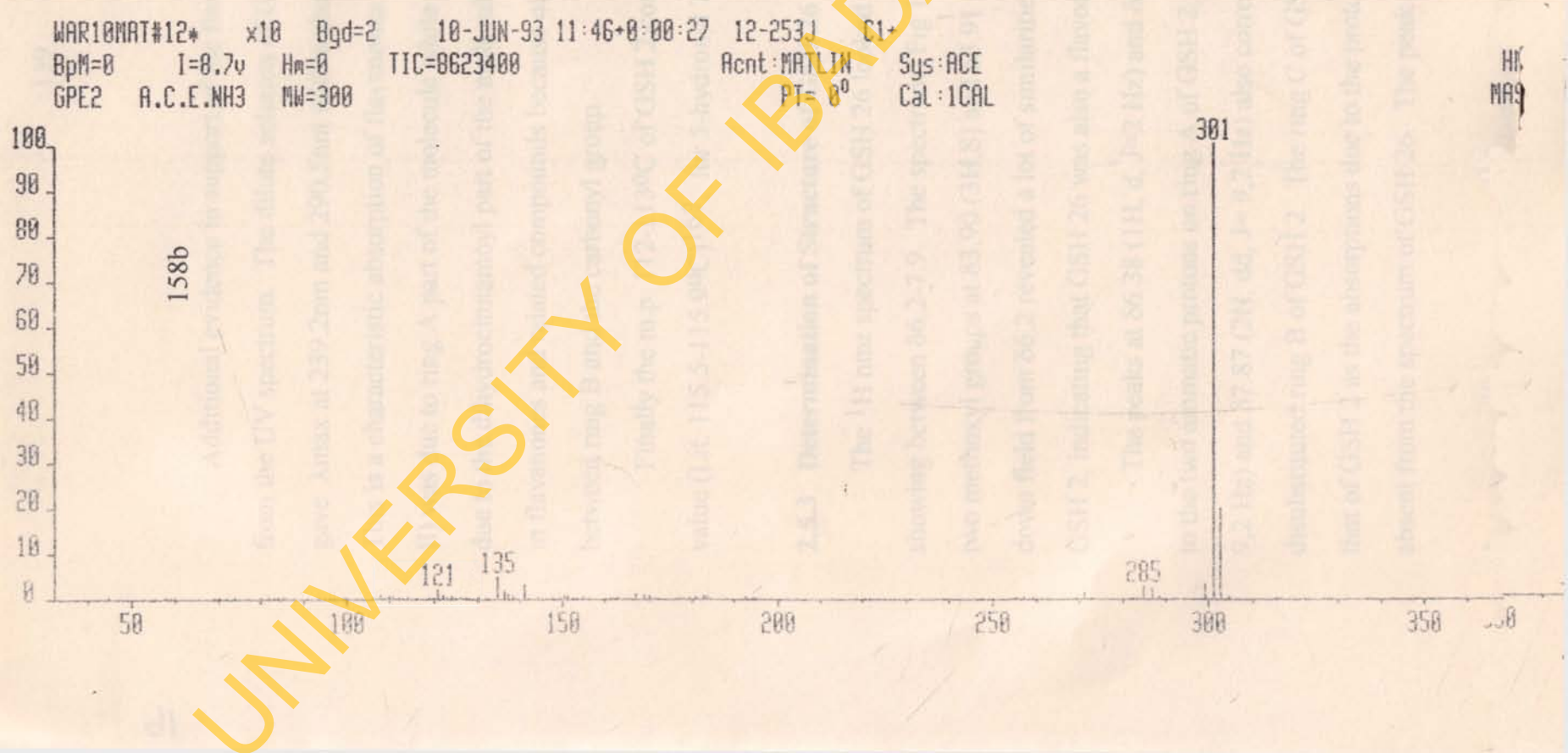


Fig. 16. EIMS of GSH 2



Additional evidence in support of the flavanone structure was also obtained from the UV spectrum. The dilute solution of GSH 2 in spectroscopic chloroform gave λmax at 239.2nm and 290.5nm with a shoulder at around 335 nm (Fig 17). This is a characteristic absorption of flavanones. The absorption at 290.5nm (Band II) was due to ring A part of the molecule while the band at 335 nm (Band I) was due to the dihydrocinnamoyl part of the molecule. Band I is usually less prominent in flavanones and related compounds because of the absence of conjugation between ring B and the carbonyl group.

Finally the m.p. 112-113°C of GSH 2 compared very well with the literature value (Lit. 115.5-115.9°C)[167], for 5-hydroxyl 7,4'-dimethoxyflavanone, 266.

2.5.3 Determination of Structure of GSH 26

The ^{1}H nmr spectrum of GSH 26 looked simple with most of the peaks showing between δ6.2-7.9. The spectrum (Fig 18) also revealed the presence of two methoxyl groups at δ3.90 (3H,S) and δ3.91 (3H,S). Inspection of the peaks down field from δ6.2 revealed a lot of similarities with the ^{1}H nmr spectrum of GSH 2, indicating that GSH 26 was also a flavonoid compound.

The peaks at δ6.38 (1H, d, J=2 Hz) and δ6.50 (1H, d, J= 2 Hz) correspond to the two aromatic protons on ring A of GSH 2, while protons at δ7.02 (2H, dd, J-9,2 Hz) and δ7.87 (2H, dd, J= 9,2 Hz) also correspond to the four protons on para disubstituted ring B of GSH 2. The ring C of GSH 26 is obviously different from that of GSH 2 as the absorptions due to the protons in the C-ring of GSH 2 were absent from the spectrum of GSH 26. The peak at δ6.60 (1H, S) suggested the

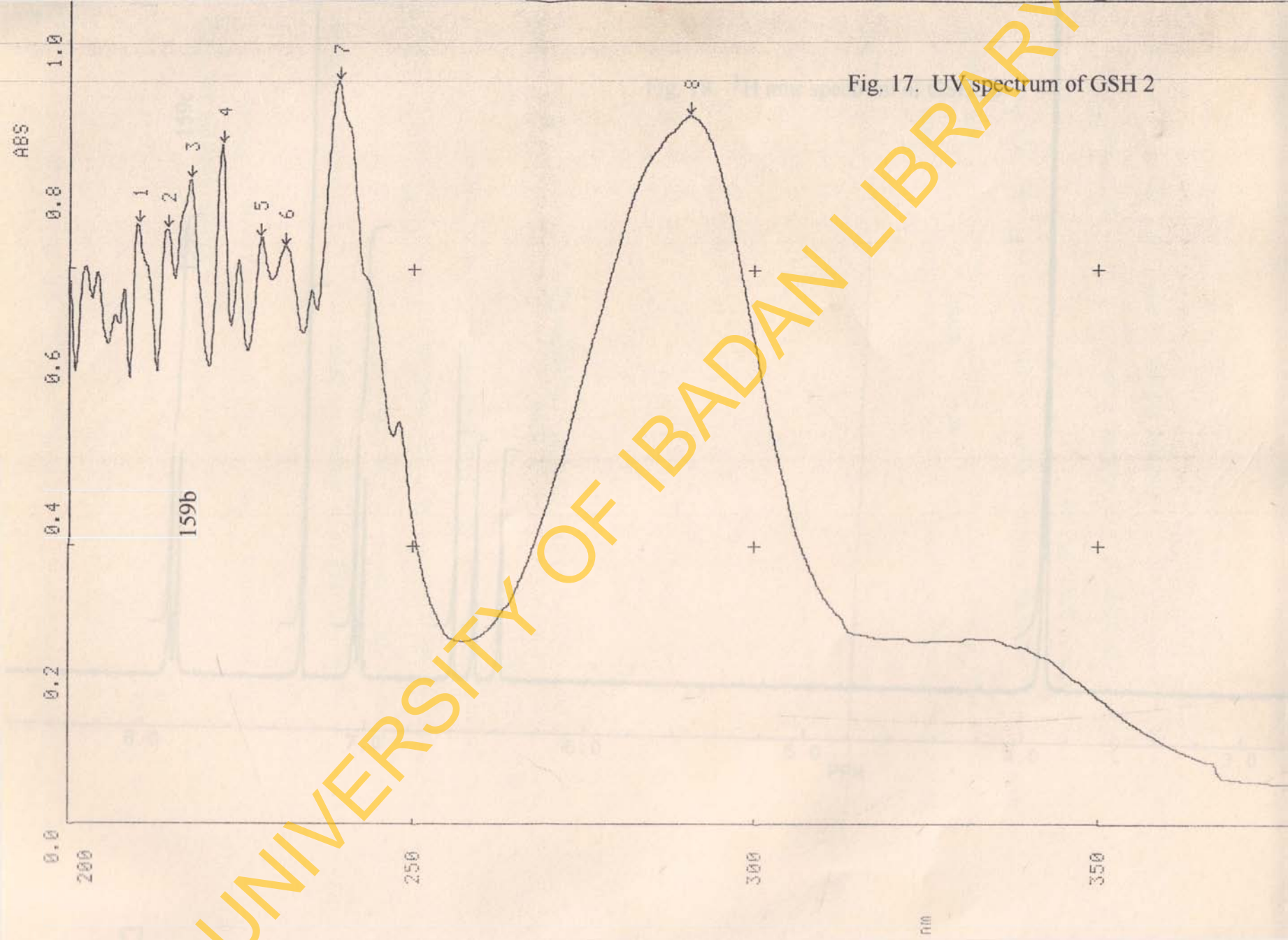
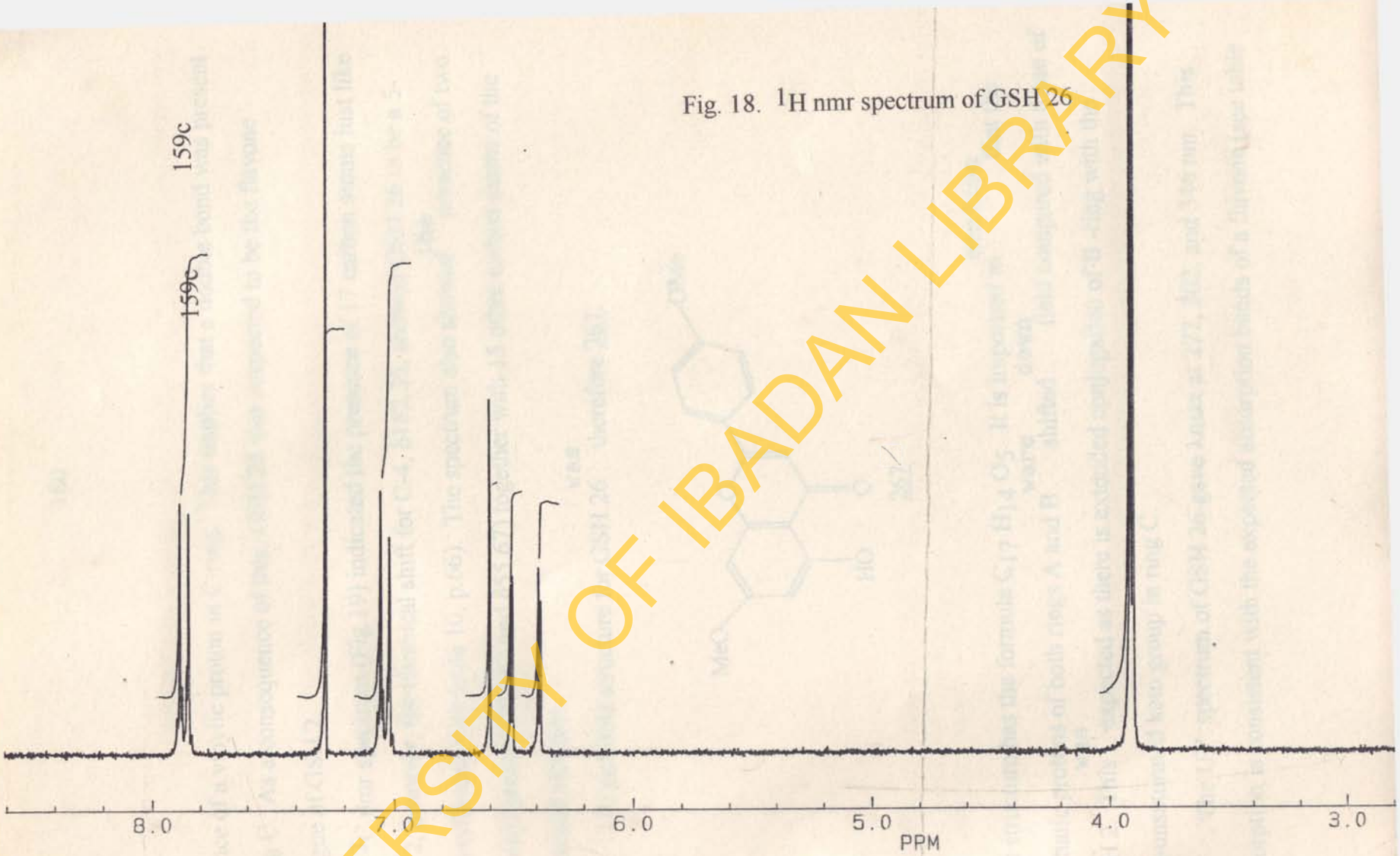


Fig. 17. UV spectrum of GSH 2

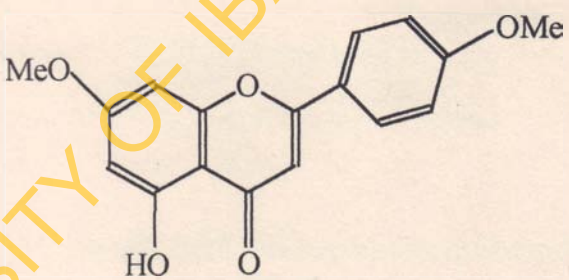
Fig. 18. ^{1}H nmr spectrum of GSH 26



presence of a vinylic proton in C-ring. This implies that a double bond was present in ring C. As a consequence of this, GSH 26 was suspected to be the flavone analogue of GSH 2.

The ^{13}C nmr spectrum (Fig 19) indicated the presence of 17 carbon atoms just like GSH 2, however, the chemical shift for C-4, δ182.35, showed GSH 26 to be a 5-hydroxyflavone (see table 10, p.66). The spectrum also showed the presence of two methoxyl groups (δ55.42 and δ55.67) together with 15 other carbon atoms of the flavnonoid skeleton.

The possible structure for GSH 26 was therefore 267.

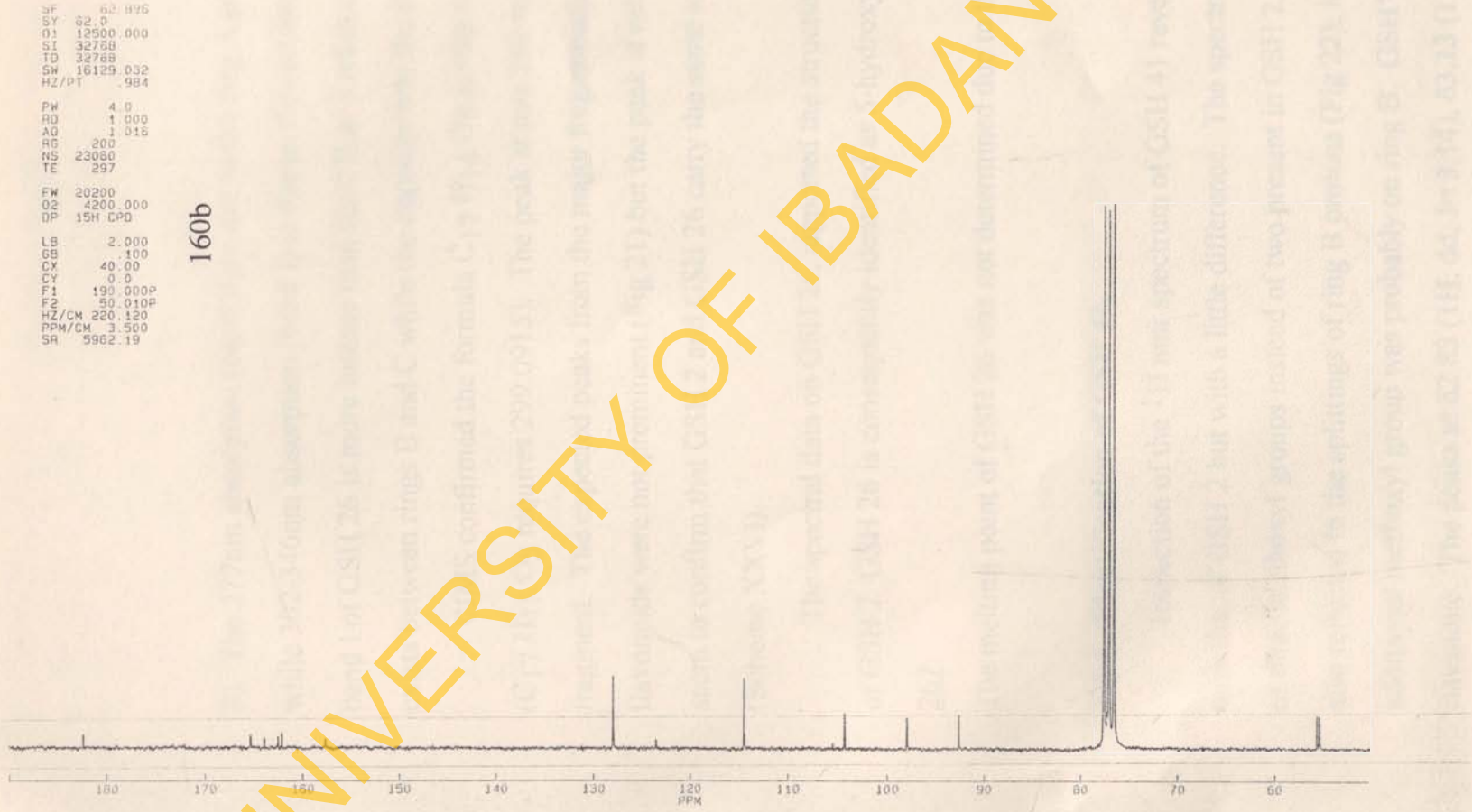


267

This structure has the formula $C_{17}H_{14}O_5$. It is important to mention that the aromatic protons of both rings A and B were shifted down field compared with those of GSH 2. This was expected as there is extended conjugation of B -ring with the α,β-unsaturated keto group in ring C.

The UV. spectrum of GSH 26 gave λmax at 277, 302, and 346 nm. This absorption is consistent with the expected absorption bands of a flavone (see table

Fig. 19. ^{13}C nmr spectrum of GSH 26



8). The 277nm absorption (band II) is due to the ring A portion of the molecule while 302-346nm absorption (band I) is due to the cinnamoyl part (Fig 20). The band I of GSH 26 is more intense than band II as a result of the conjugation which exists between rings B and C while the opposite was the case with GSH 2.

CIMS confirmed the formula $C_{17}H_{14}O_5$ giving m/z 299.0919 $[M+1]^+$ ($C_{17}H_{15}O_5$ requires 299.0915). The peak at m/z 269 represented [M-1]-CO fragment. The expected peaks from the major fragmentation pathway for flavonoids were not prominent (Fig 21) but the peak at m/z 166 (fragment A_1^+) seem to confirm that GSH 2 and GSH 26 carry the same substituents on ring A (Scheme XXVI).

The spectral data on GSH 26 supported the structure as a flavone analogue of GSH 2. GSH 26 is consequently identified as 5-hydroxy 7,4'-dimethoxyflavone, 267.

The melting point of GSH 26 was not determined due to the trace amount isolated.

2.5.4 Identification of GSH 41.

Inspection of the ^1H nmr spectrum of GSH 41 revealed marked similarity with that of GSH 2 but with a little difference. The spectrum showed the presence of three methoxyl groups instead of two present in GSH 2. This difference was also reflected in the splittings of ring B protons (Fig 22), implying that the additional methoxyl group was probably on ring B. GSH 41 is undoubtedly a flavanone. The peaks at δ2.83 (1H, dd, J= 3,14), δ3.13 (1H, dd, J=12,13) and

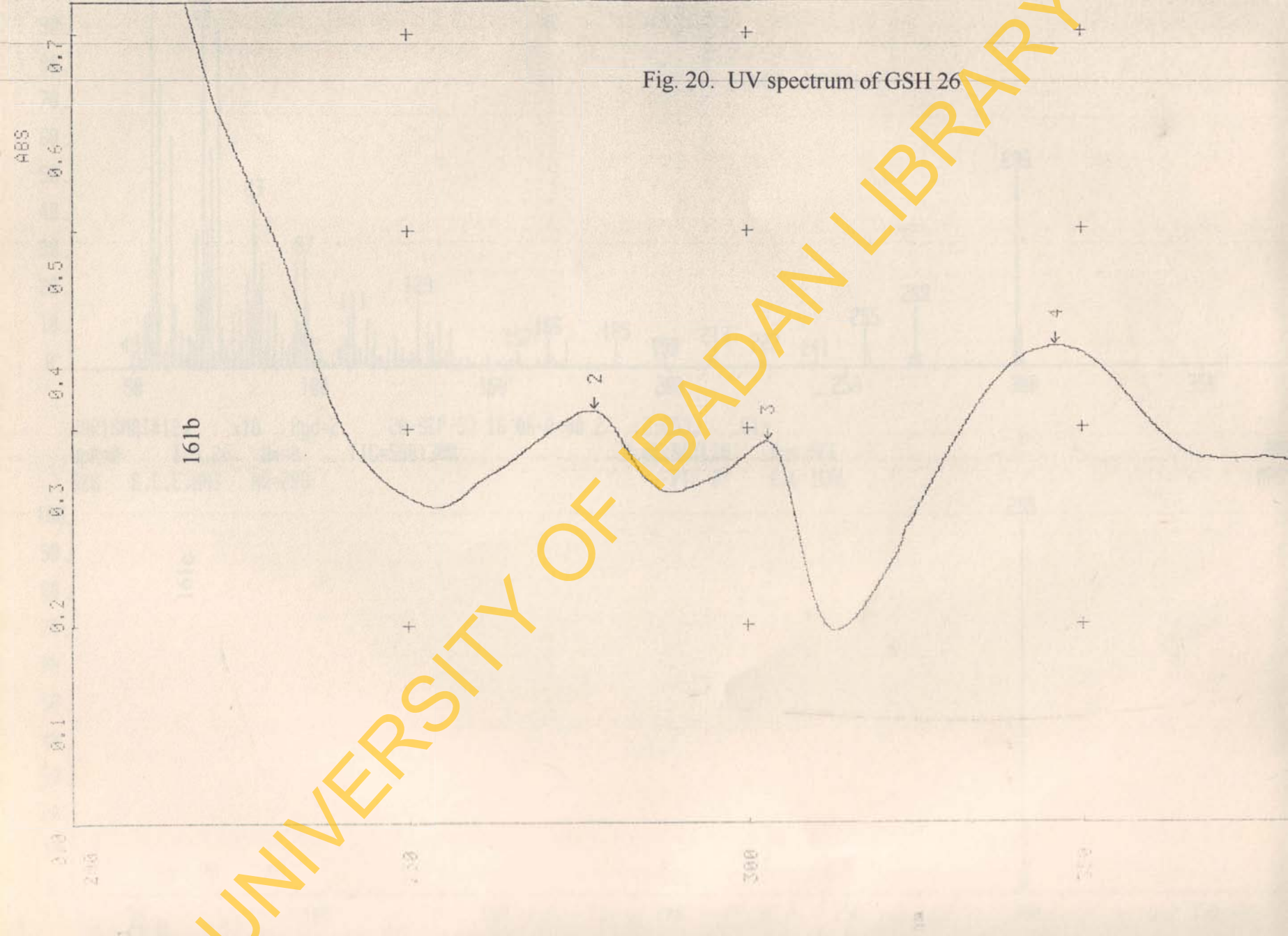


Fig. 20. UV spectrum of GSH 26

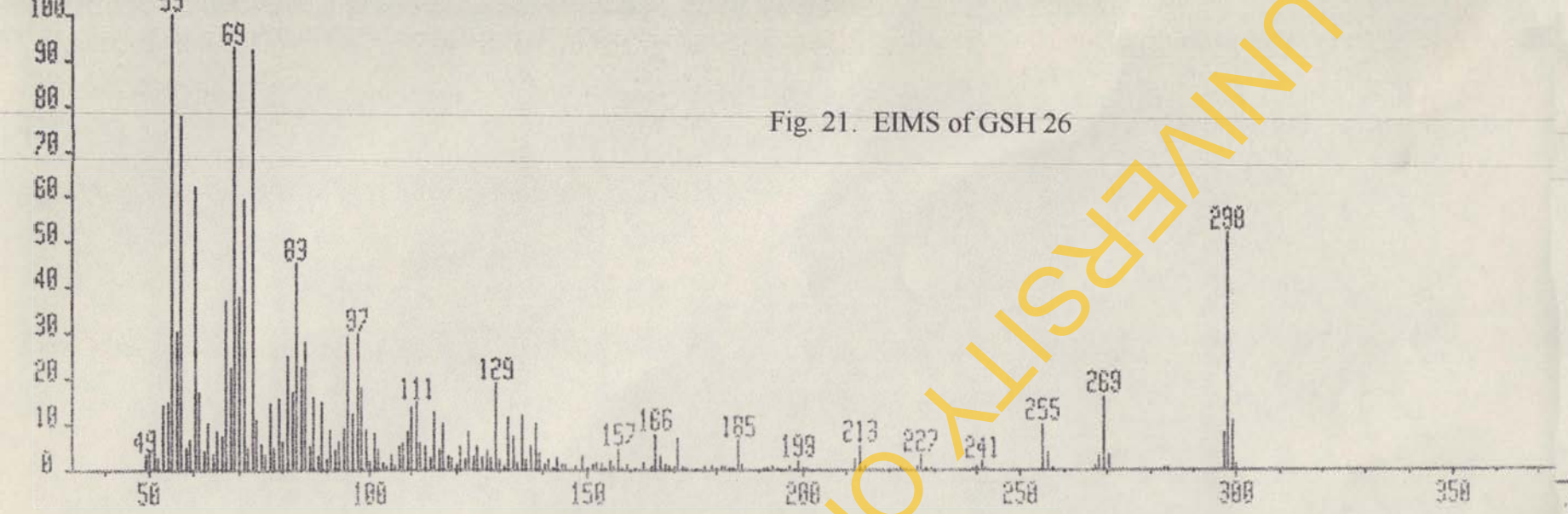
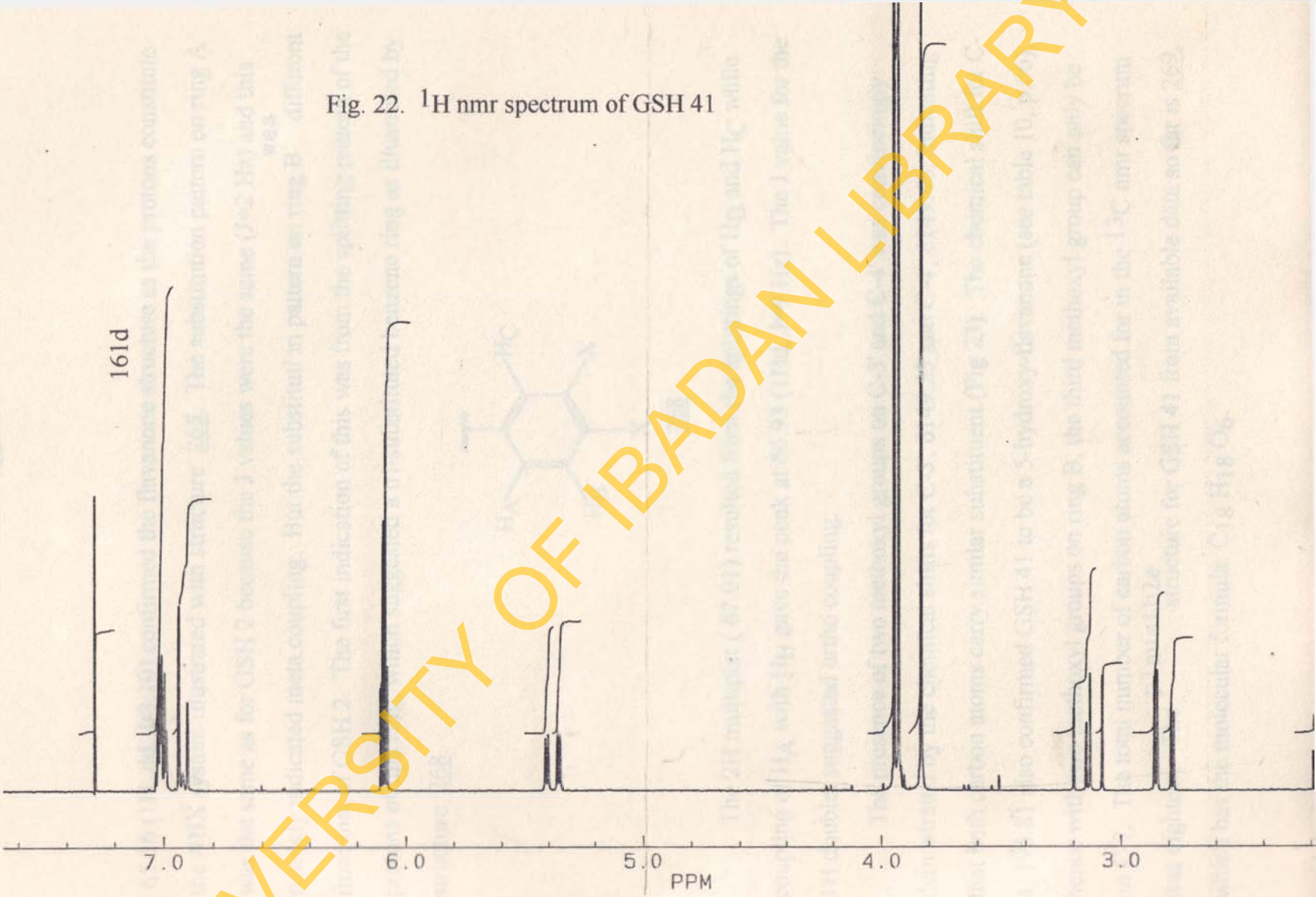
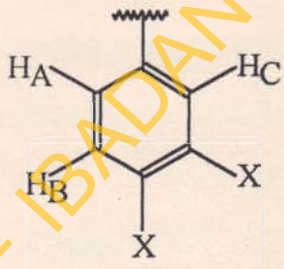


Fig. 21. EIMS of GSH 26

Fig. 22. ^{1}H nmr spectrum of GSH 41



δ5.36 (1H, dd, J=3,10) confirmed the flavanone structure as the protons constitute the ABX system illustrated with structure 265. The substitution pattern on ring A was the same as for GSH 2 because the J values were the same (J=2 Hz) and this strongly indicated meta coupling. But the substitution pattern on ring B was different from that of GSH 2. The first indication of this was from the splitting pattern of the protons of this ring, which suggested a tri-substituted benzene ring as illustrated by structure 268.



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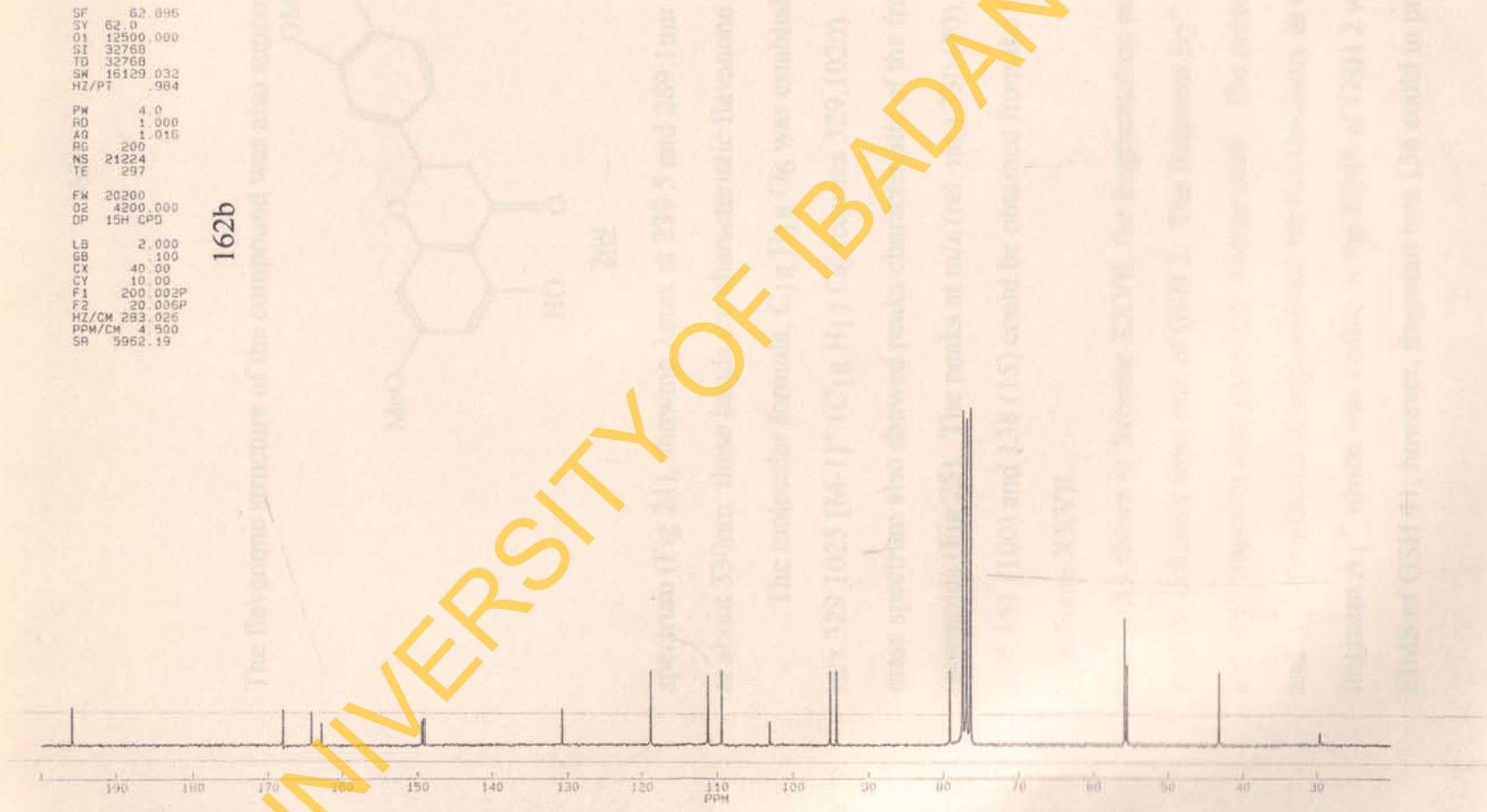
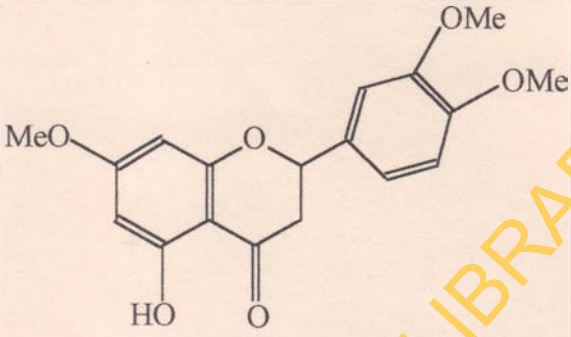
The 2H multiplet (δ7.01) resulted from the splittings of H_B and H_C while coupling of H_A with H_B gave the peak at δ6.93 (1Hd, J=9 Hz). The J value for the 1H doublet suggested ortho coupling.

The presence of two methoxyl groups on C-3' and C-4' was convincingly demostrated by the chemical shifts for C-3', δ149.23 and C-4', δ149.46, indicating that both carbon atoms carry similar substituent (Fig 23). The chemical shift for C-4, 195.81 also confirmed GSH 41 to be a 5-hydroxy-flavanone (see table 10, p.66), hence with two methoxyl groups on ring B, the third methoxyl group can only be on C-7. The total number of carbon atoms accounted for in the ^{13}C nmr specrum was eighteen. The plausible structure for GSH 41 from available data so far is 269, which has the molecular formula. $C_{18}H_{18}O_6$.

Fig. 23. ^{13}C nmr spectrum of GSH 41



The flavanone structure of the compound was also supported by the UV



269

spectrum (Fig 24), showing λmax at 239.5 and 289.1nm together with a weak band at about 330nm. these bands are characteristic flavanone absorptions.

The molecular formula, $C_{18}H_{18}O_6$ was established by EIMS which gave m/z 329.1025 $[M-1]^+$ ($C_{18}H_{17}O_6$ requires 329.1020). In addition to this, the mass spectrum also showed peaks characteristic of the fragmentation pattern of flavonoids (Fig 25). The peaks at m/z (rel. int.) 330 (20), 298 (2), 193 (10), 164 (60) 151 (100) and 138 (15) could be obtained from the fragmentations represented in Scheme XXVII.

As shown in Scheme XXVII, the fragmentation pattern of GSH 41 was slightly different from that of GSH 2. The fragment B_2^+ appeared as the base peak and B_3^+ fragment also gave very intense peak. The presence of the fragments B_3^+ and A_2^+ provided a strong evidence for the similarity in GSH 2 and GSH 41. The fragment A_1^+ which was visible in the EIMS of GSH 2 was not observed in the EIMS of GSH 41, however, fragment m/z 138 could in principle

Fig. 24. UV spectrum of GSH 41

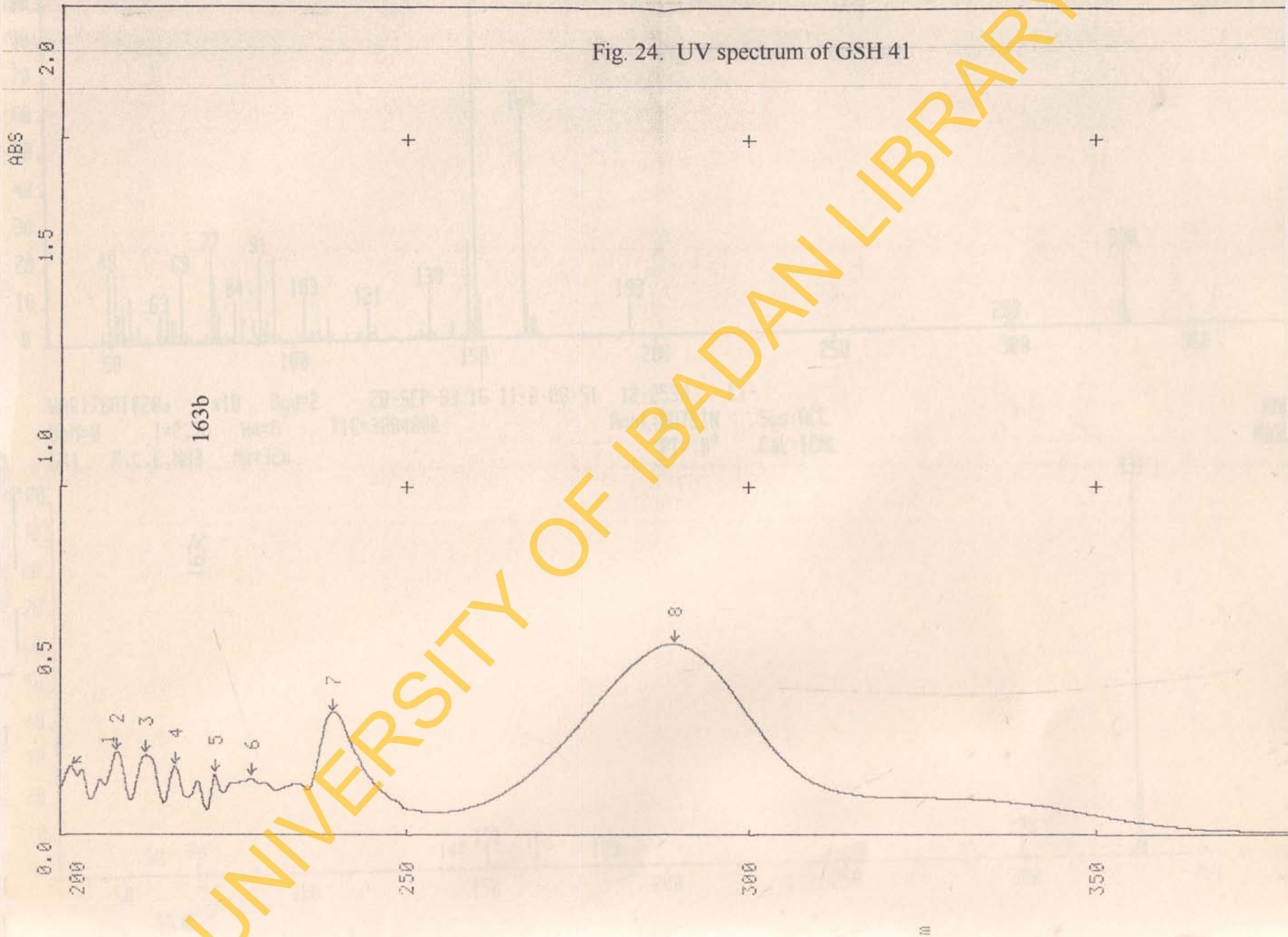
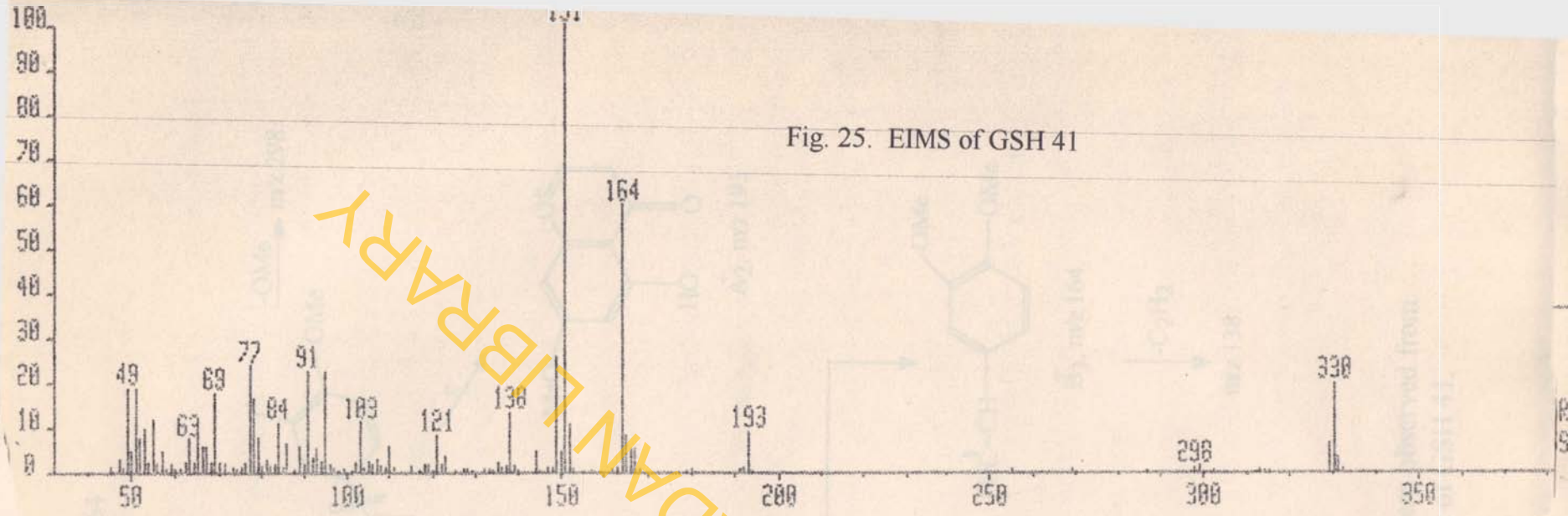
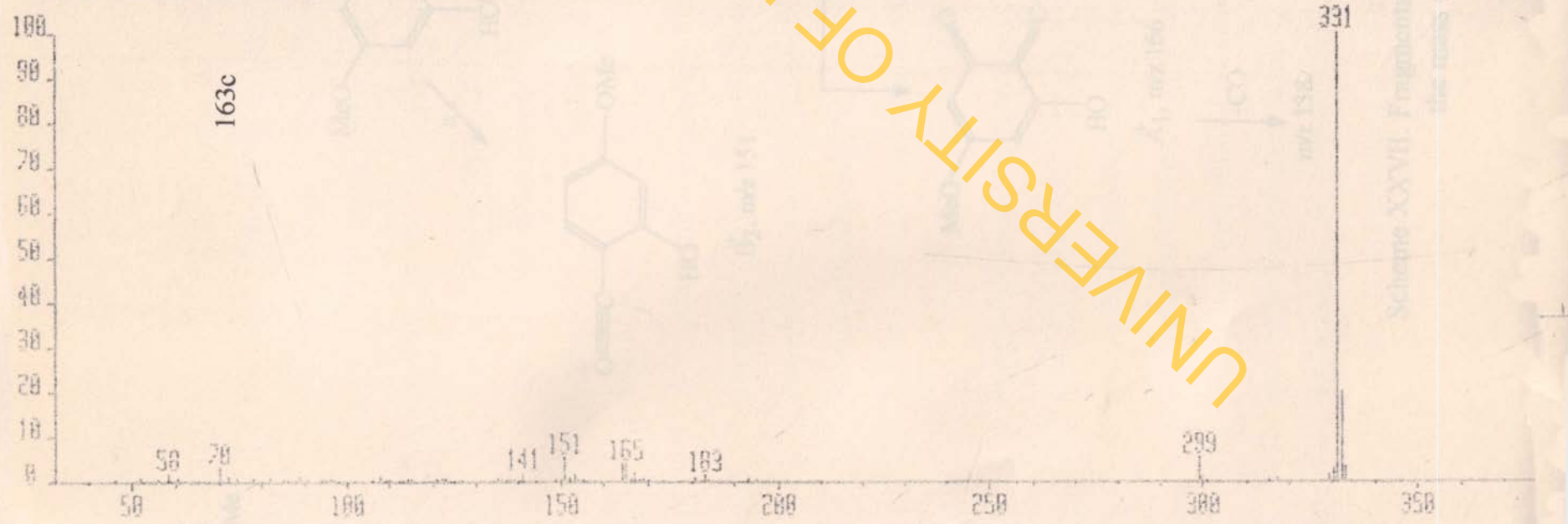
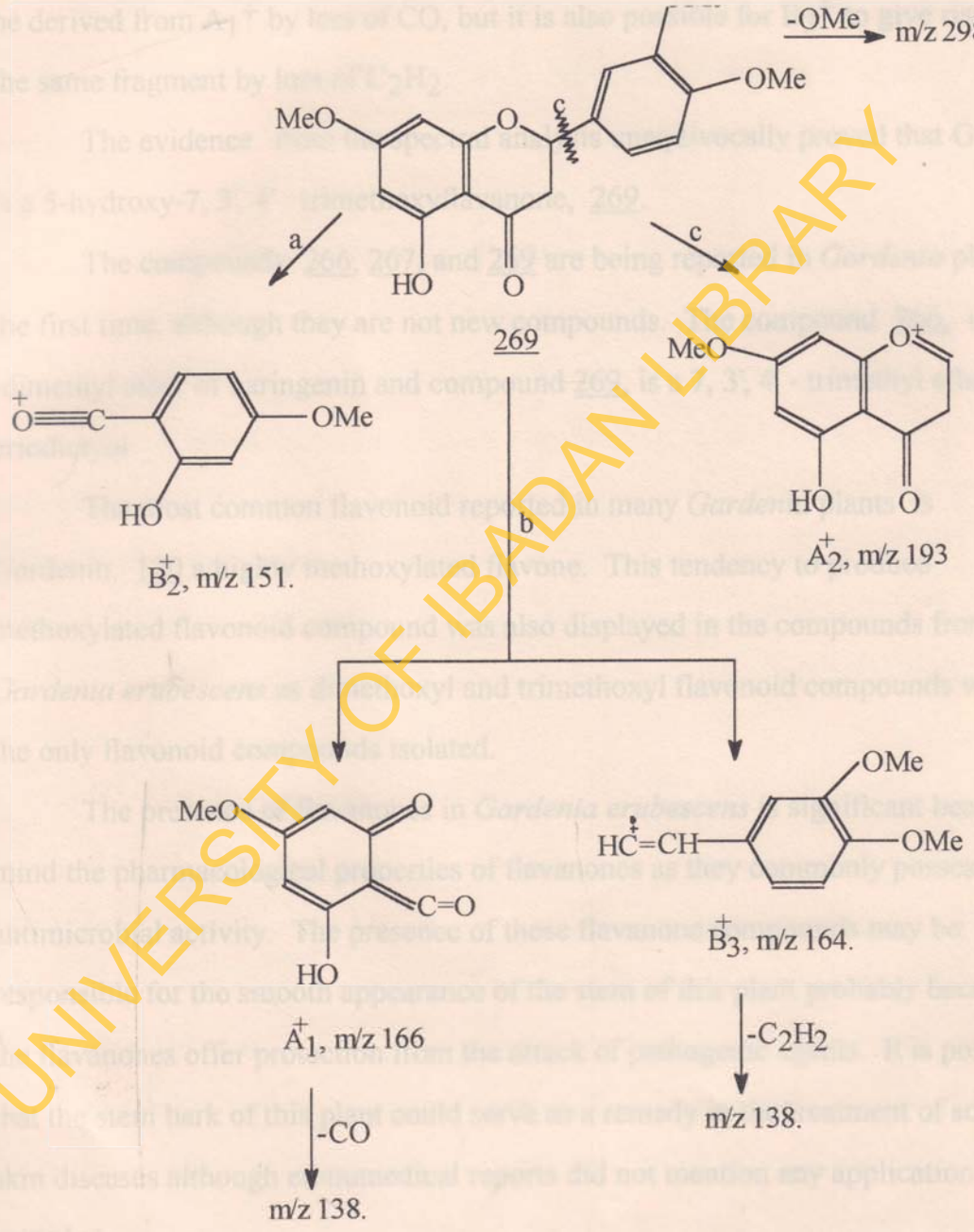


Fig. 25. EIMS of GSH 41





OMe



Scheme XXVII. Fragmentation pattern observed from the mass spectrum of GSH 41.

be derived from A_1^+ by less of CO, but it is also possible for B_3^+ to give rise to the same fragment by loss of C_2H_2.

The evidence from the spectral analysis unequivocally proved that GSH 41 is a 5-hydroxy-7, 3', 4' - trimethoxyflavanone, 269.

The compounds 266, 267, and 269 are being reported in *Gardenia* plant for the first time, although they are not new compounds. The compound 266, is a 7,4' -dimethyl ether of naringenin and compound 269, is a 7, 3', 4' - trimethyl ether of eriodictyol.

The most common flavonoid reported in many *Gardenia* plants is Gardenin, 120 a highly methoxylated flavone. This tendency to produce methoxylated flavonoid compound was also displayed in the compounds from *Gardenia erubescens* as dimethoxyl and trimethoxyl flavonoid compounds were the only flavonoid compounds isolated.

The presence of flavanones in *Gardenia erubescens* is significant bearing in mind the pharmacological properties of flavanones as they commonly possess antimicrobial activity. The presence of these flavanone compounds may be responsible for the smooth appearance of the stem of this plant probably because the flavanones offer protection from the attack of pathogenic agents. It is possible that the stem bark of this plant could serve as a remedy in the treatment of some skin diseases although ethnomedical reports did not mention any application in this regard.

The report on the treament of gonorrhoea could not possibly be attributed to the flavanone component because aqueous extracts were reportedly used for such treatment.

2.5.5 Determination of Structure of GSH 14.

The mass spectrum of GSH 14 (fig 26) established the formula $C_{29}H_{48}O$ with m/z 412.3705 [M^+] ($C_{29}H_{48}O$ requires 412.3693). The compound gave a positive Liebermann-Burchard reaction, suggesting a steroid structure. The ^1H nmr spectrum (Fig 27) gave further support for the steroidal structure as majority of the peaks were found upfield. The peaks at δ5.15 (2Hm) and δ5.36 (1H,d, J=5) revealed the presence of olefinic protons while the signal at δ3.57 (1H, m) indicated the presence of carbinolic proton. The 2H multiplet at δ5.15 suggested a symmetrically substituted double bond.

The ^{13}C nmr spectrum (Fig 28) revealed the presence of a secondary hydroxyl carbon (δC, 71.7) and four olefinic carbon atoms (δC, 121.6, 129.2, 130.2, and 140.6) The four olefinic carbon atoms confirmed the presence of two double bonds in the compou nd. The number of carbon signals in the ^{13}C nmr spectrum added up to only twenty-six as it is common for some peaks to overlap.

