SECONDARY METABOLITES OF MARINE ORGANISMS FROM THE INDIAN OCEAN AREA

A THESIS SUBMITTED TO THE GOA UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MARINE SCIENCE

BY

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DEDICATED TO

MY PARENTS

STATEMENT REQUIRED TO BE SUBMITTED UNDER ORDINANCE 19.8 OF THE GOA UNIVERSITY

The thesis is based entirely on the experimental work carried out by me under the guidance of Dr.S.Y.Kamat. The literature concerning the problems investigated has been surveyed and a list of references is appended. Due acknowledgement has been made whenever outside facilities have been availed of.

(S.Y.KAMAT)

Research Guide

(P.S.PARAMESWARAN)

Candidate

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GENERAL REMARKS

1. All the figure numbers, table numbers, structure numbers and references in a chapter/section refer to that particular chapter/section only.
2. Petroleum ether refers to the fraction boiling between the the range 60-80°C unless otherwise mentioned.
3. Silica gel used for column chromatography was of 60-120 mesh size and activated at 110°C for 5 hours before use.
4. Thin layer chromatography was done on glass plates (20X20 cms) coated with 0.25 mm layer of TLC grade silica gel containing 13% $CaSO_4$ as binder. The plates were activated at 110°C for 1 hour before use.
5. Spectral data reported for different compounds were mainly obtained through the courtesy of various institutions and are duly acknowledged. The details regarding the instruments and the experimental conditions employed are given wherever possible.
6. The chemical shifts in the ^{1}H and ^{13}C NMR spectra are expressed in 'δ' or 'ppm' dounfield to TMS, which is used as the internal standard.
7. The compounds were identified from a comparison of their spectral data with those of similar or identical compounds reported in literature. Whereever possible, their molecular formulae were arrived at from elemental analysis or HREIMS.

INTRODUCTION

It is widely believed that life originated first in sea, which subsequently migrated onto land. Whatever be the truth behind this, it is a fact that all ancient civilizations have sprung up alongside coastal areas or rivers. Faced with ever increasing population and depleting resources on land, man is, now once again forced to look into the potentialities of ocean to provide him with adequate food, energy, minerals and medicines. This is only natural, given the fact that ocean, our immediate neighbour, comprises of more than two-third of earths crust. It is believed that about 80% of earths animal life is in the ocean, numbering about 5-10 lakh species, distributed among 30 phyla.

Since time immemorial man was familier with several classical drugs of marine origin. For example, carrageenan, isolated from red seaweeds was in use as a laxative since Roman times. Similarly, cod liver oil from the fish *Gadus morrhus* is known as a good source of vitamin A and D for a long time. Also in use were spermacetti, from the head of sperm whale and protamine sulfate from the sperm of salmon[1].

Inspite of these early favourable leads, systematic studies in marine pharmacology / marine natural products could be started only about three decades ago. Many are the reasons for this rather late awakening. Perhaps the most important among them is the difficulty in collecting these samples from a relatively hostile environment and ensuring their repeat

collections as and when required. It is estimated that greater than 10,000 terrestrial plants out of a total number of 4,00,000 have been systematically studied chemically and / or pharmacologically. This figure might be even many times more if we consider the large number of plants that might have been less than thoroughly screened and prematurely rejected. As compared to this, only less than 1% of marine organisms have so far been similarly examined[1].

Recent advances in sample collection techniques including SCUBA diving and use of submercibles as well as the modern developments in extraction, isolation and purification of compounds and the ability of chemists, aided by modern spectroscopic aids such as IR, UV, NMR and MS to determine the structures of compounds available in submilligram quanties have lent a tremendous boost for research in this area. This revival was pioneered by Bergmann and Nigrelli during 1950's. The former isolated several cytotoxic nucleosides[2] which eventually led to the development of anti-leukemic drug Ara-C and antiviral drug Ara-A, while the latter isolated several glycosides from various sea cucumbers and studied their biological properties. Another important compound is nereistoxin, a natural insecticide, isolated from the worm *Lumbriconereis heteropoda*. It was first isolated in 1934 by Nitta, who also determined its elemental composition as $C_5H_{11}NS_2$. It was subsequently reisolated by Hashimoto and Okaichi in 1960, who proposed its correct structure. The

structure was finally confirmed by its synthesis by Konishi[3]. Later a synthetic analog of this compound under the brand name "cartap" was marketed as an insecticide against rice stem borers by Takeda Chemical Industries Ltd. during 1970's. This insecticide is harmless to warm-blooded animals.

Besides, several compounds with useful biological properties have been isolated from various marine plants and animals during the past three decades. Many among them have since been accepted as useful drugs against various ailments. This includes compounds such as cephalosporin C, kainic acid, carrageenan, tetradotoxin, saxitoxin, tedanolide, halitoxin, *etc*. Among these, cephalosporin C is an antibacterial compound active against a number of penicillin-resistant *Staphylococci* and several Gram negative bacteria. It is currently being marketed by Lilly Pharmaceutical Co. under the brand name Cephalothin[4]. Similarly, kainic acid in combination with santonin is marketed under the name Digesan by Takeda Pharmaceutical Co. Ltd., Japan as a useful anthelmintic and vermifuge against tapeworm *Taenia* sp., the parasitic round worm *Ascaris Lumbricoides* and the whipworm *Trichuris trichura*. Carrageenan has been found to cause growth of connective tissues. Sodium alginate from the brown seaweeds is able to remove radio-strontium (^{90}Sr) from the body without affecting calcium metabolism. Tetrodotoxin and Saxitoxin are potent neurotoxins, toxic even at very low doses. Saxitoxin is 10^4 times more potent than cocaine in

blocking action potentials. Its lethal dosage for man, based on studies of accidental death may be as low as 0.54 ng. The potent cytotoxin, tedanolide, isolated from a sponge *Tedania* sp. inhibits tumor cells at a concentration of $2.5X10^{-4}$ (KB) and $1.6X10^{-5}$ µg/ml (PS) arresting cell division and accumulating the cells in the S-phase[4-5]. Another interesting compound is halitoxin, isolated from the sponges *Haliclona rubens, H.viridis and H.erina*. It is a biopolymer of 1,3-dialkylated pyridine and exhibits potent cytolytic, hemolytic and toxic properties towards mice[6].

The list is thus endless and is ever growing as vouched by the plethora of publications appearing on these topics lately. As part of our ongoing program on "Drugs from sea" we have screened a large number of marine organisms from Indian waters for various biological properties and also studied in detail the chemical constituents of a few active ones among them as described in TABLE 1 below.

TABLE 1: LIST OF ORGANISMS SELECTED FOR DETAILED CHEMICAL INVESTIGATIONS AS PART OF THIS STUDY AND THEIR PHARMACOLOGICAL PROPERTIES.

Organism	Pharmacological Activities
Padina tetrastromatica (alga)	Spasmogenic, antifertility, hypotensive & antiamoebic
Ircinia ramosa KELLER (sponge)	Antiviral, CNS stimulant & diuretic
Haliclona sp (sponge)	Antiviral & antimicrobial
Lobophytum strictum (soft coral)	Hypotensive & antifertility
Actinopyga mauritiana (Sea cucumber)	Hypotensive, antimicrobial, hypoglycaemic & antiamoebic
Tedania anhelans (sponge)	Spasmogenic & Hypoglycaemic

During our systematic chemical investigations, several secondary metabolites, such as fatty acids, sterols, terpenoids, alkaloids, peptides and a reduced sugar have been isolated from these plants and animals by using modern chromatographic techniques (HPLC, Gel chromatography *etc.*,) and their structures established from their spectral data (IR, UV-Vis, NMR & MS) and chemical transformations wherever necessary. As part of this study, we also developed a new method for quantitative analysis of fatty acid mixtures

involving their facile derivatisation into the respective *p*-nitrobenzyl esters followed by NCI mass spectral analysis. The results of these studies are described in detail in the following chapters.

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CHAPTER 1

SECTION 1

NOVEL PHYSICO-CHEMICAL METHODS FOR STRUCTURE DETERMINATIONS

Unlike the terrestrial organisms, studies on marine flora and fauna are beset with two critical problems: inadequate supply of organisms and low probability of their repeat collection for subsequent studies. These limitations effectively blocked any meaningful research on marine natural products for a long time. The classical way of structure elucidation included elaborate chemical degradations / derivatizations which required gram-quantities of materials. However, with the advancement in modern spectroscopic techniques, especially NMR and MS, it is now possible to determine not only the structure, but also the complete three dimentional picture or configuration of compounds available in mg levels. In this chapter, some novel mass spectral techniques developed in order to determine the chemical structures of compounds available only in submilligram levels will be described.

CHEMICAL IONISATION MASS SPECTROMETRY

CHEMICAL IONISATION MASS SPECTROMETRY (CIMS), which uses relatively milder ionisation energies as compared to ELECTRON IMPACT MASS SPECTROMETRY (EIMS) is very useful for determining the molecular weights of thermally labile and nonvolatile compounds. Earlier Bose *et.al.*, had used this technique quite extensively in natural products research,

especially in studying polar, underivatised compounds of biological interest[1]. They have observed that negative ion CIMS is much more sensitive than positive ion CIMS for many compounds. Subsequently, these workers improved this method and obtained the chloride / bromide adduct ions of various molecules by adding traces of NH_4Cl or NH_4Br to the samples before introducing them into the mass spectrometer. These pseudomolecular ions could be easily identified due to the presence of the corresponding isotope peaks. Thus, chloride adduct ion will show two molecular ions differing by 2 units corresponding to $[M+^{35}Cl]^-$ and $[M+^{37}Cl]^-$ in the ratio 3:1. Similarly, use of NH_4Br would generate twin peaks due to $[M+79]^-$ and $[M+81]^-$ respectively of equal intensity. When neither of these compounds are used, strong $[M-H]^-$ ions are observed. The CIMS may be carried out using several reagent gases; *viz.*, ammonia, methane, argon, *etc.* It has been observed that if ammonia is used in the positive CIMS it gives rise to strong $[M+NH_4]^+$ pseudomolecular ions whereas CH_4 or Ar generate strong $[M+H]^+$ ions. Another interesting observation was that use of Ar as reagent gas while little NH_4Cl is added to the sample gives rise to fragmentation, akin to EIMS[2]. This method becomes particularly valuable for observing McLafferty type fragmentations using CIMS instruments. Any uncertainity in the assignment of $[M+NH_4]^+$ peak could be checked by recording the spectrum using $^{15}NH_4Cl$, thereby exchanging the $[M+18]^+$ ion with $[M+19]^+$ ion.

THIN LAYER CHROMATOGRAPHY-CHEMICAL IONISATION MASS SPECTROMETRY (TLC-CIMS)

Mass spectrometer is a powerful tool in the research on marine natural products. It gives accurate information about the molecular weights of organic compounds present as little as a few µg. Different methods such as electron impact mass spectra (EIMS), fast atom bombardment mass spectra (FABMS), field desorption mass spectra (FDMS), and chemical ionisation mass spectra (CIMS) are employed for ionising a given molecule depending on its nature and the type of information required. Among these, EIMS uses electron beams of about 70 ev for ionising molecules while all other techniques use much lower energy and are termed 'soft ionisation methods'. Consequently, EIMS yields relatively smaller molecular ions and more of fragment ions and are more suited for studying relatively less polar compounds. On the other hand, the other methods are more useful in studying polar compounds such as sugars, glycosides, proteins and peptides. Normally samples, as little as a few µg or less are introduced in small vials into the mass spectrometer. However, for meaningful results, it is imperative that these samples be very pure. A further instrumental development was in coupling a gas chromatograph with a mass spectrometer, in what is popularly known as GCMS. This enabled organic chemists to analyse impure mixtures such as fatty acid esters, sterols, *etc.* The major drawback of this method is that it involves purification using GC, which,

due to the high temperatures involved in separation, limits its use to relatively low polar, heat-stable compounds. With the development of new LCMS techniques, these drawbacks were mostly overcome. This method enables initial LC purification of impure samples and recording the mass spectra of purified compounds immediately. But modern LCMS instruments are very costly, which, at times prevent its widespread use.

As part of our studies on marine natural products, we happened to deal with both pure and impure compounds on mg and sometimes even sub mg levels. GCMS could not be adapted in many such situations due to sample volatility problems. This led us to develop a new instrumental technique for the routine analyses of these samples. The method described below involves introducing samples from TLC spots directly into a mass spectrometer and recording their mass spectra[3]. Thus, this method can be considered as complimentary to GCMS and as a poor man's substitute to the LCMS.

In this method, TLC of a mixture of compounds (synthetic or natural products) is carried out in the usual manner. The chromatogram is later visualized in an iodine chamber or under ultra violet light. About 2-5 mg of carbowax 20 (poly-ethylene glycol with very high molecular weight), is placed at the tip of a microvial which is in turn attached to the end of a heated solid probe of the mass spectrometer. The small flake of the carbowax is then warmed to 60°C in order

to make it soft and sticky. Touching the TLC spot of interest earlier visualized using iodine vapour or under UV light with this sticky wax enables efficient transfer of the compound of interest from the TLC plate to the microvial which is then introduced into the mass spectrometer with or without NH_4Cl, NH_4Br, *etc.* Using this technique intense CI mass spectra of several compounds have been obtained. Contamination with background peaks due to carbowax can be avoided by keeping the ion source temperature below 220°C. Some of the mass spectra obtained in this way will be described in the following chapters.

TLC-O_3-CIMS

Next, we decided to study the determination of the position of double bonds in unsaturated compounds, especially in long chain compounds such as fatty acids and alcohols, their esters, waxes, *etc.* This was always very difficult and laborious. The classical way of doing this involves ozonolysis, decomposition of the ozonides into the respective aldehydes, ketones or acids and their purification prior to identification. Evidently, this method cannot be adopted while working with limited quantities or in routine analyses.

With this view, we extended the TLC-CIMS technique for obtaing information about the position of unsaturations in the following manner[4]. Initially the molecular weight of the compound under a TLC spot is determined as described above. Later another spot of the same compound is exposed to ozonised air for 1-2 min. The unsaturated compounds on the TLC plate get ozonised in this way. Subsequently the TLC-CIMS of the spot is recorded in the usual manner. Inside the Mass Spectrometer the ozonides decompose into the respective carboxylic acids or ketones depending on the substitution pattern on the vinyl carbons. Significantly this reaction, which normally is catalysed by reagents such as dimethyl sulphide in the laboratory seems to be catalysed within the Spectrometer by the traces of water present in the silica gel. The carboxylic acids, generated in this manner, produce intense pseudo molecular ions in the negative CI mass spectra. We have found this method to be highly sensitive and very easy to perform. Satisfactory results could be obtained for samples even as low as 200 µg. Oleic acid methyl ester upon this treatment yielded azelaic acid and zuberic acid ester. Petroselinic acid ester, upon similar treatment yielded the corresponding C_6 monocarboxylic acid and the C_{11} dicarboxylic acid monoester. Cholesterol upon TLC-ozonolysis followed by the negative CIMS in presence of traces of NH_4Cl provided intense peaks at m/z 451 and 453 in the ratio 3:1. This corresponded to the combined weight [Cholesterol+O_3+Cl-H_2O]$^-$, which is expected from trisubstituted double bond

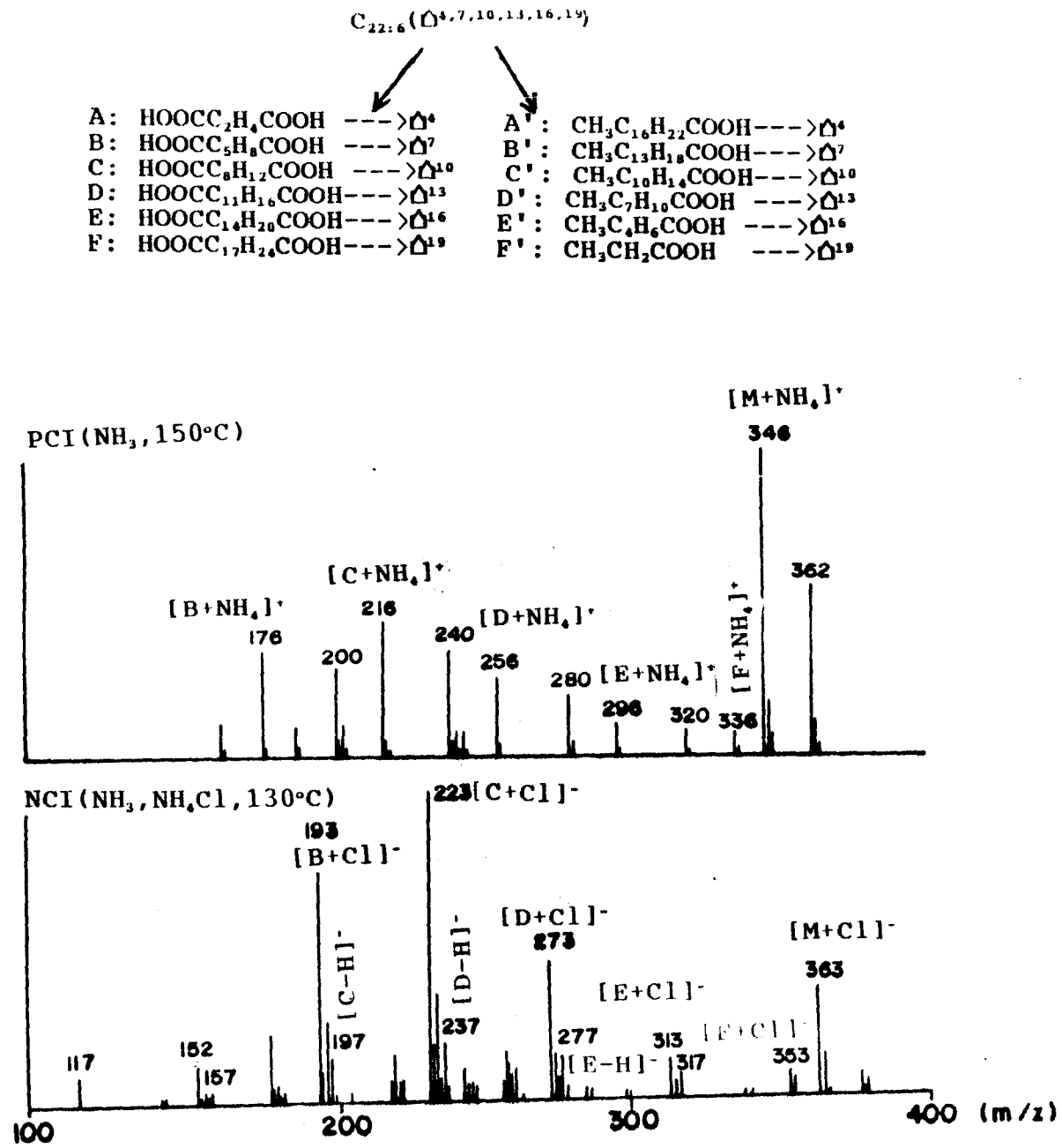

Fig. 1 TLC - O_3-CIMS of Docosahexaenoic acid.

forming part of a ring system. We have carried out this reaction further, to locate the positions of double bonds in polyenoic fatty acid esters. The NCIMS of docosahexaenoic acid methyl ester is illustrated in FIG 1.

Having successfully adapted this technique to individual compounds, we next attempted to study mixtures of fatty acids, and sterols without prior purification. All organisms contain such mixtures and it was construed as worthwhile to develop simple analytical methods for their analysis. The immediate problem as far as the fatty acids were concerned was the development of a proper technique to separate them into different spots on a TLC plate. These compounds could subsequently be analysed using the TLC-CIMS technique described earlier. Normally these fatty acids or their methyl esters are inseparable on ordinary TLC plates. They can, however, be separated on a TLC plate doped with $AgNO_3$. This argentation TLC is in fact a very powerful technique in separating close homologues such as mixtures of fatty acids, sterols etc. However, we encountered problems in visualizing the separated spots on these TLC plates. After chromatography we noticed that the entire plate turned black in colour making it impossible to visualize the spots either by iodine vapours or under UV lamp. To overcome this problem we decided to dope only half of the plate with $AgNO_3$. The other half was left intact. On the former side was spotted a mixture of unsaturated fatty acid esters, *viz.*, methyl esters of

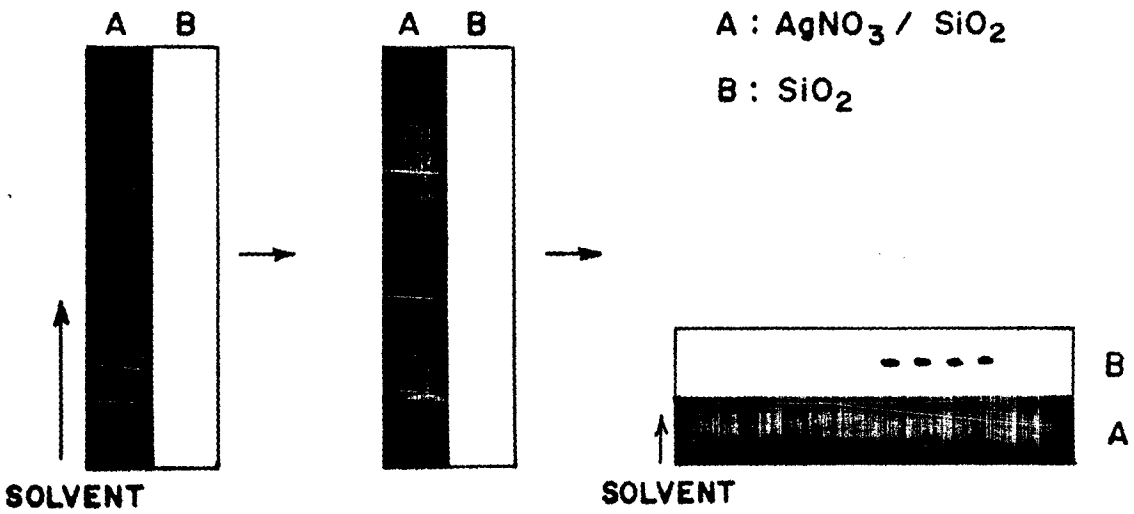

Fig. 2 Separation of a mixture of fatty acid methyl esters ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) on semi-impregnated TLC plate.

Palmitic, Stearic, Oleic, Linoleic and Linolenic acids (16:0, 18:0, 18:1, 18:2 and 18:3 respectively) and chromatographed using 15% EtOAc in pet.ether. Subsequently the plate was turned by 90° and redeveloped in a more polar solvent system such as 40-50% EtOAc-pet.ether in order to transfer these spots into the non-doped silica gel portion. They were then visualized in an iodine chamber and analysed by TLC-CIMS and TLC-O_2-NCIMS techniques. As evident from FIG 2, four separate TLC spots could be identified on the TLC plate. From the TLC-PCIMS it was clear that the least polar (uppermost) spot contained both Palmitic and Stearic acid esters while the most polar (lowest) spot contained linolenic acid ester. The other two spots of intermediate polarity were due to oleic and linoleic acid esters in the order of polarity. Subsequent TLC-O_2-NCIMS revealed the position of the double bonds in oeic acid at C-9 position. However the results with the polyenoic acids were not very satisfactory. This could be probably due to the spreading of these acids on the TLC plates, as a result of the double development or due to the masking of some of the double bonds by iodine vapours, which might have hindered the formation of the ozonides.

CHAPTER 1

SECTION 2

NEW METHOD FOR ANALYSIS OF FATTY ACID MIXTURES

CONVENIENT PREPARATIONS OF FATTY ACID DERIVATIVES FOR ANALYSES USING HPLC AND NEGATIVE CIMS TECHNIQUES.

Fatty acids and their derivatives including triglycerides, wax esters *etc.*, are important cell constituents of all living organisms. Besides serving as an efficient energy storage, many of these compounds, especially the polyunsaturated acids are important for the proper growth and normal physiological functioning of these organisms and are termed essential fatty acids. Some of these, especially arachidonic acid and homolinolenic acid also get converted *in vivo* into useful prostaglandin derivatives. Of late, great interest has been generated in determining the fatty acid constituents of various organisms from a chemotaxonomic point of view. The method is widely being used especially in identifying bacteria and other lower organisms where other methods are either cumbersome or impractical. Thus, the analysis of fatty acids is an important area of research in organic, analytical and biochemistry.

However, despite their structural simplicity, facile and accurate analysis of fatty acids in a given mixture has always been a challenge to analytical chemists. The most commonly adopted techniques involve the use of GC and GCMS after suitably derivatising them. These methods are fairly satisfactory on an analytical scale but fare badly when preparative separations involving larger quanties are

required. Besides, the high temperatures used in GC makes it rather suspect while dealing with polyunsaturated acids due to possible thermally induced isomerisations, cyclisations *etc*. These limitations led to the development of an alternate method of separation using HPLC. Here the compounds are separated at room temperature which prevents thermally induced artifact formation. However, for many years chemists could not achieve the required submicrogram level detection by this method. The commonly used RI detector was incapable of reaching these target limits[5,6]. To overcome this, attempts were made to convert them into suitable UV active derivatives and then monitor using a UV detector. To be of wide use this reaction has to be simple and at the same time yield quantitative results. Several attempts have been made in the recent past to find such a facile and reliable method of derivatisation. Earlier studies using 1-Benzyl-3-*p*-tolutriazine[7] and 1-*p*-nitro benzyl-3-tolutriazine[8] had yielded derivatives which brought down the detection limit to 1-10 nanogram levels in LC. However the necessity to vent the N_2 gas evolved during the reaction and the use of expensive reagents which has to be used in large excess for complete reaction prevented this technique from being universally accepted. Subsequent attempts were aimed at nucleophilic displacement of an active halogen atom by the respective carboxylate anion[9]. Cooper and Anders used this approach and derivatised fatty acids with 2-Naphthacyl bromide in DMF in presence of excess quantities of N,N-Diisopropyl ethyl amine[10]. But, the

use of excess amount of organic base was a disadvantage in effecting complete derivatisation as well as during the following work up. Subsequently, this reaction was improved upon by Durst *et.al.*, who derivatised fatty acids with ß,ß-dibromoacetophenone in an aprotic solvent such as CH_3CN in presence of KOH and a crown ether[11]. However, the reaction has to be carried out in extremely dry solvents and the pH of the reaction mixture scrupulously controlled to phenolphthalein end point using HCl-MeOH in order to prevent the reverse hydrolysis of the ester. These derivatives had a molar absorptivity of 17200 $mole^{-1}$ cm^{-1} in chloroform solution, permitting detection of samples as low as $0.52X10^{-6}M$. Recently Nagamuna *et.al.*, have found that S-bromomethyl-7-methoxy-1,4-benzoxazin-2-one can yield the corresponding UV active esters of fatty acids in a facile manner. The reaction was carried out at ambient temp. in CH_3CN in presence of K_2CO_3 and the crown ether, 18-crown-6[12].

The discovery that *p*-nitrobenzyl esters of fatty acids, prepared by treating the fatty acids with *p*-nitrobenzyl bromide under the same reaction conditions as above, resulted in several hundreds times enhancement of carboxylate anions in negative CIMS with minimal fragmentation and led to a reliable direct mass spectrometric method for fatty acid analysis without having to go through the cumbersome chromatographic procedures such as GC, LC *etc*[13]. Using this method quantitative estimations of various fatty acid

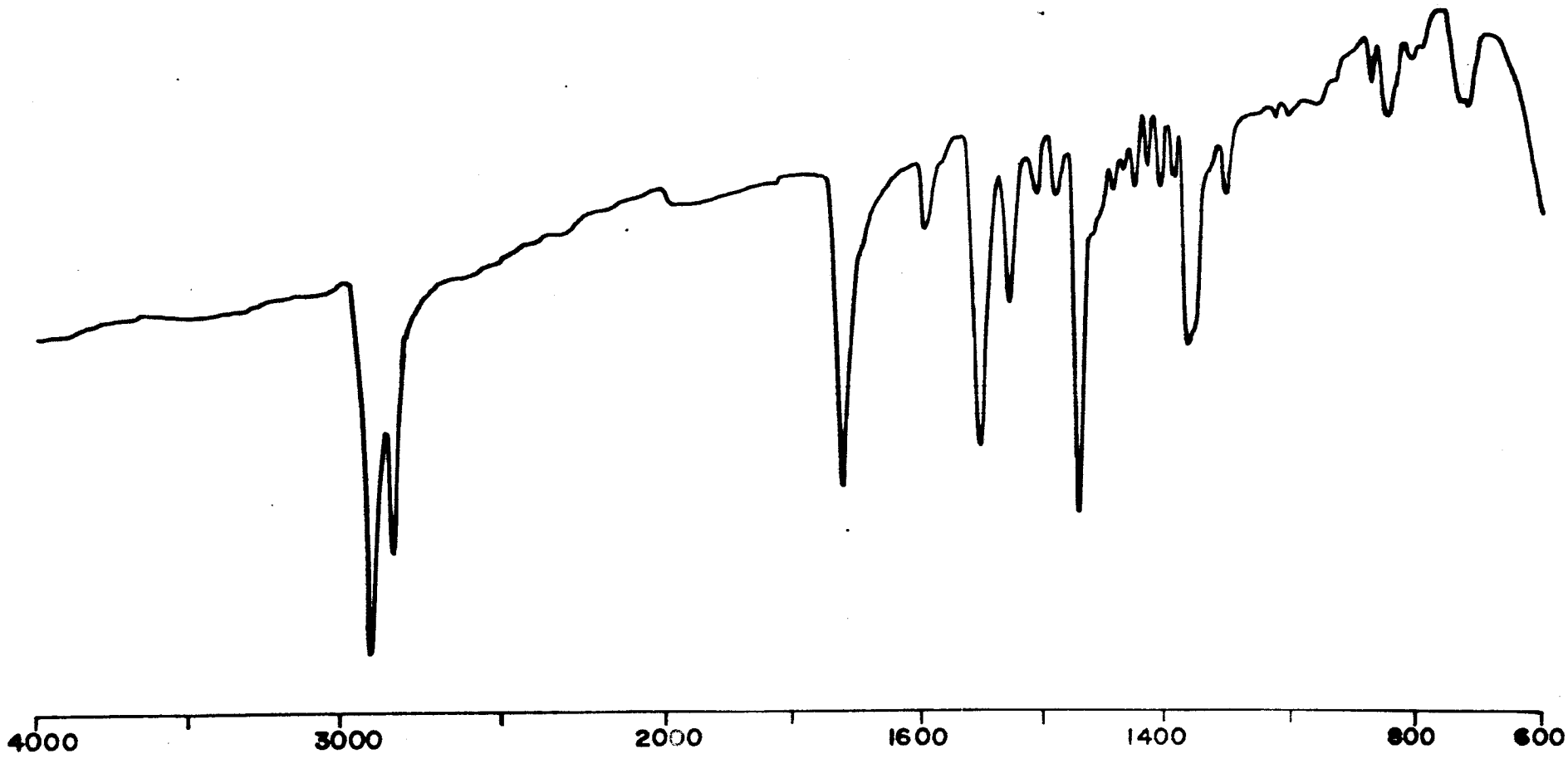

Fig. 3 IR spectrum (Neat) of p-nitrobenzyl stearate

mixtures upto 20 ng level have been achieved very accurately.

In our search for a more efficient, fast, quantitative and yet simple and cost effective method for the analysis of fatty acid mixtures from marine sources, we decided to improve upon the method of preparation of their *p*-nitro and *p*-bromo benzyl derivatives. Earlier Bose *et.al.*, had reported that several organic reactions take place at an accelerated pace when carried out in a microwave oven[14,15]. Normally these reactions are carried out in open vessels and in non-aqueous media. The key to the success of the Microwave Induced Organic Reaction Enhancement (MORE) chemistry is in the use of a solvent that absorbs microwave radiation efficiently and has a suffiently high boiling point and high vapour pressure. We found that *p*-nitrobenzyl and *p*-bromobenzyl derivatives of fatty acids or their mixtures could be prepared in a facile manner by irradiating a solution of their respective bromides and the fatty acids in DMF in presence of solid K_2CO_3 in an Erlenmeyer flask inside a microwave oven for about 4 min under medium energy settings. In order to avoid any possible pressure build up during the reaction, the flask was capped loosely. A beaker filled with water was kept in one corner of the microwave oven to serve as a heat sink. The product was isolated in quantitative yield after the usual work-up of the reaction mixture.

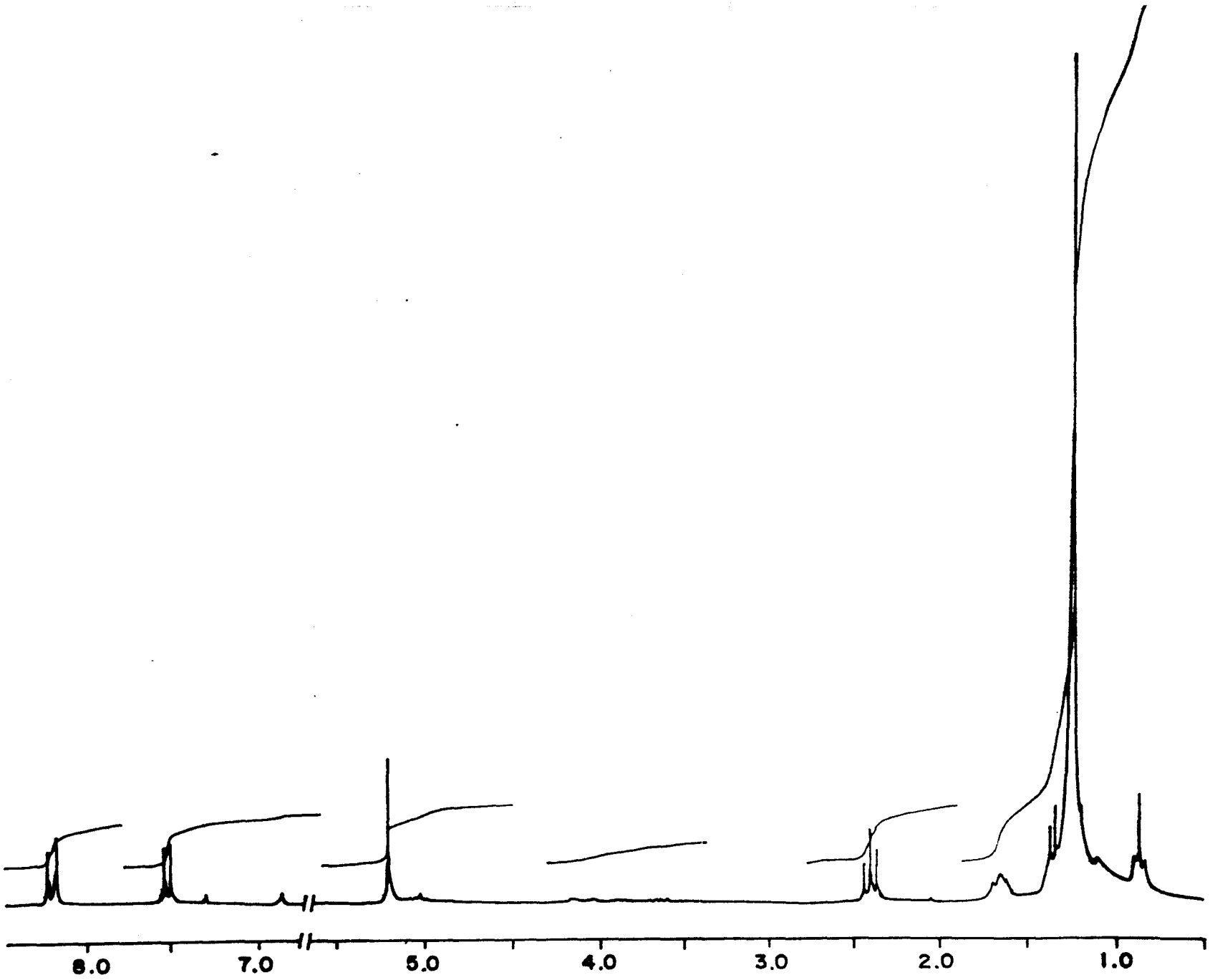

Fig. 4 ^{1}H NMR spectrum of p-nitrobenzyl stearate

Having succeeded in developing the above simplified procedure using microwave oven, we further studied the reaction under conventional conditions. We found that the reaction is complete if the reactants in DMF are heated to 140-150°C for 8-10 min. Heating at lower temp. or with lower boiling solvents or carrying out the reaction for less time failed to yield the desired products. Derivatives of stearic acid and mixtures of acids from several marine organisms were prepared by both the methods described above. The IR, ^{1}H NMR and ^{13}C NMR spectra of *p*-nitrobenzyl ester of stearic acid are given in FIGs 3, 4 and 5 respectively. The ^{1}H NMR spectrum of *p*-bromobenzyl esters of the fatty acids from the sponge *Haliclona* sp is given in FIG 6. These spectra were obtained on the crude reaction mixtures. From them, it is clear that the reaction is quantitative with no artifact formation or migration of double bonds.

The derivatives thus prepared were subsequently analysed in a mass spectrometer. The negative CIMS of *p*-nitrobenzyl derivatives produced intense carboxylate anions ($[RCOO]^-$) and relatively small molecular ions and less fragmentations. The *p*-bromobenzyl derivatives, on the other hand, produced a plethora of several ions and relatively weaker carboxylate anions. Thus, for quantitative work the former product is more desirable as it produces only one prominent ion per molecule as compared to several, produced by the latter. The EI and NCI mass spectral values of *p*-nitro and *p*-bromobenzyl

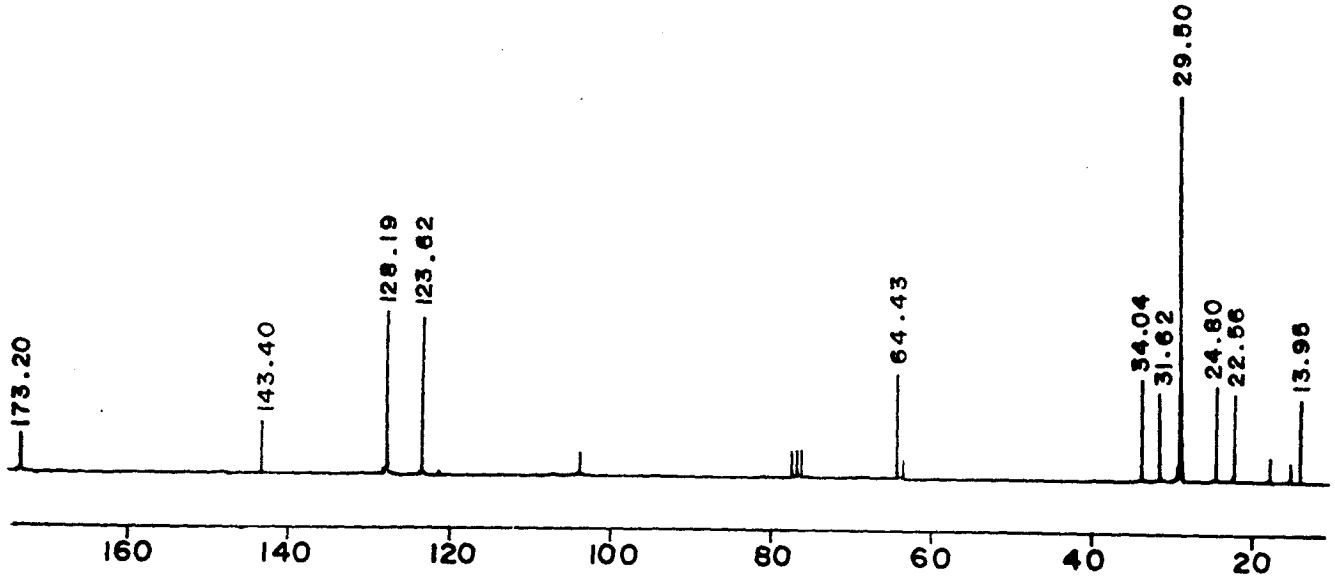

Fig. 5 ^{13}C NMR spectrum of p-nitrobenzyl stearate

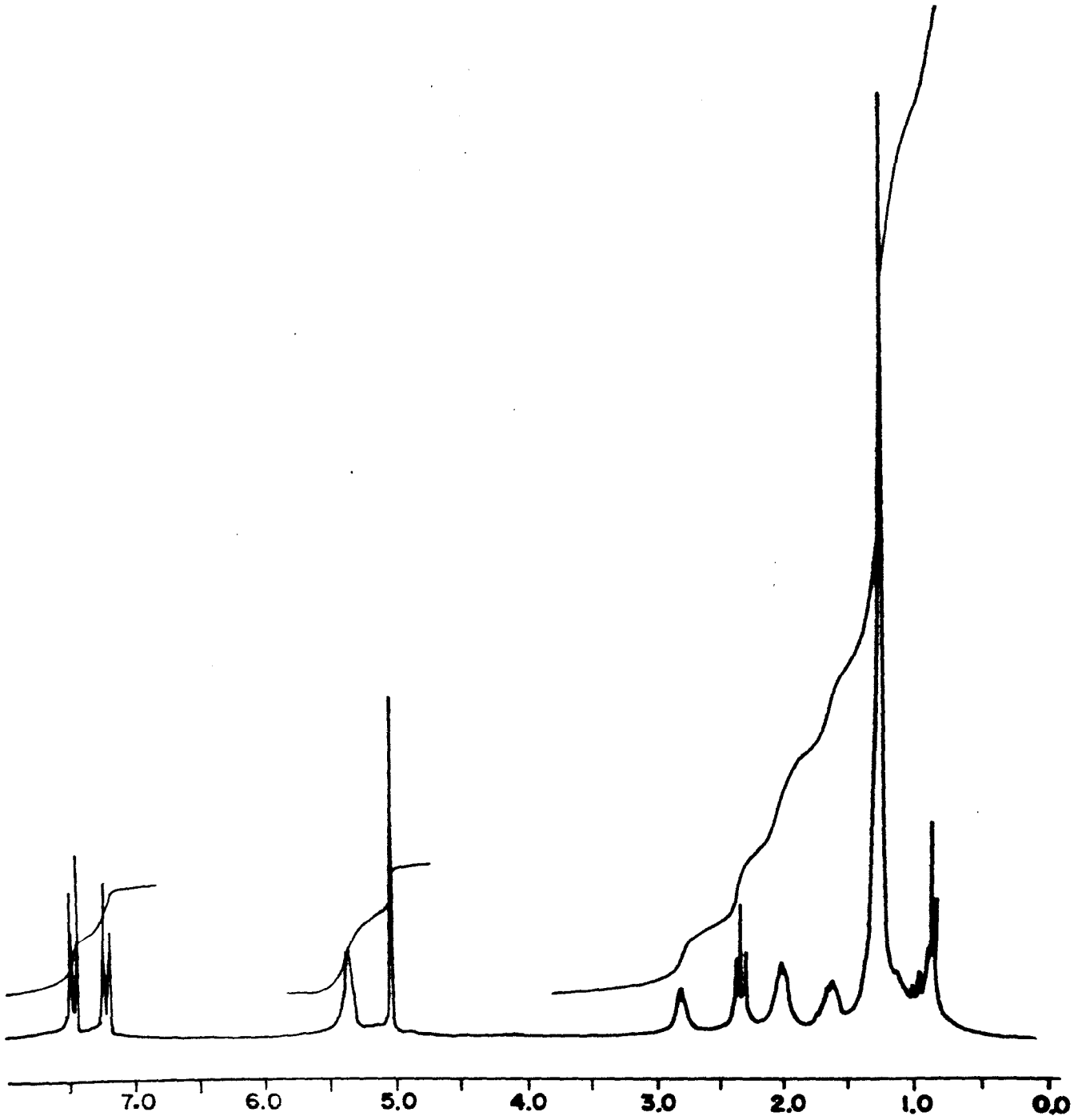

Fig. 6 ^{1}H NMR spectrum of p-bromobenzyl esters of fatty acids from Haliclona sp.

TABLE 1.

EIMS AND NCIMS DATA OF *P*-NITRO AND *P*-BROMOBENZYL STEARATES, (COMPOUNDS A AND B RESPECTIVELY).

Compound A NCIMS, m/z(%)	Compound A EIMS m/z(%)	Compound B EIMS m/z(%)	Compound B NCIMS m/z(%)
418([M-H]$^-$,1)	420([M+H]$^+$,2)	453([M+H]$^+$,1)	510(8)
285(7)	284(1)	394(3)	509(26)
284(55)	283(3)	393(5)	481(2)
283(100)	267(2)	322(15)	307(11)
269(4)	265(5)	284(2)	305(11)
256(6)	137(100)	283(8)	285(4)
255(33)	136(21)	267(4)	284(37)
166(9)	121(16)	265(8)	283(100)
136(2)	111(4)	247(4)	255(8)
89(2)	107(10)	227(3)	81(29)
---	106(12)	188(4)	79(30)
---	83(20)	186(4)	57(62)
---	57(36)	171(54)	---
---	43(70)	169(55)	---
---	---	43(100)	---

esters of stearic acid are given in TABLE 1. The result indicates that the commercial stearic acid sample contains its lower homolog, palmitic acid upto 33%. The method was successfully used in analysing the fatty acids from a seaweed, *Padina tetrastromatica* as well as a sponge *Haliclona* sp. In both these cases, the fatty acid contents were also estimated independently as their methyl esters using capillary GC (SE 30, 12.5 m,N_2, FID). During GC, the fatty acids were identified from a comparison of their retention times with those of standards. Six saturated fatty acid methyl esters, $C_{15:0}$ to $C_{20:0}$ were used as standards for this purpose. The results are presented in TABLES 2 and 3. The fatty acid estimations made by both the methods are in good agreement for the seaweed *P. tetrastromatica*. However, in the case of the sponge, *Haliclona* sp., the values disagree widely for some of the compounds. It may be mentioned here that the seaweed contains mostly saturated or monounsaturated acids, whereas the sponge has a higher content of polyunsaturated acids (PUFAs) as indicated by its ^1H NMR spectrum. Since only six saturated fatty acid standards, $C_{15:0}$ to $C_{20:0}$ were used for the GC analysis, the determination of the structures of PUFAs from the sponge might not have been very accurate. Further, during the GC separation, some of the PUFAs might have undergone artifact formation, rendering this method quite unreliable. Since the reliability of the new CIMS method is well documented[13] and has been further substantiated during these studies as shown here, we believe that this can be the

TABLE 2.

COMPARISON OF THE FATTY ACID COMPOSITION OF THE SEAWEED *PADINA TETRASTROMATICA* (HAUCK) BY GC OF THEIR METHYL ESTERS AND NCIMS OF THEIR *P*-NITROBEZYL ESTERS RELATIVE TO $C_{16:0}$.

GC R_t (min)	Structure	% Comp. by GC	$[RCOO]^-$ ion in NCIMS	% Comp. by NCIMS
3.77	14:0	10.09	227	21
---	15:1	---	239	1
5.10	15:0	2.08	241	5
---	16:1	---	253	24
6.65	16:0	100	255	100
7.70	17:1	1.63	267	2
8.19	17:0	1.04	269	2
---	18:3	---	277	1
---	18:2	---	---	2
9.26	18:1	25.37	281	27
9.84	18:0	5.04	283	5
10.27	19:1	0.50	295	2
12.54	19:0	0.50	297	1
---	20:1	---	309	1
13.08	20:0	1.78	311	1

TABLE 3; FATTY ACID COMPOSITION OF THE SPONGE *HALICLONA* SP. DETERMINED BY GC AND NCIMS METHODS (RELATIVE TO $C_{16:0}$).

R_t (min)	$RCOO^-$ ion	Structure	% by GC	% by NCIMS
3.72	227	14:0	13.51	9.72
4.56	239	15:1	36.22	8.33
5.04	241	15:0	4.32	47.22
6.15	253	16:1	96.22	91.67
6.57	255	16:0	100	100
---	267	17:1	---	16.67
8.12	269	17:0	5.95	18.06
---	277	18:3	---	9.25
8.85	279	18:2	18.92	44.44
9.32	281	18:1	95.14	91.67
9.80	283	18:0	98.92	61.11
10.86	295	19:1	8.11	25.00
11.41	297	19:0	10.81	55.56
12.06	301	20:5	12.43	38.89
---	303	20:4	---	83.33
---	305	20:3	---	11.11
---	307	20:2	---	11.11
12.49	309	20:1	14.05	11.11
13.01	311	20:0	11.89	27.78
14.15	327	22:6	2.70	36.11
---	329	22:5	---	16.67
---	331	22:4	---	11.11
---	333	22:3	---	8.33
14.80	335	22:2	1.62	5.56
15.70	337	22:1	2.16	2.78
16.68	339	22:0	7.57	11.11

method of choice for reliable and routine fatty acid analysis in future.

These novel methods were developed with an eye on simplifying the structure determination of marine natural products, especially, of those having biological importance. They enable us to obtain meaningful structural informations from miniscule quantities of compounds. This can be particularly helpful while working with limited quantities of samples with little or no guarantee of their future supply, as is the case with many marine animals and plants. Using the above methods, we have determined the structures of several compounds including fatty acids, sterols, terpenoids and alkaloids as will be seen in the following chapters.

EXPERIMENTAL

The CI mass spectral studies were carried out on a Biospect CI mass spectrometer operating at 1-2 torr pressure of the reagent gas. The instrument was calibrated using polyethylene glycol. Ammonia, argon and methane are the commonly used reagent gases, with ammonia being used in majority of the cases. When ammonia is used as the reagent gas, it gives rise to strong $[M+NH_4]^+$ followed by $[M+H]^+$ ions in the positive mode. By changing the electric charge on the continuous dynode, the mode may be switched over from positive to

negative or *vice versa*. Usually, the negative CIMS gives rise to strong $[M-H]^-$ peaks. Addition of about 10 µg of NH_4Cl or NH_4Br to the sample before recording its NCIMS spectrum gives rise to strong pseudomolecular ions, $[M+Cl]^-$ and $[M+Br]^-$ respectively. The ion source temp. was maintained in the range 100-170°C during these studies.

PREPARATION OF FATTY ACID DERIVATIVES:

i).MICROWAVE IRRADIATION METHOD

To a 500 ml Erlenmeyer flask was added sequentially the fatty acid (7.03 mmol), distilled DMF (10 ml), alkylating reagent (*p*-bromobenzyl or *p*-nitrobenzyl bromide, 7.03 mmol) and anhydrous K_2CO_3 (2 gm). The flask was covered with a glass funnel and placed in a microwave oven (GE model, 450 watts). A 500 ml beaker containing 150 ml water was also placed alongside the reaction flask to serve as a heat sink. The mixture was irradiated for 4 min at medium power level. Later, the reaction mixture was taken out, cooled and diluted with water (60 ml). The product was extracted with EtOAc (2X20 ml), dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator under vacuum, yielding the pure product in quantitative yield and which was also pure enough to be directly used for mass spectral analysis.

ii).CONVENTIONAL METHOD

To a mixture of fatty acid and the alkylating reagent (7.03 mmol each) in a round bottomed flask fitted with a water condenser, were added distilled DMF (10 ml) followed by anhydrous K_2CO_3 (2 gm). The above reaction mixture was heated to 140-150°C and maintained at that temperature for about 8 min. TLC of the reaction mixture alongwith standards indicated complete conversion of the fatty acids into the corresponding *p*-nitro or *p*-bromobenzyl esters. The reaction mixture was then cooled, diluted with water and the product extracted with EtOAc as before.

p-Nitrobenzyl ester of stearic acid, IR(neat): 2900, 2840, 1720, 1595, 1500, 1460, 1340, 1160 & 850 cm^{-1}; ^1H NMR($CDCl_3$): δ 8.2(2H,d,J=7 Hz),7.5(2H,d,J=7 Hz), 5.22(2H,s), 2.4(2H,t,7.4 Hz), 1.6(2H,m), 1.2(28H,m) and 0.87(3H,t,J=6.6 Hz); ^{13}C NMR ($CDCl_3$): δ 173.2(s), 143.4(s), 128.2(d), 123.6(d), 105(s), 64.4(t), 34-22(bunch of t's) and 13.95(q); For mass spectral data, see TABLE 1.

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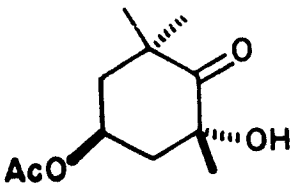
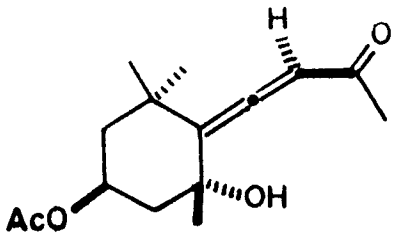
CHAPTER 2

CONSTITUENTS OF THE BROWN ALGA *PADINA TETRASTROMATICA* HAUCK

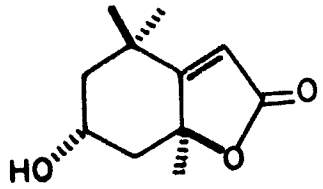
The brown alga *(Phaeophyceae)*, *Padina tetrastromatica* (HAUCK), (fam: *Dictyotaceae*), is ubiquitous in the intertidal waters of Indian coasts. During our studies on the pharmacological properties, its methanol extract exhibited promising spasmogenic (50 µg/ml), antifertility (100%,200 mg/Kg) and hypotensive activities *in vivo* in mice[1]. Follow-up studies located the activity in its pet.ether fraction. In addition, the crude extract also showed promising anti-amoebic activity[2]. From the pet.ether and $CHCl_3$ soluble fractions of this extract, we have isolated several compounds such as fatty acids, sterols and terpenoids including some halogenated compounds. They are (2R,4S)-4-acetoxy-2-hydroxy-2,6,6-trimethylcyclohexanone (1), hitherto known only as a synthetic compound or degradation product of carotenoids[3,4]; (3R),4-[(2R,4S)-2-hydroxy-4-acetoxy-2,6,6-tri-methylcyclohexylidene]-but-3-en-2-one (apo-9'-fucoxanthinone 2), previously known only as synthetic compound, being a major fragment in the oxidative degradation of the commonly occurring carotenoid, fucoxanthin[5] and which is also related to the ant-repellant "Grasshopper ketone" or "romalea allene" isolated by Meinwald *et.al.*, from the flightless grasshopper *Romalea microptera*[6], and subsequently from the leaves of a terrestrial plant, *Cissus rheifolia*[7]; loliolide (3), a versatile compound, originally isolated from the rye-grass *Lolium perenne*[8], and which was later found to be a common constituent of several angiosperms[9-11], algae[12-15], mollusc[9], sponge[16] and even marine sediment[17]; three new halogenated nor-



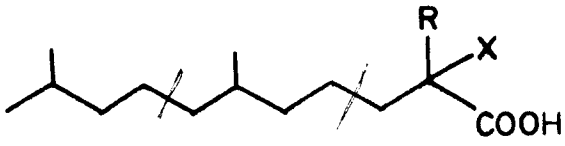
(2R,4S)-4-acetoxy-2-hydroxy-2,6,6-Trimethyl Cyclohexanone(1)



(3R),4-[(2R,4S)-2-HYDROXY-4-ACETOXY 2,6,6,TRIMETHYL CYCLOHEXYLIDENE]-but-3-en-2-one (2)



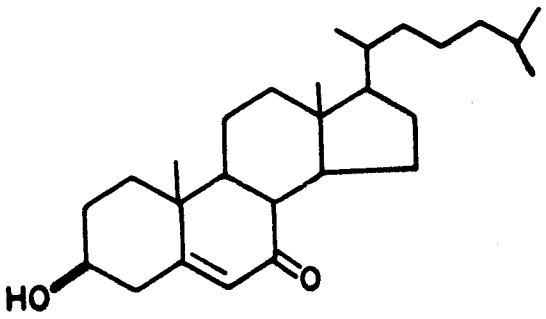
Loliolide (3)



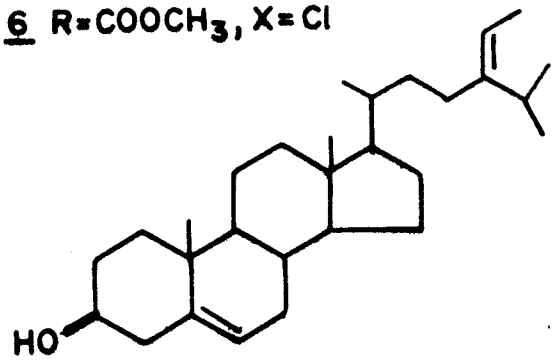
4 R=CH_3, X= Cl

5 R=CH_3, X = Br

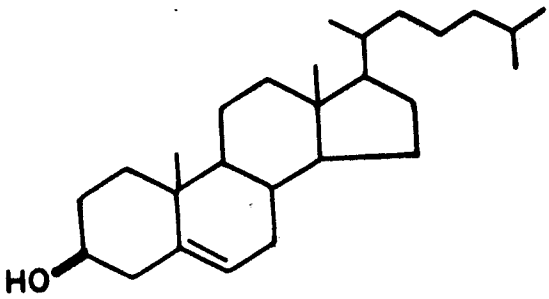
6 R=$COOCH_3$, X=Cl



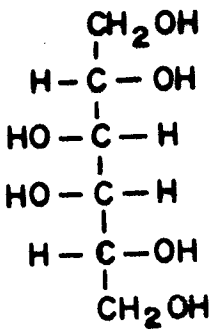
7-Ketocholesterol (7)



Fucosterol (8)



Cholesterol (9)



Galactitol (Dulcitol , 10)

Fig. 1 Compounds isolated from Padina tetrastromatica

sesquiterpenoids (4-6); besides three known sterols: 7-keto-cholesterol (7), fucosterol (8) and cholesterol (9) (FIG 1). The fatty acids range from 14:0 to 20:0 as determined by GC and the newly developed neagtive CIMS technique are described in chapter 1. Besides, a reduced hexose, dulcitol (galactitol 10) was isolated from its water-soluble fraction. Since many of the compounds were obtained in low yield, their structural elucidations were mostly based on respective spectral data (IR, UV, NMR and MS). Efforts have also been made to confirm these structures wherever possible, using our newly developed techniques such as TLC-CIMS and TLC-O_3-CIMS discussed in chapter 1.

Compound (1), (5 mg), obtained in semipure form from SiO_2 column chromatography was finally purified on preparative TLC using 25% EtOAc in Pet.ether (R_f=0.46). Its IR spectrum revealed the presence of an ester (1730 & 1250 cm^{-1}), carbonyl (1700 cm^{-1}) and hydroxyl (3400 & 1040 cm^{-1}) groups in the molecule. This was supported by the presence of peaks at δ 170, 215 & 66.7 in its ^{13}C NMR spectrum. PCI and NCI mass spectrum gave strong peaks at m/z 232$[M+NH_4]^+$ and 249$[M+^{35}Cl]^-$ respectively. Its molecular weight was deduced to be 214 from these results. This corresponded to a molecular formula $C_{11}H_{18}O_4$ which was also confirmed by HREIMS. The above molecular formula indicated that the compound has three degrees of unsaturation. Two of these could be easily accounted for by the ester and carbonyl moities. In the

absence of any olefenic bonds as indicated by its ^{1}H and ^{13}C NMR spectra, it was presumed that the compound could be monocyclic. Presence of peaks at δ 170 & 21.2 in the ^{13}C NMR as well as the 3H singlet signal at δ 2.05 in the ^{1}H NMR spectra indicated that the ester group is an acetate. This was also confirmed by the mass spectral fragmentations wherein the molecule readily lost fragments of 42 and 60 mass units. All the 11 carbon signals could be easily seen in the ^{13}C NMR spectrum. Further, with the help of DEPT spectrum, they could be easily classified into two methylenes, four methyls, one oxygen-bearing methine and four quarternary carbons. The latter signals were at δ 215, 170, 74.5 and 30, suggesting that they could be due to carbonyl, ester, oxygen-bearing quarternary carbon and tetrasubstituted aliphatic carbon atoms. Proton decoupling studies revealed the presence of the partial structure as indicated in FIG 2.

The high geminal coupling among the methylene protons (14 Hz) supported the view that these are part of a ring system. All the oxygen atoms could be accounted for as part of an ester, carbonyl and a hydroxyl group. The carbonyl absorption at 1700 cm^{-1} in its IR spectrum suggested that it might be part of a six membered ring or open chain. Cyclic ketones with lower no. of carbons would have been more strained resulting in higher carbonyl group absorption frequencies. Based on these results, the structures I-VI (FIG 3) were shortlisted as possible candidate for this compound.

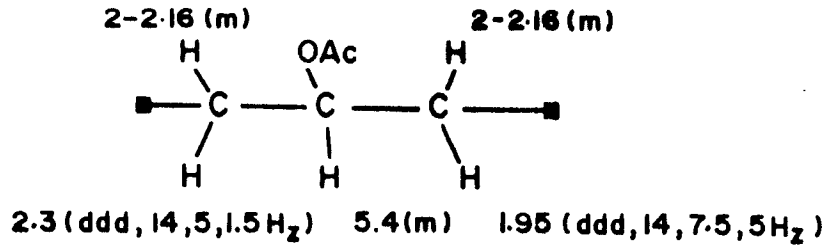


Fig. 2 Partial structure of compound (I)

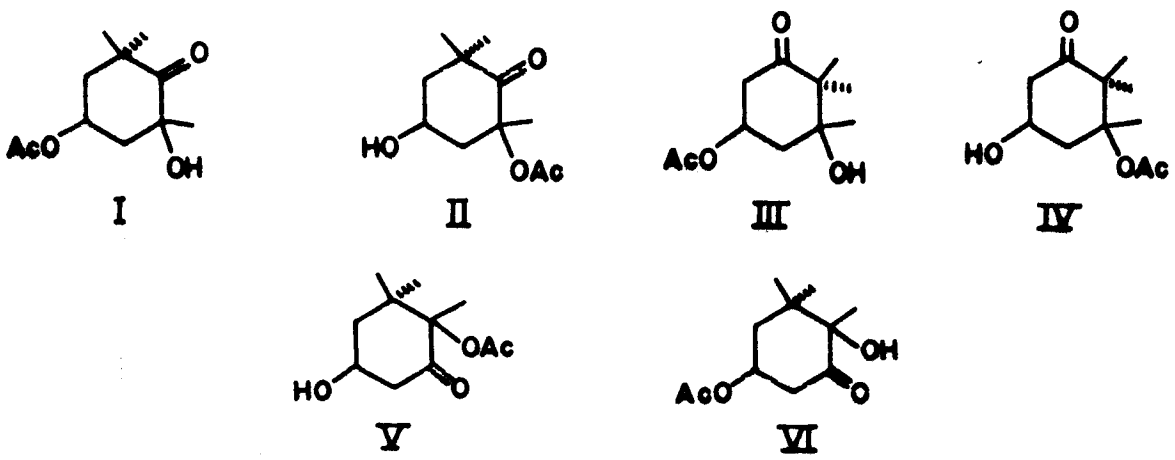
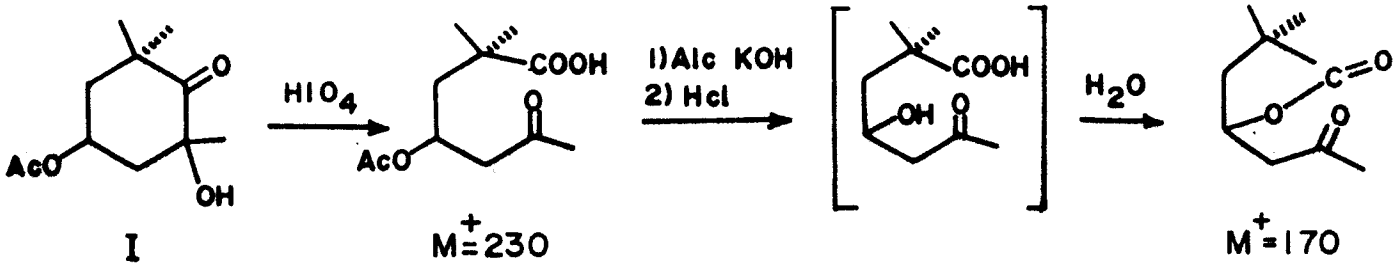


Fig 3 Possible structure of compound (I)



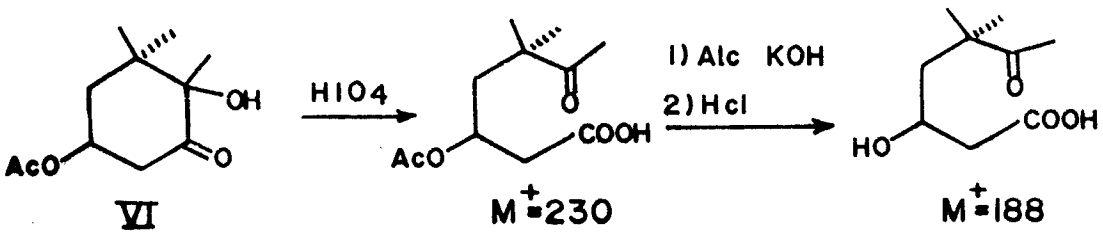


Fig. 4 Possible reaction products of structures I and VI of compound (I)

In order to conclusively prove the structure, the compound was treated with HIO_4 resulting in isolation of a product of molecular weight 230(FIG 4). This indicated that the compound has undergone cleavage which is possible only if the hydroxyl group is present in the carbon next to the carbonyl group. This reaction thus ruled out structures (II-V), leaving only (I) and (VI) as possible candidates. Hydrolysis of the above product using alc.KOH followed by acidification with dil.acid yielded a compound with a molecular weight 170 as determined by CIMS. Under these reaction conditions, compound (I) will give rise to a gamma-hydroxy acid which will then undergo facile lactonisation, yielding the above product. On the other hand, compound (VI) will give rise to a ß-hydroxy acid of molecular weight 188. The above reactions, carried out on submilligram quantities of this compound suggested structure (I) for compound (1). The hypothesis that this compound is a degradation product of the commonly occurring carotenoid, fucoxanthin further supports the above view[5].

