CHEMICAL INVESTIGATIONS OF HETERONEMA ERECTA AND AN UNIDENTIFIED SPONCE FROM THE RED SEA

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CHEMICAL INVESTIGATIONS OF *HETERONEMA ERECTA* AND AN UNIDENTIFIED SPONGE FROM THE RED SEA

by

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ABSTRACT

Chemical Investigations
of
Sponges from the Red Sea
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Dejene Shewaye
Research Advisor: Dr. Tarekegn Gebreyesus

Heteronemin, a pentacyclic sesterterpene has been characterized from the chloroform extract of an unidentified sponge sample collected from Massawa, Red Sea. The structure was established by ^{1}H nmr, ^{13}C nmr, and mass spectral interpretation.

Another C_{29} compound characterized as β-sitosterol, has been isolated from the chloroform extract of another unidentified sponge sample, collected from the same locality as the first one. The structure of the sterol was characterized by spectral interpretation, and comparing its physical constants with authentic sample.

1. INTRODUCTION

Sponges are primitive multicellular animals which are almost exclusively of marine origin. They are sessile, with only few specialized organs and tissues in which skeletal fibres or spicules usually form an important part of the body. The animals live attached to stones, shells, and other objects in water, and often form colonies of irregular form in which the various individuals are indistinguishably fused with one another.

Sponges are usually attached and stationary animals in the adult stage, distribution being brought about largely by the actively swimming ciliated larvae or by currents of water which carry the young from place to place before they become attached. The thousands of different species vary greatly in shape, size, structure, and geographical distribution. They were for centuries considered to be plants.[1]

Sponges are not easy to classify, but are arranged in four classes as follows.[2]

1) Calcarea
2) Hyalospongiae
3) Demospongiae
4) Sclerospongiae

Sponges are filter feeders, removing bacteria and other fine suspended organic matter from the water. An initial screening is provided by the pores, which permit only very small particles to enter. Filter feeding has evolved in many animals and often represents, as in sponges, an adaptation to a sessile or slow moving existence.

Inspite of their soft structure and stationary nature sponges survive the pressure from predators. Some sponges seem to derive protection from the presence of sharp spicules or tough fibrous components. Many species, however, grow exposed and are devoid of physical means of defence. It has been repeatedly suggested that they are protected from predation by toxic or noxious chemicals.[3] Only a few highly specialized fishes select these sponges as their main diet.

It is interesting to note that some sponges that produce toxic chemicals are nevertheless eaten by specific nudibranchs and molluscs. Some nudibranchs are able to store toxic chemicals produced by sponges and use them for their own defence against predators. A similar grazer-prey relationship has also been demonstrated between molluscs and algae.[4] It is premature to conclude at this point, that sponges produce secondary metabolites only as a self defence. Further studies are imperative.

Sponges could produce secondary metabolites for various reasons, all of which are not clear so far. Marine natural product chemistry deals with the isolation and structural elucidation of these metabolites. The positive feature of marine natural product chemistry is the desire to determine the function of new compounds in the marine environment.[5]

Because many sponges contain symbiotic microorganisms, there is always some uncertainity concerning the true origin of sponge metabolites.[6] Efforts have been made by

chemists to know the origin of these metabolites. However the majority of metabolites are now believed to be produced by sponges themselves.[6]

Recent studies [7,8] have shown that in tropical waters, 60-70% of the exposed sponges are toxic to gold fish in laboratory tests. The toxicity of sponges is inversely related to latitude;[7] an observation explained by the increased species diversity in tropical shallow water fish, resulting in a more intense food competition and hence the natural selection of chemically protected species.

Although a great number and variety of secondary metabolites have been isolated from both toxic and non-toxic sponges, few data have been published concerning the ecological role of these compounds. Recently however several marine chemists have reported the toxicity or antifeeding activity of sponge metabolites.[9-11]

Marine natural product chemistry is a relatively recent field of study as compared to natural product from other sources. Once it started, the development has become dramatic. Inspite of a consistant effort to disclose the secrets of life under the sea, the chemist sometimes faces problems which are difficult if not impossible to solve. One of the major problems he faces is that his ability to identify the compounds exceeds his ability to identify the marine organisms from which the compounds are obtained. This is particularly true for the identification of sponges, since expert taxonomists often fail

to agree.[12,13] This problem entails the difficulty to name a new compound from an unknown organism. So far some marine natural products have been given irrelevant names.[4] Inspite of this and other associated problems such as diving into the sea to collect samples, there are reports of sponge metabolites from various localities. But exhaustive studies have not been made especially on Red Sea sponges.

The Red Sea is located in a tropical region. It is rich in marine organisms and is peculiar in that its salinity is very high, with pH range of 7.5-8.5.[14]

Even though sponges are primitive multicellular animals they elaborate secondary metabolites of diverse nature. They produce terpenes of all type, steroids, pyrroles, indoles, polyacetylenes and peroxides.[3-17] Because of competition for survival, sponges in the tropical region are expected to produce metabolites of diverse nature, which serve the purpose of natural selection.

In the course of this research work, the chemical investigation of two unknown Red Sea sponges have been carried out. These sponge samples have yielded several fractions of which the major constituents are a pentacyclic sesterterpene and a steroidal compound as characterized by spectroscopic techniques. Lately towards the end of the work, one of the sponge samples which elaborate the sesterterpene was identified as Heteronema erecta.

2. BACKGROUND

2.1 Terpenoids

Compounds derived from the five carbon isoprene unit are among the most widely distributed in nature, more than 4000 having been isolated by 1974.[18] These isoprenoid compounds fall mainly into the classes of monoterpene (C_{10}), sesquiterpene (C_{15}), diterpene (C_{20}), sesterterpene (C_{25}), triterpene (C_{30}), carotenoids (C_{40}) and steroids.

Unlike steroids which are abundantly distributed in nature especially in marine organisms, naturally occurring sesterterpenoids were first encountered less than thirty years ago, and as a consequence they are often viewed as a rare category of natural products. Recently, an increasing number of linear and cyclic sesterterpenes have been reported. Many of the latest sesterterpenes have been obtained from sponges of the order Dictyoceratida.[17] The genera Ircinia, Spongia, Hippospongia and Cacospongia have yielded an array of sesterterpenes and a series of C_{21} terpenes postulated as degraded sesterterpenes.[19]

The sponge species which have yielded sesterterpenes and other isoprenoid compounds contain little, if any, sterols. This could imply that, in general, the order Dictyoceratida produce mevalonate derived compounds primarily by head to tail addition and that the tail-to-tail dimerization of two farnesyl residue is not a favoured process.[17]

Linear sesterterpenes have been isolated from various Ircinia species,[5,19] while tetracarbocyclic sesterterpenes often referred to as the scalaranes have been isolated from Cacospongia species.[19] Scalaranes are not distributed widely, even among sponges; they are unique to sponges of certain species.

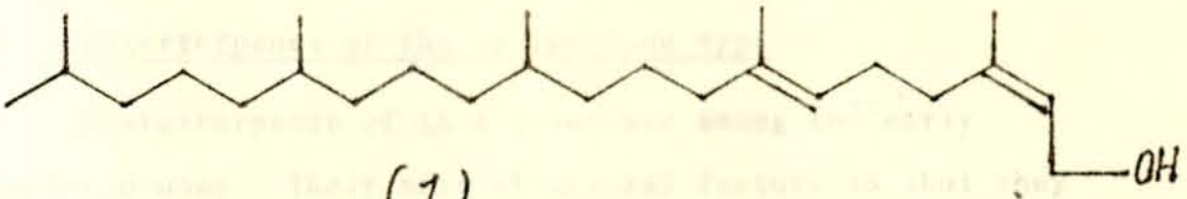
Reports on the discovery of novel sesterterpene are increasing. In 1972 only 13 sesterterpenoid compounds were known which represented one acyclic and two carbocyclic frame works. Within three years this number grew to 31.[18] Since then there have been reports of numerous other sesterterpenoids with a total count of 158 by 1985.[20]

2.1.1 Classification of Sesterterpenes

A number of trials have been made to calssify these sparsely distributed terpenoids, among which the first classification made by G.A. Cordell[18] and that made by Minale[20] are the most important ones. Since the latter classification is very detailed, for the purpose of this presentation, the former is adopted. Accordingly sesterterpenes are generally classified into five groups based on their structural relationship.

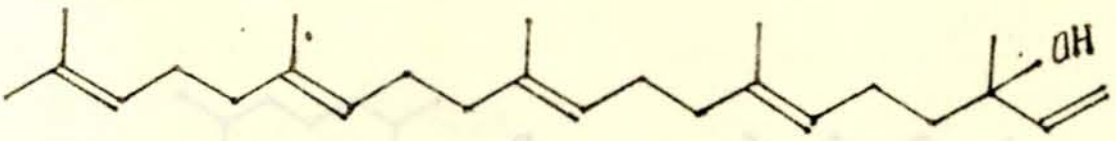
a) Sesterterpenes of the linear type

In 1969 M. Toyoda et al isolated by silicagel chromatography of unsaponifiable liquid extract of Solanum tubersum, a pale yellow liquid.[21] The isolated compound has a linear structure (1) as identified by spectroscopic techniques.



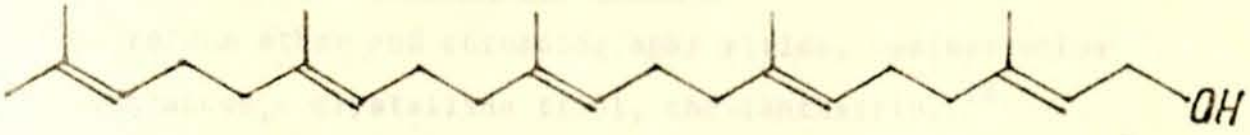
(1)

The first acyclic sesterterpene isolated, however, was all trans geranyl nerolidol (2) obtained from Cochliobolus heterostrophus,[22] the phytopathogenic fungus responsible for the leaf spot disease in maize.



(2)

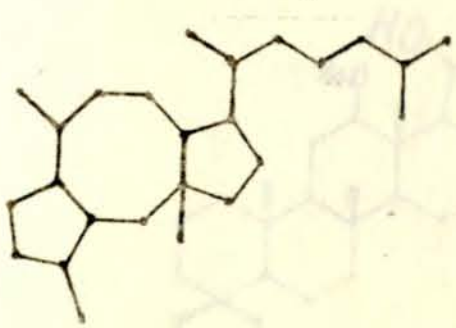
A closely related compound, geranyl farnesol (3) was isolated from the wax of the insect Ceroplastes albolineatus.



(3)

b) Sesterterpenes of the ophiobolane type

Sesterterpenes of this group are among the early reported ones. Their main structural feature is that they are based on 5-8-5 ring system (4). Ophiobolin-A (5), a compound obtained from the fungus responsible for the Helminthosporium leaf spot disease of rice, was the first in the series to be isolated; although the structure was not determined for about eight years. Its complete tricarbocyclic skeleton along with the absolute stereochemistry at the eight chiral sites was elucidated in 1965 by X-ray studies.[23]



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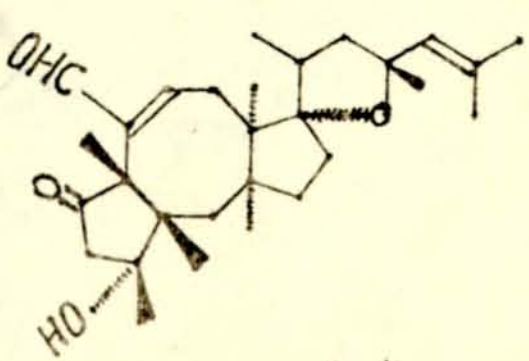
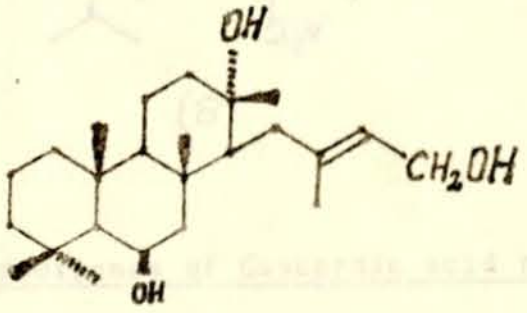


(5)

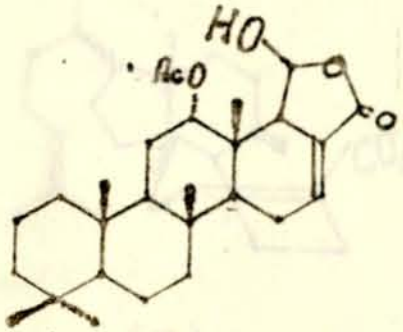
c) Sesterterpenes of cheilanthatriol types

The fern, Cheilanthes farinosa on extraction with petroleum ether and chromatography yields, besides other substances, a crystalline triol, cheilanthatriol.[24] Cheilanthatriol (6) is a tricarbocyclic compound. Biogenetic consideration and the dissimilarity to the ophiobolane type suggested a perhydrophenanthrene skeleton.



(6)

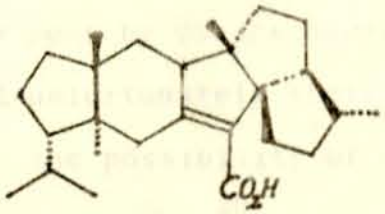
Another sesterterpene classified as cheilanthatriol type was isolated from the sponge, Cacospongia scalaris. This is a white crystalline compound with structure (7) to which the name scalarin was given.[25]



(7)

d) Sesterterpenes of the Ketigeranic acid type

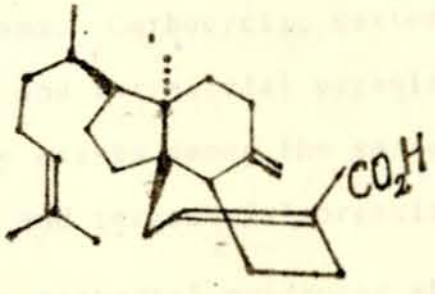
Shibata et al[25] isolated from lichen, Lobaria retigera, a compound whose structure was determined by X-ray crystallographic analysis (8) which indicated the pentacyclic structure.



(8)

e) Sesterterpenes of Gascardic acid type

Gascardic acid (9) was isolated from secretion of the insect Gascardia madagascariensis. Elegant and extensive degradative work by Arigoni's group in Zurich succeeded in elucidating the correct structure of this molecule, including the absolute stereochemistry.[18]



(9)

2.1.2 Occurrence of Sesterterpenes

Surprisingly there has been no systematic effort made to determine how the sesterterpenoids are distributed in nature. Nothing is known about their taxonomic distribution in plants or phytopathogenic fungi, or their role in the life cycle of organisms. Their discovery to date has essentially been by chance observation rather than by design, and unfortunately their structural diversity seems to preclude the possibility of a single screening test to detect their presence.[20]

As can be seen from the literatures reported so far, the range of terrestrial organisms which elaborate sesterterpenes are considerably greater than the range of marine organisms. Marine sesterterpenes have been isolated mainly from sponges, the exception being seven metabolites restricted to nudibranchs, and they may actually be derived from the sponge diets of the nudibranchs.[20]

Acyclic sesterterpenes are abundant in marine sponges but are poorly represented in each major group of terrestria organisms. Carbocyclic sesterterpenes are found in both marine and terrestrial organisms, but strikingly little overlap exists among the sesterterpene skeletons from marine and terrestrial organisms.[20]

Experimental evidences show that certain terrestrial organisms elaborate cyclic sesterterpenes by cyclization of normal head-to-tail condensed isoprenoid precursors.

By contrast no information is available on the biosynthesis of either cyclic or acyclic sesterterpenes in marine sponges or nudibranchs. In fact it has been suggested that sponges are probably not capable of de-novo terpene synthesis.[27]

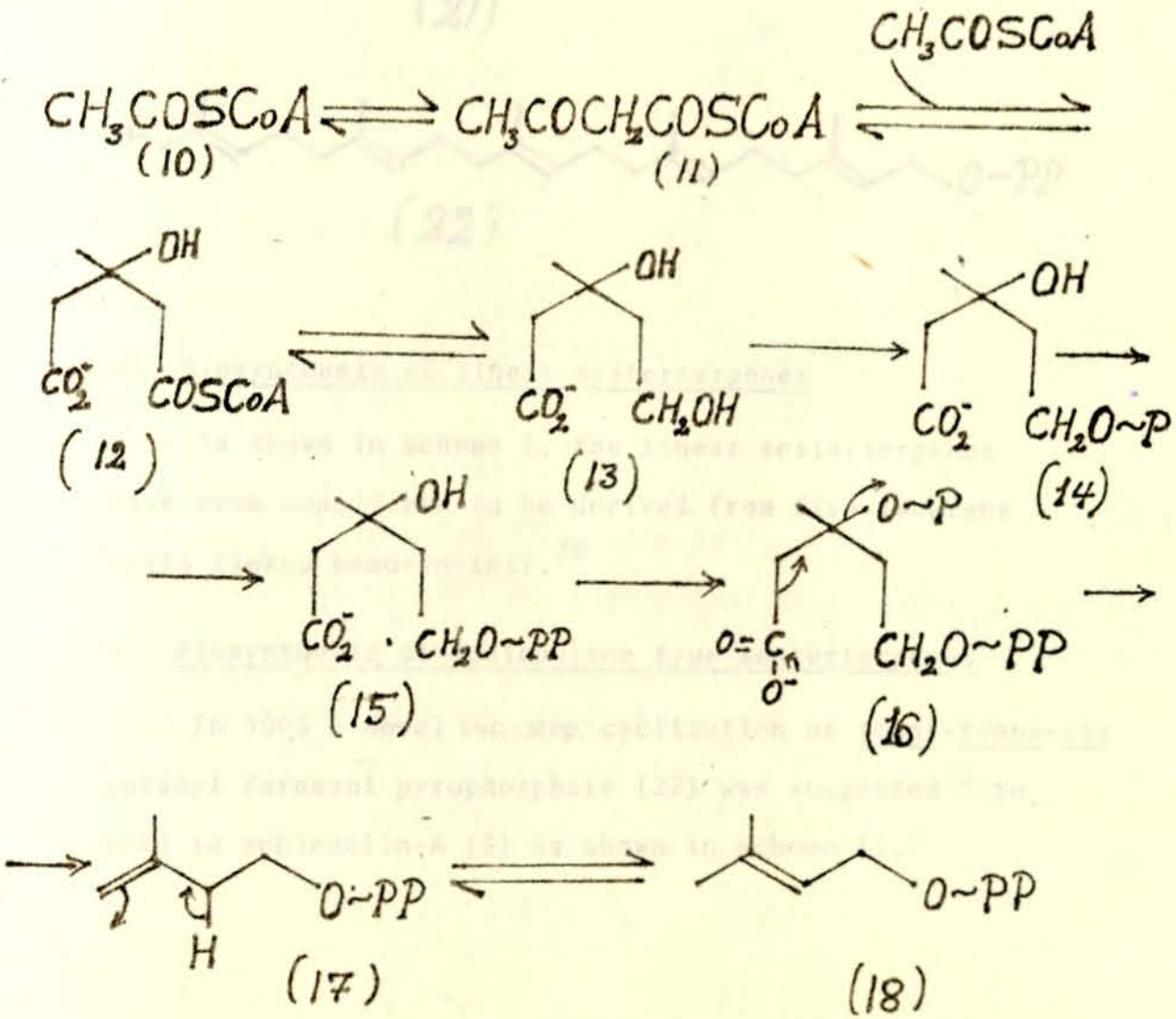
2.1.3 Biosyntheses of Sesterterpenes

It is to be expected that support for the biogenetic isoprene rule will come most directly from the invivo studies for the synthesis of terpenoid compounds in animal and plant system.[18]