The indication for the presence of hydroxyl group was also visible in the IR spectrum (Fig 29) with bands at Vmax 3426 cm^{-1} (-OH Str), 1063 cm^{-1} (C-O Str). It was also obvious from the IR spectrum that the bulk of the compound is majorly hydrocarbon in composition. The presence of double bond was also indicated by

Fig. 26. EIMS of GSH 14

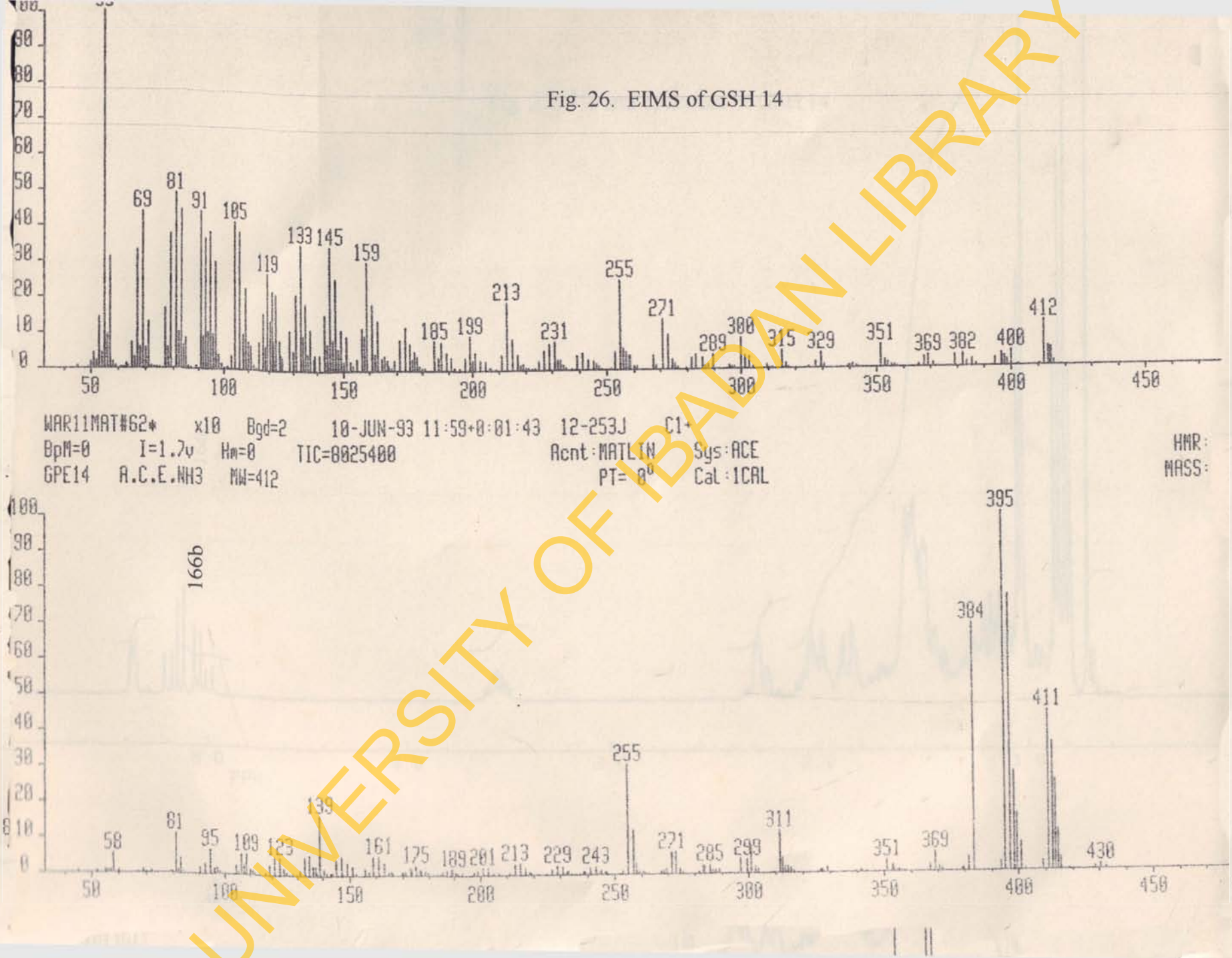


Fig. 27. ^{1}H nmr spectrum of GSH 14

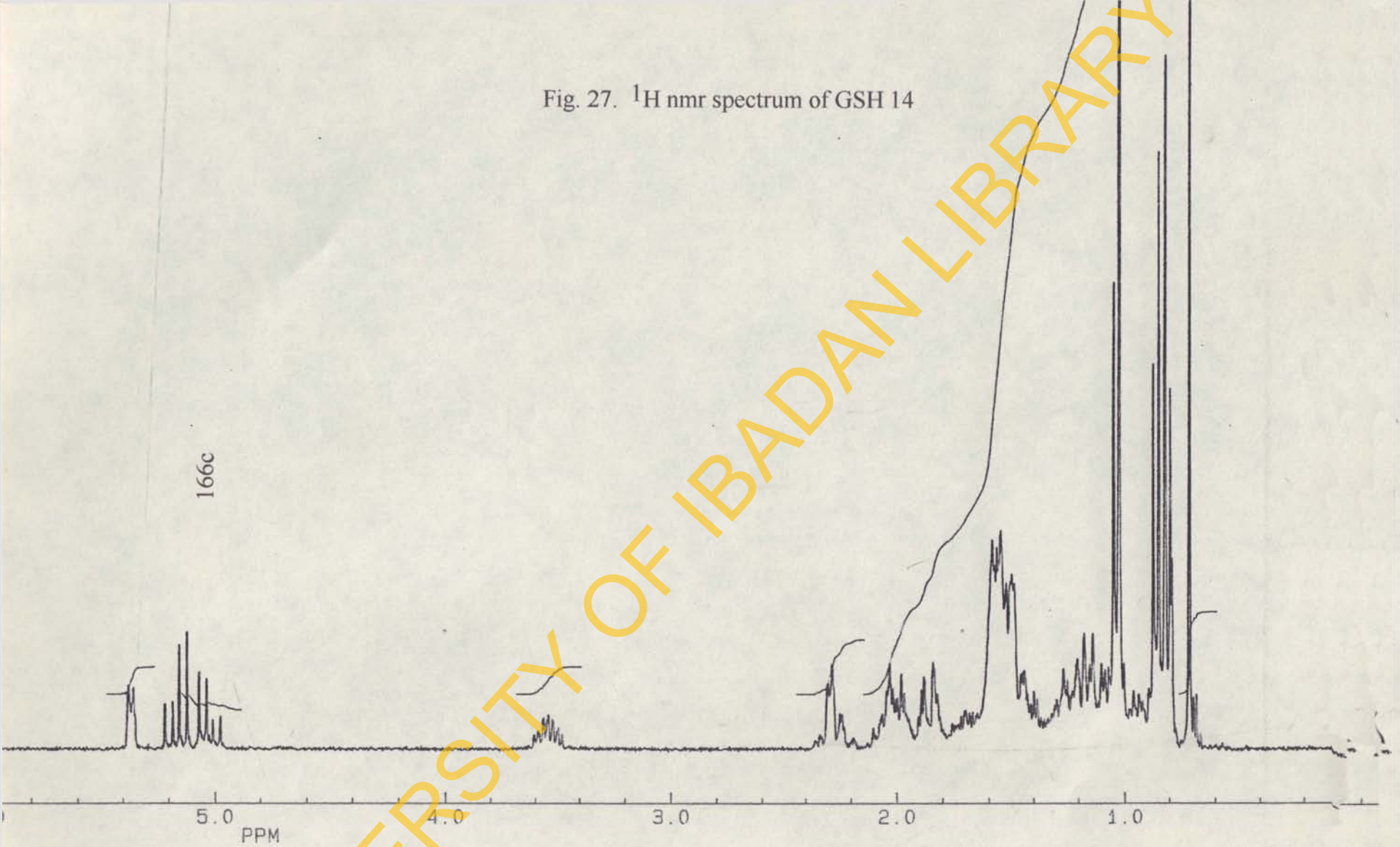
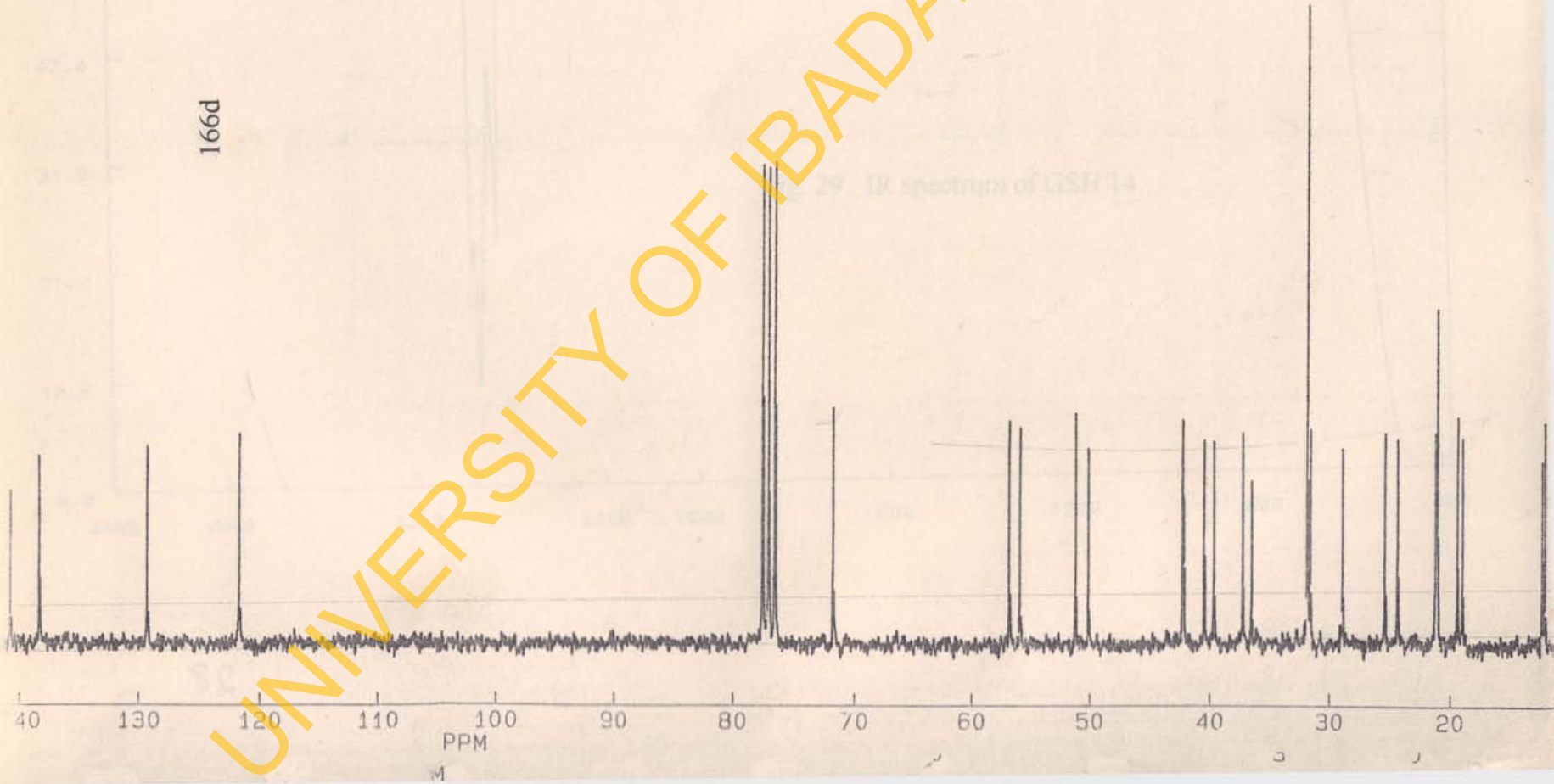


Fig. 28. ^{13}C nmr spectrum of GSH 14



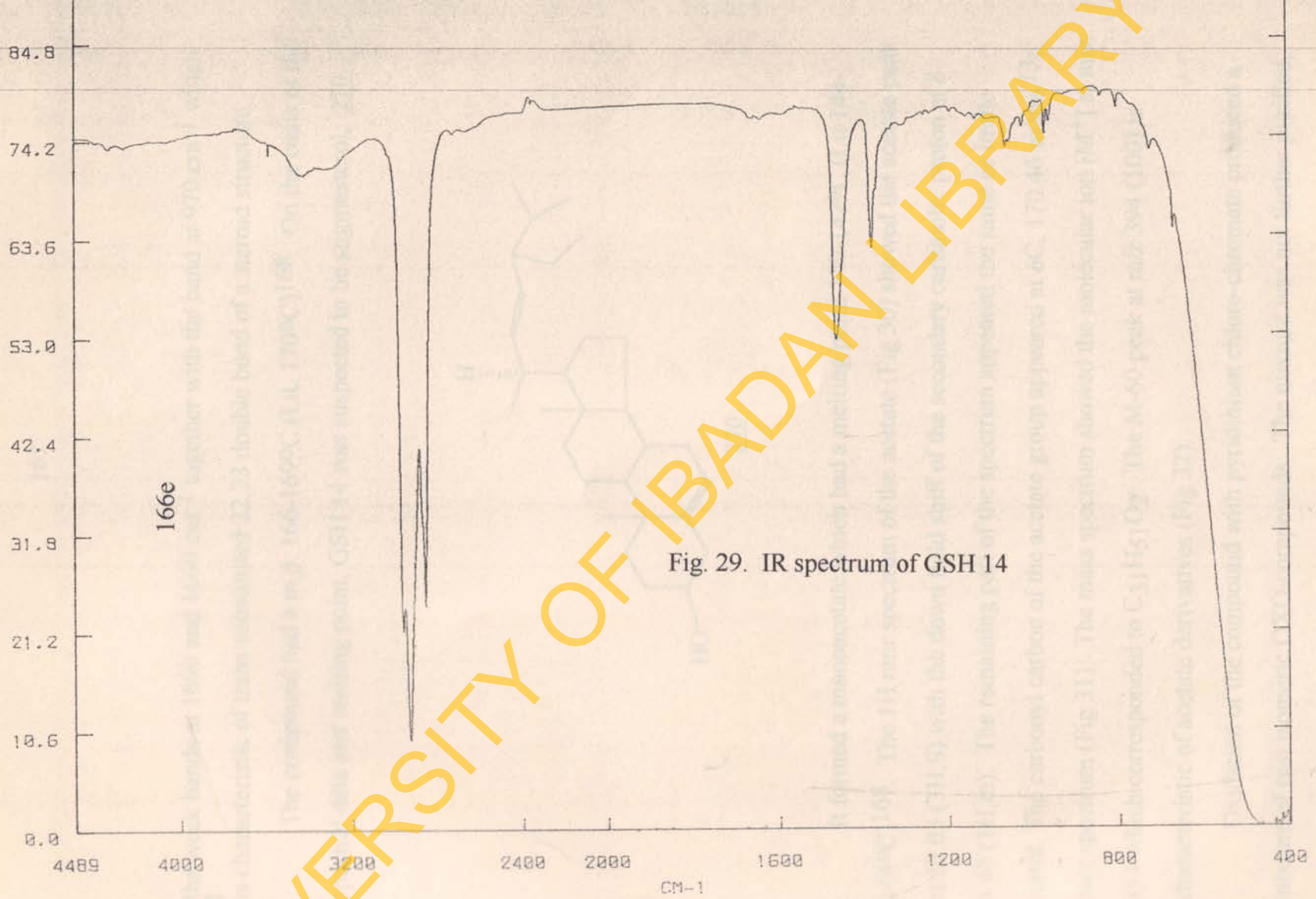
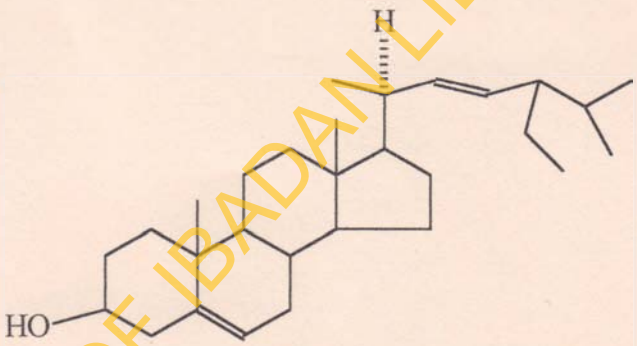


Fig. 29. IR spectrum of GSH 14

the weak bands at 1690 and 1660 cm^{-1} together with the band at 970 cm^{-1} which is characteristic of trans substituted 22,23 double bond of a steroid structure.

The compound had a m.p. 166-169°C (Lit. 170°C)[168]. On the basis of the spectral data and melting point, GSH 14 was suspected to be stigmasterol, 270.



270

It formed a monoacetate which had a melting point 140-142°C (Lit 144-144°C)[168]. The 1H nmr spectrum of the acetate (Fig 30) showed the acetate peak at δ2.05 (3H,S) with the down field shift of the secondary carbinolic proton at δ 4.63 (1H,m). The remaining part of the spectrum appeared the same as in the sterol. The carbonyl carbon of the acetate group appeared at δC, 170.46 in the ^{13}C nmr spectrum (Fig 31). The mass spectrum showed the molecular ion [M^+] at m/z 454 which corresponded to $C_{31}H_{51}O_2$. The M-60 peak at m/z 394 (100) is characteristic of acetate derivatives (Fig 32).

Oxidation of the compound with pyridinium chloro-chromate produced a mixture of two isomeric OXO-compounds. The mixture was not further purified,

Fig. 30. ^{1}H nmr spectrum of GSH 14 acetate

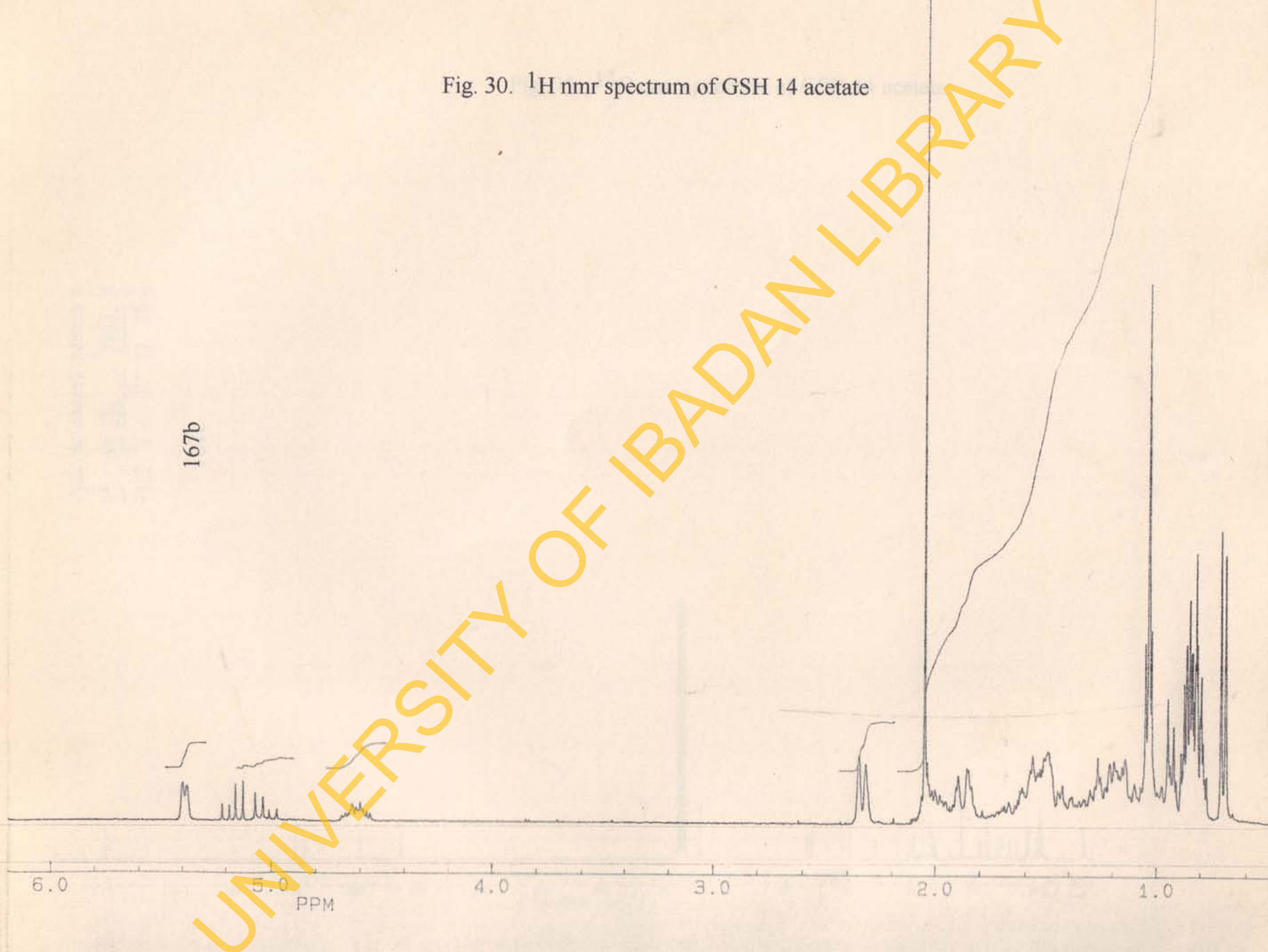


Fig. 31. ^{13}C nmr spectrum of GSH 14 acetate

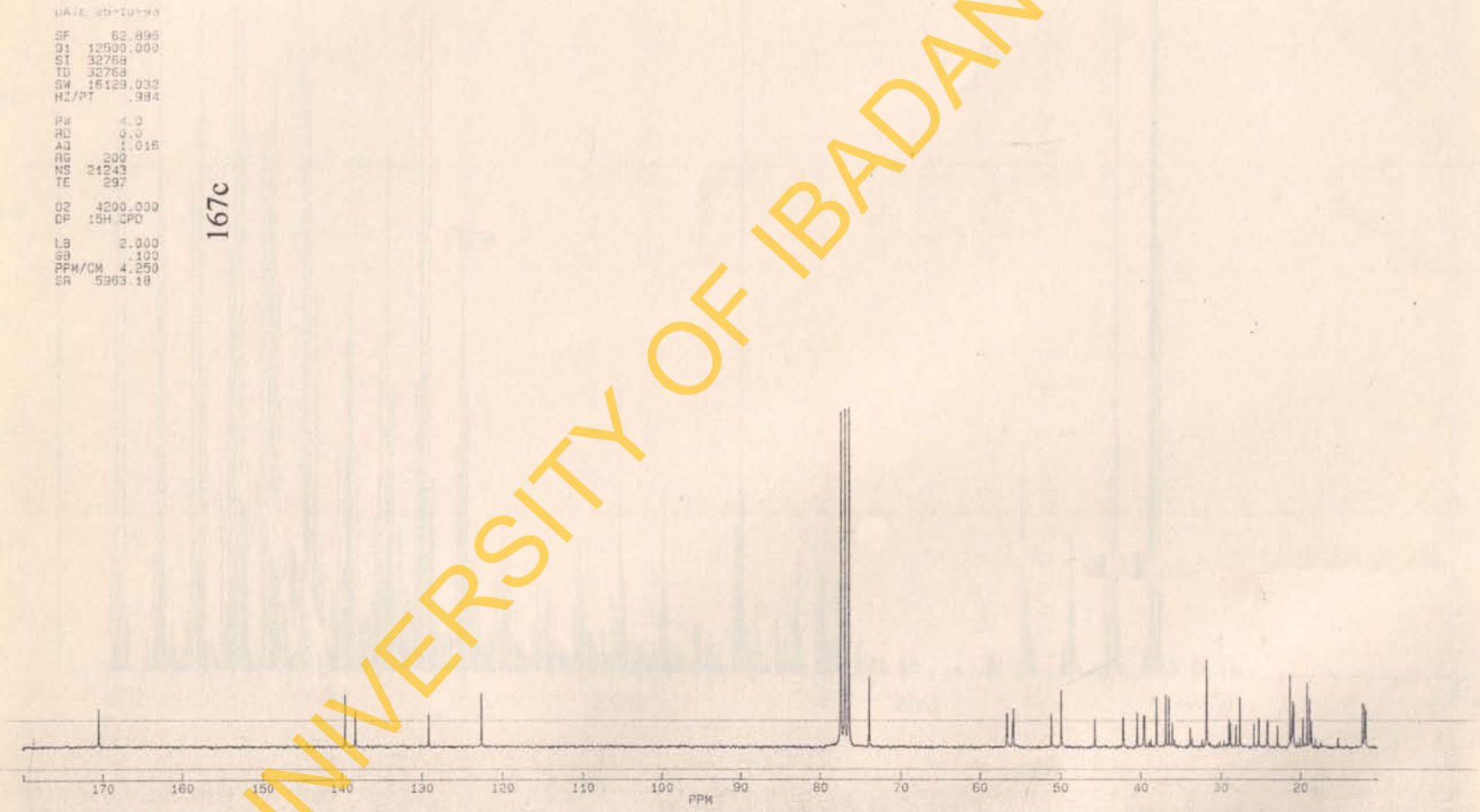
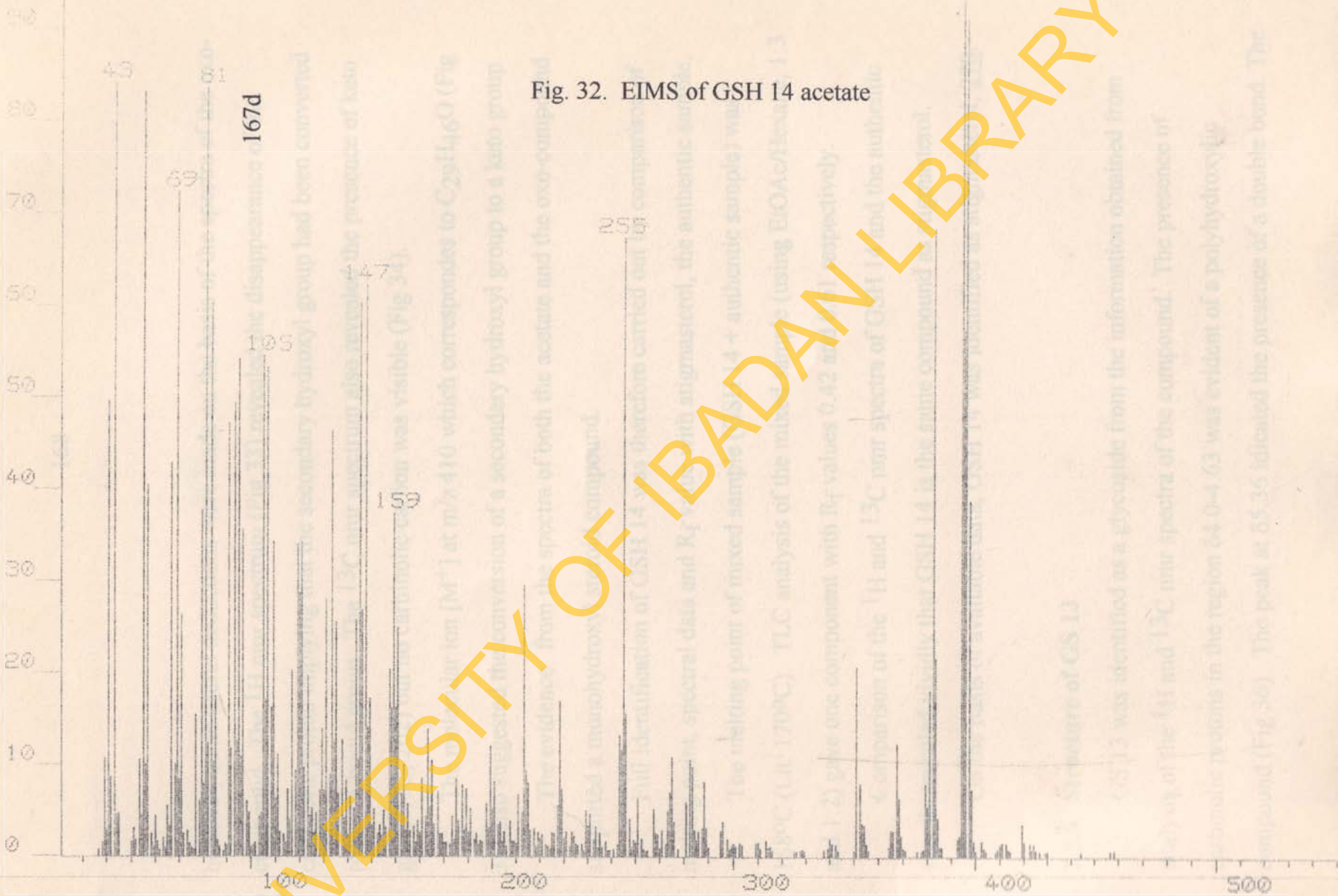


Fig. 32. EIMS of GSH 14 acetate



however, some useful deductions were made on the basis of the spectra of the oxo-compound. The ^{1}H nmr spectrum (Fig 33) revealed the disappearance of the carbinolic proton implying that the secondary hydroxyl group had been converted to the carbonyl group. The ^{13}C nmr spectrum also revealed the presence of keto group (δC, 202) but no carbinolic carbon was visible (Fig 34).

The molecular ion [M^{+}] at m/z 410 which correspondes to $C_{29}H_{46}O$ (Fig 35) also suggested the conversion of a secondary hydroxyl group to a keto group.

The evidence from the spectra of both the acetate and the oxo-compound supported a monohydroxyl steroid compound.

Full identification of GSH 14 was therefore carried out by comparison of melting point, spectral data and R_f value with stigmasterol, the authentic sample.

The melting point of mixed sample (GSH 14 + authentic sample) was 168°C (Lit. 170°C). TLC analysis of the mixed sample (using EtOAc/Hexane, 1:3 and 1:2) gave one component with R_f values 0.42 and 0.61 respectively.

Comparison of the ^{1}H and ^{13}C nmr spectra of GSH 14 and the authentic sample revealed vividly that GSH 14 is the same compound as stigmasterol.

On the basis of available data, GSH 14 was identified as stigmasterol, 270.

2.5.6 Structure of GS 13

GS 13 was identified as a glycoside from the information obtained from analysis of the ^{1}H and ^{13}C nmr spectra of the compound. The presence of carbinolic protons in the region δ4.0-4.63 was evident of a polyhydroxylic compound (Fig 36). The peak at δ5.36 idicated the presence of a double bond. The

Fig. 33. ^{1}H nmr spectrum of GSH 14 oxo-deriv.

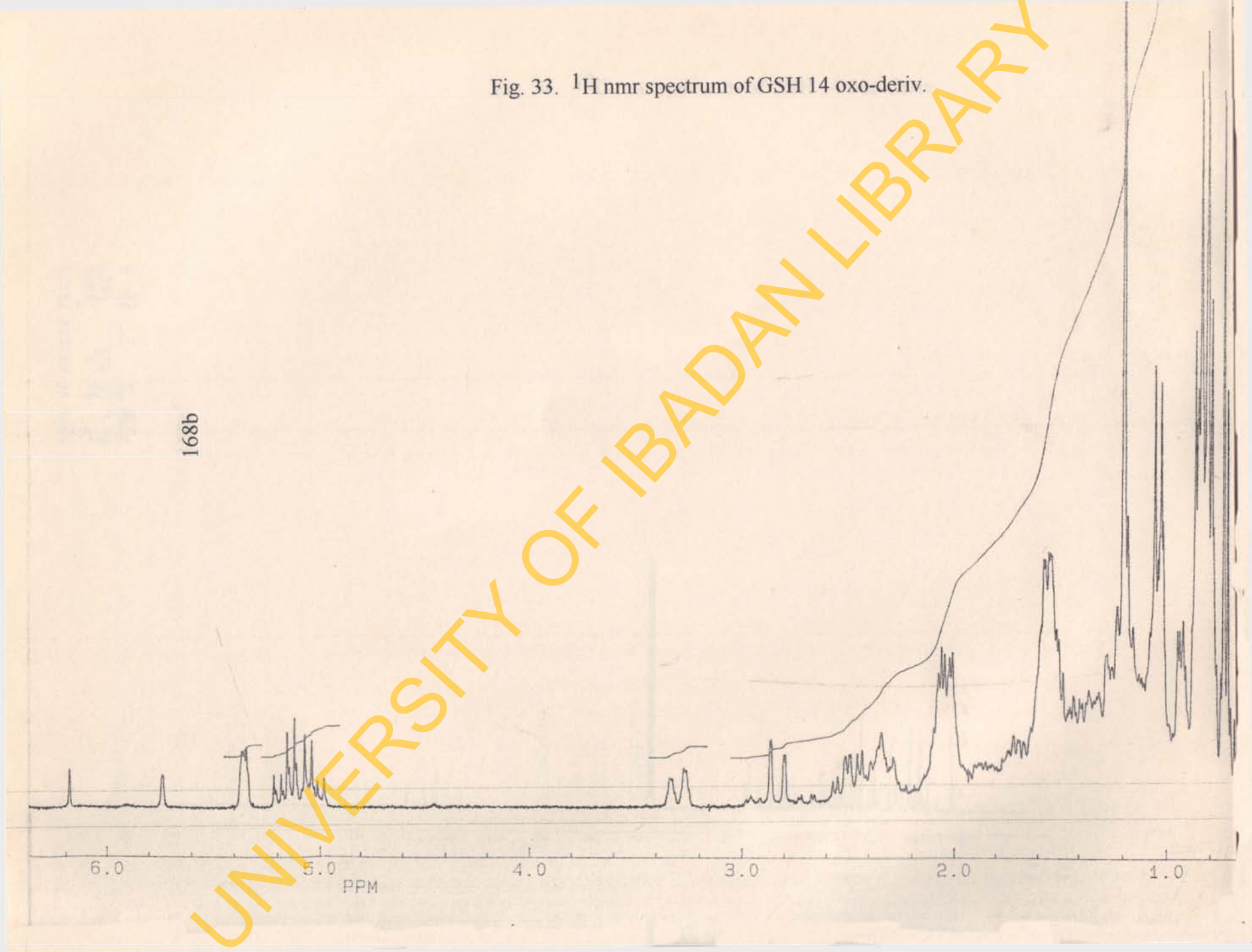


Fig. 34. ^{13}C nmr spectrum of GSH 14 oxo-deriv.

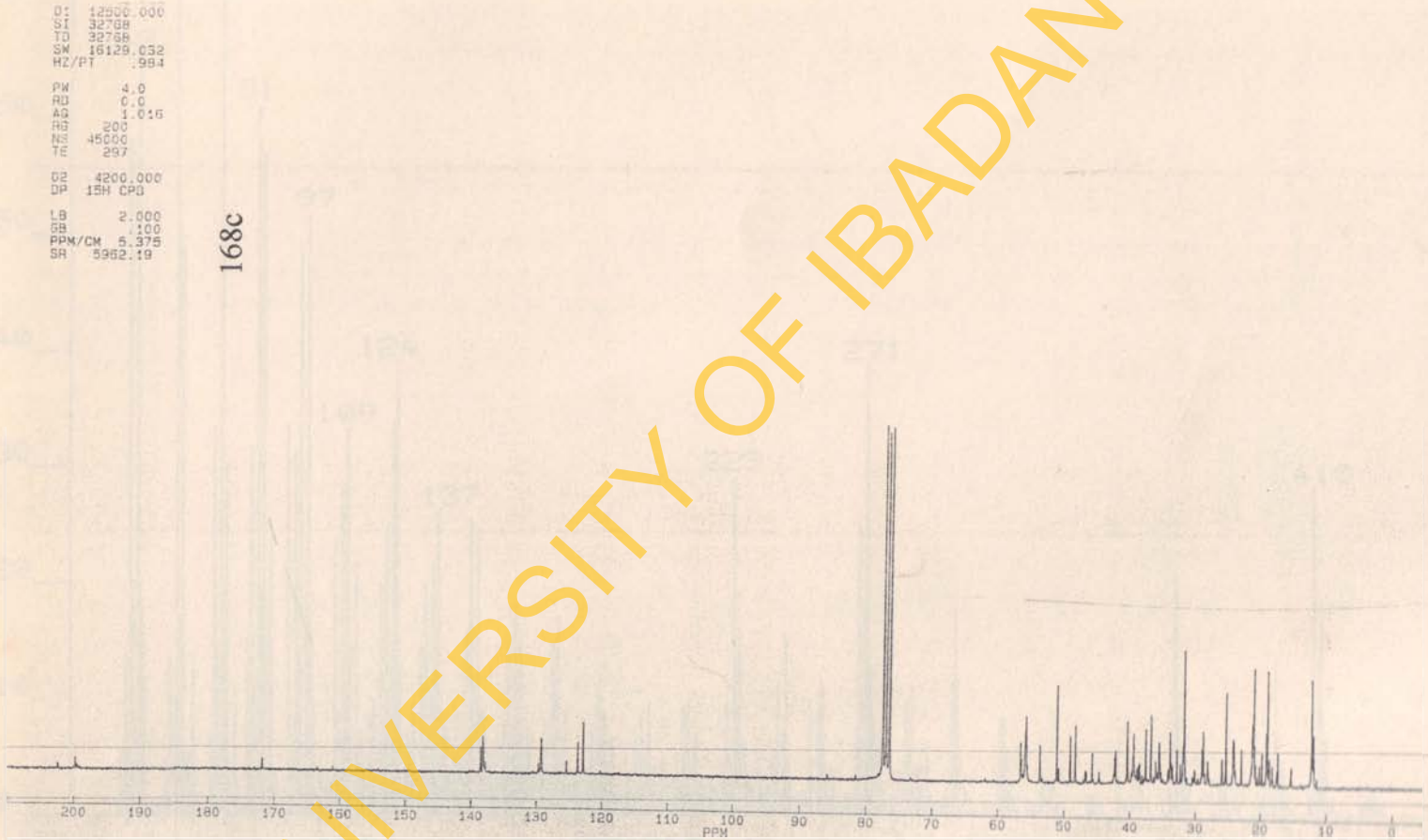


Fig. 35. EIMS of GSH 14 oxo-deriv.

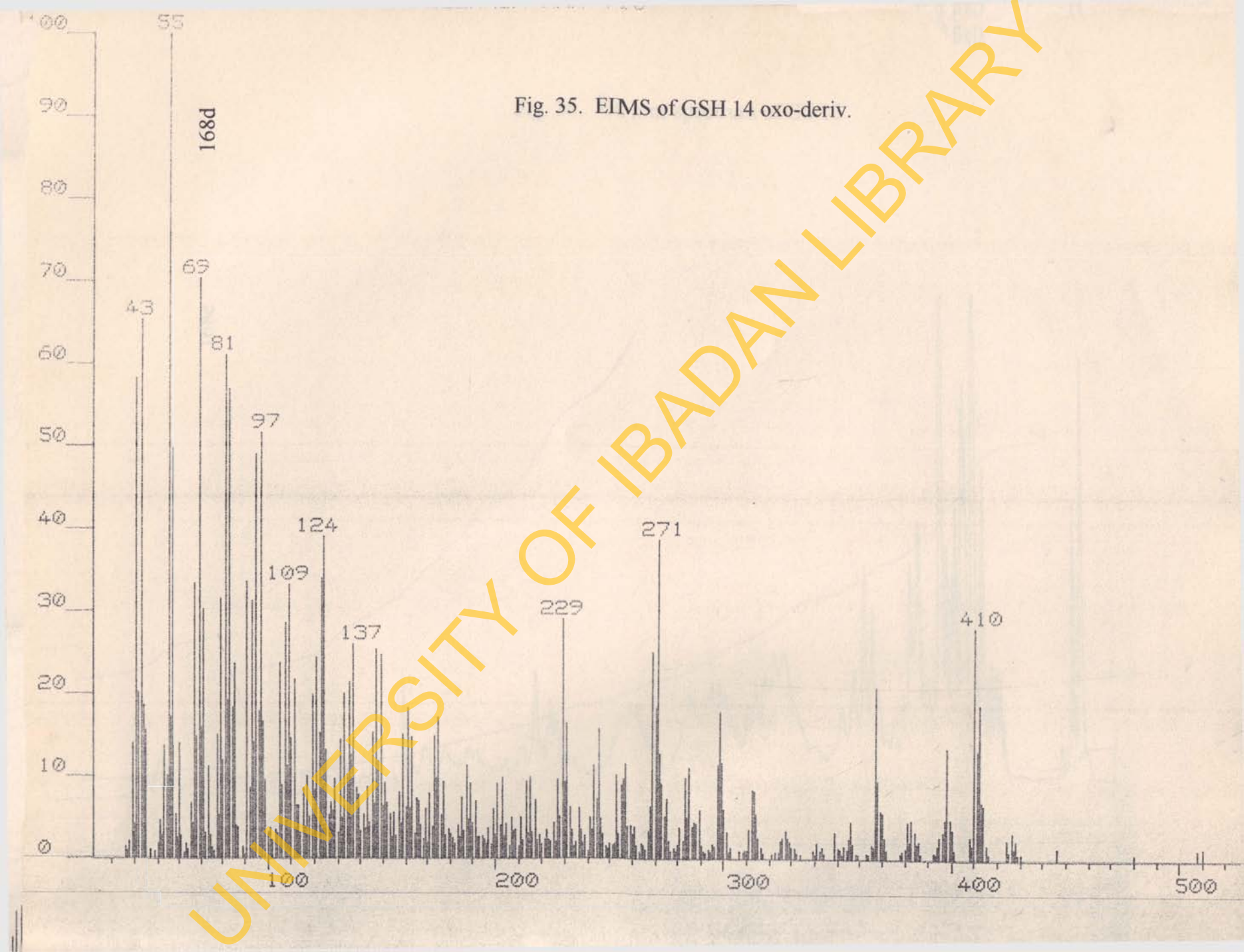
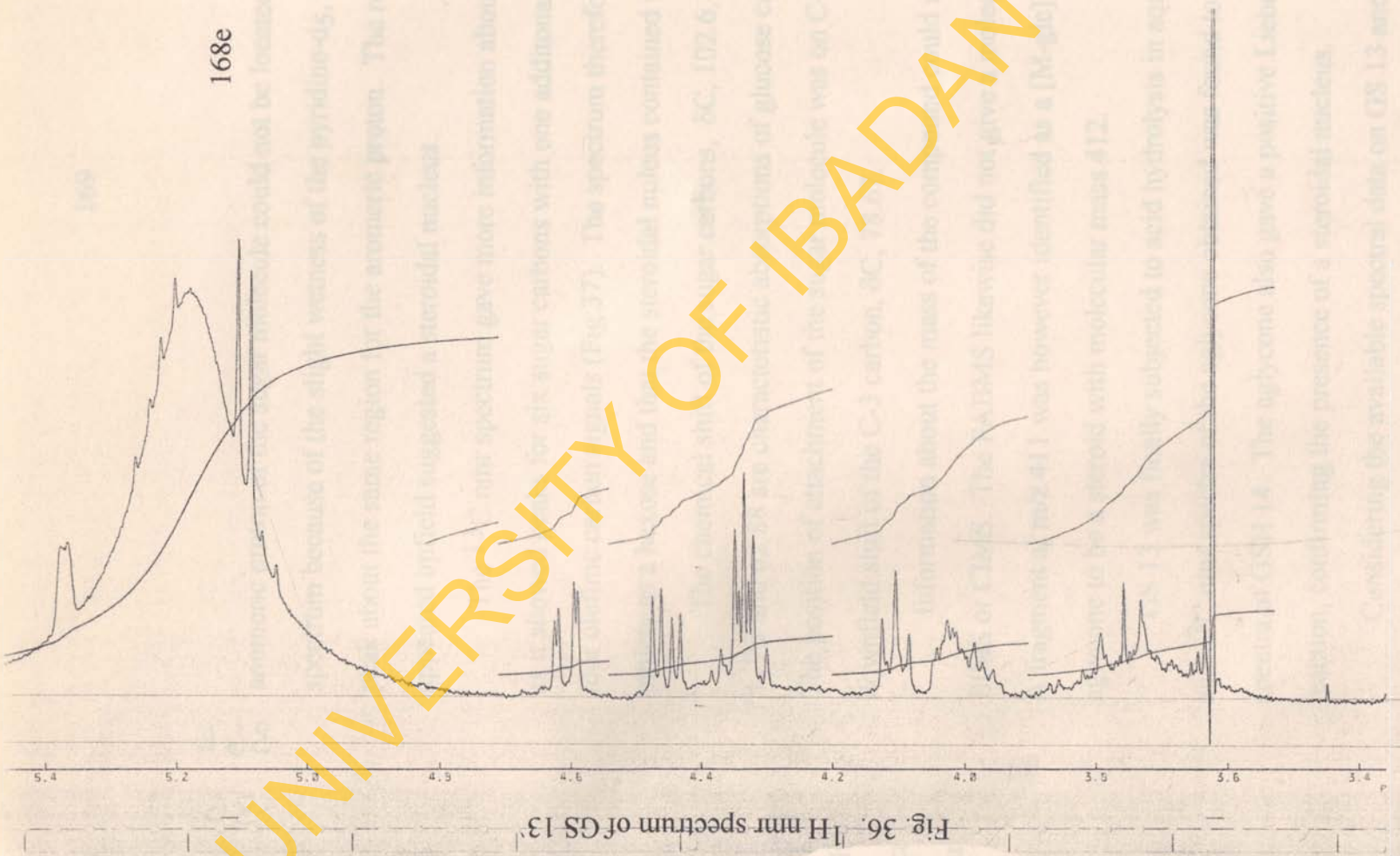


Fig. 36. ^{1}H nmr spectrum of GS 13`



anomeric proton of the sugar molecule could not be located on the ^{1}H nmr spectrum because of the slight wetness of the pyridine-d_5, giving a broad water peak about the same region for the aromeric proton. The remaining peaks which appeared upfield suggested a steroidal nucleus.

The ^{13}C nmr spectrum gave more information about the structure of GS 13 as it showed peaks for six sugar carbons with one additonal carbinolic carbon and four olefinic carbon signals (Fig 37). The spectrum therefore revealed the sugar moiety as a hexose and that the steroidal nuleus contained two double bonds.

The chemical shift of the sugar carbons, δC, 102.6, 78.56, 78.16, 75.40, 71.75 and 62.88 are characteristic absorptions of glucose carbon atoms[169-170]. The position of attachment of the sugar molecule was on C-3 and this resulted in a downfield shift of the C-3 carbon, δC, 78.67.

Information about the mass of the compound could not be obtained with EIMS or CIMS. The FABMS likewise did not give a molecular ion peak (Fig 38). A fragment at m/z 411 was however identified as a [M-glc] peak, suggesting the aglycone to be a steroid with molecular mass 412.

GS 13 was finally subjected to acid hydrolysis in aqueous methanol. ^{1}H and ^{13}C nmr spectra of the aglycone obtained was found to be identical to the spectra of GSH 14. The aglycone also gave a positive Liebermann-Burchard reaction, confirming the presence of a steroidal nucleus.

Considering the available spectral data on GS 13 and its aglycone, GS 13 was identified as stigmasterol 3β-glucopyranoside, 271.

Fig. 37. ^{13}C nmr spectrum of GS 13

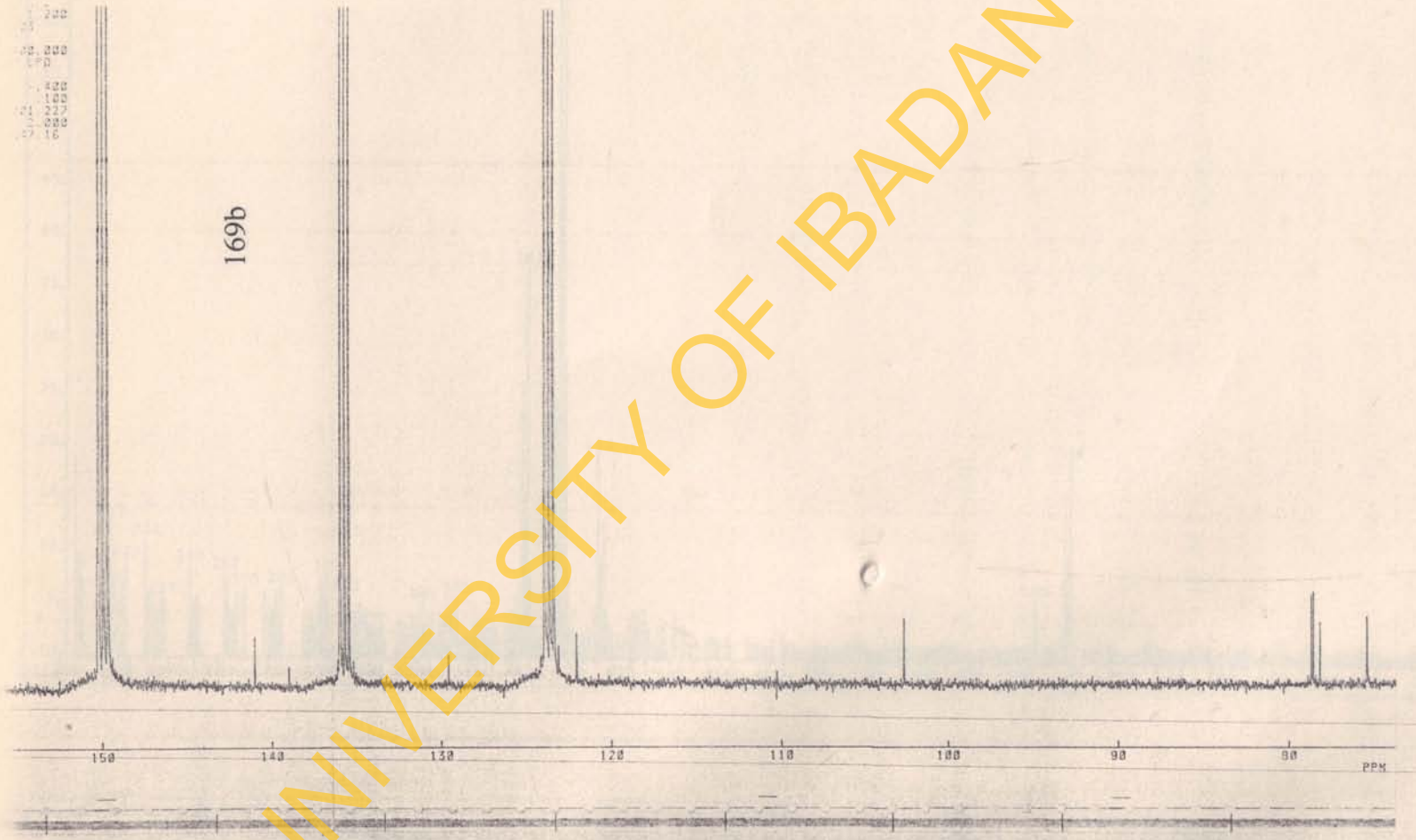
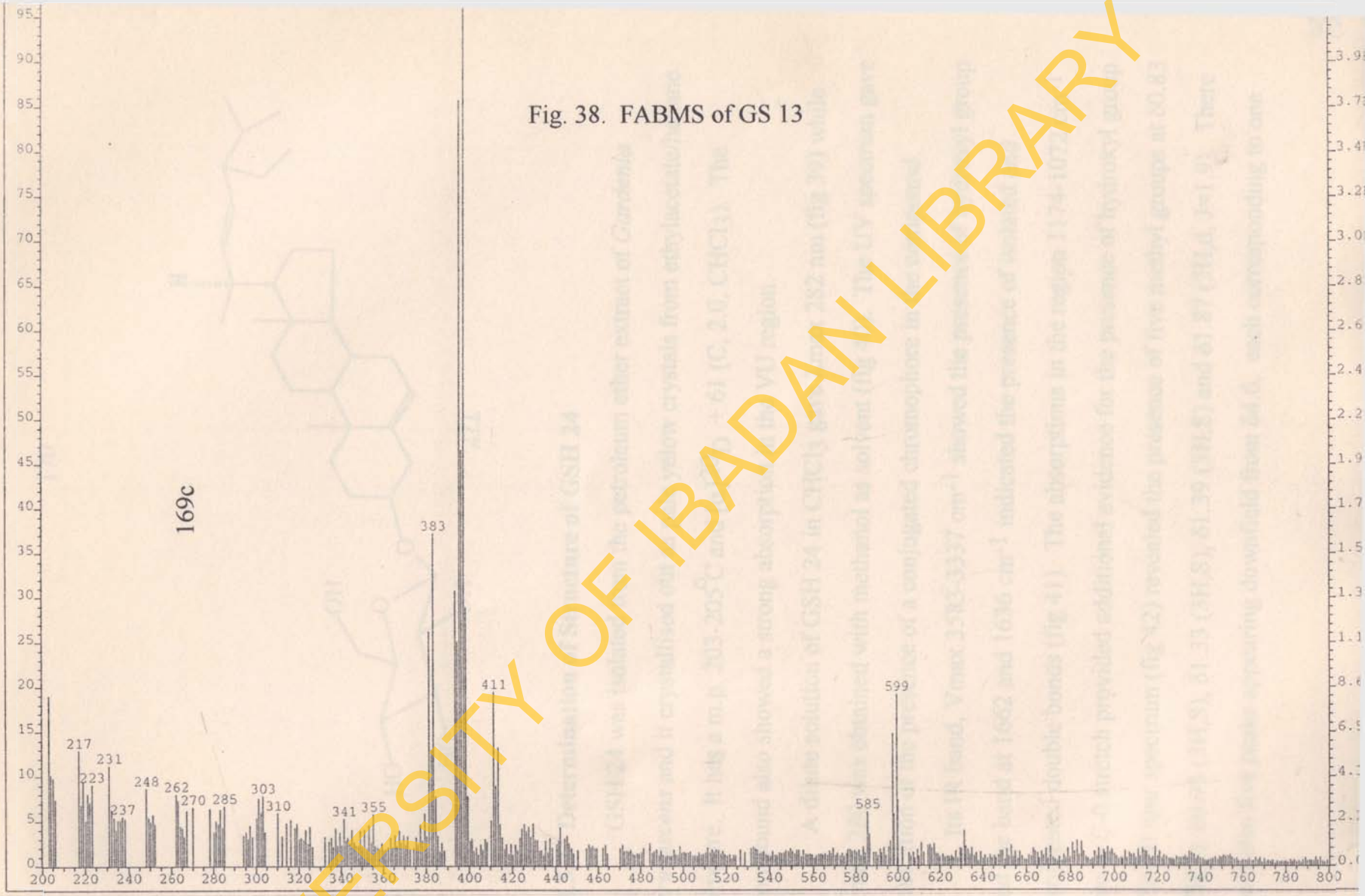


Fig. 38. FABMS of GS 13



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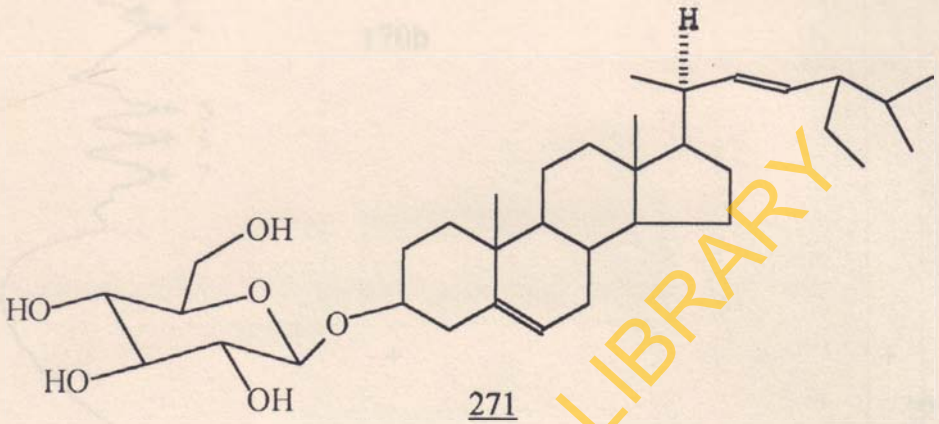

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2.5.7 Determination of Structure of GSH 24

GSH 24 was isolated from the petroleum ether extract of *Gardenia erubescens* and it crystallised out as pale yelow crystals from ethylacetate/hexane mixture. It has a m.p. 203-205°C and $[\alpha]^{20}_{D}$ + 61 (C, 2.0, $CHCl_3$). The compound also showed a strong absorption in the VU region.

A dilute solution of GSH 24 in $CHCl_3$ gave λmax 282 nm (fig 39) while λmax 280 was obtained with methanol as solvent (fig 40). The UV spectrum gave indication of the presence of a conjugated chromophore in the compound.