Compound (2), initially separated on preparative TLC alongwith the above compound, was subsequently purified on HPLC equipped with Diode Array Detector (DAD) in conjunction with a Chem-station (FIG 5). The purity of this compound was examined by novel techniques such as ratio plots, UV-Vis derivative spectra and 3D plots (Time *versus* Wavelength *versus* Absorption, FIGs 6-9) during HPLC separation and by total ion current (TIC) measurements during mass spectral

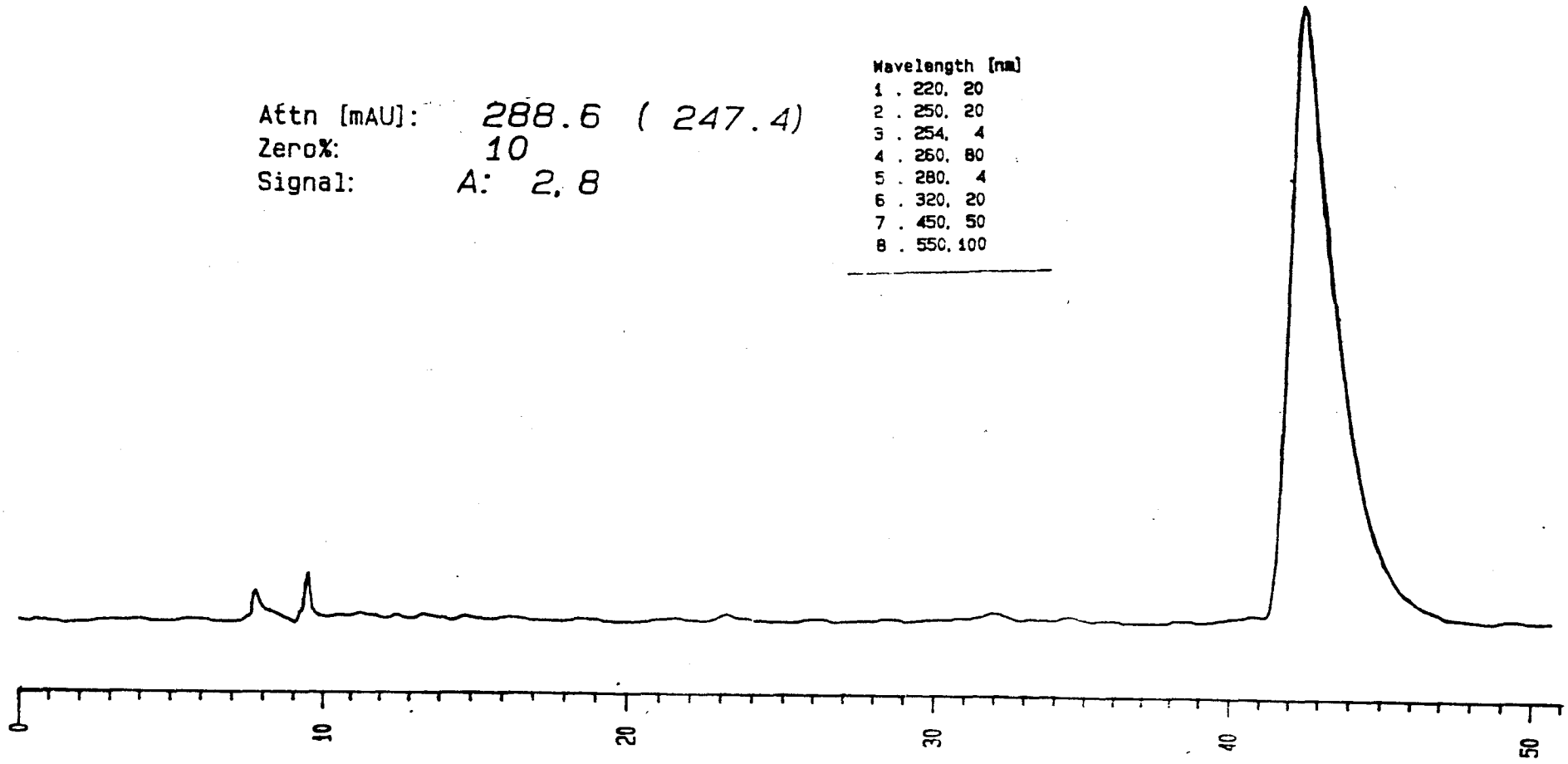


FIG 5 CHROMATOGRAM OF APO-9'-FUCOXANTHINONE (2)

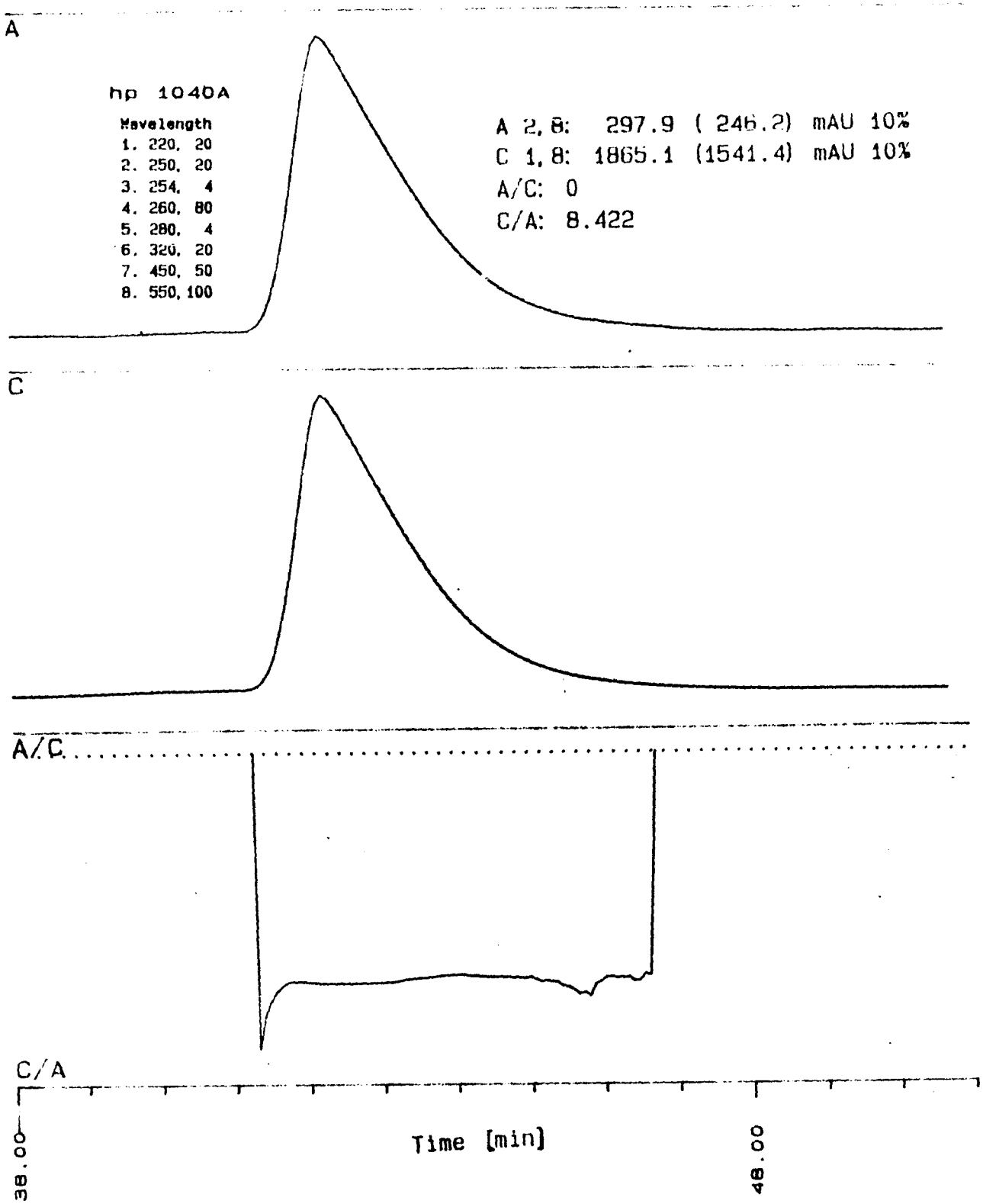


FIG 6 RATIO PLOT OF THE CHROMATOGRAM OF COMPOUND (2)

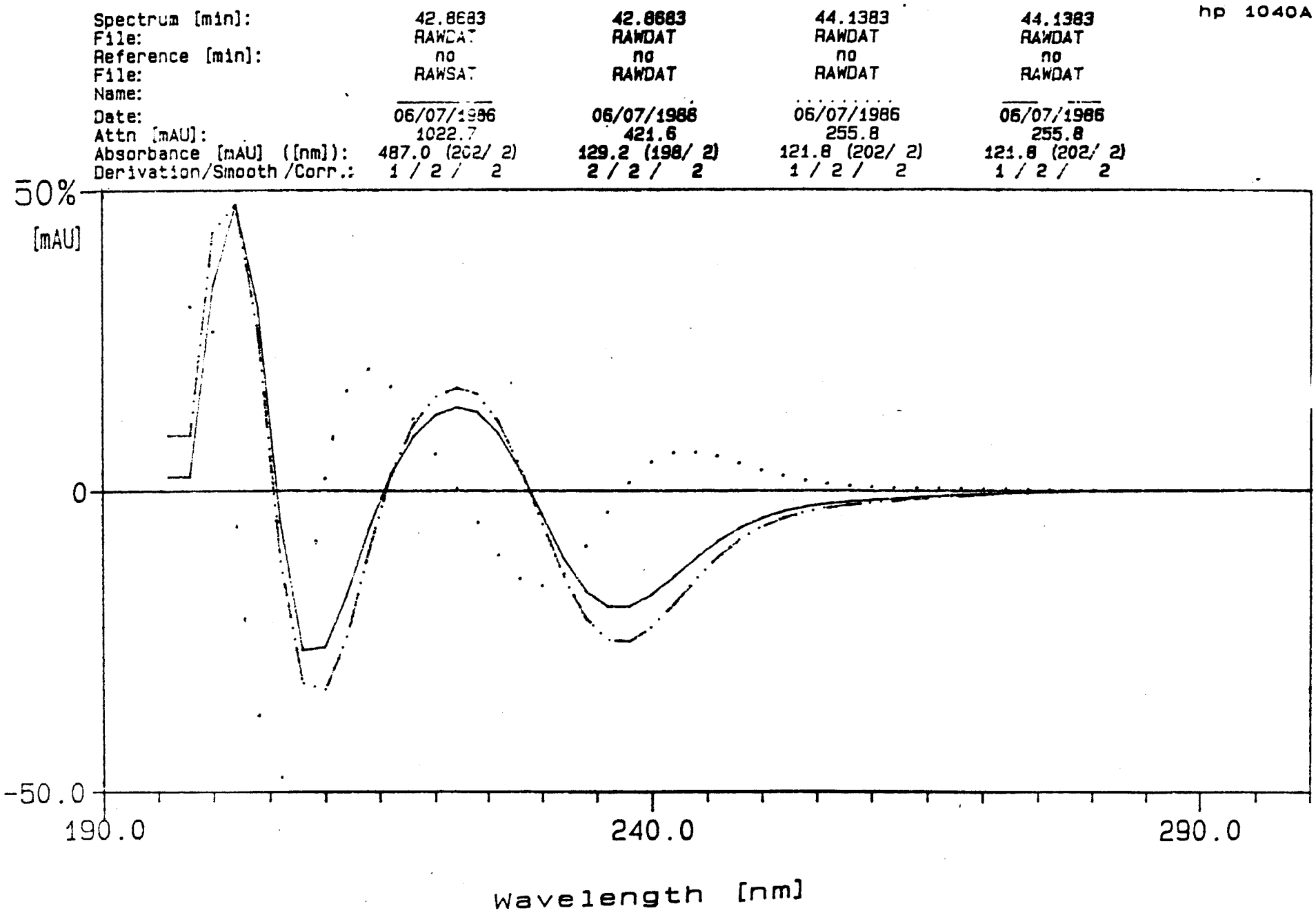


FIG 7 **UV-VIS. DERIVATIVE SPECTRA OF APO-9'-FUCOXANTHINONE (2)**

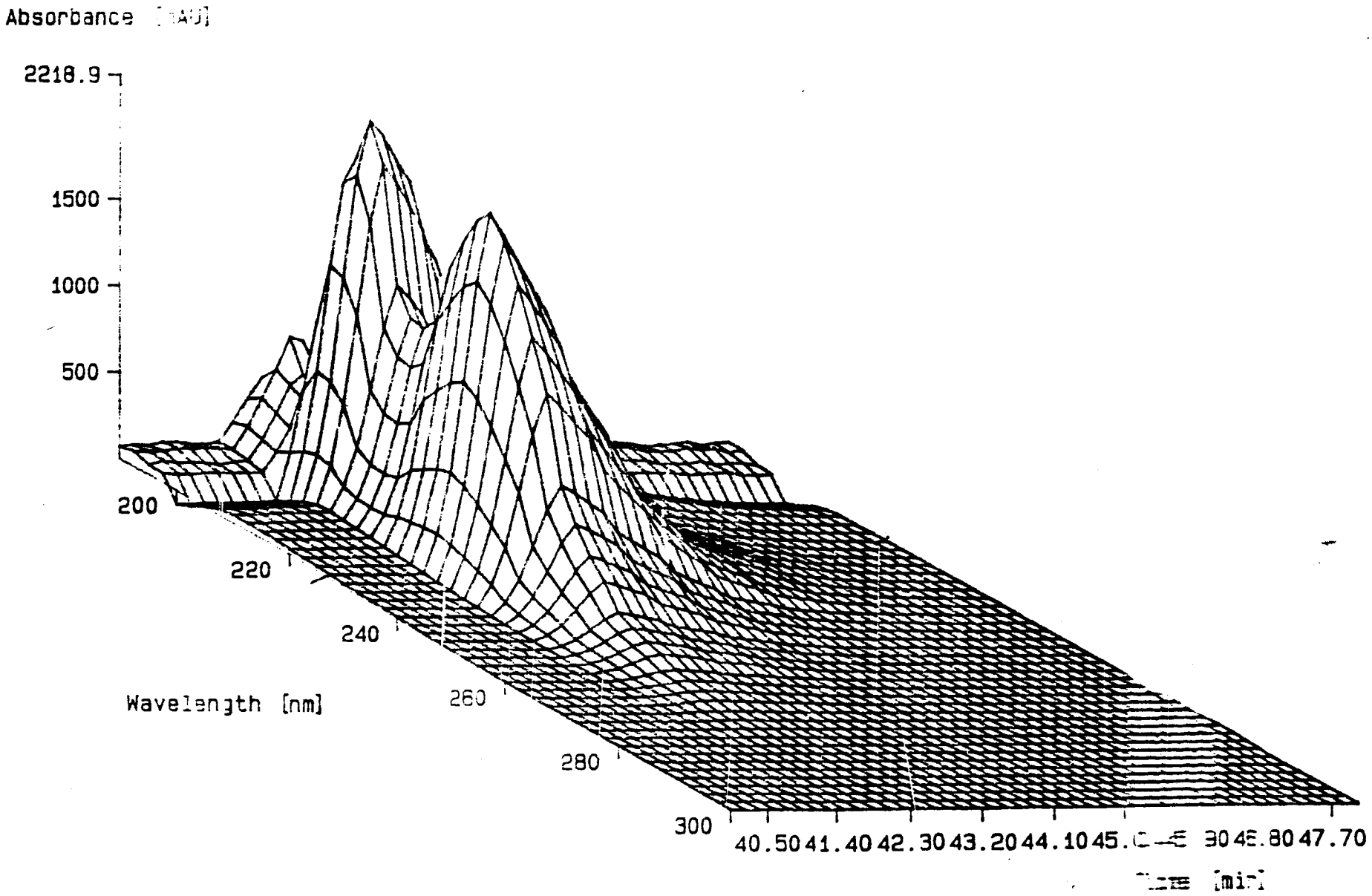


FIG 8. 3D PLOT OF COMPOUND (2)

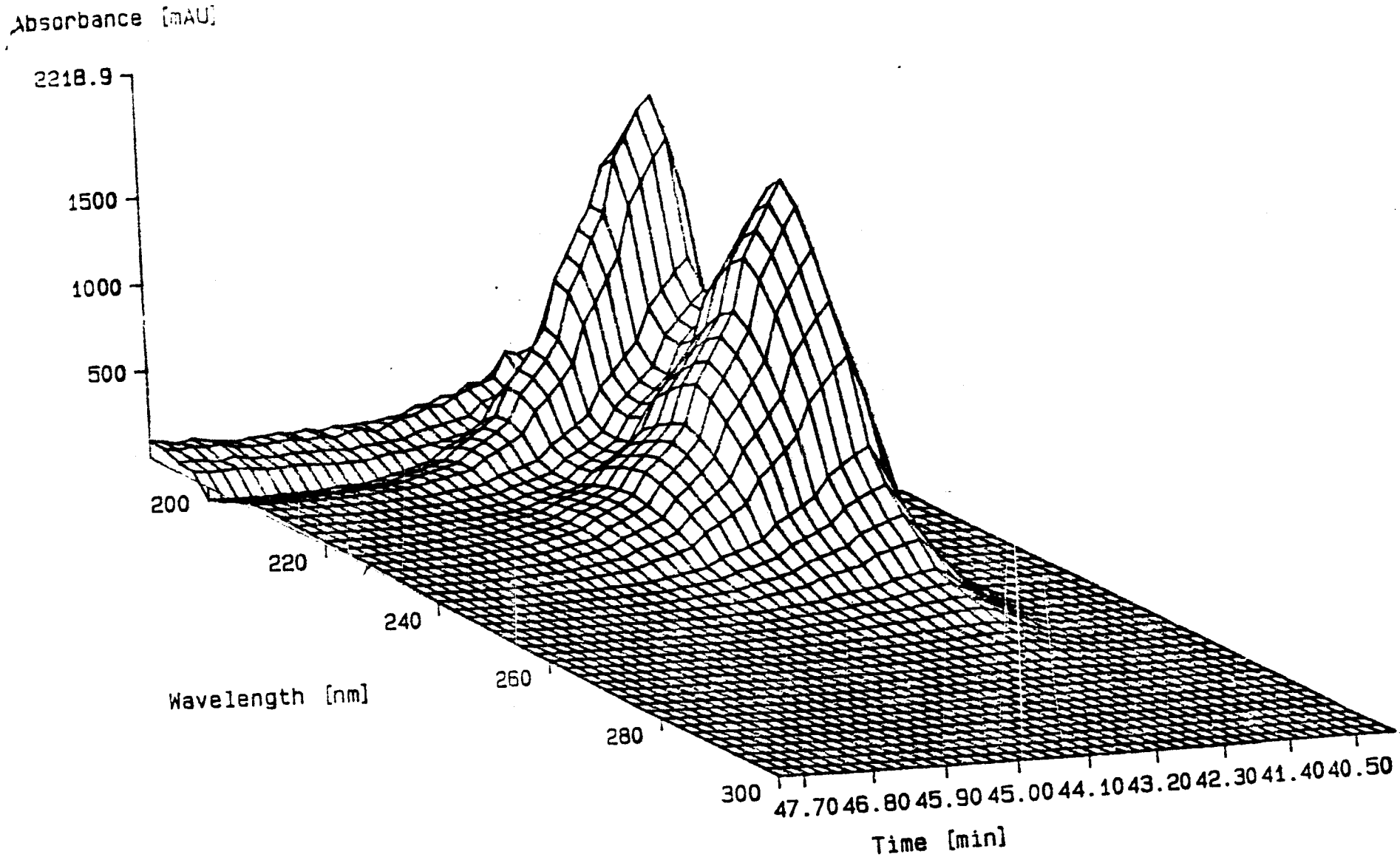


FIG 9. 3D PLOT OF COMPOUND (2) (REAR VIEW)

studies. Its IR spectrum revealed the presence of hydroxyl (3400 & 1040 cm^{-1}), allene (1930 cm^{-1}), conjugated carbonyl (1670cm^{-1}) and ester (1730 & 1250 cm^{-1}) groups in the molecule (FIG 10). This was supported by its ^{13}C NMR spectrum which had signals at δ 170.2(s) & 21.2(q,OAc), 67.52(d) and 71.8(s, hydroxyl or acetoxyl bearing carbons), 100.78(d), 118.5(s) and 197,74(s,allene carbons) and 209.2(s,carbonyl) as well as by the strong UV absorptions at 204 and 232 nm (FIGs 11-13). Particularly noteworthy was the downfield signal of its carbonyl carbon by about 6 ppm, to δ 209 as compared to 215 in the case of compound (1), which is indicative of its conjugated nature.

Positive and negative CIMS of the compound yielded the respective molecular adduct ions at m/z 284[M+18]$^+$ and 301 [M+^{35}Cl]$^-$ respectively. The latter ion was also accompanied by its characteristic chlorine isotope peak at 303 as expected. This indicated that the molecular weight of the compound is 266 which was confirmed by EIMS. HREIMS of this peak determined its elemental composition as $C_{15}H_{22}O_4$. The intense peaks at m/z 224[M-CH_2CO]$^+$) and 206([M-CH_3COOH]$^+$) in its EIMS confirmed the presence of the acetate group in this molecule (FIG 14). All the 15 carbon signals could be easily detected in the ^{13}C NMR spectrum which were distributed as five methyls, one ketone, one acetate carbonyl, two methines, four quarternary carbons and two methylene groups as revealed by its DEPT spectrum. ^1H-^1H COSY and proton decoupling studies

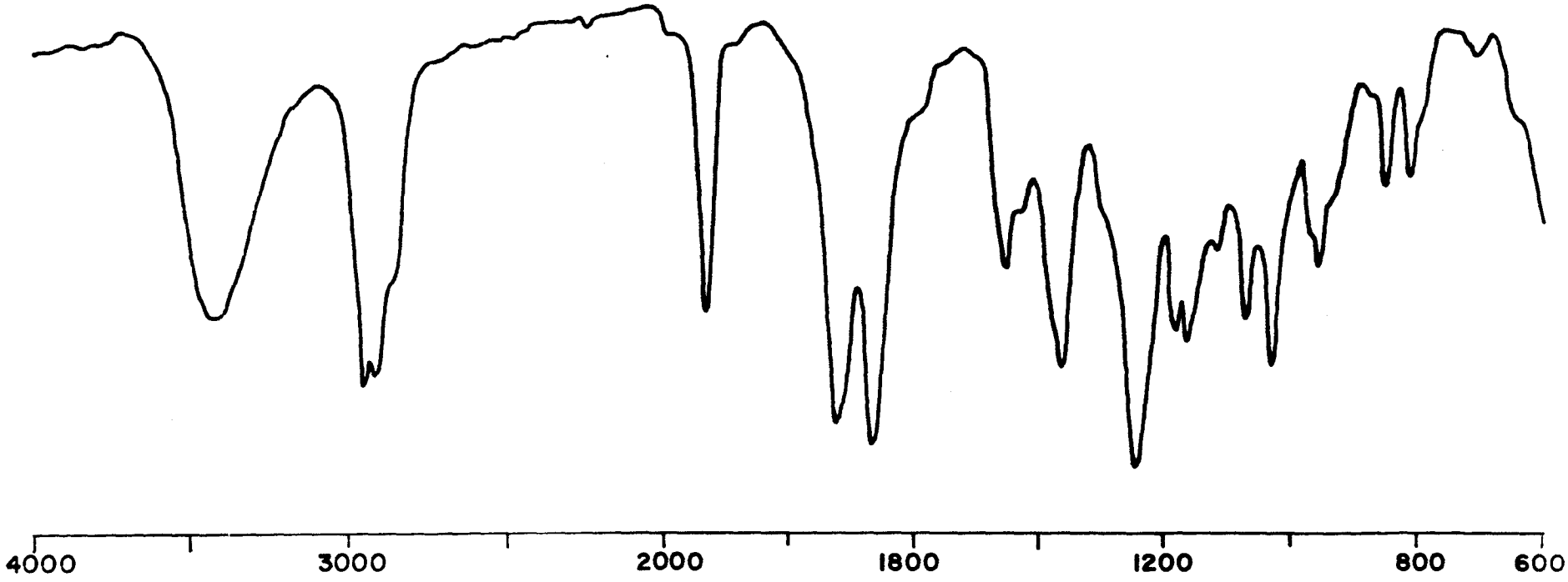


Fig. 10 IR (Neat) spectrum of apo-9'-fucoxanthinone (2)

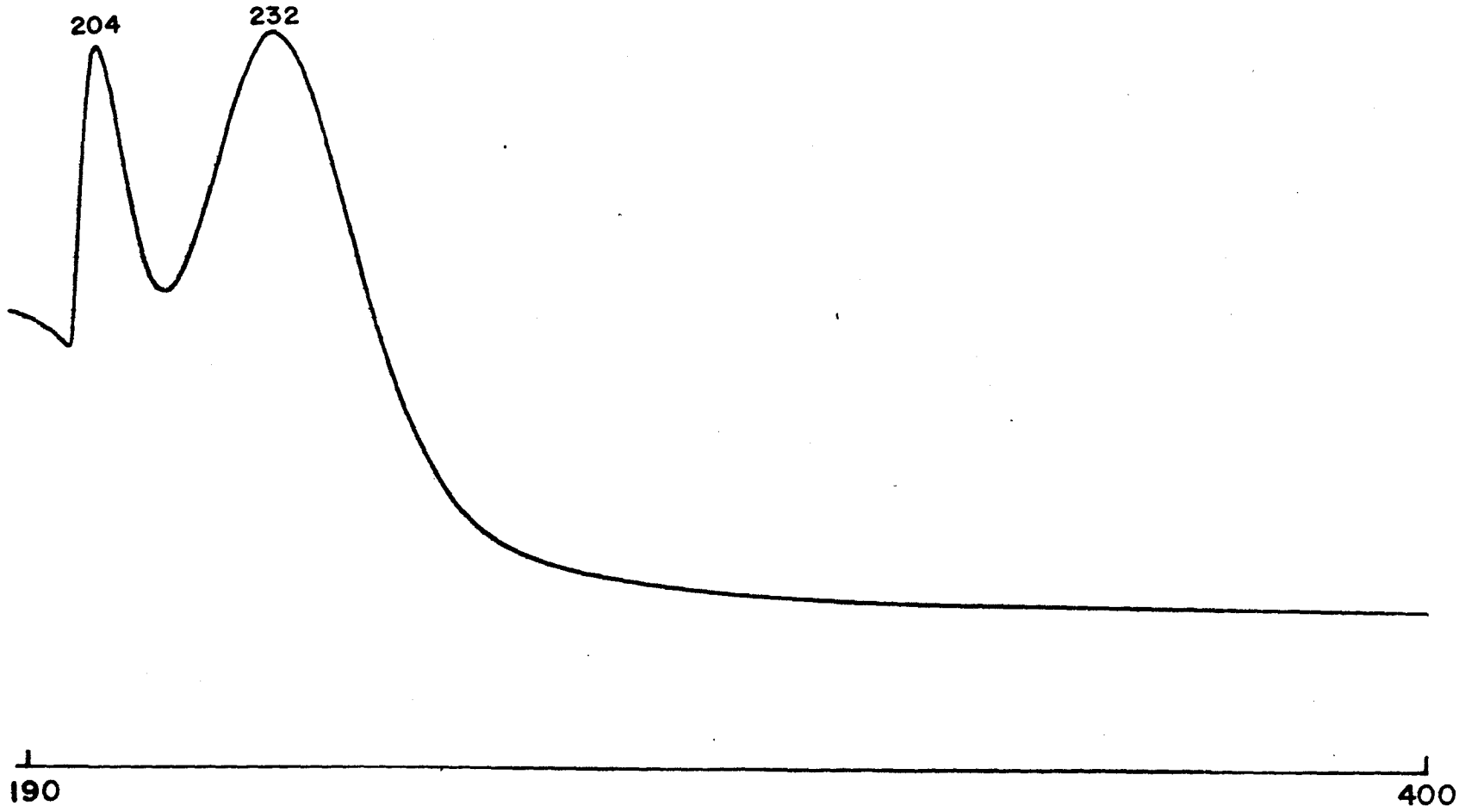


Fig. 11 UV - Vis spectrum of apo - 9'-fucoxanthinone (2).

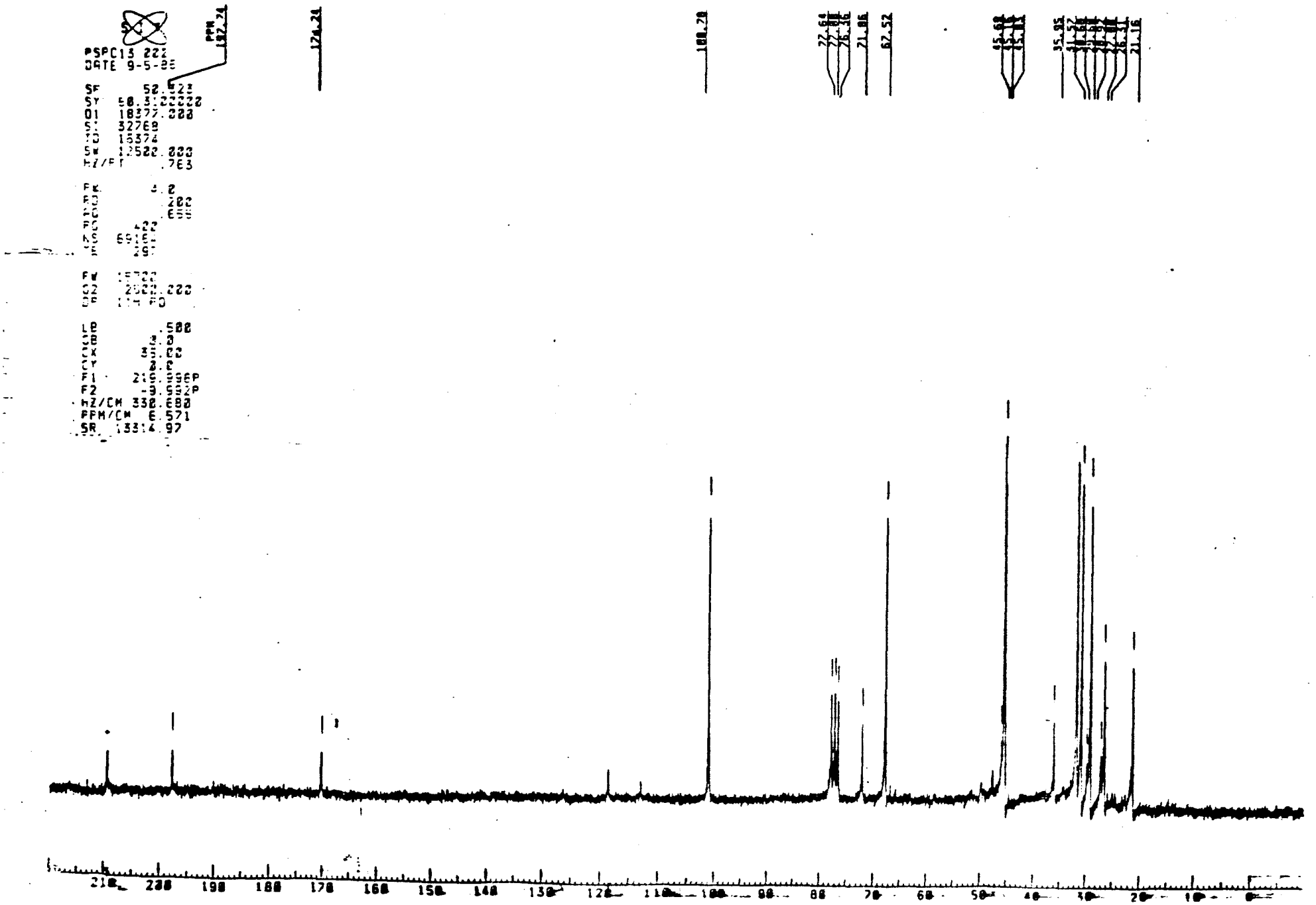


FIG 12 ^{13}C NMR SPECTRUM OF APO-9'-FUCOXANTHINONE (2)

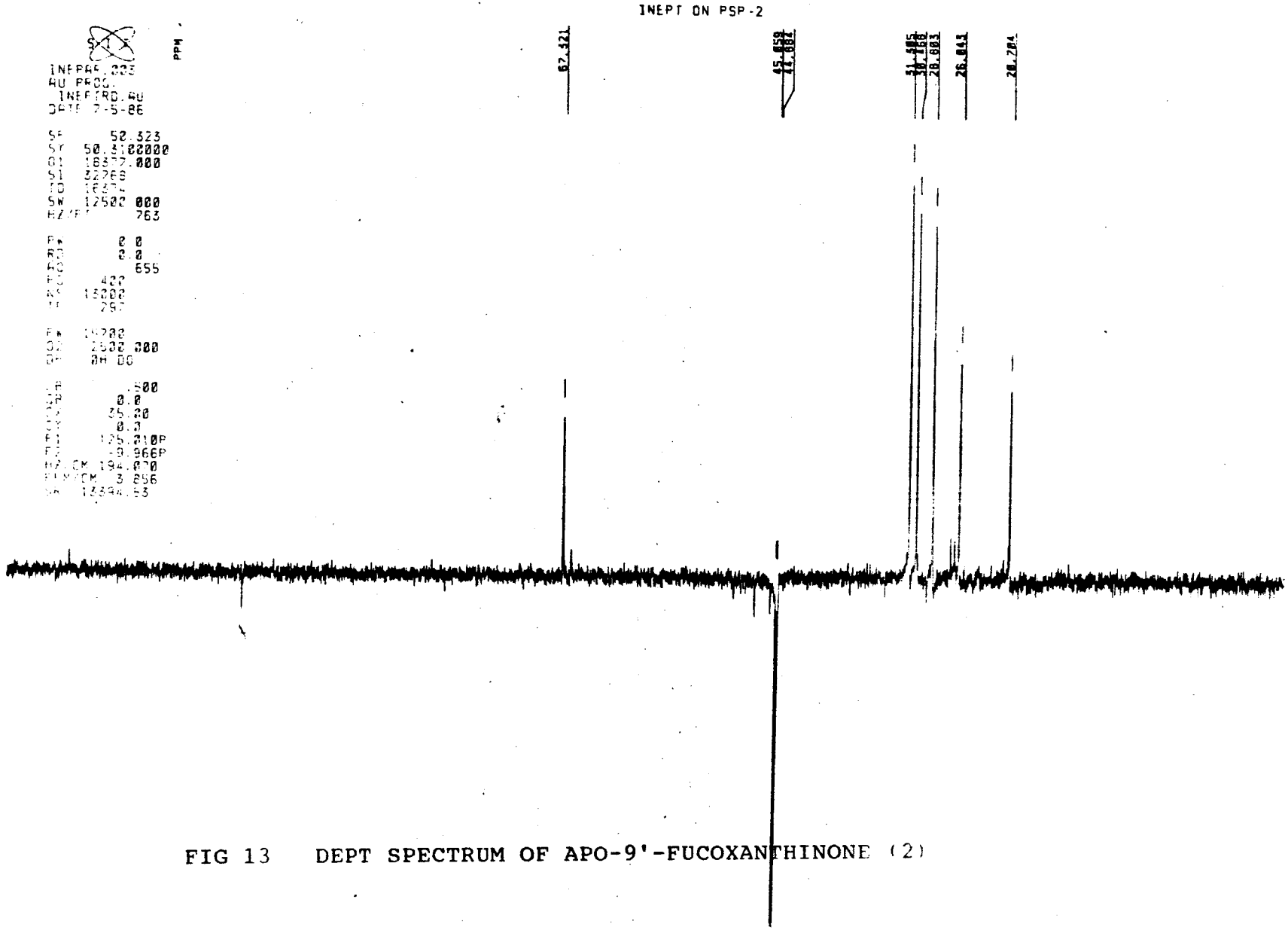


FIG 13 DEPT SPECTRUM OF APO-9'-FUCOXANTHINONE (2)

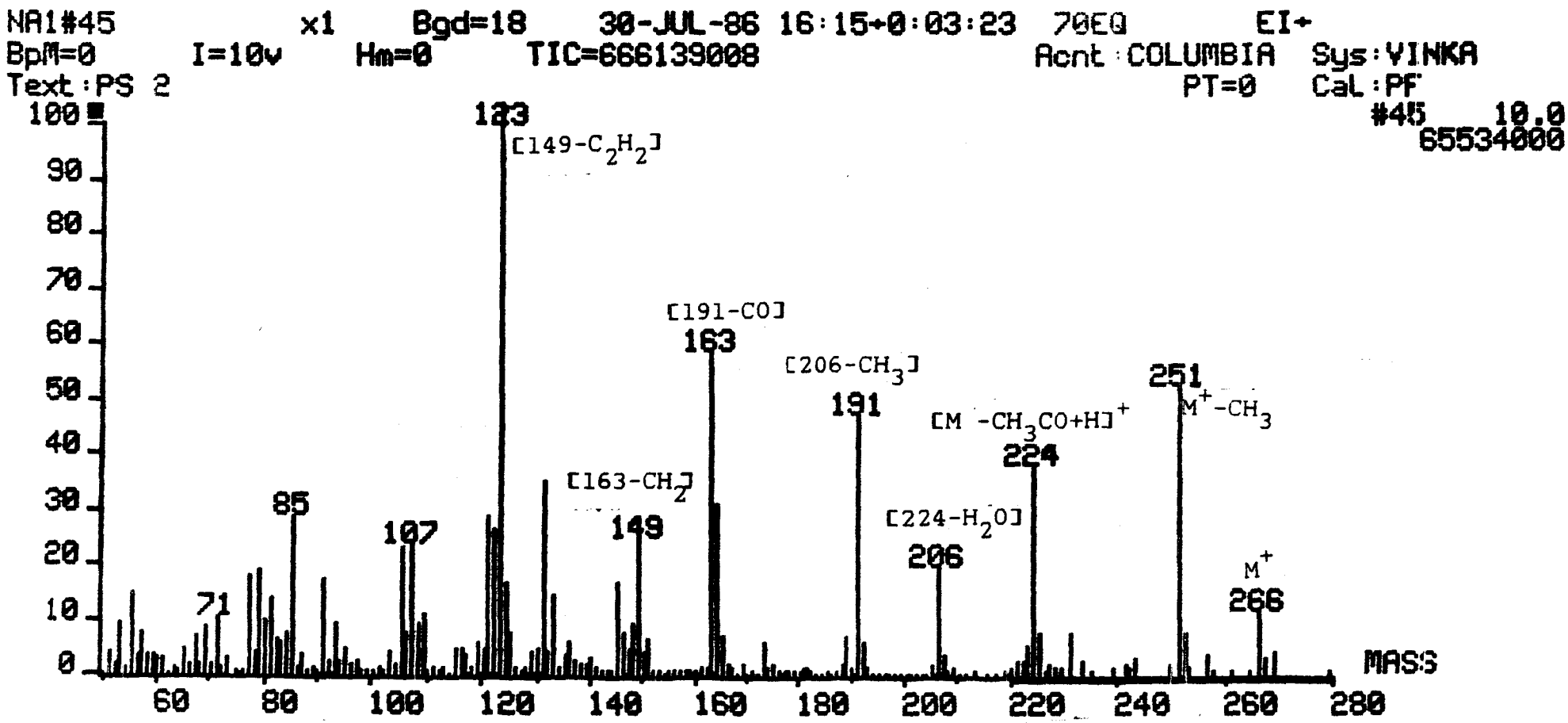


FIG 14 EIMS OF APO-9'-FUCOXANTHINONE (2)

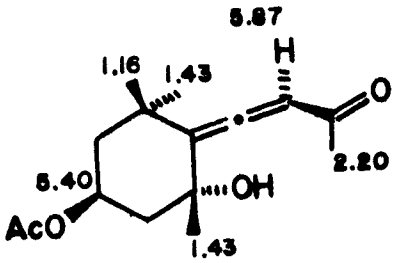
revealed the presence of $-CH_2-CHOH(OAc)-CH_2-$ group in this compound as in compound (1). As in the case of the earlier compound the high geminal couplings of the methylene protons (14 Hz) indicated that they might be part of a cyclic system. However, the absence of the carbonyl signal at 215 ppm suggested that probably the keto group of compound (1) has been replaced by the allene moiety in this molecule. On the other hand, the presence of carbonyl carbon signal at δ 209.2 in the ^{13}C NMR spectrum and the strong UV-Vis absorption at 232 nm were indicative of a conjugated carbonyl moiety. The ^1H NMR signal at δ 2.20(3H,s) suggested that a methyl group is probably attached to the carbonyl group. Since the compound had no other unsaturations apart from the allene group, it may be presumed that the above methyl ketone is attached to the allene. This indicates that the following partial structure, $=C=C=C(H)COCH_3$ has replaced the carbonyl group of compound (1), leading to the tentative structure (2) for this compound. This structure was also supported by its mass spectral fragmentations, which were in good agreement with that of apo-9'-fucoxanthinone prepared synthetically[3,18].

This structure was further confirmed by analysis of the products of its reaction with lithium aluminium hydride (LAH) as well as using the newly developed TLC-O_3-CIMS. IR and ^1H NMR spectra of the product obtained after treating the compound (2) with LAH indicated it to be a mixture of acetylenic and allenic triols. Thus its IR spectrum had

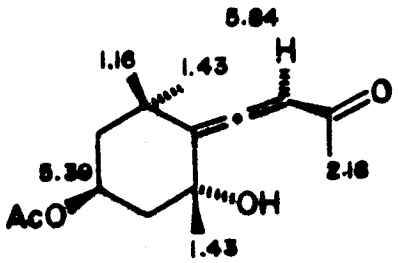
absorptions at 1930 & 2240 cm^{-1}, while the ^1H NMR spectrum of the crude reaction mixture had peaks corresponding to these compounds as evident from its comparison with the literature values[3,18]. During the TLC-O_3-CIMS studies, a TLC spot of compound (2) was ozonised by exposing to ozonised oxygen for 2-3 min. The mass spectral studies of the products of this reaction were carried out as described before. The molecular weight of the product was found to be 214, indicating that ozone has cleaved the allenic bond, forming compound (1).

The assignment of the stereochemistry of the compound was based on the comparison of its ^1H NMR spectral data with that of its natural and synthetic analogs (FIG 15)[3,18-20]. Thus, the allene (2) has been reported to be a key degradation product of fucoxanthin[3], whereas its deacetoxy derivative, romale allene (2H) could be produced from the corresponding carotenoid, neoxanthin. Russel and Weedon had synthesized three stereoisomers (2C-E) of allenic ketones in order to establish its absolute configuration unambiguously[18]. Previously Isoe *et.al.*, had synthesized two allenic compounds (2F and 2G) by photosensitized oxygenation of ß-ionol[21].

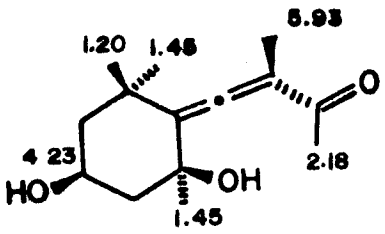
Among these, the stereochemistry of (2C) and (2E) was determined by X-ray crystallographic studies of their *p*-bromo benzoyl derivatives[18]. The ^1H NMR chemical shifts for 4-H, and the ring methyl groups of compound (2) are identical to those of apo-9'-fucoxanthinone (2C). From this, it was assumed that



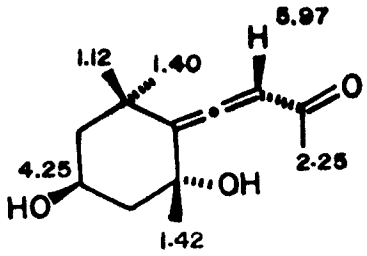
Compound (2)



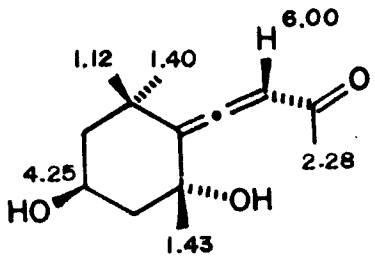
Apo-9'-fucoxanthinone (2C) (Ref 18)



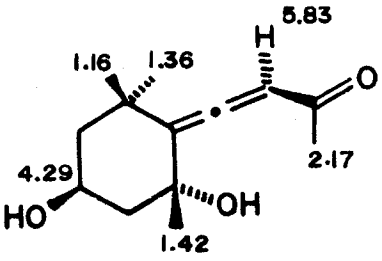
Compound (2D) (Ref 18)



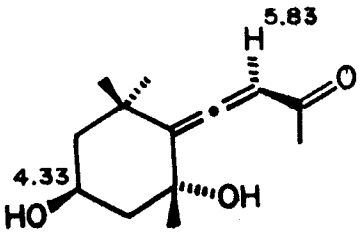
Compound (2E) (Ref 18)



Compound (2F) (Ref 20)



Compound (2G) (Ref 20)



Grasshopper ketone (2H) (Ref 4)

Fig. 15 ^{1}H NMR spectral values of grasshopper ketone and other related compounds

the relative stereochemistry of C-2 and C-4 in compound (2) are the same as in compound (2C), *i.e.*, (2R,4S). The allenic proton, which is (α) in compound (2C) as established by X-ray crystallographic studies appears at δ 5.84 in the ^{1}H NMR spectrum[19]. In compounds (2D & 2E), where these protons are (ß), the corresponding signals appear at δ 5.93 & 5.97 respectively[19]. Similarly, in compounds (2F) and (2G), where the allenic protons are (ß) and (α) respectively, their signals appear at δ 6.00 and 5.83 respectively. In compound (2), this peak appears at δ 5.87, very close to that of compounds (2C) and (2G), thereby confirming the stereochemistry of this proton as (α). Meinwald *et. al.*, had subsequently synthesized the grasshopper ketone in which this proton signal is reported at δ 5.86[3]. The structure of compound (2) was thus established as (3R)-4-[(2R,4S)-2-hydroxy-4-acetoxy-2,6,6-trimethylcyclohexylidene]-but-3-en-2-one, which is the same as that of grasshopper ketone (2H) and apo-9'-fucoxanthinone (2C)[19]. However, it must be noted that the above relative stereochemistry of the allene group is opposite to that occurring in common carotenoids. This might be the result of stereomutation of isomeric allenes formed initially. In support of this view, it was reported that irradiation of (2F) with a high pressure mercury lamp gives a product which, on the basis of ^{1}H NMR spectrum appears to be an equilibrium mixture (1:1) of (2F) and (2G)[21].

The compound (3), isolated by preparative TLC (R_f=0.40, 25% EtOAc/pet.ether) was a crystalline compound, m.p.153°C. HREIMS determined its elemental composition to be $C_{11}H_{16}O_3$ (Molecular weight 196.1105). Its IR spectrum indicated the presence of an ester or α:ß unsaturated, gamma-lactone (1730 & 1680 cm^{-1}), carbon-carbon unsaturation (1620 cm^{-1}) and hydroxyl (3440 & 1030 cm^{-1}) groups. This was also supported by its ^{13}C NMR spectrum which had signals at δ 182.4(s,ester or lactone carbonyl), 171.0 & 112.9(s & d respectively, olefenic carbons), 86.65(s, oxygenated carbon) and 66.83(d, oxygenated carbon). The signals at δ 47.3(t) and 45.6(t) could be assigned to two methylene groups. Proton decoupling studies also indicated the presence of $-CH_2-CHOH-CH_2-$ group. Presence of acetate group was ruled out as no corresponding methyl signal was found at δ 20-21. Strong UV-Vis absorption at 214 nm suggested that the compound is an α:ß unaturated ester/lactone. The structure of this compound was finalised as loliolide (3) after comparing its ^1H and ^{13}C NMR spectral values with those reported in literature[8-14]. This was also confirmed by its mass spectral fragmentations (FIG 16).

The elemental composition of the peaks at m/z 178 and 111 which were determined by HRMS confirmed the fragmentation pattern described in FIG 16. Three isomeric loliolides, *viz.*, loliolide, epiloliolide and isoloiolide are known in literature[15,16,20]. Their structures as well as some of their physical and biological properties are given in FIG 17.

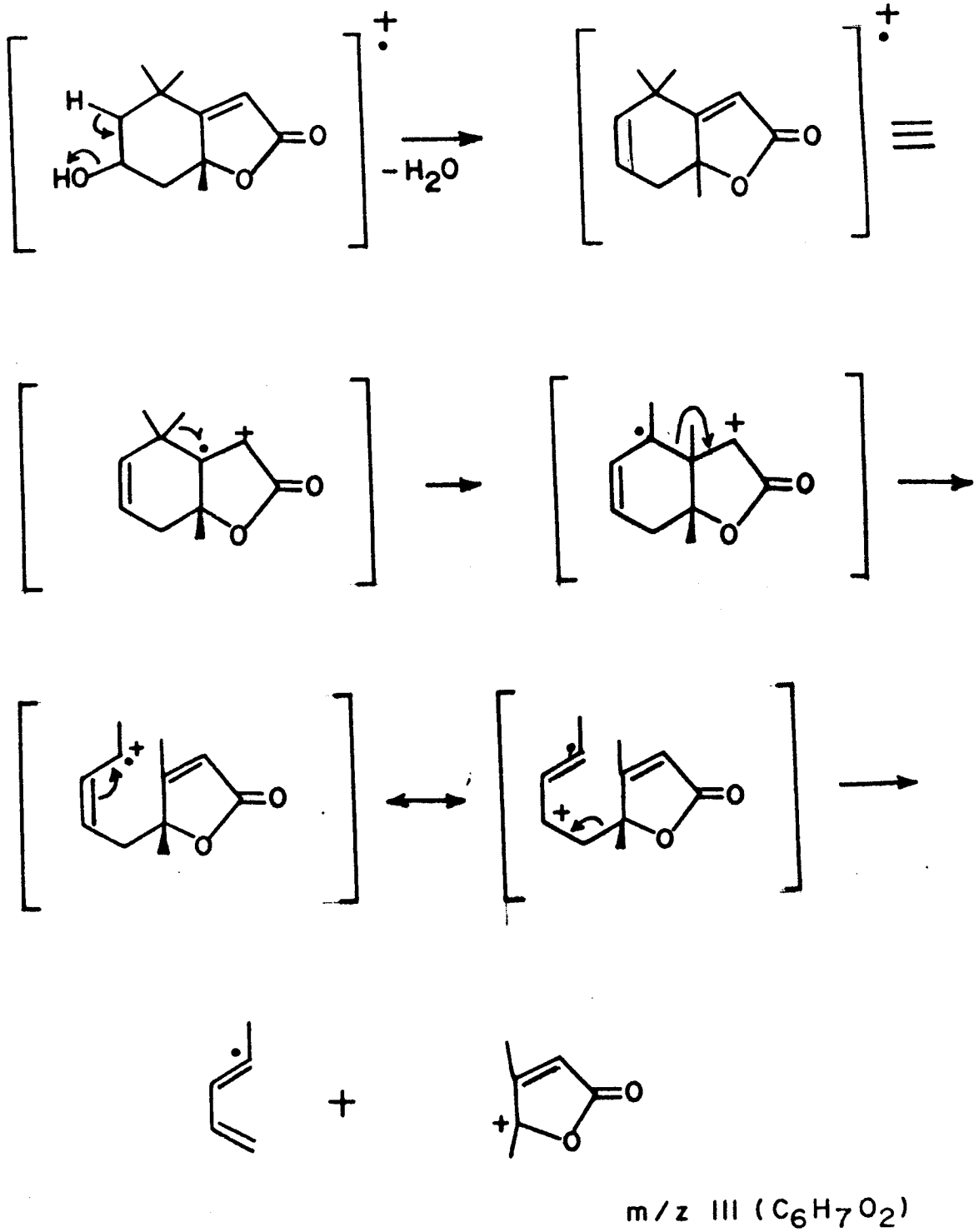
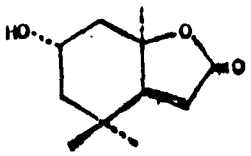
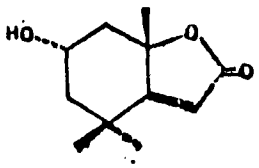


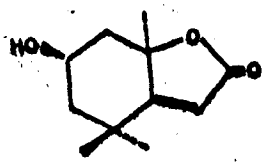
Fig. 16 Mass spectral fragmentation of loliolide



LOLIOLIDE
m.p. 155.5-156.5°C
$[\alpha]_D$= -96.3(c=0.095,$CHCl_3$)



ISOLOLIOLIDE



EPILOLIOLIDE
m.p. 119-121°C
$[\alpha]_D$= +90.8(c=0.197,$CHCl_3$)

FIG 17. STRUCTURES OF LOLIOLIDE, ISOLOLIOLIDE AND EPILOLIOLIDE

The structure of epiloliolide was ruled out for compound (3) from a comparison of their melting points. The ^1H NMR spectral data of (-)loliolide, (DL)isololiolide and that of compound (3) are given below (TABLE 1). From these results, it is clear that the compound (3) is loliolide. However, its optical rotation could not be obtained for want of adequate sample.

TABLE 1:

^{1}H NMR SPECTRAL DATA OF (DL)ISOLOLIOLIDE, (-)LOLIOLIDE AND COMPOUND (3)[21].

(-)Loliolide	(DL)Isololiolide	Compound (3)
1.28(3H,s)	1.26(3H,s)	1.35(3H,s)
1.48(3H,s)	1.30(3H,s)	1.58(3H,s)
1.51(1H,dd,14.5,3.8)	---	---
1.74(1H,dd,13.9,3.8)	---	1.70(2H,m)
1.78(3H,s)	1.58(3Hs)	1.89(3H,s)
2.02(1H,ddd,14.5,3.5 &2.5)	---	2.05(1H,m)
2.38(1H,ddd,13.9,3.8 &2.5)	---	2.53(1H,m)
2.90(OH)	3.35(OH)	---
4.3(1H,m)	4.15(1H,m)	4.42(1H,m)
5.68(1H,s)	5.73(1H,s)	5.82(1H,s)

Isoe *et.al.*, had studied the biogenesis of compounds (1-3) in great detail. They have found that photooxygenation of ß-carotene yields dihydroactinidiolide, ß-ionone and 2-hydroxy-2,6,6-trimethyl cyclohexanone, whereas zeaxanthin under similar conditions yields 2,4-dihydroxy-2,6,6-trimethyl cyclohexanone (deacetoxy 1), loliolide (3) and isololiolide[26]. On the other hand, ß-ionol, under identical conditions yields a mixture of loliolide (3), isololiolide, and romale allene

(2G). The comparison of the ^1H NMR spectrum of compound (1) with the deacetoxy compound obtained in the above study as well as by other synthetic means[3] and our finding that the allene upon ozonolysis yields the cyclohexanone (1) enabled us to confirm the stereochemistry at C-2 and C-4 as (2R,4S). Similarly, the stereochemistry assigned for loliolide (3) was confirmed by comparison of its melting point and ^1H NMR spectral values with those of all three possible isomers: loliolide, isololiolide and epiloliolide as described previously (FIG 17 and TABLE 1).

The pet.ether soluble compounds were chromatographed over silica gel columns yielding several fatty acids, their methyl esters, sterols and terpenoids. The fatty acids were analysed as described in chapter 1. The fraction rich in sterols and terpenoids was purified on HPLC (ODS column, RI detection, MeOH), yielding several pure compounds with retention times ranging from 8 to 53.5 min.

The HPLC fraction eluting at 10.28 min (1 mg), had strong IR absorption at 3400 & 1716 cm^{-1} (FIG 18), indicating the presence of carboxylic acid group in it. In addition, the presence of peaks of almost equal intensities at 1460 & 1375 cm^{-1} suggested that the compound might be of terpenoid nature. Its ^1H NMR spectrum revealed the presence of an isopropyl [δ 0.865(6H,d,6.5 Hz), collapsing into a singlet upon irradiation of the 1H multiplet at δ 1.521], one secondary

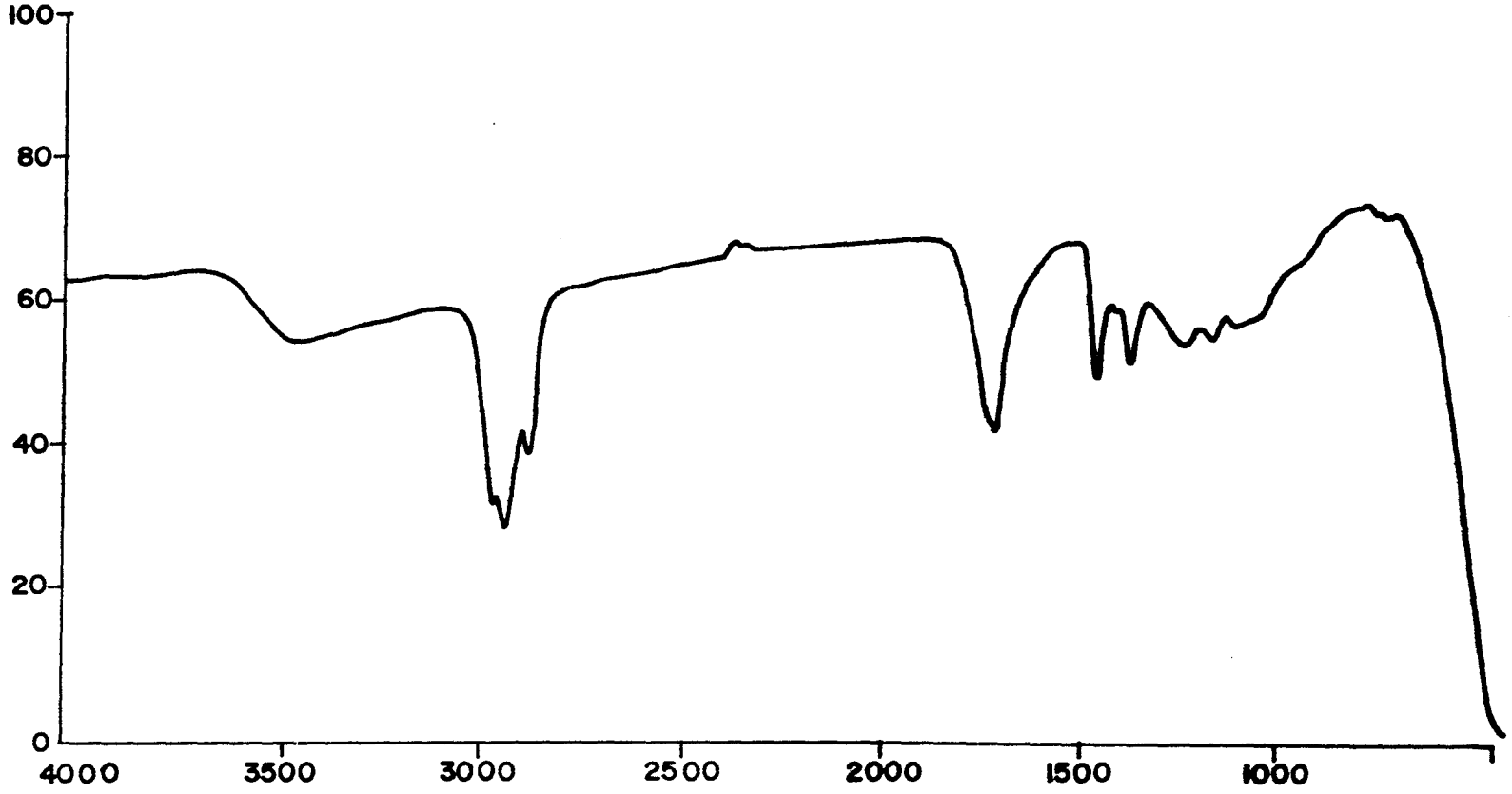


Fig. 18 IR (Neat) spectrum of 2-chloro-2,6,10 trimethyl undecanoic acid (4) and its 2-bromo analog (5)

methyl [δ 0.84(3H,d,6.5 Hz)] and one tertiary methyl [δ 1.25 (3H,s)] groups in the molecule (FIG 19). Both EI and PCI mass spectra of the sample failed to yield any strong molecular ion peak. On the other hand, NCI mass spectrum showed presence of two sets of peaks differing by two mass units each at m/z 261 & 263 and 305 & 307 and in the ratio 3:1 and 1:1 respectively. This suggested that this fraction contains two halogenated compounds, one having a chlorine atom, while the other, a bromine atom in the nuclei. Since these compounds were inseparable even on HPLC and their ^{1}H and ^{13}C NMR spectra looked identical it is possible that their remaining parts are identical. The elemental composition of these compounds were deduced as $C_{14}H_{27}ClO_2$ and $C_{14}H_{27}BrO_2$ from the above results. These molecular formulae indicate only one degree of unsaturation which could be easily explained as due to the carboxylic acid group. Thus, the rest of the molecules are acyclic and saturated. These results as well as the detailed examination of their ^{1}H NMR spectra and the assumption that the compounds are of terpenoid origin led to the structures (4) and (5) respectively. This was further supported by their ^{13}C NMR and DEPT spectra which clearly showed the presence of four methyl, two methine and three methylene groups in these compounds. However, the fourth methylene carbon as well as the quarternary carbon signals were not clear in the spectrum, perhaps due to their overlappings with some other signals or due to the inadequacy of the samples.