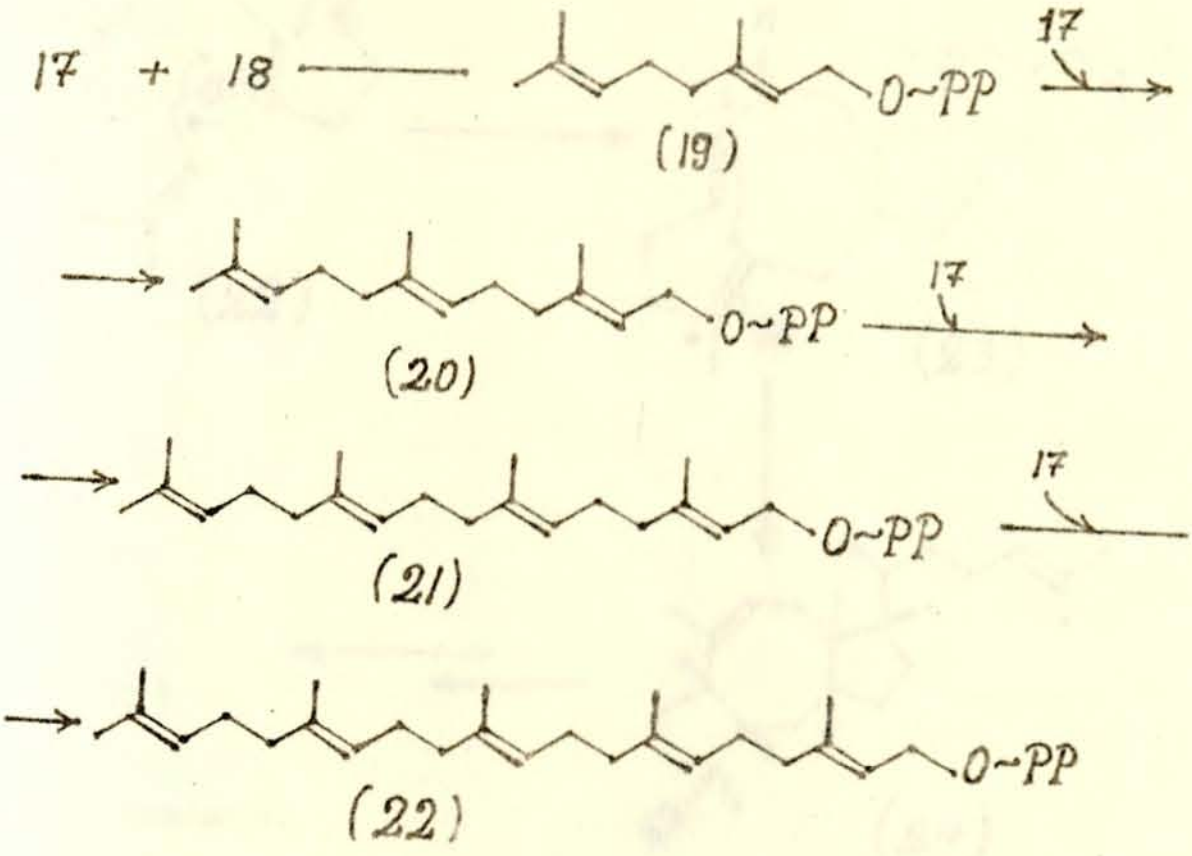
Compared with, for instance, biosynthetic studies in alkaloids, invivo studies in the terpene field have so far not been numerous. The reason for this is partly to be found in the formidable experimental difficulties met in attempts to incorporate the simple precursors of terpene biosynthesis (acetic acid and mevalonic acid) into higher plants. The most effective investigations for the biosynthesis of terpenes have been concerned with fungal metabolites.[18,20]

The discovery of the sesterterpenes has brought forth another area of experimentation, and experimenters quickly realized this. "Indeed knowledge of the biosynthesis of the sesterterpenes is surprisingly advanced for such a rare group of compounds. Clearly the fact that many of these compounds can be isolated from culture media has been of significant value.[18]

The basic precursor for all terpenoids and steroids is acetyl CoA, which inturn is obtained from acetic acid. Acetic acid in an organism is produced in the catabolic process; i.e. in the process of degradation of fuel molecules to generate energy required for life activities. The fuel molecules consist of carbohydrates, (which are produced in plants by the process of photosynthesis) fats and proteins (which are produced from carbohydrate by various biochemical conversions).



Scheme I

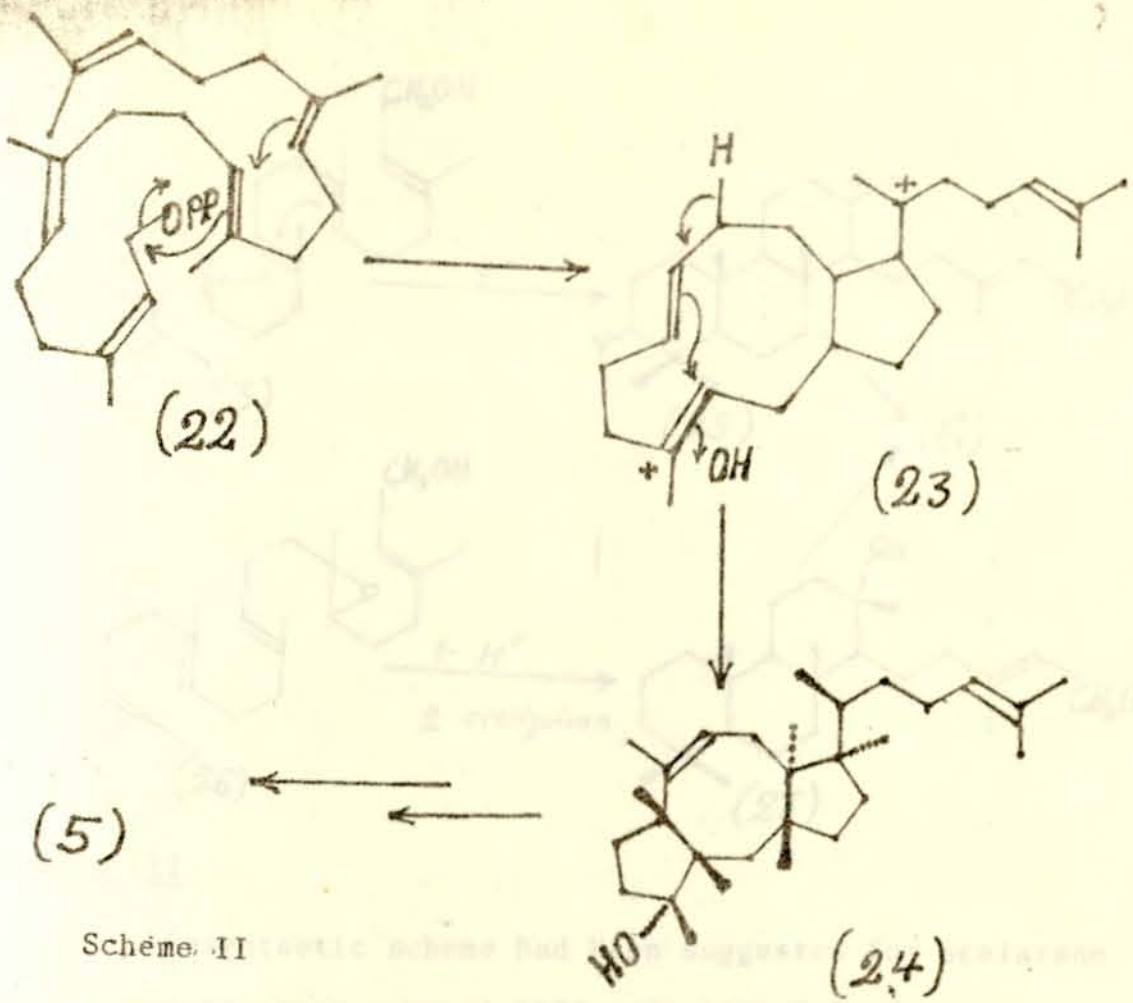


a) Biosynthesis of linear sesterterpenes

As shown in scheme I, the linear sesterterpenes have been considered to be derived from five isoprene units linked head-to-tail.[28]

b) Biosynthesis of Ophiobolane type sesterterpenes

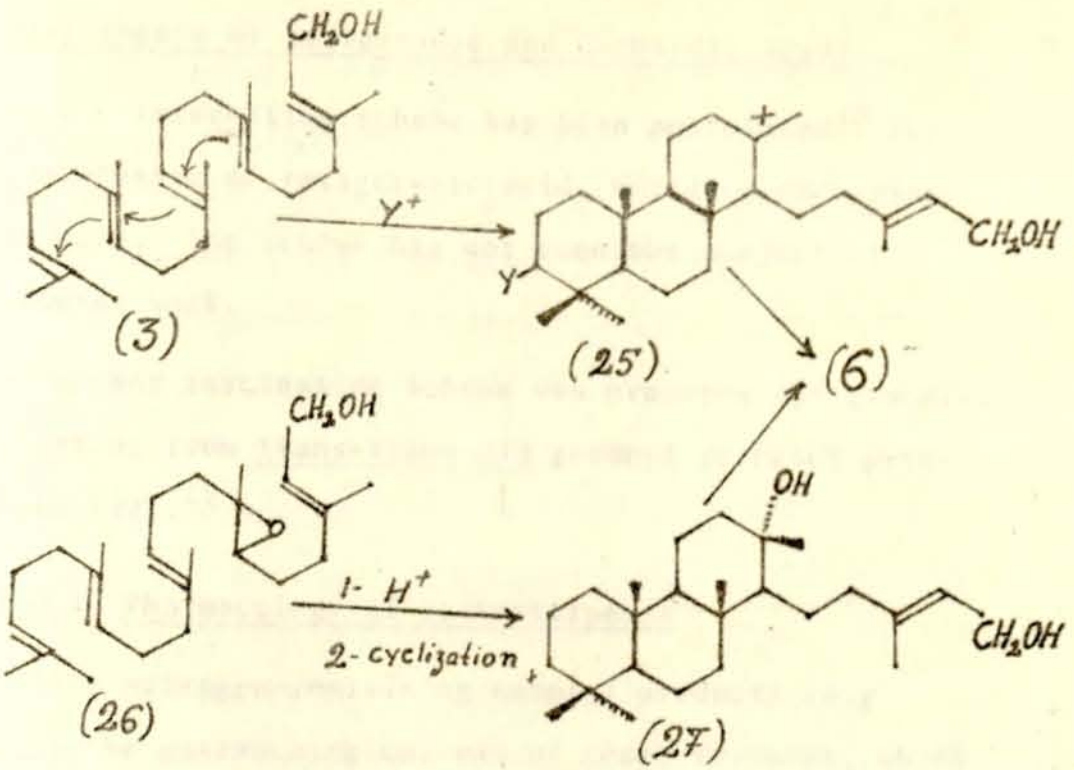
In 1965 a novel two-step cyclization of trans-trans-cis geranyl farnesol pyrophosphate (22) was suggested[23] to lead to ophiobolin-A (5) as shown in scheme II.



Scheme II

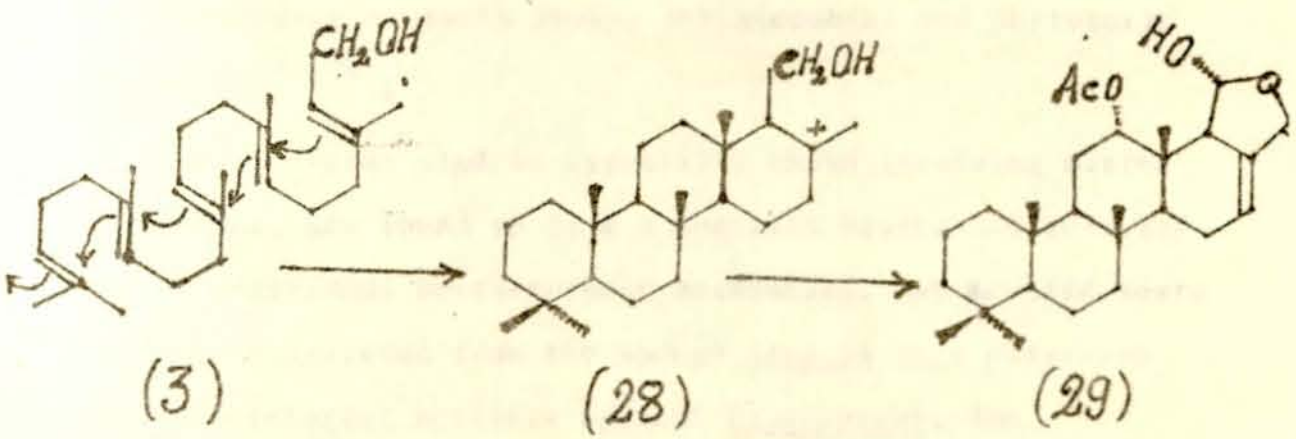
c) Biosynthesis of cheilanthatriol and scalarane type sesterterpenes

The biosynthesis of cheilanthatriol is speculated[18] to take place from geranyl farnesol (3) by standard steroid type cyclization to give the cation (25) which picks up a hydroxyl ion. An alternative cyclization may begin with the epoxy-geranyl farnesol derivative (26) which spontaneously cyclizes upon protonation as shown in scheme III.



Scheme III

No biosynthetic scheme had been suggested for scalarane type sesterterpenes until 1974. In 1974 G.A. Cordell suggested[18] that, formation of all four rings takes place in a concerted fashion from all trans-geranyl farnesol (3); oxidation then affords scalarin (29) as shown in scheme IV



Scheme IV

d) Biosyntheses of Retigeranic and Gascardic acids

A very interesting scheme has been postulated[25] for the biosynthesis of retigeranic acid, building each ring sequentially. The scheme has not been the subject of experimental work.

A further fascinating scheme was proposed for gascardic acid starting from trans-trans-cis geranyl farnesol pyrophosphate (22).[25]

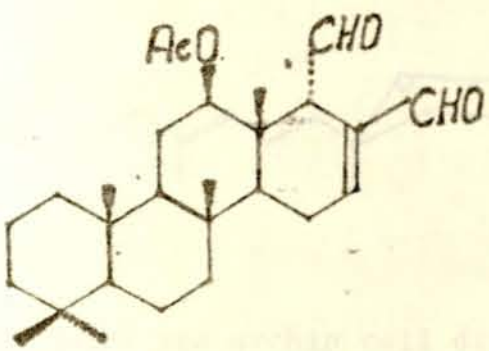
2.1.4 Pharmacology of sesterterpenes

Unlike nitrogen-containing natural products (e.g. alkaloids) the pharmacological use of sesterterpenes, which have oxygen as a prominent heteroatom, is not common. With only a few exceptions each sesterterpene metabolite is oxygenated at one or more locations. Interesting physiological properties have sometimes been described for the extract of organisms which yield sesterterpenes. These range from marine sponges which are toxic,[1,11,29] to nudibranchs with a pleasant fruity odor,[30] and fungi which produce extracts having antimicrobial and phytotoxic activity.[31,32]

Many recent studies especially those involving marine organisms, are found to link a specific biological activity to an individual sesterterpene metabolite. An acyclic sesterterpenoid isolated from the sponge Ircinia oris possesses an antibacterial activity against Diplococcus, and

Staphylococcus aureus.[33] Another acyclic compound isolated from Ircinia variabelis found to possess antibacterial activity against Staphylococcus aureus.[34] 12, 18-diepiS-calaradial (30), isolated from Spongia idia is found to be toxic to numerous predatory marine organisms including a sea star, abalone larvae and brine shrimp.[11] Among scalaranes, a tetracyclic nor-sesterterpene isolated from the sponge Hyrtios erecta[35] and a tetracyclic homosester-terpene isolated from Carteriaspongia dendyi[36] were found to have antifungal and antiinflammatory activities. Antibacterial activity is the most commonly recorded property of many sesterterpenes, but antiinflammatory activity and inhibition of cell division has also been reported.[37]

According to a report by P. Crews and S. Naylar, only two sesterterpenes, ophiobolin-A (5) (as an antibiotic) and monoalide (31) (as an antiinflammatory agent) have been the subject of patent.[20]



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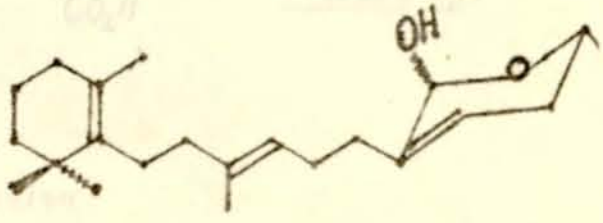


(31)

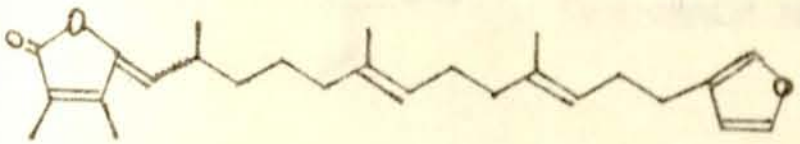
Table 1: Structure of some sesterterpenes from sponges and their biological activity

Compounds | Source | refer

(32)

. Ircinia oros

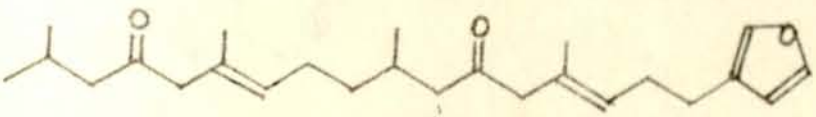
Antibacterial against Diplococcus and S. aureus



(33)

. Ircinia variabilis

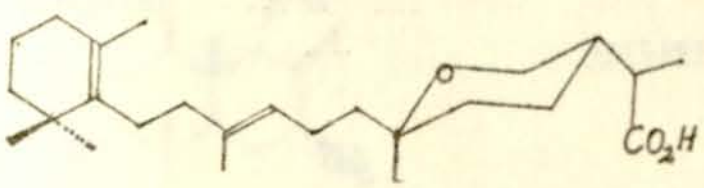
Antibacterial against S. aureus



(34)

Spongia idia

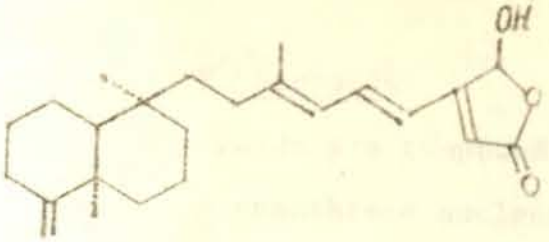
Toxic to numerous preditory marine organisms



(35)

Prianos sp.