Its IR band, Vmax 3583-3337 cm^{-1} showed the presence of hydroxyl group and the band at 1662 and 1636 cm^{-1} indicated the presence of isolated and conjugated double bonds (fig 41). The absorptions in the region 1174-1072 cm^{-1} due to C-0 stretch provided additional evidence for the presence of hydroxyl group. The ^1H nmr spectrum (fig 42) revealed the presence of five methyl groups at δ0.83 (3H,S), δ0.93 (3H,S), δ1.33 (3H,S), δ1.39 (3H,S) and δ1.87 (3H,d, J=1.9). There were also five peaks appearing downfield from δ4.6, each corresponding to one

Fig. 39. UV spectrum of GSH 24 in $CHCl_3$

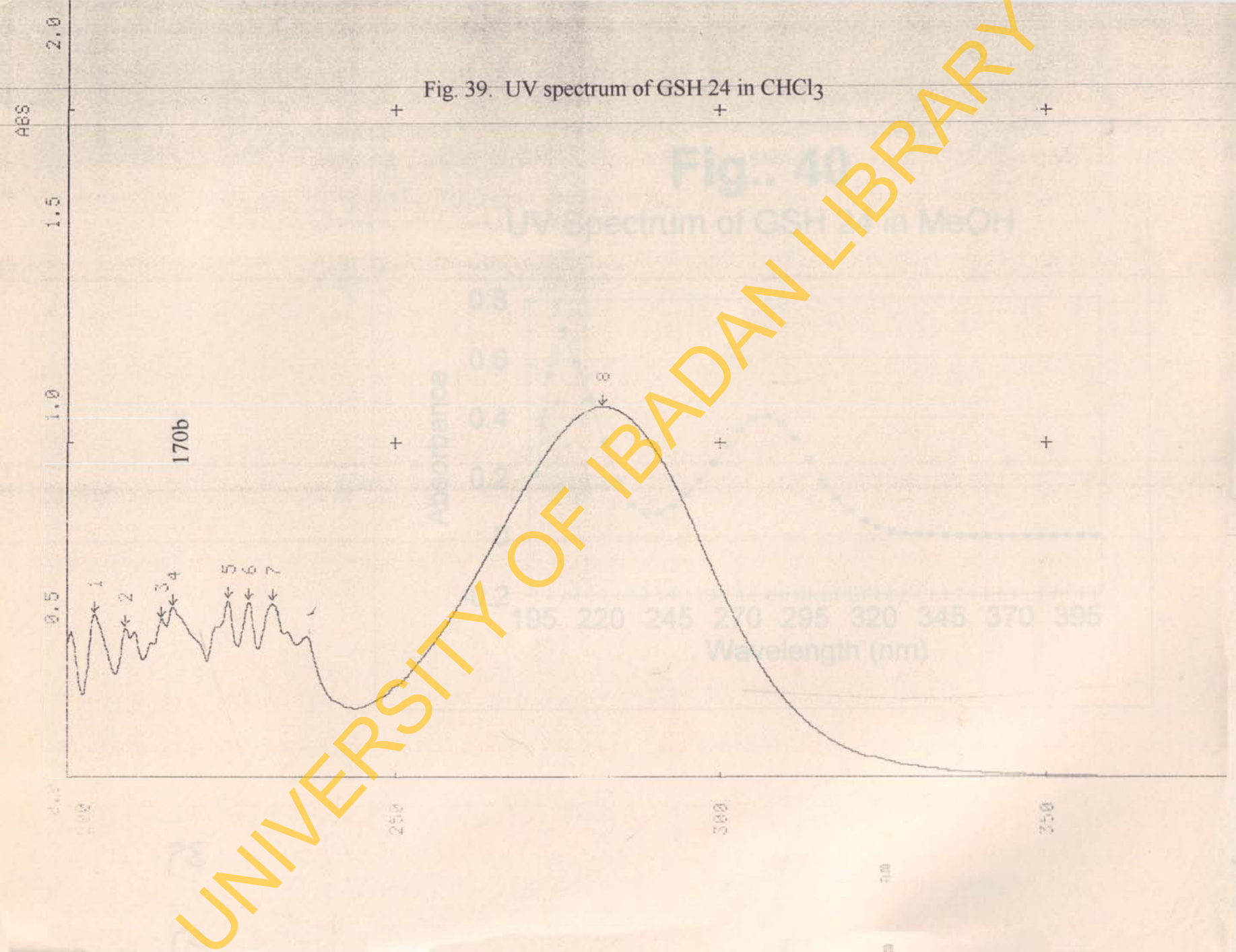
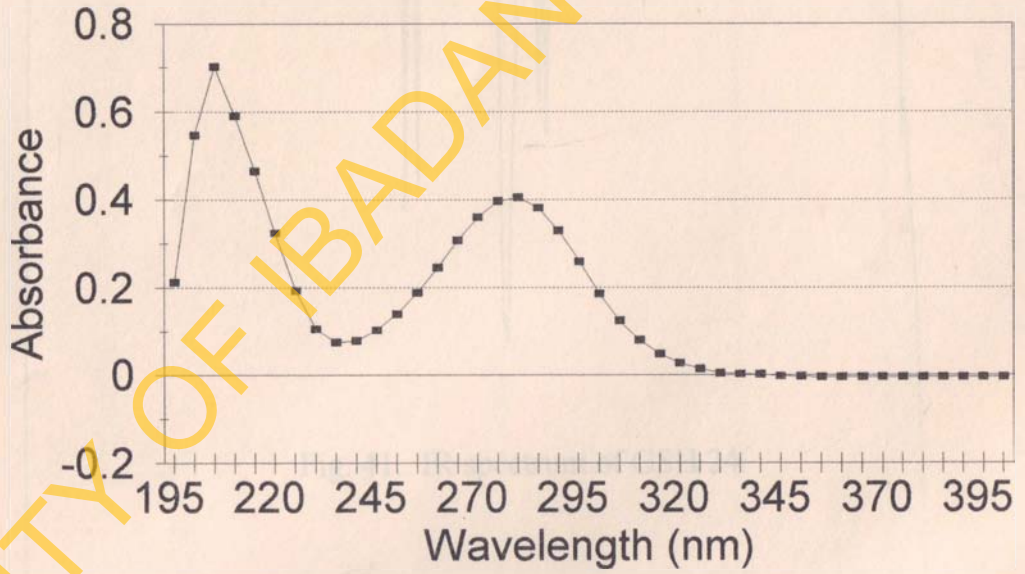


Fig.. 40

UV Spectrum of GSH 24 in MeOH



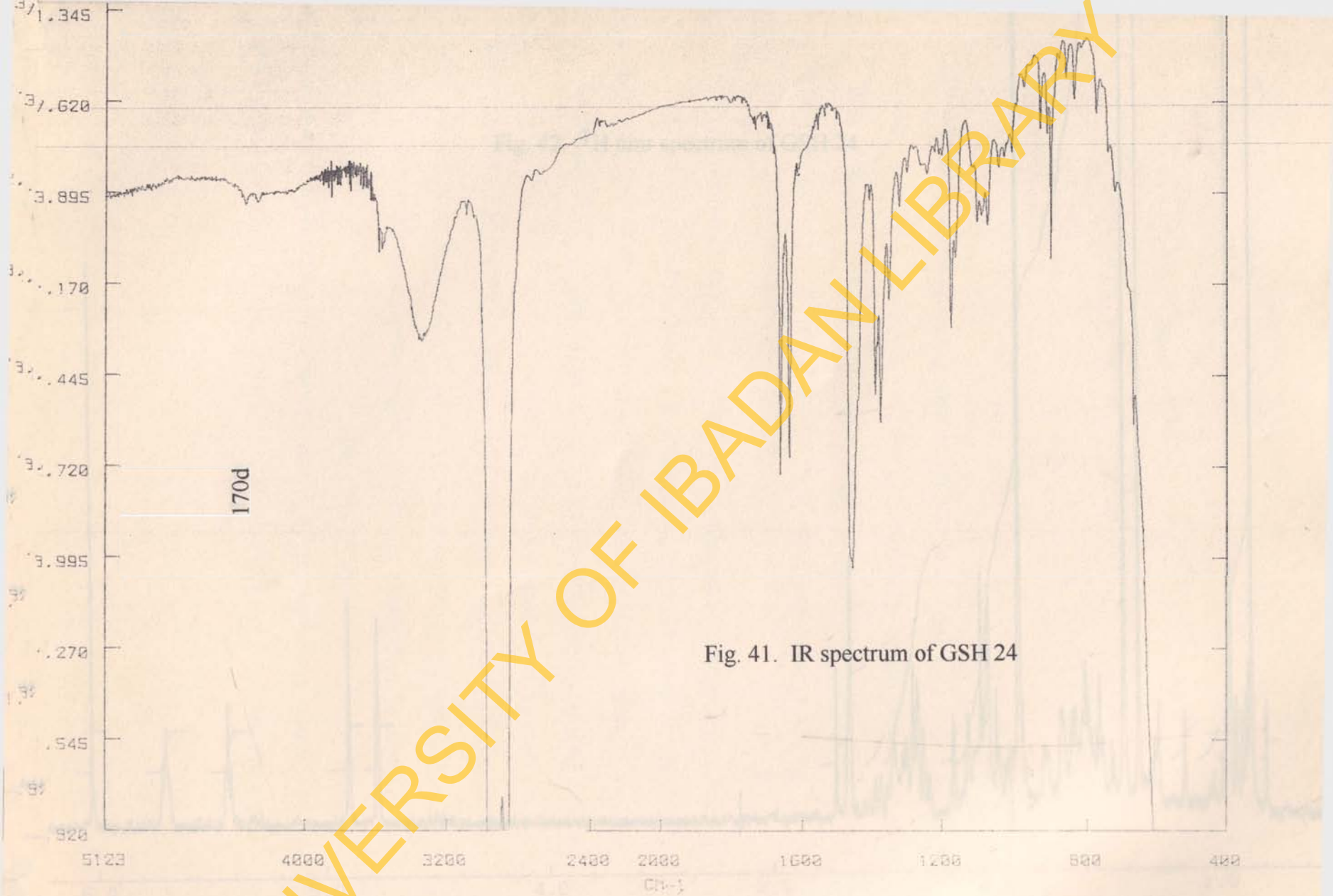
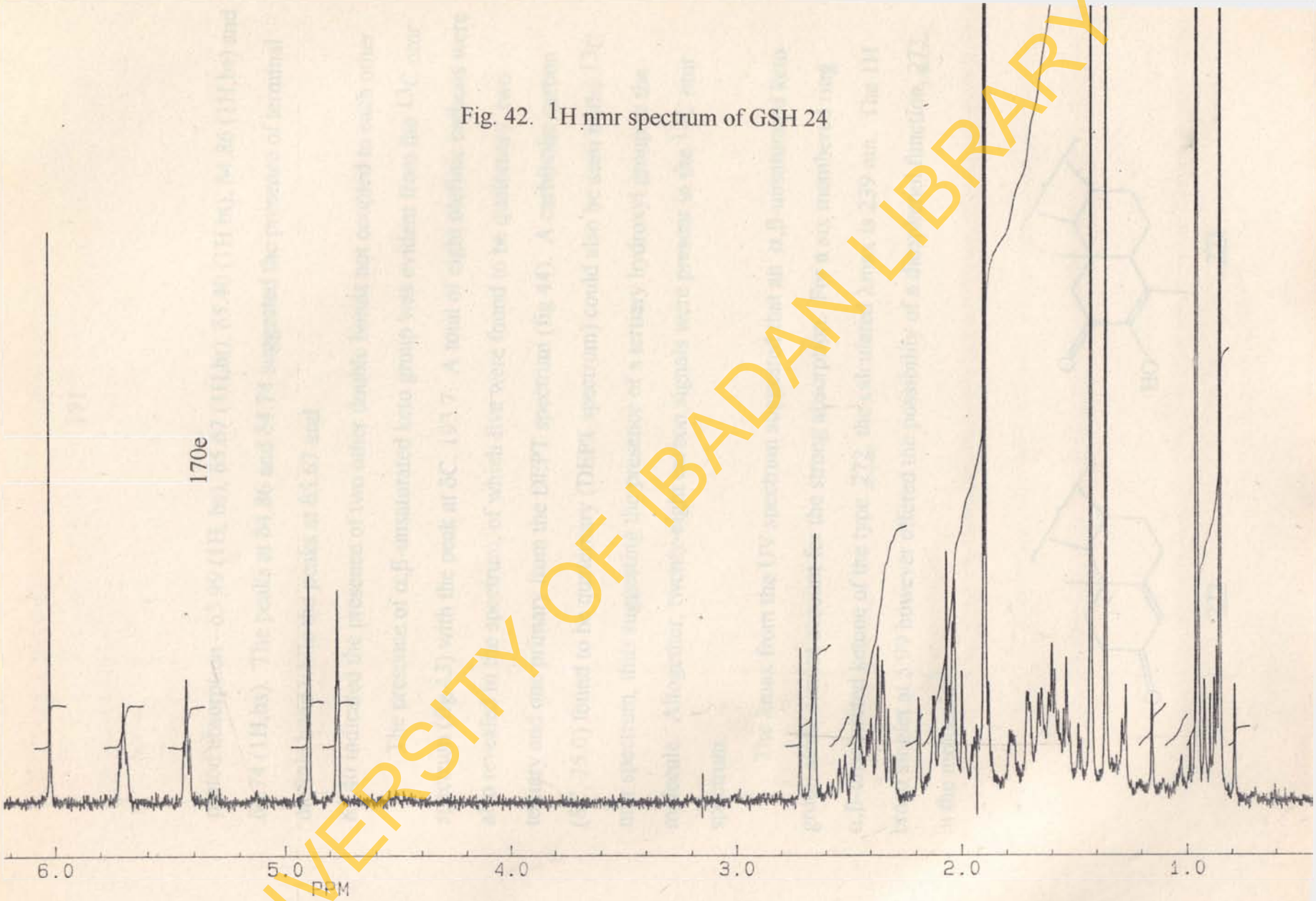


Fig. 41. IR spectrum of GSH 24

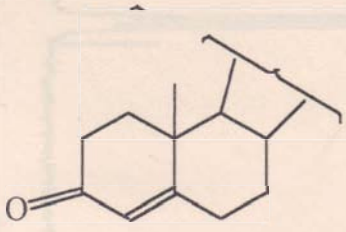
Fig. 42. ^{1}H nmr spectrum of GSH 24



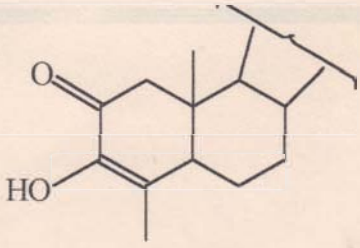
proton absorption - δ5.99 (1H, bs), δ5.67 (1H,bt), δ5.40 (1H,bt), δ4.86 (1H,bs) and δ4.74 (1H,bs). The peaks at δ4.86 and δ4.74 suggested the presence of terminal olefinic bond while the peaks at δ5.67 and
δ5.40 indicated the presence of two other double bonds not coupled to each other.

The presence of α,β-unsaturated keto group was evident from the ^{13}C nmr spectrum (fig 43) with the peak at δC, 193.7. A total of eight olefinic carbons were also revealed in the spectrum, of which five were found to be quaternary, two tertiary and one primary from the DEPT spectrum (fig 44). A carbinolic carbon (δC, 75.0) found to be quaternary (DEPT spectrum) could also be seen in the ^{13}C nmr spectrum, thus suggesting the presence of a tertiary hydroxyl group in the molecule. Altogether, twenty-eight carbon signals were present in the ^{13}C nmr spectrum.

The λmax from the UV spectrum suggested that an α,β-unsaturated keto group alone cannot account for the strong absorption. For a six membered ring α,β-unsaturated ketone of the type 272, the calculated λmax is 239 nm. The 1H broad singlet at 5.99 however offered the possibility of a diosphenol function, 273, in the molecule.



272



273

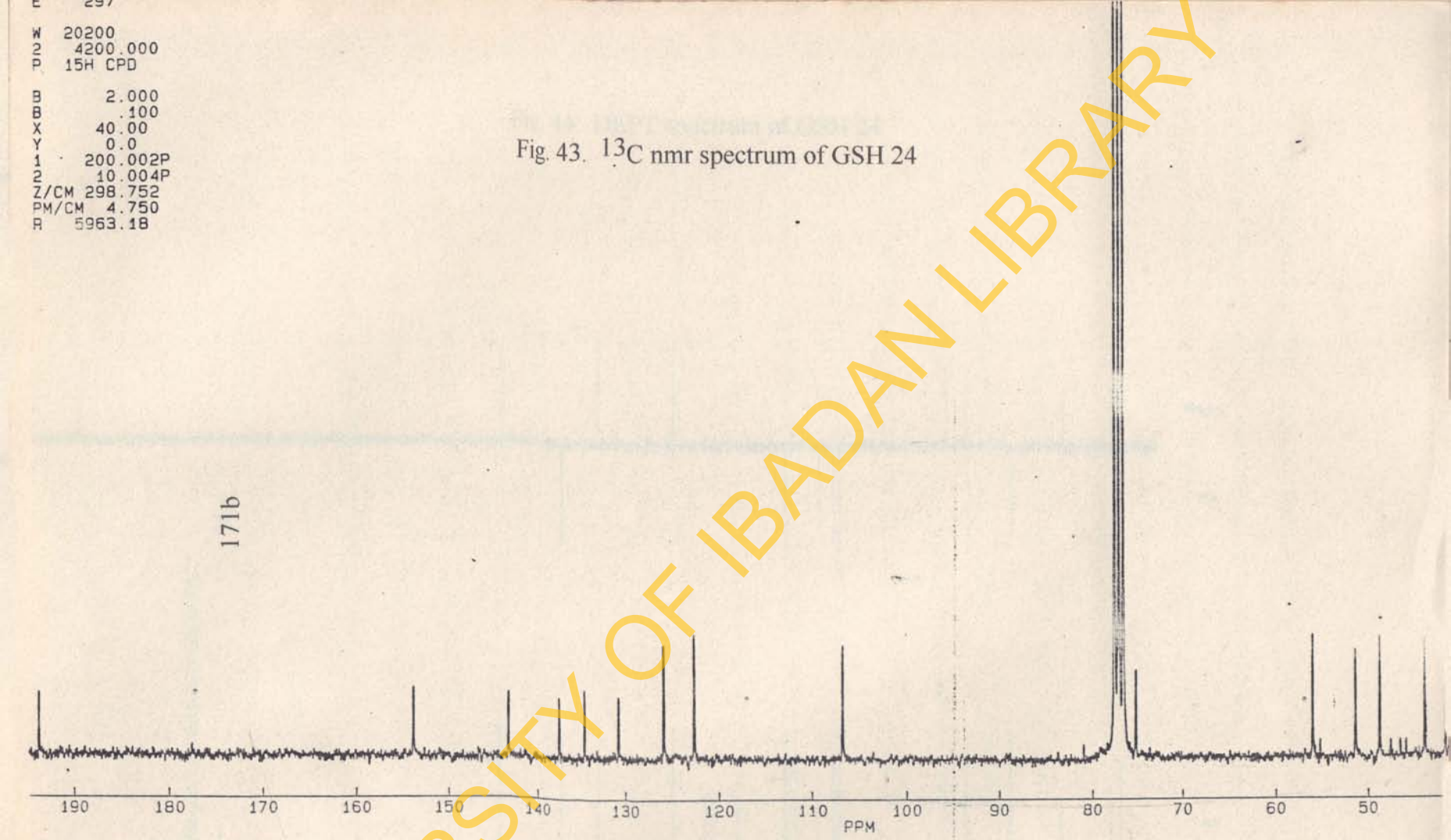
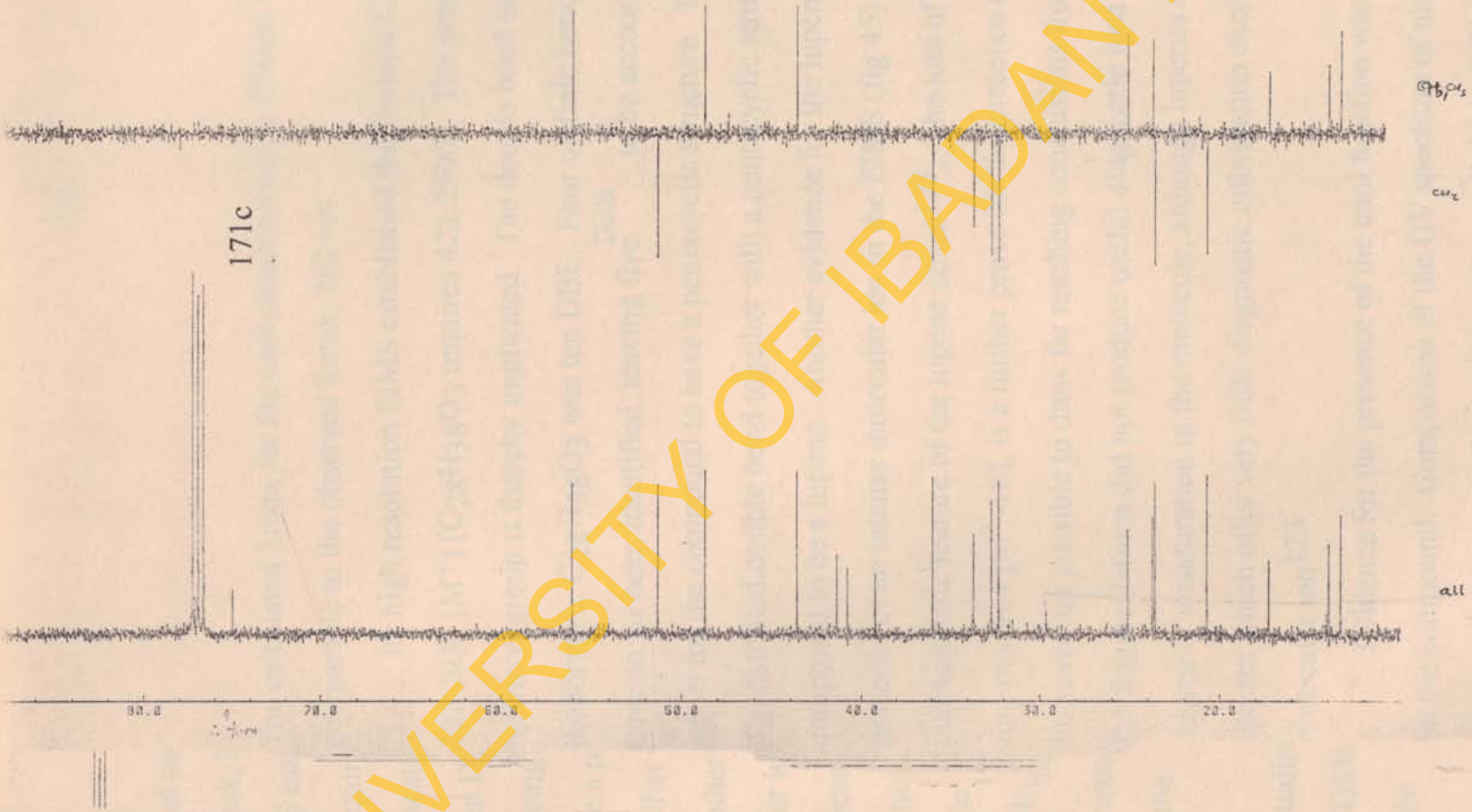


Fig. 43. ^{13}C nmr spectrum of GSH 24

Fig. 44. DEPT spectrum of GSH 24



The calculated λmax for the enol chromophore is 274nm, this obviously is comparable to the observed λmax, 282 nm.

Its high resolution EIMS established the formula $C_{28}H_{38}O_3$ with m/z 422.2930 [M^+] ($C_{28}H_{38}O_3$ requires 422.2900). The presence of a second hydroxyl group is thereby implicated. The double bond equivalent found, based on the formula, $C_{28}H_{38}O_3$ was ten DBE. Four olefinic bonds and one keto group have so far been identified, leaving five DBE to be accounted for. The only option left is for the compound to have a pentacyclic structure. The presence of a 1,1-disubstituted double bond together with a pentacyclic structure suggested the compound to be a lupene. Another evidence for the lupene skeleton was the presence of an intense molecular ion in the EIMS (fig 45) and this is a characteristic feature of the lupene series[123]. The loss of water from the molecular ion, m/z 404 [M^+-18], is a further prove for the presence of hydroxyl group. It is however not possible to draw far reaching conclusions from the mass spectrum as the fragmentations did not produce useful diagnostic ions probably due to the high degree of unsaturation in the molecule, although lupenes are known to give mass spectra which offer very little diagnostic information except for the mass of the molecular ion[123].

Evidence for the presence of the enol function was sought from UV studies on the compound. Comparison of the UV spectrum of methanol solution of GSH 24 and that obtained after addition of a small drop of dilute solution of sodium hydroxide produced no clue. A shift of the λmax to longer wavelength was

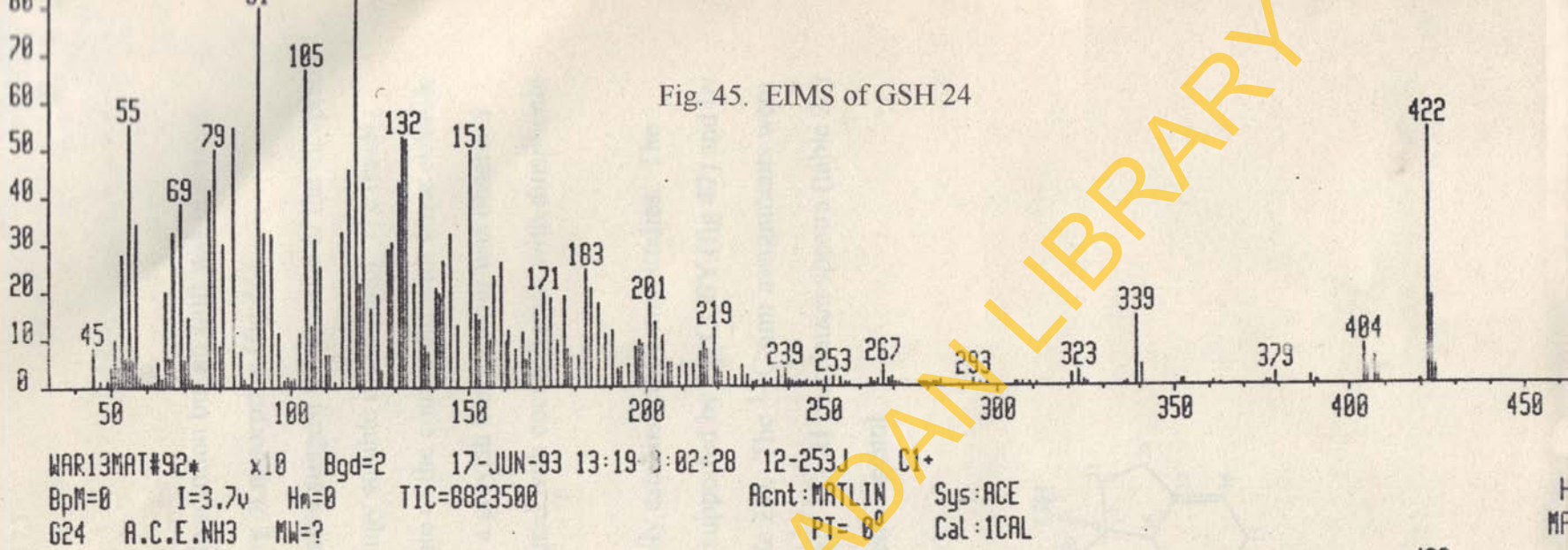


Fig. 45. EIMS of GSH 24

expected on addition of sodium hydroxide solution but no shift was observed, although there was an increase in intensity of absorption (fig 46).
However, this observation can not be used to suggest the absence of the diosphenol function as the compound was only sparingy soluble in methanol. The result obtained by treatment of the chromatoplate of the compound with ferric chloride did not also give any useful clue because a greyish black colour was observed instead of the brown colour usually obtained with cucurbitacins with diosphenol function[171].

The structure of GSH 24 was finally established by nmr studies. The assignment of the ^{1}H nmr spectrum was supported by 2D COSY (fig 47) and by use of NOE difference spectroscopy (table 25). The ^{13}C nmr assignments were supported by CPD, 1-bond and long range ^{13}C-^{1}H correlation spectra (table 26). GSH 24 was identified as a bisnortriterpene lupenol.
This we have named erubescenone, 274.

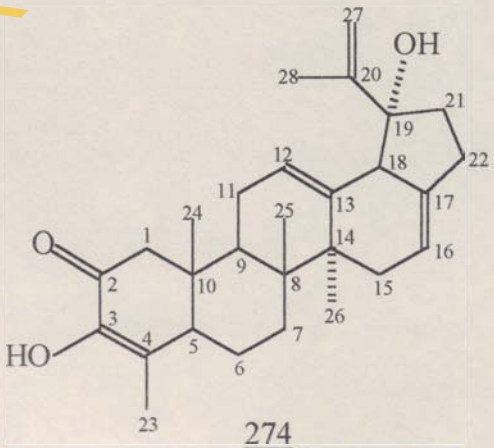


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Fig. 46

UV Spectrum of GSH 24

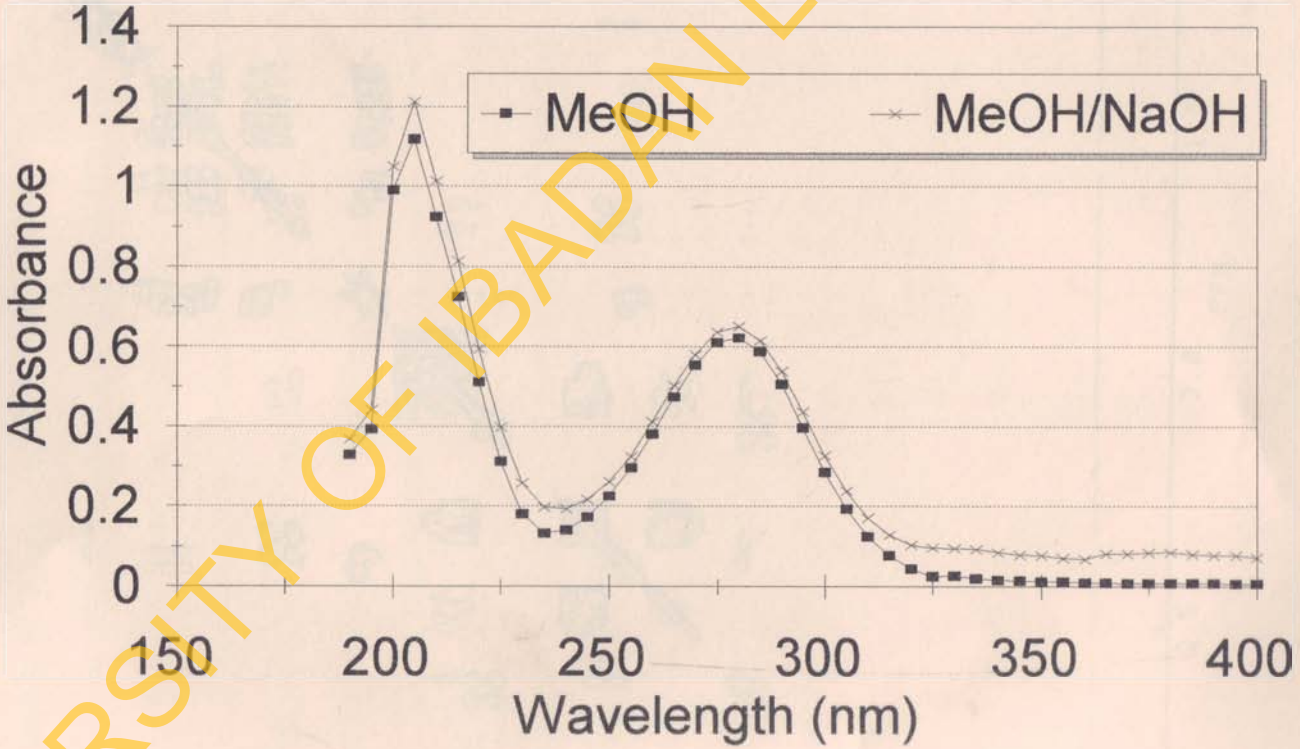
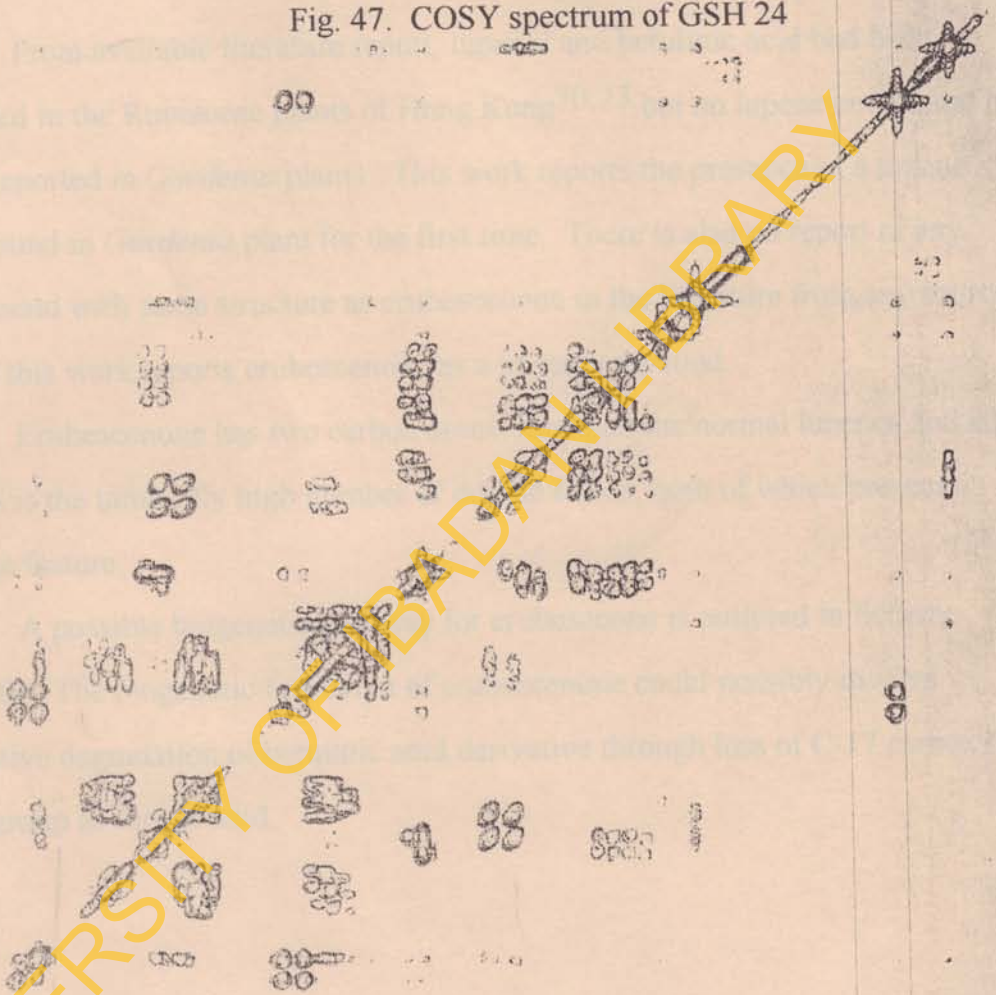


Fig. 47. COSY spectrum of GSH 24



From available literature report, lupenol and betulinic acid had been reported in the Rubiaceae plants of Hong Kong[70,73] but no lupene compound had been reported in *Gardenia* plants. This work reports the presence of a lupene compound in *Gardenia* plant for the first time. There is also no report of any compound with same structure as erubescenone in the literature from any source, hence this work reports erubescenone as a novel compound.

Erubescenone has two carbon atoms less than the normal lupenes and added to this is the unusually high number of double bonds, both of which present a unique feature.

A possible biogenetic pathway for erubesceone is outlined in Scheme XXVIII. The biogenetic formation of erubescenone could possibly involve oxidative degradation of betulinic acid derivative through loss of C-17 carboxylic acid group as formic acid.

Table 25 - 400MHz ^{1}H NMR spectrum and noes for erubescenone in $CDCl_3$.

Position.	δ/Multiplicity.	J(Hz).	Couplings to	NOEs to
1α	2.12 d	16.5	1β, 11, 24	1β, 5α, 9α, 11
1β	2.65 d	16.5	1α	1α, 24
3-OH	5.99 bs			5α, 6α, 6β, 23
5α	2.40 m		6α, 6β, 11	
6α	1.88 m		5α, 6β, 7α, 7 β	
6β	1.47 ddd	2.9,3.3	5α, 6α, 7α	
7α	1.65 dd	3.7,13.2	6α, 6β, 7β	6α, 7β, 26
7β	1.54 dm	3.3,13.2	6α, 7α	
9α	1.97 s		11α/β, 25	1α, 5α, 12, 24 25, 26
11α	2.01 m		1α, 5α, 9α	
11β	2.05			
12	5.40 bt		18β 27Z	11,18β, 28
15α	1.71 dm	17	15β, 16, 18β 22β	
15β	2.36 m		15α, 16, 18β, 26	
16	5.67 bt		15α, 15β, 18β 22β	15α, 15β, 22α

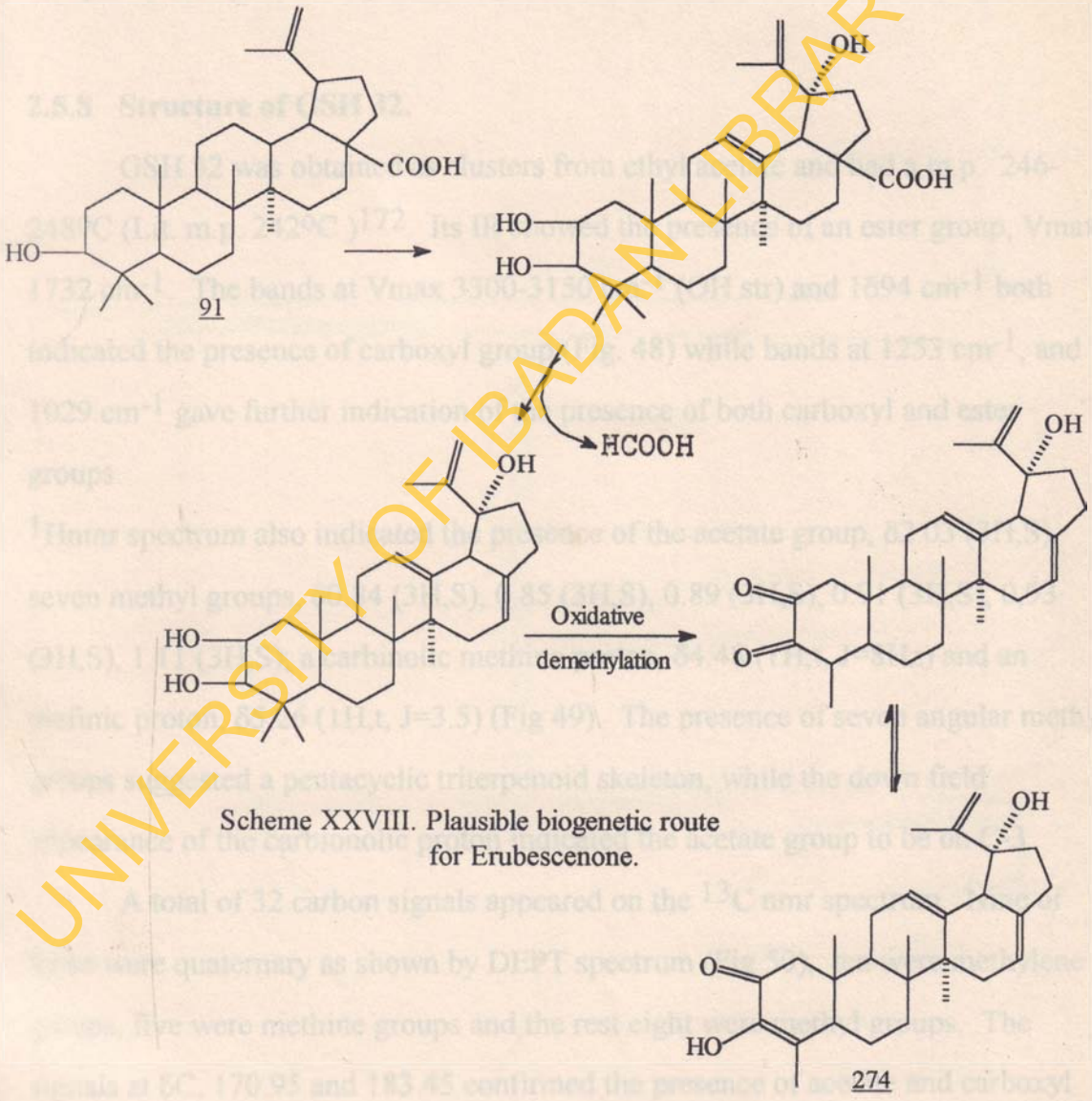
Table 25 Contd.

Position.	δ/Multiplicity.	J(Hz).	Couplings to	NOES to
18β	2.63 bs		12 15α, 15β, 16, 22β	12, 22β, 28
19β	1.65 bs		21β, 22α, 22β 27E, 27Z	
-OH				
21α	2.50 m	6.13	21β, 22α, 22β 27E, 27Z	
21β	2.31 m		21α,22α, 22β 27E, 27Z	
22α	2.33 m		21α, 21β, 22β	
22β	2.01 m		15α, 16, 18β 21α, 21β, 22α	
23	1.87 d	1.9	5α	
24	0.93 m		1α	1β, 6β, 9α, 25
25	0.83 s		9α	6β, 7β, 9α, 11 15β, 18β, 24
26	1.33 s		15β	7α, 9α, 15α
27Z	4.74 bs		21α, 21β, 27E	21β, 27E
27E	4.86 bs		21α, 21β. 27E	28, 27Z
28	1.39 s			12, 18β, 27E

Table 26- 100MHz ^{13}C NMR spectrum and ^{13}C-^{1}H correlations for erubescenone in $CDCl_3$.

C number	δ	type	coupling to 1-bond	^{1}H long range
1	51.2	CH_2	1α,2β	24
2	193.7	C=O		1α,2β
3	143.4	C=		23,1β,3-OH
4	130.6	C=		23,5,3-OH
5	48.6	CH	5	6α,6β,23,24
6	20.7	CH_2	6α,6β	1α,5
7	32.2	CH_2	7α,7β	25
8	39.2	C		7α,7β,9,25,26
9	43.6	CH	9	7β,11,24,25
10	41.4	C		1α,1β,5,9,24
11	23.6	CH_2	11α,11β	12
12	125.7	HC=	12	18
13	137.2	C=		11,18,26
14	40.8	C		12,25,26
15	32.6	CH_2	15α,15β	26
16	122.5	HC=	16	15α,15β
17	134.3	C=		18,22β
18	56.0	CH	18	12,16,28
19	75.0	C-OH		18,27E,27Z, 28
20	153.7	C=		21α,21β,28
21	33.6	CH_2	21α,21β	27E,27Z
22	35.9	CH_2	22α,22β	16,21α
23	13.2	CH_3	23	
24	13.9	CH_3	24	1α,5
25	17.2	CH_3	25	7α,11α
26	25.1	CH_3	26	
27	106.7	$=CH_2$	27E,27Z	
28	23.7	CH_3	28	

One of the C-4 methyl groups is subsequently lost. The enol function in ring A is probably formed en route to C-4 demethylation via the α-diketone.



Scheme XXVIII. Plausible biogenetic route for Erubescenone.

Erubescenone has ring A which carries a diosphenol function and this is a characteristic feature of some cucurbitacins. It is therefore logical to regard this compound as a potential cytotoxic agent.

2.5.8 Structure of GSH 32.

GSH 32 was obtained as clusters from ethyl acetate and had a m.p. 246-248°C (Lit. m.p. 242°C)[172]. Its IR showed the presence of an ester group, Vmax 1732 cm^{-1}. The bands at Vmax 3300-3150 cm^{-1} (OH str) and 1694 cm^{-1} both indicated the presence of carboxyl group (Fig. 48) while bands at 1253 cm^{-1}, and 1029 cm^{-1} gave further indication of the presence of both carboxyl and ester groups.

^{1}Hnmr spectrum also indicated the presence of the acetate group, δ2.03 (3H,S), seven methyl groups, δ0.84 (3H,S), 0.85 (3H,S), 0.89 (3H,S), 0.91 (3H,S), 0.93 (3H,S), 1.11 (3H,S); a carbinolic methine proton, δ4.48 (1H,t, J=8Hz) and an olefinic proton, δ5.26 (1H,t, J=3.5) (Fig 49). The presence of seven angular methyl groups suggested a pentacyclic triterpenoid skeleton, while the down field appearance of the carbionolic proton indicated the acetate group to be on C-3

A total of 32 carbon signals appeared on the ^{13}C nmr spectrum. Nine of these were quaternary as shown by DEPT spectrum (Fig 50), ten were methylene groups, five were methine groups and the rest eight were methyl groups. The signals at δC, 170.95 and 183.45 confirmed the presence of acetate and carboxyl groups. The signals at δC,122.43 and 143.48 were absorptions of the olefinic

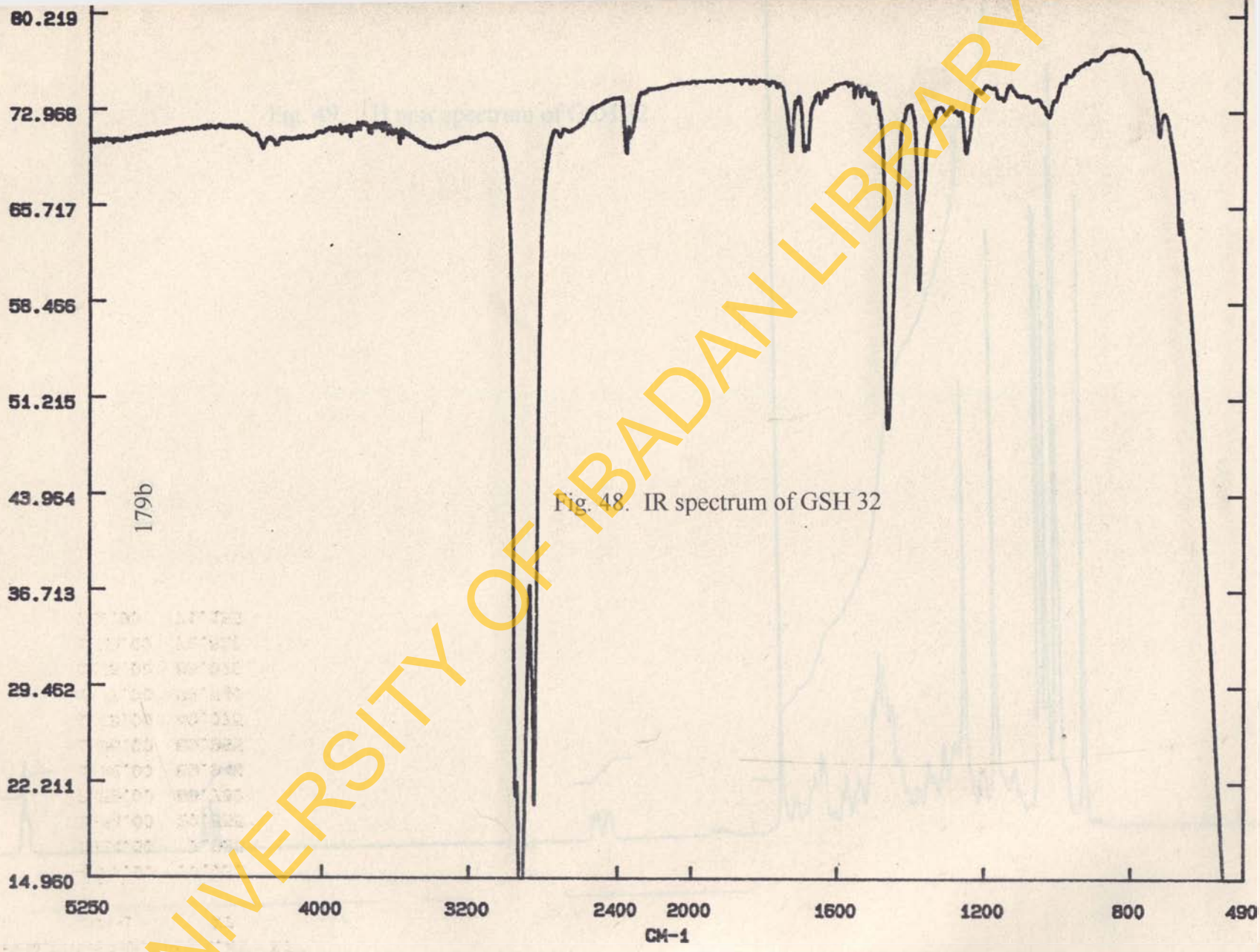
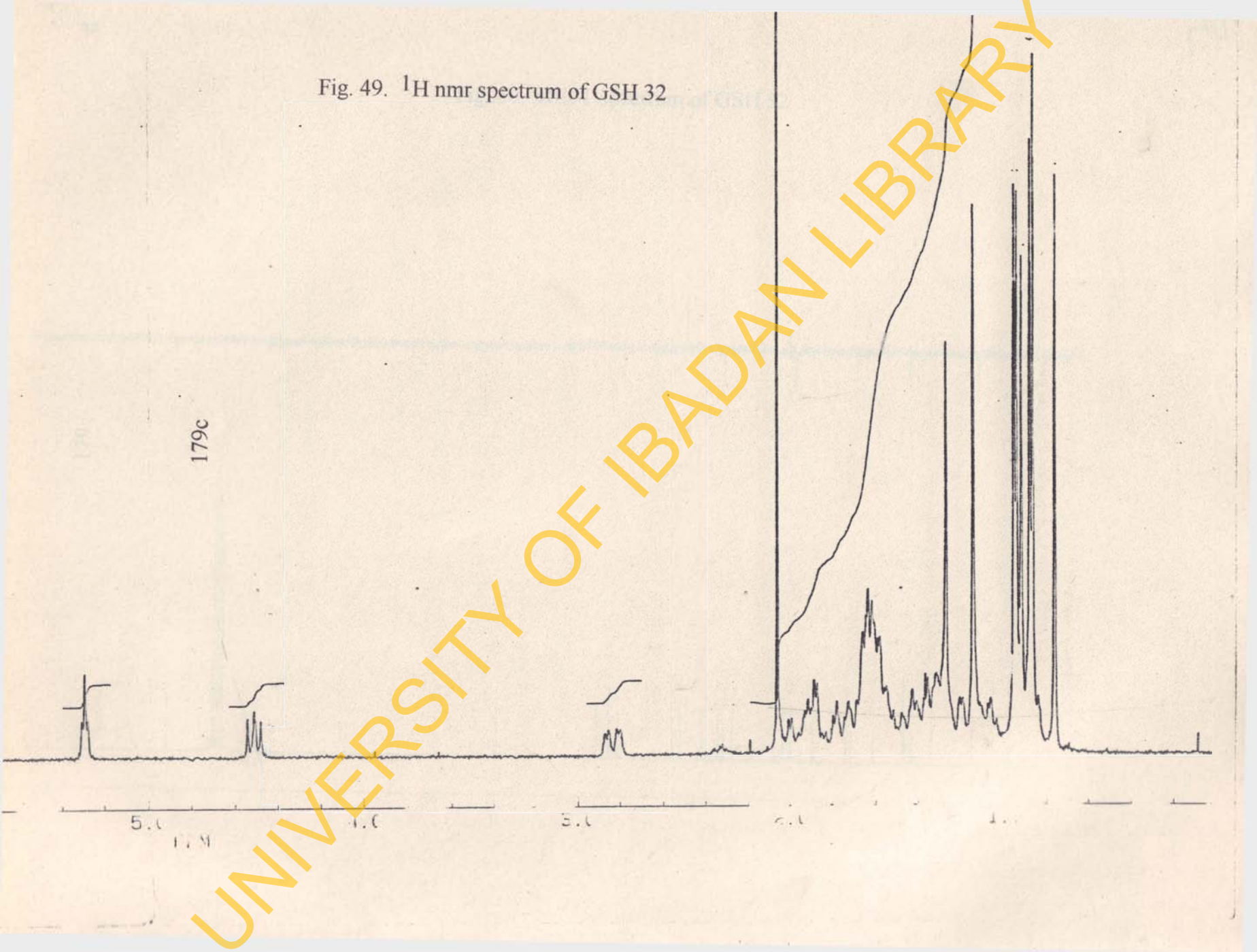


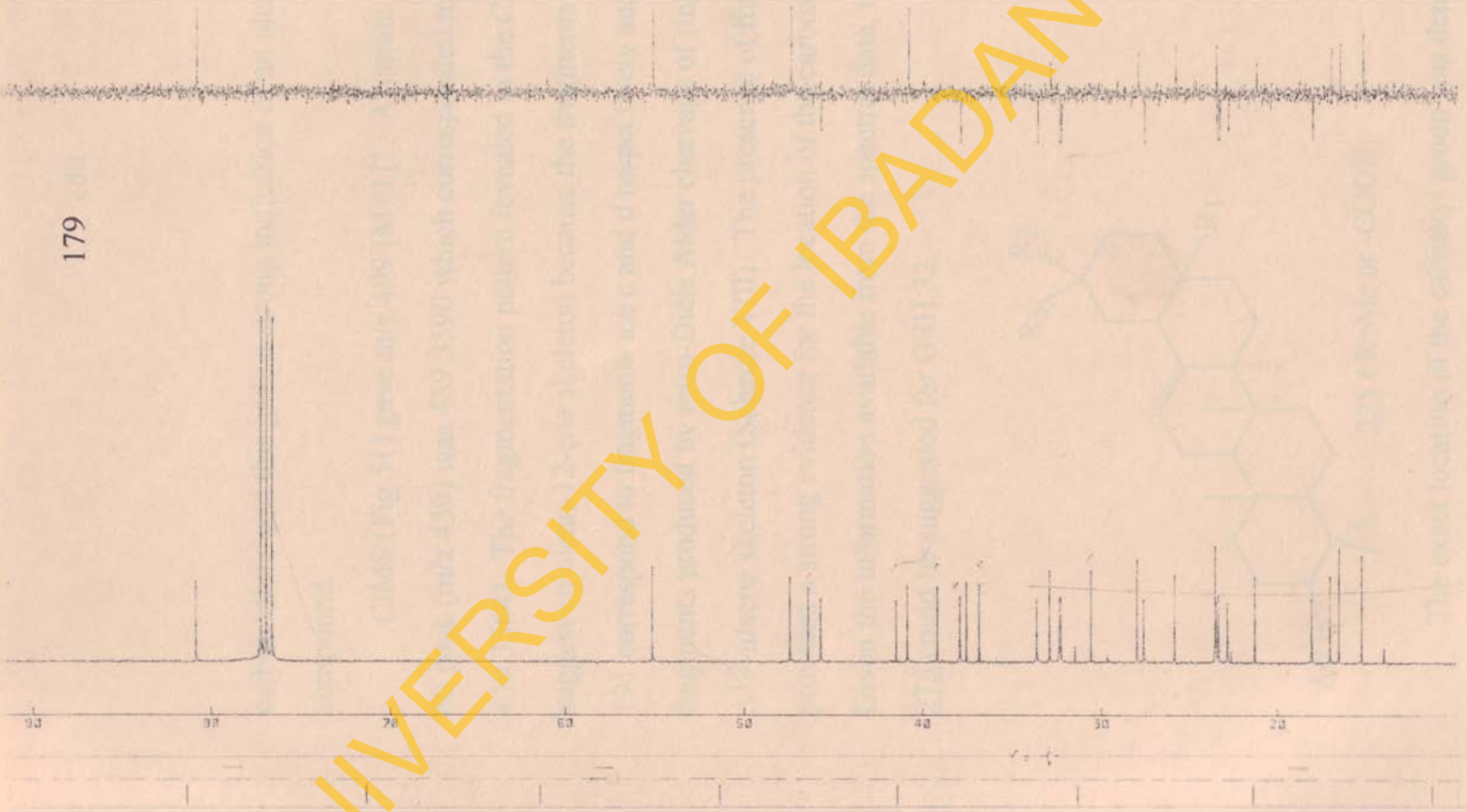
Fig. 48. IR spectrum of GSH 32

Fig. 49. ^{1}H nmr spectrum of GSH 32



d

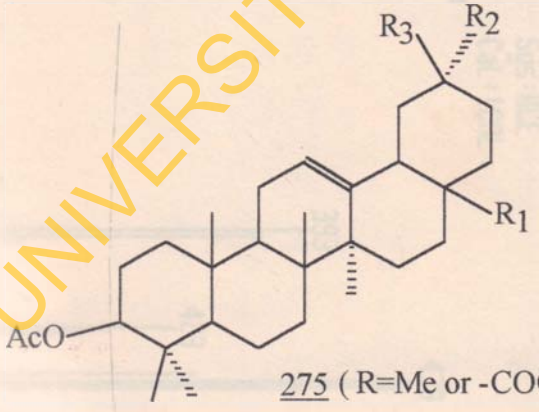
Fig. 50. DEPT spectrum of GSH 32



179

carbon atoms and this gave a strong indication of an oleanen-type skeleton for the compound.