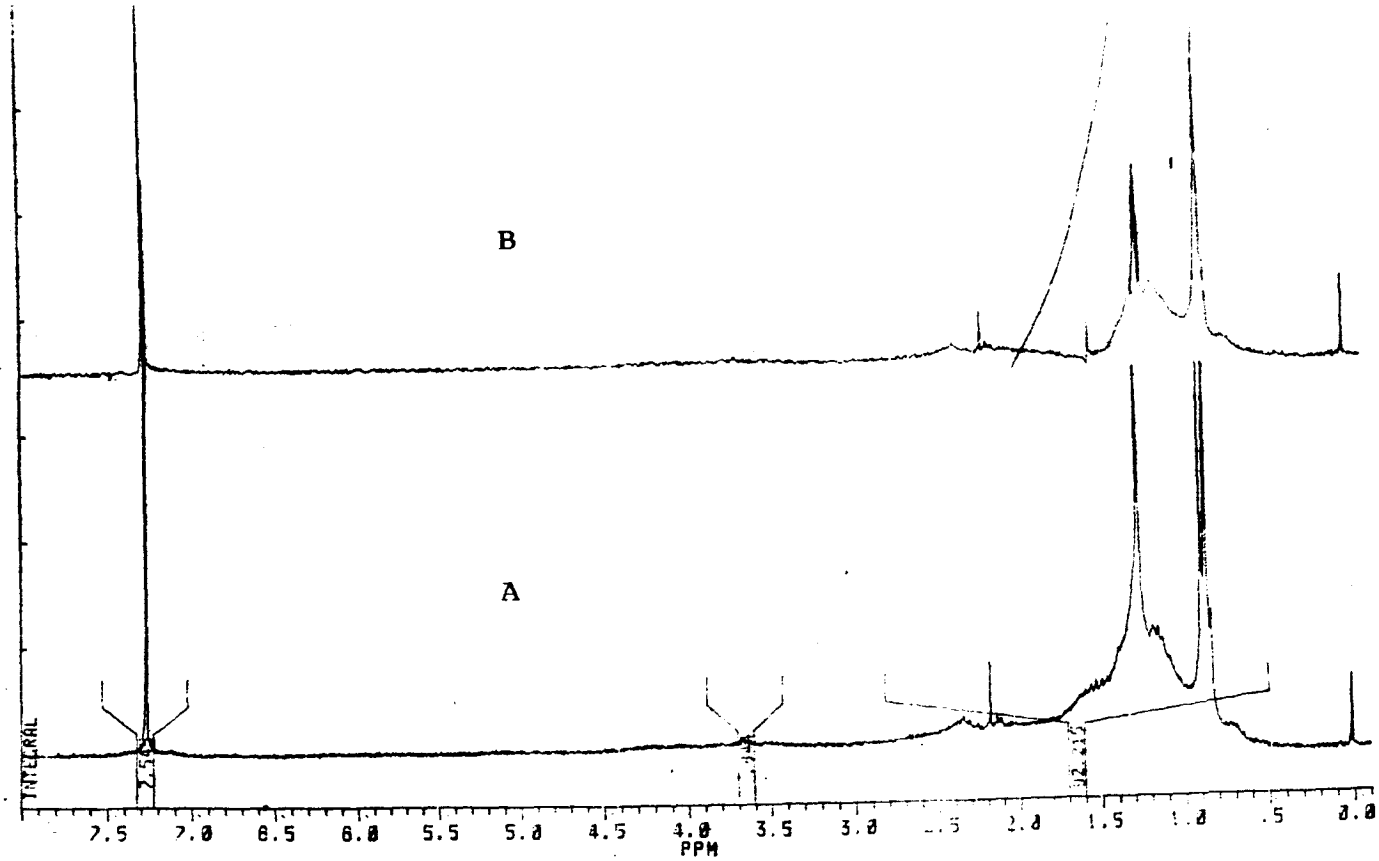


FIG 19A. ^{1}H NMR SPECTRUM OF COMPOUNDS 4 & 5
FIG 19B. DECOUPLED SPECTRUM OF COMPOUNDS 4 & 5 AT δ 1.5

The IR spectrum of the compound (6), R_t 8.27 min., had strong absorptions at 3400 & 1700-1740 cm^{-1} which indicated the presence of a carboxylic group in addition to an ester moiety. The presence of twin peaks of equal intensity at 1460 & 1380 cm^{-1} suggested its terpenoid nature. The ^1H NMR spectrum of this compound was quite similar to that of compounds (4) and (5). However, the quarternary methyl signal at δ 1.257(3H,s) of the latter compounds was absent here. Instead, a new signal appeared at δ 3.5(3H,s), possibly due to a methoxy/ or cabomethoxy group. Neither EIMS nor PCIMS yielded any molecular ion peak. But its NCIMS readily revealed the strong pseudomolecular $[M-H]^-$ ion at m/z 305 followed by its isotope peak at 307 in the ratio 3:1, indicating the presence of a chlorine atom in the molecule (FIG 20). From this, its molecular weight was deduced to be 306, corresponding to a molecular formula $C_{15}H_{27}ClO_4$. The above formula has two degrees of unsaturation, which could be easily accounted for by the ester and the free acid groups, leaving the rest of the molecule saturated and acyclic. The structure of this compound was finalised as methyl,2-chloro-2-carboxy-6,10-dimethyl undecanoate (6) from a comparison of its spectral data with that of compounds (4) and (5).

The compound (7), R_t=15.5 min, was found to be a sterol derivative by its spectral data. Its IR spectrum had absorptions at 3450 & 1700 cm^{-1}, indicating the presence of hydroxyl and carbonyl moieties. The molecular weight of this

compound was determined to be 400 by EIMS. This was 14 mass units higher than cholesterol. Thus the compound might be a methyl or a keto cholesterol derivative. Careful examination of its ^1H NMR spectrum indicated that the 3α-H which normally comes at δ 3.5 in cholesterol and similar compounds has been shifted slightly downfield, to δ 3.65 in this case. Other notable changes were: absence of the broad doublet at δ 5.35 due to 6-H in cholesterol, appearance of a new vinyl proton signal at δ 5.70(s) and a downfield shift by 0.2 ppm of 19-H signal to δ 1.20. The signals due to 18-H, 21-H, 26-H & 27-H were found in their usual places as compared to cholesterol. The structure of this compound was finalised as 7-ketocholesterol from these results. This was also supported by its ^{13}C NMR (including DEPT)[23] and mass spectra (FIGs 20 & 21)[24].

The Compound (8), R_t=49.5 min, m.p. 200°C, had strong IR absorptions at 3400, 3050, 1464 & 1380 cm^{-1}, indicating the presence of hydroxyl and vinyl groups in it. The molecular weight of the compound was found to be 412 by EI mass spectrum, corresponding to a molecular formula $C_{28}H_{44}O$. The sterol nature of the compound was indicated by its mass spectral fragmentations which had prominent peaks at m/z 397 [M-15]$^+$, 379[M-33]$^+$, 315, 314[M-C_7H_{14}]$^+$, 313, 299, 273[M-sc]$^+$, 255(273-18), 231(273-42) and 213(231-18). Examination of its ^1H NMR spectrum revealed the presence of 3β-hydroxy-Δ^5-system in the molecule. The general sterol nature of the molecule was also confirmed by the presence of the 18 and 19-angular

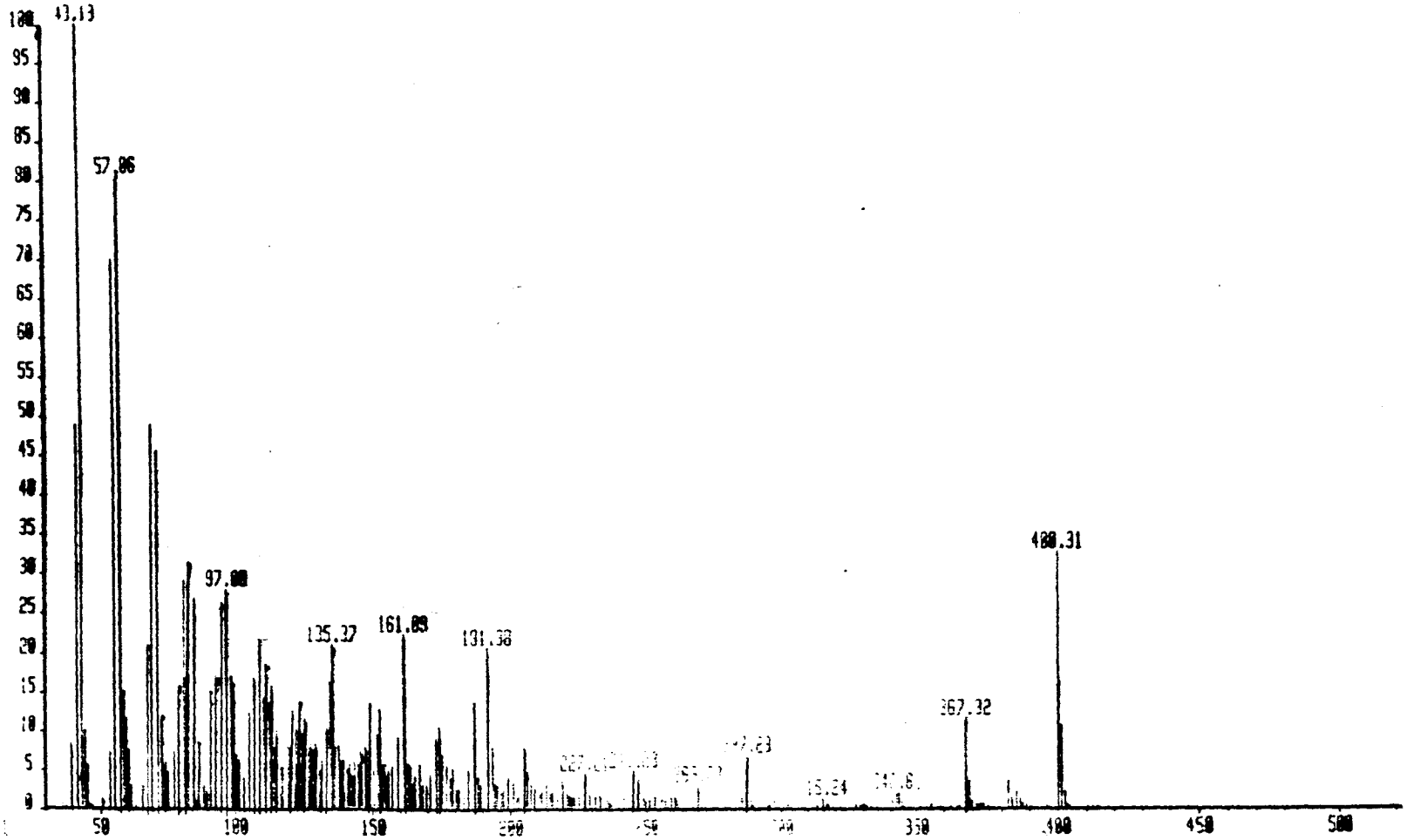


FIG 20. ELECTRON IMPACT MASS SPECTRUM OF COMPOUND (7)

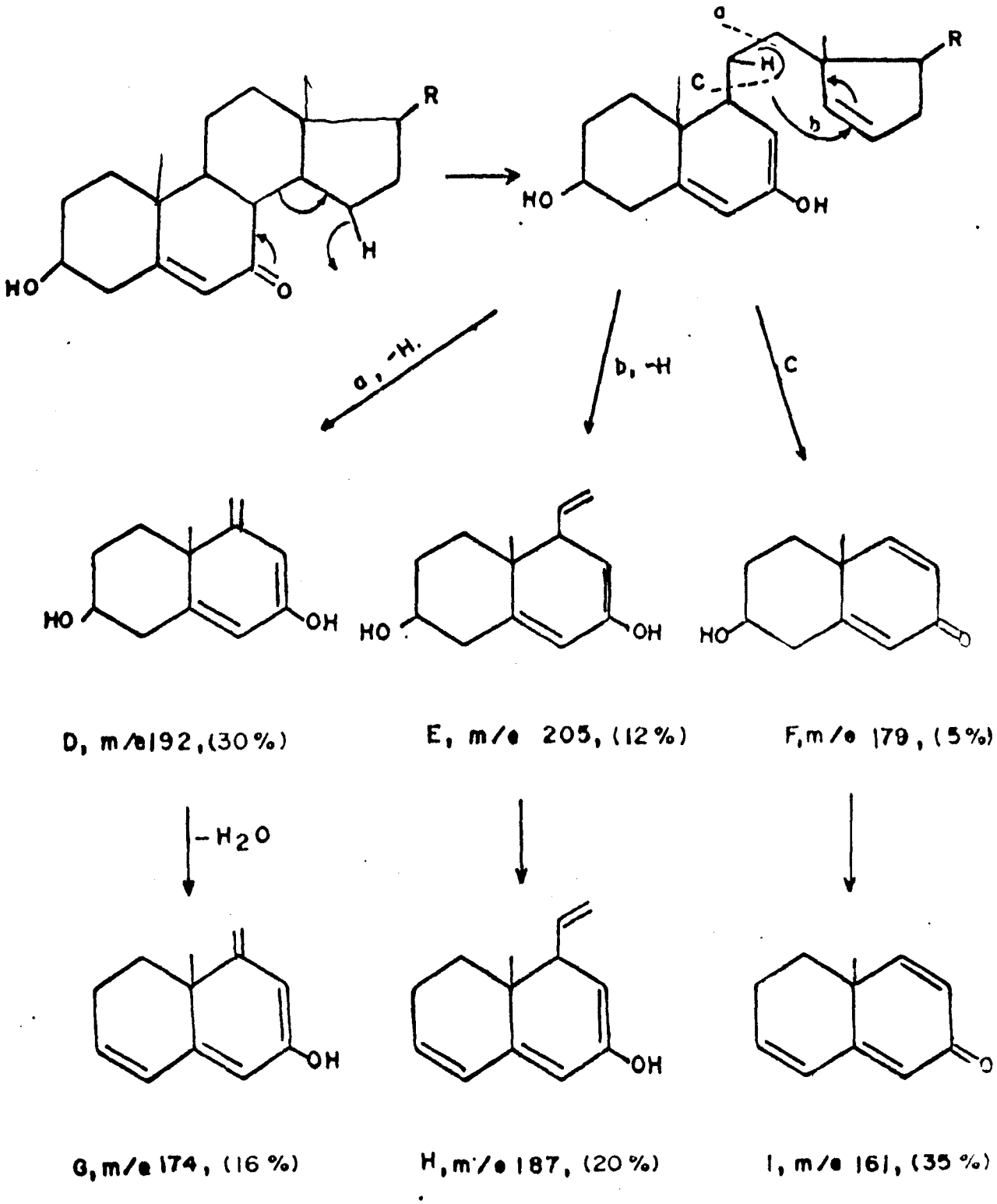


FIG 21 SOME IMPORTANT EIMS FRAGMENTS OF COMPOUND (7)

methyls at δ 0.69 and 1.01(3H each,s) respectively. In addition, the signals at δ 5.18(1H,q, 6.6Hz) and 1.57(3H,d, 6.6 Hz) suggested the presence of an ethylidene group in the side chain, possibly at C-24. This was also supported by the chemical shifts of 26 & 27-H as well as 21-H, which were seen at δ 0.975(6H,d,6 Hz) and 0.985(3H,d,5.4 Hz) respectively. The stucture of this compound was finalised as fucosterol (8) from these results. Further confirmation of the position of side chain unsaturation was provided by the strong fragment ion at m/z 313(67%), which arises out of allylic cleavage of C22-23 bond, *i.e.*, $[M-C_7H_{15}]^+$.

The spectral data of the compound (9), R_t=53.5 min, M^+=386, were similar to that of cholesterol. Thus, its ^1H NMR spectrum had peaks at δ 5.35(1H,br.d,6-H), 3.5(1H,m,3α-H), 1.01(3H,s,19-H), 0.92(3H,d,6.6 Hz), 0.86(6H,d,J=6.8 Hz,26 & 27-H) and 0.68(3H,s,18-H) which were typical of cholesterol. Similarly, the mass spectral fragment ions at m/z 371[M-15]$^+$, 368[M-18]$^+$, 358,301, 275,273, 255, 231 and 213 also confirmed the identity of this compound as cholesterol (9).

The water-soluble fraction left behind from the original methanol extract after removal of their lipophyllic compounds using pet.ether and $CHCl_3$ was purified over Sephadex G-10 and LH-20, yielding about 10 gm of a white powder, highly soluble in water. Strong absorptions at 3550-3150,1070,1045 & 1030cm^{-1} in its IR spectrum (FIG 22) as well as the cluster of peaks

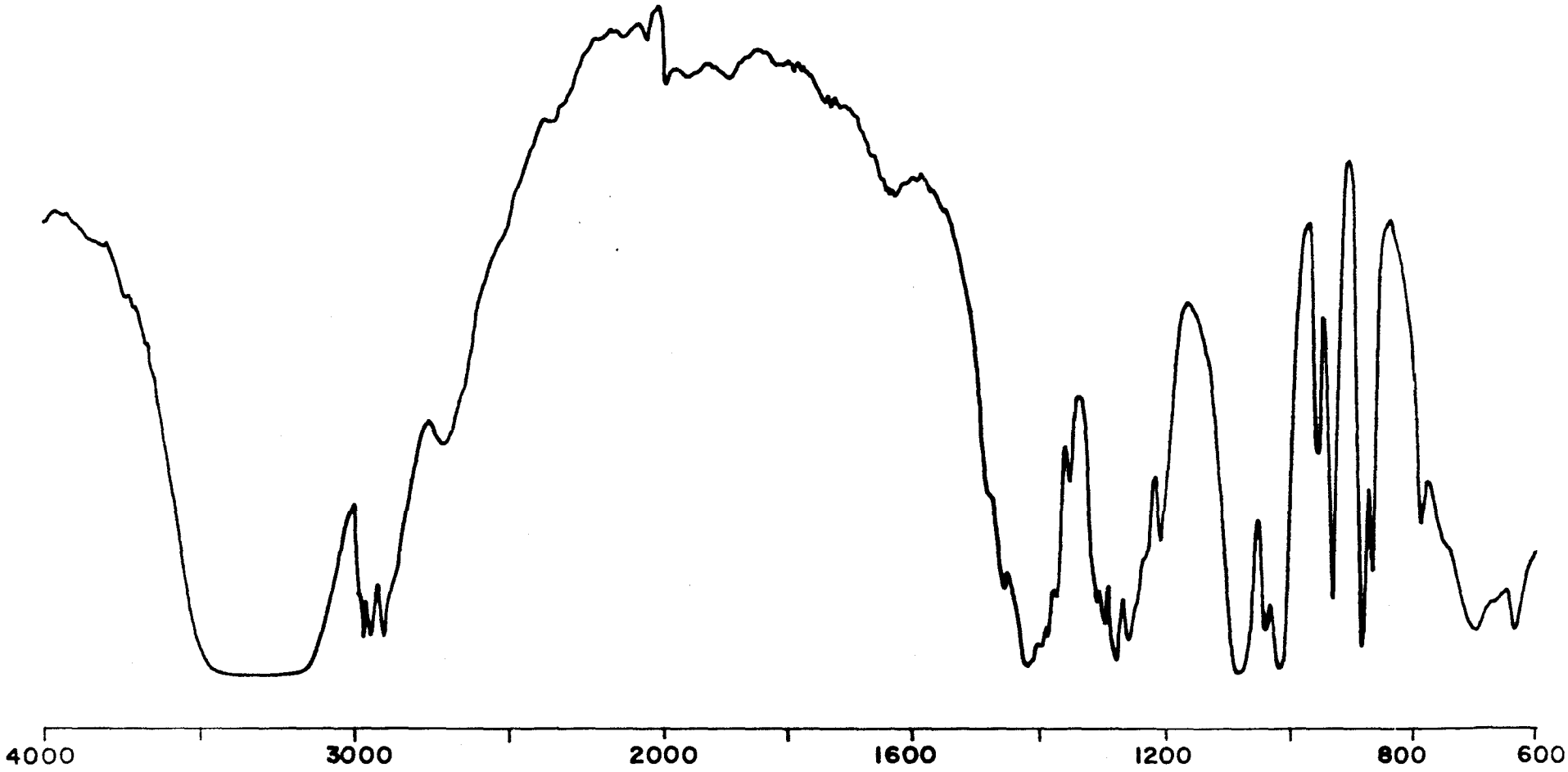


Fig. 22 IR (KBr) Spectrum of galactitol (dulcitol, 10)

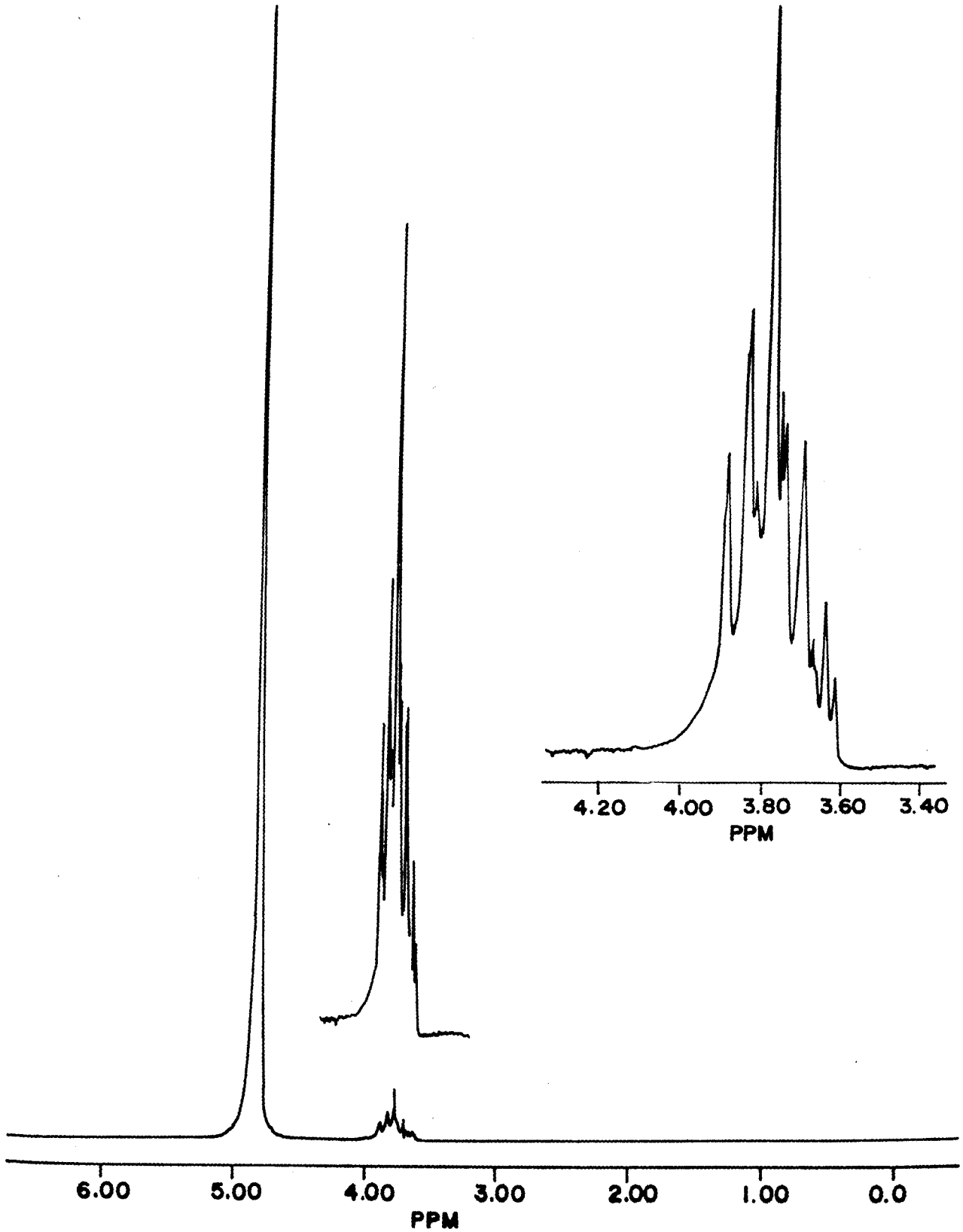


Fig. 23 ^{1}H NMR spectrum of Galactitol (10)

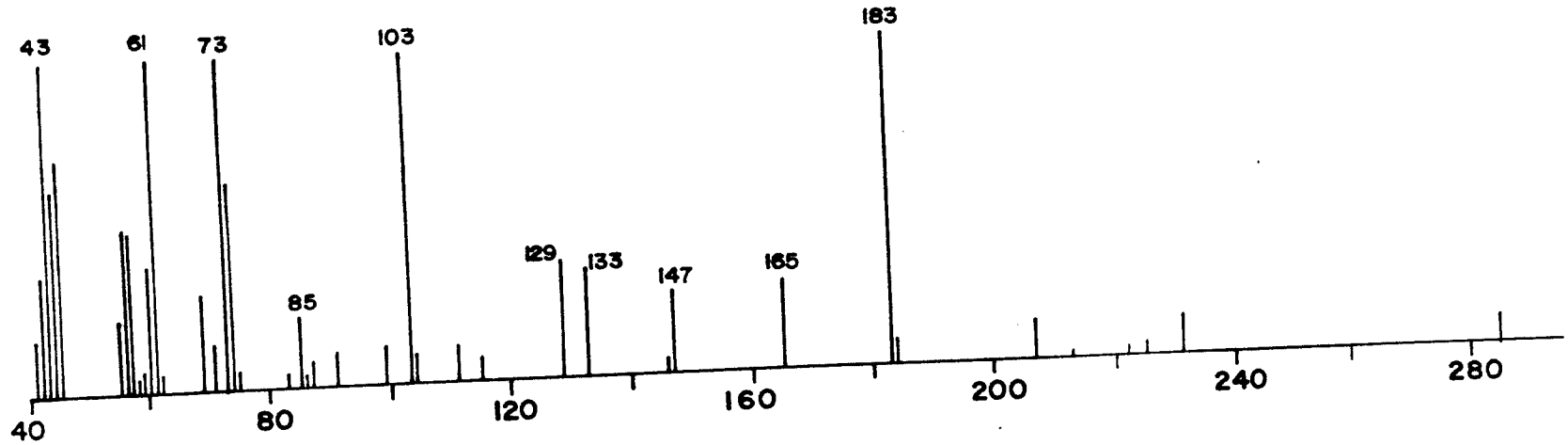


Fig. 24 Mass spectrum of dulcitol (10)

in the range of 3.6-4 ppm in the ^1H NMR spectrum (FIG 23) indicated the polyhydroxylated nature of this molecule. The molecular ion was observed in its EI mass spectrum at m/z 183, presumably due to $[M+H]^+$(FIG 24). This indicated the molecular formula to be $C_6H_{14}O_6$. Thus the compound has to be a hexol. Its ^{13}C NMR spectrum had only three peaks, at δ 70.08(d), 69.14(d), and 63.17(t). This as well as its nil optical rotation were indicative of the symmetric nature of this molecule. The ^{13}C NMR chemical shifts of various symmetric stereoisomers of hexols are as given below: mannitol (δ 76.0, 75.3 & 73.6), iditol (δ 72.16, 71.16 & 63.17), allitol (δ 65.8, 74.5, 72.9, 74.3, 76.1 & 66.1) and galactitol (δ 70.15, 69.25 & 63.25). From this, as well as from a comparison of the IR and ^1H NMR spectra of the above isomers with that of compound (10), its structure was finalised as galactitol[25].

GENERAL DISCUSSION

The compounds (1), (2), (4), (5) and (6) are reported for the first time from a natural source. However, among them, compounds (1) and (2), are previously known as synthetic products[3,18&19] or as photo-oxidation products of carotenoids or ß-ionols[4,20-22]. It is known that oxidation of fucoxanthin with $Zn(MnO_4)_2$ yields compound (2) in good yield[19]. Further, the deacetoxy analog of compound (2) has been previously isolated

from a grasshopper *R. microptera*[6] as well as from the leaves of the plant *C. rheifolia*[7]. In the former case it was the major constituent of its defensive secretion having very strong ant-repellent properties. Isoe *et al.* had obtained 2,4-dihydroxy-2,6,6-trimethyl cyclohexanone by exhaustive photooxygenation of zeaxanthin[4]. They subsequently converted it into the corresponding 4-monoacetate (1) by treatment with acetic anhydride and pyridine. This compound has also been prepared by Meinwald and Hendry during their synthesis of the grasshopper ketone[3].

Loliolide (3), also an oxidation product of carotenoids, was first isolated from the rye grass *Lolium perenne*[8]. Subsequently it was isolated from several angiosperms[9-11], algae[12-15], mollusc[9], sponge[16] and even from a marine sediment[17]. Thus, it has been isolated from the brown alga *Undaria pinnatifida* (HARVEY) by the Japanese workers Takemoto and Takeshita in 1970[12]. Subsequently, during 1982, Ravi *et.al.*, reported this compound from another brown alga *Cystophora moniliformis*[14]. Around the same time Bheemasankara Rao *et.al.*, also isolated this compound from *Padina tetrastromatica* from east coast of India[13]. In 1985, Kuniyoshi reported both (-) loliolide and (+)epiloliolide from another brown alga *Sargassum crassifolium* from Okinawa[15]. He found them to be active inhibtors of germination of the seeds of head lettuce *Lactuca sativa* var. *capitata* (LINN). First report of the occurrence of this compound from marine invertebrates was by

Schmitz *et.al.* They reported (+)loliolide and (+)epiloliolide from the sponge *Tedania ignis* in 1983[16]. Interestingly, the latter compound was found to be an active cytotoxic agent, whereas the former was inactive. Later, in 1984 Klok *et.al.*, isolated loliolide, isololiolide and dihydroactinidiolide from marine sediments[17]. These compounds constituted upto 2% of the organic matter extracted from the sediment samples from Namibian shelf at a depth of 106 meter. In their opinion, the origin of these compounds might be *via* oxidation of carotenoids such as fucoxanthin, zeaxanthin *etc.*, in the oxic zone of the water column. Incidently, both loliolide and dihydroactinidiolide are well known as flavour compounds in tea[26] and tobacco[27]. *In vitro* studies had revealed (-) loliolide to be active against human nasopharynx carcinoma (KB) and murine lymphocytic leukemia (P388) (ED_{50} 10µg/ml and 3.5-22 µg/ml respectively[28]). However, it was inactive at doses of 2.5-10 mg/Kg against P388 in *in vivo* assays[29]. In 1980, Petit *et.al.*, had isolated (-) loliolide from the mollusc *Dolabella ecaudata* from Indian waters[9]. In their paper, they have also listed 16 plants from which this compound has been reported earlier. Subsequently, Okunade and Weimer isolated (-) Loliolide from a plant identified as *Xanthoxylum setulosum* P.Wilson (*Rutaceae*) during a biassay-guided purification against the highly polyphagous leaf cutter ants *Atta cephalotes* (*Hymenoptera, Formicidae,* Attini)[10]. The occurrence of loliolide in higher plants is natural, given the chances of its formation from zeaxanthin,

the yellow pigment of corn and the petals of *Physalis* sp[4]. Similarly, photolysis of zeaxanthin diepoxide (violaxanthin), from yellow pansies (Viola tricolor) yields a mixture containing loliolide[30]. Both the above carotenoids form a major part of the pigments in several plants. Hence it may be possible that loliolide is an artifact produced by air oxidation during the extraction process. However, there is evidence to show that loliolide may, after all, be a genuine natural product. Thus, the roots of *Canscora decussata* (Gentianaceae) which is free of carotenoids, contains loliolide[31]. As pointed out by Ghosh *et al.*, in this case, loliolide might have been initially formed in its leaves which then migrated to the roots during growth.

Halogenated terpenoids occur very rarely in brown algae whereas they are abundant in green and red algae. To the best of our knowledge, this is the first report of natural occurrence of compounds (4), (5) and (6). However, saturated long chain terpenoids have previously been reported from this alga. Among the sterols, cholesterol and fucosterol are common to several marine fauna and flora. Recently there are also reports of occurrence of 7-keto and 7-hydroxysterols as well as their $\Delta^{5,8}$-7-keto analogs from several marine organisms[32]. The latter compounds might be artifacts, produced by dehydration of the former keto derivatives. We too have isolated several 7-hydroxy and 7-ketosterols from a sponge *Ircinia ramosa* (KILLER) collected from Lakshadweep islands,

as will be seen in chapter 3. It is conceivable that in these organisms 3ß-OH,Δ^5-sterols are selectively oxidised at C-7 yielding the corresponding hydroxyl or keto derivatives.

EXPERIMENTAL

HPLC separations were carried out on SPECTRAPHYSICS MODEL 8800 fitted with an RI detector or HEWLETT PACKARD (HP 1090) instrument fitted with a Diode Array Detector (DAD) in conjunction with a chem-station. Reverse and normal phase separations were carried out using ODS (5 µm, 250X8 mm^2) and µ-Porasil (10 µm, 250X4.6 mm^2) columns with MeOH/aq.MeOH and Hexane-THF(85:15) as mobile phases respectively. The IR spectra of these compounds were recorded on a PERKIN ELMER SPECTROPHOTOMETER, MODEL 1640. ^1H and ^{13}C NMR spectra were recorded on a BRUKER WM 200 machine using $CDCl_3$ as solvent and TMS as internal standard. EIMS were recorded on a VG-70 ER at a temp. gradient of 5°C/sec and 1 spectrum/2 secs. The TIC curves too were similarly recorded. CIMS was recorded on a BIOSPECT instrument using ammonia as reagent gas.

Sea weeds (5 kg,dry weight), collected from Anjuna, Goa were washed with fresh water, dried in shade, and soaked in MeOH at ambient temperature. After 3 weeks the solvent was drained off, and concentrated to one-tenth of its original

volume under vacuum. The above concentrate was then successively extracted with pet.ether and $CHCl_3$, yielding the respective fractions. The lipid fractions as well as the water-soluble fraction were subsequently concentrated under vacuum to dryness.

The pet.ether and the $CHCl_3$ fractions were purified by repeated column chromatography over silica gel using various pet.ether-EtOAc gradient systems, yielding subfractions rich in fatty acids, their methyl esters, sterols and terpenoids from the former and a few more polar terpenoids including compounds (1-3) from the latter. Thin layer chromatography (TLC) of the purfied $CHCl_3$ fraction indicated it to be a mixture of three major components having R_f values 0.77, 0.46 & 0.37 (SiO_2, 20%EtOAc-Pet.ether). They were separated by preparative TLC on 1.0 mm thick plates and about 25% EtOAc-Pet.ether system. Analytical HPLC (μ-Porasil, 15% THF in hexane, DAD) and Total Ion Curve (TIC) obtained during their high resolution EIMS studies indicated that these compounds are relatively pure, In addition, the purity of compound (2) was also checked using the new techniques such as ratio plots, 3D-plot (time *vs.* wavelength *vs.* absorption), and UV-Vis derivative spectrum during its HPLC analysis (FIGs 6-9). In ratio plot, the chromatogram is monitored at two wavelengths simultaneously and subsequently their ratio over the range taken. If the given compound is pure, its ratio plot will be a horizontal line as seen in this case. In the

derivative spectra, both first and second derivatives of the UV-Vis absorption spectra of the compound are recorded. Here as well as in the 3D-plot, the purity of the compound is checked from the shape of respective spectra. A portion of the fatty acids purified from the pet.ether fraction was esterified using diazomethane. The methyl esters thus produced were mixed with the esters previously isolated and analysed for their fatty acid composition on capillary GC(SE 30,12.5 m,N_2,5 ml/min,FID). The oven temperature was gradually raised from 150°C to 200°C at the rate of 5°C/min, while the detector temperature was maintained at 250°C during the analysis. The mixture of sterols and terpenoids initially separated on silica gel columns were subsequently purified on HPLC (ODS,250X8 mm^2,MeOH,2ml/min), fitted with a RI detector, leading to the isolation of the halogenated terpenoids(4-6), 7-ketocholesterol(7), fucosterol (8) and cholesterol (9).

The water-soluble part of the extract, remaining after successive removal of pet.ether and $CHCl_3$ soluble compounds as described above, was initially desalted over Sephadex G-10 using 1:1 mixture of MeOH-H_2O and then purified over Sephadex LH-20 using MeOH as eluent. This yielded about 10 gm of a white amorphous powder, homogenous on TLC plate using various solvents. The structure of this compound was finalised as galactitol (dulcitol 10) from its spectral data.

(2R,4S)-4-Acetoxy-2-hydroxy-2,6,6-trimethyl cyclohexanone (1), IR(neat): 3400, 1740, 1710, 1460, 1370, 1250 & 1030 cm^{-1}; ^1H NMR($CDCl_3$): δ 5.25-5.45(1H,m,4-H), 2.3(1H,ddd,J=14,5 & 1.5 Hz), 2.08(3H,s,acetate methyl), 2.0-2.16(2H,m,3-H & 5-H), 1.95(1H,ddd,J=14,7.5 & 5 Hz,5-H), 1.46(3H,s,2-CH_3) and 1.26 (6H,s,6 & 6'-CH_3); ^{13}C NMR($CDCl_3$): δ 215(s,C-1), 170(s,OAc), 74.47(s,C-2), 66.68(d,C-4), 43.19 and 43.03(t's,C-3 & C-5), 30.0(s,C-6), 27.87, 27.56, & 27.46(q's,C-2,6,6-methyls) and 21.23(q,OAc); PCIMS(NH_3): 232[M+NH_4]$^+$; NCIMS(NH_4Cl): 249 and 251 in the ratio 3:1,[M+^{35}Cl]$^-$ and [M+^{37}Cl]$^-$ respectively. HREIMS confirmed the molecular formula as $C_{11}H_{18}O_4$ (observed 215.1242 for [M+H]$^+$) and that of the major fragment at m/z 185 as $C_{10}H_{17}O_3$ [M-CO-H]$^+$ (experimental value 185.1177).

$NaIO_4$ OXIDATION OF COMPOUND (1).

About 0.2 mg of the compound (1) was dissolved in 0.5 ml EtOH and treated with a saturated solution of $NaIO_4$ in 50%aq.EtOH(0.5 ml) for one hour at 50°C. The reaction mixture was then diluted with water and the product extracted with ether. The CIMS of the product at positive and negative ionizations gave the pseudomolecular ion peaks at m/z 248 [M+NH_4]$^+$ and 265[M+Cl]$^+$ respectively, indicating its molecular weight to be 230, corresponding to the structure (1A, FIG 4).

HYDROLYSIS OF (1A) TO (1B, FIG 4).

To the keto acid (1A) dissolved in EtOH(0.5 ml), was added 0.5 ml of 0.1N alc.KOH and the mixture maintained at 50°C for 1 hr. in a water bath. The reaction mixture was subsequently diluted with water (1 ml), acidified with dil.HCl and the product extracted with ether. The positive and negative CIMS of the product yielded the corresponding pseudomolecular ion peaks at m/z 188[M+18]$^+$ and 205[M+Cl]$^-$ respectively, indicating the formation of the lactone (1B).

3R,4-[(2R,4S)-2-Hydroxy-4-acetoxy-2,6,6-trimethylcyclohexylidene]-but-3-en-2-one (Apo-9'-fucoxanthinone 2), IR(neat): 3440, 2960, 2930, 2870, 1940, 1730, 1710, 1670, 1450, 1360, 1250, 1180, 970, 960 & 860 cm^{-1}; UV(MeOH): 232 and 204 nm; ^{1}H NMR($CDCl_3$): δ 5.87(1H,s,8-H), 5.38(1H,dd,J=10 & 3.3 Hz,4-H), 2.34(1H,ddd,J=13,4.3 & 1.7 Hz,3α-H), 2.2(3H,s,10-H), 2.05(3H, s,acetate methyl), 1.95(1H,ddd,J=13,3.3 & 1.7 Hz,5α-H), 1.55 (1H,m,3β-H), 1.45(1H,m,5β-H), 1.43(6H,s,12 & 13-H) and 1.16 (3H,s,11-H); ^{13}C NMR($CDCl_3$): δ 209.2(s,C-7), 197.74(s,C-9), 170.24(s,acetate carbonyl), 118.5(s,C-5), 100.78(d,C-1), 71.86(s,C-2), 67.52(d,C-4),45.16 & 45.03(t's,C-3 & C-5), 35.95(s,C-6), 31.57, 30.68, 28.92 & 26.31(all q's,C-10,11,12 & 13) and 21.16(q,acetate methyl), see FIGs 11 & 12; PCIMS: 284[M+18]$^+$; NCIMS: 301 and 303 in the ratio 3:1 due to the pseudomolecular ions [M+^{35}Cl]$^-$ and [M+^{37}Cl]$^-$ respectively. EIMS m/z,%): 266(M$^+$,1), 252(1), 224(4), 207(4), 206([M-60]$^+$,22),

191(52), 164(34), 163(65), 149(29), 145 (18), 131(40) and 123(100), see FIG 15; From the HREIMS the formulae of the molecular ion as well as the fragment ion at m/z 224 were determined to be $C_{15}H_{22}O_4$ (266.1477 as against the expected value of 266.1518) and $C_{13}H_{20}O_3$ (224.1393 as against the expected value of 224.1421) respectively.

LAH REDUCTION OF COMPOUND (2) INTO ALLENIC AND ACETYLENIC TRIOLS

About 0.5 mg of the compound (2) was dissolved in 1 ml of alcohol and stirred with LAH (10 mg) under N_2 atmosphere overnight. Later the mixture was diluted with 0.5 ml of water and then treated with 0.1N HCl(0.5 ml) to decompose the unreacted LAH. The product was later extracted from this mixture using ether. IR(neat): 3400,2940, 2840, 2220 & 1940 cm^{-1}; ^1H NMR($CDCl_3$): 5.38(1H,d,J=5.5 Hz,4-H), 4.3(1H,m,9-H), 3.5(1H,m,4-H), 2.23(1H,ddd,J=14,7 & 4.5 Hz,3-H), 1.92(1H, ddd,J=14,4.5 & 1.7 Hz,5-H), 1.8-1.4(2H,m,3'& 5'-H), 1.32(6H, s), 1.29(3H,d,J=6.5 Hz) and 1.09(3H,s). These values agreed well with those of analogous allenic triols reported in literature[3,18].

OZONOLYSIS OF COMPOUND (2) INTO (1).

About 0.25 mg of the allenic compound (2) was spotted on a silica gel plate, which was then exposed to ozonised oxygen for 2 min inside a partly covered TLC chamber. The positive and negative CIMS of the compound was recorded in the usual way. The prominent pseudo molecular ions found in PCIMS were at m/z 232[M+18]$^+$ and 215[M+H]$^+$ while the NCIMS yielded ions at m/z 249 and 251 in the ratio 3:1 due to [M+^{35}Cl]$^-$ and [M+^{37}Cl]$^-$ adduct ions. These results indicated the cleavage of the allenic bond by ozone, leading to the formation of the cyclohexanone derivative (1).

Loliolide (3), m.p. 153°C; IR(neat): 3440,3020, 2980, 2950, 2920, 2880, 1730,1680,1620, 1470,1390, 1260,1230, 1160, 1030, 960 & 860 cm^{-1}; ^{1}H NMR($CDCl_3$): δ 5.82(1H,s,7-H),4.42(1H,m,3-H), 2.53(1H,m,4β-H), 2.05(1H,m,2β-H), 1.89(3H,s,5-Me),1.7 (2H,m,4 & 2α-H), 1.58(3H,s,1α-Me) and 1.35(3H,s,1β-H); ^{13}C NMR($CDCl_3$): δ 182.39(s,C-8), 171.0(s,C-6), 112.93(d,C-7), 86.65 (s,C-5), 66.83(d,C-3), 47.31 & 45.63 (t's,C-2 & C-4), 35.90 (s,C-1), 30.64(q,5-Me), 27.0 & 26.48(q's,1&1'- Me's); EIMS(M$^+$,%): 196 (M$^+$,11), 178(53), 163(19), 149(15), 140 (45), 135(28) and 111 (100); HREIMS of ions at m/z 196, 178 and 111 indicated their formulae to be $C_{11}H_{16}O_3$ (Experimental:196.1105 as against the expected: 196.1095), $C_{11}H_{14}O_2$ (Experimental:178.0965 as against the expected: 178.0990) and $C_6H_7O_2$ (Experimental:111.0430 as against the expected: 111.044) respectively.

2-Chloro-2,6,10-trimethylundecanoic acid (4) and 2-Bromo-2,6,10-trimethylundecanoic acid (5), R_t=10.28 min, IR(neat): 3440, 2950, 2920, 2865, 1716, 1460, 1375, 1240 & 1170 cm^{-1}; ^1H NMR($CDCl_3$): δ 1.52(1H,m), 1.25(3H,s), 1.4-1.0(13H,m), 0.865 (6H,d,J=6.5 Hz) and 0.84(3H,d,6.5 Hz); ^{13}C NMR($CDCl_3$): δ 39.03 (t), 37.3(t), 24.4(t), 32.8(d), 27.97(d&t), 22.7(q), 22.6(q), 19.7(q); NCIMS m/z(%): 307(53),305(49), 261(100) and 263(33), indicating the presence of a chloro and a bromo dereivatives of molecular weights 308 and 262 respectively; EIMS m/z(%): 227([M-X]$^+$,1), 167(2), 149(4), 139(2), 99(7), 98(4), 97(9), 85(10) and 83(11).

Methyl,2-chloro-2-carboxy-6,10-dimethylundecanoate (6), R_t=8.27 min; IR(neat): 3400, 1740-1700, 1460 and 1375 cm^{-1}; ^1H NMR($CDCl_3$): δ 3.5(3H,s), 1.5(1H,m), 1.4-1.0(13H,m), 0.87(6H, d,J=6.4 Hz) & 0.83 (3H,d,J=6.5 Hz); ^{13}C NMR($CDCl_3$): δ 50.25 (q), 38.97(t), 36.9(t), 32.4(d), 27.5(d), 24.4(t), 22.3(q), 22.2(q) & 19.3(q); NCIMS m/z(%): 307(33) and 305(100). This indicated that the molecular weight of this chloro derivative is 306.

7-Ketocholesterol (7), R_t=15.5 min; ^1H NMR($CDCl_3$): δ 5.7(1H, s), 3.65(1H,m), 1.20(3H,s), 0.92(3H,d,J=6.4 Hz), 0.865(6H,d, J=6.4 Hz) and 0.69(3H,s); ^{13}C NMR($CDCl_3$): δ 36.4(C-1), 31.2(C-2), 70.54(C-3), 38.7(C-4),126.1(C-6), 45.42(C-8), 49.98(C-9), 38.3(C-10), 21.22(C-11), 28.53(C-12),43.1(C-13), 49.98(C-14), 26.31(C-15),39.48(C-16), 54.81(C-17),11.97(C-18),18.87(C-19),

35.7(C-20), 19.72(C-21), 36.2(C-22), 23.8(C-23), 41.8(C-24), 27.99(C-25),22.7(C-26) and 22.5(C-27); EIMS m/z(%): 400(M^+, 45), 382(8), 367(15), 315(3), 287(10), 269(5), 245(8), 205 (12), 192(30), 187(20), 179(5), 174(16), 161(35), *etc.*

Fucosterol (8), m.p. 200°C; R_t=49.5 min; IR(neat): 3500, 3050, 2940-2800, 1380, 1264 and 1049 cm^{-1}; ^1H NMR($CDCl_3$): δ 5.35(1H,br.d), 5.17(1H,q,J=6.3 Hz), 3.52(1H,m), 1.57(3H,d, J=6.7 Hz), 1.01(3H,s), 0.985(3H,d,J=5.4 Hz), 0.975(6H,d, J=6.05 Hz) and 0.69(3H,s); EIMS m/z(%): 412(M^+,3.85), 398 (2.57), 397(3.13), 379(2.43), 315(2.32), 314(18.21), 313 (67.45), 299, 273, 255, 231, 213, *etc.*

Cholesterol (9), R_t=53.5 min; IR(neat): 3500,3050, 2940, 1455, 1380, 1047 & 894 cm^{-1}; ^1H NMR($CDCl_3$): δ 5.35(1H,d), 3.48(1H,m), 1.01(3H,s), 0.918(3H,d,J=6.4 Hz), 0.867(6H,d, J=6.5 Hz); EIMS m/z(%): 386(16.35), 372(2.3), 371(7.66), 368(9.48), 353(8.57), 275(15.6), 273(5.06), 231, 213, *etc.*

Galactitol (dulcitol 10), m.p. 168°C; IR(KBr): 3550-3150, 2970, 2940, 2910, 1420, 1280, 1260, 1210, 1070, 1045, 1030, 955, 930, & 890 cm^{-1}; ^{13}C NMR(D_2O): 70.78(d), 69.14(d) and 63.17(t); EIMS m/z(%): 183(M^+,100), 165(28.8), 146(25.1), 132(33.5), 128(36.5), 110(12), 102(100), 85(22.5), 73(100), 61(100), 43(100), *etc.* The IR, ^1H NMR and EI mass spectra are reproduced in FIGs 22, 23 and 24 respectively.

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CHAPTER 3

STEROLS FROM THE SPONGE

IRCINIA RAMOSA KELLER

Marine sponges are a rich source for many novel chemical compounds including pigments, fatty acids, sterols, terpenoids, nucleosides *etc.* Taxonomists have classified these animals into mainly two types: (a) those having mineral skeleton and (b) those not having mineral skeleton. The animals belonging to the latter group have, instead, a fibrous skeleton (keratosa). They are further subdivided into the following two orders: *Dictyoceratida* and *Dendroceratida*. All animals with an "obviously dentritic skeleton and large sac-shaped choanocyte chambers or forms lacking a fibrous skeleton compeletely are placed in the *Dendroceratida*." while others are placed in *Dictyoceratida*. In the systematic classification, the latter *Dictyoceratida* sponges are further subdivided into three families: the *Spongiidae, Thorectidae* and *Dysideidae*. The sponge *Ircinia ramosa* belong to the family: *Thorectidae* in this classification[1]. Chemotaxonomists, studying the chemical constituents of various *Dictyoceratida* sponges have noticed an abundance of mainly six types of terpenoids in them. Accordingly, sponges of the family *Thorectidae* in general and genus *Ircinia* in particular are rich in several linear furanosesterpenes and some C_{21} and C_{22} truncated sesterpenes. These latter compounds are believed to be produced from the former. Altogether, seven *Ircinia* sponges have been investigated chemotaxonomically. All of them were found to have linear furanosesterpenes, while one sponge, *Ircinia oros* had some truncated, C_{21} and C_{22} linear furanosesterpenes in addition to the former[1].

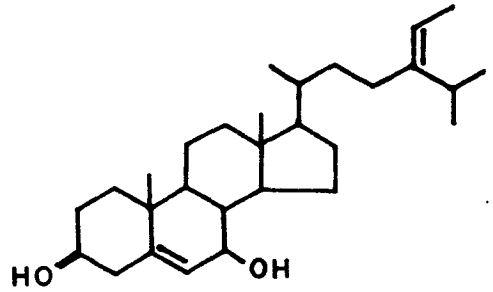
During our screening programme of marine flora and fauna for bioactive compounds, we studied the MeOH extract of the sponge *Ircinia ramosa* KELLER collected from Bangaram island, Lakshadweep. Initial pharmacological screening of the above extract indicated it to have promising antiviral[2], CNS stimulant[2] and diuretic[3] properties. Continuing our chemical investigations of this extract, we isolated several sterols (1-11), the structures of which were finalised from their spectral data including IR, NMR and MS.

The sponge was thoroughly washed with fresh water and immediately soaked in MeOH before transportation to the laboratory. After about 2 wks the solvent was decanted off, filtered and concentrated under vacuum to one-tenth of its volume. The lipid soluble compounds were extracted from this partially concentrated extract using pet.ether and $CHCl_3$ (three times each). These lipid fractions, which are rich in several fatty acids, sterols and terpenoids were repeatedly chromatographed over silica gel columns using gradient pet.ether-EtOAc solvent systems. This yielded about 100mg of a fraction rich in sterols. The above fraction, upon purification on an analytical HPLC (ODS column, 250X4.6 mm, MeOH-water (100:8), 1.3 ml/min, RI detection), yielded 11 subfractions. Examination of their spectral data revealed that fractions 3-11 represent pure compounds, whereas fractions 1 and 2, are mixtures of at least two compounds each. Since these compounds were isolated in very low yield,

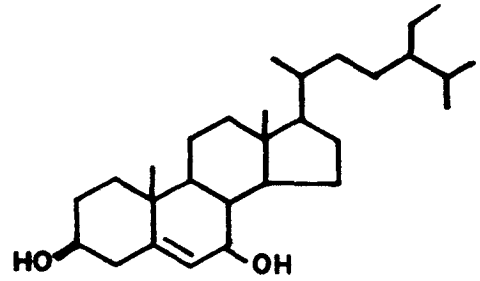
they were analysed without further purification. The names of sterols thus isolated together with their respective HPLC retention times and molecular weights are listed in TABLE 1, while their structures are provided in FIG 1.

TABLE 1. STEROL COMPOSITION OF THE SPONGE *IRCINIA RAMOSA*

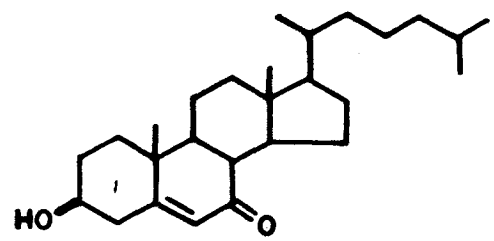
Ser. No	R_t (min)	Name of the compound	M^+
1	11.3	7-Hydroxy fucosterol(1A)	428
2	11.3	7-Hydroxy sitosterol(1B)	430
3	12.4	7-Ketocholesterol(2A)	400
4	12.4	7-Ketodesmosterol(2B)	398
5	17.4	7-ketositosterol(3)	428
6	28.2	24R-Methylcholest-4,22E-dien-3-one(4)	396
7	30.0	Cholest-4-en-3-one(5)	384
8	33.6	24R-Methylcholest-5,22E-dien-3ß-ol(6)	398
9	35.4	Cholesterol(7)	386
10	36.2	24S-Methylcholest-4-en-3-one(8)	398
11	41.4	ß-Sitosterone(9)	412
12	43.8	24S-Methylcholesterol(10)	400
13	51.6	ß-Sitosterol(11)	414



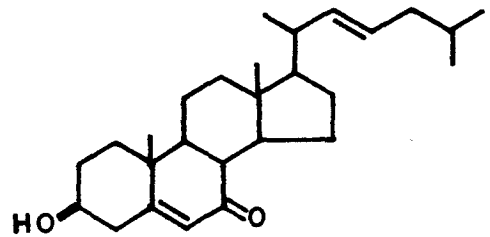
7-hydroxy fucosterol (1A)



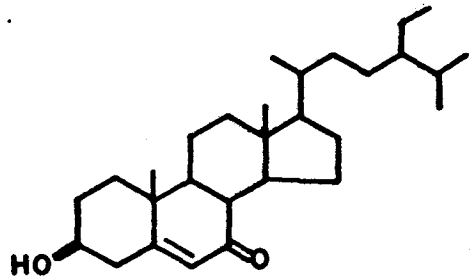
7 hydroxy sitosterol (1B)



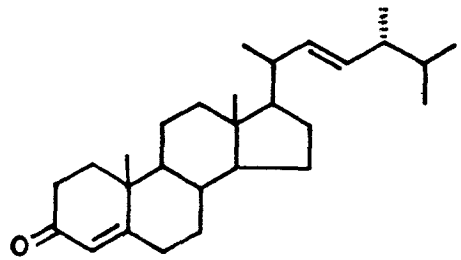
7 Keto cholesterol (2A)



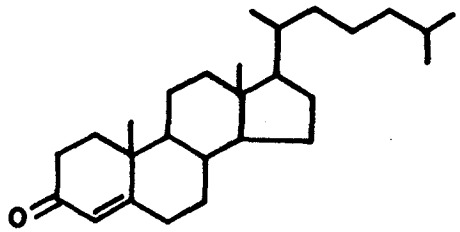
7 Keto desmosterol (2B)



7 Keto sitosterol (3)

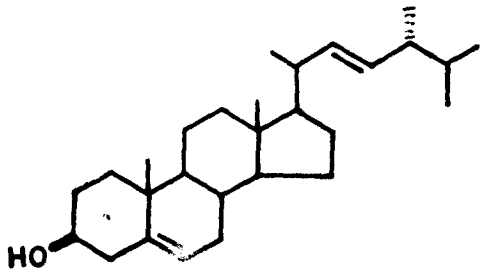


24 (R) - Methylcholest-4,22E dien-3-one (4)

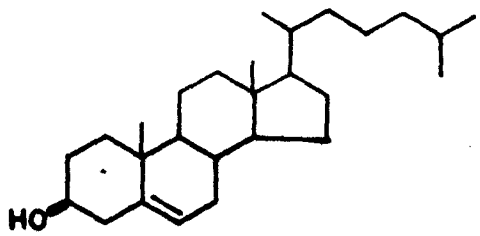


Cholest-4-en-3-one (5)

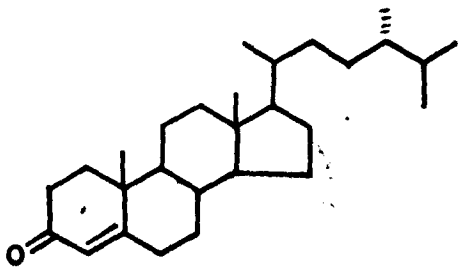
Fig. 1. Sterols isolated from the sponge Ircinia ramosa



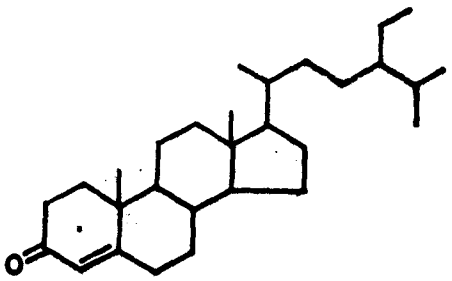
24 (R) - Methylcholest -5 ,
22 E - dien - 3 β - ol (6)



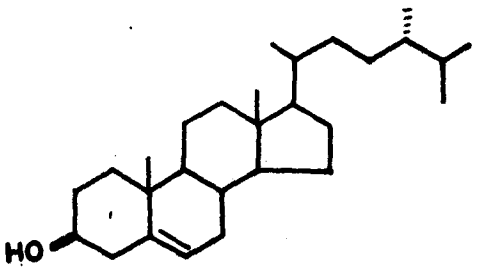
Cholesterol (7)



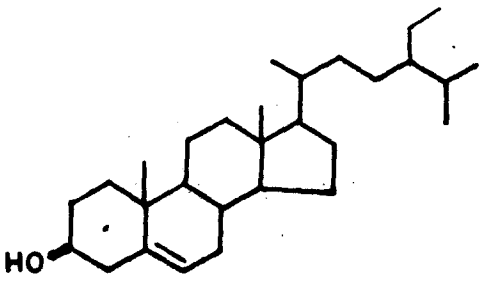
24 (S) - Methylcholest - 4 -
en - 3 - one (8)



β - Sitosterone (9)



24 (S) Methylcholesterol (10)



β - Sitosterol (11)

Fig. 1 Continued .

From this table, it is clear that this sponge contains mainly four types of sterols. They may be classified as shown in TABLE 2 below.

TABLE 2. CLASSIFICATION OF STEROLS FROM THE SPONGE *IRCINIA RAMOSA*

Group No	Structural type
Group I	3ß-hydroxy-Δ^5-sterols
Group II	3-keto-Δ^4-sterols
Group III	3ß-hydroxy-Δ^5-7-keto sterols
Group IV	3ß,7-dihydroxy-Δ^5-sterols

As is obvious from the above two tables, compounds (4), (5), (8) and (9) were found to belong to group II, (6), (7), (10) and (11) to group I, (2) and (3) to group III and (1) to group IV in the above classification.

All compounds belonging to group II had strong absorptions at 1700 & 1660 cm^{-1} in their IR spectra, indicating the presence of conjugated carbonyl groups in them. This was also supported by their strong UV absorptions at 232 nm. These UV absorptions also indicated the presence of ß,ß-dialkyl substitution of the chromophore. Another significant observation was the lack of absorption at 3500 & 1040 cm^{-1} in their IR spectra which ruled out the presence of hydroxyl

groups in these molecules. The above results indicated the presence of 3-keto-Δ⁴-moiety in all these compounds. This was supported by their ^1H NMR spectra where the methyl signal, 19-H, was found at δ 1.18-1.20 as compared to their position at δ 1.02 in cholesterol-type compounds. This downfield shift by 0.2 ppm of 19-H is expected for 3-keto-Δ⁴-steroids[4]. This was also confirmed by their mass spectral fragmentations[5] and ^{13}C NMR spectra[6]. Djerassi had noted earlier that 3-keto-Δ⁴-steroidal compounds generally produce an intense fragment ion at m/z 124 which at times will be their base peak[6]. All the compounds in group II: (4), (5), (8) and (9), had this peak in their mass spectra in very high intensity (60-100%).

The molecular weight of compound (5) was determined to be 384 by EIMS. This was two mass units lesser than cholesterol. Comparison of the ^1H NMR chemical shifts of this compound with that of cholesterol indicated their structural similarity. Thus the chemical shifts of 18-H, 21-H, 26-H & 27-H of this compound and cholesterol were nearly identical. This, as well as the absence of 3-H and 6-H signals of cholesterol in this compound, the appearance of a new vinyl proton singlet at δ 5.76 and the downfield shift by 0.2 ppm of H-19 signal which now appeared at δ 1.18 indicated its structure to be cholest-4-en-3-one (5). Compound (4), M^+=396, had 12 mass units more than its analog (5). This indicated the presence of an additional carbon atom and a double bond in it. Indeed, its ^1H NMR spectrum which had additional vinyl

proton signals at δ 5.17(2H) and an extra methyl doublet in the high field region confirmed these assumptions. The signals due to 18 and 19-H (14-Me and 10-Me respectively) were found at δ 0.72 and 1.18 as expected for similar compounds[4]. However, the side chain methyl groups appeared at different places as compared to the previous compound. This as well as the presence of two additional vinyl protons and a methyl group in the ^{1}H NMR spectrum led to the conclusion that these groups are present in the side chain of the molecule. All the four methyl signals of the side chain appeared as doublets at δ 1.01, 0.92, 0.834 & 0.817. The latter two signals could be from 26 and 27-H. Their appearance at higher field compared to compound (5) indicated that the additional methyl group is at C-24 and consequently the double bond between C22-23. This also explains the downfield shift of 21-H to δ 1.01 from its usual place at 0.92 in cholesterol-type compounds. The methyl signal at δ 0.92(d) is assigned to 28-H. These assignments agree very well with those of similar compounds reported in literature[7]. The stereochemistry of the double bond was established to be E(trans), and that of 24-methyl substitution (R) or (α) based on the comparison of the ^{1}H NMR data with those of similar compounds (TABLE 3).

TABLE 3. ^{1}H NMR CHEMICAL SHIFTS OF Δ^{22R},24α & Δ^{22R},24ß-METHYL STEROIDS[7a], COMPOUND (4) AND COMPOUND (6)

H-No	Δ^{22R},24ß	Δ^{22R},24α	Cmpd (4)	Cmpd (6)
21-H	1.02(7)	1.00(7)	1.01(6.7)	1.0(6.8)
22,23-H	5.21(m)	5.16(m)	5.17(m)	5.16(m)
26-H	0.85(7)	0.84(7)	0.834(6.7)	0.834(6.6)
27-H	0.83(7)	0.82(7)	0.816(6.7)	0.816(6.6)
28-H	0.92(7)	0.91(7)	0.91(6.7)	0.91(6.5)

It may be noted that the stereochemistry of 24-methyl group in the above compound is different from that of the common terrestrial sterol, ergosterol where it is known to be 24(S) or 24(ß).

The molecular weight of the third compound (8) in this group was determined to be 398 by mass spectrometry. This is two mass units more than that of compound (4), which indicated that this compound might be a partially saturated derivative of the latter. Its ^{1}H NMR spectrum was almost similar to that of compound (4) but for the absence of the vinyl proton multiplets at δ 5.17 and the consequent slight changes in positions of the side chain methyl group signals, which now appear at δ 0.905, 0.771, 0.851 & 0.78. They were assigned to 21, 28, 26 & 27-H respectively after comparing with the spectra of similar compounds. The stereochemistry of

24-methyl substitution was finalised to be α or (S), after comparing the chemical shifts of side chain methyl groups with those of 24(R) and 24(S) isomers (TABLE 4)[11].

TABLE 4. ^{1}H NMR CHEMICAL SHIFTS OF 24α & 24ß METHYL CHOLESTEROLS, COMPOUND (8) AND (10)[11]

H-No:	24ß-Me	24α-Me	Cmpd (8)	Cmpd (10)
21-H	0.897(s)	0.905(6.5)	0.90(7)	0.902(7)
26-H	0.848(6.8)	0.851(6.8)	0.86(6.7)	0.866(6.8)
27-H	0.799(6.8)	0.780(6.8)	0.780(6.5)	0.781(6.5)
28-H	0.769(6.5)	0.771(6.5)	0.773(6.4)	0.771(6.4)

The fourth compound in this group, (9), M$^+$=412 differed from the previous compound (8) by 14 mass unts. However, there was no consequent increase in the no. of methyl groups in this molecule. This indicated that probably the additional carbon has added to one of the existing methyl groups, thereby converting it into an ethyl substituent. The evidence for this was found upon examining the ^{1}H NMR spectrum of this compound, which had two methyl singlets, three doublets and one triplet. The singlet signals arise from the 18 and 19-H (the angular methyls at C-13 and C-10 respectively). The doublets must be from 21, 26 and 27-H. This leaves only the position C-24 for the possible attachment of the ethyl group.

This is also supported by biogenetic considerations, as well as from a comparison of its spectral data with those of similar compounds reported in literature[12]. Comparison of the chemical shifts of 21, 26, 27 and 29-H signals of this compound with those of 24(R) and 24(S) isomers suggested that the stereochemistry of this compound is probably 24(R) or (ß) (TABLE 5)[12]. The structure of this compound was finalised as ß-sitosterone (9) from these results.

TABLE 5. ^{1}H NMR CHEMICAL SHIFTS OF 24α & 24ß-ETHYL CHOLESTEROLS, COMPOUNDS (3), (9) AND (11).