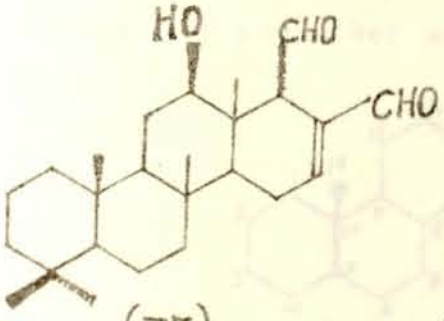
Inhibition of sea urchin cell division



(36)

Unidentified sponge 33

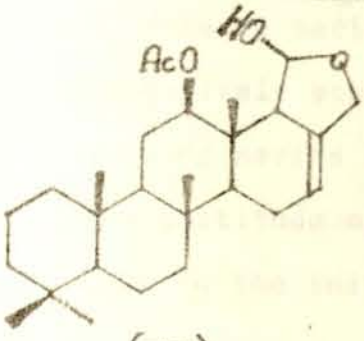
Inhibit growth of S. aureus



(37)

Cacospongia scalaris 66

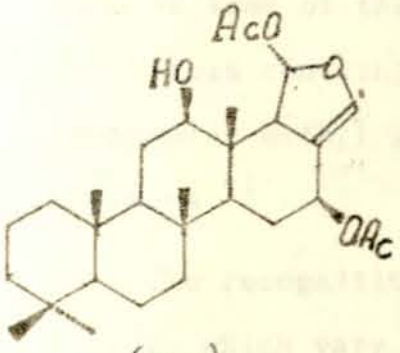
Cytotoxic



(38)

Spongia nitens 11

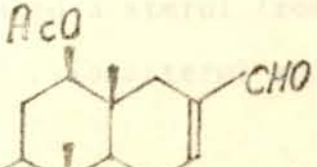
Toxic to brine shrimp



(39)

Hyrtios erecta 17

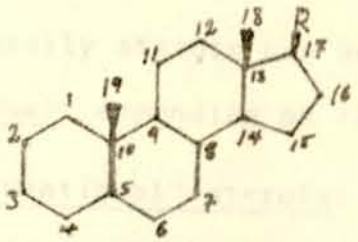
Toxic to brine shrimp



Hyrtios erecta 35

2.2 Steroids

Steroids are compounds containing the perhydrocyclopentanophenanthrene nucleus (41). They include a wide range of naturally occurring compounds, among which are the sterols proper, the bile acids, the sex hormones, the adrenocortical hormones, the cardiac glycosides, the sapogenins, some alkaloids and other minor groups.[40]



(41)

Among marine natural products, steroids are the most extensively studied. A large number of literature concerning marine steroids can be found scattered throughout the multitude of chemical and biological journals dating back to the initial report of Henze in 1908.[41]

Sterols derived from marine sources are interesting, becuase some of them possess very unusual structures such as sterols containing cyclopropane ring, sterols devoid of 19-angular methyl group or unique 3β hydroxymethyl-A-nor-5α-steranes.[41]

The recognition that sponges contain a variety of sterols which vary from one species to another was first reported in a classical paper by Doree.[42] This author isolated a sterol from the sponge Cliona celata which he named clionasterol and showed it to be different from both

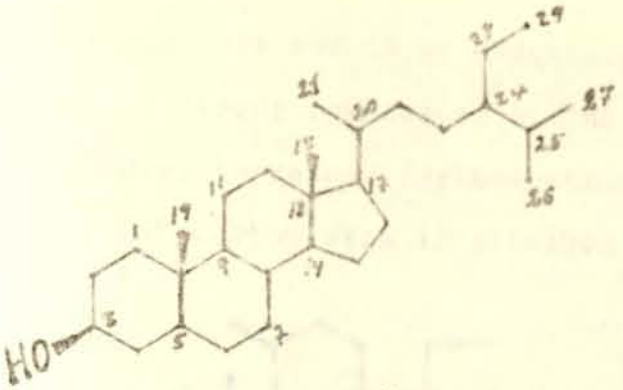
cholesterol and spongosterol. The latter, earlier isolated from a mediterranean sponge,[42] was the first animal sterol to be recognized as different from cholesterol. Since 1972 modern reinvestigations of sterols of sponges have begun to appear, which confirmed the complexity of the sterol compositi in this phylum.

2.2.1 Classification of Sterols

Generally sterols can be classified as "conventional" and "unusual" depending on their structure.[42]

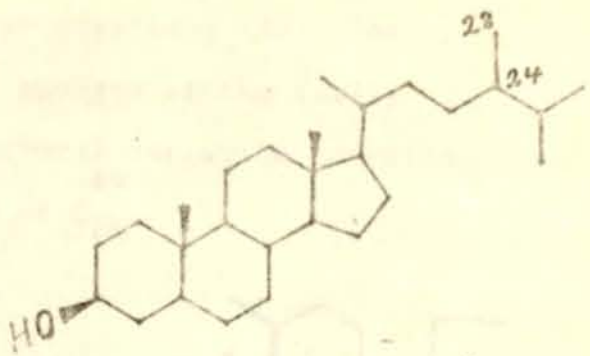
"Conventional" sterols: The term "conventional" is used to cover sterols with the conventional C_{19} tetracyclic nucleus having the usual C_8 side chain orelse modified by the addition of one or two carbon atoms at C_{24}. The C_{24} nor-sterols are also included in this group.

A C_{29} sterol, chondrillasterol (42), which seems to be confined solely to porifera in the animal kingdom, was reported as the major sterol component of Chondrilla nucula.[43] Another C_{29} sterol, β-sitosterol ((24-R)-24-ethyl cholest-5-en-3β-ol) (43) has been isolated from the sponge Cliona celata.[44] Among the C_{28} sterols, 24-methylene cholesterol (44) which is widely distributed in marine organisms was found in the sponges Chalina arbuscala and Tetilla laminaris.[4



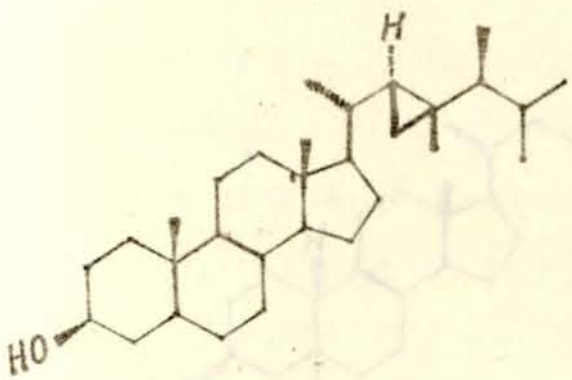
(42); (24R) $\Delta^{7,22}$

(43); (24R) Δ^{5}



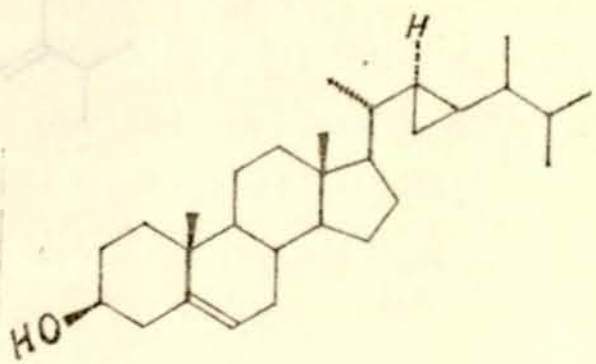
(44); $\Delta^{5,24(28)}$

"Unusual" Sterols: Marine invertebrates have proved to be sources of unusual sterols having the cholesterol nucleus with side chains involving new alkylation patterns. Thus gorgosterol (45) from gorgonians,[46,47] acanthasterol (46) from the star fish, Acanthaster planci and 23-demethyl gorgosterol (47) isolated from two gorgonians,[48] exemplify the attachment of "extra" carbons at C_{22} and C_{23}.



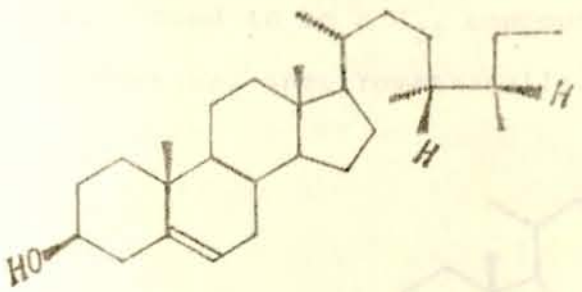
(45); Δ^{5}

(46); Δ^{7}

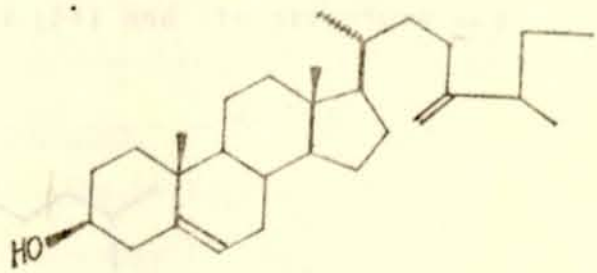


(47)

Aplysterol (48) and 24,28-didehydro-aplysterol (49), the principal sterol components of the sponges of the family verongidae, represent further structural variation in which an "extra" carbon atom is attached at C_{26}.[49]

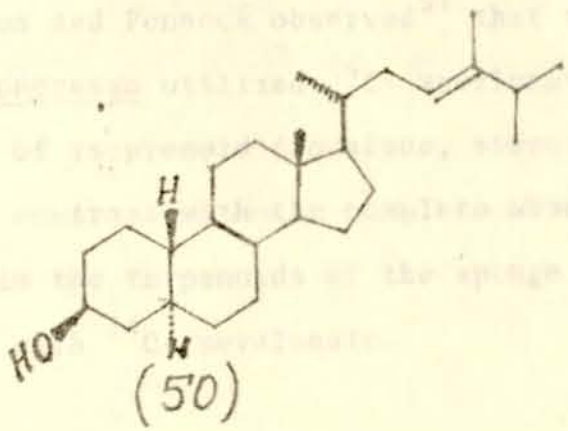


(48)



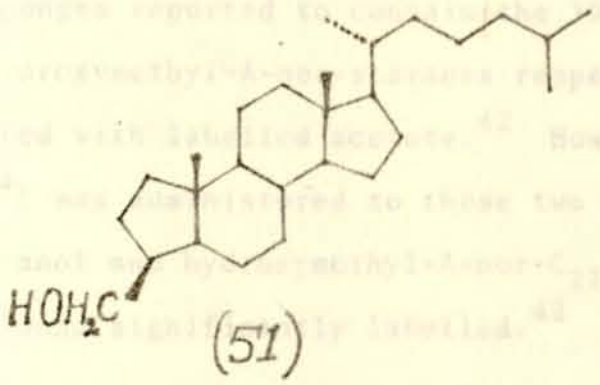
(49)

Modifications of the sterol nucleus have also been found in marine organisms. The sponge Axinella polypoides was found to contain stanol mixtures, having a 19-nor-cholestanol nucleus carrying conventional saturated and monounsaturated C_7 (24-nor), C_8, C_9 and C_{10} side chains.[50] The major resolved component has been fully characterized as 19-nor-5α,10β-ergost-trans-22-en-3β-01 (50)



(50)

The total sterol content of Axinella verrucosa is a mixture of unique stanols with a new 3β-hydroxymethyl-A-nor-5α-cholestane nucleus carrying conventional C_8, C_9 and C_{10} side chains.[42] The major fraction of the mixture was found to be a C_{27} compound (51) and its structure was determined spectrometrically.



(51)

2.2.2 Sterol Biosynthesis in Sponges

Though the knowledge about sterols present in sponges has rapidly increased in the past, the understanding about their metabolism is very meagre.

Walton and Pennock observed[51] that the sponge Grantia compressa utilized ^{14}C- mevalonate for the biosynthesis of isoprenoid (squalene, steroids and ubiquinones) in direct contrast with the complete absence of ^{14}C- radioactivity in the terpenoids of the sponge Suberites domuncula incubated with ^{14}C- mevalonate.

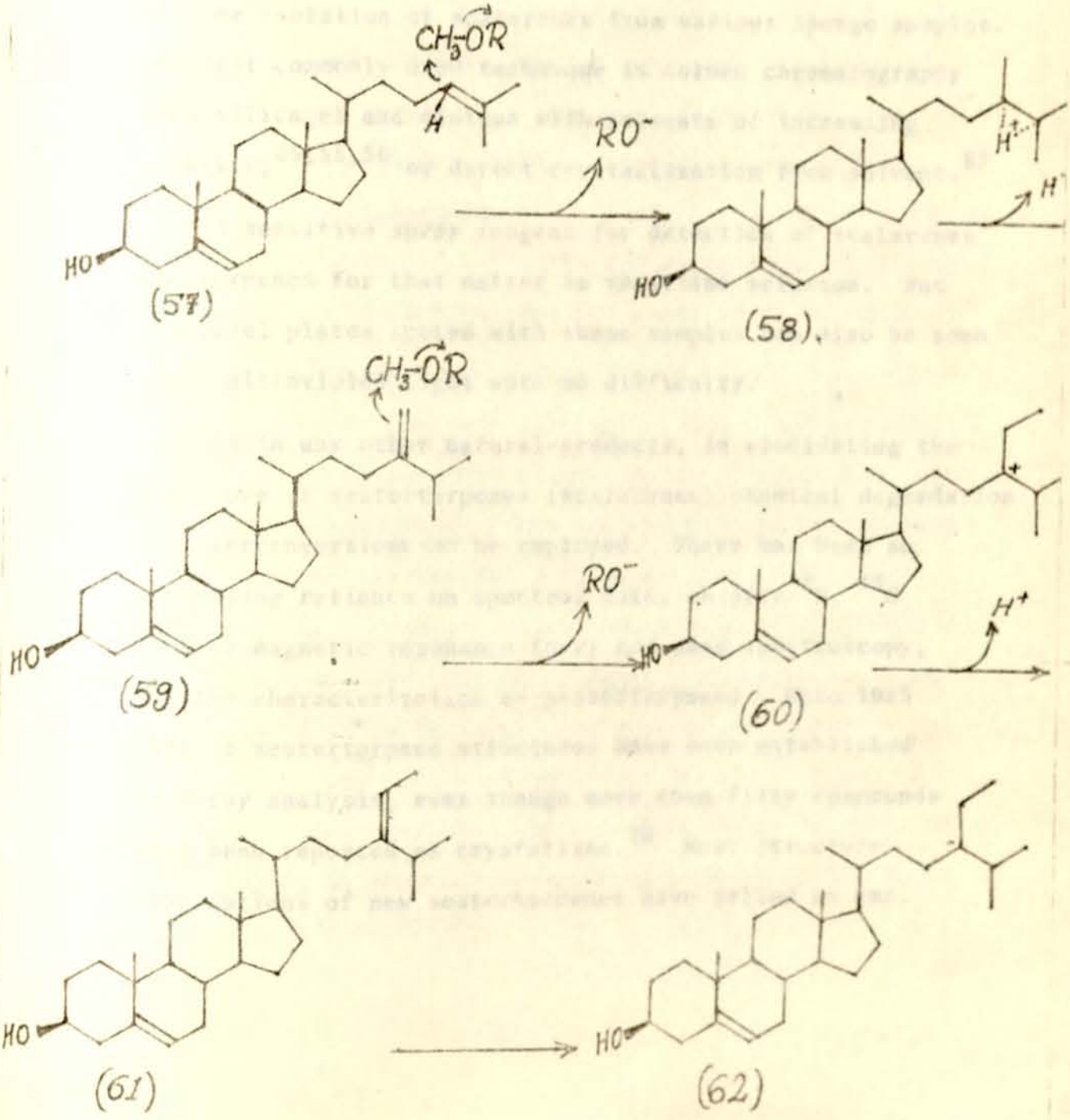
The feeding of labelled acetate, mevalonate and methionine to the sponge Vorongia aerophoba yielded no radioactive aplysterol (48). Radioactivity from all precursors used was incorporated to some extent into the fatty acids, thus proving that the administered substrates were taken up and metabolized by the animals. A similar situation arose in Axinella polypoides and A. verrucosa, the sponges reported to contain the 19-nor-stanols and the hydroxymethyl-A-nor-steranes respectively, when they were fed with labelled acetate.[42] However when cholesterol 26- ^{14}C was administered to these two sponges, the 19-nor-C_{27} stanol and hydroxymethyl-A-nor-C_{27} sterane fractions were found significantly labelled.[42]

It thus appears that in A. polypoides and A.verrucosa, the sterols cannot originate from denovo biosynthesis, but arise by modification of dietary sterols. Much work is required before a definite conclusion can be drawn about sterol biosynthesis in these primitive multicellular animals. The exogeneous sterol requirement is a characteristic of many lower organisms, the majority being invertebr

Generally sterols are biosynthesized by the tail-to-tail condensation of two farnesyl pyrophosphate (20) units, as shown in scheme V.

In the biosynthesis of β-sitosterol, the cholesterol skeleton is first formed. The ethyl group at C_{24} arises by double transmethylation, from the methyl group of methionine.[52] A possible mechanism for the introduction of an alkyl group into cholesterol for the biosynthesis of β-sitosterol as proposed by Castle is shown in scheme VI.[53,54]

Scheme VI



2.3 General isolation and characterization

In most common isolation procedures a sample is extracted with an appropriate solvent. Extraction could be cold or hot. The sponge sample could be extracted with a number of solvents ranging in polarity from n-hexane to methanol. Many isolation procedures have been described for the isolation of scalaranes from various sponge samples. The most commonly used technique is column chromatography with silicagel and elution with solvents of increasing polarity,[25,55,56] or direct crystallization from solvent.[57]

A sensitive spray reagent for detection of scalaranes and terpenes for that matter is vanilline solution. But silicagel plates spoted with these samples can also be seen under ultraviolet light with no difficulty.

As in any other natural products, in elucidating the structure of sesterterpenes (scalaranes), chemical degradation or interconversions can be employed. There has been an increasing reliance on spectral data, chiefly ^{1}H, ^{13}C nuclear magnetic resonance (nmr) and mass spectroscopy, for the characterization of sesterterpenes. Upto 1985 only 16 sesterterpene structures have been established by X-ray analysis, even though more than fifty compounds have been reported as crystalline.[20] Most structure elucidations of new sesterterpenes have relied on nmr.