CIMS (Fig 51) gave m/z 499 $[M+1]^+$. Accurate mass obtained for [M+1] - 60 peak (m/z 439) was 439.3590 which corresponded to $C_{30}H_{47}O_2$ (requires 439.3564). The fragmentation pattern revealed in the CIMS spectrum seem to suggest an olean-12-ene skeleton because the fragments at m/z 248, 235, 205 and 191 correspond to fragments a,e c and d respectively and these are diagnostic fragments produced by retro-Diels Alder cleavage of ring C of Δ^{12}-oleanene or Δ^{12}-ursene skeleton (Scheme VIII). The presence of fragment at m/z 248 also provided a strong evidence for the location of the carboxyl group on D/E rings Given the information available from the spectral data, the structure, 275 could be suggested for GSH 32.



275 (R=Me or -COOH)

The exact location of the carboxyl group was determined from NOE and ^1H-^{13}C correlation spectra (table 27) and C-30 was identified as the position occupied

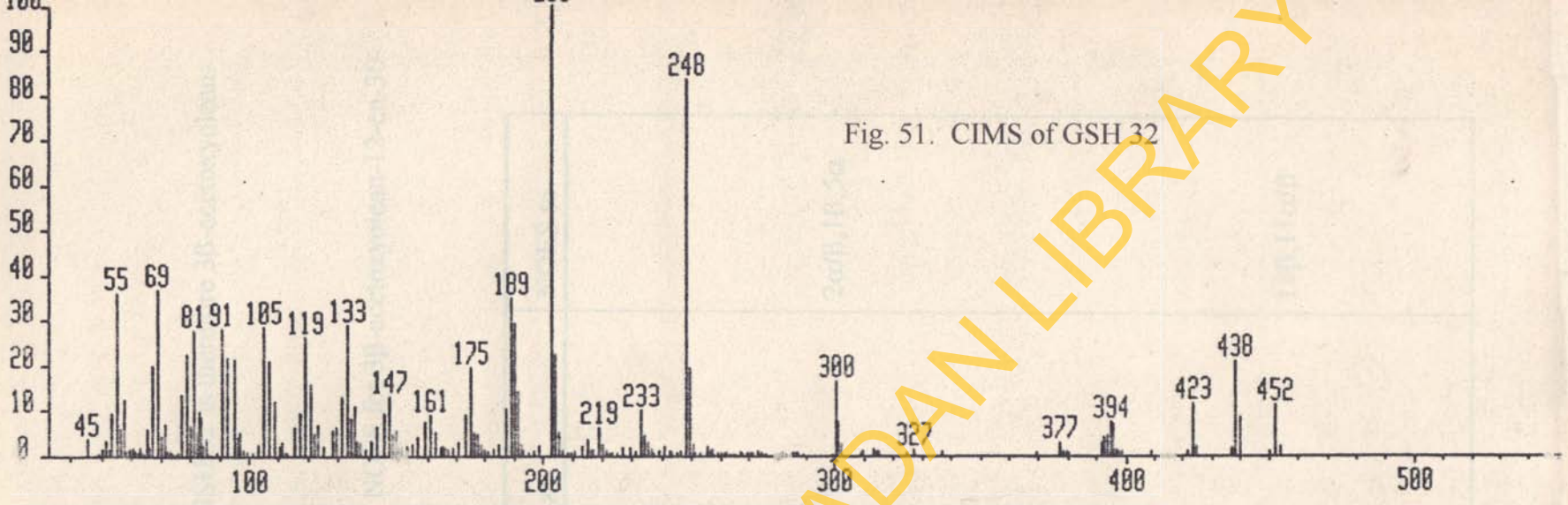
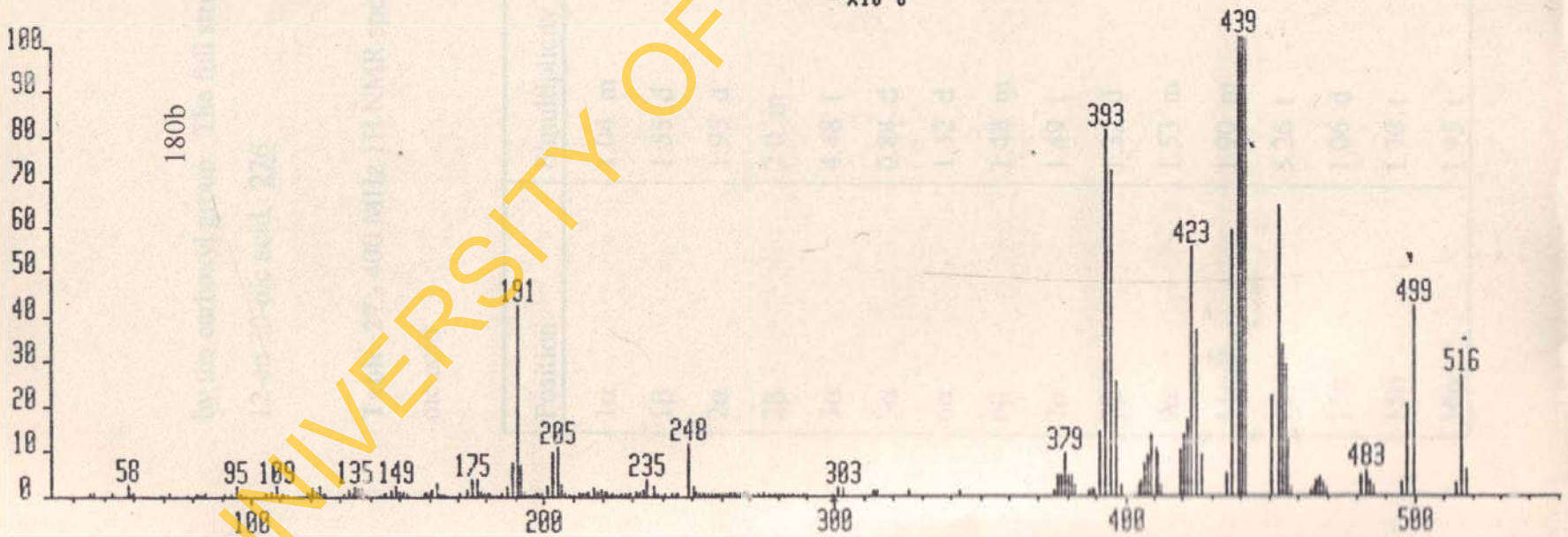


Fig. 51. CIMS of GSH 32

WAR14MAT#76* x10 Bgd=2 17-JUN-93 13:27+0:02:04 12-253J C1+
BpM=0 I=2.5v Hm=0 TIC=6455800 Acnt:MATLIN Sys:ACE
G32 A.C.E.NH3 MW=? PT= 0° Cal:ICAL
*X10*0



by the carboxyl group. The full structure of GSH 32 is therefore 3β-acetoxyolean-12-en 30-oic acid, 276.

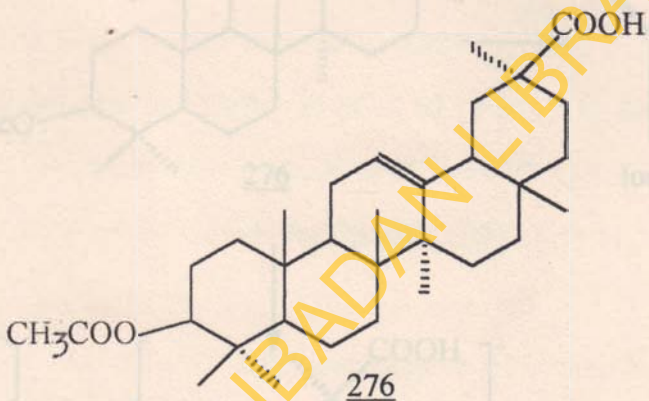
Table 27 - 400 MHz 1H NMR spectrum and NOES for 3β-acetoxyolean-12-en-30-oic acid

Position	δ/multiplicity	J(Hz)	NOES to
1α	1.04 m		
1β	1.55 d	12	
2α	1.95 d	12	
2β	2.0 m		
3α	4.48 t	11	2α/β,1β,5α
5α	0.84 d		
6α	1.52 d		
6β	1.48 m		
7α	1.49 t	11	
7β	1.32 d	11	
9α	1.53 m		
11α/β	1.90 m		
12	5.26 t	3.5	18β,11α/β
15α	1.06 d	12	
15β	1.70 t	12	
16α	1.95 t	12	

Table 27 Contd.

Position.	δ/Multiplicity.	J(Hz).	NOES to
16β	1.62 m		
18β	2.80 dd	4,10	12,11,19
19α	1.14 t	12	
19β	1.68 d	12	
21α	1.16 t	12	
21β	1.40 d	12	
22α	1.80 d	12	
22β	1.60 t	12	
23	0.85 s		3α,6α,7α,7β
24	0.84 s		6β,25,CH3COOH
25	0.93 s		1β,6β,11α/β ,24,26
26	0.73 s		7β,11α/β,15β,25
27	1.11 s		7α,12,16α
28	0.89 s		22β,21β
29	0.91		18β,19α,21α,22α
CH_3COO	2.03 s		

The spectral data and melting point of GSH 32 compared fairly well with the literature data on 3β-acetoxyolean-12 en-30-oic acid. The fragmentation pattern observed in the CIMS spectrum of GSH 32 is illustrated in Scheme XXIX.



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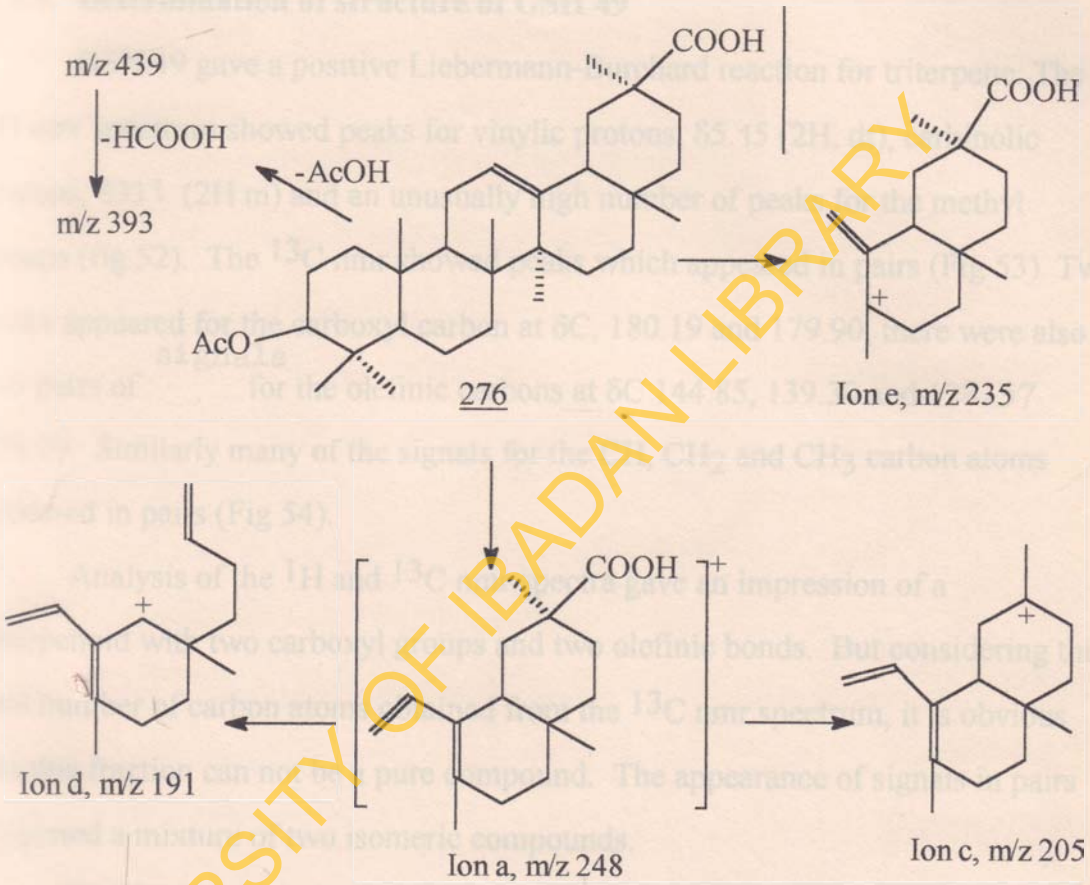
The presence of 3β-acetoxyolean-12-en-30-oic acid is being reported for the first time in a *Gardenia* plant. As the Gardenia plants have been shown to be rich sources of oleanene-type triterpenoiods, the presence of this compound is an indication of the possibilities for a variety of secondary modifications on the oleanene skeleton.



Scheme XXIX. Fragment ions identified in the mass spectrum of GSH 32.

2.5.9. Determination of structure of GSH 49

GSH 49 gave a positive Liebermann-Burchard reaction for triterpene. The ^{1}H nmr spectrum showed peaks for vinylic protons, δ5.15 (2H, dt), carbinolic protons, δ3.11 (2H m) and an unusually high number of peaks for the methyl groups (fig 52). The ^{13}C nmr showed peaks which appeared in pairs (Fig 53). Two peaks appeared for the carboxyl carbon at δC, 180.19 and 179.90, there were also two pairs of signals for the olefinic carbons at δC 144.85, 139.30 and 122.57 124.29. Similarly many of the signals for the CH, CH_2 and CH_3 carbon atoms appeared in pairs (Fig 54).

Analysis of the ^{1}H and ^{13}C nmr spectra gave an impression of a triterpenoid with two carboxyl groups and two olefinic bonds. But considering the total number of carbon atoms obtained from the ^{13}C nmr spectrum, it is obvious that this fraction can not be a pure compound. The appearance of signals in pairs suggested a mixture of two isomeric compounds.

The CIMS of this fraction however did not show two molecular ions. Only one molecular ion, m/z 457 $[M+1]^+$ peak was produced (Fig 55). Accurate mass measurement gave m/z 457.3680 for $[M+1]^+$ peak. This corresponds to $C_{30}H_{49}O_3$ (requires 457.3669). The fragments m/z 248, 235, 205, and 191 are characteristic of Δ^{12}-oleanene or Δ^{12}-ursene skeleton and suggested that this fraction contained oleanolic acid and one other triterpene compound. The peaks at m/z 439 [M-18], 411 [M-46] and 393 [M-60] supported the presence of hydroxyl and carboxyl groups.

b

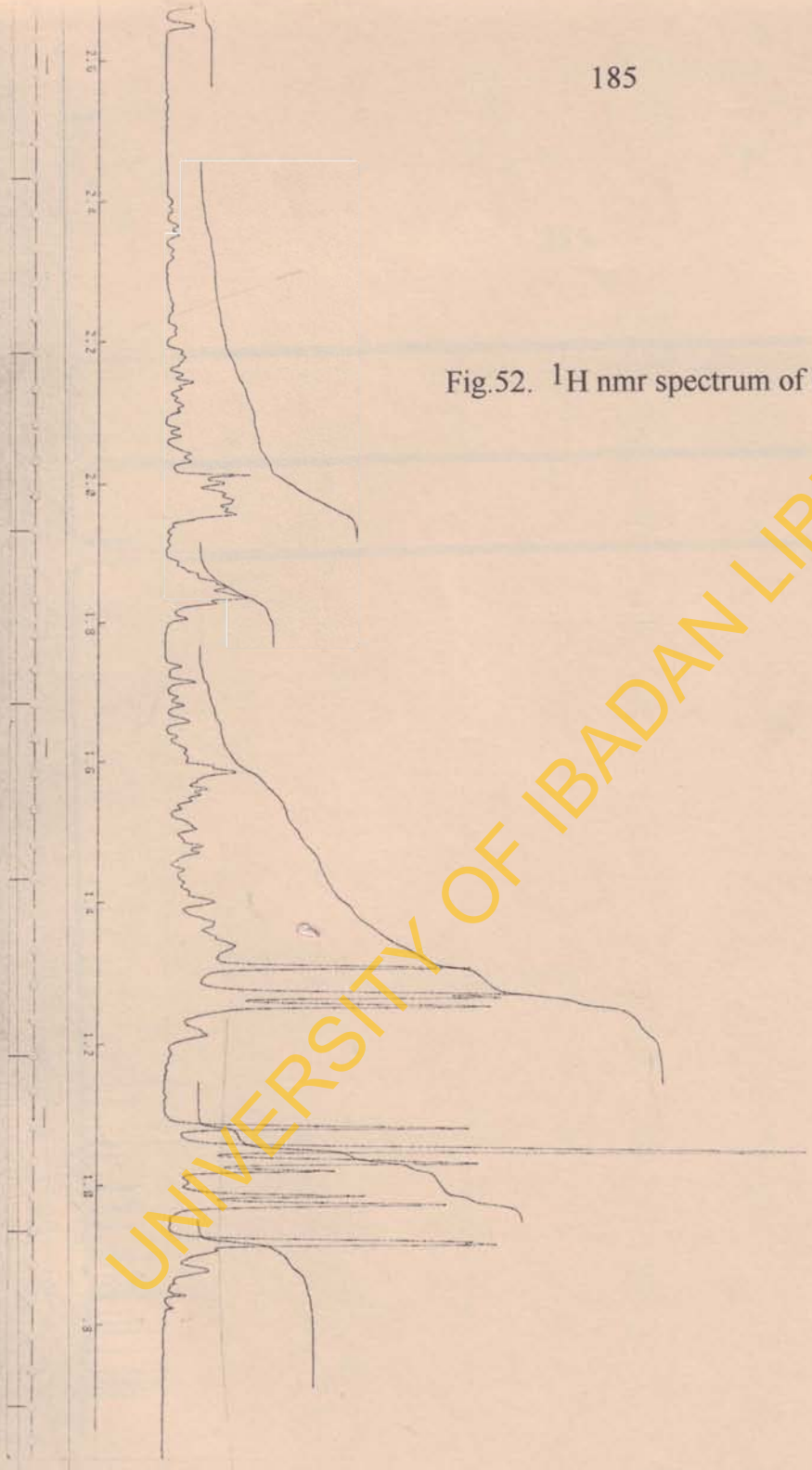


Fig.52. ^{1}H nmr spectrum of GSH 49

Fig. 53. ^{13}C nmr spectrum of GSH 49

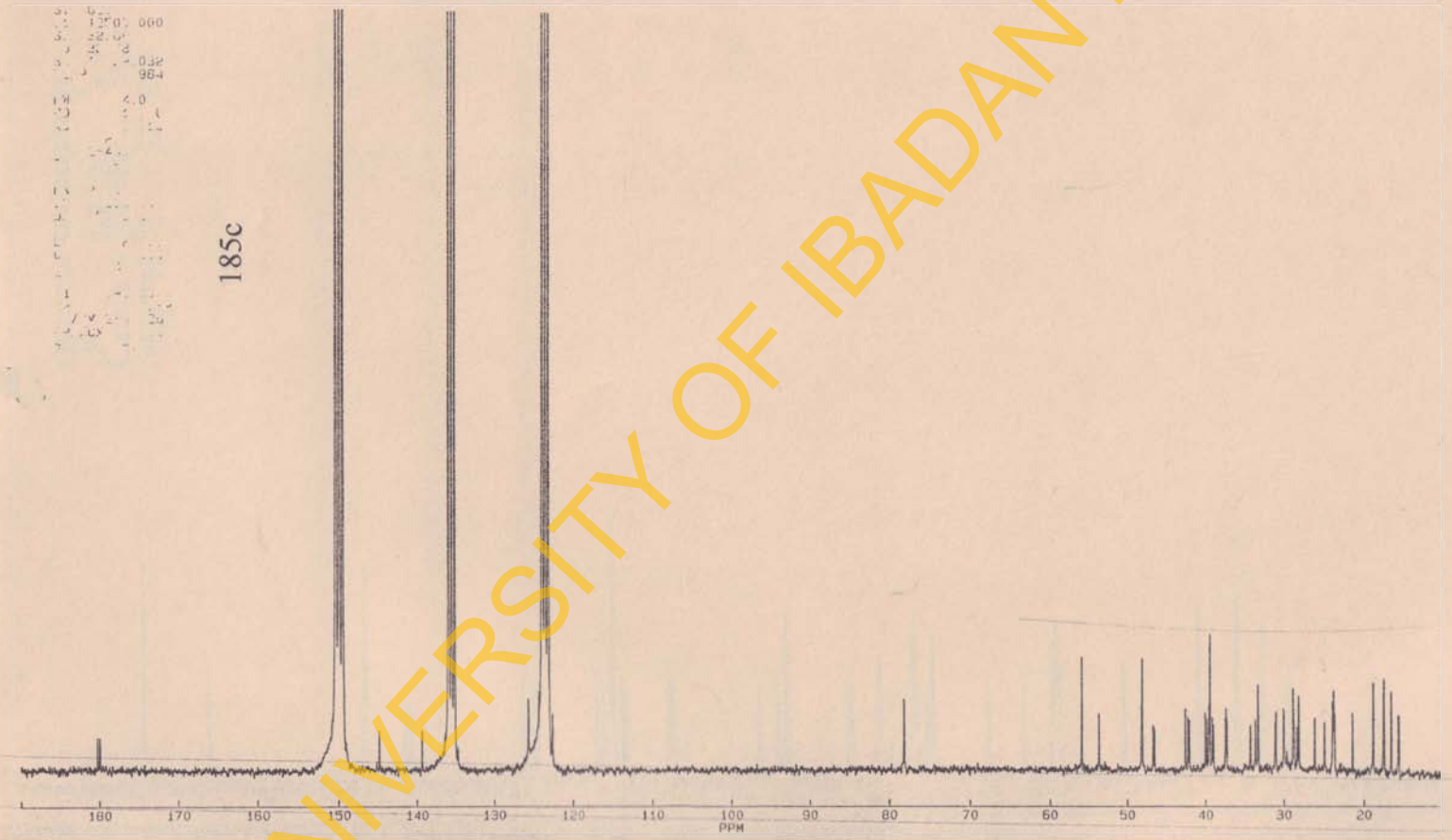
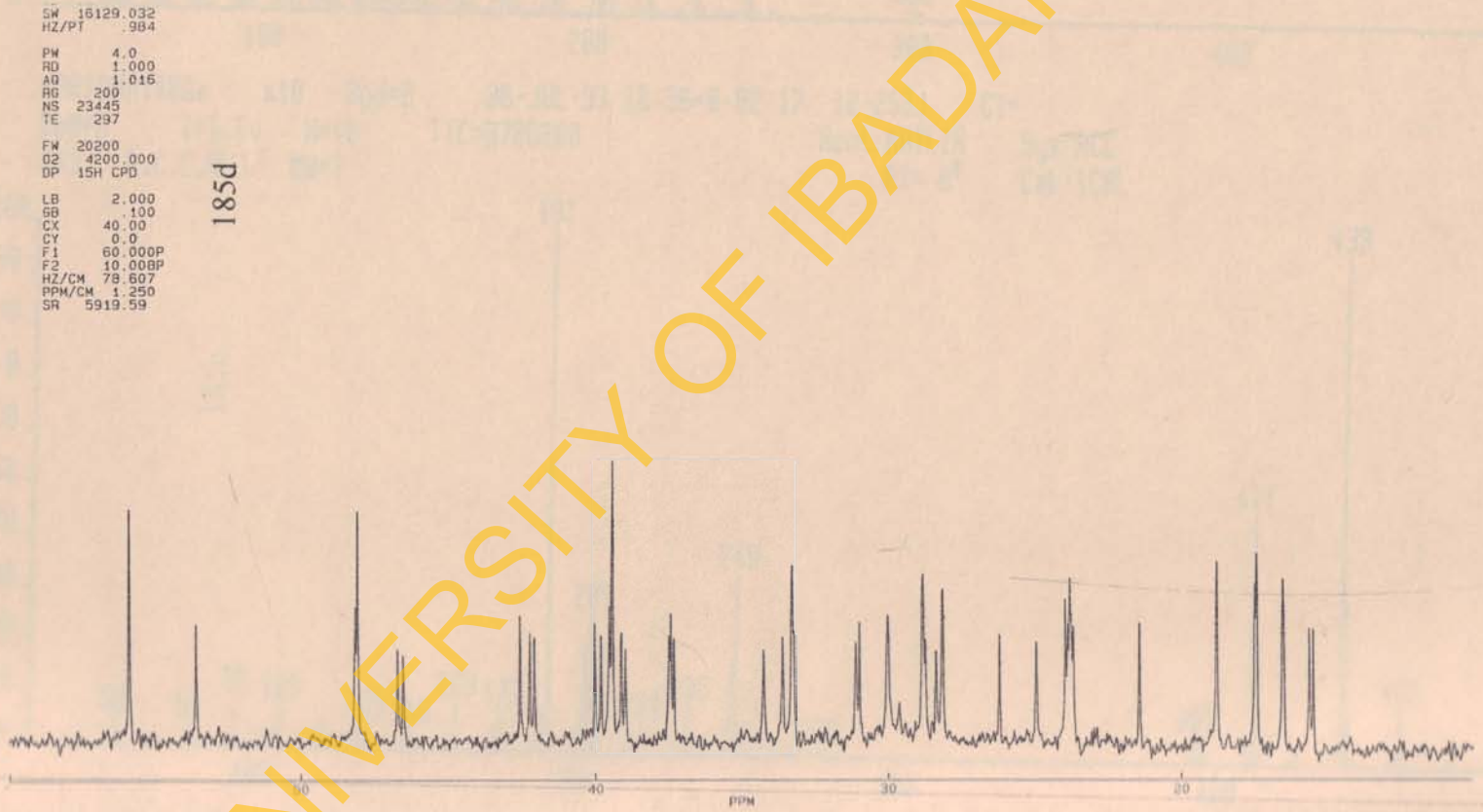


Fig. 54. ^{13}C nmr spectrum of GSH 49



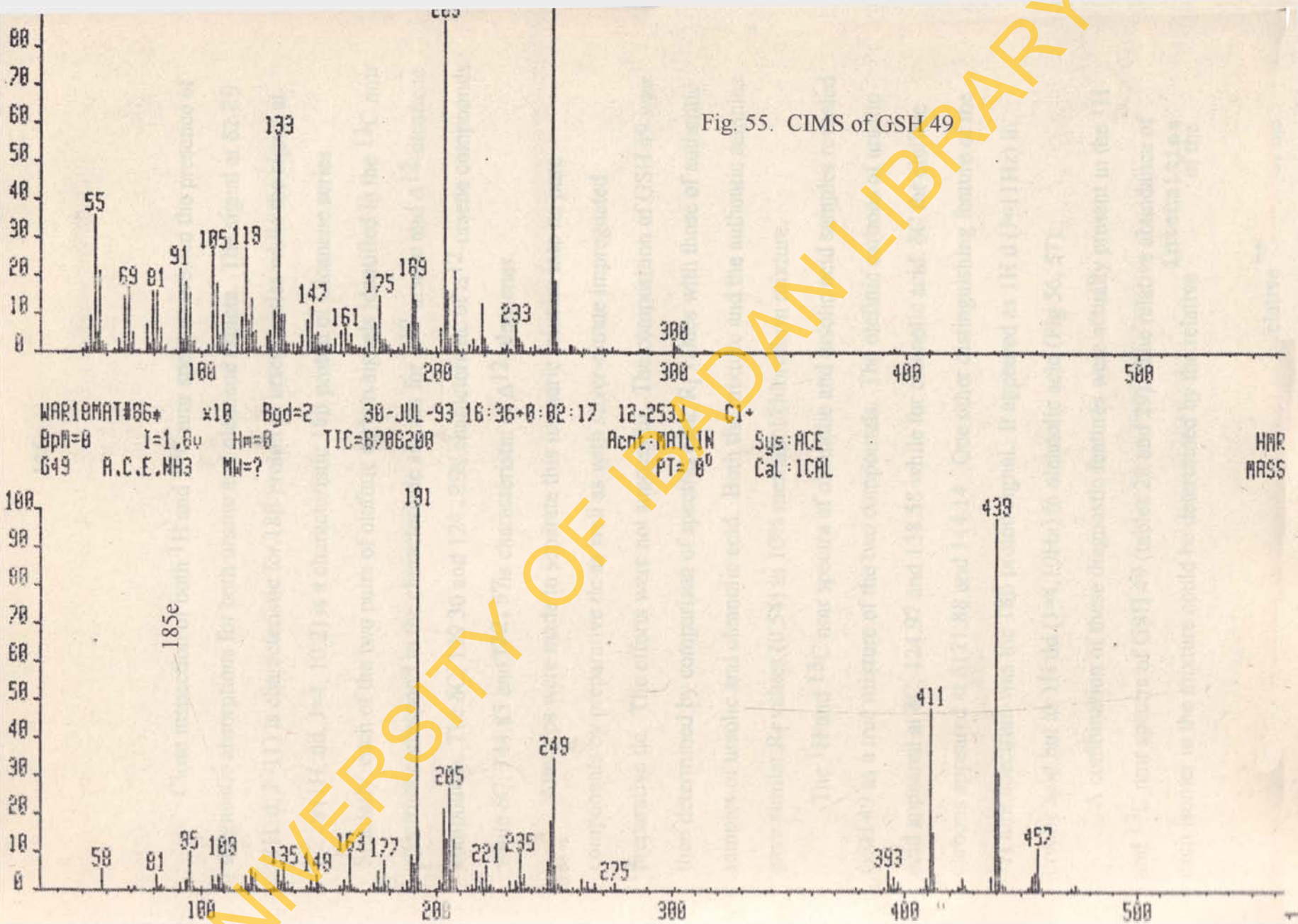


Fig. 55. CIMS of GSH 49

Close inspection of both ^{1}H and ^{13}C nmr spectra revealed the presence of diagnostic absorptions for both ursene and oleanene skeleta. The signal at δ2.29 (1H, d, J=11) is characteristic for 18β proton of ursene series while the signal at δ2.9 (1H, dd, J=4, 10.2) is a characteristic 18β proton of Oleanene series. Similarly, each of the two pairs of olefinic carbon signals identified in the ^{13}C nmr spectrum correspond to the characteristic signals for Δ^{12}-ursene and Δ^{12}-oleanene compounds. The δC, 139.30 and 124.29 is characteristic of Δ^{12}-ursene compounds while δC, 144.85 and 122.57 is characteristic of Δ^{12}-oleanenes.

Attemtps were made to separate this isomeric mixture into its pure components by preparative tlc as well as with silver-nitrate impregnated preparative tlc. The efforts were not successful. The composition of GSH 49 was then determined by comparison of spectral data, R_f values with those of authentic samples of ursolic and oleanolic acid. Both the mixture and the authentic samples gave similar R_f values (0.54) in 10% methanol/chloroform mixture.

The ^{1}H and ^{13}C nmr spectra of oleanolic and ursolic acid samples revealed GSH 49 as a true mixture of the two compounds. The olefinic carbons of ursolic acid appeared at δC, 124.97 and 138.58 while for oleanolic acid. δC for olefinic carbons appeared at δ121.88 and 144.14. One other distinguishing feature of the ^{1}H nmr spectrum was the 18β proton signal. It appeared as 1H d (J=11Hz) in ursolic acid but as 1H dd (J=4,10Hz) in oleanolic acid (Fig 56, 57).

A combination of these diagnostic features were actually present in the ^{1}H and ^{13}C nmr spectra of GSH 49 (tables 28 and 29). The relative abundance of each isomer in the mixture could be determined by the relative intensities of the

Fig. 56. ^{1}H nmr spectrum of ursolic acid

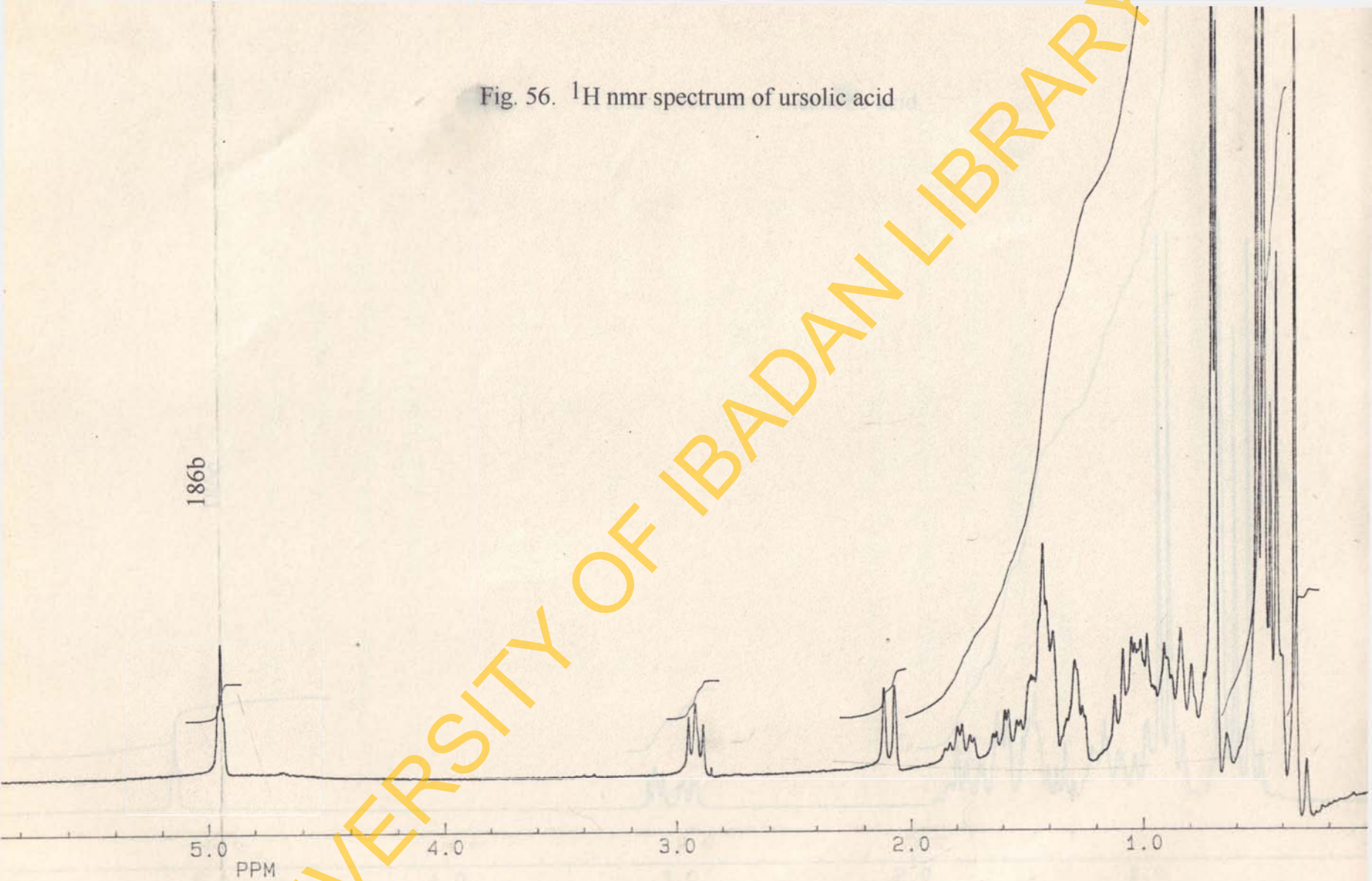
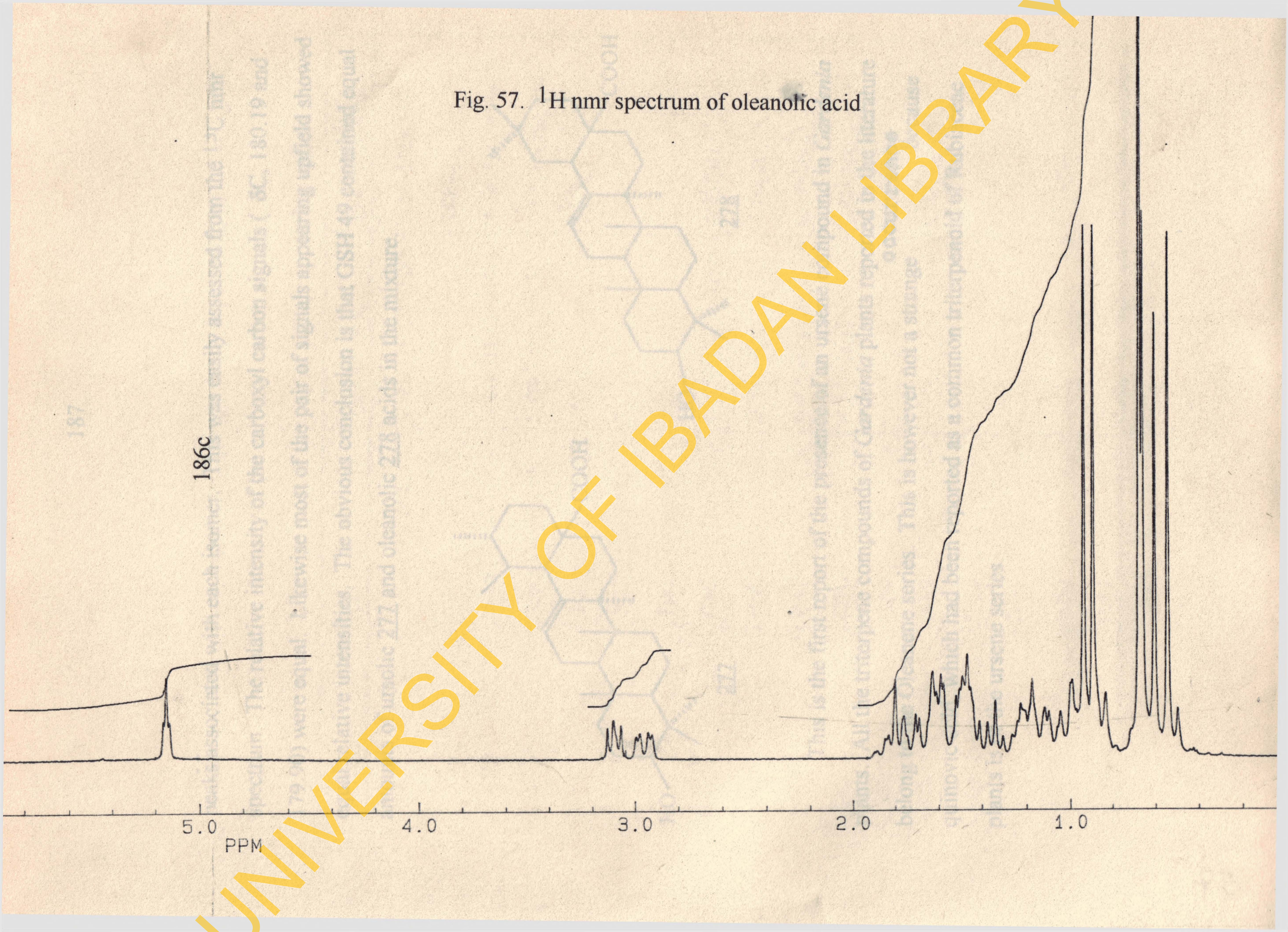
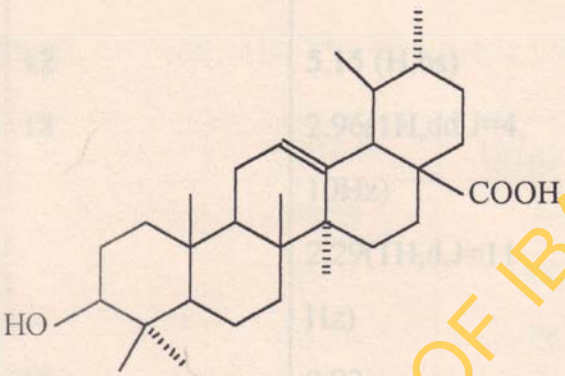


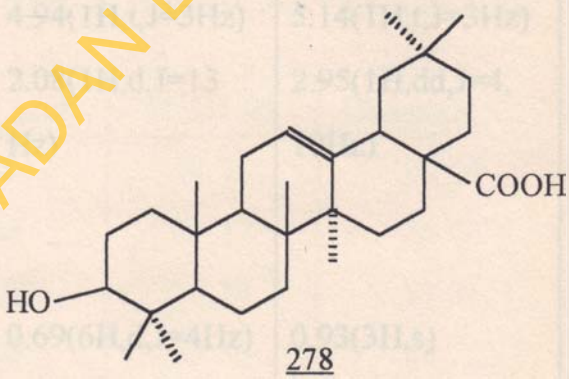
Fig. 57. ^{1}H nmr spectrum of oleanolic acid



peaks associated with each isomer. This was easily assessed from the ^{13}C nmr spectrum. The relative intensity of the carboxyl carbon signals (δC, 180.19 and 179.90) were equal. Likewise most of the pair of signals appearing upfield showed equal relative intensities. The obvious conclusion is that GSH 49 contained equal amounts of ursolic 277 and oleanolic 278 acids in the mixture.



277



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This is the first report of the presence of an ursene compound in *Gardenia* plants. All the triterpene compounds of *Gardenia* plants reported in the literature belong to the Oleanene series. This is however not a strange occurrence because quinovic acid, which had been reported as a common triterpenoid of Rubiaceae plants is of the ursene series.

Table 28- 250 MHz ^{1}H NMR Spectral data for GSH 49, Ursolic and Oleanolic acids.

Position	GSH 49	Ursolic acid	Oleanolic acid
3α	3.11(1H, m)	2.91(1H,t,J=8Hz)	3.10(1H,t,J=7-10Hz)
12	5.15 (H,bs)	4.94(1H,t,J=3Hz)	5.14(1H,t,J=3Hz)
18	2.96(1H,dd,J=4, 10Hz) 2.29(1H,d,J=11 Hz)	2.08(1H,d,J=13 Hz)	2.95(1H,dd,J=4, 10Hz)
Me_1	0.93	0.69(6H,d,J=4Hz)	0.93(3H,s)
Me_2	0.90	0.50(6H,s)	0.89(3H,s)
Me_3	0.88	0.47(6H,s)	0.67(6H,s)
Me_4	0.70	0.34(3H,s)	0.66(3H,s)
Me_5	0.68		0.60(3H,s)
Me_6	0.67		0.54(3H,s)
Me_7	0.64		
Me_8	0.61		
Me_9	0.60		
Me_{10}	0.54		

Therefore, on biogenetic grounds, the presence of ursolic acid in *G.erubescens* is a possibility, as plants of the same family naturally possess inherent potential to produce similar natural products, although a number of factors, such as geographical location and genetic variation may influence the production of certain natural products in preference to some other compounds.

Table 29- 100 MHz ^{13}C NMR data for GSH 49, Ursolic and Oleanolic acids.

Carbon	^{u}GSH 49	Ursolic acid	^{o}GSH 49	Oleanolic acid
1.	39.09	38.4	38.95	38.25
2.	24.93	24.44	26.17	25.50
3.	78.14	77.43	78.14	77.38
4.	39.50	38.80	39.39	38.71
5.	55.83	55.13	55.83	55.13
6.	17.52	18.10	18.80	18.12
7.	33.59	32.90	34.24	33.54
8.	39.50	38.80	39.77	39.07
9.	48.06	47.35	48.06	47.44
10.	37.30	36.60	37.40	36.69
11.	23.64	22.95	23.92	23.10
12.	124.29	124.97	122.57	121.88
13.	139.27	138.58	144.85	144.14
14.	42.52	41.81	42.19	41.48
15.	28.80	28.15	28.70	28.11

Table 29 Cont'd

[o]GSH 49 Chemical Shifts of Carbons for the Oleanolic acid component of the mixture.

[u]GSH 49 Chemical shifts of Carbons for the Ursolic acid component of the mixture.

The ^{13}C NMR data for GSH 49 and the pure compounds - oleanolic and ursolic acids, as presented in table 29 unequivocally shows that the ^{13}C NMR spectrum of GSH 49 contained signals for the carbons of both oleanolic and ursolic acids. The data shown in table 29 compares fairly well with a similar table for the ^{13}C NMR data of the methyl ester of a mixture containing oleanolic and ursolic acids and those of the methyl esters of pure oleanolic and ursolic acids as reported by Ngonela, S.A. in his thesis.[173-176] The obvious differences were the upfield shift of the C-28 carbon signal and the presence of a carbon signal for the methoxyl group in the methyl ester derivatives.[173]

However, table 29 shows consistent downfield shifts in the δ-value of the carbon signals for the mixture as compared with the pure components. This trend was not observed in the table for the methyl ester derivatives, as the δ-values for the carbon signals of the mixture and the pure compounds were almost the same.[173]

The shifts as they appear in table 29 could be attributed to the possibility of intermolecular H-bonding between the Oleanolic acid molecules and ursolic acid molecules in the mixture. This type of interaction is not likely to be strong in the methyl ester derivatives as they do not contain carboxyl groups. Hence, there were no shifts observed in the δ-values for the carbon atoms of the mixture of methyl ester derivatives of oleanolic and ursolic acids when compared with the δ-values of the carbon atoms of the pure methyl oleanoate and methyl ursolate.

Table 29 contd.

Table	ᵘGSH 49	Ursolic acid	ᵒGSH 49	Oleanolic acid
16.	17.52	18.10	18.80	18.12
17.	46.69	47.35	48.13	48.98
18.	53.56	52.86	42.03	41.32
19.	39.98	39.28	46.50	45.79
20.	39.39	38.71	30.00	30.29
21.	31.08	30.40	33.27	32.51
22.	37.46	36.77	30.00	30.30
23.	28.34	28.00	28.12	27.63
24.	16.57	15.92	16.57	15.90
25.	15.68	15.01	15.55	14.90
26.	17.52	18.10	17.47	16.75
27.	23.76	22.95	23.83	23.10
28.	179.90	179.23	180.19	179.51
29.	17.07	16.87	30.97	30.30
30	21.41	20.77	18.80	18.12

2.5.10 Elucidation of Structure of GSA 5

The first indication that GSA 5 is a triterpenoid was from the positive Liebermann-Burchard reaction. The absorptions at Vmax 3450 and 3330 cm^{-1} in the IR Spectrum suggested the presence of hydroxyl group. The Strong peak at

Vmax 1698 cm^{-1} also suggested the presence of carboxyl group and the bands at 1304-1016 cm^{-1} supported the presence of hydroxyl and carboxyl groups (Fig 58)

The ^{1}H nmr spectrum showed six methyl groups as singlets (Fig 59) Three carbinolic protons were also visible at δ3.57 (1H, d, J=10.4Hz) and δ3.86 (2H,m). The peak at δ5.15 (1H, t,J=3Hz) represent an olefinic proton. The ^{13}C nmr spectrum gave signals for thirty carbon atoms (Fig 60) and as shown by DEPT spectrum (Fig 61), they consist of eight quaternary, five methine, eleven methylene and six methyl carbon atoms. The two carbi nolic carbons were also revealed as one primary and the other secondary. The signals at δC, 121.06 and 143.33 supported the presence of olefinic bond while the signal at δC, 178.72 also supported the presence of carboxyl group.

From the chemical shifts of the olefinic carbons δ121.06 and δ143.33 (see table 20, p.97) and taking into consideration the fact that thirty carbon atoms were present, GSA 5 appeared to be a dihydroxyl triterpenoid with oleanene skeleton. The ^{1}H nmr signal at δ3.57 (1H,d, J=10.4Hz) could be attributed to one of the methylene protons of the primary hydroxyl group. The signal for the second proton seem to overlap with the carbinolic methine proton at δ3.86 (2 H,m).