H-No:	24α-Ethyl	24ß-Ethyl	Cmpd 3	Cmpd 9	Cmpd11
21-H	0.924 (6.5)	0.919 (6.5)	0.915 (6.8)	0.92 (6.7)	0.92 (6.4)
26-H	0.829 (7.1)	0.833 (7.4)	0.82 (6.9)	0.82 (6.8)	0.82 (6.8)
27-H	0.809 (7)	0.813 (7.5)	0.80 (6.6)	0.805 (6.8)	0.80 (6.4)
29-H	0.853 (7.3)	0.843 (7.5)	0.835 (7)	0.835 (6.8)	0.84 (6.8)

The IR spectra of compounds (6), (7), (10) and (11) had strong absorptions at 3450 & 1040 cm^{-1} indicating the presence of hydroxyl groups. At the same time, the absence of peaks in the region 1700 cm^{-1} ruled out the presence of any carbonyl groups in these molecules. This was also supported by their ^1H NMR spectra which had signals at δ 3.5(1H,m,3-H), 5.35(1H, brd,6-H), 1.01(3H,s,19-H) and 0.69(3H,s,18-H), characteristic of 3ß-hydroxy-Δ^5-sterols[4]. This suggested that all of them have identical ring substituents, with differences confined to side chains only. Further support for this assumption was lent by the mass spectral studies. Thus it was noted that peaks at m/z 273(M^+-side chain), 255(273-18), 231(273-42 or 273-ring D), and 213(231-18) were also common to all these compounds. Comparison of the ^1H NMR spectra of these compounds with those of group II indicated that the side chains of compounds (6), (7), (10) and (11) are identical with those of compounds (4), (5), (8) and (9) respectively. Mass spectral studies revealed that compounds of the former group have two mass units more than the corresponding compounds from the latter group. Based on the above results, these compounds were identified as 24(R)-methyl-5,22E-dien-3ß-ol (6), cholesterol (7), 24(S)-methyl cholesterol (10) and 24(R)-ethyl cholesterol or ß-sitosterol (11).

The compound (2A) R_t=12.4 min, M^+=400 was found to be a conjugated ketone from its IR and UV-Vis absorption spectra. Thus, its IR spectrum had absorptions at 3450 & 1040 (OH) and

1700 & 1660 cm^{-1} (conjugated carbonyl). This and the strong UV-Vis absorption at 232 nm indicated the presence of a conjugated carbonyl group in the molecule. The molecular weight, determined to be 400 by EIMS was subsequently confirmed by positive and negative CIMS. The former had strong peak at m/z 418 due to $[M+18]^+$, whereas the latter had two peaks at m/z 435 and 437 in the ratio 3:1 due to $[M+^{35}Cl]^-$ and $[M+^{37}Cl]^-$ respectively. The above molecular weight is 14 mass units more than cholesterol, which indicated that either one proton of cholesterol has been replaced by a methyl group or a methylene group by a carbonyl group. The latter probability was more probable from its IR and UV-Vis spectra. These results, thus, confirmed the presence of a conjugated carbonyl and hydroxyl groups in this molecule. All that now remained was to fix their positions in it. The usual signals due to 3-H and 6-H of cholesterol were absent in the ^1H NMR spectrum of this molecule. Instead, the CHOH signal was observed at δ 3.68, downfield by 0.18 ppm, while the vinyl proton (6-H) signal appeared at δ 5.76(1H,s). The latter signal may be attributed to the vinyl proton adjacent to a carbonyl group. The 19-H signal, which is seen at δ 1.01 in cholesterol appeared slightly downfield, at δ 1.20 in this molecule. Other methyl signals appeared at their usual places as in cholesterol. This indicated that the keto group is present somewhere in the A or B ring of the molecule. Two possible structures short-listed for this molecule from the above spectral data are: 3β-hydroxy-Δ^5-7-ketocholesterol or

3-keto-Δ^5-7-hydroxy cholesterol. Examination of its EI mass spectrum revealed the presence of peaks at m/z 205, 192, 187, 179 and 161, which are characteristic of 3ß-hydroxy-Δ^5-7-keto sterols[13]. The structure of this compound was thus finalised as 3ß-Hydroxy-Δ^5-7-ketocholesterol (2A).

The mass spectrum of this compound also indicated the co-occurrence of a minor component alongwith it having a molecular ion at m/z 398. Since this is 2 mass units lesser than that of compound (2A), its structure must be similar to it with an additional double bond in the molecule. The mass spectral peaks at m/z 315 and 297 could not have originated from compound (2A). They could be explained as $[398-C_6H_{11}]^+$ & $[398-C_6H_{11}-H_2O]^+$ respectively, which helped in locating the additional double bond at C-23. The structure of this compound was finalised as Cholest-5,23-dien-3ß-ol-7-one or 7-ketodesmosterol (2B) from these results.

The IR and UV-Vis spectra of compound (3) closely resembled that of compounds (2A) and (2B), indicating presence of similar substituents in this molecule also. CI mass spectrum indicated its molecular weight to be 428, 28 units more than compound (2A), which indicated presence of either two more methyl or an ethyl group in this molecule. The ^1H NMR spectrum confirmed presence of similar substitutions in rings A and B as in compound (2A). Further, it revealed the presence of 6 methyl groups: two singlets, three doublets and

one triplet (see experimental). The latter triplet signal indicated the presence of an ethyl group in the molecule. Comparison of the chemical shifts of these methyl and ethyl groups with those of compounds (2A), (9) and (11) led to the conclusion that compound (3) is 7-ketositosterol. The stereochemistry of C-24 was found to be (ß) from the ^{1}H NMR signals of 26, 27 & 29-H, which were similar to that of 24(ß)-ethyl cholest-4-en-3-one (9) and ß-sitosterol (11) (TABLE 5)[12].

Fraction 1, R_t=11.3 min was a mixture of at least two compounds as evident from its chromatogram and EIMS data. Since the quantity available was very limited, they were not purified further, but analysed as such. Their IR spectral absorptions at 3450 & 1040 cm^{-1} indicated the presence of hydroxyl group in it. Further, the spectrum had no peaks in the carbonyl region (1700 cm^{-1}). The EI mass spectrum had the molecular ions at m/z 430 & 428, corresponding to the formulae $C_{29}H_{50}O_2$ and $C_{29}H_{48}O_2$ respectively. This suggested that these are dihydroxy sterols, with the latter having an additional unsaturation probably in the side chain. This was also supported by the major fragment ions at m/z 289[M-sc]$^+$, 271(289-18,3.16), 247(289-42,8.05), 229(247-18,6.43) and 211 (229-18,6.43). As compared to this, the major fragment ions of cholesterol appear at m/z 273[M-sc]$^+$, 255(273-18), 231 (273-42) and 213(231-18). Further, the above mass spectral fragmentations also indicated that both the hydroxyl groups are present within the ring systems. Based on biogenetic

considerations they may be placed on C-3 and C-7, thereby completing the pathway 3ß-hydroxy-Δ^5-sterols--> 3ß,7-dihydroxy-Δ^5-sterols--> 3ß-hydroxy-Δ^5-7-ketosterols. This leaves a saturated $C_{10}H_{21}$ and an unsaturated $C_{10}H_{19}$ group in their side chains, in all probability, as in sitosterol and fucosterol. The mass spectral fragments at m/z 331[M-C_7H_{15}]$^+$, 317[M-C_8H_{17}]$^+$ and 299(317-18), confirmed the presence of fucosterol side chain. These compounds were tentatively identified as 7-hydroxy sitosterol (1A) and 7-hydroxy fucosterol (1B) from the above results.

GENERAL DISCUSSION

3-Keto-Δ^4-steroids occur rarely in marine organisms. They were first reported from a sponge *Stelleta charella* alongwith other sterols. Subsequently Kokke *et.al.*, have isolated these sterols from a dinoflagellate *Pyrocists lunula*[7]. On the other hand, 7-oxygenated sterols were first reported from the sponge *Patinigera magellanica* and has since been isolated from a variety of marine organisms[8-10]. The co-occurrence of 3ß-hydroxy-Δ^5-sterols and their corresponding 3-keto-Δ^4-steroids in this sponge suggests the presence of enzymes 3ß-Hydroxy dehydrogenase and Δ^4-Isomerase, essential for conversion of sterols into steroidal hormones in it. At the same time another set of enzymes are active oxidising the normal sterols selectively at C-7 yielding the corresponding hydroxy or ketoderivatives of the type (1-3).

EXPERIMENTAL

The IR spectra were recorded on a Perkin Elmer 1310 spectrophotometer, the NMR spectra on a Bruker (IBM) WP-200 instrument and CIMS data on a Biospect instrument. The samples were purified on a Perkin Elmer analytical HPLC instrument, PE 5010 fitted with an ODS column (4.6X250 mm) and an RI detector. The chromatography was performed using an isocratic MeOH-water (100:8) solvent system (1.3 ml/min).

The animals (3 kg) were collected from the lagoon of Bangaram island in Lakshadweep by skin diving. They were thoroughly washed with water and immediately soaked in MeOH before transportation to the Laboratory. After 2 wks the supernatant solution was decanted, filtered and concentrated under reduced pressure to one-tenth of its original volume. The extraction of the left over animals was continued in this way two more times. The above combined, concentrated extracts were sequentially extracted with pet.ether and $CHCl_3$ (X3) to yield their less polar and more polar lipid fractions. The fractions were then concentrated in a rotary evaporator under vacuum. The concentrated pet.ether fraction (5 gm) was subsequently purified repeatedly over silica gel columns using pet.ether-EtOAc systems to separate the fatty acids, their esters, batyl alcohol and its analogs from sterols (100 mg). From this, individual sterols were subsequently purified on reverse phase HPLC as mentioned earlier.

24-Ethylcholest-5-en-3ß,7-diol (7-hydroxysitosterol 1A), R_t=11.3 min, EIMS m/z(%): 430(M^+,5.4), 412([M-H_2O]$^+$,7.56), 397([M-H_2O-CH_3]$^+$,3.21), 394[M-2H_2O]$^+$, 379[M-2X18-15]$^+$, 289[M-sc]$^+$, 271(289-18,3.16), 247(289-42,8.05), 229(247-18,10.19) and 211(229-18,6.43).

24-Ethylcholest-5,24(28)-dien-3ß,7-diol(7-hydroxyfucosterol 1B), R_t=11.3 min, EIMS m/z(%):428(M^+,2.43), 410[M-H_2O]$^+$, 395[M-33]$^+$, 331([M-C_7H_{13}]$^+$,2.23), 317[M-C_6H_{11}]$^+$, 300(317-17), 299(317-18), 271[M-sc-H_2O]$^+$.

Cholest-5-en-3ß-ol-7-one (7-ketocholesterol 2A), R_t=12.4 min; IR (neat): 3400,2940, 2920,2860, 1700, 1660, 1460, 1380, 1260, 1050 & 740 cm^{-1}; UV(hexane): 232 nm; PCIMS: 418[M+18]$^+$, 401[M+H]$^+$ & 382 [M-H_2O]$^+$; NCIMS: 435[M+^{35}Cl]$^-$ and 437[M+^{37}Cl]$^-$; ^{1}H NMR($CDCl_3$):δ 5.76(1H,s,6-H),3.68(1H,m,3α-H),1.2(3H,s,19-H), 0.916(3H,d,J=6.5 Hz,21-H), 0.86(6H,d,J=6.8 Hz,26 & 27-H), 0.70(3H,s,18-H); EIMS m/z(%): 400(M^+,6.13), 385([M-15]$^+$,1.09), 382([M-18]$^+$,1.77), 367([M-33]$^+$,2.94), 287([M-sc]$^+$,4.59), 269([M-sc-H_2O]$^+$,3.48), 245([M-sc-42]$^+$,6.52), 227([M-sc-42-H_2O]$^+$, 3.88), 211(2.98), 205(E,3.57), 192(D,6.76), 179(F,1.71), 187(E-H_2O,7.55), 174(D-18,7.01) & 161(F-18,14.54).

Cholest-5,23-dien-3ß-ol-7-one (7-ketodesmosterol 2B), R_t=12.4 min, EIMS m/z(%): 398(M^+,3), 350[M-2X15-18]$^+$, 337[M-43-18]$^+$, 315([M-C_6H_{11}]$^+$,1.2), 297(315-180,1.14), *etc.*

24-Ethylcholest-5-en-3ß-ol-7-one (7-ketositosterol 3), R_t=17.4 min; IR(neat): 3400, 3040, 2940, 2920,2860, 1700, 1660, 1460, 1380, 1260, 1180,1060 & 740 cm^{-1}; UV-Vis(hexane): 232 nm; PCIMS: 446 $[M+18]^+$, 429$[M+H]^+$ and 410$[M-18]^+$; NCIMS: 463$[M+^{35}Cl]^-$ and 465$[M+^{37}Cl]^-$ in the ratio 3:1; ^1H NMR($CDCl_3$):δ 5.76(1H,s,6-H), 3.68(1H,s,3α-H), 1.2(3H,s,19-H), 0.915(3H,d, J=6.8 Hz,21-H), 0.835(3H,t,J=7 Hz,29-H), 0.82(3H,d,J=6.9 Hz, 26-H), 0.80(3H,d,J=6.6 Hz,27-H) and 0.69(3H,s,18-H).

24(R)-Methylcholest-4,22E-dien-3-one (4), R_t=28.2 min; IR(neat): 2940, 2920, 2860, 1700, 1660, 1450, 1370, 970 and 860 cm^{-1}; UV-Vis (hexane): 231 nm; PCIMS: 414$[M+NH_4]^+$ and 397 $[M+H]^+$; NCIMS: 395$[M-H]^-$; ^1H NMR($CDCl_3$): δ 5.76(1H,s,4-H), 5.17 (2H,m,22 & 23-H),1.18(3H,s,19-H),1.01(3H,d,J=6.7Hz,21-H), 0.91(3H,d,J=6.7 Hz,28-H), 0.834(3H,d,J=6.7 Hz,26-H), 0.816 (3H,d,J=6.7 Hz,27-H) & 0.72(3H,s,18-H); ^{13}C NMR($CDCl_3$):δ 199.7 (C-3), 171.7(C-5),130.9(C-22), 128.8(C-23), 123.7(C-4), 68.2, 56.1, 55.8, 53.8, 50.9, 42.4, 39.6, 39.5, 38.7, 38.6, 36.1, 35.74, 35.69, 35.63, 33.98, 32.96, 32.05, 30.36, 29.7, 28.93, 28.17, 28.0, 24.16,23.81, 23.75, 22.98, 22.80, 22.55, 21.03, 18.63, 17.38, 14.04, 11.95 and 10.0.

Cholest-4-en-3-one (5), R_t=30.0 min; IR(neat): 2940, 2920, 2860,1700, 1660,1610,1460, 1370,1260,950,860,800 and 730 cm^{-1}; UV-Vis(hexane): 232 nm; PCIMS:402$[M+NH_4]^+$ and 385$[M+H]^+$; ^1H NMR ($CDCl_3$):δ 5.76(1H,s,4-H), 1.18(3H,s,19-H), 0.91(3H,d,J=6.4Hz, 21-H), 0.86(6H,d,J=6.5 Hz,26 & 27-H) and 0.70(3H,s,18-H).

24(R)-Methylcholest-5,22-dien-3β-ol (6), R_t=33.6 min; IR (neat): 3400,2920, 2860,1450, 1370, 1040 & 800 cm^{-1}; PCIMS:416 $[M+NH_4]^+$, 399$[M+H]^+$, 381$[M+H-H_2O]^+$; NCIMS: 433$[M+^{35}Cl]^-$ and 435 $[M+^{37}Cl]^-$ in the ratio 3:1; ^1H NMR($CDCl_3$): δ 5.36(1H,brd,6-H), 5.16(2H,m,22 & 23-H), 3.5(1H,m,3α-H), 1.01(3H,s,19-H), 1.0 (3H,d,J=6.8 Hz,21-H), 0.91(3H,d,J=6.5 Hz,28-H), 0.834(3H,d, J=6.6Hz,26-H), 0.816(3H,d,J=6.6 Hz,27-H) and 0.69(3H,s,18-H).

Cholesterol (7), R_t=35.4 min; IR(neat): 3450,2920,2860,1050 and 800 cm^{-1}; ^1H NMR($CDCl_3$):δ 5.36(1H,brd,6-H),3.56(1H,m,3α-H), 1.01(3H,s,19-H),0.92(3H,d,J=6.4 Hz,21-H),0.863(6H,d,J=6.7 Hz, 26 & 27-H) and 0.70(3H,s,18-H); PCIMS: 404$[M+18]^+$,386$[M]^+$ and 369$[M+H-H_2O]^+$; EIMS m/z(%): 386$[M]^+$,371$[M-15]^+$, 368$[M-18]^+$, 353 $[M-33]^+$, 313$[M-H_2O-C_4H_7]^+$,273$[M-sc]^+$, 255(273-18), 231(273-42), 213(231-18), *etc.*

24(S)-Methylcholest-4-en-3-one (8), R_t=36.2 min; IR(neat): 3020, 2940, 2860, 1700, 1670, 1610, 1460, 1380, 1260, 800 and 735 cm^{-1}; ^1H NMR ($CDCl_3$):δ 5.74(1H,s,4-H),1.18(3H,s,19-H), 0.90 (3H,d,J=6.7 Hz,21-H), 0.86(3H,d,6.7 Hz,26-H), 0.78(3H,d,J=6.5 Hz,27-H), 0.773(3H,d,6.4 Hz,28-H) and 0.67(3H,s,18-H); PCIMS: 416$[M+18]^+$,399$[M+H]^+$; EIMS: 398(M^+,7.7),384(3.47), 383($[M-15]^+$, 2.93), 356($[M-42]^+$,3.5), 341($[M-57]^+$,2.33), 327($[M-71]^+$,3.34), 313($[M-85]^+$,3.84), 275($[M-123]^+$,13.83), 274($[M-124]^+$,9.71), 229(271-42,24.85) and 124(63.09)).

24ß-Ethylcholest-4-en-3-one (9), R_t=41.4 min; IR(neat): 2940, 2920, 2860, 1700, 1670, 1610, 1460, 1370, 1325, 1260, 1220, 1180, 860 & 730 cm^{-1}; UV-Vis(Hex): 232 nm; ^1H NMR($CDCl_3$): δ 5.74(1H,s,4-H), 1.19(3H,s,19-H), 0.92(3H,d,6.7 Hz,21-H), 0.835(3H,t,6.8 Hz,29-H), 0.82(3H,d,6.8 Hz,26-H), 0.805(3H,d, 6.8 Hz,27-H), 0.71(3H,s,18-H); PCIMS: 430$[M+NH_4]^+$ and 413 $[M+H]^+$; EIMS m/z(%):412($[M]^+$,19.6),397($[M-15]^+$,4.52),370(M-42, 7.62), 355(M-57,3.35), 327(M-85,3.82), 299(M-113,2.2), 289 (M-123,15.5), 288(M-124,13.61), 271($[M-sc]^+$,10.13), 229 and 124(100%).

24S-Methylcholesterol (10), R_t=43.8 min; IR(neat): 3400, 2930, 2860, 1460,1370 and 1050 cm^{-1}; ^1H NMR($CDCl_3$): δ 5.36(1H, brd,6-H), 3.54(1H,m,3α-H), 1.03(3H,s,19-H), 0.902(3H,d,J=7Hz, 21-H), 0.866(3H,d,J=6.8 Hz,26-H), 0.781(3H,d,J=6.5 Hz,27-H), 0.771(3H,d,J=6.4 Hz,28-H) and 0.68(3H,s,18-H); PCIMS: 418 $[M+18]^+$, 401$[M+H]^+$ and 383 $[M+H-18]^+$; EIMS m/z(%): 400(M^+, 7.07), 385($[M-15]^+$,3.26), 382($[M-18]^+$,4.66), 367($[M-33]^+$, 3.97), 273(M-sc)$^+$,8.42), 255(273-18,14.25), 231(273-42,9.1), 213(231-18,17.05) and 55(100).

24R-Ethylcholesterol(ß-sitosterol 11), R_t=51.6 min; IR (neat): 3400,2920, 2860, 1450,1370 and 1040cm^{-1}; ^1H NMR($CDCl_3$): δ 5.36(1H,brd,6-H), 3.5(1H,m,3α-H), 1.03(3H,s,19-H), 0.92 (3H,d,J=6.4 Hz,21-H), 0.84(3H,t,J=6.8 Hz,29-H), 0.82(3H,d, J=6.8 Hz,26-H), 0.80(3H,d,6.4 Hz,27-H) and 0.68(3H,s,18-H); PCIMS: 432$[M+NH_4]^+$, 414(M^+) and 397$[M+H-H_2O]^+$; EIMS m/z(%):

414($M^{\cdot}$,67.9),399([M-15]$^{\cdot}$,25.67),396([M-H_2O]$^{\cdot}$,38.5),381([M-33]$^{\cdot}$, 29.87), 354([M-18-42]$^{\cdot}$,7.44), 329([M-C_6H_{13}]$^{\cdot}$,48.83), 303([M-C_8H_{15}]$^{\cdot}$,37), 273([M-sc]$^{\cdot}$,28.55), 255(273-18,37.23), 231([M-H-sc-D]$^{\cdot}$,34.66), 213(231-18,51.15) and 57(100).

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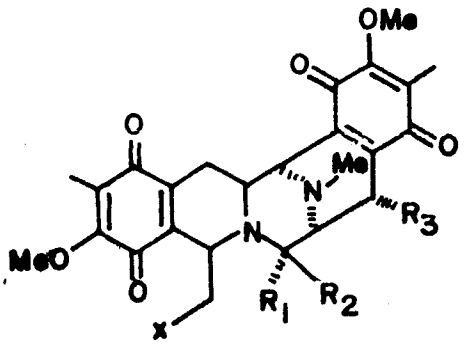
CHAPTER 4

CONSTITUENTS OF THE SPONGE

HALICLONA SP.

Bright blue sponge belonging to the genus *Haliclona*, fam: *Haliclonidae*, order: *Haplosclerida* is ubiquitous in the coastal regions of India. These sponges have a spiculation of oxeas, occurring in a range of sizes without clear distinction into megascleres and microscleres nor any ectosomal spiculation[1]. Formerly they used to be classified under different genera: *Haliclona*, *Reniera*, *Chalina* and *Pachychalina*. Later, Burton has clubbed all of them under the genus, *Haliclona*[1]. All these animals have the renierine or isodictyal skeleton composed of a meshwork of single or parallel oxeas united by spongin or more or less enclosed in spongin. These sponges are also known to be a good source for several new alkaloids, exhibiting interesting biological properties and have been the subject of extensive chemical and biological studies. For example, its extract was found to be extremely toxic to mice and haemolytic towards mouse red blood cells[2]. The latter property, however, was destroyed within a few days on storage at 0°C. A variety of novel compounds having promising cytotoxicity and antibacterial properties have been isolated from these animals till today. Important among them are: halitoxin, a biopolymer, present in *Haliclona rubens*, *H.viridis* and *H.erina*[2a] and the isoquinoline alkaloids such as renierone, mimosamycin, renieramycin A-F, and their analogs (FIG 1)[3-5], obtained from *Reniera* sp. from two divergent localities as Isla Grande, Mexico and a lake in Palau, western Caroline islands. Among these, halitoxin was cytotoxic, hemolytic and toxic to mice while the compounds

Activity : Antibiotic , Antiviral , toxic , hemolytic



Saframycins x = b

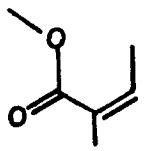
A. R_1 = CN, R_2 = R_3 = H

B. R_1 = R_2 = R_3 = H

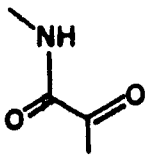
C. R_1 = R_2 = H, R_3 = OMe

S. R_1, R_2 = OH, H ; R_3 = H

(a) x =



(b) x =



Renieramycins x = a

A. R_1 = R_2 = H R_3 = OH

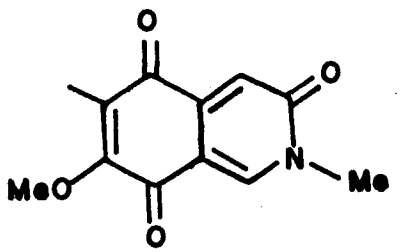
B. R_1 = R_2 = H, R_3 = OEt

C. R_1, R_2 = O, R_3 = OH

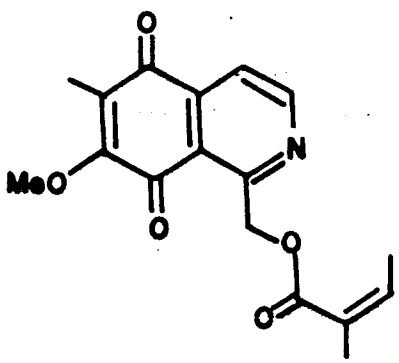
D. R_1 , R_2 = O, R_3 = OEt

E. R_1 = OH, R_2 = R_3 = H

F. R_1 = OH , R_2 = H , R_3 = OMe

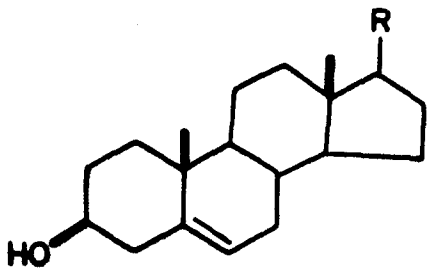


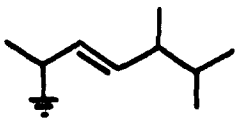
Mimosamycin

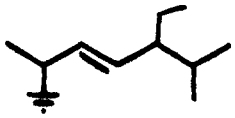


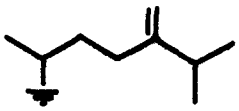
renierone

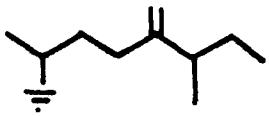
Fig. 1 Compounds from the sponge Reniera sp. and Saframycins

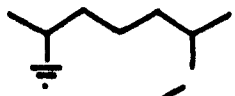


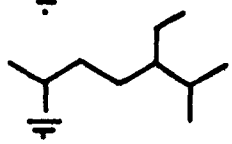
1. R = 

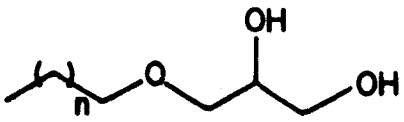
2. R = 

3. R = 

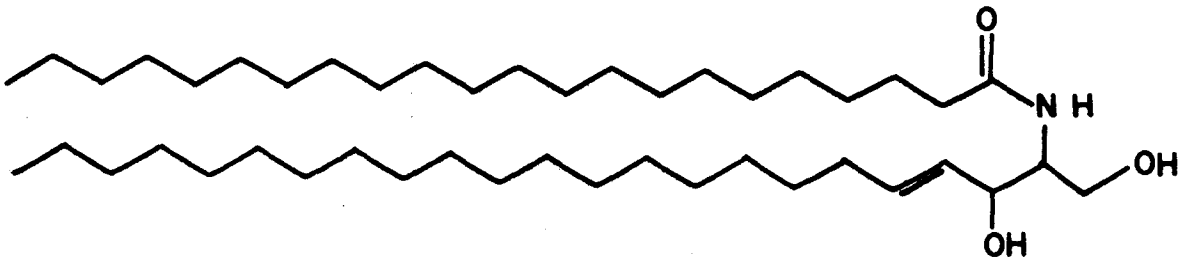
4. R = 

5. R = 

6. R = 

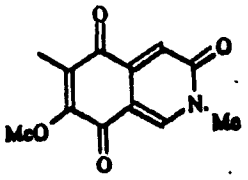


7. n = 14
8. n = 15
9. n = 16
10. n = 22:1
11. n = 24:1

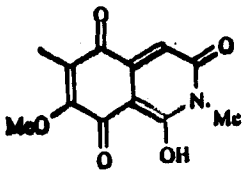


N - docosanoyl - 2- amino - 1,3— dihydroxy tricosa - 4E - ene (12)

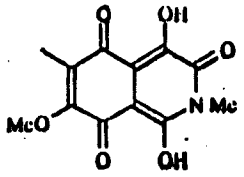
FIG. 2 STRUCTURE OF COMPOUNDS (1-12)



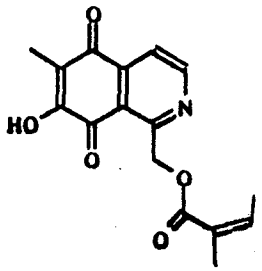
MIMOSAMYCIN (13)



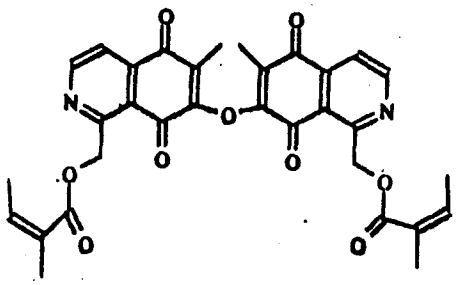
1-HYDROXY MIMOSAMYCIN (14)



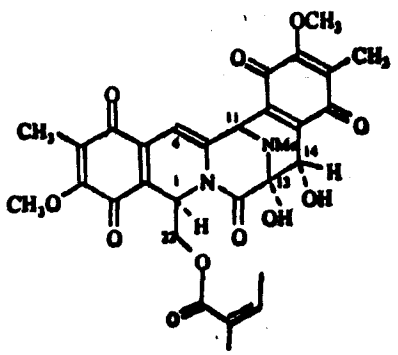
1,4-DIHYDROXY MIMOSAMYCIN (15)



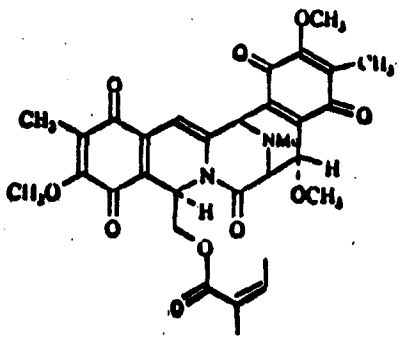
O-DEMETHYL RENIERONE (16)



DIMER OF O-DEMETHYL RENIERONE (17)



RENIERAMYCIN G (18)



RENIERAMYCIN H (19)

Fig. 2 Continued The alkaloids (13-19) from the sponge Haliclona sp.

from the latter sponge exhibited strong antibacterial properties. Interestingly, two of these metabolites, *viz.*, mimocin and mimosamycin are also produced by the bacteria *Streptomyces lavendulae* No 314, while the renieramycins closely resemble in structure to the antibiotics saframycins produced when the streptothricin-producing culture media of the above bacteria is treated with NaCN[4] (FIG 1). In 1988, Baker *et.al.*, isolated a symmetrical alkaloid, papuamine, having antibacterial properties from a sponge belonging to the genus *Haliclona* sp from Palau region[6a]. Subsequently, Fahy *et.al.*, isolated this compound as well as its diastereomer, haliclonamine from another *Haliclona* sp. from the same region[6b]. Another interesting group of compounds are 1-ß-carboline derivatives, manzamine A-C, having potent antitumor activity from a sponge *Haliclona* sp. from Okinawa[7]. Among these, manzamine B and C were active against P 388 in *in vitro* studies (PC_{50}=6 and 3 µg/ml respectively). Subsequently, other manzamine analogs were reported from different sponges: *Xestospongia* sp[7a], *Pellina* sp[7a] and *Pachypellina* sp[8]. Compounds related to N-formyl-1,2-dihydrorenierone[4], were also isolated from the sponge *Xestospongia* sp and its predator nudibranch *Joruna funebris*[9].

As part of our ongoing programme for isolation of marine bioactive substances from the Indian Ocean region, we undertook a detailed chemical examination of the deep blue sponge *Haliclona* sp. collected from the intertidal waters of

Gujarat coast. Our preliminary studies on the biological properties of the methanol extract of this sponge had shown it to be very active against several Gram positive and negative bacteria as well as the *Encephalomyocarditis* virus (75%, *in vitro*). This extract was later partitioned into pet.ether, EtOAc, n-BuOH and aqueous fractions in the usual manner. Bioassay of these fractions indicated the pet.ether, EtOAc and water-soluble fractions to be very active against several bacteria whereas the BuOH fraction was mostly inactive (see TABLE 1). Further, none of these fractions had any antifungal activity. The antibacterial tests were carried out against the following test cultures: *Salmonella typhimurium* MTCC-98, *Micrococcus luteus* MTCC-106, *Klebsiella pneumoniae* MTCC-109, *Escherichia coli* MTCC-118, *Bacillus subtilis* MTCC-121, *Staphylococcus aureus* MTCC-96, *Proteus mirabilis* MTCC-425 and *Vibrio parahaemolyticus* MTCC-451 whereas the antifungal tests were carried out using the yeast cultures *Candida albicans* MTCC-230, *Rhodotorula rubra* MTCC-248 and *Saccharomyces cerevisiae* MTCC-249.

The EtOAc fraction contained mostly the alkaloids whereas the pet.ether fraction had several lipids including fatty acids, sterols and sphingosine derivatives besides the alkaloids. Both these fractions were initially purified by column chromatography over silica gel using pet.ether-EtOAc solvent systems and the subfractions having identical TLC spots were combined. The fatty acids were analysed as their

TABLE 1

ANTI-MICROBIAL ACTIVITY OF THE VARIOUS FRACTIONS OF THE SPONGE EXTRACT AS WELL AS THE ALKALOIDS O-DEMETHYL RENIERONE (16) AND ITS DIMER (17) AGAINST VARIOUS BACTERIA (B1-B11)*:

No	Fraction	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
1	PE Fr	20	26	20	11	19	30	28	12	18	NT	NT
2	EtOAc Fr	20	10	18	8	20	12	10	11	16	NT	NT
3	BuOH Fr	--	--	8	--	--	--	--	--	--	NT	NT
4	H_2O Fr	8	16	12	--	12	16	16	15	12	NT	NT
5	Cmpd(16)	--	--	--	--	--	--	--	9	--	7	--
6	Cmpd(17)	--	--	10	--	8	--	--	--	--	--	12

* In the table above, the activity is expressed as inhibition diameter against 6 mm dia. disks containing 50 µg of the compounds or fractions per disk. The observed activity is given against the bacteria B1-B11.

NT- not tested.

Names of the bacteria used in the above study are given below:

Code	name of the bacteria	Code	Name of the bacteria
B1	*Salmonella typhimuram*	B2	*Escherichia coli*
B3	*Bacillus subtilis*	B4	*Proteus mirabilis*
B5	*Vibrio parahaemolyticus*	B6	*Staphylococcus aureus*
B7	*Arthrobacter sp*	B8	*Bacillus polymix*
B9	*Micrococcus aureus*	B10	*Flavobacterium so*
B11	*Klebsiella pneumoniae*		

methyl esters on GC and also as their *p*-nitrobenzyl esters using negative CIMS techniques (see CHAPTER 1). The sterols and batyl alcohol analogs were separated on reverse phase HPLC fitted with a RI detector, using MeOH as eluant. Several sterols (1-6) including 24(28)-didehydroaplysterol (4) as well as batyl alcohol and its analogs (7-12) were isolated in this manner. The polar fraction obtained from the silica gel column was found to be a mixture of sphingosines, which were further purified in the same manner, leading to the isolation of a dihydroxy sphingosine (12) in pure form. The fraction containing alkaloids was purified on HPLC (ODS, 8X250 mm), fitted with a UV detector yielding the following alkaloids, in pure form: mimosamycin (13), 1-hydroxy mimosamycin (14), 1,4-dihydroxy mimosamycin (15), O-demethylrenierone (16), its dimer (17), renieramycin G (18) and renieramycin H (19) (FIG 2). Among these, O-demethylrenierone (16) has been reported earlier from the sponge *Reniera* sp[4,5], while mimosamycin (13) has been isolated from the above sponge as well as the bacteria *S. lavendulae*[6]. All other alkaloids reported here have been isolated for the first time from a natural source.

A small portion of the fatty acid-rich fraction isolated by repeated column chromatography of the pet.ether fraction was esterified with diazomethane and analysed on a capillary GC fitted with a flame ionisation detector (FID). The individual compounds were identified from a comparison of their retention times with standards. The remaining portion of the

acids were converted into their *p*-nitrobenzyl derivatives and analysed on the chemical ionisation mass spectrometer as discussed in CHAPTER 1. The major fatty acids found using the GC method are 16:1 (17.8%), 16:0(18.5%), 18:1 (17.6%) & 18:0(18.3%). These values, expressed relative to that of $C_{16:0}$ acid, as well as the results from the mass spectral techniques are presented in CHAPTER 1.

The fractions rich in sterols and glyceryl ethers of fatty alcohols were purified on HPLC (ODS, 250X8 mm, MeOH, 1ml/min) fitted with a refractive index detector. Four subfractions, (A-D, 14.7, 21.24, 54.06 and 3.9% respectively) were isolated from the former fraction with R_t varying from 32.34 to 45.14 min in this way . The glyceryl ethers upon HPLC purification yielded five pure compounds with R_t between 17.7 and 50.3 min (FIG 3).

Fraction A, R_t=32.34 min, isolated from the sterol-mixture was found to be a mixture of three compounds from a detailed analysis of their ^1H NMR and ^{13}C NMR spectra. The IR spectrum of this fraction had absorptions at 3450 and 1040 cm^{-1}, indicating the presence of hydroxyl groups. Absence of IR peaks in the region of 1700 cm^{-1} ruled out the presence of any carbonyl groups in these molecules. The peaks at δ 5.35(1H, brd,6-H), 3.52(1H,m,3α-H), 0.695(3H,s,18-H) & 1.01(3H,s,19-H) in the ^1H NMR spectrum and the corresponding signals at δ 140.8(C-5) & 121.7(C-6),71.8(C-3), 11.8(C-19) and 18.7(C-18)

in the ^{13}C NMR spectrum of this fraction indicated that the 3ß-hydroxy-Δ^5-system is common to all the three compounds. The molecular weights indicated by the mass spectrum were 398 and 412, which were 12 and 26 mass units more than cholesterol respectively. The vinyl signals at δ 5.25(2H,m) and 5.18(2H,m) indicated the presence of unsaturations in the side chain, probably at C-22, consequent to the presence of a methyl and an ethyl group at C-24. The broad singlets at δ 4.72 and 4.66 indicated the presence of an exomethylene group in the side chain, preferably at C-24. From these results, the fraction might be considered to be a mixture of 24-methyl cholest-5,22E-dien-3ß-ol (1), 24-ethylcholest-5,22E-dien-3ß-ol (2) and 24-methylene cholesterol (3). This was also supported by the presence of the respective side chain vinyl carbons at 131.8 & 126.3, 136.1 & 131.9 and 156.9 & 106.0 in the ^{13}C NMR spectrum[10].

The HPLC fraction B, R_t=36.8 min, had absorptions at 3450 & 1040 cm^{-1} in its IR spectrum indicating the presence of hydroxyl group. Its ^1H NMR spectrum had signals at δ 5.37(1H, brd,6-H), 3.5(1H,m,3α-H),1.01(3H,s,19-H) and 0.68(3H,s,18-H), which are very diagnostic of 3ß-OH-Δ^5-sterols. The broad singlet at δ 4.67(2H), was indicative of an exomethylene group. However, the chemical shift and the nature of these protons were different from those of similar protons in 24-methylene cholesterol (3), in which the vinyl protons appear as two broad singlets at δ 4.72 and 4.66 (1H each). Another

interesting feature in the ^1H NMR spectrum of this compound was that it had only two secondary methyl groups: 0.99(3H,d, 6.4 Hz) and 0.945 (3H,d,6.5 Hz), as compared to three such groups present in ordinary steroidal derivatives. A third methyl signal was seen as a triplet at δ 0.83(3H,t,J=7.4 Hz). This indicated that one of the methyl groups, *viz.*, C-21, 26, or 27, might have been replaced by an ethyl group. The above observations were also confirmed by its ^{13}C NMR and DEPT spectra, which had signals at δ 155.32(s) & 107.08(t) (exomethylene), 140.76(s,C-5) & 121.7(d,C-6), 71.81(d,C-3), 19.81, 19.38, 18.73, 11.95 and 11.85(q's)[10].

The molecular weight of this compound was determined to be 412 by EI mass spectrum. This was 26 units more than cholesterol which agreed well with our earlier observation that it has an extra methyl and an exomethylene group as compared to cholesterol. The major fragment ions at m/z 273 $[M-sc]^+$, 255(273-18), 231(273-42) and 213(231-18 or 255-42) also confirmed the cholesterol-type ring system in this molecule. Thus, all that was left in order to finalise its structure was to fix the positions of the exomethylene and the ethyl group in it. The strong McLafferty peak at m/z 314 $[M-C_7H_{14}]^+$ in its EIMS indicated that the exomethylene group is present at C-24. From biogenetic considerations, as well as from a comparison of its above spectral values with those reported in literature, its position could be fixed at C-26, leading to its structure as 24(28)-didehydroaplysterol (4).

TABLE 2

HPLC, ^1H NMR AND MASS SPECTRAL DATA OF THE STEROLS (1-6).

Cmpd	R_t min	% yld	^1H NMR data	^{13}C NMR data	CIMS/EIMS
Cmpd (1-3)	32.34	14.7	5.35(1H,d,5.2) 5.25(2H,m), 5.16(2H,m), 4.72(1H,brs), 4.66(1H,brd), 3.52(1H,m), 0.695,0.682, 0.67, *etc*	140.8&121.7, 131.8&126.3, 136.1&131.9, 156.9&106.0, 71.8,*etc*	399[M+H]$^+$ 381[M-17]$^+$ 413[M+H]$^+$ 395(413-18)
Cmpd (4)	36.8	21.24	5.37(1H,brs), 4.67(2H,s,28-H),3.5(1H,m), 1.01(3H,19-H) 0.99(3H,d,21-H),0.945(3H,d) 0.83(3H,t)& 0.68(3H,s).	155.32(s), 140.76(s), 121.7(d), 107.08(t), 71.81(d), 19.81(q), 19.38(q), 18.73(q), 11.95(q) & 11.85(q).	412[M$^+$], 396[M-H-15]$^+$, 384, 328,314, 299,271, 255,229, 213,*etc.*
Cmpd (5)	42.40	54.06	5.35(1H,brd), 3.5(1H,m), 1.01(3H,s), 0.92(3H,d), 0.86(6H,d), 0.68(3H,s).	---	387[M+H]$^+$, 368[M-18]$^+$ *etc*
Cmpd (6)	45.14	3.90	1.01(3H,s), 0.92(3H,d,6.4) 0.86(3H,d,6.4) 0.84(3H,t,6.5) 0.81(3H,d,6.9) 0.68(3H,s)	---	414[M$^+$], 399[M-15]$^+$ 396,388, 373,354, 329,273, 255,231, 213,*etc*

The presence of fragment ion at m/z 382[M-H-C_2H_5]$^+$ in the EIMS, which indicates that the ethyl group is present in an allylic position further supports the above view.

The HPLC fractions C and D, eluting at R_t=42.4 and 45.4 min respectively had spectral data typical of cholesterol-type compounds. They were identified as cholesterol (5) and sitosterol (6) respectively after comparing their ^{1}H NMR and mass spectra with those of authentic samples (TABLE 2).

^{1}H and ^{13}C NMR of the more polar fraction were similar to those of batyl alcohol. This includes the characteristic proton signals at δ 3.5-3.9(7H,m), 1.2(methylenes) and 0.88 (3H,t) (FIG 3A) and the carbon signals at δ 64.2(t), 70.5(d), 71.8(t) 72.4(t), 22.65,26.05, 27.2, 28.9-29.7 & 31.8 (t's) and 14(q) (FIG 3B). Five pure compounds were separated from this mixture using HPLC. Among these, three were saturated while two were mono-unsaturated, as indicated by the additional signals at δ 5.35 (2H,m), in their ^{1}H NMR and δ 129.85 in the ^{13}C NMR spectra. The molecular weights of these compounds were determined to be 316, 330, 344, 426 and 454 by CI mass spectrometry. Based on these results, their structures were finalised as glyceryl ethers of following fatty alcohols: $C_{16:0}$ (hexadecanol 7), $C_{17:0}$ (heptadecanol 8), $C_{18:0}$ (octadecanol 9), $C_{24:1}$ (10) and $C_{26:1}$ (11) respectively. The stereochemistry of the double bond in compounds (10) and (11) were finalised as *cis*(Z), from the coupling pattern of the

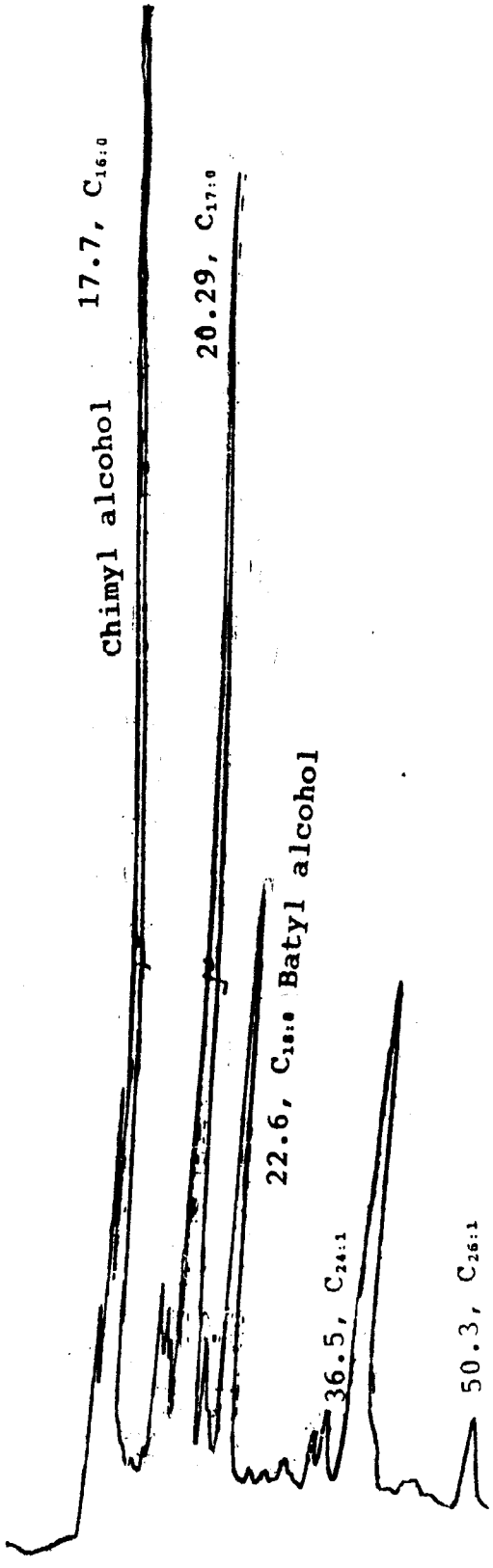


FIG 3. HPLC SEPARATION OF COMPOUNDS (7-11)

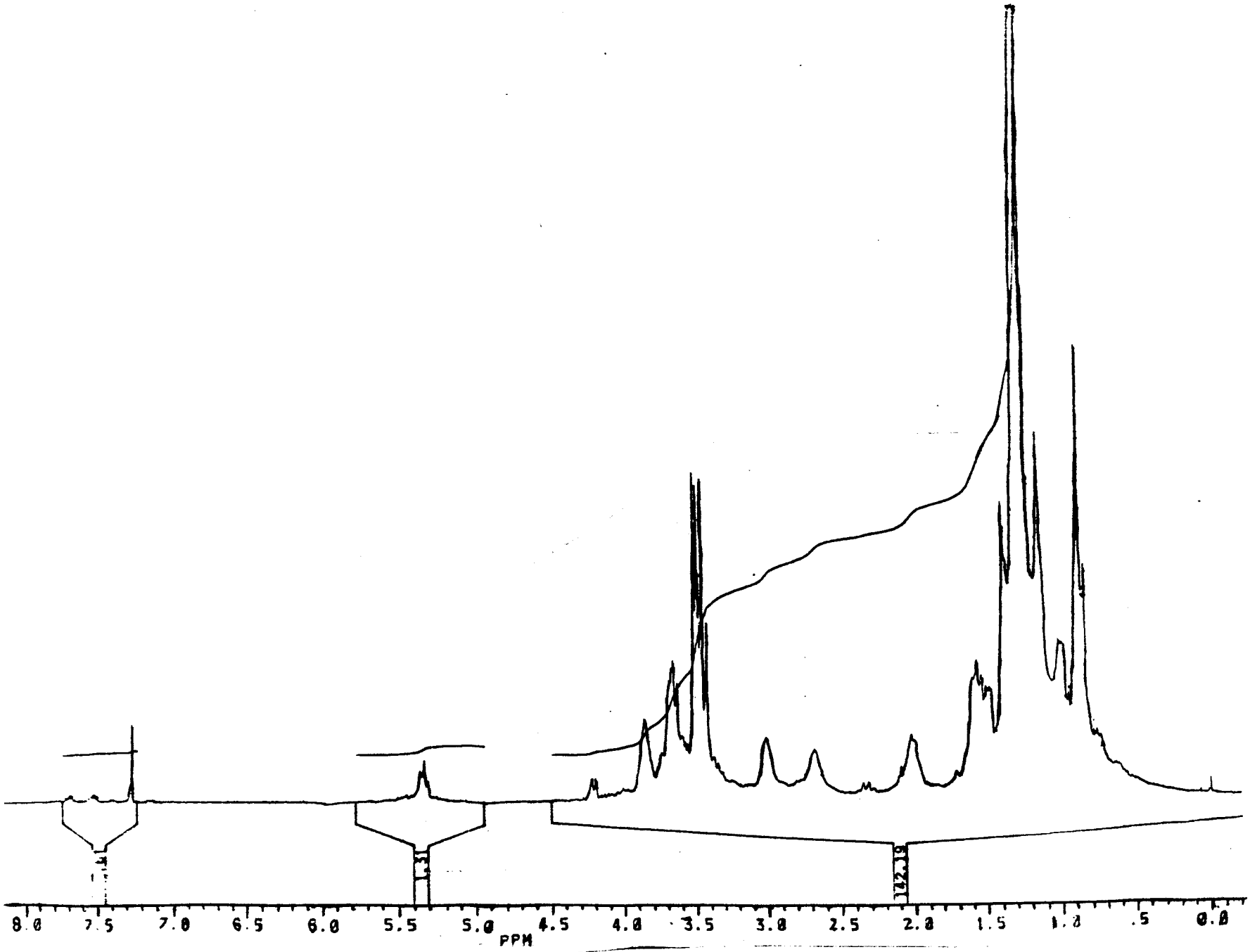


FIG 3A ^{1}H NMR SPECTRUM OF THE MIXTURE OF COMPOUNDS (7-11).

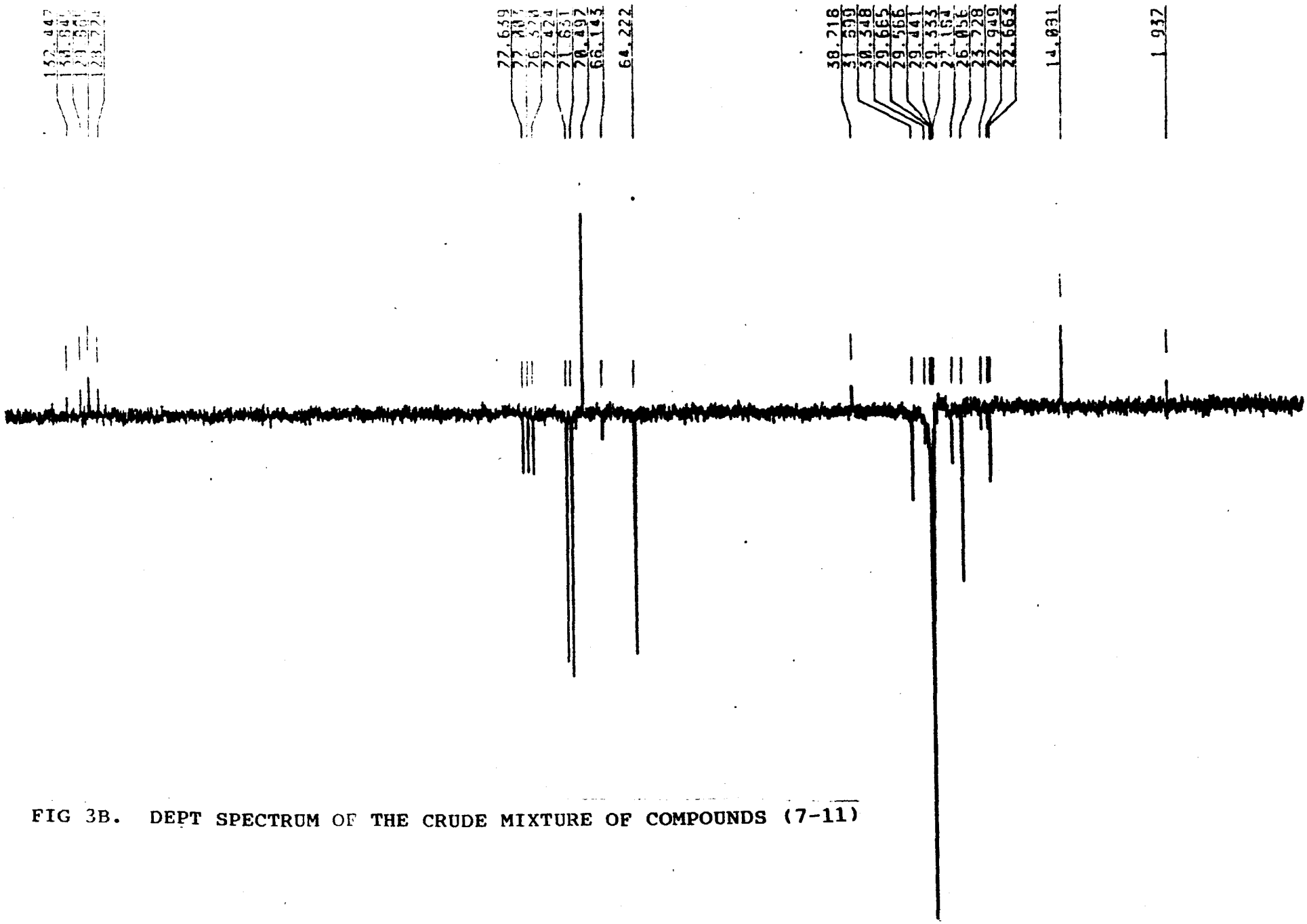


FIG 3B. DEPT SPECTRUM OF THE CRUDE MIXTURE OF COMPOUNDS (7-11)

TABLE 3.

HPLC, NMR AND MS DATA OF BATYL ALCOHOL AND ANALOGS.

Cmpd, Chain length, R_t min & %	^1H NMR (ppm)	^{13}C NMR (ppm)	CIMS
Cmpd(7), $C_{16:0}$, 17.7min, 35.3%	3.85(1H,m), 3.69(2H,m), 3.50(4H,m), 1.60(26H,m) 0.88(3H,m)	72.45,71.84, 70.5,31.90, 29.7-29.3, 26.05,22.66, &14.07	317[M+H]$^+$, 285[M-31]$^+$, 255[M-61]$^+$, 225[M-91]$^+$, *etc*
Cmpd(8), $C_{17:0}$, 20.29min, 2.45%	" "	" "	331[M+H]$^+$,299, 296 & 239.
Cmpd(9), $C_{18:0}$, 22.6min, 15.6%	" "	" "	345[M+H]$^+$,313, 283 & 253.
Cmpd(10), $C_{24:1}$ 36.5min, 17.25%	5.35(2H,t), 2.02(4H,m)	129.85(d), 28.94,27.16, *etc*	426[M]$^+$, 409, *etc.*
Cmpd(11), $C_{26:1}$ 50.3min, 3.54%	" "	" "	454[M]$^+$, 405, *etc.*

vinyl protons as well as the chemical shift of the vinyl and allyl carbons (δ 129.85 and 27.16 respectively). A *trans* stereochemistry would have given rise to the allylic carbon signals further downfield at around 32 ppm. The NMR and MS values of these compounds are given in TABLE 3.

The most polar fraction (100 mg) eluted from the silica gel column was insoluble in pet.ether and EtOAc and only sparingly soluble in $CHCl_3$ and MeOH. It was repurified on silica gel columns using pet.ether-$CHCl_3$-MeOH gradient systems, resulting in the isolation of about 50 mg of a white amorphous powder, m.p.70°C. This compound had IR absorptions at 3450 and 1630 cm^{-1}, indicating the presence of hydroxyl and amide groups in it (FIG 4). Its ^1H NMR spectrum had peaks at δ 6.4(1H,d,7.2 Hz), 5.7(1H,dt,15.4 & 6.4 Hz), 5.46(1H,dd,15.4 & 6.7 Hz), 4.2(1H,m), 3.8(2H,m) and 3.65(1H,m) in addition to the usual signals in the range δ 1-1.6 due to methylenes and the terminal methyl groups at δ 0.88(6H,t). The latter signal indicated the presence of two long chains per molecule. The signal at δ 6.4 could be assigned to an amide NH signal. Decoupling of this signal led to a simplification of the multiplet at δ 3.8, whereas decoupling of the latter signal led to a simplification of the multiplets at δ 3.65 and 4.2. At the same time, irradiation at δ 3.65 affected only the signals at δ 3.8. Similarly, irradiation of the multiplet at δ 4.2 led to the collapse of the doble doublet signal at δ 5.46 into a doublet with coupling constant of 15.4 Hz.

Fig. 4 IR (KBr) Spectrum of the sphingosine derivative (12)

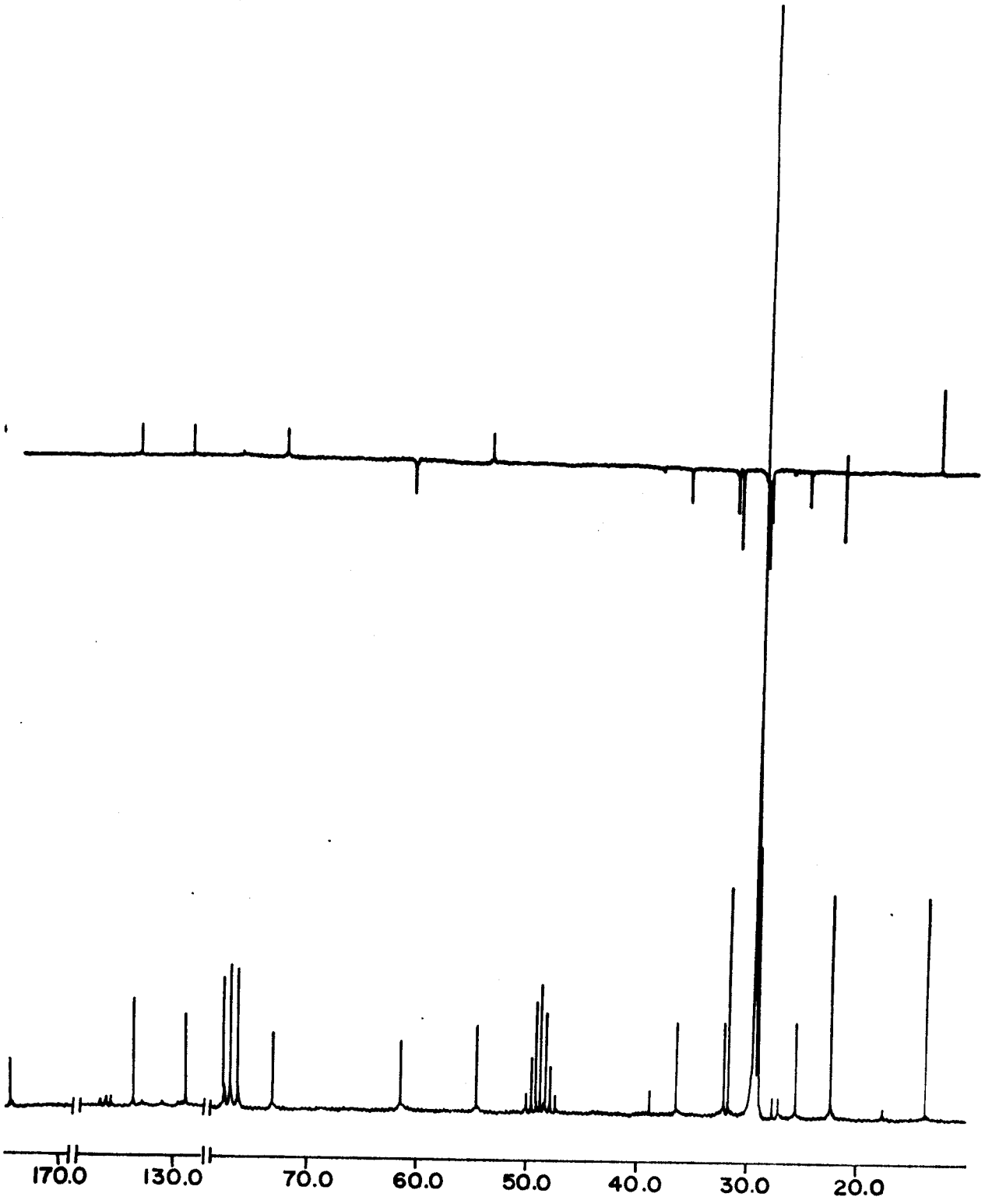


Fig. 5 ^{13}C NMR and dept spectra of the Ceramide (12)

Further, decoupling of the vinyl proton signal at δ 5.7 simplified the peaks at δ 5.46 and 2.0. The corresponding carbon peaks were observed in the ^{13}C NMR spectrum of the molecule at δ 175.4(s,amide carbonyl), 134.4(d) & 129.7(d, vinyl carbons), 73.68(d), 61.92 (t) and 54.94(d) (FIG 5). From these results, the partial structure as shown in FIG 6 was arrived at for this molecule.

FIG 6. PARTIAL STRUCTURE AND SOME ^{1}H AND ^{13}C NMR CHEMICAL SHIFTS OF THE CERAMIDE (12)

H no.	$CDCl_3$	Py-d_5	C no.	$CDCl_3$	Py-d_5
1-H_2	3.65, 3.8(m)	4.43(dd, 10.75,5.1), 4.28(dd, 10.75,4.2)	C-1	61.92	61.59
2-H	3.8(m)	4.7(m)	C-2	54.94	56.25
3-H	4.2(m)	4.83(t, 6.05)	C-3	73.68	72.76
4-H	5.46(dd, 15.4,6.7)	6.0(m)	C-4	129.7	131.64
5-H	5.7(dt, 15.4,6.4)	6.0(m)	C-5	134.4	131.74
6-H_2	2.0(m)	2.07(m)	C-6	---	---
2'-H_2	2.2(t,7.1)	2.45(t,7.5)		---	---
NH	6.4(d,7.2)		NHCO	175.4	173.86

This was further confirmed by AROMATIC SOLVENT INDUCED SHIFTS (ASIS) experiments wherein the ^1H and ^{13}C NMR spectra of the compound were recorded in pyridine-d_5(FIG 6). This led to a further spreading and hence simplification of the proton signals. The NH signal now appeared at δ 8.3, downfield by about 1.9 ppm as compared to its position earlier at δ 6.4. Similarly, the 2-H and 3-H signals were found at δ 4.70(1H,m) & 4.83(1H,t,J=6.05 Hz), down from its earlier position at δ 3.8 & 4.2 respectively. In addition, the 1-H_2 now appeared as two dintinct signals at δ 4.43(dd,10.75 & 5.1 Hz) and 4.28 (dd,10.75 & 4.2 Hz). The corresponding carbon peaks appeared at δ 172.86 (s,carbonyl), 131.74 & 131.64(d's,vinyl carbons), 72.76(d), 61.59(t) and 56.25(d).

The molecular ion was not seen either in EIMS or in FABMS. The molecular weight was inferred to be 691 from the analysis of the EI mass spectrum of the parent compound as well as that of its hydrolysis and LAH reaction products. The highest fragment ion observed in its EIMS was at m/z 644[M-18-29]$^+$. The peaks at 410([M-$C_{20}H_{41}$]$^+$,10%) and 393([M-$C_{20}H_{40}$-H_2O]$^+$,60%) could be due to the McLafferty fragmentations from a $C_{22:0}$ fatty acid residue. Alkaline hydrolysis of the ceramide yielded dodecanoic acid as one of the products. Similarly treatment of the ceramide with lithium aluminium hydride (LAH) yielded a free sphingosine and an alcohol of molecular formulae $C_{23}H_{47}NO_2$ and $C_{22}H_{46}O$ respectively as determined from their spectral data. The structure of this molecule was

finalised as N-docosanoyl-2-amino-1,3-dihydroxytricosa-4E-ene (12) (FIG 2) from these studies. The stereochemistry of the double bond could be easily assigned as *trans*(E) from the observed high vicinal coupling of the vinyl protons in its ^1H NMR spectrum recorded in Py-d_5 (10.75 Hz). The stereochemistry of C-2 and C-3 are not clear at the moment.