Not unexpectedly sesterterpenes gave ^{13}C nmr which will be completely assigned, especially when nmr spin echo procedures are employed. Reliable ^{13}C nmr chemical shift data can provide a powerful and rapid means for characterization of new members of a series and for pinpointing differences in stereochemistry or the existence of isomers. The introductions of new techniques such as NOE, and two dimensional nmr (2D-nmr) spectroscopy have opened possibilities for the analysis of spectra. These techniques are able to provide information previously not accessible. Measurement of homonuclear ^{1}H- ^{1}H shift correlated spectroscopy (COSY) greatly simplifies the assignments of signals and structural determinations for complicated molecules.[58]

The mass spectra of scalaranes (a tetracarbocyclic sesterterpenes) are very distinctive and often permit structural assignments to be made by comparison with those having known structures. Thus scalaranes display characteristic fragment ion for their AB ring. For example all scalaranes having normal substituents give characteristic peak at m/z 191 for ring AB fragment. If there are some substituents on these rings, the mass of the fragment increases by the weight of the substituents.[56] Some scalaranes are acetylated and their molecular ions cannot be detected, as they are unstable especially in electron impact ionization.[17,57]

3. RESULTS AND DISCUSSION

3.1 Extraction and fractionation of sponge samples

Dark brown sponge samples 3B and 4 were collected in June 1987, from the Red Sea, around the marine biology station of Asmara University (AU) at Massawa. Samples were collected at a depth of 2-5 meters with the help of specimenspreserved in 10% formalin salt water solution, in the Department of Biology, AU. They were given identification numbers, since they were not identified taxonomically. The collected samples were sun-dried and stored in a cold room.

500 gms of sponge sample 3B from the cold room was pulverized and soxhlet extracted with chloroform. The extract when concentrated gave 6 gms of a gummy residue which was column chromatographed on silicagel to give several fractions. DS-2 was isolated from fractions collected by eluting with petroleum ether: chloroform mixture as detailed in the experimental section.

Extraction and purification of an unidentified brown sponge sample 4, by the same method, gave DS-1, along with other uncharacterized fractions. DS-2 and DS-1 were characterized by spectroscopic techniques.

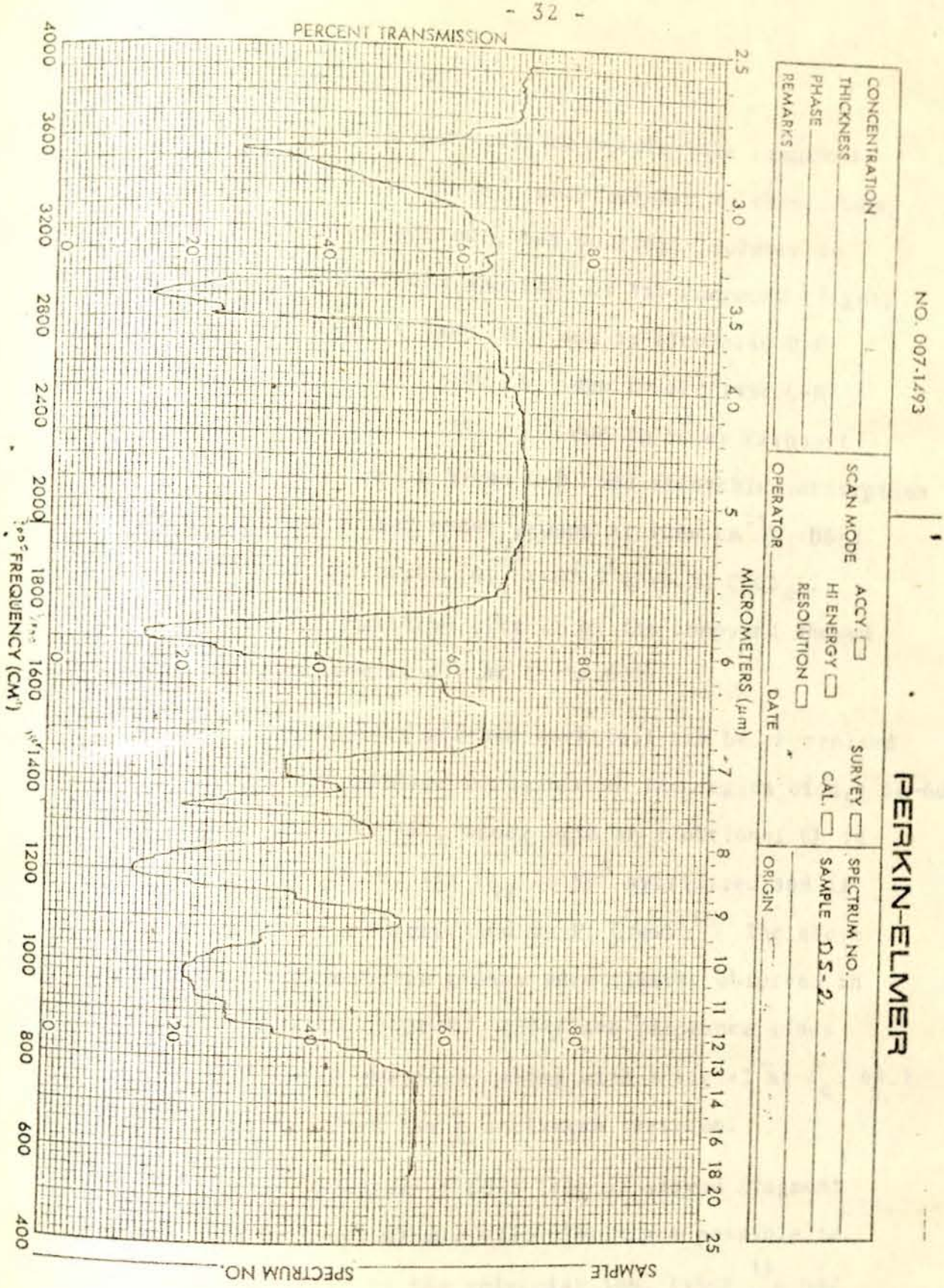


Fig. 1, IR spectrum of DS-2

3.2 Structure of DS-2

DS-2 is a white crystalline needle-like compound with m.p 172-174°C. It is highly soluble in chloroform, ethyl acetate, and acetone, but sparingly soluble in methanol. The infrared spectrum of the compound (fig-1) showed absorption at 3600 cm^{-1} due to alcoholic O-H stretching vibration, 3100 cm^{-1} due to olefinic C-H stretching vibration, 1750 cm^{-1} due to ester carbonyl (C=O) stretching and a broad C-O bond stretching absorption for both hydroxyl and ester groups at 1260 cm^{-1}. DS-2 has an optical rotation $[\alpha]_D^{20} = -67.5^\circ$ (c =0.2, $CHCl_3$). The ultraviolet spectrum (fig-2) of the compound showed maximum absorption at 230 nm (ε=14,000).

A normal scalarane frame work (65) can be recognized by three ^{13}C nmr methine resonance in the region of δ_5: 50-60 δ_9 : 52-59, δ_{14}: 49-55 , along with an additional CH in the region, δ_c: 51-65 (if C_{18} is SP^3 hybridized and if C_{25} is present), as described by P. Crews.[59] The above mentioned three methine groups were clearly observed in the DEPT spectrum of DS-2. These are resonance lines at δ_c: 58.4, 56.3 and 54.6; along with a signal at δ_c: 64.1 indicating that DS-2 has a scalarane skeleton.

The mass spectrum of DS-2 (fig-3) showed fragment ion of highest mass at m/z 428. It is not possible to conclude that this is the molecular ion, since ^{13}C nmr showed 29 carbon atoms, ir and ^{13}C nmr showed the presence

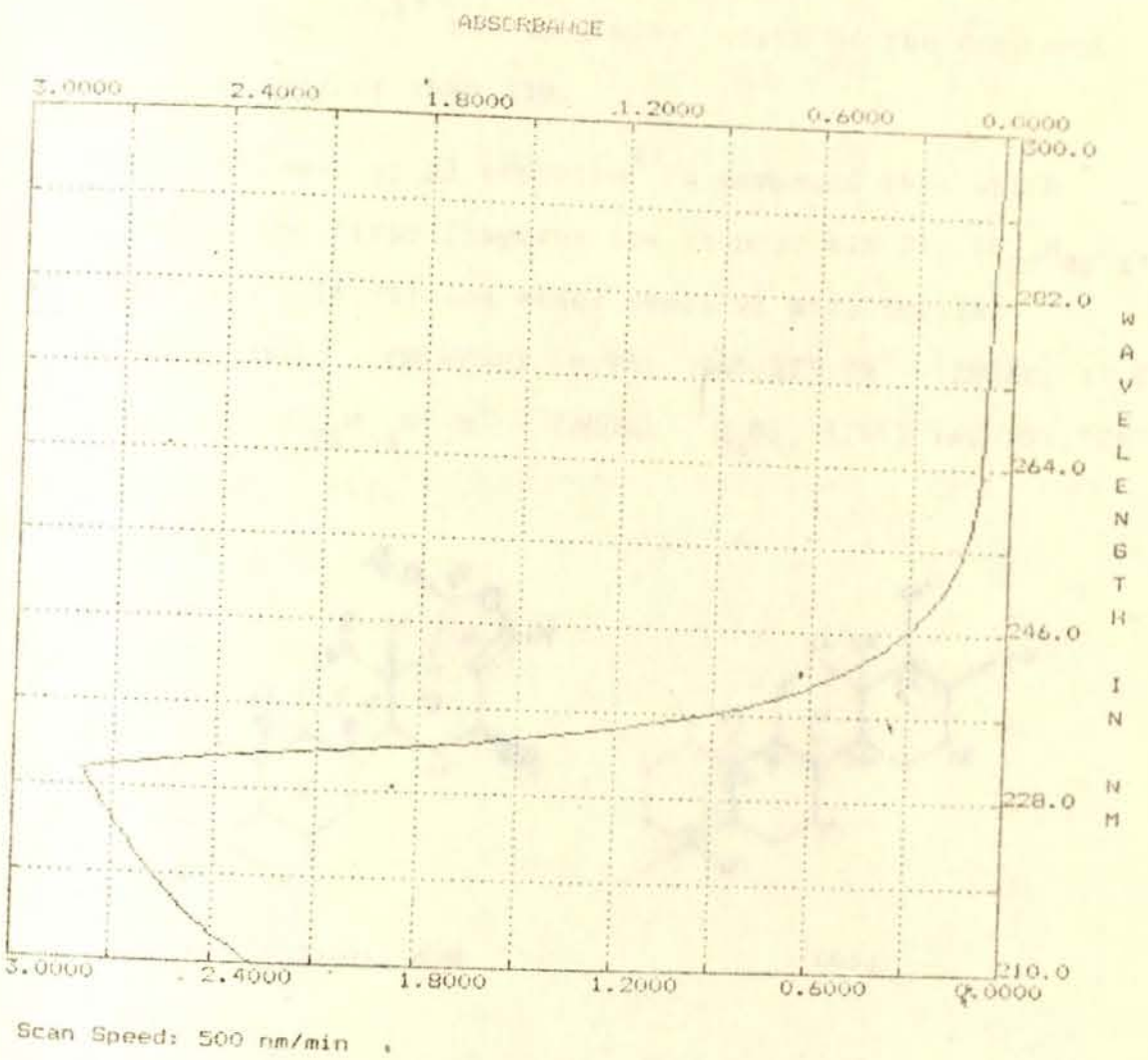
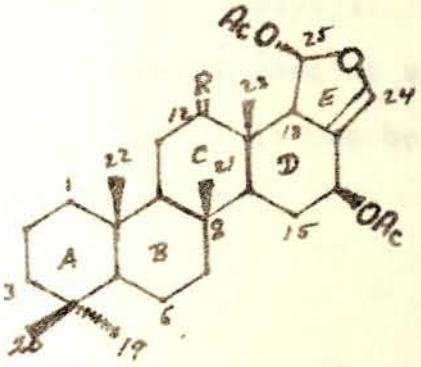


Fig-2 UV-spectrum of DS-2

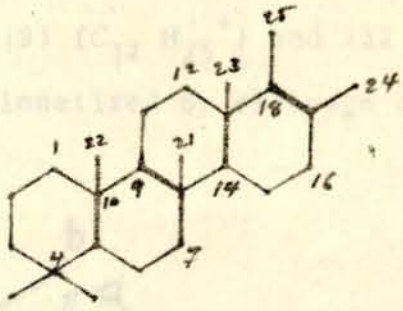
of at least five oxygen atoms (for two ester and one hydroxyl groups) and the compound is almost saturated. Since esters are usually unstable to electron impact ionization,[17,57] the molecular weight of the compound must be higher than 428.

Kashman et al reported[57] a compound (63) which showed the first fragment ion at m/z: 428.219 ($C_{27}H_{40}O_4$, M^+ - HOAc, 14.7%) and other peaks at m/z: 386.282 (M^+ - (HOAc + CH_2=C=O), 8.3%), 368.273 (M^+ - 2HOAc, 17.0%), 350.259 ($C_{25}H_{34}O$, M^+ - (2HOAc + H_2O), 3.6%) and 191.179 ($C_{14}H_{23}$, 7.5%).



(63); R=αH, βOH

(64); R=O



(65)

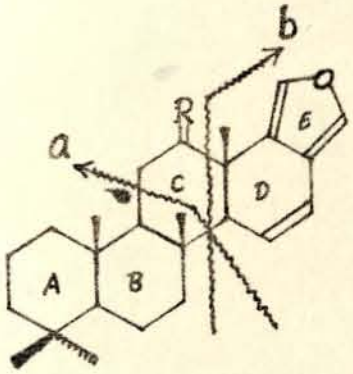
Microanalysis of heteronemin (63) as first reported by Kazlauskas et al[17], showed that it has a molecular formula $C_{29}H_{44}O_6$, whereas no molecular ion was observed in its mass spectrum. According to this report, the first fragment ion appeared at m/z: 428 ($C_{27}H_{40}O_4$, M^+ - HOAc).

DS-2 has two acetate groups (^{1}H-nmr, ^{13}C-nmr and ir), and it is logical to assume that, it has lost an acetic acid molecule to give the first fragment ion at m/z: 428, as in the above two reports.[17,57] Thus the molecular weight of DS-2 should be 488.

Compounds with scalarane skeleton (65) are reported[17,57] to give the characteristic fragment ion at m/z: 191, which corresponds to the AB ring system of the molecule. The same fragment was observed in the mass spectrum of DS-2 with 13% intensity.

The base peak of DS-2 appeared at m/z: 368, which correspond to the loss of the second acetic acid molecule from the fragment ion of highest mass.

Further support for the structure of heteronemin (63) reported [17], came from the high resolution mass spectrum of the compound (66), obtained by oxidation of heteronemin, followed by pyrolysis. This pyrolytic product showed the major fragment ions at m/z: 191 ($C_{14}H_{23}^{+}$) and 132 ($C_9H_8O^{+}$). This can be rationalized by cleavage of 'a' and 'b' in (66)



(66) R=O

(67) R=αH, β OH

If cleavage occurred at 'a' as shown, with charge retention on ring AB, then the fragment ion should have the mass at m/z: 191, but if cleavage occurred at 'b' with retention of charge on ring DE, then the fragment should have its mass at m/z: 132. This suggests that the ketonic moiety must be on ring C, and hence the hydroxyl group in heteronemin to be on ring C.

MASS	MASS	%
60	0	3.0
151	0	2.9
152	0	5.3
154	0	9.7
154	1	1.1
165	0	2.9
167	9	6.4
181	9	3.3
191	1	13.9
193	0	3.8
215	0	1.4
244	0	4.0
315	0	1.9
327	9	2.1
340	1	2.0
350	0	30.2
350	9	6.8
353	0	2.6
368	1	100.0
368	7	4.0
369	0	26.7
370	0	16.5
371	2	3.3
386	0	1.2
400	2	1.6
428	0	10.4
429	1	3.0