An insight into the probable location of the carboxyl and primary hydroxyl groups was obtained from analyis of the mass spectrum. Further evidence for the presence of hydroxyl groups was provided by the loss of mass units 17 and 18 consecutively from the molecular ion - m/z 455 [M-17] and 437 [455-H_2O] and the loss of CO group (Fig 62). The accurate mass measurement for M-17, m/z 455.3532 corresponds to C_{30} H_{47} O_3 (requires 455.3513). The EIMS also showed

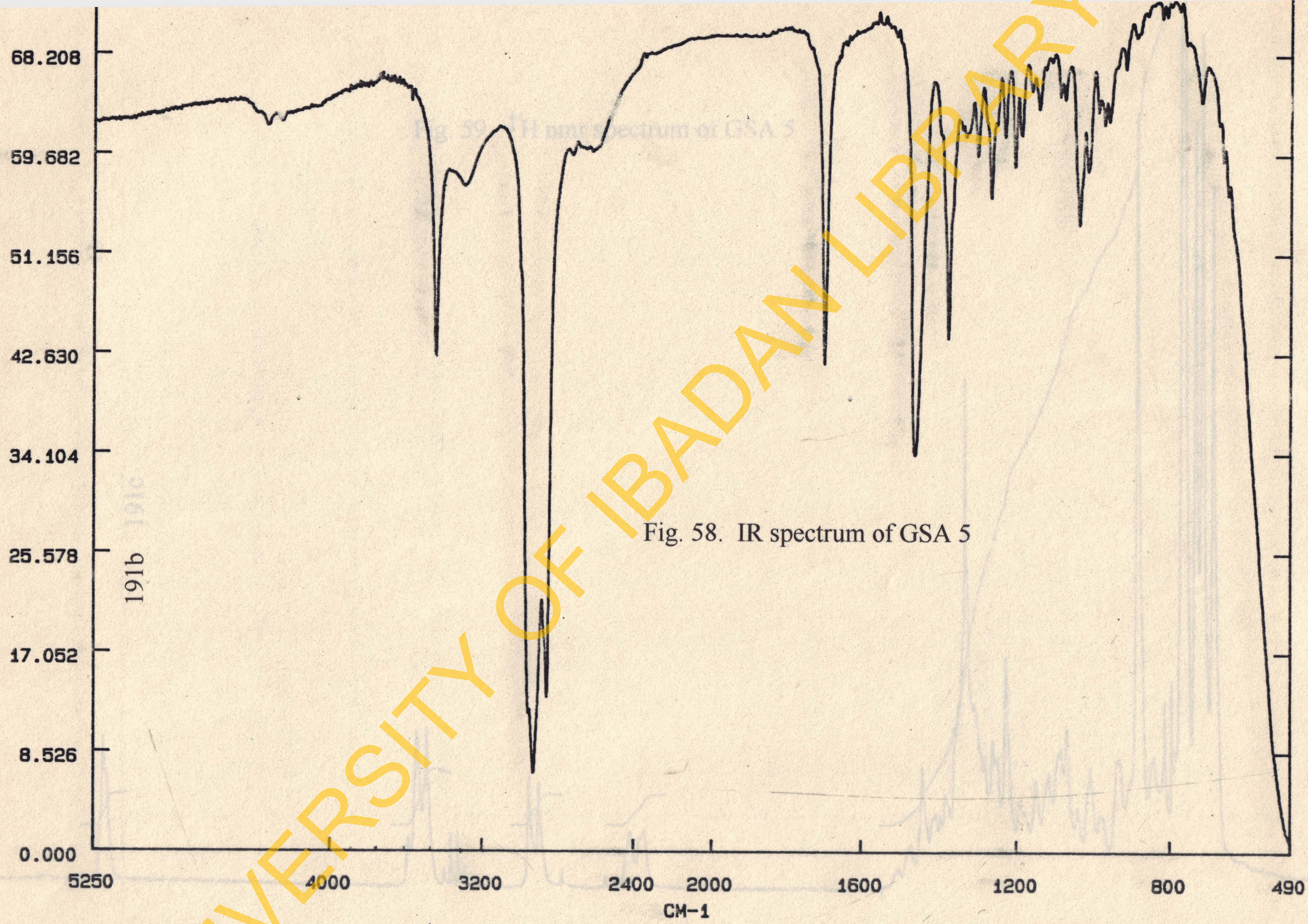


Fig. 58. IR spectrum of GSA 5

Fig. 59. ^{1}H nmr spectrum of GSA 5

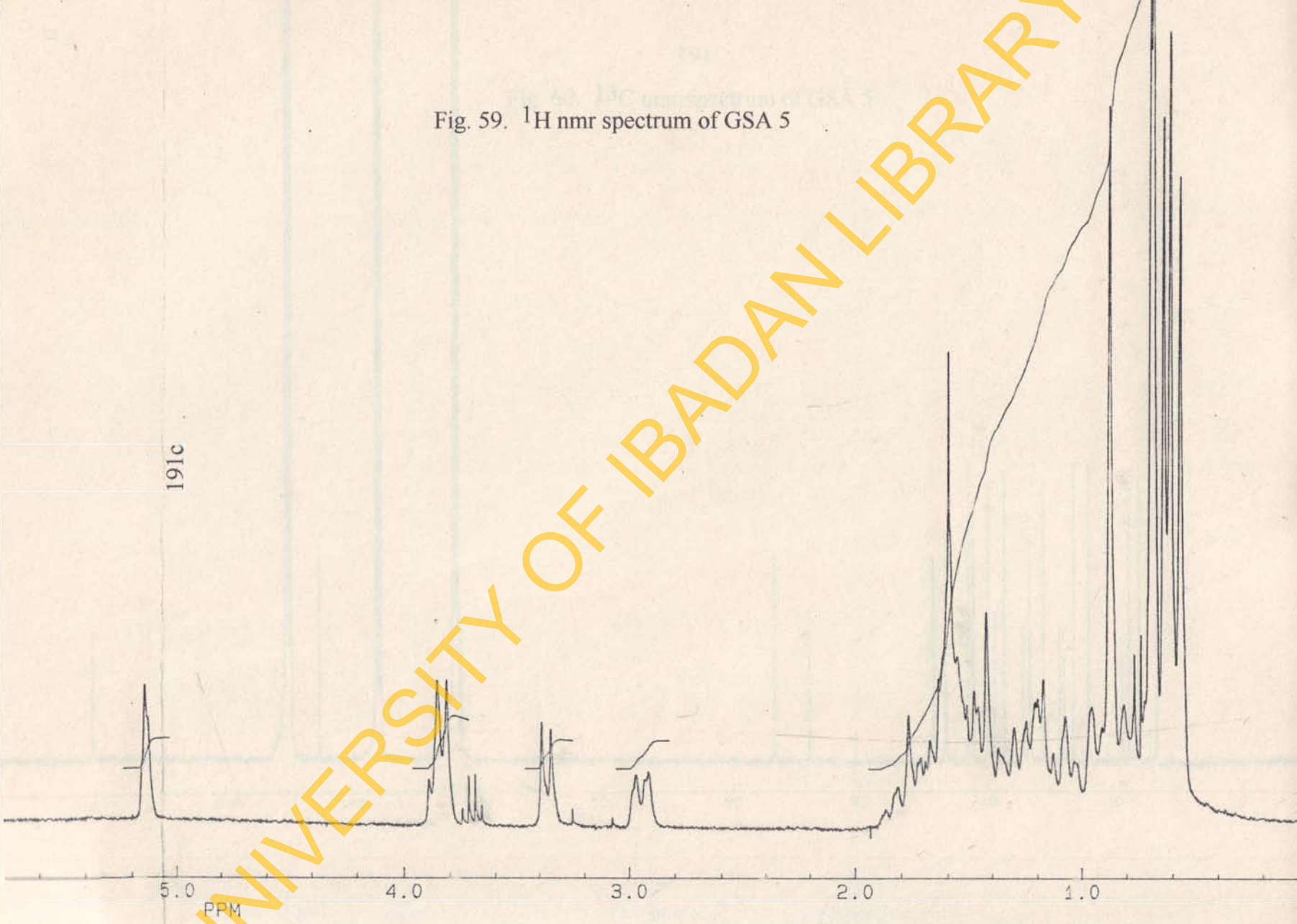
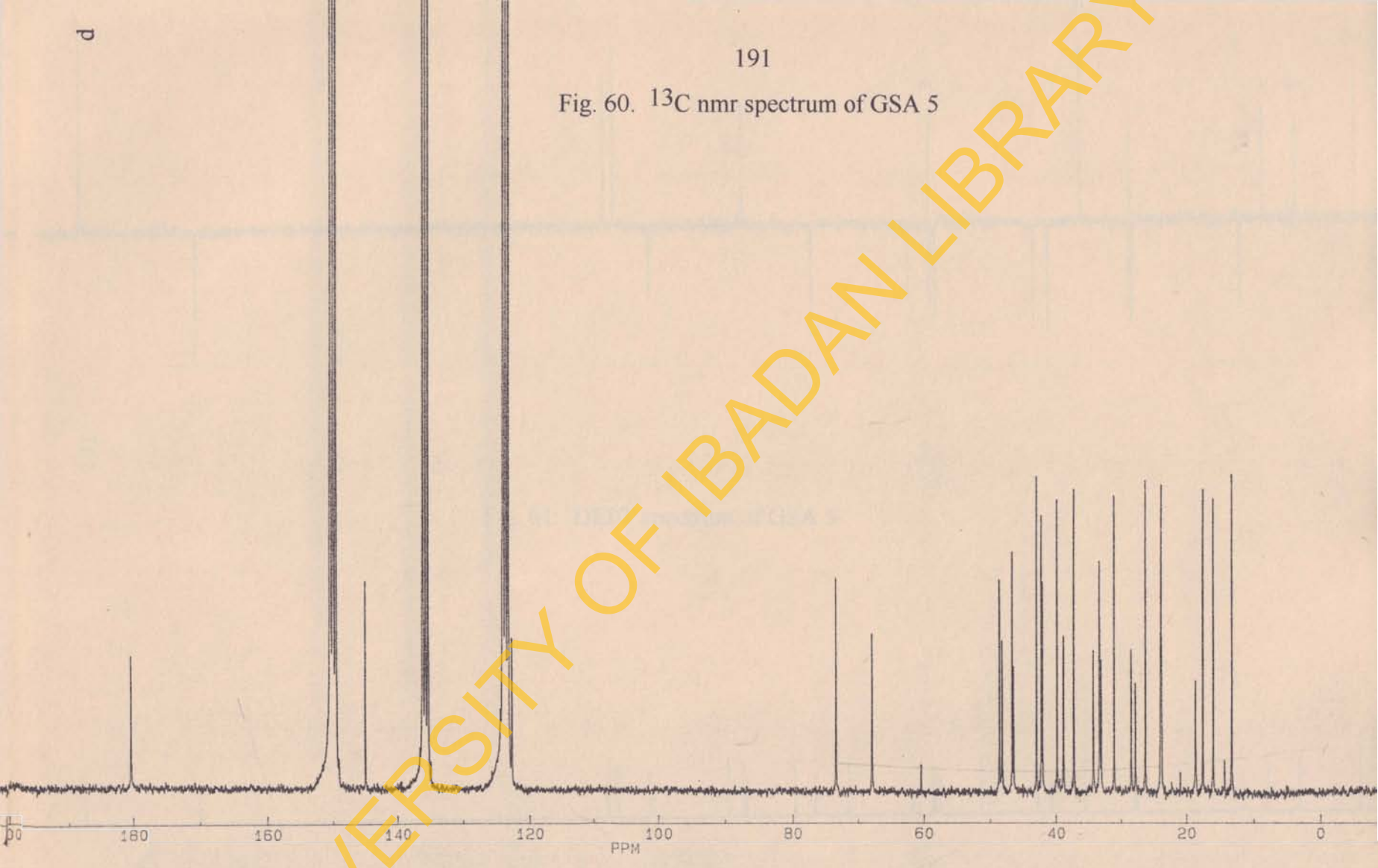


Fig. 60. ^{13}C nmr spectrum of GSA 5



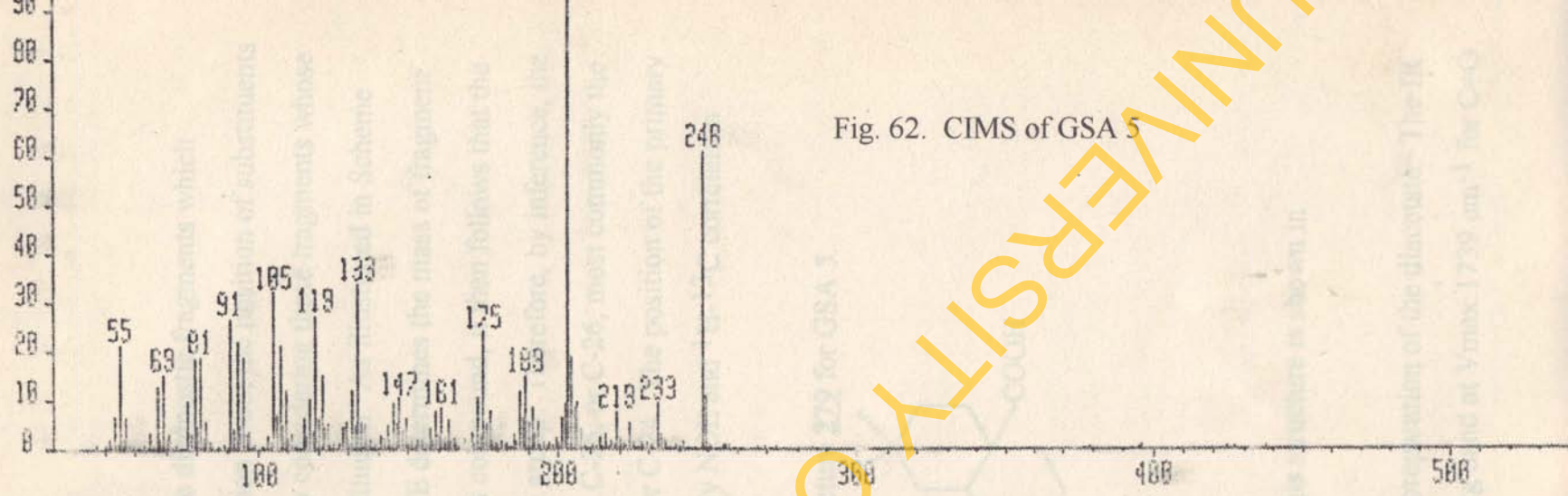
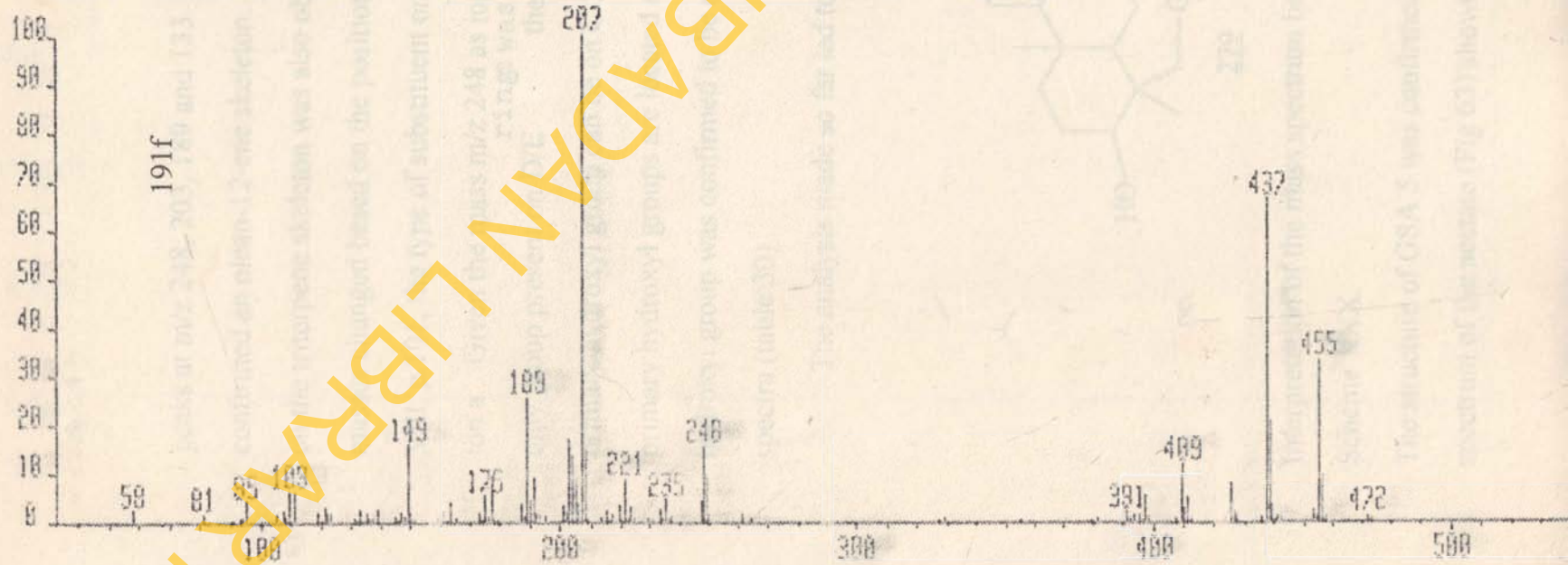
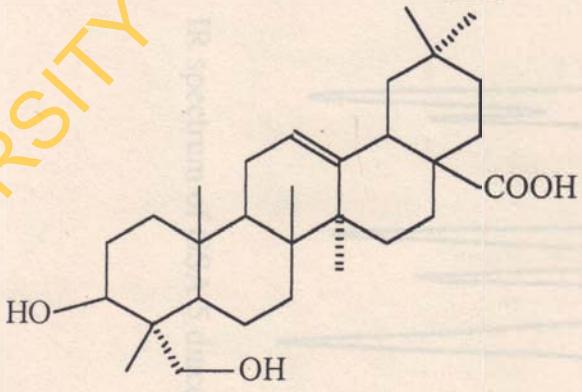


Fig. 62. CIMS of GSA 5



peaks at m/z 248, 203, 189 and 133. These are diagnostic fragments which confirmed an olean-12-ene skeleton. Information about the position of substituents on the triterpene skeleton was also obtained by considering these fragments whose masses changed based on the position of substituents. As illustrated in Scheme VIII, p.101, the type of substituent on rings D/E determines the mass of fragment ion a . Given the mass m/z 248 as ion a in this compound, it then follows that the only group present on D/E rings was the carboxyl group. Therefore, by inference, the primary hydroxyl group can be on C-23, C-24, C-25, or C-26, most commonly the primary hydroxyl groups are located on C-23 or C-24. The position of the primary hydroxyl group was confirmed to be on C-23 by NOE and ^{1}H-^{13}C correlation spectra (table 30).

The analysis made so far led to the structure 279 for GSA 5.



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Interpretation of the mass spectrum based on this structure is shown in Scheme XXX.

The structure of GSA 5 was confirmed by the preparation of the diacetate. The IR spectrum of the acetate (Fig 63) showed a strong band at Vmax 1739 cm^{-1} for C=O

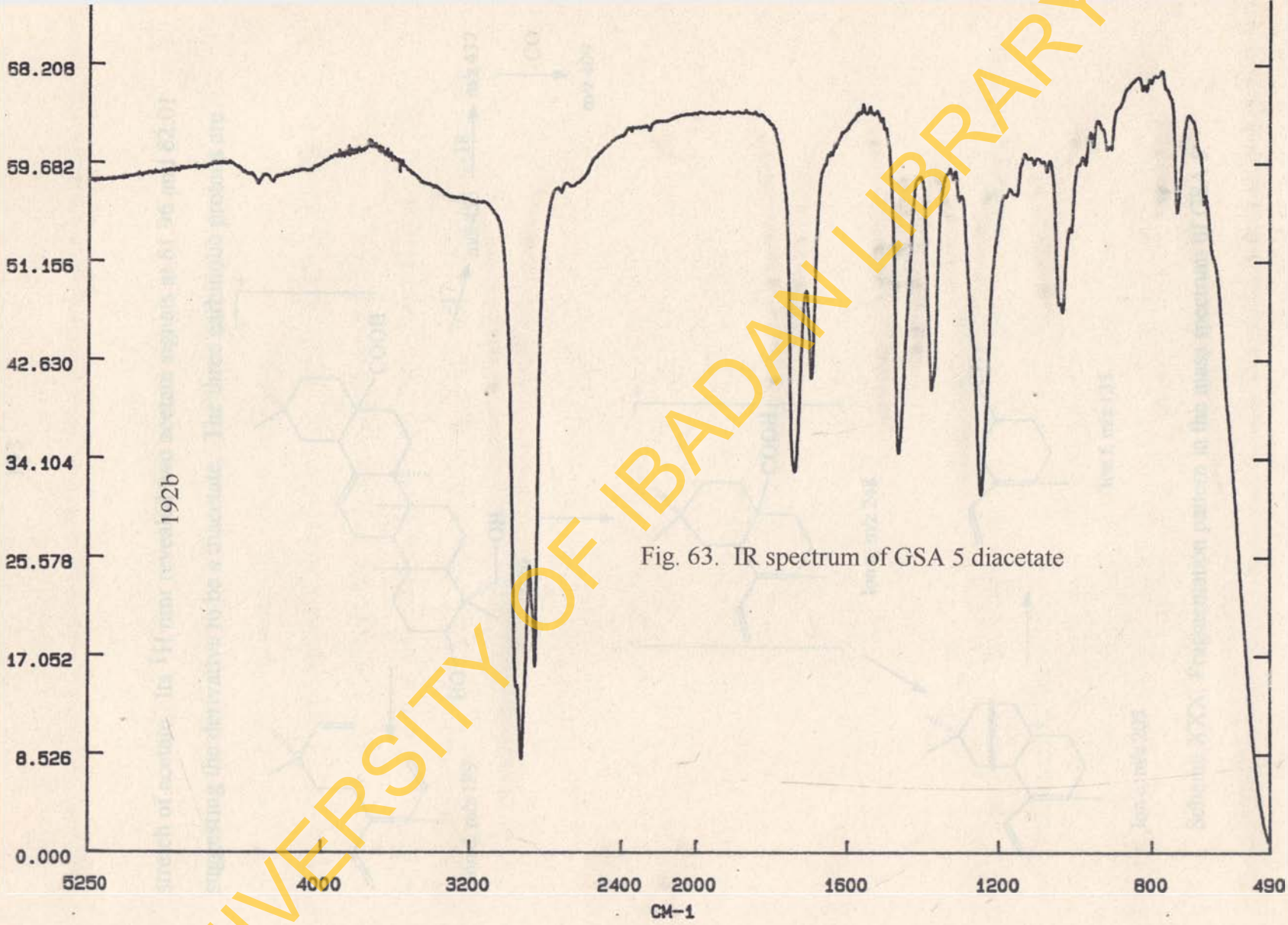
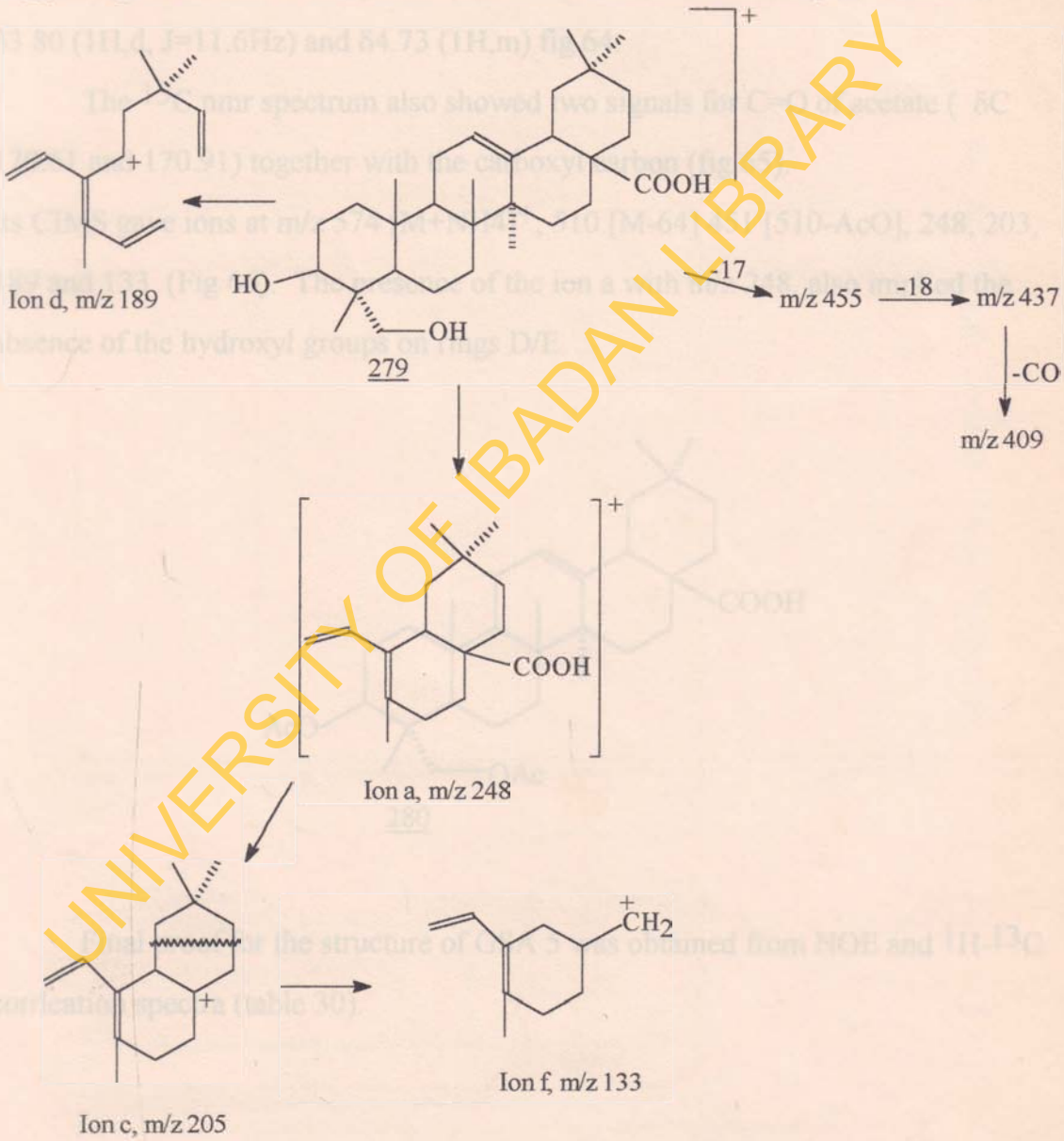


Fig. 63. IR spectrum of GSA 5 diacetate

stretch of acetate. Its ^1H nmr revealed two acetate signals at δ1.96 and δ2.01 suggesting the derivative to be a diacetate. The three carbinolic protons are

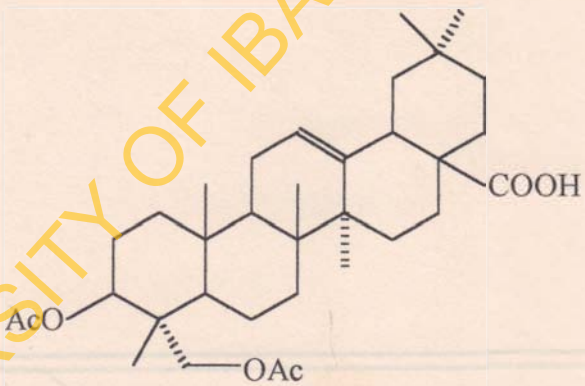


Scheme XXX. Fragmentation pattern in the mass spectrum of GSA 5.

now clearly identified and appeared more downfield - δ3.63 (1H,d, J=11.6Hz), δ3.80 (1H,d, J=11.6Hz) and δ4.73 (1H,m) fig 64.

The ^{13}C nmr spectrum also showed two signals for C=O of acetate (δC 170.61 and 170.91) together with the carboxyl carbon (fig 65).

Its CIMS gave ions at m/z 574 $[M+NH4]^+$, 510 [M-64] 451 [510-AcO], 248, 203, 189 and 133. (Fig 66). The presence of the ion a with m/z 248, also implied the absence of the hydroxyl groups on rings D/E.



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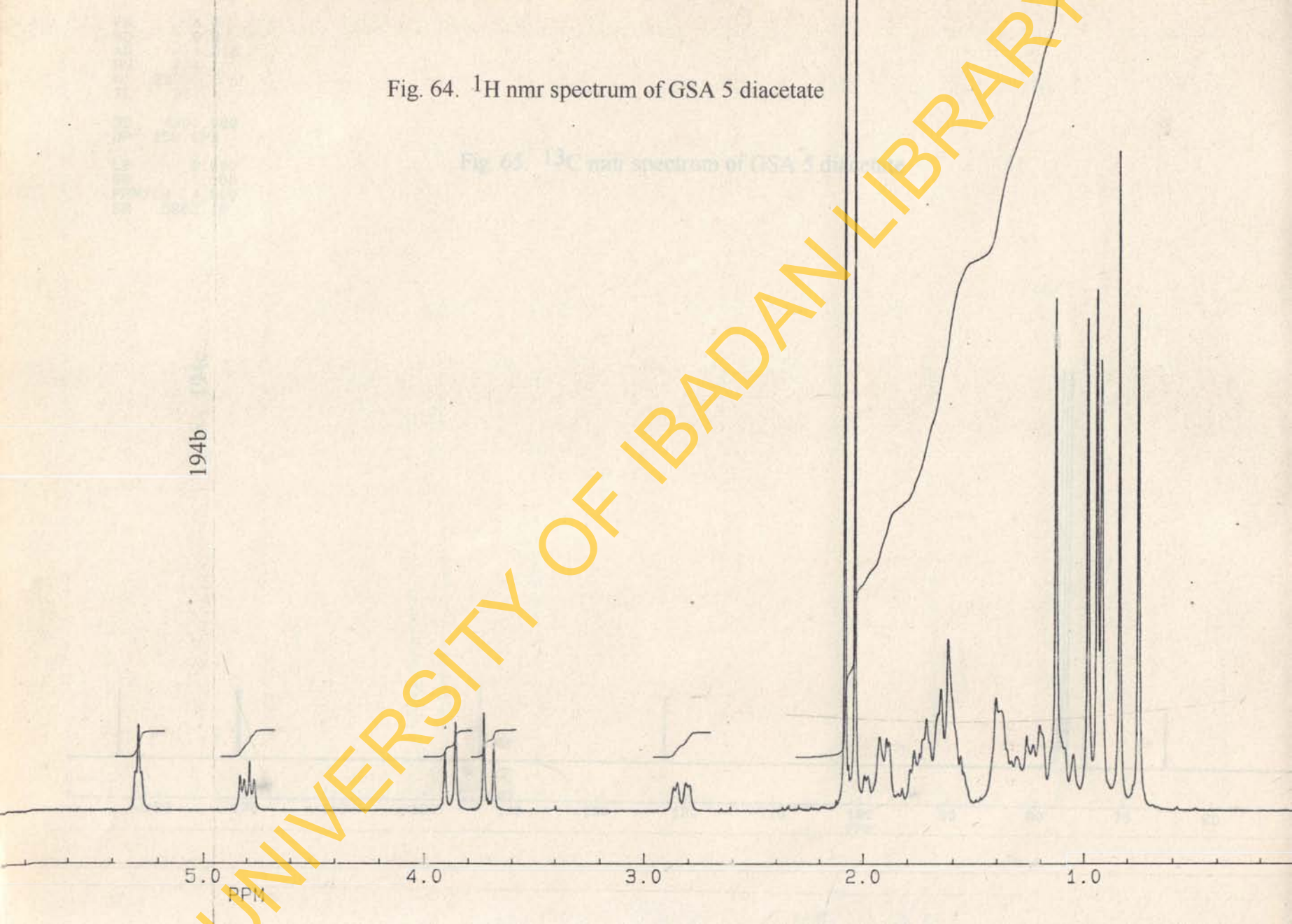
Final proof for the structure of GSA 5 was obtained from NOE and ^{1}H-^{13}C corrleation spectra (table 30).

Fig. 64. ^{1}H nmr spectrum of GSA 5 diacetate



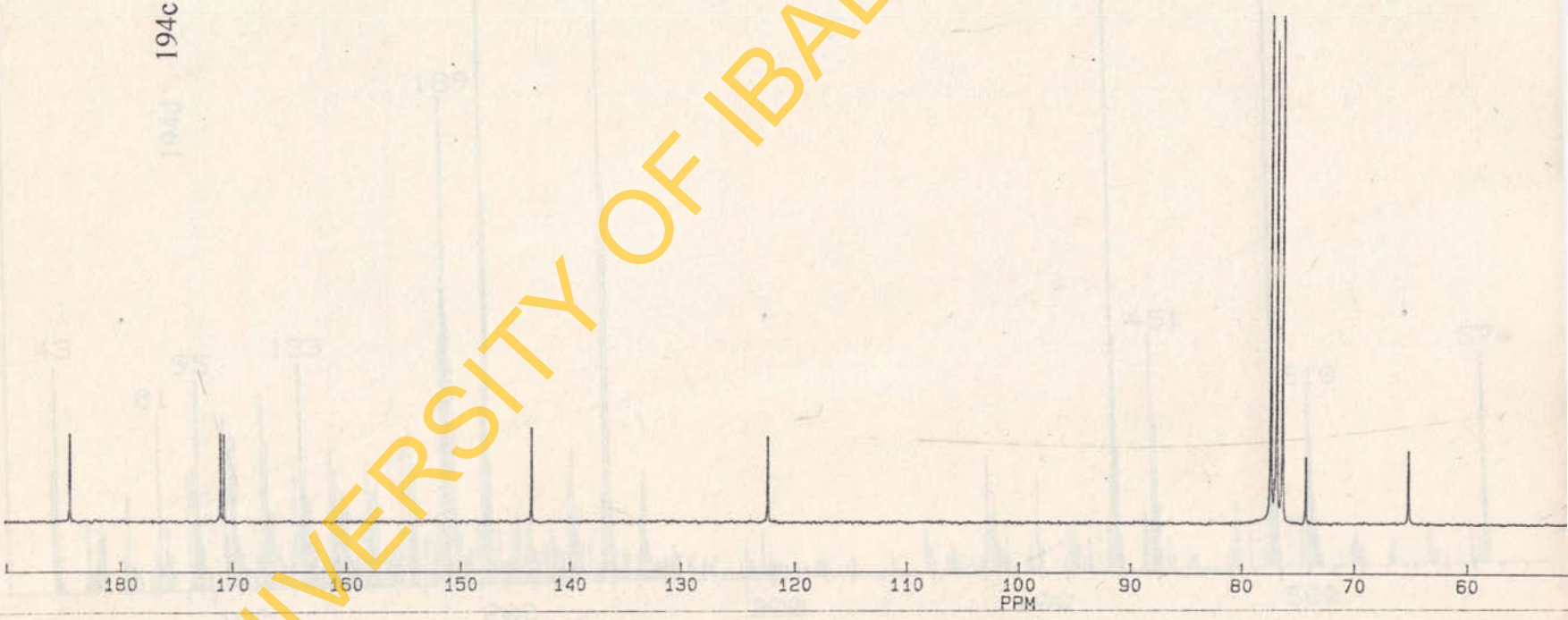
HZ/PT .984

PW 4.0
RD 0.0
AG 1.016
RG 2
NS 23351
TE 297

O2 4200.000
DP 15H CPD

LB 2.000
GB .100
PPM/CM 4.500
SR 5962.19

Fig. 65. ^{13}C nmr spectrum of GSA 5 diacetate



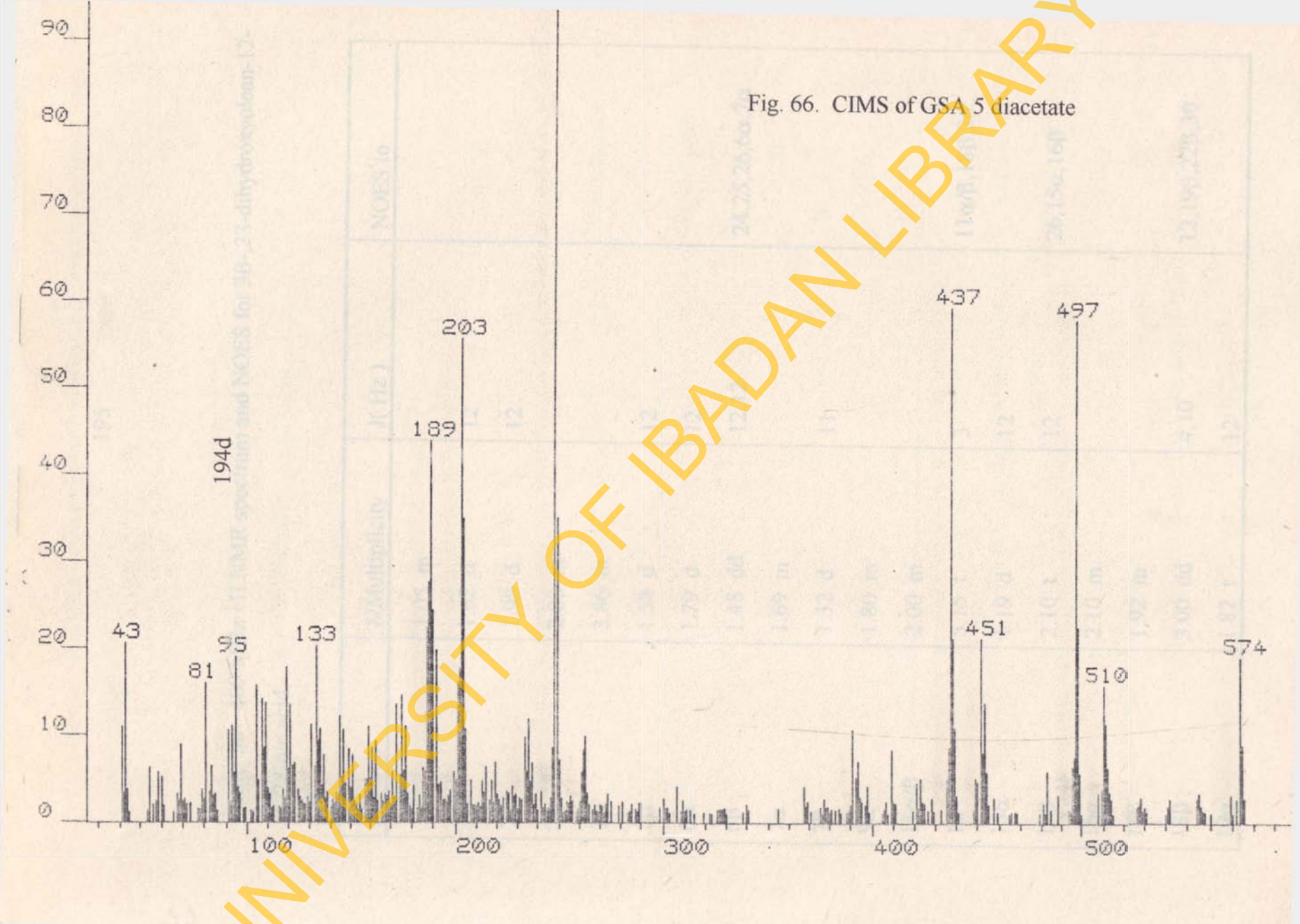


Fig. 66. CIMS of GSA 5 diacetate

Table 30- 400 MHz ^{1}H NMR spectrum and NOES for 3β-,23-dihydroxyolean-12-en-28-oic acid.

Position	δ/Multiplicity	J(Hz)	NOES to
1α	1.05 m		
1β	1.62 d	12	
2α	1.98 d	12	
2β	2.00 m		
3α	3.86 m		
5α	1.58 d	12	
6α	1.79 d	12	
6β	1.48 dd	12,12	24.25,26,6α,7α
7α	1.69 m		
7β	1.32 d	11	
9α	1.80 m		
11α/β	2.00 m		
12	5.15 t	3	11α/β,18β,26
15α	1.19 d	12	
15β	2.10 t	12	26,15α,16β
16α	2.10 m		
16β	1.92 m		
18β	3.00 dd	4,10	12,19β,22β,30
19α	1.82 t	12	

Table 30 Contd.

Position.	δ/Multiplicity.	J(Hz).	NOES to
19β	1.30 d	12	
21α	1.45 d	12	29,19α
21β	1.21 d	12	
22α	1.90 t	12	
22β	2.08 d	12	
23a	3.86 d	10.4	
23b	3.57 d	10.4	23a,5α,6α,3α
24	0.71 s		23a,23b,3α
25	0.64 s		1β,2β,11α/β
26	0.70 s		23a &b
27	0.88 s		12,16α,9α,7α, 18β
29	0.61 s		19α,21α
30	0.69 s		18β,22β

From the analysis of the spectral data available on GSA 5 and its diacetate 280, it was identified as 3β, 23-dihydroxyolean 12-en-28-oic acid, 279.

2.5.11. Structure of GSA 8

This compound also gave a positive Liebermann-Burchard reaction for triterpenoid.

Its ^{1}H nmr spectrum revealed the presence of seven methyl groups which appeared as singlets (Fig 67). There were also two carbinolic protons at δ3.12 (1H,d, J=4Hz) and δ4.06 (1H, dt, J=3 Hz) together with an olefinic proton at δ5.18 (1Ht, J=3 Hz).

The ^{13}C nmr spectrum confirmed the presence of two carbinolic carbons at δC, 70.76 and 70.68, indicating the presence of two hydroxyl groups (Fig 68). DEPT spectrum showed the two carbinolic carbons to be methine carbon atoms (Fig 69), suggesting that the two hydroxyl groups were secondary. The presence of carboxyl and olefinic groups were also supported by signals at δC 179.54, δ122.03, and δ144.19.

The CIMS showed evidence for the presence of hydroxyl and carboxyl groups with fragment ions m/z 455 [M-17], 437 [455-H_2O] and 409 [437-CO] with the molecular ion peak at m/z 472 (Fig 70). The fragment ions m/z 248, 203, 189 and 133 supported olean-12-ene skeleton and at same time gave an indication of the presence of only the carboxyl group on rings D/E. The molecular mass m/z 472 corresponds to the formula $C_{30}H_{48}O_4$.

The multiplicity of the carbinolic protons suggested a vicinal diol. If there are no hydroxyl groups on rings D/E, then the other alternative location would be on ring A since C-3 always carry a hydroxyl group. The location of the hydroxyl groups on ring A is illustrated with structure 281. The two hydroxyl groups being located on C-2 and C-3.

Fig. 67. ^{1}H nmr spectrum of GSA 8

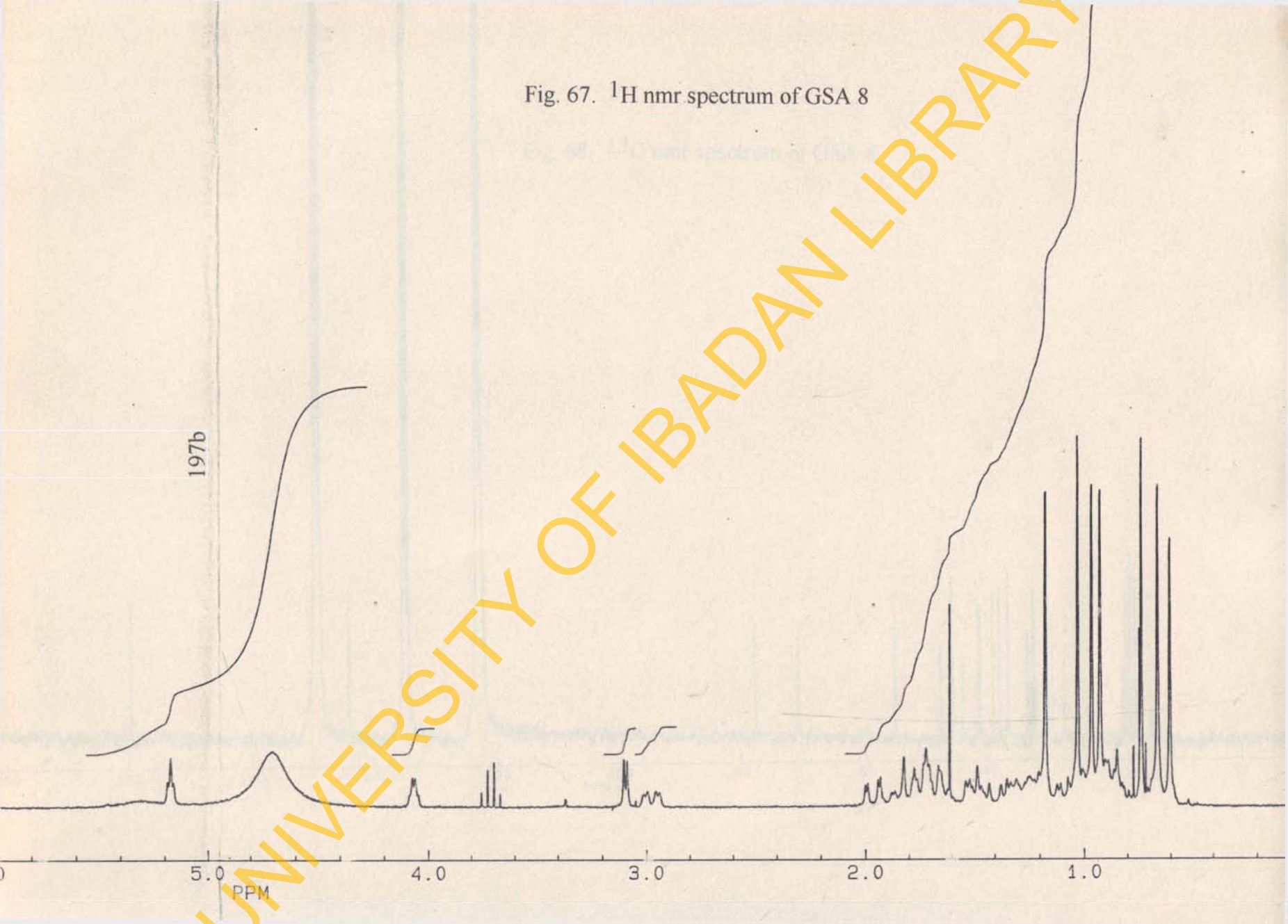


Fig. 68. ^{13}C nmr spectrum of GSA 8

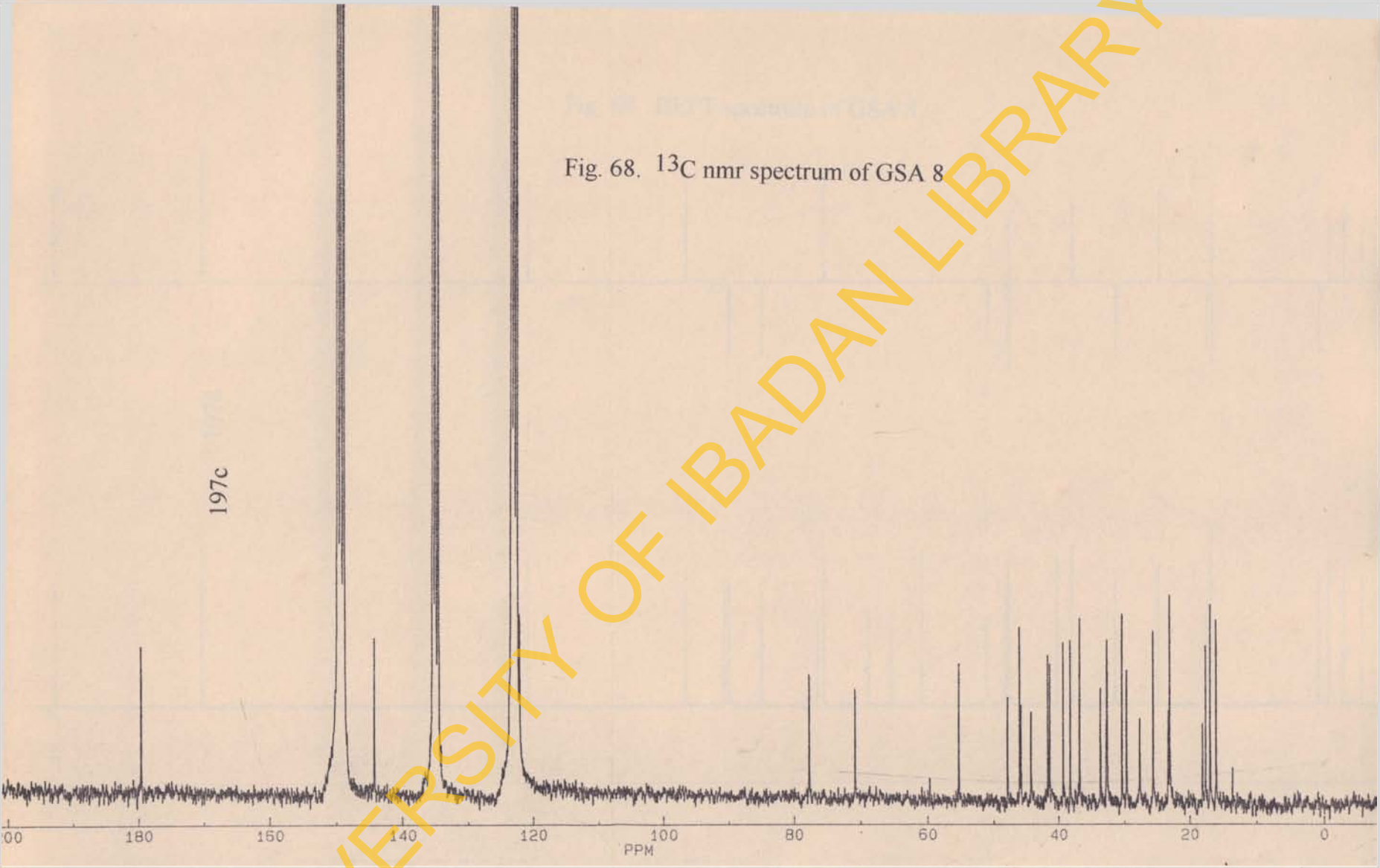
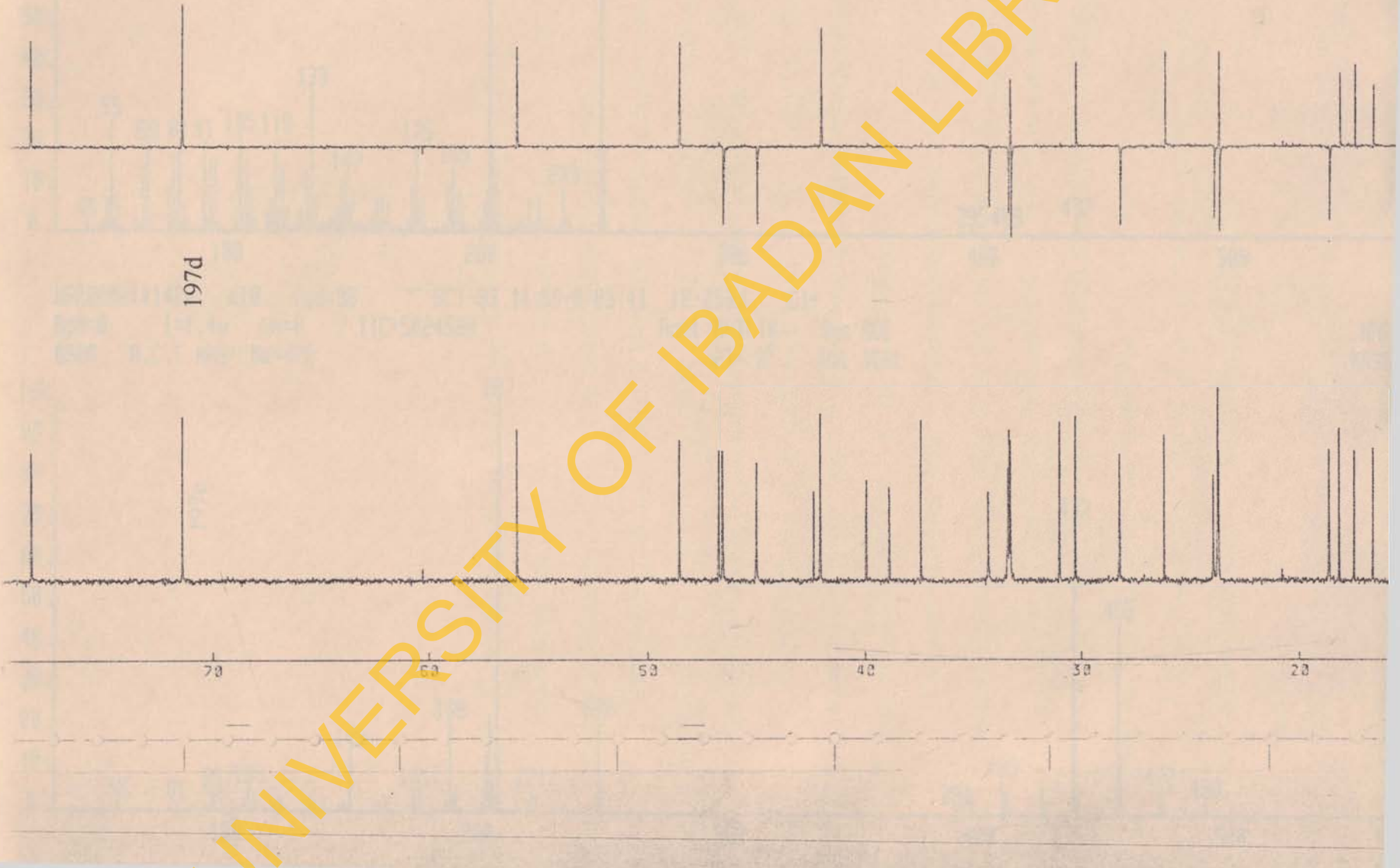


Fig. 69. DEPT spectrum of GSA 8



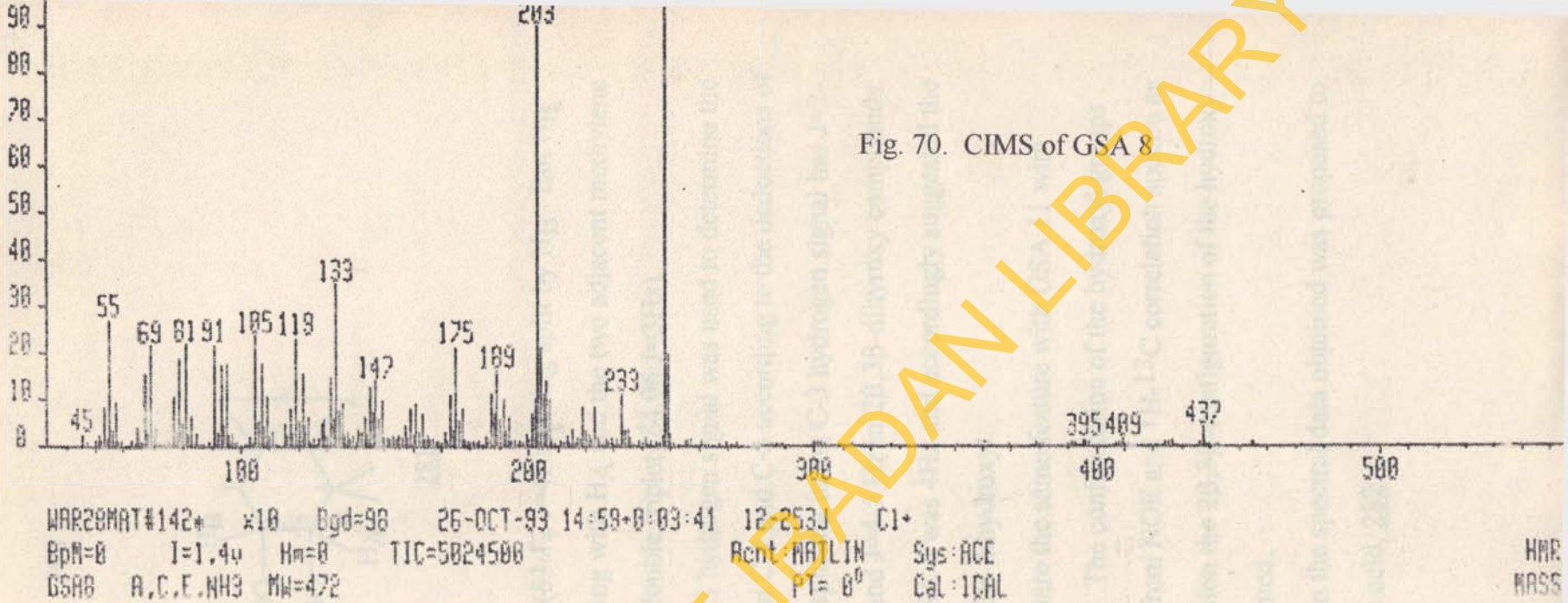
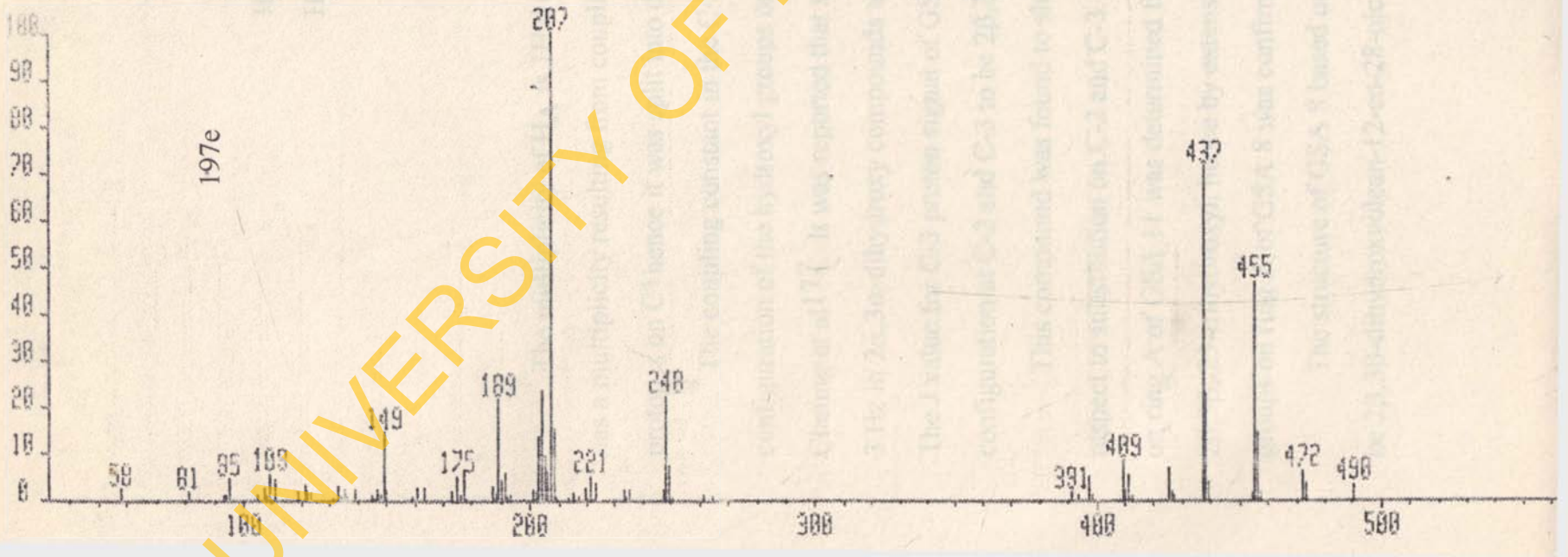
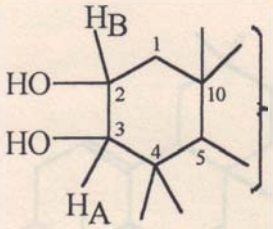


Fig. 70. CIMS of GSA 8





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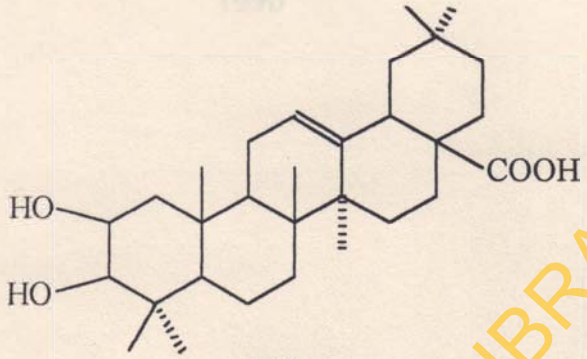
The multiplicity of H_A is 1H,d (δ3.12 J=4Hz) being split by H_B. But H_B has a multiplicity resulting from coupling with H_A and the two adjacent methylene protons on C1 hence it was split into double triplet (δ4.06 J=3Hz).

The coupling constant in the C-3 hydrogen signal was used to determine the configuration of the hydroxyl groups on C-2 and C-3 according to the deductions of Cheung et al[177]. It was reported that splitting in the C-3 hydrogen signal has J=2-3 Hz in 2α,3α-dihydroxy compounds and J=4-5 Hz in 2β,3β-dihyroxy compounds. The J value for C-3 proton signal of GSA 8 was 4Hz and accordingly suggested the configuration at C-2 and C-3 to be 2β,3β-dihydroxyl.

This compound was found to share the same feature with GSA 11 with respect to substitution on C-2 and C-3. The configuration of the hydroxyl groups on ring A of GSA 11 was determined from NOE and ^1H-^{13}C correlation spectra as 2β,3β, 23-trihydroxyl, hence by extension the 2β,3β-configuration of the hydroxy groups on ring A of GSA 8 was confirmed.

The structure of GSA 8 based on the spectral data obtained was proposed to be 2β,3β-dihydroxyolean-12-en-28-oic acid, 282.



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This structure could not be confirmed further by derivatization due to the small quantity isolated. However, it is most probable that the structure is correct because GSA 8 shares similar features with GSA 5 and GSA 11, which have been identified as olean-12-ene-28-oic acid derivatives.