The alkaloids were initially separated on silica gel columns using pet.ether-$CHCl_3$-EtOAc systems. Their final purification was carried out on HPLC (ODS column,5μ,250X8 mm) fitted with a UV detector using MeOH-H_2O(3:1,1 ml/min) as mobile phase. List of pure compounds isolated in this way as well as a brief summary of their physical properties are given in TABLE 4 below:

TABLE 4. ISOQUINOLINE ALKALOIDS ISOLATED FROM THE SPONGE *HALICLONA* SP USING HPLC

No	Name of the compound	m.p.(°C)	R_T(min)	Mol Wt
1	Mimosamycin (13)	---	12.2	233
2	1-Hydroxy mimosamycin (14)	225	14.5	249
3	1,4-Dihydroxy mimosamycin (15)	---	9.3	265
4	O-Demethyl renierone (16)	133	11.0	301
5	Dimer of O-Demethyl renierone (17)	174	19.6	584
6	Renieramycin G (18)	---	41.7	594
7	Renieramycin H (19)	---	52.4	592

Compound (13), is an yellow solid which also gave a bright yellow spot on TLC plates. The IR spectrum of this compound had multiple peaks in the region 1600-1700 cm^{-1}, indicating the presence of quinone and amide groups in it. The prominent peak in its EI mass spectrum was at m/z 234(100), presumably due to $[M+H]^+$ion. Since this compound had no IR absorptions around 3500 cm^{-1}, the possibility of hydroxyl groups being present in it could be discounted. This was supported by its EI mass spectrum in which no peaks due to loss of elements of water were observed. The ^1H NMR spectrum indicated the presence of two vinyl protons: δ 8.3 & 7.15 (1H each,s) and three methyls: δ 4.2(OMe,s), 3.7(OMe or NMe,s) & 2.1(allyl Me,s) in this molecule. These results were in agreement with that of mimosamycin, previously reported from the sponge *Reniera* sp[4] and the bacteria *S. lavendulae*[6]. The structure of this compound was thus established as mimosamycin (13).

Compound (14), isolated as an orange-red crystalline solid had absorptions at 3400,1680 and 1630 cm^{-1} in its IR spectrum, suggesting the presence of hydroxyl, quinone and amide groups in the molecule (FIG 7). The quinonoid nature of the compound was also supported by its strong UV absorptions at 227,345 & 483 nm. EI mass spectrum revealed its molecular weight to be 249, *i.e.*, 16 units more than that of mimosamycin (13). This suggested that this molecule may be a hydroxymimosamycin. Its ^1H NMR spectrum was almost identical to that of mimosamycin but for the absence of 1-H signal at δ 8.3 and a downfield

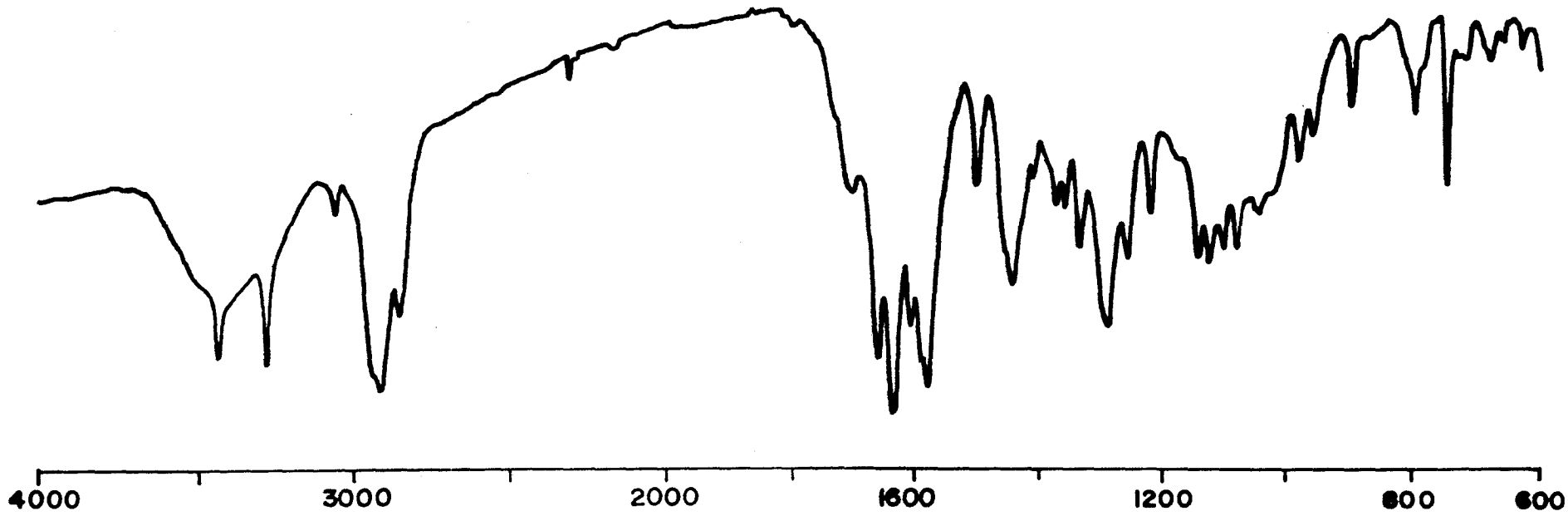


Fig. 7 IR (KBr) spectrum of compound (14)

shift by 0.45 ppm of 4-H signal to δ 7.60. Another notable difference was in the appearance of a new D_2O-exchangeable proton signal at δ 8.85(br), presumably from the new hydroxyl group in this compound. The structure of this compound was finalised as 1-hydroxymimosamycin (14) from these results.

The structure of compound (15) was found to be similar to that of compounds (13) and (14) from its UV-Vis absorption and ^1H NMR spectra. Thus this compound had strong absorptions at 220, 255 and 500 nm in its UV-Vis absorption spectrum. Comparison of its ^1H NMR spectrum with that of compounds (13) and (14) revealed the presence of all the three methyl signals in their usual places, but the absence of both 1-H and 4-H in this molecule. Instead, two new D_2O-exchangeable signals appeared at δ 8.85 & 13.20, indicating the replacement of 1-H & 4-H by OH groups in this molecule. Both EIMS and CIMS revealed its molecular weight to be 265, which was 32 mass units more than that of mimosamycin (13). Its structure was finalised as 1,4-dihydroxy mimosamycin (15) from the above data.

Compound (16), an yellow crystallline solid, m.p. 133°C (Lit:135-136°C)[3,4] had absorptions at 1648 and 1576 cm^{-1} in its IR and at 252, 297, 317 and 374 nm in its UV-Vis spectra, indicating the presence of quinone moiety. The peak at 1713 cm^{-1} in its IR spectrum could be attributed to an unsaturated ester moiety. The presence of quinone was also supported by

the peaks at δ 181.28 and 183.5 in its ^{13}C NMR spectrum. The empirical formula of the molecule was determined to be $C_{16}H_{15}NO_5$ by elemental analysis. EIMS revealed its molecular weight to be 301. Thus, it was clear that the above empirical formula represents its molecular formula itself. The base peak at m/z 82 in its mass spectrum as well as the typical peaks in the ^1H NMR spectrum of the molecule indicated the presence of angelate ester group in it. The structure of this compound was finalised as O-demethyl renierone (16) from these results. This was also confirmed by DEPT & ^1H-^{13}C COSY spectra and comparison of these values with those reported in literature[3-4].

Compound (17), R_t=19.6 min, (m.p. 174°C), was obtained as red crystals after HPLC purification. The quinonoid nature of this molecule was revealed by its IR absorptions at 1673, 1606 & 1570cm^{-1} (FIG 8) and UV-Vis absorptions at 231, 256, 304.5, 324.5, 379.5 & 485.5 nm. As in the case of compound (16), the peak at 1710 cm^{-1} could be assigned to the presence of an unsaturated ester moiety. Its ^1H NMR spectrum and mass spectral fragmentations were almost identical to those of compound (16). This prompted us to conclude that compound (17) is probably a dimer of compound (16). The above view was confirmed when the product obtained by treatment of compound (16) with dicyclohexyl carbodiimide (DCC) in dry methylene chloride was found identical to compound (17) with respect to TLC, HPLC, IR and melting point analyses.

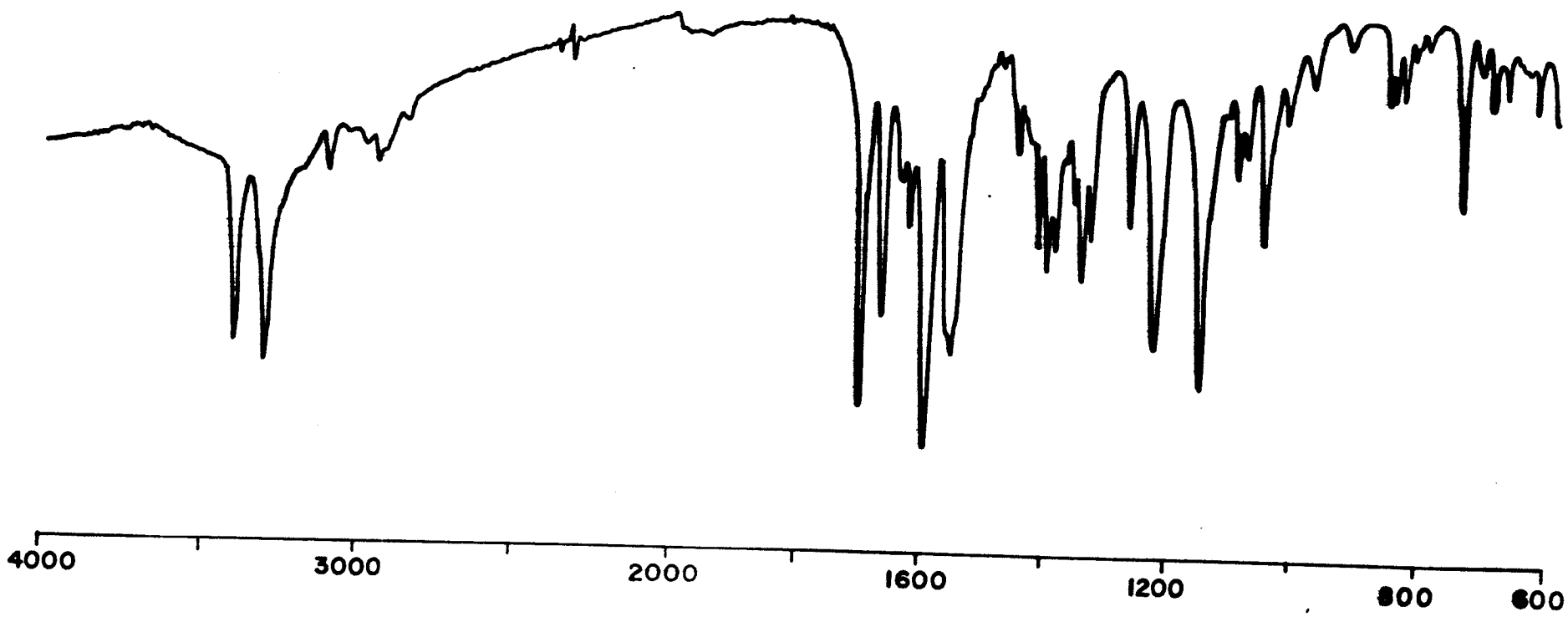


Fig. 8 IR (KBr) spectrum of compound (17)

Compound (18), R_t=41.7 min, was isolated as a light red amorphous powder after HPLC purification. Its IR spectrum had absorptions at 3425,1726,1690,1648,1279 & 1067cm^{-1}, indicating the presence of hydroxyl, ester, quinone and amide groups in the molecule (FIG 9). The quinonoid nature of the molecule was also indicated by its strong UV-Vis absorptions at 225, 275 & 375 nm and the peaks at δ 179,184 and 192 in its ^{13}C NMR spectrum (TABLE 5). Its ^1H NMR spectrum had signals due to two vinyl Me's(δ 2.15 & 1.95), two O-Me's(δ 4.05 & 3.85), one N-Me(δ 2.56), one angelate moiety (δ 1.46 (3H,d,1.5 Hz), 1.74 (3H,dq,7.25 & 0.5 Hz) and 5.91(1H,qq,7.28 & 1.5 Hz) and two D_2O-exchangeable protons(δ 5.7 & 11.34) (TABLE 6, FIG 10). Comparison of these values with those of renieramycins[4,8] and saframycins[11,12] indicated its close structural similarity with them. This was supported by the presence of characteristic pairs of carbon signals between 119 and 192 ppm in its ^{13}C NMR spectrum (TABLE 5 & FIG 11). A weak molecular ion was present at m/z 594 in both CI and EI mass spectra (TABLE 7 & FIG 12). However, the prominent peak in the EIMS was seen at m/z 580, probably due to $[M-OH+3H]^+$. The elemental composition of the above ion was determined to be $C_{30}H_{32}N_2O_{10}$ by HREIMS. From this, the molecular formula was deduced to be $C_{30}H_{30}N_2O_{11}$.

Preliminary examination of the ^1H NMR spectrum indicated it to be structurallly similar to renieramycins A-F[4-8]. However, the signals due to 3-H, 4-H, 13-H & 21-H of renieramycins A-F were absent in compound (18). In renieramycin A and B, 21-H_2

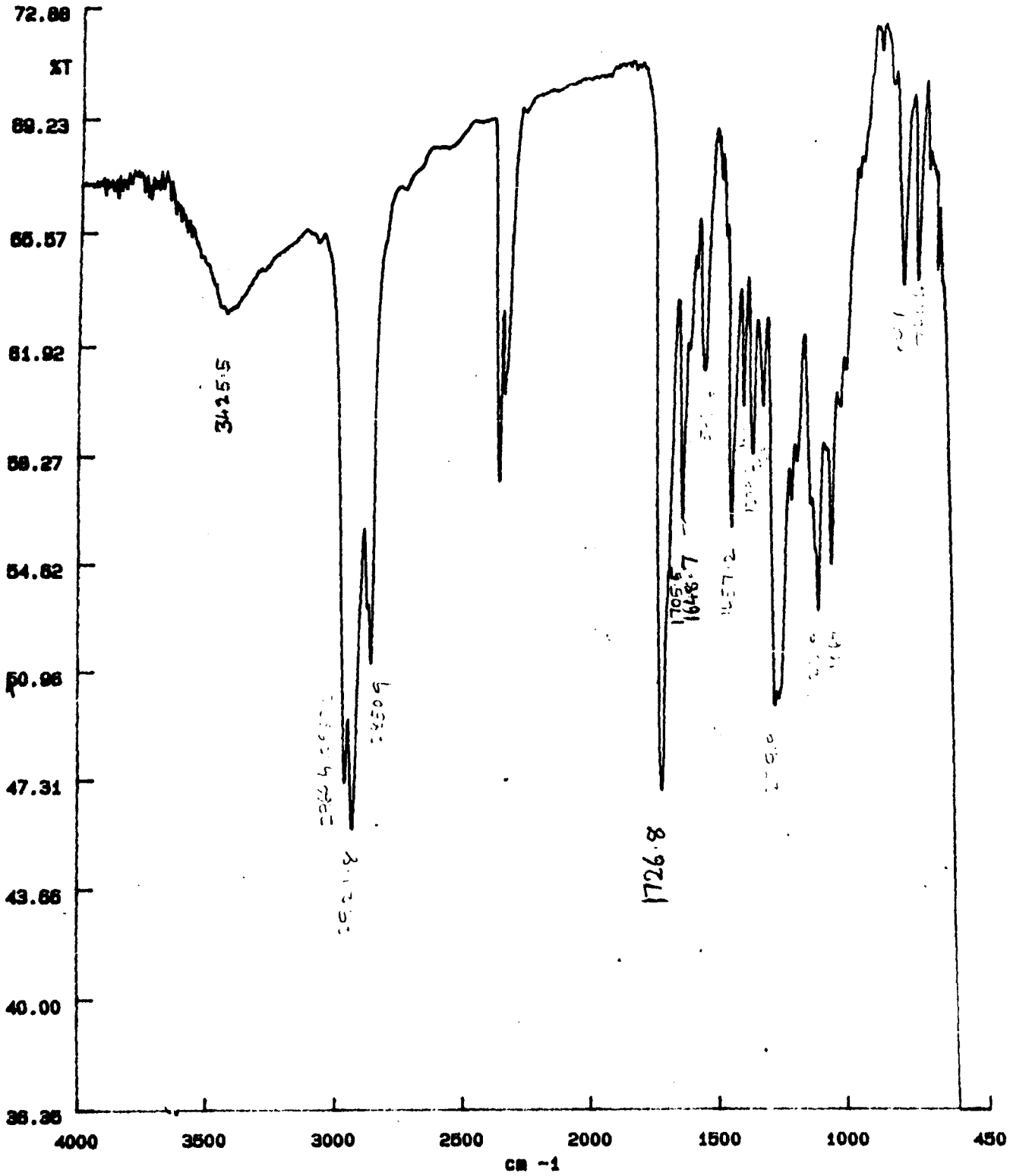


FIG 9 IR(KBr) SPECTRUM OF RENIERAMYCIN G (18)

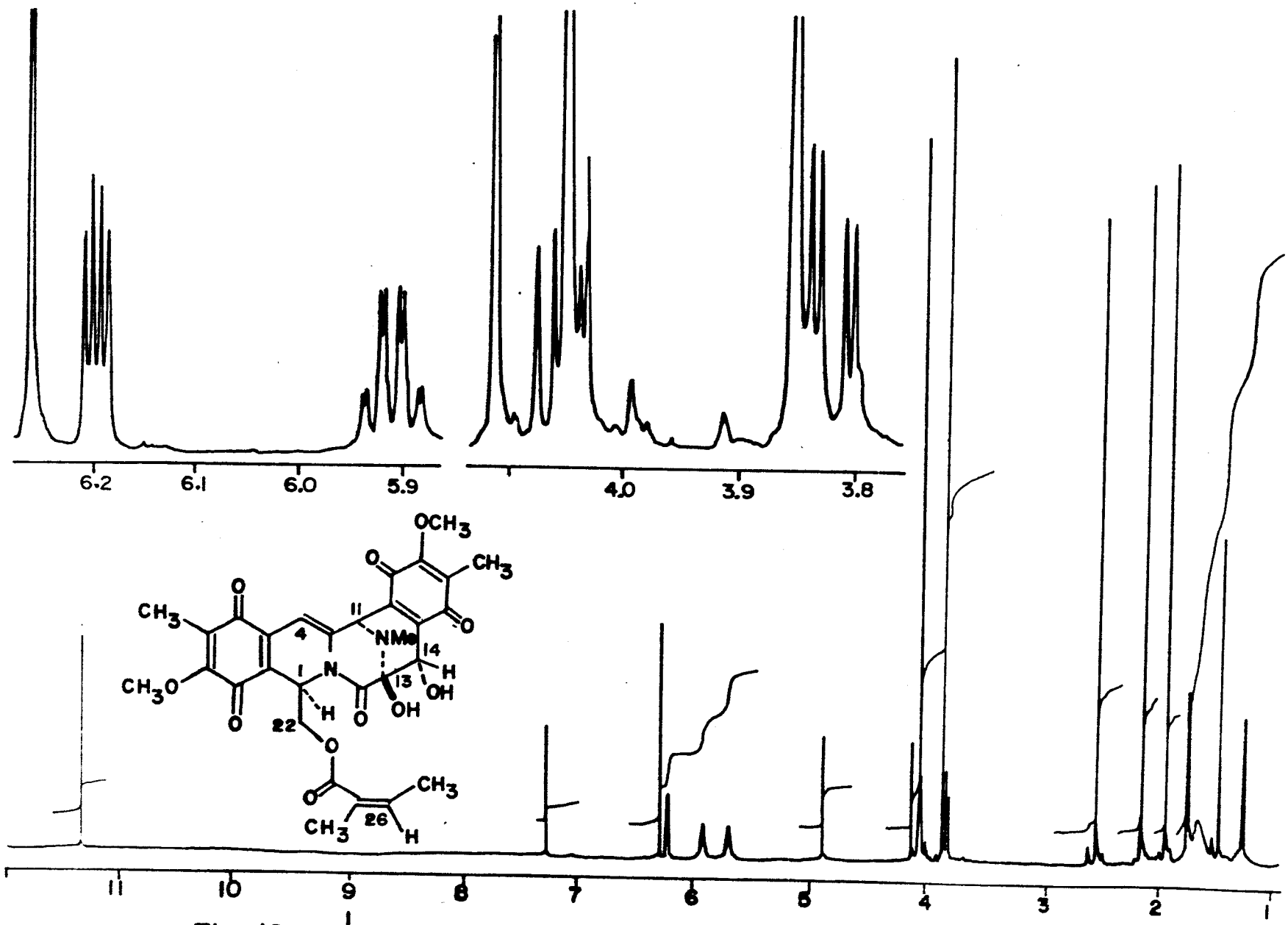


Fig. 10 ^{1}H NMR spectrum of renieramycin G (18)

TABLE 5.

^{13}C NMR Assignments of Renieramycin G (18)[a]

Carbon No	Chemical shift(δ)	Carbon No	Chemical shift(δ)
C-1	46.96(d)	C-17	156.42(s)[d]
C-3	134.70(s)	C-18	Not obsvd
C-4	100.00(d)	C-18a	124.28(s)[e]
C-4a	139.95(s)[b]	C-21	161.17(s)
C-5	184.00(s)	C-22	62.12(t)
C-6	119.93(s)[c]	C-23	Not obsvd
C-7	153.36(s)[d]	C-24	127.10(s)
C-8	179.00(s)	C-25	15.39(q)
C-8a	126.74(s)[e]	C-26	139.09(d)
C-11	56.32(d)	C-27	19.85(q)
C-13	108.00(s)	ArMe	8.54(q)
C-14	72.60(d)	ArMe	8.93(q)
C-14a	138.18(s)[b]	ArOMe	61.10(q)
C-15	192.00(s)	ArOMe	61.20(q)
C-16	119.16(s)[c]	N-Me	41.22(q)

[a] NMR spectrum was obtained on Bruker WM-400 instrument in $CDCl_3$ solution with TMS as internal standard and the multiplicities confirmed by 2D heteronuclear correlation experiments.

[b-d] Values with identical superscripts may be interchanged.

TABLE 6

^{1}H NMR Spectral Data of Renieramycins G (18) and H (19).

Proton	Renieramycin G(18), Chem shift(ppm)	Renieramycin H(19), Chem Shift(ppm)
1-H	6.20(dd,6.3&2.7Hz)	6.07(dd,5.28&2.67Hz)
4-H	6.26(s)	6.25(s)
11-H	4.80(D,1.3Hz)	4.54(d,0.93Hz)
13-H	---	3.73(br s)
14-H	4.10(d,1.3Hz)	4.34(d,1.83Hz)
25-Me	1.46(d,1.5Hz)	1.55(d,1.60Hz)
26-H	5.91(qq,7.28&1.5Hz)	5.91(qq,7.23&1.5Hz)
26-Me	1.74(dq,7.25&1.5Hz)	1.73(dq,7.25&1.55Hz)
ArMe	2.15(s)	1.96(s)
ArMe	1.95(s)	1.94(s)
22-H	4.05(dd,11.8&6.80Hz)	4.16(dd,12.1&2.67Hz)
22-H'	3.82(dd,11.8&3.1Hz)	4.01(dd,12.1&2.67)
ArOMe	4.05(s)	4.05(s)
ArOMe	3.85(s)	3.98(s)
OMe	---	3.62(s)
NMe	2.56(s)	2.55(s)
OH	11.34(s)	---
OH	5.70(s)	---

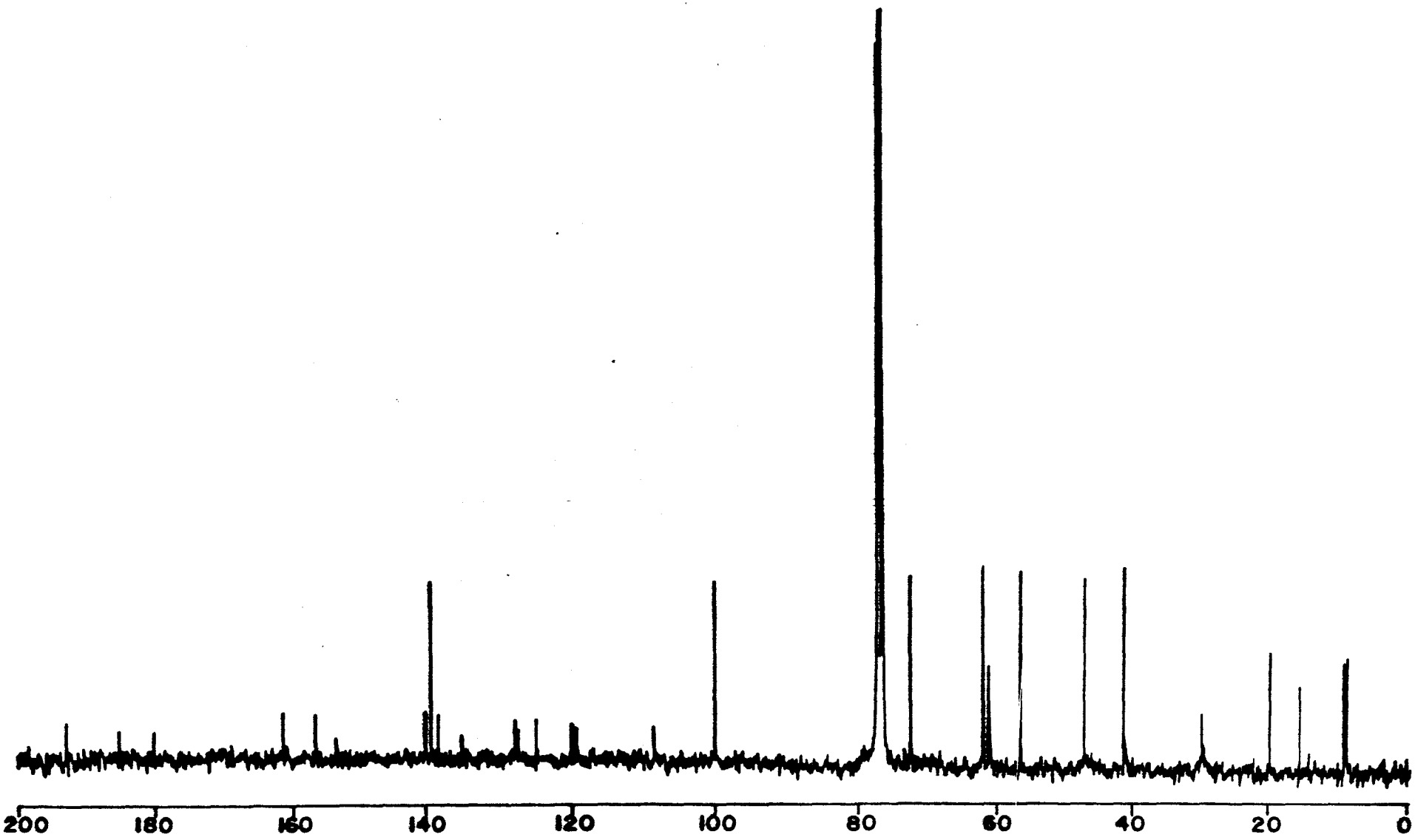


Fig. 11 ^{13}C NMR spectrum of renieramycin G (18)

TABLE 7:

EIMS DATA OF RENIERAMYCINS G (18) AND H (19).

Renieramycin G m/z(%)	Renieramycin H m/z(%)
594($M^{\cdot}$,1.5)	594([M+2H]$^{\cdot}$,5)
580([M-18+4H]$^{\cdot}$,12)	593([M+H]$^{\cdot}$,4)
550([M-28-18+2H]$^{\cdot}$, or [M-30-18+4H]$^{\cdot}$,1)	562([M-28-2H]$^{\cdot}$, or [M-30]$^{\cdot}$, 12.5)
522([M-2X28-18+2H]$^{\cdot}$, or [550-28],1.5)	534([M-2X28-2H]$^{\cdot}$ or [562-28],3.5)
481([M-4X28-H]$^{\cdot}$,2.5)	482([M-4X28]$^{\cdot}$,3)
467([M-4X28-15]$^{\cdot}$ or [550-83],11)	479([M-4X28-H]$^{\cdot}$ or [562-83],15)
453([M-5X28-H]$^{\cdot}$,10.5)	451([M-5X28-H]$^{\cdot}$ or [479-28],75)
439([M-5X28-15]$^{\cdot}$ or [467-28],75)	421([M-6X28-3H]$^{\cdot}$ or [451-30],100)
396([M-7X28-2H]$^{\cdot}$,4.5)	---
279(31)	250(57)
234(5)	218(43)
167(40)	---
149(100)	149(11)

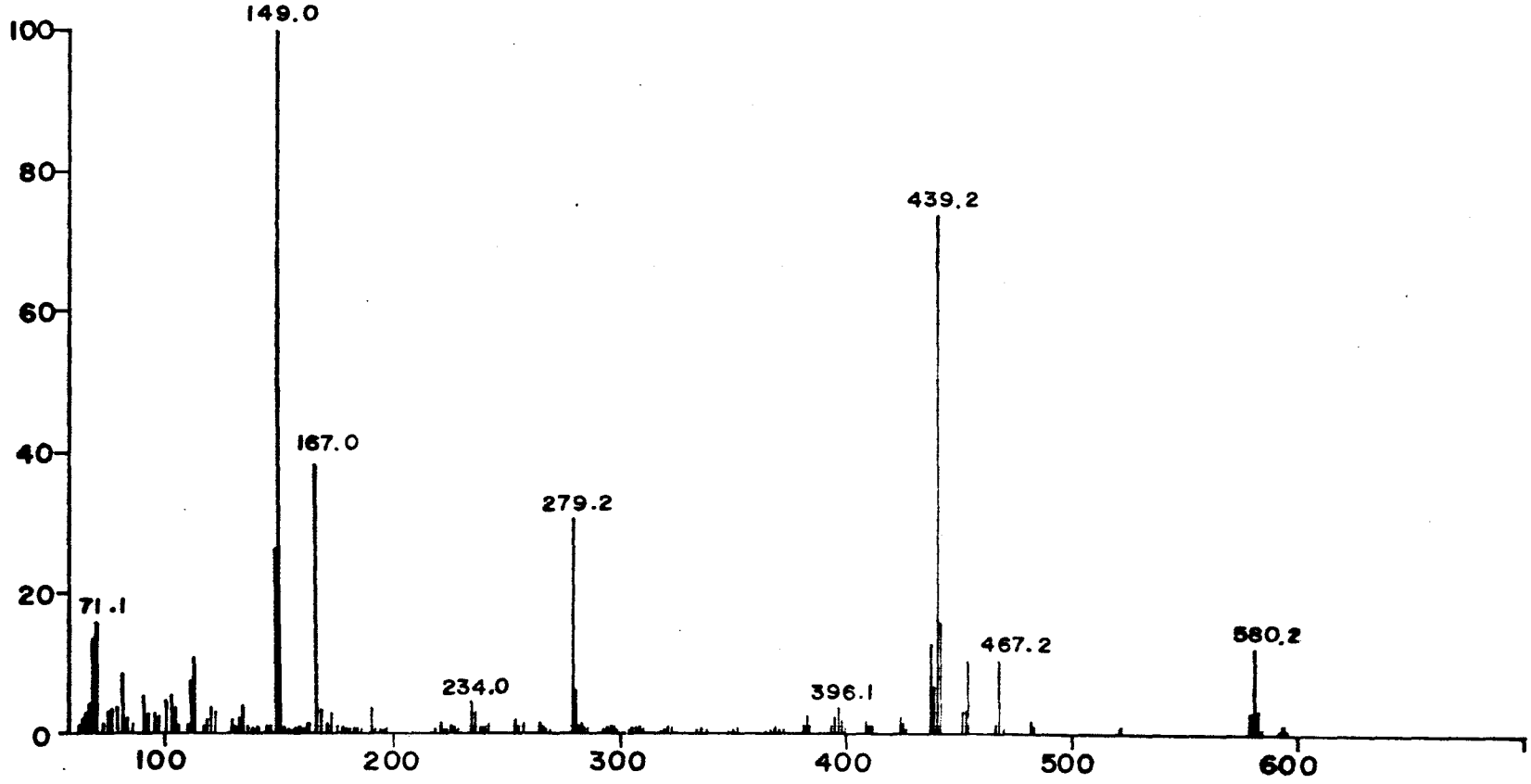


Fig. 12 Electron impact mass spectrum of renieramycin-G (18)

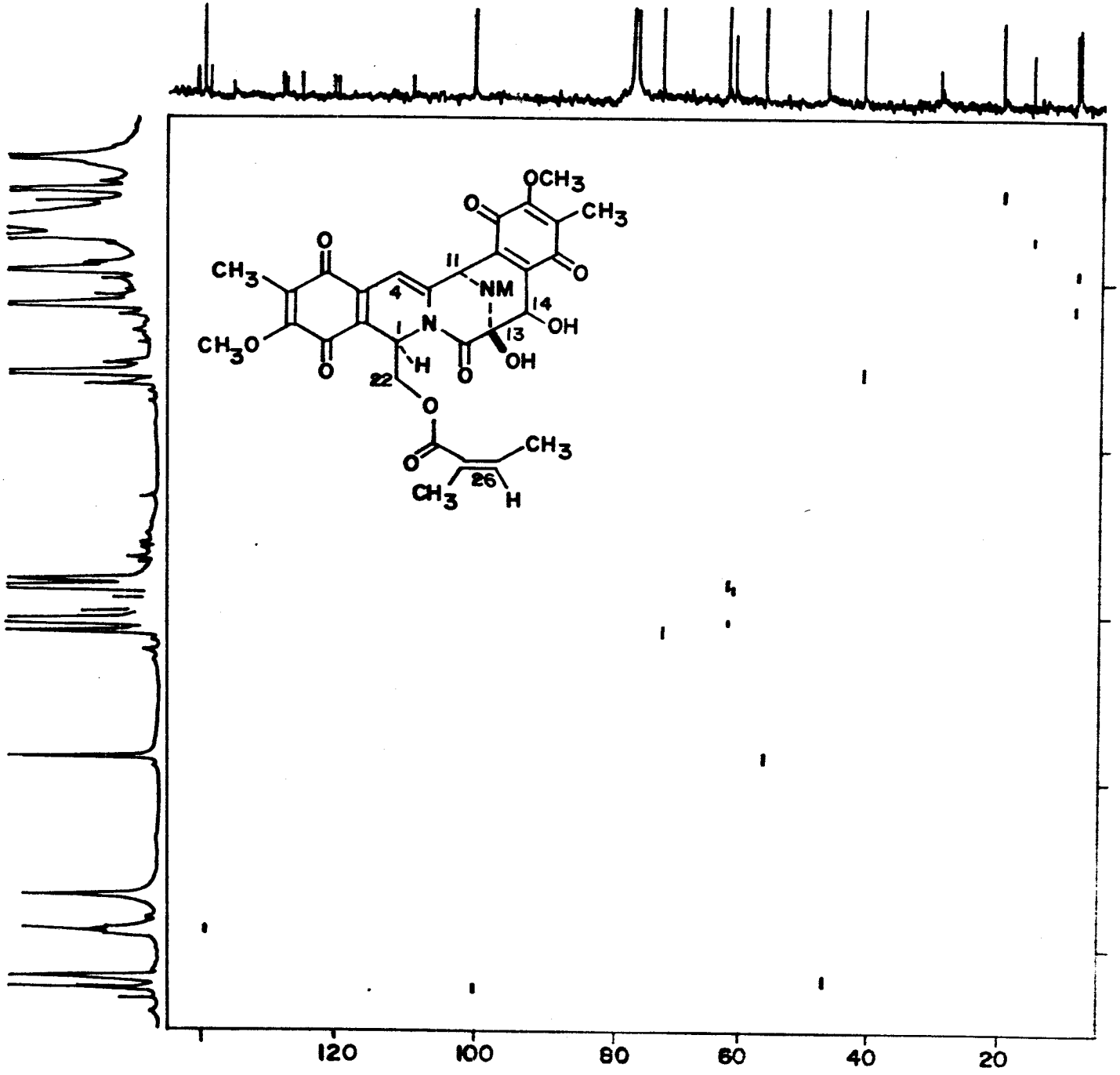


Fig. 13 $^{13}C-^{1}H$ COSY spectrum of renieramycin G (18)

appear at δ 2.71 & 3.18 and 2.76 & 3.10 respectively[4]. In renieramycins E and F, which have hydroxyl groups at C-21, the corresponding methine proton signals appear at δ 4.44 & 4.57 respectively[5]. On the other hand, renieramycins C and D have a carbonyl group at C-21[4]. Absence of proton signals due to 21-H in compound (18) is thus indicative of the presence of an amide carbonyl group at C-21 as in renieramycins C and D. This was also supported by its IR absorption at 1648 cm^{-1} and the ^{13}C NMR signal at δ 161.17. The signal at δ 6.26(1H,s) could be due to 4-H subsequent to the presence of a double bond at C-3. ^{1}H-^{13}C COSY experiment located the corresponding vinyl carbon signal at δ 100.0(d,C-4) (FIG 13). Similarly, the carbon signal at δ 134.7(s) could be due to C-3. These assignments are in agreement with the values reported for similar carbon and hydrogen signals in N-formyl-1,2-didehydrorenierone as shown in FIG 13a[4].

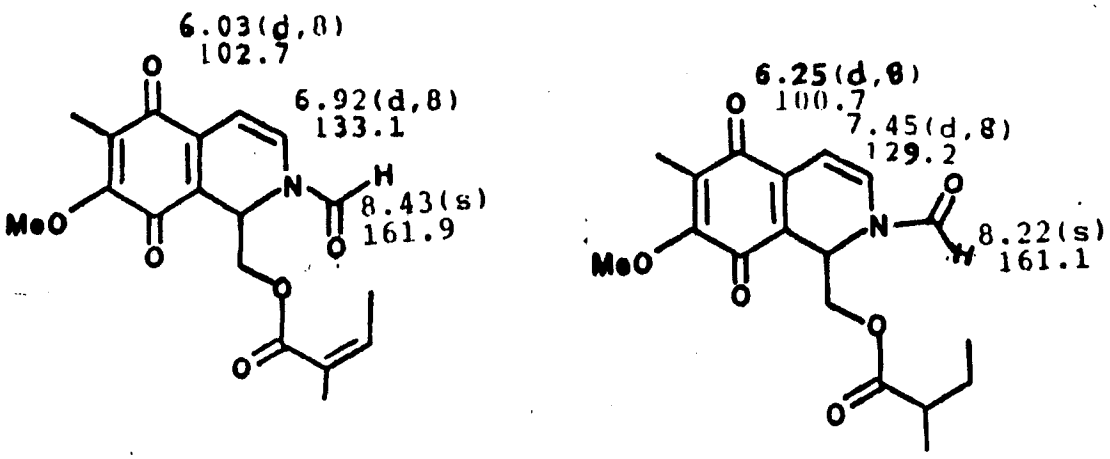


FIG 13a. CHEMICAL SHIFTS IN N-FORMYL-1,2-DIHYDRORENIERONES

From the above discussion, it is clear that the structure of compound (18) is closer to that of renieramycins C and D, with an additional double bond at C-3. Another difference was in the absence of the proton signal due to 13-H in this compound. This peak appears at δ 3.73 and 3.67 respectively in latter two compounds[4]. On the other hand, compound (18) had two D_2O-exchangeable protons at δ 5.7 and 11.34 as compared to only one such proton in renieramycin C (14-OH). These results led us to believe that probably the 13-H of renieramycin C and D has been replaced by a hydroxyl group in this molecule. This also agrees with the observed downfield shift of its C-13 signal to δ 108.0 (s) which is expected since this carbon is now directly attached to a carbonyl, hydroxyl and nitrogen groups. The proton signals at δ 6.20 (1H,dd,6.3 & 2.7 Hz), 4.80(1H,d,1.3 Hz) and 4.1(1H,d,1.3 Hz) could be assigned to 1-H, 11-H & 14-H respectively. The corresponding carbon signals were observed at δ 46.96(d,C-1), 72.6(d,C-11) & 56.32(d,C-14) in its ^1H-^{13}C COSY spectrum. The 1-H and 11-H signals appear at δ 5.48 & 5.49 and δ 4.19 & 4.22 respectively in renieramycins C and D. This downfield shift of these two signals to δ 6.20 and 4.80 in compound (18) might be due to the influence of the new double bond at C-3. With this new double bond, the 11-H becomes diallylic. Incidently, this is one of the rare cases involving coupling between protons separated by five bonds. Thus, the 11-H and 14-H signals, which otherwise should appear as singlets, appear as clear doublets with a coupling constant of 1.3 Hz

each. The allylic coupling between 11-H and 4-H is ruled out as the latter signal appears as a sharp singlet in the ^1H NMR spectrum. The ^1H & ^{13}C NMR as well as the COSY spectra of this compound are reproduced in FIGs 10, 11 and 13 respectively. The assignment of the ^1H and ^{13}C NMR spectra are provided in TABLES 5 and 6 respectively.

Preliminary examination of the spectral data of compound (19), R_t=52.4 min (yield 2 mg) indicated its structural similarity with renieramycin G (18). Thus, this compound had strong peaks at 1716, 1652 and 1600-1500 cm^{-1}, (carbonyl, amide and vinyl groups respectively) in its IR (FIG 14), and at 220, 260, 340 and 520 nm in its UV-Vis absorption spectra. At the same time, absence of a peak at 3400 cm^{-1}, in the IR spectrum ruled out the presence of hydroxyl groups in it. From the strong $[M+H]^+$ peak at m/z 593 observed in its FABMS (FIG 16) and the peaks at m/z 593 and 594 of almost equal intensities in its EIMS (FIG 17), molecular weight of this compound was deduced to be 592. HREIMS of the peak at m/z 592 established its elemental composition as $C_{31}H_{32}N_2O_{10}$ (Experimental: 592.2086 as against the calculated: 592.2057).

Its ^1H NMR spectrum was similar to that of renieramycins C, D[4] and G. Thus, this compound had signals at δ 4.05, 3.98 & 3.62 (3H each,s,O-methyls), 1.96 & 1.94 (3H each,s,methyls on vinyl carbons) and one N-methyl (δ 2.55) groups in it. Among these, the signal at δ 3.62 was attributed to 14-OMe group by

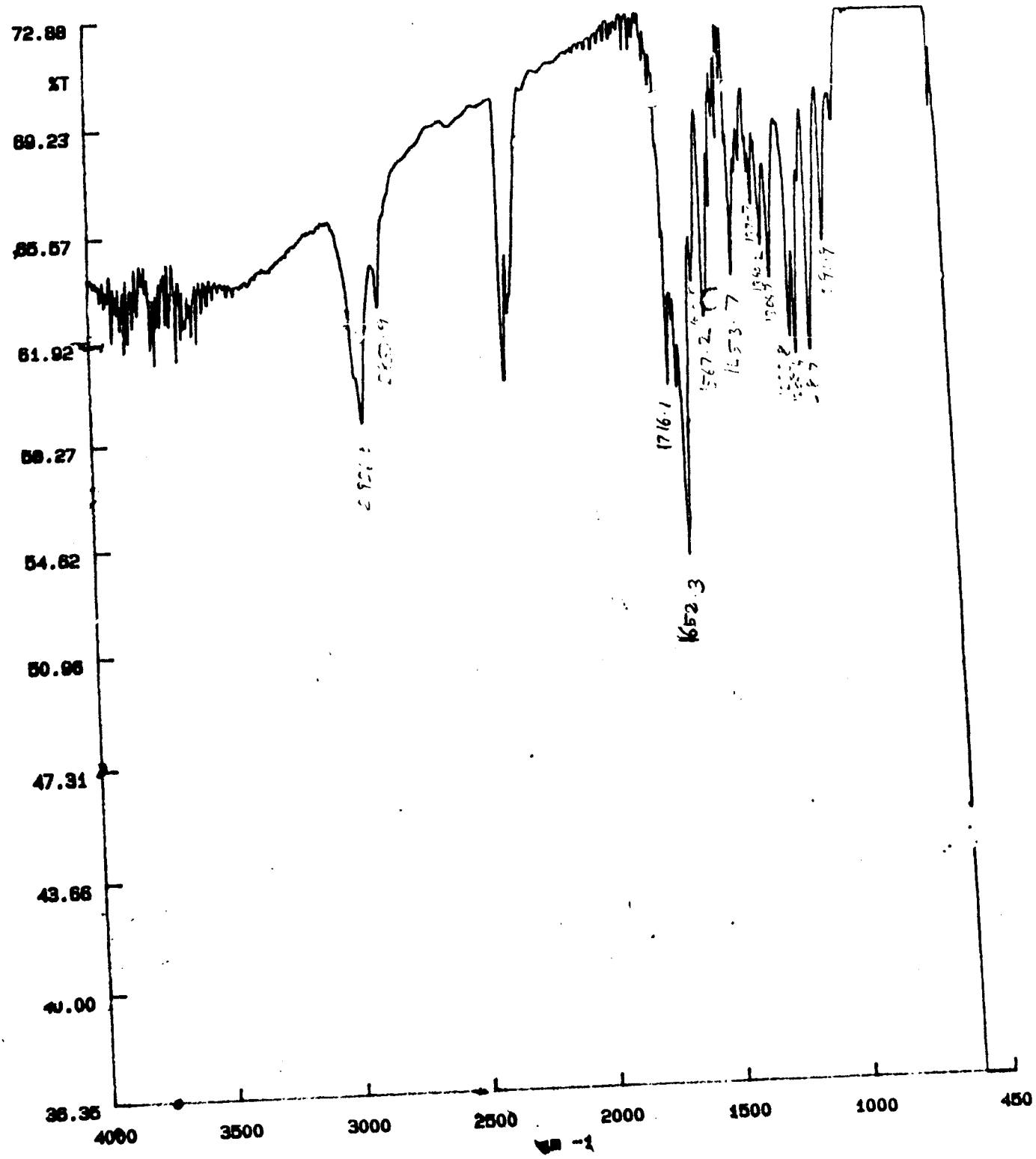


FIG 14 IR(KBr) SPECTRUM OF RENIERAMYCIN H (19)

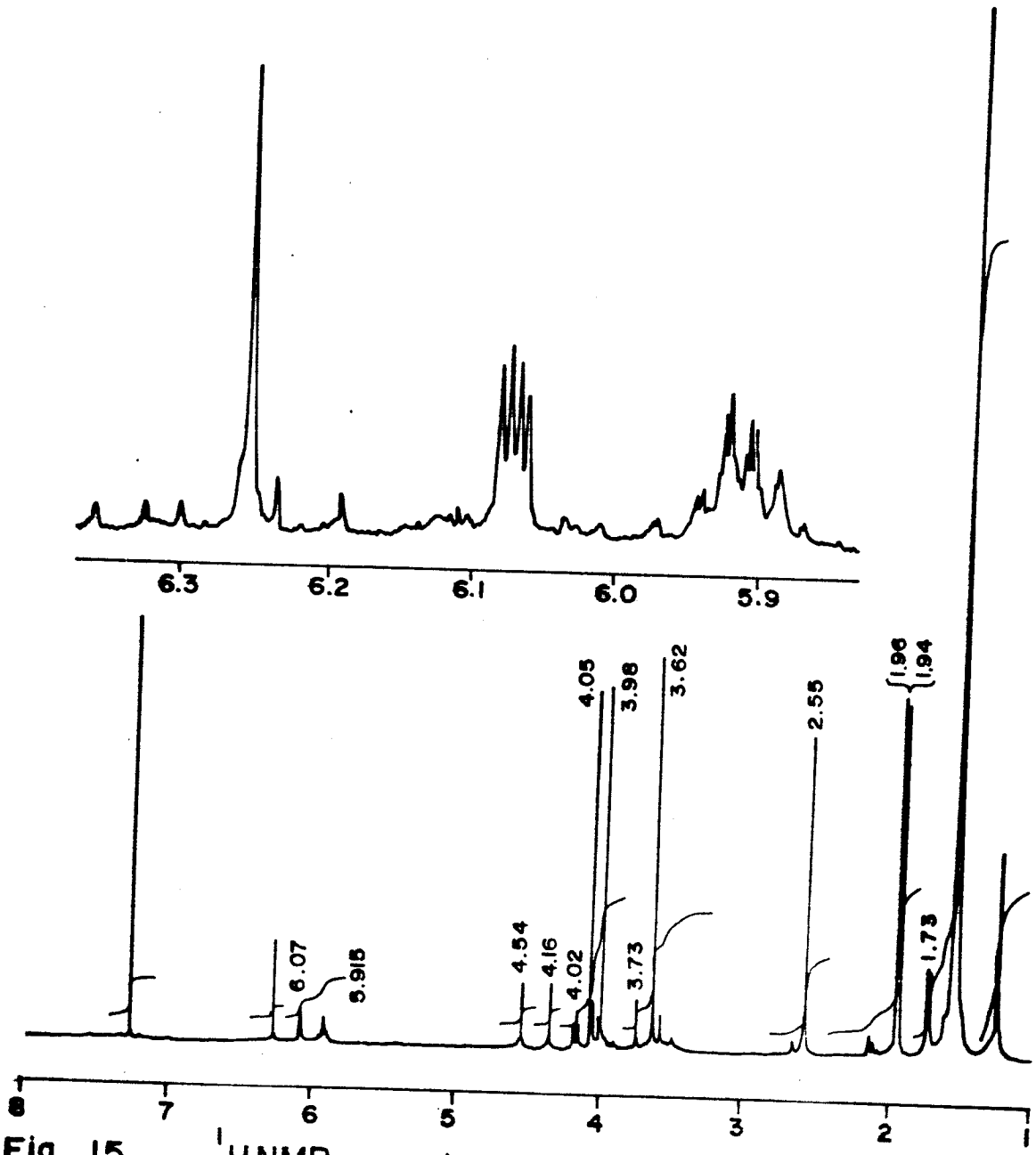


Fig. 15 ^{1}HNMR spectrum of Renieramycin H (19)

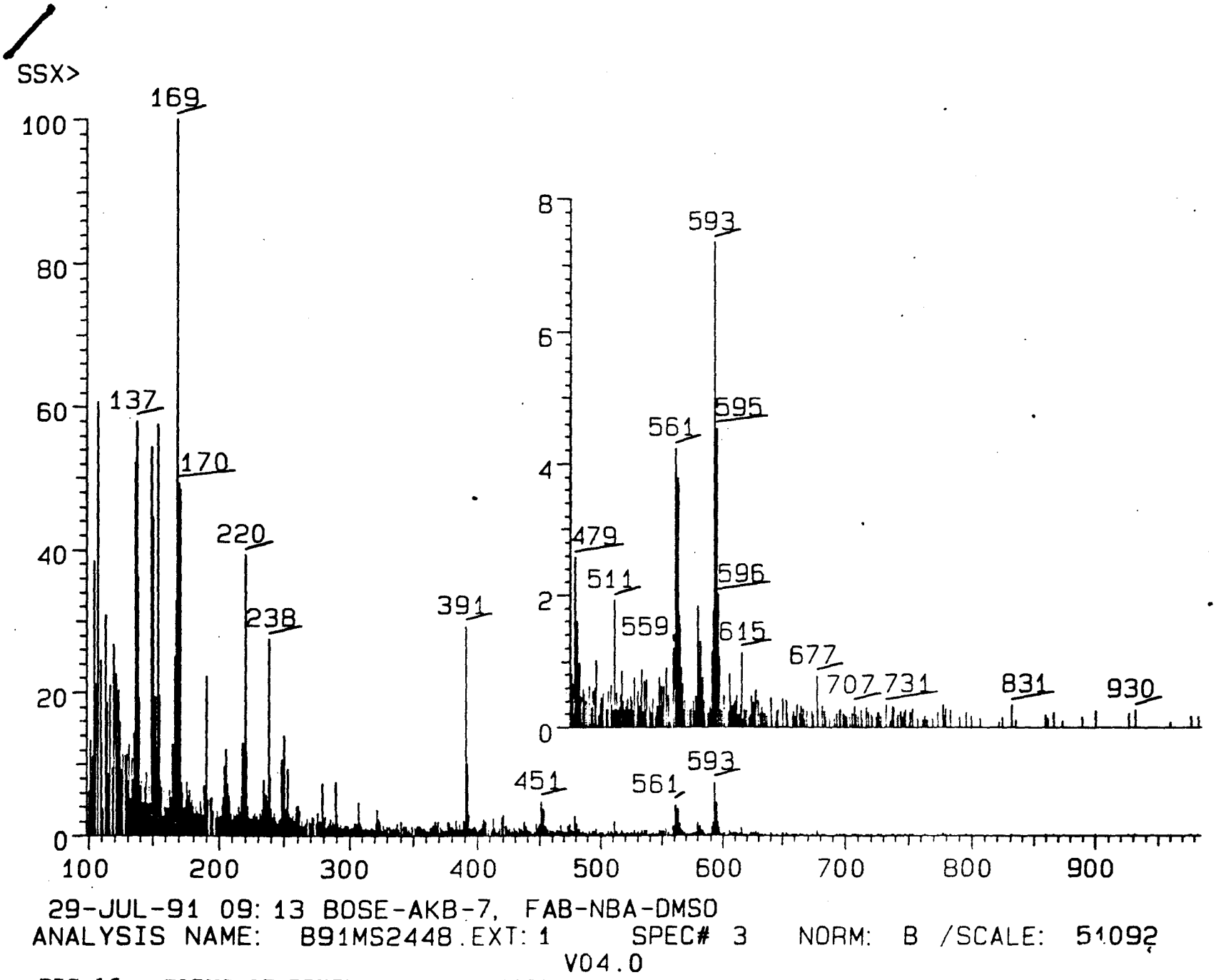


FIG 16 FABMS OF RENIERAMYCIN H (19)

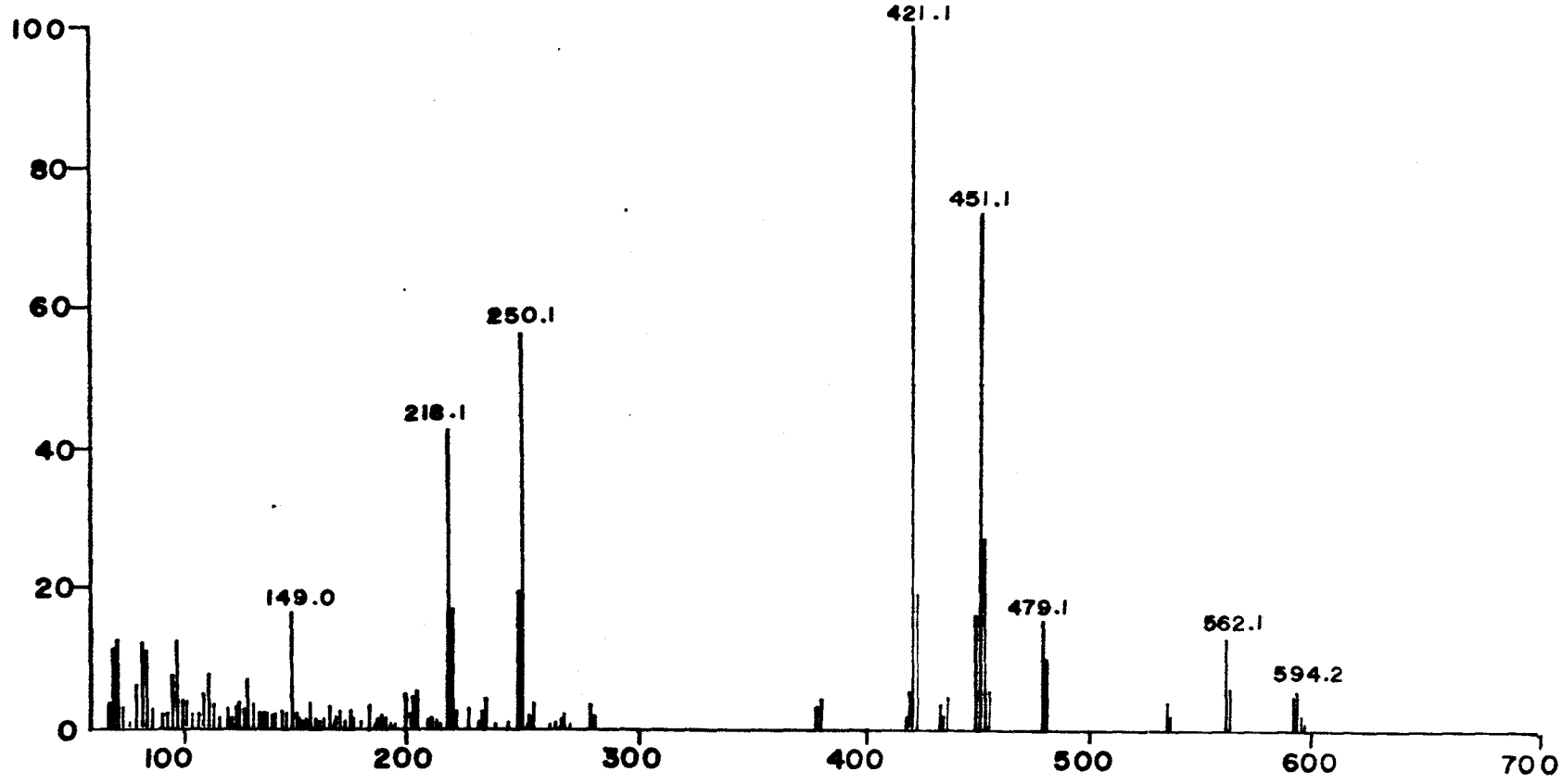


Fig. 17 Electron impact mass spectrum of renieramycin - H (19)

analogy with renieramycins B, D & F[4-5]. Detailed comparison of these values with that of renieramycin G indicated that the signals due to 1-H, 4-H, 11-H, 14-H & 22-H are all present in their expected places (see TABLE 5). The proton signals at δ 4.54(1H,d,0.93 Hz) and 4.34(1H,d,1.83 Hz) were assigned to 11-H and 14-H. The corresponding values for renieramycin G were observed at δ 4.80 and 4.10 respectively (TABLE 5). The 14-H signal appears at δ 4.78(brs) and 4.36(brs) respectively in renieramycins C and D[4]. The two methyl peaks at δ 1.55(d, 1.6 Hz) and 1.73(dq,7.25 & 1.55 Hz) as well as the single proton signal at 5.91(qq,7.23 & 1.5 Hz) could be easily assigned to the angelate ester group[4]. The additional signal at δ 3.73(1H,brs) present in this compound as compared to renieramycin G (18) must be due to 13-H[4]. In renieramycins C and D the corresponding proton signals appear at δ 3.73(s) & 3.67(brs) respectively. The very small vicinal coupling between 13-H and 14-H indicated them to be orthogonal to each other[5]. The structural similarity of compounds (18) and (19) is also evident from their near identical mass spectral fragmentations (TABLE 7). Both these compounds, *i e*, Renieramycin G (18) and H (19), are novel compounds, reported for the first time.

Renieramycins are very labile compounds[4,5]. Due to this, and partly due to their limited quantities available, further NMR or X-ray crystallographic studies to establish the stereochemisty of compounds (18) and (19) could not be carried out.

In their earlier paper, Faulkner and his group had indicated the 1-H and 3-H in renieramycins A-D[4] to be *trans* whereas they are reported to be *cis* in saframycin C as determined by X-ray crystallographic studies[11]. They had arrived at this stereochemistry mainly from NOEDS expts., wherein irradiation of 14-H had resulted in enhancement of 1-H. However, they later realised that probably this could have been due to too high power used which probably irradiated both 14-H as well as 22-H[5]. Subsequent irradiation expts. using lower power levels indicated that both 1-H and 3-H are *cis* to each other. The same results were arrived at by Fukuyama *et al*, from synthetic studies of saframycins[13] and renieramycins[14]. Since the ^{1}H and ^{13}C NMR spectra of renieramycins G (18) and H (19), are very similar to that of other renieramycins[4,5] and saframycins[11,12] we believe that their stereochemistry may also be identical.

GENERAL DISCUSSION AND CHEMOTAXONOMY

It is known that sponges belonging to the family *Aplysinidae* and *Aplysinellidae* (Order: *Verongida*) contain aplystane sterols[15]. In fact, based on some chemotaxonomic studies De Rosa *et al.*, opined that aplystane sterols are restricted only to the sponges of this order[15]. However, later Bergquist *et al.*, reported 24(28)-didehydroaplysterol from *Haliclona* sp[16]. Subsequently, this compound was isolated as

a minor component from the sponge *Jaspis stellifera* (Family: *Jaspidae*, order: *Choristida*)[17]. These results proved that this sterol is not restricted to the animals of the *Verongida* order alone, which was further supported by our work. Recent biosynthetic studies have cast serious doubt about the very origin of this sterol. Thus, studies using radiolabelled acetate, methionine and mevalonate have shown that the sponge *Verongia aerophoba* is incapable of biosynthesising aplystane sterols, leading to a speculation that, perhaps, this sterol is of planktonic origin[18].

Ceramides are fatty acid amides of the base sphingosine (2-amino-1,3-dihydroxy-4-octadecene) and form an important constituent of sphingolipids present in brain and nerve tissues. Of late, several such compounds have been isolated from various marine flora and fauna. While some of them differ only in the chain length or number of methylene groups, others have additional hydroxyl groups, double bonds *etc*. Prominent among them is caulerpicin, isolated from the edible green alga *Caulerpa lamourouxin*[19] as well as some other *Caulerpa* sp. and also another green alga *Ulva fasciata*. It causes mild anesthesia when placed in mouth and is responsible for the mild peppery taste of these algae. From the latter alga, Garg *et al.*, have isolated a tetrahydroxy sphinganine (saturated sphingosine) derivative[20]. This compound was active, both *in vitro* and *in vivo*, against Semiliki Forest Virus (SFV) and Encephalomyocarditis Virus

(EMCV). It was found to be nonverucidal but effective interferon inducer. A third compound isolated from this alga was 4,8-diene-N-palmitate derivative of sphingosine[21]. This ceramide was inactive against EMCV but active against JEV (Japanese encephalitis virus). It has been noted that the algal sphingosines have normally 18 carbon atoms in it while no such generalisations can be made regarding their counterparts in marine fauna. Thus, ceramides having C_{16} and C_{18} sphingosine residues were isolated from the lipid fraction of oysters[22]. Similarly, a ceramide having 22 carbons in their sphingosine residue was isolated from a sea anemone *Anemonia sulcata*[23]. A linear terpenoid sphingosine having potent anti-HSV-1 (*Herpes Simplex Virus*-1) and anticancer activity, besides being active against several Gram positive and Gram negative bacteria, was isolated from a tunicate *Aplidium* sp[24]. This is perhaps, the first example of a ceramide produced from a diterpene and the amino acid serine.

So far no reports have appeared about the presence of ceramides in sponges. Thus, ours is the first observation of these compounds in *Porifera*. Again, true to the earlier generalisation, the sphingosine residue of the ceramide (12) has 23 carbon atoms while the acyl group is docosanoic acid. As cited before, ceramides are active against the viruses SFV and EMCV[20]. During our initial studies it was noticed that the MeOH extract of this sponge was active against EMCV. Hence it is possible that the activity is due to the ceramide

reported here or their analogs not yet identified.

The alkaloids (13-19) have close structural similarity to those reported from similar sponges[3-5], the bacteria *S. Lavendulae*[12] and their predator nudibranch[9]. The preliminary examination of the spectral data indicated their structures to be closer to renieramycins C and D. The most important structural difference between compounds (18) and (19) and renieramycins A-F[4,5] is in the presence of an additional double bond at C-3 in the former two as compared to the latter. In addition, in renieramycin G (18), the 13-H has been replaced by a hydroxyl group. The major monomeric alkaloids, O-demethyl renierone (16) and mimosamycin (13) are found both in this sponge and in *Reniera* sp[3,4]. Faulkner had noticed that the 'dimeric' renieramycins are unstable, readily decomposing to the stabler monomers such as renierone, mimosamycin, *etc.*, when kept dissolved in chloroform for some time[5]. The 1-hydroxymimosamycin (14) may have been formed from mimosamycin or directly from renieramycins. During our studies, it was observed that compound (14) gradually undergoes air-oxidation, forming 1,4-dihydroxy mimosamycin (15).

Analyses of chemical constituents of marine organisms are important from biomedical and chemotaxonomic point of view. In this regard, it is very interesting that the origin of many of these compounds reported here are rather

controversial. For example, as mentioned earlier, it is suspected that aplysterol is probably not a genuine sponge metabolite, but is of planktonic origin[18]. Our studies indicate that 24(28)-didehydroaplysterol (4) is a major sterol constituent (21.24%) in this sponge. The biosynthetic studies, which indicated the dietary origin of this sterol was restricted to sponges of *Verongida* order only[18]. Later, however, this compound has been isolated from other sponges also, *viz.*, *Haliclona* sp[16], *J Stellifera*[17], *etc.* From the available results so far, it is thus very difficult to arrive at a firm conclusion regarding the origin of this compound.