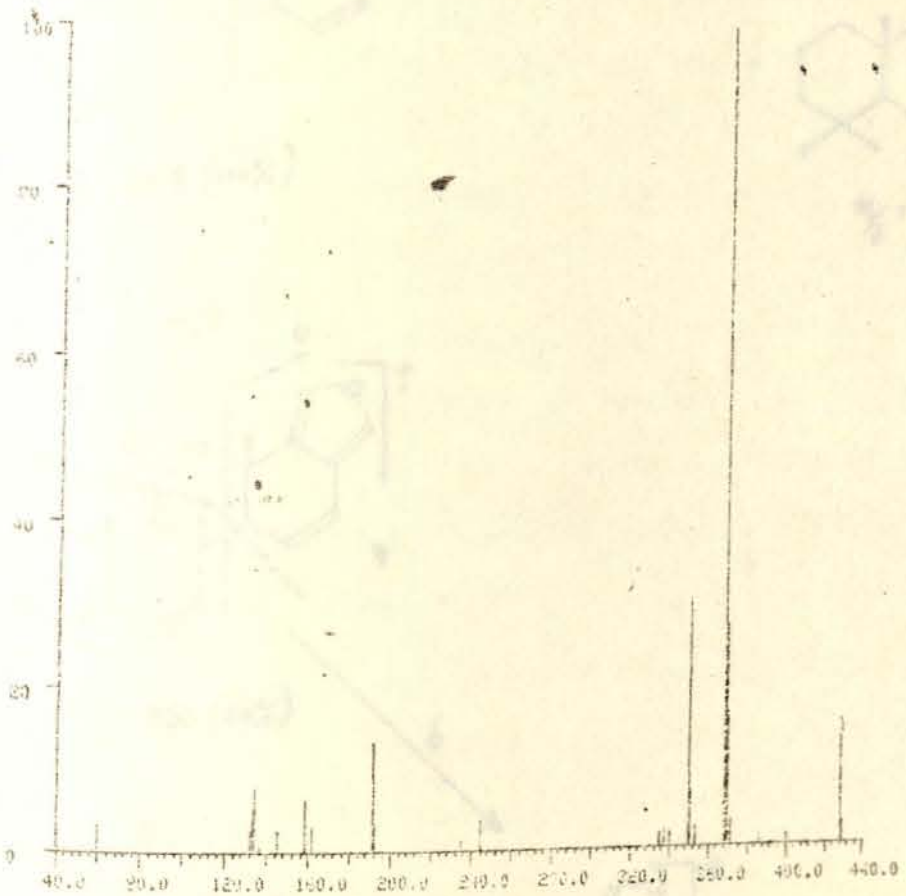
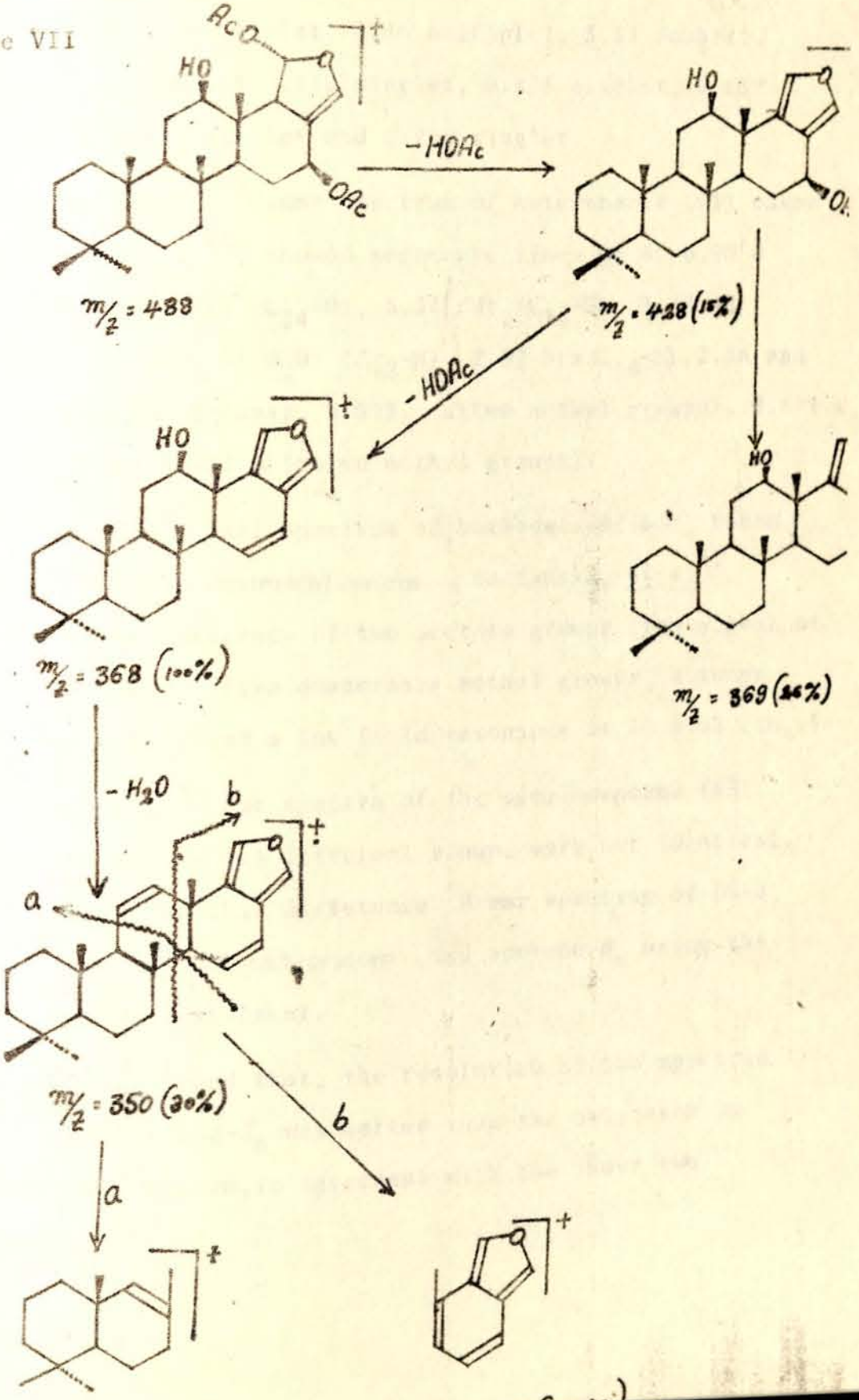


Fig-3 Mass spectrum of DS-2

In addition to the fragment ions described above (i.e 428, 368 and 191), DS-2 has major fragment ions at m/z: 369, 350, 132 and 60, which can be accounted for, as shown in Scheme VII.

Scheme VII



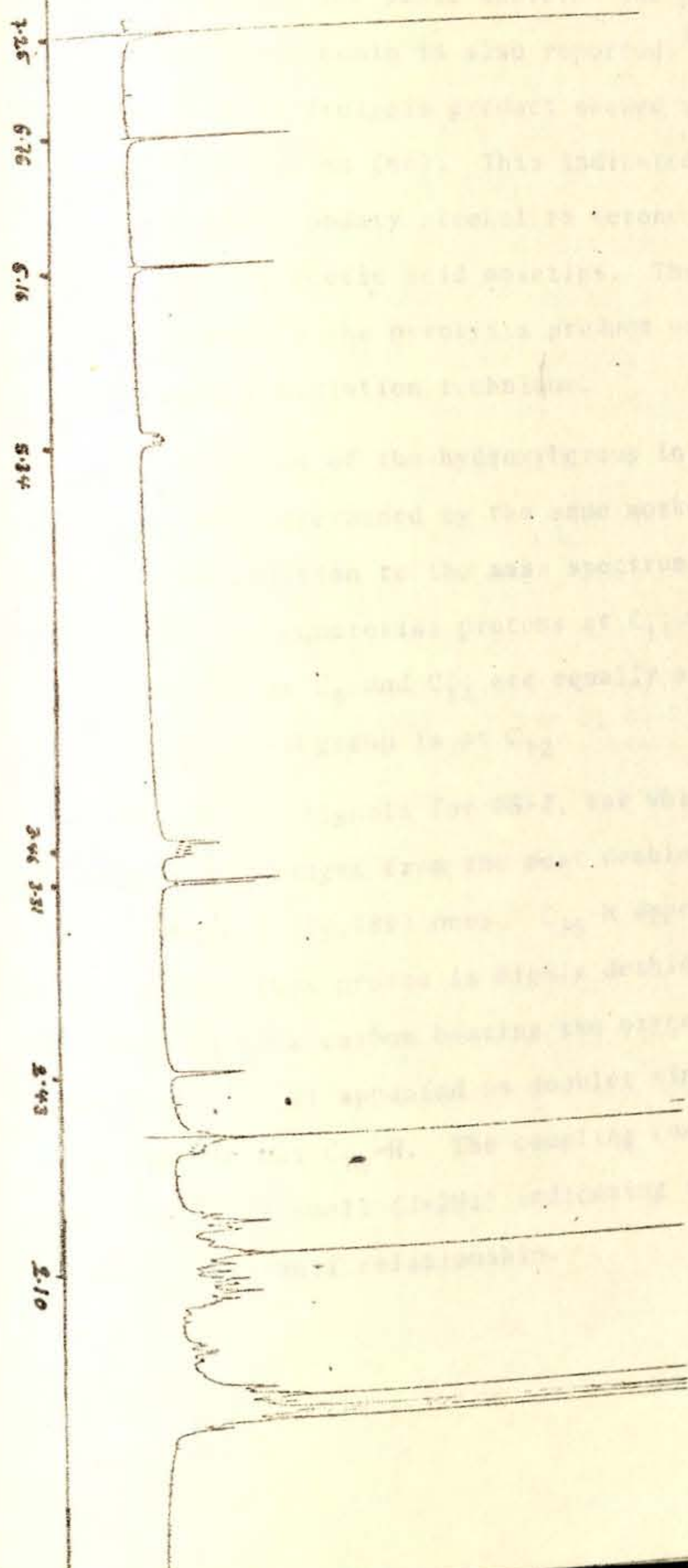
The 360 MHz ^{1}H-nmr spectrum of DS-2 (fig-4) taken in deuterochloroform showed signals at δ: 0.76 doublet, 6.16 triplet, 5.34 multiplet, 3.46 multiplet, 3.31 doublet, 2.43 broad singlet, 2:10 singlet, 0.895 singlet, 0.832 singlet, 0.814 singlet and 0.789 singlet.

The 270 MHz ^{1}H-nmr spectrum of heteronemin (63) taken in acetone - d_6[57], showed resonance lines at δ: 6.90 d (C_{25}-H), 6.16 brt (C_{24}-H), 5.34 ddt (C_{16}-H), 3.45 dd (after addition of D_2O) (C_{12}-H), 2.57 brs (C_{18}-H), 2.06 and 1.99 (two OAc groups), 0.893. s(two methyl groups), 0.871 s, 0.866 s and 0.883 s (three methyl groups).

In the ^{1}H- nmr spectrum of heteronemin (63), taken on 100 MHz, in deuterochloroform , Kazlauskas et al[17] observed the presence of two acetate groups from a peak at δ: 2.08 (6H,s), five quaternary methyl groups, between δ: 0.83 - 0.74 and a low field resonance at δ: 6.68 (1H,bs .

But the ^{1}H nmr spectra of the same compound (63) reported by the two different groups were not identical. To account for this difference ^{1}H nmr spectrum of DS-2 was taken in deuterochloroform and acetone-d_6 using the same 90 MHz instrument.

It was found that, the resolution of the spectrum taken in acetone-d_6 was better than the one taken in deuterochloroform, in agreement with the above two reports.


7·25
6·76
6·16
5·34
3·46
3·31
2·43
2·10

To establish the basic skeleton the pyrolytic product of oxidized heteronemin is also reported.[17] The ^1H nmr spectrum of the pyrolysis product showed it to be the oxidized vinyl furan (66). This indicated that oxidation converted the secondary alcohol to ketone and the pyrolysis removed the two acetic acid moieties. The chemical shifts of the protons in the pyrolysis product were assigned using proton irridiation technique.

The position of the hydroxyl group in the parent molecule (63) was determined by the same workers[17], using shift reagent, in addition to the mass spectrum. The shift of both axial and equatorial protons at C_{11} were highly affecte while protons at C_9 and C_{14} are equally affected indicating that the hydroxyl group is at C_{12}.

The ^1H nmr signals for DS-2, for which structure (63) is suggested, ranges from the most deshielded (δ: 6.76) to the shielded (0.789) ones. C_{25}-H appeared downfield at δ: 6.76. This proton is highly deshielded, because it is attached to a carbon bearing two oxygen atoms, (acetal carbon).[17,57] It appeared as doublet since it couples with the vicinal C_{18}-H. The coupling constant, as reported[57], is small (J=2Hz) indicating that these protons have anti relationship.

The olefinic proton at C_{24} of DS-2 (63), appeared at δ: 6.16. It appeared as a triplet, because it has allylic relationships to both C_{18}-H and C_{16}-H. Similarly C_{16}-H, which is attached to a carbon bearing the second acetoxy group, is deshielded, and appeared at δ: 5.34, as a broad multiplet, due to its coupling with the protons at C_{24} and C_{15}. The C_{12}-H bearing a hydroxyl group appeared at δ: 3.46 as a multiplet, because of its coupling with C_{11}-H_2 and the proton on oxygen. The signal for the proton on oxygen of the secondary alcohol appeared at δ: 3.31, as doublet, since it couples with C_{12}-H. C_{18}-H appeared at δ: 2.43, as a broad singlet. It is broad, because it couples with C_{24}-H, C_{25}-H, and one may also expect a long range coupling with C_{16}-H. The two acetate groups appeared at δ: 2.10 as a singlet. The five methyl groups appeared as four singlets at δ: 0.895, 0.832, 0.814 and 0.789. The signal at δ: 0.832 is broader representing two methyl groups.

P. Crews and Bescasa[59] obtained ^{13}C-^{1}H COSY nmr ($CDCl_3$, 100-400 MHz, ^{13}C and ^{1}H shift in ppm) over the aliphatic region of heteronemin (63), and found four singlets for the five methyl groups. These are resonance lines at δ: 0.82 (21-H_3), 0.80 (19-H_3 and 22-H_3), 0.77 (20-H_3) and 0.68 (23-H_3). Based on this observation chemical shifts at δ:0.895 in the ^{1}H nmr spectrum of DS-2 is assigned to 21-H_3, 0.832 to 19-H_3 and 22-H_3, 0.814 to 20-H_3 and 0.789 to 23-H_3.

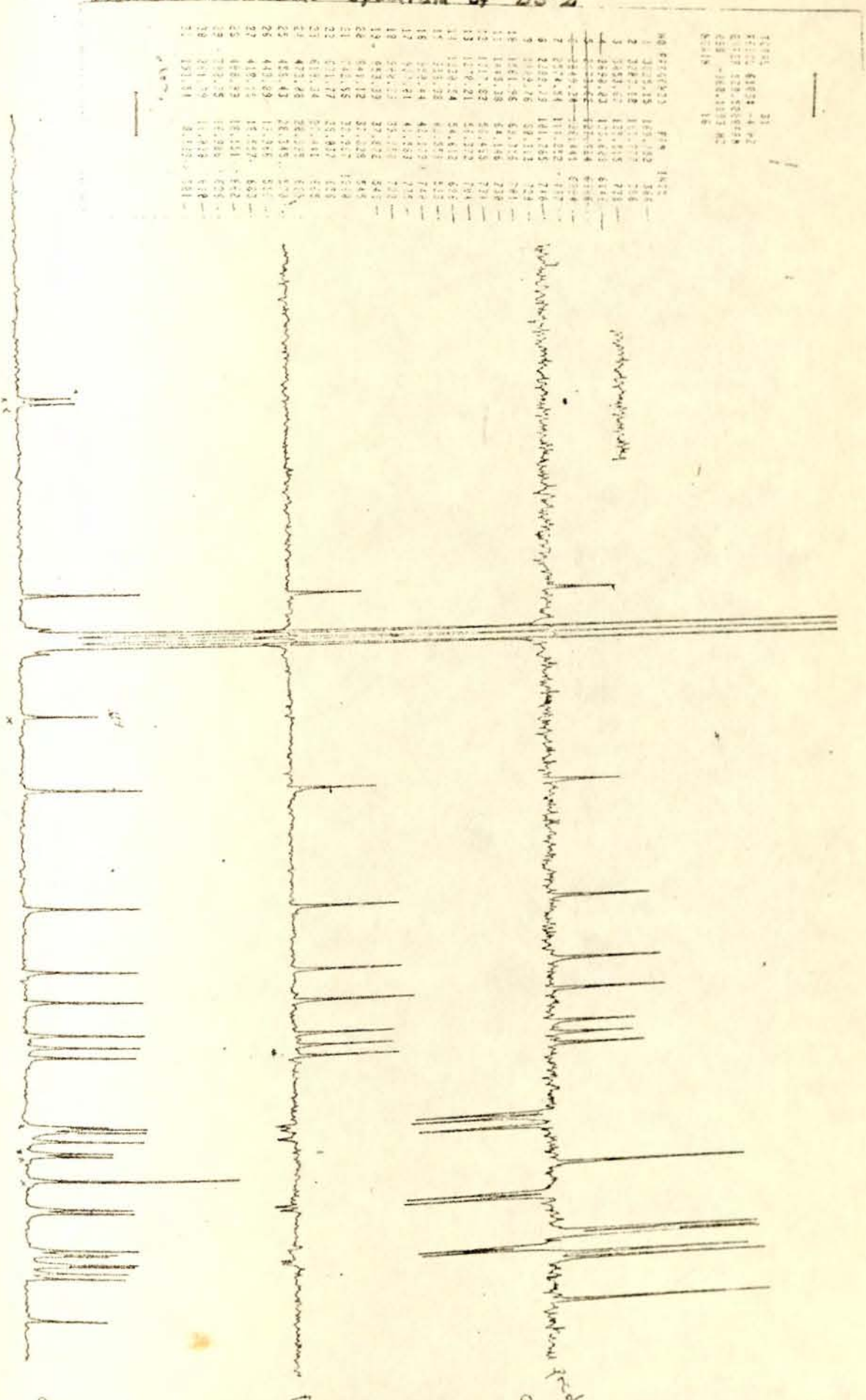
As there are more 1, 3 diaxial interactions, the protons on C_{21} are more deshielded as compared to other methyl groups; while protons on C_{23} are shielded by γ-equatorial substituents at C_{12} and C_{18}.

The ^{13}C nmr spectrum of DS-2 (fig-5), taken in deuterochloroform (22.25 MHz), gave 27 resonance lines; but when taken in benzene-d_6, the spectrum showed 28 resonance lines. The intense peak at δ: 32.9 most likely represents two carbon atoms. When a distortionless enhancement by polarization transfer (DEPT) spectrum of the compound was taken, the intensity of the peak at δ: 32.9 became equal to the rest of the methyl signals, but the other carbon which intensified the methyl signal at δ: 32.9 neither appeared as CH nor as CH_2, indicating that it has the same chemical shift as the methyl group and is a quaternary carbon atom. Thus the total number of carbon atoms for DS-2 is 29.

The DEPT spectrum of the compound showed that it has $7CH_3$, $7CH_2$, 8CH and 6 quaternary carbon atoms; the "extra" quaternary carbon atom has overlapped with one of the methyl groups.

In addition to the number of carbon atoms, information about the hybridization of carbon atoms involved, can also be obtained. The ^{13}C nmr spectrum of DS-2 showed resonance signals for carbonyl carbons, appearing at δ: 169.5 and 168.7, which are characteristics for esters. Other non

Fig. 5 ^{13}C nmr Spectrum of DS-2



aliphatic resonance signals appeared at δ: 135.5, 114.2, 102.3, 80.3 and 69.3. Resonance lines at δ: 135.5 and 114.2 are assigned to olefinic (SP^2-hybridized) carbon atoms at C_{24} and C_{17} respectively in (63). The signals at δ: 102.3, 80.3 and 69.3 are assignable to carbon atoms to which one or more oxygen atoms are directly attached. Accordingly they are assigned to C_{25}, C_{12} and C_{16} respectively.

As stated on page 33 the characteristic ^{13}C-nmr methine resonance signals of scalaranes which appeared in the region of δ_c: 50-60 are observed, along with an additional methine resonance signal of C_{18}. These are resonance lines at δ_c: 58.4, 56.3 and 54.6 which are assigned to C_9, C_5 and C_{14} respectively in (63), and the resonance signal at δ_c: 64.1 is assigned to C_{18}.

The ^{13}C nmr shift of methyl groups or methine carbons are especially useful in assessing the position and stereochemistry of the scalarane ring substituents. In addition, cis-ring junction methyl shifts can be used to determine cis- versus-trans ring fusions.

To accomplish this, data from models, along with an understanding of substituent effects, as reported by P. Crews and Wiseman[60] is required. These can be stated briefly as follows.

1- Ring junction methyls on a trans - decalin are shielded (δ: 15.8) by 11-15 ppm relative to those on cis decalin (δ:28.2)

2- Equatorial or axial angular methyls experience a 3-7 ppm shielding when a γ-substituent is added, but axial methyls experience a 2-ppm deshielding when an added carbon γ-substituent is axial.

3- Axial methyl experiences a 3-ppm deshielding when an axial δ-substituent is added.

4- Ring junction axial methyls are shielded by 4-5 ppm, when they join trans fused rings in a chair conformation in comparison to those on trans fused rings in a boat conformation.

Base values for decalin and its derivatives, when added to appropriate substituent increment, reproduce experimental methyl ^{13}C nmr chemical shift for scalaranes. Based on these experimental findings, chemical shift assignments have been made for the rest of the carbon atoms in DS-2 as shown in table 2.