2.5.12 Structure of GSA 11

GSA 11 was the third triterpene aglycone isolated from the stems of *G. erubescens*. It also gave a positive Liebermann-Burchard reaction, The IR spectrum (Fig 71) showed bands for hydroxyl groups at Vmax 3500, 3465 cm^{-1} and the carboxyl group at Vmax 1677 cm^{-1}. ^1H nmr spectrum revealed signals for six angular methyl groups and four carbinolic protons (Fig 72). The proton signals at δ3.38 (1H, d, J=11Hz) and δ3.83 (1H, d, J=11Hz) appeared to be that of geminal protons (large coupling constant). The other two carbinolic protons at δ3.93 (1H,d, J=4Hz) and δ4.18 (1H, dt, J=3Hz) were similarly coupled to each other and the J

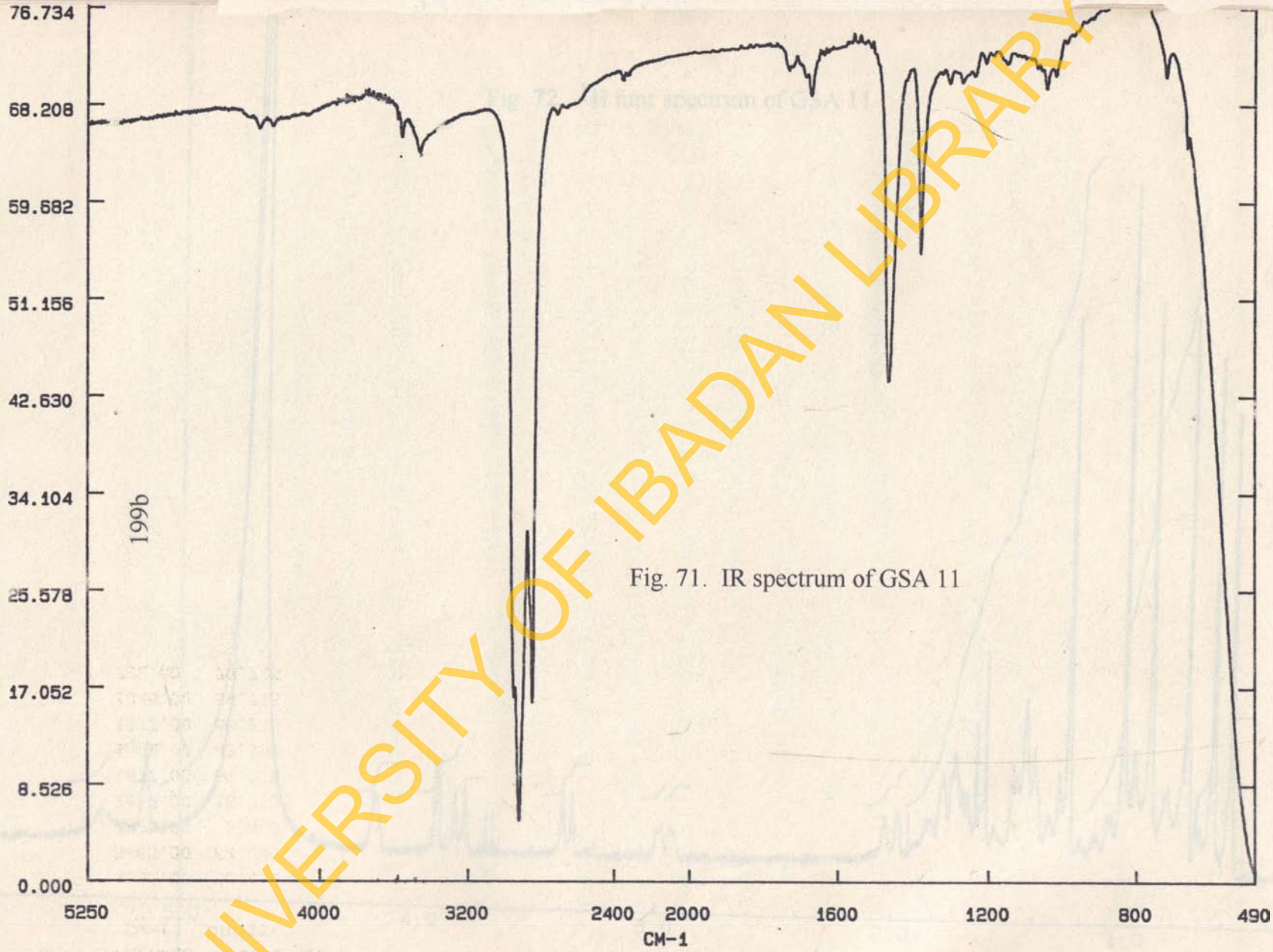
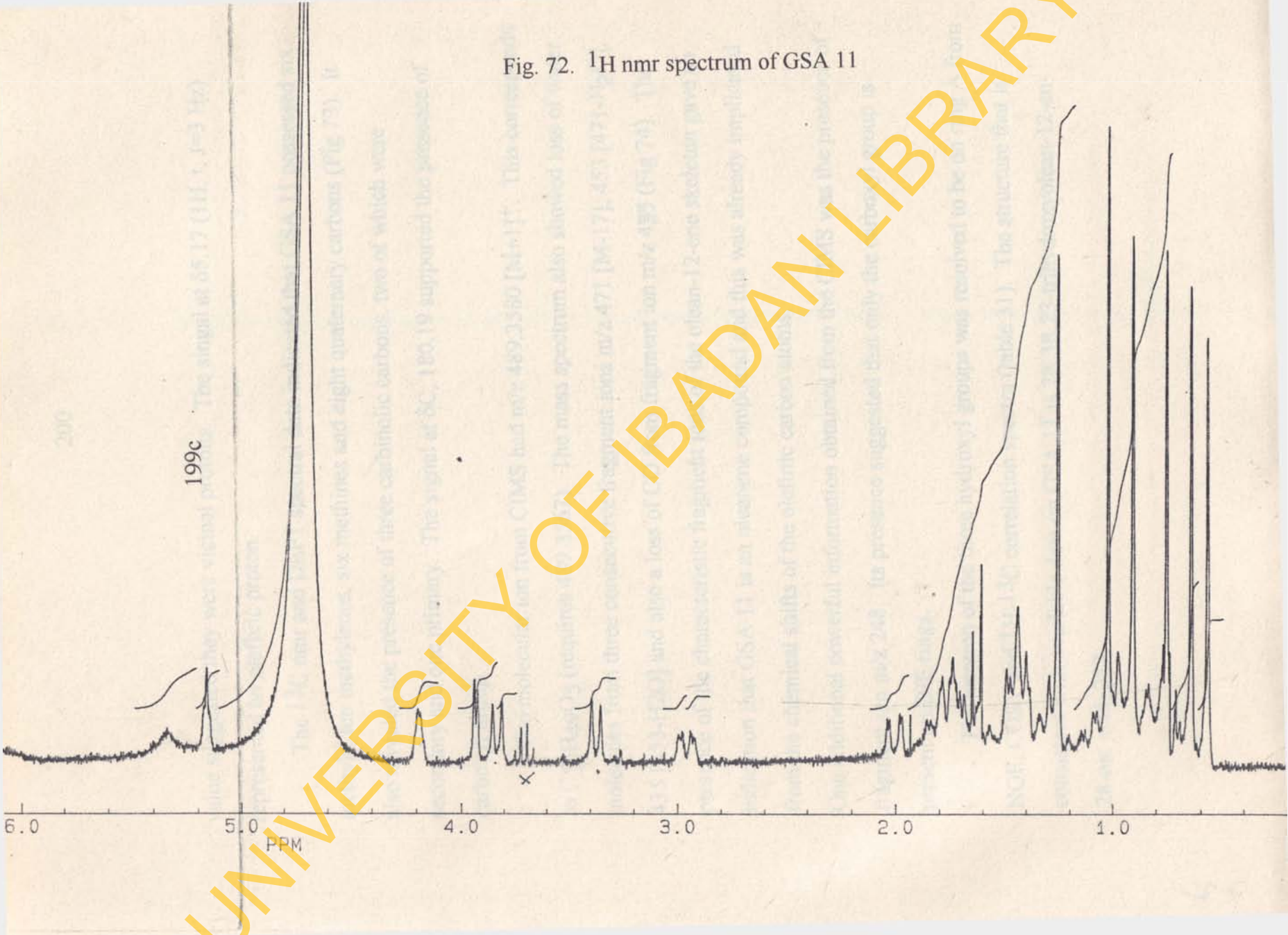


Fig. 71. IR spectrum of GSA 11

Fig. 72. ^{1}H nmr spectrum of GSA 11



value suggested they were vicinal protons. The singal at δ5.17 (1H, t, J=3 Hz) represented an olefinic proton.

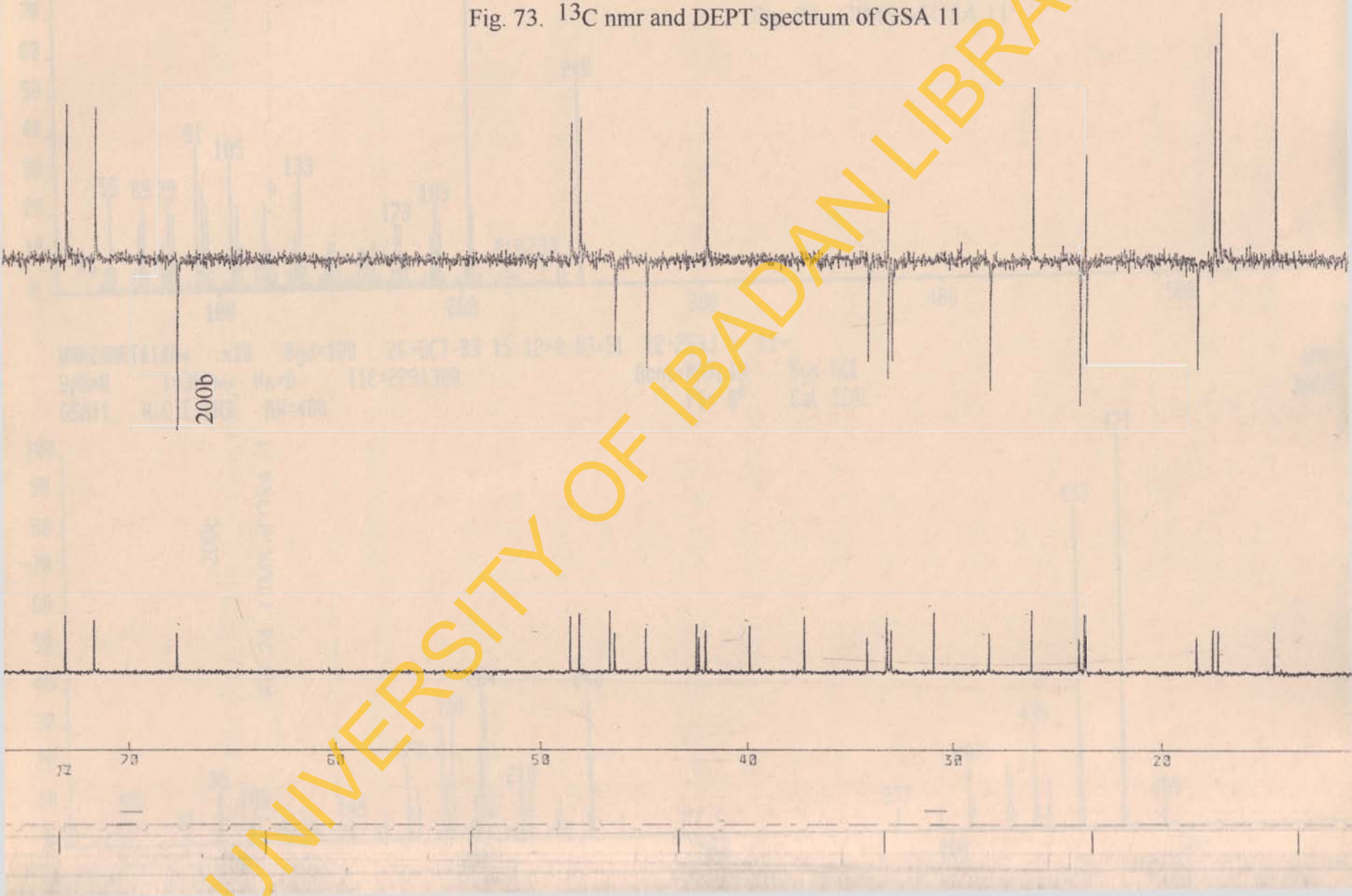
The ^{13}C nmr and DEPT spectral data indicated that GSA 11 possessed six methyls, ten methylenes, six methines and eight quaternary carbons (Fig 73). It also revealed the presence of three carbinolic carbons, two of which were secondary and one primary. The signal at δC, 180.19 supported the presence of carboxyl group.

The molecular ion from CIMS had m/z 489.3580 $[M+1]^+$. This corresponds to $C_{30}H_{49}O_5$ (requires 489.3567). The mass spectrum also showed loss of water molecules from three consecutive fragment ions m/z 471 [M-17], 453 [471-H_2O], 435 [453-H2O] and also a loss of CO from fragment ion m/z 435 (Fig 74). The presence of the characteristic fragment ions of the olean-12-ene skeleton gave an indication that GSA 11 is an oleanene compound and this was already implicated from the chemical shifts of the olefinic carbon atoms.

One additional powerful information obtained from the CIMS was the presence of fragment ion m/z 248. Its presence suggested that only the carboxyl group is present on D/E rings.

The location of the three hydroxyl groups was resolved to be on ring A from NOE, COSY and ^{1}H-^{13}C correlation spectra (table 31). The structure that is consistent with available data on GSA 11 is 2β,3β,23-trihydroxyolean-12-en-28-oic acid, 283.

Fig. 73. ^{13}C nmr and DEPT spectrum of GSA 11



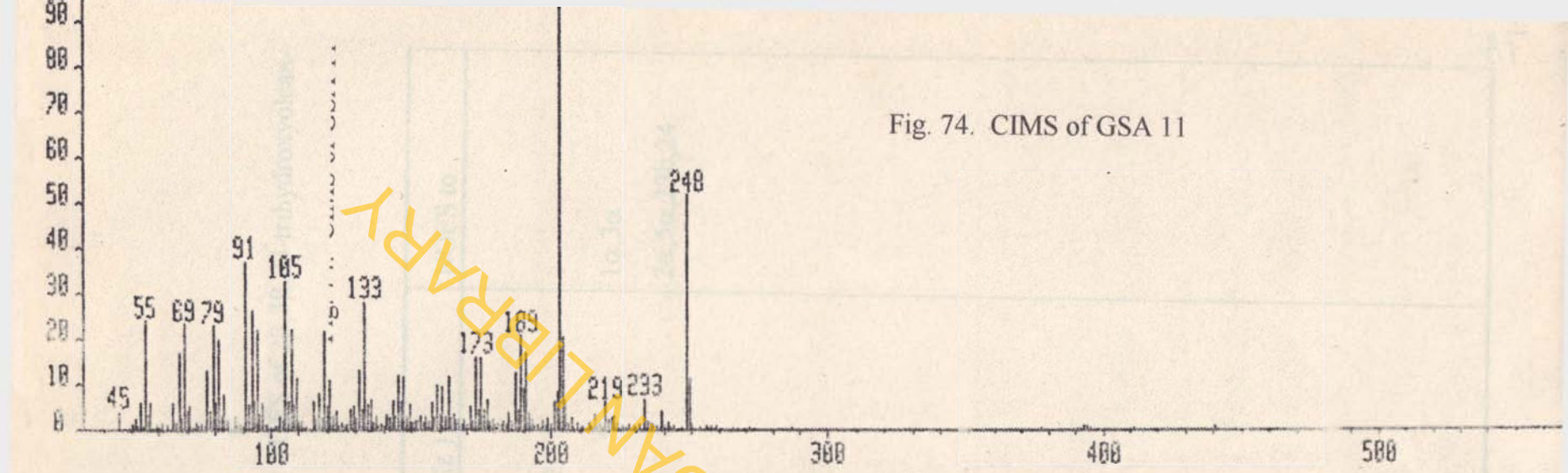


Fig. 74. CIMS of GSA 11

WAR23MAT#140* x10 Bgd=100 26-OCT-93 15:12+0:03:51 12-253J C1+
BpA=0 I=387mv Hw=0 TIC=2291380 Mont:MATLIN Sys:ACE
GSA11 A.C.E.NH3 MW=488 PT= 0° Cal:1CAL

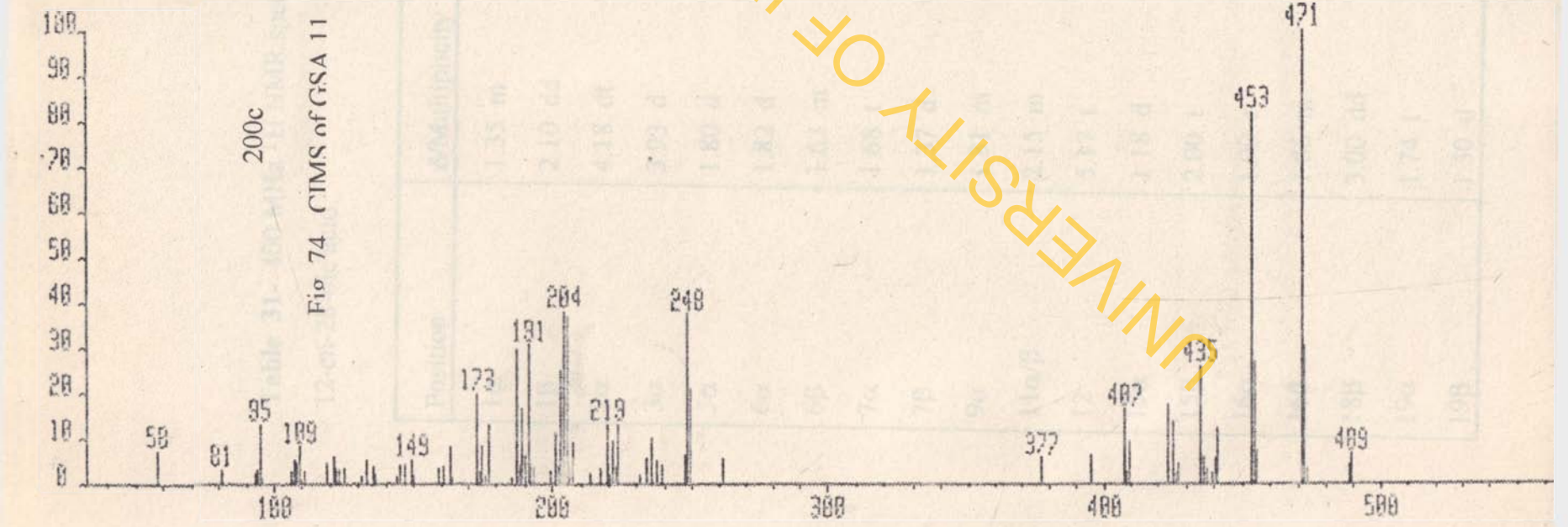


Table 31- 400 MHz ^{1}H NMR spectrum and NOES of 2β,3β,23-trihydroxyolean-12-en-28-oic acid.

Position	δ/Multiplicity	J(Hz)	NOES to
1α	1.35 m		
1β	2.10 dd	4,12	
2α	4.18 dt	3.3	1α,3α
3α	3.93 d	4	2α,5α,23b,24
5α	1.80 d	10	
6α	1.82 d	11	
6β	1.63 m		
7α	1.68 t	11	
7β	1.37 d	12	
9α	1.81 m		
11α/β	2.15 m		
12	5.17 t	3	
15α	1.18 d	12	
15β	2.00 t	12	
16α	1.90 r	12	
16β	1.86 m		
18β	3.00 dd	4,10	
19α	1.74 t	12	
19β	1.30 d	12	

Table 31 Contd.

Position.	δ/Multiplicity.	J(Hz).	NOES to
21α	1.48 t	12	
21β	1.22 d	12	
22α	1.82 d	12	
22β	1.88 t	12	
23a	3.83 d	11	23b,24
23b	3.38 d	11	23a,24
24	1.02 s		3α,23a &b,25
25	1.25 s		
26	0.75 s		
27	0.90 s		
29	0.56 s		
30	0.64 s		

The treatment of GSA 11 with Ac_2O/pyridine mixture afforded a triacetate derivative 284. This confirmed the presence of three hydroxyl groups. The IR spectrum of the acetate showed a strong band at Vmax 1747 cm^{-1} for C=O stretch of ester and a band at Vmax 1696 cm^{-1} for C=O stretch of the carboxyl group (Fig 75). The three acetate groups appeared in the ^1H nmr spectrum at δ1.94 (3H,S), δ 1.99 (3H,S) and δ2.01 (3H,S). The signals for the carbinolic protons were moved downfield (Fig 76). The ^{13}C nmr spectrum showed the three acetate carbonyl carbons and the methyl groups (Fig 77). The CIMS of the triacetate gave the

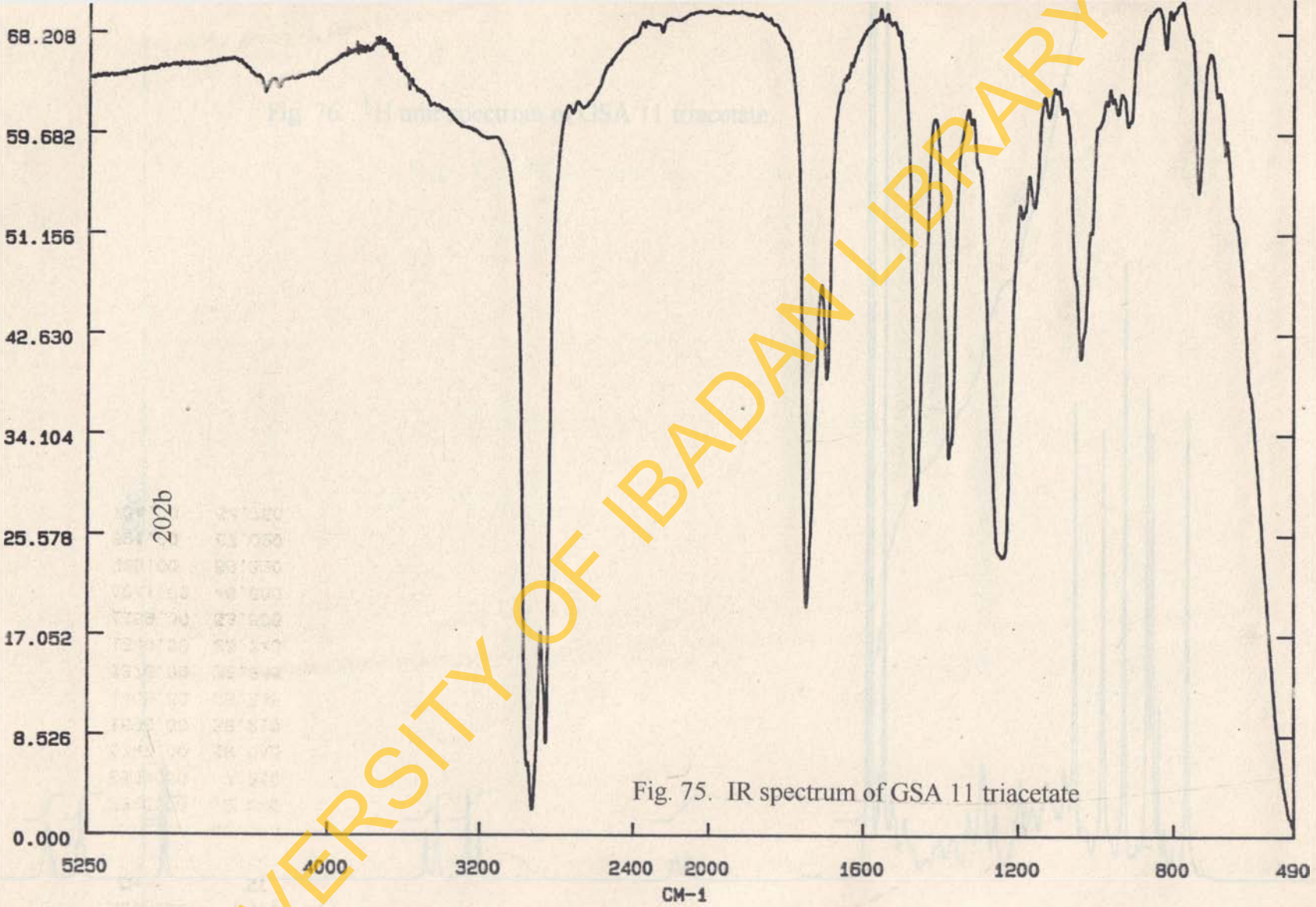
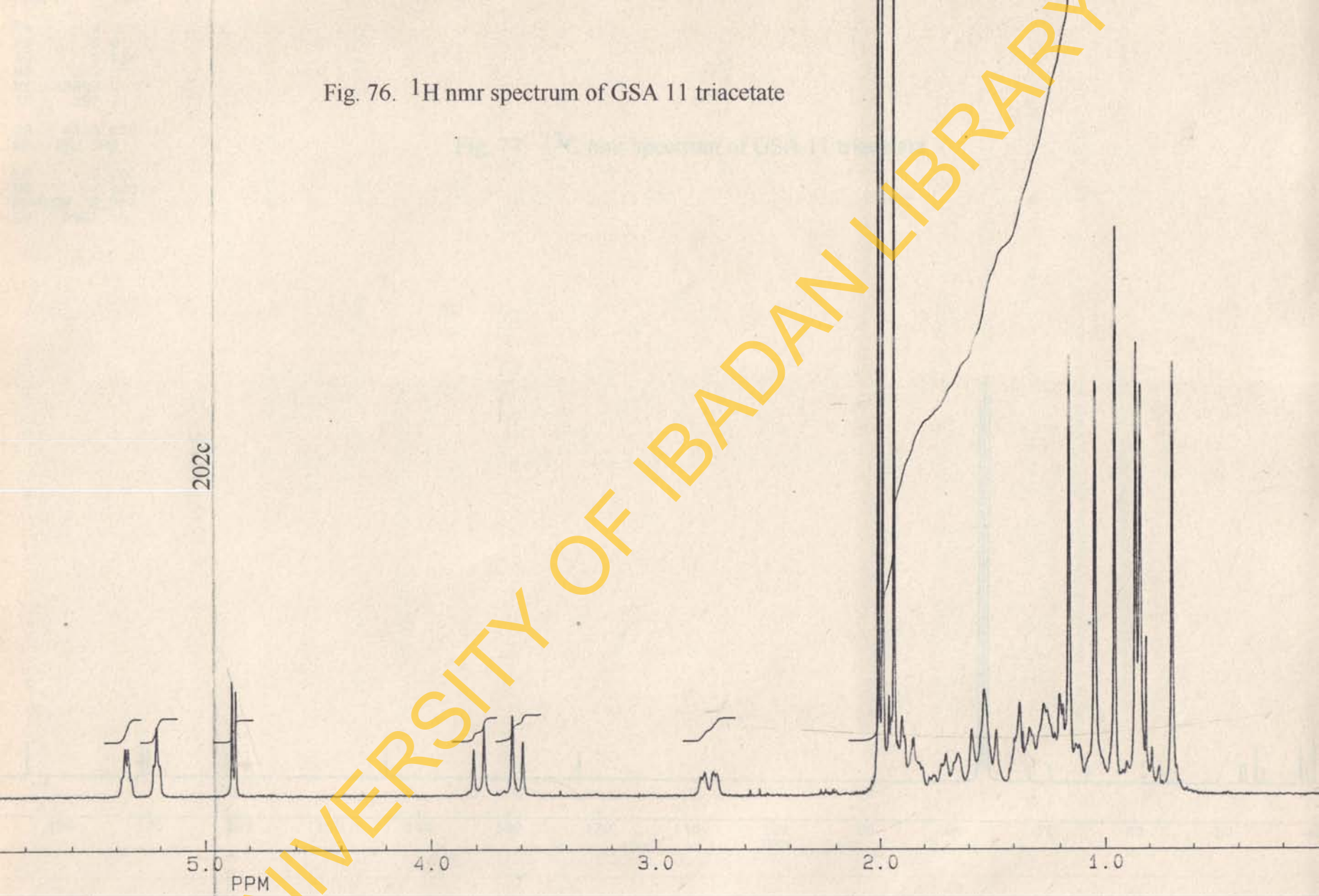


Fig. 75. IR spectrum of GSA 11 triacetate

Fig. 76. ^{1}H nmr spectrum of GSA 11 triacetate



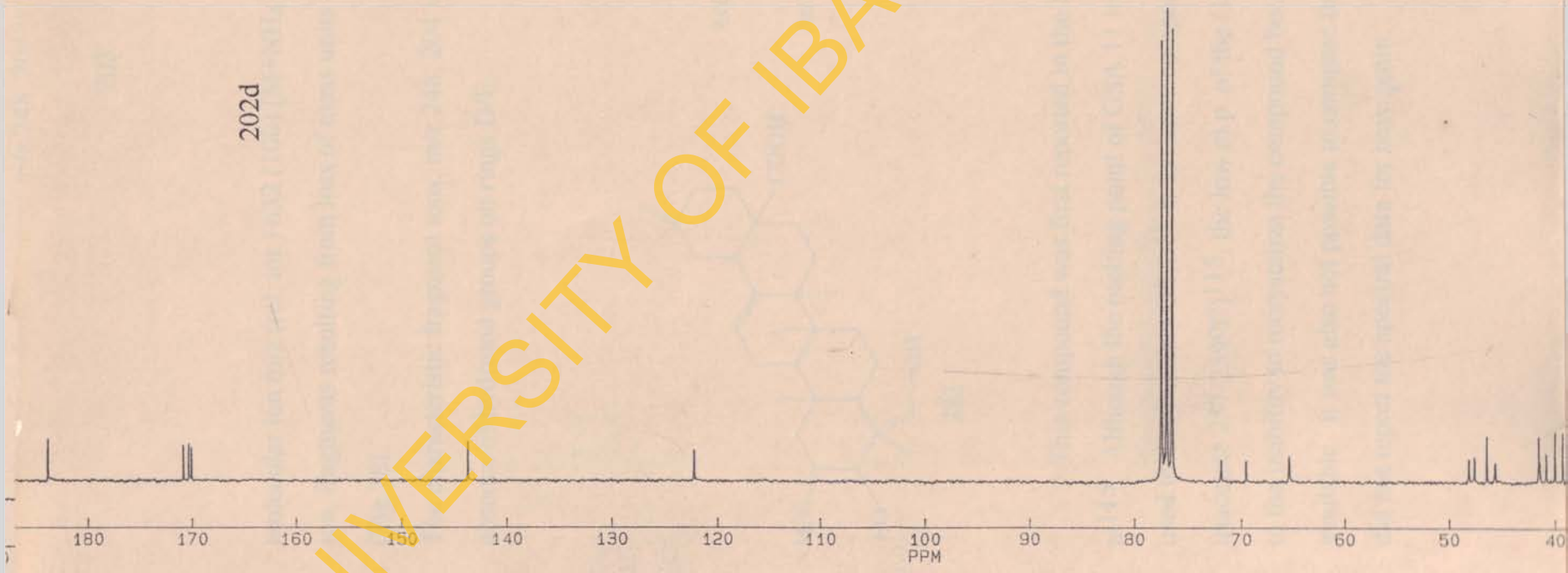
SW 16129.032
HZ/PT .984

PW 4.0
RD 0.0
AQ 1.016
RG 200
NS 22969
TE 297

O2 4200.000
DP 15H CPD

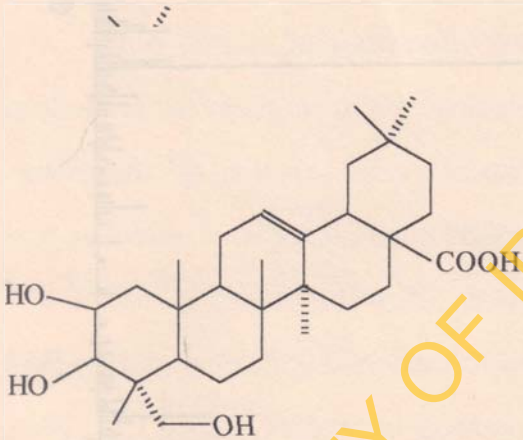
LB 2.000
GB .100
PPM/CM 4.500
SR 5962.19

Fig. 77. ^{13}C nmr spectrum of GSA 11 triacetate



molecular ion m/z (rel. int.) 632 (100) $[M+NH_4]^+$. In addition to the molecular ion, fragments resulting from loss of mass units 46, 73, 78 were prominent (Fig 78).

The characteristic fragment ions, m/z 248, 204 were present and they confirmed the absence of hydroxyl groups on rings D/E.



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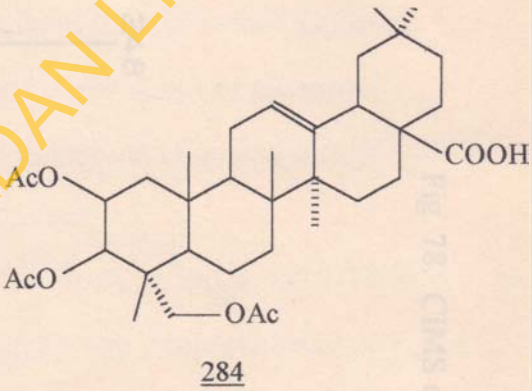


284

This compound was first reported in the literature as bayogenin by Eade et al[115]. Although the melting point of GSA 11 triacetate, 248-251°C could not be used to confirm that GSA 11 *is the same compound as bayogenin (Lit m.p. of* triacetate 257-259°C)[115], the low m.p. of the GSA 11 triacetate was probably due to the inability to recrystalize the compound because of the very small quantity available. It was also not possible to compare their spectral data because Eade et al did not report the spectral data for bayogenin.

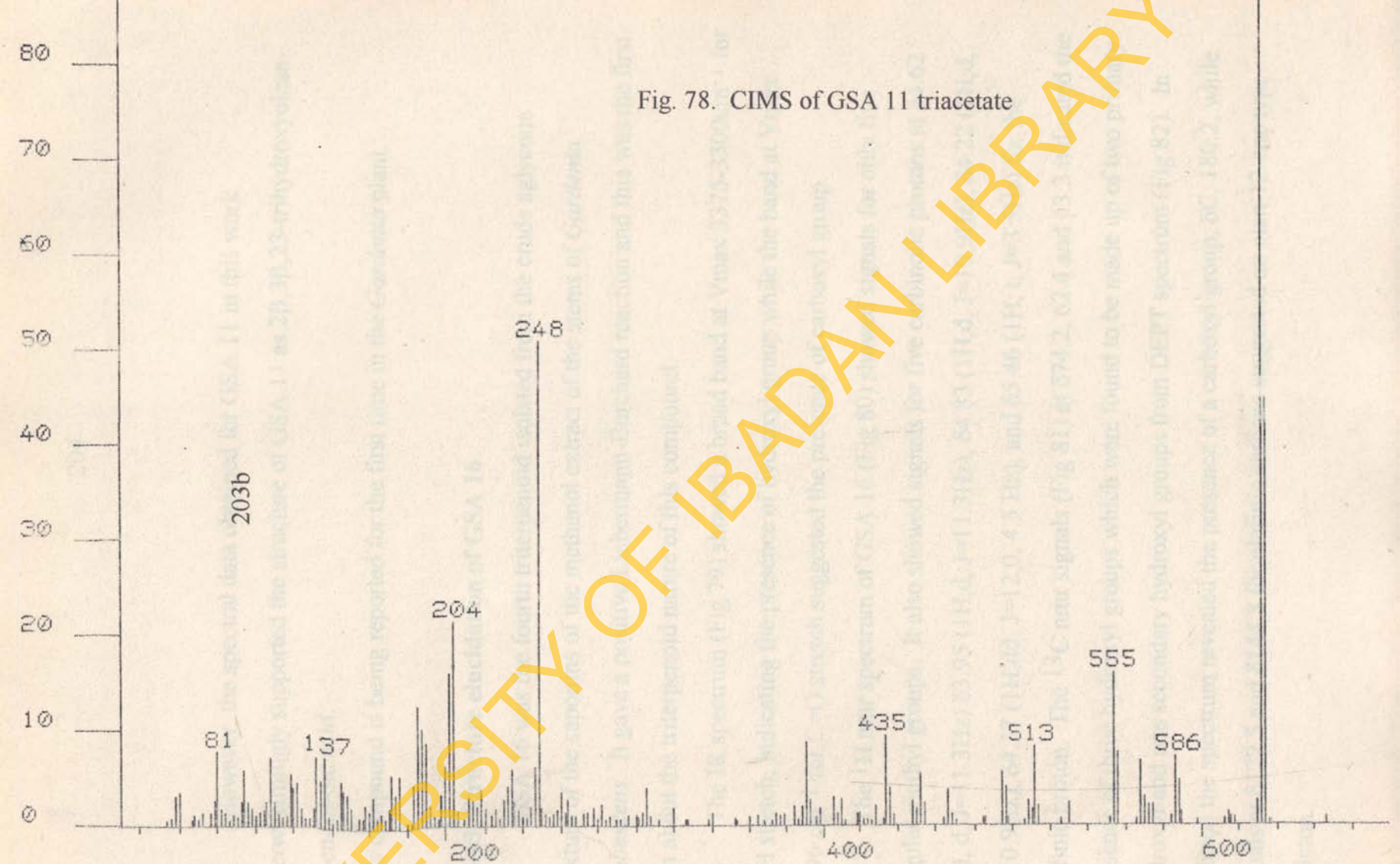


Fig. 78. CIMS of GSA 11 triacetate

However, the spectral data obtained for GSA 11 in this work overwhelmingly supported the structure of GSA 11 as 2β,3β,23-trihydroxyolean-12-en-28-oic acid.

This compound is being reported for the first time in the *Gardenia* plant.

2.5.13. Structure elucidation of GSA 16

GSA 16 was the fourth triterpenoid isolated from the crude aglycone mixture of the saponins of the methanol extract of the stems of *Gardenia erubescens*. It gave a positive Liebermann-Burchard reaction and this was the first hint about the triterpenoid nature of this compound.

The IR spectrum (Fig 79) showed a broad band at Vmax 3375-3300cm^{-1} for O-H stretch, indicating the presence of hydroxyl group while the band at Vmax 1696 cm^{-1} for C=O stretch suggested the presence of carboxyl group.

The ^{1}H nmr spectrum of GSA 16 (Fig 80) showed signals for only five angular methyl groups. It also showed signals for five carbinolic protons at δ4.62 (1H, d, J=11.3Hz) δ3.95 (1H,d, J=11.3Hz), δ4.83 (1H,d, J=10.9Hz), δ4.22 (1H,d, J=10.9Hz), δ4.37 (1H,dd, J=12.0, 4.5 Hz), and δ5.46 (1H, t, J=3.5Hz) for the olefinic proton. The ^{13}C nmr signals (Fig 81) at δ74.2, 62.4 and 63.3 indicated the presence of three hydroxyl groups which were found to be made up of two primary hydroxyl and one secondary hydroxyl groups from DEPT spectrum (Fig 82). In addition the spectrum revealed the presence of a carboxyl group, δC, 180.2, while signals at δ122.5 and δ144.8 for olefinic carbons suggested an olan-12-ene type skeleton.

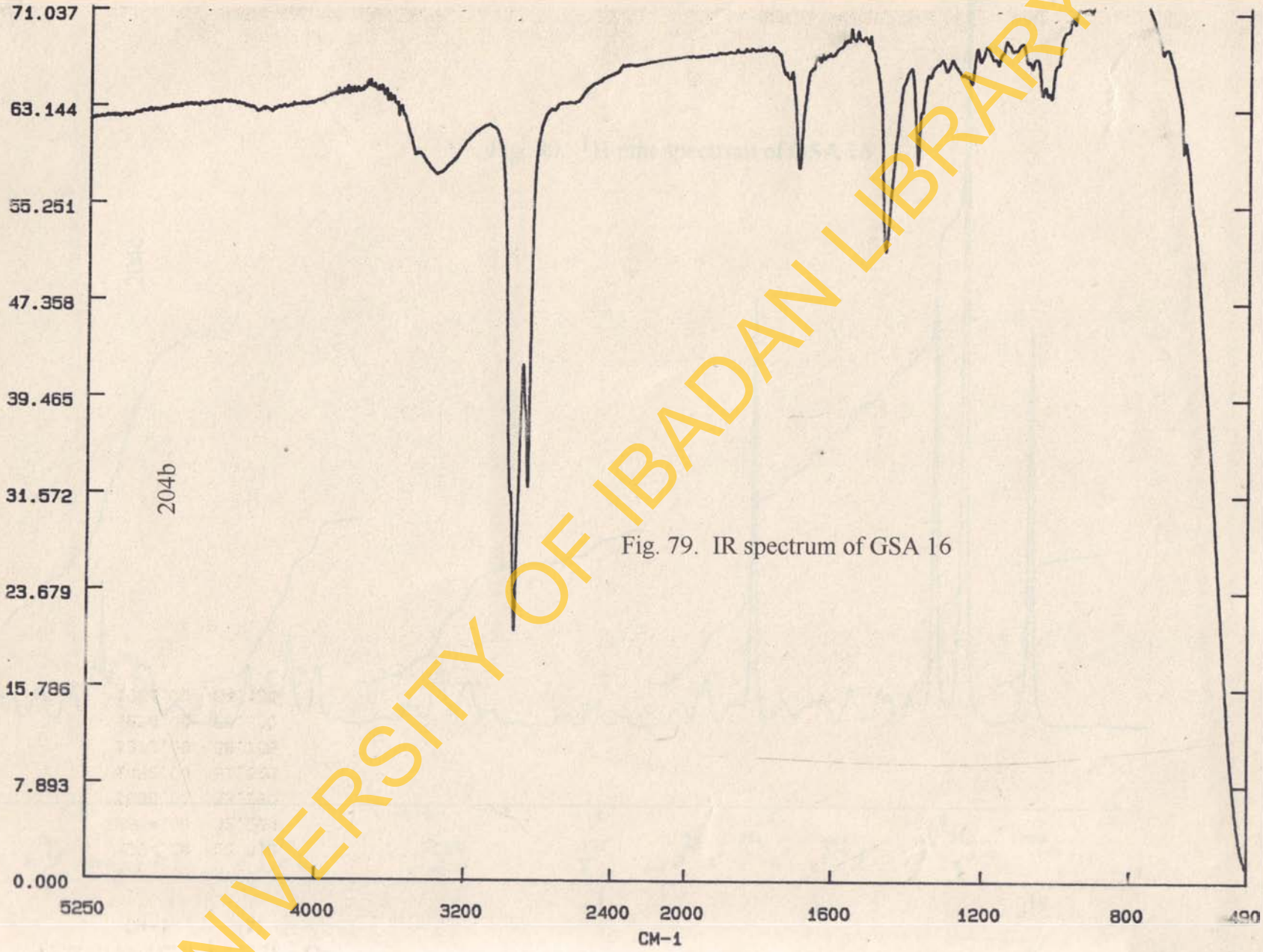


Fig. 79. IR spectrum of GSA 16

Fig. 80. ^{1}H nmr spectrum of GSA 16

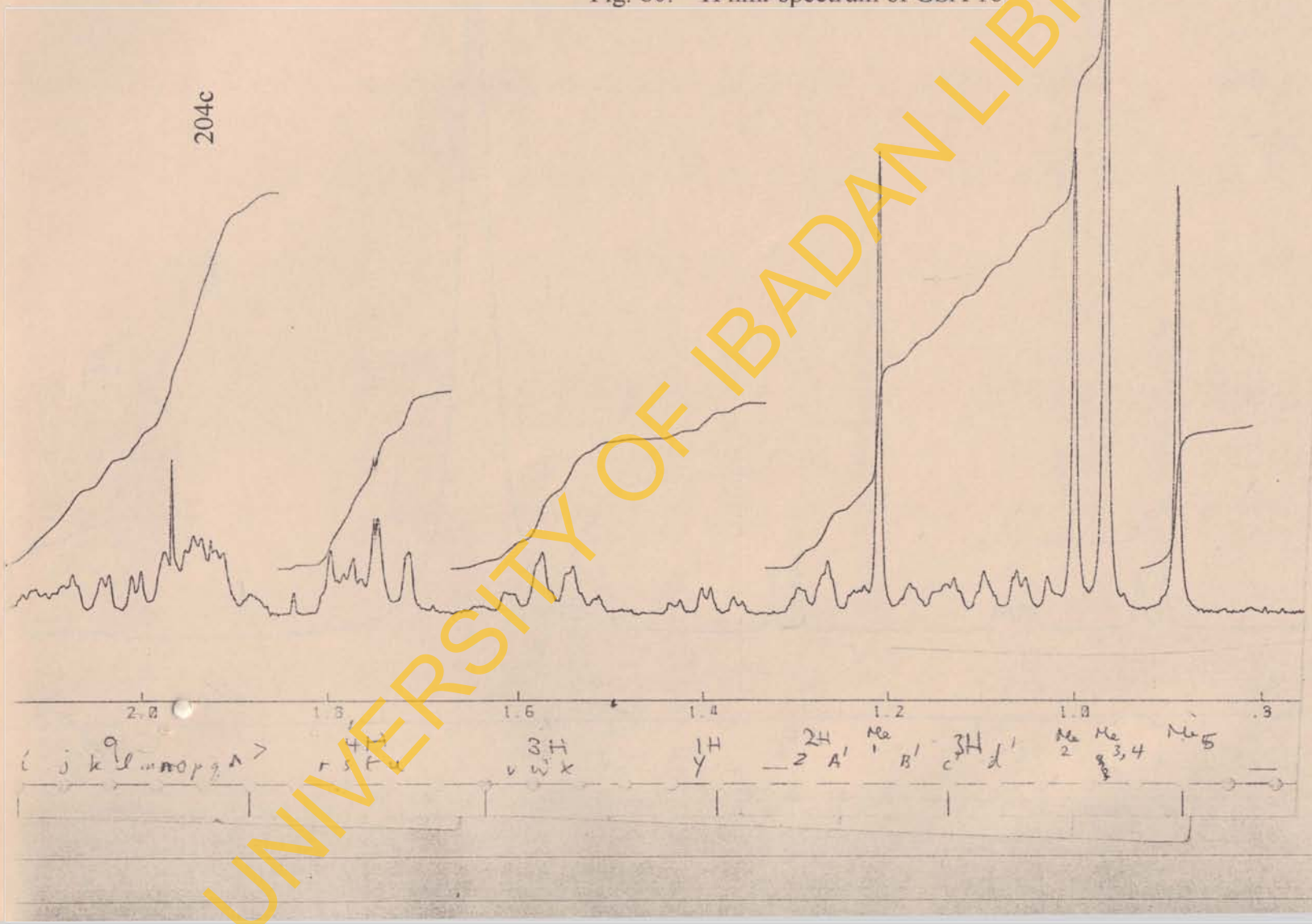
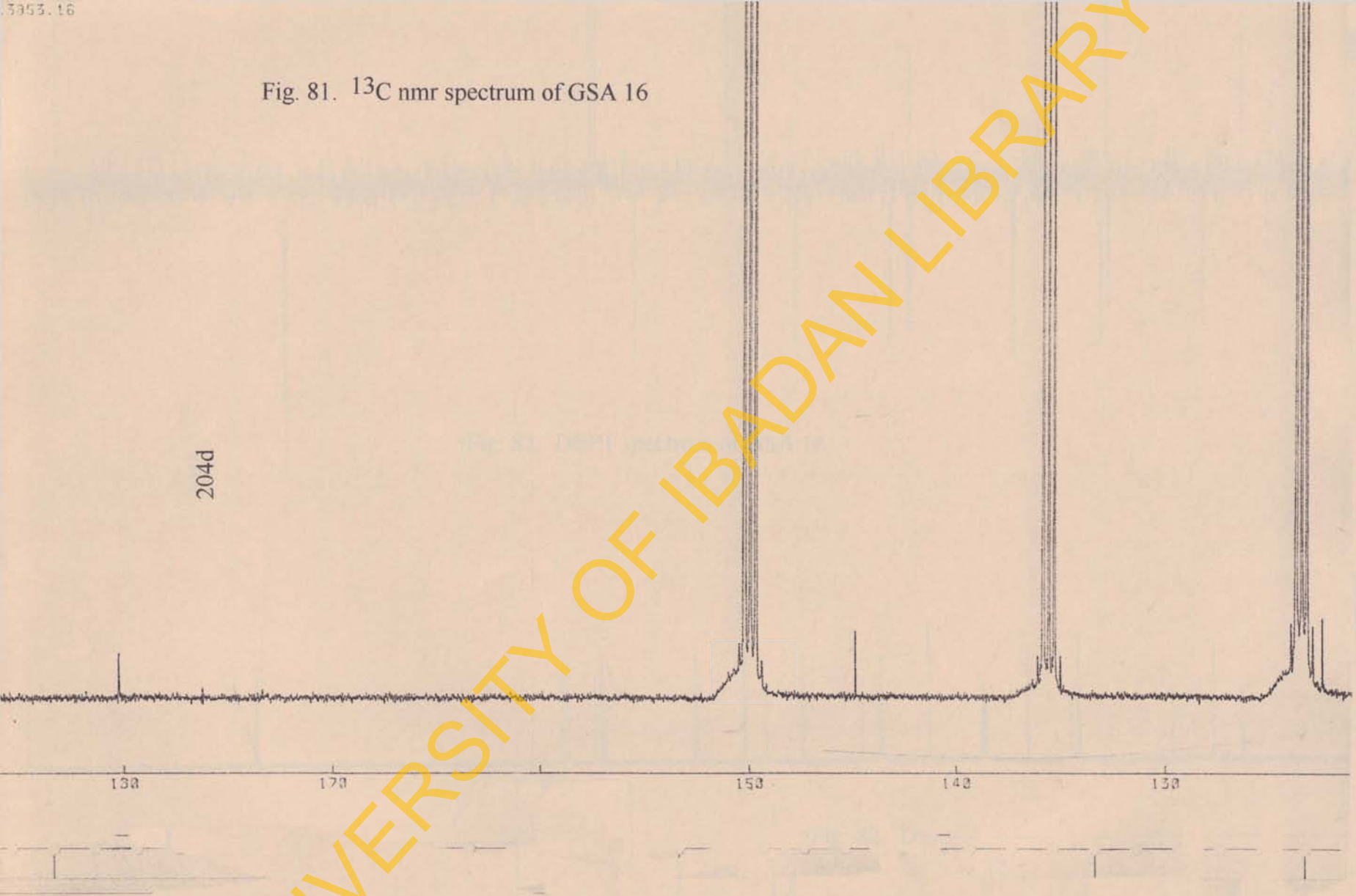


Fig. 81. ^{13}C nmr spectrum of GSA 16



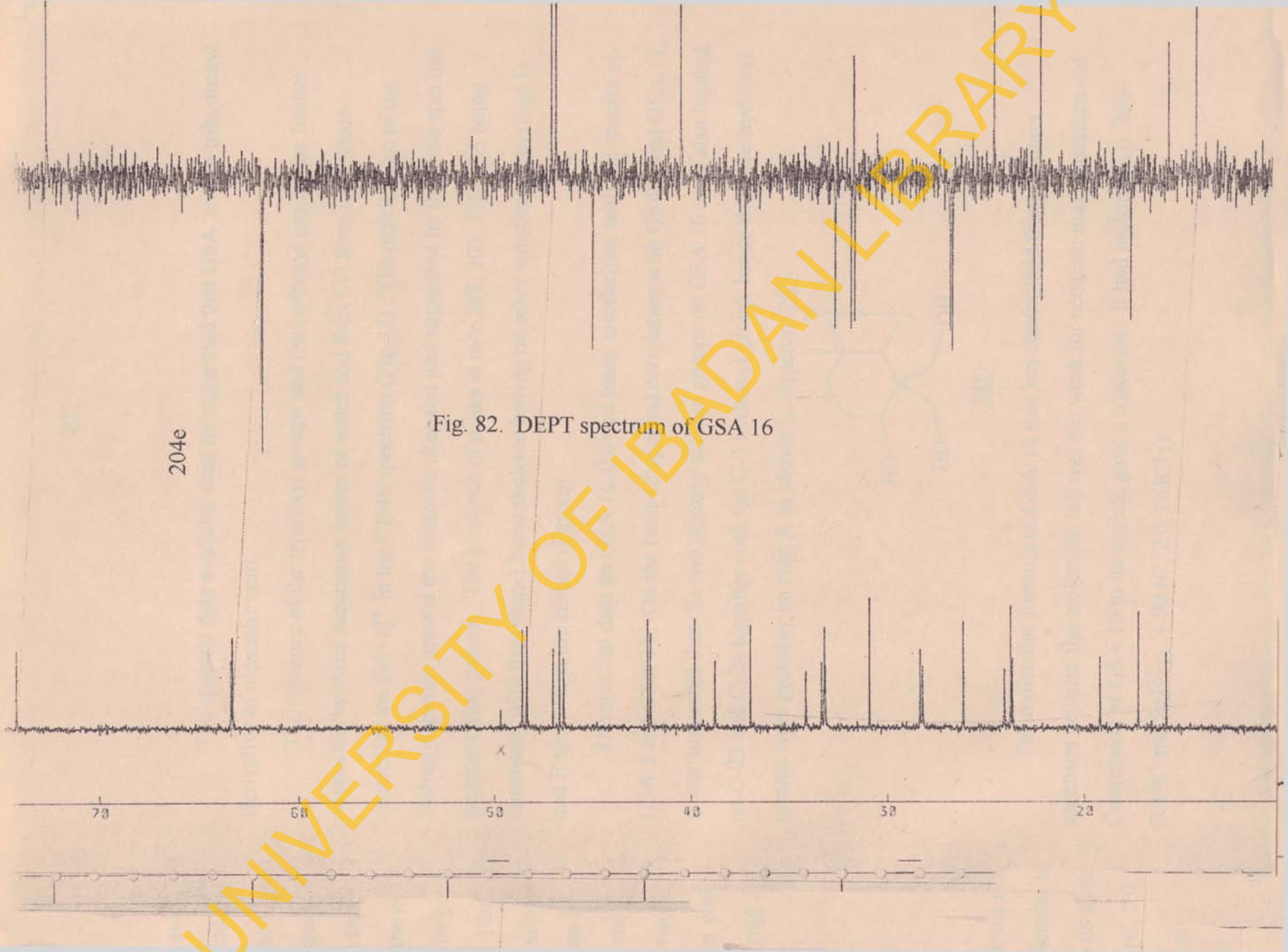
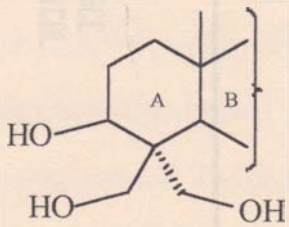


Fig. 82. DEPT spectrum of GSA 16

The spectral data available thus far suggested that GSA 16 as a trihydroxyl derivative of oleanolic acid.

The presence of the hydroxyl groups and the carboxyl group was further supported by three successive losses of water and then CO from the pseudo-molecular ion $[M+1]^+$ in the CIMS spectrum (Fig 83). The distribution of the hydroxyl groups around the oleanene skeleton was suggested by the mass spectral fragmentation pattern. The presence of peaks at m/z 248, 203, 189, 133 being consistent with the olean-12-ene skeleton having no other substituent on rings D and E apart from the carboxyl group.

The spectral data on GSA 16 showed many similarities with the spectra on GSA 5 and GSA 11. On the basis of spectral correlation with GSA 5 and GSA 11, it was suggested that the two primary alcohol groups on GSA 16 were also located on C-23 and C-24 together with the C-3 hydroxyl group, hence the three hydroxyl groups were probably on ring A as shown in structure 285.



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The molecular formula of GSA 16 was not determined from its mass spectrum because the molecular ion was too weak for accurate mass measurement. Conversion of GSA 16 to its acetate gave a triacetate. It had melting m.p. 206-208^{o}C and $[\alpha]^{22}_{D}$ + 90 (C, 2.0, $CHCl_3$).