Similarly, the alkaloids reported here, or their close analogs have been isolated from sponges of *Reniera* genus from various localities around the world[4,5] and also from the bacteria *S Lavendulae*[12]. Biologists are of the opinion that the names *Reniera* and *Haliclona* denote one and the same genus[1]. This means that similar bacteria are endemic to these sponges all over the world or that these compounds are genuine sponge metabolites. Interestingly, several isoquinoline alkaloids related to N-formyl-1,2-dihydro-renierone were also isolated from a blue sponge *Xestospongia sp* and its predator nudibranch *J funebris*[9]. But none of the above compounds had the angelate ester moiety. Instead, the acyl moieties in these molecules were acetate or propionate groups. Further, they also do not have any 'dimeric' renieramycins. From the available results so far, it is very

difficult to arrive at a firm conclusion regarding the origin of these alkaloids.

Recently there have been reports on isolation of cytotoxic ß-carboline derivatives, manzamines A-C from a sponge *Haliclona sp* from Okinawa[7]. Curiously, no renieramycins or other related isoquinoline compounds were found in this animal. Several manzamine analogs have subsequently been reported from sponges of other genera also[7-8], leading to the suggestion that probably these compounds are produced by some associated organisms[7a].

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURE

HPLC separations were carried out on a SPECTRAPHYSICS MODEL 8800. The sterols and batyl alcohol and its analogs were separated on reverse phase columns (ODS,5µ,250X8 mm) using MeOH as eluant and an RI detector. The alkaloids were separated on the same column using MeOH-H_2O (3:1) as eluant. The chromatogram was monitored at 254 nm using an UV detector. The IR and UV-Vis absorption spectra were recorded on PERKIN ELMER FTIR MODEL 1650 and BECKMAN MODEL DU-6 spectrophotometers. ^1H and ^{13}C NMR spectra were recorded on BRUKER WM-200 and 400 MHz instruments using $CDCl_3$ or Py-d_5 as

the solvent and TMS as internal standard. CIMS was recorded on a BIOSPECT MASS SPECTROMETER using CH_4 as reagent gas at a source temperature of 180°C. EIMS & LSIMS (Liquid secondary ion mass spectra) were obtained on a FINNIGAN MAT-90 SPECTROMETER, having ultra high resolution double focussing facilities and operating at an accelerating voltage of 5 Kev. EIMS was recorded at 70 ev at a probe temperature of 150°C while the source temperature was maintained at 250°C. For LSIMS experiments, samples dissolved in DMSO (2-5 µg/ml) was deposited on a stainless steel probe tip and then mixed with a thin layer of glycerol-thioglycerol (1:1) mixture. These samples were ionised by bombardment with cesium ions of an energy of 20Kev. The temperature of the source was maintained at 25°C. The fatty acid methyl esters were analysed on capillary GC, while their *p*-nitrobenzyl esters were analysed on the BIOSPECT MASS SPECTROMETER as described in CHAPTER 1.

EXTRACTION AND ISOLATION OF COMPOUNDS

The animals (10 Kg), collected from the intertidal region in Okha, Gujarat, were immediately soaked in MeOH and transported to the laboratory. After about 2 wks, the solvent was drained off from these carboys, concentrated and fractionated into pet.ether, EtOAc, BuOH and water-soluble fractions in the usual way. The fractions were tested for antimicrobial properties which indicated the pet.ether, EtOAc and aqueous fractions to be very active, whereas the n-BuOH

fraction was inactive (TABLE 1). Repeated chromatography of the lipid fractions over silica gel columns yielded fractions rich in fatty acids, their methyl esters, sterols, alkaloids, batyl alcohol and its analogs and ceramides (N-acylated sphingosines). The fatty esters were analysed on capillary GC as well as by the newly developed NCIMS techniques. The results of these are provided in CHAPTER 1.

ANTI-MICROBIAL SCREENING:

The antibiotic assays were done using the agar diffusion method. The test cultures (The bacteria: *Salmonella typhimurium* MTCC-98, *Micrococcus luteus* MTCC-106, *Klebsiella pneumoniae* MTCC-109, *Escherichia coli* MTCC-118, *Bacillus subtilis* MTCC-121, *Staphylococcus aureus* MTCC-96, *Proteus mirabilis* MTCC-425, and *Vibrio parahaemolyticus* MTCC-451, and the fungii: *Candida tropicalis* MTCC-230, *Rhodotorula rubra* MTCC-248 and *Saccharomyces cerevisiae* MTCC-249) were grown overnight in nutrient broth which was then spread on nutrient agar plates to form an uniform lawn. Paper discs (HIMEDIA, Bombay; 6 mm dia) bearing 50 µg of the compounds were placed on the surface of the media and incubated at 37°C in a bacteriological incubator. After 24 hrs, the diameter of the zone of inhibition (in mm) was recorded. All the test cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

N-docosoylsphingosine (12), m.p. 70°C, IR(neat): 3450,2860, 1630, 1540, 1460, 1380 & 1040 cm^{-1}; ^1H NMR($CDCl_3$): δ 6.4 (1H,d, J=7.2 Hz,NH), 5.70(1H,dt,J=15.4 & 6.4 Hz,5-H), 5.46 (1H,dd, J=15.4 & 6.7 Hz,4-H),4.2(1H,m,3-H), 3.8(2H,m,1-H & 2-H), 3.65 (1H,m,1-H), 2.2(2H,t,J=7.1 Hz), 2.0(2H,m), 1.6-1.0(70H,m) and 0.88(6H,t,J=6.85 Hz,CH_3); ^1H NMR(Py-d_5): δ 8.3(1H,d,J=8.09 Hz, NH), 6.0(2H,m,4-H & 5-H), 4.83(1H,t,6.05 Hz,3-H), 4.70(1H,m, 2-H), 4.43(1H,dd,J=10.75 & 5.1 Hz,1-H), 4.28(1H,dd,J=10.75 & 4.2 Hz,1-H),2.45(2H,t,J=7.5 Hz,$COCH_2$),2.07(2H,m,6-H), 1.9-1.0 (70H,m), and 0.87(6H,t,6.4 Hz); ^{13}C NMR($CDCl_3$): δ 175.4(s), 134.4(d), 129.7(d),73.68(d), 61.92(t),54.94(d), 39.09, 36.65, 32.31,31.9, 29.66, 29.6, 29.5, 29.3, 29.3, 29.2, 27.9, 25.75, 22.6, 22.5(all t's) & 13.9(q); ^{13}C NMR(Py-d_5): δ 172.86(s), 131.74(d),131.64(d),72.76(d), 61.59(t),56.25(d),32.27, 32.12, 31.52, 29.39, 29.16,29.0, 25.81, 22.32, 22.17 (all t's) and 13.65(q); EIMSm/z(%): 672([M-H-H_2O]$^+$,0.1), 644([M-H_2O-C_2H_4]$^+$, 0.54),631([M-18-42]$^+$,2.16),602([M-18-71]$^+$,3.2),588([M-18-85]$^+$, 2), 574([M-18-99]$^+$,1.7), 560([M-18-113]$^+$,1), 546([M-18-127]$^+$, 0.9), 532([M-18-141]$^+$,0.65), 518(532-14,0.65), 504(518-14 or [M-H_2O-$C_{12}H_{28}$]$^+$,0.65), 490(504-14,0.54), 476(490-14,0.54), 462 (476-14,0.86), 448(462-14,0.86), 444([M-2H_2O-$C_{18}H_{31}$]$^+$,1.62), 434(448-14,0.92), 430(444-14,0.92), 421([M-H_2O-$C_{18}H_{36}$]$^+$ or [M-CH_2OH-$C_{17}H_{35}$]$^+$,2.5), 411([M-$C_{20}H_{40}$]$^+$,5.1, McLafferty fragment), 410([M-$C_{20}H_{41}$]$^+$,13.5), 407(421-14,6.2), 393([M-H_2O-$C_{20}H_{40}$]$^+$,51.2), 351(393-42, 4.5), 337(351-14,6.4), 323(393-70 or [$C_{22}H_{43}O$]$^+$, 4.8), 309(323-14,4.6), 295(309-14,3.6), 282([393-C_8H_{18}]$^+$,7.2), 264([M-2H_2O-$C_{20}H_{40}$-C_8H_{18}]$^+$,12), 250(264-14,7.2), 236(250-14,3.6),

136, 109, 69 and 60(100); FABMS m/z(%): 397([M+H-$C_{21}H_{43}$]$^+$, 34.58), 369{[M+H-$CH_3(CH_2)_{20}CO$]$^+$,48.47}, 282(369-17-69,37.14), 280(369-18-71,39.28), 278(369-2X18-55,43.66), 264(278-14 or 282-18,71.85), 262(280-18,63.84),250(65.17), 236(50.94), *etc.*

HYDROLYSIS OF THE CERAMIDE (12): About 10 mg of the ceramide was refluxed in a water bath with 5% Alc.KOH (2 ml) for 6 hrs.The reaction mixture was then cooled, diluted with water (5 ml), and acidified with 2N HCl. The liberated fatty acid was extracted with pet.ether (3X5 ml). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. ^{1}H NMR ($CDCl_3$): δ 2.2(2H,t,J=7 Hz), 1.2-1.0(38H, m) and 0.85(3H,q,6.5Hz); PCIMS(CH_4)m/z: 341[M+H]$^+$, corresponds to the mol. formula: $C_{22}H_{44}O_2$ and the structure: $CH_3(CH_2)_{20}COOH$.

LITHIUM ALUMINIUM HYDRIDE REDUCTION OF THE CERAMIDE (12): About 5 mg of the ceramide and 20 mg of LAH were mixed with 5 ml of dry Alcohol in a round bottomed flask containing a magnetic stirrer. The mixture was refluxed in an atmosphere of N_2 for 2hrs over a water bath. Later the excess of LAH was destroyed with dil.HCl, and the products extracted using $CHCl_3$. The fatty alcohol and the sphingosine were separated by chromatography over silica gel using pet.ether-EtOAc systems. Fractions 2-5 and 14-15 were found to contain the alcohol and the sphingosine respectively from their CIMS and NMR spectal data.

Dodecanol, ^{1}H NMR($CDCl_3$): δ 3.57(2H,t,J=6.5 Hz), 1.5(2H,m), 1.2(38H,m) & 0.81(3H,t,6.78 Hz); PCIMS(CH_4): 326[M]$^+$ and 308[M-H_2O]$^+$. Sphingosine, ^{1}H NMR($CDCl_3$): δ 6.5(2H,m,NH), 5.75 (1H,m,5-H), 5.57(1H,m,4-H), 4.32(1H,m,3-H), 3.95(2H,m,1-H & 2-H), 3.69(1H,m,1-H), 2.03(2H,m,6-H), 1.2(32H,m,CH_2's) and 0.85(3H,t,CH_3). PCIMS(CH_4): 369[M]$^+$,340[M-29]$^+$ & 323[M-28-18]$^+$.

Mimosamycin (13), R_t=12.2 min, was isolated as an yellow solid. IR(KBr): 3049.5, 2956.8, 1686, 1646, 1636, 1618, 1584, 1544.5, 1368, 1320, 1284, 1236, 1204, 1160, 1102, 1063, 982, 966, 938, 901, 753 & 680 cm^{-1}; ^{1}H NMR($CDCl_3$): δ 8.3(1H,s,1-H), 7.15(1H,s,4-H), 4.2(3H,s,OMe), 3.7(3H,s,NMe) and 2.1(3H,s, vinyl Me); EIMS m/z(%): 235(M+2H]$^+$,19), 234([M+H]$^+$,100), 219 ([M+H-CH_3]$^+$,16.1), 218([M-CH_3]$^+$,36.8), 205([M-CO]$^+$,48.2), 190 ([M-CO-CH_3]$^+$,44.6), 177([M-2CO]$^+$,27.3), 176([M-2CO-H]$^+$ or [M-CO-NMe]$^+$,10.2), 162(15.2),160(13), 149(M-84,13.3), 148(23.5), 134(23.8), 119(15.4), 108(19), 106(16) & 104(11).

1-Hydroxy mimosamycin (14), R_t=14.5 min, m.p.225°C, IR(KBr): 3420, 3290, 3030, 2920,2840, 1660,1640, 1580,1500,1440, 1280, 1220, 900,800 & 745 cm^{-1}; UV-Vis(MeOH,logϵ): 227.5(4.05), 485 (3.58); ^{1}H NMR($CDCl_3$): δ 8.85(1H,brs,exchanged with D_2O, OH), 7.62(1H,s,4-H), 4.06(3H,s,OMe), 3.64(3H,s,NMe) and 2.07(3H, s,vinyl Me); PCIMS(CH_4): 250[M+H]$^+$.

1,4-Dihydroxy mimosamycin (15), UV-Vis(MeOH,logϵ): 220 (4.11), 255(3.75), 500(3.58); EIMS m/z(%): 265($M^{\cdot}$,59), 247 (8.3), 222(265-43,9.1), 219(265-2X28,7.8),181(265-3X28,18.4), 152(265-4X28-H,17.9), *etc*; ^{1}H NMR($CDCl_3$): δ 13.28 & 8.85 (1H each,s,exchanged with D_2O), 4.0(3H,s,O<u>Me</u>), 3.46(3H,s,N<u>Me</u>) and 2.16(3H,s,vinyl <u>Me</u>).

O-Demethyl renierone (16), R_t=11.0 min, m.p. 133°C, Analytical: cacld. for $C_{16}H_{15}NO_6$: C 63.79%, H 4.99% and N 4.65%; Found: C 63.295%, H 4.82% and N 4.55%; IR(KBr): 3324, 2922, 1713, 1648, 1576, 1388, 1328, 1238, 1209, 1166, 1103, 1063 & 747 cm^{-1}; UV-Vis(MeOH,ϵ): 215(3X10^4), 252(11715), 297 (6065), 317(5320) & 376(2830) nm; ^{1}H NMR($CDCl_3$): δ 8.98(1H,d, J=4.8 Hz,3-H),7.95(1H,d,J=4.8 Hz,4-H), 6.13(1H,br.q,J=6.8 Hz, 12-H),5.80(2H,s,9-H),2.1(3H,s,15-H),2.01 (3H,d,J=7.1 Hz,14-H) and 1.97(3H,br.s,13-H); ^{13}C NMR($CDCl_3$): δ 183.6(s,C-8), 181.3 (s,C-5), 167.9(s,C-10), 157.1(d,C-1), 155.3(d,C-3), 153.8(s, C-8a), 140.03(s,C-4a), 138.2(d,C-12), 127.8(s,C-11), 120.7 (s,C-6), 119.1(d,C-4), 65.2(t,C-9),20.6(q,C-14), 15.7(q,C-13) and 8.5(q,C-15), These assignments are based on ^{1}H-^{13}C COSY and DEPT experiments and comparison with the earlier results[3,4]; EIMS m/z(%): 301($M^{\cdot}$,44.86), 273([M-CO]$^{\cdot}$,3.55), 219([M-82]$^{\cdot}$,5.1), 218(10), 203([M-98]$^{\cdot}$,7.71), 202(19), 83(62), 82(100) and 55(68).

Dimer of O-demethyl renierone (17), R_t=19.6 min, m.p. 174°C, IR(KBr): 3412, 3314, 1710,1673, 1606, 1570, 1410, 1354, 1274, 1233, 1158, 1058 & 746 cm^{-1}; UV-Vis(MeOH,ϵ): 231(31850), 256.5 (29972),304.5(6247),324.5(8000),379.5(5141) & 485.5(2170) nm; ^1H NMR ($CDCl_3$): δ 8.90(1H,d,J=4.94 Hz), 7.93(1H,d,J=4.94 Hz), 6.11(1H,qq,J=7.23 & 1.28 Hz), 5.77(2H,s), 2.01(3H,s), 2.01 (3H,dq,J=7.14 & 1.5 Hz) and 1.98(3H,brq,J=1.5 Hz); EIMS m/z(%): 439,430, 428, 426, 415, 400, 384, 383, 369, 367, 355 (minor peaks), 302(58), 301(100), 300 (100), 285, 282, 272 (17.5), 271(19.2), 269(13.0), 243(14.3), 219 (19.2), 218 (67.4), 217(100),202(55.71), 201(100), 200(18.3), 190(23.4), 189(35.8), 173(40.5),162(10.5), 161(24), 146(10.1), 145(13), 131(10.4), 118(13), 117(17.5), 91(11.6), 84(12.1), 83(100), 82(100) and 55(100).

Conversion of O-demethyl renierone (16) into its dimer (17): About 5mg of the compound (16) was mixed with 15 mg DCC in dry CH_2Cl_2 in a round bottomed flask fitted with a dry $CaCl_2$ guard tube and the mixture kept stirred at room temp. using a magnetic stirrer. The progress of the reaction was periodically monitored by TLC. The reaction was complete within 3 hrs. At the end of the reaction, the excess catalyst was decomposed with a few drops of MeOH and the product purified by prep.TLC. It was identical to compound (17) with respect to melting point, HPLC retention time and IR spectrum.

Renieramycin G (18), R_t=41.7 min, IR(KBr): 3425,2964, 2921, 2850, 1726,1705, 1648, 1563, 1457,1379, 1279, 1123, 1027, 801 & 744 cm^{-1}(FIG 9); UV-Vis(MeOH,logϵ): 225(4.46), 275(3.92), 365(3.46) & 520(2.98) nm; The ^1H and ^{13}C NMR spectra are reproduced in FIGs 10 and 11 respectively, while the spectral values are given in TABLEs 3 & 2 respectively. For ^1H-^{13}C COSY SPECTRUM, see FIG 13. PCIMS(m/z): 594[M$^+$], 580[M-H_2O+4H]$^+$; LSIMS m/z: 580 (3.53),579(3.85),466([M+H-C_8H_7O-H_2O-CO]$^+$,2.6), 453([M+2H-C_8H_7O-2CO]$^+$,2.85), 438([M+H-C_8H_7O-2CO-H_2O]$^+$,4.94), 414(20.31) and 413 (29.80); HREIMS of the prominent peak at m/z 580 determined its elemental composition as $C_{30}H_{32}N_2O_{10}$ (calculated: 580.2057, as against the observed: 580.2066). For the EI mass spectrum and spectral values, see FIG 12 & TABLE 7.

Renieramycin H (19), R_t=51.4 min, IR(KBr): 2936,2850, 1716, 1652, 1567, 1453, 1375, 1233, 1205, 1148 & 1091 cm^{-1}; UV-Vis (MeOH,logϵ): 220(4.38), 260(4.10), 340(3.54) & 520(3.08) nm; For ^1H NMR spectrum and the spectral values, see FIG 15 & TABLE 3 respectively; PCIMS m/z: 592[M$^+$], 564[M-CO]$^+$; LSIMS m/z(%): 595([M+3H]$^+$,4.53), 594([M+2H]$^+$,4.2), 593(7.34), 563([M+H-CO]$^+$,3.77),561(4.22), 453(3.7) and 451(4.74); For FABMS, see FIG 16. The molecular ion at m/z 594.2086 in the HREIMS confirmed its composition as $C_{31}H_{32}N_2O_{10}$ (Expected: 594.2057). For the EI mass spectrum and mass spectral values, see FIG 17 & TABLE 7 respectively.

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CHAPTER 5

CHEMICAL CONSTITUENTS OF THE SPONGE *TEDANIA ANHELANS* LIEBERKUHN

The fire sponge, *Tedania anhelans* LIEBERKUHN (family: *Tedaniidae*, order: *Poecilosclerida*) is commonly found in the intertidal waters of Gujarat coast. Sponges belonging to the family *Tedaniidae* are generally devoid of epizoites and are known to induce necrosis of the tissues of other sponges kept in contact. Three important chemical metabolites reported from these sponges are 1-methylguanosine, a purine nucleoside isolated from *Tedania digitata*[1], atisane-3ß,16α-diol[2], a diterpenoid and tedanolide[3], a macrolide of acetate-propionate biogenesis, from *Tedania ignis*. The purine derivative causes profound muscle relaxation and hypothermia. It also reduces blood pressure, blocks polysynaptic responses in the mouse spinal chord, activates adenosine receptors and interact with benzodiazepine receptors, decreasing the cerebral level of cyclic GMP[4]. The diterpenoid was mildly cytotoxic against KB cells (ED_{50}=21 µg/ml), while the macrolide was active against both KB (ED_{50}=2.5X10^{-4} µg/ml) and P 388 (ED_{50}=1.6X10^{-5} µg/ml) cell lines in *in vitro* studies. However, the results of its *in vivo* evaluations were very disappointing as it was not active against ovarian cancer cells[5]. In addition, tedanolide was also very toxic to mice at 12-40 µg/Kg level. Other interesting compounds reported from *Tedania* sp. are some diketopiperazines[6], loliolide & isololiolide[6], *p*-hydroxy benzaldehyde, phenylacetamide and a sulphur-containing diketopiperazine[7].

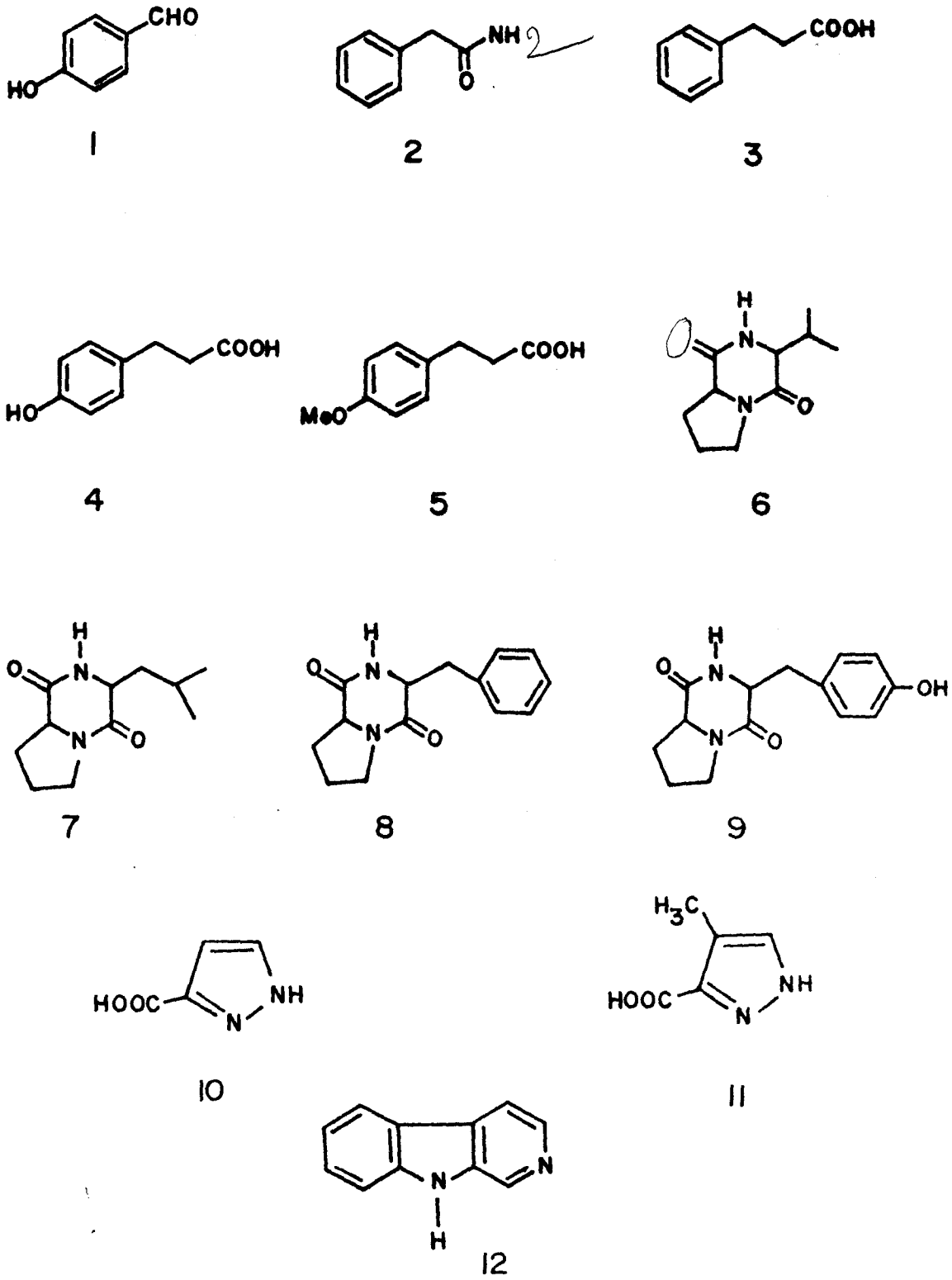


Fig. 1 Secondary metabolites from the sponge Tedania anhelans

Preliminary pharmacological screening of the methanol extract of the sponge *Tedania anhelans*, collected from Okha, Gujarat indicated it to be spasmogenic and hypoglycaemic (the latter activity: 23%, at 250 mg/kg level)[a]. This extract was non-toxic, the LD_{50} value against rats being 1000 µg/kg.

Systematic chemical investigation of the methanol extract of *T. anhelans* led to the isolation of several fatty acids, *p*-hydroxybenzaldehyde (1), phenylacetamide (2), three aromatic acids (deaminated amino acids, 3-5), four diketo-piperazines (6-9), two novel heteroaromatic acids: pyrazole-3-carboxylic acid (10) & 4-methyl pyrazole-3-carboxylic acid (11) and ß-carboline (12) (FIG 1). The fatty acids were analysed on capillary GC alogwith standards, while the compounds (1-12) were separated by chromatography over SiO_2 columns and HPLC. The structures of these compounds were finalised from their spectral data including IR, ^1H & ^{13}C NMR and MS.

The crude MeOH extract was fractionated into pet.ether, EtOAc and water-soluble fractions in the usual manner. The pet.ether fraction, upon chromatography over silica gel columns yielded a mixture of fatty acids, their methyl esters and compounds (1 & 3-5). The fatty acids were analysed as their methyl esters on a capillary GC and the individual compounds identified from a comparison of their retention times with those of standards (see FIG 2 and TABLE 1).

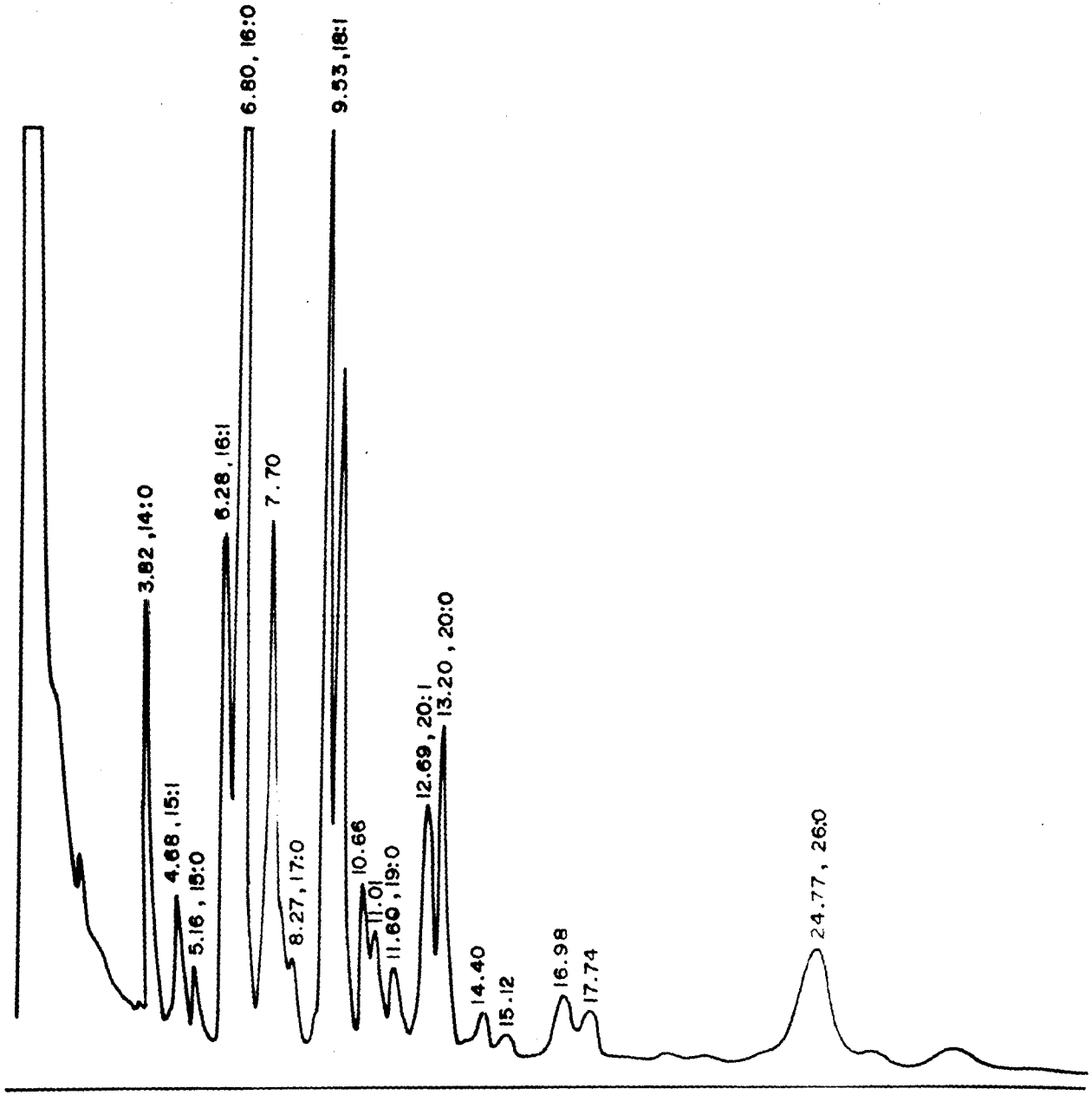


Fig. 2 Gas chromatogram of fatty acid methyl esters from Tedania anhelans

TABLE 1; FATTY ACID CONTENT OF THE SPONGE *T ANHELANS* AS DETERMINED BY CAPILLARY GC ANALYSIS:

Ret.time (min)	Structure	% Composition
3.82	14:0	0.53
4.37	---	0.23
4.68	15:1	0.264
5.16	15:0	0.118
6.28	16:1	1.114
6.80	16:0	4.120
7.77	18:3?	1.763
8.27	17:0	0.155
9.53	18:1	2.230
9.97	18:0	1.278
10.66	20:4?	0.37
11.01	20:3	0.299
11.60	19:0	0.235
12.69	20:1	0.773
13.20	20:0	0.721
14.40	21:1?	0.187
15.12	21:0	0.076
16.98	22:1?	0.31
17.74	22:0?	0.228
20.23	---	0.044
21.38	---	0.044
24.77	26:0?	1.16
26.41	---	0.123
29.03	---	0.242

Chromatography of the EtOAc fraction using gradient pet.ether-$CHCl_3$-MeOH systems yielded compounds (1-12). The compound (1), M^+=122, had the following ^1H and ^{13}C NMR data: δ 7.78(2H,d,8.6 Hz), 6.97(2H,d,8.6 Hz), 132.5(d) & 116.05(d). These values indicated a *p*-disubstituted benzene nucleus. The substituents were identified as an aldehyde [δ 9.8(1H,s) and 191.7(d)] and hydroxyl groups from its molecular formula and spectral data, thereby confirming its structure as *p*-hydroxy benzaldehyde (1).

Compound (2), M^+=135, had peaks at 3500, 3250, 2600 and 1700 cm^{-1}, in its IR spectrum, indicating the presence of a carbonyl and hydroxyl / or amine groups in this molecule. The odd molecular weight indicated the presence of a nitrogen atom in it. The ^1H and ^{13}C NMR spectra indicated it to be a monosubstituted benzene derivative. All the five aromatic hydrogens were observed as a multiplet at δ 7.2-7.5. The corresponding carbon signals were found between δ 135 and 127, as expected. The peaks at δ 3.58(2H,s) in the ^1H NMR and at δ 43.35(t) in the ^{13}C NMR and DEPT spectra could be due to a diallylic methylene group. The peaks at δ 5.85(1H,br) and 5.45(1H,br), which were readily exchangeable with D_2O must be from the NH_2 of the amide group. The signal at δ 173.47(s) in its ^{13}C NMR spectrum is attributed to the corresponding carbonyl group. The structure of this compound was finalised as phenylacetamide (2) from these results.

The compounds (3-5), M^+=150, 166 and 180 respectively, were isolated in pure form by silica gel column chromatography. Their IR spectral absorptions at 3500,1700, 1580 and 1500 cm^{-1} indicated them to be aromatic acids. This was also supported by their ^1H and ^{13}C NMR spectral data. The former had signals at δ 7.2-7.5(5H,m) for compound (3), δ 7.25(2H,d,J=8.5 Hz) & 7.08(2H,d,J=8.5 Hz) for compound(4) and 7.23(2H,d,J=8.5 Hz) & 6.72(2H,d,J=8.5 Hz) for compound (5). The corresponding carbon signals were observed at δ 140.07(s), 128.4(d), 128.1 (d) & 126.25(d) for compound (3) and at δ 129.4(d) & 115.4(d) for compound (4). From these results, it was clear that compound (3) is a monosubstituted benzene derivative, while the compounds (4) and (5) are *para* disubstituted benzene derivatives. Besides, their ^1H NMR spectra also had pairs of mutually coupled triplets at δ 2.90(2H,J=7.5 Hz) & 2.65(2H, J=7.5 Hz), indicating the presence of $-CH_2-CH_2-$ system. The corresponding carbon signals were observed at δ 35 & 30 in their ^{13}C NMR spectra. These values were indicative of 3-substituted propionic acid residues. The second substituent of the aromatic ring in compounds (4) and (5) was found to be hydroxyl and methoxyl groups respectively from their IR, MS and NMR data. The structures of these compounds were finalised as phenylpropionic acid (3), *p*-hydroxy phenyl propionic acid (4) and *p*-methoxy phenylpropionic acid(5) respectively from the above data.

The compounds (6-9) were eluted from the silica gel column with gradient $CHCl_3$-MeOH solvent systems. Among these, the compound (6) had a molecular weight of 192 as revealed by its EI and CI mass spectra. The ^{13}C NMR and DEPT spectra of this compound had two carbonyl (δ 170.26 & 164.99), two secondary nitrogen-bearing carbons (δ 61.18 & 58.76), three CH_2's (δ 45.57, 28.46 & 28.04), one methine having no attached oxygen or nitrogen atoms (δ 28.42) and two methyl groups (δ 19.06 & 16.06). The methyl signals were observed at δ 1.09(3H,d,J=7.2 Hz) and 0.92(3H,d,J=6.8 Hz) and the methine peaks at δ 4.05 (1H,m), 3.94(1H,br) and 2.62(1H,doublet of septet,J=7.1 & 2.1 Hz) respectively in its ^1H NMR spectrum. The methylene protons were observed δ 3.6(2H,m), 2.32(1H,m) and 2.00(3H,m). These spectral values were in agreement with those of cyclo [pro-val]. The structure was further confirmed by extensive decoupling studies. The detailed proton assignments arrived at, based on these studies, are given in the experimental section.

The compound (7), M^+=210, had strong IR absorptions at 3500 and 1650 cm^{-1}, indicating the presence of amide group in it. This was also supported by its ^{13}C NMR spectrum, which had peaks at δ 170.5 & 166.26, due to carboxyl / amide groups. Other peaks were at δ 58.8(d) & 53.3(d) (nitrogen-bearing methine carbons), 23.1(d), 45.15, 38.11, 27.95 & 22.6 (all t's) and 24.5 & 21.2(q). These values were in agreement with those of amino acids proline and leucine. All the proton

signals were found between δ 4.05-0.89, which were assigned based on extensive decoupling studies (see experimental section for these assignments). Based on these studies, its structure was finalised as cyclo[pro-leu] (7).

The compound (8), M^+=244, was found to be a cyclic dipeptide of proline and phenyl alanine. Its aromatic protons were seen at δ 7.20(5H,brs) in ^1H NMR spectrum. The two methine protons attached to nitrogen-bearing carbons were observed at δ 4.2(1H,dd,J=10 & 3 Hz) & 4.10(1H,t,J=7 Hz). The remaining methyl and methine proton signals were present at δ 3.65(1H,dd,J=15 & 3 Hz),3.60(2H,m), 2.75(1H,dd,15 & 10 Hz), 2.30(1H,m) and 1.93(2H,m).

The compound (9), M^+=260, had similar ^1H NMR spectral properties as the previous compound. The molecular weight of this compound was 16 units more than compound (8), which can be easily explained as due to the replacement of a hydrogen by a hydroxyl group. In the ^1H NMR spectrum, four aromatic protons were seen as AB quartets at δ 7.05(2H,d,J=8 Hz) & 6.75(2H,d,J=8 Hz), instead of the broad singlet signal due to five protons in compound (8). This suggested that the aromatic amino acid in this case is tyrosine. This was also supported by its ^{13}C NMR spectrum (see experimental). The structure of this compound was finalised as cyclo[pro-tyr] (9) from these results.

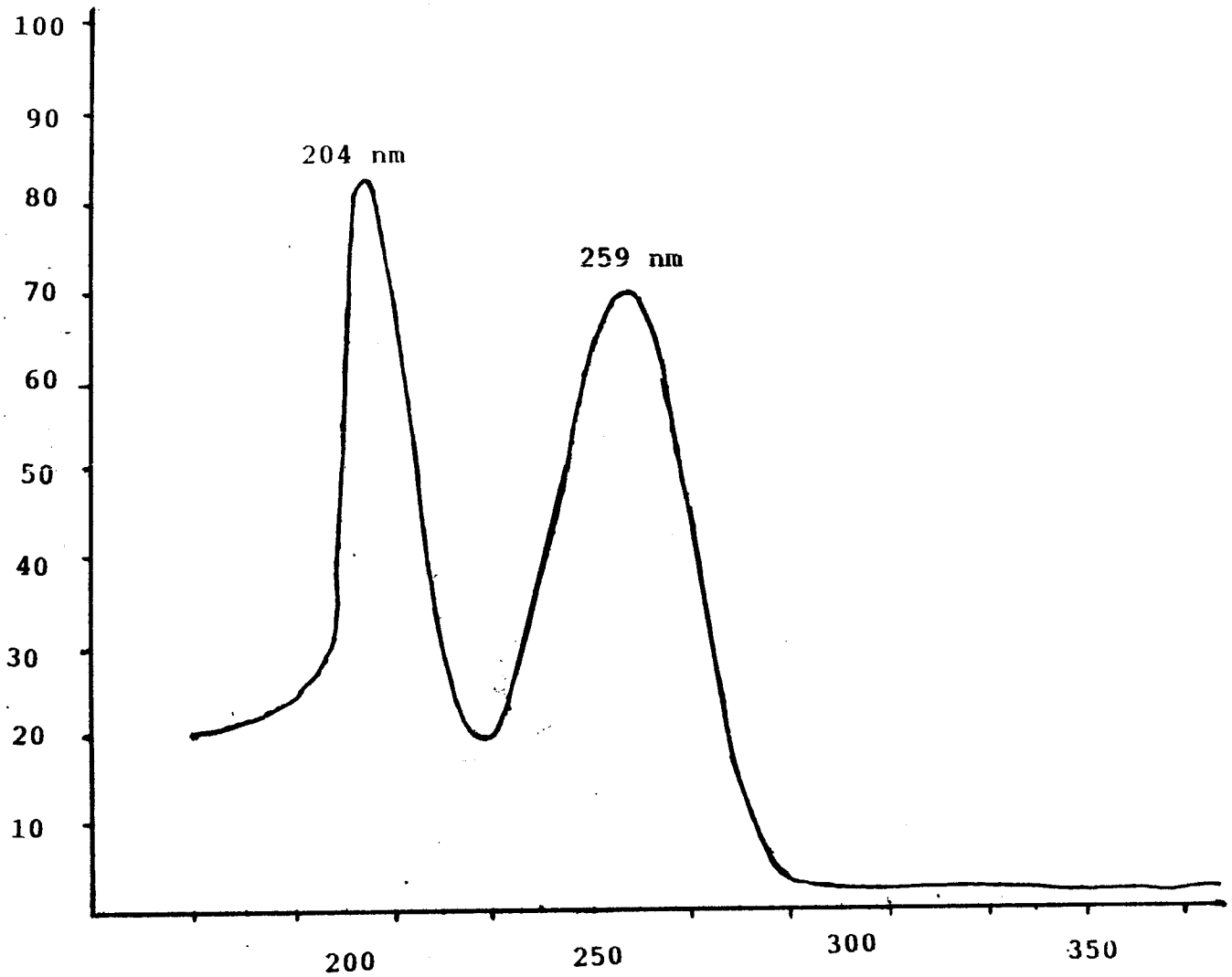


FIG 3. UV-Vis ABSORPTION SPECTRUM OF COMPOUND (10)

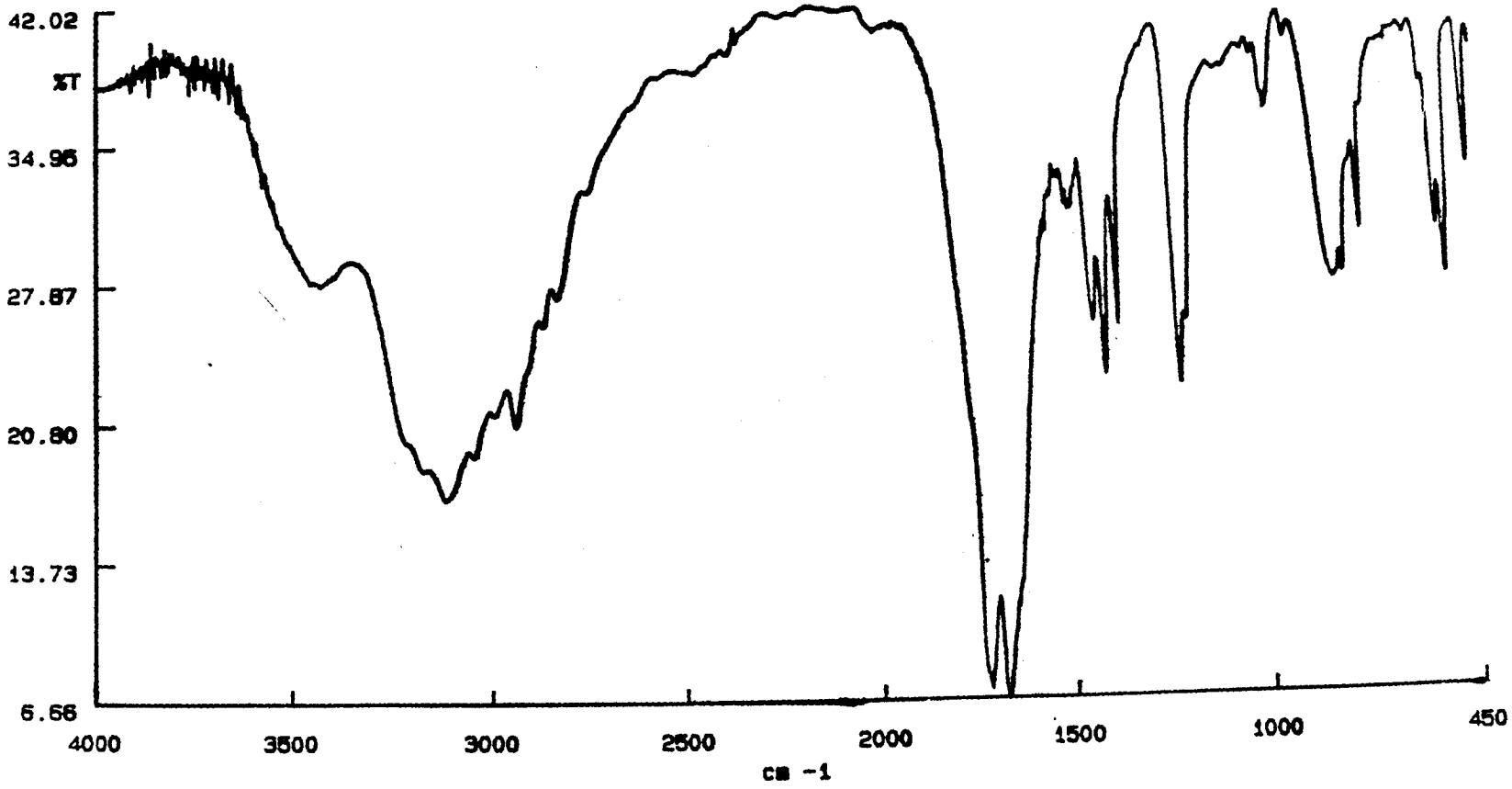


FIG 4. IR(KBr) SPECTRUM OF COMPOUND (10)

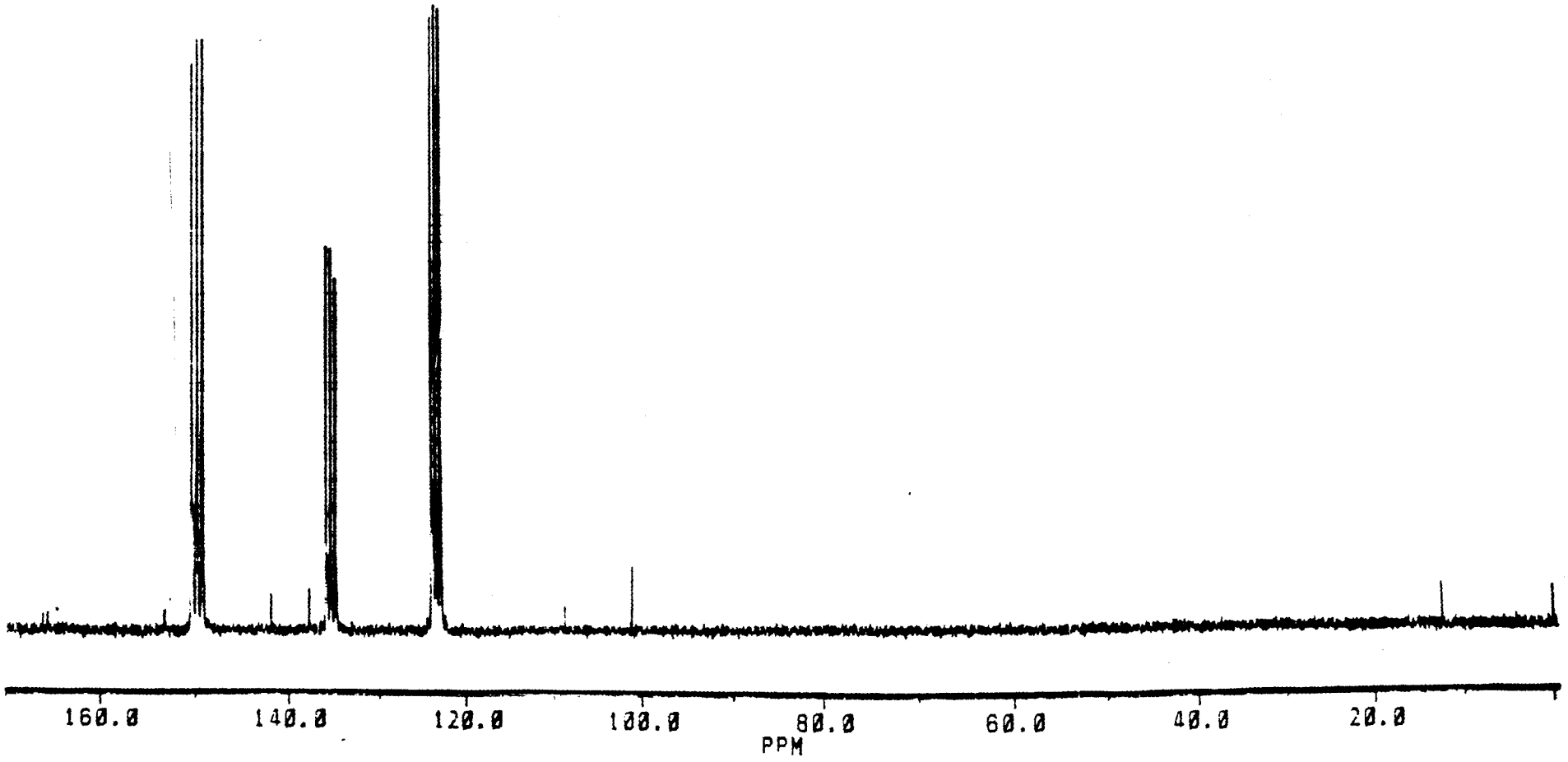


FIG 5. ^{13}C NMR SPECTRUM OF COMPOUND (10)

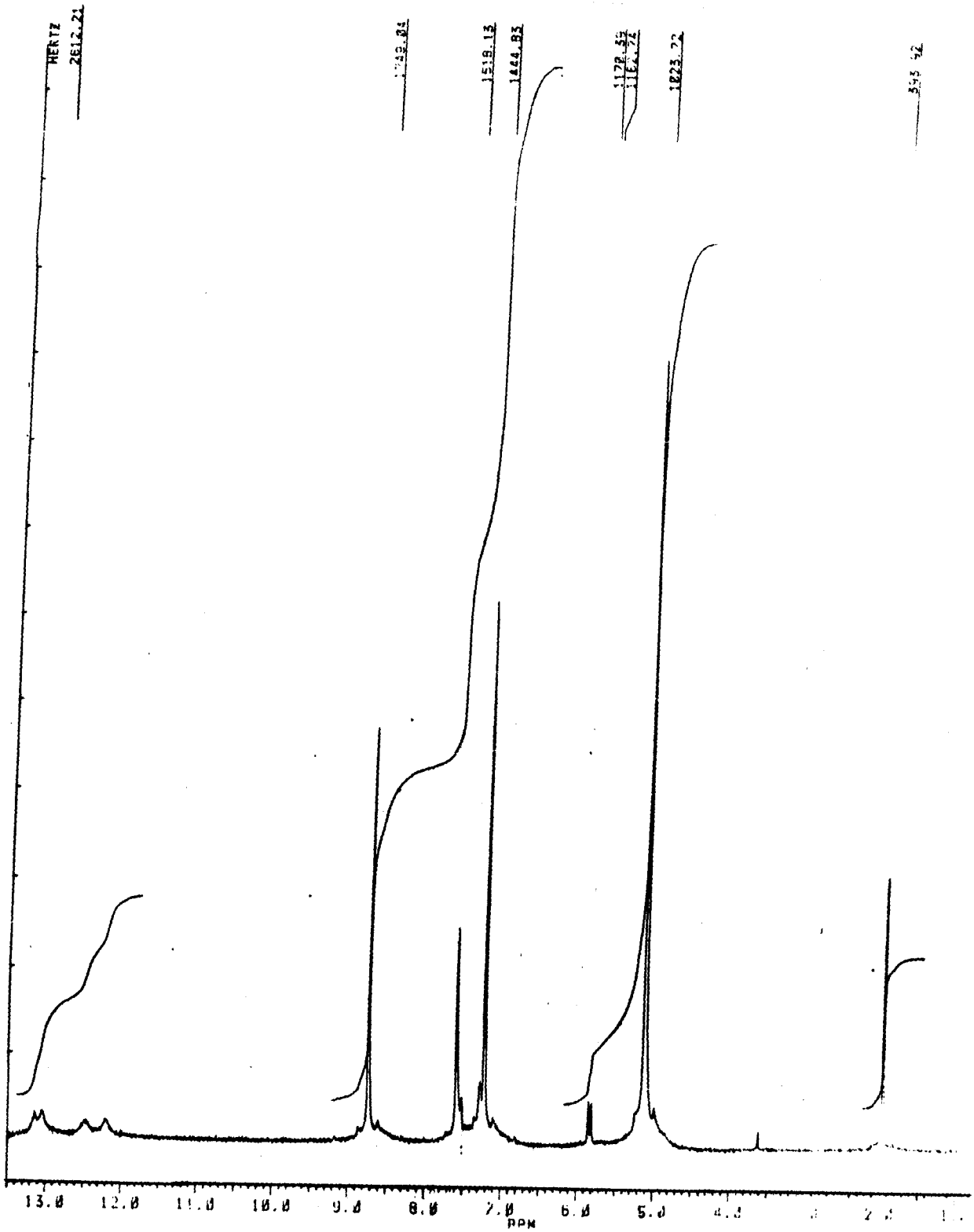
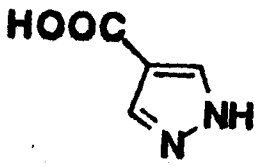


FIG 6. ^{1}H NMR SPECTRUM OF COMPOUND (10)

Continued elution of the polar fraction with 20% MeOH/$CHCl_3$ yielded two new heteroaromatic acids (10) and (11) as crystalline solids, very sparingly soluble in $CHCl_3$ but freely soluble in MeOH and acetone. The compound (10), m.p. 293°C, had UV-Vis maximas at 205 & 260 nm, indicative of its aromatic nature (FIG 3). The broad nature of absorptions in the range 3500-2700 cm^{-1} in its IR spectrum indicated the amino acid-type structure (FIG 4). Its absorptions at 1723 & 1674 cm^{-1} might be due to unionized COOH & COO^- carbanion respectively. CIMS revealed its molecular weight to be 112, corresponding to the molecular formulae $C_5H_4O_2$ or $C_4H_4N_2O_2$. The latter was more plausible, considering the high melting point and limited solubility of this compound in $CHCl_3$, EtOAc, *etc*, as well as from ^{13}C NMR spectral evidences. All the four carbon signals, expected in the latter formula were seen at δ 165.7(s,COOH), 142.3(s), 135.2(d) and 101.4(d) (FIG 5). These results as well as its UV-Vis spectrum indicated its aromatic nature. Besides, these values indicated that the compound is either imidazole or pyrazole carboxylic acid. Both the above structures could account for all the four degree of unsaturations in it. The former is ruled out from its ^{13}C NMR spectral data as well as the chemical shifts and coupling constants of the vinyl protons (FIG 6). The structure (10a) (FIG 7), too, may be ruled out from the observed ^1H NMR coupling constant of the vinyl protons (7.75 Hz), leaving the structure (10) *i.e.*, pyrazole-3-carboxylic acid as its correct structure.

TAUTOMERIC STRUCTURES OF COMPOUND 10



10a

FIG 7 ISOMERIC STRUCTURES OF PYRAZOLE CARBOXYLIC ACIDS

The compound (11), M^+=126, had similar UV-Vis and IR spectral characteristics as pyrazole-3-carboxylic acid (10); UV-Vis(MeOH): 207(7320) & 262(5770) nm, IR (KBr): 3300-2700, 1730, 1673 cm^{-1}, *etc.*, (FIGs 8 & 9 respectively). EI mass spectrum indicated its molecular weight to be 126, which is 14 mass units more than that of compound (10) (FIG 10). The two labile protons due to COOH and NH groups were found at δ 13.04 and 12.22 in its ^1H NMR spectrum (FIG 11). However, the two doublet signals due to the vinyl protons of the previous compound were absent here. Instead, it had a vinyl proton signal at δ 7.33(1H,s) and a methyl group on an SP^2 carbon at δ 1.97(3H). This indicated that one of the ring protons of the previous compound has been replaced by a methyl group in

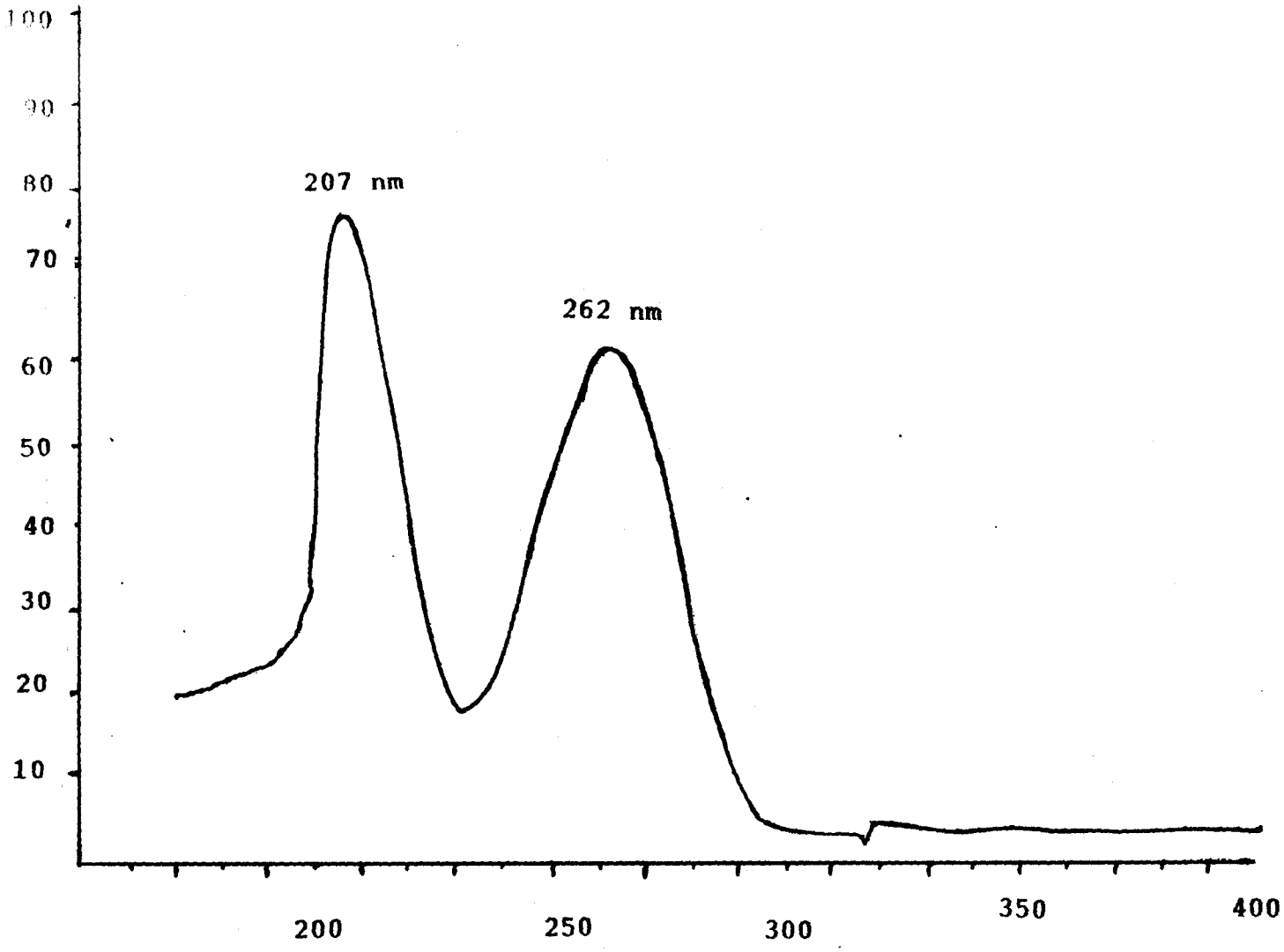


FIG 8. UV-Vis ABSORPTION SPECTRUM OF COMPOUND (11)

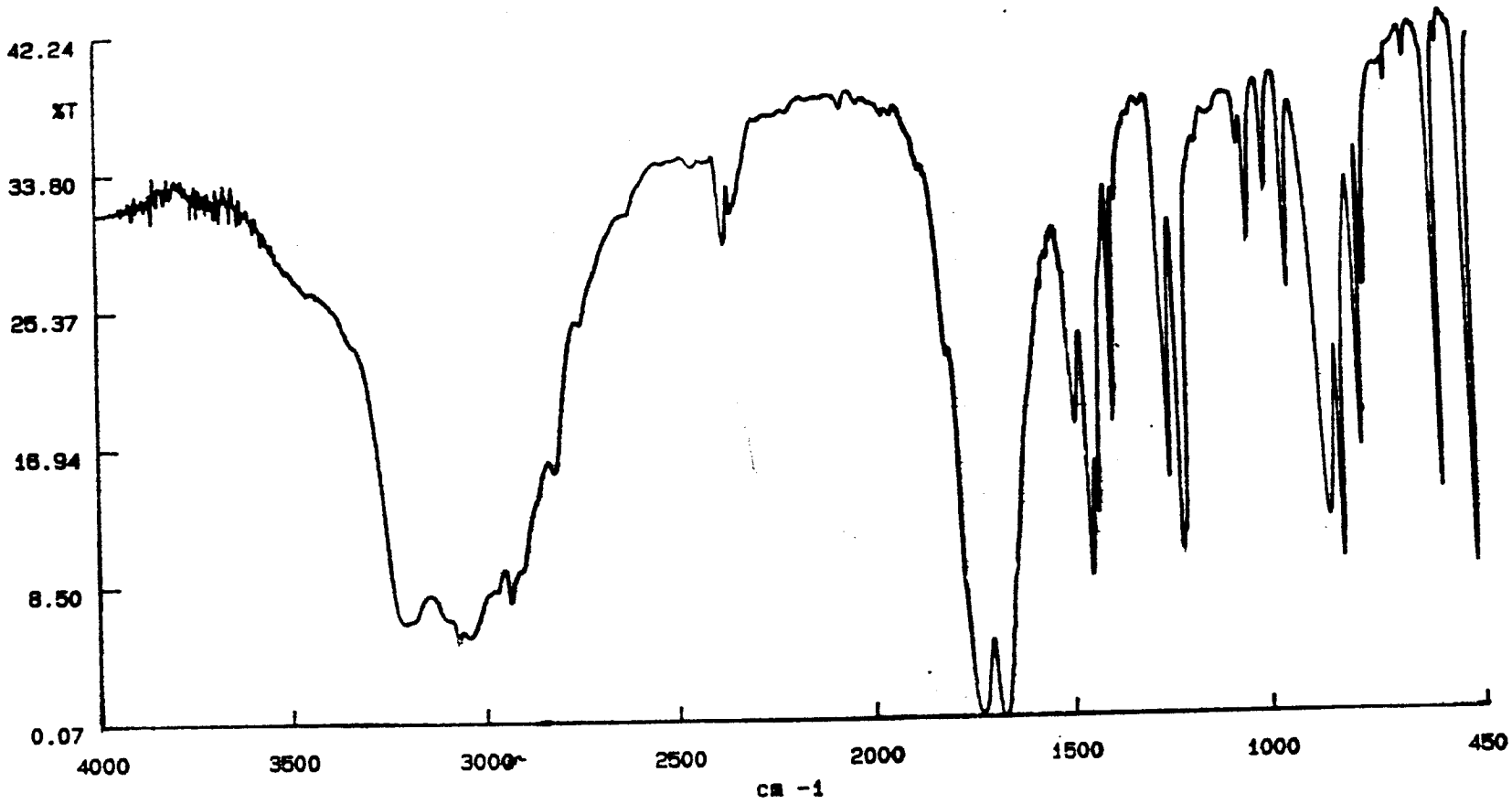


FIG 9. IR(KBr) SPECTRUM OF COMPOUND (11)

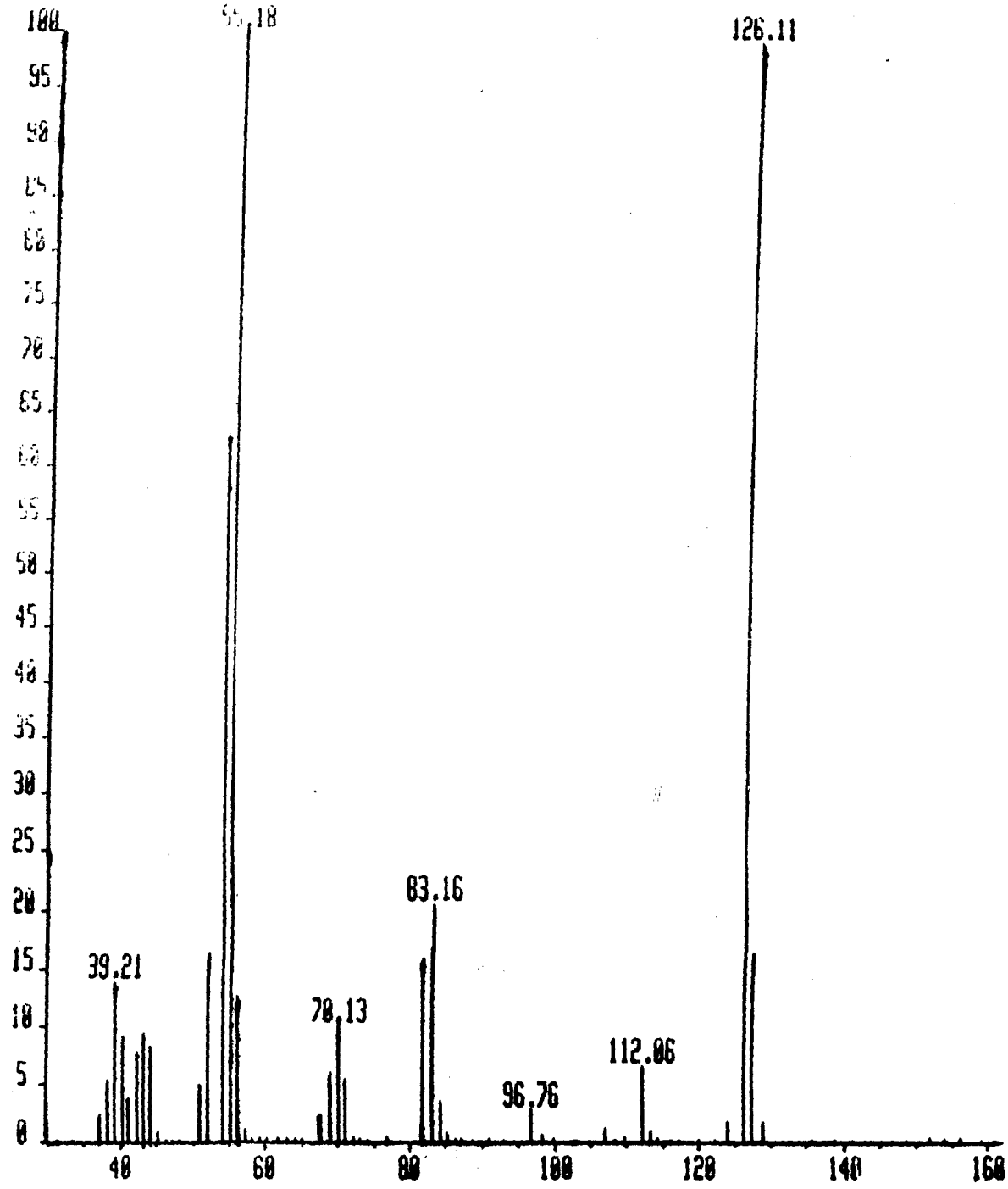


FIG 10. EI mass spectrum of compound (11)

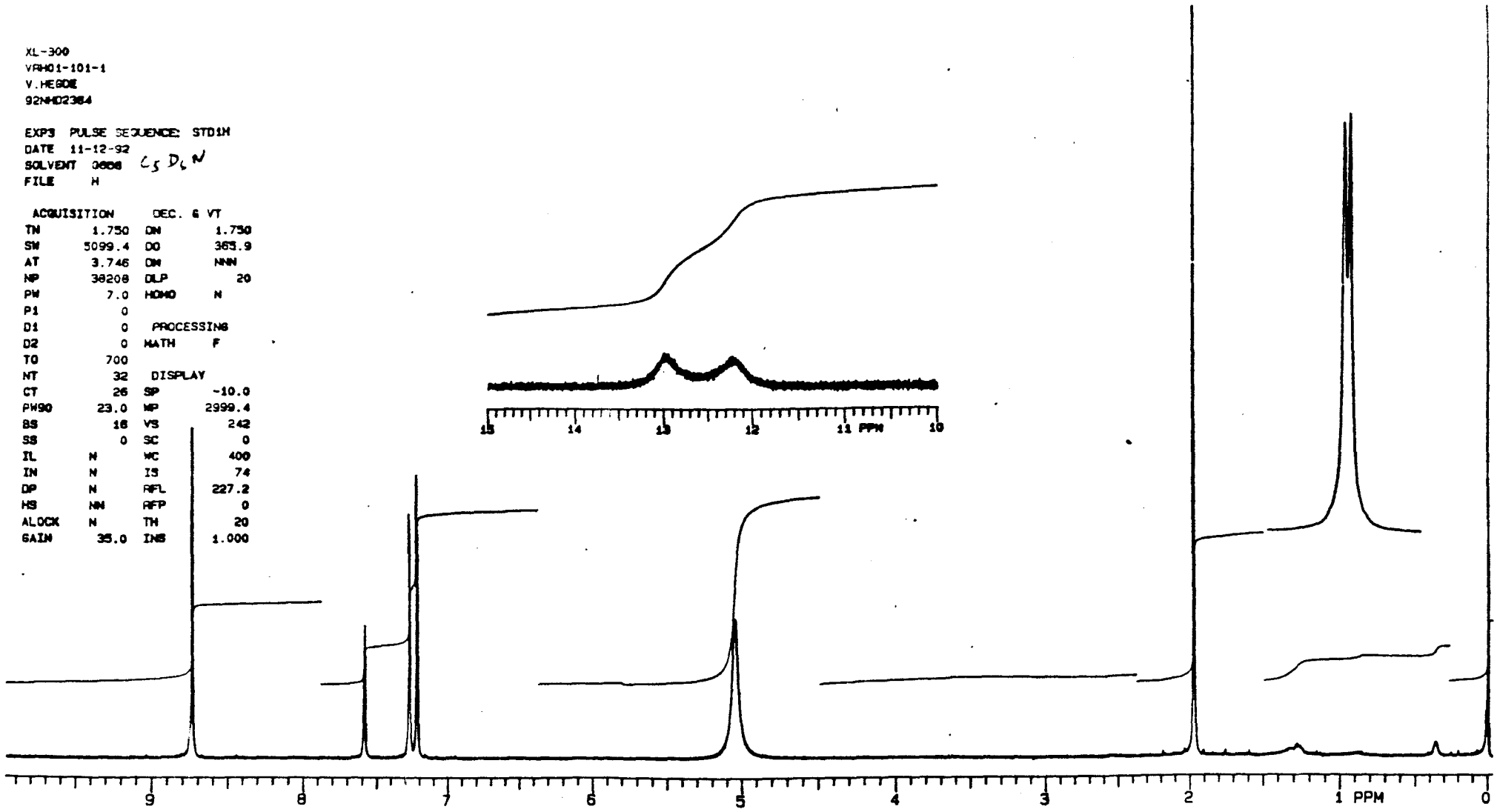
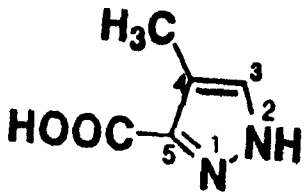
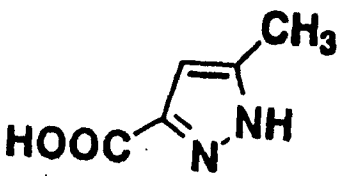


FIG 11. ^{1}H NMR SPECTRUM (Py-d_5) OF COMPOUND (11)

this molecule. Out of the two possible structures, (11) & (11a) (FIG 12), the former was favoured based on its ^{1}H and ^{13}C NMR spectral data. Thus, the lone vinyl proton of this compound resonates at rather downfield region at 7.33 ppm which is comparable to that of 5-H (δ 7.55) in compound (10). In its ^{13}C NMR spectrum, the signal due to C-4 now appears at δ 108.7(s), downfield by 7.3 ppm while the C-3 & C-5 signals appear at δ 144.3(s) & 137.7(d) respectively, upfield by 2.00 & 2.50 ppm each from their places in compound (10) (FIG 13). In addition, the upfield resonance of the methyl carbon in compound (11), at δ 11.6, also indicates its proximity to the carboxyl group. The structure of this compound was finalised as 4-methylpyrazole-3-carboxylic acid(11) from these results.