The ^{13}C nmr spectrum of heteronemin (63) taken in deutrochloroform exhibited 27 - resonance signals for 29 carbon atoms (25 - skeleton and 4 of the two acetate groups) as reported by Kashman et al.[57]

The multiplicity of the different resonance lines was established by several off resonance decoupling experiments by the same group.[57] Assignments of chemical shifts for the reported 29 carbon atoms were also carried out. In this assignment there are overlap of chemical shifts at δ: 33.1 and 21.2. The overlap of the shifts at δ: 33.1 is due to a methyl group and a quaternary carbon atom, which corresponds to the overlap of the same two carbon atoms at δ: 32.9 for DS-2; but the overlap at δ: 21.2 in heteronemin reported[57], has been resolved in DS-2 and appeared at δ: 20.9 and 20.3.

When one goes from the most deshielded to the shielded signals (table-2), the 12th signal which appeared at δ: 42.5 in the DEPT spectrum of DS-2, is quaternary and assigned to C_{13}. But Kashman *et al* reported[57] it to be a methylene group and assigned it to C_3, with a chemical shift of δ: 42.6. Also the group assigned the next three consecutive signals at δ: 41.9, 41.7 and 39.9 to C_7, C_1 and C_8 respectively. In the off - resonance experiment conducted this group reported that the resonance lines at δ: 41.9 and 41.7 are triplets while that at δ: 39.9 is singlet. But the DEPT spectrum of DS-2 showed that all the three signals are chemical shifts for methylene groups.

P. Crews et al[59] have reassigned the chemical shifts for heteronemin (63). These workers have assigned the chemical shift at δ: 42.8 to C_{13} which agrees with the DEPT spectrum of DS-2. In addition this group confirmed that the consecutive three chemical shifts at δ: 42.2, 41.9 and 40 in their ^{13}C nmr spectrum, correspond to methylene groups and assigned to C_3, C_7 and C_1 respectively.

Kashman et al[57] assigned the chemical shift signals at δ: 21.2 to C_{19} (equatorial) and δ: 33.1 to C_{20} (axial) methyl groups, that are attached to C_4 of heteronemin (63). P. Crews et al[59] reversed the assignments, which implies that axial methyl carbon is shielded as compared to the equatorial one. If one uses a model of heteronemin, the axial methyl has got 1,3 diaxial interaction with axial proton at C_2 and the angular methyl group at C_{10}. Due to Van der Waal deshieldings axial protons are deshielded, but the electron cloud density on axial methyl carbon will be increased, as that from the deshielded protons get accumulated on this carbon. Thus axial methyl carbon should be shielded as compared to equatorial one. Accordingly the chemical shift at δ: 32.9 is assigned to the equatorial methyl while that at δ: 20.9 to the axial methyl group in DS-2, assignments that agree with the latter group.[59]

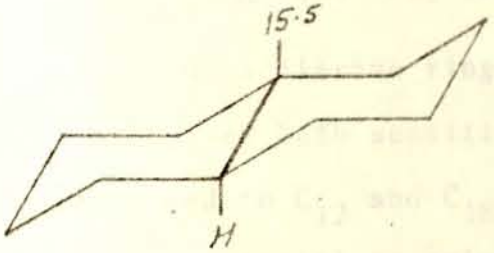
Experiment with shift reagent has also been reported.[5] According to this report, 11 signals that are not shifted upon addition of Eu $(fod)_3$ to a deutrochloroform solution of heteronemin (63) could be related to carbon atoms C_1-C_7, C_{10}, C_{19}, C_{20} and C_{22}. These carbon atoms are relatively far away from C_{12}-OH group, expected to be the main complexation site, the signals could hence be essentially uninfluenced by Eu$(fod)_3$ addition. The 11 unaffected resonance signals are found to be in agreement with the corresponding A/B ring carbon atom signals of α and β amyrin, after correction for the expected substituent effect of an additional C_8-methyl group.

Coming to conclusion, concerning ^{13}C nmr spectrum of heteronemin (63), Kashman et al[57] who did the first chemical shift assignments, indicated that their assignments may be reversed for carbon numbers 1, 2, 3, 6, 7, 19, 20, 24 and one of the two OAc groups which appeared at δ: 20.9. Latter on P. Crews et al[59] have made reassignments using COSY, and reversed some of the chemical shifts. The chemical shift assignments made for DS-2 is in complete agreement with that of P. Crews et al.[59]

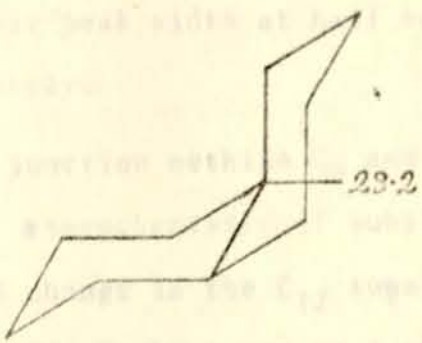
Table-2 Chemical shift assignments

		Heteronemin	
	DS-2	P. Crews et.al. [17]	Kashman et.al. [27]
1	169.7 - C_{16}'	170.6 - C_{16}'	—
2	168.7 - C_{25}'	169.7 - C_{25}'	—
3	135.5 - C_{24}	134.9 - C_{24}	134.9 - C_{24}
4	114.2 - C_{17}	114.0 - C_{17}	113.9 - C_{17}
5	102.3 - C_{25}	101.2 - C_{25}	101.2 - C_{25}
6	80.3 - C_{12}	80.4 - C_{12}	80.3 - C_{12}
7	69.3 - C_{16}	69.1 - C_{16}	69.1 - C_{16}
8	64.1 - C_{18}	64.3 - C_{18}	64.0 - C_{18}
9	58.4 - C_{9}	58.9 - C_{9}	58.6 - C_{9}
10	56.3 - C_{5}	56.6 - C_{5}	56.4 - C_{5}
11	54.6 - C_{14}	54.8 - C_{14}	54.6 - C_{14}
12	42.5 - C_{13}	42.8 - C_{13}	42.6 - C_{3}
13	42.1 - C_{3}	42.2 - C_{3}	41.9 - C_{7}
14	41.5 - C_{7}	41.9 - C_{7}	41.7 - C_{1}
15	39.9 - C_{1}	40.0 - C_{1}	39.9 - C_{8}
16	37.8 - C_{10}	38.2 - C_{10}	38.0 - C_{13}
17	37.3 - C_{8}	37.5 - C_{8}	37.3 - C_{10}
18	32.9 - C_{19e}	33.3 - C_{19e}	33.1 - C_{20a}
19	32.9 - C_{4}	33.1 - C_{4}	33.1 - C_{4}
20	28.0 - C_{15}	28.1 - C_{15}	27.9 - C_{11}
21	27.4 - C_{11}	27.3 - C_{11}	27.2 - C_{15}
22	20.9 - C_{20a}	21.1 - C_{20a}	21.2 - C_{19e}
23	20.3 - OAc_{16}'	21.2 - OAc_{16}'	21.2 - OAc
24	19.9 - OAc_{25}'	20.8 - OAc_{25}'	20.9 - OAc
25	18.5 - C_{6}	18.7 - C_{6}	18.6 - C_{6}
26	18.1 - C_{2}	18.3 - C_{2}	18.2 - C_{2}
27	16.9 - C_{21}	17.4 - C_{21}	17.3 - C_{21}
28	16.0 - C_{22}	16.4 - C_{22}	16.3 - C_{22}
29	8.5 - C_{23}	8.9 - C_{23}	8.8 - C_{23}

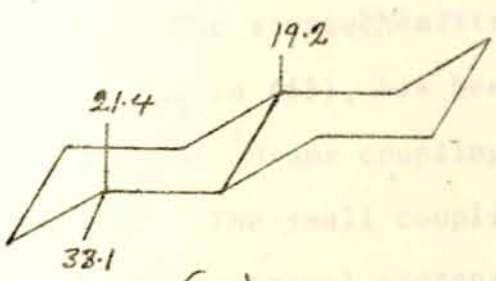
The stereochemistry of heteronemin (63) has been clearly established through a number of experiments and observations.[17] It has ten assymetric centers. Stereochemistry at the ring junctions were established based on the chemical shifts in the ^{13}C nmr spectrum. The ^{13}C nmr chemical shifts of methyl groups or methine carbons at the junctions are especially useful in assessing the position of substituents and stereochemistry of scalaranes (65) in general. In addition ring junction methyl shifts can often be used to determine cis- versus - trans ring fusion, since methyl group on trans ring fusion are shielded by about 11-15 ppm relative to those on cis ring fusion as shown in the compounds (68)-(71).



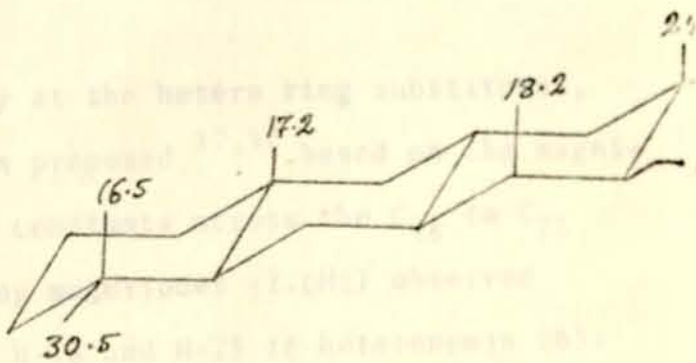
(68)



(69)



(70)



Thus an array of an all trans A,B and C rings are indicated by heteronemin's (63), Me-21 (δ: 17.4) & Me-22 (δ:16.4), which have close chemical shift values to the reference compound(71). Likewise a trans C D ring junction with an equatorial substituents γ- to the ring junction methyl is indicated by heteronemin (63). The C_{23} methyl group is shielded by the two γ-equatorial substituents at C_{12} and C_{18}, thus it appeared at δ: 8.5.

Another report[17] showed that, protons at C_{12} and C_{16} are axial as inferred from their peak width at half height ($W_{\frac{1}{2}}$), 15 Hz and 16Hz respectively.

The scalarane ring (65) junction methine C_9 and C_{14} shifts are both sensitive to sterochemistry of substituents attached to C_{12} and C_{18}.[20] A change in the C_{12} substituent from equatorial to axial, shields C_9 by 6 ppm or a change in the C_{18} carbon substituent from equatorial to axial shields C_{14} by 4 ppm.

The stereochemistry at the hetero ring substituent, at C_{25} in (63), has been proposed [17,57] based on the magnitude of ^{1}H-nmr coupling constants across the C_{18} to C_{25} bond. The small coupling magnitudes (2.0Hz) observed between vicinal protons H-18 and H-25 in heteronemin (63) demonstrated the relative trans stereochemistry.

Also the ease with which an acetic acid molecule is lost from the molecule during mass spectral fragmentation requires a cis relationship between the acetoxy group at C_{25} and hydrogen at C_{18}.[17] This strengthens the preceding argument about the stereochemistry at these centers.

5.3 Structure of DS-1

DS-1 is a white crystalline (needles) solid with melting point of 127-128°C. It is soluble in chloroform and ethyl acetate but sparingly soluble in methanol.

The ultraviolet spectrum of DS-1 (fig-6) showed maximum absorption at 215 nm (C=12500). The infrared spectrum (fig-7) showed strong absorbance for O-H stretching vibration at 3400 cm^{-1}, weak absorbance for an unconjugated C=C stretching vibration at 1610 cm^{-1} and an olefinic C-H stretching and bending vibrations at 3030 cm^{-1} and 1450 cm^{-1} respectively.

Since the compound was eluted with a relatively non-polar solvent, petroleum ether-chloroform (4:1) mixture, and sponges are well known for their sterol metabolites, Liebermann - Burchard test for 3β - hydroxy sterols was conducted, and was found to be positive.

The 400 MHz ^1H nmr spectrum taken in deuterochloroform showed a doublet at δ: 5.31, for one hydrogen corresponding to an olefinic proton. This implies that the other olefinic carbon atom is quaternary since there is no signal corresponding to other olefinic proton. The signal for the proton on oxygen appeared at δ: 5.2 coupled with C_3-H. The other deshielded resonance signal of the spectrum appeared at δ: 3.5, as multiplet, for a single proton which corresponds to the hydrogen