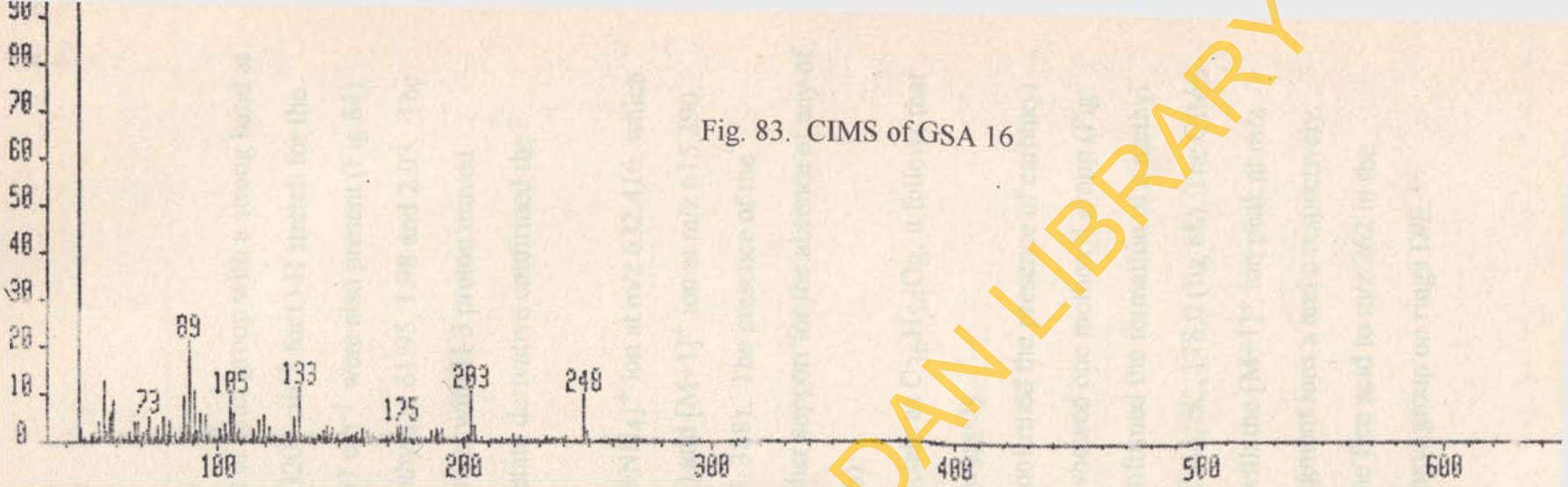
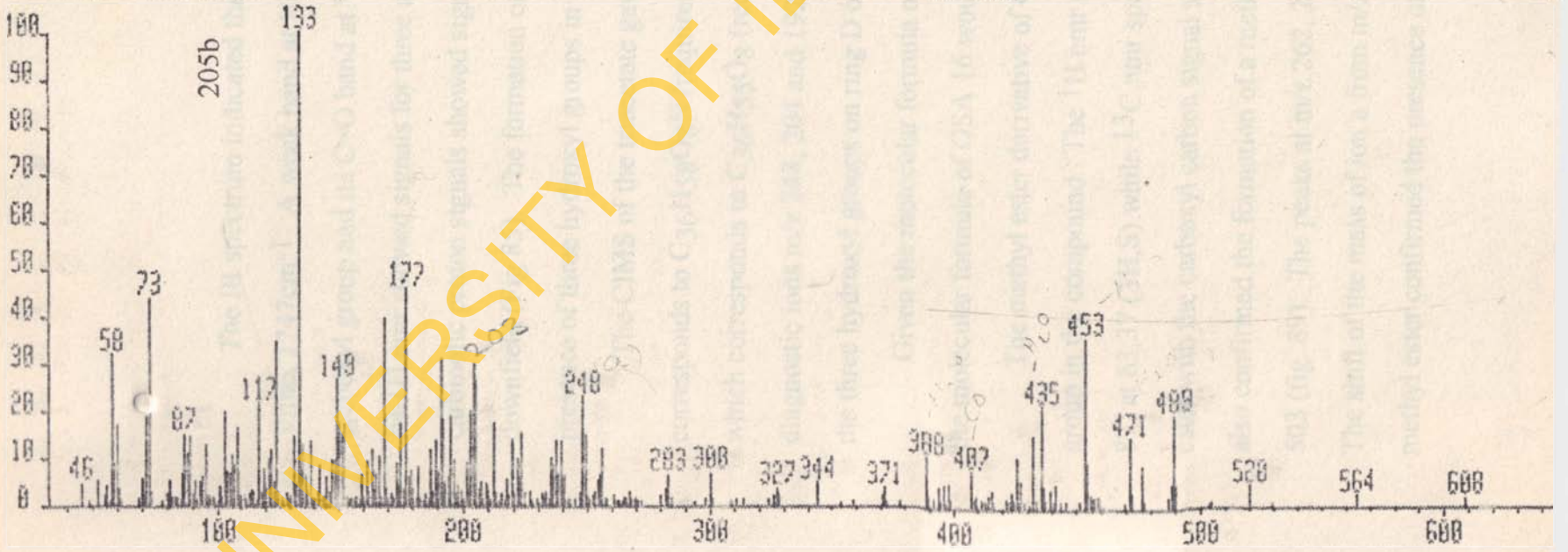


Fig. 83. CIMS of GSA 16



The IR spectrum indicated the presence of ester group with a strong band at Vmax 1747cm^{-1}. A weak band at Vmax 330-3200 cm^{-1} for O-H stretch for the carboxyl group and its C=O band at Vmax 1697 cm^{-1} were also present (Fig 84). Its ^{1}H nmr showed signals for three acetate methyls at δ1.95, 1.98 and 2.03. The carbinolic proton signals showed significant shifts, with H-3 proton moved downfield (Fig 85). The formation of the triacetate derivative confirmed the presence of three hydroxyl groups in GSA 16.

The CIMS of the triacetate gave the $[M+NH_4]^+$ ion at m/z 632.416, which corresponds to $C_{36}H_{58}O_8N$ (requires 632.415) and $[M+1]^+$ ion at m/z 615.390, which corresponds to $C_{36}H_{55}O_8$ (requires 615. 388). The presence of the diagnostic ions m/z 248, 204 and 192 gave further support for the absence of any of the three hydroxyl groups on ring D or E (fig 86).

Given the molecular formula of the triacetate as $C_{36}H_{54}O_8$, it follows that the molecular formula of GSA 16 would be $C_{30}H_{48}O_5$.

The methyl ester derivative of GSA 16 confirmed the presence of carboxyl group in the compound. The ^{1}H nmr spectrum showed one methoxyl group (Fig 87) at δ3.37 (3H,S) while ^{13}C nmr spectrum confirmed the formation of a methyl ester with the carboxyl carbon signal shifted upfield, δC, 178.0 (fig 88). The CIMS also confirmed the formation of a methyl ester with the $[M+1]^+$ ion peak at m/z 503 (fig. 89). The peaks at m/z 262, 203 are fragment ions a and c respectively. The shift of the mass of ion a from m/z 248 in the free acid to m/z 262 in the methyl ester confirmed the presence of the carboxyl group on rings D/E.

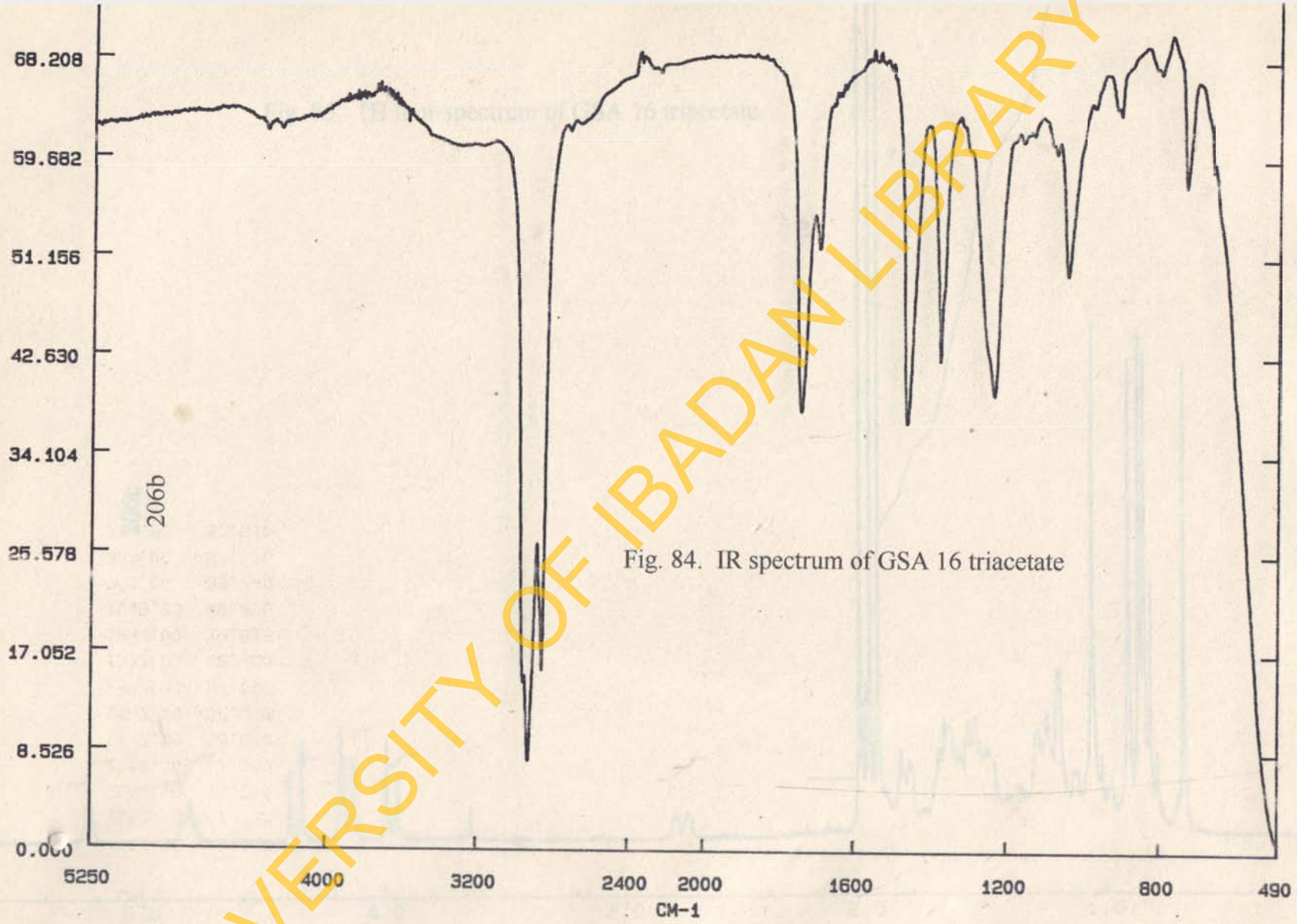


Fig. 84. IR spectrum of GSA 16 triacetate

Fig. 85. ^{1}H nmr spectrum of GSA 16 triacetate

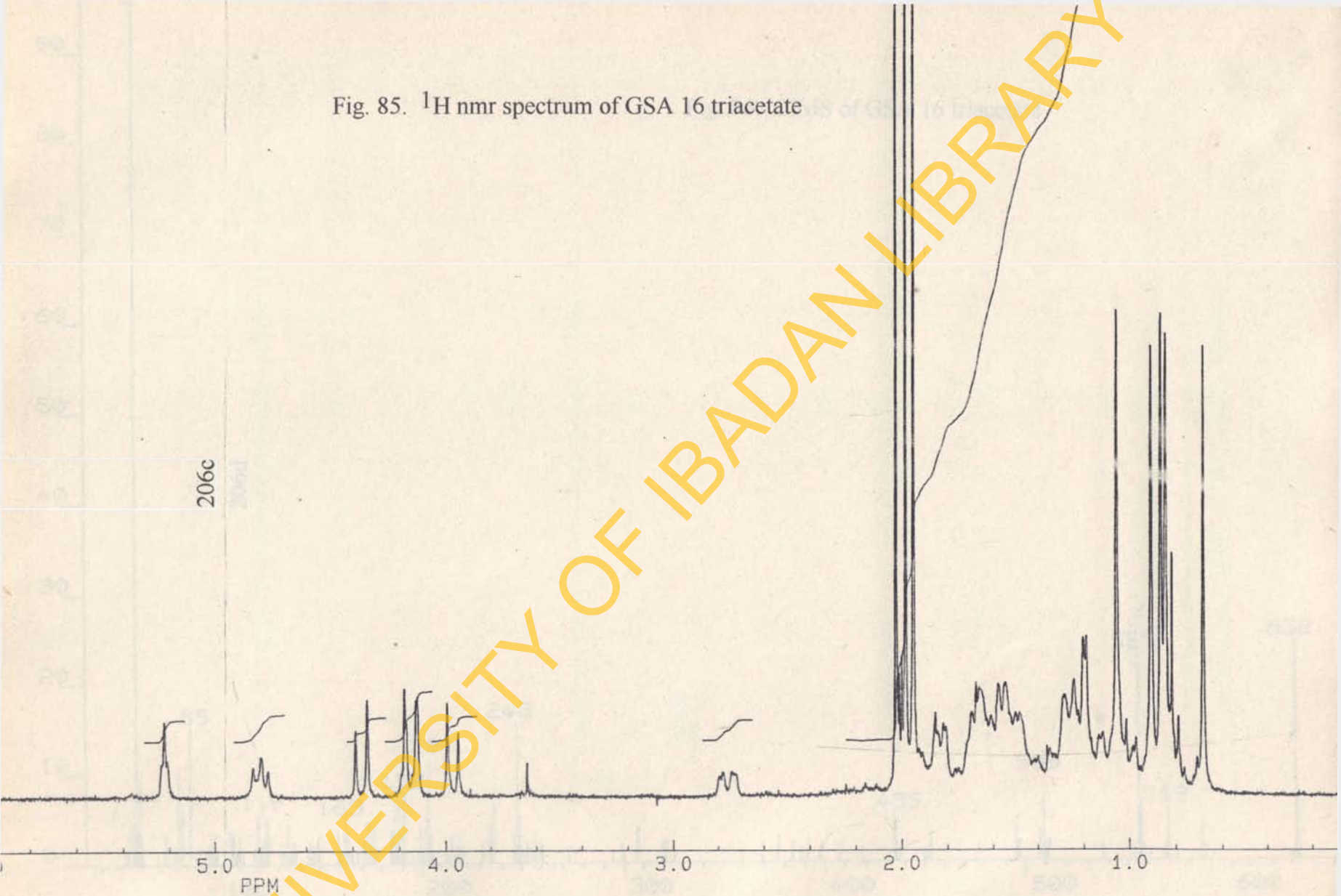


Fig. 86. CIMS of GSA 16 triacetate

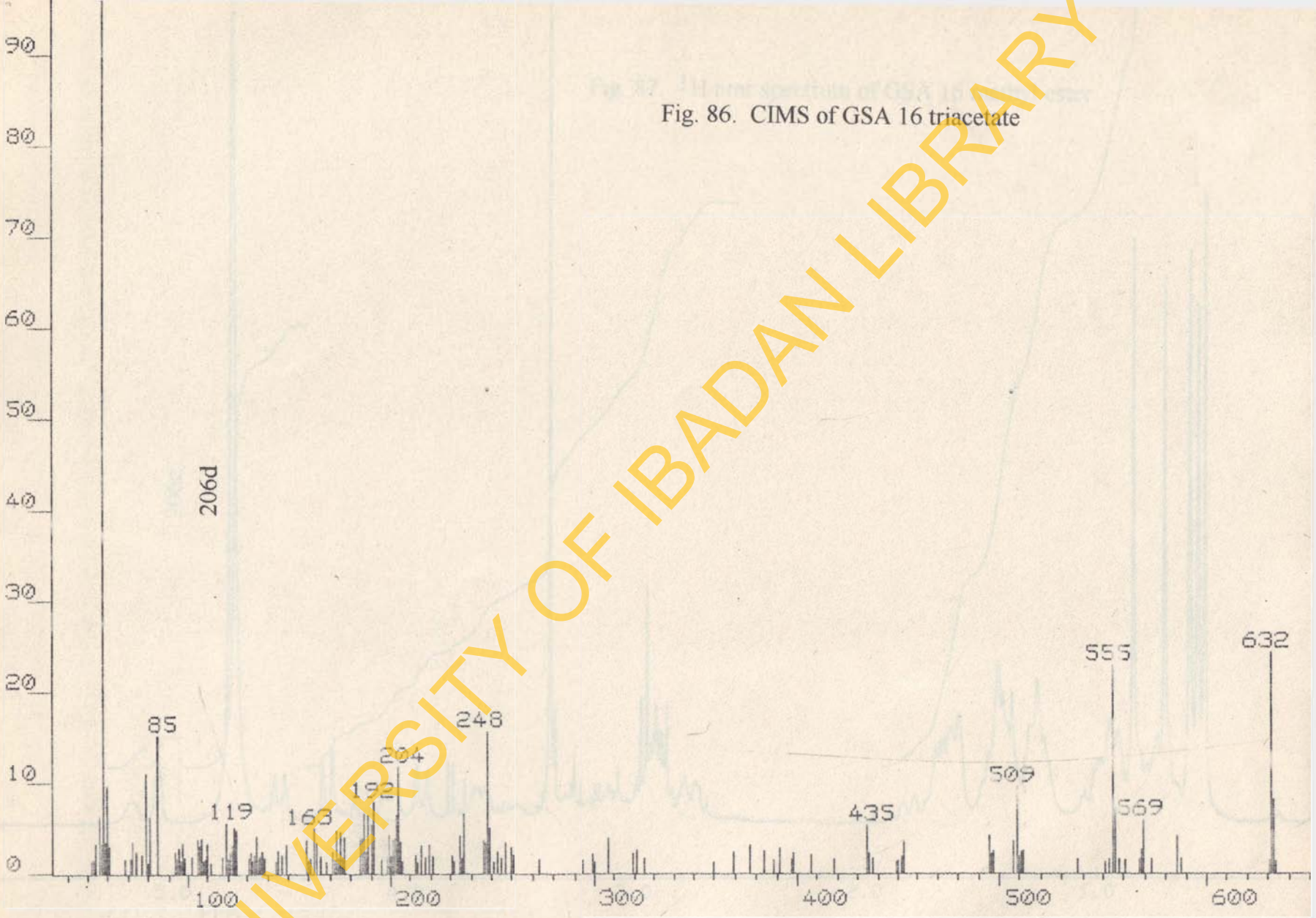


Fig. 87. ^{1}H nmr spectrum of GSA 16 methyl ester

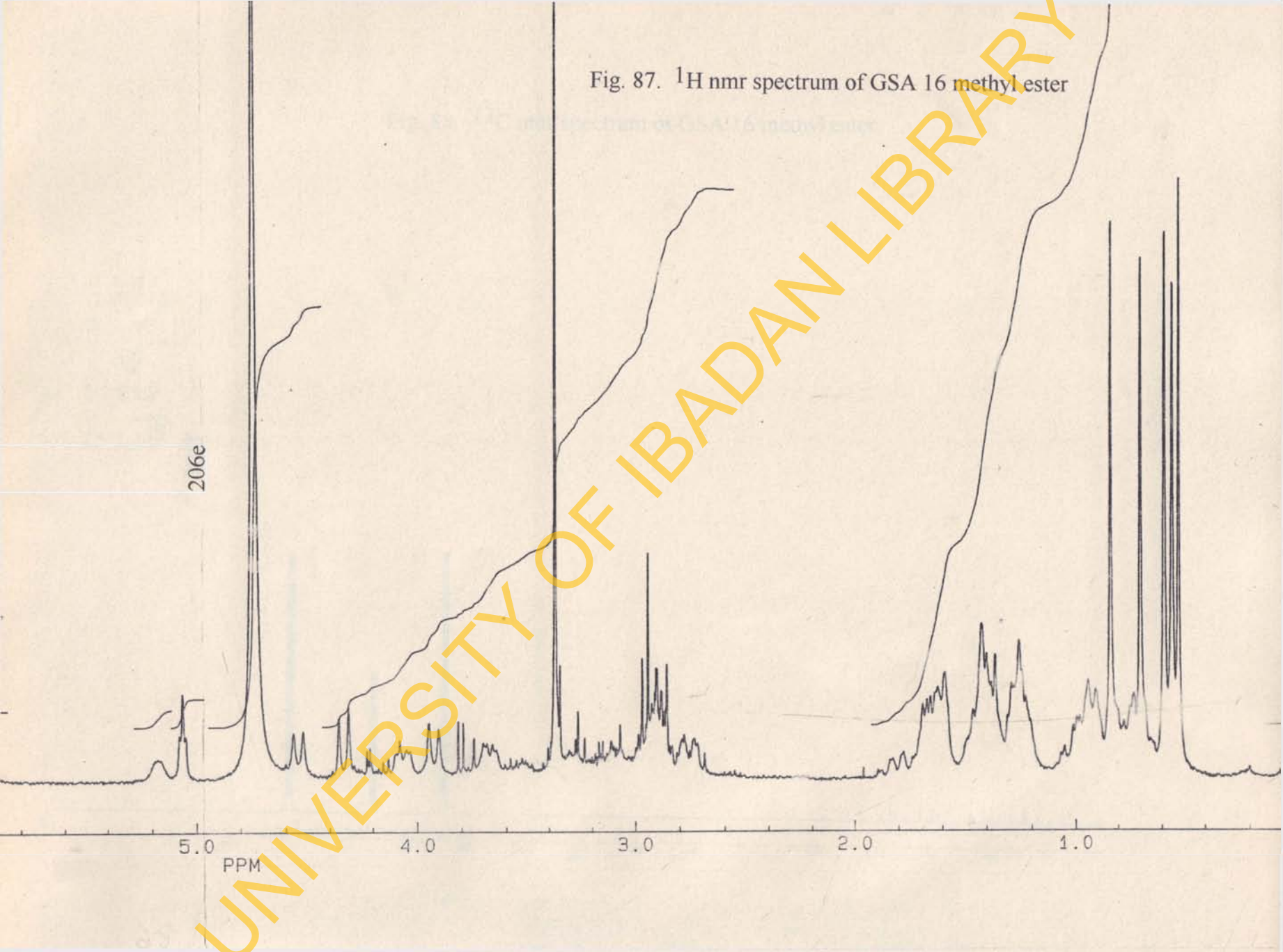
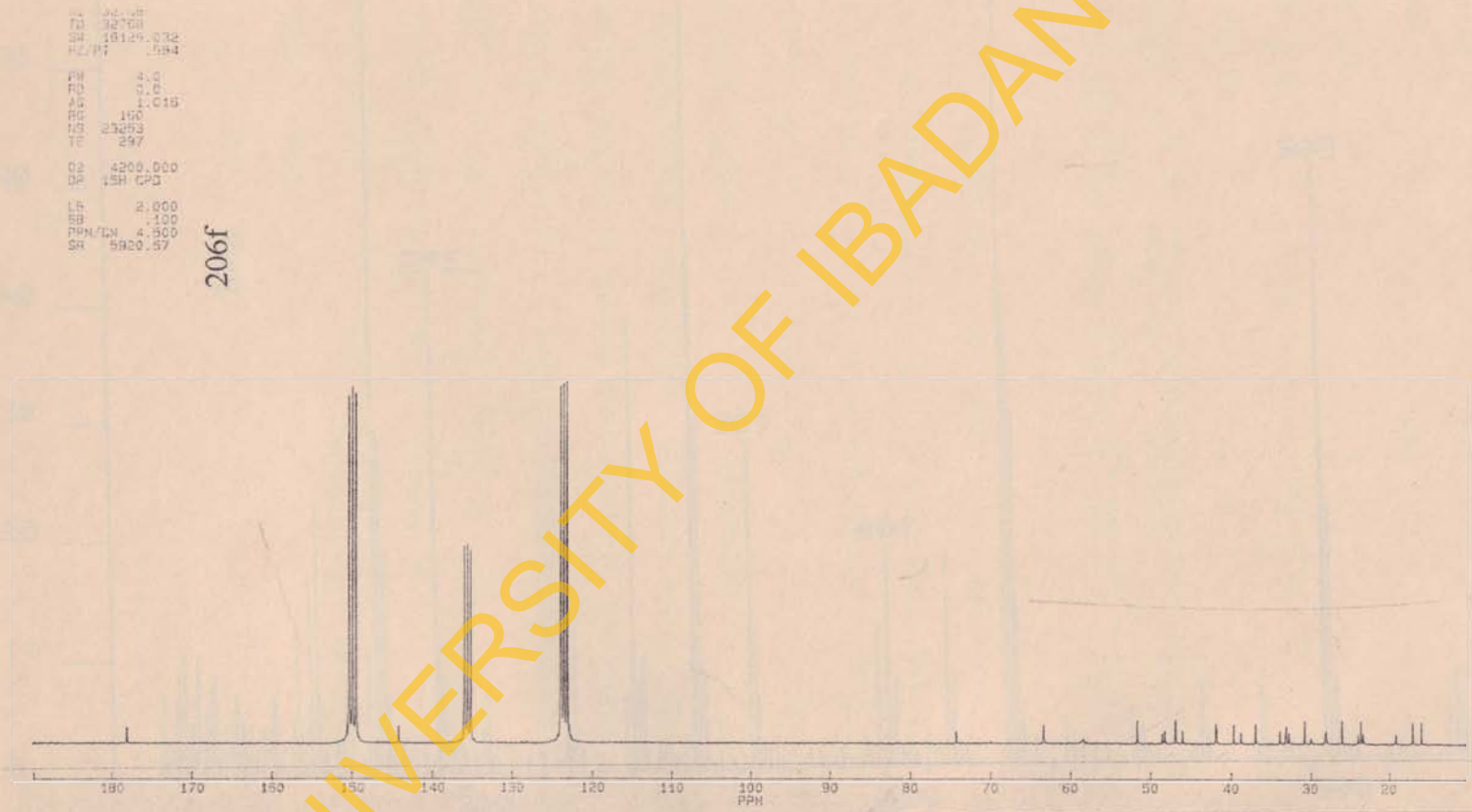


Fig. 88. ^{13}C nmr spectrum of GSA 16 methyl ester



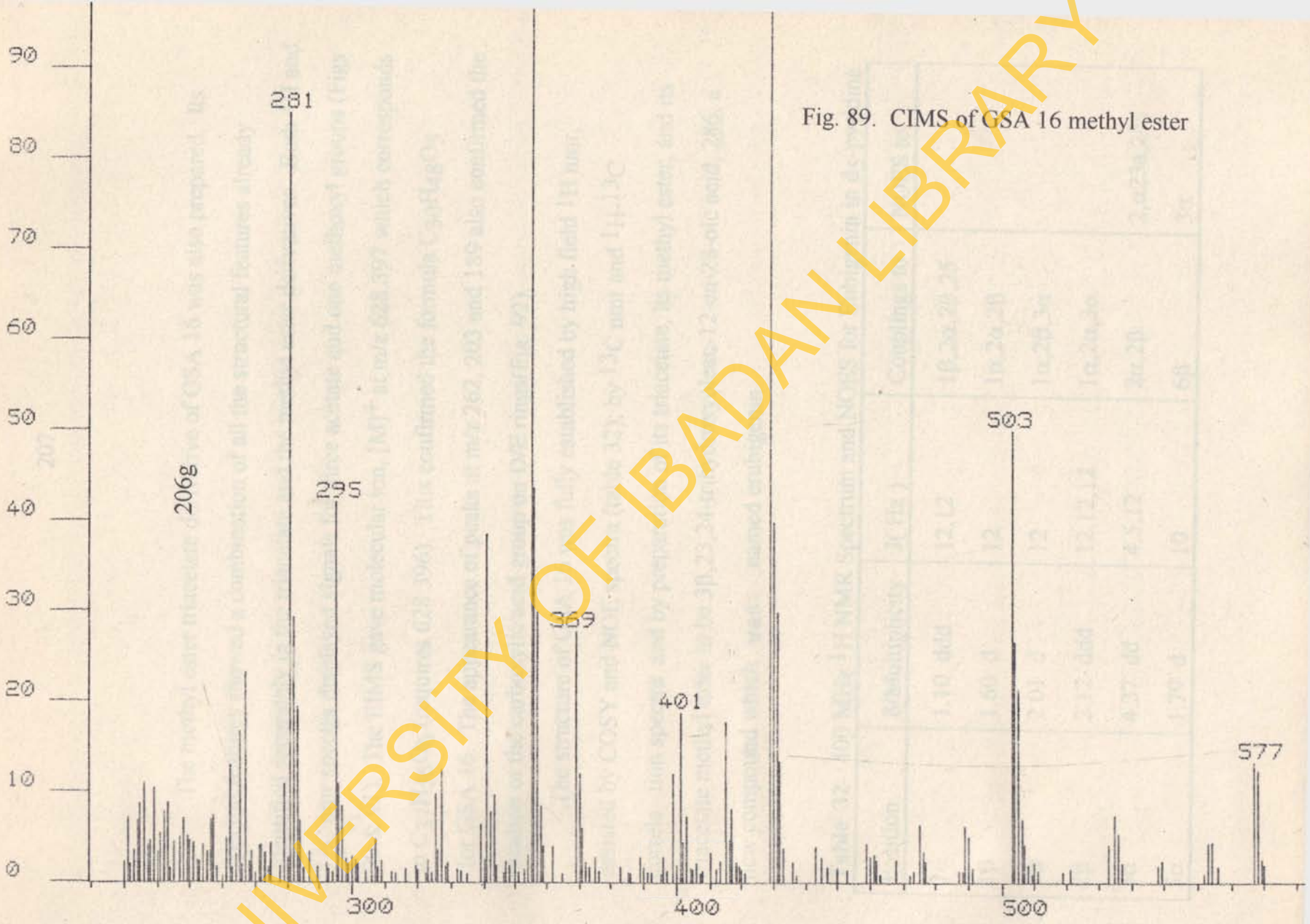


Fig. 89. CIMS of GSA 16 methyl ester

The methyl ester triacetate derivative of GSA 16 was also prepared. Its spectral features showed a combination of all the structural features already identified separately in the triacetate and the methyl ester derivatives. Both ^{1}H and ^{13}C nmr spectra displayed signals for three acetate and one methoxyl groups (Figs 90 & 91). The EIMS gave molecular ion, $[M]^{+}$ at m/z 628.397 which corresponds to $C_{37}H_{56}O_6$ (requires 628.396). This confirmed the formula $C_{30}H_{48}O_5$ for GSA 16. The appearance of peaks at m/z 262, 203 and 189 also confirmed the location of the carboxylic acid group on D/E rings(Fig. 92).

The structure of GSA 16 was fully established by high field ^{1}H nmr, assisted by COSY and NOE spectra (table 32); by ^{13}C nmr and ^{1}H-^{13}C correla tion spectra and by preparation of its triacetate, its methyl ester, and its triacetate methyl ester to be 3β,23,24-trihydroxyolean-12-en-28-oic acid, 286, a new compound which was named erubigenin.

Table 32- 400 MHz ^{1}H NMR Spectrum and NOES for Eubigenin in d_5-pyridine.

Position	δ/Multiplicity	J(Hz)	Couplings to	NOES to
1α	1.10 ddd	12,12	1β,2α,2β,25	
1β	1.60 d	12	1α,2α,2β	
2α	2.01 d	12	1α,2β,3α	
2β	2.12 ddd	12,12,12	1α,2α,3α	
3α	4.37 dd	4.5,12	2α,2β	2,α23a,24a
5α	1.79 d	10	6β	3α

Fig. 90. ^{1}H nmr spectrum of GSA 16 triacetate methyl ester

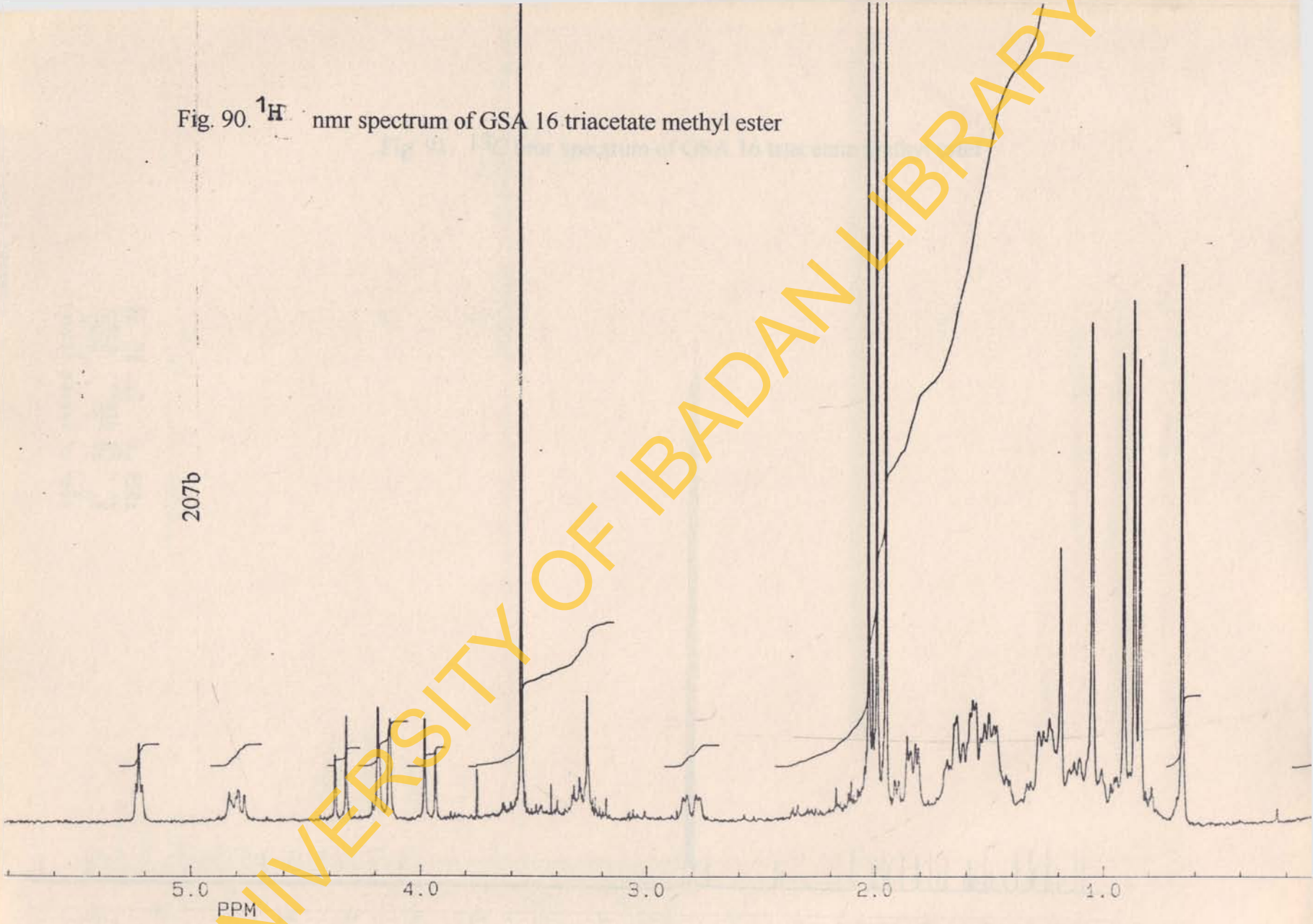


Fig. 91. ^{13}C nmr spectrum of GSA 16 triacetate methyl ester

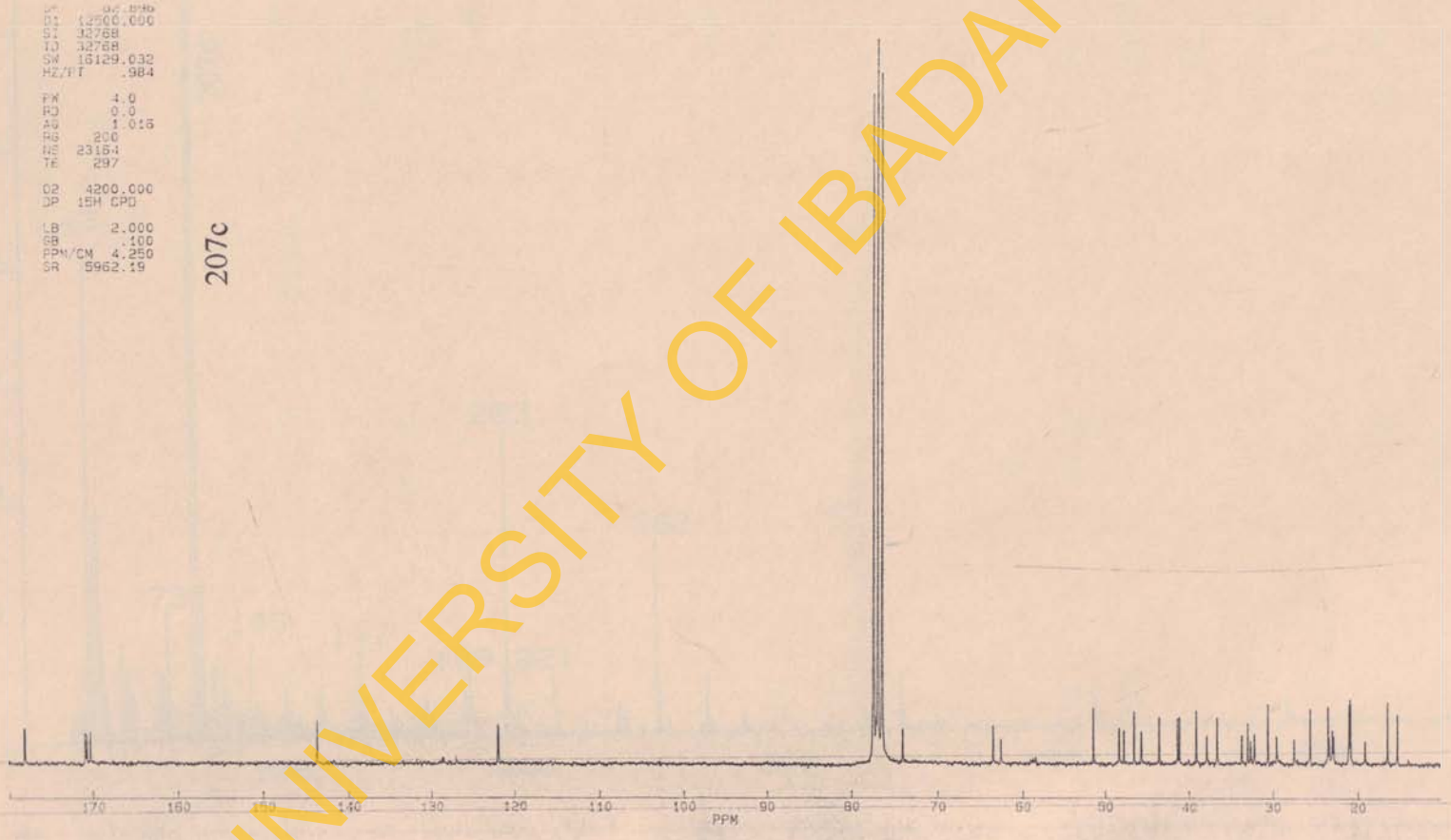


Fig. 92. EIMS of GSA 16 triacetate methyl ester

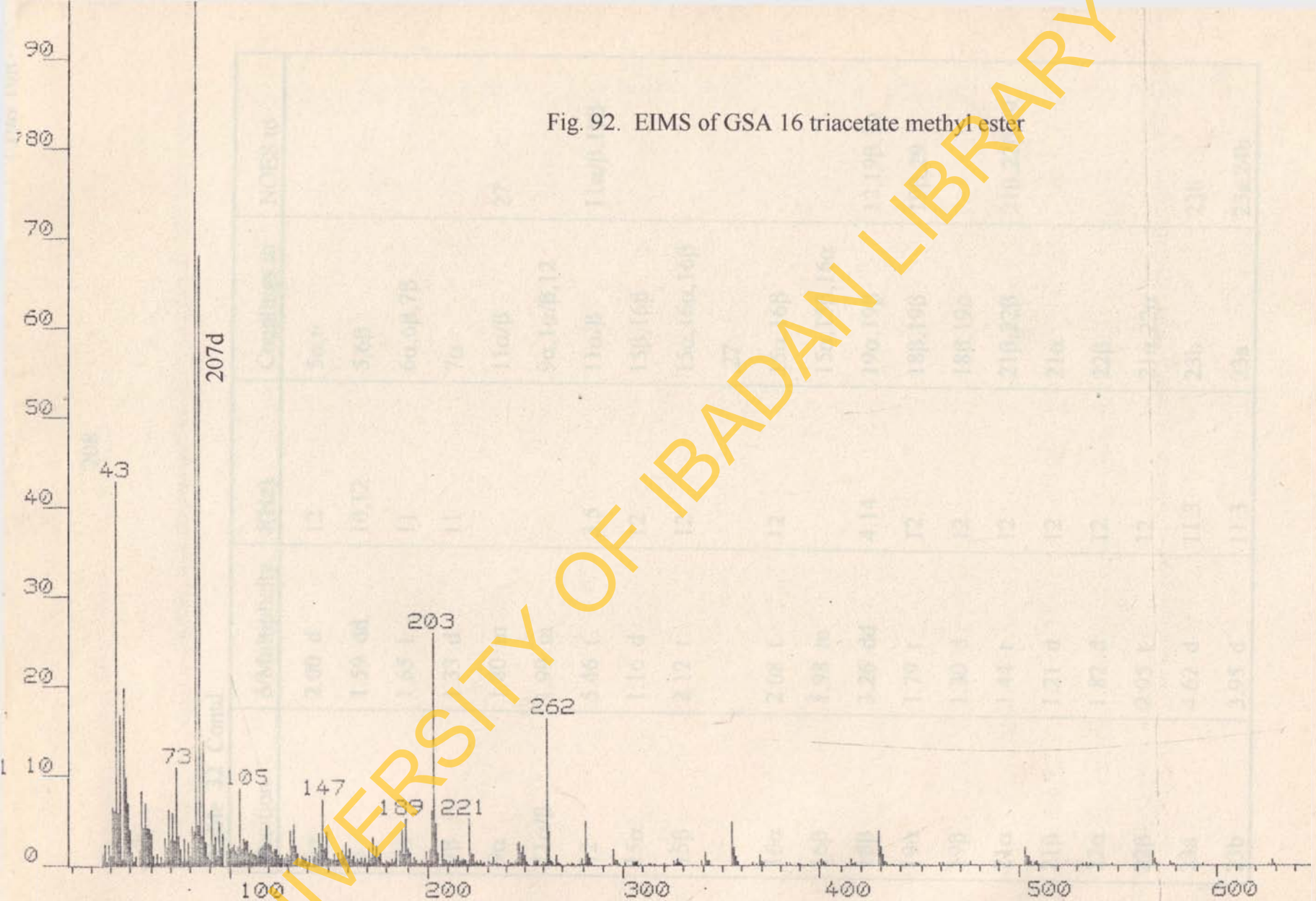


Table 32 Contd.

Position.	δ/Multiplicity.	J(Hz).	Couplings to	NOES to
6α	2.00 d	12	5α,6	
6	1.59 dd	10,12	5,6β	
7α	1.65 t	11	6α,6β,7β	
7β	1.33 d	11	7α	
9α	1.80 m		11α/β	27
11α/β	1.99 m		9α,1α/β,12	
12	5.46 t	3.5	11α/β	11α/β,18β
15α	1.16 d	12	15β,16β	
15β	2.12 t	12	15α,16α,16β	
			,27	
16α	2.08 t	12	15α,16β	
16β	1.98 m		15α,15β,16α	
18β	3.26 dd	4.14	19α,19β	12,19β,30
19α	1.79 t	12	18β,19β	19β,29
19β	1.30 d	12	18β,19α	
21α	1.44 t	12	21β,22β	21β,22β,29
21β	1.21 d	12	21α	
22α	1.82 d	12	22β	
22β	2.05 t	12	21α,22α	
23a	4.62 d	11.3	23b	23b
23b	3.95 d	11.3	23a	23a,24b

Table 32 Contd.

Position.	δ/Multiplicity	J(Hz).	Couplings to	NOES to
24a	4.83 d	10.9	24b	3α,23a,24b
24b	4.22 d	10.9	24a	23a,23b,24a
25	0.97 s			1β,2β,11α/β 6β,7β, 23a,23b
26	1.00 s		7α,9α,27	7α,9α,15α
27	1.21 s		15β,26	6α,6β,7α,9α, 26
29	0.89 s		30	
30	0.97 s		19α,21α,29	22β
3(OH)	5.39 bs			
	6.19 bs			
	6.62 bs			

The ^{13}C nmr data for compounds 286-289 is shown in table 33 and Scheme XXXI illustrates the fragment ions identified in the mass spectra of these compounds.

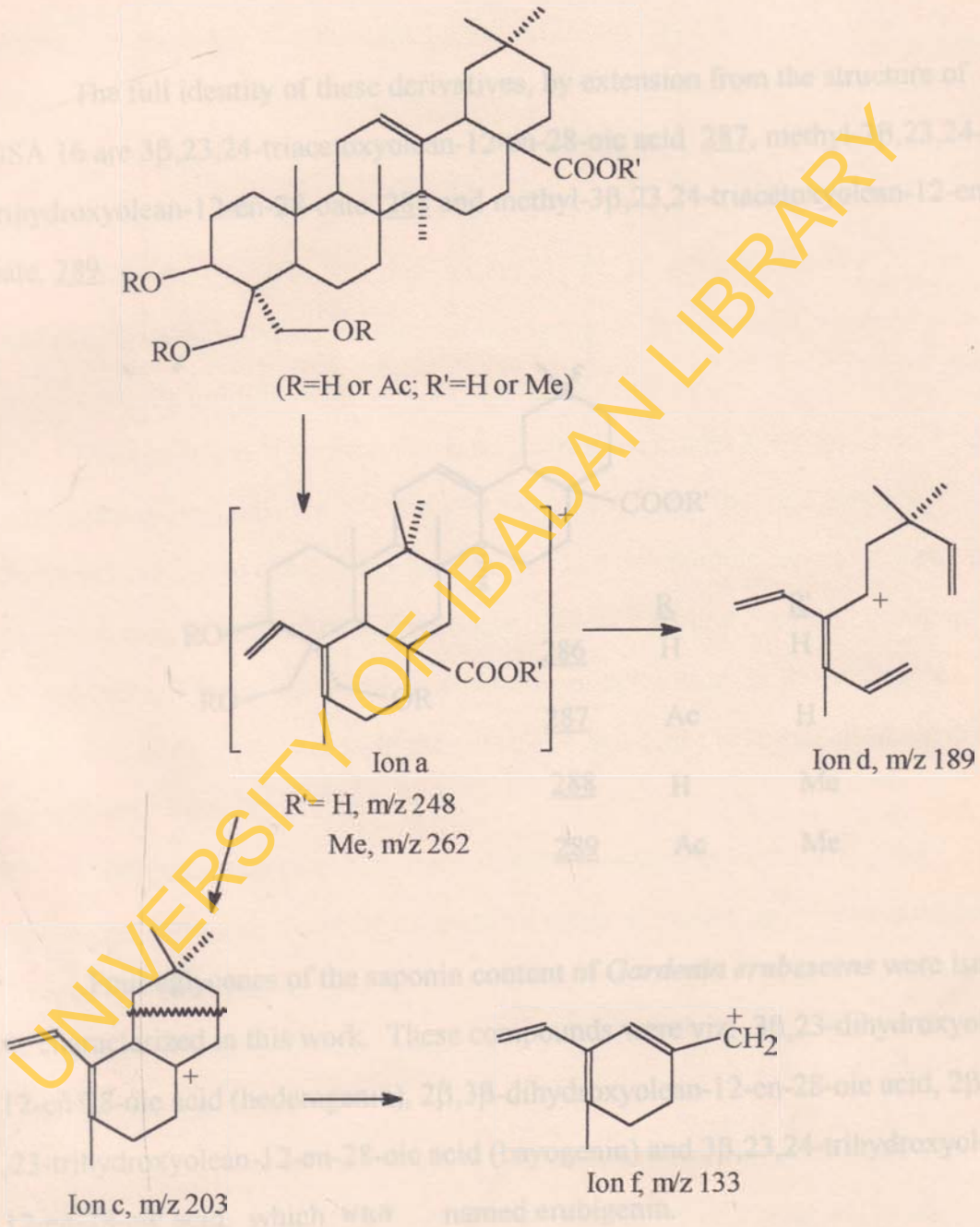
The derivatives of GSA 16 are also new compounds and they are being reported for the first time in this work.

Table 33- 100 MHz ^{13}C NMR Spectra of Erubigenin and its derivatives.

Carbon	Erubigenin (d_5-pyridine)	Erubigenin triacetate ($CDCl_3$)	Erubigenin methyl ester (d_5-pyridine)	Erubigenin triacetate methyl ester ($CDCl_3$)
1	38.7	37.8	38.7	37.8
2	26.1	27.4	26,1	27.2
3	74.2	74.0	74.2	74.0
4	38.5	48.4	48.5	48.4
5	48.3	47.8	48.2	47.8
6	19.2	19.1	19.1	19.2
7	33.2	32.2	32.7	32.7
8	39.7	39.1	39.7	39.1
9	46.9	46.4	46.9	46.6
10	37.0	36.6	36.9	36.6
11	23.7	23.4	23.9	23.4
12	122.2	122.2	122.7	121.9
13	144.8	143.4	144.1	143.6
14	41.9	40.9	41.8	41.1
15	28.2	27.4	28.1	27.4
16	23.7	23.4	23.6	23.4
17	46.6	45.7	46.9	45.7

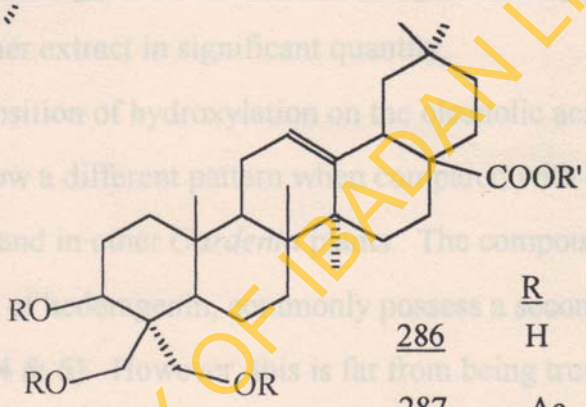
Table 33 Contd.

Carbon.	Erubigenin (d_5 pyridine)	Erubigenin triacetate ($CDCl_3$)	Erubigenin methyl ester (d_5-pyridine)	Erubigenin triacetate methyl ester ($CDCl_3$).
18	42.1	41.4	41.9	41.4
19	46.4	43.6	46.0	43.6
20	30.9	30.5	30.7	30.6
21	34.1	33.7	33.9	33.7
22	33.3	32.9	33.1	33.0
23	63.3	63.4	63.3	63.5
24	63.3	62.9	63.3	62.6
25	15.9	15.4	15.9	15.3
26	17.3	16.5	17.0	16.5
27	26.1	27.4	26.1	27.5
28	180.2	180.7	178.0	178.1
29	30.9	31.5	32.7	32.3
30	24.0	23.0	23.3	25.6
-OMe	-	-	51.6	51.5
-OCOMe	-	170.8	-	170.8
-OCOMe	-	170.6	-	170.6
-OCOMe	-	170.2	-	170.2



Scheme XXXI. Fragment ions identified in the mass spectrum of Erubigenin and its derivatives.

The full identity of these derivatives, by extension from the structure of GSA 16 are 3β,23,24-triacetoxyolean-12-en-28-oic acid 287, methyl-3β,23,24-trihydroxyolean-12-en-28-oate 288 and methyl-3β,23,24-triacetoxyolean-12-en-28-oate, 289.



	R	R'
286	H	H
287	Ac	H
288	H	Me
289	Ac	Me

Four aglycones of the saponin content of *Gardenia erubescens* were isolated and characterized in this work. These compounds were viz: 3β,23-dihydroxyolean-12-en-28-oic acid (hederagenin), 2β,3β-dihydroxyolean-12-en-28-oic acid, 2β,3β,23-trihydroxyolean-12-en-28-oic acid (bayogenin) and 3β,23,24-trihydroxyolean-12-en-28-oic acid, which was named erubigenin.

All the four triterpenoids belong to the olean-12-ene series and this is consistent with the type of triterpenes which had been reported in other Gardenia

plants (see table 6). The most abundant of the triterpene aglycones was hederagenin while erubigenin was the only new triterpenoid aglycone identified. Although 2β,3β-dihydroxyolean-12-en-28-oic and bayogenin acids are not new compounds, they are being reported for the first time in *Gardenia* plant.

It is interesting to note that neither ursolic acid nor oleanolic acid was isolated from the aglycone mixture of the saponins despite their presence in the petroleum ether extract in significant quantity.

The position of hydroxylation on the oleanolic acid skeleton in the triterpene aglycones show a different pattern when compared with the triterpenoids reported in the family and in other *Gardenia* plants. The compounds reported so far, with the exception of hederagenin, commonly possess a second hydroxyl group on ring E (see tables 4 & 6). However, this is far from being true for the triterpene aglycones of *G. erubescens*. In the triterpene aglycones isolated, all the hydroxyl substituents were located on ring A.

Talking about possible pharmacological activities of the triterpene aglycones, it may be tempting to predict a range of interesting pharmacological properties if one considers the biological acitivities which range from analgesic, antifertility, molluscidal and hypotensive to cytotoxicity as reported for some triterpenoids (see table 21).

In the interim however, it may suffice to say that since the crude saponin extract was reported by Hussain et al to possess sedative, analgesic, hypo tensive and diuretic effects, it is logical to extend these activities to the triterpenoid

aglycones. But this blanket extension calls for caution as it is necessary to evaluate the medicinal potential of each triterpene aglycone.

For the new compound, erubigenin, an evalution of its therapeutic properties needs to be carried out in order to assess and establish its medicinal potential. Considering the known compounds, hederagenin is the only known compound which had been reported for its pharmacological activity by Ahmad et al[151]. They reported a glycoside of hederagenin for its hypotensive activity in anaesthetized rats. In the light of this report, it could be suggested that the hypotensive activity of the crude saponin extract of *G. erubescens* may indeed be due to the hederagenin component of the saponin extract.

CONCLUSION

The results of the phytochemical investigation carried out on the petroleum ether and methanol extracts of the stems of *Gardenia erubescens* in this work suggested the absence of alkaloids and anthraquinones in the plant. This result is in consonance with reports available on *Gardenia* plants. However, the absence of these compounds seem to make the inclusion of *Gardenia* plants in the Rubiaceae family questionable.

This work reports for the first time isolation and characterization of fourteen compounds from the stems of *Gardenia erubescens*. All the compounds with the exception of stigmasterol and hederagenin are also being reported for the first time in *Gardenia* plants. Two of these compounds - erubescenone and erubigenin are new compounds. Three of the compounds 266, 267, 269 are flavonoids, compounds 270 and 271 are steroids while the other eight compounds are triterpenoids. The compounds isolated in this work from the stems of *Gardenia erubescens* are presented in Table 34.

It has also come to light, in the course of this research as it is visibly shown in table 34, that the saponin constituents of the plant is composed of only oleanene type triterpenoids.

The totality of the results obtained in this work highlight the presence of flavonoids, steroids and triterpenoids as the major natural products constituents of the stems of *G. erubescens* and further suggested that the reported folkloric uses and pharmacological activities of the plant may indeed be attributed to these constituents. The flavonoids for anti microbial properties and hederagenin for the hypotensive property.

Although this work reports an extensive chemical analysis of the stems of *G erubescens*, it is nevertheless not exhaustive. Areas which should attract further investigation include isolation of the triterpene constituents of the stems as their glycosides, the isolation and characterization of the constituents of the roots and also bioassay work with the isolated pure extractives of the plant.

Table 34- Compounds isolated from the stems of *Gardenia erubescens* Stapf. & Hutch.