11



11a

FIG 12 4-METHYL PYRAZOLE-3(5)-CARBOXYLIC ACID (11) AND ITS 3(5)-METHYL ISOMER (11a)

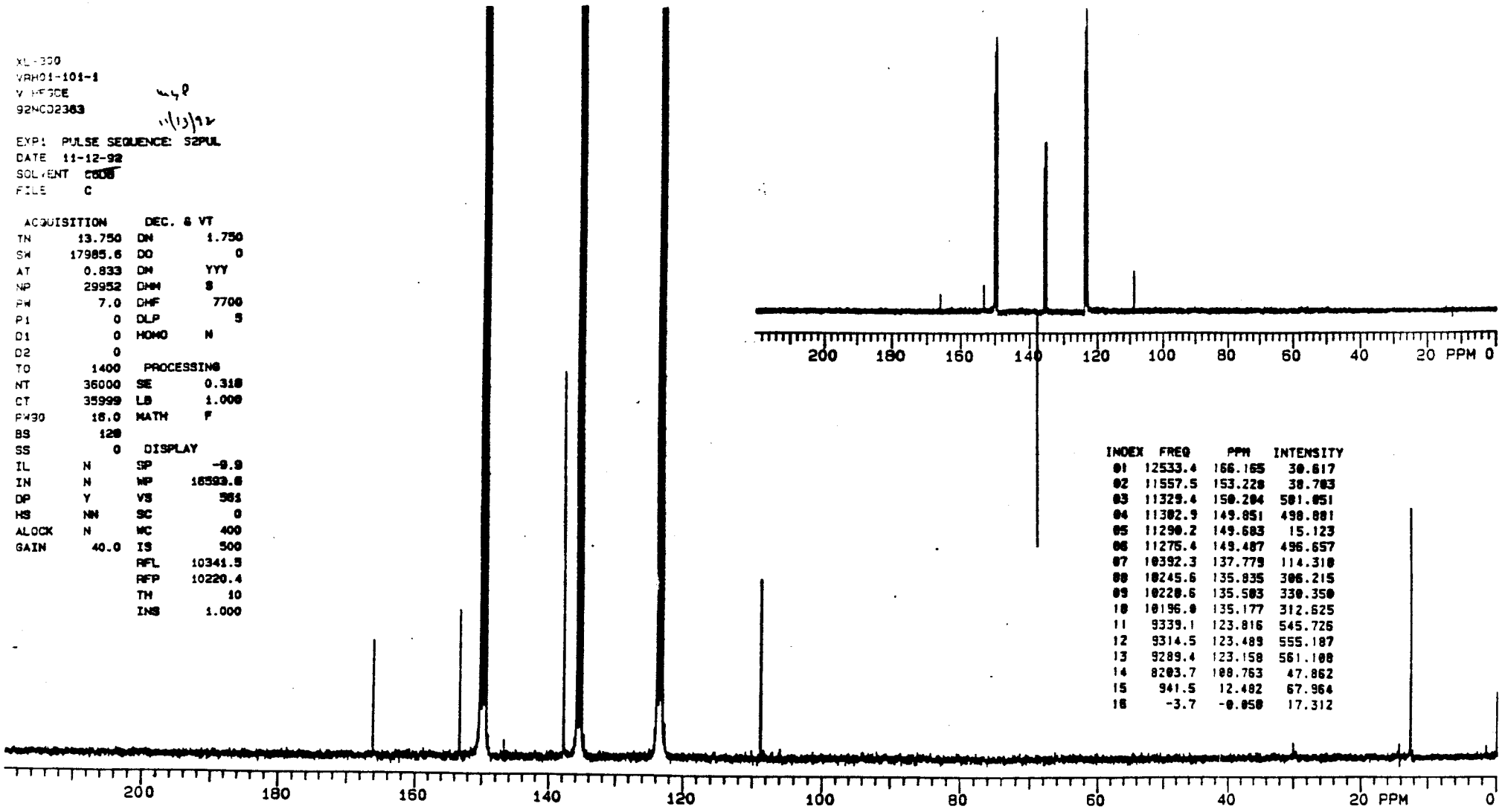


FIG 13. ^{13}C NMR AND DEPT SPECTRA OF COMPOUND (11)

Thin Layer chromatography of the EtOAc fraction using silica gel plates followed by visualisation of the chromatogram in an iodine chamber indicated the presence of an intense violet spot (R_f=0.75, $CHCl_3$-MeOH=93:7) in it. This compound was subsequently isolated in an impure form by column chromatography using $CHCl_3$-MeOH solvent systems which was finally purified on reverse phase HPLC (ODS,5 µm, 250X8 mm^2, UV detection, MeOH, 1 ml/min, R_t=10.6 min) to yield a crystalline solid (M P 196°C). The IR spectrum of this compound was very much different from that of aliphatic compounds, amino acids, *etc.* (FIG 14). The multiple peaks in the region 3150-2650 & 1650-1300 cm^{-1} indicated the presence of aromatic groups in it. EIMS determined its molecular weight to be 168, corresponding to a molecular formula $C_{13}H_{12}$ or $C_{11}H_8N_2$, having eight and nine degree of unsaturations respectively (FIG 15). The ^1H NMR spectrum had signals for eight protons. Among these, the peak at δ 9.43(1H,br) could be attributed to an NH group (FIG 16), while the remaining signals: δ 8.97(1H,s), 8.45(1H,d,J=5 Hz), 8.13(1H,d,J=7.77 Hz), 7.97(1H,d,J=5 Hz), 7.55(2H,brs) & 7.30 (1H,m), might be from aromatic protons. Comparison of these values with that of manzamines[9-12] and especially with manzamine B[10] indicated its structure to be ß-carboline (12). This was also supported by its ^{13}C NMR spectrum, which had 11 signals, out of which, 7 carbons appeared to be from methine and the remaining 4 signals, from quarternary carbons from the signal intensities (FIG 17).

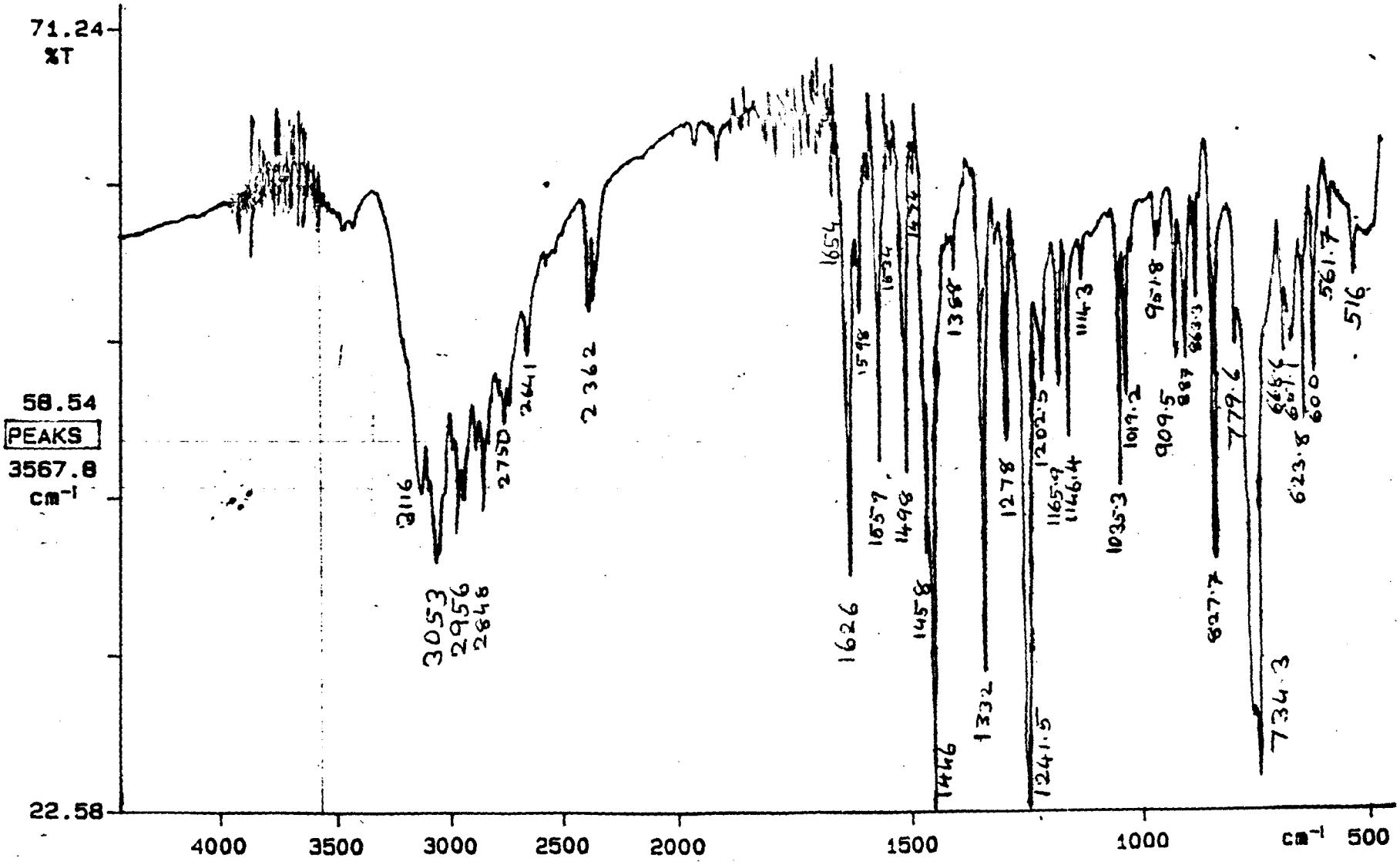
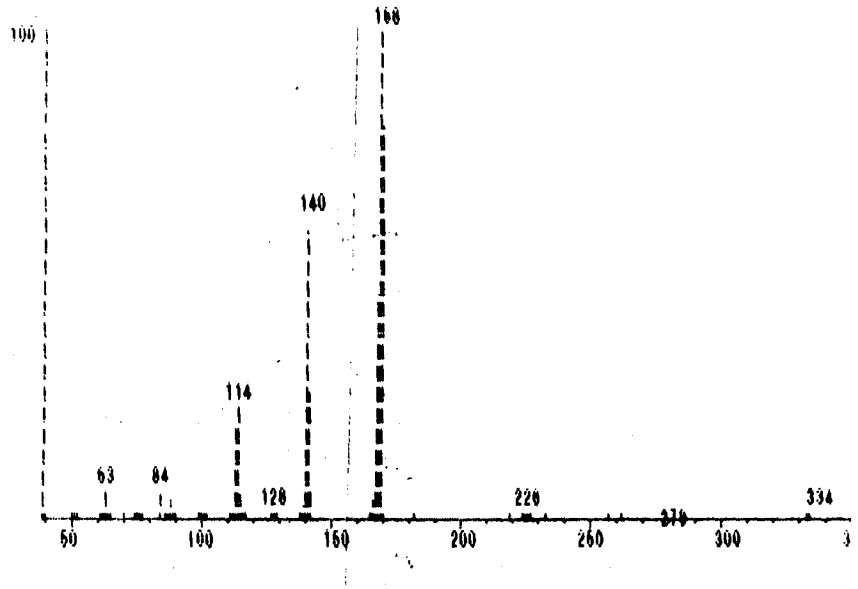


FIG 14 IR(KBr) SPECTRUM OF ß-CARBOLINE (12)



MASS	%BASE	MASS	%BASE
334	2.9	115	8.2
333	1.0	114	25.1
282	1.0	113	20.9
279	1.7	112	2.3
262	1.2	111	1.0
257	1.3	102	1.9
233	1.1	101	2.1
227	1.1	100	1.1
226	5.2	99	1.0
225	1.0	90	2.3
224	1.3	89	3.1
219	1.5	88	7.2
182	1.2	87	5.7
170	6.0	86	3.8
169	80.4	85	1.1
168	1.3	84	8.6
168	100.0	77	1.5
167	47.3	76	2.8
166	7.3	75	5.1
165	1.8	74	4.3
142	9.4	71	4.6
141	28.4	65	1.6
140	59.9	64	3.1
139	6.3	63	9.1
138	2.1	62	5.4
129	1.1	61	1.1
128	5.6	52	2.2
127	1.0	51	4.4
117	6.0	50	3.1

FIG 15. EIMS OF ß-CARBOLINE (12)

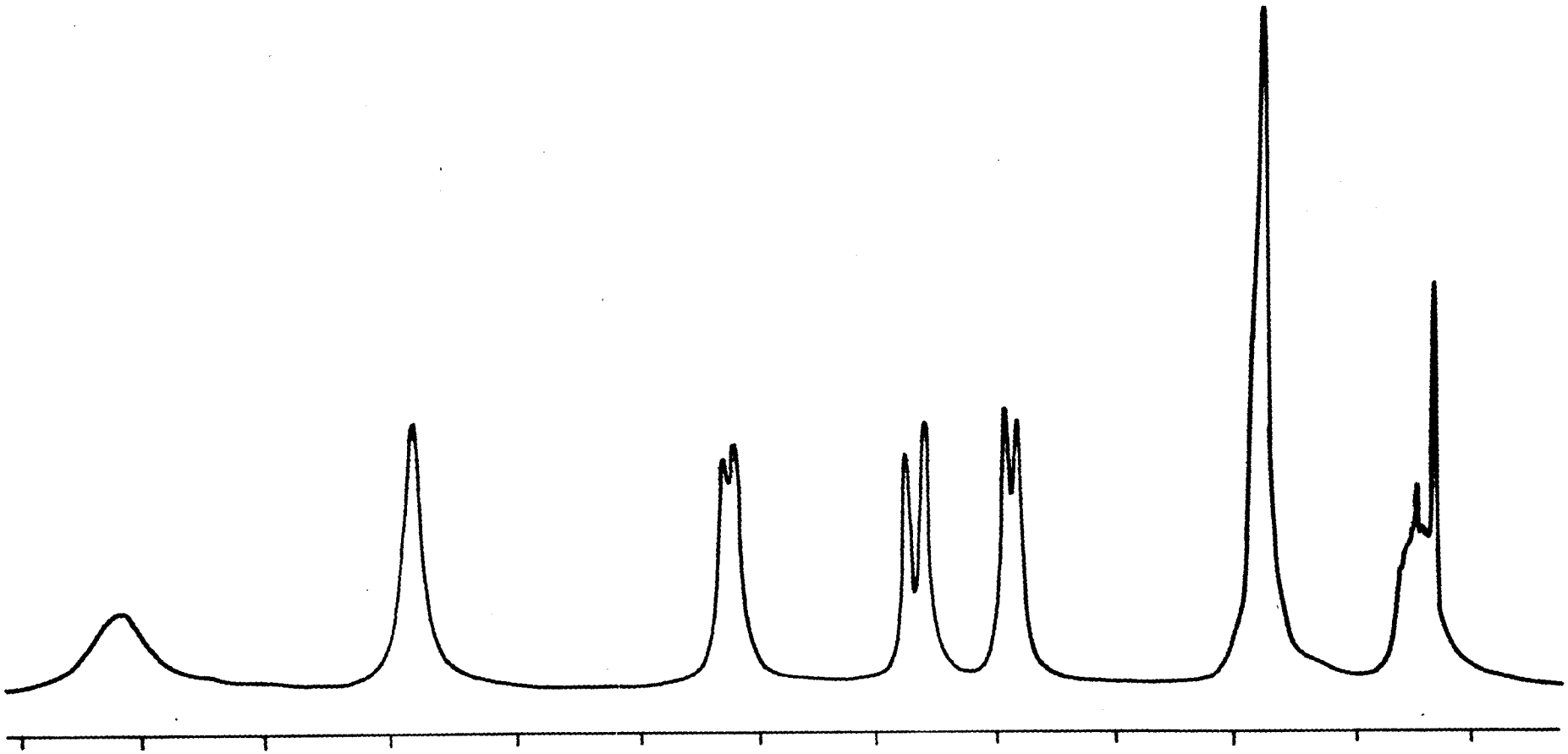


Fig. 16 ^{1}H NMR spectrum of β - Carboline (12)

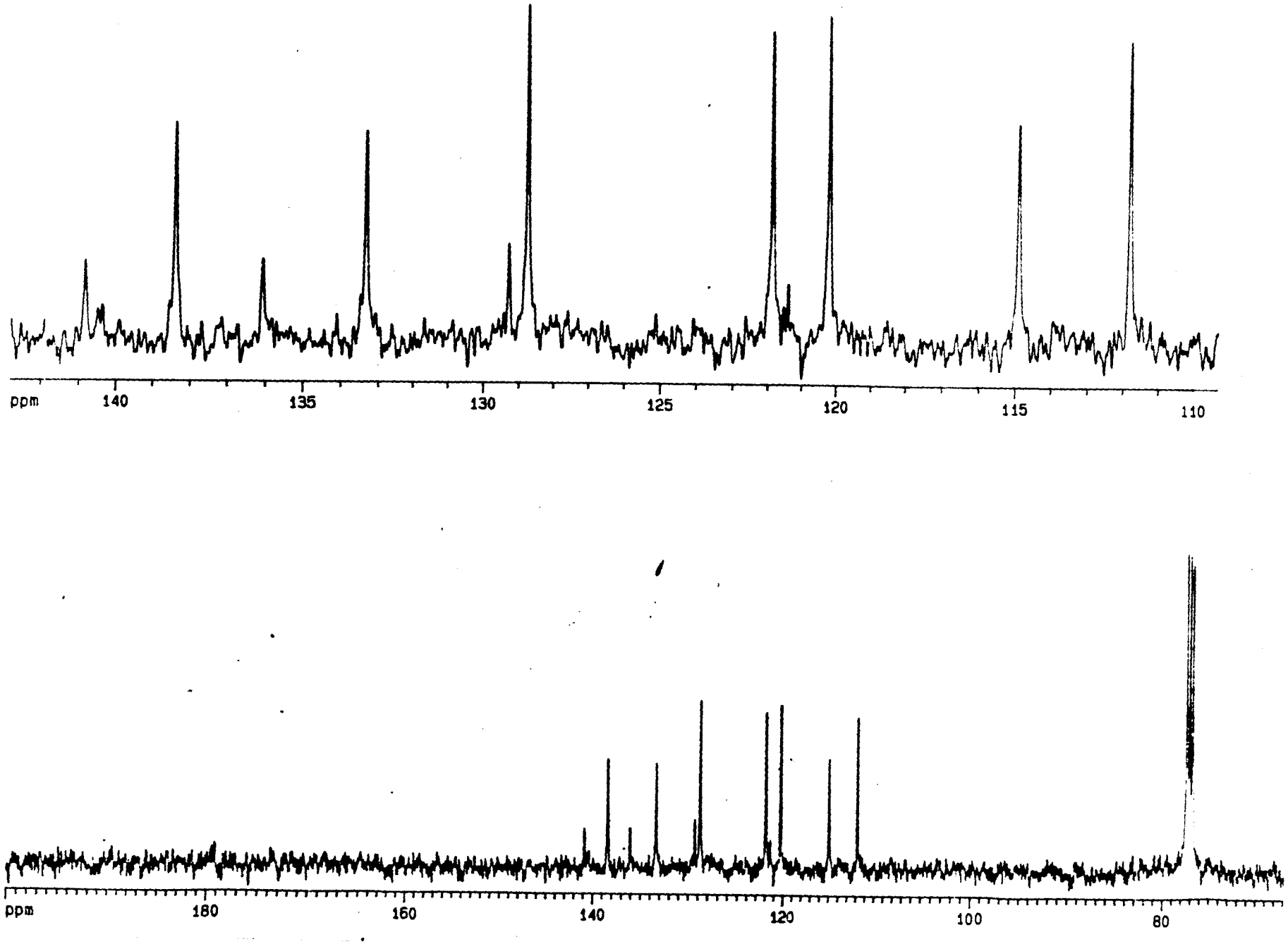


FIG 17. ^{13}C NMR SPECTRUM OF ß-CARBOLINE (12)

GENERAL DISCUSSION

Unlike other *Porifera*, sponges of the genera *Tedania* contain relatively small amounts of sterols. However, they contain lot of polyunsaturated fatty acids and several deaminated aliphatic and aromatic amino acids, and diketo piperazines. Among these, *p*-hydroxybenzaldehyde (1) and phenylacetamide (2) had been previously reported from *Tedania ignis*[7]. The compound (1) is also known as a metabolite of the fungus *Ceratocystis clavigera* which is associated with the blue disease of pine[13]. The compound (2) is known for long as a natural product, having been isolated from several terrestrial plants[14-18] and a fungus *Streptoverticillium olivoreticuli*[19]. The compounds (3-5), which are derivable from the amino acids phenylalanine and tyrosine have not been reported as natural products so far.

Diketopiperazines (cyclic dipeptides) (6), (7), (8) and (9) have been previously isolated from fungi and marine sponges. Compounds (6), (7) and (8) were reported to be produced by the sponge *Tedania ignis*[6]. Compound (7) is a metabolite of fungi[13,20] and microorganism[21]. Diketopiperazines(8) & (9) are predominantly produced by fungi[20,22]. The heteroaromatic acids (10) and (11) have not so far been isolated from the nature. However, some synthetic analogs of these compounds having promising hypoglycemic activity have been studied earlier. These studies indicated that 5-caboxy-3-methylpyrazole, when

administered orally reduced free fatty acids and triglyceride levels in plasma,liver, heart and kidney of rats[23]. In a related study, it was established that N-acyl-3,5-dimethyl pyrazoles, when given orally to rats, at 0.25 mg/kg level, had hypoglycaemic activity greater than tolbutamide[24]. The acyl group may be aromatic or aliphatic acid. It was also established that 3-methyl pyrazole-5-carboxylic acid reduces plasma FFA induced by nicotine[25]. In another significant study, Smith *et.al.*, treated rats with 3,5-dimethylpyrazole and noticed that the urine of such animals had pronounced hypoglycaemic activity[26]. They observed that the urine contains 5-methyl pyrazole-3-carboxylic acid, metabolized within the body from the dietary 3,5-dimethylpyrazole which is responsible for this property. Further, they also found that the dimethylpyrazole and the 5-methylpyrazole-3-carboxylic acid are 50 and 200 times more active than tolbutamide. The UpJohn group of workers further studied the effect of the above acid on carbohydrate and free fatty acid metabolism. As a result they established that this compound increases the oxidation rate of glucose and decreases the release of free fatty acids from adipose tissue. In these activities, this compound is 116 times more active than tolbutamide[27]. A facile synthesis of the above acid was achieved by Dulin *et.al.*, when they obtained the title compound in 32% yield by treating acetyl pyruvic acid (3.9gm) with an aqueous solution of hydrazine sulphate(3.9gm) at 50°C[28]. It is probable that the observed hypoglycaemic activity of the crude extract of

this sponge is due to compounds (10) and (11). These results, however, need to be confirmed by studying the hypoglycaemic activity of pure compounds, which could not be done due to the paucity of these compounds.

ß-carboline (12) is also a new natural product. At present no biological activities of this compound are known. Several 1-ß-carboline derivatives known as manzamines have however been reported from sponges of other genera, *viz., Haliclona, Pellina Xestospongia, Pachypellina etc*[9-12]. These compounds are known to be cytotoxic and anti-tumor agents.

EXPERIMENTAL

The general experimental conditions as well as the instruments used for obtaining the spectral data are almost the same as those described in the previous chapters and hence are not being explained further here.

The animals (2 kg) were collected from the intertidal regions of Gujarat coast. They were immediately soaked in MeOH and transported to the laboratory as such. After about 3 weeks, the solvent was filtered, concentrated under vacuum, to yield about 15 gms of an extract. The extract was

resuspended in 10% aq.MeOH and sequentially extracted with pet.ether, EtOAc and n-BuOH. The pet.ether fraction contained mostly the fatty acids and their methyl esters. It was completely converted into the respective methyl esters using diazomethane and analysed on capillary GC (SE 30, 12.5 m, carrier gas: N_2, 5 ml/min, FID, Oven Temperature: 150-200°C at the rate of 2.5°C/min). The individual acids were identified from a comparison of its retention time with those of standards comprising of $C_{18:0}$-$C_{20:0}$ (6 compounds). The chromatogram of the fatty acid mixture is given in FIG 1.

The EtOAc fraction was chromatographed over silica gel column resulting in the isolation of deaminated amino acids, ß-carboline, peptides and phenyl acetamide and finally , the polar fraction rich in the heteroaromatic acids. The above subfractions were rechromatographed over separate silica gel columns using pet.ether-$CHCl_3$-MeOH gradient systems in order to isolate the individual compounds in almost pure form. The final purifications of ß-carboline, the dipeptides and the heteroaromatic acids were carried out on reverse phase HPLC (ODS column). The ß-carboline was purified using MeOH as eluant while the peptides and heteroaromatic acids were purified using 0.1% TFA as mobile phase.

p-Hydroxy benzaldehyde (1), CIMS: 123$[M+H]^+$, ^{1}H NMR($CDCl_3$): δ 9.8(1H,s,CHO), 7.78(2H,d,8.6 Hz), 6.97(2H,d,8.6 Hz), ^{13}CNMR ($CDCl_3$): δ 191.7(d,CHO),132.5(d,C-2 & C-6),116.05(d,C-3 & 5).

Phenyl acetamide (2), IR(KBr): 3500, 3250,2600, 1700, 1600, 1400, 1270, 1170, 1105 and 1060 cm^{-1}; CIMS 136$[M+H]^+$; ^1H NMR ($CDCl_3$):δ 7.5-7.2(5H,m),5.85(1H,br), 5.45(1H,br), 3.58(2H,s); ^{13}C NMR($CDCl_3$):δ 173.47(s,C=O), 134.87(s,C-1), 129.38(d,C-2 & 6), 129.06(d,C-3 & 5), 127.44(d,C-4) and 43.35(t,CH_2).

Phenyl propionic acid (3), IR(KBr): 3500, 2800, 1690, 1580, 1420, 1280, 1200,1140, 1060, 825, 720 and 690 cm^{-1}; CIMS: 151 $[M+H]^+$; ^1H NMR($CDCl_3$): δ 10.0(1H,br,COOH), 7.6-7.2(5H,m), 2.9 (2H,t,J=7 Hz), 2.65 (2H,t,J=7 Hz); ^{13}C NMR($CDCl_3$):δ 179.06(s), 140.07(s),128.4(d,C-2 & C-6),128.1(d,C-3 & 5), 126.25(d,C-4), 35.5(T) and 30.5(t).

p-Hydroxyphenyl propionic acid (4), IR(KBr): 3500, 2900, 2800, 1700, 1500, 1440, 1220, 1100 and 825 cm^{-1}; CIMS: 167 $[M+H]^+$; ^1H NMR($CDCl_3$): δ 7.25(1H,s,exchanged by D_2O), 7.08(2H, d,J=8.5 Hz),6.76(2H,d,J=8.5 Hz), 2.90(2H,t,J=7.5 Hz) and 2.65 (2H,d,J=7.5 Hz); ^{13}C NMR($CDCl_3$): δ 129.4(d,C-2' & 6'), 115.4 (d,C-3' & C-5'), 35.3 (t,C-2) and 29.5(t,C-3).

p-Methoxyphenyl propionic acid (5), ^1H NMR($CDCl_3$): δ 7.23 (2H,d,J=8.5 Hz), 6.72(2H,d,J=8.5 Hz), 3.7(3H,s,OMe), 2.96(2H, t,J=7.7 Hz) and 2.69(2H,t,J=7.7 Hz).

Cyclo[pro-val] (6), CIMS: 193[M+H]$^+$; ^{1}H NMR($CDCl_3$): δ 4.05 (1H,m,1-H), 3.94(1H,br,1'-H), 3.6(2H,m,4-H), 2.62(1H, doublet of septet, J=7.1 & 2.5 Hz,2'-H),2.32(1H,m,2-H),2.00(3H,m,2-H_2 & 3-H), 1.09(3H,d,J=7.2 Hz) and 0.92(3H,d,J=6.8 Hz); ^{13}C NMR ($CDCl_3$):δ 170.26 (s), 164.99(s), 61.18(d,C-1'), 58.76(d,C-1), 45.57(t,C-4), 28.46(t,C-2),28.42(d,C-2'), 24.04(t,C-3), 19.06 (q,CH_3) and 16.06(q,CH_3).

Cyclo[pro-leu] (7), IR(KBr): 3500, 2860, 2700, 1650, 1620, 1460, 1420,1380,1300,1150 and 700 cm^{-1}; CIMS: 211[M+H]$^+$; ^{1}H NMR ($CDCl_3$):δ 7.17(1H,br,NH), 4.05(1H,brt,J=8 Hz,1-H), 3.95(1H, dd,J=3.7 & 8.8 Hz,1'-H), 3.5(2H,m,4-H), 2.26(1H,m,2-H), 1.92 (1H,m,2'-H), 1.9(2H,3-H), 1.82(1H,m,2-H), 1.78(1H,m,3'-H), 1.46(1H,ddd,J=13,8.8 & 4 Hz, 2'-H), 0.92(3H,d,J=8.1 Hz) and 0.89(3H,d,J=8.1 Hz); ^{13}C NMR ($CDCl_3$): δ 170.5(s,C=O), 166.26 (s,C=O),58.8(d,C-1'),53.3(d,C-1),45.15(t, C-4),38.11(t,C-2'), 27.95(t,C-2), 23.1(d,C-3'), 22.6(t,C-3), 24.5 (q,C-5') and 21.2(q,C-4').

Cyclo[pro-phe] (8), CIMS: 245[M+H]$^+$; ^{1}H NMR($CDCl_3$): δ 7.20 (5H,m), 5.70(1H,s), 4.2(1H,dd,J=10 & 3 Hz), 4.10(1H,t), 3.65 (1H,dd,15 & 3 Hz), 3.60(2H,m), 2.75(1H.dd,J=15 & 10 Hz), 2.3 (1H,m) & 1.93(1H,m).

Cyclo[pro-tyr] (9), CIMS: 261[M+H]$^+$; ^{1}H NMR($CDCl_3$): δ 7.05 (2H,d,J=8 Hz), 6.75(2H,d,J=8 Hz), 5.89(1H,s), 4.20(1H,dd,J=10 & 3 Hz), 4.10(1H,t,J=7 Hz), 3.58(2H,m), 3.45(1H,dd,J=14.5 & 3 Hz), 2.76(1H,dd,14.5 & 10z), 2.30(1H,m) and 1.95(2H,m); ^{13}C NMR($CDCl_3$): δ 169.7(s), 165.3(s), 155.9(s,C-4"), 130.3(d,C-2" & C-6"), 126.7(s,C-1"), 116.1(d, C-3" & C-5"), 59.0(d,C-1'), 45.3(t,C-4), 35.9(t,C-2), 28.2 (t,C-2) and 22.3(t,C-3).

Pyrazole-5(3)-carboxylic acid (10), m.p. 293°C(dec); CIMS: 113[M+H]$^+$; IR(KBr): 3432-2700(br),1724,1674, 1447,1418, 1383, 1237, 1216, 993, 837, 560, 540 and 475 cm^{-1}(FIG 4); UV-Vis (MeOH): 204 & 259 nm (FIG 3); ^{1}H NMR(Py-d_5): δ 13.0(1H,br, COOH), 12.1(1H,br,NH), 7.55(1H,d,J=7.75 Hz,3-H) and 5.83(1H, d,J=7.75 Hz,4-H) (FIG 6); ^{13}C NMR(Py-d_5): 165.7(s), 142.3(d), 135.2(d) and 101.4(d) (FIG 5).

4-Methylpyrazole-5-carboxylic acid (11), m.p.299°C(dec); UV-Vis (MeOH): 262(5766) and 207(7319) nm (FIG 8); IR(KBr): 3300-2700, 1730, 1673, 1482, 1446,1425,1382, 1240,1205, 1028, 985,935, 843,815, 758, 737, 556 and 471 cm^{-1}(FIG 9); PCIMS: 127[M+H]$^+$: EIMS m/z(%): 126.11(M$^+$,100), 112.06(7), 96.76(4), 83.16(20), 70.13(12) and 55.18(100) (FIG 10); ^{1}H NMR(Py-d_5): δ 13.04(1H,br),12.22(1H,br),7.33(1H,s) & 1.97(3H,s) (FIG 11); ^{13}C NMR(Py-d_5): δ 166.4(s), 144.3(s,C-5), 137.7(d,C-3), 108.7 (s,C-4) and 11.6(q,CH_3) (FIG 13).

ß-Carboline (12), R_t=10.6 min, m.p.196°C, IR(KBr): 3116, 3053, 2956,2848, 2641,1626, 1598,1559,1498,1458, 1446, 1332, 1278, 1242,1203,1166, 1146,1114, 1035,1019,952,910, 887, 863, 828, 780, 734, 651, 624 and 600 cm^{-1}(FIG 14); ^1H NMR($CDCl_3$): δ 9.43(1H,br,NH), 8.97(1H,s,1-H), 8.45(1H,d,J=5 Hz,3-H), 8.13 (1H,d,7.77 Hz), 7.97(1H,d,J=5 Hz,4-H), 7.55(2H,brs) and 7.30 (1H,m) (FIG 16); ^{13}C NMR($CDCl_3$):δ 140.83(s), 138.40(d), 136.07 (s), 133.34(d), 129.29(s), 128.75(d), 121.86(d), 121.40(s), 120.20(d), 114.92(d) and 111.81(d) (FIG 17); EIMS m/z(%): 169 ($[M+H]^+$,80.4), 168(M^+,100), 167($[M-H]^+$,47.3), 141(28.4), 140 (59.9), 139(6.3), 128(5.6), 117(5), 115(8.2), 114(25.1), 113 (20.9), 87(5.7), 84(8.6), 75(5.1) and 63(9.1) (FIG 12).

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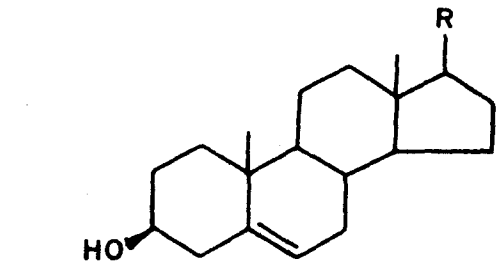
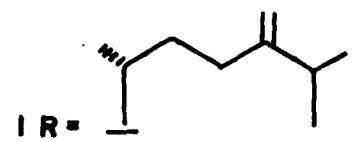
CHAPTER 6

SECTION 1

CHEMICAL CONSTITUENTS OF THE SOFT CORAL *LOBOPHYTUM STRICTUM* TIXIER-DURIVAULT (1957)

Soft corals belong to the order *Alcyonaria* of the phylum or class *Coelenterata*. In systematic classification, this phylum is further divided into three subclasses: *Hydrozoa*, *Scyphozoa* and *Anthozoa*. The latter subclass, *Anthozoa*, is again divided into two orders, *viz.*, *Alcyonaria* and *Zoantharia*. In general, animals belonging to this subclass, especially those belonging to the order *Alcyonaria*, have large amounts of extractable matter, rich in many novel secondary metabolites. Soft corals and gorgonians are two important groups of animals belonging to the order *Alcyonaria*. Both of them are rich in several novel sterols, terpenoids and prostaglandins having widespread ecological, pharmacological and biodynamic properties[1]. Many of the sterols have unique alkylation and diverse oxygenation patterns, modified ring systems, *etc.* These include several polyhydroxy sterols, sterol glycosides, and sterols with keto, aldehyde, carboxylic acid and acetate groups. Recently, pregnane-type sterols, 9(11)-secosterols too have been reported from these sources. In all, over a hundred such unusual sterols have been isolated from these animals[1]. Many of them exhibit promising cytotoxic, anti-leukemic, antirheumatic, anticancer, ichthyotoxic, hypo-cholesterolemic, antiinflammatory and antifouling properties. Most of these *Alcyonarian* sterols are found to occur either in free state or as their sulfate esters or glycosides[1-3].

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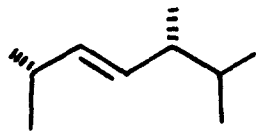
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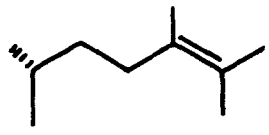
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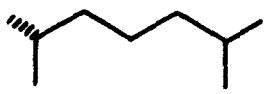
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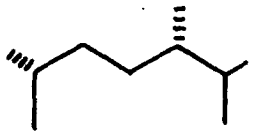
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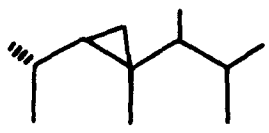
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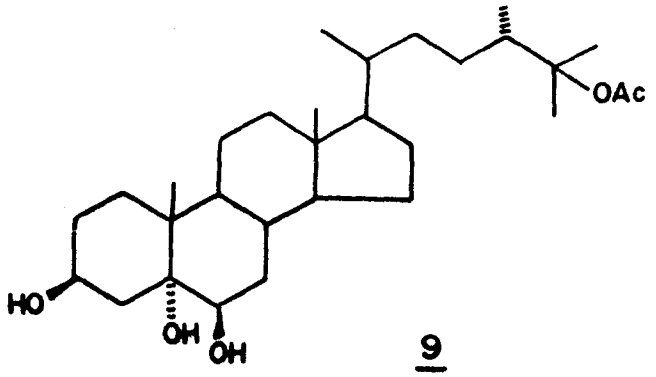

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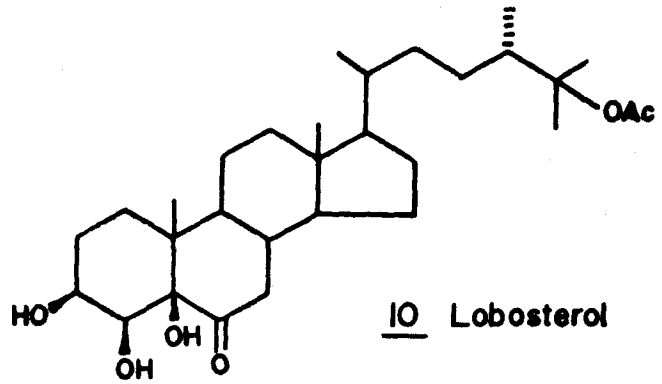
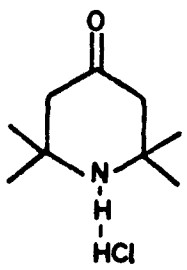
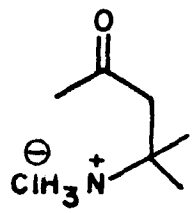


10 Lobosterol



Triacetoneamine hydrochloride

12



Diacetone amine hydrochloride

11

Fig. 1 Secondary metabolites of Lobophytum strictum

As part of our ongoing program on isolation of marine natural products having promising pharmacological properties, we studied the secondary metabolites of the soft coral *Lobophytum strictum* TIXIER DURIVAULT (1957), family: *Alcyonaceae*, collected from Lakshadweep waters by SCUBA diving. Initial pharmacological studies of its methanol extract revealed promising anti-implantation and hypotensive properties *in vivo* (latter activity: 25 mg/kg, 27-30 min)[4]. Follow up studies yielded six monohydroxy sterols (1-6), batyl and chimyl alcohols (7 and 8 respectively) and two polyhydroxy sterols: 24(S)-methyl cholestane-3ß,5α,6ß,25-tetrol-25-monoacetate (9) and 24(S)-methylcholestane-3ß,4ß,5ß,25-tetrol-6-one-25-monoacetate (lobosterol 10) from the $CHCl_3$ soluble fraction of the above extract, while the aqueous fraction yielded two amine hydrochlorides: 1-amino-1,1-dimethyl-3-ketobutane (diacetonamine 11) and 2,2,6,6-tetramethyl piperidone (triacetonamine 12) hydrochlorides (FIG 1). Among these, compound (12) is known to be a hypotensive agent having pronounced vasodilator effects besides being useful in stabilizers and anti-oxidants for various polymers, fuels, lubricants, oil additives, *etc.*

The lipid soluble compounds of the crude methanol extract were extracted using $CHCl_3$ in the usual manner. Later it was fractionated into less polar, medium polar and more polar fractions by chromatography over silica gel columns (pet. ether-EtOAc-acetone solvent systems). The low polar fraction

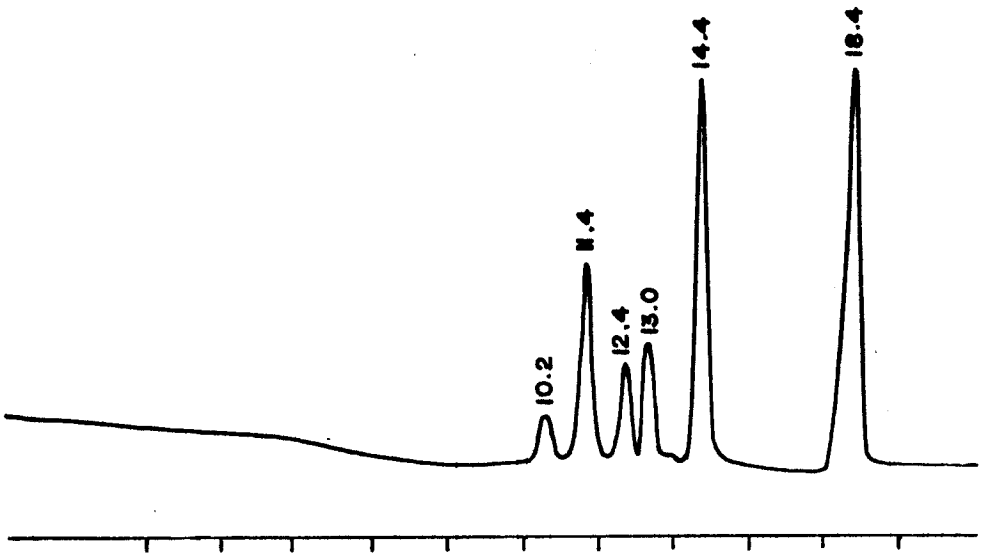


Fig. 2 HPLC of monohydroxy sterols of Lobophytum strictum.
(Me OH, 1 ml / min)

contained several hydrocarbons, waxes, fatty acids and their methyl esters as determined by IR and ^1H NMR spectroscopy. The medium polar fraction, after repeated chromatographic purifications, yielded about 50 mg of a fraction rich in several monohydroxy sterols. This fraction, upon HPLC purification (ODS column, 5μ, MeOH, 2 ml/min, RI detection) yielded six compounds (1-6) in pure form (FIGs 1 & 2). List of these compounds including their strucures, HPLC data and their percentage composition are listed in table I.

TABLE I

COMPOSITION OF MONOHYDROXY STEROL FRACTION OF THE SOFT CORAL *LOBOPHYTUM STRICTUM*

No.	Name of the sterol	R_t (min)	M⁺	% comp
1	24-Methylene cholesterol	10.2	398	3.3
2	24(R)-Methyl cholest-5,22E-dien-3ß-ol	11.4	398	14
3	24-Methyl cholest-5,24-dien-3ß-ol	12.4	398	8.7
4	Cholesterol	13.0	386	9.3
5	24(S)-Methyl cholesterol	14.4	400	32
6	Gorgosterol	18.4	426	33.7

Compound (1), R_t=10.2 min, M⁺=398, was identified as a 3β-hydroxy-Δ⁵-sterol derivative from its ¹H NMR data: δ 1.02(s, 19-H), 0.69(s,18-H), 3.5(1H,m,3α-H) and 5.35(1H,br.d,6-H). The proton signals at δ 4.66(1H,br) and 4.71(1H,br), could be from an exomethylene group, preferably at C-24. This was also supported by the presence of 21, 26 & 27-H signals at δ 0.97 (d,6.6 Hz), 1.03(d,6.6 Hz) & 0.93(d,6.6 Hz) respectively. Its molecular weight is 12 mass units more than cholesterol, indicating the presence of one more carbon atom in it. The cholesterol-type ring system was also confirmed by its mass spectral fragment ions at m/z 273[M-sc]⁺, 255(273-18), 231 [M-sc-ringD]⁺, and 213(231-18), while the unsaturation at C-24(28) was supported by the strong fragment ion at m/z 314[M-C_6H_{12}]⁺, in its EI mass spectrum[12]. Its structure was finalised as 24-methylene cholesterol (1) from these results[6-11].

The ¹H NMR data of compound (2), *viz.*, δ 1.01(s,19-H), 0.70 (s,18-H), 3.5(1H,m,3α-H) and 5.35(1H,brd,6-H) indicated its ring system to be identical to that of cholesterol. Its molecular weight was found to be 398 by CIMS. This indicated its molecular formula to be the same as that of compound (1). The ¹H NMR spectrum had signals due to four methyl groups at δ 0.82, 0.84, 0.91 & 1.01(all doublets). The exomethylene signals at δ 4.66 & 4.71 of compound (1) were absent in this compound. Instead, a multiplet appeared at δ 5.17(2H), indicating the presence of a double bond at C-22. These values were indicative of a *trans* (E) double bond at C-22 and

a methyl group at C-24[5-11]. The proton signals at δ 0.82 & 0.84 could be assigned to 26 & 27-H, while those at δ 0.91 & 1.01 must be due to 28 & 21-H respectively. The stereochemistry at C-24 was finalised as (R) after comparing the chemical shifts of 22-H, 23-H,28-H, 26-H, 27-H and 21-H of this compound with literature values of similar compounds as well as their 24(S) isomers[7,9], leading to its structure as 24(R)-methylcholest-5,22E-dien-3ß-ol (2).

The ^{1}H NMR spectrum of compound (3) was identical to those of earlier two compounds as far as the ring system is concerned. This included the two methyl singlets at δ 0.68 and 1.01 due to 18-H and 19-H, besides the methine proton multiplet at δ 3.5 due to 3α-H and the vinyl proton signal (6-H) at δ 5.35(br.d). The signals due to the diagnostically important side chain methyl groups were observed at δ 0.912 (3H,d,J=6.71 Hz) and 1.59 (9H,brs). The usual doublets at δ 0.86 due to 26 & 27-H of cholesterol were absent in this molecule. The molecular weight of this compound was found to be 398 by CIMS, which is the same as that of compounds (1) and (2). At the same time, the ^{1}H NMR spectrum ruled out the possibility of side chain unsaturations at C-24(28) or C-22. This prompted us to consider C-24(25) as an alternate site for the unsaturation. The ^{1}H NMR signal at δ 1.59(9H,s), which could be due to 26, 27 & 28-H, which are now allylic, supports this view. The signal at δ 0.912(3H,d,6.7 Hz) could be assigned to 21-H. The structure of this compound was

finalised as 24-methylcholest-5,24-dien-3ß-ol (3) from these results.

The ^{1}H NMR spectral data of compound (4), *viz.*, δ 1.01(3H, s), 0.912(3H,d,J=6.5 Hz),0.865(3H,6H,d,J=6.53 Hz),0.68(3H, s), 3.5(1H,m) and 5.35(1H,br.d) was identical to that of cholesterol. CI mass spectrum revealed its molecular weight to be 386, confirming its identity as cholesterol (4)[9].

The compound (5), M^+=400, too was found to be a sterol derivative from its ^{1}H NMR spectrum. The molecular weight was 14 mass units more than cholesterol and 2 units more than compounds (1-3). This indicated the presence of an additional methyl group, possibly at C-24, in this molecule, as compared to cholesterol. The signals at δ 1.007(3H,s), 0.68(3H,s), 3.5(1H,m) and 5.35(1H,brd) could be assigned to 19-H, 18-H, 3α-H and 6-H. Signals due to the other methyl groups were located at δ 0.916(d,6.45 Hz), 0.853(d,J=6.81 Hz), 0.778(d, J=6.81 Hz), and 0.771(d,J=6.68 Hz), which were assigned to 21, 26, 27 and 28-H respectively, in comparison with the data for similar sterols[6]. The stereochemistry at C-24 was finalised as (S) or (α), after comparing the chemical shifts of the side chain methyl groups with those of 24(S) and 24(R)-methylcholesterol derivatives, thereby, confirming its structure as 24(S)-methylcholesterol (5)[6].

The compound (6), R_t=18.4 min, was also found to be a 3ß-hydroxy-Δ^5-sterol derivative as indicated by its ^{1}H NMR spectral data: δ 3.5(1H,m,3α-H), 5.35(1H,br.d,6-H), 0.66(3H, s,18-H) and 1.005(3H,s,19-H). The prominent peaks in the CI mass spectrum at m/z 444[M+18]$^+$, 426[M$^+$], 408[M-18]$^+$, 393[M-18-15]$^+$ & 367[M-H-43-15]$^+$, revealed its molecular weight to be 426. This was 40 mass units more than cholesterol with the possible molecular formula $C_{30}H_{50}O$. The above molecular formula has six degrees of unsaturations. Five of them could be accounted for by four rings and the double bond at C-5. Thus, the second unsaturation has to be somewhere in the side chain. But there were no vinyl proton or any allyl methyl proton signals in the ^{1}H NMR spectrum, indicating the possibility of a cyclic group being present in the side chain. The four protons in the upfield region in its ^{1}H NMR spectrum: δ 0.46(1H,dd,J=8.45 Hz), 0.19(2H,m) & -0.12(1H,m), indicated the presence of a cyclopropyl group. In addition, the ^{1}H NMR spectrum also had signals due to five methyl groups. These signals were observed at δ 1.005(brs), 0.95(d, J=6.63 Hz), 0.938(d,J=6.9 Hz), 0.859(d,J=6.6 Hz) & 0.896(s). Comparison of these values with those of other marine sterols established its identity as gorgosterol[7,8]. Accordingly, the five methyl groups could now be assigned to 21-H, 26-H, 27-H, 28-H & 30-H of gorgosterol. Musmar *et.al.*, have shown that ^{1}H NMR spectrum of gorgosterol will have four upfield proton signals even though this compound has only three cyclopropyl protons. The fourth one, at δ -0.12, is from the hindered

proton at C-24[46]. The structure of this compound was finalised as gorgosterol (6) from these results.

The more polar fraction eluting from the silica gel columns after the monohydroxy sterols was found to be a mixture of batyl and chimyl alcohols (7 & 8 respectively) from its IR (FIG 3), ^1H NMR and mass spectral data as well as their comparison with of commercial batyl alcohol. The PCIMS of this sample had prominent ions at m/z 362 and 334 due to $[M+NH_4]^+$ pseudomolecular ions, leading to their molecular formulae as $C_{21}H_{44}O_3$ and $C_{19}H_{40}O_3$ respectively, in agreement with their proposed structures.

Continued elution of the silica gel column with EtOAc-acetone gradient system led to the isolation of two more crystalline compounds (9 and 10). The IR spectrum of compound (9), m.p. 233°C, had peaks at 3450 and 1730 cm^{-1}, indicating the presence of hydroxyl and ester groups in it (FIG 4). Its ^1H NMR spectrum had peaks at δ 4.09(1H,m) and 3.54(1H,brd) indicating the presence of two -CHOH- groups in this molecule (FIG 5). The signal at δ 1.98 (3H,s) and the IR absorption at 1730 and 1250 cm^{-1} were indicative of an acetate group. This was also supported by the presence of peaks at δ 170.61 and 21.15 in its ^{13}C NMR spectrum (FIG 6). The other methyl group signals were found at δ 1.18(3H,s), 0.67(3H,s), 0.87(3H,d,6.5 Hz), 0.91(3H,d,6.5 Hz) and 1.38(6H,s). The molecular weight of this compound was found to be 492 by CIMS. Its ^{13}C NMR

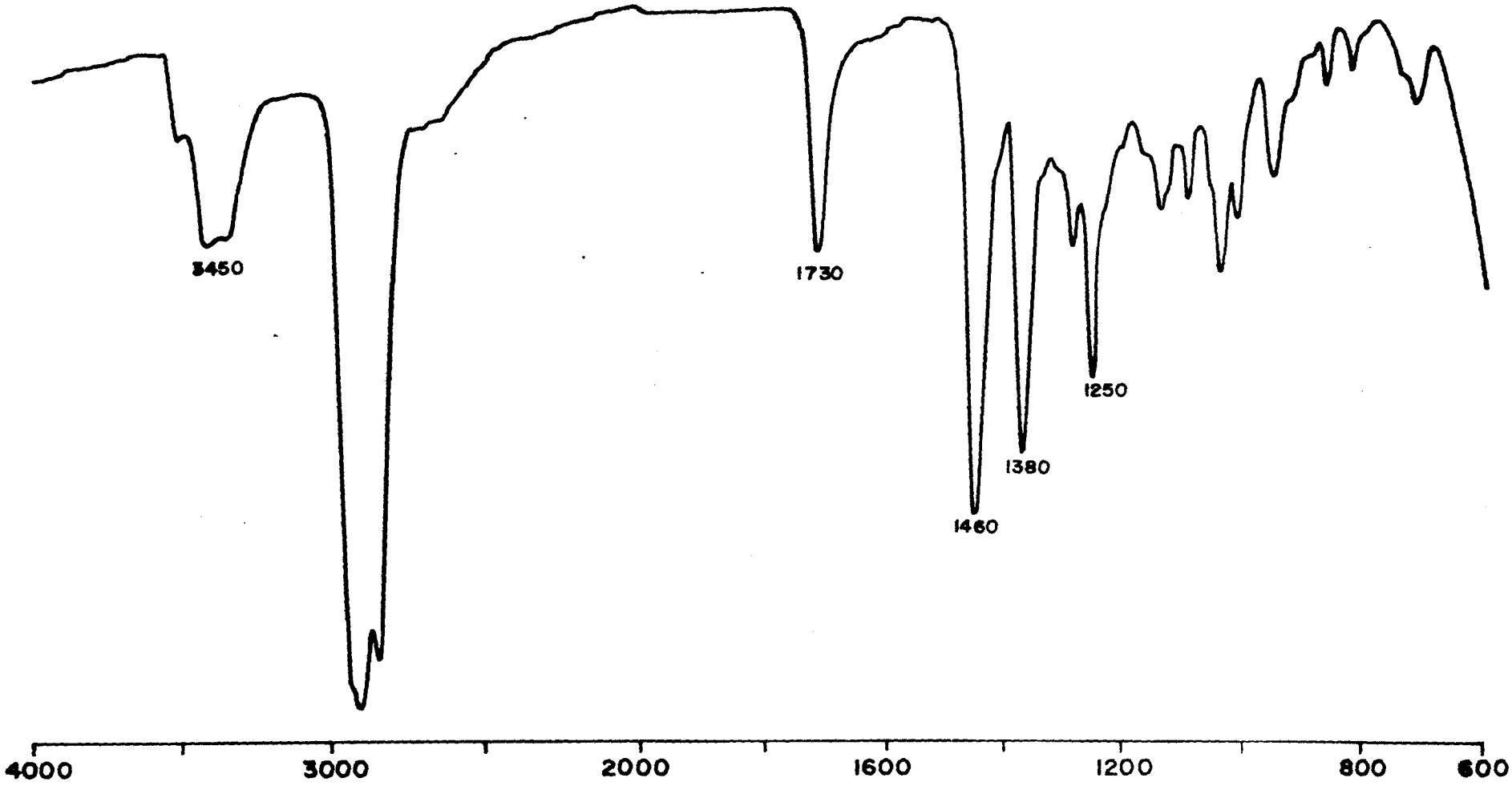


Fig. 4 IR (KBr) of 24 (S) - methylcholestane - 3β, 5α, 6 β, 25 - tetrol - 25-monoacetate (9)

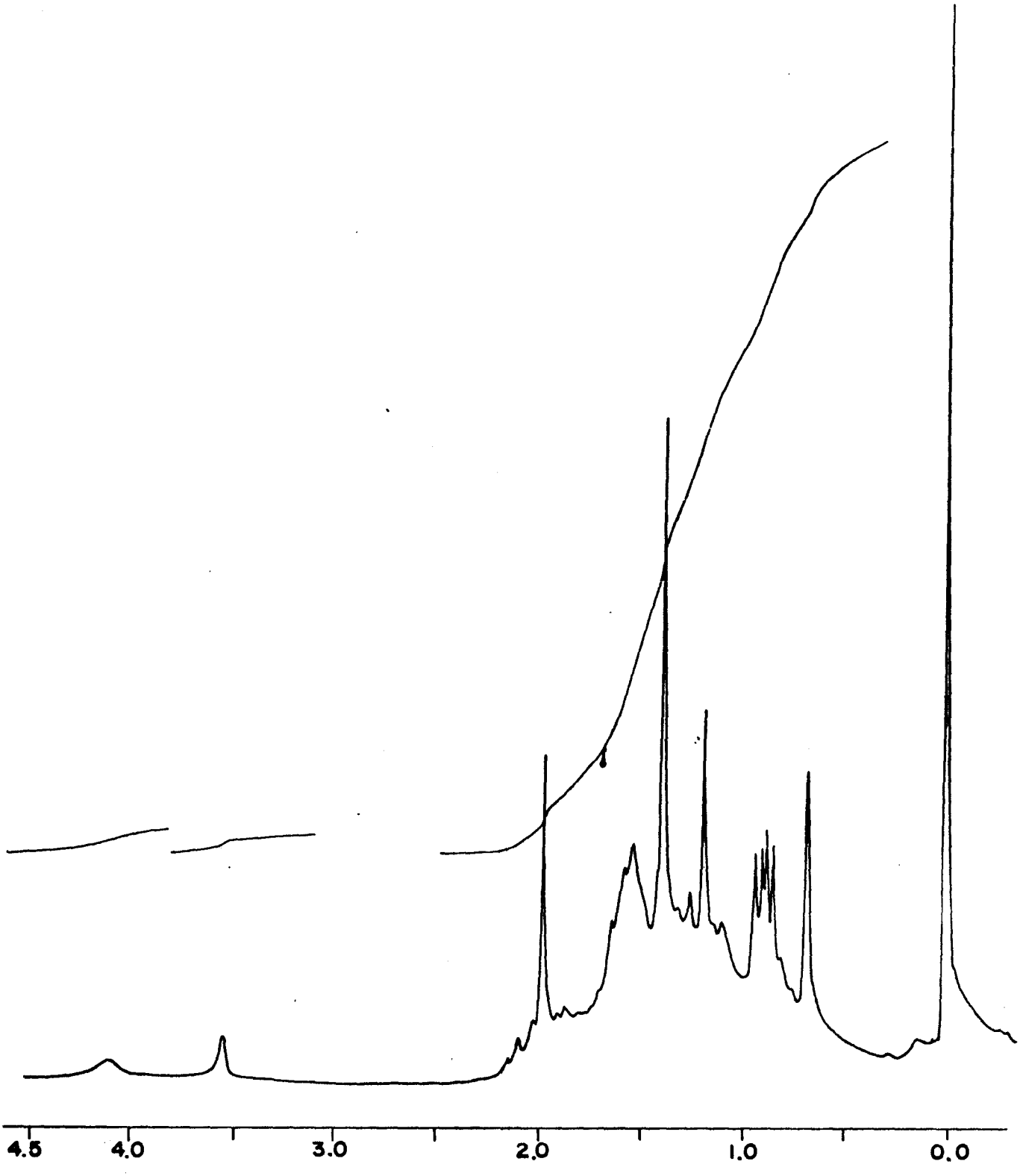


Fig. 5 ^{1}H NMR spectrum of 24(S)-methyl cholestane-3β, 5∝, 6β, 25-tetrol-25-monoacetate (9)

spectrum had four downfield carbon signals (oxygenated carbons), at δ 85.93(s), 76.06(s), 76.06 (d) and 67.63(d). At the same time the proton signal at δ 5.35 due to 6-H in cholesterol was absent in this molecule. These results suggested its molecular formula to be $C_{30}H_{52}O_5$. This was also in agreement with the ^1H and ^{13}C NMR results discussed above. The possibility of the acetate being present at a secondary carbon is ruled out since the corresponding methine signals were found at relatively high field region, *i.e.*, δ 4.09 and 3.54. Thus the acetate as well as one of the hydroxyl groups must be attached to tertiary carbons. From the proton signal at δ 1.38(6H,s), it was presumed that the acetate group is present at C-25. The signal at δ 0.68(3H,s), due to 18-H is quite normal when the rings C and D are not substituted. But the 19-H signal appeared rather downfield, at δ 1.18 from δ 1.01 in cholesterol. This, as well as the absence of the double bond at C-5 suggested that probably the other two hydroxyl groups too are present in ring A or B. Comparison of these spectral data with those of other polyhydroxy sterols from literature indicated its structural similarity with that of24(S)-methylcholestane-3ß,5α,6ß,25-tetrol-25-monoacetate, previously reported from the soft coral *Sarcophyton elegans*[13]. This structure was also confirmed by its ^{13}C NMR spectrum[14]. The stereochemical assignments of C-3, 5 and 6 are based on the chemical shift and coupling pattern of their proton and carbon signals[13-15], while that of C-24 was based on biogenetic considerations.