DS-1

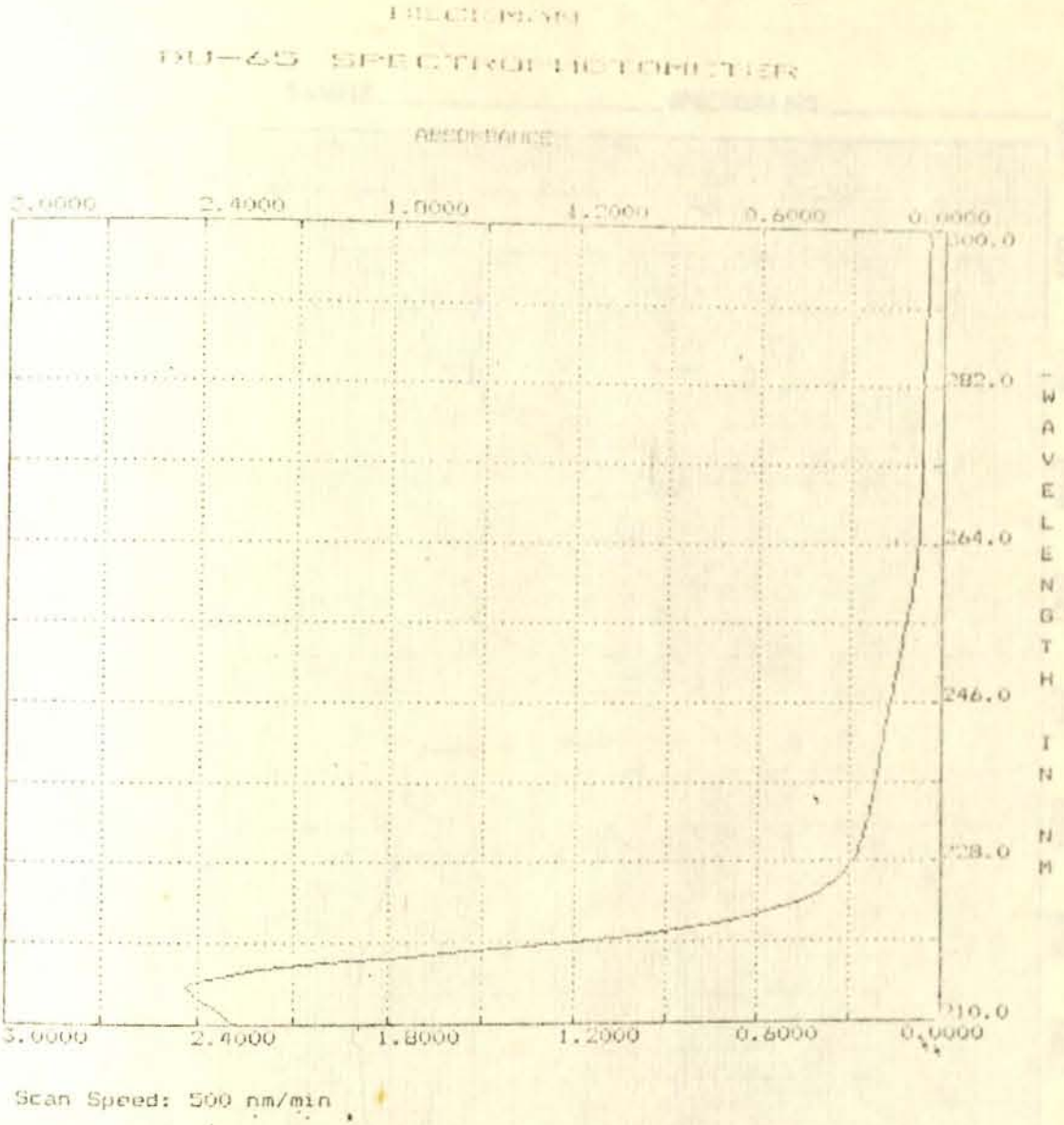


Fig-6 UV spectrum of DS-1

NO. 007-1493

PERKIN-ELMER

CONCENTRATION
THICKNESS
PHASE
REMARKS

SCAN MODE ACCY. ☐ SURVEY ☐
HI ENERGY ☐ CAL. ☐
RESOLUTION ☐
OPERATOR DATE

SPECTRUM NO.
SAMPLE DS-1
ORIGIN

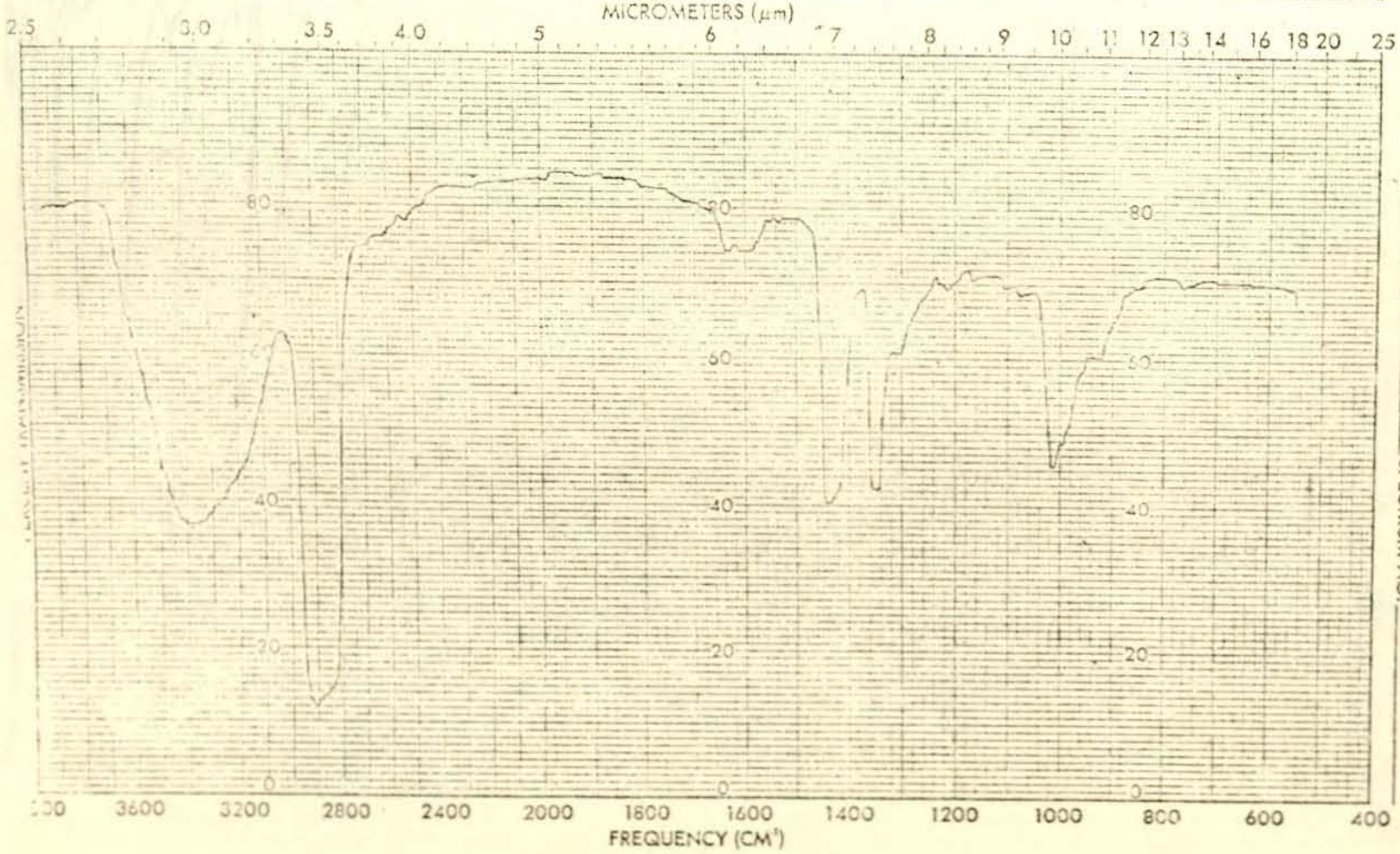
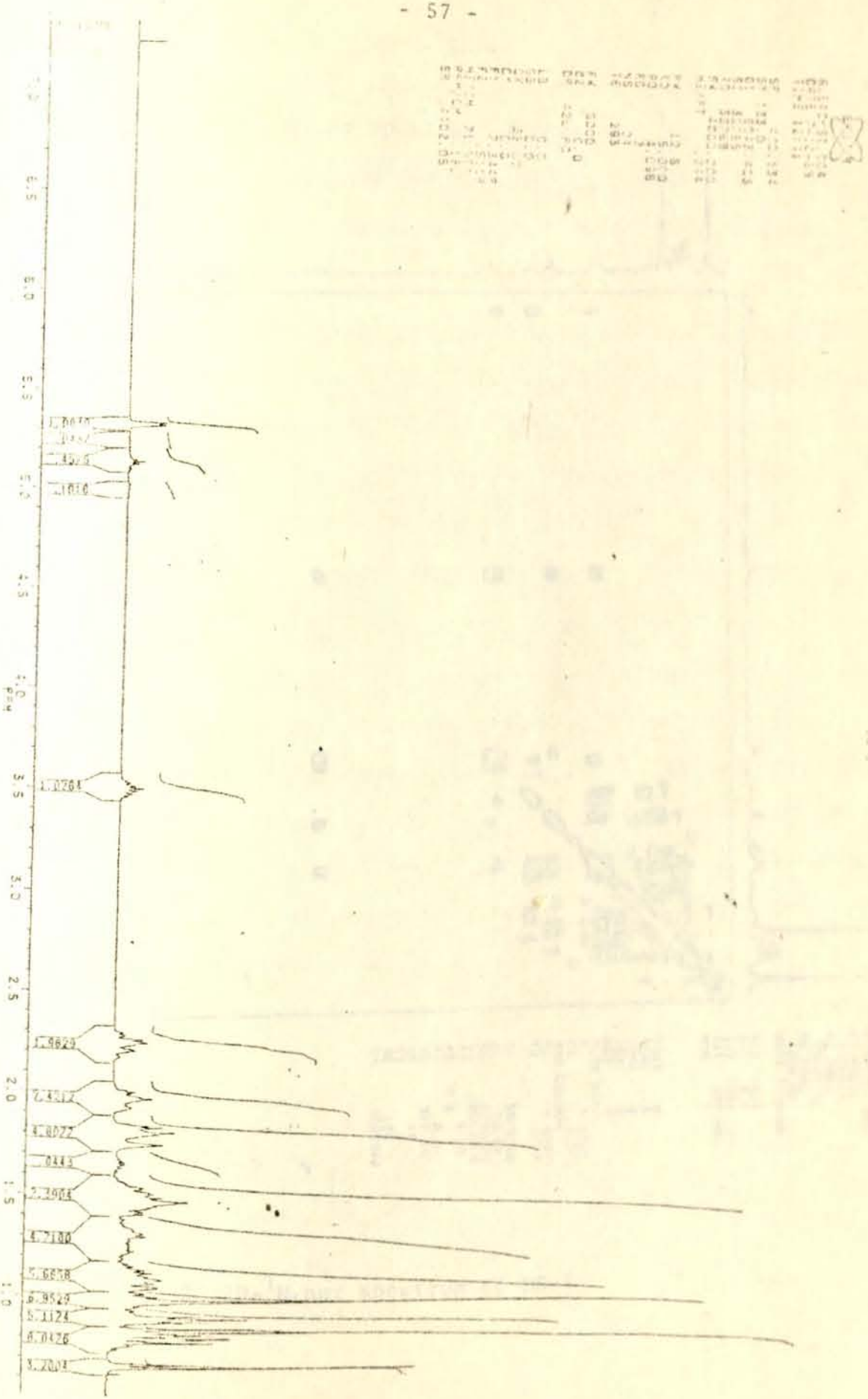


Fig-7 IR spectrum of DS-1


4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

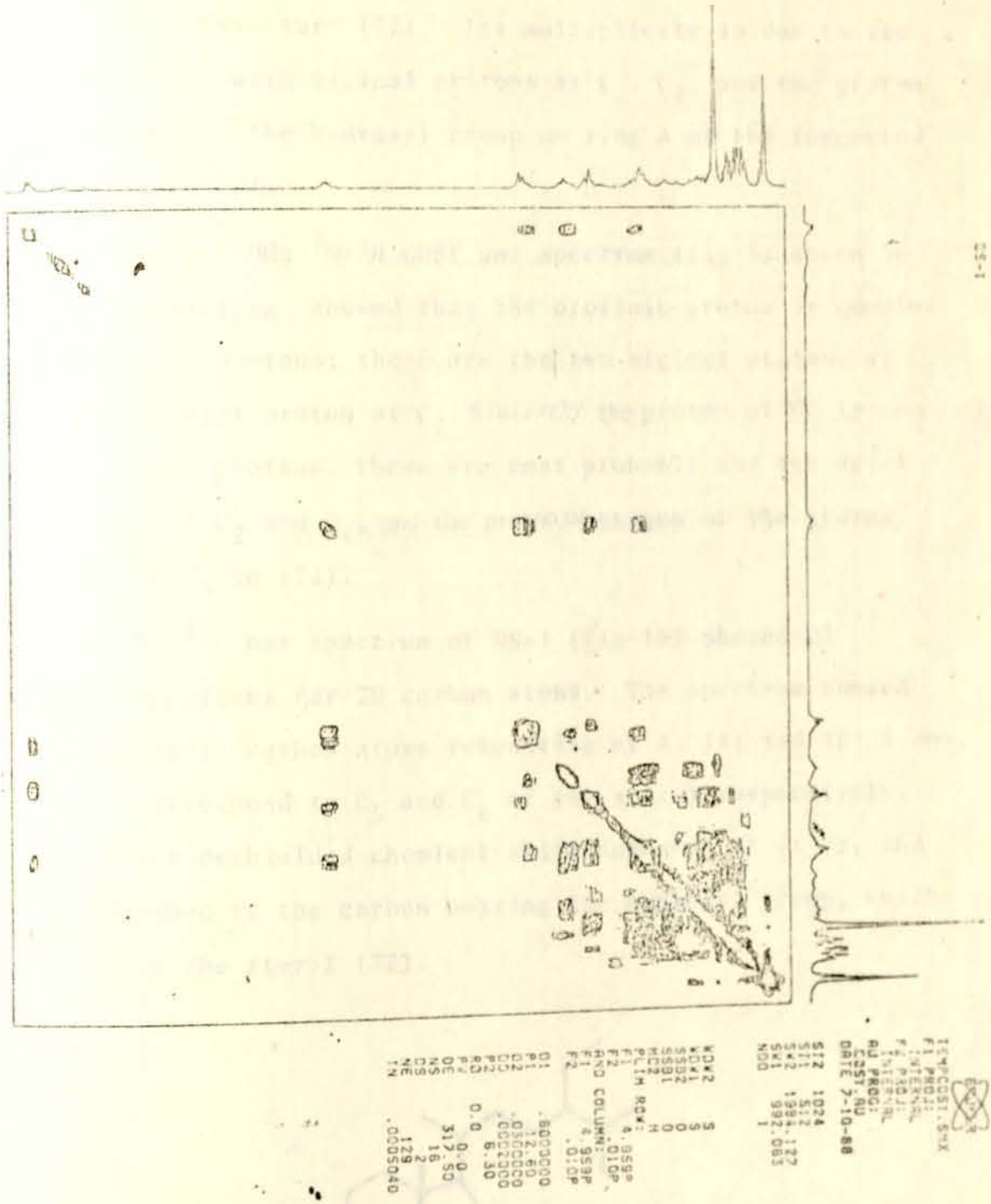
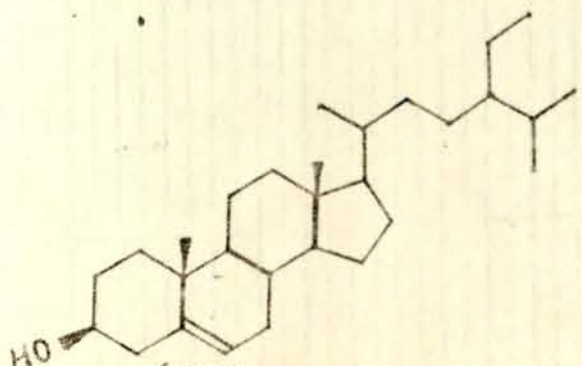


Fig-9 2D-^{1}H-nmr spectrum of DS-1

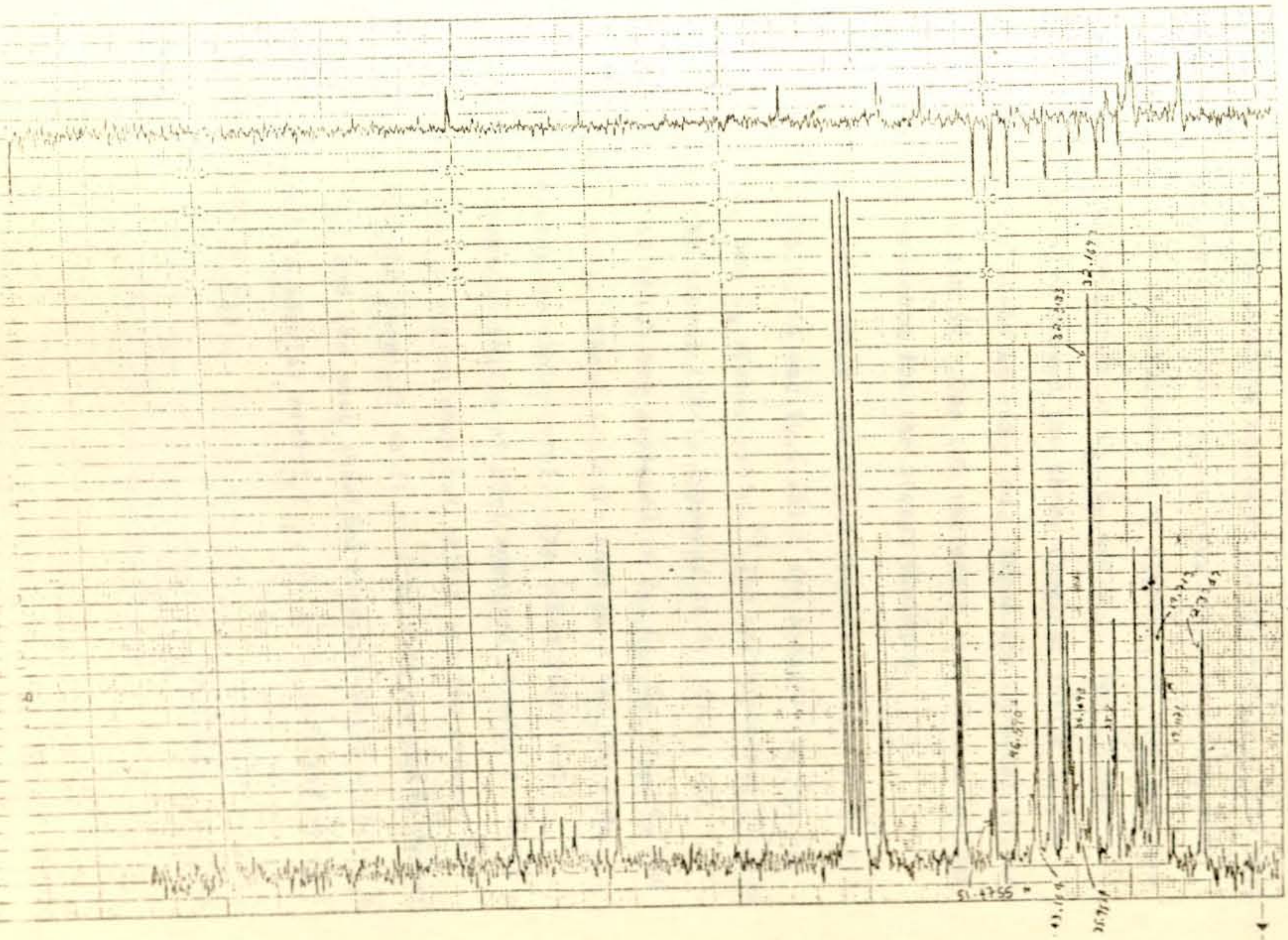
attached to a carbon bearing the hydroxyl group in the suggested structure (72). Its multiplicity is due to its interaction with vicinal protons at C_2, C_4, and the proton on oxygen of the hydroxyl group on ring A of the suggested structure (72).

The 400 MHz ^1H-^1H COSY nmr spectrum (fig-9) taken in deuterochloroform showed that the olefinic proton is coupled with three protons; these are the two vicinal protons at C_7 and the axial proton at C_4. Similarly the proton at C_3 is coupled with three protons; these are most probably the two axial protons at C_2 and C_4, and the proton on oxygen of the hydroxy group at C_3 in (72).

The ^{13}C nmr spectrum of DS-1 (fig 10) showed 27 resonance lines for 29 carbon atoms. The spectrum showed two olefinic carbon atoms resonating at δ: 141 and 121.7 ppm. These correspond to C_5 and C_6 of the sterol respectively. The other deshielded chemical shift appeared at δ: 72, and is assigned to the carbon bearing the hydroxyl group, which is C_3 of the sterol (72).



(72)



NWIDTH 14

NO	FREQUENCY	PPM	INT
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2	[illegible]	121.724	[illegible]
3	[illegible]	78.593	[illegible]
4	[illegible]	77.169	[illegible]
5	[illegible]	75.669	[illegible]
6	[illegible]	72.905	[illegible]
7	[illegible]	57.197	[illegible]
8	1276.92	56.621	[illegible]
9	1142.45	50.708	[illegible]
10	[illegible]	46.558	[illegible]
11	[illegible]	43.247	[illegible]
12	[illegible]	40.214	[illegible]
13	[illegible]	37.651	[illegible]
14	[illegible]	36.847	[illegible]
15	[illegible]	36.515	[illegible]
16	[illegible]	34.453	[illegible]
17	[illegible]	32.169	[illegible]
18	[illegible]	[illegible]	[illegible]
19	[illegible]	28.325	[illegible]
20	[illegible]	[illegible]	[illegible]
21	[illegible]	24.548	[illegible]
22	[illegible]	23.556	[illegible]
23	[illegible]	22.782	[illegible]
24	462.47	21.411	[illegible]
25	[illegible]	19.384	[illegible]
26	[illegible]	12.888	[illegible]

AMPLITUDE
DECOUPLING
NOISE
DATE
OPERATOR
REMARKS: ADRES

Ds-1
$CDCl_3$

JEOL

In the study of heteronemin (DS-2), it has been stated that the normal scalarane framework (65) can be recognized by three ^{13}C nmr methine resonance in the region of δc: 50-6 for C_5, C_9 and C_{14}. This basic idea can be extended and made use of in the elucidation of sterols, as they may also contain methine groups at C_8, C_9 and C_{14}. The ^{13}C nmr spectrum of DS-1 showed the three chemical shifts in the region of δc: 50-60 as expected. These are chemical shifts at δ: 57, 56 and 50. One can therefore take this as strong supporting evidence for the sterol skeleton.

Cholesterol shows deshielded chemical shifts at δ: 141, 121, 73.3, 56.9, 56 and 50.5 which are assigned to C_5, C_6, C_3, C_{14}, C_8 and C_9 respectively.[65] The corresponding chemica shifts in the sterol (DS-1) appeared at δ: 141.1, 121.7, 72, 57, 56 and 50 which are assigned to C_5, C_6, C_3, C_{14}, C_8 and C_9 respectively. These indicate that DS-1 has the same basic skeleton as cholesterol. The other relatively shielded chemical shifts of DS-1 appeared between δ: 46-12 p These aliphatic region chemical shifts are too crowded to assign; but the chemical shift at δ:46.5 is assigned to the met carbon (C_{17}) and the most shielded chemical shift at δ: 12.0 is assigned to angular methyl, C_{18}, as it is shielded by γ-substituent at C_{17}.

The first reported application of mass spectrometry in the identification of steroids, was by de Mayo and Reed[45], who showed that spectra of steranes recorded at low electron energy (10-15 eV), contained well defined molecular ion, affording a direct measurement of molecular weight. Accordingly much of the information used to elucidate the structure of the sterol isolated (DS-1) was obtained from its mass spectrum (fig-11). Mass of the compound was found to be 414 which requires $C_{29}H_{50}O$. The double bond equivalent (r+db) of the formula is equal to 5.

The mode of fragmentations of steroids under electron impact are quite complex, as indicated by the detailed and extensive investigation by Budzikiewicz et al.[61] The majority of applications of mass spectrometry to steroid analysis have so far been made with low resolution instrument Information as to structural features have been obtained from the mass spectrum mainly in the following ways[45]:

1- by recognizing ions arising from well characterized fragmentation processes;

2- by correlating the mass spectrum of an unknown steroid with that of reference samples;

3- by converting an unknown compound to a derivative known to be effective in influencing the mode of fragmentation in a structurally informative manner;

4- by recording mass spectra at low electron energies, so as to isolate only the most energetically facile fragmentation processes and thus, for example, to aid in distinguishing closely similar or steroisomeric steroids;

5- the combination of mass spectrometery with gas chromatography (GC-MS) made possible the analytical characterization of steroids in submicrogram amounts and remains the most powerful technique for identifying steroids in biological materials. Comparing the compatibility of the proposed structure with the gas chromatographic retention time at which the mass spectrum is recorded will also give information about the structure of the compound.