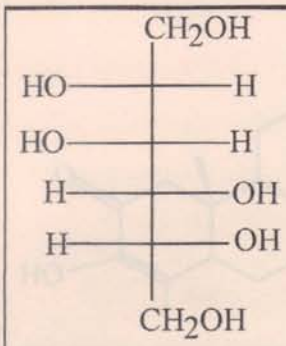
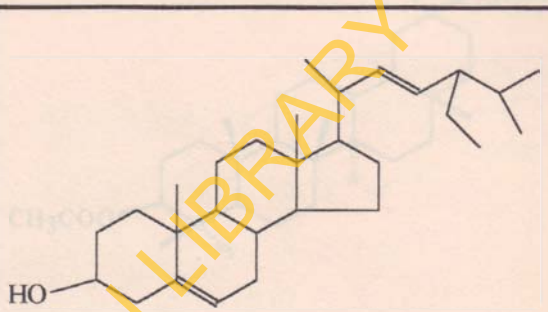
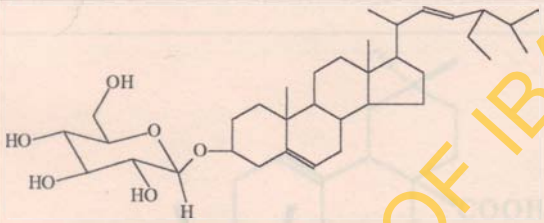
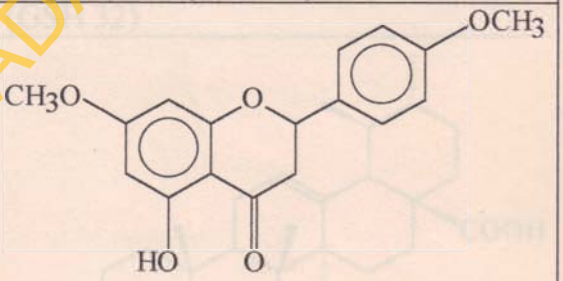
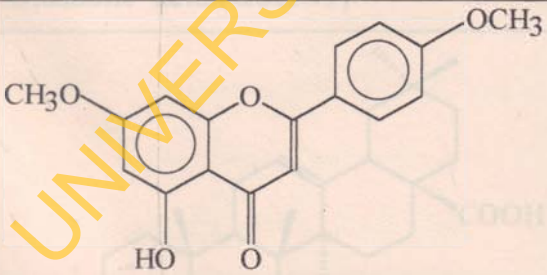
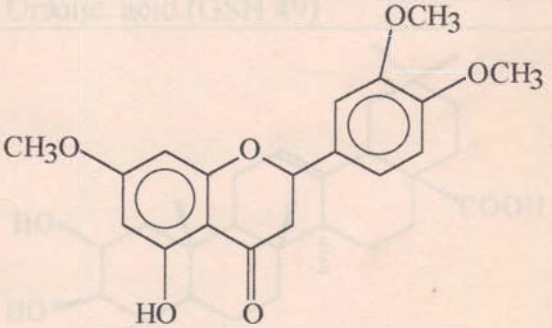
 D- Mannitol.(GS 1)	 Stigmasterol.(GSH 14)
 Stigmasterol-3β-D-glucopyranoside. (GS 13)	 5-Hydroxy-7,4'-dimethoxyflavanone.(GSH 2)
 5-Hydroxy-7,4'-dimethoxyflavone. (GSH 26)	 5-Hydroxy-7,3',4'-trimethoxyflavanone.(GSH 41)

Table 34 Contd.

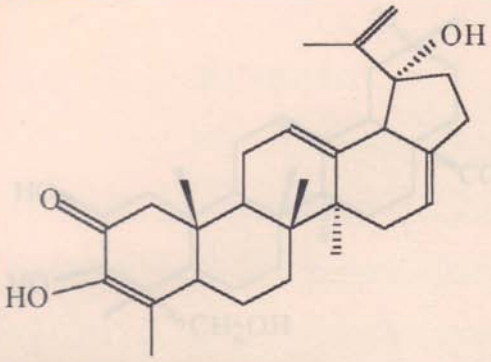
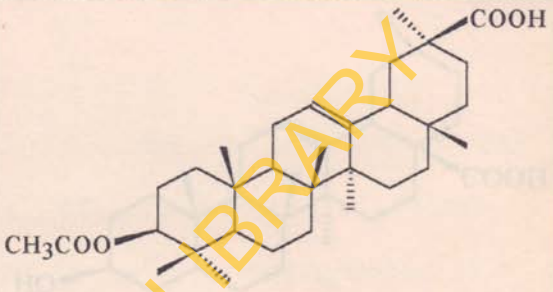
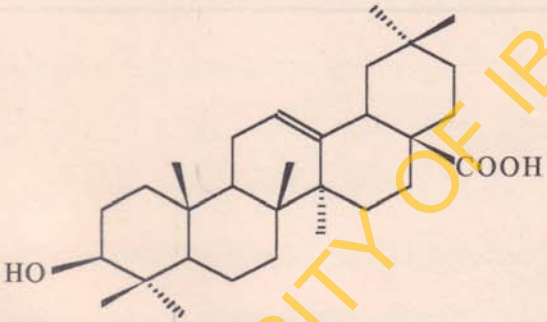
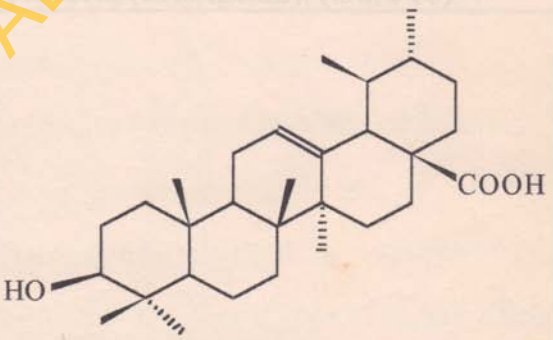
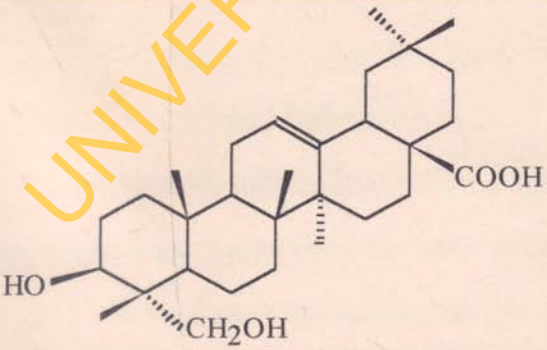
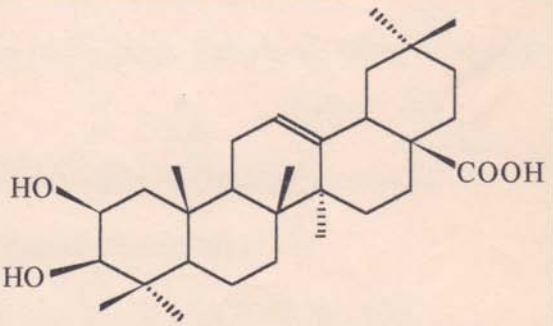
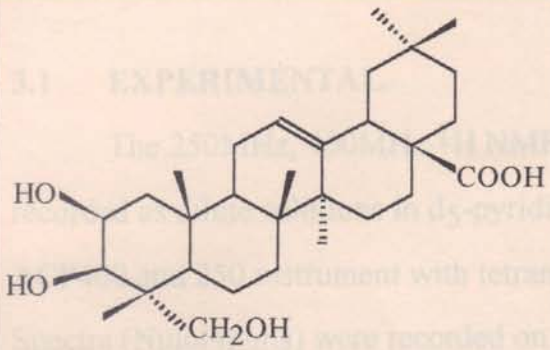
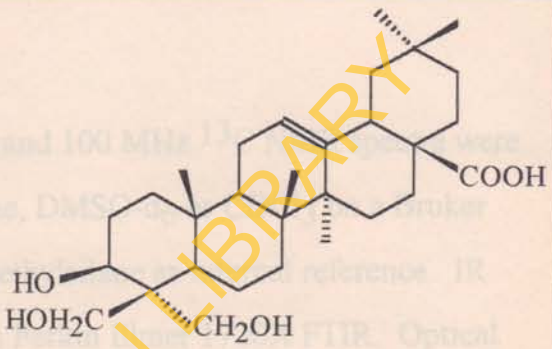
 Erubescenone.(GSH 24)	 3β-Acetoxyolean-12-en-30-oic acid. (GSH 32)
 Oleanolic acid.(GSH 49)	 Ursolic acid.(GSH 49)
 3β,23-Dihydroxyolean-12-en-28-oic acid.(GSA 5)	 2β,3β-Dihydroxyolean-12-en-28-oic acid.(GSA 8)

Table 34 Contd.

 2β,3β,23-Trihydroxyolean-12-en-28-oic acid.(Bayogenin). (GSA 11)	 3β,23,24-Trihydroxyolean-12-en-28-oic acid (Erubigenin). (GSA 16)

CHAPTER THREE

3.1 EXPERIMENTAL.

The 250MHz, 400MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra were recorded as dilute solutions in d_5-pyridine, DMSO-d_6 or $CDCl_3$ on a Bruker ACP400 and 250 instrument with tetramethylsilane as internal reference. IR Spectra (Nujol mulls) were recorded on a Perkin Elmer 1720X FTIR. Optical activity was measured on an AA-1000 polarimeter. UV spectra were recorded on Unicam 8700 series UV/VIS spectrometer and melting points were taken on Gallenkamp apparatus and were uncorrected.

EI, CI and FAB mass spectrometer spectra were run on a Kratos MS-902 double focusing instrument at 70 eV.

Precoated silica gel Kieselgel 60 F_{254} plates were used for analytical TLC, silica gel a for thinlayer chromatography was used for preparative TLC and silica gel G. (230-400 mesh) was used for column chromatography.

All solvents used in this work were redistilled. Petroleum ether used refers to the 60-80^{o}C boiling range.

The chemicals, sodium hydroxide, Aluminium chloride, magnesium sulphate, bismuth nitrate and ferric chloride were obtained from suppliers.

Detection reagents used for phytochemical test for alkaloid, was Dragendorff's reagent, chlorosulphoric acid reagent and Acetic anhydride/H_2SO_4 reagent for steroids. Flavonoid tests were carried out with NH_3 vapour, aluminium chloride reagent and magnesium/concentrated hydrochloric acid reagent. Ferric

3.2.4. Aluminium Chloride Reagent

5% solution of aluminium chloride in methanol was prepared by dissolving 5g of aluminium chloride in 10 ml of distilled water and the solution made up to 100ml with methanol.

3.2.5. Sulphuric Acid Reagent

Equal volume of methanol and concentrated sulphuric acid were mixed together to give 50% methanolic sulphuric acid.

3.2.6 Liebermann-Burchard Reagent (adapted for TLC)

2 ml concentrated sulphuric acid, 40ml acetic anhydride and 100 ml chloroform were mixed together to make the spray reagent.

.3.3 EXTRACTION OF PLANT MATERIAL

The stems of *Gardenia erubescens* were airdried and then pulverized. 3kg of the pulverised stems was extracted in a Soxhlet extractor with petroleum ether for 24hr. This was followed by extraction with methanol for 24hr.

Subsequent extractions were carried out with some modifications. For one batch of the extraction, 2kg of the pulverized stems was extracted with petroleum ether for 24hrs, then ethylacetate for 24hr and finally methanol for 24hr.

Another extraction was carried out with 4kg of the pulverised stem. The plant material was first extracted with petroleum ether for 24hr after which the

defatted plant material was soaked in 60% aqueous methanol for 72hr. The aqueous methanol extract was filtered before further treatment.

3.4 TREATMENT OF EXTRACTS

3.4.1 Petroleum ether Extract

Three sets of extractions were carried out and they were concentrated in vacuo as at when extration was carried out.

The first petroleum ether extract was concentrated on a water-bath to a dark greenish-yellow oil which became a dark greenish-yellow gum on cooling.

Before the extracts were subjected to analysis, they were taken up into chloroform and the insoluble component filtered off. The filtrate was rotary evaporated and these were used as the petroleum ether extracts. The yield of the extracts was about 3-5g.

3.4.2 Ethyl acetate Extract.

Concentration of the ethyl acetate extract gave a thick dark green oil (syrup) (2.5g). This extract was obtained once as it proved to be uninteresting

3.4.3 Methanol Extract.

The first extraction carried out with methanol yielded on concentration on a water-bath a thick dark brown gum (300g).

The extracts that were obtained from the subsequent extractions carried out with 60% aqueous methanol were concentrated in vacuo to a reddish brown solid weighing about 200g and 250g respectively.

Trituration of the concentrated methanol extracts with methanol precipitated a white crystalline solid which was filtered off and washed several times with methanol and then recrystallized from MeOH/H_2O mixture. The methanol mother liquor of the extract was again concentrated in vacuo.

100g of the extract was dissolved in 250ml of distilled water and the mixture was extracted with 3x 100ml n-butanol. The n-butanol soluble fractions were combined and concentrated in vacuo to a yellowish brown solid (~15g). This was used as n-butanol soluble fraction of methanol extract in subsequent analysis. The aqueous phase was again also extracted with 50ml portions of ethylacetate, four times and the fractions combined and concentrated in vacuo to a brown gum (~1 g). The aqueous phase was finally discarded as it contained water soluble substances which are difficult to isolate.

3.5 PHYTOCHEMICAL TESTS[178-179].

The petroleum ether, ethyl acetate, n-butanol soluble and ethyl acetate soluble extracts were separately tested for the presence of flavonoids, steroids, alkaloids, anthraquinones and saponins.

3.5.1 Phytochemicl analysis of Petroleum ether Extract.

i. Flavonoid test

A dilute solution of the extract in a mixture of chloroform/methanol was made. To this solution magnesium turnings (few pieces) were added followed by addition of concentrated hydrochloric acid. A dark red colour was observed.

ii. Steroid test

Liebermann-Burchard test was carried out. A small quantity of extract was dissolved in 2ml acetic anhydride in a test-tube. This was cooled in an ice-bath before sulphuric acid was carefully added. There was a colour change from violet to green in the upper layer.

iii. Alkaloid test

A dilute solution of the extract was made with chloroform/methanol mixture. Few drops of Dragendorff's reagent was added. There was no turbidity or precipitation observed.

iv. Anthraquinone test

The Borntrager's test was used. About 0.2g of the extract was dissolved in 10ml of benzene. The solution was filtered and 5ml dilute ammonia solution was added. The mixture was transfered into a 50ml separating funnel and shaken. The ammoniacal phase remained yellowish green.

3.5.2 Phytochemical analysis of Ethyl acetate extract.

i. Steroid test

Liebermann-Burchard test was carried on a dilute solution of the extract in acetic anhydride. The upper layer of the reaction mixture turned green.

ii. Other tests

The test for alkaloid, flavonoid, and anthraquinones were also carried out. The observations were negative for these compounds.

3.5.3 Phytochemical analyses of methanol extract, n-Butanol soluble and ethyl acetate soluble extracts.

i. Flavonoid test.

Dilute solution of each extract was prepared in a test-tube and the Shinoda test carried out.

For the methanol extract, colour of the solution remained brownish. The n-butanol soluble extract also did not give any colour change while there was a colour change from pale brown solution to yellowish brown in case of the ethylacetate soluble extract.

ii. Steroid test

Liebermann-Burchard test was carried out on each of the extracts.

The methanol extract gave a pale pinkish colouration at the interface which latter turned reddish, while a violet colour was observed for the n-butanol soluble extract which also turned reddish with time. There was no visible reaction with the ethylacetate soluble extract.

iii. Alkaloid test.

Dilute solutions of each of the extracts were tested for alkaloid. The results were negative.

iv. Tannins test

A few drops of 1% solution of ferric chloride was added to 1ml methanol solution of each extract in a test tube. A dark green colouration was observed for the methanol extract. The n-butanol soluble did not show any visible reaction but a green colouration was observed for the ethyl acetate soluble extract.

v. Saponin test

Frothing test was carried out on the crude methanol extract and the n-butanol soluble extract. A small quantity of each extract was disolved in 2ml distilled water in a test-tube and shaken. Both extracts produced frothing which persisted on warming in a hot water-bath.

3.6 ANALYTICAL THIN LAYER CHROMATOGRAPHY (TLC).

3.6.1 TLC analysis of Petroleum ether extract.

A moderately concentrated solution of the petroleum ether extract in chloroform was applied on precoated silica gel plates (cut to size 2.5 by 10 cm) with the aid of capillary tube and developed in the following solvent mixtures. (a) $CHCl_3$:Hexane, 1:3; (b) di isopropyl ether; (c) diisopropyl ether: acetone, 75:30; (d) ethylacetate:Hexane, 1:3 and (e) ethylacetate:hexane, 1:1.

The chromatoplates from each solvent system were separately viewed under the UV, in iodine tank and also sprayed with detection reagents for alkaloids, steroids and flavonoids (fresh plates were prepared for the different analyses).

In each case one or two spots were visible as flavonoid spots which appeared as dark spots in UV but changed to bright yellow spots after treatment while a number of spots appeared as steroids but there was no visible alkaloid spot. For all the solvent systems used, the extract showed several spots on the chromatoplate but the ethylacetate: Hexane (1:3) mixture gave the best resolution showing not less than ten overlapping spots with iodine vapour.

3.6.2 TLC analysis of n-butanol soluble extract.

The composition of n-butanol soluble fraction of methanol extract was examined by subjecting the extract to TLC analysis in a number of solvents. A reasonably concentrated solution of the extract in methanol was spotted on the TLC plates and developed in the following solvent systems:

a) Chloroform:methanol,1:3,1:1, and 65:45
b) Chloroform-methanol-water 65:45:12 and 65:35:10
c) Dichloromethane-methanol-water 40:10:1
d) Ethyl acetate-pyridine-water 10:4:3
e) Ethyl acetate-pridine-water methanol 16:4:2:1
f) n-Butanol-acetic acid-water 3:1:1 and 4:1:5

The spots on the chromatoplates from the different solvent systems were detected by:

i) Spray reagents for alkaloids, steroids and flavonoids.

ii) Visualising in UV and

iii) Iodine vapour.

The alkaloid and flavonoid spray reagents gave negative results, while many of the spots reacted positively with chlorosulphonic acid reagent giving grey to brown colours. Most of the steroid spots observed were concentrated at around R_f values 0.4 and below.

On each chromatoplate several spots were visible with many overlapping. Most of the solvent systems dragged the components in a tailing fashion but the chromatoplates developed in chloroform-methanol-water 65:45:12 and 65:35:10 showed the best resolution.

3.7 COLUMN CHROMATOGRAPHIC ANALYSIS OF PETROLEUM ETHER EXTRACT.

10g of the petroleum ether extract was purifed on a column of silica gel (400g) packed in hexane. The colum was developed in hexane. Elution continued with 2% EtOAc/Hexane mixture.

5% EtOAc/Hexane mixture eluted a white grease and further elution gave the carotenoid fraction as orange-red oil(~3g). Elution with 8% ethyl acetate/hexane gave a white solid. This fraction, GSH 2 (~50mg) was lightly coloured by the carotenes but the orange colouration was successfully removed by washing the solid with hexane and TLC in 5%, 8%, 10% EtOAc/Hexane showed only one spot. When the solvent mixture was changed to 10% ethyl acetate

hexane, fractions 9-20 collected contained one major steroid spot (TLC) with other minor contaminants which were mostly the carotenes. These fractions were then combined as GSH 14 and subjected to further purification on preparative TLC. 15% ethyl acetate-hexane gave fractions 22-38 which contained three components. Fractions 22-25 contained two UV active spots (TLC) with the spot having higher Rf value (R_f 0.63) as the major component, GSH 24. The minor contaminant which gave a positive flavonoid test (TLC) was removed from GSH 24 by preparative TLC. Fractions 26 and 27 were pure (TLC) R_f value 0.50 in 10% EtOAc/Hexane and found to be similar to the contaminant of GSH 24.

The TLC showed one spot which tested positive for flavonoid. This component was labelled GSH 26. The quantity obtained was in trace amount. Fractions 31-38 also happened to be contaminated with GSH 26. Therefore further purification of this fraction, GSH 32 , which has steroid (TLC, R_f value 0.42) as the major component was carried out by preparative TLC.

The chlorophylls came down with 20% ethyl acetate/hexane and with the chlorophylls also came a component which was UV active and also gave positive flavonoid test (TLC). Fractions 40-43 contained this compound as the only component but with heavy colouration with chlorophyll pigment. The solution of this fraction in chloroform was treated with activated charcoal to give a pale green solid - GSH 41 (~25mg).

The column was continued with 25% ethylacetate/hexane and the fractions collected, 48-60, showed one major steroid spot (TLC) together with chlorophyll pigment. These fractions were combined and rechromatographed on a short column.

of silica gel eluted with 20% and 25% ethyl acetate/hexane mixtures. The pure fractions collected were still slightly coloured with chlorophyll. This component was labelled as GSH 49. GSH 49 was later found (^{1}H and ^{13}C NMR) to be a mixture of two isomeric compounds and an attempt was made to separate the components. 30%-35% ethyl acetate/Hexane eluted an orange oily material. The column was terminated with 50% ethylacetate-hexane as fractions collected were uninteresting.

3.8 PREPARATIVE THIN LAYER CHROMATOGRAPHIC PLATES.

The 20 by 20 cm standard plates were used to prepare the preparative plates.

A slurry of silica gel G for thinlayer chromatography was prepared by mixing silica gel (250g) with 450 ml distilled water was used for coating up to seven standard plates with 1mm thickness. The prepared plates were activated overnight at 110 C and allowed to cool before use. All the preparative TLC carried our were with freshly prepared plates.

3.8.1. Prep. TLC for GSH 14

About 1g of GSH 14 was purified by Pred. TLC. Each plate was loaded with about 200mg of the substance as it contained only coloured contaminant. The plates were developed in 5% ethylacetate/hexane mixture. The orange colour moved with the solvent front and was easily removed from the plate. The steroid bands were scrapped and extracted with chloroform. The chloroform solution was evaporated to dryness in vacuo and the compound recrystallized from

hexane/ethylacetate mixture as white needles (~0.6g). Purity was checked by TLC in 5% and 10% ethylacetate/hexane mixture.

3.8.2. Prep. TLC for GSH 24

Less than 40 mg of this substance was available. The whole quantity was dissolved in 1ml chloroform and loaded onto two preparative plates. The plates were developed with 10% ethylacetate/hexane mixture. The major UV active band (R_f value = 0.61) was scrapped and extracted with chloroform. The chloroform extract was concentrated to a small volume, then transfered into a small sample val where the solvent was completely removed in vacuo. TLC in 10% ethyl acetate/hexane showed one spot. The chromatoplate was also sprayed with ferric chloride reagent and heated in the oven for about five minutes. A greyish black colour developed. Weight of GSH 24 recovered was ~25mg.

3.8.3. Prep TLC for GSH 32

The amount of substance available was less than 50mg. This was dissolved in 1ml chloroform and loaded on two preparative plates. The plates were developed in 10% ethyl acetate/hexane mixture. The UV active bands (minor contaminant) were removed and steroid bands (R_f value = 0.42) were scrapped and extracted with chloroform. Complete evaporation of solvent gave GSH 32 as white solid (35mg). Purity was confirmed by TLC in 10% ethyl acetate/hexane mixture.

3.8.4. Prep TLC for GSH 49

i) By normal preparative plates.

Four preparative plates were each loaded with about 50mg of GSH 49 dissolved in chloroform/methanol (1:1). The plates were allowed to run continuously in 10% MeOH/$CHCl_3$ mixture for 3hrs. The reference plate was treated with 50% methanolic sulphuric acid reagent. It was observed that there was no resolution.

ii) By silver nitrate-impregnated preparative plates

The silica gel slurry was made by adding $AgNO_3$ (10% w/w) to the silica gel powder. This was used to prepare the silver nitrate impregnated preparative plates.

The purification was repeated as for the normal preparative plates. This mixture could not be resolved.

3.9. CHROMATOGRAPHIC ANALYSES OF ETHYL ACETATE AND N-BUTANOL SOLUBLE EXTRACTS.

3.9.1. Column chromatographic analysis of Ethyl acetate Extract.

2g of ethylacetate extract was chromatographed on a column of silica gel (150g) packed in Hexane. The column was eluted with ethylacetate/hexane mixtures 10%, 15%, 20%, 25%, 35%, 50%, 70% and 100% ethylacetate. The fractions collected contained greenish oily material.

Elution of the column was continued with 5% MeOH/EtOAc, 10% MeOH/EtOAc and was terminated with 15% MeOH/EtOAc.

5% MeOH/EtOAc eluted fractions containing one major component. The fractions were combined to give a pale brown solid. This was titurated with acetone to give a pale white solid (~10mg). It gave only one spot on TLC (methanol: chroform: water (65:45:12) and 30% MeOH/$CHCl_3$). This compound was later found to be identical to GS 13 (NMR).

3.9.2. Column chromatographic analysis of n-butanol soluble extract.

The n-butanol soluble fraction (6g) of methanol extract was subjected to chromatographic analysis on a column packed with silica gel (300g) (230-400 mesh) in chloroform and the column run with mixtures of $CHCl_3$/MeOH in increasing order of polarity. Elution with 2% MeOH/$CHCl_3$ brought down chlorophyll pigment while 4% MeOH/$CHCl_3$ eluted some yellowish oily substance.

A brownish solid substance came down with 15% MeOH/$CHCl_3$. TLC analysis in 30% MeOH/$CHCl_3$ showed it as one component. The colouration was removed by tituration with ethylacetate. This was labelled as GS 13 (quantity was very small).

The column elution continued with 20%, 25% 30%, 35%, 40%, 45%, 50% and 75 MeOH/$CHCl_3$ mixtures, but the fractions eluted contained brownish gummy material which contained mixtures of substances (TLC). The fractions eluted with 50% MeOH/$CHCl_3$ showed three major components on TLC when sprayed with

chlorosulphonic acid reagent and the ^1H NMR of this impure fraction indicated the presence of triterpenoid components. The column was thus not successful.

The column was also rep eated using mixtures of solvents: (i) chloroform-methanol-water, 65:45:12 and (ii) chloroform-methanol-water, 65:35:10.

These two solvent mixtures were used to run. two separate columns. Both columns were not successful.

3.9.3. Treatment of n-butanol soluble Extract.

The n-butanol soluble extract (10g) was further subjected to treatment in order to rid the saponins of pigments and less important components. This was achieved by precipitation of the saponins from the methanol solution by using chloroform. The precipitate (saponin content) was collected by filtration and sucked dried to a dark brown solid (6g). TLC analysis of the treated n-butanol soluble fraction showed the presence of 4-6 saponin components.

3.9.4. Column chromatographic analysis of the treated n-butanol soluble extract.

The treated n-Butanol soluble fraction was subjected to column chromatography. 3g of the extract was chromatographed on a column of silica gel (250g) packed in chloroform. Elution was carried out with chloroform/methanol mixtures. The fractions collected were brownish gumy substances containing mixtures of components.

The column was repeated with chloroform-methanol-water, 65: 35: 10 mixture but the result was not satisfactory.

3.9.5 Preparative TLC for treated n-BuOH Soluble Extract.

3g of the treated n-butanol was dissolved in 10ml methanol. Each preparative plate was loaded with about 50mg of extract. The plates were developed in chloroform-methanol-water, 65: 45: 12 (best mixture, see TLC analysis of methanol extract). A reference plate which was sprayed with chlorosulphonic acid reagent was used as a maker for each set of plates run. Two bands were only visible from the preparative plates. The two bands (R_f values 0.36 and 0.44) were removed into separate flasks and were extracted with MeOH. The methanol extracts on concentration did not contain any saponin (TLC and NMR).

Another set of preparative plates were prepared and used in the solvent system chloroform-methanol-water, 65: 35: 10 containing 20% ammonium hydroxide. The bands also overlapped. When they were removed, extracted with MeOH and the solution concentrated, there was no trace of any saponins. Apparently the saponins were not being extracted from the silica gel.

3.9.6 Acid Hydrolysis of the treated n-butanol soluble fraction.

10g of the extract was dissolved in 100ml methanol and 100ml 4% methanolic sulphuric acid was added to give 2% sulphuric acid in the methanol solution. The mixture was refluxed on a steam bath for 6hr. After cooling, the reaction mixture was poured into 500ml distilled water and the aqueous mixture

extracted with ethylacetate. The ethylacetate extract was washed with water, dried with $MgSO_4$ and then concentrated in vacuo. About 3.8g of aglycone was recovered. The process was repeated for four sets of methanol extracts obtained in the course of this work. The various weights of aglycone obtained were 3.8g, 3.6g, 2.1g and 5.4g.

3.9.7 Acetylation of n-butanol soluble Aglycone.

2g of the crude aglycone was acetylated with 60ml pyridine/acetic anhydride (1:1) mixture for four days. The reaction was stopped by addition of 30ml methanol to destroy excess anhydride. The reaction mixture was concentrated, diluted with water and then extracted with chloroform. The chloroform extract was washed with water, dried with $MgSO_4$ and the solvent removed in vacuo giving 1.5g of crude aglycone acetate.

3.9.8. Column chromatographic analysis of n-butanol soluble aglycones.

5g of crude aglycone mixture was chromatographed on silica gel (300g) packed in chloroform. Column elution was carried out with chloroform/methanol mixtures. For 2% methanol/chloroform the fractions collected contained brown pigment. Fractions 5-10 were collected from elution with 4% methanol/chloroform. Fractions, 5,6 and 7 were combined on the basis of TLC (in 2% and 5% MeOH/$CHCl_3$). These fractions contained one major component with minor contaminants (pigment). Fractions 8,9,10 however showed only one pure component with similar R_f value (0.32) to the component in 5,6 and 7. Combined

fractions, 8,9,10 gave GSA 8 (~15mg). The combined fractions 5,6 and 7 were later purified by preparative TLC to give GSA 5.

Further elution with 6% methanol/chloroform gave fractions 11-25. Fractions 11, 12, 13 and 14 showed one single component (TLC) but was contaminated by brown pigment. Recrystallization in 50% methanol/chloroform gave white salt-like crystals labelled as GSA 11 (~20mg). Fractions 16, 17 and 18 contained another brownish solid which has similar R_f value (0.25 in 10% MeOH/$CHCl_3$) with the component of Fractions 11, 12 13 and 14. The combined fractions 16, 17 and 18 were further purified by preparative TLC as GSA 16.

3.9.9 Preparative TLC for GSA 5 and GSA 16

The combined fractions for GSA 5 and GSA 16 were concentrated and redissolved in 1ml 50% methanol/chloroform. Each compound was loaded separately on two preparative plates and the plates were developed in 30% methanol/chloroform mixture. The pigment contaminants did not move from the origin. The area above the pigment band was scrapped and extracted with 50% methanol/chloroform mixture giving GSA 5 (~50mg) as white crystals and GSA 16 (~40mg) as white powder.

3.9.10. Column Chromatographic analysis of Acetylated Crude Aglycones.

About 1.4g of crude aglycone acetate was chromatographed on silica gel (150g) column. The column was developed in hexane and elution continued with ethylacetate/hexane mixtures. Elution with 5%, 10%, 15%, 20%, 25% up to 75%

ethylacetate/hexane gave fractions containing mixtures of components (TLC). The column was discontinued.

3.10. Preparation of Derivatives.

i) GS 1 hexaacetate

1g of GS 1 was dissolved in 50ml pyridine/acetic anhydride (1:1) mixture. The reaction mixture was kept for three days. Excess acetic anhydride was destroyed by addition of 20ml methanol. The solvent was removed and residue diluted with water. The aqueous mixture was then extracted with chloroform. The chloroform extract was washed with distilled water, dried with magnesium, sulphate and the solvent removed completely. The product was redissolved in chloroform and left to crystallize out, giving white heavy crystals of GS 1 hexaacetate (1.2g).

ii) GSH 14 acetate

0.5g of GSH 14 was dissolved in 1:1 mixture of pyridine/Ac_2O (30ml). The reaction mixture was left overnight and the usual work-up carried out. The acetate (0.3g) was redissolved in chloroform and left to crystallize out.

iii) GSH 14 Oxo-compound

50mg of GSH 14 was added to 50mg of pyridinium chlorochromate in 10ml dried dichloromethane. The reaction was stirred at room temperature for 2hr and the reaction was minotored by TLC (in ethylacetate/hexane, 1:3). The reaction

mixture was filtered when reaction had been completed (TLC) and the filtrate diluted with water. The aqueous mixture was extracted with diethyl ether.

iv) GSA 5 diacetate

25mg of GSA 5 was dissolved in 20ml 1:1 mixture of pyridine/AC_2O mixture. The reaction was left for two days and the usual work-up carried out. The crude acetate (15mg) was redissolved in chloroform and left to crystallize out.

v) GSA 11 triacetate

20mg of GSA 11 was dissolved in 20ml 1:1 mixture of pyridine/AC_2O. The same procedure as for GSA 5 diacetate was used. The crude GSA 11 triacetate (10mg) was redissolved in $CHCl_3$ and left to crystallize out.

vi) GSA 16 triacetate

20mg of GSA 16 was acetylated according to the procedure for GSA 5 and GSA 11. The crude GSH 16 triacetate was further purified by preparative TLC. The preparative plate was run in ethyl acetate/hexane, 1:3 mixture. The band was extracted with chloroform and the solvent removed in vacuo. The product (12 mg) was then redissolved in chloroform and left to crystallize out.

vii) GSA 16 methyl ester

The methyl ester was prepared by reacting the acid with diazomethane. The diazomethane was generated from the diazogen, N-methyl-nitroso-p-toluene sulphonamide by the method described by Paolo Lombardi[180]. The diazomethane was carried into the reaction flask containing 10mg of GSA 16, dissolved in 5ml methanol, by a stream of nitrogen gas. The reaction was monitored by TLC (10% MeOH/$CHCl_3$ and EtOAc/Hexane 1:3) and stopped when all the acid molecules had reacted. The solvent was then removed and GSA 16 methyl ester was recovered as white solid.

vii) GSA 16 triacetate methyl ester

The GSA 16 methyl ester was acetylated by the usual method. The GSA 16 triacetate methyl ester (3-5mg) was obtained as a pale yellow gum. Further purification was hindered by the small quantity available.

3.11. Determination of Specific rotation for GSH 24, GSA 16 and GSA 16 triacetate.

The weighed samples GSH 24 (20mg), GSA 16 (30mg) and GSA 16 triacetate (20mg) were dissolved in 1ml spectroscopic chloroform or methanol as appropriate.

The observed rotation was measured with the AA-1000 polarimeter.

The specific rotation was then calculated from the observed rotation using the expression,

$$[\alpha]_D = \frac{\alpha_D}{2 \times C}$$

C= Conc. in g/ml,

α_D is observed rotation

length of tube = 2 decimeter

GSH 24

$\alpha^{20}_D = +2.4401$

C = 0.02g /ml ($CHCl_3$)

$$[\alpha]^{20}_D = \frac{2.4401}{2 \times 0.02}$$

$[\alpha]^{20}_D = +61$

GSA 16

$\alpha^{22}_D = +4.5604$

C = 0.03g/ml (MeOH)

$$[\alpha]^{22}_D = \frac{4.5604}{2 \times 0.03}$$

$[\alpha]^{22}_D = +76$

GSA 16 triacetate

$\alpha^{22}_D = +3.6022$

C = 0.02g/ml ($CHCl_3$)

$$[\alpha]^{22}_D = \frac{3.6022}{2 \times 0.02}$$

$[\alpha]^{22}_D = +90$

3.12. SPECTRAL DATA ON THE COMPOUNDS ISOLATED FROM *G. erubescens*.

3.12.1 GS 1

D-Mannitol (GS 1) white crystals, m.p. 170°C (Lit. 166-168°C)[93]. IR Vmax (Nujol). 3300-3100, 2927, 2825, 1377, 1351, 1262, 1147, 1092, 1026, 933, 891 and 171 cm^{-1}

^1H nmr (DMSO-d_6) δ4.42 (2H,d,J=6.0Hz) 4.35 (2H,t,J=6.0Hz) 4.13 (2H,d,J=8.9Hz) 3.50 (8H, m)

^{13}C nmr (DMSO-d_6): δ72, 70, 64

EIMS M/Z (rel. int.) 183.0869 (100) $[M+1]^+$ ($C_6H_{15}O_6$ requires 183.0864), 165(10), 147(5), 111(2), 99(2)

Mannitol hexa acetate. White crystals, mp. 117-119°C (Lit. 121°C)[93].

IR Vmax (Nujol) 2926, 2854, 1742, 1456, 1373, 1225, 1089, 1071, 1059, 1034, 992, 907, 863, 722 and 620 cm^{-1}.

^1H nmr ($CDCl_3$) δ1.98 (6H,S) 2.0 (6H,S) 2.2 (6H,S) 4.03 (2H,dd, J=5,7Hz) 4.18 (2H,dd, J=3,10Hz), 5.03 (2H,m) 5.37 (2H, dd, J=1,9Hz)

^{13}C nmr ($CDCl_3$) 20 ($CO-CH_3$), 62, 67, 68, 169, 170, 171.

EIMS m/z (rel. int.) $[M+1]^+$ 435(10), 375 (100), 333 (5), 289(3), 273(12), 213(8), 153(55), 136(48), 77(55).

3.12.2 GSH 2 m.p. 111-113°C (lit. 115.5-115.9°C)[167].

IR Vmax (nujol) 4331, 3096, 2925, 2854, 1629, 1581, 1521, 1490, 1436, 1374, 1255, 1184, 1159, 1090, 961, 804, 733cm^{-1}

U.V. λmax ($CHCl_3$) 239.2nm and 290.5nm

^{1}H nmr ($CDCl_3$) δ2.76(1H,dd,J=3Hz), 3.10(1H,dd,J=14,4Hz), 3.79(3H,S), 3.82(3H,S), 5.35(1H,dd,J=3-6,10Hz), 6.97(2H, dd, J=9,2Hz), 7.40(2H,dd,J=9,2Hz), 10.90(1H,S); 6.02(1H,d,J=3Hz), 6.05(1H,d,J=3Hz).

^{13}C nmr. δC2, 78.8; C3, 43.1; C4, 195.9; C5, 164.0; C6, 94.9; C7, 167.8; C8, 94.1; C9, 162.8; C10, 103.0; C1, 130.3; C2' & C6', 127.6; C3' & C5', 114.1.

EIMS/mz (rel. int) [M^+] 300 (45) (Acc. mass 300.0998), 283(5) 272(2), 257(3), 193(20), 166(20), 134(100), 121(60), 91(45).

3.12.3 GSH 14 m.p. 166-168°C (Lit. 170°C)[168].

IR Vmax (Nujol) 3426, 2954, 2925, 2854, 1690, 1660, 1462, 1378, 1063, 970 cm^{-1}

^{1}H nmr ($CDCl_3$) δ3.57(1H,m), 5.15(2H,m), 5.36(1H,d,J=5Hz), 0.69(3H,S), 0.71(3H,S)

^{13} Cnmr (CDCl3). δ11.9, 12.1, 19.3, 19.7, 20.9, 21.1, 24.3, 25.3, 29.1, 31.6, 31.8, 37.2, 38.7, 39.7, 40.4, 42.1, 42.2, 50.0, 51.1, 56.7, 56.8, 71.7, 121.6, 129.2, 130.2, 140.6.

EIMS. m/z (rel. int) [M^+] 412 (10) (acc. mass 412.3705), 408(5), 351(5), 255(20), 213(15), 159(30) 105(40), 81(50) 55(100).

GSH 14 acetate

m.p. 140-142°C (Lit = 144-144.6°C)[168].

^{1}H nmr ($CDCl_3$) δ0.68 (3H,S) 0.71 (3H,S) 2.05 (3H,S), 4.63 (1H,m), 5.15 (2H,m) 5.40(1H,d,J=5Hz)

^{13}C nmr ($CDCl_3$) δ11.74, 1187, 11.92, 12.15, 18.87, 19.20, 20.90, 20.99, 21,35, 27.64, 31.73, 31.76, 36.47, 36.86, 37.99, 40.40, 49.89, 55.79, 73.87, 122.53, 138.21, 139.52, 170.46

EIMS m/z (rel. int.): 454(1) [M^+], 394(100), 255(70), 159(40), 147(62), 105(55), 81(84).

GSH 14 - Oxo-compound

^{1}H nmr ($CDCl_3$) δ5.15 (2H,m) 5.35 (1H,d,J=5Hz) 5.73 (1H,S), 6.18 (1H,S).

^{13}C nmr ($CDCl_3$): δ122.81, 129.23, 138.13, 171.76, 199.72 and others.

EIMS. m/z (rel. int.): 410(30) [M^+], 27(40), 229(30), 124(40), 81(60), 55(100).

3.12.4 GSH 26

UV λmax (MeOH): 277.6, 303.0, 346.1 nm

^{1}H nmr ($CDCl_3$). δ3.90(3H,S) 3.91(3H,S), 6.38(1H,d,J=2Hz), 6.50 (1H,d,J=2Hz), 6.60 (1H,S) 7.02(2H,dd,J=9,2Hz), 7.87(2H,dd,J=9,2 Hz)

^{13}C nmr ($CDCl_3$) δ55.42 ($0CH_3$), 55.67 ($0CH_3$), 92.52(C6), 97.92(C8), 104.27(C3,C10), 114.40, C3', C5'), 123.50(C1'), 127.94(C2', C6'), 157.61 (C9), 162, 162.11 (C5), 162.49 (C2), 163.93 (C4'), 165.33 (C7), 182.35 (C4).

EIMS. m/z (rel. int.) [M^+] 298(60) (acc. mass 299.0910), 269(20), 255(10), 241(2), 213(5), 166(5), 129(20), 97(30), 83(45), 69(90), 55(100).

3.12.5 GSH 24

M.P. 203-205°C., [α]$^{20}_D$ + 61 (C,2.0, $CHCl_3$). IR Vmax (Nujol): 3583, 3565, 3337, 1662, 1636, 1457, 1377, 1244, 1174, 1162, 1072, 930, 900, 836 and 774 cm^{-1}. UV λmax ($CHCl_3$) 282 nm, λmax (EtOH) 280nm

^{1}H nmr ($CDCl_3$): δ0.83(3H, S), 0.93(3H, S), 1.33(3H, S), 1.39(3H, S), 1.87(3H,d,J=1.9Hz), 4.74(1H,bs), 4.86(1H,bs), 5.40(1H,bt), 5.67(1H,bt), 5.99(1H,bs).

^{13}C nmr ($CDCl_3$): δ13.2, 13.9, 17.2, 20.7, 23.6, 23.7, 25.1, 32.2, 32.6, 33.6, 35.9, 39.2, 40.8, 41.4, 43.6, 48.6, 51.2, 56.0, 75.0, 106.7, 122.5, 125.7, 130.6, 134.3, 137.2, 143.4, 153.7, 193.7.

EIMS m/z (rel. int.): 422.2930 [M^+] ($C_{28}H_{38}O_3$ requires 422.2900) (60), 404(12), 379(4), 339(20), 219(18), 201(20), 183(28), 151(50), 132(50), 119(100), 105(70), 91(80), 55(58).

3.12.6 GSH 32

Clusters from ethyl acetate, m.p. 246-248°C (Lit. 242°C)[169].

IR Vmax (Nujol). 2924, 2854, 1732, 1696, 1463, 1377, 1253, 1029, 722 cm^{-1}

^{1}H nmr ($CDCl_3$) δ0.73(3H,S), 0.84(3H,S), 0.85(3H,S), 0.89(3H,S), 0.91(3H,S), 0.93(3H,S), 1.11(3H,S), 2.03(3H,S), 2.80(1H,dd,J=3,10Hz) 4.48(1H,t,J=8Hz) 5.26(1H,t,J=3.5Hz).

^{13}C nmr ($CDCl_3$) δ15.27, 16.54, 17.04, 18.05, 21.21, 22.76, 23.27, 23.40, 23.46, 25.78, 27.54, 27.92, 30.55, 32.31, 32.40, 32.94, 33.65, 36.86, 37.57, 37.93, 39.14, 40.81, 41.43, 45.70, 46.41, 47.42, 55.16, 80.80, 122.43, 143.48, 170.95, 183.45.
CIMS m/z m/z (rel. int.) 499 $[M+1]^+$, 439(100) ($[M+1]^+$ - 60) acc. mass 439.3590($C_{30}H_{47}O_2$ requires 439.3564), 393(10), 248(12), 205(12), 191(45).

3.12.7 GSH 41

m.p. - 122 - 123°C. U.V. λmax ($CHCl_3$): 239.5, 289.1nm.
^1H nmr ($CDCl_3$): δ2.83 (1H,dd., J=3,14Hz), 3.13(1H, dd, J=13,4Hz), 3.82(3H,S), 3.92(3H,S), 3.94(3H,S), 5.36(1H,dd,J=3,10Hz), 6.93(1H,dd, J=9), 7.01(2H,m), 6.07 (1H,d,J=2Hz), 6.08(1H,d,J=2Hz).
^{13}C nmr ($CDCl_3$): δ43.23(C3), 55.58 ($0CH_3$), 55.89 (2x $0CH_3$) 79.12 (C2), 94.18 (C8), 95.03(C6), 103.03 (C10), 109.33 (C2), 111.10 (C5'), 118.75 (C6'), 130.71 (C1'), 149.23 (C3'), 149.45(C4'), 162.71(C9), 164.06 (C5), 167.89 (C7), 195.81 (C4).
EIMS m/z (rel. int.) $[M^+]$ 330 (20), 329.1025 $[M-1]^+$($C_{18}H_{17}O_6$ requires 329.1020), 298(2), 193(10), 164(60), 151(100), 138(15), 103(15), 91(25), 77(25).

3.12.8 GSH 49

^1H (nmr) (d_5-pyridine). See table 28, p.188.
^{13}C (nmr) (d_5-pyridine). See table 29, p.189.
CIMS m/z (rel. int.) 457.3680 (15) $[M+]^+$ ($C_{30}H_{49}O_3$ requires 457.3669), 439 (95), 411 (50), 393 (5), 249(40), 205(30), 191(100).

3.12.9 GS 13

Amorphous powder, m.p. 315-318°C.

^{1}H nmr (pyridine-d_5): δ0.67 (3H,S), 0.69(3H,S), 0.89 (6H,d,J=1Hz), 0.95 (6H,S), 4.0-4.63 (sugar protons), 5.36 (1H, m).

13C nmr (pyridine-d5) sugar carbons - δ102.62 (C1'), 75.40 (C2'), 78.16 (C3'), 71.75 (C4'), 78.56 (C5'), 62.88 (C6').

Aglycone carbons - δ12.03, 12.20, 12.59, 19.27, 19.48, 20.04, 21.34, 21.53, 23.45, 24.57, 25.77, 26.42, 29.51, 30.31, 32.11, 32.23, 37.53, 39.39, 40.00, 46.09, 50.39, 56.29, 56.88, 78.67, 121.99, 129.51, 138.89, 140.96.

FABMS m/z: 411 [M-glc], 383, 355, 303, 248, 217.

3.12.10 GSA 5

Needles from methanol/chloroform mixture, m.p. 295-297°C.

IR Vmax 3450, 3330, 2926, 2854, 1698, 1462, 1377, 1304, 1268, 1208, 1190, 1076, 1038, 1016, 973, 722 cm^{-1}.

^{1}H nmr (pyridine-d_5): δ0.61(3H,S), 0.64(3H,S), 0.69(3H,S) 0.70(3H,S), 0.71(3H,S), 0.88(3H,S), 2.96(1H,dd,J=4,10Hz) 3.57 (1H,d,J-10.4Hz), 3.86(2H,m), 5.15(1H,t,J=3Hz).

^{13}C nmr (pyridine-d_5): δ11.61, 14.45, 15.97, 17.07, 22.18, 22.24, 22.33, 24.64, 26.16, 26.82, 29.43, 31.45, 31.69, 31.72, 32.69, 35.71, 37.26, 38.24, 40.48, 40,66, 41.37, 44.93, 45,13, 46.63, 47.08, 66.37, 71.87, 121.06, 143.33, 178.72.

CIMS m/z (rel. int.): 472(2) [M^+], 455(40), 437(70) and EIMS m/z (rel. int.) 248(20), 203(100), 189(15), 133(30).

GSA 5 Diacetate

Melting point. 184°C

IR Vmax (Nujol): 2925, 2854, 1739, 1696, 1463, 1376, 1376, 1245, 1034, 920, 735 cm^{-1}.

^1H nmr ($CDCl_3$): δ0.68(3H,S), 0.76(3H,S), 0.84(3H,S), 0.86(3H,S), 0.90(3H,S), 1.05(3H,S), 1.96(3H,S), 2.01(3H,S), 2.75(1H,dd,J=4,10Hz) 3.63(1H,d,J=11.6Hz), 3.80(1H,d,J=11.6Hz), 4.73(1H,m) 5.21(1H,t,J=3Hz).

^{13}C ($CDCl_3$): δ12.96, 15.70, 16.96, 17.77, 20.84, 21.13, 22.82, 23.25, 23.45, 25.67, 27.47, 30.54, 32.12, 32.29, 32.94, 33.64, 36.68, 37.55, 39.12, 40.38, 40.84, 41.38, 45.65, 46.38, 47.55, 47.69, 65.29, 74.38, 122.33, 143.47, 170.61, 170.91, 183.37.

CIMS m/z (rel. int.): 574(20) $[M+NH_4]^+$, 510(18), 497(60), 451(22), 437(60), 248(100), 203(60), 189(45), 133(20).

3.12.11 GSA 8

Needles from methanol/chloroform mixture, m.p. 273-275°C

^1H nmr (pyridine-d_5): δ0.56(3H,S), 0.64(3H,S), 0.75(3H,S), 0.90(3H,S), 0.94(3H,S), 1.02(3H,S), 1.25(3H,S). 2.97 (1H,dd, J=4,9Hz), 3:12(1H,d,J=4Hz), 4.06(1Hd,t,J=3Hz), 5.18(1H,t,J=3Hz).

^{13}C nmr (pyridine-d_5): δ15.94, 16.81, 17.50, 17.96, 23.08, 23.29, 25.57, 27.58, 29.59, 30.29, 32.60, 33.55, 36.70, 38.14, 39.23, 41.36, 41.66, 44.26, 45.80, 46.02, 47.85, 55.30, 70.76, 77.68, 122.03, 144.19, 179.54.

CIMS m/z (rel. int.): 472(5) $[M^+]$ 455(50), 437(75), 409(10),

EIMS, m/z (rel. int.): 248(100), 203(90), 189(20), 133(40).

3.12.12 GSA 11

White crystals from methanol/chloroform mixture, m.p. 320-325°C (lit. 328-330°C)[115].

IR Vmax 3465, 3280, 2925, 3280, 2925, 2854, 1677, 1462, 1377, 1046, 722 cm^{-1}

^1H nmr (pyridine-d_5): δ0.56(3H,S), 0.64(3H,S), 0.75(3H,S), 0.90(3H,S), 1.02(3H,S), 1.25(3H,S), 2.0(1H,dd,J=2,11.4Hz), 2.96(1H,dd,J=4,9Hz), 3.98(1H,d,J=11.0Hz), 3.83(1H,d,J=11Hz), 3.93(1H,d,J=4Hz), 4.18(1H,dt,J=3.3Jz), 5.17(1H,t,J=3Hz).

^{13}C nmr (pyridine-d_5)): δ14.56, 17.25, 17.50, 18.28, 23.67, 23.73, 23.98, 26.21, 28.24, 30.92, 32.99, 33.20, 34.18, 37.22. 39.86, 41.99, 42.31, 42.42, 44.88, 46.40, 46.64, 48.13, 48.55, 67.62, 71.60, 72.99, 122.72, 144,84, 180.19.

CIMS m/z (rel. int.): 489(10) $[M]^+$ (acc. mass 489.3580), 471(100), 453(85), 435(30), 407(20), 248(40), 204(40), 191(35), 173(20).

GSA 11 triacetate

Melting point, 248-251°C (Lit 257-259°C)[115].

IR Vmax. 2926, 2854, 1747, 1696, 1463, 1376, 1240, 1159, 1041, 920, 821, 734 cm^{-1}

^1H nmr ($CDCl_3$): δ0.70(3H,S), 0.84(3H,S), 0.86(3H,S), 0.96(3H,S), 1.04(3H,S), 1.16(3H,S), 1.94(3H,S), 1.99(3H,S), 2.01(3H,S), 2.78(1H,dd,J=4,9Hz),

3.61(1H,d,J=11Hz), 3.80 (1H,d,J=11Hz), 4.86(1H,d,J=4Hz), 5.21(1H,t,J=3Hz), 5.34(1H,bd,J=3Hz)

^{13}C nmr ($CDCl_3$): δ13.67, 16.38, 17.13, 17.53, 20.71, 20.83, 21.19, 22.64, 23.31, 23.79, 27.37, 30.56, 32.08, 32.31, 32.95, 33.64, 36.57, 39,23, 39.95, 40.80, 41.41, 41.52, 45.59, 46.38, 47.54, 48.07, 65.42, 69.52, 71.86, 122.20, 143.62, 170.03, 170.28, 170.79, 183.69.

CIMS m/z (rel. int.): 632(100), $[M+NH_4]^+$, 586(10), 555(20), 513(10), 435(10), 248(50), 204(25), 81(10).

3.12.13 GSA 16

Erubigenin: White powder, m.p. 300-302°C, $[\alpha]^{22}_D$ = +76(C,3.0, MeOH).

IR Vmax (Nujol): 3375, 3300, 2924, 2854, 1696, 1462, 1378, 1238, 1021 cm-1

^1H nmr (d_5-pyridine) see table 32, p.207.

^{13}C nmr (d_5-pyridine) see table 33, p.210.

CIMS m/z (rel. int.): 489(20) $[M+1]^+$, 471(18), 453(40) 435(20), 407(10), 338(10), 248(24), 203(30), 191(30), 177(45), 133(100), 73(45), 58(35).

Triacetate: m.p. 206-208°C, $[\alpha]^{22}_D$ = + 90 (C, 2.0, $CHCl_3$).

IR Vmax (Nujol): 3300-3200, 2924, 2854, 1742, 1697, 1462, 1377, 1234, 1046, 909, 803, 736 cm^{-1}

^1H nmr ($CHCl_3$-d): δ0.68(3H,S), 0.84(3H,S), 0.86(3H,S), 0.90(3H,S), 1.05(3H,S), 3.96(1H,d,J=11.8Hz), 4.15(2H,d,J=13.7Hz), 4.35(1H,d,J=12Hz), 5.22(1H,bt, J=3-5Hz).

^{13}C nmr ($CHCl_3$-d): see table 33, p.210.

CIMS (NH_3) m/z: 632.416 $[M+NH_4]^+$ ($C_{36}H_{58}O_8N$ requires 615.338), 569, 555, 509, 435, 248, 204, 192, 163, 119, 85, 58 (100).

Methyl ester

^1H nmr (d_5-pryridine): δ0.52 (3H,S), 0.55 (3H,S), 0.58(3H,S), 0.70(3H,S), 0.83(3H,S), 3.37(3H,S), 3.68(1,H,m), 3.92(1H,d,J=10.9Hz), 4.05(1H,m), 4.32(1H,d,J=11Hz), 5.06 (1H,bt, J=34Hz), 3(0H) at δ5.17, 5.97 and 6.39.

^{13}C nmr (d_5-pyridine): see table 33, p.210.

CIMS m/z: 503 $[M+1]^+$, 453, 429, 393, 355, 295, 281, 262, 221, 203, 147, 84(100), 73, 56.

Methyl ester triacetate

^1H nmr ($CHCl_3$-d): δ0.65 (3H,S), 0.83(3H,S), 0.86(3H,S), 0.91(3H,S), 1.04(3H,S), 1.94(3H,S), 1.99(3H,S), 2.02(3H,S), 3.55(3H,S), 2.95(1H,d,J=11.8Hz), 4.15(2H,d,J=10.3Hz), 4.36(1H,d,J=12Hz), 5.22(1H,bt, J=3Hz).

^{13}C nmr ($CHCl_3$-d) - see table 33, p.210.

EIMS m/z 628.397 $[M^+]$ ($C_{37}H_{56}O_8$ requires 628.396), 568, 503, 429, 355, 262, 221, 203, 189, 105, 83(100), 47, 43.

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