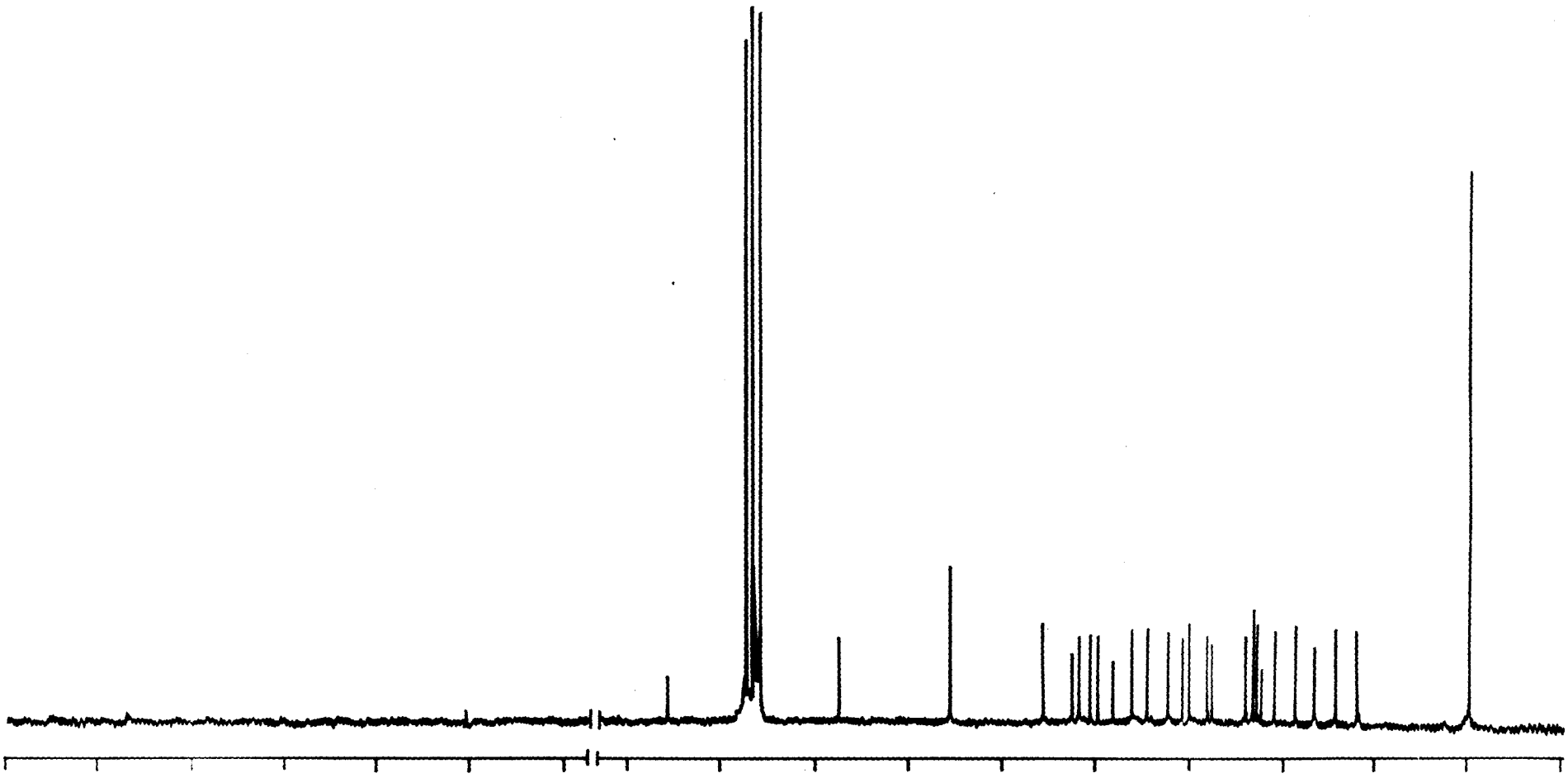


Fig. 6 ^{13}C NMR spectrum of 24(S)-methyl cholestane -3β, 5∝,6β,25- tetrol - 25- monoacetate (9)

This structure was further confirmed by peracetylation of compound (9) (Ac_2O/Pyridine), which yielded its diaceatyl derivative, M^+=576 (PCIMS m/z: 594$[M+NH_4]^+$ and NCIMS m/z: 611 $[M+Cl]^-$), as expected. The ^1H NMR spectrum of this derivative had signals due to all the three acetate methyl groups around 2 ppm. The corresponding methine signals are found at δ 5.15 and 4.68 respectively.

The compound (10), m.p. 210°C, had peaks at 3450, 1730-1700 and 1250 cm^{-1}, in its IR spectrum indicative of the presence of hydroxyl, acetate and carbonyl groups in it (FIG 7). Comparison of its ^1H NMR spectrum with that of compound (9) indicated that both these compounds have identical sterol side-chains (FIG 8). Thus, peaks at δ 2.0(3H,s,acetate), 0.92 (3H,d,J=6.8 Hz,21-H), 1.40(6H,s,26 & 27-H) and 0.86(3H,d, J=6.8 Hz,28-H) were common to both. This meant that the carbonyl and the hydroxyl groups are present within the rings of this molecule. CI mass spectrum revealed its molecular weight to be 506, corresponding to the formula $C_{30}H_{50}O_6$. Thus, this compound has six degrees of unsaturation, four of which could be due to the rings, the fifth, due to the acetate group, while the sixth must be from the new carbonyl group. The 18-H signal of this compound was found at δ 0.66, while, the 19-H signal appeared slightly upfield at δ 0.74, probably due to the shielding effect of the new carbonyl group. The signals at δ 70.22(d), 69.79(d), 85.61(s) and 85.88(s) in its ^{13}C NMR spectrum suggested the presence of four oxygenated

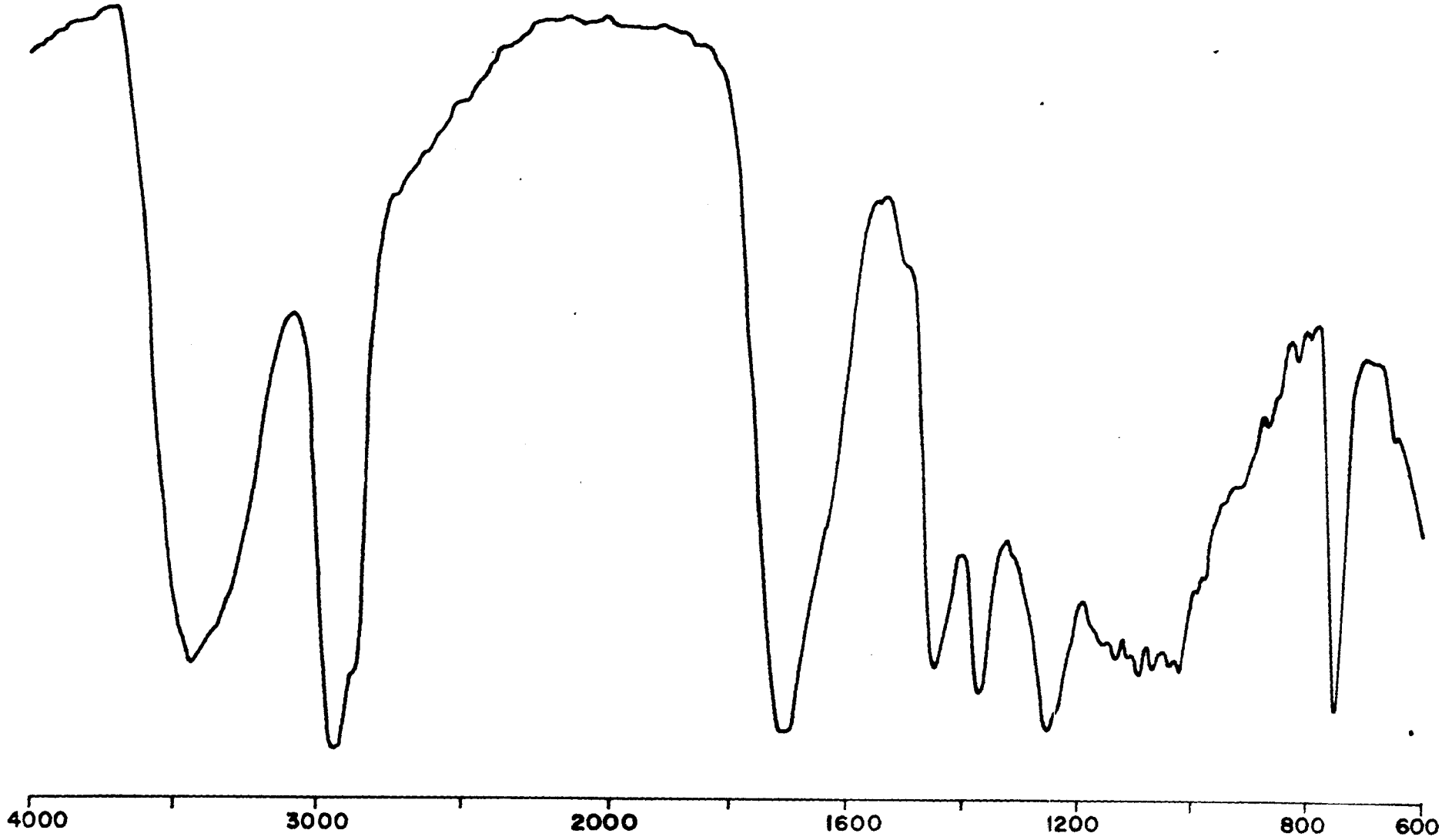


Fig. 7 IR (KBr) spectrum of lobosterol (10)

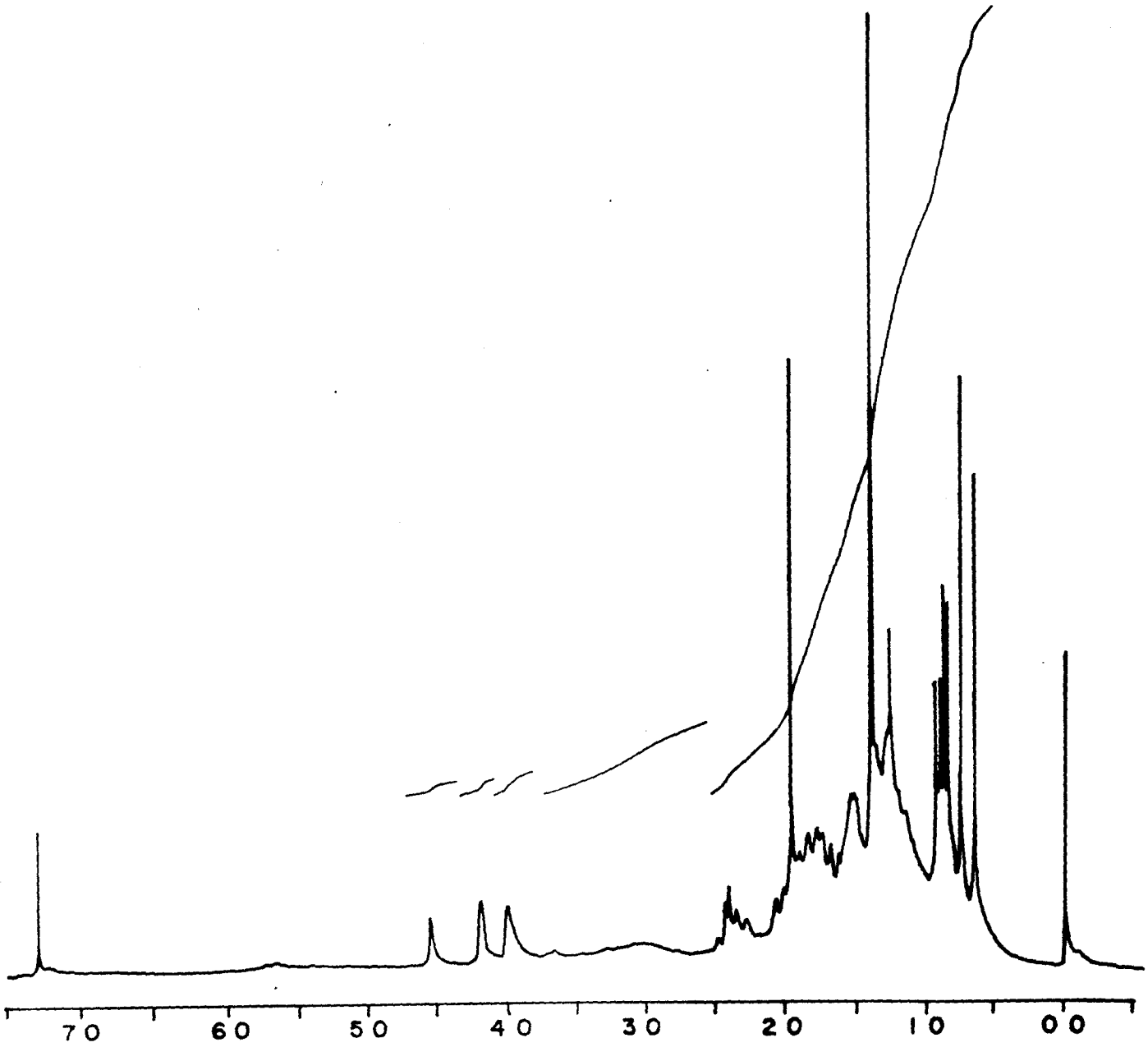


Fig. 8 ^{1}H NMR spectrum of Lobosterol (10)

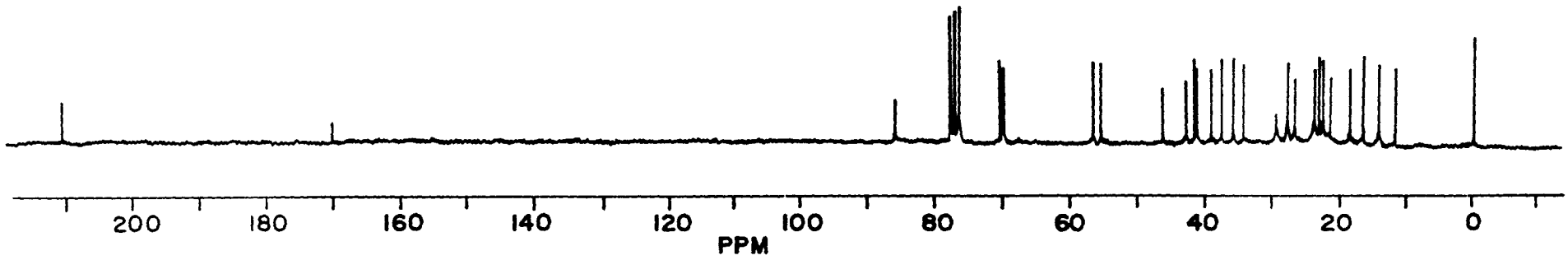


Fig. 9 ^{13}C NMR spectrum of Lobosterol (10)

carbons in it (FIG 9). Comparison of the above ^{1}H and ^{13}C NMR spectral data with the values reported in literature for similar compounds from soft corals indicated its structure to be 24(S)-methyl cholestane-3ß,4ß,5ß,25-tetrol-6-one,25-mono-acetate (lobosterol 10)[15]. The stereochemistry of the original compound had been determined by X-ray crystallographic methods. Both the ^{1}H and ^{13}C NMR values of compound (10) agreed very well with the reported values of lobosterol, which confirmed its identity.

The aqueous fraction, remaining after the removal of the lipid fraction from the methanol extract using $CHCl_3$ was partly concentrated under vacuum and the smaller molecules other than proteins, carbohydrates, *etc.*, were extracted with large excess of acetone. The acetone solution was later concentrated under vacuum. TLC of this fraction using $CHCl_3$-MeOH-AcOH (60:10:5) solvent system indicated it to be mixture of two prominent compounds, having R_f values of 0.3 and 0.5. They were separated by preparative TLC using the same solvent system.

Compound (11), R_f=0.3, had strong IR absorptions in the region 3250-2600cm^{-1} indicating the presence of amino salts. The peak at 1710cm^{-1} was indicative of the presence of a carbonyl group. An EtOH solution of this compound gave a white precipitate with $AgNO_3$ solution indicating it to be a hydrochloride salt. Its elemental composition was determined

to be $C_6H_{14}NOCl$ by analytical methods. The ^1H NMR spectrum of this molecule indicated the presence of three methyls [δ 2.2 (3H,s) and 1.55(6H,s)], one methylene [δ 3.0(2H,s)] and three hydrogens attached to a nitrogen [δ 8.45(3H,br)] in it. This was further confirmed by its ^{13}C NMR spectrum which had the corresponding carbon signals at δ 25.84(q), 31.25(q) and 50.41(t). The singlet signals at δ 207.98 and 53.54 were indicative of the presence of the carbonyl and quarternary carbons respectively. Its structure was finalised as 1-amino-1,1-dimethyl-3-ketobutane hydrochloride (11) from these results.

The relatively low polar compound (12), R_f=0.5, isolated by preparative TLC alongwith compound(11) from the water-soluble fraction of this soft coral was a crystalline solid with sharp melting point of 188°C (Lit:188-190°C)[16]. Like the diacetonamine hydrochloride (11), this compound also yielded a white precipitate with silver nitrate solution, indicating it to be a hydrochloride salt. Its IR spectrum had very broad peaks in the range 3000-2400 cm^{-1}, characteristic of ammonium salts. The peaks at 1705 cm^{-1} in the IR spectrum and its UV-Vis absorption at 276 nm clearly indicated the presence of a carbonyl group in this molecule (FIG 10). Its ^1H NMR spectrum revealed the presence of four methyls: δ 1.74 (12H,s), two methylenes (δ 2.81,4H,s) besides two protons at δ 9.76, exchangeable with D_2O. These results indicated its probable structure as 2,2,6,6-tetramethyl piperidone

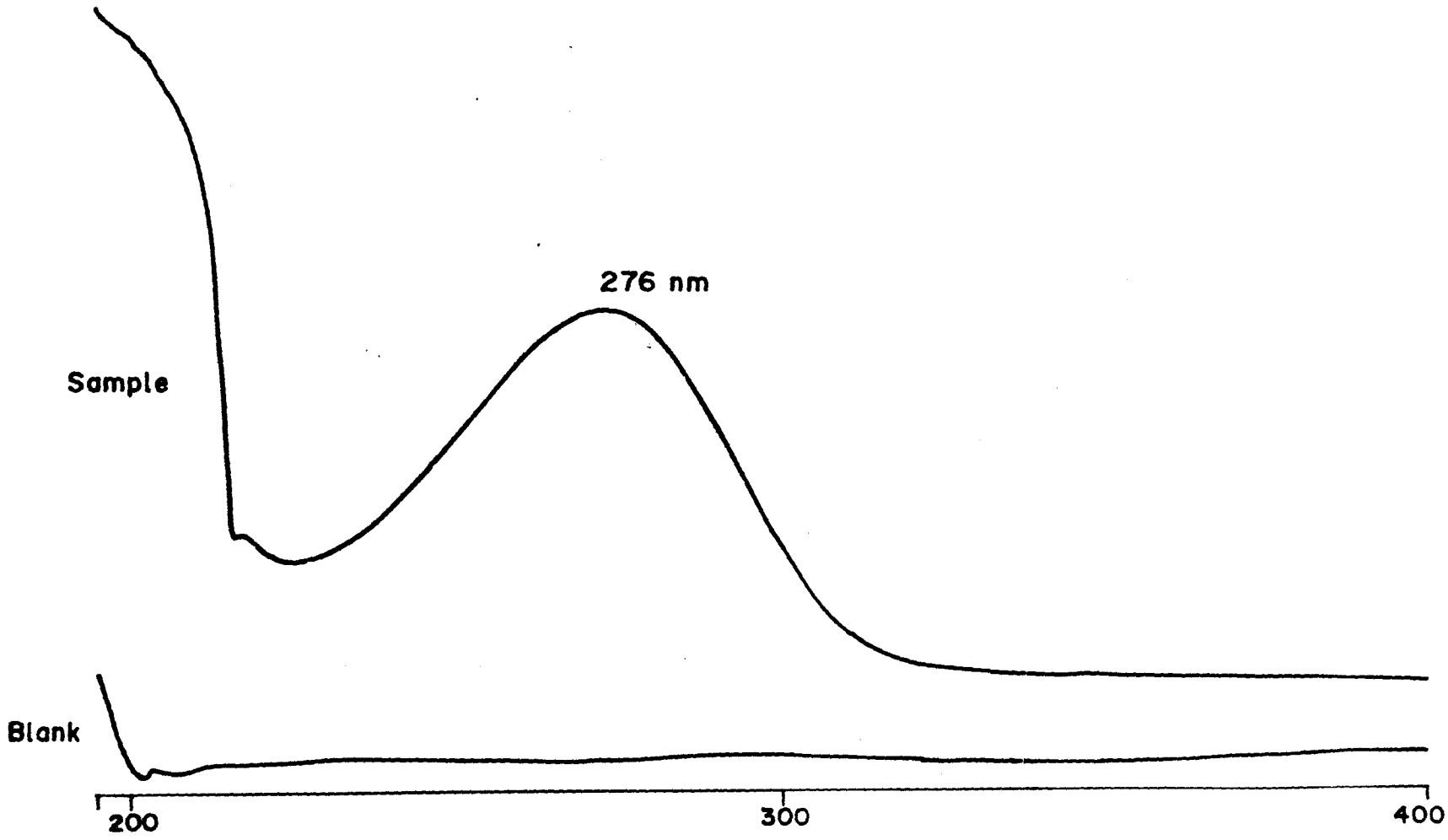


Fig. 10 UV – Vis spectrum of triacetonamine hydrochloride (12) in MeOH solution

hydrochloride (12). The structure was further confirmed by the comparison of its IR, ^{13}C NMR and mass spectral values with those reported in literature[16,17]. The carbon signals of compound (12) were seen at 203.05(s,C=O), 60.54(s), 50.4(t) and 27.63(q).

GENERAL DISCUSSION

Sterols, fatty acids, their esters including waxes and glycerides as well as batyl alcohol and its analogs are common metabolites of most of the living organisms. We have isolated many of these compounds from several corals and sponges earlier. It is interesting that most of the sterols in this animal have 28 carbons. This is the common feature of all soft corals and gorgonians. Another major sterol found in this animal is gorgosterol, a C_{30} sterol, which forms about one-third of the total monohydroxy sterols present in it. In contrast, C_{29} sterols were conspicuous by their absence while cholesterol, a C_{27} sterol, is only a minor constituent. The stereochemistry of 24-methyl substitution in compounds (2) and (5) has been determined to be (α) after comparison of their ^1H NMR values with those reported for the respective 24(α) and 24(ß) isomers[6-9]. The isolation of compound (3), having a double bond at C-24(25) is also very significant as it indicates the possible biogenetic route for 25-hydroxy or acetoxy sterols, present in this animal as well as in several other soft corals[17].

Several polyhydroxy sterols, including compounds (9) and (10) or their analogs, have been reported from soft corals, star fishes as well as from bryozoans[1-3]. Compound (9), 24-methylcholestane-3ß,5α,6ß,25-tetrol-25-monoacetate, was first reported from a soft coral *Sarcophyton elegans* as early as in 1974[13]. At that time, the stereochemistry of 24-methyl substitution was not determined. Subsequently, Tursch *et.al.*, isolated lobosterol (10) from another soft coral *Lobophytum paciflorum* and established its complete structure with the help of NMR and X-ray crystallographic studies[15]. They found that the methyl substitution at C-24 is (α) or (S). Our isolation of both the compounds (9) and (10) from this soft coral, *L. strictum* for the first time leads to a hypothesis that probably the former is the biogenetic precursor of the latter. This, as well as our earlier observation that 24α-methyl substitution is favoured in sterols in this organism suggests that probably the same is true in the case of compound (9) also, leading to the tentative stereostructure given for it. This is also supported by the findings of Kobayashi *et.al.* and Rao *et.al.*, who have reported compound (9) from the soft coral *Lobophytum* sp[17] and *Sinularia* sp[17a] respectively.

Very little is known about the pharmacological properties of compounds (1-10). On the other hand, piperidine type of compounds are known to exhibit wide range of biological activities such as antibacterial, antifungal, phytotoxic,

insecticidal, *etc.* At low concentrations piperidines inhibit Na^+ and K^+ ATPases, block neuromuscular junctions, release histamine from mast cells, besides being ant-repellants. They also have powerful lytic activity, completely hemolysing rabbit erythrocytes in seconds. Dialkyl piperidines exhibiting these properties have been isolated from certain fire ants of the genus *Solenopsis*, a taxon in the subfamily *Myrmicinae*[18]. Tetramethyl piperidone has been previously reported from a plant *Juncella squamata*[19]. In this case, it was noticed to lower blood pressure when administered *i.v.*(0.1-0.3 mg/Kg) and dilate arterioles of spinotrapezius muscle exteriorized *in situ*. The mechanism of these actions, too, has been studied in detail. Besides, it is also a starting material for stabilizers, anti-oxidants, lubricating oil additives, fuels, pharmaceuticals, *etc.*

EXPERIMENTAL

The general experimental conditions have been explained in the preceeding chapters and are not being elaborated here. The soft coral was collected by SCUBA diving from the coral reefs of Lakshadweep Sea during Dec,1985. A reference specimen is kept at the biology museum in NIO. Freshly collected animals (5 Kg,wet weight), were soaked in MeOH and transported to the laboratory on mainland. After about 3 wks,

the solvent was drained off and concentrated under vacuum. The residue was partitioned between $CHCl_3$ and 10% aq.MeOH (3 times). The combined $CHCl_3$ fractions were dried (anhydrous Na_2SO_4), and concentrated in a rotary evaporator. The residue was loaded over a silica gel column and eluted with pet.ether-EtOAc-acetone systems, yielding a mixture of hydrocarbons, long chain fatty esters (waxes), methyl esters of fatty acids, fatty acids, monohydroxy sterols, batyl alcohol and its analogs and a mixture of several polyhydroxy sterols in that order. Among these, the batyl alcohol and its analogs were analysed without further purification, using NMR and mass spectral techniques. The fraction rich in monohydroxy sterols was purified on reverse phase HPLC (ODS column, MeOH, 2 ml/min, RI detection), yielding six sterols as shown in table 1. The mixture of polyhydroxy sterols was purified on repeated silica gel columns, yielding compounds (9) and (10) in pure form. The polar, water-soluble fraction was concentrated to about one-tenth of its original volume under vacuum. To this was added large excess of acetone and the mixture shook well and allowed to settle. Carbohydrates and proteins are insoluble in acetone. On the other hand, several smaller molecules dissolve in acetone. The acetone solution was clearly separated and concentrated in a rotavapor. TLC of this fraction on silica gel using $CHCl_3$-MeOH-AcOH (60:10:5) solvent system indicated it to contain two major compounds, later identified as (11) and (12), having R_f values of 0.3 and 0.5 respectively.

24-Methylene cholesterol (1), ^{1}H NMR($CDCl_3$): δ 5.35(1H, brd,6-H), 4.71(1H,br,28-H),4.66(1H,br,28-H'), 3.5(1H,m,3α-H), 1.03(3H,d,J=6.6 Hz,26-H), 1.02(3H,s,19-H), 0.97(3H,d,J=6.6 Hz,27-H), 0.93(3H,d,J=6.6 Hz,21-H) and 0.69(3H,s,18-H); PCIMS m/z: 416[M+18]$^+$, 398[M]$^+$ and 380[M-18]$^+$; NCIMS m/z: 397[M-H]$^-$; EIMS m/z: 398, 380, 314, 301, 299, 273, 255, 231, 213, *etc.*

(24R,22E)-24-Methylcholest-5,22-dien-3ß-ol (2), ^{1}H NMR ($CDCl_3$): δ 5.35(1H,brd,6-H), 5.17(2H,m,22 and 23-H), 3.5(1H, m,3α-H), 1.01(3H,s,19-H), 1.01(3H,d,J=6.5 Hz,21-H), 0.912(3H, d,J=6.7 Hz,28-H), 0.84(3H,d,J=6.5 Hz,26-H), 0.82(3H,d,J=6.5 Hz,27-H)and 0.70(3H,s,18-H); PCIMS m/z: 416[M+18]$^+$, 398[M]$^+$ and 380[M-18]$^+$.

24-Methylcholest-5,24-dien-3ß-ol (3), ^{1}H NMR($CDCl_3$): δ 5.35 (1H,brd,6-H), 3.5(1H,m,3α-H), 1.59(9H,brs,26,27 & 28-H), 1.01 (3H,s,19-H), 0.92(3H,d,J=6.7 Hz,21-H) and 0.68(3H,s,18-H); PCIMS m/z: 416 [M+18]$^+$, 398, 380, *etc.*

Cholesterol (4), ^{1}H NMR($CDCl_3$): δ 5.35(1H,brd,6-H), 3.5(1H, m), 1.01(3H,s,19-H), 0.912(3H,d,J=6.5 Hz,21-H), 0.865(6H,d, J=6.5 Hz,26 & 27-H) & 0.68(3H,s,18-H); PCIMS m/z: 404[M+18]$^+$, 386, 369,*etc.*

24(S)-Methylcholesterol(5), ^{1}H NMR(CDC_3): δ 5.35(1H,brd,6-H, 3.5(1H,m,3α-H), 1.01(3H,s,19-H), 0.912(3H,d,J=6.45 Hz,21-H), 0.853(3H,d,J=6.8 Hz,26-H), 0.778(3H,d,J=6.8 Hz,27-H), 0.771

(3H,d,J=6.7 Hz,28-H) & 0.673(3H,s,18-H); PCIMS m/z: 418 $[M+18]^+$, 400, 381, 367, 342 & 311.

Gorgosterol (6), ^{1}H NMR($CDCl_3$):δ 5.35(1H,brd,6-H), 3.5(1H, m,3α-H), 1.005(6H,brs,19-H & 21-H), 0.95(3H,d,J=6.6 Hz,26-H), 0.938(3H,d,J=6.9 Hz,27-H), 0.897(3H,s,30-H), 0.859(3H,d,J=6.6 Hz,28-H), 0.66 (3H,s,18-H), 0.46(1H,dd,J=8 & 4,5 Hz), 0.19 (2H,m) and -0.12(1H,m); PCIMS m/z: 444$[M+18]^+$, 426, 408, 393, 367, 342 and 311.

24S-Methylcholestane-3ß,5α,6ß,25-tetrol-25-monoacetate(9), M P 233°C, IR(nujol): 3450, 1730,1380, 1280,1250, 1135, 1090, 1040, 1010, 950, 860, 820 and 720 cm^{-1}; ^{1}H NMR($CDCl_3$): δ 4.09 (1H,m,3-H), 3.54(1H,brs,6-H), 1.98(3H,acetate),1.386(6H,s,26 & 27-H), 1.183(3H, s,19-H), 0.916(3H,d,J=6.5 Hz,21-H), 0.861 (3H,d,J=6.8 Hz,28-H), and 0.68(3H,s,18-H); ^{13}C NMR($CDCl_3$): δ 32.6(t,C-1), 34.51(t,C-2), 67.63 (d,C-3), 41.89(t,C-4), 6.06 (s,C-5),34.63(t,C-7),30.83(d,C-8),45.84(d,C-9),39.89(s,C-10), 22.55(t,C-11), 40.72(t,C-12), 42.72(s, C-13), 55.89(d,C-14), 24.11(t,C-15), 28.11(t,C-16), 55.89(d,C-17), 12.15(q,C-18), 16.87(q,C-19), 36.2 (d,C-20), 18.92(q,C-21), 30.19 (t,C-22), 27.7(t,C-23),38.28(d,C-24),85.93(s,C-25),23.31(q,C-26), 22.89 (q,C-27), 14.48(q,C-28), 170.61(s,OAc) and 21.15 (q,OAc); PCIMS m/z: 510 $[M+18]^+$, 446$[M-28-18]^+$,408$[M-2X15-3X18]^+$, 390 $[M-102]^+$ or $[M-H-C(CH_3)_2-OOCCH_3]^+$, 372(390-18), 362(390-28), 356(390-34), 325(390-2X18-29) or (362-H-2X18), 306[M-sc]$^+$, 297$[M-2X18-H-CH_2CH_2CH(CH_3)C(CH_3)_2OOCCH_3]^+$, 280(297-17), *etc.*

Acetylation of compound (9): About 10 mg of the compound (9) was mixed with 1 ml each of pyridine and acetic anhydride in a round bottomed flask fitted with a condenser. The reaction mixture was initially heated to 80°C for 1 hr and then kept overnight at ambient temperature. Usual work-up of this reaction mixture yielded about 7 mg of a product, identified as 24-methyl cholestane-3ß,5α,6ß, 25-tetrol-3,6,25-triacetate from its spectral data. ^{1}H NMR($CDCl_3$): δ 5.15(1H,m,3α-H), 4.68(1H,brs,6-H), 2.08, 2.05 and 1.98(3H each,s,acetate methyls),1.40(6H,s,26 & 27-H),1.18(3H,s,19-H), 0.92(3H,d,J=6.5 Hz,21-H), 0.87(3H,d,J=6.4 Hz,28-H) and 0.68 (3H,s,18-H); PCIMS m/z: 594[M+18]$^+$, 577[M+H]$^+$, 544[M-33]$^+$, 534[M-42]$^+$,476[M-60-42]$^+$, *etc*; NCIMSm/z: 611[M+Cl]$^-$, 575[M-H]$^-$, 551[M+Cl-60]$^-$, 395, *etc.*

24(S)-methylcholestane-3ß,4ß,5ß,25-tetrol-6-one-25-monoacetate, (lobosterol 10); M P 210°C; IR(nujol): 3450,1730, 1700, 1450,1380, 1250, and 1050 cm^{-1}; ^{1}H NMR($CDCl_3$): δ 4.52 (1H,br,3α-H), 4.17(1H,d,4-H), 2.0(3H,s,OAc), 1.40(6H,s,26 & 27-H), 0.92(3H,d,J=6.8 Hz,21-H), 0.86(3H,d,J=6.8 Hz,28-H), 0.74(3H,s,19-H) and 0.66(3H,s,18-H); ^{13}C NMR($CDCl_3$): δ 37.86 (t,C-1), 29.66(t,C-2),70.22(d,C-3),69.79(d,C-4),85.61(s,C-5), 41.83(t,C-7), 41.15(d,C-8), 46.48(d,C-9), 42.79(s,C-10), 22.52 (t,C-11), 37.86(t,C-12), 43.16(s,C-13), 56.81(d,C-14), 24.15(t,C-15), 27.86(t,C-16), 55.74(d,C-17), 11.95(q,C-18), 16.84 (q,C-19), 36.06(d,C-20), 18.85(q,C-21), 29.47(t,C-22), 27.67(t,C-23), 39.3(d,C-24), 85.88(s,C-25), 23.32(q,C-26),

23.94(q,27-H), 170.0(s, OAc) and 21.62(q,OAc).

1-amino-1,1-dimethyl-3-ketobutane hydrochloride (diacetonamine hydrochloride 11); IR(KBr): 3250-2600, 1710, 1610, 1580, 1500, 1370 cm^{-1}; ^{1}H NMR($CDCl_3$): δ 8.35(3H,br), 3.0(2H,s), 2.2 (3H,s) and 1.55(6H, s); ^{13}C NMR($CDCl_3$): δ 207.98(s), 53.54(s), 50.41(t), 31.25(q) and 25.84(q); Anal. calcd for $C_6H_{14}NOCl$: C 47.68, H 9.27, N 9.27, Found: C 46.91, H 9.495 and N 8.611.

2,2,6,6-tetramethylpiperidone hydrochloride (triacetonamine hydrochloride 12), M P 188°C (lit.188-190°C); IR(KBr): 3500, 3420, 3000-2400, 1705,1540, 1460, 1420,1410, 1380,1365, 1305, 1280, 1245,1210, 915,850 and 750 cm^{-1}; ^{1}H NMR($CDCl_3$): δ 9.76 (2H,br), 2.81(4H,s), 1.74 (12H,s); ^{13}C NMR($CDCl_3$): δ 203.05 (s), 60.54(s), 50.40(t) and 27.63(q); EIMS m/z: 191([M]$^{+}$, v.weak), 155([M-HCl]$^{+}$, 139,111,97, *etc*.

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CHAPTER 6

SECTION 2

ECHINOSIDE B FROM TIIE THE SEA CUCUMBER *ACTINOPYGA MAURITIANA*

Sea cucumbers (*Holothuroidea*) are long, cucumber-shaped animals found in almost all coastal waters. The medicinal use of these animals were known to mankind for a long time. For example, in China, *Holothuria leucospilota*, commonly known as *Yuzu haishen* is being used in the treatment of convulsion caused by paralysis, cerebral concussion or spinal injury since time immemmorial[1]. Besides, many holothurins are also food delicacies, being widely consumed in Japan, China and other oriental countries.

These animals, being devoid of any calcareous skeleton, often resort to chemical defence against predation. Many of them possess special defence organs known as cuverian tubules, which arise from a common stem of the respiratory tree. When disturbed or irritated, they emit these tubules through the anus. Once in water, these tubules become elongated by hydrostatic pressure and form very sticky threads in which the predators become ensnared. It is presumed that during the elongation process the outer cells of these tubules break down thereby releasing a proteinaceous material which forms an amorphous mass having strong adhesive properties. The elongated threads may attain a length of almost a metre. They separate from their attachment and are left behind as the holothurian crawls away. The autotomized parts are regenerated in time[2]. In some sea cucumbers the tubules do not become sticky nor do they elongate, but the exudates are very toxic to both fishes and mammals. Yet some

others do not have the cuverian glands at all. They release the toxin through their body walls[2]. It is reported that the extracts of these animals or their cuverian tubules possess various pharmacological properties such as toxicity[3], antiviral[4], antifungal[4], cytotoxic[5], hemolytic[6,7], piscicidal[6,7] and antitumor activities.

As part of our investigation of marine flora and fauna from the Indian Ocean area for bioactive compounds, we decided to investigate the secondary metabolites of the sea cucumber, *Actinopyga mauritiana* from Lakshadweep waters. Our preliminary pharmacological studies indicated its MeOH extract to have hypotensive[8], antimicrobial[8], anti-amoebic[9] and hypoglycemic[9] properties. Besides it was also extremely toxic to mice (LD_{50}=0.825 mg/Kg), the fish *Thelopia* sp. (LD_{50}=7.5 ppm) and brine shrimps (LD_{50}=1.03 mg/ml, 105 min)[8]. Purification of this extract, guided by brine-shrimp bioassay yielded echinoside B (R_f=0.6,$CHCl_3$-MeOH=3:1,50 mg,m.p. 203°C), a triterpene glycoside, previously reported from *Actinopyga echinites*[10] (FIG 1). Echinoside B was highly toxic to brine shrimps (LD_{50}=0.293 mg/ml, 90 min).

The IR spectrum of this compound had absorptions at 3400, 1735, 1230 and 830 cm^{-1}, indicating the presence of hydroxyl, ester or lactone and sulfate groups (FIG 2). Transparency above 210 nm in its UV-Vis absorption spectrum ruled out the presence of any chromophores in it. ^1H and ^{13}C NMR spectra

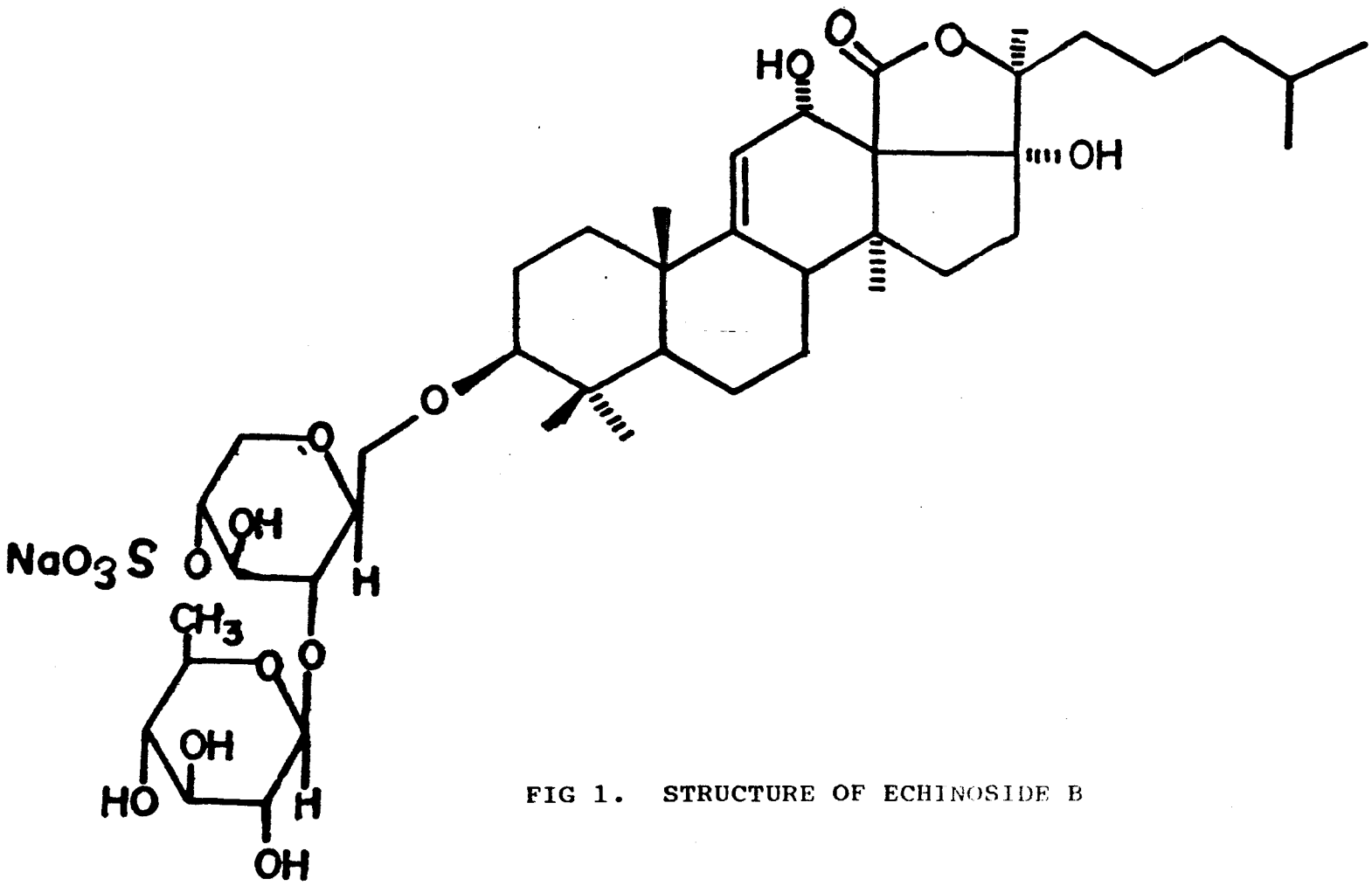

FIG 1. STRUCTURE OF ECHINOSIDE B

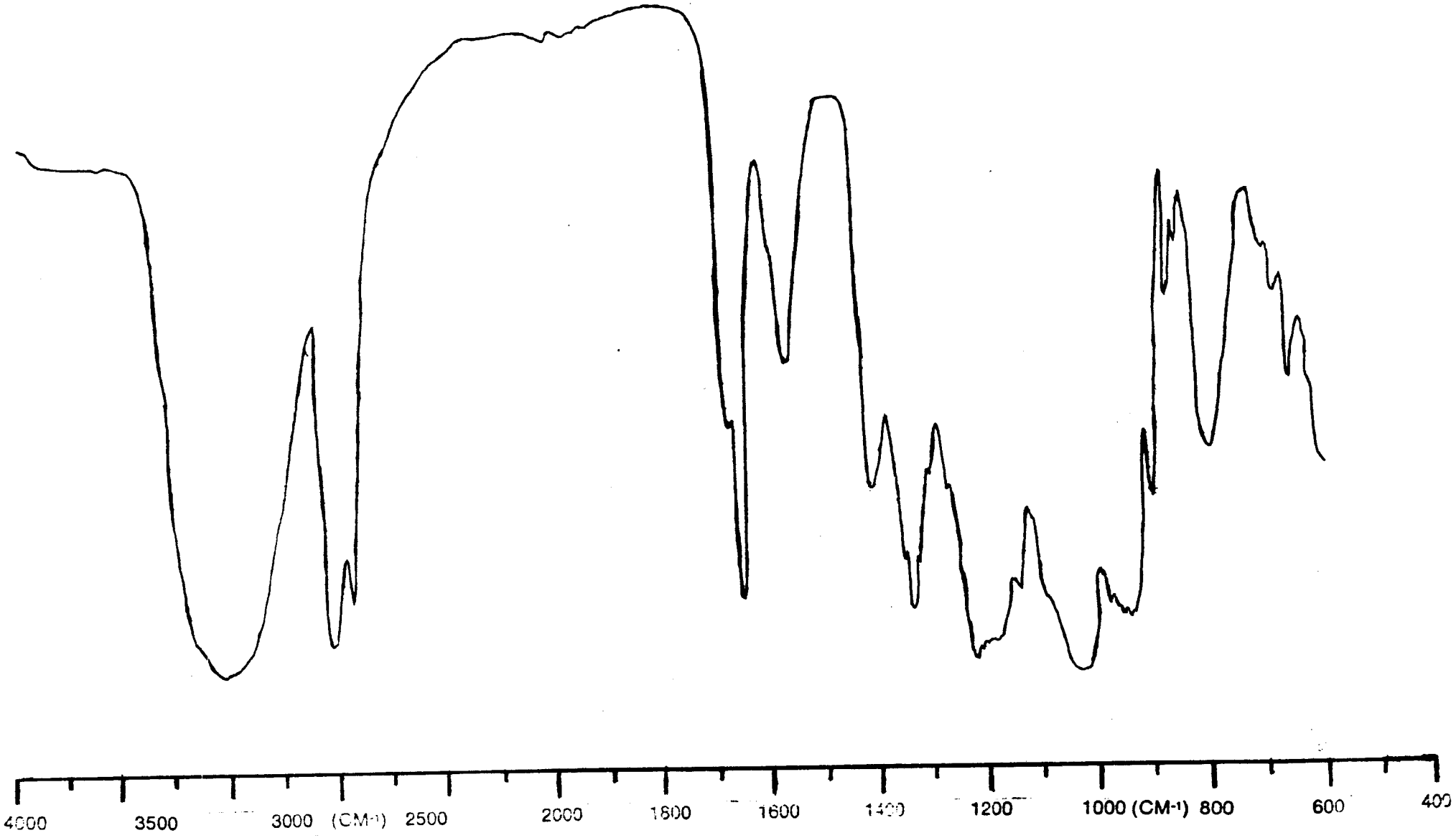

FIG 2. IR(KBr) SPECTRUM OF ECHINOSIDE B

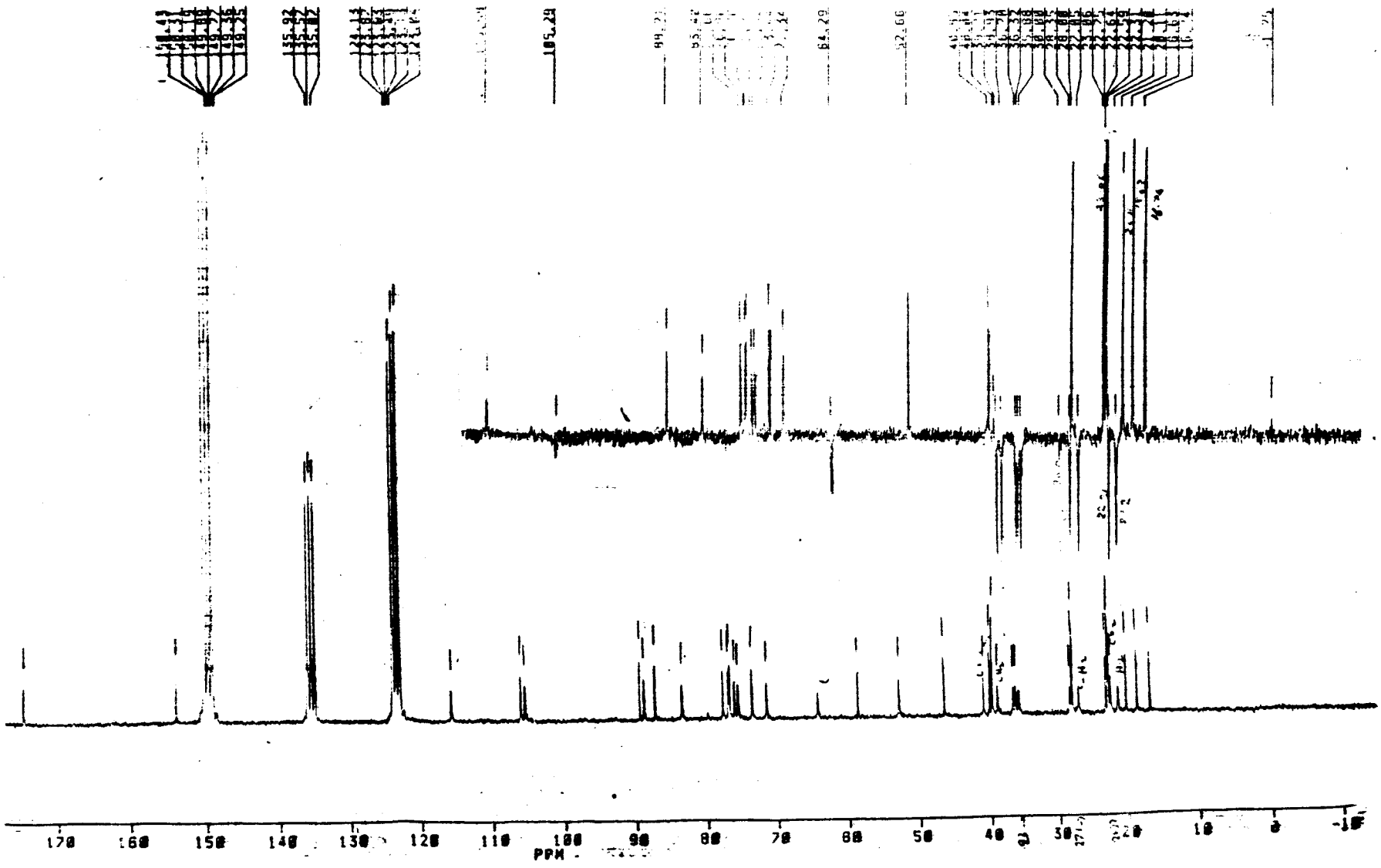

FIG 3. ^{13}C NMR AND DEPT SPECTRA OF ECHINOSIDE B

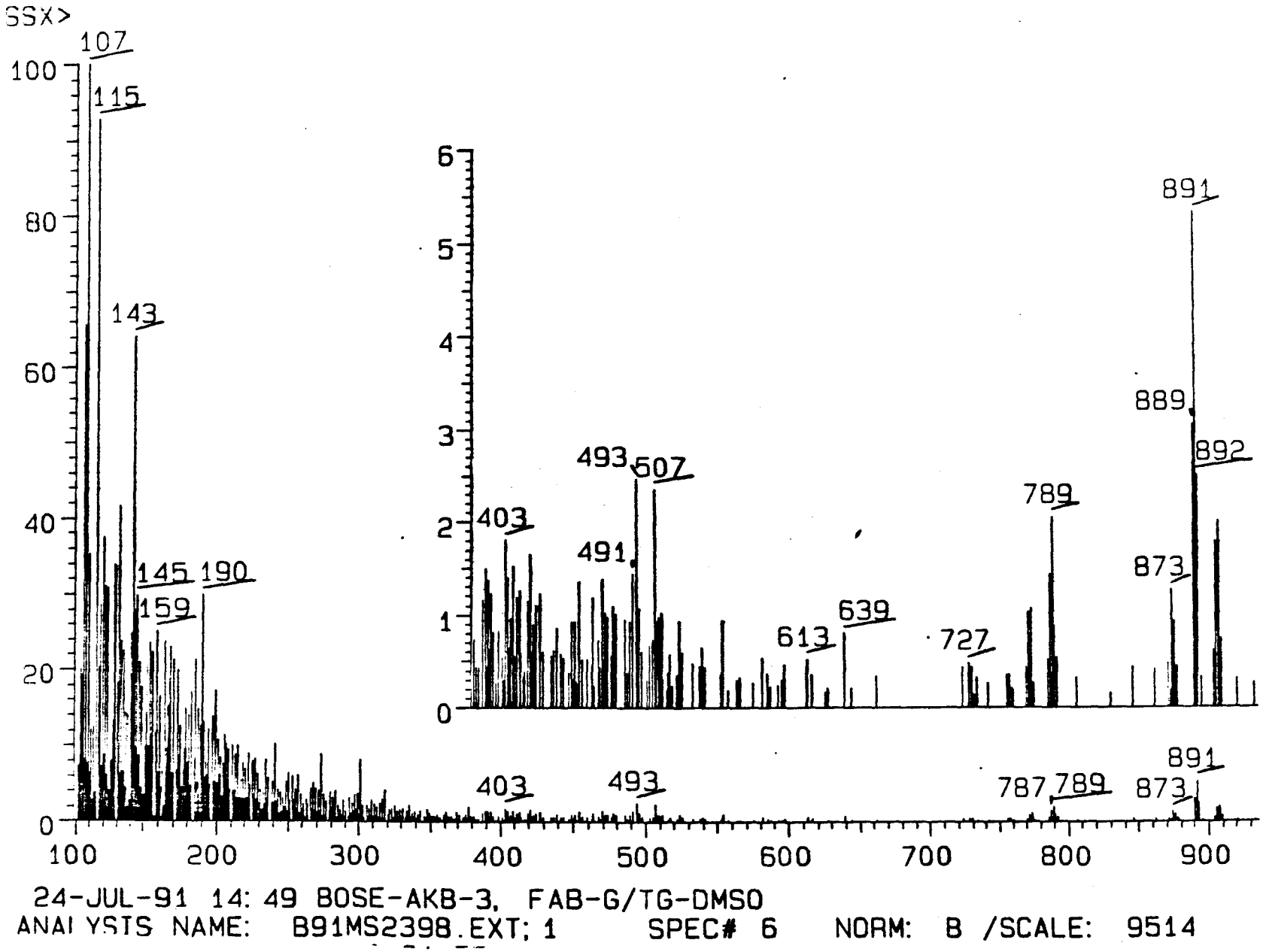

FIG 4. FABMS OF ECHINOSIDE B

revealed its polyhydroxylated / glycosidic nature. The former had several peaks in the region δ 3.3-5.10, while the latter had peaks at δ 174.87(s), 154.12(s), 115.67(d), 105.33 (d) and 105.94(d), and many peaks in the region 64-90 (FIG 3). These values indicated the presence of ester carbonyl, trisubstituted double bond and two sugar moieties in this molecule. The pseudomolecular ion peaks at m/z 891[M+Na]$^+$ and 869[M+H]$^+$ in its FAB mass spectrum indicated its molecular weight to be 868 (FIG 4). Other prominent ions in the FABMS were at m/z 789[M+Na+H-SO_3Na]$^+$ and 771[M+Na-$NaHSO_4$]$^+$, which indicated the presence of a sulfate group in this molecule.

In the EI mass spectrum, the highest peak was observed at m/z 470[M-sugar-H]$^+$. This was followed by the prominent peaks at m/z 452[470-H_2O,51]$^+$, 437([470-H_2O-CH_3]$^+$,100), 411([470-CO_2-CH_3]$^+$, 50), 393(437-44,82), *etc.*, indicating the loss of H_2O, CH_3, and CO_2 groups. Comparison of the above spectral data with the literature values for similar compounds established its structure as 3-O-(2'-O-ß-D-quinovopyranosyl-ß-D-glucopyranosyl)-holost-9(11)-en-3ß,12α,17α-triol-4'-OSO_3Na, (echinoside B 1)[10] (FIG 1).

EXPERIMENTAL

The animals (5 kg) were collected from Kavaratti island in Lakshadweep Sea. They were immediately cut into pieces and soaked in MeOH and transported to the laboratory. After about 2 weeks, the solvent was drained off, and concentrated to dryness (187 gm, LD_{50}= 1.03 mg/ml in brine-shrimp assay). The extract was resuspended in MeOH and stirred well. Part of the extract dissolved in the MeOH. The resulting suspension was then filtered under vacuum and the brine shrimp bioassay repeated with the MeOH soluble and insoluble fractions. It was found that the toxic compounds are more concentrated in the MeOH soluble fraction (64 gm) (LD_{50}=0.66 mg/ml, 120 min and 0.33 mg/ml, 240 min). This was further purified on a short celite column, the active compounds being eluted using $CHCl_3$-MeOH (1:1 & 1:2) systems (10 gm, LD_{50}=0.344 mg/ml, 10 min, all animals dead in 50 min).

Thin layer chromatography of the above toxic fraction indicated it to be a mixture of many glycosides. Echinoside B was isolated from this by chromatography over silica gel using gradient $CHCl_3$-MeOH solvent systems ($CHCl_3$-MeOH=3:1, R_f=0.6, LD_{50}=0.293 mg/ml, 90 min).

Echinoside B, m.p. 203°C, IR(KBr): 3400, 1730, 1625, 1230, 1050 and 830 cm^{-1}; UV-Vis(MeOH): Transparent above 210 nm; ^1H NMR(Py-d_5): δ 5.616(1H,d,J=5Hz,11-H), 5.01(1H,d,J=5Hz,12ß-H);

4.816(1H,d,7.5Hz,1-H_{xyl}),4.746(1H,d,1-H_{Qui}),4.316(1H,brt,3α-H), 4.086(2H,m), 3.766 (2H,m), 3.366(1H,m), 3.156(1H,m), 1.76 (3H,s,21-H), 1.68(3H,s,28-H),1.38(3H,s,19-H),1.30(3H,s,29-H), 1.14(3H,s,30-H) & 0.846(6H,d,J=7Hz,26 & 27-H); ^{13}C NMR(Py-d_5): δ 174.87(s,C-18), 154.12(s,C-9), 115.67(d,C-11), 105.94(d, Xyl-1), 105.33(d,Qui-1), 89.39(s,C-17), 88.71(d,C-3),87.26(s, C-20), 83.4(d,Xyl-2), 77.66(d,Qui-3), 76.90 (d,Xyl-3), 76.70 (d,Qui-3),75.95(d,Qui-2),75.52(d,Xyl-4), 73.44(d,Qui-5), 71.34(d,C-12), 64.29(t,Xyl-5), 58.67(s,C-13), 52.79(d,C-5), 46.46(s,C-14), 40.85(d,C-8), 40.13(s,C-4), 39.78(s,C-10), 38.93(t, C-24), 36.7(t,C-22), 36.32(t,C-1), 35.88(t,C-16), 28.42(d,C-25), 28.17(t,C-16), 28.07(q,C-29), 27.05(t,C-2), 23.12(q,C-21), 22.81(q, C-26), 22.71(q,C-28), 22.66(q,C-27), 22.36(t,C-23), 21.20(t,C-6), 20.16(q,C-19), 18.67(q,Qui-6) and 16.74(q,C-30); FABMS m/z: 891 $[M+Na]^+$, 869$[M+H]^+$, 789 $[M+Na+H-NaSO_3]^+$, 771$[M+Na-NaHSO_4]^+$, 610$[M+Na-CH_3-H\text{-}qui]^+$, *etc*; EIMS m/z(%): 488(Nil,Aglycone), 470($[M-C_{11}H_{18}SO_{12}Na-H]^+$,24), 452 $[470-H_2O,51]^+$, 437($[470-H_2O-CH_3]^+$,100), 411($[470-CO_2-CH_3]^+$,50), 393(437-44,82),383($[470-87]^+$,44),375(393-18,17.6),350(383-33, 65), 323($[470-H_2O-128(sc)-H]^+$,16.5), 297($[470-H_2O-sc-CO+H]^+$, 40.5) and 281($[470-H_2O-sc-CO-CH_3]^+$,38.39).

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summary

Part of chapter 1 of this thesis is devoted to some novel instrumental techniques such as TLC-CIMS, TLC-O_3-CIMS developed for structure-determination of organic compounds. Besides, this chapter also deals with the development of a new method for estimation of fatty acid mixtures. Accordingly, the mixture of acids were converted into their *p*-nitrobenzyl esters in a facile manner which were then analysed by either HPLC or NCIMS techniques.

Chapters 2-6 deal with the chemistry and pharmacological properties of marine natural products isolated from the brown alga *Padina tetrastromatica*, sponges *Ircinia ramosa*, *Haliclona* sp. and *Tedania anhelans*, soft coral *Lobophytum strictum* and sea cucumber *Actinopyga mauritiana*.

Several known compounds including fatty acids, sterols and terpenoids were isolated from these organisms. Some of the more important secondary metabolites isolated are galactitol, 7-ketocholesterol and three halogenated norsesquiterpenoids from the sea weed *P. tetrastromatica*, several 3ß-hydroxy-Δ^5-sterols, their 3-keto-Δ^4-analogs, 7-hydroxy and 7-ketosterols from the sponge *I. ramosa*, a sphingosine derivative, 24(28)-didehydroaplysterol and several isoquinoline alkaloids from the sponge *Haliclona* sp., four diketo piperazines, some

deaminated aminoacids, ß-carboline and two heteroaromatic acids from the sponge *T. anhelans*, several monohydroxy and two polyhydroxy sterols besides two simple amine derivatives, *viz.*, diacetonamine and triacetonamine hydrochlorides from the soft coral *L. strictum* and echinoside B, a toxic triterpene glycoside from the sea cucumber *A, mauritiana*.