Information about the structural feature of the sterol (DS-1) was obtained by recognizing ions, arising from well characterized fragmentation process. Loss of water, methyl group or both are known. Also sterols are known to lose side chains along with groups which are vulnerable to fragmentation. Cyclopentane rings in sterols are known to be liable to fragment in electron impact ionization. Sterols with Δ^5 - give characteristic fragments. Signals corresponding to all these were observed in the mass spectrum of DS-1.

The spectral data presented so far suggested a cholesterol skeleton; a 27 carbon compound. But DS-1 is a 29 carbon compound. The position of the additional two carbon atoms on the side chain is stablished from biogenetic consideration and the mass fragmentation (Scheme VII)

If there is no substituent at C_{24} on the side chain, the mass spectrum should give fragment ion at m/z 357 ($M^+-C_4H_9$); but this is not observed, indicating that a substituent has to be at C_{24}. A fragment ion at m/z 343 ($M^+-C_5H_{11}$) which appears if a methyl group is at C_{24} is not also observed indicating that the substituent at this position has to be an ethyl group. The presence of an ethyl group at C_{24} is confirmed by the presence of fragment ions at m/z 329 ($M-C_6H_{13}$) and 385 ($M-C_2H_5$) with fairly intense peak.

Thus the sterol isolated (DS-1) is either β-sitosterol (24R-stigmast-5-en-3β-ol) or clionasterol, the 24S isomer. To choose between these two possibilities:

1- DS-1 was cospotted with an authentic sample of β-sitosterol, and was found to have the same Rf value (0.78).

2- DS-1 was acetylated and the melting point of the acetylated product was found to be 126°C. The literature value of the melting point of clionasterol acetate is 144°C, while that of β-sitosterol acetate is 127°C.

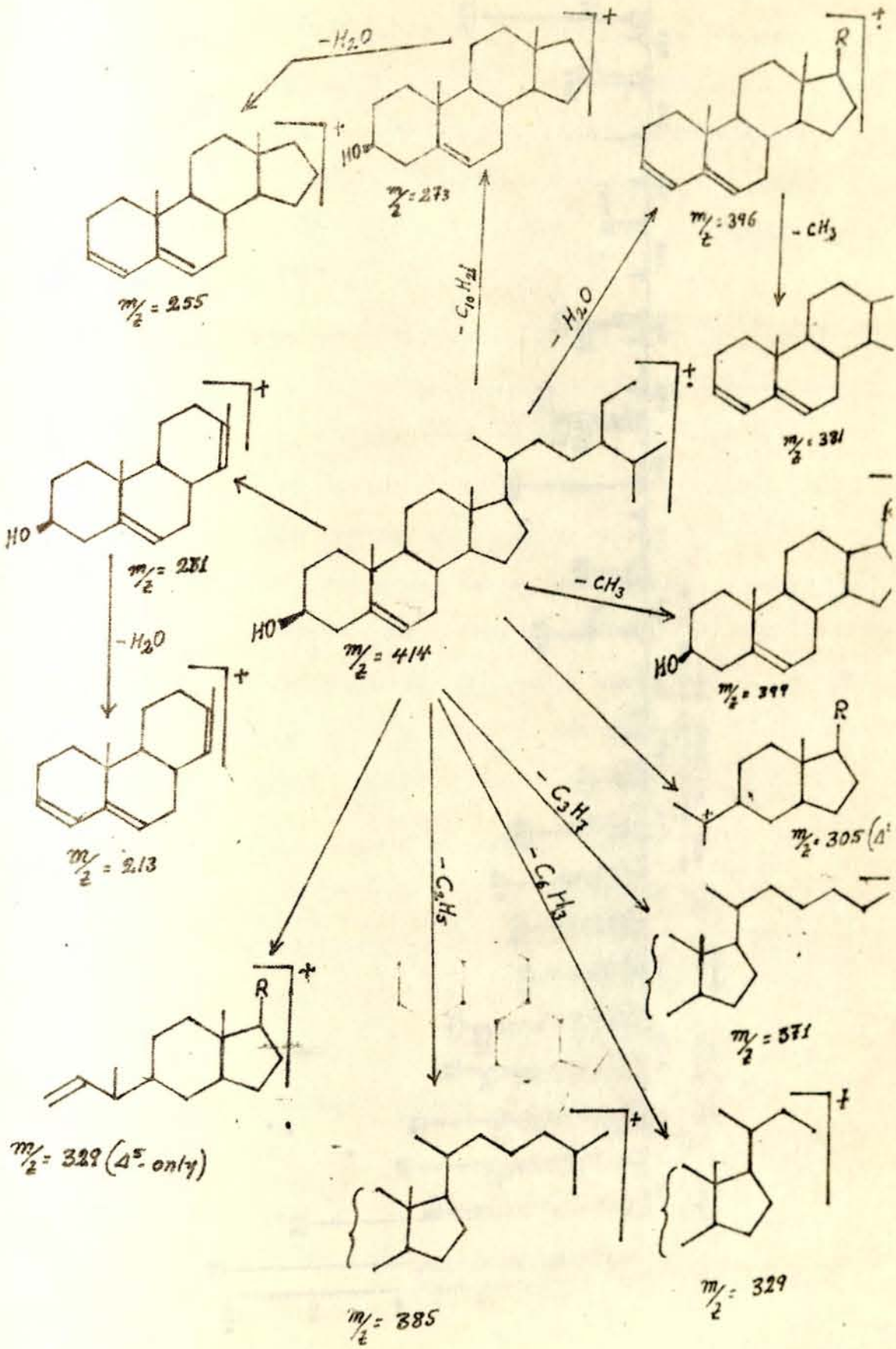
3- The optical rotation of DS-1 is found to be $[\alpha]_D^{20}$ = -36.9° (c=0.146, $CHCl_3$), while the literature value for β-sitosterol is $[\alpha]_D^{20}$ = -35°.

4- Reported melting point of β-sitosterol is 134°C while the observed melting point of DS-1 is 127-128°C.

All these evidences support the suggestion that DS-1 is β-sitosterol and not clionasterol.

DS-1 has major fragment ions at 414, 399, 398, 396, 386, 381, 355, 341, 329, 303, 301, 296, 282, 275, 273, 271, 255, 231, 229, 213. β-sitosterol from an annelide Pseudopotamilla occelata as reported by Kobayeshi[63] has major fragment ions at m/z 414, 399, 396, 381, 341, 329, 303, 296, 282, 275, 273, 271, 255, 231, and 229 which are identical to fragment ions of the sterol DS-1. This with the other data confirms that DS-1 is β-sitosterol.

Scheme VIII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-H2O
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-CH3
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m/z = 281
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m/z = 213
m/z = 414
-CH3
m/z = 399
-C3H7
-C4H9
-C6H13
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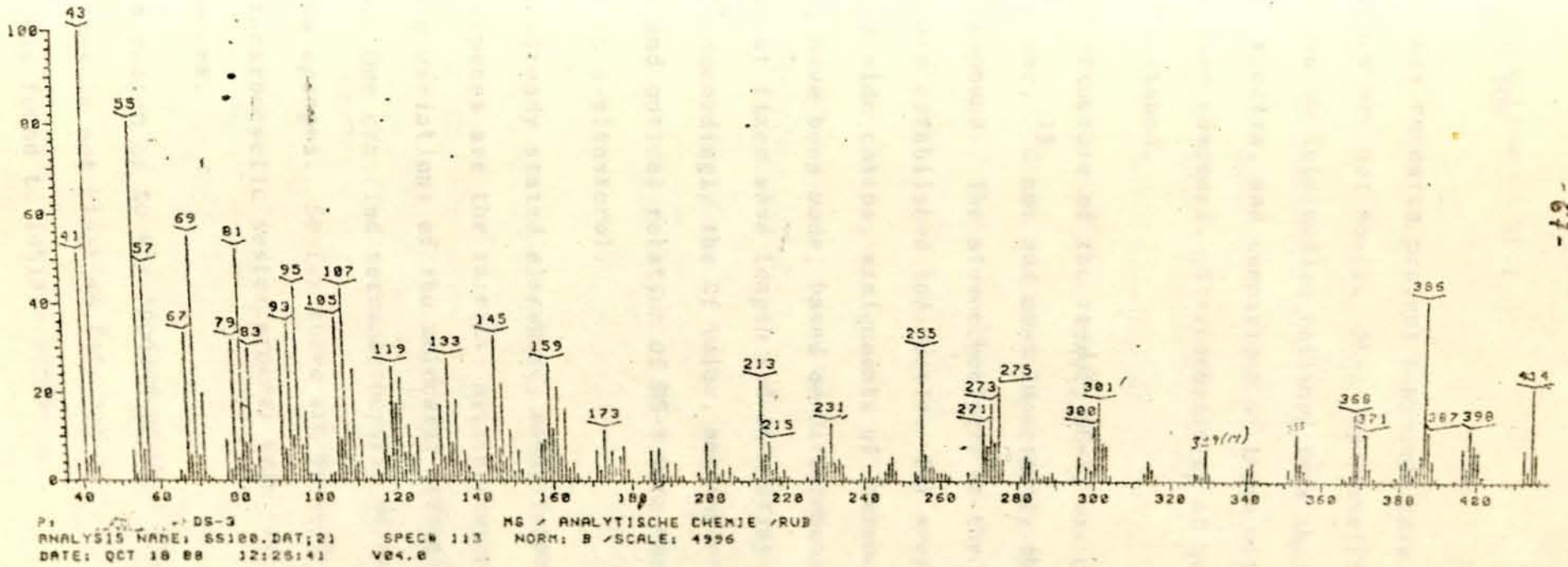
m/z = 571
m/z = 329 (Δ5 only)
m/z = 385
m/z = 329


43
55
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301
300
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387
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414
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20
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120
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220
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260
280
300
320
340
360
380
400
DS-3
MS / ANALYTISCHE CHEMIE /RUB
ANALYSIS NAME: SS100.DAT;21 SPEC# 113 NORM: B /SCALE: 4996
DATE: OCT 10 88 12:26:41 V04.0

4. CONCLUSION

In this research project compounds have been isolated though they are not novel. DS-2 was identified as heteronemin based on information obtained from the ^{1}H nmr, ^{13}C nmr and mass spectra, and comparison of these with reported data for the same compound. Stereochemistry of heteronemin is well established.

The structure of the sterol, DS-1, was characterized using ^{1}H nmr, ^{13}C nmr and most importantly the mass spectrum of the compound. The stereochemistry at the assymmetric centers are established indirectly. For sterols containing saturated side chains, assignments of stereochemistry of isomers, have been made, based on differences in specific rotation at fixed wave length and on melting point differences.[64] Accordingly the Rf value, melting point of the acetate and optical rotation of DS-1 were used to confirm that it is β-sitosterol.

As already stated elsewhere, among terpenoids sesterterpenes are the rarest. Within sesterterpenes there are variations of the molecular arrangement with sources. One can find tetracarbocyclic sesterterpenes only from sponges. So far there are no reports indicating the tetracarbocyclic sesterterpenes from sources other than sponges.

The reason as to why sponges produce these secondary metabolites is not clear so far, but in most cases the crude extract was found to inhibit predators.

5. EXPERIMENTAL

Melting points were determined on a Boetius hot-stage apparatus. Infrared spectra were recorded in KBr pellets using a Perkin Elmer 727 B spectrometer. Ultraviolet spectra were recorded using a Beckman DU-65 spectrophotometer in methanol. Optical rotations were measured in chloroform on a Perkin Elmer 241 polarimeter. NMR spectra measurements were performed in deuterochloroform, acetone-d_6 or benzene-d_6 on a JOEL 90 MHz, Bruker MA 360 MHz or Bruker SF 400 MHz spectrometer. The chemical shifts were referenced to the solvent signals. Mass spectra were recorded on either a CH-5 or a Finnigan - 4021 mass spectrometer. Analytical thin layer chromatography (t l.c) were run on a 0.20 mm thick layer silicagel-60 F254, precoated sheets and the products were detected by UV or vanillin spray reagent.

Collection of sponge samples

Dark brown sponge samples 3B and 4 were collected in June 1987, from the Red Sea, around the marine biology station of Asmara University (AU) at Massawa. Samples were collected at a depth of 2-5 meters with the help of specimens preserved in 10% formalin, salt water solution in the Department of Biology, AU. The collected samples were sun-dried and stored in a cold room.

Extraction of sponge sample 3B and isolation of DS-2

The initial work was started with a dark brown sponge sample 3B. 500 gms of the sponge from the cold room was pulverized using a blender. The pulverized sponge sample was soxhlet extracted with 2.5 liters of distilled chloroform for 48 hrs. The extract was concentrated under reduced pressure which gave 6 gms of a paste like residue. The residue was mixed with 6 gms of silicagel and dried to powder under reduced pressure. The powdered sample was applied to a 2.5 cm diameter column packed with 200 gms of silicagel. The column was first eluted with petroleum ether, followed by petroleum ether chloroform mixtures. The polarity of the solvent was increased by increasing the proportion of chloroform. 100 ml fractions were collected, and tested on TLC plates for similarity.

Fraction 3-15 which were collected with petroleum ether - chloroform (4:1) mixture were found to have three components on TLC. These were combined and concentrated under reduced pressure. The concentrate was rechromatographed on a small column with silicagel and eluted with petroleum ether - chloroform (4:1) mixture. This helped in isolating the compound DS-2, a white needle - like crystalline solid from an oily liquid mixture. DS-2 was further purified by recrystallization, first from methanol: chloroform (9:1) mixture, followed by recrystallization from n-hexane: ethyl acetate (9:1) mixture. The recrystallized product was suction filtered and dried to give 200 mg, 0.04% of

The purified compound showed a single spot in ethanol:chloroform (2:5) mixture (Rf = 0.7) and also in ethylacetate - petroleum ether (1:4) mixture on TLC.

After each recrystallization the melting point was checked. Reproducible melting point of 172-174 was obtained for DS-2.

Characterization of the purified compound was carried out by collecting various spectral data.

Mass spectrum - m/z (rel. int): 386.0 (M^+ - HOAc - CH_2=C=O, 1.2%), 368.1 (M^+ - 2HOAc, 100%), 350.0 ($C_{25}H_{34}O$, M^+ - 2HOAc - H_2O, 30%), 191.1 ($C_{14}H_{23}$, 13%), 161.9 (3.3%), 157.9 (6.4%), 134.0 (7.7%), 132.9 (C_9H_8O, 2.9%), 60 ($C_2H_4O_2$, 3.5%).

Proton-nuclear magnetic resonance -(Bruker MA 360 MHz, $CDCl_3$): (δ: ppm): 6.76 d(H_{25}, J = 2Hz), 6.16 t(H_{24}, J≅1.7 Hz), 5.34 brm (H_{16}), 3.46 m(H_{12}), 3.31 d(O-H_{12}, J=5.6 Hz), 2.43 brs(H_{18}), 2.10 s(two OAc groups), 0.895 s, 0.814 s, 0.789 s(3Me groups), 0.832 s(2Me groups).

^{13}C nmr spectrum - (22.5MHz, benzene-d_6) see table 2.

Ultraviolet spectrum - (c: 0.01, MeOH) UV spectrum showed maximum absorbance at 230 nm (ε = 14000)

Infrared spectrum - ν_{max}^{KBr} cm^{-1}): 3400, 3100, 1750, 1260, 1550, 1400, 1050.

Optical Rotation (c=0.2, chloroform)

λnm	589	578	546	436	365
$[\alpha]_D^{20}$	-67.5	-70.5	-80	-140	-232.5

Extraction of sponge sample 4 and isolation of DS-1

450 g of unidentified brown sponge sample (No 4), extracted in the same way as 3B, gave 5 g of gummy crude extract. Following the same procedure as for the extract from 3B, the sample was column chromatographed and eluted with solvents of increasing polarity. Fraction 9, 10 and 11 which were eluted with petroleum ether: chloroform (9:1) mixture showed that they are mixtures of mainly two components.

These fractions were combined and purified after concentration; on smaller column packed with silicagel, by eluting with petroleum ether: ethylacetate (9:1) mixture. The major component was further purified by recrystallization from chloroform, methanol (1:9) mixture. The recrystallized product was suction filtered to dryness to give 500 mg of DS-1; 0.11% of dry weight.

TLC of DS-1 showed one spot in ethanol: toluene (2:5) mixture (Rf=0.78) and petroleum ether: ethyl acetate (4:1) mixture. DS-1 gave reproducible melting point of 127-128°C after each recrystallization, and was characterized by spectroscopic techniques.

Mass spectrum - m/z (rel. int): 414 ($C_{29}H_{50}O$, 22%), 399 (M^+-CH_3, 9.1%), 396 (M^+-H_2O, 7.8%), 385 ($M^+-C_2H_5$, 6.5%), 381 ($M^+-H_2O-CH_3$, 5.2%), 371 ($M^+-C_3H_7$, 11.7%), 329 ($M^+-C_6H_{13}$, 6.5%), 305 (1.3%), 255(31%), 231 (13%), 215 (9.1%), 213 (23.4%).

Ultraviolet spectrum (c=0.01, MeOH) - The UV-spectrum showed maximum absorbance at 215 nm (ε=12,500)

Infrared spectrum - (ν_{max}^{KBr} cm^{-1}): 3400, 3030, 1610, 1450, 1350, 1050.

Optical rotation

$[\alpha]_D^{20}$= -36.9° (C=0.146, $CHCl_3$)

Proton nuclear magnetic resonance (Bruker SF 400 MHz, $CDCl_3$) (δ: ppm): 5.34 d(H_6, 4Hz), 5.2 d(O-H_3, 5Hz), 3.5 m(H_3).

^{13}C nmr spectrum (22.5 MHz, $CDCl_3$)

(δ: ppm), 141.1 (C-5), 121.7 (C-6), 72 (C-3), 57 (C-14), 56 (C-8), 50 (C-9), 46 (C-17), 12 (C-18), 42.7, 40.2, 37.6, 36.5, 34.4, 32.1, 29.6, 28.3, 27.2, 24.5, 23.5, 22.7, 21.4, 19.5.

Test for 3β-hydroxy-sterol (Liebermann-Burchard test)[62]

About 2 mg of the sample (DS-1) was dissolved in chloroform. To the solution, 1-ml of acetic anhydride and 1-drop of sulphuric acid were added. After 5-minutes, a characteristic color of sterol which passed from brown to deep green was observed.

Acetylation of DS-1[14] - To 100 mg of DS-1, 30 ml of acetic anhydride and two drops of pyridine were added. The mixture was stirred for 72 hrs. 40 ml of water was added to the reaction mixture and extracted with 50 ml of chloroform twice. The combined chloroform extract was washed twice with 40 ml of water, and dried over sodium sulphate. Finally the solvent was evaporated at reduced pressure and the acetate of DS-1 (mp 126°C) was recrystallized from methanol.

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DECLARATION

I; the under signed, declare that this thesis is my work and that all sources of material used for the thesis have been duly acknowledged.

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